Locomotor-Reducing Effects and Structural Characteristics of Inhaled Zerumbone and Tetrahydrozerumbone Derivatives

Kakuyou Ogawa, Takashi Miyoshi, Takashi Kitayama, and Michiho Ito

MATERIALS AND METHODS

Materials Compounds 1–9 shown in Fig. 1 were prepared as below. Zerumbone was extracted from Z. zerumbet rhizome, and the extract was refined. 2,3,10,11-Tetrahydrozerumbone (THZ) stereoisomers (3–5) and tetrahydrozerumbone derivatives (6–9) were synthesised from compound 1. First, 1 was reduced with H₂ on Pd/C to give racemic THZ. Then, 5 was reduced with LiAlH₄ to give 6 and 7, which were acetylated with acetic anhydride in pyridine solution to obtain 8 and 9. Furthermore, 6 and 7 were treated with lipase and isopropenyl acetate to obtain (2S)-tetrahydrozermoibum acetate and (2R)-tetrahydrozermoibum in order to resolve the racemic THZ into 3 and 4 by oxidation following deacylation. Compounds 1, 3, 4, and 7–9 were refined to more than 99.9% purity, as evaluated by ¹H-NMR, and 3 and 4 were obtained with 97% and 95% e.e., respectively. The purity of 6 was 85%. α-Humulene (2) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The odorless solvent triethyl citrate used to dissolve and dilute the odorant compounds for inhalation was purchased from Merck KGaA (Darmstadt, Germany). The reagents used in this study were of the highest grade available.

Animals The animal studies were designed according to the guidelines of the Kyushu University Committee for Animal Experimentation. All animal studies were conducted under the supervision of a veterinary surgeon, and all efforts were made to minimize suffering.

Key words Zerumbone; tetrahydrozerumbone; sedative effect; inhalation; structure–activity relationship

Zerumbone is an 11-membered cyclic sesquiterpene obtained from the rhizomes of Zingiber zerumbet Smith (Zingiberaceae). In this study, we investigated the structure–activity relationship of 1, α-humulene (2), tetrahydrozerumbone stereoisomers (3–5), and tetrahydrozerumbone derivatives (6–9). The oxygen-containing functional groups and the configurations at C1 and C2 contributed to the spontaneous locomotor activity reduction of zerumbone and derivatives.

Fig. 1. Structures of Zerumbone and Its Derivatives Examined in This Study

© 2014 The Pharmaceutical Society of Japan
to the recommendations of the Animal Research Committee of Kyoto University, Kyoto, Japan (authorisation numbers: 2013–17). Four-week-old male ddY mice (about 25 g each) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in colony cages (4 mice per cage) at an ambient temperature of 25°C under a 12 h light–dark cycle. They were fed standard pellet chow and water ad libitum. All behavioural observations were conducted from 09:00 to 17:00.

**Methods** Each compound was dissolved in triethylcitrate (400 µL) and dropped onto four filter paper disks attached to four corners of the glass cage (61.2 L). The vapor from the solution was allowed to fill the cage by natural diffusion for 60 min. A mouse was then placed in the center of the cage and monitored with a video camera for 60 min. The total spontaneous locomotor activity (TLA) is the area under the curve (AUC) which is calculated from a graph with time (min) on the x-axis, and the y-axis was the number of times per 5 min the mouse crossed the lines drawn at 10 cm intervals on the bottom of the cage.11) Most of the effective compounds showed a two-phase effect and the effects at lower doses were considered as the true activity. This is because the mice displayed excited activities, such as jumping and rearing, at higher doses.12)

**Statistical Analysis** Results were expressed as the mean±standard error of the mean (S.E.M.). Statistical analyses were performed by Dunnett’s test and Student’s t-test using GraphPad Instat (GraphPad Software, San Diego, CA, U.S.A.).12) A probability level of p<0.05 was considered statistically significant.

**RESULTS**

**Effect of the Presence of a Ketone Group on Locomotor Activity** The effect of a ketone group on the reduction of locomotor activity was examined by comparing 1 and 2. Locomotor activity was reduced in mice that inhaled compound 1, because the TLA was substantially reduced at a dose of 4.5×10^−2 mg/cage. In contrast, the TLA was not reduced in mice that inhaled compound 2 at any dose (Fig. 2). Therefore, the presence of the ketone group at C1 was important for re-

---

**Fig. 2. Spontaneous Motor Activity of Mice Treated with Zerumbone 1 (a) and α-Humulene 2 (b)**

Data are expressed as the mean±S.E.M. for 5 mice. The statistical analysis was performed using one-way ANOVA followed by Dunnett’s test. **p<0.01 vs. the control group.

**Fig. 3. Total Spontaneous Motor Activity of Mice Treated with (2R)-THZ 3 (a), (2S)-THZ 4 (b), and (2RS)-THZ 5 (c)**

Data are expressed as the mean±S.E.M. for 5 mice. The statistical analysis was performed using one-way ANOVA followed by Dunnett’s test. *p<0.05 and **p<0.01 vs. the control group.
Effect of Double Bonds Near the Ketone Group on Locomotor Activity

Inhalation of 3 and 4, and mixture 5 (1:1 mixture of 3 and 4) significantly reduced TLA at a dose of 4.5×10⁻²mg/cage. Moreover, the reductions in the TLA at different doses were similar for 3–5, suggesting that the reduction of locomotor activity for THZ enantiomers or racemates may be similar (Fig. 3). Significant reductions in locomotor activity were observed for 3–5 at a dose of 1/100th that required for compound 1 (Figs. 2, 3). Therefore, the loss of the two double bonds near the C1 ketone in compound 1 lowered the dose required to reduce locomotor activity.

Effect of cis–trans Isomerism on Locomotor Activity

Compounds 6–9 greatly reduced the TLA. Compound 7 achieved the same TLA reduction as compound 6 at a dose of 1/10th that required for 6. The TLA reduction achieved by 9 also occurred at a dose of 1/10th that required for 8. Thus, trans-isomers 7 and 9 had similar activities at doses 1/10th those of cis-isomers 6 and 8 in rac-cis/trans-tetrahydrozermubol (6 and 7) (THZol) and rac-cis/trans-tetrahydrozermubol acetate (8 and 9) (THZAc).

Effect of Acetylation of THZol Hydroxyl Group on Locomotor Activity

Comparing 6 and 8 or 7 and 9 shows that 6 and 7 produced a significant reduction of locomotor activity at a dose of 1/10th that of 8 and 9 for both cis–trans isomers (Fig. 4). The acetylation of the C1 hydroxyl group increased the dose necessary to achieve a significant reduction of locomotor activity.

DISCUSSION

Contribution of Ring Structure and Oxygen-Containing Groups to Locomotor Activity

α-Humulene (2) showed a weak reduction of locomotor activity (Fig. 2). Differences in the activity curves for 1 and 2 were observed at a dose of 4.5×10⁻²mg. X-Ray structural analyses of 1 and 2 were compared to clarify the relationship between the ring structure and the reduction of locomotor activity. The ring structures of both compounds did not show any notable differences except in the dihedral angles near the ketone. The ketone at C1 of 1 contributed to the decrease of the TLA. THZ 3–5 reduced locomotor activity to the greatest extent, and each required a dose of 1/100th that of 1. Therefore, the absence of double bonds at C2–C3 and C10–C11 of zerumbone 1 contributed to the significant reduction of locomotor activity. The results for THZ 3–5, THZol 6 and 7, and THZAc 8 and 9 (Figs. 3, 4) all reduced locomotor activity significantly. Comparing 1 and 2 with 3–9 indicated that the oxygen-containing groups may contribute to a significant decrease in TLA. Moreover, comparing 6 and 7 with 8 and 9 suggested that acetylating the hydroxyl groups diminished the reduction of locomotor activity. A similar reduction of activity by acetylation was reported by Miyoshi et al. [12]

Influence of Chirality on Locomotor Activity

The reduction of locomotor activity properties of THZ 3–5 were not affected by the absolute configuration at C2. Limonene enantiomers that show similar activity have been reported by Ito and Ito. [13]

Influence of cis–trans Isomers on Locomotor Activity

Vernet-Maury et al. published a comparison of the bioactivities of inhaled cis–trans isomeric cyclic compounds. [14] The cis- and trans-isomers of 8-mercaptomenthones did not show a difference in stress-inducing activity. In contrast, comparing the activity of 6 with 7, and that of 8 with 9, showed that the effective dose of trans-isomers were 10-fold less than that of the cis-isomers in this study.

Putative Mechanisms of Action

To explain the reduction in TLA induced by the inhalation of compounds vari-
cordingly, the amount of compounds inhaled by mice in 1 h
safe, because they were about 0.002% of the LD
mice, and the volume of air that the mice breathed per hour
ring, and the

Effects on locomotor activity, depending on the functional

Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, Ohigashi H. Identification of zerumbone in Zingiber zerumbet Smith as a potent inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced Epstein–Barr virus activation. Biosci. Biotechnol. Biochem., 63, 1811–1812 (1999).

Mixture 5 was dissolved in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and injected intraperitoneally (10^{-5} mg/kg) into mice. The locomotor activity of the mice decreased compared with the vehicle group, which received corn oil (Fig. 5). This may indicate that the compounds exert their activities not only by stimulation of olfactory receptors but also by absorption through the mucous membranes of the lung and nose. The reduction of locomotor activity may indicate sedative, anxiolytic, relaxative, or soporic effects. However, further experiments are required to clarify the mechanisms for the reduction of locomotor activity.

Toxicity Ibrahim et al. have reported that zerumbone has an LD_{50} of 1.84 g/kg. The dose of zerumbone that produced the strongest reduction of locomotor activity was 4.5 \times 10^{-2} mg/cage. The dose of compound 1 was 1% of the LD_{50} for the mice, and the volume of air that the mice breathed per hour was 1408.7 mL, which was 2.3% of the cage volume. Accordingly, the amount of compounds inhaled by mice in 1 h was estimated to about 1.0 µg. These quantities are likely to be safe, because they were about 0.002% of the LD_{50} of zerumbone.

CONCLUSION

Zerumbone (1) and its derivatives (2–9) showed different effects on locomotor activity, depending on the functional groups at C1, the loss of double bonds in the 11-membered ring, and the cis–trans isomers. The presence of oxygen-containing groups at C1 was important for reducing locomotor activity, whereas the absolute configuration at C2 of THZs 3–5 did not affect the reduction of locomotor activity.

Thus, 1 and 3–9 could be used as functional incenses. Furthermore, small doses were required to achieve a reduction of locomotor activity. These results and the low toxicity of THZs mean that Z. zerumbet should be a useful material for medicines and incenses.

The odors of some compounds tested in the study have been reported by Sawada et al. They reported that THZ is easy to produce industrially, and that synthetic incenses with relaxing properties could be produced without optical resolu-

Fig. 5. Effect of the Equimolar Mixture of Tetrahydrozerumbone 5 by Intrapentoneal Injection

Data are expressed as the mean±S.E.M. for 5 mice. The statistical analysis was performed by Student’s t-test. ***p<0.005 vs. the vehicle group.

REFERENCES

1) Ghosh S, Majumder PB, Sen Mandi S. Species-specific AFLP markers for identification of Zingiber officinale, Z. montanum and Z. zerumbet (Zingiberaceae). Genet. Mol. Res., 10, 218–229 (2011).

2) Perry LM. Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. The MIT Press, Cambridge, MA, p. 444 (1980).

3) Sulaiman MR, Tengku Mohamad AS, Shaik Mossadeq WM, Moin S, Yusof M, Mohktar AF, Zakaria ZA, Israf DA, Lajis N. Antinociceptive activity of the essential oil of Zingiber zerumbet. Planta Med., 76, 107–112 (2010).

4) Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, Ohigashi H. Identification of zerumbone in Zingiber zerumbet Smith as a potent inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced Epstein–Barr virus activation. Biosci. Biotechnol. Biochem., 63, 1811–1812 (1999).

5) Chien TY, Chen LG, Lee CJ, Lee FY, Wang CC. Anti-inflammatory constituents of Zingiber zerumbet. Food Chem., 110, 584–589 (2008).

6) Sawada S, Yokoi T, Kitayama T. Woody fragrance made from a wild ginger: The chemistry of zerumbone. Aroma Research, 3, 34–39 (2002).

7) Couic-Mariner F, Lobstein Annelies. Les huiles essentielles en pratique à l’officine. Actual. Pharm., 52, 31–33 (2013).

8) Kumar V. Characterization of anxiolytic and neuropharmacological activities of silexan. Wien. Med. Wochenschr., 163, 89–94 (2013).

9) Kitayama T, Ohta S, Kawai Y, Nakayama T, Awata M. Synthesis of optically active tetrahydrozerumbone. Tetrahedron Asymmetry, 21, 11–15 (2010).

10) Kitayama T, Saito A, Ohta S. Efficient synthesis of an optically active tetrahydrozerumbone exhibiting a fragrance and the application of zerumbone derivatives with a medium ring structure. Tetrahedron Asymmetry, 23, 1490–1495 (2012).

11) Takemoto H, Ito M, Shiraki T, Yagura T, Honda G. Sedative effects of vapor inhalation of agarwood oil and spikenard extract and identification of their active components. J. Nat. Med., 62, 41–46 (2008).

12) Mayoshi T, Ito M, Kitayama T, Isumori S, Yamasita F. Sedative effects of inhaled benzylacetone and structural features contributing to its activity. Biol. Pharm. Bull., 36, 1474–1481 (2013).

13) McPhail AT, Sim GA. Sesquiterpenoids. Part IV. The stereochemistry of humulene: X-Ray analysis of the humulene-silver nitrate adduct. J. Chem. Soc. B, 1966, 112–120 (1966).

14) Hall SR, Nimigirawath S, Raston CL, Sittartrakul A, Thadantith S, Thirassama N, White AH. Crystal structure of zerumbone ([E,E,E]-2,6,9-trimethylepoxycyclooctene-2,6,10-trien-1-one]. Aust. J. Chem., 34, 2243–2247 (1981).

15) Ito K, Ito M. The sedative effect of inhaled terpinolene in mice and its structure–activity relationships. J. Nat. Med., 67, 833–837 (2013).

16) Vernet-Maury E, Polak EH, Demael A. Structure–activity relationship of stress-inducing odorants in the rat. J. Chem. Ecol., 10, 1007–1018 (1984).

17) Tasaka T, Aoshima H. Effect of monoterpene alcohols on the expression of GABA receptors in the brain of mice. Aroma Research, 11, 345–351 (2010).

18) Fleming MF, Mihic SJ, Harris RA. Goodman & Gilman’s The Pharmacological Basis of THERAPEUTICS, eleventh edition, Section III, Chap. 22 (Brunton LL, Lazo JS, Parker KL, ed.) The McGraw-Hill Companies, Inc., New York, NY, U.S.A., p. 600
19) Niijima A. Physiological background of aromatherapy. Journal of Japanese Society of Aromatherapy, 6, 13–21 (2007).

20) Ibrahim MY, Abdul AB, Ibrahim TAT, Abdelwahab SI, Elhassan MM, Syam MM. Evaluation of acute toxicity and the effect of single injected doses of zerumbone on the kidney and liver functions in Sprague-Dawley rats. Afr. J. Biotechnol., 9, 4442–4450 (2010).

21) Tanaka I. Respiratory tract deposition and clearance of inhaled particles in laboratory animals. Earozoru Kenkyu, 3, 104–110 (1988).

22) Sawada S, Kitayama T, Yokoi K, Kato M. Japan Patent 2002–155001, A (2002).

23) Chane-Ming J, Vera R, Chalchat J. Chemical composition of the essential oil from rhizomes, leaves and flowers of Zingiber zerumbet Smith from reunion island. J. Essent. Oil Res., 15, 202–205 (2003).

24) Kitayama T, Komori T, Nakayama T. Seasonal variation of zerumbone contents in Zingiber zerumbet Smith. Japanese Journal of Pharmacognosy, 61, 86–88 (2007).