**COX-2 Inhibitory Activity of Homoisoflavanones and Xanthenes from the Bulbs
of the Southern African *Ledebouria socialis* and *Ledebouria ovatifolia*
(Hyacinthaceae: Hyacinthoideae)**

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Abstract: The bulbs of *Ledebouria socialis* (Hyacinthaceae) yielded the novel benzocyclobutene homoisoflavanoid, (*R*)-2',5-dihydroxy-3',4',7-trimethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one, socialinone (**1**). *Ledebouria ovatifolia* yielded (2*ε*,3*R*)-2,5-dihydroxy-7-methoxyspiro[2*H*-1-benzopyran-3(4*H*), 5'(6'*H*)-cyclobuta[*f*][1,3]benzodioxol]-4-one (**2**) and the novel homoisoflavanone, (*E*)-3-(3',4'-dihydroxybenzylidene)-5,7-dihydroxychroman-4-one, ovatifolionone (**5**), the novel dihydrochalcone, 4,4'-dihydroxy-2',6'-dimethoxydihydrochalcone (**3**), and xanthone, 1,6-dihydroxy-2,3,5-trimethoxy-8-methyl-9*H*-xanthen-9-one (**4**) along with twenty-one known compounds. Structures were determined using spectroscopic techniques. The anti-inflammatory activities of the homoisoflavanoids and xanthenes isolated were evaluated against cyclooxygenase-1 and -2 isoenzymes. (*R*)-3-(3',4'-Dihydroxybenzyl)-7-hydroxy-5-methoxychroman-4-one (**7**), (*E*)-3-(3',4'-dihydroxybenzylidene)-7-hydroxy-5-methoxychroman-4-one (**10**), 1,3,6-trihydroxy-2-methoxy-8-methylxanthen-9-one (**6**) and ovatifolionone acetate (**5Ac**) exhibited significant activity against cyclooxygenase-2 at <10 μM.

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1. Introduction:

Ledebouria Roth of the Hyacinthaceae is a genus consisting of approx. 60 species, mostly sub-Saharan, with one or two species each in Madagascar and India, and with its centre of diversity in northeastern South Africa (Venter, 2008). *Ledebouria* was previously considered congeneric with a broadly conceived *Scilla* L. but subsequently reinstated by Jessop (1970). In previous pharmacological investigations, homoisoflavanones, commonly occurring constituents in the Hyacinthoideae (Speta 1998; Pohl *et al.*, 2000), have been found to have anti-inflammatory activity (Della Loggia *et al.*, 1989; Abegaz *et al.*, 2007).

The phytochemistry of several members of *Ledebouria* have been investigated previously (Pohl *et al.*, 2000, 2001; Mutanyatta *et al.*, 2003), including both species reported in the current account. The widespread *Ledebouria ovatifolia* (Bak.) Jessop exhibits high polymorphism, with several entities currently synonymised under this name. This prompted a re-investigation of this species. The bulbs of *L. ovatifolia* are used in African traditional medicine to treat backache, influenza and gastro-enteritis (Hutchings *et al.*, 1996); it is further used as a soap and for ethnoveterinary purposes (Gerstner, 1938). Contrasting reports on the toxicity of this species to mammalian test subjects exist (Steyn, 1936; Van der Walt and Steyn, 1941). The second subject of this investigation, *Ledebouria socialis* (Bak.) Jessop is localised in the Eastern Cape Province of South Africa, and exists as both green and silver-leaved forms. From the ethyl acetate extract of *Ledebouria socialis* (as *Scilla socialis* Bak.), Kato *et al.*, (2007) yielded eleven hyacinthacines, two pyrrolidines and three piperidines. The methanol extract of *Ledebouria ovatifolia* earlier yielded two compounds 4,4'-dihydroxy-2',6'-dimethoxychalcone and 5,7-dihydroxy-3-(4'-hydroxybenzyl)-4-chromanone (Pohl *et al.*, 2001).

Cyclooxygenase is responsible for the synthesis of prostaglandins in acute inflammatory conditions (O'Banion *et al.*, 1992). Non-selective inhibitors of cyclooxygenase decrease the inflammatory response but have also been linked to gastro-intestinal tract bleeding at high doses (Smith *et al.*, 2000). Selective COX-2 inhibitors decreased this effect but were found to have cardiac implications (Cipollone *et al.*, 2008). It has also been demonstrated in several studies that cyclooxygenase inhibitors can have cancer preventative effects (Giardiello *et al.*, 1995; Levy, 1997) especially when specific against cyclooxygenase-2.

As the Hyacinthoideae has been shown to contain anti-inflammatory homoisoflavanones using the mouse oedema test (Della Loggia *et al.*, 1989), the homoisoflavanones and xanthenes isolated from the two plants were screened for cyclooxygenase activity. Reported herein is the isolation of five novel compounds along with twenty-five known compounds from the bulbs of both *L. socialis* and *L. ovatifolia*. The anti-inflammatory activities of selected isolates were evaluated.

2. Results and discussion:

The dichloromethane extract of the bulbs of *L. socialis* was separated by normal phase column chromatography over silica gel to afford one novel homoisoflavanone, socialinone (**1**) as well as (*R*)-5-hydroxy-7,2',3',4'-tetramethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]-triene}-4-one, isolated previously from *Drimiopsis barteri* (Ngamga *et al.*, 2008), and phytol and stigmasterol. The methanol extract was separated using normal phase column chromatography and yielded the known compound polybotrin (Zou *et al.*, 2007), which has not been reported previously from the Hyacinthaceae. The dichloromethane, ethyl acetate and methanol extracts of bulbs of *L. ovatifolia* were separated by normal phase column chromatography over silica gel to afford twenty-five compounds. The dichloromethane extract yielded compound **2**, reported previously (Kawasaki *et al.*, 1973) as a mixture of diastereomers, which we separated after acetylation and were able to assign the respective absolute configurations using 2D NMR and CD spectroscopy, a novel dihydro-chalcone (**3**), and a novel xanthone (**4**). Also isolated from this extract were the known compounds (*R*)-5,7-dihydroxy-3-(4'-hydroxybenzyl)chroman-4-one (Adinolfi *et al.*, 1987) and (*E*)-4,4'-dihydroxy-2',6'-

dimethoxychalcone (Sun *et al.*, 1989) which had both been isolated previously from *Ledebouria ovatifolia* (Pohl *et al.*, 2001), scillascillin (Kawasaki *et al.*, 1973), 1,6-dihydroxy-3-methoxy-8-methyl-9*H*-xanthen-9-one (McMaster *et al.*, 1960; Mutanyatta *et al.*, 2003), (*R*)-4',5,7-trihydroxy-3'-methoxyspiro{2*H*-1-benzopyran-3(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (Adinolfi *et al.*, 1985), (*R*)-3',5,7-trihydroxy-4'-methoxyspiro{2*H*-1-benzopyran-3(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (Adinolfi *et al.*, 1987), (*R*)-5,7-dihydroxy-3-(4'-methoxybenzyl)chroman-4-one (Heller *et al.*, 1976), eucomol (Bohler and Tamm, 1967), and (*R*)-7-hydroxy-3-(4'-hydroxybenzyl)-5-methoxychroman-4-one (Finckh and Tamm, 1970) which had been isolated previously from the Hyacinthaceae, and coniferaldehyde (Seikel *et al.*, 1971) and chondrillasterol (Bergmann and Feeney, 1948) which had not been isolated previously from this family. The ethyl acetate extract yielded one novel homoisoflavanone, ovatifolionone (**5**), and the known compounds, drimiopsin C (Mulholland *et al.*, 2004), norlichexanthone (Aghoramurthy and Seshadri, 1953; Mutanyatta *et al.*, 2003), 22-acetoxy-15-deoxoeucomol (Amschler *et al.*, 1997; Pohl *et al.*, 2001), (*R*)-(3-(3',4'-dihydroxybenzyl)-5,7-dihydroxychroman-4-one (Adinolfi *et al.*, 1987, 4'-demethyl-5-*O*-methyl-dihydroeucomin (Finckh and Tamm, 1970), (*R*)-3-(3',4'-dihydroxybenzyl)-7-hydroxy-5-methoxychroman-4-one (Adinolfi *et al.*, 1987), and (*E*)-3-(3',4'-dihydroxybenzylidene)-7-hydroxy-5-methoxychroman-4-one (Masterova *et al.*, 1991) which have all been isolated from the Hyacinthaceae previously and *p*-anisic aldehyde (Gottlieb *et al.*, 1970) and 4-hydroxyphenylethanol (Gharbo *et al.*, 1970) which have not been reported from this family. The methanol extract also yielded polybotrin (Zou *et al.*, 2007). Interestingly, this compound was isolated from both species in this study. The known compounds were identified by analysis of their spectroscopic and spectrometric data and comparison with literature data.

Compound **1**, (*R*)-2',5-dihydroxy-3',4',7-trimethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one, was obtained as a yellow oil. The IR absorption bands at 3455 and 1737 cm⁻¹ indicated the presence of hydroxyl and carbonyl functional groups, respectively. This compound was identified as the 2'-hydroxylated derivative of the known 5-hydroxy-7,2',3',4'-tetramethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (Ngamga *et al.*, 2008), also isolated in this work. The ¹H NMR spectrum showed a sharp resonance at δ

12.05 ascribable to the hydrogen bonded hydroxyl group proton at C-5, two *meta*-coupled proton resonances (δ 6.09, d, $J = 2.3$ Hz, δ 6.05, d, $J = 2.3$ Hz) ascribable to H-6 and H-8, resonances ascribed to the two H-2 (2H, δ 4.55) and two H-9 (δ 3.62, d, $J = 13.1$ Hz, δ 3.02, d, $J = 13.1$ Hz) resonances of a scillascillin-type of homoisoflavonoid. The H-5' resonance (δ 6.29, s) showed correlations in the HMBC spectrum with C-3, C-3' and C-1' resonances. A hydroxyl group was placed at C-2' due to correlations between the hydroxyl group proton resonance (δ 5.84, s) and the C-1' and C-3' resonances in the HMBC spectrum. Methoxy groups (δ 3.84, δ 3.80, δ 3.86) were placed, in agreement with the NOESY spectrum, at the remaining C-7, C-4' and C-3' positions. The ECD spectrum of Compound **1** showed a positive Cotton effect at 297 nm and a negative Cotton effect at 340 nm confirming the (*R*)-configuration at C-3 (Adinolfi *et al.*, 1990).

Compound **2** is the C-2 hydroxylated, C-3'/C-4'-methylenedioxy derivative of Compound **1**. The hemiacetal ring C opens and closes in solution, making it impossible to obtain a pure compound. Acetylation yielded compounds **2(R)** and **2(S)** which could be separated, and the absolute configuration at C-2 of the diastereomers was confirmed using NMR and CD experiments. Compound **2** has previously been reported from *Barnardia japonica* (as *Scilla scilloides*) (Kawasaki *et al.*, 1973).

Compound **2(R)**, the 2,5-diacetoxy derivative of **2**, was isolated as a clear oil. The ^1H NMR spectrum showed the H-2 resonance (δ 6.48, s) and the typical H-9 proton resonances (δ 3.33, d, $J=13.5$ Hz, δ 3.20, d, $J=13.5$ Hz). Resonances at δ 6.67 and δ 6.61 appeared as singlets indicating their *para*-relationship and were assigned as H-2' and H-5' respectively. Resonances due to protons of a C-3'/C-4' methylenedioxy group were seen at δ 5.91 and δ 5.89 (both d, $J=1.3$ Hz). The NMR spectra of compound **2(S)**, differed slightly in the chemical shifts for H-2, 2H-9, H-2' and H-5' as shown in Table 1. The absolute configuration at C-2 of **2(R)** and **2(S)** was established using ECD experiments. Compound **2(R)** showed a negative Cotton effect at 300 nm and Compound **2(S)** a positive Cotton effect at 307 nm. The experimental ECD spectra of the two compounds were compared to their calculated spectra. Conformational analysis of compounds **2(R)** and **2(S)** were performed on Spartan08 using MMFF (Devlin *et al.*, 2009) and the global minimum conformers subjected to TDDFT calculations employing the functional B3LYP and a basis set of

6-31G (d, f), built into Gaussian09 software (Burgueno-Tapia *et al.*, 2006; Felipe *et al.*, 2012). The calculated ECD spectra of the global minimum conformers were compared to the experimental spectra [Figure 2]. The TDDFT simulated and experimental ECD spectra of compound **2(R)** were compared and both showed negative Cotton effects at 230-400 nm confirming that compound **2(R)** had the 2*R*, 3*R* configuration. The TDDFT simulated ECD spectrum obtained for Compound **2(S)** was UV corrected and compared to the experimental ECD spectrum supporting a (2*S*, 3*R*) configuration for Compound **2(S)** [Figure 2].

Compound **3**, 4,4'-dihydroxy-2',6'-dimethoxydihydrochalcone, was isolated as a white powder from the dichloromethane extract of *L. ovatifolia*. HRESIMS indicated a molecular formula of C₁₇H₁₈O₅ and IR absorption bands at 3377 and 1736 cm⁻¹ indicated hydroxyl and carbonyl groups. Two coupled methylene proton resonances (2H, δ 2.92, t, $J=7.7$ Hz; 2H, δ 3.01, t, $J=7.7$ Hz) indicated a dihydrochalcone structure. *Para*-substitution on ring C was confirmed by the presence of a pair of doublets, each integrating to 2H (δ 7.07, d, $J= 8.5$ Hz; δ 6.73, d, $J= 8.5$ Hz). Two equivalent methoxy groups (6H, δ 3.71, s) were placed at C-2' and C-6' in agreement with correlations seen in the NOESY spectrum with the H-3'/5' resonance (2H, δ 6.02, s), and 2H_a. The remaining two hydroxyl groups were placed at C-4 and C-4'.

Compound **4**, 1,6-dihydroxy-2,3,5-trimethoxy-8-methyl-9*H*-xanthen-9-one, was isolated as a yellow powder from the CH₂Cl₂ extract of *L. ovatifolia*. HRESIMS indicated a molecular formula of C₁₇H₁₆O₇ and IR absorption bands 3417 and 1643 cm⁻¹ indicated the presence of hydroxyl and carbonyl groups. The ¹H NMR spectrum showed hydrogen bonding between the C-9 carbonyl group and a C-1 hydroxyl group proton (δ 13.26, s, H-1-OH). The C-8 methyl group proton resonance (δ 2.80, s) (Mulholland *et al.*, 2004), showed correlations in the COSY and NOESY spectra with the H-7 resonance (δ 6.75, s). The NOESY spectrum showed correlations between the H-7/C-6-OH (δ 6.24, s), H-6-OH/C-5-OMe (δ 4.05, s), C-5-OMe/H-4 (δ 6.46, s) and H-4/C-3-OMe (δ 3.97, s) proton resonances. The remaining methoxy group (δ 3.91, s) was placed at C-2. This was confirmed by an HMBC correlation between the C-2 (δ 132.2) and the H-4 resonances.

Compound **5**, ovatifolionone, a 3-benzylidene-4-chromanone homoisoflavanone, was isolated as a yellow powder from the EtOAc extract of *L. ovatifolia*. Compound **5**

was acetylated to aid purification and formed a mono-acetate (**5Ac**). The molecular ion was not observed in the HRESIMS, but a peak at 299.0553, corresponding to a $[M-OAc]^+$ ion was observed. The IR absorption bands at 3415, 1745 and 1638 cm^{-1} indicated hydroxyl, and acetate and ketone carbonyl stretches respectively. ^1H NMR and ^{13}C NMR spectra indicated a C-3(9)-double bond, with the H-9 resonance occurring as a singlet at δ 7.58. The H-2 resonance (2H, δ 5.30, d, $J=1.4$ Hz) showed a correlation in the COSY spectrum with the H-9 resonance and the C-3 resonance was observed as a quaternary carbon at δ 128.9. As no hydrogen-bonded 5-OH proton resonance was present, the acetate group was placed at C-5. This placement was confirmed by the chemical shift of the C-4 carbonyl carbon resonance which occurred at δ 180.4 compared to the usual value of δ 186 for 5-OH benzylidene homoisoflavanones (Bangani *et al.*, 1999). Resonances due to the *meta*-coupled H-6 and H-8 protons occurred at δ 6.19 and δ 6.26 respectively (both d, $J=2.3$ Hz). The ring C protons exhibited an ABX pattern (δ 6.80, d, $J=1.8$ Hz, H-2'; δ 6.85, d, $J=8.2$ Hz; δ 6.76, dd, $J=8.2, 1.8$ Hz), and the remaining hydroxyl groups were placed at C-3' and C-4'. The configuration of the double bond was established as *E* from the distinctive chemical shift of the H-9 resonance (δ 7.58) (Masterova *et al.*, 1991).

Isolates **1-5Ac**, as well as ten known compounds re-isolated in this work (**6-15**) were screened for cyclo-oxygenase-2 (COX-2) inhibitory activity. Two COX isoenzyme-specific inhibitors were used as positive controls, namely SC-560 and DuP-697 (Cayman Chemical Company, 2011), and these compounds were shown to be selective for COX-1 and COX-2 isoenzymes in the assay system that was utilised (Figure 2 and Table 3). Compounds **5Ac**, **6**, **7** and **10**, showed significant COX-2 inhibitory activity when screened at 10 μM . The compounds which showed inhibitory activity for COX-2 were screened against cyclooxygenase-1 (COX-1) to measure their selectivity for the COX-2 enzyme. Compounds **6** and **7** were found to be non-specific inhibitors of both the COX-1 and COX-2 enzyme during these studies. Compound **10**, (*E*)-3-(3',4'-dihydroxybenzylidene)-7-hydroxy-5-methoxychroman-4-one, however, was completely selective for COX-2 at a concentration of 10 μM , and had an IC_{50} for COX-2 of $2.87 \mu\text{M} \pm 1.2$. Compound **5Ac** was partially selective, inhibiting COX-1 with an IC_{50} of $2.56 \mu\text{M} \pm 1.2$ and COX-2 with an IC_{50} of $1.12 \mu\text{M} \pm 0.56 \mu\text{M}$. The COX-2 specific inhibitor DuP-697 had an IC_{50} value of 40 nM, which is substantially lower than those shown above. However, the isolates show IC_{50}

values which are within the range of widely used and non-selective inhibitors such as paracetamol (acetaminophen) (IC_{50} 26 μ M for COX-2) (Hinz *et al.*, 2008).. It can therefore be concluded that the isolates above could be therapeutically valuable and that compound **10** could be developed into a clinically relevant and COX-2 specific inhibitor.

3. Experimental

3.1 General Experimental Procedures

1D and 2D NMR spectra were recorded in $CDCl_3$ and CD_3OD on a 500 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced to the solvent references at 7.26 and 77.23 ppm for $CDCl_3$, 4.87 and 49.15 ppm for CD_3OD for 1H NMR and ^{13}C NMR respectively. ESI mass spectra were recorded on a Bruker MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford), optical rotations were determined in $CHCl_3$ on a JASCO P-1020 polarimeter (University of Surrey), IR spectra were recorded using a Perkin-Elmer 2000 spectrometer, and UV and CD spectra were obtained on a Chirascan spectropolarimeter at room temperature using a 1 cm cell in MeOH.

3.2 Plant Material

Ledebouria ovatifolia (Bak.) Jessop bulbs (1.0 kg) were purchased from the medicinal plant market in Johannesburg, Gauteng in November 2007 and a flowering voucher retained (N. Crouch 1278, NH). Bulbs of cultivated *Ledebouria socialis* (Bak.) Jessop (silver-leaved form)(1.0 kg) were harvested during January 2007 and a voucher retained (N. Crouch 1118, NH). Plant materials were identified by Professor N. Crouch of the South African National Biodiversity Institute (SANBI).

3.3 Extraction and Isolation of Compounds

The air-dried bulbs of *L. socialis* were chopped and extracted using dichloromethane and methanol successively on a shaker for 48 hours. Evaporation of the solvents *in vacuo* yielded dichloromethane (1.47 g) and methanol (7.50 g) extracts. Column chromatography of both extracts was performed over silica gel (Merck 9385) using a 5 cm diameter gravity column, collecting fractions (75 ml each) using a hexane/dichloromethane stepwise gradient followed by a dichloromethane/ methanol

stepwise gradient. Preparative TLC on 0.2 mm silica gel, aluminium backed plates (Merck Art. 5554) was subsequently used. Compounds were detected using a UV-lamp or, where necessary, a section of the plate was developed using anisaldehyde spray reagent, with subsequent heating. Final purification was undertaken using a 1 cm diameter column. The dichloromethane extract yielded compound **1** (Fraction 189-198, 53.9 mg, followed by preparative TLC in 10% EtOAc/DCM, eluted twice) as well as known compounds (*R*)-5-hydroxy-7,2',3',4'-tetramethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]-triene}-4-one (34.7 mg), phytol and stigmasterol. The methanol extract yielded only the known compound polybotrin (1.8 mg).

The bulbs of *L. ovatifolia* were extracted using dichloromethane, ethyl acetate and methanol successively on a shaker for 48 hours. After evaporation of the solvents *in vacuo* the following extracts were obtained: dichloromethane (19.65 g), ethyl acetate (11.56 g) and methanol (54.95 g). Column chromatography over silica gel (Merck 9385) using a 5 cm diameter gravity column, collecting fractions (75 ml each) using a hexane/dichloromethane stepwise gradient followed by a dichloromethane/ methanol stepwise gradient, was used to separate the constituents. Final purification was undertaken using a 1 cm diameter column. The dichloromethane extract yielded compound **2** (Fractions 140-145, 20 mg, 98% CH₂Cl₂/ 2% diethyl ether, compound **3** (Fraction 237-248, 2.1 mg, 20% diethyl ether/ 80% CH₂Cl₂), and compound **4** (Fractions 140-145, 6.7mg, 90% CH₂Cl₂/ 10% diethyl ether) as well as known compounds (*R*)-5,7-dihydroxy-3-(4'-hydroxybenzyl)chroman-4-one (10.2 mg), (*E*)-4,4'-dihydroxy-2',6'-dimethoxychalcone (6.3 mg), scillascillin (1.3 mg), 1,6-dihydroxy-3-methoxy-8-methyl-9*H*-xanthen-9-one (2.2 mg), (*R*)-4',5,7-trihydroxy-3'-methoxyspiro{2*H*-1-benzopyran-3(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (2.6 mg), (*R*)-3',5,7-trihydroxy-4'-methoxyspiro{2*H*-1-benzopyran-3(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (4.5 mg), (*R*)-5,7-dihydroxy-3-(4'-methoxybenzyl)chroman-4-one (7.3 mg), eucomol (4.4 mg), (*R*)-7-hydroxy-3-(4'-hydroxybenzyl)-5-methoxychroman-4-one (6.9 mg), coniferaldehyde (0.5 mg) and chondrillasterol (18.7 mg). The ethyl acetate extract yielded compound **5** (Fraction 61-75, 3 mg, 100% CH₂Cl₂) and known compounds drimiopsin C (12.8 mg), norlichexanthone (2 mg), 22-acetoxy-15-deoxoeucosterol (6 mg), (*R*)-(3-(3',4'-dihydroxybenzyl)-5,7-dihydroxychroman-4-one (7.8 mg), 4'-demethyl-5-*O*-methyl-

dihydroeucumin (13.3 mg), (*R*)-3-(3',4'-dihydroxybenzyl)-7-hydroxy-5-methoxychroman-4-one (370 mg) (*E*)-3-(3',4'-dihydroxybenzylidene)-7-hydroxy-5-methoxychroman-4-one (7.5 mg), *p*-anisic aldehyde (5.2 mg) and 4-hydroxyphenylethanol (1.1 mg). The methanol extract yielded only the known compound polybotrin (7.0 mg).

3.3.1 (*R*)-2',5-dihydroxy-3',4',7-trimethoxyspiro{2*H*-1-benzopyran-3-(4*H*)-9-bicyclo[4.2.0]octa[1,3,5]triene}-4-one (**1**)

Yellow oil; $[\alpha]_D^{23.6} +40$ (*c* 0.005, CHCl₃); CD data (*c* 0.001, MeOH) 297 nm (+1.51), 340 nm (-1.75); UV (CDCl₃) λ_{max} 240, 290 nm; IR ν_{max} (NaCl) cm⁻¹ 3455, 2959, 2929, 2860, 1737, 1462, 1380, 1242, 1174, 1078, 665; ¹H (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) spectroscopic data, see Table 1; HRESIMS *m/z* 381.0948 [M+Na]⁺ (calc. for C₁₉H₁₈O₇Na 381.0945).

3.3.2 (2*R*,3*R*)-2,5-diacetoxy-7-methoxyspiro[2*H*-1-benzopyran-3(4*H*), 5'(6'*H*)-cyclobuta[*f*][1,3]benzodioxol]-4-one (**2(R)**)

Clear oil; $[\alpha]_D^{23.6} -172$ (*c* 0.006, CHCl₃); CD data (*c* 0.001, MeOH) 275 nm (-2.22), 300 nm (-6.80); UV (CDCl₃) λ_{max} 290 nm; IR ν_{max} (NaCl) cm⁻¹ 2975, 2927, 2898, 1758, 1649, 1624, 1571, 1455, 1381, 1317, 1271, 1233, 1152, 1088, 1048, 880, 805, 739, 666; ¹H (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) spectroscopic data, see Table 1; HRESIMS *m/z* 449.0843 [M+Na]⁺ (calc. for C₂₂H₁₈O₉Na 449.0843).

3.3.3 (2*S*,3*R*)-2,5-diacetoxy-7-methoxyspiro[2*H*-1-benzopyran-3(4*H*), 5'(6'*H*)-cyclobuta[*f*][1,3]benzodioxol]-4-one (**2(S)**)

Clear oil; $[\alpha]_D^{23.6} +173$ (*c* 0.010, CHCl₃); CD data (*c* 0.001, MeOH) 275 nm (-1.68), 307 nm (+8.92); UV (CDCl₃) λ_{max} 300, 270 nm; IR ν_{max} (NaCl) cm⁻¹ 2922, 2850, 1758, 1686, 1619, 1570, 1457, 1369, 1302, 1269, 1233, 1194, 1152, 1106, 1036, 1007, 971, 937, 886, 846, 811, 754, 666; ¹H (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) spectroscopic data, see Table 1; HRESIMS *m/z* 449.0842 [M+Na]⁺ (calc. for C₂₂H₁₈O₉Na 449.0843).

3.3.3.1 Acetylation of 2-hydroxy-7-*O*-methylscillascillin (**2**)

Compound **2** (20 mg) was dissolved in pyridine (3 ml), acetic anhydride (3 ml) was added and the mixture was left to stand for 12h. MeOH (2 x 5 ml) was added and

solvent was removed under vacuum. The residue was subsequently purified using preparative TLC (40% hexane: 58% CH₂Cl₂: 2% diethyl ether) to yield **2(R)** (6 mg) and **2(S)** (10 mg).

3.3.4 4',4-Dihydroxy-2',6'-dimethoxydihydrochalcone (**3**)

White amorphous powder; UV (CDCl₃) $\lambda_{\max}$ 280 nm; IR $\nu_{\max}$ (NaCl) cm⁻¹ 3377, 2953, 2924, 2854, 2356, 2336, 1736, 1676, 1598, 1513, 1463, 1370, 1247, 1220, 1166, 1126, 1032, 996, 827, 756; ¹H NMR (500 MHz, CDCl₃) δ 7.07 (2H, d, J = 8.5 Hz, 2/6), δ 6.73 (2H, d, J = 8.5 Hz, 3/5), δ 6.02 (2H, s, 3'/5'), δ 3.71 (3H, s, 2'/6'-OCH₃), δ 3.01 (2H, t, 7.7 Hz, α), δ 2.92 (2H, t, 7.7 Hz, β) and ¹³C NMR (125 MHz, CDCl₃), δ 203.1 (C=O), δ 158.7 (C, 2'/6'), δ 158.7 (C, 4'), δ 153.6 (C, 4), δ 133.7 (C, 1), δ 129.9 (2-CH, 2/6), δ 112.8 (C, 1'), δ 115.5 (2-CH, 3/5), δ 92.3 (2-CH, 3'/5'), δ 56.2 (CH₃, 2'/6'-OCH₃), δ 46.9 (CH₂, α), δ 29.4 (CH₂, β); HRESIMS m/z 325.1045 [M+Na]⁺ (calc. for C₁₇H₁₈O₅Na 325.1046).

3.3.5 1,6-dihydroxy-2,3,5-trimethoxy-8-methyl-9H-xanthen-9-one (**4**)

Yellow amorphous powder; UV (CDCl₃) $\lambda_{\max}$ 310, 250 nm; IR $\nu_{\max}$ (NaCl) cm⁻¹ 3417, 2922, 2850, 2101, 1643, 1311, 1454, 1511, 1209, 1181, 1153, 1123, 1072, 1035, 987; ¹H NMR (500 MHz, CDCl₃) spectroscopic data δ 13.26 (1H, s, 1-OH), δ 6.75 (1H, s, 7), δ 6.46 (1H, s, 4), δ 6.24 (1H, s, 6-OH), δ 4.05 (3H, s, 5-OMe), δ 3.97 (3H, s, 3-OMe), δ 3.91 (3H, s, 2-OMe), δ 2.80 (3H, s, 8-CH₃); ¹³C NMR (125 MHz, CDCl₃) spectroscopic data δ 182.9 (C, 9); δ 159.4 (C, 3), δ 155.0 (C, 1), δ 153.2 (C, 6), δ 152.3 (C, 4a), δ 151.2 (C, 5a), δ 138.5 (C, 8), δ 132.2 (C, 5/2), δ 114.9 (CH, 7), δ 104.5 (C, 1a), δ 90.1 (CH, 4), δ 62.1 (CH₃, 2-OMe), δ 61.1 (CH₃, 3-OMe), δ 56.5 (CH₃, 5-OMe), δ 23.4 (CH₃, 8-CH₃); HRESIMS m/z 355.0787 [M+Na]⁺ (calc. for C₁₇H₁₆O₇Na 355.0788).

3.3.6 (E)-3-(3',4'-dihydroxybenzylidene)-5-acetoxy-7-hydroxychroman-4-one (ovatifolionone acetate) (**5Ac**)

Yellow amorphous powder; UV (CDCl₃) $\lambda_{\max}$ 280, 360 nm; IR $\nu_{\max}$ (NaCl) cm⁻¹ 3582, 3415, 2957, 2920, 2361, 2336 1745, 1638, 1512, 1461, 1447, 1370, 1259, 1202, 1162, 1115; ¹H NMR (500 MHz, CDCl₃) and ¹³C NMR (125 MHz, CDCl₃) spectroscopic data for **5Ac**, see Table 1; HRESIMS m/z 299.0553 [M-OAc]⁻ (calc. for C₁₆H₁₁O₆ 299.0561).

3.3.6.1 Acetylation of (*E*)-3-(3,4-dihydroxybenzylidene)-5,7-dihydroxychroman-4-one (5)

Compound **5** (3 mg) was acetylated using the method described in section 3.3.4.1 and purified using normal phase column chromatography (100% CH₂Cl₂) to yield Compound **5Ac** (1.8 mg).

3.4 Assay for inhibition of COX activity

Assays were performed in a 96-well plate with purified COX-1 from ram seminal vesicles (Sigma Aldrich UK, C0733, >40000 units/mg) or human purified COX-2 (Sigma Aldrich UK, C0858, >8000 units/mg). COX enzymes were used after a 1/3 dilution into buffer (0.1 M Tris. HCl, pH 8) and 5 µl of the diluted solution was used in a 200 µl total volume.

Cyclooxygenase inhibition was assayed using the Cayman Fluorescence activity kit © guidelines (Cayman Chemical Company, 2011) measured by the conversion of PGG₂ to PGH₂ *via* the fluorescence of an additive called Amplex Red (Cayman Chemical Co., A1111, 5 mg, final assay concentration of 100 µM). Inhibition of COX refers to a reduction of PGH₂ formation, demonstrated by a reduction in fluorescence, in comparison to a non-inhibitor control (Batchelor *et al.*, 2003). DuP-697 (Cayman Chemical Co., 760158, 5 mg, 304 nM final assay concentration) and SC-560 (Cayman Chemical Co., 70340, 5 mg, 354 nM final assay concentration) were used as positive inhibitor controls for COX-1 and COX-2 respectively (Cayman Chemical Company, 2011).

Other additives included the substrate, arachidonic acid (Sigma Aldrich, A9673, 10 mg, 100 µM final assay concentration), the cofactor hematin (Sigma Aldrich, H3281, 250 mg, 100 µM final assay concentration) and buffer (0.1 M, Tris. HCl, pH 8).

The study was performed on a SpectraMax Gemini XS (Molecular Devices) well-plate fluorimeter using SoftMax Pro software. The fluorimeter takes 96-well plates and was set to read at 535 nm and 595 nm excitation and emission wavelengths, with a cut-off filter at 550 nm, set manually. Automix was turned on for two seconds before each run. The readings were taken in the 'normal' range (5), at medium sensitivity when using the COX-2 enzyme and low sensitivity when using the COX-1

enzyme. Each assay was run for 300 seconds with intervals ranging from 3-10 seconds depending on the number of wells measured.

3.4.1 Sample Preparation

Samples were dissolved to make a 20 mM solution in ethanol, of which 1 μ l was diluted with 100 μ l buffer to give a 200 μ M solution. 10 μ l of the latter solution was used in a total assay volume of 200 μ l to give a final concentration of 10 μ M. Compounds were tested at a screening concentration of 10 μ M and compounds showing activity and no inhibitory activity for COX-1 at this concentration were subjected to IC₅₀ determination.

3.5 Computational methods

The conformational searches for Compounds **2(R)** and **2(S)** were carried out at the molecular mechanics level of theory employing MMFF force field incorporated in the Spartan08 (Wavefunction, Irvine, CA) software package. Global minimum conformers were subjected to TDDFT calculations using a B3LYP method at 6-31G (d, f) level built in to Gaussian09 software (Frisch, *et al.*, 2010). The simulated ECD curves were UV-corrected where necessary and compared to the experimental ECD curves.

4. Supplementary information

Supplementary data associated with this article can be found, in the online version, XXXXXXXXXXXX.

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Figure 1. Compounds isolated from *Ledebouria socialis* and *Ledebouria ovatifolia* and screened in COX-1 and COX-2 assay

Fig. 2 The experimental and modelled circular dichroism analysis of Compound 2

Table 2: The percentage cyclooxygenase-2 activity of Compounds 1-3 and 5Ac-13

Compound	COX-2 % activity at 10 μ M	COX-1 % activity at 10 μ M
1	101.1% $\pm$ 32.6%	
2(<i>R</i>)	66.7% $\pm$ 17.5%	
2(<i>S</i>)	89.0% $\pm$ 29.2%	
3	54.4% $\pm$ 25.7%	
5 acetate	0% $\pm$ 7.8%	57% $\pm$ 10.2%
6	0% $\pm$ 21.3%	56% $\pm$ 10.7%
7	0% $\pm$ 5.4%	54% $\pm$ 15.1%
8	73.8% $\pm$ 21.4%	
9	62.2% $\pm$ 21.4%	
10	0% $\pm$ 8.3%	73.8% $\pm$ 8.0%
11	13.3% $\pm$ 13.3%	
12	20.9% $\pm$ 13.0%	
13	71.4% $\pm$ 20.0%	
Controls*:		
DuP-697	47.0% $\pm$ 15.5%	83.8 $\pm$ 0.9%
SC-560	76.7 $\pm$ 16.0%	56.7% $\pm$ 3.5%

*Footnote

Compounds SC-560 and DuP-607 are positive controls for COX-1 and COX-2 respectively (Cayman Chemical Company, 2011). SC-560 at a concentration of 354 nM had a significant inhibitory effect on COX-1, but not COX-2, enzyme activity. Similarly, DuP-607 at 304 nM had a significant effect on COX-2 and not COX-1.

Fig. 3 The percentage cyclooxygenase-2 activity of Compounds 1-3 and 5 acetate-13

Table 1: ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for compounds 1, 2(*R*) and 2(*S*) (in CDCl_3) and 5 acetate (in MeOD)

No.	1		2(<i>R</i>)		2(<i>S</i>)		5 acetate	
	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR	^1H NMR
2A 2B	73.7 CH_2	4.55* 4.54*	97.1 CH	6.48, s	96.7 CH	6.58, s	67.9 CH_2	5.30, d, $J = 1.4$ Hz
3	54.5 C		56.6 C		57.3 C		128.9 C	
4	196.2 C		188.2 C		185.9 C		180.4 C	
4a	102.4 C		107.2 C		107.2 C		165.4 C	
5	164.9 C		151.9 C		152.1 C		170.4 C	
6	95.4 CH	6.09, d, $J = 2.3$ Hz	105.8 CH	6.33, d, $J = 2.4$ Hz	105.8 CH	6.36, d, $J = 2.4$ Hz	105.9 CH	6.19, d, $J = 2.3$ Hz
7	168.2 C		165.9 C		166.0 C		153.1 C	
8	94.4 CH	6.05, d, $J = 2.3$ Hz	100.6 CH	6.43, d, $J = 2.4$ Hz	100.5 CH	6.48, d, $J = 2.4$ Hz	101.4 CH	6.26, s**
8a	163.3 C		160.0 C		160.0 C		164.5 C	
9A 9B	33.6 CH_2	3.02, d, $J = 13.1$ Hz 3.62, d, $J = 13.1$ Hz	37.6 CH_2	3.33, d, $J = 13.5$ Hz 3.20, d, $J = 13.5$ Hz	31.1 CH_2	3.84, d, $J = 13.9$ Hz 2.74, d, $J = 13.9$ Hz	137.5 CH	7.58, s
1'	118.0 C		133.8 C		135.5 C		147.9 C	
2'	145.2 C		105.7 CH	6.67, s	103.9 CH	6.42, s	123.6 CH	6.80, d, $J = 1.8$ Hz
3'	137.1 C		149.3 C		148.9 C		145.8 C	
4'	154.0 C		148.0 C		147.2 C		126.8 C	
5'	99.4 CH	6.29 s	105.2 CH	6.61, s	106.1 CH	6.67, s	115.7 CH	6.85, d, $J = 8.2$ Hz
6'	139.2 C		131.8 C		134.8 C		117.4 CH	6.76, dd, $J = 8.2, 1.8$ Hz
7'A 7'B			100.3 CH_2	5.91, d, $J = 1.3$ Hz 5.89, d, $J = 1.3$ Hz	100.3 CH_2	5.86, d, $J = 1.2$ Hz, 5.82, d, $J = 1.2$ Hz,		
7-OCH ₃	56.0 CH_3	3.84 s	56.2 CH_3	3.84, s	56.2 CH_3	3.86, s		
5-COOCH ₃			21.1 CH_3	2.09, s	21.1 CH_3	2.06, s	20.3 CH_3	2.33, s
5-COOCH ₃			169.5 C		169.5 C		170.4 C	
5-OH		12.05 s						
2-COOCH ₃			21.3 CH_3	2.32, s	21.3 CH_3	2.33, s		
2-COOCH ₃			169.6 C		169.7 C			
2'-OH		5.84, s						
3'-OCH ₃	61.3 CH_3	3.86, s						
4'-OCH ₃	56.5 CH_3	3.80, s						

*non-first order coupling

** doublet unresolved