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New Platelet Aggregation Inhibitors from Tan-Shen; Radix of *Salvia miltiorrhiza* Bunge

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A potent inhibitor (Ro 09-0680) of rabbit platelet aggregation induced by collagen was isolated from a Chinese medicine, Tan-Shen, (radix of *Salvia miltiorrhiza*) and identified as a novel component, 2-isopropyl-8-methylphenanthrene-3,4-dione (I). IC_{50} of I in the previously established assay was 6.6×10^{-6} M, which is 30 times more potent than papaverine. Related diketophenanthrene derivatives such as tanshinone I, tanshinone II and cryptotanshinone were also found to have the inhibitory activity, though to lesser extents.

Keywords——platelet aggregation; inhibitor; *Salvia miltiorrhiza*; tanshinone; diketophenanthrene; Ro 09-0680.

Introduction

In the previous paper,[1] we reported that several *in vitro* biochemical systems including collagen induced platelet aggregation (CPA) and could be applicable to microbial culture broths and plant extracts as a preliminary screening probe for anti-inflammatory and cardiovascular agents.

Recently the extracts of *Artemisia capillaris*,[2] *Paeonia albiflora var trichocarpa*, *Perillae Herba*,[3] and of *Tetrapanax papyrifera*,[4] were reported to show *in vivo* anti-inflammatory activity. On the other hand, cyclic phosphodiesterase inhibitory activities were found in many extracts from Chinese medicine such as *Anemarrhena asphodeloides*, and *Polygala*,[5] while flavonoids from *Scutellaria baicalensis* and sesquiterpenes from *Cyperi Rhizoma* were reported to be prostaglandin synthetase inhibitors.[6] These findings suggest that Chinese medicines might be good sources for platelet anti-aggregant screening aimed at identifying antiinflammatory or cardiovascular agents.[1]

During the course of such screening, we have found that the methanol extract of a Chinese medicine, Tan-Shen (the radix of *Salvia miltiorrhiza*) exhibited a high inhibitory activity against rabbit CPA. Isolation and identification study revealed that the extract contained several active diketophenanthrene derivatives, and the most active principle among them has been found to be novel. Details of this work are presented here.

Results and Discussion

Isolation and Characterization of the Platelet Aggregation Inhibitors from Tan-Shen (*Salvia miltiorrhiza*)

The methanol extract of Tan-Shen showed potent inhibitory activity in rabbit CPA (IC_{50} was 150—450 μg/ml). When this extract was concentrated, dissolved in water and reextracted with ethyl acetate at pH 8, the activity was mostly transferred into the ethyl acetate fraction, which showed more than 10 times higher specific activty (27% of the total weight; IC_{50}, less than 17—30 μg/ml; thin-layer chromatography (TLC) profile, Fig. 1b), while the rest (water-soluble fraction) showed little activity (IC_{50}>800 μg/ml). Fig. 1c shows the elution profile of the alkaline ethyl acetate extract on silica gel chromatography with methylene chloride.

Eight components were isolated after purification by thin layer chromatography and 5 major pigments were crystallized and named I, II, III, IV, and V (Fig. 1).

structures of platelet anti-aggregants from Tan-Shen

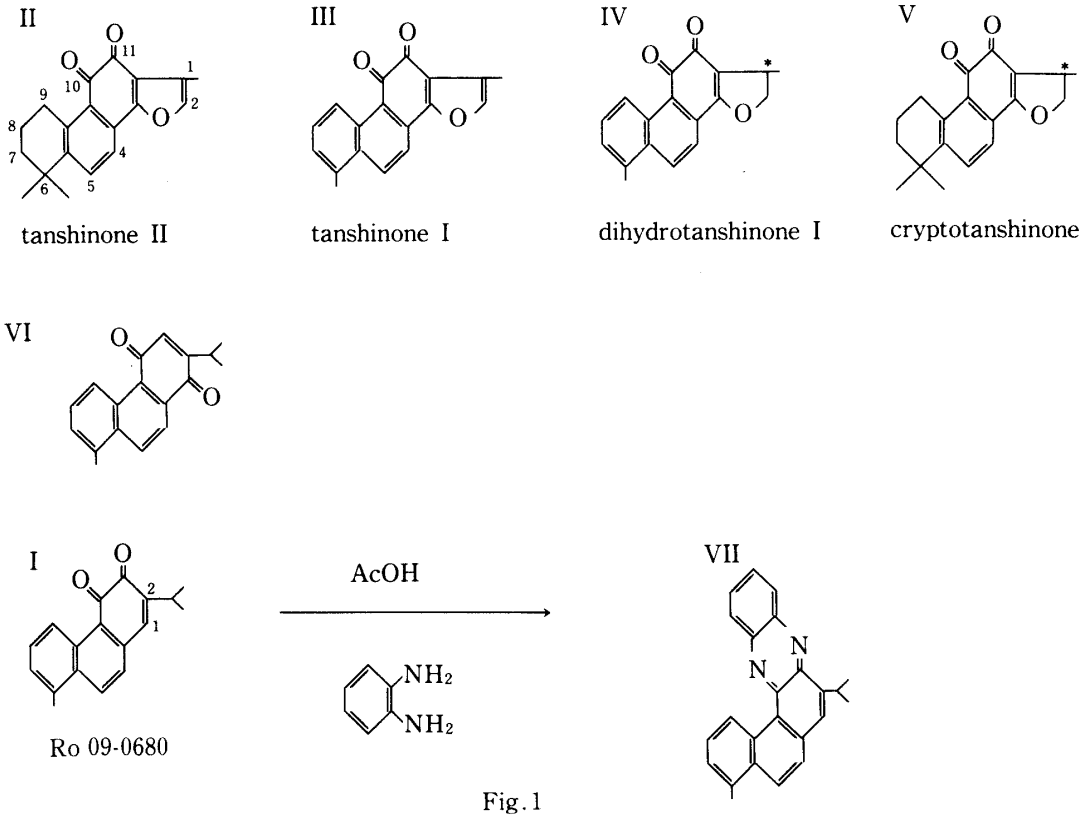

Fig. 1

Identification of the Platelet Aggregation Inhibitors II—V

Physico-chemical properties of these compounds indicated that II—V are homologous components, related to tanshinones,[7–13] and having a diketone chromophore as characterized by ultraviolet (UV) absorption measurement at 220—460 nm. By comparison with the physical constants in the literature, II, III, and V were identified as tanshinone II, tanshinone I and cryptotanshinone, respectively.

Physico-chemical properties of component IV were not identical with those of any known tanshinones from Tan-Shen. High resolution mass spectra (MS) of IV revealed a molecular ion peak at *m/e* 278 and gave the composition $C_{18}H_{14}O_3$. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of IV indicated the presence of a dihydromethyl furan system. The structure of component IV was deduced to be 1,2-dihydro-1,6-dimethylphenanthro[1,2-*b*]-furan-10,11-dione (1,2-dihydrotanshinone I). Comparison of UV, infrared (IR), NMR and MS data of component IV with those of previously reported synthetic *dl*-1,2-dihydrotanshinone I[13] revealed that the two were identical except for the optical rotation and mp (224—225°C, *cf.* lit. 200—201°C). Therefore, component IV is concluded to be an optically active 1,2-dihydrotanshinone newly isolated from Tan-Shen. This is the first report of the presence of optically active dihydrotanshinone I in nature.

Structure Determination of Compound I (Ro 09-0680)

Compound I (Ro 09-0680) obtained from the most active fraction was found to be a novel component having the molecular formula $C_{18}H_{16}O_2$; it appeared to be closely related to IV but lacked the furan moiety.

The UV absorption of compound I (λ_{max}^{MeOH}: 237, 290, 425) was similar to but rather simpler than that of the known tanshinones. The MS showed fragment ions characteristic of the tanshinone group at *m/e* 178, 165 and 152.[11]

Furthermore, a strong M+2 ion peak was observed at *m/e* 266 (20%) which was more intense than the parent ion *m/e* 264 (5%). Kakisawa[11] and Goto[14] reported that very strong M+2 ion peaks were observed in the MS of some 1,2-quinones. They concluded that the additional two hydrogens were derived from moisture in the mass spectrometer, on the basis of an experiment with D_2O. A similar D_2O experiment with compound I produced a strong M+4 peak (*m/e* 268), as expected.

The presence of a 1,2-quinone system in compound I was further confirmed by the conversion to a quinoxaline derivative (VII), $C_{24}H_{20}N_2$, by treatment with *O*-phenylenediamine under acidic conditions.

In the ^{1}H-NMR spectrum of compound I, the signal pattern in the aromatic region, δ 7.00—9.20, closely resembled those of compounds III and IV. The presence of the signals at δ 1.28 (6H, d) and δ 3.15 (1H, m) showed that compound I has an isopropyl group attached to a double bond. The linking position of the isopropyl group was deduced to be at C-2 from the following evidence. In the ^{1}H-NMR spectra of 1,2-naphthoquinones, Hα and Hβ of the α,β-unsaturated carbonyl system are generally observed at δ 6.27—6.79 and δ 7.22—7.81, respectively,[15] while in the case of compound I, C_1–H was observed at δ 7.00—7.70, overlapping with the aromatic protons.

All the evidence presented above and biogenetic considerations in relation to other tanshinones are consistent with the structure I, not with VI, for this compound (Fig. 2). Thus, the novel platelet aggregation inhibitor I was determined to be 2-isopropyl-8-methylphenanthrene-3,4-dione.

Biological Activities of Five Tan–Shen Components

Table I shows the *in vitro* inhibitory activities of the characterized α-diketophenanthrene derivatives on the rabbit platelet aggregation induced by soluble collagen (CPA).[1] The IC_{50} value of the most active component I was 6.6 μM, which is similar to that of TILCOTIL®, a potent platelet inhibitor, and 30 times more active than papaverine, an adenosine 3′,5′-cyclic monophosphate phosphodiesterase inhibitor (both used as controls). Unlike TILCOTIL®, compound I also inhibited the (primary) aggregation induced by adenosine diphosphate (ADP) (ADP-induced platelet aggregation (APA)) (IC_{50}: 32 μM for I, 290 μM for papaverine). The other 4 components, II—V in Fig. 2, showed moderate activities with IC_{50} values in the range of $2—15\times10^{-5}$ M. Among them, cryptotanshinone (V) exhibited the highest activity, accounting for about two-thirds of the total activity of Tan–Shen methanol extract on a weight basis. The other two main components, tanshinone I and II, also have weak but significant CPA inhibition activities. This is the first report on the CPA inhibition activity of Tan–Shen components; tanshinone I, tanshinone II, cryptotanshinone (V) and dihydrotanshinone I (IV).

The IC_{50} values are collected in Table I together with those of typical diketone derivatives. 1,4- or 1,2-Naphthoquinone and 1,2-phenanthrenequinone were as active as crysptotanshinone, while others were less active. Non-diketone derivatives such as phenanthrene and compound VII were not active.

The fact that compound VII and phenanthrene are inactive seems to suggest a fundamental role of the diketone moiety in the activity, in view of the fairly high activity of plain naphthoquinones and phenanthrenequinone *in vitro*. *p*-Quinone is less active than naphthoquinones and phenanthrenequinone. On the other hand, *p*-quinone and naphthoquinones are toxic while phenanthrenequinone and tanshinones are not. Comparisons among these components further suggest that saturation at the A-ring and the lack of a furan ring adjacent to the α-diketone lead to higher activity.

When *ex vivo* inhibition activity was tested in Wistar rats by oral administration at 50 mg/

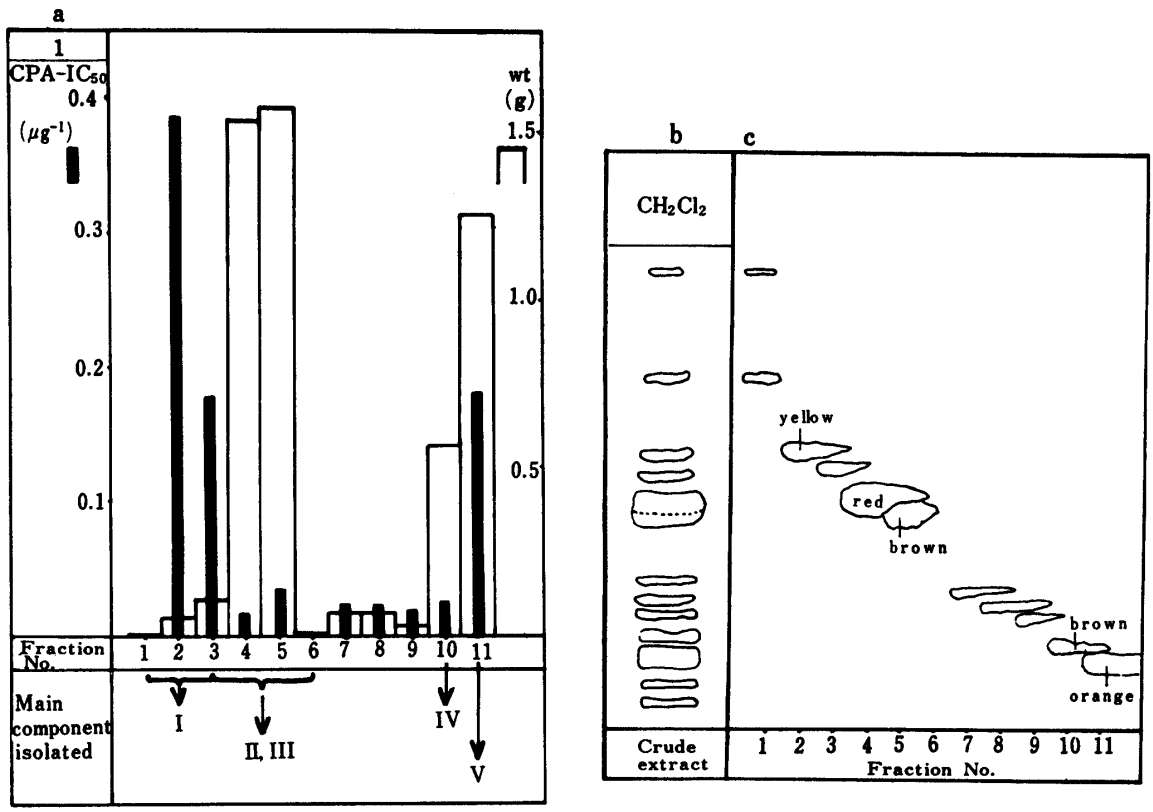

Fig. 2. a; Elution Profile on Silica Gel Chromatography, b,c; TLC Pattern of Each Fraction from the Silica Gel Column

TABLE I. CPA-inhibitory Activities of the Components from Tan-Shen Related Diketone Derivatives

	Compound	CPA IC_{50} (μM)
Components from Tan-Shen	I	6.6
	V	20
	IV	100
	II	100
	III	150
	VII	>130
Diketophenanthrene derivatives	*p*-Quinone	130
	α-Naphthoquinone	11
	β-Naphthoquinone	48
	Phenanthrenquinone	13
	Phenanthrene	1300
References	Tilcotil®[a] (Ro 12-0068)	2.4
	Papaverine	180

a) 4-Hydroxy-2-methyl-*N*-2-pyridyl-2*H*-thieno[2,3-*e*]-1,2-thiazine-3-carboxamide-1,1-dioxide.

kg, I exhibited a weaker activity than expected from its *in vitro* activity (approx. 20% inhibition). The activity was weaker than that of the alkaline ethyl acetate extract powder (34% inhibition at 50 mg/kg, 45% at 100 mg/kg), suggesting a low oral absorption or synergistic effects by other components. No toxic symptoms were observed in mice and rats on oral administration of 100 mg/kg of both compound I and cryptotanshinone (V).

Although Tan-Shen has been used in oriental medicine as a remedy for infarction and coronary pains,[16] mode of action studies will be necessary to determine any possible contra indications of these derivatives, since platelet aggregation is a complex process involving arachidonic acid release, production of prostaglandins, dependence on the pool of adenosine 3',5'-

cyclic monophosphate and Ca^{2+}, morphological change through microtubule systems, release of various stimulant factors, *etc.*

Experimental

All melting points are uncorrected. UV spectra were obtained with a Hitachi 124 spectrophotometer. IR spectra were recorded on a Hitachi 260-10 spectrophotometer. MS were obtained with a JEOL JMS-DX 300 mass spectrometer. ^{1}H-NMR spectra were determined on a Varian EM-360 (60 MHz) spectrometer. Chemical shifts are reported in parts per million downfield from tetramethylsilane as an internal standard. Specific optical rotations were obtained with a JASCO DIP-140 digital polarimeter.

Chemicals——Collagen reagent "HORM" (HORMON-CHEMIE MÜNCHEN GMBH) was used as a platelet aggregation inducer.

Assay Methods for the Inhibition of Rabbit Platelet Aggregation——Platelets were obtained as platelet-rich plasma prepared by centrifugation ($1500 \times g$ for 5 min from fresh rabbit blood with 1/15 vol. of acid-citrate-dextrose (ACD) buffer, as described previously[2]). ACD buffer contained 65 mM citric acid, 85 mM sodium citrate and 2% dextrose. The reaction mixture (250 μl) contained $1—2 \times 10^8$ platelets, 3 μg/soluble collagen or 1 μM ADP and 1 to 5 μl of sample dissolved in methanol or dimethylsulfoxide. Assay was carried out using a multi-channel platelet aggregometer (Rikadenki Kogyo). The IC_{50} value for CPA was calculated as described before.[1]

Ex vivo evaluation was done as follows. Chemicals were suspended in 0.5% carboxymethyl cellulose-saline solution and orally administrated to a Wistar rat (male, 7 weeks old). After 1 h, platelet-rich plasma was obtained by centrifugation from fresh cardiac blood and the CPA assay was performed within one hour.

Extraction and Purification of the Components from Tan-Shen——Tan-Shen (*Salvia miltiorrhiza*: 900 g) was mechanically crushed and extracted with methanol for 2 d at room temperature. The concentrated extract (90 g) was dissolved in water and reextracted with ethyl acetate at pH 8.0. The alkaline ethyl acetate extract (22 g, having *ca.* 10 times higher specific activity than that of the methanol extract) was subjected to chromatography on Wakogel C-300, which was eluted with methylene chloride. Reddish-orange pigments were recovered in each fraction eluted and were further purified through TLC (Kieselgel 60F254, developed with methylene chloride) then crystallized from methanol. Five main components were obtained as crystals: Compound I (Ro 09-0680), 26 mg of dark red needles; II, 1.0 g of reddish-orange needles; III, 1.1 g of dark red needles; IV, 370 mg of dark red needles; V, 830 mg of reddish-orange needles.

Physico-chemical Data for Compounds I—V——Compound I (2-Isopropyl-8-methylphenanthren-3,4-dione): mp 221—223°C. UV $\lambda_{max}^{methanol}$ nm (log ε): 222sh (4.41), 237 (4.54), 290 (4.06), 425 (3.69). IR ν_{max}^{KBr} cm^{-1}: 1645, 1623, 1582, 1447, 1360, 1220, 942, 785. MS *m/e*: 266 (M+2), 264 (M^+), 236 (M^+-CO), 178, 165, 152. ^{1}H-NMR (in $CDCl_3$) δ: 1.28 (6H, d, J=6 Hz $(CH_3)_2CH$–), 2.68 (3H, s, C_8–CH_3), 3.15 (1H, m, J=6 Hz $(CH_3)_2CH$–), 7.00—7.70, (3H, $C_{6,7,9}$–H), 8.23 (1H, d, J=8 Hz, C_{10}–H), 9.20 (1H, d, J=8 Hz, C_5–H). High resolution MS: Calcd for $C_{18}H_{16}O_2$: 264.1150. Found: 264.1141.

Compound II (Tanshinone II): mp 196—198°C. UV $\lambda_{max}^{methanol}$ nm (log ε): 225 (4.31), 251 (4.30), 268 (4.41), 350 (3.22), 460 (3.47). IR ν_{max}^{KBr} cm^{-1}: 1690, 1670, 1580, 1535, 1460, 1381, 1284, 1191, 1159. MS *m/e*: 294 (M^+), 279 (M^+-CH_3), 261, 251, 178, 165, 152. ^{1}H-NMR (in $CDCl_3$) δ: 1.32 (6H, s, C_6 gem-CH_3), 1.72 (4H, br m, $C_{7,8}$–H), 2.27 (3H, d, J=2 Hz, C_1–CH_3), 3.20 (2H, br t, C_9–H), 7.21 (1H, q, J=2 Hz, C_2–H).

Compound III (Tanshinone I): mp 233—234°C. UV $\lambda_{max}^{methanol}$ nm (log ε): 244 (4.60), 260sh, 325 (3.66), 417 (3.68). IR ν_{max}^{KBr} cm^{-1}: 1688sh, 1660, 1593, 1430, 1190, 1166, 917, 834, 790, 760, 707. MS *m/e*: 276 (M^+), 248 (M^+-CO), 178, 165, 152. ^{1}H-NMR (in $CDCl_3$) δ: 2.35 (3H, s, C_1–CH_3), 2.78 (3H, s, C_6–CH_3), 7.30—8.00 (4H, m, $C_{2,5,7,8}$–H), 8.28 (1H, d, J=8 Hz, C_4–H), 9.26 (1H, d, J=8 Hz, C_9–H).

Compound IV (1,2-Dihydrotanshinone I): mp 224—225°C. UV $\lambda_{max}^{methanol}$ nm (log ε): 240 (4.16), 266sh, 290 (3.96), 330 (2.92), 410 (3.27). IR ν_{max}^{KBr} cm^{-1}: 1688, 1653, 1626, 1593, 1478, 1180, 935, 843, 790. MS *m/e*: 278 (M^+), 250 (M^+-CO), 178, 165, 152. ^{1}H-NMR (in $CDCl_3$) δ: 1.45 (3H, d, J=6 Hz, C_1–CH_3), 2.72 (3H, s, C_6–CH_3), 3.70 (1H, m, C_1–Hx), 4.37 (1H, dd, 9 Hz, J_{AX}=5 Hz, C_2–H_A), 5.00 (1H, t, $J_{AB}=J_{BX}$=9 Hz, C_2–H_B) 7.20—7.80 (3H, m, $C_{5,7,8}$–H), 8.28 (1H, d, J=8 Hz, C_4–H), 9.30 (1H, d, J=8 Hz, C_9–H). $[\alpha]_D^{24}$ −328 (c=0.11; chloroform). High resolution MS: Calcd for $C_{18}H_{14}O_3$: 278.0943. Found: 278.0929.

Compound V (Cryptotanshinone): mp 191—192°C. UV $\lambda_{max}^{methanol}$ nm (log ε): 263 (4.46), 271 (4.41), 292 (3.95), 355 (3.41), 477 (3.48). IR ν_{max}^{KBr} cm^{-1}: 2950, 1680, 1648, 1620, 1553, 1460, 1400, 1333, 1193, 1140, 1167, 941, 840, 700. MS *m/e*: 296 (M^+), 281 (M^+-CH_3), 178, 165, 152. ^{1}H-NMR (in $CDCl_3$) δ: 1.38 (6H, s, C_6 gem-CH_3), 1.45 (3H, d, J=6 Hz, C_1–CH_3), 1.80 (4H, m, $C_{7,8}$–H), 3.65 (1H, m, C_1–Hx), 4.37 (1H, dd, J_{AB}= 9 Hz, J_{AX}=5 Hz, C_2–H_A), 4.92 (1H, t, $J_{AB}=J_{BX}$=9 Hz, C_2–H_B), 7.55 (2H, AB q, J=8 Hz, $C_{4,5}$–H). $[\alpha]_D^{24}$ −79.9 (c=0.18, chloroform).

References and Notes

1) Y. T. Ninomiya, Y. Yamada, M. Onitsuka (née Ono), Y. Tanaka, T. Maeda, and H. B. Maruyama, *Chem. Pharm. Bull.*, **28**, 9, 2553 (1980).

2) J. Yamahara, H. Matsuda, T. Sawada, H. Fujimura and H. Mibu, "Third symposium on the development and application of naturally occurring drug materials, abstracts," 10, 1980.
3) T. Kobuchi, S. Tsuda, and A. Sugaya, The 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April 1981.
4) E. Sugishita, N. Kakei, S. Ametani, Y. Ogiwara, and T. Nakajima, The 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumomoto, April 1981.
5) T. Nikaido, T. Ohmoto, H. Noguchi, T. Kinoshita, H. Saito, Y. Aida, U. Sankawa, S. Sakuma, J. Shoji, S. Seibu and S. Hisada, "Third symposium on the development and application of naturally occurring drug materials, abstracts," 1980.
6) U. Sankawa, *Faruaw*, **17** (5), 367 (1981).
7) Y. Okumura, H. Kakisawa, M. Kato and Y. Hirata, *Bull. Chem. Soc. Jpn.*, **34**, 895 (1961); K. Takiura and K. Koizumi, *Chem. Pharm. Bull.*, **10**, 112 (1962).
8) K. Takiura, *J. Pharm. Soc., Jpn.*, **61**, 482 (1941).
9) Y. Inouye and H. Kakisawa, *Bull. Chem. Soc. Jpn*, **42**, 3318 (1969).
10) A.C. Baillie and R.H. Thomson, *J. Chem. Soc. (C)*, 48, (1968).
11) T. Hayashi, Y. Inoue, M. Ohashi, and H. Kakisawa, *Org. Mass Spectrometry*, **3**, 1293 (1970).
12) H. Kakisawa, T. Hayashi, I. Okazaki, and M. Ohashi, *Tetrahedron. Lett.*, **28**, 3231 (1968).
13) H. Kakisawa and Y. Inouye, *J. Chem. Soc., Chem. Commun.*, **1968**, 1327.
14) S. Ukai, K. Hirose, A. Tatematsu, and T. Goto, *Tetrahedron. Lett.*, **49**, 4999 (1967).
15) St. Berger and A. Reiker, "The chemistry of the quinonoid compounds," S. Patai ed., part 1, Jonh Wiley & Sons, Ltd., Chichester, 1974, p. 176.
16) Chuzan Igakuin (ed.), "Kanyaku no Rinsho Ohyo," Ishiyaku-shuppan, Tokyo, 1975, p. 257.