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EXOCHROMONE: STRUCTURALLY UNIQUE CHROMONE DIMER WITH ANTIFUNGAL AND ALGICIDAL ACTIVITY FROM *EXOPHIALA* SP.‡

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‡*Biologically Active Secondary Metabolites from Fungi, Part 33. Part 32 ref.[1]*

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Dedicated to Prof. E. Winterfeldt on the occasion of his 75th birthday

Abstract – A unique highly substituted chromone dimer, named exochromone (**1a**), in addition to the known steroids ergosterol (**2**) and 5α,8α-epidioxyergosterol (**3**) were isolated from endophytic fungus *Exophiala* sp. The structure of the new compound was elucidated on the basis of spectroscopic data. Preliminary studies showed that exochromone is antifungal and antialgal against *Microbotryum violaceum* and *Chlorella fusca*, respectively.

Chromones, a fused [6-6] system are a group of naturally occurring compounds that are widely distributed in nature. They are part of a class of compounds which are now commonly called "privileged structures" in drug discovery. These structures represent molecules capable of binding to multiple receptors with high affinity. Whether the privileged nature of these molecules arises out of a common structural element or is due to independent molecular characteristics is open to debate.[2] Molecules containing the chromone or benzopyranone ring have a wide range of biological activities viz., tyrosine and protein kinase C inhibitors, anticancer, antifungal, antiviral, antitubulin, and antihypertensive agents.[2] As part of our systematic search for new bioactive lead compounds from fungal sources, one new chromone dimer, exochromone (**1a**), together with ergosterol (**2**) and 5α,8α-epidioxyergosterol (**3**)[3,4] were isolated from the culture of the fungus *Exophiala* sp.

RESULTS AND DISCUSSION

The endophytic fungus *Exophiala* sp. (internal strain no. 7110), was isolated from the plant *Adenocarpus foliolosus* and was cultivated at room temperature on biomalt solid agar media for 28 days.[5] The 12 L of culture media were then extracted with ethyl acetate to afford 3.5 g of a residue which was subjected to column chromatography. Three metabolites including one new chromone dimer exochromone (**1a**) of unusual connection mode were isolated from the culture extract.

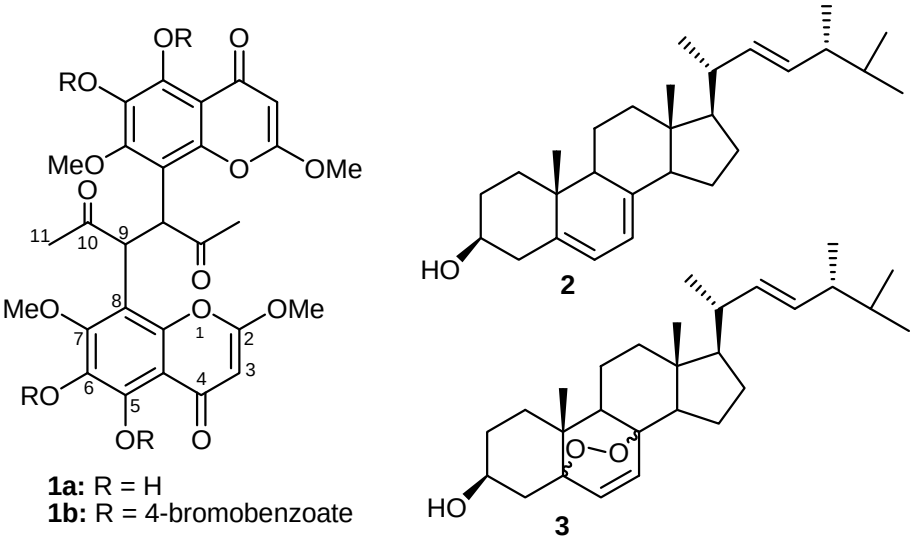

Figure 1. Structures of compounds isolated from *Exophiala* sp.

Exochromone (**1a**) has the molecular formula $C_{28}H_{28}O_{14}$ as indicated by HREIMS and in agreement with the ^{1}H and ^{13}C NMR spectra. The UV spectrum displayed absorptions at 214, 265, 295, 315, and 370 nm, which are indicative of a chromone system.[6] The molecular formula $C_{28}H_{28}O_{14}$ suggested 16 double bond equivalents. Thus, four rings must form the basic molecular skeleton of compound **1a**, considering the interconversion of the twelve double bond equivalents for four carbonyl groups and eight double bonds. Compound **1a** had a very simple ^{1}H NMR spectrum showing only six singlets: two methine signals; one downfield at (δ 6.65) and the other at (δ 5.21), two methoxy groups (δ 4.39 and 4.10), one methyl group (δ 2.05), and a low field signal at δ 16.16 for a strongly deshielded hydroxyl hydrogen. The presence of two carbonyl groups was indicated by typical signals at δ 179.7[6,7] (C-4) and 204.7 (C-10) in agreement with the IR absorption at 1664 cm^{-1}. The ^{13}C and DEPT spectra of **1a** showed signals for 14 carbons indicating a highly symmetrical molecule, comprising one methyl, two methoxy, two methine, and nine quaternary carbon signals.

The HREIMS $[M]^{+}$ molecular ion of **1a** with a *m*/*z* 586.1318 indicated that the simple NMR spectra showed only one part of a symmetric dimer. The 1D, 2D NMR and MS data presented above supported

the conclusion that compound **1a** consisted of two identical chromone units linked through a bis acetylmethine bridge [(CH–COMe)$_2$]. Thus, the major problem was the establishment of the linkage of the two chromone subunits. The signal for H-9 was the key entry into the diagnostic spin system. It occurs at a distinct chemical shift (δ 5.21) and was easily recognizable by its multiplicity compared with those of the other protons. The signal could also be identified by its HMQC correlation to a carbon at δ 48.6 and its important HMBC correlations with the acetyl carbonyl group (δ 204.7, C-10) and C-11. The signal for H-9 showed diagnostic long-range HMBC correlations with C-8, C-8a and C-7, confirming the linkage to C-8 on the aromatic ring. In addition, HMBC correlations were also observed for both the methoxy group at δ 4.39 and the benzylic hydrogen at δ 5.21 (H-9) with the carbon at δ 150.1 (C-7), establishing the location of that methoxy group at C-7. This was further confirmed by NOE correlation between H-9 and methoxy group at δ 4.39 (Figure 2).

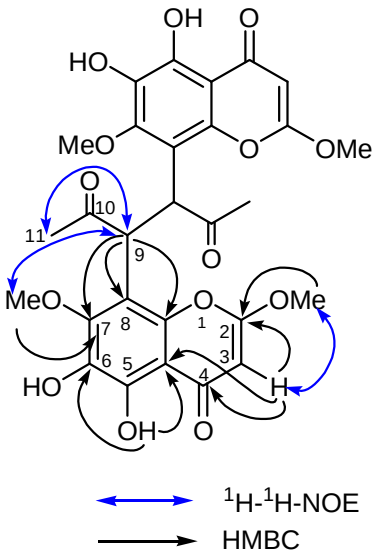

Figure 2. HMBC and DPFGSE-NOE correlations for exochromone (**1a**).

Furthermore, the assignments of the one remaining methoxy group (δ 4.10) at C-2 and hydrogen at C-3 were made based on the HMBC correlations of the methoxy group at δ 4.10 to C-2 and the hydrogen at δ 6.65 with C-2, C-3, and C-4 as well as from the NOE correlations (Figure 2).

The present data allow discussion of two remaining possibilities: either a chromone (subunit **A**) or a coumarin (subunit **B**) (Figure 3). However, detailed analysis of the ^{1}H and ^{13}C NMR spectral data allowed the unambiguous assignment of subunit **B** in **1a** due to characteristic spectroscopic differences between the units **A** and **B**.

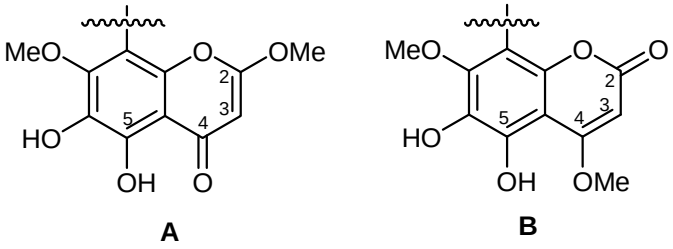

Figure 3. Structures of putative subunits **A** and **B**.

In the ^{13}C NMR spectrum, a carbonyl resonance in compound **1a** appeared at ca. δ 179.7, consistent with the reported values for the chromone carbonyl (subunit **A**).[7-11] By contrast, the chemical shift for the C-2 carbonyl in subunit **B** (coumarins) generally appears at significantly higher field (ca. δ 160)[8,9,12-15]. This remarkable difference in resonance for the carbonyl carbon (ester vs. ketone type) presents a convincing proof for the distinction between the two series of compounds. A further difference was also evident in the ^{1}H NMR spectrum. The appearance of a low field resonance for the strongly chelated proton at C-5 at δ 16.16 in compound **1a** is characteristic for **A** and excludes the possibility of a *peri* hydroxyl group as present in subunit **B**; weekly chelated protons, as present in subunit **B** (C-5 hydroxyl and C-4 OMe), usually resonate at 9 - 11.00 ppm.[16]

A reaction between compound **1a** and 4-bromobenzoyl chloride was carried out in the expectation to confirm the structure by X-ray single crystal analysis with a heavy atom incorporated. The DMAP-catalyzed perbenzoylation gave a tetra-4-bromobenzoate **1b** but, unfortunately, the compound did not afford suitable crystals (CH_2Cl_2 : MeOH and EtOAc : *n*-hexane) for X-ray analysis. However, the NMR spectra of tetra-4-bromobenzoate **1b** were in total agreement with the proposed structure and thus reconfirmed the presence of two phenolic hydroxyl groups in one chromone subunit of compound **1a**.

Exochromone (**1a**) did not show any optical activity. This suggests that **1a** exists either as a meso compound or as a racemic mixture as suggested by Floss et al.[17], Keller-Schierlein et al.,[18] and Kim et al.[19] for phenazine dimers with a related mode of linkage.

In contrast to the more frequently occurring benzoquinone, naphthoquinone, and anthraquinone dimers,[20] the dimeric chromone pigments are less common. A literature survey revealed that there is only one example of C–C linked dichromone reported by Franck et al.[21] Later, some dimeric or trimeric derivatives of 2-(2-phenylethyl)chromone were reported in the literature.[22,23] However, the linkage by an acetylmethine [$(CH–COMe)_2$ bridge between two chromone units is reported here for the first time in naturally occurring chromones.

The two additionally isolated compounds were identified as the known compounds ergosterol (**2**) and 5α,8α-epidioxyergosterol (**3**) by comparison of their physical and spectroscopic data with those reported in the literature.[3,4]

Exochromone (**1a**) was tested for herbicidal, antibacterial, and antifungal activities and showed good antifungal activity against *Microbotryum violaceum* and the algae *Chlorella fusca*, respectively (Table 1).

Table 1. Biological activity of exochromone A (**1a;** 50 μg applied to a filter disc) in an agar diffusion test.

Compound	algicidal	antifungal	antibacterial
	Chl[a]	Mb	Bm
	1mg/mL	1mg/mL	1mg/mL
Exochromone (**1a**)	5	9 gi	0

[a]*Chlorella fusca* (Chl), *Microbotryum violaceum* (Mb) and *Bacillus megaterium* (Bm). The radius of zone of inhibition was measured in mm. gi = growth inhibition, meaning that there was weak growth within the zone of inhibition.

EXPERIMENTAL

General: The endophytic fungus *Exophiala* sp., internal strain No. 7110, was isolated from *Adenocarpus foliolosus* that had been growing on the hills of Baranco de Los Jargus, Gomera, and was cultivated on 12 L of 5% w/v biomalt solid agar media at room temperature for 28 days.[5,24] For general methods and instrumentation see ref.[5,25] Melting points were determined with a Gallenkamp micro-melting point apparatus and are uncorrected. NMR spectra were run with a Bruker Avance-500 NMR spectrometer with TMS as internal standard. EIMS data were obtained with an MAT 8200 mass spectrometer.

Extraction and Isolation: The culture media were extracted with EtOAc to afford 3.5 g of a residue after removal of the solvent under reduced pressure. The extract was separated into three fractions by column chromatography (CC) on silica gel, using gradients of *n*-hexane/ EtOAc (90:10, 50:50, 0:100). The fraction E (120 mg) was separated by silica gel column chromatography eluting with *n*-hexane-EtOAc (3:7) to give crude compound **1a**. The crude compound **1a** was then purified eluting with *n*-hexane-EtOAc (3:7) to give the pure natural product **1a** (40 mg). Fraction B (400 mg) was separated by CC on silica gel (98 mg) with *n*-hexane- EtOAc (8.5:1.5) to give compounds **2** (6.5 mg) and **3** (51 mg).

Exochromone (**1a**): Red pigment, mp 224 °C (uncorrected); $[\alpha]_D^{20}$ + 0.00 (*c* 0.92, $CHCl_3$); UV λ_{max}, nm (log ε): 370 (4.20), 315 (4.67), 295 (3.65), 265 (3.15), 214 (2.60) nm; IR ν_{max} ($CHCl_3$): 3400 (OH), 1720, 1664, 1580 (C=C); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 16.16 (s, OH-5), 6.65 (s, 1H, H-3), 5.21 (s, 1H, H-9), 4.39 (s, 3H, OCH_3-7), 4.10 (s,3H, OCH_3-2), 2.05 (s, 3H, CH_3CO); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 204.7 (C-10), 179.7 (C-4), 162.0 (C-5), 167.4 (C-2), 150.1 (C-7), 149.3 (C-8a), 130.2 (C-6), 122.7 (C-8), 107.8 (C-4a), 102.4 (C-3), 61.1 (CH_3-7), 56.4 (CH_3-2), 48.6 (C-9), 27.9 (CH_3CO); HREIMS: *m/z* 586.1318

(Calcd. 586.1328 for $C_{28}H_{28}O_{14}$); EIMS (rel. int.): *m/z* 586 $[M]^+$ (100), 544.3 (88), 501.3 (77), 401.2 (66), 372.2 (15), 298.1 (14), 83.1 (17), 43.0 (47), 31.0 (25).

4-Bromobenzoylation of 1a to 1b: A solution of phenol **1a** (8 mg, 0.5 mmol) in pyridine (2 mL) was treated with 4-bromobenzoyl chloride (0.5 mg, 1 mmol) and 4-(dimethylamino)pyridine (DMAP) (2 mg). The mixture was stirred for 10 h at 21 °C and then poured into cold 2 N HCl (5 mL) and stirred for 1 h to hydrolyze the excess 4-bromobenzoyl chloride. The aqueous phase was extracted three times with CH_2Cl_2 (5 mL); the organic phase was washed with 2 N $NaHCO_3$ (5 mL) to remove 4-bromobenzoic acid, and dried with Na_2SO_4. The solvent was removed at reduced pressure followed by separated with chromatographed on silica gel (solvent EtOAc/petrol ether, 4:6 to 6:4) to afford **1b**. Red solid, mp 207 °C (uncorrected); UV λ_{max}, nm (log ε): 365 (4.12), 317 (4.13), 265 (3.18), 220 (2.90) nm; IR ν_{max} ($CHCl_3$): 1720, 1585 (C=C); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.90 (d, *J* = 8.1 Hz, 2H, H-2', H-6'), 7.88 (d, *J* = 8.1 Hz, 2H, H-2", H-6"), 7.68 (d, *J* = 8.1 Hz, 2H, H-3', H-5'), 7.65 (d, *J* = 8.1 Hz, 2H, H-3", H-5"), 6.64 (s, 1H, H-3), 5.18 (s, 1H, H-9), 4.35 (s, 3H, OCH_3-7), 4.07 (s, 3H, OCH_3-2), 2.02 (s, 3H, CH_3CO).

Bioactivity Tests: Agar diffusion test:

The tested compound **1a** was dissolved in acetone at a concentration of 1 mg/mL. 50 µL of the solution were pipetted onto a sterile filter disc, which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism.[26] The test organisms were *Escherichia coli* (NB), *Bacillus megaterium* (NB), *Microbotryum violaceum* (MPY) and *Chlorella fusca* (MPY). The radius of zone of inhibition was measured in mm.

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