Trypanocidal Terpenoids from Laurus nobilis L.

Nahoko UCHIYAMA, a Keiji MATSUNAGA, a Fumiyuki KUCHI, a, b Gisho HONDA, a Akiko TSUBOUCHI, b Junko NAKAJIMA-SHIMADA, b and Takashi AKI b

a Graduate School of Pharmaceutical Sciences, Kyoto University; 46–29 Yoshida, Sakyo-ku, Kyoto 606–8501, Japan; and b School of Medicine, Juntendo University; 2–1–1 Hongo, Bunkyo-ku, Tokyo 113–8421, Japan.

Received July 4, 2002; accepted August 10, 2002

Trypanocidal constituents of dried leaves of Laurus nobilis L. (Lauraceae) were examined. Activity-guided fractionation of the methanol extract resulted in the isolation of two guaianolides, dehydrocostus lactone (1) and zaluzanin D (2), and a new p-menthane hydroperoxide, (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate (3). The minimum lethal concentrations of these compounds against epimastigotes of Trypanosoma cruzi were 6.3, 2.5, and 1.4 μM, respectively.

Key words Laurus nobilis; Trypanosoma cruzi; p-menthane hydroperoxide; guaianolide; Lauraceae

Trypanosoma cruzi, a parasitic protozoan of Zoomastigophoria, is the etiologic agent of Chagas’ disease which is endemic in Central and South America.1) In our screening work on medicinal plants used in Asian counties for trypanocidal activity, extracts of the dried leaves of Laurus nobilis L. (Lauraceae) showed strong trypanocidal activity against epimastigotes of T. cruzi. Leaves of L. nobilis are used as a spice known as laurel or bay leaf. They are also used as a folk medicine for rheumatism and scabies.2) Although some biological activities such as inhibition of elevation of blood ethanol level in ethanol-loaded rat,3) inhibition of nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages,4) induction of glutathione S-transferase activity,5) and nematocidal activity6) were reported for the constituents of L. nobilis, nothing has been reported on anti-protozoan constituents of this plant. Here, we report the isolation and identification of trypanocidal constituents of L. nobilis.

Results and Discussion

Dried leaves of Laurus nobilis were successively extracted with hexane, CHCl3 and MeOH, and each extract was tested for anti-trypanosomal effect by the HeLa cell infection assay.7) As the CHCl3 and MeOH extracts inhibited infection and proliferation of T. cruzi, and the MeOH extract formed the major part of the extracts, the MeOH extract was further fractionated under the guidance of in vitro trypanocidal activity against epimastigotes of T. cruzi.8) The MeOH extract was suspended in aqueous MeOH and fractionated into hexane soluble, AcOEt soluble, and water soluble fractions. Since the AcOEt soluble fraction showed the strongest activity, it was fractionated by silica gel column chromatography to give three trypanocidal compounds (1—3). These compounds were also isolated from the CHCl3 extracts.

Compounds 1 and 2 were obtained as colorless needles from hexane, mp 58—60 °C, and as a colorless oil, respectively. The 1H- and 13C-NMR spectra indicated that both compounds were guaiane-type sesquiterpene with three exo-cyclic methane moieties. Based on comparisons of the spectral data with those reported, compounds 1 and 2 were identified with dehydrocostus lactone (1)9) and zaluzanin D (2),10) respectively.

Compound 3 was obtained as colorless needles from petroleum ether, mp 87.5—88.5 °C. The 13C-NMR spectrum showed that 3 was a monoterpenoid with two oxygen-bearing carbons (δ C 78.6, 84.4) and an acetoxy group (δ C 22.5, 170.6). The 1H-NMR spectrum showed the presence of three singlet methyls [δ H 1.34, 1.39, 1.44 (each 3H, s)], an acetoxy group [δ H 2.00 (3H, s)], and a cis olefin [δ H 5.65 (1H, ddd, J = 10.0, 2.5, 1.5 Hz), 5.92 (1H, dd, J = 10.0, 1.5 Hz)]. The molecular formula C18H24O4, suggested by a weak molecular ion at m/z 229 (MH+) in CI-MS, together with the relatively large chemical shift of the non-acetoxy oxygen-bearing carbon (δ C 78.6) indicated the presence of a hydroperoxy group. Comparison of 13C-NMR data with those of known monoterpenes suggested that 3 had a p-menthane-type skeleton and analyses of 1H–1H correlation spectroscopy (COSY), 1H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBBC) spectra (Fig. 1) revealed the structure to be 1-hydroperoxy-p-menth-2-en-8-ol acetate. Treatment of 3 with NaBH4 gave an acetoxy alcohol 4, which showed oxygen-bearing carbons at δ C 67.0 (C-1) and δ C 84.4 (C-8). This confirmed that 3 had a hydroperoxy and an acetoxy group at positions 1 and 8, respectively. Since nuclear Overhauser effect (NOE) experiments did not provide conclusive stereochemical information, the stereochemistry of 3 was determined by chemical correlations.

Photo-oxidation11) of d-limonene followed by reduction with PPh3 gave (1S,4R)-p-menth-2-en-8-ol (5, [α]D = +60.8°) and (1R,4R)-p-menth-2,8-dien-1-ol (6, [α]D = +186.4°). Oxymercuration of 5 followed by treatment with NaBH4 gave (1S,4R)-p-menth-2-en-1,8-diol (7, [α]D = −28.0°). The 1H- and 13C-NMR spectra of 7 were identical with those of the diol 8, which was derived from 3 by alkaline hydrolysis followed by reduction with PPh3.12) This result established that the relative stereochemistry of 3 is identical with that of 7. However, the diols 7 and 8 showed different retention times on GC-MS analysis with chiral capillary column, revealing an enantiomeric relationship between them.13) Thus, the structure of 3 was concluded to be (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate. A sesquiterpene hydroperoxide, verlotorin, has been isolated from L. nobilis.14) However, this is the first report of the isolation of a monoterpenoid hydroperoxide from this plant.

Compounds 1—3 showed strong trypanocidal activity against epimastigotes of T. cruzi with minimum lethal concentrations (MLCs) of 6.3 μM (1), 2.5 μM (2), and 1.4 μM (3). The activities were stronger than that of the anthelmintic
monoterpene endoperoxide, ascaridole (MLC=23 μm) and were comparable to those of monoterpene hydroperoxides isolated from Chenopodium ambrosioides. In the HeLa cell infection assay, compound 3 very strongly inhibited the infection of the trypanomastigotes at 1 μg/ml (Table 1). Dehydrocostus lactone (1) also significantly inhibited the infection at 1 μg/ml, whereas zaluzanin D (2) did not inhibit the infection at the same concentration.

Several sesquiterpene lactones having an α,β-unsaturated γ-lactone moiety (15-deoxyglycyzazene, dehydrozaluzanin C, lycnocholinol, naematolin, dehydroleucodine) have been reported to show trypanocidal activity. Brengio et al. reported that the effect of dehydroleucodine was irreversible, but could be blocked by simultaneous addition of a thiol compound. This suggests that the trypanocidal activity of this group of compounds relies on covalent bond formation of the α,β-unsaturated γ-lactone moiety with nucleophiles which are essential for the life of the parasite. One possible target is the trypanothione (1,8-bis-glutathionyl nucleophile which are essential for the life of the parasite.

Experimental

General

Melting points were determined on a Yanagimoto micro melting point apparatus. Optical rotation ([α]D) was measured on a JASCO DIP-370 polarimeter. 1H- and 13C-NMR spectra were measured on a JEOL JNM-LA500 spectrometer with TMS as an internal standard and chemical shifts were recorded in δ ppm. Fuji Silysia BW-127ZI silica gel was used for column chromatography.

Plant Materials

Dried leaves of Laurus nobilis were purchased from Tochimoto Tenkaido.

Extraction and Isolation

The dried leaves (225 g) were successively extracted with hexane, CHCl3, MeOH (3 times×3) at room temperature overnight. Each extract was concentrated to dryness to give hexane (3.6 g), CHCl3 (5.8 g), and MeOH (53.4 g) extracts. The MeOH extract (53.4 g) was suspended in 700 ml of aqueous MeOH and extracted with hexane (250 ml×3 times). The aqueous MeOH layer was concentrated to ca. 100 ml, diluted with water and extracted with AcOEt (250 ml×3 times) to give AcOEt soluble (13.5 g) and water soluble (34.3 g) fractions. The AcOEt soluble fraction was applied to a silica gel column eluted with hexane-acetone 9:1 (fr. 1, 127 mg; fr. 2, 40 mg; fr. 3, 403 mg; fr. 4, 187 mg), 4:1 (fr. 5, 408 mg; fr. 6, 1.1 g; fr. 7, 330 mg), 7:3 (fr. 8, 646 mg; fr. 9, 2.4 g), acetone (fr. 10, 3.8 g), and MeOH (fr. 11, 3.3 g), among which fractions 2, 3, 5, 6, and 8 showed trypanocidal activity at 25 μg/ml. Fractions 3, 5, and 6 were separately purified by silica gel column chromatography to give compounds 1 (dehydrocostus lactone, 83 mg), 2 (zaluzanin D, 245 mg), and 3 (15 mg) respectively. Fraction 8 also gave zaluzanin D (2, 239 mg).

Compound 3: Colorless needles from petroleum ether, mp 87.5—88.5 °C. [α]D = −7.9° (c= 0.61, EtOH). 1H-NMR (CDCl3, 500 MHz) δ: 1.34 (3H, s, CH3-7), 1.39 (3H, s, CH-9), 1.41 (1H, m, H-6), 1.44 (3H, s, CH-10), 1.58 (2H, m, CH-5), 2.00 (3H, s, Ac), 2.20 (1H, ddd, J=14.0, 3.5, 1.5 Hz, H-6), 2.80 (1H, m, H-4), 5.65 (1H, ddd, J=10.0, 2.5, 1.5 Hz, H-2, 3), 5.92 (1H, br dd, J=10.0, 1.5 Hz, H-3), 7.94 (1H, s, OOH). 13C-NMR (CDCl3, 125 MHz) δ: 20.1 (C-5), 22.5 (COCH3), 23.1 (C-10), 23.7 (C-9), 24.8 (C-7), 31.7 (C-6), 43.4 (C-4), 67.3°. 11) 1H-NMR (CDCl3, 500 MHz) δ: 1.29, 1.41, 1.58 (each 3H, s, CH3-7, 9, 10), 1.48 (1H, m), 1.58 (1H, m), 1.68 (1H, m), 1.87 (1H, m), 2.00 (1H, Ac), 2.75 (1H, m), 5.73 (2H, t-like, J=11 Hz, H-2, 3). 13C-NMR (CDCl3, 125 MHz) δ: 20.3 (C-5), 22.6 (COCH3), 23.1 (C-10), 23.8 (C-9), 29.9 (C-7), 37.1 (C-6), 44.5 (C-4), 78.6 (C-1), 84.4 (C-8), 129.9 (C-2), 133.7 (C-3), 170.6 (COCH3). CI-MS: m/z: 229 (M+), 221 (MH+-18, 4), 195 (10), 153 (17), 151 (13), 155 (100).

Reduction of 3 with PPh3

Compound 3 (4.5 mg) dissolved in ether (1 ml) was treated with PPh3 (8.5 mg) at room temperature for 20 min. The mixture was concentrated to dryness and the residue was chromatographed over silica gel to give an acetylo alcohol (4) (3.4 mg).

Compound 4: Colorless oil. [α]D = +11.5° (c=0.17, CHCl3). 1H-NMR (CDCl3, 500 MHz) δ: 1.29, 1.41, 1.46 (each 3H, s, CH3-7, 9, 10), 1.48 (1H, m), 1.58 (1H, m), 1.68 (1H, m), 1.87 (1H, m), 2.00 (1H, Ac), 2.75 (1H, m), 5.73 (2H, t-like, J=11 Hz, H-2, 3). 13C-NMR (CDCl3, 125 MHz) δ: 20.3 (C-5), 22.6 (COCH3), 23.1 (C-10), 23.8 (C-9), 29.9 (C-7), 37.1 (C-6), 44.5 (C-4), 67.0 (C-1), 84.4 (C-8), 129.8 (C-2), 134.7 (C-3), 170.6 (COCH3). CI-MS: m/z: 229 (M+), 221 (MH+-18, 4), 195 (10), 153 (17), 151 (13), 155 (100).

Photo-oxidation and Reduction of d-Limonene

A stirred solution of d-limonene (2 g) and Rose Bengal (29 mg) in MeOH (29 ml) was irradiated by a high pressure mercury lamp (Riko Kagaku, UV-100HA) under oxygen atmosphere at 0 °C for 8.5 h. The mixture was concentrated to dryness, and the residue was dissolved in ether (30 ml) and treated with PPh3 (2.6 g) at room temperature for 60 min. The mixture was concentrated to dryness and the residue was fractionated by repeated silica gel column chromatography with hexane–AcOEt (4:1) to give (1S,4R)-p-methyl-2,8-dien-1-ol (5) (29 mg), 13.4% and (1R,4R)-p-methyl-2,8-dien-1-ol (6) (53 mg, 5.5%).

Photo-oxidation of 3

A stirred solution of 3 in acetone (1 ml) was treated with PPh3 (8.5 mg) at room temperature for 20 min. The mixture was concentrated to dryness, and the residue was fractionated by repeated silica gel column chromatography with hexane–AcOEt (4:1) to give (1S,4R)-p-methyl-2,8-dien-1-ol (5) (29 mg, 13.4%) and (1R,4R)-p-methyl-2,8-dien-1-ol (6) (53 mg, 5.5%).
(1R,2R)-p-Mentha-2,8-dien-1-ol (6): Colorless oil. [\alpha]_D^{20} = +186.4° (c = 0.97, CHCl_3, lit. +163.8°). \text{H-NMR} (CDCl_3, 500 MHz): δ 1.29 (3H, s, CH-7), 1.56 (1H, m, Ha-5), 1.66 (1H, dd, J = 12.8, 2.8, 9.5 Hz, Ha-6), 1.74 (3H, br s, CH-10), 1.79 (1H, dd, J = 12.8, 8.9, 2.8 Hz, Hb-6), 1.90 (1H, m, Hb-5), 2.74 (1H, m, H-4), 4.67 (1H, br s, Ha-9), 4.78 (1H, quintet, J = 1.6 Hz, Hb-9), 5.61 (1H, dd, J = 10.1, 3.4 Hz, H-3), 5.70 (1H, dd, J = 10.1, 2.1 Hz, H-2). \text{13C-NMR} (CDCl_3, 125 MHz): δ 21.2 (C-10), 25.0 (C-5), 28.9 (C-7), 36.1 (C-6), 42.5 (C-4), 68.6 (C-1), 110.9 (C-9), 130.8 (C-3), 134.5 (C-2), 147.2 (C-8).

Oxymercuration of (1S,4R)-p-Mentha-2,8-dien-1-ol (5) To a solution of mercury (II) acetate in water (2 ml), THF (2 ml) and then a solution of NaBH_4 in 3 M NaOH (1 ml) were added and the mixture was stirred for 40 min. The mixture was saturated with NaCl and the THF layer was separated, dried over anhydrous MgSO_4 and concentrated to dryness. The residue was purified by column chromatography on silica gel to give (1S,4R)-p-mentha-2-en-1,8-diol (7, 14 mg, 16%).

GC-MS Analysis with Chiral Capillary Column GC-MS analysis was performed on a Hitachi G-7000/M/M-9000 system equipped with a Cyclodex-B chiral capillary column (0.25 mm i.d., 25 m) and a coldex-B chiral capillary column (0.25 mm i.d., 25 m, which was eluted with AcOEt to give an alcohol. The alcohol was purified by column chromatography on silica gel to give a diol, whose \text{1H-} and \text{13C-NMR spectra were identical with those of (1S,4R)-p-mentha-2-en-1,8-diol (7).}

Hydrolysis and Reduction of Compound 3 A mixture of compound 3 (0.5 mg) in MeOH (0.1 ml) and 10% NaOH (0.1 ml) was stirred at room temperature for 1 h. The mixture was neutralized and applied to a silica gel column, which was eluted with AcOEt to give an alcohol. The alcohol was dissolved in ether (1 ml) and stirred with PPh_3 (1.4 mg) at room temperature for 40 min. The mixture was concentrated to dryness and the residue was purified by silica gel column chromatography with hexane:AcOEt (5:4) to give the diol 8, whose \text{1H} and \text{13C-NMR spectra were identical with those of (1S,4R)-p-mentha-2-en-1,8-diol (7).}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>27) Schirmer R. H., Müller J. G., Krauth-Siegel R. L., Anke T., Fournet A., Muñoz V., Roblot F., Hocquemiller R., Cavé A., Gantier J.</td>
</tr>
<tr>
<td>4</td>
<td>26) Kiuchi F., Nakamura N., Miyashita N., Nishizawa S., Tsuda Y., Kondo K., Shoyakugakka Zasshi, 43, 279—287 (1989).</td>
</tr>
<tr>
<td>6</td>
<td>24) Schenk G. O., Gollnick K., Buchwald G., Schroeter S., Ohloff G., Liebigs Ann. Chem., 674, 93—118 (1964).</td>
</tr>
<tr>
<td>7</td>
<td>23) D’Silva C., Daunes S., Expert Opin. Invest. Drugs, 11, 217—231 (2002).</td>
</tr>
</tbody>
</table>

Acknowledgements The authors thank Professor K. Tomioka of the

Graduate School of Pharmaceutical Sciences, Kyoto University, for allowing us to use the NMR facilities, and Dr. N. Akimoto of the Faculty of Pharmaceutical Sciences, Kyoto University, for MS measurements. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 11672101) from the Japan Society for the Promotion of Science.