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Biologically-active sterol sulfates from the marine sponge *Toxadocia zumi*

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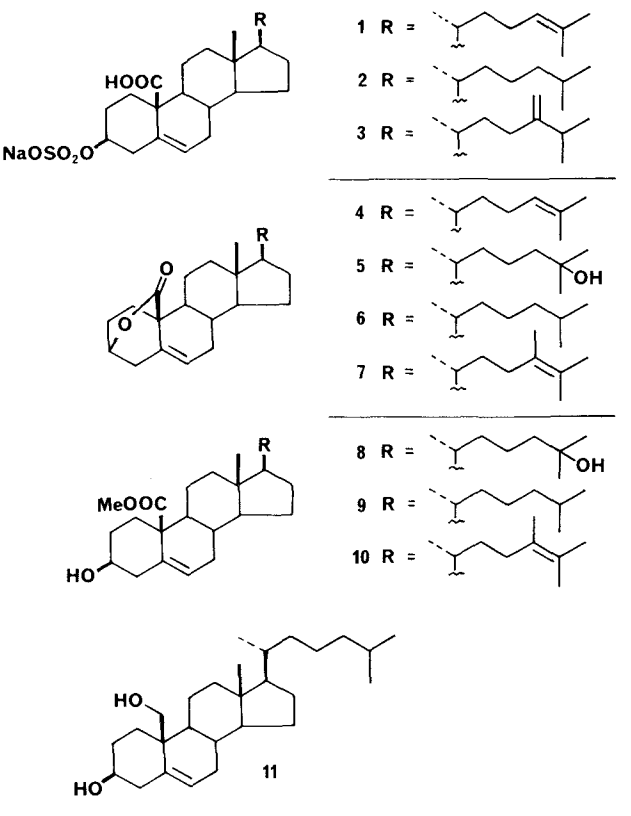
Summary. Three sterol sulfates **1**–**3** having a wide variety of biological activities were isolated from the local marine sponge *Toxadocia zumi*. The structures were determined from spectral data.

In temperate waters, sponges often dominate those assemblages of sessile organisms found on the undersides of rock formations where there is insufficient light for vigorous algal growth. Within these sponge-dominated assemblages, some species are always heavily fouled by epibionts such as hydroids, ectoprocts and algae while other species are remarkably free of epiphytic growth[3]. There does not appear to be any obvious physical rationale for the differences in fouling by epibionts. We have therefore investigated the hypothesis that certain sponges produce biologically active compounds that inhibit the growth of fouling organisms or the settling of their larval forms. Although we recognize that antimicrobial activity may not parallel antifouling activity, it is interesting to note that there is a negative correlation between the antimicrobial activity of sponges and the degree of fouling that they suffer[4].

In order to test the hypothesis that lack of fouling organisms might indicate the presence of biologically-active compounds in a sponge, we chose to study those sponges that are seldom, if ever, fouled and that did not belong to genera usually associated with production of secondary metabolites. *Toxadocia zumi* (Adociidae, Poeciloserina)[5] is such a sponge. In this paper we report the isolation and identification of 3 steroidal sulfates **1**–**3** that have antimicrobial, antifeedant and cytotoxic properties.

Toxadocia zumi was collected at Pt. Loma, San Diego. The freeze-dried sponge was extracted sequentially with hexane, chloroform and methanol and each extract was screened for antimicrobial activity. The methanol-soluble material, the major fraction (3.3% dry weight) from the extraction, was highly active against *Staphylococcus aureus* and *Bacillus subtilis*. A fractionation of the methanol soluble material was performed using Sephadex LH-20 with methanol as eluant, followed by chromatography on silica gel using 6:3:1 chloroform:methanol:ammonia as eluant and finally by reversed phase HPLC on C-18 Partisil-using 30% aqueous methanol as eluant, to obtain 3 steroidal sulfates, 3β-hydroxy-cholest-5,24-dien-19-oic acid sulfate sodium salt (**1**, 0.12% dry weight), 3β-hydroxy-cholest-5-en-19-oic acid sulfate sodium salt (**2**, 0.05% dry weight) and 3β-hydroxy-24-methylene-cholest-5-en-19-oic acid sulfate sodium salt (**3**, 0.05% dry weight) as the major antimicrobial metabolites.

The major sulfate **1** had the molecular formula $C_{27}H_{41}O_6SNa \cdot 2H_2O$ and gave a positive barium nitrate/sodium rhodizonate test for sulfate[6]. The EI mass-spectrum did not contain a molecular ion peak; the highest mass peak was at *m/z* 396 (M-$NaHSO_4$, $C_{27}H_{40}O_2$) with fragmentation peaks at 352 ($C_{26}H_{40}$), 285 ($C_{19}H_{23}O_2$) and 241



($C_{18}H_{23}$) indicating facile loss of CO_2 and a C_8H_{15} fragment. The presence of the *m/z* 285 and 241 peaks in the mass spectra of all 3 sulfates was the first indication that the molecules were sterol sulfates that differed only in the side chain composition. The IR-spectrum contained a strong sulfate band at 1250 and bands at 1695 and 3000–3500 cm^{-1} assigned to the carboxylic acid group. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.63 (s, 3H, C-18), 0.93 (d, 3 H, $J=6$ Hz, C-21), 1.60 (s, 3H, C-26) and 1.67 (s, 3 H, C-27), instead of the 5 usually found in C_{27} sterols such as cholesterol and desmosterol. We therefore proposed that the 'missing' methyl group, presumably that at C-19, had been oxidized to a carboxylic acid group. The ^1H NMR-spectrum also contained olefinic signals at δ 5.68 (m, 1 H, C-6) and at 5.08 (br t, 1 H, $J=6$ Hz, C-24) and a signal at 4.24 (m, 1 H, $w_{1/2} > 30$ Hz), partially obscured by a solvent peak, that was assigned to an axial proton at C-3, the most probable point of attachment of the O-sulfate group. The ^{13}C NMR-spectrum confirmed the presence of the carboxylic acid group [δ 174.6 (s)], 2 trisubstituted olefinic moieties [135.2 (s), 130.2 (s), 124.7 (d), 123.4 (d)] and a carbon bearing the sulfate group [74.8 (d)]. Other signals were assigned by analogy with suitable models as shown in the table. On the basis of the spectral data, the structure of sulfate **1** was assigned as the sodium 3β-sulfate of cholest-5,24-dien-19-oic acid.

Hydrolysis of sulfate **1** in refluxing methanolic hydrochloric acid gave sulfate ion and a mixture of products that was treated with excess diazomethane. Two lactones **4** and **5** and a methyl ester **6** were isolated from the complex product mixture. The lactone **4**, $[\alpha]_D^{23} = 89.1°$ (c=0.96, $CHCl_3$) had the molecular formula $C_{27}H_{40}O_2$. The IR-spectrum contained a δ-lactone band at 1740 cm^{-1}. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.84 (s, 3 H, C-18), 0.94 (d, 3 H, $J=6$ Hz, C-21), 1.60 (s, 3 H, C-26), and 1.68 (s, 3 H, C-27), indicating that no change had occurred in the side chain. The olefinic signals at δ 5.63 (m, 1 H, C-6) and 5.08 (br t, 1 H, $J=6$ Hz) were effectively unchanged but the C-3 proton signal at 4.70 (m, 1 H, $w_{1/2}=8$ Hz) was a broad singlet with small coupling constants to vicinal protons, typical of a bridgehead proton in a bicyclo[2,2,2] ring system. A detailed analysis of the ^1H NMR-spectrum of the related lactone **7** allowed the assignment of all signals for protons on rings A and B (vide infra).

A 2nd lactone **5** $[\alpha]_D$ −97° (c=1.04, $CHCl_3$) had the molecular formula $C_{27}H_{42}O_3$. The IR-spectrum contained both hydroxyl and lactone bands at 3450 and 1730 cm^{-1} respectively. The ^1H NMR-spectrum of lactone **5** was very similar to that of lactone **4** except that the 2 methyl signals at δ 1.68 and 1.60 in **4** were replaced by a signal at 1.21 (s, 6 H) due to 2 methyl groups on a carbon bearing hydroxyl. The lactone **3** must therefore contain a 25-hydroxy group resulting from addition of water across the $^{24}\Delta$ olefinic bond during the acid-catalyzed hydrolysis of the sulfate **1**. The ester **6**, $[\alpha]$ −67° (c=0.21, $CHCl_3$), was a minor product having the molecular formula $C_{28}H_{46}O_4$. The IR-spectrum contained hydroxyl and ester bands at 3320 cm^{-1} and 1725 cm^{-1} respectively. The ^1H NMR-spectrum contained methyl signals at δ 0.61 (s, 3 H), 0.89 (d, 3 H, $J=6$ Hz) and 1.13 (s, 6 H), indicating the presence of the 25-hydroxy side chain, a methoxy signal at 3.71 (s, 3 H) and signals at 3.54 (m, 1 H, $w_{1/2}=30$ Hz, C-3) and 5.66 (br d, 1 H, $J=6$ Hz, C-6). These spectral data were assigned to a dihydroxy ester **6** resulting from hydrolysis of the hydroxy-lactone **5** to a dihydroxy acid and subsequent esterification.

The sulfate **2** had the molecular formula $C_{27}H_{43}O_6SNa \cdot 2H_2O$. The EI mass-spectrum contained a peak at *m/z* 398 with fragmentation peaks at 354, 285 and 241. The IR-spectrum contained bands at 3450. 3150 (broad), 1690 and 1230 cm^{-1} for the sulfate and carboxylic acid groups. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.65 (s, 3 H), 0.86 (d, 6 H, $J=7$ Hz) and 0.97 (d, 3 H, $J=6$ Hz), a signal at 4.28 (m, 1 H) due to the 3α-proton and an olefinic proton signal at 5.68 (m, 1 H, C-6). These data suggested that the only difference between sulfate **1** and sulfate **2** was the absence of the $^{24}\Delta$ olefinic bond.

Hydrolysis of the sulfate **2** followed by methylation of the product mixture with ethereal diazomethane solution gave the lactone **7** and the hydroxy-ester **8**. The lactone **7**, $[\alpha]_D$ −93° (c=0.69, $CHCl_3$) had the molecular formula $C_{27}H$. The IR-spectrum (KBr) contained 2 carbonyl bands at 1735 and 1745 cm^{-1} but this anomaly was attributed to the crystal structure. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.84 (s, 3 H), 0.85 (d, 3 H, $J=7$ Hz), 0.86 (d, 3 H, $J=7$ Hz) and 0.92 (d, 3 H, $J=6$ Hz). As a result of careful spin-decoupling and decoupling-difference experiments, the signals for protons around rings A and B were assigned as follows: δ 1.10 (m, 1 H, $J=14$, 14, 5.5, 2.5 Hz, C-8β), 1.57 (m, 1 H, $J=14$, 12, 7 Hz, C-1α), 1.81 (m, 1 H, $J=14$, 12, 3, 3 Hz, C-2α), 2.04 (m, 1 H, $J=14$, 12, 7, 3, 2 Hz, C-2β), 2.07 (m, 1 H, $J=18$, 5.5, 4 Hz, C-7α), 2.21 (m, 1 H, $J=14$, 12, 3 Hz, C-1β), 2.39 (m, 1 H, $J=17$, 3, 2 Hz, C-4β), 2.42 (m, 1 H, $J=18$, 2, 2 Hz, C-7β), 2.66 (br d, 1 H, $J=17$, 1, 1 Hz, C-4α), 4.70 (m, 1 H, $w_{1/2}=9$ Hz, C-3) 5.63 (m, 1 H, $J=4$, 2, 1 Hz, C-6). These data provide convincing evidence for the 3β-hydroxy-5-en-19-oic acid lactone structure proposed.

Reduction of the lactone **7** with lithium aluminum hydride in ether gave the diol **11**, identical in all respects, including optical rotation, with an authentic sample prepared by the method of Kalvoda et al.[7].

The hydroxy-ester **8**, $[\alpha]_D$ −61° (c=0.23, $CHCl_3$) had the molecular formula $C_{28}H_{46}O_3$. The IR-spectrum contained a hydroxyl band at 3300 cm^{-1} and an ester band at 1725 cm^{-1}. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.61 (s, 3 H), 0.85 (d, 3 H, $J=7$ Hz), 0.86 (d, 3 H, $J=7$ Hz) and 0.88 (d, 3 H, $J=7$ Hz), a methoxyl signal at 3.71 (s, 3 H), a signal at 3.54 (m, 1 H, $J=12$, 12, 4, 4 Hz) due to a 3α

^{13}C-NMR-data for sterol sulfates **1**–**3**

Carbon No.	**1**	**2****	**3**
1	33.3	33.3	33.2
2	27.7	27.7	27.7
3	74.8	74.7	74.5
4	*	*	*
5	135.2	135.3	135.2
6	123.4	123.3	123.3
7	30.3[a]	30.6	30.3[a]
8	31.5	31.5	31.5
9	47.9	*	47.8
10	49.9	*	49.8
11	22.8	22.7[c]	22.7
12	30.5[a]	30.6	30.5[a]
13	42.0	*	42.0
14	55.7[b]	55.7	55.7[b]
15	23.7	23.7	23.7
16	41.3	*	41.9
17	55.4[b]	55.7	55.2[b]
18	11.6	11.6	11.6
19	174.6	174.5	174.6
20	35.0	34.9	35.7
21	18.4	18.5	18.4
22	35.7	*	*
23	24.1	23.1[c]	34.1
24	124.8	*	155.6
25	130.2	27.8	32.9
26	25.4	22.4[c]	21.5[d]
27	17.4	22.5[c]	21.7[d]
28			106.3

*Signals obscured by solvent (d_6-DMSO). **Multiplicities are not known for compound 2. [a–d]Signals may be exchanged.

proton and a vinyl proton signal at 5.65 (br d, 1 H, $J=6$ Hz).

The sulfate **3** had the molecular formula $C_{28}H_{43}O_6SNa$. The EI mass-spectrum contained a peak at m/z 410 with fragmentation peaks at m/z 366. 285 and 241, indicating the presence of a C_9H_{17} side chain. The ^1H NMR-spectrum contained 4 methyl signals at δ 0.66 (s, 3 H), 0.94 (d, 3 H, $J=7$ Hz) and 1.02 (d, 6 H, $J=7$ Hz). The methyl signal at δ 1.02 was coupled to a vinyl proton signal at 2.23 (septet, 1 H, $J=7$ Hz, C-25). The methylene group attached at C-24 gave rise to signals at 4.65 (br s, 1 H) and 4.70 (br s, 1 H), indicating the presence of the 24-methylene cholesterol carbon skeleton. The ^{13}C NMR-spectrum contained signals at δ 21.5 (q), 21.7 (q), 32.9 (s), 106.3 (t) and 155.6 (s) for carbons 24–28 in the 24-methylene sterol side chain. The remaining spectral data were all consistent with the proposed structure for sulfate **3**.

During the hydrolysis of the sulfate **3**, the double bond in the side chain migrated to give lactone **9** and ester **10**, both of which have a tetrasubstituted $^{24}\Delta$-olefinic bond. The lactone **9**, $[\alpha]_D$ $-96°$ (c=0.45, $CHCl_3$), had the molecular formula $C_{28}H_{42}O_2$. The ^1H NMR-spectrum contained 5 methyl signals at δ 0.85 (s, 3 H), 0.96 (d, 3 H, $J=7$ Hz), 1.57 (s, 6 H) and 1.62 (s, 3 H) together with signals at 4.70 (m, 1 H, C-3) and 2.62 (m, 1 H, C-6). The methyl ester **10** could not be obtained completely pure. However, the EI mass-spectrum contained a peak at m/z 442 corresponding to the molecular formula $C_{29}H_{46}O_3$. The ^1H NMR-spectrum contained 5 methyl signals at δ 0.61 (s, 3 H), 0.98 (d, 3 H, $J=7$ Hz), 1.60 (s, 3 H) and 1.62 (s, 6 H), a methoxy signal at 3.71 (s, 1 H) and signals at 3.54 (m, 1 H, $J=11$, 11, 4, 4 Hz) and 5.65 (br d, 1 H, $J=4$ Hz).

The hydrolysis of each of the sulfates **1–3** gave a mixture of products from which we isolated and characterized the major products. Among the minor products from each hydrolysis were the corresponding *i*-sterols, as indicated by the presence of cyclopropyl proton signals in the ^1H NMR-spectrum, but none of these were fully characterized.

The biological activity of the sterol sulfates presents a rather complicated picture. Each of the pure sterol sulfates **1–3** and a mixture of the sterol sulfates in the proportions found in the sponge inhibited the growth of *B. subtilis* at 100 µg/disc and *S. aureus* at 50 µg/disc but did not inhibit growth of 2 representative marine bacteria. Neither the methyl esters **8–10**, the corresponding hydroxy-acids nor sodium cholesteryl sulfate showed antimicrobial activity at 100 µg/disc, indicating that both the 19-carboxylic acid and 3β-sulfate groups were required for antimicrobial activity. The sterol sulfate mixture inhibited cell division in the fertilized sea urchin egg assay[8] at 5 µg/ml but did not cause cell lysis. The sterol sulfates were toxic to brine shrimp, *Artemia* sp. and goldfish, *Carassius auratus* at 100 µg/ml but not at 10 µg/ml. Even at 150 µg/mg of food pellet, the steroidal sulfates did not act as feeding inhibitors toward goldfish, *C. auratus*, or sergeant major fish, *Abudefduf saxatilis*[9].

In order to determine whether the compounds were being released into seawater, some bioassays were performed using the seawater in which *Toxadocia zumi* had been maintained for 1 h. The water showed antimicrobial properties and caused considerable distress to a keyhole limpet *Megathura crenulata* placed in the water but did not affect the starfish *Pisaster giganteus* or the nudibranch *Anisodoris nobilis*. The seawater also prevented successful development of the polychaete *Salmacina tribranchiata*, as defined by the failure to produce a calcareous tube, but did not affect the larvae of the abalone *Haliotis rufescens* or the ectoproct *Phidolopora pacifica*. The sterol sulfate mixture prevented development of *S. tribranchiata* at 10 µg/ml.

Although it is not feasible to assay compounds against all possible fouling organisms, these results suggest that the sterol sulfates **1–3** might be in part responsible for the lack of fouling organisms on *Toxadocia zumi*. This particular chemical mechanism for protection against fouling is very attractive from a bioenergetic viewpoint, for it involves a 'low cost' synthesis of the biologically active compounds from sterols that are readily available from dietary sources. The only other report of sterol sulfates from sponges is that of the antimicrobial metabolite, halistanol sulfate from *Halichondria* cf. *moorei*[9].

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Effects of bunaphtine on ^{45}Ca movements in rat aortic smooth muscle

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Summary. The effect of bunaphtine (BNA, 5×10^{-5} M) on La^{3+}-resistant ^{45}Ca content and ^{45}Ca efflux was studied on rat aortic smooth muscle. BNA decreased both control and norepinephrine-stimulated La^{3+}-resistant ^{45}Ca content and increased the ^{45}Ca efflux. These effects could explain the inhibition of the contractile responses induced by BNA.

Bunaphtine (BNA) is a new antiarrhythmic drug which has been shown to be a membrane stabilizer[1,2]. In isolated rat thoracic aorta it has been reported that BNA inhibited the contractile responses induced by different agonists and shifted the concentration-response curve of Ca^{++} downwards and to the right[3]. Therefore, these authors suggested