Studies on the Constituents of *Gliricidia sepium* (Leguminosae) Leaves and Roots: Isolation and Structure Elucidation of New Triterpenoid Saponins and Aromatic Compounds

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Our research program on the Central American fooder plant *Gliricidia sepium* led to the discovery of two new triterpene saponins (**1** and **2**) and known aromatic compounds. The new compounds possess 3β,21β,24-trihydroxy-22-oxoolean-12-ene as an aglycon. The oligosaccharide moiety linked to C-3 of the aglycon contained two pyranoses (glucuronic acid and xylose); in addition the glucose residue of both **1** and **2** is also linked to C-21. Structure elucidation of these new compounds through the extensive use of 1D and 2D NMR techniques have provided detailed information about the sapogenin and the saccharide chains, inclusive of sugar sequence and the position of glycosylation.

Keywords: *Gliricidia sepium; leguminosae; leaves; root; fooder source; triterpenoid saponins; flavonol glycosides; and ^{13}C NMR analysis*

INTRODUCTION

Gliricidia sepium (Jacq.) Steud (vernacular name, "madrecacao") is a tree, 3−10 m high, belonging to the Leguminous family. It is native to both coasts of Mexico from above the middle of the country southward and through Central America to Colombia and Venezuela. *G. sepium* is used both medicinally and for cattle feeding on the Pacific coast of Mexico, Central America, and in tropical regions of South America and Asia (Bennison and Paterson, 1993).

Its leaves are generally considered to be one of the most digestible of the tropical leguminous forages (Glover, 1989) and contain a high content of readily degradable proteins and carbohydrates. The usefulness of this species for feed and feed supplement purposes (Whetton et al., 1997) was determined through the in vitro study of degradation products of both crude leaf and soluble protein extracts of the leaf by rumen microbes. The presence of pinitol (Calle et al., 1986), coumarins, hydrocarbons, and flavonoid glycosides in the leaves has been previously surveyed (Herath et al., 1998). Previous studies have also revealed that all parts of this plant have insecticidal activity and have the capacity to protect tea plantation of Sri Lanka against tea pests. This property of *G. sepium* has been attributed to its heartwood and some organic extract exhibiting olfactive attraction and high toxicity toward the termite *Glyptotermes dilatatus* which is responsible for the great damage to tea plantations. Therefore most of the previous chemical investigations on *G. sepium* have focused on the isolation of potential allelopathic and toxic compounds from the heartwood. These investigations have led to the isolation of isoflavenes, isoflavans, and pterocarpans (Jurd and Manner, 1977; Manner and Jurd, 1979; Herath et al., 1998).

Although triterpenoid saponins are widespread in leguminous plants (Miyao et al., 1996), only a report on triterpenoid content of *G. sepium* has been reported until now (Kojima et al., 1998). We have examined the constituents of the leaves and roots of *G. sepium* and have isolated two new oleanene glycosides from the roots and a series of known aromatic compounds from the leaves. Their structures were elucidated by spectral methods, with two-dimensional NMR techniques being especially helpful.

EXPERIMENTAL PROCEDURE

Material. The leaves and the root of *Gliricidia sepium* was collected at Chimaltenango, Guatemala, in July 1995 and identified by J. Castillo. A voucher sample is deposited at the Herbario of the Facultad de Agronomia, Universidad de San Carlos de Guatemala, Guatemala.

Apparatus. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell in 0.1% solution of MeOH. A Bruker DRX-600 spectrometer operating at 599.19 MHz for ^1H and 150.858 for ^{13}C using the UXNMR software package was used for NMR measurements in CD_3OD solutions. 2D experiments ($^1H-^1H$ DQF−COSY (Double Quantum Filtered Direct Chemical Shift Correlation Spectroscopy), inverse detected $^1H-^{13}C$ HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Quantum Coherence), and ROESY (Rotating-frame Overhauser Enhancement Spectroscopy)) were obtained by employing the conventional pulse sequences and as described previously. The selective excitation spectra, 1D TOCSY, were acquired using waveform generator-based GAUSS shaped pulses, mixing time ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. Fast atom bombardment mass spectra (FABMS) were recorded in a glycerol matrix in the negative ion mode on a VG ZAB instrument (Xe atoms of energy of 2−6 kV). HPLC separations were performed with a Waters Model 6000A pump equipped with a U6K injector and a Model 401 refractive index detector.

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Extraction and Isolation. The dried and powdered roots (500 g) were defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 19 g of residue. The MeOH extract was chromatographed (6 g) on a Sephadex LH-20 column (100 × 5 cm), with MeOH as eluent, and fractions of 8 mL were collected. The fractions obtained were combined according to TLC (silica gel, *n*-BuOH−HOAc−H_2O, 60:15:25) to give three main fractions I−III. Fraction I (150 mg) was submitted to HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.5 mL min^{-1}) using MeOH−H_2O (40:60) as eluent to yield pure compounds **1** (11 mg, R_t 15.5 min) and **2** (15 mg, R_t 19 min). Air dried and powdered leaves of *G. sepium* (500 g) were sequentially extracted at room temperature with petroleum ether, $CHCl_3$, $CHCl_3$/MeOH (9:1), MeOH, and H_2O to afford 7.85, 7.03, 19.46, and 11.03 g of residue, respectively. Part of the MeOH residue (6 g) was chromatographed on a Sephadex LH-20 column (100 × 5 cm) eluting with MeOH. Fractions of 8 mL were collected and combined by TLC similarity [SiO_2 plates, *n*-BuOH−AcOH−H_2O (60:15:25) and $CHCl_3$−MeOH− H_2O (80:18:2)] in three main fractions A−C. Fractions A (161 mg), B (420 mg), and C (329 mg) were further chromatographed by RP-HPLC (μ-Bondapak C-18 column, 30 cm × 7.8 mm, flow rate 1.5 mL/min) with MeOH−H_2O (1:1) as solvent system. Fractions B, containing aromatic constituents, provided phenyl derivatives 3-*O*-β-D-glucopyranosylbenzoic acid methyl ester (**3**) (t_R = 11.5 min, 15 mg) and *m*-*O*-β-D-glucopyranosylhydrocinnamic acid (**4**) (t_R = 20.5 min, 9.5 mg), the pterocarpan medicarpin (**5**) (t_R = 21.5 min, 11.5 mg), the isoflavan vestitol (**6**) (t_R = 19.5 min, 7.5 mg), and 7,4′-dihydroxy-3′-methoxyisoflavan (**7**) (t_R = 18.5 min, 16 mg). Fraction C, containing a mixture of flavonoid glycosides, provided kaempferol-3-robinobioside-7-*O*-α-L-rhamnopyranoside (**8**) (t_R = 16 min, 10 mg), kaempferol-3-(2^{GAL}-rhamnosylrobinobioside) (**9**) (t_R = 15 min, 15 mg), kaempferol-3-rutinoside-7-*O*-α-L-rhamnopyranoside (**10**) (t_R = 13 min, 19 mg), and kaempferol-3-(2^{GLU}-rhamnosylrutinoside) (**11**) (t_R = 11 min, 21 mg).

Compounds **3**−**11** were identified by comparison of their NMR spectra with literature data (Scott, 1972; Herath et al., 1998; Harborne, 1986).

Compound 1: $[\alpha]^{25}_D$ +15.5; negative FABMS *m/z* [M − H]$^-$ 941, [(M − H) − 162]$^-$ 779, [(M − H) − (132 + 162)]$^-$ 647; NMR data for the aglycon moiety are reported in Table 1, for the sugar moiety in Table 2.

Compound 2: $[\alpha]^{25}_D$ +18.1; negative FABMS *m/z* [M − H]$^-$ 983, [(M − H) − 42]$^-$ 941, [(M − H) − 162]$^-$ 821, [(M − H) − (132 + 162)]$^-$ 689; NMR data for the aglycon moiety are reported in Table 1, for the sugar moiety in Table 2.

RESULTS AND DISCUSSION

Two saponins, gliricidoside A (**1**) and B (**2**), were isolated and purified by Sephadex LH-20 column chromatography and reversed-phase HPLC from the MeOH extract of the roots of *G. sepium*.

Gliricidoside A (**1**) ($C_{47}H_{74}O_{19}$) and B (**2**) ($C_{49}H_{76}O_{20}$) gave quasimolecular ion peaks at *m/z* 941 [M − H]$^-$ and *m/z* 983 [M − H]$^-$, respectively, and prominent peaks due to the loss of a hexose for **1** and a hexose plus acetyl for **2** in their negative FABMS spectra. The NMR spectral data of saponins **1** and **2** (Tables 1 and 2) revealed the feature of a triterpene of the oleane series and three sugar units.

The ^{1}H NMR spectra of **1** and **2** displayed signals for six tertiary Me groups (δ 0.82, 0.91, 1.01, 1.03, 1.17, and 1.23), one −CH_2OH group (δ 3.25 and 4.11, each d, J = 12 Hz), a signal typical of H-3ax (δ 3.30, dd, J = 11.1 and 4.5 Hz) due to the presence of β-OH group at C-3 position, and an olefinic proton signal at δ 5.34 (dd, J = 6.0 and 1.5 Hz) in the aglycon moiety indicating a pentacyclic triterpene skeleton of the oleanene series. In addition it was possible to observe in the NMR spectra a signal at δ_H 4.62 (s) and δ_C 86.60 ascribable to an oxymethine and a signal for a keto group (δ_C 215.26). Full assignments of the proton and carbon signals of the aglycon part of major compound **1** were secured by ^{1}H−^{1}H DQF−COSY and HSQC spectra. A combination of COSY, which showed isolated spin system for H-3−H-1, H-5−H-7, H-16−H-15, H-18−H-19, and H-9−H-12, and HSQC experiments indicated that the proton and carbon signals due to the A, B, C, and D rings are typical of a 3β,24-dihydroxyolean-12-ene skeleton glycosylated at C-3 as in Soyasapogenol A and B, compounds widespread in leguminous plants (Miyao et al., 1996; Kinjo et al., 1994, 1995). Ring E must contain the oxymethine and the keto groups such as in Sitakisoside VIII (Yoshikawa et al., 1994).

This interpretation was unambiguously confirmed by the HMBC (8 Hz) spectrum of **1**. The long-range correlation between the singlet at δ 4.62 (H-21) and C-29/C-30 methyls and C-20 quaternary carbon signals and an NOE between the former and Me-29 at δ 1.17 in the ROESY spectrum indicated the presence of 21β-OH. A long-range correlation between H-21 and carbonyl carbon signal at δ 215.26 established the site of the carbonyl at C-22. Significant cross peaks, due to $^2J_{C-H}$ and $^3J_{C-H}$ correlations, were also seen between H-18 (δ 2.32) and C-22 C-19 and C-17 and between the protons

Table 1. ^{13}C NMR and ^{1}H NMR Assignments and ^{13}C−^{1}H Long-Range Correlations of Aglycon of Compound 1[a] by ^{1}H−^{1}H COSY, HSQC, and HMBC Experiments in CD_3OD

carbon	δ_C	DEPT	δ_H (J_{HH} in Hz)	cross peaks (δ_C) in HMBC spectrum
1	39.76	CH_2	1.07 m, 1.67 m	
2	27.86	CH_2	1.08 m, 1.82 m	
3	91.45	CH	3.30 dd (11.1, 4.5)	C-23, C-24, C-4
4	44.71	C		
5	57.37	CH	0.99 m	C-23, C-6, C-7
6	19.42	CH_2	1.43 m, 1.68 m	
7	33.92	CH_2	1.42 m, 1.61 m	
8	40.84	C		
9	47.80	CH	1.68 m	
10	37.44	C		
11	24.84	CH_2	1.73 m, 1.96 m	
12	125.40	CH	5.34 dd (6.0, 1.5)	
13	142.00	C		
14	43.00	C		
15	26.91	CH_2	1.82 m, 2.17 m	
16	28.50	CH_2	1.78 ddd (13.0, 12.0, 5.0) 2.36 ddd (12.0, 5.0, 3.1)	
17	48.00	C		
18	48.60	CH	2.32 dd (11.5, 2.5)	C-17, C-19, C-20, C-22
19	47.35	CH_2	1.42 dd (12.0, 5.0) 2.43 dd (12.0, 11.0)	
20	41.32	C		
21	86.60	CH	4.62 s	C-29, C-30, C-20, C-22
22	215.26	C		C-18, C-21, C-20
23	22.67	CH_3	1.23 s	C-2, C-4, C-5
24	63.89	CH_2	3.25 d (12), 4.11 d (12)	C-23, C-4, C-5
25	16.04	CH_3	0.91 s	C-5, C-9
26	17.30	CH_3	1.01 s	C-8, C-9, C-14
27	25.94	CH_3	1.33 s	C-15, C-8, C-14
28	21.21	CH_3	1.03 s	C-16
29	28.42	CH_3	1.17 s	C-19, C-20, C-21
30	19.42	CH_3	0.82 s	C-19, C-20, C-21

[a] Values for the aglycon of compound **2** are superimposable to those of **1** except for H-21 as indicated in the text.

Table 2. ^{13}C NMR and ^{1}H NMR Data[a] of Sugar Moiety of Compounds 1 and 2 in CD_3OD

	1		2	
position[c]	δ_C	δ_H (J_{HH} in Hz)[b]	δ_C	δ_H (J_{HH} in Hz)[b]
glucur-1′	104.43	4.47 d (7.8)	105.18	4.48 d (7.8)
glucur-2′	81.11	3.53 dd (9.0, 7.8)	81.18	3.52 dd (9.0,7.5)
glucur-3′	78.43	3.63 t (9.0)	76.77	3.58 t (9.0)
glucur-4′	73.49	3.50 t (9.0)	73.53	3.49 t (9.0)
glucur-5′	76.76	3.59 d (9.0)	78.45	3.64 d (9.0)
glucur-6′	178.68		178.80	
Xyl-1″	104.81	4.66 d (7.5)	105.10	4.66 d (7.5)
Xyl-2″	75.56	3.21 dd (7.5, 8.5)	75.69	3.29 dd (7.5, 8.5)
Xyl-3″	78.13	3.29 t (8.5)	78.16	3.32 t (8.5)
Xyl-4″	69.06	3.51 m	69.04	3.51 m
Xyl-5″	66.90	3.13 dd (11.0, 2.5)	66.93	3.15 dd (11.0, 2.5)
		3.83 dd (11.0, 5.0)		3.85 dd (11.0, 5.0)
CO			176.60	
Me			21.22	2.03 s
Glc-1‴	105.00	4.34 d (7.6)	105.27	4.34 d (7.5)
Glc-2‴	74.60	3.28 dd (9.0, 7.5)	74.21	3.35 dd (9.5, 7.5)
Glc-3‴	77.83	3.35 t (9.0)	77.89	3.37 t (9.5)
Glc-4‴	71.13	3.39 t (9.0)	71.80	3.29 t (9.5)
Glc-5‴	77.83	3.23 m	78.11	3.37 m
Glc-6‴	62.41	3.69 dd (12.0, 5.0)	64.76	4.14 dd (12.2, 4.5)
		3.78 dd (12.0, 3.5)		4.43 dd (12.2, 3.5)

[a] Assignments confirmed by 1D TOCSY and 2D COSY, HSQC, and HMBC experiments. [b] $^{1}H-^{1}H$ coupling constants in the sugar spin−spin were measured from TOCSY and COSY spectra in Hz. [c] Glc = β-D-glucopyranosyl, Xyl = β-D-xylopyranosyl, and glucur = β-D-glucuronopyranosyl.

of Me-29 and Me-30 (δ 1.17 and 0.82) and C-21, C-20, and C-19 as well as between the protons of Me-28 (δ 1.03) and C-16. Hence the aglycon of saponin **1** and **2** was formulated as 3β,21β,24-trihydroxy-22-oxoolean-12-ene. To the best of our knowledge this is the first report of a naturally occurring pentacyclic triterpene of the oleanene series with a keto group at C-22 and −OH group at C-21. This seems to be an oxidation product at C-22 of soyasapogenol A (Miyao et al., 1996) and is named gliricidogenin A.

Three anomeric signals at δ_H 4.34, 4.47, and 4.66 were easily identified in the ^{1}H spectrum of **1**. They correlated to carbons at δ_C 105.00, 104.43, and 104.81 respectively, in the HSQC spectrum. The sugars were determined to be a disaccharide chain formed by β-D-glucuronopyranosyl acid and β-D-xylopyranosyl linked to C-3 of the aglycon and β-D-glucopyranosyl at C-21 by use of monodimensional TOCSY, 2D DQF−COSY, and HSQC experiments. Even at high field (600 MHz) the 1D sugar spectral region of **1** was complex as most of the shifts were overlapped and found between δ 3.83 and 3.23. The isolated anomeric proton signals resonating at uncrowded region of the spectrum (between δ 4.34 and 4.66) have been the starting point for the 1D TOCSY experiments. Because of the selectivity of the multistep coherence transfer, the 1D TOCSY subspectra of the single monosaccharide unit could be extracted from the crowded overlapping region. Each subspectrum could be attributed to one set of coupled protons such as H−C (1) to H−C (5) (for xylose and glucuronic acid) or H−C (6) (for glucose) of an individual monosaccharide. The interpretation of the 1D TOCSY subspectra of the three types of monosaccharide units was accomplished, while the type of sugar, its configuration, and conformation was assigned by the 2D COSY spectrum summarized in Table 2. The HSQC spectrum led to the establishment of the position of the interglycosydic linkages by comparison of observed resonances with those of the corresponding methylpyranosides, which accounted for the known effects of glycosylation (Breitmaier and Voelter,

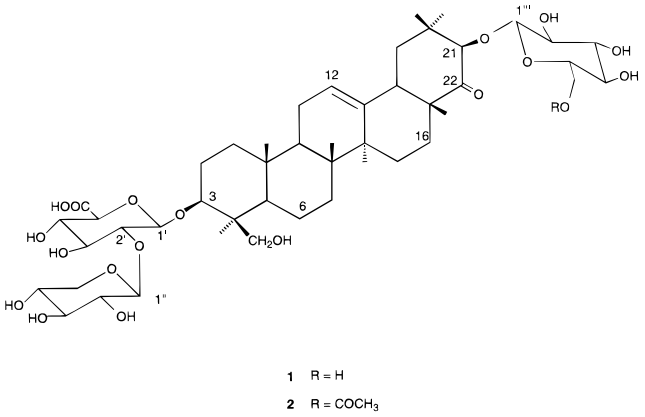

Figure 1. Structure of triterpene saponins **1** and **2** isolated from *G. sepium* roots.

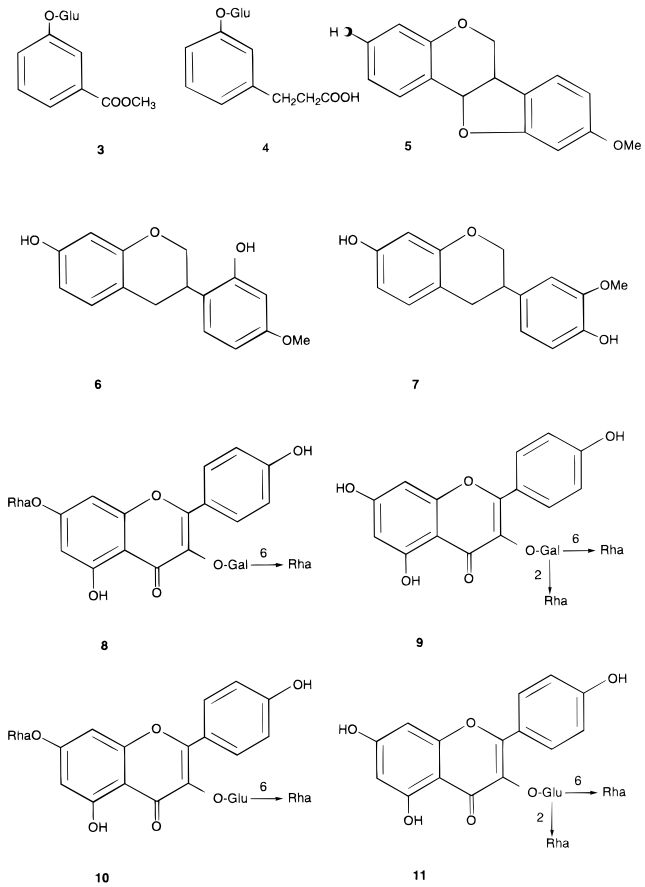

Figure 2. Aromatic compounds **3**−**11** isolated from *G. sepium* leaves.

1989). Once the proton and carbon spectra had been completely assigned, an unambiguous determination of the sequence and linkage sites was obtained from the long-range C−H correlations (HMBC spectrum) and from the interesidue ROEs in the 2D ROESY spectrum.

The esterified sugar substituent at C-21 was identified from the following evidences: a 1D TOCSY subspectrum obtained by irradiating at δ 4.34 showed a set of coupled protons at δ 3.39, 3.35, 3.28, 3.23 (all CH) and 3.69 and 3.78 (CH_2) assigned as H-1‴ to H_2-6‴ of a glucopyranose by the COSY spectrum. Analysis of the correlated ^{13}C NMR signals in the HSQC spectrum led to the identification of a terminal glucopyranose. The cross peak of the ^{3}J long-range coupling between H-1‴ (δ 4.34) and C-21 (δ 86.60) of the aglycon as well as NOE observed between H-1‴ and H-21 (δ 4.62) in the ROESY

spectrum provided evidence for the position of this sugar moiety. In a similar manner the other two monosaccharide units were connected together and located at C-3 of the aglycon. The absence of any glycosylation shift for the carbon resonances of the xylopyranose suggested this sugar to be the terminal unit, while glycosylation shift (ca. 6 ppm) was observed for C-2 (δ 81.11) of the glucuronopyranosyl unit. Key correlation peaks in the HMBC spectrum between H-1′ of the glucuronopyranosyl unit at δ 4.47 and C-3 (δ_C 91.45) of the aglycon and between H-1″ of xylopyranosyl (δ_H 4.66) and C-2′ (δ_C 81.11) of the inner glucuronopyranosyl unit were allowed to determine the sequence of the disaccharide chain as shown in Figure 1. The ^{1}H NMR data of the anomeric proton signals (J_{H1-H2} = 7.5 Hz) as well as ^{13}C NMR data of key carbons (C-2, C-3, and C-5) indicated a β-configuration at the anomeric positions of the three pyranosyl units.

Thus **1** was formulated as 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl-3β,21β,24-trihydroxy-22-oxoolean-12-en-21-*O*-β-D-glucopyranoside.

The NMR data indicated the same aglycon and sugar chain at C-3 for both compounds **1** and **2**. The NMR spectrum of **2** was very similar to that of **1** except for the presence of a signal at δ_H 2.03 (3H, s) and δ_C 21.22 suggesting a Me linked to a carbonyl (δ_C 176.76) and for chemical shift of H-6‴ signal (δ 4.43 and 4.14 in **2** and 3.69 and 3.78 in **1**) of the glucose moiety at C-21, suggesting an esterification at C-6″. Also the carbon resonances of C-6‴ (+2.35 ppm) was indicative of an acetylation. It is interesting to note that the presence of the acetyl group at C-6‴ induced an upfield shift (−0.08 ppm) of the resonance of C-21 proton (δ_H 4.54) on the aglycon of compound **2** with respect to compound **1**. From the above data **2** was determined to be 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl-3β,21β,24-trihydroxy-22-oxoolean-12-en-21-*O*-β-D-(6-*O*-acetyl)glucopyranoside.

The aromatic compounds isolated from the leaves of *G. sepium* were identified as known aromatic acids (**3** and **4**), a pterocarpan (**5**), two isoflavans (**6** and **7**), and four favonol glycosides (**8**−**11**) by comparison with the literature (Scott, 1972, Herath et al., 1998; Harborne, 1986).

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