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Antialgal *ent*-labdane diterpenes from *Ruppia maritima*

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Abstract

Seven *ent*-labdane diterpenes have been isolated from *Ruppia maritima*. The structures 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-al; 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-ol acetate; methyl 15,16-epoxy-12-oxo-*ent*-labda-8(17),13(16),14-trien-19-oate; 15,16-epoxy-*ent*-labd-8(17),13*E*-dien-15-ol and 13-oxo-15,16-bis-nor-*ent*-labd-8(17)-ene have been assigned to the five new compounds by spectroscopic means and chemical correlations. The phytotoxicity of the diterpenes has been assessed using the alga *Selenastrum capricornutum* as organism test.

Keywords: *Ruppia maritima* L.; Potamogetonaceae; *Selenastrum capricornutum*; Diterpenes; *ent*-Labdanes; Toxicity; Microbiotests

1. Introduction

Our studies on metabolites of aquatic plants have shown that many of them have a strong in vitro antialgal effect (DellaGreca et al., 1998), which could justify the reduction of phytoplankton in natural ecosystems (Rice, 1984). In pursuing our chemical investigation of aquatic plants distributed in Italy, as well as the assessment of the antialgal properties of their components, we are now examining two species of Potamogetonaceae, which grow in the river Volturno near Naples. The first one *Potamogeton natans* is a fresh water species while *Ruppia maritima*, commonly known as sea hay, lives at the mouth of the river in brackish waters. In this paper we report the chemical and phytotoxicological investigation of *R. maritima*. This plant has been already studied and the presence of flavonoids (Boutard et al., 1973), sterols (Attaway et al., 1971) and phenolic compounds (Charriere et al., 1991) has been reported.

The plant, collected in the Summer, was air dried and extracted with solvents with increasing polarity. Chromatographic processes of the light petrol extract led to the isolation of seven diterpenes with the *ent*-labdane skeleton, five of them isolated for the first time.

2. Results and discussion

The known compounds have been identified as 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-ol (**1**) and methyl 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-oate (**2**) by comparison of their physical data with those reported by Canonica et al. (1969) and Heauser and Lombard (1961) respectively.

Compound **3**, $[\alpha]_D$ −10.0°, was assigned structure 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-al. The molecular peak at *m*/*z* 300 in the EI mass spectrum and the elemental analysis defined the molecular formula $C_{20}H_{28}O_2$. The ^{1}H–NMR spectrum (Table 1) showed the aromatic protons H-14– H-16 at δ 6.24, 7.20 and 7.38, the H-20 and H-18 methyl singlets at δ 0.60 and 1.01, the H-17 methylene protons as two singlets at δ 4.60 and 4.95 and the H-19 formyl proton at δ 9.78. In the ^{13}C-NMR spectrum (Table 2) 20 carbon signals were present, which were defined by a DEPT experiment. The signals at δ 13.5 and 24.3 were attributed to the C-20 and C-18 methyl carbons, the signal at δ 205.7 was attributed to the C-19 formyl carbon, while the signals at δ 125.3, 110.8, 142.7 and 138.7 corresponded to the C-13–C-16 furan carbons. The NOE interaction of the H-19 with the H-20 methyl justified the α-orientation of the formyl group. According to the assigned structure, $NaBH_4$ reduction of **3** gave 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-ol (**1**).

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Table 1
Selected ^{1}H NMR spectral data of compounds **1–7**

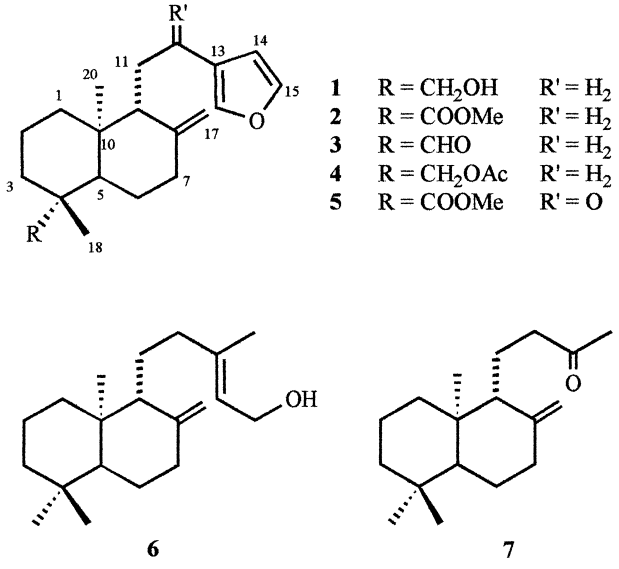
H	1[a]	2[a]	3	4	5	6	7
14	6.25 *dd* (0.9, 1.9)	6.28 *dd* (0.9, 1.9)	6.24 *dd* (1.0, 1.9)	6.26 *dd* (0.9, 2.0)	6.78 *dd* (0.9, 1.8)	5.41 *t* (7.4)	2.11 *s*
15	7.35 *dd* (1.3, 1.9)	7.35 *dd* (1.4, 1.9)	7.38 *dd* (1.2, 1.9)	7.34 *dd* (1.3, 2.0)	7.43 *dd* (1.4, 1.8)	4.17 *d* (7.4)	–
16	7.20 *dd* (0.9, 1.3)	7.20 *dd* (0.9, 1.4)	7.20 *dd* (0.9, 1.3)	7.18 *dd* (0.9, 1.3)	8.13 *dd* (0.9, 1.4)	1.68 *s*	–
17	4.56 *s* 4.86 *s*	4.58 *s* 4.48 *s*	4.60 *s* 4.95 *s*	4.60 *s* 4.88 *s*	4.38 *s* 4.78 *s*	4.46 *s* 4.82 *s*	4.44 *s* 4.83 *s*
18	0.97 *s*	1.17 *s*	1.01 *s*	0.96 *s*	1.20 *s*	0.87 *s*	0.88 *s*
19	3.43 *d* (11.5) 3.74 *d* (11.5)	–	9.78 *s*	3.85 *d* (11.2) 4.22 *d* (11.2)	–	0.80 *s*	0.81 *s*
20	0.66 *s*	0.51 *s*	0.60 *s*	0.70 *s*	0.59 *s*	0.68 *s*	0.70 *s*
OAc	–	–	–	2.04 *s*	–	–	–
OMe	–	3.60 *s*	–	–	3.63 *s*	–	–

[a] Data taken from Canonica et al. (1969) and Heauser and Lombard (1961), respectively.

Table 2
^{13}C NMR spectral data of compounds **1–7**

C	1[a]	2[b]	3	4	5	6	7
1	38.7	39.1	38.3	38.8	39.4	39.1	39.0
2	18.9	19.9	19.2	18.9	19.9	19.4	19.3
3	35.5	38.2	38.3	36.2	38.0	42.2	42.1
4	38.9	44.0	48.6	39.4	44.0	33.6	33.6
5	56.1	56.3	55.9	56.1	56.0	56.3	56.3
6	24.1	26.3	24.2	24.1	25.8	24.4	24.4
7	38.5	38.7	34.3	38.5	38.2	38.3	38.2
8	147.9	147.9	147.2	147.8	149.0	148.6	148.3
9	56.0	55.2	54.5	56.0	50.4	55.5	55.9
10	38.9	40.2	39.9	39.4	39.6	39.7	39.7
11	23.5	23.6	23.5	23.5	36.5	21.7	17.5
12	24.3	24.2	24.0	24.4	194.0	33.6	42.9
13	125.4	125.0	125.3	125.5	125.0	140.7	209.5
14	110.9	110.9	110.8	110.9	108.8	122.9	30.0
15	142.6	142.6	142.7	142.7	144.1	59.4	–
16	139.6	138.7	138.7	138.7	146.6	16.4	–
17	106.5	106.3	107.2	106.7	106.4	106.3	106.2
18	27.0	28.8	24.3	27.5	28.8	38.3	33.6
19	65.0	177.7	205.7	66.8	181.1	21.7	21.7
20	15.3	12.6	13.5	15.3	13.1	14.5	14.3
OMe	–	51.5	–	–	51.5	–	–
Ac1	–	–	–	171.0	–	–	–
Ac2	–	–	-	21.0	–	–	–

[a] Taken from supplementary data of Hasegawa and Hirose (1985).
[b] Data taken from Hasegawa and Hirose (1985).



Scheme 1.

Compound **4**, $[\alpha]_D$ −23.8°, showed in the EI mass spectrum the molecular ion at *m/z* 344. The elemental analysis defined the molecular formula $C_{22}H_{32}O_3$. The comparison of the ^{1}H and ^{13}C NMR data of **4** with those of **1** showed a downfield shift of the H-19 protons and the C-19 carbon. These shifts, along with the presence of an acetyl group, suggested the structure 15,16-epoxy-*ent*-labda-8(17),13(16),14-trien-19-ol acetate. Accordingly, acetylation of **1** gave a product identical with **4**.

Compound **5**, $[\alpha]_D$ +8.0°, was identified as methyl 15,16-epoxy-12-oxo-*ent*-labda-8(17),13(16),14-trien-19-oate. It had spectral data identical to those reported for 15,16-epoxy-12-oxo-labda-8(17),13(16),14-trien-19-oate, $[\alpha]_D$ −6.5°, isolated from *Sciadopitys verticillata* (Hasegawa and Hirose, 1985). The opposite rotation agreed with the appurtenance of **5** to the *ent*-labdane series.

Compounds **6**, $[\alpha]_D$ −15.5°, and **7**, $[\alpha]_D$ −38.8°, were easily identified as *ent*-labd-8(17),13*E*-dien-15-ol and 13-oxo-15,16-bis-nor-*ent*-labd-8(17)-ene respectively. Their physical properties were identical with those of labd-8(17),13*E*-dien-15-ol and 13-oxo-15,16-bis-nor-labd-8(17)-ene, already known as synthetic compounds obtained by McCreadie and Overton (1968) and Do Khac et al. (1975) respectively. The opposite rotations of the synthetic compounds and the diterpenes from *R. maritima* agreed with the appurtenance of these latter to the *ent*-labdane series.

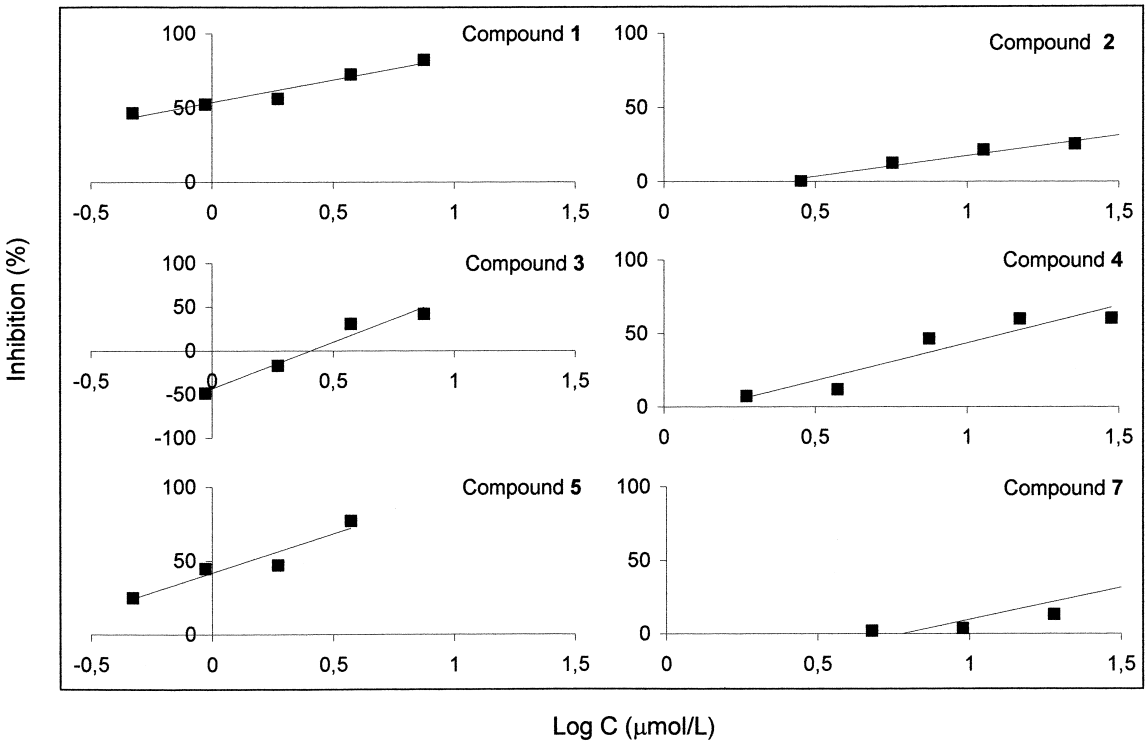


Fig. 1. Inhibition (%) of algal growth of the compounds **1**–**5** and **7**.

The biological activity of labdane diterpenes as antimicrobial (Ulubelen et al., 1985), insect antifeedant (Bohlmann et al., 1982) and cytotoxic (Zani et al., 2000) properties was extensively reported, but no much data were known for their phytotoxicity (Munesada et al., 1992).

We now tested the toxicity of diterpenes **1**–**7** on the unicellular green alga *S. capricornutum* (CCAP 278/4), renamed *Raphidocelis subcapitata*. This strain is the recommended species for toxicity testing in international guidelines (Pipe and Shubert, 1984). The seven diterpenes showed a considerable variability in toxicity. As described in Fig. 1, compound **1** was the most toxic ($IC_{50}=0.8$ μmol/l) with high level of toxicity starting from low concentrations. Also compound **5** showed a high inhibitory effect on algal growth ($IC_{50}=1.45$ μmol/l), while compound **4** revealed a clear dose-response relationship as **1** and **5**, but had a lower effect of inhibition ($IC_{50}=13.62$ μmol/l). The toxicity of compounds **2** and **7** was significant only for concentrations >9.5 μmol/l and their IC_{50} could not be registered at the highest tested concentration. The revealed toxicity for compound **6** was insignificant and without a correspondence between concentration and effect. Compound **3** at low concentration showed a stimulation of growth and the toxic effect was revealed only increasing the concentrations ($IC_{50}=7.57$ μmol/l). The growth stimulation could represent the response of the alga to the toxicant as biological systems counteract often the effects of contaminants (Calabrese, 1994).

3. Experimental

3.1. General experimental procedures

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AC 400 spectrometer with 0.05 M solns in $CDCl_3$ at 37 °C. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. IR spectra were determined in $CHCl_3$ solns on a FT–IR Perkin-Elmer 1740 spectrometer. EI mass spectra were obtained with a Kratos MS 80 apparatus. UV spectra were obtained on a Perkin-Elmer Lambda 7 spectrophotometer in EtOH solns. HPLC apparatus consisted of a pump (Varian Vista 5500), and a reflective index detector (Varian RI3) equipped with Hibar LiChrosorb RP-18 (7 μm, 250×10 mm i.d., Merk). Analytical TLC was performed on Merk Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm film thickness. Preparative TLC was performed on Merk Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Column chromatography (CC) was performed on Merk Kieselgel 60 (230–400 mesh) at a medium pressure (1–2 bar) or on Sephadex LH-20® (Pharmacia).

3.2. Plant material

Ruppia maritima was collected in the river Volturno near Naples in June 1998 and was identified by professor Gabriele Pinto. A voucher specimen is deposited at the Dipartimento di Biologia Vegetale of University Federico II of Naples.

3.3. Extraction and isolation

Air-dried plants (10 kg) were extracted with light petrol at room temperature for 7 days. The crude extract (36 g) was separated by conventional procedures into an acidic (2 g) and a neutral fraction (30 g). The neutral fraction was chromatographed on neutral Al_2O_3 (grade III) and the elution with hexane–Et_2O (19:1) gave fractions A–D. Fraction A was purified on Sephadex LH-20 [hexane–$CHCl_3$–MeOH (4:1:1)] to give compound **1** (23 mg). Fraction B was chromatographed on silica gel eluting with hexane–$CHCl_3$ (4:1) to give pure **3** (31 mg) and **2** (37 mg). Fraction C, was chromatographed on silica gel eluting with hexane–benzene mixtures. Hexane-benzene (49:1) gave crude **6** which was purified by prep. TLC [hexane:benzene (9:1)]. The fraction eluted with hexane–Et_2O (9:1) was chromatographed by RP18-HPLC [MeOH–MeCN (19:1)] to give compounds **4** (8 mg) and **7** (35 mg). Fraction D was chromatographed on Sephadex LH-20 with hexane–$CHCl_3$–MeOH (4:1:1) to give compound **5** which was purified by RP-18 HPLC [MeOH–MeCN (19:1)] (8 mg).

3.4. Compound characterizations

3.4.1. 15,16-Epoxy-ent-labda-8(17),13(16),14-trien-19-ol (*1*)

Colourless oil; $[\alpha]_D$ −28.0° ($CHCl_3$, *c* 0.8102); UV (EtOH) λ_{max} nm (log ϵ): 205.807 (3.92); IR ($CHCl_3$) ν_{max} cm^{-1}: 877, 3392; ^1H and ^{13}C-NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 302.46 $[M]^+$.

3.4.2. Methyl 15,16-epoxy-ent-labda-8(17),13(16),14-trien-19-oate (*2*)

Colourless oil; $[\alpha]_D$ −16.7° ($CHCl_3$, *c* 0.5214); UV (EtOH) λ_{max} nm (log ϵ): 206.847 (3.97); IR ($CHCl_3$) ν_{max} cm^{-1}: 874, 1713; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 330.47 $[M]^+$.

3.4.3. 15,16-Epoxy-ent-labda-8(17),13(16),14-trien-19-al (*3*)

Colourless oil; $[\alpha]_D$ −10.0° ($CHCl_3$, *c* 0.6408); UV (EtOH) λ_{max} nm (log ϵ): 205.800 (3.88); IR ($CHCl_3$) ν_{max} cm^{-1}: 870, 1647, 1735; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 300.44 $[M]^+$; elemental analysis: found: C, 79.8; H, 9.3. $C_{20}H_{28}O_2$ requires: C, 79.9, H, 9.4%.

3.4.4. 15,16-Epoxy-ent-labda-8(17),13(16),14-trien-19-ol acetate (*4*)

Colourless oil; $[\alpha]_D$ −23.8° ($CHCl_3$, *c* 0.7450); UV (EtOH) λ_{max} nm (log ϵ): 206.057 (4.05); IR ($CHCl_3$) ν_{max} cm^{-1}: 874, 1712; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 344.50 $[M]^+$; elemental analysis: found: C, 76.8; H, 9.2. $C_{22}H_{32}O_3$ requires: C, 76.7, H, 9.4%.

3.4.5. Methyl-15,16-epoxy-12-oxo-ent-labda-8(17),13(16),14-trien-19-oate (*5*)

Colourless oil; $[\alpha]_D$ +8° ($CHCl_3$, *c* 0.7856); UV (EtOH) λ_{max} nm (log ϵ): 203.852 (4.10), 242.901 (2.0); IR ($CHCl_3$) ν_{max} cm^{-1}: 875, 1721, 1755; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 344. 45 $[M]^+$; elemental analysis: found: C, 73.1; H, 8.3. $C_{21}H_{28}O_4$ requires: C, 73.2, H, 4.2%.

3.4.6. ent-Labd-8(17),13E-dien-15-ol (*6*)

Colourless oil; $[\alpha]_D$ −15.5° ($CHCl_3$, *c* 0.4589); IR ($CHCl_3$) ν_{max} cm^{-1}: 3350, 1650; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 290.49 $[M]^+$; elemental analysis: found: C, 82.6; H, 11.7. $C_{20}H_{34}O$ requires: C, 82.7, H, 11.7%.

3.4.7. 13-Oxo-14,15-bis-nor-ent-labd-8(17)-ene (*7*)

Colourless oil; $[\alpha]_D$ −38.8° ($CHCl_3$, *c* 0.4698); IR ($CHCl_3$) ν_{max} cm^{-1}: 1720, 1640; ^1H and ^{13}C NMR spectral data: see Tables 1 and 2; EI–MS (probe 70 eV) *m*/*z*: 262.44 $[M]^+$; elemental analysis: found: C, 82.3; H, 11.5. $C_{18}H_{30}O$ requires: C, 82.4, H, 11.5%.

3.5. Algal growth inhibition test

Tests were performed following the Toxkit™ technology in microbiotest. The Algaltoxkit (Creasel, Belgium) is based on the test species *S. capricornutum* immobilised in algal beads of alginate that can be set free "on demand" immediately prior to performing the toxicity test. The assay was performed in accordance with testing conditions and culturing media prescribed by international standard organisations (OECD, 1984; ISO, 1987). A preliminary screening in tenfold concentration increments (range finding test) was performed to determine the 0–100% tolerance range of organisms to toxicants before definitive tests to determine exactly 50% effect threshold.

Single chemicals of high purity were initially dissolved in DMSO and then diluted further in double-deionised water to make the final stock solns. Highest DMSO concentration in the test samples did not exceed 0.01% (v/v). Two series of controls were carried out at the same time as the test, one without solvent and the other with its maximum concentration. The alga was inoculated (1×10^4 cells/ml) in couvettes already containing 25 ml of test solns that were prepared in five toxicant concentration for each compound. Three replications were used for each concentration and control. Couvettes were placed in a growth chamber at 25 °C under continuos illumination (8000 lux). The cell density reached during three-day static exposure was determined every 24 h by an electronic particle dual threeshold counter

(Coulter Counter mod. Z2 with a 100 μm capillary). Growth inhibition data were processed by probit analysis to estimate the 72 h IC_{50} within 95% confidence limits.

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