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

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Zerumbone 1 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 1 and derivatives 2–9.

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

Zingiber zerumbet Smith is a member of the ginger family (Zingiberaceae) and is native to India. This plant grows widely from India and Southeast Asia to the Hawaiian Islands.[1)] *Z. zerumbet*, which has a flowery scape, is cultivated in Europe and the United States mainly as a garden plant. In contrast, the rhizome of *Z. zerumbet* is used as a traditional folk medicine for pain in Southeast Asia.[2)] Zerumbone **1** is a main component of the essential oil obtained from *Z. zerumbet* rhizome, and has antinociceptive,[3)] antiviral, and anti-inflammatory effects. Furthermore, zerumbone **1** is a valuable natural raw material for medicinal compounds, because of its high reactivity.[4–6)] Terpenoids, in particular monoterpenes and sesquiterpenes, often have characteristic odors, and are used in incense. In European countries, essential oils that contain odorant terpenoids are used in home remedies and medical treatments for conditions such as anxiety.[7,8)] Zerumbone, a highly volatile compound with a distinct odor,[6)] may show biological activity when inhaled. In this study, the reduction of locomotor activity in mice given zerumbone and its derivatives by inhalation was examined. The structure–activity relationship among the compounds was also investigated.

MATERIALS AND METHODS

Materials Compounds **1**–**9** shown in Fig. 1 were prepared as below. Zerumbone **1** 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 H_2 on Pd/C to give racemic THZ **5**. Then, **5** was reduced with $LiAlH_4$ 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 (2*S*)-tetrahydrozerumbyl acetate and (2*R*)-tetrahydrozerumbol in order to resolve the racemic THZ into **3** and **4** by oxidisation following deacylation.[9,10)] Compounds **1**, **3**, **4**, and **7**–**9** were refined to more than 99.9% purity, as evaluated by ^{1}H-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

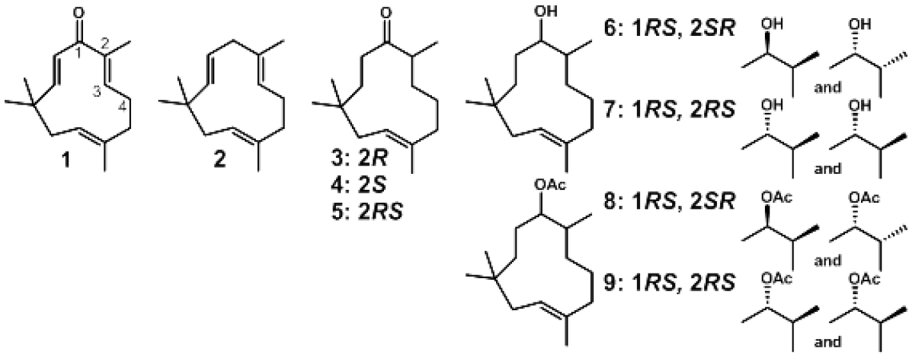

Fig. 1. Structures of Zerumbone and Its Derivatives Examined in This Study

1: zerumbone; **2**: α-humulene; **3**: (2*R*)-tetrahydrozerumbone (*R*-THZ); **4**: (2*S*)-tetrahydrozerumbone (*S*-THZ); **5**: (2*RS*)-tetrahydrozerumbone (*rac*-THZ); **6**: (1*RS*,2*SR*)-*cis*-tetrahydrozerumbol (*rac-cis*-THZol); **7**: (1*RS*,2*RS*)-*trans*-tetrahydrozerumbol (*rac-trans*-THZol); **8**: (1*RS*,2*SR*)-*cis*-tetrahydrozerumbyl acetate (*rac-cis*-THZAc); **9**: (1*RS*,2*RS*)-*trans*-tetrahydrozerumbyl acetate (*rac-trans*-THZAc).

The authors declare no conflict of interest.

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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-

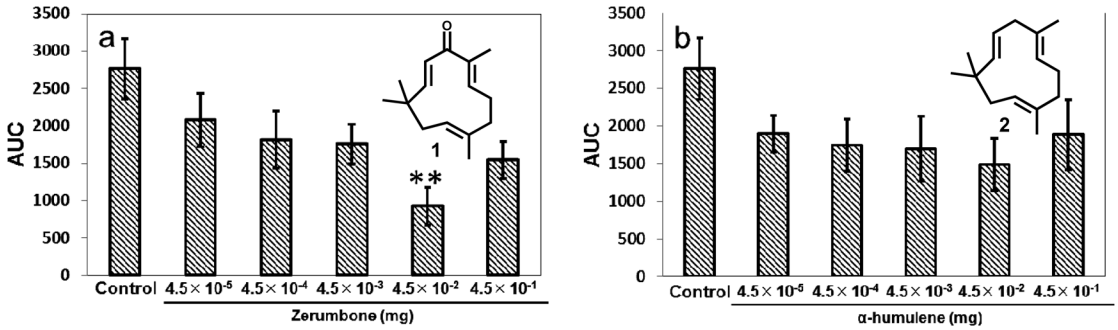

Fig. 2. Spontaneous Motor Activity of Mice Treated with Zerumbone **1** (a) and α-Humulene **2** (b)

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.

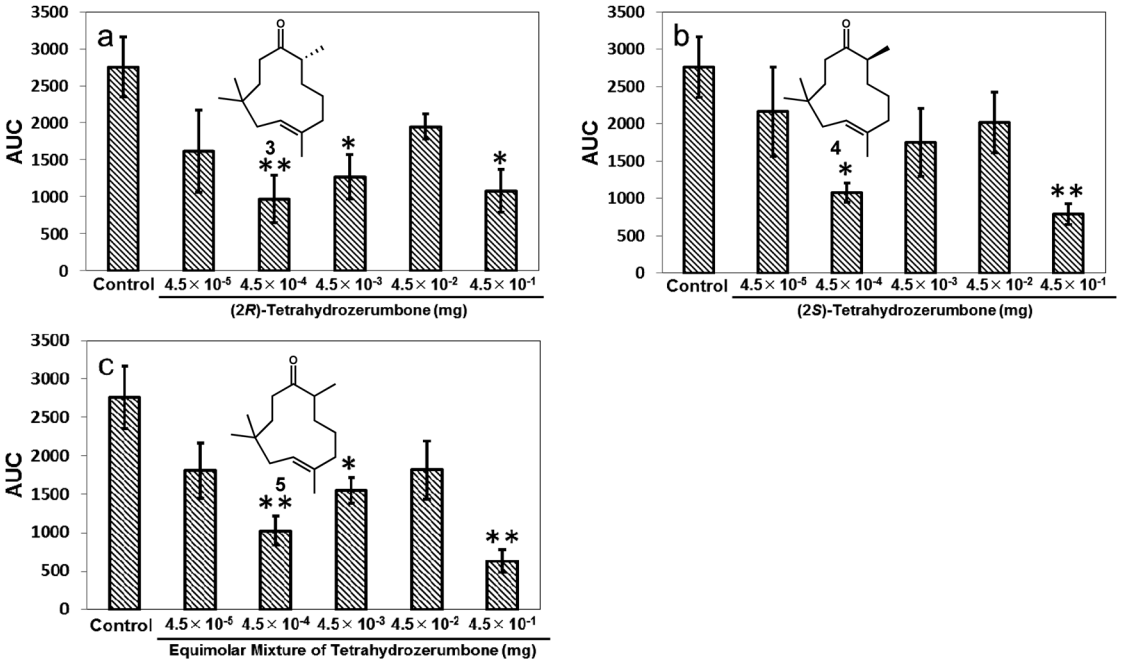

Fig. 3. Total Spontaneous Motor Activity of Mice Treated with (2*R*)-THZ **3** (a), (2*S*)-THZ **4** (b), and (2*RS*)-THZ **5** (c)

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.

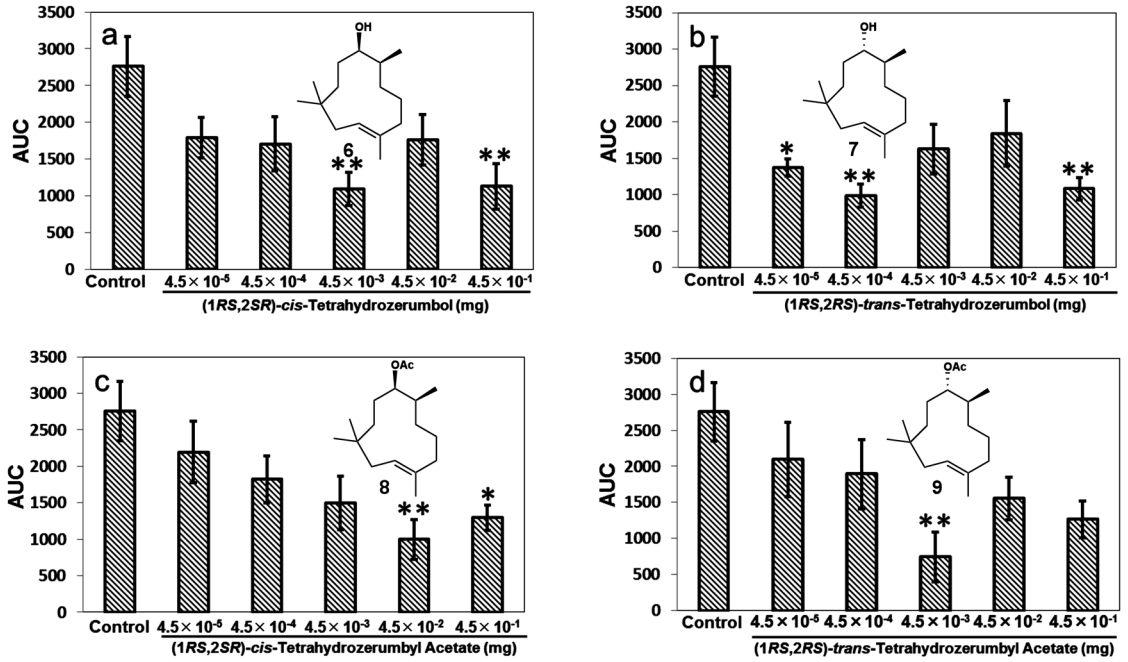

Fig. 4. Total Spontaneous Motor Activity of Mice Treated with *rac-cis*-THZol **6** (a), *rac-trans*-THZol **7** (b), *rac-cis*-THZAc **8** (c), and *rac-trans*-THZAc **9** (d)

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.

ducing locomotor activity.

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^{-4} mg/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*-tetrahydrozerumbol (**6** and **7**) (THZol) and *rac-cis*/*trans*-tetrahydrozerumbyl 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^{-2} mg. 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.[13,14] 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.[15]

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.[16] 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-

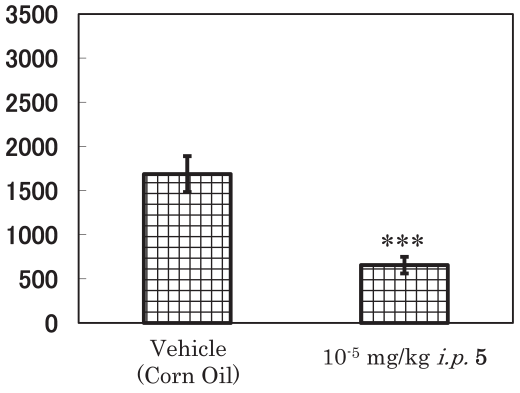

Fig. 5. Effect of the Equimolar Mixture of Tetrahydrozerumbone **5** by Intrapenitoneal Injection

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.

ous sedation mechanisms have been suggested, including the regulation of receptors in the central nervous system[17,18] and in the autonomic nervous system (ANS).[19] 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 soporific 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.[20] The dose of zerumbone that produced the strongest reduction of locomotor activity was 4.5×10^{-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,[21] 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 THZ 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.*[22] They reported that THZ is easy to produce industrially, and that synthetic incenses with relaxing properties could be produced without optical resolution. Compound **1** is contained in rhizomes of *Z. zerumbet*[23] and seasonal changes in the amounts of zerumbone contained in the rhizome have been reported[24]; therefore, cultivating *Z. zerumbet* should provide a good source of raw materials for medicines and incenses.

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