New 12a-Hydroxyrotenoids from *Gliricidia sepium* Bark

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The investigation of a methanolic extract of *Gliricidia sepium* bark afforded, in addition to vestitol and 2′-*O*-methylvestitol, three new 12a-hydroxyrotenoids, gliricidol (**1**), 2-methoxygliricidol (**2**), and gliricidin (**3**). The structures of **1**−**3** were elucidated by analysis of their spectroscopic data. Compounds **1**−**3** exhibited activity against *Artemia salina* larvae.

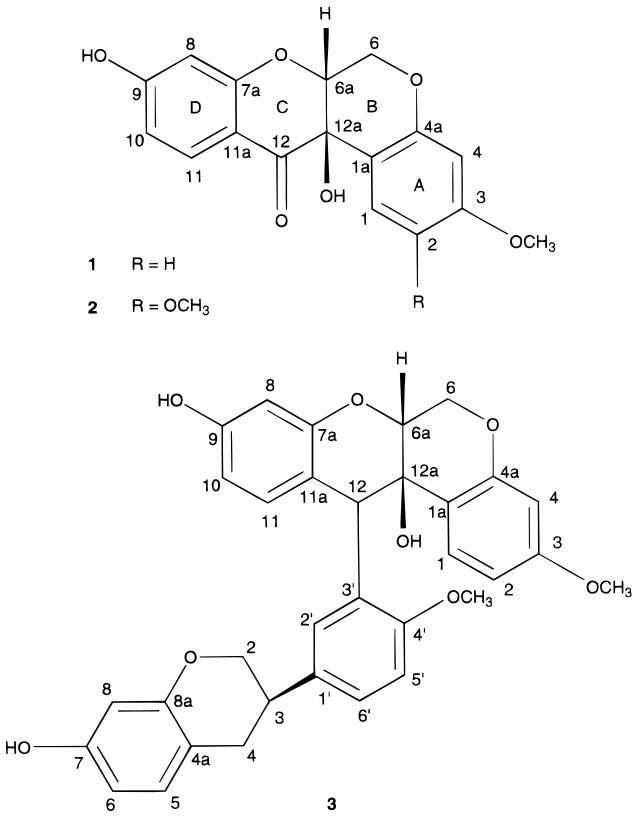
Gliricidia sepium (Jacq.) Steud (vernacular name, "madrecacao") is a tree, 3−10 m high, belonging to the family Leguminosae and native to both coasts of Mexico from above the middle of the country southward and through Central America to Colombia and Venezuela. In Guatemala and Costa Rica, the bark decoction is used against protozoal diseases and for the treatment of impetigo and other skin diseases.[1] In a continuing search for new bioactive metabolites from Central American species,[2,3] we describe here the isolation and identification of three new 12a-hydroxyrotenoids (**1**−**3**) from the bark of *G. sepium* (Chart 1). The insecticidal activity of rotenoids is regarded as their most important phytochemical property, although more recently a variety of rotenoids isolated have been found to be antitumor promoters.[4] The toxicity against the larvae of brine shrimp (*Artemia salina*) of the extracts, partially purified fractions and isolated pure compounds were assessed.

The bark of *G. sepium* was extracted successively with hexane, $CHCl_3$, and MeOH, with each extract tested against *A. salina* nauplii.[5] Both the $CHCl_3$ and MeOH extracts showed activity against the larvae of *A. salina* with LC_{50} values of 777 and 465 μg/mL, respectively. Since the MeOH extract was more active, it was submitted to further purification on a Sephadex LH-20 column with MeOH as eluent and five main fractions were collected and tested against *A. salina* nauplii. Fraction IV exerted significant toxicity after 24 h of incubation against the larvae of *A. salina* [LC_{50} (μg/mL) 427].

By means of reversed-phase HPLC, the new compounds **1**−**3**, in addition to vestitol and 2′-*O*-methylvestitol, were isolated from fraction IV. Compounds **1**-**3** were all toxic to the larvae of brine shrimp with LC_{50} (μg/mL) values of 50.7, 284.5, and 243.7.

The molecular formulas of compounds **1**−**3** ($C_{17}H_{14}O_6$ for **1**, $C_{18}H_{16}O_7$ for **2**, $C_{33}H_{30}O_8$ for **3**) were determined from EIMS, ^{13}C NMR, and DEPT NMR analysis. The general features of its NMR spectral data suggested a rotenoid structure for compound **1**. Comparison of ^1H and ^{13}C NMR spectral data of **1** and 6-deoxyclitoriacetal,[6] a compound with a typical 12a-hydroxyrotenoid skeleton, showed that the B−C ring proton and carbon resonances were similar but the A−D rings provided points of difference. The EIMS

Chart 1



of **1** gave a molecular ion at *m*/*z* 314. The 600 MHz ^1H NMR spectrum of **1** exhibited the presence of 12 protons, each of which was identified with the aid of the DQF−COSY spectrum. A methoxy signal (3H, s) resonated at δ 3.75 (3H, s), and signals at δ 7.20 (d, J = 8.6 Hz, H-1), 6.46 (dd, J = 8.6 and 2.7 Hz, H-2), 6.41 (d, J = 2.7 Hz, H-4), and δ 6.33 (d, J = 2.7 Hz, H-8), 6.50 (dd, J = 8.6 and 2.7 Hz, H-10), 7.32 (d, J = 8.6 Hz, H-11), suggested the presence of two trisubstituted aromatic rings (Table 1). A pair of nonequivalent methylene proton signals at δ 3.55 (d, J = 12.0 Hz) and 4.25 (dd, J = 12.0 and 2.5 Hz) was assigned to H-6α and H-6β, respectively, and a small doublet at δ 5.49 (d, J = 2.5 Hz) was ascribed to H-6a. The chemical shifts, multiplicity and the coupling constant values of H-6a were consistent with an equatorial proton, and, on irradiation of H-6a, a significant NOE was observed only at the methylene proton (H-6β) at δ 4.25, further

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Table 1. ^{1}H and ^{13}C NMR Chemical Shifts for Compounds **1** and **2** (in CD_3OD)[a]

position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1a	–	121.6	–	108.0
1	7.20 (d, 8.6)	128.6	6.66 (s)	108.6
2	6.46 (dd, 8.6, 2.7)	107.6	–	143.7
3	–	158.2	–	152.2
4	6.41 (d, 2.7)	97.3	6.48 (s)	97.3
4a	–	146.6	–	146.6
6α	3.55 (d, 12.0)	67.3	3.54 (d, 12.0)	67.3
6*β*	4.25 (dd, 12.0, 2.4)		4.25 (dd, 12.0, 2.5)	
6a	5.49 (d, 2.4)	77.1	5.48 (d, 2.5)	77.0
7a	–	157.8	–	157.8
8	6.33 (d, 2.7)	103.9	6.32 (d, 2.7)	103.8
9	–	157.4	–	157.4
10	6.50 (dd, 8.6, 2.7)	110.5	6.48 (dd, 8.6, 2.7)	110.3
11	7.32 (d, 8.6)	128.2	7.30 (d, 8.6)	128.2
11a	–	103.6	–	103.6
12	–	195.5	–	195.5
12a	–	67.7	–	67.6
OMe	3.75 (s)	56.0	3.73 (s)	56.2
OMe			3.82 (s)	55.5

[a] Chemical shift values are in ppm from TMS, and *J* values in Hz are presented in parentheses. Carbon multiplicities were determined using DEPT experiments. All signals were assigned by DQF−COSY, HSQC, and HMBC studies.

indicating the *β* configuration of H-6a. The configuration of compound **1** at the B/C fusion was *cis* (thermodynamically most stable form), which was easily established by the value of the shielded proton at the C-1 position (*δ* 7.20 ppm).[6] A HSQC experiment established the correlation of the protons with the corresponding carbons and allowed the assignment of all carbon resonances (Table 1). The position of the methoxy group in ring A was determined by homonuclear NOE experiments. On irradiation of the methoxy protons a significant NOE was observed at H-4 and at H-2, while irradiation of H-11 caused an appreciable NOE only at H-10. These observations clearly revealed that the methoxyl group was located at C-3 and the hydroxy group was therefore placed at C-9. The HMBC correlations for H-1/C-12a, C-4a and C-3; H-2/C-1a and C-4; OMe/C-3; H-6/C-4a and C-12a; H-6a/C-1a, C-7a and C-12; H-11/C-12 and C-9; H-8/C-11a and C-10 confirmed the assignments of all proton and carbon resonances and the location of methoxyl and hydroxyl groups. Comparison of the optical rotation of **1** with those of other 12a-hydroxyrotenoids[4] indicated that the absolute configuration at C-6a and C-12a were *R*,*R*, respectively. From all these data the structure of compound **1**, named gliricidol, was assigned as reported.

The EIMS of **2** gave a molecular ion at *m*/*z* 344 which was 30 mass units higher than that of **1**. Comparison of the NMR data of **2** with those of **1** indicated the presence in **2** of a further methoxyl group (δ_H 3.82 and δ_C 55.5) (Table 1). The substitution at the C-2 position by this methoxy group was consistent with the ^{1}H NMR aromatic signals at *δ* 6.66 (s, H-1) and 6.48 (s, H-4) and in full agreement with the observed carbon resonance of C-1, C-2, C-3, and C-4 (Table 1). Definitive evidence of the structure of compound **2** was derived from the HMBC spectrum which showed clearly cross-peaks due to long-range couplings between OMe (*δ* 3.82) and C-2; OMe (*δ* 3.73) and C-3; H-1 and C-12a, C-4a and C-3; H-4 and C-1a and C-2; H-8 and C-10 and C-11a; and H-11 and C-7a, C-9 and C-12. Based on the DQF−COSY, HSQC and HMBC data, the ^{1}H-^{13}C NMR assignments were established uneanivocally (Table 1). From all these data the structure of compound **2** was assigned as 2-methoxygliricidol.

Compound **3** showed a molecular ion in EIMS at *m*/*z* 554. The ^{13}C NMR spectrum (Table 2), showed signals due to 33 carbons, including three signals at 67.3, 79.9, and 63.7 ppm assignable to C-6, C-6a, and C-12a of a 12a-hydroxyrotenoid moiety, three signals at 70.9, 32.9, and 31.2 ppm assignable to C-2, C-3, and C-4 of a isoflavan unit, two methoxyl signals at 55.4 and 55.7 ppm and a tertiary carbon signal at 40.7 ppm. Moreover, in the sp^2 carbon region, seven oxygenated carbon signals, five quaternary carbon signals, and twelve tertiary carbon signals were present. From the above evidence, **3** was assumed to be a dimer of a 12a-hydroxyrotenoid and an isoflavan unit. The ^{1}H NMR spectrum (Table 2) of **3** exhibited signals for two methoxyl group (*δ* 3.75 and 3.96, s), two oxygenated methylene protons at C-6 of a 12a-hydroxyrotenoid moiety and at C-2 of an isoflavan unit, a hydroxymethine proton at C-6a of a 12a-hydroxyrotenoid unit, and two methine protons at C-12 of the 12a-hydroxyrotenoid and C-3 of isoflavan moiety. Moreover, in the aromatic proton region, the signals observed were consistent with the presence of four trisubstituted aromatic rings (Table 2). The DFQ-COSY spectrum of **3** indentified six different spin systems corresponding to the four aromatic rings (1,3,4-substituted) and to the -OCH_2-CHO- and -OCH_2-CH-CH_2- sequences, the last one typical of the C-2, C-3, and C-4 of an isoflavan derivative. A HSQC NMR experiment established the association of the protons of the upper (**u**) and lower (**l**) units with the corresponding carbons, as listed in Table 2. From the above evidence, and taking compound **1** as a model compound, a linkage through C-12 of the hydroxyrotenoid unit and ring C of the isoflavan unit could be proposed. This structure of **3** and bonding through the C-12 of the upper and C-3′ of the lower units was confirmed by a HMBC experiment which showed clearly cross-peaks between H-1**u** and C-3**u**, C-4a**u**, C-12a**u**; H-6a**u** and C-1a**u**, C-7a**u**, C-12**u**; H-12**u** and C-1a**u**, C-6a**u**, C-7a**u**, C-11**u**, C-2′**l**, C-4′**l**; H-2**l** and C-4**l**, C-8a**l**, C-1′**l**; H-3**l** and C-4a**l**, C-2′**l**, C-6′**l**; H-4**l** and C-2**l**, C-1′**l**, C-5**l**, C-8a**l**; H-2′**l** and C-12**u**, C-3**l**, C-4′**l**, C-6′**l**; −OMe at *δ* 3.75 and C-3**u**; −OMe at *δ* 3.96 and C-4′**l**. The stereochemistry at the C-3**l** chiral center of isoflavan moiety was assigned in accordance with the magnitude of the H-3, H_2-2, and H-3 H_2-4 proton coupling constants and by NOE difference spectra. Thus, on irradiation of the signals at *δ* 4.24 (H-2eq, α) a NOE was observed on the signals at *δ* 3.44 (H-3ax, α) and *δ* 2.73 (H-4eq, α). Moreover, irradiation at *δ* 3.96 (H-2ax, *β*) resulted in a NOE enhancement of the signal at *δ* 2.96 (H-4ax, *β*). The results of the NOE experiments led to the determination of the *β*-orientation of substituent at C-3**l** position. The stereochemistry at C-12 in compound **3** was not defined. Compound **3** is a new natural product, named gliricidon and assigned as reported.

These new rotenoids (**1**−**3**) were isolated together with vestitol and 2′-*O*-methylvestitol, which is of interest from a biogenetic consideration as it establishes evidence for a link between rotenoids and 2′-methoxyisoflavones in nature.

Compounds **1**−**3** were all toxic to the larvae of brine shrimp, *A. salina*; LC_{50} (*μ*g/mL) 50.7 (**1**), 284.5 (**2**), 243.7 (**3**).

Experimental Section

General Experimental Procedures. Melting points were determined using a Bausch & Lomb apparatus. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were obtained from a Beckman DU 670 spectophotometer. IR spectra were performed with a Bruker IFS-

Table 2. ^{1}H NMR and ^{13}C NMR Chemical Shifts for Compound **3** (in CD_3OD)[a]

	3u			**3l**	
position	δ_H	δ_C	position	δ_H	δ_C
1a	–	121.6	2 eq	4.24 (dd, 3.4, 10.2)	70.9
1	7.18 (d, 8.6)	128.6	2 ax	3.96 (t, 10.2)	
2	6.47 (dd, 8.6, 2.7)	107.4	3	3.44 (m)	32.9
3	–	160.6	4 eq	2.73 (dd, 5.4, 15.5)	31.2
4	6.41 (d, 2.7)	97.3	4 ax	2.96 (dd, 10.2, 15.5)	
4a	–	146.4	4a	–	114.6
6α	3.55 (d, 12.0)	67.3	5	6.86 (d, 8.6)	131.0
6*β*	4.25 (dd, 12.0, 2.5)		6	6.33 (dd, 8.6, 2.7)	108.8
6a	5.48 (d, 2.5)	79.9	7	–	156.2
7a	–	157.8	8	6.25 (d, 2.7)	103.6
8	6.33 (d, 2.7)	103.9	8a	–	157.0
9	–	157.3	1′	–	120.6
10	6.52 (dd, 8.6, 2.7)	110.5	2′	6.40 (d, 2.7)	105.5
11	7.31 (d, 8.6)	133.0	3′	–	121.7
11a	–	112.6	4′	–	160.8
12	3.54 (s)	40.7	5′	6.42 (d, 8.6)	102.2
12a	–	63.7	6′	6.99 (dd, 8.6, 2.7)	125.8
OMe	3.75 (s)	55.4	OMe	3.96 (s)	55.7

[a] Chemical shift values are in ppm from TMS, and *J* values in Hz are presented in parentheses. Carbon multiplicities were determined using DEPT experiments. All signals were assigned by DQF−COSY, HSQC, and HMBC studies.

48 spectophotometer. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, using the UXNMR software package was used for NMR experiments in CD_3OD. ^{1}H-^{1}H DQF−COSY (double quantum filtered COSY),[7] ^{1}H−^{13}C HSQC,[8] and HMBC[9] experiments were obtained using conventional pulse sequences as described in the literature. The EIMS were obtained from a VG-PROSPEC mass spectrometer (70 eV). HPLC separations were performed on a Waters 590 series pumping system with a Waters R401 refractive index detector equipped with a Waters μ-Bondapak C_{18} column.

Plant Material. The bark of *G. 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.

Extraction and Isolation. The dried and powdered bark (1.0 kg) was defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 39 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 five main fractions I−V. Fraction IV (800 mg), the only one that showed a toxic effect (LC_{50} = 427.3 μg/mL) against *A. salina*, 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 (65:35) as eluent, to yield pure compounds **1** (11 mg, t_R 9.5 min), **2** (15 mg, t_R 11 min), and **3** (9 mg, t_R 12 min).

Gliricidol (1). Isolated as an amorphous yellow solid: mp 118−121 °C; $[\alpha]^{25}_D$ +230° (*c* 1, MeOH); UV (MeOH) λ_{max} (log ϵ) 292 (4.56) nm; IR (KBr) ν_{max} 3456, 3002, 2850, 1640 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; EIMS *m*/*z* 314 [M]$^+$ (13), 178 (21), 136 (46).

2-Methoxygliricidol (2). Isolated as an amorphous yellow solid: mp 125−128 °C; $[\alpha]^{25}_D$ +228° (*c* 1, MeOH); UV(MeOH) λ_{max} (log ϵ) 292 (4.56) nm; IR (KBr) ν_{max} 3454, 3000, 2850, 1638 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 1; EIMS *m*/*z* 344 [M]$^+$ (18), 208 (24), 136 (48).

Gliricidin (3). Isolated as an amorphous yellow solid: mp 168−170 °C; $[\alpha]^{25}_D$ −66° (*c* 1, MeOH); UV (MeOH) λ_{max} (log ϵ) 283 (4.26), 215 (4.69) nm; IR (KBr) ν_{max} 3416, 1640 cm^{-1}; ^{1}H and ^{13}C NMR data are shown in Table 2; EIMS *m*/*z* 554 [M]$^+$ (65).

Brine Shrimp Bioassay for Compounds 1−3.[5] Newly hatched nauplii of the brine shrimp, *A. salina*, were used in this bioassay. The eggs were hatched in artificial seawater (37 g/L of "Instant Ocean" in deionized water) in a two-compartment Pyrex crystallizing dish divided by a vertical plastic strip that left a gap near the bottom of the dish. The eggs were placed in one compartment, and, after 24 h, the free swimming nauplii swam were harvested from the second compartment after phototaxis in the direction of a 60 W incandescent light.[10] The nauplii were used in the bioassay 24−36 h after the hatching of the eggs. The extracts, fractions and pure compounds to be tested were disolved or suspended in seawater to prepare stock solutions of 2 mg/mL. Water insoluble extracts were previously dissolved in 50 μL DMSO prior to adding seawater. The toxicity test was performed twice in triplicate in a well of a 48-well tissue culture plate at concentration of the test material of 1000, 500, 250, 125, 62.5, and 31.25 μg/mL. A minimum of 25 nauplii were transferred to the well in the tip of Pasteur pipet, using a minimal amount of hatching fluid in the transfer. Control wells with DMSO were included in each experiment. After 24 h of incubation at room temperature the plates were examined under a binocular microscope and the number of dead (nonmotile) nauplii in each well were counted. A 100 μL amount of methanol was then added to each well and after 15 min the total number of shrimp in each well was counted. LC_{50} values were then calculated by Probit analysis.[11]

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