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ent-Labdane diterpenes from the aquatic plant *Potamogeton pectinatus*

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Abstract

Four new *ent*-labdane diterpenes were isolated from the freshwater aquatic plant *Potamogeton pectinatus*, together with two known furano-*ent*-labdanes. The new compounds were assigned the structures methyl-15,16-epoxy-12(*R*)-acetoxy- 8(17),13(16),14-*ent*-labdatrien-19-oate, 15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oic acid, 8(17),13-*ent*-labdadien-15 → 16-lactone-19-oic acid and 16-hydroxy-8(17),13-*ent*-labdadien-15,16-olid-19-oic acid by spectroscopic means. Some of these labdanes showed a strong algicidal activity against *Raphidocelis subcapitata*.

Keywords: *Potamogeton pectinatus*; Potamogetonaceae; Spectroscopic analysis; LC-MS; LC-NMR; *ent*-Labdane diterpenes; Antialgal activity

1. Introduction

Because of their peculiar habitat, in-between aquatic and terrestrial life, aquatic plants should produce secondary metabolites with original chemical or biological features. In comparison with terrestrial plants, few papers have however dealt with hydrophytes. In our ongoing research of new active compounds in freshwater plants of Switzerland, the investigation of the aquatic macrophyte *Potamogeton pectinatus* L. (Potamogetonaceae) was undertaken. This plant is the most abundant species of the Lake Léman and its repartition was stable since 1975, in spite of the water eutrophication, as it could grow over a large range of water quality (Demierre and Durand, 1999; Lehmann and Lachavanne, 1999). This tolerance was perhaps partly mediated by allelochemicals, as some authors have pointed out that hydrophytes could produce compounds to inhibit algal growth as fatty acids, sterols and phenylpropanoids (reviewed by Aliotta et al., 1996). In their research of chemical defences in aquatic plants, Ostrofsky and Zettler (1986) detected the presence of alkaloids by TLC in *P. pectinatus* and others *Potamogeton* species. Diverse flavonoids were also identified in eighteen *Potamogeton* species and used for their chemotaxonomy (Les and Sheridan, 1990). Some papers showed more recently the presence of labdane diterpenes in *Potamogeton natans* (Della-Greca et al., 2001; Cangiano et al., 2001). *Potamogeton pectinatus* was however not yet exhaustively investigated.

In this paper, we describe the chemical investigation of *P. pectinatus* apolar extract and the isolation of six labdane diterpenes (**1, 3**–**7**), together with some other known compounds from various classes (**8**–**10**). Four of these labdanes (**1**, **3, 6**–**7**) are new and their structure elucidations are more exhaustively described.

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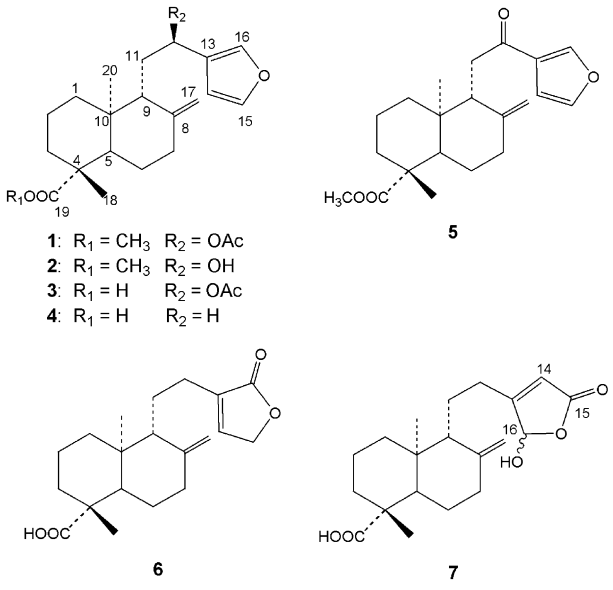

2. Results and discussion

The apolar extract (61 g) of *P. pectinatus* was obtained by the maceration of plant material (3 kg dry wt) with CH_2Cl_2. The dark green colour of the extract revealed an important proportion of chlorophyll pigments. A preliminary chemical evaluation of the extract composition was realised by LC–UV–DAD and LC–APCI–MS according to our standard procedure (Wolfender et al., 1998; Hostettmann et al., 2001) (Fig. 1). The LC–UV–DAD analysis revealed the presence of many constituents having absorption maxima between 200 and 250 nm. They were well ionized and presented protonated molecules between 250 and 450 Da in LC–APCI–MS (positive ion mode). Several pigments (UV maxima at ca. 400 nm) were also detected after rt 40 min. Because of the lack of characteristic chromophores, and the relatively restricted information obtained from the LC–MS spectra, it was difficult at this point to draw any conclusion on the chemical nature of the lipophilic constituents of *P. pectinatus*.

A complementary on-flow LC–NMR analysis (Wolfender et al., 2001) was thus performed on the enriched CH_2Cl_2 crude extract (Fig. 2). For a sensitive on-flow LC-^{1}H-NMR detection, the amount of extract injected was increased to 4 mg. The HPLC conditions were optimised in order to achieve a similar separation to that obtained by LC–UV–MS. A C_{18} column with an important internal diameter (8 mm) was used to enhance the loading capacity. Water was replaced by deuterated water, the flow-rate was reduced to 0.1 ml/min for a separation over 750 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 with good resolution on the main constituents of the extract.

The LC-^{1}H-NMR spectra of the compounds eluting at rt 480 and rt 510 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 1.0 ppm). The protonated molecules at m/z 279 (rt 35.6 min) and m/z 303 (rt 36.4 min) associated in the LC–UV–MS analysis to these latter compounds were consistent with the molecular weight of known fatty acids.

As these compounds were present in significant amounts in the extract, based on a semi-quantitative estimation provided by LC-NMR, they were analyzed by GC–MS (Weber et al., 1997). The fatty acid profile of *P. pectinatus* was obtained after derivatisation as methyl esters. The saturated myristic, pentadecanoic, palmitic, heptadecanoic and stearic acids were identified by this way. The GC–MS analysis allowed also the identification of unsaturated palmitoleic, oleic and linolenic (**11**) acids. The pentadecanoic, palmitic, heptadecanoic and oleic acids were previously identified in *P. pectinatus* lipids by Haroon et al. (1995). In that study, the arachidic acid was also detected, but it was not identified in *P. pectinatus* as free fatty acid by our analyses.

In the more polar section of the LC–NMR contour plot (rt 250–450 min), several compounds showing specific ^{1}H-NMR signals of exocyclic methylenes (δ 4.0–5.0 ppm), methyl groups (δ 0.5–1.5 ppm) and furano moieties (δ 6.0–8.5 ppm) were also detected. These groups could be related to putative furanoid labdane diterpenes since this type of constituents has already been reported in species of the *Potamogeton* genus (DellaGreca et al., 2001; Cangiano et al., 2001). The peaks eluting at rt 390 and rt 420 min presented protonated molecules at m/z 345 and 317 which was consistent with the molecular weight of known labdane diterpenes. In particular compound **5** (LC–UV–MS: rt 34.5 min, LC–NMR: rt 390 min) was most probably 12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate (M_r 344). This compound was previously reported in the aquatic plant *Ruppia maritima*, a macrophyte included by some authors in the Potamogetonaceae familiy (DellaGreca et al., 2000). In the on-flow LC-NMR analysis, several other constituents were also sharing similar structural features, but they could not be fully identified on the basis of the on-line data only.

In order to obtain more precise information on the chemical composition of the lipophilic extract of *P. pectinatus*, a targeted isolation of the labdanes was undertaken. The dichloromethane extract (17 g) was first chromatographed on a silica open column to yield fifteen fractions. Some of them were further fractionated in several steps on reverse phase C_{18} columns with different preparative chromatography methods, such as LPLC, MPLC and semi-preparative HPLC, to finally afford ten pure compounds.

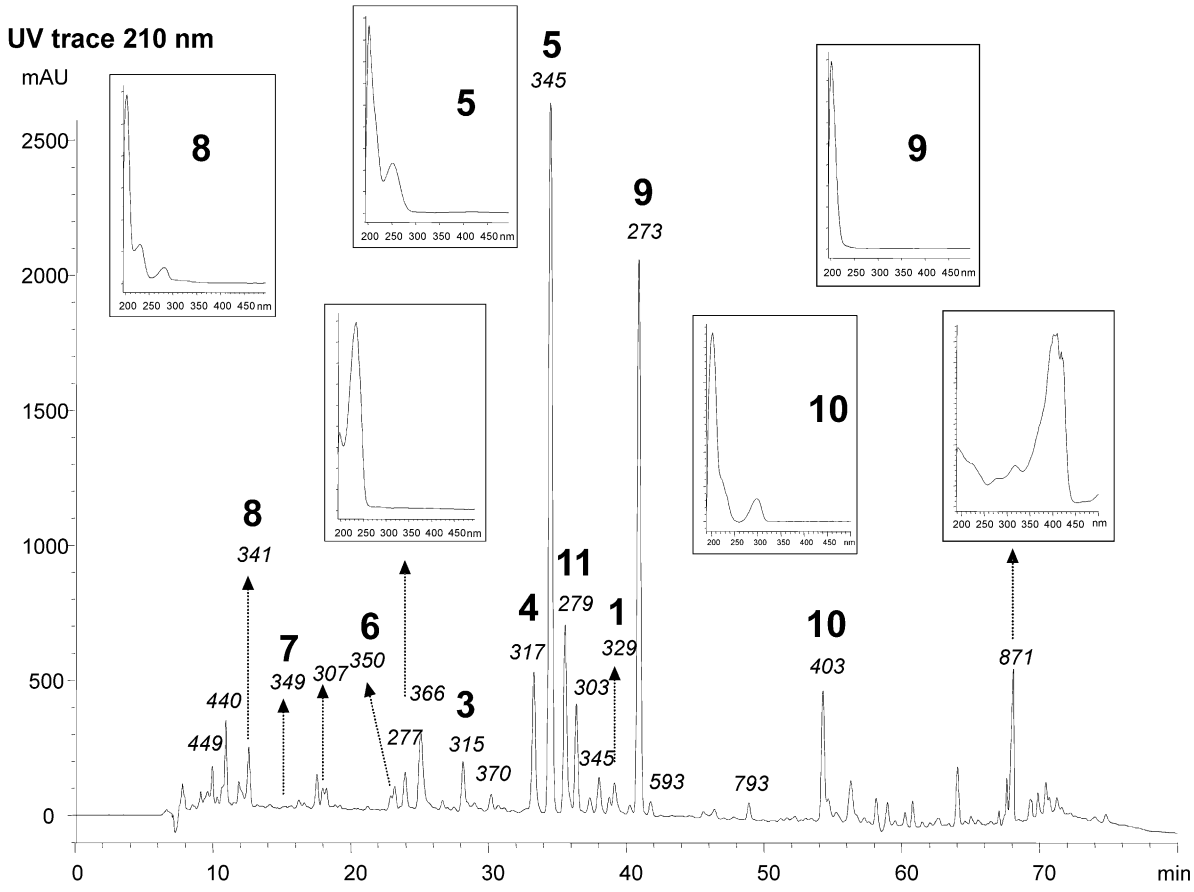

Fig. 1. LC–UV–APCI–MS analysis of the dichloromethane extract of *Potamogeton pectinatus* (m/z values of some compounds are indicated in italics).

Three compounds (**8**–**10**) were identified as known natural products by comparison of their spectroscopic and physical properties with the published data. They consisted of a lignan, pinoresinol (**8**) (Agrawal and Thakur, 1985), a linear diterpene, (3*S*)-geranyllinalool (**9**) (Guella and Pietra, 2000) and δ-tocopherol (**10**) (Matsuo and Urano, 1976; Vieira et al., 1983).

The HR–ESI–MS analysis of compound **1** (m/z 411.2176, $[M+Na]^+$) indicated the molecular formula $C_{23}H_{32}O_5$. The complete assignment of all ^1H and ^{13}C NMR signals was realised by a combination of COSY, NOESY, HSQC and HMBC experiments (Tables 1 and 2). The ^{13}C signals at δ 141.1, 143.3, 108.6 and the ^1H shifts of the attached protons at δ 7.38, 7.40 and 6.40 showed the presence of a furano moiety. An exocyclic methylene gave a ^{13}C NMR signal at δ 107.4 ascribed to C-17. Its attachment to C-8 was confirmed by the HMBC spectrum. The methyl signals at δ 12.7 and 28.6 were respectively attributed to the C-20 and C-18 of a labdane structure, while the ^{13}C signal at δ 51.1 indicated a methyl ester. The methyl at δ 21.3 was ascribed to an acetyl group, as evidenced by HMBC correlation with the carbonyl at δ 170.2. The attachment of this group to C-12 was supported by a long-range correlation between H-12 at δ 5.83 and the carbonyl at δ 170.2. All these data were in good agreement with those reported for methyl 15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-labdatrien-19-oate isolated from *Sciadopytis verticillata* (Taxodiaceae) (Hasegawa and Hirose, 1985). The relative configuration was confirmed by NOE effects between H-5 and H-9, and between H-20 and the methyl of the ester at C-19. However the optical rotation value as well as the ^1H-NMR shifts of methylene H-17 protons differ indicating a probable change in configuration.

Chemotaxonomical information indicated that all labdanes from *Potamogeton* genus belong to the *ent* series and by analogy compound **1** could be assigned to this series (Hasegawa and Hirose, 1983; Kittakoop et al., 2001; DellaGreca et al., 2001; Cangiano et al., 2001). This hypothesis was also supported by the negative optical rotation measured for **1**. Indeed negative optical rotations were reported for *ent*-labdane diterpenes and positive for those of the normal series (Hasegawa and Hirose, 1985; DellaGreca et al., 2001; Cangiano et al., 2001). This rule is inverted for the furano labdanes with a ketone at C-12.

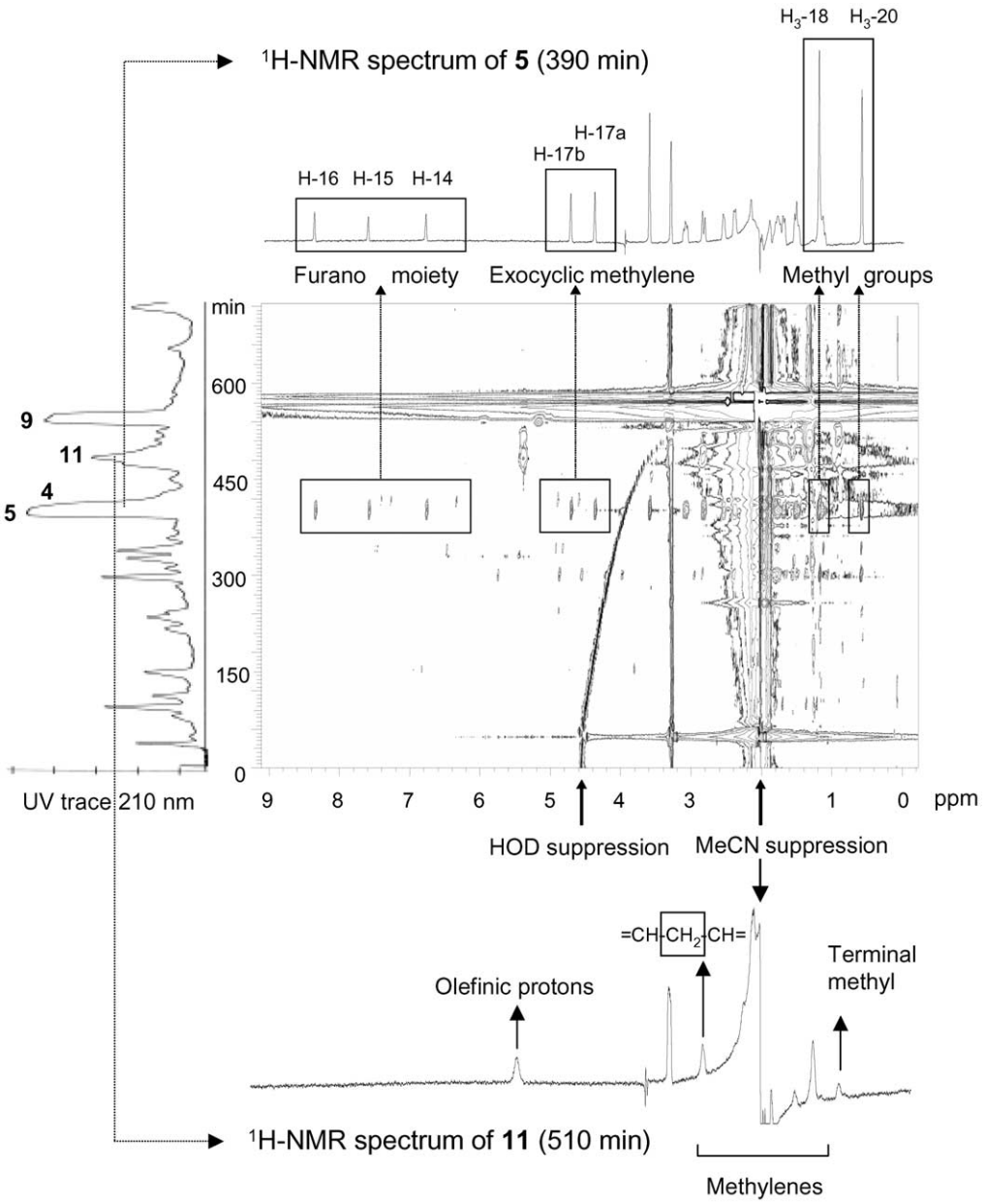

Fig. 2. On-flow LC–UV–^{1}H-NMR analysis of the dichloromethane extract of *Potamogeton pectinatus* (4 mg injected). Flow rate: 0.1 ml/min, 256 scans/increment.

For the determination of the absolute configuration at C-12, **1** was hydrolysed to give the hydroxy derivative 15,16-epoxy-12-hydroxy-8(17),13(16),14-*ent*-labdatrien-19-oate (**2**: $[\alpha]_D^{25}$ −38.3). Bell et al. (1972) reported the synthesis of the corresponding two epimeric hydroxy derivatives of the normal labdane series and showed some differences in the shifts of the H-17 protons depending of the configuration at C-12 (*R:* δ H-17a: 4.48, H-17b: 4.86; *S*: δ H-17a: 4.72, H-17b: 4.88). Because of the inversion of the whole chemical environment of H-17a and H-17b in the *ent*-labdane series, the rule described by Bell et al. (1972, 1975) has to be adapted, and the most deshielded protons correspond in this case to the *R* configuration. For **2**, the chemical shifts of H-17a at δ 4.74 and H-17b at δ 4.91 indicated thus *R* configuration at C-12.

Based on this information **1** was finally identified as methyl 15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-*ent*-labdatrien-19-oate. Its optical rotation ($[\alpha]_D^{25}$ −18.0°) confirms that **1** is not an enantiomer, but a diastereoisomer of the methyl 15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-labdatrien-19-oate ($[\alpha]_D^{25}$ + 79.4°) isolated by Hasegawa and Hirose (1985), and is thus a new natural product.

Compound **3**, $[\alpha]_D^{25}$ −10.8°, was assigned the structure 15,16-epoxy-12(*R*)-acetoxy-8(17),13(16),14-*ent*-labda-

trien-19-oic acid. ^{1}H and ^{13}C chemical shifts were very similar to those reported for **1**, but there was no signal corresponding to a methyl ester at C-19. The presence of a free acid was confirmed by downfield shifts of C-19 (δ 182.8 ppm) and of H-20 (δ 0.61 ppm). This is the first report of the isolation of a 12-acetoxy furano labdane diterpene as a free acid.

Two other furanoid *ent*-labdanes were identified as the common 15,16-epoxy-8(17),13(16),14-*ent*-labdatrien-19-oic acid (daniellic acid) (**4**), first isolated from *Daniellia oliveri* (Leguminosae) (Haeuser et al., 1961), and the methyl 15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate (**5**), previously reported from the aquatic plant *Ruppia maritima* (Ruppiaceae) (Della-Greca et al., 2000). These two main compounds were already partially identified based on the on-flow LC-^{1}H-NMR analysis performed on the extract of *P. pectinatus* (Fig. 2).

The HR–ESI–MS analysis of compound **6** (*m*/*z* 333.2061, $[M+H]^+$) indicated a molecular formula $C_{20}H_{28}O_4$. NMR data (Tables 1 and 2) showed that **6** was a labdane diterpene and its attribution to the *ent* series was supported by a negative optical rotation, $[\alpha]_D^{25}$ −33.0°, and the same biogenetic argument as exposed for **1**. The NMR spectra showed the presence of an α,β unsaturated γ-lactone moiety with two coupled ^{1}H signals at δ 7.10 (H-14, *t*, J = 1.5 Hz) and 4.76 (H-15, *dd*, J = 1.73, 3.7 Hz), and with ^{13}C at δ 134.9 (C-13), 143.8 (C-14), 70.1 (C-15) and 174.3 (C-16). The structure 8(17), 13-*ent*-labdadien-15→16-lactone-19-oic acid was finally assigned to **6**. The comparison with the spectral data of pinusolidic acid, its enantiomer isolated from *Brickellia glomerata* (Asteraceae) (Calderon et al., 1987) and from *Biota orientalis* (Cupressaceae) (Yang and Han, 1998), supported this structure. The relative configuration was confirmed by NOE effects between H-5 and H-9, and between H-18 and H-5.

The HR–ESI–MS analysis of compound **7**, $[\alpha]_D^{25}$ −36.6°, indicated the molecular formula $C_{20}H_{28}O_5$ (*m*/*z* 371.18316, $[M+Na]^+$). NMR data (Tables 1 and 2) showed that **7** was a labdane diterpene similar to **6** except for the lactone moiety. The NMR spectra showed the presence of a γ-hydroxy-butenolide group with two ^{1}H signals at δ 5.89 (H-14) and 6.02 (H-16), and with ^{13}C signals at δ 173.6 (C-13), 117.6 (C-14), 173.6 (C-15) and 101.5 (C-16). The structure 16-hydroxy-8(17),13-*ent*-labdadien-15,16-olid-19-oic acid was finally assigned to **7**. This structure was supported by the comparison with the spectral data of its enantiomer isolated from *Calocedrus formosona* (Cupressaceae) (Fang et al., 1989) and of its epimer, 16-hydroxy-

Table 2
^{13}C NMR spectral data of compounds **1**, **3**, **6** (in $CDCl_3$) and **7** (in CD_3OD)[a]

^{13}C	**1**	**3**	**6**	**7**
1	38.9 (CH_2)	38.9 (CH_2)	39.2 (CH_2)	40.5 (CH_2)
2	19.9 (CH_2)	19.8 (CH_2)	19.9 (CH_2)	21.2 (CH_2)
3	38.0 (CH_2)	37.8 (CH_2)	37.9 (CH_2)	39.4 (CH_2)
4	44.2 (C)	44.1 (C)	44.3 (C)	45.1 (C)
5	56.0 (CH)	56.0 (CH)	56.3 (CH)	57.4 (CH)
6	25.7 (CH_2)	25.9 (CH_2)	26.0 (CH_2)	27.6 (CH_2)
7	38.4 (CH_2)	38.4 (CH_2)	38.6 (CH_2)	39.8 (CH_2)
8	147.0 (C)	146.9 (C)	147.4 (C)	149.3 (C)
9	51.6 (CH)	51.7 (CH)	55.7 (CH)	57.2 (CH)
10	40.0 (C)	40.2 (C)	40.5 (C)	41.6 (C)
11	29.1 (CH_2)	29.2 (CH_2)	21.9 (CH_2)	22.4 (CH_2)
12	68.1 (CH)	68.1 (CH)	24.7 (CH_2)	27.9 (CH_2)
13	124.1 (C)	124.2 (C)	134.9 (C)	173.6 (C)
14	108.6 (CH)	108.7 (CH)	143.8 (CH)	117.6 (CH)
15	143.3 (CH)	143.4 (CH)	70.1 (CH_2)	173.6 (C)
16	141.1 (CH)	141.2 (CH)	174.3 (C)	101.5 (CH)
17	107.4 (CH_2)	107.5 (CH_2)	106.8 (CH_2)	107.0 (CH_2)
18	28.6 (CH_3)	28.8 (CH_3)	29.0 (CH_3)	29.6 (CH_3)
19	177.6 (C)	182.8 (C)	183.2 (C)	181.1 (C)
20	12.7 (CH_3)	12.9 (CH_3)	12.8 (CH_3)	13.3 (CH_3)
OMe	51.1 (CH_3)	–	–	–
Ac-1	170.2 (C)	170.3 (C)	–	–
Ac-2	21.3 (CH_3)	21.3 (CH_3)	–	–

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

Table 1
^{1}H NMR spectral data of compounds **1**, **2**, **3**, **6** (in $CDCl_3$) and **7** (in CD_3OD)

^{1}H	**1**	**2**	**3**	**6**	**7**
9	1.39 *t* (6.6)		1.41 *dd* (5.4, 6.4)	1.62 *m*	1.71 *m*
11	1.95 *m*		1.95 *m*	1.61 *m*	1.70 *m*
	1.95 *m*		1.96 *m*	1.79 *m*	1.85 *m*
12	5.83 *t* (7.6)	4.71 *dd* (4.6, 9.5)	5.83 *dd* (6.3, 9.3)	2.14 *m*	2.26 *m*
				2.47 *m*	2.56 *m*
14	6.40 *d* (1.1)	6.41 *d* (1.0)	6.40 *d* (2.0)	7.10 *t* (1.5)	5.89 *brs*
15	7.40 *t* (1.6)	7.40 *t* (1.7)	7.40 *t* (1.5)	4.76 *dd* (1.7, 3.7)	–
16	7.38 *s*	7.34 *s*	7.38 *s*	–	6.02 *brs*
17	4.86 *s*	4.74 *s*	4.86 *s*	4.59 *s*	4.56 *s*
	4.93 *s*	4.91 *s*	4.94 *s*	4.89 *s*	4.90 *s*
18	1.14 *s*	1.14 *s*	1.19 *s*	1.24 *s*	1.20 *s*
20	0.52 *s*	0.53 *s*	0.61 *s*	0.61 *s*	0.67 *s*
OMe	3.60 *s*	3.60 *s*	–	–	–
OAc	2.02 *s*	–	2.02 *s*	–	–

8(17),13-*ent*-labdadien-15,16-olid-18-oic acid, isolated from *Brickellia lemmonii* (Asteraceae) (Zdero et al., 1991). As for **6**, the relative configuration was confirmed by NOE effects between H-5 and H-9, and between H-18 and H-5. The absolute configuration at C-16 could not be determined but the presence of both epimers was suspected, as the similar compounds from *Calocedrus formosona* and *Brickellia lemmonii* were isolated as a mixture of H-16 epimers. Both **6** and **7** have not been reported with the *ent*-configuration, and are thus new natural products.

The biological activity of the extract was estimated by several bioassays to evaluate its molluscidal, fungicidal, and larvicidal activities (Hostettmann et al., 1982; Homans and Fuchs, 1970; Rahalison et al., 1991; Cepleanu et al., 1994), but it did not show any significant activity against the organisms tested. As Cangiano et al. (2002) reported an algicidal effect of the *ent*-labdane diterpenes from *Potamogeton natans* and *Ruppia maritima*, this activity was also investigated with the diterpenes isolated from *P. pectinatus*.

The algal growth inhibitory effect of the diterpenes **1-7** and **9** was tested on microplate with the alga *Raphidocelis subcapitata* (Environment Canada, 1992). No effect was displayed by the linear diterpene (**9**), while a significant activity was observed with the compounds **1**, **3-6** and their IC_{50} values could be estimated (Table 3). The labdane diterpene **5** was the most toxic and showed a high growth inhibitory effect with an IC_{50} of 6.1 μmol/l. This result is consistent with the activity estimated by DellaGreca et al. (2000) for the same compound isolated from *Ruppia maritima* (IC_{50} = 1.45 μmol/l). Compounds **1** and **4** showed also an important inhibitory activity with an IC_{50} of 18.2 and 17.2 μmol/l, but the effect of **6** was more moderate.

As underlined by DellaGreca et al. (2001), the toxicity of the labdane diterpenes is dependent on the skeleton and the kind of substituent. After hydrolysis of the acetate group at C-12 of **1**, the growth inhibitory activity disappears (**2**). The algicidal effect can be increased by the substitution of the C-12 acetoxy moiety (**1**) by an oxo group (**5**). A free carboxyl group increases the IC_{50} from 18.2 (**1**) to 107.8 μmol/l (**3**). But with the suppression of the whole acetoxy group, compound **4** displays a similar activity as **1**. The replacement of the furano moiety in **4** by a lactone in **6** diminishes also significantly the activity. These results confirm the importance of the C-12 substitution and of the presence of the furano group for the biological activity. This is in good agreement with the results reported by Cangiano et al. (2002).

Our results and the previous papers cited (Hasegawa and Hirose, 1983; Kittakoop et al., 2001; DellaGreca et al., 2001, Cangiano et al., 2001) seem to confirm the general occurrence of *ent*-labdanes, as furanoid or lactone diterpenes, in the Potamogetonaceae family. A furanoid labdane isolated from *P. nodosus* by Qais et al. (1998) seems to represent an exception as it is reported to belong to the normal labdane series. But the positive sign of the optical rotation suggests that this compound is the same enantiomer as an *ent*-labdane isolated from *P. natans*, the 12-oxo derivative of potamogetonin (DellaGreca et al., 2001). Similar compounds were isolated from *Ruppia maritima* (DellaGreca et al., 2000), which was previously considered as a member of the Potamogetonaceae family and is at present classified in the Ruppiacae family (Haynes et al., 1998). The presence of *ent*-labdane diterpenes in some close families of aquatic plants as the Zannichelliaceae, the Zosteraceae and the Juncaginaceae can therefore be suspected.

This study suggests that some of the labdanes present in *P. pectinatus* play an ecological function as allelochemicals in the aquatic ecosystems. This conclusion is also supported by the results of Cangiano et al. (2002), who demonstrated that almost all *ent*-labdanes isolated from *Ruppia maritima* and *P. natans* were significantly active against one or several aquatic organisms as alga, rotifer and crustaceans. Further investigations of *Potamogeton* species will be undertaken to have a better view of the labdane diversity and activity in this genus.

Table 3
Growth inhibition of the alga *Raphidocelis subcapitata* by diterpenes from *Potamogeton pectinatus*

Compound	72 h IC_{50} (μmol/l)[a]	95% Confidence limits
1	18.2	9.6–34.5
2	ND (50)	
3	107.8	81.6–142.4
4	17.2	12.2–24.1
5	6.1	4.0–9.3
6	47.1	34.1–65.2
7	ND (77)	
9	ND (161)	

[a] ND: not determined (at the highest concentration tested).

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 500 MHz for ^1H and 125 MHz for ^{13}C on a Varian Unity Inova spectrometer. 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 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 Bruker or a Micromass LCT instrument. Solid phase

extraction was realised with Chromabond® C_{18} (1 g) prepacked columns. Preparative LPLC was performed with a Duramat® pump equipped with a LKB 2238 UVICORD SII UV detector at 206 nm using a Lobar Lichroprep® RP-18 prepacked column (40-63 μm, 310×25 mm i.d., Merck). Preparative MPLC was realised with 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 μBondapak® C_{18} prepacked radial-compression columns (10 μm; 100×8, 100×25 or 200×40 mm i.d.; Waters). A Shimadzu LC-8A pump equipped with a Knauer UV detector was used with 100×25 mm and 200×40 mm i.d. columns, and a Shimadzu LC-10AD pump equipped with a LKB Bromma UV detector was used with a 100×8 mm i.d. column. HPLC–UV–MS analyses were realised on a Hewlett-Packard series 1100 apparatus with two Zorbax Eclipse XDB-Phenyl column connected in series (250×4.6 mm i.d.) using a CH_3CN–H_2O solvent system containing 0.05% TFA: 50 to 100% CH_3CN in 60 min at 0.7 ml/min, then 20 min with 100% CH_3CN. The spectra and UV trace 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 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 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 gradient (25 psi He column head pressure): 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 (0.1 ml/min) was used with the following gradient: 20:80 to 80:20 CH_3CN : D_2O in 450 min., 80:20 to 100% CH_3CN in 100 min., 100% CH_3CN during 200 min). The HPLC apparatus was coupled to a 500 MHz Varian Unity Inova spectrometer via a ^1H probe with a 60 μl flow cell of 3 mm i.d. Solvent suppression was realised with a WET sequence (Smallcombe et al., 1995). In the on-flow mode each increment consisted of 256 transients.

3.2. Plant material

Potamogeton pectinatus L. (Potamogetonaceae) was collected in Lake Léman, St-Sulpice (Switzerland), in June 1999. It was identified by Professor Jean-Bernard Lachavanne (LEBA, University of Geneva, Switzerland). A voucher specimen is deposited at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland (No. 2000042).

3.3. Extraction and isolation

The whole plant material (3.020 kg) was air-dried, powdered and extracted at room temperature four times with CH_2Cl_2 (4×15 l) for 24 h to give 61 g of extract. This extract (17 g) was chromatographed on Silica gel 60 (0.063–0.200 mm) with a petrol ether–EtOAc gradient (100:0 → 50:50), then a $CHCl_3$-MeOH gradient (90:10 → 50:50) to give the fractions A–O. Fraction E was separated by MPLC with a CH_3CN–H_2O gradient (70:30 → 100:0) to give the pure compounds **1** (33 mg), **5** (432 mg), **9** (331 mg) and **10** (51 mg). Fraction H was eluted with CH_3CN on a SPE C_{18} column to remove the major part of the apolar pigments. The eluate was then separated by semi-preparative HPLC on a μBondapak® C_{18} radial-compression column (200×40 mm i.d.) with a CH_3CN-H_2O gradient (5:95 → 100:0 in 60 min then 90 min at 100% CH_3CN) to give fractions 1–10. Further purification of fractions 6-8 by semi-preparative chromatography using a μBondapak® C_{18} radial-compression column (100×25 mm i.d.) with CH_3CN–H_2O (65:35) as the mobile phase led to the isolation of compound **4** (5 mg). Fractions M and N were pooled and separated on Silica gel 60 (0.035–0.070 mm) with a $CHCl_3$–MeOH gradient (100:0 → 80:20) to afford fractions 1–18. Fractions 7 and 8 were then chromatographed on Sephadex LH-20 with $CHCl_3$–MeOH (1:1) as mobile phase to give fractions I–IV. Further purification of fraction IV by semi-preparative HPLC using a μBondapak® C_{18} radial-compression column (100×8 mm i.d.) with a CH_3CN–H_2O gradient (15:85 → 50:50 in 30 min then 30 min at 50:50) led to the isolation of compounds **6** (8 mg) and **8** (6 mg). Fraction J was separated by LPLC with a CH_3CN–H_2O gradient (20:80 → 80:20) and led to the isolation of compounds **3** (10 mg), **7** (4 mg) and the re-isolation of compound **6** (8 mg).

3.4. Compound characterization

3.4.1. Methyl 15,16-epoxy-12(R)-acetoxy-8(17),13(16),14-ent-labdatrien-19-oate (1)

White gum; $[\alpha]_D^{25}$ −18.0° ($CHCl_3$; *c* 0.60); UV (EtOH) λ_{max} nm (log ε): 201 (3.89); ^1H NMR: see Table 2; ^{13}C NMR: see Table 1; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 346 [M-42(Ac)]$^+$(2), 328 [M–AcOH]$^+$ (52), 313 [M–

AcOH–Me]$^+$ (4), 269 [M-119]$^+$ (8), 121 [M-267]$^+$ (30), 71 [M-317]$^+$ (100); HR–ESI–MS: *m/z* 411.2176 ($C_{23}H_{32}O_5Na$: [M + Na]$^+$, requires 411.2147).

3.4.2. 15,16-Epoxy-12(R)-acetoxy-8(17),13(16),14-ent-labdatrien-19-oic acid (3)

Yellow oil; $[\alpha]_D^{25}$ −10.8° ($CHCl_3$; *c* 0.93); UV (EtOH) λ_{max} nm (log ε): 201 (3.85); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 1; EIMS (probe) 70 eV, *m/z* (rel. int.): 332 [M-42(Ac)]$^+$·(6), 314 [M–AcOH]$^+$ (98), 299 [M–AcOH–Me]$^+$ (10), 253 [M-121]$^+$ (11), 147 [M-227]$^+$ (100), 121 [M-267]$^+$ (76); HR–ESI–MS: *m/z* 397.1992 ($C_{22}H_{30}O_5Na$: [M + Na]$^+$, requires 397.1985).

3.4.3. 8(17),13-Ent-labdadien-15 → 16-lactone-19-oic acid (6)

Yellow oil; $[\alpha]_D^{25}$ −33.0° (MeOH; *c* 0.74); UV (MeOH) λ_{max} nm (log ε): 201 (3.81); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 1; EIMS (probe) 70 eV, *m/z* (rel. int.): 332 [M]$^+$ (7), 314 [M–H_2O]$^+$ (14), 286 [M-46]$^+$ (46), 271 [M-61]$^+$ (37), 161 [M-171]$^+$ (46), 121 [M-211]$^+$ (100); HR–ESI–MS: *m/z* 333.2061 ($C_{20}H_{29}O_4$: [M + H]$^+$, requires 333.2066).

3.4.4. 16-Hydroxy-8(17),13-ent-labdadien-15,16-olid-19-oic acid (7)

Pale-yellow gum; $[\alpha]_D^{25}$ -36.6° (MeOH; *c* 0.32); UV (MeOH) λ_{max} nm (log ε): 201 (3.89); ^{1}H NMR: see Table 2; ^{13}C NMR: see Table 1; EIMS (probe) 70 eV, *m/z* (rel. int.): 348 [M]$^+$ (1), 330 [M–H_2O]$^+$ (10), 312 [M–2H_2O]$^+$ (9), 284 [M-64]$^+$ (13), 235 [M-113]$^+$ (26), 189 [M-159]$^+$ (30), 121 [M-227]$^+$ (100); HR–ESI–MS: *m/z* 371.1831 ($C_{20}H_{28}O_5Na$: [M + Na]$^+$, requires 371.1829).

3.4.5. Hydrolysis of 1

Compound **2** was prepared according to Hasegawa and Hirose (1985) by alkaline hydrolysis of compound **1**. A solution of **1** (6 mg) in 2 M ethanolic KOH (2.5 ml) was stirred for 3 h at room temperature. After a 10-fold dilution with H_2O, the solution was extracted with Et_2O. The solvent was then evaporated to give the compound **2** (3 mg).

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). The algae were provided immobilized on beads of alginate from an Algaltoxkit F^{TM} (Creasel, Deinze, Belgium) and were set free before each toxicity test. The preparation of the algae and of the algal culturing medium was performed according to the Standard Operational Procedure of the Algaltoxkit F^{TM} (Creasel, 1996).

Single chemicals were initially dissolved in DMSO and then diluted in the algal culturing medium at the different test concentrations. Highest DMSO level in the test wells did not exceed 0.33% (v/v). Ten µl of each toxicant concentration were diluted in 290 µl of algal culturing medium containing 2×10^4 algal cells/ml. A 96-well microplate allowed the testing of two compounds with three replications of five toxicant concentrations. Every replication was associated with a control containing the maximal DMSO concentration. Four series of controls with algae only were carried out in the same microplate and the peripheral wells were filled with algal culturing medium for minimizing evaporating losses from the inner wells.

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 relation of the absorbance versus the number of cells was established to check the minimum algal growth required in the control wells. The number of algae in the control wells must have increased by at least a factor of 16 during the 72 h period for the toxicity test to be acceptable (Creasel, 1996). The raw data were processed by Microsoft Excel 5.0: 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^{TM} producer was 0.38 mg/l.

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