Elucidating the Chemical Structure of Black Tea Thearubigins

By

James Warren Drynan

Division of Chemical Sciences
Faculty of Health and Medical Sciences, University of Surrey, UK

In collaboration with
Unilever R&D, Colworth, UK

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STATEMENT OF ORIGINALITY

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ABSTRACT

A solid-phase synthesis protocol was developed, that enabled phenol-protected (-)-epicatechin, and (-)-epigallocatechin to be selectively linked to functionalised resins, as a method to investigate the mechanism of catechin oxidation, which during black tea manufacturing, leads to the ubiquitous and partially characterised thearubigins. Solid-phase synthesis was chosen because model oxidation of catechins can be undertaken, without the solution-phase disadvantage of producing undefined intermediates, and products, which are difficult to isolate. Although (-)-epigallocatechin has a lower redox potential than (-)-epicatechin, and is therefore required to be linked to the resin during oxidation to prevent uncontrolled oxidation-reduction dismutation, or oligomerisation, (-)-epicatechin was used as the model catechin to develop aspects of the synthetic protocol.

The four phenol groups in (-)-epicatechin were selectively protected with the allyl protecting group. Phenol-protected (-)-epicatechin was then selectively linked, via the secondary, aliphatic 3-OH position, via a symmetrical diisopropylsilyl linker, to hydroxytentagel resin. The selective linkage was relatively high yielding (84.6%); however, solid-phase synthesis requires yields greater than 97%, rendering the protocol uneconomical. The five phenol groups in (-)-epigallocatechin were selectively protected with the allyl protecting group. Phenol-protected (-)-epigallocatechin was selectively linked, via the aliphatic, secondary 3-OH position, via a symmetrical diisopropylsilyl linker, a 1,4-disubstituted 1,2,3-triazole, and an amide bond, to aminotentagel resin in high yield (> 97%). The five allyl protecting groups were selectively removed with a palladium(0) catalyst. Subsequent chemical oxidation, with potassium hexacyanoferrate(III) and co-substrate (-)-epicatechin, led to one major, resin-linked product. The product was selectively cleaved from the resin by fluoride, and preliminary FT-IR, NMR and MS data were acquired.

By substituting aminotentagel resin with PEGA resin, the synthetic protocol developed (linkage of (-)-epigallocatechin to a resin), can be used for the biomimetical oxidation of resin-linked catechins, using polyphenol oxidase and/or peroxidase.
PUBLICATIONS


LIST OF ABBREVIATIONS

acetic acid AcOH
acetonitrile MeCN
acetone Me2CO
ammonium acetate NH4OAc
atmospheric-pressure chemical-ionisation APCI
atomic-force microscopy AFM
atomic mass units amu
attenuated total reflectance ATR
n-butanol n-BuOH
caffeine-precipitated thearubigins CTR
carbon-13 isotope 13C
chemical-ionisation CI
circular dichroism CD
controlled-pore glass CPG
1H-1H correlation spectroscopy COSY
cross-polarisation CP
crude soluble tea CST
crush-tear-curl CTC
deuterium D
1,2-dichloroethane DCE
dichloromethane DCM
diethyl ether Et2O
3,4-dihydro-2H-pyran DHP
4-dimethylaminopyridine DMAP
N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-N-
methylmethanaminium hexafluorophosphate N-oxide HATU
N,N-dimethylformamide DMF
dimethyl sulfoxide DMSO
2,4-dinitrophenyldiazine DNP
diode-array detector DAD
<table>
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# LIST OF COMPOUNDS

1: (−)-epigallocatechin gallate  
2: (−)-epicatechin gallate  
3: (−)-gallocatechin  
4: (−)-epigallocatechin  
5: (−)-epicatechin  
6: (+)-catechin  
7: (−)-catechin gallate  
8: (−)-gallocatechin gallate  
9: (−)-epigallocatechin 3-O-(3-O-methyl)gallate  
10: (−)-epigallocatechin 3-O-(4-O-methyl)gallate  
11: (−)-4′-methylepigallocatechin 3-O-(4-O-methyl)gallate  
12: theaflavin  
13: theaflavin 3-O-monogallate  
14: theaflavin 3′-O-monogallate  
15: theaflavin 3, 3′-O-digallate  
16: theaflavin 3-O-(3-O-methyl)-gallate  
17: theaflavin 3-O-(3-O-methyl)-gallate-3′-gallate  
18: theaflavin 3-O-(4-O-methyl)-gallate  
19: theaflavin 3-O-(4-O-methyl)-gallate-3′-gallate  
20: isotheaflavin  
21: (±)-gallocatechin  
22: isotheaflavin 3′-O-gallate  
23: neotheaflavin 3-O-gallate  
24: epitheaflagallin 3-O-gallate  
25: epitheaflagallin  
26: theaflagallin  
27: purpurogallin  
28: pyrogallol  
29: gallic acid
30: purpurogallincarboxylic acid
31: theaflavate A
32: theaflavate B
33: epitheaflavic acid
34: theaflavic acid
35: epitheaflavic acid 3-O-gallate
36: catechol
37: neotheaflavate B
38: benzotropolone derivative (i) (gallate ester)
39: benzotropolone derivative (i)
40: neotheaflavin
41: benzotropolone derivative (ii)
42: theadibenzotropolone A
43: theadibenzotropolone B
44: theadibenzotropolone C
45: theatribenzotropolone A
46: theadibenzotropolone-type trimer?
47: theanaphthoquinone
48: bistheaflavin A
49: bistheaflavin B
50: dehydrotheaflavin intermediate (oxidised theaflavin)
51: dihydrotheanaphthoquinone
52: dehydrotheaflavin (Tanaka)
53: dehydrotheaflavin (O’Coincneainn)
54: dehydrotheaflavin diastereomer (O’Coincneainn)
55: theabenzoquinone
56: (-)-epigallocatechin gallate dimer
57: dehydrotheasinensin
58: desgalloyloolongtheanin
59: theasinensin C
60: theasinensin E
61: galloyloolongtheanin
62: theasinensin A
63: theasinensin D
64: (-)-epigallocatechin gallate quinone dimer A
65: dehydrotheasinensin A
66: gallated flavan elimination product
67: oxidation product
68: dehydrotheasinensin AQ
69: (-)-epigallocatechin gallate quinone dimer B
70: theasinensin-type trimer of (-)-epigallocatechin gallate
71: dehydrotheasinensin C
72: proepitheaflagallin
73: desgalloyl analogue of (-)-epigallocatechin gallate quinone dimer A
74: oolongtheanin
75: hydroxytheaflavin
76: flavan elimination product
77: theogallinin
78: theaflavonin
79: desgalloyltheaflavonin
80: isomyricitrin
81: ethylpyrrolidinonyltheasinensin A
82: L-theanine
83: Strecker aldehyde of L-theanine
84: condensation epimer (R-series)
85: condensation epimer (S-series)
86: theacitrin A
87: theacitrin B
88: theacitrin C
89: speculative structure for theacitrin 3-gallate
90: tricetinidin
91: 1,4,6-tri-O-galloyl-β-D-glucose
92: theogallin
93: strictinin
94: kaempferol
95: quercetin
96: myricetin
97: A-ring model oxidation product of (-)-epigallocatechin gallate 1
98: A-ring model oxidation product of (-)-epigallocatechin gallate 1
99: A-ring model oxidation product of (-)-epigallocatechin 4
100: A-ring model oxidation product of 15
101: A-ring model oxidation product of 15
102: rutin (quercetin-3-O-rutinoside)
103: malvin (malvidin-3,5-diglucoside)
104: cyanidin
105: delphinidin
106: pelargonidin
107: (-)-epigallocatechin-resin (representative structure)
108: (-)-epigallocatechin o-quinone-resin (representative structure)
109: resorcinol mono-benzoate
110: catechol mono-benzoate
111: (-)-epicatechin 5-monobenzoate
4a: pentaallyl(-)-epigallocatechin
4b: pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether
4c: pentaallyl(-)-epigallocatechin diisopropylsilyl dimer
4d: pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid
5a: tetraallyl(-)-epicatechin
5b: tetraallyl(-)-epicatechin diisopropylsilyl methyl ether
5c: tetraallyl(-)-epicatechin diisopropylsilyl dimer
5d: (-)-epicatechin diisopropylsilyl methyl ether
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<td>intermediate in the biosynthesis of epitheaflagallin, hydroxytheaflavin and flavan elimination product</td>
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<td>radical intermediate in the biosynthesis of theacitrin A</td>
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<td>XVIII</td>
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</table>
LIST OF SCHEMES

Scheme 1: proposed mechanism for the acid-catalysed epimerisation of 4 to 3

Scheme 2: dehydrogenation action of PPO

Scheme 3: α-quinones of 5 and 4

Scheme 4: proposed mechanism for the biosynthesis of 12

Scheme 5: alternative mechanism for the biosynthesis of 12

Scheme 6: turnover of the α-quinones of 4 and 5 and their influence on TFs

Scheme 7: proposed coupling scheme for the biosynthesis of 42

Scheme 8: proposed mechanism for the biosynthesis of 47

Scheme 9: proposed mechanism for the biosynthesis of 48

Scheme 10: proposed mechanism for the biosynthesis of 49

Scheme 11: proposed mechanism for the biosynthesis of 52

Scheme 12: general scheme for the reaction of 12 and Fe^{3+}

Scheme 13: proposed mechanism for the biosynthesis of 56

Scheme 14: proposed scheme for the biosynthesis of 64 and 65

Scheme 15: proposed mechanism for the biosynthesis of 64

Scheme 16: proposed mechanism for the biosynthesis of 65

Scheme 17: proposed equilibrium tautomers of compound 68

Scheme 18: proposed mechanism for the biosynthesis of 69

Scheme 19: proposed coupling scheme for the biosynthesis of 70

Scheme 20: proposed dimerisation and dismutation pathway of 4

Scheme 21: proposed mechanism for the formation of 25 and 75

Scheme 22: proposed mechanism for the biosynthesis of 86

Scheme 23: alternative mechanism for the biosynthesis of 86

Scheme 24: formation of p-quinone-methide

Scheme 25: structural proposals of TR biogenesis

Scheme 26: proposed SPS methodology utilising 4 and 5 as precursors

Scheme 27: proposed linkage of Ellman’s resin to 5 (arbitrary linkage position)

Scheme 28: proposed protection of 5 (arbitrary point of attachment)

Scheme 29: proposed deuterium exchange mechanism for proton at position 6

Scheme 30: deprotonation equilibria of 5
Scheme 31: proposed structures of negative-ions and negative-ion fragments  
Scheme 32: allylation of the phenolic hydroxyl groups in 5  
Scheme 33: proposed mechanism for the formation of the C-alkylated species  
Scheme 34: allylation of the phenolic hydroxyl groups in 4  
Scheme 35: silylation of 5a and methyl ether, 5b formation  
Scheme 36: silylation and linkage of 5a to hydroxytengel resin, and formation of methyl ether (5b), and symmetrical dimer (5c)  
Scheme 37: scenario 1  
Scheme 38: scenario 2  
Scheme 39: silylation and formation of 4b, and dimer 4c  
Scheme 40: peptide-type SPS of 4-azidobenzoyl aminotentagel resin  
Scheme 41: Cu(I) catalysed SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin  
Scheme 42: Cu(I) catalysed synthesis of 4d  
Scheme 43: peptide-type SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin  
Scheme 44: Pd(0)-catalysed deallylation of 5b  
Scheme 45: Pd(0)-catalysed deallylation of 5b  
Scheme 46: deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin  
Scheme 47: deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin  
Scheme 48: attempted SPS of theaflavin 12  
Scheme 49: attempted SPS of theaflavin 12
LIST OF FIGURES

Figure 1: $^1$H-NMR spectrum of (−)-epicatechin 5 (d$_6$-DMSO) 265
Figure 2: $^{13}$C-NMR spectrum of (−)-epicatechin 5 (d$_6$-DMSO) 266
Figure 3: $^1$H-NMR spectrum of (−)-epigallocatechin 4 (d$_6$-DMSO) 267
Figure 4: $^{13}$C-NMR spectrum of (−)-epigallocatechin 4 (d$_6$-DMSO) 268
Figure 5: minimum energy conformation (MM2 force-field) for 4 and 5 129
Figure 6: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin 5a (d$_6$-DMSO) 269
Figure 7: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin 5a (d$_6$-DMSO/D$_2$O) 270
Figure 8: $^{13}$C-NMR spectrum of tetraallyl-(−)-epicatechin 5a (d$_6$-DMSO/D$_2$O) 271
Figure 9: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin 5a (CDCl$_3$) 272
Figure 10: $^{13}$C-NMR spectrum of tetraallyl-(−)-epicatechin 5a (CDCl$_3$) 273
Figure 11: LC-ESI-MS chromatogram of tetraallyl-(−)-epicatechin 5a, with mono- and di-C-alkylated by-products 274
Figure 12: $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (d$_6$-DMSO) 275
Figure 13: $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (d$_6$-DMSO/D$_2$O) 276
Figure 14: $^{13}$C-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (d$_6$-DMSO/D$_2$O) 277
Figure 15: $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (CDCl$_3$) 278
Figure 16: $^{13}$C-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (CDCl$_3$) 279
Figure 17: LC-ESI-MS, UV-Vis chromatogram of MeOH quenched, hydroxytentagel resin supernatant (270-280 nm) 280
Figure 18: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (CDCl$_3$) 281
Figure 19: $^{13}$C-NMR spectrum of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (CDCl$_3$) 282
Figure 20: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin diisopropylsilyl dimer 5c (CDCl$_3$) 283
Figure 21: $^{13}$C-NMR spectrum of tetraallyl-(−)-epicatechin diisopropylsilyl dimer 5c (CDCl$_3$) 284
Figure 22: LC-ESI-MS, UV-Vis chromatogram of fluoride cleaved products (275 nm)

Figure 23: \(^1\)H-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b (CDCl\(_3\))

Figure 24: \(^{13}\)C-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b (CDCl\(_3\))

Figure 25: Gel-phase \(^{13}\)C-NMR spectrum of 4-azidobenzoyl aminotentagel resin (CDCl\(_3\))

Figure 26: FT-IR (ATR) transmission spectrum of 4-azidobenzoyl aminotentagel resin

Figure 27: FT-IR (ATR) transmission spectrum of aminotentagel resin

Figure 28: Gel-phase \(^{13}\)C-NMR, pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (CDCl\(_3\))

Figure 29: Gel-phase \(^{13}\)C-NMR spectrum of ‘cleaved’ resin (CDCl\(_3\))

Figure 30: \(^1\)H-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (\(d_6\)-DMSO)

Figure 31: \(^{13}\)C-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (\(d_6\)-DMSO)

Figure 32: Gel-phase \(^{13}\)C-NMR, (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (CDCl\(_3\))

Figure 33: \(^1\)H-NMR spectrum of the oxidation product produced from the attempted SPS of theaflavin 12 (CDCl\(_3\))

Figure 34: \(^{13}\)C-NMR spectrum of the oxidation product produced from the attempted SPS of theaflavin 12 (CDCl\(_3\))
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Binary eluent gradient for 45 minute elution profile</td>
<td>221</td>
</tr>
<tr>
<td>2</td>
<td>Binary eluent gradient for 60 minute elution profile</td>
<td>221</td>
</tr>
<tr>
<td>3</td>
<td>Binary eluent gradient for 75 minute elution profile</td>
<td>221</td>
</tr>
<tr>
<td>4</td>
<td>Binary eluent gradient for 90 minute elution profile</td>
<td>222</td>
</tr>
<tr>
<td>5</td>
<td>Binary eluent gradient for 120 minute elution profile</td>
<td>222</td>
</tr>
</tbody>
</table>
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# CONTENTS

Statement of originality i  
Abstract ii  
Publications iii  
List of abbreviations iv  
List of compounds viii  
List of intermediates and tautomers xii  
List of schemes xiii  
List of figures xv  
List of tables xvii  
Acknowledgements xviii  
Contents xix

## CHAPTER 1: INTRODUCTION 1

1.1 General introduction 2

1.1.1 Introduction 3

1.1.2 Fresh tea leaf polyphenols and black tea production 3

1.1.3 Composition of fresh tea leaves 4

1.1.4 Catechin profile of fresh tea leaves 4

1.1.5 Methylated catechins 8

1.1.6 Epimerisation of catechins 8

1.1.7 Enzyme profile of fresh tea leaves 9

1.1.8 Flavonoids in black tea 11

1.1.9 General comment on enzymatic and chemical oxidative transformations of tea catechins 12

1.2 The chemistry of low molecular mass black tea polyphenols 14

1.2.1 Theaflavins 14

1.2.2 Methylated theaflavins 19

1.2.3 Isotheaflavins 22

1.2.4 Theaflagallins 25

1.2.5 Theaflavates 28

1.2.6 Theaflavic acids 31

xix
CHAPTER 2: RESULTS AND DISCUSSION

2.1 Introduction

2.1.1 \(^1\)H- and \(^13\)C-NMR assignments of (-)-epigallocatechin 4 and (-)-epicatechin 5

2.2 Solution-phase protecting groups

2.2.1 Introduction

2.2.2 DHP protecting group

2.2.3 Attempted solution-phase preparation of THP ether of (-)-epicatechin 5

2.2.4 Other protecting groups (solution-phase)

2.2.5 Stability of (-)-epicatechin 5 in aqueous alkali

2.2.6 Methylation of (-)-epicatechin 5

2.2.7 Benzoylation of (-)-epicatechin 5

2.2.8 Conclusion

2.3 Linkage of protected (-)-epicatechin 5 to hydroxytentagel resin

2.3.1 Introduction

2.3.2 Selective phenol protection of (-)-epicatechin 5 by allylation

2.3.3 Selective phenol protection of (-)-epigallocatechin 4 by allylation

2.3.4 Silylation and MeOH quench of tetraallyl(-)-epicatechin 5a

2.3.4.1 Cleavage of the diisopropylsilyl methyl ether

2.3.5 Stability of (-)-epigallocatechin 4 in the presence of fluoride

2.3.6 Silylation and linkage of tetraallyl(-)-epicatechin 5a to hydroxytentagel resin

2.3.6.1 Chromatography of MeOH-quenched supernatant

2.3.6.2 Cleavage of tetraallyl(-)-epicatechin diisopropylsilyl ether tentagel resin

2.3.7 Conclusion

2.4 Linkage of protected (-)-epigallocatechin 4 intermediate to aminotentagel resin

2.4.1 Introduction
2.4.2 Synthesis of pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether 4b

2.4.2.1 Chromatography and isolation of pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether 4b

2.4.3 SPS of 4-azidobenzoyl aminotentagel resin

2.4.4 'Click'-chemistry with 4-azidobenzoyl aminotentagel resin and pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether 4b

2.4.4.1 Cleavage of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

2.4.5 'Click'-chemistry synthesis of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d

2.4.6 SPS of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

2.4.7 Conclusion

2.5 SPS deallylation and oxidation

2.5.1 Introduction

2.5.2 Deallylation of tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b (with formic acid/butylamine)

2.5.3 Deallylation of tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b (with sodium benzenesulfinate)

2.5.4 SPS deallylation of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate)

2.5.4.1 SPS re-deallylation of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate)

2.5.4.2 Cleavage of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

2.5.5 SPS deallylation of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with 2,4,6-collidine p-toluenesulfonate)
2.5.6 SPS oxidation of (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-
triazolebenzoyl aminotentagel resin (deallylation with sodium benzenesulfinate) with co-substrate, (−)-epicatechin 5

2.5.6.1 Solubility-test for SPS oxidation 208
2.5.6.2 SPS oxidation 210
2.5.6.3 Cleavage of the diisopropylsilyl linker 211

2.5.7 SPS oxidation of (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-
triazolebenzoyl aminotentagel resin (deallylation with 2,4,6-collidine p-
toluenesulfonate)

2.5.7.1 Cleavage of the diisopropylsilyl linker 213

2.5.8 Conclusion 215

2.6 Future Research 217

CHAPTER 3: EXPERIMENTAL 218

3.1 Instruments 219
3.1.1 QTOF-MS 219
3.1.2 NMR 219
3.1.3 FT-IR and FT-IR (ATR) 220
3.1.4 LC-ESI-MS 220
3.1.5 HPLC 222

3.2 Chemicals and suppliers 224

3.3 Solution-phase protecting groups 227
3.3.1 Attempted solution-phase preparation of THP ether of (−)-epicatechin 5 227
3.3.2 Stability of (−)-epicatechin 5 in aqueous alkali 227
3.3.3 Solution-phase methylation of (−)-epicatechin 5 228
3.3.4 Solution-phase equimolar benzoylation of (−)-epicatechin 5 228
3.3.5 Solution-phase optimised benzoylation of (−)-epicatechin 5 229

3.4 Linkage of protected (−)-epicatechin 5 to hydroxytentagel resin 231
3.4.1 Selective phenol protection in (−)-epicatechin 5 by allylation 231
3.4.2 Selective phenol protection in (−)-epigallocatechin 4 by allylation 232
3.4.3 Silylation and MeOH quench of tetraallyl-(−)-epicatechin 5a 234
3.4.3.1 Cleavage of the diisopropylsilyl methyl ether 235
3.4.4 Stability of (−)-epigallocatechin 4 in the presence of fluoride 236
3.4.5 Silylation and linkage of tetraallyl-(−)-epicatechin 5a to hydroxytenta
gel resin 236
3.4.5.1 Chromatography of the MeOH-quenched supernatant 239
3.4.5.2 Cleavage of tetraallyl-(−)-epicatechin diisopropylsilyl ether tenta
gel resin 240

3.5 Linkage of protected (−)-epigallocatechin 4 intermediate to
aminotentagel resin 241
3.5.1 Synthesis of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether
4b 241
3.5.1.1 Chromatography and isolation of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b 242
3.5.2 SPS of 4-azidobenzoyl aminotentagel resin 243
3.5.3 ‘Click’-chemistry with 4-azidobenzoyl aminotentagel resin and
pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b 244
3.5.3.1 Cleavage of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-
1,2,3-triazolebenzoyl aminotentagel resin 246
3.5.4 ‘Click’-chemistry synthesis of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d 247
3.5.5 SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-
triazolebenzoyl aminotentagel resin 248

3.6 SPS deallylation and oxidation 251
3.6.1 Deallylation of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b
(with formic acid/butylamine) 251
3.6.2 Deallylation of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b
(with sodium benzenesulfinate) 252
3.6.3 SPS deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy
ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate) 254
3.6.3.1 SPS re-deallylation of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate)

3.6.3.2 Cleavage of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

3.6.4 SPS deallylation of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with 2,4,6-collidine p-toluenesulfonate)

3.6.5 SPS oxidation of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (deallylation with sodium benzenesulfinate) with co-substrate, (-)-epicatechin

3.6.5.1 Solubility-test for SPS oxidation

3.6.5.2 SPS oxidation

3.6.5.3 Cleavage of the diisopropylsilyl linker

3.6.6 SPS oxidation of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (deallylation with 2,4,6-collidine p-toluenesulfonate)

3.6.6.1 Cleavage of the diisopropylsilyl linker

APPENDIX

REFERENCES

Reference List
CHAPTER 1

INTRODUCTION
1.1 General introduction

The initial part of the introduction summarises the chemistry of low molecular mass black tea polyphenols. Low molecular mass, in this case, includes compounds with molecular masses of less than 1000 g mol\(^{-1}\). The term ‘low molecular mass’ was chosen to differentiate between the compounds described in the initial part of the introduction and the higher molecular mass black tea polyphenols, termed ‘thearubigins’ and so far, of unknown structure. At the same time, the term ‘low molecular mass’ also refers to all the black tea polyphenols that have been purified and structurally characterised, information clearly missing on the higher molecular mass black tea polyphenols.

These polyphenolic compounds are only in part, secondary plant metabolites. They are usually not encountered in healthy plant tissue and are only formed after injury to the tissue. The compounds possibly act as a chemical defence mechanism against grazing and insect attack. Beside their natural occurrence, these compounds are also formed during the ‘fermentation’ process of converting fresh tea leaves into black tea, via a series of oxidation-cascades. The introduction summarises the literature currently available on these oxidative processes, focusing on products of enzymatic reactions and products obtained from model oxidation studies, if relevant to the natural products.

Aspects of general tea chemistry have been reviewed by Harbowy and Balentine in 1997 [1] and the chemistry of TRs reviewed by Haslam in 2003 [2] but no up-to-date and comprehensive literature on the chemistry and characterisation of low and higher molecular mass black tea polyphenols has been made available.

The introduction is organised by class of compounds, and therefore, automatically by mechanism of formation. At the same time, during the progression of the introduction, the structural complexity of the compounds described, gradually increases. From the data currently available, conclusions will be drawn on the likely mechanisms and structures involved in the formation of the more complex thearubigin fraction whose chromatographic, chemical and spectroscopic characterisation is reviewed in the final part of the introduction. Compounds have been numbered in Arabic numerals, while intermediates have been numbered in Roman numerals.
1.1.1 Introduction

Black tea is, after water, the most consumed beverage worldwide. Annual production of dried tea in 2004 was 3.2 million tonnes. The average amount of black tea consumed per person per year in the United Kingdom is currently 3.2 kg. Production of dried tea comprises 20% green, 2% oolong, and the remainder (78%) black. In 2004, tea prices were US$ 1.56 kg\(^{-1}\) in January and had increased to US$ 1.73 kg\(^{-1}\) by December [3].

This makes black tea one of the most economically important agricultural products; however, despite its importance; the majority of black tea’s chemical composition remains unresolved. An aqueous infusion of black tea is particularly rich in polyphenol compounds whose composition can be crudely characterised as follows; (1) a low molecular mass ‘monomeric’, ‘dimeric’ or ‘trimeric’ polyphenol fraction containing compounds with molecular masses less than 1000 g mol\(^{-1}\) and whose structures are known. (2) a structurally ambiguous ‘oligomeric’ and ‘polymeric’ polyphenol fraction, historically termed ‘thearubigins’ (TRs) [4]. The components of the aptly named reddish-brown TR most likely have molecular masses greater than 1000 g mol\(^{-1}\). It has also been established that the heterogeneous TRs constitute more than 70% of the dry mass of an average black tea aqueous infusion and their chemical structure remains to be elucidated [2].

1.1.2 Fresh tea leaf polyphenols and black tea production

Fresh green tea leaves (Camellia sinensis) (which are rich in flavan-3-ols) are converted to black tea leaves by a manufacturing process. There are two major types, the orthodox and the ‘crush-tear-curl’ (CTC) process [5,6]. The orthodox process consists of withering, rolling, enzymatic oxidation (fermentation), drying and sorting of the leaf. The CTC process consists of the withered leaf initially being cut to a uniform size (e.g. tea-bag production) and then being fed into a machine where they are crushed, torn and curled by metal rollers. The enzymes that are released, in particular, tea polyphenol oxidase (PPO), are collected and added to the leaves again. After the required amount of oxidation has occurred the leaves are dried and sorted. In both processing methods, the principal aim is to bring into contact tea leaf enzymes and tea leaf constituents so that enzymatic
oxidation of the constituents may occur. The method of preparation in withering, length of oxidation stage and process of deactivating the leaf enzymes (drying) vary in black tea production.

1.1.3 Composition of fresh tea leaves

Common fresh green tea leaf components include polyphenols (including flavonoids), methyl xanthines (such as caffeine and theobromine), amino acids, organic acids, carotenoids, volatiles (low molecular mass aldehydes and alcohols), carbohydrates, proteins, lignin, lipids, chlorophyll and inorganic material (ash) [7].

Flavonoids are polyphenolic compounds (carbon and heteroatom numbering shown below) and are plant secondary metabolites. They are divided into six sub-classes. The sub-classes are flavones, flavanones, isoflavones, flavan-3-ols, flavonols and anthocyanidins [8]. The two main sub-classes of flavonoids found in green tea leaves are flavanols and flavonols. However, fresh green tea leaves contain primarily, flavan-3-ols (catechins) and these are characterised by meta-5,7-dihydroxy substitution of the A-ring and di- or trihydroxy substitution of the B-ring. However, (-)-epiafzelechin is also present, but has only a single hydroxyl group on the B-ring. Throughout the text, the terms; ‘flavan-3-ols’ and ‘catechins’ will be used interchangeably.

1.1.4 Catechin profile of fresh tea leaves

Freshly picked tea leaves contain a group of low molecular mass catechins. These may constitute up to 30% of the dry leaf weight [9,10]. The relative amounts of catechins and their gallates (the gallate ester moiety, if present is at position C-3 and the aromatic-ring is designated the ‘D-ring’) are genetically controlled. The catechins are highly soluble in water and many organic solvents, therefore, their exact location within the leaf, both
tissue distribution and intracellular localisation, has in the past proven difficult to establish.

Recently, however, Suzuki et al. have investigated the tissue and cellular tea leaf morphology for catechin distribution using light- and electron-microscopy [11]. A method was employed which utilised osmium tetroxide post-fixation (the leaf was fixed with a mixture of glutaraldehyde and formaldehyde in pH 7 phosphate buffer at 4 °C) to 'locate' the catechins. It was established via light-microscopy that the catechins were not distributed evenly throughout the leaf structure, but were concentrated mainly in the mesophyll tissue, regardless of leaf age. There was no evidence of catechins being present in the epidermal cells. This finding is of interest as it contradicts the hypothesis that catechins provide protection to the leaf from insect attack.

It was also observed that the catechin distribution in the mesophyll tissue was not homogenous. The majority of catechins were located in mesophyll cells in close proximity to epidermal cells and there was evidence (lack of osmiophilic material) that the central areas of the mesophyll cells were devoid of catechins.

Intracellular catechin distribution was determined via electron-microscopy. As observed via light-microscopy, the catechin distribution in the mesophyll tissue was not homogenous. In the cells which did contain catechins, the catechins were localised in large vacuoles, usually referred to as 'central vacuoles'. In some of these cells, the catechins were present as an aggregated mass attached to the membrane of the central vacuole. However, the site of catechin synthesis and mode of transport to the vacuoles was not elucidated.

The four major catechins present in the green leaf have been established as (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5. It has also been established that individual catechin concentration is dependent on leaf age. Usually (-)-epigallocatechin gallate 1 dominates as the most abundant catechin present in the bud and first leaf [12] but in some teas it is (-)-epicatechin gallate 2 that dominates [13].
In the past, chemical methods for the determination of polyphenols in the fresh tea leaf were generally non-specific and could not distinguish between flavonoid classes. The types of methods employed were: Folin-Denis reagent, vanillin reagent, diazotised amines, Folin-Ciocalteau and Price and Butler reagent [14].

A variety of chromatographic techniques have established themselves as more reliable and reproducible methods for the determination of catechins present in the fresh leaf.

For instance, Roberts and Myers used paper chromatography (PC) to confirm the identity and presence of (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5 in green tea and unfermented leaf [15]. Roberts and Myers also proposed the epimerisation of (-)-epicatechin 5 to (+)-catechin 6 and of (-)-epigallocatechin 4 to (-)-gallocatechin 3 in the presence of heat. The presence of (-)-
epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5 in the green leaf were previously established by Tsujimura [16-20], Oshima [21] and Bradfield and co-workers [22-24].

Singh et al. have reported the use of diazotised sulfanilamide as an economical, catechin specific, visualisation spray reagent for the selective detection of fresh or dried green leaf catechins on two-dimensional thin-layer chromatograms (TLCs) or PCs without the need for expensive equipment such as high-pressure/performance liquid-chromatography (HPLC) [14]. The reagent did not react with flavonols, their glycosides and other leaf constituents. The sensitivity of the reagent was reported as < 1 µg of 6 over the concentration range 0.4-8.0 µg ml⁻¹ of 6. They also reported that if a leaf extract (Me₂CO) is applied volumetrically to the stationary phase of a TLC or PC plate, quantitative measurement of individual catechins was possible spectrophotometrically based on the formation (under acidic conditions) of the yellow catechin A-ring-diazotized sulfanilamide adduct (λ_max = 425 nm). Results for the recovery of individual catechins varied between 80-89%. This was based on the standard addition of 6. With their method, Singh et al. detected the presence of (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, 3, (-)-epigallocatechin 4 and 6. They also reported that in fresh tea shoot extracts, (-)-epigallocatechin gallate 1 was the major catechin derivative and 6 the minor. Singh et al. have also reported the variation in total catechin concentration in different parts of the tea shoot in the order: stem < second leaf < first leaf < bud.

An isocratic elution system for the resolution and detection of catechins in a sample of the dried fresh leaf utilising HPLC has been established by Wang et al. [25]. Individual catechin resolution was achieved on a C_{18} reversed-phase column and the mobile phase comprised MeOH/H₂O/orthophosphoric acid. With this method, seven catechins could be resolved and detected, viz. (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, 3, (-)-epigallocatechin 4, (-)-epicatechin 5, 6 and (-)-gallocatechin gallate 8. It was observed that (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4 were the most abundant catechins present while 6 was the least. Interestingly, (-)-catechin gallate 7, a C-2 epimer
of (-)-epicatechin gallate does not seem to be present in any significant amount in fresh green tea leaves.

### 1.1.5 Methylated catechins

Two O-methylated derivatives of (-)-epigallocatechin gallate, (-)-epigallocatechin 3-O-(3-O-methyl)-gallate 9 and (-)-epigallocatechin 3-O-(4-O-methyl)-gallate 10 have been resolved and detected by Chiu and Lin during an HPLC analysis of various fresh tea leaves and commercial tea samples including longjing, oolong, puerh and black tea [26]. Resolution was achieved using an isocratic mobile phase (0.1 M sodium dihydrogen phosphate buffer at pH 2.5 containing 0.1 mM Na2EDTA;MeCN (87:13 v/v)) and a reversed-phase Cosmosil 5 C18-MS column. The O-methylated derivatives were detected using an electrochemical detector (ECD) at an applied potential of 600 mV versus Ag/AgCl. The HPLC conditions were previously established using a mixture of pure catechin standards comprising; (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4, (-)-epicatechin 5, 6, 8, 9, 10 and (-)-4'-methylepigallocatechin-3-O-(4-O-methyl)-gallate 11.

![Chemical structures](image)

### 1.1.6 Epimerisation of catechins

Molecular rearrangements of tea catechins, including the epimerisation of (-)-epigallocatechin gallate 1 to 8, and its subsequent oxidative degallation, have been observed by Wang et al. [27]. In their studies, they found that heating slightly acidic aqueous solutions of (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, 3, (-)-epigallocatechin 4, (-)-epicatechin 5, 6 and 8 above 80 °C caused epimerisation to occur.
at the C-2 position and not the C-3 position as had been reported previously by Seto et al. [28].

The acid catalysed epimerisation of (-)-epigallocatechin 4 to 3, for example, would presumably occur via the following proposed mechanism (scheme 1). The intrinsic pH of black tea is 5.6 and during the final stages of production (enzyme deactivation), temperatures may reach 90 °C and above, therefore the acid catalysed epimerisation of catechins may lead to catechin diastereomers. These configurational subtleties may be incorporated into TRs during the final stages of their synthesis and would contribute to TR heterogeneity within a bulk structural domain (the bulk structural domain characterised, for example, as a defined CD curve).

Scheme 1: proposed mechanism for the acid catalysed epimerisation of 4 to 3

1.1.7 Enzyme profile of fresh tea leaves

In the fresh green leaf, the catechins and enzymes are spatially separated. It has been established that the enzymes are located in the epidermis and around the vascular bundles [10].

The enzymes that are involved in the oxidation of the catechins to form, for example, the black tea polyphenol monomer, tricetanidin (a 3-desoxyanthocyanidin) [27] and the black tea polyphenol dimeric species have been established as polyphenoloxidase (PPO),
peroxidase (POD) and catalase. It has been established by Davies et al. [29] that PPO, POD and catalase are present in the leaf and are all able to catalyse the oxidation of catechins, but under differing conditions, and that PPO is the most important enzyme in tea polyphenol oxidation, with POD possibly involved in oxidation to a lesser extent.

PPO [PPO; EC 1.14.18.1; monophenol mono-oxygenase (tyrosinase) or EC 1.10.3.2; o-diphenol: O₂ oxidoreductase] is a copper-containing enzyme that has the ability to convert an o-dihydroxyphenol to an o-benzoquinone using the substrate O₂ (scheme 2) [30].

\[
2 \text{O} + \text{O}_2 \xrightarrow{\text{PPO}} \text{O} + \text{H}_2\text{O}
\]

\(o\text{-dihydroxyphenol} \quad o\text{-benzoquinone}

Scheme 2: dehydrogenation action of PPO (adapted) [30]

Ravichandrin and Parthiban have studied the enzymic profile and change in enzyme activities of PPO and phenylalanine ammonia-lyase (PAL), another important enzyme, with type of leaf and also their respective change in activity during the manufacture of black tea [31]. PAL is a plant enzyme that converts L-phenylalanine into trans-cinnamic acid. This is a precursor to lignins, coumarins and flavonoids [32,33]. Ravichandrin and Parthiban found variations in enzyme activity with regards to shoot maturity, different clones and seasonal variations. They observed that during black tea manufacturing, the loss of enzyme activity during withering could be restored by rehydration and that residual activity of PPO, and not PAL, was observed in dried black tea and that this may alter the product during storage.

Clougle has reported the effects of temperature on enzyme activity during the oxidation stage in the processing of black tea [34]. It was observed that the activities of both PPO and POD decreased as a function of time but that PPO activity decreased the most. The effect of temperature on inactivation was also greater for PPO. It was also observed that at high temperature, POD activity is little affected and that the ratio POD:
PPO increases during black tea processing. Cloughley suggested that the alteration in the ratio of enzyme activity could play an important role in determining the oxidation products formed.

Pruidze et al. have reported the presence of multiple forms (isoforms) of POD in *Camellia sinensis* and their possible role in black tea production [35]. It was found that different isoforms of POD with RMM 118000 ± 3000 and 58000 ± 2000 do not have hydroxylase activity but catalyse oxidation of *o*-diphenols. RMM 250000, 118000 and 58000 isoforms were able to catalyse the oxidation of catechins, (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4. Theaflavins (TFs) and TRs produced during oxidation of (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4 in their studies inhibited the high molecular mass isoforms of POD. When the TF and TR concentrations were high enough, they completely inhibited the enzymes. RMM 28000 and 41000 isoforms were shown to catalyse hydroxylation of monophenols to produce *o*-diphenols.

### 1.1.8 Flavonoids in black tea

Following the fermentation process, the majority of flavonoids remain unchanged. However, the majority of flavan-3-ols in the green leaf are transformed and are not present in black tea leaves. This observation has been rationalised by the assumption that after enzymatic oxidation, flavan-3-ols are chemically converted into more complex polyphenols. This complex polyphenol mixture is characterised chromatographically (reversed-phase HPLC) by a large Gaussian-shape hump that is overlaid by a small number of well resolved sharp peaks. The Gaussian-shape hump is usually referred to as the TR hump. The fact that this hump has thus far not been resolved chromatographically has lead to a number of hypotheses rationalising the unusual chromatographic behaviour including: a large number of similar or isomeric compounds being present, the presence and interference of chelating metal cations and strong non-covalent interactions between the individual tea components through hydrogen-bonding, van der Waal’s forces, hydrophobic and *π*-*π* stacking interactions.
1.1.9 General comment on enzymatic and chemical oxidative transformations of tea catechins

The fundamental chemistry of black tea is characterised by oxidation processes occurring in aqueous media, mediated by PPO, which utilises flavanol polyphenols such as (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5 as substrates. Oxidation of the catechol moiety on the B-ring affords highly electrophilic o-quinones, which subsequently react with nucleophilic flavanols, (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5, initially forming a C-C bond, followed by various cascades of reactions to yield dimeric species such as theaflavins, theanaphthoquinones, theasinsensins and theacitrins. These dimeric species may yet again be subjected to oxidation or can act as nucleophiles themselves in reactions with o-quinones derived from (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5.

Generally the formation of these dimeric species can be classified mechanistically in the following three ways; Firstly, the compounds can be classified according to the substitution pattern on the B-ring, i.e. whether they are di-hydroxy or tri-hydroxy substituted hence, giving after oxidation, o-quinone I or II respectively. o-Quinone II can be in equilibrium with its tautomer III. However, both IR-spectroscopic investigations and ab initio quantum chemical calculations indicate that II is energetically favoured over III (scheme 3) [36].

Reactivity trends can be rationalised by taking into account the number of electron-donating OH functionalities on the B-ring or the resulting o-quinone. The more electron-rich, the aromatic nucleus, the more facile is one-electron oxidation, leading to the
respective o-quinones. Similarly, the more electron-rich, is the aromatic nucleus, the more nucleophilic it is. On the other hand, an additional OH functionality such as that present in II, when compared with I, renders the o-quinone of II less electrophilic making I the better electrophile.

Secondly, the compounds can be classified according to the nature of the nucleophile reacting with either I, or II, or in some cases III. It has also been shown that the nucleophile can react with the o-quinone II of the D-ring. In principle any of the flavan-3-ols such as (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5 can act as nucleophiles yielding a wide variety of products. The nucleophilic flavan-3-ols, (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4 and (-)-epicatechin 5 are ambident nucleophiles offering a large number of nucleophilic sites, including most of their oxygen and carbon atoms. Therefore, a large number of theoretically possible reaction products could arise. As it will be shown, the nucleophilic attacks are rather chemo- and regioselective resulting in a relatively small number of possible bond forming combinations.

Thirdly, the compounds can be classified according to the reaction mechanism that follows the initial C-C bond formation after the initial nucleophilic attack onto the o-quinone. The description of the compounds in the initial part of the introduction broadly follows this classification with compounds being grouped according to the nature of the dimerisation/oligomerisation linking moiety, i.e. benzotropolone, biphenyl (including redox dismutation) or cyclopenta[α]indene-type.
1.2 The chemistry of low molecular mass black tea polyphenols

1.2.1 Theaflavins

Compounds possessing one or more bicyclic undecane benzotropolone \((6E,8Z)-3,4,6\text{-tri}hydroxy-5H\text{-benzo[7]annulen-5-one})\) moieties \((R_2C_{11}H_{16}O_4)\).

\[
\begin{array}{c}
R = \text{A- and C-rings of catechins} \\
R_1 = \text{H or OH}
\end{array}
\]

A key reaction in tea fermentation involves the conversion of \((-\)-epigallocatechin 4 and \((-\)-epicatechin 5 and their gallates via \(\alpha\)-quinones to theaflavins (TFs), compounds comprising a benzotropolone moiety. The original term ‘theaflavin’ was introduced by Roberts who discovered this class of compounds to be neutral pigments present in the EtOAc extract of black tea [4]. The structure of theaflavin 12 was elucidated simultaneously by the groups of Takino [37] and Ollis [38]. It is the presence of the benzotropolone chromophore that results in the characteristic yellow-orange colour of the TFs and their derivatives. The four main TF derivatives found in black tea are; theaflavin 12, theaflavin 3-monogallate 13, theaflavin 3'-monogallate 14 and theaflavin 3,3'-digallate 15, with total concentrations up to 2\% [39].
Due to the complexity of black tea polyphenols compared with those of green tea, model fermentations, utilising pure catechins have been useful for studying the mechanism of benzotropolone-ring formation. En route to the structural elucidation of 12, and in an attempt to shed light on the mechanism of TF-type formation, Takino and Imagawa performed model oxidation experiments by treating catechins, (-)-epigallocatechin 4 and (-)-epicatechin 5 with tea oxidase (PPO). The subsequent formation of a reddish-orange pigment, characteristic of the benzotropolone chromophore, was observed [40]. The reddish-orange pigment was not present when either (-)-epigallocatechin 4 or (-)-epicatechin 5 was treated individually with tea oxidase (PPO) and it was proposed the benzotropolone-ring was formed via the oxidative dimerisation of the two catechin B-rings following a similar reaction course to the formation of 3',4'-dihydroxy-3,4-benzotropolone as described by Marukami et al. [41]. Takino and Imagawa also compared the PC chromatogram of their reddish-orange oxidation product with that of the EtOAc-soluble fraction of black tea, both displayed the same chromatographic behaviour and it was proposed the biosynthesised compound was 12 as previously
described by Roberts and Myers [42]. Takino et al. subsequently proposed a mechanism for the formation of the 3',4'-dihydroxybenzotropolone moiety (scheme 4) [43]. Following initial oxidation to o-quinone I and then C-C bond formation via an intermolecular aldol reaction to yield IV, a second intramolecular aldol reaction furnishes the tri-cyclic diketone intermediate V, which after a benzylic acid-type rearrangement yields the benzotropolone moiety. Interestingly, the epicatechin derived o-quinone I acts as the electrophile in this coupling reaction, whereas the epigallocatechin derived o-quinone II acts as the nucleophile. As a critical remark it should be pointed out that it is unclear whether (-)-epigallocatechin 4 acts as a nucleophile itself, prior to oxidation to the o-quinone II or whether o-quinone II acts as the nucleophile in the C-C coupling step.

Scheme 4: proposed mechanism for the biosynthesis of 12 (adapted) [43]

An alternative mechanism for benzotropolone-ring formation has been proposed by Haslam (scheme 5) [39]. The mechanism includes a regioselective cyclisation directed via a self-assembled intermolecular quinhydrone-type π-π complex between the catechin B-rings. (-)-Epicatechin 5 is the electron-rich donor (nucleophile) and II the electron-deficient acceptor (electrophile).
This mechanism was proposed in order to rationalise the unusual regioselectivity of the initial C-C bond formation which then yields the tri-cyclic diketone intermediate V. It is hypothesised that intermolecular hydrogen-bonding between the two carbonyl oxygen atoms in II and the two catechol-type hydrogen atoms in (-)-epicatechin 5 stabilises the quinhydrone-type π-π complex and merges the two intermolecular reaction centres into the correct orientation that results in generation of intermediate V.

It can be assumed from the two mechanisms proposed and from inspection of their respective chemical structures that 13 is produced from the oxidative dimerisation of (-)-epigallocatechin gallate 1 and (-)-epicatechin 5; similarly, 14 is produced from the oxidative dimerisation of (-)-epicatechin gallate 2 and (-)-epigallocatechin 4, while 15 is produced from the oxidative dimerisation of (-)-epigallocatechin gallate 1 and (-)-epicatechin gallate 2.

It has been observed that during black tea processing, TF formation by PPO is higher than consumption of TF by POD (destructive oxidation of phloroglucinol rings present in TFs) and that after inactivation of PPO due to high temperature, there is a reduction in the generation of o-quinones and hence a reduction in TF formation [34]. At some stage, the rate of TF degradation exceeds their formation and these degraded products may be involved in TR formation as observed by an increase in TR-type material at this stage.
The effects of the presence of (-)-epigallocatechin 4 and (-)-epicatechin 5 on oxidation of the TFs by PPO from tea leaves using equimolar concentrations of (-)-epigallocatechin 4 or (-)-epicatechin 5 and the TF substrates 12, 13, 14 and 15 have been examined by Bajaj et al. in a model aerobic fermentation system [44]. It was observed that oxidation reactions of the monogallates were more rapid than the oxidation of digallate 15 when (-)-epicatechin 5 was present in the fermentation system. It was also observed that (-)-epigallocatechin 4 was not effective in oxidising the TFs, but instead prevented their oxidation. It was hypothesised that as (-)-epicatechin 5 has the highest redox potential of the major catechins, the o-quinone of (-)-epicatechin 5, II acts as an electron donor for substrates such as the TFs and then reduces back to (-)-epicatechin 5. It was also observed that the redox potential of (-)-epigallocatechin 4 is not higher than the redox potential of TFs and that the o-quinone of (-)-epigallocatechin 4, III does not act as an electron donor. Bajaj et al. suggested that as a consequence of the relatively low redox potential of (-)-epigallocatechin 4, it was polymerised to a TR-like form (scheme 6).

Scheme 6: turnover of the o-quinone of 4 and 5 and their influence on TFs [44]

It was concluded by Bajaj et al. that their experimental results might explain why (-)-epicatechin 5 is detected in fair amounts in black tea, whereas generally, the concentration of (-)-epicatechin 5 in the leaf is the lowest of the major catechins. In contrast to this was the disappearance of the majority of (-)-epigallocatechin 4 during their model black tea fermentation. Furthermore, it was observed that the amounts of TFs in black tea were less than expected when compared with the amounts of the catechins.
present in the leaf. It was therefore assumed that large amounts of TFs synthesised during black tea manufacturing were oxidised by (−)-epicatechin 5.

To this effect, Opie et al. have reported the role of PPO and (−)-epicatechin 5 in the coupled oxidative breakdown of TFs [45]. It was observed that 12, 13, 14 and 15 were not substrates for PPO [EC 1.14.18.1], but (−)-epicatechin 5 or mixtures of (−)-epicatechin 5 and a TF were rapidly converted to TR-like compounds in the presence of PPO, with the distinctive TR hump present in the reversed-phase HPLC chromatogram. Hence, TFs act only as nucleophiles, rather than electrophiles in TR formation. It was concluded that TFs are degraded by a coupled oxidation with the o-quinone of (−)-epicatechin 5 and Opie et al. hypothesised that certain mechanistic aspects of TR formation might follow this pathway.

1.2.2 Methylated theaflavins

Theaflavin 3-O-(3-O-methyl)-gallate 16, theaflavin 3-O-(3-O-methyl)-gallate-3′-gallate 17 and the four major TFs 12-15 have been isolated from an aqueous ethanolic extract of black tea leaves (solid-phase C18) and characterised (reversed-phase LC-ESI-MS) by Nishimura et al. while in the process of refining chromatographic techniques for TF analysis and quantification in black tea with respect to biological activity [46]. The aim of their work was to quantify TFs in black tea leaves using differentiating adsorbents to exclude major contaminating oxidation products and hence to provide evidence for the hypothesised existence of methylated TFs such as 16 and 17. In general, TF profiling for biological activity has been concerned with aqueous infusions of black tea leaves. Nishimura et al. observed that after aqueous extraction of black tea leaves, TFs and other oxidation products are to a small extent still present in the leaves. Therefore their extraction technique employed aqueous ethanol as a more efficient extraction solvent than H2O for TF quantification.
Pulverised black tea leaves were extracted with 50% aqueous EtOH containing 2% ascorbic acid (w/v) as antioxidant. The leaves were extracted three times and the extracts combined and centrifuged. The supernatant was diluted four-fold with H₂O and applied to a C₁₈ column set in a vacuum manifold apparatus. The column was washed with H₂O and 15% aqueous EtOH to eliminate polar contaminating oxidation products and ascorbic acid. The majority of TF material was eluted with 40% aqueous EtOH. The eluent was concentrated in vacuo and the residue redissolved in 50% aqueous EtOH for LC-ESI-MS analysis (280 nm). The TFs 12-15, compounds 16 and 17 were separated on a linear gradient composed of 80% aqueous MeCN with 0.5% AcOH as additive. The assignments for 16 and 17 were based on retention times relative to previously synthesised authentic standards and confirmed using SIM. The negative-polarity ESI spectrum yielded the molecular ion of 16 [M-H]⁻ at m/z 729 and of 17 [M-H]⁻ at m/z 881.

As in the proposed mechanism for the formation of 12 from the oxidative dimerisation of (−)-epigallocatechin 4 and (−)-epicatechin 5, Nishimura et al. proposed that 16 was formed from the oxidative dimerisation of (−)-epicatechin 5 and 9 while 17 was formed from the oxidative dimerisation of (−)-epicatechin gallate 2 and 9.

Interestingly, during the course of their investigations, Nishimura et al. observed that during the process of converting oolong tea to black tea, the quantity of 17 produced from
(-)-epicatechin gallate 2 and 9 was higher than the quantity of 16 produced from (-)-epicatechin 5 and 9. Furthermore, the production ratio of 17 to 15 in black tea leaves was approximately 25%, but the ratio of 9 to (-)-epigallocatechin gallate 1 in green tea was approximately 14%. This observation implies that 9 is more oxidatively labile in its dimerisation reaction with (-)-epicatechin gallate 2 than is (-)-epigallocatechin gallate 1.

It is almost certain that theaflavin 3-O-(4-O-methyl)-gallate 18 and theaflavin 3-O-(4-O-methyl)-gallate-3'-gallate 19 are present in black tea by analogy, considering the presence of 10 in the fresh green leaf. Compound 18 would presumably be formed from the oxidative dimerisation of (-)-epicatechin 5 and 10 while 19 would presumably be formed from the oxidative dimerisation of (-)-epicatechin gallate 2 and 10. However, a methylated catechin such as 11 with the 4'-OH position protected against oxidation by a methoxy group would not be expected to form a B-ring to B-ring type TF with the current understanding of benzotropolone-ring formation.

TRs may, to a small extent, contain these methoxy oxidation 'dead-ends' in their precursors preventing potentially active sites from further benzotropolone-ring formation. It can be inferred that the use of a methylated catechin such as 11 in model oxidation studies involving tea fermentation may prove useful in elucidating certain mechanistic aspects of TR formation.
1.2.3 Isotheaflavins

Isotheaflavin 20 has been isolated from an aqueous infusion of black tea (Sephadex LH-20, TLC and PC) and characterised (IR spectroscopy, UV-Vis spectroscopy, MS, \(^1\)H-NMR spectroscopy and ORD spectroscopy) by Coxon et al. while investigating the apparent non-stereospecific enzymatic oxidation of green leaf flavanols, and their gallates, during black tea manufacturing [47]. The aim of their work was to identify and isolate stereoisomers of 12.

![Compound 20](image)

Compound 20 was analytically isolated by column chromatography from an isolated fraction of black tea (established method) containing 12. Compound 20 was further separated from 12 by TLC (cellulose; \(n\)-butanol:formic acid:H\(_2\)O, 100:25:60 v/v). Subsequently, it was established that 20 could be isolated preparatively from the mother liquor remaining after crystallisation of 12 [48]. The method involved isolation of a mixture of 12 and 20 by column chromatography (Sephadex LH-20; propan-2-ol:AcOH:H\(_2\)O, 4:1:4 v/v), PC (3MM papers; \(n\)-BuOH:AcOH:H\(_2\)O, 4:1:5 v/v) and column chromatography (Sephadex LH-20; 20-60\% Me\(_2\)CO\(_{aq}\) v/v).

The IR and UV-Vis spectra of 20 were almost identical to 12. Six UV-Vis absorption maxima for 20 were observed at 207, 231, 270, 297, 378 and 462 nm. The high resolution mass spectrum of the heptamethyl ether of 20 established its empirical formula and fragmentation patterns of the heptamethyl ether of 12 and heptamethyl ether of 20 were similar, indicating that 20 was a diastereomer of 12.
Analyses of $^1$H-NMR coupling constant data for 12 and 20, established the relative configuration of 20 to be 2,3-trans-2',3'-cis and also provided the first direct deduction of a 2,3-cis-2',3'-cis relative configuration for 12. The relative and absolute configurations of 12 were deemed compatible on the basis that 12 was formed via the oxidative coupling of (-)-epigallocatechin 4 and (-)-epicatechin 5. Similarly, it was proposed that 20 was formed via the oxidative coupling of (-)-epicatechin 5 and (+)-gallocatechin 21. Compound 12 did not undergo isomerisation when kept in acidic solvents for several months and it was therefore considered unlikely that 20 was an artefact derived from 12 during isolation employing acidic solvents.

The absolute configuration of 12 and 20 were independently correlated by similarity of their ORD curves, the characteristics of which indicated that they both possessed the same configuration at their benzylic 2 and 2' positions.

Isotheaflavin 3'-O-gallate 22 and neotheaflavin 3-O-gallate 23 have been isolated (Sephadex LH-20, reversed-phase preparative and semi-preparative HPLC) from a MeOH extract of black tea leaves by Lewis et al. [49]. Compounds 22 and 23 were characterised via 1D/2D NMR spectroscopy, negative-polarity ESI-MS and chemical synthesis.
Compound 22 was isolated from a mixture that also contained epithealagallin 3-O-gallate 24, in approximate molar proportions of 2:1 respectively. Further chromatographic purification of the mixture was not successful. UV-Vis absorption maxima (HPLC-PDA) for 22 were observed at 284, 375 and 455 nm, similar to those observed for 12 and 15 (273, 375 and 455 nm). Negative-polarity ESI-MS data revealed the RMM of 22 to be 716 amu, identical to that of 13 or 14. It was therefore proposed that 22 was a stereoisomer of 13 or 14.

$^1$H-NMR data for 22 confirmed that it was a TF-type compound possessing a galloyl ester group (expected double intensity singlet present at approx. 7.0 ppm) with one flavanol moiety possessing cis-2,3 geometry ($J_2, 3' = 0.8$ Hz) and the other, trans-2,3 geometry ($J_2, 3 = 8.6$ Hz). HMQC and HMBC $^{13}$C-$^1$H correlations established the location of the benzotropolone ring, epicatechin and catechin-like moieties and galloyl ester group.

It was proposed that 22 was formed via the oxidative coupling of (−)-epicatechin gallate 2 with 21 (spectroscopic data). Further evidence for the suggested combination of catechins involved in the formation of 22, and the designation of its absolute configuration, was attained when Lewis et al. chemically oxidised (−)-epicatechin gallate 2 with 21. The reaction mixture contained a compound with identical reversed-phase HPLC retention time and UV-Vis spectrum as measured for 22.
UV-Vis absorption maxima (HPLC-PDA) for 23 were observed at 277, 373 and 451 nm, also similar to those observed for 12 and 15 (273, 375 and 455 nm). Negative-polarity ESI-MS data revealed the RMM of 23 to be 716 amu, identical to that of 13 or 14. It was therefore proposed that 23 was a stereoisomer of 13 or 14.

\[^1\text{H-NMR}\] data indicated that 23 was a TF-monogallate with characteristic benzotropolone, galloyl ester and A- and C-ring proton resonances being observed. Coupling constants for C-ring resonances indicated one flavanol moiety possessed \textit{cis}-2,3 geometry and the other, \textit{trans}-2,3 geometry. \[^{13}\text{C-NMR}\] chemical shifts for 23 were analogous to the TFs, the HMBC spectrum confirmed the presence of a benzotropolone moiety and the location of the epicatechin and catechin-like moieties. However, no correlations were observed to the galloyl ester carbon in the HMBC spectrum, but it was proposed that the galloyl ester group was attached at C-3 (H-3 chemical shift increase of approx. 1.5 ppm on introduction of a galloyl ester group at C-3) [50].

Additional evidence for the proposed structure of 23 was attained when Lewis \textit{et al.} chemically oxidised (−)-epigallocatechin gallate 1 with 6. The reaction mixture contained a compound with identical HPLC retention time and UV-Vis spectrum as measured for 23.

Compounds 22 and 23 were present in black tea at low levels (< 0.01% dry wt). Lewis \textit{et al.} hypothesised that a combination of contributing factors lead to the low level of 22 and 23 found in black tea in comparison to their stereoisomeric counterpart 12 i.e. the low concentration of catechins detected in black tea leaves which contain \textit{trans}-2,3 stereochemistry, evidence that \textit{trans}-2,3 precursors yield TFs less favourably than \textit{cis}-2,3 precursors, and the fact that it has been shown from model chemical and enzymatic oxidation studies that gallated precursors yield TFs less readily than non-gallated precursors [51,52].

1.2.4 Theaflagallins

Epithealagallin 3-O-gallate 24, epitheaflagallin 25 and theaflagallin 26, all of which contain a theaflavin-type benzotropolone moiety with 1',2',3'-trihydroxy substitution,
have been isolated (Sephadex LH-20, MCI-gel CHP-20P and Bondapak C\textsubscript{18}) from black tea by Nonaka \textit{et al.} [53]. The isolation of 24-26 was the first reported evidence for benzotropolone-type dimerisation arising from pyrogallol-pyrogallol oxidative dimerisation during the manufacture of black tea.

An 80\% aqueous Me$_2$CO extract of black tea was chromatographed over Sephadex LH-20 (H$_2$O with increasing proportions of MeOH) to yield three fractions. The third fraction was chromatographed over Sephadex LH-20, MCI-gel CHP-20P and Bondapak C\textsubscript{18} to yield 24 and 15. Similar chromatography of the first fraction yielded 25 and 26, while the second fraction yielded 12, 13 and 14.

Compound 24 was isolated as a red crystalline powder. Positive-polarity FAB-MS yielded the molecular ion of 24 [M+H]$^+$ at \textit{m/z} 553 and combined with elemental analysis, the molecular formula was provisionally deduced as C$_{27}$H$_{20}$O$_{13}$. The $^1$H-NMR spectrum revealed four aliphatic proton resonances, with similar chemical shifts to those of the C-ring protons in (−)-epigallocatechin gallate 1, while other resonances were attributed to galloyl and phloroglucinol-type protons. The $^{13}$C-NMR spectrum revealed carbon resonances which closely corresponded to the galloyl, A- and C-ring resonances present in (−)-epigallocatechin gallate 1. Eleven other carbon resonances were detected including a carbonyl carbon and ten sp$^2$ carbon atoms. Their chemical shifts were similar to those of purpurogallin 27 except for one upfield shift. The combined $^1$H- and $^{13}$C-NMR data suggested the presence of a benzotropolone moiety with hydroxyl substitution such as that proposed for 24. Compound 24 was further characterised via mp and polarimetry.
Confirmation for the proposed structure was provided by chemical synthesis, via oxidative dimerisation of (-)-epigallocatechin gallate 1 and pyrogallol 28 using K$_3$Fe(CN)$_6$ in a weakly alkaline medium (NaHCO$_3$). The oxidation reaction yielded 24 and 27. Compound 27 was produced from oxidative self-dimerisation of 28 (with loss of CO$_2$). A similar synthesis with (-)-epigallocatechin gallate 1 and gallic acid 29 (both present in green tea leaves) yielded 24 and purpurogallincarboxylic acid 30.

![Chemical structures](image)

Compound 25 was isolated as red needle-like crystals. Positive-polarity FAB-MS yielded the molecular ion of 25 [M+H]$^+$ at m/z 401 and combined with elemental analysis, the molecular formula was provisionally deduced as C$_{20}$H$_{16}$O$_9$. The $^1$H-NMR spectrum of 25 was similar to that of 24 except for the absence of the galloyl resonances suggesting 25 to be a desgalloyl derivative of 24. Compound 25 was further characterised via mp and polarimetry.

Confirmation for the proposed structure was provided by enzymatic hydrolysis of 24 with tannase which yielded 25 and 29.

Compound 26 was isolated as a red crystalline powder. Positive-polarity FD-MS yielded the molecular ion of 25 [M]$^+$ at m/z 400 and combined with elemental analysis, the molecular formula was provisionally deduced as C$_{20}$H$_{16}$O$_9$. The $^{13}$C-NMR spectrum of 26 was almost identical to that of 25, but the chemical shifts of the C-2 and C-3 resonances were at lower field than those of 25, indicating trans-stereochemistry. Furthermore, the $^1$H-NMR spectrum revealed the C-2 and C-3 proton resonances to contain relatively large coupling constants, $J = 9$ Hz and $J_{ax} = 16$ Hz, respectively. From the combined spectral data, 26 was considered to be an epimer of 25. Compound 26 was further characterised via mp and polarimetry.
Confirmation for the proposed structure was provided by chemical synthesis, via oxidative condensation of (+)-gallocatechin and 28 using K$_3$Fe(CN)$_6$ in a weakly alkaline medium (NaHCO$_3$). The oxidation reaction yielded optically inactive 26 and 27. However, the absolute configurations at C-2 and C-3 were not determined, but were presumed to be 2R and 3S on the basis that 21 is the more abundant epimer in natural products.

From their investigations, Nonaka et al. concluded that theaflavilin-type black tea pigments represent the product of an alternative enzymatic oxidation system involving commonly occurring tea leaf pyrogallol-type compounds such as (−)-epigallocatechin 4 and 29. This is in contrast to the mechanism for their biotransformation during the production of TF and theasinensin / hexahydroxydiphenic acid-type species.

1.2.5 Theaflavates

Theaflavate A 31 has been synthesised via the chemical oxidation of (−)-epicatechin gallate 2 with K$_3$Fe(CN)$_6$ in weakly alkaline medium (NaHCO$_3$). The black tea pigment was isolated (Sephadex LH-20 and semi-preparative HPLC) and characterised (1D/2D NMR spectroscopy and positive-polarity ESI-MS) by Wan et al. while investigating the chemical oxidation of (−)-epicatechin gallate 2. HPLC at 390 nm also confirmed the presence of 31 in black tea [54].

An ice-cooled aqueous solution of K$_3$Fe(CN)$_6$ and NaHCO$_3$ was added to an ice-cooled aqueous solution of (−)-epicatechin gallate 2 and allowed to stand for fifteen minutes in an ice-bath. The pH was decreased to approximately pH 2 with addition of citric acid and the solution extracted into EtOAc. The EtOAc extract was dried over anhydrous MgSO$_4$ and the solvent evaporated to yield an orange powder.

The crude extract was dissolved in Me$_2$CO and chromatographed over Sephadex-LH20 (50% aqueous Me$_2$CO) to yield six fractions. The sixth fraction was further chromatographed using reversed-phase semi-preparative HPLC to yield 31.
Compound 31 was isolated as a yellow solid. The UV-Vis spectrum yielded three absorption maxima at 235, 281 and 402 nm. Positive-polarity ESI-MS yielded [M+H]^+ and [M+Na]^+ pseudomolecular ions at m/z 853 and m/z 875 respectively. The ^13C-NMR spectrum revealed the presence of forty three carbon atoms. With these data the empirical formula was provisionally deduced as C_{43}H_{32}O_{19}. This is twice the sum of the RMM of (-)-epicatechin gallate 2 less 32 amu (CH_{4}O) which suggested oxidative inter-molecular coupling of (-)-epicatechin gallate 2, the product containing a characteristic (theaflavin-type) benzotropolone moiety.

Analyses of ^1H- and ^13C-^1H-NMR data confirmed that inter-molecular oxidative dimerisation had occurred between the B- and D-rings of (-)-epicatechin gallate 2. Additional structure confirmation was attained via HMBC, ROESY and HMQC correlations. The stereochemistry at C-2, C-3, C-2’ and C-3’ was expected to be that of cis-2,3 geometry (epi-like) and this was confirmed via ^1H coupling constant data.

Reversed-phase HPLC with UV-Vis detection at 390 nm established 31 as being present in black tea infusions; however, the amount detected was negligible. The location of the benzotropolone moiety (D- to B-ring) was of mechanistic interest and provided evidence that galloyl ester groups are able to participate ‘directly’ in oxidative coupling reactions. Wan et al. hypothesised that this type of oxidative coupling, an alternative regio-selective pathway for extending molecular size, could be implicated in the production of TR.

![Diagram of 31 and 32](image-url)
Theaflavate B 32 has been isolated (Sephadex LH-20 and reversed-phase preparative HPLC) from a MeOH extract of black tea leaves by Lewis et al. [49]. Compound 32 was characterised via 1D/2D NMR spectroscopy, positive-polarity ESI-MS and chemical synthesis.

Compound 32 was isolated as an orange-red amorphous solid which was soluble in MeOH and Me2CO. UV-Vis absorption maxima (HPLC-DAD) were observed at 284 and 406 nm, similar to those measured for 31 and epitheaflavic acid 33. This was an indication that 32 may contain a carboxy-substituted benzotropolone nucleus as present in TF-type derivatives. Negative-polarity ESI-MS data revealed the RMM of 32 to be 700 amu. This differs from the RMM of 31 (852 amu) by 152 amu which indicated the absence of a galloyl ester group (152 amu) in 32. With these data the empirical formula was provisionally deduced as C_{36}H_{28}O_{15}.

^1H- and ^13C-NMR data revealed similarities between the spectra of 31 and 32 except for the absence of galloyl ester signals and a relatively large downfield shift of H-3' (loss from C-3') in 32. C-ring chemical shifts and coupling constants (1.2 Hz or less) were similar to those observed for 31 and it was suggested the relative stereochemistry of 32 at C-2, C-3 and C-2', C-3' was cis-2, 3 geometry based on the small J_{2,3} and J_{2',3'} coupling constants (epi-like). This was an indication that 32 was formed via oxidative coupling of (-)-epicatechin gallate 2 with (-)-epicatechin 5 with the characteristic mass loss of 32 amu (CH_{4}O).

The absolute stereochemistry of 32 at C-2, C-3 and C-2', C-3' was concluded to be cis-2, 3 geometry following the synthesis and isolation of 32, from the chemical oxidation (K_{3}Fe(CN)_{6} / NaHCO_{3}) of (-)-epicatechin gallate 2 with (-)-epicatechin 5. The isolated compound displayed identical NMR, UV-Vis and chromatographic properties to those of 32. On the basis of its structure, Lewis et al. proposed that 32 and other theaflavate-type pigments were formed during black tea processing by oxidative coupling of two catechins, with one containing a galloyl ester group as prerequisite. It was not established if PPO oxidises the galloyl ester of (-)-epicatechin gallate 2 directly during the formation of 32.
during black tea manufacturing. However, this mechanism was suggested as being kinetically not feasible as it was known that PPO does not readily oxidise 29 [55,56].

A mechanism for the formation of 32, based on that proposed for the formation of the theaflavic acids, was proposed by Lewis et al. [57], whereby oxidation of the galloyl ester group to a quinone occurs via a chemical redox reaction between the PPO induced quinone of an accompanying catechin. Subsequently, coupling between the galloyl ester quinone and the catechin occurs to form the theaflavate. It was suggested that similar interflavanol linkages may occur in the heterogeneous TRs, formed from the oxidative coupling of (prerequisite) galloyl ester groups and catechin B-ring precursors.

The isolation and characterisation of 32 from black tea is of mechanistic interest as it was generally considered that galloyl ester groups were insensitive to oxidation during black tea manufacturing.

1.2.6 Theaflavic acids

Epitheaflavic acid 33 and theaflavic acid 34 were chemically synthesised and characterised (mp, MS, chemical derivatisation, IR spectroscopy, UV-Vis spectroscopy and $^1$H-NMR spectroscopy) by Coxon et al. while investigating the chemical composition of the components of Roberts’ ‘substance Q’ found in black tea [58]. Roberts had suggested that ‘substance Q’ was possibly a mixture of oxidation products formed from the dimerisation of green leaf flavan-3-ols and their gallates with 29 [42,59,60]. Coxon et al. were interested specifically in the TF fraction of black tea.

![Chemical structures](33.png)
![Chemical structures](34.png)
![Chemical structures](35.png)
An aqueous bicarbonate solution of 6 and 29 at 0 °C was treated with K$_3$Fe(CN)$_6$ to yield 34, from EtOAc (extraction). Similarly, the treatment of (-)-epicatechin 5 and 29 with K$_3$Fe(CN)$_6$ yielded 33, from EtOAc (extraction).

The empirical formula of 33 and 34 was deduced as C$_{21}$H$_{16}$O$_{10}$ from analyses of their high-resolution mass spectra (RMM = 428.0743 amu). The IR spectra of 33 and 34 were similar and both showed characteristic carbonyl absorption bands attributable to benzotropolone (1630 cm$^{-1}$) and carboxylic acid groups (1700 cm$^{-1}$). The UV-Vis absorption maxima for 34 were observed at 208, 280 and 404 nm, while those for 33 were observed at 208, 280 and 400 nm. Comparison of the UV-Vis absorption maxima of 33 and 34 between 400 and 500 nm and those for 20 (462 nm) indicated that the benzotropolone chromophore in 33 and 34 had been modified (blue shift) by its direct bonding to a carboxylic acid group. A similar effect was shown to occur by comparison of the UV-Vis absorption maxima for the benzotropolone chromophore of 27 (434 nm) and 30 (400 nm) [61,62]. Comparison of the $^1$H-NMR spectra of 33 and 34 and that of 20 clearly indicated (relative coupling constant data) that 34 had the 2',3'-trans configuration and 33 had the 2',3'-cis configuration.

Compound 33 and epitheaflavic acid-3-gallate 35 have been biosynthesised in model tea fermentation systems containing a crude soluble tea (CST) enzymes preparation [63] by Berkowitz et al. [57] while investigating the chemical composition of the components of Roberts’ ‘substance Q’. Berkowitz et al. observed that model tea fermentation systems containing (-)-epicatechin gallate 2, or (-)-epicatechin 5 and another gallated flavan-3-ol always formed a compound that appeared to be the same as ‘substance Q’. Compounds 33 and 35 were isolated (Sephadex LH-20) and 33 characterised by comparison with authentic material by co-chromatography, NMR spectroscopy and IR spectroscopy.

A short oxidation period (~fifteen minutes) of (-)-epicatechin 5 and 29 in a model tea fermentation system using a CST enzymes preparation was shown by PC to contain a number of reaction products with the major product identified as 33. When the substrates used in the short oxidation period were (-)-epicatechin gallate 2 and 29, two major
reaction products were formed, 33 and a compound which Berkowitz et al. proposed to be 35.

It was observed that when (-)-epicatechin 5 was the only substrate in the model fermentation system, TRs and two unidentified substances were produced, but there was no production of 33. However, when (-)-epicatechin gallate 2 was the only substrate in the model fermentation system, a number of oxidation products was formed, including 33 and 35. Compound 29 was also detected in the mixture, as had previously been found in model tea fermentation systems, whenever gallated flavan-3-ols such as (-)-epigallocatechin gallate 1 and (-)-epicatechin gallate 2 were present [64] and during the manufacture of black tea [48]. When 29 was the only substrate in the model fermentation system, no reaction products were formed, indicating that 29 was not a substrate for tea catechol oxidase. Respirometric studies on the model fermentation systems were summarised as follows (adapted) [57]:

(a) \(5 + 29 + \frac{1}{2}O_2 \rightarrow 33 + CO_2\)
(b) \(2 + 29 + \frac{1}{2}O_2 \rightarrow 35 + CO_2\)
(c) \(5 + O_2 \rightarrow \text{unidentified} + \text{unidentified} + \text{TRs}\)
(d) \(2 + O_2 \rightarrow \text{streaking} + \text{TRs}\)
(e) \(2 \rightarrow 5 + 29 \rightarrow (a) + (b), \text{as above}\)

The requirement of an o-dihydroxy phenolic moiety, present in (-)-epicatechin gallate 2 and (-)-epicatechin 5, and a vic-trihydroxy phenolic moiety, present in 29, for the production of the red-coloured biosynthesised compounds suggested that the reaction leading to their formation was analogous to the reaction leading to the formation of TFs. The structures for 33 and 35 were proposed based on the observation they were the primary products of the oxidations with (a) and (b) dominating and (e) leading to the same product as (a). The biosynthesised compound proposed to be 33 was found to be identical to authentic 33 prepared by co-oxidation of (-)-epicatechin 5 and 29 by \(K_3Fe(CN)_6\) in dilute bicarbonate solution [58,65].
Interestingly, the model fermentation system with (-)-epicatechin 5 and 29 as substrates yielded the maximum concentration of 33 after approximately fifteen minutes, with the concentration of 33 decreasing and completely disappearing after an oxidation period of approximately forty minutes. Additionally, both 33 and 35 accumulated in the first few minutes of the oxidation period of the model fermentation with (-)-epicatechin gallate 2 and 29 as substrates, but their concentration began to decrease while the concentration of TRs appeared to increase until (-)-epicatechin gallate 2 was depleted.

In both model fermentation systems, TRs appeared to accumulate at the expense of the low molecular mass polyphenols present. The biotransformation of 33 into TRs was further investigated using a pure sample of 33. When 33 was the only substrate in the model fermentation system, no reaction products were formed. However, when (-)-epicatechin 5 was added to the model system containing 33, it was observed that the concentration of 33 and (-)-epicatechin 5 rapidly decreased with the simultaneous increase in the concentration of TRs.

Berkowitz et al. observed that although 33 and 35 resembled the TFs by containing a benzotropolone moiety, with both types of compounds being formed via the oxidative condensation of an o-dihydroxy phenolic moiety and a vic-trihydroxy phenolic moiety, tea catechol oxidase could catalyse the oxidation of both substrate molecules in the formation of TFs but only one of the substrates in the formation of 33 or 35. It was also observed that oxidation of 29, a requirement for the formation of 33 and 35, was induced by the oxidation of (-)-epicatechin 5. The results of the model tea fermentations showed that 33 and 35 were ultimately converted to TRs and that their biotransformation was dependent on the tea catechol oxidase catalysed oxidation of flavan-3-ols. Also, the high reactivity of 33 and 35 was thought to explain why they were present in black tea in only trace amounts [60].

1.2.7 Theaflavin/benzotropolone derivatives

Eighteen benzotropolone derivatives have been biosynthesised, isolated (EtOAc extraction and Sephadex LH-20) and characterised (positive-polarity APCI-MS and
1D/2D NMR spectroscopy) by Sang et al. [66] using a horseradish POD/H₂O₂ model oxidation system operating on selected pairs of compounds, one with a vic-trihydroxyphenyl moiety ((−)-epigallocatechin gallate 1, (−)-epigallocatechin 4, 28 and 29) and the other with an o-dihydroxyphenyl moiety ((−)-epicatechin gallate 2, (−)-epicatechin 5, 6 and catechol 36). Sang et al. were interested in the health benefits of TFs and TF-type compounds and therefore biosynthesised the benzotropolone derivatives for biological screening.

The generic method for the biosyntheses of the eighteen benzotropolone derivatives involved dissolving non-stoichiometric amounts of the selected pairs of compounds in a mixture of Me₂CO and phosphate-citrate buffer (1:10 v/v) (pH 5.0), which contained horseradish POD. Four aliquots of 3.13% H₂O₂ were added over forty-five minutes whilst stirring. The mixture was then extracted with EtOAc and the combined organic fractions concentrated in vacuo. The residue was chromatographed over Sephadex LH-20 with a mixture of Me₂CO and H₂O as eluent.

Among the eighteen compounds isolated and characterised were two novel benzotropolone derivatives, neoeaflavate B 37 and benzotropolone derivative (i) (gallate ester), compound 38.

Compound 37 was biosynthesised from the oxidative dimerisation of (−)-epicatechin gallate 2 and 6. Positive-polarity APCI-MS yielded the molecular ion of 37 [M+H]⁺ at m/z 701 and combined with ¹³C-NMR data, which were the same as those for 32, its molecular formula was provisionally deduced as C₃₆H₂₈O₁₅. The ¹³C-NMR spectrum of 37 revealed the presence of thirty-six carbon resonances, eighteen of which were assigned to the A- and C-rings of the flavanol moieties. Additionally, ¹H-NMR data indicated that the A- and C-rings did not undergo change during the oxidation with two sets of resonances assigned to the protons at the 2-, 3-, 4-, 6- and 8-positions of the flavanol moieties. By comparison of its ¹H-NMR spectrum with the ¹H-NMR spectra of (−)-epicatechin gallate 2 and 6, Compound 37 was distinguishable by the absence of the galloyl ester resonances of (−)-epicatechin gallate 2 and the B-ring resonances of 6. The ¹³C-NMR spectrum revealed eighteen carbon resonances (besides the A- and C-rings),
comprising a carbonyl, an ester carbonyl and sixteen olefinic carbon atoms which indicated the presence of a benzotropolone moiety in 37. It was deduced from the above NMR data that the galloyl ester group of (−)-epicatechin gallate 2 had reacted with the B-ring of 6 to form the benzotropolone moiety. This assumption was supported by HMBC data and the complete interpretation of NMR results, with structure assignment, was based on HMQC and HMBC correlations.

It was proposed by Sang et al. that along with 31 and 32, compound 37 may be present as a minor oxidation product in black tea and at the time of publication, investigations using LC-ESI-MS/MS were in progress to determine whether or not 37 is present in the TF fraction of black tea.

Compound 38 was biosynthesised from the oxidative condensation of (−)-epigallocatechin gallate 1 and 36. Positive-polarity APCI-MS yielded the molecular ion of 38 \([M+H]^+\) at \(m/z\) 537 and combined with \(^{13}\)C-NMR data, its molecular formula was provisionally deduced as \(C_{27}H_{20}O_{12}\). The NMR spectra of 38 and benzotropolone
derivative (i), compound 39 differed in that 38 contained characteristic gallate resonances, similar to the difference between the NMR spectra of (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4; therefore 38 was proposed to be the gallate ester of 39.

The other sixteen benzotropolone derivatives biosynthesised were; 12 ((-)epigallocatechin 4 with (-)-epicatechin 5), 13 ((-)epigallocatechin gallate 1 with (-)-epicatechin 5), 14 ((-)epicatechin gallate 2 with (-)-epigallocatechin 4), 15 ((-)epigallocatechin gallate 1 with (-)-epicatechin gallate 2), 23 ((-)epigallocatechin gallate 1 with 6), 24 ((-)epigallocatechin gallate 1 with 29), 27 (28 with 36), 30 (6 with 29 or (-)-epicatechin 5 with 29 or (-)-epicatechin gallate 2 with 29), 31 ((-)epicatechin gallate 2 with itself), 32 ((-)epigallocatechin gallate 1 with 6), 33 ((-)epicatechin 5 with 29), 34 (6 with 29), 35 ((-)epicatechin gallate 2 with 29), 39 ((-)epigallocatechin 4 with 36), neotheaflavin 40 ((-)epigallocatechin 4 with 6) and benzotropolone derivative (ii), compound 41 (29 with 36).

Interestingly, from a mechanistic point of view, 27, 30, 40, and 41 have been previously synthesised via chemical oxidation of their monomers but have not been found in black tea [53,67,68].

The benzotropolone nature of the oxidation products formed during the model oxidation reactions led Sang et al. to hypothesise that POD plays an important role in the formation of TF-like compounds during black tea manufacturing.

1.2.8 Theadi/tribenzotropolones

Theadibenzotropolone A 42, a TF-type trimer has been biosynthesised (POD/H₂O₂), isolated (Sephadex LH-20 and C₁₈) and characterised (negative-polarity ESI-MS and 1D/2D NMR spectroscopy) by Sang et al. while investigating the mechanism of enzymatic oxidation of catechins with respect to TF-type benzotropolone ring formation during black tea manufacturing [69]. The model fermentation system comprised horseradish POD in the presence of H₂O₂ using (-)-epigallocatechin gallate 1 and (-)-epicatechin 5 as substrates. In addition to 42, not surprisingly, 13 was also isolated. The
The presence of 42 was also established in a TF sub-fraction of black tea using LC-ESI-MS/MS with selected-ion monitoring (SIM).

\[ \text{PHH} \quad \text{O} \quad \text{PH} \]

\[ \text{H O} \quad \text{H O} \quad \text{H O} \quad \text{H O} \]

\[ \text{PH} \quad \text{H O} \quad \text{H O} \]

\[ (-)-Epigallocatechin gallate 1 \quad \text{and} \quad (-)-epicatechin 5 \]

were dissolved in a Me\textsubscript{2}CO-pH 5 buffer mixture containing horseradish POD. H\textsubscript{2}O\textsubscript{2} was added over forty-five minutes and the formation of 13 was observed. Further addition of H\textsubscript{2}O\textsubscript{2} over a period of thirty minutes resulted in a decrease in 13 with the concurrent formation of 42. The oxidation mixture was chromatographed over Sephadex LH-20 (40% Me\textsubscript{2}CO/H\textsubscript{2}O) and C\textsubscript{18} (50% MeOH/H\textsubscript{2}O) to yield 13, compound 42 and non-reacted (-)-epigallocatechin gallate 1 and (-)-epicatechin 5.

\[ \text{42} \]

Negative-polarity ESI-MS yielded the molecular ion of 42 [M-H] at \textit{m/z} 973 and the molecular formula was provisionally deduced as C\textsubscript{50}H\textsubscript{38}O\textsubscript{21} after complementary analysis \textit{via} \textsuperscript{13}C-NMR data. The \textsuperscript{13}C-NMR spectrum exhibited fifty carbon resonances with twenty-seven of these assigned to catechin A- and C-rings. Similarly, the \textsuperscript{1}H-NMR spectrum exhibited three sets of proton resonances corresponding to the 2-, 3-, 4-, 6- and 8-positions of the three catechin moieties. The \textsuperscript{13}C-NMR spectrum of 42 also revealed the presence of one ester and two ketone carbonyl resonances in addition to twenty olefinic carbon resonances. In comparison with the \textsuperscript{1}H-NMR spectrum of 13, the \textsuperscript{1}H-NMR spectrum of 42 was distinguished by an additional set of A- and C- ring resonances, the absence of galloyl ester resonances and the presence of three extra olefinic resonances.
Additional structure confirmation was attained via HMBC, COSY and HMQC correlations.

The presence of 42 in black tea was confirmed using LC-ESI-MS/MS with SIM of the TF fraction of black tea that was isolated (80% Me$_2$CO/H$_2$O) from a black tea extraction that was followed by Sephadex LH-20 gel permeation chromatography (40% Me$_2$CO/H$_2$O). A sample of enzymatically biosynthesised 42 was employed as the internal standard. Not only did the standard and the LC-peak in the TF fraction have the same retention times and molecular masses but their fragmentation ions (m/z) were also the same.

It has been established that it is mainly PPO enzymatic oxidation in tea leaves that produces the TFs and Sang et al. proposed that the formation of 42 was via the reaction between (-)-epicatechin 5 and 13 by the action of POD in the presence of H$_2$O$_2$. Their hypothesis was confirmed when they reacted 13 with (-)-epicatechin 5 using the same conditions as those for the reaction of (-)-epigallocatechin gallate 1 with (-)-epicatechin 5. It was observed that all of 13 could be converted to 42 if (-)-epicatechin 5 was in excess. These findings were significant as it provided the first evidence that suggested the galloyl ester group of 13 is as reactive as the B-ring (vic-trihydroxy) of (-)-epigallocatechin gallate 1 or (-)-epigallocatechin 4 and the galloyl ester group of (-)-epicatechin gallate 2, mimicking the same reaction mechanism (scheme 7) as described during the production of TFs, 31 [54] and 32 [49].
The dibenzotropolone B 43 and dibenzotropolone C 44, TF-type trimers and a tribenzotropolone tetramer, tribozotropolone A 45 have been biosynthesised (POD/H₂O₂), isolated (Sephadex LH-20 and C₁₈) and characterised (negative-polarity LC-APCI-MS and 1D/2D NMR spectroscopy) by Sang et al. while further investigating the mechanism of enzymatic oxidation of catechins with respect to TF-type benzotropolone ring formation [70]. As in the case of 42, the presence of 43 was also established in a TF sub-fraction of black tea using LC-ESI-MS/MS.
The biosynthesis, isolation and characterisation methods for 42 have been previously described [69]. However, during the LC-ESI-MS/MS sub-fraction characterisation of the EtOAc fraction of black tea which led to the discovery of 42, three additional peaks with the same relative molecular mass were observed in a subsequent sub-fraction. In order to identify these compounds and possibly characterise further unknown components of black tea, Sang et al. engaged in further model enzymatic oxidation experiments using various combinations of tea catechins and TFs. Horseradish POD/H$_2$O$_2$ was chosen as the model oxidation system to investigate whether or not POD contributes to the formation of the TRs. The experimental method used to biosynthesise 43, 44 and 45 was the same as that used to biosynthesise 42 [69].

Compound 43 was biosynthesised from the oxidative coupling of 13 with 6 (an epimer of (-)-epicatechin 5). Negative-polarity LC-APCI-MS yielded the molecular ion of 43 [M-H]$^-$ at m/z 973 and the molecular formula was provisionally deduced as C$_{50}$H$_{38}$O$_{21}$ after complementary analysis via $^{13}$C-NMR data. The $^1$H- and $^{13}$C-NMR spectra resembled those of 42. The $^{13}$C-NMR spectrum exhibited fifty carbon resonances with twenty-seven of these assigned to catechin A- and C-rings. Similarly, the $^1$H-NMR spectrum exhibited three sets of proton resonances corresponding to the 2-, 3-, 4-, 6- and 8-positions of the three catechin moieties. This was evidence that the A- and C-rings of 13 did not undergo
structural change during the oxidation experiment. The $^{13}\text{C}-\text{NMR}$ spectrum of 43 also revealed the presence of one ester and two ketone carbonyls in addition to twenty olefinic carbon resonances. In comparison with the $^1\text{H}-\text{NMR}$ spectrum of 13, the $^1\text{H}-\text{NMR}$ spectrum of 43 was distinguished by an additional set of A- and C-ring resonances, the absence of galloyl ester resonances and the presence of three extra olefinic resonances. Complete structure confirmation was attained via HMBC and HMQC correlations. These NMR data suggested that the galloyl ester group present in 13 had reacted with the B-ring of 6 to form a new benzotropolone-ring.

Compound 44 was biosynthesised from the oxidative coupling of (-)-epicatechin 5 with 23. Negative-polarity LC-APCI-MS yielded the molecular ion of 44 [M-H]$^-$ at $m/z$ 973 and the molecular formula was deduced as C$_{56}$H$_{38}$O$_{21}$. The $^1\text{H}$- and $^{13}\text{C}-\text{NMR}$ spectra resembled those of 43. The $^{13}\text{C}-\text{NMR}$ spectrum exhibited fifty carbon resonances with twenty-seven of these assigned to catechin A- and C-rings. Similarly, the $^1\text{H}-\text{NMR}$ spectrum exhibited three sets of proton resonances corresponding to the 2-, 3-, 4-, 6- and 8-positions of the three catechin moieties. The $^{13}\text{C}-\text{NMR}$ spectrum of 44 also revealed the presence of one ester and two ketone carbonyl resonances in addition to twenty olefinic carbon resonances. Complete structure confirmation was attained via HMBC and HMQC correlations. The NMR data suggested that the galloyl ester group on 23 had reacted with the B-ring of (-)-epicatechin 5 to form a new benzotropolone ring. It was observed however, that 23 did not couple with 6 to form the theadibenzotropolone-type trimer 46 as was expected.
Compound 45 was biosynthesised from the oxidative coupling of \((-\)-epicatechin 5 with 15. Negative-polarity LC-APCI-MS yielded the molecular ion of 45 \([\text{M-H}]^-\) at \(m/z\) 1383 and the molecular formula was provisionally deduced as \(\text{C}_{71}\text{H}_{52}\text{O}_{30}\) after complementary analysis via \(^{13}\text{C}\)-NMR data. The \(^1\text{H}\)-NMR spectrum exhibited four sets of proton resonances corresponding to the 2-, 3-, 4-, 6- and 8-positions of the four catechin moieties as well as three sets of characteristic benzotropolone resonances. The \(^{13}\text{C}\)-NMR spectrum exhibited seventy-one carbon resonances with thirty-six of these assigned to catechin A- and C-rings and the remainder attributed to two ester and three ketone carbonyls in addition to thirty olefinic carbon resonances. The NMR data suggested that the two galloyl ester groups present in 15 had reacted with two equivalents of the B-ring of \((-\)-epicatechin 5 to form two additional benzotropolone-rings. Interestingly, the two isomers of theadibenzo[tropolon]-type were not produced as expected (i.e. the spectroscopic and spectrometric data suggest both galloyl D-rings in 15 had been involved in oxidative coupling). Complete structure confirmation was attained via HMBC and HMQC correlations. However, it should be pointed out that the structure of 45 as shown and published by Sang \textit{et al.}, if biosynthesised from \((-\)-epicatechin 5 and 15 should possess the alternative configuration \((R\)-series\) at position C-3" because 15, a precursor, possesses epi-like stereochemistry at this position.

Black tea was extracted (80% \(\text{Me}_2\text{CO}/\text{H}_2\text{O}\)) and the extract was concentrated \textit{in vacuo}. The residue was dissolved in \(\text{H}_2\text{O}\) and partitioned against \(\text{CHCl}_3\), \(\text{EtOAc}\) and \(n\)-BuOH.
The EtOAc fraction was chromatographed over Sephadex LH-20 (30-60% Me₂CO/H₂O) to give fourteen sub-fractions.

Comparison of the LC-ESI-MS/MS spectra of the pure standards (42-44) with those of the thirteenth sub-fraction of black tea containing the three unknown peaks confirmed the presence of 42 (220 µg kg⁻¹ dry leaf) and 43 (70 µg kg⁻¹ dry leaf) in black tea. Not only did the standards and two of the LC-peaks have the same retention times and molecular masses but their fragmentation ions also corresponded. The third unknown peak could not be characterised as it displayed a retention time different from any of the standards, although the MS/MS spectrum suggested it was an isomer of the known theadibenzotropolone-type compounds.

In an effort to establish the identity of the compound responsible for the uncharacterised LC peak, Sang et al. attempted to biosynthesise the isomers of 42, 43 and 44 by reacting 14 with (-)-epicatechin 5 and 6 in the POD/H₂O₂ system but they were not successful. It was previously observed that no dibenzotropolone-type compounds were produced when 15 was reacted with (-)-epicatechin 5 and that the expected compound 46 was not produced when 23 was reacted with 6. Therefore, without the required standards, the identity of the compound responsible for the third unknown LC peak was not elucidated.

It was hypothesised [70] that the reason for the coupled oxidation reactions proceeding with (-)-epicatechin 5 but not 6 was that the relative redox potential of 6 (79 mV at pH 13.5) is higher than that of (-)-epicatechin 5 (48 mV at pH 13.5) and therefore 6 is not as susceptible to oxidation and coupling as (-)-epicatechin 5 [71].

The presence of 45 in black tea was sought using LC-ESI-MS/MS with SIM but no evidence for its presence in any of the black tea extractions (CHCl₃, EtOAc and n-BuOH) was found.

POD can catalyse the oxidation of o-diphenols in the presence of H₂O₂, but the exact role of POD in black tea manufacturing is still not clear. The results of these model biosynthetic oxidations show that TFs can couple via galloyl ester groups (D-rings) with catechins to form di- and tribenzotropolone-type structures. This type of oxidation
reaction may be important in extending molecular size, leading to TR formation and the presence of the epimers of the four major catechins in the fresh green leaf may add to complicate TR analysis through the inherent production of diastereomers.

1.2.9 Theanaphthoquinone

Theanaphthoquinone 47 has been isolated (Sephadex LH-20) and characterised (1D/2D NMR spectroscopy and negative-polarity FAB-MS) by Tanaka et al. while investigating model enzymatic oxidation reactions of a mixture of (-)-epigallocatechin 4 and (-)-epicatechin 5 [72]. The aim of their work was to yield insight on the oxidative metabolism of flavan-3-ols during tea fermentation and to aid the characterisation of TRs.

During an initial experiment, using a homogenised and filtered aqueous extract of fresh tea leaf as the oxidant matrix, they observed (HPLC-PDA) the accumulation of 12 at the early stages of the biosynthesis and subsequently the production of a previously unknown polyphenol 47 (UV-Vis maximum at 440 nm). Compound 47 was difficult to isolate due to the presence of many minor reaction products. An experiment using banana fruit homogenate as the oxidant matrix yielded 12 and 47 and their chromatographic resolution and isolation was less complex (compared to the former oxidant matrix).

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{O} & \quad \text{OH}
\end{align*}
\]

An aqueous solution (-)-epigallocatechin 4 and (-)-epicatechin 5 was combined with banana fruit homogenate and the mixture stirred for five hours at 20°C. The mixture was poured into Me$_2$CO and filtered. The filtrate was concentrated, extracted with EtOAc and the extracts chromatographed over Sephadex LH-20 with EtOH as eluent to yield 47.

![Image of compound 47]
Compound 47 was isolated as a red amorphous solid. Negative-polarity FAB-MS yielded the pseudo-molecular ion of 47 at \( m/z \) 535. This was two mass units larger than expected and was attributed to the reduction product of 47. \(^1\)H- and \(^{13}\)C-NMR spectra were similar to those of 12 with the two characteristic sets of A-ring and C-ring resonances identified. The \(^{13}\)C-NMR spectrum indicated the presence of two conjugated carbonyl groups at 180.62 and 183.38 ppm and eight \( sp^2 \) carbon atoms, excluding those of the A- and C-rings. HMBC correlations revealed the presence of a 1,2-naphthoquinone moiety. A down-field proton resonance at 12.28 ppm suggested an intermolecular hydrogen bond with one of the carbonyl groups in the 1,2-naphthoquinone moiety.

Condensation of 47 and \( o \)-phenylenediamine, yielded the phenazine derivative of 47 ([M-H]\(^-\) at \( m/z \) 605) to give confirmation of the proposed structure.

It was observed that 12 and 47 were not produced when either (−)-epigallocatechin 4 or (−)-epicatechin 5 were treated separately with banana fruit homogenate, and the hypothesis was that 47 was biosynthesised from 12 with the mediation of PPO (scheme 8). The mechanism includes oxidation of 12 to dehydrotheaflavin intermediate 50 which undergoes rearrangement to yield intermediate VI. Subsequent hydration, and a benzylic acid-type rearrangement, with loss of \( CO_2 \), yields dihydrotheanaphthoquinone 51, which undergoes further oxidation to yield 47. Interestingly, it was found that a mixture of (−)-epigallocatechin 4 and (−)-epicatechin 5 with extracts of apple, potato, sweet potato, persimmon and black mushroom produced 12 while there was no evidence (HPLC-PDA) for the production of 47.
The hypothesis that 47 was generated by oxidation of 12 was of mechanistic interest and during a prolonged experiment using an aqueous extract of fresh tea leaf as the oxidant matrix (flavan-3-ols were removed prior to reaction by washing with Polyclar AT PVP and filtration), a decrease of 12 and 47 was observed with a concurrent increase in polymeric components (HPLC-broad peak). These observations suggested that 47 was a transient species being further transformed during tea fermentation, and was most likely to be implicated in TR formation.

**1.2.10 Bistheaflavins**

Bistheaflavin A 48 and bistheaflavin B 49 have been isolated (Sephadex LH-20 and MCI-gel CHP20P) and characterised (1D/2D NMR spectroscopy, FAB-MS, UV-Vis spectroscopy, polarimetry and elemental analysis) by Tanaka et al. while investigating model enzymatic and auto-oxidation products of 12 [73]. The aim of the study was to gain insight into the reactivity of 12 under model conditions with the hypothesis that 12 may be an intermediate in TR formation.
An aqueous solution of (-)-epigallocatechin 4, and (-)-epicatechin 5, were combined
with banana fruit homogenate and the mixture stirred for five hours at 20 °C. The mixture
was poured into Me₂CO and filtered. The filtrate was concentrated, extracted with EtOAc
and the extracts chromatographed over Sephadex LH-20 with EtOH as eluent to yield 12
and 47. The column was further eluted with 50% aqueous Me₂CO. The fraction obtained
was chromatographed over MCI-gel CHP20P with H₂O containing increasing
proportions of MeOH as eluent to yield 48.

Compound 48 was isolated as a reddish-brown solid. Negative-polarity FAB-MS yielded
the molecular ion of 48 [M-H]⁻ at m/z 1143. The ¹³C-NMR spectrum revealed the
presence of twenty-nine carbon atoms. Eighteen of these were attributed to two sets of A-
and C-ring resonances. However, the molecular mass obtained for 48 suggested the
presence of four flavan-3-ol units and the combined ¹³C-NMR and negative-polarity
FAB-MS data indicated the four flavan-3-ol units had a C₂ axis of symmetry. This was
tentatively confirmed when the proposed structure was assigned via HSQC, HMBC,
COSY and NOESY NMR correlations.

It was hypothesised that 48 was produced via oxidative C-C coupling between two anti-
parallel π-π stacked benzotropolone rings of 12 (scheme 9). To elaborate the chemical
mechanism of POD activity during the formation of TRs, it was attempted to
biosynthesise 48 from 12 with a horseradish POD-H₂O₂ oxidation system or fresh tea leaf
homogenate. Both attempts were unsuccessful.
Tanaka et al. investigated the auto-oxidation of 12 in phosphate buffer (0.1M and pH 7.3) by atmospheric oxygen. The solution was stirred at 25 °C for twelve hours, acidified with 0.1M HCl and applied to a MCI gel CHP20P column. The stationary phase was washed with H₂O and the column subsequently eluted with H₂O containing increasing proportions of MeOH to yield unaltered 12 (based on HPLC UV-Vis maxima) and 49.

Compound 49 was isolated as a reddish-brown solid. UV-Vis measurements (HPLC) yielded absorption maxima at 274 and 378 nm. Negative and positive-polarity FAB-MS yielded the molecular ions of 49 [M-H]⁻ at m/z 1097 and [M+H]^+ at m/z 1099 respectively (corresponding to the sum of the RMM of 12 and 47).

¹H- and ¹H-¹H COSY NMR spectra exhibited four sets of resonances attributed to protons at the 2-, 3-, 4-, 6- and 8-positions of the four flavan nuclei. The ¹³C-NMR spectrum revealed the presence of twenty-one carbon resonances besides those attributed to the four flavan nuclei. These were assigned to two carbonyl, three aliphatic and sixteen
olefinic-type carbon atoms. These data suggested the A- and C-rings of 12 did not undergo any structural changes during the autoxidation. The remainder of the structure was assigned via HMBC and NOESY correlations. However, the absolute configuration of the bicyclooctene moiety was not assigned.

Tanaka et al. proposed that 49 was formed via an intermolecular cyclisation between dehydrotheaflavin intermediate 50 (oxidised theaflavin) and dihydroanaphthoquinone 51. The mechanism (scheme 10) includes a regioselective cyclisation directed via a self-assembled intermolecular quinhydrone-type charge-transfer complex. Compound 50 is the electron-deficient acceptor and 51 is the electron-rich donor with the equilibrium quinhydrone-type complex being electronically represented as \([50:51] \leftrightarrow [50^*:51^+]\) [39]. However, the conformation of the quinhydrone-type assembly proposed in this scheme would presumably incur a relative decrease in stabilisation energy from the loss of one potentially accessible intermolecular hydrogen bond.

![Scheme 10: proposed mechanism for the biosynthesis of 49 (adapted)](image)

The isolation of 48 and 49 by Tanaka et al. demonstrated that 12 was susceptible to oxidative dimerisation via two independent routes. With respect to mechanistic understanding of TR formation, Tanaka et al. demonstrated the plausibility that following
oxidation, the anti-parallel self-associated dimer conformation could be a prerequisite for the directed formation of 48 while the quinhydrone-type complex could be a prerequisite for the directed formation of 49. It was hypothesised that these reaction types may participate in the formation of TRs, leading to similar structural C-C connectivity during oxidative dimerisation.

1.2.11 Dehydrotheaflavins

Dehydrotheaflavin 52 has been isolated (Sephadex LH-20, MCI-gel CHP 20P and Chromatorex ODS) and characterised (polarimetry, FT-IR spectroscopy, UV-Vis spectroscopy, CD spectroscopy, negative-polarity FAB-MS, elemental analysis and 1D/2D NMR spectroscopy) by Tanaka et al. while investigating the oxidation mechanism of green tea polyphenols under model fermentation conditions. In their model fermentation experiments, Tanaka et al. used polyphenol-free tea leaf homogenate as the enzyme matrix. The aim of their work was to investigate further the mechanism of oxidation of TFs prepared in situ [75].

![Chemical structure of 52](image)

An aqueous solution of (-)-epigallocatechin 4 and (-)-epicatechin 5 was treated with fresh leaf homogenate whilst stirring. The homogenate was deemed polyphenol-free by prior treatment with PVP. The resulting fermentation mixture was poured into Me₂CO. The precipitate was filtered off and the filtrate was concentrated and then extracted with EtOAc. The EtOAc extract was chromatographed over Sephadex LH-20 with EtOH as eluent containing increasing ratios of H₂O (0-20%) and finally Me₂CO (50%) to yield four fractions. The second fraction was chromatographed over MCI-gel CHP 20P and Chromatorex ODS with H₂O containing increasing ratios of MeOH (0-80%) to yield non-reacted (-)-epicatechin 5 and 52.
Compound 52 was isolated as a reddish brown amorphous solid. Negative-polarity FAB-MS yielded the molecular ion of 52 [M-H]' at \( m/z \) 577 and combined with elemental analysis and NMR data the molecular formula was provisionally deduced as \( \text{C}_{29}\text{H}_{22}\text{O}_{13} \). UV-Vis absorption maxima (EtOH) for 52 were observed at 360, 296 and 279 nm and the CD spectrum (EtOH) exhibited two positive Cotton effects at 232 and 293 nm and two negative Cotton effects at 279 and 353 nm. The FT-IR spectrum yielded a characteristic benzotropolone carbonyl absorption at 1676 cm\(^{-1}\). The \(^1\text{H}-\) and \(^{13}\text{C}-\)NMR spectra exhibited two sets of resonances attributed to the A- and C-rings of catechins which were similar to those observed in 12. The remaining eleven carbon resonances in the \(^{13}\text{C}-\)NMR spectrum and two methine proton resonances in the \(^1\text{H}-\)NMR spectrum were assigned to a moiety originating from a benzotropolone ring. Additional structure confirmation was attained via HMBC, HMQC and COSY correlations. The stereochemistry of 52 was assigned by NOESY correlations, and combined with the known absolute configurations of (-)-epigallocatechin 4 and (-)-epicatechin 5, the absolute configuration of C-1'' and C-5'' was designated as belonging to R-series.

A mechanism for the formation of 52 was proposed (scheme 11). However, the presence of 52 in black tea was not confirmed.

Scheme 11: proposed mechanism for the biosynthesis of 52 (adapted) [75]
A compound suggested as being dehydrotheaflavin 53, shown below, and its diastereomeric analogue 54 have been isolated (HPLC) and characterised (ESI-MS) from the reaction between excess Fe$^{3+}$ and 12 at pH < 3.0 by O’Coinceanainn et al. [76]. In their work they were interested in gaining insight into the mechanistic interaction of Fe$^{3+}$ and TFs as a method of elucidating the bioavailability and enzyme inhibition action of TFs.

It should be noted that 54 has the same structure and stereochemistry as 52, [75] which O’Coinceanainn et al. propose to be the diastereomer of 54. The structure of 53 was proposed on the basis of mass spectrometry data and available literature [75]. It would however have been difficult for O’Coinceanainn et al. to spectroscopically distinguish between 52, 53 and 54 without the aid of CD spectroscopy or $^1$H-NMR NOESY correlations. The stereochemistry at C-2 in 53 and 54 is presumably cis as this is their configuration in 12. Additional oxidation products of the Fe$^{3+}$-induced oxidation of structural interest was 47 and theabenoquinone 55.

A general scheme for the formation of 47, 53, 54 and 55 was proposed (scheme 12) which involves the initial formation of an Fe$^{3+}$-benzotropolone complex followed by decomposition via different modes of electron transfer.
Scheme 12: proposed scheme for the reaction of 12 and Fe$^{3+}$ (adapted) [76]

It was noted that the major oxidation product of the reaction of Fe$^{3+}$ with 12 was a polymeric polyphenol component, which O'Coinceanain et al. were unable to characterise. It was concluded that TFs, such as 12, are unstable under the oxidative conditions employed and they formed polymeric products of varying molecular masses. The formation of the polymeric components with respect to an increase in time was not accompanied by significant spectral changes and it was concluded that the benzotropolone ring of 12 had been transformed, with no further structural changes, after initial complexation and reaction with Fe$^{3+}$.

1.2.12 (-)-Epigallocatechin gallate dimer

An (-)-epigallocatechin gallate dimer, compound 56 is of structural interest and has been isolated (Sephadex LH-20, MCI-gel CHP 20P, Chromatorex ODS and TSK-gel Toyopearl) and characterised (polarimetry, FT-IR spectroscopy, UV-Vis spectroscopy, CD spectroscopy, negative-polarity FAB-MS, elemental analysis and 1D/2D NMR spectroscopy), by Tanaka et al. while investigating the oxidation mechanism of green tea polyphenols under model fermentation conditions [75]. In their model fermentation experiments, Tanaka et al. used polyphenol-free tea leaf homogenate as the enzyme matrix. The aim of their work was to investigate further, the mechanism of oxidation of TFs prepared in situ.
An aqueous solution of (-)-epigallocatechin gallate 1 and (-)-epicatechin 5 was treated with fresh leaf homogenate whilst stirring. The homogenate was deemed polyphenol-free by prior treatment with PVP. The resulting fermentation mixture was poured into Me$_2$CO. The filtrate was concentrated and then extracted with EtOAc and $n$-BuOH. The $n$-BuOH extract was chromatographed over Sephadex LH-20 with EtOH as eluent containing increasing ratios of H$_2$O and finally Me$_2$CO (50%). The phenolic fraction was chromatographed over MCI-gel CHP 20P (H$_2$O/MeOH), Chromatorex ODS (H$_2$O/MeOH) and TSK-gel Toyopearl HW-40F to yield non-reacted (-)-epigallocatechin gallate 1 and 56.

Compound 56 was isolated as a brown amorphous solid. Negative-polarity FAB-MS yielded the molecular ion of 56 [M-H]$^-$ at $m/z$ 885. The UV-Vis absorption maximum (EtOH) for 56 was observed at 275 nm and the CD spectrum (EtOH) exhibited a positive Cotton effect at 280 nm and a negative Cotton effect at 231 nm. The FT-IR spectrum (KBr) yielded absorption bands at 3300, 1753, 1707, 1628 and 1615 cm$^{-1}$. The $^1$H-NMR spectrum exhibited two singlet resonances due to gallate ester groups and two sets of resonances attributed to the A- and C-rings of catechins. The $^{13}$C-NMR spectrum confirmed that these gallate groups were located at C-3 and C-3' on the catechin A-rings. Additional structural NMR data, attained via HMBC and NOESY correlations, revealed the presence of the tricyclo[5.2.2.0]undec-4-en-3,8,11-trione moiety. The existence of the cyclopentenone ring was also supported by FT-IR data which exhibited a characteristic
carbonyl absorption band at 1753 cm\(^{-1}\) and this was distinguishable from the ester carbonyl absorption band (1707 cm\(^{-1}\)).

The absolute configuration of the tricycloudecacentrine moiety was suggested to be S-series at C-8" based on NOESY NMR data. These data confirm the hypothesis that 56 was formed from dimerisation of \((-\)-epigallocatechin gallate 1 with the respective catechin C-ring, C-2 and C-2' positions belonging to the R-series.

A mechanism for the formation of 56 was proposed which includes hydration of energetically unfavourable \(\sigma\)-quinone tautomer III followed by ring contraction, decarboxylation, oxidation and intermolecular cycloaddition to finally yield 56 (scheme 13).

![Scheme 13: proposed mechanism for the biosynthesis of 56 (adapted)](image_url)

Interestingly, besides the formation of compounds 52 and 56, the respective by-products of these generic biosynthetic reactions included, in the case of the mixture of \((-\)-epigallocatechin 4 and \((-\)-epicatechin 5, following EtOAc extraction: 12, 47, dehydrotheasinensin 57, desgalloyloolongtheanin 58, theasinensin C 59 and theasinensin E 60 [75]. Ambiguity exists with the structural designation of 57 as Tanaka et al. present the same structure as that of 57, and term it dehydrotheasinensin E, in a later publication [77].
Similarly, compounds of structural interest isolated from the reaction of (−)-epigallocatechin gallate 1 and (−)-epicatechin 5 following EtOAc extraction were 13, 29, galloyloolongtheanin 61, theasinensin A 62, theasinensin D 63 and (−)-epigallocatechin gallate quinone dimer A 64.

The results of these model fermentation experiments indicate that B-ring enzymatic oxidation of catechins such as (−)-epigallocatechin gallate 1, (−)-epigallocatechin 4 and (−)-epicatechin 5 is an important mechanistic step in tea polyphenol chemistry leading to, but not limited to, oxidative dimerisation resulting in compounds with large structural variations. However, the presence of 56 in black tea was not confirmed.
1.2.13 Theasinensins

Compounds possessing one or more biphenyl-2,2',3,3',4,4'-hexaol moieties (R_2C_{12}H_8O_6).

\[
\begin{align*}
&\text{R} = \text{A- and C-rings of catechins} \\
\end{align*}
\]

Theasinensins are a class of flavan-3-ol dimers with two flavan units linked 2-2' (B-rings), forming a biphenyl function. As in the case of theacitrins, theasinensins are composed of structures that are obtained via dimerisation of two epigallocatechin-type derivatives (i.e. (−)-epigallocatechin gallate 1 or (−)-epigallocatechin 4). Besides their occurrence in black tea, theasinensins have been found to occur in the fresh green tea leaf [78] and in semi-fermented oolong tea [79].

One significant outcome following the formation of the biphenyl function from two pyrogallol-type catechin B-rings is the chirality induced from hindered rotation about the biphenyl C-C bond due to steric congestion. This characteristic structural variation among theasinensins is termed atropisomerism (conformational isomerism that among other factors is temperature dependant).

For example, 62 and 63 are atropisomers and are termed theasinensins A and D respectively. Their existence was first reported by Roberts and Myers who isolated three model enzymatic-oxidation products from mixtures of (−)-epigallocatechin gallate 1 and (−)-epigallocatechin 4 using PC and suggested them to be bisflavanoids [80]. This suggestion was confirmed by later work [81] and final structural elucidation was carried out by the group of Nishioka [53,79,82,83].
At first inspection, the biosynthesis of the theasinensins seems to involve a straightforward nucleophilic addition reaction onto o-quinone III. However, mechanistic studies by the group of Tanaka reveal a much more complex series of reactions for their biosynthesis; these will be outlined in the following section.

1.2.14 (-)-Epigallocatechin gallate quinone dimer A and dehydrotheasinensin A

(-)-Epigallocatechin gallate quinone dimer A 64, dehydrotheasinensin A 65 (a quinone acetal) and a gallated flavan elimination product, compound 66, have been biosynthesised (Japanese pear fruit homogenate), isolated (MCI-gel CHP20P and Sephadex LH-20) and characterised (polarimetry, positive-polarity FAB-MS, elemental analysis, reversed-phase HPLC and 1D/2D NMR spectroscopy) by Tanaka et al. while investigating the mechanism of theasinensin production during black tea manufacturing [84]. The focus of
the investigation was based on the hypothesis that hydrated unstable theasinsin o-quinones are converted via heat-activated oxidation-reduction dismutation (the compound is simultaneously oxidised and reduced to yield two new compounds) to yield theasinsins during the enzyme deactivation and drying stage [85]. The model fermentation system utilised (−)-epigallocatechin gallate 1 as substrate. Interestingly, compound 66 was also isolated from black tea (MCI-gel CHP20P, Chromatorex ODS, Sephadex LH-20 and TSK-gel Toyopearl HW-40F).

An aqueous solution of (−)-epigallocatechin gallate 1 was treated with Japanese pear fruit homogenate. The mixture was stirred for two hours at room temperature and the resulting fermentation mixture was filtered and the filtrate chromatographed over MCI-gel CHP20P with 0.1% TFA as eluent containing increasing ratios of MeOH (20-30%) to yield 65. The fractions containing 65 were concentrated in vacuo and further chromatographed over MCI-gel CHP20P with 0.1% TFA as eluent containing increasing ratios of MeOH (30-40%) to yield a fraction containing 64. This fraction was chromatographed over Sephadex LH-20 with 80% MeOH to yield 64 and 66.

A scheme for the formation of 64 and 65 was proposed where these compounds are produced as major products as a result of stereoselective dimerisation of the o-quinone of
(-)-epigallocatechin gallate I, II (scheme 14). However, a mechanism for the formation of 66 based on an elimination reaction was only put forward in later work by Tanaka et al. [86].

Scheme 14: proposed scheme for the biosynthesis of 64 and 65 (adapted) [77]

Compound 64 was identified by comparison of its $^1$H-, $^{13}$C-NMR data and COSY, HSQC and HMBC correlations to those in the literature, as 64 had formerly been synthesised by Valcic et al. by treatment of (-)-epigallocatechin gallate 1 with 2,2'-azobis(2,4-dimethylvaleronitrile) in MeCN, via a proposed radical-coupling mechanism [87].

A more detailed mechanism for the formation of compound 64 (scheme 15) from (-)-epigallocatechin gallate 1 was proposed that includes a nucleophilic addition between two o-quinones II to produce a tricyclic substructure containing two sterically hindered carbonyl groups. Hydration subsequently releases the steric strain with the ensuing formation of the ether bridge [75]. The stereochemistry of 64 is not provided in the literature.
Compound 65 was isolated as an off-white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 65 [M+H]^+ at m/z 931 and combined with elemental analysis and $^{13}$C-NMR data the molecular formula was deduced as C$_{44}$H$_{34}$O$_{23}$. The UV-Vis absorption maximum (EtOH) for 65 was observed at 276 nm. The FT-IR spectrum yielded absorption bands at 3365, 1696, 1610, 1518 and 1456 cm$^{-1}$. Interestingly, 65 was observed as a broad peak during reversed-phase HPLC analysis and this was most likely a result of the equilibrium between hydrated quinone isomers.

The $^1$H- and $^{13}$C-NMR spectra were similarly indicative of the proposed hydrated quinone equilibrium as they exhibited complex resonances, however, signals arising from a dominant isomer (>80%) were used in the spectral interpretation. Two sets of A- and C-ring resonances and two gallate ester resonances were observed which were similar to those observed for ($\sim$)-epigallocatechin gallate 1. The remaining twelve carbon resonances in the $^{13}$C-NMR spectrum were attributed to an aliphatic methine, two olefinic, six aromatic, a carbonyl and two acetal carbon atoms, these constituted the catechin B-rings. Additional structure confirmation was attained via HMBC and COSY correlations. The absolute configuration at the C-2' methine carbon atom was determined as belonging to the $S$-series as chemical reduction of 65 with a mild reducing agent such as dithiothreitol yielded 62, which contains a biphenyl bond belonging to the $R$-series.

The structure of 65 incorporates an unusual hydrate moiety at the bridge of the [2.2.1] oxo-bicycloheptane substructure. Due to the energetic preference of a carbonyl group over a hydrate, this occurrence can only be rationalised by assuming stabilisation of the
hydrate functionality through intramolecular hydrogen-bonding. Molecular modelling at the MM2 force-field level suggests that this particular hydrate is stabilised by two hydrogen bonds, one between the bridge hydrate (HO-C-OH) and the hemiacetal oxygen atom in the bicyclic structure and the other between the bridge hydrate and the B-ring galloatechin-type carbonyl oxygen atom [88].

A more detailed mechanism for the formation of compound 65 from (-)-epigallocatechin gallate 1 was proposed (scheme 16) that involves the 1,4-addition of the nucleophilic carbon atom of II onto the unsaturated carbonyl (β-position) of another molecule of II [84].

Scheme 16: proposed mechanism for the biosynthesis of 65 (adapted) [84]

An aqueous solution of 65 was unstable upon heating at 80 °C for fifteen minutes and reversed-phase HPLC analysis yielded evidence for the presence of oxidation-reduction dismutation products, 61 and 63. However, 62 was not detected and this observation indicated that isomerisation occurred prior to the oxidation-reduction dismutation reaction.

Interestingly, unstable intermediate 65 was also shown, via reversed-phase HPLC, to decompose in neutral phosphate buffer (pH 6.8) at 20 °C after fifteen hours via oxidation-reduction dismutation into compounds 61, 62, 63 and a precursor to oxidation product 67. The precursor to 67 was unstable and was converted to 67 during chromatographic isolation (MCI-gel CHP20P and Chromatorex ODS).
Compound 67 was isolated as an off-white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 67 [M+H]^+ at m/z 931 and combined with elemental analysis and ^13^C-NMR data the molecular formula was deduced as C_{44}H_{34}O_{23}. The UV-Vis absorption maximum (EtOH) for 67 was observed at 277 nm. The FT-IR spectrum yielded absorption bands at 3367, 1737, 1697 and 1607 cm\(^{-1}\).

The ^1H- and ^13^C-NMR spectra exhibited two sets of resonances attributed to the A- and C-rings of gallated catechins which were similar to those observed for 65. From analysis of ^13^C-NMR data, the remaining carbon resonances were attributed to a pyrogallol-type B-ring and a modified B-ring containing two carbonyl carbons, two methine, one oxygenated quaternary carbon and one methylene carbon atom. Additional structure confirmation was attained via HMBC and NOESY correlations.

The absolute configuration of the 8-lactone ring was assigned on the basis that the configuration of C-3' was retained during the reaction. Characterisation of the unstable precursor is considered to be important in understanding the mechanism of the dismutation of 65. Additionally, the formation of 65 was shown to occur by autoxidation of (-)-epigallocatechin gallate 1 in a phosphate buffer (pH 7.4) and due to its instability was only detected in the initial stages of the autoxidation. This observation indicated that the formation of 62 and 63 from 65 during black tea manufacturing was a spontaneous process rather than an enzymatic process, occurring when the leaves are heated and dried during the enzyme deactivation stage.
Compound 66 (see page 60) was isolated as an off-white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 66 \([M+H]^+\) at \(m/z\) 351. The UV-Vis absorption maximum (EtOH) for 66 was observed at 277 nm. The FT-IR spectrum yielded absorption bands at 3389, 1694 and 1632 cm\(^{-1}\). Interestingly, compound 66 was observed as a broad peak during reversed-phase HPLC analysis and \(^1\)H- and \(^13\)C-NMR resonances were observed in duplicate. These observations were attributed to equilibrium between epimers. In the \(^1\)H-NMR spectrum the galloyl group at C-3 was evident from chemical shift data. Additional structure confirmation was attained via COSY, HSQC and HMBC correlations.

Black tea was extracted six times with boiling H\(_2\)O and the extract concentrated to 50% volume. The aqueous solution was partitioned against CHCl\(_3\) to remove caffeine and then successively against EtOAc and n-BuOH to yield two respective fractions. The n-BuOH fraction was chromatographed over Sephadex LH-20 with H\(_2\)O as eluent containing increasing ratios of MeOH to yield eleven fractions. The seventh least polar fraction was further chromatographed over MCI-gel CHP20P (H\(_2\)O-MeOH), Chromatorex ODS (H\(_2\)O-MeOH), Sephadex LH-20 (EtOH) and TSK-gel Toyopearl HW-40F (H\(_2\)O-MeOH) to yield 66.

Heating an aqueous solution of 64 at 100 °C yielded 66 along with (-)-epigallocatechin gallate 1 and this suggested that 66 could be a product of oxidation-reduction dismutation of II that is formed during black tea manufacturing.

### 1.2.15 Dehydrotheasinensin AQ

Dehydrotheasinensin AQ 68, a theasinensin-type intermediate containing a diketone moiety, has been biosynthesised (Japanese pear fruit homogenate), isolated (MCI-gel CHP20P, Sephadex LH-20 and Chromatorex ODS), characterised (polarimetry, positive-polarity FAB-MS, UV-Vis spectroscopy, FT-IR spectroscopy, elemental analysis and 1D/2D NMR spectroscopy) and its presence in black tea confirmed (reversed-phase HPLC) by Tanaka et al. while investigating oxidation-reduction dismutation chemistry of pyrogallol-type quinone intermediates during black tea manufacturing [77]. The model fermentation system utilised (-)-epigallocatechin gallate 1 as substrate and the mixture
was chemically reduced after oxidation (2-mercaptoethanol) to yield, besides 68, (−)-epigallocatechin gallate quinone dimer B 69 and a theasinensin-type trimer of (−)-epigallocatechin gallate 1, compound 70. An initial reversed-phase HPLC investigation of the fermentation mixture produced evidence for the presence of (−)-epigallocatechin gallate 1, 64, 65 and 69. After chemical reduction it was shown that 65 was completely converted to 62. The reduction mixture was chromatographed over MCI-gel CHP20P, Sephadex LH-20 and Chromatorex ODS to yield unreacted (−)-epigallocatechin gallate 1, 24, 62, 64, 68, 69 and 70. The production of 24 is an interesting observation as this can be rationalised as being produced from (−)-epigallocatechin gallate 1 and the decarboxylation derivative of gallic acid 29, compound 28. Compound 29 is presumably produced from the acid catalysed hydrolysis of (−)-epigallocatechin gallate 1.

It is hypothesised that as a consequence of oxidation-reduction dismutation of tea polyphenols many chromatographically unresolved minor compounds may be produced and these ‘by-products’ inherently make black tea components difficult to characterise structurally. Therefore, as an aid to structure elucidation and to determine the role of a compound in a mechanistic pathway, chemical reduction of unstable oxidation intermediates during in vitro processes allows classes of compounds, that would otherwise most likely be structurally transient and ultimately incorporated in the TRs, to be isolated and characterised.
An aqueous solution of (-)-epigallocatechin gallate 1 was treated with Japanese pear fruit homogenate as this homogenate was previously shown to produce 12 from a mixture of (-)-epigallocatechin 4 and (-)-epicatechin 5 with ease [89]. The mixture was stirred for three hours and the resulting fermentation mixture was poured into 8% 2-mercaptoethanol in EtOH at -20 °C. After stirring at RT for eighteen hours, the precipitates were filtered off and the filtrate was concentrated in vacuo. The aqueous solution was chromatographed over MCI-gel CHP20P with H2O as eluent containing increasing ratios of MeOH (0-80%) to yield two FeCl3-positive fractions. The first relatively more polar fraction was chromatographed over Sephadex LH-20 with H2O containing increasing ratios of MeOH (0-100%) to yield seven fractions. The sixth least polar fraction was chromatographed over MCI-gel CHP20P with H2O as eluent containing increasing ratios of MeOH (20-60%) to yield 68.

Compound 68 was isolated as a yellow amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 68 [M+H]+ at m/z 913 and combined with elemental analysis and 13C-NMR data the molecular formula was deduced as C44H32O22. UV-Vis absorption maxima for 68 were observed at 447 and 277 nm. The FT-IR spectrum yielded absorption bands at 3355, 1698, 1686, 1604 and 1521 cm⁻¹.

The 1H- and 13C-NMR spectra exhibited two sets of resonances attributed to the A- and C-rings of catechins and the 1H coupling patterns were similar to those observed in (-)-epigallocatechin gallate 1. The presence of two gallate ester groups was also established and the large downfield shift of the C-ring H-3 and H-3'' protons confirmed that these were at C-3 and C-3'' respectively. Confirmation for the planar structure of 68 was attained via HMBC correlations. The presence of the diketone moiety was confirmed by the condensation reaction of 68 with o-phenylenediamine to yield the quinoxaline derivative. The stereochemistry of C-1' was designated as belonging to the R-series by analysis of 1H-NMR chemical shift data.

Black tea leaves were extracted with 70% Me2CO. The organic solvent was removed in vacuo and the aqueous solution successively partitioned against Et2O, CHCl3 and EtOAc. The EtOAc layer was concentrated in vacuo and chromatographed over Sephadex LH-20
with 80-100% MeOH and then 50% Me₂CO to yield fractions mainly containing TFs. Reversed-phase HPLC analysis of one of these fractions showed a peak with same retention time as 68 and its UV-vis spectrum was identical to that of 68 (absorption maxima at 277 and 447 nm) providing evidence for the existence of 68 in black tea.

The structure of 68 is closely related to that of 65 and it was suggested that 68 was most likely produced by intramolecular addition of the phenolic hydroxyl group at C-3‴ to the conjugated double bond of 65. The R-series configuration of C-1‴ of 68 is consistent with that of 65 and of the atropisomer of its reduction product, compound 62. From a steric congestion point of view, however, it seems unlikely for C=C conjugation to occur adjacent to the biphenyl axis (tautomer VII) and the most dominant tautomer would most likely be IX with tautomer VIII closely resembling the relative instability of tautomer III (Scheme 17).

Scheme 17: proposed equilibrium tautomers of compound 68

It was also noted that 68 was structurally related to 57. Compound 57 has been previously characterised and found to be formed via oxidative self-dimerisation of (-)-epigallocatechin 4 [89].

A sub-fraction of the fourth least polar fraction (of the seven) obtained from the pear fruit homogenate-catalysed bioproducts from (-)-epigallocatechin gallate 1 was chromatographed over MCI-gel CHP20P with EtOH and then Me₂CO (50%) to yield two fractions. The Me₂CO (50%) fraction was chromatographed over MCI-gel CHP20P with 0-50% MeOH to yield 62, 64 and a quinone dimer with an interesting cage-like structure, compound 69.
Compound 69 was isolated as a white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 69 [M+H]^+ at m/z 931 and positive-polarity MALDI-TOF-MS yielded a pseudo molecular ion of 69 [M+Na]^+ at m/z 953. Combined with elemental analysis, the molecular formula was deduced as C_{44}H_{34}O_{23}. The UV-Vis absorption maximum for 69 (EtOH) was observed at 278 nm. The FT-IR spectrum yielded absorption bands at 3405, 1770, 1683, 1608, 1519 and 1465 cm^{-1}.

The ^{1}H- and ^{13}C-NMR spectra exhibited two sets of resonances attributed to the A- and C-rings of gallated catechins. From ^{13}C-NMR data the remaining twelve carbon resonances were attributed to two carbonyl, two olefinic, three aliphatic methine, one acetal and four oxygenated quarternary carbon atoms. These data supported the designation of 69 being a dimer of (−)-epigallocatechin gallate 1. Additional structure confirmation was attained via HMBC and NOESY correlations. However, the absolute configuration of 69 was not determined.

The structure of 69 is formally related to 64 and a mechanism was proposed for the formation of 69 from (−)-epigallocatechin gallate 1 whereby dimerisation occurs through two successive aldol reactions between the extended enol moiety in o-quinone II and the o-quinone carbonyl functionality in energetically unfavourable tautomer III. Following formation of two new C-C bonds in intermediate X, a 6-exo-trig cyclisation initiated by nucleophilic attack of hydroxide ion onto one of the carbonyls leads to enolate intermediate XI, which is again followed by a 5-exo-trig cyclisation via an aldol reaction,
ultimately leading to formation of the cage-like structure (scheme 18) [77]. It is worthwhile stating that the chemical synthesis of 69 would require two equivalents of hydroxide ion as nucleophile.

Scheme 18: proposed mechanism for the biosynthesis of 69 (adapted) [77]

The fifth least polar fraction (of the seven) obtained from the pear fruit homogenate-catalysed bioproducts from (-)-epigallocatechin gallate 1 was chromatographed over Sephadex LH-20 with 0-20% H₂O in EtOH to yield 62, a theasinensin-type trimer of (-)-epigallocatechin gallate 1, compound 70 and isomers of compound 70. This fraction was further chromatographed over Chromatorex ODS (0-40% MeOH) and Sephadex LH-20 (80-100% MeOH) and then MeOH:H₂O:Me₂CO (90:5:5 v/v/v) to yield crude 70. Compound 70 was further chromatographed over MCI-gel CHP20P (0-40% MeOH) to yield 70 in high purity.
Compound 70 was isolated as a white amorphous powder. Positive-polarity MALDI-TOF-MS yielded a pseudo molecular ion of 69 [M+Na]^+ at m/z 1393, while positive-polarity FAB-MS yielded the molecular ion of 70 [M+H]^+ at m/z 1371 and negative-polarity FAB-MS yielded the molecular ion of 70 [M-H]^− at m/z 1369. This observation indicated that 70 was a trimer of (-)-epigallocatechin gallate 1 (458 amu x 3) = 1370 amu. High-resolution positive-polarity FAB-MS yielded the molecular ion of 70 [M+H]^+ at m/z 1371.2312 and the molecular formula was deduced as C_{66}H_{50}O_{33}. The UV-Vis absorption maximum for 70 (EtOH) was observed at 275 nm.

The $^1$H- and $^{13}$C-NMR spectra were related to those of 62 and 63 but three sets of A- and C-ring resonances and two gallate ester resonances were observed. $^1$H-NMR data revealed four one-proton aromatic singlet resonances as well as two two-proton singlets attributed to gallate ester groups. These data suggested that a B-ring C-2 was covalently bonded to the C-2 of a D-ring (gallate ester) theasinensin-type compound.
The absolute configuration of the B-B'-ring biphenyl bond in 70 was suggested to be the R-series on the basis that 62, which also contains a biphenyl bond, was stereo-selectively produced by chemical reduction of 65. Additional confirmation for the designated absolute configuration was that the presence of atropisomer 63 was not detected after chemical reduction. It was presumed that 70 was produced by reduction of XIII, which was formed via oxidative coupling of quinones, XII and II. A coupling scheme was proposed for the biosynthesis of 70 (scheme 19) and is analogous to the scheme proposed for the biosynthesis of 62 and 63 from 65 [84].

Scheme 19: proposed coupling scheme for the biosynthesis of 70 (adapted) [77]

Evidence for the production of 70 suggests that besides B-ring quinones, galloyl quinones couple intermolecularly to yield minor polyphenols such as theasinensin-type trimers.

As a further elaboration of the oxidation-reduction dismutation chemistry forming for example theasinensins 62 and 63 from intermediate 65, the group of Tanaka observed similar intermediates from the oxidative dimerisation of (−)-epigallocatechin 4.
1.2.16 Dehydrotheasimensin C and proepitheflagallin

Dehydrotheasimensin C 71 (the desgalloyl analogue of 65) and proepitheflagallin 72 have been biosynthesised, isolated (MCI-gel CHP20P) and characterised (positive-polarity FAB-MS, elemental analysis and 1D/2D NMR spectroscopy) by Matsuo et al. (Tanaka group), while investigating pyrogallol-type catechin oxidation, but with the aim of limiting production of minor oxidative-coupling products by utilising the pyrogallol-type B-ring of (−)-epigallocatechin 4 as substrate as opposed to the pyrogallol-type B- and D-rings present in (−)-epigallocatechin gallate 1 [86].

![Chemical Structures](image)

An aqueous solution of (−)-epigallocatechin 4 was treated with Japanese pear fruit homogenate whilst stirring at RT for ninety minutes. The mixture was cooled and acidified with TFA. The precipitates were filtered off and the filtrate was chromatographed over MCI-gel CHP20P with 0-50% MeOH in 0.1% TFA (stabilising additive) to yield a fraction containing 71. The remaining fractions were chromatographed over Chromatorex ODS (H₂O/MeOH) to yield 72 and the desgalloyl analogue of 64, compound 73.

Compound 71 was isolated as a white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 71 [M+H]⁺ at m/z 627. The UV-Vis absorption maximum for 71 (MeOH) was observed at 269 nm and the FT-IR spectrum yielded absorption bands at 3365, 2920, 1696, 1627 and 1606 cm⁻¹.
The $^1$H- and $^{13}$C-NMR spectra were similar to those of 65. The $^{13}$C-NMR spectrum exhibited carbon resonances attributed to a conjugated ketone, a benzyl methine, a trisubstituted double bond and also two hemiacetal carbon atoms, indicating the presence of the hydrated form of the cyclohexenetrione moiety. When 71 was hydrogenated with dithiothreitol it was converted to 59 which contains an R-series biphenyl bond, providing evidence that the stereochemistry at the benzyl methine carbon (C-2') of 71 was S-series.

Compound 72 was isolated as a white amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 72 [M+H]$^+$ at $m/z$ 625 and combined with elemental analysis the molecular formula was deduced as C$_{30}$H$_{24}$O$_{15}$. The UV-Vis absorption maximum for 72 (MeOH) was observed at 271 nm and the FT-IR spectrum yielded absorption bands at 3403, 1706, 1631, 1519 and 1468 cm$^{-1}$.

The $^1$H- and $^{13}$C-NMR spectra exhibited two sets of resonances attributed to the A- and C-rings of non-gallated catechins. From $^{13}$C-NMR data the remaining carbon resonances were attributed to an acetal, a methylene, a methine, a quaternary carbon, two conjugated carbonyl carbon atoms, four olefinic and a carboxyl carbon atom. The presence of the carboxyl group was also supported by positive-polarity FAB-MS with an ion signal [M-CO$_2$+H]$^+$ at $m/z$ 581. However, the $^{13}$C-NMR spectrum only exhibited twenty-nine of the expected thirty carbon resonances and this observation was attributed to possible line broadening from keto-enol tautomerism or hydration. Additional structure confirmation was attained via HMBC and COSY correlations.

Interestingly, unstable intermediate 71 (the desgalloyl analogue of 65) was shown to decompose in neutral phosphate buffer via oxidation-reduction dismutation into compounds 58, 59 and 60. The unusual dimeric compoundoolongtheanin 74 (C-3 gallate ester analogue of 58) had been previously isolated from oolong tea by the group of Nishioka [79].
Compound 57 was also present in the decomposition mixture, but was proposed to be formed via isomerisation at C-2' in 71 followed by intramolecular acetal formation (scheme 20) [86]. Compounds 71 and 73 were shown to be dimerisation products of II by conversion into their phenazine derivatives via addition of o-phenylenediamine to the fermentation mixture. The phenazine derivatives of 71 and 73 were the major products while that of II was a minor product, indicating that stereoselective dimerisation of II was probably the most important oxidation route of (-)-epigallocatechin 4. It should be noted that the stereochemistry of 73 and 74 is not provided in the literature.
Scheme 20: proposed dimerisation and dismutation pathway of 4 (adapted) [86]

Heat-labile intermediate 72 was expected to be a precursor of minor black tea pigments and therefore Matsuo et al. heated an aqueous solution of 72 at 80 °C for ten minutes (heating occurs during enzyme deactivation in the final stages of black tea manufacturing) and the solution was then analysed by reversed-phase HPLC for evidence of minor pigment production. PDA detection revealed the presence of 25 and hydroxytheaflavin 75. A scale-up experiment allowed 25 and 75 to be isolated. Compound 25 was identified as such by comparison of its $^1$H- and $^{13}$C-NMR, UV-Vis and MS spectra with an authentic standard.

Compound 75 was isolated as a red amorphous powder. High-resolution positive-polarity FAB-MS yielded the molecular ion of 75 [M+H]$^+$ at m/z 581.1294. The molecular formula was therefore deduced as C$_{29}$H$_{25}$O$_{13}$. UV-Vis absorption maxima for 75 (EtOH)
were observed at 286, 309, 378 and 428 nm, similar to those observed for 25. The FT-IR spectrum yielded absorption bands at 3366, 1627, 1604, 1517, 1467 and 1430 cm⁻¹.

The ¹H- and ¹³C-NMR spectra were also similar to those of 25, exhibiting characteristic trihydroxybenzotropolone resonances. Additionally, two sets of resonances attributed to the A- and C-rings of catechins were observed with the ¹³C-NMR data distinguishing between 25 and 75 by an extra set of A- and C-rings present in 75, extending from the benzene ring of the benzotropolone moiety. Additional structure confirmation was attained via HMBC correlations.

The production of 25 and 75 from unstable precursor 72 is of mechanistic interest as most benzotropolone ring formations described so far occur via oxidative dimerisation between a catechol-type and pyrogallol-type hydroxy-substituted catechin B-ring. However, theaflagallins and compound 75 are unique in so far as they are produced via oxidative dimerisation between two pyrogallol-type hydroxy-substituted catechin B-rings. It is thought that theaflagallins such as 24 and 25 are produced via oxidative dimerisation between the respective catechin B-ring and 29 via decarboxylation [53,90]. The fact that 25 was produced solely from (−)-epigallocatechin 4 without any gallated derivative present is mechanistically intriguing and a mechanism for the formation of 25 and 75 was proposed (scheme 21) [86]. The mechanism includes dimerisation, decarboxylation and key intermediate XIV undergoing a flavan C-ring 1,2-migration to yield 75 or undergoing a flavan C-ring elimination to yield 25 and flavan elimination product 76, the desgalloyl analogue of compound 66 which has previously been found in black tea [84].
Scheme 21: proposed mechanism for the formation of 25 and 75 (adapted) [86]

1.2.17 Theogallinin, theaflavonin and desgalloyltheaflavonin

Theogallinin 77, theaflavonin 78 and desgalloyltheaflavonin 79 have been isolated from commercial black tea (Sephadex LH-20, MCI-gel CHP 20P and Bondapak Porasil B) and characterised (polarimetry, positive-polarity FAB-MS, elemental analysis, UV-Vis, CD spectroscopy, $^1$H- and $^{13}$C-NMR spectroscopy) by Hashimoto et al., while investigating the chemical composition of black tea [90]. Hashimoto et al. were specifically interested in products of pyrogallol-pyrogallol-type oxidative coupling. However, the terminology applied in naming these three compounds has been used ambiguously [91].
Black tea was extracted with 80% aqueous Me₂CO and after removal of Me₂CO under reduced pressure the remaining aqueous solution was extracted with Et₂O. The aqueous layer was chromatographed over Sephadex LH-20 (H₂O with increasing proportions of MeOH) to yield five fractions. The second fraction was further chromatographed over Sephadex LH-20 with EtOH as eluent. Purification of the first-eluted compound by further chromatography over Sephadex LH-20 with 80% aqueous Me₂CO yielded 78. The remainder of the second fraction was further chromatographed over MCI-gel CHP 20P and Bondapak Porasil B with H₂O containing increasing proportions of MeOH to yield 77 and 79.

Compound 77 was isolated as a pale brown amorphous powder. Positive-polarity FAB-MS yielded the molecular ion of 77 [M+H]⁺ at m/z 801. The CD spectrum exhibited negative Cotton effects at 228 and 275 nm and a positive Cotton effect at 245 nm.

¹H-NMR data of 77 exhibited characteristic C-ring resonances; however, a small coupling constant for the H-2 resonance and a low-field shift of the H-3 resonance indicated the presence of a 3-O-acylated cis-2,3 flavan-3-ol group. Additionally, the ¹H-NMR spectrum exhibited resonances attributed to three oxygen-bearing methine protons and two methylene protons and their chemical shifts and coupling constants were closely
correlated to those of theogallin 92. Analyses of the aromatic region of the spectrum indicated the presence of a gallate ester group and two di-substituted B-rings. The $^{13}$C-NMR spectrum indicated 77 contained thirty-six carbon atoms, the same amount present in (-)-epigallocatechin gallate 1 and 92 in total. The $^{13}$C-NMR spectrum of 77 was also similar to the combined $^{13}$C-NMR spectra of (-)-epigallocatechin gallate 1 and 92 except for the chemical shifts of the flavan-3-ol C-2'/C-6' and galloyl C-2/C-6 signals. These data suggested that 77 contained moieties present in (-)-epigallocatechin gallate 1 and 92, connected via a C-C bond at the B-ring of (-)-epigallocatechin gallate 1 and the galloyl group of 92.

Hydrolysis of 77 with tannase yielded 29, and the desgalloyl derivative of 77. The $^1$H-NMR spectrum of the desgalloyl derivative of 77, exhibited resonances similar to those of the combined spectra of (-)-epigallocatechin 4, and 92 (excluding the above mentioned substituted B-ring and gallate ester resonances).

Complementary confirmation for the proposed structure of 77 was gained via chemical synthesis of the desgalloyl derivative of 77 by treating (-)-epigallocatechin 4 and 92 with $K_3Fe(CN)_6$ in a weakly alkaline medium. The products isolated from the reaction were 59 and the desgalloyl derivative of 77. This was a justified indication that the asymmetric centres in the flavan and quinic acid moieties present in 77 possessed the same absolute configurations as those in 59 and 92. The atropisomerism of the biphenyl bond was determined by comparison of the CD spectrum of the desgalloyl derivative of 77 with those of 59 (R-series) and 60 (S-series). The sign of the Cotton effects for the desgalloyl derivative of 59 and 77 were similar suggesting the chirality of the biphenyl bond was R-series. Hashimoto et al. hypothesised that 77 was produced from the oxidative coupling of (-)-epigallocatechin gallate 1 and 92, linked via their pyrogallol-type B-rings. The absence, in black tea, of the diastereomer with the S-biphenyl configuration suggested that formation of 77 proceeded stereospecifically during black tea processing.

Compound 78 was isolated as yellow-brown amorphous solid. Positive-polarity FAB-MS yielded the molecular ion of 78 [M+H]$^+$ at $m/z$ 937. Its UV-Vis spectrum yielded absorption maxima at 253, 260, 301 and 345 nm (MeOH). The CD spectrum exhibited
negative Cotton effects at 222 and 276 nm and a positive Cotton effect at 253 nm (MeOH).

A chemical test (anisaldehyde-sulfuric acid reagent) indicated the presence of a catechin moiety. The $^1$H-NMR spectrum of 78 exhibited catechin C-ring signals whose chemical shifts and coupling patterns were similar to (-)-epigallocatechin gallate 1. $^1$H-NMR data also revealed meta-coupled A-ring and a two-proton galloyl singlet signals. Furthermore, an additional set of meta-coupled A-ring signals and a pyranose anomic signal were observed, suggesting the presence of a flavonol glycoside moiety. The $^{13}$C-NMR spectrum of 78 exhibited forty-three carbon signals. Seven of these were attributed to the gallate ester moiety, six were attributed to the $\beta$-D-glucose moiety and the thirty remaining carbon signals were attributed to the catechin and flavonol moieties. $^1$H-NMR data also revealed the pyrogallol-type B-rings of the catechin and flavonol moieties (each B-ring exhibiting a one-proton singlet) were linked at the respective C-2' positions via a C-C bond.

Compound 79 was isolated as yellow-brown amorphous solid. Positive-polarity FAB-MS yielded the molecular ion of 79 [M+H]$^+$ at m/z 785. Its UV-Vis spectrum yielded absorption maxima at 254, 261, 297 and 345 nm (MeOH). The CD spectrum exhibited a negative Cotton effect at 224 nm and a positive Cotton effect at 256 nm (MeOH).

A chemical test (anisaldehyde-sulfuric acid reagent) indicated the presence of a catechin moiety. The $^1$H- and $^{13}$C-NMR spectra of 79 resembled those of 78, except for the absence of galloyl signals and the upfield shift of the catechin H-3 and C-3 signals. The desgalloyl structure of 79 was confirmed when treatment of 78 with tannase yielded 29 and 79 as the products of hydrolysis. Compound 78 was also subjected to enzymatic hydrolysis with crude hesperidinase. The products were the hydrolysate of 78 and $\beta$-D-glucose. Examination of $^{13}$C-NMR data of the hydrolysate concluded that the location of the glucose moiety in 78 was at the flavonol C-3 position and the flavonol moiety was also established as isomyricitrin 80 (myricetin 3-O-$\beta$-D-glucoside) by comparison of available literature. Further evidence for the myricetin moiety was gained from EI-MS fragmentation data of the undecamethyl ether derivative of the hydrolysate of 78.
The chirality of the biphenyl bond in both 78 and 79 was proposed to be the R-series by comparison of the CD spectrum of the hydrolysate of 78 with the CD spectra of atropisomers 59 (R-series) and 60 (S-series).

(-)-Epigallocatechin gallate 1, (-)-epigallocatechin 4 and 80 are present in the fresh green tea leaf and it was proposed that 78 and 79 were formed during the manufacture of black tea via the oxidative coupling of (-)-epigallocatechin gallate 1 with 80 and (-)-epigallocatechin 4 with 80, respectively. Hashimoto et al. observed that 78 and 79 were the first isolated bisflavanols linked through a C-C bond at the B-rings of flavonol and catechin moieties.

1.2.18 Ethylpyrrolidinyltheasinensin A

Ethylpyrrolidinyltheasinensin A 81, containing a N-ethyl-2-pyrrolidinone moiety, has been isolated from relatively polar fractions of commercial black tea (solvent partitioning and column chromatography) and characterised (polarimetry, UV-Vis, 1D/2D NMR spectroscopy, FAB-MS, CD spectroscopy, elemental analysis and partial synthesis - condensation of 62 with N-ethyl-5-hydroxy-2-pyrrolidinone) by Tanaka et al., while investigating the proposed production of cross-condensation products, formed between reactive polyphenolic metabolites and other important tea leaf components, for example, the major tea amino acid, L-theanine 82 [92]. Compound 82 is a derivative of L-glutamic acid.
Black tea was extracted with boiling H₂O, concentrated and decaffeinated by partitioning against CHCl₃. The aqueous layer was further extracted with EtOAc and n-BuOH, the combined n-BuOH extracts were chromatographed over Sephadex LH-20 (H₂O with increasing proportions of MeOH) to yield ten fractions. Reversed-phase analytical HPLC and TLC analysis showed that the eighth fraction contained (−)-epigallocatechin gallate 1, (−)-epicatechin gallate 2, 62 and 81. Fraction eight was further chromatographed over MCI-gel CHP20P (H₂O-MeOH), Chromatorex ODS (H₂O-MeOH) and TSK gel Toyopearl HW-40F (H₂O-MeOH) to yield pure (−)-epigallocatechin gallate 1, (−)-epicatechin gallate 2, 62 and 81. Similar chromatography of the ninth fraction yielded 63.

Compound 81 was isolated as an amorphous brown powder. Its UV-Vis spectrum yielded an absorption maximum at 276 nm. The CD spectrum exhibited three negative Cotton effects at 221, 247 and 287 nm. Positive-polarity FAB-MS yielded the molecular ion of 81 [M+H]+ at m/z 1026, with the odd number molecular mass (1025 amu) suggesting the incorporation of a nitrogen atom in the compound.

The ¹H- and ¹³C-NMR spectrum of 81 revealed two characteristic sets of resonances assigned to flavan-3-ol and gallate ester groups. ¹H-NMR chemical shifts and coupling constants were related to those of 62 and 63, which are both atropisomeric symmetrical dimers of (−)-epigallocatechin gallate 1, but the chemical shifts were more similar to
those of 62 than those of 63 [79]. However, only three proton resonances were observed for the two A-rings (excluding phenolic protons) which usually contain four. One of the resonances was a singlet (no meta-coupling) suggesting substitution at C-6 or C-8. Additionally, $^1$H- and $^{13}$C-NMR data revealed the presence of a methine, two methylene, a carbonyl and an ethyl group. $^1$H-$^1$H COSY data revealed the partial ring structure (-CH$_2$-CH$_2$-CH-) while the ethyl group was shown to be attached to a nitrogen atom by $^1$H and $^{13}$C chemical shift data. HMBC correlations indicated the presence of an N-ethyl-2-pyrroolidinone ring with the methine proton exhibiting correlations with aromatic carbon resonances attributable to A-ring carbons in one of the flavan-3-ol units. The location of substitution was assigned at C-8 by observation of NOE correlations between the N-ethyl protons and the B-ring aromatic protons.

The atropisomerism of the biphenyl bond was proposed to be R-series by comparison of the CD spectra of 62 (R-series), 63 (S-series) and 81 which exhibited negative Cotton effects at 219, 219 and 221 nm respectively. However, the molar ellipticity of 63 was much smaller than that of 62 and 81, which were similar. The relative configuration at the methine carbon (N-ethyl-2-pyrroolidinone ring) was not determined.

As a complementary method of structure elucidation, and to yield mechanistic and stereochemical information, 81 was partially synthesised via an acid catalysed condensation between N-ethyl-5-hydroxy-2-pyrroolidinone and 62. Initially, the Strecker aldehyde of 82, compound 83, was synthesised and underwent spontaneous intramolecular cyclisation to yield N-ethyl-5-hydroxy-2-pyrroolidinone.

![82 and 83](image)

The product formed from the condensation between N-ethyl-5-hydroxy-2-pyrroolidinone and 62 possessed a reversed-phased HPLC retention time the same as that measured for 81, while the retention time of the product formed from the condensation between N-ethyl-5-hydroxy-2-pyrroolidinone and 63 was different from that measured for 81. Post
purification (column chromatography), the partially synthesised compound (condensation of N-ethyl-5-hydroxy-2-pyrrolidinone and 62) displayed spectra identical to that of 81. Furthermore, substitution was shown to occur at C-8 of the A-ring of 62. However, the relative configuration at the methine carbon (N-ethyl-2-pyrrolidinone ring) could not be determined. HPLC analysis of the reaction mixture showed production of other minor compounds, and although Tanaka et al. only isolated one diastereomer, it was proposed that another epimer of 81 may have been present in the mixture. Additional credibility was given to this proposal when it was found that acid-catalysed condensation between (-)-epigallocatechin gallate 1 and N-ethyl-5-hydroxy-2-pyrrolidinone yielded two enantiomers, compound 84 (R-series) and compound 85 (S-series) in a ratio of 4:1 respectively.

![Chemical structures](image)

To determine whether or not N-ethyl-5-hydroxy-2-pyrrolidinone was present in black tea or other tea products, Tanaka et al. derivatised N-ethyl-5-hydroxy-2-pyrrolidinone with 2,4-dinitrophenylhydrazine (DNP) and compared its properties (reversed-phase HPLC) with those of DNP derivatised fresh tea leaf, green tea, roasted green tea, oolong tea and black tea. The DNP derivative was not present in any of the extracts and it was proposed that 83 may have reacted with co-existing polyphenols or evaporated during the manufacturing stages.

Compound 81 is the first nitrogen-containing dimeric polyphenol isolated from black tea. Tanaka et al. hypothesised that Strecker aldehydes of tea amino acids (i.e. 82) may be produced during the enzyme deactivation and drying stage of black tea processing. The
resulting aldehydes, which are reactive electrophiles, are thought to subsequently attack the A-rings of localised catechins.

1.2.19 Theacitrins

Compounds possessing a 5,7,8a-trihydroxycyclopenta[a]indene-1,6,8(3aH,3bH,8aH)-trione moiety (R_2C_{12}H_6O_6).

\[ R = A- \text{ and C-rings of catechins} \]

Theacitrin A 86, theacitrin B 87 and theacitrin C 88 were isolated by Powell et al. [93-95] during an investigation into the nature of the products formed from the antioxidative depolymerisation of two crude TR fractions, obtained from Assam black tea - the CTR [93] and theafulvin (TFu) fractions [96].

The three novel compounds were isolated from Sephadex LH-20 chromatographed TFu sub-fractions using semi-preparative reversed-phased HPLC (UV-Vis detection at 380 nm). At the time they were not fully characterised due to the small quantities obtained and no definitive structures were assigned. Nevertheless, these ‘intense’ yellow compounds represented a new class of black tea pigments and were termed ‘theacitrins’ derived from the Greek *thea* (‘goddess’ but adapted to refer to tea) and *kitrino* (yellow).
The CTR was obtained free of TFs utilising the method of caffeine-induced precipitation of the EtOAc-insoluble material from black tea [97] while the TFu was obtained relatively free of FGs utilising cellulose adsorption chromatography as a method of TR fractionation introduced by Bailey et al. [96].

The two crude TR fractions (characteristically brown in colour) were further fractionated using gel permeation chromatography (Sephadex LH-20, 30-40 % aqueous Me₂CO). For both the CTR and TFu (30 % aqueous Me₂CO loading) the fractionation procedure was essentially arbitrary, based primarily on the poor visual chromatographic resolution between the yellow and brown bands spread throughout the column. After evaporation of Me₂CO under reduced pressure, the aqueous sub-fractions were lyophilised and subsequently analysed by reversed-phase analytical HPLC.

UV-Vis absorbance data at 380 nm indicated that most of the sub-fractions obtained from the ‘arbitrary’ fractionation remained irresolvable, bearing the hallmarks of the reversed-phase HPLC TR ‘hump’. However, in some sub-fractions, peaks were found to elute ‘above’ the irresolvable hump material. Spectral analyses determined that FGs were responsible for some of these ‘floating’ peaks while others were attributed to novel compounds. The TFu sub-fractions were shown to contain less flavonol material than the CTR sub-fractions and were therefore subjected to further spectral analyses. Two theacitrins, TC-6.4 and TC-7.3 were detected at 380 nm in the third TFu sub-fraction while the third, TC-9.4 was detected at 380 nm in the sixth TFu sub-fraction. Based on comparisons of the UV-Vis spectra of the three theacitrins with a TF, a theaflavic acid and a FG, it was realised that the three compounds did not belong to a class of pigments that were well known in black tea. Similarities between the UV-Vis spectra of the theacitrins suggested they belonged to the same novel class of black tea pigments.

Compared with characterised black tea pigments such as TFs, theaflavic acids and FGs, the theacitrins were relatively hydrophilic, eluting much earlier on the HPLC gradient [97]. This chromatographic characteristic of theacitrins was attributed to carboxyl functionality and it was observed that the UV-Vis spectra of theacitrins resembled theaflavic acids more than TFs. A diagnostic method was developed for assigning the
number of gallate esters present in TFs. The method is based on the 360:280 nm absorbance ratio of TFs which have maxima at 360 nm while gallate esters have maxima at 280 nm. The method was applied to the theacitrins, the data suggesting that TC-6.4 and TC-7.3 contained a mono-gallate ester, while TC-9.4 contained di-gallate esters.

Using a semi-preparative reversed-phase column and a modified HPLC gradient, it was possible to isolate theacitrins in small quantity, free of the underlying TR hump material. With the amounts available, it was possible only to acquire preliminary NMR and MS data. Powell et al. hypothesised that theacitrins may be structurally related to TFs and drew some conclusions to aid structural assignment, by comparison of their respective $^1$H-NMR and ESI spectra.

Based on $^1$H-NMR data, there was evidence for the presence of a mono or digallate ester as appropriate (signals at 6.88 and 6.92 ppm) and there was also evidence of a change in the structure associated with the dihydroxy-benzene component of the bicyclic undecane benzotropolone moiety.

ESI-MS revealed that there were fragmentation peaks common to both TFs and theacitrins, however, no molecular ions were observed. LSIMS using Cs$^+$ for TC-6.4 gave a weak ion [M+H]$^+$ at $m/z$ 749. Based on the acquired spectral and spectrometric data, a dicarboxylic acid structure for theacitrin-3-gallate 89 was initially proposed.
Powell et al. speculated that theacitrins may be precursors to TRs or intermediates in the conversion of TFs to TRs [93]. The stereochemistry of 89 is incomplete in the literature.

The chemical structure of 86 was formally assigned by Davis et al. (therefore the structure 89 corrected) who isolated 86 from the MeOH/Me2CO extract of decaffeinated Assam black tea leaves using a combination of column chromatography and semi-preparative reversed-phase HPLC [98]. The compound was found to be highly soluble in H2O and MeOH but less so in Me2CO. Compound 86 was unstable in solution, particularly in the presence of acid whereby rapid structural changes were observed to occur.

Structural characterisation was accomplished using 1H/13C-NMR spectroscopy, UV-Vis, ESI-MS and FT-IR data. Negative-polarity ESI-MS yielded the molecular ion of 86 [M-H]⁻ at m/z 759 and the 1H-13C-NMR spectrum revealed the presence of thirty-seven carbon atoms. With these data the empirical formula was provisionally deduced as C37H28O18. The 1H-NMR spectrum indicated the presence of two flavan-3-ol units, part of which had been transformed. Two sets of A-ring aromatic resonances between 6.0 and 6.4 ppm (two pairs of doublets each with J = 2.3 Hz, consistent with meta-substitution) and two sets of C-ring resonances were readily identified. The coupling patterns of H-2 and H-2' indicated that the flavan-3-ol moieties had retained epi-like stereochemistry. A proton singlet at 6.94 ppm indicated the presence of a gallate ester. This was established to be attached at C-3' as deduced from HMBC connectivities. The assignment for the chemical structure of the proposed 5,7,8a-trihydroxycyclopenta[a]indene-1,6,8(3aH,3bH,8aH)-trione moiety was based on HMBC connectivities and FT-IR data. The UV-Vis spectrum for 86, with maxima at 277 and 379 nm was obtained via the PDA detector of the HPLC instrumentation.

The characterisation of 86 was of great interest as it had certain structural features in common with 74 such as a fused, three-ring system with their mutually inclusive cyclopent-2-enone moieties. The major structural difference between 74 and 86 is compound 86 has a molecular mass of 28 amu (a carbonyl group) more than 74.
A mechanism was proposed (scheme 22) for the formation of 86, whereby the B-rings of (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4 are oxidised to produce two radical species, XV and XVI respectively, which subsequently form a new C-C bond [98]. The intermediate undergoes intramolecular cyclisation, rearrangement and hydration, to yield the product. Davis et al. hypothesised that formation of dimeric polyphenolic compounds such as 86 via oxidation of flavanol B-rings, with further oxidation of the dimers, might give rise to more structurally diverse (heterogeneous) oligomers, such as those which may be present in the TRs.

Scheme 22: proposed mechanism for the biosynthesis of 86 (adapted) [98]

From a mechanistic perspective, it seems unlikely (based on the life-time and reactivity of the induced radicals) that homolytic bond formation (radical combination) would occur under the oxidation conditions prevailing during the manufacture of black tea (low concentration of monomers in the exposed leaf matrix).

A more realistic mechanism is suggested below (scheme 23) that involves attack of the nucleophilic radical XVI onto the electrophilic o-quinone II, resulting in an oxygen-stabilised radical XVII after C-C bond formation. The newly formed radical could
participate in propagation of a radical chain regenerating XVI or participate in intramolecular C-C bond formations.

Scheme 23: alternative mechanism for the biosynthesis of 86

Davis et al. also isolated, purified and partially characterised the two structurally related compounds, 87 and 88 which were present in the same MeOH/Me₂CO extract [98]. The compounds exhibited similar UV-Vis spectra to 86, but were present in smaller quantities.

The proposed structure of 87 was based on reversed-phase HPLC data, with 87 having the same UV-Vis absorption as 86 (maxima at 277 and 379 nm), but eluting 4.93 minutes later. Davis et al. postulated that 87 was an isomer of 86, with the galloyl ester linked to C-3 rather than C-3'.

Compound 88 eluted 11.27 minutes later than 86 and had a relative molecular mass of 912 amu as determined by ESI-MS suggesting the presence of one galloyl ester more than 86. Treatment of 88 with tannase yielded 86 amongst the products and led Davis et al. to believe that 88 had the same structure as 86 but with an additional galloyl ester group at C-3.

Based on the mechanism that was proposed; 86 and 87 are produced from the oxidative dimerisation of (-)-epigallocatechin gallate 1 and (-)-epigallocatechin 4, while 88 is produced from the oxidative self-dimerisation of (-)-epigallocatechin gallate 1. The stereochemistry of the tertiary hydroxyl group of 86, 87 and 88 is not provided in the literature.
1.2.20 Tricetinidin

Tricetinidin 90, an intense red-coloured compound (a 3-desoxyanthocyanidin) has been isolated from black tea and fully characterised (1H-NMR spectroscopy) by Wang et al. while investigating molecular rearrangements of catechins during the manufacturing of black tea [27]. The structure of 90 was first proposed by Roberts (compound P) [60]. Wang et al. also showed, using acid hydrolysis, that 90 was not a breakdown product of fresh leaf proanthocyanidins, but rather the product of oxidative degallation of (-)-epigallocatechin gallate 1. This hypothesis was also supported by the observed enzymatic (PPO) production of 90 and 29 from (-)-epigallocatechin gallate 1 in model oxidation studies conducted by Coggon et al. [99].

\[
\text{[Diagram of 90]}
\]

Kuhnert et al. have synthesised and characterised (spectroscopic) 90 and a range of 3-desoxyanthocyanidins via a BF₃·Et₂O-mediated aldol condensation between a substituted acetophenone and a salicylaldehyde derivative [100]. The compounds were prepared as standards for further investigation into the hypothesis that the reactive electrophilic 3-desoxyanthocyanidins play a crucial role in the formation of the TRs during black tea manufacturing. Bruschi et al. have previously observed that the addition of certain monomeric flavonoids to a crude TR fraction produces an unexpected hyperchromic effect similar to anthocyanin copigmentation and postulate that this might indicate a disturbance in the desoxyanthocyanidin equilibrium in the TR fraction [101].

1.2.21 Galloyl glucose and galloylquinic acids

Besides the typical catechin derivatives, a small number of hydrolysable tannins, in particular 1,4,6-tri-O-galloyl-β-D-glucose 91, theogallin 92 (a galloylquinic acid) and strictinin 93 have been identified in black tea [90]. Roberts and Myers initially isolated
(solvent extraction, cellulose-acetate column chromatography and preparative PC) and characterised (elementary analysis, spectrophotometry, PC, electrometric and permanganate titration) compound 92 from dried green tea leaf and identified 29 and quinic acid as products of hydrolysis (3N HCl). The regio-chemical identity of 92 was designated as the 3-galloylquinic acid by analogy with 3-caffeoylquinic acid which is the most abundant of the tea chlorogenic acids [102]. It should be noted that this numbering system predates the IUPAC recommendations for cyclitols including quinic acids, where 3-galloylquinic acid is now 5-galloylquinic acid in the IUPAC system [103].

![Chemical structures](image)

Subsequent investigations using tandem LC-ESI-MS has demonstrated the presence also of 3-galloylquinic acid and 4-galloylquinic acid (IUPAC) [104]. It is interesting to note that no chlorogenic acids other than these caffeoyl and galloyl esters, and the previously discussed theogallinin 77 [90], have ever been reported in tea, despite the large number of chlorogenic acids widely distributed elsewhere in nature [105,106].

1.2.22 Flavonol glycosides

Additionally, apart from the previously mentioned hydrolysable tannins, black tea also contains a mixture of glycosides of three major flavonols, kaempferol 94, quercetin 95 and myricetin 96. Seven quercetin, five kaempferol and two myricetin glycosides have been extracted (boiling H₂O), isolated (polyamide SPE cartridge) and characterised (reversed-phase C₁₈ HPLC with internal standards) from black tea by Price et al. while investigating the quantitative FG profile of tea as a prerequisite to understanding the role and bioavailability of flavonol glycosides (FGs) in the diet of UK tea drinkers [107]. It
was observed that a substantial amount of relative variation occurred in the concentrations of FGs between the different teas analysed (36.5 to 88.3 mg dm\(^{-3}\) in an average tea infusion).

Similarly, and prior to the above mentioned quantification of FGs in black tea, a reversed-phase HPLC analysis (Hypersil-ODS/Nucleosil-C\(_{18}\)) of fresh tea shoots and green and black tea by Engelhardt et al. revealed the presence (reversed-phase HPLC, NMR spectroscopy and MS) of fourteen glycosides of 94, 95 and 96 [108]. The glycoside moieties consisted of glucose, rhamnose, galactose and fructose and were configured in mono-, di- and tri-glycoside units. Fructosides are comparatively rare in nature.

The presence of structurally intact FGs in black tea is a mechanistically interesting observation as their presence in the fresh green tea leaf evidently plays a precursory role in the production of the theasinensin-like theaflavonins during black tea manufacturing, i.e. compounds 78 and 79. However, the production of the theaflavonins is most likely a relatively minor oxidative dimerisation pathway involving catechin and flavonol B-rings. The low abundance of the theaflavonins is in contrast to the prolific production of the closely related theasinensins. Theasinensins in black tea are thought to be constructed entirely from the oxidative dimerisation of catechin pyrogallol-type B- or D-rings. However, from a mechanistic point of view, and of possible relevance to TR formation, the aforementioned theaflavonins are thought to be biosynthesised from the oxidative dimerisation of the pyrogallol-type B-ring of a mono-glycoside analogue of compound 96 and the pyrogallol-type B-ring of a catechin such as (-)-epigallocatechin gallate 1 or (-)-epigallocatechin 4. The question arises; can an FG with a catechol-type B-ring, such as 95, oxidatively dimerise with a catechin containing a pyrogalloyl-type B- or D-ring?
Besides the generic theaflavin-type analogues one could expect from a flavonol pyrogallol-type B-ring precursor, it is probable that in black tea there are theaflavonin-type theasinensin dimers constructed from the oxidative dimerisation of 94 or 95, both containing differing mono-, di- and tri-glycoside moieties. An extra mechanistic complexity manifests itself when the biosynthesis incorporates catechin molecules of differing B-ring substitution ((-)-epigallocatechin gallate 1 or (-)-epigallocatechin 4, and more interestingly (-)-epicatechin gallate 2 or (-)-epicatechin 5). These compounds, if they exist, have yet to be discovered in black tea and they most likely contribute to the components of the chromatographically non-resolvable TR.

1.2.23 Catechin A-ring model oxidations

Ho et al. have synthesised, isolated (Sephadex LH-20 and silica gel), and characterised (1H-13C-NMR spectroscopy and FAB-MS) three novel compounds, formed from the chemical oxidation of (-)-epigallocatechin gallate 1 or (-)-epigallocatechin 4 with H2O2 (50% dilution of commercial grade) [109]. The oxidation of (-)-epigallocatechin gallate 1 produced compounds 97 and compound 98, while the oxidation of (-)-epigallocatechin 4 produced compound 99. However, the stereochemical configurations for 97, 98, and 99 as published, do not imply that 97 and 98 were produced from (-)-epigallocatechin gallate 1 or that 99 was produced from (-)-epigallocatechin 4. It is unlikely that epimerisation occurred during oxidation.

(-)-Epigallocatechin gallate 1 was oxidised with H2O2 (50%) and the crude reaction mixture chromatographed over Sephadex LH-20 with 95% EtOH as eluent. Five fractions were collected with two major products present in the fourth fraction. This fraction was...
chromatographed over silica gel using a mixture of EtOAc:MeOH:H₂O, 7:1:1 v/v/v as eluent to yield 97 and 98.

Compound 97 was isolated as a colourless amorphous solid. Positive-polarity FAB-MS yielded the molecular ion [M+H]^+ at m/z 479 and combined with ^1^H-NMR data, the empirical formula was deduced as C_{21}H_{18}O_{13}. The ^1^H- and ^1^C-NMR spectra of 97 were similar to those of (−)-epigallocatechin gallate 1 although there was evidence that the A-ring had been transformed. Indications that only the A-ring had been altered were supported by HMOC, HMBC, ^1^H-^1^H COSY and NOESY correlations for the B-, C-ring and galloyl moiety. The ^1^H-NMR spectrum of the transformed A-ring exhibited two doublets at 3.64 and 3.86 ppm (J = 16 Hz) having HMQC correlations with C-8 at 41.2 ppm. The ^1^C-NMR spectrum revealed two carbonyl carbon atoms at 171.9 and 175.0 ppm, indicative of carboxylic acid groups, their presence confirmed by FAB-MS data.

Compound 98 was isolated as a colourless amorphous solid. Positive-polarity FAB-MS yielded the molecular ion [M+H]^+ at m/z 465 and combined with ^1^C-NMR data, the empirical formula was deduced as C_{20}H_{16}O_{13}.

The ^1^H- and ^1^C-NMR spectra of 97 and 98 were similar, with the only difference being the loss of H-8 and C-8 of 97. Additionally, the ^1^C-NMR spectrum of 98 revealed C-7 had decreased its chemical shift to 169.8 ppm, indicative of conjugation with a double bond.

(−)-Epigallocatechin 4 was oxidised with H₂O₂ (50%) and the crude reaction mixture chromatographed over Sephadex LH-20 with 95 % EtOH as eluent. Four fractions were collected with a major product present in the third fraction. This fraction was chromatographed over silica gel using a mixture of EtOAc:MeOH:H₂O, 6.5:1:1 v/v/v as eluent to yield 99.

Compound 99 was isolated as a colourless amorphous substance. The empirical formula was deduced as C_{14}H_{14}O_{9}. The FAB-MS and NMR spectra of 99 indicated the difference between 97 and 99 was the loss of the galloyl moiety in 97.
Valcic et al. proposed that the B-ring was the principal site of antioxidant activity in (-)-epigallocatechin gallate 1 [87], while Nanjo et al. [110] and Wan et al. (synthesis of theaflavate A) [54] suggest the gallate moiety present in (-)-epigallocatechin gallate 1 and (-)-epicatechin gallate 2 is the principal site of antioxidant activity. The synthesis of dicarboxylic acids 97, 98 and 99 is chemical evidence that the A-ring of flavan-3-ols can be oxidised but the mechanism of oxidation is not known. These types of oxidation reactions are unlikely to occur during black tea processing. There are no previous reports on direct A-ring oxidation reactions of catechins, however, p-quinone-methide formation is possible which includes one-electron oxidation of the A- and the C-ring (scheme 24).

Scheme 24: formation of p-quinone-methide

Compounds 97, 98 and 99 are unusual, because their precursors generally undergo oxidative transformations in their B- and D-rings and there seems to be no rational reason why these rings remain untransformed during the production of compounds 97, 98 and 99. Additionally, it has been established that catechol (B-ring model) has a lower relative redox potential than resorcinol (A-ring model) [71,111,112].

1.2.24 A-ring model oxidations of theaflavin 3',3'-O-digallate

The reaction products of 15 with hydroxyl radicals generated by H₂O₂ have been characterised by Sang et al. with the intention of gaining insight into mechanisms of antioxidant reactions in physiological systems [113]. Two oxidation products, compound 100 and compound 101, were isolated and identified on the basis of their spectrometric and spectroscopic properties (positive-polarity APCI-MS and 1D/2D NMR spectroscopy).
Compound 100 was isolated as reddish amorphous solid. Positive-polarity APCI-MS yielded the molecular ion of 100 [M+H]^+ at m/z 889 and combined with^{13}C-NMR data the empirical formula was provisionally deduced as C_{42}H_{32}O_{22}.

The ^1H-NMR spectrum of 100 was similar to that of 15 with three characteristic benzotropolone resonances at 7.42, 7.64 and 7.66 ppm. Additionally, two sets of C-ring resonances and two sets of gallate group singlets (6.88 and 6.86 ppm) were identifiable. Only two sets of two of A-ring resonances (6.00 and 6.09 ppm) were observed instead of two sets of four A-ring resonances. Additionally, resonances at 3.97 and 3.88 ppm were assigned to a methylene moiety based on HMQC data. The complete interpretation of NMR data was based on HMBC and HMQC experiments. Indications from spectral data were that the C-rings, gallate groups and benzotropolone moiety of 15 did not undergo structural change during the oxidation experiment.

Compound 101 was isolated as reddish amorphous solid. Positive-polarity APCI-MS yielded the molecular ion of 100 [M+H]^+ at m/z 889. The ^13C-NMR spectrum revealed the presence of forty-two carbon atoms. With these data the empirical formula was provisionally deduced as C_{42}H_{32}O_{22}. The NMR spectra of 101 were similar to 100 with respect to the characteristic benzotropolone, C-ring, methylene and gallate group resonances, although evidence for isomeric A-ring modification was attained from^{13}C-
NMR data. The complete interpretation of NMR data was based on HMBC and HMQC correlations.

A mechanism was proposed for the formation of 100 and 101, whereby there is initial attack by hydroxyl radical on the respective A-rings of 15 with subsequent ring cleavage. The mechanism was not further elaborated. These data provide evidence that (depending on the oxidation system used) it is possible to activate the A-rings of catechins as the initial site for oxidation reactions. However, the conditions that prevail during black tea manufacturing are unlikely to lead to A-ring oxidation as described.

1.2.25 Conclusion

This initial part of the introduction has comprehensively summarised the current knowledge on the chemistry of low molecular mass black tea polyphenols. Approximately one hundred compounds have been extracted, purified and fully characterised. However, one can be sure that the compounds so far described constitute only the ‘tip of the iceberg’ of black tea chemistry.

It is interesting to note that the large majority of low molecular mass polyphenols isolated from black tea are formed from catechins as the main ‘building-blocks’. With few exceptions, the compounds are derived from the reactions between two or more catechin ‘building-blocks’ following the identical first mechanistic step; oxidation of the catechin B- or D-ring. Surprisingly the nucleophile attacking the o-quinone intermediate is, in most cases, another catechin. Few examples of exception to this rule have been reported, although many other potentially biogenic nucleophiles such as amino acids, carbohydrates or even water, are present in the fresh tea leaf.

As an organic chemist, one can only be amazed by the structural diversity, and complexity, of the compounds so far discussed. The polyphenol chemistry of black tea incorporates a strategy commonly encountered in nature; a small library of structurally defined ‘building-blocks’, in the current case, catechins, are elaborated via simple organic transformations (oxidations, condensations, benzilic-acid rearrangements etc.) into more complex structures. The theme of using small, well defined ‘building-blocks’ ultimately
to be elaborated into larger structures, with function, can be found in amino acid and protein chemistry, nucleic acid and DNA chemistry and carbohydrate chemistry. Importantly, during the chemistry of all these primary metabolites named above, their chemical structure remains largely unchanged as a result of the oligomerisation process and only one or two functional groups undergo chemical transformation, e.g. loss of water during DNA, or peptide formation. In polyphenol chemistry, however, a large part of the chemical structure undergoes dramatic changes. Based on simple reaction sequences, compounds of enormous structural complexity are obtained via a series of inter- and intramolecular reaction cascades initiated by a few relatively simple oxidation processes. It can be said that plants such as *Camellia sinensis* have achieved structural and chemical diversity through the inherent reactivity of the catechin ‘building-blocks’ rather than through a ‘mix-and-match’ strategy of alternatively assembling structurally different ‘building-blocks’ (*cf.* the status of amino acids in protein synthesis). This concept and strategy, adopted by fresh tea leaves, is reminiscent of Whitehead’s concept of predisposed chemistry, which is defined as “kinetic reaction preferences bestowed upon the functional groups in their specific molecular context”. Thus, black tea polyphenols have programmed, or built into their structure, these kinetic preferences that lead to compounds of enormous structural complexity and diversity.

The reason behind this strategy is subject to speculation. However, a myriad of structurally diverse compounds emerges that can be viewed as an excellent chemical library, able to potentially serve a wide variety of functions. Medicinal chemists in their search for new drug candidates and lead compounds, in a world that is rich in targets but poor in lead compounds, would be advised to investigate nature’s polyphenol chemistry to seek inspiration on how a small set of compounds, if correctly selected, leads efficiently to a vast array of diverse products.

Whether it is the process of oxidation, or the biological function of the resulting polyphenols that has led to the evolution of polyphenol chemistry is uncertain and remains the topic of debate. Both arguments have their value. The process of polyphenol oxidation allows the plant to synthesise efficiently, *via* parallel synthesis, a series of compound libraries aimed at deterring insects and herbivores or combating disease.
Additionally, it can be speculated that this combinatorial chemistry approach of plants takes a role similar to that of the immune response in higher organisms. *Camellia sinensis* and many other plants that employ this oxidative-transformation strategy (otherwise referred to as ‘browning’ or ‘tanning’) produce a large number of structurally complex compounds to cover all eventualities of life in a potentially dangerous environment. The chemistry employed is rather non-selective and seems to have been replaced in the course of evolution by the more controlled chemistry of peptides in the immune system of higher organisms. Some of the low molecular mass black tea polyphenols, such as TFs, have been shown to be biologically active and are responsible for some positive health effects of black tea. A summary of these effects is beyond the scope of this introduction and has been discussed elsewhere [114-118].

The chemistry described in the initial part of this introduction has been useful for drawing conclusions on the types of mechanisms most likely to be implicated in the formation of the TRs, since TRs are believed to be more complex variants of the low molecular mass black tea polyphenols. Beside tea chemistry itself, the chemistry outlined here might serve as a helpful guideline in understanding other polyphenol oxidation processes in the plant kingdom. For example, a product such as chocolate might be based on very similar basic chemistry, only with a variation in the chemical ‘building-blocks’ utilised. Additionally, most ‘browning’ processes, the best known being apple or banana browning, are based on the enzymatic oxidation of polyphenols, most likely leading to compounds via mechanisms similar to those described so far, with resultant structures closely related to the ones presented in the initial part of the introduction.
1.3 Characterisation of higher molecular mass black tea polyphenols

1.3.1 Introduction

The literature concerned with higher molecular mass black tea polyphenols, most specifically the thearubigins, is vast. It is beyond the scope of this introduction to cover all aspects of the biogenesis and characterisation of the TRs that have been published. Selected examples have been chosen in order to exemplify what the author considers to be some important milestones in the understanding of these elusive polyphenolic compounds. Nomenclature, instrumental methods for TR isolation and characterisation, and chemical degradation (as an aid to chemical structure elucidation) will be discussed.

1.3.2 Initial characterisation of TRs

Solvent fractionation, and 2D-PC, (BuOH:AcOH:H₂O, 4:1:2.2 v/v/v and 2% aqueous AcOH) of the H₂O-soluble substances in a black tea infusion, enabled Roberts et al. to detect nine unknown compounds (compact spots on their PC chromatograms) and additionally, two characteristic PC streaks [119].

The black tea infusion was extracted with EtOAc to yield fraction 1A. The aqueous layer was precipitated with saturated lead acetate, the precipitate was suspended in MeOH and then decomposed by hydrogen sulphide. The filtrate obtained from this procedure was evaporated to dryness, under reduced pressure, to yield fraction 1B.

Fraction 1A was analysed by 2D-PC and the most characteristic part of the PC was an orange-brown streak which Roberts termed SI. Fraction 1B was analysed by 1D-PC and the most characteristic part of the PC was a heavy, brown streak of zero Rf in 2% aqueous AcOH. Fraction 1B was further partitioned in BuOH and H₂O. The BuOH layer was evaporated to dryness, dissolved in MeOH and precipitated by Et₂O. The precipitate was termed SII. The filtrate from this procedure was evaporated to dryness, dissolved in Me₂CO and precipitated by Et₂O. The precipitate obtained was termed Sia. The most characteristic part of the 2D-PC of the SII fraction was a short, pennant-like, dark-brown streak and its position was almost completely unaffected by the subsequent run with 2% aqueous AcOH. The SII fraction was soluble in MeOH, EtOH, BuOH and hot H₂O, but
insoluble in EtOAc and dry Me$_2$CO. The SII fraction was found to be acidic (pH 3.2) and dissolved readily in cold aqueous sodium bicarbonate. The most characteristic part of the 2D-PC of the SIA fraction was an orange-brown streak which broadened slightly during the 2% aqueous AcOH elution. Unlike the SII fraction, the length of the streak was proportional to the length of the solvent run. The SIA fraction was soluble in MeOH, EtOH, Me$_2$CO and hot H$_2$O but less soluble in EtOAc. The SIA fraction was found to be acidic (pH 3.2) and hence dissolved readily in cold aqueous sodium bicarbonate. The yields of the SI, SIA and SII fractions amounted to more than 10% of the total mass of the tea extracted. However, recent indirect estimates, based on caffeine precipitation of black tea phenolic pigments, and monitoring by reversed-phase HPLC, are that the ambiguous TRs are responsible for ~80% of the phenolic compounds present in a black tea infusion [97].

It was proposed by Roberts that the SI, SIA and SII fractions should be classed together and termed ‘thearubigins’, along with all the brown pigments with acidic properties extractable from black tea [4]. The UV-Vis absorption spectrum of the SIA fraction exhibited $\lambda_{\text{max}}$ at ~275nm. At longer wavelengths, the absorption decreased steadily with slight ‘shoulders’ at 360 and 510 nm. The UV-Vis absorption spectrum of the SI and SIA fractions were almost identical, while the UV-Vis absorption spectra of the SIA and SII fractions were almost identical below 360 nm. Above 360 nm, the curve for the SII fraction followed a similar trend to that of the curve of the SIA fraction, but the intensity of absorption decreased less, indicating that the SII fraction absorbs more than the SIA fraction in the visible region [62].

Roberts and Myers carried out model tea-fermentations, using mixtures of catechin substrates treated with tea oxidase, while investigating the effect of coupled-oxidations during black tea manufacturing and their role in TR production. Mixtures of substrates with similar, and differing oxidation potentials were investigated. It was shown that TFs and theasinensins (produced in situ), formed from (−)-epigallocatechin gallate 1 or (−)-epigallocatechin 4, underwent further transformations if the substrate mixture also contained (−)-epicatechin gallate 2 or 6 [59]. An interesting observation during the coupled-oxidation experiments was the fact that (−)-epigallocatechin gallate 1 or (−)-
epigallocatechin 4 was totally consumed before any appreciable decrease in, or oxidation of, (−)-epicatechin gallate 2 or 6. Additionally, when a substrate mixture containing (−)-epigallocatechin gallate 1 and (−)-epicatechin gallate 2 was treated with tea oxidase, it was observed, via PC, that after five hours, (−)-epicatechin gallate 2 remained unoxidised while the only trace of (−)-epigallocatechin gallate 1 was a streak with similar chromatographic characteristics to that of the TR, SI fraction. Interestingly, it was observed that at the end of the coupled-oxidation experiment utilising (−)-epicatechin gallate 2 as a carrier, the enzyme had become completely inactivated, most likely through an inhibition mechanism initiated by (−)-epicatechin gallate 2.

Coupled-oxidation experiments were also undertaken utilising TFs, theasinensins and the TR, SI and SII fractions as substrates. The former two substrates were shown not to be oxidised by tea oxidase at an appreciable rate prior to the addition of the catechol-type catechins, and this supported the hypothesis that TFs and theasinensins were oxidation end-products. Individually, compounds 12 and 15 were shown to undergo coupled-oxidations with (−)-epicatechin 5 and 6 acting as carriers. However, (−)-epicatechin gallate 2 was unable to act as a carrier, most likely due to enzyme inhibition and (−)-epigallocatechin 4 did not act as a carrier in the attempted coupled-oxidation of 15. Increased O₂ uptake and CO₂ output was indicative of the coupled-oxidations of 12 and 15 and these manometric observations were accompanied by the loss of the characteristic orange-red colour of the TFs. PC analyses of the products formed from the coupled-oxidation reactions showed that the TFs had totally disappeared. Similar experiments using a substrate mixture of a theasinensin and 12, and utilising 6 as the carrier, showed that there was a relative rate increase in substrate oxidation compared with 12 as the only substrate in the fermentation mixture. The PCs of the oxidation products also showed, that, besides the total transformation and disappearance of 12, there was a marked decrease in the concentration of the theasinensin. Similarly, enhanced oxidation was shown to occur to the TR, SI and SII substrates, utilising 6 as carrier.

The results of these experiments suggested that TFs, and theasinensin-type dimers, can undergo coupled-oxidations with the catechol-type B-ring catechins (acting as carriers) to
form the TRs. However, it was concluded that this may not be the sole pathway for TR production. As the TRs themselves were observed to undergo further coupled-oxidation reactions, it was suggested that the TRs changed continually during black tea manufacturing. The implication of this observation is that TRs present in black tea which has received a relatively long oxidation treatment are likely to be chemically different from those produced from a short oxidation treatment.

Model tea-fermentations, with tea-enzymes acting on the substrates (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4, (-)-epicatechin 5 and 6 have also been performed by Sanderson et al. [120]. Generally, it was found that TRs were formed by the oxidation and ‘polymerisation’ of the individual catechins (except for (-)-epigallocatechin 4) and they were also formed by the oxidation and polymerisation of mixtures of the catechins. The results obtained by Sanderson et al. indicated that TFs were not necessarily precursors for TR formation as had been previously proposed by Roberts [121]. It was shown that during short oxidation periods, and depending on which catechins were being oxidised, the TR fractions on their PCs, differed in position and concentration, but after long oxidation periods, the fractions tended to merge and their Rf values decreased. These results implied that the TRs are a heterogeneous mixture of oxidation products and they undergo continuous oxidation and polymerisation with respect to time. As mentioned previously, continual change of the TR fraction during tea fermentation was observed by Roberts and Myers in their model tea-fermentation experiments [59].

1.3.3 Instrumental methods for TR isolation and characterisation

Reversed-phase HPLC, utilising LC columns with three different packing materials has been utilised during a comparative study of black tea liquors by Bailey et al. as means to overcome reversed-phase chromatographic difficulties encountered during the resolution of TRs, i.e. resolving mixtures of low and high molecular mass components and the phenomenon of secondary retention via the interaction of TRs with hydroxyl groups or metals on the surface of silica-based reversed-phase packing material [122]. The three reversed-phase LC columns used in their study consisted of a Hypersil ODS (C\textsubscript{18}),
Hypersil octyl wide-pore (C$_{18}$) and Hamilton PRP-1 (styrene-DVB copolymer) column. The Hypersil ODS column was chosen on the basis that the narrow-pore and high surface area of the packing had previously been shown to resolve low molecular mass polyphenols. The Hypersil octyl wide-pore column was chosen on the basis that the wide-pore packing has a low surface area and is less retentive than narrow-pore packing. It was therefore considered useful for chromatography of polymers. The Hamilton PRP-1 column was chosen on the basis that the packing has no surface hydroxyl groups or surface metals, thus eliminating the effects associated with secondary retention.

From the results of their HPLC data, Bailey et al. classified the black tea polyphenol pigments by their chromatographic behaviour, into three groups as follows: [122] Group I pigments ran close to the void volumes of all three columns. It was suggested that these pigments may have been charged, hydrogen-bonded, associated, polymeric or a combination of these, with the basis for exclusion being strong electrostatic repulsion, molecular size, or both factors. Group II pigments were resolved pigments and were further sub-divided into four groups. Sub-group II.1 pigments were assigned to eight theaflavin-type compounds, including the four TFs. Sub-group II.2 pigments were assigned to the three known theaflavic acids. Sub-group II.3 pigments consisted of eight resolved peaks and were assigned to type I resolved pigments. It was suggested that these pigments may have been TRs of low molecular mass or oxidation products of TFs, theaflavic acids, and/or catechins. Sub-group II.4 pigments consisted of thirteen resolved peaks that were brown in colour and were assigned to type II resolved pigments. It was suggested that these pigments may have been TRs of low molecular mass or oxidation products of TFs, theaflavic acids, and/or catechins. However, they could not be ruled out as products of non-enzymatic browning reactions. Group III pigments were unresolved pigments whose LC spectra yielded convex broad bands on the Hypersil octyl wide-pore and Hamilton PRP-1 columns. PDA data showed a band at 280 nm and additional absorption which covered the entire spectral region. It was suggested that these pigments were polymeric TRs, composed of polydisperse flavanol polymers incorporating monomeric units with chromophores, rather than being composed of proanthocyanandin
polymers. It was concluded by Bailey et al. that the HPLC data they obtained could further aid in instrumental measures of tea quality.

An alternative type of chromatography, micellar electrokinetic capillary chromatography (MEKC) has been investigated by Larger et al. for its potential in the analysis of polyphenols in black tea, with particular reference to TRs. Resolution was accomplished via electrophoretic migration in the positive-voltage mode at 30 kV [123]. An initial electropherogram of a whole black tea infusion was acquired; however, the identification of individual compounds was made difficult by the fact that the underlying TR hump contributed to the spectra of most peaks. Therefore, to aid in resolution, the whole black tea infusion was fractionated into three fractions via solvent extraction (EtOAc, MeOH and H$_2$O) before analysis. The EtOAc fraction produced an electropherogram that showed the underlying TR hump had been greatly reduced in size. TFs were not observed in the electropherogram; however, it was suggested that the TFs had possibly interacted with the silanol groups of the capillary wall via the benzotropolone-ring. While catechins were not observed in the whole black tea infusion electropherogram, (−)-epigallocatechin gallate 1, (−)-epicatechin gallate 2, (−)-epigallocatechin 4, (−)-epicatechin 5 and 6 were clearly identified in the EtOAc fraction. This observation indicated that the catechins are not completely transformed during black tea manufacturing, but are present in low concentration, too low to permit detection via MEKC, in the whole black tea infusion. Additionally, a theasinensin-type compound was also detected (not specified) along with a flavonoid (not specified).

The MeOH and H$_2$O fractions produced electropherograms containing the same absorption peaks. However, the H$_2$O fraction also contained two additional peaks. The most significant difference between the two electropherograms was the size of underlying TR hump and its relative migration time. In the MeOH fraction, the hump rose sharply after ten minutes, reached a maximum after fourteen minutes and then tailed off after thirty minutes. In the H$_2$O fraction the hump displayed a higher absorbance, with a later maximum and possessed a long tail which extended past thirty-five minutes. It was hypothesised that due to the longer migration time, the H$_2$O-TR may contain a higher negative charge and this would also explain its relative lower solubility in MeOH. It was
noted that there were two categories of brown pigments present in the MeOH and H₂O fractions. The first category resembled the Group 1 pigments described by Bailey et al. [122] and the second category comprised the two peaks (exclusive to the H₂O fraction) which also resembled Group 1 pigments, but with greater absorption at 400 nm. A further set of three peaks was observed in both the MeOH and H₂O fractions but these could not be identified from the literature.

It was concluded by Larger et al. that MEKC could be considered a chromatographic technique complementary to reversed-phase HPLC for resolution of tea components. However, the technique could not resolve the TRs. An additional drawback was the adsorption of TFs by the capillary wall. It was not established whether the presence of metal ions, adsorbed on the capillary silanol groups, catalysed TF-type compound degradation or whether the benzotropolone-ring was strongly retained. It was suggested that a way to alleviate this problem was to physically or dynamically coat the inner capillary wall.

High-speed countercurrent chromatography (HSCCC), a chromatographic method that does not employ a solid-phase for resolution has been utilised by Degenhardt et al. to isolate a polymeric fraction from black tea [124]. The principle of HSCCC incorporates the partitioning of solutes between two immiscible liquid phases and this potentially eliminates the observed problem of TRs interacting with many solid supports used in column chromatography. As a pre-treatment step, black tea was infused with boiling H₂O, the H₂O solution was passed over Amberlite XAD-7 resin and the phenolic compounds eluted from the resin with MeOH. The methanolic eluate was concentrated in vacuo and freeze-dried. The lyophilysate was subsequently separated by HSCCC using EtOAc:n-BuOH:H₂O, 2:3:5 v/v/v. The less dense organic phase was utilised as the stationary phase. The TR eluted with, and shortly after, the solvent front. Reversed-phase HPLC demonstrated that prior to HSCCC, the black tea extract contained a range of flavonoids, whereas after HSCCC resolution, the UV spectrum (280 nm) showed a broad, convex Gaussian hump, free of all monomers and no peaks were ‘floating’ on top of the hump. Two small peaks were present shortly after the dead volume of the reversed-phase HPLC column (Nucleosil RP 18) and it was suggested that these may be Group 1 TRs [122].
The yield of TR from the HSCCC separation was relatively low compared with yields from other reported methods of isolation and it was hypothesised that HSCCC allowed isolation of a specific proportion of the total TR.

The properties of the isolated TR were investigated and it was found that in aqueous solution, the TR had a pH value of 7.6 while that of the black tea infusion from where it originated, had a pH value of 4.9. The pH value measured for the HSCCC-TR is in contrast to that observed by Roberts who described the TRs as being weakly acidic [4]. Additionally, the colour of the HSCCC-TR was grey. Co-pigmentation effects were investigated. Addition of catechins and caffeine produced no change in colour, but addition of 1 M HCl resulted in a lighter colour. Further addition of HCl resulted in a purple colour. Addition of 0.5 M NaOH produced the opposite effect. The colour changed from grey to brown and then olive-green. It was suggested that anthocyanins may have been liberated from anthocyanidin-like polymers after lowering the pH, accounting for the purple colour observed.

The HSCCC-TR was fractionated with solvents, according to Roberts’s scheme and after fractionation was classified as being comprised mainly of SII-TR (50% SII and 25% of SI and Slα each). Roberts’s fractionation procedure was applied to the freeze-dried black tea extract (before HSCCC) and SI-TR was found to be the dominant TR, indicating that the HSCCC-TR only represented a portion of the vast TR ‘spectrum’.

It was concluded that HSCCC was an ideal method to isolate black tea pigments since it is amenable to high sample load with a relatively short separation time and produces high-purity isolated fractions. It was proposed that the use of more hydrophilic solvent systems had the potential to fractionate TRs further, as an aid to structure elucidation. Furthermore, the method was shown to be reproducible.

Positive-polarity MALDI-TOF-MS, with delayed pulsed ion extraction, has been utilised to analyse black tea fractions by Menet et al. during investigations into the chemical structure of TRs [125]. MALDI-TOF-MS is a useful analytical method for polyphenol analysis, as it is extremely sensitive, is tolerant of impurities and allows the simultaneous
determination of molecular masses in complex mixtures of low and high molecular mass compounds. Depending on the configuration of the instrument, a pulsed-ion extraction delay allows ions to fragment into smaller ions and neutral species, before extraction and acceleration into the TOF-tube. Fragmentation patterns may yield structural information.

Black tea was extracted with 80% Me$_2$CO at room temperature for two weeks, the solvent removed in vacuo and the residue partitioned against CHCl$_3$, EtOAc and n-BuOH. The EtOAc fraction was chromatographed over Sephadex LH-20 with Me$_2$CO/H$_2$O (30-60%) as eluent to yield fourteen fractions. It should be noted that the extraction process employed (80% Me$_2$CO over a period of two weeks) would most likely allow many black tea components to undergo autoxidation. However, in an attempt to elucidate the structure of TRs, known TF standards were initially analysed for their MALDI-TOF-MS characteristics. The main fragmentation patterns, involved cleavage of the gallate ester functions and retro-Diels-Alder (RDA) fission of the C-ring. The EtOAc fractions and the n-BuOH fraction were analysed. The EtOAc fractions yielded evidence, via degallation and RDA fragmentation, for the presence of compounds 12, 13, 14, 15, 31 and 32.

The n-BuOH fraction, taking fragmentation into account, yielded evidence for compounds with molecular masses of 1172, 1430, 1598 and 1750 amu. However, it should be noted that the mass spectra Menet et al. present in their work appear to be too 'clean'. It is the experience of the author that utilising α-cyano-4-hydroxycinnamic acid as a MALDI matrix (as utilised by Menet et al.) gives rise to a large number of oxidation-reduction dismutation species, centred around the molecular ion and this diminishes the S/N significantly, producing a mass spectrum that is difficult to calibrate [126]. However, based on the molecular ions, and fragmentation ions observed in the mass spectrum, Menet et al. proposed structural fragments for TRs, derived from catechins, such as a proanthocyanidin, or a theasinensin (those for 1172 (proanthocyanidin-type) and 1430 amu (theasinensin-type) are shown below). Menet et al. suggested that the formation of TRs consisted, in part, of the oxidative coupling between a 3,4,5-trihydroxyl moiety of gallate esters of a dimer (theasinensin- or proanthocyanidin-type), and a catechin derivative ((-)epigallocatechin gallate 1, (-)-epicatechin gallate 2, 3, (-)-epigallocatechin 4, (-)-epicatechin 5, 6, 7 and 8).
It was concluded by Menet et al. that, using MALDI-TOF-MS with the pulsed-ion extraction delay configuration, was a powerful technique for the analyses of TFs and TRs and it had allowed them, via fragmentation patterns, to assign structural features of TRs.

Recently, Drynan et al. have investigated a range of MALDI matrices (337 nm) for the high-resolution MALDI-TOF-MS analyses of tannic acid, TFs and TRs (isolated by caffeine precipitation from the twelve ‘world’ teas) with the pulsed-ion extraction delay configuration. Utilising tannic acid and TFs as model analytes, detailed mechanisms of secondary ionisation were proposed and this led to the successful development of a ‘redox-silent’ matrix for TR applications. Employing the ‘redox-silent’ matrix for TR analyses greatly reduced the oxidation-reduction dismutation aspects of the MALDI-TOF mass spectra (initially acquired via ‘conventional’ matrices), allowed assignment of molecular formulae and yielded insight into the degree of TR ‘polymerisation’ [126]. Interestingly, the mass spectra indicated that the molecular masses of TRs were not significantly greater than ~2000 amu.
Solid-state CP-MAS $^{13}$C-NMR spectroscopy has been utilised to analyse commercial samples of green and black tea by Martínez-Richa and Joseph-Nathan as a method of bulk chemical characterisation of these two types of teas [127]. The CP-MAS $^{13}$C-NMR spectrum (75 MHz) of black tea yielded evidence for catechin-type derivatives, terpene constituents, alkaloids and carbohydrate components. Spectral assignments were based on data obtained from model compounds and data presented in the literature. Of interest, and with specific reference to catechins, the observed $^{13}$C-NMR carbonyl resonances provided evidence for gallate ester derivatives of catechins and TFs ($^{13}$C-carbonyl resonance at 168.44 ppm). Additionally, a distinctive $^{13}$C resonance was observed for a tropolone-type carbonyl that did not belong to TFs ($^{13}$C-carbonyl resonance 11.1 ppm upfield with respect to the benzotropolone carbonyl measured for 12 in solution). From these data, it was hypothesised that TRs contain a tropolone-type carbonyl group. However, the gallate ester groups could not unequivocally be assigned to a structural fragment of TRs.

1.3.4 Theafulvin fraction

Column chromatography of black tea liquor on Solka-Floc cellulose, enabled Bailey et al. to isolated a tawny-coloured TR fraction which they termed, the ‘theafulvin’ fraction (TFu), the word was derived from the Latin word fulvus, meaning tawny [96]. Black tea liquor was applied to a Solka-Floc cellulose column and the column developed stepwise with MeOH, Me$_2$CO and 50% aqueous Me$_2$CO. Brown pigments, containing the TFu, eluted after the 50% aqueous Me$_2$CO wash. The brown pigments were dried in vacuo, dissolved in MeOH, precipitated by the addition of Et$_2$O and the precipitate filtered and washed with light petroleum (60-80 °C fraction) to yield the TFu.

The reversed-phase HPLC chromatogram, utilising 3D-PDA detection showed the TFu to be free from FGs. Direct-infusion thermospray MS showed the TFu to be complex and polymer-like. Additionally, the thermospray spectra varied with respect to time, additional evidence that the TFu was polymeric and polydisperse. The observed ions could not be correlated with intact flavanol oligomers and were suggested to be most likely fragment ions of larger polymers. Elemental analysis showed that the TFu
contained no nitrogen and these data ruled out the presence of incorporated caffeine or protein. IR data yielded evidence for phenol groups and aromatic rings. However, the wavenumber of the aromatic out-of-plane, C-H bending vibration was different from that observed for proanthocyanidin polymers, indicating that the intermonomer linkages in the TFu differed from those present in proanthocyanidin polymers. $^{13}$C-NMR data indicated that the TFu were galloylated flavanol polymers, containing intermonomer linkages, similar to those found in theasinensin-type dimers.

Additional reversed-phase HPLC fractionation of black tea has enabled Bailey and Nursten to reclassify Roberts' TR fractions [128]. The SI- and SII-TR fractions were shown to be mixtures of pigments classified as group I, II and III pigments (pigments excluded from, resolved by and unresolved by HPLC, respectively), including TFs, theaflavic acids, resolved TRs and unresolved TRs. Type I resolved pigments were yellow and exhibited an absorption maximum at ~400 nm while type II resolved pigments were brown and exhibited an absorption that decreased into the visible region. Type III pigments were designated as being those of the TFu [96]. 'Polar' (SI-like) and 'non-polar' (SII-like) fractions were separated on a Sep-Pak $C_{18}$ cartridge and were established as such by reversed-phase HPLC analyses. Fractions enriched in group I pigments were isolated using a Bond-Elut SCX cation exchange cartridge. The group I pigments were shown by PC and reversed-phase HPLC to be mixtures of anthocyanidin pigments of varying molecular masses. Also present in the group I pigment mixture was compound 90, as designated by Roberts [60,119]. Compound 90 was identified by reversed-phase HPLC (Hamilton PRP-1 column) and thermospray mass spectrometry. It was concluded by Bailey and Nursten that their reversed-phase HPLC methods were useful for the qualitative analysis of black tea pigments and that Sep-Pak cartridge-isolation could determine the amount of TRs in black tea.

Unexpected hyperchromic interactions have been observed by Bruschi et al. during reversed-phase HPLC studies on model solutions of isolated TFu, containing known amounts of pure flavanols, flavonols and caffeine [101]. The aim of their study was to validate a proposed method that would allow the quantification of classes of compounds associated with most isolates of the TRs (29, 92, theacitrins, flavanols, FGs and TFs), that
elute as relatively well-resolved peaks, and are situated above the reversed-phase HPLC-TR ‘Gaussian hump’ [13,95,129,130].

In previous, related studies, Bruschi et al. had quantified the HPLC-TR-associated peaks by calculating the peak-area above the hump. Their proposed quantification method would also include the area under the resolved peak to the horizontal baseline, incorporating the area of the underlying TFu, reversed-phase hump. Chromatographic methods for the isolation of TFu, which elute on reversed-phase columns as a hump, free of 29, 92, theacitrins, flavanols, FGs and TFs were devised by Bailey et al. [96] and this provided an opportunity for Bruschi et al. to test their proposed method using ‘pure’ TFu for control purposes.

Employing a previously published protocol for TFu isolation, [94,97] related to the method of Bailey et al., [96] Bruschi et al. isolated two TFu fractions under identical conditions. Aqueous TFu solutions were prepared which were individually spiked with (−)-epicatechin gallate 2, (−)-epicatechin 5, 6, 95, caffeine and rutin 102 (quercetin-3-O-rutinoside).

Statistical analysis of the reversed-phase HPLC chromatograms of the spiked TFu solutions, and the reversed-phase HPLC chromatograms of the pure test-compounds revealed that the presence of (−)-epicatechin gallate 2, (−)-epicatechin 5 and 95 produced a significant hyperchromic shift, whereby the peak-area above the TFu hump was larger than expected, compared with the peak-area of the pure test-compound alone. The hyperchromic effects were associated with the peaks, rather than the TFu hump.
Compound 6 and caffeine produced no effect while 102 produced a weak and inconsistent response. Interestingly, (-)-epigallocatechin gallate 1 was unstable under the experimental conditions and useful data could not be obtained. Although the two TFu isolates were prepared under the same conditions, Bruschi et al. observed that data from replicate experiments on the two TFu isolates were similar, but not identical, suggesting chemical differences between the two TFu fractions. They postulated that the hyperchromic effect they observed may involve interactions with certain components of the TFu which may vary in concentration from one isolate to another.

It was also observed that the model-solution test phenols differed in their retention times and it was suggested this variation may be an indication of variation in the composition of the TFu hump underlying the peaks, as well as variation in the intrinsic properties of the test-compounds. A mechanism for the hyperchromic effect could not be determined and it was postulated that the effect may be a manifestation of co-pigmentation. The electronic effect of co-pigmentation is to alter the molar extinction coefficient of the affected species (hyperchromism) and its $\lambda_{\text{max}}$ (bathochromism). Anthocyanins provide good examples of co-pigmentation in nature, and the presence of 90, a desoxyanthocyanidin, has been established in black tea. However, it was observed that there was no bathochromic shift (using a rapid scanning detector) associated with the observed hyperchromism, suggesting the bathochromic effect was very small or not present. Additionally, the hyperchromic effect observed in their study was limited to the colourless co-pigment (UV-region of the spectrum). Interestingly, hyperchromic effects below 400 nm have been reported for mixtures of 102 with malvin 103 (malvidin 3,5-diglucoside) [131]. The lack of hyperchromic response with 102 in the study conducted by Bruschi et al. compared with the hyperchromic response induced by (-)-epicatechin 5 led them to suggest that the effect they observed differed from classical co-pigmentation and that anthocyanin-like structures may not be involved in the process. As an aside, it was noted that the colour of black tea was unlikely to be influenced by this effect as the hyperchromism was limited to the UV-region of the spectrum.

Clearly, the hyperchromic effect observed by Bruschi et al. with regards to structural characteristics of the TFu (and hence TRs) is complex, and most likely involves a
multitude of species, synergistically, enhancing or diminishing the effect. Although these observed effects are scientifically interesting, they do not point to a well-defined characteristic of the TR structure.

1.3.5 Chemical degradation of TRs

Purified, EtOAc-soluble TRs, prepared from an aqueous EtOH extract of black tea have been fractionated by Sephadex LH-20 gel-permeation chromatography and then hydrolysed by alcoholic mineral acid by Cattell and Nursten during investigations into the nature of the interflavanoid linkages in the TRs [132]. Three TR sub-fractions were isolated via Sephadex LH-20 chromatography and each had a molecular mass of ~1500 amu (proposed pentameric oligomers). The three fractions were degradable by 0.5 M propanolic HCl to yield (-)-epigallocatechin gallate 1, (-)-epicatechin gallate 2, (-)-epigallocatechin 4, (-)-epicatechin 5, gallic acid 29, cyanidin 104 and delphinidin 105, although each sub-fraction contained different proportions of the aforementioned compounds. Evidence for the presence of benzotropolone moiety, in one of the sub-fractions, was based on the UV-Vis absorption spectrum of the sub-fraction which exhibited a small peak at 380 nm and a broad shoulder at ~455 nm. Similarly the TFs contain an absorption maximum at 380 nm and a small peak at ~460 nm. The EtOAc-soluble TRs were viewed as pentameric catechins, and their gallates, containing hydrolysable and non-hydrolysable interflavanoid links as well as benzotropolone moieties.

![Chemical structures](image)

Two TR fractions, the caffeine-precipitatable (CTR) and the TFu have been subjected to chemical antioxidative depolymerisation, using Fe$^{3+}$ salts dissolved in mineral acid (HCl) and $n$-BuOH (Porter's Reagents) by Powell and Clifford [133]. Their work was concerned with refining reversed-phase HPLC methods for monitoring, quantitatively,
additional transformations induced by Porter’s reagents during quantitation of proanthocyanidins via antioxidative depolymerisation.

The UV-Vis spectrum (530 nm) of the depolymerisation products of the CTR fraction yielded 104, 105 and pelargonidin 106. Using a predetermined conversion factor for the integrated peak areas, an original proanthocyanidin content (as procyanidin dimer equivalents) of 167-196 g kg\(^{-1}\) in the CTR was calculated. The TFu similarly yielded 104, 105 and 106, but the percentage of 105 was relatively larger than that observed for 104 and 106. Application of the predetermined conversion factor indicated an original proanthocyanidin content (as procyanidin dimer equivalents) of 84.5-103.0 g kg\(^{-1}\). However, the origin of the proanthocyanidins could not be determined. Three scenarios were speculated; (1) They may be fresh tea leaf proanthocyanidins that had survived the fermentation process and were present as contaminants or (2) they may have been products of the fermentation process and were present as contaminants or (3) they may have been novel products of the fermentation process and were incorporated in the structure of the TRs.

The UV-Vis spectrum (380 nm) of the antioxidative depolymerisation products of the TFu fraction showed the TFu to be essentially free of FGs. However, the CTR fraction contained small amounts of FGs. Interestingly, the reversed-phase-unresolvable CTR hump survived the antioxidative depolymerisation treatment, but eluted later than a non-treated CTR control, indicating the CTR fraction was more hydrophobic than before treatment. Two scenarios were speculated; (1) Transformation of certain polymeric units to anthocyanidins, and the simultaneous formation of aglycones from certain FGs, despite degallation, had rendered the hump material more hydrophobic and/or (2) carboxylic acid residues in the hump, whether in their original form, or formed during antioxidative depolymerisation had been converted to n-butyl esters via transesterification.

Additionally, the UV-Vis spectrum (280 nm) of the antioxidative depolymerisation products of the TFu and CTR fractions yielded evidence of n-butyl gallate (via spiking and spectral matching). Statistical analyses of calibration curves, utilising standards, indicated that the amount of galloyl esters associated with the untreated TFu and CTR
fractions was relatively low. Additional characterisation of the two TR fractions, via micro-analysis, determined the presence of elemental nitrogen at 5.6-6.3 g kg\(^{-1}\) in the CTR fraction; however, reversed-phase HPLC, before and after antioxidative depolymerisation treatment of the CTR fraction yielded no evidence of free or bound caffeine, respectively. The detection of elemental nitrogen led Powel and Clifford to hypothesise the presence of protein, nucleotides or a combination thereof. No elemental nitrogen was detected in the TFu fraction.

Neutral and acidic TRs (\(\text{n-BuOH-soluble}\)) have been prepared by a combination of solvent extraction, fractional precipitation and Toyopearl\(^*\) column chromatography and subsequently derivatised by Ozawa et al. during investigations into the nature of the intermonomer bonds that exist in the TRs [134]. Toyopearl\(^*\) is a hydrophilic, porous polymer-packing for gel-filtration. Both neutral and acidic TRs were methylated, degallated, and chemically degraded with KMnO\(_4\) under alkaline conditions and the degradation products were analysed by GC-MS and GC-SIM.

Among the methylated degradation products, detected by GC-MS and GC-SIM were methyl esters of 4-methoxybenzoic acid, 3,4-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, 3,4-dimethoxy-1,2-benzenedicarboxylic acid, 3,4-dimethoxy-1,5-benzenedicarboxylic acid, 3,4-dimethoxy-1,6-benzenedicarboxylic acid, 3,4,5-trimethoxy-1,2-benzenedicarboxylic acid, 4,5,6-trimethoxy-1,2,3-benzenetricarboxylic acid, 4,5,4',5'-tetramethoxy-2,2'-diphenic acid and 3,4,5,3'4',5'-hexamethoxy-2,2'-diphenic acid. The \(\text{n-BuOH-soluble}\), neutral TRs were hydrolysed with butanolic HCl and compounds 104 and 105 were identified in the hydrolysed mixture.

The results of these derivatisation and degradation experiments suggested that the TRs were heterogenous polymers composed of flavanols and their gallates which have a bond at C4, C6, C8, C2', C5' and C6' in the flavan-3-ol units. Additionally, apart from the C4-C8 or C4-C6 interflavanoid linkage, a C6'-C6' linkage was found. The linkages thus proposed are in general agreement to the linkages considered present by other workers; however, since there are variations in methods for preparing TRs, and TRs constitute a heterogeneous mixture by definition, this general agreement seems rational.
A partial structure of the polymeric TRs was proposed by Ozawa et al. that contained procyanidin-type, theasinensin-type and theaflavin-type moieties as well as an o-quinone functionality. Although there was no direct evidence for the presence of a benzotropolone-ring, or the o-quinone functionality, it was hypothesised by Ozawa et al. that the presence of these groups was conceivable since the brown colour of TRs cannot emanate from theasinensin-type or procyanidin-type moieties, whose respective chromophores (aromatic rings) are UV-active at ~280 nm. However, it is highly unlikely that an o-quinone functionality, being highly electrophilic, would survive in the presence of the nucleophilic flavanols.

1.3.6 Additional theoretical considerations

A relatively recent review by Haslam, has thrown additional light onto theoretical aspects that may be considered when attempting to understand the biogenesis of TRs [2]. Haslam paralleled the formation of TRs (by analogy with the oxidative self-condensation of B-ring model, pyrogallol 28) with melanogenesis. Eumelanin, which is the smallest polymeric structural unit considered part of the melanin structure, has been proposed to be formed from precursor L-tyrosine, via L-dopaquinone and a cyclic indolequinone. The bicyclic indolequinone contains an o-quinone moiety, exactly the same as present in o-quinone I (page 12). Haslam proposed a scheme for the self-condensation of 28, in solution, whereby II represents the hydroxyquinone of 28 (R = H) as an important intermediate (scheme 25). This idea was extended to include II representing the pyrogallol-type B-ring of catechins, such as (-)-epigallocatechin gallate 1 or (-)-epigallocatechin 4 (R = A- and C-rings of catechins). The relatively simple mechanism comprised the self-condensation of II, while a more complex mechanism was elaborated using a hexahydroxy-theasinensin-type compound as the precursor. Both mechanisms ultimately yield, via further intramolecular oxidation, the respective tetrameric/dimeric, cationic structure presented in scheme 25. Haslam suggested that the representative, conjugated cationic structure may be brown in colour as are the TRs.
Scheme 25: structural proposals of TR biogenesis (adapted) [2]

Although an interesting hypothesis, it seems unlikely that such a tetramer/dimer could form during black tea manufacturing, considering the large, debilitating steric hindrance associated with the catechin A- and C-rings. Concurrently, the unfavourable decrease in entropy would presumably require a high temperature for the formation of such a compound, much higher than the temperatures involved during black tea manufacturing.

1.3.7 Conclusion

It can be safe to pronounce that the term ‘TR’ has lost its intrinsic significance and these elusive, black tea pigments should rather be referred to as ‘uncharacterised higher molecular mass black tea polyphenols’. The hydrophilic theacitrins were most likely present in the TR fractions isolated by Roberts. As analytical, and chromatographic technologies evolve, more and more compounds that were most likely in the SI, S1a and
SII fractions, have been amenable to characterisation and therefore, by definition, are not considered to be TRs.

Additionally, it can be argued that TRs cannot be classified as being polymeric either, since there is no well-defined repeat-unit as is the case with ‘true’ polymers. A suggested schematic linear ‘pentameric’ structure, one of many possibilities that may be proposed for these ‘uncharacterised higher molecular mass black tea polyphenols’, based on some of the information summarised so far during the introduction to this thesis, is shown below.

Clearly, there is still an immense amount of experimental work that needs to be carried out with respect to elucidating the chemical structure of the higher molecular mass black tea polyphenols, and, appropriately, Haslam stated in his review: “theory guides, but experiment decides” [2].
1.4 Concluding remarks

1.4.1 Significance of structure elucidation

The literature has shown that attempts to chromatographically resolve the TR in black tea have generally failed. Arguments have been put forward for their unusual chromatographic behaviour, including: the presence of a large number of isomers, metal complexation and non-covalent interactions. The TRs have been spectroscopically and chemically characterised only in-part; they continue to remain a chemically elusive and ambiguous entity.

It is commercially important to elucidate the chemical structure of the TRs for a number of reasons. Structural characterisation and elucidation will improve the understanding of what contributes to the taste, colour and shelf-life of black tea-based products. TR elucidation will also allow for the identification of chemical compounds responsible for beneficial biological activity arising from the human consumption of black tea. Additionally, structure elucidation will allow unhindered registration of innovative black tea-based products.

1.4.2 Preliminary TR analyses

Fortunately, some progress has been made at the University of Surrey into structure elucidation by Kuhnert et al. [135]. Size-exclusion chromatography (SEC), Atomic-force microscopy (AFM) and Diffusion-NMR spectroscopy studies have indicated that the TRs are polyphenol compounds, with molecular masses ranging from 800–2000 amu.

ESI-MS, utilising an ion-trap for tandem experiments, presented further useful information. When a TR fraction was infused, the mass spectrum revealed a series of ion clusters between $m/z$ 800–2000. It was hypothesised that each cluster seemed to arise from an oligomerisation process, utilising the catechins as the most likely precursors. Three major types of dimerisation processes have been described in the introduction to the thesis which leads to the formation of TFs, theasinensins and theacitrins.
Reversed-phase LC-ESI-MS data suggested that the components that make up the TR fraction fall into two categories. The first category is a series of compounds that are resolved chromatographically, yielding mass spectra of defined ion clusters, and the second category is a group of compounds with similar masses that cannot be resolved chromatographically.

Both categories were studied in-depth using tandem ESI-MS. The main fragmentation patterns observed were retro-Diels-Alder fission and degallation. These fragmentation patterns are, again, indicative of catechin precursors. Although the advances in structure elucidation using tandem mass spectrometry provided a working hypothesis for the chemical structure of the TRs, none of the individual components could be chemically identified unambiguously for the following reasons:

- The presence of mass clusters suggested the presence of isomers.
- The presence of mass clusters suggested aggregation of compounds by non-covalent interactions (i.e., \( \pi-\pi \) stacking and hydrogen-bonding).
- The presence of mass clusters suggested redox processes involving catechol-\( \sigma \)-quinone redox couples.
- It was difficult to unambiguously distinguish between poly-gallates and polyphenol monomeric units due to mass coincidences.

1.5 Aim of project

The primary aim of the project was to synthesise, via solid-phase synthesis (SPS), resin-linked theaflavin 12, as this is one of the most important oxidative dimerisation processes encountered during black tea manufacturing. From the introduction to the thesis, which was concerned with the literature that has been made available on the chemistry of low molecular mass black tea polyphenols, it can be inferred that for the successful SPS of theaflavin 12, the precursor, \((-\)-epigallocatechin 4, needs to be linked to the resin for two reasons: (1) \((-\)-epicatechin 5 has the higher redox potential of the two precursors and, when \((-\)-epigallocatechin 4 was in solution during the oxidation step, it may undergo uncontrollable oligomerisation. (2) \((-\)-epigallocatechin 4 is prone to undergo oxidation-
reduction dismutation in solution and this may lead to undefined solution-phase intermediates.

However, compounds of mechanistic interest such as theadibenzotropolones and theasinensin-type trimers are not attainable via this route because (−)-epigallocatechin 4 and (−)-epicatechin 5 are devoid of the gallate ester, pyrogallol-type D-ring which is a prerequisite for the formation of theadibenzotropolones and theasinensin-type trimers.

The proposed linkage of (−)-epigallocatechin 4 to a resin, to yield the resin-linked immobilised catechin, can be represented by structure 107 (scheme 26). However, the actual linkage site varies depending on the functionality of the resin, the reaction types and conditions employed.

Scheme 26: proposed SPS methodology utilising 4 and 5 as precursors

Post-linkage, a one-electron oxidising agent (at least two equivalents) is applied, to yield resin-linked o-quinone 108. The o-quinone thus formed, may be oxidatively coupled with
(−)-epicatechin 5 to produce representative intermediate XVIII. This may lead to theaflavin 12, or a theasinensin- or a theacitrin-type dimer.

To the best of the author’s knowledge, there are no reports of linking an intact catechin directly to a resin. However, Tanaka et al. have described the SPS of (±)-epigallocatechin gallate, and the subsequent combinatorial synthesis of the methoxy protected derivatives, in an attempt to biologically evaluate the derivates for their antiallergic effect. Additionally, it was desired by Tanaka et al. to evaluate (±)-epigallocatechin gallate for its anticancer properties. The racemic catechin mixture was synthesised on Wang-linker resin [136].
CHAPTER 2

RESULTS AND DISCUSSION
2.1 Introduction

The theme adopted for the project was one of method development, rather than method optimisation, as this type of SPS chemistry has not been reported in the literature and the primary aim was the mechanistic understanding of the oxidative dimerisation processes of catechins, mimicking the process of black tea manufacturing.

To begin with, (-)-epicatechin 5 was used as a model compound to pave the way for SPS method development utilising (-)-epigallocatechin 4. It was envisaged that (-)-epigallocatechin 4 would behave in a similar manner to (-)-epicatechin 5 with respect to general reactivity, save oxidation.

The project relied, in part, on NMR data for reaction monitoring, and for final structure elucidation. It was therefore imperative to assign $^1$H- and $^{13}$C-NMR data for the original starting compounds, (-)-epigallocatechin 4 and (-)-epicatechin 5. As the project focused on method development, 2D-NMR spectroscopy was not routinely employed for structure elucidation.

2.1.1 $^1$H- and $^{13}$C-NMR assignments of (-)-epigallocatechin 4 and (-)-epicatechin 5

![Chemical structures of (-)-epigallocatechin 4 and (-)-epicatechin 5](image)

Although NMR measurements for reaction-monitoring were performed in $d_7$-MeOH and CDCl$_3$, and those of the synthetic derivatives of both (-)-epigallocatechin 4 and (-)-epicatechin 5 in $d_7$-MeOH, CDCl$_3$ and $d_6$-DMSO, the initial $^1$H- and $^{13}$C-NMR characterisation of (-)-epigallocatechin 4 and (-)-epicatechin 5 was performed in $d_6$-DMSO as (-)-epigallocatechin 4 and (-)-epicatechin 5 were found to be insoluble in CDCl$_3$. Also, the eventual synthetic strategy involved chemical protection of the phenolic hydroxyl groups in (-)-epigallocatechin 4 and (-)-epicatechin 5, and $d_6$-DMSO was considered the most suitable $^1$H-NMR solvent to check their disappearance as residual
protons in $d_6$-DMSO undergo only very slow exchange (cf. CDCl$_3$ and $d_6$-Me$_2$CO). Davis et al. have reported $^1$H- and $^{13}$C-NMR data for (-)-epigallocatechin 4 and (-)-epicatechin 5 in $d_6$-Me$_2$CO [50] and the $^1$H- (excluding those for phenolic hydroxyl protons) and $^{13}$C-NMR assignments for (-)-epicatechin 5 in $d_6$-DMSO using 2D methods have been reported by Shen et al. [137]. These data (Shen et al.) were used to assign the $^1$H- and $^{13}$C-NMR spectra of (-)-epicatechin 5 in $d_6$-DMSO, shown in figure 1 and figure 2, respectively (appendix). The $^1$H- and $^{13}$C-NMR assignments for (-)-epigallocatechin 4 in $d_6$-DMSO are shown in figure 3 and figure 4, respectively (appendix), and have currently been assigned by difference of the respective $^1$H- and $^{13}$C-NMR spectra of (-)-epigallocatechin 4 in comparison with those of (-)-epicatechin 5.

ChemDraw Ultra 9.0 [138] $^{13}$C-NMR chemical shift predictions were found to be useful when assigning $^{13}$C resonances of gel-phase $^{13}$C-NMR spectra (SPS reactions). ChemDraw Ultra 9.0 “estimates chemical shifts for all hydrogen or carbon atoms for which additivity rules are available. Following a hierarchical list, it first identifies key substructures of a molecule. A substructure provides the base value for the estimated shift. The data set for the $^1$H-NMR Shift tool contains 700 base values and about 2000 increments. The $^{13}$C-NMR Shift tool is based on 4000 parameters”. The software is capable of implementing models for ethylenes (cis/trans) and cyclohexanes (equatorial/axial).

However, ChemDraw Ultra 9.0 [138] has some limitations, and in the case of $^{13}$C-NMR, “it estimates over 95% of the shifts with a mean deviation of 0.29 ppm and standard deviation of 2.8 ppm”.

Figure 5 (below) shows the minimum-energy conformations (low level, MM2 force-field calculation) of (-)-epicatechin 5 and (-)-epigallocatechin 4 as calculated by Chem3D Ultra [138]. These conformations (both with a total steric energy of -16.4210 kcal mol$^{-1}$) have been included to reiterate the diastereotopic relationship of the heterocyclic C-ring hydrogen atoms, H-4$\alpha$ and H-4$\beta$ that renders them magnetically non-equivalent. In CDCl$_3$ and $d_6$-DMSO, H-4$\alpha$ generally resonates at a relatively lower frequency, while H-4$\beta$ generally resonates at a relatively higher frequency. In figure 5, carbon atoms are
depicted in grey, hydrogen atoms in white, and oxygen atoms are depicted in red, while their lone pairs of electrons are depicted in pink.

Figure 5: minimum-energy conformations (MM2 force-field) of 5 and 4 [138]
2.2 Solution-phase protecting groups

2.2.1 Introduction

Within the context of the initial SPS synthetic proposal attempted with \((-\)-epicatechin 5, solution-phase protection (and therefore a mimic for linking \((-\)-epicatechin 5 to a resin) was required to be at a hydroxyl group of the A-ring; this would leave the 3' and 4' hydroxyl groups free for subsequent oxidation reactions; as is mentioned in the introduction, the 4' hydroxyl group is fundamentally implicated in TR formation. So if the solution-phase protection chemistry could be optimised to yield single derivatives, linked via the hydroxyl groups at positions 5 or 7, a resin containing this functionality could be utilised, in principle (excluding steric hindrance and solvent effects), to immobilise \((-\)-epicatechin 5 for further oxidation. Being non-phenolic, linkage via the hydroxyl group at position 3 was not anticipated.

2.2.2 DHP protecting group

The rationale for considering the use of dihydropyran was that it is the active part of Ellman’s resin. Oxidation products of the catechins in basic media are highly reactive and so the first idea was to link \((-\)-epicatechin 5, under acidic conditions to prevent oxidation, via an ether bond [139], to commercially available Ellman’s resin. The proposed linkage of \((-\)-epicatechin 5 to the resin to produce the immobilised catechin is shown in scheme 27.

Scheme 27: proposed linkage of Ellman’s resin to 5 (arbitrary linkage position)
The strategy included acetylation of the free hydroxyl groups present in the immobilised catechin, to produce the fully protected acetyl analogue, which when followed by acidic cleavage of the ether linkage would produce the mono-hydroxyl derivative and hence elucidate the point of attachment of the ether linkage to (-)-epicatechin 5.

Initially, four preliminary solution-phase attempts using DHP (including a variation in reaction temperature) and utilising pyridinium p-toluenesulphonate (PPTS) as the acid catalyst, were undertaken in order to produce the THP ether of (-)-epicatechin 5. All attempts failed to produce the respective THP ethers as judged by $^1$H-NMR analyses in $d_6$-DMSO (spectra not shown). Additionally, the complexity of the crude $^1$H-NMR spectra due to THF contamination, made the analyses unreliable. It was therefore decided to monitor, by $^1$H-NMR spectroscopy, the progress of the reaction between (-)-epicatechin 5 and DHP. The solvent utilised in the monitoring experiment was $d_6$-DMSO. A stronger acid than that used by Pearson et al. was employed, i.e., PPTS ($pK_a$ ~5) was substituted for toluene-4-sulfonic acid (TsOH) ($pK_a$ ~7). However, it is known that catechins are susceptible to epimerisation under acidic conditions and substituting TsOH for PPTS was undertaken with reluctance.

2.2.3 Attempted solution-phase preparation of THP ether of (-)-epicatechin 5

A solution of (-)-epicatechin 5 in $d_6$-DMSO was treated with DHP and a catalytic amount of TsOH at 66 °C. The reaction was monitored by $^1$H-NMR spectroscopy and TLC (DCE:MeOH:Me$_2$CO, 60:30:26 v/v/v). TLC reaction-monitoring was inconclusive with streaking on the plate obscuring any possible product formation, however, $^1$H-NMR spectra were acquired (spectra not shown) on aliquots of the reaction mixture at two hours thirty minutes, twenty-two hours and ninety-eight hours thirty minutes of reaction time. The most significant $^1$H-NMR detail was the appearance of three new proton resonances after twenty-two hours, at 5.94, 6.89 and 6.94 ppm. These new resonances suggested the production of a new compound.

The reaction was allowed to proceed and a final $^1$H-NMR spectrum of the mixture was acquired after ninety-eight hours thirty minutes, whereupon it was observed that the three additional signals had disappeared. Two fractions were isolated by column
chromatography. It was interesting to note that in the $^1$H-NMR spectrum of the less polar fraction, in $d_7$-MeOH (spectrum not shown), there was the presence of a non-exchangeable or very slowly exchangeable peak at ~10 ppm. This was possibly indicative of the formate ester of (-)-epicatechin 5. The $^1$H-NMR spectra the two isolated fractions (spectra not shown) were assigned with reference to the $^1$H-NMR spectrum of (-)-epicatechin 5 in $d_6$-DMSO (figure 1). Mass spectral analyses (QTOF) were performed via both negative and positive-polarity (spectra not shown). The respective mass spectra indicated a RMM for the compound as 318 amu. This is 28 amu greater than the RMM of (-)-epicatechin 5. A formate ester derivative of (-)-epicatechin 5 contains this atomic mass unit difference. Although the data were not conclusive for the formate derivative, the THP of (-)-epicatechin 5 was clearly was not isolated from the reaction. The $^1$H-NMR spectrum of the second fraction, in $d_7$-MeOH (spectrum not shown), contained broad resonances which were not well defined. This was possibly indicative of an oligomeric component. This fraction was not fractionated any further. An attempt was made to scale-up the reaction and isolate, by column chromatography, the product from the reaction of (-)-epicatechin 5 with DHP. However, attempts to chromatographically resolve the components of the reaction mixture failed.

Additionally, attempting SPS using Ellman’s resin for the formation of the immobilised catechin gave no evidence for the addition of (-)-epicatechin 5 to Ellman’s resin either. Two attempts were made to produce the immobilised catechin. The methods used were a variation of that of Pearson et al., whereby THF was substituted for DCM as the polymer swelling-solvent and EtOH was substituted for MeOH in the PPTS cleavage-solvent mixture. Alternative cleavage solvent mixtures were also investigated, but the washings did not yield evidence for the addition of (-)-epicatechin 5 to Ellman’s resin.

It was hypothesised that the THP reaction failed because the acid catalyst (PPTS) utilised in the initial attempts to catalyse the reaction was perhaps not strong enough. There was also the possibility that acid-catalysed (TsOH) pyran ring-opening (C-ring) had adversely affected the integrity of the carbon backbone of (-)-epicatechin 5.
2.2.4 Other protecting groups (solution-phase)

The initial SPS experimental work concentrated on the attempted synthesis of the THP ether of (−)-epicatechin 5 under acidic conditions. After many attempted syntheses, it was concluded that (−)-epicatechin 5 did not add to DHP under the acidic conditions attempted (PPTS and TsOH). Therefore, the strategy was altered to find alternative protecting groups and hence, resin linkers. An investigation into NaOH-deprotonation of (−)-epicatechin 5, by Cren-Olive et al. [140] established that 82% B-ring deprotonation occurs with 0.25 eq NaOH and 63% B-ring deprotonation occurs with 1 eq NaOH. It was also observed that B-ring deprotonation seems increasingly favoured H₂O > MeOH > DMF > DMSO. Most surprisingly, their results suggested that (−)-epicatechin 5 was stable under alkaline conditions for at least several hours. Therefore, if a base could be used to facilitate protection (and ultimately linkage to a resin), the following protecting-group experiments become relevant (scheme 28).

Scheme 28: proposed protection of 5 (arbitrary point of attachment)
2.2.5 Stability of (-)-epicatechin 5 in aqueous alkali

In light of the work by Cren-Olive et al., preliminary experiments were carried out to establish whether (-)-epicatechin 5 is stable to deprotonation by alkali in H₂O/D₂O, and for what length of time. It was also hoped that information on the site(s) of deprotonation could be gained.

(-)-Epicatechin 5 was treated with 1.0 eq NaOD in D₂O in an NMR tube at 0.089 M. ¹H-NMR spectra were acquired fifteen minutes, forty-eight hours, three hundred and sixty hours and five hundred hours after mixing the reagents (spectra not shown). ¹H-NMR deprotonation spectra of (-)-catechin 5 in D₂O were assigned by difference of the ¹H-NMR spectrum of (-)-epicatechin 5 in ｄ₆-DMSO (figure 1). After fifteen minutes, it was observed in the ¹H-NMR spectrum that there was a loss of the resorcinol (A-ring) proton resonances attributed to H₆ and H₈. This observation suggested that deprotonation had occurred on the A-ring. The proposed deuterium exchange mechanism is shown in scheme 29. Deprotonation of the hydroxyl group at position 7 and/or position 5 renders the protons H₆ and H₈ appreciably more exchangeable than in their former environment due to resonance stabilisation of the phenoxy anion that involves carbanionic forms.

![Scheme 29: proposed deuterium exchange mechanism for proton at position 6](image)

It was also observed that fifteen minutes after mixing of reagents, there was little change in the catechol (B-ring) proton resonances at positions 2', 5' and 6'. This observation also suggested that it is the A-ring phenolic hydroxyl sites that become deprotonated. There
was also little change in the pyran ring (C-ring) proton resonances at position 4. It was observed that after forty-eight hours, a new set of pyran ring proton signals had appeared, suggesting that a new compound(s) had been formed. It was also hypothesised (due to signal broadening in the spectrum) that perhaps oxidation and polymerisation of (−)-epicatechin 5 had taken place.

A similar result was observed in NaOD/d₆-MeOH. However, in hindsight, NaOMe in d₆-MeOH could have been utilised instead of NaOD/d₆-MeOH to avoid the added complexity of the aqueous-phase, proton resonances. So, in the presence of 1.0 eq NaOD in D₂O or d₆-MeOH, deprotonation of (−)-epicatechin 5 occurs within minutes of the reactants being mixed. There is also evidence to suggest that it is the A-ring phenolic hydroxy site(s) that become deprotonated. While encouraging, this was surprising. The microscopic pKₐ values have been estimated for (−)-epicatechin 5 at 9.02−9.58 (with B-ring hydroxyl groups more acidic) [140]. Therefore the proportion of deprotonation should reflect the Kₐ of each site, under equilibrium conditions (scheme 30).

![Scheme 30: deprotonation equilibria of 5](image)

To begin with, attempts were made to trap the phenoxide group using aqueous or methanolic hydroxide with r-butyl chloride or acetyl chloride. Neither the r-butyl chloride nor the acetyl chloride gave any evidence by ¹H-NMR spectroscopy, of reacting with (−)-epicatechin 5.

2.2.6 Methylation of (−)-epicatechin 5

In the case of dimethyl sulfate with NaOD in D₂O and (−)-epicatechin 5, ¹H-NMR spectra were acquired seventeen minutes, eighteen hours thirty minutes and ninety-two hours thirty minutes after mixing the reagents (spectra not shown). The ¹H-NMR experiment gave evidence (seventeen minutes after the reactants being mixed) of two new methyl
(methoxy) singlets at 4.03 and 3.77 ppm. This observation suggested that two new methoxy derivatives of (−)-epicatechin 5 had been produced. The reaction was allowed to proceed to ninety-two hours thirty minutes, whereby the $^1$H-NMR spectrum revealed that the methyl singlet at 4.03 ppm had disappeared. The reaction mixture was acidified, salted-out and extracted into EtOAc. TLC revealed the presence of two new, less polar spots, in addition to a relatively small amount of non-reacted (−)-epicatechin 5. The extract was recovered as a yellow residue (11.9 mg). The $^1$H-NMR results were not unambiguous and negative-polarity LC-ESI-MS resolved four components with a mass corresponding to mono-methoxy derivatives of (−)-epicatechin 5 at m/z 303.1 (spectrum not shown), along with five possible di-methoxy derivatives of (−)-epicatechin 5 at m/z 317.1 and four possible tri-methoxy derivatives of (−)-epicatechin 5 at m/z 331.1. There was no MS evidence of a tetra-methoxy species. The LC-ESI-MS data suggested that the reaction was not selective, in that all the hydroxyl groups underwent methylation to varying extents. Although the methyl group is a good protecting group, it was postulated that the reaction was not selective due to the relatively small size of the methyl group. Therefore, the benzylation of (−)-epicatechin 5 was attempted as an alternative SPS method.

2.2.7 Benzylation of (−)-epicatechin 5

Initially, (−)-epicatechin 5 was treated with 1.0 eq NaOD/D$_2$O in d$_4$-MeOH at 0.089 M and 1.0 eq benzyol chloride, in an NMR-tube. $^1$H-NMR spectra were acquired at 300 MHz, thirty-five minutes, twenty-six hours fifteen minutes, seventy-four hours fifteen minutes and ninety-six hours thirty-six minutes after mixing the reagents (spectra not shown). It was observed in the $^1$H-NMR spectrum that after thirty-five minutes of the reactants being mixed, there was some non-reacted (−)-epicatechin 5. However, the increased complexity of the pyran ring (C-ring) proton resonances and the new proton resonance at 8.19 ppm (o-benzoate protons) suggested the presence of a benzoate ester in approximately 50% yield. A further new proton resonance at 8.00 ppm was indicative of the presence of benzoic acid (hydrolysis of benzyol chloride). After ninety-six hours thirty-six minutes of reaction time, in this first $^1$H-NMR spectroscopy experiment, the spectrum was much simpler, and the proton resonance at 8.19 ppm had almost
disappeared suggesting the decomposition of the benzoate ester, resulting in a majority of (−)-epicatechin 5 and benzoic acid. TLC analysis (DCE:MeOH:Me₂CO, 80:5:15 v/v/v) revealed a new, less polar spot (possibly the benzoate ester of (−)-epicatechin 5) as well as a relatively small amount of benzoic acid and (−)-epicatechin 5. Whether or not this was non-reacted (−)-epicatechin 5 or the decomposition product from the benzoate ester of (−)-epicatechin 5 was not ascertained.

The reaction was repeated three times (with varying reaction times) in an effort to isolate, by column chromatography and preparative TLC, the benzoate ester of (−)-epicatechin 5. However, the component with the proton resonance at 8.19 ppm, although prominent in crude ¹H-NMR spectra, was not amenable to isolation under the chromatographic conditions attempted (page 229).

In order to maximise the yield of the compound(s) containing the proton resonance at 8.19 ppm in the crude product, the mole ratios of reagents (benzoyl chloride and NaOD in D₂O (d₆-MeOH as NMR solvent)) were varied, keeping (−)-epicatechin 5 constant. Six ¹H-NMR spectroscopy experiments were performed and it was found that the mole ratio of reactants that produced the "cleanest" benzylation with the maximum proton-resonance intensity at 8.19 ppm was: (−)-epicatechin 5 (1.0 eq): benzoyl chloride (0.5 eq): NaOD (0.5 eq).

The optimised reaction was scaled-up. (−)-Epicatechin 5 was treated with NaOH(aq) and benzoyl chloride. After twenty-three minutes, the reaction was terminated. The crude reaction mixture was recovered as a reddish-orange crystalline material (146.1 mg). The crude mixture was subjected to column chromatography (EtOAc:DCM, 3:2 v/v). Inspection of their respective ¹H-NMR spectra, acquired in d₆-MeOH (spectra not shown), revealed that the least polar fraction (72.5 mg) contained the least amount of benzoyl by-products (i.e., benzoic acid or possibly sodium benzoate). This assumption was based on the almost absent proton resonance at 8.00 ppm. There was evidence that (−)-epicatechin 5 was still present in this fraction, and there was evidence for the presence of a mono-benzoate ester of (−)-epicatechin 5. TLC analyses (DCE:MeOH:Me₂CO, 60:30:26 v/v/v)
of the seven fractions revealed the presence of (-)-epicatechin 5 in small amounts in all fractions, along with benzoic acid in the two least polar fractions. All fractions displayed (in varying amounts) a spot less polar than (-)-epicatechin 5, which was attributed to the possible presence of the benzoate ester of (-)-epicatechin 5.

It was attempted to resolve chromatographically (reversed-phase, LC-ESI-MS) the components of the least polar fraction and to obtain alternative evidence (molecular ion) for the existence of the mono-benzoate ester of (-)-epicatechin 5 (calculated as 394.358 amu). It was also hoped that fragmentation patterns would provide insight into the point of attachment of the benzoate ester.

A sample of the least polar fraction was subjected to negative-polarity LC-ESI-MS. The chromatogram was comprised of three major components (from 13 to 33 minutes on a sixty minute binary eluent gradient) (spectrum not shown). One (relatively hydrophilic) early eluting component was well resolved (UV-Vis and TIC) and was assigned to (-)-epicatechin 5 with an observed negative-ion at m/z 289.1 (-85% IC) with retention time of 13.49 minutes. Of the two relatively hydrophobic components with an observed negative-ion at m/z 393.2 (100% IC), corresponding to the same m/z value as a mono-benzoate ester of (-)-epicatechin 5, the component with the retention time of 30.22 minutes displayed a broad peak. The other component, with a retention time of 32.31 minutes, was well resolved. An additional negative-ion in the TIC was observed at m/z 452.8 (~40% IC) and this was attributed to a molecule of AcOH hydrogen-bonded, to the B-ring of the mono-benzoate ester of (-)-epicatechin 5 (see scheme 31 below). Interestingly, there was no MS evidence to suggest the production of the di-, tri- and tetra-benzoate derivatives of (-)-epicatechin 5.

Column-overloading (reversed-phase) was eliminated as being responsible for the broad peak with the retention time of 30.22 minutes, because a sample, twice as concentrated yielded the same characteristic broad peak in a subsequent run. Surprisingly, consistency in its negative-polarity MS$^2$ analysis (m/z 243.1) revealed that the component that was responsible for the broad peak was chemically homogenous; therefore degradation of the compound was also eliminated as being responsible for the broad peak. The possibility of
proton exchange equilibrium, re-equilibrium being responsible for the broad peak was investigated using HPLC. TFA was substituted for AcOH as an ionisation suppression additive. However, due to a dilution error, the pH of the eluent (pH 2.5) was similar to the pH of the eluent used in the LC-ESI-MS analysis utilising AcOH as additive (pH 2.7).

The HPLC chromatogram was obtained under the same gradient conditions and the broad peak was well resolved in the UV-Vis chromatogram (retention time of 30.07 minutes). This observation casts doubt on proton-exchange being responsible for the broad peak, because, if it were (both analyses at similar pH), the HPLC chromatogram (UV-Vis) would not contain the well resolved peak. In an attempt to investigate further the nature of the broad peak, the sample was subjected to positive-polarity LC-ESI-MS. The UV-Vis and TIC chromatograms were relatively more complex with the broad peak at m/z 395.03 distinguishable as two components, overlapped, with retention times of 28.53 and 29.15 minutes. However, it is not clear why a change in polarity should chromatographically resolve the broad LC peak. These LC-ESI-MS data suggested that there were possibly three benzoate esters of (-)-epicatechin 5 present in the least polar fraction. The chemical structures of the negative-ions responsible for the negative-polarity LC-ESI-MS component, resolved at 30.22 minutes at m/z 393.2, are proposed to be those shown in scheme 31.
Scheme 31: proposed structures of negative-ions and negative-ion fragments

It is hypothesised that the negative-ion observed at m/z 452.8 (calculated as m/z 453.12) is attributed to a molecule of AcOH (present in the LC eluent as an ion-suppression additive), hydrogen bonded, to the B-ring of the mono-benzoate ester of (-)-epicatechin 5. This hypothesis could be further investigated using resorcinol and catechol as model A- and B-rings respectively. If resorcinol exhibits this hydrogen-bonding interaction, it would limit the likelihood of benzylation having occurred on the A-ring of (-)-epicatechin 5. It is proposed that the negative-ion observed at m/z 393.2 (calculated as m/z 393.10) is attributed to the mono-benzoate ester of (-)-epicatechin 5. However, from these MS data, it is not possible to deduce whether benzylation occurs at position 5 or 7, benzylation has been arbitrarily assigned to position 5. Furthermore, it is proposed that this negative-ion undergoes retro-Diels-Alder fission and the negative-ion fragment (calculated as m/z 241.05), containing the A-ring, is subsequently reduced [141]. The reduced negative-ion undergoes a toluene-type rearrangement to yield the resonance-stabilised anion responsible for the observed negative-ion at m/z 243.1 (calculated as m/z 243.10).
Alternative evidence (FT-IR carbonyl stretch) for the proposed existence (^H-NMR spectroscopy and LC-ESI-MS) of the mono-benzoyl ester of (−)-epicatechin 5 was sought. The least polar chromatographic fraction was subjected to FT-IR spectroscopy (spectrum not shown). The FT-IR spectrum of the proposed mono-benzoyl ester of (−)-epicatechin 5 was acquired as a nujol mull and was compared with the FT-IR spectrum of (−)-epicatechin 5 (acquired as a nujol mull). The most significant aspect of the spectrum is as follows: the introduction of a band at 1708 cm^{-1} (mono-benzoyl ester of (−)-epicatechin 5), which is evidence for the presence of a carbonyl group (C=O stretch). The literature states that in a nujol mull, resorcinol mono-benzoate 109 (a model for (−)-epicatechin 5 benzoylated in the A-ring) exhibits a carbonyl stretch at 1700 cm^{-1} (no intramolecular hydrogen-bonding) [142] whereas in CCl₄, catechol mono-benzoate 110 (a model for B-ring benzoylation) exhibits a carbonyl stretch at 1753 cm^{-1} (five-member intramolecular hydrogen-bonded ring) [143].

Based on the FT-IR and LC-ESI-MS data, it appears that benzoylation of (−)-epicatechin 5 occurs on the A-ring, probably at either hydroxyl sites and to a minor extent on one other hydroxyl site (most likely on the B-ring). It is proposed that the major product obtained for the benzoylation of (−)-epicatechin 5 under the optimised conditions is (−)-epicatechin-5-mono-benzoate 111. The comparatively high-frequency carbonyl absorption band at 1708 cm^{-1} observed in the FT-IR spectrum (cf. resorcinol mono-benzoate 109 at 1700 cm^{-1}) is evidence of a nine-member intramolecular hydrogen-bonded ring, incorporating the ester carbonyl oxygen atom (benzoylation at position 5) and the hydrogen atom of the secondary alcohol at position 3. The minimum-energy conformation (low level, MM2 force-field calculation) of compound 111, as shown, also
indicates the presence of this stabilising, intramolecular hydrogen-bonded, nine-member ring [138].

\[
\text{HO-}\begin{array}{c} \\
\text{HO} \\
\text{O} \\
\text{O} \\
\end{array}-\text{H} \\
\text{HO} \\
\text{OH} \\
\text{HO}
\]

2.2.8 Conclusion

In comparison with the methylation of (−)-epicatechin 5 with respect to selectivity, benzylation appears to be more selective. However, the ~100% regio-selectivity required for linkage to a solid-phase resin, bearing the same functionality as benzoyl chloride, was considered unattainable, and coupled with the added possibility that the benzoate ester linkage may hydrolyse under reaction conditions required for the oxidation step, other linkages/procedures were sought.
2.3 Linkage of protected (−)-epicatechin 5 to hydroxytentaigel resin

2.3.1 Introduction

The similar pKₐ’s (9.02–9.58) of the four phenolic hydroxyl groups in (−)-epicatechin 5 [140], rendered them unsuitable for their use as a potential site for the regio-selective, covalent linkage of (−)-epicatechin 5 to a SPS resin. A more suitable method was chosen that included the aliphatic, secondary 3-OH group (pKₐ ~ 16). Besides the aforementioned regio-selective advantage, linkage via the aliphatic, secondary 3-OH group in (−)-epicatechin 5, and later (−)-epigallocatechin 4, was considered important with respect to steric congestion; the mechanistically important B-ring would be extended outwards from the carbon ‘back-bone’ of the resin, hence facilitating, relatively more easily, chemical oxidation. In order to accomplish the regio-selective SPS attachment to a resin, the four phenolic hydroxyl groups required chemical ‘protection’ in order to render the aliphatic, secondary 3-OH group free for further reaction.

2.3.2 Selective phenol protection of (−)-epicatechin 5 by allylation

The allyl group is a well-documented protecting group for hydroxyl protection [144]. The ease of its introduction, the many mild reaction conditions for its removal and its general chemical stability, rendered it a suitable candidate for an orthogonal set. It was envisaged that the relative ease in deprotonation of the phenolic hydroxyl groups, compared with deprotonation of the aliphatic, secondary 3-OH group, would allow for the regio-selective protection, yielding tetraallyl-(−)-epicatechin 5a (scheme 32).

\[
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} \\
\text{HO} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} \\
\end{align*}
\]

\[
\text{R=CH}_2\text{CH}=\text{CH}_2
\]

j) BrCH₂CH=CH₂, K₂CO₃, DMF, rt, 170 hours (N₂).

Scheme 32: allylation of the phenolic hydroxyl groups in 5
The allylation reaction was performed under positive N$_2$ pressure, under mildly alkaline conditions (anhydrous potassium carbonate), in deoxygenated N,N-dimethylformamide. The chemically inert atmosphere was considered necessary, in order to prevent the oxidation of (−)-epicatechin 5 during allylation. A relatively large stoichiometric excess of allyl bromide (2.5 eq per phenolic hydroxyl group) was employed to maximise the reaction yield.

It was observed that nineteen hours after the addition of reagents, the initial yellow colour of the heterogeneous reaction mixture had become lighter (off-white-beige), indicating the reaction was proceeding. One hundred and eighteen hours, twenty minutes after the addition of reagents, the progress of the reaction was assessed via negative- and positive-polarity LC-ESI-MS (60 minute binary eluent gradient with acetic acid as negative-polarity ionisation-suppression additive) (spectra not shown) and TLC (DCE:MeOH:Me$_2$CO, 60:30:26 v/v/v). Both negative- and positive-polarity LC spectra yielded almost exact LC (retention time) and UV-Vis (PDA) data (260-280 nm). However, MS data for the respective allyl derivatives of (−)-epicatechin 5 were inconsistent and it was found, in general, that the negative-polarity spectrum yielded a TIC of less intensity, but higher sensitivity (S/N), than the positive-polarity spectrum, and therefore, the negative-polarity MS spectrum was considered more amenable to interpretation.

The negative-polarity mass spectrum yielded evidence of one, almost negligible, minor hexaallyl-(−)-epicatechin species at m/z 529.65 and two, minor distinguishable pentaallyl-(−)-epicatechin species at m/z 489.17. Both these observations were of interest, as it was expected that if a pentaallyl-(−)-epicatechin species were present, there would only be one, with additional single allylation expected to occur by conversion of the secondary, aliphatic 3-OH group. The fact that a hexaallyl species was also encountered suggested that, besides the possible conversion of the secondary, aliphatic 3-OH group, there was an additional mono-allylated species present. Fortunately, however, there was no evidence of a heptaallyl-(−)-epicatechin species. Not surprisingly, the negative-polarity spectrum also revealed the presence of one tetraallyl-(−)-epicatechin species at m/z 449.19. This
was the dominant UV-Vis and MS peak. There was also evidence of two minor triallyl-(-)-epicatechin species at \( m/z \) 409.16 and two, minor diallyl-(-)-epicatechin species at \( m/z \) 369.12. There was no evidence of a monoallyl-(-)-epicatechin species.

The progress of the reaction was simultaneously checked by a relatively polar TLC system (DCE:MeOH:Me₂CO, 60:30:26 v/v/v); originally designed to analyse (-)-epicatechin 5 and (-)-epigallocatechin 4, but not their allylation-transformation products. This system yielded evidence of one large spot, less polar than (-)-epicatechin 5. Experimentation with TLC systems led to the development of a more suitable eluent (EtOAc:hexane, 50:50 v/v) for distinguishing the aforementioned mono-, di-, tri-, tetra- and pentaallyl species. Utilising this system, five spots were resolved, being spread almost evenly along the length of the plate, with the most intense spot being the second least polar one, and this was attributed to tetraallyl-(-)-epicatechin 5a. The other four spots were of negligible intensity. Interestingly, the TLC system did not yield any evidence of the aforementioned hexaallyl species, with chromatographic resolution of isomers being limited, and thus establishing LC-ESI-MS as a more useful method of reaction monitoring. However, the instrument was not always available for use and in these instances, TLC and/or NMR spectroscopy was utilised.

After additional TLC monitoring (EtOAc:hexane, 50:50 v/v), and after one hundred and seventy hours of stirring, the reaction mixture was worked up to yield 913.8 mg crude product. The column chromatography-purification protocol was uncertain, and initially, only 441.0 mg of crude product was chromatographed over silica gel. The eluent consisted of a mixture of EtOAc and hexane with increasing proportions of EtOAc to yield eighty-six fractions. After TLC analysis (EtOAc:hexane, 50:50 v/v), fractions 1–52 (21.4 mg) were combined to yield a white solid and fractions 55–74 (220.9 mg) were combined to yield a colourless viscous liquid.

The \(^1\)H-NMR spectrum of fractions 1–52 was acquired in \( d_6 \)-DMSO (spectrum not shown). \(^1\)H-NMR spectrum indicated the presence of at least one pentaallyl-(-)-epicatechin species. The presence of two doublets of a doublet between 3.00 and 3.03 ppm, indicated the presence of a diastereotopic, alkyl \(-\text{CH}_2-\) of an allyl group
(represented by a, a' in figure 6) (appendix), being split by the single, vinyl-type proton (represented by 'b' in figure 6) (appendix). The vinyl-type proton was present as two singlets at 8.05 and 8.01 ppm. The presence of a doublet between 7.38 and 7.36 ppm, indicated the presence of the diastereotopic –CH₂ protons of an allyl group (represented by c, c' in figure 6) (appendix) being split by the single, vinyl-type proton. Of interest, was the fact that all phenolic protons had disappeared (8.70–9.09 ppm), indicating total phenolic protection by allylation. Additionally, the 3-OH doublet was still present between 4.81 and 4.82 ppm, and had disappeared after a D₂O exchange experiment, indicating that additional allylation had occurred at either position H6 or H8 of the A-ring. In combination with the reaction-monitoring LC-ESI-MS spectrum, it was proposed that the hexaallyl-(–)-epicatechin and two, pentaallyl-(–)-epicatechin species observed, were attributed to a di- and two mono- C-alkylated A-ring by-products, respectively.

The proposed mechanism for the formation of the O-allylated and C-alkylated species, under alkaline conditions, is shown below (scheme 33). During the initial stages of the reaction, and after deprotonation of the resorcinol-type A-ring hydroxyl groups, nucleophilic attack of an A-ring phenoxide anion onto allyl bromide yields the tetra-O-allylated species, 5a (route a). However, as a consequence of phenoxide anion tautomerism, nucleophilic attack of the carbanion, C-8, onto allyl bromide yields the di-C-alkylated and tetra-O-allylated species (route b). It is proposed that the di-C-alkylated species at m/z 529.65 occurs from alkylation at both C-8 and C-6 of the A-ring.
Scheme 33: proposed mechanism for the formation of the C-alkylated species

The $^1$H-NMR spectrum of fractions 55–74 in $d_6$-DMSO is shown in figure 6 (appendix). The spectrum was tentatively assigned as being that of tetraallyl-($-$)-epicatechin 5a (~95% purity). The spectrum was assigned by difference of the $^1$H-NMR spectrum of ($-$)-epicatechin 5 in $d_6$-DMSO. The two sets of nominally diastereotopic allyl protons, have been assigned as a, a’ ($-$CH$_2$-) and c, c’ (=CH$_2$).

Quenching the contents of the NMR-tube, with 50 µL D$_2$O (10% v/v) for a deuterium-exchange experiment, led to the disappearance of a doublet resonance (4.80–4.81 ppm). This doublet was attributed to the 3-OH proton (indicated in figure 7) (appendix). The deuterium-exchange experiment indicated that the secondary, aliphatic 3-OH was not allylated and was therefore free for further reaction.
The $^{13}$C-NMR spectrum of tetraallyl-(-)-epicatechin 5a in $d_6$-DMSO and 50 µL D$_2$O (10% v/v) is shown in figure 8 (appendix). The spectrum was assigned by difference of the $^{13}$C-NMR spectrum of (-)-epicatechin 5 in $d_6$-DMSO.

The $^1$H-NMR spectrum of tetraallyl-(-)-epicatechin 5a was also acquired in CDCl$_3$ (figure 9) (appendix), and its $^{13}$C-NMR spectrum acquired in CDCl$_3$ (figure 10) (appendix), to aid in further reaction monitoring and structure elucidation. These two spectra were assigned by comparison of their respective $^1$H- and $^{13}$C-NMR spectra with those of (-)-epicatechin 5 and tetraallyl-(-)-epicatechin 5a in $d_6$-DMSO.

A section (45–55 minutes) of the LC-ESI-MS chromatogram at 270 nm (75 minute binary eluent gradient) of the isolated product, tetraallyl-(-)-epicatechin 5a, after column chromatography, is shown in figure 11 (appendix). It can be seen that besides tetraallyl-(-)-epicatechin 5a ($m/z$ 449.19), with a LC retention time of 49.08 minutes, there is a relatively small LC peak ($m/z$ 489.17) with a retention time of 49.81 minutes and an almost negligible LC peak ($m/z$ 529.65) with a retention time of 50.28 minutes. Although these two additional peaks show evidence of C-alkylated impurities, based on $^1$H- and $^{13}$C-NMR data, these impurities were estimated to be approximately 5%.

The remainder of the crude reaction mixture (472.8 mg) was chromatographed over silica gel with a mixture of EtOAc and hexane with increasing amounts of EtOAc to yield seventy-two fractions. After TLC analysis, fractions 18–28 were combined to yield a white solid (13.9 mg), and fractions 50–59 were combined to yield a colourless viscous liquid (242.7 mg).

TLC analysis of combined fractions 55–74 from first column, and that of combined fractions 50–59 from the second column, yielded two spots with the same R$_f$ values, although TLC was unable to resolve the minor, C-alkylated impurities. These two sets of fractions were combined (463.6 mg) with a yield of 51.8% and utilised for the remainder of the project involving the synthesis of derivatives of tetraallyl-(-)-epicatechin 5a.
2.3.3 Selective phenol protection of (-)-epigallocatechin 4 by allylation

![Scheme 34: allylation of the phenolic hydroxyl groups in 4]

The reaction conditions employed for the allylation of (-)-epigallocatechin 4 were relatively similar to those employed for the allylation of (-)-epicatechin 5. The reaction was performed under positive N₂ pressure, under mildly alkaline conditions (anhydrous potassium carbonate) in deoxygenated N,N-dimethylformamide. Again, a relatively large stoichiometric excess of allyl bromide (2.4 eq per phenolic hydroxyl group) was employed in order to maximise the reaction yield. Additionally, the effect of adding twice as much K₂CO₃ relative to that used during the allylation of (-)-epicatechin 5, was also investigated (scheme 34).

It was observed that twenty-one hours five minutes, after the addition of reagents, the initial colour of the yellow heterogeneous reaction mixture had slightly darkened in colour (light-brown). This was in contrast to observations during the early stages of the allylation of (-)-epicatechin 5. Forty-eight hours, ten minutes after the addition of reagents, the progress of the reaction was assessed via negative-polarity LC-ESI-MS (75 minute binary eluent gradient with acetic acid as negative-polarity ionisation-suppression additive) (spectra not shown) and TLC (EtOAc:hexane, 50:50 v/v).

The negative-polarity mass spectrum yielded evidence of one, almost negligible, heptaallyl(-)-epigallocatechin species at m/z 585.32, and one minor and one major distinguishable hexaallyl(-)-epigallocatechin species at m/z 545.26. Fortunately, there was no evidence of an octaallyl(-)-epigallocatechin species. The presence of an
octaallyl(-)-epigallocatechin species would imply di-C-alkylation and di-O-allylation of the A-ring and allylation of the aliphatic, secondary 3-OH group. Not surprisingly, the negative-polarity spectrum also revealed the presence of one pentaallyl(-)-epigallocatechin species at \( m/z \) 505.22. This was the dominant UV-Vis and MS peak. There was also evidence for one minor, and one major, tetraallyl(-)-epigallocatechin species at \( m/z \) 465.19, one major triallyl(-)-epigallocatechin species at \( m/z \) 425.15, one negligible diallyl(-)-epigallocatechin species at \( m/z \) 385.08, and one negligible monoallyl(-)-epigallocatechin species at \( m/z \) 345.09.

TLC analysis resolved six spots, with the three least polar spots occupying the lower half of the plate, and the three most polar spots occupying the top half of the plate. These were attributed to the mono, di, tri, tetra, penta and hexaallyl(-)-epigallocatechin species. The heptaallyl(-)-epigallocatechin species was not resolved by the TLC system. The intensity of spots was approximately the same for the five least polar spots, with the most polar spot, of minimum intensity, being attributed to hexaallyl(-)-epigallocatechin.

After additional TLC (EtOAc:hexane, 50:50 v/v) and negative polarity LC-ESI-MS monitoring, and after one hundred and eighty-nine hours of stirring, the reaction mixture was worked up to yield 1236.9 mg crude product. The column chromatography-purification protocol was uncertain, and initially, only 740.7 mg of crude product was chromatographed over silica gel. The eluent consisted of a mixture of EtOAc and hexane with increasing proportions of EtOAc to yield seventy-two fractions. After TLC analysis (EtOAc:hexane, 50:50 v/v), fractions 20–26 were combined to yield a white solid (19.5 mg) and fractions 45–61 were combined to yield a colourless viscous liquid (400.2 mg).

The \(^1\)H-NMR spectrum of fractions 20–26 was acquired in \( d_6\)-DMSO (spectrum not shown). The \(^1\)H-NMR spectrum indicated the presence of at least one hexaallyl(-)-epigallocatechin species. The vinyl-type proton (represented by 'b') was present as two singlets at 8.06 and 8.01 ppm. The other two sets of allyl protons, \(-\text{CH}_2\)- and \(=\text{CH}_2\), were not readily observed due to low concentration of fractions 20–26 in the NMR-tube. Of interest was the fact that all phenolic protons had disappeared (7.96–9.09 ppm),
indicating total phenolic protection by allylation. Additionally, the 3-OH doublet was still present at ~4.9 ppm, and had disappeared after a D₂O exchange experiment, indicating that additional allylation had occurred at either position H6 or H8 of the A-ring. In combination with the reaction-monitoring LC-ESI-MS spectrum, it was proposed that the heptaallyl-(−)-epigallocatechin, and two, hexaallyl-(−)-epigallocatechin species observed, be attributed to a di- and two mono- C-alkylated A-ring by-products, respectively. It is proposed that the formation of these species follows the same mechanism as that proposed for the formation of the hexaallyl-(−)-epicatechin, and the two, pentaallyl-(−)-epicatechin species.

The ¹H-NMR spectrum of fractions 45–61 in d₆-DMSO is shown in figure 12 (appendix). The spectrum was tentatively assigned as being that of pentaallyl-(−)-epigallocatechin 4a (~95% purity). The spectrum was assigned by difference of the ¹H-NMR spectrum of (−)-epigallocatechin 4 in d₆-DMSO.

Quenching the contents of the NMR-tube, with 50 μL D₂O (10% v/v) for a deuterium-exchange experiment, led to the disappearance of a doublet resonance (4.79–4.80 ppm). This doublet was attributed to the 3-OH proton (indicated in figure 13) (appendix). The deuterium-exchange experiment indicated that the secondary, aliphatic 3-OH was not allylated and was therefore free for further reaction. The ¹³C-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a in d₆-DMSO and 50 μL D₂O (10% v/v) is shown in figure 14 (appendix). The spectrum was assigned by difference of the ¹³C-NMR spectrum of (−)-epigallocatechin 4 in d₆-DMSO.

The ¹H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a was also acquired in CDCl₃ (figure 15) (appendix), and its ¹³C-NMR spectrum, acquired in CDCl₃ (figure 16) (appendix) to aid in further reaction monitoring and structure elucidation. These two spectra were assigned by comparison of their respective ¹H- and ¹³C-NMR spectra with those of (−)-epigallocatechin 4 and pentaallyl-(−)-epigallocatechin 4a in d₆-DMSO.

The remainder of the crude reaction mixture (496.2 mg) was chromatographed over silica gel with a mixture of EtOAc and hexane with increasing polarity to yield sixty-five
fractions. After TLC analysis, fractions 46–57 were combined to yield a colourless viscous liquid (279.0 mg).

TLC analysis of combined fractions 45–61 from first column, and that of combined fractions 46–57 from the second column, yielded two spots with the same Rf values, although TLC was unable to resolve the minor, C-alkylated impurities. These two sets of fractions were combined (679.2 mg) with a yield of 67.0% and utilised for the remainder of the project involving the synthesis of derivatives of pentaallyl-(−)-epigallocatechin 4a.

Interestingly, adding twice as much K₂CO₃, relative to that used during the allylation of (−)-epicatechin 5, seemed to have no real effect on the reaction time.

2.3.4 Silylation and MeOH quench of tetraallyl-(−)-epicatechin 5a

\[ \begin{align*}
5a & \\
R &= \text{CH}_2\text{CH=CH}_2
\end{align*} \]

i) Pr₂Si(OtF)₂, pyridine, CDCl₃, rt, 45 minutes (N₂).
ii) CH₃OH, rt.

Scheme 35: silylation of 5a and methyl ether 5b formation

The use of the diisopropylsilyl group, to protect hydroxyl functions, was first reported by Corey and Hopkins, who used the group to successfully protect a range of diols, in quantitative yield, using non-commercially available diisopropylsilyl bis(trifluoromethanesulfonate), as the symmetrical starting compound [145]. The diisopropylsilyl group was later employed in the synthesis of hexanucleotide analogues containing diisopropylsilyl internucleotide linkages, by Cormier and Ogilvie [146]. However, the symmetrical nature of diisopropylsilyl bis(trifluoromethanesulfonate), and its high reactivity, led to a need to minimise, or avoid, production of symmetrically-linked dinucleotides which would arise from the diisopropylsilyl monotriflate intermediate reacting with the nucleotide starting compound. The dimerisation process
described above, would significantly diminish the yield of product, and also render isolation of the product more difficult.

The use of the diisopropylsilyl group, as a linker, to attach a hydroxyl function to a resin, has also been documented by Saha et al. [147], and was successfully utilised in linking oligodeoxynucleotide analogues to controlled-pore glass (CPG), via solid-phase automated synthesis, using the hindered base, 2,6-di-tert-butyl-4-methylpyridine (compared with imidazole) as proton scavenger. The use of the hindered base greatly diminished the production of symmetrical dimers, by increasing the activation energy of this undesired, reaction pathway.

In light of its ease of introduction, relative stability towards hydrolysis, and ease of removal using fluoride ion under neutral conditions [148,149], the diisopropylsilyl group was deemed the linker of choice, to covalently link tetraallyl-(−)-epicatechin 5a, to a commercially available hydroxytentagel resin - NovaSyn® TG hydroxy resin.

Initially, a small scale silylation reaction was attempted, to test the hypothesis that it was possible to link tetraallyl-(−)-epicatechin 5a, via the diisopropylsilyl linker, to hydroxytentagel resin. MeOH was chosen to represent the resin hydroxyl functionality due to the simplicity of $^1$H-NMR monitoring of the kinetics of formation of the expected product, tetraallyl-(−)-epicatechin silyl methyl ether 5b, and, any symmetrical dimer formation (scheme 35). Although MeOH is a relatively small molecule, compared with hydroxytentagel resin, it was envisaged that the resin hydroxyl functionality would ultimately react in a similar manner, although, it was borne in mind that steric hindrance may affect the kinetics of the resin reaction.

The amount of diisopropylsilyl bis(trifluoromethanesulfonate) utilised was kept to a minimum, to try and prevent dimer formation (2.0 eq). A large excess of pyridine (5.6 eq) was utilised, to prevent the formation of a benzylic cation intermediate (see scheme 1). Commercial diisopropylsilyl bis(trifluoromethanesulfonate) contains a trace amount of trifluoromethanesulfonic acid, as an impurity, and neutralisation of the acid with pyridine, would prevent protonation of the oxygen atom on the C-ring, which could lead to ring-
cleavage, with the concurrent formation of the undesired benzylic cation. Although pyridine is a relatively unhindered base, it was utilised in the initial investigation to see how it would fare against symmetrical dimer formation. It was also hoped that after fluoride-ion treatment, tetraallyl-(-)-epicatechin 5a would be isolated unchanged. This would pave the way for further reactions on the resin, using the diisopropylsilyl linker.

MeOH (HPLC grade) and distilled DCM (CaH$_2$) were stored over activated A3 molecular sieves under a dry, inert atmosphere (N$_2$) prior to use. Additionally, anhydrous CDCl$_3$ was stored over CaH$_2$ before use. It is essential to remove all traces of H$_2$O to maximise the reaction yield (to prevent hydrolysis of diisopropyl bis(trifluoromethanesulfonate)). All glassware was dried in an oven at 120 °C for at least twenty-four hours prior to the reaction.

It was observed that during the initial addition of pyridine to a flask, containing diisopropylsilyl bis(trifluoromethanesulfonate), white vapour was released, possibly due to neutralisation of trifluoromethanesulfonic acid. The first $^1$H-NMR spectrum was acquired sixteen minutes after initiation of the reaction (spectrum not shown). The most significant change in the spectrum, compared with that of tetraallyl-(-)-epicatechin 5a, was the down-field shift of H3 from approximately 4.27 ppm to 4.77 ppm, and that of H2 from 4.94 ppm to 5.04 ppm, indicating the formation of the monotriflate intermediate.

A further twelve, $^1$H-NMR spectra of the reaction mixture were acquired over a period of twenty-nine minutes (spectra not shown). Forty-five minutes after initiation of the reaction, and after no change in $^1$H-NMR spectra, a large stoichiometric excess of anhydrous MeOH (15 µL) was added to the NMR-tube via a pre-N$_2$ flushed 100 µL gas-tight syringe to quench the reaction mixture (active monotriflate intermediate). The NMR-tube was gently shaken to mix the contents and again, the generation of a small amount of white precipitate was observed.

The first $^1$H-NMR spectrum, of the MeOH quenched reaction mixture, was acquired three minutes after addition of MeOH. The most significant aspect of the $^1$H-NMR spectrum, was the generation of a singlet at 3.27 ppm. The singlet was hypothesised to belong to the
CH₃ group, of a methyl ether species. Another significant aspect of the ¹H-NMR spectrum, was the up-field shift of H₃ from 4.77 ppm to 4.45 ppm. Furthermore, ¹H-NMR spectra (not shown) of the MeOH quenched mixture were acquired twenty minutes after addition of MeOH, and one week after addition of MeOH and no change in the ¹H-NMR spectra were observed (and no visual change in contents of the NMR-tube), indicating that methyl ether formation occurs rapidly, and its formation is complete within three minutes of addition of MeOH.

As the ¹H-NMR spectrum of the MeOH-quenched NMR-tube was being acquired, the reaction mixture was similarly quenched with an excess of anhydrous MeOH (20 uL) via a pre-N₂ flushed 100 μL gas-tight syringe.

After work-up, the mixture was analysed by TLC (EtOAc:hexane, 50:50 v/v). It was confirmed that all tetraallyl-(-)-epicatechin 5a had been consumed. The translucent, light-yellow, crude mixture was present as a single spot, less polar than tetraallyl-(-)-epicatechin 5a. This spot was attributed to tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b.

The composition of the worked-up mixture was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient with acetic acid) (spectrum not shown). Positive-polarity ionisation was utilised as there are no sites readily available for deprotonation on the proposed product, tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b, or the expected dimer, tetraallyl-(-)-epicatechin diisopropylsilyl dimer 5c. Utilising positive-polarity conditions, it was envisaged that protonation of an oxygen atom would facilitate ionisation. UV-Vis (PDA) data (270-280 nm) and MS detection (m/z 150-1200) yielded evidence for the formation of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b, at retention time 15.15 minutes at m/z 595.31. This was the most dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the expected, mono-C-alkylated A-ring by-products were present at retention times of 15.97 and 16.16 minutes at m/z 635.37 (with a relative absorbance of 22 and 16%, respectively). The di-C-alkylated A-ring by-product was present at a retention time of 16.76 minutes at m/z 675.41 (with a relative absorbance of 5%). Also present, in surprisingly insignificant
amount (with a relative absorbance of <5%, assuming all species have the same molar extinction coefficient), was a species attributed to the symmetrical dimer, tetraallyl-(−)-epicatechin diisopropylsilyl dimer 5e, at a retention time of 17.09 minutes at m/z 1013.44, and its mono-C-alkylated A-ring by-product, present at retention time of 18.18 minutes at m/z 1053.51 (with a relative absorbance of 2%). The observed mono- and di-C-alkylated A-ring by-products were contaminants in the starting compound, tetraallyl-(−)epicatechin 5a, which was initially isolated by column chromatography.

2.3.4.1 Cleavage of the diisopropylsilyl methyl ether

Cleavage of the diisopropylsilyl group has been reported by Routledge et al. [150], utilising 1 M tetra-n-butylammonium fluoride (TBAF) in THF, by Kobori et al. [151], utilising 1 M TBAF/AcOH in THF, by Paterson et al. [149], using HF/pyridine, and by Lindsley et al. [148], utilising HF-pyridine/THF with TMSOMe as HF scavenger, or 20% TFA/DCM, or TBAF. In the current work, neutral conditions were deemed essential to minimise the generation of side-products or acid-catalysed C-ring cleavage.

The deprotection method consisted of treating the worked-up reaction mixture with triethylamine trihydrofluoride, buffered with two equivalents of triethylamine in anhydrous and deoxygenated THF. The mixture was stirred for thirty minutes, after which, an aliquot (~10 μL) of the colourless, homogenous reaction mixture was extracted into EtOAc. The EtOAc layer was washed with saturated NaHCO₃ and analysed by TLC (EtOAc:hexane, 50:50 v/v). The organic layer was present as a single spot. This spot was attributed to tetraallyl-(−)epicatechin 5a (standard).

The ¹H-NMR spectrum of the diisopropylsilyl-cleaved product, was acquired in CDCl₃ (spectrum not shown) to yield the exact same ¹H-NMR spectrum as that acquired for tetraallyl-(−)epicatechin 5a (figure 9) (appendix). The four, allyl protecting groups, were present, indicating that they are stable to fluoride treatment.
2.3.5 Stability of (−)-epigallocatechin 4 in the presence of fluoride

Following the success of fluoride deprotection/cleavage of the diisopropylsilyl linker, to yield tetraallyl-(−)-epicatechin 5a, the stability of (−)-epigallocatechin 4 in the presence of fluoride was checked. This was deemed necessary as this would emulate final cleavage conditions (buffered triethylamine trihydrofluoride) after SPS of the potential TR-type intermediates, bearing their reactive phenolic hydroxyl functional groups.

(−)-Epigallocatechin 4 was subjected to the diisopropylsilyl linker cleavage regime for a total of two hours. It was observed that all the (−)-epigallocatechin 4 was dissolved after one hour. After two hours, and with slight modification to the original cleavage regime, trimethylethoxysilane was added to the solution to consume any excess fluoride ion. The solution was stirred with a magnetic stirrer bar for thirty minutes. An aliquot of the translucent, light-yellow solution, was analysed by TLC (EtOAc:hexane:AcOH, 10:2:0.1 v/v/v). The mixture was present as two spots, one on the baseline which was attributed to triethylamine, and a less polar spot, with the same Rf value as (−)-epigallocatechin 4 (standard). This TLC result implied that (−)-epigallocatechin 4, with its five, free phenol groups, is stable to the modified diisopropylsilyl linker cleavage regime.

2.3.6 Silylation and linkage of tetraallyl-(−)-epicatechin 5a to hydroxytentaigel resin

\[ \text{Scheme 36: silylation and linkage of 5a to hydroxytentaigel resin, and formation of methyl ether (5b), and symmetrical dimer (5e)} \]
Hydroxytenta-gel resin (0.28 mmol g\(^{-1}\) loading) was dried over anhydrous P\(_2\)O\(_5\) in a vacuum-desiccator for six days prior to use, as it is essential to remove all traces of H\(_2\)O to maximise the reaction yield (prevention of diisopropyl bis(trifluoromethanesulfonate) hydrolysis).

MeOH (to quench excess diisopropylsilyl monotriflate, and to facilitate characterisation of off-resin products, such as symmetrical dimer 5e formation), and distilled DCM (CaH\(_2\)) were stored over activated A3 molecular sieves under a dry, inert atmosphere (N\(_2\)) prior to use.

The reaction was performed in a purpose-constructed, 'quick-fit' apparatus, under dry N\(_2\). The resin was treated with tetraallyl-(\(\rightarrow\))-epicatechin 5a, pyridine as base, and an excess of diisopropylsilyl bis(trifluoromethanesulfonate) in anhydrous DCM, for one hour and forty-two minutes (scheme 36). After the reaction, the resin was a pale, yellow colour, similar to the colour it was before the reaction. Any potentially active resin-diisopropylsilyl monotriflate sites were 'capped' with anhydrous MeOH and so was the supernatant, which contained excess reagents. After the reaction, the resin was washed with a variety of organic solvents and it was observed that the resin was almost white in colour, indicating that no detrimental side-reactions had taken place.

The MeOH-quenched, translucent, light-yellow supernatant was partitioned against saturated NaHCO\(_3\) and EtOAc. After work-up, the vacuum-dried organic layer was analysed by TLC (EtOAc:hexane, 50:50 v/v). It was confirmed that all tetraallyl-(\(\rightarrow\))-epicatechin 5a had been consumed. The organic residue was present as a single spot, relatively less polar than tetraallyl-(\(\rightarrow\))-epicatechin 5a. This observation suggested that tetraallyl-(\(\rightarrow\))-epicatechin diisopropylsilyl dimer 5e, or tetraallyl-(\(\rightarrow\))-epicatechin diisopropylsilyl methyl ether 5b, was present in the organic layer, having been formed during the reaction, or after the MeOH-quench, respectively.

The composition of the MeOH-quenched supernatant was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient without AcOH). A section of the LC-ESI-MS chromatogram (270-280 nm) is shown in figure 17 (appendix). Positive-polarity
ionisation was utilised as there are no sites readily available for deprotonation on the expected products, tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b and/or tetraallyl-(-)-epicatechin diisopropylsilyl dimer 5e. Utilising positive-polarity conditions, it was envisaged that protonation of an oxygen atom would facilitate ionisation. In contrast to the previous small-scale silylation of tetraallyl-(-)-epicatechin 5a, the LC-ESI-MS spectrum was attained without AcOH. This was rationalised on the basis that AcOH, as ion-suppression additive, may possibly assume a role in the hydrolysis of the diisopropylsilyl linker. UV-Vis (PDA) data (270-280 nm), and MS detection (m/z 150-1200), yielded evidence for the production of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b at retention time 14.11 minutes at m/z 595.32. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the expected two, mono-C-alkylated A-ring by-products of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b were present at retention times of 14.74 and 14.88 minutes at m/z 635.36 (with a relative absorbance of 22 and 15%, respectively). The di-C-alkylated A-ring by-product of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b was present at a retention time of 15.39 minutes at m/z 675.35 (with a relative absorbance of 4%). Also present, in a relatively significant amount (with a relative absorbance of 17%, assuming all species have the same absorbance), was the proposed symmetrical dimer, tetraallyl-(-)-epicatechin diisopropylsilyl dimer 5c, at retention time of 15.74 minutes at m/z 1013.49. Also present were its two, mono-C-alkylated A-ring by-products, at retention times of 16.26 and 16.41 minutes at m/z 1053.43 (with a relative absorbance of 4 and 9%, respectively). The mono- and di-C-alkylated A-ring by-products were contaminants in the starting compound, tetraallyl-(-)-epicatechin 5a, which was initially isolated by column chromatography. Interestingly, only one, mono-C-alkylated A-ring by-product of tetraallyl-(-)-epicatechin diisopropylsilyl dimer 5c, was observed in the small-scale diisopropylsilylation experiment during LC-ESI-MS analysis. This may have been due to different concentrations of analyte mixture in the preparation of samples to be analysed.

Additionally, compared with the small-scale diisopropylsilylation reaction of tetraallyl-(-)-epicatechin 5a (with 1H-NMR spectroscopy monitoring), and the LC-ESI-MS
conditions used to analyse the products (with AcOH additive), there was no apparent difference in the stability of the disisopropylsilyl linker; however, the effect of omitting AcOH was made evident by a decrease in component elution times, by approximately one minute. For the remainder of the LC-ESI-MS analyses of the synthetic products, containing a disisopropylsilyl linker, it was decided that AcOH should be omitted from the LC eluent, because its presence had no beneficial effect on the LC-ESI-MS spectrum, and, as a precaution, any potential artefacts that may arise from its use, may be avoided.

2.3.6.1 Chromatography of MeOH-quenched supernatant

The translucent, light-yellow residue was chromatographed over silica gel, with a mixture of EtOAc and hexane with increasing amounts of EtOAc to yield fifty-four fractions. After TLC analysis, fractions 1–17 (11.2 mg), fractions 19–29 (143.9 mg) and fractions 33–35 (14.7 mg) were combined to yield three, colourless viscous liquids, respectively.

The $^1$H-NMR spectrum of fractions 1–17 in CDCl$_3$ (spectrum not shown) suggested that the fractions were composed of a mixture of the two, mono-C-alkylated A-ring by-products and the di-C-alkylated A-ring by-product of tetraallyl(--)-epicatechin diisopropylsilyl methyl ether 5b. This was made evident by the presence of three sets of diisopropyl resonances between 0.77 and 1.07 ppm and the presence of three prominent methoxy CH$_3$ singlets at 3.42, 3.49 and 3.55 ppm.

The $^1$H-NMR spectrum of fractions 19–29 in CDCl$_3$ (figure 18) (appendix) was assigned as being that of tetraallyl(--)-epicatechin diisopropylsilyl methyl ether 5b (~95% purity). The spectrum was assigned by difference of the $^1$H-NMR spectrum of tetraallyl(--)-epicatechin 5a in CDCl$_3$. The $^{13}$C-NMR spectrum of tetraallyl(--)-epicatechin diisopropylsilyl methyl ether 5b in CDCl$_3$, is shown in figure 19 (appendix). The spectrum was assigned by difference of the $^{13}$C-NMR spectrum of tetraallyl(--)-epicatechin 5a in CDCl$_3$.

The $^1$H-NMR spectrum of fractions 33–35 in CDCl$_3$ (figure 20) (appendix) was assigned as being that of tetraallyl(--)-epicatechin diisopropylsilyl dimer 5e (~95% purity).
spectrum was assigned by difference of the $^1$H-NMR spectrum of tetraallyl-(-)-epicatechin 5a in CDCl$_3$. The $^{13}$C-NMR spectrum of tetraallyl-(-)-epicatechin diisopropylsilyl dimer 5c in CDCl$_3$, is shown in figure 21 (appendix). The spectrum was assigned by difference of the $^{13}$C-NMR spectrum of tetraallyl-(-)-epicatechin 5a in CDCl$_3$.

2.3.6.2 Cleavage of tetraallyl-(-)-epicatechin diisopropylsilyl ether tentagel resin

To analyse the on-resin products, a portion of the vacuum-dried resin (250.0 mg, 0.0656 mmol) was subjected to the diisopropylsilyl linker cleavage regime for one hour and five minutes, using buffered fluoride. At the end of the cleavage regime, trimethylethoxysilane was added to mixture to consume excess fluoride. The vacuum-dried, colourless residue (25.0 mg, 0.0555 mmol) was present in 84.6% yield and was analysed by TLC (EtOAc:hexane, 50:50 v/v). The residue contained one major component spot, with the same R$_f$ value as tetraallyl-(-)-epicatechin 5a (standard), and two less polar spots in almost negligible quantity. These two, less polar components, were attributed to the two mono-C-alkylated A-ring by-products of tetraallyl-(-)-epicatechin 5a, which the TLC system was unable to resolve, and the one, di-C-alkylated A-ring by-product of tetraallyl-(-)-epicatechin 5a (see LC-ESI-MS result below). It is possible that previous TLC analyses involving tetraallyl-(-)-epicatechin 5a, and its mono- and di-C-alkylated A-ring by-products were resolved, but their low concentration on the TLC plate may have eluded detection.

The composition of the fluoride-cleaved, tetraallyl-(-)-epicatechin diisopropylsilyl ether tentagel resin mixture, was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient without AcOH). A section of the LC-ESI-MS chromatogram (275 nm) is shown in figure 22 (appendix). UV-Vis (PDA) data, and MS detection ($m/z$ 150-1200), yielded evidence for the presence of tetraallyl-(-)-epicatechin 5a, at retention time 11.24 minutes at $m/z$ 451.21. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the two, mono-C-alkylated A-ring by-products of tetraallyl-(-)-epicatechin 5a, were present at a retention time of 12.00
minutes at \( m/z \) 491.25 (with a relative absorbance of 34%). These two isomers were not resolved on this occasion (relatively broad peak). Also present in the LC-ESI-MS spectrum was the di-C-alkylated A-ring by-product of tetraallyl-(−)-epicatechin 5a, at retention time 12.52 minutes at \( m/z \) 531.27 (with a relative absorbance of ~1%).

The \(^1\)H-NMR spectrum of the fluoride-cleaved, tetraallyl-(−)-epicatechin diisopropylsilyl ether tentagel resin mixture was acquired in CDCl\(_3\) (spectrum not shown). There was evidence for the presence of tetraallyl-(−)-epicatechin 5a, with its phenol groups still protected by the allyl protecting groups. There was evidence of minor contamination (~5%) evident in the spectrum from the various silyl species (fluoride and trimethylsilylsilane treatment). All the relevant proton resonances in the spectrum were present by comparison with the \(^1\)H-NMR spectrum of tetraallyl-(−)-epicatechin 5a in CDCl\(_3\).

The FT-IR (ATR) spectrum of the vacuum-dried, tetraallyl-(−)-epicatechin diisopropylsilyl ether tentagel resin, was acquired, and compared with that of the FT-IR (ATR) spectrum of hydroxytentagel resin (spectra not shown). The most significant aspects of the spectra are as follows: (1) The decrease in the wavenumber of the band at 2879 cm\(^{-1}\) (hydroxytentagel resin) to 2864 cm\(^{-1}\) (tetraallyl-(−)-epicatechin diisopropylsilyl ether tentagel resin), indicating the introduction of a methyl C-H asymmetrical/symmetrical stretch, as present in an isopropyl group. (2) The decrease in intensity of the band at 1467 cm\(^{-1}\), prominent in hydroxytentagel resin (attributed to an aromatic ring-stretch), indicating a modification of the environment of the resin ‘backbone’ and the PEG tether. (3) The disappearance of the band at 1360 cm\(^{-1}\) (hydroxytentagel resin), indicating the loss of a primary alcohol, in-plane bend. (4) The decrease in intensity of the band at 1145 cm\(^{-1}\), prominent in hydroxytentagel resin (attributed to an alkyl-substituted ether, C-O stretch), indicating a modification of the PEG tether, as would occur on substitution. (5) The modification and broadening of the band at 1099 cm\(^{-1}\) (hydroxytentagel resin), and its decrease to 1097 cm\(^{-1}\) (tetraallyl-(−)-epicatechin diisopropylsilyl ether tentagel resin), indicating the presence of an organic siloxane bond, R-C-O-Si-O-C-R [152,153], as present in the diisopropylsilyl linker. (6)
The disappearance of the band at 1061 cm\(^{-1}\) (hydroxytenta
gel resin), indicating the loss of a primary alcohol C-O stretch. (7) The decrease in the wavenumber of the band at 961 cm\(^{-1}\) (hydroxytenta
gel resin) to 951 cm\(^{-1}\) (tetraallyl-(\(-\))-epicatechin diisopropylsilyl ether tent
gel resin), indicating the introduction of a vinyl C-H out-of-plane bend, as present in the allyl protecting groups. Although this vinyl C-H out-of-plane bend is present in hydroxytenta
gel resin (1% DVB cross-linking), it is relatively hindered, and this may increase the frequency of the vibration. Furthermore, the introduction of the relatively ‘free’ allyl protecting groups, may therefore significantly lower the frequency of the vibration. (8) The introduction of a band at 885 cm\(^{-1}\) (tetraallyl-(\(-\))-epicatechin diisopropylsilyl ether tenta
gel resin), indicating the presence of a vinylidene C-H out of plane bend, as present in the allyl protecting groups. (9) The introduction of a band at 638 cm\(^{-1}\) (tetraallyl-(\(-\))-epicatechin diisopropylsilyl ether tenta
gel resin), indicating the presence of an aromatic C-H out-of-plane bend, as present in the A- and B-rings of the catechin moiety. Although these characteristic vibrations were diagnostic of an organic siloxane linkage, and provided evidence for the linkage of tetraallyl-(\(-\))-epicatechin 5a to hydroxytenta
gel resin, FT-IR (ATR) data should be analysed with caution during interpretation (presence of overtones, combination bands and hydrogen-bonding).

2.3.7 Conclusion

Although attachment of tetraallyl-(\(-\))-epicatechin 5a to hydroxytenta
gel resin was determined a success, the low resin loading (84.6%-based on mass of resin-cleaved product, with minor contamination from the various silyl species, i.e., from fluoride and trimethylethoxysilane treatment), and the formation of tetraallyl-(\(-\))-epicatechin diisopropylsilyl dimer 5c, rendered the synthetic method uneconomical. It is essential during SPS to maximise the yield of the linkage step (> 97%), as low resin loading negatively affects the overall yield, and the final SPS cleavage step may not yield any significant amount of product. Therefore, an alternative SPS strategy was sought which would produce a more efficient linkage step. No further SPS work, utilising (\(-\))-epigallocatechin 4 and hydroxytenta
gel resin was attempted, as it was hypothesised that
pentaallyl-(−)-epigallocatechin 4a would react in a similar manner to tetraallyl-(−)-epicatechin 5a, with respect to resin-loading and symmetrical dimer formation.
2.4 Linkage of protected (-)-epigallocatechin 4 intermediate to aminotentagel resin

2.4.1 Introduction

Solid-phase peptide synthesis is well established, and the general method employed for the stepwise synthesis of linear, di-, tri-, or oligopeptides, begins with the covalent linkage of an amino acid, through its C-terminus, via a linker, to a resin. The N-terminus is chemically protected during linkage of the C-terminus. The N-terminus is subsequently, selectively deprotected. The peptide-coupling step (to produce a dipeptide for example) consists of a condensation reaction, between the deprotected primary amine, of the resin-linked amino acid, and the C-terminus (N-terminus, protected) of the next amino acid in the sequence, with the concurrent formation of an amide bond.

Methods have been developed that produce highly efficient SPS peptide-coupling steps (amide bond formation), using sophisticated coupling reagents such as HATU. Based on the established success of SPS peptide chemistry, it was envisaged that the new synthetic method, for linking (-)-epigallocatechin 4 to a resin, in high yield, should employ the ease of introduction of the peptide bond, and also benefit from its general chemical robustness. However, it should be noted that the amide bond is susceptible to hydrolytic cleavage under extreme conditions (low or high pH).

An additional, high-yielding, and well established chemical reaction was needed that would complete the SPS method, without the requirement of additional, and synthetically cumbersome, protecting groups. ‘Click’-chemistry is a modular approach to organic syntheses that utilises only the most practical and reliable chemical transformations. A ‘click’-reaction, by definition, should be easy to perform (using readily available reagents), producing high reaction yields, without the need for chromatographic work-up. A number of reaction types fits this profile, including, 1,3-dipolar cycloadditions, hetero-Diels-Alder reactions, nucleophilic ring-opening reactions, carbonyl chemistry of the non-aldol type and addition to carbon-carbon multiple bonds [154]. An example of an ‘almost perfect’ ‘click’-reaction, is the Huisgen 1,3-dipolar cycloaddition of terminal
alkynes, with azides, to form 1,2,3-triazoles (five-member nitrogen-containing heterocycles) [155]. A major advantage of the reaction is the chemical orthogonality of the reaction conditions, without the need for protecting groups, and the inertness of the product heterocycle to strong bases, oxidation and reduction. During the ‘spring-loaded’, irreversible 1,3-dipolar cycloaddition, the azide acts as the 1,3-dipole whilst the alkyne acts as the dipolarophile.

Although alkynes and azides are highly energetic species, they are also among the least reactive of functional groups (on a kinetic basis) and, even under elevated temperatures, react very slowly. The dramatic rate increase in the regio-selective, copper(I) catalysed formation of 1,4-disubstituted 1,2,3-triazoles (as opposed to a mixture of 1,4- and 1,5-disubstituted 1,2,3-triazoles produced without the presence of the copper catalyst) has been utilised by Rostovtsev et al. to produce a wide range of 1,2,3-triazoles, under a range of pH (4–12) and solvent conditions (aqueous tert-butyl alcohol or ethanol, and H₂O without an organic co-solvent) [156].

In light of the versatility of this particular ‘click’-reaction, it seemed that another important aspect of the synthetic method should include the formation of the 1,2,3-triazole heterocycle, as part of the linking process. The combination of the two aforementioned reactions (peptide coupling, and 1,3-dipolar cycladdition), as a method to link (–)-epigallocatechin 4 to a resin, leads to two scenarios; (1) the amide bond can be introduced, and utilised to functionalise the required resin (aminotentagel) with an azide group, and the terminal alkyne can be introduced to functionalise the allyl-protected (–)-epigallocatechin 4 moiety, with the ‘click’-chemistry being performed as a SPS reaction (scheme 37). (2) the allyl-protected (–)-epigallocatechin 4 moiety can be functionalised, via a 1,3-dipolar cycladdition, with a carboxylic acid (the ‘click’ chemistry performed in solution utilising an azide-functionalised carboxylic acid), and the peptide-type coupling, performed as a SPS reaction (scheme 38).
Scheme 37: scenario 1

Scheme 38: scenario 2

The first scenario described above, requires the least effort in terms of reaction-monitoring (FT-IR (ATR) spectroscopy and gel-phase $^{13}$C-NMR spectroscopy), ease of introduction of the amide bond, and azide-functionalisation of the aminotentagel resin. The method also utilises one less solution-phase reaction (which requires work-up for
product characterisation). Therefore, the first scenario was initially attempted and is described below.

2.4.2 Synthesis of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b

![Chemical structure](image)

1) Pr₂Si(OEt)₂, pyridine, CH₂Cl₂, rt, 15 minutes (N₂).
2) HC≡C(CH₂)₂OH, rt, 1 hour.

Scheme 39: silylation and formation of 4b, and dimer 4c

1-Butynol (as the source of the precursor terminal alkyne) was stored over activated A4 molecular sieves under a dry, inert atmosphere (N₂) prior to use, while distilled DCM (CaH₂) was stored over activated A3 molecular sieves under a dry, inert atmosphere (N₂) prior to use. The amount of pyridine, used as base, was approximately double that used during silylation and MeOH-quenching of tetraallyl-(−)-epicatechin 5a (¹H-NMR spectroscopy monitoring), to prevent the formation of the unwanted benzylic cation intermediate (from traces of trifluoromethanesulfonic acid). The amount of diisopropylsilyl bis(trifluoromethanesulfonate) used was also increased by ~10%, as diisopropylsilyl bis(trifluoromethanesulfonate) decomposes/hydrolyses with time. All glassware was dried in an oven at 120 °C for at least twenty-four hours prior to the reaction.

The silylation reaction (scheme 39) was performed using the same experimental method as that used during the silylation and MeOH-quenching of tetraallyl-(−)-epicatechin 5a (¹H-NMR spectroscopy monitoring) (scheme 36).

168
After work-up, the organic residue was analysed by TLC (EtOAc:hexane, 50:50 v/v). It was confirmed that all pentaallyl-(-)-epigallocatechin 4a had been consumed. The crude mixture was present as a single spot, less polar than pentaallyl-(-)-epigallocatechin 4a and this spot was attributed to pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b.

The composition of the organic residue was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient without AcOH) (spectrum not shown). UV-Vis (PDA) data (270-280 nm) and MS detection (m/z 150-1200) yielded evidence for the production of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b at a retention time of 14.50 minutes at m/z 689.36. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the expected two, mono-C-alkylated A-ring by-products were present at retention times of 15.07 and 15.22 minutes at m/z 729.40 (with a relative absorbance of 18 and 12%, respectively). The di-C-alkylated A-ring by-product was present at a retention time of 15.70 minutes at m/z 769.42 (with a relative absorbance of 3%). Also present, in almost insignificant amount (with a relative absorbance of 1%, assuming all species have the same absorbance), was the symmetrical dimer, pentaallyl-(-)-epicatechin diisopropylsilyl dimer 4c at a retention time of 16.53 minutes at m/z 1125.54. Also present was its mono-C-alkylated A-ring by-product, present at a retention time of 16.85 minutes at m/z 1165.56 (with a relative absorbance of <0.1%). The mono- and di-C-alkylated A-ring by-products were contaminants in the starting compound, pentaallyl-(-)-epigallocatechin 4a, which was initially isolated by column chromatography. To be satisfied that no de-allylation occurs during silylation, or work-up, a mono-deallylation species was sought at m/z 649.32, but none was present in the LC-ESI-MS spectrum.

2.4.2.1 Chromatography and isolation of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b

The translucent, light-yellow organic residue was chromatographed over silica gel with a mixture of EtOAc and hexane, with increasing amounts of EtOAc to yield forty fractions.
A light-yellow band remained at the top of the column throughout the chromatographic separation (possibly attributable to oxidised/oligomerised material).

After TLC analysis, fractions 18–28 (568.7 mg) were combined to yield colourless pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b in 73.1% yield. Pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b was not as viscous as pentaallyl-(-)-epigallocatechin 4a. The symmetrical dimer, pentaallyl-(-)-epicatechin diisopropylsilyl dimer 4c, present in extremely low concentration, was not isolated.

The $^1$H-NMR spectrum of fractions 18–28 in CDCl$_3$ (figure 23) (appendix) was assigned as being that of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (~95% purity). The spectrum was assigned by difference of the $^1$H-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a. The $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b, in CDCl$_3$, is shown in figure 24 (appendix). The spectrum was assigned by difference of the $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a in CDCl$_3$.

2.4.3 SPS of 4-azidobenzoyl aminotentagel resin

![Scheme 40: peptide-type SPS of 4-azidobenzoyl aminotentagel resin](image)

i) 4-N$_3$C$_6$H$_5$CO$_2$H, HATU, Pr$_3$NEt, DMF, rt, 1 hour 55 minutes.

Aminotentagel resin (0.24 mmol g$^{-1}$ loading) was used, as supplied from the supplier, without further washing or drying.

4-Azidobenzoic acid was treated with peptide-coupling reagents, HATU and $N,N$-diisopropylethylamine in DMF, and then poured over, pre-swelled aminotentagel resin (DMF) that was contained in a sintered glass SPS column (scheme 40). The
heterogeneous reaction mixture was allowed to stir gently for one hour and fifty-five minutes, during which there was no change in observations. At the end of this period, a small sample of the resin was transferred to a small, sintered glass vacuum-funnel. The sample was washed with DMF and then MeCN to yield a slightly off-white resin (similar to the colour of the resin before reaction). A colour test was undertaken which detects free, primary amine groups, such as those present in aminotentagel resin. The colour-test is sensitive to 5 \( \mu \text{mol g}^{-1} \) resin with the presence of free, primary amine groups turning the resin, a brick-red colour [157]. However, at the end of the colour test, and after washing the resin with DMF, the colour of the resin was slightly off-white. It was therefore concluded that the peptide-type coupling reaction had proceeded to at least 97.9% completion.

Any remaining, free primary amine groups (maximum of 2.1%) were ‘capped’ with NMI, 40% 2,6-lutidine and 20% acetic anhydride in THF as a precaution. After completion of the ‘capping’ reaction, the resin was washed with a variety of organic solvents and it was observed that the resin was a slightly off-white colour, indicating that no detrimental side-reactions had taken place.

The advancement of SPS has required the development of techniques to monitor the progress of reactions, and to characterise the products of the reactions. FT-IR (ATR) spectroscopy can provide direct, semi-quantitative and qualitative information on the extent of a reaction, but, as stated previously, FT-IR (ATR) data should be analysed with caution.

Tentagel resins are polystyrene based and contain chains of PEG, grafted onto the sparsely cross-linked (1% DVB) hydrophobic core. The core provides mechanical stability, while the flexible PEG chains allow the resin to swell in a variety of organic solvents. The chains are generally considered chemically inert. The flexible PEG chains also allow the molecules that are covalently linked to them, via the linker, to possess molecular mobility, and in some cases, their mobility can approach that of solution-state mobility [158].
High-resolution MAS $^1$H-NMR spectroscopy has been utilised to study the spectral quality of a small molecule (aspartic acid), covalently linked to a range of commercially available resins [159]. It was found that the choice of resin, and solvent, can radically affect the $^1$H-NMR spectra; however, the extent of resin swelling had little effect on spectral quality. Additionally, it was found that the chemical structure of the resin played the dominant role in influencing $^1$H line-widths, while choice of solvent played a secondary role. Each resin analysed, exhibited a characteristic set of broad resonances, attributed to the polymer core and to the tether (extending chain), whilst the solvents DMF, CD$_2$Cl$_2$ and $d_6$-DMSO (in order), generated the 'best' $^1$H-NMR spectra. The optimal solvent should allow the resin to swell, and, concurrently, disperse the resin in a homogenous form within the RF coils. Vidal-Ferran et al. have used gel-phase $^{13}$C-NMR spectroscopy to monitor the progress of SPS reactions, using no more than a regular NMR spectrometer [160]. Vidal-Ferran et al. found that the relative distance of a carbon atom from the polymer core (which is an indication of its mobility), had a profound effect on the $^{13}$C line-widths, with those resonances of carbon atoms closer to the core, having broader signals. In view of this information, and the fact that no specialised NMR instrumentation was routinely available during this project, the progress, and characterisation of subsequent SPS reactions was, in-part, monitored/analysed by gel-phase $^{13}$C-NMR spectroscopy.

The gel-phase $^{13}$C-NMR spectrum of 4-azidobenzoyl aminotentagel resin was acquired in CDCl$_3$ (figure 25) (appendix). The most significant feature of the spectrum is the evidence for an amide bond, with the carbonyl carbon atom present at 173.1 ppm. Of interest and concern is the benzene-ring ‘back-bone hump’, situated between ~125 and 145 ppm, which is a consequence of the back-bone mobility in CDCl$_3$. This was considered a negative aspect of the spectrum as interpretation of the carbon resonances, resulting from the eventual synthesis of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, may be obscured and over-lapped, by the back-bone ‘hump’. The spectrum was assigned by difference of the gel-phase $^{13}$C-NMR spectrum of aminotentagel resin (spectrum not shown) and with the partial aid of ChemDraw Ultra 9.0 [138].
To investigate whether or not replacing CDCl₃ with d₆-DMSO would decrease the mobility of the benzene-ring back-bone [159], and hence allow for more accurate interpretation of further acquired gel-phase ¹³C-NMR spectra, the gel-phase ¹³C-NMR spectrum of aminotentagel resin was acquired in d₆-DMSO (spectrum not shown). However, besides all the resonances attributed to the ‘fine-structure’ of aminotentagel resin (PEG tether and vinyl cross-linker), the presence of the benzene-ring ‘back-bone hump’ was still evident, albeit with a slight up-field shift, and broadening (situated between ~115 and 135 ppm). As the majority of the NMR spectra acquired during the analyses of the synthetic products were acquired in CDCl₃, and no apparent benefit was obtained from replacing CDCl₃ with d₆-DMSO during acquisition of the aforementioned gel-phase ¹³C-NMR spectra, the remainder of the gel-phase ¹³C-NMR spectra were acquired in CDCl₃.

As a method of attaining additional supporting spectral data, the FT-IR (ATR) spectrum of the vacuum-dried, 4-azidobenzoyl aminotentagel resin was acquired (figure 26) (appendix) and compared with that of the FT-IR (ATR) spectrum of aminotentagel resin (figure 27) (appendix). The most significant aspects of the spectra are as follows; (1) the introduction of a band at 2123 cm⁻¹ (4-azidobenzoyl aminotentagel resin), indicating the presence of an azide group [161-163]. (2) The introduction of a band at 1655 cm⁻¹ (4-azidobenzoyl aminotentagel resin), indicating the presence of a carbonyl C=O stretch (amide ii), attributed to the amide link. (3) The increase in wavenumber, and intensity, of the band occurring at 1602 cm⁻¹ (aminotentagel resin), attributed to the primary amine, N-H bend, to 1603 cm⁻¹ (4-azidobenzoyl aminotentagel resin), indicating the presence of an amide N-H bend (amide i), which was also attributed to the amide link [152]. (4) The decrease, in intensity, of the band occurring at 1061 cm⁻¹ (aminotentagel resin), attributed to the primary amine, C-N stretch. However, the corresponding introduction of a band between 1130 cm⁻¹ and 1190 cm⁻¹ (4-azidobenzoyl aminotentagel resin) was not observed, most likely as a consequence of being overlapped by the band occurring at 1146 cm⁻¹, attributed to an alkyl-substituted ether (C-O stretch), as present in the dominating PEG tethers. Additionally, no differentiation could be made between the aliphatic primary amine, N-H stretch (aminotentagel resin), and the aliphatic amide, N-H stretch (4-
azidobenzoyl aminotentagel resin), which would normally occur between 3400 cm$^{-1}$ and 3300 cm$^{-1}$.

The combination of gel-phase $^{13}$C-NMR and FT-IR (ATR) data, and the results of the colour-test, were sufficient evidence for the successful synthesis of 4-azidobenzoyl aminotentagel resin, in high yield.

2.4.4 ‘Click’-chemistry with 4-azidobenzoyl aminotentagel resin and pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b

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\text{Scheme 41: Cu(I) catalysed SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin}
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In an effort to link pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b to 4-azidobenzoyl aminotentagel resin, the regio-selective SPS synthesis of the 1,4-disubstituted 1,2,3-triazole, via a Cu(I) catalysed Huisgen 1,3-dipolar cycloaddition of the resin-bound azide (4-azidobenzoyl aminotentagel resin) and the terminal alkyne of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b, was attempted (scheme 41), as described in the introduction to section 2.4.
The available literature generally recommends 0.25–10 mol% Cu(I) as catalyst, with respect to mole azide group [154,156]. Interestingly, even Cu(0) has been used as the regio-selective catalyst; however, reaction times are somewhat longer than the Cu(I) catalysed reactions [156]. Rostovtsev et al. have prepared the Cu(I) catalyst in situ, in aqueous media (1 mol%), utilising CuSO₄·5H₂O as the copper source and ascorbic acid and/or sodium ascorbate as the reductant. However, these were solution-phase reactions and did not contain the added complexity of inhomogeneity manifested during SPS. Somewhat more appropriate, a Cu(I) salt, CuI in THF, has successfully been utilised by Löber et al. to synthesise ‘click’ backbone amide linkers, using SPS [163]. However, THF is a relatively poor swelling-solvent for tentagel resins, and so the synthetic method involved utilising a Cu(I) salt that was soluble in DMF or MeCN. It is known that DMF is a relatively better solvent for swelling tentagel resins (by about 10%) compared with MeCN. However, tetrakis(acetonitrile)copper(I) hexafluorophosphate was chosen as the catalyst, and MeCN as solvent, as it was envisaged that the MeCN ligands would facilitate dissolution of the Cu(I) cation in MeCN, while the non-nucleophilic hexafluorophosphate anion would not participate in the reaction. An intermediate amount of Cu(I) catalyst (~5 mol%), to that recommended, was considered suitable for the initial attempt of the 1,3-dipolar cycloaddition.

As a precaution, deoxygenated MeCN was used during the SPS, as it was not known whether or not the presence of O₂ may oxidise the Cu(I) catalyst, forming unwanted oxidation-reduction dismutation copper products. The reduction product, Cu(0), if produced, may become trapped within the PEG tethers, negatively affecting the performance of the resin (forming a suspension and perhaps limiting solvent accessibility), and also rendering the washing procedure for the next synthetic step inefficient. Additionally, the oxidation product, Cu(II), if produced, would undermine the catalytic capability of the system.

4-Azidobenzoyl aminotentagel resin was treated with pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b and 4.5 mol% tetrakis(acetonitrile)copper(I) hexafluorophosphate in MeCN under an inert atmosphere (N₂), with stirring, in the dark. Light was excluded from the reaction as a precaution, as it was not known whether or not,
photochemically induced side-reactions would occur. Importantly, all the resin was immersed in solvent (4-azidobenzoyl aminotentagel resin sinks in MeCN). The supernatant was colourless, and there was no evidence of a precipitate being formed after the mixing of reagents. However, as anticipated, the resin took a while to swell (relative to using DMF as the reaction solvent).

As an aid to reaction monitoring, and due to a relatively quick sample preparation protocol (as opposed to gel-phase $^{13}$C-NMR spectroscopy), the FT-IR (ATR) spectrum of a sample of the slightly off-white, vacuum-dried ‘click’ resin was acquired after eighteen hours and fifteen minutes of reaction (spectrum not shown), and compared with that of the FT-IR (ATR) spectrum of 4-azidobenzoyl aminotentagel resin. The most significant aspect of the spectra are as follows: (1) The decrease in intensity of the band at 1466 cm$^{-1}$, prominent in 4-azidobenzoyl aminotentagel resin (attributed to an aromatic ring-stretch), indicating a modification of the environment of the resin ‘backbone’ and the 4-azidobenzoyl aminotentagel PEG tether. (2) The decrease in intensity of the band at 1146 cm$^{-1}$, prominent in 4-azidobenzoyl aminotentagel resin (attributed to an alkyl-substituted ether, C-O stretch), indicating a modification of the PEG tether as would occur on substitution. (3) The decrease in the wavenumber of the band at 962 cm$^{-1}$ (4-azidobenzoyl aminotentagel resin) to 948 cm$^{-1}$ (vacuum-dried ‘click’ resin), indicating the introduction of an aromatic C-H in-plane bend, as present in the A- and B-rings of the (−)-epigallocatechin 4 moiety. Although this aromatic C-H in-plane bend is present in 4-azidobenzoyl aminotentagel resin (1% DVB cross-linking), it is relatively hindered, and this may increase its frequency of vibration. The introduction of the relatively ‘free’ A- and B-rings of the (−)-epigallocatechin 4 moiety may significantly lower the frequency of the vibration. However, there was no apparent decrease in the azide stretch, observed at 2123 cm$^{-1}$ (4-azidobenzoyl aminotentagel resin). It is possible that the azide stretch has a relatively large infrared absorption cross-section [152], and, if this is the case, the FT-IR (ATR) spectrum can only provide, at the most, semi-quantitative data.

As an aside, fortunately, there was no change in the colour of the supernatant. A change from colourless to green would indicate detrimental Cu(I) oxidation.
Additional FT-IR (ATR) spectra of the vacuum-dried ‘click’ resin were acquired after one hundred and fourteen hours and fifty minutes, and also after one hundred and sixty-two hours and forty-five minutes (spectra not shown). However, there was no noticeable change in the spectra, compared with the spectrum of the vacuum-dried ‘click’ resin, which was acquired after eighteen hours and fifteen minutes. Of concern was the presence of the unaltered azide stretch at 2123 cm\(^{-1}\). Fortunately, there was no change in the colour of the supernatant and the vacuum-dried ‘click’ resin remained an off-white colour.

Unsure whether the reaction was proceeding at a suitable rate, it was attempted to increase the reaction rate by heating the mixture at 60 °C. Unfortunately, due to the experimental assembly, it was not possible to stir the mixture.

The FT-IR (ATR) spectrum of a sample of the slightly yellow-white, vacuum-dried ‘click’ resin was acquired after one hundred and eighty-five hours and fifty-five minutes of reaction time, or twenty-three hours and ten minutes at 60 °C (spectrum not shown), and compared with that acquired after eighteen hours and fifteen minutes. The most significant aspect of the spectrum is as follows: the increase of a band at 641 cm\(^{-1}\) (vacuum-dried ‘click’ resin after one hundred and eighty-five hours and fifty-five minutes), indicating the presence of an aromatic C-H out-of-plane bend, as present in the A- and B-rings of the catechin moiety. This observation was encouraging; however, the azide stretch at 2126 cm\(^{-1}\) was still prominent, but was slightly modified, with a shoulder at approximately 2110 cm\(^{-1}\) (the later, solution-phase synthesis of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d would show that the 1,4-disubstituted 1,2,3-triazole stretch occurs at 2111 cm\(^{-1}\). At the time of the current SPS ‘click’ reaction, various data bases were searched, but no literature was found that supplied this information).

At this point in time it was also evident that a small amount of MeCN had evaporated from the flask. There was also evidence of a slight precipitate, with yellow crystals present on the walls of the flask. Deoxygenated MeCN (500 uL) was added to the flask under positive N\(_2\) pressure, the crystals dissolved, and the precipitate disappeared.
(perhaps attributable to tetrakis(acetonitrile)copper(I) hexafluorophosphate). The pear-shaped flask was flushed with N₂, sealed with a glass stopper, and reheated to 60 °C for further reaction monitoring.

However, before being reheated, an aliquot of the supernatant was analysed by TLC (EtOAc:hexane, 50:50 v/v). The supernatant mixture was present as two spots, one on the baseline being attributed to Cu(I) and the other exhibiting the same R_f value as pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b. This was encouraging, as, after the ‘click’ reaction, it would be possible to isolate, non-reacted, pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b for further use.

As the reaction continued to proceed, and after two hundred and ten hours and twenty-five minutes (forty-seven hours and forty minutes at 60 °C) of reaction time, a small sample of the ‘click’ resin was washed and visually observed for a change in colour. However, there was no significant change in observations, with the ‘click’ resin still being a slightly, yellow-white colour. The reaction was allowed to continue, and after two hundred and thirty-four hours and twenty-five minutes (seventy-one hours and forty minutes at 60 °C) of reaction time, a small sample of the ‘click’ resin was washed and visually observed for a change in colour. At this stage it was observed that the ‘click’ resin was a deep-yellow colour. This provided visual evidence that the reaction may be complete.

The ‘click’ resin was washed with MeCN to remove tetrakis(acetonitrile)copper(I) hexafluorophosphate. The filtrate was dried in vacuo (removal of MeCN) to constant weight, to yield the crude-filtrate residue of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (86.1 mg), presumably containing tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.8 mg). Assuming the SPS of 4-azidobenzoyl aminotentagel resin proceeded to 100% completion, the yield of the current reaction, as calculated by the mass of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b consumed, was 98.5%.
The vacuum-dried MeCN-filtrate was analysed by TLC (EtOAc:hexane, 50:50 v/v). TLC analysis indicated the presence of two compounds in the filtrate, one on the baseline which was attributed to Cu(I), and the other, exhibited the same Rf value as pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b.

The ‘click’ resin was further washed with DMF and a large excess of 0.5 M sodium diethylidithiocarbamate in DMF. Sodium diethylidithiocarbamate is generally an efficient ligand for sequestering metals such as Fe, Zn and Mn. It was hoped that it would also sequester any copper species remaining ‘trapped’ in the PEG tethers, if any. After the sequestering treatment, the resin was washed with a variety of organic solvents and it was observed that the resin still retained its deep-yellow colour, an indication that the ‘click’ reaction may be complete.

The FT-IR (ATR) spectrum of a sample of the deep-yellow, vacuum-dried ‘click’ resin was acquired (spectrum not shown), and compared with that of the FT-IR (ATR) spectrum acquired after one hundred and eighty-five hours and fifty-five minutes of reaction time, or twenty-three hours and ten minutes at 60 °C. The most significant aspects of the spectrum are as follows: (1) A drastic increase in intensity of the band at 641 cm⁻¹. This band was considered an indication of the presence of an aromatic C-H out-of-plane bend, as present in the A- and B-rings of the catechin moiety. (2) The azide stretch at 2124 cm⁻¹ was still present, but the intensity of the shoulder at approximately 2110 cm⁻¹ had increased (evidence for the production of the 1,4-disubstituted 1,2,3-triazole). The FT-IR (ATR) spectrum the yellow, vacuum-dried ‘click’ resin was also compared with that of the FT-IR (ATR) spectrum of 4-azidobenzoyl aminotentagel resin. The most significant aspect of the spectrum is as follows: the modification and broadening of the band at 1099 cm⁻¹ (4-azidobenzoyl aminotentagel resin) and its decrease to 1095 cm⁻¹ (vacuum-dried ‘click’ resin), indicating the presence of an organic siloxane bond, R-C-O-Si-O-C-R [152,153], as present in the diisopropylsilyl linker.

The gel-phase ¹³C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin was acquired in CDCl₃ (figure 28) (appendix). The most significant features of the spectrum are: (1) The carbonyl resonance
at 173.1 ppm. (2) The 1,2,3-triazole resonances at 141.3 and 126.4 ppm. (3) The diisopropyl resonances at 6.7, 7.7, 18.8 and 23.8 ppm. (4) The allyl protecting group resonances at 134.9, 124.0, and 70.2–75.5 ppm. However, it is not certain that the resonance at 134.9 ppm emanates from the allyl group, and this particular carbon resonance (labelled ‘b’), may be suppressed by the background resonance of the benzene-ring back-bone ‘hump’.

The $^{13}$C resonances were assigned by comparison with the gel-phase $^{13}$C-NMR spectrum of 4-azidobenzoyl aminotentagel resin in CDCl$_3$, by comparison with the solution-phase $^{13}$C-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyloyx ethyl-1,2,3-triazolebenzoic acid 4d in $d_6$-DMSO (see later synthesis via the second scenario), and with the partial aid of ChemDraw Ultra 9.0 [138]. It should be noted that pentaallyl-(−)-epigallocatechin diisopropylsilyloyx ethyl-1,2,3-triazolebenzoic acid 4d is not soluble in CDCl$_3$ and therefore, $^{13}$C-NMR spectra were not easily correlated. An additional complication arose when assigning the $^{13}$C resonances, as it was not known whether unidentified peaks were attributed to the baseline. Unfortunately, 2D-NMR spectroscopic methods were not possible to perform because the $^1$H line-widths were extremely broad. The $^1$H line-broadening phenomenon may be alleviated, to a certain extent, utilising MAS.

2.4.4.1 Cleavage of pentaallyl-(−)-epigallocatechin diisopropylsilyloyx ethyl-1,2,3-triazolebenzoyl aminotentagel resin

To analyse the on-resin products, and the cleaved resin (efficiency of cleavage protocol), a portion of the vacuum-dried resin (109.4 mg, 0.0263 mmol) was subjected to the diisopropylsilyl linker cleavage regime for two hours and thirty minutes (in an attempt to maximise the yield) using buffered fluoride. At the end of the cleavage regime, trimethylethoxysilane was added to the mixture to consume excess fluoride. The vacuum-dried colourless residue was analysed by TLC (EtOAc:hexane, 50:50 v/v). The residue contained only one spot with same $R_f$ value as pentaallyl-(−)-epigallocatechin 4a. It is possible that the mono- and di-C-alkylated A-ring by-products of pentaallyl-(−)-epigallocatechin 4a were present, but their low concentration on the TLC plate may have
eluded detection (see LC-ESI-MS analysis below). Pentaallyl-(−)-epigallocatechin 4a (8.8 mg, 0.0174 mmol) was present in 66.1% yield.

The composition of the fluoride-cleaved pentaallyl-(−)-epigallocatechin disisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin mixture was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient without AcOH) (spectrum not shown). UV-Vis (PDA) data (275 nm) and MS detection (m/z 150-1200) yielded evidence for pentaallyl-(−)-epigallocatechin 4a at a retention time 12.12 minutes at m/z 507.23. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the expected two, mono-C-alkylated A-ring by-products of pentaallyl-(−)-epigallocatechin 4a were present at a retention time of 12.96 minutes at m/z 547.26 (with a relative absorbance of 24%). These two isomers were not resolved on this occasion (relatively broad peak). Also present in the LC-ESI-MS spectrum was the di-C-alkylated A-ring by-product of pentaallyl-(−)-epigallocatechin 4a at a retention time 13.57 minutes at m/z 587.30 (with a relative absorbance of 8%).

The $^1$H-NMR spectrum of the fluoride-cleaved pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin mixture, was acquired in CDCl$_3$ (spectrum not shown). There was evidence for the presence of pentaallyl-(−)-epigallocatechin 4a, with its phenol groups still protected by the allyl protecting groups, with minor contamination (~5%) evident in the spectrum from the various silyl species (fluoride and trimethylethoxysilane treatment). All the relevant proton resonances in the spectrum were present by comparison with the $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a in CDCl$_3$.

The gel-phase $^{13}$C-NMR spectrum of the ‘cleaved’ pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, was acquired in CDCl$_3$ (figure 29) (appendix). The most significant feature of the spectrum is the disappearance of the resonances attributed to the allyl-protected (−)-epigallocatechin moiety, assigned in figure 28. However, there was still evidence for the presence of the
diisopropyl resonances, indicating that the cleavage regime was not 100% efficient. It appears there may be a mixture of two ‘cleaved’ resins (see structures below).

![Chemical Structures](image)

The FT-IR (ATR) spectrum of the vacuum-dried, fluoride-cleaved, pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (spectrum not shown) was acquired, and compared with the FT-IR (ATR) spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (spectrum not shown). The most significant aspect of the spectrum is as follows: the disappearance of the band at 641 cm\(^{-1}\) (pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin). This band was considered an indication of the presence of an aromatic C-H out-of-plane bend, as present in the A- and B-rings of the catechin moiety. However, there was also evidence for an organic siloxane band at 1094 cm\(^{-1}\), an indication that the cleavage protocol was not 100% efficient.

The results presented above indicate that the SPS ‘click’ chemistry was successful. However, the yield of the reaction was considered too low (based on the mass of fluoride-cleaved residue; however, gel-phase \(^{13}\)C-NMR and FT-IR (ATR) data suggested that not all the 1,2,3-triazole-linked pentaallyl-(-)-epigallocatechin 4a was cleaved during the fluoride-cleavage protocol). Additionally, the time taken for the reaction to proceed was considered ‘excessive’.
The yield of the reaction, based on the mass of fluoride-cleaved residue, is in contradiction to the yield of the reaction proposed by gravimetric analysis of the mass of pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether 4b consumed during the SPS ‘click’ reaction. This observation suggests that further refinement of quantitative analysis techniques was required.

It should be noted that heating the reaction mixture to 60 °C seemed to have a significant, enhancing effect on the rate of reaction. As an alternative method for linkage of pentaallyl(-)-epigallocatechin 4a to the resin, and to compare the two scenarios outlined in the introduction to section 2.4, the second scenario was attempted, and is described below, beginning with the Cu(I) catalysed synthesis of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d.

2.4.5 'Click'-chemistry synthesis of pentaallyl(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d

![Scheme 42: Cu(I) catalysed synthesis of 4d](image)

*Scheme 42: Cu(I) catalysed synthesis of 4d*

SPS ‘click’ chemistry was shown to be successful; however, a low yield (66.1%) and an excessive amount of reaction time rendered the first scenario, described in the introduction to section 2.4, uneconomical. The Cu(I) catalysed, Huisgen 1,3-dipolar cycloaddition was therefore attempted as a solution-phase reaction (scheme 42).
4-Azidobenzoic acid was treated with a 10% mole excess of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b and 5.1 mol% tetrakis(acetonitrile)copper(I) hexafluorophosphate in MeCN under an inert atmosphere (N₂) with stirring, in the dark. There was no evidence of a precipitate being formed in the translucent, light-yellow reaction mixture on addition of reagents. A 10% mole excess of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b, relative to 4-azidobenzoic acid, was utilised as this would ensure a maximum yield of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d, with any remaining pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b being rendered inert under the SPS peptide-type coupling conditions. Fortunately, this protocol avoids the need for chromatographic isolation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d.

After forty-six hours and twenty-five minutes of reaction time, an aliquot (~20 µL) of the reaction mixture was taken via a pre-N₂ flushed 100 µL gas-tight syringe for assessment via TLC (EtOAc:hexane, 50:50 v/v). The crude mixture was present as four spots. One spot was present on the baseline and was attributed to Cu(I). Two of the non-polar spots had the same Rf values as pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b (0.62) and 4-azidobenzoic acid (0.23) (standards), respectively. The fourth spot, which was also the most intense/concentrated and which was the most polar of the three spots (Rf 0.19), was attributed to pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d. This was encouraging, and was evidence that the reaction was proceeding smoothly. Due to the unavailability of the LC-ESI-MS instrument, the LC-ESI-MS spectrum was not acquired as part of the reaction monitoring process.

The reaction mixture was visually inspected after ninety-three hours and twenty-five minutes of reaction time; the translucent, light-yellow coloured reaction mixture had become a translucent, pale-straw colour. The reaction was allowed to continue, and after one hundred and seventeen hours and fifteen minutes, with no visually observed change, the progress of reaction was assessed via TLC (EtOAc:hexane, 50:50 v/v). The crude mixture was present as four spots. One spot was present on the baseline and was
attributed to Cu(I). Two of the non-polar spots exhibited the same Rf values as pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (0.62) and 4-azidobenzoic acid (0.23), respectively. The intensity/concentration of the spot attributed to pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b was very low, while the intensity of the spot attributed to 4-azidobenzoic acid was negligible and hence was difficult to observe. The fourth spot, which was also the most polar of the three spots above the base-line (Rf 0.19) and which was attributed to pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d, had drastically increased in intensity/concentration. This was an encouraging observation and the reaction was deemed complete.

The translucent, pale-straw coloured reaction mixture was partitioned against EtOAc and 0.1M sodium thiosulfate. Sodium thiosulfate was utilised to sequester Cu(I) into the aqueous phase. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo to yield an off-white waxy solid (372.7 mg, 0.438 mmol) in an approximate yield of 95.8% (small presence of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b). The product was found to be insoluble in DCM and CDCl₃ but soluble in Me₂CO and d₅-DMSO.

The ¹H-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d in d₅-DMSO is shown in figure 30 (~90–95% purity) (appendix). As expected, the spectrum is ‘contaminated’ with a small amount (~5%) of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b. The spectrum was assigned by difference of the ¹H-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b in CDCl₃.

The ¹³C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d in d₅-DMSO is shown in figure 31 (appendix). The spectrum was assigned by difference of the ¹³C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b in CDCl₃, by difference of the ¹³C-NMR spectrum of 4-azidobenzoyl aminotentagel in CDCl₃.
The FT-IR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d was acquired as a film on NaCl plates (spectrum not shown). The most significant aspects of the spectrum are as follows: (1) the presence of a band at 2111 cm\(^{-1}\), attributed to the 1,2,3-triazole stretch. (2) The presence of a band at 1678 cm\(^{-1}\), attributed to the C=O stretch of the carbonyl group of the carboxylic acid. (3) The presence of a band at 1116 cm\(^{-1}\), attributed to an organic siloxane bond, \(R-C-O-Si-O-C-R\) [152].

2.4.6 SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

\[
\begin{align*}
\text{R} & = \text{CH}_2\text{CH}=&\text{CH}_2 \\
\end{align*}
\]

i) HATU, \(\text{Pr}_2\text{NEt}, \text{DMF}, \text{rt}, 4 \text{ hours 10 minutes.}

Scheme 43: peptide-type SPS of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

With the successful synthesis of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d, peptide-type coupling with aminotentagel resin was the next synthetic step.

Aminotentagel resin (0.24 mmol g\(^{-1}\) loading) was used, as supplied from the supplier, without further washing or drying.
Pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d was treated with peptide-coupling reagents, HATU and \(N,N\)-diisopropylethylamine, and then poured over aminotentagel resin, contained in a sintered glass SPS column, utilising DMF as solvent (scheme 43). The synthetic method set pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d at 3.0 eq over aminotentagel resin, in an attempt to force the reaction to completion.

It was also hoped that it would be possible to obtain unchanged, excess pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d after the reaction, via decomposition of its active-ester analogue, for further use in SPS (2.0 eq maximum recovery). The equivalents of HATU utilised, compared with the equivalents of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d, was kept to a minimum (< 1.0 eq) to prevent potential side reactions occurring during formation of the active-ester analogue.

The translucent, light-orange reaction mixture was allowed to stir gently for four hours and ten minutes, without any change in observations. At the end of this period, a small sample of the resin was transferred to a small, sintered glass vacuum-funnel. The sample was washed with DMF to yield a slightly off-white, yellow resin (similar to the colour of the resin before reaction). The free, primary amine, colour-test was undertaken. However, at the end of the colour test, and after washing the resin with DMF, the colour of the resin was slightly off-white, yellow. It was therefore concluded that the peptide-type coupling reaction had proceeded to at least 98.1% completion.

The resin was washed with MeCN, and any remaining free primary amine groups (maximum of 1.9%) were 'capped' with NMI, 40% 2,6-lutidine and 20% acetic anhydride in THF as a precaution. After completion of the 'capping' reaction, the resin was washed with a variety of organic solvents and it was observed that the resin was a slightly off-white, yellow colour, indicating that no detrimental side-reactions had taken place.
The FT-IR (ATR) spectrum of a sample of the off-white, yellow vacuum-dried resin was acquired (spectrum not shown) and compared with that of the FT-IR (ATR) spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, synthesised via SPS ‘click’ chemistry. As was expected, the two spectra contained exactly the same transmission bands and so the two resins were considered to be the same.

The gel-phase $^{13}$C-NMR spectrum of a sample of the off-white, yellow vacuum-dried resin was acquired in CDCl$_3$ (spectrum not shown). As was expected, the spectrum contained exactly the same $^{13}$C resonances as those present in the gel-phase $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (figure 28), synthesised via SPS ‘click’ chemistry.

The SPS peptide-type coupling reaction was shown by the colour-test to have proceeded to at least 98.1% completion, and this was considered a major success in linking allyl-protected (-)-epigallocatechin 4 to a resin. Subjecting a portion of the ‘loaded’ resin to the fluoride-cleavage regime, and analysis of the cleaved product, was shown by LC-ESI-MS, to yield pentaallyl-(-)-epigallocatechin 4a and its two, mono-C-alkylated A-ring by-products, as was expected. The aliquot of the cleaved mixture, subjected to LC-ESI-MS, was extremely dilute and therefore the di-C-alkylated A-ring by-product was not detected. Analysis of the cleaved resin, by FT-IR (ATR) spectroscopy, yielded a spectrum with exactly the same transmission bands as the FT-IR (ATR) spectrum acquired after cleaving pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, synthesised via the first scenario described in the introduction to section 2.4. Pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, synthesised via the second strategy, was utilised for the remainder of the SPS synthetic work involving allyl-protected (-)-epigallocatechin 4.

In an attempt to recover the excess pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (via decomposition of the active-ester), the translucent, light-orange filtrate obtained from the first MeCN wash was partitioned against EtOAc and H$_2$O.
A small aliquot of the translucent, brown aqueous layer was analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v). TLC analysis revealed the presence of one spot that was present on the baseline; this was attributed to N,N-diisopropylethylamine (standard).

A small aliquot of the translucent, pale-yellow organic layer was analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v). TLC analysis revealed the presence of three spots, the least polar and most concentrated spot being attributed to non-reacted pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b (standard). The remaining two spots were slightly more polar than pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b, with the more polar of the two, present in a very low concentration. There was no evidence for the presence of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d, and it was hypothesised that the entire amount of this compound had been converted into its active-ester.

To test this hypothesis, an aliquot of the translucent, light-yellow organic layer was treated with N-benzylamine for thirty minutes in an attempt to derivatise the active-ester. As the N-benzylamine was added to the organic layer, it was observed that the solution became an opaque-white colour and then became colourless. The reaction of the active-ester with N-benzylamine would, in theory, yield a product with similar characteristics to the derivatised resin (a reasonable mimic). However, the disadvantage of utilising N-benzylamine for derivatisation is the presence of its UV-active aromatic ring which could possibly complicate the TLC analysis for the presence of a new product. A more suitable amine would have been an alkylamine; however, N-benzylamine was the only primary amine available at the time.

TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v) of the reaction mixture, after thirty minutes of reaction time, revealed the presence of four spots, three being attributed to those previously described, and one, very polar spot (just off the baseline) was attributed to N-benzylamine (standard). These TLC results suggested that none of the N-benzylamine had reacted. It was therefore assumed that the active-ester may have decomposed during work-up of the MeCN filtrate, and was not amenable to recovery.
The $^1$H-NMR spectrum of the vacuum-dried organic layer was acquired in CDCl$_3$, and $d_6$-DMSO. (spectra not shown). The spectra contained evidence for the presence of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b, N,N-diisopropylethylamine and a negligible amount of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d. This observation suggested that HATU had converted the maximum amount of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d into its active-ester, on a molar basis. Fortunately, there was no evidence for a species containing free phenolic protons, indicating that the allyl protecting groups were still intact.

Additionally, the composition of the vacuum-dried organic layer was analysed by positive-polarity LC-ESI-MS (45 minute binary eluent gradient without AcOH) (spectrum not shown). UV-Vis (PDA) data (270-280 nm) and MS detection (m/z 150-1200) yielded evidence for the presence of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b at a retention time 15.43 minutes at m/z 689.36. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, the two, mono-C-alkylated A-ring by-products of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b were present at retention times of 16.31 and 16.39 minutes at m/z 729.39 (with a relative absorbance of ~22 and ~13%, respectively). The di-C-alkylated A-ring by-product of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b was present at a retention time of 17.44 minutes at m/z 769.41 (with a relative absorbance of ~3%). Also present, in almost insignificant amount, and difficult to detect because of its extremely low IC, was pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d. Other very minor 'contaminating' UV-active species were also present in the spectral region of the aforementioned peaks (<5%); however, these were not analysed further.

2.4.7 Conclusion

The total amount of reaction time taken to synthesise the high-yielding pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, via the second scenario, was one hundred and twenty-one hours and twenty-five minutes. In
contrast, the total amount of reaction time taken to synthesise pentaallyl-(−)-
epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin
(66.1% yield), via the first scenario described, was two hundred and thirty-six hours and
twenty minutes (including seventy-one hours and forty minutes at 60 °C). However, it
should be noted that discrepancies exist between the reaction yield calculated from the
gravimetric analysis of the amount of pentaallyl-(−)-epigallocatechin diisopropylsilylethyl
butynyl ether 4b consumed during the SPS ‘click’ reaction (98.5% yield) and the amount
of pentaallyl-(−)-epigallocatechin 4a obtained from cleavage and work-up of the ‘loaded’
resin (66.1% yield).

Additionally, compared with the synthesis of tetraallyl-(−)-epicatechin diisopropylsilylethertentagel resin (84.6% yield, calculated from the gravimetric analysis of tetraallyl-
(−)-epicatechin 5a, obtained from cleavage and work-up of the ‘loaded’ resin), it can be
said that the combination of peptide-type coupling, and ‘click’-chemistry, yields far
superior results with respect to covalently linking a phenol-protected catechin (containing
an aliphatic, secondary 3-OH group) to a resin. Additionally, the chemistry presented in
section 2.4 provides an interesting combination of orthogonal reactions that may prove
useful in future polyphenol research.
2.5 SPS deallylation and oxidation

2.5.1 Introduction

The allyl group is a frequently utilised protecting group for amino, carboxyl and hydroxyl functional groups in organic synthesis and has found good use in carbohydrate chemistry, too, being stable under reasonably strong basic and acidic conditions [164]. Concurrently, Pd(0)-catalysed deallylation, in the presence of a nucleophile acting as an allyl cation scavenger, has been developed and is a well established method for deprotecting allyl-protected functions. Pd(0)-catalysed deallylation, comprising π-allyl activation, has been utilised successfully with, and without, an additional proton source. In some instances, it has been essential to carry out the Pd(0)-catalysed deallylation reaction in the presence of a protonic agent to avoid possible side reactions [165]. To this end, and with the wealth of literature that is available on Pd(0)-catalysed allyl-deprotection, this method was considered the method of choice for the allyl-deprotection strategy.

2.5.2 Deallylation of tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b (with formic acid/butylamine)

Initially, a small-scale Pd(0)-catalysed deallylation of model compound, tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b, using a neutral pH system, comprising formic acid (1.0 eq):butylamine (1.0 eq) as the allyl cation scavenger, was attempted (scheme 44). It was envisaged that reaction conditions could be transferred for SPS
deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin. Advantageously, the allyl scavenger mixture is chemically reducing until reaction work-up, and this would serve to minimise undesired oxidation of the deprotected catechin moiety. Additionally, the neutral pH allyl scavenger system was chosen in order to minimise any potential silyl linker cleavage, which may otherwise occur under reaction conditions of varying pH. Hayakawa et al. have employed this system to deprotect, in solution, an allyloxycarbonyl-protected nucleoside with 94% yield [166]. In their work, 2 eq of scavenger mixture, per allyl group, 5 mol% of tetrakis(triphenylphosphine)palladium(0) (Pd(PPh₃)₄) as catalyst, and 20 mol% triphenylphosphine (PPh₃), in THF was used. However, it is generally accepted that SPS reactions require excess reactants to maximise product yield, and, for this reason, the small-scale Pd(0)-catalysed deallylation was attempted, utilising 5 eq scavenger mixture, per allyl group, and ~10 mol% Pd(PPh₃)₄. Excess PPh₃ was not employed as there was a concern that solubility of reactants may adversely affect the homogeneity of SPS reaction conditions. Additionally, the solvent employed was a mixture of THF and MeCN, as THF is generally a poor swelling solvent for tentagel resins.

Tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (1.0 eq) and Pd(PPh₃)₄ (9.8 mol%) were treated with formic acid (5.3 eq):butylamine (5.1 eq) in a mixture of anhydrous and deoxygenated THF and MeCN (pH 7), under N₂, to yield a homogeneous brown-coloured solution.

After one hour of reaction time, an aliquot (~20 μL) of the reaction mixture was taken, via a pre-N₂ flushed 1000 μL gas-tight syringe, and diluted with MeCN (50 μL) for TLC analysis (EtOAc:hexane, 50:50 v/v). The crude mixture was present as a single spot on the baseline. A relatively more polar TLC system was investigated and utilised for the analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). The crude mixture was resolved as six spots. The spot on the baseline was attributed to Pd(0) and the sixth spot, which was the least polar was attributed to PPh₃ (standard). There was no evidence for the presence of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (standard). This was an encouraging observation and the remaining four spots of intermediate polarity were attributed to tri-, di- and monoallyl-(−)-epicatechin diisopropylsilyl methyl ether and (−)-
epicatechin diisopropylsilyl methyl ether 5d. It was observed that thirty minutes after the TLC analysis, the aliquot had become purple in colour. This may have been due to Pd(0) oxidation.

An aliquot of the reaction mixture was similarly analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v) after nineteen hours and thirty minutes of reaction time. The crude mixture was present as three spots. The spot on the baseline was attributed to Pd(0). However, there was no evidence of free PPh₃. The second least polar and most relatively concentrated spot was hypothesised as being attributed to (−)-epicatechin diisopropylsilyl methyl ether 5d, while the least polar (third) spot, of lowest relative concentration, was attributed to a monoallyl-(−)-epicatechin diisopropylsilyl methyl ether species and possibly, non-resolved, monoallyl-(−)-epicatechin diisopropylsilyl methyl ether, positional-isomers. Again, there was no evidence for the presence of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (standard). This observation was encouraging. Interestingly, it was observed that twenty minutes after the TLC was developed, the initially non-visible, least polar spot on the TLC plate had become yellow in colour.

An aliquot of the reaction mixture was similarly analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v) after twenty-five hours and thirty minutes of reaction time. The result was similar; however, the spot attributed to (−)-epicatechin diisopropylsilyl methyl ether 5d had increased in relative concentration, while that attributed to the monoallyl-(−)-epicatechin diisopropylsilyl methyl ether species had decreased in relative concentration. A similar TLC analysis, after sixty-six hours thirty minutes of reaction time, revealed the presence of only two spots. The spot on the baseline was attributed to Pd(0) and the other was attributed to (−)-epicatechin diisopropylsilyl methyl ether 5d. At this point the reaction was considered complete. The reaction mixture was worked-up to yield a translucent, light-yellow solid (38.4 mg). The solid was kept under N₂, to prevent oxidation, and stored at -20 °C between analyses.

The composition of the worked-up mixture was analysed by positive- and negative-polarity LC-ESI-MS (75 minute binary eluent gradient with AcOH as ionisation-
suppression additive) (spectra not shown). Utilising positive-polarity conditions, it was envisaged that protonation of an oxygen atom would facilitate ionisation, whilst utilising negative-polarity conditions, it was envisaged that deprotonation of a phenol group would facilitate ionisation. Both positive- and negative-polarity spectra ultimately yielded the same data, and therefore, only the negative-polarity data will be discussed forthwith. UV-Vis (PDA) data (270-280 nm) and MS detection (m/z 150-1200) yielded evidence for the production of (-)-epicatechin diisopropylsilyl methyl ether 5d at retention time 39.16 minutes at m/z 433.16. This was the dominant species present in the spectrum (with a relative absorbance of 100%). Additionally, a monoallyl(-)-epicatechin diisopropylsilyl methyl ether species was present at a retention time of 45.49 minutes at m/z 473.20 (with a relative absorbance of 16%). However, of concern, was the presence of (-)-epicatechin 5 at a retention time 13.73 minutes at m/z 289.07 (with a relative absorbance of ~20%), indicating cleavage of the diisopropylsilyl linker in tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b, or in (-)-epicatechin diisopropylsilyl methyl ether 5d. From these LC-ESI-MS data, it is evident that the TLC system utilised during reaction monitoring failed to resolve (-)-epicatechin 5 in the reaction mixture.

Additional evidence for the presence of (-)-epicatechin 5, (-)-epicatechin diisopropylsilyl methyl ether 5d, and the monoallyl(-)-epicatechin diisopropylsilyl methyl ether species was sought. The $^1$H-NMR spectrum of the worked-up mixture was acquired in $d_6$-DMSO (spectrum not shown). The most significant aspects of the spectrum were assigned by comparison with the $^1$H-NMR spectrum of (-)-epicatechin 5 in $d_6$-DMSO, and by comparison with the $^1$H-NMR spectrum of tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b in CDCl$_3$.

The $^1$H-NMR spectrum yielded evidence for the presence of (-)-epicatechin diisopropylsilyl methyl ether 5d, and a monoallyl(-)-epicatechin diisopropylsilyl methyl ether species, because two sets of isopropyl resonances were clearly discernable between 0.70 and 1.00 ppm. Additionally, three sets of phenolic proton resonances were discernable between 8.70 and 9.13 ppm. Two of these sets were attributed to the aforementioned diisopropylsilyl methyl ether species, while the third set, was attributed
to (-)-epicatechin 5. Also present was a small amount of contaminating PPh$_3$, with residual aromatic proton resonances located between 7.49 and 7.64 ppm. Due to overlapping of signals, it was difficult to establish the relative proportion of the aforementioned species.

While it is evident that the deallylation protocol employed was successful, the undesired diisopropylsilyl linker cleavage encountered would undermine the SPS deallylation reaction yield. A yield of less than 95% would be considered unacceptable. Therefore, an alternative deallylation method, at neutral pH, was sought.

2.5.3 Deallylation of tetraallyl(-)-epicatechin diisopropylsilyl methyl ether 5b (with sodium benzenesulfinate)

![Scheme 45: Pd(0)-catalysed deallylation of 5b](image)

$\text{R} = \text{CH}_2\text{CH} = \text{CH}_2$

i) Pd(PPh$_3$)$_4$, PhSO$_2$Na, DMF, rt, 216 hours (N$_2$).

The use of sulfinic acid, or its sodium, lithium, or tetrabutylammonium salt, in the presence of a catalytic amount of Pd(PPh$_3$)$_4$ (~5 mol%), with and without added PPh$_3$, has been found to be highly effective for facilitating carbon-oxygen bond cleavage in allyl esters of unstable penems, during investigations by Honda et al. into mild allyl-deprotection methods [167]. Additionally, it was also found that this system facilitated cleavage of O-allyl ethers, and N-allyl amines in good yield. This system was particularly appealing because utilising a neutral salt, as allyl scavenger, would potentially minimise, or eliminate, diisopropylsilyl linker cleavage.
Again, an initial small-scale Pd(0)-catalysed deallylation of model compound, tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b was attempted (scheme 45). It was preferred to use DMF as the solvent, so that homogeneous reaction conditions could be transferred for SPS deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin. Ultimately this would be a heterogeneous system, but would only incorporate two phases.

Tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b (1.0 eq), Pd(PPh₃)₄ (10.3 mol%) and sodium benzenesulfinate (2.1 eq) were dissolved in anhydrous and deoxygenated DMF under N₂ to yield a translucent, brown reaction mixture.

After forty minutes of reaction time, an aliquot (~20 μL) of the reaction mixture was taken, via a pre-N₂ flushed 1000 μL gas-tight syringe, for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). A TLC pre-treatment was performed, whereby the aliquot was washed with EtOAc and 0.1 M diaminoethanetetra-acetic acid disodium salt in order to sequester Pd(0) into the aqueous-phase. The organic layer was further washed with distilled H₂O and 20% NaCl(aq) and stored under N₂. The organic layer was resolved as five spots. The least polar spot was attributed to PPh₃ (standard). There was no evidence for the presence of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b (standard). This was an encouraging observation and the remaining four spots, of intermediate polarity, were attributed to tri-, di- and monoallyl-(-)-epicatechin diisopropylsilyl methyl ether and the most polar, and most concentrated spot, was attributed to (-)-epicatechin diisopropylsilyl methyl ether 5d.

An aliquot of the reaction mixture was similarly analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v) after two hours, twenty-three hours forty minutes, forty-eight hours, ninety-three hours ten minutes, one hundred and twenty-three hours forty minutes, one hundred and forty hours twenty minutes and one hundred and sixty-eight hours thirty minutes of reaction time. The three spots, attributed to tri-, di- and monoallyl-(-)-epicatechin diisopropylsilyl methyl ether, had significantly decreased in intensity (becoming gradually weaker in relative concentration), whilst the most polar spot,
attributed to (-)-epicatechin diisopropylsilyl methyl ether 5d had become relatively more concentrated.

After two hundred and sixteen hours of reaction time, TLC analysis (EtOAc:hex:AcOH, 50:50:1 v/v/v) revealed that the organic layer exhibited two spots. The least polar spot was attributed to PPh3 (standard), while the other was attributed to (-)-epicatechin diisopropylsilyl methyl ether 5d. At this point the reaction was considered complete. The reaction mixture was worked-up to yield a translucent, light-yellow viscous liquid (88.3 mg). The solid was kept under N2, to prevent oxidation, and stored at -20 °C between analyses. After work-up, a small sample of the liquid was analysed by TLC (EtOAc:hex:AcOH, 50:50:1 v/v/v). The liquid was present as one spot, with no evidence of PPh3 being present. Three very faint spots of intermediate polarity, hardly noticeable, were also observed.

The composition of the liquid was analysed by positive- and negative-polarity LC-ESI-MS (75 minute binary eluent gradient with AcOH as ionisation-suppression additive) (spectra not shown). Both positive- and negative-polarity spectra ultimately yielded similar data; however, the TIC produced during the negative-polarity run was unexplainably weak, and, therefore, only the positive-polarity data will be discussed forthwith. UV-Vis (PDA) data (270-280 nm) and MS detection (m/z 150-1200) yielded evidence for the production of (-)-epicatechin diisopropylsilyl methyl ether 5d at a retention time 38.17 minutes at m/z 435.19. This was the dominant species present in the spectrum (with a relative IC of 100%). Fortunately, there was no evidence for the presence of (-)-epicatechin 5 in the worked-up mixture, indicating that the diisopropylsilyl linker was still intact, and had not undergone hydrolysis during deallylation. Interestingly, the spectrum also yielded evidence for the presence of allyl phenyl sulfone at retention time 37.99 minutes at m/z 183.04. The presence of allyl phenyl sulfone was additional evidence for successful deallylation. Additionally, a monoallyl-(−)-epicatechin diisopropylsilyl methyl ether species was present at a retention time of 45.02 minutes at m/z 475.20 (with a relative IC of ~24%). However, it was also observed that between retention times, 47.42 minutes and 52.88 minutes, there were
numerous di- and triallyl-(-)-epicatechin diisopropylsilyl methyl ether species present. These were not resolved with the binary eluent gradient employed, but made up no more than ~20% of the composition of the liquid (as judged by IC intensity). Instrumental UV-Vis calibration was not operating correctly, therefore IC's were utilised in the analysis. The IC data can initially be judged as semi-quantitative because ionisation potentials for the various diisopropylsilyl species were not known; however, it can be assumed that the data are comparative because the chemical structures of the analytes are relatively similar.

The $^1$H-NMR spectrum of the worked-up mixture was acquired in $d_6$-DMSO (spectrum not shown). The most significant aspects of the spectrum were assigned by comparison with the $^1$H-NMR spectrum of (-)-epicatechin $5$ in $d_6$-DMSO, and by comparison with the $^1$H-NMR spectrum of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether $5b$ in CDCl$_3$ (figure 18) (appendix). The $^1$H-NMR data corroborated the data attained via LC-ESI-MS, with the most significant aspects of the spectrum indicating the presence of tri-, di- and monoallyl-(-)-epicatechin diisopropylsilyl methyl ether. Numerous sets of diisopropyl resonances were clearly discernable between 0.72 and 1.07 ppm. Additionally, numerous overlapping sets of phenolic proton resonances were discernable between 8.70 and ~9.55 ppm. Furthermore, evidence for partial deallylation was exhibited by the presence of broad proton resonances between 4.50 and 4.53 ppm (attributed to those labelled ‘a, a” in figure 18), broad proton resonances between 5.22 and 5.37 ppm (attributed to those labelled ‘c, c” in figure 18), and broad proton resonances between 6.00 and 6.02 ppm (attributed to those labelled ‘b’ in figure 18). Also present in the spectrum were two sets of aromatic resonances located between 7.52 and 7.82 ppm. These were most likely attributable to the presence of allyl phenyl sulfone, and a small amount of contaminating PPh$_3$; however, PPh$_3$ was not readily observed in the LC-ESI-MS spectrum, most likely due to elution occurring during the latter part of the reversed-phase run. Again, due to overlapping signals, it was difficult to establish the relative proportions of the aforementioned species.

It was hypothesised that the excessive reaction time, and the presence of tri-, di- and monoallyl-(-)-epicatechin diisopropylsilyl methyl ether, was attributed to the lack of a
proton source in the deallylation reagent. It is most likely that the liberated phenoxide ions were trapping allyl cations and regenerating the O-allyl functions.

2.5.4 SPS deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate)

![Diagram of deallylation reaction]

R=CH₂CH=CH₂

i) Pd(PPh₃)₄, PhSO₂Na, PPh₃, DMF, 45°C, 238 hours 45 minutes (N₂).

Scheme 46: deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

Deallylation of the allyl-protected phenol groups in tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b was shown to be relatively successful. However, it was evident that the lack of a proton source (to assume the role as a phenoxide ion trap) hindered the reaction rate and also had an influence on the equilibrium products, which consisted of tri-, di- and monoallyl-(-)-epicatechin diisopropylsilyl methyl ether. However, for consistency, the small-scale removal of the allyl protecting groups in pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, utilising sodium benzenesulfinate as allyl scavenger, was attempted (scheme 46).
Reaction conditions were slightly modified, in that the mole ratio of allyl scavenger, per allyl group, was increased to ~2.5 eq. Additionally, the reaction temperature was increased to 45 °C, and additional PPh₃ was introduced, in order to shift the ligand-exchange equilibrium in favour of regenerating the Pd(PPh₃)₄ catalyst.

Pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (1.0 eq) was treated with sodium benzenesulfinate (2.6 eq), Pd(PPh₃)₄ (10.3 mol%) and PPh₃ (21.8 mol%), in anhydrous and deoxygenated DMF, under N₂ at 45 °C. The supernatant was homogeneous, being a translucent, golden-yellow colour, while the resin was a light-yellow colour. Due to the apparatus assembly, stirring was not possible.

The progress of the reaction was visually monitored by observation of the reaction mixture after twenty-three hours fifty minutes, forty-two hours twenty minutes, seventy hours thirty minutes and ninety-three hours forty minutes of reaction time. However, there was no apparent change in observations, although the resin may have darkened in colour slightly (from light-yellow to light-orange). After one hundred and sixty-seven hours forty minutes of reaction time, there was a noticeable change in the colour of the resin, by which time it had become a reddish-brown colour. There was also evidence of a slight precipitate in the supernatant solution, this may possibly have been attributed to colloidal Pd(0)-allyl complex. Overall, observations indicated that the reaction was progressing.

At this point, an aliquot (~20 μL) of the translucent, golden-yellow supernatant was taken, via a pre-N₂ flushed 1000 μL gas-tight syringe, for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). A TLC pre-treatment was performed, whereby the aliquot was washed with EtOAc and distilled H₂O and stored under N₂. The organic layer was resolved as two spots. The least polar spot was attributed to PPh₃ (standard), while the more polar spot was attributed to allyl phenyl sulfone. Fortunately, there was no evidence for the presence of (−)-epicatechin 5 (standard). These observations were encouraging as they yielded evidence that the deallylation reaction had proceeded smoothly. At this point, the resin was washed under vacuum with DMF and 0.1 M sodium diethyldithiocarbamate in
DMF and 10% H₂O (v/v) to sequester Pd(0). The resin was further washed with a variety of organic solvents and it was observed that the resin was a light, orange-brown colour, indicating that no detrimental side-reactions had taken place.

The composition of the organic layer, utilised for TLC analysis, was analysed by positive- and negative-polarity LC-ESI-MS (75 minute binary eluent gradient with AcOH as ionisation-suppression additive) (spectra not shown). Both positive- and negative-polarity spectra ultimately yielded similar data, and therefore, only the positive-polarity data will be discussed forthwith. UV-Vis (PDA) data (274-275 nm) and MS detection (m/z 150-1200) yielded evidence for the production of allyl phenyl sulfone at a retention time 37.36 minutes at m/z 183.04. This was the dominant species present in the spectrum (with a relative absorption of 100%). Fortunately, there was no evidence for the presence of (–)-epicatechin 5, indicating that the diisopropylsilyl linker was still intact and had not undergone hydrolysis during deallylation. Also present, in a very small amount (almost not visible in the UV-Vis spectrum) was PPh₃ at a retention time 65.61 minutes at m/z 263.10. These LC-ESI-MS results were encouraging; however, additional evidence, and perhaps relative quantification for the extent of deallylation was sought.

The gel-phase ¹³C-NMR spectrum of the vacuum-dried resin was acquired in CDCl₃ (figure 32) (appendix). The most significant feature of the spectrum is the virtual absence (background level) of allyl group resonances around 124.0, and 70.2–75.5 ppm (see figure 28 for reference) and 133.8, 117.2–116.7 and 73.1–66.7 ppm (see figure 31 for reference).

2.5.4.1 SPS re-deallylation of pentaallyl–(–)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate)

The gel-phase ¹³C-NMR spectrum of the vacuum-dried ‘deallylated’ resin indicated that deallylation was partially complete, therefore it was attempted to maximise the SPS yield, by subjecting the partially deallylated resin to further Pd(0)-catalysed deallylation.
Partially-deallylated pentaallyl-\((-\)epigallocatechin diisopropylsiloxy ethyl-1,2,3-triazolebenzoyl aminotentaegel resin (1.0 eq) was treated with sodium benzenesulfinate (2.6 eq), Pd(PPh\(_3\))\(_4\) (10.0 mol%) and PPh\(_3\) (22.3 mol%) in anhydrous and deoxygenated DMF, under N\(_2\) at 45 °C. The supernatant was homogeneous, being a golden-yellow colour, and the resin was a light-orange colour.

The progress of the reaction was visually monitored by observation of the reaction mixture after twenty hours fifteen minutes and forty-six hours twenty-five minutes of reaction time. However, there was no change in observations, although the resin may have darkened in colour slightly (from light-orange to a darker orange-brown).

After sixty-nine hours twenty-five minutes of reaction time (total deallylation reaction time of two hundred and thirty-eight hours forty-five minutes), with no further change in observations, the resin was washed under vacuum with DMF and 0.1 M sodium diethyldithiocarbamate in DMF and 10% H\(_2\)O (v/v) to sequester Pd(0). The resin was further washed with a variety of organic solvents and it was observed that the resin was a light, orange-brown colour, indicating that no detrimental side-reactions had taken place.

An aliquot (~20 \(\mu\)L) of the clear, golden-yellow supernatant was collected from the initial resin filtrate for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). A TLC pre-treatment was performed, whereby the aliquot was washed with EtOAc, 0.1 M diaminoethanetetra-acetic acid disodium salt, distilled H\(_2\)O and 20% NaCl\(_{aq}\). The organic layer was stored under N\(_2\). TLC analysis of the organic layer revealed the presence of two spots. The least polar spot was attributed to PPh\(_3\), while the more polar spot was attributed to allyl phenyl sulfone. This observation was encouraging as it yielded evidence that additional deallylation had occurred, and that deallylation of the resin was most likely optimised for oxidation.

The gel-phase \(^{13}\)C-NMR spectrum of the vacuum-dried resin was acquired in CDCl\(_3\). (spectrum not shown). The most significant feature of the spectrum was, again, the negligible amount of allyl, carbon resonances.
2.5.4.2 Cleavage of (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagonel resin

Gel-phase $^{13}$C-NMR spectroscopy suggested the presence of a negligible amount of allyl protecting groups remaining on the resin. Therefore, to analyse the on-resin product/s, in the hope that unchanged (−)-epigallocatechin 4 would be released from the resin, a portion of the vacuum-dried resin (20.0 mg, 0.00480 mmol) was subjected to the diisopropylsilyl linker cleavage regime, for two hours and thirty minutes. At the end of the cleavage regime, trimethylsilyl silane was added to the mixture so as to consume excess fluoride ion. The vacuum-dried colourless residue (25.0 mg) was stored under N$_2$ and analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v). The residue contained only one spot with the same R$_f$ value as (−)-epigallocatechin 4 (standard). However, there was also evidence of very slight streaking (less polar than this spot). This observation suggested that there may still be positional, mono-, di- and tri-allylated derivatives/isomers of epigallocatechin 4 to a minor extent.
2.5.5 SPS deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with 2,4,6-collidine p-toluenesulfonate)

Scheme 47: deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin

The SPS deallylation, utilising sodium benzenesulfinate as allyl scavenger, was considered a success (as judged by TLC and gel-phase $^{13}$C-NMR spectroscopy). However, an alternative SPS deallylation protocol was sought that would consist of the deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin in the presence of a suitable proton source. It was hypothesised that 2,4,6-collidine p-toluenesulfonate may be a good proton source, and perhaps an allyl acceptor as well; therefore an SPS deallylation was attempted with 2,4,6-collidine p-toluenesulfonate [168]. Additionally, in an attempt to increase the reaction rate, the amount of Pd(0) catalyst was increased to ~25 mol% (scheme 47).

Pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (1.0 eq) was treated with 2,4,6-collidine tosylate (2.3 eq), Pd(PPh$_3$)$_4$ (23.3 mol%) and PPh$_3$ (4.1 mg, 0.0156 mmol, 13.0 mol%), in anhydrous and
deoxygenated DMF, under N\textsubscript{2} at 45 °C with stirring. The supernatant was homogeneous, being a golden-yellow colour, and the resin was a light-yellow colour.

The progress of the reaction was visually monitored by observation of the reaction mixture after twenty-one hours, sixty-five hours and ninety-four hours. However, there was no apparent change in observations, although the resin may have darkened in colour slightly (from light-yellow to light-orange).

After ninety-four hours of reaction time, an aliquot (~20 μL) of the translucent, golden-yellow supernatant was taken, via a pre-N\textsubscript{2} flushed 1000 μL gas-tight syringe, for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). A TLC pre-treatment was performed whereby the aliquot was washed with EtOAc, 0.1 M diaminoethanetetra-acetic acid disodium salt, distilled H\textsubscript{2}O and 20% NaCl(aq). The organic layer was stored under N\textsubscript{2}. TLC analysis of the organic layer revealed the presence of one spot, which was not attributed to PPh\textsubscript{3} (standard). It may be attributed to 2,4,6-collidine tosylate, because in hindsight, 2,4,6-collidine tosylate is a poor allyl acceptor.

However, after one hundred and eighty-seven hours and twenty-five minutes of reaction time, it was observed that the supernatant was still a golden-yellow colour. The fact that no precipitate was observed was an indication that some phenolate anions (23.3 mol\%) may have been quenched by protons from 2,4,6-collidine tosylate. After one hundred and eighty-nine hours twenty minutes of reaction time, the resin was washed under vacuum with DMF and 0.1 M sodium diethyldithiocarbamate in DMF and 10% H\textsubscript{2}O (v/v/v) to sequester Pd(0). The resin was further washed with a variety of organic solvents and it was observed that the resin was a light, orange colour, indicating that no detrimental side-reactions had taken place.

The gel-phase \textsuperscript{13}C-NMR spectrum of the vacuum-dried resin was acquired in CDCl\textsubscript{3} (spectrum not shown). Although the spectrum displayed similar characteristics to the gel-phase \textsuperscript{13}C-NMR spectrum shown in figure 32, the resolution of the spectrum (S/N) was extremely poor and no accurate assignments could be made. The resin was not cleaved for further analysis and was directly subjected to SPS oxidation. It should be noted that it
is not 100% certain that the 2,4,6-collidine tosylate method worked on the grounds of gel-phase $^{13}$C-NMR spectroscopy, alone.

2.5.6 SPS oxidation of \((-\)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotengel resin (deallylation with sodium benzenesulfinate) with co-substrate, \((-\)-epicatechin 5

Many variations in solution-phase methods, including nature of oxidant and stoichiometry of reactants, have been utilised to synthesise theaflavins and benzotropolones. Only two selected examples, with specific reference to the chemical synthesis of benzotropolones, under mildly alkaline conditions, from catechin-type and gallicatechin-type precursors will be discussed forthwith.

The methods of chemical synthesis have employed the use of at least two equivalents of potassium hexacyanoferrate(III) as the (one-electron) oxidising agent. The amount of equivalents of potassium hexacyanoferrate(III) utilised for oxidation, compared with the amount of equivalents of pyrogallol- and catechol-type B-rings, present in the respective precursors, has varied from worker to worker. For example, Collier \textit{et al.} employed 1.0 eq \((-\)-epigallocatechin 4 (pyrogallol-type B-ring), 2.0 eq \((-\)-epicatechin 5 (catechol-type B-ring) and 2.0 eq of potassium hexacyanoferrate(III), in aqueous sodium hydrogen carbonate, to synthesise chemically theaflavin 12 [68], while Bailey and Nursten employed 1.0 eq gallic acid 29 (pyrogallol-type B-ring), 1.9 eq \((-\)-epicatechin gallate 2 (catechol-type B-ring) and 20.2 eq of potassium hexacyanoferrate(III), in aqueous sodium hydrogen carbonate, to synthesise chemically epitheafavic acid-3-gallate 35 [67]. However, from an empirical perspective, the amounts of component-equivalents utilised in the method employed by Collier \textit{et al.}, was considered a good starting point for chemical oxidation, and formed the basis for the initial attempt at the SPS oxidation of \((-\)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotengel resin, with the co-substrate, \((-\)-epicatechin 5, in solution. As SPS reactions generally require an excess of reagents to force them to completion, the amounts of equivalents considered suitable for the initial attempt were; 1.0 eq \((-\)-epigallocatechin diisopropylsilyloxy ethyl-
1,2,3-triazolebenzoyl aminotentagel resin (pyrogallol-type B-ring), ~5.0 eq (–)-epicatechin 5 (catechol-type B-ring), and ~5.0 eq of potassium hexacyanoferrate(III).

An obvious question raised by employing potassium hexacyanoferrate(III) as the polyphenol oxidant is: to what extent does the oxidant mimic the role of PPO and POD enzymes during their role in polyphenol oxidation during black tea manufacturing? To this end, Opie et al. have compared the products of *in vitro* oxidations utilising PPO, with the products of *in vitro* oxidations utilising potassium hexacyanoferrate(III) [169]. The results obtained by Opie et al. suggest that, although the products of PPO-driven oxidation resemble those of potassium hexacyanoferrate(III)-oxidation, it would be premature to suggest that the mechanisms for the formation of the products are the same. However, as will be outlined in the section on ‘future research’, the SPS strategy employed throughout this research project, is amenable to biomimetical SPS utilising PPO and POD, and those results could be compared with the results obtained herein.

### 2.5.6.1 Solubility-test for SPS oxidation

In their chemical oxidation experiment, Collier *et al.* mixed the two catechin precursors in a minimum of H₂O and treated the precursor solution, with a solution of potassium hexacyanoferrate(III) in aqueous sodium hydrogen carbonate. Therefore, the initial task was to ascertain the solubility of the respective reagents in a suitable solvent system and thus enable the transfer of homogeneous conditions for SPS oxidation. Although in general, solution-phase chemical oxidations have employed H₂O as the solvent, tentagel-type resins require an organic solvent, at least in part, to swell the cross-linked hydrophobic core. It was therefore envisaged that H₂O:1,4-dioxane, 50:50 v/v would, to some extent, resemble the aqueous system used by Collier *et al.*, but also allow for the resin to swell. However, the solubility of sodium hydrogen carbonate in the solvent system was expected to be lower with the addition of 1,4-dioxane. The pH of the oxidation-solution utilised by Collier *et al.* was 6.5-7.5; however, a supernatant of pH ~8.5 was considered suitable to aid the SPS oxidation. Additionally, Collier *et al.* allowed their oxidation reaction to proceed for fifteen minutes; however, due to the two-phase nature of SPS, it was hypothesised that SPS oxidation would require at least two hours to
complete. The mole calculations for the solubility-test were based on 80 mg (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (0.0192 mmol, 1.0 eq).

Sodium hydrogen carbonate (40.7 mg, 25.2 eq) was incrementally and concurrently dissolved in 30 µL portions of H₂O and 1,4-dioxane with stirring, and it was found that a homogeneous solution required H₂O:1,4-dioxane (840 µL:360 µL, 70:30 v/v). Although this volume of solvent was relatively large for SPS oxidation of 80 mg of resin, and the relative proportions of H₂O:1,4-dioxane were 70:30 v/v, these solvent conditions were considered acceptable, and were probably the most attainable for the SPS system. The resulting sodium hydrogen carbonate solution was pH 8.5. As a large volume of solvent mixture was required to dissolve the sodium hydrogen carbonate, (−)-epicatechin 5 (27.5 mg, 4.9 eq) was dissolved in this solution, without additional dilution, to yield a translucent, light-yellow solution.

Potassium hexacyanoferrate(III) (31.7 mg, 5.0 eq) was dissolved in 100 µL distilled H₂O and added to the sodium hydrogen carbonate/(−)-epicatechin 5 solution, drop-wise over a period of one minute, with stirring, to yield a deep-red solution. After one hour of stirring, there was no evidence of any precipitate being formed and these stoichiometric conditions were deemed suitable for the SPS oxidation.
2.5.6.2 SPS oxidation

i) $(-)$-epicatechin 5, NaHCO$_3$, H$_2$O/1,4-dioxane, rt, 20 minutes.

ii) K$_3$Fe(CN)$_6$, H$_2$O, rt, 2 hours.

Scheme 48: attempted SPS of theaflavin 12

$(-)$-Epigallocatechin diisopropylsiloxo ethyl-1,2,3-triazolebenzoyl aminotentagel resin (80 mg, 1.0 eq) was treated with sodium hydrogen carbonate (40.2 mg, 24.9 eq)/$(-)$-epicatechin 5 (28.3 mg, 5.1 eq), in H$_2$O (840 µL) and 1,4-dioxane (360 µL), with stirring. This mixture was treated with potassium hexacyanoferrate(III) (32.1 mg, 5.1 eq) in H$_2$O (100 µL) to yield a translucent, deep-red supernatant (scheme 48). After the addition of potassium hexacyanoferrate(III), it was observed that the mixture became gel-like, with the evolution of gas (most likely CO$_2$). This could be attributed to the slightly acidic potassium hexacyanoferrate(III) reacting with sodium hydrogen carbonate. However, during the solubility-test, the evolution of gas was not observed. The other possibility that may be proposed, for the evolution of gas, is the release of CO$_2$ during benzotropolone-ring formation. After continued stirring, the gel-like phase disappeared to yield free-flowing resin beads. After one hour of reaction time, the colour of the supernatant solution had become orange in colour, suggesting some form of oxidation was proceeding.
After two hours of reaction time, the resin was washed with H₂O:1,4-dioxane 70:30 v/v and then H₂O, to extract inorganic material. The resin was further washed with a variety of organic solvents and it was observed that the resin was a light orange-red colour.

The gel-phase ¹³C-NMR spectrum of the vacuum-dried resin was acquired in CDCl₃ (spectrum not shown). The spectrum was of extremely poor quality (S/N) and interpretation was not attempted.

2.5.6.3 Cleavage of the diisopropylsilyl linker

To analyse the on-resin product, the vacuum-dried resin (~80.0 mg) was subjected to the diisopropylsilyl linker cleavage regime for two hours and thirty minutes.

The vacuum-dried translucent, light-orange residue was analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v). The residue was present as one spot. Interestingly, and of concern, was the observation that the spot was less polar than (-)-epigallocatechin 4 (standard), (-)-epicatechin 5 (standard), and theaflavin 12 (standard), but was found to be more polar than pentaallyl-(−)-epigallocatechin 4a. Surprised by this TLC result, the material that was cleaved from the resin was stored under N₂ until further TLC analysis (see page 213).
2.5.7 SPS oxidation of (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotagentgel resin (deallylation with 2,4,6-collidine p-toluencesulfonate)

![Chemical structure](image)

i) K$_3$Fe(CN)$_6$, H$_2$O, rt, 50 minutes.

ii) (−)-epicatechin 5, NaHCO$_3$, H$_2$O/1,4-dioxane, rt, 2 hours 45 minutes.

Scheme 49: attempted SPS of theaflavin 12

As an alternative SPS oxidation protocol, it was attempted to first oxidise the deallylated resin, without the presence of (−)-epicatechin 5, as a means to investigate the mechanism of oxidation. Additionally, the amount of potassium hexacyanoferrate(III), relative to (−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotagentgel resin was increased to ~10 equivalents, (−)-epicatechin 5 being also increased to ~10 equivalents.

(−)-Epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotagentgel resin (99.7 mg, 1.0 eq) was allowed to swell in 1,4-dioxane (500 µL) with stirring, over a period of forty minutes. The resin was then treated with potassium hexacyanoferrate(III) (79.7 mg, 10.1 eq) in distilled H$_2$O (300 µL), to yield a translucent, deep-yellow supernatant (scheme 49). After the addition of the potassium hexacyanoferrate(III) solution, it was observed that the mixture became gel-like, with the evolution of gas (most likely CO$_2$). As there was no sodium hydrogen carbonate present, this observation
could not be attributed to the slightly acidic potassium hexacyanoferrate(III) reacting with sodium hydrogen carbonate. After twenty minutes of continued stirring, the gel-like phase had disappeared, to yield free-flowing resin beads. After fifty minutes (without a change in the colour of the supernatant), the oxidation reaction was presumed to be complete and the resin was treated with sodium hydrogen carbonate (43.1 mg, 21.5 eq)/(-)-epicatechin 5 (70.4 mg, 10.2 eq), in H₂O (1870 µL) and 1,4-dioxane (386 µL) (pH 8.5), with stirring, to yield a deep-red supernatant. After this addition, it was again observed that the mixture became gel-like with the evolution of gas. After five minutes of continued stirring, the gel-like phase had disappeared to yield free-flowing resin beads.

Two hours and fifty-five minutes after the addition of the sodium hydrogen carbonate/(-)-epicatechin 5 solution, the resin was washed with H₂O:1,4-dioxane, 70:30 v/v, 1,4-dioxane and then H₂O to extract inorganic material, to yield a deep-red coloured resin. The resin was further washed with a variety of organic solvents, the resin remaining a deep-red colour.

2.5.7.1 Cleavage of the diisopropylsilyl linker

To analyse the on-resin product, a portion of the vacuum-dried resin (25.2 mg) was subjected to the diisopropylsilyl linker cleavage regime for two hours and thirty minutes. At the end of the cleavage regime, the resin was a light, orange-red colour.

The vacuum-dried translucent, light-orange residue was analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v). The residue was present as one spot. Again, the spot was less polar than (-)-epigallocatechin 4 (standard), (-)-epicatechin 5 (standard) and theaflavin 12 (standard), but was found to be more polar than pentaallyl-(−)-epigallocatechin 4a. Interestingly, the spot possessed exactly the same Rf as the material produced via the SPS 'one-pot' oxidation reaction (see page 211).

Attempts were made to obtain the MS spectrum of the material cleaved from the resin via EI-MS, positive-polarity CI-MS and positive- and negative-polarity, infusion ESI-MS (EPSRC National Mass Spectrometry Service Centre, Swansea, UK). All methods attempted, exhibited poor ionisation and it was suggested that sample impurity may have
resulted in ion suppression. However, ESI-MS yielded the most useful information, although ions produced from negative- and positive-polarity analyses were inconsistent. Two positive-polarity spectra were acquired, with and without NH$_4$OAc (NH$_4$OAc, for positive-ion generation), and both produced consistent results. The dominant ion (100%) was $m/z$ 391.2844. Two prominent ions were $m/z$ 279.1592 and $m/z$ 163.1326. However, it could not be ascertained whether these two prominent ions were fragment ions. Positive-polarity CI-MS with NH$_3$, suggested the presence of two species, one with $m/z$ 594.20 (low IC) and the other with $m/z$ 393.81 (high IC). A common ion, to both species, was $m/z$ 279.11. These data imply that $m/z$ 279.1592 is a fragment ion from $m/z$ 391.2844 and that $m/z$ 391.2844 is a molecular ion.

The FT-IR spectrum of the vacuum-dried material, cleaved from the resin, was acquired as a film on NaCl plates (spectrum not shown). The most significant aspects of the spectrum are as follows: (1) the presence of a narrow, weak band at 3583 cm$^{-1}$, indicative of a phenol or tertiary alcohol stretch. (2) The presence of a broad, strong band at 3379 cm$^{-1}$, indicative of a hydrogen-bonded OH stretch. (3) The presence of a narrow, strong band at 2950 cm$^{-1}$, indicative of a methyl C-H asymmetrical stretch, suggesting the presence of the fluoride cleaved diisopropylsilyl linker, non-volatile, trisiloxane by-product [-O-Si-(i-Pr)$_2$-]. NMR data (see below) also yields evidence for the presence of diisopropyl resonances. (4) The presence of a narrow, strong band at 2604 cm$^{-1}$, indicative of a secondary absorption (over tone) of a hydrogen-bonded OH of a carboxylic acid. (5) The presence of a narrow, strong band at 2497 cm$^{-1}$, indicative of a hydrogen-bonded OH of a carboxylic acid. (6) The presence of a broad, strong band at 1723 cm$^{-1}$, indicative of a carbonyl stretch of an aldehyde, ketone, carboxylic acid or ester. (7) The presence of a broad, strong band at 1474 cm$^{-1}$, indicative of a methyl C-H asymmetrical stretch, suggesting the presence of the cleaved diisopropylsilyl linker, non-volatile, trisiloxane by-product. (8) The presence of a narrow, weak band at 1260 cm$^{-1}$, indicative of a C-O stretch of a phenol or carboxylic acid. (9) The presence of a narrow, strong band at 1173 cm$^{-1}$, indicative of a cyclic ether C-O stretch. (10) The presence of a narrow, strong band at 1066 cm$^{-1}$, indicative of a primary alcohol C-O stretch. (11) The presence of a narrow, weak band at 850 cm$^{-1}$, indicative of a hydrogen-bonded carboxylic acid OH,
out-of-plane bend. (12) The presence of a narrow, strong band at 720 cm⁻¹, indicative of an alcohol OH out-of-plane bend or a (CH₂)ₙ rock.

The ¹H-NMR spectrum of the material cleaved from the resin was acquired in d₆-DMSO (spectrum not shown). The most significant aspect of the spectrum, which was not present in the ¹H-NMR spectrum acquired in CDCl₃, was a resonance at ~11.68 ppm. This is an indication of one, or more, acidic protons. As it seems there is a mixture of two products, with one of them being dominant, ¹H integrals cannot be utilised to assign the number of protons responsible for the resonances. Additionally, of interest was the absence of phenolic protons.

The ¹H-, and ¹³C-NMR spectrum of the material cleaved from the resin was acquired in CDCl₃ (figure 33 and figure 34, respectively) (appendix). The ¹H-NMR spectrum yielded evidence for isopropyl resonances at ~1.28 ppm. Of interest is the absence of aromatic protons. The ¹³C-NMR spectrum yielded evidence for isopropyl resonances at 8.6 and 8.2 ppm. However, there is no evidence of a carbonyl resonance, which would suggest that the product is not an aldehyde, ketone, carboxylic acid or ester. These data are intriguing, and inconclusive, and the structure of the major product cannot be deduced from the three NMR spectra that were acquired.

SPS oxidation was only attempted twice because of time and money constraints (end of three year’s laboratory work). However, theaflavin 12 can be ruled out as the oxidation product but the production of a thearubigin precursor, or a member of the thearubigins, cannot unequivocally be ruled out. It is uncertain why theaflavin 12 was not obtained from the SPS oxidation reactions. This needs to be investigated by additional experiments (see ‘future research’ on page 217, point 3).

2.5.8 Conclusion

Pd(0)-catalysed deallylation of the allyl protecting groups in tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b and pentaallyl-(−)-epigallocatechin diisopropyloxyethyl-1,2,3-triazolebenzoyl aminotentagel resin was shown to be a successful method for their orthogonal removal. However, Pd(0)-catalysed deallylation, with formic
acid/butylamine, as allyl cation scavenger, was not 100% selective in that the diisopropylsilyl linker was cleaved, and in the context of the SPS method, rendered it an unsatisfactory scavenger system. Sodium benzenesulfinate was shown to be a selective allyl cation scavenger, both in solution, and during SPS deallylation. Unfortunately, gel-phase $^{13}$C-NMR spectroscopy, although a useful method per se, was not informative due to poor spectral quality. The successful, selective SPS allyl-deprotection, could only be confirmed by fluoride cleavage of a small portion of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin, with identification of free, unchanged (-)-epigallocatechin 4 being made possible by comparison with an authentic sample.

Although one major product was obtained from both SPS oxidation routes, the key piece of information, its molecular mass, is not 100% certain. From the data available (MS, FT-IR spectroscopy and NMR spectroscopy), it is not possible to deduce whether the product is a monomeric or dimeric species, and this requires further investigation (see ‘future research’ on page 217, point 3).
2.6 Future Research

There are opportunities to progress the work that has been discussed in this thesis:

1. Column-chromatography conditions could be optimised to yield pure pentaallyl-(−)-epigallocatechin 4a, and tetraallyl-(−)-epicatechin 5a, for subsequent reaction.

2. Both solution-, and solid-phase, reaction conditions could be optimised to increase reaction yield and efficiency.

3. Aminotentagel resin could be substituted with PEGA resin (which also contains an amino functionality) during linkage (peptide-type coupling) of pentaallyl-(−)-epigallocatechin 4a. However, PEGA resin swells in a range of organic solvents, as well as 100% H₂O, and, therefore, biomimetical SPS oxidation becomes possible, utilising PPO and/or POD enzymes. Additionally, further attempts at chemical oxidation may help to elucidate the structure of the product obtained from the SPS oxidations described herein.

4. An orthogonally, phenol-protected, derivative of (−)-epicatechin gallate 2 could be synthesised from (−)-epicatechin 5 (A- and B-ring phenol groups protected with β-(trimethylsilyl) ethoxy] methyl (SEM) protecting group), and benzoyl chloride (pseudo D-ring protected with O-allyl function). The O-SEM functions could subsequently be selectively removed in the presence of fluoride to liberate the free A- and B-ring phenol groups, and allow for B-ring co-oxidation with (−)-epigallocatechin 4 (tethered to either aminotentagel or PEGA resin). This strategy would allow for chemical, or biomimetical, SPS of theadibenzotropolone-type structures. Furthermore, the retained D-ring, O-allyl functions could then be selectively removed with Pd(0) to allow for the chemical, or biomimetical, SPS of thetribenzotropolone-type structures etc.
CHAPTER 3

EXPERIMENTAL
3.1 Instruments

3.1.1 QTOF-MS

The instrument used was a QTOF Premier (Micromass MS technologies). The material of interest (~1 mg) was dissolved in 70% MeOH (1000 μL), infused into the instrument and the MS spectrum acquired.

3.1.2 NMR spectroscopy

All NMR experiments were performed using a 'Bruker DRX 500' (500.15 MHz) or a 'Bruker Avance II' 300' (300.13 MHz) instrument.

All ¹H- and ¹³C-NMR spectra were internally calibrated with reference to solvent peaks for consistency, irrespective of whether or not the deuterated solvent was spiked with TMS. For ¹H-NMR spectroscopy, the d₅-MeOH pentet was referenced at 3.31 ppm. For ¹H-NMR spectroscopy, the d₅-DMSO triplet was referenced at 2.50 ppm and for ¹³C-NMR spectroscopy, the d₅-DMSO heptet was referenced at 39.5 ppm. For ¹H-NMR spectroscopy, the CDCl₃ singlet was referenced at 7.26 ppm (residual CHCl₃) and for ¹³C-NMR spectroscopy, the CDCl₃ triplet was referenced at 77.2 ppm.

All ¹H-NMR spectra were acquired at 500.15 MHz (5 mm broad-band, inverse, multinuclei probehead) or 300.13 MHz (5 mm DUL z-gradient high-resolution probe) at 298.0 K. ¹H-NMR spectra acquired at 300.13 MHz are stated as having been so in the text. All ¹³C-NMR spectra (including gel-phase) were acquired at 75.5 MHz and 298.0 K.

Gel-phase ¹³C-NMR spectra were obtained by weighing out the appropriate amount of vacuum-dried resin (70-100 mg) into a 5 mm NMR-tube. The deuterated NMR solvent (~300 μL) was added via a syringe, connected to a 300 mm needle, with gentle flushing/pumping at the bottom of the NMR-tube to remove any trapped air bubbles in the gel, if present. In cases where the resin was sensitive to air/oxygen, the NMR-tube was flushed with N₂ prior to sealing. The resin was allowed to swell for at least one hour prior to the first FID being acquired. Standard acquisition parameters were: 7.70 μsec pulse width with an acquisition time of 1,442 sec and a 0.56 sec delay between pulses,
using the zgpg45 pulse programme. The total number of FIDs acquired, ranged from 24576 to 153600 where appropriate.

3.1.3 FT-IR and FT-IR (ATR) spectroscopy

All infra-red experiments were carried out using a ‘Perkin Elmer System 2000 FT-IR’ instrument. Spectra obtained were an average of sixteen scans. The wavenumber resolution was 1 cm\(^{-1}\) with an interval of 0.5 cm\(^{-1}\). For SPS work, an instrument-supplied ATR crystal was utilised for data acquisition.

3.1.4 LC-ESI-MS

The LC equipment (ThermoFinnigan) comprised a Surveyor MS Pump, autosampler with 50 µL loop and a PDA detector with a light-pipe flow cell (recording at 450, 320 and 280 nm, and scanning from 200 to 600 nm with a bandwidth of 1 nm). This was interfaced with a LCQ Deca XP Plus mass spectrometer fitted with an ESI source (ThermoFinnigan). The spectrometer was operating in the data-dependent, full scan, MS\(^0\) mode to obtain fragment ion \(m/z\). The MS operating conditions (negative-polarity) were optimised using (−)-epicatechin 5 as the analyte and comprised: collision energy of 35%, ionisation voltage of 5 kV, capillary temperature of 275 °C, sheath gas (N\(_2\)) flow rate of 90 arbitrary units, and an auxiliary gas flow rate of 20 arbitrary units. The reproducibility of the conditions was confirmed using a variety of hydroxyphenylpropanoic and hydroxyphenylethanolic acid standards [170]. Positive-polarity analysis was performed under the same MS operating conditions, the only difference being a change in instrumental ionisation polarity. LC resolution was achieved utilising a 150 × 3 mm column containing Gemini 5 µm phenylhexyl packing (Phenomenex, Macclesfield, UK). Solvent A comprised H\(_2\)O:MeCN:AcOH (975:20:5 v/v/v, pH 2.7). Solvent B comprised MeCN:AcOH (995:5 v/v). In the case of LC-ESI-MS analysis without AcOH as an ionisation suppression additive, the solvent system was as follows: Solvent A comprised H\(_2\)O:MeCN: (980:20 v/v). Solvent B comprised MeCN (100%). Solvents were delivered at a total flow rate of 300 µL min\(^{-1}\) and the auto-sampler injection volume was set at 20 µL. The auto-sampler tray temperature control was set at 10 °C. Solvents were degassed by an on-line degasser. ‘Milli-Q’ H\(_2\)O (with resistivity 18.2 MΩ) was used in the
preparation of the respective solvent systems. The binary eluent gradients are given in Tables 1-5.

**Table 1: binary eluent gradient for 45 minute elution profile**

<table>
<thead>
<tr>
<th>Time / min</th>
<th>Solvent A / %</th>
<th>Solvent B / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td>5.00</td>
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<td>4</td>
</tr>
<tr>
<td>45.00</td>
<td>96</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 2: binary eluent gradient for 60 minute elution profile**

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<th>Time / min</th>
<th>Solvent A / %</th>
<th>Solvent B / %</th>
</tr>
</thead>
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<tr>
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</tr>
</tbody>
</table>

**Table 3: binary eluent gradient for 75 minute elution profile**

<table>
<thead>
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<th>Time / min</th>
<th>Solvent A / %</th>
<th>Solvent B / %</th>
</tr>
</thead>
<tbody>
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<td>0.00</td>
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</tr>
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</tbody>
</table>
Table 4: binary eluent gradient for 90 minute elution profile

<table>
<thead>
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<th>Time / min</th>
<th>Solvent A / %</th>
<th>Solvent B / %</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>96</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5: binary eluent gradient for 125 minute elution profile

<table>
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<th>Time / min</th>
<th>Solvent A / %</th>
<th>Solvent B / %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>12.00</td>
<td>75</td>
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</tr>
<tr>
<td>40.00</td>
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</tr>
<tr>
<td>125.00</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

As a typical procedure, for structure elucidation, the material of interest (~1 mg) was dissolved in 100% MeCN (1000 µL), placed in an auto-sampler vial and the LC-ESI-MS spectrum was acquired. As a typical procedure for reaction monitoring, the mixture/solution of interest (~20 µL) was dissolved in 100% MeCN (1000 µL), placed in an auto-sampler vial and the LC-ESI-MS spectrum was obtained. In cases where a precipitate/colloid was observed, the MeCN mixture was filtered via a syringe filter (0.45 µm) prior to analysis.

3.1.5 HPLC

The HPLC equipment (Thermoseparations) comprised a P2000 Binary Gradient Pump, SCM1000 Degasser, AS 1000 autosampler and a UV6000 PDA detector (recording at 320 and 280 nm). Resolution was achieved on a 150 x 3 mm column containing Gemini 5 µm phenylhexyl packing (Phenomenex, Macclesfield, UK). Solvent A was H2O:MeCN:TFA (980:20:0.5 v/v/v, pH 2.5). Solvent B was MeCN:TFA (995:5 v/v).
Solvents were delivered at a total flow rate of 300 μL min⁻¹. The solvent gradient utilised was that presented in Table 2. As a typical procedure, the material of interest (~1 mg) was dissolved in 70% MeOH (1000 μL), placed in an auto-sampler vial and the HPLC spectrum acquired.

Note: Infusion ESI-MS, CI-MS, EI-MS and FAB-MS were acquired by the EPSRC National Mass Spectrometry Service Centre, Swansea, UK.
3.2 Chemicals and suppliers

Acetic acid ~ Fisher Scientific (HPLC grade)
Acetic anhydride ~ BDH (AnalaR®)
Acetone ~ Fisher Scientific (Analytical reagent grade)
Acetonitrile ~ Fisher Scientific (HPLC grade)
Allyl bromide ~ Aldrich (99%)
4-Azidobenzoic acid ~ TCI (>97.0%)
Benzenesulfonic acid, sodium salt ~ Aldrich (98%)
Benzoyl chloride ~ Aldrich (99%)
Benzyllamine ~ Acros Organics (99%)
Butylamine ~ Fluka (>99.5%)
3-Butyn-1-ol ~ Aldrich (97%)
Calcium chloride (fused, granular) ~ Fisher Scientific (General purpose grade)
Carbon tetrachloride ~ Fluka
CDCl₃ ~ Aldrich (99.8 atom% D) 0.05% (v/v) TMS
Chloroform ~ Fisher Scientific (Analytical reagent grade)
2-Chloro-2-methylpropane ~ Lancaster (98+%)  
Citric acid ~ BDH (AnalaR®)
2,4,6-Collidine p-toluenesulfonate ~ Aldrich (98%)
Deuterium oxide ~ Aldrich (99.9 atom% D)
Diaminoethanetetra-acetic acid disodium salt ~ Fisher Scientific (Analytical reagent grade)
1,2-Dichloroethane ~ Fisher Scientific
Dichloromethane ~ Fisher Scientific (Analytical reagent grade)
Diethyl ether ~ Fisher Scientific (Laboratory reagent grade)
3,4-Dihydro-2H-pyran (DHP) ~ Lancaster (97%)
N,N-Diisopropylethylamine ~ Aldrich (99.5%)
Diisopropylsilyl bis(trifluoromethanesulfonate) ~ Fluka (>97%)
N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) ~ Applied Biosystems
N,N-Dimethylformamide ~ Fluka (99.8%)
Dimethyl sulfate ~ Acros Organics (99+\%)
d_{6}-DMSO ~ Euriso-top (H_{2}O<0.02\%) + 0.03\% TMS
1,4-Dioxane ~ Sigma (99+\%)
(\textpm)-Epicatechin ~ Unilever (>97\%)
(\textpm)-Epigallocatechin ~ Unilever (>97\%)
Ethyl acetate ~ Fisher Scientific (Analytical reagent grade)
Filter paper ~ Whatman N° 1
Formic acid ~ Fluka (≥98.0\%)
Hexane ~ Fisher Scientific (HPLC grade)
Light petroleum (40-60 °C) ~ Fisher Scientific (Laboratory reagent grade)
2,6-Lutidine ~ Fluka (98\%)
d_{6}-MeOH ~ Euriso-top (HDO + D_{2}O<0.03\%)
Methanol ~ Fisher Scientific (HPLC grade)
N-Methylimidazole (NMI) ~ Aldrich (99.5\%)
3A molecular sieves ~ BDH
4A molecular sieves ~ Fisons scientific equipment
NovaSyn® TG amino resin ~ Novabiochem (90 μm)
NovaSyn® Ellman's resin (3,4-dihydro-2H-pyran-2-ylmethoxymethyl polystyrene) ~
Novabiochem (100-200 mesh)
NovaSyn® TG hydroxy resin ~ Novabiochem (130 μm)
pH indicator paper ~ Fisher Scientific
Phosphorus pentoxide ~ Fisher Scientific (Laboratory reagent grade)
Picrylsulfonic acid dihydrate ~ Aldrich (97\%)
Potassium carbonate (anhydrous) ~ Fisher Scientific
Potassium hexacyanoferrate(III) ~ Fluka (≥99\%)
Pyridine (anhydrous) ~ Sigma-Aldrich (99.8\%)
Pyridine ~ Fisher Scientific (Analytical reagent grade)
Pyridinium p-toluenesulfonate (PPTS) ~ Acros Organics (98\%)
Silica 60 A Particle size 40-63 micron ~ Fisher Scientific (Chromatography grade)
Sodium chloride ~ Fisher Scientific (Laboratory reagent grade)
Sodium deuterioxide ~ Acros Organics (99+ atom% D)
Sodium diethyldithiocarbamate trihydrate ~ Fluka (>99%)
Sodium hydrogen carbonate ~ Fisher Scientific (Analytical reagent grade)
Sodium hydroxide ~ Fisher Scientific (Analytical reagent grade)
Sodium sulfate (anhydrous) ~ Fisher Scientific
Sodium thiosulfate, 0.1 N ~ Acros Organics (pure)
Tetrahydrofuran ~ Fisher Scientific (HPLC grade)
Tetrakis(acetonitrile)copper(I) hexafluorophosphate ~ Aldrich
Tetrakis(triphenylphosphine)palladium(0) ~ Sigma-Aldrich (99%)
Theaflavin ~ Unilever (>97%)
TLC plates (AlO₁₂) ~ Merck 60 F₂₅₄ (neutral)
TLC plates (SiO₂) ~ Macherey-Nagel Alugram® Sil G/UV₂₅₄
Toluene ~ Fisher Scientific (Analytical reagent grade)
Toluene-4-sulfonic acid (TsOH) ~ Fisher Scientific (98+%) 
Triethylamine ~ Aldrich (99%)
Triethylamine trihydrofluoride ~ Aldrich (98%)
Trimethylethoxysilane ~ ABCR (97%)
Triphenylphosphine ~ Aldrich (99%)
3.3 Solution-phase protecting groups

3.3.1 Attempted solution-phase preparation of THP ether of (-)-epicatechin 5 (page 131)

A solution of (-)-epicatechin 5 (50.20 mg, 0.1729 mmol, 1.0 eq) in d_6-DMSO (2 ml) was placed in a dry 50 ml one-neck round-bottomed flask. DHP (16.00 µL, 0.1754 mmol, 1.0 eq) and a catalytic amount of toluene-4-sulfonic acid (1.8 mg, 0.009463 mmol, 0.05 eq) were added to the flask. A cold-water condenser with a drying tube containing CaCl_2 was fitted to the neck of the flask. The solution was stirred, using a magnetic follower at 66 °C for ninety-eight hours thirty minutes. The temperature of the reaction was maintained using a silicone oil bath coupled to a programmable thermocouple. At the end of the reaction, the mixture was subjected to column chromatography without solvent removal. The column was 500 mm in length (~28 g silica gel to a height of 420 mm) and had i.d. of 12 mm. The column was eluted with DCE:MeOH:Me_2CO, 60:30:26 v/v/v. Twelve fractions, each of 4.9 ml, were collected. After TLC analysis, fractions 1–6 were combined and fractions 8–13 were combined and the solvent removed in vacuo to yield two brown residues. The least polar fraction (1–6) was triturated with DCM to yield ~10 mg of a brown precipitate. The precipitate was analysed by 1H-NMR spectroscopy and QTOF-MS.

3.3.2 Stability of (-)-epicatechin 5 in aqueous alkali (page 134)

(-)-Epicatechin 5 (15.1 mg, 0.052 mmol, 1.0 eq) was placed in a 2 ml glass vial and D_2O (600 µL) was added to the vial. The vial was swirled for twenty seconds and it was observed that the (-)-epicatechin 5 had not dissolved. The mixture was beige-coloured. NaOD in D_2O (10.7 M) (5.00 µL, 0.054 mmol, 1.0 eq) was added to the vial. The vial was swirled and it was observed that the solution became an intense yellow colour. It appeared that all of the (-)-epicatechin 5 had dissolved. The solution was dispensed into a NMR-tube for 1H-NMR spectroscopy reaction monitoring.
3.3.3 Solution-phase methylation of (-)-epicatechin 5 (page 135)

(-)-Epicatechin 5 (15.2 mg, 0.052 mmol, 1.0 eq) was placed in a 2 ml glass vial and D$_2$O (500 µL) was added to the vial. The vial was swirled for twenty seconds and it was observed that the (-)-epicatechin 5 had not dissolved. The mixture was beige coloured. NaOD in D$_2$O (10.7 M) (5.00 µL, 0.054 mmol, 1.0 eq) was added to the vial. The vial was swirled and it was observed that the solution became an intense yellow colour. It appeared that all of the (-)-epicatechin 5 had dissolved. Dimethyl sulfate (4.74 µL, 0.050 mmol, 1.0 eq) was added to the vial. The solution was dispensed into a NMR-tube for $^1$H-NMR spectroscopy reaction monitoring. It was observed that a white precipitate had formed at the bottom of the NMR-tube after mixing of the reagents. The contents of the NMR-tube were washed into a 5 ml beaker by rinsing with distilled H$_2$O (2 ml). The diluted solution was yellow and no further precipitation occurred upon dilution. HCl(aq) (3 M) was added until pH 5.5-6.0 (indicator paper). The colour of the solution changed from yellow to straw-coloured. NaCl(s) was added to the aqueous solution until it became saturated. The aqueous solution was extracted with EtOAc (3 x 2 ml). An aliquot of the organic layer was analysed by TLC (DCE:MeOH:Me$_2$CO, 60:30:26 v/v/v). The organic layer was dried over anhydrous Na$_2$SO$_4$, the Na$_2$SO$_4$ was filtered off and the filtrate placed in a 25 ml round-bottomed flask. The EtOAc was removed in vacuo. The flask was subsequently placed on a high-vacuum line for two hours. A sample of the residue was analysed by LC-ESI-MS.

3.3.4 Solution-phase equimolar benzoylation of (-)-epicatechin 5 (page 136)

(-)-Epicatechin 5 (15.0 mg, 0.052 mmol, 1.0 eq) was placed in a 2 ml glass vial. d$_r$-MeOH (600 µL) was added to the vial. The vial was swirled for twenty seconds and it was observed that all the (-)-epicatechin 5 dissolved. The solution was straw-coloured. NaOD in D$_2$O (10.7 M) (5.00 µL, 0.054 mmol, 1.0 eq) was added to the vial. The vial was swirled and it was observed that the solution became an intense yellow colour. Benzoyl chloride (5.81 µL, 0.050 mmol, 1.0 eq) was added to the vial. The solution was dispensed into a NMR-tube for $^1$H-NMR spectroscopy reaction monitoring. The tube was
flushed with N₂ and sealed. At the end of the reaction, an aliquot of the contents of the NMR-tube was analysed by TLC (DCE:MeOH:Me₂CO, 80:5:15 v/v/v).

3.3.5 Solution-phase optimised benzoylation of (−)-epicatechin 5 (page 137)

(−)-Epicatechin 5 (100.8 mg, 0.347 mmol, 1.0 eq) was placed in a one-necked 25 ml round bottom flask. MeOH (2 ml) was added to the flask. The flask was swirled for twenty seconds and it was observed that all the (−)-epicatechin 5 had dissolved. The solution was straw-coloured. NaOH (10.7 M) (16.1 µL, 0.172 mmol, 0.5 eq) was added to the flask. The flask was swirled and it was observed that the solution became an intense yellow colour. Benzoyl chloride (19.9 µL, 0.172 mmol, 0.5 eq) was added to the flask. A cold-water condenser with a N₂ bubbler was fitted to the neck of the flask. The mixture was stirred, using a magnetic follower, at room temperature. After twenty-three minutes, the reaction was terminated. It was observed that a small amount of white precipitate was present at the bottom of the flask. The MeOH was removed in vacuo. A small sample (~7 mg) of the crude reaction mixture was analysed by ¹H-NMR spectroscopy. The flask containing the crude mixture was placed on a high-vacuum line for three hours. The crude mixture was obtained as a red-orange crystalline residue (146.1 mg). A sample of this mixture was utilised via TLC, to optimise chromatographic resolution conditions.

The mixture was subjected to column chromatography. The column was 300 mm in length (~6 g silica to a height of 115 mm) and had i.d. of 10 mm. The column was eluted with EtOAc:DCM, 3:2 v/v. Seven fractions (15 ml each) were collected. Each fraction was analysed by TLC (DCE:MeOH:Me₂CO, 60:30:26 v/v/v). Each fraction was dispensed into a 25 ml round-bottomed flask. The solvent mixture was removed in vacuo. Each flask was subsequently placed on a high-vacuum line for two hours. The least polar fraction was recovered as a colourless residue (72.5 mg). The other fractions of increasing polarity were recovered as a white residue (7.8 mg), a colourless residue (1.2 mg), a whitish residue (1.3 mg), a whitish residue (1.0 mg), a whitish residue (2.2 mg) and a whitish residue (2.0 mg). Each fraction was dissolved in d₄-MeOH (600 µL) and dispensed into an NMR-tube (the tubes were not flushed with N₂) for ¹H-NMR
spectroscopy analysis. An aliquot of the $d_{r}$-MeOH solution containing the least polar fraction was analysed by LC-ESI-MS and HPLC. Additionally, the contents of the NMR-tube containing the least polar fraction were dispensed into a 25 ml round-bottomed flask. The $d_{r}$-MeOH was removed in vacuo. The flask was placed on a high-vacuum line for two hours. A small sample was analysed by FT-IR spectroscopy. The least polar fraction (72.5 mg) was tentatively identified as (−)-epicatechin-5-monobenzoate 111. $^1$H-NMR (500 MHz, $d_{r}$-MeOH, δ): 8.19 (d, $J$ = 8 Hz, 2H, o-benzoate-H). FT-IR (Nujol, NaCl, cm$^{-1}$): 3500 (OH), 1708 (C=O ester), 1140 (C-O-C pyran ring ether).
3.4 Linkage of protected (−)-epicatechin 5 to hydroxytentagel resin

3.4.1 Selective phenol protection in (−)-epicatechin 5 by allylation (page 143)

Deoxygenated DMF (20 ml) was added to a two-necked, 50 cm³ round-bottomed flask, containing a magnetic follower, which was equipped with an air condenser and a N₂ bubbler. (−)-Epicatechin 5 (580.2 mg, 1.99 mmol, 1.0 eq) was added to the flask under positive N₂ pressure with stirring, at room temperature, to yield a yellow homogenous solution. Anhydrous K₂CO₃ (1.3823 g, 10.0 mmol, 5.0 eq) and allyl bromide (1730 µL, 20.0 mmol, 10.0 eq) were similarly added, and the side neck was stoppered. The solution was allowed to stir at room temperature.

After stirring for one hundred and seventy hours, the reaction mixture was filtered, poured into EtOAc (200 ml) contained in a 500 ml separating-funnel, and washed with distilled H₂O (3 x 120 ml) and 20% NaCl(aq) (1 x 120 ml). The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo to yield crude product (913.8 mg).

A portion of the crude worked-up mixture (441.0 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 285 mm) and had i.d. of 20 mm. Silica (45 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 5:95 v/v (100 ml). The crude mixture was dissolved in EtOAc (300 µL) and hexane (400 µL) and applied to a filter paper disc at the top of the column. The column was washed with EtOAc:hexane, 5:95 v/v (50 ml) before the first fraction was collected. Eighty-six fractions, each of 15.4 ml were collected. Fractions 1–10 were eluted with EtOAc:hexane, 5:95 v/v. Fractions 11–40 were eluted with EtOAc:hexane, 10:90 v/v and fractions 41–86 were eluted with EtOAc:hexane, 15:85 v/v. Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis, fractions 1–52 (21.4 mg) were combined to yield a white solid and fractions 55–74 (220.9 mg) were combined to yield a colourless viscous liquid. Fractions 55–74 contained tetraallyl-(−)-epicatechin 5a. §H-NMR (500 MHz, CDCl₃, δ): 7.09 (d, J = 2 Hz, 1H), 7.01 (d, J = 1.5 Hz, 1H), 6.94 (d, J = 8.5 Hz, 1H), 6.19 (d, J = 2.5 Hz, 1H), 6.14 (d,
\[ J = 2.5 \text{ Hz, 1H}, \ 6.08-6.02 \text{ (m, 4H)}, \ 5.44-5.39 \text{ (m, 4H)}, \ 5.29-5.26 \text{ (m, 4H)}, \ 4.94 \text{ (s, 1H)}, \ 4.65-4.62 \text{ (m, 4H)}, \ 4.52-4.48 \text{ (m, 4H)}, \ 4.27 \text{ (s, 1H)}, \ 2.97-2.93 \text{ (m, 2H)}. \]

\[^1\text{H}-\text{NMR} \text{ (300 MHz, CDCl}_3\text{, } \delta): 158.7, 158.4, 155.4, 148.8, 148.6, 133.6, 133.6, 133.4, 133.4, 131.3, 119.3, 117.9, 117.8, 117.3, 114.4, 112.7, 101.0, 94.7, 94.0, 78.6, 70.2, 70.1, 69.1, 69.0, 66.6, 28.3 \text{ (see figures 9 and 10, respectively).} \]

Another portion of the crude, worked-up mixture (472.8 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 287 mm) and had i.d. of 20 mm. Silica (46.7 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 5:95 v/v (100 ml). The crude mixture was dissolved in EtOAc (200 µL) and hexane (400 µL) and applied to a filter paper disc at the top of the column. The column was washed with EtOAc:hexane, 10:90 v/v (50 ml) before the first fraction was collected. Seventy-two fractions, each of 15.4 ml were collected. Fractions 1–35 were eluted with EtOAc:hexane, 10:90 v/v and fractions 36–72 were eluted with EtOAc:hexane, 15:85 v/v. Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis, fractions 18–28 (13.9 mg) were combined to yield a white solid and fractions 50–59 (242.7 mg) were combined to yield a colourless viscous liquid. Fractions 50–59 contained tetraallyl-(−)-epicatechin 5a.

3.4.2 Selective phenol protection in (−)-epigallocatechin 4 by allylation (page 149)

Deoxygenated DMF (20 ml) was added to a two-necked 50 ml round-bottomed flask, containing a magnetic follower, which was equipped with an air condenser and a N\(_2\) bubbler. (−)-Epigallocatechin 4 (610.9 mg, 2.00 mmol, 1.0 eq) was added to the flask under positive N\(_2\) pressure with stirring, at room temperature, to yield a yellow homogenous solution. Anhydrous K\(_2\)CO\(_3\) (3.3180 g, 24.0 mmol, 12.0 eq) and allyl bromide (2080 µL, 24.0 mmol, 12.0 eq) were similarly added, and the side neck was stoppered. The solution was allowed to stir at room temperature.

After stirring for one hundred and seventy hours, the reaction mixture was filtered, poured into EtOAc (200 ml) in a 500 ml separating-funnel, and washed with distilled H\(_2\)O (4 x 120 ml) and 20% NaCl(aq) (1 x 120 ml). The organic layer was dried over
anhydrous Na$_2$SO$_4$ and the solvent removed \textit{in vacuo} to yield the crude product (1.2369 g).

A portion of the crude worked-up mixture (740.7 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 285 mm) and had i.d. of 20 mm. Silica (45 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 5:95 v/v (100 ml). The mixture was dissolved in EtOAc (300 µL) and hexane (600 µL) and applied to a filter paper disc at the top of the column. The column was washed with EtOAc:hexane, 10:90 v/v (50 ml) before the first fraction was collected. Seventy-two fractions, each of 15.4 ml were collected. Fractions 1–35 were eluted with EtOAc:hexane, 10:90 v/v and fractions 36–72 were eluted with EtOAc:hexane, 15:85 v/v. Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis (EtOAc:hexane, 50:50 v/v), fractions 20–26 were combined to yield a white solid (19.5 mg) and fractions 45–61 were combined to yield a colourless viscous liquid (400.2 mg). Fractions 45–61 contained pentaallyl-(-)-epigallocatechin 4a. $^1$H-NMR (500 MHz, CDCl$_3$, δ): 6.73 (d, $J = 4$ Hz, 2H), 6.19 (d, $J = 1.5$ Hz, 1H), 6.14 (d, $J = 2.5$ Hz, 1H), 6.13–6.02 (m, 5H), 5.44–4.93 (m, 10H), 4.90 (s, 1H), 4.61–4.49 (m, 10H), 4.25 (s, 1H), 3.01–2.93 (m, 2H). $^{13}$C-NMR (300 MHz, CDCl$_3$, δ): 158.7, 158.4, 155.3, 153.0, 134.8, 133.9, 133.8, 133.6, 133.4, 117.9, 117.8, 117.7, 117.4, 105.9, 101.0, 94.7, 94.1, 78.8, 74.4, 70.3, 70.1, 69.2, 69.0, 66.7, 28.2 (see figures 15 and 16, respectively).

Another portion of the crude, worked-up mixture (496.2 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 285 mm) and had i.d. of 20 mm. Silica (45 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 5:95 v/v (100 ml). The mixture was dissolved in EtOAc (200 µL) and hexane (400 µL) and applied to a filter paper disc at the top of the column. The column was washed with EtOAc:hexane, 10:90 v/v (100 ml) before the first fraction was collected.

Sixty-five fractions, each of 15.4 ml were collected. Fractions 1–33 were eluted with EtOAc:hexane, 10:90 v/v and fractions 34–65 were eluted with EtOAc:hexane, 15:85 v/v.
Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis, fractions 46-57 (279.0 mg) were combined to yield a colourless viscous liquid. Fractions 46-57 contained pentaallyl-(-)-epigallocatechin 4a.

3.4.3 Silylation and MeOH quench of tetraallyl-(-)-epicatechin 5a (page 152)

Tetraallyl-(-)-epicatechin 5a (50.0 mg, 0.111 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask A). Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask and the DCM removed in vacuo on a rotary evaporator. This process was repeated twice to ensure that tetraallyl-(-)-epicatechin 5a was rendered free from H₂O. As the vacuum was released at the end of this process, a dry, magnetic follower was placed in the pear-shaped flask. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Anhydrous CDCl₃ (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to this flask with stirring. Once all the tetraallyl-(-)-epicatechin 5a had dissolved, anhydrous pyridine (15 μL, 0.186 mmol, 1.7 eq) was added, via a 100 μL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring, to yield a translucent, light-yellow solution.

Anhydrous CDCl₃ (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to a pre-dried, and pre-N₂ flushed, 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Diisopropylsilyl bis(trifluoromethanesulfonate) (65 μL, 0.220 mmol, 2.0 eq) was added to this flask, with stirring, to yield a colourless solution. Anhydrous pyridine (35 μL, 0.435 mmol, 3.9 eq) was added, via a 100 μL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring, to yield a colourless solution.

The solution in flask A was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask B over a period of thirty seconds, with continued stirring, to yield a translucent, light-yellow solution. A small amount of white precipitate was observed.
An aliquot (600 µL) of the reaction mixture was taken, via a pre-N₂ flushed 1000 µL gas-tight syringe, and placed in a pre-dried NMR-tube and the ¹H-NMR spectra acquired. The contents of the NMR-tube were subsequently quenched with a large stoichiometric excess of anhydrous MeOH (15 µL), via a pre-N₂ flushed 100 µL gas-tight syringe.

As the ¹H-NMR spectrum of the MeOH-quenched NMR-tube was being acquired, the contents of flask B were similarly quenched with a stoichiometric excess of anhydrous MeOH (20 µL), via a pre-N₂ flushed 100 µL gas-tight syringe.

The MeOH-quenched mixture (3.4 ml) was added to saturated NaHCO₃ (4 ml), contained in a 10 ml separating-funnel. The contents of the separating-funnel were shaken and allowed to separate, the organic layer was drained and the saturated NaHCO₃ layer was decanted out the top. The organic layer was returned to the separating-funnel and further washed with saturated NaHCO₃ (2 x 4 ml) and then 20% NaCl(aq) (1 x 4 ml). The colourless organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The organic residue was further treated in vacuo (40 °C) with DCM (1 x 2 ml) and then toluene (2 x 2 ml) to remove traces of pyridine. The organic residue was further dried on a high-vacuum line for six hours and forty minutes to yield a translucent, light-yellow, viscous liquid (35.7 mg).

3.4.3.1 Cleavage of the diisopropylsilyl methyl ether (page 156)

The crude methyl ether, hypothesised as being tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether Sb (10.0 mg, 0.0168 mmol, 1.0 eq), was placed in a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (670 µL) was added to the eppendorf-tube with stirring. Triethylamine (94.8 µL, 0.680 mmol, 60.7 eq) was added to the eppendorf-tube, via a 200 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (72.3 µL, 0.444 mmol, 39.6 eq). The mixture was stirred for thirty minutes, after which, an aliquot (~10 µL) of the colourless, homogenous reaction mixture was added to a 5 ml separating-funnel, containing EtOAc (100 µL). The organic layer was washed with saturated NaHCO₃ (2 x 200 µL) and then analysed by TLC (EtOAc:hexane, 50:50 v/v).
After TLC analysis, the reaction mixture was added to a 10 ml separating-funnel containing EtOAc (3 ml). The organic layer was washed with saturated NaHCO₃ (3 x 2 ml), with light effervescence being observed. The organic layer was dried over anhydrous Na₂SO₄. The organic layer was filtered into a 25 ml round-bottomed flask and the solvent removed in vacuo to yield a colourless liquid (weight not measured).

The organic residue was further treated with DCM (3 x 2 ml) and the solvent removed in vacuo. The organic residue was further dried on a high-vacuum line for two hours. The ¹H-NMR spectrum was acquired.

3.4.4 Stability of (−)-epigallocatechin 4 in the presence of fluoride (page 157)

(−)-Epigallocatechin 4 (19.6 mg, 0.0640 mmol, 1.0 eq) was weighed into a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (750 µL) was added to the eppendorf-tube with stirring. Triethylamine (240 µL, 1.72 mmol, 40.3 eq) was added, via a 1000 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (140 µL, 0.859 mmol, 20.1 eq), which after addition, yielded a translucent, light-yellow heterogeneous solution. The mixture was stirred for two hours. Trimethylethoxysilane (126 µL, 0.807 mmol, 18.9 eq) was added to the solution in the 2 ml eppendorf-tube. The solution was stirred with a magnetic follower for thirty minutes, after which, it was analysed by TLC.

3.4.5 Silylation and linkage of tetraallyl-(−)-epicatechin 5a to hydroxytentagel resin (page 157)

Hydroxytentagel resin (530.5 mg, 0.149 mmol, 1.0 eq) was added, via a polypropylene funnel (prevents resin adhering to glassware), onto the sintered glass disc of a purpose constructed ‘quick-fit’ glassware-assembly, part of which consisted of a N₂ line (CaCl₂ guard-tube), connected via a stop-cock, to a two-necked still-head. The first neck of the still-head (female) was attached to a N₂ bubbler, and the second neck of the still-head (female) was sealed with a septum. The single (male) outlet was connected to a sintered glass column, which contained a stop-cock (male), connected to a ‘quick-fit’ conical vacuum-flask (female) that contained a side-arm outlet. Anhydrous DCM (2.5 ml) was
added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the sintered glass column. The volume of DCM used, was enough to just cover the resin. The resin was allowed to swell for five minutes.

Tetraallyl-(-)-epicatechin 5a (345.9 mg, 0.768 mmol, 5.2 eq) was weighed into a 5 ml pear-shaped flask (flask A). Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask and the DCM removed in vacuo on a rotory evaporator. This process was repeated twice to ensure that all the tetraallyl-(-)-epicatechin 5a was rendered free from H₂O. As the vacuum was released at the end of this process, a dry, magnetic follower was placed in the pear-shaped flask. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Anhydrous DCM (1 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to this flask with stirring. Once all the tetraallyl-(-)-epicatechin 5a had dissolved, anhydrous pyridine (100 µL, 1.24 mmol. 8.3 eq) was added, via a 100 µL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring to yield a translucent, light-yellow solution.

Anhydrous DCM (1 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to a predried, and pre-N₂ flushed, 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Diisopropylsilyl bis(trifluoromethanesulfonate) (420 µL, 1.42 mmol, 9.5 eq) was added, via a 1000 µL pre-N₂ flushed gas-tight syringe, to this flask with stirring to yield a translucent, light-yellow solution. Anhydrous pyridine (200 µL, 2.48 mmol. 16.6 eq) was added, via a 1000 µL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring to yield a colourless solution.

The solution in flask A was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask B with continued stirring to yield a translucent, light-yellow solution. There was no evidence of a precipitate being formed. The translucent, light-yellow solution was stirred vigorously for fifteen minutes and then injected, via the septum, over the top of the preswelled resin, via a pre-N₂ flushed 2 ml gas-tight syringe, to leave a ‘dead’ volume of
~750 μL above the resin. Due to the apparatus assembly, it was not possible to stir the mixture with a magnetic follower. The mixture was allowed to stand for one hour and forty-two minutes. At the end of this period, the translucent, light-yellow supernatant was drained under positive N₂ pressure (without the need for vacuum) into the conical vacuum-flask, with N₂ exiting through the side-arm. A small trail of yellow crystals was observed on the walls of the conical vacuum-flask as draining commenced. The resin was washed with anhydrous DCM (3 x 2 ml) under positive N₂ pressure and the filtrate collected in the conical vacuum-flask. The stopcock was closed and any potentially active sites on the resin (diisopropylsilyl monotriflate) were then quenched/capped with a solution containing anhydrous MeOH (500 μL) and anhydrous pyridine (100 μL) in anhydrous DCM (1.5 ml). The ‘capping’ solution was allowed to stand for five minutes before being drained, under positive N₂ pressure, into the conical vacuum-flask.

As the resin was being ‘capped’, the translucent, light-yellow supernatant in the conical vacuum-flask was quenched with anhydrous MeOH (500 μL) and then stirred for thirty minutes using a magnetic follower.

After ‘capping’, the resin was washed with MeOH, DCM and Et₂O (3 x 5 ml each, in order) to swell and shrink the resin prior to drying. The resin was dried under vacuum for fifteen minutes, placed in the dark, and the vacuum held overnight. The resin was further dried for six hours under high-vacuum. The resin was identified as tetraallyl-(-)-epicatechin diisopropylsilyl ether tentagel resin. FT-IR (ATR) (cm⁻¹): 2864 (methyl C-H asymmetrical/symmetrical stretch), 1097 (R-C-O-Si-O-C-R), 951 (vinyl C-H out-of-plane bend), 885 (vinylidene C-H out of plane bend), 638 (aromatic C-H out-of-plane bend).

The MeOH-quenched supernatant was poured into saturated NaHCO₃ (15 ml), contained in a 50 ml separating-funnel. The contents of the separating-funnel were shaken and allowed to separate. The organic layer was drained and the saturated NaHCO₃ layer was decanted out the top. The organic layer was returned to the separating-funnel and further washed with saturated NaHCO₃ (2 x 15 ml) and then 20% NaCl(aq) (1 x 15 ml). The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The
organic residue was further treated *in vacuo* (40 °C) with toluene (3 x 2 ml) to remove traces of pyridine, to yield a translucent, light-yellow residue (283.3 mg).

### 3.4.5.1 Chromatography of the MeOH-quenched supernatant (page 160)

The translucent, light-yellow residue (~283.3 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 190 mm) and had i.d. of 20 mm. Silica (25 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 2:98 v/v (25 ml). The translucent, light-yellow residue was dissolved in EtOAc (200 μL) and hexane (200 μL) and applied to a filter paper disc at the top of the column.

Fifty-four fractions, each of 10.5 ml, were collected. Fractions 1–12 were eluted with EtOAc:hexane, 5:95 v/v. Fractions 13–23 were eluted with EtOAc:hexane, 10:90 v/v. Fractions 24–36 were eluted with EtOAc:hexane, 15:85 v/v. Fractions 37–48 were eluted with EtOAc:hexane, 20:80 v/v and fractions 49–54 were eluted with EtOAc:hexane, 25:75 v/v. Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis, fractions 1–17 (11.2 mg), fractions 19–29 (143.9 mg) and fractions 33–35 (14.7 mg) were combined to yield three, colourless viscous liquids, respectively. The combined fractions were analysed by NMR spectroscopy. Fractions 1–17 contained a mixture of the two, mono-C-alkylated A-ring by-products of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b. Fractions 19–29 contained tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b. ESI-MS [M+H]+ m/z 595.3074. 1H-NMR (500 MHz, CDCl₃, δ): 7.08 (d, J = 2 Hz, 1H), 6.98 (d, J = 1.5 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 6.17 (d, J = 2.5 Hz, 1H), 6.10–6.04 (m, 5H), 5.43–5.37 (m, 4H), 5.29–5.25 (m, 4H), 4.98 (s, 1H), 4.60–4.59 (m, 4H), 4.50–4.48 (m, 4H), 4.44 (d, J = 1.5 Hz, 1H), 3.26 (s, 3H), 2.91–2.88 (m, 2H), 0.92–0.89 (m, 7H), 0.83–0.81 (m, 7H). 13C-NMR (300 MHz, CDCl₃, δ): 158.5, 158.0, 155.8, 148.4, 148.2, 133.8, 133.6, 132.6, 119.9, 117.9, 117.7, 117.1, 114.2, 113.6, 101.6, 94.4, 93.3, 79.1, 70.3, 70.2, 69.1, 68.9, 67.5, 50.9, 28.5, 17.5, 17.3, 12.5, 12.2 (see figures 18 and 19, respectively).
Fractions 33–35 contained tetraaryl-(−)-epicatechin diisopropylsilyl dimer 5c. FAB-MS [M+H]+ m/z 1013.40. 1H-NMR (500 MHz, CDCl₃, δ): 6.97–6.97 (m, 2H), 6.89–6.87 (m, 2H), 6.81–6.80 (m, 2H), 6.14–6.14 (m, 2H), 6.09–6.02 (m, 10H), 5.42–5.34 (m, 8H), 5.28–5.22 (m, 8H), 4.81 (s, 2H), 4.57–4.55 (m, 8H), 4.49–4.43 (m, 8H), 3.97–3.97 (m, 2H), 2.69 (s, 4H), 0.70–0.65 (m, 14H). 13C-NMR (300 MHz, CDCl₃, δ): 158.5, 157.9, 155.7, 148.3, 133.8, 133.7, 133.6, 132.4, 120.0, 117.9, 117.7, 117.1, 114.0, 113.7, 101.6, 94.4, 93.3, 78.9, 70.3, 69.1, 69.0, 67.0, 28.5, 17.3, 17.0, 13.6 (see figures 20 and 21, respectively).

3.4.5.2 Cleavage of tetraaryl-(−)-epicatechin diisopropylsilyl ether tentagel resin (page 161)

A portion of the vacuum-dried resin (234.3 mg, 0.0656 mmol, 1.0 eq) was placed in a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (900 µL) was added to the eppendorf-tube. The volume of THF used was enough to just cover the resin. The resin was allowed to swell for fifteen minutes with stirring. At the end of this period, triethylamine (336 µL, 2.42 mmol, 55.4 eq) was added, via a 1000 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (198 µL, 1.21 mmol, 27.7 eq). The mixture was stirred for one hour and five minutes, after which it was filtered through a sintered glass vacuum-funnel and the colourless filtrate collected in a 5 ml round-bottomed flask. The resin was washed with THF (3 x 1 ml) and the washings collected in the 5 ml round-bottomed flask. Trimethylthoxysilane (300 µL, 1.92 mmol, 44.0 eq) was added to the solution in the 5 ml round-bottomed flask. The solution was stirred with a magnetic follower for fifteen minutes. The solution was concentrated in vacuo to yield a colourless residue. The residue was further dried on a high-vacuum line for three hours to yield a colourless residue (25.0 mg, 0.0555 mmol). The residue was analysed by TLC (EtOAc:hexane, 50:50 v/v), positive-polarity LC-ESI-MS and 1H-NMR spectroscopy.
3.5 Linkage of protected (-)-epigallocatechin 4 intermediate to aminotentagel resin

3.5.1 Synthesis of pentaallyl(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (page 168)

Pentaallyl-(-)-epigallocatechin 4a (573.9 mg, 1.13 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask A). Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask and the DCM removed in vacuo on a rotary evaporator. This process was repeated twice to ensure that pentaallyl(-)-epigallocatechin 4a was rendered free from H₂O. As the vacuum was released at the end of this process, a dry, magnetic follower was placed in the pear-shaped flask. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to this flask with stirring. Once all the pentaallyl(-)-epigallocatechin 4a had dissolved, anhydrous pyridine (280 µL, 3.48 mmol, 3.1 eq) was added, via a 1000 µL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring to yield a translucent, light-yellow solution.

Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to a predried, and pre-N₂ flushed, 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Diisopropylsilyl bis(trifluoromethanesulfonate) (740 µL, 2.50 mmol, 2.2 eq) was added to this flask with stirring to yield a translucent, light-yellow solution. Anhydrous pyridine (640 µL, 7.95 mmol, 7.0 eq) was added, via a 1000 µL pre-N₂ flushed gas-tight syringe, to this flask with continued stirring to yield a colourless solution.

The solution in flask A was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask B over a period of thirty seconds, with continued stirring, to yield a translucent, light-yellow solution. There was no evidence of a precipitate being formed. The translucent, light-yellow solution was stirred vigorously for fifteen minutes and then quenched with
1-butynol (1.7 ml, 22.5 mmol, 19.9 eq), added, via a pre-N₂ flushed 2 ml gas-tight syringe. The translucent, light-yellow solution was stirred vigorously for one hour.

The quenched mixture was poured into saturated NaHCO₃ (5 ml), contained in a 10 ml separating-funnel. The contents of the separating-funnel were shaken and allowed to separate. The organic layer was drained and the saturated NaHCO₃ layer was decanted out the top. The organic layer was returned to the separating-funnel and further washed with saturated NaHCO₃ (3 x 5 ml) and then 20% NaCl(au) (1 x 5 ml). The organic layer was an opaque yellow colour, but became translucent-yellow after the 20% NaCl(au) wash. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The organic residue was further treated in vacuo (40 ºC) with toluene (3 x 5 ml) to remove traces of pyridine. The organic residue was then dried on a high-vacuum line for six hours forty minutes, to yield the translucent, light-yellow crude product (926.9 mg). The organic residue was analysed by TLC (EtOAc:hexane, 50:50 v/v) and positive-polarity LC-ESI-MS.

3.5.1.1 Chromatography and isolation of pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b (page 169)

The translucent, light-yellow residue (~926.9 mg) was subjected to column chromatography. The column was 300 mm in length (silica gel to a height of 285 mm) and had i.d. of 20 mm. Silica (45 g) was made up as a slurry in hexane. The column was washed with EtOAc:hexane, 5:95 v/v (50 ml). The translucent, light-yellow residue was dissolved in EtOAc (300 µL) and hexane (150 µL) and applied to a filter paper disc at the top of the column.

Forty fractions, each of 15.4 ml, were collected. Fractions 1–15 were eluted with EtOAc:hexane, 5:95 v/v. Fractions 16–29 were eluted with EtOAc:hexane, 10:90 v/v and fractions 30–40 were eluted with EtOAc:hexane, 15:85 v/v. Each fraction was analysed by TLC (EtOAc:hexane, 50:50 v/v). After TLC analysis, fractions 18–28 (568.7 mg) were combined to yield colourless, pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b.¹H-NMR (500 MHz, CDCl₃, δ): 6.72 (d, J = 4 Hz, 2H), 6.18 (d, J = 0.5
Hz, 1H), 6.11–6.03 (m, 6H), 5.43–5.38 (m, 5H), 5.30–5.24 (m, 5H), 4.93 (s, 1H),
4.57–4.56 (m, 5H), 4.53–4.49 (m, 5H), 4.47 (d, J = 2 Hz, 1H), 3.58–3.55 (m, 2H),
2.31–2.30 (m, 2H), 1.91 (s, 1H), 0.89–0.86 (m, 7H), 0.82–0.77 (m, 7H). 13C-NMR (300
MHz, CDCl3, δ): 158.6, 158.0, 155.6, 152.5, 134.9, 134.9, 134.0, 133.8, 133.6, 117.9,
117.6, 117.4, 117.2, 106.6, 101.5, 94.5, 93.3, 81.6, 79.2, 74.4, 70.3, 69.6, 69.1, 69.0, 67.6,
61.3, 28.7, 22.8, 17.4, 17.3, 12.6, 12.4 (see figures 23 and 24, respectively).

3.5.2 SPS of 4-azidobenzoyl aminotentagel resin (page 170)

Aminotentagel resin (498.5 mg, 0.119 mmol, 1.0 eq) was added, via a polypropylene
funnel (prevents resin adhering to glassware), to a SPS sintered glass column, fitted with
a stopcock.

4-Azidobenzoic acid (95.5 mg, 0.585 mmol, 4.9 eq) and HATU (217.5 mg, 0.572 mmol,
4.8 eq) were weighed into a 5 ml pear-shaped flask. DMF (2 ml) was added to the flask
with stirring. N,N-Diisopropylethylamine (105 μL, 0.603 mmol, 5.1 eq) was subsequently
added to the flask to yield a translucent, yellow solution. After five minutes, another
volume of N,N-diisopropylethylamine (105 μL, 0.603 mmol, 5.1 eq) was added to the
flask. The resulting solution was poured over the aminotentagel resin contained in the
SPS glassware. DMF (500 μL) was used to wash the resin beads off the sides of the
glassware. The solution was allowed to stir gently for one hour fifty-five minutes, using a
magnetic follower.

At the end of this period, a colour test was undertaken, which detects the presence of free,
primary amine groups. A small sample of the resin was transferred, via a 20 μL pipette, to
a small sintered glass vacuum-funnel. The sample was washed with DMF (2 ml) and then
MeCN (2 ml). Picrylsulfonic acid dihydrate (9.2 mg) was added to a 2 ml pear-shaped
flask. DMF (500 μL) and N,N-diisopropylethylamine (50 μL) was added to this flask with
stirring. The resulting solution was poured over the sample of resin and allowed to stand
for ten minutes. After this period, the sample of resin was washed with DMF (2 x 0.5 ml)
under vacuum.

243
Any remaining, free primary amine groups (maximum 2.1%) present in the resin contained in the SPS glassware were ‘capped’. 20% NMI, 40% 2,6-lutidine in THF (1.5 ml) was placed into a 5 ml pear-shaped flask. 20% acetic anhydride in THF (1.5 ml) was added to this flask and the resulting solution poured over the pre-washed (3 x 5 ml MeCN), and filtered resin, contained in the SPS glassware. The heterogeneous mixture was allowed to stir for twenty minutes. The ‘capping’ solution was then filtered from the resin under vacuum.

The resin was washed with MeCN, MeOH, DCM and Et₂O (3 x 5 ml each, in order) to swell and shrink the resin prior to drying. The resin was dried under vacuum for twenty minutes, placed in the dark, and the vacuum held overnight. The resin was further dried for six hours under high-vacuum. The resin was identified as 4-azidobenzoyl aminotentagel resin. Gel-phase $^{13}$C-NMR (300 MHz, CDCl₃, δ): 173.1, 149.6, 137.8, 135.8, 125.5, 84.6, 84.1, 83.7, 83.2, 76.4, 46.6 (see figure 25). FT-IR (ATR) (cm$^{-1}$): 2123 (N$_3$ stretch, azide), 1655 (C=O stretch, amide ii), 1603 (NH stretch, amide i), 1146 (C-O stretch, PEG tether) (see figure 26).

3.5.3 ‘Click’-chemistry with 4-azidobenzoyl aminotentagel resin and pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (page 174)

The general trend for using MeCN as a swelling-solvent during SPS, utilising tentagel resins, is 2.5 ml MeCN to 0.5 g resin. Therefore, before commencing the reaction, the solubility of tetrakis(acetonitrile)copper(I) hexafluorophosphate in MeCN had to be checked. From calculations based on the scale of the reaction, the amount of tetrakis(acetonitrile)copper(I) hexafluorophosphate required was 1.04 mg in 1.5 ml MeCN. Tetrakis(acetonitrile)copper(I) hexafluorophosphate (1.1 mg) was weighed into a 2 ml eppendorf tube and MeCN (1.5 ml) was added with gentle shaking. All the tetrakis(acetonitrile)copper(I) hexafluorophosphate appeared to dissolve.

Pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (117.9 mg, 0.171 mmol, 3.6 eq) was weighed into a 5 ml pear-shaped flask (flask A). Anhydrous DCM (2 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask and the DCM removed in
vacuo on a rotary evaporator. This process was repeated twice to ensure that all the pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b was rendered free from O₂. As the vacuum was released at the end of this process, a dry, magnetic follower was placed in the pear-shaped flask, and the flask flushed with dry N₂. The flask was sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Deoxygenated MeCN (1 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to this flask with stirring. All the pentaallyl-(−)-epigallocatechin diisopropylsilyl butynyl ether 4b appeared to dissolve.

4-Azidobenzoyl aminotentagel resin (200.0 mg, 0.048 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. Tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.8 mg, 0.00215 mmol, 4.5 mol%) was added to this flask, and the flask was flushed with dry N₂. The flask was sealed with a glass stopper.

The solution in flask A was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask B under positive N₂ pressure, with continued stirring, to yield a colourless solution. The stopper was reapplied and the mixture was allowed to stir at room temperature, and later in an oven, at 60 °C in the dark.

At certain intervals, the reaction was monitored by FT-IR (ATR) spectroscopy: a small sample of the resin was removed, under positive N₂ pressure, and transferred, via a 20 μL pipette to a small sintered glass vacuum-funnel. The pear-shaped flask was flushed with N₂, sealed with a glass stopper and allowed to continue stirring in the dark. The sample of resin was washed with MeCN, MeOH, DCM and B₃O (3 x 5 ml each, in order) to swell and shrink the resin prior to drying. The resin was dried in vacuo for at least one hour prior to FT-IR (ATR) spectroscopy.

When the reaction was deemed complete, the deep-yellow coloured resin was washed with MeCN (3 x 5 ml). The filtrate was dried in vacuo (removal of MeCN) to constant weight to yield the crude filtrate residue of pentaallyl-(−)-epigallocatechin
diisopropylsilyl butylnyl ether 4b (86.1 mg), presumably containing tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.8 mg).

Finally, the resin was washed with DMF (3 x 5 ml), and a large excess of 0.5 M sodium diethylldithiocarbamate in DMF, to sequester any ‘trapped’ Cu(I) species in the PEG tethers. During each sequestering treatment, the diethylldithiocarbamate solution was allowed to stand over the resin for ten minutes. After the sequestering treatment, the resin was washed with DMF, MeOH, DCM and Et₂O (3 x 5 ml each, in order) to swell and shrink the resin prior to drying. The resin was dried under vacuum for fifteen minutes, placed in the dark, and the vacuum held overnight. The resin was further dried for six hours under high-vacuum, to yield a deep-yellow coloured resin. The resin was identified as pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin. FT-IR (ATR) (cm⁻¹): 2110 (1,4-disubstituted 1,2,3-triazole), 1095 (R-C-O-Si-O-C-R).

3.5.3.1 Cleavage of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (page 180)

A portion of the vacuum dried ‘click’ resin (109.4 mg, 0.0263 mmol, 1.0 eq) was placed in a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (1000 µL) was added to the eppendorf-tube. The volume of THF used was enough to just cover the resin. The resin was allowed to swell for fifteen minutes with stirring. At the end of this period, triethylamine (98 µL, 0.703 mmol, 40.2 eq) was added, via a 200 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (57 µL, 0.350 mmol, 20.0 eq). The mixture was stirred for two hours and thirty minutes (in an attempt to obtain maximum cleavage of the diisopropylsilyl linker), after which, it was filtered through a sintered glass vacuum-funnel and the colourless filtrate collected in a 25 ml round-bottomed flask. The resin was washed with THF (1 x 10 ml) and the washings collected in the 25 ml round-bottomed flask. Trimethylethoxysilane (101 µL, 0.647 mmol, 37.0 eq) was added to the solution in the 25 ml round-bottomed flask. The solution was stirred with a magnetic follower for one hour. The solution was concentrated in vacuo to yield a colourless residue. The residue was
further dried on a high-vacuum line for one hour and the vacuum held overnight to yield a colourless viscous residue (8.8 mg).

The resin was washed with MeOH and DCM (4 x 0.5 ml each, in order). Et₂O would normally have been utilised to shrink the resin prior to drying; however, the gel-phase \(^{13}\)C-NMR acquisition required CDCl₃ as solvent, and, therefore, the final wash was DCM. The resin was dried under vacuum for one hour and fifteen minutes, placed in the dark, and the vacuum held overnight. The resin was further dried for six hours under high-vacuum. However, the resin was ‘sticky’ after the final DCM wash, and vacuum-drying stage, and could not readily be placed into the NMR-tube for gel-phase \(^{13}\)C-NMR spectroscopic analysis. Therefore, the resin was washed with Et₂O (2 x 5 ml) and vacuum-dried for six hours before gel-phase \(^{13}\)C-NMR spectroscopy.

3.5.4 ‘Click’-chemistry synthesis of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (page 183)

Pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (346.1 mg, 0.502 mmol, 1.1 eq) was weighed into a 25 ml round-bottomed flask (flask A). Anhydrous DCM (10 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask and the DCM removed \textit{in vacuo} on a rotary evaporator. This process was repeated twice to ensure that pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b was rendered free from O₂. As the vacuum was released at the end of this process, a dry, magnetic follower was placed in the round-bottomed flask. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum.

4-Azidobenzoic acid (74.6 mg, 0.457 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was inserted into the septum. Tetrakis(acetonitrile)copper(I) hexafluorophosphate (8.6 mg, 0.0231 mmol, 5.1 mol%) was added to a 2 ml eppendorf-tube under light positive N₂ pressure. Deoxygenated MeCN (2 ml) was added, \textit{via} a pre-N₂ flushed 2 ml gas-tight syringe, to the eppendorf-
tube. The contents of the eppendorf-tube were shaken, and the resulting solution was added to flask B, via a pre-N₂ flushed 2 ml gas-tight syringe, with stirring. A further portion of MeCN (3 x 1 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask, with stirring, to enable all the 4-azidobenzoic acid to dissolve.

The solution in flask B was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask A under positive N₂ pressure with continued stirring to yield a translucent, colourless solution. The residual contents of flask B were rinsed with MeCN (4 x 2 ml) and added to flask A, via a pre-N₂ flushed 2 ml gas-tight syringe. There was no evidence of a precipitate being formed. The translucent, pale-yellow reaction mixture was allowed to continue stirring in the dark.

At the end of the reaction, the translucent, pale-straw coloured reaction mixture was added to EtOAc (22 ml), contained in a 100 ml separating-funnel. The organic layer was washed with 0.1 M sodium thiosulfate (3 x 15 ml) to sequester Cu(I) into the aqueous layer. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The organic layer was further dried on a high-vacuum line for three hours forty minutes to yield an off-white, waxy solid (372.7 mg, 0.438 mmol). The product was identified as a mixture of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (> 90%) and pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b. FT-IR (film, NaCl, cm⁻¹): 2111 (1,4-disubstituted 1,2,3-triazole), 1678 (C=O stretch, amide ii), 1116 (R-C-O-Si-O-C-R).

3.5.5 SPS of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (page 186)

Aminotentagel resin (559.8 mg, 0.134 mmol, 1.0 eq) was added, via a polypropylene funnel (prevents resin from 'sticking' to glassware), to a SPS sintered glass column fitted with a stopcock.

Pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d (343.1 mg, 0.403 mmol, 3.0 eq) and HATU (146.9 mg, 0.386 mmol, 2.9 eq) were weighed into a 10 ml pear-shaped flask. DMF (2 ml) was added to the flask with stirring.
to yield a translucent, light-orange solution. *N,N*-Diisopropylethylamine (70.15 μL, 0.403 mmol, 3.0 eq) was subsequently added to the flask. After ten minutes, another volume of *N,N*-diisopropylethylamine (70.15 μL, 0.403 mmol, 3.0 eq) was added to the flask. There was no change in observations. The resulting solution was poured over aminotentagel resin, contained in the SPS glassware. DMF (500 μL) was used to wash the resin beads off the sides of the glassware. The resin was totally submerged, and was swelled within five minutes. The translucent, light-orange mixture was allowed to stir gently for four hours and ten minutes using a magnetic follower.

At the end of this period, a colour-test was undertaken which detects the presence of free primary amine groups. A small sample of the resin was transferred, *via* a 20 μL pipette, to a small sintered glass vacuum-funnel. The sample was washed with DMF (10 x 0.5 ml). Picrylsulfonic acid dihydrate (54.2 mg) was added to a 2 ml pear-shaped flask. DMF (450 μL) and *N,N*-diisopropylethylamine (50 μL) was added to this flask with stirring. The resulting solution was poured over the sample of resin and allowed to stand for fifteen minutes. After this period, the sample of resin was washed with DMF (2 x 0.5 ml).

Any remaining, free primary amine groups (maximum 1.9%) present within the resin contained in the SPS glassware were ‘capped’. 20% NMI, 40% 2,6-lutidine in THF (1.5 ml) was placed into a 5 ml pear-shaped flask. 20% acetic anhydride in THF (1.5 ml) was added to this flask and the resulting solution poured over the pre-washed (4 x 5 ml MeCN), and filtered resin, contained in the SPS glassware. The heterogeneous mixture was allowed to stir for twenty minutes. The ‘capping’ solution was then filtered off the resin under vacuum. The resin was washed with MeCN, DMF, MeOH, DCM and Et₂O (4 x 5 ml each, in order) to swell and shrink the resin prior to drying. The resin was dried under vacuum for thirty minutes, placed in the dark, and the vacuum held overnight. The resin was further dried for six hours under high-vacuum.

The translucent, light-orange filtrate obtained from the first MeCN wash (4 x 5 ml) was added to EtOAc (30 ml) and H₂O (30 ml) in a 100 ml separating-funnel. The contents of the separating-funnel were shaken and allowed to separate. The organic layer was dried
over anhydrous Na₂SO₄. The translucent, brown aqueous layer was analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v).

An aliquot of the translucent, light-yellow organic layer (100 μL) was placed in a 2 ml round-bottomed flask containing a magnetic follower. Benzylamine (3 μL) was added to the flask and the solution was allowed to stir for thirty minutes. The reaction mixture was analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v).

The remainder of the translucent, pale-yellow organic layer was dried *in vacuo* to yield a non-viscous, yellow-orange liquid (934.2 mg). The organic residue was further treated *in vacuo* (40 °C) with toluene (3 x 2 ml) to remove N,N-diisopropylethylamine. The organic residue was then dried on a high-vacuum line for four hours and fifteen minutes to yield a viscous, orange liquid (421.1 mg). The liquid was analysed by ¹H-NMR spectroscopy and positive-polarity LC-ESI-MS.
3.6 SPS deallylation and oxidation

3.6.1 Deallylation of tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (with formic acid/butylamine) (page 192)

Tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (73.2 mg, 0.492 mmol allyl, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask A). Tetrakis(triphenylphosphine)palladium(0) (Pd(PPh₃)₄) (55.0 mg, 0.0480 mmol, 9.8 mol%) was added to the flask. A dry, magnetic follower was placed in the flask, the flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum.

Anhydrous and deoxygenated THF (0.5 ml) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, into a pre-N₂ flushed 5 ml pear-shaped flask (flask B) containing a dry magnetic follower. The flask was then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Formic acid (93 μL, 2.46 mmol, 5.0 eq) was added to the flask, via a pre-N₂ flushed 100 μL gas-tight syringe, with stirring. Butylamine (235 μL, 2.37 mmol, 4.8 eq) was added, dropwise to the flask, via a pre-N₂ flushed 1000 μL gas-tight syringe, with stirring, to yield two colourless immiscible layers. The pH of the solution was checked by taking 2 μL of the contents of flask B and adding this to a 1.5 ml eppendorf-tube containing H₂O (18 μL). The mixture was then poured over pH paper (pH 5.5). The pH was adjusted so that the final volumes of formic acid (99 μL, 2.62 mmol, 5.3 eq) and butylamine (247.5 μL, 2.50 mmol, 5.1 eq) yielded an immiscible solution at pH 7. Anhydrous and deoxygenated MeCN (5 x 100 μL) was added, via a pre-N₂ flushed 1000 μL gas-tight syringe, to this mixture to yield a colourless homogeneous solution.

The solution in flask B was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask A under positive N₂ pressure, with vigorous stirring to yield a colourless solution. However, it was observed that not all the Pd(PPh₃)₄ had dissolved (~20%). However, the reaction mixture was allowed to continue stirring and after one hour had become homogeneous.
The deallylation reaction was considered complete after sixty-six hours thirty minutes, as judged by TLC. At this point, the crude reaction mixture was added to distilled H₂O (2 ml) contained in a 25 ml separating-funnel, to yield a milky-white opaque mixture. EtOAc (5 ml) was added to the separating-funnel. The contents of the separating-funnel were shaken and allowed to separate. The organic layer had become purple and the aqueous layer had become colourless. The aqueous layer was drained and the organic layer was further washed with distilled H₂O (2 x 2 ml) and 0.1 M diaminooethane-tetra-acetic acid disodium salt (3 x 2 ml) to sequester and transfer Pd(0) into the aqueous phase. At this stage, the organic layer had retained its purple colour and the aqueous layer was translucent pale-brown. The organic layer was washed with ice-cooled citric acid (5%) (3 x 2 ml), to neutralise any remaining butylamine, and to transfer the resultant salt into the aqueous phase. After the first addition of citric acid, the opaque, purple organic layer became a yellow-orange colour and also contained a small amount of yellow precipitate. The aqueous layer was pale-yellow. The opaque yellow-orange organic layer was finally washed with 20% NaCl(aq) (3 x 2 ml) by which time it had become translucent-orange. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The residue was further dried on a high-vacuum line for six hours to yield a translucent, light-yellow solid (38.4 mg). The solid was kept under N₂ and stored at -20 °C between analyses. The organic residue was analysed by positive- and negative-polarity LC-ESI-MS and ¹H-NMR spectroscopy.

3.6.2 Deallylation of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b (with sodium benzenesulfinate) (page 196)

The solubility of sodium benzenesulfinate in DMF (2.5 ml as used in SPS) was checked. An arbitrary amount of sodium benzenesulfinate (60 mg) was weighed into a 5 ml pear-shaped flask. DMF (in 100 µL portions) was added with heating, and the mixture allowed to cool to room temperature. It was established that the solubility was sodium benzenesulfinate:DMF, 1:11.7 w/v or 0.086 mg sodium benzenesulfinate µL⁻¹ DMF.

Tetraallyl-(−)-epicatechin diisopropylsilyl methyl ether 5b (68.0 mg, 0.457 mmol allyl, 1.0 eq) was weighed into a 5 ml pear-shaped flask. Pd(PPh₃)₄ (54.2 mg, 0.0469 mmol,
10.3 mol%) and sodium benzenesulfinate (154.5 mg, 0.941 mmol, 2.1 eq) were added to the flask. A dry, magnetic follower was placed in the flask. The flask was flushed with dry N\textsubscript{2} and then sealed with a septum. A balloon containing dry N\textsubscript{2} was connected to a needle and the needle was inserted into the septum.

Anhydrous and deoxygenated DMF (1.5 ml) was added, \textit{via} a pre-N\textsubscript{2} flushed 2 ml gas-tight syringe, to the flask with vigorous stirring. It was observed that the sodium benzenesulfinate had not dissolved. The mixture was heated with a hot-air gun without change. DMF (500 \mu L) was added with heating and all the sodium benzenesulfinate appeared to dissolve. However, it was observed that after ten minutes, when the flask had cooled to room temperature, the sodium benzenesulfinate had again precipitated out of solution. More DMF (5 x 500 \mu L) was added with heating to finally yield a brown homogeneous solution, at room temperature. The volume of DMF used was approximately three times larger than calculated from the solubility test. The test was undertaken without the presence of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b, and the decrease in solubility of the components was attributed to the additional presence of tetraallyl-(-)-epicatechin diisopropylsilyl methyl ether 5b.

At various intervals, aliquots (~20 \mu L) of the translucent, homogeneous brown reaction mixture were taken, \textit{via} a pre-N\textsubscript{2} flushed 1000 \mu L gas-tight syringe, for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). As a typical procedure, the aliquot of interest was placed in a 10 ml test-tube and EtOAc (2 ml) was added to the test-tube. At this point, the contents of the test-tube became cloudy and a precipitate formed. 0.1 M diaminooctane-tetra-acetic acid disodium salt (2 ml) was added to the test-tube with shaking and the contents of the test-tube were allowed to separate. The top, organic layer (EtOAc) was transferred, \textit{via} a glass pipette, into a clean 10 ml test-tube. Distilled H\textsubscript{2}O (2 ml) was added to the test-tube with shaking and the contents of the test tube were allowed to separate. The top, organic layer (EtOAc) was transferred, \textit{via} a glass pipette, into a clean 10 ml test-tube. 20\% NaCl\textsubscript{(aq)} (2 ml) was added to the test-tube with shaking and the contents of the test-tube were allowed to separate. The top, organic layer (EtOAc) was transferred, \textit{via} a glass pipette, into a clean vial, dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}, and flushed with N\textsubscript{2} prior to analysis.
The deallylation reaction was considered complete after two hundred and sixteen hours, as judged by TLC. At this point, the crude reaction mixture was washed into a 100 ml separating-funnel with 0.1 M diaminoethanetetra-acetic acid disodium salt (10 ml) to yield an opaque, pale-orange mixture. EtOAc (30 ml) was added to the separating-funnel. The contents of the separating-funnel were shaken and allowed to separate, by which time the organic and aqueous layer had become translucent-orange. The aqueous layer was drained and the organic layer was further washed with 0.1 M diaminoethanetetra-acetic acid disodium salt (2 x 10 ml), distilled H₂O (3 x 10 ml) and 20% NaCl(aq) (3 x 10 ml) to yield a translucent-orange organic layer, and a colourless aqueous layer. The organic layer was dried over anhydrous Na₂SO₄ and the solvent removed in vacuo. The residue was further dried on a high-vacuum line for three hours to yield a translucent, light-yellow viscous liquid (88.3 mg). The liquid was kept under N₂ and stored at -20 °C in between analyses. The liquid was analysed by TLC (EtOAc:hexane:AcOH, 50:50:1 v/v/v), positive- and negative-polarity LC-ESI-MS and ¹H-NMR spectroscopy.

3.6.3 SPS deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate) (page 200)

Pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (99.4 mg, 0.119 mmol allyl, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask A). The flask was flushed with dry N₂ and sealed with a glass stopper.

Sodium benzenesulfinate (50.8 mg, 0.309 mmol, 2.6 eq), Pd(PPh₃)₄ (14.1 mg, 0.0122 mmol, 10.3 mol%) and PPh₃ (6.8 mg, 0.0259 mmol, 21.8 mol%) were weighed into a 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was flushed with dry N₂ and then sealed with a septum. A balloon containing dry N₂ was connected to a needle and the needle was inserted into the septum. Anhydrous and deoxygenated DMF (600 μL) was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to the flask, with vigorous stirring. This volume of DMF was calculated from the solubility test. However, it was observed that the sodium benzenesulfinate had not dissolved. The light-yellow heterogeneous mixture was heated with a hot-air gun, without change. DMF (3 x 200 μL)
was added with heating and all the sodium benzenesulfinate appeared to dissolve, and remained dissolved after the flask had cooled to room temperature.

The solution in flask B was added, via a pre-N₂ flushed 2 ml gas-tight syringe, to flask A under positive N₂ pressure (overhead funnel). The stopper was reapplied. There was no evidence of a precipitate being formed. It was observed that the resin was totally swelled after five minutes. The flask was placed in an oven at 45 °C, without stirring.

At the end of the reaction, an aliquot (~20 μL) of the translucent, golden-yellow supernatant was taken, via a pre-N₂ flushed 1000 μL gas-tight syringe, for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). The aliquot was subjected to a TLC pre-treatment. The aliquot was placed in a 10 ml test tube. EtOAc (500 μL) and H₂O (500 μL) were added to the test-tube, with shaking, and the contents of the test-tube were allowed to separate. The organic layer was dried over anhydrous Na₂SO₄ and flushed with N₂ prior to analysis.

The deallylation reaction was considered complete after one hundred and sixty-seven hours forty minutes, as judged by TLC. The resin was washed under vacuum with DMF (3 x 5 ml) and 0.1 M sodium diethyldithiocarbamate in DMF and 10% H₂O (v/v) (3 x 5 ml) to sequester Pd(0), to yield a light-brown resin. The resin was washed with MeOH, DCM and Et₂O (4 x 5 ml each, in order) to swell and shrink the resin prior to drying. After the Et₂O wash, it was observed that the resin was a light, orange-brown colour. The resin was dried under vacuum for fourteen hours in the dark. The resin was further dried for six hours under high-vacuum. The resin was analysed by gel-phase ¹³C-NMR spectroscopy.

3.6.3.1 SPS re-deallylation of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (with sodium benzenesulfinate) (page 202)

Partially deallylated pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (80.0 mg, ~0.0960 mmol allyl, 1.0 eq) was washed from the NMR-tube with anhydrous and deoxygenated DMF (160 μL) into a 5 ml pear-
shaped flask (flask A) containing a dry, magnetic follower. The flask was flushed with dry N\textsubscript{2} and then sealed with a glass stopper.

Sodium benzenesulfinate (40.4 mg, 0.246 mmol, 2.6 eq), Pd(PPh\textsubscript{3})\textsubscript{4} (11.1 mg, 0.00961 mmol, 10.0 mol%) and PPh\textsubscript{3} (5.6 mg, 0.0214 mmol, 22.3 mol%) was weighed into a 5 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was flushed with dry N\textsubscript{2} and then sealed with a septum. A balloon containing dry N\textsubscript{2} was connected to a needle and the needle was inserted into the septum. Anhydrous and deoxygenated DMF (700 \textmu L) was added, \textit{via} a pre-N\textsubscript{2} flushed 2 ml gas-tight syringe, to the flask, with vigorous stirring. However, it was observed that the sodium benzenesulfinate had not dissolved. The light-yellow heterogeneous mixture was heated with a hot-air gun without change. DMF (100 \textmu L) was added with heating and all the sodium benzenesulfinate appeared to dissolve, and remained dissolved after the flask had cooled to room temperature.

The solution in flask B was added, \textit{via} a pre-N\textsubscript{2} flushed 2 ml gas-tight syringe, to flask A under positive N\textsubscript{2} pressure (overhead funnel) with stirring. The stopper was reapplied. There was no evidence of a precipitate being formed. It was observed that the resin was totally swelled after five minutes. The solution was stirred at 45 °C. The temperature of the reaction was maintained using a silicone oil bath coupled to a programmable thermocouple.

The deallylation reaction was considered complete after sixty-nine hours twenty-five minutes (total reaction time of two hundred and thirty-eight hours and forty-five minutes). The resin was washed under vacuum with DMF (1 x 10 ml) and 0.1 M sodium diethylthiocarbamate in DMF and 10% H\textsubscript{2}O (v/v) (1 x 10 ml) to sequester Pd(0), to yield a light-brown resin. The resin was washed with MeOH, DCM and Et\textsubscript{2}O (2 x 5 ml each, in order) to swell and shrink the resin prior to drying. After the Et\textsubscript{2}O wash, it was observed that the resin was a light, orange-brown colour. The resin was dried under vacuum for four hours in the dark. The resin was further dried for two hours under high-vacuum.
An aliquot (~20 µL) of the translucent, golden-yellow supernatant was collected from the initial resin filtrate for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). The TLC pre-treatment consisted of the aliquot being placed in a 10 ml test-tube. EtOAc (500 µL) and 0.1 M diaminooetanetetra-acetic acid disodium salt (500 µL) was added to the test-tube with shaking, and the contents of the test-tube were allowed to separate. The organic layer was transferred, via a glass pipette, into a clean 10 ml test-tube. The organic layer was then washed with H2O (500 µL), and finally 20% NaCl(aq) (500 µL) and then dried over anhydrous Na2SO4.

3.6.3.2 Cleavage of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (page 204)

Following the gel-phase 13C-NMR spectroscopy experiment, the resin (~80 mg) was washed with Et2O (2 x 5 ml) under vacuum. A portion of the vacuum dried resin (20.0 mg, 0.00480 mmol, 1.0 eq) was weighed into a 2 ml eppendorf-tube containing a small, magnetic follower. Deoxygenated and anhydrous THF (220 µL) was added to the eppendorf-tube. The volume of THF used was enough to just cover the resin. The resin was allowed to swell for fifteen minutes with stirring. At the end of this period, triethylamine (17.84 µL, 0.128 mmol, 40.0 eq) was added, via a 20 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (10.4 µL, 0.0638 mmol, 19.9 eq). The mixture was stirred for two hours and thirty minutes, after which, it was filtered through a sintered glass vacuum-funnel, and the colourless filtrate collected in a 25 ml round-bottomed flask. The resin was washed with THF (1 x 10 ml) and the washings collected in the 25 ml round-bottomed flask. Trimethylethoxysilane (9.28 uL, 0.0594 mmol, 18.5 eq) was added to the solution in the 25 ml round-bottomed flask. The solution was stirred with a magnetic follower for forty minutes. The solution was concentrated in vacuo to yield a colourless residue. The residue was further dried on a high-vacuum line for thirty minutes to yield a colourless residue (25.0 mg). This residue was stored under N2 and analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v).
3.6.4 SPS deallylation of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotengel resin (with 2,4,6-collidine p-toluenesulfonate) (page 205)

Pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotengel resin (99.7 mg, 0.120 mmol allyl, 1.0 eq) was weighed into a 5 ml pear-shaped flask (flask A). The flask was flushed with dry N\(_2\) and sealed with a glass stopper. 2,4,6-Collidine p-toluenesulfonate (82.4 mg, 0.281 mmol, 2.3 eq), Pd(PPh\(_3\))\(_4\) (32.4 mg, 0.0280 mmol, 23.3 mol\%) and PPh\(_3\) (4.1 mg, 0.0156 mmol, 13.0 mol\%) were weighed into a 10 ml pear-shaped flask (flask B) containing a dry, magnetic follower. The flask was flushed with dry N\(_2\) and sealed with a septum. A balloon containing dry N\(_2\) was connected to a needle and the needle was inserted into the septum. Anhydrous and deoxygenated DMF (600 \(\mu\)L) was added, via a pre-N\(_2\) flushed 2 ml gas-tight syringe, to the flask, with vigorous stirring. This volume of DMF was calculated from a solubility test. However, it was observed that the 2,4,6-collidine p-toluenesulfonate had not dissolved. The brown heterogeneous mixture was heated with a hot-air gun with no change. DMF (1 x 200 \(\mu\)L) was added with heating and all the 2,4,6-collidine p-toluenesulfonate appeared to dissolve, and remained dissolved after the flask had cooled to room temperature.

The solution in flask B was added, via a pre-N\(_2\) flushed 2 ml gas-tight syringe, to flask A under positive N\(_2\) pressure (overhead funnel) with stirring. The stopper was reapplied. There was no evidence of a precipitate being formed. It was observed that the resin was totally swelled after five minutes. The mixture was stirred, using a magnetic follower at 45 °C. The temperature of the reaction was maintained using a silicone oil bath coupled to a programmable thermocouple.

Reaction monitoring consisted of a small aliquot (~20 \(\mu\)L) of the clear, golden-yellow supernatant being taken, via a pre-N\(_2\) flushed 1000 \(\mu\)L gas-tight syringe for TLC analysis (EtOAc:hexane:AcOH, 50:50:1 v/v/v). The TLC pre-treatment consisted of the aliquot being placed in a 10 ml test-tube. EtOAc (500 \(\mu\)L) and 0.1 M diaminooethanetetra-acetic acid disodium salt (500 \(\mu\)L) were added to the test-tube, with shaking, and the contents of
the test-tube were allowed to separate. The organic layer was transferred, via a glass pipette, into a clean 10 ml test-tube. The organic layer was then washed with H₂O (500 µL) and finally 20% NaCl(aq) (500 µL), and dried over anhydrous Na₂SO₄.

The deallylation reaction was considered complete after one hundred and eighty-nine hours, twenty minutes. The resin was washed under vacuum with DMF (2 x 10 ml) and 0.1 M sodium diethylthiocarbamate in DMF and 10% H₂O (v/v) (1 x 10 ml) to sequester Pd(0), to yield a light-orange resin. The resin was washed with MeOH, DCM and Et₂O (4 x 5 ml each, in order) to swell and shrink the resin prior to drying. After the Et₂O wash, it was observed that the resin was a light, orange colour. The resin was dried under vacuum for two hours in the dark. The resin was further dried for six hours under high-vacuum.

3.6.5 SPS oxidation of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (deallylation with sodium benzenesulfinate) with co-substrate, (-)-epicatechin 5

3.6.5.1 Solubility-test for SPS oxidation (page 208)

NaHCO₃ (40.7 mg, 0.484 mmol, 25.2 eq) was weighed into a 10 ml pear-shaped flask containing a magnetic follower (flask A). Distilled H₂O (7 x 30 µL) was added to the flask with stirring. All the NaHCO₃ appeared to dissolve. 1,4-Dioxane (210 µL) was added to the flask with stirring, and a large amount of precipitated NaHCO₃ was observed. Distilled H₂O (2 x 60 µL) was added to the flask with stirring; however, the precipitate remained. An equal volume of 1,4-dioxane (120 µL) was added with stirring with no change. Distilled H₂O (5 x 60 µL) was added to the flask, with stirring, and the mixture became translucent and homogeneous. To increase the relative proportion of organic solvent, 1,4-dioxane (1 x 30 µL) was added with stirring; however, the precipitate reformed. Distilled H₂O (3 x 70 µL) was again added to the flask with stirring and the mixture became translucent and homogeneous. Although the volume of solvent was large, the relative proportions of H₂O:1,4-dioxane (840 µL:360 µL, 70:30) were considered suitable. The pH of the resulting solution was checked by taking 20 µL of the solution
and dispensing over pH indicator-paper (pH 8.5). (-)-Epicatechin 5 (27.5 mg, 0.0947 mmol, 4.9 eq) was added to this flask with stirring, without additional dilution, to yield a translucent, light-yellow solution.

K₃Fe(CN)₆ (31.7 mg, 0.0963 mmol, 5.0 eq) was weighed into a 5 ml pear-shaped flask containing a magnetic follower (flask B). H₂O (5 x 20 µL) was added to the flask, with stirring, to yield a translucent, homogeneous deep-yellow solution. The contents of flask B were added to flask A, via a 100 µL gas-tight syringe, drop-wise over one minute, with stirring, to yield a deep-red solution. After one hour of stirring, there was no evidence of any precipitation.

3.6.5.2 SPS oxidation (page 210)

(-)-Epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (80 mg, 0.0192 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask containing a magnetic follower (flask A).

NaHCO₃ (40.2 mg, 0.479 mmol, 24.9 eq) was weighed into a 10 ml pear-shaped flask containing a magnetic follower (flask B). Distilled H₂O (840 µL) and 1,4-dioxane (360 µL) were added to the flask with stirring. (-)-Epicatechin 5 (28.3 mg, 0.0975 mmol, 5.1 eq) was added to this flask with stirring, to yield a translucent, light-yellow solution.

K₃Fe(CN)₆ (32.1 mg, 0.0975 mmol, 5.1 eq) was weighed into a 5 ml pear-shaped flask containing a magnetic follower (flask C). Distilled H₂O (100 µL) was added to the flask with stirring, to yield a translucent, deep-yellow solution.

The contents of flask B were added to flask A, via a 2 ml gas-tight syringe, with stirring, to yield a translucent, light-yellow supernatant. It was observed that the resin was totally swelled after twenty minutes. The contents of flask C were added to flask A, via a 100 µL gas-tight syringe, drop-wise over a period of one minute with stirring, to yield a deep-red coloured solution. After this addition, it was observed that the mixture became gel-like with the evolution of gas. After five minutes of continued stirring, the gel-like phase had disappeared to yield free-flowing resin beads. After one hour of stirring, it was observed
that the supernatant was an orange colour. After two hours of reaction time, the resin was filtered and washed under vacuum with distilled H2O:1,4-dioxane 70:30 v/v (2 x 20 ml). The resin was further washed with distilled H2O (1 x 20 ml) to extract inorganic material, and finally washed with DMF, DCM and Et2O (1 x 20 ml each, in order) to swell and shrink the resin prior to drying. After the Et2O wash, it was observed that the resin was a light, orange-red colour. The resin was dried under high-vacuum for six hours and thirty minutes.

3.6.5.3 Cleavage of the diisopropylsilyl linker (page 211)

The vacuum-dried resin (~80.0 mg, 0.0192 mmol, 1.0 eq) was placed in a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (400 µL) was added to the eppendorf-tube. The volume of THF used was enough to just cover the resin. The resin was allowed to swell for fifteen minutes with stirring. At the end of this period, triethylamine (72 µL, 0.517 mmol, 40.4 eq) was added, via a 100 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (42 µL, 0.258 mmol, 20.2 eq). The mixture was stirred for two hours thirty minutes, after which, it was filtered through a sintered glass vacuum-funnel and the light-orange filtrate collected in a 25 ml round-bottomed flask. The resin was washed with THF (1 x 10 ml) and the washings collected in the 25 ml round-bottomed flask. Trimethylethoxysilane (37 µL, 0.237 mmol, 18.6 eq) was added to the solution in the 25 ml round-bottomed flask. The solution was stirred, with a magnetic follower, for forty minutes. The solution was concentrated in vacuo to yield a translucent, light-orange residue. The residue was further dried on a high-vacuum line for twenty minutes to yield a translucent, light-orange residue. The resin was a light-orange colour after cleavage. The residue was analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v).
3.6.6 SPS oxidation of (-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (deallylation with 2,4,6-collidine p-toluenesulfonate) (page 212)

(-)-Epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel resin (99.7 mg, 0.0239 mmol, 1.0 eq) was weighed into a 5 ml pear-shaped flask containing a magnetic follower (flask A). 1,4-Dioxane (500 μL) was added to the flask, with stirring for forty minutes, to swell the resin.

K₃Fe(CN)₆ (79.7 mg, 0.242 mmol, 10.1 eq) was weighed into a 5 ml pear-shaped flask containing a magnetic stirrer bar (flask B). Distilled H₂O (300 μL) was added to the flask with stirring to yield a translucent, deep-yellow solution.

NaHCO₃ (43.1 mg, 0.513 mmol, 21.5 eq) was weighed into a 10 ml pear-shaped flask containing a magnetic stirrer bar (flask C). Distilled H₂O (1870 μL) and 1,4-dioxane (386 μL) were added to the flask with stirring. (-)-Epicatechin 5 (70.4 mg, 0.243 mmol, 10.2 eq) was added to this flask with stirring, to yield a translucent, light-yellow solution. The pH of the resulting solution was checked by taking 20 μL of the solution and dispensing over pH indicator-paper (pH 8.5).

The contents of flask B were added to flask A, via a 2 ml gas-tight syringe, drop-wise over a period of one minute, with stirring, to yield a deep-yellow coloured solution. After this addition, it was observed that the mixture became gel-like with the evolution of gas. After twenty minutes of continued stirring, the gel-like phase had disappeared to yield free-flowing resin beads. After fifty minutes of stirring, the oxidation reaction was presumed to be complete. The contents of flask C were added to flask A, via a 2 ml gas-tight syringe, drop-wise over a period of one minute, with stirring, to yield a deep-red coloured solution. After this addition, it was observed that the mixture became gel-like, with the evolution of gas. After five minutes of continued stirring, the gel-like phase had disappeared to yield free-flowing resin beads.

After two hours and forty-five minutes of reaction time, the resin was filtered and washed under vacuum with distilled H₂O:1,4-dioxane, 70:30 v/v (2 x 20 ml). The resin was
further washed with 1,4-dioxane (1 x 15 ml) and distilled H₂O (1 x 15 ml) to extract inorganic material, to yield a deep-red coloured resin. The resin was finally washed with MeOH, DCM and Et₂O (4 x 5 ml each, in order) to swell and shrink the resin prior to drying. After the Et₂O wash, it was observed that the polymer was a deep, red colour. The resin was dried under high-vacuum for five hours prior to cleavage.

3.6.6.1 Cleavage of the diisopropylsilyl linker (page 213)

A portion of the vacuum-dried resin (25.2 mg, 0.00605 mmol, 1.0 eq) was placed in a 2 ml eppendorf-tube containing a small magnetic follower. Anhydrous and deoxygenated THF (225 µL) was added to the eppendorf-tube. The volume of THF used was enough to just cover the resin. The resin was allowed to swell for fifteen minutes with stirring. At the end of this period, triethylamine (22.0 µL, 0.158 mmol, 39.2 eq) was added, via a 100 µL pipette, immediately followed by the addition of triethylamine trihydrofluoride (13.2 µL, 0.0810 mmol, 20.1 eq). The mixture was stirred for two hours thirty minutes, after which, it was filtered through a sintered glass vacuum-funnel and the light-orange filtrate collected in a 25 ml round-bottomed flask. The resin was washed with THF (1 x 10 ml) and the washings collected in the 25 ml round-bottomed flask. Trimethylethoxysilane (11.6 µL, 0.0743 mmol, 18.4 eq) was added to the solution in the 25 ml round-bottomed flask. The solution was stirred, with a magnetic follower, for forty minutes. The solution was concentrated in vacuo to yield a translucent, light-orange residue. The residue was further dried on a high-vacuum line for twenty minutes to yield a translucent, light-orange residue. The resin was a light, orange-red colour after cleavage. The residue was analysed by TLC (EtOAc:hexane:AcOH, 11:1:0.1 v/v/v), ¹H- and ¹³C-NMR spectroscopy (see figures 33 and 34, respectively), FT-IR spectroscopy and MS. FT-IR (film, NaCl, cm⁻¹): 3583, 3379, 2950, 2739, 2604, 2531, 2497, 1723, 1474, 1398, 1260, 1173, 1066, 1037, 850, 720, 666. ESI-MS: [M+H]⁺, m/z 391.2844.
APPENDIX
Figure 1: $^1$H-NMR spectrum of (-)-epicatechin 5 ($d_6$-DMSO)
Figure 3: $^1$H-NMR spectrum of (-)-epigallocatechin 4 (d$_5$-DMSO)
Figure 4: $^{13}$C-NMR spectrum of (-)-epigallocatechin 4 ($d_0$-DMSO)
Figure 6: \(^1\)H-NMR spectrum of tetryllyl-\((+)-\)epicatechin 5a (d_6-DMSO)
Figure 7: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin 5a ($d_6$-DMSO/D$_2$O)
Figure 11: LC-ESI-MS chromatogram of tetraallyl-(-)-epicatechin 5a, with mono- and di-C-alkylated by-products
Figure 12: $^1$H-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a ($d_6$-DMSO)
Figure 13: $^1$H-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a ($d_6$-DMSO/D$_2$O)
Figure 14: $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a ($d_6$-DMSO/D$_2$O)
Figure 15: $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin 4a (CDCl$_3$)
Figure 16: $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin 4a (CDC3)
Figure 17: LC-ESI-MS, UV-Vis chromatogram of MeOH quenched, hydroxytentagel resin supernatant (270-280 nm)
Figure 20: $^1$H-NMR spectrum of tetraallyl-(−)-epicatechin diisopropylsilyl dimer 5e (CDCl$_3$)
Figure 21. $^{13}$C-NMR spectrum of tetraallyl-(−)-epicatechin dianisopropyl dimer 5c (CDCl$_3$)
Figure 22: LC-ESI-MS, UV-Vis chromatogram of fluoride cleaved products (275 nm)
Figure 23: $^1$H-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (CDCl$_3$)
Figure 24: $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyl butynyl ether 4b (CDCl$_3$)
Figure 25: Gel-phase $^{13}$C-NMR spectrum of 4-azidobenzoyl aminotengel resin (CDCl$_3$)
Figure 26: FT-IR (ATR) transmission spectrum of 4-azidobenzoyl aminotentagel resin
Figure 27: FT-IR (ATR) transmission spectrum of aminotentagel resin

- NH\(_2\) (amine)
- CN (1'-amine)
Figure 28: Gel-phase $^{13}$C-NMR, pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoyl aminotentagel (CDCl$_3$)
Figure 30: $^1$H-NMR spectrum of pentaallyl-(−)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d ($d_6$-DMSO)
Figure 31: $^{13}$C-NMR spectrum of pentaallyl-(-)-epigallocatechin diisopropylsilyloxy ethyl-1,2,3-triazolebenzoic acid 4d ($d_6$-DMSO)
Figure 32: Gel-phase $^{13}$C-NMR, (-)-epigallocatechin diglycosylxy ethyl-1,2,3-triazolebenzoyl amiontransfer resin (CDCl$_3$)
Figure 33: 1H-NMR spectrum of the oxidation product produced from the attempted SPS of theaflavin 12 (CDCl3)
Figure 34: $^{13}$C-NMR spectrum of the oxidation product produced from the attempted SPS of the flavin 12 (CDCl₃)
Reference List


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