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Identification of the polar constituents of *Potamogeton* species by HPLC-UV with post-column derivatization, HPLC-MS^n and HPLC-NMR, and isolation of a new *ent*-labdane diglycoside

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Abstract

The polar extracts of *Potamogeton pectinatus*, *P. lucens*, *P. perfoliatus* and *P. crispus* (Potamogetonaceae) were analyzed by HPLC-UV-MS and their chromatographic profiles were very similar. The polar constituents of *P. pectinatus* were more exhaustively investigated by HPLC-UV with post-column derivatization, HPLC-MS^n and HPLC-NMR, which allowed the on-line identification of various known flavones (dereplication). One of these compounds, luteolin 3′-*O*-glucoside, has never been characterized in the *Potamogeton* genus. The HPLC-UV-MS and HPLC-NMR analyses revealed also the presence of *ent*-labdane diterpene glycosides in the polar extracts of *P. pectinatus* and *P. lucens* and led to the isolation of a new *ent*-labdane diglycoside from *P. pectinatus*, β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate.

Keywords: *Potamogeton pectinatus*; *P. lucens*; *P. perfoliatus*; *P. crispus*; Potamogetonaceae; Spectroscopic analysis; HPLC-MS; HPLC-NMR; *ent*-Labdane diterpenes; Flavonoids

1. Introduction

As a part of our ongoing research on new active compounds from aquatic macrophytes of Switzerland, we have previously investigated the non-polar extracts of pondweeds *Potamogeton perfoliatus* L., *P. crispus* L., *P. pectinatus* L. and *P. lucens* L. (Potamogetonaceae) (Waridel et al., 2003, 2004). The presence of *ent*-labdane diterpenes was demonstrated in these latter two species, as well as in other *Potamogeton* species by other authors (DellaGreca et al., 2001; Cangiano et al., 2001; Qais et al., 1998; Kittakoop et al., 2001; Smith et al., 1976), and several of these diterpenes showed an algicidal activity. Some labdanes were isolated as glycosides from *P. lucens* and *P. natans* (Cangiano et al., 2001; Waridel et al., 2004), but up to now the polar extracts of *Potamogeton* have been preferentially studied for their flavonoid composition (Boutard et al., 1972, 1973; Roberts and Haynes, 1986; Les and Sheridan, 1990).

This paper describes the chemical investigation of the polar extracts of *P. perfoliatus*, *P. crispus*, *P pectinatus* and *P. lucens*. Most of the polar constituents, known glycosylated labdanes (**2**–**3**) and flavones (**4**–**15**), were efficiently dereplicated based on on-line identification

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by various hyphenated methods. The HPLC-NMR analysis of the extract of *P. pectinatus* revealed also the presence of a new diglycosylated *ent*-labdane (**1**). The targeted isolation of this compound was undertaken for a complete structure assignment.

2. Results and discussion

The polar extracts of *P. pectinatus*, *P. lucens*, *P. perfoliatus* and *P. crispus*, obtained by maceration of powdered plant material with MeOH after extraction with CH_2Cl_2, were subjected to HPLC-UV/DAD-APCI/MS in the positive ion mode according to our standard procedure (Wolfender et al., 1998; Hostettmann et al., 2001). The chromatographic profiles were very similar, since many constituents were common to all species investigated, in particular compounds **4**, **5**–**7**, **9**–**11**, **14** and two peaks at *m/z* 734 and 419 (Fig. 1). This latter compound, displaying UV maxima at 219, 291 and 330 nm and a fragment ion at *m/z* 163, was most probably a caffeic acid ester derivative. Most of the constituents (**4**–**13**) displayed UV spectra characteristic of flavonoids, flavones or flavonols, with maxima between 251–271 nm (band II) and 335–350 nm (band I) (Table 1). The corresponding protonated molecules between *m/z* 271 and 463 were consistent with the molecular weight of known flavonoids. In the MS spectra of **7**–**10**, main fragment ions at *m/z* 271 $[M-162+H]^+$, 287 $[M-162+H]^+$ or 287 $[M-176+H]^+$, indicating the loss of hexosyl or uronic acid residues, were characteristic of flavonoid *O*-glycosides. The MS spectra of **11**–**13** showed an unique protonated molecule at *m/z* 271 $[M+H]^+$, 287 $[M+H]^+$ or 301 $[M+H]^+$, revealing the presence of flavonoid aglycones. Spectra of **4**–**6** displayed fragment ions with losses of 90 and 120 Da, characteristic of *C*-glycoside derivatives. In order to get more structural information, MS^2 experiments were performed on the precursor ion $[M-120+H]^+$ of **4**–**6**, and on the aglycone ions of **7**–**13** at *m/z* 271, 287 or 301

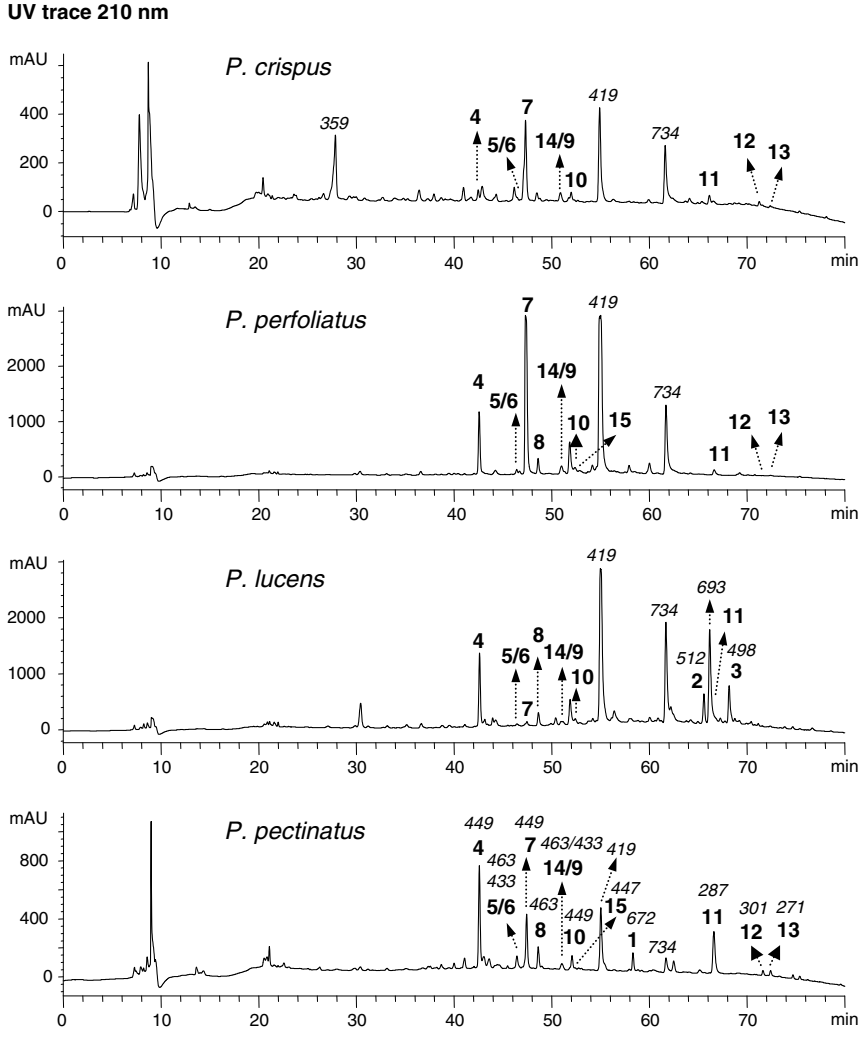


Fig. 1. HPLC-UV-MS analysis of the methanol extracts of *P. crispus*, *P. lucens*, *P. perfoliatus* and *P. pectinatus* (*m/z* values of compounds are indicated in italics).

Table 1
HPLC-MS^n and HPLC-UV spectral data of compounds **4**–**13**[a]

	HPLC-MS^n (m/z (%))		HPLC-UV (λ_{max} band II/band I)			
	Full MS[b]	MS^2	Eluent	+NaOAc	+$AlCl_3$	+$AlCl_3/H^+$
4	329 (100), 359 (38), 395 (16), 413 (23), 431 (44), 449 (74) $[M+H]^+$	283 (100), 300 (68), 311 (73)	259^{sh}, 269/350	269, 275^{sh}/333^{sh}, 405	275, 297^{sh}/407	273, 297^{sh}/351, 381^{sh}
5	343 (100), 409 (17), 427 (13), 445 (13), 463 (43) $[M+H]^+$	297 (32), 314 (25), 325 (49), 328 (100)[c]	–	–	–	–
6	313 (100), 343 (22), 379 (15), 397 (8), 415 (16), 433 (36) $[M+H]^+$	267 (100), 284 (59), 295 (99)	271/337	269^{sh}, 277/331, 399	277, 297^{sh}/339, 379^{sh}	277, 299^{sh}/339, 381^{sh}
7	287 (100), 449 (47) $[M+H]^+$	135 (24), 137 (8), 153 (100), 161 (13), 171 (25) 241 (26), 269 (27)	255, 269^{sh}/349	263/403	271, 293^{sh}/409	271, 293^{sh}/355^{sh}, 381
8	287 (100), 463 (14) $[M+H]^+$	135 (13), 137 (12), 153 (100), 161 (16), 171 (26), 179 (15), 241 (20), 269 (16)	254^{sh}, 268, 350	267/401	275, 337/407	271, 293^{sh}/353, 379
9	271 (100), 433 (70) $[M+H]^+$	119 (21), 121 (15), 145 (14), 153 (100), 163 (15), 171 (21), 225 (15), 243 (15)	268/336	269/401	273, 293^{sh}/335, 377^{sh}	277, 295^{sh}/335, 379^{sh}
10	287 (100), 449 (72) $[M+H]^+$	135 (21), 137 (24), 153 (100), 161 (21), 171 (33), 241 (38), 269 (26)	251, 271/339	269/381	277, 293^{sh}/341, 379^{sh}	277, 291^{sh}/343, 379^{sh}
11	287 (100) $[M+H]^+$	135 (30), 137 (6), 153 (100), 161 (27), 171 (37), 241 (36), 269 (25)	253, 267^{sh}/349	269, 333^{sh}/403	271, 295^{sh}/355^{sh}, 401	271, 293^{sh}/351, 379^{sh}
12	286 (18), 301 (100) $[M+H]^+$	258 (5), 286 (100)	251, 269^{sh}/345	269/407	273, 291^{sh}/345, 383^{sh}	263/347, 379^{sh}
13	271 (100) $[M+H]^+$	119 (26), 121 (11), 145 (37), 153 (100), 163 (7), 171 (56), 225 (29), 243 (27)	267/335	272/327, 395	273, 297^{sh}/339, 377^{sh}	277, 299^{sh}/341, 375^{sh}

[a] **4**, Isoorientin; **5**, isoscoparin; **6**, isovitexin; **7**, luteolin 7-*O*-glucoside; **8**, luteolin 7-*O*-glucuronide; **9**, apigenin 7-*O*-glucoside; **10**, luteolin 3′-*O*-glucoside; **11**, luteolin; **12**, chrysoeriol; **13**, apigenin.
[b] MS^2 experiments were performed on underlined ions.
[c] Ions obtained in a different HPLC-MS^n analysis with a MS^3 fragmentation of 463→343.

(Table 1), as described in our previous papers about HPLC-MS^n of flavonoids (Wolfender et al., 2000; Waridel et al., 2001). The three diagnostic fragment ions obtained in the MS^2 spectra of **4**–**6**, $[M-120-H_2O+H]^+$ (*m/z* 311, 325 and 295), $[M-120-CHO+H]^+$ (*m/z* 300, 314 and 284) and $[M-120-CH_2O_2+H]^+$ (*m/z* 283, 297 and 267), indicated that these compounds were 6-*C*-glycosides (Waridel et al., 2001), namely isoorientin (M_r 448), isoscoparin (M_r 462) and isovitexin (M_r 432). They were already identified in several species of *Potamogeton* (Boutard et al., 1972, 1973; Roberts and Haynes, 1986; Les and Sheridan, 1990). The fragment ions observed in the MS^2 spectra of the aglycone ions of **7**–**11** and **13**, $^{1,3}A^+$ (*m/z* 153), $^{1,3}B^+$ (*m/z* 119, 135) and $^{0,4}B^+$–H_2O (*m/z* 145, 161) (Fig. 2), showed the presence of two hydroxyl substituents on ring A and one (**9**, **13**) or two hydroxyl groups (**7**, **8**, **10**, **11**) on ring B of flavones (Wolfender et al., 2000). They were most probably apigenin (M_r 270) (**13**) and luteolin (M_r 286) (**11**), respectively, as these aglycones and their glycosylated derivatives have previously been identified by TLC in various species of *Potamogeton* (Boutard et al., 1973; Roberts and Haynes, 1986; Les and Sheridan, 1990). The fragment ion at *m/z* 286 $[M-15+H]^+$ observed in the MS^2 spectra of **12** was produced by the neutral loss of CH_3, indicating the presence of a methoxyl group in this structure (Wolfender et al., 2000). This compound was identified as chrysoeriol (M_r 300) (**12**) as this aglycone and its glycosylated derivatives have also already been characterized in several species of *Potamogeton* (Boutard et al., 1973; Roberts and Haynes, 1986; Les and Sheridan, 1990).

In order to confirm these preliminary results and to elucidate the structures of flavone *O*-glycosides **7**–**10**, HPLC-UV/DAD analyses of the methanol extract of *P. pectinatus* were carried out with post-column addition of UV-shift reagents (Table 1) (Hostettmann et al., 1984; Wolfender and Hostettmann, 1993). With the addition of a weak base (NaOAc), $AlCl_3$ and acidic $AlCl_3$, a precise localization of hydroxyl groups and sugars was possible. Bathochromic UV shift of band I (29–44 nm) obtained with acidic $AlCl_3$ showed the presence of a hydroxyl substituent at C-5 for all flavones **4**–**13**. Further bathochromic shift of band I (22–28 nm) observed after addition of $AlCl_3$ without acid indicated the presence of an *ortho* dihydroxyl group on ring B for **4**, **7**–**9** and **11**. Bathochromic shift of band I (42–65 nm) obtained after addition of NaOAc revealed the presence of a hydroxyl group at C-4′ for all flavones and allowed thus the localisation of the *ortho* dihydroxyl group of **4**, **7**–**9**, **11** at C-3′ and C-4′. For **7**–**9**, the absence of UV shift of band II after addition of NaOAc indicated a glycosylation at C-7. In contrast, a shift of band II (5–18 nm) was observed for **4**, **6**, **10**–**13** with NaOAc, revealing a free hydroxyl at C-7. For **10**, it indicated a glycosylation at C-3′, as the presence of only one free hydroxyl was demonstrated on ring B at C-4′. The flavones luteolin 7-*O*-glucoside (**7**), luteolin 7-*O*-glucuronide (**8**) and apigenin 7-*O*-glucoside (**9**) were thus identified in accordance with previous results (Roberts and Haynes, 1986; Les and Sheridan, 1990). Luteolin 3′-*O*-glucoside (**10**) could also be identified here in *P. pectinatus*, *P. lucens*, *P. perfoliatus* and *P. crispus*. It is the first report of this compound in *Potamogeton*. HPLC-MS analyses revealed also the presence of minor flavones chrysoeriol 7-*O*-glucoside (**14**, *m/z* 463 $[M+H]^+$ and 301 $[M-162+H]^+$) in all species and apigenin 7-*O*-glucuronide (**15**, *m/z* 447 $[M+H]^+$ and 271 $[M-162+H]^+$) in *P. perfoliatus* and *P. pectinatus*. Both compounds were already characterised in the genus (Les and Sheridan, 1990), but their identity could not be confirmed here by HPLC-UV. Moreover, the presence of isomers of apigenin 7-*O*-glucoside (rt 52.4 min) and chrysoeriol 7-*O*-glucoside (rt 52.7 min) in *P. pectinatus* and of a chrysoeriol glucuronide (rt 52.3, *m/z* 477 $[M+H]^+$ and 301 $[M-176+H]^+$) in *P. perfoliatus* was demonstrated (Fig. 1). These products have never been identified in *Potamogeton*, but their structure could not be completely elucidated based on the on-line data only. An acidic hydrolysis of the polar extract of *P. pectinatus* was performed in a methanol solution of 2N HCl and the analysis of the hydrolysate by HPLC-UV-MS confirmed that all the flavone *O*-glycosides derived from luteolin, apigenin and chrysoeriol.

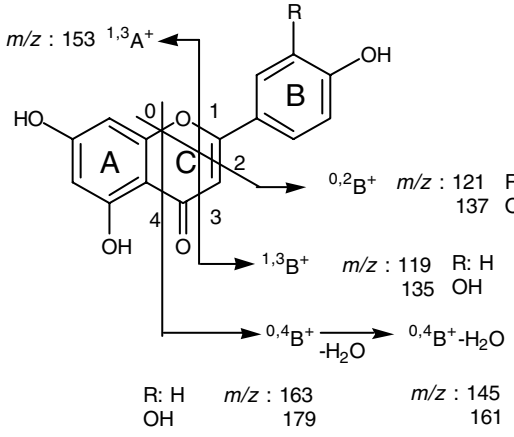
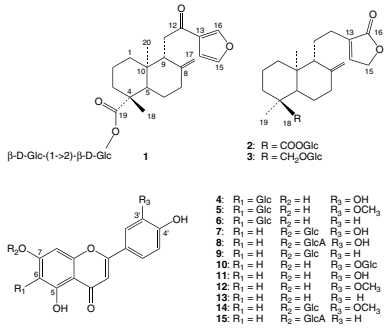


Fig. 2. Fragmentation pathway of flavones by CID tandem MS.



Besides these flavonoids, labdane diterpenes could be dereplicated in the polar extract of *P. lucens* by HPLC-UV-MS (Fig. 1). Compounds **2** and **3** at *m/z* 512 and 498 $[M+NH_4]^+$were identified as β-D-glucopyranosyl-8(17),13-*ent*-labdadien-16,15-olid-18-oate (**2**) and 18-β-D-glucopyranosyloxy-8(17),13-*ent*-labdadien-16,15-olide (**3**) in comparison with standards previously isolated as minor constituents from the dichloromethane extract of *P. lucens* (Waridel et al., 2004). In the HPLC-UV-MS analysis of *P. pectinatus*, the MS spectrum of **1** displayed an ammonium adduct at *m/z* 672, a protonated molecule at *m/z* 655 and two fragments at *m/z* 493 $[M-162+H]^+$ and 331 $[M-2*162+H]^+$ indicating the successive losses of two hexosyl moieties. The same spectrum showed also two ions, produced by the fragmentation of the aglycone (A), at *m/z* 221 $[A–C_6H_6O_2]^+$ and 175 $[A–C_6H_6O_2–CH_2O_2]^+$. These fragments were consistent with the successive losses of a furan moiety and a carboxylic group from a furano-labdane molecule (Fig. 3) (Sethi et al., 1988).

As the information obtained from UV and MS did not allow the identification of **1**, complementary on-flow and stop-flow HPLC-^{1}H NMR analyses were performed on the polar extract of *P. pectinatus* (Fig. 4). For a sensitive HPLC-^{1}H NMR detection, pigments were removed from the samples by SPE and the amounts of enriched extract injected were increased to about 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 and a fast solvent suppression technique (WET) was applied (Smallcombe et al., 1995). With these HPLC-NMR conditions, 32 scans per increment were sufficient in the on-flow mode to obtain HPLC-^{1}H NMR spectra of the main constituents of the extract with good resolution. Compound **1** eluting at rt 44 min showed ^{1}H NMR signals for an exocyclic methylene (δ 4.4–4.7 ppm), methyl groups (δ 0.5–1.2 ppm), a furan moiety (δ 7.5–8.4 ppm) and glycosidic protons (δ 3.1–5.5 ppm). These functionalities could be related to a glycosylated furano-labdane diterpene. The signals of two anomeric protons at δ 4.80 and 5.47 ppm confirmed the presence of two hexosyl moieties in the structure of **1**, while the most downfield ^{1}H at δ 8.35 ppm revealed a ketone at C-12 (DellaGreca et al., 2000, 2001). The aglycone signals were very similar to those of methyl-15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate, previously isolated from the non-polar extract of *P. pectinatus* (Waridel et al., 2003). Based on HPLC-MS and HPLC-^{1}H NMR information, **1** was thus identified as a dihexoside ester of 15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oic acid. As **1** was recognized as a new natural product, its targeted isolation was undertaken for the complete structure elucidation of the disaccharide

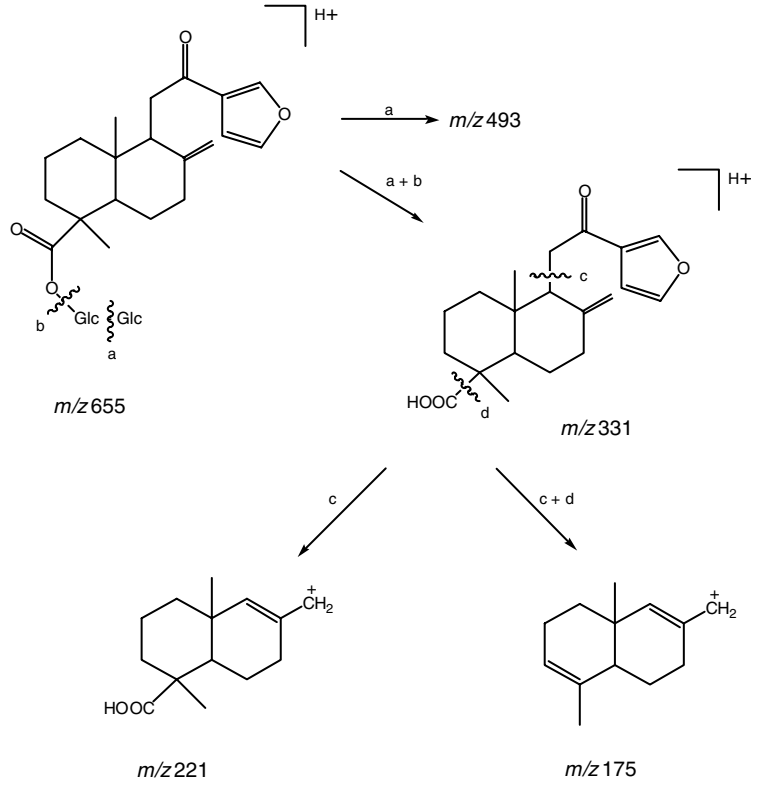


Fig. 3. Fragmentation pathway of **1** by MS.

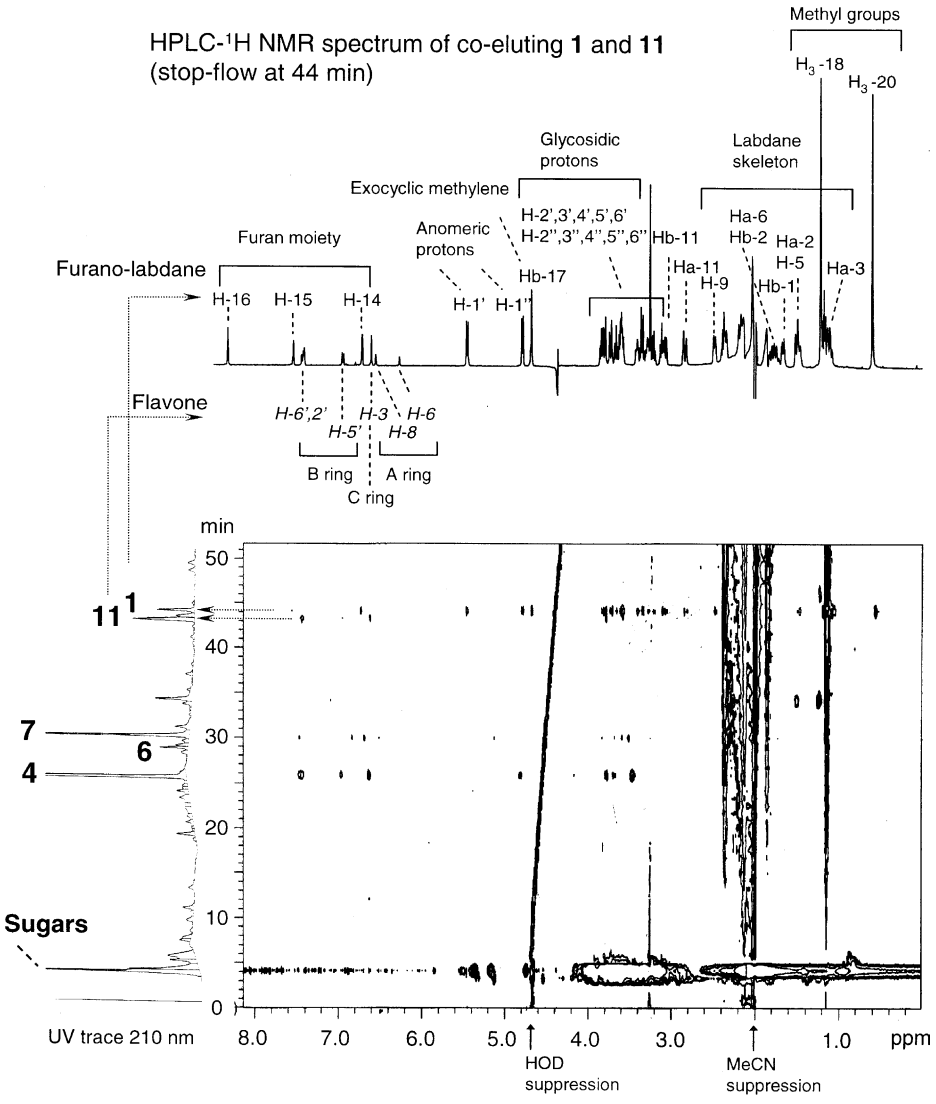


Fig. 4. On-flow HPLC-UV-^{1}H NMR analysis of the methanol extract of *P. pectinatus* (11 mg injected). Flow rate: 0.9 ml/min, 32 scans/increment. The inset represents the ^{1}H spectrum of **1** recorded in the stop-flow mode with 10,000 scans. The signals of flavone **11**, partly co-eluting with **1**, were also noticed (see labels below ^{1}H spectrum).

moiety and the measurement of its physical and chemical parameters.

In addition to **1**, the HPLC-^{1}H NMR spectra of the compounds eluting at rt 26 (**4**), rt 29 (**6**), rt 30 (**7**) and rt 43 min (**11**) showed characteristic signals for flavone glycosides (**4**, **6**, **7**) or aglycone (**11**) (sugars ^{1}H at 3.4–3.9, anomeric protons at 4.8–5.1, A- and C-ring ^{1}H at δ 6.5–6.9, B-ring ^{1}H at δ 7.0–7.9 ppm). The chemical shift values confirmed the previous identification of these compounds by HPLC-MS^n and HPLC-UV with post-column derivatization. The semi-quantitative estimation provided by HPLC-NMR (Godejohann et al., 1998) showed that the most abundant polar compounds of *P. pectinatus* were the flavones **4**, **7**, **11** and the labdane **1**.

For the isolation of **1**, the methanol extract of *P. pectinatus* was first fractionated by liquid–liquid extractions between hexane, $CHCl_3$ and $MeOH_{aq}$. The $MeOH_{aq}$ phase was further chromatographed on a Sephadex column to yield seven fractions. From pooled fractions 5 and 6, **1** was then isolated by MPLC on a reversed phase C_{18} column. The HR-ESI-MS analysis of **1** (*m*/*z* 653.2797, $[M-H]^-$) indicated the molecular formula $C_{32}H_{46}O_{14}$. The assignment of all ^{1}H and ^{13}C NMR signals was accomplished by a combination of COSY, NOESY, TOCSY, HSQC and HMBC experiments (Table 2). The NMR data showed that **1** was a labdane diterpene with an exocyclic methylene at C-17 (δ 107.3 ppm), a carboxyl carbon attached to C-4 (δ 177.7 ppm) and a methyl group at C-20 (δ 14.3 ppm). The ^{13}C chemical shift (δ 29.4 ppm) of the methyl group attached to C-4 indicated its equatorial position ((*R*)-configuration at C-4). The NMR spectra showed also the presence of a furan moiety with three ^{1}H signals at

Table 2
^{1}H and ^{13}C NMR spectral data of compound **1** (in CD_3OD)

	^{13}C	^{1}H
1	40.50	ax 1.20, *m*; eq 1.72, *m*
2	21.15	ax 1.94, *m*; eq 1.50, *m*
3	39.07	ax 1.13, *m*; eq 2.31, *m*
4	45.71	–
5	57.76	1.50, *m*
6	26.58	ax 2.01, *m*; eq 2.01, *m*
7	39.15	ax 2.06, *m*; eq 2.43, *m*
8	150.22	–
9	52.07	2.60, *d*, 8.79 Hz
10	40.89	–
11	37.52	a 2.82, *dd*, 2.93, 17.09 Hz b 3.11, *dd*, 9.77, 17.09 Hz
12	197.16	–
13	129.35	–
14	109.32	6.76, *t*, 0.97 Hz
15	145.85	7.58, *d*, 1.46 Hz
16	149.58	8.41, *d*, 1.46 Hz
17	107.26	a 4.41, *s*; b 4.73, *s*
18	29.38	1.28, *s*, 3H
19	177.66	–
20	14.32	0.71, *s*, 3H
1′	94.20	5.52, *d*, 7.81 Hz
2′	78.23	3.86, *m*
3′	78.93	3.66, *m*
4′	71.18	3.40, *m*
5′	78.65	3.37, *m*
6′	62.43	a 3.68, *m*; b 3.81, *m*
1″	103.66	4.85, *d*, 7.82 Hz
2″	75.82	3.19, *dd*, 7.81, 9.28 Hz
3″	78.04	3.35, *m*
4″	72.11	3.27, *m*
5″	78.27	3.27, *m*
6″	63.30	a 3.71, *m*; b 3.89, *m*

δ 6.76 (H-14, *t*, $J=1.0$ Hz), 7.58 (H-15, *d*, $J=1.5$ Hz) and 8.41 (H-16, *d*, $J=1.5$ Hz) ppm, and with ^{13}C at δ 129.4 (C-13), 109.3 (C-14), 145.9 (C-15) and 149.6 (C-16) ppm. The HMBC spectrum indicated that the furan moiety was attached to the labdane bicyclic skeleton by a methylene at C-11 (37.5 ppm) and a ketone at C-12 (δ 197.2 ppm). The other ^{13}C (δ 60–100 ppm) and ^{1}H signals (δ 3.3–5.5 ppm) were assigned to two glucopyranosyl residues. The coupling constants of H-1′ (δ 5.52 ppm, *d*, $J=7.8$ Hz) and H-1″ (δ 4.85 ppm, *d*, $J=7.8$ Hz) were characteristic for β-anomeric protons. The HMBC correlation between H-1′ and C-19 indicated an ester linkage between a glucose and the carboxyl group of the labdane. The HMBC correlations between H-1″ and C-2′ and between H-2′ and C-1″ revealed a β1→2 linkage between both glucoses, indicating that the carboxylic group was esterified by a sophorose residue. Compound **1** was therefore β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-15,16-epoxy-12-oxo-8(17),13(16),14-*ent*-labdatrien-19-oate, which confirmed the partial structure elucidation based on the on-line information. This is the first report of the isolation of a furano-labdane diglycoside ester. Its attribution to the enantiomeric series was based on biogenic arguments as all labdanes characterized in *Potamogeton* are *ent*-labdanes (Waridel et al., 2004).

In this study, various hyphenated techniques allowed an efficient dereplication of known flavones in *Potamogeton*, complementing the results of previous authors based on TLC (Roberts and Haynes, 1986; Les and Sheridan, 1990). Moreover, some flavonoids never reported in this genus, luteolin 3′-*O*-glucoside, a chrysoeriol glucuronide and isomers of apigenin 7-*O*-glucoside and chrysoeriol 7-*O*-glucoside, were observed. These latter flavonoids could be only partially identified, which underlines some limitations of the dereplication process in the case of minor compounds. When compared with TLC, HPLC-MS enabled a precise and sensitive comparison of species of *Potamogeton* and revealed a homogenous flavonoid composition between them. The major part of flavones previously reported in this genus was indeed identified in all species investigated here. This homogeneity seemed to be in contradiction with the results of Les and Sheridan (1990), who reported a reduced flavonoid profile in submersed foliage of *Potamogeton*. These authors linked this observation to an evolutionary loss of flavonoids as the UV filtering properties of these compounds were less important in submersed foliage protected by natural water. As we have studied only submersed species, it is currently not possible to confirm or refute this hypothesis before investigating with the same analytical techniques some heterophyllous species of *Potamogeton* containing both floating and submersed foliage.

Our results and previous investigations demonstrated that *ent*-labdanes with furan or lactone groups are present in *Potamogeton* both as aglycones and glycosides (Cangiano et al., 2001; Waridel et al., 2003, 2004). The HPLC-UV-MS data indicated that other glycosylated *ent*-labdanes are probably present in *P. lucens*, *P. pectinatus*, *P. crispus* and *P. perfoliatus*, but their structures could not be ascertained based on the on-line data only. In accordance with our previous conclusions (Waridel et al., 2004), the *ent*-labdane glycoside profiles of the polar extracts of *Potamogeton* species confirm that the labdane diversity in this genus is higher than that of flavonoids. These diterpenes can therefore be regarded as more interesting chemotaxonomic markers than flavonoids.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Perkin–Elmer 241MC polarimeter. UV spectra were determined on a Perkin–Elmer Lambda20 UV/VIS spectrophotometer.

HR-ESI-MS spectra were recorded on a Micromass LCT instrument. Solid phase extraction was carried out using Chromabond® C_{18} (1 g) prepacked columns. NMR spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian Unity Inova spectrometer with a $^{1}H[^{13}C]$ pulse field gradient indirect detection probe. TMS was used as internal standard for ^{1}H spectra. The same instrument equipped with a $^{1}H[^{13}C]$ pulse field gradient indirect detection microflow probe (flow cell 60 μl; 3 mm i.d.) was used for HPLC-NMR analyses, along with a Varian 9012 pump, a Valco injection valve and a Waters 490-MS UV detector. For HPLC-MS detection, a Finnigan triple quadrupole (TSQ 700) equipped with an APCI interface was used. The HPLC separation was carried out with a HP-1100 HPLC system (Hewlett–Packard) equipped with a binary pump system, a photodiode array high speed spectrophotometer detector (HP 1050), all controlled by the HP Chemstation software. A PAL autosampler (CTC Analytics) controlled by its own software was used for injections. For HPLC-MS^n analyses, a Finnigan LCQ ion trap equipped with an APCI interface was used. The HPLC separation was carried out with a HP-1100 HPLC system (Hewlett–Packard) equipped with a binary pump system, a photodiode array high speed spectrophotometric detector and an autosampler, all controlled by the HP Chemstation software. For HPLC-UV analyses with post-column derivatization, the separation was performed on a HP-1050 HPLC system (Hewlett–Packard) equipped with a quaternary pump system, a photodiode array high speed spectrophotometric detector and an autosampler, all controlled by the HP Chemstation software. The post-column neutralisation of eluent was carried out with a Gilson 303 pump and the UV shift reactants were added by a Shimadzu LV-9A pump. Preparative MPLC was carried out with a Büchi 681 pump equipped with a Knauer UV detector at 254 nm using a Lichroprep® RP-18 (15–25 μm) user-packed column (460×15 mm i.d., Büchi).

3.2. Plant material

P. lucens L. and *P. perfoliatus* L. (Potamogetonaceae) were collected in Lake Léman, near Morges, Switzerland, in July 1999. *P. 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 Prof. Jean-Bernard Lachavanne (LEBA, University of Geneva, Switzerland). Voucher specimens are deposited at the Laboratory of Pharmacognosy and Phytochemistry, University of Geneva, Switzerland (*P. pectinatus* No. 2000042, *P. lucens* No. 2000072, *P. perfoliatus* No. 2000073 and *P. crispus* No. 2000071).

3.3. HPLC-MS and HPLC-MS^n analyses

For HPLC-MS analyses on triple quadrupole, the separation was performed on two Symmetry® C_{18} columns (250×4.6 mm i.d., 4 μm; Waters) connected in series using gradient conditions with MeCN–H_2O (+0.05% TFA) at 0.7 ml/min (5:95–50:50 in 60 min, 50:50–75:25 in 10 min, 75:25–100:0 in 10 min and 100:0 during 10 min). The APCI parameters were: vaporizer 400 °C, capillary 150 °C, corona 5 μA, sheath gas N_2. Analyses were performed in the positive ion mode. For HPLC-MS^n analyses on ion trap, the separation was performed on a Symmetry® C_{18} column (250×4.6 mm i.d., 4 μm; Waters) using gradient conditions with MeCN–H_2O (+0.05% TFA) at 1.0 ml/min (5:95–30:70 in 40 min, 30:70–65:35 in 20 min, 65:35–100:0 in 5 min and 100:0 during 5 min). The APCI parameters were: vaporizer 450 °C, capillary 200 °C, corona 5 μA, sheath gas N_2, collision gas He, collision energy 50%. Analyses were performed in the positive ion mode. For fragmentations, scan dependent MS^2 experiments were automatically conducted on the most intense ions.

3.4. HPLC-UV analyses with post-column addition of UV shift reagents

For HPLC-UV analyses with post-column derivatization, the separation was realised using the same column and the same gradient conditions as in HPLC-MS^n analyses with MeCN–H_2O as mobile phase at 0.6 ml/min. The method used for post-column addition of UV shift reagents was based on the protocol described by Wolfender and Hostettmann (1993). The eluent was neutralised post-column with a NaOH solution (0.01 M, 0.2 ml/min) before the addition of UV shift reactants, NaOAc (0.5 M, 0.2 ml/min) or $AlCl_3$ (0.3 M, 0.2 ml/min). The addition of $AlCl_3$ (0.3 M, 0.4 ml/min) in acidic conditions was carried out with a mobile phase containing TFA (0.05%) without prior neutralisation. The reactions of derivatization were performed in a reaction coil at ambient temperature (NaOAc) or at 90 °C ($AlCl_3$).

3.5. HPLC-NMR analysis

The separation of the extract was performed on a μBondapak® C_{18} prepacked radial-compression column (10 μm; 100×8 mm i.d.; Waters) using gradient conditions with CH_3CN–D_2O at 0.9 ml/min (5:95–50:50 in 60 min, then 50:50–100:0 in 1 min and 100:0 during 10 min). In the on-flow mode, an amount of 11 mg was injected and the number of transients per increment was set to NT = 32. In the stop-flow mode, an amount of 14.5 mg was injected and ^{1}H NMR spectra were acquired with NT = 400–10,000. The UV trace was measured at 210 nm for monitoring the chromatographic

separation. References of the solvent signals were set at δ 2.00 ppm for acetonitrile. Solvent suppression was performed on-line by the use of the WET sequence (Smallcombe et al., 1995). During gradient elution, the shape of the selective pulses was automatically calculated on the fly based on a scout scan recorded before each increment.

3.6. Extraction and isolation

The whole plants were air-dried, powdered and extracted at room temperature three times with CH_2Cl_2 and three times with MeOH for 24 h. After filtration, the methanol extracts were evaporated to dryness under reduced pressure and lyophilized. Twenty mg of the samples were dissolved in 1 ml of MeOH, and after centrifugation, the supernatants were used for the HPLC-UV-MS analyses. Five hundred mg of the methanol extract of *P. pectinatus* were dissolved in 20 ml of MeOH and eluted on a 3 ml Chromabond® C_{18} column previously stabilized with MeOH. The percolate was then evaporated to dryness under reduced pressure and lyophilized before dissolution in 2 ml of deuterated methanol and analysis by HPLC-UV-NMR. A portion of the methanol extract (2 g) of *P. pectinatus* was 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. After extraction by $CHCl_3$, the polar phase was evaporated to dryness and this fraction was chromatographed on Sephadex® LH-20 with MeOH as mobile phase to give fractions 1–7. Further purification of fractions 5 and 6 by MPLC with a CH_3CN–H_2O gradient (20:80 → 100:0) led to the isolation of compound **1** (3 mg).

3.7. Compound characterisation

3.7.1. β-D-Glucopyranosyl-(1→2)-β-D-glucopyranosyl-15,16-epoxy-12-oxo-8(17),13(16)-14-ent-labdatrien-19-oate (1)

Yellow gum; $[\alpha]_D^{25} -2.0°$ (CH_3OH, *c* 0.25); UV (MeOH) λ_{max} nm (log ε): 202 (4.35); ^1H and ^{13}C NMR: see Table 2; HR-ESI-MS: *m/z* 653.2797 ($C_{32}H_{45}O_{14}$: $[M-H]^-$, requires 653.2809).

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