Two New Curine-Type Bisbenzylisoquinoline Alkaloids from the Roots of *Cyclea wattii* with Cytotoxic Activities

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Received February 6, 2010; accepted April 13, 2010; published online April 16, 2010

Two new curine-type bisbenzylisoquinoline alkaloids, wattisines A (1) and B (2) along with three known alkaloids were isolated from the roots of *Cyclea wattii*. Their structures were established by interpretation of NMR and high-resolution electrospray ionization (ESI)-MS data. Absolute configuration of wattisines A and B were determined by single-crystal X-ray diffraction and circular dichroism (CD) spectra, respectively. *In vitro*, wattisine A (1) showed significant cytotoxic activities with IC_{50} value of 1.74 μM against HCT-8, and 7.29 μM against Bel-7402.

Key words *Cyclea wattii*; bisbenzylisoquinoline alkaloid; wattisine A; wattisine B; cytotoxic activity

The bisbenzylisoquinoline (BBI) alkaloids are dimers built up of two benzylisoquinoline units linked by one or more ether bridges or direct bonding. They constitute an important group of isoquinoline alkaloids because of their favourable biological activities, including cytotoxicity and antiplasmodial activity.[1)] Among over 430 of these dimers, about ten percent are head-to-tail bisbenzylisoquinoline alkaloids.[2,3)] *Cyclea wattii* DIELS (Menispermaceae) was grown in the southwest of China. The dried roots of this plant have been used in the fork medicine for treatment of gastroenteritis and toothache. In our chemical investigation on this plant, two new head-to-tail bisbenzylisoquinoline alkaloids, wattisine A (**1**) and wattisine B (**2**), together with three known alkaloids curine,[4,5)] steponine,[6)] and α-cyclanoline[6)] were isolated. Herein, we report the isolation and structural elucidation of wattisines A and B, as well as their biological activities.

Results and Discussion

Wattisine A (**1**) was isolated as colorless needles, whose molecular formula was deduced to be $C_{36}H_{38}N_2O_6$ from the HR-electrospray ionization (ESI)-MS at *m*/*z* 595.2806 $[M+H]^+$. Its IR spectrum suggested the presence of hydroxyl (3517 cm^{-1}), aromatic rings (1608, 1507 cm^{-1}), and ether bonds (1218, 1254 cm^{-1}).

The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) data of compound **1** (Table 1) indicated the existence of fourteen quaternary, twelve methine, six methylene, and four methyl carbons. Twelve of these signals could be characterized as non-aromatic carbons, four of which represent two *N*-methyl (δ_C 42.7; δ_H 2.28, s; δ_C 42.4; δ_H 2.52, s) and two aromatic methoxyl groups (δ_C 60.9; δ_H 3.74, s; δ_C 55.8; δ_H 3.86, s). According to the NMR data shown in Table 1, coupled with the biogenetic consideration, it can be concluded that two tetrahydrobenzylisoquinoline units presented in compound **1**. Of the 19 unsaturation degrees, two tetrahydrobenzylisoquinolines moieties accounted for 18, while the remaining one degree of unsaturation can be contributed to an internal macrocycle by formation of two bridges. The ESI-MS peak at *m*/*z* 298, formed due to the facile double benzylic cleavage, indicated that compound **1** should be a head-to-tail type bisbenzylisoquinoline alkaloid.[7)] The coupling patterns in the ^{1}H-NMR data suggested the signals for one *p*-disubstituted aromatic ring (δ_H 6.60, 1H, dd, J=7.6, 2.0 Hz; δ_H 6.88, 1H, dd, J=7.6, 2.4 Hz; δ_H 6.75, 1H, dd, J=8.0, 2.4 Hz; δ_H 7.15, 1H, dd, J=8.0, 2.0 Hz) and a 1,2,4-trisubstituted benzene (δ_H 6.37, 1H, d, J=1.6 Hz; δ_H 6.80, 1H, d, J=8.0 Hz; δ_H 6.86, 1H, dd, J=8.0, 1.6 Hz). The other three isolated aromatic protons (δ_H 5.87, 6.61, 6.65, each 1H, s) could be assigned to the two tetrahydroisoquinoline moieties.

The methine carbon signals at δ_C 59.3 and 64.7 were attributed to C-1 and C-1′, which implied that C-8 was oxygenated and a hydrogen atom was attached to C-8′. As a result of the γ-gauche effect of oxygen on C-8, the δ value of C-1 was shifted upfield.[8)] The methoxyl group at δ_C 55.8 was connected to C-6′, while the corresponding signal (δ_C 60.9) was assigned as 7-OCH_3, which was sterically hindered and shifted downfield.[9)] The heteronuclear multiple bond correlation (HMBC) data (Fig. 1) exhibited long-range correlations from H-1 to NCH_3, C-4a, C-8a, C-3, and C-8, from H-8′ to C-1′, C-6′, C-7′, and C-4a′, as well as from 7-OCH_3 to C-7 and from 6′-OCH_3 to C-6′. Comparison of the NMR data of **1** with those of the known compound chondrocurine[8)] (**3**) revealed they were structurally similar except for the substituents on ring B. All of these evidences confirmed the planar structure of **1**.

After carefully checking the 2D NMR data including the nuclear Overhauser effect spectroscopy (NOESY) spectrum (Fig. 1), it was still not sufficient to establish the ether linkage and the stereochemistry of compound **1**. Therefore, a single X-ray crystallographic analysis was conducted, which not only verified the planar structure but also clarified the stereochemistry of compound **1** as shown in Fig. 2. The crystal of wattisine A (**1**) was used on diffractometer with a mirror Cu$K\alpha$ (λ=1.54184 Å) radiation (ω scans, $2\theta_{max}$=140.12°). The absolute configuration was determined by anomalous dispersion effects, Flack x=0.04 (12). The configuration of C-1 and C-1′ was deduced as *S* and *R*, respectively.

Compound **2** was obtained as a white powder. The molecular formula was determined as $C_{35}H_{36}N_2O_6$ by the positive HR-ESI-MS at *m*/*z* 581.2654 $[M+H]^+$. Its IR spectrum showed the presence of hydroxyl groups (3439 cm^{-1}), aromatic systems (1605, 1505 cm^{-1}), and ethers (1219, 1273 cm^{-1}). The NMR features of compound **2** (Table 1)

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Table 1. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR Data of **1** and **2** (δ ppm, J in Hz)

No.	**1**[a)]		**2**[b)]		**3**[a)]
	δ_C	δ_H	δ_C	δ_H	δ_C
1	59.3 d	4.02 br d (9.2)	59.4 d	4.09 br d (9.9)	59.3
3	44.2 t	3.38 m, 2.90 m	43.8 t	3.44 m, 2.75 m	44.2
4	23.3 t	2.84 m, 2.52 m	22.7 t	2.90 m, 2.64 m	23.2
4a	130.6 s		129.6 s		124.6
5	111.4 d	6.61 s	112.3 d	6.65 s	107.7
6	147.6 s		148.4 s		146.7
7	137.9 s		138.5 s		137.2
8	143.4 s		143.2 s		137.9
8a	125.0 s		124.0 s		125.4
α	40.2 t	2.91, 2.55 (hidden)	39.9 t	2.75, 2.60 (hidden)	40.1
9	132.8 s		131.8 s		132.6
10	121.3 d	6.37 d (1.6)	121.9 d	6.43 d (1.6)	121
11	143.2 s		142.7 s		143.1
12	145.9 s		147.2 s		145.9
13	115.3 d	6.80 d (8.0)	117.0 d	6.82 d (8.0)	115.4
14	125.9 d	6.86 dd (8.0, 1.6)	126.2 d	6.87 dd (8.0, 1.6)	125.7
2-NMe	42.7 q	2.28 s	42.2 q	2.33 s	42.5
6-OMe					56
7-OMe	60.9 q	3.74 s	60.6 q	3.79 s	
1′	64.7 d	3.54 dd (10.4, 3.6)	64.7 d	3.52 dd (10.4, 3.2)	64.4
3′	46.6 t	3.33 m, 2.80 m	45.7 t	3.35 m, 2.75 m	46.5
4′	25.3 t	2.94 m, 2.76 m	24.5 t	2.65 m, 2.55 m	25.2
4a′	129.5 s		127.6 s		128.6
5′	111.7 d	6.65 s	117.0 d	6.73 s	111.9
6′	148.2 s		145.9 s		148
7′	143.2 s		143.1 s		143.1
8′	118.4 d	5.87 s	115.0 d	5.73 s	117.9
8a′	128.7 s		126.1 s		128.1
α'	39.3 t	3.26 dd (12.8, 3.6), 2.80 (hidden)	38.4 t	3.08 dd (12.8, 3.6), 2.68 (hidden)	39
9′	132.2 s		131.4 s		131.7
10′	132.5 d	6.60 dd (7.6, 2.0)	132.6 d	6.54 dd (8.4, 2.0)	132.2
11′	115.1 d	6.88 dd (7.6, 2.4)	115.6 d	6.96 dd (8.4, 2.4)	113.2
12′	155.2 s		155.3 s		155.4
13′	113.2 d	6.75 dd (8.0, 2.4)	112.8 d	6.76 dd (8.4, 2.4)	114.9
14′	130.1 d	7.15 dd (8.0, 2.0)	129.9 d	7.17 dd (8.4, 2.0)	129.9
2′-NMe	42.4 q	2.52 s	42.0 s	2.51 s	42.4
6′-OMe	55.8 q	3.86 s			55.8

a) $CDCl_3$. *b*) $CDCl_3$–CD_3OD.

suggested that it had the same skeleton as **1**, with the difference in the C-6′ substitute of ring B′. A hydroxyl was attached to C-6′ in **2** other than a methoxy, which was confirmed by 14 mass units lost in the MS data of **2** compared to that of compound **1**. The circular dichroism (CD) spectrum of **2** was closely similar to that of **1**, which leading to conclusion that the former has the same 1-*S*, 1′-*R* configuration with wattisine A. Finally, the structure of compound **2** was established by careful analyses of the 1D-NMR and 2D-NMR (^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and HMBC) spectra, and was thus given the trivial named wattisine B.

The two new alkaloids (**1**, **2**) were evaluated for their cytotoxic activities aginst HCT-8, Bel-7402, and A2780 cancer cell lines *in vitro* by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 2). Wattisine A (**1**) exhibited significant cytotoxic activities with IC_{50} value of 1.74 μM against HCT-8, 7.29 μM against Bel-7402, and 7.13 μM against A2708.

Experimental

General Experimental Procedures Melting points were determined on a micro melting point apparatus (Kexing-X4) without correction. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were measured on a PuXi Tu 1800 PC spectropolarimeter. IR spectra were obtained on a Nicolet FT-IR 200 SXV spectrophotometer. CD spectra were obtained with a JASCO J-810 spectropolarimeter. NMR spectra were recorded on a Varian Unity INOVA 400/45 NMR spectrometer. Mass spectra were carried out on Waters Q-time-of-flight (TOF)-Premier spectrometers. Elemental analysis was conducted with a Carlo Erba 1106 analyzer. X-Ray analysis was used for measurements on an Oxford Diffraction Gemini S Ultra CCD diffractometer with a mirror Cu$K\alpha$ (λ=1.54184 Å) radiation at room temperature. Silica gel H (Qindao Sea Chemical Factory, China) was used for column chromatography. Spots on TLC plates (silica gel G, Qindao Sea Chemical Factory) were visualized with modified Dragendorff's reagent.

Plant Material *Cyclea wattii* Diels was collected from Gulin, Sichuan Province, China, in June, 2008 and authenticated by Professor Guang-Hua Lu of Chengdu University of Traditional Chinese Medicine. A voucher specimen (No. 20080816) was deposited in the West China College of Pharmacy, Sichuan University.

Extraction and Isolation The powdered dried roots (4.8 kg) of *C. wattii* were extracted with 95% ethanol at room temperature three times. After removal of the solvent under reduced pressure, the crude extract (305 g) was dissolved in 1.0 l of H_2O to give a suspension and adjusted with 2 N hydrochloric acid to pH 3. The acidic mixture was defatted with EtOAc (600 ml×2), and the aqueous phase was basified with 10% aqueous ammonium hydroxide in water to pH 10, extracted with $CHCl_3$ (600 ml×3) to afford 19.0 g of alkaloids (E1), and then extracted with 600 ml $CHCl_3$–CH_3OH (8 : 2) to give 3.0 g of alkaloids (E2). E1 were subjected to a silica gel column eluted with $CHCl_3$/CH_3OH/Et_2NH (99 : 1 : 0.5 to 20 : 10 : 0.5) to give four major fractions (F1—F4). Fraction 2 (7.0 g) was chromatographed on a silica gel column eluted with EtOAc/CH_3OH/Et_2NH (95 : 5 : 0.5→50 : 50 :

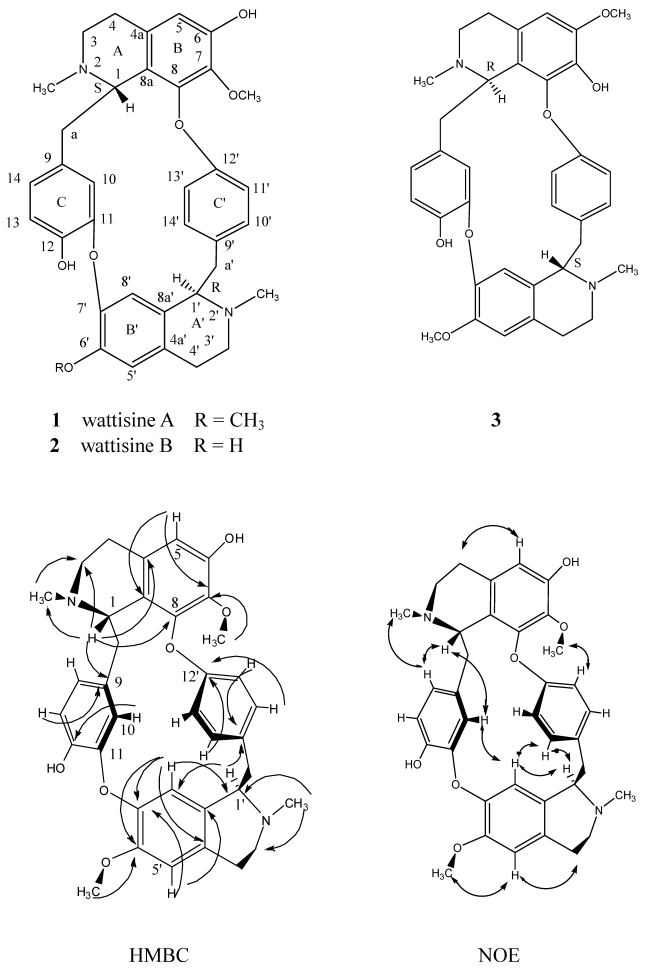

Fig. 1. The Key HMBC and NOE Correlations of Wattisine A (**1**)

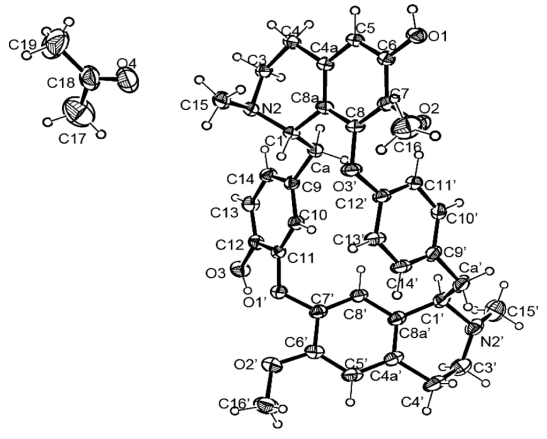

Fig. 2. The ORTEP Drawing of Wattisine A (**1**)

0.5) to afford six subfractions (A—F). Fraction B (1.8 g) was further chromatographed on a silica gel column eluted with chloroform–methanol (95 : 5→8 : 2) to give wattisine A (**1**, 260 mg) and curine (170 mg). Fraction C (1.1 g) was further chromatographed on a silica gel column eluted with chloroform–methanol (9 : 1→6 : 4) to give wattisine B (**2**, 33 mg). E2 were subjected to a silica gel column eluted with CH_3OH/H_2O (99 : 1→8 : 2) to give steponine (53 mg) and α-cyclanoline (47 mg).

Wattisine A (**1**): Colorless needles ($CHCl_3$–Me_2CO), mp 214—216 °C. $[\alpha]_D^{20}$ −168 (c=0.64, MeOH). UV (MeOH) λ_{max} (log ε) nm: 212 (4.29), 225 (4.23), 280 (3.85), CD (MeOH) λ_{max} ($\Delta\varepsilon$) nm: 212 (−202), 232 (−30.4), 258 (−2.55), 289 (−8.87), 307 (−0.802). IR (KBr) ν_{max} cm^{-1}: 3517, 2937, 1608, 1507, 1440, 1254, 1218, 1116, 1016, 812, 753. ^{1}H-NMR (400 MHz, $CDCl_3$), see Table 1. ^{13}C-NMR (100 MHz, $CDCl_3$), see Table 1. ESI-MS m/z: 595 $[M+H]^+$, 298, 297, 190; HR-ESI-MS m/z: 595.2806 $[M+H]^+$ (Calcd for $C_{36}H_{39}N_2O_6$, 595.2808). *Anal.* Calcd for $C_{36}H_{38}N_2O_6$: C, 72.71; H, 6.44; N, 4.71. Found: C, 72.82; H, 6.44; N, 4.69. X-Ray crystallographic data: $C_{39}H_{44}O_7N_2$, monoclinic, space group $P2_1$, a=9.85860(10) Å, b=14.7828(2) Å, c=12.43800(10) Å, V=1755.78(3) Å^3, Z=2, d=1.235 g/cm^3, crystal dimensions 0.38×0.36×0.32 mm was used for measurements on an Oxford Diffraction Gemini S Ultra CCD diffractometer with a mirror Cu$K\alpha$ (λ=1.54184 Å) radiation at room temperature (ω scans, $2\theta_{max}$= 140.12°). The total number of independent reflections measured was 6207, of which 6046 were observed ($|F|^2 \geq 2\sigma|F|^2$). Final indices ($|F|^2 \geq 2\sigma|F|^2$): R_1=0.0315, wR_2=0.0848, S=1.061, $(\Delta/\sigma)_{max}$=0.029, $(\Delta\rho)_{min}$=−0.149 e/Å^3, $(\Delta\rho)_{max}$=0.234 e/Å^3. Flack x=0.04(12). Crystallographic data for compound **1** have been deposited in the Cambridge Crystallographic Data Centre (deposition no.: CCDC 759825).

Wattisine B (**2**): White amorphous powder, mp 231—234 °C. $[\alpha]_D^{20}$ −564 (c=0.15, EtOH). UV (MeOH) λ_{max} (log ε) nm: 212 (4.23), 223 (4.19), 277 (3.98). CD (MeOH) λ_{max} ($\Delta\varepsilon$) nm: 212 (−74.1), 232 (−20.1), 258 (−1.32), 275 (−0.615), 290 (−4.03), 307 (−0.515). IR (KBr) ν_{max} cm^{-1}: 3439, 2923, 1605, 1505, 1441, 1365, 1273, 1219, 1114, 1045, 1008, 836. ^{1}H-NMR (400 MHz, $CDCl_3$–CD_3OD), see Table 1. ^{13}C-NMR (100 MHz, $CDCl_3$–CD_3OD), see Table 1. ESI-MS m/z: 581 $[M+H]^+$, 297, 296, 284, 190, 177. HR-ESI-MS m/z: 581.2654 $[M+H]^+$ (Calcd for $C_{35}H_{37}N_2O_6$, 581.2652).

Table 2. Cytotoxicity of Wattisine A (**1**) and Wattisine B (**2**) against Cultured HCT-8, Bel-7402 and A2780 Cancer Cell Lines

Compounds	Growth inhibition constant (IC_{50}) [μM]		
	HCT-8	Bel-7402	A2780
1	1.74	7.29	7.13
2	>10	>10	>10

Antitumoral Cytotoxic Bioassays The cytotoxic activities of the compounds were evaluated *in vitro* by measuring cell viability by the MTT assay. The HCT-8, Bel-7402, BGC-823, A549, A2780 cells were maintained in RPMI-1640 medium supplied with 5% fetal bovine serum. Cell in logarithmic phase were cultured at a density of 50000 cells/ml per well in a 24-well plate. The cells were exposed to various concentrations of the test compounds for 72 h, and each compound was tested in triplicate at every concentration. The methylene blue dye assay was used to evaluate the effects of the tested compounds on cell growth. The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with the control.

Acknowledgment We are grateful to Prof. Xiao-Guang Chen from the Chinese Academy of Medical Sciences and Peking Union Medical College for the bioactivity screenings. We also thank Dr. Shi-Ming Zhou in University of Science and Technology of China in Hefei for the X-ray crystallographic analysis of **1**.

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