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^{1}H and ^{13}C-NMR and Biological Activity Investigations of Four Lichen-derived Compounds

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The lichen-derived natural products, atranorin (1), hopane-6α, 22-diol (2), usnic acid (3), and vulpinic acid (4) were analysed by both one and two-dimensional (^{1}H, ^{13}C)-NMR. Experiments employed included COSY, NOESY, XHCO, HMQC and HMBC. For 1 and 2, fully assigned proton NMR data are reported for the first time; the reassigned ^{13}C NMR data for both 1 and 2 are also reported. For 3, cross-peaks were observed in the HMBC spectrum that suggest that CH long-range coupling through H bonds is occurring. Biological activity investigations of each compound indicated hopane-6α, 22-diol (2) to have anti-tubercular activity (MIC 8 μg/mL) and usnic acid (3) to be very weakly cytotoxic (ED_{50} 13 μg/mL).

Keywords: ^{1}H-NMR; ^{13}C-NMR; two-dimensional NMR; lichens; atranorin; usnic acid; hopane-6α, 22-diol; vulpinic acid; biological activity.

INTRODUCTION

Lichens are known to contain interesting secondary metabolites in relatively high levels. Some of these well-known chemicals are, however, not fully characterized with respect to their NMR data. We thus collected lichen samples from various locations, where they are present in abundance, for later chemical and biological investigations. From three different species, *Stereocaulon vesuvianum* Pers. (Laconorales, Stereocaulaceae) (Lanzarote, Spain), *Lecanora muralis* (Schreber) Rabenh. (Lecanoraceae, Lecanorales) (Braunschweig, Germany), and *Letharia columbiana* (Nutt.) Thoms. (Usneaceae, Lecanorales) (California, USA), the four known natural products, atranorin (**1**) (Sundholm and Huneck, 1981), hopane-6α, 22-diol (**2**) (Yosioka *et al.*, 1967; Elix *et al.*, 1982), usnic acid (**3**) (Culberson, 1969), and vulpinic acid (**4**) (Culberson, 1969; Brassy *et al.*, 1985) were isolated. None of these compounds had previously been the subject of the detailed two-dimensional NMR investigations that form the basis of the current study. Each compound was also tested for its cytotoxic, antimicrobial, anti-algal, anti-tubercular, and anti-malarial activity, as well as for its ability to inhibit the enzymes HIV-1 reverse transcriptase (RT) and tyrosine kinase (TK).

EXPERIMENTAL

Natural materials. All of the lichens were identified by Dr. C. Scheidegger (Swiss Federal Institute for Forest, Snow and Landscape Research, Zurich, Switzerland). *Stereocaulon vesuvianum*, the source of compound **1**, was collected during April 1991 from volcanic areas around Lanzarote, Canary Islands, Spain. *Lecanora muralis*, which yielded compounds **2** and **3**, was collected from roof tiles in Braunschweig, Germany. *Letharia columbiana* was collected from the bark of a large redwood (*Sequoia* sp.) which had recently been felled in the vicinity of Yosemite National Park, California, USA, and yielded compounds **1** and **4**.

Extraction of natural materials. General laboratory procedures were as described in Wright *et al.*, (1996). *S. vesuvianum* was air-dried and a sample (10 g) exhaustively extracted with dichloromethane (200 mL) to yield an extract (80 mg; 0.80%) that was essentially pure atranorin (**1**). This compound was isolated as a white powder and identified by comparison of its IR, UV and ^{1}H-NMR data with those of an authentic sample; the fully assigned ^{1}H- and ^{13}C-NMR are presented in Table 1.

Lecanora muralis, which was dry on collection (563 g), was extracted with dichloromethane (2.5 L) to yield a dichloromethane soluble fraction (1.83 g; 0.33%). Separation of this material by vacuum liquid chromatography (VLC), employing step gradient elution from hexane to ethyl acetate, yielded 11 fractions each of 90 mL. VLC fractions *5* and *6* were pure **3;** fractions *10* and *11* were mainly **2**, which was purified by washing with methanol. Hopane-6α, 22-diol (**2;** 50 mg; 0.009%) was isolated as an amorphous white powder and had comparable physical and spectroscopic data to those reported (Yosioka *et al.*, 1967; Elix *et al.*, 1982); for fully assigned ^{1}H- and ^{13}C-NMR data see Table 2. Usnic acid (**3;** 43 mg; 0.008%) was isolated as an amorphous white powder and showed physical and spectroscopic data

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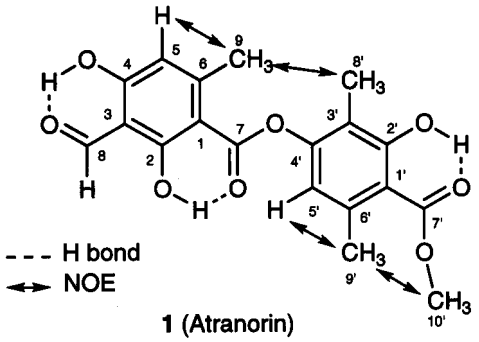

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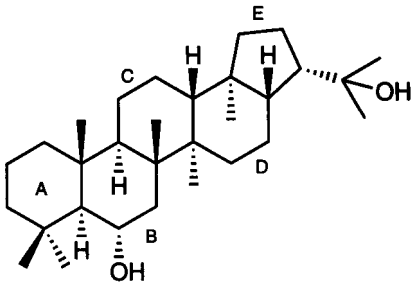

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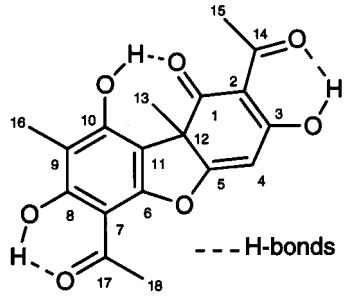

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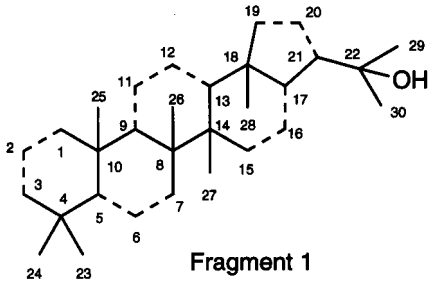
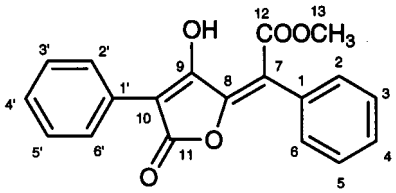


Connectivities deduced from HMBC
Connectivities deduced from COSY



4 (Vulpinic acid=Methylpulvinic acid)

comparable with those reported (Huneck and Yoshimura, 1996); for fully assigned ^{1}H- and ^{13}C-NMR data see Table 3.

Letharia columbiana was also air-dried and a sample (5.8 g) of dry tissue was extracted with dichloromethane (0.5 L) to yield 31 mg (0.54%) of extract. By ^{1}H-NMR and TLC examination the extract was shown to be composed of two main components and a small amount of lipid. Compounds **1** and **4** were separated by fractional crystallization from a 1:1 mixture of dichloromethane and acetone. Compound **1** (9 mg; 0.16%) was identical to atranorin isolated from *S. vesuvianum*. Vulpinic acid (**4;** 19 mg; 0.33%) was isolated as yellow crystals; ^{13}C-NMR[a] (75.5 MHz, deutero-acetone) 54.9 (*q*, C-13), 104.8 (*s*, C-10), 117.0 (*s*, C-7), 128.4 (*d*, 2 × C[b]), 128.7 (*d*, 2 × C[b]), 129.0 (*d*, 2 × C), 129.2 (*d*, 2 × C[b]), 130.2 (*s*), 130.9 (*d*, 2 × C[b]), 133.5 (*s*, C-1), 155.0 (*s*, C-8), 161.7 (*s*, C-11), 166.4 (*s*, C-9), and 172.7 (*s*, C-12) ppm; the remaining physical and spectroscopic data were identical to those reported (Pattenden *et al.*, 1991).

Biological activity assessment. The antifungal, anti-bacterial, anti-algal (Schulz *et al.*, 1995), anti-malarial and cytotoxic (Angerhofer *et al.*, 1992; Likhitwitayawuid *et al.*, 1993) properties of compounds **1–4** were assessed. Their abilities to inhibit the enzymes HIV-1 reverse transcriptase (RT) (Eberle and Seibl, 1992) and tyrosine kinase (TK) (Wessels *et al.*, 1999) were also investigated in ELISA-based assays.

[a] All assignments are tentative.
[b] One of these resonances is 3 × C.

RESULTS AND DISCUSSION

Atranorin

The dichloromethane extract of *S. vesuvianum* was essentially pure atranorin (**1**); >95% as judged by TLC and ^{1}H-NMR analysis). This compound is a significant taxonomic maker for various lichen species (Elix, 1993) and has interesting ecological and biological properties (Rundel, 1978; Elix, 1993). Detailed NMR investigation of this molecule was considered worthwhile as no complete spectral assignments could be found for its ^{1}H-NMR data, and also the original ^{13}C-NMR spectral assignment work on this type of compound had not employed any two-dimensional techniques (Sundholm

Table 1. ^{13}C and ^{1}H-NMR (300 MHz, $CDCl_3$) data for atranorin (1)

Carbon	δ (^{1}H)[a]	δ (^{13}C)[a,b]	^{1}H–^{13}C long-range correlations
1		102.8 *s*[c]	
2		169.1 *s*	
3		108.6 *s*	
4		167.4 *s*	
5	6.40 *s*	112.8 *d*	25.5, 102.8, 108.6, 167.4
6		152.4 *s*	
7		169.7 *s*	
8	10.36 *s*	193.8 *d*	108.6, 112.8, 169.1
9	2.69 *s*	25.5 *q*	102.8, 112.8, 152.4
1′		110.3 *s*	
2′		162.9 *s*	
3′		116.8 *s*	
4′		152.0 *s*	
5′	6.52 *s*	116.0 *d*	24.0, 110.3, 116.8, 152.0
6′		139.9 *s*	
7′		172.2 *s*	
8′	2.09 *s*	9.3 *q*	116.8, 152.0, 162.9
9′	2.54 *s*	24.0 *q*	110.3, 116.0, 139.9
10′	3.99 *s*	52.3 *q*	172.2
2-OH	12.50 *s*		102.8, 108.6, 169.1
4-OH	12.55 *s*		108.6, 112.8, 167.4
2′-OH	11.95 *s*		110.3, 116.8, 162.9

[a] Solvent resonances were used as internal references: δ (^{1}H) residual $CHCl_3$, δ 7.26; δ (^{13}C) $CDCl_3$, δ 77.0.

[b] Revised ^{13}C-NMR assignments were based on short-range (HMQC; *J* 125 Hz) and long-range (HMBC; *J* 8.3 Hz) correlations.

[c] Multiplicities were determined using DEPT, *s* = C, *d* = CH, *t* = CH_2, *q* = CH_3.

Table 2. ^{13}C and ^{1}H–NMR (400 MHz, DMSO, d_6) data for hopane-6α, 22-diol (2)

Carbon	δ (^{1}H)[a]	δ (^{13}C)[a,b]	Diagnostic ^{1}H–^{13}C long-range correlations
1	0.77 *m*, 1.56 *m*	39.5 *t*[c]	
2	1.32 *m*, 1.51 *m*	18.1 *t*	
3	1.14 *m*, 1.28 *m*	43.5 *t*	
4	—	33.3 *s*	
5	0.73 (*d*, *J* 10.4 Hz)	60.0 *d*	
6	3.75 *m*	66.5 *d*	
7	1.38 *m*	44.7 *t*	
8	—	42.1 *s*	
9	1.21 *m*	49.3 *d*	
10	—	38.5 *s*	
11	1.27 *m*, 1.50 *m*	20.5 *t*	
12	1.35 *m*, 1.48 *m*	23.6 *t*	
13	1.34 *m*	48.9 *d*	
14	—	41.4 *s*	
15	1.17 *m*, 1.36 *m*	33.9 *t*	
16	1.53 *m*, 1.93 *m*	21.3 *t*	
17	1.37 *m*	53.8 *d*	
18	—	43.5 *s*	
19	0.88 *m*, 1.45 *m*	40.9 *t*	
20	1.48 *m*, 1.64 *m*	26.0 *t*	
21	2.10 *m*	50.3 *d*	
22	—	71.5 *s*	
23	1.12 *s*	36.6 *q*	22.0, 33.3, 43.5, 60.0
24	0.95 *s*	22.0 *q*	33.3, 36.6, 43.5, 60.0
25	0.82 *s*	16.9 *q*	38.5, 39.5, 49.3, 60.0
26	0.98 *s*	18.0 *q*	41.4, 42.1, 44.7, 49.3
27	0.92 *s*	16.8 *q*	33.9, 41.4, 42.1, 48.9
28	0.71 *s*	15.8 *q*	40.9, 43.5, 48.9, 53.8
29	1.04 *s*	29.0 *q*	30.8, 50.3, 71.5
30	1.08 *s*	30.8 *q*	29.0, 50.3, 71.5
6-OH	3.86 (*d*, *J* 6.4 Hz)		
22-OH	3.78 *s*		

[a] Solvent resonances were used as internal references: δ (^{1}H) DMSO, δ 2.5; δ (^{13}C) DMSO, δ 39.7.

[b] Assignments were based on short-range (HMQC; *J* 125 Hz) and long-range (HMBC; *J* 8.3 Hz) correlations.

[c] Multiplicities were determined using DEPT, *s* = C, *d* = CH, *t* = CH_2, *q* = CH_3.

and Huneck, 1981). In the ^{1}H-NMR spectrum of **1**, all of the 10 (rather than the anticipated seven) resonances are singlets. These, the extra three resonances, occur because each of the hydroxyl protons form hydrogen bonds with adjacent oxygen containing functions, giving rise to three sharp singlets with resonances above 11.8 ppm. After the assignment of all of the protons to their respective carbons via an ^{1}H-^{13}C HMQC (*J* 125 Hz) measurement, a ^{1}H-^{13}C HMBC (*J* 8.3 Hz) experiment was performed. The results of this measurement (Table 1) indicated that some ^{13}C-NMR spectral assignments originally made were incorrect. If one considers the ^{1}H-^{13}C long-range correlations from H_3-9' and H_3-8' to their neighbouring carbons, it is evident that the resonances for C-1′ and C-3′ should be interchanged. Thus, in the HMBC spectrum of **1** there are cross-peaks between the proton resonance for CH_3-8′ and the ^{13}C resonances for C-2′, C-3′ and C-4′, and between the resonance for CH-9′ and the ^{13}C resonances for C-1′, C-5′ and C-6′. Cross-peaks were also observed between the proton resonance for OH-2′ and the ^{13}C resonances for C-1′, C-2′ and C-3′, indicating the resonances assigned to C-1′ and C-3′ to be as shown in Table 1, and not as previously reported (Sundholm and Huneck, 1981). This result also suggests that a number of other compounds of this structural type may also require reassignment of their ^{13}C-NMR spectral data (Sundholm and Huneck, 1981). For details of the NMR spectral assignments see Table 1 and structural formula **1**. After this work was completed, the work of Huneck and Yoshimura (1996) was published and found to contain both ^{1}H- and ^{13}C-NMR data for atranorin; these assignments are, however, not unambiguous and in some cases are still incorrect.

From the dichloromethane extract of *Lecanora muralis* both hopane-6α, 22-diol (**2**) (Yosioka *et al.*, 1967; Elix *et al.*, 1982), and usnic acid (**3**) (Culberson, 1969) were isolated.

Hopane-6α, 22-diol

Hopane-based triterpenes as a group are of taxonomic importance (Elix *et al.*, 1982). Even though, over the years, a number of groups have undertaken NMR investigations (Wilkins *et al.*, 1987), it appears that there are no complete ^{1}H–^{13}C-NMR data for hopane-6α, 22-diol (**2**) itself. Detailed NMR investigations of **2** (also known as zeorin) indicated that the ^{13}C-NMR data reported by Elix *et al.*, (1982) were in good agreement with the present data. There were, however, no ^{1}H-NMR data, hence the relevant one-dimensional NMR spectra, two-dimensional ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC and HMBC spectra were recorded for **2** to allow the ^{1}H-NMR data to be assigned and also to enable the ^{13}C-NMR data to be verified. From the ^{1}H–^{13}C HMQC spectrum it was

Table 3. ^{13}C and ^{1}H–NMR (300 MHz, $CDCl_3$) data for usnic acid (3)

Carbon	δ (^{1}H)[a]	δ (^{13}C)[a,b]	Diagnostic ^{1}H–^{13}C long-range correlations
1		198.1 *s*[c]	
2		105.3 *s*	
3		191.8 *s*	191.8, 179.4, 105.3, 59.1, 32.2
4	5.92 *s*	98.4 *d*	
5		179.4 *s*	
6		155.2 *s*	
7		101.6 *s*	
8		163.9 *s*	
9		109.4 *s*	
10		157.6 *s*	
11		104.0 *s*	
12		59.1 *s*	
13	1.75 *s*	32.2 *q*	198.1, 179.4, 104.0, 59.1
14		201.8 *s*	
15	2.66 *s*	27.9 *q*	201.8, 105.3
16	2.10 *s*	7.6 *q*	163.9, 157.6, 109.4, 104.0, 101.6
17		200.4 *s*	
18	2.67 *s*	31.3 *q*	200.4, 101.6
3-OH	18.84 *s*		201.8, 191.8, 105.3, 98.4, 27.9
8-OH	13.31 *s*		163.9, 157.6, 109.4, 101.6
10-OH	11.02 *s*		163.9, 157.6, 109.4, 104.0

[a] Solvent resonances were used as internal references: δ (^{1}H) residual $CHCl_3$, δ 7.26; δ (^{13}C) $CDCl_3$, δ 77.0.

[b] Assignments were based on short-range (HMQC; J 125 Hz) and long-range (HMBC; J 8.3 Hz) correlations.

[c] Multiplicities were determined using DEPT, s = C, d = CH, t = CH_2, q = CH_3.

possible to associate all carbons with their directly bonded protons (see Table 2). With these associations complete, the HMBC spectral data were used to unambiguously develop major parts of fragment 1, the solid lines coming purely from long-range couplings [^{2}J (C, H) and ^{3}J (C, H)] from methyl group protons. Cross-peaks were observed in the ^{1}H–^{1}H COSY spectrum between the resonance at δ 3.75 (H-6) and the resonances for H-5 and H_2-7, thus completing ring B. Coupling between H-21 and H_2-20 [δ 2.10 (*m*) and 1.48 (*m*), 1.64 (*m*)] positioned C-20, and thus completed ring E. Coupling from H-21 to H-17, and from H-17 to H_2-16 [1.53 (*m*), 1.93 (*m*)] unequivocally positioned C-16, and completed ring D. Clear cross-peaks were also observed between the resonances for H_2-1 [0.77 (*m*), 1.56 (*m*)] and H_2-2 [1.32 (*m*), 1.51 (*m*)], confirming the position of C-2 and completing ring A. Finally, H-9 demonstrated coupling with H_2-11, leaving only H_2-12 to be positioned by deduction, thus completing ring C and all of the assignments for **2**.

Usnic acid

The proton spectrum for usnic acid (**3**) is very similar to that of atranorin (**1**) in that all of the proton resonances are singlets, and all of the OH protons are present as sharp singlet resonances, indicating that they form H-bonds with the oxygen of neighbouring keto groups. What is also noteworthy in this spectrum is the extremely deshielded nature of one of the OH protons, δ 18.84 (3-OH), suggesting that it forms a very strong H-bond. In the publication by Huneck and Yoshimura (1996), complete NMR data are provided for usnic acid (**3**), and the ^{1}H-NMR part of this information is in agreement with the present data. The ^{13}C-NMR assignments, however, bear little resemblance to those proposed (see Table 3). In the present assignments, long-range ^{1}H–^{13}C-NMR correlations involving the OH group protons proved to be very important (see Fig. 1). Thus, after measuring the relevant one-dimensional NMR spectra, ^{1}H–^{13}C HMQC and HMBC spectra were recorded for **3**. From the ^{1}H–^{13}C HMQC spectrum all of the carbons were associated with their directly bonded protons (see Table 3). With these associations complete, interpretation of the HMBC spectrum (Fig. 1) commenced. From the long-range correlations listed in Table 3 it is a relatively straightforward process to deduce the structure of **3** and hence the assignments of the ^{13}C-NMR data. Two most unusual correlations were observed in the HMBC spectrum of **3** between the 3-OH proton and C-14 and C-15. If these correlations are considered as deriving from couplings

Table 4. Biological activities of compounds 1–4

	Radius of inhibition zone (cm)						
	Fungi[a]				Bacteria[b]		Alga[c]
	U. violacea	*M. microspora*	*E. repens*	*F. oxysporum*	*B. megaterium*	*E. coli*	*C. fusca*
Compound (amount added to plate)							
1 Atranorin (50 μg)	n.a.[d]	n.a.	0.1	n.a.	n.a.	n.a.	n.a.
2 Hopane-6α, 22-diol (50 μg)	n.a.	n.a.	0.5	n.a.	n.a.	n.a.	n.a.
3 Usnic acid (50 μg)	0.1	0.3	0.1	n.a.	1.0	n.a.	n.a.
4 Vulpinic acid (50 μg)	0.2	n.a.	0.1	n.a.	0.1	n.a.	n.a.
Positive controls (amount added to plate)							
(±)-Miconazol nitrate (500 μg)	2.4	1.0	2.5	0.8			
Cycloheximide (60 μg)	Total[e]	0.4	0.5	0.3			
Benzylpenicillin, potassium salt (85 μg)					2.0	0	
Streptomycin sulphate (65 μg)					0.5	0.3	

[a] Test fungi were *Ustilago violacea, Mycotypha microspora, Eurotium repens*, and *Fusarium oxysporum*.

[b] Test bacteria were *Bacillus megaterium* and *Escherichia coli*.

[c] Test alga was *Chlorella fusca*.

[d] n.a. = not active.

[e] Total = complete inhibition of fungal growth on the test plate.

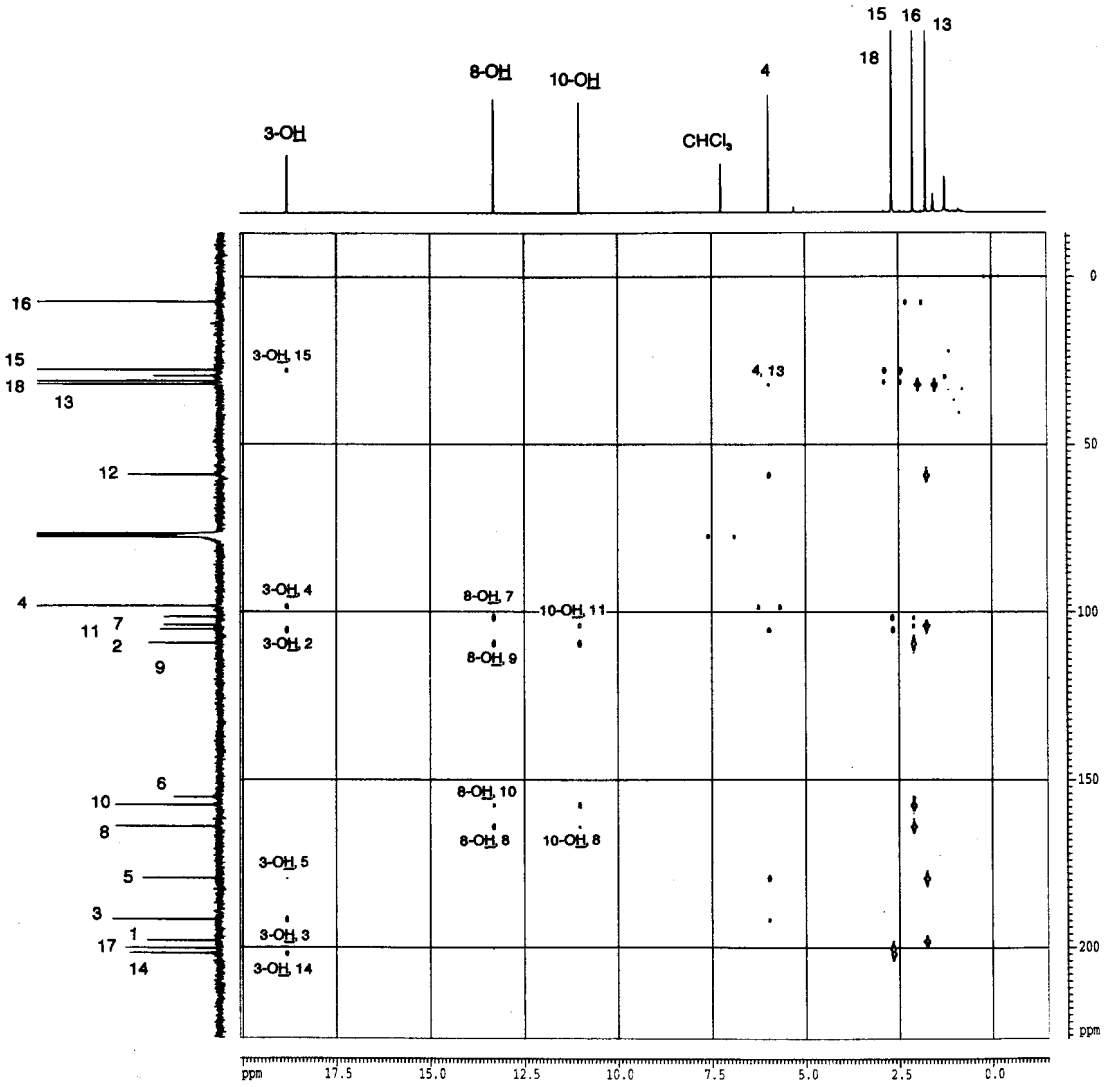


Figure 1. PFG HMBC spectrum of compound **3** at 300 MHz in deutero-chloroform at 25°C. For the acquisition, 40 scans were made for each of 512 individual experiments. All delays were optimized for $J = 8.3$ Hz. Processing was done with sine filters in both dimensions. The final matrix had dimensions 1K × 2K.

through covalent bonds, then the first is ^4J (C, H), and the second ^5J (C, H). The ^4J (C, H) coupling in such aromatic systems is possible, but the intensity, and hence the magnitude, of the ^5J (C, H) is less probable. It is far more likely that the observed correlations are actually due to ^2J (C, H), and ^3J (C, H) couplings through the hydrogen bond, thus making this observation unusual, but not unique (Hilton and Sutcliffe, 1975).

From the dichloromethane extract of *Letharia columbiana* both **1** (Sundholm and Huneck, 1981), and vulpinic acid (**4**) (Culberson, 1969; Brassy *et al.*, 1985) were isolated.

Table 5. Antitubercular activities of compounds 1–4[a]

Compound	MIC (μg/ml)
1 Atranorin	—
2 Hopane-6α, 22-diol	8
3 Usnic acid	16
4 Vulpinic acid	64
Control (Rifampin)	0.25

[a] Test organism was *Mycobacterium tuberculosis* strain $H_{37}Rv$ (ATCC 7294).

Vulpinic acid

Similar to atranorin, vulpinic acid (**4**) is a significant taxonomic marker. The proton spectrum, measured in deutero-acetone, for **4** (methylpulvinic acid), contains the resonances for the acetate methyl [δ 3.86 (*s*)], for the 10 aromatic protons [δ 7.2–7.7 (8H), 8.1–8.3 (2H)], and for the 9-OH, the latter being a very broad signal centred on δ 13.95, and is comparable to that reported by Pattenden *et al.*, (1991). The ^{13}C-NMR spectrum recorded in the same solvent contained 14 resonances and is also essentially identical to that reported previously (Pattenden *et al.*, 1991). As the ^{13}C-NMR data for this structurally quite unusual molecule were not assigned, this was attempted, but unfortunately the results obtained did not allow unambiguous assignments to be made due to the highly congested (degenerate) nature of many of the ^{13}C-NMR resonances. The results of the ^{13}C-NMR investigations are reported in the Experimental section.

Biological activity

All compounds were tested for their antimicrobial, antialgal (see Table 4), antitubercular (see Table 5), and antimalarial activities, as well as for their cytotoxicity and their abilities to inhibit the enzymes HIV-1-RT and TK. From these results it is evident that in the applied test systems only **3** has any, albeit weak, antimicrobial properties. As well as being weakly antibacterial, **3** (see Table 4) was also found to be very weakly cytotoxic (ED_{50} 13 μg/mL against KB cells). Anti-tubercular activity was shown by **2** and to a lesser extent by **3** (see Table 5). Anti-malarial activity was shown by none of the isolates. All compounds were also found to be inactive in both HIV-1-RT and TK inhibition assays. The above findings tend to support the contention that many of the highly conjugated molecules found in lichens have other functions for the organism, more than likely a UV absorbing and/or protective role (Rundel, 1978; Elix, 1993).

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