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Guaianolides from two subspecies of *Amphoricarpos neumayeri* from Montenegro

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Abstract

Quantitative ^{1}H NMR measurements revealed $\Delta^{11(13)}$ sesquiterpene γ-lactones as the main constituents (⩾ 1% per weight of dried plant material) in the crude extracts of the aerial parts of *Amphoricarpos neumayeri* ssp. *neumayeri* and ssp. *murbeckii* from mountains Orjen and Visitor (Montenegro), respectively. Preparative silica gel chromatography afforded thirteen guai-11(13)-en-12,6α-olides, named amphoricarpolides (**1**–**13**), with the same relative (1αH,4βH,5αH,7βH) configuration of the basic skeleton. The common structural feature of lactones **2**–**13** was 3β,15-dioxygenation pattern. The only exception was **1** (3-deoxyamphoricarpolide), containing a single oxygen substituent (15-OH). Eight of them exhibited an additional oxygen substituent, 9β-OH (**5** and **6**), 2α-OH (**8**–**12**), or 2α-OAc (**13**). Compound **7** was epoxydated at 10α(14)-position, whereas the remaining lactones contained a 10(14) double bond.

Keywords: Amphoricarpos neumayeri; Asteraceae; Sesquiterpene lactones; Guaianolides; Amphoricarpolides

1. Introduction

The genus *Amphoricarpos* Vis. (Asteraceae), whose chemical examination is reported in this paper, is the endemic species of west part of Balkan peninusula, inhabiting cracks of carbonate rocks from central Bosnia to north-west Greece (Blečić and Mayer, 1967; Webb, 1972). Some species of the genus, *A. elegans* and *A. exsul*, can also be found in west Caucasus and south west Anatolia, respectively (Schwarz, 1970). Taxonomic position of the genus is still ambiguous. According to morphological and palynological characteristics it was assigned to following tribes: Carlineae Cass. (Dittrich, 1977), Carduae Cass. subtribe Carlininae Dumort. (Bremer, 1994) or Carduae *sensu lato* tribe Cardueae Cass. subtribus Carduinae Dumort. *Xeranthemum* group (series 1) (Petit et al., 1996). In their recent molecular cytogenetic study of the genera of *Xeranthemum* group, involving *Amphoricarpos*, *Chardinia* and *Siebera*, Garnatje et al. (2004) claimed that *Amphoricarpos*, with its most frequent chromosome number ($2n = 24$) appeared to be the most primitive in the group. In their examination of European *Amphoricarpos* complex Blečić and Mayer (1967) reported two endemic species: *A. neumayeri* Vis. and *A. autariatus* Blečić et Mayer, the latter comprising two subspecies, ssp. *autariatus* and ssp.

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bertisceus Blečić et Mayer. The occurrence of *A. neumayeri* is limited to coastal Montenegro mountains over Boka Kotorska Bay, Orjen and Lovćen, whereas *A. autariatus* could be found throughout the wider area. The taxon growing on mountains of Bosnia, Herzegovina and north west Montenegro was assigned as *A. autariatus* ssp. *autariatus* and the remaining one, mostly inhabiting mountain group Prokletije (situated between Montenegro, Kosovo and Albania) and the mountains of north Greece was denoted as *A. autariatus* ssp. *bertisceus*. On the other hand, Webb (1972) recognized only a single species, *A. neumayeri* Vis., divided in two subspecies, i.e. ssp. *neumayeri* and ssp. *murbeckii* Bošnjak (syn. *Amphoricarpos autariatus* Blečić & E. Mayer).

There is no report regarding the chemotaxonomic study of genus *Amphoricarpos* insofar. Since the knowledge of secondary metabolites could provide additional information about the systematics of this taxon, a phytochemical study of the genus *Amphoricarpos*, as part our examinations of the wild-growing highland species of Montenegro, was undertaken. In this paper the identification of the secondary metabolites of the aerial parts of *A. neumayeri* ssp. *neumayeri* and *A. neumayeri* ssp. *murbeckii* (Webb, 1972), collected at the mountains Orjen and Visitor, respectively, is reported.

2. Results and discussion

The ^{1}H NMR spectrum and HPLC (Fig. 1) of the crude extracts of the aerial parts, of both samples, obtained by the usual procedure (Bohlmann et al., 1984), revealed $\Delta^{11(13)}$ sesquiterpene lactones as the major constituents. According to quantitative ^{1}H NMR measurement, based on integrals of a low-field exomethylene one-proton doublet centered at $\delta \sim 6.2$ and two-proton singlet, δ 7.0, of 2,6-di-*t*-butyl-4-methylphenol (BHT), used as internal standard, the extracts contained $\geqslant 1\%$ of the lactones (calculated per weight of the dried plant material).

The isolation procedure, involving silica gel CC and prep TLC afforded 11 guaianolides (**1**–**5** and **7**–**12**) from *A. neumayeri* ssp. *neumayeri*. The application of 2D NMR methods applied on the majority of the isolated lactones enabled their ^{1}H and ^{13}C NMR spectral assignments. All lactones showed $[M + H]^+$ ions in DCIMS. The ^{1}H and ^{13}C NMR spectral data of **1**–**11** (Tables 1–3) indicated the same guaian-12,6α-olide skeleton. All compounds showed double doublet signals at the almost same chemical shift ($\delta \sim 4$), assigned as a lactone proton (H-6), exhibiting vicinal couplings ($J > 9$ Hz), usually observed in 5αH,6βH,7αH-guaianolides. The similar frequency of lactone carbonyl band (<1770 cm^{-1}), typical for α,β-unsaturated γ-lactones, together with two characteristic narrow doublets ($J \sim 3.0$–3.6 Hz) in the olefinic region ($\delta \sim 6.55$–5.55) of their ^{1}H NMR spectra, assigned to exomethylene protons (H_2-13), revealed the 11(13)-double bond. A pair of characteristic one-proton olefinic broad singlets in **1**–**5** and **8**–**12**, overlapped in some cases, ($\delta \sim 5.5$–5.2), identified as H_2-14, according to correlations observed in HMBC of **3**, **5** and **12** (see Fig. 1) and NOEs of one of these pro-

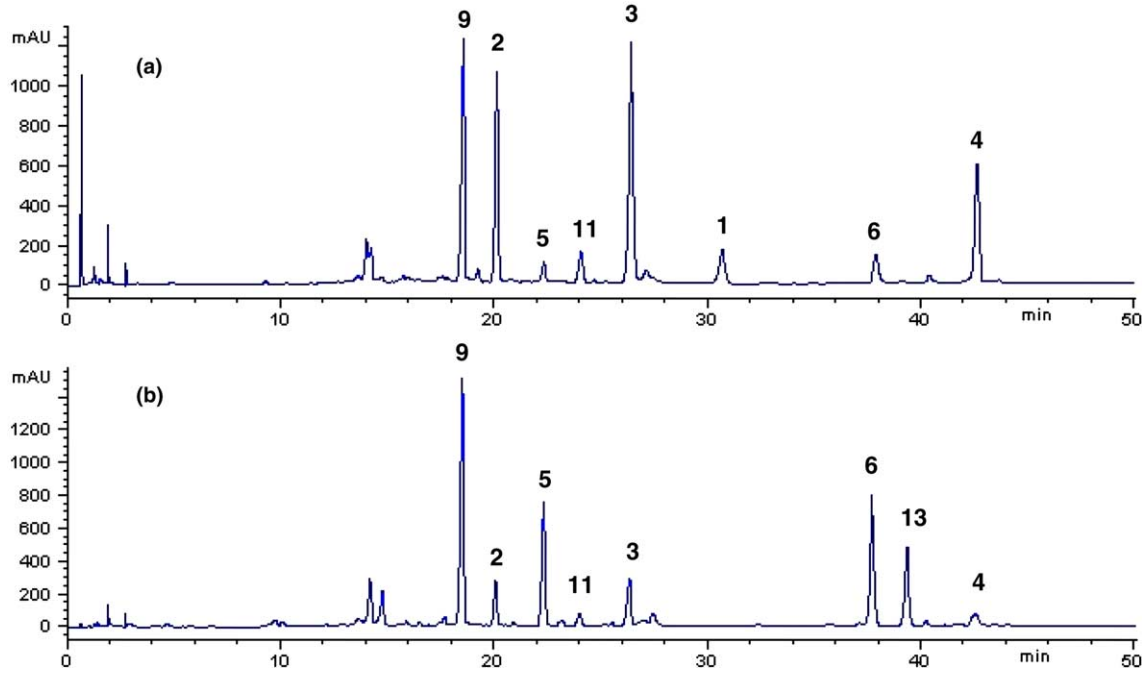

Fig. 1. HPLC profiles of crude extracts of *Amphoricarpos neumayeri* ssp. *neumayeri* from Orjen (a) and *A. neumayeri* ssp. *murbeckii* fromVisitor (b), both collected in July 2002; for chromatographic conditions see Section 3.1.

Table 1
^{1}H NMR ($CDCl_3$) chemical shifts, multiplicities and couplings (in parentheses) of compounds **1**–**7**

H	1 (500 MHz)	2 (200 MHz)	3 (200 MHz)	4 (500 MHz)	5 (6)[a] (500 MHz)	7 (500 MHz)
1	2.84 *brq* (~6)[b]	2.80 *brq* (~8.8)[b]	2.79 *brq* (~8.8)[b,d]	2.90 *brq* (~8.5)[b]; *ddd* (6.8, 9.5, 10.5)[c]	2.87 *brq* (~8.8)[b]; *ddd* (7.5, 8.0, 9.0)[c]	2.47 *ddd* (8.5, 10.0, 10.0)
2 α	1.91 *dddd* (3.0, 7.1, 7.1, 12.8)	2.0–2.4[d]	2.1–2.3[d]	2.41 *ddd* (6.7, 6.8, 12.8)	2.50 *ddd* (7.5, 7.5, 13.5)[d]	2.24–2.33[d]
2 β	1.72 *dddd* (7.0, 9.0, 10.4, 12.8)	1.83 *ddd* (10.5, 10.5, 12.2)	1.83 *brddd* (10.2, 10.2, 12.2)[b]	1.77 *ddd* (8.8, 10.5, 12.8)	1.83 *ddd* (8.0, 8.4, 13.5)	1.41 *ddd* (8.0, 10.0, 13.8)
3	1.99 *dddd* (3.1, 7.2, 7.2, 12.4) 1.46 *dddd* (7.4, 10.3, 10.3, 12.4)	4.0–4.2[d]	4.00[d,e]	5.03 *ddd* (6.7, 8.8, 8.8)	5.05 *q* (~8.5)[b]	4.95 *ddd* (7.0, 8.0, 8.0)
4	~2.24[d]	2.0–2.4[d]	2.1–2.3[d]	2.50 *dddd* (4.0, 4.8, 8.0, 8.8)	2.48 *m*[d]	2.42 *m*
5	2.15 *ddd* (7.5, 7.5, 10.1)	2.0–2.4[d]	2.1–2.3[d]	~2.30[d]	2.28 *ddd* (9.0, 9.5, 10.0)	2.24–2.33[d]
6	3.91 *dd* (9.2, 10.4)	3.99 *dd* (9.8, 9.8)	4.00[d,e]	3.99 *dd* (9.2, 10.6)	3.91 *dd* (9.5, 10)	4.16 *dd* (9.0, 11.0)
7	2.76 *m*	~2.7[d]	2.69[d]	2.74 *ddddd* (3.2, 3.5, 4.2, 9.2, 11.5)	2.78 *ddddd* (3.0, 3.5, 4.0, 9.5, 12.0)	2.85 *m*
8α	2.27 *dddd* (4.4, 4.4, 4.4, 13.1)	2.0–2.4[d]	~2.3[d]	~2.30[d]	2.60 *ddd* (3.5, 4.0, 12.5)	2.24–2.33[d]
8β	1.39 *dddd* (4.4, 11.5, 11.5, 13.1)	1.37 *dddd* (4.0, 12.0, 12.0, 12.0)	1.37 *dddd* (4.0, 11.6, 11.6, 12.5)	1.42 *dddd* (4.2, 11.5, 11.5, 13.2)	1.46 *ddd* (12.0, 12.0, 12.5)	1.48 *m*
9α	~2.05 *m*[d]	1.98 *ddd* (4.0, 12.0, 13.0)	1.98 *ddd* (4.0, 11.6, 13.0)	2.06 *ddd* (4.2, 11.5, 13.2)	4.21[d]	1.92 *dddd* (~1.5, 5.5, 9.3, 14.0)
9β	2.57 *ddd* (4.4, 4.4, 12.7)	2.64 *ddd* (4.2, 4.2, 13.0)	2.63[d]	2.63 *ddd* (4.4, 4.5, 13.2)		1.80 *ddd* (5.5, 6.5, 14.0)
13	6.19 *d* (3.5) 5.48 *d* (3.2)	6.18 *d* (3.4) 5.48 *d* (3.2)	6.17 *d* (3.6) 5.48 *d* (3.2)	6.21 *d* (3.5) 5.50 *d* (3.2)	6.25 *d* (3.5) 5.55 *d* (3.0)	6.25 *d* (3.5) 5.54 *d* (3.5)
14	4.93 *brs* 4.90 *brs*	4.98 *brs* 4.98 *brs*	4.98 *brs* 4.98 *brs*	5.00 *brs* 4.95 *brs*	5.56 *brs* 5.16 *d* (~1)	2.77 *dd* (1.5, 4.5) 2.62 *dd* (~0.5, 4.5)
15	3.68 *dd* (5.8, 10.7) 3.74 *dd* (5.9, 10.8)	3.7 *dd* (7.5, 10.4) 4.0–4.2[d]	4.54 *dd* (3.6, 11.4) 4.20 *dd* (5.0, 11.4)	4.32 *dd* (4.0, 11.4) 4.22 *dd* (4.8, 11.4)	4.38 *dd* (3.5, 11.5) 4.20[d]	4.28 *dd* (4.0, 11.0) 4.19 *dd* (5.5, 11.0)
OAc			2.10 *s*	2.08 *s* 2.09 *s*	2.11 *s* 2.08 *s*	2.05 *s* 2.05 *s*
OH	not visible	3.3 *brs*, 2H	~2.6[d]			

[a] The spectral data of **6** are almost identical to those of **5**, only differing in the signals of the ester residues, OAc: δ 2.10 (3H, *s*); O-*i*-Val: $\delta \sim$ 2.10 (1H, *m*), 2.22 (2H, *d*, 7.0 Hz), 0.98 (6H, *d*, 6.5 Hz).
[b] Peak separations (in parenteheses), not coupling constants.
[c] Multiplicities and couplings obtained by spectral simulation, using ACD/HNMR DB, version 3.0.
[d] Overlapped (partly or completely).
[e] Resolved in C_6 D_6, H-3: δ 3.74 *ddd* (6.6, 9.0, 9.0), H-6: 3.25 *dd* (9.2, 10.6).

Table 2
^{1}H NMR ($CDCl_3$) chemical shifts, multiplicities and couplings (in parentheses) of **8**–**13**

H	**8** (500 MHz)	**9** (200 MHz)	**10** (500 MHz)	**11** (500 MHz)	**12** (500 MHz)	**13** (200 MHz)
1	~2.79[a]	~2.7[a]	~2.65[a]	2.82 *brt* (~9.0)[b]	2.80 *brt* (~9.0)[b]; *ddd* (0.5, 8.2, 9.3)[c]	2.92 *brt* (~9.4)[b]
2	4.22 *brt* (~7.5)	4.06 *dd* (8.4, 9.8)	4.07 *dd* (~8.5,~10)	4.17 *brt* (~7.2)	4.15 *brt* (~7.5)[b]; *ddd* (0.7, 6.8, 9.0)[c]	5.42 *dd* (7.0, 9.0)
3	4.89 *dd* (7.1, 9.7)	3.71 *dd* (8.4, 9.8)	3.69 *dd* (8.4, 9.6)	4.84 *dd* (6.8, 9.2)	4.82 *dd* (6.8, 9.0)	5.20 *dd* (7.2, 9.0)
4	~2.20 *m*	~2.1	~2.1[a]	~2.45 *m*[a]	~2.45 *m*[a]	~2.50 *m*[a]
5	2.52 *q* (~10.5)	~2.3	~2.3[a]	~2.42 *m*[a]	~2.39 *m*[a]	~2.42 *m*[a]
6	4.00 *dd* (9.2, 10.4)	4.02 *dd* (9.0, 10.8)	4.02 *dd* (9.0, 10.4)	4.00 *dd* (9.2, 10.0)	3.99*dd* (9.0, 10.2)	4.08 *dd* (9.2, 10.2)
7	~2.7[a]	~2.7[a]	~2.7[a]	2.74 *m*	2.72 *m*	2.74 *m*
8α	2.29 *dq* (~13.0, ~5.0)	~2.3	~2.3[a]	2.28 *dq* (~13.0, ~5.0)[b]	2.26 *dq* (~13.2, ~5.0)[b]; *dddd* (4.5, 4.8, 5.0, 13.2)[c]	~2.30 *m*[a]
8β	1.43 *m*	1.42 *m*	1.42 *m*	1.45 *m*	1.43 *dddd* (4.8, 9.7, 11.4, 13.2)	~1.5 *m*
9α	2.11 *m*	~2.1	~2.1[a]	~2.12 *m*[a]	~2.12 *m*[a]	~2.12 *m*[a]
9β	2.59 *dt* (~13.0, ~5.0)	~2.7[a]	~2.7[a]	2.56 *dt* (~13.5, ~5.0)	2.54 *dt* (~13.2, ~5.0)[b]; *ddd* (4.8, 5.0, 13.2)[c]	~2.65 *m*[a]
13	6.22 *d* (3.4) 5.51 *d* (3.4)	6.20 *d* (3.6) 5.42 *d* (3.4)	6.20 *d* (3.4) 5.49 *d* (3.2)	6.21 *d* (3.6) 5.50 d (3.2)	6.21 *d* (3.6) 5.50 *d* (3.2)	6.22 *d* (3.4) 5.50 *d* (3.2)
14	5.09 *brs* 5.01 *brs*	5.10 *brs* (2H)	5.10 *brs* (2H)	5.09 *brs* 5.04 *brs*	5.07 *brs* 5.02 *brs*	5.03 *brs* 4.98 *brs*
15	3.87 *dd* (3.1, 11.7) 3.73 *dd* (3.6, 11.7)	4.60 *dd* (3.7, 11.4) 4.24 *dd* (4.3, 11.4)	4.63 *dd* (3.2, 11.4) 4.23 *dd* (4.4, 11.4)	4.34 *dd* (3.4, 11.4) 4.23 *dd* (4.0, 11.4)	4.33 *dd* (3.3, 11.4) 4.23 *dd* (4.2, 11.4)	4.31 *dd* (3.8, 11.6) 4.22 *dd* (4.3, 11.6)
OAc	2.16 *s* (3H)	2.12 *s* (3H)		2.09 *s* (3H)	2.15 *s* (3H)	2.03 *s* (3H) 2.07 *s* (3H) 2.09 *s* (3H)
O-*i*-Val			0.98 *d* (6.6) (6H) ~2.1 *m* (1H) 2.26 *d* (~7.0) (2H)		0.95 *d* (6.6) (6H) ~2.1 *m* (1H) 2.21 *d* (7.2) (2H)	
OH	2.30[b] 3.10 *brs*	1.80 *brs* 2.90 *brs*	1.60 *brs* 2.70 *brs*	3.49 *brs*	3.50 *brs*	

[a] Overlapped (partly or completely).
[b] Peak separations (in parentheses), not coupling constants.
[c] Multiplicities and couplings obtained via spectral simulation using ACD/HNMR DB, version 3.0.

Table 3
^{13}C NMR data of lactones **1**–**5**, **7**, **9**, **11**–**13** ($CDCl_3$, 50 MHz)

C	1	2	3	4	5	7	9	11	12	13
1	47.7	42.5	42.2	43.1	40.6	42.8	48.8[a]	50.8	51.0	48.8
2	30.3	37.9	37.7	35.6	35.6	32.8	77.0[b]	77.8	78.2	76.7[c]
3	29.3	76.0	73.2	74.7	74.8	74.7	77.3[b]	82.0	82.4	77.6[c]
4	46.9	52.5	50.5	47.4	47.6	48.3	42.1	43.5	43.8	43.8
5	48.7	45.8	45.2	45.3	45.0	45.4	47.8[a]	44.1	44.3	44.3
6	86.0	85.6	85.2	84.9	84.9	84.7	85.0	84.8	84.9	84.4
7	47.4	48.4	48.3	48.0	43.8	45.9	45.8	46.6	46.6	46.1
8	30.9	31.0	30.7	30.7	38.6	26.4	30.5	30.4	30.4	30.4
9	36.0	36.4	35.6	35.3	73.9	33.5	34.7	34.5	34.6	33.8
10	149.4	148.3	148.0	147.9	152.7	57.7	145.2	145.3	145.4	144.2
11	139.9	139.6	139.5	139.5	138.4	139.2	139.3	139.2	139.3	139.2
12	169.9	170.0	169.8	169.6	169.4	169.3	169.6	169.5	169.0	169.3
13	120.0	120.2	119.9	120.1	120.7	121.0	120.4	120.6	120.7	120.8
14	112.8	113.6	113.6	113.9	111.0	50.9	114.9	115.3	115.4	115.8
15	65.7	65.6	64.7	63.8	63.0	63.6	63.9	63.2	62.9	63.2
OAc			171.5	171.0	171.0	170.9	171.9	172.9	170.0	170.8
			20.8	170.8	170.9	170.5	21.0	170.9	21.0	170.5[d]
				21.0	21.0	21.1		20.9		20.8[d]
				20.8	21.0	20.9		20.8		23.8
O-*i*-Val									172.1	
									43.3	
									25.6	
									22.4	

[a–c] The assignments can be interchanged.
[d] Relative intensities of δ 170.5 and 20.8 indicate *ca* two carbons for each signal.

tons to H-9β or H-2β (**1**, **3**–**5**, **8**, **10**–**12**) was typical for exocyclic double bond (i.e. $\Delta^{10(14)}$). Instead of this, lactone **7** contained two mutually coupled one-proton doublets (δ 2.70 and 2.55, $J = 4.5$ Hz) assigned to H_2-14 of the 10(14)-epoxy moiety. Another common feature of all isolated lactones was a diastereotopic methylene (AM portion of an AMX system), coupled to a single proton (H-4, δ 2.0–2.5), assigned according to HMBC and NOESY as C(15)H_2–OH (δ 3.7–4.2) (**1**, **2**, **8**) or C(15)H_2–OAcyl (δ 4.2–4.6) (**3**–**5**, **7**, **9**–**12**).

Lactone **1** ($C_{15}H_{20}O_3$, ν_{max} 3397 cm^{-1}), 3-deoxyamphoricarpolide, exhibited the same gross structure as the previously reported (without relative stereochemistry) 15-hydroxydehydrocostus lactone (Saxena and Dixit, 1992), but the ^1H NMR spectral data of this lactone did not match those of **1** (see Table 1), which could be explained by different relative configuration. The NOEs H-6/H-4, H-6/H-8β , H-5/H-1 and H-5/H-7 were fully in accordance with 1αH,4βH,5αH,6βH,7αH-relative stereochemistry in **1**.

The molecular formula of lactone **2** ($C_{15}H_{20}O_4$), together with a strong OH absorption in the IR (3408 cm^{-1}), and the ^1H NMR signals assigned to CH_2OH and CHOH moieties ($\delta \sim 3.3$, *brs*, $2 \times$ OH; δ 3.6–4.2, 3H) indicated a diol structure. This was also confirmed by acetylation of **2** (Ac_2O/pyridine), yielding the co-occurring diacetate **4**. The gross structure and stereochemistry of **2**, i.e. 3βOH, 4βH, was determined indirectly via 2D NMR study of **4** (vide infra). It should also be noted that the gross structure of **2** was identical to that of the previously reported 15-hydroxyzaluzanin C (Spring et al., 1997, 1999). However, different chemical shifts of H-3 and H_2-15 in comparison to those reported for 15-hydroxyzaluzanin could be interpreted in terms of different relative configuration at C-4.

Diacetate **4** ($C_{19}H_{24}O_6$) exhibited two three-proton singlets (δ 2.08 and 2.09) and protons α-positioned to the OAc groups, assigned as H-3 (δ 5.03 *ddd*, 6.7, 8.8, 8.8) and H_2-15 (δ 4.32, *dd*, 4.0, 11.4 Hz; δ 4.22, *dd*, 4.8, 11.4 Hz; AM pattern mentioned above), according to their COSY correlations to the same proton (i.e. H-4, δ 2.50 *dddd*, 4.0, 4.8, 8.0, 8.8). The remaining COSY correlations of H-3 to the adjacent methylene (H_2-2), as well as its multiplicity, was in accordance with the proposed substitution pattern of the five-membered ring. NOESY correlations of **4**, such as H-6/H-4, H-3/H-1, H-3/H-2α and H-15/H-5, revealed 3β-position of the acetoxy group and α-orienation of C(15)H_2OAc moiety.

The ^1H NMR spectrum of **3** ($C_{17}H_{22}O_5$) was rather similar to that of diacetate **4**, differing from the latter in the presence of a hydroxyl (3446 cm^{-1}) instead of one OAc group. A diamagnetic shift of H-3 ($\Delta\delta \sim 1$ ppm) (in comparison to the chemical shift of H-3 in **3**) indicated C-3 as the hydroxylation site. At the same time, an acetoxy singlet (δ 2.10 *s*, 3H), associated with the chemical shift of H_2-15 (δ 4.20 and 4.54), typical for the methylene bearing OAc group, revealed 15-OAc substitution. Scalar couplings of H-3 in **3** (observed in C_6D_6 spectrum, see footnote 'e' under Table 1),

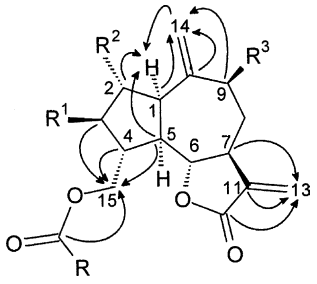

Fig. 2. Significant HMBC (C → H) correlations of **3** (R = Me, R^1 = OH, R^2 = R^3 = H), **5** (R = Me, R^1 = OAc, R^2 = H, R^3 = OH), **6** (R = CH_2CHMe_2, R^1 = OAc, R^2 = H, R^3 = OH) and **12** (R = CH_2CHMe_2, R^1 = OAc, R^2 = OH, R^3 = H).

almost identical to those in **4**, were in agreement with 3αH,4βH relative configurations, same as in **2** and **4**. This was also supported by occurrence of NOEs such as H-3/H-1 and H-6/H-4. The structures of **2**–**4** were also supported by their ^{13}C NMR data (Table 3).

Lactone **5** ($C_{19}H_{24}O_7$) contained two acetoxy functions (2 × 3H, 2 × *s*, δ 2.11, 2.08) and a hydroxyl group (3463 cm^{-1}). Occurrence of low-field signals, assigned using COSY and INEPT edited HSQC, as H-3 (δ 5.05, *q*, ∼8.5) and H_2-15 (δ 4.38 and ∼4.20) indicated C-3 (δ 74.8) and C-15 (δ 63.0) as those bearing the ester groups. The signal of the proton α-positioned to the hydroxyl (δ ∼ 4.21, overlapped with the high-field signal of H_2-15) was assigned as H-9 on the basis of its COSY correlations to H_2-8, as well as comparison of ^{1}H and ^{13}C NMR data of the seven-membered ring with those of the co-occurring lactones. Substitution of H-9β with OH resulted in simplification of multiplicity of H-8β (δ 1.46, *ddd*) and H-8α (δ 2.60, *ddd*) due to the removal of vicinal couplings with H-9β (Table 1). Couplings of H-9 to the (pseudo)axial H-8β ($J_{9,8\beta}$ = 12.0 Hz) and (pseudo)equatorial H-8α ($J_{9,8\alpha}$ = 3.5 Hz), as well as the NOESY correlations of H-9 to α-positioned protons (i.e. H-1, H-7 and H-8α) fully accorded with (pseudo)equatorial 9β-OH geometry. Accordingly, lactone **5** was assigned as 3,15-di-*O*-acetyl-9β-hydroxyamphoricarpolide.

The ^{1}H and ^{13}C NMR of **7** ($C_{19}H_{24}O_7$) indicated, in addition to 10(14) epoxide (mentioned above), the presence of two acetoxy groups (2 × 3H, 2 × *s*, δ ∼ 2.05). As evident from the conversion of **4** into **7** (using *m*-CPBA), the substitution pattern in **7**, as well as the relative configuration were identical in these lactones. In addition, the epoxidation of **4** afforded 10-epimer of **7** as the minor product. The configuration of the 10(14)-epoxy ring in **7** was established as α, according to the NOESY correlation of one of the H_2-14 protons (δ 2.77) to β-positioned H-6. The remaining ^{1}H and ^{13}C NMR data of **7** (Tables 1 and 3) also fit to the proposed structure.

The coupling patterns of the ring protons in **8**–**12** were almost identical, thus indicating the same basic structure and relative configurations of these compounds. As evident from the occurrence of one-proton broadened triplet (δ 4.06–4.22, Table 2), coupled (according to COSY) to H-1 and H-3, characteristic for a carbinol proton, these lactones contained a hydroxyl positioned at C-2. Hydroxylation of C-2 also affected multiplicity of the signals of H-3 (*dd*, 6.8–8.4, 9.0–9.8 Hz) and H-1 (*brt*, ∼9.0 Hz), causing their simplification in comparison to their multiplicity in **2**–**7**. The NOEs such as H-2/H-4, H-2/H-9β, H-6/H-4, H-3/H-1 (observed in the NOESY of **12**) indicated 2βH,3αH,4βH-configuration. According to ^{1}H and ^{13}C NMR evidence (Tables 2 and 3) compounds **9** ($C_{17}H_{22}O_6$) and **10** ($C_{20}H_{28}O_6$) contained a 3β-hydroxyl (δ ∼ 3.70, H-3), and an ester function at C-15 (δ ∼4.60, 4.20, H_2-15). The nature of the ester residues in **9** (OAc) and **10** (O-*i*-Val), was evident from the characteristic NMR signals of these groups (Tables 2 and 3) and the molecular formulas of these lactones. Thus, the structures of 15-*O*-acetyl- and 15-*O*-isovaleroyl-2α-hydroxyamphoricarpolide could be assigned to **9** and **10**, respectively. Lactone **8** with the same molecular formula as monoacetate **9**, was assigned as 3-*O*-acetyl-2α-hydroxyamphoricarpolide on the basis of upfield shift of H_2-15 (δ 3.73, 3.83) and downfield shift of H-3 (δ 4.89) in comparison to the chemical shifts of the same protons in **9**. Occurrence of two acetoxy methyl singlets (δ 2.09) and in the ^{1}H NMR spectrum of **11** ($C_{19}H_{24}O_7$), along with the chemical shifts of H_2-15 (4.34, 4.23) and H-3 (δ 4.84), typical for the protons in α-position to the ester group, revealed the structure of 3,15-di-*O*-acetyl-2α-hydroxyamphoricarpolide. Lactone **12** ($C_{22}H_{30}O_7$), a mixed diester, exhibited almost identical NMR pattern for H_2-15 and H-3 as in **11**, which indicated the same positions of the ester groups. The nature of the ester side chains was evident from the ^{1}H and ^{13}C NMR spectra exhibiting signals of acetate and isovalerate (Tables 2 and 3). The three-bond HMBC correlation of the isovalerate carbonyl carbon (δ 172.0) to H_2-15 (Fig. 2) was in accordance with the structure of 15-*O*-isovaleroyl-3β-*O*-acetyl-2α-hydroxyamphoricarpolide.

The identification of lactones **2**–**5** and **9** in *A. neumayeri* ssp. *murbeckii* was mostly based on HPLC (Fig. 1), assigned using coinjection of the compounds isolated from *A. neumayeri* ssp. *neumayeri*. Lactones **6** and **13** were isolated preparatively by the combination of chromatographic techniques (silica gel CC and TLC) same as those applied in the previous case and identified by the spectral data.

The mixed diester **6** showed $[M + H]^+$ ion in ESIMS at *m*/*z* 407, corresponding to the molecular formula $C_{22}H_{30}O_7$. The ^{1}H and ^{13}C NMR spectra of **6** were almost identical to those of diacetate **5**, thus indicating the same basic structure of 3,15-di-*O*-acyl-9β-hydroxyamphoricarpolide. The ester side chains were identified as acetate (3H, δ 2.10, *s*) and isovalerate (6H, δ 0.95, *d* 6.5 Hz; 1H, δ ∼ 2.1, *m*; 2H, δ 2.22 *d* 7.0 Hz). The three-bond HMBC

correlations of the ester carbonyl carbons, i.e. the isovalerate (δ 173.0) to H_2-15 and the acetate ($\delta \sim 171.0$) to H-3 (Fig. 2) were in agreement with the structure of 15-*O*-isovaleroyl-3β-*O*-acetyl-2α-hydroxyamphoricarpolide. Although lactone **6** was not isolated from *A. neumayeri* ssp. *neumayeri*, according to HPLC (Fig. 1), its presence is also evident in this subspecies.

The overall similarity of ^{1}H and ^{13}C NMR spectra of **13** ($C_{21}H_{26}O_8$) to those of diesters **11** and **12**, together with the occurrence of three-proton singlets from the acetates (δ 2.05, 2.07, 2.09) and the paramagnetic shift of H-2 to δ 5.42 in comparison to $\delta \sim 4.1$–4.2 observed in 2α-OH derivatives (Table 3, **8**–**12**), indicated the structure of *3,15-di-O-acetyl-2α-acetoxyamphoricarpolide* for this lactone.

2.1. Chemotaxonomic aspects

According to ^{1}H NMR and HPLC (Fig. 1), the both studied taxa of *Amphoricarpos*, exhibited very similar sesquiterpene lactone pattern, differing in the relative amounts of the constituents. All identified lactones are of guaianolide type, representing one of the largest groups among the sesquiterpene lactones from Asteraceae, which is generally characteristic of the sesquiterpene lactones with a low complexity on biogenetic level. Superimposed on the type of skeleton, more or less significant for the taxonomy, is a set of taxon-specific substituents. The common feature of all guaianolides isolated from the studied taxa was a free or esterified (as acetate or isovalerate) hydroxyl group at α-positioned C(15)H_2. At the same time, guainolides **2**–**13** were oxygenated at 3β-position. Since, according to our knowledge, this 15,3β-disubstitution pattern together with 3αH,4βH-relative configuration is rather unique (not detected before in Cardueae, as well as in other tribes of Asteraceae) these guaianolides are named amphoricarpolides. According to HPLC (Fig. 1), among the major lactones in both taxa was trioxygenated 15-*O*-acetyl-2α-hydroxyamphoricarpolide (**9**). The observed differences between the taxa, such as predominance of 15,3β-dioxygenated guaianolides **2**–**4** in ssp. *neumayeri*, higher abundance of 3β,9β,15-trioxygenated lactones (**5** and **6**) in ssp. *murbeckii* and absence of **1** in the latter (Fig. 1), are not sufficient to enable conclusion about the classification of the studied taxa. The chemotaxonomic examination of the members of *Amphoricarpos* complex from other localities within the habitat will be necessary.

3. Experimental

3.1. General

Dry-column flash chromatography (DCFC) and column chromatography (CC): silica gel 60 (Merck), under 0.063 mm. TLC: Kieselgel 60 GF_{254}, layer thickness 0.25 mm. IR: transparent dry films (Perkin–Elmer FT IR spectrometer 1725X). ^{13}C and ^{1}HNMR: at 50 and 200 MHz, respectively (Varian Gemini 2000) and 125/500 MHz (Bruker DMX 500). DCIMS: double focusing mass spectrometer (Finnigan MAT 8230). ESIMS double focusing mass spectrometer + electro spray interface (Finnigan MAT 900). Elemental analysis (Elementar). Analytical HPLC (HP1090) + Diode array detector (HP 1040), $\lambda = 210$ nm, column, Lichrosorb RP 18 (5 μm), 250 × 4 mm ID flow rate, 1 ml/min, mobile phase, A (H_2O) + B(MeCN), elution, combination of gradient and isocratic modes: 90% A, 0–5 min, 90–65% A, 5–20 min, 65% A, 20–30 min, 65–50% A, 30–40 min, 50% A, 40–60 min. The samples, prepared by dissolving 7 mg of the extracts (obtained by the procedure described in 3.3, first paragraph) in 1 ml of MeOH were injected through a sample loop (20 μl).

3.2. Plant material

Two samples (#1 and #2) of the aerial parts of *A. neumayeri* ssp. *neymayeri* were collected during the flowering (July) 2001 and 2002, respectively, at exactly the same locality at mountain Orijen (altitude of ca. 1750 m), situated at the Adriatic coast (Montenegro). Voucher specimen (BEOU AN72001) was deposited in the herbarium of the Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade. The sample of *A. neumayeri* ssp. *murbeckii* (Voucher, BEOU Anm72002) was also collected during the flowering (July) 2002 at mountain Visitor (altitude of ca. 1900 m), situated over the town Plav, Montenegro.

3.3. Extraction and isolation (*A. neumayeri ssp. neumayeri*)

A crude extract (16 g) of air-dried aerial parts (sample #1, 500 g) was obtained by two successive extractions with freshly distilled solvents (4.5 l): Et_2O–petrol–MeOH (1:1:1) at room temperature, followed by treatment with MeOH to remove long chain saturated hydrocarbons by the usual procedure (Bohlmann et al., 1984).

The whole quantity of the crude extract was applied to a dry-column flash chromatography and the elution was started with petrol. The polarity of the solvent was gradually increased first by addition of Et_2O (up to 100%) and than with MeOH (up to 30%).

Lactone **1** (6 mg) was isolated from the fraction eluted with Et_2O–MeOH, 9.5:0.5, followed by CC (CH_2Cl_2–MeOH, 9.5:0.5) and prep TLC (CH_2Cl_2–MeOH, 9.5:0.5, twice). Lactone **2** (40 mg) was isolated from the fraction eluted with Et_2O–MeOH, 8:2, after two CCs ($CHCl_2$–MeOH, 9.5:0.5). Lactone **3** (30 mg), eluted with Et_2O–MeOH, 9:1, was purified by silica gel CC (elution started with CH_2Cl_2 and polarity was

gradually increased by addition of MeOH), followed by prep TLC (CH_2Cl_2–MeOH, 9.5:0.5). Lactone **4** (180 mg) crystallised directly from the fraction eluted with neat Et_2O. Lactone **5** (30 mg) was isolated from the fraction eluted with Et_2O–MeOH, 8:2, after CC (CH_2Cl_2–MeOH, 9.5:0.5) followed by prep TLC (CH_2Cl_2–MeOH, 9.4:0.6). Lactone **7** (3 mg), isolated from the fraction eluted with Et_2O–MeOH, 8.5:1.5, was purified by means of CC ($CHCl_2$–MeOH, 9.5:0.5) followed by prep TLC ($CHCl_2$–MeOH, 9.6:0.4). Lactones **8** (3 mg) and **9** (3 mg) were isolated from fraction eluted with Et_2O–MeOH, 8.5:1.5 after two CCs (elution in the first CC started with CH_2Cl_2, polarity increased with MeOH and in the second CC CH_2Cl_2–MeOH, 9.5:0.5 was used), followed by prep TLC (petrol–EtOAc–MeOH, 4.5:4.5:1). Lactone **9** (17 mg) was isolated after CC (CH_2Cl_2–MeOH, 9.5:0.5), followed by prep TLC (petrol–EtOAc–MeOH, 4.5:4.5:1, twice), from the fraction eluted with Et_2O–MeOH, 8:2. Lactones **11** (6 mg) and **12** (12 mg), eluted with Et_2O–MeOH, 9:1, were purified by CC (elution started with CH_2Cl_2 and polarity was gradually increased by addition of MeOH) followed by prep TLC (CH_2Cl_2–MeOH, 9.5:0.5).

3.4. Extraction and isolation (*A. neumaery ssp. murbeckii*)

The same extraction procedure as in 3.3, applied on the air-dried ground plant material (190 g), followed by treatment wit MeOH, yielded 9.5 g of the crude extract. The application dry-column flash chromatography (as in 3.3) afforded several fractions of different polarity.

Silica gel CC (CH_2CCl_2–MeOH, 9.5:0.5) of the fraction (Et_2O–MeOH, 9:1) from dry-column flash chromatography, yielded **6** (74 mg). Lactone **13** (38 mg) was isolated in form of gum from the fraction eluted with petrol–Et_2O, 1.5:8.5, after prep TLC (CH_2Cl_2–MeOH, 9.5:0.5).

3.5. 3-Deoxymphoricarpolide (*1*)

Colourless oil; $[\alpha]_D^{25} + 12.4°$ ($CHCl_3$; *c* 0.25); IR ν_{max}^{film} cm^{-1}: 3397 (OH), 3077, 1666, 1637 (C=CH), 1765 (C=O, conjugated γ-lactone); ^1H and ^{13}C NMR (see Tables 1 and 3, respectively; DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 497 $[2M + H]^+$ (27), 249 $[M + H]^+$ (100), 431 $[M + H\text{-}18]^+$ (15)); HRCIMS: *m/z* 249.1481 (calc. for $C_{15}H_{21}O_3$ 249.1491).

3.6. Amphoricarpolide (*2*)

Colourless gum; $[\alpha]_D^{25} + 24.5°$ ($CHCl_3$; *c* 0.22); IR ν_{max}^{film} cm^{-1}: 3408 (OH), 3070, 1665, 1639 (C=CH), 1761 (C=O, conjugated γ-lactone); ^1H and ^{13}C NMR (see Tables 1 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 265 $[M + H]^+$ (100); Elemental analysis (Found: C, 68.31; H, 7.42. $C_{15}H_{20}O_4$ requires: C, 68.16; H, 7.63%).

3.7. 15-O-Acetylamphoricarpolide (*3*)

Colourless oil; $[\alpha]_D^{25} - 40.5°$ ($CHCl_3$; *c* 0.44); IR ν_{max}^{film} cm^{-1}: 3456 (OH), 3088, 1665, 1639 (C=CH), 1767 (C=O, conjugated γ-lactone), 1735, 1250 (OAc); ^1H and ^{13}C NMR (see Tables 1 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 613 $[2M + H]^+$ (25), 307 $[M + H]^+$ (100), 289 $[M + H\text{-}18]^+$ (10), 247 $[M + H\text{-}60]^+$ (7.5), 229 $[M + H\text{-}60\text{-}18]^+$ (6); Elemental analysis (Found: C, 66.41; H, 7.31. $C_{17}H_{22}O_5$ requires: C, 66.65; H, 7.24%).

3.8. 3,15-Di-O-acetylamphoricarpolide (*4*)

Colourless crystals (from Et_2O), m.p. 105–106 °C; $[\alpha]_D^{25} + 16.5°$ ($CHCl_3$; *c* 0.20); IR ν_{max}^{film} cm^{-1}: 1751 (C=O, conjugated γ-lactone), 1740, 1247 (OAc), 1665, 1639 (C=CH); ^1H and ^{13}C NMR (see Tables 1 and 2, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 697 $[2M + H]^+$ (3), 349 $[M + H]^+$ (100), 289 $[M + H\text{-}60]^+$ (15); Elemental analysis (Found: C, 66.20; H, 7.38. $C_{19}H_{24}O_6$ requires: C, 65.50; H, 6.94%).

3.9. 3,15-Di-O-acetyl-9β-hydroxyamphoricarpolide (*5*)

Colourless gum; $[\alpha]_D^{25} + 3.4°$ ($CHCl_3$; *c* 0.25); IR ν_{max}^{film} cm^{-1}: 3443(OH), 1764 (C=O, conjugated γ-lactone), 1736, 1248 (OAc); ^1H and ^{13}C NMR (see Tables 1 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 365 $[M + H]^+$ (90), 289 $[M + H\text{-}18]^+$ (8), 305 $[M + H\text{-}60]^+$ (100), 245 $[M + H\text{-}2 \times 60]^+$ (33); Elemental analysis (Found: C, 62.75; H, 6.47. $C_{19}H_{24}O_7$ requires: C, 62.63; H, 6.64%).

3.10. 15-O-Isovaleroyl-3β-O-acetyl-9β-hydroxyamphoricarpolide (*6*)

Viscous oil; $[\alpha]_D^{25} + 7.9$ ($CHCl_3$; *c* 0.9); IR ν_{max}^{film} cm^{-1}: 3454(OH), 1768 (C=O, conjugated γ-lactone), 1737, 1248 (*O-i*-Val, *OAc*); ^1H NMR (see Table 1); ESIMS (MeOH–H_2O, 1:1 + 1% NH_4OAc), *m/z* (rel. int.): 835.4 $[2M + NH_4]^+$ (16), 813.4 $[2M + H]^+$ (39), 429.2 $[M + Na]^+$ (31), 424.2 $[M + NH_4]^+$ (18), 407.2 $[M + H]^+$ (65), 347.2 $[M + H\text{-}60]^+$ (100), 305.1 $[M + H\text{-}102]^+$ (13), 245.1 $[M + H\text{-}60\text{-}102]^+$; Elemental analysis (Found: C, 65.09; H, 7.32. ($C_{22}H_{30}O_7$) requires: C, 64.99; H, 7.44%).

3.11. 3,15-Di-O-acetyl-10α(14)-epoxyamphoricarpolide (*7*)

Colourless gum; $[\alpha]_D^{25} + 12.3$ ($CHCl_3$; *c* 0.13); IR ν_{max}^{film} cm^{-1}: 3443 (OH), 1764 (C=O, conjugated γ-lac-

tone), 1737, 1246 (OAc); ^{1}H and ^{13}C NMR (see Tables 1 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 365 $[M + H]^+$ (100), 305 $[M + H\text{-}60]^+$ (62.5), 245 $[M + H\text{-}2 \times 60]^+$ (13); HRCIMS: *m/z* 365.1608 (calc. for $C_{19}H_{25}O_7$, 365.1600).

3.12. 3-O-Acetyl-2α-hydroxyamphoricarpolide (**8**)

Colourless oil; $[\alpha]_D^{25} + 13.6°$ ($CHCl_3$; *c* 0.28); IR ν_{max}^{film} cm^{-1}: 3456 (OH), 3088, 1665, 1639 (C=CH), 1760 (C=O, conjugated γ-lactone), 1725sh, 1254 (OAc); ^{1}H NMR (see Table 2); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 323 $[M + H]^+$ (100), 305 $[M + H\text{-}18]^+$ (∼2), 263 $[M + H\text{-}60]^+$ (20); HRCIMS: *m/z* 323.1489 (calc. for $C_{17}H_{23}O_6$, 323.1495).

3.13. 15-O-Acetyl-2α-hydroxyamphoricarpolide (**9**)

Colourless gum; $[\alpha]_D^{25} - 60.3°$ ($CHCl_3$; *c* 0.34); IR ν_{max}^{film} cm^{-1}: 3427(OH), 3019, 1642 (C=CH), 1761 (C=O, conjugated γ-lactone), 1740sh, 1257 (OAc); ^{1}H and ^{13}C NMR (see Tables 2 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 323 $[M + H]^+$ (100), 305 $[M + H\text{-}18]^+$ (9.5), 263 $[M + H\text{-}60]^+$ (47), 245 $[M + H\text{-}60\text{-}18]^+$ (11.5); Elemental analysis (Found: C, 63.47; H, 7.01. $C_{19}H_{24}O_7$ requires: C, 63.34; H, 6.88%).

3.14. 15-O-Isovaleroyl-2α-hydroxyamphoricarpolide (**10**)

Colourless gum; $[\alpha]_D^{25} - 7.5°$ ($CHCl_3$; *c* 0.28); IR ν_{max}^{film} cm^{-1}: 3416 (OH), 1674, 1652 (C=CH), 1763 (C=O, conjugated γ-lactone), 1736 (O-*i*-Val); ^{1}H NMR (see Table 2); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 365 $[M + H]^+$ (100), $[M + H\text{-}102]^+$ (41); HR CIMS *m/z* 365.1971 (calc. for $C_{20}H_{29}O_6$, 365.1964).

3.15. 3,15-Di-O-acetyl-2α-hydroxyamphoricarpolide (**11**)

Colourless gum; $[\alpha]_D^{25} + 21.9°$ ($CHCl_3$; *c* 0.36); IR ν_{max}^{film} cm^{-1}: 3453 (OH), 3062, 1671, 1641 (C=CH), 1761 (C=O, conjugated γ-lactone), 1740sh, 1257 (OAc); ^{1}H and ^{13}C NMR (see Tables 2 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 729 $[2M + H]^+$ (67), 365 $[M + H]^+$ (100), 347 $[M + H\text{-}18]^+$ (11), 305 $[M + H\text{-}60]^+$ (27), 287 $[M + H\text{-}60\text{-}18]^+$ (16), 245 $[M + H\text{-}2\times60]^+$ (9.5); HRCIMS: *m/z* 365.1594 (calc. for $C_{19}H_{25}O_7$, 365.1600).

3.16. 15-O-Isovaleroyl-3β-O-acetyl-2α-hydroxyamphoricarpolide (**12**)

Colourless gum; $[\alpha]_D^{25} + 14°$ ($CHCl_3$; *c* 0.25); IR ν_{max}^{film} cm^{-1}: 3463 (OH), 1674, 1650 (C=CH), 1768 (C=O, conjugated γ-lactone), 1736 (O-*i*-Val); ^{1}H and ^{13}C NMR (see Tables 2 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 813 $[2M + H]^+$ (13) 407 $[M + H]^+$ (100), 347 $[M + H\text{-}60]^+$ (19), 305 $[M + H\text{-}102]^+$ (18.5), 245 $[M + H\text{-}102\text{-}60]^+$ (27.5); Elemental analysis (Found: C, 64.80; H, 7.63. $C_{22}H_{30}O_7$ requires: C, 65.01; H, 7.44%).

3.17. 3,15-Di-O-acetyl-2α-acetoxyamphoricarpolide (**13**)

Colourless gum; $[\alpha]_D^{25}17.5°$ ($CHCl_3$; *c* 0.85); IR ν_{max}^{film} cm^{-1}: 1643 (C=CH), 1766 (C=O, conjugated γ-lactone), 1744, 1245 (OAc); ^{1}H and ^{13}C NMR (see Tables 2 and 3, respectively); DCIMS (*iso*-butane probe), 150 eV, *m/z* (rel. int.): 813 $[2M + H]^+$ (28), 407 $[M + H]^+$ (100), 347 $[M + H\text{-}60]^+$ (16.5), 287 $[M + H\text{-}2x60]^+$ (<1%), 227 $[M + H\text{-}3 \times 60]^+$ (2); HRCIMS: *m/z* 407.1715 (calc. for $C_{21}H_{27}O_8$, 407.1706).

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