



Published in final edited form as:

Fitoterapia. 2020 March ; 141: 104479. doi:10.1016/j.fitote.2020.104479.

Bufadienolides and anti-angiogenic homoisoflavonoids from *Rhodocodon cryptopodus*, *Rhodocodon rotundus* and *Rhodocodon cyathiformis*

Hannah Whitmore^a, Kamakshi Sishtla^b, Walter Knirsch^c, Jacky L. Andriantiana^d, Sianne Schwikkard^e, Eduard Mas-Claret^a, Sarah M. Nassief^a, Sani M. Isyaka^{a,f}, Timothy W. Corson^{b,*}, Dulcie A. Mulholland^{a,f,**}

^aNatural Products Research Group, Department of Chemistry, University of Surrey, Guildford GU2 7XH, United Kingdom

^bEugene and Marilyn Glick Eye Institute, Department of Ophthalmology, Indiana University School of Medicine, 1160 W. Michigan St., Indianapolis, IN 46202, USA

^cInstitute of Plant Sciences, NAWI Graz, Karl-Franzens University Graz, Holteigasse 6, A-8010 Graz, Austria

^dParc Botanique et Zoologique de Tsimbazaza, Rue Fernand Kassanga, Antananarivo 101, Madagascar

^eSchool of Life Sciences, Pharmacy and Chemistry, Kingston University, Kingston-upon-Thames KT1 2EE, United Kingdom

^fSchool of Chemistry and Physics, University of KwaZulu-Natal, Durban, South Africa

Abstract

Background: Homoisoflavonoids have been shown to have potent anti-proliferative activities in endothelial cells over other cell types and have demonstrated a strong antiangiogenic potential *in vitro* and *in vivo* in animal models of ocular neovascularization. Three species of *Rhodocodon* (Scilloioideaea subfamily of the Asparagaceae family), endemic to Madagascar, *R. cryptopodus*, *R. rotundus* and *R. cyathiformis*, were investigated.

Purpose: To isolate and test homoisoflavonoids for their antiangiogenic activity against human retinal microvascular endothelial cells (HRECs), as well as specificity against other ocular cell lines.

Methods: Plant material was extracted at room temperature with EtOH. Compounds were isolated using flash column chromatography and were identified using NMR and CD spectroscopy and HRESIMS. Compounds were tested for antiproliferative effects on primary human

*Corresponding author. tcorson@iu.edu (T.W. Corson). **Corresponding author at: Natural Products Research Group, Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guildford GU2 7XH, United Kingdom. d.mulholland@surrey.ac.uk (D.A. Mulholland).

Declaration of Competing Interest
There are no conflicts to declare.

Appendix A. Supplementary data
Supplementary data to this article can be found online at <http://doi.org/10.1016/j.fitote.2020.104479>.

microvascular retinal endothelial cells (HRECs), ARPE19 retinal pigment epithelial cells, 92–1 uveal melanoma cells, and Y79 retinoblastoma cells. HRECs exposed to compounds were also tested for migration and tube formation ability.

Results: Two homoisoflavonoids, 3*S*-5,7-dihydroxy-(3'-hydroxy-4'-methoxybenzyl)-4-chromanone (**1**) and 3*S*-5,7-dihydroxy-(4'-hydroxy-3'-methoxybenzyl)-4-chromanone (**2**), were isolated along with four bufadienolides. Compound **1** was found to be non-specifically antiproliferative, with GI₅₀ values ranging from 0.21–0.85 μM across the four cell types, while compound **2** showed at least 100-fold specificity for HRECs over the other tested cell lines. Compound **1**, with a 3*S* configuration, was 700 times more potent than the corresponding 3*R* enantiomer recently isolated from a *Massonia* species.

Conclusion: Select homoisoflavonoids have promise as antiangiogenic agents that are not generally cytotoxic.

Keywords

Rhodocodon; Homoisoflavonoids; Bufadienolides; Anti-angiogenic; Neovascularization; Endothelial cells

1. Introduction

Abnormal formation of new blood vessels in the eye is associated with blindness in many ocular diseases such as retinopathy of prematurity (ROP) affecting children, proliferative diabetic retinopathy (PDR), the wet form of age-related macular degeneration (AMD) and neovascular glaucoma affecting working-age and older adults, respectively [14]. Small molecule antiangiogenic drugs are urgently needed to supplement the existing available biologics, including drugs such as bevacizumab, ranibizumab, and aflibercept, which target the vascular endothelial growth factor (VEGF) [4]. Homoisoflavonoids, a class of compounds commonly found in the Scilloideae subfamily of the Asparagaceae family, have been shown previously to have potent anti-proliferative activities in endothelial cells over other cell types [17]. Moreover, they demonstrated a strong antiangiogenic potential *in vitro* and *in vivo* in animal models of ocular neovascularization [23].

The genus *Rhodocodon* (Asparagaceae) has been the topic of much taxonomic debate and, in this work, we report on the phytochemical investigation into *Rhodocodon cryptopodus* (H. Perrier), *R. rotundus* (Baker) and *R. cyathiformis* (var. giganteus). The genus is endemic to Madagascar and thirteen species have been identified based on morphological, biogeographical and molecular evidence [7]. In the 1990s, Speta [20] integrated the genus *Rhodocodon* into that of *Rhadamanthus*, effectively replacing the *Rhodocodon* genus, while *Rhadamanthus* was subsequently combined with *Drimia* by Manning et al., giving rise to the synonym *Drimia rotunda* [10]. *Hyacinthus cryptopodus* (Baker) (syn. *Rhodocodon cryptopodus*), which had been placed in the Hyacinthoideae subfamily by Speta [19] as the only Madagascan species in the *Hyacinthus* genus, was tentatively moved by Manning et al. [10] to the *Ledebouria* genus, pending further evidence, becoming *Ledebouria cryptopoda* (Baker) J. C. Manning and Goldblatt. This was due to characteristics unique to the *Ledebouria* genus such as the fibres produced when the bulb scales are torn. However, in

2006, this move was re-evaluated by Pfosser et al. [15], who re-assigned *L.cryptopodus* to the subfamily Urgineoideae, which brought about a necessary transfer from the genus *Ledebouria* to *Drimia* as *D. cryptopoda* (Baker) Pfosser, Wetschnig & Speta. *Rhodocodon* Baker has recently been reinstated as a genus within the Scilloideae subfamily of the Asparagaceae (*sensu* APG III) by Knirsch et al. [7] based on morphological, biogeographical and molecular evidence and *Drimia cryptopoda* was transferred to this new genus. No medicinal use of any species of *Rhodocodon* has been reported although the crushed bulbs of *R. cryptopodus* are used for epilation.

The aim of this work was to investigate the phytochemistry of the three *Rhodocodon* species and to evaluate the homoisoflavonoids isolated for their antiangiogenic activity.

2. Materials and methods

2.1. Instrumentation

NMR spectra were recorded on a 500 MHz Bruker AVANCE NMR spectrometer in either CDCl₃ or CD₃OD, UV-VIS spectra were recorded on a Libra Biochrom spectrometer in CH₃OH in a 1 cm cell, IR spectra were obtained using an Agilent (Cary 600 series) FTIR spectrometer (University of Surrey), ESIMS analysis was performed using an Alliance 2695 Quattro Ultra mass spectrometer, HRESIMS data were recorded on an Agilent 6550 iFunnel Q-TOF LC/MS with samples dissolved in CH₃OH. Optical rotations were measured at room temperature in CH₃OH using a JASCO P-1020 polarimeter and CD spectra were measured on a Chirascan CD spectrometer using a 1 mm cell in CH₃CN. Solvents were reagent grade and purchased from Sigma-Aldrich.

2.2. Plant material

Plant material of *Rhodocodon cryptopodus* (Baker), *Rhodocodon rotundus* (H. Perrier) and *R. cyathiformis* was collected by Dr. Walter Knirsch in Madagascar. Collection permit: 215/13/MEF/SG/DGF/DCB.SAP/SCB, collected 27/12/2016–12/01/2017. Acquisition numbers of the collected plants are *R. cryptopodus*: 02442, *R. rotundus*: 04953 and *R. cyathiformis*: WK3. *R. cryptopodus* was collected from Ambatondradama, Madagascar, and purchased in the market of Antananarivo. *R. rotundus* was collected from along the roadside on the way to Sakalalina, Madagascar and *R. cyathiformis* was collected near Mahajanga at the Grotte d'Anjohibe. Voucher specimens have been retained at the Karl-Franzens-University, Graz, Austria.

2.3. Extraction and isolation

The dried bulbs of *R. cryptopodus* (67.5 g) were extracted with EtOH (550 mL) by shaking at room temperature for 24 h. The EtOH extract (1.806 g) was obtained after solvent evaporation. The extract was separated using gravity column chromatography over silica gel (Merck 9385) to yield **1** (5.4 mg), **3** (3.5 mg), **6** (6.2 mg), **7** (2.9 mg), and **10** (3.7 mg). Dried bulbs of *R. rotundus* (269.2 g) were extracted at room temperature with continuous agitation over 24 h with EtOH (200 mL). The resulting EtOH extract (18.1 g) was separated using a flash column chromatography system to yield compounds **2** (40.0 mg), **4** (7.8 mg), **5** (4.3 mg), **8** (40.7 mg) and **9** (11.4 mg). The fresh bulbs of *R. cyathiformis* (450 g) were extracted

with CH₂Cl₂ (1 L) by shaking at room temperature for 42 h. The extract (2.0 g) was separated using a flash column chromatography system (Biotage SP1 Flash Chromatography Purification System) to yield compounds **1** (1.0 mg) and **2** (1.1 mg). A detailed separation scheme of each extract can be found in the Supplementary Data, along with spectra.

2.4. Compound characterization

Compound **2** yellow oil; $[\alpha]_D^{23} + 7.95$ (*c* 0.86, MeOH); ECD (CH₃CN) λ (ϵ) 230 nm (−0.2) 293 nm (+2.7), 310 nm (−0.1); ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) data are given in Table 1. HRESIMS *m/z* 315.0876 [M-H][−] (calcd. for [C₁₇H₁₆O₆ - H], *m/z* 315.0874).

Compound **9** white crystals; $[\alpha]_D^{23} + 40.9$ (*c* 0.44, MeOH); ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) data are given in Table 3. HRESIMS *m/z* 643.2728 [M + Na]⁺ (calcd. for [C₃₂H₄₄O₁₂ + Na], *m/z* 643.2725).

Compound **10** white powder; ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz) data are given in Table 2. ESIMS *m/z* 190.0631 [M]⁺, (calcd. for [C₁₁H₁₀O₃]⁺, *m/z* 190.0629).

2.5. Biological assay materials

Endothelial Growth Medium (EGM-2) was prepared by mixing the contents of an EGM-2 “Bullet Kit” (Cat. no. CC-4176) with Endothelial Basal Medium (EBM) (Lonza). The EGM-2 “Bullet Kit” contains hydrocortisone, human fibroblast growth factor (hFGF), VEGF, R3-insulin like growth factor (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), gentamycin and heparin along with 2% foetal bovine serum (FBS). Human Retinal Endothelial Cells (HRECs) and Attachment Factor were purchased from Cell Systems (Kirkland, WA, USA). HRECs used for cell proliferation studies were of varying passages (P6-P8) while cells used in tube formation assays were P4 and migration assays utilised P5. The complete medium used for culturing ARPE-19 cells (ATCC, Manassas, VA, USA) contained Ham’s-F10 growth medium (Thermo Scientific, Waltham, MA, USA) + 10% FBS + 1% penicillin-streptomycin (pen–strep). 92–1 uveal melanoma cells (a kind gift of Dr. Martine Jager, University of Leiden) were grown in RPMI medium containing 10% FBS and 1% pen–strep. Y-79 retinoblastoma cells (a kind gift of Dr. Brenda L. Gallie, Ontario Cancer Institute) were grown in RB medium (IMDM +10% FBS + 55 μ M β -mercaptoethanol +10 μ g/mL insulin +1% pen–strep). Matrigel was from Corning (Corning, NY, USA), while alamarBlue was from AbD Serotec (Raleigh, NC, USA).

2.6. Cell proliferation assays

Cells (2,500) in growth medium (100 μ L) were incubated in the centre 48 wells of 96-well clear bottom black plates overnight, with the surrounding wells containing deionized, sterilised water (100 μ L). This was followed by treatment of cells with 1 μ L of different concentrations of each test compound. Compounds were tested in triplicate over the range of 1 mM to 1 nM (1% v/v final DMSO concentration). Treated cells were incubated for a further 44 h. At the end of this incubation period, alamarBlue reagent (11.1 μ L) was added and after 4 h of incubation, fluorescence readings were taken with excitation and emission

wavelengths of 560 nm and 590 nm respectively. Data were analysed and dose response curves generated using GraphPad Prism software (v. 7.0).

2.7. Migration assays

The scratch wound migration assay was performed as previously described [2] with HRECs grown in EGM-2 to confluency in a 12 well plate. A scratch was introduced with a sterile 10 μ L pipette tip and medium was replaced with EGM-2 containing the indicated concentrations of compound with 1% DMSO/well and wells were imaged by brightfield microscopy to establish scratch width at $t = 0$. Cells that migrated into the scratch after 11 h were manually counted and normalized to control. Statistical analysis using one-way ANOVA with Dunnett's *post hoc* tests to compare treatment with DMSO control was completed using GraphPad Prism. *P*-values $<.05$ were considered significant.

2.8. Tube formation assays

The Matrigel based tube formation assay was performed as previously described [2]. Briefly, 50 μ L Matrigel was allowed to solidify in a 96 well black, clear bottom plate at 37 °C for 20 min. HRECs were added to the solid Matrigel at 15,000 cells/well in 100 μ L EGM-2 and dosed with appropriate concentrations of compound with 1 μ L DMSO/well. Tube formation was observed every 2 h by brightfield microscopy and images were taken after 8 h of tube formation. Six images per treatment were analysed with AngiogenesisAnalyzer plugin for ImageJ (<http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ>), and total tubule length for treated cells was normalized to DMSO control. Statistical analysis using one-way ANOVA with Dunnett's *post hoc* tests to compare treatment with DMSO control data was completed using GraphPad Prism.

3. Results

3.1. Isolation and characterization of compounds from *R. cryptopus*

The EtOH extract of the bulbs of *R. cryptopus* yielded a 3*S*-3-benzyl homoisoflavonoid, identified as 3*S*-(5,7-dihydroxy-(3'-hydroxy-4'-methoxybenzyl)-4-chromanone (**1**), previously reported from *Rhodocodon campanulatus* but not tested previously for angiogenic activity [16], the cinnamic acid derivative, *p*-hydroxyphenylethyl *p*-coumarate (**3**), previously reported from *Dendrobium falconeri* (Orchidaceae), a species of orchid native to Asia, which showed a marginal inhibitory effect (EC_{50} of 352.1 μ M) against Herpes simplex virus type 1 (HSV-1) [21], two known bufadienolide glycosides, deglucohellebrin (**6**) and hellebrigenin (**7**), both of which have been isolated from the *Helleborous* genus (Ranunculaceae) [26] and the previously unreported coumarin (**10**). Compound **6** has been tested previously against oral human squamous carcinoma cells (HSC-2) and human melanoma cells (A 375) and was found to have a potent cytotoxic effect against both ($GI_{50} = 2.8$ nM and 6.3 nM respectively) [26]. It was also found to have an inotropic effect on cat heart *in situ* and isolated guinea pig and rat hearts, while compound **7** is known to be cytotoxic against oral human squamous carcinoma cells (HSC-2) ($GI_{50} = 2.9$ nM) and human melanoma cells (A 375) ($GI_{50} = 8.6$ nM) [26]. The structures of the compounds were determined using NMR spectroscopy and structures of known compounds were confirmed by comparison against literature values as referenced above. Complete

NMR assignments for bufadienolides **6–9** (which are not available in the literature) are given (Table 3).

Compound **2**, 3*S*-5,7-dihydroxy-(4'-hydroxy-3'-methoxybenzyl)-4-chromanone, gave the same molecular formula (C₁₇H₁₆O₆), ESI-MS peak at m/z 315.0876 ([M-H]⁻) and NMR spectrum (Table 1) as a homoisoflavonoid isolated previously from *Drimia delagoensis* [8]. However, an ECD study showed Cotton effects of +2.8 at 290 nm and – 0.2 at 230 nm indicating the unusual 3*S* configuration [11]. This agrees with the reported configurations of other 3-benzyl homoisoflavonoids isolated from *Rhodocodon* which have also been found to have the 3*S* configuration [16]. Hence compound **2** is the *S*-enantiomer of the previously reported 3*R* isomer.

Compound **9**, 6 α -acetoxy-3 β ,8 β ,14 β -trihydroxy-10,13-dimethylbufa-4,20,22-trienolide 3-*O*- β -D-glucopyranoside, was found to be the 6 α -acetoxy-analogue of scilliroside (6 β -acetoxy-3 β ,8 β ,14 β -trihydroxy-10,13-dimethylbufa-4,20,22-trienolide 3-*O*- β -D-glucopyranoside), a toxic compound from *Urginea maritima*, which has been used as a rodenticide [1]. ESI-MS analysis gave a [M + Na]⁺ peak at m/z 643.2728, indicating a molecular formula of C₃₂H₄₄O₁₂ for the compound. Resonances attributable to carbons and protons of the bufadienolide lactone ring were present (Table 3) and correlations were seen between the H-17 resonance (δ_{H} 2.56, dd, J = 9.2, 6.5 Hz) and the oxygenated C-14 resonance (δ_{C} 86.5) and the C-18 methyl carbon resonance (δ_{C} 19.8). The 3H-19 (δ_{C} 1.36) resonance showed correlations in the HMBC spectrum with the C-10 (δ_{C} 38.1), C-9 (δ_{C} 52.2) and alkene C-5 (δ_{C} 143.2) resonances. The C-10 resonance showed correlations with the H-4 alkene (δ_{H} 5.94, br s) and H-6 oxymethine resonances (δ_{H} 5.47, dd, J = 8.6, 2.6 Hz). Coupling was seen in the COSY spectrum between the H-4 and H-3 (δ_{H} 4.26, bs) resonances. An acetate group was placed at C-6 due to a correlation seen between an acetate group carbonyl carbon resonance (δ_{C} 171.7) and the H-6 resonance. The orientation of H-6 was established as β as correlations were seen in the NOESY spectrum between the H-H-4/H-6, H-4/3H-19 and H-6/3H-19 resonances. The H-3 resonance showed a correlation with the acetate methyl group proton resonance. The specific rotation of compound **9** was found to be +40.9, while the literature value for scilliroside has been reported as –59.4 [1].

LRMS of compound **10** indicated a molecular ion at m/z = 190.2 corresponding to a molecular formula of C₁₁H₁₀O₃ and seven degrees of unsaturation. The NMR spectra indicated the presence of a substituted coumarin with the C-2 lactone carbonyl resonance occurring at δ_{C} 167.5 and the H-3 and H-4 resonances appearing as a pair of doublets at δ_{H} 5.64 and δ_{H} 7.41 (both d, J = 7.8 Hz) respectively. The corresponding C-4 resonance (δ_{C} 143.7) showed a correlation with the H-5 resonance at δ_{H} 6.69 (d, J = 8.0 Hz), which showed coupling in the COSY spectrum with the H-6 (δ_{H} 6.54, dd, J = 8.0, 2.1 Hz) and H-8 (δ_{H} 6.66, d, J = 2.1 Hz) proton resonances. Both the H-6 and H-8 resonances showed correlations in the HMBC spectrum with the C-9 methylene carbon resonance (δ_{C} 39.9) and the corresponding 2H-9 triplet (δ_{H} 2.68, t, J = 7.4 Hz) showed coupling with the 2H-10 oxymethylene proton resonance (δ_{H} 3.69, t, J = 7.4 Hz), indicating a hydroxy group at C-10, in accordance with the molecular formula. Although 7-methylated and 7-prenylated coumarins and their derivatives are known, this is the first example of a 7-hydroxyethyl

coumarin. It could possibly arise from the oxidative degradation of a prenyl group [3,24,25,28]. Coumarins have not been reported previously from the Hyacinthaceae [12].

3.2. Isolation and characterization of compounds from *R. rotundus* and *R. cyathiformis*

The EtOH extract of the bulbs of *R. rotundus* yielded five compounds, two of which, a rare 3*S*-type 3-benzyl homoisoflavonoid (**2**) and a bufadienolide glycoside (**9**), have not been reported previously (Fig. 1). Two known cinnamic acid derivatives, *p*-hydroxyphenethyl-*trans*-ferulate (**4**), first extracted from *Heracleum lanatum* [13] and 2-hydroxyethyl-*trans*-ferulate (ariscucurbin A) (**5**), previously isolated from *Aristolochia cucurbitifolia* [27], and 3β,14β-dihydroxy-19-oxo-5β-bufa-20,22-dienolide 3-O-β-D-glucopyranoside (**8**), reported once previously from the related Madagascan *Rhodocodon campanulatus* [16] were isolated. Compound **4** is a known free-radical scavenger [5] which has also been shown to have an affinity toward serotonin (5-HT₇) receptors [6].

The CH₂Cl₂ extract of *R. cyathiformis* yielded compounds **1** and **2**, also isolated in this work from *R. cryptopodus* and *R. rotundus* respectively.

3.3. Antiproliferative effects of homoisoflavonoids

Homoisoflavonoids are known to inhibit angiogenesis with some selectivity for blocking the proliferation of endothelial cells over other ocular cell types [2,9]. Thus, they have appeal as a basis for new treatments for neovascular eye diseases such as wet age-related macular degeneration [22]. Because of this, compounds **1** and **2** were tested for their anti-proliferative activities against human retinal endothelial cells (HRECs), giving excellent GI₅₀ results of 0.13 μM and 0.49 μM respectively in this assay (Fig. 2). In comparison, the *R*-enantiomer of compound **1**, recently isolated from *Massonia bifolia* and tested for anti-proliferative activity against HRECs gave a GI₅₀ of 93.2 μM (*R*), > 700-fold less potent than *S*-enantiomer **1** [18].

Although compounds **1** and **2** are very similar in structure and gave similar GI₅₀ results against the endothelial cells, HRECs, the compounds showed very different activities against other cell lines. Compounds were tested for their specificity against human retinal pigmented epithelial cells (ARPE-19) as well as retinoblastoma (Y-79) and uveal melanoma (92-1) cell lines (Fig. 2). Compound **1** was found to be nonspecific, with GI₅₀ values ranging from 0.21–0.85 μM across the four cell types, while compound **2** showed at least 100-fold specificity for HRECs over the other tested cell lines (Fig. 2).

3.4. Antiangiogenic effects of homoisoflavonoids

Both compounds **1** and **2** were tested for inhibition of key *in vitro* angiogenic properties of HRECs, migration and tubule formation, and showed dose-dependent blockade of both these properties (Figs. 3 and 4).

4. Discussion

The limitations of existing therapies for neovascular eye diseases like wet age-related macular degeneration provide a compelling need for novel pharmacological approaches.

There is a growing body of evidence showing that homoisoflavonoids have antiangiogenic activity relevant to these diseases, in some cases with limited effects on nontarget cells. Thus, in this study we sought novel homoisoflavonoids from a plant genus known to produce this class of compounds, and tested isolated compounds for antiangiogenic activity.

Despite the similarity in structure of compounds **1** and **2**, compound **1** was non-specific for antiproliferative effects on endothelial cells, while compound **2** was selective for HRECs. However, both compounds blocked migration and tubule formation of HRECs; these assays provide a good *in vitro* model of antiangiogenic activity. Taken together, these results suggest that compound **2** shows promise for the development of future small molecule treatments for ocular neovascularization, due to its specificity and its excellent activity against HRECs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

H. Whitmore acknowledges a PhD scholarship from the University of Surrey. Sianne Schwikkard acknowledges support from the Daphne Jackson Trust, the University of Surrey and the Royal Society of Chemistry for funding. T. W. Corson acknowledges support from NIH/NEI R01EY025641, the BrightFocus Foundation, and an unrestricted grant from Research to Prevent Blindness, Inc.

Abbreviations:

AMD	age-related macular degeneration
CD	circular dichroism
COSY	correlation spectroscopy
EBM	endothelial basal medium
EGM	endothelial growth medium
FBS	foetal bovine serum
FTIR	Fourier-transform infrared
HMBC	heteronuclear multiple bond spectroscopy
HRECs	human retinal microvascular endothelial cells
HRESIMS	high- resolution electrospray ionization mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
NMR	nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
PDR	proliferative diabetic retinopathy

ROP	retinopathy of prematurity
TOF	time of flight
VEGF	vascular endothelial growth factor

References

- [1]. Bahri L, Djegham M, Makhlouf M, Urginea maritima a poisonous plant of North Africa, *Vet. Hum. Toxicol* 42 (2000) 108–110. [PubMed: 10750179]
- [2]. Basavarajappa H, Lee B, Lee H, Sulaiman H, An H, Magaña C, Shadmand M, Vayl A, Rajashekhar G, Kim E, Suh Y, Lee K, Seo S, Corson T, Synthesis and biological evaluation of novel homoisoflavonoids for retinal neovascularization, *J. Med. Chem* 58 (2015) 5015–5057. [PubMed: 26035340]
- [3]. El-Sharkawy ER, Mahmoud K, Cytotoxicity of two new coumarin derivatives isolated from *Launaea mucronata*, *Nat. Prod. Res* 30 (2016) 394–398. [PubMed: 25751124]
- [4]. Folk JC, Stone EM, Ranibizumab therapy for Neovascular age-related macular degeneration, *N. Engl. J. Med* 363 (2010) 1648–1655. [PubMed: 20961248]
- [5]. Hirano H, Tokuhira T, Yokoi T, Shingu T, Isolation of free radical scavenger from *Coptidis rhizoma*, *J. Nat. Med* 51 (1997) 539–540.
- [6]. Kaewamatawong R, Ruangrunsi N, Likhitwitayawuid K, Chemical constituents of *Polyalthia parviflora* stem, *J. Nat. Med* 61 (2007) 349–350.
- [7]. Knirsch W, Martínez-Azorín M, Pfosser M, Wetschnig W, The reinstatement and rediagnosis of the Madagascan genus *Rhodocodon* (Asparagaceae, Scilloideae), with validation and remarks on H. Perrier's taxa, *Phytotaxa* 195 (2015) 101–134.
- [8]. Koorbanally C, Mulholland D, Crouch N, A novel homoisoflavonoid from *Drimia delagoensis* (Urgineoideae: Hyacinthaceae), *Biochem. Syst. Ecol* 33 (2005) 743–748.
- [9]. Lee B, Basavarajappa H, Sulaiman R, Fei X, Seo S, Corson T, The first synthesis of the antiangiogenic homoisoflavone, cremastranone, *Org. Biomol. Chem* 12 (2014) 7673–7677. [PubMed: 25167470]
- [10]. Manning JC, Goldblatt P, Fay M, Revised generic synopsis of Hyacinthaceae in sub-Saharan Africa, based on molecular evidence, including new combinations and the new tribe Pseudoprosperaeae, *Edinb. J. Bot* 60 (2003) 533–568.
- [11]. Moodley N, Crouch N, Mulholland D, Slade D, Ferreira D, 3-Benzyl-4-chromanones (homoisoflavanones) from bulbs of the ethnomedicinal geophyte *Ledebouria revoluta* (Hyacinthaceae), *S. Afr. J. Bot* 72 (2006) 517–520.
- [12]. Mulholland DA, Schwikkard SL, Crouch NR, The chemistry and biological activity of the Hyacinthaceae, *Nat. Prod. Rep* 30 (2013) 1165–1210. [PubMed: 23892453]
- [13]. Nakata H, Sashida Y, Shimomura H, A new phenolic compound from *Heracleum lanatum* MICHX. var. *nippinicum* HARA. II, *Chem. Pharm. Bull* 30 (1982) 4554–4556.
- [14]. Penn JS, Madan A, Caldwell RB, Bartoli M, Caldwell RW, Hartnett ME, Vascular endothelial growth factor in eye disease, *Prog. Retin. Eye Res* 27 (2008) 331–371. [PubMed: 18653375]
- [15]. Pfosser M, Wetschnig W, Speta F, *Drimia cryptopoda*, a new combination in Hyacinthaceae from Madagascar, *Linz. Biol. Beitr* 38 (2006) 1731–1739.
- [16]. Schwikkard S, Alqahtani A, Knirsch W, Wetschnig W, Jaksevicus A, Opara E, Langat M, Andriantiana J, Mulholland D, Phytochemical investigations of three *Rhodocodon* (Hyacinthaceae sensu APG II) species, *J. Nat. Prod* 80 (2017) 30–37. [PubMed: 28051316]
- [17]. Schwikkard S, Whitmore H, Sishitla K, Sulaiman RS, Shetty T, Basavarajappa HD, Waller C, Alqahtani A, Frankemoelle L, Chapman A, Crouch N, Wetschnig W, Knirsch W, Andriantiana J, Mas-Claret E, Langat MK, Mulholland D, Corson TW, The Antiangiogenic activity of naturally occurring and synthetic Homoisoflavonoids from the Hyacinthaceae (sensu APGII), *J. Nat. Prod* 82 (2019) 1227–1239. [PubMed: 30951308]

- [18]. Schwikkard SL, Whitmore H, Corson TW, Sischtla K, Langat MK, Carew M, Mulholland DA, Antiangiogenic activity and cytotoxicity of Triterpenoids and Homoisoflavonoids from *Massonia pustulata* and *Massonia bifolia*, *Planta Med.* 84 (2018).
- [19]. Speta F, Kubitzki K (Ed.), *The Families and Genera of Vascular Plants III Flowering Plants, Monocotyledons: Lillianaes (Except Orchidaceae)*, Springer, Berlin, 1998, pp. 261–285.
- [20]. Speta F, *Systematische analyse der Gattung Scilla L. s. l. (Hyacinthaceae)*, *Phyton* 38 (1998) 1–141.
- [21]. Sritularak B, Likhitwitayawuid K, New Bisbibenzyls from *Dendrobium falconeri*, *Helv. Chim. Acta* 92 (2009) 740–744.
- [22]. Sulaiman R, Basavarajappa H, Corson T, Natural product inhibitors of ocular angiogenesis, *Exp. Eye Res* 129 (2014) 161–171. [PubMed: 25304218]
- [23]. Sulaiman RS, Merrigan S, Quigley J, Qi X, Lee B, Boulton ME, Kennedy B, Seo SY, Corson TW, A novel small molecule ameliorates ocular neovascularisation and synergises with anti-VEGF therapy, *Sci. Rep* 6 (2016) 25509. [PubMed: 27148944]
- [24]. Tantray MA, Bhat BA, Khuroo MA, Shawl AS, Two new coumarins from *Euonymus hamiltonianus*, *Chem. Nat. Compd* 44 (2008) 10–12.
- [25]. Tesso H, König WA, Kubeczka K-H, Bartnik M, Glowniak K, Secondary metabolites of *Peucedanum tauricum* fruits, *Phytochemistry* 66 (2005) 707–713. [PubMed: 15771895]
- [26]. Watanabe K, Mimaki Y, Sakagami H, Sashida Y, Bufadienolide and spirostanol glycosides from the rhizomes of *Helleborus orientalis*, *J. Nat. Prod* 66 (2003) 236–241. [PubMed: 12608856]
- [27]. Wu T, Leu Y, Chan Y, Constituents of the fresh leaves of *Aristolochia cucurbitifolia*, *Chem. Pharm. Bull* 47 (1999) 571–573.
- [28]. Yang X-L, Awakawa T, Wakimoto T, Abe I, Induced production of novel prenyldepside and coumarins in endophytic fungi *Pestalotiopsis acaciae*, *Tetrahedron Lett.* 54 (2013) 5814–5817.

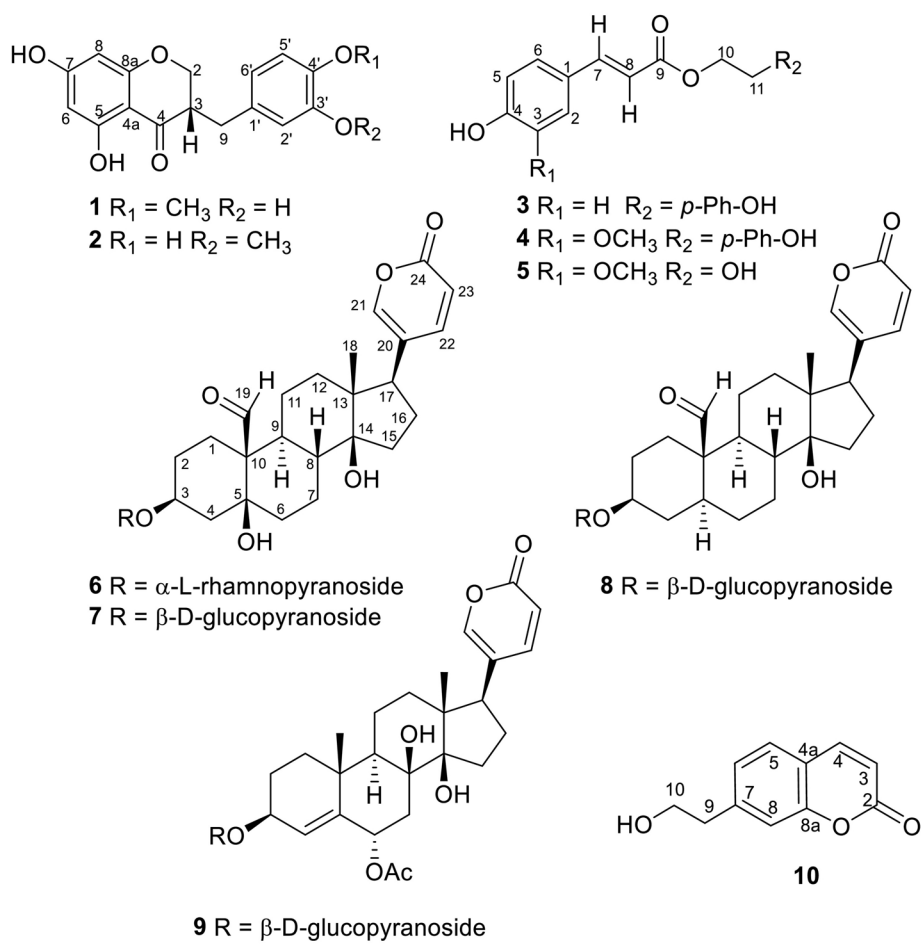


Fig. 1. Compounds isolated from *R. cryptopus*, *R. rotundus* and *R. cyathiformis*.

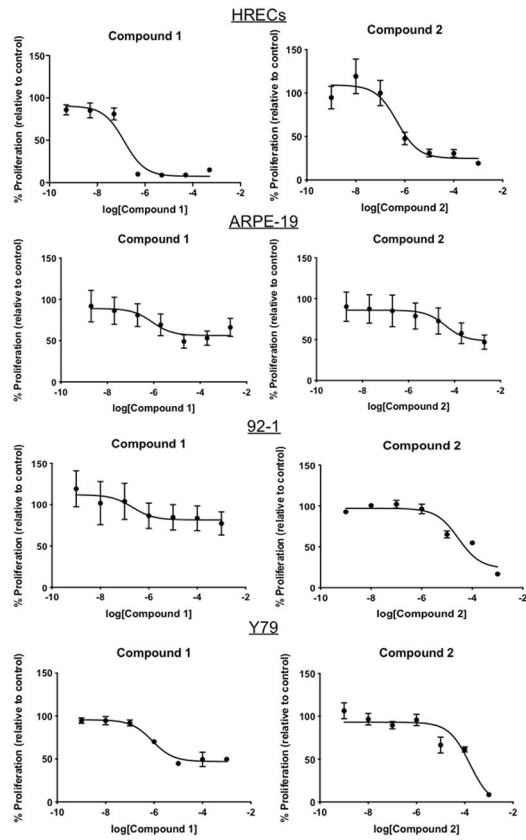


Fig. 2. Dose-response curves for inhibition of proliferation of indicated cell types by compounds **1** and **2**. Mean \pm SEM, $n = 3$.

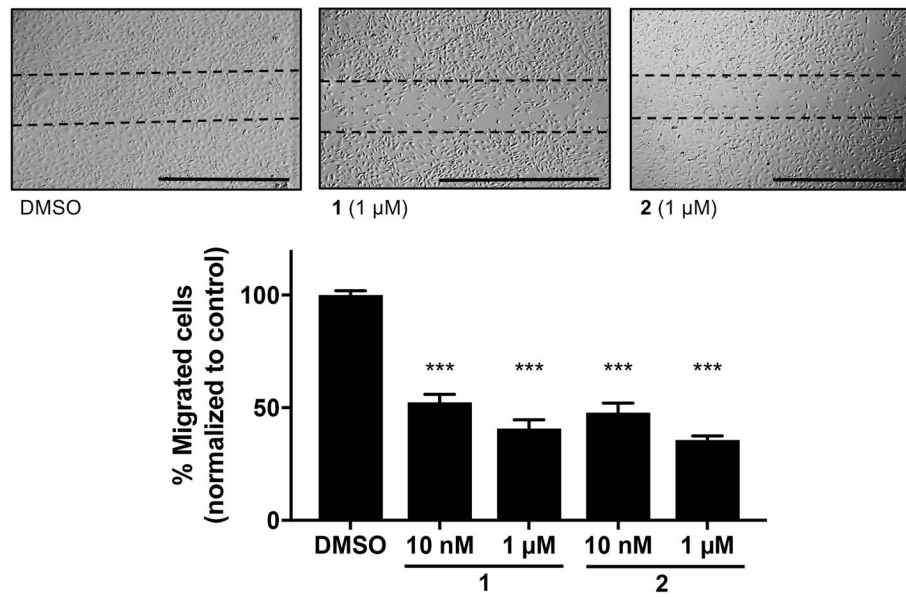


Fig. 3. Migration assays for HRECs treated with compounds **1** and **2**. Representative images of the highest concentration treatment of each compound or DMSO control shown (scale bars = 1 mm) and quantification of migrated cells. Mean \pm SEM, $n = 3$. ***, $P < .001$ compared to DMSO, ANOVA with Dunnett's *post hoc* tests.

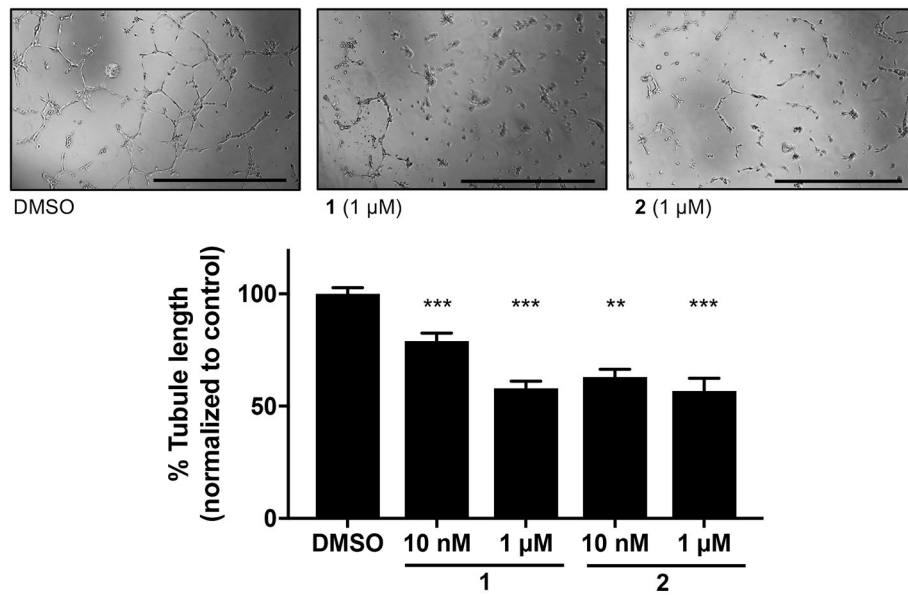


Fig. 4. Tube formation assays for HRECs treated with compounds **1** and **2**. Representative images of highest concentration treatment of each compound or DMSO control shown (scale bars = 1 mm) along with quantification of tubule length, Mean \pm SEM, $n = 6$. **, $P < .01$; ***, $P < .001$, ANOVA with Dunnett's *post hoc* tests.

Table 1NMR data for compounds **1** and **2** (500 MHz, CDCl₃, *J* in Hz).

No.	1		2	
	δ_C	δ_H	δ_C	δ_H
2	69.3	(α) 4.11 (dd, 11.4, 7.4) (β) 4.27 (dd, 11.4, 4.3)	69.1	(α) 4.10 (dd, 11.5, 7.0) (β) 4.26 (dd, 11.5, 4.2)
3	46.9	2.83 (m)	46.8	2.80 (m)
4	198.1	-	198.3	-
4a	103.0	-	102.6	-
5	164.9	-	165.3	-
6	96.8	5.98 (d, 2.2)	96.9	6.01 (d, 2.5)
7	164.4	-	164.7	-
8	95.2	5.91 (d, 2.2)	95.4	5.93 (d, 2.5)
8a	163.5	-	163.4	-
9	32.4	(a) 3.16 (dd, 14.0, 4.6) (b) 2.65 (dd, 14.0, 10.6)	32.4	(a) 3.14 (dd, 13.9, 4.5) (b) 2.65 (dd, 13.9, 7.0)
1'	131.1	-	131.2	-
2'	115.4	6.81 (d, 1.9)	115.4	6.80 (d, 2.3)
3'	146.0	-	145.8	-
4'	145.7	-	145.7	-
5'	111.0	6.80 (d, 8.1)	111.1	6.79 (d, 8.2)
6'	120.8	6.70 (dd, 8.1, 1.9)	120.9	6.70 (dd, 8.2, 2.3)
3'-OMe	-	-	56.2	3.88 (s)
4'-OMe	56.2	3.88 (s)	-	-
5-OH	-	12.13 (s)	-	12.15 (s)

Table 2NMR data for compound **10** (500 MHz, CDCl₃, *J* in Hz).

10						
No.	¹³ C	Type	¹ H (<i>J</i> in Hz)	HMBC (H → C)	COSY	NOESY
2	167.5	C				
3	101.9	CH	5.64 (d, 7.8)	4	4	4
4	143.7	CH	7.41 (d, 7.8)	2, 3, 4a	3	3
4a	144.8	C				
5	116.4	CH	6.69 (d, 8.0)	4, 4a, 6, 7	6	6
6	121.3	CH	6.54 (dd, 8.0 2.1)	4a, 5, 8	5	5, 9
7	131.9	C				
8	117.3	CH	6.66 (d, 2.1)	4a, 6		6, 9
8a	151.7	C				
9	39.9	CH ₂	2.68 (t, 7.4)	6, 7, 8, 10	10	6, 8, 10
10	64.8	CH ₂	3.69 (t, 7.4)	7, 9	9	9

Table 3

NMR data for compounds **6–9** (500 MHz, CDCl₃, *J* in Hz).

No.	7			8			9		
	δ_C	δ_H	HMBC (H → C)	δ_C	δ_H	HMBC (H → C)	δ_C	δ_H	HMBC (H → C)
1	23.7	(α) 1.57 (d, 2.5) (β) 1.18 (s)	(α) 1.54 br s (β) 1.17 (d, 2.1)	32.8	(α) 2.03 (m) ^d (β) 1.69 (m)	39.2	(α) 1.87 (m) (β) 1.38 (d) ^d	3, 5, 9, 10	
2	29.8	(α) 2.23 (m) (β) 1.77 (t, 3.7)	(α) 2.22 (d, 3.5) (β) 1.77 (d, 1.92)	28.8	(α) 2.27 (dd, 12.8, 2.7) (β) 1.32 (d, 5.0) ^d	27.7	(α) 2.08 (m) (β) 1.77 (m)		
3	75.1	4.17 (bs, <i>W</i> _{1/2} = 8.4 Hz)	4.26 (bs, <i>W</i> _{1/2} = 9.1 Hz)	78.6	3.78 (m, <i>W</i> _{1/2} = 24.5 Hz)	77.0	4.29 (bt, <i>W</i> _{1/2} = 20.7)	4, 5, 1'	
4	37.4	(α) 2.17 (d, 4.3) (β) 1.71 (d, 4.7)	(α) 2.17s (β) 1.76 (d, 3.7)	36.9	(α) 1.95 (m) (β) 1.31 (d, 5.0) ^d	131.7	5.94 (br s)	2, 6, 10-	
5	75.3	-	75.0	44.3	1.63 (m)	143.2	-	4, 5, 8, 10, 6-OAc(a)	
6	36.4	(α) 2.22 (d, 4.9) (β) 1.64 (t, 2.8)	(α) 2.14 (d, 2.8) (β) 1.71 (d, 5.0)	32.3	(α) 2.43 (dt, 13.6, 3.2) (β) 1.05 (td, 14.0, 3.5)	77.6	5.47 (dd, 8.6, 2.6)	5, 6, 8, 9, 14	
7	19.2	(α) 2.09 (m) (β) 1.78 (m)	(α) 2.18 (d, 3.0) (β) 1.71 (d, 4.9)	30.3	(α) 2.58 (s) (β) 2.02 (m) ^d	38.7	(α) 2.39 (dd, 15.5, 2.3) (β) 1.69 (m)	10, 19	
8	40.7	1.74 (d, 3.3)	1.73 m	44.1	1.42 (m)	77.5	-	-	
9	43.2	1.98 (d, 2.8)	1.98 (d, 2.9)	49.9	1.30 (m)	52.2	1.43 (m)	9, 10	
10	56.3	-	56.4	53.0	-	38.1	-	8, 13	
11	25.4	(α) 2.21 (br s) (β) 1.42 (d, 3.8)	(α) 2.20 (d, 1.77) (β) 1.40 m	23.3	(α) 1.74 (dd, 13.0, 5.0) (β) 1.21 (d, 7.1)	19.4	(α) 1.86 (m) (β) 1.49 (m)	9, 11, 13, 14 13	
12	41.6	(α) 1.52 (d, 3.2) (β) 1.47 (d, 4.1)	(α) 1.52 (d, 2.0) (β) 1.44 br s	41.5	(α) 1.51 (t, 3.1) (β) 1.40 (d, 3.0)	42.1	(α) 1.45 (m) (β) 1.62 (m)	- -	
13	49.0	-	49.0	49.6	-	50.5	-	8, 16	
14	85.8	-	85.8	85.6	-	86.5	-	14, 16, 17	
15	32.5	(α) 2.12 (d, 2.9) (β) 1.69 (d, 5.7)	(α) 2.10 (d, 5.0) (β) 1.70 (d, 3.7)	31.7	(α) 2.01 (m) ^d (13) 1.34 (m)	35.1	(α) 2.05 (d) ^d (β) 1.68 (d, 8.4)	13, 14, 15, 20 13, 14, 17, 20	
16	26.2	(α) 1.86 (m) (β) 1.72 (d, 4.9)	(α) 1.90 m (β) 1.69 (d, 4.0)	29.8	(α) 2.19 (dt, 12.6, 9.8) (β) 1.62 (d, 9.4)	30.3	(α) 2.19 (m) (β) 1.72 (m)	12, 13, 14, 16, 20, 22 12, 13, 14, 17	

No.	6		7		8		9		HMBC (H → C)
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	
17	52.1	2.58 (dd, 9.4, 6.5)	52.1	2.57 (dd, 9.6, 6.7)	52.2	2.55 (dd, 9.4, 6.5)	52.9	2.56 (dd, 9.2, 6.5)	1, 5, 9, 10
18	17.2	0.70 (s)	17.2	0.70s	17.2	0.67 (s)	19.8	0.92 (s)	-
19	209.8	10.10 (s)	210.2	10.08s	210.7	10.01 (s)	22.4	1.36 (s)	17, 20, 22, 24
20	125.0	-	125.0	-	125.0	-	125.3	-	17, 21, 24
21	150.7	7.45 (dd, 2.4, 0.8)	150.7	7.44 (d, 1.7)	150.6	7.44 (d, 1.7)	150.6	7.43 (d, 1.6)	20, 24
22	149.4	8.01 (dd, 9.7, 2.4)	149.4	8.00 (dd, 12.3, 2.0)	149.4	7.99 (dd, 9.7, 2.4)	149.4	8.03 (dd, 9.7, 2.5)	-
23	115.6	6.30 (dd, 9.7, 0.6)	115.6	6.30 (d, 9.7)	115.6	6.30 (d, 9.7)	115.6	6.31 (d, 9.7)	3, 3'
24	164.9	-	164.9	-	164.9	-	165.0	-	1', 4'
1'	101.1	4.87 m ^a	102.0	4.43 (d, 7.8)	102.5	4.40 (d, 7.8)	103.8	4.43 (d, 7.7)	1', 2', 4', 5'
2'	72.6	3.63 (d, 3.3)	75.2	3.19 (dd, 9.2, 7.9)	75.2	3.14 (dd, 9.0, 7.8)	75.2	3.19 (dd, 9.2, 7.7)	3', 5'
3'	72.7	3.80 (dd, 3.2, 1.8)	78.4	3.37 m	78.1	3.37 (m)	78.2	3.38 (t, 8.8)	4', 6'
4'	73.9	3.42 (t, 9.4)	78.3	3.29 ^a m	78.0	3.28 (d, 1.5)	78.1	3.29 (br s)	4', 5'
5'	70.8	3.65 m	71.8	3.30 ^a m	71.8	3.29 ^a m	71.8	3.31 (d, 7.6)	4', 5'
6'	18.1	1.29 (d, 6.2) ^a	62.9	(a) 3.98 (d, 11.6) (b) 3.70 (dd, 11.6, 5.1)	62.9	(a) 3.88 (d, 11.4) (b) 3.67 (m)	62.9	(a) 3.88 (m) (b) 3.68 (dd, 11.7, 5.2)	
6-OAc	-	-	-	-	-	-	171.7	-	
	-	-	-	-	-	-	21.7	2.04 (s) ^a	

^a Overlapped resonances.