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Haplosamates A and B: New Steroidal Sulfamate Esters from two Haplosclerid Sponges

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Abstract: Two sponges from the Philippines, one a *Xestospongia* sp. (Haplosclerida, Petrosiidae) and the other an unidentified haplosclerid sponge, both contained the unusual steroidal sulfamate esters, haplosamates A (1) and B (2), the structures of which were determined by interpretation of spectral data. Haplosamates A (1) and B (2) inhibit HIV-1 integrase with IC_{50}'s of 50μg/mL and 15μg/mL, respectively. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Sponges, marine metabolites, steroids and sterols, NMR.

A recent review of sulfated compounds from marine organisms recorded about 100 sulfates from sponges, 35 of which were sterol sulfates.[1] Sponges of the family Petrosiidae are known to contain a variety of sterols, notably petrosterol from *Petrosia ficiformis*,[2] weinbersterol disulfates A and B and orthoesterol disulfates A, B and C from *Petrosia weinbergi*,[3,4] contignasterol from *Petrosia contignata*,[5] and the aragusterols from *Xestospongia* sp.[6] Of these, the disulfates from *P. weinbergi* showed *in vitro* activity against feline leukemia virus, mouse influenza virus, mouse coronavirus and HIV. Although sulfated sterols have gained a reputation as "nuisance compounds", due largely to the broad spectrum of biological activity associated with more common sulfates such as halistanol sulfate,[7] we believe that they may represent an opportunity for drug discovery.

As part of an ongoing investigation to isolate biologically active marine metabolites, we found that methanolic extracts of two sponges from the Philippines, a *Xestospongia* sp. (Haplosclerida, Petrosiidae) and an unidentified haplosclerid sponge, displayed mild *in vitro* activity against prostrate, human colon, colon carcinoma and mouse lung carcinoma. In this paper we report the isolation and structure elucidation of haplosamates A (1) and B (2), which were obtained from both sponges. These compounds represent the first examples of marine sterols with the sulfamate functionality and an unprecedented six-membered ether ring.

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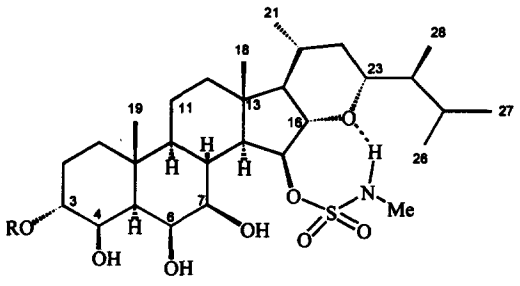
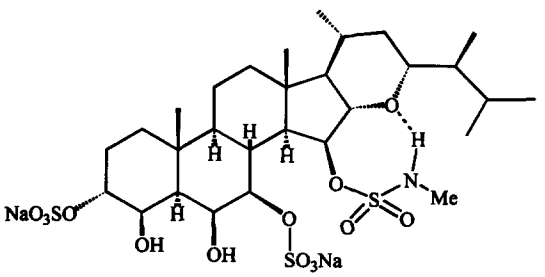
Although the haplosamates did not display useful activity in the antitumor assays used to select the sponges for investigation, they inhibited HIV integrase with IC_{50} values of 50 μg/mL and 15 μg/mL respectively.



1 R = SO_3Na
3 R = H



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The presence of the sulfamate functionality in marine sterols was hitherto unknown and there have been few chemical and spectroscopic studies of sulfamates. The most notable study of sulfamates was that of Maryanoff and co-workers[8] on the structure-activity studies of anticonvulsant sugar sulfamates related to topiramate, a synthetically derived antiepileptic drug. Structure-activity studies were also carried out on sulfamate sweeteners in the 1970's,[9] following the accidental discovery of the sweetening properties of cyclamate by Sveda in 1937.[10] Six-membered ether rings are also rare in marine sterols, although the cyclic hemiacetal functionality has recently been described.[5]

Results and Discussion

Specimens of *Xestospongia* sp. and an unidentified haplosclerid sponge were collected by hand using SCUBA at Hagakhak and Haningad Islands, Philippines, 1997, and were kept frozen until extraction. Both sponges were processed in the same manner. The concentrated methanolic extract of the *Xestospongia* sp., obtained by soaking the diced sponge tissue, was partitioned between ethyl acetate and water. The aqueous extract, which had shown mild activity in the initial screens against prostrate, human colon, colon carcinoma and mouse lung carcinoma, was chromatographed on Amberlite XAD-2 resin, followed by Sephadex LH-20 using 2:1 methanol:water as eluent. Final purification on TSK HW40F gel afforded haplosamate A (**1**, 0.041% wet wt.) and haplosamate B (**2**, 0.004% wet wt.) as colorless solids.

Haplosamate A (**1**) had a molecular formula of $C_{29}H_{51}NO_{11}S_2Na$, established by HRFABMS and NMR data (Table 1), which requires five degrees of unsaturation in addition to the sulfate groups. The FAB mass spectrum showed an $[M-H]^-$ ion at *m/z* 675 and a base peak at *m/z* 653 $[M-Na]^-$. Tandem ESI mass spectrometry displayed not only the $[M-Na]^-$ ion at *m/z* 653, but also fragments at *m/z* 573 $[M-Na-SO_3]^-$, 559

$[M\text{-}Na\text{-}SO_2NHCH_3]^-$ and 543 $[M\text{-}Na\text{-}SO_3NHCH_3]^-$, providing the first evidence for the presence of an *N*-methyl sulfamate group. The presence of eleven oxygen atoms, two sulfur atoms and one sodium atom in the molecular formula suggested the presence of both a sulfate ester group, present as the sodium salt, in addition to the sulfamate. Haplosamate A (**1**) gave a positive sulfamate test (white precipitate of barium sulfate on boiling an acidified aqueous-dioxane solution of **1** for 1 hr and then adding barium chloride solution).[9] Strong IR bands at 1210 and 1055 cm^{-1}, and 1370 and 1140 cm^{-1} confirmed the presence of the sulfate and sulfamate groups, respectively. A weak N-S band was observed at 710 cm^{-1}, with a NH band at 1640 cm^{-1} and bands at 3440 and 3260 cm^{-1} that were indicative of OH and NH groups.

The high field section of the ^1H NMR spectrum (Table 1) contained two methyl singlet resonances (δ 0.99 and 1.32) and four methyl doublet resonances (0.77, 0.81, 0.89, and 0.97). These data, together with the ^{13}C NMR and HRMS data, suggested that haplosamate A was a C_{28} sterol with the additional carbon being that of the *N*-methyl sulfamate. The low field portion of the NMR spectrum (Figure 1) contained seven one proton signals between δ 3.34 and 4.71, assigned to protons attached to carbons bearing oxygen atoms and two singlets at 3.59 and 3.63, that integrated to one and a half protons each.

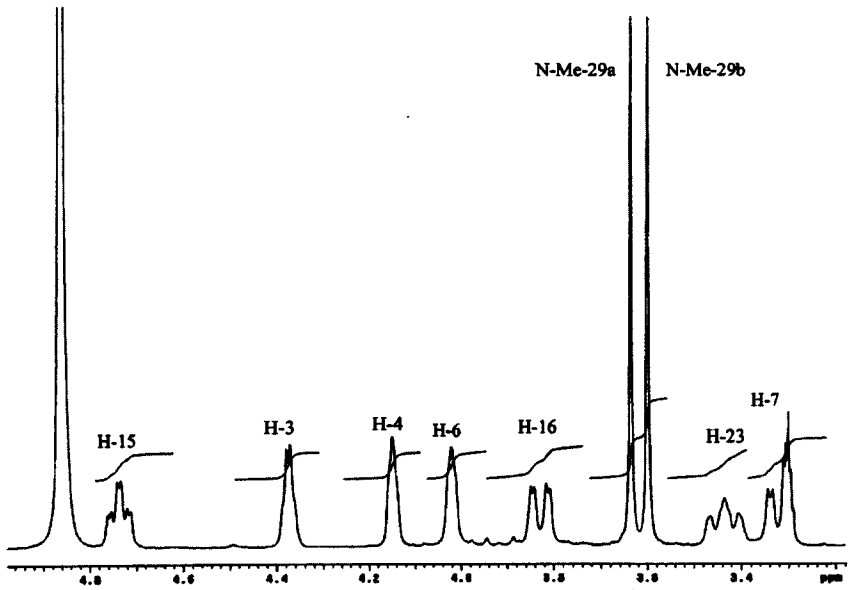

Figure 1. Partial ^1H NMR spectrum (methanol-d_4, 300 MHz) of haplosamate A (**1**)

We initially assigned these signals as an *N*-methyl doublet at δ 3.61 (J = 11 Hz) but this possibility was eliminated when the HMQC experiment indicated that the two proton signals were correlated to two carbon signals at slightly different chemical shifts. Since *N*-methyl sulfamates normally give rise to methyl singlets,[8] we had to account for an *N*-methyl sulfamate group that adopted two different conformations (see

Table 1. ^{1}H (300 MHz, CD_3OD) and ^{13}C (100 MHz, CD_3OD) NMR assignments for Haplosamate A (1)

C#	δC	δH	mult, *J* (Hz)	HMBC
1	34.9	1.33	m, 2 H	
2	23.8	1.82	m, 1 H	
		2.11	m, 1 H	
3	77.5	4.38	d, 1 H, 2.5	C-4, C-5
4	76.0	4.14	br s, 1 H	C-2, C-3, C-10
5	45.4	1.50	m, 1 H	C-4, C-6, C-7, C-9, C-10, C-19
6	78.0	4.01	br s, 1 H	C-4, C-5, C-10
7	80.0	3.34	m, 1 H	C-6, C-8, C-14
8	34.7	2.26	q, 1 H, 11	C-7, C-9, C-10, C-14
9	54.2	0.90	m, 1 H	
10	36.6	-		
11	19.9	1.54	m, 2 H	
12	41.0	1.19	m, 1 H	C-13, C-18
		1.82	m, 1 H	
13	43.0	-		
14	58.7	1.41	m, 1 H	C-7, C-8, C-13, C-18
15	81.8	4.71	m, 1 H	C-13, C-16
16	92.2	3.86	dd, 1 H, 10, 2	C-17, C-20, C-23
17	62.8	0.72	m, 1 H	C-16, C-18, C-20, C-21
Me-18	15.8	0.99	s, 3 H	C-11, C-12, C-13, C-14, C-17
Me-19	17.7	1.32	s, 3 H	C-1, C-5, C-9, C-10
20	34.2	1.86	m, 1 H	C-13*
Me-21	20.8	0.97	d, 3 H, 6.5	C-17, C-20
22	40.0	1.64	br d, 1 H, 11.5	
		0.86	m, 1 H	
23	82.7	3.45	t, 1 H, 9	
24	45.1	1.43	m, 1 H	C-23, C-25, C-26, C-28
25	28.4	2.03	m, 1 H	C-24, C-26, C-27, C-28
Me-26	17.6	0.81	d, 3 H, 7	C-24, C-25, C-27
Me-27	21.8	0.89	d, 3 H, 6.5	C-24, C-25, C-26
Me-28	10.7	0.77	d, 3 H, 7	C-23, C-24, C-25
N-Me 29a	53.3	3.63	br s, 1.5 H	
N-Me 29b	53.2	3.59	br s, 1.5 H	

* with 1 drop TFA

below). The ^{13}C NMR spectrum (Table 1) revealed the presence of twenty-nine carbon atoms, seven of which were oxygen-bearing carbons in the region between δ 76.0 and 92.2. Other characteristic signals observed, together with the results of the ^{1}H NMR, DEPT and GHMQC experiments, strongly suggested the presence of a C_{28} sterol skeleton which, in order to account for the five degrees of unsaturation, must also contain an additional cyclic ether ring.

Building on from a basic steroid structure, the structure of haplosamate A (1) was established using a combination of homonuclear and heteronuclear two-dimensional NMR experiments. Three pairs of vicinal oxygen-substituted carbons were assigned from the COSY correlations between signals at δ 4.38 (H-3) and 4.14 (H-4), 4.01 (H-6) and 3.34 (H-7), and 4.71 (H-15) and 3.86 (H-16), and the wealth of HMBC correlations (Table 1) allowed their positions on the sterol nucleus to be assigned. The final oxygen-substituted carbon was located at C-23 of a 24-methyl substituted side chain on the basis of HMBC correlations from both Me-26 and Me-27 to C-24 and C-25 and from Me-28 to C-23, C-24 and C-25. Furthermore, a correlation between H-16 and C-23 provided evidence for the position of the ether ring. Chemical shift data suggested that the sulfate and sulfamate groups were at C-3 and C-15. Hydrolysis of haplosamate A in 1:1 dioxane-pyridine at 130°C for 2 hr. gave the desulfated derivative **3** that retained the *N*-methyl sulfamate group (δ_H 3.59 and 3.63). The observed shift of the H-3 signal from δ 4.38 in **1** to 3.87 in **3** indicated that the sulfate ester was at C-3 and thus the sulfamate group was at C-15. By default, there are three hydroxyl groups at C-4, C-6 and C-7. Placement of the *N*-methyl sulfamate group at C-15 explains the presence of two *N*-methyl signals in the NMR spectra. Hydrogen bonding of the NH proton to the ether oxygen allows the *N*-methyl group to adopt two conformations as illustrated in Figure 2.

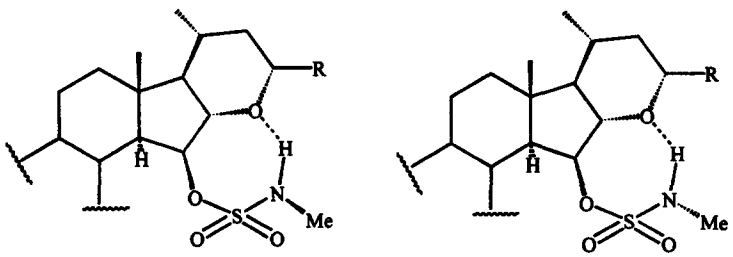

Figure 2. The two isomers present as a result of the sulfamate functionality

The relative stereochemistry of haplosamate A (1) was assigned primarily on the basis of NOESY data. Very strong correlations were observed between the signal at δ 1.50 (H-5) and those at 4.14 (H-4), 4.01 (H-6) and 3.34 (H-7), thereby placing all four protons on the same face of the molecule. The small coupling constants associated with H-3, H-4 and H-6 are consistent with an equatorial conformation for each of these

protons and the unusual chemical shift of the Me-19 signal at δ 1.32 is typical of a 4β,6β-dihydroxy sterol.[11] The three large coupling constants associated with H-8 indicate that H-7, H-8, H-9 and H-14 are all axial and the hydroxyl group at C-7 then has the equatorial conformation. The w-coupling between Me-19 and H-5 and H- 9 and between Me-18 and H-14 established the normal all-*trans* sterol nucleus. The NOESY correlations between H-14 and both H-15 and H-17 and between Me-18 and H-16 established the stereochemistry about the D-ring while correlations from H-16 to H-20 and H-23 defined the stereochemistry about the ether ring. The stereochemistry at C-24 was assigned with reasonable confidence on the basis of the large coupling constant between H-23 and H-24, which required a preferred *anti* conformation, and NOESY data. Correlations from H-22β to H-22α (strong), Me-21 (weak) and Me-28 (medium) and between the *N*-methyl signals and Me-27 required a 24β-methyl group on the side chain.

Haplosamate B (**2**) had a molecular formula of $C_{29}H_{50}NO_{14}S_3Na_2$, established by HRFABMS and NMR data, which again required five degrees of unsaturation, in addition to the sulfate-containing groups. The FAB mass spectrum showed ions at *m/z* 755 $[M - Na]^-$, 733 $[M + 1 - 2Na]^-$ and 653 $[M + 1 - 2Na - SO_3]^-$, which suggested that **2** was related to haplosamate A (**1**) by replacement of a hydroxyl group by a sulfate ester sodium salt. Haplosamate B (**2**) gave rise to strong IR bands at 1195 and 1040 cm^{-1}, and 1380 and 1090 cm^{-1}, indicative of sulfate and sulfamate moieties. The ^1H NMR spectrum differed from that of (**1**) in the region δ 3.4 – 4.9, where the shifts for protons attached to carbons bearing oxygen were observed somewhat downfield compared to those in (**1**). Most notable were the shifts for H-6 (δ 4.51 in **2**: 4.01 in **1**), H-7 (3.96 in **2**: 3.34 in **1**), H-8 (2.53 in **2**: 2.26 in **1**) and H-15 (4.85 in **2**: 4.71 in **1**). In the ^{13}C NMR data, there were notable differences in the chemical shifts for C-6 (δ 76.8 in **2**: 78.0 in **1**) and C-7 (84.2 in **2**: 80.0 in **1**). Based on these chemical shift considerations and comparison with literature values for other hydroxylated and sulfated sterols,[11] the sulfate group was placed at C-7. All other data, including COSY, HMQC, HMBC and NOESY spectra, were compatible with the structure proposed for haplosamate B (**2**).

Haplosamates A (**1**) and B (**2**) did not possess useful activity against tumor cell lines but were shown to inhibit HIV-1 integrase with IC_{50} values of 50 μg/mL and 15 μg/mL respectively. The desulfated compound **3** did not inhibit HIV-1 integrase. From these results and previous research,[12] it appears that the sulfate groups are important for activity.

Experimental

General: Optical rotations were measured on a Rudolph Research Autopol III polarimeter. IR and UV spectra were recorded on Perkin-Elmer 1600 FT-IR and Lambda 3B instruments, respectively. The ^1H, G(radient)COSY, GHMQC and GHMBC spectra were recorded on a Varian Inova 300 MHz spectrometer and the ^{13}C and DEPT experiments on a Varian Gemini 400 MHz spectrometer. The GHMBC experiment was recorded twice with *jnxh* set at 8 and 4 Hz. All NMR data were recorded in CD_3OD. HRMS were obtained

from the UC Riverside Regional Mass Spectrometry Facility. All solvents were distilled prior to use. All TLC was carried out on silica gel 60 F254 aluminium sheets with 12:3:5 butanol:acetic acid:water as eluent, and visualized with 2N cerium sulfate in H_2SO_4.

Animal Material: The sponges NCI 2350 (*Xestospongia* sp.) and NCI 2418 (unknown genus) were collected by hand using SCUBA at a depth of 10-17 m at Hagakhak Island (SSW Dinagat Island), Philippines, and Haningad Island, Philippines, respectively, in May 1997. Voucher specimens have been deposited in the SIO Benthic Invertebrate Collection as P1177 and P1178, respectively. The sponges were frozen immediately after collection, and kept frozen until extracted.

Extraction and Purification: Two contrasting extraction procedures were used for the unknown sponge (NCI 2418) but only the first procedure was used to extract *Xestospongia* sp. (NCI 2350), with very similar results to those reported for NCI 2418.

In the first procedure, frozen sponge (290 g wet wt.) was diced and extracted with MeOH (3 x 1L). The combined methanol extracts were concentrated and partitioned between 1:1 ethyl acetate/water (2 x 300 mL). At this stage it was already obvious from the ^{1}H NMR spectra of the crude extracts that the aqueous extracts of both sponges were very similar. The aqueous extract was lyophilized and then chromatographed on a C_{18} SepPak using a gradient elution of 100% water to 100% methanol as eluent. Fractions obtained with 30% and 70-80% methanol were further purified using countercurrent chromatography with $CHCl_3$/MeOH/H_2O (4:4:2) as eluent, followed by column chromatography with TSK HW40F gel (ToSoHaas) to obtain haplosamate B (**2**, 8mg, 2.8 x 10^{-3} % wet wt.) and haplosamate A (**1**, 104 mg, 0.04 % wet wt.). From NCI 2350 (290 g wet wt.), we obtained haplosamate B (**2**, 11mg, 3.8 x 10^{-3} % wet wt.) and haplosamate A (**1**, 120 mg, 0.04% wet wt.).

In the second procedure, the frozen sponge (297 g wet wt.) was again diced and extracted with methanol (3 x 1L). The combined methanol extracts were concentrated and partitioned between 1:1 ethyl acetate/water. The aqueous extract was lyophilized, then re-dissolved in water (25 ml) and chromatographed on Amberlite XAD-2 resin with copious amounts of water as eluent. The eluent was then changed to 100% methanol and the column washed thoroughly until no further color remained. The methanolic wash was concentrated under vacuum and then chromatographed on Sephadex LH-20 (2:1 methanol/water as eluent) followed by chromatography on TSK HW40F gel with 1:1 methanol/water as eluent to give haplosamate B (**2**, 26mg, 8.8 x 10^{-3} % wet wt.) and haplosamate A (**1**, 122mg, 0.04 % wet wt.).

Haplosamate A (1): colorless solid; mp. 210 °C (dec.); $[\alpha]_D$ -32° (*c* 0.4, MeOH); UV (MeOH) λ_{max} 269 nm (ε 9100); IR (film) 3440, 3260, 1640, 1370, 1210, 1140, 1055, 710 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 1; FABMS *m/z* (rel. int.) 675 $[M-H]^-$ (30.2), 653 $[M-Na]^-$ (100); ESI-MS *m/z* 653 $[M-Na]^-$, 573 $[M-Na-SO_3]^-$, 559 $[M-Na-SO_2NHCH_3]^-$, 543 $[M-Na-SO_3NHCH_3]^-$; HRFABMS *m/z* 653.2898 (calcd. for $C_{29}H_{51}NO_{11}S_2$, 653.2904).

Haplosamate B (2): colorless solid; mp. 213 °C (dec.); $[\alpha]_D$ -8° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 269 nm (ε 5200); IR (film) 3410, 3215, 3070, 2885, 1640, 1380, 1195, 1090, 1080, 1040, 710 cm^{-1}; ^{1}H NMR (CD_3OD, 300 MHz) δ 4.85 (m, 1 H, H-15), 4.51 (br s, 1 H, H-6), 4.36 (d, 1 H, *J* = 3 Hz, H-3), 4.14 (br s, 1 H, H-4), 3.96 (m, 1 H, H-7), 3.76 (dd, 1 H, *J* = 10, 2 Hz, H-16), 3.62 (br s, 1.5 H, *N*-Me), 3.58 (br s, 1.5 H, *N*-Me), 3.41 (t, 1 H, *J* = 9 Hz, H-23), 2.53 (q, 1 H, *J* = 11 Hz, H-8), 2.02 (m, 1 H, H-25), 1.87 (m, 2 H, H-2), 1.86 (m, 1 H, H-

20), 1.85 (m, 1 H, H-12a), 1.63 (m, 1 H, m, H-22), 1.56 (m, 1 H, H-5), 1.51 (m, 2 H, H-11), 1.45 (m, 1 H, H-14), 1.41 (m, 1 H, H-24), 1.37 (m, 2 H, H-1), 1.36 (s, 3 H, Me-19), 1.21 (m, 1 H, H-12b), 0.98 (m, 1 H, H-9), 0.98 (s, 3 H, Me-18), 0.97 (d, 3 H, J = 6 Hz, Me-21), 0.88 (d, 3 H, J = 7 Hz, Me-27), 0.85 (m, 1 H, H-22b), 0.80 (d, 3 H, J = 7 Hz, Me-26), 0.76 (d, 3 H, J = 8 Hz, Me-28), 0.72 (m, 1 H, H-17); ^{13}C NMR (CD_3OD, 100 MHz) δ 92.5 (C-16), 84.2 (C-7), 82.8 (C-23), 81.4 (C-15), 77.6 (C-3), 76.8 (C-6), 75.9 (C-4), 62.3 (C-17), 57.2 (C-14), 53.9 (C-9), 53.1 (N-Me), 53.0 (N-Me), 45.5 (C-5), 45.0 (C-24), 43.0 (C-13), 40.7 (C-12), 39.8 (C-22), 36.8 (C-10), 35.0 (C-1), 34.2 (C-20), 33.4 (C-8), 28.4 (C-25), 23.7 (C-2), 21.8 (Me-27), 20.8 (Me-21), 19.8 (C-11), 17.8 (Me-19), 17.7 (Me-26), 15.7 (Me-18), 10.7 (Me-28); FABMS *m/z* (rel. int.) 755 $[M\text{-}Na]^-$ (26.2); ESI-MS *m/z* 733 $[M + 1 - 2Na]^-$, 653 $[M + 1 - 2Na - SO_3]^-$; HRFABMS *m/z* 755.2295 (calcd. for $C_{29}H_{50}NO_{14}S_3Na$, 755.2291).

Solvolysis of Haplosamate A (1): Haplosamate A (**1**, 10 mg, 0.01 mmole) was stirred with freshly distilled pyridine (1 mL) and dry dioxane (1 mL) in a sealed vial at 130 °C for 2 hours. On cooling, the solvent was removed under a stream of N_2. The residue was purified on TSK HW40F gel using 50% aqueous methanol as eluent to obtain the desulfated derivative **3**: ^{1}H NMR (CD_3OD, 300 MHz, selected signals) δ 4.73 (H-15), 3.99 (H-6), 3.89 (H-16), 3.87 (H-3), 3.75 (H-4), 3.63 (br s, *N*-Me), 3.59 (br s, *N*-Me), 3.45 (H-23), 3.29 (H-7), 2.30 (H-8), 1.50 (H-5); ESI-MS *m/z* 597 $[M+Na]^+$, 573 $[M-H]^-$. HRFAB-MS *m/z* 597.3311 $[M + Na]^+$ (calcd. for $C_{29}H_{52}NO_8SNa$, 597.3313).

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