Cytotoxic Flavonoids from *Platymiscium floribundum*

Maria José C. Falcão,[†] Yvone Brígido M. Pouliquem,[†] Mary Anne S. Lima,[†] Nilce Viana Gramosa,[‡] Letícia V. Costa-Lotufo,[†] Gardênia Carmen G. Militão,[†] Cláudia Pessoa,[†] Manoel Odorico de Moraes,[†] and Edilberto R. Silveira*[,†]

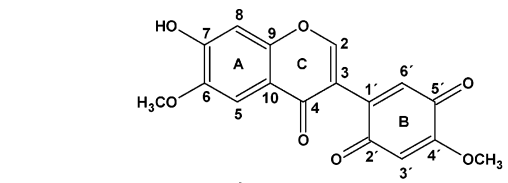
Curso de Pós-Graduação em Química Orgânica, Departamento de Química Orgânica e Inorgânica, Centro de Ciências, Curso de Pós-Graduação em Farmacologia, Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, CP 12200, Fortaleza-Ce, 60021-940, Brazil, and Departamento de Química, Universidade Estadual do Ceará, Fortaleza-Ce, 60740-000, Brazil

Received April 23, 2004

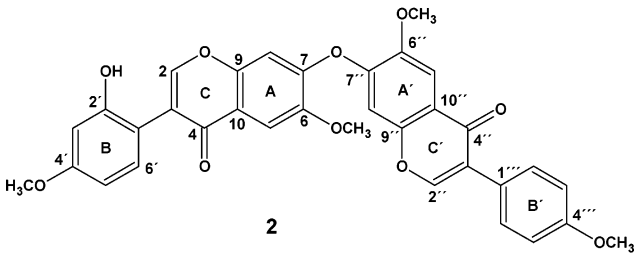
Two new isoflavonoids, 7-hydroxy-6,4′-dimethoxy-isoflavonequinone (**1**) and 2′-hydroxy-6,4′,6″,4‴-tetramethoxy-[7-*O*-7″]-bisisoflavone (**2**), and seven other known flavonoids, 3-hydroxy-9-methoxypterocarpan (medicarpin), 3,10-dihydroxy-9-methoxypterocarpan, 3,9-dimethoxypterocarpan (homopterocarpin) (**3**), 2,3,9-trimethoxypterocarpan (**4**), 3,4-dihydroxy-9-methoxypterocarpan (vesticarpan) (**5**), 2′,4,4′-trihydroxychalcone (isoliquiritigenin), and 7,4′-dihydroxyflavanone (liquiritigenin) (**6**), were isolated from the heartwood of *Platymiscium floribundum.* The structures of compounds **1** and **2** were established by spectroscopic methods. Compounds **3**−**6** showed cytotoxic activity when evaluated against five human cancer cell lines in vitro.

Platymiscium (Leguminosae-Papilionoideae) is a small genus containing 33 species with a restricted distribution in the Americas.[1] The chemistry of this genus is limited to the phytochemical investigation of only four species so far and has revealed flavonoids, isoflavonoids, and coumarins as principal constituents.[2−5] *Platymiscium floribundum* Vog. is a tree that occurs in Northeast Brazil, where it is known as "sacambu" and "jacaranda-do-litoral". This species is used by the local population as an antiinflamatory agent, and its wood has high commercial value in carpentry as a structural timber.[1]

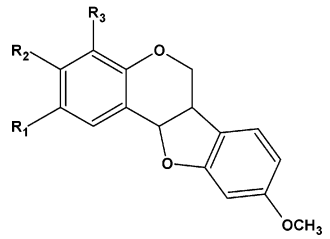
Previous studies of roots of *P. floribundum* led to the isolation of isoflavonoids, coumarins, and triterpenes.[5] In our work from the heartwood, we have now isolated nine flavonoid derivatives: the new isoflavonoids, 7-hydroxy-6,4′-dimethoxy-isoflavonequinone (**1**) and 2′-hydroxy-6,4′,6″,4‴-tetramethoxy-[7-O-7″]-bisisoflavone (**2**), and the previously known 3,9-dimethoxypterocarpan (homopterocarpin) (**3**),[6] 2,3,9-trimethoxypterocarpan (**4**),[8] 3-hydroxy-9-methoxypterocarpan (medicarpin),[6] 3,4-dihydroxy-9-methoxypterocarpan (vesticarpan) (**5**),[9] 2′,4,4′-trihydroxychalcone (isoliquiritigenin),[10] and 7,4′-dihydroxyflavanone (liquitirigenin) (**6**).[11] Although several isoflavonoids have been obtained from roots of *P. floribundum*,[5] none of the above-mentioned compounds have been isolated previously from this species. The cytotoxicity of the isolates obtained herein from *P. floribundum* has been evaluated against a small panel of cancer cell lines.



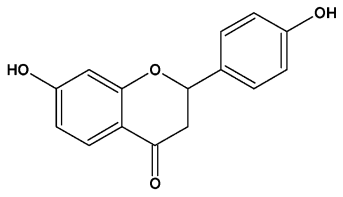
1



2



3 $R_1 = R_3 = H$; $R_2 = OMe$
4 $R_1 = R_2 = OMe$; $R_3 = H$
5 $R_1 = H$; $R_2 = R_3 = OH$



6

Compound **1** was obtained as a yellow powder, mp > 300 °C, and was found to possess the molecular formula $C_{17}H_{12}O_7$ by HREIMS *m/z* 328.0466 (calcd for $C_{17}H_{12}O_7$, 328.0583 [M^+]). The IR spectrum showed bands for two carbonyl groups at 1671 and 1625 cm^{-1} and for hydroxy groups at 3500 cm^{-1}. The ^{13}C NMR spectrum displayed signals for 17 carbon atoms, including three carbonyl groups (δ 185.3, 181.3, and 173.1) and two methoxyl groups (δ 56.5 and 55.9). The presence of an upfield oxymethine carbon at δ 156.2 (C-2), typical of the isoflavone skeleton,[6] besides the three carbonyl groups, was indicative of the presence of a benzoquinone system, suggesting an isoflavonequinone structure for compound **1**. This was confirmed by analysis of the ^1H NMR spectrum, which showed a deshielded signal for a hydrogen at δ 8.33 (s, H-2), related

* To whom correspondence should be addressed. Tel: 55 85 32889971. Fax: 55 85 32889971. E-mail: edil@ufc.br.
[†] Universidade Federal do Ceará.
[‡] Universidade Estadual do Ceará.

10.1021/np049854d CCC: $30.25
Published on Web 02/12/2005

Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** and **2** (DMSO-d_6)[a]

	1			**2**		
carbon	δ_H	δ_C	HMBC	δ_H[b]	δ_C	HMBC
2	8.33 s	156.2		8.33 s	155.0	
3		116.2	H-2, H-6′		123.2	H-2, H-6′
4		173.1	H-2, H-5		177.2	H-2, H-5
5	7.39 s	104.6		7.84 s	105.6	
6		147.3	H-5, H-8		148.2	H-5, H-8, OMe-6
7		153.3	H-5		155.2	H-5, H-8
8	6.99 s	103.0		7.19 s	104.0	
9		151.5	H-2, H-5, H-8		153.4	H-2, H-5, H-8
10		115.8			117.1	H-5, H-8
1′		139.4	H-2, H-3′, H-6′		114.1	H-2, H-3′, H-5′
2′		185.3			158.7	H-6′
3′	6.24 s	108.0		6.94 d (2.5)	104.0	H-5′
4′		158.4	H-3′, H-6′		162.0	H-6′, OMe-4′
5′		181.3		6.69 dd (2.5, 8.5)	106.2	H-3′
6′	7.07 s	132.8		7.49 d (8.5)	132.6	
2″				8.16 s	152.7	
3″					124.2	H-2″, H-2‴, H-6‴
4″					175.5	H-2″, H-5″
5″				7.91 s	105.1	
6″					148.0	H-5″, H-8″, OMe-6″
7″					154.8	H-5″, H-8″
8‴				7.21 s	104.2	
9‴					153.3	H-2″, H-5″, H-8″
10‴					117.7	H-5″, H-8″
1‴					125.6	H-2‴, H-3‴, H-5‴
2‴/6‴				7.80 d (8.7)	131.0	
3‴/5‴				7.07 d (8.7)	114.4	H-2‴, H-6‴
4‴					160.1	OMe-4‴
OMe-4′	3.83 s	56.5		3.68 s	55.4	
OMe-4‴				3.68 s	55.4	
OMe-6	3.88 s	55.9		3.72 s	56.1	
OH-7	10.73 s					

[a] The ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively. [b] Coupling constants (in parentheses) are in Hz.

to an oxyolefinic proton characteristic of the 3-phenylchromone C-ring of isoflavones.[12] Also observed were one nonchelated, but conjugated, hydroxyl group at δ 10.73 (s, OH-7), two methoxyl groups at δ 3.89 and 3.83, and four singlets, one proton each, of aromatic-like protons at δ 6.24 (s, H-3'), 6.99 (s, H-8), 7.07 (s, H-6'), and 7.39 (s, H-5). This information supported a 6,7-disubstituted A-ring of an isoflavonoid moiety linked to a 2,5-disubstituted *para*-quinone unit.

Analysis of the HMQC and HMBC NMR spectra of **1** allowed the assignments of all carbons and hydrogens (Table 1). The long-range correlations in the HMBC spectrum confirmed the isoflavone skeleton by the three-bond coupling of the proton at δ 8.34 (H-2) with the carbonyl group at δ 173.1 (C-4), and also with the carbons at δ 151.5 (C-9) and 139.4 (C-1′). The absorptions of hydrogens and the methoxyl group at the *para*-benzoquinone system were defined by the ^3J correlations of the hydrogen at δ 7.07 (H-6′) with the deshielded carbonyl at δ 181.3 (C-2′) and with the carbon at δ 116.3 (C-3). ^3J correlations of the hydrogen at δ 6.24 (H-3′) were observed with the most deshielded carbonyl group at δ 185.3 (C-5′) and with the carbon at 139.4 (C-1′). The correlations of both protons (H-3′ and H-6′) and the methoxyl group at δ 3.83 with the carbon at δ 158.4 (C-4′) were also diagnostic.

The substitution pattern of the aromatic ring A in **1** was established definitively by HMBC correlations between the proton at δ 6.99 (H-8) and the carbons at δ 151.5 (C-9, ^2J), 153.3 (C-7, ^2J), 147.3 (C-6, ^3J), and 115.8 (C-10, ^3J). The remaining proton at δ 7.39 (H-5), *peri* to the carbonyl, showed correlations with the signals at δ 173.1 (C-4, ^3J), 153.3 (C-7), 151.5 (C-9, ^3J), and 147.6 (C-6, ^2J). Further, correlations observed of the methoxyl group at δ 3.88 and the hydroxyl group at δ 10.73 with the carbons at δ 147.6 (C-6) and 153.3 (C-7), respectively, allowed the positions of these groups to be determined at C-6 and C-7, respectively. Comparison with ^{13}C NMR data of 5-hydroxybowdichione, an isoflavonequinone isolated from *Dalbergia candenatensis*,[13] supported the determination of the structure of **1** as the new 7-hydroxy-6,4′-dimethoxy-isoflavonequinone, the third isoflavonequinone so far reported in the literature. EIMS fragments at *m/z* 166 (7.0%) and 162 (2.0%), arising by the retro-Diels−Alder rearrangement involving the B-ring, and an intense peak at *m/z* 69 (37.0%), characteristic of methoxybenzoquinones, also supported the proposed structure for **1**.

Compound **2** was isolated as a yellow powder, mp 213−215 °C. The IR spectrum showed absorption bands at 3443, 1622 (broad), 1571, and 1516 cm^{-1}, suggesting the presence of hydroxyl, carbonyl, and aromatic ring functionalities. Support for a biflavonoid structure for compound **2** came from the presence of two very close carbonyl groups (δ 177.2 and 175.5) in the ^{13}C NMR spectrum, in addition to two methoxyl groups (δ 55.4 and 56.1), and 25 signals for sp^2 carbons, of which nine were oxygen-bearing non-hydrogenated carbons. Analysis of the ^{1}H NMR spectrum established the presence of two intense signals at δ 3.72 and 3.68, integrating for six protons each, related to two sets of superimposable aromatic methoxyl groups, and 13 signals relative to aromatic or olefinic protons resonating between δ 6.67 and 8.33. A COSY experiment showed the presence of an AMX coupling system, through correlations of the hydrogen at δ 6.69 (dd, J = 2.5, 8.5 Hz, H-5′) with the signals at δ 7.49 (d, J = 8.5 Hz, H-6′) and 6.94 (d, J = 2.5 Hz, H-3′). In addition, an A_2B_2 aromatic system was established by the correlations of the signals at δ 7.80 (d, J = 8.7 Hz, H-2‴ and H-6‴) and at δ 7.07 (dd, J = 8.7 Hz, H-3‴ and H-5‴), both integrating for two protons. Further,

Table 2. Cytotoxic Activity of Flavonoids **3**−**6** Isolated from *Platymiscium floribundum* for Tumor Cell Lines[a]

compound	cell line (IC_{50} μg/mL)				
	CEM	HL-60	HCT-8	MCF-7	B16
doxorubicin	0.02	0.02	0.04	0.20	0.03
	0.01−0.02	0.01−0.02	0.03−0.05	0.17−0.24	0.02−0.04
3	5.5	3.9	6.4	18.5	5.2
	4.4−6.7	2.6−5.7	3.9−10.5	17.8−19.2	3.7−7.3
4	0.6	0.1	0.6	0.7	2.9
	0.5−0.7	0.1−0.2	0.2−1.6	0.4−1.4	1.6−5.3
5	7.3	6.9	12.4	10.	2.7
	6.9−7.8	4.4−10.8	9.7−15.8	8.0−13.1	2.0−3.7
6	>25.0	22.5	19.5	>25.0	5.0
		17.5−28.9	16.8−22.6		3.5−7.1

[a] Data are presented as IC_{50} values and 95% confidence interval obtained by nonlinear regression for leukemias (HL-60 and CEM), breast (MCF-7), colon (HCT-8), and skin (B16) cancer cells from three independent experiments. Doxorubicin was used as positive control. Only compounds with IC_{50} values lower than 5 μg/mL at least for one cell line were considered active.

the remaining four aromatic singlets at δ 7.84 (H-5), 7.19 (H-8), 7.91 (H-5″), and 7.21 (H-8″) were ascribed as comprising two tetrasubstituted aromatic rings, with the protons situated *para* to one another, corresponding to two 6,7-disubstituted A aromatic rings.

The HMQC NMR spectrum of **2** allowed the assignments of all hydrogens and carbons (Table 1) and revealed correlations of the signals relative to two sets of superimposable methoxyl groups at δ 3.72 and 3.68 with the carbons at δ 56.1 and 55.4, respectively. Correlations of the monohydrogenated carbons at δ 155.0 and 152.7, with protons in the downfield region at δ 8.33 (1H, s) and 8.16 (1H, s), suggested that both flavonoid units were isoflavones. Correlations of the methoxyl group at δ 3.68 and the hydrogens of the A_2X_2 system at δ 7.80 (H-2‴ and H-6‴) and 7.05 (H-3‴ and H-5‴), with the carbon at δ 160.1 (C-4‴) in the HMBC spectrum, established the position of this methoxyl group at C-4‴ on the B′-ring. On the other hand, correlations of the second methoxyl group at δ 3.68 and hydrogens of the AMX system at δ 6.69 (H-5′), 6.94 (H-3′), and 7.49 (H-6′), with the carbon at δ 162.0 (C-4′), supported the placement of this methoxyl group at the C-4′ position of the B-ring. The relative positions of the two remaining methoxyls at δ 3.72 (C-6, C-6′) were determined by their correlations with the oxygenated carbons at δ 148.0 (C-6′) and 148.2 (C-6), of the two tetrasubstituted aromatic rings A and A′, respectively.

All these observations allowed the establishment of the connection of the isoflavonoid moieties through a C-7−O−C-7″ ether linkage and permitted the final structure of compound **2** to be proposed as 2′-hydroxy-6,4′,6″,4‴-tetramethoxy-[7-*O*-7″]-bisisoflavone**.** This structure was corroborated by its EIMS,[14] from the presence of ions at *m/z* 298 and 314, indicating two isoflavone subunits generated through mass fragmentation.

Pterocarpans are abundant in Leguminosae and are well represented in the genus *Platymiscium*, particularly *P. yucatanum*[3,4] and *P. trinitatis*.[3] On the other hand, biflavonoids in this family have been limited to the genus *Acacia*, chiefly proanthocyanidin C−C and C−O−C linked dimers.[6] However, this is the first report of the occurrence of an ether-linked isoflavonoid from plants. Isoflavonequinones such as **1** are rare and were previously isolated only from the legume species *Dalbergia candenatensis*[13] and *Bowdichia nitida.*[15]

The isolated compounds were tested for their cytotoxicity using five tumor cell lines: two human leukemias (HL-60 and CEM), human breast adenocarcinoma (MCF-7), human colon adenocarcinoma (HCT-8), and murine melanoma (B16). The IC_{50} values are shown in Table 2. The pterocarpans were found to be the most active compounds among the isolated flavonoids. Compound **4** displayed strong activity against the human cell lines and a moderate activity against murine melanoma.

Experimental Section

General Experimental Procedures. Melting points were determined with a Metler micromelting apparatus and are uncorrected. UV spectra were obtained as methanol solution on a Varian Cary 50 Conc UV−visible spectrophotometer. IR spectra were recorded in KBr disks on a Perkin-Elmer 1000 FT-IR spectrometer. ^1H and ^{13}C NMR data were obtained on a Bruker Avance DRX-500 spectrometer at room temperature in DMSO-d_6 and $CDCl_3$, observing ^1H at 500.13 and ^{13}C at 125.77 MHz, respectively. HMQC and HMBC spectra were obtained using Bruker's standard pulse sequences.[16] Chemical shifts are given on the δ scale and were referenced to residual DMSO or $CHCl_3$. HREIMS and EIMS were obtained on VG Autospec Fisons instruments. Silica gel 60 (Merck, 70−230 mesh) was used for column chromatography, and precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical chromatography. Centrifugal preparative TLC was performed using a Harrison Research Chromatotron 7429T. Thin-layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm thickness), and the compounds were visualized by spraying with vanillin/percloric acid/EtOH solution and then heating on a hot plate.

Plant Material. The heartwood of *Platymiscium floribundum* Vog. was collected in December 1999, in Acarape County, Ceará, Brazil. A voucher specimen (No. 31052) was identified by Dr. A. G. Fernandes (Universidade Federal do Ceará) and deposited at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Ceará, Brazil.

Extraction and Isolation. The air-dried heartwood (1.7 kg) was pulverized and extracted with hexane (4000 mL) at room temperature. The solvent was removed under reduced pressure, yielding a viscous brown oil (7 g). The marc obtained after hexane extraction was extracted with $CHCl_3$ (4000 mL) to afford a dark brown resinous extract (76.0 g) and later extracted with EtOH (4000 mL) to give a dark brown resinous extract (35.0 g).

Part of the $CHCl_3$ extract (50 g) was adsorbed onto silica gel (5 g) and coarsely fractionated over an initial silica gel (150 g) column, by elution with hexane, CH_2Cl_2, EtOAc, and MeOH, as binary mixtures of increasing polarity, yielding 10 pooled fractions after TLC analysis. Fraction *G* (CH_2Cl_2−EtOAc 1:1, 3.0 g) yielded small crystals, which were collected by removal of the supernatant liquid and recrystallized from MeOH, yielding **1** (18 mg). Fraction *H* (CH_2Cl_2−EtOAc 1:1, 1.8 g) was rechromatographed on a silica gel (30.0 g) column, by elution with hexane followed by CH_2Cl_2, EtOAc, and MeOH, which were combined according to TLC analysis to give **2** (15 mg) as a pale yellow solid. Evaporation of the mother liquor of fraction *G* gave 3-hydroxy-9-methoxypterocarpan[6] (17 mg) as a white solid. Fraction *C* (hexane−CH_2Cl_2 1:1, 3.1 g) yielded white

crystals, which were filtered to give 3,4-dihydroxy-9-methoxypterocarpan[9] (1.0 g) as a white solid. Fraction *E* (CH_2Cl_2, 0.39 g) was rechromatographed on silica gel (20.0 g) by elution with hexane, CH_2Cl_2, EtOAc, and MeOH. Six fractions of progressively increasing polarity were subsequently obtained and pooled according to TLC profiles. Fraction E_1 (hexane, 38 mg) was submitted to centrifugal chromatography using a mixture of hexane−EtOAc, 1:1, as eluent to yield 3,9-dimethoxypterocarpan[6] (**3**, 20 mg) as a yellowish solid. Fraction E_3 (CH_2Cl_2−EtOAc 7:3, 0.19 g) was further purified over Sephadex LH-20 by elution with $CHCl_3$−MeOH, 1:1, to give 2,3,9-trimethoxypterocarpan[8] (**4**, 30 mg) and 3,4-dihydroxy-9-methoxypterocarpan[9] (**5**, 8 mg) as yellowish solids. Fraction *F* (CH_2Cl_2, 12.0 g) was rechromatographed on silica gel (120.0 g) and eluted with hexane, $CHCl_3$, EtOAc, and MeOH as binary mixtures with increasing polarity, to yield 2′,4,4′,-trihydroxychalcone[10] (isoliquiritigenin) (14 mg) and 7,4′-dihydroxyflavanone[11] (liquiritigenin) (**6**, 12 mg) as colorless solids.

7-Hydroxy-6,4′-dimethoxy-isoflavonequinone (1): mp > 300 °C, UV (MeOH) λ_{max} (log ϵ) 230 (4.43), 256 (4.14), 331 (3.96) nm; IR (KBr) ν_{max} 3431, 2925, 1671, 1625, 1514, 1477, 1442, 1292, 1028 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 328 (62), 302 (2), 300 (3), 285 (100), 272 (2), 216 (3), 166 (5), 164 (20), 84 (5), 63 (27), 55 (15); HREIMS *m/z* 328.0466 (calcd for $C_{17}H_{12}O_7$, 328.0583).

2′-Hydroxy-6,4′,6″,4‴-tetramethoxy-[7-*O*-7″]-bisisoflavone (2): mp 213−215 °C; UV (MeOH) λ_{max} (log ϵ) 215 (4.55), 231 (4.30), 257 (4.19), 325 (3.87) nm; IR (KBr) ν_{max} 3443, 1622, 1570, 1476, 1410, 1282, 1247, 1207, 1159, 1023 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 314 (49), 298 (100), 296 (37), 259 (7), 227 (2), 166 (21), 148 (43), 132 (7), 58 (9).

Determination of Cytotoxicity. All compounds (0.39 to 25 μg/mL) were tested for cytotoxic activity against five tumor cell lines (National Cancer Institute, Bethesda, MD): B16 (murine melanoma), HCT-8 (human colon), MCF-7 (human breast), CEM and HL-60 (leukemia) after 72 h of incubation. Doxorubicin (0.01 to 0.58 μg/mL) was used as a positive control. The general viability of cultured cells was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product as described by Mosmann.[17]

Acknowledgment. The authors are grateful to CNPQ/CAPES/PADCT/PRONEX/FINEP/FUNCAP for the fellowships and financial support.

References and Notes

(1) Lorenzi, H. *Árvores Brasileiras*; Plantarum: São Paulo, 1992.
(2) Ahluwalia, V. K.; Seshadri, T. R.; Gupta, V. N. *Tetrahedron* **1974**, *5*, 90−102.
(3) Craveiro, A. A.; Gottlieb, O. R. *Phytochemistry* **1974**, *13*, 1629−1630.
(4) Chipa, R. R.; Garibaldy, F. G.; Torres, G. M.; Estrada, M. J.; Vasquez, R. I. Q. *Holzforschung* **1998**, *52*, 459−460.
(5) Sannomiya, M. Estudo Fitoquimico das Raizes de *Platymiscium floribundum* Vog. e *Lonchocarpus montanus* A. Z. Tozzi (Leguminosae). M.S. Thesis, Universidade Estadual de Campinas, São Paulo-Brazil, 2000.
(6) Agrawal, P. K. *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, 1989.
(7) Kurosawa, K.; Ollis, W. D.; Redman, B. T.; Sutherland, I. O. *Phytochemistry* **1978**, *17*, 1413−1415.
(8) Pueppke, S. G.; VanEtten, H. D. *J. Chem. Soc., Perkin Trans. 1* **1975**, 946−947.
(9) Ingham, L. J. *Phytochemistry* **1976**, *15*, 1489−1495.
(10) Markham, K. R. *Tetrahedron* **1976**, *32*, 2607−2612.
(11) Oliveira, A. B.; Fonseca e Silva, L. G.; Gottlieb, O. R. *Phytochemistry* **1972**, *11*, 3515−3519.
(12) O'Neill, M. J.; Adesanya, S. A.; Roberts, M. F.; Pantry, I. R. *Phytochemistry* **1986**, *25*, 1315−1322.
(13) Hamburger, M. O.; Cordell, G. A.; Tantivatana, P.; Ruangrungsi, N. *J. Nat. Prod*. **1987**, *50*, 696−699.
(14) Attempts to obtain the HREIMS data for the molecular ion peak of compound **2** were unsuccessful.
(15) Brown, M.; Thomson, R. H. *Liebigs Ann. Chem*. **1974**, 1295−1300.
(16) Braun, S.; Kalinowski, H. O.; Berger S. *150 and More Basic NMR Experiments*, 2nd expanded ed.; Wiley-VHC: New York, 1998.
(17) Mosmann, T. *J. Immunol. Methods* **1983**, *16*, 55−63.

NP049854D