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Studies on the Activities of Tannins and Related Compounds from Medicinal Plants and Drugs. I. Inhibitory Effects on Lipid Peroxidation in Mitochondria and Microsomes of Liver

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The inhibitory effects of 25 tannins and related compounds on the lipid peroxidation induced by adenine 5′-diphosphate (ADP) and ascorbic acid in rat liver mitochondria, and on that induced in rat liver microsomes by ADP and nicotinamide adenine dinucleotide phosphate (NADPH), were determined. All of the tannins, except for some polyphenols of low molecular weight and methylated polyphenols, showed significant inhibition in these two systems at the concentration of 1 μg/ml. Almost complete inhibition of the lipid peroxidation in the two systems was shown by some ellagitannins such as pedunculagin, isoterchebin, *etc.*, at the dose of 5 μg/ml. Marked differences of the inhibitory activities were observed among the tannins depending on the tannin structure, including the stereoisomerism in the monomer of condensed tannin, and on the experimental system used. The inhibitory effects of most of the hydrolyzable tannins were higher than those of the condensed tannins in both systems. Some monomeric polyphenols, represented by (−)-epigallocatechin gallate, showed inhibitory effects stronger than those of the condensed tannins in these systems, particularly on the lipid peroxidation induced by ADP and NADPH. The inhibitory effects of these tannins in both systems were very much stronger than that of α-tocopherol.

Keywords——tannin; hydrolyzable tannin; ellagitannin; condensed tannin; lipid peroxidation; inhibition; ADP; ascorbic acid; NADPH; structure–activity correlation

A number of oriental medicinal plants and drugs are rich in tannins, which are regarded as the active principles. Various tannins have been isolated recently from these medicinal plants and drugs,[1] and some effects of these tannins on coexisting substances, including the inhibitory effect of tannins on cupric ion-catalyzed autoxidation of ascorbic acid, have been investigated.[2–5]

Several of these medicinal plants and drugs have been regarded as effective against diseases induced by lipid peroxides, which are known to injure the liver, kidney and blood vessels.[6] It is possible that the tannins in medicinal plants and drugs, which are known to have a reducing effect on coexisting substances, or to prevent their oxidation,[2,3] are effective against diseases such as liver injury and arteriosclerosis in higher animals, by inhibiting the formation of lipid peroxides and thus protecting the tissues against injury.

The present paper describes the inhibitory effects of tannins and related compounds on the lipid peroxidation in rat liver mitochondria stimulated by ADP and ascorbic acid,[7] and on that in rat liver microsomes stimulated by ADP and NADPH.[7] The correlation between the structures of tannins and their inhibitory effects is discussed.

Materials and Methods

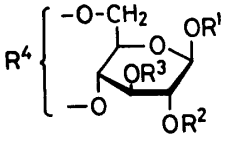
Materials——Twenty-five tannins and related polyphenols used in this study were as follows: tellimagrandin I (**1**),[8a] tellimagrandin II (**2**),[8a,b] pedunculagin (**3**),[8a] isoterchebin (**4**),[8b] alnusiin (**5**),[8c] geraniin (**6**),[8d-f] mallotusinic acid (**7**),[8g] dehydrogeraniin (**8**),[8h] furosinin (**9**),[8h] corilagin (**10**),[8i] chebulinic acid (**11**),[8j] chebulagic acid (**12**),[8j] agrimoniin (**13**),[8k] gemin A (**14**),[8l] RSF-tannin H (**15**) ($\overline{M}$n 3100, 81% galloylated at *O*-3),[8m] Ss-tannin 1 (**16**) ($\overline{M}$n 2300, 96% galloylated at O-3),[8m] (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, gallic acid, methyl gallate, ellagic acid, 3,3′-di-*O*-methylellagic acid, and methyl tetra-*O*-methylluteate (Fig. 1). These polyphenols were dissolved or suspended in Krebs–Ringer phosphate buffer (pH 7.4).

Animals——Young male Wistar-King strain rats weighing 150—200 g were housed in a room at 25±1°C with 60% relative humidity, and were given free access to food and water. The room was illuminated for 12 h a day starting at 7: 00 a.m.

Preparation of Mitochondria and Microsomes in Rat Liver——Rats were killed by decapitation, and their liver tissue was quickly removed. Microsomes and mitochondria were isolated from the liver tissue by the method of Oda *et al.*[9] The protein in the isolated mitochondria and microsomes was determined by the method of Lowry *et al.*[10] using bovine serum albumin as a standard. Mitochondria and microsomes were suspended in Krebs–Ringer phosphate buffer (pH 7.4).

Estimation of Adenine 5′-Diphosphate (ADP) plus Ascorbic Acid-, and ADP plus Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-induced Lipid Peroxidation in Mitochondria and Microsomes——A mixture of mitochondrial suspension (0.5 ml, equivalent to 12 mg protein), Krebs–Ringer phosphate buffer (pH 7.4) (0.2 ml), Krebs–Ringer phosphate buffer containing 40 mM ADP solution (0.1 ml), 12 mM ascorbic acid solution (0.1 ml) and 1, 5 or 20 μg/ml of various tannins or related compounds was incubated at 37°C for 30 min in a final volume of 1 ml. The reaction was then terminated by cooling the mixture to 4°C, and the lipid peroxides of mitochondria were determined by the method of Yagi *et al.*[11] using malondialdehyde (MDA) as a standard.

The lipid peroxidation induced by ADP and NADPH in microsomes was determined as described above except that Krebs–Ringer phosphate buffer (pH 7.4) containing 40 mM ADP and 4 mM NADPH was used instead of ADP and ascorbic acid.



	R^1	R^2	R^3	R^4
tellimagrandin I (**1**):	H (α,β)	gall	gall	(*S*)-HHDP
tellimagrandin II (**2**):	gall	gall	gall	(*S*)-HHDP
pedunculagin (**3**):	H (α,β)	(*S*)-HHDP		(*S*)-HHDP
isoterchebin (**4**):	gall	gall	gall	(1′*S*)-DHHDP
alnusiin (**5**):	H (α,β)	(*S*)-HHDP		[A]

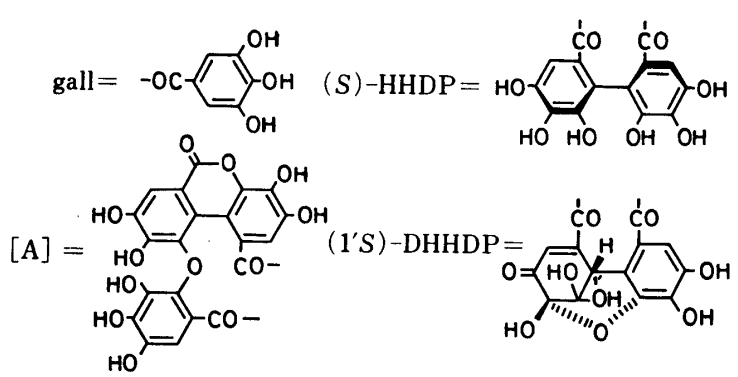
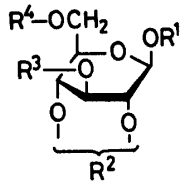
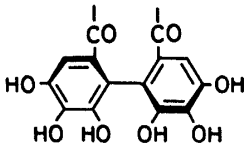
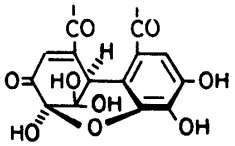


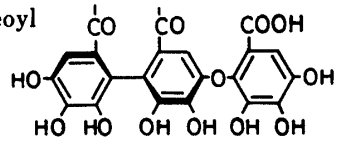
Fig. 1 (1)

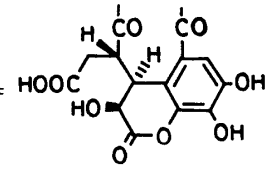


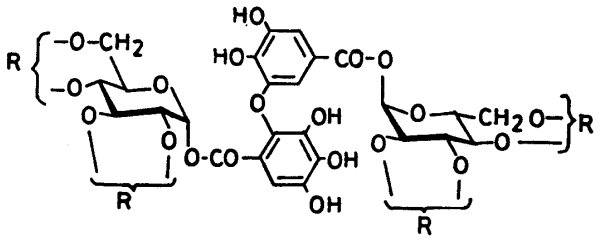
	R^1	R^2	R^3	R^4
geraniin (**6**)	: gall	(1'*R*)-DHHDP	(*R*)-HHDP	
mallotusinic acid (**7**)	: gall	(1'*R*)-DHHDP	(*R*)-valoneoyl	
dehydrogeraniin (**8**)	: gall	(1'*R*)-DHHDP	(1'*R*)-DHHDP	
furosinin (**9**)	: H (α,β)	(1'*R*)-DHHDP	(1'*R*)-DHHDP	
corilagin (**10**)	: gall	H,H	(*R*)-HHDP	
chebulinic acid (**11**)	: gall	[B]	gall	gall
chebulagic acid (**12**)	: gall	[B]	(*R*)-HHDP	

(*R*)-HHDP= 

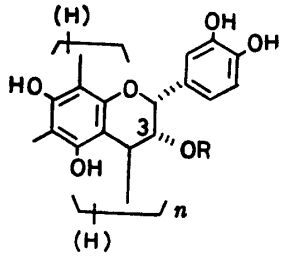
(1'*R*)-DHHDP= 

(*R*)-valoneoyl = 

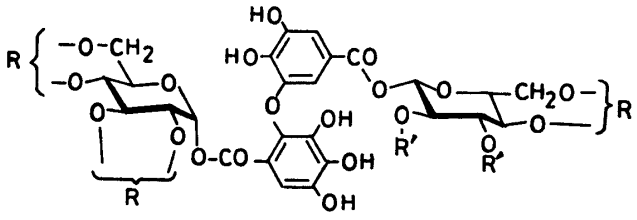
[B]= 



agrimoniin (**13**) R=(*S*)-HHDP



RSF-tannin H (**15**)
$\bar{M}$n 3100, R=81% gall
terminal unit:
(+)-catehin (28%)
(−)-epicatechin (7%)
(−)-epicatechin gallate (65%)



gemin A (**14**) R=(*S*)-HHDP, R'=gall

Ss-tannin 1 (**16**)
$\bar{M}$n 2300, R=96% gall
terminal unit:
(−)-epicatechin gallate (100%)

Fig. 1(2)

Results

Effects of Tannins and Related Compounds on ADP plus Ascorbic Acid-induced Lipid Peroxidation in Mitochondria

The basal value of lipid peroxidation was 0.46 nmol per 1 mg protein of mitochondria in

liver. It was increased to 1.64 in the presence of ADP (4 mM) and ascorbic acid (1.2 mM). As shown in Table I, hydrolyzable tannins such as pedunculagin (**3**), penta-*O*-galloylglucose and isoterchebin (**4**) showed 96%, 97% and 92% inhibition, respectively, at the concentration of 5 μg/ml, of the lipid peroxidation induced by ADP and ascorbic acid in mitochondria. Condensed tannins such as RSF-tannin H (**15**) and Ss-tannin 1 (**16**) showed 14% and 16% inhibition at the dose of 1 μg/ml, and showed 47% and 52% inhibition at the dose of 5 μg/ml, respectively. Marked differences of inhibitory effect were observed among the tannins and also among the polyphenols of low molecular weight. The difference of inhibitory activity between (+)-catechin and (−)-epicatechin indicates the significance of the monomer stereostructure in relation to the inhibitory effect. The inhibitory effects of gallic acid and ellagic acid were almost negligible. The methylated polyphenols, *e.g.*, methyl tetra-*O*-methylluteate and 3,3′-di-*O*-methylellagic acid, exhibited no activity. (−)-Epigallocatechin gallate, which is the main constituent of the green-tea polyphenol, and is a galloylated monomer, significantly inhibited the ADP plus ascorbic acid-induced lipid peroxidation in spite of its small molecular size.

At the concentration of 20 μg/ml, all of the hydrolyzable tannins, condensed tannins and related compounds, except for ellagic acid, 3,3′-di-*O*-methylellagic acid and methyl tetra-*O*-methylluteate, exhibited over 50% inhibition of the lipid peroxidation induced by ADP and ascorbic acid in mitochondria. Significant inhibition was also seen at the concentration of 1 μg/ml [46% by gemin A (**14**) and 41% by pedunculagin (**3**) and chebulagic acid (**12**)].

TABLE I. Effects of Various Tannins and Related Compounds on ADP plus Ascorbic Acid-induced Lipid Peroxidation in Fresh Mitochondria[a)]

Compound	ID_{50} (μg/ml)	inhibition (5 μg/ml)
ADP(4 mM), ascorbic acid(1.2 mM)		
+ pedunculagin (**3**)	1.2	96
+ penta-*O*-galloylglucose	1.2	97
+ isoterchebin (**4**)	1.6	92
+ gemin A (**14**)	1.8	67
+ chebulagic acid (**12**)	2.2	66
+(−)-epigallocatechin gallate	2.4	74
+ agrimoniin (**13**)	2.6	64
+ furosinin (**9**)	2.8	64
+ dehydrogeraniin (**8**)	2.8	51
+ tellimagrandin I (**1**)	3.0	65
+ (+)-catechin	3.4	61
+ tellimagradin II (**2**)	3.7	58
+ Ss-tannin 1 (**16**)	4.5	52
+ RSF-tannin H (**15**)	5.7	47
+ methyl gallate	6.4	41
+ geraniin (**6**)	6.6	42
+(−)-epicatechin	6.7	43
+ chebulinic acid (**11**)	7.5	37
+ alnusiin (**5**)	8.2	35
+ mallotusinic acid (**7**)	8.5	38
+ corilagin (**10**)	9.9	34
+ gallic acid	11.6	20
+ ellagic acid	32.0	10
+ methyl tetra-*O*-methylluteate	>100	1
+ 3,3′-di-*O*-methylellagic acid	>100	− 27
+ α-tocopherol	65.2	4

a) Inhibition ratio was evaluated as the mean ± standard error of 4—6 replicate determinations for the control and experiments with 1, 5, or 20 μg/ml of polyphenol, and was calculated by subtracting the basal value (MDA nmol/mg protein) of lipid peroxidation from the value of the stimulated system in the presence and absence of tannins and polyphenols.

TABLE II. Effects of Various Tannins and Related Compounds on ADP plus NADPH-induced Lipid Peroxidation in Rat Liver Fresh Microsomes

Compound	ID_{50} (μg/ml)	% inhibition (5 μg/ml)
ADP(4 mM), NADPH(0.4 mM)		
+ chebulinic acid (**11**)	0.1	101
+ pedunculagin (**3**)	0.2	96
+ mallotusinic acid (**7**)	0.4	99
+ corilagin (**10**)	0.5	79
+ tellimagrandin I (**1**)	0.7	95
+ alnusiin (**5**)	0.8	88
+ penta-*O*-galloylglucose	0.9	95
+ (−)-epigallocatechin gallate	0.9	98
+ geraniin (**6**)	1.0	96
+ isoterchebin (**4**)	1.2	93
+ agrimoniin (**13**)	1.4	82
+ dehydrogeraniin (**8**)	1.6	68
+ tellimagrandin II (**2**)	2.0	71
+ gemin A (**14**)	2.0	89
+ Ss-tannin I (**16**)	2.2	87
+ RSF-tannin H (**15**)	2.4	77
+ furosinin (**9**)	2.4	74
+ ellagic acid	5.8	47
+ chebulagic acid (**12**)	7.0	42
+ (+)-catechin	11.2	30
+ methyl gallate	12.5	32
+ (−)-epicatechin	13.4	22
+ gallic acid	19.0	19
+ methyl tetra-*O*-methylluteate	>100	1
+ 3, 3′-di-*O*-methylellagic acid	>100	−5
+ α-tocopherol	43.7	6

Effects of Tannins and Related Compounds on ADP plus NADPH-induced Lipid Peroxidation in Microsomes

The basal value of lipid peroxidation was 0.32 nmol per 1 mg protein of rat liver microsomes. Upon stimulation by ADP (4 mM) and NADPH (0.4 mM), 2.28 nmol of lipid peroxide was formed from 1 mg protein of microsomes. As shown in Table II, some hydrolyzable tannins such as chebulinic acid (**11**) and mallotusinic acid (**7**) almost completely inhibited the lipid peroxidation induced by ADP and NADPH in microsomes at the concentration of 5 μg/ml. Inhibition of over 90% was observed with tellimagrandin I (**1**), geraniin (**6**), isoterchebin (**4**), penta-*O*-galloylglucose and (−)-epigallocatechin gallate. The inhibition by the condensed tannins such as Ss-tannin 1 (**16**) and RSF-tannin H (**15**) was lower than that by (−)-epigallocatechin gallate. Methylated polyphenols such as 3,3′-di-*O*-methylellagic acid and methyl tetra-*O*-methylluteate did not inhibit the lipid peroxidation induced by ADP and NADPH in microsomes, although the small polyphenols having free phenolic hydroxyl groups, *e.g.*, (+)-catechin and (−)-epicatechin, showed weak inhibition.

Discussion

The present investigation has demonstrated that various tannins and related compounds from medicinal plants and drugs significantly affect lipid peroxidation in rat mitochondria and microsomes. The degree of inhibition of ADP plus ascorbic acid-induced lipid peroxidation in rat mitochondria was in the order penta-*O*-galloylglucose>**3**>**4**>(−)-epigallocatechin gallate>**14**>**12**>**1**>**9**≈**13**>(+)-catechin>**2**>**8**≈**16**>**15**>(−)-epicatechin>**6**>methyl gallate >**7**>**11**>**5**>**10**>gallic acid>ellagic acid, at the concentration of 5 μg/ml.

The absence of inhibitory activity of 3,3′-di-*O*-methylellagic acid and methyl tetra-*O*-methylluteate shows that the free phenolic hydroxyl groups are essential for the activity. The inhibitory effects of the hydrolyzable tannins, which are generally higher than those of the condensed tannins such as Ss-tannin 1 (**16**) and RSF-tannin H (**15**), and the polyphenols of low molecular weight indicate that structures in which several polyphenol groups such as galloyl, hexahydroxydiphenoyl (HHDP) and dehydrohexahydroxydiphenoyl (DHHDP) group located around the molecular center of the tannin could be an important requirement for activity for the inhibition of lipid peroxidation. Comparisons of the activities among the tannins which have the same skeleton in their structures, and have DHHDP and HHDP groups (**8**>**6**, **4**>**2**), and also among the tannins which have HHDP and galloyl groups (**3**>**1**, **12**>**11**) suggest that the differences of the activities could be due to the structural differences among these polyphenolic groups. It is remarkable that the inhibitory effects on the ADP plus ascorbic acid-induced lipid peroxidation of the small-molecular polyphenols such as (+)-catechin, (−)-epicatechin and (−)-epigallocatechin gallate are higher than the inhibitory effects of the condensed tannins such as RSF-tannin H (**15**) and Ss-tannin 1 (**16**) which are composed of these monomeric polyphenols.

Marked differences in the order of degree of inhibitory effects were observed between the lipid peroxidation induced by ADP plus NADPH in rat liver microsomes, and that induced by ADP plus ascorbic acid in mitochondria. In the former case, the inhibitory effects were in the order **11**>**7**>(−)-epigallocatechin gallate>**6**≈**3**>**1**≈penta-*O*-galloylglucose>**4**>**14**>**5**>**16**>**13**>**10**>**15**>**9**>**2**>**8**>ellagic acid>**12**→methyl gallate>(+)-catechin>(−)-epicatechin>gallic acid at the concentration of 5 μg/ml. The inhibitions by all of these tannins, except for those by the polyphenols of low molecular weight, are over 40%. It is noticeable that the inhibitory effect of (−)-epigallocatechin gallate on the formation of lipid peroxide induced by ADP and NADPH in microsomes is particularly strong, while small-molecular polyphenols such as (+)-catechin and gallic acid showed only weak effects, and methylated polyphenols, *e.g.*, 3,3′-di-*O*-methylellagic acid and methyl tetra-*O*-methylluteate, showed no effect. It was also observed that the inhibitory effects of the hydrolyzable tannins on the ADP plus NADPH-induced lipid peroxidation are stronger than those of the condensed tannins such as RSF-tannin H (**15**) and Ss-tannin 1 (**16**), as was the case for the lipid peroxidation induced by ADP and ascorbic acid in mitochondria. Trends of the strength of the inhibitory effects on the ADP plus NADPH-induced lipid peroxidation among some tannins with the same skeleton and different polyphenolic groups (**11**>**12**, **6**>**8**, **13**>**14**) are different from those observed for the ADP plus ascorbic acid-induced lipid peroxidation. These trends also seem to be different from the trends of the fundamental activities of tannins determined by relative astringency (RA)[12] and relative activity on methylene blue (RMB)[13] measurements, since peduculagin (**3**), which showed the highest activities in the present experiment gave a low value in the RA measurement (RA 0.22). In general, these inhibitory effects of the hydrolyzable tannins on ADP plus NADPH-induced lipid peroxidation in microsomes are stronger than those on the ADP plus ascorbic acid-induced lipid peroxidation in mitochondria.

It is noteworthy that the inhibitory effects of these tannins in both experimental systems are very much stronger than that of α-tocopherol.

These tannins and related compounds thus markedly inhibited the lipid peroxide formation induced by ADP plus ascorbic acid in mitochondria, and that induced by ADP plus NADPH in microsomes, and may prevent the destructive effects of lipid peroxide in liver cells[14] by lowering the level of lipid peroxide in the cells. Further work to clarify the physiological significance of tannins and related compounds is in progress.

References and Notes

1) *a*) T. Okuda, T. Yoshida, K. Mori, and T. Hatano, *Heterocycles*, **15**, 1323 (1981); *b*) T. Okuda, *Kagaku*

no Ryoiki, **35**, 28 (1981); *c*) I. Nishioka, Abstract Papers, 102nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April 1982, p. 55.
2) T. Yoshida, S. Koyama, and T. Okuda, *Yakugaku Zasshi*, **101**, 695 (1981).
3) T. Okuda, K. Mori, M. Shiota, and K. Ida, *Yakugaku Zasshi*, **102**, 735 (1982).
4) T. Okuda, K. Mori, and M. Shiota, *Yakugaku Zasshi*, **102**, 854 (1982).
5) T. Okuda, T. Yoshida, T. Hatano, M. Kuwahara, and S. Iida, *Proc. Symp. Wakan-Yaku*, **15**, 111 (1982).
6) *a*) H. Okuda, *Igaku to Yakugaku*, **2**, 411 (1979); *b*) H. Ohminami, H. Okuda, T. Hamai, I. Kitagawa, M. Yoshioka, S. Arichi, and T. Hayashi, *Eiyo to Shokuryo*, **34**, 105 (1981); *c*) H. Izumida, T. Yasugi, T. Shimizu, I. Kobayashi, H. Konno, G. Mizuno, T. Tochohara, S. Tabuchi, and H. Kishi, *The Journal of Japan Atherosclerosis Society*, **8**, 295 (1980); *d*) Y. Kimura, M. Kubo, T. Tani, S. Arichi, and H. Okuda, *Chem. Pharm. Bull.*, **29**, 2610 (1981); *e*) Y. Kimura, H. Okuda, T. Tani, and S. Arichi, *Chem. Pharm. Bull.*, **30**, 1792 (1982); *f*) T. Mizunuma, Y. Takahashi, Y. Kishino, F. Sato, and H. Okuda, *Lipid Peroxide Research*, **2**, 88 (1978).
7) *a*) P. Hochstein and L. Ernster, *Biochem. Biophys. Res. Commun.*, **12**, 388 (1963); *b*) A. Ottolenghi, *Arch. Biochem. Biophys.*, **79**, 355 (1959); *c*) E.H. Thile and J.W. Huff, *Arch. Biochem. Biophys.*, **88**, 203 (1960).
8) *a*) T. Okuda, T. Yoshida, and M. Ashida, *Heterocycles*, **16**, 1681 (1981); *b*) T. Okuda, T. Hatano, and T. Yasui, *Heterocycles*, **16**, 1321 (1981); *c*) T. Yoshida, M.U. Memon, and T. Okuda, *Heterocycles*, **16**, 1085 (1981); *d*) T. Okuda, T. Yoshida, and H. Neyeshiro, *Chem. Pharm. Bull.*, **25**, 1682 (1977); *e*) T. Okuda, T. Yoshida, and T. Hatano, *Tetrahedron Lett.* **21**, 2561 (1980); *f*) T. Okuda, T. Yoshida, and T. Hatano, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 9; *g*) T. Okuda and K. Seno, *Tetrahedron Lett.*, **1978**, 139; *h*) T. Okuda, T. Hatano, and K. Yazaki, *Chem. Pharm. Bull.*, **30**, 1113 (1982); *i*) T. Okuda, T. Yoshida, and K. Mori, *Phytochemistry*, **14**, 1877 (1975); *j*) T. Yoshida, T. Okuda, T. Koga, and N. Toh, *Chem. Pharm. Bull.*, **30**, 2655 (1982); *k*) T. Okuda, T. Yoshida, M. Kuwahara, M.U. Memon, and T. Shingu, *J. Chem. Soc., Chem. Commun.*, **1982**, 163; *l*) T. Yoshida, T. Okuda, M.U. Memon, and T. Shingu, *J. Chem. Soc., Chem. Commun.*, **1982**, 351; *m*) T. Okuda, T. Hatano, M. Kuwahara, Y. Higashiyama, and Y. Maruyama, Abstract Papers, 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April 1981, p. 511, 512.
9) T. Oda and S. Seki, *J. Electron Microscopy*, **14**, 210 (1965).
10) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) H. Ohkawa, N. Ohishi, and K. Yagi, *Anal. Biochem.*, **95**, 351 (1979).
12) T. Okuda, K. Mori, and K. Aoi, *Yakugaku Zasshi*, **97**, 1267 (1977).
13) T. Okuda, K. Mori, and R. Murakami, *Yakugaku Zasshi*, **97**, 1273 (1977).
14) *a*) N. Shibata, T. Kinumaki, H. Okuda, and S. Fujii, *Agric. Biol. Chem.*, **37**, 1899 (1973); *b*) N.R. di Luzio and A.D. Hartman, *Fed. Proc., Fed. Am. Sec. Exp. Biol.*, **26**, 1436 (1967); *c*) T.F. Salter and B.C. Sawyer, *Biochem. J.*, **123**, 823 (1971).