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# Specificity of Esterases and Structures of Prodrug Esters. III. Activity of Rat Tissue Homogenates, Rat Plasma and Porcine Liver Esterase for the Hydrolysis of 3′,5′-Bis-dicarboxylic Acid Hemiesters of 5-Fluoro-2′-deoxyuridine

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Five acidic 3′,5′-diesters of 5-fluoro-2′-deoxyuridine (FUdR) including 3-carboxypropionate, 4-carboxybutyrate, 5-carboxypentanoate, 6-carboxyhexanoate and 7-carboxyheptanoate were synthesized, and their susceptibility to porcine liver, rat liver homogenate, rat intestinal homogenate and rat plasma esterase preparations was studied. The susceptibility of the derivatives to porcine liver esterase preparation increased as the number of methylene groups in the ester promoiety increased in both acidic (pH 5.0) and neutral (pH 7.0) solutions. The derivatives showed about 100 times higher reactivity to the esterase at pH 5.0 than at pH 7.0. With the rat tissue homogenates and plasma preparations, the longer the ester chain the higher the susceptibility, except for 3′,5′-bis(3-carboxypropionyl)FUdR (I) with rat liver homogenate. Though the reactivity of I was the lowest with porcine liver esterase preparation and not measurable with rat intestinal homogenate and plasma, I showed the highest reactivity with the rat liver homogenate.

**Keywords**——5-fluoro-2′-deoxyuridine; ester prodrug; acidic promoiety; enzymatic hydrolysis; tissue homogenate; esterase

Appropriate control of drug levels at a target site is required for effective cancer chemotherapy, and many attempts have been made to improve drug delivery by chemical transformation of a parent compound.[1–5] In a prodrug approach, not only the physicochemical properties but also the biological lability is important to control the regeneration of the parent drug. Thus, the biological lability of many promoieties has been investigated in various enzyme systems.[6–10]

In previous studies, we found a close relationship between the bioactivation characteristics and the chemical structure of 3′,5′-diacyl-5-fluoro-2′-deoxyuridine esterified with a saturated aliphatic acid.[11,12] Biological experiments revealed that some of these ester prodrugs showed up to 100 times higher antitumor activity than the parent drug, and that the high activity was related to a slow bioactivation rate of the prodrugs. In the present study, five 3′,5′-bis-dicarboxylic acid hemiesters of 5-fluoro-2′-deoxyuridine (FUdR) having negative charges under physiological conditions were synthesized to determine their susceptibility to various enzyme systems.

The purpose of the study was to determine the relative selectivity of crude gut, liver and blood preparations obtained from the rat and of a preparation of porcine liver esterase for the hydrolysis of certain anionic esters of FUdR. The data obtained may help in the selection of an acyl function allowing release of the drug at a selected point. The methods of study employed were admittedly crude in that no serious efforts were made to separate soluble and cell-bound enzymes when tissue homogenates were used. The results nevertheless provide

interesting insights into the degree of structural specificity in these reactions.

## Experimental

**General Procedures**——Ultraviolet (UV) absorption spectra were recorded on a Shimadzu 260 UV-VIS spectrophotometer. Field desorption (FD)-mass spectral (MS) data were obtained on a Hitachi M-80B mass spectrometer. Proton nuclear magnetic resonance ($^1$H-NMR) spectra were taken on a Varian EM 360A. Thin-layer chromatography (TLC) was carried out on TLC aluminum sheets pre-coated with a 0.25 mm layer of Silica gel 60 $F_{254}$ (E. Merck), using the solvent system of chloroform–ethanol–acetic acid (89.5 : 9.9 : 0.6).

**Materials**——FUdR was purchased from Heinrich Mach Nachf. All other chemicals were of reagent grade and were obtained commercially.

**Synthesis of Derivatives**——3′,5′-Bis(3-carboxypropionyl)FUdR (I) was prepared as follows. Succinic anhydride 2.5 (mol eq) and 4-dimethylaminopyridine (0.1 mol eq) were added to 300 mg of FUdR in 10 ml of anhydrous pyridine at 0 °C. The mixture was held at room temperature for 12 h, then the solvent was removed *in vacuo*. The residue was added to 50 ml of ice-cooled water. The aqueous layer was adjusted to pH 3.0 with 1 N HCl and extracted three times with 50 ml of ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The residue was chromatographed on a silica gel column, and developed with a mixture of chloroform–ethanol. Evaporation of the eluate *in vacuo* gave I (530 mg, 88% yield) as an amorphous solid. TLC *Rf* 0.14. $^1$H-NMR (methanol-$d_4$) $\delta$: 2.25—2.65 (10H, m, $C_2$·H, $COCH_2CH_2CO$), 4.10—4.35 (3H, m, $C_4$·H, $C_5$·H), 5.05—5.20 (1H, m, $C_3$·H), 6.15 (1H, t, $C_1$·H), 7.75 (1H, d, $J$=6.5 Hz, $C_6$H). MS *m/e*: 447 (M+1). UV $\lambda_{max}^{ethanol}$ ($\varepsilon \times 10^3$): 208.1 (7.1), 266.7 (7.2).

3′,5′-Bis(4-carboxybutyryl)FUdR (II) was prepared from FUdR with glutaric anhydride in a similar manner. (II); 80% yield; oil. TLC *Rf* 0.22. $^1$H-NMR (chloroform-*d* : methanol-$d_4$ (4 : 1)) $\delta$: 1.70—2.10 (4H, m, $COCH_2CH_2$), 2.15—2.60 (10H, m, $C_2$·H, $COCH_2CH_2CH_2CO$), 4.25—4.50 (3H, m, $C_4$·H, $C_5$·H), 5.15—5.40 (1H, m, $C_3$·H), 6.20 (1H, t, $C_1$·H), 7.90 (1H, d, $J$=6.5 Hz, $C_6$H). MS *m/e*: 475 (M+1). UV $\lambda_{max}^{ethanol}$ ($\varepsilon \times 10^3$): 208.5 (7.1), 266.5 (7.2).

3′,5′-Bis(5-carboxypentanoyl)FUdR (III) was prepared as follows. Adipic acid (10 mol eq) and dicyclohexylcarbodiimide (DCC) (2.0 mol eq) were added to 300 mg of FUdR in 20 ml anhydrous pyridine at room temperature. The mixture was held at room temperature for 12 h, then the solvent was removed *in vacuo*. The residue was added to 50 ml of ice-cooled water. The aqueous layer was adjusted to pH 3.0 with 1 N HCl, and extracted three times with 50 ml of ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate and evaporated *in vacuo*. The residue was chromatographed on a silica gel column and eluted with a mixture of chloroform–ethanol. Evaporation of the eluate *in vacuo* gave III (257 mg, 42% yield) as an amorphous solid. TLC *Rf* 0.35. $^1$H-NMR (chloroform-*d* : methanol-$d_4$ (4 : 1)) $\delta$: 1.45—1.95 (8H, m, $COCH_2CH_2CH_2$), 2.05—2.55 (10H, m, $C_2$·H, $COCH_2$–$CH_2CO$), 4.15—4.45 (3H, m, $C_4$·H, $C_5$·H), 5.10—5.30 (1H, m, $C_3$·H), 6.20 (1H, t, $C_1$·H), 7.60 (1H, d, $J$=6.5 Hz, $C_6$H). MS *m/e*: 503 (M+1). UV $\lambda_{max}^{ethanol}$ ($\varepsilon \times 10^3$): 208.8 (7.0), 266.9 (7.1).

3′,5′-Bis(6-carboxyhexanoyl)FUdR (IV) and 3′,5′-bis(7-carboxyheptanoyl)FUdR (V) were prepared from FUdR with pimelic acid and suberic acid, respectively, in a manner similar to that used for III.

IV; 40% yield; amorphous solid. TLC *Rf* 0.45. $^1$H-NMR (chloroform-*d*) $\delta$: 1.40—1.90 (12H, m, $COCH_2CH_2CH_2CH_2$), 2.05—2.60 (10H, m, $C_2$·H, $COCH_2$–$CH_2CO$), 4.10—4.40 (3H, m, $C_4$·H, $C_5$·H), 5.00—5.25 (1H, m, $C_3$·H), 6.15 (1H, t, $C_1$·H), 7.60 (1H, d, $J$=6.5 Hz, $C_6$H). MS *m/e*: 531 (M+1). UV $\lambda_{max}^{ethanol}$ ($\varepsilon \times 10^3$): 208.9 (7.1), 266.5 (7.2).

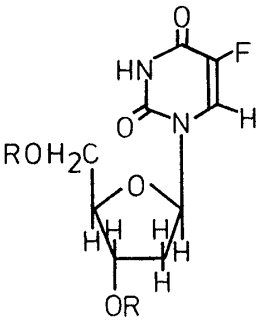
V; 43% yield; amorphous solid. TLC *Rf* 0.63. $^1$H-NMR (chloroform-*d*) $\delta$: 1.25—1.95 (16H, m, $COCH_2CH_2CH_2CH_2CH_2$), 2.10—2.55 (10H, m, $C_2$·H, $COCH_2$–$CH_2CO$), 4.15—4.40 (3H, m, $C_4$·H, $C_5$·H), 5.10—5.25 (1H, m, $C_3$·H), 6.20 (1H, t, $C_1$·H), 7.70 (1H, d, $J$=6.5 Hz, $C_6$H). MS *m/e*: 553 (M+1). UV $\lambda_{max}^{ethanol}$ ($\varepsilon \times 10^3$): 208.8 (7.0). 266.5 (7.4).

**Stability Study in Aqueous Solution and Biological Media**——Stock solutions of all derivatives were prepared in ethanol to give a concentration of $4 \times 10^{-3}$ M, and 10 $\mu$l of the stock solution was mixed with 1 ml of aqueous buffer or enzyme preparation for kinetic studies.

Chemical hydrolysis rates were determined in 0.1 M phosphate buffer (pH 7.0, $\mu$=0.22) at 37 °C. Degradation was initiated by addition of the stock solution to preheated buffer to give a concentration of $4 \times 10^{-5}$ M. Aliquots of the solution were withdrawn at appropriate time intervals and analyzed by high performance liquid chromatography (HPLC).

Male Sprague Dawley rats weighing 250—280 g were used to obtain blood, liver and small intestine. The rats were killed by exsanguination with a heparinized syringe *via* the descending aorta. The blood was centrifuged at 1000 ***g*** for 15 min, and the resulting plasma was used for the experiments. The small intestine was washed with saline and the mucosa membrane was removed by scraping with a cover glass. A half gram of the liver or the intestinal mucosa was homogenized with 5 ml of isotonic phosphate buffer (pH 7.0) containing 0.19 M sucrose at 0 °C by using a glass homogenizer and a Teflon pestle. The homogenates were centrifuged at 600 ***g*** for 10 min, and the supernatant was used for the experiments. A 1 ml aliquot of an esterase preparation of porcine liver (Sigma, #E-9627, 1500

TABLE I. Structures and Properties of 3′,5′-Bis-dicarboxylic Acid Hemiesters of FUdR

| General structure | Compound No. | –R | Formula | $M_r$ | HPLC Condition (0.2% $CH_3COOH$ : $CH_3CN$) |
|---|---|---|---|---|---|
|  | I | –CO–$(CH_2)_2$–COOH | $C_{17}H_{19}FN_2O_{11}$ | 446 | 85 : 15 |
| | II | –CO–$(CH_2)_3$–COOH | $C_{19}H_{23}FN_2O_{11}$ | 474 | 75 : 25 |
| | III | –CO–$(CH_2)_4$–COOH | $C_{21}H_{27}FN_2O_{11}$ | 502 | 70 : 30 |
| | IV | –CO–$(CH_2)_5$–COOH | $C_{23}H_{31}FN_2O_{11}$ | 530 | 65 : 35 |
| | V | –CO–$(CH_2)_6$–COOH | $C_{25}H_{35}FN_2O_{11}$ | 552 | 60 : 40 |
| | FUdR | –H | $C_9H_{11}FN_2O_5$ | 246 | 95 : 5 |

units/ml) was diluted with 4.0 ml of the isotonic buffer (pH 7.0), then this solution was filtered through a membrane filter (Millipore, 0.45 $\mu$m), and used for the experiments. The temperature was maintained at 0—5 °C during the preparation and storage of the enzyme systems. All enzyme preparations were kept for no more than 50 h.

The enzymatic hydrolysis rates of the derivatives were determined in the presence of various enzyme preparations diluted appropriately with isotonic phosphate buffer (pH 7.0) or 0.1 M acetate buffer (pH 5.0, $\mu=0.09$). The experiments were performed at 37 °C and initiated by adding stock solution of the derivatives to give a final concentration of $4\times10^{-5}$ or $8\times10^{-5}$ M. The decrease in concentration of the FUdR derivatives and the increase in concentration of released FUdR were followed by HPLC analysis of samples taken periodically from the reaction mixture.

**Analysis**——Degradation of the FUdR derivatives was monitored by HPLC (LC-3A, Shimadzu) with a variable-wavelength UV detector (SPD-2A, Shimadzu). Mixtures of acetonitrile and 0.2% acetic acid in the ratios shown in Table I were used as the mobile phase at a flow rate of 0.8 ml/min. A 10 $\mu$l sample was injected directly into a $\mu$-Bondapack C-18 column fitted with a guard precolumn (Lichrosorb RP-18, Brownlee). Detection was achieved by UV absorption measurement at 270 nm.

## Results and Discussion

### Stability and Aqueous Buffer Solution

The half-lives of the derivatives of FUdR in 0.1 M phosphate buffer (pH 7.0, $\mu=0.22$) at 37 °C were well over 5000 h for compounds II—V, and 1020 h for I. Compound I was less stable than the other derivatives presumably because of intramolecular nucleophilic attack of the carboxyl group on the ester linkage. Since the experiments on enzymatic hydrolysis were carried out within 2 h, these derivatives can be regarded as chemically stable in evaluating their susceptibility to enzymes.

### Activity of Porcine Liver Esterase

The rate of enzymatic degradation of the derivatives showed a significant dependence on the esterase concentration, and did not decrease even when the substrate concentration was doubled from $4\times10^{-5}$ to $8\times10^{-5}$ M (data not shown). The high stability of FUdR in porcine liver esterase solution was established in the previous study.[12] The susceptibility of the derivatives to hydrolysis by the esterase was studied in a similar manner to that reported previously.[11] That is, to evaluate the rate constants of enzymatic hydrolysis of the monoesters, the time courses of appearance of FUdR from each diester prodrug were measured at various esterase concentrations. Since the regeneration of FUdR by esterase should occur *via* two separate hydrolytic cleavages, diester to monoester and monoester to FUdR, the overall decomposition reaction may be described by the following equations:

$$C(\mathrm{A}) = C_0(\mathrm{A})e^{-K_1 t} \quad (1)$$

$$C(\mathrm{C}) = C_0(\mathrm{A})[1 + 1/(K_1 - K_2) \times (K_2 e^{-K_1 t} - K_1 e^{-K_2 t})] \quad (2)$$

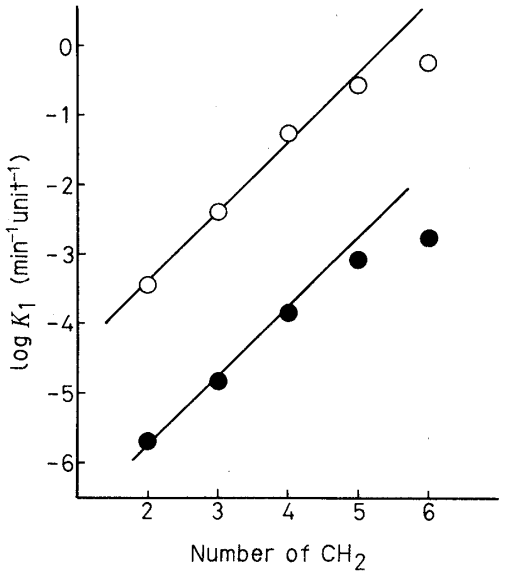



Fig. 1. Relationship between $K_1$ Values and Structures of the Ester Promoiety at pH 5.0 (—○—) and pH 7.0 (—●—)

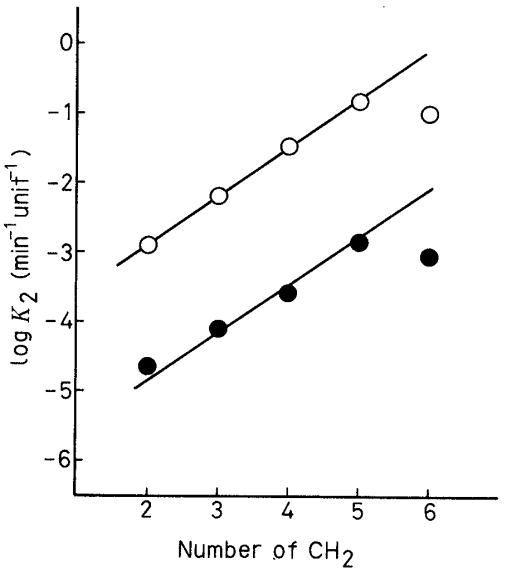



Fig. 2. Relationship between $K_2$ Values and Structures of the Ester Promoiety at pH 5.0 (—○—) and pH 7.0 (—●—)

where $C_0$(A) represents the initial concentration of diester prodrug, and $C$(A) and $C$(C) are the concentrations of diester and FUdR at time $t$.

Based on these equations, curve-fitting and parameter estimation were done by using a non-linear least-squares program.[13]

Figures 1 and 2 show the relationship between the number of methylene groups in the ester promoiety and the susceptibility to the esterase. For the two hydrolytic cleavages, whose rate constants are represented by $K_1$ and $K_2$, the reactivity of the derivatives increases as the number of methylene groups increases. A reasonably good linear relationship between the number of methylene groups and log rate constant was observed for both $K_1$ and $K_2$ values in compounds I through IV, but the increasing trend of reactivity was less marked in the case of V.

Since the derivatives involved in this study bear carboxyl groups, separate experiments were carried out in an acidic solution (pH 5.0) in order to evaluate the susceptibility of the neutral form of the derivatives relative to that of the corresponding ionic form. In order to evaluate the change of the enzyme activity under acidic conditions, the reactivity of a neutral derivative (3′,5′-diacetyl-FUdR) was measured in both the neutral and acidic solutions. The activity of the porcine liver esterase towards the neutral substrate at pH 5.0 was 5.2 times lower than that at pH 7.0. Therefore, the hydrolytic rate constants of the acidic derivatives measured at pH 5.0 were increased 5.2-fold compared with those measured at pH 7.0 (Figs. 1 and 2). As shown in Figs. 1 and 2, the relative susceptibility of the derivatives at pH 5.0 is about 100 times higher than that at pH 7.0. A more detailed study is required to correct for the change of enzyme activity between pH 7.0 and 5.0, but this observation suggests that it is mainly the unionized form of the acidic derivatives that is involved in the reaction.

**Activity of Rat Tissue Homogenates and Plasma**

The relative susceptibility of the FUdR derivatives to enzymatic hydrolysis was examined at pH 7.0 and 37 °C in the post nuclear fraction of 0.5% rat liver and 0.5% rat intestinal homogenates and in 20% rat plasma. For both the homogenates and the plasma, the decrease in concentration of the derivatives showed pseudo-first-order kinetics. The rate constants were calculated by linear regression analysis of a logarithmic plot of concentration against

Table II. Rate Constants for Enzymatic Hydrolysis of FUdR Derivatives in 20% Rat Plasma (pH 7.0, 37 °C)

| Compound No. | Rate constants ($min^{-1}$) | S.D. ($min^{-1}$) | Number of exp. |
|---|---|---|---|
| I | $<1.0 \times 10^{-5}$ | | 5 |
| II | $<1.0 \times 10^{-5}$ | | 4 |
| III | $<1.0 \times 10^{-5}$ | | 4 |
| IV | $1.32 \times 10^{-4}$ | $3.26 \times 10^{-5}$ | 5 |
| V | $5.28 \times 10^{-4}$ | $1.23 \times 10^{-4}$ | 7 |

S.D., standard deviation.

Table III. Rate Constants for Enzymatic Hydrolysis of FUdR Derivatives in 0.5% Rat Intestinal Homogenate (pH 7.0, 37 °C)

| Compound No. | Rate constants ($min^{-1}$) | S.D. ($min^{-1}$) | Number of exp. |
|---|---|---|---|
| I | $<1.0 \times 10^{-5}$ | | 5 |
| II | $<1.0 \times 10^{-5}$ | | 5 |
| III | $2.86 \times 10^{-4}$ | $1.92 \times 10^{-4}$ | 6 |
| IV | $1.24 \times 10^{-3}$ | $9.49 \times 10^{-4}$ | 5 |
| V | $1.22 \times 10^{-2}$ | $7.09 \times 10^{-3}$ | 6 |

Table IV. Rate Constants for Enzymatic Hydrolysis of FUdR Derivatives in 0.5% Rat Liver Homogenate (pH 7.0, 37 °C)

| Compound No. | Rate constants ($min^{-1}$) | S.D. ($min^{-1}$) | Number of exp. |
|---|---|---|---|
| I | $2.76 \times 10^{-2}$ | $6.38 \times 10^{-3}$ | 12 |
| II | $<1.0 \times 10^{-5}$ | | 4 |
| III | $<1.0 \times 10^{-5}$ | | 4 |
| IV | $5.99 \times 10^{-4}$ | $1.44 \times 10^{-4}$ | 7 |
| V | $1.53 \times 10^{-2}$ | $4.75 \times 10^{-3}$ | 7 |

time and the results are summarized in Tables II—IV.

Rat Plasma: As can be seen in Table II, compounds I, II and III were hardly hydrolyzed in 20% rat plasma. Compounds IV and V showed degradation with half-lives of 87.5 and 21.9 h, respectively.

Rat Intestine: As can be seen in Table III, the enzymatic hydrolysis rate increases as the number of methylene groups in the ester promoiety increases from four (III) to six (V). This order of susceptibility is the same as that obtained for the porcine liver esterase. Again, degradation was not observed in the case of compounds I and II.

Rat Liver: As can be seen in Table IV, compounds I and V showed significant reactivity to rat liver homogenate. The specificity of the rat liver homogenate towards compound I was markedly different from that seen with rat intestinal homogenate, rat plasma and porcine liver esterase, where the compound showed the lowest reactivity or no measurable reactivity. In contrast to I, compounds II and III were hardly hydrolyzed in rat liver homogenate. The reactivity of the derivatives with the liver homogenate increases from III to V, as observed in other enzyme systems.

Although it was not possible to determine the relative susceptibilities of II and III in the liver homogenate, I and II in the intestinal homogenate, and I, II and III in the plasma because of the low reactivity in these systems, an increasing number of methylene groups in the ester promoiety clearly tends to increase the reactivity with the enzyme systems except for compound I with rat liver homogenate. Whether such specific reactivity of I also exists in human liver has not yet been tested, but the observation seems to merit further exploration.

**References and Notes**

1) A. Rosowsky, R. A. Forsch, C. S. Yu, H. Lazarus, and G. P. Beardsley, *J. Med. Chem.*, **27**, 605 (1984).
2) T. Tsuruo, H. Iida, S. Tsukagoshi, and Y. Sakurai, *Cancer Res.*, **39**, 1063 (1979).
3) S. Ozaki, Y. Ike, H. Mizuno, K. Ishikawa, and H. Mori, *Bull. Chem. Soc. Jpn.*, **50**, 2406 (1977).
4) F. Kanzawa, A. Hoshi, K. Kuretani, M. Saneyoshi, and T. Kawaguchi, *Cancer Chemother. Pharmacol.*, **6**, 19 (1981).
5) H. Sasaki, E. Mukai, M. Hashida, T. Kimura, and H. Sezaki, *Int. J. Pharmaceut.*, **15**, 61 (1983).
6) M. Johansen, H. Bundgaard, and E. Falch, *Int. J. Pharmaceut.*, **13**, 89 (1983).
7) V. H. L. Lee, R. E. Stratford, Jr., and K. W. Morimoto, *Int. J. Pharmaceut.*, **13**, 183 (1983).
8) S. Babhair and A. Hussain, *Int. J. Pharmaceut.*, **13**, 273 (1983).
9) D. C. Baker, S. D. Kumar, W. J. Waites, G. Arnett, W. M. Shannon, W. I. Higuchi, and W. J. Lambert, *J. Med. Chem.*, **27**, 270 (1984).
10) H. Sasaki, M. Fukumoto, M. Hashida, T. Kimura, and H. Sezaki, *Chem. Pharm. Bull.*, **31**, 4083 (1983).
11) T. Kawaguchi, Y. Suzuki, Y. Nakahara, N. Nambu, and T. Nagai, *Chem. Pharm. Bull.*, **33**, 1652 (1985).
12) T. Kawaguchi, M. Saito, Y. Suzuki, N. Nambu, and T. Nagai, *Chem. Pharm. Bull.*, **33**, 1652 (1985).
13) K. Yamaoka, Y. Tanigawa, T. Nakagawa, and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).