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ON THE COMPOSITION OF SULFATED POLYHYDROXYSTEROIDS IN SOME OPHIUROIDS AND THE STRUCTURE DETERMINATION OF SIX NEW CONSTITUENTS

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ABSTRACT.—Nine ophiuroid species from different geographical areas have been analyzed for their polar steroid content. Six new sulfated polyhydroxysteroids have been isolated, together with six known compounds of this type previously reported from brittle stars. The new steroids possess C-21 and 3α- sulfate groups, which are distinctive features of the steroid sulfates of ophiuroids. Hydroxylation at C-2, C-4, and C-11 and the presence of double bonds at C-5 are further features of the new steroids. The structures of the new compounds were solved by spectroscopic analysis.

In the course of our systematic study on the secondary metabolism of echinoderms, we have had the opportunity to investigate a number of ophiuroid species (1,2). They have shown a general lack of saponins, even though two steroidal glycosides were isolated from the Mediterranean species, *Ophioderma longicaudum* (Ophiodermataceae) (3), whereas several disulfated polyhydroxylated steroids were isolated from all species investigated. The large majority of polar steroids isolated from ophiuroids are characterized by the presence of C-21 and 3α- sulfate groups, cis A/B ring fusion, and additional hydroxy groups located in rings A and C (1,2).

The recent discovery of the antiviral properties of sulfated polyhydroxysteroids has increased interest in these compounds. Halistanol sulfate, isolated from the marine sponge *Halichondria moorei* (4) showed marked activity against HIV (5), and weibesterol disulfates, isolated from the sponge *Petrosia weinbergi* (6), exhibited in vitro activity against both feline leukemia virus and HIV-1. A selected group of disulfated sterols from ophiuroids was tested in the NCI's primary anti-HIV screen and all were shown to inhibit the cytopathic effects and replication of HIV-1 (7).

In this paper we report the results of a systematic investigation of nine ophiuroids species collected in different geographical areas (Table 1). This study has confirmed the ubiquitous occurrence of disulfated polyhydroxysteroids in ophiuroids and has led to the isolation of six more new steroids [**2–7**], whose structures were determined by spectral analysis.

RESULTS AND DISCUSSION

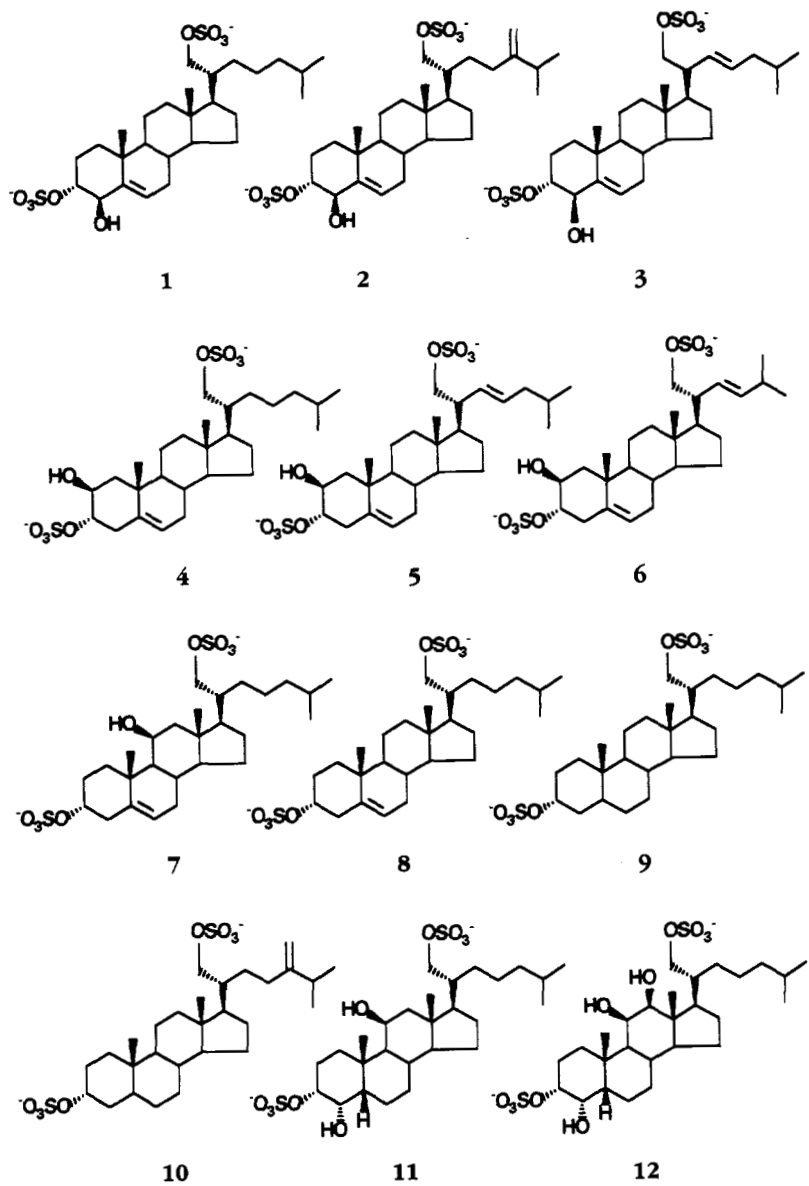
The samples of ophiuroids were extracted with MeOH and the extracts were partitioned between hexane and H_2O. The aqueous phase was then extracted with *n*-BuOH and the *n*-BuOH-soluble portions were chromatographed on Sephadex LH-20, followed by reversed-phase hplc. Table 1 reports the composition of sulfated polyhydroxysteroids in each ophiuroid species investigated.

The known compounds [**1**, **8–12**] were identified based on direct comparison (^{1}H-nmr, fabms, and hplc mobilities) with authentic samples (8–10). The structures of the new compounds [**2–7**] were determined by interpretation of spectral data (nmr and fabms) as well as by comparison of their spectral data with those of related compounds.

(20*R*)-Cholest-5-ene-3α,4β,21-triol 3,21-disulfate [**1**] was found to be the major component of *Ophiotrix fragilis, Ophiura texturata,* and *Ophionotus victoriae.* It was previously isolated by Stonik's group from the ophiuroid *Ophiura sarsi* (8). Negative-ion

TABLE 1. Composition of Disulfated Polyhydroxysteroids **1–12** in Ophiuroids.

Species and Collection Location	Compound											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Ophiocomina nigra Abildgaard (Galicia, Spain)								+	++	+		
Ophiotrix fragilis Abildgaard (Naples, Italy)	++	+	+	+				+	+			
Ophiura texturata Lamark (Naples, Italy)	++	+	+	+	+	+		+	+			
Ophiocoma scolopendrina Lamark (Okinawa, Japan)												+
Ophionereis reticulata Saj (Little San Salvador, Bahamas)							+				++	+
Ophiozona impressa Lutken (Little San Salvador, Bahamas)								+			+	+
Ophiocoma wendti Muller & Troshel (Little San Salvador, Bahamas)											+	+
Ophiocoma echinata Lamark (Little San Salvador, Bahamas)									+		+	+
Ophionotus victoriae Bell (Antarctic)	+			+	+			+	+			



fabms exhibited molecular ion species at *m/z* 615 [$M(SO_3K)(SO_3^-)$] and 599 [$M(SO_3Na)(SO_3^-)$], compatible with a disulfated trihydroxycholestene structure. The ^{1}H-nmr spectrum (Table 2) showed signals ascribable to a 21-sulfoxy group [δ_H 3.98 (1H, dd, J=10.0 and 6.5 Hz) and 4.24 (1H, dd, J=10.0 and 3.7 Hz)], a trisubstituted double bond [δ_H 5.65 (1H, br d, J=3.8 Hz)], and two >CHO- protons [δ_H 4.20 (1H, br d, J=2.7 Hz) and 4.52 (1H, q, J=2.7 Hz)]. The 3α,4β-diol-5-ene structure of **1** was deduced mainly from double resonance nmr experiments. Irradiation of the olefinic proton at δ 5.65 sharpened the H-4 proton at δ 4.20, whereas irradiation of this latter resonance collapsed the signal at δ 4.52 for H-3 to a doublet. The narrowing of the signal for H-3 is consistent with its equatorial orientation, while its downfield shift to δ 4.52 was in agreement with the location of a sulfate at that position. The configuration of the hydroxyl group at C-4 was assigned as β due to the downfield shifts observed in the ^{1}H-nmr spectrum for the olefinic proton (δ_H 5.65) and for the CH_3-19 protons (δ_H 1.24),

TABLE 2. Selected ^{1}H-Nmr Data for Compounds **1–7** (500 MHz, CD_3OD).[a]

Proton(s)	Compounds						
	1	**2**	**3**	**4**	**5**	**6**	**7**
H-2				4.10 br d (2.0)	4.10 br d (2.0)	4.13 br d (2.0)	
H-3	4.52 q (2.7)	4.52 q (2.7)	4.51 q (2.7)	4.45 m	4.45 m	4.45 m	4.64 m
H-4	4.20 br d (2.7)	4.20 br d (2.7)	4.19 br d (2.7)	2.34 br d (15.0)	2.30 br d (15.0)	2.34 br d (15.0)	
				2.85 dt (15.0, 2.0)	2.85 dt (15.0, 2.0)	2.88 dt (15.0, 2.0)	
H-6	5.65 br d (3.8)	5.65 br d (3.8)	5.65 br d (3.8)	5.38 br d (5.5)	5.38 br d (5.5)	5.38 br d (5.5)	5.19 m
H-18	0.78 s	0.78 s	0.80 s	0.78 s	0.80 s	0.82 s	1.02 s
H-19	1.24 s	1.24 s	1.24 s	1.19 s	1.19 s	1.22 s	1.31 s
H-21	3.98 dd (10.0, 6.5)	3.98 dd (10.0, 6.5)	3.84 t (10.0)	3.96 dd (10.0, 6.5)	3.84 t (10.0)	3.90 dd (10.0, 6.4)	3.99 dd (10.0, 6.5)
	4.24 dd (10.0, 3.7)	4.26 dd (10.0, 3.7)	4.25 dd (10.0, 3.7)	4.23 dd (10.0, 3.8)	4.22 dd (10.0, 3.8)	4.23 dd (10.0, 3.5)	4.25 dd (10.0, 3.6)
			5.27 dd (15.0, 8.0)			5.27 dd (15.0, 8.7)	
H-22			5.45 m			5.42 dd (15.0, 6.2)	
H-23							
H-26, 27	0.91 d (7.0)	1.07 d (7.0)	0.92 d (7.0)	0.91 d (7.0)	0.92 d (7.0)	1.02 d (7.0)	
Other signals		H-28 4.69 br s		H-1 1.85 dd		H-20 2.39 m	H-11α 4.37 q (2.2)
		4.75 br s		(13.2, 2.4)		H-24 2.28 m	
				1.56 dd (13.2, 4.6)			

[a]Data are expressed in δ values (ppm). For stated multiplicities, *J* values are provided in parentheses.

TABLE 3. ^{13}C-Nmr Spectral Data for Compounds **1**, **4**, **6**, and **7** (125 MHz, CD_3OD).[a]

Carbon	Compound			
	1	**4**	**6**	**7**
1	33.6	40.1	40.3	33.4
2	24.5	69.5	69.5	27.4
3	78.5	78.9	78.9	76.4
4	76.7	33.5	33.7	37.2
5	142.3	139.6	139.5	140.7
6	129.6	123.0	123.1	139.0
7	33.2	32.7	32.8	32.8
8	33.2	32.5	32.7	29.6
9	51.6	51.8	51.9	54.7
10	37.3	37.4	37.5	38.1
11	21.4	21.8	21.9	68.5
12	40.2	41.0	41.2	49.8
13	43.4	43.1	43.3	42.2
14	58.2	58.1	58.2	60.1
15	25.2	25.2	25.0	25.0
16	28.5	28.8	28.7	28.4
17	52.1	51.7	51.7	52.1
18	12.5	12.5	12.9	14.8
19	21.5	22.4	22.6	22.4
20	41.4	41.4	46.3	41.5
21	69.8	69.5	71.5	69.1
22	31.0	31.0	129.6	30.7
23	24.5	23.3	135.9	24.2
24	40.7	40.5	30.8	40.6
25	29.1	29.0	22.9	29.0
26	23.1	23.1	23.1	22.9
27	23.0	23.0		23.1

[a]Assignments aided by DEPT measurements.

consistent with a hydroxyl function that is axially oriented. The ^{13}C-nmr spectrum (Table 3) gave further support for the identification of **1** as (20*R*)-cholest-5-ene-3α,4β,21-triol 3,21-disulfate.

Compound **2**, (20*R*)-24-methylcholesta-5,24(28)-diene-3α,4β,21-triol 3,21-disulfate, isolated from *Ophiotrix fragilis* and *Ophiura texturata*, $[\alpha]_D$ −14.0°, fabms *m/z* 611 $[M(SO_3Na)(SO_3^-)]$ and 627 $[M(SO_3K)(SO_3^-)]$, is related to the more abundant **1** by the introduction of an exo-methylene at C-24. The ^{1}H-nmr spectrum showed two one-proton olefinic signals at δ 4.69 (br s) and 4.75 (br s) ascribable to the 28-methylene protons, and the signals for H-25 and -26 and CH_3-27 were observed downfield shifted to δ 2.31 (1H, heptet, J=7 Hz) and 1.07 (6H, d, J=7 Hz), respectively.

(20*R*,22*E*)-Cholesta-5,22-diene-3α,4β,21-triol 3,21-disulfate, **3**, $[\alpha]_D$ −6.36°, was also isolated from both the Mediterranean *Ophiotrix fragilis* and *Ophiura texturata*. The fabms exhibited molecular ion species at *m/z* 575 $[M(SO_3H)(SO_3^-)]$, 597 $[M(SO_3Na)(SO_3^-)]$ (major) and 613 $[M(SO_3K)(SO_3^-)]$, two mass units shifted relative to **1**. An analysis of the ^{1}H-nmr spectrum revealed that this compound had the same nucleus as **1**, with a Δ^{22} cholesterol side-chain. The ^{1}H-nmr spectrum included two well-separated olefinic protons at δ 5.27 (1H, dd, J=15.0 and 8.0 Hz, H-22) and 5.45 (1H, m, H-23). The presence of the Δ^{22} double bond also caused the expected slight downfield shift of CH_3-18 to δ 0.80 (δ 0.78 in **1**) and the upfield shift of one of two C-21 protons to δ 3.84 (δ 3.98 in **1**).

(20*R*)-Cholest-5-ene,2β,3α,21-triol 3,21-disulfate [**4**], [α]D +8.7°, isolated from *Ophiotrix fragilis, Ophiura texturata,* and *Ophionotus victoriae,* showed fabms molecular ion species at *m/z* 599 [$M(SO_3Na)(SO_3^-)$] and 615 [$M(SO_3K)(SO_3^-)$], corresponding to a disulfated trihydroxycholestene structure. The ^{1}H- and ^{13}C-nmr data (Tables 2 and 3) indicated the presence of a 21-sulfoxycholestane side-chain and a trisubstituted double bond (δ_H 5.38, δ_C 139.6 and 123.0), which was located at the C-5–C-6 position. Also observed were two >CH-O- signals as narrow multiplets at δ 4.10 and 4.45 coupled to each other with J=2.0 Hz. The location and stereochemistry of two secondary oxygenated functions at C-2 and C -3 of the tetracyclic nucleus were determined from double-resonance nmr experiments and coupling constant analysis. Irradiation of the olefinic proton at δ 5.38 collapsed the C-4 proton at δ 2.85 (dt, J=15.0 and 2.0 Hz) to a doublet of doublets (J=15.0 and 2.0 Hz). Irradiation of the signal resonating at δ 4.45 collapsed both the C-4 geminal protons at δ 2.85 and 2.34 (br d, J=15.0 Hz) to a doublet of doublets (J=15.0 and 2.0 Hz) and to a sharp doublet, respectively, whereas irradiation of the signal resonating at δ 4.10 collapsed the two geminal protons, assigned to H_2-1, at δ 1.56 (1H, dd, J=13.2 and 4.6 Hz) and δ 1.85 (1H, dd, J=13.2 and 2.4 Hz) into two doublets with J=13.2 Hz. Protons at C-2 and C-3 were both assigned as equatorial due to the absence of any detectable large vicinal coupling constants, and the sulfate was placed at C-3 based on the downfield shift observed for the C-3 proton compared to the desulfated derivative (see Experimental). Comparison of nmr spectral data (Tables 2 and 3), and especially the chemical shifts of CH_2-1 and CH_2-4 of **4** with those of the isomeric (20*R*)-cholest-5-ene-2β,3α,21-triol 2,21-disulfate [CH_2-1: δ_H 2.16 dd, 1.61 dd, δ_C 39.1; CH_2-4: δ_H 2.84 dd, 2.00 dd, δ_C 35.8 ppm], recently isolated from the Antarctic ophiuroid *Ophiosparte gigas* (11), gave strong support to the location of the sulfate at C-3 in **4**. The 20*R*-configuration assigned to **4** was further supported by the ^{1}H-nmr chemical shifts of the 21-hydroxymethylene protons of the desulfated derivative at δ 3.70 ($CDCl_3$); in the reference (20*R*)- and (20*S*)-cholest-5-ene-3β,21 diols, the signals for the protons at C-21 are reported at δ 3.70 s and at 3.62 m, respectively (12).

(20*R*,22*E*)-Cholesta-5,22-diene-2β,3α,21-triol 3,21-disulfate [**5**], [α]D −7.4°, isolated from *Ophiura texturata* and *Ophionotus victoriae*, had the same tetracyclic nucleus as **4**. The data from its fabms spectrum (*m/z* 597 and 613) and from the ^{1}H-nmr spectrum (Table 2) clearly indicated **5** to be the Δ^{22E} derivative of **4**.

(20*R*,22*E*)-24-Norcholesta-5,22-diene-2β,3α,21-triol 3,21-disulphate [**6**], [α]D −10.4°, isolated from *Ophiura texturata*, had the same tetracyclic nucleus as **4** with a shorter side-chain, which was implied by the negative fabms [*m/z* 599 [$M(SO_3K)(SO_3^-)$], 583 [$M(SO_3Na)(SO_3^-)$], and *m/z* 561 [$M(SO_3H)(SO_3^-)$], and nmr data. The ^{13}C-nmr spectrum was consistent with the presence of 26 carbon atoms, and DEPT measurements revealed the presence of four methyl groups, seven methylenes, six methines, two quaternary carbons, two -OCH< units, one -OCH_2- unit, and a disubstituted and a trisubstituted double bond. The structure of the side-chain was determined from double-resonance nmr experiments. One of the well-separated *E*-olefinic protons at δ 5.27 was coupled to H-20 at δ 2.39, which was in turn coupled to CH_2-21 at δ 3.90 and 4.23. The other olefinic proton at δ 5.42 was coupled to H-24 at δ 2.28, which was coupled with both the CH_3-25 and -26 protons at δ 1.02 (d, J=7.0 Hz). The ^{13}C-nmr data gave further support to the proposed structure (see Table 3).

(20*R*)-Cholest-5-en-3α,11β,21-triol 3,21-disulfate [**7**], [α]D −7.60°, was isolated from the ophiuroid *Ophionereis reticulata*. The fabms showed molecular ion species at *m/z* 599 and 615, corresponding to a trihydroxycholestene disulfate structure. The ^{1}H-nmr spectrum contained the usual signals at δ 3.99 dd and 4.25 dd, coupled to each other by 10 Hz, indicative of a 21-sulfoxy cholestane side-chain, an olefinic proton at δ 5.19 (br

s, H-6), and one methine proton at δ 4.64 (m, H-3), assigned to a 3α-sulfoxy-Δ^5 steroid by comparison with ^{1}H- and ^{13}C-nmr data for the (20*R*)-cholest-5-ene-3α,21-diol 3,21-disulfate previously isolated from other ophiuroid species. Also present in the ^{1}H-nmr spectrum of **7** was a narrow hydroxymethine signal at δ 4.37 (q, J=2.2 Hz), which was assigned to an 11β-hydroxy group, in agreement with the nmr resonances of CH_3-18 and -19 [both shifted downfield to δ_H 1.02, δ_C 14.8; δ_H 1.31, δ_C 22.4, respectively, due to the 1,3-diaxial interaction with 11β-OH]. In confirmation, the resonances of nuclear carbons of the rings C and D in **7** matched those of the known 5β-cholestane-3α,4α,11β,21-tetrol 3,21-disulfate [**11**].

EXPERIMENTAL

ANIMAL MATERIAL.—*Ophiotrix fragilis* and *Ophiura texturata* were collected in the Bay of Naples in 1989, and identified by Dr. Flegra Bencivenga (Stazione Dohrn, Naples; voucher specimens are preserved there). *Ophiocomina nigra* was collected in 1987, along the coasts of Galicia, Spain, and identified by Carlos Duran of Centro Investigaciones Submarinas (a voucher specimen is preserved at the Departemento de Quimica Organica, Universidad Santiago de Compostela, Spain). *Ophiocoma scolopendrina* was collected at Manza, Japan, in 1986, and identified by the zoologists of the Department of Marine Sciences, University of the Ryukyus, Okinawa, Japan, where a voucher specimen is preserved. *Ophionotus victoriae* was collected during the 1989–90 Italian-Antarctic expedition and identified by Prof. Gordon Hendler of the Natural History Museum of Los Angeles (a voucher specimen is preserved at Dipartimento di Chimica delle Sostanze Naturali, Naples, under the reference number Mor 68). *Ophionereis reticulata, Ophiozona impressa, Ophiocoma wendti,* and *Ophiocoma echinata* were collected during the 1990 Fenical expedition in the Bahamas Islands (Little San Salvador Island) and identified by the zoologist on the ship. Reference specimens are preserved at Dipartimento di Chimica delle Sostanze Naturali.

EXTRACTION AND FRACTIONATION.—Each sample was stored frozen and then chopped in small pieces and extracted three times with MeOH (1 liter for 1 kg of animal). Removal of solvent under reduced pressure left a residue in each case, which was partitioned between H_2O and *n*-hexane. These aqueous residues were then extracted twice with *n*-BuOH. The combined *n*-BuOH layers were filtered and taken to dryness under reduced pressure to give viscous oils (the amount of each *n*-BuOH extract from the respective wet organism is reported in Table 4), which by tlc analysis (Si gel, *n*-BuOH-AcOH-H_2O, 4:3:5) were found to contain polar metabolites (R_f 0.3–0.5). Each *n*-BuOH extract was chromatographed on a column of Sephadex LH-20 (2×60 cm, MeOH, 6 ml fractions were collected) to give the major polar compounds dispersed in the fractions listed in Table 4. The polar fractions obtained from chromatography on the Sephadex LH-20 column were dissolved in H_2O and subjected to prep. reversed-phase hplc on a C_{18} μ-Bondapak column with MeOH-H_2O (45:55) to give the pure compounds **1–12**. The known compounds [**1**, **8–12**] were identified based on direct comparison (fabms, ^{1}H-nmr, and hplc mobilities) with authentic samples (8–10).

TABLE 4. Sulfated Polyhydroxysteroids Present in Sephadex LH-20-Eluted Fractions Obtained from *n*-BuOH-Soluble Extracts

Species	Fraction Number	Combined Fraction Weight (mg)	Compound(s) Present[b]
Ophiocomina nigra (1.8 g)[a]	60–93	108	**8, 9, 10**
Ophiotrix fragilis (1.6 g)[a]	78–118	122	**1, 2, 3, 4, 8, 9**
Ophiura texturata (1.8 g)[a]	107–134	103	**1, 2, 3, 4, 5, 6, 8, 9**
Ophiocoma scolopendrina (18 g)[a]	68–120	1026	**12**
Ophionereis reticulata (0.6 g)[a]	63–85	61	**7, 11, 12**
Ophiozona impressa (1.1 g)[a]	100–129	38	**8, 11, 12**
Ophiocoma wendti (5.1 g)[a]	121–151	136	**11, 12**
Ophiocoma echinata (6.4 g)[a]	110–147	234	**9, 11, 12**
Ophionotus victoriae (4.7 g)[a]		280	**1, 4, 5, 8, 9**

[a]Amount of *n*-BuOH extract obtained from the following wet wts of the animals: *Ophiocomina nigra*, 1.1 kg; *Ophiotrix fragilis*, 0.38 kg; *Ophiura texturata*, 0.48 kg; *Ophiocoma scolopendrina*, 3.4 kg; *Ophionereis reticulata* 0.15 kg; *Ophiozona impressa*, 0.15 kg; *Ophiocoma wendti*, 3.0 kg; *Ophiocoma echinata*, 3.2 kg; *Ophionotus victoriae*, 2.1 kg.

[b]As observed by tlc analysis (see Experimental).

The ^{1}H-nmr spectra for compounds **1–7** are reported in Table 2, with the ^{13}C-nmr spectra of compounds **1**, **4**, **6**, and **7** reported in Table 3. The fab mass spectrometry (negative-ion) data of the new compounds **2–7** are indicated in the text.

SOLVOLYSIS OF COMPOUND **4**.—A solution of **4** (1 mg) in pyridine (0.1 ml) and dioxane (0.1 ml) was heated at 140° for 24 h in a stoppered reaction vial. Removal of the solvent left a crude reaction mixture that was chromatographed by reversed-phase hplc to afford 0.6 mg of a desulfated derivative: hreims *m/z* 418.6015; ^{1}H nmr (CD_3OD) δ_H 0.75 (3H, s, CH_3-18), 0.92 (6H, d, J=6.8 Hz, CH_3-26 and CH_3-27), 1.18 (3H, s, CH_3-19), 3.55 (1H, dd, J=10.5 and 5.5 Hz, H-21), 3.73 (1H, dd, J=10.5 and 3.4 Hz, H-21), 3.78 (1H, br d, J=3.4 Hz, H-3), 3.80 (1H, br d, J=3.4 Hz, H-2), 5.35 (1H, m, H-6).

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