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The Antiangiogenic Activity of Naturally Occurring and Synthetic Homoisoflavonoids from the Hyacinthaceae (*sensu* APGII)

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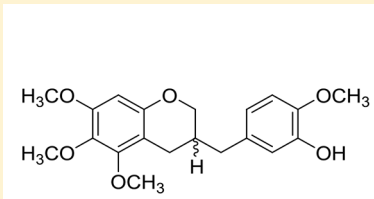
Supporting Information

ABSTRACT: Excessive blood vessel formation in the eye is implicated in wet age-related macular degeneration, proliferative diabetic retinopathy, neovascular glaucoma, and retinopathy of prematurity, which are major causes of blindness. Small molecule antiangiogenic drugs are strongly needed to supplement existing biologics. Homoisoflavonoids have been previously shown to have potent antiproliferative activities in endothelial cells over other cell types. Moreover, they demonstrated a strong antiangiogenic potential in vitro and in vivo in animal models of ocular neovascularization. Here, we tested the antiangiogenic activity of a group of naturally occurring homoisoflavonoids isolated from the family Hyacinthaceae and related synthetic compounds, chosen for synthesis based on structure−activity relationship observations. Several compounds showed interesting antiproliferative and antiangiogenic activities in vitro on retinal microvascular endothelial cells, a disease-relevant cell type, with the synthetic chromane, **46**, showing the best activity (GI_{50} of 2.3×10^{-4} μM).



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 adults and elderly people, respectively.[1] The newly formed vasculature is fragile and leaky, causing hemorrhage and accumulation of fluids in the retina. If left untreated, the resulting edema and fibrotic scarring can lead to irreversible vision loss.[2] The current pharmacotherapeutic mainstays for these diseases are biologics targeting the vascular endothelial growth factor (VEGF) such as bevacizumab, ranibizumab, and aflibercept.[3] Despite being successful in suppressing disease progression, these large molecule therapies are associated with some undesirable ocular and systemic side effects as well as the time-consuming regular visits to hospitals required for the intravitreal injections.[4,5] Moreover, resistance is a problem: about 30% of wet AMD patients are resistant to these biologics.[6] Currently, there is no FDA approved small molecule for the treatment of ocular neovascularization. Therefore, there is an unmet need to develop novel and specific antiangiogenic small molecule therapies to complement and combine with existing drugs for ROP, PDR, and wet AMD.

Natural products continue to provide appealing lead compounds for treatment of diseases.[7] Naturally occurring homoisoflavonoids are reported to have diverse biological activities including antioxidant, anti-inflammatory, and anti-

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angiogenic activities.[8] The Hyacinthaceae is one of the families rich in homoisoflavonoids distributed in different parts of the plants. The Hyacinthaceae (*sensu* APGII) consists of approximately 900 species, in about 70 genera. The family can be divided into four subfamilies, the widespread Hyacinthoideae, Urgineoideae, and Ornithogaloideae and the small subfamily Oziroëoideae which is restricted to South America. For the most part, the compounds found are subfamily restricted. Homoisoflavonoids and spirocyclic nortriterpenoids characterize the Hyacinthoideae, bufadienolides the Urgineoideae, and cardenolides and steroidal glycosides the Ornithogaloideae. To date, no phytochemical investigations have been reported of the Oziroëoideae.[9]

Members of the Hyacinthoideae subfamily have a long tradition of being used by traditional healers to treat a wide range of complaints. Phytochemical investigations of the genus *Eucomis* L'Hér.,[10] *Ledebouria revoluta* (L.F.) Jessop,[11] *Ledebouria ovatifolia* (Baker) Jessop,[12] *Merwilla plumbea* (Lindl.) Speta,[13] all widely used in African traditional medicine, have been reported.

Previously, a homoisoflavanone, cremastranone, isolated from the orchid *Cremastra appendiculata* (D.Don) Makino, was shown to have antiangiogenic activity in the ocular context, but unfortunately, the configuration at C-3 was not reported.[14−16] This compound was also previously isolated from the Hyacinthoideae genera *Muscari* Mill.[17] and *Merwilla* Speta.[13] More recently, a synthetic racemate of this compound showed strong antiangiogenic activity against human retinal microvascular endothelial cells (HRECs), with lesser effects on nonendothelial cell types.[18] A synthetic regioisomer of cremastranone (SH-11052) also had antiangiogenic activities in vitro.[19] Subsequently, our SAR campaign identified a promising synthetic analogue of cremastranone, named SH-11037, with more potent and selective antiproliferative effects on HRECs compared to endothelial cells of macrovascular origin.[20] Moreover, SH-11037 demonstrated strong antiangiogenic potential in vivo in animal models of ROP[20] and choroidal neovascularization.[21] We have also isolated known and novel homoisoflavonoids from *Massonia* Houtt. species, some of which showed selective antiproliferative effects on HRECs.[22] Given the selective and potent antiangiogenic activity of cremastranone and derivatives, the search for further antiangiogenic homoisoflavonoids is warranted. In parallel with our medicinal chemistry approach,[20] we have here further explored natural-source and related synthetic homoisoflavonoids as antiangiogenic agents.

Homoisoflavonoids and related compounds isolated from seven species of Hyacinthaceae have been investigated in this study for antiangiogenic activity. The isolation and structure determination of compounds from *Ledebouria socialis* (Baker) Jessop (**1**, **2**), *Ledebouria ovatifolia* (Baker) Jessop (**3**−**14**), *Rhodocodon campanulatus* Knirsch (**25**, **26**), Mart-Azorín and Wetschnig, *Rhodocodon* aff. *intermedius* Knirsch, Mart-Azorín and Wetschnig (**27**−**29**), have been reported by us previously, and compounds from these sources were screened as part of this work.[23,24] In addition, *Chionodoxa luciliae* Boiss, *Eucomis autumnalis* (Mill.) Chitt., and *Eucomis bicolor* Baker were reinvestigated to provide material for screening and a previously unreported homoisoflavonoid, **35**, along with previously reported ones were isolated, some for the first time from these sources. These species were chosen for re-extraction due to large amounts of bulb material being available from a commercial supplier enabling us to isolate large amounts of compounds for screening purposes.

In addition, on the basis of our cell-based structure−activity observations, a number of known (**44**, **47**, **48**) and previously undescribed homoisoflavonoids (**42**, **43**, **45**, **46**, **49**, and **50**) were synthesized and investigated for antiangiogenic activity, including the enantioselective synthesis of compound **45** with the 3*R* configuration. Compound **46**, a previously undescribed compound with a chromane skeleton, showed significantly greater activity than the related chromanones (**45** and **47**).

RESULTS AND DISCUSSION

The EtOH extract of the bulbs of *Chionodoxa luciliae* Boiss yielded compound **16**, *para*-hydroxybenzaldehyde, and homoisoflavonoids **15, 17** and **21**-**24**. Compound **17** was acetylated and yielded the 7-acetate (**19**) and 3,7-diacetate (**20**). Compound **16** is likely to be a degradation product of compound **21**. All compounds have been reported previously from several sources[9,25] but this is the first report of the isolation of compounds **17**, **22** and **23** from a *Chionodoxa* species. Structures were determined using NMR techniques and were confirmed by comparison against literature data; the configurations at C-3 were confirmed using circular dichroism spectroscopy and optical rotation measurements.

The MeOH and butanol extracts of the bulbs of *Eucomis autumnalis* yielded compounds **22** and **30**−**39**, and the EtOH extract of the bulbs of *E. bicolor* yielded compounds **40** and **41**. Compounds **31** and **32** have been isolated previously from both *E. autumnalis* and *E. bicolor*,[26] compound **36** from *E. autumnalis*,[27−29] compounds **30**, **40**, and **41** from *E. bicolor*,[27,30,31] compound **33** from *E. montana*,[32] compound **38** from *Muscari armeniacum*,[17] compound **39** from *Scilla persica*,[33] and compound **37** from *Ledebouria ovatifolia*.[23]

Compound **35**, 3*R*-(4′-hydroxybenzyl)-6,8-dihydroxy-5,7-dimethoxy-4-chromanone, is a homoisoflavonoid that has not been reported previously with a fully substituted ring A. HRMS showed a protonated molecular ion at m/z 347.11254 corresponding to a molecular formula of $C_{18}H_{18}O_7$. Characteristic peaks at δ_H 4.37 (H-2α, dd, 11.4, 4.1 Hz), δ_H 4.20 (H-2β, dd, 11.4, 7.2 Hz), δ_H 2.79 (H-3, m), δ_H 3.17 (H-9A, dd, 14.0, 4.4 Hz), and δ_H 2.70 (H-9B, dd, 14.0, 10.6 Hz) were indicative of a 3-benzyl-4-chromanone structure, and resonances at δ_H 6.79 (2H, 3′ and 5′, 8.1 Hz) and δ_H 7.11 (2H, 2′ and 6′, 8.1 Hz) showed that the B ring was *para*- disubstituted. The two H-2 and two H-9 resonances showed correlations with the C-4 keto group carbon resonance at δ_C 192.0. Two methoxy group proton resonances and three exchangeable hydroxy group proton resonances (δ_H 5.59, 6-OH; δ_H 5.39, 8-OH and δ_H 4.74, 4′-OH) could be assigned from correlations seen in the HMBC spectrum (Figure 1). A specific rotation of −55.6 and negative Cotton effect at 290 nm indicated a 3*R*-configuration.[11] Homoisoflavonoids with a fully substituted ring A

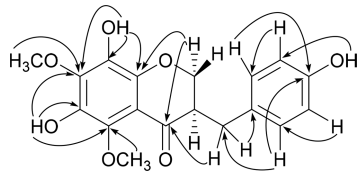

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Figure 1. HMBC correlations (H → C) for compound **35**.

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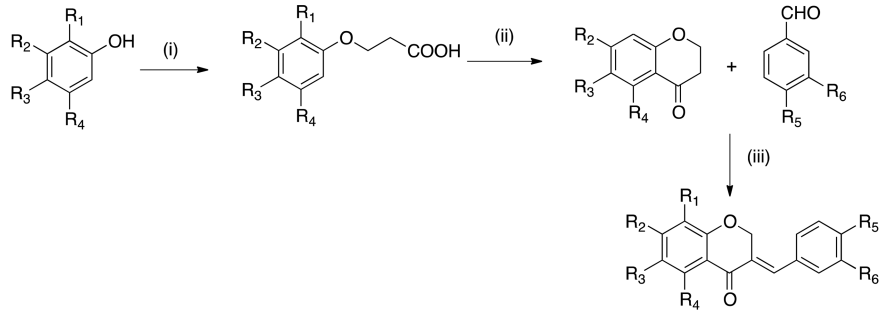
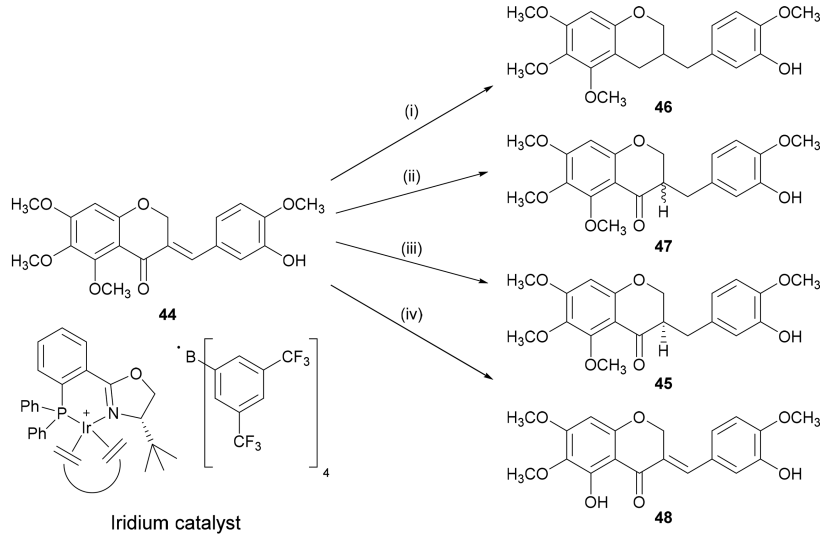
Scheme 1. General Scheme for Synthesis of 3-Benzylidene Homoisoflavonoids



Reagents: (i) NaH; dry Dimethylformamide; 3-Bromopropionic acid (ii) Polyphosphoric acid; 80^{0}C (iii) Piperidine; 80^{0}C

42 $R_1 = R_3 = R_6 = H$; $R_2 = OCH_3$; $R_4 = OCH_3$; $R_5 = SCH_3$
43 $R_1 = R_2 = OCH_3$; $R_3 = R_4 = R_6 = H$; $R_5 = SCH_3$
44 $R_1 = H$; $R_2 = R_3 = R_4 = R_5 = OCH_3$; $R_6 = OH$

Scheme 2. Reactions of Compound 44[a]



(i) Pd/C; Ethanol; H_2 atmospheric pressure; room temperature
(ii) Wilkinson's catalyst; Toluene; H_2 atmospheric pressure; room temperature
(iii) Iridium catalyst; dry Toluene; H_2 3 bar; room temperature
(iv) Trimethylsilyl chloride (8 eq.); NaI (8 eq.); dry Dichloromethane; 0 °C

[a]Iridium catalyst = (*S*)-(−)-2-[2-(diphenylphosphino)phenyl]-4-isopropyl-2-oxazolium-(1,5)-cyclooctadiene iridium(I) tetrakis(3,5-bis-(trifluoromethyl)phenyl borate).

are rare with only two similar compounds being reported previously: (4′-hydroxybenzyl)-5,6-dihydroxy-7,8-dimethoxy-4-chromanone, from *Bellevalia eigii*,[34] and (4′-hydroxy)-5,8-dihydroxy-6,7-dimethoxy-4-chromanone, from *Muscari comosum*.[35]

Previous work on synthetic analogues of cremastranone demonstrated that much potential remained for the improvement of activity. Previously, 3-benzyl analogues had been synthesized as racemic mixtures and we wished to investigate the possibility of synthesizing a single enantiomer via selective hydrogenation of the 3,9-double bond of a 3-benzylidene analogue. In addition, the effect of producing the chromane derivative of 5,6,7-trimethoxy-3-(3′-hydroxy-4′-methoxybenzylidene)-4-chromanone was of interest. Homoisoflavonoids **42**−**49** were synthesized following known methods[36] (Schemes 1 and 2). An iridium catalyst, the (*S*)-4-*tert*-butyl-2-[2-(diphenylphosphino)phenyl]-2-oxazoline complex, also known as iridium-phosphino-oxazoline or phox, used to hydrogenate compound **44** to form **45**, was synthesized using a known methodology[37] and is shown in Scheme 2. Chalcone **50** was synthesized via a Claisen−Schmidt condensation, using known methods.[38]

The syntheses of compounds **44**, **47**, and **48** have been reported,[20,39] while the synthesis of compounds **42**, **43**, **45**, **46**, **49**, and **50** has not been reported before. Compounds **42** and **43** were both synthesized in low yield (3 and 3.5%, respectively). The first two steps proceeded well, with the low yield being a result of poor reaction completion in step three and extensive chromatography to produce the pure product. The structures were confirmed by high resolution mass spectrometry and NMR analysis.

Compound **44** was used as a starting point for our investigation into the asymmetric hydrogenation of the 3,9-double bond in the benzylidene-type homoisoflavonoids using the iridium catalyst (Scheme 2) for the hydrogenation. The reaction proceeded quantitatively to give compound **45**.

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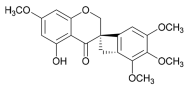
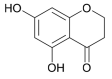
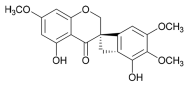
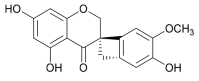
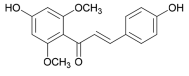
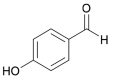
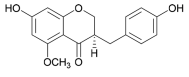
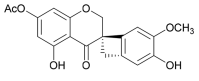
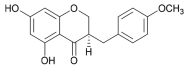
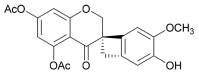
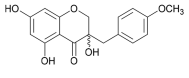
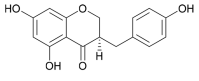
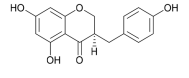
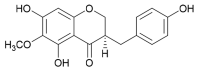
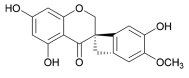
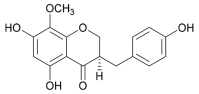
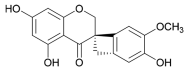
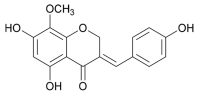
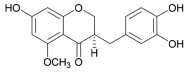
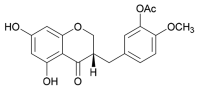
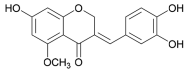
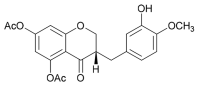
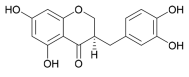
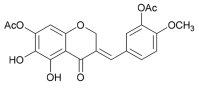
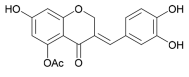
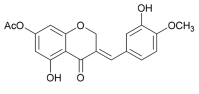
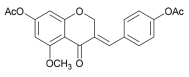
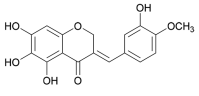
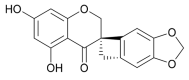
Table 1. Antiproliferative Activity of Homoisoflavonoids and Co-Isolated Compounds against Endothelial and Ocular Tumor Cells (Growth Inhibitory Concentration, GI_{50}, Shown in μM)[a]

No.	Structure	Source	GI_{50} HREC	GI_{50} HUVEC	GI_{50} 92-1	GI_{50} Y79	No.	Structure	Source	GI_{50} HREC	GI_{50} HUVEC	GI_{50} 92-1	GI_{50} Y79
1		*Ledebouria socialis*	>100	>100	>100	>100	**16**		*Chionodoxa luciliae*	>100	>100	>100	>100
2		*Ledebouria socialis*	>100	>100	>100	>100	**17**		*Chionodoxa luciliae*	>100	>100	>100	>100
3		*Ledebouria ovatifolia*	>100	>100	ND	>100	**18**		*Chionodoxa luciliae*	>100	>100	>100	>100
4		*Ledebouria ovatifolia*	>100	>100	>100	>100	**19**		*Chionodoxa luciliae*	>100	>100	>100	>100
5		*Ledebouria ovatifolia*	>100	>100	>100	>100	**20**		*Chionodoxa luciliae*	>100	>100	>100	>100
6		*Ledebouria ovatifolia*	>100	>100	>100	>100	**21**		*Chionodoxa luciliae*	>100	>100	>100	>100
7		*Ledebouria ovatifolia*	>100	>100	>100	>100	**22**		*Chionodoxa luciliae Eucomis autumnalis*	>100	>100	>100	>100
8		*Ledebouria ovatifolia*	>100	>100	>100	>100	**23**		*Chionodoxa luciliae*	>100	>100	>100	>100
9		*Ledebouria ovatifolia*	>100	>100	>100	>100	**24**		*Chionodoxa luciliae*	40	>100	>100	>100
10		*Ledebouria ovatifolia*	>100	14	>100	25	**25**		*Rhodocodon campanulatus*	>100	>100	>100	>100
11		*Ledebouria ovatifolia*	>100	>100	>100	>100	**26**		*Rhodocodon campanulatus*	>100	92	>100	>100
12		*Ledebouria ovatifolia*	>100	>100	>100	>100	**27**		*Rhodocodon* aff. *intermedius*	16	11	69	29
13		*Ledebouria ovatifolia*	1.4	5.0	>100	2.5	**28**		*Rhodocodon* aff. *intermedius*	18	26	67	58
14		*Ledebouria ovatifolia*	96	43	>100	>100	**29**		*Rhodocodon* aff. *intermedius*	17	41	>100	23
15		*Chionodoxa luciliae*	>100	>100	>100	>100							

[a]ND, no data.

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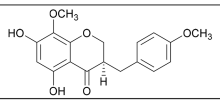
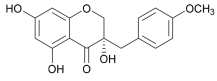
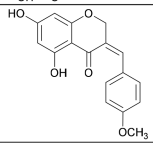
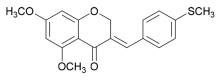
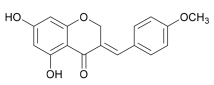
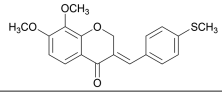
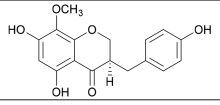
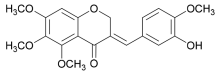
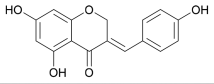
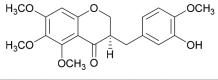
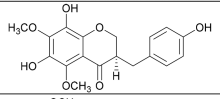
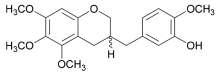
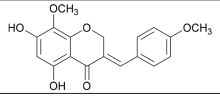
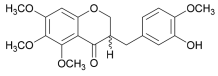
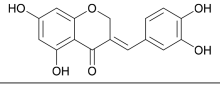
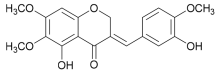
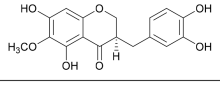
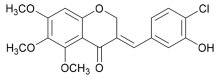
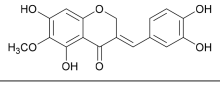
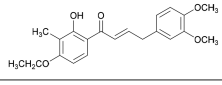
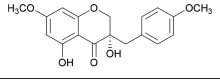
Table 2. Antiproliferative Activity of Natural-Source and Synthetic Homoisoflavonoids and Co-Isolated Compounds against HRECs and Other Ocular Cell Types (Growth Inhibitory Concentration, GI_{50}, Shown in μM)

No.	Structure	Source	GI_{50} HREC	GI_{50} ARP E-19	GI_{50} 92-1	GI_{50} Y79	No.	Structure	Source	GI_{50} HREC	GI_{50} ARP E-19	GI_{50} 92-1	GI_{50} Y79
30		*Eucomis autumnalis*	>100	>100	>100	>100	**41**		*Eucomis bicolor*	74	>100	>100	>100
31		*Eucomis autumnalis*	12	83	>100	>100	**42**		*synthetic*	2.5	48	>100	2
32		*Eucomis autumnalis Eucomis bicolor*	>100	>100	>100	>100	**43**		*synthetic*	3.6	>100	>100	11
33		*Eucomis autumnalis*	64	>100	>100	>100	**44**		*synthetic*	2.2	1.3	8.2	23
34		*Eucomis autumnalis*	60	>100	>100	>100	**45**		*synthetic*	0.035	>100	>100	85
35		*Eucomis autumnalis*	>100	>100	>100	>100	**46**		*synthetic*	2.3×10^{-4}	3.4×10^{-4}	52	18
36		*Eucomis autumnalis*	>100	>100	>100	>100	**47**		*synthetic*	0.21	>100	>100	>100
37		*Eucomis autumnalis*	>100	>100	>100	>100	**48**		*synthetic*	0.26	4.8	>100	2.4
38		*Eucomis autumnalis*	>100	>100	>100	>100	**49**		*synthetic*	0.11	0.73	3.6	13
39		*Eucomis autumnalis*	>100	>100	>100	>100	**50**		*synthetic*	3.9	>100	>100	>100
40		*Eucomis bicolor*	64	>100	>100	>100							

A systematic conformational search of 3*R*- and 3*S*-isomers of 5,6,7-trimethoxy-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone was performed *via* molecular mechanics force field calculation (MMFF) built in the software Spartan'16. Conformers under 3.0 kJ/mol relative energy cutoff were selected and optimized using density functional theory (DFT) calculations at the B3LYP/6-31G+(d,p) level built into Gaussian 09. Once optimized, their ECD spectra were simulated using time dependent density functional theory (TDDFT) at the B3LYP/6-31+G(d,p) level. A polarizable continuum model (IEFPCM) was applied during the TDDFT calculation to simulate the presence of acetonitrile, which was the solvent used in the experimental ECD. The ECD spectra obtained for each conformer were Boltzmann weighted and compared to the experimental ECD spectrum of **45**.[40] The 6-31+G(d,p) basis set was applied, as it provides a fine balance between accuracy and computational time needed. In addition, it has been applied in the past by our group with satisfactory results.[41]

The measured ECD spectrum of **45** showed a positive Cotton effect at λ ($\Delta\varepsilon$) 326 (+1.3) and a negative Cotton effect at λ ($\Delta\varepsilon$) 284 (−1.9), which is in accordance with the theoretical calculated spectrum for the 3*R*-enantiomer, indicating the 3*R* configuration for compound **45** (Supporting Information).[42] For comparative purposes, compound **44** was hydrogenated using Pd/C to give the racemic mixture, **46**. This reaction resulted in the reduction of both the 3,9-double bond as well as the carbonyl group at C-4, as shown by the loss of the fully substituted carbon resonance at δ_C 179.8 (C-4) and the new methylene resonance at δ_C 37.5 (C-4). Coupling could clearly be seen in the COSY spectrum between the resonance at δ_H 2.14 (1H, m, H-3) and the resonance at δ_H 2.53 (2H, dd, J = 4.1 and 7.4 Hz, H-4). Compound **44** was then successfully hydrogenated using Wilkinson's catalyst (tris-(triphenylphosphine)rhodium(I) chloride) to give compound

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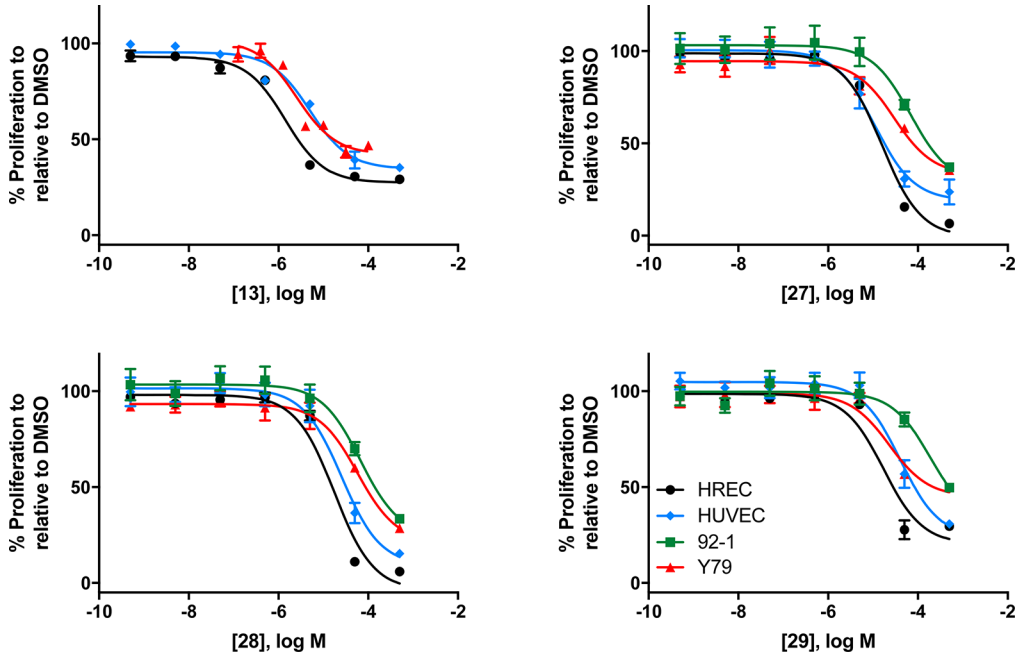

Figure 2. Homoisoflavonoids block the proliferation of HRECs. Dose−response curves for representative active compounds' effects on proliferation of the indicated cell types as assessed by alamarBlue fluorescence. Mean ± SEM, n = 3−6 wells.

47, the previously synthesized racemate.[20] Compound **48** was synthesized to determine whether the presence of a 5-OH group was significant for activity. Many natural homoisoflavonoids have a hydroxyl group at C-5. The selective demethylation was confirmed by the appearance of a sharp singlet at δ_H 12.45, due to the proton of a H-bonded hydroxyl group at C-5. In addition, three methoxy groups were noted instead of four.

Compound **49** was synthesized from 3,4,5-trimethoxyphenol and 4-chloro-3-hydroxybenzaldehyde, and the expected product was formed. A HRESIMS value of m/z 377.0786 $[M + H]^+$ indicated a molecular formula of $C_{19}H_{17}O_6Cl$. The structure of the product was confirmed by key ^{1}H NMR resonances at δ_H 4.99 (2H, d, J = 1.8 Hz, H-2), 7.75 (1H, bs, H-9), and 6.16 (1H, s, H-8) and three methoxy group three-proton resonances at δ_H 3.75 (3H, s), 3.82 (3H, s), and 3.91 (3H, s). The B-ring resonances at δ_H 7.15 (1H, dd, J = 2.4 and 8.6 Hz, H-2′), 6.91 (1H, d, J = 2.4, H-6′), and 6.83 (1H, d, J = 8.6, H-3′) supported the 4′,5′-substitution pattern.

Compound **50**, a chalcone, was synthesized from 3,4-dimethoxybenzaldehyde and 4′-ethoxy-2′-hydroxy-3′-methylacetophenone via a Claisen−Schmidt condensation. The HRESIMS gave a $[M + H]^+$ ion at m/z 343.1545, giving a molecular formula of $C_{20}H_{22}O_5$ for the compound. The chalcone structure was confirmed by a pair of doublets in the ^{1}H NMR spectrum at δ_H 7.47 (1H, d, J = 15.2 Hz, H-2) and 7.84 (1H, d, J = 15.2 Hz, H-3) indicative of E geometry.

Antiproliferative Activities of Homoisoflavonoids. To find novel antiangiogenic homoisoflavonoids, compounds were tested for their antiproliferative activities on HRECs. To assess effects on other normal cell types, we tested compounds against primary macrovascular human umbilical vein endothelial cells (HUVECs; a nontarget endothelial cell type) or the retinal pigment epithelial cell line, ARPE-19, a nontarget ocular cell type. Ideal antiangiogenic candidates should have lesser effects on these nontarget cell types than on HRECs. To further assess potential cytotoxic effects against ocular cell types, we also employed two ocular cancer cell lines, uveal melanoma 92-1 and retinoblastoma Y79. Again, ideal candidates for further investigation as antiangiogenic homoisoflavonoids should show lower activity against these other ocular cell lines derived from uveal melanocytes and photoreceptor precursors, respectively.

While many of the compounds lacked activity against the cell types, some compounds were reasonably potent and selective for the endothelial cell types (Tables 1 and 2; Figure 2). The results would suggest some structural features are significant in determining the activity of the homoisoflavonoid. Optimal activity was observed when the A-ring was either 5,6,7-trisubstituted or 5,7-disubstituted. If only two substituents were present, at least one of them would need to be an acetyl rather than a hydroxy group. 3′,4′-Disubstitution in ring B produced the highest activity, in particular when combined with the presence of a 3/9 double bond. Homoisoflavonoids with the bulkier 3′-acetyl group were more active than ones with the 3′-hydroxyl group. The presence of a cyclobutane ring rendered the homoisoflavonoid inactive, regardless of the substituents on rings A and B. The importance of a 5,6,7-trisubstituted A-ring was confirmed by the synthetic samples, with the trisubstituted compounds (**44**, **45**, **46**, **47**, **48**, and **49**) showing better activity than the disubstituted compounds (**42** and **43**). Interestingly, the 3R-enantiomer (**45**) showed greater activity than the precursor with a double bond in the 3,9-position (**44**) as well as greater activity than the racemic mixture (**47**). This serves to emphasize the importance of being able to synthesize these homoisoflavonoids enantioselectively. The complete reduction of both the 3,9-double bond and the carbonyl group at C-4 produced a highly active compound (**46**) (Table 2, GI_{50} (HREC) of 2.3×10^{-4} μM, GI_{50} (ARPE-19) of 3.4×10^{-4} μM, and GI_{50} (92-1) of 52 μM). This significant increase in activity with the loss of the carbonyl group at C-4 has not been reported before, although this compound was synthesized simultaneously by the Seo lab (personal communication). Compound **49**, with a 4′-chloro-

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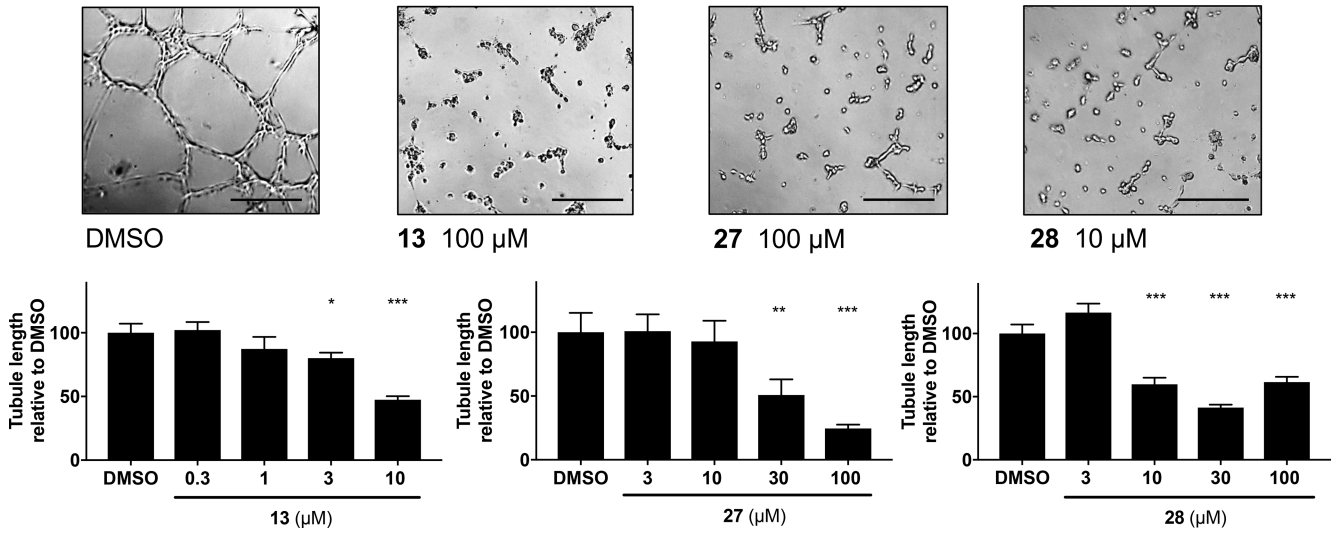

Figure 3. Potent natural-source homoisoflavonoids significantly block the ability of HRECs to form tube-like structures. Top: Representative images of the tube formation of HRECs on Matrigel at the highest concentrations of each compound tested plus DMSO control, scale bars = 250 μm. Bottom: Quantification of the extent of tube formation was measured as the tubule length of HRECs treated with compounds compared to DMSO control. Mean ± SEM, n = 3−6 wells. Representative results from duplicate experiments. *P < 0.05, ***P < 0.001 compared to DMSO control (one-way ANOVA with Dunnett's *posthoc* tests).

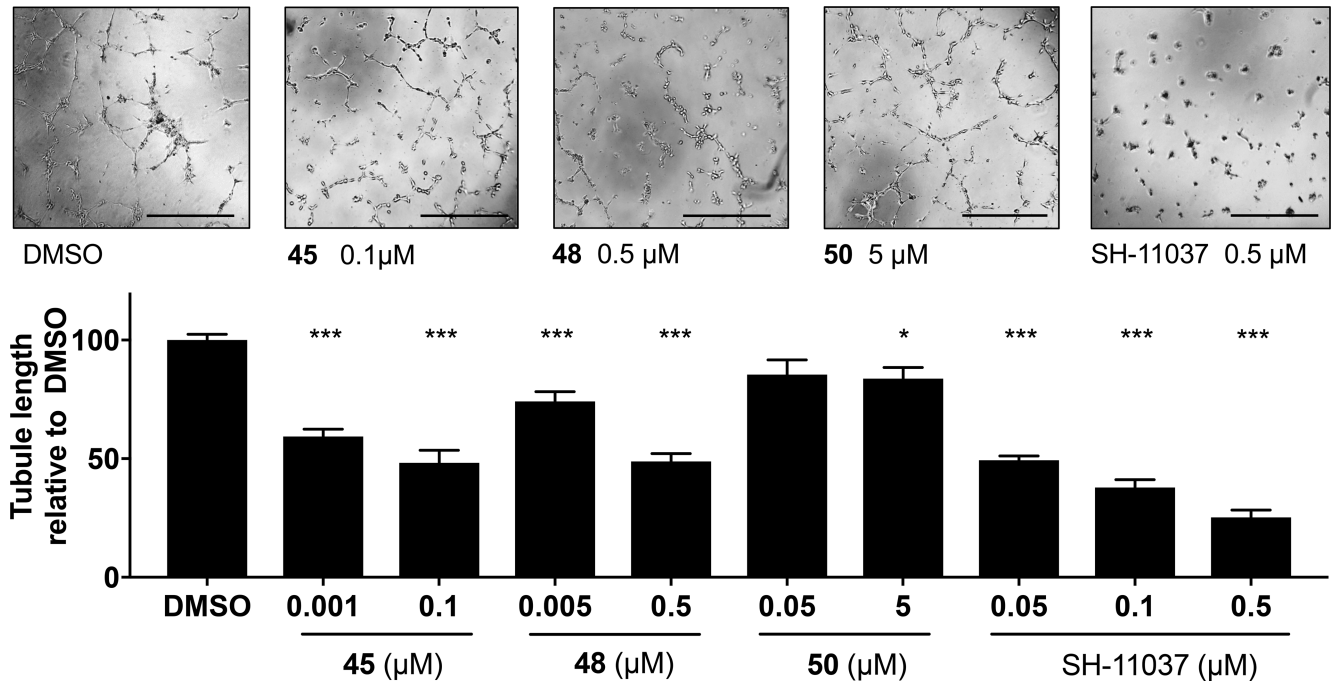

Figure 4. Potent synthetic homoisoflavonoids significantly block the ability of HRECs to form tube-like structures. Top: Representative images of the tube formation of HRECs on Matrigel at the highest concentrations of each compound tested plus DMSO control, scale bars = 500 μm. Bottom: Quantification of the extent of tube formation was measured as the tubule length of HRECs treated with each compound compared to DMSO control. SH-11037 is a positive control. Mean ± SEM, n = 6 wells. Representative results from duplicate experiments. *P < 0.05, ***P < 0.001 compared to DMSO control (one-way ANOVA with Dunnett's *posthoc* tests).

3′-hydroxyphenylbenzylidene group at C-3, produced good activity with selectivity for HRECs (Table 2, GI_{50} (HREC) of 0.11 μM, GI_{50} (92-1) of 3.6 μM), compared to that of compound **44** (Table 2, GI_{50} (HREC) of 2.2 μM, GI_{50} (92-1) of 8.2 μM). By way of comparison, cremastranone as reported was substantially less potent but still selective (GI_{50} (HREC) of 0.22 μM, GI_{50} (HUVEC) of 0.38 μM, GI_{50} (92-1) of 48 μM, GI_{50} (Y79) of 9.8 μM).[20]

In Vitro Antiangiogenic Efficacy of the Potent Homoisoflavonoids. After identifying some of the most potent antiproliferative compounds with some selectivity for HRECs—compounds **13**, **27**, **28**, **45**, **46**, **48**, and **50**—we further investigated their potential in inhibiting the ability of HRECs to form tubes. Matrigel based tube formation is the gold-standard in vitro assay that recapitulates most of the physiological angiogenesis events such as cell proliferation, migration, and cell−cell adhesion, as endothelial cells form

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three-dimensional tube-like structures.[43] Interestingly, all three of the natural-source compounds (**13**, **27**, and **28**), inhibited the ability of HRECs to form tubes in a concentration-dependent manner (Figure 3). Three potent synthetic compounds, **48**, **45**, and **50**, were also effective (Figure 4), while the most potent antiproliferative compound, **46**, maintained this low-dose efficacy in the tube formation assay (Figure 5).

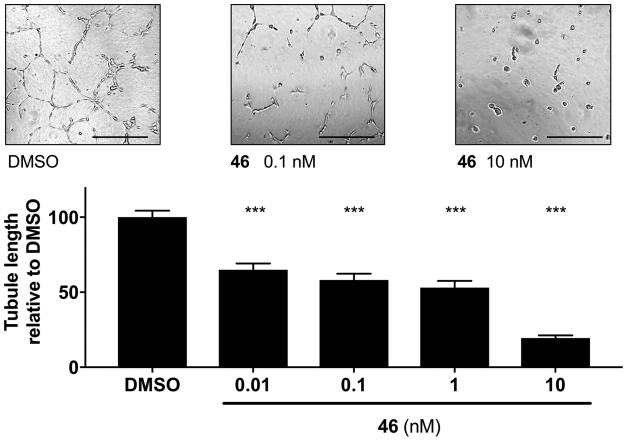

Figure 5. Highly potent synthetic homoisoflavonoid **46** significantly blocks the ability of HRECs to form tube-like structures. Top: Representative images of the tube formation of HRECs on Matrigel, scale bars = 500 μm. Bottom: Quantification of the extent of tube formation was measured as the tubule length of HRECs treated with compound compared to DMSO control. Mean ± SEM, n = 6 wells. Representative results from duplicate experiments. ***P < 0.001 compared to DMSO control (one-way ANOVA with Dunnett's *posthoc* tests).

CONCLUSIONS

A group of naturally occurring homoisoflavonoids isolated from the Hyacinthaceae as well as compounds synthesized have been tested for their antiangiogenic activities. The most potent compounds, **13**, **28**, **29**, **42**, **43**, **45**, **46**, **48** and the chalcone **50**, demonstrated selective antiproliferative activities on endothelial cells compared to nonendothelial cell types, with concentration-dependent antiangiogenic effects in vitro on HRECs, a disease-relevant cell type. Recently, intracellular target proteins for some other bioactive homoisoflavonoids have been identified: ferrochelatase as a target of cremastranone,[44] soluble epoxide hydrolase as a target of SH-11037 (a synthetic Boc-Phe-derivatized homoisoflavonoid),[45] and inosine monophosphate dehydrogenase 2 as a target of sappanone A.[46] The relative effects of the natural products described here on each of these enzymes will be a valuable topic for future exploration. Our results will open the doors to the development of further synthetic analogues with higher potency and better antiangiogenic activities to treat blinding eye diseases caused by pathological neovascularization.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded on a 500 MHz Bruker AVANCE NMR instrument in either $CDCl_3$ or CD_3OD at room temperature. All chemical shifts (δ) are in ppm and referenced to the relevant solvent references, 7.26 ppm ($CDCl_3$) and 4.87 ppm (CD_3OD) for ^{1}H NMR spectra and 77.23 ppm ($CDCl_3$) and 49.15 ppm (CD_3OD) for ^{13}C NMR spectra. ESI mass spectra were either recorded on a Bruker MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford), a Micromass Quattro Ultima mass spectrometer using a Waters Alliance HPLC to introduce samples (University of Surrey), or a Waters Xevo G2-S (National Mass Spectrometry Facility, Swansea). Optical rotations were recorded in $CDCl_3$ on a JASCO P-1020 polarimeter (University of Surrey), IR spectra were recorded on a PerkinElmer 2000 spectrometer (University of Surrey), and UV spectra were recorded using a Biochrom libraS60 in MeOH or acetonitrile in a 1 cm cell (University of Surrey). CD spectra were recorded using a Chirascan spectropolarimeter at room temperature in a 1 cm cell in MeOH (University of Surrey). Reagents were purchased from Sigma-Aldrich Company Ltd., Gillingham, U.K., and used without further purification.

Plant Material. The sourcing of plant material, extraction methodology, and isolation of compounds from the bulbs of *L. ovatifolia* (Bak.) Jessop, *L. socialis* (Bak.) Jessop, *Rhodocodon campanulatus* Knirsch, Mart-Azorín and Wetschnig and *Rhodocodon aff. intermedius* have been described before.[24,25] Bulbs of *Chionodoxa luciliae* Boiss. (Gigantea Hort), *Eucomis autumnalis* (Mill.) Chitt., and *E. bicolor* Baker were purchased from the Dix Export Company (Netherlands) in 2012 and grown to produce a flowering specimen to confirm identification. Voucher specimens (DAM 2012 CL, EA and EB) are retained at the University of Surrey.

Extraction and Isolation. Dried bulbs of *Chionodoxa luciliae* (3.0 kg) were extracted in EtOH at room temperature for 24 h on a shaker. The resulting extract (119 g) was separated using gravity column chromatography over silica gel (Merck 9385) and solvent mixtures of 80:20 CH_2Cl_2:EtOAc (75 cm^{-3}, 75 fractions) and 70:30 CH_2Cl_2:EtOAc (75 cm^{-3}, 10 fractions). Further purification was necessary in some cases using PTLC (Macherey-Nagel 0.25 mm, silica gel 60 with fluorescent indicator UV_{254}), further column chromatography or acetylation of complex mixtures to obtain pure compounds. Fraction 68 yielded **15** (11 mg), fraction 70 yielded, after further purification, **16** (2 mg) and **17** (10 mg), fraction 72 yielded **18** (17 mg) and after acetylation of a mixture containing **17**, the monoacetate, **19** (15 mg), and diacetate (12 mg), **20**. Fraction 74 yielded, after further purification, **21** (20 mg) and **22** (20 mg), fraction 77 yielded **23** (50 mg), and fraction 80 yielded **24** (15 mg). Compounds were identified using 2D NMR and CD methods and by comparison against literature data as referenced earlier.

Chopped bulbs of *E. autumnalis* (4 kg) were extracted with MeOH (3 L) for 48 h, yielding an extract which was partitioned with CH_2Cl_2 (300 mL) to yield extract 1 (5.1 g) after solvent evaporation. Bulb material was further extracted in 1-butanol (2.5 L) for 24 h, yielding extract 2 (26.9 g) after solvent evaporation. Extract 1 was separated using gravity column chromatography over silica gel (Merck 9385) and a gradient solvent elution method (40 cm^{-3}, 63 fractions) using solvent mixtures of 50:50 hexane:CH_2Cl_2 (40 cm^{-3}, 6 fractions), 20:80 hexane:CH_2Cl_2 (40 cm^{-3}, 9 fractions), 100% CH_2Cl_2 (40 cm^{-3}, 4 fractions), 50:50 CH_2Cl_2:EtOAc (40 cm^{-3}, 27 fractions), and 50:50 CH_2Cl_2:MeOH (20 cm^{-3}, 17 fractions). Fraction 24 yielded, after further purification, **30** (3.5 mg), and fractions 26 and 27 yielded, after further purification **31** (139.5 mg) and **32** (1.9 mg). Fractions 34−38 yielded, after further purification, **22** (101 mg), fractions 41−43 yielded, after further purification, **33** (15.7 mg) and **34** (7.1 mg). Fraction 45 yielded, after further purification, the previously unreported compound **35** (37.5 mg). Red-brown oil; $[\alpha]^{27}_D$ −55.6 (c 0.03, CH_3OH); ECD (c 0.03, MeOH) 290 nm (−5), 230 nm (−2); IR (NaCl) ν_{max} 3327, 3007, 2938, 2400, 2000 1668 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz), ^{13}C NMR ($CDCl_3$, 125 MHz) Table 3; HRESIMS m/z 347.1125 $[M + H]^+$ (calcd for $[C_{18}H_{18}O_7 + H^+]$, 347.1130).

Extract 2 was separated using gravity column chromatography over silica gel (Merck 9385) and a gradient solvent elution method (40 cm^{-3}, 54 fractions) using solvent mixtures of 100% CH_2Cl_2 (40 cm^{-3}, 20 fractions), 50:50 CH_2Cl_2:EtOAc (40 cm^{-3}, 20 fractions), and 50:50 CH_2Cl_2:MeOH (20 cm^{-3}, 14 fractions). Fractions 5−11 yielded after further purification **36** (1.5 mg). Fractions 31−33

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Table 3. NMR Data (^{1}H 500 MHz, ^{13}C 125 MHz) for Compound 35 (in $CDCl_3$, J in Hz)

position	^{1}H	^{13}C
2	(α) 4.37 (dd, 11.4, 4.1)	69.9, CH_2
	(β) 4.20 (dd, 11.4, 7.2)	
3	2.79 m	49.3, CH
4		192.0, C
4a		110.0, C
5		139.5, C
6		137.2, C
7		140.6, C
8		134.1, C
8a		143.3, C
9	(a) 3.17 (dd, 14.0 4.4)	32.2, CH_2
	(b) 2.70 (dd, 14.0, 10.6)	
1′		130.5 C
2′	7.11 (d, 8.1)	130.6 CH
3′	6.79 (d, 8.1)	115.8 CH
4′		154.6 C
5′	6.79 (d, 8.1)	115.8 CH
6′	7.11 (d, 8.1)	130.6 CH
5-OCH_3	3.88 s	62.3
6-OH	5.59 br s	
7-OCH_3	4.11 s	61.3
8-OH	5.39 br s	
4′-OH	4.74 s	

yielded, after further purification, **37** (1.2 mg), **38** (6.4 mg), and **39** (1.2 mg). Compounds were identified using 2D NMR and CD methods and by comparison against literature data as referenced earlier.

Bulbs of *E. bicolor* (5 kg) were chopped and extracted EtOH (3 L) for 48 h. The EtOH extract was partitioned with CH_2Cl_2 (300 mL), yielding extract 3 (12.7 g) after evaporation of the CH_2Cl_2 and the remaining EtOH extract, extract 4 (12.0 g). The bulbs were re-extracted in 1-butanol (2.5 L) for 24 h, yielding extract 5 (14.8 g) after solvent evaporation. Extract 3 (12.7 g) was coated onto silica, loaded into a column, and separated using flash column chromatography using a gradient solvent elution method (20 cm^{-3}, 282 fractions) using solvent mixtures of 100% hexane (20 cm^{-3}, 50 fractions), 50:50 hexane:CH_2Cl_2 (20 cm^{-3}, 50 fractions), 100% CH_2Cl_2 (20 cm^{-3}, 50 fractions), 50:50 CH_2Cl_2:EtOAc (20 cm^{-3}, 100 fractions), and 50:50 CH_2Cl_2:MeOH (20 cm^{-3}, 32 fractions). Fractions 160−170 yielded **40** (193.8 mg), and fractions 224−225 yielded **41** (321.9 mg). Extract 4 (12.0 g) was separated using gravity column chromatography over silica gel (Merck 9385) and a solvent mixture of 80:20 CH_2Cl_2:EtOAc (100 cm^{-3}, 9 fractions). Fraction 2 yielded **40** (60.5 mg), and fractions 4−6 yielded **41** (322.7 mg). Extract 5 (14.8 g) was separated using gravity column chromatography over Sephadex using a solvent mixture of 50:50 CH_2Cl_2:MeOH (150 cm^{-3}, 6 fractions). Fractions 5 and 6 yielded, after further purification, **41** (186.7 mg) and **32** (28.2 mg). Compounds were identified using 2D NMR and CD methods and by comparison against literature data as referenced earlier.

Synthesis of Compounds 42−50. *Preparation of (E)-5,7-Dimethoxy-3-(4′-methylthiobenzylidene)-4-chromanone (**42**).* Under inert conditions, NaH (60% in mineral oil, 13 mmol) was added to 3,5-dimethoxyphenol (1 g, 6 mmol) in dry DMF (10 mL) at 10 °C. After stirring for 60 min, 3-bromopropionic acid (1.1 g, 7.2 mmol) was added and the reaction was further stirred at room temperature for 60 min. The mixture was acidified with HCl to pH 3−5, extracted into EtOAc, and washed with brine (1 × 30 mL) and water (1 × 30 mL). The product (3-(3,5-dimethoxyphenoxy)-propanoic acid) was used without further purification.

Polyphosphoric acid (10 g) was added to 3-(3,5-dimethoxyphenoxy)-propanoic acid, and the resulting mixture was heated at 80 °C for 2 h. The mixture was poured into ice water and extracted into diethyl ether. The extract was washed with 3 N NaOH (30 mL) and water (50 mL) and dried over $MgSO_4$. The product (3,5-dimethoxychromone) was heated at 80 °C with 4-methylthiobenzaldehyde (1:1 mol equiv) and piperidine (4−5 drops), and the reaction was monitored by TLC (3:7, EtOAc:*n*-hexane). The mixture was diluted with water and extracted into EtOAc. This was washed with water (30 mL) and brine (30 mL), before drying over $MgSO_4$. The resulting product was purified by flash column chromatography (3:7, EtOAc:*n*-hexane) to give (*E*)-5,7-dimethoxy-3-(4′-methylthiobenzylidene)-4-chromanone (**42**) as a yellow gum (61 mg, 3.0% overall yield).

UV (MeOH) λ_{max}(log ε) 346 (−3.78); IR (ATR) ν_{max} 1659, 1583, 1495, 1463, 1343, 1198, 1093, 830 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS *m*/*z* 343.0989 $[M + H]^+$ (calcd for $[C_{19}H_{18}O_4SH + H]^+$, 343.0999).

*Preparation of (E)-7,8-Dimethoxy-3-(4′-methylthiobenzylidene)-4-chromanone (**43**).* Under inert conditions, NaH (60% in mineral oil, 13 mmol) was added to 2,3-dimethoxyphenol (1 g, 6 mmol) in dry DMF (10 mL) at 10 °C. After stirring for 60 min, 3-bromopropionic acid (1.1 g, 7.2 mmol) was added and the reaction was further stirred at room temperature for 60 min. The mixture was acidified with HCl to pH 3−5, extracted into EtOAc, and washed with brine (1 × 30 mL) and water (1 × 30 mL). The product (3-(2,3-dimethoxyphenoxy)-propanoic acid) was used without further purification.

Polyphosphoric acid (10 g) was added to 3-(2,3-dimethoxyphenoxy)-propanoic acid (from above), and the resulting mixture was heated at 80 °C for 2 h. The mixture was poured into ice water and extracted into diethyl ether. The extract was washed with 3 N NaOH (30 mL) and water (50 mL) and dried over magnesium sulfate. The product (2,3-dimethoxychromone) was heated at 80 °C with 4-methylthiobenzaldehyde (1:1 mol equiv) and piperidine (4−5 drops), and the reaction was monitored by TLC (3:7, EtOAc:*n*-hexane). The mixture was diluted with water, extracted into EtOAc, and washed with water (30 mL) and brine (30 mL), before drying over $MgSO_4$. The product was purified by flash column chromatography (3:7, EtOAc:*n*-hexane) to give (*E*)-7,8-dimethoxy-3-(4′-methylthiobenzylidene)-4-chromanone (**43**) as a yellow gum (72 mg, 3.5% overall yield).

UV (MeOH) λ_{max}(log ε) 343 (−4.02); IR (NaCl) ν_{max} 2921, 1665, 1580, 1572, 1493, 1425, 1306, 1264, 1159, 820 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS *m*/*z* 343.0995 $[M + H]^+$ (calcd for $[C_{19}H_{18}O_4SH + H]^+$, 343.0999).

*Preparation of (E)-5,6,7-Trimethoxy-3-(3′-hydroxy-4′-methoxybenzylidene)-4-chromanone (**44**).* Under inert conditions, NaH (60% in mineral oil, 13 mmol) was added to 3,4,5-trimethoxyphenol (1 g, 5.4 mmol) in dry DMF (10 mL) at 10 °C. After stirring for 60 min, 3-bromopropionic acid (1.1 g, 7.2 mmol) was added and the reaction was further stirred at room temperature for 60 min. The mixture was diluted with methanol (20 mL) acidified with HCl to pH 3−5, extracted into EtOAc, and washed with brine (1 × 30 mL) and water (1 × 30 mL). The product (3-(3,4,5-trimethoxyphenoxy)-propanoic acid) was used without further purification.

Polyphosphoric acid (10 g) was added to 3-(3,4,5-trimethoxyphenoxy)-propanoic acid (from above), and the resulting mixture was heated at 80 °C for 2 h. The mixture was poured into ice water and extracted into diethyl ether. The extract was washed with 3 M NaOH (30 mL) and water (50 mL) and dried over $MgSO_4$. The product (3,4,5-trimethoxychromone) was heated at 80 °C with 3-hydroxy-4-methoxybenzaldehyde (1:1 mol equiv) and piperidine (4−5 drops), and the reaction was monitored by TLC (3:7, EtOAc:*n*-hexane). The mixture was diluted with water, extracted into EtOAc, and washed with water (30 mL) and brine (30 mL), before drying over $MgSO_4$. The resultant product was purified by flash column chromatography (3:7, EtOAc:*n*-hexane) to give (*E*)-5,6,7-trimethoxy-3-(3′-hydroxy-4′-methoxybenzylidene)-4-chromanone (**44**) as a yellow gum (0.1053 g, 5.2% yield).

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Table 4. ^{1}H NMR (400 MHz) Data for Compounds 42−50 (in $CDCl_3$, J in Hz)

position	42	43	44	45	46	47	48	49	50
2	5.17, d (2.0)	5.34, d (1.8)	5.21, bs	4.04, dd (7.6, 11.4)	3.65, dd (3.1, 10.7)	4.09, dd (7.6, 11.4)	5.25, d (1.8)	4.99, d (1.8)	7.47, d (15.2)
				4.21, dd (4.1, 11.4)	4.02, dd (10.1, 10.7)	4.27, dd (4.1, 11.4)			
3				2.68, m	2.14, m	2.74, m			7.84, d (15.2)
4					2.53, dd (4.1, 7.4)				
5		7.75, d (8.8)							
OH-5							12.45, s		13.51
6	6.05, d (2.3)	6.63, d (8.8)							
8	6.00, d (2.3)		6.22, s	6.17, s	6.11, s	6.25, s	5.98, s	6.16, s	6.48, d (9.5)
									7.78, d (9.5)
9	7.69, bs	7.74, bs	7.71, s	2.54, dd (10.4, 14.5)	2.25, dd (7.6, 16.2)	2.58, dd (10.4, 14.5)	7.70, bs	7.75, bs	
				3.12, dd (4.0, 14.5)	2.70, ddd (1.5, 5.4, 16.2)	3.18, dd (4.0, 14.5)			
2′	7.12, d (7.8)	7.18, d (8.5)	6.84, dd (2.0, 8.4)	6.62, dd (2.0, 8.3)	6.58, dd (2.1, 8.4)	6.70, dd (2.0, 8.3)	6.79, dd (1.9, 7.9)	7.15, dd (2.4, 8.6)	7.25, dd (1.9, 8.4)
6′	7.12, d (7.8)	7.18, d (8.5)	6.79, d (2.0)	6.73, d (2.0)	6.72, d (2.1)	6.81, d (2.0)	6.81, d (1.9)	6.91, d (2.4)	7.16, d (1.9)
3′	7.20, d (7.8)	7.20, d (8.5)	6.86, d (8.4)	6.71, d (8.3)	6.71, d (8.4)	6.79, d (8.3)	6.85, d (7.9)	6.83, d (8.6)	6.91, d (8.4)
5′	7.20, d (7.8)	7.20, d (8.5)							
SCH_3-4′	2.45, s	2.46, s							
OCH_3	3.76, s	3.79, s	3.80	3.79, s	3.72, s	3.81, s	3.78, s	3.75, s	3.94, s
	3.84, s	3.87, s	3.85	3.86, s	3.72, s	3.88, s	3.82, s	3.82, s	3.96, s
			3.88	3.86, s	3.78, s	3.88, s	3.89, s	3.91, s	
			3.95	3.99, s	3.81, s	3.93, s			
CH_3									2.15, s
CH_2CH_3									4.13, q (7.1)
CH_2CH_3									1.47, t (7.1)

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Table 5. ^{13}C NMR (100 MHz) Data for Compounds 42−46 and 48−50 (in $CDCl_3$)

position	42	43	44	45	46	48	49	50
2	67.6, CH_2	68.2, CH_2	67.8, CH_2	69.1, CH_2	69.6, CH_2	67.4, CH_2	67.2, CH_2	118.4, CH
3	131.4, C	130.3, C	130.2, C	48.3, CH	33.2, CH	127.8, C	129.9, C	144.2, CH
4	179.4, C	181.2, C	179.8, C	191.4, C	37.5, CH_2	185.8, C	179.7, C	192.3, C
4a	162.8, C	158.4, C	154.8, C	154.4, C	107.2, C	155.9, C	159.7, C	163.2, C
5	164.5, C	124.0, CH	159.3, C	159.5, C	132.7, C	160.8, C	154.9, C	162.5, C
6	93.6, CH	106.1, CH	147.9, C	154.7, C	151.6, C	130.7, C	153.3, C	114.0, C
7	107.3,C	117.3, C	137.9, C	137.8, C	135.8, C	156.6, C	138.3, C	162.9, C
8	93.5, CH	136.6, C	96.3, CH	96.0, CH	96.2, CH	91.9, CH	96.5, CH	102.5, CH
8a	165.7, C	154.7, C	159.5, C	159.2, C	152.2, C	158.1, C	159.9, C	128.9, CH
9	136.3, CH	136.3, CH	136.5, CH	32.2, CH_2	25.5, CH_2	138.1, CH	129.8, CH	
1′	131.3, C	129.1, C	128.1, C	131.6, C	132.7, C	127.6, C	133.4, C	127.5, C
2′	131.3, CH	131.3, CH	123.3, CH	120.5, CH	120.4, CH	123.3, CH	130.4, CH	123.7, CH
3′	128.5, CH	128.5, CH	110.8, CH	110.8, CH	110.8, CH	110.7, CH	118.1, CH	111.3, CH
4′	140.5, C	140.5, C	147.9, C	145.6, C	152.3, C	145.9, C	125.3, C	148.6, C
5′	128.5, CH	128.5, CH	145.8, C	145.3, C	145.5, C	145.6, C	125.4, C	152.1, C
6′	131.3, CH	131.3, CH	116., CH	116.2, CH	115.4, CH	115.7, CH	129.2, CH	109.9, CH
OCH_3	57.0, CH_3	56.2, CH_3	56.1, CH_3	56.0, CH_3	55.8, CH_3	56.1, CH_3	56.1, CH_3	56.1, CH_3
OCH_3	56.4, CH_3	61.2, CH_3	56.2, CH_3	56.1, CH_3	56.0, CH_3	56.2, CH_3	61.2, CH_3	56.1, CH_3
OCH_3			61.4, CH_3	61.3, CH_3	60.6, CH_3	60.9, CH_3	61.6, CH_3	
OCH_3			61.7, CH_3	61.6, CH_3	61.0, CH_3			
SCH_3	15.3, CH_3	15.2, CH_3						
Ph-CH_3								7.7, CH_3
CH_2CH_3								64.0, CH_2
CH_2CH_3								13.6, CH_3

UV (MeOH) λ_{max}(log ε) 360 (−3.77), 348 (−3.76), IR ν_{max} (NaCl) 3650−3590, 3376, 2936, 2841, 1596 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS m/z 395.1096 $[M + Na]^+$ (calcd for $[C_{20}H_{20}O_7 + Na]^+$, 395.1107).

*Preparation of 5,6,7-Trimethoxy-3(R)-(3′hydroxy-4′-methoxybenzyl)-4-chromanone (**45**).* Compound **44** (34.6 mg, 0.093 mmol) was dissolved in deuterated toluene (1 mL). Iridium catalyst 1, (*S*)-(−)-2-[2-(diphenylphosphino)phenyl]-4-isopropyl-2-oxazolium-(1,5)-cyclooctadiene iridium(I) tetrakis(3,5-bis-(trifluoromethyl)phenyl borate (Scheme 2), was added (1%), and the reactor was pressurized to 5 bar with hydrogen. The reaction was stirred overnight at room temperature. The mixture was filtered to remove the catalyst, and the NMR data were obtained directly in deuterated toluene (quantitative yield).

Yellow gum; $[\alpha]^{23}_D$ −43.8 (c 0.9, CH_3OH); ECD (c 0.03, ACN) 326 nm (+1.3), 284 nm (−1.9); UV (MeOH) λ_{max} (log ε) 277 (−4.20), 277 (−3.66); IR (NaCl) ν_{max} 2936, 1674, 1580, 1567, 1485, 1454, 1412, 1200, 1179, 1116, 822 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS m/z 397.1259 $[M + Na]^+$ (calcd for $[C_{20}H_{22}O_7 + Na]^+$, 397.1263).

*Preparation of 5,6,7-Trimethoxy-3-(3′-hydroxy-4′-methoxybenzyl)-chromane (**46**).* Compound **44** (50.0 mg, 0.134 mmol) was dissolved in methanol (2 mL). Pd/C (10%) was added, the flask was saturated with H_2 (1 atm), and the mixture was stirred overnight at room temperature. The catalyst was filtered and the solvent removed under reduced pressure to yield **46** (quantitative yield).

Yellow gum; UV (MeOH) λ_{max} (log ε) 282 (−3.14); ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS m/z 383.1462 $[M + Na]^+$ (calcd for $[C_{20}H_{24}O_6 + Na]^+$, 383.1471).

*Preparation of 5,6,7-Trimethoxy-3-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone (**47**).* Compound **44** (5.9 mg, 0.0158 mmol) was dissolved in toluene (2 mL). Wilkinson's catalyst (1%) was added, the flask was saturated with H_2 (1 atm), and the mixture was stirred overnight at room temperature. The catalyst was filtered and the solvent removed under reduced pressure. The mixture obtained was subject to column chromatography over silica gel (8:2 EtOAc:CH_2Cl_2) to yield **47** (2.4 mg, 40% yield). Yellow gum; UV (MeOH) λ_{max} (log ε) 278 (−3.02); ^1H NMR ($CDCl_3$, 400 MHz) Table 4; HRESIMS m/z 397.1254 $[M + Na]^+$ (calcd for $[C_{20}H_{22}O_7 + Na]^+$, 397.1263).

*Preparation of (E)-5-Hydroxy-6,7-trimethoxy-3-(3′-hydroxy-4′-methoxybenzylidene)-4-chromanone (**48**).* Compound **44** (7.9 mg, 0.021 mmol) was dissolved in dry CH_2Cl_2 (2 mL) and cooled to 0 °C. TMSCl (8 equiv) and NaI (8 equiv) were added, and the mixture was heated to 60 °C for 2 h. The solvent was removed under reduced pressure and the product purified using column chromatography over silica gel (2:8, EtOAc:CH_2Cl_2) to give 5-hydroxy-6,7-trimethoxy-3-(3′hydroxy-4′-methoxybenzylidene)-4-chromanone (**48**) (4.5 mg, 60% yield).

Yellow gum; UV (MeOH) λ_{max} (log ε) 368 (−4.03); IR (NaCl) ν_{max} 3525, 2935, 2848, 1643, 1579, 1511, 1455, 1359, 1272, 1202, 1106, 813, 736 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS m/z 381.0946 $[M + Na]^+$ (calcd for $[C_{19}H_{18}O_7 + Na]^+$, 381.0950).

*Preparation of (E)-5,6,7-Trimethoxy-3-(4′-chloro-3′-hydroxybenzylidene)-4-chromanone (**49**).* NaH (60% in mineral oil, 13 mmol) was added to 3,4,5-trimethoxyphenol (1 g, 5.4 mmol) in dry DMF (10 mL) at 10 °C under inert conditions. After stirring for 60 min, 3-bromopropionic acid (1.1 g, 7.2 mmol) was added and the reaction was stirred at room temperature for a further 60 min. The mixture was acidified with HCl to pH 3−5, extracted into EtOAc, and washed with brine (1 × 30 mL) and water (1 × 30 mL). The product (3-(3,4,5-trimethoxyphenoxy)-propanoic acid) was used without further purification.

Polyphosphoric acid (10 g) was added to 3-(3,4,5-trimethoxyphenoxy)-propanoic acid (from above), and the resulting mixture was heated at 80 °C for 2 h. The mixture was poured into ice water and extracted with diethyl ether. The extract was washed with 3 M NaOH (30 mL) and water (50 mL) and dried over magnesium sulfate. The product (3,4,5-trimethoxychromone) was heated at 80 °C with 4-chloro-3-hydroxybenzaldehyde (1:1 mol equiv) and piperidine (4−5 drops), and the reaction was monitored by TLC (3:7, EtOAc:*n*-hexane) and was found to be complete after 24 h. The mixture was diluted with water and extracted into EtOAc, then washed with water (30 mL) and brine (30 mL), before drying over $MgSO_4$. The product was purified by flash column chromatography (3:7, EtOAc:*n*-hexane)

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to give 5,6,7-trimethoxy-3-(4′-chloro-3′- hydroxybenzylidene)-4-chromanone (**49**) as a yellow powder (155 mg, 7.8% yield).

UV (MeOH) λ_{max} (log ε) 295 (−3.94), 352 (−3.77); IR (NaCl) ν_{max} 3328, 2939, 1661, 1650, 1488, 1470, 1415, 1391, 1258, 1201, 1171, 1100, 818 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS *m/z* 377.0786 [M + H]$^+$ [calcd for ($C_{19}H_{17}O_6Cl$ + H)$^+$, 377.0792].

*Preparation of (E)-3-(3,4-Dimethoxylphenyl)-1-(4-ethoxy-2-hydroxy-3-methyl-phenyl)prop-2-en-1-one (**50**).* 4-Ethoxy-2-hydroxy-3-methyl acetophenone (0.1 g, 0.51 mmol) was added to 3,4-dimethoxybenzaldehyde (0.85 g, 0.51 mmol) and 10% NaOH (1 mL) in methanol (5 mL) and stirred at room temperature for 2 h. Upon standing, (*E*)-3-(3,4-dimethoxylphenyl)-1-(4-ethoxy-2-hydroxy-3-methyl-phenyl)prop-2-en-1-one (**50**) precipitated out as a yellow powder and was isolated by vacuum filtration (25.9 mg, 15% yield).

UV (MeOH) λ_{max} (log ε) 368 (−4.62); IR (ATR) ν_{max} 1650, 1570, 1550, 1499, 1419, 1364, 1265, 1221, 1080, 847 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) Table 4; ^{13}C NMR ($CDCl_3$, 100 MHz) Table 5; HRESIMS *m/z* 343.1545 [M + H]$^+$ [calcd for ($C_{20}H_{22}O_5$+ H)$^+$, 343.1545].

BIOLOGICAL METHODS

Materials. Human retinal microvascular endothelial cells (HRECs; Cell Systems, Seattle, WA) and human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD) were used between passage 5 and 8. Endothelial growth medium (EGM-2) was prepared by mixing the contents of an EGM-2 "Bullet Kit" (Cat no. CC-4147) with endothelial basal medium (EBM) (Cat no. CC-3156) (Lonza). 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% penicillin−streptomycin (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 μM β-mercaptoethanol + 10 μg/mL insulin + 1% pen−strep). ARPE-19 retinal pigment epithelial cells (ATCC, Manassas, VA) were grown in DMEM containing 10% FBS and 1% pen−strep. AlamarBlue reagent (product code BUF012B) was from AbD Serotec (Raleigh, NC). Matrigel matrix basement membrane (Cat no. 354234) was purchased from Corning (Corning, NY).

Cell Proliferation Assay. The antiproliferative activities of different compounds were monitored by an alamarBlue based fluorescence assay as described previously.[20] Five cell types were used: the target endothelial cell type (HRECs), a nontarget endothelial cell type (HUVECs), a nontarget normal retinal pigment epithelial cell line (ARPE-19), a nontarget uveal melanoma cell line (92-1), and a nontarget retinoblastoma cell line (Y79). Briefly, 2500 cells in 100 μL of growth medium were incubated in 96-well clear bottom black plates for 24 h at 37 °C, 5% CO_2; different concentrations of each test compound were then added (range: 0.5 nM to 500 μM) followed by 44 h of incubation. A 11.1 μL portion of alamarBlue reagent was then added, and after 4 h, fluorescence readings were taken on a Synergy H1 plate reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 560 and 590 nm, respectively. GraphPad Prism software (v. 7.0) was used for data analysis. Compounds that reduced the cell number by 50% or more at the highest concentration tested (relative to DMSO control) were reported as having a value of GI_{50} < 100 μM.

In Vitro Tube Formation Assay. A Matrigel based tube formation assay was performed to monitor the ability of HRECs to form tube-like structures in the presence of different concentrations of **13**, **27**, **28**, **45**, **46**, **48**, and **50**, as described previously.[20] Briefly, 15,000 cells in 100 μL of EGM-2 medium were incubated in the absence (DMSO treated) and presence of different concentrations of each compound in 96-well clear plates coated with 50 μL of Matrigel basement membrane. Known antiangiogenic homoisoflavonoid SH-11037[21] was a positive control. After 8 h, the images were recorded using an EVOS FL microscope (AMG, Mill Creek, WA) and the tube length was measured using Angiogenesis Analyzer macros in ImageJ (http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ).

Statistical Analysis. The data obtained from tube formation experiments were analyzed by one-way ANOVA with Dunnett's post hoc tests for comparisons between compounds' treatments and control. All analyses were performed using GraphPad Prism software (v. 7.0). A *p* value of <0.05 was considered statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00989.

Flowcharts showing how the compounds were isolated and NMR spectra of isolated and synthetic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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