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BACE inhibitory flavanones from *Balanophora involucrata* Hook. f.☆

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ABSTRACT

One new flavanone (S)-5,7,3′,5′-tetrahydroxy-flavanone-7-*O*-(6″-galloyl)-*β*-D-glucopyranose (**1**), together with one known flavanone and four known dihydrochalcones were isolated from the ethyl acetate partitions from an alcoholic extract of the whole plant of *Balanophora involucrata* Hook. f. All of the compounds were characterized by spectroscopic methods. Their *in vitro* BACE inhibitory effects were evaluated. Compounds **1**, **2**, **4**, and **5** were found to be a little more active than the positive control, although all of which are below 50% inhibition at 10 μM.

1. Introduction

The family *Balanophoraceae* consists of about 18 genera and 120 species, mostly distributed in tropical and subtropical countries. About 20 species have been found in China, and some of which are used as traditional medicines [1]. *Balanophora involucrata* Hook. f. is a parasitic plant growing on the roots of *Rhododendron* plants distributed in southern China. The whole plant has been used as an analgesic and anti-inflammatory agent in China. Previous chemical studies revealed the presence of triterpenes, phenylpropanoids, tannins [2], flavonoids and their glycosides [3,4] in this plant.

During the screening of BACE inhibitory plant extracts, we found that the ethyl acetate partitions of alcoholic extracts of the whole plant of *B. involucrata* showed significant activities in BACE1 (β-secretase) inhibition. Subsequent phytochemical investigation on this plant led to the isolation and identification of one new flavanone, one known flavanone, and four known dihydrochalcones.

Since professor Szent-Györgyi reported the isolation of vitamin P in the year 1936 [5], more than other 4000 flavonoids have been identified and studied. Flavonoids were reported as a wealth of BACE1 non-competitive inhibitors. Flavonoids with 5′,7′-dihydroxy on A ring, and two hydroxyl substitutions at C3′ and C4′ positions on C ring, exhibited the most potent BACE1 activity so far, with non-competitive mode [6]. A series of dihydroflavonoids with a lipophilic carbon chain has reported with BACE1 inhibitory activity [7].

2. Experimental

2.1. General

Melting points were determined with an XT5B Digital Display Micro-Melting point apparatus (Beijing Keyi Electro-optic Instrument Plant Co., Ltd, Beijing, China), uncorrected. Optical rotation was recorded in CH_3OH using a JASCO P-2000 digital polarimeter (Jasco Analytical Instruments, Easton, USA). UV spectra were measured on a JASCO V-650 UV–vis/NIR spectrophotometer instrument (Jasco Analytical Instruments, Easton, USA). IR spectra were recorded with a Nicolet-5700 FT-IR spectrophotometer (Thermo Fisher Scientific, MA, USA). ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and 2D NMR were recorded on a Bruker AV500-III NMR spectrometer (Bruker AXS Inc., Madison, USA) in acetone-d_6 with TMS as the internal standard. HR-ESI-MS was recorded on a Bruker APEX II mass

☆ We congratulate Prof. Atta-ur-Rahamn on his 70th year birthday.

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spectrometer (Bruker AXS Inc., Madison, USA). Silica gel (200–300 mesh) used for column chromatography and silica gel (GF254) for TLC were supplied by the Qingdao Marine Chemical Factory in China. TLC was detected at 254 nm, and spots were visualized by spraying with 10% H_2SO_4 in ethanol (v/v) followed by heating.

2.2. Plant material

The plant material was collected from Dali, Yunnan Province of P.R. China and was identified by Prof. Xiaokuang Ma, School of Medicine, Dali College, Yunnan Province, P.R. China. A voucher specimen (No. ID-S-2369) was deposited in the herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College.

2.3. Extraction and isolation

The air-dried whole plant of *B. involucrata* Hook. f. (2.0 kg) was powdered and extracted with 95% ethanol (3×5 L, 2 h each time). The combined EtOH extracts (336 g) were suspended in water and then partitioned successively with petroleum ether (m.p. 60–90 °C), EtOAc, and n-BuOH. The EtOAc partitions (56 g) were subjected to silica gel column chromatography using chloroform–methanol gradients (v/v, 98:2, 96:4, 94:6, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50) as eluent to give ten fractions (Fr. 1–10). Fr. 5 (3.5 g) was further chromatographed over a reversed phase ODS column, eluted with methanol/water (v/v, 10:90) to furnish compound **2** (100 mg). Fr. 6 (4.9 g) was also chromatographed over a reversed phase ODS column, eluted with methanol/water (v/v, 30:70) to furnish compounds **1** (110 mg), **4** (120 mg), and **5** (100 mg), while eluted with methanol/water (v/v, 40:60) to yield compound **3** (9 mg). Repeated chromatography of Fr. 9 (3.9 g) over ODS column (v/v, methanol/water 40:60) was followed by sephadex LH-20 to furnish compound **6** (40 mg). For purity determination, compounds **1**, **3**, **4**, and **5** were detected at 254 nm on TLC, and the spots were also visualized by spraying with 10% H_2SO_4 in ethanol (v/v) followed by heating.

(S)−5,7,3′,5′−tetrahydroxy−flavanone−7−*O*−(6″−galloyl)−β−D−glucopyranose (1)

White powder; mp 182–184 °C. $[\alpha]_D^{20}$ +22.3°(*c* 0.305, MeOH). UV (MeOH) λ_{max} (log ε): 215 (1000), 282 (567), and 334 (sh) nm. IR (KBr cm^{-1}) ν_{max}: 3385, 1696, 1635, 1580, 1528, 1446, 1344, 1242, 1073, and 857. CD (MeOH): $[\theta]_{229.5\ nm}$ -1.495×10^4, $[\theta]_{264.5\ nm}$ $+0.389\times10^4$, $[\theta]_{290\ nm}$ -2.225×10^4, $[\theta]_{340.5\ nm}$ $+0.370\times10^4$. HR-ESI-MS: revealed m/z: 603.1354 $[M+H]^+$, calcd. for $C_{28}H_{26}O_{15}$, 602.1272. ^1H and ^{13}C NMR: see Table 1.

2.4. Determination of BACE1 inhibitory activity

The ability of the compounds to inhibit BACE1 cleavage of APP was assessed using a fluorescent resonance energy transfer (FRET) peptide cleavage assay. The FRET peptide substrate is Mca-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-Lys(Dnp)-Arg-Arg-NH (where Mca is (7-methoxycoumarin-4-yl)acetyl, and Dnp is 2, 4-dinitrophenyl) and recombinant human BACE1 was purchased from R&D Systems, Inc. A commercially available BACE1 inhibitor N-benzyloxycarbonyl-Val-Leu-leucinal (Z-Val-Leu-Leu-CHO, CALBIOCHEM® 565749) was purchased from EMD Biosciences, Inc. and used as a positive control. The assay was carried out according to the supplier's protocol with modifications [8]. Briefly, assays were performed in triplicate in 96-well black plates with 100 μL of 50 mM sodium acetate buffer (pH 4.0), containing 4 μM substrate, 2 mg/mL recombinant human BACE1 and certain concentration of inhibitors (dissolved in small volumes of DMSO prior to addition to the buffer). The fluorescence intensity was measured using a SpectraMAX Gemini XS plate reader (Molecular Devices Co.) at Ex320/Em405 both at zero time and after 60 min incubation at 25 °C. The inhibition percentage was calculated with the following equation: Inhibition (%) = $[1-(F_S-F_{S0})/(F_C-F_{C0})]\times100\%$, where F_{S0} and F_S are the fluorescence of samples at zero time and 60 min, F_{C0} and F_C are the fluorescence of control at zero time and 60 min, respectively. All data presented are the mean values of triplicate experiments. The purities of samples were determined by HPLC, and compounds **1–6** and the positive control were found to have purities of more than 98%.

In a previous report [8], the chromone glycoside C-2′-decoumaroyl-aloeresin G has showed significant BACE inhibitory activity, which IC_{50} = 20.5 μM. In this article, we chose C-2′-decoumaroyl-aloeresin G as the positive control.

Table 1
^1H NMR and ^{13}C NMR spectral data for compound **1**.[a]

	^1H-NMR	^{13}C-NMR
2	5.41 (1H, ddd, *J* = 2.5, 4.5, 12.5 Hz)	80.1
3	3.17 (1H, ddd, *J* = 2.5, 3.0, 19.5 Hz) 2.74 (1H, td, *J* = 3.0, 17.0 Hz)	43.5
4		197.9
5		164.6
6	6.15 (1H, s)	97.3
7		166.4
8	6.15 (1H, s)	96.3
9		166.6
10		104.5
1′		131.3
2′, 6′	6.85 (2H, br s)	119.3, 119.4
3′, 5′		164.0, 164.1
4′	7.01 (1H, s)	114.7
1″	5.17 (1H, t, *J* = 8.0 Hz)	100.7
2″	3.52–3.61 (1H, m)	74.3
3″	3.52–3.61 (1H, m)	79.9
4″	3.52–3.61 (1H, m)	70.6
5″	3.94 (1H, m)	77.4
6″	4.61 (1H, dd, J_1 = 1.5, 12.0 Hz) 4.39 (1H, dd, *J* = 4.5, 12.0 Hz)	63.9
1‴		121.6
2‴, 6‴	7.12 (2H, d, *J* = 1.0 Hz)	109.9
3‴, 5‴		145.9
4‴		138.7
7‴		166.6

[a] In acetone-d_6, 500/125 MHz.

3. Results

Compound **1** (Fig. 1) was obtained as white powder, $[\alpha]_D^{20}$ +22.3° (*c* 0.305, MeOH). The HR-ESI-MS of compound **1** gave an $[M+H]^+$ peak at m/z 603.1354 and $[M+Na]^+$ peak at m/z 625.1178 (calcd. for $C_{28}H_{26}O_{15}$, 602.1272). The