ORGANIC LETTERS

2012
Vol. 14, No. 8
1954–1957

Incarvilleatone, a New Cyclohexylethanoid Dimer from *Incarvillea younghusbandii* and Its Inhibition against Nitric Oxide (NO) Release

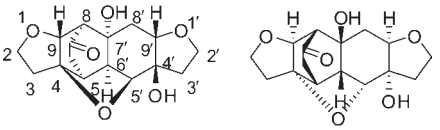
Yan-Ping Gao,[†,‡] Yun-Heng Shen,[*,†] Shou-De Zhang,[∥] Jun-Mian Tian,[†] Hua-Wu Zeng,[†] Ji Ye,[†] Hui-Liang Li,[†] Lei Shan,[†] and Wei-Dong Zhang[*,†,‡,§,∥]

Department of Phytochemistry, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, P. R. China, Department of Traditional Chinese Medicine, Chinese Pharmaceutical University, Nanjing 210009, P. R. China, King Saud University, Riyadh 11451, Saudi Arabia, and School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, P. R. China

wdzhangy@hotmail.com; shenyunheng9217018@yahoo.com.cn

Received December 5, 2011

ABSTRACT



(-)-**1** (+)-**1**

Incarvilleatone (1), an unprecedented dimeric cyclohexylethanoid analog with a racemic nature, was isolated from the whole plant of *Incarvillea younghusbandii*. HPLC chiral separation of 1 gave two enantiomers (−)-incarvilleatone and (+)-incarvilleatone. The structure of 1 was established by spectroscopic methods and single crystal X-ray diffraction. The absolute configurations of enantiomers were determined by quantum mechanical calculation. (−)-Incarvilleatone exhibited a potent inhibitory effect against NO production in LPS-induced RAW264.7 macrophages.

Incarvillea younghusbandii, one member of the genus *Incarvillea* (Bignoniaceae), is a perennial herb native to the Qinghai and Tibet provinces of China.[1] As a Chinese folk medicine, this plant is traditionnally used to treat dizziness and anemia and to stimulate lactation.[2,3] Previous investigations of *I. younghusbandii* have led to the isolation of coumarins,[4] volatile oil,[5] and phenolic glycosides.[6] In earlier efforts, we studied several *Incarvillea* species, including *I. delavayi*,[7] *I. mairei var. granditlora*,[8] and *I. arguta*.[9] As part of continuing efforts focused on *Incarvillea* species, we isolated an unusual racemic dimeric cyclohexylethanoid derivative, incarvilleatone (**1**), from the titled plant. The formation of the novel carbon skeleton of **1** appears to be biogenetically involved in an intramolecular Diels–Alder reaction between two linked cyclohexylethanoid monomeric units. Incarvilleatone (**1**) was further proved to be a natural product by detecting its existence in the freshly prepared 95% EtOH extract of *I. younghusbandii* using LC-MS analysis (see Supporting Information). HPLC separation of **1** on a chiral phase led to two individual enantiomers, (−)-incarvilleatone

[†] Second Military Medical University.
[‡] Chinese Pharmaceutical University.
[§] King Saud University.
[∥] Shanghai Jiao Tong University.

(1) Editor committee. *Flora of China*; Science Press: Beijing, China, 1990; Vol. 69, p 46.
(2) Ni, Z. C. *Economic Plants of Tibet*; Beijing Science and Technology Press: Beijing, China, 1990; p 606.
(3) Editor committee. *Chinese Herb*; Shanghai Science and Technology Publisher: Shanghai, China, 1999; Vol. 7, p 428.
(4) Fu, Y.; Bai, Y.; Dawa, Z. M.; Bai, B. R.; Ding, L. S. *Chin. J. Chin. Mater. Med.* **2010**, *35*, 58–62.
(5) Fu, Y.; Li, P. J.; Bai, Y.; Dawa, Z. M.; Bai, B. R.; Ding, L. S. *J. Instrum. Anal.* **2008**, *27*, 70–71.
(6) Pan, W. G.; Jiang, S. P.; Luo, P.; Gao, P.; Chen, B.; Bu, H. T. *Acta Pharm. Sin.* **2011**, *46*, 422–427.
(7) Lu, T.; Shen, Y. H.; Lu, M.; Tang, J.; Shan, L.; Liu, R. H.; Li, H. L.; Zhang, W. D. *Helv. Chim. Acta* **2009**, *92*, 768–773.
(8) Su, Y. Q.; Shen, Y. H.; Lin, S.; Tang, J.; Tian, J. M.; Liu, X. H.; Zhang, W. D. *Helv. Chim. Acta* **2009**, *92*, 165–170.
(9) Fu, J. J.; Jin, H. Z.; Shen, Y. H.; Zhang, W. D.; Xu, W. Z.; Zeng, Q.; Yang, S. K. *Helv. Chim. Acta* **2007**, *90*, 2151–2155.

10.1021/ol3004639
Published on Web 04/06/2012

((−)-**1**) and (+)-incarvilleatone ((+)-**1**). Below, we describe the isolation and structural elucidation of **1**, as well as its inhibition of NO production in LPS-induced RAW264.7 macrophages.

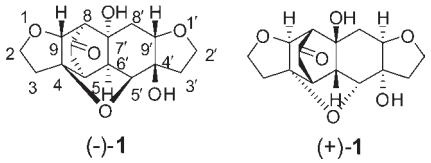


Figure 1. Structure of incarvilleatone (**1**).

Incarvilleatone (**1**)[10] (Figure 1), isolated as a colorless block, has a UV spectrum that contains maximum absorptions at 278, 280, and 282 nm. The IR spectrum of **1** exhibited two characteristic bands at 3344 (—OH) and 1733 (C═O) cm^{-1}. In addition, a pesuomolecular ion peak $[M+Na]^+$ is observed at *m*/*z* 331.1175 (calcd 331.1152) in the HRESIMS spectrum of this substance, corresponding to a molecular formula $C_{16}H_{20}O_6$ with seven degrees of unsaturation.

In the ^1H NMR spectrum of **1**, three oxygenated protons were observed at δ_H 4.49, 4.01, and 3.88, respectively (Table 1). The ^1H NMR spectrum also displayed two oxymethylene proton resonances at δ_H 4.01, 3.88, 4.07, and 4.03. Other signals occurred in a relatively high-field region, resonating from either a methine or methylene group. The ^{13}C and DEPT spectra revealed the presence of 16 carbon resonances, which were sorted into 6 methylenes, 6 methines, and 4 quarternary carbons. In addition, four pairs of carbon resonances with chemical shifts of δ_C 68.6 (t) and 65.7 (t), 32.3 (t) and 36.1 (t), 88.3 (s) and 79.7 (s), and 83.1 (d) and 79.9 (d) were observed pretty close to characteristic resonances of the respective C-2, C-3, C-4, and C-9 carbons of the known cyclohexylethanoid compound rengyolone.[11] On the basis of 1D and 2D NMR experiments, all proton and carbon resonances were divided into two sets of data (C-2−C-9, C-2′−C-9′). Each set of NMR data seemed to be due to a rengyolone derivative. Based on these data, we hypothesized incarvilleatone likely is a dimeric rengyolone derivative containing a polycyclic ring system.

More detailed information about the structure of incarvilleatone came from $^1H-^1H$ COSY and HMBC analyses. The $^1H-^1H$ COSY spectrum of **1** was observed to exhibit proton correlations (Figure 2) between two methylenes H_2-2/H_2-3, between H-5 (δ_H 2.83) and H_2-6, and between H-9 (δ_H 3.88) and H-8 (δ_H 2.92). Moreover, the HMBC

Table 1. ^1H and ^{13}C NMR Data (D_2O Containing 1% CD_3OD) for **1**[a]

no.	δ_H mult. (*J* in Hz)	δ_C	no.	δ_H mult. (*J* in Hz)	δ_C
2a	4.01 (overlap)	68.6	2′a	4.07 (ddd, 2.7, 9.5, 18.5)	65.7
2b	3.88 (overlap)		2′b	4.03 (dd, 7.4, 18.5)	
3a	2.35 (ddd, 3.4, 9.5, 14.4)	32.3	3′a	2.26 (dd, 9.5, 13.7)	36.1
3b	2.27 (dd, 8.0, 14.4)		3′b	2.03 (ddd 2.7, 7.4, 13.7)	
4		88.3	4′		79.7
5	2.83 (dt, 3.0, 4.4)	44.3	5′	4.49 (d, 4.4)	80.7
6a	2.65 (dd, 3.0, 20.3)	33.3	6′	2.58 (t, 4.4)	46.2
6b	2.39 (dd, 4.4, 20.3)		7′		72.5
7		213.9	8′a	2.27 (dd, 5.5, 14.7)	41.6
8	2.92 (d, 5.1)	59.5	8′b	1.85 (dd, 9.7, 14.7)	
9	3.88 (overlap)	83.1	9′	4.01 (overlap)	79.9

[a] All proton signals integrate to 1 proton, unless otherwise indicated.

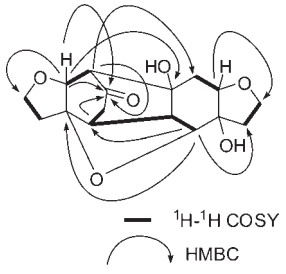


Figure 2. Key $^1H-^1H$ COSY and HMBC correlations of incarvilleatone (**1**).

spectrum of this substance displayed long-range correlations (Figure 2) from H-8, H-9, and H-5 to a carbonyl resonance at δ_C 213.9, attributing the carbonyl resonance to C-7. Also, H-8, H_2-2, and H_2-6 (δ_H 2.65, 2.39) were correlated with an oxygenated quarternary carbon at δ_C 88.3 in the HMBC spectrum. These findings suggested that one cyclohexylethanoid unit in **1** should have a 4-oxygenated, 7-oxo-cyclohexylethanoid structure.

Three structural fragments of **1**, corresponding to H_2-2′/H_2-3′, H-5′/H-6′, and H_2-8′/H-9′, were identified by using $^1H-^1H$ COSY correlation data. Two oxymethines and two oxygenated quarternary carbons were unambiguously assigned to C-5′, C-9′, C-4′, and C-7′, based on the observed HMBC correlations from H_2-3′, H-6′, and H-9′ (δ_H 4.01) to the oxymethine carbon at δ_C 80.7, from the oxygenated proton at δ_H 4.01 to C-2′, from H_2-2′, H-6′ (δ_H 2.58), and H_2-8′ (δ_H 2.27, 1.85) to the oxygen-bearing quarternary carbon at δ_C 79.7, and from H-5′ (δ_H 4.49), H-6′, and H_2-8′ to the oxygen-bearing quarternary carbon at δ_C 72.5. These data suggested that the second structural

(10) Incarvilleatone (**1**): $C_{16}H_{20}O_6$; colorless block; $[\alpha]^{20}_D$ −13.0 (*c* 0.30, MeOH), (−)-incarvilleatone; $[\alpha]^{20}_D$ +17.3 (*c* 0.30, MeOH), (+)-incarvilleatone; UV (MeOH) λ_{max} (log ε): 278 (1.59), 280 (1.55), 282 (1.57); for ^1H and ^{13}C NMR data, see Table 1; ESIMS (positive) *m*/*z* 331.1 $[M+Na]^+$; HRESIMS (positive) *m*/*z* 331.1175 $[M+Na]^+$ (calcd for $C_{16}H_{20}O_6Na$, 331.1152).

(11) Tian, J.; Zhao, Q. S.; Zhang, H. J.; Lin, Z. W.; Sun, H. D. *Chin. Chem. Lett.* **1997**, *8*, 129–132.

(12) CCDC 848831 contains the crystallographic data for incarvilleatone (**1**). These data can be obtained free of charge from The Cambridge Crystallographic Data Centra via www.ccdc.cam.ac.uk/data_request/cif.

(13) Ding, Y.; Li, X. C.; Ferreira, D. *J. Org. Chem.* **2007**, *72*, 4707–4715.

unit in **1** is a C-4′, C-5′, and C-7′ oxygen-substituted cyclohexylethanoid.

Finally, the presence of key HMBC correlations between H-9 and C-7′, between H-8 and C-8′, between H_2-8′ and C-8, between H-5 and C-5′, between H-5′ and C-5, and between H-6′ and C-4 indicated that the two cyclohexylethanoid units in **1** are connected by two C−C bonds (between C-8 and C-7′, and C-5 and C-6′) as part of an additional six-member ring. In addition, the HMBC correlation between H-5′ with C-4 carbon resonance at δ_C 88.3 suggested the presence of an epoxyl ring between C-5′ and C-4. The structure of **1** was thus established as shown in Figure 1 and named incarvilleatone.

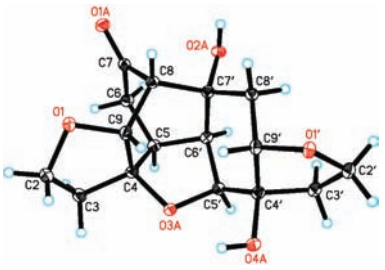


Figure 3. Single crystal X-ray diffraction of incarvilleatone (**1**).

In the NOESY spectrum of **1**, H-9 was observed to correlate with H-8 and H-9′, while H-5 correlates with H_2-6, H-6′, and H-5′. These findings led to the assignment of the relative configuration of **1**, which was verified by using single crystal X-ray diffraction (Figure 3).[12] However, the crystal of **1** is in the space group $P2_1/c$, which is indicative of its racemic nature. The deduction was also proved by circular dichroism (CD) and optical rotation. Subsequent HPLC separation of **1** on a chiral stationary phase led to isolation of the individual enantiomers (−)-**1** and (+)-**1**, which displayed opposite Cotton effects in their CD spectra (see Supporting Information) and opposite optical rotations. Unfortunately, we were not able to obtain single crystals of either enantiomer.

Recently, quantum mechanical computation of electronic circular dichroism (ECD) has been successfully used to determine the absolute configuration of natural products.[13,14] Thus, to determine the absolute configuration of the enantiomers of **1**, we compared the experimentally observed ECD spectrum of (−)-incarvilleatone ((−)-**1**) with a calculated spectrum obtained by using a time-dependent DFT method in Gaussian 03 software.[15] Because of the rigid structure of (−)-**1** and the fact that its relative configuration had been established by using NOESY experiment and single crystal X-ray diffraction, the arbitrarily assigned 4*R*, 5*S*, 8*S*, 9*R*, 4′*R*, 5′*S*, 6′*R*, 7′*R*, 9′*S* absolute configuration of (−)-**1** was geometrically optimized by using density functional theory (DFT) at the B3LYP/6-31G* level to afford a preferred conformer (Figure 4). The ECD spectra of (−)-incarvilleatone ((−)-**1**) was calculated at the B3LYP/6-31*// B3LYP/6-31* level in the gas phase and in the methanol solution with the COMSO model (for data, see Supporting Information). As depicted in Figure 5, the calculated ECD spectrum for the (4*R*, 5*S*, 8*S*, 9*R*, 4′*R*, 5′*S*, 6′*R*, 7′*R*, 9′*S*) enantiomer matched well with the experimentally determined spectrum of (−)-**1**.

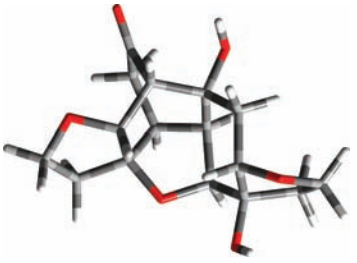


Figure 4. Optimized geometry of (−)-**1** at the B3LYP/6-31G* level in the gas phase.

Molecular orbital (MO) analysis (see Supporting Information) of (−)-incarvilleatone ((−)-**1**) at the B3LYP/6-31G*//B3LYP/6-31G* level with the COSMO model in MeOH yielded more information to better understand the ECD spectrum of (−)-**1**. The diagnostic, strongly negative CE at 280 nm is mainly contributed from the electronic transition from MO82 (HOMO) to MO83 (LUMO) in the carbonyl of (−)-**1**. Therefore, the absolute configuration of (−)-incarvilleatone ((−)-**1**) was assigned as 4*R*, 5*S*, 8*S*, 9*R*, 4′*R*, 5′*S*, 6′*R*, 7′*R*, 9′*S*, while the absolute configuration of (+)-incarvilleatone ((+)-**1**) was assigned as 4*S*, 5*R*, 8*R*, 9*S*, 4′*S*, 5′*R*, 6′*S*, 7′*S*, 9′*R*.

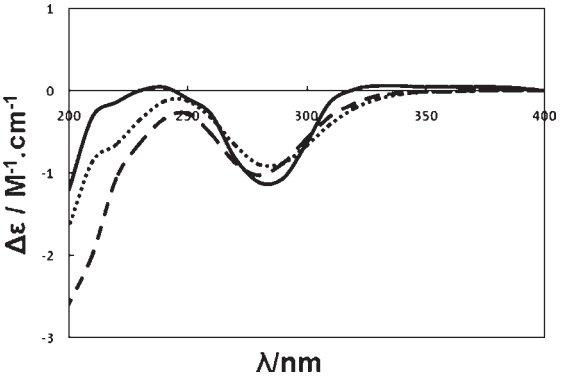


Figure 5. Comparison of the experimental and calculated ECD spectra of (−)-**1** (—) experimental in MeOH; (•••) at B3LYP/6-31G* level in the gas phase; (---) at B3LYP-SCRF/6-31G*//B3LYP/6-31G* level with the COSMO model in methanol solution).

(14) Stephens, P. J.; Pan, J. J.; Devlin, F. J. *J. Org. Chem.* **2007**, *72*, 2508–2524.

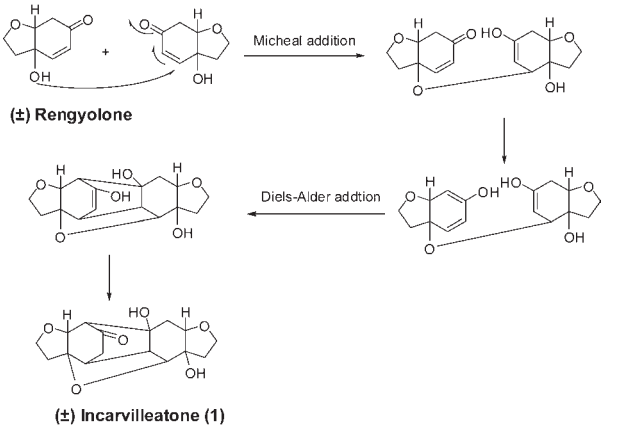
(15) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B. et al. *Gaussian 03*, revision D.01; Gaussian, Inc.: Wallingford, CT, 2004; see Supporting Information for full reference.

(16) Calixto, J. B.; Otuki, M. F.; Santos, A. R. *Planta Med.* **2003**, *69*, 973–983.

(17) Lee, H. J.; Kim, N. Y.; Jang, M. K.; Son, H. J.; Kim, K. M.; Sohn, D. H.; Lee, S. H.; Ryu, J. H. *Planta Med.* **1999**, *65*, 104–108.

Rengyolone is a common cyclohexylethanoid derivative coexisting in *I. younghusbandii* as a racemic mixture (for data see Supporting Information). A plausible biogenetic pathway (Scheme 1) based on the dimerization of two rengyolone monomers was proposed shown in Scheme 1. The key step of this pathway is a Micheal addition reaction between two rengyolones and subsequently a Diels–Alder reaction to form a six-membered ring between two rengyolone units.

Scheme 1. Proposed Biogenetic Pathway for Incarvilleatone (**1**)



Nitric oxide (NO) is known to play an important role in the inflammatory process,[16] and substances that inhibit NO release have a potential therapeutic effect in inflammatory diseases.[17] Therefore, two enantiomers (−)-incarvilleatone and (+)-incarvilleatone were tested for inhibition against NO production in LPS-induced RAW264.7 macrophages, respectively, as described previously.[18] The results (see Figure 6) exhibited that both (−)-incarvilleatone and (+)-incarvilleatone could remarkably inhibit NO release. Interestingly, (−)-incarvilleatone showed stronger inhibition than that of (+)-incarvilleatone, with 49.2%, 37.7%, and 22.4% of inhibition rates at 25.0, 12.5, and 6.3 μM, respectively.

(18) Diaz, A. M.; Abad, M. J.; Fernandez, L.; Silvan, A. M.; Santos, J. D.; Bermejo, P. *Life Sci.* **2004**, *74*, 2525–2526.

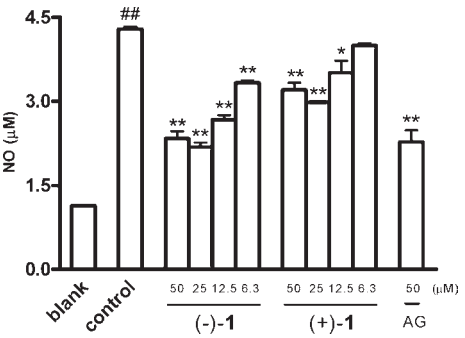


Figure 6. Inhibitory effects of two enantiomers (−)-**1** and (+)-**1** against nitric oxide (NO) production in LPS-induced RAW264.7 macrophages (Control: 1 μg/mL LPS; AG: aminoguanidine, as positive control).

In conclusion, incarvilleatone (**1**) is the first cyclohexylethanoid dimer connected by a six-membered ring, which yields a new carbon skeleton, and forms a spacial approximate cage-shaped structure. The structure of **1** affords not only a interesting synthetic target but also a potent inhibitor of nitric oxide release.

Acknowledgment. The work was supported by program NCET Foundation, NSFC(30725045), partially supported by Global Research Network for Medicinal Plants (GRNMP) and King Saud University, Shanghai Leading Academic Discipline Project (B906), FP7- PEOPLE-IRSES-2008 (TCMCANCER Project 230232), Shanghai Engineering Research Center for the Preparation of Bioactive Natural Products (10DZ2251300), and the Scientific Foundation of Shanghai China (09DZ1975700, 09DZ1971500, 10DZ1971700).

Supporting Information Available. Detailed isolation procedure, chiral separation, experimental ECD spetra, NMR spectra, crystallographic data of **1**, quantum mechanical ECD calculations, and NO release inhibition assay. This information is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.