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SOIL BACTERIAL HYDROLYSIS LEADING TO GENUINE AGLYCONE—VIII

STRUCTURES OF A GENUINE SAPOGENOL PROTOBASSIC ACID AND A PROSAPOGENOL OF SEED KERNELS OF *MADHUCA LONGIFOLIA* L.

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Abstract—By virtue of the soil bacterial hydrolysis method, a genuine sapogenol named protobassic acid was obtained from the saponins of seed kernels of *Madhuca longifolia* L. (*Sapotaceae*), and the structure has been established as 2β, 3β, 6β, 23 - tetrahydroxy - olean - 12 - en - 28 - oic acid (**2a**). In addition, the structure of a prosapogenol designated as Mi-glycoside I, which was isolated concurrently during the above microbiological procedure, has been elucidated as 3 - O - β - D - glucopyranosyl - protobassic acid (**3a**). It has been suggested that protobassic acid (**2a**) may be a common sapogenol of several sapotaceous plants in place of hitherto approved bassic acid (**1a**).

To split the glycoside linkage of saponin avoiding the secondary alteration of sapogenol which often occurs during the ordinary chemical hydrolysis procedure, the soil bacterial hydrolysis method has been developed in this laboratory.[1,2a] The method has initially been shown to be useful for the elucidation of the genuine sapogenols of triterpenoid saponins[2] and further the usefulness of the method has been extended to clarify the genuine aglycones of triterpenoid glycoside,[3] diterpenoid glycoside,[4] monoterpenoid glucosides,[1] and steroidal glycoside.[5]

As described in a preliminary communication,[6] we have employed the microbiological method for the saponins isolated from the seed kernels of *Madhuca longifolia* L. (*Sapotaceae*), and elucidated that a new oleanene triterpenoid named protobassic acid (**2a**) is a genuine sapogenol in place of bassic acid (**1a**) which was known as a common sapogenol of several sapotaceous plants[7,8] and whose structure was initially studied by Heywood and Kon[7] and later established as **1a** by King and Yardley.[9,10] In addition, the method has led to the isolation of a prosapogenol named Mi-glycoside I† whose structure has now been shown to be 3 - O - β - D - glucopyranosyl - protobassic acid (**3a**). The present paper deals with the details of the studies.

Acid hydrolysis followed by silica gel column chromatography of the crude saponin mixture obtained from the seed kernels (Chart 1) afforded bassic acid (**1a**), m.p. 289–291°, as a major sapogenol. Since the direct comparison with an authentic sample was impossible, the identification was made by comparison of the physical properties of methyl ester (**1b**), methyl ester triacetate (**1c**), and methyl ester monoacetonide (**1d**) with those reported in the literature[7] as described in the Experimental. The identity of our material with bassic acid has been further assured on the basis of PMR and mass spectral evidences as shown later.

On the other hand, the soil bacterial hydrolysis applied to Mi-saponin† (saponin mixture), which was prepared by charcoal-Celite column chromatography of the crude saponin mixture (Chart 1), furnished a new compound as a sole sapogenol being unidentical with bassic acid (**1a**) and designated as protobassic acid (**2a**). Furthermore, a compound, having a more polar property and designated as Mi-glycoside I (**3a**), was obtained simultaneously from the hydrolysate. It should be pointed here that there has been no indication of bassic acid in the total hydrolysate.

Protobassic acid (**2a**), m.p. 310–312°, gave a methyl ester (**2b**), m.p. 198–201°, with ethereal diazomethane. Acetylation of **2b** with Ac_2O and pyridine at room temperature furnished a methyl ester triacetate (**2c**), in which one OH function was left unattacked as shown by the IR absorption band at 3540 cm^{-1}.

On treatment with $POCl_3$ in pyridine followed by mild alkaline hydrolysis, **2c** afforded bassic acid methyl ester (**1b**) as an only product. Acid treatment of **2b** under the same reaction conditions as for the hydrolysis of saponin yielded **1b** as a major

†Named after the Sinhalese name "Mi" of the seed.

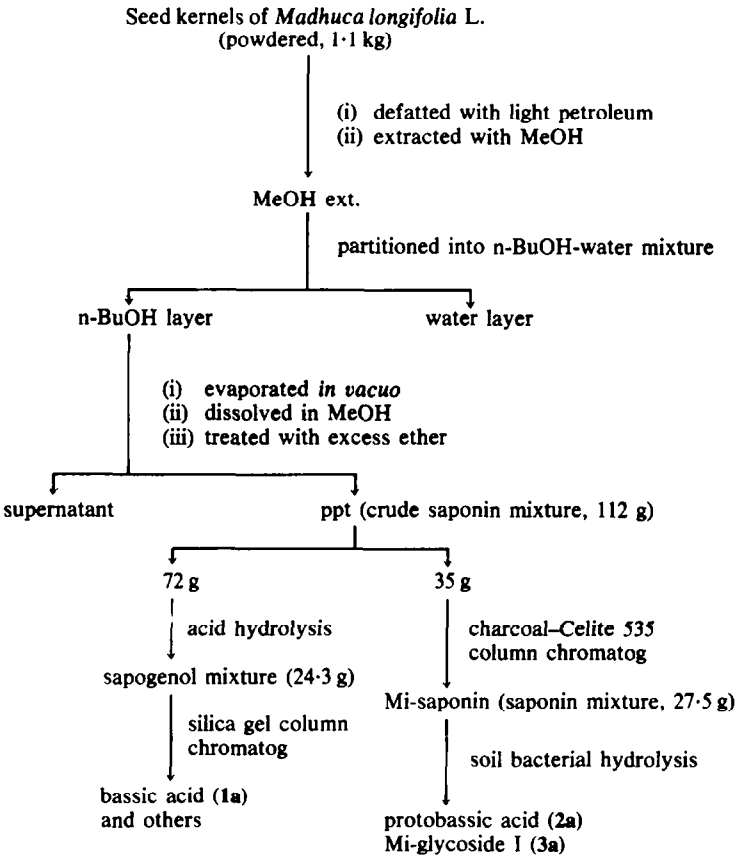


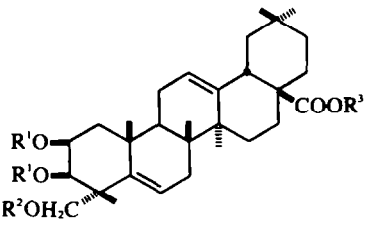
CHART 1

product along with some minor secondary products. These observations indicate not only the close relation between protobassic acid and bassic acid, but also genuineness of the former towards the latter. CrO_3-pyridine complex oxidation of **2c** furnished a carbonyl compound (**2d**), m.p. 164–167°, whose CO function resisted against the ordinary Huang-Minlon reduction.

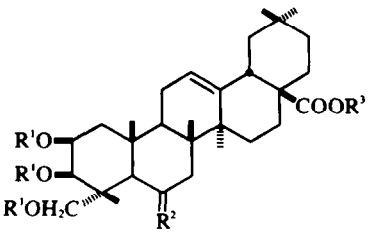
The PMR spectrum of **2c** in comparison with that of bassic acid methyl ester triacetate **1c** (Table 1) demonstrates resemblance and difference of the both compounds. The data show the presence of six tertiary Me's (all observed as singlets), three AcO functions, one carbomethoxyl group and one acetoxymethylene function in **1c** and **2c**. However, they are distinguished from each other by a broad multiplet at δ 5·50–5·70 (1 H) due to the C-6 olefinic proton in **1c** and by a multiplet (1 H, $W_{h/2} = 8$ Hz) at δ 4·41 assignable to an equatorial proton attached to a carbon bearing an OH function[11] in **2c**.

Examination of the mass spectra of **1b** and **2b** (Table 2) also discloses the close relation of both methyl esters. Although the parent ion peak of **2b** (*m/e* 518) is observed at 18 mass unit higher than that of **1b** (*m/e* 500), the fragment ions of *m/e* 262(ii) and 238(iii) due to the reverse Diels–Alder type fragmentation[12] of ring C are observed both in **1b** and **2b**, and a common base peak of *m/e* 203(ii-$COOCH_3$) derivable from ion (ii) is observed. The other significant features noticed in the mass spectra are that **2b** exhibits a fragment ion peak at *m/e* 500 being coincided with the parent ion of **1b** and another common fragment ion peak of *m/e* 302(i) due to the reverse Diels–Alder type fragmentation in ring B[6,13] is observed in both **1b** and **2b**. These assignments have been substantiated by the high resolution mass spectrometry (Table 2). Therefore, it has been suggested that an OH group in **2b** is located at C-5 or C-6 and is readily dehydrated giving rise to a double bond at C-5.

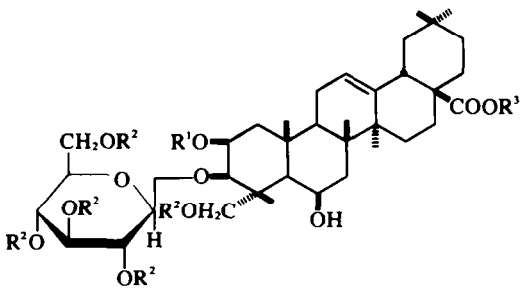
In connection with resistance of an OH function in **2b** to the ordinary acetylation, the PMR signal pattern of the geminal proton as mentioned above (δ 4·41 in **2c**, Table 1) defines the configuration of the OH as C-6β. Furthermore, the presence of three paramagnetically shifted Me signals assignable to C-4β Me, C-8β Me, and C-10β Me supports the C-6β OH assignment. Consequently, the structure



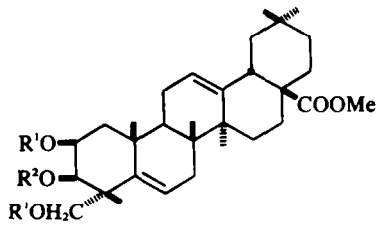
1a: $R^1 = R^2 = R^3 = H$ (bassic acid)
1b: $R^1 = R^2 = H$, $R^3 = Me$
1c: $R^1 = R^2 = Ac$, $R^3 = Me$
1d: $R^1 = (CH_3)_2C$<, $R^2 = H$, $R^3 = Me$



2a: $R^1 = H$, $R^2 = \beta$-OH, α-H, $R^3 = H$ (protobassic acid)
2b: $R^1 = H$, $R^2 = \beta$-OH, α-H, $R^3 = Me$
2c: $R^1 = Ac$, $R^2 = \beta$-OH, α-H, $R^3 = Me$
2d: $R^1 = Ac$, $R^2 = O$, $R^3 = Me$

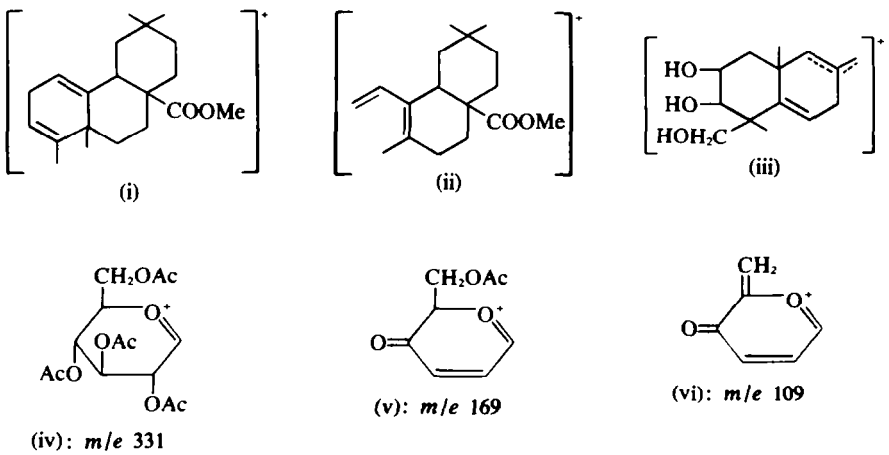


3a: $R^1 = R^2 = R^3 = H$ (Mi-glycoside I)
3b: $R^1 = R^2 = H$, $R^3 = Me$
3c: $R^1 = H$, $R^2 = Ac$, $R^3 = Me$
3d: $R^1 = R^2 = R^3 = Me$



4: $R^1 = Me$, $R^2 = Ac$
5: $R^1 = R^2 = Me$

CHART 2



(iv): *m/e* 331
(v): *m/e* 169
(vi): *m/e* 109

CHART 3

of protobassic acid has been expressed as 2β, 3β, 6β, 23 - tetrahydroxy - olean - 12 - en - 28 - oic acid (**2a**).

Taking into consideration of the chemical correlation of protobassic acid (**2a**) and bassic acid (**1a**), the former is considered to be a genuine form of the latter and it is reasonably inferred that **2a** is a common sapogenol of some sapotaceous plants rather than previously approved **1a**.

A prosapogenol named Mi-glycoside I (**3a**), m.p. 300–304°, obtained by the soil bacterial hydrolysis method along with **2a** as mentioned above, was also

Table 1.*

	1c†	2c‡
—C(—CH₃)—	0·94 (6H), 0·95, 1·05, 1·26 1·34 (3 H each) (all s)	0·91, 0·94, 1·03, 1·09, 1·42 1·58 (3 H each, all s)
—OCOCH₃	1·99, 2·04, 2·08 (3 H each, all s)	2·00, 2·02, 2·06 (3 H each, all s)
—COOCH₃	3·63 (3 H, s)	3·63 (3 H, s)
—C(23)H₂OAc	3·81, 4·22 (2 H, ABq, J = 12 Hz)	3·87, 3·91 (2 H, ABq, J = 13 Hz)
C(6)HOH		4·41 (1 H, m, $W_{\frac{1}{2}}$ = 8 Hz)
=C(6)H	5·50–5·70 (1 H, m)	
C(3)HOAc	5·02 (1 H, d, J = 4 Hz)	4·86 (1 H, d, J = 4 Hz)
C(2)HOAc		
=C(12)H	5·32–5·48 (2 H)§	5·24–5·46 (2 H)§

*Abbreviation: ABq = AB type quartet, d = doublet, m = multiplet, s = singlet
†Measured at 100 MHz.
‡Measured at 90 MHz.
§Signal patterns are unclear due to the overlapping.

Table 2.

		1b		2b	
	Calcd.	Obsvd.	Relative Intensity (%)	Obsvd.	Relative Intensity (%)
$C_{31}H_{50}O_6$ (M^+, **2b**)	518·361			518·360	2
$C_{31}H_{48}O_5$ (M^+, **1b**)	500·350	500·348	7	500·353	4
$C_{20}H_{30}O_2$ (i)	302·225	302·222	8	302·226	5
$C_{17}H_{26}O_2$ (ii)	262·193	262·193	40	262·191	52
$C_{14}H_{22}O_3$ (iii)	238·157	238·155	28	238·159	14
$C_{15}H_{23}$ (ii-$COOCH_3$)	203·180	203·179	100	203·181	100

isolated in a good yield through the mild acid hydrolysis of the saponin mixture.

Mi-glycoside I gave a methyl ester (**3b**), m.p. 293·5–295·5°, with ethereal diazomethane. Acetylation of **3b** with Ac_2O and pyridine at room temperature furnished a methyl ester pentaacetate (**3c**), m.p. 237–239°, which exhibits the OH absorption bands at 3560 and 3435 cm^{-1} in its IR spectrum. The mass spectrum of **3c** shows the prominent fragment ions at *m/e* 331(iv), 169(v), and 109(vi) derived from the aldohexopyranose moiety of glycoside[14] along with the fragment ions at *m/e* 262(ii) and 203(ii-$COOCH_3$) due to the sapogenol moiety (Chart 3).

On periodate oxidation followed by alkaline treatment,[15] **3b** afforded protobassic acid methyl ester (**2b**), while acid hydrolysis of **3b** gave bassic acid methyl ester (**1b**) as a sapogenol and glucose as a sugar portion.

The PMR spectrum of **3c** indicates the presence of five AcO functions and six tertiary Me's, and the coupling constant (J = 7 Hz) of a doublet at δ 4·55 due to the anomeric proton reveals that D-glucose is linked with β-orientation.[16]

Methylation of Mi-glycoside I after the Hakomori's procedure[17] or the Kuhn's procedure[18] afforded a methyl ester hexamethyl ether (**3d**) which still retains an OH function as shown by its IR spectrum (3600 cm^{-1}) and yielded methyl 2, 3, 4, 6 - tetra - O - methyl - D - glucopyranoside upon methanolysis.

The PMR spectrum of **3d** indicates the presence of six MeO groups and six tertiary Me functions,

and one proton appearing at δ 4·41 (m, $W_{h/2}$ = 9 Hz) is assignable to C-6α H geminal to a free OH function of the protobassic acid moiety.

All this evidence demonstrates that Mi-glycoside I is a monoglucoside of protobassic acid (**2a**). Methanolysis of **3d** followed by acetylation yielded 3 - 0 - acetyl - 2, 23 - di - 0 - methyl - bassic acid methyl ester (**4**) as a result of concomitant dehydration of C-6 axial OH function of protobassic acid moiety during the methanolysis. For a comparison purpose, methylation of protobassic acid methyl ester (**2b**) after Hakomori's procedure and subsequent acid treatment of the product under the same methanolysis condition as for **3d** were undertaken. In this case, 2, 3, 23 - tri - 0 - methyl - bassic acid methyl ester (**5**) was obtained as a major product.

The formulation of **4** has been based on the PMR spectrum which indicates the presence of one AcO, two MeO groups and one carbomethoxyl group along with six tertiary Me's. The PMR spectrum of **4** shows a doublet at δ 5·14 (J = 4 Hz) ascribable to C-3α H geminal to an AcO, while in that of **5**, the corresponding proton signal is observed in the region of δ 3·70–3·90* showing that the C-3β OH of **5** is methylated.

These combined evidences have led to a conclusion that Mi-glycoside I is formulated as 3 - 0 - β-D - glucopyranosyl - protobassic acid (**3a**).

The saponin mixture designated as Mi-saponin comprises three major saponins as revealed by TLC and the separation was effected by repeated silicia gel column chromatography to afford Mi-saponin A (amorphous), Mi-saponin B, m.p. 250–253°, and Mi-saponin C (amorphous). The soil bacterial hydrolysis of each saponin furnished protobassic acid (**2a**) and Mi-glycoside I (**3a**) respectively, thus substantiating **2a** and **3a** to be a common sapogenol and prosapogenol of these saponins. The structures of these saponins are now under investigation.

Very recently, Hariharan *et al.* reported[19] the structures of two saponins isolated from the seed of *Bassia* (= *Madhuca*) *latifolia* Roxb. to be the glycosides of bassic acid (**1a**). Although the direct comparison has not yet been made, the proposed structures appear to be inconsistent with our saponins since alkaline treatment of Mi-saponin B furnishes only Mi-glycoside I (**3a**) and two glucose moieties are attaching to the C-2 and C-3 OH functions of bassic acid moiety in their proposed structures.

EXPERIMENTAL

The following instruments were used for the physical data: m.p. (Yanagimoto Micro-m.p. Apparatus; recorded uncorrected); specific rotation (Rex Photoelectric Polarimeter, measured at room temp with l = 1 dm); IR spectra (Hitachi IR Spectrometer EPI-S2, EPI-G21, or EPI-G31); mass spectra (Hitachi RMU-6D Spectrometer); PMR spectra (Hitachi R-22, Varian A-60, or HA-100 NMR Spectrometer, in $CDCl_3$ and TMS as the internal standard). The chemical shifts are given in δ values and coupling constants (J) are in Hz. Silica gel D-5 (Camag) was used for TLC and detection by 1% $Ce(SO_4)_2$ in 10% H_2SO_4. For column chromatography, silica gel (Merck, 0·05–0·2 mm) was used.

Isolation of bassic acid (**1a**) *from* Madhuca longifolia L. Powdered seed kernels of *Madhuca longifolia* L. (1·1 kg) collected at Jaffna in Ceylon was defatted 4 times with light petroleum and extracted 4 times with MeOH at reflux. A residue obtained by evaporation of MeOH was partitioned into n-BuOH-water as usual. Upon evaporation under reduced pressure, the n-BuOH soluble portion furnished a dark reddish-brown residue which was dissolved in small amount of MeOH and treated with excess ether repeatedly to precipitate a crude saponin mixture (112 g). A soln of the crude saponin mixture (72 g) in 15% H_2SO_4-EtOH (1 : 1, 1·5 l) was refluxed for 15 h and poured into water to afford a sapogenol mixture which was collected by filtration and dried (24·3 g). The crude sapogenol mixture was then chromatographed on silica gel (1·2 kg) eluting with benzene, benzene-$CHCl_3$, $CHCl_3$, and $CHCl_3$-MeOH successively. The eluate with $CHCl_3$-MeOH (49 : 1) which contained mainly bassic acid was recrystallized from MeOH to give fine colourless crystals (5 g) of **1a**, m.p. 289–291°; $[\alpha]_D$ + 87·5° (c = 1·0, pyridine); ν_{max} (KBr): 3430 (br.) (OH), 1689 (COOH) cm^{-1}; mass spectrum *m/e* (%): 486 (M^+, 6), 288 (12), 248 (100), 238 (88), 203 (95), 189 (43) (Found: C, 73·80; H, 9·68. $C_{30}H_{46}O_5$ requires: C, 74·03; H, 9·53%). The identity of our material with bassic acid was further confirmed by preparation of the following derivatives in the usual manner. *Methyl ester* (**1b**), m.p. 218–219°; $[\alpha]_D$ + 55·0° (c = 1·0, $CHCl_3$); ν_{max} (KBr); 3425 (br.) (OH), 1725, 1710 (COOMe) cm^{-1} (Found: C, 74·54; H, 9·65. $C_{31}H_{48}O_5$ requires: C, 74·36; H, 9·66%). *Methyl ester triacetate* (**1c**), m.p. 146–148°; $[\alpha]_D$ + 69·6° (c = 0·7, $CHCl_3$); ν_{max} (CCl_4): 1743, 1236 (br.) (OAc, COOMe) cm^{-1} (Found: C, 71·15; H, 8·57. $C_{37}H_{54}O_8$ requires: C, 70·90; H, 8·68%). *Methyl ester monoacetonide* (**1d**), m.p. 209–210°; $[\alpha]_D$ + 67·5° (c = 1·1, $CHCl_3$); ν_{max} (KBr): 3505 (OH), 1710 (COOMe) cm^{-1} (Found: C, 75·62; H, 9·69. $C_{34}H_{52}O_5$ requires: C, 75·51; H, 9·69%). In lit:[7] **1a**, m.p. 316°; $[\alpha]_D$ + 82·9° (pyridine); **1b**, m.p. 216–217°; $[\alpha]_D$ + 56·2° ($CHCl_3$); **1c**, m.p. 148–149°; **1d**, m.p. 205–206°.

Soil bacterial hydrolysis of Mi-saponin. The crude saponin mixture (35 g) was purified by passing through an active charcoal–Celite column (70 g of charcoal, Tokuseishirasagi, Takeda Chem. Ind.; 70 g of Celite 535, Wako Pure Chem. Ind.) with an aid of MeOH. The combined MeOH eluates were concentrated *in vacuo* and treated with excess ether to precipitate a saponin mixture (Mi-saponin), which was collected by filtration, dried (27·5 g) and used for the soil bacterial hydrolysis. Necessary soil bacterial strain was selected by the procedure as described before.[2a] A strain (YSB-15*) thus selected was cultivated stationarily in a larger scale at 33° for 27 days on the same synthetic medium as used for the selection ($(NH_4)_2HPO_4$ 4 g, KH_2PO_4 1 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0·7 g, $FeSO_4 \cdot 7H_2O$ 0·03 g, Mi-saponin 3 g (only carbon source), water 1 liter, and adjusted to pH 6 by dil HCl). The total culture broth was extracted with ether and *n*-BuOH successively. The ether extract, after the usual work-up followed by recrystallization from MeOH, furnished colourless crystals (140 mg) of protobassic acid

*Signal patterns are unclear due to the overlapping.

*Unidentified yet. The microorganism gave **2a** as a major hydrolysate.

(**2a**), m.p. 310–312°; $[\alpha]_D$ + 22·7° (*c* = 0·11, pyridine); ν_{max} (KBr): 3560, 3470 (OH), 1680 (COOH); mass spectrum *m/e* (%): 504 (M^+, 2), 486 ($M^+ - H_2O$, 2), 288 (4), 248 (100), 238 (iii, 16), 203 (ii-$COOCH_3$, 78), 189 (22) (Found: C, 71·53; H, 9·75. $C_{30}H_{48}O_6$ requires: C, 71·39; H, 9·59%). The n-BuOH extract (1·21 g) was found by TLC to comprise mainly Mi-saponin and a trace amount of Miglycoside I (**3a**).

Protobassic acid methyl ester (**2b**). Diazomethane treatment of **2a** (100 mg) in ether-MeOH mixture followed by recrystallization from acetone furnished colourless needles (70 mg) of **2b**, m.p. 198–201°; $[\alpha]_D$ + 42·4° (*c* = 0·5, MeOH); ν_{max} (KBr): 3400 (br.) (OH), 1725 (COOMe) cm^{-1} (Found: C, 70·59; H, 9·83. $C_{31}H_{50}O_6 \cdot \frac{1}{2}H_2O$ requires: C, 70·59; H, 9·67%).

Protobassic acid methyl ester triacetate (**2c**). A soln of **2b** (30 mg) in pyridine (3 ml) and Ac_2O (2 ml) was left standing at 31° for 24 h, poured into ice-water and extracted with ether. Treatment of the ether extract in the usual manner gave **2c** (25 mg, single spot on TLC, but the crystallization being without success); ν_{max} (Nujol): 3540 (OH), 1755, 1247 (br.) (OAc, COOMe) cm^{-1} (Found: C, 69·10; H, 8·73. $C_{37}H_{56}O_9$ requires: C, 68·91; H, 8·75%).

Bassic acid methyl ester (**1b**) *from protobassic acid methyl ester triacetate* (**2c**). To a soln of **2c** (25 mg) in pyridine (5 ml), was added $POCl_3$ (3 ml) and the total mixture was left standing at room temp for 24 h, diluted with ice-water, and extracted with ether. After the usual work-up of the ether extract, the product (26 mg) was dissolved in EtOH (2 ml) and treated with 10% KOH aq (2 ml) at 50° for 5 h. A product obtained by the usual work-up was purified by preparative TLC developing with $CHCl_3$-acetone (1:1) mixture followed recrystallization from acetone to furnish colourless needles (5 mg), which were found identical with bassic acid methyl ester (**1b**) by m.m.p., IR (KBr), and TLC.

Acid treatment of protobassic acid methyl ester (**2b**). Treatment of **2b** (TLC scale) with 15% H_2SO_4-EtOH (1:1) mixture at reflux for 12 h followed by the usual work-up furnished a mixture, whose major component was identified with bassic acid methyl ester (**1b**) by TLC using different combinations of the solvent systems.

Oxidation of protobassic acid methyl ester triacetate (**2c**). To an ice-cooled pyridine soln (2 ml) of **2c** (30 mg) was added CrO_3-pyridine complex (15 mg-2 ml) and the total mixture was left standing at room temp for 2 h and treated as usual. The product was purified by preparative TLC followed by recrystallization from n-hexane–light petroleum mixture to furnish colourless plates (13 mg) of **2d**, m.p. 164–167°; ν_{max} ($CHCl_3$): 1745 (br.) (OAc, COOMe), 1715 (sh.) (CO) cm^{-1} (Found: C, 69·30; H, 8·43. $C_{37}H_{54}O_9$ requires: C, 69·13; H, 8·47%).

Attempted modified Huang-Minlon reduction[20] *of* **2d**. To a triethyleneglycol soln (3·1 g) of **2d** (20 mg) were added 98·5% hydrazine (2·7 g) and hydrazine dihydrochloride (450 mg), and the soln was heated at 150° for 7 h. After addition of KOH (600 mg), the reaction temp was gradually raised to 200° while distilling off a low boiling material and then the mixture was heated at 220° for 3 h, diluted with water and extracted with ether. The ether extract, after the usual work-up, yielded a mixture which was methylated with ethereal diazomethane and treated as usual to furnish a final product (15 mg). Since the product consisted of a complex mixture as revealed by TLC developing with $CHCl_3$–acetone (1:1), further purification was abandoned.

Isolation of Mi-glycoside I (**3a**)

(1) *Via acid hydrolysis of Mi-saponin.* A soln of Mi-saponin (10 g) in 0·1 N HCl-EtOH (1:1, 100 ml)-benzene (100 ml) mixture was refluxed on a boiling water-bath for 6 h. Benzene layer, after the usual work-up, gave a residue (35 mg), while aqueous portion, after concentration to a half volume *in vacuo*, yielded a ppt which was collected by filtration, washed and dried to give crude Mi-glycoside I (3·9 g). The filtrate was extracted with AcOEt and the AcOEt extract, after the usual work-up, furnished another crop of crude Mi-glycoside I (1·6 g). The combined Mi-glycoside I was then chromatographed on silica gel (400 g). The combined eluates with $CHCl_3$-MeOH (83:17–80:20) mixture furnished mainly Mi-glycoside I (1·9 g), which was recrystallized from MeOH-acetone to give fine crystals of Mi-glycoside I (**3a**), m.p. 300–304°; $[\alpha]_D$ + 38·5° (*c* = 0·78, MeOH); ν_{max} (KBr): 3400 (br.) (OH), 1698 (COOH) cm^{-1} (Found: C, 63·67; H, 8·88. $C_{36}H_{58}O_{11} \cdot \frac{1}{2}H_2O$ requires: C, 64·01; H, 8·74%).

(2) *Via soil bacterial hydrolysis of Mi-saponin.* A soil bacterial strain (YSB-16*) was cultivated at 31° for 27 days on the synthetic medium (1 liter) containing Mi-saponin (3g) as an only carbon source as described above. The total culture broth was extracted with ether, AcOEt, and n-BuOH successively. The ether extract after the usual work-up furnished protobassic acid **2a** (23 mg). Treatment of the AcOEt extract in the usual manner furnished a product (72 mg) which was purified by preparative TLC developing with $CHCl_3$-MeOH (4:1) to give Mi-glycoside I (**3a**; 30 mg). The n-BuOH extract recovered Mi-saponin (2·43 g).

Mi-glycoside I methyl ester (**3b**). A combined fraction containing mainly Mi-glycoside I (1·8 g), obtained by above mentioned column chromatography of acid hydrolysate of Mi-saponin, was treated with ethereal diazomethane to give a product (1·9 g) which was purified by column chromatography on silica gel (100 g). Elution with $CHCl_3$-acetone (1:4) mixture followed by recrystallization from MeOH gave colourless needles (570 mg) of **3b**, m.p. 293·5–295·5°; $[\alpha]_D$ +33·3° (*c* = 0·54, MeOH); ν_{max} (KBr): 3365 (br.) (OH), 1734 (COOMe) cm^{-1} (Found: C, 65·21; H, 8·92. $C_{37}H_{60}O_{11}$ requires: C, 65·29; H, 8·82%).

Mi-glycoside I methyl ester pentaacetate (**3c**). A soln of **3b** (115 mg) in pyridine (3 ml) and Ac_2O (2 ml) was kept at 31° for 20 h and treated as usual. Recrystallization of the product from acetone furnished colourless needles (105 mg) of **3c**, m.p. 237–239°; $[\alpha]_D$ +32·7 (*c* = 0·7, $CHCl_3$); ν_{max} (Nujol): 3560, 3435 (OH), 1760 (br.) (OAc, COOMe) cm^{-1}; mass spectrum *m/e* (%): 331 (iv, 38), 262 (ii, 60), 203 (ii-$COOCH_3$, 100), 169 (v, 77), 109 (vi, 34), 43 (89); PMR (100 MHz): 0·89, 0·93, 1·02, 1·04, 1·36, 1·58 (3 H each, s, six Me's), 1·99, 2·02, 2·04 (15 H, totally five AcO's), 3·59 (3 H, s, COOMe), 4·55 (1 H, d, J = 7, $C_{(12)}$-H), 4·84–5·20 (3 H, m, $C_{(2', 3', 4')}$-H_3), 5·20–5·38 (1 H, m, $C_{(12)}$-H) (Found: C, 63·62; H, 7·85. $C_{47}H_{70}O_{16}$ requires: C, 63·37; H, 7·87%).

Periodate oxidation of Mi-glycoside I methyl ester (**3b**) *giving protobassic acid methyl ester* (**2b**). To a soln of **3b** (60 mg) in MeOH (8 ml) was added a soln of $NaIO_4$ (30 mg) in water (0·5 ml) under N_2 atmosphere at 0°. The total soln was allowed to stand at 0° for 4 h and at room temp overnight, and concentrated *in vacuo*, diluted with water and extracted with ether. After washing with 5% $Na_2S_2O_3$ aq,

*Unidentified yet. The microorganism gave **3a** as a major hydrolysate.

the ether extract was evaporated *in vacuo* to give a product which was dissolved in a mixture of EtOH (8 ml), water (2 ml), and KOH (50 mg). The mixture was then refluxed for 3 h under N_2 atmosphere, diluted with water, acidified with 5% H_2SO_4 aq to pH 3, and extracted with ether. After the usual work-up, the ether extract gave a product (51 mg), which was purified by preparative TLC developing with $CHCl_3$-MeOH (4:1) mixture and recrystallized repeatedly from MeOH-acetone to furnish colourless needles (9 mg) of **2b**. The methyl ester obtained here was identified with protobassic acid methyl ester (**2b**) by m.m.p., IR (KBr), and TLC.

Acid hydrolysis of Mi-glycoside I methyl ester (**3b**). A soln of **3b** (18 mg) in 1 N H_2SO_4-50% EtOH (1:1) mixture (6 ml) was refluxed for 10 h, diluted with water and the ppt was taken up with AcOEt. The aqueous portion was neutralized by treatment with ion exchange resin (Dowex 44 (OH^-), 10 g) and evaporated to dryness. The paper chromatography of the product (10 mg) (solvent system: i-PrOH–n–BuOH–water (7:1:2); detection: aniline hydrogenphthalate) showed the presence of glucose.

Methylation of Mi-glycoside I methyl ester (**3b**).

(1) *Via the Hakomori's procedure.* A stirred mixture of NaH (1 g, washed with light petroleum 3 times beforehand) and DMSO (10 ml) was heated at 50–60° for 1 h under N_2. After cooling to room temp, a soln of **3b** (212 mg) in DMSO (5 ml) was added to the above soln (5 ml) and the mixture was kept stirring at room temp for 4 h, and added with MeI (4 ml) while keeping the reaction temp at 31°. The total mixture was stirred further for 16 h at room temp, poured into water, and extracted with AcOEt and the extract was treated in the usual manner to give a product. The product was methylated 2 more times under the same conditions as above and the final product (210 mg) was purified by preparative TLC developing with benzene-AcOEt (3:1) mixture to give a colourless solid (43 mg) of **3d** (single spot on TLC, but crystallization being without success).

(2) *Via the Kuhn's procedure.* A soln of **3b** (300 mg) in DMF (10 ml) was treated with Ag_2O (3 g) and MeI (5 ml) and the total mixture was kept stirring at 31° for 20 h. After the usual work-up, the crude product was methylated again in the same way and the final product (250 mg) was chromatographed on silica gel (10 g) developing with benzene, benzene-AcOEt, AcOEt successively. The eluate with benzene-AcOEt (10:1) mixture furnished colourless solid (98 mg) of **3d**; $[\alpha]_D$ +28·9° (c = 0·18, $CHCl_3$); ν_{max} (CCl_4): 3600 (OH), 1728 (COOMe), 1100 cm^{-1}; PMR (90 MHz): 0·90, 0·94, 1·04, 1·10, 1·32, 1·56 (3H each, s, six Me's), 3·30, 3·35, 3·38, 3·54 (3H each, s), 3·64 (9H, s) (six MeO's, one COOMe), 3·11 (2H, s-like, $C_{(23)}$-$\underline{H}_2$OMe), 4·41 (1H, m, $W_{\frac{1}{2}}^h$ = 9 Hz, $C_{(6)}$-$\underline{H}$OH), 5·24–5·42 (1H, m, $C_{(12)}$-$\underline{H}$). Since the purity of **3d** was assured by TLC, it was submitted to further reactions described below.

Methanolysis of Mi-glycoside I methyl ester hexamethyl ether (**3d**). A soln of **3d** (40 mg) in 2 N HCl-MeOH (3 ml) was refluxed for 5 h. After cooling, MeOH was removed under reduced pressure and a resulting product was treated with water and extracted with ether. After the usual work-up, the product (36 mg) was chromatographed on silica gel eluting with hexane–ether mixture and ether successively. An oily product (20 mg) obtained by elution with hexane–ether (1:1) mixture was acetylated with Ac_2O and pyridine at room temp and the product was purified by preparative TLC to furnish an oily product of **4** (9 mg, single spot on TLC, but crystallization being without success); $[\alpha]_D$ +27·1° (c = 0·24, $CHCl_3$); ν_{max} (CCl_4): 1732 (COOMe), 1238, 1104 cm^{-1}; PMR (90 MHz): 0·86, 0·89, 0·92, 1·17, 1·26, 1·33 (3H each, s, six Me's), 2·08 (3H, s, OAc), 3·24, 3·33 (3H each, s, two MeO's), 3·63 (3H, s, COOMe), 5·14 (1H, d, J = 4, $C_{(3)}$-$\underline{H}$), 5·35 (1H, t-like, $C_{(12)}$-$\underline{H}$), 5·54 (1H, t-like, $C_{(6)}$-$\underline{H}$) (Found: C, 73·52; H, 9·56. $C_{35}H_{54}O_6$ requires: C, 73·64; H, 9·54%). From the fractions eluted with ether was identified methyl 2, 3, 4, 6-tetra-O-methyl-D-glucopyranoside by GLC (conditions: 3% SE-30 on chromosorb W, column temp 190°, using Hitachi Gas Chromatograph 063 equipped with hydrogen flame ionization detector, detector temp: 215°, N_2 gas flow rate: 30 ml/min), retention time: 1 min 55 sec.

Methylation of protobassic acid methyl ester (**2b**) *followed by acid hydrolysis giving* **5**. Compound **2b** (110 mg) was methylated after the Hakomori's procedure as described above and the crude product was purified by preparative TLC to give an oily product (68 mg); ν_{max} (CCl_4), 3600 (OH), 1720, 1105 cm^{-1}. A soln of the product (48 mg) in 2 N HCl-MeOH (5 ml) was refluxed for 5 h. After the usual work-up, the product was purified by preparative TLC to furnish an oily product of **5** (22 mg, single spot on TLC); $[\alpha]_D$ +70·9° (c = 0·26, $CHCl_3$); ν_{max} (CCl_4): 1728 (COOMe), 1110 cm^{-1}; PMR (90 MHz): 0·84, 0·89, 0·92, 1·05, 1·12, 1·31 (3H each, s, six Me's), 3·30 (3H, s), 3·37 (6H, s) (three MeO's), 3·62 (3H, s, COOMe), 3·70–3·90* (1H, m, $C_{(3)}$-$\underline{H}$), 5·34 (1H, t-like, $C_{(12)}$-$\underline{H}$), 5·52 (1H, t-like, $C_{(6)}$-$\underline{H}$) (Found: C, 75·17; H, 9·66. $C_{34}H_{54}O_4$ requires: C, 75·23; H, 10·03%).

Isolation of Mi-saponin A, B, and C. The crude saponin mixture (50 g) was subjected to an active charcoal–Celite column (100 g of charcoal: 100 g of Celite 535) chromatography with an aid of MeOH to furnish Mi-saponin (45·7 g). Mi-saponin (40 g) was then chromatographed on silica gel (800 g) developing with n-BuOH sat. with water to furnish (in the order of elution) Mi-saponin A(3·0 g), Mi-saponin B(3·1 g), Mi-saponin C(0·7 g) and mixtures of them (totally 24·4 g). Mi-saponin A(amorphous), $[\alpha]_D$ −33·1° (c = 1·10, MeOH); ν_{max} (KBr): 3375 (br.) (OH), 1736, 1040 (br.) cm^{-1}. Mi-saponin B, m.p. 250–253° (crystallized from MeOH-n-BuOH sat. with water); $[\alpha]_D$ −45·0° (c = 1·22, MeOH); ν_{max} (KBr): 3380 (br.) (OH), 1754, 1050 (br.) cm^{-1}. Mi-saponin C (amorphous), $[\alpha]_D$ −37·2° (c = 0·95, MeOH); ν_{max} (KBr): 3355 (br.) (OH), 1730, 1050 (br.) cm^{-1}.

Soil bacterial hydrolysis of Mi-saponin A, B, and C. The soil bacterium (YSB-16) was cultivated at 31° for 20 days on the above described synthetic medium (100 ml) containing Mi-saponin A(0·3 g) as an only carbon source and the total culture broth was extracted with ether and AcOEt successively. Treatment of the ether extract in the usual manner gave a product (30 mg) which was methylated with ethereal diazomethane and recrystallized from acetone to furnish colourless needles being identical with protobassic acid methyl ester (**2b**) by m.m.p., IR (KBr), and TLC. The AcOEt extract, after the usual work-up, gave a product (22 mg) which was treated with ethereal diazomethane followed by acetylation to furnish colourless needles being identical with Mi-glycoside I methyl ester pentaacetate (**3c**) by m.m.p., IR (KBr), and TLC. The same hydrolysis method was applied to Mi-saponin B and Mi-saponin C respectively and protobassic acid (**2a**) and Mi-glycoside I (**3a**) were obtained by the ordinary procedures and identified with au-

*Unclear due to the overlapping.

thentic samples such as **2b**, **3b**, or **3c** by m.m.p., IR, (KBr), and TLC respectively.

Alkaline treatment of Mi-saponin B. A soln of Mi-saponin B(100 mg) in 20% KOH-EtOH (1:1) mixture (60 ml) was refluxed for 3 h, acidified with 5% H_2SO_4 aq, and diluted with water while evaporating EtOH under reduced pressure. The resulting aqueous mixture was extracted with AcOEt and n-BuOH successively. The AcOEt soluble portion (30 mg), which was shown by TLC to contain mainly Mi-glycoside I, was treated with ethereal diazomethane and acetylated with Ac_2O (2 ml) and pyridine (5 ml) at 31° for 40 h to give a product (28 mg). The product was then crystallized repeatedly from MeOH and identified with Mi-glycoside I methyl ester pentaacetate (**3c**) by m.m.p., IR (KBr), and TLC ($CHCl_3$–MeOH = 4:1). The n-BuOH soluble portion was revealed by TLC (n-BuOH–AcOH–water = 4:1:5, upper layer) to contain mainly sugar-like substances along with a trace amount of Mi-glycoside I.

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