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BIOSYNTHESIS OF ASTEROSAPONINS FROM CHOLESTEROL AND OTHER STEROLS UNDER THE CONDITIONS OF *IN VITRO* HOMOGENATES OF STARFISH GONADS AND PYLORIC CECA

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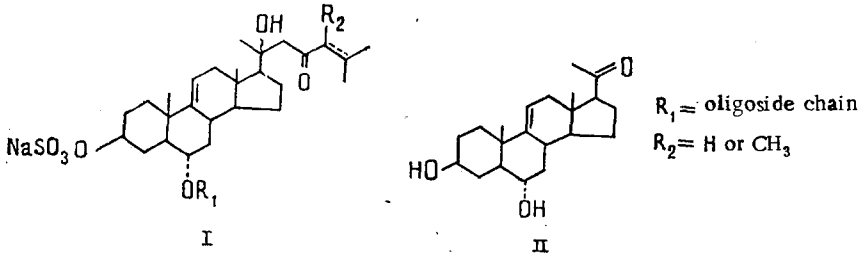
Conditions have been selected for performing the *in vitro* biosynthesis of asterosaponins from cholesterol and other sterols in homogenates and cultures of the gonads and pyloric ceca of Far Eastern starfish. It has been shown that the aglycone moiety of an asterosaponin can be biosynthesized from cholesterol, cholesterol sulfate, 5α-cholestanol, and 3β,6α-dihydroxy-5α-cholestane but not from 3β, 6β-dihydroxy-5α-cholestane. Of the give precursors studied, cholesterol was transformed into asterosaponins most completely.

The steroid glycosides of starfish (asterosaponins) form the only group of steroid oligosides known at the present time that are biosynthesized not by higher plants but by animals. The majority of representatives of this group of compound have the general formula (I) [1-3] and on acid hydrolysis form mainly the pregnane aglycone (II) [3-6].



I II

The **biogenesis** of the asterosaponins has been little studied. Although it has been shown previously that these compounds are formed by two routes — de novo synthesis from mevalonic acid and the biotransformation of exogenous cholesterol [2] — it has remained unknown in what organs or tissues these transformations take place, whether steroid alcohols other than cholesterol are capable of acting as precursors, and what is the sequence of biosynthetic reactions leading from cholesterol to the asterosaponins. It was possible to answer these questions only by using either homogenates of individual organs or cultures of the corresponding cells or tissues.

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TABLE 1. Biotransformation of Cholesterol in Homogenates of the Starfish *Asterias amurensis* and *Patiria pectinifera*

Animal	Tissue	Radioactivity introduced, pulses/min	Fraction isolated	Weight of the fraction, g	Specific activity, pulses/min · mg	% biotransformation
A. amurensis	Gonads, 335 g	$352 \cdot 10^6$	Sum of the water-soluble substances	42 7468	$1 \cdot 10^3$	12
			Sum of the butanol-soluble substances	2,5569	$53 \cdot 10^3$	28 5
			Steroid fraction	1,1388	$54 \cdot 10^3$	17,3
			Glycoside fraction	0.2056	34	0,002
A. amurensis	Pyloric ceca, 524 g	$423 \cdot 10^6$	Sum of the water-soluble substances	39,7007	—	—
			Sum of the butanol-soluble substances	3,8833	$87 \cdot 10^3$	79,9
			Steroid fraction	1,7178	$57 \cdot 10^3$	23
			Glycoside fraction	0.3874	35	0,003
P. pectinifera	Gonads, 297 g	$70 \cdot 10^6$	Sum of the water-soluble substances	36,9899	—	—
			Sum of the butanol-soluble substances	2,4 92	$28 \cdot 10^3$	100
			Steroid fraction	1,0092	$38 \cdot 10^3$	54,4
			Glycoside fraction	0 0642	70	0,006

TABLE 2. Distribution of Radioactivity in the DNPHs of Pregnane Aglycones from Five Parallel Experiments on the *in Vitro* Biosynthesis in Homogenates of *A. amurensis* Pyloric Ceca

Precursor	Radioactivity introduced, pulses/min	Weight of the DNPHs, mg	Weight of sample for radioactivity determination, mg	Absolute count, pulses/min	Specific activity, pulses/min · mg	Σ activity, pulses/min	% inclusion of the label
III	$150 \cdot 10^6$	1,30	1,163	345	261	339	$2,1 \cdot 10^{-4}$
IV	$248 \cdot 10^6$	0,65	0,288	88	160	104	$4,2 \cdot 10^{-5}$
V	$408 \cdot 10^6$	0,95	0 900	137	105	100	$2,5 \cdot 10^{-5}$
VI	$427 \cdot 10^6$	2,4	1,717	111	40	96	$2,2 \cdot 10^{-5}$
VII	$184 \cdot 10^6$	2,5	1,054	—	—	—	—

We have shown (Table 1) that when labeled cholesterol is incubated with homogenates of the gonads or the pyloric ceca of Far Eastern starfish catabolism of the sterol to gaseous or low-molecular-weight water-soluble compounds takes place to an appreciable extent only in homogenates of the gonads of *Asterias amurensis*. On the other hand, both homogenates of this starfish and homogenates of *Patiria pectinifera* leave a considerable part of the precursor (17-54%) is unchanged form or convert it into the $\Delta^{7(8)}$-isomer which is characteristic for starfish sterols [2]. The amount of cholesterol added to the system that is transformed into the glycosidic fraction is 0.002-0.006%, which is comparable with the results obtained in a study of the transformation of cholesterol into asterosaponins in *in vivo* experiments [2].

To confirm the conclusion of the possibility of using homogenates of starfish tissue in a study of the biosynthesis of the asterosaponins and to determine the most suitable sterol precursor for such a biosynthesis, we have added the following sterols and sterol derivatives to homogenates: cholesterol (III), cholesterol sulfate (IV), 5α-cholestanol (V), 3β,6α-dihydroxy-5α-cholestane (VI), and 3β,6β-dihydroxy-5α-cholestane (VII). The sum of the glycosides isolated from these homogenates was hydrolyzed, and the pregnane aglycone (II) so obtained was converted into the crystalline 2,4-dinitrophenylhydrazone (DNPH) and its radioactivity was measured. The results of these experiments are given in Table 2.

TABLE 3. Distribution of Radioactivity in the DNPHs of the Pregnane Aglycones in Two Parallel Experiments on *in Vitro* Biosynthesis in Cultures of the Gonads of *A. amurensis*

Precursor	Radioactivity introduced, pulses/min	Weight of the DNPHs, mg	Weight of the sample for radioactivity determination, mg	Absolute count, pulses/min	Specific activity, pulses/min·mg	Σ activity, pulses/min	% inclusion of the label
III	$354 \cdot 10^6$	30 First crystallization	0,2	595	$2,8 \cdot 10^3$	$8,5 \cdot 10^4$	$2,4 \cdot 10^{-2}$
		9,6 Second crystallization	0,9	155	146	$1,4 \cdot 10^3$	$4,0 \cdot 10^{-4}$
		5,4	2,8	413	139	750	$2,1 \cdot 10^{-4}$
IV	$78 \cdot 10^6$	43.4 First crystallization	0,6	163	220	$9,5 \cdot 10^3$	$1\,2 \cdot 10^{-2}$
		4,6 Second crystallization	0,6	53	48	222	$2,8 \cdot 10^{-4}$
		1,3	1,3	93	53	69	$0,9 \cdot 10^{-4}$

It could be assumed that the sterols used were potential precursors of (II). Only in the case of (VII) did the hydroxy group at carbon atom 6 have the β orientation and not the α orientation as in (II), which gave grounds for assuming that the direct transformation of (VII) into (II) was impossible.

Cholesterol (III) underwent the greatest conversion into the aglycone (II). It was followed by its sulfate (IV). 5α-Cholestanol (V) and the 3β,6β-diol (VI) were transformed into (II) less intensively and to approximately equal degrees. And, finally, as was assumed, the 3β,6β-diol (VII) was not transformed into (II) at all.

We repeated the experiments with the precursors (III) and (IV) on cultures of the gonads of *A. amurensis*, and the DNPHs of the aglycone (II) obtained were crystallized to constant specific radioactivity. As can be seen from Table 3, cholesterol (III) was actually transformed more completely into the pregnane aglycone (II). The sulfate (IV) was only slightly inferior to it.

Thus, of the compounds investigated the best biosynthetic precursors of the asterosaponins are cholesterol (III) and cholesterol sulfate (IV).

EXPERIMENTAL

GLC analysis was performed on a Pye-Unicam instrument under the conditions given in [6], PMR spectra were recorded on Bruker HX-90E and M-250 instruments in $CDCl_3$ and C_5D_5N with TMS as internal standard (δ scale). Radioactivities were measured in SBS-2 and Mark II counters in toluene (4 g of PPO and 0.2 g of POPOP in 1 liter of toluene) and dioxane (4 g of PPO, 0.2 g of POPOP and 60 g of naphthalene in 1 liter of dioxane) scintillators.

[7-^{3}H]- and [4-^{14}C]Cholesterols (Izotop) were diluted with unlabeled freshly crystallized cholesterol to specific activities of from $2 \cdot 10^6$ to $4 \cdot 10^7$ pulses/min·mg.

Cholesterol sulfate (IV) in the form of the sodium salt was obtained by the reaction of labeled cholesterol with an excess of pyridine/sulfur trioxide in absolute benzene with heating [7]. The reaction mixture was separated on a column of dry silica gel (L 40/100), the (IV) being eluted with the chloroform–ethanol (1:1) system.

5α-Cholestanol (V) was obtained by the hydrogenation of labeled cholesterol over Adams catalyst in the presence of perchloric acid [8]. The completeness of the reaction was followed from the PMR spectra of the reaction mixture.

3β,6α-Dihydroxy-5α-cholestane (VI) and 3β,6β-Dihydroxy-5α-cholestane (VII). At 1°C in an atmosphere of argon, 2 ml of a 1 M solution of diborane in THF was added dropwise to a solution

of 0.1083 g of [4-^{14}C]cholesterol in 3 ml of thrice-redistilled THF. The mixture was stirred and cooled until the evolution of hydrogen ceased. On the following day. 0.8 ml of MeOH, 0.8 ml of 10% NaOH, and 1.7 ml of 30% H_2O_2 were added dropwise to the reaction mixture at room temperature. After the reaction solution had been stirred at 40°C for 1.5 h, it was concentrated in vacuum and the residue was diluted twofold and was extracted with ethyl acetate and diethyl ether. The extracts were combined, washed with water, dried over $MgSO_4$, and evaporated to dryness. The total reaction products were separated on a silica gel column in the benzene and benzene–diethyl ether (2.5:1) systems. This gave 0.0291 g (25.7%) of (VI) with $[\alpha]_D$ +38° and 0.0281 g (24.8%) of (VII) with $[\alpha]_D$ + 13°.

Preparation of the Homogenates. The starfish *A. amurensis* (15-55 animals) and *P. pectinifera* (200 animals) were trapped in Posyet Bay, Sea of Japan, in August, 1980 (Table 1), May, 1982 (Table 2), and August, 1983 (Table 3). The gonads (162-335 g) and pyloric ceca (239-971 g) were separated out, homogenized, and centrifuged (K-70, 4600 × g, 15 min) at 0°C. The volumes of the supernatants were brought to 500 ml with boiled and filtered seawater. In the 1983 experiments (Table 3), the volume of each was 2.5 liters. The gonads were not homogenized. NADP·H (Reanal, c = 0.1 mM), EDTA (Reakhim, c = 1 mM), and sucrose (c = 1 mM) were added to the incubation mixture. All the precursors with the exception of (IV) were added dropwise in the form of suspensions in a 5% solution of Tween-60. The cholesterol sulfate was dissolved in water. The mixtures were aerated with air and stirred at 25°C. The time of incubation was 6-8 h.

Isolation of the Pregnane Aglycone (II). The reaction mixture was extracted with butanol three times. The extracts were evaporated to dryness. The glycoside and sterol fractions were separated on a column of Polikhrom-1 in the butanol–saturated water and water–ethanol (1:1) systems. The isolation of the (II) was performed as described in [5, 6]. It was identified by comparison with a standard sample in TLC (silica gel L 40/100; benzene–acetone (1.5:1)) and GLC.

The 2,4-dinitrophenylhydrazone of the pregnane aglycone was obtained as described in [9]. The purification of this derivative was performed on a column of silica gel in the chloroform–ethanol (17:3) system. The compound obtained was identified by comparison with a standard sample by PMR spectroscopy. It was crystallized from ethanol.

In the calculation of the total percentage inclusion of the label into (II) and its derivative the amount of radioactivity introduced into the experiment was taken as 100%.

SUMMARY

It has been shown that the aglycone moiety of an asterosaponin can be biosynthesized from cholesterol, cholesterol sulfate, 5α-cholestanol, and 3β,6α-dihydroxy-5α-cholestane but not from 3β,6β-dihydroxy-5α-cholestane. Of the five precursors investigated, cholesterol was transformed into asterosaponins most completely.

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