Carbonic Anhydrase Inhibitors from the Pericarps of *Punica granatum* L.

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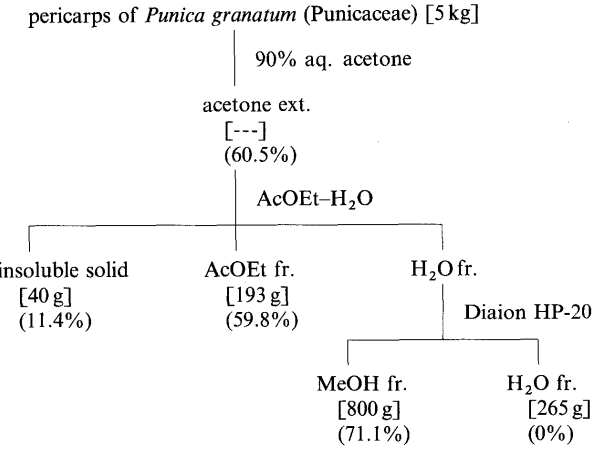
Seven highly active inhibitors against carbonic anhydrase (CA, EC 4.2.1.1), punicalin (2), punicalagin (3), granatin B (5), gallagyldilactone (7), casuarinin (8), pedunculagin (9) and tellimagrandin I (10), and four weakly active inhibitors, gallic acid (1), granatin A (4), corilagin (6) and ellagic acid (11), were isolated from the pericarps of *Punica granatum* L. (Punicaceae). They are ellagitannins. The type of inhibition by 3 and 7 using *p*-nitrophenyl acetate as a substrate, is noncompetitive. The structure–activity relationship of inhibitory effects on CA is discussed.

Keywords carbonic anhydrase; inhibitor; *Punica granatum*; noncompetitive inhibition; *p*-nitrophenyl acetate; gallagyldilactone

Inhibition of carbonic anhydrase (CA, EC 4.2.1.1) is expected to be therapeutically useful for the treatment of glaucoma and diuresis.[1] In *in vitro* screening tests aimed at finding CA inhibitors from many crude oriental drugs, a crude 90% aqueous acetone extract of the pericarps of *Punica granatum* L. (Punicaceae) showed high inhibitory activity. *Punica granatum* has been used since ancient times as an anthelmintic and antidiarrheic.[2] The constituents of the pericarps have been reported to be gallic acid (**1**),[3] isoquercitrin[4] and several ellagitannins, such as, punicalin (**2**),[5] punicalagin (**3**),[5] granatin A (**4**),[6,7] granatin B (**5**)[7,8] and corilagin (**6**).[7,8] Many biological actions of tannins have been investigated, including their inhibition of xantine oxidase,[9] their ability to destroy the larvae of dog roundworm (*Toxocara canis*),[10] and their antioxidant effect.[11] In this paper, we report the isolation and inhibitory effects of ellagitannins on CA from the pericarps of *Punica granatum*.

Results and Discussion

CA mediates the reversible hydration of carbon dioxide and carbonyl compounds and also has esterase activity.[12] So, in this study, the esterase activity of CA *in vitro*, using *p*-nitrophenyl acetate as substrate was assayed spectrophotometrically at 348 nm by a modification of the method of Armstrong *et al.*[13a] The Japanese commerical pericarps of *Punica granatum* were extracted with 90% aqueous acetone. The extract was fractionated as shown in Chart 1. The yield and inhibitory activity of each fraction is also shown in Chart 1.



[ ]: yields
( ): percent inhibition (test sample 0.5 mg/ml)

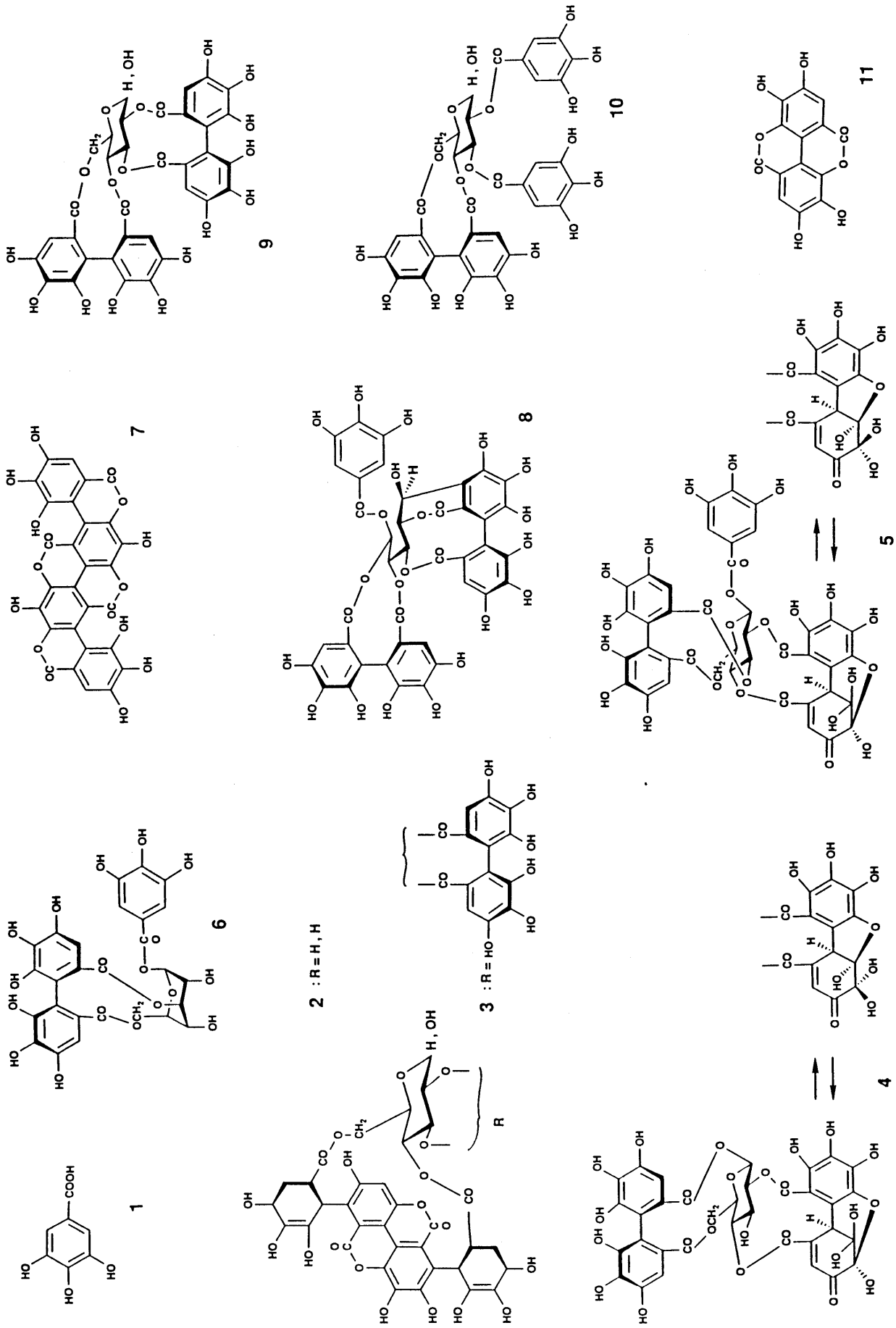
Chart 1

A methanol fraction, with the most potent activity, was subjected to column chromatography using Sephadex LH-20, MCI-gel CHP 20P and Toyopearl HW-40C, and followed by high performance liquid chromatography (HPCL) using Develosil 60-5 and ODS-7 with monitoring by measurement of the inhibitory activity against CA, to obtain **2**, **3**, **4** and **5**. From the ethyl acetate fraction, **1**, **6**, gallagyldilactone (**7**),[5,6] casuarinin (**8**),[14,15] pedunculagin (**9**)[14,16] and tellimagrandin I (**10**)[16,17] were similarly isolated. From the ethyl acetate–H_2O insoluble solids, ellagic acid (**11**) and **7** were purified by fractional recrystallization from MeOH followed by HPLC on ODS-7. The structures of the purified constituents were identified by comparison of their physical and spectral data with the literature or authentic samples. Compounds **2**—**11** are ellagitannins. This is the first report of the isolation of **8**, **9** and **10** from *Punica granatum*.

The inhibitory activities of **1**—**11** on CA are shown in Table I. As an active control, acetazolamide (**12**)[18] was used. The compounds demonstrating considerable activity against CA are **2**, **3**, **5**, **7**, **8**, **9** and **10**. The most active compound in this study was **7**. The concentration of **7** required under our assay conditions to give 50% inhibition

TABLE I. Inhibitory Activities of Constituents from the Pericarps of *Punica granatum* L. on Carbonic Anhydrase

Compound	IC_{50} (M)
Gallic acid (**1**)	$>1.0\times10^{-5}$
Punicalin (**2**)	1.0×10^{-6}
Punicalagin (**3**)	2.3×10^{-7}
Granatin A (**4**)	$>6.2\times10^{-6}$
Granatin B (**5**)	3.7×10^{-7}
Corilagin (**6**)	$>5.0\times10^{-6}$
Gallagyldilacton (**7**)	2.2×10^{-7}
Casuarinin (**8**)	2.7×10^{-7}
Pedunculagin (**9**)	5.5×10^{-7}
Tellimagrandin I (**10**)	3.2×10^{-7}
Ellagic acid (**11**)	$>1.0\times10^{-5}$
Acetazolamide (**12**)	2.0×10^{-7}



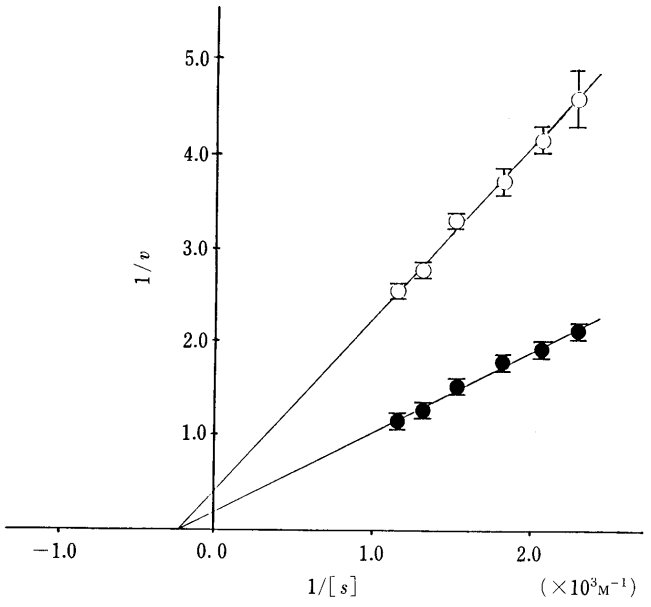


Fig. 1. Inhibitory Effects of **7** on Carbonic Anhydrase

Lineweaver–Burk plots in the absence (0 M, ●—●) and presence (3.1×10^{-7} M, ○—○) of **7** with *p*-nitrophenyl acetate as the substrate. *v*: unit/mg enzyme/min. *s*: substrate.

(IC_{50})[19] was 2.2×10^{-7} M. The inhibitory activities of **1**, **4**, **6** and **11** are low. From the point of structure–activity relationships, the tetramer (**7**) of the galloyl group shows a higher inhibitory activity than the dimer (**11**) or monomer (**1**). In the case of esterification with glucose, the inhibitory activity of **7** decreases, but further addition of a galloyl group to the glucosyl ester of **7**, causes the inhibitory activity to increase [**7**>**3**>**2**]. Two galloyl groups in one molecule show higher activity than one hexahydroxydiphenoyl (HHDP) or one dehydrohexahydroxydiphenoyl (DHHDP) moiety, [**10**>**9**, **4**]; the HHDP moiety shows higher activity than the DHHDP moiety [**8**>**5**; **9**>**4**]. Addition of a further galloyl group to the molecules increases their inhibitory activity [**5**>**4**; **8**>**9**].

Kinetic studies, using Lineweaver–Burk plots,[20] were carried out on the effect of **3** or **7** on the hydrolysis of *p*-nitrophenyl acetate by CA, under the assay conditions described. The results for **7** are shown in Fig. 1. The type of inhibition by both **3** and **7** is noncompetitive. The K_i values for **3** and **7** for the hydrolysis of *p*-nitrophenyl acetate by CA were 3.9×10^{-7} M and 2.4×10^{-7} M, respectively. Thus, we have found a new biological activity for ellagitannins.

Materials and Methods

The following instruments were used to obtain physical data. The optical rotations were determined with a JASCO DIP-360 digital polarimeter. FAB-Mass spectra were recorded on a JEOL JMS-SX-102 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FX-90Q spectrometer (89.55 and 22.50 MHz, respectively), JEOL JNX-GSX-270 spectrometer (270.05 and 67.80 MHz, respectively) and JEOL JNX-GSX-500 spectrometer (500.0 and 125.7 MHz, respectively). HPLC was carried out on a JASCO 880-PU. Normal-phase HPLC was performed on a Develosil 60-5 column, with hexane–MeOH–tetrahydrofuran–formic acid (55 : 33 : 11 : 1, by vol.) containing oxalic acid (1 g/l) as the mobile phase, and reversed-phase HPLC was carried out on a column of YMC R-ODS-7 with 0.05M H_3PO_4 aq.–0.05 M KH_2PO_4 aq.–EtOH–EtOAc (8 : 8 : 3 : 1, by vol.) and 0.05 M H_3PO_4–0.05 M KH_2PO_4–MeOH (13 : 13 : 14, by vol.) as mobile phases. Detection was by UV absorption at 254 nm or 280 nm. Analytical or preparative thin-layer chromatography (PLC) was performed on Funakoshi cellulose plates with 7% acetic acid as mobile phase, and the spots were visualized by UV irradiation (254 nm) or by spraying with $FeCl_3$ solution. Sephadex LH-20 (Pharmacia), MCI-gel CHP 20P (Mitsubishi Chemical Industries Ltd.), Toyopearl HW-40C (Tosoh) and Daiaion HP-20 (Mitsubishi Chemical Industries Ltd.) were used for column chromatography with H_2O–EtOH and H_2O–MeOH gradient solvent elution. The spectrophotometric measurements were carried out on a Hitachi U-1100 spectrophotometer.

Enzyme and Chemicals Carbonic anhydrase (EC 4.2.1.1) from bovine erythrocytes was obtained from Sigma Chemical Co. *p*-Nitrophenyl acetate was obtained from Tokyo Kasei Kogyo Co., Ltd. Dimethyl sulfoxide (DMSO), 2-amino-2-hydroxymethyl-1,3-propanediol and gallic acid were obtained from Kanto Chemical Co., Inc. Ellagic acid and acetazolamide were obtained from Aldrich Chemical Company, Inc.

Substrates The substrate of CA is 1.5 mM *p*-nitrophenyl acetate (*p*-nitrophenyl acetate, 136.0 mg, to be dissolved in acetone, 10.0 ml, and to be added to water to give 100 ml solution). It is prepared immediately before use.

Enzyme Solution The enzyme solution of CA, containing about 200 units per ml in 15 mM Tris–sulfate buffer (pH 7.6), is prepared immediately before use.

Test Solution Samples were dissolved in EtOH and DMSO, and diluted with water to give final concentrations of 2% (w/v) for EtOH and 1% (w/v) for DMSO in the assay solution. At these concentrations of EtOH and DMSO, enzyme activities were little affected.

Assay of CA Activity The CA activities, with *p*-nitrophenyl acetate as a substrate, were measured spectrophotometrically by the reported method[13] with the following modification. The test solution (1.0 ml), 15 mM Tris–sulfate buffer (pH 7.6) (2.9 ml) and the enzyme solution of CA (0.1 ml) were mixed and preincubated at 37 °C for 15 min. After this, the substrate of CA (2.0 ml) was added to assay mixture and then incubated at 37 °C for 40 min. Then, 1 N HCl (1.0 ml) was added to stop the reaction. The absorbance of the reaction mixture was measured spectrophotometrically at 348 nm. The activity was given as the mean of four experiments.

Calculation of CA Activity unit/mg = $\Delta A_{348\,nm} \times$ ml reaction mixture (7.0 ml) × 1000/5.4 × mg enzyme × min (40 min). The molar absorption coefficient for *p*-nitrophenol had been determined to be 5.4×10^3.

Estimation of CA Inhibitory Activity and IC_{50} Enzyme inhibitory activity was expressed as the percentage of enzyme inhibition in the above assay system, calculated as $(1 - B/A) \times 100$, where A is the activity of the enzyme without the test material and B is the activity of the enzyme with the test material. The IC_{50} was obtained from the probit-logarithmic scale graph[19] using six measured points.

Extraction and Isolation The dried pericarps (5 kg, commercial products) of *Punica granatun* L. were extracted at room temperature with 90% aqueous acetone. After removal of the acetone by evaporation under reduced pressure (*ca.* 40 °C), the residue was partitioned between EtOAc and H_2O. The insoluble solids were filtered off. The aqueous solution was chromatographed on a Diaion HP-20 column with H_2O and MeOH successively to give H_2O and MeOH fractions. The yields and inhibitory activities are shown in Chart 1. From the MeOH fraction with highest activity (3 g), four tannins, **2** (80.0 mg), **3** (1.0 g), **4** (40.0 mg) and **5** (17.9 mg), were isolated by Sephadex LH-20 (H_2O–MeOH), MCI-gel CHP 20P (H_2O–MeOH) and Toyopearl HW-40C (H_2O–EtOH) column chromatography, followed by ODS-7 HPLC using the solvent system described above. From the EtOAc fraction (3 g), six tannins, **1** (13 mg), **6** (20.0 mg), **7** (34.2 mg), **8** (19.4 mg), **9** (20.0 mg) and **10** (130.7 mg) were isolated by Sephadex LH-20 (H_2O–EtOH), MCI-gel CHP 20P (H_2O–MeOH), and Toyopearl HW-40C (H_2O–MeOH) column chromatography followed by ODS-7 and Develosil 60-5 HPLC using the solvent systems described above. From the EtOAc–H_2O insoluble solids (5 g), **11** (1 g) and **7** (3 g) were purified by fractional recrystallization from MeOH, followed ODS-7 HPLC using the solvent system described above. Each purified component was identified by comparison with authentic samples and/or the data from the literatures: punicalin (**2**),[5] punicalagin (**3**),[5] granatin A (**4**),[6,7] granatin B (**5**),[7,8] corilagin (**6**),[7,8] gallagyldilactone (**7**),[5,6] casuarinin (**8**),[14,15] pedunclagin (**9**),[14,16] and tellimagrandin I (**10**).[16,17]

Lineweaver–Burk Plots The Lineweaver–Burk plots[20] for CA, with *p*-nitrophenyl acetate as a substrate, were made under our assay conditions in the absence and presence of **3** or **7**. The type of inhibition by both **3** and **7** is noncompetitive. The K_i values of **3** and **7** for the hydrolysis of *p*-nitrophenyl acetate by CA were 3.9×10^{-7} M ($i = 1.8 \times 10^{-7}$ M) and 2.4×10^{-7} M ($i = 3.1 \times 10^{-7}$ M), respectively (Fig. 1).

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References

1) T. Shimizu, *Nippon Rinsho*, **468**, 1208 (1981); S. Morimoto, *ibid.*, **468**, 964 (1981).
2) Chiang Su New Medicinal College (ed.), "Dictionary of Chinese Crude Drugs," Shanghai Scientific Technologic Publisher, Shanghai, 1977, p. 619.
3) E. Flaccomio, *Prog. Terap. Sez. Farm.*, **18**, 183 (1929) [*Chem. Abstr.*, **24**, 1931[8] (1930)].
4) S. Rajadurai, Y. M. Theresa, K. N. S. Sastry, *Leather Sci.*, **10**, 141 (1963).
5) T. Tanaka, G. Nonaka, I. Nishioka, *Chem. Pharm. Bull.*, **34**, 650 (1986).
6) E. A. Haddock, R. K. Gupta, E. Haslam, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 2535.
7) T. Tanaka, G. Nonaka, I. Nishioka, *Phytochemistry*, **24**, 2075 (1985).
8) T. Yoshida, T. Okuda, *Heterocycles*, **14**, 1743 (1980).
9) T. Hatano, T. Yasuhara, R. Yoshihara, I. Agata, T. Noro, T. Okuda, *Chem. Pharm. Bull.*, **38**, 1224 (1990).
10) F. Kiuchi, Y. Tsuda, K. Kondo, H. Yoshida, I. Nishioka, G. Nonaka, *Chem. Pharm. Bull.*, **36**, 1796 (1988).
11) Y. Fujita, I. Uehara, Y. Morimoto, M. Nakashima, T. Hatano, T. Okuda, *Yakugaku Zasshi*, **108**, 129 (1988).
12) J. A. Verpoorte, S. Mehta, J. T. Edsall, *J. Biol. Chem.*, **244**, 4221 (1967).
13) *a*) M. J. Armstrong, D. V. Meyers, J. A. Verpoorte, J. T. Edsell, *J. Biol. Chem.*, **241**, 5137 (1966); *b*) T. Noro, M. Ehara, A. Ueno, *Chem. Pharm. Bull.*, **38**, 1403 (1990).
14) T. Okuda, T. Yoshida, M. Ashida, K. Yazaki, *J. Chem. Soc., Perkin Trans. 1*, **1983**, 1765.
15) T. Tanaka, G. Nonaka, I. Nishioka, *Chem. Pharm. Bull.*, **34**, 656 (1986).
16) T. Hatano, T. Yoshida, T. Shingu, T. Okuda, *Chem. Pharm. Bull.*, **36**, 2925 (1988).
17) R. K. Gupta, S. M. K. Al-Shhafi, K. Layden, E. Haslam, *J. Chem. Soc., Perkin Trans. 1*, **1982**, 2525.
18) *a*) Y. Pocker, J. T. Stone, *J. Am. Chem. Soc.*, **87**, 5498 (1965); *b*) Society of Japanese Pharmacopoeia (ed.), "Commentary of the Japanese Pharmacopoeia," 12th ed., Hirokawa Publishing Co., Inc., Tokyo, 1991, p. c-57.
19) K. Yamada, N. Cyujyo, "Seibutsugaku-teki Shikenhou (The Methods of Bioassay)," Hirokawa Publishing Co., Inc., Tokyo, 1979, p. 47.
20) H. Lineweaver, D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934); M. Dixon, E. C. Weff, "Enzymes," 3rd ed., Longman Group Ltd., London, 1979, p. 341.