Glycolipids from Sponges. 6.[1] Plakoside A and B, Two Unique Prenylated Glycosphingolipids with Immunosuppressive Activity from the Marine Sponge *Plakortis simplex*

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Abstract: Plakoside A (**1a**) and B (**2a**), two unique glycosphingolipids belonging to a new class of prenylated glycolipid, have been isolated from the marine sponge *Plakortis simplex*, and their structures have been determined by spectroscopic and chemical means. Plakosides are strongly immunosuppressive on activated T cells and proved to be useful natural models for an improved comprehension of the structural requirements for immunomodulating activity of glycosphingolipids.

The fundamental task of the mammalian immune system is to provide protection against pathogens. In some cases it is advisable to modulate the immune response pharmacologically by inducing a non-antigen dependent stimulus either to elicit or to reduce the immune response. Therefore, there is currently an increasing interest in finding natural or synthetic compounds possessing immunostimulating or immunosuppressive activity. Natural glycosphingolipids (GSLs), which are mainly located in the cell membrane and are frequently involved in immunological processes, are a particularly suitable target for this kind of research.

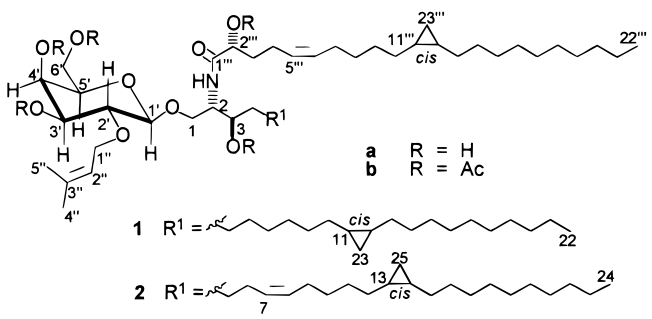
Agelasphins, α-galactosylceramides isolated from the sponge *Agelas mauritiana*, were recently shown to possess an interesting immunostimulating activity.[2] Subsequent studies investigated the relationship between structure and activity of agelasphins and their synthetic analogues. In particular, both α- and *β*-glycosylceramides were shown to be immunostimulating agents, the α-isomers being more active.[3]

More recently, several additional cerebrosides have been isolated by our research group from sponges of the genus *Agelas*. They differ from agelasphins in having a more complex sugar head with a further glycosylated α-galactose. The immunostimulating properties of these compounds were studied, and their bioactivity has been found to be affected by a structural feature, namely the glycosylation at position 2 of the inner sugar, which cancels the immunostimulation.[4,5] These results prompted us to extend our search for new biologically active GSLs to species belonging to different genera.

In this paper we wish to report the isolation, the structural determination, and the biological activity of plakoside A (1a) and B (**2a**) from *Plakortis simplex*, two unique *β*-galactosylceramides whose galactose residues are alkylated at O-2 by a 3,3-dimethylallyl group. Plakosides are very interesting from a structural point of view, since they are the first example of GSLs with a prenylated sugar and also the first natural GSLs with a cyclopropane-containing ceramide. In addition, they are strong immunosuppressive agents and appear to be suitable model compounds for a more precise definition of the structural requirements for immunomodulating activity of cerebrosides.

Results

Specimens of *P. simplex* were extracted sequentially with MeOH and $CHCl_3$, and the combined extracts were partitioned between water and *n*-BuOH. The organic layer was subjected to chromatography on a RP-18 column and then on a SiO_2 column, yielding a fraction enriched in GSLs. In spite of our efforts, we were not able to obtain **1a** and **2a** in a pure state from this fraction. Therefore, the GSL fraction was subjected to acetylation, and subsequently to HPLC chromatography on a SiO_2 column, to give 47 mg of a mixture containing peracetylated plakosides, and also a completely different glycolipid, whose structure determination is currently in progress. Separation of these two kinds of glycolipids required Et_3N/MeOH deacetylation of the mixture followed by direct-phase HPLC chromatography, and this yielded 11 mg of a fraction entirely composed of plakoside A (**1a**), plakoside B (**2a**), and minor amounts of their homologues.



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(1) Part 5: Cafieri, F.; Fattorusso, E.; Mangoni, A.; Taglialatela-Scafati, O. *Gazz. Chim. It.* **1996**, *126*, 711.

(2) Natori, T.; Morita, M.; Akimoto K.; Koezuka, Y. *Tetrahedron* **1994**, *50*, 2771.

(3) Motoki, K.; Kobayashi, E.; Uchida, T.; Fukushima, H.; Koezuka Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 705.

(4) Cafieri, F.; Fattorusso, E.; Mahajnah, Y.; Mangoni, A. *Liebigs Ann. Chem* **1994**, 1186. Costantino, V.; Fattorusso, E.; Mangoni, A. *Liebigs Ann.* **1995**, 1471. Cafieri, F.; Fattorusso, E.; Mangoni, A.; Taglialatela-Scafati, O. *Liebigs Ann.* **1995**, 1477. Costantino, V.; Fattorusso, E.; Mangoni, A. *Liebigs Ann.* **1995**, 2133.

(5) Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A.; Maffia, P. *Tetrahedron* **1996**, *52*, 1573.

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In contrast with GSLs from *Agelas* species, isolated as inseparable mixtures of homologues, the composition of this mixture was relatively simple, so that reversed phase HPLC chromatography allowed separation of the components as chemically homogeneous and homologous GSLs. Compounds **1a** (5 mg) and **2a** (2 mg) were by far the most abundant components. The additional homologues, present in the mixture only in trace amounts, could not be characterized. The whole purification procedure was monitored using ^{1}H NMR spectroscopy in order to make sure that no functionalities were present in compounds **1a** and **2a** that could be affected by the acetylation and deacetylation steps.

The more abundant plakoside A **1a** was examined first. The negative-ion FAB mass spectrum of compound **1a** displayed a pseudomolecular ion peak at *m/z* 946, in accordance with the molecular formula $C_{57}H_{105}NO_9$. The ^{1}H NMR spectra as well as all the NMR experiments directed to structure elucidation were performed on the peracetylated derivative **1b** because of its more favorable NMR features, most of all less signal overlapping. A large signal at δ 1.26 (alkyl chain methylene protons) and several multiplets between δ 5.42 and 3.49 were indicative of a glycolipid. A D_2O exchangeable doublet at δ 6.59, attributable to the ceramide NH proton, suggested that **1b** was a glycosphingolipid. Only one anomeric carbon signal (δ 103.8) was present in the ^{13}C NMR spectrum of **1b**, indicative of a monoglycosylceramide.

Two methyl singlets at δ 1.76 and 1.68 (H_3-4″ and H_3-5″, respectively), very unusual for a glycosphingolipid, were also present in the ^{1}H NMR spectrum of **1b**. The COSY spectrum showed them to be long-range coupled with an olefinic proton at δ 5.34 (H-2″), which in turn was coupled with two oxymethylene protons at δ 4.26 and 4.21 (H_2-1″). These data were clearly indicative of a 3,3-dimethylallyl group that was ether-linked to a hydroxyl group of the glycolipid.

The nature of the sugar unit was unequivocally determined by NMR analysis. The anomeric proton doublet (δ 4.30, J=7.8 Hz) was identified from its correlation peak with the relevant carbon C-1′ in the HMQC spectrum. It was used in the analysis of the COSY spectrum as a starting point for the sequential assignment of the proton resonances in the monosaccharide unit, which turned out to be a hexose as one set of methylene protons and five methine protons were identified. As usual in acetylated glycolipids, the acylated oxymethines (H-3′, δ 4.90 and H-4′, δ 5.33) were readily distinguishable from the alkylated ones (H-2′, δ 3.49 and H-5′, δ 3.83) on the basis of their chemical shifts. These data were strongly suggestive of a 2−*O*-substituted pyranose. Coupling constant analysis (see Table 1) showed all the ring protons to be axial except for H-4′; thus the sugar residue was definitively identified as a β-galactopyranoside.

The unusual presence of the 3,3-dimethyallyl group as the substituent at O-2′ was established by the long range ^{1}H−^{13}C coupling constants of H-2′ with C-1″ detected through a HMBC experiment and by the dipolar coupling between H-2′ with H-1″b observed by a ROESY spectrum.

As for the ceramide part of the molecule, NMR spectroscopic analysis allowed us to determine its broad structural features, but full characterization required degradative chemical analysis. The long-chain base is a saturated dihydroxylated sphinganine (2-amino-1,3−alkanediol). In fact, the COSY spectrum showed the amide methine H-2 (δ_H 4.33, δ_c 50.8) to be coupled with the NH doublet at δ 6.59, the oxymethylene protons at δ 4.06 and 3.56 (H_2-1), and the oxymethine proton at δ 4.99 (H-3). The latter proton was in turn coupled with the methylene protons at δ 1.66 and 1.57 (H_2-4). The COSY and ROESY spectra also allowed identification of the fatty acid residue as a Δ^5-unsaturated α-hydroxyacid. The multiplet at δ 5.03 was attributed to H-2‴ because of its correlation peak with the doublet signal for NH-2 in the ROESY spectrum. Starting from this signal, the COSY spectrum allowed us to assign, in sequence, H_2-3‴ (δ 1.89, m), H_2-4‴ (δ 2.12, m), H-5‴ (δ 5.31, submerged), and H-6‴ (δ 5.42, m). The *cis* stereochemistry of the double bond was deduced from the chemical shift of C-7‴ (δ 27.1).[6] Both the fatty acid and the long chain base had to be unbranched, as only a 6H triplet at 0.88 was present in the methyl region of the ^{1}H spectrum of **1b**.

Table 1. NMR Data of Plakoside A Pentaacetate (**1b**) ($CDCl_3$)

pos.		δ_H (mult, J (Hz))	δ_C (mult)	
1	a	4.06 (dd, 11.3, 3.1)	68.9	(CH_2)
	b	3.56 (dd, 11.3, 3.7)		
2		4.33 (dddd, 9.3, 7.5, 3.2, 3.2)	50.9	(CH)
2-N*H*		6.59 (d, 9.3)		
3		4.99 (m)	72.6	(CH)
4	a	1.66 (m)	31.2	(CH_2)
	b	1.57 (m)		
5		1.32[a]	25.0	(CH_2)
6−8, 15−19, 14‴-19‴		1.26[a]	29.9−29.3	(CH_2)
9, 14, 8‴, 13‴		1.37[a]	29.9	(CH_2)
10, 13, 9‴, 12‴	a	1.37[a]	28.6	(CH_2)
	b	1.13 (m)		
11, 12, 10‴, 11‴		0.65 (m)	15.8	(CH)
20, 20‴		1.26[a]	31.8	(CH_2)
21, 21‴		1.28[a]	22.7	(CH_2)
22, 22‴		0.88 (t, 7.1)	14.1	(CH_3)
23, 23‴	a	0.56 (ddd, 8.3, 8.3, 4.3)	11.0	(CH_2)
	b	−0.34 (ddd, 5.1, 5.1, 4.3)		
1′		4.30 (d, 7.8)	104.1	(CH)
2′		3.49 (dd, 10.2, 7.8)	76.0	(CH)
3′		4.90 (dd, 10.2, 3.5)	72.7	(CH)
4′		5.33 (dd, 3.5, 1.0)	67.5	(CH)
5′		3.83 (ddd, 6.6, 6.6, 1.0)	70.6	(CH)
6′	a	4.11 (m)	61.4	(CH_2)
	b	4.09[a]		
1″	a	4.26 (dd, 11.2, 7.0)	69.8	(CH_2)
	b	4.21 (dd, 11.2, 7.1)		
2″		5.34[a]	120.7	(CH)
3″			137.6	(C)
4″		1.76 (s)	25.7	(CH_3)
5″		1.68 (s)	18.0	(CH_3)
1‴			169.7	(C)
2‴		5.03 (m)	73.9	(CH)
3‴		1.89 (m)	31.9	(CH_2)
4‴		2.12 (m)	22.6	(CH_2)
5‴		5.31[a]	127.4	(CH)
6‴		5.42 (m)	131.5	(CH)
7‴		2.00 (m)	27.3	(CH_2)
Ac's		2.14, 2.13, 2.07, 2.04, 2.02 (s)	170.4−169.8 21.0−20.6	(C) (CH_3)

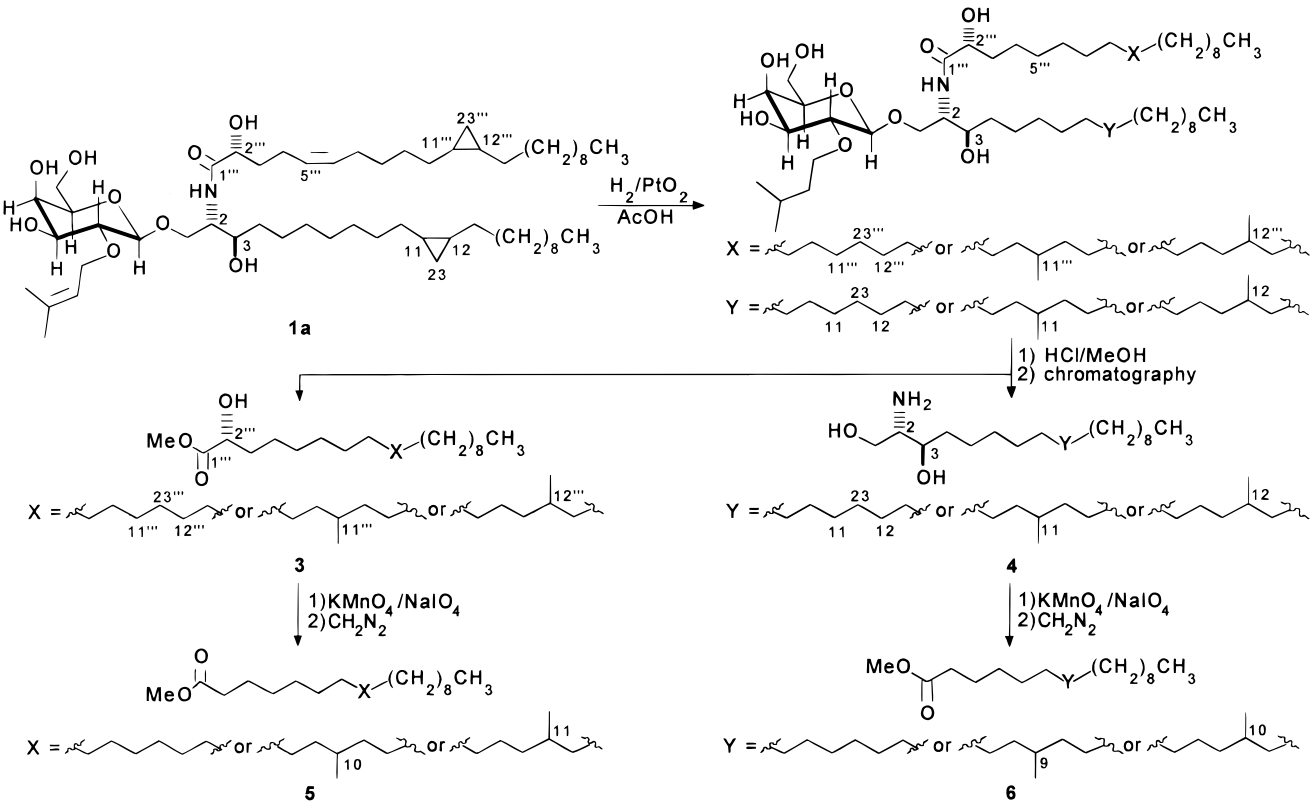
[a] Submerged by other signals.

In addition, the alkyl chains of the ceramide contained two cyclopropane rings, as shown by the three upfield shifted multiplets at δ −0.34 (2H), 0.56 (2H), and 0.65 (4H) in the proton spectrum of **1a**. Analysis of COSY and HMQC spectra permitted assignment of protons of the cyclopropane rings and of the two couples of adjacent methylene groups as reported in Table 1. The large difference between chemical shifts of the ring geminal methylene protons is a clear indication of the *cis* stereochemistry of the ring substituents.

Chemical degradation was necessary in order to locate the two cyclopropane rings, establish the length of the alkyl chains, and determine the stereochemical details of the molecule. To achieve this goal, we developed the submilligram scale degradation procedure outlined in Scheme 1, based on the widely used

(6) Gunston, F. D.; Pollard, M. R.; Scrimgeour, C. M.; Vedanayagam, H. S. *Chem. Phys. Lipids* **1977**, *18*, 115.

Scheme 1. Submilligram-Scale Procedure for Degradation of Plakoside A (**1a**)



procedure for determining the position of a cyclopropane ring in fatty acid methyl esters described by McCloskey and Law.[7] First, the cyclopropane rings were opened to give methyl branched alkyl chains; then, the GSL was subjected to methanolysis, and the α-hydroxy acids and the sphinganines were converted into methyl fatty esters to allow location of the methyl branching by GLC-MS. Thus, a small amount (200 μg) of plakoside A was subjected to reduction with H_2 and PtO_2 in acetic acid at room pressure. Under these conditions, the two double bonds are reduced, and each cyclopropane ring is reductively cleaved to give a mixture of one unbranched and two methyl-branched chains.[7] The reduced GSL was treated with methanolic HCl,[8] and the methanolysis product was separated into three fractions, containing α-hydroxyacid methyl esters (**3**), sphinganines (**4**), and methyl glycosides, respectively (see Experimental Section for details).

Lemieux oxidation ($KMnO_4/NaIO_4$) of the α-hydroxy acid methyl esters **3** caused the cleavage of the 1‴-2‴ bond, and the resultant fatty acids with one less carbon atom were methylated with diazomethane to give the esters **5**. The methyl ester mixture **5** was analyzed by GLC-MS and found to be composed of three esters derived from reductive cleavage of one cyclopropane ring, viz., methyl docosanoate, identified by comparison of its retention time and mass spectrum with those of an authentic sample, and an inseparable mixture of two methyl branched methyl heneicosanoates. Mass spectra of such mixtures have been studied in details, and it has been found that the methyl branches can be located unambiguously.[7] The relatively intense fragment peaks at *m/z* 199 and 171 originate from the favored α-cleavage with respect to the tertiary carbon atom of methyl 10-methylheneicosanoate. The latter fragment ion is accompanied by ions arising from rearrangement of one and two protons to this part of the molecule, resulting in diagnostic peaks at *m/z* 172 and 173. In addition, subsequent loss of methanol and water from the fragment at *m/z* 199 gives rise to characteristic peaks at *m/z* 167 and 149. Likewise, peaks at *m/z* 213, 187, 186, 185, 181, and 163 are indicative of methyl 11−methylheneicosanoate. Therefore the original α-hydroxy acid, which has one more carbon atom, had to be 11,12-methylene-2-hydroxy-5-docosenoic acid. The absolute configuration at C-2 was determined as *R* on the basis of the CD spectrum of the methyl esters **3** showing a negative Cotton effect at λ_{max} 214.5 nm ($\Delta\epsilon$ −0.95), which is a general feature of the 2*R*-hydroxy fatty acid derivatives.[9]

The positive-ion FAB mass spectrum of the sphinganine mixture 4 showed a pseudomolecular ion peak at *m/z* 372, in accordance with the molecular formula $C_{23}H_{49}NO_2$. A portion of **4** was subjected to oxidative cleavage with loss of two carbon atoms using $KMnO_4/NaIO_4$, and the obtained fatty acids converted to methyl esters with diazomethane. GLC-MS analysis of the reaction mixture allowed identification of methyl heneicosanoate, methyl 9−methyleicosanoate, and methyl 10-methyleicosanoate. The two branched esters were eluted together and were identified from their mass spectra (peaks at *m/z* 175, 159, 158, 157, 153, 135 and 199, 173, 172, 171, 167, 149, respectively).[7] These data allowed us to locate the second cyclopropane ring on the sphinganine chain at position 11,12. The 2*S*,3*R* stereochemistry of the long-chain base was established using a recently developed CD method.[10] The remaining portion of the sphinganine mixture **4** was converted into the naphthimido/bisnaphthoate derivative **7**, the CD spectrum of which proved to be identical with that reported for the same

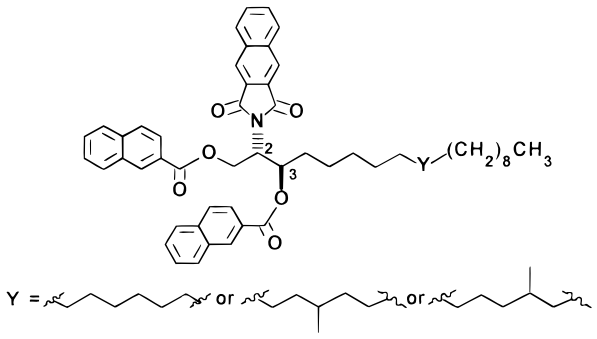
(7) McCloskey, J. A.; Law, H. L. *Lipids* **1967**, *2*, 225.

(8) We chose to perform the reduction prior to the methanolysis because methanolysis of the intact GSL led to incorporation of methanol in both the sphingosine and the fatty acid, apparently by electrophilic addition to the cyclopropane rings. This was also an indication that both the sphingosine and the fatty acid contained a cyclopropane ring.

(9) Cymerman Craig, J.; Pereira, W. E., Jr. *Tetrahedron* **1970**, *26*, 3457.

(10) Kawamura, A.; Berova, N.; Dirsch, V.; Mangoni, A.; Nakanishi, K.; Schwartz, G.; Bielawska, A.; Hannun, Y.; Kitagawa, I. *Bioorg. Med. Chem.* **1996**, *4*, 1035.

derivative of (2*S*,3*R*)-dihydrosphingosine [(2*S*,3*R*)-2-amino-1,3-octadecanediol].



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Therefore, the structure of plakoside A (**1a**) was established as (2*S*,3*R*,11*R**,12*S**)-1−*O*-[2−*O*-(3-methyl-2-butenyl)-β-D-galactopyranosyl]-2-[(1*R*,11*R**,12*S**)-1-hydroxy-11,12-methylene-5-docosenamido]-11,12-methylene-1,3-docosanediol. After structure determination was concluded, an extensive NMR analysis based on COSY and HMQC two-dimensional experiments was performed in order to assign all the resonances in the ^{1}H and ^{13}C NMR spectra of the nonacetylated **1a** (see Experimental Section).

The less abundant plakoside B (**2a**), which showed a pseudomolecular ion peak at *m/z* 972 in the negative ion FAB mass spectrum (molecular formula: $C_{59}H_{107}NO_9$), has two more carbon atoms and one more unit of unsaturation than **1a**. Its ^{1}H NMR spectrum was very similar to that of plakoside A, except for two olefinic proton signals at δ 5.52 and 5.50 which accounted for the additional degree of unsaturation. Degradation analysis of **2a** was performed with the same procedure used for **1a**, i.e., catalytic reduction (of double bonds and cyclopropane rings) followed by methanolysis and oxidation. The α-hydroxy acyl chain of plakoside B (**2a**) was found to be identical with that of plakoside A (**1a**). The fatty ester mixture obtained from oxidative cleavage of the sphinganine fraction was analyzed by GLC-MS as described above and found to be composed of methyl tricosanoate, methyl 11-methyldocosanoate, and methyl 12-methyldocosanoate. This pointed to a sphinganine with a C_{24} chain and a cyclopropane at position 13,14.

Analysis of the COSY spectrum of plakoside B (**2a**) allowed us to identify the sequential coupling of H-3 (δ 4.20), H_2-4 (δ 1.99 and 1.90), H_2-5 (δ 1.99 and 1.69), H_2-6 (δ 2.18), and the olefinic proton at δ 5.52 (H-7), thus locating the additional double bond at position 7 of the sphinganine. The long chain base of **2a** was therefore identified as 13,14-methylene-2-amino-7-tetracosene-1,3-diol. This was confirmed by $KMnO_4/NaIO_4$ oxidation of a small amount of the intact (nonreduced) plakoside B (**2a**). After diazomethane methylation, the reaction mixture was analyzed by GLC-MS, and the chromatogram displayed a single peak for a compound whose mass spectrum showed a molecular ion peak at *m*/*z* 296. This was attributed to the expected methyl 6,7-methyleneheptadecanoate, which was derived from the cleavage of both the double bond at position 5 of the acyl chain and the double bond at position 7 of the sphinganine chain.

Plakosides A and B were tested for immunomodulation activity using the T cell proliferation assay.[11] Surprisingly, while all the GSLs tested so far were found to be either immunostimulating or inactive, plakosides present an immunosuppressive activity. As shown in Figure 1, both compounds

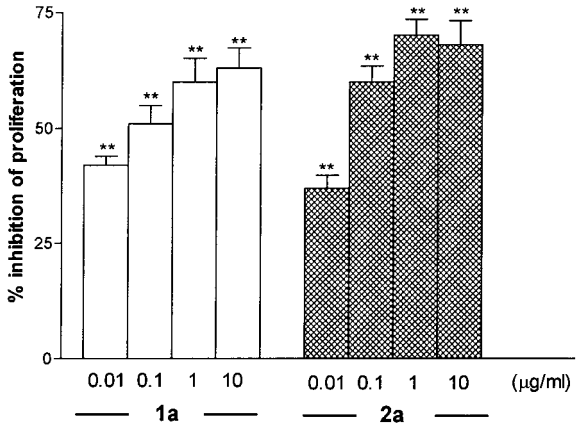

Figure 1. Inhibitory effect of plakoside A (**1a**) and B (**2a**) on lymph node cells proliferation. Cells were stimulated with Con A at 0.5 μg/mL for 24 h. Data are expressed as percent of inhibition of lymph node cells proliferation evaluated as [^{3}H]thymidine incorporation (cpm ± s.e.m., $n = 4$). Mean total cpm of Con A stimulated cells in absence of test compounds (control cells) was 575 ± 16, $n = 4$. **$P < 0.001$ vs control cells.

significantly ($P < 0.01$) inhibited the proliferative response of lymph node cells to Con A (0.5 μg/mL) at all doses tested (0.01−10 μg/mL). No significant difference was found between the two compounds. In addition, the MTT assay[12] was used in order to evaluate a possible cytotoxic activity of plakosides. Both compounds were found to be inactive, as no significant difference of respiratory metabolism was observed in lymph node cells treated with plakosides compared to control cells (data not shown).

Discussion

GSLs with a prenylated sugar have not been reported so far. Like the α-galactosyl ceramides from *Agelas* species, plakosides could be members of a very characteristic new class of GSLs from marine sponges, whose glycolipid metabolism is showing a surprising variety.[5,13]

The presence of cyclopropane rings on the ceramide part is another unprecedented feature of plakosides. In this connection, it is worth noting that, on account of the commonly accepted biogenesis of the sphinganines (addition of serine to a fatty acid followed by decarboxylation and reduction),[14] the fatty acyl and the sphinganine parts of plakoside B (**2a**) should be biosynthesized from the same very unusual 11,12-methylene-5-docosenoic acid. In the same way, the sphinganine of plakoside A (**1a**) could be derived from the more common 9,10-methyleneeicosanoic acid; however, the 2-hydroxy derivative of this acid is not found as the acyl part in any GSL from *P. simplex*. This seems to indicate a very high degree of specificity of the enzymes responsible of either α-hydroxylation or sphinganine acylation in *P. simplex*.

The presence of a prenylated galactose in plakosides is of great consequence with respect to their biological activity. All the unsubstituted monoglycosylceramides tested so far have proved to be immunostimulating,[3] irrespective of the nature of the sugar (glucose or galactose) and of the α or β stereochemistry of the glycosidic linkage. Immunostimulation activity was displayed also by GSLs from *Agelas* with an oligosaccharidic

(11) Ianaro, A.; Xu, D.; O′Donnel, C. A.; Di Rosa, M.; Liew, F. Y. *Immunology* **1995**, *84*, 8.

(12) Mosmann, T. *J. Immunol. Meth.* **1983**, *65*, 55.

(13) Costantino, V; Fattorusso, E.; Mangoni, A.; Aknin, M.; Fall, A.; Samb, A.; Mirailles, J. *Tetrahedron* **1993**, *49*, 2711. Costantino, V.; Fattorusso, E.; Mangoni, A. *J. Org. Chem.* **1993**, *58*, 186.

(14) Kanfer, J. N. In *Handbook of Lipid Research. 3. Sphingolipid Biochemistry*; Kanfer, J. N., Hakomori, S., Eds.; Plenum Press: New York, 1983; p 167.

carbohydrate chain, as the hydroxyl group at position 2 of the first sugar was not involved in a glycosydic linkage. In contrast, those GSLs from *Agelas* species in which this hydroxyl group is glycosylated were found to be inactive.[5] The immunological behavior of plakosides is different yet. Not only are they not immunostimulating agents but also they are effective immunosuppressors. This reversal of activity, which can reasonably be ascribed to the presence of the 2′-*O*-dimethylallyl group, demonstrates that not only glycosylation of the 2′-OH group but also its alkylation dramatically modifies the immunological activity of a monoglycosylceramide, confirming the crucial importance of this hydroxyl group for the immunological behavior of GSLs. Therefore, the interest toward GSL immunoactivity is now further increased, since they can act either as immunostimulators or as immunosuppressors depending on their carbohydrate moiety.

The immunosuppressive activity of plakosides is also interesting in itself, since unlike most immunosuppressors used clinically, it does not appear to be related to a cytotoxic effect, as shown by the negative response to the MTT assay. This suggests that plakosides as well as their analogues may be of potential interest as therapeutic agents.

Experimental Section

General Methods. Optical rotations were measured at 589 nm using a 10-cm microcell. ^{1}H and ^{13}C NMR spectra were determined at 500.13 and 125.77 MHz, respectively; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$; CD_5N: $\delta_H =$ 8.71, 7.56, and 7.19, $\delta_C = 149.8$, 135.3, and 123.4). Homonuclear ^{1}H connectivities were determined by COSY and HOHAHA (mixing time 100 ms) experiments. 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. The identification of fatty acid methyl esters was based on their GLC-MS retention times and GLC-MS spectra. A fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25% Ph Me silicone, 0.33-mm film thickness) was used. The temperature of the column was varied, after a delay of 5 min from the injection, from 220 to 300 °C with a slope of 3 °C min^{-1}. Quantitative determination was based on the area of the GLC peaks.

Collection, Extraction, and Isolation Procedure. Specimens of *Plakortis simplex* were collected in the summer of 1992 along the coast of Little San Salvador Island and identified by Prof. M. Pansini (University of Genoa). 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 (57 g of dry weight after extraction) was homogenized and extracted with methanol (3 × 2 L) and then with chloroform (3 × 2 L); the combined extracts were partitioned between H_2O and *n*-BuOH. The organic layer was concentrated *in vacuo* and afforded 15 g of a dark brown oil, which was chromatographed on a column packed with RP-18 silica gel. Three fractions (A−C) were collected [eluents: MeOH/H_2O (9:1), MeOH/EtOAc (9:1), and $CHCl_3$, respectively]. Fraction C (1.85 g), containing plakosides, was further chromatographed on a SiO_2 column. A fraction (230 mg) eluted with EtOAc/MeOH (9:1) was peracetylated with Ac_2O in pyridine for 12 h and separated by HPLC on a SiO_2 column [eluent: *n*-hexane/EtOAc (6:4)], thus affording a mixture (47 mg) containing plakosides. This fraction was deacetylated by keeping it in a mixture of 9 mL of MeOH and 1 mL of Et_3N for 72 h at 60 °C. HPLC separation on a SiO_2 column [eluent: *n*-hexane/*i*-PrOH (7:3)] of the deacetylated glycolipids yielded 11 mg of a mixture mainly composed of **1a** and **2a**. Finally, pure **1a** (5 mg) and **2a** (2 mg) were obtained by reversed-phase HPLC using MeOH as eluent.

Plakoside A (1a). Amorphous solid; $[\alpha]^{25}_D = +7$ (*c* 0.5, MeOH); FABMS (negative ion mode, triethanolamine matrix) *m/z* 946 ([M −H]$^-$), 878 ([M − isopentenyl]$^-$), 716 ([ceramide − H]$^-$); ^{1}H NMR (Py-d_5, 300 K) δ 8.29 (1H, d, $J = 9.3$ Hz, NH-2), 7.82 (1H, d, $J = 5.3$ Hz, OH-2‴), 6.78 (1H, d, $J = 5.4$ Hz, OH-3′), 6.59 (2H, m, H-1′, OH-6′ and OH-4′), 6.52 (1H, d, $J = 6.4$ Hz, H-3), 5.69 (1H, t, J = 6.5 Hz, H-2″), 5.60 (1H, ddd, $J = 10.5$, 7.3, and 7.3 Hz, H-5‴), 5.52 (1H, ddd, $J = 10.5$, 7.3, and 7.3 Hz, H-6‴), 4.82 (1H, dd, $J = 10.0$ and 4.7 Hz, H-1a), 4.74 (partly overlapped, d, $J = 7.7$ Hz, H-1′), 4.73 (overlapped, H-1″a), 4.73 (overlapped, H-2), 4.63 (1H, m, H-2‴), 4.58 (1H, dd, $J = 11.7$ and 7.3 Hz, H-1″b), 4.49 (1H, br.s, H-4′), 4.39 (2H, m, H-6′a and H-6′b), 4.20 (1H, m, H-3), 4.09 (overlapped, H-1b), 4.08 (overlapped, H-2′), 4.05 (overlapped, H-3′), 3.94 (1H, br t, $J = 5.9$ Hz, H-5′), 2.60 (2H, m, H_2−4‴), 2.33 (1H, m, H-3‴a), 2.18 (3H, overlapping H-3‴b and H_2-7‴), 1.93 (overlapped, H-4a), 1.93 (overlapped, H-5a), 1.89 (overlapped, H-4b), 1.60 (overlapped, H-5b), 1.58 (6H, s, H_3-4″ and H_3-5″), 1.34 (large band, H_2-8‴ and protons β to the cyclopropane ring), 1.25 (large band, long chain methylene protons), 0.85 (6H, t, $J = 7.3$ Hz, H_3-22 and H_3-22‴), 0.71 (4H, m, H-11, H-12, H-11‴, and H-12‴), 0.65 (2H, m, H-23a and H-23‴a), −0.23 (2H, m, H-23b and H-23‴b); ^{13}C NMR (Py-d_5) δ 174.9 (C, C-1‴), 134.9 (C, C-4″), 130.8 (CH, C-6‴), 129.6 (CH, C-5‴), 123.1 (CH, C-2″), 105.5 (CH, C-1′), 79.7 (CH, C-2′), 76.9 (CH, C-5′), 74.4 (CH, C-3′), 71.8 (CH, C-2‴), 71.2 (CH, C-3), 70.3 (CH, C-4′), 69.8 (CH_2, C-1), 69.5 (CH_2, C-1″), 62.2 (CH_2, C-6), 54.3 (CH, C-2), 35.7 (CH_2, C-3‴), 34.8 (CH_2, C-4), 32.8 (CH_2, C-20 and C-20‴), 30.1−29.5 (several CH_2), 28.9 (CH_2, C-10, C-13, C-10‴, and C-13‴), 27.6 (CH_2, C-7‴), 26.5 (CH_2, C-5), 25.6 (CH_3, C-4″), 23.7 (CH_2, C-4‴), 22.8 (CH_2, C-21 and C-21‴), 18.1 (CH_3, C-5″), 16.1 (CH_2, C-11, C-12, C-11‴, and C-12‴), 14.2 (CH_3, C-22 and C-22‴), 11.3 (CH_2, C-23 and C-23‴).

Plakoside A Pentaacetate (1b). Colorless oil; $[\alpha]^{25}_D = 0 \pm 1$ (*c* 0.5, MeOH); FABMS (negative ion mode, triethanolamine matrix) *m/z* 1156 ([M − H]$^-$); ^{1}H and ^{13}C NMR: Table 1.

Catalytic Reduction and Methanolysis of Plakoside-A (1a). A small amount (200 μg) of compound **1a** was dissolved in 1 mL of AcOH, and 3 mg of PtO_2 was added. The suspension was kept under H_2 at 1 atm for 12 h, then filtered, and taken to dryness. The residue was dissolved in 1 mL of 1 N HCl in 92% MeOH, and the obtained solution was kept for about 12 h at 80 °C in a sealed tube. The reaction mixture was dried under nitrogen and dissolved in a small quantity of $CHCl_3$, and the solution was passed through a SiO_2 (70−230 Mesh) column. Elution with 10 mL of 0.1% pyridine in $CHCl_3$ gave a mixture of α-hydroxy acid methyl esters (**3**), and subsequent elution with 0.1% pyridine in MeOH afforded sphinganines and methyl glycosides. The mixture was partitioned between $CHCl_3$ and H_2O/MeOH (8:2), and the organic and aqueous layers were separated and concentrated to give a mixture of sphinganines (**4**) and a mixture of methyl glycosides, respectively.

α-Hydroxy Fatty Acid Methyl Esters from 1a (3). EIMS *m/z* 384 ([M]$^+$); CD (EtOH) λ_{max} 214.5 nm ($\Delta\epsilon$ −0.95).

Oxidation of α-Hydroxy Fatty Acid Methyl Esters (3) and GLC-MS Analysis. To a solution of mixture **3** in 1 mL of *t*-BuOH were added 0.2 mL of a 0.04 M solution of K_2CO_3 (0.2 mL) and 0.8 mL of an aqueous solution of 0.023 M in $KMnO_4$ and 0.09 M in $NaIO_4$. The reaction was allowed to proceed at 37 °C for 18 h. After acidification with 5 N H_2SO_4, the solution was decolorized with a saturated solution of Na_2SO_3 and extracted twice with 4 mL of Et_2O. The combined extracts were dried over Na_2SO_4, and the resulting mixture of carboxylic acids was methylated with CH_2N_2 and analyzed by GLC-MS. The results were as follows. Methyl docosanoate: 16.3%; $t_R = 14.35$; t_R and MS spectrum were identical with those of an authentic sample. Methyl 10-methylheneicosanoate and methyl 11−methylheneicosanoate: 83.7%; $t_R = 13.05$; MS *m/z* (relative intensities) 354 (18), 213 (20), 199 (17), 187 (3), 186 (7), 185 (26), 181 (7), 173 (1), 172 (3), 171 (6), 167 (5), 163 (9), 149 (6).

Analysis of Sphinganines from 1a (4). A portion of the sphinganine mixture **4** from compound **1a** (positive ion FABMS: *m/z* 372) was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ as described above. GLC-MS analysis of the the resulting methyl esters gave the following results. Methyl heneicosanoate: 17.2%; $t_R = 12.25$; t_R and MS spectrum were identical with those of an authentic sample. Methyl 9-methyleicosanoate and methyl 10-methyleicosanoate: 82.8%; $t_R =$

11.01; MS *m/z* (relative intensities) 340 (16), 199 (16), 185 (6), 173 (2), 172 (4), 171 (6), 167 (7), 159 (2), 158 (3), 157 (11), 153 (6), 149 (9), 135 (5).

Derivatization of Sphinganines from 1a (4). A portion of the sphinganine mixture **4** and 3 mg of freshly sublimed 2,3-naphthalenedicarboxylic acid anhydride[10] were dissolved in 200 μL of anhydrous pyridine and refluxed under stirring for 12 h. The reaction mixture was purified by preparative TLC (*n*-hexane/EtOAc 1:1, strongly fluorescent band at $R_f = 0.60$). The obtained *N*-naphthimido derivative of sphinganines was dissolved in 200 μL of anhydrous MeCN, and 10 mg of 2-naphthoylimidazole and a catalytic amount of 1,8−diazabicyclo-[5.4.0]undec-7-ene (DBU) were added. The reaction mixture was stirred under argon for 2 h, then was dried up, and purified by preparative TLC (*n*-hexane/EtOAc 3:1, strongly fluorescent band at $R_f = 0.65$), thus obtaining the pure naphthimido/bisnaphthoate derivative **7**: CD (MeCN) λ_{max} 238.5 nm ($\Delta\epsilon$ +33.3), 260.0 (-42.3).

Plakoside B (2a). Amorphous solid; $[\alpha]^{25}_D = +7$ (*c* 0.2, MeOH); FABMS (negative ion mode, triethanolamine matrix) *m/z* 972 ([M −H]$^-$), 904 ([M - isopentenyl]$^-$), 742 ([ceramide - H]$^-$); ^{1}H NMR (Py-d_5, 300 K) Same resonances as those reported for **1a**, except for: two additional signals at δ 5.52 (overlapped, H-7) and 5.50 (overlapped, H-8), the multiplet at δ 2.18 which comprises four more protons (H_2-6 and H_2-9), and the shifted resonances at δ 1.99 (overlapped, H-4a), 1.99 (overlapped, H-5a), 1.90 (1H, m, H-4b), 1.69 (1H, m, H-5b); ^{13}C NMR (Py-d_5). Same resonances as those reported for **1a**, except for two additional CH signals at δ 130.2 and 130.3 (C-7 and C-8), the more intense CH_2 signal at δ 27.6 (CH_2, C-7‴, C-9, and C-6), and the shifted resonances at δ 34.4 (CH_2, C-4) and 26.7 (CH_2, C-5).

Analysis of α-Hydroxy Fatty Acid Methyl Esters from 2a. Same results as those reported for **1a**.

Analysis of Sphinganines from 2a. A portion of the sphinganine fraction from compound **2a** was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ and methylation as described above. GLC-MS analysis of the the resulting methyl esters gave the following results. Methyl tricosanoate: 18.6%; $t_R = 16.48$; t_R and MS spectrum were identical with those of an authentic sample. Methyl 11-methyldocosanoate and methyl 12-methyldocosanoate: 81.4%; $t_R = 15.10$; MS *m/z* (relative intensities) 368 (13, M$^+$), 227 (11), 213 (12), 201 (3), 200 (13), 199 (63), 195 (6), 187 (2), 186 (6), 185 (19), 181 (5), 177 (8), 163 (5).

Oxidation of Plakoside B (2a). Plakoside B **2a** (100 μg) was subjected to oxidative cleavage with $KMnO_4$/$NaIO_4$ and methylation as described above. Only one methyl ester was formed, as showed by GLC-MS analysis: $t_R = 6.28$; MS *m/z* (relative intensities) 296 (17, M$^+$).

T Cell Proliferation Assay. Male Swiss mice, 6−8 weeks old, obtained from Nossan (Italy), were housed in temperature-controlled rooms (22 ± 1 °C) and received food and water *ad libitum*. Single lymph node cell suspension was obtained from popliteus lymph nodes removed from mice killed with CO_2. Cells were suspended (2.5×10^6 cells/mL) in the RPMI-1640 culture medium containing 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/mL), streptomicin (100 μg/mL), and 2-mercaptoethanol (50 μM). The cell suspension was dispensed at 100 μL/well in 96-well flat-bottomed plates (Nunk, Roskilde, Denmark), stimulated with Concanavalin A (Con A; 0.5 μg/mL) in the presence or in the absence of the test compounds and incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% O_2. Cultures, in triplicates, were pulsed with 1 μCi/well [^{3}H]-thymidine (47 Ci/mmol, Amersham Intl., Amersham, UK) for the final 6 h of incubation, then harvested, and counted in a β-scintillation counter. Data are expressed as the mean ± s.e. mean; statistical analysis of the data was preformed using a Pharm/PCS computer program. Means were compared by Student's test for unpaired data.

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Supporting Information Available: ^{1}H and ^{13}C NMR and related COSY, HMQC, GE-HMBC, and ROESY of plakoside A pentaacetate **1b**; ^{1}H and ^{13}C NMR, COSY, and HMQC spectra and FABMS spectra of plakoside A (**1a**) and B (**1b**); MS spectra of the mixtures of methyl branched fatty acid methyl esters; CD spectra of the sphinganine derivative **7** and of the 2*R*-hydroxyacid methyl ester mixture **3** (18 pages). See any current masthead page for ordering and Internet access information.

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