Antibacterial Neoclerodane Diterpenoids from *Ajuga lupulina*

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The whole plants of *Ajuga lupulina* afforded five compounds, including three new clerodane diterpenes, lupulins A−C (**1**−**3**), whose structures were elucidated by spectral methods. Among these compounds, lupulins A (**1**) and B (**2**) as well as the acid hydrolysate (**5**) of lupulin D (**4**) showed antibacterial activities against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.*

Several clerodane diterpenes from *Ajuga* species possess potential insect antifeedant activity.[1,2] Recently, we have investigated *Ajuga lupulina* (Maxim.) (Lamiaceae), which is common in northwestern China and is used in folk medicine, and have found it contains novel clerodane diterpenes with antibacterial activity. This paper reports the isolation and structure elucidation of the new diterpenoids, lupulins A−C (**1**−**3**), as well as their antibacterial activity.

Guided by in vitro antibacterial activity against *Escherichia coli*, a petroleum ether−Et_2O−MeOH (1:1:1) extract of the air-dried and powdered whole plants of *A. lupulina* was subjected to column chromatography over Si gel, to yield β-sitosterol and lupulin D (**4**),[3] as well as three new clerodane diterpenoids, lupulins A−C (**1**−**3**).

Compound **1** was obtained as colorless needles upon recrystallization from hexane−Me_2CO. The IR spectrum showed absorptions attributable to free hydroxyl (3452 cm^{-1}), oxirane ring (3030 cm^{-1}), and ester (1739 and 1241 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** exhibited two acetate groups (δ 2.11 and 1.93), a MeO group (δ 3.32), and a 2-methylbutyric ester functionality (δ 0.87, t, J = 5.2 Hz; δ 1.10, d, J = 7.0 Hz; and δ 2.24, sextet, J = 5.6 Hz). The presence of this last functionality was also confirmed by signals due to protons on carbon atoms bearing oxygen at δ 4.68 (1H, dd, J = 11.4, 4.8Hz), 4.78, and 4.40 (AB system, J = 12.2 Hz), 2.79 (1H, d, J = 4.0 Hz), and 2.55 (1H, d, J = 4.0 Hz). In addition, the characteristic signals of a methyl singlet at δ 0.92 and of a methyl doublet at δ 0.88 (J = 6.2 Hz) were also observed. Likewise, the presence of a hexahydrofurofuran ring moiety was confirmed by the signals at δ 5.81 (d, J = 5.3 Hz), 4.96 (d, J = 5.6 Hz), 4.38 (dd, J = 11.5, 5.7 Hz), and 2.81 (m). On the basis of the above observations and from the EIMS spectrum (see Experimental Section), the presence of a clerodane diterpene skeleton could be easily deduced.[4] The lowfield ^{13}C-NMR resonance at δ 104.8 (CH) indicated that the methoxyl group was located at C-15. This was also confirmed by the presence of significant peaks at m/z 143, 111, 81, and 69 in the mass spectrum.[5] Furthermore, the peak correlating signals at δ 3.61 (ddd, J = 9.8, 8.8, 5.3 Hz) and 5.22 (d, J = 9.8 Hz) in the ^{1}H−^{1}H COSY NMR spectrum of **1**, as well as their proton coupling pattern, indicated that hydroxyl and 2-methylbutyric groups were located at C-2 and C-3, respectively.

The sole remaining structural problems were to determine the sterochemistry of H-2, H-3, and H-15. On comparison with some neoclerodane diterpenes previously isolated from *Ajuga* species, such as ajugapitin [6] and 15-ethoxy-14-hydroajugapitin,[7] and by considering the large coupling constant (J = 9.8 Hz) between H-2 and H-3, the hydroxyl group at C-2 was assigned as being α-oriented, with the 2-methylbutyric ester function being β-oriented. In the 2D ^{1}H−^{1}H NOESY spectrum of **1**, these assignments were reinforced by the NOE correlations of H-3 to two diastereotopic protons at C-18. By comparison with the H-15 ^{1}H-NMR signal of clerodinin A,[3] the chemical shift and coupling pattern were closely comparable to **1**; thus, the methoxyl group at C-15 in **1** was assigned with β-orientation. The EIMS of **1** showed a molecular ion at m/z 582. From the ^{13}C-NMR data (Table 2) and elemental analysis, the molecular formula of **1** was confirmed as $C_{30}H_{46}O_{11}$, in accordance with the proposed structure.

The structure of compound **2** clearly followed from its ^{1}H-NMR data (Table 1), which were closely comparable to those of **1**. However, in the ^{1}H-NMR spectrum of **2**, the signal of the geminal proton of the free hydroxyl at δ 3.5−4.0 was absent and the doublet of H-3 at δ 5.22 in that of **1** was replaced by double doublets at δ 5.30 (J = 12.0, 4.7 Hz). Furthermore, no hydroxyl absorption band was apparent in the IR spectrum of **2**. From elemental analysis and ^{13}C-NMR data, the molecular formula of **2** was determined as $C_{30}H_{46}O_{10}$, which was in agreement with the molecular ion at m/z 566 (16 mass units lower than that of **1**) in the EIMS of **2**. These observations indicated that compound **2** was a 2-deoxygenated analogue of **1**. In the 2D NOESY spectrum of **2**, correlation of H-3 with the methylene protons at H-18 established the α-orientation of H-3. In addition, the chemical shift (δ 5.11) and coupling constant (J = 4.8 Hz) of H-15 in **2** were also different from those of **1**, with such data coincident with those of the known compound **4**. The methoxyl group at C-15 in **2** was therefore assigned as being α-oriented.

The ^{1}H-NMR spectrum of compound **3** showed a methyl doublet at δ 0.86 (J = 6.6 Hz), a methyl singlet

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Table 1. ^{1}H-NMR Data of Compounds **1**−**5**[a]

	compound				
proton	**1**	**2**	**3**	**4**	**5**
2	3.61 ddd (9.8, 8.8, 5.4)[b]			1.86 m	
3	5.22 d (9.8)	5.30 dd (12.0, 4.7)		1.87 m	
6	4.68 dd (11.4, 4.8)	4.74 dd (11.6, 4.6)	5.03 dd (10.9, 4.9)	4.66 dd (11.5, 4.2)	4.65 dd (11.4, 4.3)
11	4.37 dd (11.5, 5.7)	4.02 dd (11.8, 4.6)	4.08 dd (11.4, 5.2)	3.99 dd (11.7, 4.2)	4.03 dd (11.3, 4.6)
13	2.81 m	3.01 m	2.84 m	2.98 m	2.95 m
15	4.96 d (5.6)	5.11 d (4.8)	3.86 m	5.08 d (4.7)	5.54 dd (10.3, 4.7)
16	5.81 d (5.3)	5.72 d (5.6)	5.61 d (5.0)	5.68 d (5.3)	5.79 d (4.8)
17	2.79, 2.55 (AB, 12.3)	2.81, 2.59 (AB, 12.3)	4.73, 4.43	2.95, 2.19 (AB, 4.0)	2.94, 2.17 (AB, 4.1)
18	4.78, 4.40 (AB, 12.3)	4.79, 4.39 (AB, 12.3)	4.83, 4.24 (AB, 12.1)	4.87, 4.38 (AB, 12.2)	4.84, 4.36 (AB, 12.1)
19	0.92 s	0.96 s	0.97 s	0.94 s	0.95 s
20	0.88 d (6.2)	0.87 d (6.1)	0.86 d (6.6)	0.84 d (6.2)	0.86 d (6.6)
OMe	3.32 s	3.33 s		3.30 s	
OAc	2.12 s	2.12 s	1.98 s	2.08 s	2.07 s
OAc	1.93 s	1.95 s	1.94 s	1.92 s	1.94 s

[a] Recorded at 400 MHz in $CHCl_3$. [b] *J* values (Hz) are provided in parentheses.

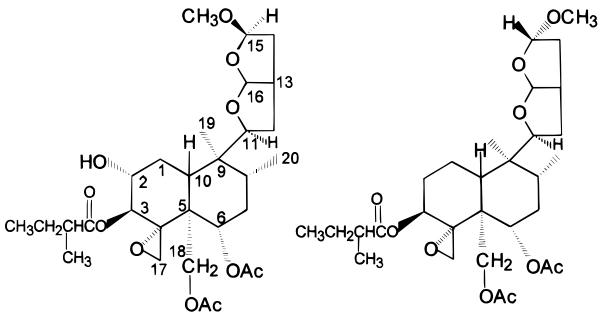
Table 2 . ^{13}C-NMR Data for Compounds **1-4**[a]

	compound			
carbon	**1**	**2**	**3**	**4**
1	30.2	29.7	22.7	22.1
2	72.4	31.0	28.6	24.9
3	71.4	66.6	32.2	38.0
4	62.9	65.3	151.6	65.0
5	45.6	46.3	48.7	45.6
6	71.8	71.3	75.4	71.9
7	33.3	33.2	32.6	33.4
8	35.8	36.1	35.8	36.1
9	42.4	40.2	40.9	40.1
10	43.4	47.9	42.1	48.5
11	82.8	83.4	85.5	83.5
12	32.7	32.2	32.6	32.2
13	40.5	39.9	49.9	39.9
14	39.5	38.1	34.0	32.7
15	104.8	104.9	68.4	104.8
16	109.2	107.2	107.7	107.2
17	43.4	42.5	106.4	48.4
18	61.5	61.5	61.2	61.7
19	13.8	13.9	14.4	13.9
20	16.5	16.3	16.4	16.4
OMe	54.5	54.7		54.6
OAc	170.1	170.2	170.5	170.0
	21.1	21.2	21.2	21.1
OAc	171.1	171.1	170.9	170.8
	20.9	21.0	21.2	21.1

[a] Recorded at 100.6 MHz in $CDCl_3$.

at δ 0.97, a methylene AB system at δ 4.83 and 4.24 (J = 12.1 Hz), and a methine double doublet at δ 5.03 (J = 10.9, 4.9 Hz). These features, as well as the significant fragment peaks at m/z 111, 81, and 69 in the EIMS, indicated that **3** was also a clerodane diterpene with a hexahydrofuranofuran ring.[5] Two singlets at δ 4.73 and 4.43 in the ^{1}H-NMR spectrum of **3** suggested the presence of an exocyclic double bond that was associated with ^{13}C-NMR resonances at δ 151.6 (C) and 106.4 (CH_2) (Tables 1 and 2). Considering the structural features of clerodane diterpenes, the exocyclic double bond must be located at C-4. Moreover, the absence of any signals for an oxirane ring at δ 2.0−3.0 in the ^{1}H-NMR spectrum also supported the above inferences. The EIMS of **3** showed a molecular ion at m/z 420. From its ^{13}C- and DEPT NMR spectra, as well as by elemental analysis, the molecular formula $C_{24}H_{36}O_6$ was deduced for **3**.

The antibacterial activity of compounds **1**, **2**, **4**, and **5** was tested by using a paper-diffusion method. In vitro antibacterial evaluation of **1** showed strong activity against *Pseudomonas aeruginosa* and *Escherichia coli* (inhibitory zone 3−5 mm), and weak activity against *Staphylococcus aureus* (1.5 mm). Compounds **2** and **5** exhibited weak antibacterial activity against *S. aureus* and *E. coli* (1.2 mm), with no activity against *P. aeruginosa*. Compound **4** showed no activity against any of the list organisms at the concentrations used.



Experimental Section

General Experimental Procedures. The melting points were determined on a Kofler hot-stage instrument and are uncorrected. ^{1}H-, ^{13}C-, and 2D NMR spectra were measured on a Bruker AM 400 FT-NMR spectrometer with TMS as internal standard. IR spectra were recorded on a Nicolet 170 SX-FTIR spectrometer using KBr disks. EIMS were obtained on a VG ZAB-HS spectrometer at 70 eV. Elemental analysis was carried out on a Perkin-Elmer elemental analyzer.

Plant Material. Whole plants of *A. lupulina* were collected from the southern area of Gansu Province, China. The plant was identified by Prof. G. L. Zhang, Department of Biology, Lanzhou University, and a

voucher specimen is deposited in the Herbarium of the Department of Biology, Lanzhou University.

Extraction and Isolation. The air-dried whole plants of *A. lupulina* (0.94 kg) were powdered and extracted with petroleum ether (60−90 °C)−Et_2O−MeOH (1:1:1) three times (2 days each) at room temperature. The resultant extract (19 g), which was subjected to column chromatography over (500 g) Si gel with a petroleum ether/Me_2CO gradient, separated into five crude fractions (fractions 1−5). Fraction 2 was further separated by column chromatography over Si gel with a petroleum ether−EtOAc (10:1−1:1) gradient and was purified by preparative TLC using a hexane−EtOAc (4:1) system, and finally gave **3** (10 mg) and **4** (70 mg). Fraction 3 was rechromatographed with a petroleum ether−Me_2CO (15:1−2:1) system followed by preparative TLC with *n*-hexane−EtOAc (3:1) to afford 50 mg of β-sitosterol, 26 mg of **1**, and 11 mg of **2**.

The known compound **4** was identified by comparing its corresponding properties (MP, MS, IR, ^{1}H, and ^{13}C NMR) with literature values.[3]

Lupulin A (1): colorless needles (recrystallized from *n*-hexane−Me_2CO), mp 172−174 °C; IR (KBr) ν max 3452 (OH), 3030 (oxirane ring), 1739 (C=O, ester), 1463, 1366, 1241, 910, 610 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2, respectively; EIMS *m/z* [M]$^+$ 582 (0.5), 551 (0.8), [M − MeOH]$^+$ 550 (0.6), 490 (0.2), 449 (0.5), 388 (1), 315 (1), 278 (1), 265 (11), 218 (5), 200 (4), 187 (8), 143 (80), 111 (100), 85 (17), 83 (19), 81 (10), 69 (14), 57 (62), 43 (58). Anal. Calcd for $C_{30}H_{46}O_{11}$: C, 61.86, H, 7.90. Found: C, 61.87; H, 7.88.

Lupulin B (2): amorphous powder; IR (KBr) ν max 3043 (oxirane ring), 1740 (C=O), 1463, 1367, 1238, 1020, 613 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) data, see Table 1; 2-methylbutyric unit [δ 2.20 (1H, sextet, J = 3.9 Hz), 1.66 (2H, m), 0.88 (3H, t, J = 7.4 Hz), 1.09 (3H, d, J = 7.3 Hz)]; ^{13}C-NMR ($CDCl_3$, 100.6 MHz) data, see Table 2; 2-methylbutyric unit [δ 175.1 (C=O), 41.1 (CH), 26.6 (CH_2), 11.2 (CH_3), 16.3 (CH_3)]; EIMS *m/z* [M]$^+$ 566 (0.5), 534 (1), 433 (0.3), 325, (2), 278 (0.6), 265 (1), 218 (10), 187 (5), 187 (11), 143 (57), 111 (100), 81 (18), 69 (9), 57 (70), 43 (81). Anal. Calcd for $C_{30}H_{46}O_{10}$; C, 63.59; H, 8.18. Found: C, 63.54; H, 8.21.

Lupulin C (3): oily gum; IR (KBr) ν max 2940, 2879, 1738, 1710 br (C=O), 1610 (C=C), 1275, 1251, 1026, 970, 785 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2; EIMS *m/z* [M]$^+$ 420 (10), 309 (21), 171 (8), 111 (100, hexahydrofuranofuran fragment ion), 85 (17), 83 (11), 81 (13), 69 (11), 57 (69), 43 (61). Anal. Calcd for $C_{24}H_{36}O_6$: C, 68.55; H, 8.63. Found: C, 68.59; H, 8.68.

Preparation of Compound 5. A solution of 20 mg of **4** in Me_2CO (3 mL) was hydrolyzed with 2 mL of HOAc by stirring for 6 h at room temperature. The reaction mixture was evaporated under a vacuum, and the residue (14 mg) was purified by preparative TLC [*n*-hexane−EtOAc (3:1)] to afford 8 mg of **5**; ^{1}H-NMR data, see Table 1.

Antibacterial Activity. A paper disk diffusion method was used,[8,9] with the organisms used consisting of *S. aureus*, *P. aeruginosa* (NJ94), and *E. coli*. Fresh cultures and nutrient agar at 37 °C were used. Bacteria were prepared according to standard techniques [8] and their concentrations adjusted to 10^7 cell/mL.[8,10] The samples were dissolved in distilled H_2O and their concentrations were adjusted to 0.02 mg/mL. Then, paper disks (5-mm diameter) were saturated with each solution and placed into the petri dishes after seeding the test organisms (1%) into the cooled specific medium described. The inhibition zones (mm) were compared with controls.

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