Tetrahedron Vol. 48, No. 32, pp. 6739-6746, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

Santiagoside, the first Asterosaponin from an Antarctic Starfish (*Neosmilaster georgianus*).

M. Jesús Vázquez[1], Emilio Quiñoá[1], Ricardo Riguera[1]*, Aurelio San Martín[2] and José Dárias[3].

[1]Departamento de Química Orgánica, Facultad de Química, Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain.

[2]Departamento de Química, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

[3]C.S.I.C., La Laguna, Tenerife, Spain.

(*Received in UK* 18 *June* 1992)

Abstract: A new asterosaponin, named santiagoside (**1**), has been isolated from the Antarctic starfish *Neosmilaster georgianus* and, its structure elucidated on the basis of extensive spectroscopic experiments and chemical correlations. Santiagoside is the first asterosaponin possessing a tetrasaccharide sugar chain and a 1,4-disubstituted glucose unit.

INTRODUCTION

Equinoderm saponins are a well established class of marine metabolites with complex structures of up to sixty carbons, and a wide range of biological activities. Apart from a couple of examples from sponges[1] and gorgonians[2], the only known sources of saponins are Asteroideae and Holothuridae[3]. Though difficult to isolate and purify, in the last few years the use of sophisticated separation procedures and powerful spectroscopic techniques has allowed the direct study of the structures on the intact compounds.

The geographic isolation and climatic characteristics of the Antarctic ocean, with low temperatures and high U. V. irradiation levels produce living conditions quite different from those of other seas. The adaptation of marine organisms to a habitat with different predators and feeding possibilities may reasonably be expected to have led to the synthesis of different or modified secondary metabolites.

As part of our work marine organisms, we describe in this paper the structure of a tetrasaccharide sulphated steroid (**1**, Figure 1) that we have named santiagoside[4]. It is the first saponin isolated from an Antarctic starfish. We used COSY,TOCSY, HMQC and ROESY (Figures 3 and 5) nmr spectroscopy for complete signal assignment and structural analysis. Chemical degradation results corroborated the spectroscopic findings.

The freeze-dried starfish *Neosmilaster georgianus* was extracted with acetone and the extracts were successively partitioned with hexane, dichloromethane and methanol. The methanol extract was partitioned into a mixture of n-butanol and water to afford the n-butanol soluble fraction. Polar material was recovered from the butanolic extract on a column of Amberlite XAD-2, which was followed by Sephadex LH-20 column chromatography.

Final isolation was accomplished by DCCC (7:13:8, $CHCl_3$/MeOH/H_2O, ascending mode) and C_{18} reverse-phase

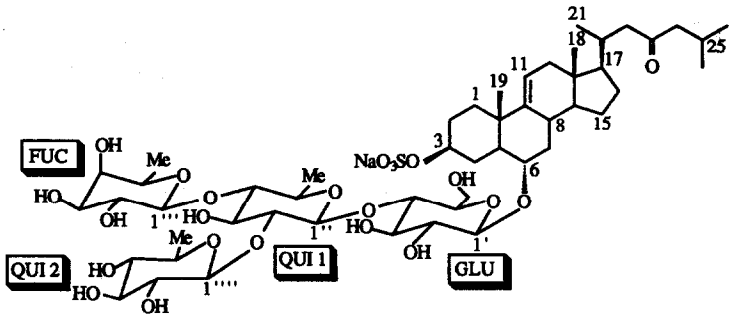

Fig. 1. Santiagoside, **1**.

HPLC (60:40, MeOH/H2O) to give pure santiagoside (**1**), a glassy material $[\alpha]_D^{20}$ = -50 (MeOH, c= 1.45 mg/ml) which upon treatment with HCl gave a 1:2:1 ratio of D-glucose, D-quinovose and D-fucose (identified by GC-MS in the form of the peracetates of the corresponding alditols).

The FAB mass spectrum (positive-ion mode) of **1** showed the molecular species $[(M+Na)]^+$ at m/z 1142 and $[(M-H+2Na)]^+$ at m/z 1164, where M is the molecular weight of 1119 daltons corresponding to the molecular formula $C_{51}H_{83}NaO_{23}S$. The fragment observed at m/z 1022, $[(M+Na)\text{-}NaHSO_4]^+$, indicated that santiagoside has a sodium sulphated group. The fragments corresponding to the loss of sugars from $[(M+Na)]^+$ were observed at m/z 996 $[(M+Na)\text{-}C_6H_{10}O_4]^+$, 703 $[(M+Na)\text{-}C_{18}H_{31}O_{12}]^+$ and 525 $[(M+Na)\text{-}C_{24}H_{41}O_{18}]^+$.

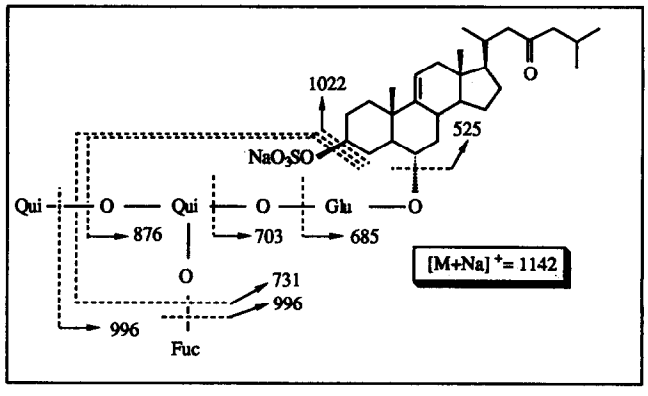

Fig. 2. Positive ion FAB mass spectroscopy results for santiagoside (**1**).

The difference between the fragments at m/z 703 [(M+Na)-trisaccharide]+ and 525 [(M+Na)-tetrasaccharide]+ is 178 amu ($C_6H_{10}O_6$), showing that glucose is the last sugar to be lost in the sequential cleavage of the glycoside chain and must thus be directly bonded to the aglycone (m/z 525, Figure 2). Although little further information about the sequence can be deduced from these data because of the identical molecular formulae of the other sugar components, fucose and quinovose, the absence of peaks corresponding to the loss of two other sugar monosaccharides can be taken as an indication that the chain is not linear but branched.

The ^{13}C nmr and DEPT spectra of **1**, recorded in pyridine-d_5, showed peaks for 51 carbons. It featured four quaternary carbons, ten methylenes, twenty-nine methines and eight methyl groups. There was a ketone peak at 210.4, signals at δ_C 116.3 and 145.7 indicative of a double bond, and a peak at 78.0 ppm typical of a carbon bonded to a sulphate group.

The ^1H nmr spectrum in the same solvent contained singlet signals for two quaternary methyl groups (0.52 and 0.85 ppm), two secondary methyls at δ_H 0.90 and another at 0.98 (J= 7.0 Hz), one olefinic proton at 5.75 ppm and a broad multiplet at 4.85 ppm. The chemical shifts of all the signals due to the aglycone coincided with those reported by Minale *et al.* for the steroidal part of the saponins from *Marthasterias glacialis*[5]. As for the signals due to the oligosaccharide chain, the ^1H nmr spectrum clearly shows the presence of five β-anomeric protons appearing as doublets (J= 7-8 Hz) in the 4.7-5.2 ppm region. Other significant signals were the three doublets at δ_H 1.45 (d, J= 6.0 Hz), 1.60 (d, J= 5.7 Hz) and 1.82 (d, J= 6.1 Hz) due to the methyl groups of D-fucose and D-quinovose, and two signals at 4.30 ppm (dd) and 4.46 ppm (dd) corresponding to the O-methylene protons of D-glucose. The presence of these sugars is confirmed by the ^{13}C nmr spectrum, which has four signals for the anomeric carbons in the 106-103 ppm region and a peak at δ_C 62.1 for the CH_2OH of D-glucose.

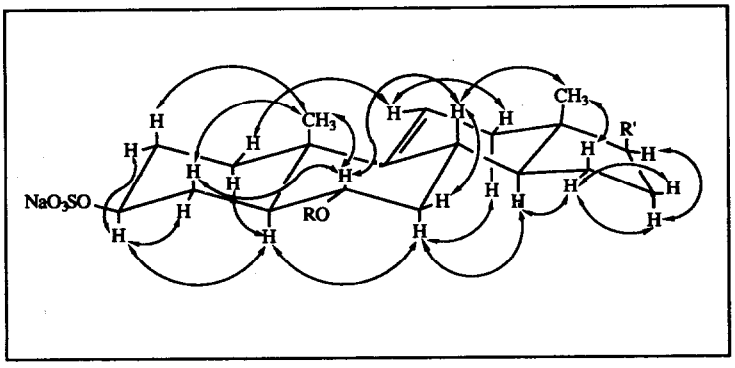

Fig. 3. Interpretation of ROESY data for the aglycone.

To deduce the sequence of the sugars, the location and stereochemistry of the interglycoside linkages and the stereochemistry of the aglycone, we used a combination of COSY, TOCSY, HMQC and ROESY experiments. COSY and TOCSY were used first to study the spin systems of the molecule. Starting from the easily distinguished anomeric proton of a particular sugar unit, COSY showed the correlations between successive vicinal spin systems until completion of that sugar unit. TOCSY[6], is one of the most powerful tools for the structural elucidation of glycosides in which the magnetization of one H is transferred sequentially to the next, since it allows the immediate determination of networks of mutually coupled protons. In our case, each sugar unit, as shown in Figure 4.

Having identified all the spin systems of **1**, we proceeded to use the results of a direct C-H correlated spectrum (HMQC) for the unambiguous assignment of all carbon and proton resonances. This showed the presence in **1** of one D-fucose and one D-quinovose unit as unsubstituted glycosides, one D-glucose as a monosubstituted glycoside, and another D-quinovose unit as a disubstituted glycoside. The aglycone signals were studied similarly. All assignments

are shown in Table 1.

The exact sequence of the sugars, their points of attachment and the stereochemistry of the compound were solved by means of ROESY experiments (Figures 3 and 5). The nOe's between H6 of the aglycone and H2' of glucose confirms that β-D-glucose is bound to C6 of the aglycone. That C4' of glucose is bound to the anomeric carbon of β-D-quinovose 1 may be deduced from the nOe's observed between H2" and H4' and between H2" and H2'. Finally, the existence of nOe's between H3" and H1"" and between H3" and H1"' leads to the conclusion that quinovose 1 is bonded to β-D-quinovose 2 and to β-D-fucose. On the basis of the above results, and of an analogous analysis of the signals for the aglycone[7], the structure of santiagoside is established as 3β-O-sulphated-6α-[β-D-fucopyranosyl (1→4) β-D-quinovopyranosyl ((2→1) β-D-quinovopyranosyl) (1→4) β-D-glucopyranosyl]-5α-cholest-9(11)-en-23-one.

To confirm the spectroscopic results, and in particular to corroborate the presence of an unusual C4' substituted glucose, we subjected 1 to the Hakomori procedure for oligosaccharides[8], which as expected gave 1, 4, 5-tri-O-acetyl-2, 3, 6-tri-O-methylglucitol; 1, 2, 4, 5-tetra-O-acetyl-3-O-methylquinovitol; 1, 5-di-O-acetyl-2, 3, 4-tri-O-methylfucitol and 1, 5-di-O-acetyl-2, 3, 4-tri-O-methylquinovitol (identified by GC-MS). Santiagoside is the first of the 60-odd known asterosaponins to have been isolated from a starfish that has a 1→4 bond between D-glucose and the branching sugar D-quinovose.

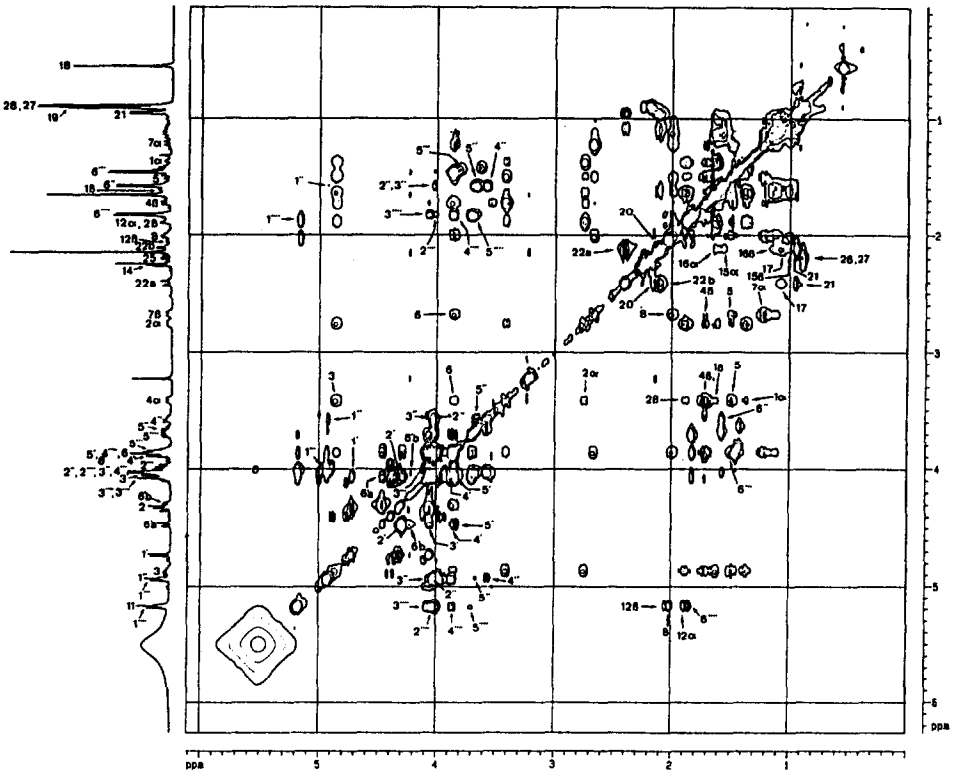

Fig. 4. Selected TOCSY assignments for santiagoside (1).

Table 1. ^{1}H and ^{13}C nmr Chemical Shifts of santiagoside in C_5D_5N (298 K).

CARBON	δ_C	DEPT	δ_H, mult., J (Hz)	CARBON	δ_C	DEPT	δ_H, mult., J (Hz)
1	36.7	CH_2	α=1.40, β=1.65	1'	105.3	CH	4.71, d, 7.6
2	29.8	CH_2	α=2.75, β=1.90	2'	71.7	CH	4.32, dd
3	78.0	CH	4.85, bm	3'	69.3	CH	4.06
4	31.0	CH_2	α=3.40, β=1.75	4'	91.0	CH	3.89
5	50.0	CH	1.58	5'	71.8	CH	3.85
6	80.2	CH	3.90	6'	62.1	CH_2	4.30, dd; 4.46, dd
7	42.0	CH_2	α=1.25, β=2.68				
8	35.4	CH	2.00, m	1''	103.5	CH	4.93, d, 7.9
9	145.7	C		2''	83.3	CH	4.02
10	38.2	C		3''	75.3	CH	4.02
11	116.3	CH	5.75	4''	85.0	CH	3.58
12	41.7	CH_2	α=1.88, β=2.05, dd	5''	71.4	CH	3.65
13	41.2	C		6''	17.5	CH_3	1.60, d, 5.7
14	53.6	CH	2.15				
15	25.1	CH_2	α=1.00, β=1.65	1'''	104.4	CH	4.92, d, 7.9
16	28.5	CH_2	α=1.60, β=1.15	2'''	73.8	CH	4.02
17	56.1	CH_2	1.13	3'''	74.9	CH	4.08
18	11.5	CH_3	0.56, s	4'''	72.2	CH	4.02
19	19.1	CH_3	0.95, s	5'''	77.5	CH	3.87
20	32.3	CH_2	2.13	6'''	16.7	CH_3	1.45, d, 6.0
21	19.5	CH_3	0.98, d, 7				
22	50.7	CH_2	2.05, 2.40, dd	1''''	105.8	CH	5.18, d
23	210.4	C		2''''	76.4	CH	3.95
24	52.3	CH_2	1.15, 1.60	3''''	77.0	CH	4.05
25	24.4	CH	2.15, m	4''''	75.7	CH	3.89
26	22.5	CH_3	0.90, d	5''''	73.8	CH	3.70
27	22.4	CH_3	0.90, d	6''''	18.1	CH_3	1.82, d, 6.1

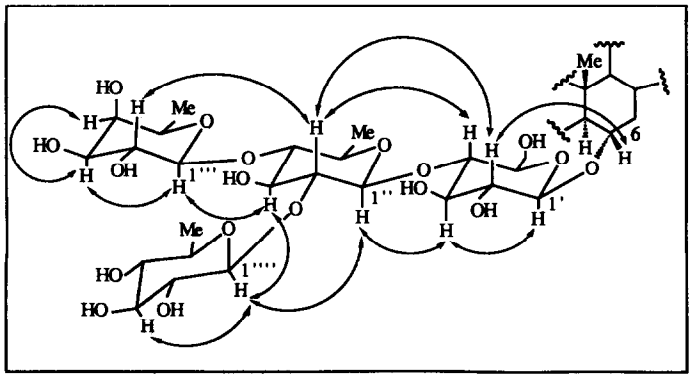

Fig. 5. Interpretation of ROESY data for the oligosaccharide.

The asterosaponins are known to play an important role in the defensive mechanism and toxicity of equinoderms. It remains to be seen whether other Antarctic starfish saponins present the same structural characteristics as **1**, and whether they have any precise biological significance.

EXPERIMENTAL

General.

Optical rotation was measured in MeOH in a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. One-dimensional ^1H and ^{13}C nmr spectra were recorded in C_5D_5N at 298 K on a Bruker WM-250 spectrometer at 250 and 60.13 MHz. HPLC was performed with a Waters Model 6000A apparatus equipped with an R401 differential refractometer. DCCC was carried out with an Eyela Model 300-S apparatus equipped with 300 tubes. Fast atom bombardment mass (FAB MS) spectra were obtained on a Kratos MS50 mass spectrometer with a Kratos FAB source by dissolving the samples in a 1:1 glycerol/thioglycerol matrix with NaCl and KCl as additives and bombarding with Xe atoms of 2-8 kV energy. GC-MS analyses were carried out with a Hewlett-Packard 5890 mass spectrometer and a Supelco SP^{TM}-2330 capillary column (15m, i. d. 0.25 mm, film thickness 0.20 μm, carrier gas He 1 ml/min, 240 °C) using the following experimental conditions:

a) alditol acetates: T_1= 240 °C maintained for 10 min; b) partially methylated alditol acetates: T_1 =80 °C maintained for 2 minutes, then 2 °C/min up to 100 °C, 30 °C/min up to 170 °C and 4 °C/min up to 240 °C, maintained for 10 minutes.

Two-dimensional nmr experiments.

All two-dimensional nmr measurements were performed on a Bruker AMX-400 spectrometer with a Bruker X-32 computer running the UXNMR software package. Two-dimensional ^1H-^1H COSY spectra (348 x 1K) were obtained accumulating 8 scans per t_1, with a relaxation delay of 1.5 s; the data were zero-filled to 1024 in F_1 and subjected to a QSINE transformation and symmetrized. The ^1H-^1H TOCSY experiments were performed in the phase sensitive mode (tppi) using the MELV-17 sequence for mixing; the spectral width (t_2) was 5050, 50 Hz, 512 experiments of 8 scans each (relaxation delay 1.5 s, mixing time 23.8 ms) were adquired in 2 K data points and subjected to QSINE transformation. The ^1H-^1H ROESY spectra was obtained accumulating 32 scans per t_1, with a relaxation delay of 1.5 s, and a the mixing time (tm of 1-2 s); the data were zero filled to 512 in F_1, subjected to QSINE transformation and symmetrized. The two-dimensional ^1H-^{13}C heteronuclear shift correlation (HMQC) spectra (512 x 1 K) for directly bonded protons and carbons were obtained by accumulating 8 scans per t_1, with a relaxation delay of 1.5 s and J_{CH}= 130 Hz.

Extraction and isolation.

Neosmilaster georgianus was collected by divers, from a rocky bottom at a depth of 35 m off Greenwich island, in the proximity of the Chilean Antarctic base Arturo Prat, and was immediately extracted with acetone. Specimens have been deposited at the Museo de Historia Natural, Santiago, Chile. The extract was evaporated to dryness under reduced pressure. The dry extract (72.5 g) was dissolved in MeOH/H_2O (90:10), defatted with hexane (2 x 200 ml), CCl_4 (2 x 200 ml) and CH_2Cl_2 (2 x 200 ml), and partitioned between n-BuOH and H_2O. The

n-BuOH extract was concentrated and passed through a column of Amberlite XAD-2 which was then washed with water (3 l) and eluted with MeOH (4 l). The methanol eluates were dried under reduced pressure at room temperature to give 2.6 g of a glassy material which was chromatographed on Sephadex LH-20 (flow rate 60 ml/h) using methanol as eluant and TLC monitoring (silica gel, 12:3:5, n-BuOH/AcOH/H_2O) giving five fractions. The second fraction contained 0.87 g of glycosides, which were rechromatographed by DCCC (ascending mode, 7:13:8, $CHCl_3$/MeOH/H_2O, flow rate 12 ml/h) into five fractions. The second fraction (0.23 g) was subjected to repetitive reverse phase HPLC on a 30 cm x 19 mm i. d. C_{18} μ-Bondapack column, eluting with 60:40 MeOH/H_2O, affording 17 mg of santiagoside (1) (retention time 20 min).

Santiagoside (1): $C_{51}H_{83}NaO_{23}S$. $[\alpha]_D^{20}$ = -50 (MeOH, c= 1.45 mg/ml). ^1H and ^{13}C nmr see Table 1. FAB-MS (positive ion mode; m/z %): 1164 ($[(M-H+2Na)]^+$,30), 1142 ($[(M+Na)]^+$, 100), 1022 ($[(M+Na)-NaHSO_4]^+$, 90), 996 ($[(M+Na)-C_6H_{10}O_4]^+$, 40), 731 ($[(M+Na)-C_{12}H_{20}NaO_{12}S]^+$, 40), 703 ($[(M+Na)-C_{18}H_{31}O_{12}]^+$, 45), 685 ($[(M+Na)-C_{18}H_{33}O_{13}]^+$, 38) and 525 ($[(M+Na)-C_{24}H_{41}O_{18}]^+$, 22).

Acid hydrolysis of santiagoside (1).

Compound 1 (2.9 mg) was refluxed in 2N HCl (1 ml) containing MeOH (1 ml) for 2h. The solution was neutralized with Ag_2CO_3, filtered, dried under reduced pressure at room temperature, and partitioned between CH_2Cl_2 and H_2O. The sugar fraction was dissolved in MeOH (2 ml), $NaBH_4$ (1 mg) was added, and the mixture was stirred at room temperature for 2 h. After addition of AcOH to eliminate excess $NaBH_4$, the mixture was concentrated to dryness under reduced pressure and the resulting alditols were acetylated with Ac_2O-pyridine (1:1, 2 ml) by refluxing overnight. The solution was washed with satd. aqueous $CuSO_4$ solution and extracted with CH_2Cl_2, and the alditol acetates were then identified as those of fucose, glucose and quinovose by GC-MS.

Permethylation of saponins and preparation of the partially methylated alditol acetates of santiagoside (1).

A solution of 1 (2.3 mg) in anhydrous dimethyl sulphoxide (DMSO) (1 ml) was stirred with dimsyl carbanion at 60 °C for 2 h under an Ar atmosphere. The mixture was treated with CH_3I (0.25 ml) under ice-cooling and stirred at room temperature for a further 1.5 h in the dark. The reaction mixture was then poured into ice-water and extracted with $CHCl_3$ (3 x 2 ml). The $CHCl_3$ was washed with satd. aqueous NH_4Cl solution, and then dried over Na_2SO_4 and evaporated under reduced pressure to give the permethylated compounds as an oily residue. A solution of the permethylated compounds in MeOH (1 ml) and 2N HCl (1 ml) was stirred at 60 °C for 2 h. After cooling, it was neutralized with Ag_2CO_3 and filtered, the solvent was evaporated in vacuo, and the resulting residue was reduced with $NaBH_4$ at room temperature for 4 h. The reaction was terminated by adding a few drops of acetic acid, and the resulting alditols were acetylated and analysed by GC-MS.

ACKNOWLEDGEMENTS

This work was supported by the Plan Nacional de Investigación (FAR 88-0512), the Xunta de Galicia (XUGA 20906B91 and XUGA 20904A90), the Plan Antártico Nacional (PAE) and the Instituto Antártico Chileno (INACH). M. J. V. acknowledges a fellowship from the Fundación Segundo Gil Dávila and the Xunta de Galicia. We are

grateful to Dr. Domingo Domínguez and BRUKER (Wiesembourg) for nmr experiments. We wish to thank Dr. John Dearborn (University of Maine, USA) for identifying the starfish.

REFERENCES

1. Valeria D' Auria, M.; Gómez Paloma, L.; Minale, L. and Riccio, R., *Tetrahedron*, **1992**, 48, 491-498.

2. Wasylyk, J. M.; Martin, G. E.; Weinheimer, A. J. and Alam, M., *J. Nat. Prod.*, **1989**, 52, 391.

3. **a** Faulkner, D. J., *Nat. Prod. Rep.*, **1984**, 1, 251-280; **b** *Nat. Prod. Rep.*, **1986**, 3, 1-33; **c** *Nat. Prod. Rep.*, **1987**, 4, 539-576; **d** *Nat. Prod. Rep.*, **1988**, 5, 613-663; **e** *Nat. Prod. Rep.*, **1990**, 6, 269-309; **f** *Nat. Prod. Rep.*, **1991**, 7, 97-147.

4. Named after the cities of Santiago (Chile) and Santiago de Compostela (Spain).

5. De Simone, F.; Dim, A.; Minale, L. and Pizza, C., *Tetrahedron Lett.*, **1979**, 11, 959-962.

6. Bax, A. and Davis, D.G., *J. Mag. Res.*, **1985**, 65, 355.

7. Although all the carbon signals and selected protons had been assigned by comparison with model compounds, no direct corroboration of the structure and stereochemistry of this aglycone has been reported till now.

8. Hakomori S., *J. Biochem.*, **1964**, 55, 205.