GLYCOLIPIDS FROM MARINE SPONGES: MONOGLYCOSYLCERAMIDES AND ALKYLDIGLYCOSYLGLYCEROLS: ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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ABSTRACT

Monoglycosylceramides have been isolated from the sponges *Axinyssa djiferi* and *Aaptos papillatus*, and alkyldiglycosylglycerols have been isolated from *Trikentrion laeve* and *Myrmekioderma dendyi*. Glycolipids from *Axinyssa djiferi* corresponded to a mixture of galactosylceramides, named axidjiferosides, with different α-hydroxy fatty acids and long-chain unsaturated bases, and showed an antimalarial activity. *Aaptos papillatus* contained glycolipids, named aaptopapillosides, possessing *N*-acetylglucosamine, a mixture of long-chain bases linked to hydroxylated or non hydroxylated fatty acyl chains. Acid methanolysis of mixtures of glycosylceramides homologues allowed to characterize their three parts by GC-MS: sugar unit, fatty acyl chains and bases. Glycolipids from *M. dendyi* had xylose, *N*-acetylglucosamine, a glycerol backbone and two alkyl long-chains with a terminal primary alcohol group. An *O*-alkyl-*O*-glycosylglycerol, known in *T. laeve,* was isolated in order to perform pharmacological screening, and was found to be associated with a series of glycolipids differing by chain length and unsaturation pattern. Ether glyceroglycolipids showed antitumor activity.

KEY WORDS

Sponge glycolipids, glycosylceramides, alkylglycosylglycerols, antimalarial activity, antitumor activity.

INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitous membrane constituents in animals, which play fundamental roles in major phenomena such as cell-cell recognition and

antigenic specificity. Glycolipids from marine sponges are known to possess immunomodulating and antitumor activity (COSTANTINO *et al.,* 1994, 1997, 2001; NATORI *et al.,* 1994; LI *et al.,* 1995). The first report of immunomodulating and antitumor glycosphingolipids came from a Japanese group which isolated agelasphins from the sponge *Agelas mauritiana* (NATORI *et al.,* 1994). These metabolites were the first members of a new class of glycolipids having an α-galactosylceramide structure. Another sponge belonging to the genus *Agelas* was also reported (CAFIERI *et al.,* 1994). Their synthetic analogue 1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol, namely KRN-7000, was selected for a clinical trial in patients with solid tumors (MORITA *et al.,* 1995; KOBAYASHI *et al.,* 1998; NATORI *et al.,* 2000). The mono-*O*-alkyl-*O*-diglycosylglycerols are a new type of sponge glycolipids, which have been isolated from *Trikentrion laeve* (COSTANTINO *et al.,* 1993) and *Myrmekioderma* sp. (AOKI *et al.,* 1999). The latter showed antitumor activity. We report the isolation and characterization of two types of sponge glycolipids: glycosphingolipids from *Axinyssa djiferi* and *Aaptos papillatus,* and alkyldiglycosylglycerols from *T. laeve* and *M. dendyi*.

MATERIAL AND METHODS

Axinyssa djiferi Boury-Esnault *et al.,* 2002 (Demospongiae, Halichondrida, Halichondriidae) was collected on mangrove tree roots, namely *Rhizophora mangle* (South of Senegal), Sept. 1996. *Trikentrion laeve* Carter, 1879 (Demospongiae, Poecilosclerida, Raspailliidae) was collected by scuba diving, off Dakar in depth 40 m, Sept. 1996. *Myrmekioderma dendyi* (Burton, 1959) (Demospongiae, Halichondrida, Desmoxyidae) was collected in South Pacific, near Vanuatu during the European Res. Progr. MAST III, by ORSTOM/IRD, Nouméa, New-Caledonia, in 1998. *Aaptos papillatus* (Keller, 1880) (Demospongiae, Hadromerida, Suberitidae) was collected in the Mediterranean Sea, off Montpellier. Sponges were steeped in CH_2Cl_2-MeOH (1:1, v/v) and the combined extracts yielded the crude total lipids. Glycolipids were separated from other lipids by column chromatography on silica gel with hexane, dichloromethane, acetone (glycolipids) and methanol. Individual glycolipids were refluxed in MeOH/H_2O/HCl, 29:4:3, v/v/v. Resulting fatty acids were analysed as methyl esters and *N*-acyl pyrrolidides, long-chain bases as *N*-acetylated-*O*-trimethylsilylated, and methylglycosides as peracetates.

FAB-MS measurements were obtained with a JEOL 700 mass spectrometer (Xe atoms) in the mixture 1,4-dithio-1-threitol/dithioerythritol (1:4), with NaI. HR-ESI-MS were obtained with a MS/MS ZabSpec TOF MICROMASS, positive mode, 4.5kV, source temperature 60° C, methanol/water (70:30, v/v), 1 % CH_3COOH. Peracetylated axidjiferosides were separated by reverse-phase HPLC (MeOH). Gas chromatography-mass spectrometry (GC-MS) was performed on a HP 5890II chromatograph, DB-1 column (30 m x 0.25 mm, 0.33 μm phase thickness) linked to a HP 5989A spectrometer and a HP 98785A integrator.

RESULTS

Monoglycosylceramide

The major components of the glycolipid mixture, isolated in this work, have been separated as peracetates by HPLC and studied by high resolution FAB-MS, electrospray ESI-MS, and NMR. Acid methanolysis of the mixture of gycosylceramides homologues afforded their three parts as useful derivatives for analysis by gas chromatography-mass spectrometry (GC-MS): sugar as a methyl glycoside, fatty acid chains as methyl esters (then converted into *N*-acyl pyrrolidides),

and sphingoid bases (then converted into *N*-acetyl-*O*-trimethylsilyl bases). Structural studies of isolated intact glycolipids were performed by high resolution FAB-MS, electrospray ionisation ESI-MS, and NMR.

Thus, the galactosylceramides named axidjiferosides were isolated from *Axinyssa djiferi*, sponge first identified in 1983 (BOURY-ESNAULT *et al.,* 2002). The major mixture of galactosylceramides (pure by TLC, m.p. 139 - 140° C) contained 9 components axidjiferosides A-I (Fig. 1).

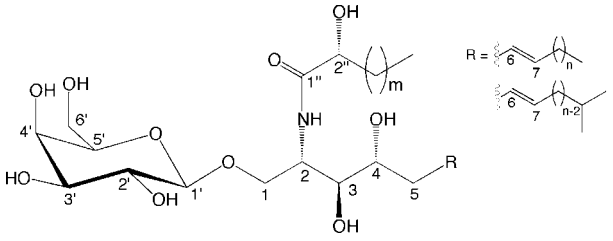

Fig. 1. Axidjiferosides from *Axinyssa djiferi*: 9 glycosylceramides (A-I): m = 19 - 23, n = 6 - 14.

The five major glycolipids (E-I) were isolated as peracetates by reverse-phase HPLC (MeOH). Structural studies of axidiferosides were performed by HR-FAB-MS. Mass spectra of peracetylated glycolipids E, F and H were analysed by electrospray ionisation MS and by NMR (Tab. I). The mass spectrum of the major glycolipid E showed an adduct $[M+Na]^+$ at m/z 1160.7402 (calcd for $C_{49}H_{82}O_{17}Na$: 1160.7437). The other major glycolipids showed at low resolution the peaks $[M+Na]^+$ at m/z 1160.8 and 1174.8, isomer and homologue respectively. The glycosyl ceramides contained C_{22}-C_{26} α-hydroxy fatty acids and C_{14}-C_{22} sphingoid bases, with an unusual double bond between C-6 and C-7. The double bond was assigned in the sphingoid long chain base through extensive analysis of 2D NMR data. The following correlations have been observed in a COSY experiment for the axidjiferosides. The multiplet at 4.33 ppm (1H) is correlated with the signal of N-H at 6.77 ppm (amide). It was attributed to H-2, also correlated with the H-1 protons (δ 3.89, dd; δ 3.70, dd). Starting from the H-2 signal, the COSY spectrum allowed us to assign, in sequence, H-3 (1H, δ 5.13, m), H-4 (1H, δ 5.02, m), H-5 (1H, δ 2.40, m; 1H, d 2.28, m), H-6 (1H, δ 5.48, dt), and H-7 (1H, δ 5.27, dt). The C-6/C-7 alkene bond was determined to be *trans*, as evidenced by the large coupling constant (15.3 Hz). Such unsaturation pattern in sphingoid base is quite rare, but has been formerly observed (ENDO *et al.,* 1986; HIRSCH & KASHMAN, 1989). The major components of the glycolipid mixture were separated by HPLC as peracetates and will be studied chemically and biologically.

Tab. I. Selected NMR data for peracetylated axidjiferosides.

H or C	δ ^{1}H (ppm) mult. (*J* in Hz)	δ ^{13}C (ppm)
H-1' anomeric	4.46 d (*J* = 8.0)	101
H-6 olefinic	5.48 dt (*J* = 15.3, 6.5)	134
H-7 olefinic	5.27 dt (*J* = 15.3, 6.9)	124
Isopropyl term.	0.86 d (*J* = 6.6)	22
Methyl term.	0.88 t (*J* = 6.7)	15
Long chain	1.30	30
7 Ac	2.23, 2.16, 2.10, 2.06, (6H), 2.04, 1.97	22
NH	6.77 dd (*J* = 8.9, 9.3)	--

The β-anomeric linkage was deduced from the value *J* = 8.0 Hz of coupling constant between H-1' and H-2'.

Aaptos papillatus contained two families of *N*-acetylglucosaminyl ceramides, namely aaptopapillosides A and B (colored oil), possessing hydroxylated or non hydroxylated fatty acyl chains. In NMR spectra, anomeric protons were at 4.46 ppm, d (*J* = 8.0 Hz) and 5.48 ppm, d (*J* = 15.3, 6.5 Hz), respectively coupled with anomeric carbons at 101 and 104 ppm. Other NMR data and all MS measurements gave the structures showed in Fig. 2.

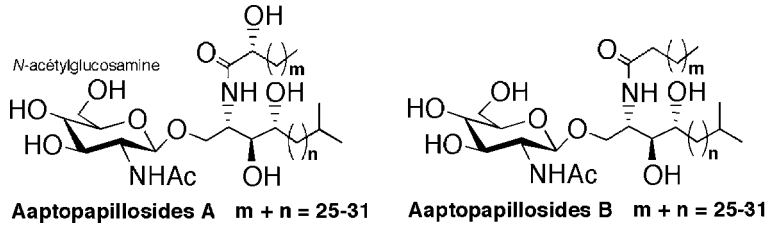

Fig. 2. Aaptopapillosides A and B from *Aaptos papillatus.*

Alkyldiglycosylglycerols

A glycerol ether glycolipid, already reported in *T. laeve* (COSTANTINO *et al.,* 1993) (Fig. 3) has been isolated in order to perform pharmacological screening. This unusual glycolipid includes a glycerol unit, two xylopyranoses, and a C_{24} alkenyl ether chain. It was associated with eight closely related compounds, differing by chain length and unsaturation pattern as shown by ESI-MS studies. Tab. II gives selected data for major glycolipid components as peracetates. Alkyl chains of the other components of the series differ by additional carbon atoms and double bonds.

Tab. II. Positive ESI-MS of the major peracetylated glycolipids (GL) from *T. laeve.*

$[M+Na]^+$ *m/z*	Formula	Calculated	Structural features
965.5441	$C_{49}H_{82}O_{17}Na$	965. 5450	Major GL, see Fig. 3
963.5249	$C_{49}H_{80}O_{17}Na$	963. 5293	+ 1 double bond
989.5457	$C_{51}H_{82}O_{17}Na$	989. 5450	+ 2 carbon atoms + 2 double bonds
991.5634	$C_{51}H_{84}O_{17}Na$	991. 5606	+ 2 carbon atoms + 1 double bond

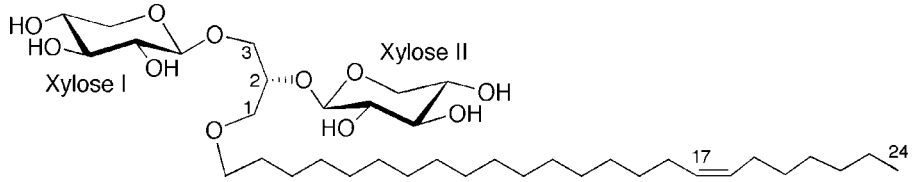

Fig. 3. Trikentroside from *Trikentrion laeve* (COSTANTINO *et al.,* 1993).

T. laeve contained some unusual secondary metabolites such as trikentramine (AKNIN *et al.,* 1990) and interesting phospholipid fatty acids including several new compounds (BARNATHAN *et al.,* 1996; BARNATHAN & KORNPROBST, 2000).

M. dendyi contained two alkyldiglycosylglycerols as major glycolipids, namely Myrmekiosides C and D, including xylose and *N*-acetylglucosamine, a glycerol backbone and alkyl long-chains with a terminal primary alcohol group (Fig. 4).

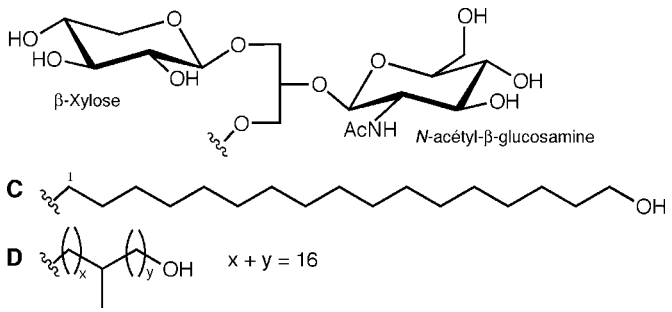

Fig. 4. Myrmekiosides from *Myrmekioderma dendyi.*

Similar glycolipid isolated from another *Myrmekioderma* sponge exhibited an antitumor activity (AOKI *et al.,* 1999). ^{1}H-NMR and ^{13}C-NMR, COSY, HMQC, HMBC correlations allowed to establish the structures. Two anomeric protons were observed at 4.25 ppm, d (J = 5.9 Hz) and 4.43 ppm, d (J = 7.9 Hz) for Myrmekioside C, and at 4.25 ppm, d (J = 5.8 Hz) and 4.40 ppm, d (J = 7.7 Hz) for Myrmekioside D. The alkyl moiety of Myrmekioside D was shown to contain a secondary methyl signal (δ 0.86, 3H, d, J = 6.5 Hz). In addition, a HMBC experiment showed a correlation from the secondary methyl group to methylene carbons at δ 37.1 and δ 33.1. Unfortunately, the small amount of this compound did not allow to determine the location of the methyl branch.

MS experiments (HR-ESI-MS,TOF) showed that they differ only by a CH_2 group as shown below in Table 3. MS/MS Experiments (positive FAB) confirmed the presence of the two different sugar units.

Tab. III. Positive ESI-MS of the major peracetylated Myrmekiosides C and D from *Myrmekioderma dendyi.*

Glycolipid	$[M+Na]^+$ m/z	Formula	Calculated
C	732.4504	$C_{35}H_{67}NO_{13}Na$	732.4510
D	718.4344	$C_{34}H_{65}NO_{13}Na$	718.4354

Acid methanolysis yielded the expected methyl glycosides and glycerol ether, identified as the peracetates by GC-MS. It should be noted that another species *Myrmekioderma* sp. (AOKI *et al.,* 1999) contained antitumor glycolipids closely related to those found in *M. dendyi* (Fig. 5).

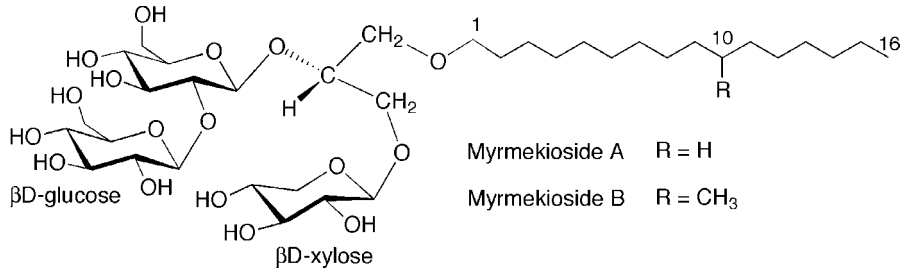

Fig. 5. Myrmekiosides from *Myrmekioderma* sp. (AOKI *et al.,* 1999).

Biological activity of the isolated glycolipids

Several biological tests have been performed (group GDR G1206, CNRS, ICSN): cytotoxicity, antifungal, antibacterial, antiviral and immunomodulation activities, activity against *Plasmodium*, *Leishmania* and *Trypanosoma*. Relationships between lipoprotein secretion and body cholesterol turnover (three cells models with ^{3}H-cholesterol: intestinal, hepatic, lipoproteins secretion) have been studied (Service Biochimie, Faculté de Pharmacie, Nantes). Immunomodulating properties (proliferation of the lymphocytes NKT) have also been studied (Institut Pasteur, Service Immunologie, Paris).

Total glycolipids from sponges *T. laeve* and *A. djiferi* were shown to be cytotoxic on KB cells (47 and 75 % inhibition activity at 10 μg/ml) and to possess an antimalarial activity (IC_{50}: 9.5 μg/ml and 3.1 μg/ml, respectively). Axidjiferosides showed an increasing activity against *Plasmodium falciparum* (IC_{50} = 0.45 μg/ml). Glycolipids from *M. dendyi* showed an antitumor activity on THP1 cells, and glycolipids from *T. laeve* on NSCLC-L16 cell line.

DISCUSSION AND CONCLUSIONS

Thus, this work showed a large structural diversity of sponge glycolipids as already observed. The pharmacological importance of sponge glycolipids is noteworthy as various active compounds have been isolated. This work confirms previous results that showed the interest of glycosphingolipids and glycoglycerolipids as potentially useful therapeutic agents.

The C-6/C-7 insaturation of the long-chain bases of axidjiferosides is quite unusual and it allows structural modulations, grafting of immunofluorescence markers, and isotopic labelling. The isolation of separated axidjiferosides, and of major gycolipids from *T. laeve* is in progress in our laboratory in order to select the most active component.

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REFERENCES

AKNIN M., MIRALLES J., KORNPROBST J.M., FAURE R., GAYDOU E.M., BOURY-ESNAULT N., KATO Y., CLARDY J., 1990 - Trikentramine, an original pyrrole derivative from the sponge *Trikentrion loeve* Carter. *Tetrahedron Lett.,* **31**: 2979-2982.

AOKI S., HIGUCHI K., KATO A., MURAKAMI N., KOBAYASHI M., 1999 - Myrmekiosides A and B, novel mono-*O*-alkyl-diglycosylglycerols reversing tumor cell morphology of ras-transformed cells from a marine sponge *Myrmekioderma* sp. *Tetrahedron,* **55**: 14865-14870.

BARNATHAN G., KORNPROBST J.M., 2000 - Fatty acids from marine organisms: recent research developments. In G. Baudimant, J. Guezennec, P. Roy, J.F. Samain, (eds), *Marine Lipids.* International Symposium on Marine Lipids, IFREMER Press, Brest, France: 35-43.

BARNATHAN G., KORNPROBST J.M., DOUMENQ P., MIRALLES J., 1996 - New unsaturated long-chain fatty acids in the phospholipids from the Axinellidae sponges *Trikentrion loeve* and *Pseudaxinella* cf. *lunaecharta*. *Lipids,* **31**: 193-200.

BOURY-ESNAULT N., MARSCHAL C., KORNPROBST J.M., BARNATHAN G., 2002 - A new species of *Axinyssa* Lendenfeld, 1897 (Porifera, Demospongiae, Halichondrida) from the Senegalese coast. *Zootaxa,* **117**: 1-8.

CAFIERI F., FATTORUSSO E., MAHAJNA Y., MANGONI A., 1994 - Longiside, a novel digalactosylceramide from the Caribbean sponge *Agelas longissima. Liebigs Ann. Chem.*: 1477-1481.

COSTANTINO V., FATTORUSSO E., MANGONI A., AKNIN M., FALL A., SAMB A., MIRALLES J., 1993 - An unusual ether glycolipid from the Senegalese sponge *Trikentrion loeve* Carter. *Tetrahedron,* **49**: 2711.

COSTANTINO V., FATTORUSSO E., MANGONI A., AKNIN M., GAYDOU E.M., 1994 - Two novel tri-α-galactosylceramides from the marine sponge *Axinella* sp. *Liebigs Ann. Chem.*: 1181-1185.

COSTANTINO V., FATTORUSSO E., MANGONI A., DI ROSA M., IANARO A., 1997 - Glycolipids from sponges. VII. Simplexides, novel immunosuppressive glycolipids from the Caribbean sponge *Plakortis simplex. Bioorg. Med. Chem. Lett.,* **9**: 271-276.

COSTANTINO V., FATTORUSSO E., MANGONI A., 2001 - Glycolipids with immunomodulating activity from marine sponges. In C. Tringali (ed.), *Bioactive Compounds from Natural Sources.* Taylor & Francis Inc., London and New York, Chap. 14: 557-575.

ENDO M., NAKAGAWA M., HOMAMOTO Y., ISHIHAMA M., 1986 - Pharmacologically active substances from southern Pacific marine invertebrates. *Pure Appl. Chem.,* **58**: 387-394.

HIRSCH S., KASHMAN Y., 1989 - New glycosphingolipids from marine organisms. *Tetrahedron,* **45**: 3897-3906.

KOBAYASHI E., MOTOKI K., UCHIDA T., FUKUSHIMA H., KOEZUKA Y., 1998 - KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res.,* **7**: 529-534.

LI H.-Y, MATSUNAGA S., FUSETANI N., 1995 - Halicylindrosides, antifungal and cytotoxic cerebrosides from the marine sponge *Halichondria cylindrata*. *Tetrahedron,* **51**: 2273-2280.

MORITA M., NATORI T., AKIMOTO K., SAKAI T., SAWA E., YAMAJI K., KOEZUKA Y., KOBAYASHI E., FUKUSHIMA H., 1995 - Structure-activity relationship of α-galactosylceramides against B-16-bearing mice. *J. Med. Chem.,* **38**: 2176-2187.

NATORI T., MORITA M., AKIMOTO K., KOEZUKA Y., 1994 - Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritianus*. *Tetrahedron,* **50**: 2771- 2784.

NATORI T., MOTOKI K., HIGA T., KOEZUKA Y., 2000 - KRN7000 as a new type of antitumor and immunostimulatory drug. In N. Fusetani (ed.), *Drugs from the Sea.* Karger AG., Basel, Switzerland: 86-97.