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Annotinolides A−C, Three Lycopodane-Derived 8,5-Lactones with Polycyclic Skeletons from *Lycopodium annotinum*

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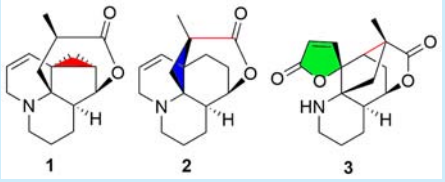
Supporting Information

ABSTRACT: Three novel 7,8-*seco*-lycopodane-derived 8,5-lactones (annotinolides A−C, **1**−**3**) were isolated from *Lycopodium annotinum*. Their structures were elucidated by spectroscopic methods and single-crystal X-ray diffraction. Compound **1** possesses an unusual cyclopropane ring constructed through a hitherto unknown C-6/C-12 bond. Compound **2** represents the first 7,8-*seco*-lycopodane-derived alkaloid with a rare cyclobutane ring formed by a new C-12/C-15 linkage, while the C-8/C-15 bond remains. Compound **3** contains an unprecedented 12-spiro-9,12-γ-lactone moiety. Their plausible biosynthetic pathways and antiaggregation effects on amyloid-β_{1-42} are also presented.



The club mosses in the Lycopodiaceae family have been well-documented to be rich in *Lycopodium* alkaloids with intriguing carbon skeletons, including the four major types of fawcetimine, lycopodine, lycodine, and phlegmarine.[1] This class of alkaloids has attracted broad interest from chemists and pharmacologists worldwide.[2] Club moss (*Lycopodium annotinum* Linn) samples of European, Canadian, and Japanese origins have been extensively studied, and lycopodine-related alkaloids were found to be the main secondary metabolites.[1,3] Although this perennial herb is easily found in the colder, higher elevation forests in Northwestern and Southwestern China,[4,5] no alkaloids have been isolated from *L. annotinum* samples native to China. In our continuing research toward the discovery of bioactive *Lycopodium* alkaloids,[6] three novel 7,8-*seco*-lycopodane-derived 8,5-lactones, annotinolides A−C (**1**−**3**) (Figure 1), were

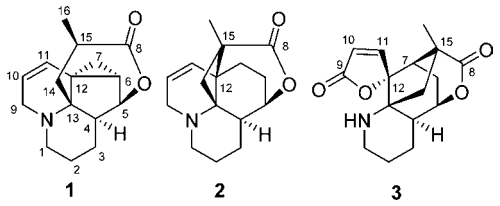


Figure 1. Chemical structures of compounds **1**−**3**.

obtained from the title plant collected from a plateau of the Taibai Mountains in the Qinling Area, Shaanxi of China. Their structures were characterized by extensive spectroscopic data and X-ray crystallography. We herein present the isolation, structural elucidation, and plausible biosynthetic pathways of **1**−**3** as well as their inhibitory effects against the amyloid-β $(A\beta)_{1-42}$ peptide aggregation.

The 90% MeOH extract of the dried whole plant (10 kg) of *L. annotinum* was partitioned between EtOAc and 3% aq tartaric acid. The water-soluble layer, adjusted to pH 9 with $NH_3{\cdot}H_2O$, was then extracted with $CHCl_3$. After concentration, the alkaloid-containing $CHCl_3$ fraction was subjected to column chromatography over silica gel, Sephadex LH-20, and semipreparative HPLC to afford alkaloids **1** (0.8 mg, 0.000008% yield), **2** (1.5 mg, 0.000015% yield), and **3** (1.6 mg, 0.000016% yield) (Figure 1).

Annotinolide A (**1**), obtained as colorless crystals from MeOH, has a molecular formula $C_{16}H_{21}NO_2$ as established by a quasi-molecular ion at m/z 260.1647 ($[M+H]^+$, calcd 260.1645) in its HRESIMS and its ^{13}C NMR data (Table 1), implying seven degrees of unsaturation. The IR absorption band at 1723 cm^{-1} gave hints of the presence of an ester carbonyl group. The ^{13}C NMR data of **1** (Table 1), with the aid of an HSQC NMR experiment (see Supporting Information), showed a total of 16 carbon resonances comprising one ester carbonyl at δ 176.8, one methyl, six methylenes, six methines (inter alia, one oxymethine at δ 80.5 and two olefinic at δ 127.6 and 126.6), and two quaternary carbons. In the ^1H NMR spectrum of **1**, signals attributable to one secondary methyl group at δ 1.21 (d, J = 6.6 Hz, Me-16), one oxymethine at δ 4.54 (br d, J = 4.8 Hz, H-5), and two olefinic protons at δ 5.75 (H-10) and 5.81 (H-11) were observed (Table 1). The above spectral data were similar to those of lannotinidine G (**5**), a known 7,8-*seco*-lycopodine-derived 8,5-lactone previously isolated from a Hokkaido *L. annotinum*,[3,7] implying these two alkaloids are structurally related.

Detailed interpretation of the 2D NMR ($^1H-^1H$ COSY, HSQC, and HMBC) data of **1** (Figure 2) allowed the construction of its gross structure. As expected, the 8,5-lactone group was verified by the HMBC correlation from H-5 to C-8, while the Δ^{10} double bond was confirmed by the COSY correlations among the spin system of H_2-9/H-10/H-11 and the

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Table 1. ^{1}H and ^{13}C NMR Data (δ in ppm, J in Hz) for Compounds 1−3 (in $CDCl_3$)[a]

	1		2		3	
no.	δ_H (J in Hz)[b]	δ_C[c]	δ_H (J in Hz)[b]	δ_C[d]	δ_H (J in Hz)[b]	δ_C[d]
1a	2.74, ddd, overlapped	47.8	2.72, ddd (11.8, 3.2, 3.2)	50.0	2.90, ddd (13.5, 3.8, 3.8)	43.2
1b	2.43, ddd (12.9, 12.9, 3.4)		2.24, ddd (11.8, 11.8, 5.3)		2.56, ddd (13.5, 13.5, 2.8)	
2a	1.86, m	25.7	1.92, m	24.2	1.72, m	27.2
2b	1.67, m		1.73, m		1.43, m	
3a	1.70, m	19.0	1.91, m	25.8	1.83, dddd (12.7, 12.7, 12.7, 4.0)	23.6
3b	1.54, m		1.74, m		1.75, m	
4	1.48, m	41.8	1.92, ddd, overlapped	44.7	2.25, br d (12.7)	42.2
5	4.54, br d (4.8)	80.5	4.35, br dd (5.0, 1.4)	78.3	4.40, br ddd (3.0, 2.5, 1.2)	77.9
6a	2.12, dd (8.3, 1.8)	27.5	2.08, m	28.4	2.61, ddd (13.0, 3.0, 1.2)	31.5
6b			2.03, m		1.96, ddd (13.0, 5.1, 2.5)	
7a	1.05, br d (6.0)	15.3	1.91, ddd, overlapped	25.1	2.19, ddd (5.1, 1.2, 1.2)	47.3
7b	0.71, dd (8.3, 6.0)		1.74, ddd, overlapped			
8		176.8		178.2		176.6
9a	3.58, br d (17.4)	50.9	3.26, dd (17.4, 5.4)	48.48		170.6
9b	3.02, br d (17.4)		2.83, br d (17.4)			
10	5.75, ddd (10.0, 3.0, 2.5)	127.6	5.86, br dd (10.4, 5.4)	124.1	6.16, d (5.8)	121.3
11	5.81, br d (10.0)	126.6	5.61, br d (10.4)	129.6	7.60, d (5.8)	156.0
12		34.0		44.9		94.6
13		59.7		59.5		65.3
14a	1.84, dd, overlapped	24.0	2.19, d (11.7)	30.4	2.76, d (14.0)	41.2
14b	1.79, dd (13.0, 13.0)		1.87, d (11.7)		1.68, dd (14.0, 1.9)	
15	2.74, m	34.7		48.51		42.9
16	1.21, d (6.6)	18.6	1.23, s	19.5	1.43, s	24.2

[a]Free base of each; assignments were made by a combination of 1D and 2D NMR experiments. [b]400 MHz. [c]150 MHz. [d]100 MHz.

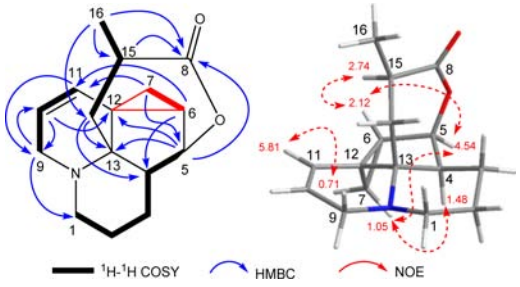

Figure 2. ^{1}H−^{1}H COSY, key HMBC, and NOE correlations of **1**.

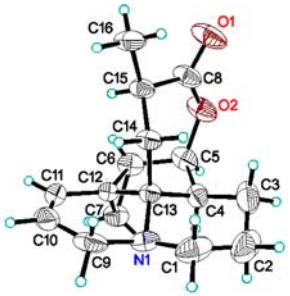

Figure 3. ORTEP drawing of **1**.

HMBC correlations of H-10/C-9, H-10/C-12, H-11/C-9, and H-9b/C-10. However, taking the molecular formula and the presence of a single double bond into account, one more ring system is required for **1** when compared with lannotinidine G (**5**).[3,7] This could be accounted for by a linkage between C-6 and C-12, which was supported by the abnormal upfield-shifted signals (δ_H 0.71 and 1.05, δ_C 15.3) observed for CH_2-7, and the key ^{3}J-HMBC correlations from H-5 to C-12 and from H-6/H-7b to C-11. Therefore, the planar structure of **1** was elucidated to possess an unprecedented [6/5/6/3]-tetracyclic backbone, together with a seven-membered 8,5-lactone ring (Figure 2).

The relative configuration of **1** was established by analysis of the NOESY data (Figure 2). The distinctive NOE correlations of H-7a (δ 1.05) with H-4 (δ 1.48) and H-5 (δ 4.54) suggested that the cyclopropane ring, H-4, and H-5 were all α-oriented. In addition, the NOE cross-peaks of H-15 (δ 2.74) with H-6 (δ 2.12) indicated that Me-16 adopted the β-orientation. The structure and absolute configuration (4*S*,5*R*,6*S*,12*S*,13*S*,15*R*) of **1** were confirmed by single-crystal X-ray diffraction [Cu Kα, at 296 K; Flack parameter: 0.01 (11)][8] analysis (Figure 3).

The HRESIMS (m/z 260.1646, $[M + H]^+$) and ^{13}C NMR data of annotinolide B (**2**) indicated that it possesses the same molecular formula ($C_{16}H_{21}NO_2$) as compound **1**. The ^{1}H and ^{13}C NMR data of **2** (Table 1) closely resembled those of **1**, indicative of a similar 7,8-*seco*-Δ^{10}-lycopodine-derived 8,5-lactone skeleton for **2**. Differing from **1**, the C-16 methyl group appeared as a singlet (δ 1.23, 3H, s) in **2** and was found to have a long-range HMBC correlation with C-12 (δ 44.9) (Figure 4). This, together with the remaining one degree of unsaturation,

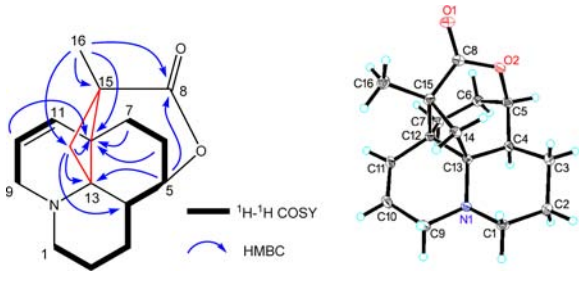

Figure 4. COSY, key HMBC correlations, and ORTEP drawing (right, colorless crystals obtained from MeOH) of **2**.

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required a direct connection between C-12 and C-15. Consequently, a cyclobutane ring encompassing C-12/C-13/C-14/C-15 was proposed. To our knowledge, only three *Lycopodium* alkaloids (i.e., lannotinidines E and F[3a] and annotinine[9]) with such a cyclobutyl ring formed by a C-12/C-15 linkage have been so far reported, but they all feature a common 8,15-*seco*-lycopodine 8,5-lactone skeleton. Herein, annotinolide B (**2**), possessing a sterically congested [6,6,6,4]-tetracyclic ring system, is reported as the first 7,8-*seco*-lycopodine-related alkaloid with such a unique cyclobutane ring. The relative configurations of stereogenic centers C-4, C-5, C-13, and C-15 in compound **2** were assigned to be the same as those of **1** according to the related proton−proton coupling constants (Table 1) and NOE correlations (see Supporting Information). A single-crystal X-ray diffraction study [Cu Kα, at 130 K; Flack parameter: 0.04 (7)][8] (Figure 4) confirmed the structure of **2** and established its absolute configuration (4*S*,5*R*,12*S*,13*S*,15*R*) as shown in Figures 1 and 4.

Annotinolide C (**3**), colorless crystals obtained from $CHCl_3$/MeOH (5:1, v/v), exhibited an $[M + H]^+$ ion peak at m/z 290.1394 in its positive HRESIMS, which was consistent with the molecular formula $C_{16}H_{19}NO_4$. Similar to compounds **1** and **2**, signals for the C-16 methyl (δ_H 1.43, s; δ_C 24.2) and 8,5-lactone [δ_H 4.40 (br ddd, J = 3.0, 2.5, 1.2 Hz, H-5); δ_C 176.6 (C-8) and 77.9 (C-5)] (Table 1) groups could be easily recognized from the ^1H and ^{13}C NMR spectra of **3**. In addition, chemical shifts typical of a conjugated carbonyl group [δ_H 6.16 (d, J = 5.8 Hz, H-10), 7.60 (d, J = 5.8 Hz, H-11); δ_C 121.3 (C-10), 156.0 (C-11), 170.6 (C-9)] were observed for **3**. This, together with a strong IR absorption band at 1748 cm^{-1} and the UV maximum at 212 nm,[10] hinted at an α,β-unsaturated γ-lactone in the structure of **3**. Moreover, the significantly downfield-shifted carbon resonance at δ 94.6, assigned to C-12, suggested that the γ-lactone ring was formed between C-9 and C-12, and consequently, the unusual γ-lactone ring was fused with ring B via the spiro carbon C-12. This inference was verified by the COSY and HMBC correlations depicted in Figure 5, from which a long spin system from CH_2-1

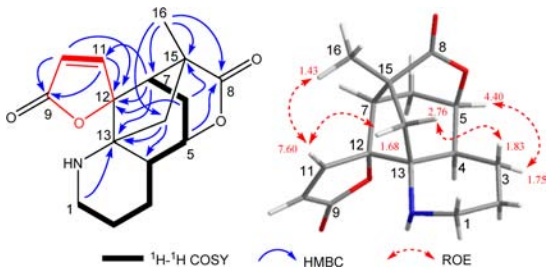


Figure 5. COSY, key HMBC, and ROE correlations of **3**.

to CH-7 and the diagnostic HMBC correlations from H-10/H-11 to C-9 and C-12 were observed. The linkage between C-7 and C-15 was deduced from the key HMBC correlations from H_2-6 (δ 2.61, 1.96) to C-15 (δ 42.9), from H-7 (δ 2.19) to C-16, and from Me-16 to C-7 (δ 47.3).

The relative configuration of **3** was determined by the observed proton−proton coupling constants (Table 1) and the ROE correlations (Figure 5). In particular, the large J value (12.7 Hz) between H-3a and H-4 and the smaller ones observed for H-5 and H-7 implied that H-3a and H-4 were both in the axial position, whereas H-3b, H-5, and H-7 were equatorial. Clear ROE correlations of H-3a/H-14a, H-3b/H-5, and H-5/H-4 suggested the α-orientation for H-4 and H-5, while the cross-peaks of H-11/H-14b and H-11/Me-16 established the relative stereochemistry of C-12, as shown in Figure 5. An obvious W-coupling (J = 1.2 Hz) between H-5 and H-7 supported their orientations. The absolute configuration of **3** (4*S*,5*R*,7*R*,12*R*,13*S*,15*R*) was unequivocally assigned by a single-crystal X-ray diffraction experiment using Cu Kα radiation at 296 K [Flack parameter: 0.05 (10)][8] (Figure 6).

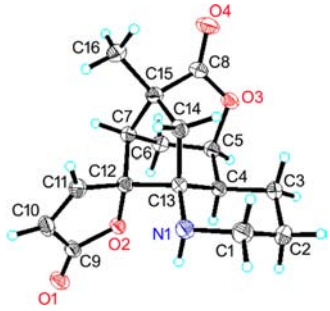
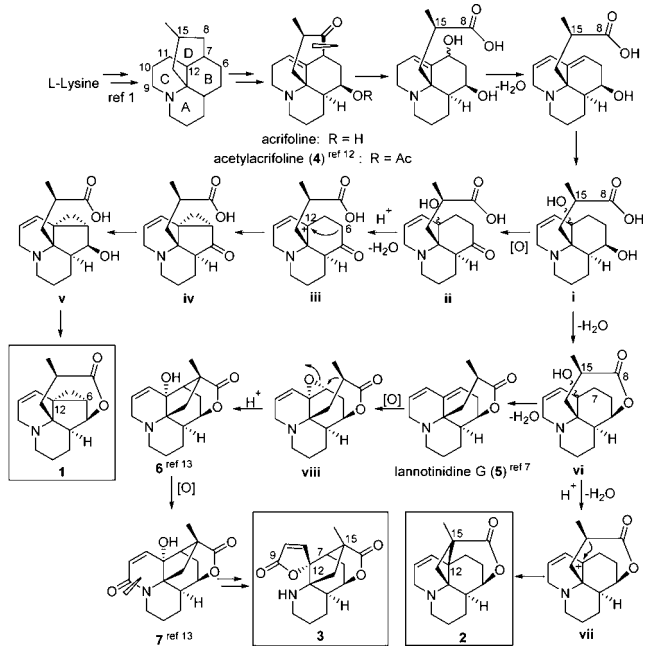


Figure 6. ORTEP drawing of **3**.

Annotinolides A−C (**1**−**3**) feature a common 7,8-*seco*-lycopodane-derived 8,5-lactone architecture, but each comprises a novel and distinctive oligocyclic ring system. Their plausible biosynthetic pathways were proposed, as shown in Scheme 1.

Scheme 1. Plausible Biosynthesis of Compounds 1−3[12,13]



The conventional lycopodine-type alkaloid (e.g., acrifoline[9,11]) could be considered as a precursor. In fact, the tetracyclic acrifoline-like compounds could originate from L-lysine[1] (for details, see Supporting Information), which could then furnish the key intermediate **i** via an oxidative cleavage of the C-7/C-8 bond. After the 5-OH oxidized to a ketone group, the cyclopropane ring would be readily constructed by the attack from C-6 to the C-12 carbocation. A reduction reaction and a lactonization would then give compound **1**. For alkaloid **2**, the cyclobutane ring might be formed by an intramolecular nucleophilic addition between C-15 and C-12. Compound **3** could also be generated from the 8,5-lactone derivative **vi**, which

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could be easily eliminated to its stable conjugated lannotinidine G (**5**) under acidic conditions. Then, compound **3** could be formed (via **5**, **6**, and **7**) by a cascade process of epoxidation, oxidation with concomitant C ring cleavage, and lactonization reactions.

The abnormal aggregation of Aβ peptides, especially peptides with 40 or 42 residues, is regarded as a key factor in the pathogenesis of Alzheimer's disease.[14] In this study, alkaloids **1**−**3** were evaluated for their inhibitory effects against the aggregation of Aβ_{1-42} peptide using thioflavin T (ThT) fluorescence.[15] As shown in Figure 7, all three alkaloids exhibited

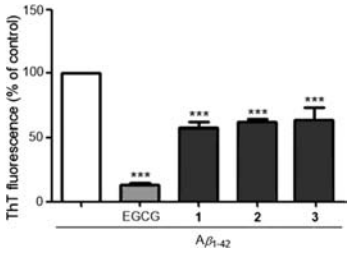

Figure 7. Inhibition of Aβ_{1-42} aggregation by compounds **1**−**3** at 50 μM monitored by ThT fluorescence. Three independent experiments were carried out; ***$p < 0.001$ vs Aβ_{1-42} group.

considerable antiaggregation activities at 50 μM, with inhibitory ratios of 42.4, 38.1, and 36.1%, respectively. Epigallocatechin-3-gallate[15a] was used as a positive control (inhibitory ratio: 86.6% at 10 μM). To our knowledge, this is the first report on the inhibitory potency of *Lycopodium* alkaloids against the Aβ_{1-42} aggregation. In addition, compounds **1**−**3** were also tested for their antiacetylcholinesterase and neuroprotective effects,[6] but none of them was active.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02132.

Experimental procedures, compound characterization, crystallographic data, and copies of spectra (PDF)
X-ray data for Annotinolide A (CIF)
X-ray data for Annotinolide B (CIF)
X-ray data for Annotinolide C (CIF)
X-ray data for Lannotinidine G (CIF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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(13) Compounds **6** (annotinolide D, 26.0 mg) and **7** (annotinolide E, 3.4 mg) were isolated and identified as new structures in this work (see Supporting Information).
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