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Isolation of Hepatic Drug Metabolism Inhibitors from the Rhizomes of *Curcuma zedoaria**

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Abstract □ The methanolic extract of the Rhizome of *Curcuma zedoaria* exhibited a significant prolongation of hexobarbital (HB)-induced hypnosis. Through liquid chromatography of an ether soluble fraction, monitoring by bioassay, three sesquiterpenes, germacrone(A), curzerenone(B) and germacrone epoxide(C) were isolated as active constituents. A single treatment (100-200mg/kg, i.p.) of each compound showed not only a significant prolongation of HB-induced sleeping time but also a significant inhibition of aminopyrine N-demethylase activity in mice, and further exhibited a typical type I binding spectra with oxidized rat hepatic cytochrome P-450 induced by phenobarbital. All of the compounds provoked a sleep episode at a subhypnotic dose of HB, implying that they possess CNS depressant properties.

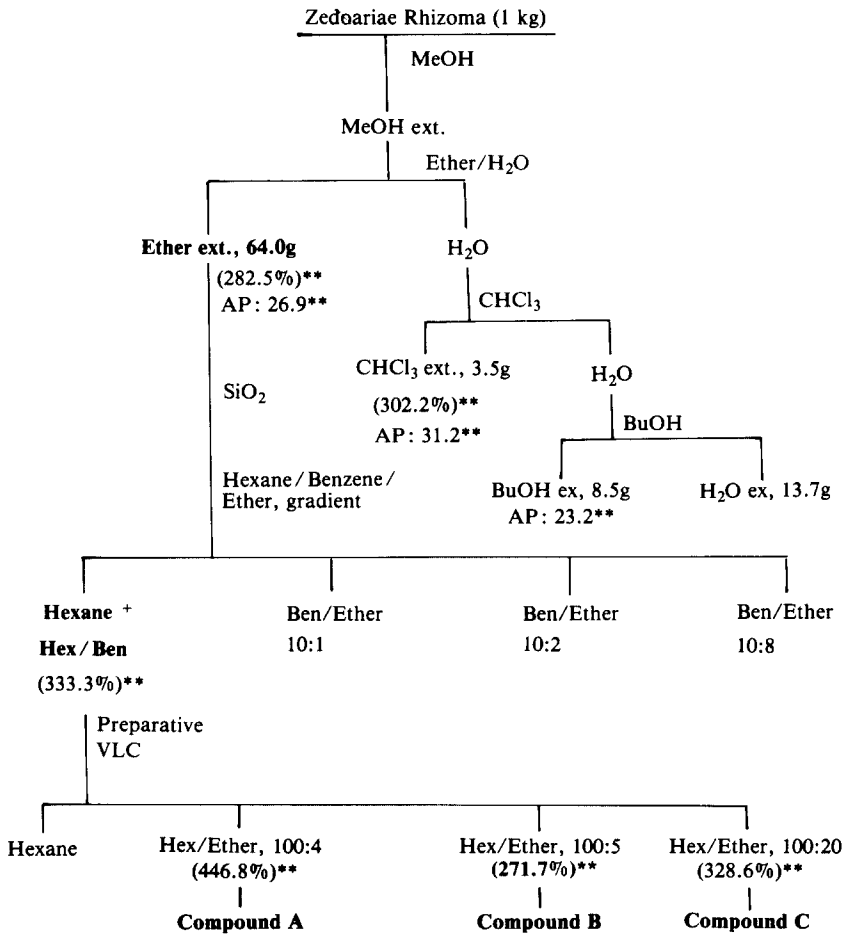
Keywords □ *Curcuma zedoaria,* Zingiberaceae, sesquiterpene, germacrone, curzerenone, germacrone epoxide, drug-metabolizing enzyme inhibitors, sedative activity.

In the course of a search for herbal medicines with hepatic drug-metabolizing enzyme (DME) modifying activities, the 95% methanol extract of the rhizome of *Curcuma zedoaria* being used for aromatic stomachic in traditional medicine was revealed to cause a marked prolongation in hexobarbital (HB)-induced sleeping time as well as potentiation of strychnine (ST)-induced mortality when administered i.p., suggesting the presence of hepatic DME inhibitors[2]. In this paper, we report the separation and identification of active principles from the drug along with their effect on HB-induced sleeping time and on aminopyrine N-demethylase activity and the interaction with hepatic microsomal cytochrome P-450 *in vitro*.

The methanol extract was suspended in water and extracted with ether, chloroform and then n-butanol as shown in Scheme 1, to afford ether, chloroform, butanol and water soluble fractions. The effect of each fraction thus obtained on the barbiturate hypnosis and hepatic microsomal aminopyrine N-demethylase activity were examined and the ether, chloroform and butanol fractions were found to be active (Scheme 1). In order to isolate the active principles from the ether fraction which was a main fraction (64g, yield), column chromatography on silica gel with a solvent system of hexane, benzene, ether (gradient) was carried out with monitoring by TLC to give four subfractions. Among them, hexane together with hexane-benzene (10:1) subfraction gave the most potent activity. Subsequent preparative VLC of the subfraction with solvent system of hexane:ether (gradient) gave compounds A to C which were identified by their physicochemical and spectral data as germacrone[3] (A), curzerenone[4] (B), germacrone-4,5-epoxide[3,5] (C), respectively. All of three compounds at a dose of 100 mg, i.p. caused a marked potentiation of sleeping time, which strongly reflected that they possess CNS depressant activity[6] and/or hepatic microsomal DME inhibitory activity (Table I).

Table II shows the results of the direct effects on hepatic DME activities in mice sacrificed 30 min after a single i.p. administration of the compounds (200 mg/kg). The activity of aminopyrine N-demethylase were markedly decreased by 30-58% after treatment of the compounds, among which the most potent inhibitory activity was observed in animals treated with compound C. And furthermore, these compounds, *in vitro,* showed a distinct typical type I difference spectrum in rat liver microsomes (absorption max. at 380-388; trough at

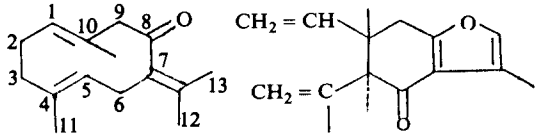
*Part 13 in the series "Studies on crude drugs acting on drug-metabolizing enzyme". For part 12 see ref. 1



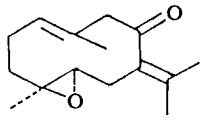
Scheme 1. Fractionation and isolation of the active principles from Zedoariae Rhizoma and their activities.

*Figures in parentheses, sleeping time prolongation % of control; AP, aminopyrine N-demethylase inhibition %.

Control sleeping time, 27 min at HB-Na, 60 mg/kg, i.p.; Sample dose, 200 mg/kg i.p.; **$p<0.01$.



germacrone (A) curzerenone (B)



germacrone-4,5-epoxide (C)

Fig. 1. Structures of germacrone (A), curzerenone (B) and germacrone-4,5-epoxide (C).

Table 1. Effect of the constituents of Zedoariae Rhizoma on hexobarbital-induced sleeping time

Treatment	Dose (mg/kg, i.p.)	Hexobarbital sleeping time (min)[a]	% of control
Control	0.5% CMC	24.8 ± 1.7	—
Compound A	100	93.3 ± 2.0*	376.2
	200	135.3 ± 11.7*	545.6
Compound B	100	94.3 ± 5.7*	380.2
Compound C	100	105.0 ± 19.1*	423.4

Mice were treated 30 min before injection of hexobarbital-Na (50 mg/kg, i.p.).

Significantly different from control: *$p<0.001$.

a) Mean ± S.E. of 5 animals.

Table II. Effect of the constituents of Zedoariae Rhizoma on aminopyrine N-demethylase activities in mice

Treatment	Dose (mg/kg, i.p.)	Aminopyrine N-demethylase (μmoles/30 min/g prot.)[a]
Control	0.5% CMC	19.83 ± 1.34
Compound A	200	13.98 ± 1.08* (70.5)
Compound B	200	12.07 ± 0.72** (60.9)
Compound C	200	8.28 ± 2.67* (41.8)

Mice were treated 30 min before estimation of enzyme activity.
Significantly different from the control: * $p<0.01$, ** $p<0.001$. Figures in parentheses indicate % of control.
a) Mean ± S.E. of 3 separate determinations.

420-425 mm) induced with phenobarbital as shown in Fig. 2. This result strongly indicates that the sesquiterpenes interact with cytochrome P-450 to inhibit drug metabolism[7].

All compounds, on the other hand, caused a distinct sleeping episode and furthermore elicited remarkable long duration of sleeping time even at a subhypnotic dose at HB (40 mg/kg), even though the control mice showed a severe ataxia but none of animals fell asleep. This result gives an evidence that they also possess a distinct sedative activity[6]. The contents of the sesquiterpenes in this plant were found to be relatively high that their inhibitory effect on DME and/or sedative action may contribute to the action of Zedoariae Rhizoma as a crude drug. Further study on the mode of their biological as well as biochemical activities is in progress.

EXPERIMENTAL

Melting points were determined on a Mitamura-Riken (No. 4204) melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were taken with a varian FT-80A NMR spectrometer. EI-MS spectra were determined on a Hewlett Packard (5985) B) GS/MS spectrometer. IR spectra were taken with Perkin-Elmer 283 A IR spectrophotometer. TLC was carried out on precoated Kiselger 60 F254 Plates (Merck).

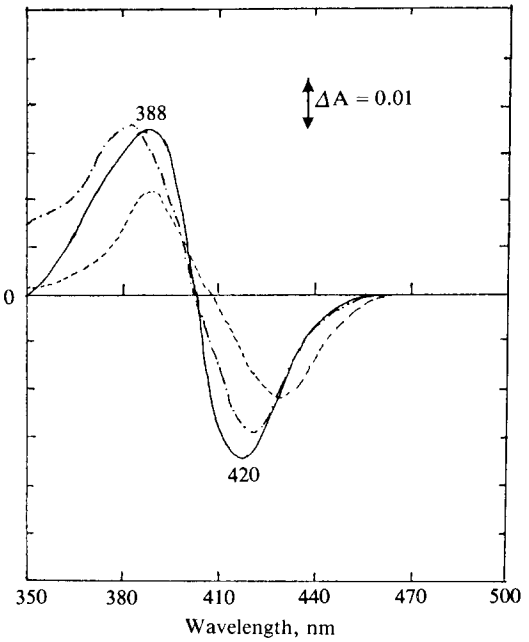


Fig. 2. Type I spectral change on binding of compound A, B and C to rat liver microsomes.
Microsomes obtained from rat liver induced by phenobarbital (80 mg/kg, i.p. for 3 days) were suspended in 0.01 M K^+/Na^+ phosphate buffer (pH 7.4) at a concentration of 3 mg protein/ml. The concentrations of Comp. A. (—), Comp. B. (-·-·-·) and Comp. C. (----) were 0.3 mM.

Plant material

The rhizomes of *Curcuma zedoaria* cultivated in Korea were purchased at a local market and were botanically identified. Voucher specimens are deposited in this Institute.

Isolation of compounds

The dried rhizomes (1kg) were coarsely powdered, extracted three times with hot 95% methanol and the combined methanol solution was concentrated under reduced pressure. The methanol extract was suspended in water and extracted with ether to afford ether soluble fraction (64.0g). Column chromatography of the ether fraction as shown in Scheme 1 gave compounds A to C.

Compound A (germacrone)

Colorless needles, mp 50-52°C; MS m/z (rel. int.): 218 [M^+, 12.7], 203 [M^+-CH_3, 6.8], 185 (5.8), 175 (20.2), 167 (19.4), 149 (15.2), 148 (12.3), 135 (70.9), 121 (39.2), 107 (100), 91 (50.7); UV (me-

Table III. Effect of the constituents of Zedoariae Rhizoma on subhypnotic dose of hexobarbital in mice

Treatment	Dose (mg/kg, i.p.)	Duration of sleep (min) (mean ± S.E.)	No. of animals slept/ No. of animals dosed
Control	0.5% CMC	0	(0/3)
Compound A	100	14.0	(1/3)
	200	22.7 ± 3.7	(3/3)
Compound B	100	8.7 ± 2.9	(3/3)
	200	19.7 ± 2.7	(3/3)
Compound C	100	4.5	(2/3)
	200	41.7 ± 7.3	(3/3)

Mice were injected with 40 mg/kg of hexobarbital-Na 30 min post sample treatment.

thanol) λ_{max} nm(log ε): 217.5 (4.03); IR ν_{max} (KBr) cm^{-1}: 1658 (α,β-unsaturated ketone), 1440, 1380, 1283, 1250, 1175, 1000, 860; ^{1}H-NMR ($CDCl_3$, 80 MHz) δ: 1.43 (3H, s, H-14), 1.61 (3H, s, H-15), 1.71 (3H, s, H-12), 1.76 (3H, s, H-13), 2.0-2.4 (4H, m, H-2, H-3), 2.86 (1H, dd, J = 11.3, 13.5, H-6α), 2.95 (1H, d, J = 10.5, H-9α), 2.97 (1H, dd, J = 4.0 and 135 Hz, H-6β), 3.41 (1H, d, J = 10.5, H-9β), 4.71 (1H, dd, J = 4.0 and 11.3 Hz, H-5), 4.99 (1H, dd, J = 3.5 and 12.2 Hz, H-1); ^{13}C-NMR ($CDCl_3$) δ: 15.5 (C-14), 16.7 (C-15), 19.8 (C-12), 22.2 (C-13), 24.0 (C-2), 28.9 (C-6), 37.9 (C-3), 55.9 (C-9), 125.5 (C-5), 126.8 (C-4), 129.0 (C-7), 132.2 (C-1), 134.9 (C-10), 137 (C-11), 207.2 (C-8).

Compound B (Curzerenone)

Colorless oil, $[\alpha]_D^{20} = 0°$; Lieberman-Burchard, blue; Erlich, yellowish-red; MS *m/z* (rel. int.): 230 [M^+, 34.1], 215 [M^+-CH_3, 13.4], 122 [M^+-C_7H_8O, 100], 107 [215-C_7H_8O, 23.9], 94 [122-CO, 67.9]; UV (MeOH) λ_{max} nm(log ε): 215 (4.08), 270 (3.44); IR ν_{max} (KBr) cm^{-1}: 3095 (=CH_2), 2975, 2930, 2870, 1670, 1675 (C=O), 1640 (C=C), 1610, 1570 (C=C, furan), 1450, 1430, 1385, 1375, 1255, 1240, 1070 (C-O-C, furan), 995, 915 (-CH=CH_2, monosubstitute), 895(1,1-disubstitute); ^{1}H-NMR($CDCl_3$, 80 MHz) δ: 1.19 (3H, s, H-15), 1.82 (3H, s, H-14), 2.16 (3H, s, H-13), 2.73 (1H, d, J = 17 Hz, H-9β), 2.95 (1H, d, J = 17 Hz, H-9α), 3.01 (1H, s, H-5), 4.75-4.99 (4H, m, H-2 and H-3), 5.81 (1H, dd, J = 10.5 Hz and 18 Hz, H-1), 7.09 (1H, br.s, H-12).

Compound C (Germacrone-4,5-epoxide)

Colorless needles, mp 58-60°; MS *m/z* (rel. int.): 234 [M^+, 4.0], 219 [M^+-CH_3, 3.2], 191 (6.4), 167 (70.7), 149 (13.3), 121 (54.5), 107 (33.5), 68 (100); UV λ_{max} (methanol), nm(log ε): 214 (4.11), 240 (3.59); IR ν_{max} (KBr) cm^{-1}: 1705, 1660, 1620, 1440, 1230; ^{1}H-NMR ($CDCl_3$, 80 MHz) δ: 1.03 (3H, s, H-14), 1.20 (2H, m, H-3), 1.72 (3H, s, H-15), 1.81 (6H, s, H-12 and 13), 2.0-2.5 (4H, m, H-2 and 6), 3.03 (1H, d, J = 10.3 Hz, H-9α), 3.43 (1H, d, J = 10.3 Hz, H-9β), 3.45 (1H, dd, J = 8 and 14 Hz, H-5), 5.20 (1H, m, H-1); ^{13}C-NMR ($CDCl_3$) δ: 15.9 (C-14), 17.0 (C-15), 20.4 (C-12), 22.8 (C-13), 24.6 (C-2), 29.7 (C-6), 37.7 (C-3), 55.5 (C-9), 60.7 (C-4), 64.5 (C-5), 126.8 (C-7), 129.7 (C-1), 133.6 (C-10), 134.3 (C-11), 204.6 (C-8).

Bioassay

Measurement of HB-induced sleeping time, aminopyrine N-demethylase activity and preparation of Cytochrome P-450 were carried out as previously described[8].

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