MRC Letters

Magnetic Resonance in Chemistry

Received: 5 June 2011 Revised: 25 July 2011 Accepted: 1 August 2011 Published online in Wiley Online Library: 16 October 2011

(wileyonlinelibrary.com) DOI 10.1002/mrc.2803

Structure determination of baeckeins C and D from the roots of *Baeckea frutescens*

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Baeckeins C (compound 1) and D (compound 2), a pair of new diastereomeric biflavonoids, were isolated from the roots of *Baeckea frutescens*. Their structures were elucidated on the basis of spectroscopic analysis, including HR-ESI-MS and one- and two-dimensional NMR spectra (^{1}H and ^{13}C NMR, HSQC, HMBC, and ROESY). The absolute configurations of the 2,3-epoxide moiety for compounds 1 and 2 were determined by circular dichroism spectrometry combined with quantum chemical calculations. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: NMR; ^{1}H; ^{13}C; two-dimensional NMR; circular dichroism; biflavonoids; baeckein C; baeckein D; *Baeckea frutescens*; Myrtaceae

Introduction

Baeckea frutescens L. (Myrtaceae) is an aromatic low-growing shrub distributing from Southeast Asia to Australia. The roots of *B. frutescens*, known as "Pu-Lao-Zhong," have been used for treating rheumatism and snake bites in the southern part of China.[1] Many active constituents have been isolated from this species, including essential oil, some sesquiterpenes, phloroglucinols, chromones, flavones, and their derivatives.[2–10] In our search for structurally and pharmacologically interesting natural products, a phytochemical study of *B. frutescens* was undertaken. As a result, baeckeins C (compound **1**) and D (compound **2**), a pair of new diastereomeric biflavonoids with a 2,3-epoxide ring, were isolated from the roots of this plant (Fig. 1). Herein, their isolation and structural elucidation are presented. The one- and two-dimensional NMR techniques and HR-ESI-MS were extensively applied to characterize their structures and to establish the ^{1}H and ^{13}C resonance assignments. Furthermore, the absolute stereochemistry of the 2,3-epoxide moiety for compounds **1** and **2** were determined by circular dichroism (CD) spectrometry combined with quantum chemical calculations.

Results and Discussion

Baeckein C (compound **1**) was obtained as a yellow amorphous powder and gave positive results for the Mg–HCl reaction and Molish reagent, indicating it to be a flavonoid glycoside. The HR-ESI-MS exhibited a $[M–H]^-$ ion at *m/z* 791.1463 (calculated 791.1465), corresponding to the molecular formula $C_{38}H_{32}O_{19}$, with 23 degrees of unsaturation. The UV spectrum (λ_{max} = 275, 315, and 370 nm) suggested the presence of a conjugated system. The IR spectrum showed absorption bands for hydroxyl (3377 cm^{-1}) and carbonyl (1633 cm^{-1}) groups and aromatic functionalities (1599 and 1488 cm^{-1}, respectively).

The ^{13}C NMR spectra (Table 1) of compound **1** displayed a group of signals (δ_C = 101.6, 77.2, 75.9, 73.2, 69.8, and 60.7) belonging to a hexosyl unit and 32 carbon signals attributable to a biflavonoid structure, among which are 2 carbonyl groups (δ_C = 188.0 and 176.0), 2 aromatic methyl groups (δ_C = 6.9 and 7.3), 8 CH (δ_C = 122.3, 119.0, 117.1, 116.6, 115.8, 115.5, 95.7, and 92.7), and 20 quaternary carbons. The ^{1}H NMR spectrum (Table 1) of compound **1** showed signals for two sets of typical ABX coupling systems [δ_H 7.03 (1H, d, *J* = 8.7 Hz, H-2′), 7.19 (1H, d, *J* = 2.0 Hz, H-5′), and 7.10 (1H, dd, *J* = 8.7, 2.0 Hz, H-6′)] and [δ_H 7.28 (1H, d, *J* = 8.7 Hz, H-2*′), 7.82 (1H, d, *J* = 2.0 Hz, H-5*′), and 7.85 (1H, dd, *J* = 8.7, 2.0 Hz, H-6*′)], corresponding to the 3′,4′-dihydroxy-substituted B ring of flavonoids (rings B and B*). Also, two aromatic protons [δ_H 6.08 (1H, s, H-8) and 6.53 (1H, s, H-8*)] and two aromatic methyl signals [δ_H 1.88 (3H, s, Me-6) and 2.0 (3H, s, Me-6*)] were observed, revealing the presence of the 6-methyl-substituted A ring of quercetin (rings A and A*). In addition, a series of signals in the range of approximately δ_H 5.5–3.0 related to a sugar moiety were presented. Taken together, the ^{1}H and ^{13}C NMR data revealed that compound **1** was composed of two 6-C-methylquercetin[10,11] moieties and a hexosyl residue.

A comprehensive analysis of the one-dimensional NMR and HSQC spectra of compound **1** made the assignments of segment 1, a 6-C-methylquercetin unit, referring to rings A*, B*, and C*, which was confirmed by HMBC correlations (Fig. 2) from Me-6* to [δ_C = 157.6 (C-5*), 106.3 (C-6*), and 162.4 (C-7*)], from H-8* to [δ_C = 176.0 (C-4*), 106.3 (C-6*), 162.4 (C-7*), 154.0 (C-9*), and 102.8 (C-10*)], from H-2*′ to [δ_C = 44.8 (C-2*), 125.9 (C-1*′), 140.4 (C-3*′), and 122.3 (C-6*′)], from H-5*′ to [δ_C = 125.9 (C-1*′), 140.4 (C-3*′), and 141.5 (C-4*′)], and from H-6*′ to [δ_C = 144.8 (C-2*), 125.9 (C-1*′), 141.5 (C-4*′), and

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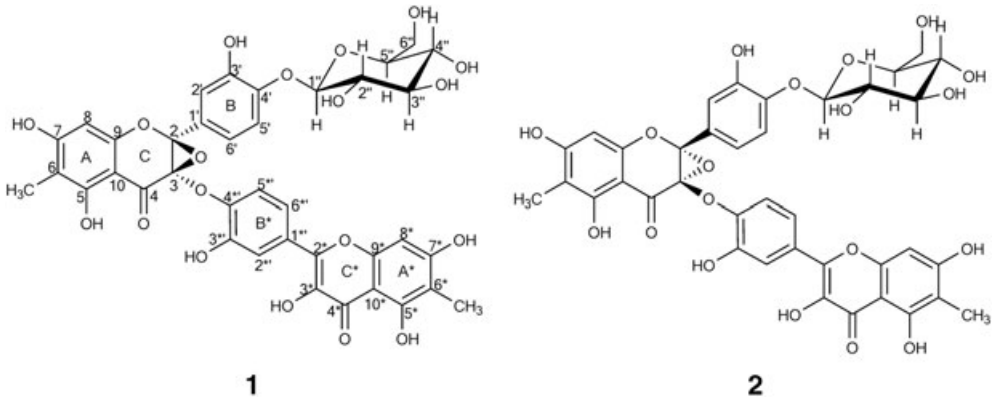

Figure 1. Structures of compounds **1** and **2**.

Table 1. ^{1}H and ^{13}C NMR data for compounds **1** and **2** (δ in ppm, J in Hz)

Position	Compound **1**		Compound **2**	
	δ_H	δ_C	δ_H	δ_C
2		100.0		99.8
3		90.6		90.5
4		188.0		186.7
5		160.5		160.3
6		105.1		105.1
CH_3-6	1.88 s	6.9	1.83 s	7.0
7		166.8		169.0
8	6.08 s	95.7	5.93 s	95.7
9		156.3		156.2
10		99.0		98.2
1′		128.1		128.4
2′	7.03 d (8.7)	119.0	7.03 d (8.4)	118.9
3′		146.1		146.0
4′		146.5		146.4
5′	7.19 d (2.0)	115.8	7.18 d (2.0)	115.7
6′	7.10 dd (8.7, 2.0)	115.5	7.08 dd (8.4, 2.0)	115.4
2*		144.8		144.8
3*		136.7		136.6
4*		176.0		176.0
5*		157.6		157.5
6*		106.3		106.2
CH_3-6*	2.00 s	7.3	2.00 s	7.3
7*		162.4		162.4
8*	6.53 s	92.7	6.53 s	92.6
9*		154.0		153.9
10*		102.8		102.7
1*′		125.9		125.8
2*′	7.28 d (8.7)	117.1	7.25 d (8.7)	117.0
3*′		140.4		140.4
4*′		141.5		141.5
5*′	7.82 d (2.0)	116.6	7.79 d (2.0)	116.5
6*′	7.85 dd (8.7, 2.0)	122.3	7.83 dd (8.7, 2.0)	122.2
Glc-1″	4.75 d (7.0)	101.6	4.73 d (6.9)	101.5
2″	3.28 m	73.2	3.27 m	73.2
3″	3.33 m	77.2	3.31 m	77.1
4″	3.17 m	69.8	3.14 m	69.7
5″	3.27 m	75.9	3.26 m	75.8
6″	3.47 dd (11.2, 5.0)	60.7	3.45 dd (11.1, 5.1)	60.6
	3.71 dd (11.3, 2.0)		3.69 dd (11.0, 2.0)	

^{1}H NMR was recorded at 500 MHz and ^{13}C at 125 MHz (in DMSO-d_6).

116.6 (C-5*′)]. Segment 1 was further identified by comparison with literature values of known compound 6-C-methylquercetin.[10,11]

Inspection of the signals of segment 2 revealed some structural characteristics of segment 1 and also suggested the presence of rings A and B. The major differences between segments 1 and 2 were in the ring C. Particularly, the signals assigned to two quaternary carbons [δ_C = 100.0 (C-2) and 90.6 (C-3)] and a carbonyl carbon [δ_C = 188.0 (C-4)] were observed, which indicated that the carbon skeleton of the ring C was converted into a dihydropyrone ring from a pyrone ring and new substituents were bound to C-2 and C-3 positions (both of them bearing oxygen atoms).[12] From these evidence, the presence of a 2,3-epoxide ring was proposed according to the molecular weight and the remaining degrees of unsaturation. Segment 2, including rings A, B, and C, was confirmed by HMBC correlations from Me-6 to [δ_C = 160.5 (C-5), 105.1 (C-6), and 166.8 (C-7)], from H-8 to [δ_C = 105.1 (C-6), 166.8 (C-7), 156.3 (C-9), and 99.0 (C-10)], from H-2′ to [δ_C = 100.0 (C-2), 146.5 (C-4′), and 115.5 (C-6′)], from H-5′ to [δ_C = 128.1 (C-1′), 146.1 (C-3′), and 146.4 (C-4′)], and from H-6′ to [δ_C = 128.1 (C-1′) and 115.8 (C-5′)].

A C–O–C linkage between segments 1 and 2 was deduced from the above spectral analysis. For segment 1, all ^{1}H and ^{13}C resonance assignments were established, and the carbon signals of C-3*′ (δ_C = 140.4) and C-4*′ (δ_C = 141.5) were shifted δ_C = 5.0–7.5 low frequency against those of 6-C-methylquercetin, suggesting one available binding position for segment 2. Elucidated in the same manner, the 2,3-epoxide ring provided another available position for a connection between two segments, which was supported by related literature values of known compound 1,3,11a-trihydroxy-9-(3,5,7-trihydroxy-trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-4-on-2-yl)-5a-(3,4-dihydroxyphenyl)-5,6,11-hexahydo-5,6,11-trioxanaphthacene-12-one.[13] A comparative evaluation of the chemical shifts of quaternary carbons C-2 (δ_C = 100.0) and C-3 (δ_C = 90.6) revealed that the C–O–C linkage should be bound to C-3, which was confirmed by an HMBC correlation from H-2′ to C-2. As a result, the (C-3)–O–(C-4*′) ether linkage was determined to be the only possible option for a linkage between segments 1 and 2 of compound **1**. Furthermore, the unusual 2,3-epoxide moiety and the (C-3)–O–(C-4*′) bond were verified by the NMR data presented by Cherviakovsky *et al.*[14] for the product named as product B.

The D-glucose was obtained from the acid hydrolysis experiment of compound **1** and was identified by thin layer chromatograph (TLC) and gas chromatograph (GC) analysis.[15,16] The large $^{3}J_{H\text{-}1,H\text{-}2}$ coupling constant of the anomeric proton [δ_H 4.75 (1H, d, J = 7.0 Hz, H-1″)] suggested a β-configuration for the glucose unit, and the location of the glucose residue should be at C-4′ (segment

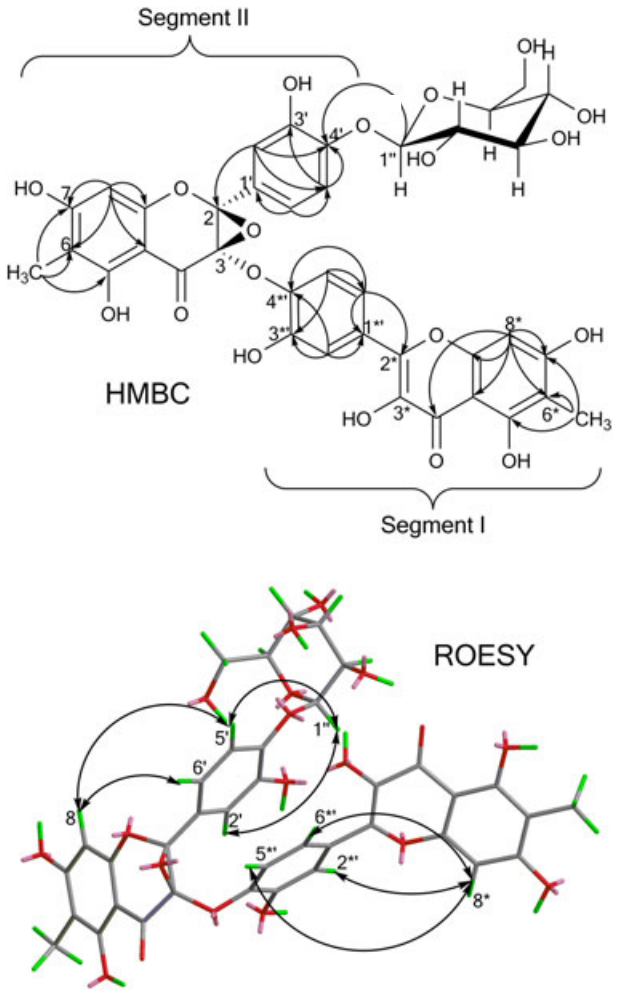

Figure 2. Key HMBC and ROESY correlations for compound **1**.

2), which was supported by the HMBC cross peak from H-1″ to C-4′ and the NOE correlations (Fig. 2) between H-1″ and aromatic protons of ring B (H-2′, H-5′, and H-6′). Moreover, the β-D-glucose moiety was further confirmed by the related data in the literature.[10]

The stereochemical assignment of epoxide rings in complex natural structures is not an easy task, especially when the oxiranes are tetrasubstituted.[17] The CD spectrum (Fig. 3A) of compound **1** showed positive Cotton effects at 307 and 378 nm and negative Cotton effects at 281 and 332 nm, but these spectral data (including NMR) were not sufficient enough to determine the absolute stereochemistry of the 2,3-epoxide moiety. In this case, quantum chemical CD calculations were used.[18] The geometry was built on a three-dimensional structure of compound **1** with configurations (2S and 3S). The conformational analysis was analyzed using the semiempirical PM3 method, as implemented in the Q-Chem program package, starting from preoptimized geometries generated by the MM2 force field in Chem three-dimensional software. The electronic circular dichrism (ECD) spectrum was computed in the gas phase using time-dependent density-functional theory (TD-DFT) at the B3LYP/6-31 G (d) level. The shapes of the computed CD (Fig. 3B) were in good agreement with those of the experimental CD spectrum, which allowed the assignment of the absolute configuration of compound **1** as depicted. On the basis of the results, the structure of baeckein C (compound **1**) was unambiguously established as (1aS,7aS)-4,6-dihydroxy-

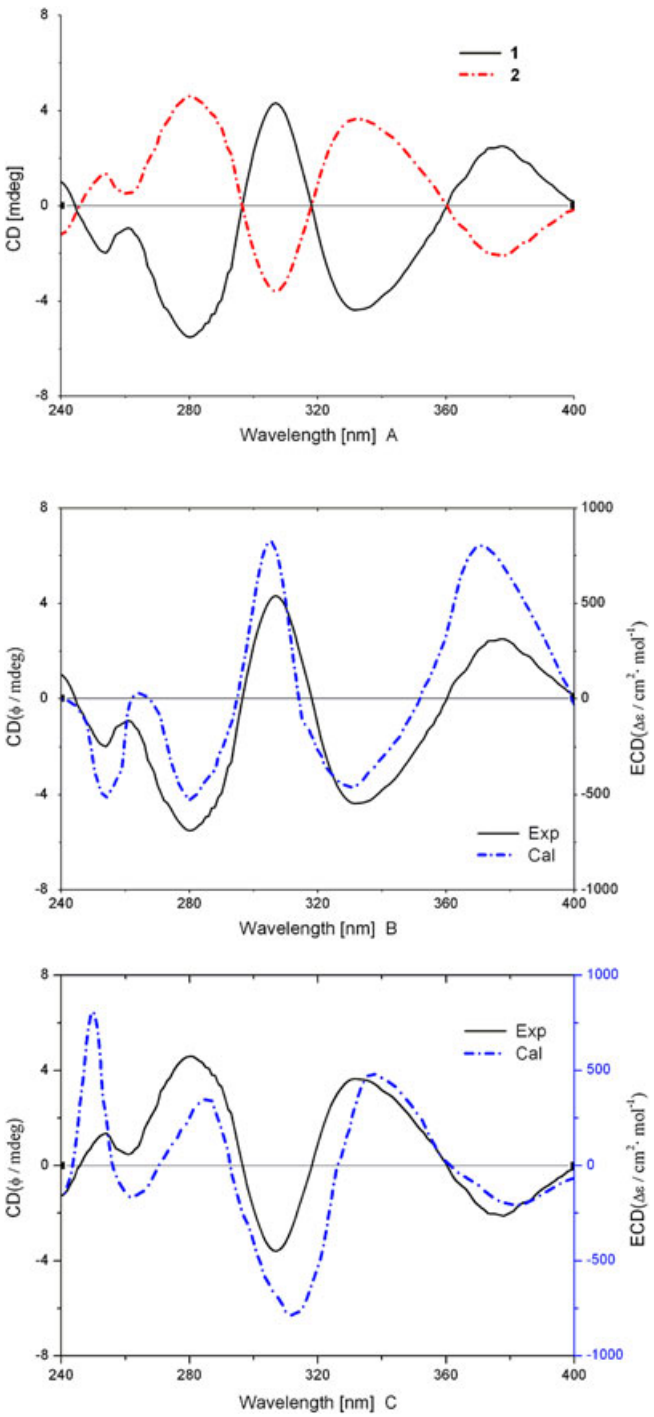

Figure 3. (A) Experimental CD spectra and (B, C) calculated CD spectra of compounds **1** and **2**.

1a-(3-hydroxy-4-((2R,3R,4S,5R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yloxy)phenyl)-7a-(2-hydroxy-4-(3,5,7-trihydroxy-6-methyl-4-oxo-4H-chromen-2-yl)phenoxy)-5-methyl-1aH-oxireno[2,3-b]chromen-7(7aH)-one.

The HR-ESI-MS and ^{13}C NMR analysis of baeckein D (compound **2**) indicated that it possessed the molecular formula of $C_{38}H_{32}O_{19}$, which was the same as that of compound **1**. The ^{1}H and ^{13}C NMR data (Table 1) as well as the two-dimensional correlations (Fig. 4) disclosed that both the skeleton and the functional groups presented in compound **2** were similar to those of compound **1**,

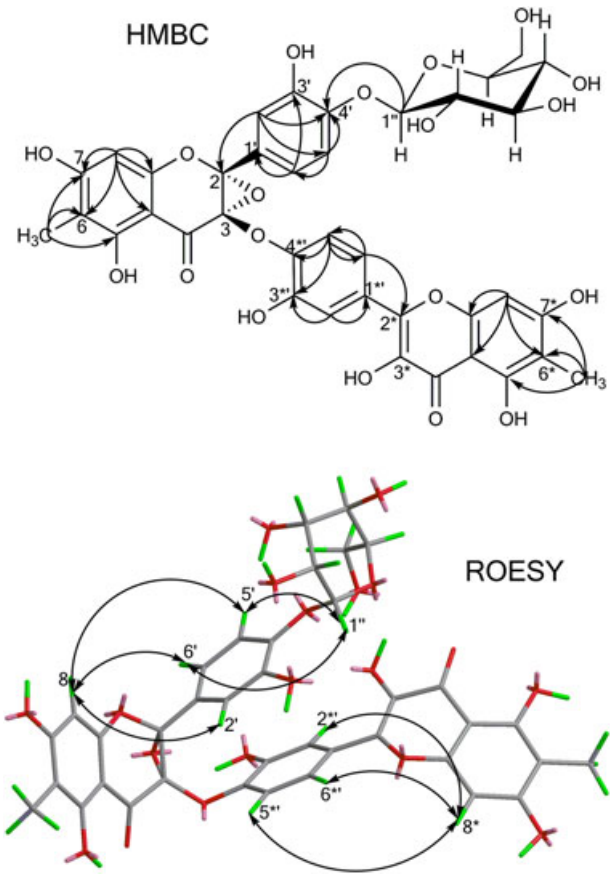

Figure 4. Key HMBC and ROESY correlations for compound **2**.

suggesting that compound **2** was a diastereomer of compound **1**. This deduction was verified by the CD spectrum of compound **2** (Fig. 3A), which displayed positive Cotton effects at 280 and 332 nm and negative Cotton effects at 307 and 377 nm, nearly a mirror image of the CD spectrum of compound **1**. Simultaneously, a TD-DFT calculation of the ECD data for compound **2** was also carried out following similar procedures that compound **1** used. In addition, the shapes of the computed CD and experimental CD of compound **2** are almost the same (Fig. 3C). Accordingly, the absolute configuration of the 2,3- epoxide ring for compound **2** was assigned as (2R and 3R), and the full assignments of ^{1}H and ^{13}C NMR data were achieved in combination with HMBC, HSQC, and ROESY experiments.

Experimental

General procedures

Column chromatography was carried out using silica gel (SiO_2; Qingdao Haiyang Chemical Industry, 200–300 mesh), ODS (40–63 μm, Fuji), and Sephadex LH-20 (20–100 μm, Pharmacia). Optical rotations were measured with a JASCO P-1020 polarimeter. UV and CD spectra were determined on a JASCO J-810 CD spectrometer. IR spectra (KBr discs) were recorded with Nicolet Impact-410 spectrometer. MS spectra were obtained on an Agilent LC/MSD TOF. GC analysis was carried out on a Agilent 6890 Plus gas chromatography using a DB-5 capillary column (30 m × 0.25 mm, i.d.); detection, FID; detector temperature, 280 °C; injection temperature, 250 °C; initial temperature of 180 °C was maintained for 5 min and then raised to 260 °C at the rate of 8 °C/min; carrier gas, He.

NMR spectra

NMR spectra were recorded at room temperature on a Bruker AV-500 spectrometer equipped with a QNP 5-mm probe. ^{1}H, ^{13}C NMR spectra (500 and 125 MHz, respectively), and two-dimensional NMR experiments (HSQC, HMBC, and ROESY) were recorded at 303 K using standard Bruker pulse programs. Approximately 3.0- to 5.0-mg samples were dissolved in 0.5 ml DMSO-d_6, which was used as the internal lock. The chemical shifts were given in δ (ppm) scale and referenced to residual solvent peaks of DMSO-d_6 at δ_H 2.50 for ^{1}H NMR and $\delta_C = 39.51$ for ^{13}C NMR. Coupling constants (J) were given in hertz.

For ^{1}H NMR, 8–16 transients were acquired using spectral widths of 7861 and 8620 Hz for compounds **1** and **2** and 90° pulses (14.80 μs at −1.00 dB). For ^{13}C NMR, 400–20 000 transients were acquired using spectral widths of 30303 Hz and 90° pulses (9.50 μs at 2.00 dB). Data points (32 K) were used for the processing with an exponential function (LB = 0.20 Hz for ^{1}H NMR, LB = 2.00 Hz for ^{13}C NMR).

The two-dimensional spectra used 1024 × 256 (HSQC), 1024 × 512 (HMBC), and 1024 × 128 (ROESY) data point matrices, which were zero filled to 2048 × 1024, 2048 × 1024, and 1024 × 1024, respectively. A relaxation delay of 2.0 s was used in all experiments. HSQC data were acquired with 16 transients per t_1 increment, and spectral widths of 12570 Hz/2750 Hz (compound **1**) and 27500 Hz/4000 Hz (compound **2**) were used for ^{13}C and ^{1}H, respectively. The HMBC experiment was performed with 32 transients per t_1 increment. Spectral widths of 12570 Hz/2750 Hz (compound **1**) and 30050 Hz/4000 Hz (compound **2**) were used for ^{13}C and ^{1}H, respectively. For ROESY, a mixing time of 800 ms and a spectral width of 4006 Hz for both dimensions were used, and the number of transients collected for each time increment in the indirect dimension was eight.

Plant material

The roots of *B. frutescens* were collected from Nanning, Guangxi Province, China, and identified by Q. Wang, China Pharmaceutical University. A voucher sample (No. GS001) is deposited in the Department of Chinese Materia Medica Analysis, China Pharmaceutical University, Nanjing, China.

Extraction and isolation

The dried roots of *B. frutescens* (10 kg) were extracted three times with 90% ethanol and concentrated in vacuum to give residue (600 g), which was suspended in water and partitioned with petroleum ether, ethyl acetate, and *n*-butanol successively. The ethyl acetate soluble part (200 g) was first subjected to chromatography over a silica gel column and eluted with gradient solvent $CHCl_3$/MeOH (1 : 0–1 : 1) to yield eight fractions on the basis of TLC analyses. Fraction 6 was then submitted to a Sephadex LH-20 column eluted with $CHCl_3$/MeOH (1 : 1) to afford six subfractions (F6.1–6.6), of which subfraction F6.3 was repeatedly chromatographed over ODS columns eluted with MeOH/H_2O (60 : 40) to yield compound **1** (20 mg) and compound **2** (5 mg).

*Baeckein C (compound **1**)*

Yellow amorphous powder, $[\alpha]_D^{30}$ −100° (c = 0.10, MeOH). UV (MeOH) nm λ_{max} (log ε): 272 (2.95), 313 (3.20), and 375 (2.80); IR (KBr) ν_{max}: 3377, 2921, 2354, 1633, 1599, 1488, 1435, 1274, 1198,

1160, 1075, 1010, 948, 896, 808, 731, and 623; CD ($c = 0.10$, MeOH): 281 nm ($\Delta\varepsilon$ -5.50), 307 nm ($\Delta\varepsilon$ +4.32), 332 nm ($\Delta\varepsilon$ -4.38), and 378 nm ($\Delta\varepsilon$ +2.51); ^{1}H- and ^{13}C-NMR data, see Table 1; ESI-MS *m/z* 791 $[M-H]^-$, HR-ESI-MS *m/z* 791.1463 $[M-H]^-$ (calculated for $C_{38}H_{31}O_{19}$, 791.1465).

*Baeckein D (compound **2**)*

Yellow amorphous powder, $[\alpha]_D^{30}$ -37.5° ($c = 0.11$, MeOH). UV (MeOH) nm λ_{max} (log ε): 272 (2.93), 312 (3.20), and 374 (2.81); IR (KBr) ν_{max}: 3408, 2919, 2352, 1633, 1599, 1504, 1435, 1273, 1199, 1145, 1076, 1011, 949, 895, 808, 732, and 624; CD ($c = 0.11$, MeOH): 280 nm ($\Delta\varepsilon$ +4.60), 307 nm ($\Delta\varepsilon$ -3.60), 332 nm ($\Delta\varepsilon$ +3.65), and 378 nm ($\Delta\varepsilon$ -2.11); ^{1}H- and ^{13}C-NMR data, see Table 1; ESI-MS *m/z* 791 $[M-H]^-$, HR-ESI-MS *m/z* 791.1463 $[M-H]^-$ (calculated for $C_{38}H_{31}O_{19}$, 791.1465).

Acid hydrolysis and GC analysis

Each compound (2 mg) was hydrolyzed with 10% HCl–dioxane (1 : 1, 5 ml) at 80 °C for 4 h. After extraction with ethyl acetate (5 ml × 3), the sugar component in the aqueous layer was evaporated under reduced pressure and then examined by silica gel TLC with $CHCl_3$–MeOH–H_2O (8 : 5 : 1) by comparison with the authentic sample.[15] Furthermore, the sugar residue was dissolved in 1 ml of dry pyridine, and 2 mg of L-cysteine methyl ester hydrochloride was added, followed by heating at 60 °C for 2 h. The reaction mixture was concentrated to dryness with N_2 gas, and then trimethylsilyl imidazole was added to the residue, followed by stirring at 60 °C for 1 h. Finally, the resulted solution was extracted with cyclohexane and H_2O, and the combined organic phase was analyzed by GC.[16] The standard D-glucose (Sigma, USA) was subjected to the same reaction and GC analysis under the same conditions.

Computational

The Q-Chem program package and the Chem three-dimensional software were used for calculations. Conformation searching was performed at MM2 molecular mechanics force field. All ground-state geometries were optimized at the B3LYP/6-31 G(d) level, and harmonic vibrational frequencies were calculated to confirm the minima. TD-DFT calculations for excitation energy and rotatory strength *R* were carried out at the same level in the gas phase. The ECD spectra were then simulated by overlapping Gaussian functions for each transition.

Acknowledgements

This work was supported by grants from a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors appreciate the kind help of Dr K. Zan (State Key Laboratory of Natural and Biomimetic Drugs, Peking University Health Science Center), Dr J. Luo (Department of Natural Medicinal Chemistry, China Pharmaceutical University), and Mr. L. Feng (Simcere Pharmaceutical Group) for providing assistance in technology and equipment.

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