Three Triterpenoids and one Flavonoid from the Liverwort *Asterella blumeana* Grown *In Vitro*

M. Neves[1], R. Morais[1]*, S. Gafner[2], K. Hostettmann[2]

[1] Escola Superior de Biotecnologia- UCP, Rua Dr. Bernardino de Almeida, 4200 Porto, Portugal
[2] Institut de Pharmacognosie et Phytochimie, Université de Lausanne, B.E.P., CH-1015, Lausanne, Switzerland

This is the first report on the chemical constituents of *Asterella blumeana.* Three triterpenoids (22-hydroxyhopane, 22,29-dihydroxyhopane, 6α,22-dihydroxyhopane) and one flavonoid (isoscutellarein-7,8,4′-trimethyl ether) were isolated from the CH_2Cl_2 extract of the liverwort *Asterella blumeana* grown *in vitro.* The structures of isolated compounds were established by spectroscopic methods (UV, EI mass spectroscopy, ^{1}H- and ^{13}C-NMR). 22,29-dihydroxyhopane is a new natural compound. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

Liverworts are known to be a rich source of terpenoids and phenolic compounds which constitute the oil bodies. From these plants a high diversity of substances has been isolated, including new structure types, some of them with interesting biological activities (Asakawa, 1989). Since only a few species of liverworts have been phytochemically investigated (less than 5% of known species), many interesting substances remain to be isolated. However, advances on the study of the chemistry of these plants are hindered by the difficulty in collecting a sufficient quantity of pure material of certain species. Their small size and the fact that liverworts usually grow mixed with other bryophytes and are strongly attached by their rhizoids to the soil, make their purification a difficult and time consuming task (Becker and Harrison, 1987; Asakawa, 1989). *In vitro* cultures of liverworts can be a good alternative, producing sufficient and homogenous amounts of plant material for subsequent analysis (One *et al*., 1992; Takazi *et al*., 1995).

The genus *Asterella* contains approximately 80 species (Frahm and Frey, 1983) but only a few have been studied phytochemically: *Asterella augusta, A. odora; A. australis; A. tenera, A. lindenbergiana; A. venosa* (Asakawa and Heidelberger, 1982). In this work, we report the isolation and structural elucidation of three hopane type triterpenoids and one flavonoid, from *in vitro* cultures of *Asterella blumeana.* The structures are shown in Figures 1 and 2.

* Correspondence to: R. Morais, Escola Superior de Biotecnologia- UCP, Rua Dr. Bernardino de Almeida, 4200 Porto, Portugal.
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MATERIAL AND METHODS

Plant material and culture conditions. Plants of the liverwort *Asterella blumeana* (Nees) Pandé Srivastava et Khen grown *in vitro*, obtained from the culture collection of autotrophic organisms of the Institute of Botany, Trebon, Czech Republic, were subcultured on fresh B5 medium (Gamborg *et al*., 1968) every 2 months and maintained in a photoperiod of 16 h light (30 mol/m^2/s) at 22°C.

Plant material extraction. Air dried *Asterella blumeana* plants (147 g) were ground and then extracted at room temperature with CH_2Cl_2 to give 7.51 g of extract.

Isolation procedure. A portion of the CH_2Cl_2 extract of *Asterella blumeana* plants (7 g) was submitted to CC on silica gel using mixtures of petrol, EtOAc and MeOH of increasing polarity, giving frs 1–20. Fr 8 (400 mg) was fractionated by CC on Sephadex LH-20 with MeOH-$CHCl_3$ (1:1), giving fractions A–E. Compound **1** (100 mg) was isolated from frs C–D by crystallization with $CHCl_3$ and MeOH. Fraction 14 (500 mg) was fractionated by CC on Sephadex LH-20 with MeOH-$CHCl_3$ (1:1) giving fractions A–F. Fr C (210 mg) was further chromatographed over CC silica gel with petrol-EtOAC (9:1) resulting in the isolation of compound **2** (50 mg). Frs 15–16 were subjected to CC on Sephadex

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LH-20 with MeOH-$CHCl_3$ (1:1), to afford fractions A–H. Fr F (36 mg) was further fractionated on CC silica gel with MeOH-$CHCl_3$ (99:1) resulting in the isolation of the compounds **3** (29 mg) and **4** (2.6 mg).

General. ^{1}H and ^{13}C spectra were measured in $CDCl_3$ at 200.06 and 50.30 MHz respectively. TMS: int. standard. TLC silica gel 60 F254 Al sheets (Merck). CC silica gel (40–63 μm, Merck, 600 × 55mm i.d., 250 × 21 mm i.d. and 115 × 15 mm i.d.) ^{1}H and ^{13}C NMR: Varian VXR 200, EI- MS and D/CI-MS: Finnigan MAT TSQ-700 Triple stage quadrupole instrument.

RESULTS AND DISCUSSION

Dried plants of *Asterella blumeana* grown *in vitro* were extracted with CH_2Cl_2. The fractionation of the extract by a combination of silica gel column chromatography, gel filtration on Sephadex LH-20 and recrystallization, resulted in the isolation of three triterpenoids containing the hopane skeleton (**1–3**) and one flavonoid (**4**).

Compound 1

White crystals. On SiO_2 TLC (EtOAc- petrol, 1:1) R_f 0.61 and on Diol TLC (hexane- isopropanol, 95:1) R_f 0.28. This compound was not UV active on the TLC plate and acquired a violet colouration with Godin reagent. MP 229.0°C–230.5°C. $[\alpha]_D = +39.6°$ (c = 1, $CHCl_3$). EI-MS and DCI-MS gave molecular weight for **1** of *m/z* = 428. In ^{13}C-NMR and DEPT, 30 carbons were found: 11 CH_2; 8 CH_3; 5 CH and 6 quaternary carbons (Table 1). With these data, a molecular formula of $C_{30}H_{52}O$ was proposed. Finally, **1** was identified as the triterpenoid 22-hydroxyhopane by comparison of ^{1}H- and ^{13}C-NMR data with literature values (Mahato and Kundu, 1994; Grammes *et al*., 1994; Ageta *et al*., 1993; Kamaya *et al*., 1990). Tsuda *et al.* (1961) described the isolation of 22-hydroxyhopane from the fern *Diplopterygium glaucum.* Grammes *et al.* (1994) described the isolation of this compound from the liverworts *Fossombronia alaskana* and *F. pussilla.*

Compound 2

White crystals. On SiO_2 TLC (EtOAc- petrol, 1:1) R_f 0.45 and on Diol TLC (hexane- isopropanol, 95:1) R_f 0.19. On TLC plate it was not UV active and acquired a pink colouration with Godin reagent. MP 193,7°C–195.1°C. $[\alpha]_D = +60.3°$ (c = 1, $CHCl_3$). EI-MS and DCI-MS data gave a molecular weight for **2** of *m/z* = 444. In ^{13}C-NMR and DEPT were detected 30 carbons: 10 CH_2,8 CH_3,6 CH and 6 quaternary carbons (Table 1). Thus, the molecular formula obtained was $C_{30}H_{52}O_2$. The ^{1}H- and ^{13}C-NMR of this compound were very similar to the spectra of **1,** but in the case of **2,** in ^{13}C-NMR a carbon at 69.3 ppm (corresponding to an oxygenated methine) was detected. By comparison with literature data (Mahato and Kundu, 1994; Wong *et al*., 1986; Elix *et al*., 1982), **2** was identified as zeorine (6α, 22-dihydroxyhopane). Elix *et al.* (1982) reported the isolation of this compound from the lichen *Heterodermia tremulans* and Morais (1990) described its isolation from the liverwort *Reboulia hemispheaerica.* Although **2** did not present biological activity against *Cladosporium cucumerinum* and *Candida albicans* (data not shown), Wong *et al.* (1986) reported cytotoxic activity toward cultured P-388 cells (ED = 1.1 μg/mL).

Table 1. ^{13}C-NMR chemical shifts of compounds 1, 2 and 3

Carbon	1[a]	2[a]	3[b]
1	40.3	40.4	40.5
2	18.6	18.5	19.0
3	42.1	43.8	42.3
4	33.2	33.6	33.4
5	56.1	61.1	56.3
6	18.6	69.4	19.0
7	33.2	45.5	33.5
8	41.8	42.9	42.1
9	50.3	49.5	50.7
10	37.4	39.4	37.6
11	20.9	21.1	213
12	24.1	24.0	24.5
13	49.8	49.8	50.1
14	41.9	41.9	42.1
15	34.4	34.4	34.6
16	21.9	21.9	22.8
17	53.9	54.0	54.4
18	44.1	44.0	44.5
19	41.2	41.2	419
20	26.6	26.6	25.8
21	51.1	51.1	47.0
22	73.9	73.9	75.0
23	33.4	36.7	33.5
24	21.6	22.1	21.8
25	16.1	17.1	16.0
26	15.8	18.3	16.9
27	17.02	17.1	17.2
28	16.7	16.1	16.2
29	28.7	28.8	70.1
30	30.8	30.9	25.5

[a] The values given are ppm from internal Me_4Si in $CDCl_3$ solution.
[b] C_5D_5N solution.

Compound 3

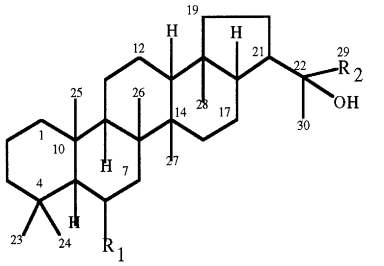
White crystals. On SiO_2 TLC (EtOAc- petrol, 1:1), **3** had R_f 0.34 and on Diol TLC (hexane- isopropanol, 95:1) R_f 0.11. On the TLC plate was not UV active and acquired violet colouration with Godin reagent. MP 230.9°C–232.7°C. $[\alpha]_D = -6.5°$ (c = 1, $CHCl_3$). EI-MS and DCI-

Table 2. Long-range correlations detected in the HMBC spectrum of compound 3

^{1}H	^{13}C
CH_2-29	21, 22, 30
(1H 3.88 *d, J*=10.3 Hz	
1H 3.85 *d, J*=10.3 Hz)	
CH- 21	17, 20, 22, 29, 30
(1H 2.68 *q, J*=10 Hz)	
CH_3- 23 (3H 0.89 *s*)	3, 4, 5, 24
CH_3- 24 (3H 0.83 *s*)	3, 4, 5, 23
CH_3- 25 (3H 0.83 *s*)	1, 9, 10
CH_3- 26 (3H 0.98 *s*)	8, 9, 14
CH_3- 27 (3H 1.00 *s*)	7, 8, 14, 15
CH_3- 28 (3H 1.02 *s*)	13, 17, 18, 19

Table 3. ^{1}H-NMR and ^{13}C-NMR data of compound 4

	^{1}H	^{13}C
2		163.9
3	6.58 s	103.8
4		182.6
5		157.5
6	6.43 s	95.7
7		158.5
8		128.4
9		149.5
10		104.0
1′		123.6
2′	7.91 (*dd, J* = 17 Hz, 2 Hz)	128.1
3′	7.04 (*dd, J* = 7 Hz, 2 Hz)	114.6
4′		162.2
5′	7.04 (*dd, J* = 7 Hz, 2 Hz)	114.6
6′	7.91 (*dd, J* = 7 Hz, 2 Hz)	128.1



	R_1	R_2
Compound 1	H	CH3
Compound 2	OH	CH3
Compound 3	H	CH2-OH

Figure 1. Structure of compounds 1, 2 and 3.

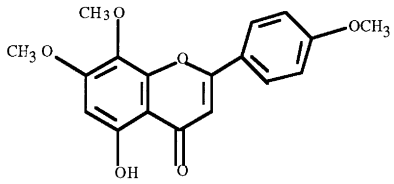


Figure 2. Structure of compound 4.

MS gave a molecular weight for **3** of *m/z* = 444. In ^{13}C-NMR and DEPT, 30 carbons were found: 7 CH_3, 12 CH_2, 5 CH and 6 quaternary carbons. With these data, a molecular formula of $C_{30}H_{52}O_2$ was proposed. An oxygenated methylene at 70.1 ppm was detected in ^{13}C-NMR (Table 1). As this compound had one methyl group less than **1** and **2,** although the spectra were similar, it was possible to conclude that a methyl group was substituted by a -CH_2-OH. In order to determine the exact position of the CH_2-OH in molecule **3,** HMBC and HMQC experiments were carried out. Finally, based on the long-range correlations detected in HMBC spectrum (Table 2), the structure of 22,29-dihydroxyhopane was established. Galbraith *et al.* (1964) described the chemical synthesis of this compound, but to our knowledge it was never reported to have been isolated from a natural source.

Compound 4

Yellow powder. R_f (SiO_2 TLC EtOAc-petrol 1:1) = 0.38. R_f (Diol TLC hexane-isopropanol 95:5) = 0.17. On the TLC plate it was UV active at 254 nm and acquired a yellow colouration with Godin reagent. EI-MS spectrum gave a molecular weight for **4** of m/z = 328. In ^{13}C-NMR spectrum of **4** were detected 18 carbons: 3 CH_3 (at 61.65, 56.32 and 55.53 corresponding to methoxy groups), 6 CH and 9 quaternary carbons. Its UV spectrum was characteristic of an aromatic compound. Three singlets at 3.96 ppm, 3.95 ppm and 3.94 ppm were detected in ^{1}H-NMR spectrum of **4** corresponding to three methoxy groups. In the aromatic region of the spectrum two signals at 7.91 ppm (2H, *dd*, *J* = 7 Hz, 2 Hz) and at 7.04 ppm (2H, *dd*, *J* = 7 Hz, 2 Hz) gave evidence of the presence of a *para* substituted aromatic ring. Two singlets corresponding to two aromatic hydrogens were also present at 6.59 ppm and at 6.43 ppm (Table 3). By comparison of ^{13}C-NMR spectrum of **4** (Table 3) with literature data, a great similarity to those of salvigenin (Ring B) and wigertin (ring A and C) was found (Markham *et al*., 1982). The existence of a methoxy group in position 8, was deduced from the EI-MS data. In the case of 8-OCH_3 flavone, the intensity of the peak $M^+ - 15$ is higher than peak M^+ (molecular ion), whereas this is the opposite for 6-OCH_3 flavones (Goudard *et al*., 1978, 1979). Finally, this compound was identified as 5-OH-7,8,4′-trimethoxyflavone (isoscutellarein-7,8,4′-trimethyl ether) by comparison of its EI-MS, ^{1}H-NMR and UV spectra with literature data (Morais, 1990). Isoscutellarein-7,8,4^{1}-trimethyl ether was first isolated from *Citrus reticulata* (Iimuma *et al*., 1980). Morais (1990) described the isolation of this compound from the liverwort *Reboulia hemispheaerica.*

This is the first report on the chemical constituents of *Asterella blumeana* and the first time that triterpenoids have been isolated from this genus. 22,29-dihydroxyhopane is a new natural compound.

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