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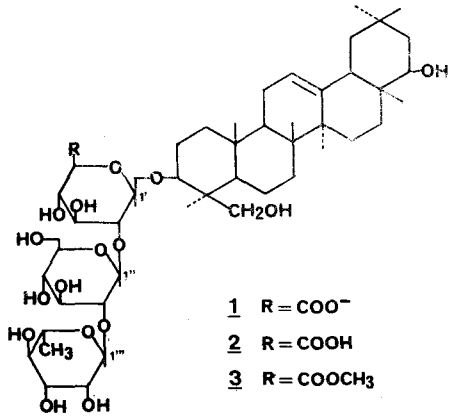
Isolation of Azukisaponin V Possessing Leucocyte Migration Inhibitory Activity from *Melilotus officinalis*

Sam Sik Kang, Young Soon Lee and Eun Bang Lee
Natural Products Research Institute, Seoul National Universisty, Seoul 110, Korea

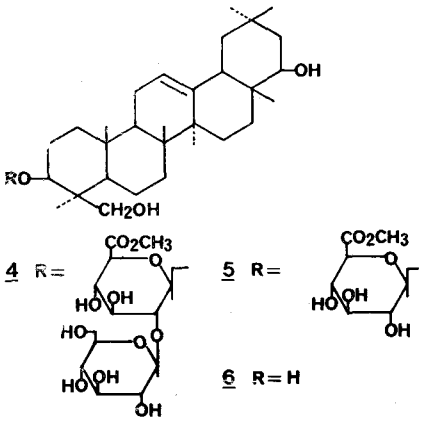
Abstract—Chemical investigation of the inhibitory compound on leucocyte migration from *Melilotus officinalis* has led to the isolation and characterization of azukisaponin V (3-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyransosyl(1→2)-β-D-glucuronopyranosyl]-soyasapogenol B) as the carboxylate form, which exhibits potent leucocyte migration inhibitory activity at a dose of 6mg/rat.

Keywords—*Melilotus officinalis* • Leguminosae • Azukisaponin V • Leucocyte migration

The Melilotus extract obtained from *Melilotus officinalis* Lam. (Leguminosae) is used as a medicine to relief of inflammation. We found that water insoluble fraction from the commercially available Melilotus extract showed potent inhibitory activity on leucocyte migration. This paper deals with the isolation and characterization of chemical constituents from *Melilotus officinalis* with monitoring for inhibitory activity on leucocyte migration in animals. Commercially available Melilotus extract was fractionated and bioassayed. The dose of each fractions was applied in proportion to the yield. As shown in Table I, fractions B and C showed inhibitory effect on leucocyte migration. It is noted that fraction B showed inhibitory action at a small doses of 2.2 and 4.4 mg/rat. In a preliminary screening, this fraction comprised a mixture of saponins and flavonoid glycosides.[1] On the basis of the above results, the BuOH fraction of MeOH extract from the aerial parts of *Melilotus officinalis*, which was equal to the fraction B[1], was chromatographed over SiO_2 column and eluted with $CHCl_3$-MeOH-H_2O(520 : 280 : 80, lower phase) to yield pure compound. The main fraction was recrystallized from MeOH to amorphous white (**1**), mp<300°. The physical and IR spectrum



1 R=COO⁻
2 R=COOH
3 R=COOCH3



4 R=

5 R=

6 R=H

Table I. The effect of fractions B, C and D and aspirin on leucocyte migration in CMC pouch of rats

Sample	Dose(mg/rat, sc)	No. of animals	No. of leucocytes/ml ($\bar{M}\pm$S.E.)	Inhibition percent
Control	—	5	8343.8±685.9	—
Fraction B	2.2	5	4303.4±619.8*	48.4
	4.4	5	3678.4±538.0*	55.9
Fraction C	26	5	4718.8±575.6*	43.5
	52	5	3500.0±392.4*	58.1
Fraction D	172	5	8976.8±337.0	−7.6
	344	5	5964.8±941.0	28.5
Aspirin	15	5	5039.5±272.9*	39.6

* Significantly different from the control group ($p<0.01$).

of **1** was suggested that it consisted of the carboxylate form and therefore treated with weak sulfuric acid to give pure acidic compound (**2**)[2], mp 232~3°. Its IR spectrum showed strong peak at 3,410 cm^{-1} for hydroxyl groups and carboxyl at 1,728 cm^{-1}. Acidic hydrolysis

Table II. ^{13}C-NMR spectra of compounds **2-6** in pyridine-d_5

Carbon	6[2]	2	3	4	5	Carbon	6[2]	2	3	4	5
1	39.0	38.7	38.9	39.0	39.0	26	17.1	17.2	17.2	17.2	17.2
2	28.5	26.6	26.7	26.6	27.0	27	25.7	25.8	25.8	25.8	25.8
3	80.3	91.4	91.4	91.1	89.5	28	28.7	28.8	28.8	28.8	28.8
4	43.3	43.9	44.0	44.0	44.6	29	33.3	33.3	33.3	33.3	33.3
5	56.5	56.5	56.5	56.5	56.4	30	21.2	21.1	21.2	21.2	21.2
6	19.2	18.8	18.9	18.8	18.8						
7	33.6	33.5	33.5	33.5	33.7	1′		105.3	105.3	105.2	106.6
8	40.1	40.1	40.2	40.2	40.2	2′		78.3	78.3	81.9	75.4
9	48.2	48.0	48.0	48.0	48.1	3′		78.4	78.3	78.2	78.0
10	37.1	36.6	36.7	36.7	36.9	4′		73.6	73.5	72.6	73.3
11	24.1	24.2	24.3	24.2	24.2	5′		77.8	76.8	77.1	77.4
12	122.7	123.3	122.9	123.3	123.3	6′		172.4	170.3	170.3	170.3
13	145.0	144.9	145.0	145.0	145.0	(OCH_3)			52.2	52.2	52.2
14	42.4	42.6	42.6	42.6	42.6	1″		102.2	102.2	104.9	
15	26.5	26.6	26.7	26.6	27.0	2″		79.0	78.6	75.8	
16	28.8	28.8	28.8	28.8	28.8	3″		78.0	78.0	78.6	
17	38.1	38.1	38.2	38.1	38.2	4″		72.4	72.4	70.1	
18	45.5	45.6	45.6	45.6	45.6	5″		78.5	78.4	78.3	
19	46.9	46.8	47.0	47.0	47.0	6″		62.9	61.5	61.8	
20	30.9	30.9	31.0	31.0	31.0	1‴		102.0	102.0		
21	42.5	42.6	42.6	42.6	42.6	2‴		72.4	72.4		
22	75.7	75.8	75.8	75.8	75.8	3‴		72.7	72.8		
23	23.6	22.9	22.9	22.8	23.4	4‴		74.4	74.4		
24	64.7	63.7	63.7	63.5	63.5	5‴		69.4	69.5		
25	16.3	15.8	15.8	15.8	15.8	6‴		18.9	18.9		

Table III. The effect of azukisaponin V and aspirin on leucocyte migration in CMC pouch of rats

Sample	Dose(mg/rat, sc)	No. of animals	No. of leucocytes/ml ($\bar{M}\pm$S.E.)	Inhibition percent
Control	—	5	6060±695	—
Azukisaponin V	2	5	4675±272	29.6
	6	5	3060±662*	49.5
Aspirin	20	5	1867±296**	69.2

Significantly different from the control group at *$p<0.01$ and **$p<0.001$.

of the saponin(**2**) yielded soyasapogenol B(**6**), mp 259~260°, as the genin indentified by direct comparison with an authentic sample[1] (tlc, mmp and ^{13}C-NMR) and glucuronic acid, glucose and rhamnose. It gave a methylester(**3**), mp 241~3°, on CH_2N_2 methylation. Partial hydrolysis of the methylester(**3**) afforded prosapogenins I(**5**) and II(**4**) together with soyasapogenol B(**6**). Prosapogenin I(**5**), mp 246~7°, gave glucuronic acid and soyasapogenol B(**6**) on acid hydroloysis and identified as 3-O-β-D-glucuronopyranosyl soyasapogenol B methylester[3] on the basis of ^{13}C-NMR spectral data (see Table II). It was confirmed by comparison with the published data[3]. Prosapogenin II(**4**), mp 249~253°, was acid hydrolized to give glucose and glucronic acid besides soyasapogenol B(**6**). The ^{13}C-NMR spectrum of **4** showed signals for glucose, glucuronic acid methylester and soyasapogenol B. The C-2′ signal of glucuronic acid moiety was deshielded (6.5 ppm) than prosapogenin I(**5**). Consequently, prosapogenin II(**4**) was suggested to possess a terminal β-D-glucopyranosyl residue attached to the C-2′ hydroxyl function in the prosapogenin I(**5**)[4]. In the light of these observations, structure of prosapogenin II(**4**) has been assigned as 3-O-[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-soyasapogenol B methylester. Prosapogenin II(**4**) was identified as azukisaponin II methylester by comparison with the published data[5] which isolated from the seeds of *Vigna angularis* as desmethyl form[5]. The presence of significant glycosidation shift of C-2″ signal of the glucopyranoside moiety (3.2 ppm in **2** and 2.8 ppm in **3**, respectively) together with a set of signals for terminal rhamnose moiety in the ^{13}C-NMR spectra of **2** and **3** indicated that the terminal rhamnose moiety in **2** was bound to C-2″ hydroxyl group in the prosapogenin II (**4**)[6]. All spectral data indicated the saponin(**2**) to be 3-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-soyasapogenol B, which is identical to azukisaponin V[7]. This saponin was previously isolated from azuki beans, the seeds of *Vigna angularis* as free form.[5] The carboxylate form of azukisaponin V is the first isolation from the plant source and the present report confirms the assigned structure from ^{13}C-NMR spectral data.

The effect of azukisaponin V on leucocyte migration was showed in Table III. This compound was the main constituent of the fraction B and showed remarkable inhibition at a dose of 6 mg/rat. However, it is assumed that any other potent inhibitory substance(s) in fraction B might be included. Further studies on this fraction are in progress.

Experimental

Melting points were determined on a Mitamura-Riken apparatus and are uncorrected. The IR spectra were obtained with a Perkin-Elmer 283B spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded in CD_3OD and pyridine-d_5 at 80 MHz and 20 MHz, respectively, with a Varian FT-80A spectrometer, and chemical

shifts are given as ppm with TMS as an internal standard.

Plant Materials

Dried powder of the Melilotus extract imported from West Germany was obtained from Sam Jin Pharmaceutical Co., Ltd., Seoul and the aerial parts of *M. officinalis* were purchased from Apotheke im Stathaus, Bonn, West Germany. The plant material was identified by Prof. H.J. Chi of our institute.

Fractionation of Melilotus extract

Melilotus extract(100 g) was refluxed with anhydrous EtOH(700 ml) for 3hr(×3) and filtered. The filtrate was concentrated *in vacuo*(fraction A; 14.1 g). The fraction A was suspended in H_2O and filtered. The precipitate was dried to yield powder(fraction B; 1.2 g). The H_2O soluble portion was concentrated *in vacuo* and lyophilized(fraction C; 12.9 g). The marc after refluxing with EtOH was dried to afford fraction D(85.9 g).

Isolation of Saponin

The chopped aerial parts of *M. officinalis* (800 g) was extracted and fractionated as previously described[1]. A portion of BuOH fraction was chromatographed over a flash column(SiO_2) and eluted with $CHCl_3$-MeOH-H_2O (520 : 280 : 80, lower phase) to give subfractions. The main subfraction was concentrated and recrystallized from MeOH to afford amorphous white(**1**).

Mp<300°;

IR ν_{max}^{KBr}: 3400(OH), 1610(COO^-), 1070, 1040(C—O), 805(C=O).

The compound(**1**) was hardly soluble in MeOH, EtOH, H_2O, pyridine and dioxane. The saponin(**1**) was dissolved in 0.02 N-H_2SO_4 in 60% dioxane at room temperature and filtered to remove the precipitate. The filtrate was concentrated to the half volume *in vacuo* and added to the crushed ice. The precipitate was filtered, washed and dried. The dried precipitate was dissolved in EtOH followed by recrystallization to yield pure saponin(**2**) as flakes.

Mp 232~3°(Lit.[7] mp 228~9°);

IR ν_{max}^{KBr}: 3410(OH), 1728(COOH), 1070, 1045(C—O), 1635, 805(C=C).

Acid Hydrolysis of 2

A solution of **2**(40 mg) in 5%-H_2SO_4 in dioxane-H_2O(2 : 1, 25 ml) was refluxed for 4 hr. After cooling, $BaCO_3$ was added with stirring and filtered. The filtrate was extracted with ether. The ether layer was concentrated and recrystallized from MeOH to give pure sapogenin(**6**) as needles.

Mp 259~260°(Lit.[1] mp 259~260°).

It was identified as soyasapogenol B(**6**) by direct comparison with an authentic sample(tlc, mmp and ^{13}C-NMR)[1]. The aqueous layer was concentrated *in vacuo*. Rhamnose, glucose and glucuronic acid were detected by tlc(precoated cellulose plate, pyridine-EtOAc-HOAc-H_2O= 36 : 36 : 7 : 21, Rf 0.67 for rhamnose, 0.46 for glucose and 0.15 and 0.56 for glucuronic acid).

Methylation of 2

A sample of **2**(30 mg) was methylated in MeOH solution with ethereal CH_2N_2. After usual workup, the reaction product was crystallized from MeOH to give **3** as shining plates.

Mp 241~3°;

IR ν_{max}^{KBr}: 3400(OH), 1745($COOCH_3$), 1640, 810(C=C), 1072, 1040(C—O).

^1H-NMR(80 MHz, CD_3OD)δ: 0.83, 0.88, 0.90, 0.98, 1.00, 1.12, 1.21(3H, s, CH_3), 1.25(3H, d, J=6.2Hz, rha-CH_3), 3.77(3H, s, OCH_3), 5.21(1H, t-like, J=3Hz, H-12).

Partial Hydrolysis of 3

A solution of **3**(210 mg) in 0.1 N-H_2SO_4 in 75% dioxane(15 ml) was refluxed for 1.5 hr, to which crushed ice was added and filtered. The precipitate was washed with H_2O and chromatographed over SiO_2 column. Elution with $CHCl_3$-MeOH(6 : 1) gave 10 subfractions. Subfraction No. 7 was recrystallized from MeOH

to afford prosapogenin II(**4**) as plates(50 mg).

Mp 249~253°(Lit.[5] mp 247~250°);

IR ν_{max}^{KBr}: 3460, 3400(OH), 1735($COOCH_3$), 1650(C=C), 1090, 1080, 1053, 1031(C—O).

Subfraction No. 5 was rechromatographed over SiO_2 column, followed by elution with $CHCl_3$-MeOH(9 : 1) and crystallized from MeOH to prosapogenin I(**5**) as needles(20 mg).

Mp 246~7°(Lit.[3] mp 242~6°);

IR ν_{max}^{KBr}: 3420(OH), 1745($COOCH_3$), 1640 (C=C), 1092, 1052, 1030(C—O).

Acid Hydrolysis of 4 and 5

Acid hydrolysis of **4** and **5** was separately performed by refluxing each prosapogenin(4 mg) with 5% H_2SO_4 in 60% dioxane for 4 hr. Soyasapogenol B(**6**) was identified as the genin in each case. Glucuronic acid from **5** and glucuronic acid and glucose from **4** were detected by tlc as described above.

Leucocyte Migration into CMC Pouch in Rats

The effect of the test samples on leucocyte migration into CMC pouch in rats was tested with modification of the method of Ishikawa *et al.*[8] Two percent CMC solution in physiological saline was sterilized. Five ml of CMC solution was injected into the air sac which was made before 24 hr by injecting subcutaneously on the dorsum of rat with 7 ml of air. In the treatment group, the samples suspended in sterilized 2% CMC solution was injected likewise into the air sac. Six hr after the treatment, the animal was sacrificed and the whole fluid in the sac was collected. For staining the leucocytes 0.5 ml of pouch fluid was mixed with 4.5 ml of Türk solution for 10 min and the number of leucocytes was counted with hemocytometer.

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