A NEW TRITERPENOID SAPONIN FROM THE FLOWERS OF *MELILOTUS ALBA*, WHITE SWEET CLOVER[1]

Gilles Nicollier

Department of Biochemistry, Mississippi State University,
Mississippi State, MS 39762

and

A. C. Thompson

Boll Weevil Research Laboratory, USDA, ARS, P. O. Box 5367,
Mississippi State, MS 39762

ABSTRACT.—A new triterpenoid saponin, 2-β-[L-rhamnopyranosyl-(1→4)-β-D-gluco-pyranosyloxy-(1→2)]-3-β-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyloxy-(1→3)]-28-trihydroxyolean-12-ene, has been isolated and identified from the methanolic extract of *Melilotus alba*.

Melilotus alba, white sweet clover (Leguminosae), a widely distributed plant in the United States, is a source of feed for animals, a cover crop, and a source of nectar for the honey bee. The plant has been shown to be phytotoxic to itself and other plants [1–3]. The major compounds found were coumarins, their derivatives and flavonoids [3–5]. Also, a compound, assumed to be a saponin, was isolated from white sweet clover seed [6].

TABLE 1. Carbon-12 chemical shifts of triterpenoid (4) (melilotin) and 2, 3β, 28-trihydroxyolean-12-ene in C_5D_5N and $CDCl_3$.

Compound	Carbon No.					
	1	2	3	4	5	6
2, 3β, 28-trihydroxyolean 12-ene...	47.70	66.00	78.90	38.80	48.20	18.60
Triterpenoid (4) = Melilotin.......	48.80	91.83	92.62	40.98	57.46	17.91
	7	8	8	10	11	12
2, 3β, 28-trihydroxyolean 12-ene...	33.80	38.20	47.60	37.14	22.57	122.0
Triterpenoid (4) = Melilotin.......	33.69	39.70	47.83	38.76	23.55	122.0
	13	14	15	16	17	18
2, 3β, 28-trihydroxyolean 12-ene...	144.0	41.48	27.83	22.88	46.30	43.0
Triterpenoid (4) = Melilotin.......	144.1	43.09	27.26*	26.37	46.43	43.39
	19	20	21	22	23	24
2, 3β, 28-trihydroxyolean 12-ene...	46.38*	31.00	34.03	33.20	28.34	21.90
Triterpenoid (4) = Melilotin.......	44.70	31.46	37.46	33.69	29.74*	16.53
	25	26	27	28	29	30
2, 3β, 28-trihydroxyolean 12-ene...	16.90	17.00	26.12	72.00	34.03	22.49
Triterpenoid (4) = Melilotin.......	17.91	19.58	27.26	72.24	34.22	21.46

*Can be reversed.

RESULTS AND DISCUSSION

The aglycone (1) gave a M+ 458 on CI and FD-MS. Electron impact ms gave a strong M-18 (440) with the most abundant ions at m/z 234, 224, 217, 203 and 233. These fragments suggest either a trihydroxyolean-12-ene or an urs-12-ene analog, with two OH substituents in rings A, B and one in rings D, E. Both structures will give a retro-Diels Alder cleavage of ring C [8]; however, the oleanene structure was confirmed by ^{13}C-nmr (table 1) with a shift of C–12 and C–13 signals

at 122–143 ppm.   Doddrel et al. [9] found the value of the C–12 and C–13 signals of ursene to be 125–138 ppm.   Proton resonance of the aglycone gave a triplet δ 5.25–5.20), which was ascribed to H–12.   A quartet at δ 4.27–4.13 ($W_{1/2}$ 8 Hz) is due to the C–2 hydrogen in the 2β, 3β according to Cheung and Yan [10], and the downfield shifts of 1 ppm on acetylation.   At δ 3.44–3.25, the signal characteristic of an AB (q, $J=11$ Hz) can be attributed to CH_2–OH (C–28).   A broad signal at δ 3.35–3.30) resolved to a doublet on D_2O exchange ($J=4$ Hz) is observed for H–3.   The attribution of the hydroxyls groups in C–3 and C–2 were made by use of the ¹H-nmr spectra of aglycone (3) as well as 2β-hydroxy-3β, 28-diacetoxyolean-12-ene and 3β-hydroxy-2β, 28-diacetoxyolean-12-ene.   All these results and the melting point are in agreement with the structure 2β, 3β, 28-trihydroxyolean-12-ene called 3-*epi*-maslinol, found by Ramaiah and Bai [11], and the ¹³C-nmr data (table 1) are very close to those of Seo *et al.* [12] for methyl 3-epimaslinate.

In order to determine the position of attachment of the sugar moieties, the saponin (4, fig. 1) was methylated with diazomethane then hydrolyzed by 6N
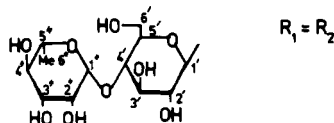
AGLYCONE

(1) : R_1 , R_2 , R_3 = H

(2) : R_1 , R_2 = H R_3 = CH_3

(3) : R_1 , R_2 = H R_3 = acetyl
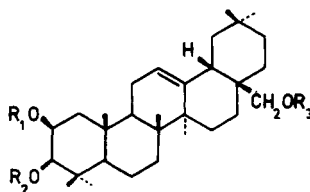
Triterpenoid saponin

(4) R_3=H R_1,R_2=rhamnose (1—4) glucose

(5) R_3=H R_1,R_2=glucose

(6) R_1,R_3=H R_2=glucose

HCl.   Ei-ms of the product gave M^+ 472 and fragments at m/z 248, 216 indicative of rings D+E, which shows that the 28–OH group of melilotin is not glycosylated. Mild hydrolysis of the acetylated saponin (4) gave a fragment m/z 224 corresponding to ring A+B indicating sugar moieties at C–2 and C–3.   A downfield shift of the signal for C–2 and C–3 of melilotin to 91.83 and 92.62 was observed in the ¹³C-nmr spectrum indicative of sugars in the C–2 and C–3 position.   The ¹³C-nmr spectrum of triterpenoid (6), with only the C–3 position occupied, showed signals at 89.63 ppm for C–3 and 75.94 ppm for C–2; whereas triterpenoid (5) produced resonances at 91.9 ppm for C–3 and 83.18 ppm for C–2.   These facts show that the more sugars there are attached at C–2 the greater the downfield shift of the C–3 signal is and that the steric effect determines the magnitude of this shift. Another example of this finding is the fact that C'-6 (glucose in C–3) has a signal at 63.56 ppm in triterpenoid (6), and 64.18 ppm in triterpenoid (5) (table 2).

Assignments of the carbon shifts of the sugar moieties were made according to Hostettman *et al.* [13] and Tori *et al.* [14].   The signal values of 105.38 for the anomeric carbons show the steric effect due to the presence of two sugars each in C–2 and C–3 (triterpenoid 4, table 2) when these values are compared to the normal value of 106.46 found in triterpenoid (6) (table 2).   The linkage between glucose and rhamnose in triterpenoid (4) is also indicated by the fact that any C' (X)-glucosidic linkage (X=position in the sugar) is shifted downfield by 7–9 ppm (β-effect), in pyridine-d_5 when sugars are attached [15].   As no signal between 80 and 86 ppm was observed, the glucosidic linkage must be at C' (4) (70.5→ 79.87).   The structure of the triterpenoid saponin (4) is 2-β-[L-rhamnopyranosyl-

TABLE 2. ^{13}C-nmr chemical shift values (ppm) of sugar
moieties of compounds **4, 5, 6.**

Sugars	Triterpenoid saponin		
	4	**5**	**6**
2–Glucose C–1	105.38	105.42*	
C–2	76.37	76.53	
C–3	78.60	79.04	
C–4	79.50	71.12	
C–5	78.95	79.04	
C–6	62.32	62.55	
Rhamnose C–1	102.07		
C–2	72.62		
C–3	73.71		
C–4	73.71		
C–5	70.06		
C–6	19.28		
3–Glucose C–1	105.38	105.99*	106.46
C–2	76.37	76.53	75.44
C–3	77.53	77.91	77.54
C–4	79.87	73.70	73.38
C–5	78.95	79.30	78.19
C–6	64.33	64.18	63.56
Rhamnose C–1	102.89		
C–2	72.62		
C–3	73.27		
C–4	74.99		
C–5	70.42		
C–6	19.28		

*Can be reversed.

(1→4)-β-D-glucopyranosyloxy   (1→2)]-3-β-L-rhamnopyranosyl-(1→4)-β-D-gluco-
pyranosyloxy-(1→3)-28-trihydroxyolean-12-ene, named melilotin.

MATERIALS AND METHODS

EXTRACTION AND SEPARATION OF THE SAPONIN.—Fresh flowers of *Melilotus alba* (1 kg) were
heated in 1 liter of boiling water for 5 min, then extracted with 2 liters of methanol (60–80°)
for 6 hr.   The methanol extract was chromatographed on a silica gel column (1=50 cm, id=3
cm) first with benzene, then ethylacetate and finally with methanol.   The methanol fraction
was separated on dcc.   The lower phase of a mixture of chloroform-methanol-propanol-water
(5:6:1:4) was the moving phase, and the upper phase was the stationary phase.   The separation
was monitored by silica gel tlc of the fractions in the lower phase of the dcc solvent.   The
combined fractions containing the saponin, evaporated to dryness and recrystallized from
methanol, gave a white solid, mp 296° (melilotin).

HYDROLYSIS OF TRITERPENOID SAPONIN.—Melilotin was hydrolyzed by refluxing with 4N
HCl for 6 hr.   The aglycone (**1**) was separated from the hydrolysate by filtration, washed with
water, and recrystallized in chloroform-methanol (1 to 1) to give a solid mp 248°.   The hy-
drolysate, after neutralization, was chromatographed on thin-layers of cellulose (pentan-2-ol-
pyridine-water 7:7:2); glucose and rhamnose,which were shown to be in the ratio 1:1 by gc
[7], were obtained.
Partial hydrolysis of melilotin (200 mg) with 10 ml 6% H_2SO_4 at reflux for 4 hr gave a
white precipitate when cooled.   The solid was separated by dcc into 4 fractions: triterpenoid
saponins (**4**) 50 mg, (**5**) mg and (**6**) 50 mg; and aglycone (**1**) 35 mg.   Saponins (**5**) and (**6**) were
further hydrolyzed with 2N HCl.

METHYLATION OF MELILOTIN (**4**).—Compound **4** (2 mg) in methanol was treated with an
ether soln. of CH_2N_2 until a permanent color was obtained.   The reaction was kept at 0° over-
night and hydrolyzed with 4N HCl to give the aglycone (**2**).   Mp 191°; ms m/z: 472 (M⁺), 440,
248, 216, 203, 175.

ACETYLATION OF MELILOTIN (**4**).—Compound **4** (10 mg) was dissolved in 15 ml of acetic an-
hydride containing 1.5 ml of pyridine and let stand overnight at 25°.   The solvent was removed
by vacuum and the product hydrolyzed with 4N HCl to give the aglycone (**3**) mp 164°; ms
m/z: 500 (M⁺), 317, 273, 234, 216, 201.
Identification: Aglycone (**1**) mp: 248° ir: γ Max(KBr) 3500–3200, 2920, 2840, 1725, 1460,
1270–1380, 1030, 760.   ms: m/z 458 (M⁺), 440, 425, 234, 216, 203, 187, 175, 161, 133, 119.   ¹H-nmr
(CDCl₃ 100 MHz) 0.75 (s, 3H, CH₃–26); 0.83 (s, 3H, CH₃–29); 0.95 (s, 3H, CH₃(–30; 1.00 (s, 3H,
CH₃–24); 1.10 (s, 3H, CH₃–23); 1.15 (s, 3H, CH₃–27); 1.25 (s, 3H, CH₃–25); 2.35–2.28 (bs, 2H,

resolved to doublet after D_2O exchange, H–18, $J=11$ Hz, OH–C(3)); 2.70 (q, 1H, H–19, $J=11$ Hz); 3.35–3.30 (bs, 2H, resolved to a doublet after exchange with D_2O, H–3, $J=4$ Hz, OH–C(2)): 3.44–3.35 (q, 2H, $J=11$ Hz, CH$_2$OH(28)); 4.27–4.13 (m, 1H, H–2), 5.26–5.24 (t, 1H, H–12).

ACETYLATION OF AGLYCONE (1).—Aglycone (1) (40 mg) was dissolved in 1 ml of acetic anhydride containing 1.2 ml of pyridine and let stand for 3 hrs at 0°. The product was poured into water, taken to dryness and chromatographed on thin-layer chromatography prep. silica gel (benzene-ethylacetate 9:1). Three bands were separated and chromatographed on a sephadex column (methanol). The first one gave $2\beta, 3\beta,$ 28-triacetoxyolean-12-ene. Ci-ms of the acetylated aglycone (1) m/z 585 (M+1), 525, 501, 465, 441. Methane was used as the ionization gas. ^{1}H-nmr (CDCl$_3$, 60 MHz): 5.24 (m, 2H, H–2 and H–12); 4.60–4.55 (bs, 1H, H–3); 4.28–4.17 (d, 2H, CH$_2$–OAc); 2.03 (s, 9H, 3 x OAc); 1.25 (s, CH, CH$_3$-25); 1.15 (s, 3H, CH$_3$-27); 1.10 (s, 3H, CH$_3$-23); 1.01 (s, 3H, CH$_3$-24); 0.98 (s, 3H, CH$_3$-30); 0.85 (s, 3H, CH$_3$-29); 0.77 (s, 3H, CH$_3$-26). The second one gave 2β-hydroxy-$3\beta,$28-diacetoxyolean-12-ene, ^{1}H-nmr (CDCl$_3$, 60 MHz): 5.25 (m, 1H, H–12); 4.60–4.69 (d, 1H, $J=4.5$ Hz, H–3); 4.30–4.19 (d, 2H, CH$_2$OAc); 3.85–4.10 (bs, 2H, resolved to a multiplet after D_2O exchange, H–2 and OH–C(2)); 2.03 (s, 6H, 2x OAc). The third band gave a 3β-hydroxy-$2\beta,$ 28-diacetoxyolean-12-ene, ^{1}H-nmr (CDCl$_3$, 60 MHz): 5.26 (m, 2H H–2, and H–12); 4.30–3.19 (d, 2H, CH$_2$OAc); 3.43 (m, 1H, resolved after D_2O exchange in a doublet $J=4$ Hz, H–3); 2.40 (bs, 1H, disappeared on D_2O exchange OH–3); 2.05 (s, 3H, 1 x OAc); 2.03 (s, 3H, 1 x OAc).

Aglycone (3) mp 164°; ms m/z: 500 (M$^+$), 317, 273, 249, 216, 201; ^{1}H-nmr (CDCl$_3$, 60 MHz) 5.25 (m, 1H, H–12); 4.30 (d, 2H, CH$_2$OAc); 4.15 (m, 1H, H–2); 3.47-3.30 (m, 2H, resolved to a doublet on D_2O exchange, $J=4$ Hz, H–3).

EXPERIMENTAL[1]

The ^{1}H-nmr spectra were measured in CDCl$_3$ and DMSO-d^6 with TMS as an internal standard on a Bruker 200 MHz machine, and on a Varian CFT-20 at 100°. EI and CI-MS were made on a HP-5985. Droplet counter current (dcc) separations were made on an apparatus manufactured by Tokyo Rikakikai, Nishipama Bldg., Toyama-Cho, Kanda, Chiyoda, Tikyo, Japan.

LITERATURE CITED

1. T. M. McCalla, F. L. Duley, *Science*, **108**, 163 (1948).
2. J. P. San Antonio, *Botanical Gazette*, **82**, 79–95 (1952).
3. G. Nicollier, A. C. Thompson, *J. Agr. Food Chem.*, in print (1982).
4. O. C. Huisman, T. Kosuge, *Phytochemistry*, **9**, 131–137 (1970).
5. M. Torck, L. Bezanger-Beauquesne, M. Pinkas, *Ann. Pharm. fr.*, **29**, 297–304 (1941).
6. K. Blaim, M. Preszlakowska, *Rocz Nauk Roln Roca*, **95**, 75–81 (2969).
7. C. Chen, and C. D. McGinnis, *Carbohydr. Res.*, 90, 127–13- (1981).
8. H. Budzikiewiez, J. M. Wilson and C. Djerassi, *J. Amer. Chem. Soc.*, **85**, 3688 (1963).
9. D. M. Doddrell, P. W. Khoug and K. G. Lewis, *Tetrahedron Lett.*, 2381 (1974).
10. H. T. Cheung, and T. C. Yan, *Chemical Communications*, 370 (1970).
11. T. S. Ramaiah, and V. Vimala Bai, *Curr. Sci.*, **46**, 252–3 (1977).
12. S. Seo, Y. Tomita and K. Tori, *Tetrahedron Lett.*, 7 (1975).
13. K. Hostettmann, M. H. Kaldas and K. Nakanishi, *Helv. Chim. Acta*, **61**, 1990 (1978).
14. K. Tori, S. Seo, Y. Yoshimuna, M. Nakamura, Y. Tomita, and H. Ishic, *Tetrahedron Lett.*, 4167 (1976).
15. T. Konishi, A. Tada, J. Shoji, R. Kasai and O. Tanaka, *Chem. Pharm. Bull.*, **26**, 668 (1978).