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195. Toshio Kawasaki and Tatsuo Yamauchi : Saponins of Timo (知母 : Anemarrhenae Rhizoma). II.*1 Structure of Timosaponin A-III.

(*Institute of Pharmaceutical Sciences, Faculty of Medicine, Kyushu University**2)

Timosaponin A-Ⅲ is the saponin which was isolated*1 as crystals, m.p. 317~322° (decomp.), $[\alpha]_D^{27}$ −41.6°, from a commercial crude drug Timo (知母) (Anemarrhenae Rhizoma) (the dried and stored rhizomes of *Anemarrhena asphodeloides* Bunge). When hydrolyzed with 2*N* hydrochloric acid in 50% ethanol, it afforded sarsasapogenin, D-galactose and D-glucose, and the elemental analysis of the saponin suggested it to be a diglycoside.*1 The present paper deals with its structure elucidation.

The condition of complete hydrolysis of Timosaponin A-Ⅲ with aqueous hydrochloric acid was first determined and then the quantitative analyses of the hydrolytic products were carried out. The yields of sapogenin and total sugar from the saponin were in agreement with those calculated from the molecular formula $C_{39}H_{64}O_{13}$ (sarsasapogenin dihexoside) and the molar ratio of glucose to galactose in the hydrolyzate was found to be 1:1. Therefore a diglycoside structure of the saponin was confirmed.

Since it had been known*1 that timosaponin A-Ⅲ was hydrolyzed under a mild condition to provide, as the water-insoluble products, sarsasapogenin and a prosapogenin which was identical with timosaponin A-I (one of the six saponins found in Timo),*1 the partial hydrolysis was conducted in a larger scale and the prosapogenin was isolated as colorless prisms, m.p. 240~245° (decomp.), $[\alpha]_D^{31}$ −68.5°. The prosapogenin (timosaponin A-I) which gave on hydrolysis sarsasapogenin and galactose was identified with synthetic sarsasapogenin β-D-galactopyranoside (I) prepared by Koenigs-Knorr method.

Although timosaponin A-Ⅲ was not cleaved*1 with emulsin, the M_D difference between A-Ⅲ and A-I indicated the β-linkage of D-glucose to A-I and hence timosaponin A-Ⅲ was regarded as sarsasapogenin O-β-D-glucosyl-β-D-galactopyranoside.

A paper chromatographic examination of the water soluble portion of the partial hydrolyzate showed the existence of a polar substance besides glucose and galactose, and the substance was isolated by means of cellulose powder chromatography as colorless needles, m.p. 164~170°, $[\alpha]_D^{17}$ +42.0→+40.6°. It was splitted by emulsin to yield glucose and galactose, while on oxidation with bromine followed by acid hydrolysis it provided only glucose. Accordingly the substance is nothing but the biose which forms the sugar moiety of timosaponin A-Ⅲ. Since the biose gave neither the phenylosazone nor positive triphenyltetrazolium test[1] and its physical constants agreed with those

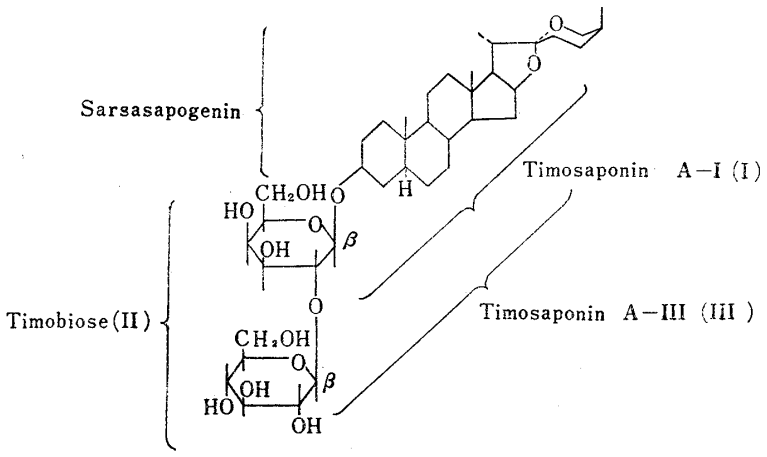
*1 Part I : T. Kawasaki, T. Yamauchi, N. Itakura : Yakugaku Zasshi, 83, 892 (1963).
*2 Katakasu, Fukuoka (川崎敏男, 山内辰郎).
1) K. Wallenfels : Naturwissenschaften, 37, 491 (1950).

(m.p. 171~172°, $[\alpha]_D^{20}$ +42.6°) of the synthetic one reported,[2] it was assumed to be O-β-D-glucopyranosyl-(1→2)-D-galactopyranose (II).

Timosaponin A-III, on permethylation by Kuhn method[3] followed by acid hydrolysis, gave a mixture of two methylated sugars, which was fractionated with the aid of carbon-celite chromatography. Each fraction was purified by repeated recrystallizations and one was identified as 2,3,4,6-tetra-O-methyl-D-glucose by comparison with synthetic sample and the other as 3,4,6-tri-O-methyl-D-galactose by the mixed melting point determination of its phenylosazone with the authentic specimen[4] kindly donated by Prof. Dr. Kuhn.

Consequently the assumed structure (II) of the biose was verified and timosaponin A-III was proved to be sarsasapogenin O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside (III).

Although three sarsasapogenin glycosides[5] with known sugar components were reported, none of them has been investigated to elucidate the detailed structure of its sugar moiety. Timosaponin A-III is the first one of which structure has been established. The sugar which forms the glycidic part of the saponin is a new naturally occurring biose*3 and named timobiose.



Experimental

Paper Chromatography (P. C.)——Paper: Toyo Roshi No. 50. Ascending, at 10~25°C. Solvent system (developing time): Solv. I, Benzene-BuOH-H_2O (10:2:10) (3~4 hr.); Solv. II, Benzene-BuOH-H_2O(10:5:5)(4~5hr.); Solv. III, BuOH-AcOH-H_2O (4:1:5) (17 hr.); Solv. IV, BuOH-Pyridine-H_2O (6:2:3) +Pyridine (1) (17 hr., double development); Solv. V, BuOH-EtOH-H_2O (5:1:4) (17 hr.). Spray reagent: $SbCl_3$ in $CHCl_3$+anisaldehyde in EtOH (for saponin and sapogenin), aniline hydrogenphthalate (for sugar).

Complete Hydrolysis of Timosaponin A-III (T) and Quantitative Determination of the Products——(T)*1 (10 mg.) was refluxed with 2*N* HCl (1 ml.) for 1 hr. and the hydrolyzate was treated and examined

*3 Only two, sophorose (O-β-D-glucopyranosyl-(1→2)-D-glucopyranose)[6] and kojibiose (O-α-D-glucopyranosyl-(1→2)-D-glucopyranose),[7] have been reported as the 1→2 linked reducing biose which occurred in nature.

2) A.M. Gakhokidze, N.D. Kutidze: Zhur. Obshchei Khim. (J. Gen. Chem.), **22**, 139 (1950). (C.A., **46**, 11116 (1952)).
3) R. Kuhn, I. Löw, H. Trischmann: Ber., 88, 1492, 1690 (1955).
4) R. Kuhn, H.H. Baer, A. Gauhe: *Ibid.*, 88, 1135 (1955).
5) *a*) A.W. van der Haar: Rec. trav. chim., **48**, 726 (1929). (sarsasaponin: sarsasapogenin+2 glucose +1 rhamnose). *b*) M.M. Krider, J.R. Branaman, M.E. Wall: J. Am. Chem. Soc., **77**, 1238 (1955). (sarsasapogenin, glucose, galactose). *c*) A. Stabursvik: Acta Chem. Scand., 8, 1304 (1954). (sarsasapogenin, arabinose, galactose, xylose, glucose).
6) J. Rabaté, *et al.*: Bull. soc. chim. biol., **20**, 454, 459, 467 (1938).
7) K. Shibasaki: Tohoku J. Agrc. Res., **6**, 171 (1955). S. Sato, K. Aso: Nature, **180**, 984 (1957).

as described before.[8] On paperchromatograms (solv. II for sapogenin and solv. IV for sugar), sarsasapogenin, glucose and galactose were detected and neither prosapogenin nor biose was found. (T) (71.1 mg.) was refluxed with 2*N* HCl (7.0 ml.) for 1 hr. and the yields of aglycone and total sugar and the molar ratio of glucose to galactose in the hydrolyzate were determined essentially*4 in the same manner as reported before.[10] Yield: aglycone, 39.1 mg. (55.0%); total sugar, 33.8 mg. (47.5%). Molar ratio of glucose to galactose: 0.92:1, 0.83:1, 1.04:1. Calcd. for $C_{39}H_{64}O_{13}$ (sarsasapogenin+1 glucose+1 galactose): aglycone, 56.2%; sugar, 48.9%.

Prosapogenin of Timosaponin A-III (T)——(T) (4.5 g.) was refluxed with 0.5*N* H_2SO_4-50% EtOH (400 ml.) for 2 hr., EtOH was evaporated and H_2O was added. A H_2O-insoluble product was collected by filtration, washed with H_2O and crystallized from BuOH saturated with H_2O. Colorless prisms (2.5 g.) (identified with (T)) was filtered off and the mother liquor was evaporated *in vacuo* to give a solid residue (1.57 g.). Rf (solv. I): 0.97, 0.75, 0.08 (sarsasapogenin 0.97, timosaponin A-I 0.73, T 0.08). The residue (1.12 g.) was chromatographed on a column of silica gel (150 g.) impregnated with 40 ml. of H_2O saturated with AcOEt. Fr. 1~2: AcOEt-EtOH-H_2O (20:1:1), 500 mg., mainly sarsasapogenin. Fr. 3: AcOEt-EtOH-H_2O (10:1:1), 180 mg., prosapogenin. Fr. 4: AcOEt-EtOH-H_2O (5:1:1), 400 mg., (T). Fr. 3 (180 mg.) was chromatographed again on an Al_2O_3 (Woelm, grade IV, 48 g.) column. The first fraction ($CHCl_3$-MeOH (30:1), 40 mg., Rf (solv. I) 0.97, 0.73) was removed and the following fractions ($CHCl_3$-MeOH (30:1 and 20:1), total 130 mg., Rf 0.73) were combined and recrystallized three times from MeOH to give prosapogenin as colorless prisms, m.p. 240~245° (decomp.), $[\alpha]_D^{31}$ −68.5° (c=0.72, dioxane). *Anal.* Calcd. for $C_{33}H_{54}O_8$ (sarsasapogenin monohexoside): C, 68.48; H, 9.40. Found: C, 68.51; H, 9.44. M_D −395.1. Timosaponin A-III: M_D −384.5.*1 Δ +10.6 (methyl α-D-glucopyranoside M_D +309, methyl β-D-glucopyranoside M_D −66).

Prosapogenin (50 mg.) was acetylated on standing for 24 hr. with 1 ml. each of Ac_2O and pyridine and the acetate was crystallized from MeOH. Colorless plates, m.p. 240~242°, $[\alpha]_D^{18}$ −52.4° (c=0.50, $CHCl_3$). *Anal.* Calcd. for $C_{41}H_{62}O_{12}\cdot H_2O$ (sarsasapogenin monohexoside tetraacetate·H_2O): C, 64.37; H, 8.43. Found: C, 64.69, 64.73; H, 8.16, 8.30.

Prosapogenin (5 mg.) was refluxed for 2 hr. with 2*N* HCl-50% EtOH (1 ml.) and the products were examined by P.C. H_2O-insoluble product: Rf 0.92 (solv. I, sarsasapogenin 0.93) H_2O-soluble product: $R_{glucose}$ 0.89 (solv. IV, galactose 0.89).

Synthesis of Sarsasapogenin β-D-Galactopyranoside by Koenigs-Knorr Method——The solution of 300 mg. each of sarsasapogenin and α-bromotetraacetyl-D-galactose in benzene (10 ml.) was stirred with Ag_2O (350 mg.) and drierite (450 mg.) at room temperature for 3 days. The reaction mixture was filtered and the filtrate was evaporated to dryness. The residue (300 mg.) was refluxed with 5% KOH-MeOH (40 ml.) for 45 min., the solvent was distilled off, and H_2O was added. The H_2O-insoluble substance was collected by filtration, and submitted to Al_2O_3 chromatography followed by crystallization from MeOH to give sarsasapogenin β-D-galactopyranoside as colorless prisms, m.p. 242~246° (decomp.), $[\alpha]_D^{23}$ −68.2° (c=0.56, dioxane). *Anal.* Calcd. for $C_{33}H_{54}O_8$: C, 68.48; H, 9.40. Found: C, 68.48; H, 9.68. On admixture with the prosapogenin of timosaponin A-III, it showed no melting point depression.

Timobiose——(T) (5 g.) was refluxed with 0.5*N* H_2SO_4-50% EtOH (400 ml.) for 2 hr., EtOH was evaporated and H_2O was added. The H_2O-insoluble substance was removed by filtration, and the filtrate was neutralized with $BaCO_3$, filtered and evaporated *in vacuo*. The residue (540 mg.) was chromatographed on a cellulose powder column. Fr. 1: BuOH saturated with H_2O, 440 ml., 150 mg., Rf 0.15~0.13. Fr. 2: 240 ml., 60 mg., Rf 0.14, 0.07. Fr. 3: MeOH, 120 ml., 110 mg., Rf 0.06. (solv. III, D-glucose 0.15, D-galactose 0.13). Fr. 3 was recrystallized from MeOH to give timobiose as colorless needles, m.p. 164~170°*5 (immediately after recrystallization), $[\alpha]_D^{17}$ +42.0° → +40.6° (3 hr.) (c=0.72, H_2O) (synthetic O-β-D-glucopyranosyl-(1→2)-D-galactopyranose,[3] m.p. 171~172°, $[\alpha]_D^{20}$ +42.6° (H_2O)). $R_{lactose}$ 1.30 (solv. III). *Anal.* Calcd. for $C_{12}H_{22}O_{11}\cdot H_2O$ (hexobiose·H_2O): C, 40.00; H, 6.71. Found: C, 39.70; H, 6.62. Triphenyltetrazolium test[2]: negative.

Timobiose (10 mg.) in H_2O (2 ml.) was left with emulsin[10] (20 mg.) and toluene (3 drops) at 25° for 42 hr. The precipitates were filtered off, the filtrate was evaporated *in vacuo*, and examined by P.C. Rf (solv. IV): 0.28, 0.24 (D-glucose 0.29, D-galactose 0.25, timobiose 0.15).

The mixture of timobiose (10 mg.) in H_2O (2 ml.) and 10% Br_2-AcOH (0.5 ml.) was allowed to stand for 3 days at room temperature. The solution was debrominated with HCOOH, evaporated *in vacuo*, and the residue was heated with 2*N* H_2SO_4 (2 ml.) for 2 hr. on a water bath. The hydrolyzate was worked up as usual and sugars were examined by P.C. Rf (solv. IV): 0.28, 0.23 (trace) (D-glucose 0.27, D-galactose 0.24).

*4 Determination of sugars was carried out according to the Momose method[9] in place of the Fehling-Lehmann-Schoorl method and the Borel method employed before.[10]

*5 m.p. 170~190° (after standing for a few days in the atmosphere).

8) T. Tsukamoto, T. Kawasaki, T. Yamauchi: This Bulletin, **4**, 35 (1956).
9) T. Momose, Y. Mukai, M. Watanabe: Talanta, **5**, 275 (1960).
10) T. Kawasaki, T. Yamauchi, R. Yamauchi: This Bulletin, **10**, 698 (1962).

Permethylation of Timosaponin A-III (T) (by Kuhn Method[4])——(T) (3.4 g.) in dimethylformamide (70 ml.) was shaken with Ag_2O (20 g.) and MeI (20 ml.) for 42 hr. The precipitates were filtered off and the filtrate was shaken again with additional Ag_2O (20 g.) and MeI (20 ml.) for 24 hr., and worked up according to the Kuhn's procedure. Crude (T) permethylate (syrup, 3.4 g.) was passed through an Al_2O_3 column using benzene as a solvent, and crystallized from MeOH to give pure (T) permethylate (2.0 g.) as colorless needles, m.p. 167~170°, $[\alpha]_D^{18}$ −51.6° (c=2.46, $CHCl_3$). *Anal.* Calcd. for $C_{46}H_{78}O_{13}$ (sarsasapogenin glucosyl-galactoside heptamethylate): C, 65.84; H, 9.37. Found: C, 65.41; H, 9.41.

Hydrolysis of Timosaponin A-III Permethylate——Timosaponin A-III permethylate (1.87 g.) was refluxed with 5% HCl-MeOH (80 ml.) for 5 hr. MeOH was evaporated and the residue was heated with *N* HCl (75 ml.) for 5 hr. on a water bath. The precipitates were filtered off, the filtrate was treated with Ag_2CO_3 and H_2S and evaporated *in vacuo* to give a syrup (0.9 g.). The syrup was submitted to a chromatography on carbon-celite (1:1) using 10~90% EtOH as a solvent. Fr. 1: 10~15% EtOH, trace, Rf 0.45 (trace), 0.53. Fr. 2: 15% EtOH, 270 mg., 0.53. Fr. 3: 20~30% EtOH, 30 mg., 0.64 (trace), 0.79. Fr. 4: 90% EtOH, 370 mg., 0.83 (solv. V, 2,3,4,6-tetra-O-methyl-D-glucose 0.83).

2,3,4,6-tetra-O-methyl-D-glucose: Fr. 4 was recrystallized four times from petr. ether to give colorless needles, m.p. 80~82°, $[\alpha]_D^9$ +85.5°(c=1.1, H_2O). *Anal.* Calcd. for $C_{10}H_{20}O_6$(tetra-O-methylglucose): C, 50.83; H, 8.53. Found: C, 51.08; H, 8.73. Tetramethylglucose (200 mg.) in abs. EtOH (5 ml.) was refluxed with aniline (150 mg.) for 2 hr. and then EtOH was evaporated. The residue was sublimed in reduced pressure, and the sublimate was recrystallized from petr. ether to give colorless needles, m.p. 135~139°, $[\alpha]_D^{29}$ +238°(c=0.42, Me_2CO). *Anal.* Calcd. for $C_{16}H_{25}O_5N$ (N-phenyl-tetra-O-methylglucosylamine): C, 61.71; H, 8.09. Found: C, 61.56; H, 8.13. Mixed melting point determination with synthetic sample of N-phenyl-2,3,4,6-tetra-O-methyl-D-glucosylamine(m.p. 130~135°) showed no depression.

3,4,6-tri-O-methyl-D-galactose: Fr. 2 was purified by carbon-celite chromatography using 20% EtOH as a solvent. The eluate was evaporated and recrystallized twice from CCl_4 to give prisms, m.p. 83~87°, $[\alpha]_D^{11}$ +157°→+99.3° (c=1.41, H_2O). *Anal.* Calcd. for $C_9H_{18}O_6$ (tri-O-methylgalactose): C, 48.64; H, 8.16. Found: C, 48.25; H, 8.25. Triphenyltetrazolium test: positive (pink).

To the solution of trimethyl galactose (190 mg.) in H_2O (5 ml.) Br_2 (0.1 ml.) was added and the mixture was allowed to stand for 7 days at room temperature. Excess Br_2 was removed by air bubbling and the solution was evaporated *in vacuo*. Recrystallization of the residue from hexane-Et_2O gave needles, m.p. 131~133° (3,4,6-tri-O-methyl-D-galactonic acid,[5] m.p. 127~128°). *Anal.* Calcd. for $C_9H_{18}O_7$ (tri-O-methylgalactonic acid): C, 45.37; H, 7.62. Found: C, 45.74; H, 7.89.

Trimethylgalactose (220 mg.) in H_2O (10 ml.) was heated with phenylhydrazine·HCl (1 g.) and AcONa (0.7 g.) for 2 hr. The H_2O-insoluble product was triturated with ice-water and crystallized from 50% EtOH to give yellow needles, m.p. 132°, $[\alpha]_D^{11}$ +89.1°(c=2.3, pyridine). *Anal.* Calcd. for $C_{21}H_{28}O_4N_4$ (tri-O-methylgalactose phenylosazone): C, 62.98; H, 7.05; N, 13.99. Found: C, 63.25; H, 7.00; N, 13.71. On admixture with authentic sample[4] of 3,4,6-tri-O-methyl-D-galactose phenylosazone (m.p. 130~132°) kindly donated by Prof. Dr. Kuhn, it gave no melting point depression.

The authors thank Prof. Dr. R. Kuhn for providing us with an authentic sample of 3,4,6-tri-O-methyl-D-galactose phenylosazone, Mr. M. Shido and his associates for microanalyses, and Misses N. Itakura and C. Omura for technical assistance.

Summary

Timosaponin A-III, the crystalline steroid saponin isolated from a commercial crude drug Timo (Anemarrhenae Rhizoma), has been shown to be sarsasapogenin O-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside.

The biose which forms the sugar moiety of timosaponin A-III was obtained as crystals from the partial hydrolyzate of the saponin and named timobiose.

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