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Antiangiogenic Activity and Cytotoxicity of Triterpenoids and Homoisoflavonoids from *Massonia pustulata* and *Massonia bifolia*

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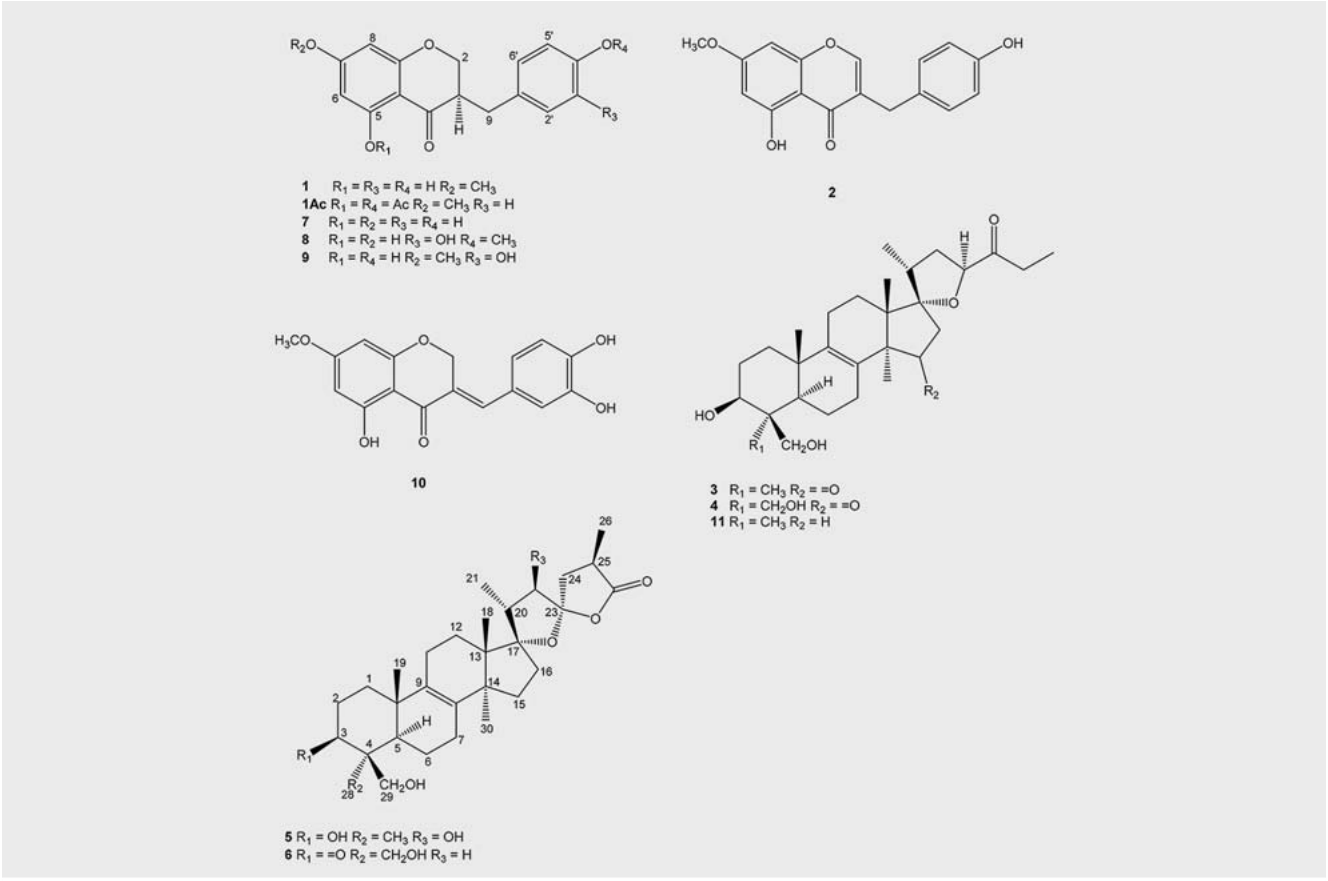
ABSTRACT

The Hyacinthaceae family (*sensu* APGII), with approximately 900 species in around 70 genera, plays a significant role in traditional medicine in Africa as well as across Europe and the Middle and Far East. The dichloromethane extract of the bulbs of *Massonia pustulata* (Hyacinthaceae *sensu* APGII) yielded two known homoisoflavonoids, (*R*)-5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** and 5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromone **2** and four spirocyclic nortriterpenoids, eucosterol **3**, 28-hydroxyeucosterol **4** and two previously unreported triterpenoid derivatives, (*17S, 23S*)-17α,23-epoxy-3β,22β,29-trihydroxylanost-8-en-27,23-olide **5**, and (*17S, 23S*)-17α,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide **6**. Compounds **1, 2, 3**, and **5** were assessed for cytotoxicity against CaCo-2 cells using a neutral red uptake assay. Compounds **1, 2**, and **5** reduced cell viability by 70 % at concentrations of 30, 100, and 100 µM, respectively. *Massonia bifolia* yielded three known homoisoflavonoids, (*R*)-(4′-hydroxy)-5-hydroxy-7-methoxy-4-chromanone **1**, (*R*)-(4′-hydroxy)-5,7-dihydroxy-4-chromanone **7** and (*R*)-(3′-hydroxy-4′-methoxy)-5,7-dihydroxy-4-chromanone **9**, two previously unreported homoisoflavonoids, (*E*)-3-benzylidene-(3′,4′-dihydroxy)-5-hydroxy-7-methoxy-4-chromanone **8** and (*R*)-(3′,4′-dihydroxy)-5-hydroxy-7-methoxy-4-chromanone **10,** and a spirocyclic nortriterpenoid, 15-deoxoeucosterol **11**. Compounds **1, 1Ac, 7, 8, 9,** and **10** were screened for antiangiogenic activity against human retinal microvascular endothelial cells. Some compounds showed dose-dependent antiproliferative activity and blocked endothelial tube formation, suggestive of antiangiogenic activity.

Introduction

The genus *Massonia* Houttuyn (Hyacinthaceae, subfamily Hyacinthoideae, tribe Massonieae) [1] is found in the dry areas of South Africa and southwestern Namibia [2]. The genus was named after a Scottish student gardener at Kew, Francis Masson, who collected seeds in the Cape in 1772 [3]. The genus is not known to be used by traditional healers in the region [4]. *Massonia pustulata*, first described in 1791 by Nikolaus von Jacquin, flowers in winter and a dry summer dormancy is required [3]. *Massonia bifolia* (syn. *Whiteheadia bifolia*) was originally described by von Jacquin in 1791 as *Eucomis bifolia*; however, the plant was "rediscovered" as *Melanthium massoniifolium* in 1804 and as *Whiteheadia latifolia* in 1865 [5]. It was soon after renamed as *W. bifolia* due to similarities between the collected specimen and that described by Jaquin [6]. It remained the only species in the *Whiteheadia* genus until 2004



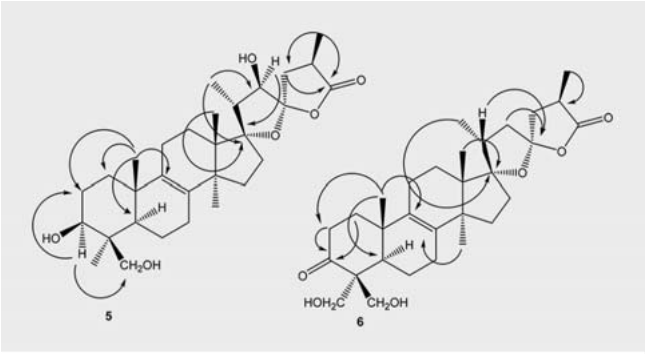
▶ **Fig. 1** Structures of compounds **1**–**11**.

when it was transferred to *Massonia* by Manning et al. due to the results of its DNA sequence analysis [6]. This analysis was prompted by the discovery of a second species, *Whiteheadia etesionamibensis*, which was tentatively placed in the *Whiteheadia* genus. However, DNA analysis indicated that this species fell between the genera *Whiteheadia* and *Massonia*, suggesting it was an evolutionary stepping stone from *Massonia* to *Whiteheadia*. As such, *Massonia* was given a broader scope to include *Whiteheadia* rather than to create a second, new single-species genus for *W. etesionamibensis* [5]. The phytochemistry of the genus *Massonia* has not been investigated previously.

The subfamily Hyacinthoideae (*sensu* APGII) is characterized by the presence of homoisoflavonoids and spirocyclic nortritepenoids, usually of the lanostane type [7]. Genera from this family are widely used by traditional healers [7], and homoisoflavonoids isolated from this subfamily have shown anti-inflammatory activity [8–10] and activity against colon (HT-29) and breast cancer (MDA-MB-435) [11, 12] cells. Spirocyclic nortriterpenoids have shown activity against HeLa cells [13] and HSC-2 oral squamous carcinoma cells [14–16]. Homoisoflavonoids **1** and **2** and spirocyclic nortriterpenoids **3, 4, 5**, and **6** were isolated from the dichloromethane extract of the bulbs of *M. pustulata* and were assessed for antiproliferative activity against CaCo-2 cells. Homoisoflavonoids **1, 7, 8, 9**, and **10** and the spirocyclic nortriterpenoid **11** were isolated from the ethanol extract of the bulbs of *M. bifolia*. Compound **1** was acetylated to aid in separation, forming compound **1Ac**. Since some homoisoflavonoids have been shown to have antiangiogenic activity [17–20], the ability of compounds **1, 1Ac, 7, 8, 9**, and **10** to block *in vitro* angiogenesis of human retinal microvascular endothelial cells (HRECs) was assessed. Structures are provided in ▶ **Fig. 1**.

Results and Discussion

(*R*)-5-Hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** and 5-hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromone **2** have been isolated previously from *Lachenalia rubida* Jacq. [21]. This species, like *M. pustulata,* is from the subfamily Hyacinthoideae and endemic to the dry areas of South Africa and Namibia. (*R*)-5-Hydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone **1** has also been isolated from *Ledebouria graminifolia* Bak. (Jessop) [22]. Eucosterol **3** was first isolated from *Eucomis autumnalis* [23] and later from *Eucomis bicolor* [24], (17*S*, 23*S*)-23,17-epoxy-3*β*,28,29-trihydroxy-27-norlanost-8-en-24-one **4** has been isolated previously from *Eucomis zambesiaca* [24], (*R*)-(4′-hydroxy)-5,7-dihydroxy-4-chromanone **7** has been extracted from *Scilla scilloides* [25], and *Leopoldia* (*Muscari*) *comosa* [26] and several *Ledebouria* species [10] and (*R*)-(3′-hydroxy-4′-methoxy)-5,7-dihydroxy-4-chromanone **9** has been extracted from *Scilla nervosa* [12]. 15-Deoxoeucosterol **11** has been isolated from several plant



▶ **Fig. 2** Key correlations seen in the HMBC spectra for compounds **5** and **6**.

species including *Scilla scilloides* [27] and Pseudoprospero firmifolium [28].

Compound **5** was isolated as an amorphous white powder. HRESIMS indicated a formula of $C_{30}H_{46}O_6$ with $[M^+ + 1]$ at *m/z* 503.33666 ($C_{30}H_{46}O_6$ + H requires 503.33726). The FTIR spectrum showed absorption peaks at 3391 cm^{-1} and 1758 cm^{-1} due to hydroxyl and carbonyl stretches respectively. NMR spectra indicated a lanosterol-type triterpenoid, with a spirocyclic γ-lactone side chain, typical of the subfamily Hyacinthoideae. The spectroscopic data obtained was very similar to that obtained for the co-isolated eucosterol **3**. As with eucosterol, an 8,9-double bond (δ_c 134.7 and 134.6, respectively), a 3β-hydroxyl group (δ_c 80.7 and δ_H 3.50 dd, J = 6.0 Hz and 12.2 Hz, $W_{1/2}$ = 20.3 Hz) [24] and an oxymethylene group at C-29 (δ_c 64.3 and δ_H 4.27, δ_H 3.36) were noted. Four tertiary methyl groups were present at δ_H 0.94 (3H-18), δ_H 0.96 (3H-19), δ_H 1.25 (3H-28), and δ_H 2.24 (3H-30), together with two secondary methyl group resonances at δ_H 1.31, (3H-21) and δ_H 1.39, (3H-26). The presence of the C-17 carbon resonance at δ_c 99.4 together with C-23 fully substituted carbon resonance at δ_c 115.5 indicated a spirocyclic ring system as shown in ▶ **Fig. 2**. [29]. An oxymethine proton resonance ascribed to H-22 was noted (δ_H 3.99), and the corresponding carbon resonance (δ_c 84.0) showed correlations in the HMBC spectrum with the H-21 (δ_H 1.31) resonance. Further correlations were seen between the H-22 (δ_H 3.99) and C-17 (δ_c 99.4) resonances, the 3H-26 (δ_H 1.39) and C-27 (δ_c 178.8) and C-24 (δ_c 42.0) resonances, and the H-24 (δ_H 2.45) and C-27 (δ_c 178.8) and C-23 (δ_c 115.5) resonances. A correlation was seen between the 3H-21 (δ_H 1.31) and H-22 (δ_H 3.99) resonances in the NOESY spectrum, indicating the hydroxyl group at C-22 was β. The compound was identified as the previously unreported (*17S,23S*)-17α,23-epoxy-3β,22β,29-trihydroxylanost-8-en-27,23-olide.

Compound **6** was isolated as an amorphous white powder. The FTIR spectrum showed absorption peaks at 3353 cm^{-1} and 3250 cm^{-1} indicative of the presence of hydroxyl groups as well as absorption peaks at 1772 cm^{-1} and 1723 cm^{-1} indicative of carbonyl groups. The molecular ion was not seen in the LCMS. The NMR data for compound **6** was similar to that obtained for compound **5,** but differed in showing the presence of a keto group at C-3 (δ_c 213.6), an oxymethylene group at C-28 (δ_c 63.4), and the absence of the hydroxyl group at C-22 (δ_c 32.3 instead of δ_c 84.0). Correlations were seen in the HMBC spectrum between the H-24α (δ_H 2.00) and H-24β (δ_H 2.72) and C-23 (δ_c 113.6) resonances, and between 3H-26 (δ_H 1.28) and C-25 (δ_c 35.8) resonances. Correlations were also noted in the HMBC spectrum between the 3H-30 (δ_H 1.05) and C-8 (δ_c 133.5) resonances and between the 3-H-19 (δ_H 0.98) and C-9 (δ_c 135.4) resonances. Various glycoside derivatives of compound **6** have been isolated from *Scilla peruviana* [13] but the aglycone (*17S,23S*)-17α,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide has not been reported previously. Key correlations seen in the HMBC spectra of compounds **5** and **6** are shown in ▶ **Fig. 2**.

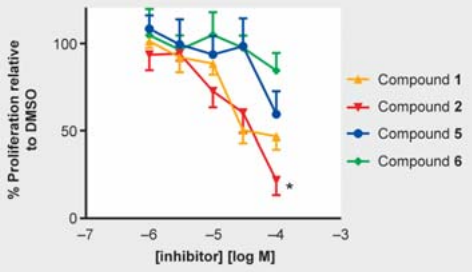
HRESIMS of compound **9** indicated a $[M + H]^+$ ion at *m/z* 317.1020 corresponding to a molecular formula of $C_{17}H_{16}O_5$ for the compound. The ^{1}H NMR spectrum indicated this compound was a 3-benzyl-4-chromanone-type homoisoflavonoid due to characteristic proton peaks and coupling patterns, including the two H-2 proton resonances at δ_H 4.30 (dd, J = 11.3, 4.4 Hz) and δ_H 4.14 (dd, J = 11.3, 7.4 Hz), the H-3 resonance at δ_H 2.84 (m) and the two H-9 resonances at δ_H 3.08 (dd, J = 14.4, 5.3 Hz) and δ_H 1.62 (dd, J = 14.4, 10.1 Hz). Also seen in the proton spectrum was a singlet peak at δ_H 3.83 integrating to 3H, indicating that a single methoxy group was present.

The C-4 carbonyl carbon resonance was present at δ_C 200.1 and a H-bonded OH group proton resonance was seen at δ_H 12.10, confirming the placement of a hydroxy group at C-5. Two meta-coupled proton resonances were present (δ_H 6.05, J = 2.3 Hz, H-6 and 6.02 J = 2.3 Hz, H-8). Both resonances showed a correlation with the single methoxy group proton resonance in the NOESY spectrum, so a methoxy group was placed at C-7.

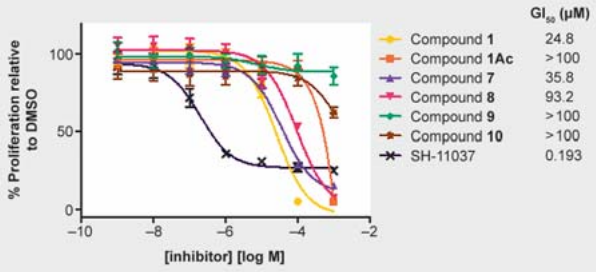
The B-ring was 1,3,4-trisubstituted as shown by the typical splitting patterns of H-2′ (δ_H 6.70, d, J = 2.1 Hz), H-5′ (δ_H 6.73, d, J = 8.0 Hz) and H-6′ (δ_H 6.57, dd, J = 8.0, 2.1 Hz) in the ^{1}H NMR spectrum. Hydroxy groups were placed at the remaining C- 3′ and C-4′ positions. CD analysis confirmed the configuration at C-3 as *R* due to the negative Cotton effect seen at 290 nm [30], and the compound was identified as the previously unreported 3*R*-(3′,4′-dihydroxybenzyl)-5-hydroxy-7-methoxy-4-chromanone.

Compound **10** was isolated as a brown oil. HRESIMS indicated a protonated molecular ion at *m/z* 315.0863, indicating a molecular formula of $C_{17}H_{14}O_6$ for the compound. This compound was identified as the *E*-3-benzylidene homoisoflavonoid analogue of compound **9**, *E*-3-(3′,4′-dihydroxybenzylidene)-5-hydroxy-7-methoxy-4-chromanone. The two equivalent H-2 resonances occurred at δ_H 5.39 (J = 1.8 Hz) and the H-9 resonance occurred at δ_H 7.72 (br s). The *E*-configuration of the double bond was confirmed by the characteristic H-9 proton shift. For the *E*-configuration H-9 occurs in the δ_H 7.58–7.79 region while for the *Z*-configuration, the H-9 resonance occurs at about δ_H 6.86 [31].

Compounds **1, 2, 3**, and **5** were assessed for activity against CaCo-2 cells using a neutral red uptake assay [32, 33] (▶ **Fig. 3**). Healthy cells take up neutral red into lysosomes whereas dead or damaged cells do not. The amount of neutral red recovered from cells at the end of the assay (as determined by absorbance) is related to the number of viable cells after exposure to a test compound. Cell viability was reduced below 70% by compound **1** at 30 µM and for compounds **2** and **5** at 100 µM. Compound **3** had



▶ **Fig. 3** Effect of test compounds on Caco-2 proliferation after 48 h as measured by neutral red uptake. Mean values are shown with SEM. n = 5 independent experiments. Effect of positive control (actinomycin D) was 23% ± 8% (not shown). * $p < 0.05$ compared to DMSO control.



▶ **Fig. 4** Effect of compounds on HREC cell proliferation, normalized to DMSO control. Mean values are shown with SEM, n = 3 wells, representative data from at least triplicate experiments. Synthetic homoisoflavonoid SH-11037 was positive control.

no effect on cell viability. Compound **2** at 100 μM ($p < 0.05$) was of similar activity (22% viability) to actinomycin D (10 μg/mL or 7.96 μM, purchased from Sigma-Aldrich, cat no: A1410, 98% purity), a known cytotoxic agent, but at 13× concentration (▶ **Fig. 3**). Overall, the reported activity was moderate for these compounds.

Compounds **1**, **1Ac**, and **7**–**10** were tested for their effect on proliferation of HRECs (▶ **Fig. 4**). GI_{50} values for the compounds, the concentration at which the proliferation of cells is reduced by 50% relative to the DMSO control, were calculated, and compounds that only reduced cell proliferation at the highest concentration tested (relative to DMSO control) were reported as having a $GI_{50} > 100$ μM. Compound **1** was reported to have the most potent GI_{50} value of 24.8 μM, and compounds **7** and **8** were found to have GI_{50} values of 35.8 μM and 93.2 μM, respectively. Compounds **1Ac**, **9**, and **10** had negligible effect on cell proliferation.

We then tested these compounds for anti-angiogenic activity in an *in vitro* Matrigel tube formation assay. This assay models the endothelial cells' ability to form vascular structures. HRECs overall tubule formation decreased significantly and in a dose-dependent manner when compared to DMSO (▶ **Fig. 5**). Compounds **1**, **7**, and **8** again proved most potent of the compounds to block vascular tube formation by HRECs.

The majority of (synthetic) antiangiogenic homoisoflavonoids described previously have trisubstituted A-rings [19]; thus, the finding that compound **1**, disubstituted on this ring, had reasonably potent activity is novel. Taken together with previous structure activity relationship (SAR) studies on synthetic homoisoflavonoids, these findings help identify homoisoflavonoids that could be pursued towards therapies for neovascular eye diseases such as wet age-related macular degeneration.

Photographs of plant material (**1S**), spectra for compounds **5**, **6**, **8**, and **10** (**2S**), and results of HREC cell proliferation studies (**3S**) are available as Supporting Information.

Materials and Methods

General experimental procedures

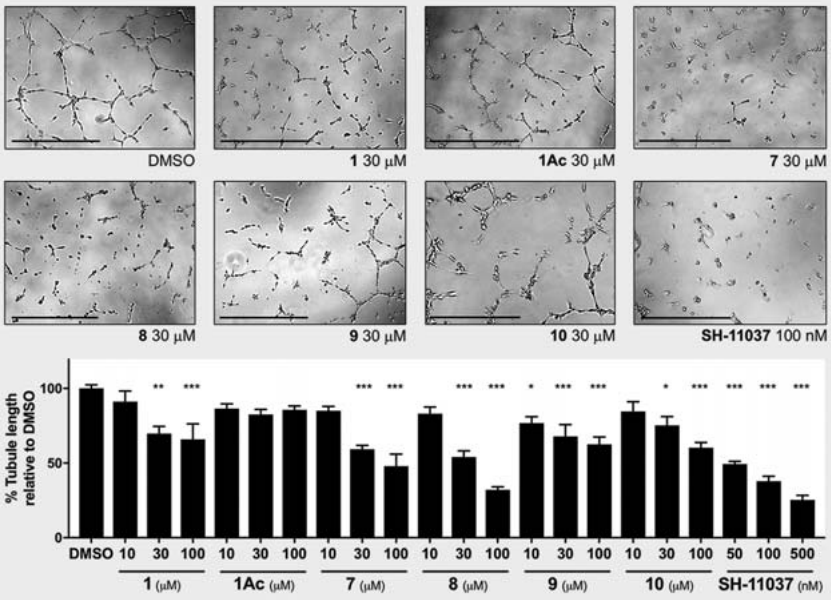
Optical rotations were measured at room temperature on a JASCO-P-1020 polarimeter and IR spectra were recorded using a Perkin-Elmer (2000 FTIR) spectrophotometer using KBr windows. Electronic circular dichroism (ECD) spectra were measured on an Applied Photophysics Chirascan CD spectrometer using a 1-mm cell and CH_3CN as the solvent. ^1H, ^{13}C, and 2D NMR spectra were recorded on a Bruker AVANCE III NMR spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C, using standard experiments from the Bruker pulse programs library. Chemical shifts are reported in ppm (δ) referencing the solvent signal ($CDCl_3$) as internal standard respect to TMS (0 ppm), and coupling constants (J) are measured in Hz. HRESIMS was performed on a Bruker MicroToF mass spectrometer, using an Agilent 1100 HPLC to introduce samples. Gravity column chromatography was performed using silica gel (Merck Art. 9385) packed 1- or 4-cm diameter columns. TLC was performed on aluminum precoated silica gel plates (Merck 9385) visualized using anisaldehyde spray reagent. Mass spectra of compounds **9** and **10** were recorded on a Thermo Q-Exactive Orbitrap mass spectrometer using direct HESI injection.

Plant material

M. pustulata and *M. bifolia* bulbs were obtained from the collection of the Royal Horticultural Society, Wisley Gardens, Wisley, Surrey, UK (W20150204A WSY and W20150850 WSY, respectively) on June 10, 2016, and identity was confirmed by comparison against voucher specimens at the Wisley Gardens Herbarium (**Section 1S**, Supporting Information).

Extraction and isolation of compounds from *M. pustulata* and *M. bifolia*

The bulbs of *M. pustulata* (672.1 g) were chopped and extracted by shaking successively for 24 h in dichloromethane (CH_2Cl_2) and then MeOH to yield 8.35 g of CH_2Cl_2 extract and 29.67 g of MeOH extract. The dichloromethane extract was fractionated over silica gel starting with EtOAc : CH_2Cl_2 (1 : 9) and increasing the concen-



▶ **Fig. 5** Tube formation ability of HRECs treated with compounds at three concentrations (10 µM, 30 µM, and 100 µM) was observed in Matrigel over 8 h and images were taken using brightfield microscopy. Tubule length was determined using ImageJ. Synthetic homoisoflavonoid SH-11037 was positive control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to DMSO, ANOVA with Dunnett's *post hoc* tests. Mean values are shown with SEM, n = 6, representative data from at least triplicate experiments. Images above are tubes formed by HRECs in the presence of 30 µM of each compound above, or 100 nM SH-11037 positive control, or DMSO control. Scale bars = 500 µm.

tration of EtOAc. One hundred ten fractions of 20 mL each were collected. Further fractionation over Sephadex LH20 (1 : 1, CH_2Cl_2 : MeOH) yielded compounds **1**–**6** (20 mg, 8.9 mg, 50 mg, 15 mg, 4.6 mg, and 28 mg, respectively).

Bulbs of *M. bifolia* (263.0 g) were chopped and extracted by shaking for 2 × 24 h in EtOH, yielding a combined 5.13 g of extract. The extract was fractionated over silica gel, starting with CH_2Cl_2 as solvent and gradually increasing the polarity by addition of use of EtOAc. Ninety-five fractions of 20 mL each were collected. Further fractionation over Sephadex LH20 (1 : 1, CH_2Cl_2 : MeOH) yielded compounds **1** and **7**–**11** (19 mg, 9.8 mg, 2.3 mg, 3.2 mg, 1.4 mg, and 6.9 mg respectively). Structures of compounds **1**–**11** are shown in ▶ **Fig. 1**.

(*17S,23S*)-17*α*,23-epoxy-3*β*,22*β*,29-trihydroxylanost-8-en-27,23-olide (**5**): Amorphous white solid (4.6 mg, 75% pure; from NMR); $[\alpha]_D^{23.6}$ + 44.4, ($CHCl_3$, *c* = 2.7 mg/mL); IR $\nu_{max} \cdot cm^{-1}$: 3391, 2934, 1758, 1455, 1375, 1034, 736; ^{1}H- and ^{13}C NMR (500 MHz, $CDCl_3$) see ▶ **Table 1** and **Section 2S** (Supporting Information). HRESIMS: *m/z* 503.33667 for $[M^+ + H]^+$ (calcd. for $C_{30}H_{47}O_6$, 503.33726).

(*17S,23S*)-17*α*,23-epoxy-28,29-dihydroxylanost-8-en-3-on-27,23-olide (**6**): Amorphous white solid (28 mg, 70% pure from NMR); $[\alpha]_D^{23.6}$ + 3.08 ($CHCl_3$, C = 20.3 mg/mL); IR $\nu_{max} \cdot cm^{-1}$: 3353, 3250, 2937, 1772, 1723, 1456, 1371, 1046, 736; ^{1}H- and ^{13}C NMR (500 MHz, $CDCl_3$) see ▶ **Table 1** and **Section 2S** (Supporting Information). HRESIMS: M^+ not detected.

3*R*-(3′,4′-dihydroxybenzyl)-5-hydroxy-7-methoxy-4-chromanone (**9**): cream/brown oil (3.2 mg); $[\alpha]_D^{20.2}$ = + 67.5 (MeOH, C = 1.6 mg/mL); CD (CH_3CN) 256 nm (Δε + 0.25), 286 nm (Δε − 9.19), 313 nm (Δε + 1.05); IR $\nu_{max} \cdot cm^{-1}$: 3349, 2949, 2925, 2848, 1639, 1449, 1014; ^{1}H- and ^{13}C NMR (500 MHz, MeOD) see ▶ **Table 2** and **Section 2S** (Supporting Information). HESIMS *m/z* 317.1020 $[M + H]^+$, (calcd. for $C_{17}H_{17}O_6$, 317.1025).

E-3-(3′,4′-dihydroxybenzylidene)-5-hydroxy-7-methoxy-4-chromanone (**10**): brown oil (1.4 mg); IR $\nu_{max} \cdot cm^{-1}$: insufficient quantity of compound; ^{1}H- and ^{13}C NMR (500 MHz, MeOD). See ▶ **Table 2** and **Section 2S** (Supporting Information). HESIMS *m/z* 315.0863 $[M + H]^+$, (calcd. for $C_{17}H_{15}O_6$, 315.0868).

Pharmacological assays

Neutral red assay

CaCo-2 human colon carcinoma cells (CACO-2 [ECACC 86010202]) from Public Health England, UK, were cultured in Eagle's Minimum Essential Medium (ATCC/LGC Standards) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1% nonessential amino acids, and 100 U/mL penicillin/100 U/mL streptomycin and maintained in 5% CO_2/95% air in an incubator at 37 °C. Cells were plated in 96-well plates at 2×10^4 cells/well for 48 h. Cells were then treated for 48 h with one of the following, in 4 replicates: culture medium alone (untreated control); 0.1% DMSO (vehicle control; maximum DMSO exposed to cells); actinomycin D 10 µg/mL (positive control); test substances at 1, 3, 10, 30, and 100 µM (diluted from a 100 mM stock in DMSO). Cells were washed with PBS, and neutral red (25 µg/mL in culture medium, diluted from a stock of 2.5 mg/mL in ultrapure water) was added for 3 h. Cells were washed with PBS, and neutral red ex-

▶ **Table 1** ^{1}H and ^{13}C NMR data for compounds **5** and **6** ($CDCl_3$).

	Compound 5		Compound 6	
No.	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	35.3	1.21 m, 1.80 m	34.8	1.25 m, 1.79 m
2	28.3	1.30 m	35.8	1.75, 1.25
3	80.7	3.50 dd (6.0, 12.2)	213.6	
4	43.5		48.3	
5	51.2	1.20 m	45.3	1.80 m
6	18.6	1.50 m, 1.80 m	19.5	1.5 m, 1.8 m
7	26.4	1.90 m	26.3	2.05 m
8	134.7		133.5	
9	134.6		135.4	
10	37.4		36.9	
11	20.6	1.99 m, 2.13 m	20.5	1.94, 2.05
12	24.9	2.23 m, 1.55 m	24.7	1.5, 2.0 m
13	49.6		46.7	
14	50.6		50.6	
15	32.0	α 1.35 m β 1.75 m	31.3	α 1.38 m β 1.68 m
16	37.7	α 1.85 dd (5.6, 13.4) β 2.80 dd (9.1, 13.4)	36.9	α 1.80 m β 2.50 m
17	99.4		98.3	
18	19.6	0.94 s	18.6	0.90 s
19	19.8	0.96 s	19.5	0.98 s
20	50.3	2.30 m	43.7	2.25 m
21	15.5	1.31 d (6.8)	18.6	1.05 d (6.8)
22	84.0	3.99 bs ($W_{1/2}$ = 5.4)	32.3	α 1.80 m β 2.55 m
23	115.5		113.6	
24	42.0	α 2.45 dd (4.5, 7.8), β 2.10 dd (7.8, 8.0)	45.2	α 2.00 m, β 2.72 m
25	35.5	2.70 m	35.8	2.97 m
26	16.5	1.39 d (7.4)	15.1	1.28 d (7.0)
27	178.8		179.6	
28	22.2	1.25 s	63.4	α 4.10 d (12.5) β 3.80 d (12.5)
29	64.3	α 3.36 d (11.2) β 4.27 d (11.2)	70.7	α 3.70 d (14.0) β 4.40 d (14.0)
30	26.0	2.24 s	24.7	1.05 s

▶ **Table 2** ^{1}H and ^{13}C NMR data for compounds **9** and **10** (CD_3OD).

	Compound 9		Compound 10	
No.	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
2	70.4	α 4.14 (dd, 11.3, 7.4) β 4.30 (dd, 11.3, 4.4)	68.9	5.39 (d, 1.79)
3	48.3	2.84 m	127.4	
4	200.1		187.2	
4a	103.8		105.0	
5	165.2		169.6	
6	95.9	6.05 (d, 2.3)	95.7	6.08 (d, 2.3)
7	169.6		169.5	
8	94.7	6.02 (d, 2.3)	94.7	6.02 (d, 2.3)
8a	164.8		163.9	
9	33.3	α 3.08 (dd, 14.4, 5.3) β 2.62 (dd, 14.4, 10.1)	139.8	7.72 s
1′	131.1		127.0	
2′	117.3	6.70 (d, 2.1)	118.5	6.86 (d, 2.1)
3′	146.7		150.3	
4′	145.2		163.8	
5′	116.6	6.73 (d, 8.0)	116.9	6.87 (d, 8.1)
6′	121.6	6.57 (dd, 8.0, 2.1)	125.1	6.81 (dd, 8.2, 2.1)
5-OH (in $CDCl_3$)		12.10 s		
7-OCH_3	56.4	3.83 s	56.1	3.84 s

tracted in 50% ethanol/water with 1% acetic acid. Absorbance was read at 540 nm. Data are reported from five independent experiments (different cell passages). The mean absorbance of wells without cells (blank) was subtracted from all other readings. Plates were accepted for analysis given adequate neutral red loading in vehicle treated cells (> 0.18 absorbance units) and positive control activity (< 60% viability); Z-factor values were in the range 0.68–0.88. Replicates were averaged to give one treatment value per plate. All plate treatment values were divided by the mean value of vehicle-treated cells on the same plate, so data was normalized as percentage of maximum cell viability. The plate treatment values were averaged to produce group means of n = 5. Analysis of multiple means was performed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test against the untreated cells (negative control) value, using GraphPad Prism 6.

HREC proliferation assay

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 (Lonza). The EGM-2 Bullet Kit contains hydrocortisone, human fibroblast growth factor, VEGF, R3-insulin like growth factor (R3-IGF-1), ascorbic acid, human epidermal growth factor, gentamycin, and heparin along with 2% FBS. HRECs and proprietary Attachment Factor were purchased from Cell Systems. HRECs were used for cell proliferation studies between passages 5 and 7.

The base of a tissue culture flask (10 cm^2) was washed with 1 mL of attachment factors and aspirated. Cryopreserved cells were removed from liquid nitrogen storage and defrosted just prior to use. Cells were transferred into EGM-2 media (5 mL), which was then centrifuged (2 min, 270 ×*g*). The liquid above the cell pellet was aspirated off, and the cell pellet was resuspended in 10 mL EGM-2 and transferred into the flask. The tube was then in-

cubated overnight (37 °C, 5 % CO_2) until cells reached confluence. Medium was aspirated and the cells were washed with PBS (3 mL). PBS was aspirated and 500 µL TrypLE (Life Technologies) added and coated over the surface of the cells for a maximum of 30 s. Trypsin was then aspirated and EGM-2 medium (2 mL) was added to the tube to wash cells from the surface. The resulting cell slurry was transferred to a Falcon tube (15 mL) and diluted with sufficient EGM-2 to seed approximately 2500 cells per well.

The required 2500 cells/well in 100 µL medium were incubated in the center 48 wells of a 96-well clear bottom black plates for 24 h, with the surrounding 48 wells containing 100 µL deionized, sterilized water. Standard 1 : 10 dilutions of compounds in DMSO ranged from 100 mM to 100 nM such that seven concentrations of each compound were tested and the eighth and final well for testing containing DMSO as a control. Cells were treated with 1 µL of different concentrations of each test compound and control, resulting in a final in-well concentration range of 1 nM to 1 mM with a final DMSO concentration of 1 % in all wells. All compounds were tested in triplicate, and our previously reported synthetic, antiangiogenic homoisoflavonoid SH-11037 [19] was included as a positive control. Treated cells were incubated for a further 48-h period.

At the end of this incubation period, AlamarBlue reagent (11.1 µL) 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 analyzed and dose response curves generated using GraphPad Prism (**Section 3S**, Supporting Information).

In vitro angiogenesis assay

A Matrigel-based tube formation assay was performed as previously described [34]. Briefly, 50 µL 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 µL EGM-2 and dosed with appropriate concentrations of compound with 1 µL 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 analyzed with AngiogenesisAnalyzer plugin for ImageJ [35], and HREC total tubule length for treated cells was normalized to DMSO. Statistical analysis was completed using GraphPad Prism.

Supporting Information

Photographs of plant material (**1S**), spectra for compounds **5, 6, 8**, and **10** (**2S**), and results of HREC cell proliferation studies (**3S**) are available as Supporting Information.

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Conflict of Interest

The authors declare there is no conflict of interest.

References

[1] Martinez-Azorin M, Pinter M, Crespo MB, Slade J, Deutsch G, Wetschnig W. Clarification of *Massonia echinata* and some other frequently misunderstood *Massonia* species (Asparagaceae, Scilloideae), with the description of *M. pseudoechinata* and *M. roggeveldensis*. Phytotaxa 2015; 239: 101–129

[2] Martínez-Azorín M, Pinter M, Crespo M, Pfosser M, Wetschnig W. *Massonia mimetica* (Hyacinthaceae, Hyacinthoideae), a new remarkable species from South Africa. STAPFIA 2013; 99: 187–197

[3] Jacquin NJ. Plantarum rariorum horti caesarei Schoenbrunnensis descriptiones et icones. Schönbrunn: Austria; 1804

[4] DeBeer J, Van Wyk BE. An ethnobotanical survey of the Agter-Hantam region, Northern Cape Province of South Africa. S Afr J Bot 2011; 77: 741–754

[5] Manning JP, Goldblatt P, Saunders R. 721. *Massonia bifolia*. Curtis's Botanical Magazine 2011; 28: 324–332

[6] 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 Pseudoprospereae. Edinb J Bot 2003; 60: 533–568

[7] Mulholland DA, Schwikkard SL, Crouch NR. The chemistry and biological activity of the Hyacinthaceae. Nat Prod Reports 2013; 30: 1165–1210

[8] Gaidamashvili A, van Staden J. Prostaglandin inhibitory activity by lectin-like proteins from South African medicinal plants. S Afr J Bot 2006; 72: 661–663

[9] du Toit K, Kweyama A, Bodenstein J. Anti-inflammatory and antimicrobial profiles of *Scilla nervosa* (Burch.) Jessop (Hyacinthaceae). S A J Sci 2011; 107: 96–100

[10] Waller CP, Thumser AE, Langat MK, Crouch NR, Mulholland DA. COX-2 inhibitory activity of homoisoflavanones and xanthones from the bulbs of the Southern African *Ledebouria socialis* and *Ledebouria ovatifolia* (Hyacinthaceae: Hyacinthoideae). Phytochemistry 2013; 95: 284–290

[11] Abegaz BM. Novel phenylanthraquinones, isofuranonaphthoquinones, homoisoflavonoids, and biflavonoids from African plants in the genera *Bulbine, Scilla, Ledebouria*, and *Rhus*. Phytochem Rev 2002; 1: 299–310

[12] Silayo A, Ngadjui BT, Abegaz BM. Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa* subsp *rigidifolia*. Phytochemistry 1999; 52: 947–955

[13] Mimaki Y, Nishino H, Ori K, Kuroda M, Matsui T, Sashida Y. Lanosterol oligosaccharides from plants of the subfamily Scilloideae and their anti-tumor-promoter activity. Chem Pharm Bull 1994; 42: 327–333

[14] Ori K, Koroda M, Mimaki Y, Sakagami H, Sashida Y. Lanosterol and tetranorlanosterol glycosides from the bulbs of *Muscari paradoxum*. Phytochemistry 2003; 64: 1351–1359

[15] Ori K, Kuroda M, Mimaki Y, Sakagami H, Sashida Y. Norlanostane and lanostane glycosides from the bulbs of *Chionodoxa luciliae* and their cytotoxic activity. Chem Pharm Bull 2003; 51: 92–95

[16] Kuroda M, Mimaki Y, Ori K, Koshino H, Nukada T, Sakagami H, Sashida Y. Lucilianosides A and B, two novel tetranor-lanostane hexaglycosides from the bulbs of *Chionodoxa luciliae*. Tetrahedron 2002; 58: 6735–6740

[17] Shim JS, Kim JH, Lee J, Kim SN, Kwon HJ. Anti-angiogenic activity of a homoisoflavanone from *Cremastra appendiculata*. Planta Med 2004; 70: 171–173

[18] Basavarajappa HD, Lee B, Fei X, Lim D, Callaghan B, Mund JA, Case J, Rajashekhar G, Seo SY, Corson TW. Synthesis and mechanistic studies of a novel homoisoflavanone inhibitor of endothelial cell growth. PLoS One 2014; 9: e95694

[19] Basavarajappa HD, Lee B, Lee H, Sulaiman RS, An H, Magana C, Shadmand M, Vayl A, Rajashekhar G, Kim EY, Suh YG, Lee K, Seo SY, Corson TW. Synthesis and biological evaluation of novel homoisoflavonoids for retinal neovascularization. J Med Chem 2015; 58: 5015–5027

[20] Sulaiman RS, Basavarajappa HD, Corson TW. Natural product inhibitors of ocular angiogenesis. Exp Eye Res 2014; 129: 161–171

[21] Langlois A, Mulholland DA, Duncan GD, Crouch NR, Edwards TJ. A novel 3-benzylchromone from the South African *Lachenalia rubida* (Hyacinthaceae). Biochem Syst Ecol 2005; 33: 961–966

[22] Mutanyatta J, Matapa BG, Shushu DD, Abegaza BM. Homoisoflavonoids and xanthones from the tubers of wild and *in vitro* regenerated *Ledebouria graminifolia* and cytotoxic activities of some of the homoisoflavonoids. Phytochemistry 2003; 62: 797–804

[23] Ziegler R, Tamm C. Isolation and structureof eucosterol and 16*β*-hydroxyeucosterol, two novel spirocyclic nortriterpenes, and of a new 24-nor-5*α*-chola-8, 16-dien-oic acid, from bulbs of several *Eucomis* species. Helv Chim Acta 1976; 59: 1997–2011

[24] Sihra JK, Thumser AE, Langat MK, Crouch NR, Mulholland DA. Constituents of bulbs of three species of the Hyacinthaceae (Hyacinthoideae): *Eucomis vandermerwei, E. zambesiaca* and *Resnova humifusa*. Nat Prod Comm 2015; 10: 1207–1209

[25] Ren F, Wang L, Wang F, Li B. Chemical constituents from *Scilla scilloides*. Chinese Trad Herbal Drugs 2014; 14: 1984–1988

[26] Adinolfi M, Barone G, Belardini M, Lanzetta R, Laonigro G, Parrilli M. Three 3-benzyl-4-chromanones from *Muscari comosum*. Phytochemistry 1984; 23: 2091–2093

[27] Nishida Y, Eto M, Miyashita H, Ikeda T, Yamaguchi K, Yoshimitsu H, Nohara T, Ono M. A new homostilbene and two new homoisoflavonoids from the bulbs of *Scilla scilloides*. Chem Pharm Bull 2008; 56: 1022–1025

[28] Koorbanally C, Sewjee S, Mulholland D, Crouch N, Dold A. Homoisoflavanones from *Pseudoprospero firmifolium* of the monotypic tribe Pseudoprospereae (Hyacinthaceae: Hyacinthoideae). Phytochemistry 2007; 68: 2753–2756

[29] Ren FC, Wang LX, Yu Q, Jiang XJ, Wang F. Lanostane-type triterpenoids from *Scilla scilloides* and structure revision of drimiopsin D. Nat Prod Bioprospect 2015; 5: 263–270

[30] 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 2006; 72: 517–520

[31] Koorbanally C, Crouch N, Langlois A, DuToit K, Mulholland D, Drewes S. Homoisoflavanones and spirocyclic nortriterpenoids from three *Eucomis* species: *E. comosa, E. schijffii* and *E. pallidiflora* subsp. *pole-evansii* (Hyacinthaceae). S Afr J Bot 2006; 72: 428–433

[32] Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 2008; 3: 1125–1131

[33] Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 1999; 4: 67–73

[34] Basavarajappa HD, Sulaiman RS, Qi X, Shetty T, Sheik Pran Babu S, Sishtla KL, Lee B, Quigley J, Alkhairy S, Briggs CM, Gupta K, Tang B, Shadmand M, Grant MB, Boulton ME, Seo SY, Corson TW. Ferrochelatase is a therapeutic target for ocular neovascularization. EMBO Mol Med 2017; 9: 786–801

[35] Carpentier G. Angiogenesis Analyzer for ImageJ. Available at http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt. Accessed November 30, 2017