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Marine Natural Products. IX.[1] Structural Elucidation of Triterpenoidal Oligoglycosides from the Bahamean Sea Cucumber *Actinopyga agassizi* Selenka

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Triterpenoidal oligoglycosides contained in the Cuvierian tubules of the Bahamean sea cucumber *Actinopyga agassizi* Selenka were shown to consist of a new lanostane-type triterpene tetraglycoside sulfate (24-dehydroechinoside A) (**10**) and holothurin A (**11**) in an approximate ratio of 2: 1. The chemical structure of 24-dehydroechinoside A (**10**) was elucidated on the basis of chemical and physicochemical evidence.

Keywords——sea cucumber; *Actinopyga agassizi*; lanostane-type triterpene; oligoglycoside; 24-dehydroechinoside A; holothurin A; HPLC

In 1969, Chanley and his group proposed a hypothetical structure (**1**)[2] for "holothurin A",[3] which was isolated from the Cuvierian tubules of the Bahamean sea cucumber *Actinopyga agassizi* Selenka. "Holothurin A" had been of considerable interest because of its various biological activities.[4] Afterwards, Elyakov and his group reported the fairly wide distribution of "holothurin A" in Holothuroidea.[5] On the other hand, the triterpenoidal oligoglycosides of the Japanese sea cucumber *Holothuria leucospilota* (Brandt) (Japanese name: nisekuro-namako) were investigated by Yasumoto *et al.*[6] and one of them, holothurin A, was suggested to be identical with "holothurin A," since both compounds showed identical behavior on thin-layer chromatography (TLC).

In the course of our studies on marine natural products,[7] we have recently elucidated the chemical structures of two bioactive oligoglycosides, holothurin B (**6**)[8] and holothurin A (**11**),[1] which we isolated from the above-mentioned sea cucumber *H. leucospilota*. As is apparent from the elucidated structure for holothurin A (**11**), holothurin A from *H. leucospilota* appeared not to be identical with "holothurin A" from *A. agassizi*. In order to shed light on the structural relationship between holothurin A (**11**) and "holothurin A," we undertook the chemical elucidation of the triterpenoidal oligoglycosides contained in the Cuvierian tubules of the Bahamean sea sucumber *A. agassizi*, since a preserved sample of the organ became available to us by courtesy of Professors L.S. Ciereszko and F.J. Schmitz, University of Oklahoma, U.S.A. As described in this paper, we finally reached the conclusion that "holothurin A" from the Cuvierian tubules of *A. agassizi*, although it showed a single spot on TLC (Fig. 1), is a mixture of a new lanostane-type triterpene tetraglycoside (24-dehydroechinoside A) (**10**) and holothurin A (**11**) in an approximate ratio of 2: 1.[9]

The methanolic extractive of the air-dried Cuvierian tubules of *A. agassizi* was partitioned into *n*-butanol–water mixture and column chromatographic purification of the *n*-butanol-soluble portion gave "holothurin A," which showed a single spot on TLC, in *ca.* 25% yield from the air-dried organ.

"Holothurin A" does not show any ultraviolet (UV) absorption maximum, but it was positive in the potassium rhodizonate test[10] indicating the presence of a sulfate group in its molecule. On acidic hydrolysis, "holothurin A" liberated three lanostadiene-γ-lactones (**2**, **3**, and **4**), among which one was found to be identical with 22,25-oxidoholothurinogenin (**2**)[1a,8b] previously obtained by acidic hydrolysis of holothurin B (**6**) and A (**11**) from *H. leucospilota*.

The major diene (3), $C_{30}H_{44}O_4$, mp 263—264°C, shows hydroxyl and γ-lactone absorption bands (3500, 1748 cm^{-1}) in its infrared (IR) spectrum. It shows characteristic UV absorption maxima at 238, 245, and 253 nm (ε=11700, 12500, and 8900) which are ascribable to the lanosta-7,9(11)-diene moiety.[1a,8b] The proton nuclear magnetic resonance (^{1}H NMR) spectrum of **3** shows signals due to 20-CH_3 (δ 1.40, s), two olefinic protons (δ 5.25 and 5.51, both 1H, m), two olefinic methyls (δ 1.61 and 1.70, both s) and one additional olefinic proton (δ 5.11, 1H,

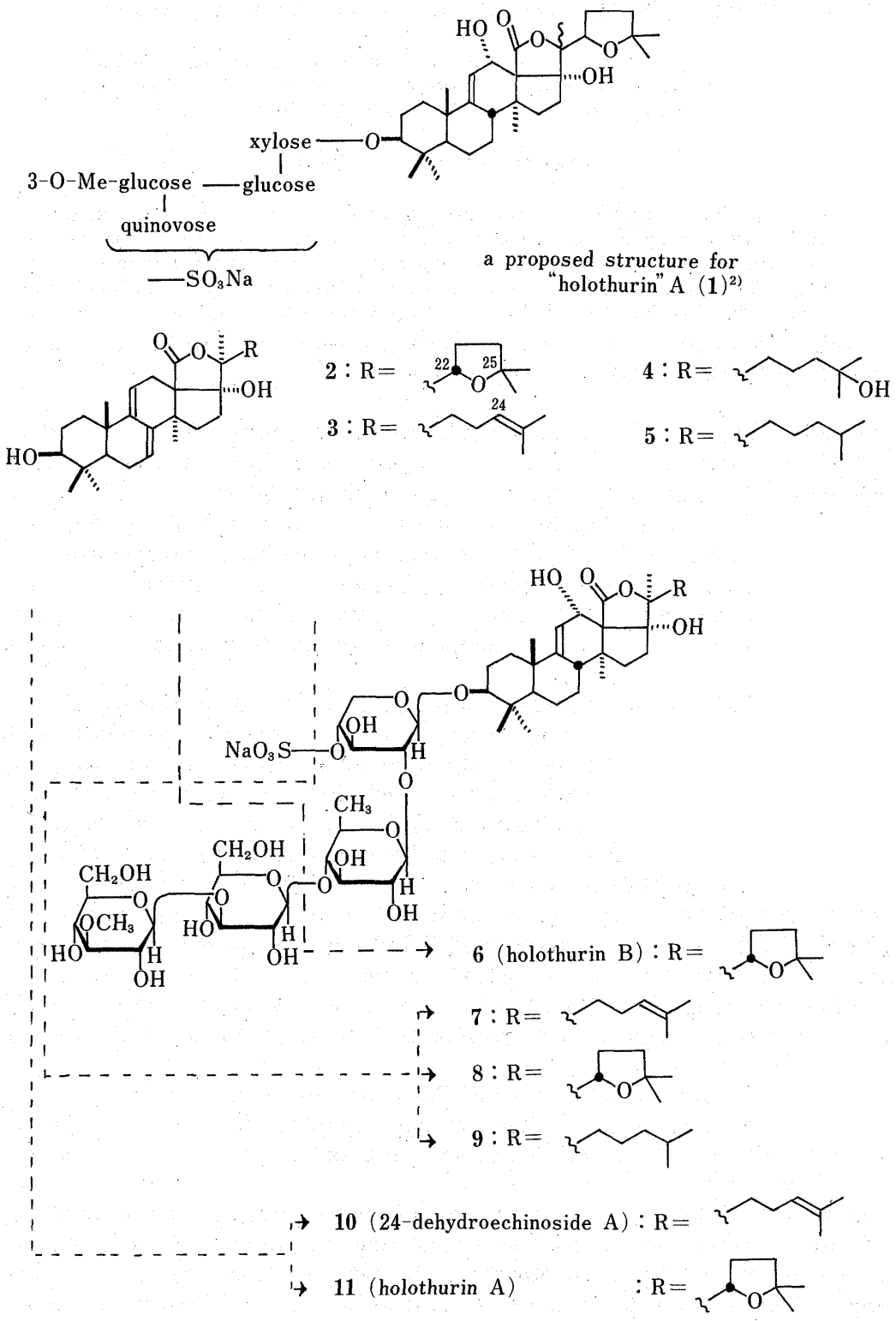

Chart 1

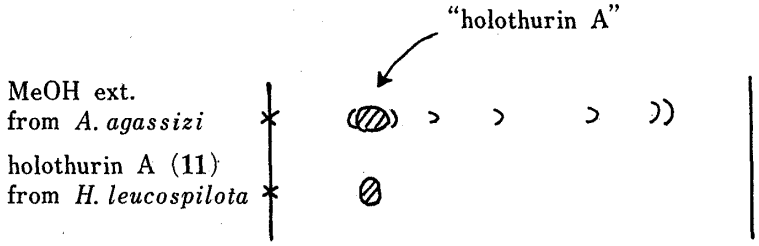

Fig. 1. TLC Diagram of the MeOH ext. and Holothurin A

Adsorbent: Kieselgel 60 F_{254} (Merck).
Solvent: $CHCl_3$–MeOH–H_2O (65: 35: 10, lower phase).
Detection: 1% $Ce(SO_4)_2$–10% H_2SO_4 (with heating).

m) which suggests the presence of the 24-ene moiety. The carbon nuclear magnetic resonance (^{13}C NMR) spectrum of **3** shows signals attributable to carbons in the γ-lactone (δ 175.6, s), the 7,9(11)-diene (δ 120.0, d; 142.2, s; 148.0, s; 112.5, d), and the 24-ene (δ 124.6, d; 131.6, s) moieties. Reduction of **3** with Raney Ni (W-2) yielded a dihydro derivative, mp 258—260°C, which was identical with a sapogenol (holothurinodiene) (**5**) recently characterized as an acidic hydrolysate of two triterpene-oligolycosides, echinosides A and B, from the Okinawan sea cucumber *Actinopyga echinites* (Jaeger).[11] Consequently, the structure of the major diene (**3**) was suggested to be the 24-dehydro derivative of **5**.

Another minor hydrolysate (**4**), mp 298—300°C, has the molecular composition $C_{30}H_{46}O_5$ corresponding to hydrated **3**, as shown by its molecular ion peak (*m/z* 486) in the mass spectrum. The IR and UV spectra of **4** indicate the presence of hydroxyl and γ-lactone groups, and a heteroannular diene moiety as in **3**. The ^1H NMR spectrum of **4** shows signals assignable to 20-CH_3, 7,9(11)-diene protons, and two methyls attached to a carbon bearing a hydroxyl group (δ 1.22, 6H, s). Furthermore, treatment of **3** with aq. 2 N hydrochloric acid in acetone furnished **4**. Thus, the structure of the third hydrolysate has been demonstrated to be the C-24 (25) hydrated derivative of **3**.

The ^{13}C NMR signals due to the sapogenol moiety of "holothurin A" suggest that "holothurin A" contains the same sapogenol side chains as in **2** and **3**, as indicated by signals at δ 80.6 (d, C-22), 81.3 (s, C-25) and δ 124.7 (d, C-24), 131.6 (s, C-25). On the other hand, the ^{13}C NMR signals due to the carbohydrate moiety of "holothurin A" are superimposable on those of holothurin A (**11**) from *H. leucospilota*.[1] Thus, "holothurin A" was presumed to be a mixture of triterpene-oligoglycosides possessing the same oligosaccharide moiety as holothurin A (**11**).

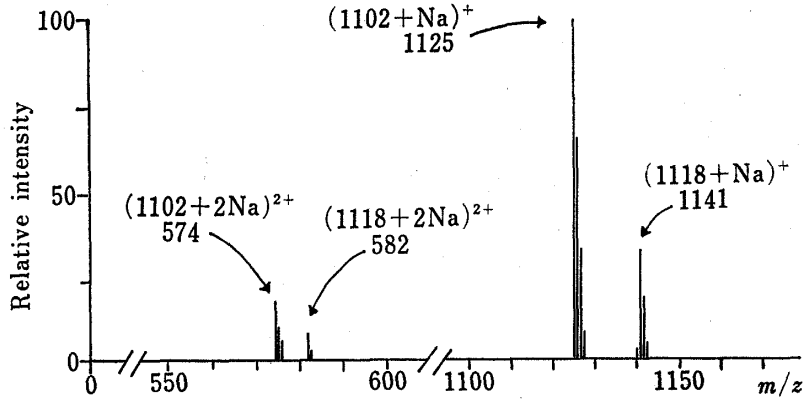

Fig. 2. FD–MS Diagram of Desulfated Holothurin A

Cathode voltage: 5 kv. Accelerating voltage: 2 kv.
Emitter heating current: 17—18 mA. Number of scans: 22.
Mass range: *m/z* 100—1200.

Heating of "holothurin A" with dioxane–pyridine[12] under reflux for one hour furnished almost quantitatively the desulfated derivative, which was separated by high performance liquid chromatography (HPLC) into two components in an approximate ratio of 2: 1. The minor component was found to be identical with the desulfated product of holothurin A (**8**). Acidic hydrolysis of the major component liberated the above-mentioned 24-dehydro sapogenol (**3**), whereas reduction of the major component over Raney Ni (W-2) quantitatively furnished the desulfated derivative of echinoside A (**9**).[11] Thus, the major component has been clarified to be **7**. Furthermore, the field desorption mass spectrum (FD–MS) of the total desulfated derivative of "holothurin A" gave two ion peaks at *m/z* 1125 (**7**+Na) and *m/z* 1141 (**8**+Na) in an approximate peak height ratio of 3: 1 (Fig. 2).[9]

Based on the accumulated evidence mentioned above, "holothurin A" from the Cuvierian tubules of *Actinopyga agassizi* was concluded to be a mixture of holothurin A (**11**) and a new sulfate-containing tetraglycoside named 24-dehydroechinoside A (**10**) in an approximate ratio of 1: 2. Furthermore, TLC examinations claiming wide distribution of "holothurin A" carried out by Elyakov *et al.*[5] seem to require detailed reinvestigation, since the TLC examination of triterpene-oligoglycosidic constituents would not necessarily give satisfactory results.

Experimental[13]

Isolation of "Holothurin A"——The air-dried powder (4 g) of the Cuvierian tubules of the Bahamean sea cxcumber *A. agassizi* was extracted with refluxing MeOH (200 ml × 3) to furnish the extract (3.5 g). The MeOH extract (2 g) was partitioned into *n*-butanol–water and the *n*-butanol-soluble portion (1.5 g) was purified by column chromatography (SiO_2 80 g; $CHCl_3$–MeOH–H_2O=7: 3: 1, lower phase) to furnish "holothurin A" (0.6 g), which gave a single spot on TLC (Fig. 1). "Holothurin A." UV (MeOH): transparent above 210 nm. Potassium rhodizonate test: positive (yellow). ^{13}C NMR (d_5-pyridine, δ): 174.5 (s, C-18), 154.1 (s, C-9), 131.6 (s, C-25), 124.8 (d, C-24), 115.6 (d, C-11), 89.4 (s, C-17), 88.8 (d, C-3), 86.8 (s, C-20), 81.3 (s, C-25), 80.6 (d, C-22), 71.6 (d, C-12), 105.7 (1′), 83.2 (2′), 76.4 (3′), 74.9 (4′), 63.8 (5′), 105.0 (1″, 1‴), 76.1 (2″), 76.4 (3″), 86.6 (4″), 71.8 (5″), 18.0 (6″), 73.9 (2‴), 88.2 (3‴), 70.8 (4‴), 77.5 (5‴), 62.3 (6‴), 104.7 (1⁗), 74.9 (2⁗), 87.8 (3⁗), 69.7 (4⁗), 78.1 (5⁗), 62.0 (6⁗), 60.5 (3⁗-OMe).

Acidic Hydrolysis of "Holothurin A"——A mixture of "holothurin A" (40 mg) in aq. 3 N HCl (4 ml) was heated under reflux for 4 h. The reaction mixture was diluted with water and extracted with EtOAc. The EtOAc extract was washed successively with aq. sat. $NaHCO_3$ and aq. sat. NaCl and dried over $MgSO_4$. Removal of the solvent under reduced pressure furnished a sapogenol mixture (14 mg) which was subjected to column chromatography (SiO_2 5 g; $CHCl_3$–MeOH=20: 1) to give **2** (4 mg), **3** (5 mg), and **4** (2 mg). **2**, colorless needles of mp 302—303°C (from EtOAc). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1745 (γ-lactone). UV λ_{max}^{MeOH} nm (ε): 238 (12900), 245 (13900), 253 (9700). CD ($c=3.0\times10^{-2}$, MeOH): $[\theta]_{280}$ 0, $[\theta]_{246}$ −45000 (neg. max.), $[\theta]_{236}$ 0, $[\theta]_{222}$ +48000 (pos. max.), $[\theta]_{200}$ 0. ^1H NMR ($CDCl_3$, δ): 0.89, 1.00 (both, 3H, s, 4-$(CH_3)_2$), 1.09 (3H, s, 10-CH_3), 1.18 (3H, s, 14-CH_3), 1.21, 1.23 (both 3H, s, 25-$(CH_3)_2$), 1.33 (3H, s, 20-CH_3), 3.24 (1H, t-like, 3α-H), 4.18 (1H, t, J=6, 22β-H), 5.25, 5.50 (both 1H, m, 7-H, 11-H). MS *m/z* (%): 484 (M^+, 23), 451 ($M^+-H_2O-CH_3$, 12), 397 (M^+−ring A, 20), 99 (side chain, 100). It was shown to be identical with an authentic sample[8b] by mixed mp determination and by IR, UV, NMR, and CD comparisons. **3**, colorless needles of mp 263—264°C (from MeOH), $[\alpha]_D^{21}$ −27° (c=0.3, $CHCl_3$), IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1748 (γ-lactone). UV λ_{max}^{MeOH} nm (ε): 238 (11700), 245 (12500), 253 (8900). CD ($c=3.4\times10^{-2}$, MeOH): $[\theta]_{350}$ 0, $[\theta]_{246}$ −41500 (neg. max.), $[\theta]_{237}$ 0, $[\theta]_{222}$ +43500 (pos. max.), $[\theta]_{205}$ +1000! ^1H NMR ($CDCl_3$, δ): 0.90, 1.02 (both 3H, s, 4-$(CH_3)_2$), 1.10 (3H, s, 10-CH_3), 1.15 (3H, s, 14-CH_3), 1.40 (3H, s, 20-CH_3), 1.61, 1.71 (both 3H, s, 25-$(CH_3)_2$), 3.25 (1H, t-like, 3α-H), 5.11 (1H, m, 24-H), 5.25, 5.51 (both 1H, m, 7-H, 11-H). ^{13}C NMR (d_5-pyridine, δ): 175.6 (s, C-18), 148.0 (s, C-9), 142.2 (s, C-8), 131.6 (s, C-25), 124.6 (d, C-24), 120.0 (d, C-7), 112.5 (d, C-11), 86.2, 86.0 (both s, C-17, C-20), 78.3 (d, C-3). MS *m/z* (%): 468 (M^+, 100), 435 ($M^+-H_2O-CH_3$, 27), 381 (M^+−ring A, 58). High resolution MS: Found: 381.243, 468.322. Calcd for $C_{25}H_{33}O_3$=381.243, $C_{30}H_{44}O_4$ (M^+)=468.324. **4**, colorless needles of mp 298—300°C (from MeOH), $[\alpha]_D^{14}$ −50° (c=0.3, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3480 (OH), 1745 (γ-lactone). UV λ_{max}^{MeOH} nm (ε): 238 (11600), 245 (12500), 253 (8800). CD ($c=3.1\times10^{-2}$, MeOH): $[\theta]_{350}$ 0, $[\theta]_{236}$ −43000 (neg. max.), $[\theta]_{236}$ 0, $[\theta]_{224}$ +44000 (pos. max.), $[\theta]_{200}$ 0. ^1H NMR ($CDCl_3$+CD_3OD, δ): 0.90, 1.01 (both 3H, s, 4-$(CH_3)_2$), 1.09 (3H, s, 10-CH_3), 1.17 (3H, s, 14-CH_3), 1.22 (6H, s, 25-$(CH_3)_2$), 1.41 (3H, s, 20-CH_3), 3.23 (1H, t-like, 3α-H), 5.27, 5.51 (both 1H, m, 7-H, 11-H). MS *m/z* (%): 486 (M^+, 50), 453 ($M^+-CH_3-H_2O$, 15), 435 ($M^+-CH_3-2H_2O$, 32), 381 (M^+−ring A–H_2O, 68), 43 (100). High resolution MS: Found 381.242, 486.334. Calcd for $C_{25}H_{33}O_3$=381.242, $C_{30}H_{46}O_5$(M^+)=486.334.

Reduction of 3 giving 5——A suspension of Raney Ni (W-2) (0.5 ml) in EtOH (10 ml) was mixed with **3** (40 mg) and the whole was heated under reflux for 6 h. Removal of the solvent from the filtrate under reduced pressure yielded crude **5** (36 mg), which was purified by recrystallization from MeOH to furnish **5**

as colorless needles of mp 258—260°C (dec.), $[\alpha]_D^{16}$ −17° (c=0.3, $CHCl_3$). Authentic **5** from echinoside B,[11] mp 258—260°C (dec.), $[\alpha]_D^{25}$ −17° ($CHCl_3$). Mixed mp 257—260°C (dec.). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 1745 (γ-lactone). ^1H NMR (d_5-pyridine, δ): 0.82 (6H, d, J=6, 25-$(CH_3)_2$). MS m/z (%): 470 (M^+, 70), 473 (M^+−H_2O−CH_3, 37), 383 (M^+−ring A, 74), 43 (100).

Acidic Treatment of 3——A solution of **3** (2 mg) in aq. 2 N HCl (0.2 ml)–acetone (0.2 ml) was heated under reflux for 15 min. After dilution with water, the reaction mixture was extracted with ether. The ether extract was subjected to TLC analysis (*n*-hexane–EtOAc=2: 3) to identify **4** (Rf=0.20) and **3** (Rf=0.65, recovered) (detected as spots having approximately equal intensities).

Treatment of "Holothurin A" in Refluxing Dioxane–Pyridine——The MeOH extract (1 g) of the Cuvierian tubules (*vide supra*) was dissolved in dioxane (9 ml)–pyridine (36 ml) mixture and the solution was heated under reflux for 1 h. The residue obtained by removal of the solvent under reduced pressure was extracted with MeOH. Purification of the MeOH-soluble portion by column chromatography (SiO_2 100 g, $CHCl_3$–MeOH–H_2O=10: 3: 1, lower phase) furnished the desulfated product (340 mg, a mixture of **7** and **8**). Treatment of "holothurin A" (4 mg) in refluxing dioxane–pyridine mixture as described above furnished a single desulfated product as indicated by a single spot on TLC ($CHCl_3$–MeOH–H_2O=7: 3: 1, lower phase). Purification of the desulfated product (100 mg) by HPLC (Waters Assoc. ALC/GPC 201, Semi Prep μBondapak C_{18}, 3/8″×1′, solvent: CH_3CN–MeOH–H_2O=1: 1: 1, flow rate: 4 ml/min, 2000 psi) furnished **7** (58 mg) and **8** (33 mg). **7**, mp 243—244°C (MeOH), $[\alpha]_D^{18}$ −5.5° (c=1.1, pyridine). *Anal.* Calcd for $C_{54}H_{88}O_{23}\cdot H_2O$: C, 57.84; H, 7.90. Found: C, 57.57; H, 8.03. IR ν_{max}^{KBr} cm^{-1}: 3417 (br), 1727 (br). UV (MeOH): transparent above 210 nm. CD (c=7×10^{-2}, MeOH): $[\theta]_{246}$ 0, $[\theta]_{224}$ −6100 (neg. max.), $[\theta]_{216}$ −1600! **8**, mp 230—232 °C (from 80% EtOH), $[\alpha]_D^{26}$ −1.0° (c=0.3, MeOH). Identical with an authentic sample prepared from holothurin A[1] by mixed mp determination, $[\alpha]_D$, and HPLC (conditions as described above).

Acid Hydrolysis of 7——A mixture of **7** (5 mg) in aq. 2 N HCl (2 ml) was heated under reflux for 2 h. Work-up of the reaction mixture as described above for "holothurin A" yielded a sapogenol mixture, which was shown to comprise **3** (major) and **4** (trace) by TLC analysis (*n*-hexane–EtOAc=2: 3).

Reduction of 7 giving 9——A suspension of Raney Ni (W-2) (1 ml)–EtOH (20 ml) was added with **7** (40 mg) and the whole mixture was heated under reflux for 5 h. After cooling, the mixture was filtered. Removal of the solvent under reduced pressure yielded the product (42 mg) which was purified by column chromatography (SiO_2 20 g, $CHCl_3$–MeOH–H_2O=8: 3: 1, lower phase) to furnish **9** (37 mg), mp 238—240°C, which was identical with an authentic sample (DS-echinoside A)[11] by mixed mp determination (mp 237—239°C), $[\alpha]_D^{10}$ −1.4° (c=0.9, pyridine), and comparison of other physical properties.

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References and Notes

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