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Article

Two 8*C*-methylated Flavonols from the Leaves of *Vellozia candida* Mikan (Velloziaceae)

***Alexsandro Branco*[#,a], *Ângelo C. Pinto*[a], *Demian R. Ifa*[b] *and Raimundo Braz-Filho*[*c]**

[a] *Instituto de Química, Departamento de Química Orgânica, Universidade Federal do Rio de Janeiro, 21949-970, Rio de Janeiro - RJ, Brazil*

[b] *Departamento de Farmacologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, 05508-900, São Paulo - SP, Brazil*

[c] *Setor de Química de Produtos Naturais - LCQUI – CCT, Universidade Estadual do Norte Fluminense, 28015-620, Campos - RJ, Brazil*

Do extrato etéreo das folhas de *Vellozia candida* Mikan (Velloziaceae) foram isolados os flavonóis 3',4',5,7-tetraidroxi-3,6-dimetoxi-8-metilflavona e 3',4',5-triidroxi-3,6,7-trimetoxi-8-metilflavona. Estes compostos foram identificados através de seus dados espectrais, incluindo RMN 2D e EM-EM. O derivado 5-hidroxi-3,3',4',6,7-pentametoxi-8-metilflavona foi obtido por metilação usando diazometano.

From the leaves of *Vellozia candida* Mikan (Velloziaceae) the new flavonol 3',4',5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone and its known 7-*O*-methyl derivative, 3',4',5-trihydroxy-3,6,7-trimethoxy-8-methylflavone, were isolated and characterized by spectral data, including 2D NMR and tandem mass spectrometry experiments. The 5-hydroxy-3,3',4',6,7-pentamethoxy-8-methylflavone derivative was obtained by methylation with diazomethane.

Keywords: Velloziaceae, *Vellozia candida*, flavonoids, ESI-MS/MS

Introduction

The flavonol aglycones constitute a very diverse group of plant secondary metabolites. A number of these natural products are lipophilic and may show close R_f-values in TLC, with overlapping spots, making it impossible to identify them in mixtures containing several related components. A preliminary MS spectrum of a plant fraction and/or crude extract, and also data from GC, HPLC, TMS (Tandem Mass Spectrometry) can supply important structural information on natural compounds and thereby allow an optimization of the investigation and avoid unnecessary isolation, especially of known compounds. The Velloziaceae family contains *C*-methyl and *C*-prenylated flavonoids which are relatively rare in nature.[1,2] Recently, we applied high temperature high resolution gas chromatography (HT-HRGC) coupled to mass spectrometry (HT-HRGC-MS) in the prospection and identification of the monoisoprenylated flavonol 3,5,4'-trimethoxy-3'-hydroxy-6,7-(2"-isopropenyldihydrofuran)-flavone from *Vellozia graminifolia*, and the importance of this technique to determine the presence of lipophilic flavonols was demonstrated.[3]

A previous phytochemical analysis of *V. candida*, collected on the Corcovado mountain, in the city of Rio de Janeiro, Brazil, resulted in the identification of several rosane diterpenoids,[4,5] including a velloziolide with a new skeleton[6] and a bioactive *seco*-rosane.[7] In this paper we describe the characterization of the 8-*C*-methylated flavonols 3',4',5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone (**1**) and its known 7-*O*-methyl derivative, 3',4',5-trihydroxy-3,6,7-trimethoxy-8-methylflavone (**2**), isolated from a specimen of *V. candida* collected in Barra da Tijuca, a coastal area of the city of Rio de Janeiro. The structural determination of these compounds as components of a mixture was based on spectral data, including 2D NMR techniques, such as heteronuclear correlation ^{1}H-^{13}C-COSY-$^{n}J_{CH}$ (n=1, HMQC = ^{1}H-detected Heteronuclear Multiple Quantum Coherence; n=2 and 3, HMBC = ^{1}H-detected Heteronuclear Multiple Bond Connectivity) and tandem mass spectrometry, together with chemical transformation and comparative analysis of

* e-mail: braz@uenf.br
# Present address: Departamento de Química, Universidade Federal de Santa Catarina, 88040 970, Florianópolis-SC, Brazil

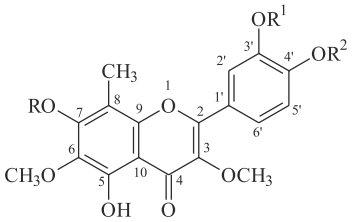
chemical shifts of previously reported analogous flavonols isolated from the Velloziaceae family.[8]

Results and Discussion

Chromatography of the Et_2O extract of *V. candida* on a silica gel column yielded initially a fraction containing aliphatic carboxylic acids and sterols, which were characterized by GC/MS analysis (see Experimental). The other fractions obtained from the same column were combined on the basis of TLC comparison and subsequently chromatographed on Sephadex LH-20 to give a mixture of two lipophilic flavonoids. This mixture was subjected to methylation with diazomethane and yielded 5-hydroxy-3,6,7,3',4'-pentamethoxy-8-methyl-flavone (**3**) as the only product of known structure.

The mixture of flavonols **1** and **2** showed IR absorption bands for a conjugated carbonyl group (ν_{max} 1651 cm^{-1}) and for aromatic ring (ν_{max} 1602 and 1559 cm^{-1}). The comparative analysis of the HBBD- and DEPT-^{13}C NMR spectra was used to recognize the number of signals corresponding to quaternary, methine and methyl carbon atoms (Table 1). The ^{1}H NMR spectrum showed signals for aromatic hydrogens H-2', H-5' and H-6' in the B-ring of a 3',4'-disubstituted flavonoid, along with signals for methoxyl, methyl and chelated hydroxyl groups (Table 1).

In accordance with the ^{1}H and ^{13}C NMR spectral data of **1**, the HMBC spectrum confirmed a completely substituted A-ring through the heteronuclear long range couplings via two ($^2J_{CH}$) and three ($^3J_{CH}$) bonds between carbon atoms C-6 (δ_C 130.24) and hydrogens of both HO-5 [δ_H 12.76, s; $^3J_{CH}$] and MeO-6 [δ_H 3.94, s; $^3J_{CH}$] groups, C-9 (δ_C 149.50) and Me-8 [δ_H 2.31, s; $^3J_{CH}$] and C-7 (δ_C 154.05) and Me-8 [δ_H 2.31, s; $^3J_{CH}$]. The presence of a cross



	R	R^1	R^2
1	H	H	H
2	CH_3	H	H
3	CH_3	CH_3	CH_3

Table 1. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) data for flavonols **1** and **2** ($CDCl_3$, δ in ppm, *J* in parentheses in Hz) Including results of heteronuclear 2D experiments ^{1}H-^{13}C-COSY-$^n J_{CH}$ (HMQC and HMBC)*

	1				**2**			
	$^1J_{CH}$		$^2J_{CH}$	$^3J_{CH}$	$^1J_{CH}$		$^2J_{CH}$	$^3J_{CH}$
C	δ_C	δ_H			δ_C	δ_H		
2	155.74	-		H-2', H-6'	156.40	-		H-2', H-6'
3	137.68	-		MeO-3	137.91	-		MeO-3
4	178.94	-			179.11	-		
5	150.82	-	HO-5		152.39	-	HO-5	
6	130.24	-		HO-5, MeO-6	135.77	-		HO-5, MeO-6
7	154.05	-		Me-8	156.74	-		Me-8, MeO-7
8	101.98	-	Me-8		108.70	-	Me-8	
9	149.50	-		Me-8	149.07	-		Me-8
10	104.90	-		HO-5	107.76	-		HO-5
1'	122.00	-		H-5'	121.75	-		H-5'
3'	144.66	-	H-2'	H-5'	144.66	-	H-2'	H-5'
4'	147.85	-	H-5'	H-2', H-6'	147.85	-	H-5'	H-2', H-6'
CH								
2'	115.30	7.74, d (2.0)		H-6'	115.30	7.74, d (2.0)		H-6'
5'	115.38	6.98, d (8.5)	H-6'		115.38	6.98, d (8.5)	H-6'	
6'	120.89	7.59, dd (8.5; 2.0)	H-5'	H-2'	121.05	7.59, dd (8.5; 2.0)		H-2'
CH_3								
MeO-3	59.67	3.84, s			59.66	3.85, s		
MeO-6	60.36	3.94, s			60.46	3.92, s		
MeO-7	-	-			60.87	3.99, s		
Me-8	7.67	2.31, s			8.07	2.34, s		
HO-5	-	12.76, s			-	12.67, s		

* Multiplicity of signals of carbon atoms deduced by comparative analysis of HBBD- and DEPT-^{13}C. 2D ^{1}H-^{1}H-COSY NMR also used for these assignments

peak correlating the signals at δ_C 137.68 (C-3) and δ_H 3.84 (MeO-3) allowed location of the remaining methoxyl group at C-3 of **2** (Table 1). The ^{13}C chemical shifts of the methoxyl groups at δ_C 60.36 (MeO-6) and 59.67 (MeO-3) are in accordance with the location of these groups at sterically hindered positions. Other heteronuclear long range spin-spin interactions of hydrogen and carbon atoms are summarized in Table 1.

The ^1H NMR spectra (1D and 2D ^1H-^1H-COSY) showed signals compatible with a 1,3,4-trisubstituted aromatic ring in flavonoids (**1** and **2**) based on the chemical shifts and coupling constants for H-2' [δ_H 7.74 (d, 2.0 Hz)], H-5' [δ_H 6.98 (d, 8.5 Hz)] and H-6' [δ_H 7.59 (dd, 2.0 and 8.5 Hz). These assignments were confirmed by the chemical shifts and multiplicities of the signals corresponding to C-1' and C-6' and by heteronuclear correlations, observed in the $^1Hx^{13}C$-COSY-$^nJ_{CH}$ NMR spectra, as shown in Table 1.

The electrospray ionization (ESI) mass spectrum at collision energies of 10 eV and 20 eV of the mixture isolated from *V. candida* shows peaks due to protonated molecular ions ($[M+H]^+$) for **1** (*m/z* 361, **1a**) and for the related minor 8-*C*-methylated flavonol **2** (*m/z* 375, **2a**) (Figure 1). The difference of 14 daltons between the protonated molecular ions $[M+H]^+$ of **1** (*m/z* 361 $[360+H]^+$) and **2** (375 $[374+H]^+$) was attributed to a biomethylation of one hydroxyl group

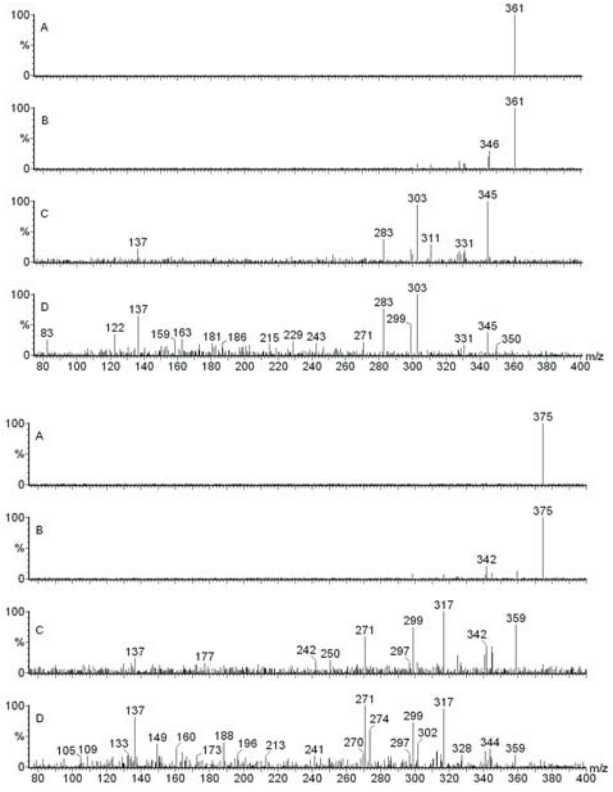


Figure 1. CID mass spectra of flavonols **1** (upper) and **2** (lower) isolated from *V. candida*. Collision energies at 10 (A), 20 (B), 30 (C) and 40 (D) eV

of **1** (dimethoxylated) to produce **2** (trimethoxylated). This is in accordance with the selective methylation of the mixture which afforded only one product containing a chelated hydroxyl group at C-5 [δ_H 12.58 (s)], a relatively strong intramolecular hydrogen bond involving a six-membered ring.

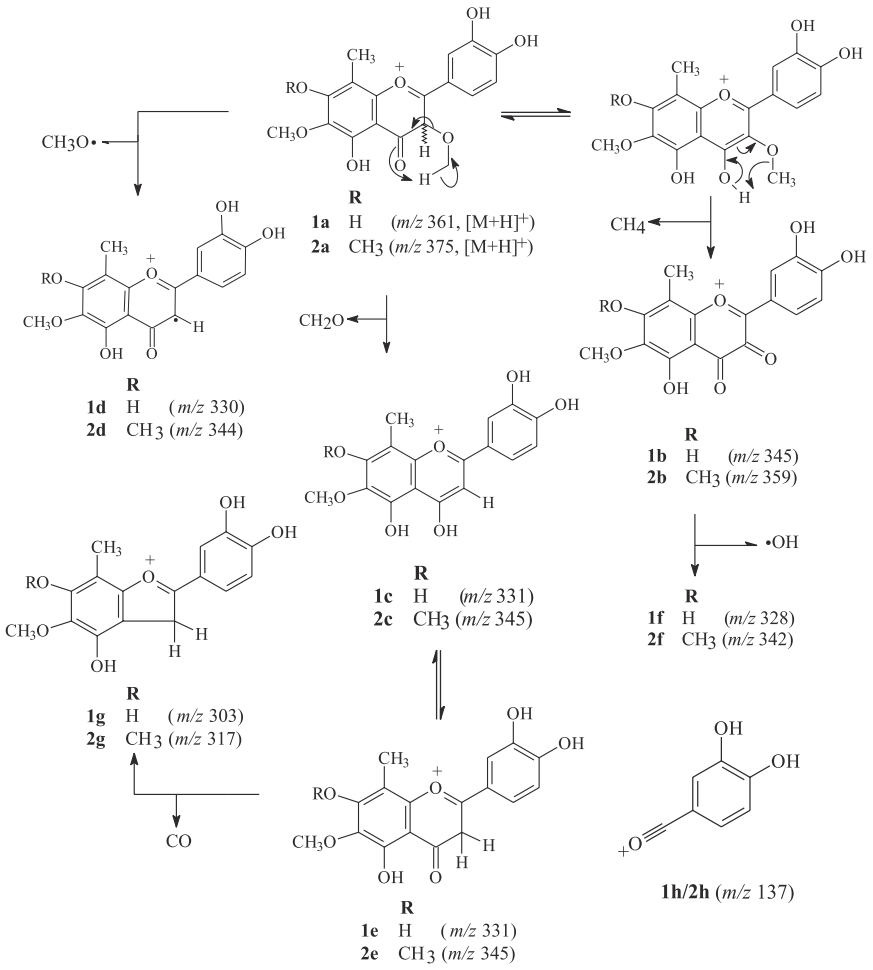
In fact, the remaining signals observed in the ^1H and ^{13}C NMR spectra together with the results obtained from heteronuclear 2D shift-correlated $^1Hx^{13}C$-COSY-$^nJ_{CH}$ (n=1, HMQC; n=2 and 3, HMBC) spectra (Table 1), allowed the definition of the structure as 3',4',5-trihydroxy-3,6,7-trimethoxy-8-methylflavone (**2**) for the minor component present in the mixture. The HMBC spectrum revealed 2D $^1H/^{13}C$ multiple bond ($^2J_{CH}$ and $^3J_{CH}$) correlation between carbon and hydrogen atoms (Table 1): a) C-6 (δ_C 135.77) and both HO-5 [δ_H 12.67, s; $^3J_{CH}$] and MeO-6 [δ_H 3.92, s; $^3J_{CH}$]; b) C-9 (δ_C 149.07) and Me-8 [δ_H 2.34, s; $^3J_{CH}$]; c) C-7 (δ_C 156.74) and both Me-8 [2.34, s; $^3J_{CH}$] and MeO-7 [δ_H 3.99, s; $^3J_{CH}$]; d) C-3 (δ_C 137.91) and MeO-3 [δ_H 3.85, s; $^3J_{CH}$]. Other heteronuclear long range spin-spin interactions of hydrogen and carbon atoms are summarized in Table 1.

The prominent peak at *m/z* 137 observed in both mass spectra obtained at 30 eV (**1h**/**2h**, 16 %/17 %) and 40 eV (**1h**/**2h**, 60 %/75 %) was also used to confirm the pattern of substitution in B ring as 3',4'-dihydroxylated ($^{0,2}B^+$ fragments in Claeys' nomenclature).[9]

Thus, the two 8*C*-methylated flavonols isolated as a mixture from *Vellozia candida* were characterized as the new flavonol 3',4',5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone (**1**) and its known 7-*O*-methyl derivative, 3',4',5-trihydroxy-3,6,7-trimethoxy-8-methylflavone (**2**).[2] On the basis of intensity of the signals corresponding to HO-5 [**2**/**3**: δ_H 12.76 (s, rel. int.=1.000)/12.67 (s, rel. int.= 0.376)] in the ^1H NMR spectrum the percentages of **1** (72.7%) and **2** (27.3%) in the mixture were estimated. Furthermore, the complete ^1H and ^{13}C chemical shift assignments of **1** and **2** were also accomplished by extensive NMR analysis (Table 1).

The CID spectra of **1a** and **2a** show peaks at *m/z* 345 (**1**) and 359 (**2**), which were attributed to ionic fragments **1b** and **2b** formed from quasimolecular ions by elimination of one molecule of methane ($[M+H]^+$ - 16 daltons), consistent with literature data for 3-methoxy flavonoids.[9] Thus, the CID spectra at collision energies of 10 and 20 eV allowed the observation of peaks corresponding to $[M+H]^+$. At 30 and 40 eV these ionic species are absent and the peaks observed can be used to determine the fragmentation mechanisms for **1a** and **2a** in the mass spectrum (Scheme 1).

Tandem mass spectrometry, a technique based in tandem mass analyzers (ions formed are separated by mass



Scheme 1. CID mass fragmentation of **1** and **2**. Elimination of CH_2O and CH_4 showed in this scheme may involve heterolytic (as indicated with full arrow corresponding to a electron par) or homolytic (fish-hook corresponding to a single electron) bond cleavages

analysis and secondary mass spectrum of each ion may be obtained, MS/MS) used for the separation and identification of ions (such as those representing components in complex mixture), proved to be a rapid and powerful technique for the characterization of flavonoids.[10] Structural information of the components of a sample may be obtained by fragmentation of selected $[M+H]^+$ in CID.

Conclusions

The flavonoid substitution patterns are useful in the determination of the generic limits within the family Velloziaceae.[11] In the genus *Vellozia* it is common to find flavonols with *C*-prenylation, 6-hydroxylation and *C*-methylation which are rarely found in the other genera of this family. In the case of *V. candida* Mikan, after the characterization of the new 3',4',5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone (**1**) by NMR spectroscopy (Table 1), it was possible to recognize its known O-methyl ether derivative (**2**) in the mixture with the aid of tandem mass spectrometry.

Experimental

General

Column chromatography was performed with silica gel 60 (Merck 70-230 mesh). Merck silica gel F254 was used for TLC plates and spots visualized by UV (254 and 360 nm). NMR spectra in $CDCl_3$ solution were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Bruker AC 300 spectrometer, TMS as internal standard or referenced to the solvent signals $C\underline{H}Cl_3$, at δ_H 7.25 and $\underline{C}HCl_3$, at δ_C 77.00. Samples were initially diluted in aqueous/acetonitrile solution and subsequently with acetic acid to provide solutions of 10 mM HOAc (50/50). The ESI/MS/

MS spectra were acquired using a Quattro II (MicroMass, UK) triple quadrupole mass spectrometer equipped with an electrospray ionization source. The spectra were recorded and processed by MassLynx v. 3.2 software (MicroMass, UK). The acquisitions were accomplished in positive ion mode (ES+) from *m/z* 50 to 550 at 2 s/scan. The cone voltage was 40V and source temperature maintained at 100°C. Each sample was delivered to the electrospray source through an infusion pump (Pump 22, Harvard Apparatus, So. Natick, MA) at a flow rate of 10 μL min^{-1}. The protonated molecule, $[M+H]^+$, was selected at Q1 as precursor ion, and product ion scans were made at Q3, after collisionally induced dissociation at q2. Argon was used as collision gas (Gas Cell pressure was 1.7x10^{-3} mBar) and different collision energies, from 10 to 40 eV, were applied.

Plant material

Vellozia candida Mikan was collected in Barra da Tijuca, Rio de Janeiro, Brazil and identified by Prof. Nanuza L. de Menezes of the Universidade de São Paulo (USP), São Paulo-SP, Brazil. A voucher specimen has been deposited at the herbarium of the Instituto de Botânica of USP.

Extraction and isolation

Dried and powdered leaves were extracted with ethyl ether at room temperature. After concentration of the solvent, under vacuum, an oily residue (6.9 g) was obtained. This extract was re-dissolved in methanol, filtered to remove waxes and evaporated at reduced pressure to yield an oily material (3.6 g), which was chromatographed on a silica gel column (45 g) with a gradient of EtOAc in hexane. A total of 12 fractions of *ca* 100 mL each were collected and combined on the basis of TLC comparison. Fraction 2, eluted with hexane/EtOAc (1:1) contained palmitic acid (40 %), stearic acid (20 %), b-sitosterol (3 %) and stigmasterol (12 %) which have been characterized through fragmentation patterns obtained by HRGC-MS.[12] Fractions 4, 5 and 6, eluted with EtOAc/MeOH (7:3 to 5:5), were combined and chromatographed on a Sephadex LH-20 column using methanol as eluent. Fractions 2-9 (15 mL each) were again combined and rechromatographed on a Sephadex LH-20 column using methanol as eluent. Fractions 2-6 (15 mL each) furnished a mixture of **1** and **2** as a yellow oil (19.6 mg).

*3',4',5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone (**1**) and 3',4',5-trihydroxy-3,6,7-trimethoxy-8-methylflavone (**2**):* IR ν_{max}/cm^{-1}: 3408, 1651, 1602, 1559 (KBr); ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$): Table 1; ESI/MS/MS: Figure 1.

*Methylation of the mixture of **1**+**2***: The mixture (9.1 mg) was treated with CH_2N_2 in the usual manner to yield 5-hydroxy-3,6,7,3',4'-pentamethoxy-8*C*-methylflavone (**3**, 9.2 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ_H 12.58 (*s*, HO-5), 7.79 (dd, *J* 2.0, 8.5 Hz, H-6'), 7.77 (d, *J* 2.0, H-2'), 7.02 (d, *J* 8.5 Hz, H-5'), 3.88 (s, MeO), 3.93 (s, MeO), 3.97 (s, MeO), 3.98 (s, MeO), 4.01 (s, MeO). ^{13}C NMR (75 MHz, $CDCl_3$): δ_C 151.49(C-2), 138.72 (C-3), 179.54 (C-4), 155.93 (C-5), 136.35 (C-6), 157.30 (C-7), 108.28 (C-8), 151.49 (C-9), 108.88 (C-10), 123.34 (C-1'), 111.14 (C-2'), 148.89 (C-3'), 149.49 (C-4'), 110.94 (C-5'), 122.14 (C-6'), 60.21 (MeO-3), 60.90 and 61.29 (MeO-6 and MeO-7), 56.08 and 56.03 (MeO-3' and MeO-4'); ESI/MS/MS: 10 eV *m/z* (rel. int.): 403 (100%, $[M + H]^+$); 30 eV: 387 (64, $[M + H]^+ - CH_4$), 373 (100, $[M + H]^+ - CH_2O$), 345 (88, $[M + H]^+ - CH_2O - CO$), 40 eV: 165 (79, $[C_7H_5O_3]^+$).

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