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Platelet Aggregation Potentiators from Cho-Rei

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Four components were isolated from Cho-Rei (*Polyporus umbellatus*), one of the traditional Chinese medicines, and three of them potentiated the rabbit platelet aggregation response induced by collagen and/or adenosine-5′-diphosphate *in vitro*.

The four components were identified as ergosta-7,22-dien-3-one, ergosta-7,22-dien-3-ol, 5α,8α-epidioxy ergosta-6,22-dien-3-ol and the already known component, ergosterol. When the C_3-hydroxy group of the active components was replaced by a ketone group, the enhancing activity disappeared, and an inhibitory tendency was observed. This suggests a relationship between bioactivity and the C_3-hydroxy group (or polarity) of these components.

Keywords——platelet aggregation potentiator; Chinese medicine; ergosterol analogue; Cho-Rei; *Polyporus umbellatus*; thrombosis

During the screening of Chinese medicines aimed at finding new hemostatic drugs, the extract of Cho-Rei (*Polyporus umbellatus*) was found to be able to potentiate the platelet aggregation induced by collagen. In this experiment, we isolated four components from Cho-Rei benzene extract and identified them as ergosterol analogues on the basis of physicochemical analysis. Three of them were found to enhance the rabbit platelet aggregation induced by collagen and/or adenosine-5′-diphosphate (ADP) *in vitro*. Although there have been several reports dealing with the enhancing effect of cholesterol, whose chemical structure

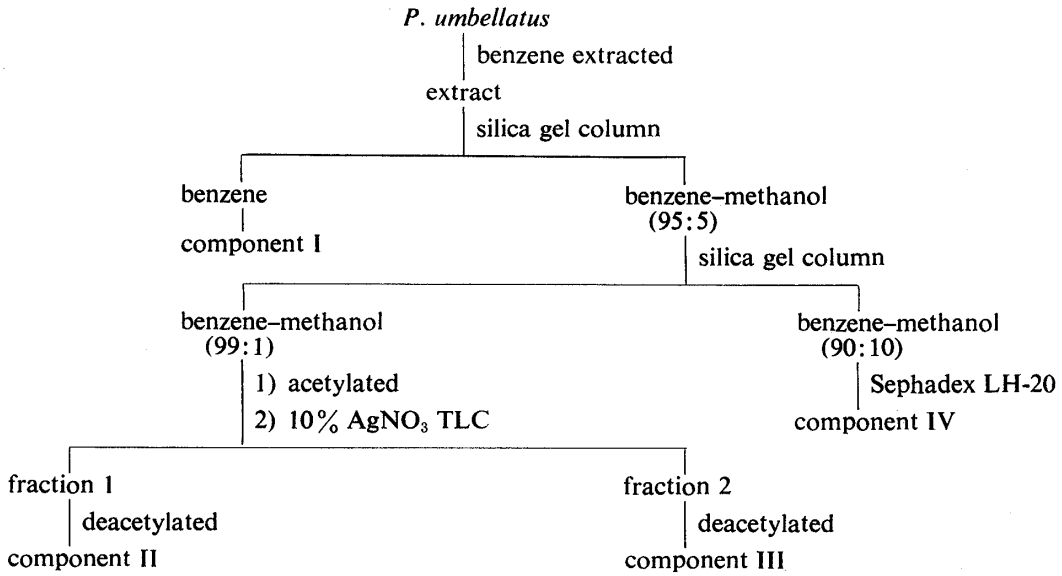

Fig. 1. Extraction and Isolation of Platelet Aggregation Potentiators from Cho-Rei

is very similar to that of ergosterol, on platelet aggregation response,[1] this is the first report dealing with the effect of ergosterol and its analogues on platelet aggregation. Furthermore, the effective concentration of these components was about 0.8 μg/ml, at which concentration, cholesterol showed no activity.

Isolation and Characterization of the Platelet Aggregation-Potentiating Substances from Cho-Rei

Cho-Rei was extracted with various solvents, and the extracts were tested for their effect on the rabbit platelet aggregation induced by collagen (data not shown). Active components existed mostly in the benzene extract, and they were identified as ergosterol analogues on the basis of the positive Liebermann–Burchardt reaction and a comparison of the infrared absorption (IR) spectrum, mass spectrum (MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectrum with those of standard ergosterol. Ergosterol is known to break down easily to vitamin D, but these analogues were more stable than ergosterol both chemically and in terms of bioactivity.

The fractionation procedure for the Cho-Rei benzene extract is shown in Fig. 1. Each component isolated was recrystallized from acetone (component I) or acetone–methanol (component II—IV) three times.

Structure Identification of the Platelet Aggregation-Potentiating Substances from Cho-Rei

Component I, mp 185.5—187 °C, was obtained as colorless flats. MS determination revealed the molecular ion fragment (M^+) at *m/z*: 396 ($C_{28}H_{44}O$). The absorption at 1715 cm^{-1} in the IR spectrum suggested the existence of a C_3-ketone group in the molecule.[2] The absorptions at 985 and 970 cm^{-1} might arise from the $^{H}\!\!>\!C=C\!<_{H}$ structure, and those at 845 and 800 cm^{-1} from the $>C=C\!<_{H}$ structure.[3] Two of the three olefinic protons (5.22 ppm, 2H) were considered to be those of the Δ^{22} double bond from a comparison with the ^{1}H-NMR spectrum of standard ergosterol, while the MS fragment at *m/z*: 271 (M^+ − side chain) indicated the other one to be located in the nucleus. On the basis of the above analysis and a comparison with literature data,[4] component I was identified as ergosta-7,22-dien-3-one.

Component II, colorless needles (mp 167—170.5 °C), was determined to have the molecular weight of 398 ($C_{28}H_{46}O$) from the MS. The IR absorption at 3400 cm^{-1} indicated the existence of a hydroxy group, and it was considered to be at C_3 based on the fact that component II reacted with equimolar acetyl reagent and gave a sharp IR absorption[2] at 1740 cm^{-1}. The IR absorptions at 965 and 935 cm^{-1} indicated the existence of $^{H}\!\!>\!C=C\!<_{H}$, and those at 830 and 800 cm^{-1} indicated the existence of $>C=C\!<_{H}$ structure. Two of the three olefinic protons were considered to be due to trans Δ^{22} from a comparison with the spectrum of standard ergosterol, and *m/z*: 161 (M^+ − side chain − ring A − CH_2) was observed in the MS. Thus, component II was identified as ergosta-7,22-dien-3-ol based on the above observations and a comparison with literature data.[5]

Component III, colorless flats (mp 150—152 °C), had a molecular weight of 396 ($C_{28}H_{44}O$) on the basis of MS determination. Component III was identified as ergosterol, a known component of Cho-Rei, based on a comparison of the melting point, ultraviolet (UV), IR, MS and ^{1}H-NMR spectra with those of standard ergosterol.

Component IV, mp 178—181 °C, was obtained as colorless needles, and MS determination revealed the molecular ion at *m/z*: 428 ($C_{28}H_{44}O_3$). A hydroxy group absorption was observed in the IR spectrum (a broad absorption at 3450 cm^{-1}) and in the ^{1}H-NMR spectrum (a multiplet peak at 4.00 ppm, 1H). In the MS, an evident peak in *m/z*: 396 was observed, due to the loss of one molecule of O_2 from the compound. Fragments below 396 were very similar to those of ergosterol. These findings indicated that component IV was ergosterol peroxide. The peak at *m/z*: 396 in MS presumably arose from a retro-Diels–Alder fragmentation.[6] During the MS determination, component IV lost the peroxy bond, and then

the 5,8-epidioxy sterol bearing a Δ^6 double bond in the nucleus fragmented to a diene structure, and gave the MS fragments characteristic of a $\Delta^{5,7}$ sterol.[7] This was confirmed by ^{1}H-NMR analysis. The C_6-H and C_7-H of component IV were found to exist as a doublet of doublets around 6.27 and 6.54 ppm. Another double bond was present, and it was considered to be Δ^{22}, based on the ^{1}H-NMR spectrum (5.21 ppm, m, 2H). Based on the above observations and a comparison with literature data,[8] component IV was identified as 5α,8α-epidioxy ergosta-6,22-dien-3-ol.

Biological Activity of the Four Components Isolated from Cho-Rei

Figure 2 shows the results of a representative experiment on the effect of ergosterol and Cho-Rei steroids on rabbit platelet aggregation induced by collagen *in vitro*. All the steroids showed stimulating activity except for component I, which had a ketone group in the C_3 position instead of a hydroxy group. The most active component was IV, the ergosterol peroxide. The experiment was repeated 5 times, but the aggregation profiles could not be compiled into a single curve. Nevertheless, the results of the different experiments were essentially identical with that described.

Table I shows the effect of Cho-Rei steroids on the platelet aggregation induced by ADP, arachidonic acid and calcium ionophore A 23187. Components II and III, which had the C_3-hydroxy group, showed a clear enhancing activity on the ADP-induced platelet aggregation ($p<0.01$), while the ketone derivative, component I, showed an inhibitory effect. No distinct effect of any of the steroids isolated from Cho-Rei was observed on arachidonic acid or calcium ionophore-induced platelet aggregation.

Each test component was dissolved in ethanol, and diluted to 1.0% with saline before being added to the platelet suspension. It was confirmed that 1.0% ethanol in saline had no effect on the platelet aggregation, and it was used as the control.

There have been some reports dealing with the enhancing effect of cholesterol on epinephrine-, ADP- and collagen-induced platelet aggregation. It is reasonable to consider

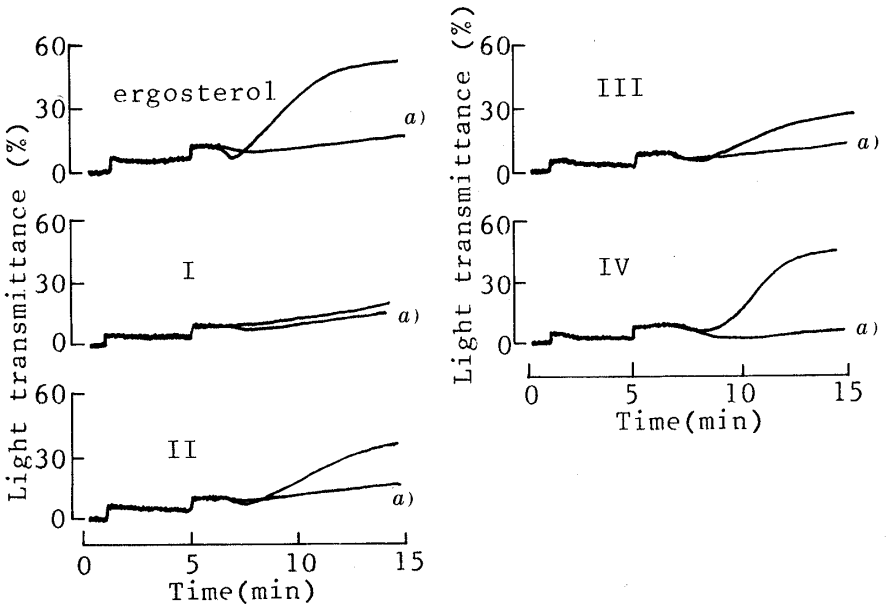

Fig. 2. Effects of Ergosterol and the Components Isolated from Cho-Rei on the Rabbit Platelet Aggregation Induced by Collagen

An aliquot of PRP (200 μl) was preincubated at 37 °C, and 20 μl of sample solution (9.6 μg/ml) was added at 1 min, then 20 μl of collagen solution (100 μg/ml) was added at 5 min. Platelet aggregation was recorded as the increase in light transmittance. The curve marked by *a*) in each column is that of the control experiment.

TABLE I. Effect of the Cho-Rei Steroids on the Rabbit Platelet Aggregation Induced by ADP, Arachidonic Acid or Calcium Ionophore A 23187

	Ergosterol	I	II	III	IV
ADP	1.50±0.07 ($p<0.01$)	0.82±0.05 ($p<0.05$)	1.65±0.15 ($p<0.01$)	1.43±0.09 ($p<0.01$)	1.08±0.04
Arachidonic acid	1.12±0.05	1.12±0.08	1.00±0.01	0.96±0.01	1.00±0.02
Calcium ionophore A 23187	1.06±0.04	1.00±0.04	1.00±0.06	1.00±0.05	1.08±0.10

An ethanol solution of each steroid was diluted 100-fold with saline, and 20 μl of each solution was added to 200 μl of PRP to make 80 μg/dl final concentration. Platelet aggregation was performed as described in Experimental. The area under the platelet aggregation curve (AUC) for 10 min after addition of each inducer was calculated. The effect of each steroid on platelet aggregation was expressed as the ratio of AUC with the sample to that of the control. Data are means±S.E. of 5 experiments. p values were obtained by using Student's t-test.

that an increase of cholesterol can perturb the platelet membrane structure, affect membrane fluidity, and thus influence platelet response.[9] Components II and III, ergosterol analogues isolated from Cho-Rei, showed behavior similar to that of cholesterol in enhancing the platelet aggregation induced by collagen and ADP. It is possible that these components acted on platelets through a similar mechanism. Like cholesterol, these components were considered to affect the platelet membrane primarily, as judged from the fact that activity was only observed in platelet aggregation induced by collagen and/or ADP, which combine with cell surface receptors, and not in that induced by arachidonic acid or calcium ionophore A 23187, whose actions are thought to be independent of membrane receptors. However, ergosterol analogues influenced platelet response at a much lower concentration as compared to the level of cholesterol in platelet-rich plasma (PRP). This suggests that a complex mechanism is involved. Moreover, the observation that different effects were observed between component I and the others could not be explained only in terms of their influence on platelet membrane fluidity.

It is interesting that enhancing activity on platelet aggregation induced by collagen and ADP was observed in components which had a C_3-hydroxy group, while component I, in which the C_3 position was replaced by a ketone group, showed an inhibitory effect. This suggests a relationship between the C_3-hydroxy group and/or the polarity of these components, and the platelet aggregation effect.

The result that Cho-Rei benzene extract potentiated the platelet aggregation induced by collagen seems to provide a partial justification for the use of Cho-Rei in traditional Chinese medicine as an auxiliary drug to cure hematuria.

Experimental

The melting points were determined on a Yanagimoto micromelting point apparatus and were uncorrected. The following instruments were used: optical rotation, Jasco DIP-140; UV spectra, Hitachi 200-20; IR spectra, Hitachi 260-50; MS, JEOL JMS-D 200; ^{1}H-NMR spectra, Varian XL-200 with tetramethylsilane ($\delta=0$) as an internal reference (s, singlet; d, doublet; m, multiplet; br, broad). Silica gel CHR-63 (Nakarai), Wakogel S-I (Wako) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography. Kieselgel 60 F_{254} plates (Merck) and silica gel plates containing 10% $AgNO_3$ were used for thin-layer chromatography (TLC) and preparative TLC, respectively.

Platelet aggregation measurement was carried out with NKK Hematracer I. Aggregation agents used were: collagen (Collagen reagent "HORM"), ADP (Sigma), arachidonic acid (Sigma) and calcium ionophore A 23187 (Calbiochem) at final concentrations of 5—20 μg/ml, 0.02—1 $\times 10^{-4}$ M, 3.5 $\times 10^{-4}$ M and 3—6 $\times 10^{-5}$ M, respectively.

Bioassay——Platelets were obtained as the PRP which was prepared by centrifugation of fresh rabbit blood

(1000 × *g* for 10 min) with 1/10 volume of 3.8% sodium citrate aq. Platelet density was adjusted to 3.0×10^5 per μl with platelet-poor plasma, which was obtained by centrifugation of the same blood at 3000 × *g* for 10 min. After a 4 min incubation of PRP (200 μl) with a test sample (20 μl) at 37 °C, 20 μl of inducer was added and the aggregation profile was recorded in terms of the change of PRP light transmittance with constant stirring.

Isolation——Dried Cho-Rei (960 g) was mechanically crushed and extracted with benzene at room temperature, and the extract was subjected to column chromatography (Silica gel CHR-63), eluted with benzene and benzene–methanol solution. The eluate was monitored by UV absorption measurement at 280 nm, and also checked by TLC. Component I was obtained from the initial benzene eluate. The benzene–methanol (95 : 5) eluate was further applied to a Wakogel S-I column, and the benzene–methanol (99 : 1) eluate was separated according to the method developed by Yokokawa *et al.*[10] The mixture was acetylated with acetic anhydride–pyridine (1 : 1) for 8 h at room temperature. Products were applied to a preparative TLC silica gel plate containing 10% $AgNO_3$ and developed with benzene. The plate was sprayed with 0.05% Rhodamine B ethanol solution, and each zone which was visible under illumination at 360 nm was collected by elution with benzene. The products were deacetylated with 1.0% NaOH ethanol solution for 2 h and components II and III were obtained. The benzene–methanol (90 : 10) eluate of the Wakogel S-I column was rechromatographed on a Sephadex LH-20 column, and component IV was obtained in the ethanol eluate.

The yields of the components from 960 g of Cho-Rei were: component I, 70 mg; II, 125 mg; III, 160 mg; IV, 102 mg.

Ergosta-7,22-dien-3-one (Component I)——mp 185.5—187 °C. $[\alpha]_D^{23}$: −5.8° (*c* = 0.20, chloroform). IR ν_{max}^{KBr} cm^{-1}: 1715 (C=O), 1650 (C=C), 985, 970, 845, 800. MS *m/z*: 396 (M^+, $C_{28}H_{44}O$), 271 (M^+ − side chain). ^{1}H-NMR (in $CDCl_3$) δ ppm: 0.59 (3H, s), 0.83 (3H, d, *J* = 7 Hz), 0.85 (3H, d, *J* = 7 Hz), 0.92 (3H, d, *J* = 7 Hz), 1.02 (3H, s), 1.03 (3H, d, *J* = 6.5 Hz), 5.22 (3H, m).

Ergosta-7,22-dien-3-ol (Component II)——mp 167—170.5 °C. $[\alpha]_D^{23}$: −25.4° (*c* = 0.20, chloroform). IR ν_{max}^{KBr} cm^{-1}: 3400 (O–H), 1650 (C=C), 965, 935, 830, 800. MS *m/z*: 398 (M^+, $C_{28}H_{46}O$), 365 ($M^+ - H_2O - CH_3$), 273 (M^+ − side chain), 161 (M^+ − side chain − $C_7H_{12}O$). ^{1}H-NMR (in $CDCl_3$) δ ppm: 0.55 (3H, s), 0.81 (3H, s), 0.82 (3H, d, *J* = 7 Hz), 0.84 (3H, d, *J* = 7 Hz), 0.91 (3H, d, *J* = 7 Hz), 1.02 (3H, d, *J* = 6.5 Hz), 4.71 (1H, br), 5.22 (3H, m).

Ergosta-5,7,22-trien-3-ol (Component III)——mp 150—152 °C. $[\alpha]_D^{23}$: −128° (*c* = 0.20, chloroform). UV: $\lambda_{max}^{ethanol}$ nm (ε): 272 (10800), 282 (11300); $\lambda_{shoulder}^{ethanol}$ nm (ε): 294 (5800). IR ν_{max}^{KBr} cm^{-1}: 3410 (O–H), 1655, 1610 (C=C), 985, 970, 835, 805. MS *m/z*: (M^+, $C_{28}H_{44}O$), 363 ($M^+ - H_2O - CH_3$), 271 (M^+ − side chain). ^{1}H-NMR (in $CDCl_3$) δ ppm: 0.62 (3H, s), 0.82 (3H, d, *J* = 7 Hz), 0.83 (3H, d, *J* = 7 Hz), 0.91 (3H, d, *J* = 7 Hz), 0.95 (3H, s), 1.03 (3H, d, *J* = 6.5 Hz), 4.72 (1H, m), 5.21 (2H, m), 5.40 (1H, m), 5.58 (1H, m).

5α,8α-Epidioxyergosta-6,22-dien-3-ol (Component IV)——mp 178—181 °C. $[\alpha]_D^{23}$: −37° (*c* = 0.20, chloroform). IR ν_{max}^{KBr} cm^{-1}: 3450 (O–H), 1625 (C=C), 970, 940. MS *m/z*: 428 (M^+, $C_{28}H_{44}O_3$), 396 ($M^+ - O_2$), 363 ($M^+ - O_2 - H_2O - CH_3$), 271 ($M^+ - O_2$ − side chain). ^{1}H-NMR (in $CDCl_3$) δ ppm: 0.82 (3H, s), 0.82 (3H, d, *J* = 6.5 Hz), 0.84 (3H, d, *J* = 6.5 Hz), 0.89 (3H, s), 0.92 (3H, d, *J* = 7 Hz), 1.00 (3H, d, *J* = 6.5 Hz), 4.00 (1H, m), 5.21 (2H, m), 6.27 (1H, d, *J* = 8.5 Hz), 6.54 (1H, d, *J* = 8.5 Hz).

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