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IRIDOIDS FROM *TOCOYENA FORMOSA*

VANDERLAN DA S. BOLZANI,* CLARA M. IZUMISAWA,† M. CLAUDIA M. YOUNG,† LÍGIA M. V. TREVISAN, DAVID G. I. KINGSTON‡ and A. LESLIE GUNATILAKA‡

Instituto de Química, Universidade Estadual Paulista, CP. 335, 14800, Araraquara, São Paulo, Brazil; † Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, CP. 4005, 10051, São Paulo, Brazil; ‡ Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0212, U.S.A.

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Key Word Index—*Tocoyena formosa*; Rubiaceae; antifungal iridoids.

Abstract—The leaves of *Tocoyena formosa* afforded two antifungal iridoids, α- and β-gardiol, and the new iridoids, mollugoside methyl ester and formosinoside. Formosinoside was characterized as its hexaacetyl derivative. © 1997 Elsevier Science Ltd

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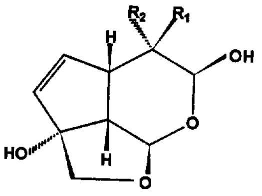
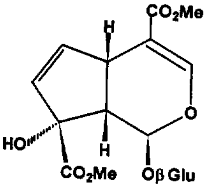
1. INTRODUCTION

Tocoyena formosa (Cham. et Sch.) K. Shun, a small ornamental tree growing in dry regions of Central Brazil, is commonly known as 'genipapo do campo' and has very impressive yellow-green flowers [1]. In the course of our continuing search for bioactive constituents from rubiaceous species that grow in 'Cerrado Regions' of Brazil [2–4], we investigated the ethyl acetate-soluble fraction of the 60% aqueous ethanol extract of leaves of *T. formosa*. In our mechanism-based bioassay for potential anticancer agents utilizing genetically-engineered yeast (*Saccharomyces cerevisiae*) strains [5], this extract exhibited moderate and nonselective activity on all the mutant strains tested suggesting the presence of antifungal agents rather than potential anticancer agents. Subsequent confirmation of its antifungal activity using *Cladosporium cladosporioides* and *C. esphaerospermum* [6] followed by bioassay-guided fractionation afforded four iridoids **1–4**. Of these, α-gardiol (**1**) and β-gardiol (**2**) which were obtained as an inseparable mixture [7] exhibited antifungal activity. The third iridoid, mollugoside methyl ester (**3**), is being described here for the first time from a natural source. The fourth iridoid found to be new and hence named formosinoside (**4**), was characterized as its hexaacetate derivative (**5**). This paper deals with the structure elucidation of **5** and the antifungal activities of **1** and **2**.



1 $R_1 = H; R_2 = CO_2Me$

2 $R_1 = CO_2Me; R_2 = H$



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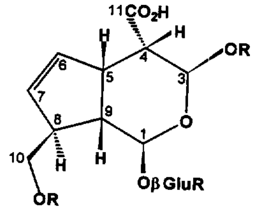

4 R = H

5 R = Ac

RESULT AND DISCUSSION

The 60% aqueous ethanol extract of the leaves of *T. formosa* was concentrated to half its original volume and was extracted successively with hexane and ethyl acetate. The ethyl acetate fraction on silica gel chromatography afforded three pure compounds identified as α-gardiol (**1**), β-gardiol (**2**) [5] (see Experimental) and mollugoside methyl ester (**3**) [8], and an inseparable mixture (3:1) of two compounds. These two compounds which were suspected to be isomers from NMR spectra of the mixture were separated via their peracetates to obtain the hexaacetate (**5**) of the major compound (**4**). Besides the iridoids, the ethyl esters of ferulic and caffeic acids were also isolated. Both are probably artifacts of aqueous ethanol extraction.

The molecular formula of **5**, mp 144–146°, $[\alpha]_D + 36.1°$, was deduced as $C_{28}H_{36}O_{17}$ by HRMS and ^{13}C NMR spectroscopy. The IR spectrum indicated the presence of a carbonyl ester function ($1710\ cm^{-1}$)

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* Author to whom correspondence should be addressed.

Table 1. ^{1}H and ^{13}C NMR chemical shifts and coupling constants (Hz) of **5**[a]

C	^{13}C[b]	^{1}H[c]	^{1}H long-range correlations (J CH = 10 Hz)
1	98.06 *d*	5.00 *d*(5.8)	5.93 (H-1), 3.00 (H-9), 3.08 (H-8), 5.92 (H-3)
3	91.52 *d*	5.92 *d*(7.6)	2.08 (Ac), 5.00 (H-1)
4	46.56 *d*	2.98 *dd*(7.6; 2.0)	5.92 (H-3), 3.53 (H-5), 3.00 (H-9), 5.80 (H-6)
5	42.44 *d*	3.53 *ddd*(2.0; 2.2; 9.0)	5.80 (H-6), 5.55 (H-7), 3.00 (H-9)
6	136.57 *d*	5.80 *dd*(6.0; 2.0)	3.53 (H-5), 5.55 (H-7), 3.08 (H-8)
7	134.62 *d*	5.55 *dd*(6.0; 1.5)	3.08 (H-8), 3.53 (H-5), 3.00 (H-9)
8	39.42 *d*	3.08 *dd*(9.0; 1.5)	5.00 (H-1), 3.00 (H-9), 5.80 (H-6), 4.22 and 4.90 (H-10)
9	42.28 *d*	3.00 *dd*(9.0; 5.8)	5.00 (H-1), 3.53 (H-5), 5.55 (H-7), 3.08 (H-8), 4.22 and 4.90 (H-10)
10	67.18 *t*	4.22 *dd*(11.0; 8.0) 4.90 *dd*(11.0; 3.0)	3.08 (H-8), 3.00 (H-9), 5.55 (H-7)
11	180.29 *s*	—	2.98 (H-4), 5.92 (H-3)
1′	99.28 *d*	5.93 *d*(8.0)	5.00 (H-1), 4.68 (H-2′), 4.42 (H-3′)
2′	73.70 *d*	4.68 *m*	5.93 (H-1′)
3′	76.08 *d*	4.42 *m*	5.93 (H-1′), 4.18 (H-5′)
4′	69.08 *d*	4.92 *m*	4.68 (H-2′), 4.18 (H-5′)
5′	74.10 *d*	4.78 *m*	4.42 (H-3′), 4.44 (H-6′)
6′	65.01 *t*	4.44 *dd*(11.9; 6.5)	4.78 (H-5′), 4.92 (H-4′)
$CH_3\underline{C}O$	168.11 to 170.45 *s*	—	—
$\underline{C}H_3CO$	20.57 to 21.87 *q*	1.98 *s* (2 × OAc), 2.01 *s*, 2.02 *s*, 2.05 *s* 2.09 *s* (4 × OAc)	—

[a] The ^{1}H NMR spectrum was recorded in $CDCl_3$ at 200 and 400 MHz. The ^{13}C NMR spectrum was recorded in $CDCl_3$ at 50 and 100 MHz.

[b] Multiplicities were based in DEPT-135 experiment, assignments were based on HETCOR. [c] Assignments from ^{1}H-^{1}H COSY-45 experiment.

and a carboxylic acid group (3378 and 1695 cm^{-1}). The ^{1}H NMR spectrum of **5** (Table 1) exhibited the presence of two olefinic protons at δ 5.55 and δ 5.80 (each 1H, *dd*, $J = 6.0$; 1.5 Hz, H-7 and *dd*, $J = 6.0$; 2.0 Hz, H-6). In addition, the ^{1}H NMR spectrum showed signals at δ 5.00 (1H, *d*, $J = 5.8$ Hz), δ 5.92 (1H, *d*, $J = 7.6$ Hz), δ 4.22 and 4.90 (2H, *dd*, $J = 11.0$; 8.0 and 11.0; 3.0 Hz), which were attributed to protons at C-1, C-3 and C-10, respectively. The remaining doublet at δ 5.93 (1H, $J = 8.0$ Hz) could be assigned to the anomeric proton from sugar moiety present in the molecule of **5**. The ^{1}H NMR also supported the presence of six acetyl groups as broad signals at δ 1.98 (corresponding two acetyl groups), 2.01, 2.02, 2.05 and 2.09. The ^{13}C NMR of **5** (Table 1) showed signals due to two olefinic carbons at δ 134.62 and 136.57; a hexopyranose moiety (δ 99.28, 76.08, 74.10, 73.70, 69.08 and 65.01); six acetyl groups; two hemiacetalic carbons at δ 91.52 and 98.06 and one —CH_2— carbon at δ 67.18. Detailed analysis of chemical shifts, with the aid of 2D NMR, mainly ^{1}H-^{1}H COSY spectrum led us to suggest that this compound also had an iridoidal structure. It could belong to a structural type quite close to apodantheroside [9] with a double bond between C-6, C-7 and hydroxyl function at C-10. By assuming a iridoidal skeleton, and by comparison with carbon assignments of known iridoids, it was possible to assign the carboxyl group (δ 180.29 *s*) to C-11. The structure of the iridoidal aglycone and the position of glycosidic linkage of **5** were further elucidated, as follows, by an extensive use of 2D NMR methods (^{1}H-^{1}H COSY, HETCOR and COLOC). By COSY correlation it was possible to observe that a doublet at δ 5.00 ($J = 5.8$ Hz) was coupled to a double doublet at δ 3.00 ($J = 9.0$; 5.8 Hz) and a doublet at δ 5.92 coupled to a double doublet at δ 2.98 ($J = 7.6$; 2.0 Hz). In addition, the COSY-45 spectrum showed that the olefinic methine H-7 at δ 5.55 was correlated with methine H-8 at δ 3.08 ($J = 9.0$; 1.5 Hz); and an ABX system at δ 4.22 ($J = 11.0$; 8.0 Hz) and 4.90 ($J = 11.0$; 3.0 Hz) were due to the hydroxymethylene H-10. The HETCOR spectrum of **5** revealed that the methine at δ 99.28 was correlated with H-1′ (δ 5.93 *d*), while the signal at δ 98.06 was correlated with H-1 (δ 5.00 *d*). The correlations between methines δ 91.52 and H-3 (δ 5.92 *d*); δ 46.56 and H-4 (δ 2.98 *dd*); δ 42.44 and H-5 (δ 3.53 *ddd*); δ 39.42 and H-8 (δ 3.08 *dd*); δ 42.28 and H-9 (δ 3.00 *dd*) were also confirmed. The COLOC spectrum (see Table 1) further confirmed the proposed structure and allowed unequivocal assignment of all ^{1}H and ^{13}C NMR signals. NOE experiments performed on compound **5**, dissolved in $CDCl_3$, confirmed the stereochemical assumptions on the relative orientation of acetoxyl, carboxyl and hydroxymethylene groups and permitted the assignment of the overall relative stereochemistry of the molecule

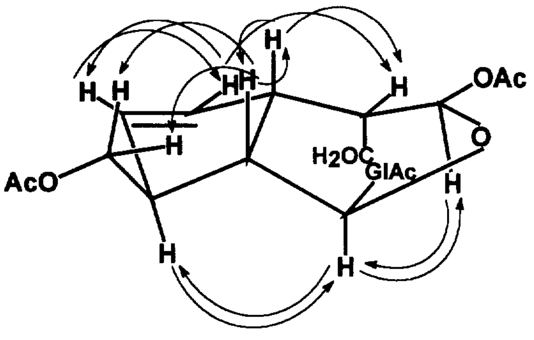

Fig. 1

as in Fig. 1. Particularly, irradiation of the signal for 9β-H at δ 3.00 produced significant enhancement of the H-10 (~20%) methylene and methine 5β-H (~35%). Furthermore, irradiation of 1α-H showed simultaneous enhancement of the signals at 8α-H (12%) and 3α-H (15%). These findings suggest that the acetoxyl group at C-3 and the hydroxymethylene at C-8 are in β disposition.

From iridoids derivatives isolated from *T. formosa* only the epimeric mixture of α- and β-gardiol showed antifungal activity (Table 2). The fungitoxic properties of some iridoidal aglycones have already been observed in the case of *Alibertia macrophylla* [4] and seem to be an interesting feature of Rubiaceae growing in 'Cerrado Regions'.

EXPERIMENTAL

General. Mp: uncorr.; IR: KBr; NMR: 200, 400 MHz (^{1}H) and 100 MHz (^{13}C) in: $CDCl_3$: EIMS: at 70 EV Finnigan Mat 4610; HREMS: at 70 EV VG 7070E-HF. CC: silica gel 60; TLC: silica gel Gel GF_{254}.

Plant material. Leaves of *Tocoyena formosa* were collected in the Estação Ecológica e Experimental de Mogi-Guaçú, São Paulo State, Brazil in July 1991 by Dr M. C. M. Young and identified by Dr S. L. J. Mendaçolli, Instituto de Botânica de São Paulo. A voucher specimen (SP1787979) has been deposited in the Herbarium of Instituto de Botânica de São Paulo, Brazil.

Biological screening procedures. The experimental methods utilized in the screening procedures with *S. cerevisiae* mutants [5] and *C. cladosporioides* [6] have been described elsewhere. The procedure used for *C. esphaerospermum* was similar to that of *C. cladosporioides*.

Extraction and isolation. Air-dried and powdered leaves (800 g) of *T. formosa* were extracted with 60% aq. EtOH. The resulting extract was concd to half its original vol. and extracted with hexane followed by EtOAc. Evapn afforded hexane (3.00 g) and EtOAc (5.5 g) extracts. The EtOAc fr. was submitted to CC on silica gel and eluted with mixts of $CHCl_3$ and MeOH. A total of 28 frs were collected. Frs 9–11 were further purified by prep. TLC [silica gel, C_6H_6–EtOAc (2:3) containing 3% HOAc] yielding an epimeric mixt. of **1** and **2** (42.5 mg) [5] and ethyl ferulate (19.4 mg). Frs 12–14 yielded, after prep. TLC (as described for 9–11) ethyl caffeoate (20 mg). Mollugoside methylester **3** (40 mg) [8] was isolated by prep. TLC [silica gel, C_6H_6–EtOAc (1:1)+1.5% HOAc], after purification of fr. 15.

Fractions 17–18 were purified by prep. TLC [silica gel, EtOAc–MeOH–H_2O (77:15:8). TLC and ^{1}H NMR investigations of this purification indicated that the resulting fr. was of interest and looked to be constituted of only one product. However, ^{13}C NMR of this compound indicated an inseparable mixt. (3:1) which was subjected to acetylation (Ac_2O/pyr. 25°, 12 Hs). The acetyl derivatives obtained, were subjected to prep. TLC (silica gel, CH_2Cl_2–EtOAc (2:3)+1% HOAc), which afforded the major compound **5** (23 mg).

*Formosinoside-*1*-hexaacetate* (**5**). Amorphous powder, mp 144–146°, $[\alpha]_D = +36.1°$ ($CHCl_3$, *c* 3.17). Found: C, 52.68; H, 4.92% $C_{28}H_{36}O_{17}$ requires C, 52.17; H, 5.59%; IR ν_{max}^{KBr} 3780, 1710, 1695, 1630, 1270, 1055 cm^{-1}; EIMS *m/z* (rel int.) $[M]^+$ 644 (8), 331 (89), 314 (35), 296 (15), 253 (9) 251 (50), 209 (5), 233 (100), 43 (70); HREIMS 644.4374 (calcd for $C_{28}H_{36}O_{17}$ 644.4321); ^{1}H NMR 200, 400 MHz and ^{13}C NMR 50 MHz, $CDCl_3$ see Table 1.

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Table 2. Bioactivity data of crude extract, fractions, α-, β-gardiol and the standards[a]

Crude extract, fractions and compounds	Organisms[b] RS321	RS167N	RS322N	RS188N
60% aqueous EtOH extract	980	950	650	680
Fractions 9–11	250	210	180	200
α- and β-Gardiol	120	126	90	105
Camptotecin	—	8.7	0.6	—
Streptonigrin	—	2.4	0.4	—

[a] Results are expressed as IC_{12} [RS321, RS167N, RS322N, RS188N (μg m^{-1})].

[b] Organisms RS321, RS167N, RS322N are DNA repair-deficient yeast strains and RS188N is the repair proficient wild-type strain of *S. cerevisiae*.

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