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STUDIES ON ASTEROSAPONINS—V[1]

A NOVEL STEROID CONJUGATE, 5α-PREGN-9(11)-ENE-3β,6α-DIOL-20-ONE-3-SULFATE, FROM A STARFISH SAPONIN, ASTEROSAPONIN A

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Abstract—A new crystalline steroid conjugate was obtained by partial acid hydrolysis of asterosaponin A, a steroidal saponin from the starfish, *Asterias amurensis*. The structure of the conjugate was established as 5α-pregn-9(11)-ene-3β,6α-diol-20-one-3-sulfate (2) on the basis of elemental analysis, IR and PMR spectra measurement and chemical reaction. Solvolysis of compound 2 yielded 5α-pregn-9(11)-ene-3β,6α-diol-20-one. Oxidation with chromium trioxide-pyridine complex followed by solvolysis afforded a new steroid, 5α-pregn-9(11)-ene-3β-ol-6,20-dione, whose structure was deduced by the measurement of ORD curve and PMR spectra. Thus, the location of carbohydrate moiety in asterosaponin A has been assigned to 6α-hydroxy group of the steroid conjugate.

Many species of sea-cucumbers in the family of holothuroidea and of starfish in the family of asteroidea, both belonging to the phylum Echinodermata, have been shown to contain poisonous saponins. From the sea-cucumber, *Actinopyga agassizi*, was obtained holothurin A which is composed of one mole each of triterpene aglycone, glucose, 3-O-methylglucose, 6-deoxyglucose and xylose sulfate.[2] Asterosaponin A was first isolated from the starfish, *Asterias amurensis* by Yasumoto and Hashimoto,[3] who reported that it contains a steroidal aglycone to which two moles each of 6-deoxy-D-galactose and 6-deoxy-D-glucose and a mole of sulfuric acid are attached. Subsequently, we isolated asterosaponin A as an inhibitor counteracting the action of the neutral spawning inducer in the same organism[5-8] and elucidated the structure of the main aglycone in the acid hydrolyzate of the saponin to be a novel steroid, 5α-pregn-9(11)-ene-3β,6α-diol-20-one (1).[9,10] Further, we established the structure of the carbohydrate moiety of the saponin as O-(6-deoxy-D-galactopyranosyl)-(1→4)-O-(6-deoxy-D-galactopyranosyl)-(1→4)-O-(6-deoxy-D-glucopyranosyl)-(1→4)-6-deoxy-D-glucose, which constitutes an O-acetal glycosidic linkage with the aglycone at the reducing terminal.[11-13] The present paper describes the details of isolation and structure elucidation of a novel steroidal sulfate, 5α-pregn-9(11)-ene-3β,6α-diol-20-one-3-sulfate (2), which led to the structure assignment of asterosaponin A. A preliminary communication on the study has been reported previously.[14]

As a provisional trial, release rates of deoxyhexoses and sulfate on acid treatment of asterosaponin A were investigated. Nearly all of the deoxyhexoses were released after a 5-hour treatment, whereas the sulfate group was liberated partially. This result indicated that the sulfate group should be attached to the steroidal aglycone but not to the carbohydrate moiety. Then, the saponin was hydrolyzed with 0·05 N HCl for 5 hr, and the hydrolyzate was neutralized with aqueous sodium hydroxide and subjected to partition between n-butanol and water. The butanol layer free from deoxyhexoses was evaporated to dryness and applied to gel-filtration on a Sephadex LH-20 column, by which clearly separated a steroidal conjugate from unconjugated steroids. The conjugate fraction was further purified by TLC on silica gel GF_{254} plates using a solvent system on n-butanol-acetic acid–water (10:1:1, v/v). The zone at R_f 0·45 was extracted with methanol. After evaporation of the solvent, the residue was dissolved in 0·6 M triethylammonium sulfate (pH 7·0) and extracted with chloroform. After evaporation of the solvent from the extract, the residual solid was recrystallized from ethyl acetate–methanol to afford triethylammonium salt of compound 2 as colorless needles.

The salt was indicated to contain a sulfate group by the presence of an adsorption band at 1235 cm^{-1} in its IR spectrum. The PMR spectrum revealed a signal due to a sulfate methine at δ 4·20, in addition to a secondary carbinol methine at δ 3·50 and an olefinic proton at δ 5·40 (Table 1). Larger peaks at δ 1·32 and 3·23 were ascribed to 15 protons constituting the triethylammonium ion. Solvolysis of the salt afforded a steroid, which was identified as 5α-pregn-9(11)-ene-3β,6α-diol-20-one (1) by the measurement of retention times on GLC and of R_f values on TLC.

Oxidation of pyridinium salt of compound 2

with chromium trioxide in pyridine followed by solvolysis solely gave a diketone (3). In the PMR spectrum of 3 in deuterochloroform (Table 1), the signal due to an olefinic proton appeared at significantly lower field (δ 5·63), as in the case of 5α-pregn-9(11)-ene-3,6,20-trione,[9] as compared with that of compound 1 (δ 5·35). This suggests that a ketonic function is located at C-6 in 3 and affects the chemical shift of the olefinic proton anisotropically. It has been known that the ORD of 3-keto-5α-steroids shows a single, positive Cotton-effect curve whereas that of 6-keto-5α-steroids shows the opposite sign.[15] Molar rotation difference obtained here by the ORD measurement on 3 and 1 revealed a single, negative Cotton-effect curve (Fig 1). Thus, 3 has been assigned as 5α-pregn-9(11)-ene-3β-ol-6,20-dione.

The chemical shifts (δ 0·58 and 0·91) of two quaternary Me groups at C-18 and C-19 of 3 showed good agreements with the values (δ 0·57 and 0·91) calculated according to the method of Zürcher.[16,17] If compound 3 possessed the alternative structure, 5α-pregn-9(11)-ene-6α-ol-3,20-dione, the calculated value for C-19 Me should be significantly lower (δ 1·16).

Since 3 was the sole product, as mentioned before, by the oxidation of 1 with chromium trioxide-pyridine complex followed by solvolysis, 2 must have the structure, 5α-pregn-9(11)-ene-3β,-6α-diol-20-one-3-sulfate. Accordingly, the carbohydrate moiety in asterosaponin A must be attached to 6α-OH group to form the acetal system. The configuration of the anomeric proton of the carbohydrate remains to be clarified.

Until now, cholesterol sulfate has been the sole steroid conjugate isolated from invertebrates: the starfish, *Asterias rubens*[18] and the sea urchin, *Anthocidaris crassispina*.[19] Compound 2 in this experiment would be the first pregnane sulfate of invertebrate origin which has been fully characterized.

EXPERIMENTAL

M.ps were determined on a microscope hot stage and reported uncorrected. IR spectra were obtained using a JEOL IR-S spectrometer. Optical rotations were measured on a JASCO DIP-S polarimeter. ORD was measured using a JEOL ORD/UV-5 automatic recording spectropolarimeter. PMR spectra were recorded on a JEOL-JNM-4H spectrometer at 100 MHz with TMS as an internal standard. Mass spectra were measured with a Hitachi RMU-6L mass spectrometer operating with an ionization energy of 70 eV. High resolution mass spectra were obtained with a Hitachi RMH-2 mass spectrometer as direct probe sample using an ionization voltage of 70 eV.

GLC were performed on a Hitachi gas chromatograph K-53, equipped with a hydrogen flame ionization detector. A glass column (0·3 × 200 cm) packed with 3% Silicone OV-1 on Chromosorb W (80–100 mesh) and a stainless steel column (0·3 × 100 cm) packed with 10% SE-30 on Chromosorb W (80–100 mesh) were used. The flow rate of N_2 as a carrier gas, was maintained at 40 ml/min. Samples were trimethylsilylated in dry pyridine according to the method of Sweeley *et al.*[20]

TLC were carried out with silica gel GF_{254} plates of 0·38 mm thickness. Solvent systems employed here were as follows: n-BuOH–AcOH–water (10:1:1, Solvent 1),* $CHCl_3$–MeOH–AcOH–water (60:20:10:4, Solvent 2), $CHCl_3$–MeOH (9:1, Solvent 3), benzene–acetone (3:2, Solvent 4) and benzene–acetone (25:10, Solvent 5). Spots on plates were detected by spraying

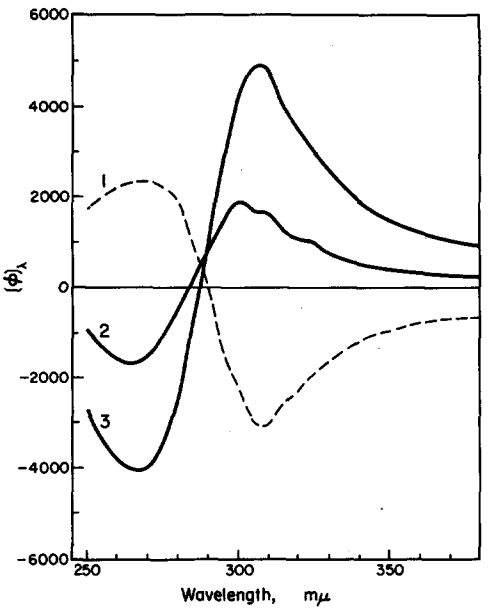

Fig 1. Molar rotation difference (1) obtained by the ord measurement on compound 3 (2) and 1 (3).

*The numerals in parentheses show v/v ratios.

Table 1. PMR spectra (δ values, J values in c/s in parentheses)

Compound	Solvent	Me groups			3H	6H	11H	Ions
		18	19	21				
1[9]	$CDCl_3$	0·59s	0·97s	2·17s	3·55m	3·55m	5·35(t,4)	
2	CD_3OD	0·54s	0·97s	2·12s	4·20m	3·50m	5·40(t,4)	1·32(t,9) 3·23(q,9)
3	$CDCl_3$	0·58s	0·91s	2·13s	3·55m		5·63(t,4)	
4	CD_3OD	0·56s	0·89s	2·12s	4·01m		5·62(t,5)	

10% (w/v) phosphotungustic acid in EtOH followed by heating at 100° for several min.

Asterosaponin A

The starfish, *Asterias amurensis*, were collected at Tokyo Bay in July, 1972. Asterosaponin A was prepared according to the method of Yasumoto *et al.*[3,21] with slight modification using silica gel instead of alumina for column chromatography. Crude saponins obtained by partition with diethyl ether-n-BuOH–water (6:2:1, v/v) were applied to a silica gel column, which was eluted with $CHCl_3$–MeOH–AcOH (6:2:1, v/v) mixture. Fractions containing asterosaponin A were combined and added with three times of their volume of diethyl ether. The resulting ppts were collected by filtration on an acid-washed Celite 545 layer, washed repeatedly with a large volume of diethyl ether, and then eluted from the Celite with water. Lyophylization of the aqueous soln afford amorphous asterosaponin A. Homogeneity of the sample was verified through TLC analyses using Solvents 1 (R_f 0·10) and 2 (R_f 0·05).

Triethylammonium salt of 5α-pregn-9(11)-ene-3β,6α-diol-20-one-3-sulfate (2). Asterosaponin A (57 mg) was treated with 0·05 N HCl (10 ml) for 5 hr at 100°. The hydrolyzate was neutralized with NaOHaq and partitioned between n-BuOH and water (1:1, v/v). The BuOH layer was separated and evaporated under reduced pressure. The residual solid (36 mg) was dissolved in 2 ml $CHCl_3$–MeOH (1:1, v/v) containing NaCl (0·01 mole/l.) and applied to a column of 8 g of Sephadex LH-20 (14 × 320 mm). The column was eluted with the same solvent system by 5-ml fractions. Unconjugated steroids were eluted in the fractions 20–30 ml and steroid sulfates at 50–70 ml of the effluent.[22] The sulfate fractions were combined, evaporated to dryness and subjected to TLC using Solvent 1. The zone at R_f 0·45 was extracted with MeOH, and the extract was evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of 0·6 M triethylammonium sulfate (pH 7·0), and the resulting aqueous soln was extracted with $CHCl_3$ according to the procedure of Mickan *et al.*[23] After evaporation of the solvent, the residual solid was recrystallized from EtOAc–MeOH to afford triethylammonium salt of 2 as colorless needles (9·7 mg). TLC using Solvent 1 or 2 showed a single spot with R_f 0·45 or 0·50, m.p. 160–161°; $[\alpha]_D^{25}$ +20° (c = 0·5, MeOH); ν_{max} (film) 3350, 1700, 1660, 1235 cm^{-1}; (Found: S, 6·28. $C_{27}H_{46}O_6NS$ requires: S, 6·25%). For PMR, see Table 1.

Solvolysis of triethylammonium salt of compound 2 to 5α-pregn-9(11)-ene-3β,6α-diol-20-one (1). The triethylammonium salt of 2 (0·2 mg) was dissolved in 1 ml EtOAc saturated with 2N H_2SO_4 and kept overnight at 37°.[24] Then, the mixture was partitioned between EtOAc and water (1:1, v/v). The EtOAc layer was separated, washed repeatedly with water and dried over Na_2SO_4. After evaporation of the solvent, the residual solid was identified on TLC as 1: R_f 0·37 with Solvent 3 and 0·38 with Solvent 4. Further, GLC on trimethylsilyl ether of the reaction product revealed a single peak whose retention time was identical with that of 1: 3·7 min with Silicone OV-1 at 240° and 10·4 min with SE-30 at 240°.

Oxidation of compound 2 with chromium trioxide pyridine complex. Triethylammonium salt of 2 (7·9 mg) was dissolved in 2 ml 0·6 M pyridinium sulfate (pH 7·0), and the soln was extracted with an equal volume of n-BuOH. The extract was evaporated to dryness under reduced pressure, and the residue was dissolved in 1 ml of anhyd pyridine to which was added dropwise 1 ml of 1% chromium trioxide-pyridine complex in anhyd pyridine and stood overnight at room temp.[25] Then the mixture was poured into 5 ml of 0·6 M pyridinium chloride (pH 7·0) and was extracted with two equal volumes of n-BuOH. The extracts were combined, washed with water and evaporated to dryness under reduced pressure. The residual oxidation product (4) showed a single spot on TLC at R_f 0·65 (Solvent 1). For PMR spectrum, see Table 1.

Solvolysis of compound 4 to 5α-pregn-9(11)-ene-3β-ol-6,20-dione (3). Compound 4 (4·0 mg) was dissolved in 4 ml dioxane containing 5% water and kept overnight at room temp. Then, the mixture was heated at 100° for 5 min.[26] After cooling, the mixture was partitioned between benzene and water (1:1, v/v; 10 ml). The benzene layer was separated, washed repeatedly with water and then dried over Na_2SO_4. Evaporation of the solvent afforded an oily residue (2·7 mg, 3), which showed a single spot on TLC at R_f 0·45 (Solvent 5). $[\theta]_{264}$ −500°, $[\theta]_{300}$ +570° (c = 0·085, EtOH); ν_{max} (KBr) 3450 and 1710 cm^{-1}. High resolution MS *m/e:* 330·2167 (M^+; calc. for $C_{21}H_{30}O_3$, 330·2193; rel. I. 6%), 297·1909 ($C_{20}H_{25}O_2$, 4%), 279·1789 ($C_{20}H_{23}O$, 4%), 259·1688 ($C_{17}H_{23}O_2$, 16%), 245·1536 ($C_{16}H_{21}O_2$, 9%), 244·1469 ($C_{16}H_{20}O_2$, 11%), 227·1437 ($C_{16}H_{19}O$, 29%), 209·1322 ($C_{16}H_{17}$, 21%), 105·0707 (C_8H_9, 29%), 95·0847 (C_7H_{11}, 15%), 91·0551 (C_7H_7, 22%), 85·0648 (C_5H_9O, 19%), 81·0706 (C_6H_9, 30%), 55·0566 (C_4H_7, 22%), 43·0152 (C_2H_3O, 100%). For PMR spectrum, see Table 1.

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