Investigation on the Epoxidation of Piperitenone, and Structure-activity Relationships of Piperitenone Oxide for Differentiation-inducing Activity

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Abstract: Piperitenone oxide, a major chemical constituent of the essential oil of spearmint, Mentha spicata, induces differentiation in human colon cancer RCM-1 cells. In this study, piperitenone oxide and trans-piperitenone dioxide were prepared as racemic forms by epoxidation of piperitenone. The relative configuration between two epoxides in piperitenone dioxide was determined to be trans by ¹H NMR analysis and nuclear Overhauser effect spectroscopy (NOESY) in conjunction with density functional theory (DFT) calculations. Optical resolution of (±)-piperitenone oxide by high-performance liquid chromatography (HPLC) using a chiral stationary phase (CSP) afforded both enantiomers with over 98% enantiomeric excess (ee). Evaluation of the differentiation-inducing activity of the synthetic compounds revealed that the epoxide at C-1 and C-6 in piperitenone oxide is important for the activity, and (+)-piperitenone oxide has stronger activity than (−)-piperitenone oxide. The results obtained in this study provide new information on the application of piperitenone oxide and spearmint for differentiation-inducing therapy. Furthermore, natural piperitenone oxide was isolated from M. spicata. The enantiomeric excess of the isolated natural piperitenone oxide was 66% ee. Epoxidation of piperitenone with hydrogen peroxide proceeded in a phosphate buffer under weak basic conditions to give (±)-piperitenone oxide. These results suggest that the nonenzymatic epoxidation of piperitenone, which causes a decrease in the enantiomeric excess of natural piperitenone oxide, is accompanied by an enzymatic epoxidation in the biosynthesis of piperitenone oxide.

Key words: piperitenone oxide, piperitenone, structure-activity relationships, differentiation-inducing activity

1 Introduction

Piperitenone oxide (1) is one of the major constituents of the essential oil of spearmint, Mentha spicata (Fig. 1)¹⁻⁻³. It was first named rotundifolone since it was isolated from the essential oil of M. rotundifolia cultivated in Japan⁴⁻⁻⁶. The absolute configuration of natural (1)-1 was determined to be 1S,6S (the carbon atoms have been labeled using the IUPAC numbering system)⁷⁻⁻⁹. The biosynthesis of natural (1)-1 involves the enzymatic epoxidation of piperitenone (2)¹⁻⁻¹⁰. Thus, optically active piperitenone...
oxide is produced from optically inactive piperitenone. The synthesis of \((-\)-piperitenone dioxide\(\text{3}\)) by epoxidation of natural\((+\)-1 was reported by Shimizu\(5\) and Katsuhara\(9\). The relative configuration between two epoxides in \((-\)-3 was determined to be cis\(5\). And the absolute configuration of \((-\)-3 was determined to be \(1S,3S,6S\)\(^{30}\).

Natural 1 has several biological activities such as antimicrobial and antinociceptive activities\(^{11}\). Recently, it was reported that piperitenone oxide isolated from \(M. spicata\) induces differentiation in human colon cancer RCM-1 cells\(^{11}\). Interestingly, structurally \((-\)-carvone and \(-\)-menthol do not show differentiation-inducing activities\(^{11}\).

The attractive biological activities of 1 stimulated us to perform both synthetic and structure-activity relationship studies on this compound. In this study, \((\pm\)-1 and \((\pm\)-trans-piperitenone dioxide\(\text{2}\)) were prepared from 2. Optical resolution of synthetic \((\pm\)-1 was achieved by high-performance liquid chromatography (HPLC) using a chiral stationary phase (CSP). In addition, the structure-activity relationships of piperitenone oxide for the differentiation-inducing activity in RCM-1 cells were examined. Furthermore, according to the results obtained by the determination of the enantiomeric excess of natural 1 and epoxidation of 2 in a phosphate buffer under basic conditions, the biosynthesis of natural 1 through both enzymatic and nonenzymatic epoxidation of 2 is also proposed.

2 Experimental Procedures

2.1 General information

Solvents and reagents were used without further purification unless otherwise noted. Analytical TLC was performed using Silica gel 60 \(F_{254}\) plates (0.25 mm, normal phase) (Merck, Darmstadt, Germany). Flash column chromatography was performed using Silica gel 60 (particle size 40–63 \(\mu\)m; 230–400 mesh ASTM) (SiliCycle, Québec, Canada). Melting point (Mp) data were determined using an MM-2 instrument (Shimadzu) and uncorrected. \(^{1}H\) and proton-decoupled \(^{13}C\)\((\text{\textsuperscript{13}C }{^1}H\text{) NMR spectra}(400 and 100 MHz, respectively) were recorded on a Bruker Avance 400 spectrometer (Bruker, Billerica, MA), using chloroform-\(d\) (CDCl\(_{3}\)) as a solvent. Chemical shift values are expressed in \(\delta\) (ppm) relative to tetramethylsilane (TMS; 0.00 ppm) for \(^{1}H\) NMR and the solvent resonance (CDCl\(_{3}\)) \(\delta\) 77.0 ppm for \(^{13}C\)\((\text{\textsuperscript{13}C }{^1}H\text{) NMR.}\) Data are reported as follows: chemical shift, multiplicity \((s\text{ = singlet, } d\text{ = doublet, } m\text{ = multiplet, } br\text{ = broad}),\) coupling constants\((J; \text{Hz}),\) and integration. IR spectra were recorded on an FT-720 spectrometer (Horiba), using KBr pellets. Mass spectra were obtained by a JEOL high-resolution double-focusing mass spectrometer (JMS-700) using fast atom bombardment (FAB). High-performance liquid chromatography (HPLC) was performed using JASCO LC-NET II system (pump: PU-2089 quaternary gradient pump; detector: UV-2075 and CD-2095). Specific rotations were recorded on a JASCO polarimeter (P-1010), and recorded as \(\alpha\) values (concentration in g/100 mL). Piperitenone (2) was prepared according to the reported procedure\(^{13,14}\).

2.2 Preparation of racemic piperitenone oxide and \textit{trans}-piperitenone dioxide

A 10\% aqueous solution of KOH (0.85 mL, 1.51 mmol), followed by a 30\% aqueous solution of \(\text{H}_2\text{O}_2\) (0.90 mL, 7.94 mmol) was added to a solution of \(2\)\(^{14}\) (1.10 g, 7.32 mmol) in 2-propanol (22 mL) at \(-10^\circ\text{C}\). The mixture was stirred at \(-10^\circ\text{C}\) for 117 h. The reaction was quenched by the addition of water. The mixture was diluted with hexane. The organic layer was collected, washed with water and brine, dried over \(\text{Na}_2\text{SO}_4\), and concentrated. The residue was purified by silica gel chromatography (hexane/ethyl acetate = 30:1) to give \((\pm\)-piperitenone oxide (1) (293 mg, 24\%) as a pale yellow oil and \((\pm\)-\textit{trans}-piperitenone dioxide (4) (222 mg, 17\%) as a white solid with recovered 2 (621 mg, 56\%). The structure for synthetic \((\pm\)-1 was confirmed by comparison of its \(^{1}H\) NMR spectroscopic data with the reported one\(^{13}\): \(^{1}H\) NMR (400 MHz, CDCl\(_{3}\)) \(\delta\) 3.24 (s, 1H), 2.50–2.45 (m, 1H), 2.38 (dd, \(J = 16.0, 5.9\) Hz, 1H), 2.14–2.09 (m, 1H), 2.10 (d, \(J = 2.8\) Hz, 3H), 1.88 (m, 1H), 1.80 (d, \(J = 1.4\) Hz, 3H), 1.48 (s, 3H).

\((\pm\)-4: Mp = 34–37\(^\circ\)C. \(^{1}H\) NMR (400 MHz, CDCl\(_{3}\)) \(\delta\) 3.35 (s, 1H), 2.37 (dd, \(J = 14.6, 10.2, 6.8\) Hz, 1H), 2.17–2.13 (m, 2H), 1.73 (dd, \(J = 14.6, 5.6, 4.5\) Hz, 1H), 1.52 (s, 3H), 1.38 (s, 3H), 1.25 (s, 3H). \(^{13}C\)\((\text{\textsuperscript{13}C }{^1}H\text{) NMR}(100 MHz, CDCl\(_{3}\)) \(\delta\) 203.7, 65.8, 64.7, 64.4, 62.9, 27.2, 25.9, 22.0, 20.5, 18.9. IR (KBr) \(\nu_{\text{max}}\) = 3039, 3010, 2966, 2933, 1709, 1433, 1400 cm\(^{-1}\).
HRMS (FAB) m/z calcd. for C_{10}H_{16}O_{3} ([M + H]^+) 183.1019, found 183.1020.

2.3 DFT calculations
Conformational analysis of cis and trans-piperitenone oxide\cite{23}, and the reduced forms of trans-piperitenone oxide (5 and 6 in Supporting Information) was performed using the MMFF94s conformer search algorithm\cite{16}. Conformers within 1.0 kcal/mol energy differences were optimized using density functional theory (DFT) calculations at the B3LYP/6-31G(d,p) level in chloroform, implemented in the Gaussian 09 program package\cite{27}. The lowest energy conformations were determined by comparing the sum of the electronic and zero-point energies of each conformer.

2.4 Optical resolution of (±)-piperitenone oxide and determination of the enantiomeric excess of natural piperitenone oxide isolated from M. spicata
Optical resolution of (±)-piperitenone oxide (1) was performed using HPLC using a CSP under the following conditions: column, Daicel CHIRALPAK IB (10 x 250 mm); mobile phase, hexane/2-propanol = 98/2; flow rate, 5.0 mL/min; temperature, room temperature; detection, UV and CD at 254 nm; 1.0 mL/min; temperature, room temperature; detection, UV and CD at 254 nm; t_R = 5.8 min for (+)-1 and t_R = 6.8 min for (-)-1. The enantiomeric excesses of (+)-1 and (-)-1 were determined to be >98%. (+)-1: [α]_D^25 = 199.6 (c 0.09, CHCl_3), (-)-1: [α]_D^25 = -214.1 (c 0.15, CHCl_3).

Natural piperitenone oxide was extracted by hexane from M. spicata, which was harvested in a greenhouse culture system at Kyoto Prefectural University. The plant source was purchased at local market in Bangkok, Thailand in 2017. The extract was purified by silica gel chromatography (hexane/ethyl acetate = 20:1) to afford natural 1 (18.8 mg). The specific rotation of natural 1 was [α]_D^25 = 186.6 (c 0.11, CHCl_3). The enantiomeric excess of natural 1 was determined to be 66% ee by chiral HPLC using Daicel CHIRALPAK IB column (4.6 x 250 mm) under the following conditions: mobile phase, hexane/2-propanol = 98/2; flow rate, 1.0 mL/min; temperature, room temperature; detection, UV and CD at 254 nm; t_R = 5.3 min for (+)-1 and t_R = 5.9 min for (-)-1.

2.5 Epoxidation of piperitenone in phosphate buffer
A 30% aqueous solution of H_2O_2 (21 μL, 185 μmol) was added to a solution of 2 (25.3 mg, 168 μmol) in 0.2 M sodium phosphate buffer (pH 8.5) (1.0 mL) at room temperature (rt). The mixture was stirred at rt for 7 days. The reaction was quenched by the addition of water. The mixture was diluted with hexane. The organic layer was collected, washed with water and brine, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (hexane/ethyl acetate = 30:1) to give (±)-1 (9.1 mg, 33%) and recovered 2 (10.4 mg, 41%). The same epoxidation of 2 (25.5 mg, 170 μmol) was performed in 0.2 M phosphate buffer (pH 7.5) for 7 days to give (±)-1 (2.0 mg, 7%) and recovered 2 (19.3 mg, 76%).

2.6 Evaluation of the differentiation-inducing activity in RCM-1 cells
The evaluation of the differentiation-inducing activity in RCM-1 cells was performed according to the protocol reported previously\cite{11}. Briefly, RCM-1 cells (1 x 10^5) were each plated into 96-multi-well plastic culture plate with 0.2 mL of 45% RPMI1640 medium with 45% Ham’s F12 medium (Gibco-Invitrogen Corporation, Grand Island, NY) and 10% fetal bovine serum (10% FBS-RPMI+F12). When RCM-1 cells were cultivated with samples at various concentrations for 2 days, the duct number was measured from digital photographs at 40 x using a Zeiss Axiovert 25 microscope equipped with a CCD camera. To judge whether the sample is effective, we used a criterion that sample increases the number of ducts above the background of spontaneous differentiation found in the control.

3 Results and Discussion
3.1 Synthesis and optical resolution of (±)-piperitenone oxide
(±)-Piperitenone oxide (1) was synthesized from piperitenone (2) (Scheme 1)\cite{15,14}. Epoxidation of 2 with hydrogen peroxide (H_2O_2) under basic conditions gave (±)-1 and (±)-trans-piperitenone dioxide (4)\cite{12} in 24% and 17% yields, respectively. In this reaction, 2 was recovered in 56% yield. The relative configuration of (±)-4 was determined by H NMR analysis, nuclear Overhauser effect spectroscopy (NOESY), and DFT calculations (Fig. 2). The coupling constant (J_{H-4ax,H-4a}) between the pseudoaxial proton (H-4ax) at C-4 and the pseudoaxial proton (H-5ax) at C-5 is 10.2 Hz (Fig. 2A). The coupling constant (J_{H-4ax,H-4a}) between the H-4ax and the pseudoequatorial proton (H-5ae) at C-5 is 6.8 Hz. NOESY correlation between 3‘-Me_a and H-4ax was observed in (±)-4. NOESY correlation between 3’-Me_a and H-4ax was not observed in (±)-4. These results indicate the distance between 3’-Me_a and H-4ax is smaller.
Fig. 2 Determination of the relative configuration of (±)-4. The carbon atoms have been labeled using the IUPAC numbering system. (A) NOESY correlation between 3'-Me_a and the pseudoaxial proton (H-4_ax) at C-4 was observed. NOESY correlation between 3'-Me_a and the pseudoequatorial proton (H-4_eq) at C-4 was not observed (the cross sign). The coupling constants between H-4_ax and the pseudoequatorial proton (H-4_eq) at C-4, between H-4_eq and the pseudoaxial proton (H-5_ax) at C-5, and between H-4_eq and the pseudoequatorial proton (H-5_eq) at C-5 are indicated as $J_{H-4 axial/H-4 equatorial}$, $J_{H-5 axial/H-5 equatorial}$, and $J_{H-5 axial/H-5 equatorial}$, respectively. (B) The two main conformations of cis-isomer and selected calculated distances between the indicated hydrogens in cis-isomer. The Boltzmann population percentages at 300 K are indicated in parentheses. (C) The lowest energy conformation of trans-isomer and selected calculated distances between the indicated hydrogens in trans-isomer. The Boltzmann population percentage at 300 K is indicated in parentheses.
than that between 3’-Me₆ and H-4_{eq}. The major conformations of cis- and trans-isomers in chloroform were calculated by DFT calculations at the B3LYP/6-31G(d,p)/level. The calculations provided two major conformations for the cis-isomer (Fig. 2B). The Boltzmann population percentages at 300 K of the cis-conformations I and II are 52% and 48%, respectively. On the other hand, only one major conformation for the trans-isomer is obtained by the DFT calculations (Fig. 2C). The trans-conformation I is estimated to be more stable than the cis-conformation I by 4.65 kcal/mol. The calculated distances between 3’-Me₆ and H-4_{eq} are longer than those between 3’-Me₆ and H-4_{ax} in both cis-conformations I and II. This result does not match the experimental NOESY correlation. The calculated distances between 3’-Me₆ and H-4_{ax} and between 3’-Me₆ and H-4_{eq} in the trans-conformation I were 2.28 Å and 2.49 Å, respectively. Because the calculated distance between 3’-Me₆ and H-4_{ax} is smaller than that between 3’-Me₆ and H-4_{eq}, the calculation results for the trans-conformation I are consistent with the NOESY correlation observed in (+)-4. Taking these results together, we concluded that (+)-4 is the trans-isomer. Carman and coworker reported the structural determination of the dioloxides of terpinolene, obtained by direct epoxidation of terpinolene, 1-methyl-4-(propan-2-ylidene) cyclohex-1-ene (Scheme S1). The major product has the trans relationship between the two epoxides. Terpinolene is the 2-deoxy derivative of piperitenone. Due to the structural similarity between terpinolene and piperitenone, the result reported by Carman and coworker strongly supports our conclusion. The relative configuration between the two epoxides in (+)-4 was also confirmed by determination of the relative configuration of the reduced forms of (+)-4 (see Supporting Information, Scheme S2, and Fig. S1).

Although several attempts were made to obtain enantiomerically pure 1 by the asymmetric epoxidation of 2, none of 1 was obtained and, in most cases, the starting material 2 was recovered. Therefore, the optical resolution of synthetic (+)-1 via HPLC using a CSP was performed. Optical resolution of synthetic (+)-1 by chiral HPLC using a Chiralpak IB column gave both enantiomers of 1, which possessed opposite signs of the specific rotations ([α]D25 + 199.6 (c 0.09, CHCl₃) and −214.1 (c 0.15, CHCl₃), respectively (Fig. S11 in Supporting Information). Natural (+)-1 has the L,S,6S configuration, and unnatural (−)-1 has the 1R,6R configuration. The value for (+)-1 is much higher than those reported for natural 1 [e.g., [α]D25 + 166.5 (MeOH)⁸, [α]D27 + 160.2 (c 0.20, EtOH)⁹, and [α]D27 + 148.6 (c 0.30, CHCl₃)¹⁰]. The enantiomeric excesses of both enantiomers were >98% ee, as determined by chiral HPLC analyses. Unfortunately, we were unable to achieve the optical resolution of (+)-4.

2 30% aq. H₂O₂
phosphate buffer
rt, 7 days
(+)–1
7% at pH = 7.5
33% at pH = 8.5
Scheme 2 Epoxidation of piperitenone (2) with hydrogen peroxide in a sodium phosphate buffer at the indicated pHs.

3.2 Enantiomeric excess and chemical speculation on the biosynthesis of natural piperitenone oxide

It has been proposed that one of the biosyntheses of natural piperitenone oxide involves the enzymatic epoxidation of piperitenone (2) catalyzed by the terpenoid epoxidase¹⁰,¹⁹–²². Ravid and Chanotiya independently reported that chiral GC–MS analyses of natural 1 showed high (> 99%) enantiomeric purity²¹,²². However, we considered the possibility of the contamination of a small amount of (−)-1 in the extract from M. spicata, thereby causing the specific rotation of natural 1 to be lower than that of the synthetic one. Thus, natural 1 in the extract was analyzed by chiral HPLC. The chiral HPLC analysis revealed that the enantiomeric excess was 66% ee, indicating that natural piperitenone oxide contains both enantiomers with a preference for (+)-1 (Fig. S12 in Supporting Information). In addition, the specific rotation of natural piperitenone oxide ([α]D25 + 186.6 (c 0.11, CHCl₃)) was lower than that of enantiomerically pure (+)-1. Thus, we considered that contamination of (−)-1 in natural 1 is due to nonenzymatic epoxidation of 2 in the plant cells. Indeed, treatment of 2 with H₂O₂ (1.1 equiv) in a 0.2 M phosphate buffer at pH 7.5 and 8.5 afforded (−)-1 in 7% and 33% yields, respectively (Scheme 2). Epoxidation did not occur under neutral (pH 7) and acidic conditions (pH < 7). These results suggest that the nonenzymatic epoxidation of piperitenone will occur in the presence of H₂O₂ under basic conditions in plant cells. H₂O₂ is one of the most abundant reactive oxidative species and acts as an important signaling molecule that mediates various physiological and biochemical processes in plants²³,²⁴.

3.3 Structure-activity relationships of piperitenone oxide

First, (±)-1, 2, and (±)-4 were used in the differentiation-inducing assay cell system (Fig. 3). Both (±)-1 and (±)-4 induced differentiation of RCM-1 cells, but 2 did not induce the differentiation of RCM-1 cells at concentrations below 200 μg/mL. Our previous studies show that (−)-carvone and (−)-menthol do not show differentiation-inducing activities¹¹. Taken together, these results indicate that the epoxide at C-1–C-6 is important for the activity. Concentrations for this assay range from 0.625 to 20 μg/mL in (±)-1 and (±)-4, which indicated that (±)-4 was on the
Fig. 3 Structures and effective or ineffective concentration ranges of (±)-piperitenone oxide (1), piperitenone (2), (±)-trans-piperitenone dioxide (4), (−)-carvone, and (−)-menthol for the differentiation-inducing activity against RCM-1 cells. The carbon atoms have been labeled using the IUPAC numbering system.

lower end of this range, thus lacking very strong differentiation potential (Figs. 4A and 4B). Next, the differentiation-inducing activity of (±)-1 and (−)-1 were investigated (Figs. 4C and 4D). Both (±)-1 and (−)-1 induced differentiation of RCM-1 cells above the background of spontaneous differentiation. In linear regression equations of the activity of (±)-1 and (−)-1, the value of the slope in (−)-1 was slightly lower than that in (±)-1. Thus, the activity of (±)-1 was slightly stronger than that of (−)-1. However, no significant difference in the potency between (±)-1 and (±)-1 was observed (Figs. 4A and 4C). (±)-1 was almost as potent as (±)-1 in spite of the contamination of the less potent (−)-1. However, the mechanism underlying the activity of (±)-1 remains unclear.

4 Conclusion

In this study, (±)-piperitenone oxide and (±)-trans-piperitenone dioxide were prepared by epoxidation of piperitenone. The trans-relationship between the two epoxides in (±)-piperitenone dioxide was determined by NMR analysis, NOESY correlation, and DFT calculations. The optical resolution of (±)-piperitenone oxide was successfully achieved by HPLC using a chiral stationary phase. Both enantiomers of piperitenone oxide were obtained in > 98% ee. The enantiomeric excess of piperitenone oxide extracted from M. spicata collected in Bangkok, Thailand was determined to be 66% by chiral HPLC analysis. Epoxidation of piperitenone proceeded in a phosphate buffer under weak basic conditions. These results suggest that the nonenzymatic epoxidation of piperitenone is accompanied by the enzymatic one in plant cells. Both (±)-piperitenone oxide and (±)-trans-piperitenone dioxide displayed differentiation-inducing activity in RCM-1 cells, however, piperitenone did not show this activity. These results indicate that the epoxide at C-1 and C-6 is important for the activity. And the activity of (−)-piperitenone oxide was stronger than that of (±)-piperitenone oxide.

Generally, the chiral center of the ligand/substrate is recognized by its specific target proteins. And the activity of (±)-piperitenone oxide in RCM-1 cells. Because differentiation-inducing agents do not kill normal cells generally, they tend to have less toxicity than conventional anticancer agents. Our results provide new insights for the application of piperitenone oxide and spearmint for differentiation-inducing therapy in cancer patients.

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Supporting Information

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Fig. 4  Differentiation-inducing activity of (±)-piperitenone oxide (1), (±)-trans-piperitenone dioxide (4), (±)-1, and (−)-1. The differentiation potential was determined by seeding $1 \times 10^5$ RCM-1 cells into a 96-multi-well plastic culture plate and then treated for 2 days with each compound (A: (±)-1, B: (±)-4, C: (+)-1, D: (−)-1). The formation of ducts was visually determined using phase contrast microscopy and the resulting images were used for quantifying the number of ducts in each culture plate. The data was plotted as a number of ducts, and each point represents the individual value. Average number of the individual value ranges from 0 to 3 in control (0 μg/mL) in different experiments. Linear regression equations were calculated in the concentration observed with a linear increase in the number of ducts (A, C, and D: 0–10 μg/mL; B: 0–20 μg/mL).
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