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Studies on Biochemical Transformation of Plant Steroids. Part II.[1] Biochemical Conversion of Gitogenin into 12-Oxygenated Sapogenins in *Hosta kiyosumiensis* F. Maek

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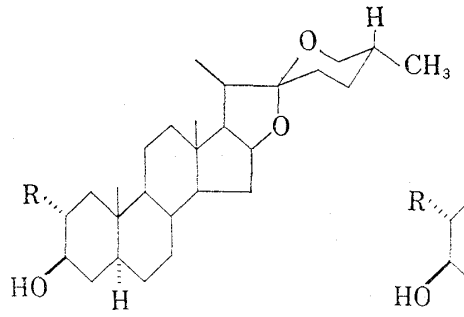
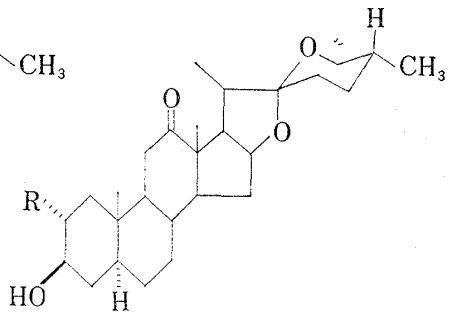
From the examination of yields of sapogenins isolated from *Hosta kiyosumiensis* F. Maek in each season, it was assumed that gitogenin is oxidized to the 12-oxygenated sapogenins in the hypogeous part of the plant in winter. In order to confirm this assumption, [27-^{14}C]-gitogenin was incubated with the homogenate from the hypogeous part of the plant at 37° for 6 hr. The result from this experiment indicates the conversion of gitogenin into the 12-oxygenated sapogenins by the action of the plant enzyme.

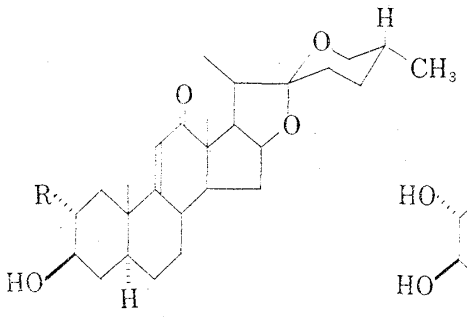
In 1947, Marker, *et al.*[3] published an article on the biogenesis of steroidal sapogenins, in which the following view was expressed. The steroidal content of the *Yucca* species soon after flowering is predominantly the saponins of one sapogenin; then saponins of other sapogenins are formed; these gradually undergo a change to give saponins of sapogenins with fewer oxygenated groups; and, finally, the latter are discarded in the flower stalks. Moreover,



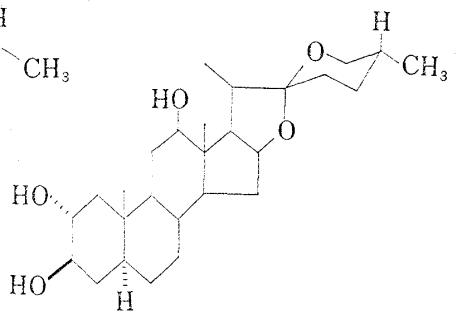
I: R=H
IV: R=OH



II: R=H
V: R=OH



III: R=H
VI: R=OH



VII

1) Part I: K. Takeda, H. Minato, and A. Shimaoka, *J. Chem. Soc.* (C), 876 (1967).
2) Location: *Fukushima-ku, Osaka.*
3) R.E. Marker, R.B. Wagner, P.R. Ulshafer, E.L. Wittbecker, D.P.J. Goldsmith, and C.H. Ruof, *J. Am. Chem. Soc.*, **69**, 2211 (1947).

these changes are also readily seen in the *Agave* species, and the sapogenins in the old plants at the flowering period (the *Agave* dies after its flowering period) are consisted of simplified and fewer oxygenated sapogenins, which are discarded with the flowers.

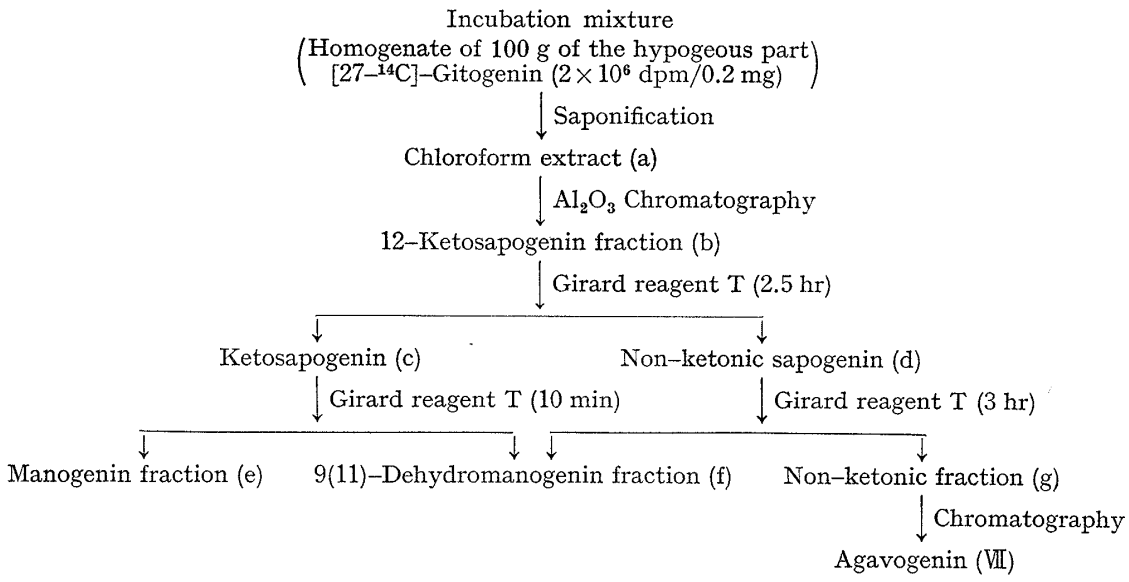
This Marker's observation stimulates our interest and prompts us to investigate the change of the steroidal contents of the *Hosta* species in each season, the steroidal sapogenins of which were already reported[4,5] to be tigogenin (I), hecogenin (II), 9(11)-dehydrohecogenin (III), gitogenin (IV), manogenin (V), and 9(11)-dehydromanogenin (VI). For this purpose, *Hosta kiyosumiensis* F. MAEK was chosen, and the ratio of yield of gitogenin (IV) to that of 12-ketosapogenin (V+VI) in each season was investigated, because the yields of tigogenin (I), hecogenin (II), and 9(11)-dehydrohecogenin (III) are very poor.

TABLE I. Yields of Gitogenin (Ⅳ) and the 12-Ketosapogenin (Ⅴ+Ⅵ) in Each Season

	Epigeous part		Hypogeous part	
	Gitogenin (Ⅳ) (%)	12-Ketosapogenins (Ⅴ+Ⅵ) (%)	Gitogenin (Ⅳ) (%)	12-Ketosapogenins (Ⅴ+Ⅵ) (%)
April (before germination)	—	—	67	33
New sprout (5—10 cm)	93	7	68	32
July (flowering period)	89	11	79	21
Flower	90	10	—	—
August (soon after flowering)	96	4	79	21
December (withering leaves)	99—100	trace	75	25
Seed-vessel	99—100	trace	—	—
Seed	75	25	—	—
February	—	—	76	24

The *Hosta* species is a perennial herb, buds in April, flowers in July, and the epigeous part withers in December. Therefore it is reasonable to suppose that the plant in April is young and the plant in December is an oldest one. As shown in Table I, in the epigeous part the amount of the 12-ketosapogenin gradually decreases from July to December and a fewer oxygenated sapogenin, gitogenin is discarded with the withering leaves and the seed-vessels.

TABLE Ⅱ



4) K. Takeda, T. Okanishi, H. Minato, and A. Shimaoka, *Chem. Pharm. Bull.* (Tokyo), **12**, 779 (1964).
5) K. Takeda, T. Okanishi, H. Minato, and A. Shimaoka, *Tetrahedron*, **21**, 2089 (1965).

This result is in good agreement with the Marker's observation. On the other hand, in the hypogeous part, although there is little change in the amount of the 12-ketosapogenin all the year round, it is maximum in April. This fact thus may be explained by assumption that according to the flowering cycle of the *Hosta* species, the steroidal components also undergo a following change that the 12-ketosapogenin is gradually replaced by gitogenin from July to December in the epigeous part and the amount of the 12-ketosapogenin in the hypogeous part gradually increases from December to April.

However, as the data of the 12-ketosapogenin in the hypogeous part as shown in Table I is insufficient to demonstrate the above-mentioned assumption, we tried by use of C-27-labelled gitogenin to confirm whether gitogenin is oxidized to the 12-ketosapogenin in the hypogeous part in December-April period.

The method[1] using plant homogenate was applied for this purpose. [27-^{14}C] Gitogenin[1] (2×10^6 dpm) was added to the homogenate and incubated.

Table III. Incubation of [27-^{14}C]-Gitogenin with Homogenate

	Hypogeous part		Control	
	Activity (dpm)	Yield (mg)	Activity (dpm)	Yield (mg)
Incubated [27-^{14}C]-gitogenin	2×10^6 (0.2 mg)	—	2×10^6 (0.2 mg)	—
$CHCl_3$ extract (a)	1.63×10^6	820	1.52×10^6	970
12-Ketosapogenin fraction (b)	1.9×10^5	85	6.9×10^4	110
Ketosapogenin (c)	1.4×10^4	28	2.0×10^3	32
Non-ketonic sapogenin (d)	1.73×10^5	51	—	—
Manogenin fraction (e)	4.9×10^3	4	2.7×10^2	5
9(11)-Dehydromanogenin (f)	8.9×10^3	19	1.6×10^3	24
Non-ketonic fraction (g)	1.64×10^5	45	—	—

As shown in Tables II and III, the incubation mixture was saponified and extracted with chloroform to give chloroform extract (a) (1.63×10^6 dpm) as already reported.[1,4] The 12-ketosapogenin fraction (b) (1.9×10^5 dpm) was collected by alumina chromatography. As this fraction (b) is contaminated with non-ketonic sapogenins, it was treated with Girard reagent T in acetic acid-ethanol for 2.5 hr to give the ketosapogenin fraction (c) (1.4×10^4 dpm) and the non-ketonic sapogenin fraction (d) (1.73×10^5 dpm). In order to separate the fraction (c) to manogenin (V) and 9(11)-dehydromanogenin (VI), it was treated with Girard reagent T in ethanol for 10 min to give manogenin fraction (e) (4900 dpm) and 9(11)-dehydromanogenin fraction (f) (6900 dpm).

Since the fraction (d) showed the presence of the 12-ketosapogenin by thin-layer chromatography, it was treated again with Girard reagent T in acetic acid-ethanol for 3 hr to

Table IV. Recrystallization of Manogenin (e) and 9(11)-Dehydromanogenin (f) in Table III

	Manogenin (e)			9(11)-Dehydromanogenin (f)		
	Activity (dpm)	Yield (mg)	Specific activity (dpm/mg)	Activity (dpm)	Yield (mg)	Specific activity (dpm/mg)
First recrystallization	2340	3	780	4160	10.5	396
Second recrystallization	1410	6[a]	(705)[b]	1920	5	384
Third recrystallization	780	3.5	(669)[b]	1510	3.5	430
Fourth recrystallization	230	1	(690)[b]	—	—	—

a) The authentic sample, unlabelled manogenin, 6 mg, was added and the mixture was recrystallized.
b) As the first recrystallization product (3 mg) was diluted with unlabelled manogenin (6 mg) and recrystallized, the value of the measured specific activity from the second recrystallization shown in parentheses has been multiplied by a factor of three.

give the non-ketonic fraction (g) (1.64×10^5 dpm) and 9(11)-dehydromanogenin fraction (2000 dpm) which was combined with the fraction (f).

The converted labelled sapogenin was recrystallized with an unlabelled authentic sample until it showed a constant specific activity. Results are shown in Table IV, and the radioactive manogenin isolated showed constant specific activity, 700 dpm /mg, and the radioactive 9(11)-dehydromanogenin isolated showed 390 dpm/mg activity. The incorporation ratios of these biogenetic conversions are 0.17 and 0.45%, respectively. (As a control, homogenate was heated at 100° for 20 min before the incubation.)

Furthermore, the non-ketonic fraction (g) (45 mg) had still an activity of 1.64×10^5 dpm, and showed the presence of two radioactive unknown compounds by radioactivity scanning of the paper chromatogram. This fraction was therefore chromatographed on alumina and then subjected to the preparative thin-layer chromatography to give unknown compounds (VII), 1 mg, 650 dpm and (VIII), 0.1 mg, 1620 dpm.

Table V. Chromatograms of Compounds (Ⅶ) and (Ⅷ)

	Rf Value of compound (Ⅶ)	*Rf* Value of agavogenin	*Rf* Value of compound (Ⅷ)
Paper chromatogram			
Xylene–$CHCl_3$–EtOAc–HOAc (150:50:20:4)	0.30	0.30	0.18
Toluene–HOAc (50:3)	0.28	0.28	0.20
Thin-layer chromatogram			
$CHCl_3$–Acetone–EtOH (15:5:1.5)	0.24	0.24	0.20
n-BuOH–Acetone (3:1)	0.32	0.32	0.26
n-BuOH–Acetone–EtOH (10:5:1.5)	0.15	0.15	0.13

Table Ⅵ. Chromatograms of Acetates of Compounds (Ⅶ) and (Ⅷ)

	Rf Value of acetate of (Ⅶ)	*Rf* Value of agavogenin triacetate	*Rf* Value of acetate of (Ⅷ)
Paper chromatogram			
Heptane–$CHCl_3$ (200:5)	0.62	0.62	0.42
Thin-layer chromatogram			
n-BuOH–Et_2O (2:1)	0.63	0.63	0.49
n-BuOH–EtOAc (2:1)	0.42	0.42	0.32

As shown in Table V and VI, according to *Rf* values of the genins and their acetates, compound (VII) was identified with agavogenin and compound (VIII) was assumed to be a tetrahydroxy sapogenin.

From these results, it was clarified that gitogenin (IV) is transformed into manogenin (V), 9(11)-dehydromanogenin (VI), and agavogenin (VII) in the hypogeous part of *H. kiyosumiensis* F. Maek in the winter season, and the tetraol (VIII) may intervene between this transformation.

Experimental

Incubation of [27-^{14}C]-Gitogenin with Homogenate of the Hypogeous Part (see Tables II, III, and IV)—— The hypogeous part (100 g) of the plant in February was homogenized with 1/15 M phosphate buffer (200 ml, pH 6.5) under nitrogen at 0—3° in an ice-bath for 5 min by use of "Satake-Homogeniser" (3000 rpm). [27-^{14}C]-Gitogenin[1] (0.2 mg, 2×10^6 dpm) was incubated with the above-mentioned homogenate at 37° for 6 hr using a "Sakaguchi's shaking flask" fitted with cotton stopper, with gentle shaking (110—120 times/min). The incubation mixture was hydrolyzed with a solution of H_2SO_4 (25 g) in MeOH (200 ml) and H_2O (100 ml) followed by saponification with 5% NaOH–MeOH (100 ml) and extracted with $CHCl_3$ to give $CHCl_3$ extract (a) (820 mg, 1.63×10^6 dpm). The extract (a) was chromatographed twice on alumina to give the 12-

ketosapogenin fraction (b) (85 mg, 1.9×10^5 dpm), which was dissolved in a solution of Girard reagent T (250 mg) in EtOH (10 ml) and AcOH (10 ml) and refluxed for 2.5 hr, to give the ketosapogenin fraction (c), a crystalline product (28 mg, 1.4×10^4 dpm), and non-ketonic sapogenin fraction (d) (51 mg, 1.73×10^5 dpm).

Fraction (d) was dissolved in a solution of Girard reagent T (100 mg) in EtOH (10 ml) and refluxed for 10 min, to give manogenin fraction (e), colorless needles, mp 240—243° (4 mg, 4900 dpm), and 9(11)-dehydromanogenin fraction (f), colorless prisms, mp 235—238° (14 mg, 6900 dpm).

Fraction (d) was dissolved in a solution of Girard reagent T (200 mg) in EtOH (10 ml) and AcOH (10 ml) and refluxed for 3 hr, to give the non-ketonic fraction (g) (45 mg, 1.64×10^5 dpm) and 9(11)-dehydromanogenin fraction (5 mg, 2000 dpm), which was combined with the fraction (f).

Manogenin (e) was recrystallized four times from MeOH with the unlabelled manogenin (V) (6 mg) to give radioactive manogenin, which has the constant specific activity, 700 dpm/mg, as shown in Table IV.

9(11)-Dehydromanogenin (f) was recrystallized three times from MeOH–AcOEt to give radioactive 9(11)-dehydromanogenin, which has the constant specific activity, 390 dpm/mg as shown in Table IV.

Fraction (g) was chromatographed on alumina to give a crystalline product having lower *Rf* values than that of gitogenin (IV) (12 mg, 1.2×10^4 dpm), which was separated into compound (VII) (1 mg, 650 dpm), compound (VIII) (0.1 mg, 1620 dpm), and 12-ketosapogenins (8.5 mg) by preparative thin-layer chromatography. Compound (VII) was identified with agavogenin and compound (VIII) was assumed to be a tetrahydroxy sapogenin as shown in Tables V and VI.

Control Test of Incubation of [27-^{14}C]-Gitogenin with Homogenate of the Hypogeous Part (see Tables II and III)——The homogenate of the hypogeous part (100 g) of the plant in February was heated at 100° for 20 min, and [27-^{14}C]-gitogenin (0.2 mg, 2×10^6 dpm) was incubated with this homogenate as described above. The result was shown in Table III, and the 12-ketosapogenin obtained was radio-inactive.