PERGAMON

Phytochemistry 55 (2000) 643–651

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Pyrano chalcones and a flavone from *Neoraputia magnifica* and their *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase-inhibitory activities

Daniela M. Tomazela [a], Mônica T. Pupo [b], Edna A.P. Passador [a], M. Fátima das G.F. da Silva [a,*], Paulo C. Vieira [a], João B. Fernandes [a], Edson Rodrigues Fo [a], Glaucius Oliva [c], José R. Pirani [d]

[a] *Departamento de Química, Universidade Federal de São Carlos, Caixa Postal 676, 13565-905 São Carlos, SP, Brazil*
[b] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil*
[c] *Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil*
[d] *Instituto de Ciências Biológicas, Departamento de Botânica, Universidade de São Paulo, São Paulo, Brazil*

Received 7 February 2000; received in revised form 16 May 2000
Dedicated to Professor Otto R. Gottlieb on the occasion of his 80th birthday

Abstract

The fruits of *Neoraptua magnifica* var. *magnifica* afforded three new flavonoids: 2′-hydroxy-4,4′,-dimethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone, 2′-hydroxy-3,4,4′-trimethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone, and 3′,4′-methylenedioxy-5,7-dimethoxyflavone which were identified on the basis of spectroscopic methods. The known flavonoids 2′-hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone, 2′-hydroxy-3,4,4′,5,6′-pentamethoxychalcone, 3′,4′-methylenedioxy-5,6,7-trimethoxyflavone, 3′,4′-methylenedioxy-5′,5,6,7-tetramethoxyflavone, 3′,4′,5′,5,7-pentamethoxyflavanone and 3′,4′,5′,5,7-pentamethoxyflavone were also identified. The latter flavone was the most active as glyceraldehyde-3-phosphate dehydrogenase-inhibitor

Keywords: *Neoraputia magnifica*; Rutaceae; Flavonoids; Biochemical systematics; *Trypanosoma cruzi*; Glycosomal glyceraldehyde-3-phosphate dehydrogenase-inhibitory activity

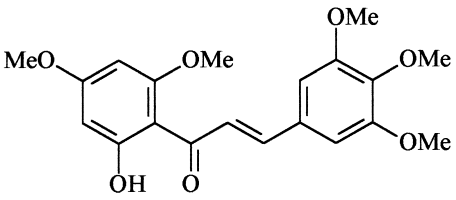
1. Introduction

As part of our continuous investigation into the chemical composition of Brazilian *Neoraputia* (Engler) Emmerich species, we recently reported the isolation of eight polymethoxylated flavones and one flavanone from *N. alba* (Engler) Emmerich (Arruda et al., 1991, 1993), four polymethoxylated flavones, 2′-hydroxy-3,4,4′,5,6′-pentamethoxychalcone (**1**) and 2′-hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**2**) from *N. magnifica* var. *magnifica* (Engler) Emmerich (Passador et al., 1997). The isolation of these interesting new chalcones combined with our taxonomic interest in the Rutaceae stimulated an investigation of other organs of *N. magnifica* var. *magnifica*.

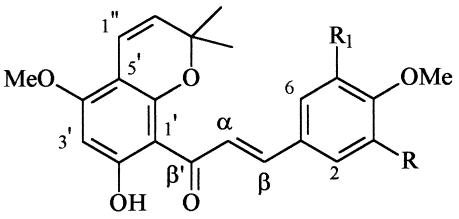
Chagas' disease, caused by the protozoan *Trypanosoma cruzi*, is estimated to affect some 16–18 million people,mostly from South and Central America, where 25% of the total population is at risk (World Health Organization). Control of the insect vector (*Triotoma infestans*) in endemic areas has led to the virtual elimination of transmission by insect bites, and, as a consequence, blood transfusion and congenital transmission are currently the major causes for the spread of the disease. Besides low efficacy, the drugs currently available, nifurtimox and benzonidazole, have strong side effects (Souza et al., 1998). The bloodstream form of the parasite *T. cruzi* has no functional tricarboxylic acid cycle, and it is highly dependent on glycolysis for ATP production (Souza et al., 1998). This great dependence on glycolysis as a source of energy makes the glycolytic enzymes attractive targets for trypanocidal drug design. Thus, the three dimensional structure of the enzyme was determined (Souza et al., 1998). GAPDH catalyses the

* Corresponding author. Tel.: +55-016-260-8208; fax: +55-16-260-8350.
E-mail address: dmfs@power.ufscar.br (M.F.G.F. da Silva).

0031-9422/00/$ - see front matter
PII: S0031-9422(00)00248-X



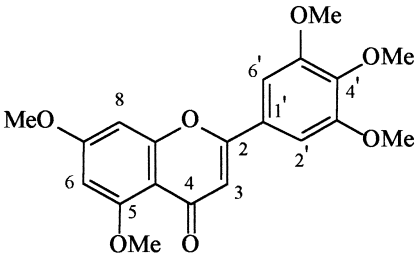
1



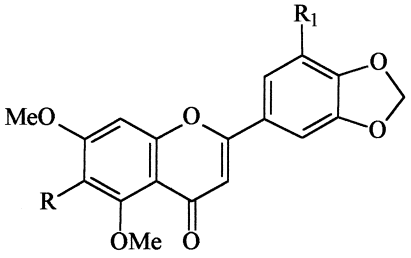
2: $R_1 = R = OMe$

3: $R_1 = R = H$

4: $R_1 = H$; $R = OMe$



5



6: $R_1 = R = OMe$

7: $R_1 = H$; $R = OMe$

8: $R_1 = R = H$

oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. Glycosomal GAPDH shows potential target sites with significant differences compared with the homologous human enzyme, and inhibitors have been designed, synthesised, obtained from natural sources, and tested. In order to find blocking agents, chalcones and flavones isolated from *N. magnifica* were assayed and evaluated by interaction with the enzyme GAPDH from *T. cruzi*.

2. Results and discussion

The hexane extract of the ripe fruits of *N. magnifica* var. *magnifica*, afforded γ-tocopherol and three chalcones. One was characterised as 2′-hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**2**) by comparison with both published data and an authentic sample (Passador et al., 1997). Placement of the chromene ring between C-5′ and O-6′ in **2** was determined on the basis of the following data. The ^{1}H NMR spectrum (Table 1) showed features of a chromene ring (δ 6.60 and 5.47 vinylic protons, and a singlet at δ 1.50, 6H, assigned to a pair of magnetically equivalent methyl groups) and signals for four methoxy groups. The assignment of one singlet to H-2 and H-6 (δ 6.86, 2H) suggested that three methoxy groups are attached to the B-ring. From a biosynthetic point of view, the remaining methoxy group could be attached to C-4′ or C-6′ of the A-ring. The spectrum also revealed a singlet at δ 14.24 corresponding to one chelated hydroxyl, thus attached to C-2′. Furthermore, a singlet at δ 6.07 (1H) clearly indicated the A-ring to be 2′,4′,5′,6′- or 2′,3′,4′,6′-tetrasubstituted. From the HMBC experiments (Table 2) the observed correlations between the hydroxyl proton at δ 14.24 and the ^{13}C signals at δ 105.9 (J^3), 167.9

Table 1
^{1}H NMR chemical shifts for chalcones **1**–**4**

H	1	2	3	4
3′	6.2 *d* (2.4)	6.07 *s*	6.06 *s*	6.07 *s*
5′	5.97 *d* (2.4)			
2	6.84 *s*	6.86 *s*	7.56 *d* (8.6)	7.16 *d* (2.0)
3			6.93 *d* (8.6)	
5			6.93 *d* (8.6)	6.90 *d* (8.3)
6	6.84 *s*	6.86 *s*	7.56 *d* (8.6)	7.20 *dd* (2.0, 8.3)
β	7.71 *d* (16)	7.72 *d* (16)	7.77 *d* (15.5)	7.76 *d* (15.5)
α	7.80 *d* (16)	8.05 *d* (16)	8.02 *d* (15.5)	8.03 *d* (15.5)
1″		6.60 *d* (12)	6.58 *d* (9.8)	6.60 *d* (9.9)
2″		5.47 *d* (12)	5.45 *d* (9.8)	5.47 *d* (9.9)
4″/5″		1.50 *s*	1.55 *s*	1.56 *s*
OMe	3.84 *s*	3.87 *s*	3.86 *s*	3.86 *s*
OMe	3.90 *s*	3.90 *s*	3.86 *s*	3.93 *s*
OMe	3.91 *s*	3.90 *s*		3.94 *s*
OMe	3.92 *s*	3.91 *s*		
OMe	3.92 *s*			
OH	14.31 *s*	14.24 *s*	14.31 *s*	14.34 *s*

(J^2) and 92.5 (J^3) led to their assignments as C-1′, C-2′ and C-3′, respectively, thus indicating that the unsubstituted carbon must be vicinal to C-2′. The ^{13}C NMR spectrum (Table 3) showed a signal for only one methoxy group attached to the *ortho*-disubstituted carbon (δ 63.1) which was assigned to the 4-OMe functionality. This implies that the methoxy group in the A-ring is located at C-4′ (δ 55.9), and therefore established the position of the chromene ring to be between C-5′ and O-6′. Moreover, the existence of any correlation between the ^{1}H signal at δ 6.07, assigned to H-3′ (by HSQC and HMBC), and the ^{13}C signal at δ 102.9 (C-5′), confirmed the position of the chromene ring between C-5′ and O-6′. The mass spectrum and elemental analysis data for **2** are reported here for the first time (see also Scheme 1 and Section 3).

Chalcone (**3**) exhibited similar NMR spectra to that of **2** (Tables 1 and 3). In addition to signals described for the A-ring of **2**, the ^{1}H NMR spectrum revealed the presence of a 1,4-disubstituted phenyl moiety (δ 7.56 *d*, $J = 8.6$ Hz, H-2 and H-6; 6.93 *d*, $J = 8.6$ Hz, H-3 and H-5). The presence of a fragment ion at m/z 161 (Scheme 1) in the mass spectrum for **3**, associated with cleavage between the A-ring and C-β′, clearly indicated the presence of one methoxy group in the B-ring. The signal of H-2/H-6 (δ 7.56) showed a one-bond correlation (by HSQC) with the ^{13}C signal at δ 130.0, and a long-range correlation (by HMBC, Table 2) with the ^{13}C signal at δ

Table 2
HMBC assignments for chalcones **1**–**4**

1		**2**		**3**		**4**	
H/C		H/C		H/C		H/C	
2/	3; β; 4; 6	2/	3; β; 4; 6	6,2/	4	2/	6; β, 3
6/	5; β; 4; 2	6/	5; β; 4; 2	5,3/	4	6/	2; β
3′/	1′; 2′; 4′; 5′	3′/	1′; 2′; 4′; 5′	3′/	5′; 1′	5/	1; 4
5′/	1′; 3′; 4′; 6′	α (8.05)/	1; β′	α (8.02)/	1; β	3′/	5′; 1′; 2′
α (7.80)/	1; β; β′	β (7.72)/	2/6; β′	β (7.77)/	6; 2	α (8.03)/	1; β′
β (7.71)/	2/6; α; β′	OH/	1′; 2′; 3′			β (7.76)/	2; 6; β′
OH/	1′; 2′; 3′	1″/	6′; 3″			1″/	3″; 6′
		2″/	5′; 3″			2″/	3″; 5′
						4″,5″/	3″; 2″

Table 3
^{13}C NMR chemical shifts for chalcones **1**–**4** and flavones **5**–**8**[a]

C	**1**	**2**	**3**	**4**	C	**5**	**6**	**7**	**8**
1	131.2	130.8	128.4	128.0	2	160.5	160.7	160.8	160.2
2	105.7	105.3	130.0	109.4	3	108.9	107.8	107.5	108.7
3	153.5	153.4	114.5	153.4	4	177.5	177.1	177.1	176.6
4	141.0	139.5	161.1	149.0	5	161.0	152.6	152.6	161.3
5	153.5	153.4	114.5	111.1	6	96.2	140.4	140.4	96.8
6	105.7	105.3	130.0	123.3	7	164.1	157.7	157.7	164.4
1′	105.8	105.9	106.4	106.3	8	92.9	96.2	96.2	93.7
2′	168.5	167.9	167.5	167.4	9	159.9	154.4	154.4	160.1
3′	93.9	92.5	92.7	92.7	10	109.3	112.9	112.9	109.7
4′	166.3	161.3	161.4	161.2	1′	126.8	126.0	125.6	126.0
5′	91.5	102.9	103.1	102.9	2′	103.5	100.4	106.1	106.6
6′	162.5	156.0	155.7	155.6	3′	153.6	149.5	148.4	148.9
β	142.4	142.3	142.3	142.6	4′	140.9	138.1	150.3	150.6
α	127.0	126.5	125.2	125.2	5′	153.6	143.8	108.7	108.9
β′	192.4	192.4	192.8	192.4	6′	103.5	106.6	121.0	121.3
1″		116.9	116.8	116.8	OMe	55.8	56.3	56.3	55.9
2″		124.2	124.5	124.4	OMe	56.4	56.9	61.6	56.2
3″		77.7	77.9	77.8	OMe	56.4	61.5	62.2	
4″/5″		28.1	28.0	27.9	OMe	56.4	62.2		
OMe	55.6	55.9	55.4	55.8	OMe	61.0			
OMe	55.8	55.9	55.8	56.1	OCH_2O		102.2	101.9	102.5
OMe	55.8	55.9		56.1					
OMe	56.2	63.1							
OMe	61.0								

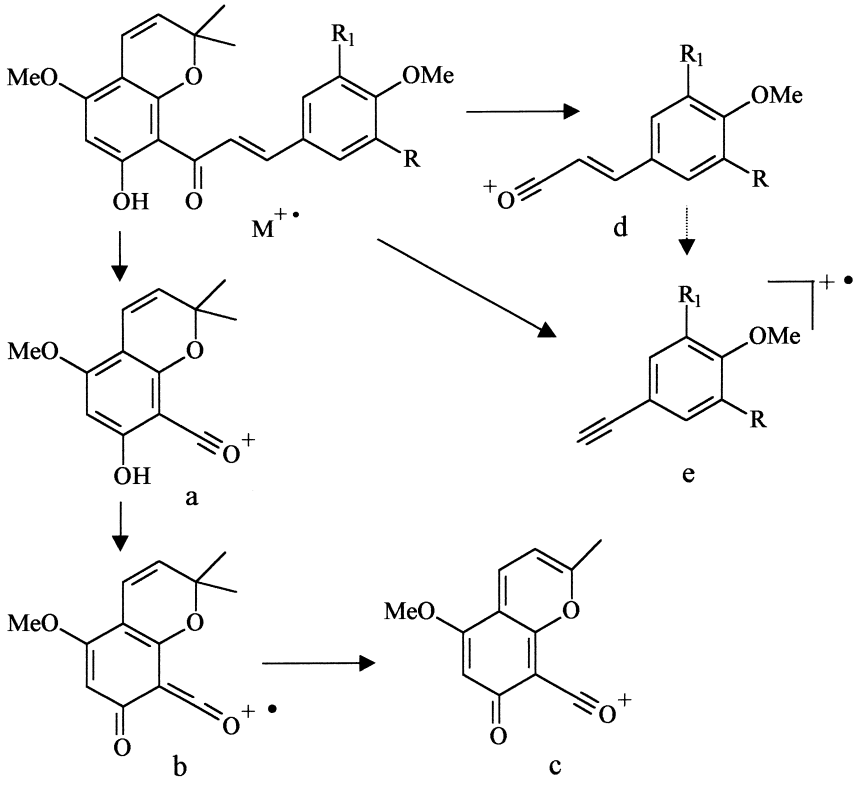
[a] Assignments based on HSQC/HMBC for **1**–**4** and **6**. ^{13}C–^{1}H COSY and long range ^{13}C–^{1}H COSY for **5** and HSQC for **8**. All in $CDCl_3$, except for **8** (in pyridine–d_6).

161.1 (J^3), thus indicating the methoxy group to be located at C-4 and permitting the assignment of the signal at δ 161.1 to C-4. HSQC experiments also showed correlations of the signal of H-3/H-5 (δ 6.93) with the ^{13}C signal at δ 114.5. In addition, the signal of H-α (δ 8.02) showed cross peaks with the ^{13}C signal at δ 128.4, indicating this signal to C-1.

For **2**, placement of the chromene ring was apparent from the J^3 couplings observed between the hydroxyl proton (2′-OH) and the ^{13}C signal for C-3′, indicating that the unsubstituted carbon must be vicinal to C-2′. HMBC experiments with **3** did not detect any correlations of the hydroxyl signal with the A-ring carbons. However, the ^{13}C NMR spectrum of **3** (Table 3) revealed resonances for C-1′ to C-6′ and C-1″ to C-5″ in close agreement with the resonances for the corresponding carbons in **2**. A singlet at δ 6.06 (1H) showed a one-bond correlation (HSQC) with the ^{13}C signal at δ 92.7, permitting the assignment of these signals to H-3′ and C-3′, respectively, when compared with **2**. The ^{13}C NMR spectrum of **3** did not show any signal for methoxy groups attached to the *ortho*-disubstituted carbon (ca δ 63); they were observed at δ 55.4 and 55.8. This implies that the methoxy in the A-ring was located at C-4′ and established the position of the chromene ring to be between C-5′ and O-6′. The signals at δ 55.4 and 55.8 were then assigned to 4′-OMe and/or 4-OMe. Based on the above evidence, the correlations from H-3′ to the ^{13}C signals of C-5′ (δ 103.1) and C-1′ (δ 106.4) were consistent with the angular structure for **3**. The new natural product is, therefore, 2′-hydroxy-4,4′-dimethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**3**).

Chalcone (**4**) also showed spectral characteristics of a 2′-hydroxy-4′-methoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (Tables 1 and 3). HMBC experiments (Table 2) with **4** also did not detect a relationship between the 2′-OH signal at δ 14.34 to the A-ring carbons. However, the chemical shifts of the A-ring and chromene carbons were comparable with those for **2** and **3** (Table 3). As discussed above, the signals of the methoxy groups at δ 55.8 and 56.1 (6H) supported the chromene ring to be between C-5′ and O-6′. This was also confirmed by the HMBC experiments, which showed correlations of the H-3′ signal (δ 6.07) with the resonances of C-2′ (δ 167.4) and C-1′ (δ 106.3).

The mass spectrum of **4** gave significant fragments for *m/z* 191 and 233 (Scheme 1) requiring the presence of two methoxy groups in the B-ring. This was supported by the ^{1}H NMR spectrum which showed signals for three methoxy groups (δ 3.94, 3H, *s*; 3.93, 3H, *s*; 3.86, 3H, *s*) and for three protons giving rise to an AB spin system (δ 7.16, *d*, J=2.0 Hz, H-2; δ 6.90, *d*, J=8.3 Hz, H-5; δ 7.20, *dd*, J=2.0 and 8.3 Hz, H-6). From the HMBC experiments (Table 2), the observed correlation



Scheme 1. Mass spectral fragmentation of 2-4. 2: R1 = R = OMe: M$^+$ (=426 (70); a = *m/z* 233 (40); b = *m/z* 232 (10); c = *m/z* 217 (100); d = *m/z* 221 (5); e = *m/z* 192 (20). 3: R1 = R = H: M+ (=366 (60); a = *m/z* 233 (25); b = *m/z* 232 (5); c = *m/z* 217 (100); d = *m/z* 161 (5); e = *m/z* 132 (10). 4: R1 = H; R = OMe: M+ (=396 (60); a = *m/z* 233 (30); b = *m/z* 232 (10); c = *m/z* 217 (100); d = *m/z* 191 (10); e = *m/z* 162 (15).

between the β-proton at δ 7.76 and the ^{13}C signals at δ 109.4 (J^3) and 123.3 (J^3) led to their assignments as C-2 and C-6, respectively. The spectrum also showed a correlation of H-6 at δ 7.20 with the ^{13}C signal at δ 149.0 (J^3), confirming a methoxy group at C-4 and showing that this signal can be attributed to C-4. The existence of a correlation between the ^1H signal at δ 7.16, assigned to H-2, and the ^{13}C signal at δ 153.4 determined the position of the other methoxy group at C-3 and permitted the assignment of this signal to C-3. The structure of the new natural product was thus established as 2′-hydroxy-3,4,4′-trimethoxy-5′,6′-(2″,2″-dimethylpyrano) chalcone (**4**).

It has been noted that the H-β and C-β of a chalcone are more deshielded than the H-α and C-α resonances (Agrawal, 1989). Both are affected by changes in the B-ring substitution and C-α also by the presence or absence of a 2′- or 6′-oxysubstituent. HSQC experiments of **1**–**4** showed correlations of ^1H signals at ca δ 8.0 and 7.7 to the ^{13}C signals at ca δ 126 and 142, respectively, indicating apparent anomalies. The methine proton at ca δ 7.7 showed cross peaks with the C-2 and C-6 signals (Table 2), so permitting the assignment of the upfield signal at ca δ 7.7 to H-β. The anisotropic effect of the 5′,O-6′-chromene substituent on the H-α caused a downfield shift above the usual range for 2′,6′-oxysubstituted chalcones, this resulting in the H-α proton being more deshielded than H-β.

The dichloromethane extract of fruits on successive chromatographic separation afforded the five flavonoids, 3′,4′,5′,5,7-pentamethoxyflavanone (Passador et al., 1997), 3′,4′,5′,5,7-pentamethoxyflavone (**5**) (Passador et al., 1997), 3′,4′-methylenedioxy-5′,5,6,7-tetramethoxyflavone (**6**) (Arruda et al., 1993), 3′,4′-methylenedioxy-5,6,7-trimethoxyflavone (**7**), and 3′,4′-methylenedioxy-5,7-dimethoxyflavone (**8**). The latter appears to be new.

Compound **6** has been isolated from *Ageratum conyzoides* (Vyas and Mulchandani, 1986) and *N. alba* (Arruda et al., 1993); however, its ^{13}C NMR data are reported here for the first time (Table 4). The HSQC and HMBC experiments on **6**, permitted the assignments of all carbons (Tables 2 and 3). A singlet at δ 6.55 (1H) showed a one-bond correlation with the ^{13}C signal at δ 107.8, and a long-range correlation with the ^{13}C signals at δ 126.0 (J^3), 160.7 (J^2), 177.1 (J^2) and 112.9 (J^3) permitting their assignments as H-3, C-3, C-1′, C-2, C-4 and C-10, respectively. Two doublets at δ 7.06 and 7.07 were coupled to each other and showed a long-range correlation with the C-2 and C-1′ signals, thus indicating these two hydrogens were attached to C-2′ and C-6′. The ^1H NMR spectrum revealed a signal for three methoxy groups at δ 3.98 (6H) and 3.99 (3H); however, in HSQC (δ 62.2, 56.9, 56.3) and HMBC experiments these signals appeared as a broad singlet. The observed correlations between the methoxy protons at δ 3.98/or 3.99 and the ^{13}C signal at δ 143.8 (J^3), which showed cross peaks with the 1H signal at δ 7.07, led to their assignments as 5′-OMe, C-5′ and H-6′. The signal at δ 7.06 was then assigned to H-2′. HSQC experiments permitted the assignments of ^{13}C signal at δ 100.4, 106.6 and 56.3/or 56.9 to C-2′, C-6′ and 5′-OMe, respectively. HMBC experiments also confirmed the B-ring to have a methylenedioxy group between C-3′ and C-4′ by the cross peaks with the ^1H signal of OCH_2O at δ 6.08 with the ^{13}C signals at δ 149.5 and 138.1, which showed correlations with H-2′ and the latter (δ 138.1) with H-6′. The signals at δ 149.5 and 138.1 were then assigned to C-3′ and C-4′, respectively. The ^{13}C signal at δ 102.2 could be attributed to OCH_2O by HSQC.

The ^{13}C NMR spectrum showed signals for two methoxy groups at δ 61.5 and 62.2. Thus implies that the methoxy groups in the A-ring are located at C-5, C-6 and C-7, since the ^1H signal at δ 6.78 (1H) for the unsubstituted methine indicated that this proton must not be vicinal to C=O (C-4). Thus, the observed one-bond correlation between the signal of H-8 (δ 6.78) and the ^{13}C signal at δ 96.2 led to the assignment of C-8 to this resonance. The methoxy protons at δ 3.92 showed a one-bond correlation with the ^{13}C signal at δ 61.5, and a long-range correlation with the ^{13}C signal at δ 140.4 (J^3), suggesting the assignment of the latter signal to C-6 or C-5. The presence of a correlation between the signal of H-8 and the ^{13}C signal at δ 140.4 (J^3) clearly indicated this signal to be at C-6. The signal for C-7 was established as δ 157.7 by the existence of a correlation

Table 4
Effect of compound **5**, mixture of **6**+**7** and **2**+**4** on TcGAPDH activity[a]

Compound	Concentration (μg/ml)	Absorbance	Specific activity (U/mg)	% Inhibitory activity
5	30	0.345	24.65	46
	50	0.136	9.72	80
	100	0.009	0.64	99
Control	30	0.641	45.80	
	50	0.670	47.87	
	100	0.560	40.01	
6+**7**	25	0.343	20.24	54
	35	0.249	14.69	68
	100	0.000	00.00	100
Control	25	0.751	44.31	
	35	0.773	45.61	
	100	0.688	40.59	
2+**4**	105	0.425	25.08	45
Control	105	0.773	45.61	

[a] Control: 50 mM Tris–HCl pH 8.6 buffer, 1 mM EDTA, 1 mM β-mercapto-ethanol, 30 mM Na_2HAsO_4, 2.5 mM NAD^+, 0.3 mM glyceraldehyde-3-phosphate, 4–9 μg protein and 10% DMSO, in a total volume of 1000 μl. Positive control: coumarin chalepin: concentration (μg/ml): 30, (U/mg): 3.50,% inhibitory activity = 75, IC_{50} = 64 μM. (Calenbergh et al., 1995).

between the H-8 resonance and this ^{13}C signal (J^2), which also showed a cross peak with the methoxy protons at δ 3.98/or 3.99 and the latter with the ^{13}C signal at δ 56.3 or 56.9. In the same way, the signal for C-5 emerged from analysis of the correlation between the methoxy signal at δ 3.98/or 3.99 (δ 62.2 by HSQC) and the ^{13}C signal at δ 152.6, which did not show a correlation with the H-8 signal. The H-8 signal also showed a cross-peak with the ^{13}C signal at δ 154.4 (J^2), which was attributed to C-9, since it was the only carbon to be assigned in the A-ring.

Flavone **8** exhibited a B-ring spin system in the ^1H NMR spectrum for 3′,4′-substitution (δ 7.54, *dd*, $J=1.8$ and 0.4 Hz, H-2′; δ 6.98, *dd*, $J=8.1$ and 0.4 Hz, H-5′; δ 7.51, *dd*, $J=8.1$ and 1.8, H-6′). This spectrum also showed signals for a *meta*-coupled A-ring protons (δ 6.55, *d*, $J=2.3$ Hz, H-6; δ 6.78, *d*, $J=2.3$ Hz, H-8), two methoxy groups (δ 3.85, *s*, 6H) and one methylenedioxy group (δ 6.07, *s*, 2H). The retro-Diels-Alder (RDA) (Chen et al., 1997) fragments of this flavone gives a good indication of the substitution patterns of the A- and B-rings (Scheme 2). Thus, a combination of ^1H NMR spectral data and the fragment ions at *m/z* 146 (70%) and 180 (5%) fully support the presence of a 3′,4′-methylenedioxy substituent in the B-ring and a 5,7-dimethoxy system in the A-ring.

HSQC experiments permitted assignments of all protonated carbons (Table 3). However, the ^{13}C signals at δ 96.8 and 93.7 were attributed to C-6 and C-8, and the ^1H signals at δ 6.55 and 6.78 to H-6 and H-8, respectively, by comparison with the resonances for the corresponding carbons and hydrogens in 3′,4′,5′,5,7-pentamethoxyflavone (**5**). The quaternary carbons of the A- and C-rings (δ 160.2, C-2; 176.6, C-4; 161.3, C-5; 164.4, C-7; 160.1, C-9; 109.7, C-10) were also assigned by comparison with those of **5** (δ 160.5, C-2; 177.5, C-4; 161.0, C-5; 164.1, C-7; 159.9, C-9; 109.3, C-10), whose assignments were aided by ^{13}C–^1H COSY and long-range ^{13}C–^1H COSY spectra (Kinoshita and Firman, 1997). The signal for C-3′ was established as δ 148.9 by its comparison with the resonance for the corresponding carbon in **6**. The signal at δ 150.6 was then assigned to C-4′. The structure of flavone **8** was thus established as 3′,4′-methylenedioxy-5,7-dimethoxyflavone. Flavone **5** was previously obtained from *N. alba* (Arruda et al., 1993) and *Murraya paniculata* (Rutaceae) (Kinoshita and Firman, 1997).

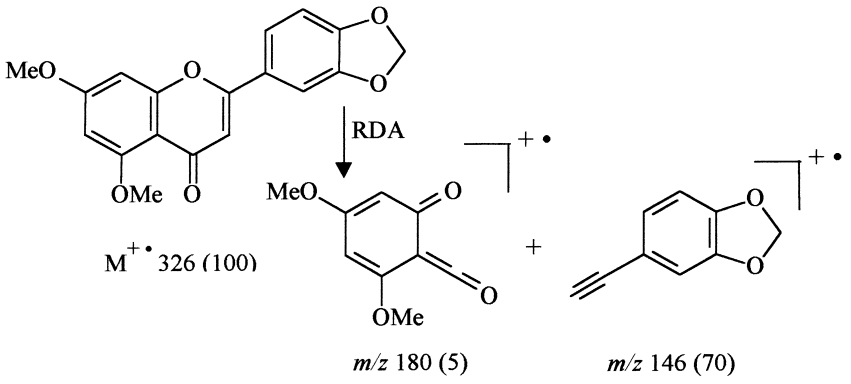
3′,4′-Methylenedioxy-5,6,7-trimethoxyflavone (**7**, ageconyflavone A) has been found previously in *Ageratum conyzoides* (Asteraceae) (Vyas et al., 1986). However, its ^{13}C NMR spectral data have not been reported previously in the literature. The chemical shifts of the A- and B-ring carbons were comparable with those for **6** and **8**, respectively (Table 3).

All phytochemical studies on *Neoraputia* genus have been undertaken in our laboratory, and isolation procedures used in these studies should have revealed rutaceous alkaloids, coumarins and limonoids if they had been present. However, it is premature to use the absence of other classes of compounds as an argument to remove *Neoraputia* to the Citroideae, which produces a considerable number of highly oxygenated flavones (Passador et al., 1997; Silva, et al., 1988). Clearly much more detailed phytochemical investigations of *Neoraputia* species will be essential for a better understanding of its chemotaxonomic position in the Rutaceae.

Some compounds were evaluated for their ability to inhibit the enzymatic activity of the protein glycosomal GAPDH from *T. cruzi* (Table 4). Chalcone **2** was separated from **4** and flavone **6** from **7** by R-HPLC; however, they were obtained in very small amount. Thus, only the initial inhibitory activity of these mixtures were evaluated. GAPDH activity was only inhibited by 45% when the mixture of **2** and **4** was added to the assay system at a concentration of 105 μg/ml, suggesting that these chalcones act as weak inhibitors. The mixture of **6** and **7** completely inhibited the enzymatic activity (by 100%) at 100 μg/ml.

The activity of flavone **5** was comparable to that of the mixture of **6**–**7**, reducing the enzymatic activity by 99% at 100 μg/ml. The 50% inhibitory concentration value (IC_{50}) was 81 μM.

Highly oxygenated flavones appear to possess the structural requirements for inhibiting trypanosomal



Scheme 2. Mass spectral fragmentation of **8**.

GAPDH. However, to develop an effective blocking agent from the natural product lead compounds, it is necessary to determine as precisely as possible, how the tested compounds occupy the active site and at the same time how they make specific interactions with the amino acids of the target enzyme. For this purpose co-crystallization experiments with flavone **5** have been undertaken, both in laboratory and under microgravity conditions, in the NASA Space Shuttle during mission STS-91, in May 1998. Unfortunately, we did not have any success with flavone **5**, and effective crystallization was observed only for a rutaceous coumarin. The related crystallographic studies are in progress and will be reported separately.

Therefore, we still have not enough experimental evidence for developing a quantitative understanding of the structural basis of the specificity in the catalytic-site-activity relationships among flavones and the enzyme GAPDH. Further crystallization experiments as well as solution studies by NMR spectral analysis are in progress.

3. Experimental

3.1. General

NMR: on a Bruker DRX 400, with TMS as int. standard, HSQC: Heteronuclear Single Quantum Coherence (Ruiz-Cabello et al., 1992); PIEIMS: 70 eV, low resolution on a VG Platform II (Fisons) instrument; IR (KBr, BOMEN-Ft/IR); UV (Perkin–Elmer); R-HPLC: Recycling High-Performance Liquid Chromatography on a model Shimadzu LC-6AD; the column used was a Shim-pack Prep-Sil (H), 250 mm×20 mm, 5 μm particle size, 100 Å pore diameter; eluant: hexane-CH_2Cl_2-*iso*-PrOH (15:5:1); flow rate: 3.0 ml/min; detection (Shimadzu SPD-6AV): UV λ 254 nm; Elemental analysis: on a EA 1108, CHNS-O (Fisons).

3.2. Plant material

Neoraputia magnifica var. *magnifica* was collected in Espirito Santo, Brazil, and a voucher specimen (SPF 81-316) is deposited in the Herbarium of Instituto de Ciências Biológicas-USP-São Paulo.

3.3. Isolation of compounds

Ground fruits (825 g) were extracted with hexane, then CH_2Cl_2 and finally with MeOH. The conc. hexane extract was submitted to vacuum chromatography over silica gel using a hexane–CH_2Cl_2–MeOH gradient to give a mixture of fatty acids followed by 16 fractions. Fr 5 was rechromatographed on silica gel using hexane–CH_2Cl_2 gradient affording further frs. Fr 5-3 was rechromatographed as above, eluting with hexane–CH_2Cl_2–MeOH (15:5:1) yielding **1** (12 mg). The fraction containing **2** and **4** was then subjected to further flash chromatography as above, eluting with a hexane–Me_2CO gradient, and then by R-HPLC (detection UV λ 254 nm) to give **2** (2nd peak, 1.9 mg) and **4** (1st peak, 2.0 mg), after recycling ×3. Fr containing **3** was applied to flash chromatography as above, eluting with a hexane–CH_2Cl_2 gradient, and then by prep. TLC (silica gel, hexane–CH_2Cl_2–MeOH (1:1:0.1) to yield pure **3** (6.1 mg).

The concd CH_2Cl_2 extract was subjected to CC over silica gel. Elution with a hexane–CH_2Cl_2–MeOH (1:1:0.2) afforded 16 frs. Fr 3 was subjected to flash chromatography on silica gel column, eluting with hexane–CH_2Cl_2–MeOH (1:1:0.1) to afford 4 new frs. Fr 3-2 was treated as above, eluting with a hexane–EtOAc gradient, to afford 3′,4′,5′,5,7-pentamethoxyflavanone (5.0 mg), after crystallization from MeOH. Fr 4 was recrystallized from MeOH and then purified by prep. TLC (silica gel, benzene–CH_2Cl_2–Me_2CO, 7:5:2) to give a mixt. of 6 and 7. This mixt. was submitted to R-HPLC (detection UV λ 254 nm) to afford **6** (2nd peak, 2.0 mg) and **7** (1st peak, 2.5 mg), after recycling ×3. Fr 6 was crystallized from MeOH yielding **5** (30 mg). Fr 11 afforded **8** (15 mg) after crystallization from MeOH.

3.3.1. 2′-Hydroxy-3,4,4′,5-tetramethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**2**)

Yellow powder; EA: Found: C, 67.62; H, 6.04; O, 26.34. Calc. for $C_{24}H_{26}O_7$: C, 67.59; H, 6.15; O, 26.26%; MS *m*/*z* (rel. int.): 426 $[M]^+$. (70), 411 $[M\text{-}Me]^+$ (75), 233 (40): associated with cleavage between C-α and C-β′, 232 (10), 217 $[233\text{-}H\text{-}Me]^+$ (100), 221 (5): associated with cleavage between A-ring and C-β′, 192 (20).

3.3.2. 2′-Hydroxy-4,4′-dimethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**3**)

Yellow powder; UV λ_{max} ($CHCl_3$) nm: 290, 362; IR ν_{max} (KBr) cm^{-1}: 3414, 1603, 1454, 1248, 615; ^1H NMR (400 MHz, $CDCl_3$): see Table 1; ^{13}C NMR (100 MHz, $CDCl_3$): see Table 2; HSQC (400/100 MHz, $CDCl_3$); HMBC (400/100 MHz, $CDCl_3$): see Table 3. EA: Found: C, 72.05; H, 6.04; O, 21.91. Calc. for $C_{22}H_{22}O_5$: C, 72.12; H, 6.05; O, 21.83%; MS *m*/*z* (rel. int.): 366 $[M]^+$. (60), 351 $[M\text{-}Me]^+$ (70), 233 (25): associated with cleavage between C-α and C-β′, 232 (5), 217 $[233\text{-}H\text{-}Me]^+$ (100), 161 (5): associated with cleavage between A-ring and C-β′, 132 (10).

3.3.3. 2′-Hydroxy-3,4,4′-trimethoxy-5′,6′-(2″,2″-dimethylpyrano)chalcone (**4**)

Yellow powder; UV λ_{max} ($CHCl_3$) nm: 294, 372; IR ν_{max} (KBr) cm^{-1}: 3427, 1640, 1215, 759; ^1H NMR (400 MHz, $CDCl_3$): see Table 1; ^{13}C NMR (100 MHz, $CDCl_3$): see Table 2; HSQC (400/100 MHz, $CDCl_3$); HMBC (400/100 MHz, $CDCl_3$): see Table 3. EA: Found: C, 69.67; H, 6.11; O, 24.22. Calc. for $C_{23}H_{24}O_6$: C, 69.68; H, 6.10; O, 24.21%. MS *m*/*z* (rel. int.): 396

$[M]^+$. (60), 381 $[M\text{-}Me]^+$ (70), 233 (30): associated with cleavage between C-α and C-β′, 232 (10), 217 [233-H-Me]$^+$ (100), 191 (10): associated with cleavage between A-ring and C-β′, 162 (15).

3.3.4. 3′,4′-Methylenedioxy-5′,5,6,7-tetramethoxyflavone (*6*)

Yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.07 (1H, *d, J* = 1.6 Hz, 6′), 7.06 (1H, *d, J* = 1.6 Hz, 2′), 6.78 (1H, *s*, 8); 6.55 (1H, *s*, 3), 6.08 (2H, *s*, OCH_2O), 3.99 (3H, *s*, OMe), 3.98 (6H, *s*, 2×OMe), 3.92 (3H, *s*, OMe); ^{13}C NMR (100 MHz, $CDCl_3$): see Table 4; HSQC (400/100 MHz, $CDCl_3$); HMBC (400/100 MHz, $CDCl_3$): H-2′→C-1′, C-3′, C-4′, C-6′, C-2; H-6′→C-1′, C-2′, C-4′, C-5′, C-2; H-8→C-6, C-7, C-9, C-10; H-3→C-1′, C-2, C-4, C-10; OCH_2O→C-3′, C-4′; OMe (δ_H 3.92, δ_C 61.5)→C-6; OMe (δ_H 3.98 or 3.99, δ_C 62.2)→C-5; OMe (δ_H 3.98 or 3.99, δ_C 56.3 or 56.9)→C-7; OMe (δ_H 3.98 or 3.99, δ_C 56.3/or 56.9)→C-5′.

3.3.5. 3′,4′-Methylenedioxy-5,7-dimethoxyflavone (*8*)

Yellow powder; UV λ_{max} ($CHCl_3$) nm: 268, 328; IR ν_{max} (KBr) cm^{-1}: 1654, 1613, 1493, 1451, 1330, 1258, 1202, 1110, 1030, 921, 844, 811; ^{1}H NMR (400 MHz, pyridine-d_6): δ 7.54 (1H, *dd, J* = 1.8, 0.4 Hz, 2′), 6.98 (1H, *dd, J* = 8.1, 0.4 Hz, 5′), 7.51 (1H, *dd, J* = 8.1, 1.8, Hz, 6′), 6.92 (1H, *s*, 3), 6.55 (1H, *d, J* = 2.3 Hz, 6), 6.78 (1H, *d, J* = 2.3 Hz, 8), 6.07 (2H, *s*, OCH_2O), 3.85 (6H, *s*, 2×OMe); ^{13}C NMR (100 MHz, pyridine-d_6): see Table 4; HSQC (400/100 MHz, pyridine-d_6). EA: Found: C, 66.24; H, 4.33; O, 29.43. Calc. for $C_{18}H_{14}O_6$: C, 66.26; H, 4.32; O, 29.42%; MS *m/z* (rel. int.): 326 $[M]^+$. (100), 180 (5): associated with retro-Diels-Alder cleavage of C-ring, 146 (70): associated with retro-Diels-Alder cleavage of C-ring.

3.4. Preparation and purification of recombinant T. cruzi GAPDH

Tc GAPDH was overexpressed and purified as reported by Souza et al. (1998).

3.5. T. cruzi GAPDH-activity

Tc GAPDH activity was determined according to a modification of a previously reported procedure (Barbosa and Nakano, 1987). Reduced NADH was measured spectrophotometrically at 340 nm at 30 s intervals. The reaction medium was 50 mM Tris–HCl pH 8.6 buffer, 1 mM EDTA, 1 mM β-mercapto-ethanol, 30 mM Na_2HAsO_4, 2.5 mM NAD^+, 0.3 mM glyceraldehyde-3-phosphate and 4–9 μg protein, in a total volume of 1000 μl. The reaction was initiated by the addition of enzyme.

The specific activity (unit = U) of the enzyme was calculated as below:

$$(\mathrm{U/mg}) = \{(\Delta\text{absorbance}/\Delta t) \times \text{volume of cell}\}/6.22 \times \text{volume of enzyme} \times [\text{enzyme}]$$

where $\Delta t = 0.5$ min; volume of cell = 1.00 ml; $^{\varepsilon}$NADH = 6.22 $(\mu\mathrm{Mol/cm^3})^{-1}$ cm^{-1}; volume of enzyme = 0.005 ml; [ ] concentration of enzyme in mg/ml.

3.6. T. cruzi GAPDH-inhibitory activity

The inhibitory activity was recorded using the reaction medium as above, in a total volume of 1000 μl. Absorbance was read at 340 nm at 30 s intervals. Compound **5** was tested at 30, 50 and 100 μg/ml in 10% DMSO using 5 μl of GAPDH-9 (prepared in January 1998) at 0.90 mg/ml. The mixture of **6** and **7** was tested at 25, 35 and 100 μg/ml in 10% DMSO using 5 μl of GAPDH-10 (prepared in February 1998) at 1.09 mg/ml. The mixture of **2** and **4** was tested at 105 μg/ml as for the mixture above.

In each case, a blank experiment was performed with 10% DMSO alone in the reaction medium and was used as a positive control. The specific activity of TcGAPDH was not significantly affected by 10% DMSO alone.

Data were means of three repetitions and values as a percent of control were used as follows:

$$\%\ \text{inhibitory activity} = \{(\mathrm{U/mg\ compound} - \mathrm{U/mg\ control})/\mathrm{U/mg\ control}\} \times 100.$$

Acknowledgements

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Financiadora de Estudos e Projetos (FINEP) and the World Health Organization (TDR grant 940854) for financial support. G.O. is an International Scholar of the Howard Hughes Medical Institute.

References

Agrawal, P.K., 1989. Carbon-13 NMR of Flavonoids. Elsevier Science, New York.

Arruda, A.C., Vieira, P.C., da Silva, M.F. das G.F., Fernandes, J.B., Francisco, R.H.P., Rodrigues, A.M.G.D., Lechat, J.R., 1991. Two pyrano-flavones from *Neoraputia alba*. Phytochemistry 30, 3157–3159.

Arruda, A.C., Vieira, P.C., Fernandes, J.B., da Silva, M.F. das G.F., 1993. Further pyrano flavones from *Neoraputia alba*. Journal of the Brazilian Chemical Society 4, 80–83.

Barbosa, V.M., Nakano, M., 1987. Muscle d-glyceraldehyde-3-phosphate dehydrogenase from *Anas* sp. 1. Purification and properties. Comparative Biochemistry and Physiology 88B, 563–568.

Calenbergh, S.V., Verlinde, C.L.M.J., Soenens, J., Bruyn, A.D., Callens, M., Blaton, N.M., Peeters, O.M., Rozenski, J., Hol, W.G.J.,

Herdewijn, P., 1995. Synthesis and structure-activity relationships of analogs of 2′-deoxy-2′-(3-methoxybenzamido)ade-nosine, a selective inhibitor of trypanosomal glycosomal glyceraldehyde-3-phosphate dehydrogenase. Journal of Medical Chemistry 38, 3838–3849.

Chen, J., Montanari, A.M., Widmer, W.W., 1997. Two new polymethoxylated flavones, a class of compounds with potential anticancer activity, isolated from cold pressed dancy tangerine peel oil solids. Journal of Agriculture and Food Chemistry 45, 364–368.

Kinoshita, T., Firman, K., 1997. Myricetin 5,7,3′,4′,5′-pentamethyl ether and other methylated flavonoids from *Murraya paniculata*. Phytochemistry 45, 179–181.

Passador, E.A.P., da Silva, M.F. das G.F., Rodrigues, Fo E., Fernandes, J.B., Vieira, P.C., Pirani, J.R., 1997. A pyrano-chalcone and a flavanone from *Neoraputia magnifica*. Phytochemistry 45, 1533–1537.

Ruiz-Cabello, J., Vuister, G.W., Moonen, C.T.W., van Gelderen, P., Cohen, J.S., van Zijl, P.C.M., 1992. Gradient-enhanced heteronuclear correlation spectroscopy. Theory and experimental aspects. Journal of Magnetic Resonance 100, 282–302.

da Silva, M.F. das G.F., Gottlieb, O.R., Ehrendorfer, F., 1988. Chemosystematics of the Rutaceae: suggestions for a more natural taxonomy and evolutionary interpretation of the family. Plant Systematics and Evolution 16, 97–134.

Souza, D.H.F., Garratt, R.C., Araújo, A.P.U., Guimarães, B.G., Jesus, W.D.P., Michels, P.A.M., Hannaert, V., Oliva, G., 1998. *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase: structure, catalytic mechanism and targeted inhibitor design. Federation of European Biochemical Societies Letters 424, 131–135.

Vyas, A.V., Mulchandani, N.B., 1986. Polyoxygenated flavones from *Ageratum conyzoides*. Phytochemistry 25, 2625–2627.

World Health Organization. Statistical Information System Website, at http://www.who.ch./whosis/whosis.htm.