The structures of four triterpenoid saponins isolated from the seed of *Trifolium incarnatum*

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Abstract

A novel bisdesmosidic saponin, 3-O-[α -L-rhamnopyranosyl(1 $\rightarrow$ 2)- β -D-galactopyranosyl(1 $\rightarrow$ 2)- β -D-glucuronopyranosyl]-22-O-[β -D-glucopyranosyl(1 $\rightarrow$ 2)- β -D-glucopyranosyl] olean-12-ene-24-ol has been isolated from the seed of the clover, *Trifolium incarnatum*. The structure of this and of three other structurally related saponins, 3-O-(β -D-glucuronopyranosyl)olean-12-ene-22, 24-diol, soyasaponin I and soyasaponin III have been assigned on the basis of spectral and chemical evidence.

Key words: clover seeds, *Trifolium incarnatum*, triterpenoid saponins, soyasapogenol B glycosides

INTRODUCTION

Clover is an important animal feedingstuff in many parts of Europe and the realisation that it may contain significant amounts of biologically active saponins has prompted many studies (Price et al. 1987). Thus saponins, or their aglycones, have been isolated and characterised in strawberry clover (*Trifolium fragiferum* L.) (Walter 1960), burr clover (*Medicago hispida* Gaertn.) (Walter 1957), bird's foot trefoil (*Lotus corniculatus* L.) (Walter 1961), ladino clover (*T. repens*) (Walter et al. 1955, Myoga and Shibata 1977) and white sweet clover (*Melilotus alba*) (Nicollier and Thompson

1983). In addition, the isolation of the triterpenoid soyasaponin I, from *T. repens* L., *T. pratense*, *T. lupinaster* L. or *T. hybridum* L. has been described (Kitagawa 1984).

As part of continuing investigation into the nature and effects of saponins in plants used as human foods or animal feedingstuffs (Johnson et al. 1986, Price et al. 1986, Jurzysta et al. 1987, and Oleszek et al. 1987), the authors have examined the seed of *T. incarnatum* for saponins. Four such compounds have been isolated and their structures determined by chemical and physico-chemical methods.

MATERIALS AND METHODS

EXPERIMENTAL

Melting points were uncorrected. ^1H - and ^{13}C -NMR spectra were recorded in CDCl_3 (400 MHz) and d_5 -pyridine (100.6 MHz), respectively using a JEOL 400 MHz spectrometer. FAB-MS were obtained as described previously (Price et al. 1988) by bombardment with xenon atoms (9KV nominal) produced by an ion-Tech 11 NF atom gun attached to a Kratos MS 9/50 TC mass spectrometer. Coupled GC-MS were obtained using a Perkin-Elmer Sigma 2 instrument linked to a MS 30 mass spectrometer (Price and Fenwick 1984). Permethylation and reduction to the partially permethylated alditol acetates were carried out as described previously (Price and Fenwick 1984). Optical rotation was determined using a 0.25 dm cell in a Jasco DIP 360 polarimeter.

ISOLATION OF SAPONINS 1-4

Three kg of ground seeds were extracted with CHCl_3 in a Soxhlet apparatus for 80 hrs. Five-hundred g portions of defatted seeds were boiled 5 times with 3 l of EtOH for 3 hrs. The combined extracts were concentrated *in vacuo* until all alcohol was removed. The syrup residue was dissolved in 1.5 l of distilled H_2O and saturated lead acetate solution was added in excess. The precipitate was filtered, washed with water and suspended in 1 l of MeOH. Hydrogen sulphide was passed through the suspension for 8 hrs. The precipitated PbS was removed and the filtrate was evaporated *in vacuo*. The residue was desiccated over P_2O_5 , dissolved in 50 ml MeOH, and 500 ml of petroleum ether was added. The precipitate was filtered, washed with Et_2O , then with a small quantity of Me_2CO and dissolved in 50 ml of EtOH. Distilled H_2O was added to the hot solution until it became turbid. The mixture was put into a hot water bath, which was allowed to cool to room temperature. The white crystalline precipitate (2.7 g) was filtered. By use of

Silica gel TLC (AcOEt-AcOH-H₂O, 7:2:2), 1, 2 and 3 were found in the precipitated fraction (PF). Compound 4 (see Fig. 1) was found in the non precipitated fraction.

Fig. 1. Formulae of isolated saponins

SEPARATION OF SAPONINS 1, 2 AND 3

Two g of the crystalline saponins were dissolved in 20 ml of CHCl₃-MeOH-H₂O, 7:3:1 (bottom phase). The mixture was chromatographed on a silica gel column using the above solvent mixture for elution. The eluted fractions were monitored by TLC (AcOEt-AcOH-H₂O, 7:2:2). Three homogenous saponins (Fig. 1), 1 (65 mg), 2 (170 mg) and 3 (1600 mg) were isolated in order of their increasing polarity. Each was treated with 1 M HCl, and immediately filtered and washed with water. Subsequently they were recrystallized from aqueous EtOH (1 fine plates, 2 and 3 needles).

PURIFICATION OF 4

From the aqueous EtOH filtrate the alcohol was distilled off and the aqueous residue was extracted exhaustively with AcOEt, and then after evaporation of water a remaining solid was dissolved in 10 ml of CHCl₃-MeOH-H₂O, 55:35:10. The solution was chromatographed on a silica gel column using the above solvent mixture for elution. A chromatographically homogenous, amorphous 4, (70 mg) was separated. The saponin was easily soluble in water and moderately soluble in alcohols.

THIN LAYER CHROMATOGRAPHY

Silica gel plates were developed in 7:2:5 nBuOH:MeOH:— ammonium, silica gel impregnated with oxalic acid in 65:35:10 CHCl₃:MeOH:H₂O (lower layer) and RP18 silica plates in 3:2 MeOH:H₂O.

COMPLETE ACID HYDROLYSIS OF SAPONINS

Ten mg each of 1-4 were boiled with 5 ml of 2M HCl in 50% aqueous MeOH for 2 hrs. After cooling and distilling the alcohol off, the aqueous residues were exhaustively extracted with AcOEt.

RESULTS AND DISCUSSION

The saponins were isolated from defatted clover seed by solvent extraction, lead salt precipitation and column chromatography based on a previously reported procedure (Jurzysta 1973). Further fractionation produced four compounds 1→4, which upon acid hydrolysis yielded olean-12-en-3,22,24-triol (soyasapogenol B), having identical chemical, TLC, GC and GC-MS characteristics to that of an authentic standard.

In line with previous studies (Curl et al. 1986) the carbohydrate portions of the saponins were studied by FAB-MS (positive and negative ion modes), which provided useful information on the molecular weight of the intact saponins and the sequence of the bound sugars, ¹³C-NMR spectroscopy of the intact saponin, ¹H-NMR spectroscopy of the permethylated saponin (which enabled the nature of the anomeric linkages to be determined) and, following hydrolysis, GC-MS analysis of the sugar-derived alditol-acetates which identified the sugar and the mode of linkage.

Substance 1 was found to possess a molecular weight of 634, FAB-MS showing negative ion mode *m/z* 633 (M-H)⁻; positive ion mode *m/z* 635 (MH)⁺. The latter spectrum also showed fragments consistent with the loss of an uronic acid from a soyasapogenol B — containing saponin (*m/z* 441 (MH-uronic acid — H₂O), 423 (MH-uronic acid — 2H₂O). The ¹H NMR spectrum of the permethylated product gave a signal at δ 4.31 (*J* = 7.8 Hz) consistent with the presence of a β-D-glucuronic acid moiety. GC-MS of the partially permethylated alditol acetate gave a single peak (Rt 204 min, *m/z* 191, 162, 118) indicative of a terminal glucuronic acid. The ¹³C NMR spectrum showed signals consistent with a substitution at C-3 on soyasapogenol B (δ 80.0 for the free aglycones (15)) to δ 89.0 for 1 while signals for the other possible sites of substitution C-22, C-24 were unaltered (Table 1). On the basis of this evidence, 1 is considered to be 3-O-(β-D-glucuronopyranosyl) olean-12-en-22,24-diol.

Table 1
 ^{13}C NMR data

Carbon atom		δ (PPM)							
		1	2	3	4	5	6	7	8
Aglycone	C-3	89.0	90.7	91.2	90.6	91.3	91.5		80.0
	C-12	122.5	122.4	122.3	122.1	122.3	122.8		122.3
	C-13	144.8	144.8	144.1	142.9	144.7	144.3		144.7
	C-22	75.4*	75.5*	75.5	81.2	75.5	80.9		75.4
	C-24	63.3	63.5	63.6	62.7	63.5	63.0		64.4
3-O-Glu- curonopy- ranoside	C-1	106.4	105.0	105.4	105.4	105.3	105.4		
	C-2	73.5	81.0	78.4	77.3*	77.9	77.8		
	C-3	77.9	77.3	77.8	76.5	76.3	76.5		
	C-4	75.6*	73.0	72.7	73.0	72.5	72.8		
	C-5	78.1	75.6*	77.6	77.2*	76.8	77.2		
	C-6	173.0	172.4	172.3	169.3	170.0	170.2		
(1+2)	C-1''		105.0	102.4	101.7	101.7	102.2		
Galacto- pyrano- side	C-2		73.7	76.6*	77.3*	77.5	77.6		
	C-3		75.6*	76.4*	75.8	74.3	74.4		
	C-4		71.1	71.1	71.1	71.0	71.3		
	C-5		77.3	76.5*	77.0*	76.8	76.9		
	C-6		62.7	61.6	62.2	61.7	62.1		
(1+2) Rham- nopyra- noside	C-1			101.7	101.0	102.0	102.0		
	C-2			74.3	73.7	72.1	72.0		
	C-3			72.3	72.1 $\pm$	72.5	72.3		
	C-4			73.7	73.3	73.3	73.4		
	C-5			69.3	68.8	69.2	69.4		
	C-6			18.5	18.6	18.7	18.9		
22-O-Glu- copyrano- side	C-1				105.4		98.3**	105.9	
	C-2				82.9		80.1	83.2	
	C-3				77.5*		71.8	78.2	
	C-4				71.1 $\pm$		66.8	71.6	
	C-5				78.0		63.7	78.2	
	C-6				62.2		—	62.7	
(1+2) Gluco- pyrano- side	C-1				104.7		105.6	104.6	
	C-2				77.2*		75.6	76.9	
	C-3				78.0		78.3	78.2	
	C-4				71.8 $\pm$		71.8	71.6	
	C-5				78.0		78.1	77.7	
	C-6				62.7		63.0	62.7	

(5) NMR signals of soyasaponin I ($\equiv$ 3) from Walter (1957).

(6), (7) NMR signals of a bisdesmoside with structural similarities to 4 from Walter (1960).

(8) NMR signals of soyasapogenol B.

* $\pm$ Signals are interchangeable within the vertical columns.

** Signals refer to a 22-O- α -L-arabinopyranoside.

Substance 2 showed ions in the FAB-MS, negative ion mode, corresponding to m/z 795 $(M-H)^-$, m/z 633 $(M-H\text{-hexose})^-$, and in the positive ion mode m/z 797 $(MH)^+$, 441 $(MH\text{-hexose-uronic acid} - H_2O)$ and 423 $(MH\text{-hexose-uronic acid} - 2H_2O)$. The 1H NMR of the permethylated saponin showed signals at δ 4.39 ($J = 7.0$ Hz; β -D-glucuronic acid) and δ 4.61 ($J = 7.6$ Hz; β -D-galactose). The presence of galactose was confirmed by sugar analysis as described previously (Price et al. 1986), the uronic acid is not detected under these conditions. Examination of the GC-MS of the partially methylated alditol acetates gave two components, the first (R_t 154 min) having ions at m/z 205, 162, 161 and 118 indicating a terminally linked galactose, the second (R_t 258 min, m/z 234, 191, 190) being consistent with a 1,2-linked glucuronic acid. The ^{13}C NMR spectrum for 2, showed a signal δ 90.7 indicative of a C-3 substitution on the aglycone (Table 1). Further comparison of the spectrum of 2 with 5 showed 2 to be 3-O- $[\beta$ -D-galactopyranosyl (1 $\rightarrow$ 2) β -D-glucuronopyranosyl] olean-12-en-22,24-diol (soyasaponin III). In agreement with this finding soyasaponin III and saponin 3 were identical when chromatographed in a variety of TLC systems.

Substance 3 exhibited positive and negative FAB-MS data consistent with that of a saponin (MW 942) possessing deoxyhexose, hexose and uronic acid sugars. The 1H NMR of the permethylated saponin showed characteristic anomeric proton signals at δ 4.28 ($J = 7.5$ Hz, β -D-glucuronic acid), δ 4.65 ($J = 7.9$ Hz, β -D-galactose) and δ 4.64 ($J = 7.8$ Hz, α -L-rhamnose). The ^{13}C NMR spectrum of 3 when compared with that of authentic soyasaponin I, 5 (see Table 1) combined with sugar analysis and GC-MS of the partially methylated alditol-acetates ($R_t = 80$ min, m/z 175, 162, 131, 118; R_t 205 min, m/z 234, 205, 190, 165; and R_t 258 min, m/z 234, 191, 190 predicts the presence of a terminal rhamnose, 1,2 linked galactose and a 1,2 linked glucuronic acid respectively), showed 3 to be 3-O- $[\alpha$ -L-rhamnopyranosyl (1 $\rightarrow$ 2) β -D-galactopyranosyl (1 $\rightarrow$ 2) β -D-glucuronopyranosyl] olean-12-en-22,24-diol. This compound and 3 also were shown to possess identical TLC characteristics in several solvent systems.

The negative ion FAB MS of substance 4 (MW 1266) clearly showed the presence of a deoxyhexose (d), uronic acid (u) and three hexose (h) units, m/z 1119 $(M-H-d)^-$, 1103 $(M-H-h)^-$, 957 $(M-H-d)^-$, 941 $(M-H-2h)^-$, 795 $(M-H-2h-d)^-$, 633 $(M-H-3h-d)^-$, 457 $(M-H-3h-d-u)$. This is consistent with two sugar chains containing terminal hexose and deoxyhexose residues and with the uronic acid linked directly to the soyasapogenol B. Inspection of the 1H NMR spectrum of the permethylated saponin confirmed the presence of five sugars, these being identified as β -D-glucuronic acid and β -D-glucose (2 proton signal, 4.28, $J = 7.0$ Hz), β -D-glucose (δ 4.62, $J = 7.9$ Hz), β -D-galactose (4.66, $J = 7.6$ Hz) and α -L-rhamnose (δ 5.24, broad singlet). The ^{13}C NMR spectrum showed the presence of signals in agreement with the above (Table 1) with the points of glycoside attachment being shown to be at

C-3 and C-22. Sugar analysis confirmed the presence of the rhamnose, galactose and glucose whilst detailed analysis of the partially-methylated alditol acetates identified two terminal residues (Rt 80 min, m/z 175, 162, 131, 118; Rt 141 min, m/z 205, 162, 161, 118) rhamnose and glucose respectively, a 1→2 linked glucose (Rt 189 min, m/z 234, 205, 190, 161), a 1→2 linked galactose (Rt 203 min, m/z 234, 205, 190, 161) and a 1,2-deuterated glucose (Rt 258 min, m/z 234, 191, 190) (i.e. 1,2-linked glucuronic acid).

Reversed-phase thin layer chromatographic analysis revealed 4 to possess similar chromatographic characteristics (Rf 0.46) to the bisdesmoside soyasaponin A₁ which possesses two sugar moieties attached to C₃ and C₂₂ of a soyasapogenol A aglycone. This is supported by the similarity of the observed ¹³C NMR spectrum of 4 to that of sophoroflavoside I 6, isolated by Yoshikawa et al. (1985) from *Sophora flavescens* Aiton. Various chemical and physicochemical procedures failed to preferentially hydrolyse the uronic acid containing oligosaccharide and the structure of 4 is suggested on the basis of its biosynthetic relationships to 1→3 and the detailed examination of the FAB-MS spectra. Under negative ion conditions, abundant ions at m/z 499 and 483 are observed. These ions, also present in the mass spectrum of soyasaponin I, are considered to be due to cleavage on either side of the aglycone oxygen atom, hence indicating the presence of rhamnose-galactose-glucuronic acid sequence. This, in turn, identifies the second sugar moiety as glucosyl-glucose, in support of which is the similarity of the ¹³C NMR spectrum with that of anchusoside-9 (Romussi et al. 1984), see Table 1. Optical activity of 4 was $[\alpha]_D^{20} = -0.92^\circ$.

The above data, together with the similarity to 6 and the obvious biosynthetic relationship between the four isolated saponins lead us to conclude that 4 has the structure 3-O-[α -L-rhamnopyranosyl (1→2) β -D-galactopyranosyl (1→2) β -D-glucuronopyranosyl β -22-O-[β -D-glucopyranosyl (1→2)- β -D-glucopyranosyl] olean-12-en-24-ol.

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Budowa czterech saponin trójtterpenoidowych wyodrębnionych z nasion Trifolium incarnatum

Streszczenie

Z nasion inkarnatki (*T. incarnatum*) wyodrębniono nową saponinę o dwu łańcuchach cukrowych: 3-O-[α -L-ramnopyranozylo(1 $\rightarrow$ 2)- β -D-galaktopyranozylo(1 $\rightarrow$ 2)- β -D-glukuronopyranozylo]-22-O-[β -D-glukopyranozylo(1 $\rightarrow$ 2)- β -D-glukopyranozylo]olean-12-en-24-ol. Budowę tej saponiny, jak też trzech innych saponin: 3-O-(β -D-glukuronopyranozylo)olean-12-en-22,24-diolu, saponinę I i sojasaponinę III, ustalono na podstawie analiz spektralnych i badań chemicznych.