Studies on Biochemical Transformation of Plant Steroids. Part III.[1] Conversion of [^{14}C]Chiograsterol A into [^{14}C]Chiogralactone by a Homogenate of *Chionographis japonica* Maxim.

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Chionographis japonica contains a C_{23} steroid, chiogralactone (3β,16β-dihydroxy-6-oxo-24-nor-5α-cholan-23-oic acid 16β,23-lactone) (I), which is the first reported example containing a δ-lactone group. In order to elucidate its biosynthesis, labelled chiograsterol A(3β,16β,23,24-tetrahydroxy-5α-cholestan-6-one) (III) and B(5α-cholestan-3β,6β,16β,23,24-pentaol) were incubated with the homogenate from the whole *Chionographis japonica* plant at 27° for 6 hr. The results indicate that chiograsterol A is converted into chiogralactone, whereas chiograsterol B is not so converted by the action of the plant enzyme.

It has been reported [2] that the steroidal components of *Chionographis japonica* Maxim. consist of steroidal sapogenins (diosgenin, bethogenin, pennogenin, and kryptogenin), sterols (cholesterol, campesterol, stigmasterol, and β-sitosterol), polyoxygenated sterols (chiograsterol A and B [3] and unknown compounds E and H), and chiogralactone.[4,5] Chiogralactone possesses the C_{23}-steroidal structure (I) containing the novel δ-lactonic function. We report its biosynthesis by the action of the plant enzyme.

Two biosynthetic routes to the δ-lactonic function of (I) were considered possible, the first analogous to that occurring in the cardenolide series [see formulae (II)]. Leete *et al.*[6] have studied digitoxigenin biosynthesis and report (*a*) that acetic acid is incorporated into its butenolide ring and (*b*) that only C-20 and C-21 in the butenolide are derived from squalene. Leete concluded that the cardenolide is formed by the condensation of a pregnane derivative derived from mevalonic acid *via* squalene with one molecule of acetic acid. Thus chiogralactone may also be formed by way of a pregnane derivative.

The second route considered involved oxidative cleavage of the side-chain of a polyoxygenated sterol, followed by lactone formation between the carboxy-group thus obtained and the C-16 hydroxy-group.

Chiogralactone is an A/B- and C/D-*trans*-steroid, whereas most of the cardenolides are A/B- and C/D-*cis*-derivatives, and coexists with sapogenins and the polyoxygenated sterols, chiograsterol A and B, in this plant. We therefore suggest that chiogralactone is biosynthesised by the second route, that is, by the series of reactions: mevalonic acid ⟶ squalene ⟶ chiograsterol A and/or B ⟶ chiogralactone.

In order to confirm the last step, the following experiments were carried out. Radioactive chiograsterol A and B were obtained by feeding (±)-[2-^{14}C]mevalonic acid to ten *Chionographis japonica* plants, which were extracted ten days later with methanol. The results are shown in Table 1.

Radioactive chiograsterol A (III) ($3{\cdot}5 \times 10^4$ d.p.m./8 mg.) was added to a homogenate of the whole plant and incubated at 27° for 6 hr. The mixture was then saponified with 5% sulphuric acid in methanol and extracted with chloroform. The extract was separated into the sterol fraction (inactive), the sapogenin and polyoxygenated sterol fraction (radioactive), and the lactone

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[2] K. Takeda, A. Shimaoka, M. Iwasaki, and H. Minato, *Chem. and Pharm. Bull.* (*Japan*), 1965, **13**, 691.
[3] K. Takeda, H. Minato, and A. Shimaoka, *Chem. Comm.*, 1968, 104.
[4] K. Takeda, M. Iwasaki, A. Shimaoka, and H. Minato, *Tetrahedron*, 1966, Suppl. 8, 123.
[5] M. Iwasaki, *Tetrahedron*, 1967, **23**, 2145.
[6] E. Leete, H. Gregory, and E. G. Gros, *J. Amer. Chem. Soc.*, 1965, **87**, 3475.

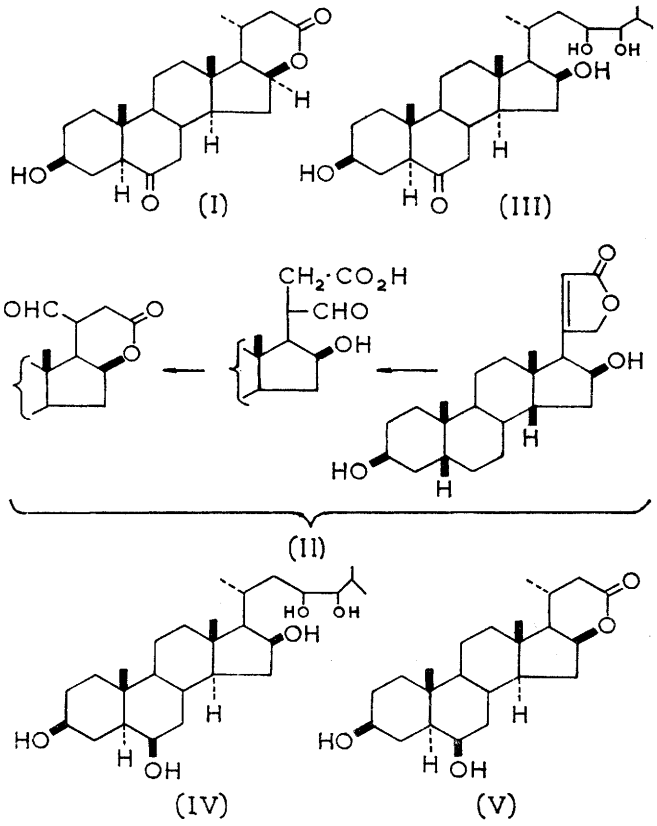
TABLE 1

Feeding (±)-[2-^{14}C]mevalonic acid ($1{\cdot}86 \times 10^8$ d.p.m.) to *Chionographis japonica* Maxim.

	Yield (mg.)	Total activity (d.p.m.)	Specific activity (d.p.m./mg.)	Incorporation * (%)
Sterols	9	$2{\cdot}1 \times 10^6$	$2{\cdot}33 \times 10^5$	2·3
Diosgenin	20	$6{\cdot}8 \times 10^5$	$3{\cdot}4 \times 10^4$	0·73
Bethogenin	7	$1{\cdot}05 \times 10^5$	$1{\cdot}5 \times 10^4$	0·113
Pennogenin	13	$1{\cdot}43 \times 10^5$	$1{\cdot}1 \times 10^4$	0·15
Compound E	5	$1{\cdot}65 \times 10^4$	3300	0·018
Kryptogenin	2	$1{\cdot}02 \times 10^4$	5000	0·011
Chiogralactone (I)	2	2660	1330	0·003
Compound H	4	$1{\cdot}77 \times 10^4$	4700	0·019
Chiograsterol A (III)	5	$3{\cdot}05 \times 10^4$	6100	0·033
Chiograsterol B (IV)	43	$9{\cdot}14 \times 10^4$	2130	0·098

* Since the racemic acid was used, the actual incorporation is twice that observed.

fraction (see Experimental section). The lactone fraction was purified by preparative t.l.c. to give a lactone, m.p. 229—234° (630 d.p.m./3 mg.), identified as chiogralactone (I) by mixed m.p. and t.l.c. comparisons. The incorporation is therefore 1·8%.



Next, labelled chiograsterol B was incubated with the homogenate under the same conditions as chiograsterol A. The lactone fraction afforded inactive chiogralactone. The radioactivity of the total lactone fraction showed up on t.l.c. in the region expected for dihydrochiogralactone (V),[4] but insufficient product could be isolated for characterisation.

The results show that chiograsterol A (III) is converted into chiogralactone, whereas chiograsterol B is not so converted by the action of the plant enzyme.

The sapogenin and polyoxygenated sterol fractions obtained on incubation of chiograsterol A with the homogenate, gave unidentified compounds E (650 d.p.m./15 mg.) and H (700 d.p.m./10 mg.), unchanged chiograsterol A (10,000 d.p.m./15 mg.), chiograsterol B (350 d.p.m./35 mg.), and sapogenins (inactive). However, the radioactivity of the same fractions obtained from chiograsterol B was almost entirely due to unchanged chiograsterol B (9000 d.p.m./26 mg.), although compounds E (240 d.p.m./6 mg.) and H (510 d.p.m./12 mg.) showed some small activity.

As shown in Table 1, the specific activities of radioactive compounds obtained from feeding (±)-[2-^{14}C]-mevalonic acid to the plant are chiograsterol A, 6100 d.p.m./mg., compound H, 4700 d.p.m./mg., compound E, 3300 d.p.m./mg., and chiograsterol B, 2130 d.p.m./mg.

These results indicate that conversions of chiograsterol A into compounds E and H and chiograsterol B occur in the plant body. We are at present investigating the structures of compounds E and H.

EXPERIMENTAL

Solvent systems for t.l.c. were (A) benzene–acetone–methanol (1 : 1 : 1), (B) chloroform–ethyl acetate–methanol (10 : 10 : 1), (C) same as (B) but (15 : 5 : 1), (D) benzene–acetone (1 : 1), (E) benzene–acetone–ethyl acetate (1 : 1 : 1), (F) hexane–chloroform–ethyl acetate (1 : 1 : 1), (G) same as (F) but (4 : 1 : 1), and (H) benzene–ether (1 : 1).

Feeding of (±)-[2-^{14}C]*Mevalonic Acid to* Chionographis japonica *Maxim.*—Aqueous (±)-[2-^{14}C] mevalonic acid (0·5 ml.; $1{\cdot}86 \times 10^7$ d.p.m. for one plant) was fed to ten plants by means of a cotton wick inserted into the stem. Ten days later, the plants (76 g.) were washed with water, dried, sliced and extracted with methanol (4 × 200 ml.) under reflux. The extract (5·5 g., $4{\cdot}56 \times 10^7$ d.p.m.) was hydrolysed with 5% sulphuric acid in methanol and extracted in turn with ethyl acetate to give a viscous oil (600 mg.; $3{\cdot}28 \times 10^7$ d.p.m.). The extract was chromatographed on alumina and then silica gel; [2] each fraction was purified by preparative t.l.c. The products are shown in Table 1.

Incubation of Labelled Chiograsterol A (III) *with the Homogenate of the Whole Plant* (see Table 2).—Whole plants (50 g.), collected in September, were homogenised with 0·066M-phosphate buffer (200 ml.; pH 6·5) under nitrogen at 0—3° for 1 min. in a Satake homogeniser (3000 r.p.m.). Labelled chiograsterol A (III) (8 mg.; $3{\cdot}5 \times 10^4$ d.p.m.) was incubated with the homogenate at 27° for 6 hr. in a Sakaguchi shaking flask fitted with a cotton stopper, with

gentle shaking (110—120 times/min.). The incubation mixture was hydrolysed with sulphuric acid (25 g.) in methanol (200 ml.) and water (100 ml.) and extracted with chloroform.[2] The extract was washed with 5% sodium hydrogen carbonate to give the neutral extract (*a*) (1·7 g.; $3{\cdot}25 \times 10^4$ d.p.m.). This was chromatographed on alumina (20 g.) to give the sterol fraction (660 mg.; inactive), the sapogenin and polyoxygenated sterol fraction (*b*) (280 mg.; $3{\cdot}1 \times 10^4$ d.p.m.), and the low R_F fraction (*c*) (27 mg.; 430 d.p.m.). Fraction (*b*) was saponified with 5% potassium hydroxide in methanol to give the acid fraction (*d*) (40 mg.; 800 d.p.m.) and the neutral fraction (*e*) (210 mg.; 3×10^4 d.p.m.). Fraction (*d*) was lactonised with 2N-sulphuric acid and chromatographed on alumina to give a lactone (15 mg.), which was purified by preparative t.l.c., affording a lactone (*f*), as colourless prisms (3 mg., 630 d.p.m.), m.p. 229—234° (from acetone–ether), identical with chiogralactone (I) [R_F values: (A) 0·52, (B) 0·39, (D) 0·46]. Its acetate was also purified by preparative t.l.c. to give colourless plates (3 mg., 640 d.p.m.), m.p. 226—229° (from ether–acetone), identical with chiogralactone acetate [mixed m.p. and t.l.c.: R_F (E) 0·57, (F) 0·10, (H) 0·20]. Fraction (*e*) was separated by alumina chromatography followed by preparative t.l.c. into sapogenins (inactive), compound E (15 mg.; 650 d.p.m.), R_F (C) 0·55, (D) 0·57 [acetate R_F (G) 0·45, (H) 0·48], compound H (10 mg.; 700 d.p.m.), R_F (C) 0·45, (D) 0·53 [acetate R_F (G) 0·33, (H) 0·30], chiograsterol B (IV) (35 mg., 350 d.p.m.), R_F (C) 0·18, (D) 0·18 [acetate R_F (G) 0·35, (H) 0·33], and recovered chiograsterol A (15 mg., 10,000 d.p.m.), R_F (C) 0·35, (D) 0·30 [acetate R_F (G) 0·20, (H) 0·27].

Incubation of Labelled Chiograsterol B (IV) *with the Homogenate of the Whole Plant* (see Table 3).—Under the same conditions as for chiograsterol A (III), whole plants (50 g.), collected in August were homogenised, and labelled chiograsterol B (IV) (10 mg.; $3{\cdot}0 \times 10^4$ d.p.m.) was incubated with the homogenate. The incubation mixture was hydrolysed with 5% sulphuric acid in methanol and extracted with chloroform. Treatment as before gave the corresponding fractions: (*a'*) (2·1 g.; $2{\cdot}13 \times 10^4$ d.p.m.), sterol fraction (710 mg.; inactive), (*b'*) (330 mg.; $1{\cdot}48 \times 10^4$), (*c'*) (168 mg.; $1{\cdot}8 \times 10^3$), (*d'*) (30 mg.; 570 d.p.m.), (*e'*) (300 mg.; $1{\cdot}0 \times 10^4$ d.p.m.), and (*f'*) (16 mg.; 570 d.p.m.), the last of which gave chiogralactone (I) (4 mg.; radioinactive). Fraction (*e'*) gave sapogenins (inactive), compound E (6 mg.; 240 d.p.m.), compound H (12 mg.; 510 d.p.m.), and recovered chiograsterol B (26 mg.; 9000 d.p.m.).

TABLE 2

Incubation of chiograsterol A (III) with homogenate

	Activity (d.p.m.)	Yield (mg.)
Incubated (III)	$3{\cdot}5 \times 10^4$ (8 mg.)	
Neutral extract (*a*) ...	$3{\cdot}25 \times 10^4$	1700
Fraction (*b*).....	$3{\cdot}1 \times 10^4$	280
Residue (*c*)	430	27
Acid fraction (*d*).....	800	40
Neutral fraction (*e*) ...	$3{\cdot}0 \times 10^4$	210
Lactone (*f*)	630	3

TABLE 3

Incubation of chiograsterol B (IV) with homogenate

	Activity (d.p.m.)	Yield (mg.)
Incubated (IV)	$3{\cdot}0 \times 10^4$ (10 mg.)	
Neutral extract (*a'*) ...	$2{\cdot}13 \times 10^4$	2100
Fraction (*b'*)	$1{\cdot}48 \times 10^4$	330
Residue (*c'*)	$1{\cdot}8 \times 10^3$	168
Acid fraction (*d'*)	570	30
Neutral fraction (*e'*) ...	$1{\cdot}0 \times 10^4$	300
Crude lactone (*f'*)	570	16

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