Note

Inhibition by (−)-Persenone A-related Compounds of Nitric Oxide and Superoxide Generation from Inflammatory Leukocytes

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We have previously reported that persenone A, isolated from avocado fruit, is an effective inhibitor of both nitric oxide (NO) and superoxide (O2−) generation in cell culture systems. In this study, we have prepared four persenone A-related compounds and examined their inhibition of NO and O2− generation from inflammatory leukocytes. Some structural importance in persenone A to attenuate free radical generation is discussed.

Key words: nitric oxide; superoxide; persenone A

The generation of nitric oxide (NO) and superoxide (O2−) is closely associated with the development of several inflammation-associated diseases. NO rapidly and spontaneously reacts with triplet oxygen to form stable anions, nitrite (NO2−) and nitrate. The protonation of NO2− leads to the formation of dinitrogen trioxide and dinitrogen tetraoxide, the latter of which is an electrophilic agent that N-nitrosates primary and secondary amines to yield carcinogenic nitrosamines.1

Tumor promoter-induced reactive oxygen species generation is considered to be important in tumor promoting pathways. In particular, 12-O-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters are reported to trigger O2− generation in epithelial cells or inflammatory leukocytes through the xanthine/xanthine oxidase and NADPH oxidase systems, respectively. Also, numerous studies have thus far demonstrated that ROS originating from O2− may form oxidative DNA bases,5 which induce a specific mutational spectrum. It is now realized that the interactions between NO and O2− may also be important in disease pathology. Namely, under inflammatory conditions, macrophages can greatly increase their ability to produce both NO and O2−. These two radical species rapidly react with each other to form a peroxynitrile anion,6,7 which may form 8-nitroguanine and nitrotyrosine.8

In our previous study, two novel phytochemicals, (−)-persenone A (1) and (−)-persenone B (2), and a known one, (−)-PA-3 (3), were isolated from avocado fruit (Persea americana P. Mill), as inhibitors of NO and O2− generation in cell culture systems (Fig. 1).9 The fifty% inhibitory concentrations (IC50) of 1–3 against NO generation were 1.2, 3.5, and 3.6 μM, respectively,6 all of which were lower than that of a synthetic iNOS inhibitor, N-iminoethyl-L-ornithine (L-NIO, IC50 = 7.5 μM, see Table 1). On the other hand, the inhibitory potencies of 1–3, and DHA against O2− generation were 1.4, 1.8, 33.7, and 10.3 μM, respectively.6

In this study, we synthesized (+)-persenone A and its related compounds to discuss the structure-activity relationships of this class of compounds. In order to synthesize (+)-persenone A, linoleic acid (4) was used as a starting material (Fig. 2). Treatment of 4 with an excess of methyl lithium (McLi) afforded a methyl ketone (5), that was converted into an α,β-unsaturated derivative (7) using phenylenclidochloride (PhSeCl). This reaction is based on the mechanism where alkylphenyl selenoxides undergo facile syn

![Fig. 1. Structures of Persenone A (1), B (2) and PA-3 (3).](image-url)
Fig. 2. Synthetic Scheme for (2R, S)-(12Z, 15Z)-2-Hydroxy-4-oxoheneicos-5,12,15-trien-1-yl acetate (10).
(a) MeLi/THF, 0°C, 2 hr, (b) PhSeCl/THF, −78°C, 2 hr, (c) 
H2O2, 0°C, 30 min (d) O3, PPh3, −78°C, 12 hr, (e) LDA/THF, −78°C, 30 min.

Table 1. The IC50 Values of Persenone A, Its Related Compounds and Known NO Generation Inhibitors

<table>
<thead>
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<th>Compounds</th>
<th>(IC50, μM)</th>
<th>Compounds</th>
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<td>8.2</td>
<td>12</td>
<td>2.3</td>
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<td>5</td>
<td>&gt;100</td>
<td>L-NIO*</td>
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</tr>
<tr>
<td>7</td>
<td>48.9</td>
<td>DHA*</td>
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</tr>
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<td>&gt;100</td>
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<td>1.2</td>
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<tr>
<td>10</td>
<td>1.4</td>
<td>3′</td>
<td>3.6</td>
</tr>
<tr>
<td>11</td>
<td>1.2</td>
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</tr>
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</table>

a N2-Imidomethyl-l-ornithine,
b Docosahexaenoic acid,
c Reference 6

Table 2. The IC50 Values of Persenone A, Its Related Compounds in the Inhibition of TPA-induced O2·− Generation in Differentiated HL 60 Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(IC50, μM)</th>
<th>Compounds</th>
<th>(IC50, μM)</th>
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<td>DHA</td>
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<td>NT*</td>
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<tr>
<td>10</td>
<td>3.3</td>
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</tbody>
</table>

* Not Tested.

Elimination to form olefins. Then, 10 was obtained by the Aldol reaction of 7 and 9. The racemate 10 had two independent peaks with retention times of 22 (11) and 24 (12) min on HPLC analysis [chiral column (Sumipax 25001, Sumika Chemical Analysis Service, Ltd., Osaka, Japan), n-hexane:chloroform: ethanol = 130:15:1, 1.0 ml/min, 244 nm]. The spectral data of 11 were consistent with those of the natural compound (1) including optical rotation ([α]D 22 = +17.6°, c 0.48, CHCl3) while those of 10 and 12 were 0° and −17.6°, respectively. Then, compounds 4-12 were tested for inhibition of NO and O2·− generation.

NO generation was evaluated by measuring the formation of both NO2 and l-citrulline. L-Citrulline is formed from an iNOS substrate L-arginine and generated NO is then converted spontaneously to NO2. RAW 264.7 cells were cultured in the presence of test compounds stimulated with both lipopolysaccharide (LPS, 100 ng/ml) and interferon-γ (IFN-γ, 100 U/ml). Cells incubated in the absence of samples and treated only with LPS and IFN-γ were used as a control.

As shown in Table 1, the less structural requirement at the alkyl chain for inhibitory activity; i.e., the presence of the double bonds at C5, C12, or C15, was not indispensable to attenuate NO generation because the activities of 2 and 3 are equivalent to that of 1. Linoleic acid (4) (IC50 = 8.2 μM) has much higher activity than both its methyl ketone counterpart 5 (IC50 > 100 μM) and 7 (IC50 = 48.9 μM) (Table 1), suggesting that a free hydroxyl group is important for inhibiting NO generation. Thus, the amphiphilic nature, which is characterized as hydrophobic long alkyl chain and hydrophilic substituents at the terminal position, may play an important role in the interactions with target molecule(s) participating in the biological pathways for NO generation. The activity of 11 (IC50 = 1.2 μM) was equivalent to those of its racemate (10, IC50 = 1.4 μM) and an enantiomer (12, IC50 = 2.3 μM), indicating that the absolute configuration at the C-2 of 1 is not important for inhibitory activity.

As for O2·− generation inhibition, the double bond at C5 conjugated to carbonyl group was an important factor because the activities of 1 (IC50 = 1.4 μM) and 2 (IC50 = 1.8 μM) are almost equal to each other and markedly exceed that of 3 (IC50 = 28.0 μM) (Table 2). Compound 7 has much higher activity than both 4 and its methyl ketone counterpart 5. This suggested that a free hydroxyl group was not required to inhibit O2·− generation, different from the case in NO generation inhibition by this class of compounds. The activity of compound 11 (IC50 = 3.0 μM) was equivalent to those of its racemate 10 (IC50 = 3.0 μM) and enantiomer 12 (IC50 = 3.1 μM), indicating that the absolute configuration at the C-2 of 2 is also not a critical factor for inhibitory activity.

In conclusion, persenone A (1) was synthesized in four steps from the linoleic acid (4). We have shown that an amphiphilic nature and the enone moiety of 1 are important to suppress the production NO and O2·−, respectively, in stimulated leukocytes.
Experimental

General procedures. Fast atom bombardment mass spectra (FAB-MS) were obtained on a Jeol HX-100 (Jeol Datum, Osaka, Japan). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-300 using tetramethylsilane (TMS) as an internal standard (δ0.00). Chromatographic materials used were as follows: Wako gel C-100, C-200 and C-300 (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan), Keiselgel 60 F254 for TLC (Merck Co. Ltd., Darmstadt, Germany), and ODS gel KC18F for TLC (Whatman, Clifton, NJ).

Chemicals. RPMI 1640 medium from Gibco BRL (NY, USA); LPS (E. coli serotype 0127, B8) from Difco Labs (MI, USA); IFN-γ from Genzyme (MA, USA). L-Arginine, (6R)-tetrahydro-L-bioperin (BH₃), linoleic acid, and allyl acetate were purchased from Sigma Chemical Co. Ltd (MI, USA); TPA, tetrahydrofurane (THF), diethyl ether (Et₂O), diisopropylamide, and chlorotrimethylsilane (Me₃SiCl) from Wako Pure Chemicals Co. (Osaka, Japan); MeLi, butyl lithium (BuLi), lithium diisopropylamide (LDA), and PhSeCl were purchased from Aldrich (Deutch); other chemicals were purchased from Wako Pure Chemicals Co. Ltd (Osaka, Japan).

Measurement of NO₃⁻ and l-citrulline. Murine macrophage cell line RAW 264.7 (2 × 10⁶ cells/ml) was inoculated onto a 24-well plate and incubated in 1 ml of RPMI medium with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂. The cells were treated with LPS (100 ng/ml), BH₄ (10 μg/ml), IFN-γ (100 U/ml), and l-arginine (2 mM). After 24 hr, both the levels of NO₃⁻ and l-citrulline were measured as reported previously.

TPA-induced O₂⁻ generation test. Inhibitory tests of TPA-induced O₂⁻ generation were done as previously reported. Human promyelocytic leukemia HL-60 cells were incubated at 4 × 10⁶ cells/ml in RPMI 1640 with 10% FBS. The cells were incubated with 1.25% DMSO at 37°C in a 5% CO₂ incubator for 6 days to induce their differentiation into granulocyte-like cells. Then the cells were washed with Hank’s buffer, and suspended at a density of 1 × 10⁶ cells/ml. The test compound, dissolved in 5 μl of DMSO, was added to the cell suspension, and the mixture thus obtained was incubated at 37°C for 15 min. The cells were washed with Hank’s buffer twice to remove extracellular test compounds. Ninety seconds after stimulation with 5 μl of TPA solution (20 μM), 50 μl of cytochrome c solution (20 mg/ml) was added to the reaction mixture, which was incubated for another 15 min. The reaction mixture was centrifuged at 2,000 g for 1 min, and the visible absorp-

(10Z, 13Z)-Nonadeca-10,13-dien-2-one (5). A stirred solution of 4 (2.0 g, 20 mmol) in dry THF (50 ml) was cooled to 0°C in a N₂ gas atmosphere and treated with MeLi (80 mmol; 1.4 m in Et₂O, 19 ml). After 2 hr, Me₂SiCl (10 ml, 120 mmol) was added to the solution. The reaction mixture was adjusted to room temperature, and 1 N HCl (50 ml) was added. After it was stirred for 1 hr, the mixture was then transferred into a separatory funnel and extracted with Et₂O. The organic layer was washed with saturated NaHCO₃, NaCl, and H₂O, and dried over MgSO₄. Reaction products were separated on silica gel column chromatography (100% hexane) to afford 5 as a colorless oil (1.4 g, 70%); 1H NMR (CDCl₃) δ1.13 (3H, s), 2.41 (2H, t), 1.29–1.37 (16H, br. s), 2.03 (4H, d), 5.32–5.37 (4H, m), 2.77 (2H, t), 0.89 (3H, t, J = 6.7 Hz). FAB-MS (mNBA as a matrix) m/z: 278 [M⁺].

2-Acetoxyacetdehyde (7). Ozone was passed through a solution of 6 (20 g, 200 mmol) in CHCl₃ (150 ml) at −78°C. After the reaction was complete, a PPh₃ solution (52.4 g, 200 mmol) in CHCl₃ (150 ml) was added dropwise at −78°C for 3 hr and the solution was stirred at room temperature overnight. After CHCl₃ was removed by evaporation, the residue was suspended in Et₂O and left overnight at −15°C. After removal of P₃O₅ by filtration and concentration from the filtrate, the residue was distilled under reduced pressure to obtain 7 (17.4 g, 85%); bp 48°C 14 Torr; 1H NMR (CDCl₃) δ2.21 (3H, s), 4.69 (2H, s), 9.61 (1H, s).

(10Z, 13Z)-Nonadeca-3,10,13-trien-2-one (9). Compound 5 (3.7 g, 81.2 mmol) in 150 ml dry THF was reacted with LDA/THF solution at −78°C (dry ice/EtOH) for 30 min, and added to PhSeCl (4.5 g, 92.9 mmol) at dry THF (100 ml). After 30 min, the dry ice/EtOH bath was removed and the reaction mixture was allowed to rise to room temperature and poured into a cold solution of 2N HCl. Next the reaction mixture was extracted with hexane/Et₂O (1:1), and the organic layer was washed with saturated NaHCO₃, NaCl, and H₂O, and dried over MgSO₄. Reaction mixtures were separated on silica gel column chromatography (hexane/EtOAc) to afford 8 as a yellowish oil. Then, 8 (623 mg, 43.1 mmol) in EtOAc: THF (2:1, 14 ml) was added to NaHCO₃ (311 mg) and 30% H₂O₂ (490 μl) at 0°C for 30 min. Continuously, reaction mixture was allowed to come to room temperature for 1 hr, and added to saturated Na₂CO₃ for reduction of excess H₂O₂. The reaction mixture was extracted with Et₂O, the organic layer was washed with saturated NaHCO₃, NaCl, and H₂O, and dried over MgSO₄. Reaction mixtures were separated on silica gel column chromatography
(hexane/EtOAc) to afford 9 as a colorless oil (81 mg, 2.1%); 1H NMR (CDCl3), δ 85.85 (1H, d, J = 16 Hz), 6.99 (1H, d, J = 16.6 Hz), 2.15 (2H, m), 1.20–1.50 (14H, m), 2.00 (4H, m), 5.24–5.40 (4H, m), 0.88 (3H, t). FAB-MS (mNBA as a matrix) m/z: 277 [M + H]+.

(12Z, 15Z)-2-Hydrorxy-4-oxoheneicosa-5, 12, 15-trien-1-yl acetate (10). (±)-Persenone A was prepared from 9 and LDA (5 mmol, 180 µl) in dry THF at −78°C. To this solution, freshly distilled 7 (41 mg, 5 mmol) in THF was added dropwise at the same temperature. After 30 min, the coolant was removed and the solution immediately neutralized with AcOH (5 mmol, 20 µl). Reaction products were separated on silica gel column chromatography (CHCl3/acetone) to afford (±)-10 as a colorless oil (26.8 mg, 30%); 1H NMR (CDCl3), δ4.13 (2H, m), 4.34 (1H, m), 2.78 (2H, m), 6.12 (1H, d, J = 16 Hz), 6.88 (1H, dt, J = 16, 6.9 Hz), 2.24 (2H, m), 1.27–1.37 (16H, m), 2.04 (4H, d), 5.29–5.41 (4H, m), 2.78 (2H, m), 0.88 (3H, t), 3.30 (1H, s), 2.09 (3H, s). FAB-MS (mNBA as a matrix) m/z: 401 [M + Na]+; 379 [M + H]+.

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References