Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 271–276

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Glycolipids from Sponges. VII.[1] Simplexides, Novel Immunosuppressive Glycolipids from the Caribbean Sponge *Plakortis simplex*

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

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

Received 5 November 1998; accepted 7 December 1998

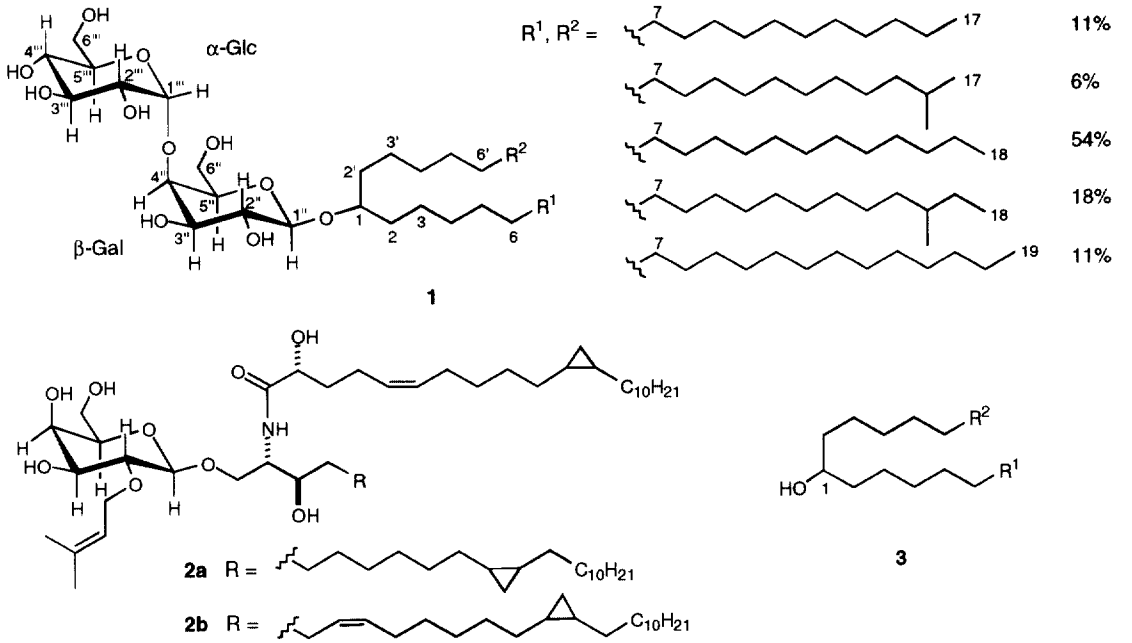
Abstract. The new glycolipids simplexides (**1**) have been isolated from the marine sponge *Plakortis simplex*, and their structure determined by spectroscopic data and microgram-scale chemical degradation. Simplexides are composed of long-chain secondary alcohols glycosylated by a disaccharide chain, and represent a new structural kind of glycolipids. Simplexides strongly inhibit proliferation of activated T-cells by a non-cytotoxic mechanism and can be regarded as simple model molecules for designing immunosuppressive drugs.

Keywords: Glycolipids, Immunosuppressive agents, Natural products, NMR.

Marine sponges are proving to be a rich source of unusual glycolipids, many of which are active on the immune system of mammals. Sponges of the genera *Agelas* and *Axinella* were shown to contain a unique class of glycosphingolipids with an α-galactose as the first sugar of the saccharide chain.[2] Such glycolipids are immunostimulating if the OH group at position 2 of the inner galactose is not glycosylated.[3] Due to their immunostimulating activity, agelasphins (α-galactosylceramides) and their synthetic analogue KRN-7000 showed a remarkable in vivo antitumor activity;[4] the latter compound is being currently tested as a candidate anti-cancer drug. More recently, our research group reported the isolation of plakoside A (**2a**) and B (**2b**), two prenylated glycosphingolipids showing potent immunosuppressive properties.[1] These unique glycolipids were isolated from the Caribbean sponge *Plakortis simplex*. The same organism is the source of simplexides (**1**), immunosuppressive glycolipids of a novel structural type.

*E-mail: fattoru@unina.it – FAX: (internat.)-39-081-7486552

0960-894X/99/$ - see front matter
PII: S0960-894X(98)00719-7



Specimens of *P. simplex* were extracted sequentially with MeOH and $CHCl_3$, and the extracts partitioned between water and *n*-BuOH. The isolation procedure, similar to that reported for plakosides,[1] involved reversed phase and then direct-phase column chromatography, acetylation, HPLC chromatography on SiO_2, and Et_3N/MeOH deacetylation. This led to a mixture entirely composed of plakosides and simplexides, which was separated by direct-phase HPLC chromatography ($CHCl_3$/*i*-PrOH 8:2), giving 3.9 mg of simplexides **1**, $[\alpha]^{25}_D$ = +13 (*c* = 0.4 in MeOH/$CHCl_3$ 1:1), pure by TLC and ^{1}H-NMR.

The ^{1}H NMR spectrum (pyridine-d_5) of simplexides **1** clearly showed them to be glycolipids. The presence of long alkyl chains was indicated by a large peak at δ 1.24, and a series of well-resolved signals in the midfield region of the spectrum were suggestive of sugars. The negative ion FAB mass spectrum of **1** (triethanolamine matrix) displayed a series of 14-amu-apart pseudomolecular ion peaks at m/z 859, 845, 831, and 817, in accordance with the molecular formula $C_{46}H_{90}O_{11}$ + nCH_2 (n = 0-3). In addition, in the methyl region of the ^{1}H NMR spectrum a complex signal at about δ 0.83 was present, originating from the superimposition of different methyl signals in a non-integral ratio. Examination of the methyl resonances in the ^{13}C NMR spectrum showed that these methyl groups belonged to linear (δ 14.2), iso (δ 22.5) and anteiso (δ 11.5 and 19.3) chains.[5] These data showed that simplexides, like most natural glycolipids, are a very complex mixture of homologues differing by the length and branching of their alkyl chains. In spite of our attempts, such a complex mixture could not be separated into individual compounds by reversed-phase HPLC. Therefore, we determined first the structure of

the polar part of the molecule from spectral data using the whole mixture, and then the exact composition of the alkyl chains by chemical degradation.

The ^{13}C NMR spectrum of simplexides **1** showed the presence of two anomeric carbon atoms (δ 104.6, C-1" and 102.6, C-1'''), and therefore of two sugar residues; the HMQC spectrum allowed identification of the respective anomeric protons 1"-H and 1'''-H. Using these protons as starting points, a COSY spectrum allowed the sequential assignment of four further methine protons and a couple of methylene protons for each sugar (Table 1), showing them to be hexoses. The large coupling constants of 2"-H with 1"-H (7.5 Hz) and 3"-H (9.5 Hz) indicated all these three protons were on a six-membered ring, in the axial orientation, while 4"-H, showing small coupling constants (2.9 and <1 Hz) was equatorial. As for the orientation of 5"-H, it was established as axial from its prominent correlation peak with the axial 1"-H in the ROESY spectrum. Therefore, the first sugar residue was a galactopyranoside in the β configuration. Also the second sugar residue was in the pyranose form, as shown by the large coupling constants between the axial protons 2'''-H and 3'''-H (9.4 Hz), 3'''-H and 4'''-H (9.4 Hz), and 4'''-H and 5'''-H (9.9 Hz); in contrast, the equatorial 1'''-H resonated as a close doublet (J = 3.8 Hz). Consequently, the second sugar residue was a glucopyranoside in the α-configuration.

Table 1. NMR Data of Simplexides **1** (500 MHz, Pyridine-d_5, 300K).

Pos.		δ_H (mult, J/Hz)[a]	δ_C (mult)[b]	
1		3.95 (q, 5.7)	79.9	(CH)
2	a	1.81 (m)	35.5	(CH_2)
	b	1.68 (m)		
2'	a	1.73[c]	34.5	(CH_2)
	b	1.73[c]		
3		1.48[c]	25.6	(CH_2)
3'		1.61[c]	25.3	(CH_2)
1"		4.83 (d, 7.5)	104.6	(CH)
2"		4.34 (dd, 9.5, 7.5)	72.9	(CH)
3"		4.15[c]	75.1	(CH)
4"		4.74 (br. d, 2.9)	79.7	(CH)
5"		4.14 (m)	75.0	(CH)
6"	a	4.69 (br.t. 9.9)	60.5	(CH_2)
	b	4.31[c]		
1'''		5.80 (d, 3.8)	102.6	(CH)
2'''		4.21[c]	74.1	(CH)
3'''		4.57 (t, 9.4)	74.9	(CH)
4'''		4.18[c]	72.1	(CH)
5'''		4.91 (ddd, 9.9, 5.9, 2.4)	74.7	(CH)
6'''	a	4.61 (dd, 11.5, 2.4)	62.5	(CH_2)
	b	4.29[c]		

[a] Additional ^1H signals: δ 1.24 [broad band, alkyl chain protons], 0.87 [t, J = 7.0 Hz, $-CH_2CH_2CH_3$], 0.87 [overlapping, $-CH(CH_3)_2$], 0.86 [overlapping, $-CH(CH_3)CH_2CH_3$], 0.85 [overlapping, $-CH(CH_3)CH_2CH_3$].

[b] Additional ^{13}C signals: δ 36.8 [$-CH_2CH(CH_3)CH_2CH_3$], 32.0 [$-CH_2CH_2CH_3$], 30.3-29.5 (several CH_2), 27.3 [$-CH_2CH_2CH(CH_3)CH_2CH_3$], 22.9 [$-CH_2CH_2CH_3$], 22.5 [$-CH(CH_3)_2$], 19.3 [$-CH(CH_3)CH_2CH_3$], 14.2 [$-CH_2CH_2CH_3$], 11.5 [$-CH_2CH_2CH_3$].

[c] Overlapping to other signals.

The two sugar residues accounted for the two formal unsaturations implied by the molecular formula and for all the oxygen atoms in the molecule. In addition, only a 1H quintet at δ 3.95 was present in the mid-field region of the proton spectrum apart from the signals from the saccharide chain. The COSY spectrum showed it to be coupled with two pairs of methylene protons (2-H_2, δ 1.81 and 1.68, and 2'-H_2, both at δ 1.73); each of them was coupled with a further methylene (δ 1.48, 3-H_2, and 1.61, 3'-H_2), which in turn was coupled with protons in the large band at δ 1.24. Therefore, the lipid part of the molecule was a long-chain secondary alcohol or,

considering that we are dealing with a mixture of homologues, a mixture of homologous long-chain alcohols, which are glycosylated by a disaccharide unit. Consideration of molecular formula indicated that the chains were very long, ranging from C_{34} to C_{37}.

Linkages between the two sugar residues and with the long-chain alcohol were determined from HMBC data. The $^3J_{CH}$ coupling of 4"-H with C-1"' indicated that the glucose residue was linked at position 4 of the galactose, which in turn glycosylated the OH group of the long chain alcohol as shown by the coupling of 1"-H with C-1.

Determination of the exact composition of the homologous alkyl chains and of the position of the hydroxyl group on the chain required a microscale degradative analysis. The free alcohols **3** were prepared by treating 200 μg of simplexides **1** with HCl in MeOH. The alcohols were converted to alkenes by tosylation with TsCl in pyridine, and subsequent treatment with DBU in toluene. Finally, the alkenes were cleaved with the Lemieux reagent ($KMnO_4/NaIO_4$) to carboxylic acids, which were methylated with CH_2N_2 and analyzed by GLC-MS. The results were as follows: methyl hexadecanoate: 5.7%; methyl 15-methylhexadecanoate: 2.5%; methyl heptadecanoate: 31.8%; methyl 16-methylheptadecanoate: 3.2%; methyl 15-methylheptadecanoate: 9.2%; methyl octadecanoate: 33.0%; methyl 16-methyloctadecanoate: 9.3%; methyl nonadecanoate: 5.3%.

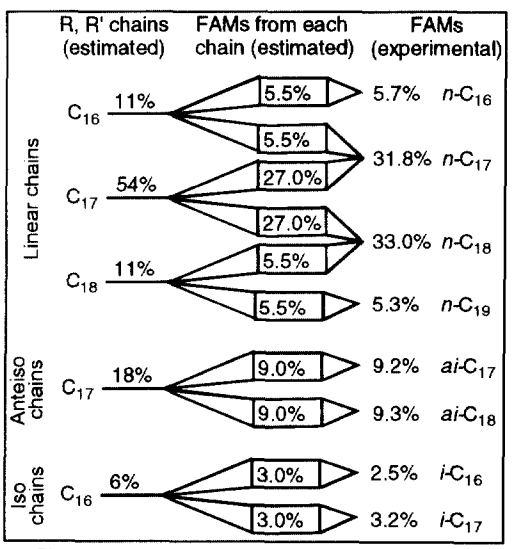
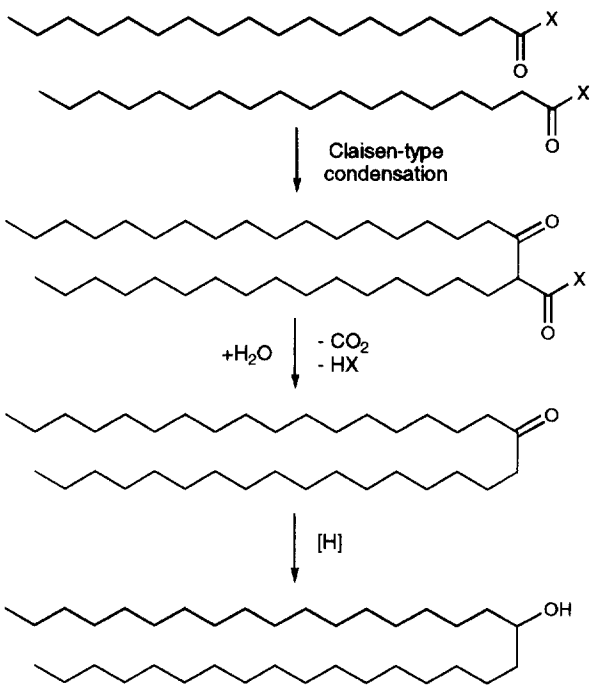


Figure 1. Estimation of composition of alkyl chains of simplexides **1** based on the fatty acid methyl esters (FAMs) isolated after degradation analysis.

Since the cleavage of **3** may occur either at the C-1/C-2 or at the C-1/C-2' bond with the same probability, any alkyl chain linked to C-1 gives rise to an equimolar amount of two carboxylic acids, one comprising and the other one not comprising the carbinol carbon atom (see Figure 1). Accordingly, since the C_{17} and C_{18} anteiso carboxylic acid methyl esters were present in comparable amounts (9.2% and 9.3%, respectively), while neither the C_{16} nor the C_{19} homologues were present, we concluded that they derived from the same anteiso C_{17} chain, whose abundance was therefore estimated as 18%; likewise, both C_{16} and C_{17} iso esters derived from one iso C_{16} chain. The four C_{16}-C_{19} unbranched esters derived from three linear chains. The amount of C_{16} and C_{19} esters, deriving only from the C_{16} and C_{18} chains, respectively, were used to quantify these latter chains; the amount of the C_{17} chain was calculated by difference from that of C_{17} or C_{18} ester. The calculated abundance of each alkyl chain is reported in Figure 1.



Scheme 1. Proposed biosynthesis of the lipid part of simplexides **1** (X = SCoA or any other acyl carrier).

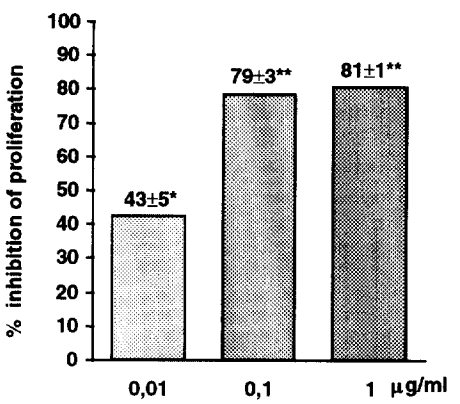


Figure 2. Inhibitory effect of simplexides **1** on lymph node cell proliferation. Cells were stimulated with ConA at 0.5 µg mL^{-1} for 72 h and proliferation was evaluated as [^{3}H]-thymidine incorporation. Data are expressed as % of inhibition of proliferation. * $P<0.05$ and ** $P<0.01$ versus control (ConA stimulated cells in absence of test compound)

Glycosylated long-chain secondary alcohols have never been reported as natural compounds, so that simplexides can be considered the first members of a new, simple class of glycolipids. The most similar examples are the glycosylated hydroxy fatty acids isolated from some yeast.[6] Another unusual feature of simplexides are the very long chains of alcohols **3**, which are composed of 34-37 carbon atoms with the hydroxyl group nearly in the middle. This suggests that the lipid part of simplexides could be biosynthesized from the coupling of two molecules of fatty acid. A reasonable biogenetic hypothesis is sketched in Scheme 1.

When tested[7] on murine immune-system T-cells stimulated with Concanavalin-A (ConA), simplexides **1** showed a potent inhibitory activity on their proliferation. Simplexides caused a 43% proliferation inhibition at a concentration as low as 10 ng/mL, which raised to 79% at 100 ng/mL (Figure 2). This immunosuppressive activity is not related to a cytotoxic activity, as shown by the negative response to the MTT assay[8] at all the concentrations tested.

The biological activity of simplexides is very similar to that of plakosides **2a-2b** from the same *P. simplex*.[1] Both compounds are potent immunosuppressors in vitro, and, more remarkably, they appear to be capable to effectively inhibit proliferation of T-cell without killing them. This similarity may appear rather surprising as plakosides and simplexides are remarkably different both in the aglycone and in the sugar chain. However, they share some broad

structural features, on which their ability to affect the immune system is based: two long alkyl chains and a hydrophilic head. While the alkyl chains are important in that they can firmly fix the molecule on the cell membrane, the specific biological activity of a glycolipid is mainly attributable to its sugar head, very probably through an interaction with the glycoproteins involved in the immune mechanisms. Even little variations in the structure and stereochemistry of the polar part of a glycolipid can be so important as to modify its immune activity.[1,4] However, the precise relationship between the structure of a glycolipid and its immunological activity is far from being fully understood.

Simplexides can be regarded as oversimplified, but still very active immunosuppressive glycolipids. Analogues of simplexides are easy to synthesize, and could be useful for the development of compounds of therapeutic interest as well as for a better comprehension of structure requirements for immunomodulating activity.

Acknowledgments. This work was sponsored by CNR and by MURST. We wish to thank Prof. W. Fenical 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.

References

1. Part VI: Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A. *J. Am. Chem. Soc.* **1997**, *119*, 12465-12470.
2. Costantino, V.; Fattorusso, E; Mangoni, A. *Liebigs Ann.* **1995**, 2133, and references therein.
3. Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A.; Maffia, P. *Tetrahedron* **1996**, *52*, 1573-78.
4. Motoki, K.; Kobayashi, E.; Uchida, T.; Fukushima, H.; Koezuka, Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 705.
5. Breitmaier, E.; Voelter, W. *^{13}C NMR Spectroscopy*, 2nd ed., VCH, Weinheim, **1978**, p.131-137.
6. Gunstone, F. D.; Harwood, J. L.; Padley, F.B. *The Lipid Handbook*, Chapman & Hall, London, **1994**, p. 39.
7. Ianaro, A.; Xu, D.; O'Donnel, C. A.; Di Rosa, M.; Liew,F.Y. *Immunology* **1995**, *84*, 8.
8. Mosmann, T. *J. Immunol. Meth.* **1983**, *65*, 55.