Bioorganic & Medicinal Chemistry 18 (2010) 5310–5315

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Terpioside B, a difucosyl GSL from the marine sponge *Terpios* sp. is a potent inhibitor of NO release ☆

Valeria Costantino [a], Ernesto Fattorusso [a], Alfonso Mangoni [a,*], Roberta Teta [a], Elisabetta Panza [b,c], Angela Ianaro [b]

[a] The NeaNAT group, Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, via D. Montesano 49, 80131 Napoli, Italy
[b] Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli Federico II, via D. Montesano 49, 80131 Napoli, Italy
[c] Department of Pharmaceutical, Toxicological and Biological Chemistry, University of Palermo, Palermo, Italy

ARTICLE INFO

Article history:
Received 19 January 2010
Revised 14 May 2010
Accepted 18 May 2010
Available online 24 May 2010

Keywords:
Fucose
Glycosphingolipid
NO release
Sponge
Structure elucidation

ABSTRACT

Terpioside B (**2a**), a unique glycolipid containing two fucose residues in the furanose form in its pentasaccharide chain, was isolated from the marine sponge *Terpios* sp. Its complete stereostructure was solved by interpretation of mass spectrometric and NMR data along with CD and GG–MS analyses of its degradation products. Terpioside B is a potent inhibitor against LPS-induced NO release, and is considerably more active than simpler glycosphingolipids such as terpioside A and monoglucosylceramide.

1. Introduction

Fucose-containing glycosphingolipids (fucosyl GSLs) are relatively common in mammals. The classical Lewis blood antigens are fucosyl GSLs, and fucosylation is one of the most common modifications of glycolipids observed in cancer cells.[2] Apart from mammals, fucosyl GSLs are mainly found in invertebrates, such as the millipede *Parafontaria laminata armigera*,[3] the brine shrimp *Artemia salina*,[4] and the sponge *Aplysinella rhax*.[5] The fucosylated triglycosylceramide produced by this last organism was shown to be an effective inhibitor of LPS-induced NO release, and this results were confirmed by a subsequent synthetic study.[6]

Most fucosylated GSLs are characterized by a terminal L-fucose residue in the pyranose form in the saccharide moiety of the molecule, and there are only three examples in the literature of natural GSLs with the fucose residue in the furanose form, all from marine invertebrates.[7,8] Among them is terpioside A, a diglycosylceramide isolated from the marine sponge *Terpios* sp.[9]

A recent reinvestigation of the GSL composition of *Terpios* sp. revealed the presence of an additional, more complex GSL, terpioside B (**2a**), which is a pentaglycosylated GSL characterized by the presence of two terminal α-L-fucofuranose units. The complete structure of terpioside B was investigated by interpretation of its MS and NMR spectra along with CD and GG–MS analyses of its degradation products. Following the previous results on inhibition of NO release by GSLs,[5,6] terpioside A and terpioside B were assayed for this activity. Terpioside B was shown to be more potent against LPS-induced NO release than previously reported GSLs.

2. Results

2.1. Isolation and purification

The extract of the marine sponge *Terpios* sp. collected around Key Largo (Florida) was partitioned between BuOH and H_2O. The organic fraction was purified by reversed-phase chromatography followed by normal-phase chromatography, yielding a crude fraction mainly composed of glycolipids. This fraction was acetylated with Ac_2O in pyridine and subjected to normal-phase HPLC, yielding terpioside B as its peracetyl derivative **2b** (3.8 mg). Because the NMR experiments directed to the structure elucidation are best performed on the peracetate derivative (it shows a wider dispersion of sugar resonances in the proton spectrum and allows discrimination between ether, δ 3.5–4.5, and ester, δ 4.7–5.7, oxymethine proton resonances) a complete set of 2D NMR experiments of **2b** was recorded. After this, the peracetylated terpioside B **2b** was deacetylated with MeOH/MeONa (8:2), giving 2.5 mg of the natural, non-acetylated terpioside B (**2a**).

☆ Glycolipids from sponges: 22. Ref. 1.

* Corresponding author. Tel.: +39 081 678532; fax: +39 081 678552.
E-mail address: alfonso.mangoni@unina.it (A. Mangoni).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.05.048

To make sure that none of the acetyl groups of **2b** had been present in the natural product **2a** before the acetylation reaction, a small portion of the glycolipid fraction was acetylated with trideuterioacetic anhydride instead of acetic anhydride. Purification of this fraction gave compound **2c**, which showed an ^{1}H NMR spectrum identical to that of **2b** except for the absence of all the acetyl methyl singlets.

2.2. Planar structure and relative configuration

The molecular formula of terpioside B **2a** was determined by interpretation of mass spectral data. The ESI mass spectrum contained a series of pseudomolecular ion peaks at *m/z* 1513.0, 1499.0, 1485.0, and 1470.9, indicating that **2a** is composed of a mixture of homologues, as observed for most glycolipids of marine origin. A high-resolution measurement provided the exact mass of the most abundant homologue at *m/z* 1498.9248, in accordance with the formula $C_{73}H_{137}NNaO_{28}$ ($[M+Na]^+$, calcd 1498.9219). The ESI mass spectrum of peracetylated terpioside B (**2b**) contained a series of $[M+H+Na]^{2+}$ pseudomolecular ion peaks at *m/z* 1104.0, 1111.2, 1118.2, and 1125.0, corresponding to the molecular weights 2162, 2176, 2190, 2204, respectively. This indicated the presence of 17 acetyl groups in compound **2b**.

The general features of the proton spectrum of peracetylated terpioside B (**2b**) clearly resembled those of glycolipids, as illustrated by (a) the large band of alkyl chain methylene protons at δ 1.27, (b) many overlapping oxymethine and oxymethylene protons between δ 3.4 and 5.6 and (c) one NH doublet at δ 6.65.

The ceramide portion of terpioside B is composed of a trihydroxylated, saturated sphinganine and a 2-hydroxy fatty acid. Using the amide proton as a starting point, analysis of the 2D COSY spectrum allowed identification of all the proton of the polar part of the sphinganine up to H_2-6. As for the ceramide fatty acid residue, a resonance at δ 5.13 (H-2^{VI}) in the ^{1}H NMR spectrum, instead of the characteristic triplet at δ 2.3 of the fatty acid α-protons, indicated the α-hydroxy substitution. This resonance showed in the ROESY spectrum an intense correlation peak with the amide NH doublet, confirming the –NHCOCHOH– functionality. In addition, both H-2^{VI} and 2-NH were shown to be coupled with the same CO carbon atom at δ 170.0 (C-1^{VI}) by the HMBC experiment.

Analysis of HSQC spectrum of terpioside B peracetate showed the sugar portion of the molecule to be composed by a pentasaccharide chain. In fact, resonances for the five anomeric protons (δ 4.41, 4.44, 4.95, 5.24, and 5.30) and the five relevant carbons (δ 100.4, 100.9, 99.0, 100.6 and 98.8, respectively) were identified in the spectrum.

Elucidation of the structure of the pentasaccharide chain was made challenging by the severe signal overlapping and the presence of two sugars in the furanose form. Decisive information came from the z-filtered version of the TOCSY 2D NMR experiment.[10] Starting from the relevant anomeric proton, the z-TOCSY experiment allowed us to identify the signals of each sugar, except for protons at positions 5 and 6 of sugar residues II and III: for these residues, the small coupling constant between H-4 and H-5 prevented coherence transfer beyond H-4. The ROESY correlation peak between H-3^{II} and H-5^{II} and that between H-3^{III} and H-5^{III} were used to complete the proton assignment for these residues.

Five methine protons and a couple of methylene protons were assigned to the first sugar residue (I) of the saccharide chain, suggesting a hexose; the shielded chemical shift of H-5^{I} showed if to be in the pyranose form. The linkage of this sugar residue with the sphinganine was demonstrated by the HMBC correlation peak of H-1^{I} (δ 4.41) with C-1 (δ 66.8) and that of H-1b (δ 3.58) with C-1^{I} (δ 100.4). This sugar is glycosylated at position 4^{I}, as shown by the high-field chemical shift of H-4^{I} (δ 3.84) and the correlation peak between H-1^{II} (δ 4.44) and C-4^{I} (δ 75.8) in the HMBC spectrum. The second sugar unit (II) also contains protons for a hexopyranoside, confirmed by the high-field chemical shift of H-5^{II}. The shielded chemical shift of H-4^{II} and H-6^{II} suggested glycosylation at these positions, which was confirmed by the HMBC correlation peaks between H-4^{II} and C-1^{III} and between H-6^{II} and C-1^{V}. The third and last hexopyranoside (III) is glycosylated at position 6, as shown by the shielded chemical shift of protons at C-6^{III} (δ 3.69 and 3.60) and by the HMBC correlation of H-1^{IV} with C-6^{III} and that of H-6$_a$^{III} and H-6$_b$^{III} with C-1^{IV}.

The glycosylating sugar is a 6-deoxyhexofuranoside (IV). In fact, the TOCSY spectrum showed for this sugar a spin system containing five methine protons and a characteristic methyl doublet at δ 1.17, which pointed to the presence at position 6 of a methyl group instead of the usual –CH_2OH group. The high-field chemical shift of H-4^{IV} (δ 3.89), together with the low-field chemical shift of H-5^{IV} (δ 4.97) clearly indicated that C-4^{IV} is involved in an acetal function rather than an ester function; thus, the sugar must be in the furanose form. The low-field chemical shifts of H-2^{IV}, H-3^{IV}, H-5^{IV} and H_2-6^{IV} showed that hydroxyl groups at positions 2, 3, 5 and 6 are all acetylated, and therefore that the sugar is a terminal one. Another 6-deoxyhexose is linked to the position 6 of the hexopyranoside (II). Its furanose form was argued by the HMBC correlation peak between H-1^{V} and C-4^{V} and by the chemical shift of H-4^{V} (δ 3.87). Also this residue is a terminal sugar, as shown by the chemical shifts of H-2^{V}, H-3^{V}, H-5^{V} and H_2-6^{V}.

The nature of the three hexopyranosides was easily ascertained by ^{1}H–^{1}H coupling constant analysis (Table 1). In the sugar residue (I), the large coupling constants between each vicinal pair of ring protons clearly indicated that all the ring proton are axial and consequently that this sugar is a β-glucopyranoside. The sugar residue (II) is a β-galactopyranoside; H-1^{II}, H-2^{II} and H-3^{II} are indeed axial (H-2^{II} is coupled to the vicinal protons with two large coupling constants), but H-4^{II} experiences two small couplings and is therefore equatorial. A similar pattern is observed for the couplings of the sugar residue (III), except for the small coupling constant between H-1^{III} and H-2^{III}, pointing to the equatorial orientation of H-1^{III} and therefore the α anomeric configuration of this sugar residue, which is therefore an α-galactopyranoside.

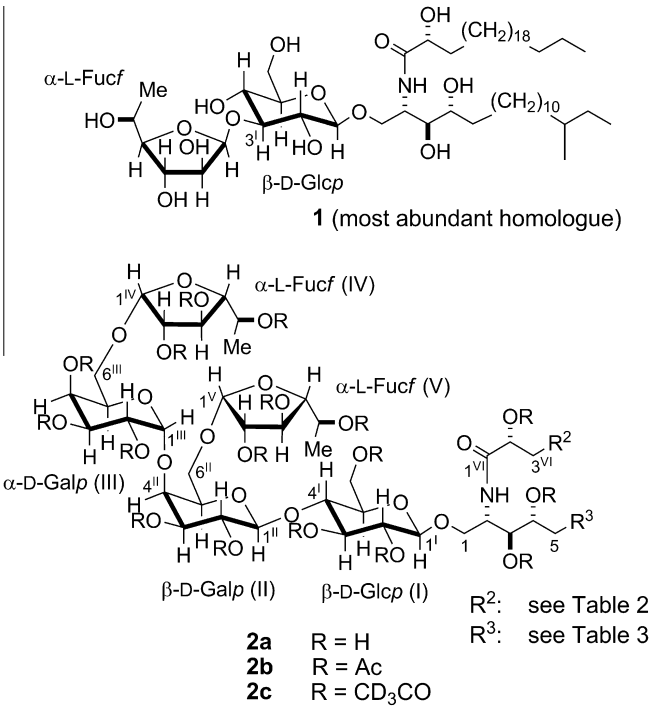


Figure 1. Structures of compounds **1** and **2a**–**2c**.

Table 1
^{1}H and ^{13}C NMR data of terpioside B peracetate (**2b**) ($CDCl_3$)

Position		δ_H (mult, J (Hz))[a]	δ_C (mult)[b]
1	a	3.85 (dd, 10.6, 2.9)[d]	66.8 (CH_2)
	b	3.58 (dd, 10.6, 3.5)[d]	
2		4.28[c]	48.0 (CH)
2-N*H*		6.65 (d, 8.9)	
3		5.11 (dd, 8.6, 3.0)[d]	71.3 (CH)
4		4.86 (ddd, 10.1, 3.0, 2.3)	73.2 (CH)
5		1.58[c]	28.0 (CH_2)
6	a	1.32[c]	25.6 (CH_2)
	b	1.18[c]	
1^I		4.41 (d, 7.9)	100.4 (CH)
2^I		4.80 (dd, 9.5, 7.9)	71.3 (CH)
3^I		5.11 (t, 9.5)[d]	72.2 (CH)
4^I		3.84 (t, 9.6)[d]	75.8 (CH)
5^I		3.59 (ddd, 9.7, 5.5, 1.9)[d]	72.9 (CH)
6^I	a	4.48 (dd, 12.0, 1.9)	62.0 (CH_2)
	b	4.10 (dd, 12.0, 5.5)	
1^{II}		4.44 (d, 7.8)	100.9 (CH)
2^{II}		5.03 (dd, 10.7, 7.8)[d]	69.0 (CH)
3^{II}		4.74 (dd, 10.7, 2.6)	73.0 (CH)
4^{II}		4.01 (br d, 2.6)	77.2 (CH)
5^{II}		3.64 (m)	74.1 (CH)
6^{II}	a	3.78 (dd, 11.6, 4.3)	66.1 (CH_2)
	b	3.73 (dd, 11.6, 6.5)	
1^{III}		4.95 (d, 3.3)	99.0 (CH)
2^{III}		5.15 (dd, 11.1, 3.3)[d]	68.9 (CH)
3^{III}		5.30[c]	67.4 (CH)
4^{III}		5.58 (br d, 3.2)	67.9 (CH)
5^{III}		4.29 (m)	67.0 (CH)
6^{III}	a	3.60 (dd, 10.6, 5.5)[d]	64.5 (CH_2)
	b	3.69 (dd, 10.6, 9.2)	
1^{IV}		5.24 (d, 4.5)	100.6 (CH)
2^{IV}		5.15 (dd, 7.0, 4.5)[d]	76.1 (CH)
3^{IV}		5.45 (dd, 7.0, 6.4)	74.4 (CH)
4^{IV}		3.89 (dd, 7.2, 6.4)[d]	81.3 (CH)
5^{IV}		4.97[c]	71.2 (CH)
6^{IV}		1.17 (d, 6.4)	15.5 (CH_3)
1^V		5.30 (d, 4.6)[d]	98.8 (CH)
2^V		4.95 (dd, 7.2, 4.6)[d]	77.0 (CH)
3^V		5.54 (dd, 7.2. 6.5)	73.6 (CH)
4^V		3.87 (dd, 7.0, 6.6)	80.9 (CH)
5^V		5.02[c]	70.4 (CH)
6^V		1.19 (d, 6.5)	15.8 (CH_3)
1^{VI}			170.0 (C)
2^{VI}		5.13[c]	74.0 (CH)
3^{VI}		1.83 (m)	31.8 (CH_2)
4^{VI}		1.33[c]	24.9 (CH_2)
Ac's		2.22–1.96	21.1–20.5
		—	171.1–168.8

[a] Additional ^{1}H signals: δ 1.24 (br, alkyl chain protons), 0.88 (t, J = 7.0 Hz, *n*-chain Me groups), 0.86 (d, J = 6.7 Hz, *iso*-chain Me groups), 0.85 (t, J = 7.1 Hz, *anteiso*-chain, Me group), 0.83 (d, J = 6.4 Hz, *anteiso*-chain, Me group).

[b] Additional ^{13}C signals: δ 39.0 (CH_2, *iso*-chain, ω-2), 36.6 (CH_2, *anteiso*-chain, ω-3), 34.4 (CH, *anteiso*-chain, ω-2), 31.9 (CH_2, *n*-chain, ω-2), 29.4 (CH_2, *anteiso*-chain, ω-1), 28.0 (CH, *iso*-chain, ω-1), 22.7 (CH_2, *n*-chain, ω-1), 22.7 (CH_3, *iso*-chain, Me group), 19.2 (CH_3, *anteiso*-chain, Me group), 14.1 (CH_3, *n*-chain, Me group), 11.4 (CH_3, *anteiso*-chain, Me group).

[c] Overlapped signal.

[d] Overlapping signal; multiplicities and coupling constants were determined from the z-filtered TOCSY spectrum.

Coupling constant analysis was not sufficient for an unambiguous assignment of the relative configuration of the two furanose deoxysugars. However, their ^{13}C chemical shifts and ^{1}H multiplicities were very close to each other and to the 6-deoxy-α-galacto-furanoside (α-fucofuranoside) residue present in terpioside A peracetate,[9] thus suggesting them to be also α-fucofuranosides. This was confirmed by chemical degradation analysis (see below).

2.3. Absolute configuration and alkyl chains

Chemical degradation allowed us to establish the nature of the two 6-deoxyhexofuranosides, the absolute configuration of all the stereogenic centers of the molecule, and the nature of the fatty acids and sphinganines present in compound **2a**. Most glycolipids from marine sponges indeed occur as complex mixtures of homologues, differing in the length and in the branching of the two alkyl chains of the ceramide. A small amount (600 μg) of terpioside B peracetate (**2b**) was subjected to acid-catalyzed methanolysis, giving a mixture of methyl glycosides, fatty acid methyl esters and phytosphingosines. A small portion of the reaction product was directly analyzed by GC–MS and was shown to contain a mixture of branched and linear 2-hydroxy fatty acid methyl esters (Table 2).

Table 2
Fatty acyl composition of terpioside B (**2a**)

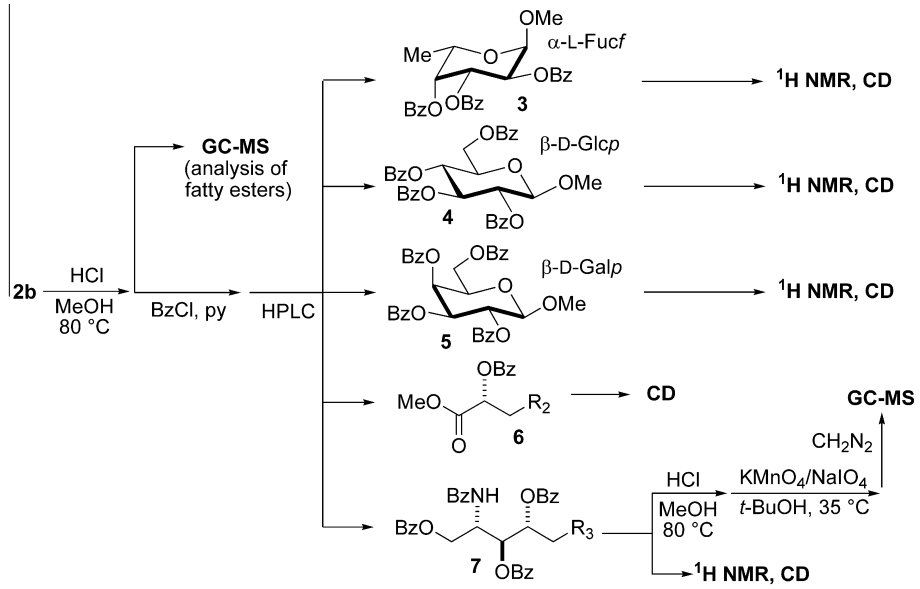
Fatty acid methyl ester	
Methyl 2-hydroxy-20-methylhenicosanoate (*iso*-C_{22})	3.4%
Methyl 2-hydroxydocosanoate (*n*-C_{22})	1.6%
Methyl 2-hydroxy-21-methyldocosanoate (*iso*-C_{23})	16.2%
Methyl 2-hydroxy-20-methyldocosanoate (*anteiso*-C_{23})	4.3%
Methyl 2-hydroxytricosanoate (*n*-C_{23})	4.4%
Methyl 2-hydroxy-22-methyltricosanoate (*iso*-C_{24})	2.1%
Methyl 2-hydroxy-21-methyltricosanoate (*anteiso*-C_{24})	5.9%
Methyl 2-hydroxytetracosanoate (*n*-C_{24})	23.3%
Methyl 2-hydroxy-23-methyltetracosanoate (*iso*-C_{25})	16.1%
Methyl 2-hydroxy-22-methyltetracosanoate (*anteiso*-C_{25})	4.7%
Methyl 2-hydroxypentacosanoate (*n*-C_{25})	10.9%
Methyl 2-hydroxy-24-methylpentacosanoate (*iso*-C_{26})	3.2%
Methyl 2-hydroxy-23-methylpentacosanoate (*anteiso*-C_{26})	1.8%
Methyl 2-hydroxyhexacosanoate (*n*-C_{26})	2.1%

The remaining portion of the methanolysis product was perbenzoylated and purified by HPLC (Scheme 1) giving compounds **3**–**7**. The obtained benzoylated methyl glycosides **3**–**5**, identified from their ^{1}H NMR and CD spectra, confirmed the presence of fucose residues in the saccharide chain and assigned them to the L series, and showed that the glucose and galactose residues belong to the common D series. In addition, compounds **6** and **7** showed that the configuration of the ceramide is that most commonly found in marine glycosphingolipids.

Finally, the benzoylated sphinganines **7** were subjected to acidic methanolysis to remove the benzoyl groups, and then to Lemieux oxidation and methylation as previously reported.[11] The obtained fatty acids methyl esters were analyzed by GC–MS. The results are described in Table 3, in the form of the corresponding sphinganines.

2.4. NO_2^- production by J774.2 macrophages

Nitric oxide (NO), a short lived mediator, is synthesized by a family of enzymes termed NO-synthase (NOS).[12] Two types of NOS are recognised: constitutive isoforms (endothelial NOS and neuronal NOS) and an inducible isoform, for which mRNA translation and protein synthesis are required.[12] Inducible NOS (iNOS) is regulated by inflammatory mediators (LPS, cytokines),[13] and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response.[14–16] We measured the production of NO_2^- (a stable metabolite of NO) as a parameter of macrophages activation and iNOS induction. Unstimulated J774 cells generated undetectable (<5 nmol/mL) amounts of NO_2^-. Stimulation of the cells with LPS (1 μg/mL) for 24 h produced a dose-dependent release of NO_2^- (15.5 ± 0.3 nmol/mL). When the cells were incubated with different concentrations of terpioside B (**2a**, 1–3–10 μM) a significant (p <0.001) inhibition of NO_2^- production was observed (Fig. 1). The simpler fucosyl GSL terpioside (**1**) was less active, showing only a slight inhibition of NO_2^- production (Fig. 2). On the other hand, a β-glucosylceramide isolated from the same sponge *Terpios* sp. was completely inactive (data not shown).



Scheme 1. Microscale chemical degradation scheme of terpioside B (**2b**).

Table 3
Sphinganine composition of terpioside B (**2a**)

Sphinganine	
(2*S*,3*S*,4*R*)-2-Amino-16-methyl-1,3,4-heptadecanetriol (*iso*-C_{18})	4.5%
(2*S*,3*S*,4*R*)-2-Amino-15-methyl-1,3,4-heptadecanetriol (*anteiso*-C_{18})	16.7%
(2*S*,3*S*,4*R*)-2-Amino-1,3,4-octadecanetriol (*n*-C_{18})	3.9%
(2*S*,3*S*,4*R*)-2-Amino-17-methyl-1,3,4-octadecanetriol (*iso*-C_{19})	69.3%
(2*S*,3*S*,4*R*)-2-Amino-1,3,4-octanonanetriol (*n*-C_{19})	5.6%

3. Discussion

Structure of terpioside B (**2a**), the first know glycolipid with two fucose residues in the furanose form, has been completely determined by spectroscopic and chemical means. Marine sponges confirm their capacity to produce a large array of unusual glycolipids, which are mostly species-specific.

The natural role of these glycolipids in sponges is not yet known, but they are possibly involved in cell recognition processes. Sponges possess a power of regeneration allowing a new individual to be generated from a sponge cell suspension. In a classical experiment,[17] dissociated cells of two different sponge species were mixed and allowed to aggregate, and only the cells of the same species combined. This clearly implies self/non-self recognition. Glycoconjugates (although not glycolipids) have been shown to be involved in these processes.[18] The species-specificity of many glycolipids from sponges may be a clue that sponge glycolipids may also be involved in self/non-self recognition, but, to our knowledge, no specific studies on this possible role of sponge glycolipids are available in the literature.

When assayed on murine J774 cells, terpioside B has been shown to possess an anti-inflammatory activity, which is related to its ability to prevent the production of proinflammatory mediators such as nitrite. The inhibitory effect of fucosyl GSLs has been first evidenced for the fucosylated triglycosylceramide from *Aplysinella rhax*,[5] containing a fucose in the pyranose form. In a subsequent paper, some analogues of this GSL were tested.[6] The results showed that the activity is retained even in analogues where the ceramide is replaced by a greatly simplified aglycon, thus suggesting that the non-polar part of the molecule is not important, while the activity is lost in an analogue which does not contain fucose, thus evidencing the importance of this sugar. Our experiments revealed that terpioside B (**2a**) is more potent than any other fucosyl GSL tested previously, being significantly active at 1 μM. The diglycosyl terpioside A (**1**) is also active, but to a lesser extent; in contrast glucosyl ceramide, which not contains fucose, is completely inactive.

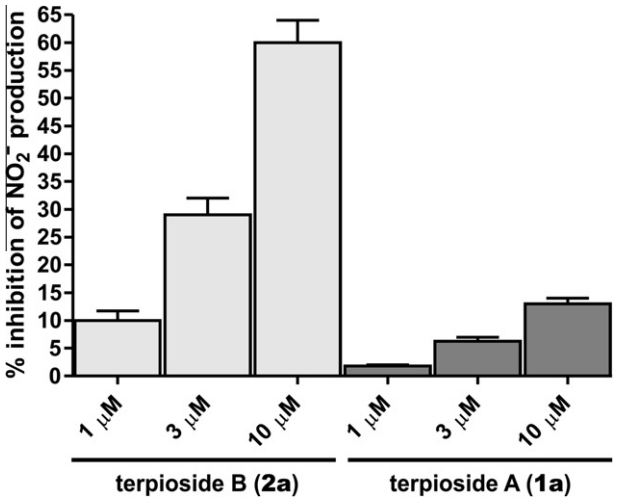


Figure 2. Effect of different concentrations of terpioside B (**2a**) and terpioside A (**1**) on the production of NO_2^- by J774 macrophages stimulated with LPS (1 μg/mL). Each bar represents the mean ± SEM of three separate experiments run in triplicate.

On one hand, this confirms and extends previous results, showing that fucosyl GSLs possess inhibitory activity even when the fucose residue is in the furanose form. On the other hand, it is quite surprising that a fucose residue in a GSL can exert a similar biological effect either in the pyranose or in the furanose form, because the two forms have completely different molecular shapes and are therefore expected to interact in different ways with their receptors. This suggests a more complex mechanism of action than the simple interaction of the intact fucosyl GSL with a receptor. Further studies are required in order to clarify the mechanism of the anti-inflammatory activity of fucosyl GSLs.

4. Experimental section

4.1. General experimental procedures

High Resolution ESI-MS spectra were performed on a Micromass QTOF Micro mass spectrometer, dissolving the sample in MeCN/H_2O (1:1) with 0.1% TFA. Standard ESI-MS experiments were performed using an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as the solvent. Optical rotations were measured at 589 nm on a Perkin–Elmer 192 polarimeter using a 10-cm microcell. NMR spectra were determined on a Varian Unity Inova spectrometers at 700 and 500 MHz; chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0; pyridine-d_5: δ_H 8.71, 7.56, and 7.19; δ_C 149.9, 135.6, and 123.6). 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 a COSY and z-TOCSY[10] experiments. Through-space ^{1}H connectivities were evidenced using a ROESY experiment with a mixing time of 450 ms. The HSQC spectra were optimized for $^1J_{CH}$ = 142 Hz, and the HMBC experiments for $^{2,3}J_{CH}$ = 8.3 Hz. GC–MS spectra were performed on a Hewlett–Packard 5890 gas chromatograph with a mass selective detector MSD HP 5970 MS, a split/splitness injector, and a fused-silica column, 25 m × 0.20 mm HP-5 (cross-linked 25% Ph Me silicone, 0.33-mm film thickness); the temperature of the column was varied, after a delay of 3 min from the injection, from 150 °C to 280 °C with a slope of 10 °C min^{-1}; quantitative determination was based on the area of the GLC peaks. High performance liquid chromatography (HPLC) separations were achieved on a Varian Prostar 210 apparatus equipped with an Varian 350 refractive index detector or a Varian 325 UV detector.

4.2. Collection, extraction and isolation

Specimens of *Terpios* sp.were collected by scuba in December 2005 along the coast of Key Largo (Florida) and identified by Professor Sven Zea (University of Colombia). The samples were stored at −20 °C until extraction. The frozen sponge sample of *Terpios* sp. (115 g of dry weight after extraction) was extracted with MeOH and $CHCl_3$, and the glycolipid fraction (405 mg) was obtained as described previously.[9] This fraction was acetylated with Ac_2O in pyridine and chromatographed by HPLC on an SiO_2 column using *n*-hexane/*i*-PrOH (8:2), affording peracetylated terpioside B (**2b**, 3.8 mg).

4.2.1. Terpioside B peracetate (2b)

Colorless oil, $[\alpha]_D^{25} = -13.2$ (*c* 0.35, $CHCl_3$). ESI-MS (positive ion mode, MeOH): the singly charged $[M+Na]^+$ ions could not be detected, being at *m/z* >2000; doubly charged $[M+2Na]^{2+}$ ions were detected at *m/z* 1125.0, 1118.2, 1111.2, and 1104.0 corresponding, respectively, to molecular weights 2204, 2190, 2176, and 2162. Figures given refer to the lowest-mass monoisotopic peak of each ion, although the respective M+1 isotopic peaks are more abundant. ^{1}H and ^{13}C NMR: Table 1. Composition in fatty acids: Table 2. Composition in sphinganines: Table 3.

4.3. Deacetylation of terpioside B peracetate (2b)

Terpioside B peracetate (**2b**, 3.8 mg) was dissolved in MeOH (950 μL), and a solution of MeONa in MeOH (0.4 M, 50 μL) was added. After 18 h at 25 °C, the solution was partitioned between *n*-BuOH and water (5 mL each) to remove the excess MeONa. After removal of the solvent, the organic phase yielded native terpioside B (**2a**, 2.5 mg, quantitative).

4.3.1. Terpioside B (2a)

Colorless amorphous solid; HRESIMS (positive ion mode, MeOH) 1498.9248 ($[M+Na]^+$, $C_{73}H_{137}NNaO_{28}$ gives 1498.9219); ESI-MS (positive ion mode, MeOH) *m/z* 1513.0, 1499.0, 1485.0, and 1470.9 ($[M+Na]^+$); ^{1}H NMR (pyridine-d_5): δ 8.57 (1H, d, *J* = 9.2 Hz, 2-NH), 7.79 (1H, br s, 2^{VI}-OH), 7.65 (overlapped, 3^V-OH), 7.47 (1H, br s, 2^{II}-OH), 7.39 (1H, br s, 3^{IV}-OH), 7.08 (1H, br s, 2^{IV}-OH), 6.95 (1H, br s, 2^{III}-OH), 6.91 (1H, br s, 3-OH), 6.48 (2H, br s, 4-OH and 6^I-OH), 6.39 (2H, br s, 3^I-OH and 4^{III}-OH), 6.16 (1H, br s, 5^{IV}-OH), 6.13 (2H, br s, 2^I-OH and 3^{II}-OH), 6.10 (1H, br s, 3^{III}-OH), 6.04 (1H, br s, 5^{IV}-OH), 5.33 (1H, d, *J* = 4.6 Hz, H-1^{IV}) , 5.31 (1H, d, *J* = 3.8 Hz, H-1^{III}), 5.28 (1H, m, H-2), 5.23 (1H, d, *J* = 4.4 Hz, H-1^V), 4.98 (1H, dd, *J* = 7.7 and 4.2 Hz, H-5^{III}), 4.96 (1H, d, *J* = 7.8 Hz, H-1^{II}), 4.93 (overlapped, H-3^{IV}) , 4.85 (1H, d, *J* = 7.8 Hz, H-1^I), 4.75 (overlapped, H1^V H-3^V), 4.70 (1H, dd, *J* = 10.4 and 6.7 Hz, H-1a), 4.62 (1H, ddd, *J* = 7.6, 7.6, and 4.6 Hz, H-2^{IV}), 4.60 (overlapped, H-2^{III}), 4.59 (overlapped, H_2-6^{II}), 4.58 (overlapped, H-2^{VI}), 4.53 (1H, ddd, *J* = 8.4, 8.4, and 4.4 Hz, H-2^V), 4.51 (1H, dd, *J* = 10.1 and 4.2 Hz, H-6a^{III}) , 4.43 (1H, dd, *J* = 10.4 and 4.4 Hz, H-1b), 4.38 (2H, m, H_2-6^I), 4.35 (overlapped, H-5^{IV}), 4.34 (overlapped, H-2^{II}) , 4.32 (overlapped, H-3), 4.29 (1H, m, H-5^V), 4.27 (overlapped, H-5^{II}), 4.21 (overlapped, H-4), 4.20 (overlapped, H-3^{III}), 4.17 (t, *J* = 8.8 Hz, H-3^I) , 4.17 (t, *J* = 6.4 Hz, H-4^{IV}), 4.17 (1H, dd, *J* = 10.1 and 7.7 Hz, H-6b^{III}), 4.14 (t, *J* = 6.8 Hz, H-4^V), 4.14 (overlapped, H-4^{II}), 4.10 (br s, H-4^{III}), 4.10 (1H, dd, *J* = 9.4 and 8.8 Hz, H-4^I) , 3.99 (overlapped, H-3^{II}) , 3.97 (1H, ddd, *J* = 8.7, 7.8, and 3.4 Hz, H-2^I), 3.81 (1H, ddd, *J* = 9.4, 3.3, and 3.3 Hz, H-5^I), 2.24 (1H, m, H-5a), 2.19 (1H, m, H-3a^V), 1.99 (1H, m, H-3b^V), 1.92 (overlapped, H-6a), 1.90 (1H, m. H-5b), 1.76 (overlapped, H-4a^V), 1.68 (overlapped, H-4b^V), 1.67 (overlapped, H-6b), 1.54 (3H, d, *J* = 6.6 Hz, H_3-6^{IV}), 1.50 (3H, d, *J* = 6.2 Hz, H_3-6^V), 1.27 (br band, alkyl chain protons), 0.85 (overlapped, *n*-chain Me group), 0.85 (overlapped, *iso*-chain Me groups) 0.83 (overlapped, *anteiso*-chain Me groups); ^{13}C NMR (pyridine-d_5): δ 175.3 (C, C-1^{VI}), 106.2 (CH, C-1^{II}), 105.2 (CH, C-1^I), 103.2 (CH, C-1^V), 102.9 (CH, C-1^{III}), 102.9 (CH, C-1^{IV}), 87.9 (CH, C-4^V), 87.8 (CH, C-4^{IV}), 83.6 (CH, C-4^I), 80.1 (CH, C-2^V), 79.9 (CH, C-2^{IV}), 79.6 (CH, C-4^{II}), 76.7 (CH, C-3^I), 76.6 (CH, C-3^V), 76.3 (CH, C-3^{IV}), 76.2 (CH, C-3), 74.5 (CH, C-2^I), 75.0 (CH, C-3^{II}), 76.3 (CH, C-5^I), 75.6 (CH, C-5^{II}), 72.5 (CH, C-2^{II}), 72.5 (CH, C-2^{VI}), 70.6 (CH_2, C-1), 72.7 (CH, C-4), 70.6 (CH, C-2^{III}), 70.1 (CH, C-3^{III}), 70.8 (CH, C-4^{III}), 71.5 (CH, C-5^{III}), 70.3 (CH, C-5^V), 69.3 (CH, C-5^{IV}), 68.4 (CH_2, C-6^{III}), 68.1 (CH_2, C-6^{II}), 62.4 (CH_2, C-6^I), 51.7 (CH, C-2), 35.8 (CH_2, C-3^{VI}), 34.3 (CH_2, C-5), 26.1 (CH_2, C-4^{VI}), 26.8 (CH_2, C-6), 23.1 (CH_3, *iso*-chain Me groups), 20.2 (CH_3, C-6^{IV}), 20.0 (CH_3, C-6^V), 19.6 (CH_3, *anteiso*-chain, Me group), 14.5 (CH_3, *n*-chain Me group), 11.9 (CH_3, *anteiso*-chain Me group). Composition in fatty acids: Table 2. Composition in sphinganines: Table 3.

4.4. Methanolysis of terpioside B (2a)

Terpioside B (**2a,** 0.6 mg) was dissolved in HCl (1 M, 0.5 mL) in 91% MeOH in a sealed tube and kept at 80 °C for 12 h. The reaction mixture was dried under nitrogen, and a small aliquot was kept for GC–MS analysis (see below). The remaining portion was benzoylated with benzoyl chloride (50 μL) and pyridine (500 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH; after 30 min, the mixture was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum with an oil pump for 24 h. The residue was purified by HPLC [column: Phenomenex Luna SiO_2, 5 μ; eluent: *n*-hexane/*i*PrOH (99:1); flow: 1 mL min^{-1}]. Five fractions were collected, which contained, respectively, methyl tri-*O*-benzoyl-α-L-fucopyranoside (**3**, t_R = 7.7 min), methyl tetra-*O*-benzoyl-β-D-glucopyranoside (**4**, t_R = 13.2 min), methyl tetra-*O*-benzoyl-β-D-galactopyranoside (**5**, t_R = 15.0 min),[19] the 2*R*-benzoyloxy fatty acid methyl esters **6** (t_R = 4.0 min), and the perbenzoylated D-*ribo*-phytosphingosines **7** (t_R = 11.0 min). All the compounds (apart for the alkyl chains of compounds **6** and **7**) were

identified by comparison of their ^{1}H NMR and CD spectra with those reported in the literature.[9,20,21]

4.5. Analysis of fatty acid methyl esters

A small amount of the methanolysis product (see above) was analyzed by GC–MS, and the methyl α-hydroxy fatty esters which were contained in the sample were identified by a comparison of their retention times and mass spectra with those of authentic samples. The results are compiled in Table 2.

4.6. Oxidative cleavage and GC–MS analysis of sphinganines

The perbenzoylated phytosphingosines **7** were debenzoylated by acidic methanolysis as described above and subjected to oxidative cleavage with $KMnO_4/NaIO_4$ as described[11] and the resulting carboxylic acids were methylated with CH_2N_2. The obtained esters were analyzed by GC–MS, and the results are compiled in Table 3, expressed in terms of original sphinganines.

4.7. Cell culture

The murine monocyte/macrophages cell line J774 was from ECACC. J774 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) and cultured at 37 °C in humidified 5% CO_2/95% air. The cells were plated in 24 well culture plates (Falcon) at a density of 2.5×10^6 cells/mL/well and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium and cells were activated by lipopolysaccharide (LPS 1 μg/mL) from *E. coli* (Fluka) for 24 h in the presence or absence of different concentrations of test compounds. The culture medium was then removed and centrifuged, and the supernatant was used for the determination of nitrite (NO_2^-) production. Cell viability (>95%) was determined with the MTT assay.[22]

4.8. NO_2^- assay

NO_2^- levels in culture media from J774 macrophages were measured 24 h after LPS with the Griess reaction as previously described.[23] Results are expressed as nmol/mL of NO_2^- and represent the mean ± SEM of *n* experiments run in triplicates.

Acknowledgments

This research work was supported by MIUR PRIN 2007 (Grant no. 2007KZ9W5J) and by the FP7 EC Project NatPharma (grant agreement no. 229893). A warm thank you is due to Professor J. R. Pawlik, University of North Carolina at Wilmington, for collecting the sponge, and Professor Sven Zea (Universidad Nacional de Colombia) for identifying it. Mass and NMR spectra were recorded at the 'Centro di Servizi Interdipartimentale di Analisi Strumentale', Università di Napoli 'Federico II'. The assistance of the staff is gratefully acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.048.

References and notes

1. Part 21: Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A.; Teta, R. *Eur. J. Org. Chem.* **2009**, 2112.
2. Miyoshi, E.; Moriwaki, K.; Nakagawa, T. *J. Biochem.* **2008**, *143*, 725.
3. Sugita, M.; Hayata, C.; Yoshida, T.; Suzuki, M.; Suzuki, A.; Takeda, T.; Hori, T.; Nakatani, F. *Biochim. Biophys. Acta* **1994**, *1215*, 163.
4. Xu, X.; Horibata, Y.; Inagaki, M.; Hama, Y.; Sakaguchi, K.; Goda, H. M.; Okino, N.; Ito, M. *Glycobiology* **2009**, *19*, 1446.
5. Borbone, N.; De Marino, S.; Iorizzi, M.; Zollo, F.; Debitus, C.; Ianaro, A.; Pisano, B. *Eur. J. Org. Chem.* **2001**, 4651.
6. Hada, N.; Nakashima, T.; Shrestha, S. P.; Masui, R.; Narukawa, Y.; Tani, K.; Takeda, T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5912.
7. Miyamoto, T.; Yamamoto, A.; Wakabayashi, M.; Nagaregawa, Y.; Inagaki, M.; Higuchi, R.; Iha, M.; Teruya, K. *Eur. J. Org. Chem.* **2000**, 2295.
8. Cafieri, F.; Fattorusso, E.; Mangoni, A.; Taglialatela-Scafati, O. *Gazz. Chim. Ital.* **1996**, *126*, 711.
9. Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A.; Teta, R. *Eur. J. Org. Chem.* **2008**, 2130.
10. Thrippleton, M. J.; Keeler, J. *Angew. Chem., Int. Ed.* **2003**, *42*, 3938.
11. Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A.; Maffia, P. *Tetrahedron* **1996**, *52*, 1573.
12. Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
13. Xie, Q.; Kashiwabara, Y.; Nathan, C. J. *Biol. Chem.* **1994**, *269*, 4705.
14. Ialenti, A.; Ianaro, A.; Moncada, S.; Di Rosa, M. *Eur. J. Pharmacol.* **1992**, *211*, 177.
15. Ianaro, A.; O'Donnell, C. A.; Di Rosa, M.; Liew, F. Y. *Immunology* **1994**, *82*, 370.
16. Ianaro, A.; Ialenti, A.; Sautebin, L.; Di Rosa, M. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *358*, 489.
17. Wilson, H. V. *J. Exp. Zool.* **1907**, *5*, 245.
18. Fernandez-Busquets, X.; Burger, M. M. *Microsc. Res. Tech.* **1999**, *44*, 204.
19. The perbenzoylated α-glycosides of glucose and galactose showed retention times very close to each other under the condition used, so we preferred to analyze the respective β-glycosides, which were less abundant but easier to separate.
20. Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A. *Eur. J. Org. Chem.* **2005**, 368.
21. Gutiérrez, M.; Capson, T. L.; Guzmán, H. M.; González, J.; Ortega-Barría, E.; Quiñoá, E.; Riguera, R. *J. Nat. Prod.* **2006**, *69*, 1379.
22. Denizot, F.; Lang, R. *J. Immunol. Methods* **1986**, *89*, 271.
23. Ianaro, A.; Ialenti, A.; Maffia, P.; Sautebin, L.; Rombolà, L.; Carnuccio, R.; Iuvone, T.; D'Acquisto, F.; Di Rosa, M. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 156.