Secondary Metabolites from Centaurea deusta with Antimicrobial Activity

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The aerial parts of Centaurea deusta Ten. afforded in addition to several known compounds, mainly sesquiterpene lactones, one new eudesmanolide and one new elemane derivative. Structures of the new compounds were elucidated by spectroscopic methods. The in vitro antifungal and antibacterial activities of the isolated compounds was tested, using the micro-dilution method. All compounds tested showed high antifungal activity.

Introduction

Continuing our research on the chemical constituents of Greek Centaurea sp. (Skaltsa et al., 1999; 2000a; 2000b), we report here the results of the investigation of secondary metabolites from C. deusta Ten. (= C. alba subsp. deusta (Ten.) Nyman), a species, belonging to section Phalolepis (Mabberlay, 1997). Isolated were the flavonoids cirsimaritin and salvigenin (Mabry et al., 1970), the nor-isoprenoid 3,5R-loliolide (11) (Hodges and Porte, 1964), two eleanolides (1, 2), two related elemanes (3, 4), four germacranolides (5–8) and two eudesmanolides (9–10). Compounds 4 and 10 are new naturally occurring sesquiterpene lactones. The in vitro antifungal and antibacterial activity of the isolated compounds were tested, using the micro-dilution method (Hanel and Raether, 1988; Daouk et al., 1995).

Results and Discussion

The crude extracts of the aerial parts of C. deusta afforded the dehydromelitensine derivatives, 8α-(3,4-dihydroxy-2-methylene-butanoyloxy) (1) (Bruno et al., 1995), 8α-(4-acetoxy-3-hydroxy-2-methylene-butanoyloxy) (2) (Bruno et al., 1994), the elemanes methyl 8α-(3,4-dihydroxy-2-methylene-butanoyloxy)-6α, 15-dihydroxy-elema-1,3, 11(13)-trien-12-oate (3) (Cardona et al., 1997) and methyl 8α-(4-acetoxy-3-hydroxy-2-methylene-butanoyloxy)-6α, 15-dihydroxy-elema-1, 3, 11(13)-trien-12-oate (4) (Bruno et al., 1998). The germacranolides cnicin (5) (Rustaiyan et al., 1982), 4‘-acetylenicin (6) (Jakupovic et al., 1986), 3‘-acetylenicin (7) (Polo, 1994) and 8α-(4-acetyl-2-hydroxymethyl-buten-2-oyl-oxy)-salonitenolide (8) (Huneck et al., 1986) and the eudesmanolides 8α-(4-acetoxy-3-hydroxy-2-methylene-butanoyloxy)-4-epi-sonchucarpolide (9) (Skaltsa et al., 2000b) and 8α-(4-acetoxy-3-hydroxy-2-methylene-butanoyloxy)-sonchucarpolide (10). Compounds 4 and 10 are new natural products.

The MS of 4 exhibited a molecular peak at m/z 452.2067, which agreed with the molecular formula C32H32O9 and its IR spectrum afforded absorptions characteristic of hydroxyl and ester groups. The signals in the 1H NMR spectrum (Table I) at δ 5.64 dd, 4.93 d, 4.89 d, 5.35 s and 4.98 s were assigned to H-1, H-2a, H-2b, H-3a and H-3b, respectively, of an elemane framework. The methyl ester chain on C-7 was evident from the two singlets for H-13a and H-13b at δ 6.27 and 5.72, respectively, and the methyl singlet at δ 3.76. A typical doublet at δ 2.13 (J = 10.0 Hz) for H-5, the signal at δ 4.18 (J = 10.6 Hz) for H-6 and the low field double triplet at δ 5.43 corresponding to H-8 indicated a trans-disposition of H-5/H-6, H-6/H-7 and H-7/H-8 and so the oxygenated functions...
at C-6 and C-8 should be α-oriented. From a pair of doublets at δ 4.07 and 3.93 a hydroxymethyl group as substituent at C-4 was also evident. The identity of a 4-acetoxy-3-hydroxy-2-methylenebutanoyl ester side chain was deduced from the chemical shifts of its protons. This side chain sustains independently to the central skeleton as it is shown by the lack of signals in the NOESY spectrum. In addition, from the NOE experiments further information was obtained (Fig. 1). The chair conformation of the cyclohexane ring derives from the NOE signals between H-8, H-6 and 10-CH₃ and H-5 with H-1, H-7 and one of the two protons of the 15-hydroxymethyl-group (H-15b). The α/β-orientation of the two vinyl groups gave rise to the NOE signals of H-2b with the 14-CH₃
and H-3b with H-6. We have assigned to compound 4 the structure of the new methyl 8α-(4-acetoxy-3-hydroxy-2-methylenebutanoyloxy)-6α, 15-dihydroxyelema-1, 3, 11(13)-trien-12-oate.

The MS of 10 showed a molecular peak at m/z 436.1715 [M]+ which agreed with the molecular formula C22H28O9. Its 1H and 13C NMR spectra showed typical signals that suggested an eudesmane framework. The analysis of the NMR spectra with the aid of 1H–1H COSY, HMOC and HMBC (Table I) showed that 10 has an eudesmanolide nucleus with an 8α-acyl side chain with identical functionalisation and stereochemistry to compound 9 (Skaltsa et al., 2000b) except for C-4. Due to the different orientation of the aldehyde group the following differences are observed: H-5 is shielded at δ 1.87 (vs δ 1.94 in compound 9) giving a triplet with a coupling constant of 11.2 Hz, showing that this proton has a trans-diaxial disposition with H-4 and H-6. This suggests a change in the configuration of C-4: H-4 is axial, 15-CHO is equatorial and appears as a doublet slightly shifted at δ 9.69 (vs 9, where appears as singlet at δ 9.90), due to the coupling with H-4, which is also shielded. The conformation of the decaline skeleton is confirmed by the NOESY spectrum. In the 1H NMR spectrum a sharp singlet at δ 2.05 (3H) suggested the presence of an acetate group. For the acetyl group we assigned the position C-4’ in the basis of the low field shift of the signals of the H-4’a (δ 4.29) and H-4’b (δ 4.19) in 10. A similar low field shift is observed for those protons in 4’-acetylcnicin (Jakupovic et al., 1986) vs cnicin (Barrero et al., 1988; Rustaiyan et al., 1982). Consequently, compound 10 is the new 8α-(4-acetoxy-3-hydroxy-2-methylenebutanoyloxy)-sonchucarpolide.

The main compounds of C. deusta Ten. are cnicin and 4’-acetylcnicin. When its chemical profile is compared to previously studied Centaurea sp. belonging to the same section, it appears that their main constituents are germacranolides: C. alba (Geppert et al., 1983; Fernández et al., 1995); C. alba ssp. caliacrae (Geppert et al., 1983).

Additionally, the chemistry of this taxon is characterized by the absence of guaianolides, common metabolites of other Centaurea species (Connolly and Hill, 1991; Fraga, 1992; 1993; 1994; 1995; 1996; 1997; 1998; 2000).

From Table II, it can be seen that 8 possessed the highest antifungal potential, while 3 is of lower activity. All compounds tested show greater antifungal potential than miconazole, a commercial fungicide, which was used as a control, except against Cladosporium cladosporioides. These results are in agreement with our previously reported results on sesquiterpene lactones (Skaltsa et al., 2000b). Concerning the antibacterial potential, only 5 is active against all bacterial strains tested, as expected (Vanhaeelen-Fastre and Vanhaelen, 1976).

**Experimental**

**General procedures**

NMR: 400, 200 MHz (1H) and 50.3 MHz (13C). Chemical shifts are reported in δ (ppm) values. MS: Fisons VG Autospec GC 8000 (CI, CH3); Hewlett-Packard mod. 5988A; (EI, 70 eV). FT-IR Spectrometer: Perkin-Elmer Paragon 500. Polar-
Table II. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations, nmol/ml.

<table>
<thead>
<tr>
<th>Fungi</th>
<th><strong>2</strong> MIC (MFC)</th>
<th><strong>3</strong> MIC (MFC)</th>
<th><strong>8</strong> MIC (MFC)</th>
<th><strong>10</strong> MIC (MFC)</th>
<th><strong>11</strong> MIC (MFC)</th>
<th>Miconazole MIC (MFC)</th>
<th>Bifonazole MIC (MFC)</th>
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<tr>
<td>A. niger</td>
<td>2.5</td>
<td>1.2</td>
<td>0.6</td>
<td>0.6</td>
<td>7.5</td>
<td>7.5</td>
<td>3.0</td>
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<tr>
<td>A. ochraceus</td>
<td>(2.5)</td>
<td>(1.2)</td>
<td>(0.6)</td>
<td>(0.6)</td>
<td>(7.5)</td>
<td>(7.5)</td>
<td>(8.0)</td>
</tr>
<tr>
<td>A. versicolor</td>
<td>2.5</td>
<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
<td>7.5</td>
<td>7.5</td>
<td>4.0</td>
</tr>
<tr>
<td>A. flavus</td>
<td>(2.5)</td>
<td>(1.2)</td>
<td>(1.2)</td>
<td>(0.6)</td>
<td>(7.5)</td>
<td>(7.5)</td>
<td>(8.0)</td>
</tr>
<tr>
<td>P. ochrochloron</td>
<td>5.0</td>
<td>1.2</td>
<td>0.6</td>
<td>1.2</td>
<td>10.0</td>
<td>10.0</td>
<td>2.0</td>
</tr>
<tr>
<td>P. funiculosum</td>
<td>(5.0)</td>
<td>(2.4)</td>
<td>(1.2)</td>
<td>(2.4)</td>
<td>(12.0)</td>
<td>(12.0)</td>
<td>(10.0)</td>
</tr>
<tr>
<td>T. viride</td>
<td>5.0</td>
<td>1.2</td>
<td>0.6</td>
<td>1.2</td>
<td>10.0</td>
<td>10.0</td>
<td>4.0</td>
</tr>
<tr>
<td>C. cladosporioides</td>
<td>(4.0)</td>
<td>(2.4)</td>
<td>(2.4)</td>
<td>(1.2)</td>
<td>(15.0)</td>
<td>(15.0)</td>
<td>(10.0)</td>
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<tr>
<td>A. alternata</td>
<td>1.25</td>
<td>0.3</td>
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<td>0.15</td>
<td>3.75</td>
<td>3.75</td>
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<tr>
<td>(1.25)</td>
<td>(0.3)</td>
<td>(0.15)</td>
<td>(0.15)</td>
<td>(3.75)</td>
<td>(3.75)</td>
<td>(3.75)</td>
<td>(1.0)</td>
</tr>
</tbody>
</table>

*The nos. on top denote the compounds shown in Fig. 1. All the components were tested in triplicate and MICs and MFCs were presented as mean values.

**Chromatography**

Vacuum liquid chromatography (VLC): silica gel (Merck; 43–63 µm), CC: silica gel (SDS; 40–63 µm), gradient elution with the solvents mixtures indicated in each case; HPLC: CE 1100 Liquid Chromatography Pump Techsil 10-C18 (250 × 10 mm).

Absorbents for TLC: Merck RP 18 F254; Merck silica gel 60 F254; solvents for TLC: mixt. of CH₂Cl₂ with MeOH; mixt. of cyclohexane with EtOAc. Detection on TLC-plates: UV-light, spray reagent (anisaldehyde-H₂SO₄ on silica gel).

**Plant material**

Aerial parts of *Centaurea deusta* were collected on Mount Pelion in June 1997 and authenticated by Dr. Th. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens). A voucher specimen is deposited in the Herbarium of the Institute of Systematic Botany, University of Patras under the number Skaltsa and Lazari 107 (UPA).

**Bioassays**

For the bioassays nine fungi were used: *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus flavus* (ATCC 9643), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 36839), *Trichoderma viride* (IAM 5061), *Cladosporium cladosporioides* (ATCC 13276) and *Alternaria alternata* (DSM 2006).

The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological research “Sinisa Stan kovic”, Belgrade, Yugoslavia.

The micromycetes were maintained on malt agar (MA) and the cultures were stored at +4 °C and subcultured once a month (Booth, 1971).

To investigate the antifungal activity of the compounds the modified microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (vol/vol). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 × 10⁶ in a final volume of 100 µl per well. The inocula were stored at +4 °C for further use. Dilutions of the inocula...
were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs), which inhibited fungal growth, were performed by a serial dilution technique using 96-well microtitre plates. Extracts of compounds investigated were dissolved in malt medium broth with fungal inoculum to achieve concentrations of 0.03–4 µg/ml. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs), were determined by serial subcultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 h at 28 °C. The lowest concentration which killed the 99.5% of the original inoculum, spores and mycelium of the fungi, was defined as the MFC. Commercial fungicides, miconazole and bifonazole, were used as control (0.03–5 µg/ml). The antimicrobial activity of the compounds against the Gram-positive bacteria Staphylococcus aureus (ATCC 27853) was evaluated using the microdilution technique. Streptomycin (solution of 1 mg/ml in H2O, kindly provided by Alkon Lab. Hellas) was used as standard for the antibacterial activity.

Column chromatography of fr C (CH2Cl2–MeOH, 10:0 to 8.2) followed by further CC and several HPLC (MeOH–H2O, 1:1) allowed the isolation of 2 (29.5 mg), 6 (386.0 mg), 7 (11.4 mg), 9 (9.5 mg) and 11 (7.8 mg). VLC of fr D (CH2Cl2–MeOH, 10:0 to 8.2) followed by further CC and several HPLC (MeOH–H2O, 4:3, 1:1) allowed the isolation of 1 (29.5 mg), 2 (14.0 mg), 4 (3.6 mg), 5 (727.5 mg), a mixture of 5 and 9 (8.0 mg), a mixture of 5 and 1 (72.2 mg), 6 (129.1 mg), 7 (3.9 mg), 8 (3.3 mg) and 10 (7.2 mg). CC of fr E (CH2Cl2–MeOH, 10:0 to 7:3) followed by further CC and several HPLC (MeOH–H2O, 1:1) allowed the isolation of 5 (283.7 mg), 6 (4.6 mg) and 10 (6.0 mg). CC of fr F (CH2Cl2–MeOH, 10:0 to 7:3) followed by further CC and several HPLC (MeOH–H2O, 1:1) allowed the isolation of 3 (3.8 mg) and 5 (39.2 mg).

Methyl 8α-(4-acetoxy-3-hydroxy-2-methylenebutanoxy)-6α,15-dihydroxyelema-1,3,11(13)-trien-12-oate (4)

Oil; [α]D 20 +11.58 (CHCl3, c 0.10); IR νmax cm−1: 3600–3300 (OH), 1773, 1764 (C=O, γ-lactone, ester), 1719, 1712 (C=O, acetate). CIMS m/z 452.2067 [M]+ (19) (C23H32O9 requires 452.2046), 420 [M–MeOH]+ (5), 374 [M–AcOH–H2O]+ (8), 360 [M–AcOH–MeOH]+ (6), 342 [360-H2O]+ (5), 241 (100). 1H NMR spectral data: see Table I.

8α-(3-hydroxy-4-acetoxy-2-methylenebutanoxy)-sonchucarpolide (10)

Oil; [α]D 20 +8.59 (CHCl3, c 0.09); IR νmax cm−1: 3600–3300 (OH), 1773, 1764 (C=O, γ-lactone, ester), 1719, 1712 (C=O, acetate, aldehyde). CIMS m/z 436.1715 [M]+ (100) (C22H28O9 requires 436.1733), 420 [M+H–H2O]+ (30), 430 [M+CO]+ (14), 376 [M–AcOH]+ (80), 363 [M–Ac–H2CO]+ (64), 279 [M–B]+ (15), 262 [M–BOH]+ (52). 1H NMR and 13C NMR spectral data: see Table I.

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