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Biosynthesis of polar steroids from the Far Eastern starfish *Patiria* (=*Asterina*) *pectinifera*. Cholesterol and cholesterol sulfate are converted into polyhydroxylated sterols and monoglycoside asterosaponin P_1 in feeding experiments

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ABSTRACT

For the first time, it is experimentally established that the dietary cholesterol and cholesterol sulfate are biosynthetic precursors of polyhydroxysteroids and related low molecular weight glycosides in starfishes. These deuterium labeled precursors were converted into partly deuterated 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptaol, 5α-cholestane-3β,4β,6α,7α,8,15β,16β,26-octaol, and steroid monoside asterosaponin P_1 in result of feeding experiments on the Far Eastern starfish *Patiria* (=*Asterina*) *pectinifera*. The incorporations of deuterium were established by MS and NMR spectroscopy. Scheme of the first stages of biosynthesis of polar steroids in these animals was suggested on the basis of inclusion of three from six deuterium atoms and determination of their positions in biosynthetic products, when [2,2,3,4,4,6-$^2H_6$]cholesterol 3-sulfate was used as precursor. It was also shown that labeled cholesterol is transformed into Δ^7-cholesterol (lathosterol) in digestive organs and gonads of the starfish.

1. Introduction

Starfishes are an extremely rich source of different steroid compounds including free sterols and polar steroid compounds such as polyhydroxysteroids, related mono- and biosides, and oligoglycosides named as asterosaponins. Polar steroid compounds from starfish have drawn attention not only by peculiar chemical structures, but also due to their wide spectrum of biological effects, including cytotoxic, antiviral, antibacterial, antibiofouling, neuritogenic, antifungal and other activities [1–4].

The starfish *Patiria* (=*Asterina*) *pectinifera* (Muller and Trochel) is a common species in the Northwestern Pacific. Earlier, our group has studied steroid constituents of this starfish as well as distribution of free sterols, polyhydroxysteroids and steroid glycosides in various body components of *P. pectinifera* [5]. It was shown that all the body components of this starfish contain free sterols and steroid oligoglycosides (asterosaponins), but polyhydroxysteroids and related low molecular weight glycosides present mainly in the digestive organs (stomach and pyloric ceca). The main steroid components of the latter fraction from steroid sum of *P. pectinifera* were identified as (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptaol (**1**), (25*S*)-5α-cholestane-3β,4β,6α,7α,8,15β,16β,26-octaol (**2**), and sulfated monoside asterosaponin P_1 (**3**). Seasonal variations in the levels of polar steroids in digestive organs of this starfish were also studied. On the basis of all the previously obtained data it was suggested that one of the biological functions of **1**–**3** and related compounds is concluded in their participation in digestive processes in starfish like bile acids and bile alcohols in vertebrates [5,6]. At the same time, biogenesis of **1**–**3** and related metabolites in starfishes was unknown, although a hypothesis that dietary sterols are biogenetic precursors of these steroids was proposed [6]. Experimentally, the biosynthesis of starfish polar steroidal compounds remained also to be insufficiently studied. Only a few data were obtained in *in vivo* experiments by Mackie et al., in which aglycons of oligoglycosides (asterosaponins) were shown to include some radioactivity from [2-^{14}C]mevalonic acid and [4-^{14}C]cholesterol [7], and low inclusion into asterosaponins was indicated in experiments with homogenates of different starfish body components, when radioactive [7-^{3}H] and [4-^{14}C]cholesterol and [4-^{14}C]6-hydroxycholesterols were used as precursors [8]. However, up to date there were no information concerning biosynthesis of polyhydroxysteroids and related low molecular weight steroid glycosides in starfishes.

Herein, we report the results of our studies on possible biosynthetic pathways of polyhydroxysteroids and related glycosides in *P. pectinifera*. With this aim, we have carried out the feeding experiments with labeled by stable isotopes precursors, namely:

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[1-^{13}C]sodium acetate, [1-^{13}C]D-glucose, [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol, and [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate. Free sterols, asterosaponins and compounds **1**–**3** from digestive organs and gonads of *P. pectinifera* were isolated and analyzed. The data concerning isotope incorporation and distribution in these steroids were obtained using GLC–MS, ESIMS, ESIMS/MS, and NMR spectroscopy.

2. Experimental

2.1. General methods

^{1}H, ^{2}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 700 spectrometer at 700.13, 107.48 and 176.04 MHz, respectively, using TMS as an internal standard. HRESI mass spectra and ESIMS/MS spectra were recorded on an Agilent 6510 Q-TOF LC/MS mass spectrometer; samples were dissolved in MeOH (c 0.01 mg/mL).

HPLC separations were carried out on an Agilent 1100 Series chromatograph equipped with a differential refractometer. Supelco Discovery C_{18} (5 μm, 250 × 10 mm) column was used. Low pressure column liquid chromatography was performed using Polychrome-1 (powdered Teflon, Biolar, Latvia) and Si gel KSK (50–160 μm, Sorbpolimer, Krasnodar, Russia). Sorbfil Si gel plates (4.5 × 6.0 cm, 5–17 μm, Sorbpolimer, Krasnodar, Russia) were used for thin-layer chromatography. GLC–MS analyses were carried out on a Hewlett Packard 6890 GC System instrument with a 5973 mass selective detector, a capillary column HP-5MS (crosslinked 5% Me siloxane) (30 m × 250 μm × 0.25 μm) at 270 °C. Helium was used as the carrier gas (1 mL/min) and the ionizing energy was of 70 eV. Relative retention times (RRT) were calculated in relation to that of cholesterol acetate.

The labeled by stable ^{13}C isotope [1-^{13}C]sodium acetate and [1-^{13}C]D-glucose were purchased from Aldrich Chem. Co., [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol (98%) and [2,2,3,4,4,6-$^{2}H_6$]cholesterol (97–98%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

2.2. Preparation of the [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate

The [2,2,3,4,4,6-$^{2}H_6$]cholesterol (100 mg) was treated with sulfur trioxide pyridine complex (Sigma–Aldrich Chem. Co.) in dry pyridine (3 mL) for 16 h at 110 °C. The TLC on Sorbfil Si gel plates in the system toluene/EtOH (9:5) was used for control of the reaction products. The [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate was precipitated by hexane (100 mL), obtained residue was washed by hexane (100 mL, twice), separated by centrifugation (3000 rpm/min, 5 min), and dried on air. The precipitate was dissolved in H_2O (100 mL) and solution was neutralized by 10% NaOH to pH = 7. This solution was passed through a Polychrome-1 column (2 × 11 cm), the column was washed by H_2O (200 mL), and then cholesterol sulfate was eluted by EtOH (150 mL) and ethanol eluate was evaporated. As a result [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate as amorphous powder was obtained (105 mg, R_f 0.53).

[2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate: $C_{27}H_{39}D_6O_4SNa$; amorphous powder; $[\alpha]_D^{25}$ −5.3° (c 0.17, MeOH); (−)HRESIMS *m/z* 471.3420 $[M-Na]^-$ (calcd for $C_{27}H_{39}D_6O_4S$, 471.3421).

2.3. Animal material

The twenty five specimens of the starfish *Asterina* (=*Patiria*) *pectinifera* (order Valvatida, family Asterinidae) were collected from a depth of 1–1.5 m at the Posiet Bay, the Sea of Japan, in May 2011. All the specimens were sexually mature and ranged in diameter from 8 to 10 cm.

2.4. Feeding experiments

Food samples, containing potential biosynthetic precursors were prepared by the modifying method used by Mollo et al. [9]. Every day during 5 days period five portions of food were used, including one control portion without labeled compounds and four portions with labeled precursors: [1-^{13}C]sodium acetate (200 mg), [1-^{13}C]D-glucose (200 mg), [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol (20 mg), and [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate (21 mg). For the preparing of a food portion, alginic acid (50 mg), dried squid (50 mg) and purified sea sand (50 mg; granular size 0.1–0.3 mm) were combined. A labeled compound was added to this mixture and carefully mixed. After it the distilled H_2O (1 mL) was added and obtained mixture was stirred, loaded into 5-mL syringe, and extruded into a 0.25 M calcium chloride solution for 2 min to harden. The resulting spaghetti-like strand was cut into 10-mm-long pellets. A control food preparation was made in the same manner without any labeled precursors.

The five groups of starfish (5 specimens per group) were kept alive in aquariums without any food 7 days. Then starfish were allowed to feed during 5 days and afterwards were maintained again without food during 14 days. In each group of starfish, animals were dissected and a sum of digestive organs (stomach and pyloric ceca) and separately gonads were isolated. As result, five preparations each of gonads (I–V) and digestive organs (VI–X) were obtained: I and VI – control, II and VII – after feeding [1-^{13}C]sodium acetate, III and VIII – after feeding [1-^{13}C]D-glucose, IV and IX – after feeding [25,26,26,26,27,27,27-$^{2}H_7$]cholesterol, and V and X – after feeding [2,2,3,4,4,6-$^{2}H_6$]cholesterol 3-sulfate.

2.5. Extraction

The homogenized preparations of fresh gonads or digestive organs (preparations I–X) were thrice extracted with EtOH (3 × 50 mL) at room temperature and the obtained precipitates were separated by centrifugation (3000 rpm/min, 5 min). The ethanol extracts were treated with benzene (1:3 mL of the ethanol extract, twice). The benzene layers were separated and concentrated in vacuo to give free sterols. The aqueous EtOH layers were evaporated in vacuo to give residues that contained polar steroids: 428 mg, 501 mg, 485 mg, 273 mg, 450 mg, 1.29 g, 1.68 g, 1.30 g, 1.54 g, and 2.35 g from preparations I–X, respectively.

2.6. Purification, acetylation and GLC–MS analysis of free sterol fractions

The isolation of free sterol fractions from benzene extracts of digestive organs and gonads was achieved by preparative TLC on glass plates with Silica gel L (5/40 μm, Chemapol, Praha, Czech Republic) in the system hexane/ethylacetate (3:1, v/v). Detection of the corresponding bands was carried out by spraying of plates with water. Free sterols were removed from Silica gel by elution with $CHCl_3$/EtOH (1:1, v/v). Each sterol mixture was treated with Ac_2O/pyridine (1:1, v/v, 0.2 mL) for 12 h at room temperature. The reaction mixtures were concentrated in vacuo and the obtained residues of sterol acetates were purified by chromatography on a Silica gel column (0.8 × 4 cm) in hexane/ethylacetate systems (20:1). The obtained sterol acetates were analyzed by GLC–MS method. Identification of sterol constituents was carried out by comparison of their RRT and MS with those reported by Kicha et al. [5]. Cholesterol (Sigma Grade: 99+%) was used as a standard for determination of RRT values.