Lyonin A, a New 9,10-Secograyanotoxin from *Lyonia ovalifolia*

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Phytochemical studies on the branches and leaves of *Lyonia ovalifolia* yielded a new grayanane diterpenoid, lyonin A (**1**), together with two known compounds, secorhodomollolides A and D (**2** and **3**, resp.). The structure of **1** was elucidated by combination of 1D- and 2D-NMR, and MS analyses. Compound **1** turned out to be a new, highly *O*-acylated grayanane diterpenoid, of which ring *B* has undergone an oxidative cleavage between C(9) and C(10), yielding a system differing from the previously reported grayanane type with a 5/7/6/5 ring system. Results of the cAMP regulation activity assay showed that compounds **2** and **3** at 50 μM induced a significantly decreased cAMP level in N1E-115 neuroblastoma cells ($p < 0.001$), indicating neuropharmacological potential.

Introduction. – Grayanane diterpenoids possess a 5/7/6/5 (*trans* or *cis*/*cis*/*cis*) ring system, formed probably by rearrangement of a kaurane skeleton. They have been found particularly in the genera *Pieris*, *Rhododendron*, *Lyonia*, and *Leucothoe* of the Ericaceae family. Up to now, nearly 110 grayanoids have been reported, of which some exhibited remarkable biological activities, including growth inhibitory, antifeedant, muscle spindle stimulant [1], and insecticidal activities [2].

Lyonia ovalifolia (WALL.) DRUDE is a deciduous tree growing mainly in hilly and valley regions of south and southwest China. *Yasue et al.* isolated lyoniols A–C as toxic components from the leaves [3], and lyoniside, which is a major component, and lyoniresinol from the bark of this plant [4][5]. In addition, *Sakakibara et al.* identified triterpene glycosides in this plant [6][7]. Recently, *Yun et al.* reported the isolation of five new lignans, *i.e.*, ovafolinins A–E, from the wood of the title plant, of which ovafolinins A–C possess a unique benzoxepin structure [8].

In our search for chemically novel and biologically active compounds from natural sources [9], the phytochemical study of *L. ovalifolia* was undertaken. Repeated column chromatography of an AcOEt extract of this plant led to the isolation of lyonin A (**1**), as well as two known compounds, secorhodomollolides A and D (**2** and **3**, resp.), which were reported recently by *Shi et al.* [10]. Here, we describe the isolation, structure eludication of compounds **1**–**3** (*Fig. 1*), and the bioactivity of compounds **2** and **3**[1]).

[1]) The lack of sufficient material prevented us from testing compound **1** for its cAMP regulation activity.

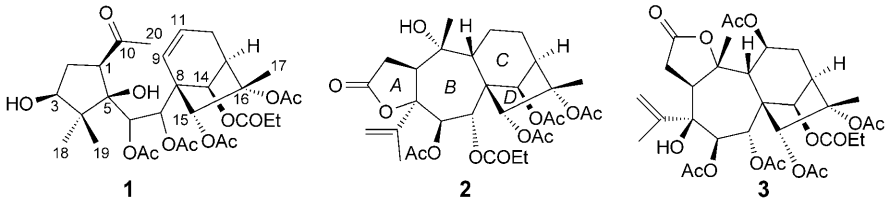

Fig. 1. *Grayanane diterpenoids* **1**–**3** *from* Lyonia ovalifolia

Results and Discussion. – Compound **1**, $[\alpha]_D^{26} = -7.78$ ($c = 0.18$, $CHCl_3$), was obtained as an amorphous powder, with a molecular formula of $C_{31}H_{44}O_{13}$ (with ten degrees of unsaturation), as derived from its HR-ESI-MS (*m/z* 659.2465 ($[M+Cl]^-$)) and confirmed by the NMR data (*Table 1*). The IR spectrum indicated the presence of OH (3433 cm^{-1}) and ester CO (1743 cm^{-1}), and a C=C bond (1629 cm^{-1}).

Table 1. *^{1}H- and ^{13}C-NMR* (125 MHz) *Data and HMBCs of Compound* **1** (in (D_5)pyridine, δ in ppm, *J* in Hz)

Position	δ(H)	δ(C)	HMBC (H→C)
1	3.61 (*dd*, *J*=10.6, 7.0)	54.8 (*d*)	2, 10, 20
2	2.36–2.45 (*m*)	35.0 (*t*)	1, 3, 5, 10
3	4.07 (*t*, *J*=3.5)	81.1 (*d*)	1, 5, 18, 19
4		50.6 (*s*)	
5		87.5 (*s*)	
6	5.96 (*s*)	74.8 (*d*)	1, 4, 8, 6-AcO
7	6.33 (*s*)	68.0 (*d*)	5, 8, 9, 14, 7-AcO
8		53.9 (*s*)	
9	6.23 (*d*, *J*=9.6)	129.0 (*d*)	8, 14
10		210.1 (*s*)	
11	5.62 (*d*, *J*=9.3)	129.2 (*d*)	
12α	2.34–2.39 (*m*)	30.6 (*t*)	14
12β	2.25–2.32 (*m*)		
13	3.44 (br. *s*)	46.3 (*d*)	
14	5.13 (overlap)	81.0 (*d*)	16, 14-propanoyl
15	5.51 (*s*)	86.3 (*d*)	9, 13, 14, 16, 17, 15-AcO
16		88.9 (*s*)	
17	1.90 (*s*)	24.0 (*q*)	13, 15, 16
18	1.26 (*s*)	22.9 (*q*)	3, 4, 5, 19
19	1.48 (*s*)	18.4 (*q*)	3, 4, 5, 18
20	2.51 (*s*)	31.0 (*q*)	1, 10
6-AcO	2.25 (*s*)	21.6 (*q*), 170.9 (*s*)	
7-AcO	2.11 (*s*)	21.3 (*q*), 169.8 (*s*)	
14-Propanoyl	1.29 (*t*, *J*=7.4), 2.64 (*q*, *J*=7.2)	9.5 (*q*), 28.5 (*t*), 173.5 (*s*)	
15-AcO	2.36 (*s*)	21.2 (*q*), 170.4 (*s*)	
16-AcO	1.96 (*s*)	22.2 (*q*), 169.3 (*s*)	
5-OH	5.95 (*s*)		1, 4, 5, 6

The ^{1}H-NMR spectrum of **1** exhibited eight Me *singlet*s at δ(H) 1.26, 1.48, 1.90, 1.96, 2.11, 2.25, 2.36, and 2.51 (each 3 H, *s*), and signals of five *O*-bearing CH groups at δ(H)

4.07, 5.13, 5.51, 5.96, and 6.33, of two olefinic CH groups at δ(H) 5.62 and 6.23, and of one *O*-propanoyl group at δ(H) 1.29 (*t*, $J=7.4$, 3 H) and 2.64 (*q*, $J=7.2$, 2 H). Close investigation on the ^{13}C- and DEPT-NMR data revealed that **1** had five AcO groups, which was confirmed by HMBCs. Apart from those of five AcO groups and one *O*-propanoyl group, 20 other C-atom signals were observed in the ^{13}C-NMR spectrum, including those for four Me, two CH_2, and nine CH groups (including five O-bearing ones, and one C=C bond), and for five quaternary C-atoms (including two O-bearing ones and one C=O group), which suggested a highly O-bearing diterpene skeleton. As required by its molecular formula, two OH groups and three carbocyclic rings of **1** should be present.

As diterpenoids previously isolated from Ericaceae were basically of the grayanane-type, it is reasonable to presume that the Me groups with signals at δ(C) 24.0, 22.9, and 18.4 correspond to Me(17), Me(18), and Me(19) of a grayanane skeleton [10]. Comparison of the ^{1}H- and ^{13}C-NMR data for **1** with those of secorhodomollolides A and D (**2** and **3**, resp.) suggested the similar ring *D* for three compounds. However, the data for the remaining portion of **1** were quite distinctive from those of known members of the grayanane class. Only two non-O-bearing CH groups (δ(C) 46.3 and 54.8) were evidenced in **1**. Another noticeable feature was the presence of an AcO group (δ(C) 31.0, 210.1) directly connected with one of the CH groups, which was confirmed by HMQCs and HMBCs.

Extensive analysis of ^{1}H,^{1}H-COSY, HMBC, and HSQC data led to the establishment of two substructures, **1a** and **1b** (*Fig. 2*), which were deduced as follows. In the HMBC spectrum, the correlations of Me(18) (δ(H) 1.26 (*s*)) with C(3), C(4), C(5), and Me(19), and of Me(19) (δ(H) 1.48 (*s*)) with C(3), C(4), C(5), and Me(18) implied that Me(18) and Me(19) should be attached to the same quaternary C-atom (C(4) (δ(C) 50.6 (*s*)). In addition, the Me(20) group (δ(H) 2.51 (*s*)) displayed HMBCs with C(1) and C(10). Other HMBCs were detected between H–C(2) (δ(H) 2.36–2.45 (*m*)), and C(1), C(3), C(5), and C(10), and between H–C(6) (δ(H) 5.96, *s*), and C(1) and

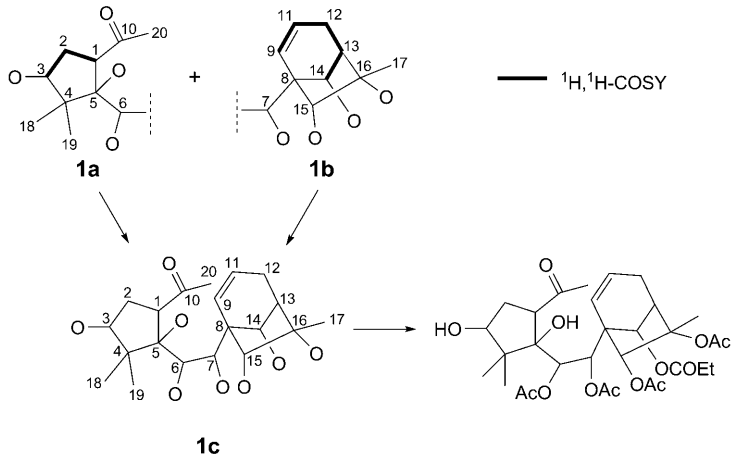

Fig. 2. *Structural fragments of* **1**

C(4). These observations, along with one H-atom spin systems (H–C(1)/H–C(3)) deduced from ^{1}H,^{1}H-COSY correlations, led to the establishment of substructure **1a**. HMBCs were observed from Me(17) (δ(H) 1.90, *s*) to C(13), C(15), and C(16), from H–C(15) (δ(H) 5.32, *s*) to C(9), C(13), C(14), C(16), and C(17), and from δ(H) 6.23 (*d*, $J = 9.6$, H–C(9)) to C(8) and C(14). The above evidence, in combination with a H-atom spin system H–C(9)/H–C(11)/H–C(12)/H–C(13)/H–C(14), provided the substructure **1b**. In the HMBC spectrum, H–C(6) showed correlations with C(1), C(4), and C(8), and H–C(7) (δ(H) 6.33 (*s*)) exhibited cross-peaks with C(5), C(8), C(9), and C(14), implying the connection of **1a** and **1b** to form **1c**.

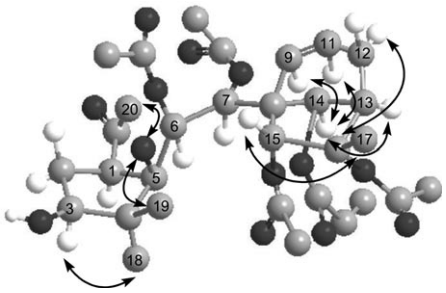

Fig. 3. *Key ROESY correlations for* **1**

Three AcO groups must be attached to C(6), C(7), and C(15), since H–C(6), H–C(7), and H–C(15) exhibited HMBC cross-peaks with the corresponding three AcO C=O C-atoms (δ(C) 169.8, 170.4, and 170.9, resp.). HMBCs of the H-atom signal at δ(H) 5.13 (overlap) with the C=O signal at δ(C) 173.5 (propanoyl) indicated that the propanoyl group is attached to C(14). Moreover, a OH group (δ(H) 5.95, *s*) was located at C(5) on the basis of its HMBC cross-peaks with C(1), C(4), C(5), and C(6). Commonly, the signals of C(16), which carries a OH group, of grayanane diterpenoids appeared at around δ(C) 79.0 in (D_5)pyridine, such as rhodomollein III [11], and the downfield shift of C(16) (from δ(C) 79.5 in rhodomollein III to 88.2 in **1**) indicated that HO–C(16) was also acetylated [12]. As required by its molecular formula, another O-bearing substituent should be a OH group at C(3). Based on the above data, the constitutional formula of **1** was established.

The relative configurations of all stereogenic centers of **1**, as well as the conformation of each ring, were elucidated by the analysis of its ROESY data, coupling constants, and analogy with **2** and **3**. In the ROESY spectrum, cross-peaks of HO–C(5) with Me(19) and Me(20), and of Me(17) with H–C(15) revealed that HO–C(5), Ac–C(1), H–C(15), and Me(17) were β-oriented. Correlations of H–C(3) with Me(18) indicated that H–C(3) was α-oriented. The relative configurations of H–C(13) and H–C(14) were assigned as α on the basis of ROESY correlations of H–C(14) with H–C(9), H–C(11), H_α–C(12), and H–C(13). Accordingly, the structure of **1** was determined as shown and named lyonin A.

The cyclic adenosine monophosphate (cAMP) is an important second messenger, which regulates several biological processes. In humans, cAMP affects not only the

higher order of thinking, but also neurogenesis, memory, emotional disorder, and cognitive function. cAMP is formed from ATP, and its intracellular concentration is strictly regulated by two membrane-bound enzymes, adenylate cyclase and phosphodiesterase. Since *L. ovalifolia* is a toxic plant, and may induce the paralysis of nerve centers and motor nerve terminals [13], the cAMP regulation activity of the compounds isolated from this plant was evaluated by AlphaScreen assay. Compounds **2** and **3** significantly decreases the cAMP level at a concentration of 50 μM ($p < 0.001$) in N1E-115 neuroblastoma cells (*Table 2*), indicating a neuropharmacological potential.

Table 2. *The cAMP Regulation Activity of Compounds* **2** *and* **3**[a])

Compound	Mean of AlphaScreen counts per second (cps)[b])	Standard error	*P* value
DMSO	6800	300	
2	9400	800	< 0.001
3	10500	1350	< 0.001
Forskolin	3400	200	< 0.001

[a]) N1E-115 Neuroblastoma cells (5,000 per well) in a 384-well plate were incubated with 50 μM of **2** or **3**, or 10 μM of forskolin, and processed for cAMP detection by AlpharScreen assay as described in *Exper. Part*. [b]) The AlphaScreen counts decrease with increasing cAMP concentration (*e.g.*, forskolin), and increase with decreasing cAMP concentration (compounds **2** and **3**).

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300, 100–200, and 80–100 mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), *Sephadex LH-20* (*Amersham Biosciences AB*, S-Uppsala) and *MCI* (*MCI*-gel *CHP-20P*, *75–150 μm*, *Mitsubishi Chemical Corporation*, Tokyo, Japan). Fractions were monitored by TLC (silica-gel GF_{254} plates, *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), and spots were visualized by heating SiO_2 plates sprayed with 5% H_2SO_4/EtOH. Optical rotations: *Jasco DIP-370* digital polarimeter. IR Spectra: *Bio-Rad FTS-135* spectrophotometer; KBr pellets. 1D- and 2D-NMR spectra: *Bruker AM-400* and *DRX-500* instruments; TMS as an internal standard. FAB-MS: *VG Auto Spec-3000* spectrometer. HR-ESI-MS: *API Qstar Pulsar* instrument.

Plant Material. The leaves and branches of *L. ovalifolia* were collected from Luchun County, Yunnan Province, P. R. China, in December 2008, and identified by Prof. *Hua Peng*, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (KMUST 20081201) was deposited with the Laboratory of Phytochemistry, Biotechnology Research Center, Kunming University of Science and Technology.

Extraction and Isolation. Air-dried branches and leaves (5 kg) of *L. ovalifolia* were ground and extracted with 75% Me_2CO/H_2O (3×28 l; each 24 h) at r.t. and filtered. The filtrate was concentrated under reduced pressure, and the resulting residue was extracted with AcOEt. The AcOEt extract (70 g) was chromatographed over *Sephadex LH-20* (MeOH/H_2O 3:7, 6:4, 9:1, 1:0) to afford four fractions, *Frs. 1–4*. *Fr. 1* (MeOH/H_2O 3:7) was subjected to CC (*MCI*; MeOH/H_2O, 3:7, 6:4, 9:1, 1:0) to give four subfractions, *Subfrs. A–D*. Mixed crystals were obtained from *Subfr. C*, and were further chromatographed over SiO_2 ($CHCl_3$/MeOH 180:1) to obtain compound **3** (45 mg). The mother liquor

was submitted to CC (*Sephadex LH-20*; $CHCl_3$/MeOH 1:1; then SiO_2; petroleum ether (PE)/Me_2CO 9:1, 8:2, 7:3, 6:4) to obtain four fractions, *Frs. C1–C4*. *Fr. C2* was finally purified by repeated CC (SiO_2; $CHCl_3$/MeOH 200:1, 180:1, 160:1, 140:1; and benzene/Me_2CO 10:1) to yield compounds **1** (1.1 mg) and **2** (6.8 mg).

AlphaScreen cAMP Assay. The effect on cAMP levels in neuroblastoma cells of the compounds **2** and **3** was determined using the *AlphaScreen* cAMP assay kit (*Perkin–Elmer*) according to the reported procedures [14]. Briefly, when neuroblastoma N1E-115 cells were grown to *ca.* 80% confluence, the cells were harvested and suspended with stimulation buffer (HBSS, pH 7.4, containing 0.5 mM IBMX, 5 mM *HEPES*, and 0.1% BSA) into 2000 cells/μl, and were further diluted by anti-cAMP acceptor bead soln. (0.2 units of anti-cAMP acceptor beads in 1 μl of stimulation buffer) into 1000 cells/μl. Then, the cell-bead mix was allocated into a 384-well white opaque microplate with 5 μl/well (*Perkin–Elmer*, No. 6007290). The test compounds (50 μM), or forskolin (10 μM), which is a generic activator of cAMP synthesis directly stimulating adenylate cyclase, were added to the wells and incubated for 30 min at r.t. Then, streptavidin donor beads/biotinylated cAMP detection mix was added to the plate with 15 μl/well and incubated for 60 min in the dark. The mixture containing 5 μl of anti-cAMP acceptor beads, 5 μl of 5 μM cAMP soln., and 15 μl of biotinylated-cAMP/streptavidin donor beads was used as a positive control. Plates were read on a *2104 EnVision® Multilabel Plate Reader* (*Perkin–Elmer*) at an excitation wavelength of 680 nm and an emission wavelength of 570 nm. The *AlphaScreen* counts decrease with increasing cAMP concentration (*e.g.*, positive control forskolin), and increase with decreasing cAMP concentration [14]. The cAMP level change due to the presence of test compounds was calculated. The statistical tests were performed using one-way ANOVA analysis in software SPSS11.5 (*SPSS*, Chicago, IL).

Lyonin A (= rel-*(1S,5R,6S,7R,8R)-1-{2-[(1R,3S,5R)-5-Acetyl-1,3-dihydroxy-2,2-dimethylcyclopentyl]-1,2-bis(acetyloxy)ethyl}-6,7-bis(acetyloxy)-6-methylbicyclo[3.2.1]oct-2-en-8-yl Propanoate*; **1**). Amorphous powder. $[\alpha]_D^{26} = -7.78$ ($c = 0.18$, $CHCl_3$). IR (KBr): 3433, 2923, 2853, 1743, 1629, 1374, 1224, 1042. ^{1}H- and ^{13}C-NMR: *Table 1*. ESI-MS (neg.): 659 ($[M+Cl]^-$). HR-ESI-MS (neg.): 659.2465 ($[M+Cl]^-$, $C_{33}H_{44}O_{13}Cl^-$; calc. 659.2470).

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