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Neo-Clerodane Diterpenes from *Ajuga turkestanica*

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Abstract

The ethyl acetate extract of the aerial parts of *Ajuga turkestanica* afforded 6 *neo*-clerodane diterpenes, including two novel compounds, 14, 15-dihydroajugachin B (**1**) and 14-hydro-15-methoxyajugachin B (**2**), in addition to the known diterpenoids chamaepitin (**3**), ajugachin B (**4**), ajugapitin (**5**) and lupulin A (**6**). Structures were established through exhaustive NMR spectroscopic analysis and chemical transformation in the case of **1**. The full ^{1}H and ^{13}C NMR assignment of the C-15 *R* and *S* configurations of 14-hydro-15-methoxyajugachin B and chamaepitin were elucidated.

Keyword index

Ajuga turkestanica; Labiatae; *neo*-clerodane diterpenes; 14, 15-dihydroajugachin B; 14-hydro-15-methoxyajugachin B

1. Introduction

The genus *Ajuga* (Labiatae), comprised of more than 40 species widely distributed in temperate regions of both hemispheres (Hedge, 1992), has been a focus of interest due to significant medicinal and economic properties. *Ajuga* contains at least three classes of potentially bioactive compounds: clerodane diterpenes, phytoecdysteroids and iridoid glycosides. Clerodane diterpenes are known for their activity as insect alleochemicals (Camps and Coll, 1993; Klein Gebbinck *et al*., 2002), and are also recognized sources of antimicrobial, antiviral, antitumor, antibiotic and amoebicidal activities (Coll and Tandron, 2007). In addition, many *Ajuga* species are well known among phytoecdysteroid-producing plants because of the great variety of such compounds they contain. Phytoecdysteroids exhibit significant physiological activities in insects and also in mammals, which might correlate with the successful applications in folk medicine of the plants that produce them. Both the clerodane diterpenoids and the phytoecdysteroids with potential insect antifeedant and moulting hormone activities, respectively, may work interactively to potentiate the bioactivity of the *Ajuga* plants (Camps and Coll, 1993). Also, *Ajuga* is known as a source of iridoid glycosides, which have demonstrated cancer chemopreventive activity (Konoshima *et al*., 2000).

Ajuga turkestanica (Regel) Briq., a perennial herb growing mainly in Central Asia, is known as a rich source of bioactive substances and is used by local people to treat heart diseases,

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muscle and stomach aches (Mamatkhanov *et al*., 1998; Abdukadirov *et al*., 2004). Several phytoecdysteroids (turkesterone, 20-hydroxyecdysone, cyasterone, cyasterone 22-acetate, ajugalactone, ajugasterone B, α-ecdysone and ecdysone 2, 3-monoacetonide) have been reported in this species along with the iridoids harpagide and harpagide 8-acetate (Usmanov *et al*., 1971, 1973, 1975, 1978; Baltaev, 2000; Ramazanov, 2005).

In this paper, we report the isolation and structural elucidation of six *neo*-clerodane diterpenes, two of which are novel compounds. This is the first study to identify *neo*-clerodane diterpenes in *A. turkestanica*.

2. Results and discussion

The ethyl acetate extract from the aerial parts of *Ajuga turkestanica* yielded two new *neo*-clerodane diterpenoids: 14, 15-dihydroajugachin B (**1**) and 14-hydro-15-methoxyajugachin B (**2**), together with the known diterpenes: chamaepitin (14-hydro-15-hydroxyajugachin B) (**3**) (Camps *et al*., 1987), ajugachin B (**4**) (Boneva *et al*., 1990), ajugapitin (**5**) (Hernandez *et al*., 1982), and lupulin A (Hao *et al*., 1996). ^{1}H and ^{13}C NMR spectra of compounds **3** and **5** were in agreement with the published data for chamaepitin and lupulin. Previous reports did not provide the full set of assignments for the compounds with *R* and *S* configuration at C-15, however we report in this manuscript the full NMR assignments, and the characteristic NMR signals, for chamaepitin and lupulin A, respectively, with *R* and *S* configuration at C-15.

Compound **1** was obtained as an optically active white amorphous substance. The IR spectrum showed absorption bands for hydroxyl (3457 cm^{-1}) and ester groups (1728 and 1245 cm^{-1}). HR-ESI-MS of **1** showed a molecular ion peak $[M+H]^{+}$ at *m/z* 611.3063, corresponding to the molecular formula $C_{31}H_{47}O_{12}$. The ^{1}H- and ^{13}C NMR spectra (Tables 1 & 2) indicated clearly that the compound is a *neo*-clerodane diterpene and, furthermore, closely related to the known compounds **3** and **4**, suggesting a *neo*-clerodane diterpene with the same 3′ acetoxy-2′-methylbutyryl functionality at C-3. This was clear from the downfield signal for H-3′ (δ 5.14, *m*) and the two methyl doublets at δ 1.20 and 1.11 (*d*, *J* = 7.1Hz, 4′ and 5′ CH_3, respectively). The presence of this functionality at C-3 was confirmed by the downfield signal due to a proton on a carbon atom bearing an oxygen at δ 5.16 (1H, *d*, *J* = 9.5 Hz, H-3). The presence of three acetate groups was evident from the ^{1}H NMR spectra (δ 2.10, 2.01 and 1.91) and ^{13}C NMR spectra (δ_c 170.3, 171.3 and 171.4). Two of these acetate groups were attached at positions 6 and 18 and the third one was a substituent at position 3′ of the 2-methylbutyryloxy moiety. Likewise, the presence of an unsubstituted hexahydrofuran ring system was confirmed by the signals at δ 5.66 (1H, *d*, *J* = 5.1 Hz, H-16 *β*), 4.09 (1H, *dd*, *J* = 11.4, 5.2 Hz, H-11*α*), 3.85 (2H, *m*, H-15) and 2.87 (1H, *m*, H-13 *β*). Furthermore, the peak correlating signals at δ 3.65 (*ddd*, *J* = 9.5, 8.8, 4.9 Hz) and 5.16 (*d*, *J* = 9.5 Hz) in the ^{1}H-^{1}H COSY NMR spectrum of **1**, as well as their proton coupling pattern, indicated that the hydroxyl and 2-methylbutyryloxy groups were located at C-2 and C-3, respectively. The relative configuration at H-2 and H-3 was deduced by comparing some *neo*-clerodane diterpenes previously isolated from *Ajuga pseudoiva* (Ben Jannet *et al*., 2000), and by considering the large coupling constant (*J* = 9.5 Hz) between H-2 and H-3. The hydroxyl group at C-2 was assigned as *α*-oriented, with the 2-methylbutyryloxy ester functionality *β*- oriented. This assignment was reinforced by NOE correlations of H-3 to the two diastereotopic protons at C-19. The ^{1}H-^{1}H COSY NMR spectra, as well as their proton coupling patterns, were sufficient to interpret all correlated protons. The structure of **1** was confirmed through catalytic hydrogenation of ajugachin B (**4**) (Barton *et al*., 1961) to afford 14, 15-dihydroajugachin B (**1**). The ^{1}H and ^{13}C NMR spectral data of the semi-synthetic and the isolated compound **1** are in total agreement, thereby supporting the structure assignment. From the above data, compound **1** was confirmed as 14, 15-dihydroajugachin B (**1**) with the structure shown in Fig 1.

Compound **2** was obtained as a 1:4, *R* to *S*, epimeric mixture of a 15-methoxy derivative of dihydroajugachin (**1**), as indicated from its ^{1}H NMR and also the occurrence of pairs of signals in its ^{13}C NMR spectrum. The epimeric mixture was obtained as an amorphous white material which was homogenous on TLC. The IR spectrum showed absorption bands for hydroxyl (3436 cm^{-1}) and ester groups (1732 and 1245 cm^{-1}). The HR-ESI-MS showed a molecular ion peak at *m/z* 641.3182 $[M+1]^{+}$, corresponding to the molecular formula $C_{32}H_{48}O_{13}$. The ^{1}H NMR spectrum of **2** was very close to that of the isolated compound **3** (chamaepitin), with an additional methoxy group at δ 3.30 (s), suggesting a methylated derivative of **3**. The presence of a non-equimolar mixture of C-15 epimers enabled us to assign the proton signals for both compounds depending on their integration values. In fact, H-11, H-13, H-15 and H-16 appeared as pairs of signals: H-11*α* at δ 3.97 and 4.35 (*dd*, *J* = 11.6, 4.2 Hz and 11.2 Hz); H-13 at δ 3.97 and 2.77 (*m*); H-15 at δ 5.07 and 4.94 (*d*, *J* = 4.9 Hz and 5.4 Hz); H-16 at δ 5.71 and 5.79 (*d*, *J* = 5.6 and 4.4 Hz), for *R* and *S* configurations, respectively. The resonance of H-11 in the 15 *R* configuration appeared at higher field than in the 15 *S* configuration.

In contrast, the resonance of H-13 occurred in the 15 *R* configuration at lower field than in the 15 *S* configuration. Similarly, several peaks in the ^{13}C NMR displayed higher shift values for the 15 *R* configuration: C-12 and C-14 resonated at δ_c 32.62 (32.83, *S*) and 38.27 (39.70, *S*), respectively, whereas the C-15 resonated at slightly lower field (δ_c 105.04 for *R* and 105.02 for *S*). However, the greatest difference was observed for C-16 where the 15 *R* epimer resonated at δ_c 107.42 while the 15 *S* configuration resonated at a much lower field (δ_c 109.37). NMR data were in full agreement with those reported in Rosselli *et al*. (2004) for the *R* and *S* configuration of the 15-methoxy-hexahydrofuran *neo*-clerodane diterpenes (see Table 1 and 2). The presence of the 2-methylbutyryloxy functionality at C-3 was evident from the two methyl doublets at δ 1.18 and 1.10 (*d*, *J* = 6.6 and 7.0 Hz, for 4′ and 5′ CH_3, respectively). The presence of this functionality was also confirmed by the downfield signal due to a proton on a carbon atom bearing an oxygen at δ 5.13 and 5.14 (1H each, *d*, *J* = 9.0 and 9.5 Hz, H-3) for the C-15, *R* and *S* configuration**,** respectively. The ^{1}H-^{1}H COSY NMR spectra, as well as their proton coupling patterns, were efficient in interpretation of all correlated protons. ^{13}C NMR assignments were determined by DEPT spectra. On the basis of the above data, compound **2** was confirmed as a C-15 epimeric mixture of 14-hydro-15-methoxyajugachin B and was assigned the structure **2** shown in Fig 1.

Compound **3** was obtained as an amorphous white material. Its NMR spectral data showed that it was an equimolar mixture of the C-15 epimers. It has a molecular formula of $C_{31}H_{46}O_{13}$, deduced from its HR-ESI-MS (*m/z* 627.3019 $[M+1]^{+}$) and its IR showed absorption bands for free hydroxyl (3436 cm^{-1}) and ester groups (1733 and 1250 cm^{-1}). ^{1}H and ^{13}C NMR were in complete agreement with the data reported for chamaepitin (C-15 equimolar epimeric mixture) previously isolated from *Ajuga chamaepitys* (Camps *et al*. 1987). The ^{1}H, ^{13}C and 2D NMR enabled us to provide precise assignment for the protons and carbons of both the *R* and *S* configurations that were not fully assigned in the previously reported study. Our spectral data were in full agreement with the rules proposed by Rosselli *et al*. (2004) for *R* and *S* configurations of the 15-hydroxy hexahydrofuran *neo*-clerodane diterpenes (see Tables 1 and 2).

Compound **4** and **5** were identified according to their physical, chemical and spectral data, which were in full agreement with the reported data for ajugachin B (Boneva *et al*., 1990) and ajugapitin (Hernandez *et al*., 1982; Boneva *et al*., 1990).

Compound **6** was obtained as 1:2 mixture of the C-15 epimers (*R* and *S*, respectively) of the known *neo*-clerodane lupulin A previously isolated from *Ajuga lupulina* (Chen *et al*., 1996). The ^{1}H NMR data for the mixture shows that H-11, -13, -15 and -16 appear as pairs which differ in their chemical shifts as observed with our isolated 14-hydro-15-methoxyajugachin B

(**2**) and chamaepitin (**3**). Since the previously identified compound was the *S* epimer, it is worthy to report here the NMR signals that showed a great deal of difference between the two epimers: H-11 resonated at δ 4.03 and 4.41, H-13 at δ3.02 and 2.82, H-15 at δ 5.12 and 4.99 and H-16 at δ5.76 and 5.84 for the C-15, *R* and *S* -configurations, respectively. Likewise, in the ^{13}C NMR: δ_c C-11 resonated at 83.06 and 82.4 and C-16 resonated at 107.09 and 109.1 for the *R* and *S* configurations, respectively. This assignment was in total agreement with the reported rule for the 15-methoxy-hexahydrofurofuran *neo*-clerodane diterpenes (Rosselli *et al*., 2004).

It is of interest to note that many of the 15-hydroxy and 15-methoxy derivatives of *neo*-clerodane diterpenes having a hexahydrofuran functionality have been reported in *Ajuga* species and other plants as epimeric mixtures (Coll and Tandron, 2007; Rosselli *et al*., 2004). The separation of individual configurations from these mixtures is difficult using ordinary chromatographic methods. Assignment of the NMR data of both epimers in the mixtures was successfully performed utilizing 2D NMR and following the reported rules for 15-hydroxy- and methoxy-hexahydrofuran *neo*-clerodane diterpenes. (Rosselli *et al*., 2004).

3. Experimental

3.1. Plant material

Shoots and leaves of *A. turkestanica* were harvested in July 2004 from the mountainous regions of Uzbekistan (UIUC; ICBG Central Asia, voucher UPL_00057, ILLS, MO). All biological materials were transferred according to the ICBG material transfer agreement signed between Rutgers University and Uzbekistan on August 30th, 2004 and approved by the National Institutes of Health.

3.2. Extraction and isolation

Dried powdered plant tissue of *A. turkestanica* (500 g) was extracted with MeOH (3 × 1 L). After evaporation of solvent and addition of water (200 mL), the aqueous suspension was partitioned with pet ether (3 × 0.5 L), then with EtOAc (4 × 0.5 L) to afford 18.5 g and 11.9 g, respectively. The EtOAc extract on purification over silica gel column using n-hexane and n-hexane-EtOAc step gradient yielded **5** (20 mg), **4** (350 mg), **1** (180 mg), **3** (100 mg), **2** (25 mg) and **6** (2.8 mg), with this sequence of separations. Later fractions eluted with a step gradient of MeOH in EtOAc afforded phytoecdysteroids and iridoid glycosides (Cheng *et al.*, 2008).

14, 15-Dihydroajugachin B (1)—White amorphous powder, $[\alpha]^{20}_{D}$ -28 ($CHCl_3$; c 0.19); IR ν_{max} ($CHCl_3$) 3457 (OH), 2971, 1728 and1245 (ester) groups, 1369, 1026, 752 cm^{-1}. ^{1}H and ^{13}C NMR (see Tables 1 and 2, respectively). HR-ESI-MS *m/z* 611.363 (calc. for $C_{31}H_{47}O_{12}$ 611.3068) $[M+1]^+$; ESI-MS m/z (rel. int.): 611 $[M+1]^+$ (55), 593 (35), 575 (25), 551 (28), 515 (22), 433 (100), 391 (85), 313 (55). Hydrogenation of compound **4** over palletized charcoal (Barton *et al*., 1961) afforded 14, 15-Dihydroajugachin B. The optical rotation and NMR data observed for the obtained compound were identical to those data observed for the naturally isolated compound **1**.

14-hydro-15-methoxyajugachin B (2)—White amorphous material, IR ν_{max} ($CHCl_3$) 3436 (OH), 2956, 1732 and1245 (ester) groups, 1372, 1080, 749 cm^{-1}. ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively. HR-ESI-MS *m/z* 641.3182 (calc. for $C_{32}H_{49}O_{13}$ 641.3173) $[M+1]^+$; ESI-MS m/z (rel. int.): 658 $[M+H_2O]^+$ (100), 641 $[M+1]^+$ (65), 591 (40), 463 60), 421 (65), 389 (50), 371 (65), 329 (45), 311 (60).

Chamaepitin (14-hydro-15-hydroxyajugachin B) (3)—White amorphous material, IR, ^{1}H and ^{13}C NMR were in agreement with the published data (Camps *et al*., 1987). See

Tables 1 and 2 for the *R* and *S* configurations. HR-ESI-MS *m/z* 627.3019 (calc. for $C_{31}H_{47}O_{13}$ 627.3017) $[M+1]^+$; ESI-MS m/z (rel. int.): 644 $[M+ H_2O]+$ (100), 627 $[M+1]^+$ (50), 591 (35), 407 (70), 389 (85), 329 (60), 311 (50).

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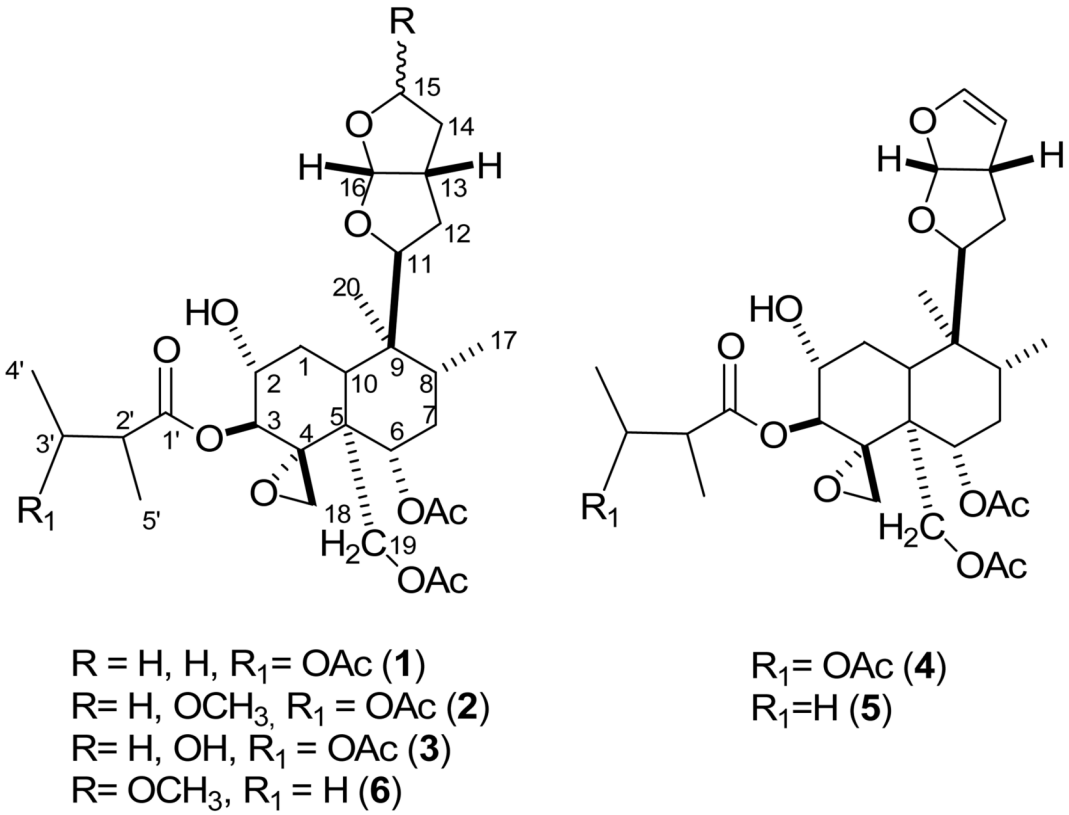

R = H, H, R_1= OAc (**1**)
R= H, OCH_3, R_1 = OAc (**2**)
R= H, OH, R_1 = OAc (**3**)
R= OCH_3, R_1 = H (**6**)

R_1= OAc (**4**)
R_1=H (**5**)

Fig. 1.
neo-Clerodane diterpenes (**1–6**) isolated from *Ajuga turkestanica*

Table 1

^{1}H NMR spectroscopic data δ_H m (*J* in Hz) for *neo*-clerodane diterpenes **1–3** ($CDCl_3$, 500 MHz) resonance assignments for methylene groups are given as "a" and "b", where a indicates the resonance with the lowest δ value.

H	(1)	2 (*R*)	2 (*S*)	3 (*R*)	3 (*S*)
1α	1.75 *m*	1.68*m*	1.68 *m*	1.70 *m*	1.70 *m*
1β	2.58 *m*	2.57*m*	2.57 *m*	2.56 *m*	2.56 *m*
2 β	3.65 *ddd* (9.5, 8.8, 4.9)	3.63*m*	3.63 *m*	3.60 *m*	3.60 *m*
3 α	5.16 *d* (9.5)	5. 13 *d* (9.0)	5.14 *d* (9.5)	5.11 *d* (9.5)	5.11 *d* (9.5)
6 β	4.67 *dd* (11.9,4.5)	4.66 *dd* (11.6,4.5)	4.66 *dd* (11.8,4.5)	4.64 *dd* (10.9,5.0)	4.64 *dd* (10.9,5.0)
7 α	1.62 *dd* (11.8,4.5)	1.60 *m*	1.60 *m*	1.57 *m*	1.57*m*
7 β	1.46 *m*	1.44 *m*	1.44 *m*	1.40 *m*	1.40 *m*
8 β	1.46*m*	1.50 *m*	1.5 *m*	1.48 *m*	1.48 *m*
10 β	1.75 *m*	1.78 m	1.78 *m*	1.70 *m*	1.70 *m*
11 α	4.09 *dd* (11.4,5.2)	3.97 *dd* (11.6,4.2)	4.35 *d* (11.3)	3.94 *dd* (11.2,4.2)	4.55 *dd* (11.2,5.7)
12a	1.82 *m*	1.60–1.75 *m*	1.60–1.75 *m*	1.52–1.78 *m*	1.52–1.78 *m*
12b	1.62 *m*	1.60–1.75 *m*	1.60–1.75 *m*	1.52–1.78 *m*	1.52–1.78 *m*
13 β	2.87 *m*	2.97 *m*	2.77 *m*	3.04 *m*	2.80 *m*
14a	2.16 *m*	2.16 *m*	2.22 *m*	2.22 *m*	2.13 *m*
14b	1.75 *m*	1.78 *m*	1.61 *m*	1.78 *m*	1.68 *m*
15	3.85 *m*	5.07 *d* (4.9)	4.94 *d* (5.4)	5.58 *d* (4.2)	5.47 *d* (5.7)
16 β	5.66 *d* (5.1)	5.71 *d* (5.6)	5.79 *d* (5.4)	5.78 *d* (5.4)	5.74 *d* (5.4)
17	0.86 *d* (6.1)	0.84 *d* (6.6)	0.85 *d* (6.6)	0.82 *d* (6.2)	0.84 *d* (6.2)
18b	2.78 *d* (4.3)	2.75 *d* (4.3)	2.77 *d* (4.3)	2.74 *d* (4.1)	2.74 *d* (4.1)
18a	2.52 *d* (4.3)	2.48 *d* (4.3)	2.48 *d* (4.3)	2.47 *d* (4.1)	2.47 *d* (4.1)
19a	4.78 *d* (12.5)	4.76 *d* (12.6)	4.76 *d* (12.6)	4.72 *d* (12.1)	4.72 *d* (12.1)
19b	4.25 *d* (12.5)	4.33 *d* (12.2)	4.33 *d* (12.2)	4.34 *d* (12.4)	4.34 *d* (12.4)
20	0.94 *s*	0.92 *s*	0.89 *s*	0.89 *s*	0.86 *s*
2′	2.58 *m*	2.57*m*	2.57 *m*	2.56 *m*	2.56 *m*
3′	5.14 *m*	5.14 *m*	5.14 *m*	5.08 *m*	5.08 *m*
4′	1.20 *d* (7.1)	1.18 *d* (6.6)	1.18 *d* (6.6)	1.16 *d* (6.4)	1.16 *d* (6.4)
5′	1.11 *d* (7.1)	1.10 *d* (7.0)	1.10 *d* (7.0)	1.07 *d* (7.0)	1.07 *d* (7.0)
OMe	-	3.30 *s*	3.30 *s*	-	-

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H	(1)	2 (*R*)	2 (*S*)	3 (*R*)	3 (*S*)
acetate	2.01 *s*	2.00 *s*	1.99 *s*	1.96 *s*	1.96 *s*
acetate	1.91 *s*	1.91 *s*	1.89 *s*	1.87 *s*	1.87 *s*
acetate	2.10 *s*	2.10 *s*	2.08 *s*	2.06 *s*	2.06 *s*

Table 2

^{13}C NMR spectroscopic data (δ_C in ppm) for *neo*-clerodane diterpenes **1–3** (125 MHz, $CDCl_3$)

C	(1)	2 (*R*)	2 (*S*)	3 (*R*)	3 (*S*)
1	29.8	30.5	29.8	30.0	29.9
2	72.9	73.1	73.2	71.6	71.6
3	71.6	71.6	71.6	72.8	72.8
4	63.0	63.0	63.1	63.0	63.0
5	46.0	45.8	45.7	45.7	45.7
6	71.2	71.2	71.4	71.1	71.1
7	33.2	33.3	33.4	33.2	33.4
8	35.8	36.1	36.0	35.9	35.7
9	42.3	40.6	40.7	41.1	41.1
10	43.4	43.8	43.6	43.6	43.5
11	84.8	83.1	82.9	83.4	83.0
12	32.7	32.6	32.8	32.3	32.9
13	40.5	40.1	40.1	40.1	40.2
14	32.7	38.2	39.7	38.9	39.9
15	68.5	105.0	105.0	98.7	98.5
16	107.9	107.4	109.3	107.5	109.4
17	16.6	16.6	16.5	16.8	16.6
18	42.1	42.3	42.4	43.5	43.6
19	61.3	61.3	61.4	61.3	61.4
20	13.9	13.9	14.0	13.9	14.0
1′	172.4	172.2	172.2	172.4	172.4
2′	45.7	46.4	46.4	46.0	46.0
3′	84.8	82.9	82.9	83.0	83.0
4′	17.3	17.6	17.6	17.3	17.3
5′	13.0	13.3	13.3	13.0	13.0
$\underline{C}H_3COO$	21.0	21.1	21.1	21.1	21.1
$\underline{C}H_3COO$	21.2	21.2	21.2	21.2	21.2
$\underline{C}H_3COO$	21.3	21.4	21.4	21.3	21.3

C	(1)	2 (*R*)	2 (*S*)	3 (*R*)	3 (*S*)
$\underline{C}H_3$-O	-	54.9	54.7	-	-
$CH_3\underline{C}OO$	170.3	170.2	170.2	170.3	170.3
$CH_3\underline{C}OO$	171.3	171.3	171.3	171.4	171.4
$CH_3\underline{C}OO$	171.4	171.4	171.4	171.4	171.4