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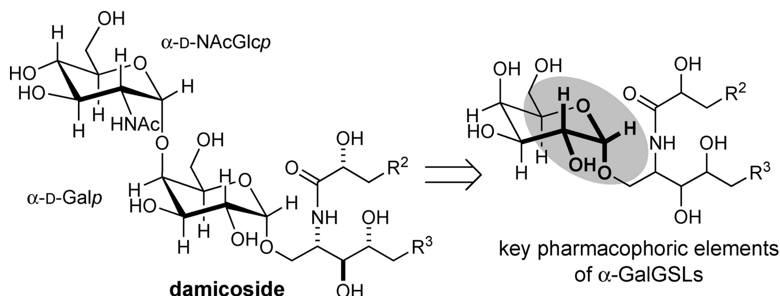

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Damicoside from *Axinella damicornis*: The Influence of a Glycosylated Galactose 4-OH Group on the Immunostimulatory Activity of α-Galactoglycosphingolipids

Valeria Costantino,[†] Monica D'Esposito,[†] Ernesto Fattorusso,[†] Alfonso Mangoni,[*,†] Nicoletta Basilico,[‡] Silvia Parapini,[‡] and Donatella Taramelli[‡]

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", via D. Montesano 49, 80131 Napoli, Italy, and Istituto di Microbiologia, Università di Milano, Via Pascal 36, 20133 Milano, Italy

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α-Galactoglycosphingolipids (α-GalGSLs) are unique immunostimulatory glycosphingolipids from marine sponges. Analysis of the glycosphingolipid composition of the marine sponge *Axinella damicornis* revealed the presence of a new α-GalGSL, damicoside (**3a**), which is the first α-GalGSL with a glycosylated galactose 4-OH group. Structure elucidation of damicoside was performed using spectroscopic and chemical methods. When tested in a spleen cell proliferation assay, **3a** exhibited a stimulatory activity comparable to that of agelasphin (**2**), showing that a free galactose 4-OH group is not essential for the immunostimulatory activity of α-GalGSLs and providing a further step toward the complete understanding of their structure−activity relationship.

Introduction

It is well-established that sponges of the genus *Agelas* and *Axinella* produce α-galactoglycosphingolipids (α-GalGSLs). The distinctive feature of these glycosphingolipids is the α-anomeric linkage of the inner galactose to the lipid, unlike the ubiquitous *β*-glycosidic bond in nearly all known natural glycosphingolipids from higher animals and plants.[1−5]

Natural α-GalGSLs possess interesting immunomodulating activities. They are potent ligands of the MHC class I-like CD1d protein, which is present on the surface of the antigen presenting cells (APCs), and are capable of activating in vitro and in vivo a specialized population of T cells, named natural killer T cells (NKT cells), that play an important role in regulating innate and adaptive immunity during infection, tumor growth, and autoimmune diseases.[6] α-GalGSLs induce a rapid production of high levels of cytokines, such as IFN-*γ* and interleukin (IL)-4, thereby activating other antitumor effector cells.[6] Very recently, it has been shown that NKT cells also recognize glycosphingolipids containing α-linked galacturonic or glucuronic acid residues. These are produced by Gram-negative bacteria that do not contain lipopolysaccharides and are suggested to be a natural target of the NKT cells in an innate-type immune mechanism.[7] As a result of these studies, α-GalGSLs have been proposed as vaccine adjuvants,[8] while KRN7000 (also known as α-GalCer, **1**), a synthetic analogue of the simplest α-GalGSL, agelasphin (**2**), is under clinical trial as a novel anticancer agent.[9]

A number of natural and synthetic analogues of agelasphin have been tested for immunomodulating activities, in the attempt to determine their structure−activity relationship. In particular, several agelasphin analogues were assayed that presented the galactose OH groups at positions 2, 3, or 6 as glycosylated or modified in other ways.[10−12] The results indicated that glycosylation or any other modification at position 2 cancels the immunomodulating activity of the GSL, while glycosylation at position 6 has no effect. The effect of a modification at the galactose 3-OH is less clear: glycosylation of this hydroxyl group cancels activity,[11] but sulfation leaves it unchanged.[12] Establishing this structure−activity relationship was complicated by the fact that the inactive α-GalGSLs with a glycosylated galactose can give positive results in some assays, due to the removal of the sugars linked to the galactose 2-OH or 3-OH by α-glycosidases present in the lysosome of APCs. Only with the use of an inhibitor of α-glycosidases was clear-cut experimental evidence obtained.[11]

Despite the extensive experimental work, the structure−activity relationship of the sugar part of α-GalGSLs is not yet fully defined, because no data are available about the effect of modifications at the galactose 4-OH. In this paper, we report the isolation and the structure elucidation of damicoside (**3a**) from *Axinella damicornis*. This is a new α-GalGSLs with a 2-deoxy-2-acetamido-α-glucopyranoside unit linked to the 4-OH of the inner galactose. The immunostimulatory properties of this new GSL are described.

Results and Discussion

Specimens of *A. damicornis* were extracted, in sequence, with MeOH and $CHCl_3$. The MeOH extract was partitioned between water and *n*-BuOH, and the organic phase was combined with the $CHCl_3$ extract. The combined organic extracts were subjected to chromatography through an RP-18 column and then through a SiO_2 column, yielding a fraction mainly composed of glycolipids. This was acetylated and purified by repeated HPLC on SiO_2 columns, using *n*-hexane/EtOAc mixtures as eluents, to give 7.4 mg of damicoside peracetate (**3b**).

* Corresponding author. Phone: +39−081−678−532. Fax: +39−081−678−552. E-mail: alfonso.mangoni@unina.it.
† Università di Napoli "Federico II".
‡ Università di Milano.

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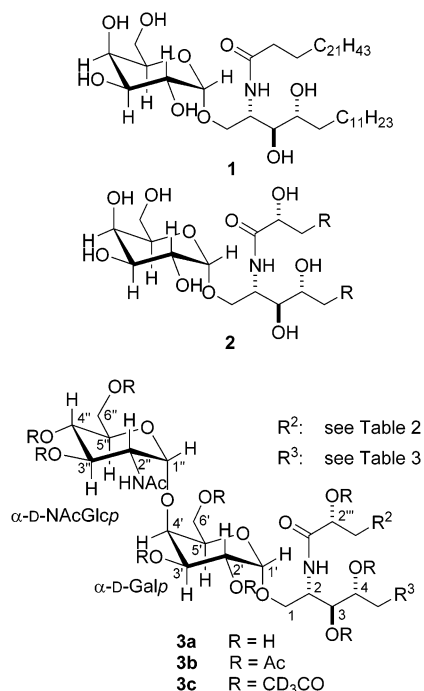

Figure 1. Structure of KRN-7000 (**1**), agelasphin (**2**), and damicoside (**3**).

Native **3a** was obtained by deacetylation of **3b** with a 0.02 M solution of MeONa in MeOH.

The ESI mass spectrum of **3a** contained a series of sodiated pseudomolecular ion peaks at *m/z* 1099, 1085, 1071, 1057, and 1043, in accordance with the molecular formula $C_{54}H_{104}N_2O_{15} + nCH_2$ ($n = 0-4$). This indicated that damicoside was composed of a complex mixture of homologues. A high-resolution measurement was performed on the most abundant ion at *m/z* 1085.7823, confirming the molecular formula $C_{57}H_{110}N_2O_{15}$ for the dominant homologue. That the alkyl chain mixture differed not only in the length but also in the branching of the alkyl chains was suggested by the methyl region of the ^{1}H NMR spectrum of **3a** (pyridine-d_5), displaying the complex set of signals of a mixture of unbranched and terminally branched (anteiso and iso) alkyl chains.

Further NMR studies devoted to the structural elucidation of damicoside were performed on the peracetylated derivative **3b**, because of the more favorable features of its proton NMR spectrum.[2] Thus, compound **3b** was readily identified as a glycosphingolipid from its ^{1}H NMR spectrum ($CDCl_3$) on account of the intense aliphatic chain signal at δ 1.24, the many signals of oxymethine and oxymethylene groups between δ 5.38 and 3.34, the 10 acetyl methyl singlets between δ 2.11 and 2.01, and the characteristic amide NH doublets at δ 7.20. An additional NH doublet at δ 5.99 suggested the presence a further amide function other than that of ceramide, tentatively that of an aminosugar.

The ceramide amide proton, resonating at δ 7.20, was used as a starting point for the sequential assignment of the protons at C-1 through C-6 by means of a series of clear COSY correlation peaks (see Table 1 and Supporting Information). The same doublet at δ 7.20 was useful to assign the signal at δ 5.12 to the proton at C-2 of the ceramide fatty acid residue on the basis of the ROESY correlation peak between these protons. These data showed the ceramide to be composed of a 4-hydroxysphinganine and a 2-hydroxyacid residue.

Elucidation of the structure of the sugar chain required collection of the entire set of one- and two-dimensional NMR experiments in two different solvents. In fact, configurational analysis in pyranose sugars is largely based on evaluation of proton−proton vicinal coupling constants, but this can be hindered by the strong overlapping of the signals of the CH−O protons. In particular, when signals belonging to coupled protons overlap, non-first-order multiplets result and the coupling constant information is lost. A way to recover this information is to modify the chemical shift of the coupled protons by changing the solvent. As shown in Table 1, C_6D_6 was selected as the alternative solvent, and all the protons in compound **3b** gave rise to clear multiplets in at least one of the two solvents.

The two anomeric protons of **3b** ($CDCl_3$, δ 4.97, d, J = 3.6 Hz and 4.88, d, J = 3.6 Hz) were identified from their correlation peaks with the anomeric carbons at, respectively, δ 96.3 and 98.6 in the HSQC spectrum. Starting from these protons, all the ^{1}H and ^{13}C signals of the two sugars were assigned using the TOCSY, COSY, and HSQC spectra, and the vicinal proton−proton coupling constants were determined. All the data are reported in Table 1.

The sugar linked to the ceramide was identified as a 4-glycosylated α-galactopyranoside. The *galacto* configuration of the sugar, as well as its α configuration, was established on the basis of the ring proton coupling constants ($CDCl_3$, $J_{1,2}$ = 3.6 Hz, $J_{2,3}$ = 10.6 Hz, $J_{3,4}$ = 2.6 Hz), showing H-2′ and H-3′ to be axial and H-1′ and H-4′ to be equatorial, and on the basis of the ROESY correlation peak between H-3′ and H-5′ due to their 1,3-diaxial relationship. Glycosylation at position 4 was deduced by the shielded chemical shift of H-4′ (δ 4.17), which indicated that the relevant oxygen atom is not acetylated. Finally, the linkage of this sugar to the ceramide was demonstrated by the three-bond ^{13}C−^{1}H couplings between C-1 and H-1′ and between C-1′ and H-1b detected through an HMBC spectrum.

The terminal sugar was identified as a 2-acetamido-2-deoxy-α-glucopyranoside. Coupling constants (C_6D_6, $J_{1,2}$ = 3.4 Hz, $J_{2,3}$ = 10.6 Hz, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 9.6 Hz) showed all the ring protons, except for H-1″, to be axial. In addition, the coupling of the NH doublet at δ 6.26 with H-2″ and the relatively high-field chemical shift of C-2″ (δ 53.8) demonstrated an acetamido group, rather that an acetoxy group, to be linked at C-2″. The (1→4) linkage between the two sugars was confirmed by the HMBC correlation peak between H-4′ and C-1″.

The nature and the amount of the fatty acid and sphingosine components of compound **3a** were determined by microscale chemical degradation of the GSL, according to the method recently developed by our group[14] (Scheme 1). Compound **3a** (200 μg) was subjected to acidic methanolysis in HCl/MeOH, and the resulting reaction mixture was separated by partitioning between $CHCl_3$ and water/MeOH into an aqueous phase containing methyl glycosides (fraction A) and an organic phase containing 2-hydroxy fatty acid methyl esters and sphinganines (fraction B).

Fraction B was analyzed for fatty acid methyl esters by GC−MS and was shown to contain five homologues

Table 1. ^{1}H and ^{13}C NMR Data of **3b** (500 MHz)

	CDCl$_3$		C$_6$D$_6$	
positn	δ_H (mult, J in Hz)[a]	δ_C (proton count)[b]	δ_H (mult, J [Hz])[c]	δ_C (proton count)[d]
1a	3.70 (dd, 10.4, 2.6)	66.0 (CH$_2$)	3.78 (br d 10.6)	67.2 (CH$_2$)
1b	3.35 (br d, 10.4)		3.33 (br d 10.6)	
2	4.3[e]	47.9 (CH)	4.57[e]	48.7 (CH)
2-NH	7.08 (d, 9.3)	–	7.67 (d 9.0)	–
3	5.34 (dd, 9.8, 2.3)	70.4 (CH)	5.71(dd 10.3, 1.6)	70.9 (CH)
4	4.88[e]	73.2 (CH)	5.24 (br d 10.2)	73.8 (CH)
5	1.60 (m)	27.6 (CH$_2$)	1.81	27.7 (CH$_2$)
1′	4.97 (d, 3.6)	96.3 (CH)	4.97[e]	97.5 (CH)
2′	5.12 (dd, 10.6, 3.6)[f]	67.7 (CH)	5.52[e]	68.6 (CH)
3′	5.19 (dd, 10.6, 2.6)[f]	68.9 (CH)	5.51[e]	69.7 (CH)
4′	4.17 (br d, 2.6)	75.9 (CH)	4.17 (br s)	77.5 (CH)
5′	3.98[e]	68.4 (CH)	4.28[e]	69.5 (CH)
6′a	4.30[e]	61.8 (CH$_2$)	4.51[e]	62.9 (CH$_2$)
6′b	4.00[e]		4.11 (dd 11.1, 4.4)	
1″	4.88 (d, 3.6)	98.6 (CH)	4.97[e]	99.7 (CH)
2″	4.34 (ddd, 10.6, 8.8, 3.6)	52.5 (CH)	4.66 (ddd, 10.6, 8.6, 3.4)	53.8 (CH)
2″-NH	5.95 (d, 8.8)		6.26 (d, 8.6)	–
2″-Ac	2.004 (s)	23.2 (CH$_3$)	1.934 (s)	22.8 (CH$_3$)
3″	5.21[e]	70.7 (CH)	5.61 (dd, 10.6, 9.6)	71.5 (CH)
4″	5.19[e]	67.7 (CH)	5.59 (t, 9.6)[f]	68.3 (CH)
5″	4.26 (m)	68.3 (CH)	4.55[e]	69.3 (CH)
6″a	4.29[e]	61.4 (CH$_2$)	4.55[e]	62.0 (CH$_2$)
6″b	4.04 (dd, 12.1, 1.4)		4.30[e]	
2‴	5.12[e]	74.1 (CH)	5.44 (dd, 7.0, 5.2)	74.7 (CH)
3‴	1.84 (m)	32.0 (CH$_2$)	2.06	32.1 (CH$_2$)
Ac's	2.228, 2.103, 2.090, 2.085, 2.080, 2.066, 2.051, 2.034, 2.013 (9 singlets)	21.1–20.8 (CH$_3$)	2.210, 1.964, 1.912, 1.816, 1.798, 1.717, 1.662, 1.638, 1.615 (9 singlets)	20.8–20.1 (CH$_3$) 169.0–170.8 (C=O)

[a] Additional ^{1}H signals (CDCl$_3$): δ 1.25 (br band, alkyl chain protons), 0.88 (t, J = 7.0, *n*-chain Me groups), 0.86 (d, J = 6.5, iso-chain Me groups), 0.85 and 0.84 (overlapped, anteiso-chain Me groups). [b] Additional ^{13}C signals (CDCl$_3$): 32.0 (CH$_2$, ω-2), 25.3 (CH$_2$, C-4‴), 23.5 (CH$_2$, ω-1), 22.8 (CH$_3$, iso-chain Me groups), 21.1–20.8 (several CH$_3$, Ac methyl groups), 19.2 (CH$_3$, anteiso-chain Me group), 14.3 (CH$_3$, ω), 11.5 (CH$_3$, anteiso-chain Me group). [c] Additional ^{1}H signals (C$_6$D$_6$): δ 1.51 (2H, m, H-4‴), 1.32 (br band, alkyl chain protons), 0.91 (overlapped, iso-chain Me groups), 0.90 (overlapped, *n*-chain Me groups), 0.90 and 0.89 (overlapped, anteiso-chain Me groups). [d] Additional ^{13}C signals (CDCl$_3$): 32.3 (CH$_2$, ω-2), 25.7 (CH$_2$, C-4‴), 23.0 (CH$_2$, ω-1), 22.9 (CH$_3$, iso-chain Me groups), 20.8–20.0 (several CH$_3$, Ac methyl groups), 19.3 (CH$_3$, anteiso-chain Me group), 14.3 (CH$_3$, ω), 11.5 (CH$_3$, anteiso-chain Me group). [e] Submerged by other signals. [f] Coupling constants were measured using 1D-TOCSY experiments.

Scheme 1

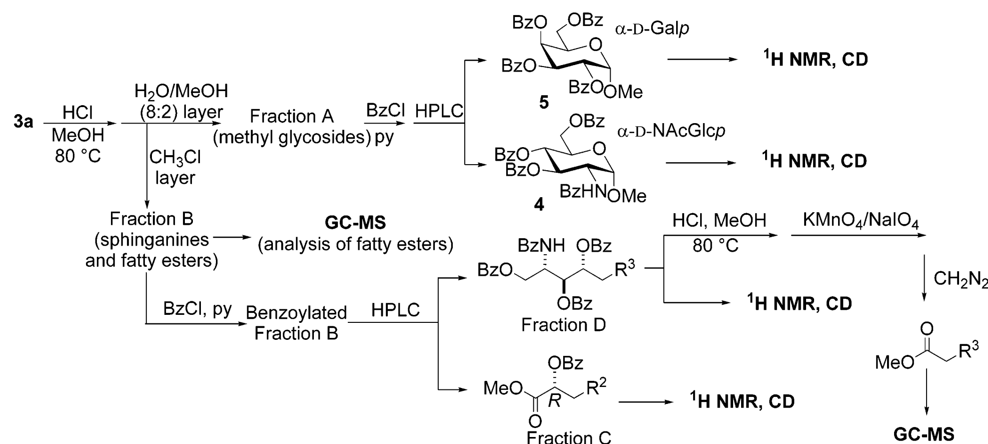

Table 2. Fatty Acyl Composition of **3a**

fatty acid methyl ester	%
methyl 2-hydroxyheneicosane (*n*-C$_{21}$)	6.9
methyl 2-hydroxydocosane (*n*-C$_{22}$)	8.5
methyl 2-hydroxytricosane (*n*-C$_{23}$)	29.2
methyl 2-hydroxytetracosane (*n*-C$_{24}$)	37.1
methyl 2-hydroxypentacosane (*n*-C$_{25}$)	18.2

of unbranched 2-hydroxy fatty esters by comparison of their retention times and mass spectra with those of authentic samples (Table 2).

Fraction B was then perbenzoylated with benzoyl chloride in pyridine, and the resulting benzoates were separated by normal-phase HPLC. Two fraction were collected, one composed of 2-benzoyloxy fatty acid methyl esters (fraction C) and the other one composed of perbenzoylated sphinganines (fraction D). Comparison of the CD and ^{1}H NMR spectra of fractions C and D with those of authentic samples of 2-(R)-benzoyloxy fatty acid methyl esters and perbenzoylated D-*ribo*-phytosphingosine (see Experimental Part for details) allowed us to assign the relative and absolute stereochemistry of the ceramide.

The sphinganine fraction D was then subjected to basic methanolysis, KMnO$_4$/NaIO$_4$ oxidative cleavage, and CH$_2$N$_2$ methylation as described[2] to give fatty acid methyl esters with three less carbon atoms. These were

Table 3. Sphinganine Composition of **3a**

sphinganine	%
(2*S*,3*S*,4*R*)-2-amino-16-methyl-1,3,4-heptadecanetriol (iso-C_{18})	14.5
(2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol (*n*-C_{18})	5.7
(2*S*,3*S*,4*R*)-2-amino-16-methyl-1,3,4-octadecanetriol (anteiso-C_{19})	36.8
(2*S*,3*S*,4*R*)-2-amino-1,3,4-nonadecanetriol (*n*-C_{19})	23.9
(2*S*,3*S*,4*R*)-2-amino-18-methyl-1,3,4-nonadecanetriol (iso-C_{20})	7.5
(2*S*,3*S*,4*R*)-2-amino-17-methyl-1,3,4-nonadecanetriol (anteiso-C_{20})	5.3
(2*S*,3*S*,4*R*)-2-amino-1,3,4-eicosanetriol (*n*-C_{20})	6.3

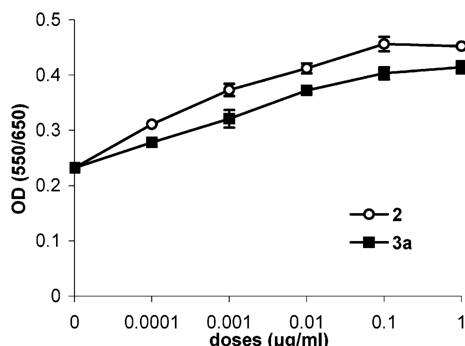

Figure 2. Dose-dependent proliferation of C57Bl/6 spleen cells to **2** or **3a**. Spleen cells (5×10^4/well) from C57Bl6 mice were stimulated for 72 h using RPMI 1640 medium + 5% FCS and different doses of test compounds. Cell proliferation was evaluated using a metabolic dye (MTT). The results are representative of three different experiments done in quadruplicate. Data are expressed as mean ± SD.

analyzed by GC−MS and shown to contain unbranched, iso, and anteiso esters, as reported in Table 3 in the form of structures of the corresponding sphinganines.

Finally, the absolute configuration of the sugar was established by subjecting the methyl glycosides in fraction A to perbenzoylation and subsequent HPLC separation. The HPLC chromatogram of the reaction product contained two peaks that were collected and identified (apart from their absolute configuration) on the basis of their respective retention times and ^{1}H NMR spectra compared to those of authentic samples of the methyl glycosides **4** and **5**. The CD spectrum of each glycoside from **3a** was recorded and matched that of the corresponding synthetic glycoside, thus showing also the absolute stereochemistry of glycosides from **3a** and reference glycosides to be the same.

After structure **3b** was fully established, it remained to be verified whether any of the acetyl groups in **3b** was already present in the natural glycolipid. This was accomplished by subjecting a small amount of the glycolipid fraction from *A. damicornis* to acetylation using trideuterioacetic anhydride instead of acetic anhydride. The isolation procedure used for **3b** was repeated, leading to the isolation of 0.5 mg of compound **3c**, chromatographically indistinguishable from **3b**. The ^{1}H spectra of the pertrideuterioacetylated compound **2c** and the peracetylated compound **3b** were very similar, but in the spectrum of **3c** only one acetyl methyl singlet (C_6D_6, δ 1.934) was present, compared to 10 in the spectrum of **3b**. This signal was assigned to the acetamido methyl group on the basis of its correlation peaks in the HMBC spectrum of **3b** with the carbonyl carbon signal at δ 169.7, which also showed an HMBC correlation peak with the NH amide doublet at δ 6.26.

To verify whether the peculiar structural features of damicoside were relevant to its immunostimulatory properties, the biological activity of compound **3a** compared to that of the nonglycosylated α-GalGSL **2** from *A. clathrodes*[13] was tested in a splenocyte proliferation test, as described.[10] The 4′-*O*-glycosylated compound **3a** stimulated the proliferation of spleen cell from C57Bl/6 mice in a dose-dependent manner. The activity peaked after 72 h of incubation at 37 °C. The response was not significantly different from that obtained with the same doses of compound **2** (Figure 2). To exclude the possibility that the activity of compound **3a** was due to the removal of the *N*-acetylglucosamine residue by the APC α-glycosidase, the experiments were repeated in the presence of increasing concentrations of DGJ (deoxygalactonojirimycin), a known inhibitor of α-glycosidases.[11] No significant inhibition of the activity of **3a** or **2** was observed at any of the doses of inhibitor tested (Figure 3). Since at the highest dose (1 μg/mL) the inhibitor DGJ showed some toxicity against the splenocytes, the experiments were repeated by preincubating the splenocytes with DGJ for 2 h, before the addition of compounds **3a** or **2**. Again, no significant difference between the activity of **3a** and that of **2** was seen in the presence of DGJ (data not shown).

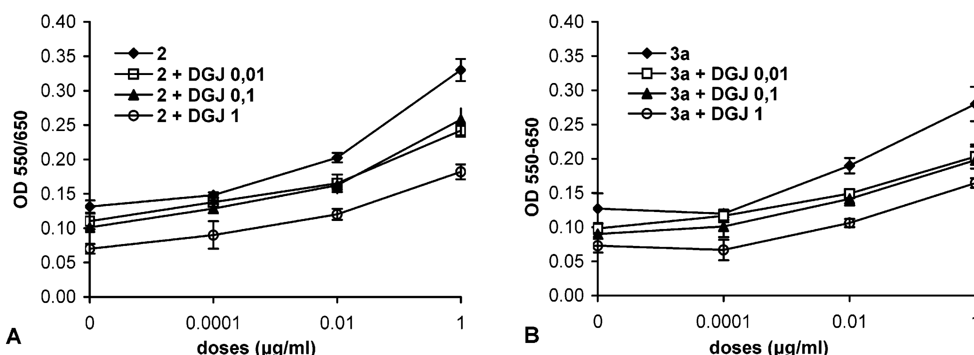

Figure 3. Proliferative response of C57Bl/6 spleen cells to different doses of **2** (panel A) or **3a** (panel B) in the presence of increasing concentrations of DGJ. The results are representative of three different experiments conducted in quadruplicate. Data are expressed as mean ± SD.

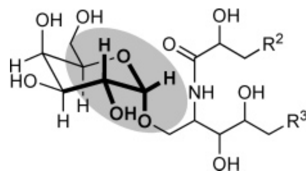

Figure 4. The key pharmacophoric elements of an α-GalGSLs.

Conclusion

The novel α-GalGSL **3a** is the first example of an α-GalGSL with a glycosylated galactose 4-OH group. Not only was the occurrence of damicoside in *A. damicornis* a further evidence of the well-defined chemotaxonomic characterization of these peculiar GSLs (limited to the genera *Agelas* and *Axinella*), but this hitherto unavailable compound also filled a gap in the SAR studies on the sugar part of α-GalGSLs.

The splenocyte proliferation tests demonstrate that the immunostimulatory activity of **3a** is similar to that of **2** and it is not affected by the presence of the additional sugar on the galactose 4-OH group. These results provided a further step toward the complete understanding of the structure−activity relationship of the sugar part of an α-GalGSL, which can be now summarized as follows. A free galactose 2-OH group is critical for the activity of an α-GalGSL, and any modification at this position results in an inactive compound; the galactose 3-OH group is also important, but some derivatives modified at this position are nevertheless active; finally, the 4-OH and 6-OH groups are less important, and glycosylation at each of these positions does not affect the activity. Therefore, it is now clear that the key pharmacophoric elements of an α-GalGSLs are located in a well-delimited part of the molecule (Figure 4), i.e., at the galactose positions 1 (α-anomeric linkage) and 2 (free OH group).

Experimental Section

General Experimental Procedures. High-Resolution ESI-MS spectra were performed on a hybrid quadrupole-TOF mass spectrometer. ESI MS/MS experiment were performed on a 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 using a 10-cm microcell.

^{1}H and ^{13}C NMR spectra were determined at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal ($CDCl_3$, δ_H 7.26, δ_C 77.0; pyridine-d_5, δ_H 8.73, 7.56, and 7.21; δ_C 149.9, 135.6, and 123.6; C_6D_6, δ_H 7.15, δ_C 128). Homonuclear ^{1}H connectivities were determined by COSY and TOCSY (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-detected single-quantum heteronuclear correlation (HSQC) spectra were optimized for an average $^1J_{CH}$ of 140 Hz. Coupling constants of signals overlapped in the ^{1}H spectrum were measured from the rows of the HSQC and/or TOCSY experiments. The gradient-enhanced multiple-bond heteronuclear correlation (HMBC) experiment was optimized for a $^3J_{CH}$ of 8 Hz.

GC−MS spectra were performed on a gas chromatograph with a mass selective detector, 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 to 280 °C with a slope of 10 °C min^{-1}; quantitative determination was based on the area of the GLC peaks.

Collection, Extraction, and Isolation. Specimens of *A. damicornis* were collected in the summer of 2002 along the coast of Sorrento (Italy). They were frozen immediately after collection and kept frozen until extraction. Reference specimens were deposited at Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli. The sponge (1140 g wet weight) was homogenized and extracted, in sequence, with methanol (3 × 1 L), methanol/chloroform (2:1) (1 L), methanol/chloroform (1:2) (1 L), and chloroform (3 × 1 L). The MeOH extracts were partitioned between H_2O and *n*-BuOH, and the BuOH layer was combined with the remaining extracts, and concentrated in vacuo to afford 25.0 g of a dark brown oil. The crude extract was chromatographed on a column packed with RP-18 silica gel, and the fraction eluted with $CHCl_3$ (7.4 g) was further chromatographed on a SiO_2 column, giving a fraction [835 mg, eluent EtOAc/MeOH (7:3)] mainly composed of glycolipids.

The glycolipid fraction was peracetylated with Ac_2O in pyridine for 12 h. The acetylated glycolipids were subjected to HPLC separation on a SiO_2 column (eluent EtOAc)], thus affording a mixture (345 mg) containing **3b** and other glycolipids. Further normal-phase HPLC purification [eluent *n*-hexane/EtOAc (1:9)] gave 10.6 mg of damicoside peracetate (**3b**).

Damicoside Peracetate (3b). The peracetylated derivative **3b** $[\alpha]^{25}_D = +102$ (c = 0.19 in $CHCl_3$) was obtained as a colorless oil. ^{1}H and ^{13}C NMR data are available in Table 1. The composition in fatty acids is listed in Table 2. The composition in sphinganines is listed in Table 2.

Deacetylation of 3b. Compound **3b** (9.7 mg) was dissolved in 950 μL of MeOH, and 50 μL of a 0.4 M solution of MeONa in MeOH was added. The reaction was allowed to proceed for 18 h at 25 °C, the reaction mixture was dried under nitrogen, and the residue partitioned between water and chloroform. After removal of the solvent, the organic layer gave 6.6 mg of the native glycosphingolipid **3a** (92% yield).

Damicoside (3a): white solid; $[\alpha]^{25}_D = +55$ (c = 0.66 in MeOH); HRESIMS (positive ion mode, MeOH) m/z 1085.7823 ($[M + Na]^+$, $C_{57}H_{110}N_2NaO_{15}$ gives 1185.7798); ESIMS (positive ion mode, MeOH) m/z 1099 (34), 1085 (100), 1071 (98), 1057 (42), 1043 (25) ($[M + Na]^+$ series); ^{1}H NMR (pyridine-d_5) δ 9.28 (d, J = 8.3 Hz, 1H, 2-NH), 8.48 (d, J = 8.9 Hz, 1H, 2″-NH), 5.82 (d, J = 3.2 Hz, 1H, H-1″), 5.62 (d, J = 3.1 Hz, 1H, H-1′), 5.28 (m, 1H, H-2), 5.14 (m, 1H, H-5″), 4.90 (ddd, J = 10.6, 8.2 and 3.2 Hz, 1H, H-2″), 4.62 (m, 1H, H-1a), 4.60 (overlapped, H-4′), 4.59 (overlapped, H-2‴), 4.53 (overlapped, H-3″), 4.51 (overlapped, H-6″a), 4.47 (overlapped, H-5′), 4.47 (overlapped, H-3′), 4.40 (overlapped, H-2′), 4.36 (overlapped, H-6″b), 4.32 (overlapped, H-4), 4.30 (overlapped, H_2-6′), 4.30 (overlapped, H-1b), 4.28 (overlapped, H-4″), 4.25 (overlapped, H-3), 2.30 (m, 1H, H-5a), 2.22 (m, 1H, H-3‴a), 2.15 (s, 1H, 2″-Ac), 2.01 (m, 1H, H-3‴b), 1.92 (overlapped, H-6a), 1.87 (overlapped, H-5b), 1.72 (overlapped, H_2-4‴), 1.65 (overlapped, H-6b), 1.29 (br band, alkyl chain protons), 0.87 (overlapped, *n*-chain Me groups), 0.87 (overlapped, iso-chain Me groups), 0.85 (overlapped, anteiso-chain Me groups); ^{13}C NMR (pyridine-d_5) δ 100.4 (CH, C-1′), 100.0 (CH, C-1″), 78.9 (CH, C-4′), 76.5 (CH, C-3), 74.2 (CH, C-5″), 73.1 (CH, C-4″), 73.0 (CH, C-5′), 72.7 (CH, C-3″), 72.4 (CH, C-2‴), 72.1 (CH, C-4), 70.9 (CH, C-3′), 69.9 (CH, C-2′), 67.4 (CH_2, C-1), 62.6 (CH_2, C-6″), 60.8 (CH_2, C-6′), 55.8 (CH, C-2″), 50.1 (CH, C-2), 35.6 (CH_2, C-3‴), 34.6 (CH_2, C-5), 32.1 (CH_2, ω-2), 26.5 (CH_2, C-4‴), 25.8 (CH_2, C-6), 23.2 (CH_3, acetyl Me group), 23.0 (CH_2, ω-1), 22.8 (CH_3, iso-chain Me groups), 19.4 (CH_3, anteiso-chain Me group), 14.3 (CH_3, ω), 11.6 (CH_3, anteiso-chain Me group). The composition in fatty acids is listed in Table 2. The composition in sphinganines is listed in Table 2.

Methanolysis of 3a. A small amount (100 μg) of **3a** was dissolved in 1 mL of 1 N HCl in 91% 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 partitioned between $CHCl_3$ and H_2O/MeOH (8:2). After removal of the solvent, the aqueous layer gave a mixture of methyl glycosides

(fraction A) and the organic layer a mixture of α-hydroxy acid methyl esters and sphinganines (fraction B).

Methyl Tri-*O*-benzoyl-2-benzamido-2-deoxy-α-D-glucopyranoside (4). 2-Amino-2-deoxy-α-D-glucose (2.5 mg) was subjected to acidic methanolysis as described above. The resulting methyl glycosides were benzoylated with benzoyl chloride (50 μL) in pyridine (500 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column, Luna SiO_2, 5 μm; eluent, *n*-hexane/*i*-PrOH 99:1; flow, 1 mL/min), affording methyl tri-*O*-benzoyl-2-benzamido-2-deoxy-α-D-glucopyranoside **4** ($t_R = 31.8$ min) as the major reaction product: ^{1}H NMR ($CDCl_3$) δ 8.06 (d, $J = 7.8$ Hz, 2H, benzoyl ortho protons), 7.93 (d, $J = 7.8$ Hz, 2H, benzoyl ortho protons), 7.90 (d, $J = 7.8$ Hz, 2H, benzoyl ortho protons), 7.68 (d, $J = 7.8$ Hz, 2H, benzoyl ortho protons), 7.57 (t, $J = 7.8$ Hz, H-1, benzoyl para proton), 7.52−7.23 (11H, benzoyl protons), 6.62 (d, $J = 9.3$ Hz, 1H, NH-2), 5.82 (t, $J = 10.1$ Hz, H-3 or 1H, H-4), 5.76 (t, $J = 10.0$ Hz, H-4 or 1H, H-3), 5.02 (d, $J = 2.8$ Hz, 1H, H-1), 4.77 (m, 1H, H-2), 4.63 (br. d, $J = 10.1$ Hz, 1H, H-6a), 4.48 (dd, $J = 12.1$ and 4.9 Hz, 1H, H-6b), 4.37 (m, 1H, H-5), 3.50 (s, 3H, OMe); CD (MeCN) $\lambda_{max} = 230$ nm ($\Delta\epsilon = +6.5$).

Absolute Stereochemistry of Methyl Glycosides from Compound 3a. Fraction A from methanolysis of compound **3a** was benzoylated with benzoyl chloride (20 μL) in pyridine (200 μL) at 25 °C for 16 h. The reaction was then quenched with MeOH and after 30 min was dried under nitrogen. Methyl benzoate was removed by keeping the residue under vacuum for 24 h with an oil pump. The residue was purified by HPLC (column, Luna SiO_2, 5 μm; eluent, *n*-hexane/*i*-PrOH 99:1; flow, 1 mL/min). The chromatogram contained two peaks, which were identified as methyl tetra-*O*-benzoyl-α-D-galactopyranoside **5**[5] ($t_R = 8.3$ min) and tri-*O*-benzoyl-2-benzamido-2-deoxy-α-D-glucopyranoside **4** ($t_R = 31.6$ min) by a comparison of their retention times, ^{1}H NMR spectra, and CD spectra with those of the authentic samples prepared, respectively, from D-galactose[5] and 2-amino-2-deoxy-α-D-glucose.

Analysis of Fatty Acid Methyl Esters. Fraction B from methanolysis of compound **1a** was analyzed by GLC−MS and its components identified (Table 2) by a comparison of their retention times and mass spectra with those of authentic samples.

Analysis of Fraction B. Fraction B from methanolysis of compounds **1a** was benzoylated as described above, and the crude of reaction was purified by HPLC (column, Luna SiO_2, 5 μm; eluent, *n*-hexane/*i*-PrOH 99:1; flow, 1 mL/min). The chromatogram contained two peaks, which were identified as a mixture of homologous (*R*)-2-benzoyloxy fatty acid methyl esters (fraction C, $t_R = 3.8$ min) and a mixture of D-*ribo*-phytosphingosines (fraction D, $t_R = 6.5$ min) by comparison of their respective ^{1}H NMR and CD spectra with those reported.[14]

Oxidative Cleavage and GC−MS Analysis of Sphinganines. Fraction D was debenzoylated by acidic methanolysis, as reported above, and subjected to oxidative cleavage with $KMnO_4/NaIO_4$ as described.[13] 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.

Lymphocyte Proliferation Test. Spleens from C57Bl/6 mice (Charles River Italia, Calco, Como) were aseptically removed and minced, and cell suspensions were incubated at 5×10^5/well in 96-well microtiter plates (3799 Costar Italia, Milan, Italy) using an RPMI 1640 medium supplemented with 100 U/mL of penicillin, 2 mM glutamine, 100 μg/mL streptomycin, 20 mM Hepes buffer (Euroclone), and 10% FCS (Euroclone). All reagents were free of endotoxin contamination by the LAL assay. Various doses of damicoside (**3b**) and agelasphin (**2**) were added. Concanavalin A (1 μg/mL) was used as positive control. The treatment with different doses of DGJ (deoxygalactonojirimycin), an α-glycosidase inhibitor (Sigma), was performed for all the length of the assay or for a 2 h pulse, before the addition of the glycolipids. Cell proliferation was measured using the MTT assay, already described.[10] Plates were incubated for 72 h at 37 °C in 5% CO_2 and then 20 μL of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) (M-2128 Sigma) in PBS was added for an additional 3 h at 37 °C. The plates were then centrifuged, the supernatants discarded, and the dark blue formazan crystals dissolved using 100 μL of lysing buffer consisting of 20% (w/v) of a solution of SDS (Sigma) and 40% of *N*,*N*-dimethylformamide (Merck) in H_2O, at pH 4.7 adjusted with 80% acetic acid. The plates were then read on a microplate reader (Molecular Devices Co., Menlo Park, CA) at a test wavelength of 550 nm and a reference wavelength of 690 nm. The results are expressed as OD 550/OD650, which means that the values at OD 650 have been subtracted from the values at OD 550. All the tests were performed at least three times in quadruplicate and the statistical analysis was performed by one-way ANOVA with the Scheffe *F*-test post hoc; *p* values less than 0.05 were considered to be significant.

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Supporting Information Available: 1D and 2D NMR spectra of damicoside (**3a**), damicoside peracetate (**3b**), and damicoside pertrideuterioacetate (**3c**). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Natori, T.; Morita, M.; Akimoto, K.; Koezuka, Y. Agelasphin, novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritiana*. *Tetrahedron* **1994**, *50*, 2771−2784.

(2) Costantino, V.; Fattorusso, E.; Mangoni, A.; Aknin, M.; Gaydou, E. M. Axiceramide-A and B, two novel tri-α-glycosylceramides from the marine sponge *Axinella* sp. *Liebigs Ann. Chem.* **1994**, 1181−1185.

(3) Pettit, G. R.; Xu, J.; Gingrich, D. E.; Williams, M. D.; Doubek, D. L.; Chapuis, J.-C.; Schmidt, J. M. Isolation and structure of agelagalastatin from the Papua New Guinea marine sponge *Agelas* sp. *Chem. Commun.* **1999**, 915−916.

(4) Costantino, V.; Fattorusso, E.; Mangoni, A.; Di Rosa, M.; Ianaro, A.; Mafia, P. Immunomodulating glycosyl ceramides from the marine sponge *Agelas dispar*. *Tetrahedron* **1996**, *52*, 1573−1578.

(5) Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A. Clarhamnoside, the first rhamnose-containing α-glycosyl ceramide from the marine sponge *Agelas clathrodes*. *J. Org. Chem.* **2004**, *69*, 1174−1179.

(6) Kronenberg, M. Recognition of bacterial glycosphingolipids by natural killer T cells. *Annu. Rev. Immunol.* **2005**, *23*, 877−900.

(7) Kinjo, Y.; Wu, D.; Kim, G.; Xing, G.-W.; Poles, M. A.; Ho, D. D.; Tsuji, M.; Kawahara, K.; Wong, C.-H.; Kronenberg, M. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* **2005**, *434*, 520−525.

(8) Gonzalez-Aseguinolaza, G.; Kaer, L. V.; Bergmann, C. C.; Wilson, J. M.; Schmieg, J.; Kronenberg, M.; Nakayama, T.; Taniguchi, M.; Koezuka, Y.; Tsuji, M. Natural killer T cell ligand α-galactosylceramide enhances protective immunity induced by malaria vaccines. *J. Exp. Med.* **2002**, *195*, 617−624.

(9) Ishikawa, A.; Motohashi, S.; Ishikawa, E.; Fuchida, H.; Higashino, K.; Otsuji, M.; Iizasa, T.; Nakayama, T.; Taniguchi, M.; Fujisawa, T. A phase I study of a-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent nonsmall cell lung cancer. *Clin. Cancer Res.* **2005**, *11*, 1910−1917.

(10) Barbieri, L.; Costantino, V.; Fattorusso, E.; Mangoni, A.; Aru, E.; Parapini, S.; Taramelli. D. Immunomodulatory α-galactoglycosphingolipids: Synthesis of a 2′-*O*-methyl-α-GalGSL and evaluation of its immunostimulating capacity. *Eur. J. Org. Chem.* **2004**, 468−473.

(11) Prigozy, T. I.; Naidenko, O.; Qasba, P.; Elawaut, D.; Brossay, L.; Khurana, A.; Natori, T.; Koezuka, Y.; Kulkarni, A.; Kronenberg, M. Glycolipid antigen processing for presentation by CD1d molecules. *Science* **2001**, *291*, 664−667.

(12) Wu, D.; Xing, Guo-Wen; Poles, M. A.; Horowitz, A.; Kinjo, Y.; Sullivan, B.; Bodmer-Narkevitch, V.; Plettenburg, O.; Kronenberg, M.; Tsuji, M.; Ho, D. D.; Wong, Chi-Huey. Bacterial glycolipids and analogues as antigens for CD1d-restricted NKT cells. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1351−1356.
(13) Costantino, V.; Fattorusso, E.; Mangoni, A. Glycosyl ceramide composition of the marine sponge *Agelas clathrodes*. *Liebigs Ann. Chem.* **1995**, 1471−1476.
(14) Costantino, V.; Fattorusso, E.; Imperatore, C.; Mangoni, A. Vesparioside from the Marine Sponge *Spheciospongia vesparia*, the First Diglycosylceramide with a Pentose Sugar Residue. *Eur. J. Org. Chem.* **2005**, 368−373.

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