Pergamon

Tetrahedron 56 (2000) 1393–1395

TETRAHEDRON

Glycolipids from Sponges. Part 8:[1] Plakopolyprenoside from the Marine Sponge *Plakortis simplex*. An Improved Procedure for Isolation of Glycolipids as Peracetyl Derivatives

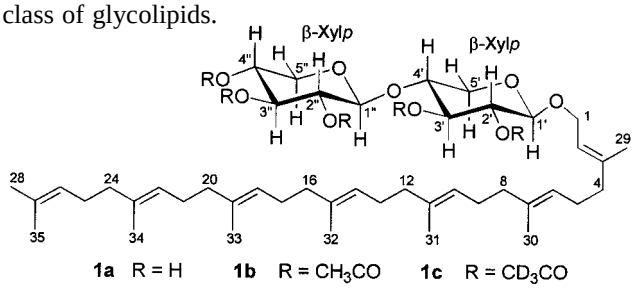
Valeria Costantino,[a] Ernesto Fattorusso,[a,*] Alfonso Mangoni,[a] Massimo Di Rosa[b] and Angela Ianaro[b]

[a]*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli 'Federico II', via D. Montesano 49, I-80131 Napoli, Italy*
[b]*Dipartimento di Farmacologia Sperimentale, Università di Napoli 'Federico II', via D. Montesano 49, I-80131 Napoli, Italy*

Received 15 September 1999; revised 8 December 1999; accepted 23 December 1999

Abstract—Plakopolyprenoside, a unique cytotoxic glycolipid composed of a C_{35} linear polyisoprenoid alcohol and a dixylosyl carbohydrate chain was isolated from the Caribbean sponge *Plakortis simplex* as its pentaacetate. Because the isolation of native plakopolyprenoside was unfeasible, acetylation with trideuteroacetic anhydride was used to ensure that no acetyl group was already present in the natural compound. The structure of plakopolyprenoside was established from spectral data. Plakopolyprenoside is cytotoxic against the J774 cell line.

An increasing number of new glycolipids from marine sponges are appearing in the literature.[1–4] Many of them are glycosphingolipids, such as the α-Gal-GSLs from *Agelas* sponges[2] and the plakosides from *Plakortis simplex*.[3] These compounds possess interesting biological properties, particularly as regulators of the immune system of mammals. In addition, a few unusual glycolipids with no counterparts in other phyla have been found in the Porifera, and these compounds are also active on the immune system. In particular, simplexides from *P. simplex* are immunosuppressors[1] and erylusamines from *Erylus placenta* are inhibitors of the interleukin-6 receptor.[4] As a part of our continuing study of sponge glycolipids, we wish to report here the isolation and structure determination of plakopolyprenoside (**1a**), a cytotoxic glycolipid from *P. simplex* composed of a C_{35} linear polyprenyl alcohol and a dixylosyl carbohydrate chain, that is the first member of another new class of glycolipids.



1a R = H **1b** R = CH_3CO **1c** R = CD_3CO

Specimens of *P. simplex* from the coast of Berry Island (Bahamas) were extracted, in sequence, with MeOH and $CHCl_3$. Following our usual procedure,[1] the glycolipid fraction was obtained by partitioning the extract between water and *n*-BuOH, and subjecting the organic phase to sequential column chromatography with RP-18 and normal silica gel.

In spite of our efforts, we were unable to isolate compound **1a** in the pure form by HPLC from this fraction. It was therefore acetylated with Ac_2O in pyridine. In our experience, working on peracetylated glycolipids is often convenient. Otherwise inseparable mixtures of glycolipids can be separated after acetylation, and also structural elucidation can be performed more easily on peracetylated glycolipids. In addition, the acetylation reaction is easily reversible, so that the natural glycolipid can be obtained after purification is completed. After acetylation, the glycolipid mixture from *P. simplex* was subjected to repeated direct-phase HPLC purification leading to 2.3 mg of pure peracetylated plakopolyprenoside **1b**.

A major drawback of this procedure, however, is the uncertainty regarding the actual structure of the natural glycolipid, because if any acetyl group is already present in the natural compound it cannot be distinguished from those introduced during the acetylation reaction. In our previous work, we could overcome this difficulty by analyzing the ^1H NMR spectrum of the non-acetylated glycolipid mixture in search of characteristic signals of the fully deacetylated glycolipid. Because of the small amounts of plakopolyprenoside in *P. simplex*, this was not possible. Therefore, we developed a simple and more general method to the same end.

Keywords: glycolipids; marine metabolites; terpenes and terpenoids; natural products.
* Corresponding author. Tel.: +39-081-7486-503; fax: +39-081-7486-552; e-mail: fattoru@unina.it

0040–4020/00/$ - see front matter
PII: S0040-4020(00)00018-1

The purification procedure of a small portion of the crude glycolipid fraction was carried out using trideuteroacetic anhydride instead of acetic anhydride in the acetylation step, and 0.5 mg of compound **1c**, chromatographically indistinguishable from **1b**, was obtained. The ^{1}H spectra of the pertrideuteroacetylated compound **1c** and the peracetylated compound **1b** were nearly identical, except that none of the five acetyl methyl singlets were present in the former spectrum. Consequently, no acetyl group was present in the natural plakopolyprenoside **1a**. Compound **1a** could be obtained from the derivatives **1b** and **1c** by deacetylation with Et_3N/MeOH.[3]

The negative-ion FAB mass spectrum of compound **1a** showed a pseudomolecular ion peak $[M-H]^-$ at *m*/*z* 757.5289, accounting for the molecular formula $C_{45}H_{74}O_9$. The ^{1}H NMR spectrum (pyridine-d_5) displayed two signals of anomeric protons at δ 4.71 and 4.57, thus showing that two sugar units were present in **1a**. Other notable signals in the spectrum were several methyl singlets between δ 1.65 and 1.56, and two prominent bands of allylic protons centered at δ 2.16 and 2.06, while the large signal of methylene protons of long, saturated alkyl chains at δ 1.29 typical of acetate-derived glycolipids was completely absent. All these data pointed to plakopolyprenoside being a diglycosylated polyprenyl compound.

All the subsequent NMR experiments devoted to structure elucidation were more conveniently performed using the peracetyl derivative **1b**.[5] The saccharide part was considered first. The COSY spectrum was used to identify protons belonging to the two sugars (Table 1), and both of them proved to be pentoses. The sugar with the anomeric proton resonating at δ 4.11 was identified as a terminal β-xylopyranose, because (a) deshielded chemical shifts of H-2″, H-3″, and H-4″ were characteristic of acetoxymethine protons, and therefore the relevant position were not involved in glycosidic linkages and (b) H-1″, H-2″, H-3″, and H-4″ showed a coupling constant pattern typical of protons axially oriented in a six-membered ring. As for the other sugar, it was also identified as a β-xylopyranose using similar reasoning; in addition, the shielded chemical shift of H-4′ (δ 3.59) indicated that this position was glycosylated, rather than acetylated. The linkage between the two sugars was confirmed by the coupling constant of H-1″ with C-4′, evidenced by the HMBC spectrum, and by the ROESY correlation of H-1″ with H-4′.

Structure of the lipid moiety of the molecule was established as follows. Consideration of the molecular formula showed that the aglycone contains 35 carbon atoms and no heteroatom, except for the oxygen atom involved in the glycosidic bond. Combined analysis of the ^{1}H and ^{13}C NMR spectra of **1b** evidenced eight methyl groups on tetrasubstituted sp^2 carbon atoms and seven trisubstituted double bonds. These latter functionalities accounted for all the remaining formal unsaturations implied by the molecular formula, so the aglycone must be acyclic. In addition, most ^{1}H and ^{13}C resonances of the aglycone were arranged in clusters of close signals, thus suggesting a repetitive structure. These data are in accordance with a geranyl-like polyprenyl chain composed of seven units, as depicted in structure **1a**, and all the ^{1}H–^{1}H and ^{1}H–^{13}C couplings evinced by the COSY and HMBC spectra were in accordance with this structure. The stereochemistry of double bonds was deduced by ^{13}C chemical shifts, all very similar to those reported for linear all-*E* terpenes.[6] Finally, the linkage between the aglycone and the sugar chain was demonstrated by the HMBC correlation peak between the anomeric proton H-1′ and the oxymethylene carbon atom C-1.

Plakopolyprenoside represents another unprecedented kind of glycolipids from marine sponges, having no close analogue in any phylum of living organisms. Glycosides of sesquiterpenes, diterpenes, and triterpenes are frequently found in plants and marine animals. Plakopolyprenoside, however, is quite different from these metabolites in that it contains a linear long-chain polyprenoid aglycone, that makes it much more similar to fatty-acid-containing glycolipids from the biological point of view.

Plakopolyprenoside **1a** was tested for cytotoxic activity on the J774 (murine monocyte/macrophage) cell line. It inhibited the cell growth evaluated at 72 h with an IC_{50} of 4.54 μg/ml. Evaluation of a possible immunological activity of plakopolyprenoside is currently in progress.

Table 1. NMR data of compound **1b** (spectra recorded in C_6D_6. Assignment based on COSY, HMQC, HMBC, and ROESY spectra)

Pos.	δ_H [mult., *J* (Hz)]	δ_C (mult.)
1		
a	4.35 (br. dd, 12.1, 6.0)	65.1 (CH_2)
b	4.13 (br. dd, 12.1, 7.4)	65.1 (CH_2)
2	5.47 (br. dd, 7.4, 6.0)	120.3 (CH)
3	–	141.0 (C)
4	2.01 (br. t, 7.1)	39.8 (CH_2)
5	2.11[a]	26.7 (CH_2)
6	5.22[a]	124.2 (CH)
7	–	135.5 (C)
8, 12, 16, 20, 24	2.09[a]	40.2 (CH_2)
9, 13, 17, 21, 25	2.20[a]	27.2 (CH_2)
10, 14, 18, 22	5.29[a]	124.8 (CH)
11, 15, 19, 23	–	135.0 (C)
26	5.23[a]	124.5 (CH)
27	–	131.0 (C)
28	1.68 (s)	25.7 (CH_3)
29	1.52 (s)	16.4 (CH_3)
30	1.57 (s)	16.2 (CH_3)
31, 32, 33, 34	1.62–1.62 (4 singlets)	16.2 (CH_3)
35	1.57 (s)	17.6 (CH_3)
1′	4.36 (d, 7.3)	100.0 (CH)
2′	5.29[a]	71.8 (CH)
3′	5.37 (t, 9.0)	73.5 (CH)
4′	3.59 (ddd, 9.8, 9.0, 5.3)	75.5 (CH)
5′		
a	3.75 (dd, 11.7, 5.3)	62.7 (CH_2)
b	2.97 (dd, 11.7, 10.0)	62.7 (CH_2)
1″	4.11 (d, 6.3)	100.1 (CH)
2″	5.02 (dd, 7.9, 6.3)	70.7 (CH)
3″	5.28[a]	70.9 (CH)
4″	4.90 (ddd, 8.0, 7.7, 4.8)	68.6 (CH)
5″		
a	3.87 (dd, 11.8, 4.8)	61.4 (CH_2)
b	3.06 (dd, 11.8, 8.0)	61.4 (CH_2)
Ac's		
CH_3	1.60, 1.67, 1.71, 1.79, 1.80	20.0–20.4
CO	–	170.0–170.2

[a] Overlapping signal.

Experimental

General methods

FAB-MS spectra were performed in a glycerol matrix on a VG Prospec-Autospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin–Elmer 192 polarimeter using a 10 cm microcell. ^{1}H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal (C_6D_6: δ_H=7.15, δ_C=128.0; CD_5N: δ_H=8.71, 7.56, and 7.19, δ_C=149.8, 135.3, and 123.4). For an accurate measurement of the coupling constants, the one-dimensional ^{1}H NMR spectra were transformed at 64 K points (digital resolution: 0.09 Hz). Homonuclear ^{1}H connectivities were determined by the COSY experiment. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the interpulse delays were adjusted for an average $^1J_{CH}$ of 142 Hz. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiment was optimized for a $^3J_{CH}$ of 8.3 Hz. High performance liquid chromatographies (HPLC) were achieved on a Varian 2510 apparatus equipped with a Varian Star 9040 refractive index detector.

Collection, extraction and isolation

Specimens of *P. simplex* were collected in the summer of 1998 near the coast of Berry Island (Bahamas), and identified by Prof. M. Pansini (University of Genoa, Italy). They were frozen immediately after collection and kept frozen until extraction. Reference specimens were deposited at the Istituto di Zoologia, University of Genoa, Italy. The sponge (64 g of dry weight after extraction) was homogenized and extracted with methanol (3×1 L) and then with chloroform (2×1 L); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated in vacuo and afforded 20.4 g of a dark brown oil, which was chromatographed on a column packed with RP-18 silica gel. A fraction eluted with $CHCl_3$ (2.7 g) was further chromatographed on a SiO_2 column, giving a fraction [450 mg, eluent EtOAc/MeOH (9:1)] mainly composed of glycolipids. This glycolipid fraction was peracetylated with Ac_2O in pyridine for 12 h and separated by repeated normal-phase HPLC [eluents: *n*-hexane/EtOAc (6:4) and *n*-hexane/*i*-PrOH (9:1)], affording pure plakopolyprenoside pentaacetate **1b** (2.3 mg). Natural (non acetylated) plakopolyprenoside **1a** was obtained in quantitative yield by keeping compound **1b** in a mixture of 800 μL of MeOH and 200 μL of Et_3N for 24 h at 60°C.

Plakopolyprenoside (1a). Amorphous solid, $[\alpha]_d$=−9 (*c*=0.1); HRFAMBS (negative ions): *m/z* 757.5289 ($[M-H]^-$, $C_{45}H_{73}O_9$ gives 757.5255); ^{1}H NMR (500 MHz, pyridine-d_5): δ 5.56 (1H, br. t, *J*=6.3 Hz, H-2), 5.26–5.18 (6H, m, H-6, H-10, H-14, H-18, H-22, and H-26), 4.93 (1H, d, *J*=7.6 Hz, H-1″), 4.71 (1H, d, *J*=7.6 Hz, H-1′), 4.57 (1H, dd, *J*=10.0 and 7.2 Hz, H-1a), 4.46 (1H, dd, *J*=11.3 and 5.2 Hz, H-5′a), 4.33 (2H, m, H-1b and H-4′), 4.30 (1H, dd, *J*=11.3 and 5.2 Hz, H-5″a), 4.17 (2H, m, H-3′ and H-4′), 4.11 (1H, t, *J*=8.3 Hz, H-3″), 4.03 (1H, t, *J*=8.0 Hz, H-2″), 4.02 (1H, t, *J*=8.0 Hz, H-2′), 3.64 (1H, t, *J*=9.4 Hz, H-5″b), 3.66 (1H, t, *J*=9.4 Hz, H-5′b), 2.20–2.07 (overlapping, H_2-5, H_2-9, H_2-13, H_2-17, H_2-21, H_2-25), 2.10–1.99 (overlapping, H_2-4, H_2-8, H_2-12, H_2-16, H_2-20, H_2-24), 1.65 (3H, s, H_3-28), 1.62 (12H, s, H_3-31, H_3-32, H_3-33, and H_3-34), 1.60 (3H, s, H_3-29), 1.58 (3H, s, H_3-30), 1.56 (3H, s, H_3-35); ^{13}C NMR (125 MHz, pyridine-d_5): δ 124.7 (CH, C-26), 124.5 (CH, C-10, C-14, C-18, and C-22), 124.3 (CH, C-6), 121.3 (CH, C-2), 103.9 (CH, C-1′), 103.7 (CH, C-1″), 77.9 (CH, C-3″), 76.6 (CH, C-4′), 76.6 (CH, C-3′), 74.6 (CH, C-2′), 73.7 (CH, C-2″), 70.8 (CH, C-4″), 67.3 (CH_2, C-5″), 65.7 (CH_2, C-1), 64.5 (CH_2, C-5′), 39.8–39.6 (CH_2, C-4, C-8, C-12, C-16, C-20, and C-24), 26.8–26.7 (CH_2, C-5, C-9, C-13, C-17, C-21, and C-25), 25.6 (CH_3, C-28), 17.5 (CH_3, C-35), 16.2 (CH_3, C-29), 15.9 (CH_3, C-30, C-31, C-32, C-33, and C-34).

Plakopolyprenoside pentaacetate (1b). Colorless oil, $[\alpha]_d$=+19 (*c*=0.1); ^{1}H and ^{13}C NMR: Table 1.

Acknowledgements

This work is the result of research supported by MURST PRIN 'Chimica dei Composti Organici di Interesse Biologico', Rome, Italy. We wish to thank Prof. Joseph R. Pawlik for giving us the opportunity to participate in an expedition to the Caribbean Sea, during which the sponge *P. simplex* was collected, and Prof. M. Pansini (University of Genoa, Italy) for identifying the sponge. Mass and NMR spectra were recorded at the 'Centro Interdipartimentale di Analisi Strumentale', Università di Napoli 'Federico II'. The assistance of the staff is gratefully acknowledged.

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