ORGANIC LETTERS

2012
Vol. 14, No. 10
2614–2617

Lycojaponicumins A−C, Three Alkaloids with an Unprecedented Skeleton from *Lycopodium japonicum*

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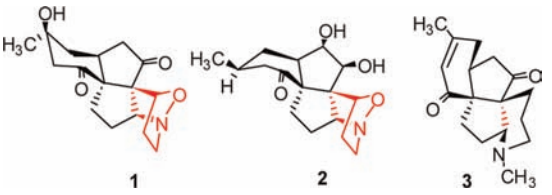
Received April 12, 2012

ABSTRACT



Lycojaponicumins A−C (1−3), three trace alkaloids isolated from *Lycopodium japonicum*, represent a unique heterocyclic skeleton formed by the new linkage C4−C9. Notably, lycojaponicumins A and B (1 and 2) are the first examples of natural products possessing a 5/5/5/5/6 pentacyclic ring system with a 1-*aza*-7-oxabicyclo[2.2.1]heptane moiety. These structures were elucidated by spectroscopic methods and X-ray diffraction analysis. A plausible biogenetic pathway was proposed.

The *Lycopodium* alkaloids are quinolizine or pyridine and α-pyridone alkaloids from plants of the genus *Lycopodium*,[1] which are well-known for their unique heterocyclic structures. Many of them continue to be of interest from a biogenetic and biological point of view, as well as providing challenging targets for total synthesis.[2]

Research on *Lycopodium* alkaloids has been active in the past decade, and nearly 300 alkaloids have been discovered so far.[1,3] However, among over 500 species in the genus *Lycopodium* (family Lycopodiaceae), the alkaloid content has been studied in just about 50 species.[1,3]

Lycopodium japonicum THUNB. ex Murray, a common club moss widely distributed in the southern parts of China, was historically used as a traditional Chinese medicine for the treatment of contusion, strains, and myasthenia.[4] Its chemical constituents have been widely investigated, and a large number of compounds such as triterpenoids, flavones, and anthraquinones have been isolated and reported.[5] However, only few *Lycopodium*

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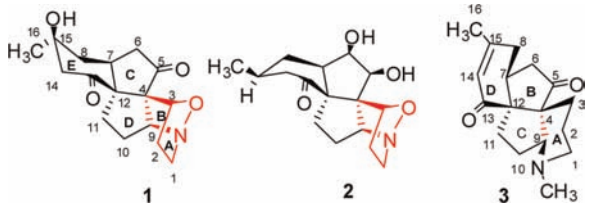
10.1021/ol3009478 © 2012 American Chemical Society
Published on Web 05/08/2012

alkaloids have been reported up to now because of the low content (0.1%−0.3%) of alkaloids in this plant.[3a,6] In our present research, three novel alkaloids in trace amounts, lycojaponicumins A and B (**1** and **2**) with a unique 5/5/5/5/6 pentacyclic ring system featured by two fused tetrahydroisoxazole rings, and lycojaponicumin C (**3**) possessing a 6/5/5/6 tetracyclic skeketon, were isolated from the alkaloidal extract of the whole plant (100 kg). It is interesting to note that the structures of **1**−**3** were remarkable for their unprecedented skeleton formed by new C−C bond linkage, since they never existed in *Lycopodium* alkaloids.[1,3] Furthermore, the nitrogen atom in compounds **1** and **2** was attached to C-3 through an oxygen atom to form a 1-*aza*-7-oxabicyclo[2.2.1]heptane moiety, which was first reported in natural products. Herein, we report the structure elucidation, plausible biogenetic pathway, and biological evaluation of **1**−**3**.



Lycojaponicumin A (**1**) was obtained as colorless prisms (mp 223−225 °C). Its molecular formula, $C_{16}H_{21}NO_4$, was evidenced from the $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ ions at *m*/*z* 292.1545 (calcd 292.1543), 314.1362 (calcd 314.1363), and 330.1099 (calcd 330.1102) respectively in HRESIMS analysis, requiring seven degrees of unsaturation. The IR spectrum showed absorptions for hydroxyl and carbonyl groups respectively at 3371, 1736, and 1697 cm^{-1}. The ^{13}C NMR and DEPT spectra of **1** (see Figures S3 and S4 in the Supporting Information (SI)) revealed 16 carbon signals due to two keto carbonyl signals (δ_C 215.9 and 210.6), three sp^3 quaternary carbon signals (δ_C 77.5, 75.0, and 66.6), three sp^3 methine signals (δ_C 88.0, 78.9, and 41.3), seven methylene signals, and one methyl signal (δ_C 32.2). All these functional groups accounted for two degrees of unsaturation, thus requiring five rings (rings A−E) in the structure, one ring more than the common tetracyclic ring system of *Lycopodium* alkaloids.[1,3]

The $^1H-^1H$ COSY and HSQC spectra (see Figures S5 and S7 in the SI) revealed the presence of the three following spin systems: $C(1)H_2-C(2)H_2-C(3)H$, $C(6)H_2-C(7)H-C(8)H_2$, and $C(9)H-C(10)H_2-C(11)H_2$ as shown by bold lines in Figure 1. The cross peaks of H_2-1/C-9 and

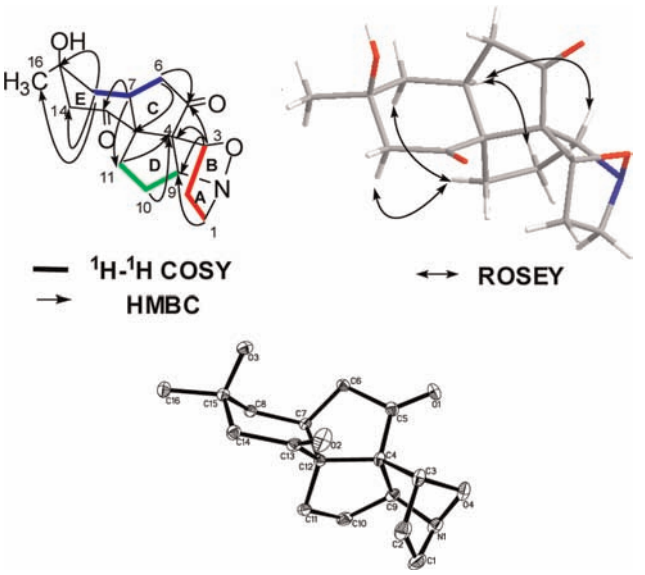


Figure 1. 2D NMR correlations and X-ray structure of **1**.

H-9/C-1 in the HMBC spectrum (see Figure S6 in the SI) established the connection of C-1 and C-9 through a nitrogen atom. A cyclopentanone ring (ring C) and a cyclohexanone ring (ring E) were validated by the fragment $C(6)H_2-C(7)H-C(8)H_2$ and the HMBC correlations from H-3 to C-4 and C-5, from H_2-6 to C-5 and C-12, from H_2-8 to C-12, from H-7 to C-11 and C-13, from H-11a to C-4, and from H_2-8 to C-14, C-15, and C-16. The new carbon linkage C4−C9 was deduced from the cross-peaks of H-10b/C-4 and H-3/C-9. At the same time, a cyclopentane ring (ring D) was constructed based on the C4−C9 connection, fragment $C(9)H-C(10)H_2-C(11)H_2$, and the above HMBC correlations. Then, to fulfill the unsaturation degrees and MS analysis, the nitrogen atom was attached to C-3 (δ_C 88.0) through an oxygen atom to form two fused tetrahydroisoxazole rings (rings A and B). Thereby, compound **1** likely possessed an unprecedented skeleton consisting of two tetrahydroisoxazole rings (C-1/C-2/C-3/O/N, C-3/C-4/C-9/N/O, rings A and B), a cyclopentanone ring (C-4/C-5/C-6/C-7/C-12, ring C), a cyclopentane ring (C-9/C-10/C-11/C-12/C-4, ring D), and a cyclohexanone ring substituted with methyl and hydroxyl groups at C-15 (C-7/C-8/C-15/C-14/C-13/C-12, ring E), and its planar structure was further validated by X-ray diffraction analysis as shown in Figure 1.[7]

The stereochemistry of **1** was elucidated by ROESY correlations (see Figure S8 in the SI) and X-ray crystallographic analysis (Figure 1). The ROESY correlations of H-7/H-9, H-7/H-10a, H-11a/H-14a, and H-8a revealed the *cis*-fused relationships of C/D and C/E rings, leading to the assignment of the relative stereochemistry of C-4, C-7, C-9,

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(7) CCDC 848526 (**1**), CCDC 848527 (**2**), and CCDC 848528 (**3**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of **1** and **2** (δ in ppm, J in Hz)

	1 (C_5D_5N)		**2** (C_5D_5N)	
no.	δ_H	δ_C	δ_H	δ_C
1a	3.06, ddd (12.0, 8.0, 4.5)	48.7	2.97[a]	48.4
1b	2.98, ddd (12.0, 12.0, 5.0)		2.97[a]	
2a	2.01[a]	30.9	2.13, m	31.8
2b	1.78, m		2.09, m	
3	5.59, d (6.0)	88.0	5.95, d (5.0)	85.4
4		75.0		71.9
5		215.9	4.38[a]	80.9
6a	3.47, dd (18.0, 14.5)	43.6	4.38[a]	71.3
6b	2.40, m			
7	2.55, m	41.3	2.43, m	52.8
8a	2.14, dd (14.5, 6.0)	34.7	1.95[a]	33.7
8b	2.02[a]		1.58, ddd (13.0, 13.0, 13.0)	
9	4.09, d (8.5)	78.9	3.72, d (8.0)	77.7
10a	2.10, m	24.3	1.85, m	23.6
10b	1.82, m		1.67, dd (14.5, 6.0)	
11a	2.38, m	34.0	1.95[a]	39.7
11b	2.04, m		1.95[a]	
12		66.6		63.7
13		210.6		212.0
14a	2.90, d (11.5)	53.7	2.74, ddd (16.5, 9.0, 1.5)	46.9
14b	2.61, dd (11.5, 2.0)		1.95[a]	
15		77.5	2.04, m	29.0
16	1.44, 3H, s	32.2	0.86, 3H, d (6.5)	23.3
5-OH			6.53, br s	
6-OH			5.59, d (6.5)	

[a] Overlapping signals.

and C-12. However, the ROESY spectrum could not provide sufficient information to determine the relative stereochemistry of C-3 and C-15. The X-ray diffraction analysis not only revealed the unique 5/5/5/5/6-pentacyclic ring system as deduced above but also established the absolute configuration of **1** to be 3*R*, 4*S*, 7*S*, 9*S*, 12*S*, 15*S* in light of the Flack parameter of 0.05(16), using anomalous dispersion with copper radiation.[7]

Lycojaponicumin B (**2**), also obtained as colorless prisms (mp 175−177 °C), has the molecular formula $C_{16}H_{23}NO_4$ deduced by HRESIMS for the $[M + H]^+$ and $[M + Na]^+$ ions at m/z 294.1703 (calcd 294.1700) and 316.1522 (calcd 316.1519) respectively, indicating six degrees of unsaturation. The IR absorptions at 3460, 3319, and 1700 cm^{-1} implied the presence of hydroxyl and carbonyl groups. Comparison of ^{1}H and ^{13}C NMR data (Table 1) of **2** with those of **1** suggested that the structure of **2** was similar to that of **1**. However, only one keto carbonyl signal (δ_C 212.0) was displayed, and the signal for C-15 upshifted to δ_C 29.0 in **2** (δ_C 77.5 in **1**) in the ^{13}C NMR spectrum (see Figure S13 in the SI). Meanwhile, a doublet of the methyl group (C-16) at δ_H 0.86 (d, 6.5 Hz) in the ^{1}H NMR spectrum of **2** (see Figure S12 in the SI) was shown

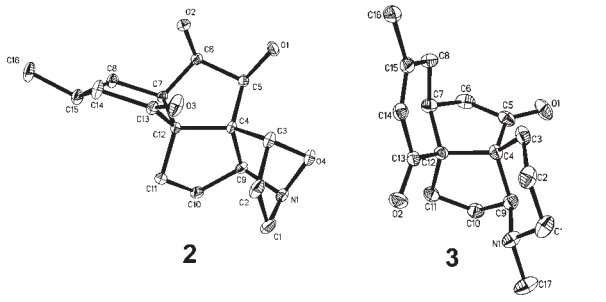


Figure 2. X-ray structures of **2** and **3**.

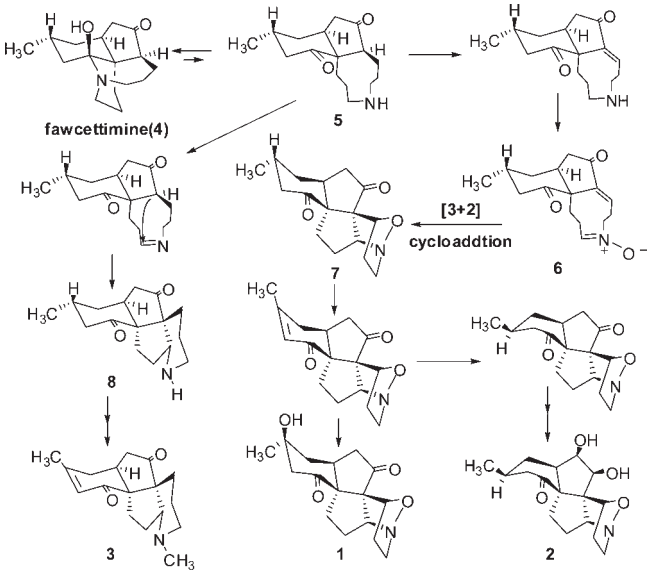
instead of a singlet at δ_H 1.44 in that of **1**. These results indicated the hydroxyl group at C-15 in **1** was replaced by one hydrogen atom in **2**. By comprehensive analysis of HRMS, ^{1}H NMR, ^{13}C NMR, and 2D NMR of **2**, the same skeleton of the 5/5/5/5/6-pentacyclic ring system was established as that of **1**. The major differences between these two compounds were that the location of the hydroxyl group was changed from C-15 to C-6 and the carbonyl group at C-5 was reduced to a hydroxyl group to form a 1,2-diol moiety in **2**.

The ROESY spectrum in C_5D_5N (see Figure S18 in the SI) could not provide sufficient information to elucidate the relative stereochemistry, because of the overlapping of some key proton signals in the ^{1}H NMR spectrum. Fortunately, a ^{1}H NMR spectrum with adequate resolution could be obtained when measured in CD_3OD (see Table S1 and Figure S19 in the SI), and difference NOE experiments in CD_3OD were carried out to solve this problem (see Figure S23 in the SI). Strong NOEs of H-5/H-6, H-6/H-7 indicated that H-5, H-6, and H-7 were on the same side of ring C. NOEs were also observed between H-5, H-6, and H-9, between H-7 and H-11a, H-15, revealing the same orientation of these protons. Thus, the relative configurations of C-4, C-5, C-6, C-7, C-9, C-12, and C-15 could be deduced from the above-mentioned NOE results.

In order to assign the absolute configuration, the suitable single crystal was obtained for X-ray diffraction, the result of which established the stereochemistry, including the *cis*-configuration of the 1,2-diol and the boat conformation of the cyclohexanone ring (C-7/C-8/C-15/C-14/C-13/C-12), as well as the absolute configuration of each chiral center (3*R*, 4*S*, 5*S*, 6*R*, 7*R*, 9*S*, 12*S*, 15*S*) (Figure 2).[7]

The HRESIMS of lycojaponicumin C (**3**) showed a pseudomolecular ion peak at m/z 274.1809 $[M + H]^+$, corresponding to a molecular formula of $C_{17}H_{23}NO_2$ (calcd for $C_{17}H_{24}NO_2$, 274.1802) with seven degrees of unsaturation. The ^{13}C NMR and DEPT spectra (see Table S1, Figures S28 and S29 in the SI) revealed 17 carbon signals ascribed to a keto carbonyl group (δ_C 219.6), a conjugated carbonyl group (δ_C 199.3), a trisubstituted double bond (δ_C 157.8 and 127.7), two sp^3 quaternary carbons (δ_C 62.8 and 60.1), two sp^3 methines (δ_C 71.0 and 39.2), seven methylenes, and two methyls (δ_C 43.8 and 24.1) including a *N*-methyl (δ_C 43.8). According to the

Scheme 1. Plausible Biogenetic Pathway of **1**–**3**



above data, a tetracyclic system was inferred to fulfill the unsaturation index. Elucidation of 2D NMR spectra (see Figures S30–S32 in the SI) validated our assumption and established the fused-tetracyclic ring system of **3**, which was composed of a 1-methylpiperidine ring (C-1/C-2/C-3/C-4/C-9/N(CH_3), ring A), a cyclopentanone ring (C-4/C-5/C-6/C-7/C-12, ring B), a cyclopentane ring (C-9/C-10/C-11/C-12/C-4, ring C), and an α,β-unsaturated cyclohexanone ring with a methyl group substituted at C-15 (C-7/C-12/C-13/C-14/C-15/C-8, ring D). A single crystal X-ray diffraction analysis was carried out to further confirm the planar structure and demonstrate the absolute configuration to be 4*S*, 7*S*, 9*S*, 12*S* as shown in Figure 2.[7]

The unique biogenetic origin of **1**–**3** can plausibly be traced back to fawcettimine (**4**),[8] a *Lycopodium* alkaloid also isolated during our study, which might exist in either an aminoacetal form or an amino ketone form (Scheme 1). The amino ketone form (**5**) underwent dehydrogenation and oxidation to form the nitrone **6**, which was followed by intramolecular 1,3-dipolar cycloaddition to produce the pentacyclic intermediate **7**. Compounds **1** and **2** might be generated from a series of dehydrogenation, addition, reduction, and oxidation of **7**, respectively. Oxidation of **5** accompanied by a subsequent attack of the α-carbonyl carbon atom (C-4) to the electron-deficient imine carbon (C-9) will accomplish the intramolecular cyclization and then form the tetracyclic structure. The intemediate **8** underwent *N*-methylation and dehydrogenation to give **3**.

Biological testing *in vitro* showed that lycojaponicumins A–C (**1**–**3**) inhibited lipopolysaccharide (LPS)-induced pro-inflammatory factors in BV2 macrophages with IC_{50} = 43.61 ± 12.79, 61.98 ± 5.98, 64.97 ± 14.09 μM, respectively (curcumin as the positive control, IC_{50} = 3.12 ± 0.56 μM).[9] However, these three compounds were inactive (IC_{50} > 200 μM) against acetylcholinsterase using the improved Ellman's method (tacrine as the positive control, $IC_{50} = 2.18 \times 10^{-7}$ M).[10]

In conclusion, three novel *Lycopodium* alkaloids, lycojaponicumins A–C (**1**–**3**), were discovered from the traditional Chinese medicine Shenjincao (*Lycopodium japonicum* THUNB.). Their structures possess an unusual connection between C-4 and C-9. Moreover, lycojaponicumins A and B (**1** and **2**), which might be the products of intramolecular 1,3-dipolar cycloaddition, are the first examples of natural products possessing a 1-*aza*-7-oxabicyclo[2.2.1]heptane moiety. These findings prompt us to pay more attention to trace components in the chemical research of medicinal plants.

Acknowledgment. The work was supported by grants from the Natural Science Foundation of China (No. 21132009) and National Science and Technology Project of China (No. 2012ZX09301002-002). The authors are grateful to the Department of Instrumental Analysis of our institute for the measurements of UV, IR, NMR, MS spectra and X-ray measurement and analysis.

Supporting Information Available. Experimental procedures, 1D and 2D NMR, MS, UV, IR spectra for compounds **1**–**3**, and X-ray crystallographic data of **1**–**3** in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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