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Chalcone–flavone tetramer and biflavones from *Aristolochia ridicula*

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Abstract

Biflavones and a chalcone–flavone tetramer were isolated from the leaves of *Aristolochia ridicula*, together with *proto*-quercitol. Their structures were determined by spectroscopic methods.

Keywords: *Aristolochia ridicula*; Aristolochiaceae; Flavonoids; Biflavonoid; Tetraflavonoid; Chalcone–flavone tetramer; Cyclitol

1. Introduction

The genus *Aristolochia* in the family Aristolochiaceae is represented in Brazil by nearly 60 species. Members of this genus are used in Brazilian traditional medicine as a stomachic, anti-inflammatory, antiasthmatic and abortifacient, as well as an antidote for snakebit, to cure several types of cancer (Lopes et al., 2001). Flavonols and dihydroflavonols have been isolated from *Aristolochia* species. Various biflavonoids (chalcone–flavone dimers and biflavones) and a tetraflavonoid (chalcone–flavone tetramer) have been isolated from stems of *Aristolochia ridicula* Brown (Carneiro et al., 2000). Reports on the natural occurrence of tetraflavonoids are rare, and are limited to *A. ridicula*, *Lophira alata* (Ochnaceae) (Murakami et al., 1992; Tih et al., 1992), and *Cephalotaxus wilsoniana* (Cephalotaxaceae) (Wang et al., 2004). This paper deals with the isolation and structural elucidation of two new biflavones (ridiculuflavone A and B), a new chalcone–flavone tetramer (ridiculuflavonylchalcone A), and a known cyclitol (*proto*-quercitol) from the leaves of *A. ridicula*.

2. Results and discussion

The acetone extract of the leaves of *A. ridicula* was fractionated by chromatographic columns to give the new flavonoids (**1**–**3**) and a cyclitol. The cyclitol was identified by comparing its physical (m.p.) and spectroscopic (IR, ^{1}H and ^{13}C NMR) data to those reported in the literature for *proto*-quercitol (Salamci et al., 1997); nOeDS and gNOESY experiments also corroborated its relative configuration.

The ^{1}H and ^{13}C NMR, IR and UV spectroscopic data for compounds **1** and **2** were very similar to those reported for biflavones **4** and **5** previously isolated from *A. ridicula* (Carneiro et al., 2000). Compounds **1** and **2** were also suggested to be biflavones based on the ESI-MS spectra, since they displayed *quasi*-molecular ions at *m*/*z* 553 $[M - H]^-$ and at *m*/*z* 569 $[M + H]^+$, respectively, which were consistent with the molecular formula $C_{30}H_{18}O_{11}$ for **1** and $C_{31}H_{20}O_{11}$ for **2**. The IR spectra of both compounds showed an absorption characteristic of aromatic ketones at 1646 cm^{-1}. The ^{1}H and ^{13}C NMR spectra of **1** and **2** (Tables 1 and 2) suggested the presence of 1,4-disubstituted (B ring), 1,3,4-trisubstituted aromatic rings (B′ ring), tetrasubstituted (A ring), and pentasubstituted (A′ ring) rings with alternating oxygenation patterns. These spectra showed signals for two

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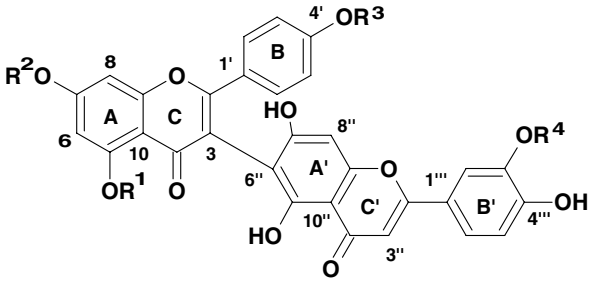
hydrogen-bonded hydroxyl groups at δ 12.92 (OH-5″) and δ 13.16 (OH-5), and two carbonyl carbons at δ 182.2 (C-4) and δ 181.6 (C-4″) for **1**, whereas the spectra of **2** showed signals for only one hydrogen-bonded hydroxyl group at δ 13.05 (OH-5″) and two carbonyl carbons at δ 174.0 (C-4) and 181.7 (C-4″). These spectra also showed additional resonances reminiscent of three tetrasubstituted sp^2 carbons and one trisubstituted sp^2 carbon (**1**: δ 103.0, **2**: δ 102.9, C-3″). These data thus suggested that the main differences between the spectra of **1** and **2** were due to the presence of a methoxyl group (δ_C 55.9, δ_H 3.79) in **2** instead of a hydrogen-bonded hydroxyl group. Furthermore, gHMBC experiments supported correlations between C-2″ (**1**: δ 164.4 and **2**: δ 163.8) and H-3″ (**1** and **2**: δ 6.66), between OH-5″ (**1**: δ 12.92, **2**: δ 13.05) and C-5″ (**1**: δ 162.9, **2**: δ 159.5), C-6″ (**1**: δ 106.2, **2**: δ 106.1) and C-10″ (**1**: δ 104.1, **2**: δ 103.4), and between H-3″ and C-10″ (**1**: δ 104.1, **2**: δ 103.4). Moreover, nOeDS and gNOESY experiments showed interactions between H-3″ and H-2‴, and H-6‴ for both compounds. Furthermore, interactions of $OC\underline{H}_3$-5 (δ 3.79) with H-6 (δ 6.40) were observed in gNOESY experiments of **2**. Therefore, C-5″ carried the only hydrogen-bonded hydroxyl group of biflavone **2** and C-5 carried a methoxyl group. These observations were confirmed by the ESI-MS of **2**, which displayed a base peak at *m/z* 429 $[M + K - 178]^+$ and ions at *m/z* 403 arising from rearrangements involving the C′ and C rings, respectively. These data led to biflavone structures in which the monomer units should be linked through positions 3-C and C-6″, and established the structures of **1** as 3‴,4′,4‴,5,5″,7,7″-heptahydroxy-3,6″-biflavone (named ridiculuflavone A) and **2** as 3‴,4′,4‴,5″,7,7″-hexahydroxy-5-methoxy-3,6″-biflavone (named ridiculuflavone B).

The ^{13}C NMR spectra showed a total of 33 signals for **3** (Table 3). Two of these signals were clearly twice as intense as other sp^2 carbons, suggesting the presence



	R^1	R^2	R^3	R^4
1	H	H	H	H
2	CH_3	H	H	H
4	H	CH_3	CH_3	CH_3
5	CH_3	H	CH_3	CH_3

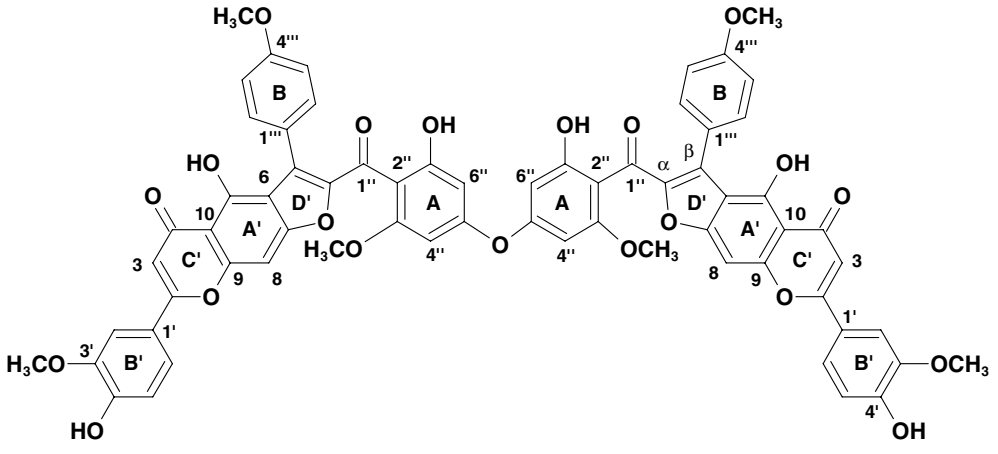


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Table 1
^{13}C NMR spectroscopic data for compounds **1** and **2** (126 MHz, DMSO-d_6)[a]

C	**1**		**2**	
	δ	gHMBC	δ	gHMBC
2	161.6 *s*[b]	H-2′, H-6′	159.0 *s*[b]	H-2′, H-6′
3	112.1 *s*		[d]	
4	182.2 *s*		174.0 *s*	
5	159.8 *s*	OH-5	160.8 *s*	OCH_3-5
6	98.8 *d*	H-8	96.5 *d*	H-8
7	164.0 *s*		161.5 *s*	H-6
8	93.6 *d*	H-6	94.9 *d*	H-6
9	156.7 *s*		162.4 *s*	H-8
10	102.8 *s*		106.5 *s*	H-6
1′	124.0 *s*	H-3′, H-5′	123.7 *s*	H-3′, H-5′
2′	129.6 *d*		129.3 *d*	
3′	115.2 *d*		115.0 *d*	
4′	161.5 *s*[b]	H-2′, H-6′	159.1 *s*[b]	H-2′, H-6′
5′	115.2 *d*		115.0 *d*	
6′	129.6 *d*		129.3 *d*	
2″	164.4 *s*	H-3″	163.8 *s*	H-3″
3″	103.0 *d*		102.9 *d*	
4″	181.6 *s*		181.7 *s*	
5″	162.9 *s*	OH-5″	159.5 *s*	OH-5″
6″	106.2 *s*	OH-5″	106.1 *s*	OH-5″, H-8″
7″	159.5 *s*[c]		159.3 *s*[c]	
8″	93.5 *d*		93.3 *d*	
9″	157.3 *s*[c]	H-8″	156.5 *s*[c]	H-8″
10″	104.1 *s*	H-3″, OH-5″	103.4 *s*	H-3″, OH-5″
1‴	121.4 *s*		121.6 *s*	H-5‴
2‴	113.4 *d*		113.4 *d*	H-6‴
3‴	145.8 *s*		145.8 *s*	H-2‴, H-5‴
4‴	149.8 *s*		149.7 *s*	H-2‴
5‴	116.1 *d*		116.1 *d*	
6‴	119.0 *d*		119.0 *d*	H-2‴
OCH_3-5			55.9 *q*	H-6

[a] The ^{13}C NMR data were assigned with the assistance of DEPT 135°, gHMQC, gHMBC and experiments.
[b,c] Assignments may be interchangeable within the same column.
[d] Signal not observed.

Table 2
^{1}H NMR spectroscopic data for compounds **1** and **2** (500 MHz, DMSO-d_6, J in Hz)[a]

H	**1**		**2**	
	δ	nOeDS	δ	gNOESY
6	6.25 (*d*, 2.0)		6.40 (*d*, 2.5)	OCH_3-5
8	6.47 (*d*, 2.0)		6.47 (*d*, 2.5)	
2′	7.38 (*d*, 8.8)	H-3′	7.35 (*d*, 9.0)	H-3′
3′	6.72 (*d*, 8.8)	H-2′	6.70 (*d*, 9.0)	H-2′, OH-4′
5′	6.72 (*d*, 8.8)	H-6′	6.70 (*d*, 9.0)	H-6′, OH-4′
6′	7.38 (*d*,8.8)	H-5′	7.35 (*d*, 9.0)	H-5′
3″	6.66 (*s*)	H-2‴, H-6‴	6.66 (*s*)	H-2‴, H-6‴
8″	6.59 (*s*)		6.50 (*s*)	
2‴	7.42 (*d*, 2.2)	H-3″	7.41 (*d*, 2.8)	H-3″
5‴	6.91 (*d*, 8.0)	H-6‴	6.91 (*d*, 8.5)	H-6‴
6‴	7.41 (*dd*, 8.0, 2.2)	H-3″	7.43 (*dd*, 8.5, 2.8)	H-3″, H-5‴
OCH_3-5			3.79 (*s*)	H-6
OH-4′	8.80 (*br s*)		8.71 (*br s*)	H-3′, H-5′
OH-5	13.16 (*s*)			
OH-5″	12.92 (*s*)		13.05 (*s*)	

[a] Multiplicities were determined with the assistance of ^{1}H–^{1}H COSY.

of a 1,4-disubstituted aromatic ring. The ^{1}H NMR spectrum and gHMQC experiments showed 30 sp^2 carbons, including 13 carbons bearing an oxygen atom and two carbonyls, and three sp^3 carbons corresponding to three aromatic methoxyl groups. Detailed analyses of ^{1}H and ^{13}C NMR, 1D- and 2D-gNOESY, ^{1}H–^{1}H gCOSY, gHMQC, and gHMBC experiments led to a suggestion of three substructures for **3** (Figs. 1 and 2, Table 3). Based mainly on the chemical shifts of the carbonyl carbon (δ 193.4) and O<u>H</u>-7″ (δ 13.50), on the chemical shifts and multiplicities of six aromatic carbons and of the two aromatic hydrogens (δ 5.90 *d*, J = 2.2 Hz; 6.08 *d*, J = 2.2 Hz), and on the respective gHMBC and gNOESY correlations observed for the methoxyl group (δ_C 55.7, δ_H 3.28) and C-3″ (δ 164.0) and H-4″ (δ 5.90), substructure **3a** was determined. Substructure **3b** corresponds to *p*-methoxyphenyl where the OCH_3-4‴ (δ_C 55.4, δ_H 3.86) was correlated to C-4‴ (δ 160.9) by gHMBC, and to H-3‴ and H-5‴ (δ 7.02) by 1D and 2D-gNOESY experiments. The trisubstituted aromatic ring in substructure **3c** was mainly supported by the multiplicities of the hydrogens and by the nOe between OC<u>H</u>$_3$-3′ (δ 4.03) and H-2′ (δ 7.62). These interactions, together with the correlations evidenced by gHMBC between H-3, H-2′, and H-6′ and C-2 (δ 164.5), as well as the nOes between H-3 (δ 6.78) and H-2′ (δ 7.62) and H-6′ (δ 7.66), allowed establishment of the B′ and C′ rings of a flavone unit. Moreover, the correlations observed between the signals at δ 6.78 (H-3) and δ 106.2 (C-10), as well as between δ 7.38 (H-8) and δ 106.2 (C-10) and δ 113.5 (C-6), by gHMBC allowed substructure **3c** to be established. Thus, the two reminiscent tetrasubstituted sp^2 carbons (δ 151.8 and 131.5) should be involved in the linkage of these substructures. Therefore, four alternative chalcone–flavone structures (**3d**–**3g**, Fig. 3) could be proposed for **3**. Only five chalcone–flavone dimers with the carbon skeletons **3d** to **3f** have already been described in the literature (Carneiro et al., 2000; Ramírez et al., 2003a,b). It is important to note that distinct chemical shifts were observed for C-α and C-β from each carbon skeleton (Fig. 3). The chemical shifts observed for C-α and C-β of **3** were δ 151.8 and 131.5, which, in principle, suggested structure **3g**, which could be consistent with an $[M + H]^+$ at *m*/*z* 597 in ESI-MS. However, while the ESI-MS spectra (+70, +35, +20 eV) of **3** did not displayed a signal for this *quasi*-molecular ion, they did display signals for significant ions at *m*/*z* 876, 743, 699, 683, and 595, together with a signal for a less abundant ion at *m*/*z* 1175 $[M + H]^+$. These data suggested that **3** could be a tetraflavonoid consisting of two chalcone–flavone units with an *–O–* linkage, since the observed *quasi*-molecular ion and the elemental analysis results were consistent with the formula

Table 3
^{13}C and ^{1}H NMR spectroscopic data for compound **3**

C and H	**3**				
	δ ^{13}C[a]	gHMBC	δ ^{1}H[c,d]	nOeDS[c]	gNOESY[c]
2	164.5	H-2′, H-6′, H-3			
3	103.5		6.78 (*s*)		H-2′, H-6′
4	184.4	H-3			
5	157.4				
6	113.5	H-8			
7	154.5[b]	H-8			
8	90.7		7.38 (*s*)		
9	154.9[b]	H-8			
10	106.2	H-3, H-8			
1′	123.2	H-3, H-5′			
2′	110.1	H-6′	7.62 (*d*, 2.2)		H-3, OCH_3-3′
3′	148.0	H-5′			
4′	150.5	H-2′, H-6′			
5′	115.5		7.05 (*d*, 8.5)		H-6′
6′	121.0	H-2′	7.66 (*dd*, 8.5, 2.2)		H-3, H-5′
α	151.8				
β	131.5				
1″	193.4				
2″	107.3	H-4″, H-6″			
3″	164.0	OCH_3-3″			
4″	91.7	H-6″	5.90 (*d*, 2.2)	OCH_3-3″	
5″	165.2				
6″	95.5	H-4″	6.08 (*d*, 2.2)		
7″	166.4	OH-7″			
1‴	122.0	H-3‴, H-5‴			
2‴	128.2	H-6‴	7.68 (*d*, 9.0)		H-3‴
3‴	114.7	H-5‴	7.02 (*d*, 9.0)		H-2‴, OCH_3-4‴
4‴	160.9	H-2‴, H-6‴, OCH_3-4‴			
5‴	114.7	H-3‴	7.02 (*d*, 9.0)		H-6‴, OCH_3-4‴
6‴	128.2	H-2‴	7.68 (*d*, 9.0)		H-5‴
OCH_3-3′	56.3		4.03 (*s*)	H-2′	H-2′
OCH_3-3″	55.7		3.28 (*s*)	H-4″	
OCH_3-4‴	55.4		3.86 (*s*)	H-3‴, H-5‴	H-3‴, H-5‴
OH-5			13.78 (*s*)		
OH-7″			13.50 (*s*)		

[a] Recorded in CD_3CN, 126 MHz.
[b] Assignments may be interchangeable within the same column.
[c] Recorded in CD_3CN, 500 MHz, *J* in Hz.
[d] Multiplicities were determined with the assistance of ^{1}H–^{1}H COSY, 500 MHz.

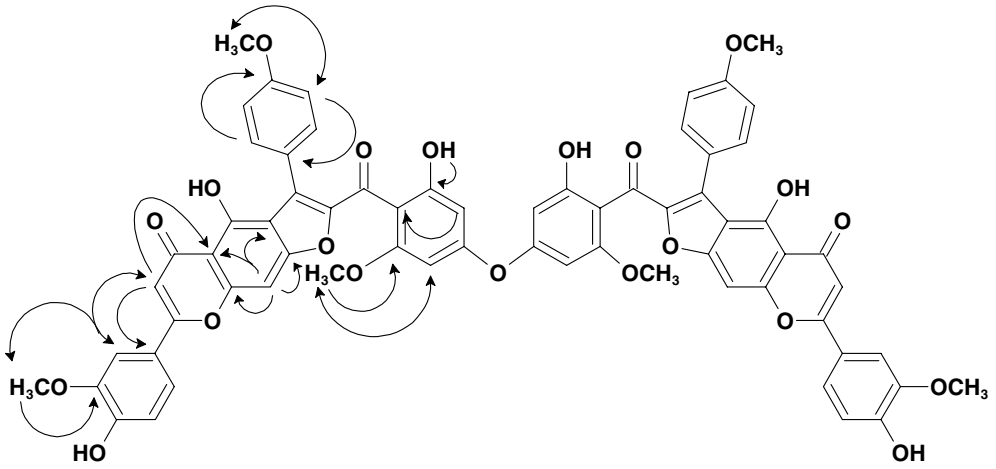

Fig. 1. Selected nOe interactions (↔) and HMBC (→) correlations for tetramer **3**.

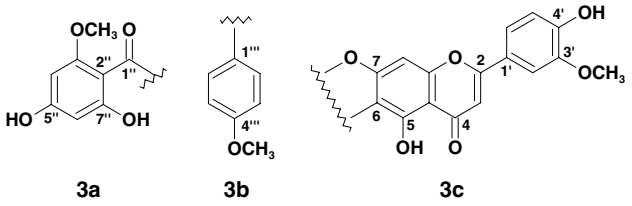

Fig. 2. Substructures for tetramer **3**.

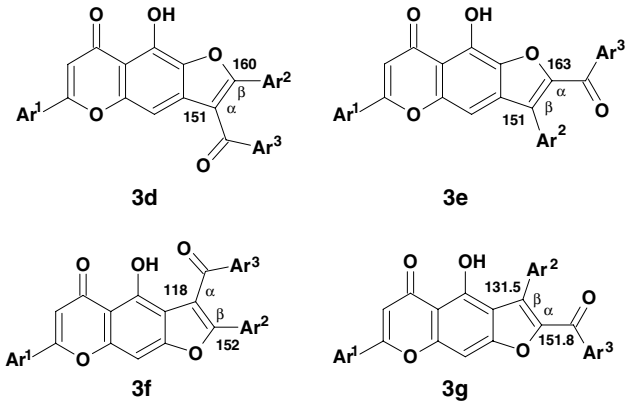

Fig. 3. ^{13}C Chemical shifts of C-α and C-β for alternative skeletons of chalcone–flavones.

$C_{66}H_{46}O_{21}$. Therefore, these two units (I–II) should be linked through C-5″ → O → C-5″, which is in accordance with a symmetric molecule and with notion that most of the ions observed by ESI-MS, including those at *m*/*z* 743, 683 and 551, originated from retro Diels Alder rearrangements involving the I-C′ and II-C′ rings. Based on the above spectral evidence, **3** was characterized as oxy{bis[5″(4′,5,7″-trihydroxy-3′,3″,4‴-trimethoxy-7-*O*-α:6-β-flavone–chalcone)]} (named ridiculuflavonylchalcone A).

3. Experimental

3.1. General experimental procedures

The 1D-(^1H, ^{13}C, DEPT, and nOeDS) and 2D-(^1H–^1H gCOSY, gHMQC, gHMBC and gNOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^1H) and 126 MHz (^{13}C), using the solvents as an internal standard. Mass spectra (ESI-MS) were obtained on a Fisons Platform II, and flow injection into the electrospray source was used. IR spectra were obtained on a Perkin Elmer 1600 FT-IR spectrometer using KBr discs. UV absorptions were measured on a Perkin Elmer UV–vis Lambda 14P spectrophotometer. Optical rotations were measured on a Polamat A Carl Zeiss Jena polarimeter. HPLC analyses were carried out using a Shimadzu liquid chromatograph 10Avp equipped with a UV–vis detector. Columns were RP-18 (Shimadzu, C18, 250 × 4.6 mm for analytical analysis and 250 × 20 mm for semi-preparative analysis), and chromatograms were acquired at 254 nm. TLC: Silica gel 60 PF_{254}. Melting points were recorded on a Microquímica MQAPF-301 melting point apparatus and were uncorr.

3.2. Plant material

The plant material was collected in São Joaquim da Barra, SP, Brazil, in February, 2000, and identified as *Aristolochia ridicula* Brown by Dr. Condorcet Aranha and as *c. s. Aristolochia ridicula* H.B.K. by Dr. Lindolpho Cappellari Júnior. A voucher specimen (ESA88276) was deposited at the herbarium of the Escola Superior de Agricultura, Luiz de Queiroz (ESALQ), Piracicaba, SP, Brazil. The material was separated by plant parts, dried (~45 °C) and ground.

3.3. Extraction and isolation

Ground leaves (297.1 g) of *A. ridicula* were extracted exhaustively at room temperature with hexane, Me_2CO and EtOH, successively, and the extracts were then individually concentrated. The crude acetone extract (10.0 g) was partially dissolved in MeOH–H_2O 4:1 to give a solution and a precipitate. The soluble fraction was adjusted to MeOH–H_2O 3:2, and washed with $CHCl_3$, concentrated, and fractionated over Sephadex LH-20 (MeOH) to give 10 fractions. Fraction 2 was crystallized from methanol to give *proto*-quercitol (137 mg). Fraction 4 was subjected to prep. HPLC (MeOH–H_2O 11:9) to give **2** (135 mg), fraction 6 by repetitive precipitation procedures from CH_3CN gave **3** (5 mg), and fraction 8 was subjected to CC (silica gel, activated carbon 3:1, $CHCl_3$–MeOH gradient) to give **1** (11 mg).

3.4. 3‴,4′,4‴,5,5″,7,7″-Heptahydroxy-3,6″-biflavone (ridiculuflavone A, *1*)

Yellow solid; m.p. 230.5–232.2 °C; $[\alpha]_D^{26}$ +47.9° (MeOH, *c* 0.046); UV λ_{max} (MeOH) nm (log ε): 274 (3.7), 325 (3.4), 347 (3.4); IR (KBr) ν_{max} 3400, 1646, 1613, 1553, 1487, 1453, 1380 cm^{-1}; ^1H and ^{13}C NMR: see Tables 1 and 2; negative ESI-MS (probe) 70 eV, *m*/*z* (rel. int.): 553 $[M - H]^-$ (14), 401 (14), 325 (21), 311 (14). Found: C, 65.0; H, 3.5. $C_{30}H_{18}O_{11}$ requires: C, 64.5; H, 3.3%.

3.5. 3‴,4′,4‴,5″,7,7″-Hexahydroxy-5-methoxy-3,6″-biflavone (ridiculuflavone B, *2*)

Yellow solid; m.p. 224.1–225.7 °C; $[\alpha]_D^{25}$ +18.2° (MeOH, *c* 0.10); UV λ_{max} (MeOH) nm (log ε): 280

(3.9), 337 (3.5); IR (KBr) ν_{max} 3373, 1646, 1613, 1520, 1446 cm^{-1} ; ^1H and ^{13}C NMR: see Tables 1 and 2; positive ESI-MS (probe) 70 eV, *m/z* (rel. int.): 569 $[M + H]^+$ (43), 429 (100), 403 (12). Found: C, 65.5; H, 3.7. $C_{31}H_{20}O_{11}$ requires: C, 65.5; H, 3.5%.

3.6. Oxy{bis[5″(4′,5,7″-trihydroxy-3′,3″,4‴-trimethoxy-7-O-α:6-β-flavone–chalcone)]} (ridiculuflavonylchalcone A, **3**)

Yellow solid; m.p. 153.6–156.5 °C; $[\alpha]_D^{28}$ −30.8° (MeOH, *c* 0.088); UV λ_{max} (MeOH) nm (log ε): 305 (4.8), 345 (4.7); IR (KBr) ν_{max} 3437, 2920, 2951, 1660, 1625, 1594, 1568 cm^{-1} ; ^1H and ^{13}C NMR: see Table 3; positive ESI-MS (probe) 20 eV, *m/z* (rel. int.): 1175 $[M + H]^+$ (<1), 876 (11), 743 (17), 699 (33), 683 (56), 595 (83), 551 (100). Found: C, 67.5; H, 4.1. $C_{66}H_{46}O_{21}$ requires: C, 67.4; H, 3.9%.

3.7. proto-Quercitol

White crystals; m.p. 231.6–234.1 °C [lit. 228–230 °C (Salamci et al., 1997)]; $[\alpha]_D^{25}$ +8.2° (H_2O, *c* 0.10); IR data agree with those reported in the literature (Salamci et al., 1997); ^1H NMR spectral data (500 MHz, D_2O): δ 1.71 (1H, *ddd*, *J* = 14.0, 11.5 and 3.3 Hz, H-6b), 1.89 (1H, *dddd*, *J* = 14.0, 5.0, 3.3 and 1.3 Hz, H-6a), 3.46 (1H, *t*, *J* = 9.3 Hz, H-2), 3.61 (1H, *dd*, *J* = 9.3 and 3.3 Hz, H-3), 3.65 (1H, *ddd*, *J* = 11.5, 9.3 and 5.0 Hz, H-1), 3.83 (1H, *dt*, *J* = 1.3 and 3.3 Hz, H-4), 3.92 (1H, *q*, *J* = 3.3 Hz, H-5); ^{13}C NMR data (126 MHz, D_2O): δ 33.5 (*t*, C-6), 68.8 (*d*, C-5), 69.1(*d*, C-1), 71.2 (*d*, C-3), 72.5 (*d*, C-4), 74.8 (*d*, C-2); positive ESI-MS (probe) 70 eV, *m/z* (rel. int.): 165 $[M + H]^+$ (100), 151 (80).

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