UDC 547.918 : 582.572.7

113. Toshio Kawasaki and Tatsuo Yamauchi : Structures of Dioscin, Gracillin, and Kikuba-saponin. (2).[1] (Saponins of Japanese Dioscoreaceae. XI.[*1])

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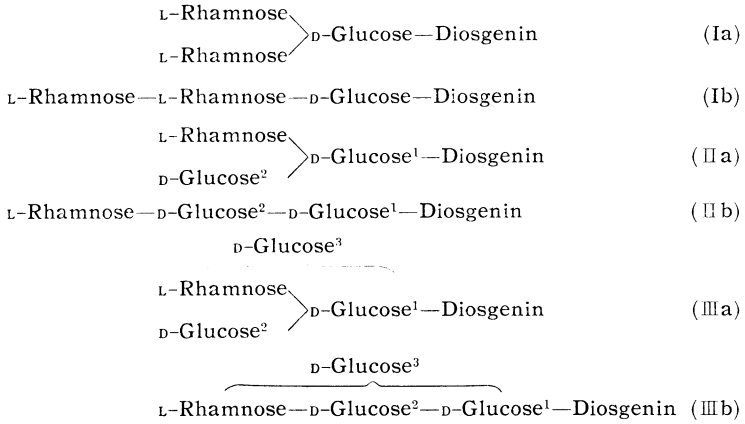
In the previous papers[1] of this series, the authors reported the isolation of three steroid saponins, dioscin, gracillin and kikuba-saponin, from the rhizomes of Japanese Dioscoreaceae plants, and assigned them partial structures (Ia) or (Ib), (IIa) or (IIb) and (IIIa) or (IIIb), respectively. Taking into account the products from partial hydrolyses of these saponins, it was suggested that dioscin should have a branched-chain trisaccharide moiety (Ia), while gracillin should have linear structure (IIb) and kikuba-saponin structure (IIIb).



The authors would now like to report the experimental results which led to the establishment of branched-chain structures of the sugar moieties of these three saponins.

Dioscin, on permethylation followed by acid hydrolysis, afforded diosgenin and a mixture of methylated sugars, the latter of which could be fractionated into only one kind of methylated rhamnose and a methylated glucose. They were purified by distillation or recrystallization, and identified, respectively, as 2,3,4-tri-O-methyl-L-rhamnose and 3,6-di-O-methyl-D-glucose by direct comparisons with authentic specimens.[*3] Therefore it is clear that dioscin has, as its sugar moiety, a branched-chain trisaccharide, bis-L-rhamnopyranosyl(1→2 and 1→4)-D-glucopyranose. Analysis of the molecular rotation (M_D) differences of diosgenin, dioscin and its two prosapogenins (D_A and D_C) and the fact that D_C (diosgenin D-glucoside[1a]) was hydrolyzed with emulsin to give diosgenin and D-glucose indicate that D_C is the β-D-glucoside and one rhamnose residue is attached to the glucose moiety of D_C with an α-linkage to form D_A (diosgenin L-rhamnosido-

*1 This paper forms part of the series, "Takeo Tsukamoto : Saponins of Japanese Dioscoreaceae." Part X : Ref. 1c).

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*3 3,6-di-O-methyl-D-glucose and 4,6-di-O-methyl-D-glucose were kindly furnished by Prof. Dr. R. Kuhn.

1) Part (1). *a*) Part V : T. Tsukamoto, T. Kawasaki, T. Yamauchi : This Bulletin, **4**, 35 (1956). *b*) Part VI : T. Tsukamoto, T. Kawasaki : *Ibid.*, **4**, 104 (1956). *c*) Part X : T. Kawasaki, T. Yamauchi, R. Yamauchi : *Ibid.*, **10**, 698 (1962).

D-glucoside[1a]). Although the M_D difference of D_A and dioscin failed to provide any evidence with regard to the mode of attachment of the second rhamnose residue to D_A to form dioscin, (see Klyne rule[2]) on the cardiac glycosides), it was also presumed to be α-linked, and consequently dioscin is formulated as diosgenin bis-α-L-rhamnopyranosyl-(1→2 and 1→4)-β-D-glucopyranoside (IV).

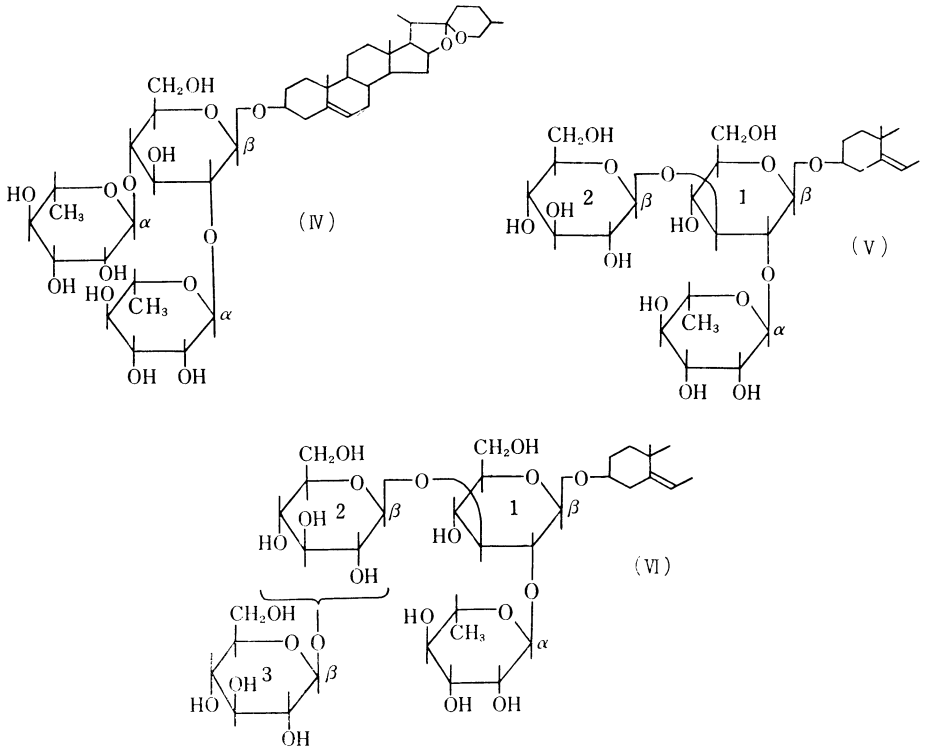

Chart 1.

Fully methylated gracillin gave on hydrolysis three kinds of methylated sugars which were identified by direct comparisons with the authentic samples[*3] as 2,3,4-trimethyl-L-rhamnose, 2,3,4,6-tetra-O-methyl-D-glucose and 4,6-di-O-methyl-D-glucose, respectively. Therefore a branched-chain structure of the trisaccharide moiety of gracillin has been verified. When prosapogenin A of gracillin (G_A) (diosgenin D-glucosido-D-glucoside[1b]), dioscin (IV) and D_C, for the sake of comparison, were oxidized with periodate, they consumed 2.3, 4.2, and 2.3 moles of periodate, respectively, indicating that G_A has two free α-glycol groupings. Furthermore, upon oxidation followed by acid hydrolysis, both G_A and dioscin gave the products which were found by paper chromatography to contain D-glucose, whereas D_A (diosgenin L-rhamnopyranosyl(1→2 or 1→4)-glucopyranoside) and D_C afforded no glucose. Hence it is obvious that the second glucose unit (Glc.^{2}) in G_A is attached at the 3-hydroxyl group of the first glucose (Glc.^{1}) bound to the aglycone. Thus, it was concluded that gracillin has structure (V), viz. diosgenin α-L-rhamnopyranosyl(1→$2_{Glc.1}$)-β-D-glucopyranosyl(1→$3_{Glc.1}$)-β-D-glucopyranoside.

Since the structure of gracillin has now been established as (V), kikuba-saponin should have a branched-chain tetrasaccharide moiety such as (IIIa) and be represented by either one of three partial formulae (IIIc), (IIId), and (IIIe). When kikuba-saponin

2) W. Klyne : Biochem. J., **47**, xli (1950).

L-Rhamnose
|
D-Glucose[3]—D-Glucose[2]—D-Glucose[1]—Diosgenin (IIIc)

D-Glucose[3]—L-Rhamnose
|
D-Glucose[2]—D-Glucose[1]—Diosgenin (IIId)

L-Rhamnose
|
D-Glucose[2]—D-Glucose[1]—Diosgenin (IIIe)
|
D-Glucose[3]

permethylate was hydrolyzed, it provided four kinds of methylated sugar. Two of them, isolated as crystals, were identified by direct comparison with authentic samples as 4,6-di-o-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose and the other two were characterized by paper chromatography as 2,3,4-tri-O-methyl-L-rhamnose and a tri-O-methyl-D-glucose. The above results indicate kikuba-saponin to have structure (IIIc) in which the third glucose unit (Glc.[3]) is bound with a β-linkage[1c] to the second glucose residue(Glc.[2]) of gracillin. Although, because of insufficient material at hand the authors could not identify the tri-O-methyl-D-glucose and determine the position at which Glc.[3] is linked to Glc.[2], it is suggested that kikuba-saponin should be represented by the formula (VI), viz. diosgenin β-D-glucopyranosyl(1→$X_{Glc.2}$)-β-D-glucopyranosyl(1→$3_{Glc.1}$)-α-L-rhamnopy-ranosyl(1→$2_{Glc.1}$)-β-D-glucopyranoside.

The structural similarity in sugar moiety as well as aglycone between these saponins and some solanum alkaloids[3] is also noted.

Experimental*4

Permethylation of Dioscin——Dioscin[1a] (3 g.) was methylated with Purdie's reagent[4] and the product (partially methylated dioscin, 2.5 g.) was shaken in dimethylformamide (50 cc.) with Ag_2O (15 g.) and CH_3I (15 cc.) at room temperature for 47 hr. The precipitates were filtered off and the filtrate was shaken again with additional Ag_2O (8 g.) and CH_3I (8 cc.) for 20 hr. After the reaction mixture was worked up by the method of Kuhn, Löw and Trischmann,[3] the crude permethylate was obtained as a yellow resinous oil (2.27 g.). Chromatography on alumina (Brockmann, 23 g.) and elution with benzene furnished dioscin permethylate (1.64 g.), which was crystallized from hydr. MeOH to give an amorphous white powder, m.p. 130~135°. *Anal.* Calcd. for $C_{53}H_{88}O_{16}$ (dioscin octamethylate) : C, 64.87; H, 9.04; OCH_3, 25.2. Found : C, 64.94; H, 8.70; OCH_3, 25.1. Almost no hydroxyl group absorption was observed in the infrared spectrum.

Hydrolysis of Dioscin Permethylate——The above permethylate (1.34 g.) was refluxed with 40 cc. of 5% HCl in MeOH for 5 hr., the solvent evaporated *in vacuo* and the residue was heated again with 40 cc. of *N* HCl on a water bath for 3 hr. The hydrolyzate was extracted with Et_2O (furnishing 550 mg. of diosgenin and $\Delta^{3,5}$-deoxytigogenin[5]), then with $CHCl_3$ (affording 200 mg. of a syrup; Rf: 0.86 (g)) and the H_2O layer was neutralized with Ag_2CO_3 followed by treatment with H_2S and evaporated *in vacuo* to give a syrupy residue (280 mg.; Rf : 0.48 (b)), 0.86 (g)). The substance obtained from the H_2O layer was dissolved in a small amount of H_2O, placed on a column of active carbon-Celite (1:1) and eluted with 5% and 10% EtOH successively. Fr.1 (5% EtOH, 50 cc.) : 40 mg. ; Rf : 0.28 (b)±, 0.48 (b)+, 0.79 (b)±, Fr.2 (5% EtOH, 50 cc.) : 110 mg.; Rf : 0.47 (b)+, 0.79 (b)±, Fr. 3 (10% EtOH, 300 cc.) : 60 mg.; Rf : 0.88 (g).

2,3,4-Tri-O-methyl-L-rhamnose : The syrup (200 mg.) from the $CHCl_3$ layer and Fr. 3 (60 mg.) were

*4 All melting points were taken on a Kofler block and are uncorrected. Paper chromatographic examinations of methylated sugars were carried out with Tōyō Roshi No. 50 using BuOH-EtOH-H_2O (5:1:4) as a developing solvent (ascending, 17 hr., at 10~25°). Letters in the parentheses next to the Rf values represent colors with aniline hydrogenphthalate : b, brown; g, green. A relative amount of each substance detected on the chromatogram was represented as +, ±, by visual comparison of the intensity and the area of the spot.

3) R. Kuhn, I. Löw, H. Trischmann : Chem. Ber., **88**, 1492, 1690 (1955).
4) T. Purdie, J.C. Irvine : J. Chem. Soc., **1903**, 1021.
5) T. Tsukamoto, T. Kawasaki, T. Yamauchi, Y. Shimauchi : This Bulletin, **5**, 492 (1957); T. Yamauchi : *Ibid.*, **7**, 343 (1959).

combined and purified by distillation to give a colorless syrup (150 mg.), b.p$_4$ 140～150° (bath temp.), $[\alpha]_D^{15}$ +27° (c=0.30, H_2O) (reported for 2,3,4-tri-O-methyl-L-rhamnose,[6] $[\alpha]_D^{21}$ +27° (H_2O)), n_D^{15} 1.4561. *Anal.* Calcd. for $C_9H_{18}O_5$ (2,3,4-tri-O-methyl-L-rhamnose): C, 52.41; H, 8.80; OCH_3, 45.2. Found: C, 52.35; H, 9.08; OCH_3, 45.8.

This compound (130 mg.) in dehyd. EtOH (2 cc.) was refluxed with aniline (100 mg.) and NH_4Cl (a few mg.) for 3 hr. The EtOH was distilled off, H_2O added, and an insoluble substance collected by filtration was sublimed *in vacuo* and recrystallized from petr. ether to colorless fine needles, m.p. 110～113°, $[\alpha]_D^{13}$ +139° (c=0.66, EtOH). *Anal.* Calcd. for $C_{15}H_{23}O_4N$ (2,3,4-tri-O-methyl-L-rhamnose anilide): C, 64.03; H, 8.24. Found: C, 63.81; H, 8.20. Mixed melting point with synthetic 2,3,4-tri-O-methyl-L-rhamnose anilide (m.p. 113°) showed no depression.

3,6-Di-O-methyl-D-glucose: Fr. 2 (110 mg.) was further fractionated by chromatography on a cellulose powder column using BuOH-ligroin (b.p. 80～100°) (2:3) saturated with H_2O as a developing solvent. Fr. 1' (140 cc.): 60 mg., Rf: 0.48 (b)+, 0.70 (b)±. Fr. 2' (340 cc.): 30 mg., Rf: 0.47 (b). The latter was crystallized from AcOEt to colorless prisms, m.p. 113～116°, $[\alpha]_D^{15}$ +62 (c=0.30, H_2O)*5 (reported for 3,6-di-O-methyl-D-glucose,[7] m.p. 113～116°, $[\alpha]_D$ +102→+61.5°). *Anal.* Calcd. for $C_8H_{16}O_2$ (3,6-di-O-methyl-D-glucose): C, 46.15; H, 7.75. Found: C, 46.22; H, 7.99. On admixture with authentic 3,6-di-O-methyl-D-glucose*3 (m.p. 112～117°) no melting point depression was observed.

Analysis of the Molecular Rotation Differences——See Table I.

TABLE I. Molecular Rotation Differences

	$[\alpha]_D$	M_D	Δ		$[\alpha]_D$	M_D	Δ
Diosgenin	−111° (dioxane)	−460°	−65°	$G_C=D_C$	−91° (dioxane)	−525	−303°
D_C	−91° (〃)	−525°	−596°	G_A	−112° (pyridine)	−828	+49°
D_A	−155° (〃)	−1121°	+43°	Gracillin	−88° (〃)	−779	−6°
Dioscin	−124° (〃)	−1078°		Kikubasaponin	−75° (MeOH)	−785	
α-methyl-D-glucopyranoside		M_D +309°		α-methyl-L-rhamnopyranoside		M_D −111	
β-methyl-D-glucopyranoside		M_D −66°		β-methyl-L-rhamnopyranoside		M_D +170	

Hydrolysis of Prosapogenin C of Dioscin (Dc) (diosgenin glucoside) with Emulsin——A mixture of D_C[1a] (10 mg.) in EtOH (10 cc.) and emulsin*6 (100 mg.) in H_2O (20 cc.) was allowed to stand for 7 days at 20～25°. The EtOH was removed *in vacuo*, H_2O added and an insoluble substance was collected by filtration, dried, extracted with $CHCl_3$-MeOH (1:1) and the extract was examined by paper chromatography. Rf: 0.32, 0 (diosgenin: 0.32, D_C: 0); solvent: petr. ether-toluene-EtOH-H_2O (40:5:1:9).[1a] The H_2O layer was found to contain D-glucose [Rf: 0.21; solvent: BuOH-AcOH-H_2O (4:1:5)].

Permethylation of Gracillin——Gracillin[1b] (5 g.) in DMF (100 cc.) was shaken with Ag_2O (35 g.) and CH_3I (35 cc.) for 70 hr. After the precipitates were filtered off, the filtrate was shaken with additional Ag_2O (25 g.) and CH_3I (23 cc.) for 50 hr. and again filtered. Further shaking of the filtrate with Ag_2O (20 g.) and CH_3I (20 cc.) for 26 hr. followed by treatment according to the Kuhn, *et al.*'s procedure gave crude gracillin permethylate (5.65 g.). Chromatography on alumina using benzene as a solvent followed by crystallization from hydr. MeOH afforded the pure permethylate as an amorphous white powder, m.p. 75～85°, $[\alpha]_D^{12}$ −54° (c=0.67, MeOH). *Anal.* Calcd. for $C_{54}H_{90}O_{17}$ (gracillin nonamethylate): C, 64.13; H, 8.97; OCH_3, 27.6. Found: C, 64.03; H, 9.16; OCH_3, 27.4.

Hydrolysis of Gracillin Permethylate——Gracillin permethylate (3.7 g.) was hydrolyzed with HCl and worked up in the similar way as in the hydrolysis of dioscin permethylate.

(1) $CHCl_3$ extract: 440 mg.; Rf: 0.82 (b+g)

(2) Substance from H_2O-layer: 880 mg.; Rf: 0.43 (b)+, 0.67 (b)±, 0.86 (b+g)+

(1) The sample (440 mg.) was dissolved in a small amount of H_2O, placed on a column of active carbon-Celite (1:1) and eluted with 5% MeCOEt in H_2O. Fr. 1 (90 cc.): 130 mg.; Rf: 0.88 (b+g). Fr. 2 (120 cc.): 60 mg.; Rf: 0.86 (b). Fr. 1 was rechromatographed in a similar manner and two fractions were obtained. Fr. 1' (45 cc.): 30 mg.; Rf: 0.87 (g). Fr. 2' (100 cc.): 60 mg.; Rf: 0.86 (b).

(2) The sample (880 mg.) was submitted to the chromatography on a cellulose powder using BuOH-ligroin (b.p. 80～100°) (2:3) saturated with H_2O as a solvent, and each fraction was examined by paper chromatography. Fr. 1 (100 cc.): 490 mg.; Rf: 0.86 (b+g). Fr. 2 (100 cc.): 180 mg.; Rf: 0.85 (b+g)+, 0.72 (b)±. Fr. 3 (50 cc.): none. Fr. 4 (300 cc.): 130 mg.; Rf: 0.46 (b). Fr. 1 (490 mg.) was

*5 Measured after standing for 2 hr.

*6 Prepared from apricot seeds (*Prunus Armeniaca* LINN. var. *Ansu* MAXIM.) according to the B. Helferich's procedure.[8]

6) W. N. Haworth, E. L. Hirst, E. J. Miller: J. Chem. Soc., **1929**, 2469.

7) D. J. Bell: J. Chem. Soc., **1935**, 175.

8) B. Helferich, *et al.*: Z. physiol. Chem., **208**, 91 (1932); **209**, 369 (1932); **215**, 277 (1933).

rechromatographed on an active carbon-Celite (1:1) column and eluted with 5% MeCOEt in H_2O. Fr. 1′(100 cc.) : 250 mg.; Rf : 0.86(g). Fr. 2′(250 cc.) : 50 mg.; Rf : 0.85(b).

2,3,4-Tri-O-methyl-L-rhamnose : Frs. 1′ from both (1) and (2) were combined (280 mg.) and purified by distillation to give a colorless syrup (150 mg.), b.p_2 140～150°(bath temp.), $[\alpha]_D^{18}$ +28°(c=0.63, H_2O), n_D^{19} 1.4589. *Anal.* Calcd. for $C_9H_{18}O_5$(2,3,4-tri-O-methyl-L-rhamnose) : OCH_3, 45.1. Found : OCH_3. 44.6.

The anilide of this compound was prepared in the same way as described before; m.p. 110°, $[\alpha]_D^{15}$ +137°(c=0.51, EtOH). *Anal.* Calcd. for $C_{15}H_{23}O_4N$ (2,3,4-tri-O-methyl-L-rhamnose anilide) : C, 64.03; H, 8.24. Found : C, 63.92; H, 8.43. Mixed melting point with an authentic specimen (m.p. 113°) showed no depression.

2,3,4,6-Tetra-o-methyl-D-glucose : Fr. 2 from (1) and Frs. 2′ from both (1) and (2) were combined (170 mg.) and crystallized from petr. ether to colorless needles, m.p. 90～92°. On admixture with synthetic 2,3,4,6-tetra-O-methyl-D-glucose (m.p. 90～93°) no melting point depression was observed. Anilide (prepared as described before) : fine needles (from petr. ether), m.p. 125～128°, $[\alpha]_D^{13}$ +227°(c=0.43, Me_2CO). *Anal.* Calcd. for $C_{16}H_{25}O_5N$ (2,3,4,6-tetra-O-methyl-D-glucose anilide) : C, 61.71; H, 8.09. Found : C, 61.86; H, 8.20. On admixture with the authentic anilide (m.p. 130°) no melting point depression was observed.

4,6-Di-O-methyl-D-glucese : Fr. 4 from (2) was crystallized from AcOEt to fine needles, m.p. 150～157°, $[\alpha]_D^{30}$ +103°→+62.6°(c=0.99, H_2O) (reported for 4,6-di-O-methyl-D-glucose,[9] m.p. 156～157°, $[\alpha]_D$ +108°→+66°(H_2O)). *Anal.* Calcd. for $C_8H_{16}O_2$(4,6-di-O-methyl-D-glucose) : C, 46.15; H, 7.75. Found: C, 46.09; H, 8.01. Mixed melting point with authentic 4,6-di-O-methyl-D-glucose*3 (m.p. 156～160°) showed no depression.

Periodate Oxidation of Prosapogenin A of Gracillin (G_A) (diosgenin-D-glucosido-D-glucoside) and Some Reference Compounds——A sample (G_A,[1b] dioscin, D_C) (13～16 mg.) in EtOH (15 cc.) was oxidized with 15.0 cc. of $NaIO_4$ solution in 0.05% AcOH ($NaIO_4$, 36.9 mg.) at 25～30° for 24 hr. and the remaining IO_4^- was titrated by iodmetry using 0.1*N* $NaAsO_2$ and 0.1*N* I_2.

G_A, dioscin and D_C were found to consume 2.3, 4.2 and 2.3 moles of $NaIO_4$, respectively (Calcd. for dioscin, 4 moles; for D_C, 2 moles).

In another run G_A, dioscin, D_C and prosapogenin A of dioscin (D_A) [diosgenin L-rhamnopyranosyl (1→2 or 1→4) D-glucopyranoside][1a] were independently oxidized as described above for 90 hr. The reaction mixture was diluted with H_2O and extracted with BuOH. The BuOH extract was then refluxed with 2*N* HCl in 50% EtOH (3 cc.) for 2 hr. and worked up in the usual manner. Sugars in the hydrolyzate were examined by paper chromatography. Both products from G_A and dioscin were found to contain D-glucose (Rf : 0.18, BuOH-AcOH-H_2O (4:1:5)), while no sugar was detected in the products from D_C and D_A.

Permethylation of Kikuba-saponin——Kikuba-saponin[1c] (300 mg.) was methylated and worked up in the same way as in the case of gracillin to give the crude permethylate (350 mg.) as a syrup. Chromatography on alumina using benzene as a solvent followed by crystallization from hydr. MeOH furnished kikuba-saponin permethylate (270 mg.) as a white powder, m.p. 50～60°, $[\alpha]_D^{10}$ −10°(c=0.56, $CHCl_3$), No absorption was found near 3500 cm^{-1} in the infrared spectrum.

Hydrolysis of Kikuba-saponin Permethylate——The permethylate (250 mg.) was hydrolyzed with HCl and worked up in the same manner as in the case of dioscin permethylate. (1) $CHCl_3$ extract: 30 mg.; Rf : 0.87(b+g). (2) material from H_2O layer : 30 mg.; Rf : 0.87(b+g), 0.71(b), 0.46(b). The sample from (1) was dissolved in a small amount of H_2O, placed on a column of active carbon-Celite(1:1)and eluted with 5% MeCOEt. Fr. 1 (10 cc.), Rf : 0.83(g) (2,3,4-tri-O-methyl-L-rhamnose, 0.83 (g)); Fr. 2 (10 cc.), Rf : 0.83(b+g); Fr. 3 (25 cc.), Rf : 0.83 (b) [2,3,4,6-tetra-O-methyl-D-glucose, 0.83 (b)]. Fr. 3 was crystallized from petr. ether to colorless fine needles, m.p. 90～94°. Mixed melting point with authentic 2,3,4,6-tetra-O-methyl-D-glucose (m.p. 90～94°) gave no depression.

The sample from (2) was chromatographed on active carbon-Celite (1:1). Fr. 1 (5% EtOH, 100 cc.), Rf : 0.46(b)+ [4,6-di-O-methyl-D-glucose, 0.46(b)], 0.68(b)± [Tri-O-methyl-D-glucose, 0,68(b)]; Fr. 2 (10% EtOH, 100 cc.), Rf : 0.67 (b)+, 0.85(b)+ [2,3,4,6-tetra-O-methyl-D-glucose, 0.84(b)]. Fr. 1 was crystallized from AcOEt to give colorless fine needles, m.p. 156～159°. Mixed melting point with authentic 4,6-di-O-methyl-D-glucose*3 showed no depression. On the basis of its Rf value and color reaction the substance of Rf 0.67～0.68 was regarded as a tri-O-methyl-D-glucose.

The authors are very grateful to Prof. Dr. T. Tsukamoto for his helpful interest in this investigation and to Prof. Dr. R. Kuhn of Max-Planck Institute, Heidelberg, for his kindness in providing us with the comparison samples. Thanks are also due to the members of the Central Analysis Room of this University for the analytical data and to Mr. H. Matsui for the infrared spectra.

9) D. J. Bell, J. Lorbell : J. Chem. Soc., **1940**, 453.

Summary

Three steroid saponins from japanese Dioscoreaceae plants, dioscin, gracillin and kikuba-saponin were proved to have branched-chain oligosaccharide moieties, and assigned structures, diosgenin bis-α-L-rhamnopyranosyl(1→2 and 1→4)-β-D-glucopyranoside (IV), diosgenin α-L-rhamnopyranosyl(1→$2_{Glc.1}$)-β-D-glucopyranosyl(1→$3_{Glc.1}$)-β-D-glucopyranoside (V) and diosgenin β-D-glucopyranosyl(1→$X_{Glc.2}$)-β-D-glucopyranosyl(1→$3_{Glc.1}$)-α-L-rhamnopyranosyl(1→$2_{Glc.1}$)-β-D-glucopyranoside (VI), respectively.

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114. Satoshi Toki, Keiko Toki, and Hisao Tsukamoto : Metabolism of Drugs. XXXI.[1] Metabolic Fate of Methylhexabital (5-Cyclohexenyl-3,5-dimethylbarbituric Acid). (10).[2] Enzymatic Oxidation of Methylhexabital.

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In the enzymatic study of methylhexabital (MHB, 5-cyclohexenyl-3,5-dimethylbarbituric acid), Cooper and Brodie[3] found out that the enzyme in the rabbit liver which catalyzed an oxidation of the cyclohexenyl ring to yield keto-MHB was located in the microsomes of liver cells and required both oxygene and TPNH. Under a similar experimental condition, Tochino[4] observed that hydroxy-MHB was produced in the rabbit liver. Very regrettable, however, was the fact that in both cases unchanged MHB was extracted and assayed by the method of Brodie, *et al.*[5] and the enzyme activity was determined only with the value of disappeared MHB without regard to the metabolites themselves. In spite of the fact that while MHB disappeared, its metabolite are produced, as shown below, Cooper and Brodie[3] discussed only about the enzymatic mechanism of formation of keto-MHB, or only about the hydroxylation of MHB by Tochino.[4]

Previous studies in this laboratory showed that two diastereoisomeric α- and β-3-OH-MHB (α- and β-5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid), 3-keto-MHB(5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid) and three additional metabolites were excreted in the urine of rabbits administered MHB,[6,7] and that 3-OH-MHB and 3-keto-MHB were interconvertible *in vivo*.[2] Furthermore, it was confirmed that 3-OH-MHB and 3-keto-MHB were hardly separable on the paper chromatogram by the solvent system which those workers adopted since both compounds gave similar Rf

*1 Katakasu, Fukuoka (土岐 智, 土岐圭子, 塚元久雄).

1) Part XXX : M. Yoshimura, H. Tsukamoto : This Bulletion, (in press).
2) H. Tsukamoto, S. Toki, K. Kaneda : *Ibid.*, **7**, 651 (1959).
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