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I. Bruno, L. Minale, R. Riccio, L. Cariello, Tatsuo Higa, and Junichi Tanaka

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# STARFISH SAPONINS, PART 50.[1] STEROIDAL GLYCOSIDES FROM THE OKINAWAN STARFISH *NARDOA TUBERCULATA*

I. BRUNO, L. MINALE,* R. RICCIO,

*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", via D. Montesano 49, 80131 Napoli*

L. CARIELLO,

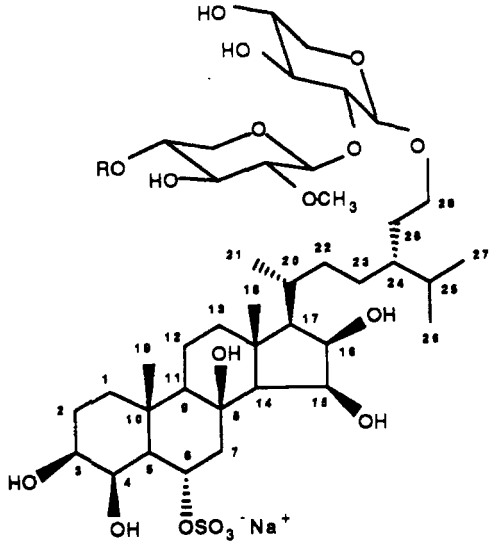
*Biochemistry Laboratory, Stazione Zoologica, Villa Communale, Napoli, Italy*

TATSUO HIGA, and JUNICHI TANAKA

*Department of Marine Sciences, University of the Ryukyus Sembaru, I Nishihara, Okinawa 903-01, Japan*

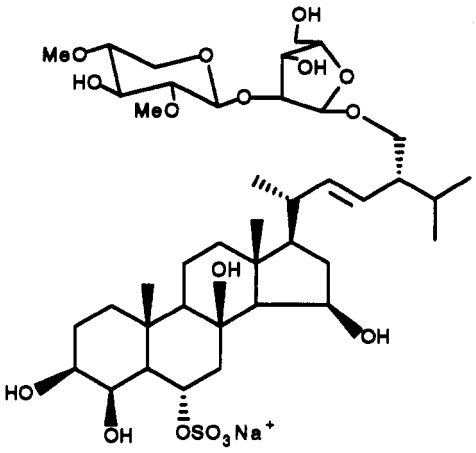
ABSTRACT.—This paper describes a complete analysis of the glycoside and polyhydroxysteroid constituents of the starfish *Nardoa tuberculata*, collected at Zampa, Okinawa, Japan. Besides a group of known non-sulfated steroidal diglycosides, halitylosides A [**5**], B [**6**], D [**7**], E [**8**], and F [**9**], and two known polyhydroxysteroids **10** and **11**, *N. tuberculata* also contains four new sulfated steroidal glycosides **1–4** closely related to the previous halitylosides. The antifungal activities and the effects on the development of fertilized sea urchin eggs of some steroid components from this starfish were examined.

Continuing our work on biologically active compounds from echinoderms, we have been working on the steroid constituents of the Pacific starfish *Nardoa tuberculata* Perrier (family Ophidiasteridae, order Valvatida), collected at Zampa, Okinawa, Japan, and have isolated four new sulfated steroidal diglycosides **1–4**. They co-occur with a group of known steroidal diglycosides, halitylosides A [**5**], B [**6**], D [**7**], E [**8**], and F [**9**], first

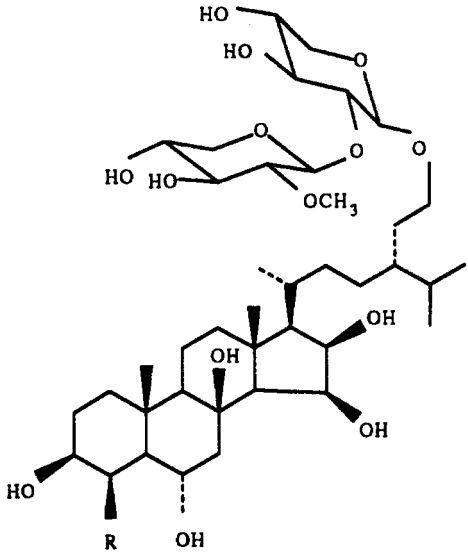



**1** R=H
**2** R=Me
**3** R=H, $\Delta^{22E}$

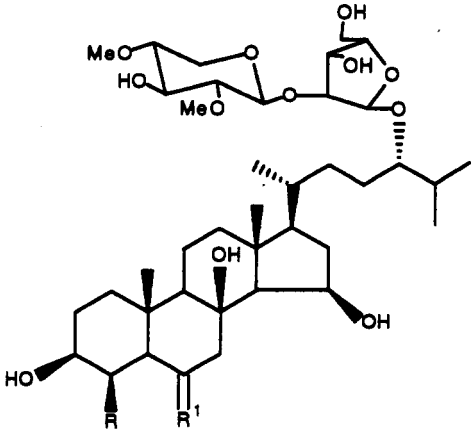
[1]For Part 49, see A. Casapullo, E. Finamore, L. Minale, F. Zollo, J.B. Carrè, C. Debitus, D. Laurent, A. Folgore, and F. Galdiero, *J. Nat. Prod.*, **50**, 105 (1993).



4



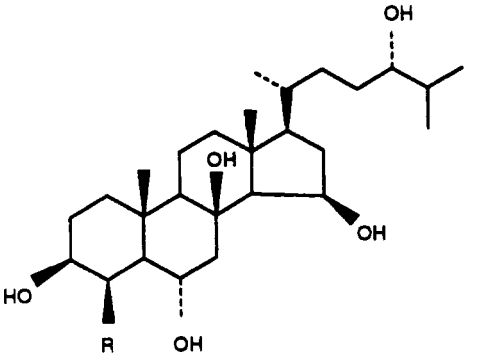
5 R=OH
6 R=H



7 R=OH, $R^1$= 

8 R=H, $R^1$= 

9 R=H, $R^1$= 




**10** R=H
**11** R=OH

isolated from *Halityle regularis* (1) and then from *Culcita novaeguineae* (2), *Sphaerodiscus placenta* (3), and two more *Nardoa* species, *Nardoa novaecaledoniae* and *Nardoa gomophia* (4) and with two known polyhydroxysteroids **10** and **11** (5).

The sulfated glycosides **1–3** are closely related to halityloside A [**5**]: **1** is its 6-*O*-sulfated derivative, whereas **2** and **3** are the 4″-*O*-methyl and the $\Delta^{22E}$ derivatives, respectively, of **1**. The remaining sulfated glycoside **4** is the $\Delta^{22E}$ derivative of culcitoside $C_7$, previously isolated from *Cu. novaeguineae* (2). Separation and isolation of the individual compounds from the aqueous extracts of the animals were carried out as previously described (2). The antifungal activity of halitylosides E [**8**] and F [**9**] and of the steroids **10** and **11** against the pathogenic fungus *Cladosporium cucumerinum* as well as the effects of halitylosides A [**5**], B [**6**], D [**7**], E [**8**], and F [**9**] and of the steroid **11** on the development of fertilized sea urchin eggs are also reported.

## RESULTS AND DISCUSSION

The results of our analysis are shown in Table 1. Identification of known compounds was achieved by direct comparison (fabms, $^1$H nmr, hplc) with authentic samples. $^1$H- and $^{13}$C-nmr assignments are listed in Tables 2 and 3, respectively.

TABLE 1. Compounds Isolated from *Nardoa tuberculata*.

| Compound | Amount[a] (mg) | $[\alpha]_D$ | Antifungal activity (μg)[b] | Sea urchin asay ($LD_{50}$ M) |
|---|---|---|---|---|
| Halityloside A 6-*O*-sulfate [**1**] | 6 | +3.7° | —[c] | — |
| 4″-*O*-Methyl halityloside A 6-*O*-sulfate [**2**] | 2 | −5° | — | — |
| 4″-*O*-Methyl$\Delta^{22E}$ halityloside A 6-*O*- sulfate [**3**] | 2 | −8.5° | — | — |
| $\Delta^{22E}$ Culcitoside $C_7$ [**4**] | 3 | −15.4° | — | — |
| Halityloside A [**5**] | 8 | | 40 | $10^{-5}$ |
| Halityloside B [**6**] | 6 | | — | $10^{-5}$ |
| Halityloside D [**7**] | 8 | | — | $10^{-6}$ |
| Halityloside E [**8**] | 18 | | 10 | $10^{-7}$ |
| Halityloside F [**9**] | 12 | | 5 | $10^{-6}$ |
| Steroid **10** | 9 | | 3 | [d] |
| Steroid **11** | 10 | | — | $10^{-6}$ |

[a]From 1.85 kg fresh wt.
[b]Minimum inhibitory amount against the fungus *Cladosporium cucumerinum*.
[c]—, not tested.
[d]No activity below $10^{-5}$ M.

TABLE 2. $^1$H-nmr Data (250 MHz, $CD_3OD$) for Steroidal Protons in **1–4** in δ (Hz).

| Proton | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| H-3 | 3.5 m | 3.5 m | 3.5 m | 3.5 m |
| H-4 | 4.34 brs | 4.34 brs | 4.34 brs | 4.34 brs |
| H-6 | 4.90 m | 4.90 m | 4.90 m | 4.90 m |
| H-7 | 2.76 dd (4.5,12) | 2.76 dd (4.5,12) | 2.76 dd (4.5,12) | 2.73 dd (4.5,12) |
| H-15 | 4.39 m | 4.39 m | 4.43 dd (7,12) | 4.4 m |
| H-16 | 4.24 t (7) | 4.24 t (7) | 4.17 t (7) | 2.27 m |
| H-18 | 1.27 s | 1.27 s | 1.30 s | 1.31 s |
| H-19 | 1.27 s | 1.27 s | 1.27 s | 1.27 s |
| H-21 | 0.98 d (6.5) | 0.98 d (6.5) | 1.10 d (6.5) | 1.05 d (6) |
| H-26 | 0.88 d (7.5) | 0.88 d (7.5) | 0.88 d (7.5) | 0.88 d (6.5) |
| H-27 | 0.91 d (7.5) | 0.91 d (7.5) | 0.93 d (7) | 0.94 d (6.5) |
| H-22 | — | — | 5.58 dd (15,7.5) | 5.27 m |
| H-23 | — | — | 5.23 dd (15,9) | 5.27 m |
| H-28 | — | — | — | 3.43 t (10) |
| H-29 | [b] | [b] | [b] | [a] |

[a]Proton overlapping in the region δ 4.0–4.1.
[b]Protons overlapping in the region δ 3.4–3.6.

For compound **1** the negative fabms gave a molecular anion peak at *m/z* 869 $[M+SO_3]^-$. The $^1$H-nmr spectrum was very similar to that of halityloside A [**5**], except for signals of H-6, H-7, and $H_3$-19, which were shifted downfield to $\delta_H$ 4.90 (4.22 in **5**), 2.76 (2.50 in **5**), and 1.27 (1.19 in **5**), respectively, and pointed to a sulfate group at C-6. This was supported by the $^{13}$C-nmr signals of C-6, shifted downfield to 74.4 ppm (64.7 in **5**), and of C-5 and C-7 shifted upfield to 56.2 (57.2 in **5**) and 47.8 (ca. 49.0 in **5**) ppm, respectively. Upon solvolysis in dioxane/pyridine, **1** afforded halityloside A [**5**].

With compound **2** the negative fabms gave the molecular anion peak at *m/z* 883 $[M+SO_3]^-$, fourteen mass units higher than **1**, and fragment peaks at *m/z* 723 and *m/z* 591, corresponding to the consecutive loss of a dimethoxylated pentose unit (160 mass units) and of a pentose (132 mass units) residue. Examination of $^1$H-nmr spectral data (Table 2) indicated the presence of the same steroidal aglycone found in **1**. Also present in the spectrum were signals assigned to the 2,4-di-*O*-methyl-β-xylopyranosyl moiety already encountered in halitylosides D [**7**], E [**8**], and F [**9**] (1). Particularly informative for the location of the methyl groups at C-2″ and C-4″ of the terminal dimethoxylated xylose unit were the signals for H-2″ and H-4″ shifted highfield to $\delta_H$ 2.97 (dd $J$=7.5, 8.7 Hz) and 3.20 m, and the signal for H-5″ eq shifted downfield to δ 4.02 (dd, $J$=8.5, 3.5 Hz) (Table 4), this latter indicative for the substitution at C-4″ in a xylopyranose structure (the usual chemical shift of H-5 eq in a xylopyranoside ring is ca. δ 3.85, dd). Furthermore, the H-1 anomeric signal of the xylopyranosyl unit at $\delta_H$ 4.40, virtually unshifted relative to **1**, is suggestive for the attachment of the terminal 2,4-di-*O*-methylxylopyranosyl unit at 2′-OH of the xylopyranosyl moiety.

For compound **3** the negative fabms gave the molecular anion peak shifted by two mass units relative to compound **1**, *m/z* 867 $[M+SO_3]^-$. Examination of its $^1$H-nmr spectral data (Table 2) indicated the presence of the same steroidal tetracyclic nucleus found in **1** and of the 2-*O*-methylxylopyranosyl (1⟼2)-β-xylopyranosyl unit. Additional $^1$H-nmr signals assigned to the side chain were: three methyl doublets at $\delta_H$ 0.88 ($J$=7 Hz, $H_3$-26) 0.93 ($J$=7 Hz, $H_3$-27), and 1.10 ($J$=6.5 Hz, $H_3$-21), two olefinic doublet doublets at $\delta_H$ 5.23 (1H, $J$=15, 9 Hz, H-23) and 5.58 (1H, $J$=15, 7.5 Hz, H-22) and a 2H broad multiplet at δ 3.65 ($H_2$-29). Solvolysis in dioxane/pyridine, followed

TABLE 3. $^{13}$C-nmr Shifts ($CD_3OD$) of Steroid Carbons in **1**, **3**, and **4**.[a]

| Carbon | Compound | | |
|---|---|---|---|
| | **3**[b] | **1**[b] | **4**[c] |
| C-1 | 39.4 | 39.5 | 39.2 |
| C-2 | 26.5 | 26.6 | 26.2 |
| C-3 | 73.3 | 73.0 | 72.6 |
| C-4 | 68.9 | 69.0 | 68.6 |
| C-5 | 56.1 | 56.2 | 55.8 |
| C-6 | 74.4 | 74.4 | 74.2 |
| C-7 | 47.8 | 47.8 | 47.9 |
| C-8 | 77.2 | 77.2 | 77.2 |
| C-9 | 58.2 | 58.3 | 57.9 |
| C-10 | 38.8 | 38.8 | 38.8 |
| C-11 | 18.8 | 18.9 | 18.9 |
| C-12 | 43.3 | 43.5 | 43.1 |
| C-13 | 44.7 | 44.7 | 44.7 |
| C-14 | 61.2 | 61.1 | 62.0 |
| C-15 | 71.2 | 71.3 | 70.7 |
| C-16 | 72.9 | 72.9 | 42.9 |
| C-17 | 63.8 | 63.1 | 57.3 |
| C-18 | 18.0 | 17.9 | 16.4 |
| C-19 | 16.9 | 16.9 | 16.6 |
| C-20 | 34.8 | 31.4 | 40.9 |
| C-21 | 20.7 | 18.7 | 20.7 |
| C-22 | 139.8 | 34.8 | 139.9 |
| C-23 | 130.3 | 28.7 | 128.8 |
| C-24 | 49.8 | 42.3 | 53.3 |
| C-25 | 33.5 | 30.7 | 29.3 |
| C-26 | 19.6 | 19.2 | 18.8 |
| C-27 | 21.3 | 20.0 | 21.4 |
| C-28 | 33.9 | 31.9 | 70.6 |
| C-29 | 69.6 | 69.7 | — |

[a]Assignments aided by comparison with known compounds.

[b]Sugar signals: xylosyl (103.6, 81.5, 76.8, 71.2, and 66.5 ppm), 2-*O*-methylxylosyl (104.3, 84.7, 77.7, 71.2, 66.5, 60.7 ppm).

[c]Sugar signals: arabinosyl (108.4, 91.7, 77.3, 84.4, and 62.9), 2,4-di-*O*-methylxylosyl (104.7, 84.9, 77.7, 81.9, 64.5, 59.0, 61.0).

by hplc purification and hydrogenation (Pd/C 10%) afforded halityloside A [**5**]. On this basis we also propose the 24*R* configuration for compound **3**.

With compound **4** the negative fabms gave the molecular anion peak shifted by two mass units relative to culcitoside $C_7$ (2), *m/z* 851 $[M+SO3]^-$. Examination of $^1$H- and $^{13}$C-nmr data (Tables 2 and 3) immediately indicated that **4** is the 22(23)-didehydroculcitoside $C_7$. The double bond was located at the usual 22 position on the basis of the $^1$H-nmr olefinic signals observed as overlapping multiplets at $\delta_H$ 5.27 and the doublet signal for the C-21 methyl protons shifted downfield to δ 1.05 [δ 0.97 in culcitoside $C_7$ (2)], typical for Me-21 in a $\Delta^{22}$-steroidal structure. The *E* configuration was assigned on the basis of the $^{13}$C-nmr data and comparison with synthetic models (24*S*)- and (24*R*)-cholesta-5,22(*E*)-dien-3β-ols (6). Particularly informative were the

TABLE 4. $^1$H-nmr (250 MHz, $CD_3OD$) Signals of Disaccharide Chains in Compounds **1–4**.

| Proton | Compound | | |
|---|---|---|---|
| | **1** and **3** | **2** | **4** |
| H-1′ | 4.41 d (7.0) | 4.42 d (7.0) | 4.99 d (1.5) |
| H-2′ | 3.21 dd (7.0, 9) | [a] | 4.07 dd (1.5, 4) |
| H-3′ | [a] | [a] | 4.03 m |
| H-4′ | [b] | [b] | 3.94 m |
| H-5′ | 3.20 dd (11.9, 9.5) | 3.26 dd (12.5, 7.5) | 3.81 dd (12.5, 3) |
| | 3.92 dd (11.9, 4.8) | 3.90 dd (12.5, 5) | 3.66 dd (12.5, 4.8) |
| H-1″ | 4.75 d (7.0) | 4.75 d (7.5) | 4.45 d (7.5) |
| H-2″ | 2.97 dd (8.7, 7.0) | 2.97 dd (7.5, 8.7) | 2.90 dd (7.5, 9) |
| H-3″ | [a] | [a] | 3.42 t (9.0) |
| H-4″ | [b] | 3.20 m | 3.22 m |
| H-5″ | [a] | 3.16 t (8.5) | 3.14 t (10.6) |
| | 3.86 dd (8.0, 4.8) | 4.02 dd (8.5, 3.5) | 4.05 dd (10.6, 4) |
| OMe | 3.65 s | 3.62 s | 3.60 s |
| OMe | | 3.50 s | 3.50 s |

[a]Protons overlapping in the region δ 3.2–3.6.
[b]Signal under solvent signal.

chemical shifts of C-20 and C-24 ($\delta_C$ 40.9 and 53.3 ppm in **4**; 41.7, 53.0 and 41.5, 52.9 ppm in the 24*S* and 24*R* model compounds, respectively), which in a structure with a 22*Z* double bond would be expected to be significantly shifted highfield, because of the γ-syn-interaction. The configuration 24*R* of C-24 in compound **4** is suggested only on the basis of comparison with coscinasteroside C (6) and pisasteroside A (7), $\Delta^{22E}$ 28-*O*-glycosides previously isolated from starfishes.

The D-series for the xylosyl and the L-series for the arabinosyl residues are assumed by analogy with the many other D-xylosides and L-arabinosides isolated from starfishes.

Halitylosides A [**5**], E [**8**], and F [**9**] and the steroid **10** were tested by using direct bioautography on tlc (8). *Cl. cucumerinum* was used as test organism for the antifungal assay. Compounds **8**, **9**, and **10** were active below 10 μg (minimum inhibition dose), whereas **5** showed a very weak activity (active at a dose of 40 μg).

Halytilosides A [**5**] and F [**9**] and the steroids **10** and **11** were tested for cytotoxic activity by using the sea urchin egg assays (9). All compounds were moderately active in the inhibition of cleavage of the fertilized eggs, except the steroid **10** which did not show any activity below $10^{-5}$ M concentration.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra, Bruker WM-250 ($^1$H at 250 MHz, $^{13}$C at 62.9 MHz), δ (ppm), *J* in Hz, spectra referred to $CHD_2OD$ signal at 3.34 ppm and to central carbon $CD_3OD$ signal at 49.0 ppm. Mass spectra, VG ZAB mass spectrometer equipped with fab source [in glycerol or glycerol-thioglycerol (3:1) matrix; Xe atoms of 2-6 kV]. Optical rotations, Perkin Elmer model 241 polarimeter. Glc, Carlo Erba Fractovap 2900 for capillary column (SE-30, 25 mt, 125°, helium carrier flow 2 ml/min). Reversed-phase hplc, $C_{18}$ μ-Bondapack column (30 cm×8 mm i.d., flow rate 5 ml/min) and $C_{18}$ μ-Bondapack column (30 cm×3.9 mm i.d., flow rate 2 ml/min), Waters model 6000 A pump equipped with a U6K injector and a differential refractometer, model 401. Dccc, DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes, and Buchi apparatus equipped with 300 tubes.

EXTRACTION AND ISOLATION.—The animals were collected at Zampa, Okinawa, in August 1986, and identified by the zoologists of the Department of Marine Sciences, University of the Ryukyus, Okinawa, Japan; a voucher specimen is preserved there.

The animals (1.85 kg) were chopped and soaked in $H_2O$ (twice, 2 liters for 8 h each); the aqueous extract was settled and, after centrifugation, passed through a column of Amberlite XAD-2 (1 kg). The column was washed with distilled $H_2O$ (1 liter) and eluted with MeOH (2 liters) to give, after removal of the solvent, 3.24 g of crude extract. This was chromatographed on a column of Sephadex LH-60 (4×100 cm) using MeOH-$H_2O$ (2:1) as eluent (flow rate ca. 10 ml/h). Fractions (5 ml) were collected and monitored by tlc on silica plates (Merck) with *n*-BuOH–HOAc–$H_2O$ (12:3:5) (detection by ceric sulfate/$H_2SO_4$). Fractions 80–124 (A, 700 mg) contained a mixture of nucleosides, fractions 125–154 (B, 710 mg) contained mainly sulfated glycosides, and the successive fractions 155–200 (C, 260 mg) contained mainly polyhydroxysteroids and nonsulfated steroid glycosides. Fractions B and C were submitted to dccc using $CHCl_3$-MeOH-$H_2O$ (7:13:8). Fractions (5 ml each) were collected and monitored by tlc. The results are summarized in Table 5.

The above fractions were then submitted to hplc on a $C_{18}$ μ-Bondapak column (30 cm×7.8) with MeOH-$H_2O$ (75:25) (flow rate 5 ml/min) to give the pure known compounds. The amount of each steroidal constituent is reported in Table 1. The more polar dccc fractions (1–61) that still contained a mixture of sulfated glycosides were submitted to dccc using the *n*-BuOH–$Me_2CO$–$H_2O$ (3:1:5) solvent system (flow ca. 10 ml/h). Fractions (5 ml each) were collected, combined according to tlc analysis (on Si gel plates with *n*-BuOH/HOAc/$H_2O$) and then submitted to hplc on a $C_{18}$ μ-Bondapak column (30 cm×7.8 mm) with MeOH-$H_2O$ (55:45) (flow rate 5 ml/min) to afford pure compounds **1–4**.

SOLVOLYSIS OF COMPOUND **1** TO GIVE HALITYLOSIDE A [**5**].—A solution of **1** (3 mg) in dioxane-pyridine (1:1) (0.2 ml) was heated at 120° for 2 h in a stoppered reaction vial. After cooling, $H_2O$ (2 ml) was added and the solution was extracted with *n*-BuOH (3×1 ml). The combined extracts were washed with $H_2O$ and evaporated under reduced pressure. The residue was purified by hplc on a $C_{18}$ μ-Bondapak column with MeOH-$H_2O$ (75:25) (flow 2 ml/min) to give 1.9 mg of halityloside A [**5**]: negative fabms *m/z* $[M-H]^-$ 789; $^1H$ nmr δ ($CD_3OD$) 1.27 (3H, s, $H_3$-18), 1.19 (3H, s, $H_3$-19), 0.99 (3H, d, $J$=6.3 Hz, $H_3$-21), 0.91 (3H, d, $J$=6.8 Hz, $H_3$-26), 0.88 (3H, d, $J$=6.9 Hz, $H_3$-27), 4.28 (1H, bs, H-4α), 4.22 (1H, dt, $J$=10.5, 4 Hz, H-6β), 2.49 (1H, dd, $J$=12.5, 4 Hz, Heq-7), 4.42 (1H, dd, $J$=5.6, 6.6 Hz, H-15α), 4.25 (1H, dd, $J$=6.6, 7 Hz, H-16α).

SOLVOLYSIS OF COMPOUND **3** FOLLOWED BY HYDROGENATION TO GIVE HALITYLOSIDE A [**5**].—A solution of **3** (1.5 mg) in dioxane/pyridine was treated as above. The residue was further purified by hplc (conditions given above) to afford 1 mg of the desulfated derivative. Negative fabms *m/z* $[M-H]^-$ 787; 1H nmr δ ($CD_3OD$) 1.30 (3H, s, $H_3$-18), 1.19 (3H, s, $H_3$-19), 0.88 (3H, d, $J$=6.5 Hz, $H_3$-26 or -27), 0.94 (3H, d, $J$=7.2 Hz, $H_3$-27 or -26), 1.1 (3H, d, $J$=7 Hz, $H_3$-21), 4.29 (1H, bs, H-4α), 4.22 (1H, dt, $J$=10.5, 4 Hz, H-6β), 2.50 (1H, dd, $J$=12.5, 4 Hz, Heq-7), 4.43 (1H, m, H-15), 4.17 (1H, t, $J$=6.5 Hz, H-16), 5.58 (1H, dd, $J$=7.3, 14.7 Hz, H-22), 5.23 (1H, dd, $J$=14.7, 8.7 Hz, H-23). This was hydrogenated in EtOH (0.5 ml) at atmospheric pressure over 10% Pt/C for 12 h. Removal of the catalyst by filtration and evaporation of solvent gave pure halityloside A [**5**] as identified by fabms and $^1H$ nmr (see above).

METHANOLYSIS OF COMPOUND: **1** SUGAR ANALYSIS.—A solution of **1** (1.5 mg) in anhydrous 2 M HCl in MeOH (0.5 ml) was heated at 80° in a stoppered reaction vial for 10 h. After being cooled, the reaction

TABLE 5. Dccc Fractionation[a] of the Mixture of Steroid Glycosides and Polyhydroxysteroids from *Nardoa tubercolata*.

| Fraction | Total amount (mg) | Compound |
|---|---|---|
| 1–61 | 200 | mixture of sulfated glycosides |
| 62–71 | 15 | **5** |
| 72–93 | 16 | **6** |
| 94–119 | 20 | **7** |
| 120–134 | 15 | **11** |
| 135–151 | 20 | **11+8** |
| 152–163 | 8 | **8** |
| 164–177 | 6 | **10** |
| 178–197 | 17 | **10** |
| Recovery from stationary phase. | 12 | **9** |

[a]Solvent system $CHCl_3$-MeOH-$H_2O$ (7:13:8), ascending mode, 250 tubes.

mixture was neutralized with $Ag_2CO_3$ and centrifuged. The supernatant was evaporated to dryness under $N_2$. A small portion of the residue was trimethylsilylated with trisil-Z (Pierce Chemical Co.) for 15 min at room temperature. Glc analysis at 140° using a 25 m column (SE-30) ($H_2$ carrier, flow 10 ml/min) gave glc peaks which co-eluted with those of silylated methyl xylosides and methyl-2-*O*-methylxylosides.

SEA URCHIN BIOASSAY.—*Spaerechinus granularis* eggs were collected from a single female for each experiment. Samples containing about 50,000 eggs/ml were treated for 10 min with different amounts of steroidal glycosides dissolved in MeOH-$H_2O$ (20:80). After 10 min, the sample were diluted five times with filtered sea water and fertilized. The controls were treated and fertilized in the same conditions as the samples. For light microscopic analysis, samples were collected and observed in vivo after 10 min and 2 h.

The antifungal tests were done in the laboratory of Professor K. Hostettmann, Université de Lousanne. The samples were spotted on tlc plates and sprayed with a suspension of the fungus *Cl. cucumerinum*. Plumbagine was used as reference compound.

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