Monoterpene Hydroperoxides with Trypanocidal Activity from *Chenopodium ambrosioides*

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Received September 12, 2001

Four monoterpene hydroperoxides were isolated from aerial parts of *Chenopodium ambrosioides* along with ascaridole (**1**), the anthelmintic principle of this plant, as anti-trypanosomal compounds. The structures of these monoterpenes were determined to be (−)-(2*S*,4*S*)- and (−)-(2*R*,4*S*)-*p*-mentha-1(7),8-dien-2-hydroperoxide (**2a** and **3a**) and (−)-(1*R*,4*S*)- and (−)-(1*S*,4*S*)-*p*-mentha-2,8-dien-1-hydroperoxide (**4a** and **5a**) on the basis of spectroscopic methods and chemical correlations. In vitro trypanocidal activities of ascaridole (**1**) and these hydroperoxides (**2a**−**5a**) against epimastigotes of *Trypanosoma cruzi* were 23, 1.2, 1.6, 3.1, and 0.8 μM, respectively. Fresh leaves of *C. ambrosioides* also contained isomeric hydroperoxides **6a** and **7a**, and the content ratio of **2a**−**7a** suggested that these hydroperoxides were formed through the singlet-oxygen oxidation of limonene.

Chenopodium ambrosioides L. var. *anthermintic*um A. Gray (Chenopodiaceae) is used to produce chenopodium oil, which has been used as an anthelmintic for the treatment of intestinal worms.[1] In our search for anti-trypanosomal compounds from natural sources, an AcOEt extract of aerial parts of this plant showed potent trypanocidal activity against the epimastigotes of *Trypanosoma cruzi*, the etiologic agent of American trypanosomiasis (Chaga's disease).[2] In this paper, we report the isolation and characterization of four *p*-menthane-type monoterpene hydroperoxides (**2a**−**5a**) together with ascaridole (**1**), the anthelmintic principle of chenopodium oil, as the trypanocidal constituents of this plant.

Results and Discussion

Fresh aerial parts of *C. ambrosioides* were extracted overnight with AcOEt at room temperature. The diethyl ether-soluble fraction of this extract, which showed strong in vitro trypanocidal activity, was separated by silica gel column chromatography to give ascaridole (**1**), the anthelmintic principle of this plant, as a major constituent. Although **1** showed strong trypanocidal activity, more potent activity was found in compounds that appeared just below the spot of **1** and gave a bright bluish-green color with *p*-anisaldehyde/sulfuric acid reagent on TLC. These compounds were separated by repeated column chromatography to give **2a**−**5a**.

Compounds **2a**−**5a** were monoterpenes with two double bonds and one oxygen function based on their ^{13}C NMR spectra. The chemical shifts (δ 79.4−85.5) of the oxygen-bound carbon of these compounds suggested that the oxygen function is a hydroperoxy group, which is consistent with their molecular formula ($C_{10}H_{16}O_2$ based on high-resolution chemical ionization mass spectrum (HRCIMS)) and their relative instability. Compounds **2a** and **3a** showed similar NMR spectra, with signals of two exocyclic methylenes: δ 4.72 (2H, br s), δ 4.84 and 4.93 (each 1H, d, J = 1.5 Hz) in **2a**; δ 4.68 and 4.70 (each 1H, br s), δ 5.00 and 5.01 (each 1H, d, J = 2 Hz) in **3a**. Compounds **4a** and **5a** also showed similar spectra, with signals of an exocyclic methylene and a *cis*-double bond: δ 4.74 and 4.76 (each 1H, br s), δ 5.62 (1H, br d, J = 9.9 Hz), δ 5.82 (1H, dd, J = 9.9, 2.6 Hz) in **4a**; δ 4.68 and 4.79 (each 1H, br s), δ 5.70 (1H, dd, J = 10.3, 2.0 Hz), δ 5.82 (1H, dd, J = 10.3, 3.4 Hz) in **5a**. Detailed analyses of their two-dimensional NMR spectra (HMQC, HMBC, COSY) revealed that **2a** and **3a** are epimers of *p*-mentha-1(7),8-dien-2-hydroperoxide at the 2-position. In compound **2a**, H-2 (δ 4.45, d with fine splittings, J = 11.6 Hz) and H-4 (δ 2.21, 1H, tt, J = 12.2, 3.2 Hz) are oriented axially, whereas in **3a**, H-2 (δ 4.51, t, J = 2.9 Hz) and H-4 (δ 2.35, 1H, tt, J = 12.9, 3.2 Hz) are in equatorial and axial positions, respectively. Thus, the structures of **2a** and **3a** were assigned as indicated. Similarly, **4a** and **5a** were considered to be epimers of *p*-mentha-2,8-dien-1-hydroperoxide at the 1-position. The stereochemistry of these compounds was determined to be 1,4-*cis* for **4a** and 1,4-*trans* for **5a**, as described below. These compounds have been reported as products of the singlet-oxygen oxidation of limonene,[3,4] and the negative optical rotation values of these compounds indicated that they have the same absolute configuration at the 4-position as *l*-limonene.[5] While these oxidation products of limonene have been analyzed by GC−MS[3] and characterized by IR and 60 MHz ^{1}H NMR,[4] they have not been fully characterized by high-field NMR spectroscopy. Thus, the structures of **2a**−**5a** were confirmed by chemical means. Compounds **2a**−**5a** were treated with PPh_3 to give the corresponding alcohols **2b**−**5b**. The negative signs of the optical rotations of these alcohols confirmed that the absolute configuration at the 4-position was the same as that in *l*-limonene.[5] The ^{13}C NMR data of the alcohol obtained from **2a** were consistent with those of the known alcohol **2b**,[6] which has a *cis* relationship between the hydroxy and isopropenyl groups as assigned above. The alcohols derived from **4a** and **5a** were further hydrogenated to give tetrahydro derivatives **8** and **9**, respectively. The ^{13}C NMR spectra of **8** and **9** were in good agreement with those of 1,4-*cis* and 1,4-*trans* alcohols, respectively,[6] which established the stereochemistries of the alcohols **4b** and **5b**, and hence those of the hydroperoxides **4a** and **5a**, as indicated in their

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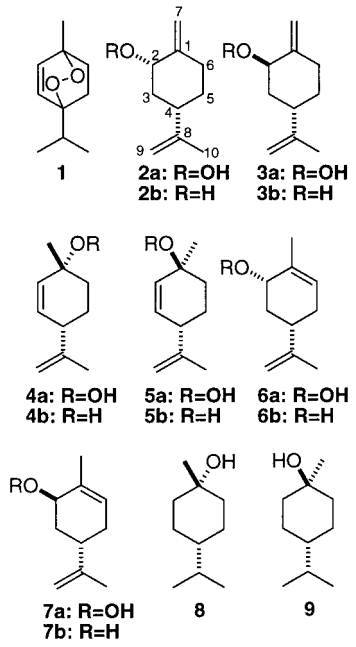
10.1021/np010445g CCC: $22.00
Published on Web 03/15/2002

structures. On the basis of these results, the structures of the hydroperoxides isolated from *C. ambrosioides* were concluded to be (−)-(2*S*,4*S*)-*p*-mentha-1(7),8-dien-2-hydroperoxide (**2a**), (−)-(2*R*,4*S*)-*p*-mentha-1(7),8-dien-2-hydroperoxide (**3a**), (−)-(1*R*,4*S*)-*p*-mentha-2,8-dien-1-hydroperoxide (**4a**), and (−)-(1*S*,4*S*)-*p*-mentha-2,8-dien-1-hydroperoxide (**5a**). Many hydroperoxides have been reported from various plant sources,[7] including *trans*-pinocarveyl-hydroperoxide from *C. ambrosioides*.[8] Although compounds **4a** and **5a** (absolute stereochemistry not reported) have been reported from *Citrus iyo*,[9] this is the first report of isolation and full characterization of these *p*-menthane hydroperoxides from natural sources.



It has been reported that the singlet-oxygen oxidation of limonene produces six isomeric hydroperoxides, **2a**−**7a**.[3,5,10] Therefore, we checked for the presence of the isomeric hydroperoxides **6a** and **7a** in *C. ambrosioides*. The peroxide fraction of the AcOEt extract was treated with PPh_3 to convert the hydroperoxides to alcohols and analyzed by GC. All of the six isomers were present: **2b** (23.8%), **3b** (21.7%), **4b** (35.7%), **5b** (7.5%), **6b** (3.1%), and **7b** (8.2%). This ratio is very similar to that reported for the products of the singlet-oxygen oxidation of limonene,[3,10] which suggests that these hydroperoxides are formed by the singlet-oxygen oxidation of *l*-limonene in *C. ambrosioides*.

Paré et al. reported the isolation of the alcohol **2b** as an antifungal compound[6] and Ahmed isolated several *p*-menthane alcohols[11] from *C. ambrosioides*. However, when the fresh plants were extracted with AcOEt, the extract did not contain any **2b**−**7b** by TLC. However, when the extract was heated, these alcohols appeared and their amounts increased, accompanied by a decrease in the amount of the hydroperoxides. Degradation of the hydroperoxides was evident when the fresh aerial part was steam-distilled. The essential oil fraction obtained by steam-distillation contained ascaridole as the major component and a considerable amount of the alcohols. However, the hydroperoxides were not detectable by TLC. Therefore, the *p*-menthane alcohols isolated from this plant might be degradation products of the hydroperoxides **2a**−**7a** and ascaridole.

Table 1. Anti-trypanosomal Activity of Compounds **1** and **3a**−**5a** against *T. cruzi* in the HeLa Infection Assay[a]

compound	infection rate (%)	number of amastigotes/ infected cell
control[b]	12.0 ± 0.9	18.8 ± 3.8
1	8.5 ± 2.5	22.8 ± 1.1
3a	4.4 ± 0.3*	18.6 ± 6.0
4a	1.5 ± 1.3	19.1 ± 5.1
5a	0.13 ± 0.11*	12.0 ± 4.8
allopurinol	7.0 ± 2.3*	10.9 ± 3.5*

[a] The mean values ± SE (* $p < 0.05$ compared with control) at 1 μg/mL. [b] Ethanol (10 μL).

The hydroperoxides **2a**−**5a** showed stronger trypanocidal activity than ascaridole (**1**). The minimum lethal concentrations (MLCs) of ascaridole (**1**) and hydroperoxides **2a**−**5a** against epimastigotes of *Trypanosoma cruzi* were 23, 1.2, 1.6, 3.1, and 0.8 μM, respectively. A few monoterpenoids, espintanol,[12] piquerol A,[13] and terpinen-4-ol,[14] all of which have OH function, have been reported to show anti-trypanosomal activity. However, the alcohols **2b**−**5b**, corresponding to the hydroperoxides **2a**−**5a**, did not show trypanocidal activity even at 400 μM, indicating that hydroperoxy group is essential for the activity of this type of compounds.

To evaluate the effect on the blood stream forms of the parasite, the anti-trypanosomal effect of the hydroperoxides **3a**−**5a** was also tested with the HeLa cell infection assay.[15] Under this assay condition, these compounds were toxic to HeLa cells at 10 μg/mL. At 1 μg/mL, **5a** almost completely inhibited the infection of HeLa cells by the trypomastigotes, and **3a** and **4a** inhibited the infection by 63% and 88%, respectively (Table 1). However, they did not inhibit the proliferation of amastigotes in infected cells.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-370 polarimeter. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-LA500 spectrometer with tetramethylsilane as an internal standard, and chemical shifts are given as δ values. Mass spectra were measured on a JEOL JMS-HX/HX110A spectrometer. GC was performed on a Hitachi G-5000 system equipped with an FID detector. *l*-Limonene was a generous gift from Takasago International Co. (−)-Carveol (a mixture of epimers **6b** and **7b**) was purchased from Kanto Chemical Co.

Extraction and Isolation. The seeds of *C. ambrosioides* L. var. *anthermin*ticum A. Gray were provided by the Kyoto Medicinal Plant Garden of Takeda Chemical Industries Ltd. The plants were cultivated, and a voucher specimen (No. 4212) was deposited at the Experimental Station of Medicinal Plants, Faculty of Pharmaceutical Sciences, Kyoto University. Fresh aerial parts including immature seeds (10 kg) were extracted with AcOEt at room temperature overnight, and the extract was concentrated to dryness under reduced pressure. The residue was suspended in water and extracted with diethyl ether. The extract was dried over anhydrous sodium sulfate and concentrated to dryness to give an ether-soluble fraction (108 g). Some of this extract (21.0 g) was fractionated by silica gel column chromatography (hexane−AcOEt, 20:1) to give three fractions: fraction 1 (1.46 g), fraction 2 (4.93 g), and fraction 3 (2.97 g), and the rest was eluted with MeOH to give fraction 4. Minimum lethal concentrations (MLCs) of these fractions were 12.5, 1.56, 0.1, and >6.25 μg/mL, respectively. Some of fraction 2 (1.31 g) was purified by silica gel column chromatography (hexane−AcOEt, 10:1) to give ascaridole (**1**, 1.13 g). Repeated column chromatography of fraction 3 on silica gel (benzene−AcOEt, 98:2, $CHCl_3$−AcOEt, 100:3) and a Lobar column (LiChroprep Si-60B, Merck, benzene−AcOEt, 98:2) gave compounds **2a** (64 mg), **3a** (151 mg), **4a** (85 mg), and **5a** (67 mg).

Ascaridole (1): pale yellow oil; $[\alpha]^{25}_D$ 0° (*c* 3.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.95 (3H, s), 0.98 (3H, s), 1.34 (3H, s), 1.48 (2H, m), 1.89 (1H, m), 1.99 (2H, m), 6.37 (1H, d, J = 8.5 Hz), 6.46 (1H, d, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 17.1, 17.2, 21.3, 25.5, 29.4, 32.1, 74.3, 79.7, 133.0, 136.3.

(−)-(2*S*,4*S*)-*p*-Mentha-1(7),8-dien-2-hydroperoxide (2a): colorless oil; $[\alpha]^{20}_D$ −18.7° (*c* 4.7, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.27 (1H, qd, J = 12.8, 4 Hz, H-5), 1.30 (1H, q, J = 11.9 Hz, H-3), 1.72 (3H, s, H-10), 1.82 (1H, d with fine splittings, J = 12.8 Hz, H-5), 2.08 (1H, t with fine splittings, J = 13.4 Hz, H-6), 2.21 (1H, tt, J = 12.2, 3.2 Hz, H-4), 2.27 (1H, d with fine splittings, J = 11.6 Hz, H-3), 2.43 (1H, ddd, J = 13.7, 4, 2.8 Hz, H-6), 4.45 (1H, d with fine splittings, J = 11.6 Hz, H-2), 4.72 (2H, br s, H-9), 4.84 (1H, d, J = 1.5 Hz, H-7), 4.93 (1H, d, J = 1.5 Hz, H-7), 8.10 (1H, s, OOH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.7 (q, C-10), 32.7 (t, C-5), 34.1 (t, C-6), 37.0 (t, C-3), 43.9 (d, C-4), 85.2 (d, C-2), 105.3 (t, C-7), 109.4 (t, C-9), 146.7 (s, C-1), 148.4 (s, C-8); CIMS (isobutane) *m*/*z* 169 [M + H$^+$] (51), 151 (100), 135 (89); HRCIMS *m*/*z* 169.1219 (calcd for $C_{10}H_{17}O_2$, 169.1228).

(−)-(2*R*,4*S*)-*p*-Mentha-1(7),8-dien-2-hydroperoxide (3a): colorless oil; $[\alpha]^{20}_D$ −78.1° (*c* 2.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.28 (1H, qd, J = 13, 4.1 Hz, H-5), 1.54 (1H, td, J = 13.6, 3.3 Hz, H-3), 1.70 (3H, s, H-10), 1.86 (1H, d with fine splittings, J = 11.9 Hz, H-5), 2.16 (1H, dq, J = 14.3, 3 Hz, H-3), 2.29 (1H, dt, J = 13.4, 3.2 Hz, H-6), 2.35 (1H, tt, J = 12.9, 3.2 Hz, H-4), 2.38 (1H, t with fine splittings, J = 13.4 Hz, H-6), 4.51 (1H, t, J = 2.9 Hz, H-2), 4.68 and 4.70 (each 1H, br s, H-9), 5.00 and 5.01 (each 1H, br s, H-7), 7.64 (1H, br s, OOH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.7 (q, C-10), 30.4 (t, C-6), 32.4 (t, C-5), 35.5 (t, C-3), 38.6 (d, C-4), 85.5 (d, C-2), 109.0 (t, C-9), 114.0 (t, C-7), 145.2 (s, C-1), 149.0 (s, C-8). ^1H NMR (C_6D_6, 500 MHz) δ 1.14 (1H, qd, J = 13.0, 4.0 Hz, H-5), 1.33 (1H, td, J = 13.6, 3.4 Hz, H-3), 1.56 (3H, s, H-10), 1.70 (1H, m, H-5), 2.03 (1H, dt, J = 13.4, 3.1 Hz, H-6), 2.16 (1H, ddd, J = 14.0, 6.1, 3.1 Hz, H-3), 2.31 (1H, t with fine splittings, J = 13.7 Hz, H-6), 2.42 (1H, tt, J = 12.5, 2.8 Hz, H-4), 4.32 (1H, t, J = 2.7 Hz, H-2), 4.71 and 4.73 (each 1H, m, H-9), 4.81 and 4.85 (each 1H, t, J = 2.1 Hz, H-7), 7.12 (1H, br s, OOH); ^{13}C NMR (C_6D_6, 125 MHz) 20.8 (q, C-10), 30.7 (t, C-6), 32.8 (t, C-5), 35.8 (t, C-3), 39.0 (d, C-4), 85.2 (d, C-2), 109.3 (t, C-9), 113.3 (t, C-7), 145.9 (s, C-1), 149.2 (s, C-8); CIMS (isobutane) *m*/*z* 169 (M + H$^+$, 17), 167 (38), 151 (100), 135 (47); HRCIMS *m*/*z* 169.1224 [M + H$^+$] (calcd for $C_{10}H_{17}O_2$, 169.1228).

(−)-(1*R*,4*S*)-*p*-Mentha-2,8-dien-1-hydroperoxide (4a): colorless oil; $[\alpha]^{20}_D$ −49.6° (*c* 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.32 (3H, s, H-7), 1.43 (1H, m, H-6), 1.68 (2H, m, H-5), 1.71 (3H, s, H-10), 2.14 (1H, dt, J = 13.8, 4.3 Hz, H-6), 2.66 (1H, br t, J = 7.3 Hz, H-4), 4.74 (1H, br s, H-9), 4.76 (1H, br s, H-9), 5.62 (1H, br d, J = 9.9 Hz, H-2), 5.82 (1H, dd, J = 9.9, 2.6 Hz, H-3), 7.29 (1H, br s, OOH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.8 (q, C-10), 24.5 (t, C-5), 24.6 (t, C-7), 31.0 (t, C-6), 43.5 (d, C-4), 79.4 (s, C-1), 111.0 (t, C-9), 129.3 (d, C-2), 135.9 (d, C-3), 147.7 (s, C-8); CIMS (isobutane) *m*/*z* 169 (M + H$^+$, 23), 167 (43), 151 (51), 135 (100); HRCIMS *m*/*z* 169.1231 [M + H$^+$] (calcd for $C_{10}H_{17}O_2$, 169.1228).

(−)-(1*S*,4*S*)-*p*-Mentha-2,8-dien-1-hydroperoxide (5a): colorless oil; $[\alpha]^{20}_D$ −164.2° (*c* 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.32 (3H, s, H-7), 1.53 (1H, m, H-5), 1.63 (1H, m, H-6), 1.74 (3H, s, H-10), 1.94 (1H, m, H-5), 2.01 (1H, m, H-6), 2.77 (1H, m, H-4), 4.68 (1H, br s, H-9), 4.79 (1H, br s, H-9), 5.70 (1H, dd, J = 10.3, 2.0 Hz, H-2), 5.82 (1H, dd, J = 10.3, 3.4 Hz, H-3), 7.35 (1H, br s, OOH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.2 (q, C-10), 24.3 (q, C-7), 24.8 (t, C-5), 30.0 (t, C-6), 42.6 (d, C-4), 80.8 (s, C-1), 111.0 (t, C-9), 130.0 (d, C-2), 134.6 (d, C-3), 147.2 (s, C-8); CIMS (isobutane) *m*/*z* 169 (M + H$^+$, 13), 167 (14), 151 (26), 135 (100); HRCIMS *m*/*z* 169.1237 [M + H$^+$] (calcd for $C_{10}H_{17}O_2$, 169.1228).

Conversion of Hydroperoxides to Alcohols. Compound **2a** (7.5 mg) and triphenylphosphine (15.3 mg) were dissolved in ether (1 mL) and stirred at room temperature for 1 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography to give the corresponding alcohol **2b** (6 mg). Each of the hydroperoxides **3a** (8.3 mg), **4a** (50 mg), and **5a** (50 mg) was treated with triphenylphosphine as above to give the corresponding alcohols **3b** (7.0 mg), **4b** (11 mg), and **5b** (28 mg), respectively.

(−)-(2*S*,4*S*)-*p*-Mentha-1(7),8-dien-2-ol (2b): colorless oil; $[\alpha]^{25}_D$ −16.4° (*c* 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.24 (2H, overlapped, H-3, 5), 1.65 (1H, br s, OH), 1.72 (3H, s, H-10), 1.83 (1H, d with fine splittings, J = 12.5 Hz, H-5), 2.06 (1H, t with fine splittings, J = 13.5 Hz, H-6), 2.18 (2H, overlapped, H-3, 4), 2.43 (1H, dt, J = 13.5, 3.2 Hz, H-6), 4.45 (1H, d with fine splittings, J = 11.6 Hz, H-2), 4.71 (2H, br s, H-9), 4.79 (1H, d, J = 1.5 Hz, H-7), 4.95 (1H, d, J = 1.5 Hz, H-7); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.8 (q, C-10), 32.7 (t, C-5), 33.8 (t, C-6), 42.1 (t, C-3), 44.1 (d, C-4), 72.2 (d, C-2), 103.9 (t, C-7), 109.1 (t, C-9), 148.6 (s, C-8), 151.2 (s, C-1).

(−)-(2*R*,4*S*)-*p*-Mentha-1(7),8-dien-2-ol (3b): colorless oil; $[\alpha]^{25}_D$ −60.5° (*c* 0.7, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.30 (1H, qd, J = 12.4, 4 Hz, H-5), 1.48 (1H, br s, OH), 1.53 (1H, ddd, J = 13.7, 12.2, 3.1 Hz, H-3), 1.73 (3H, s, H-10), 1.85 (1H, d with fine splittings, J = 12.4 Hz, H-5), 1.99 (1H, dq, J = 13.7, 3.1 Hz, H-3), 2.21 (1H, dt, J = 13.7, 3.5 Hz, H-6), 2.48 (1H, t with fine splittings, J = 13.4 Hz, H-6), 2.54 (1H, tt, J = 12.2, 3.2 Hz, H-4), 4.37 (1H, br s, H-2), 4.71 (2H, br s, H-9), 4.77 (1H, t, J = 1.8 Hz, H-7), 4.86 (1H, t, J = 1.5 Hz, H-7); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.0 (q, C-10), 29.9 (t, C-6), 32.6 (t, C-5), 38.1 (d, C-4), 39.0 (t, C-3), 72.4 (d, C-2), 108.9 (t, C-9), 109.9 (t, C-7), 149.4 (s, C-8), 149.8 (s, C-1).

(−)-(1*R*, 4*S*)-*p*-Mentha-2,8-dien-1-ol (4b): colorless oil; $[\alpha]_D$ −68.1° (*c* 0.54, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.30 (3H, s, Me-7), 1.61 (2H, m, overlapped, H-5, H-6), 1.74 (3H, br s, Me-10), 1.77 (1H, m, H-5), 1.85 (1H, m, H-6), 2.66 (1H, m, H-4), 4.75 (1H, br s, H-9), 4.78 (1H, quint, J = 1.5 Hz, H-9), 5.66 (1H, ddd, J = 10.1, 2.2, 1.0 Hz, H-3), 5.71 (1H, ddd, J = 10.1, 2.2, 1.3 Hz, H-2); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 20.9 (q, C-10), 24.9 (t, C-5), 29.4 (q, C-7), 36.7 (t, C-6), 43.4 (d, C-4), 67.5 (s, C-1), 110.6 (t, C-9), 132.2 (d, C-3), 133.9 (d, C-2), 148.1 (s, C-8).

(−)-(1*S*, 4*S*)-*p*-Mentha-2,8-dien-1-ol (5b): colorless oil; $[\alpha]_D$ −150.8° (*c* 1.07, $CHCl_3$); ^1H NMR ($CDCl_3$, 500 MHz) δ 1.29 (3H, s, Me-7), 1.56 (1H, m, H-5), 1.66 (1H, ddd, J = 12.8, 9.5, 2.8 Hz, H-6), 1.74 (3H, br s, Me-10), 1.79 (1H, ddd, J = 12.8, 8.9, 2.8 Hz, H-6), 1.90 (1H, m, H-5), 2.74 (1H, m, H-4), 4.67 (1H, br s, H-9), 4.78 (1H, quint, J = 1.6 Hz, H-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); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.2 (q, C-10), 25.0 (t, C-5), 28.9 (q, C-7), 36.1 (t, C-6), 42.5 (d, C-4), 68.6 (s, C-1), 110.9 (t, C-9), 130.8 (d, C-3), 134.4 (d, C-2), 147.2 (s, C-8).

Hydrogenation of the Alcohols 4b and 5b. The alcohols **4b** and **5b** were dissolved in AcOEt and hydrogenated in the presence of 5% Pd−C at room temperature for 6 h to give the corresponding saturated *p*-menthane alcohols **8** and **9**, respectively. ^{13}C NMR in $CDCl_3$: compound **8**, δ 69.2 (C-1), 38.9 (C-2,6), 25.0 (C-3,5), 43.4 (C-4), 31.3 (C-7), 32.6 (C-8), 19.9 (C-9,10); compound **9**, δ 71.1 (C-1), 40.3 (C-2,6), 27.2 (C-3,5), 43.4 (C-4), 25.6 (C-7), 32.3 (C-8), 20.0 (C-9,10).

GC Analysis of the Hydroperoxide Fraction of AcOEt Extract. Fresh leaves of *C. ambrosioides* were extracted with AcOEt at room temperature for 3 h. The extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was fractionated with a short silica gel column with 4:1 hexane−AcOEt to give a peroxide fraction. The peroxide fraction was dissolved in ether and treated with PPh_3 at room temperature for 5 min. The mixture was concentrated and fractionated by a short silica gel column (6:1 hexane−AcOEt then 3:1) to give the alcohol fraction, which was analyzed by GC. The GC conditions were as follows: column, TC-WAX (60 m × 0.25 mm, GL Sciences, Japan); carrier gas, helium; injector temperature, 210 °C; temperature program, 70 °C (4 min), 10 °C/min to 120 °C, 2 °C/min to 160 °C, 10 °C/min to 210 °C (10 min); detector temperature, 210 °C.

Trypanocidal Assay. Epimastigotes of *T. cruzi* (Tulahuen strain) were kept in GIT medium (Wako) supplemented with hemin (12.4 μM, Wako). The epimastigotes in GIT medium (10 μL) were incubated with a test sample dissolved in EtOH (5 μL) and autoclaved saline (185 μL). After 24 h of incubation, the movement of epimastigotes was observed under a micro-

scope (× 100). Each assay was performed in duplicate. Gentian violet, which is used to disinfect trypanosomes from transfusion blood in Latin America, is used as a positive control. MLC of gentian violet under this assay condition was 6.3 μM.

HeLa Infection Assay. The HeLa cell infection assay was performed as described previously.[15] In brief, a round coverslip was placed in each well of a 24-well plate. Exponentially growing HeLa cells (5×10^3 cells/mL/well) were added to each well, followed by incubation at 37 °C for 2 days in 5% CO_2 in air. The cells were then infected with *T. cruzi* trypomastigotes (1.2×10^4 parasites/well). A compound to be tested was dissolved in ethanol (10 μL) and added immediately after infection. HeLa cells attached to the coverslip were fixed and stained with Diff-Quik (Kokusai Shiyaku) at day 4 after infection. The coverslip was then transferred upside down to a slide glass, and the cells were finally embedded in HSR solution (Kokusai Shiyaku) for observation under a microscope. The percentage of infected host cells, i.e., those containing more than one amastigote, and the mean number of amastigotes per infected cell were determined by analyzing more than 200 host cells distributed in randomly chosen microscopic fields. Allopurinol was used as a positive control.

Acknowledgment. We are grateful to the Kyoto Medicinal Plant Garden of Takeda Chemical Industries Ltd. for providing seeds of *C. ambrosioides*. We also 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 Grants-in-Aid for Scientific Research (Nos. 11672101 and 12557199) from the Japan Society for the Promotion of Science.

Supporting Information Available: Gas chromatogram of the alcohols prepared from the hydroperoxide fraction of the AcOEt extract. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP010445G