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ent-Labdane glycosides from the aquatic plant *Potamogeton lucens* and analytical evaluation of the lipophilic extract constituents of various *Potamogeton* species

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Abstract

Two new *ent*-labdane glycosides, one known furano-*ent*-labdane and a new hydroxylated fatty acid were isolated from the dichloromethane extract of the freshwater aquatic plant *Potamogeton lucens*. The new compounds were assigned the structures of β-D-glucopyranosyl-8(17),13-*ent*-labdadien-16,15-olid-18-oate, 18-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide and 13(*R*)-hydroxy-octadeca-(9*Z*,11*E*,15*Z*)-trien-oic acid by spectroscopic means. The algicidal activity of these compounds was tested against *Raphidocelis subcapitata*. Based on our previous study of *Potamogeton pectinatus*, other constituents were identified in *P. lucens* by LC–UV–MS, LC–NMR and GC–MS. The lipophilic extract profiles of both species are presented. Two other species, *Potamogeton perfoliatus* and *P. crispus*, were also investigated by analytical comparison of their non-polar extracts. The distribution of *ent*-labdanes characterized in *Potamogeton* is summarized.

Keywords: *Potamogeton lucens*; *P. pectinatus*; *P. perfoliatus*; *P. crispus*; Potamogetonaceae; Spectroscopic analysis; LC–MS; LC–NMR; *ent*-Labdane diterpenes; Glycosides; Algicidal activity

1. Introduction

As a part of our ongoing research on new active compounds from aquatic macrophytes of Switzerland (Waridel et al., 2003), we have investigated the pondweed *Potamogeton lucens* L. from Lake Léman. This species is not as widespread in this lake as *Potamogeton pectinatus*, but its distribution has been stable since 1975 (Demierre and Durand, 1999; Lehmann and Lachavanne, 1999), which indicates that it tolerates water eutrophication, a tolerance perhaps mediated by specific allelochemicals. Various articles have shown that *ent*-labdane diterpenes occur in species of *Potamogeton* (Potamogetonaceae) (Smith et al., 1976; Hasegawa and Hirose, 1983; Qais et al. 1998; Kittakoop et al., 2001; DellaGreca et al., 2001; Cangiano et al., 2001; Waridel et al., 2003), and that several *ent*-labdanes isolated from species of *Potamogeton* and *Ruppia* (Ruppiaceae) were algicidal (DellaGreca et al., 2000, 2001; Cangiano et al., 2001; Waridel et al., 2003).

In this article, we describe the chemical investigation of the non-polar extract of *P. lucens*, which resulted in isolation of two new *ent*-labdane diterpenes (**1**–**2**), together with a new hydroxylated fatty acid (**3**) and a known furano-*ent*-labdane (**4**). *Ent*-labdanes (**5**–**9**) and some fatty acids (**10**–**11**) were also identified in the non-polar extract of this species by LC–UV–MS analysis, and the results were compared with extracts of *P. pectinatus*, *P. perfoliatus* and *P. crispus*, using LC–UV–MS and GC–MS.

A summary of the labdanes produced by these peculiar macrophytes, which may serve as chemotaxonomic markers, is also presented.

2. Results and discussion

The non-polar extract of *P. lucens* obtained by maceration of plant material with CH_2Cl_2 was subjected to LC–UV–DAD and LC–APCI–MS in the positive ion

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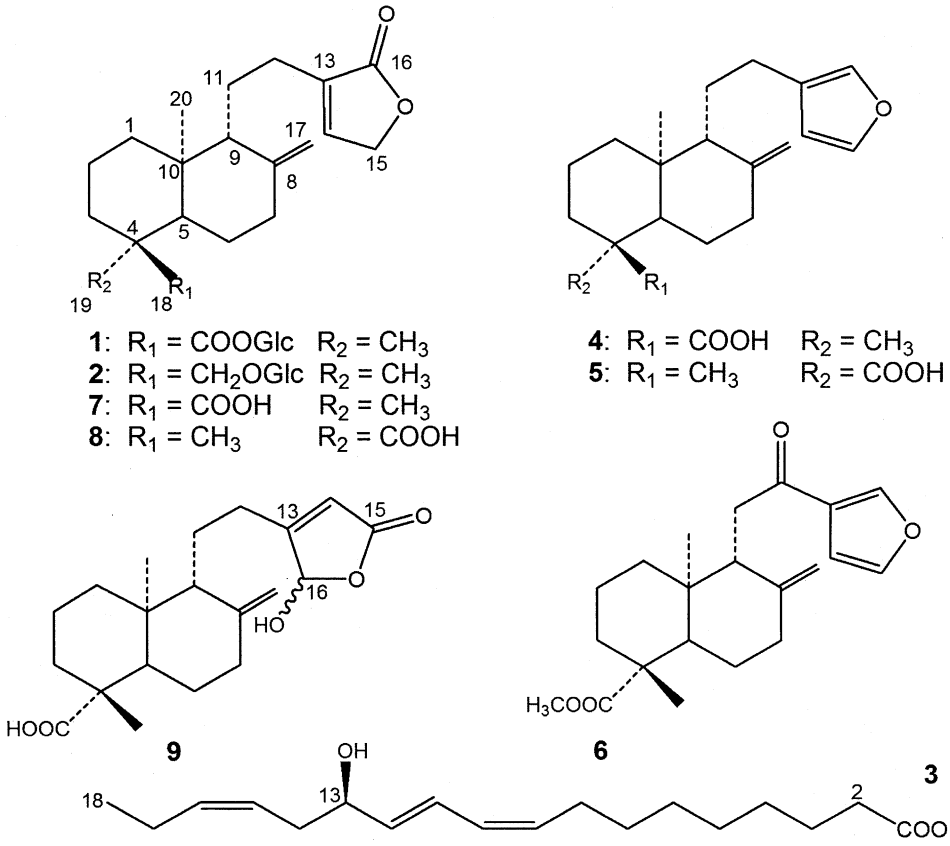


mode, according to our standard procedure (Wolfender et al., 1998; Hostettmann et al., 2001), in order to permit a precise comparison of the crude extract with that of a related previously investigated species, *Potamogeton pectinatus* (Waridel et al., 2003). The LC–UV–MS comparison showed that several constituents were present in both species, in particular compounds **3**, **5**–**6**, **8**–**9** and **10** (Fig. 1). The main constituent of *P. lucens*, as indicated by the UV trace at 210 nm, was identified as linolenic acid (**10**). Four *ent*-labdanes, previously isolated from *P. pectinatus* (Waridel et al., 2003), were also characterized: daniellic acid (**5**), its 12-oxo methyl ester derivative (**6**), 8(17),13-*ent*-labdadien-16,15-olid-19-oic acid (**8**) and 16-hydroxy-8(17),13-*ent*-labdadien-15,16-olid-19-oic acid (**9**). In addition, several adducts and protonated molecules at *m*/*z* 440, 366, 303, 593 and 793 were found to be common to both extracts. A peak at *m*/*z* 593 was attributed to a degradation product of chlorophyll, phaephorbide a (M_r 592) (Villanueva and Hastings, 2000), but the other constituents could not be identified based on the on-line data only.

As the information obtained from UV and MS analyses did not allow a partial identification of the main constituents of *P. lucens* which were not present in *P. pectinatus*, a complementary on-flow LC–^{1}H NMR was performed on the enriched dichloromethane extract (Fig. 2). For a sensitive on-flow LC–^{1}H NMR detection, the amount of extract injected was increased to 10 mg. A C_{18} column with a wide internal diameter (8 mm) was used to enhance the loading capacity. Water was replaced by deuterated water, the flow-rate was reduced to 0.15 ml/min for a separation over 800 min and a fast solvent suppression technique (WET) was applied (Smallcombe et al., 1995). With these LC–NMR conditions, 256 scans per increment were sufficient in the on-flow mode to obtain LC–^{1}H NMR spectra of the main constituents of the extract with good resolution.

The LC–^{1}H NMR spectra of the compounds eluting at rt 490 (**10**), rt 540 and rt 590 min showed characteristic signals of unsaturated fatty acids (methylenes at δ 1.3–2.8, olefinic protons at δ 5.4 and a terminal methyl at δ 0.8–1.0 ppm). The protonated molecular ions at *m*/*z* 279 (rt 35.6 min, **10**), *m*/*z* 303 (rt 36.4 min) and *m*/*z* 263 (rt 38.0 min, **11**) associated in the LC–UV–MS analysis to these latter compounds were consistent with the molecular weight of fatty acids.

The semi-quantitative estimation provided by LC–NMR (Godejohann et al., 1998) demonstrated that these fatty acids were present in significant amounts, a finding which indicates their importance for these macrophytes. Hence the fatty acid composition of *P. lucens* was further investigated by GC–MS analysis of their methyl esters (FAME) (Weber et al., 1997), and compared with the profile of *P. pectinatus* (Waridel et al., 2003). Myristic, pentadecanoic, palmitic, heptadecanoic and stearic acids were identified in this manner. The GC–MS analysis

allowed also the identification of palmitoleic, oleic, linoleic (**11**) and linolenic (**10**) acids. This fatty acid composition was very similar to that previously determined for *P. pectinatus*, which differed from the profile of *P. lucens* only by the absence of linoleic acid.

Besides fatty acids, compounds eluting at rt 420 and 550 min in the LC–NMR contour plot showed specific ^{1}H NMR signals of exocyclic methylenes (δ 4.5–5.0 ppm), methyl groups (δ 0.5–1.2 ppm) and furan moieties (δ 6.2–7.5 ppm). These groups could be related to furanolabdane diterpenes, in particular to daniellic acid (**5**, rt 420 min) identified earlier by LC–UV–MS. The presence of four methyl groups (δ 0.6, 0.7, 1.1 and 1.2 ppm) instead of two within this broad LC peak (see **4**/**5** in Fig. 2) revealed the co-elution of another furanolabdane (**4**) which, clearly separated in the LC–UV–MS analysis (Fig. 1), was found to be an isomer of daniellic acid as it shared the same protonated molecular ion at *m*/*z* 317. With the exception of the methyl groups, all LC–^{1}H NMR signals were identical with those of **5**. A literature search indicated that **4** was most probably polyalthic acid (Gopinath et al., 1961), a well-known *ent*-labdane. In the more polar section of the LC–NMR plot (rt 170–210 min), two compounds (**1** and **2**) showing ^{1}H NMR signals for exocyclic methylenes (δ 4.5–5.0 ppm), methyl groups (δ 0.5–1.2 ppm) and α,β unsaturated γ-lactone moieties (δ 4.8 and 7.3 ppm) were also detected. These groups could be related to labdane diterpene lactones since this type of constituents has already been reported in *Potamogeton* species (DellaGreca et al., 2001; Cangiano et al., 2001; Waridel et al., 2003). The LC-^{1}H NMR signals at δ 3.0–3.8 and 5.4 ppm indicated that these labdanes were glycosylated. Their ammonium adducts at *m*/*z* 512 (**1**) and *m*/*z* 498 (**2**) in LC–APCI–MS analysis, together with fragments at $[M-162+H]^+$, confirmed this hypothesis.

In order to obtain more precise information on the labdane constituents of the lipophilic extract of *P. lucens*, the targeted isolation of these compounds was undertaken. The dichloromethane extract was first fractionated by liquid–liquid extractions between hexane, $CHCl_3$ and $MeOH_{aq}$. The $CHCl_3$ phase was further chromatographed on a silica open column to yield 13 fractions. Compounds **1** and **2** were isolated from one fraction by semi-preparative HPLC on a reversed phase C_{18} column. Six other fractions were pooled and afforded **3** after successive fractionation by CPC and reversed phase semi-preparative HPLC. Isolation of **4** from the hexane phase was carried out by VLC and MPLC on reversed phase.

Compound **4** was identified as the common 15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-18-oic acid (polyalthic acid), first isolated from *Polyalthia fragrans* Benth. et Hook. (Annonaceae) (Gopinath et al., 1961). This result confirmed the earlier partial identification based on the on-flow LC–^{1}H NMR analysis (Fig. 2).

The HR–ESI–MS analysis of **1** (*m*/*z* 517.2410, $[M+Na]^+$) indicated the molecular formula $C_{26}H_{38}O_9$. The assignment of all ^{1}H and ^{13}C NMR signals was accomplished by a combination of COSY, NOESY, DEPT, HSQC and HMBC experiments (Tables 1 and 2). The NMR data showed that **1** was a labdane diterpene with an exocyclic methylene at C-17 (δ 107.7 ppm), a carboxyl carbon attached to C-4 (δ 178.9 ppm) and a methyl group at C-20 (δ 15.2 ppm). Its attribution to the enantiomeric series was supported by the negative optical

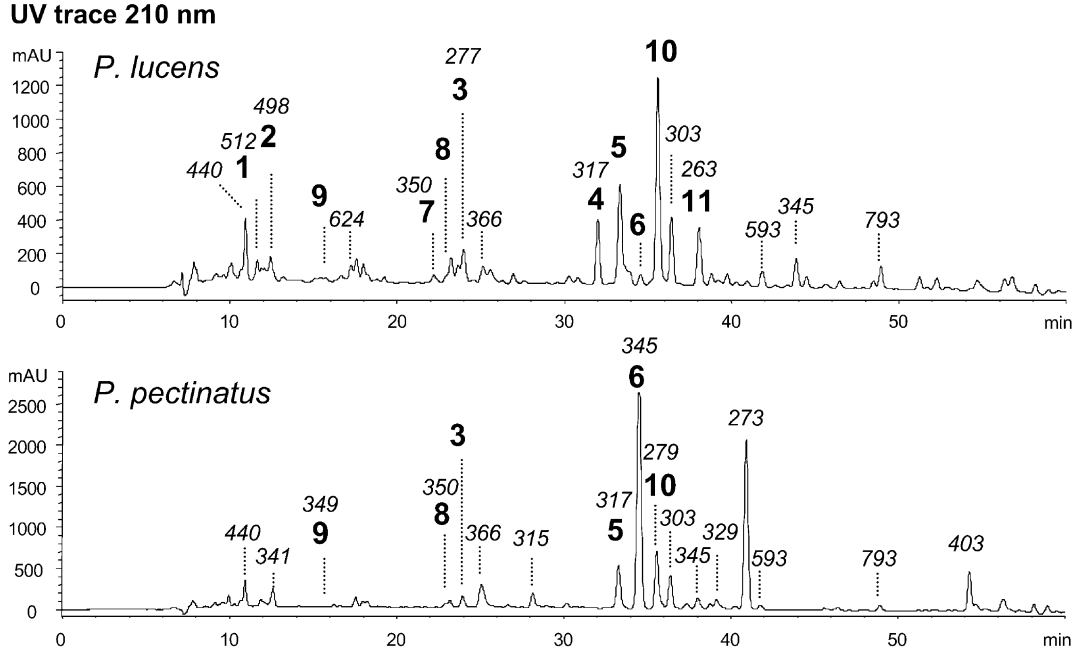


Fig. 1. LC–UV–APCI–MS analysis of the dichloromethane extract of *P. lucens* and *P. pectinatus* (*m*/*z* values of compounds are indicated in italics).

rotation, $[\alpha]_D^{25}$ −18.1°, and based on arguments discussed earlier (Waridel et al., 2003). The ^{13}C chemical shift (δ 17.1 ppm) of the methyl group attached to C-4 indicated its axial position ((*S*)-configuration at C-4) as in **4** (Carreras et al., 1998), since compounds with (*R*)-configuration at C-4, such as in daniellic acid (**5**) and in its methyl ester (DellaGreca et al., 2000), exhibit the equatorial methyl frequency at lower field (+12 ppm). The NMR spectra showed also the presence of an α,β unsaturated γ-lactone moiety with two ^1H signals at δ 7.35 (1 H, H-14, *t*, J=1.5 Hz) and 4.82 (2 H, H-15) ppm, and with ^{13}C at δ 134.8 (C-13), 147.6 (C-14), 72.1 (C-15) and 177.0 (C-16) ppm. The HMBC spectrum indicated that the lactone moiety was attached to the labdane bicyclic skeleton by the aliphatic chain C-11 and C-12. The other ^{13}C (δ 60–100 ppm) and ^1H signals (δ 3.3–4.0 ppm) were assigned to a glucopyranose residue (C-1′–C-6′, H-2′–H-6′). The coupling constant of H-1′ (δ 5.43 ppm, *d*, J=7.8 Hz) was characteristic for a β-anomeric proton. The HMBC correlation between H-1′ and C-18 revealed an ester linkage between the glucose and the carboxyl group of the labdane. Furthermore,

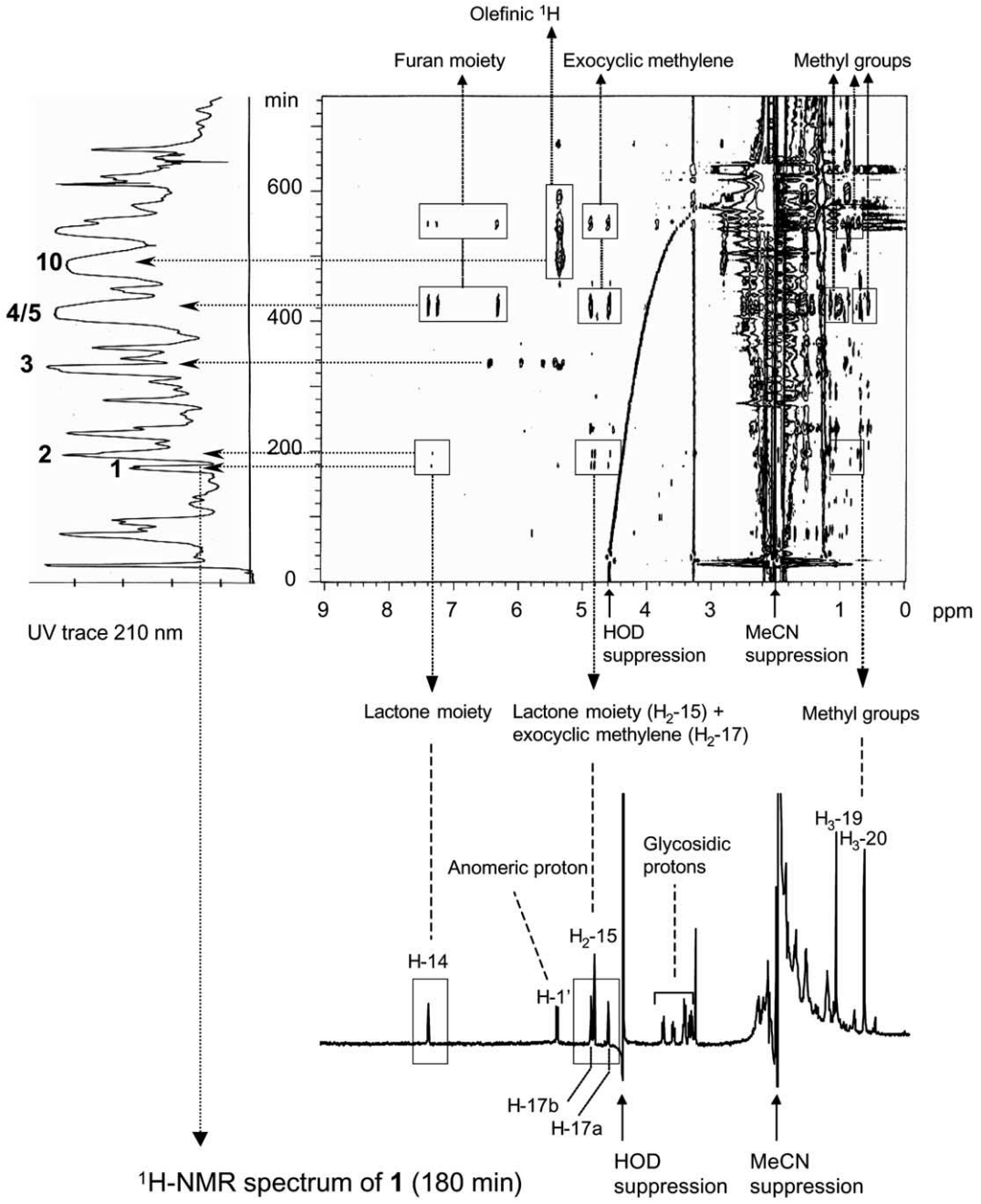


Fig. 2. On-flow LC–UV–^1H NMR analysis of the dichloromethane extract of *P. lucens* (10 mg injected). Flow rate: 0.15 ml/min, 256 scans/increment. The ^1H spectrum of **1** was recorded in stop-flow mode with 512 scans.

the successful hydrolysis of this compound by β-D-glucosidase proved that the glucopyranose unit was β-D-glucose. Compound **1** was therefore β-D-glucopyranosyl-8(17),13-*ent*-labdadien-16,15-olid-18-oate, a new natural product, which confirmed the hypothesis based on the on-line information.

The HR–ESI–MS analysis of **2**, $[\alpha]_D^{25}$ −37.8°, indicated the molecular formula $C_{26}H_{40}O_8$ (*m*/*z* 503.2606, $[M+Na]^+$). The NMR data (Tables 1 and 2) showed that it was an *ent*-labdane similar to **1**, except for the replacement of the carboxyl by an oxymethylene group (δ^{13}C: 79.9; ^{1}H: 3.26, 3.46 ppm). A HMBC correlation between H-1′ and C-18 indicated the presence of an ether linkage between the glucopyranose residue and the methylene at C-18. The coupling constant of H-1′ (δ 4.18 ppm, *d*, *J* = 7.8 Hz) was characteristic for a β-anomeric proton. Furthermore, the successful hydrolysis of **2** by β-D-glucosidase proved that the glucopyranose unit was β-D-glucose. The structure 18-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide, a new substance, could therefore be finally assigned to **2**, which confirmed our hypothesis based on the on-line information. This compound is the C-4 epimer of 19-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide (neoandrographolide), first reported in *Andrographis paniculata* Nees (Acanthaceae) (Chan et al., 1968), and isolated recently from *Potamogeton natans* L. (Cangiano et al., 2001).

Table 1
Selected ^{1}H NMR spectral data of compounds **1**, **2** (in CD_3OD)

^{1}H	**1**	**2**
9	1.78 *m*	1.75 *m*
11	1.66 *m*	1.63 *m*
	1.78 *m*	1.78 *m*
12	2.12 *m*	2.11 *m*
	2.42 *m*	2.41 *m*
14	7.35 *t* (1.5 Hz)	7.33 *t* (1.5 Hz)
15	4.82	4.82
16	–	–
17	4.66 *s*	4.62 *s*
	4.88 *s*	4.86 *s*
18	–	3.26 *m*
		3.46 *d* (9.8 Hz)
19	1.17 *s*	0.78 *s*
20	0.75 *s*	0.75 *s*
1′	5.43 *d* (7.8 Hz)	4.18 *d* (7.8 Hz)

Table 2
^{13}C NMR spectral data of compounds **1**, **2** (in CD_3OD)[a]

^{13}C	**1**	**2**
1	39.2 (CH_2)	39.7 (CH_2)
2	19.4 (CH_2)	19.8 (CH_2)
3	37.7 (CH_2)	37.1 (CH_2)
4	49.0 (C)	38.7 (C)
5	50.7 (CH)	49.3 (CH)
6	27.8 (CH_2)	25.5 (CH_2)
7	38.8 (CH_2)	39.0 (CH_2)
8	149.1 (C)	149.8 (C)
9	57.7 (CH)	57.5 (CH)
10	40.0 (C)	40.6 (C)
11	22.7 (CH_2)	22.9 (CH_2)
12	25.4 (CH_2)	25.5 (CH_2)
13	134.8 (C)	134.9 (C)
14	147.6 (CH)	147.5 (CH)
15	72.1 (CH_2)	72.1 (CH_2)
16	177.0 (C)	177.0 (C)
17	107.7 (CH_2)	106.9 (CH_2)
18	178.9 (C)	79.9 (CH_2)
19	17.1 (CH_3)	18.3 (CH_3)
20	15.2 (CH_3)	15.5 (CH_3)
1′	96.1 (CH)	105.2 (CH)
2′	74.1 (CH)	75.2 (CH)
3′	78.4 (CH)	78.2 (CH)
4′	71.1 (CH)	71.7 (CH)
5′	78.9 (CH)	77.9 (CH)
6′	62.4 (CH_2)	62.8 (CH_2)

[a] Number of hydrogens was given by a DEPT experiment

An enriched fraction containing **1** and **2** was hydrolysed by β-D-glucosidase and the resulting aglycones were used as standards for LC–UV–MS analyses. Only the aglycone of **1**, 8(17),13-*ent*-labdadien-16,15-olid-18-oic acid (nivenolide) (**7**), first isolated from *Croton niveus* Jacq. (Euphorbiaceae) (Rojas and Rodriguez-Hahn, 1978), was identified in the non-polar extract of *P. lucens*.

The DCI-MS analysis of **3** (*m*/*z* 294, $[M-H_2O+NH_4]^+$) and the NMR spectra (^{1}H, ^{13}C and DEPT) indicated the molecular formula $C_{18}H_{30}O_4$. The NMR data showed that **3** was a tri-unsaturated fatty acid with six olefinic protons, one hydroxyl group attached at C-13 (δ 72.1 ppm), a carboxyl carbon at C-1 (δ 178.3 ppm) and a terminal methyl group at C-18 (δ 14.2 ppm). The coupling constants of the olefinic protons showed that the double bond configuration was 9*Z*,11*E*,15*Z*. Comparison with the spectral data of 13(*S*)-hydroxy-octadeca-(9*Z*,11*E*,15*Z*)-trien-oic acid ($[\alpha]_D^{25}$ +4.8° or +12.0°) (Shimura et al., 1983; Yadav et al., 1992) supported the structure assigned to **3**, but with (*R*)-configuration at C-13 as indicated by the negative optical rotation ($[\alpha]_D^{25}$ −4.3°). To our knowledge this is the first report of the isolation of this enantiomer, 13(*R*)-hydroxy-octadeca-(9*Z*,11*E*,15*Z*)-trien-oic acid (**3**).

LC–UV–MS and GC–MS analyses were also carried out with the non-polar extracts of two other *Potamogeton* species occurring in Lake Léman, *P. crispus* L. and *P. perfoliatus* L. GC–MS showed the same fatty acid profiles as described previously for *P. lucens*. In the LC–UV–MS profiles the fatty acids **3**, **10** and **11** found earlier in *P. lucens* and *P. pectinatus* were also present in the non-polar extracts of *P. crispus* and *P. perfoliatus*. In addition, these analyses indicated the presence of *ent*-labdane **6** in *P. perfoliatus* and of pinoresinol in *P. crispus*. This latter lignan had already been isolated from *P. pectinatus* (Waridel et al., 2003). MS and UV spectra

Table 3
ent-Labdane structures and localization in the genus *Potamogeton*

Name	Compound	Structure							*P. pectinatus*	*P. lucens*	*P. perfoliatus*	*P. nodosus*	*P. natans*	*P. ferrugineus*
			R_1	R_2	R_3	R_4	R_5	R_6						
15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oic acid		I	IV	H	OAc	CH_3	COOH	CH_3	X[a]					
methyl-15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oate		I	IV	H	OAc	CH_3	$COOCH_3$	CH_3	X[a]					
15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-19-oic acid	**5**	I	IV	H	H	CH_3	COOH	CH_3	X[a]	X				
8(17),13-*ent*-labdadien-16,15-olid-19-oic acid	**8**	I	V	H	H	CH_3	COOH	CH_3	X[a]	X				
16-hydroxy-8(17),13-*ent*-labdadien-15,16-olid-19-oic acid	**9**	I	VI	H	H	CH_3	COOH	CH_3	X[a]	X				
methyl-15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate	**6**	I	IV	C=O (at C-12)		CH_3	$COOCH_3$	CH_3	X[a]	X	X			
15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-18-oic acid	**4**	I	IV	H	H	CH_3	CH_3	COOH		X				
8(17),13-*ent*-labdadien-16,15-olid-18-oic acid	**7**	I	V	H	H	CH_3	CH_3	COOH		X				
β-D-glucopyranosyl-8(17),13-*ent*-labdadien-16,15-olid-18-oate	**1**	I	V	H	H	CH_3	CH_3	COOGlc		X				
18-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide	**2**	I	V	H	H	CH_3	CH_3	CH_2OGlc		X				
19-acetoxy-20-hydroxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatriene (potamogetonol)		I	IV	H	H	CH_2OH	CH_2OAc	CH_3				X[b]		
19-acetoxy-20-oxo-15,16-epoxy -8(17),13(16),14-*ent*-labdatriene (potamogetonyde)		I	IV	H	H	CHO	CH_2OAc	CH_3				X[b]	X[c]	
19,20-dihydroxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatriene		I	IV	H	H	CH_2OH	CH_2OH	CH_3					X[c]	
10a,19-dihydroxy-15,16-epoxy-8(17),13(16),14-nor-*ent*-labdatriene		I	IV	H	H	OH	CH_2OH	CH_3					X[c]	
19-hydroxy-8(17),13-*ent*-labdadien-16,15-olide		I	V	H	H	CH_3	CH_2OH	CH_3					X[d]	
19-acetoxy-8(17),13-*ent*-labdadien-16,15-olide		I	V	H	H	CH_3	CH_2OAc						X[d]	
19-acetoxy-20-oxo-8(17),13-*ent*-labdadien-16,15-olide		I	V	H	H	CHO	CH_2OAc	CH_3					X[d]	
19-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide		I	V	H	H	CH_3	CH_2OGlc	CH_3					X[d]	
(6′-acetyl) 19-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide		I	V	H	H	CH_3	CH_2OGlc-6′-Ac	CH_3					X[d]	
8(17),13-*ent*-labdadien-16,15-19,20-diolide		II	V	H	H								X[d]	
15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-20,19-olide (potamogetonin)		II	IV	H	H							X[b]	X[c]	
12(*S*)-hydroxy-15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-20,19-olide		II	IV	OH	H							X[b]	X[c]	
15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-20,19-olide		II	IV	C=O (at C-12)							X[e]	X[f]	X[c]	X[e]
15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19,20-olide		III	IV	C=O (at C-12)									X[c]	

[a] Waridel et al. (2003).
[b] Kittakoop et al. (2001).
[c] DellaGreca et al. (2001).
[d] Cangiano et al. (2001).
[e] Smith et al. (1976).
[f] Qais et al. (1998).

indicated that related labdanes were probably present in *P. crispus* and *P. perfoliatus*, but their structures could not be ascertained based on the on-line data only.

All extracts were evaluated for their molluscidal, fungicidal and larvicidal activities (Hostettmann et al., 1982; Homans and Fuchs, 1970; Rahalison et al., 1991; Cepleanu et al., 1994), but did not show any significant activity against the tested organisms. The algicidal activity of compounds **1**–**4** isolated from *P. lucens* was also investigated, using microplates with the alga *Raphidocelis subcapitata* (Environment Canada, 1992), since Cangiano et al. (2002) and Waridel et al. (2003) had reported a growth inhibitory effect of the *ent*-labdane diterpenes from *P. natans* and *P. pectinatus*. While labdane **4** had a significant effect, no such inhibition was caused by labdanes **1**–**2** and fatty acid **3**. The IC_{50} value of **4** could be estimated within 95% confidence limits (33.9 μmol/l, 14.5–79.2) and the compound appeared less toxic than its epimer, daniellic acid (IC_{50} = 17.2 μmol/l, Waridel et al., 2003). Neoandrographolide, the epimer of **2**, was significantly active (80.5 μmol/l, Cangiano et al., 2002), while **2** showed no inhibitory effect at the highest concentration tested (107 μmol/l). These results indicate that the configuration at C-4 seems important for algal growth inhibitory activity of labdanes.

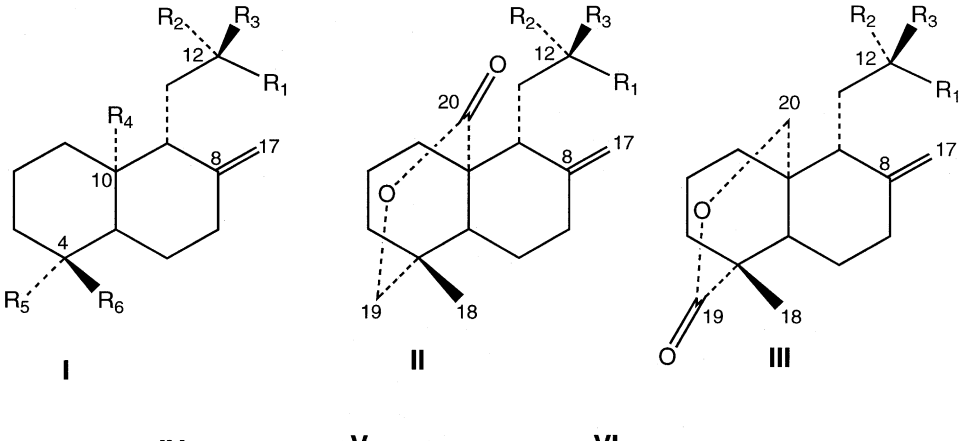
The results obtained with *P. lucens* and *P. pectinatus* demonstrated that *ent*-labdanes with furan or lactone groups are important original chemical constituents of these species and might play an ecological role as putative allelochemicals against algae. A literature survey further confirmed the importance of this class of compounds in Potamogetonaceae. As shown in Table 3 and Fig. 3, twenty-four *ent*-labanes were identified in six *Potamogeton* species investigated for their diterpene content. Only a relatively minor part of these constituents were found to be common to the species and the profiling of *P. crispus* and *P. perfoliatus*, not completely investigated in this study, also showed this trend. This variability of closely related labdanes might indicate some specific chemical adaptations of these macrophytes to the aquatic environment.

These compounds can thus be regarded as interesting chemotaxonomic markers. Their diversity contrasts with the relative homogenous fatty acid and flavonoid composition of *Potamogeton* species (Les and Sheridan, 1990). A more exhaustive investigation of these macrophytes might lead to a better understanding of their chemical features and their role in the aquatic ecosystems.

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian Unity Inova spectrometer.



R2: H, OH, C=O
R3: H, OAc, C=O
R4: CH_3, CH_2OH, OH, CHO
R5: CH_3, CH_2OH, CH_2OAc, CH_2OGlc, $CH_2OGlc^{6'}$-Ac, COOH, $COOCH_3$
R6: CH_3, CH_2OGlc, COOH, COOGlc

Fig. 3. *ent*-Labdanes in the genus *Potamogeton*.

TMS was used as internal standard for ^1H spectra. Optical rotations were measured with a Perkin-Elmer 241MC polarimeter. UV spectra were determined on a Perkin-Elmer Lambda20 UV/vis spectrophotometer. EI-MS and DCI–MS spectra were recorded on a Finnigan MAT TSQ-70 triple stage quadrupole spectrometer and HR–ESI–MS spectra on a FTMS 4.7T BioApex II Brüker or a Micromass LCT instrument. Solid phase extraction was carried out using Chromabond® C_{18} (1 g) prepacked columns. Preparative CPC was performed with an ITO apparatus (Multi-Layer-Coil-Separator-Extractor Model#1) at 800 rpm, connected to a Waters pump 6000A at a flow rate of 2 ml/min. Preparative MPLC was carried out using a Büchi 681 pump equipped with a Knauer UV detector at 210 nm using a Lichroprep® RP-18 (15–25 μm) user-packed column (460×26 mm i.d., Büchi). Semi-preparative HPLC was performed at 210 nm with a μBondapak® C_{18} prepacked radial-compression column (10 μm; 100×8 mm i.d.; Waters) or a SymmetryPrep RP-18 column (7 μm; 150×19 mm i.d.; Waters). A Shimadzu LC-8A pump equipped with a LKB Bromma 2151 UV detector was used with the SymmetryPrep RP-18 column, and a Varian 9012 pump equipped with a UV/VIS Varian ProStar 320 detector was used with the μBondapak® C_{18} column. HPLC–UV–MS analyses were performed on a Hewlett-Packard series 1100 apparatus with two Zorbax Eclipse XDB-Phenyl columns connected in series (250×4.6 mm i.d.) using a CH_3CN:H_2O solvent system containing 0.05% TFA: 50–100% CH_3CN in 60 min at 0.7 ml/min, then 20 min with 100% CH_3CN. The spectra and UV traces at 210 and 254 nm were recorded on a Hewlett-Packard 1050 DAD detector. The HPLC apparatus was coupled to a Finnigan triple-quadrupole MS instrument (TSQ 700) with APCI interface operated in the positive ion mode: vaporizer 400 °C, capillary 150 °C, corona 5 μA, sheath gas N_2. Analyses of fatty acid methyl esters (FAME) were performed by GC-MS with a Varian 3400 gas chromatograph coupled to a Finnigan MAT TSQ-70 triple stage quadrupole mass spectrometer in the EI and CI (CH_4 as ionisation gas) modes. A Chrompak (CP Select CB for FAME) capillary column (100 m×0.25 mm i.d.×0.25 μm) was used with the following temperature gradients (He column head pressure: 25 psi): 100 °C for 1 min, 100–220 °C at 3 °C/min, 220–235 °C at 0.5 °C/min, 235–280 °C at 4.5 °C/min, and 280 °C for 10 min. Injector temperature was set at 250 °C and transfer line at 280 °C. Injections (1 μl, hexane) were performed in the splitless mode. Identification of fatty acid methyl esters was carried out with a standard mixture (Supelco 37 Component FAME mix). LC–UV–NMR analysis was carried out at 210 nm with a Varian 9012 pump and a ProStar 320 Varian UV/vis detector using a μBondapak® C_{18} prepacked radial-compression column (10 μm; 100×8 mm i.d.; Waters). A CH_3CN:D_2O solvent system (0.15 ml/min) was used with the following gradients: 20:80 to 80:20 CH_3CN:D_2O in 480 min, 80:20 to 100:0 CH_3CN:D_2O in 107 min, 100:0 CH_3CN:D_2O during 213 min). The HPLC apparatus was coupled to a 500 MHz Varian Unity Inova spectrometer via a LC–NMR flow probe (60 μl, 3 mm i.d.). Solvent suppression was achieved with a WET sequence (Smallcombe et al., 1995). In the on-flow mode, each increment consisted of 256 transients.

3.2. Plant material

Potamogeton lucens L. and *Potamogeton perfoliatus* L. (Potamogetonaceae) were collected in Lake Léman, near Morges, Switzerland, in July 1999. *Potamogeton pectinatus* L. was collected in Lake Léman, near St-Sulpice, Switzerland, in June 1999. *Potamogeton crispus* L. was collected in Lake of Joux, near Le Sentier, Switzerland, in August 2000. Macrophytes were identified by Professor Jean-Bernard Lachavanne (LEBA, University of Geneva, Switzerland). Voucher specimens are deposited at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland (*P. pectinatus* no. 2000042, *P. lucens* no. 2000072, *P. perfoliatus* no. 2000073 and *P. crispus* no. 2000071).

3.3. Extraction and isolation

The whole plant material was air-dried, powdered and extracted at room temperature three to five times with CH_2Cl_2 for 24 h. Seventeen grams of non-polar extract was obtained from *P. lucens* (1.3 kg dry wt.) in this manner. A portion of this extract was further fractionated by liquid–liquid extractions with hexane, $CHCl_3$ and $MeOH_{aq.}$ according to the procedure described by Gunatilaka et al. (1998). It was first partitioned between hexane and 80% aqueous MeOH. The methanol phase was then diluted to 60% aqueous MeOH with water and extracted by $CHCl_3$. The $CHCl_3$ fraction (4 g) was chromatographed on Silica gel 60 (0.070–0.200 mm) with a $CHCl_3$:MeOH gradient (98:2→0:100) to give fractions C1–C13. Fraction C8 was eluted with CH_3CN:H_2O 50:50 on a SPE C_{18} column to remove the major part of the non-polar pigments. The eluate was then separated by semi-preparative HPLC on a μBondapak® C_{18} radial-compression column (10 μm, 100×8 mm i.d.) with CH_3CN:H_2O (32:68) as mobile phase (1 ml/min) to give compounds **1** (7 mg) and **2** (9 mg). Fractions C2–C7 were pooled and chromatographed by CPC with a solvent system heptane:AcOEt:MeOH:H_2O (6:5:6:5). The lower phase of this system was first used as the mobile phase to give fractions Ca–Cg, then in the reversed mode, the upper phase was used as mobile phase to afford fractions Ch–Cj. Fraction Ce was eluted with MeOH:H_2O 9:1 on a SPE C_{18} column and the eluate was then separated by semi-preparative HPLC on a SymmetryPrep RP-18 column (150×19 mm i.d.) with a CH_3CN:H_2O

gradient (20:80→80:20 in 60 min, 10 ml/min) to lead to the isolation of compound **3** (3 mg). The hexane fraction (6 g) was separated by VLC on Lichroprep® RP-18 phase (15–25 μm) with a CH_3CN:H_2O gradient (50:50→100:0) to give fractions H1–H9. Fractions H2–H6 were then pooled and fractionated by MPLC with the same CH_3CN:H_2O gradient to afford compound **4** (44 mg).

3.4. Compound characterization

3.4.1. β-D-Glucopyranosyl-8(17),13-ent-labdadien-16,15-olid-18-oate (1)

Yellow gum; $[\alpha]_D^{25}$ −18.1° (CH_3OH, *c* 0.73); UV (MeOH) λ_{max} nm (log ε): 202 (4.04); ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; HR–ESI–MS: *m/z* 517.2410 ($C_{26}H_{38}O_9Na$: $[M+Na]^+$, requires 517.2408).

3.4.2. 18-β-D-Glucopyranosyloxy-8(17),13-ent-labdadien-16,15-olide (2)

Yellow oil; $[\alpha]_D^{25}$ −37.8° (CH_3OH, *c* 0.87); UV (MeOH) λ_{max} nm (log ε): 202 (4.07); ^1H NMR: see Table 1; ^{13}C NMR: see Table 2; HR–ESI–MS: *m/z* 503.2606 ($C_{26}H_{40}O_8Na$: $[M+Na]^+$, requires 503.2615).

3.4.3. 13(R)-Hydroxy-octadeca-(9Z,11E,15Z)-trien-oic acid (3)

Yellow oil; $[\alpha]_D^{25}$ −4.3° ($CHCl_3$, *c* 0.37); UV (EtOH) λ_{max} nm (log ε): 201 (3.54); ^1H NMR ($CDCl_3$): δ 0.97 (3H, *t*, *J* = 7.6 Hz, H-18), 1.32 (6H, *m*, H-4, H-5 and H-6), 1.38 (2H, *m*, H-7), 1.63 (2H, *m*, H-3), 2.07 (2H, *qd*, $J_{17,16}$ = 7.3 Hz, $J_{17,18}$ = 7.5 Hz, H-17), 2.18 (2H, *m*, H-8), 2.35 (4H, *t*, *J* = 7.6 Hz, H-2 and H-14), 4.23 (1H, *dd*, $J_{13,12}$ = 6.4 Hz, $J_{13,14}$ = 5.9 Hz, H-13), 5.36 (1H, *m*, H-15), 5.44 (1H, *m*, H-9), 5.57 (1H, *m*, H-16), 5.69 (1H, *dd*, $J_{12,11}$ = 15.4 Hz, $J_{12,13}$ = 6.4 Hz, H-12), 5.98 (1H, *t*, *J* = 10.7 Hz, H-10), 6.52 (1H, *dd*, $J_{11,10}$ = 10.7 Hz, $J_{11,12}$ = 15.1 Hz, H-11); ^{13}C NMR ($CDCl_3$): δ 14.2 (C-18), 20.7 (C-17), 24.6 (C-3), 27.6 (C-8), 28.8 (C-4, C-5 and C-6), 29.3 (C-7), 33.7 (C-2), 35.2 (C-14), 72.1 (C-13), 123.7 (C-15), 125.9 (C-11), 127.8 (C-10), 132.9 (C-9), 134.9 (C-12), 135.3 (C-16), 178.3 (C-1); EIMS 70 eV, *m/z* (rel. int.): 276 $[M-H_2O]^{\cdot+}$(2), 225 $[M-69]^+$ (52), 207 $[M-87]^+$ (100), 189 $[M-105]^+$ (14), 171 $[M-123]^+$ (28), 161 $[M-133]^+$ (23), 147 $[M-147]^+$ (42), 111 $[M-183]^+$ (50); DCIMS NH_3, *m/z* (rel. int.): 312 $[M+NH_4]^+$(4), 294 $[M-H_2O+NH_4]^+$(10), 277 $[M-H_2O+H]^+$ (100).

3.4.4. Enzymatic hydrolysis of 1 and 2

An enriched fraction (5 mg) containing compounds **1** and **2** was dissolved in acetate buffer at pH 5.5 (1 ml) and incubated for 4 days at 37 °C with 1 mg of β-D-glucosidase (Sigma). The solution was then extracted by $CHCl_3$ (3 ml) and the solvent evaporated to give a mixture (2 mg) containing the aglycones of **1** (compound **7**) and **2**.

3.5. Bioassays

The algal growth inhibition test was performed on the green alga *Raphidocelis subcapitata* (previously named *Selenastrum capricornutum*), adapting the microplate technique recommended by Environment Canada (1992) as described in our previous paper (Waridel et al., 2003). The algae were obtained immobilized on beads of alginate from an Algaltoxkit F™ (Creasel, Deinze, Belgium) and were set free before each toxicity test. Preparation of the algae and of the algal culturing medium was performed according to the Standard Operational Procedure of the Algaltoxkit F™ (Creasel, 1996).

Chemicals were initially dissolved in DMSO and then diluted in the algal culturing medium at the different test concentrations (three replications of five toxicant concentrations). Highest DMSO level in the test wells did not exceed 0.33% (v/v). Ten microlitres of each toxicant concentration were diluted in 290 μl of algal culturing medium containing 2×10^4 algal cells/ml.

The microplates were sealed in transparent plastic bags to minimize evaporation during the exposure period and placed in an incubator at 24 °C under continuous illumination (8000 lux) during 72 h. The light absorbance at 450 nm of the resuspended algal cells was measured by a microplate photometer (SLT Spectra) before (t_0) and after the incubation period (t_{72}) as an indirect measure of the algal growth. The mean values of inhibition (percentage > 0%) were reported against log-transformed data of concentrations (μmol/l) (at least three concentrations). The concentration resulting in 50% growth inhibition (IC_{50}) was estimated within 95% confidence limits.

The validity of the test was controlled with a reference toxicant, potassium dichromate ($K_2Cr_2O_7$), and a 72 h IC_{50} of 0.47 mg/l (0.37–0.61) was obtained with five replications of four concentrations between 0.18 and 1.07 mg/l. The 72 h IC_{50} value given by the Algaltoxkit F™ producer was 0.38 mg/l.

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