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112. Toshio Kawasaki, Tatsuo Yamauchi, and Ryoko Yamauchi: Kikuba-saponin. (1). (Saponins of Japanese Dioscoreaceae. X.*1)

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In the previous paper[1] of this series it was reported that a new water soluble steroid saponin, named kikuba-saponin, was obtained from the air-dried rhizomes of *Dioscorea septemloba* THUNB, as an amorphous powder, which was considerably more polar than dioscin[2] and gracillin[3] [$R_{dioscin}$ 0.53, $R_{gracillin}$ 0.55; butanol ethanol-water (5:1:4)] and consisted of diosgenin, glucose and rhamnose.

The present paper describes the isolation of pure crystalline kikuba-saponin (K) and also its structure elucidation.

The improved method for the isolation of (K) from the air-dried rhizome is shown in Chart 1. The water-soluble portion of the defatted methanol extract was treated with water-saturated butanol and the saponin which was transferred to the organic phase was precipitated with acetone and purified according to the Rothman, Wall, and Walens's procedure.[4] The amorphous water-soluble substance thus obtained was subsequently chromatographed on alumina and the fraction (K) which seemed to be homogeneous (examined by paper chromatography) was repeatedly recrystallized from methanol to colorless fine needles, m.p. 249~251° (decomp.), $[\alpha]_D^{17}$ −77°. Crystalline (K) was hardly soluble*3 in water but gave the same Rf value as amorphous (K)[1] when run in parallel on a paper chromatogram. It had a sharp melting point and was paper chromatographically pure, and gave, on acid hydrolysis, diosgenin, $\Delta^{3,5}$-deoxytigogenin,[1,5] glucose and rhamnose without any contaminants.*4 It could be acetylated to the peracetate, m.p. 158°, $[\alpha]_D^{15}$ −51°, which, upon saponification, gave back the original (K), m.p. 249~251° (decomp.), $[\alpha]_D^{17}$ −75°. Therefore crystalline (K) was regarded as a pure saponin.

Quantitative analyses of the hydrolytic products were then undertaken and it was found that under the condition of complete hydrolysis determined by a preliminary experiment (Table I), (K) was cleaved to afford diosgenin (and $\Delta^{3,5}$-deoxytigogenin) and sugar in 39.5 and 63.4% yields, respectively, and the molar ratio of glucose and rhamnose in the hydrolysate was 3.2 to 1. Therefore, (K) has the molecular formula $C_{51}H_{82}O_{22}$ consisting of one mole each of diosgenin and rhamnose and three moles of glucose. The analytical date of (K) and its acetate also supported this formulation (Table II).

A mild acid hydrolysis of (K) provided three prosapogenins and their Rf values agreed, respectively, with those of gracillin and its two prosapogenins.[3] Furthermore, upon enzymatic hydrolysis with emulsin, (K) yielded glucose and only one prosapogenin whose Rf value was identical with that of gracillin. Accordingly, (K) was presumed to

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*1 This paper forms part of the series, "Takeo Tsukamoto: Saponins of Japanese Dioscoreaceae." Part IX: Ref. 5b).

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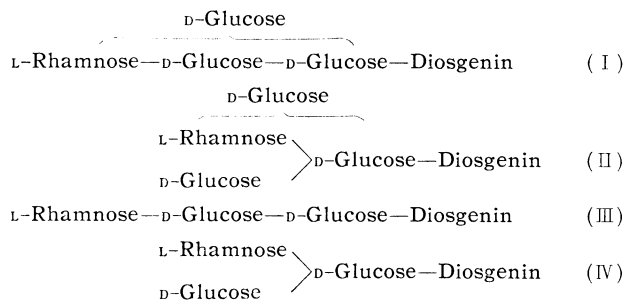
*3 Amorphous (K) was soluble in water.

*4 Amorphous (K) was hydrolyzed to give additional three unknown substances as the non-sugar product.[1]

1) T. Tsukamoto, T. Kawasaki, Y. Shimauchi: Yakugaku Zasshi, **77**, 1221 (1957).
2) T. Tsukamoto, T. Kawasaki, T. Yamauchi: This Bulletin, **4**, 35 (1956).
3) T. Tsukamoto, T. Kawasaki: *Ibid.*, **4**, 104 (1956).
4) E.S. Rothman, M.E. Wall, H.A. Walens: J. Am. Chem. Soc., **74**, 5791 (1952).
5) *a*) T. Tsukamoto, T. Kawasaki, T. Yamauchi, Y. Shimauchi: This Bulletin, **5**, 492 (1957).
b) T. Yamauchi: *Ibid.*, **7**, 343 (1959).

be a diosgenin glycoside in which additional one mole of glucose is attached to gracillin with a β-linkage. This assumption was confirmed by the quantitative determination of glucose in the enzymatic hydrolyzate and by the isolation and identification with authentic gracillin of the prosapogenin. Consequently, (K) is assigned structure (I) or (II), because gracillin is either (III) or (IV).[3]



In constrast to the experiment with air-dried rhizomes, when a frozen fresh material was used, the yield of the water-insoluble saponin (gracillin+dioscin) was markedly decreased (0.03%; from air-dried material, 0.82%), while that of a water-soluble amorphous saponin much increased (ca. 0.16%; from air-dried material ca. 0.04%). This water-soluble saponin when hydrolyzed with emulsin gave gracillin, dioscin and glucose and this fact indicates that it is a mixture of mother saponins of gracillin and dioscin having another mole (s) of glucose. Although attempts to isolate the dioscin-glycoside from the above mixture were unsuccessful, the gracillin-glycoside was obtained as fine crystals and identified as (K). Therefore (K) might be considered as a parent saponin of gracillin.

Experimental*5

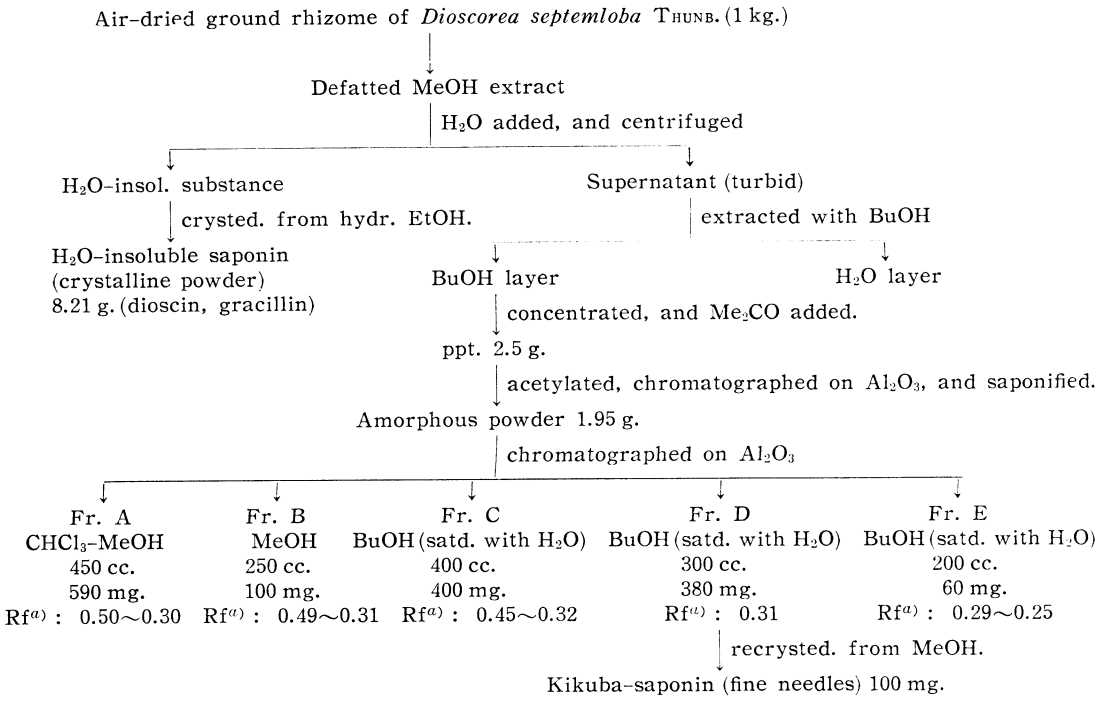
Paper Chromatography——Paper : Tōyō Roshi No. 50. Solvent system (developing time) for saponin : Solv. I, benzene-BuOH-H_2O (10:4:5) (4 hr.); Solv. II, benzene-BuOH-pyridine-H_2O (3:10:2:5) (13 hr.); Solv. III, BuOH-EtOH-H_2O (5:1:4) (15 hr.); for sapogenin : Solv. IV, petr. ether-toluene-EtOH-H_2O (40:5:1:9) (1.5 hr.); for sugar : Solv. V, BuOH-AcOH-H_2O (4:1:5) (17 hr.). All paper chromatographies were conducted at 10~20 by the ascending method. Spray reagent : $SbCl_3$ in $CHCl_3$ (for saponin and sapogenin), aniline hydrogenphthalate (for sugar).

Isolation and Purification of Kikuba-saponin (K) (Chart 1)——Air-dried*6 ground rhizome (1 kg.) of *Dioscorea septemloba* Thunb. (collected at Mikazuki-Yama, Fukuoka Prefecture in October, 1959) was extracted three times with MeOH (total 3.5 L., for 43 hr.). After the solvent was evaporated *in vacuo*, the residue was defatted with boiling benzene (1 L.) for 1 hr. and 700 cc. of H_2O was added with stirring. A H_2O insoluble substance was separated by centrifugation, washed with H_2O (centrifuged) and crystallized from hydr. EtOH to give a H_2O-insoluble saponin as a white crystalline powder, 8.21 g. (Yield, 0.82%; Rf : Solv. I, 0.26+, 0.14+ (dioscin (D), 0.27; gracillin (G), 0.13).

The supernatant solution and the washings were combined, and extracted three times with BuOH (total 900 cc.). The BuOH layer was concentrated *in vacuo* to 200 cc., Me_2CO (1.5 L.) added, and the precipitates formed were collected by filtration. Yield, 2.5 g.; Rf : Solv. III, 0.63 (tailing) +. 0.31# (D, 0.64; G, 0.60; K, 0.30). Acetylation of the above substance with 25 cc. each of pyridine and Ac_2O for 24 hr. at room temperature gave the acetate (2.8 g.), which was dissolved in benzene (30 cc.) and chromatographed on alumina (Wakō Al_2O_3, 40 g.). A colorless glassy substance (2.5 g.) was obtained from benzene (200 cc.) and benzene-MeOH (100:2, 250 cc.) eluates. It was then refluxed with 5% KOH-MeOH (100 cc.) and the hydrolyzate was concentrated, neutralized with hydr. HCl and extracted with BuOH, followed by evaporation *in vacuo* to give a yellowish amorphous substance (1.95 g.); Rf : Solv. II, 0.59+, 0.09## (D, 0.68; G, 0.56; K, 0.10); Solv. III, 0.50+, 0.30## (D, 0.58; G, 0.54;

*5 All melting points were taken on a Kofler block and are uncorrected.
*6 Sliced and dried in the air in the shade at room temperature for one week.

K, 0.31). The saponin mixture dissolved in $CHCl_3$–MeOH (1:1) was placed on an alumina column (Brockmann Al_2O_3, 25 g.), and eluted successively with $CHCl_3$–MeOH (1:1), MeOH, and BuOH satd. with H_2O. The BuOH fraction (0.38 g.) which seemed to be homogeneous (K) (Rf : Solv. III, 0.30) was recrystallized three times from MeOH to give (K) as fine needles (100 mg.), m.p. 249~251° (decomp.), $[\alpha]_D^{17}$ −77° (c=0.39, MeOH); Rf : Solv. II, 0.10; Solv. III, 0.30. (Amorphous (K), Rf : Solv. III, 0.30). *Anal.* Found : C, 57.03; H, 7.71. (cf. Table II). Hardly soluble in H_2O, $CHCl_3$ and benzene, slightly soluble in dehyd. EtOH and soluble in MeOH and hydr. EtOH. Caused lasting foam when shaken with H_2O or hydr. EtOH. Hemolytic index : ca. 20,000 (in H_2O or in 16% MeOH, 16 C, rabbit erythrocytes). Carr-Price reaction ($SbCl_3$) : orange-red; Liebermann-Burchard reaction : reddish violet; Kariyone-Hashimoto reaction (CCl_3COOH) : reddish brown at 60°. No precipitation occurred with cholesterol in 90% EtOH nor with $(AcO)_2Pb$ in 10% MeOH, but white precipitates were produced with $Pb(AcO)_2(OH)_2$ in 10% MeOH. Hydrolysis with 2*N* hydrochloric acid in 50% EtOH for 2 hr. gave diosgenin, $\Delta^{3,5}$-deoxytigogenin (Rf : Solv. IV, 0.59, 0.98), glucose and rhamnose (Rf : Solv. V, 0.13, 0.29).



a) Rf : Solv. III, Dioscin 0.58, Gracillin 0.54, Kikuba-saponin 0.31.

Chart 1. Isolation and Purification of Kikuba-saponin (K)

Kikuba-saponin Peracetate——(K) (100 mg.) was allowed to stand with 1 cc. each of pyridine and Ac_2O at room temperature for 24 hr. The reaction mixture was poured into ice water and the deposited substance was collected and washed with H_2O. Recrystallization from dehyd. EtOH gave the peracetate (100 mg.) as colorless fine needles, m.p. 155~158°, $[\alpha]_D^{15}$ −51 (c=0.47, MeOH). *Anal.* Found : C, 58.21, 57.62; H, 7.03, 7.19; CH_3CO, 33.1. (cf. Table II).

The peracetate (100 mg.) was boiled with 5% KOH-MeOH (15 cc.) for 30 min., diluted with H_2O and extracted with BuOH. Crystallization of the BuOH extract from MeOH gave (K), m.p. 249~251° (decomp.), $[\alpha]_D^{17}$ −75° (c=0.76, MeOH).

Acid Hydrolysis of (K)——5 mg. of (K) was refluxed with 1 cc. of a hydrolytic agent. The hydrolysate was treated and examined essentially in the same manner as described before.[2,3] The results are summarized in Table I.

Quantitative Determinations of Aglycon and Total Sugar of (K)——96.15 mg. of (K) was refluxed with 20 cc. of 2*N* HCl for 1 hr. and then diluted with 10 cc. of H_2O. A H_2O-insoluble product was collected by filtration, washed with 20 cc. of H_2O, and dried over $CaCl_2$ *in vacuo* to the constant weight. Yield, 37.95 mg. (39.5%). After the filtrate was diluted to 50.0 cc. with H_2O, an aliquot (45.0

Table I. Hydrolysis of Kikuba-saponin

		Acid	HCl					H_2SO_4		
Condition (Sample 5 mg. / Reagent 1 cc.)	Concn. of acid (*N*)		2	2	1	1	1/5	1	1/10	1/2
	Concn. of EtOH in the reagent (%)		0	50	50	25	50	25	50	25
	Refluxing time (hr.)		1	2	2	1/2	1	1/2	1	1
Products	Diosgenin + $\Delta^{3,5}$-deoxytigogenin	(0.99)[a]	+	+	++	+	?	±	−	?
	Prosapogenin 3	(0.95)	−	−	+	±	±	?	±	±
	〃 2	(0.78)	−	−	±	?	±	?	±	±
	〃 1	(0.44)	−	−	±	±	±	±	+	±
	Kikuba-saponin	(0.00)	−	−	−	+	++	+	+	++
	Rhamnose		±	±	+	+	+	+	±	+
	Glucose		+	+	+	+	+	+	+	+
	Oligosaccharide		−	−	−	−	−	−	−	−

a) Figures in parentheses indicate Rf values in benzene-BuOH-pyridine-H_2O system. Rf values of gracillin and its two prosapogenins (A and C)[3] run in parallel : gracillin, 0.42; prosapogenin A, 0.78; prosapogenin C, 0.96; diosgenin, 0.99.

cc.) of the solution was neutralized with *N* NaOH (indicator : phenolphthalein) and the amount of the total sugar present was determined by the method described in Part V,[2] 54.9 mg. (63.4% calcd. as glucose). (cf. Table II).

Table II. Analytical Data of Kikuba-saponin and Its Acetate

	Found	Calcd. for $C_{51}H_{82}O_{22}\cdot H_2O$	Found	Calcd. for $C_{75}H_{106}O_{34}$
	Kikuba-saponin	Diosgenin + 3 Glc. + 1 Rhamn.	(K)-peracetate	Diosgenin + 3 Glc. + 1 Rhamn. peracetate
C (%)	57.03	57.50	58.21	58.05
H (%)	7.71	7.95	7.03	6.89
CH_3CO (%)			33.1	33.3
Yield of Diosgenin (%)	39.5	38.9		
Yield of Total Sugar (as glucose) (%)	63.4	65.8		

Determination of the Molar Ratio of Glucose and Rhamnose——An aliquot (5 cc.) of the above sugar solution was deionized with Ag_2CO_3 and H_2S, and evaporated *in vacuo* to dryness. The separatory estimation of glucose and rhamnose was carried out according to the method reported in Part V,[2] and the molar ratio of glucose and rhamnose present in the hydrolyzate was found to be 3.2 to 1.

Emulsin Hydrolysis of (K)——(K) (10 mg.) was dissolved in a small amount of hot 50% EtOH, diluted with phosphate buffer (pH 4.6) to 10 cc., and 50 mg. of emulsin*7 and a few drops of toluene were added. The mixture was allowed to stand at 25~30° for 4 days and the precipitates formed were collected by filtration, washed with H_2O, dried over $CaCl_2$ *in vacuo* and extracted with $CHCl_3$-MeOH (1:1) followed by evaporation to give a white crystalline powder; Rf : Solv. II, 0.65 (gracillin, 0.65). The aqueous filtrate was extracted with BuOH and each layer was evaporated and examined by paper chromatography. BuOH layer, no spot (Solv. II); water layer, Rf : Solv. V, 0.18 (glucose, 0.18).

Quantitative Determination of Glucose in Emulsin Hydrolyzate——(K) (14.505 mg.) was hydrolyzed with emulsin (80 mg.) at 25° for 140 hr. and worked up as above. The aqueous filtrate and the washings were combined, diluted to 25.0 cc. with H_2O and an aliquot (1, 2, and 3 cc.) of the solution was subjected to the colorimetric assay of glucose according to the Borel, *et al.*'s method.[2]*8 Sample solutions, 1, 2 and 3 cc. were found to contain 0.085, 0.170 and 0.245 mg. of glucose, respectively. Yield, 13.4~14.5%. Theoretical percentage : (K) → prosapogenin + 1 glucose : 16.9%; (K) → prosapogenin + 2 glucose : 29.8%.

Identification of Prosapogenin with Gracillin——The prosapogenin (150 mg.) obtained by emulsin hydrolysis of (K) was acetylated with 2 cc. each of pyridine and Ac_2O for 24 hr. at room temperature.

*7 Prepared from apricot seeds (*Prunus Armeniaca* Linn. var. *Ansu* Maxim.) according to the B. Helferich's procedure.[6]

*8 Standard concentration-extinction curve was prepared by a parallel experiment using a known amount of glucose in place of (K).

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

The acetate (150 mg.) was crystallized from MeOH to colorless needles (60 mg.), m.p. 205~207°, $[\alpha]_D^{15}$ −73° (c=0.92, $CHCl_3$). *Anal.* Calcd. for $C_{63}H_{90}O_{26}$ (gracillin acetate): C, 59.88; H, 7.18. Found: C, 59.55; H, 7.23. Mixed melting point with authentic gracillin acetate (m.p. 205~207°, $[\alpha]_D^{17}$ −75° (c=1.23, $CHCl_3$)) showed no depression.

60 mg. of the acetate was refluxed with 3 cc. of 5% KOH-MeOH for 1 hr. After the solvent was evaporated, H_2O was added and an insoluble substance was collected by filtration and crystallized from 90% EtOH to colorless needles, m.p. 290~297° (decomp.), $[\alpha]_D^{15}$ −86° (c=0.55, pyridine). *Anal.* Calcd. for $C_{45}H_{72}O_{17}\cdot 2H_2O$ (gracillin $2H_2O$): C, 58.68; H, 8.32. Found: C, 58.72; H, 8.62. Mixed melting point with authentic gracillin [m.p. 293° (decomp.)] showed no depression. M_D difference between (K) and gracillin: −6° (α-Me-glucoside: +309; β-Me-glucoside: −66°).

Saponins from Fresh Rhizome of *Dioscorea septemloba* THUNB.——Fresh rhizome (350 g., corresponding to 100 g. of air-dried material, collected at Mikazuki-Yama, Fukuoka Prefecture in October, 1959, and stored at −5° for 1 week) was sliced and extracted three times with hot MeOH (for 21 hr., total 2.1 L.). The MeOH solutions were combined, evaporated *in vacuo* and the residue was treated with hot benzene (1 L.). To the defatted MeOH extract 500 cc. of H_2O was added and a H_2O-insoluble substance was collected by centrifugation, washed with H_2O and crystallized from hydr. EtOH to give 30 mg. of a crystalline powder; Rf: Solv. I, 0.29+, 0.13+ (D, 0.27; G, 0.11).

The H_2O layer was extracted three times with BuOH (total 300 cc.) and the BuOH phase was evaporated *in vacuo*. The residue (3 g.) was purified by the MeOH-Et_2O method followed by acetylation, chromatography of the acetate and regeneration according to the procedure as before. The crude saponin thus obtained (1 g.; Rf: Solv. III, 0.27 tailing) was chromatographed on alumina as described before and the BuOH eluates in which one spot (Rf: Solv. III, 0.26; (K), 0.27) was detected were combined and evaporated to dryness (K') (160 mg.). (K') (50 mg.) was hydrolyzed with emulsin (250 mg.) in the same manner as described earlier and the products were examined by paper chromatography. Rf: Solv. I, 0.36+, 0.19+ (D, 0.36; G, 0.19); Solv. V, 0.16 (glucose, 0.15).

Since attempted crystallization from MeOH was unsuccessful, (K') (50 mg.) was submitted to the chromatography on silica gel and eluted with benzene-BuOH-H_2O mixtures (Fr. 1, 10:4:2.5, 200 cc.; Fr. 2, 10:10:5, 200 cc.). Fr. 2 furnished on crystallization from MeOH fine needles, m.p. 225° (decomp.); Rf: Solv. III, 0.27 [(K), 0.27]. Mixed melting point with (K) showed no depression.

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Summary

Kikuba-saponin which had been obtained as an amorphous powder from the rhizome of *Dioscorea septemloba* THUNB. was isolated in pure state as crystals and found to be a diosgenin glycoside in which one mole of D-glucose is attached to gracillin with a β-linkage.

Kikuba-saponin seemed to be a parent saponin of gracillin.

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