Sulfated flavonoid isolated from *Flaveria bidentis* and its semisynthetic derivatives as potential drugs for Alzheimer´s disease

Cavallaro Valeria[1], Estévez Braun Ana[2], Gutiérrez Ravelo Ángel[2] and Murray Ana Paula[1].

[1]INQUISUR-CONICET, Departamento de Química, Universidad Nacional del Sur, Avda. Alem 1253, Bahía Blanca, Argentina. e-mail: apmurray@uns.edu.ar
[2]Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna, Avda. Astrofísico Fco. Sánchez Nº2. La Laguna, Tenerife, España.

Abstract

Flaveria bidentis (L.) Kuntze (Asteraceae), is an endemic species from Argentina. Potent AChE inhibitory activity was observed in its ethanolic extract (IC_{50}=0.12 mg/mL). Partition of this extract led to spontaneous crystallization of a sulfated flavonoid with excellent yield. It´s structure was elucidated by HRMS and mono- and bidimensional NMR, and has been identified as 6-methoxykaempferol-3-sulphate (**1**). In order to improve the moderate acetylcholinesterase (AChE) inhibitory activity of **1**, this compound has been submitted to chemical modifications which led to the semisynthethic desulfated and alkylated analogs. These derivatives have been fully characterized by NMR and MS and are reported here for the first time. AChE inhibitory activity and scavenging of DPPH free radical of these derivatives will be discussed and compared to **1**.

Keywords: acetylcholinesterase, flavonoid, analogs

Introduction

Alzheimer's disease (AD) is clinically characterized by a progressive and irreversible cognitive impairments and memory loss [1]. The treatment for AD remains a challenge for pharmaceutical scientist. According to the cholinergic hypothesis, the cognitive and memory symptoms of AD are caused by the drastic decline of acetylcholine. Increasing acetylcholine levels in the brain by AChE inhibition is one of the current strategies for AD treatment [2].
During aging, the progressive damage of the endogenous antioxidant protection system is another obvious phenomenon in AD. Moreover, increasing evidence supports the significant impact of oxidative stress in the pathogenesis and progression of AD [3]. Studies have indicated that preventing the formation of the free radicals could be useful for AD treatment.
Flavonoids are well-known natural compounds that attract increasing attention due to a wide range of pharmacological properties related to a variety of neurological disorders, like neuroprotective effect [4], AChE inhibitory activity [5] and free radical scavenging ability [6], among others. Thus, the isolation from natural sources and semi-synthesis of new effective flavonoid derivatives are interesting strategies for the research on anti-Alzheimer´s disease drugs.

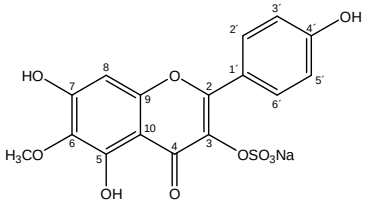
Experimental

General: NMR measurements, including COSY, HSQC, HMBC and NOESY experiments, were carried out from DMSO solutions, on a Bruker Avance 400 spectrometer (400 and 100 MHz for hydrogen and carbon respectively) and on a Bruker Avance 600 spectrometer (600 and 150 MHz for hydrogen and carbon respectively). Chemical shifts are given in ppm (δ) with TMS as an internal standard. HRMS data were recorded with a LCT Premier XE (Waters) spectrometer. UV spectra were recorded on a JASCO V-630BIO spectrophotometer. Precoated TLC plates SIL G-100 UV254 were used for preparative TLC purification. Acetylcholinesterase from *electric eel* (type VI-S), 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCI), eserine and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma.

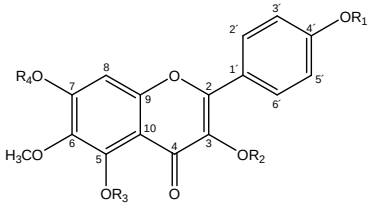
Plant Material: *Flaveria bidentis* was collected during flowering period (November 2012) in Salitral de la Vidriera, Buenos Aires, Argentina.

Sulfated flavonoid extraction: Fresh roots and aerial parts of *F. bidentis* (100 g) were extracted with ethanol (96% v/v) at room temperature for two weeks. Ethanolic extract was evaporated under reduced pressure and the residue was extracted with Hexane/H_2O several times. The aqueous layer was concentrated to minimum volume and refrigerated for 24 hs to obtain a yellow precipitate. Once filtered, the solid was recrystallized in water to obtain 200 mg of **1**. Its structure was elucidated by HRMS and mono- and bidimensional NMR.

Figure 1: Structures of compounds **1**-**5**



(1)



(2) $R_1=R_2=R_3=R_4=H$
(3) $R_1=R_2=R_3=H$, $R_4=CH_3$
(4) $R_1=R_3=R_4=H$, $R_2=CH_3$
(5) $R_2=R_3=H$, $R_1=R_4=CH_2CH_2Br$

Semisynthesis of 6-methoxykaempferol-3-sulfate analogs:
6-methoxykaempferol (2)
0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulphate (**1**) was dissolved in 3 mL of HCl (0.1 M). The solution was stirred for 12 h under reflux and cooled to room temperature. Next, the reaction mixture was extracted with AcOEt (3 x 5 mL) and dried over $MgSO_4$. Pure compound **2** was obtained as bright yellow solid in 95 % yield. HRMS (ESI) Calcd for $C_{16}H_{12}O_7$ $[M+Na]^+$ 339.0481, found 339.0478. ^1H and ^{13}C data are presented in Table 1 and 2.

6,7-dimethoxykaempferol (3)
0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulphate (**1**) was dissolved in 1 mL of ethyl ether. Then, 0.096 mmol (0.01 mL) of $C_4H_{10}N_2Si$ and 12.3 mmol (0.5 mL) of MeOH were added. The mixture was stirred at room temperature for 24 h until TLC analysis indicated total conversion. The reaction mixture was concentrated to dryness affording compound **2**. ^1H and ^{13}C data are presented in Table 1 and 2.

5,5´,6,7-tetramethoxykaempferol (4)
0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulphate (**1**) was dissolved in 1 mL of ethyl ether. Then 0.384 mmol (0.2 mL) of $C_4H_{10}N_2Si$ and 37 mmol (1.5 mL) of MeOH were added. The solution was stirred at room temperature for 24 h. The reaction mixture was concentrated to dryness and purified by preparative TLC (Silica Gel F_{254}, CH_2Cl_2: MeOH (80:20)) affording compound **3** in 66% yield. ^1H and ^{13}C data are presented in Table 1 and 2.

4´,7-di(2-bromoethoxy)-6-methoxykaempferol (5)
0.096 mmol (40 mg) of 6-methoxykaempferol-3-sulphate (**1**) was dissolved in 2 mL of DMF. Then 0.480 mmol (67.2 mg) of K_2CO_3 and 9.8 mmol (0.4 mL) of MeOH were added. The mixture was stirred at room temperature for 11 h. The reaction mixture was concentrated to dryness and purified by preparative TLC (Silica Gel F_{254}, CH_2Cl_2: MeOH (90:10)) affording compound **5** in 60% yield. ^1H and ^{13}C data are presented in Table 1 and 2.

Cholinesterase inhibition assay: Electric eel (*Torpedo californica*) AChE was used as source of cholinesterase. AChE inhibitory activity was measured *in vitro* by the spectrophotometric method developed by Ellman with slight modification [8]. The lyophilized enzyme, 500 U AChE, was prepared in buffer A (8 mM

K_2HPO_4, 2.3 mM NaH_2PO_4) to obtain 5 U/mL stock solution. Further enzyme dilution was carried out with buffer B (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 0.15 M NaCl, 0.05% Tween 20, pH 7.6) to produce 0.126/0.06 U/mL enzyme solution. Samples were dissolved in buffer B. Compounds required 2.5% of MeOH as cosolvent. Enzyme solution (300 μL) and 300 μL of sample solution were mixed in a test tube and incubated for 60 min at room temperature. The reaction was started by adding 600 μL of the substrate solution (0.5 mM DTNB, 0.6 mM ATCI, 0.1 M Na_2HPO_4, pH 7.5). The absorbance was read at 405 nm for 180 s at 27°C. Enzyme activity was calculated by comparing reaction rates for the sample to the blank. All the reactions were performed in triplicate. IC_{50} values were determined with GraphPad Prism 5. Eserine (99%) was used as the reference AChE inhibitor.

Scavenging DPPH free radical assay: 850 μL of a methanolic solution of the test compounds or extract were added to 350 μL of a 50 μM DPPH solution in MeOH. Eight concentrations, ranging from 1 to 100 μM, were prepared for each sample and analyzed in triplicate. 350 μL of MeOH plus 850 μL of each compound solution were used for blank solutions. 350 μL of a 50 μM DPPH solution plus 850 μL of MeOH were used for negative control. The absorbance at 517 nm was determined after 30 min of incubation and the percentage of DPPH reduction was calculated taking into account the absorbance of the blank solutions and the negative control. BHT was used as reference compound under the same experimental conditions. Results are summarized in Table I.

Results and discussion

Roots and aerial parts of *Flaveria bidentis* were collected in Salitral de la Vidriera during flowering period. Fresh material was macerated in EtOH immediately after its collection. It was observed that the ethanolic extract showed potent AChE inhibitory activity (IC_{50}=0.12 mg/mL) and significant activity in DPPH assay (IC_{50}=60.8 μg/mL). An activity guided fractionation led us to the isolation, after partition with Hexane/H_2O, of the active compound **1** in good yield (4% from ethanolic extract). The analysis of complete ^1H and ^{13}C NMR (Table 1 and 2) and HRMS data of compound **1** allowed us to identify it as 6-methoxykaempferol-3-sulfate. As far as we know, flavonoid **1** has only been isolated previously from *F. chloraefolia* [7] and has been characterized just by UV spectroscopy. The present study is the first report of complete NMR and MS data of **1**.

Table 1: ^1H NMR data of compounds **1**-**5** in DMSO-d^6

Position	Compound				
	1[a]	**2**[a]	**3**[a]	**4**[b]	**5**[b]
2´and 6´	8.08 d (8.9)	8.04 d (12)	8.08 d (6)	8.18 d (18)	8.23 d (12)
3´and 5´	6.85 d (8.9)	6.92 d (12)	6.93 d (6)	7.02 d (18)	7.08 d (12)
8	6.53 s	6.54 s	6.88 s	7.12 s	6.94 s
6 -OCH_3	3.74 s	3.75 s	3.73 s	3.77 s	3.78 s
7 -OCH_3			3.91 s	3.94 s	
5 - OCH_3				3.85 s	
4´- OCH_3				3.81 s	
4´-C**H**$_2$$CH_2$Br					4.43 m
4´-CH_2C**H**$_2$Br					3.86 m
7 -C**H**$_2$$CH_2$Br					4.50 m
7 -CH_2C**H**$_2$Br					3.89 m

[a] Recorded at 600 MHz. [b] Recorded at 400 MHz

Acid hidrolysis of **1** afforded the desulfated analog **2**. The molecular weight of **2** was determined by HRMS to be 339.0481 $[M+Na]^+$, consistent with a molecular formula $C_{16}H_{12}O_7$. In addition, the structure was confirmed by ^1H and ^{13}C NMR. The upfield shift of C-2 signal in ^{13}C spectrum corresponds with a sulfate loss in that position.

In order to obtain compound **3** y **4**, 6-methoxykaempferol-3-sulphate (**1**) was treated with MeOH and an equivalent and an excess of $C_4H_{10}N_2Si$ respectively, in ethyl ether. ^1H and ^{13}C NMR analysis of **3** revealed a singlet for 3H at δ 3.91 and a carbon signal at δ 56.4 related to an additional methoxy group. The formation of **4** was supported by the presence of additional signals of methoxyl groups in the ^1H NMR spectrum at δ

3.81, 3.85 and 3.91. The presence of three additional methoxyl groups was also confirmed in the ^{13}C spectrum, by the carbon signals observed at δ 55.28, 56.40 and 61.87.

Compound **5** was obtained by reaction of compound **1** with 1,2-dibromoethane, K_2CO_3 and methanol in DMF. The formation of **5** was confirmed by mono and bidimensional ^{1}H and ^{13}C NMR. The HMBC correlations to C-7 and C-4´ allowed to place the bromoethoxy chains.

The upfield shift of C-2 signal in all the analogs corresponds with the sulfate group loss in C-3 under reaction conditions.

Table 2: ^{13}C NMR data of compounds **1**-**5** in DMSO-d^6

Position	Compound				
	1[a]	**2**[a]	**3**[a]	**4**[b]	**5**[b]
1´	121.29	121.74	121.69	123.26	123.73
2´and 6´	130.82	129.59	129.67	130.20	131.15
3´and 5´	115.15	115.49	115.54	113.47	114.68
4´	159.98	159.26	159.42	160.62	160.36
2	151.38	147.01	147.40	151.44	143.34
3	132.00	135.45	135.85	139.34	133.13
4	178.09	176.18	176.21	176.21	178.60
5	156.52	151.78	151.11	152.72	151.93
6	131.16	130.87	131.30	134.45	132.21
7	157.20	157.25	158.60	157.12	157.59
8	93.83	93.81	91.28	96.77	92.57
9	152.55	151.45	151.65	152.86	152.48
10	104.57	103.46	104.41	112.41	106.28
6 -OCH_3	60.06	60.05	60.16	61.02	60.69
7 -OCH_3			56.50	56.40	
5 - OCH_3				61.87	
4´- OCH_3				55.28	
4´-**C**$H_2$$CH_2$Br					68.38
4´-CH_2**C**H_2Br					31.52
7 -**C**$H_2$$CH_2$Br					69.28
7 -CH_2**C**H_2Br					31.79

[a] Recorded at 150 MHz. [b] Recorded at 100 MHz

Compounds **1**-**5** were evaluated for their AChE inhibitory activity and for their scavenging of DPPH free radical ability. Results are reported in Table 3. Regarding enzyme inhibitory activity, synthetized analogs resulted to be less active than isolated compound **1** at the same concentration. Compound **2** elicited the highest activity in the DPPH assay, while **4** and **5** resulted to be inactive at the assayed concentrations.

Table 3: AChE inhibitory activity and scavenging of DPPH free radical ability

Compound	AChE - PI % (0.2mM)	DPPH - IC_{50} (μM)
1	50	86.6
2	38.6	4.0
3	26.9	11.6
4	17.1	>100
5	36.3	>100

Conclusions

Our results indicate that the AChE inhibition observed for the ethanolic extract of *F. bidentis* is partially ascribable to the high content of the sulfated flavonoid 6-methoxykaempferol-3-sulfate (**1**). The lost of the 3-sulfate group led to weaker AChE Inhibitors (**2**-**5**) suggesting that the presence of this group is crucial for the interaction with the enzyme. On the other hand, the best antiradical activity was observed for compounds **2** and **3**, both with a free hydroxyl group at C-3.

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