Molluscicidal Triterpenoidal Saponin from *Lysimachia sikokiana*

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The main molluscicidal activity of the methanol extract of *Lysimachia sikokiana* is due to several triterpenic saponins called sakuraso-saponins. The most active component was isolated from the aerial parts and elucidated as 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranosyl-(1→4)-[β-glucopyranosyl-(1→2)]-α-arabinopyranosyl protoprimulagenin A, named lysikoianoside 1, on the basis of ^{1}H- and ^{13}C-nuclear magnetic resonance spectral data and methylation analysis results.

Keywords molluscicidal; *Lysimachia sikokiana*; lysikokianoside 1; sakuraso-saponin; *Oncomelania nosophora*

In connection with our systematic isolation and structure studies on biologically active constituents from crude drugs and medicinal plants, we have already reported some results of preliminary screening for molluscicidal activity.[1] Among the active extracts, *Lysimachia* (*L.*) *sikokiana* MIQUEL (Japanese name: morokoshiso) was selected for further study.

L. sikokiana belongs to Primulaceae and is a perennial herb growing wild in the southern parts of Japan. It is used to repel mosquitos in some areas of the Loochoo islands. Roots of some kinds of plants belonging to Primulaceae are recognized as valuable expectorants.[2] Saponin and sapogenin constituents of the Japanese Primulaceous plants have already been investigated extensively by Kitagawa *et al.*[3] and those of European plants by Tschesche and coworkers.[4] The chemical components of *L. sikokiana* have not yet been investigated.

In this paper we describe the isolation and structure determination of the main molluscicidal active principle of *L. sikokiana* and the molluscicidal activity of related compounds against *Oncomelania nosophora*, the intermediate host of the Japanese strain of *Shistosoma japonicum*.

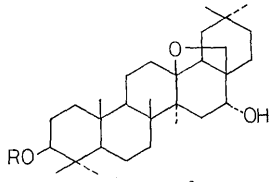
The molluscicidal activity of each fraction was checked at each stage of the isolation process. A suspension of the MeOH extract of the dried aerial parts of *L. sikokiana* was fractionated using highly porous polymer, DIAION HP-20, and successively eluted with 30% aqueous MeOH, 50% aqueous MeOH, 80% aqueous MeOH, 100% MeOH, and $CHCl_3$. The 100% MeOH fraction, which is the crude saponin fraction, showed strong molluscicidal activity, which was found to be lethal to the snail *Oncomelania nosophora* at the concentration of 800 ppm. The 100% MeOH fraction was chromatographed as described in the experimental section and was found to contain about seven main saponins. Of these saponins, only three were found to be molluscicidally active saponins and finally were purified to give the most active compound, named lysikokianoside 1 (**1**).

Inspection of the ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra (Table I) indicated that lysikokianoside 1 has four monosaccharide units. The signals due to the aglycone moiety were in good agreement with those of the aglycone of sakuraso-saponin,[3,5] which was found to have a 13β,28-oxide moiety. The carbon signals due to the sugar moiety of **1** were found to be almost superimposable on those of desglucoanagalloside A.[6] On acid hydrolysis, **1** afforded arabinose, glucose and xylose, but the genuine aglycone could not be obtained owing to acid-catalyzed modification. On enzymatic hydrolysis with crude hesperidinase,[7] **1** yielded an aglycone (**2**). Comparison of the ^{13}C-NMR spectrum (Table I) of **2** with that of authentic protoprimulagenin A revealed that **2** was protoprimulagenin A.[3] This compound has already been

TABLE I. ^{13}C-NMR Chemical Shifts of Protoprimulagenin A (PPA), Lysikokianoside 1 and the Sugar Moiety of Desglucoanagalloside B (DGA-B)[6] in C_5D_5N

	PPA	Lysikokianoside 1	3-O-Sugar moiety	Lysikokianoside 1	DGA-B
C-1	39.6	39.2	(Terminal)		
C-2	28.4	26.6	X-1	107.6	107.8
C-3	78.1	89.0	X-2	76.0[a]	76.2
C-4	39.6	39.7	X-3	77.7	77.9
C-5	55.8	55.7	X-4	70.6	70.7
C-6	18.3	18.0	X-5	67.4	67.5
C-7	31.9	31.8			
C-8	42.6	42.4	G-1	104.9	105.0
C-9	50.7	50.5	G-2	76.2[a]	76.2
C-10	37.3	36.9	G-3	78.3[b]	78.4
C-11	19.3	19.2	G-4	71.8	71.9
C-12	34.6	34.5	G-5	78.2[b]	78.4
C-13	86.5	86.4	G-6	63.0	63.1
C-14	44.7	44.6			
C-15	37.0	36.9	(Inner)		
C-16	77.2	77.1	G-1	104.6	104.9
C-17	44.7	44.6	G-2	85.3	85.5
C-18	51.6	51.5	G-3	77.5	77.7
C-19	39.1	39.0	G-4	71.1	71.1
C-20	31.9	31.8	G-5	78.6	78.7
C-21	36.9	36.8	G-6	62.3	62.4
C-22	33.0	32.9			
C-23	28.7	28.1	A-1	104.2	104.2
C-24	16.6	16.6	A-2	79.7	79.8
C-25	16.4	16.5	A-3	73.2	73.4
C-26	18.7	18.5	A-4	77.9	78.0
C-27	19.6	19.6	A-5	64.2	64.3
C-28	78.0	77.9			
C-29	33.8	33.8			
C-30	24.8	24.8			

δ (ppm) relative to TMS. *a*) and *b*) may be reversed in each column. X = xylopyranosyl; G = glucopyranosyl; A = arabinopyranosyl.



1: R = α-arabinose(p)^{2}—β-glucose
$^{1}_{4}$
β-glucose^{2}—β-xylose(p)

2: R = H

Chart 1. Structure of Lysikokianoside 1

isolated from the hydrolysate of the *Primula sieboldii* (Japanese name: sakuraso).[3]

The molecular weight of **1** was determined by negative fast-atom bombardment mass spectrometry (negative FAB-MS). A molecular weight of 1046 was confirmed by an intense peak at *m/z* 1045 in the compound's negative-ion spectrum, corresponding to the $(M-H)^-$ ion. Furthermore, negative fragment ions at *m/z* 913 and 883 may be ascribed to the loss of a terminal pentose (xylose or arabinose) and a terminal glucose unit, respectively. A fragment ion at *m/z* 751 may be attributed to the loss of a terminal glucose–pentose disaccharide and/or, though it seems less likely, both a terminal pentose and a terminal glucose unit. Fragment ions corresponding to loss of a pentose–pentose or glucose–glucose or a trisaccharide unit were not observed.

Permethylation of **1** using *tert*-BuONa–MeI in dimethylsulfoxide (DMSO) afforded the permethylated product, which on acid hydrolysis liberated methyl pyranosides of 2,3,4,6-tetra-*O*-methylglucose, 3,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylxylose, and 3-*O*-methylarabinose. The attachment of the carbohydrate moiety at the C-3 position of **2** was evident from the ^{13}C-NMR glycosylation shift[8] at C-3 of **1** (Table I). These observations led to the formulation of **1** as shown in Chart 1. Though glucuronide saponins are common in Primulaceae, **1** is not that.

Compound **1** was found to be lethal to the snail *Oncomelania nosophora* at the concentration of 2 ppm. Saikosaponins a, c and d, having the 13β,28-oxide moiety, are related compounds and are found widely in *Bupleurum* spp. Saikosaponins a and d were shown to be lethal to the snail at the concentration of 20 ppm, while saikosaponin c had no effect at the same concentration. But neither saikosaponin a nor d had molluscicidal activity at the concentration of 5 ppm.

Finally, **1** did not show a repellent effect against mosquitos. Investigation of the other saponins is in progress.

Experimental

Bioassay of Molluscicidal Activity Bioassay was done with snails of the species *Oncomelania nosophora*. The bioassay method has already been reported in the previous paper.[1] The powder-like sample obtained by lyophilization was tested for its activity at various concentrations.

General Methods Optical rotation was measured with a Union PM-101 automatic polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-400 spectrometer in C_5D_5N solution using tetramethylsilane (TMS) as an internal standard. For gas liquid chromatography (GLC), a Shimadzu GC-6A apparatus was used with a glass column of 1.5% SE-30 on Chromosorb W, 4 mm × 2 m; detector, FID; injection temperature, 200 °C; column temperature, 180 °C; carrier gas, N_2, 1.0 kg/cm^2. GC-MS was taken on a JEOL JMS-SX102 spectrometer: column, Neutra Bond-1 25 m × 0.25 mm i.d. 0.4 μm, ionizing volt 70 eV, He 1 ml/min., injection temp. 200 C; FAB-MS was performed on a JEOL JMS D-300 instrument. For column chromatography, Kieselgel 60H (Art. 7736, Merck), LiChroprep RP-18 (25—40 μm) and Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd., Tokyo, Japan) were used. All solvent systems for chromatography were homogeneous.

Extraction and Separation *L. sikokiana* was collected in Tanegashima island in 1984 and cultivated at the Experimental Station of Medicinal Plants, Hiroshima University School of Medicine. The dried aerial parts of *L. sikokiana* (1 kg) were extracted with boiling MeOH. A suspension of the MeOH extract was fractionated using highly porous polymer, DIAION HP-20, and successively eluted with 30% aqueous MeOH, 50% aqueous MeOH, 80% aqueous MeOH, 100% MeOH, and $CHCl_3$. The 100% MeOH fraction (17.6 g) was separated by silica gel column chromatography with $CHCl_3$–MeOH–H_2O (15 : 4 : 0.4 and 15 : 8 : 1.2, homogeneous, successively). One of the biologically active fractions, fr. 34, was subjected to reversed-phase (LiChroprep RP-18) column chromatography with H_2O–MeOH (25 : 75) to give 2 fractions, frs. A and B in order of elution. Fraction B was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (15 : 6 : 1, homogeneous) to give **1** (123 mg), named lysikokianoside.

Lysikokianoside 1: A white powder, $[\alpha]_D^{17}$ −10.5° (c = 0.76, C_5H_5N). *Anal.* Calcd for $C_{52}H_{86}O_{21}$: C, 59.66; H, 8.22. Found: C, 59.70; H, 8.20; ^{1}H-NMR (in C_5D_5N) δ: 0.87, 0.98, 1.07, 1.08, 1.21, 134, 1.52 (each 3H, s, *tert*-CH_3 × 7), 4.78 (1H, d, J = 5.9 Hz, anomeric H of arabinose), 4.92 (1H, d, J = 7.0 Hz, anomeric H of terminal glucose), 4.96 (1H, d, J = 7.7 Hz, anomeric H of glucose), 5.41 (1H, d, J = 7.3 Hz, anomeric H of xylose). ^{13}C-NMR data of lysikokianoside 1 are given in Table I.

Enzymatic Hydrolysis of Lysikokianoside 1 A solution of **1** (50 mg) and crude hesperidinase (50 mg, Tanabe Pharm. Co., Ltd., Osaka, Japan) in phosphate buffer (pH 4) was incubated at 40 °C for 2 d. The reaction mixture was diluted with water and then extracted with 1-BuOH. The 1-BuOH extract was washed with H_2O and evaporated to dryness, and the residue was subjected to chromatography on silica gel to give the aglycone **2** (3 mg). This product was identified as protoprimulagenin A by mixed melting point determination and by comparison of the spectral data with those of an authentic sample. The aqueous layer was passed through the column of Amberlite MB-3 and evaporated to dryness. The carbohydrate components of the hydrolysate were identified as glucose, arabinose and xylose by thin layer chromatography.

Acid Hydrolysis of Lysikokianoside 1 Hydrolysis of **1** (3 mg) was carried out with 10% HCl in H_2O–dioxane (1 : 1) at 80 °C for 2 h. The reaction mixture was concentrated to dryness by blowing N_2 gas over it at room temperature. The residue was trimethylsilylated (TMSi) with *N*-trimethylsilylimidazole (0.5 ml) in a sealed micro-tube at 80 °C for 2 h. The reaction mixture was diluted with H_2O and then extracted with *n*-C_6H_{14}. The *n*-C_6H_{14} layer was washed with H_2O and concentrated to dryness by blowing N_2 gas over it at room temperature. A solution of the residue in *n*-C_6H_{14} was subjected to GLC analysis. TMSi-glucose, TMSi-arabinose and TMSi-xylose were identified by comparison of the retention times with those of authentic samples.

Permethylation of Lysikokianoside 1 A mixture of 10 mg of **1**. 1 ml of dry DMSO, 50 mg of dry *tert*-BuONa, 10 mg of finely powdered dry NaOH and 1 ml of CH_3I was stirred at room temperature for 1 h. The reaction mixture was poured into ice water and extracted with diethyl ether. The extract was washed with saturated NaCl and water, dried over anhydrous Na_2SO_4, and evaporated to yield a gummy residue. This was chromatographed on silica gel to give a permethylated product. This was hydrolyzed by refluxing in 10% HCl in aqueous MeOH for 3 h. The reaction mixture was cooled, and evaporated to dryness under reduced pressure. The residue was dissolved in water, and the solution was filtered. The filtrate was neutralized with Ag_2CO_3 and filtered. The neutral filtrate was concentrated, and reduced with $NaBH_4$. The residue was acetylated with Ac_2O–pyridine (1 : 1) at water-bath temperature for 1 h, dried *in vacuo*, and subjected to GLC analysis. The four peaks were identified as the alditol acetates of 2,3,4,6-tetra-*O*-methylglucose, 2,3,4-tri-*O*-methylxylose, 3,4,6-tri-*O*-methylglucose and 3-*O*-methylarabinose.

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