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Synthesis and enzymatic hydrolysis of aspirin-basic amino acid ethyl esters

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Summary

Aspirin L-arginine ethyl ester (As-Arg-OEt) and aspirin *p*-guanidino-L-phenylalanine ethyl ester (As-GPA-OEt) were synthesized and the hydrolyses of these compounds in the presence of trypsin (Tp) and carboxypeptidase B (CPB) were examined in order to evaluate their availability as prodrugs for aspirin. The ester bonds of both As-Arg-OEt and As-GPA-OEt were hydrolyzed by Tp at about one-tenth the rate of *N*-α-benzoyl-L-arginine ethyl ester, a specific substrate for Tp, which suggests that they are good substrates for this enzyme. Salicylic acid was generated in the hydrolysis of aspirin L-arginine (As-Arg-OH) by CPB after the hydrolysis of As-Arg-OH to salicyl L-arginine (Sa-Arg-OH). On the other hand, aspirin and salicyl *p*-guanidino-L-phenylalanines were not hydrolyzed by CPB, and the latter competitively inhibited the CPB-catalyzed hydrolysis of hippuryl-L-arginine. These results indicate that As-Arg-OEt and As-GPA-OEt were not utilized as prodrugs for aspirin. However, L-arginine, which has alkylguanidine in its side chain, was more available than *p*-guanidino-L-phenylalanine, which has phenylguanidine in its side chain, in the CPB-mediated release of drugs from their prodrugs.

Introduction

Aspirin is the most widely used oral medication for its analgetic, anti-inflammatory and antipyretic effects. It has also been used as an antithrombic agent (Fields et al., 1977; Elwood et al., 1979). Recent studies have suggested that the efficacy of aspirin, given either intravenously or intramuscularly, is comparable to that of narcotic analgesics for the relief of postoperative pain, without producing any respiratory depressant effects (Kweekel-De Vries et al., 1974; Korttila et al., 1980). To date, many prodrugs for aspirin have been synthesized to minimize gastrointestinal irritation and bleeding associated with salicylic acid, and in addition their pharmaceutical properties have been examined (Dittert et al., 1968; Misher et al., 1968; Hussain et al., 1974; Swintosky et al., 1984). It was reported that aspirin L-phenylalanine ethyl ester (As-Phe-OEt) was a prodrug for aspirin, because it cleaved to aspirin in the presence of α-chymotrypsin and carboxypeptidase A (CPA) (Banerjee et al., 1981a–c). However, it was revealed that As-Phe-OEt did not

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cleave to aspirin in the presence of CPA (Kawahara et al., 1983; Muhi-Eldeen et al. 1985).

We synthesized the ethyl esters of aspirin L-arginine (As-Arg-OH) and aspirin *p*-guanidino-L-phenylalanine (As-GPA-OH) as water-soluble prodrugs and anticipated that these derivatives would be available as prodrugs for aspirin if aspirin was released by carboxypeptidase B (CPB) after the hydrolysis of the ester bond by trypsin (Tp) (Fig. 1). Concerning GPA, we reported that the ester, amide and anilide substrates of *N*-substituted GPA were useful for the study of the specificities of trypsin-like enzymes (Tsunematsu et al., 1980, 1985a; Hatanaka et al., 1985) and that a new enzyme which hydrolyzed the substrates of GPA in preference to those of Arg and lysine was found in human and mouse ascitic plasmas and tumor cells (Tsunematsu et al., 1985b). It is also interesting to compare the hydrolytic rate of As-Arg-OH by CPB, whose side chain is alkylguanidine, with that of As-GPA-OH, whose side chain is phenylguanidine, since the alkyl methylenes in the arginyl residue are replaced by a benzene ring in the chemical structure of GPA.

In this paper, we report the synthesis of the ethyl esters of aspirin- and salicylic acid-basic amino acids, the hydrolysis of these derivatives by Tp and CPB, and evaluate the availability in vitro of these compounds as prodrugs for aspirin.

Materials and Methods

L-Arg hydrochloride (HCl) and benzoyl(Bz)-Gly-L-Arg(Hip-Arg) were purchased from the Protein Research Foundation (Osaka, Japan). Aspirin was obtained from Hoei Chemical Co. (Osaka, Japan) and recrystallized from methanol. GPA · HCl was synthesized in the same manner as previously (Tsunematsu et al., 1985a). L-Arg ethyl ester (OEt) · 2HCl and L-GPA-OEt · 2HCl were prepared by using thionyl chloride and absolute ethanol. *p*-Nitrophenyl-*p'*-guanidinobenzoate HCl was synthesized as described by Chase and Shaw (1967). Nitroprusside-ferricyanide (NF) reagent was prepared as described by Makisumi (1952). All chemicals were of analytical or reagent grade. Optical rotations were measured on a Jasco type

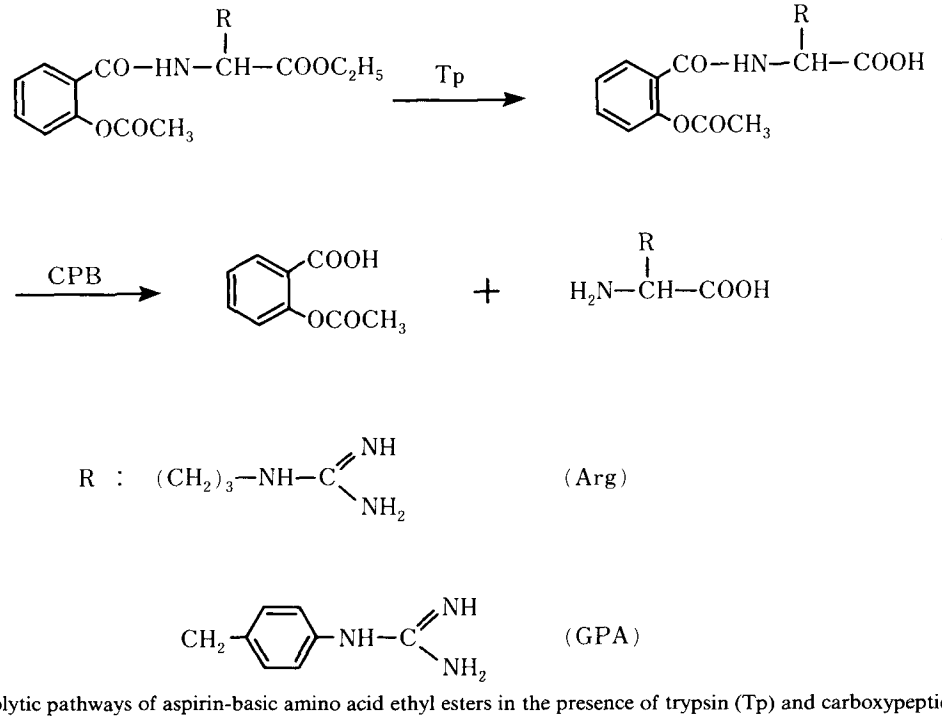

Fig. 1. Hydrolytic pathways of aspirin-basic amino acid ethyl esters in the presence of trypsin (Tp) and carboxypeptidase B (CPB).

DIP-4 digital polarimeter. All melting points were uncorrected. The purity of each compound was checked by thin-layer chromatography on Kieselgel plates 60 PF_{254} (Merck, Darmstadt, Germany) with chloroform-methanol(MeOH)-ethyl acetate (4 : 3 : 1, v/v) and 1-butanol-acetic acid-water (4 : 1 : 1, v/v) as developing systems. The structures of the synthesized compounds were confirmed with the aid of fast atom bombardment and field desorption mass spectrometry using a JEOL JMS-DX 300 with JMA 3000 data system (Tokyo, Japan).

Synthesis

Salicyl arginine (Sa-Arg-OH) Aspirin (1.8 g, 10 mmol) was dissolved in *N,N'*-dimethylformamide (DMF) (15 ml) and then cooled to about −5 °C. To the solution were added L-Arg-OEt · 2HCl (1.4 g, 5 mmol), 1-hydroxybenzotriazole (0.75 g, 5 mmol), triethylamine (0.7 ml, 5 mmol) and dicyclohexylcarbodiimide (DCC) (1 g, 5 mmol) dissolved in 5 ml of DMF. The mixture was stirred for 3 h at −5 °C and for 20 h at room temperature. After removal of the precipitate by filtration, the solvent was evaporated off and the resulting oil was washed with dry ether. After removing the solvent by decantation, the oil was dissolved in methanol, the solution was then applied to a column (2.8 × 100 cm) of Sephadex LH-20 and eluted with MeOH (flow rate, 20 ml h^{-1}, fraction volume, 5 ml). Fractions of eluate containing the desired material as judged by paper chromatography with NF reagent were combined, the solvent was evaporated off, and the resulting oil was dried in vacuo over P_2O_5 to afford a foam. However, an NF reagent positive and ultraviolet (UV) negative by-product (R_f value = 0.50, chloroform-MeOH-ethyl acetate (4 : 3 : 1, v/v)) remained in this product. Therefore, several methods for the removal of this by-product were attempted, but all were unsuccessful. Then, As-Arg-OEt · HCl was saponified at room temperature for 2 h, the reaction mixture was neutralized with 1 N HCl and the solvent was removed in vacuo. The residue was dissolved in 30% methanol solution (50 ml) and applied to a Dowex 50 (NH_4^+ form) column (2.4 × 14 cm). After being washed with 30% methanol solution, the column was eluted with 2 N NH_4OH, the NF reagent and UV positive fractions were combined and the solvent was evaporated to dryness in vacuo. The crude product was recrystallized from MeOH-ethyl acetate. Yield, 843 mg (57.3%), m.p. 180–182 °C, $[\alpha]_D^{20}$ +32.6 ° ($c = 1$, MeOH). Analysis: Calcd. for $C_{13}H_{18}N_4O_4 \cdot 1/2H_2O$: C, 51.47; H, 6.33; N, 18.47. Found: C, 51.17; H, 6.36; N, 18.09. FAB-MS *m/z*: 295 $[M + H]^+$.

Salicyl arginine ethyl ester hydrochloride (Sa-Arg-OEt · HCl) Thionyl chloride (0.16 ml, 2.2 mmol) was added dropwise to absolute ethanol (2 ml) cooled with a mixture of salt and ice. Sa-Arg-OH (590 mg, 2 mmol) was added to the mixture and refluxed for 5 h. The reaction mixture was then cooled, and the solvent was evaporated under reduced pressure. The evaporation was repeated several times after the addition of a small amount of absolute ethanol to the resulting oil. The oily crude product was purified by chromatography on Sephadex LH-20, as described above. The yield of the hygroscopic product was 558 mg (77.8%), $[\alpha]_D^{20}$ −10.4 ° ($c = 1$, MeOH). Analysis: Calcd. for $C_{15}H_{22}N_4O_4 \cdot HCl \cdot 1/2H_2O$: C, 48.98; H, 6.58; N, 15.23. Found: C, 49.27; H, 6.60; N, 15.20. FAB-MS *m/z*: 323 $[M + H]^+$.

Aspirin arginine ethyl ester hydrochloride (As-Arg-OEt · HCl) Sa-Arg-OEt · HCl (540 mg, 1.5 mmol) was dissolved in acetic anhydride (10 ml) and the reaction mixture was heated at 80 ° C for 5 h. The reaction mixture was cooled and the solvent was removed under reduced pressure. The resulting oil was washed with dry ether and purified by chromatography on Sephadex LH-20. This product was a hygroscopic material. Yield, 504 mg (83.8%), $[\alpha]_D^{20}$ −32.6 ° ($c = 1$, MeOH). Analysis: Calcd. for $C_{17}H_{24}N_4O_5 \cdot HCl \cdot 1/2H_2O$: C, 49.82; H, 6.41; N, 13.67. Found: C, 49.98; H, 6.49; N, 13.41. FAB-MS *m/z*: 365 $[M + H]^+$.

Aspirin arginine (As-Arg-OH) As-Arg-OEt · HCl (300 mg, 0.75 mmol) was dissolved in 75 ml of 0.1 M phosphate buffer solution (pH 7.5) containing Tp (30 mg). The reaction mixture was incubated at 25 ° C for about 5 min. After checking the disappearance of As-Arg-OEt · HCl by HPLC, it was frozen and subjected to lyophilization. The lyophilizate was dissolved in MeOH (2 ml), filtered and the filtrate was purified by using

Sephadex LH-20. Yield, 168 mg (66.8%), $[\alpha]_D^{20}$ −16.4° ($c = 1$, MeOH). Analysis: Calcd. for $C_{15}H_{20}N_4O_5 \cdot H_2O$: C, 50.83; H, 6.27; N, 15.81. Found: C, 51.16; H, 6.17; N, 15.54. FAB-MS m/z: 337 $[M + H]^+$.

Salicyl p-guanidino-L-phenylalanine (Sa-GPA-OH) Reaction of aspirin (1.8 g, 10 mmol) and GPA-OEt · 2HCl (1.6 g, 5 mmol) was carried out according to the same method as described for Sa-Arg-OH. Yield, 818 mg (47.8%), m.p. 191–193° C; $[\alpha]_D^{20}$ + 57.6° ($c = 1$, MeOH). Analysis: Calcd. for $C_{17}H_{18}N_4O_4 \cdot H_2O$: C, 56.65; H, 5.60; N, 15.55. Found: C, 56.93; H, 5.87; N, 15.69. FAB-MS m/z: 343 $[M + H]^+$.

Salicyl p-guanidino-L-phenylalanine ethyl ester hydrochloride (Sa-GPA-OEt · HCl) The esterification of Sa-GPA-OH (791 mg, 2.3 mmol) was carried out as described above for Sa-Arg-OEt · HCl. Yield, 752 mg (80.1%), $[\alpha]_D^{20}$ −32.2° ($c = 1$, MeOH). Analysis: Calcd. for $C_{19}H_{22}N_4O_4 \cdot HCl \cdot H_2O$: C, 53.70; H, 5.94; N, 13.19. Found: C, 53.60; H, 6.07; N, 13.47. FAB-MS m/z: 371 $[M + H]^+$.

Aspirin p-guanidino-L-phenylalanine ethyl ester hydrochloride (As-GPA-OET · HCl) The acetylation of Sa-GPA-OEt · HCl (203 mg, 0.5 mmol) was carried out by the same method as used for As-Arg-OEt · HCl. Yield, 211 mg (94.1%), $[\alpha]_D^{20}$ −35.2° ($c = 1$, MeOH). Analysis: Calcd. for $C_{21}H_{24}N_4O_5 \cdot HCl \cdot 1/2H_2O$: C, 55.07; H, 5.73; N, 12.24. Found: C, 55.22; H, 5.82; N, 12.34. FAB-MS m/z: 413 $[M + H]^+$.

Aspirin p-guanidino-L-phenylalanine (As-GPA-OH) This compound was prepared by the hydrolysis of As-GPA-OEt · HCl (300 mg, 0.66 mmol) by Tp as described for As-Arg-OH. Yield, 153 mg (60.2%), $[\alpha]_D^{20}$ +57.1° ($c = 1$, MeOH). Analysis: Calcd. for $C_{19}H_{20}N_4O_5 \cdot H_2O$: C, 56.70; H, 5.52; N, 13.92. Found: C, 56.49; H, 5.45; N. 13.66. FAB-MS m/z: 385 $[M + H]^+$.

As-Arg-OEt and As-GPA-OEt were rapidly and completely hydrolyzed by Tp in the pH-stat titration analysis. This probably indicates that no racemization took place during the synthetic steps.

Enzymes Bovine Tp (lyophilized, salt-free; Lot 10460524-66) was purchased from Boehringer Mannheim-Yamanouchi Co. (Tokyo, Japan) and used without further purification. The relative concentrations were determined by measuring the extinction at 280 nm. The operational normality was then determined by titration of a solution with NPGB as described by Chase and Shaw (1967). The enzyme concentration obtained was found to be 57% of that calculated using an optical factor of 0.695 (Green et al., 1953) and molecular weight of 24000 (Kay et al., 1961). CPB (Type I diisopropyl fluorophosphate, porcine pancreas, Lot 75F-8046) was obtained from Sigma (St. Louis, MO, U.S.A.). The relative concentrations were determined by measuring the extinction at 278 nm and using an optical factor of 0.467 and molecular weight of 34300 (Folk et al., 1960). The k_{cat} and K_m values for the hydrolysis of Hip-Arg by this enzyme were 85.9 s^{-1} and 0.263 mM, respectively. These values were almost the same as those reported previously (Wolff et al., 1962).

Kinetic studies The initial rates of hydrolysis of aspirin- and salicyclic acid-basic amino acid ethyl esters by Tp were determined at pH 7.85 at 25° C (with a water-jacketed cell) using a Radiometer RTS-5 titrator (Copenhagen, Denmark). The buffer solution used was 0.01 M Tris-HCl buffer containing 0.01 M KCl and 0.05 M $CaCl_2$ (Walsh et al., 1970). The enzyme concentrations in the assay mixture were 2.31×10^{-7} M. The substrate concentration ranges were $0.0912–2.27 \times 10^{-3}$ M for As-Arg-OEt, $0.0723–2.27 \times 10^{-3}$ M for Sa-Arg-OEt and $0.21–4.0 \times 10^{-3}$ M for As-GPA-OEt and Sa-GPA-OEt. The values of K_m and k_{cat} were calculated from a plot of E/V vs $1/S$ at seven to eight different substrate concentrations using the least-squares method (Williams et al., 1959). The hydrolysis of the acetoxyl groups of As-Arg-OEt and As-GPA-OEt was not observed during the measurement of the initial rates of hydrolysis of the esters by Tp.

The rates of hydrolysis by CPB of As-Arg-OH, Sa-Arg-OH, As-GPA-OH and Sa-GPA-OH were measured by using HPLC. The enzyme concentration was 3.13×10^{-5} M and the substrate concentrations were 4.0×10^{-3} M in the reaction mixture. Chromatography was performed on a Shimadzu LC-6A system equipped with a UV spectrometric detector (SPD-6A), chromatopac (C-R3A) and system controller (SCL-6A) (Kyoto, Japan). The UV detector was set at 254 nm. The

column was Shimadzu-Dupon Zorbax C_8 (250 × 4.6 mm i.d.). The mobile phase was MeOH-0.072% orthophosphoric acid (55 : 45, v/v) (Lo et al., 1980) and the flow rate was 1.45 ml min^{-1} at 40 ° C. The rate of hydrolysis by CPB of Hip-Arg were followed in terms of the increase in absorbance at 254 nm at 25 ° C by using 0.025 M Tris-HCl buffer (pH 7.65) (Wolff et al., 1962) with a Hitachi 340 spectrophotometer (Tokyo, Japan). The enzyme concentration in the assay mixture was 2.78×10^{-8} M. The substrate concentrations were $1.18–3.93 \times 10^{-4}$ M and the inhibitor concentrations were $0.41–1.23 \times 10^{-3}$ M for Sa-GPA-OH. Measurements were made at four different substrate concentrations and at three different inhibitor concentrations. The inhibition pattern was judged from a Lineweaver-Burk plot (Lineweaver et al., 1934) and the inhibitor constant K_i value was calculated from this plot by the least-squares method.

Results and Discussion

Hydrolysis of aspirin- and salicylic acid-basic amino acid ethyl esters by Tp

Before determining the kinetic constants, the pH-activity relationship for the hydrolysis of As-Arg-OEt by Tp was examined. The initial velocity at a fixed substrate concentration (2.0×10^{-3} M) plotted vs pH gave a bell-shaped curve with a maximum at around 7.85. Quite similar pH-activity profiles were obtained for the hydrolysis of Sa-Arg-OEt, As-GPA-OEt and Sa-GPA-OEt by this enzyme.

The action of Tp on each ester substrate followed Michaelis-Menten kinetics in the range of substrate concentration below 2.5 mM for As-Arg-OEt and Sa-Arg-OEt, and 4.0 mM for As-GPA-OEt and Sa-GPA-OEt (data not shown). The kinetic parameters for Tp with aspirin- and salicylic acid-basic amino acid ethyl esters are presented in Table 1 with those reported for the specific ester substrates of Arg and GPA. Based on the second-order rate constants (k_{cat}/K_m), the value for Sa-Arg-OEt is about 50% lower than that for Bz-Arg-OEt. The K_m and k_{cat} values for Sa-Arg-OEt are almost the same as those for Bz-Arg-OEt. According to a three-step mechanism (Eqn 1), accepted for the Tp-catalyzed hydrolysis of ester substrates, K_m and k_{cat} can be expressed by Eqns 2 and 3, respectively, where ES represents an enzyme-substrate complex, K_s its dissociation constant, EA an acyl enzyme, P_1 a leaving group, and P_2 an acid.

TABLE 1

Kinetic parameters for the hydrolysis of aspirin- and salicylic acid-basic amino acid ethyl esters by trypsin at pH 7.85 at 25 ° C

Compounds	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^{-3}$) ($M^{-1}\,s^{-1}$)
As-Arg-OEt	0.727 ± 0.027	37.4 ± 1.2	51.4 ± 1.4
As-GPA-OEt	2.39 ± 0.07	103 ± 3	43.3 ± 2.4
Sa-Arg-OEt	0.101 ± 0.005	32.3 ± 1.4	320 ± 11
Sa-GPA-OEt	1.26 ± 0.05	336 ± 18	267 ± 13
Bz-Arg-OEt [a]	0.061 ± 0.002	31.4 ± 0.5	512 ± 16
Bz-GPA-OEt [a]	0.90 ± 0.05	414 ± 19	461 ± 11

[a] Tsunematsu et al. (1980).

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} \underset{P_1}{\underset{+}{EA}} \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

$$K_m = K_s k_3/(k_2 + k_3) \qquad (2)$$

$$k_{cat} = k_2 k_3/(k_2 + k_3) \qquad (3)$$

It is generally accepted that the deacylation rate constant (k_3) is much smaller than the acylation rate constant (k_2) for the reaction involving a substrate cleaved at the ester bond. The expressions for K_m and k_{cat} can then be reduced to $K_m = K_s k_3/k_2$ and $k_{cat} = k_3$. Thus, k_{cat} represents the deacylation rate constant. It was therefore assumed that the mechanism described above would be adaptable to the Tp-catalyzed hydrolysis of aspirin- and salicylic acid-basic amino acid ethyl esters. Based on this assumption, it is clear that Sa-Arg-OEt is almost as preferable as Bz-Arg-OEt with respect to its binding ability to the specificity site of Tp. This suggests that the existence of the hydroxyl group in salicylic acid does not have much effect on the binding property of this enzyme. On the other hand, the lack of any

great difference between the k_{cat} values for the two substrates reflects the similar orientation of the scissile bond to the catalytic site of this enzyme. However, As-Arg-OEt was hydrolyzed by Tp at about one-sixth the rate of Sa-Arg-OEt. The K_m value for As-Arg-OEt is about 7 times larger than that for Sa-Arg-OEt, while the k_{cat} value for the former is almost the same as that for the latter. A high K_m value for As-Arg-OEt implies a lower binding affinity of this substrate to Tp than that of Sa-Arg-OEt. This may be due to the existence of the acetyl group in As-Arg-OEt. It is likely that the acetyl group in aspirin may give rise to unfavorable effects on the interaction between aspirin and the S_2 site of Tp.

Sa-GPA-OEt was hydrolyzed by Tp at about half the rate of Bz-GPA-OEt. The K_m and k_{cat} values for Sa-GPA-OEt are almost the same as those for Bz-GPA-OEt. On the other hand, As-GPA-OEt was hydrolyzed by this enzyme at one-sixth the rate of Sa-GPA-OEt. The K_m value for As-GPA-OEt is 2 times larger than that for Sa-GPA-OEt, while the k_{cat} value for the former is about 3 times smaller than that for the latter. These results imply that the existence of the hydroxyl group in Sa-GPA-OEt does not have any influence on the binding and catalytic properties of Tp, but the presence of the acetyl group in As-GPA-OEt provides an unfavorable orientation to the catalytic site of this enzyme.

The changes in the values of the kinetic parameters arose from the existence of the acetyl group in As-GPA-OEt in the hydrolysis of GPA derivatives by Tp, which were different from that in As-Arg-OEt in the hydrolysis of Arg derivatives. Krieger et al. (1974) have presented some data on benzamidine bound in the specificity pocket of Tp and reported fairly large movements of the main chain and some side chains bordering the pocket when the inhibitor molecule is bound. It is likely that the benzene rings in the side chains of GPA derivatives induce a conformational change in the active center of Tp during enzyme-substrate complex formation and the resulting change could be responsible for the difference between the kinetic parameters for the two substrates.

Sa-Arg-OEt was hydrolyzed by Tp as fast as Sa-GPA-OEt. The K_m value for Sa-Arg-OEt is about 12 times smaller than that for Sa-GPA-OEt, while the k_{cat} value for the former is 10 times smaller than that for the latter. Significant difference was found between the kinetic parameters for these two derivatives, and similar results were reported on the hydrolysis of Bz-Arg-OEt and Bz-GPA-OEt by Tp (Tsunematsu et al, 1980). On the other hand, As-Arg-OEt was hydrolyzed as fast as As-GPA-OEt. The K_m value for As-Arg-OEt is about 3 times smaller than that for As-GPA-OEt, while the k_{cat} value for the former is about 3 times smaller than that for the latter. As is distinct from the hydrolysis of the salicylic acid- and benzoyl-basic amino acid ethyl esters by Tp, significant difference was not observed between the kinetic parameters for As-Arg-OEt and those for As-GPA-OEt. Therefore, in the hydrolysis of aspirin-basic amino acid ethyl esters by Tp, it seems that the kinetic parameters are not significantly affected due to the existence of acetyl groups in these derivatives.

There are two absolute requirements for potential CPB substrates (Neurath, 1950; Neurath et al., 1950); the terminal carboxyl group must be free and the C-terminal residue must be of the L-configuration. Aspirin- and salicylic acid-basic amino acid ethyl esters were hydrolyzed by Tp from about one-tenth to half the rate of Bz-Arg-OEt, a specific substrate for Tp. These results indicate that these four aspirin- and salicylic acid-derivatives are good substrates for Tp. It therefore becomes apparent that the four derivatives are changed to CPB substrates by the action of Tp.

Hydrolysis of As-Arg-OH by CPB

The hydrolysis of As-Arg-OH by CPB was examined by using HPLC. Product formation was linear with time, and the initial rates were linear with enzyme concentration. Fig. 2A shows a chromatogram of a mixture of equimolar concentrations (2.70×10^{-4} M) of aspirin (3 min), salicylic acid (4.1 min), As-Arg-OH (5.4 min), Sa-Arg-OH (5.7 min), As-Arg-OEt (8.9 min) and Sa-Arg-OEt (10 min). Fig. 2B (1 and 2) shows the HPLC chromatograms for the hydrolysis of As-Arg-OH by CPB at 0 and 4 h, respectively, after the addition of CPB at 37°C. In Fig. 2B (2), two peaks corresponding to salicylic acid and Sa-Arg-

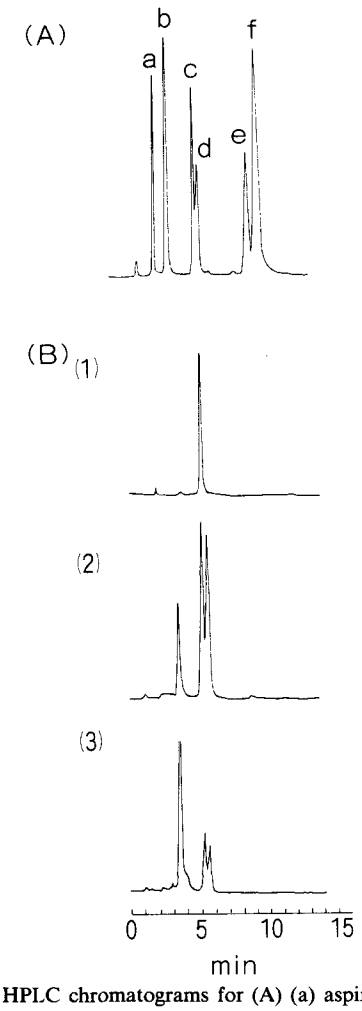

Fig. 2. HPLC chromatograms for (A) (a) aspirin, (b) salicylic acid, (c) As-Arg-OH, (d) Sa-Arg-OH, (e) As-Arg-OEt, (f) Sa-Arg-OEt, and (B) the hydrolysis of As-Arg-OH by CPB at (1) 0 h, (2) 4 h and (3) 24 h.

OH were observed. After 24 h, As-Arg-OH and Sa-Arg-OH were almost hydrolyzed to salicylic acid by CPB, but no aspirin was detected in spite of the existence of As-Arg-OH in the reaction mixture. Then, the hydrolytic rate for acetoxyl group of As-Arg-OH was compared with that for aspirin in the presence or absence of CPB in Tris-HCl buffer. Consequently, the former (1.27 ± 0.09 mM h^{-1}) was 5 times larger than the latter (0.262 ± 0.024 mM h^{-1}) in the presence of CPB, although the rate of As-Arg-OH (0.259 ± 0.022 mM h^{-1}) was almost the same as that of aspirin (0.236 ± 0.019 mM h^{-1}) in the absence of CPB. These results indicate that the faster hydrolysis of the acetoxyl group of As-Arg-OH by CPB was due to the existence of Arg. Therefore, there is one possibility that the acetoxyl group of As-Arg-OH was hydrolyzed owing to the interaction with the amino acid residue of CPB when As-Arg-OH binds to the active site of this enzyme. Further work will be required to elucidate this point in detail. Table 2 shows the salicylic acid releasing rate in the hydrolysis of As-Arg-OH and Sa-Arg-OH by CPB. The hydrolytic rate of Sa-Arg-OH was about 20 times larger than that of As-Arg-OH. Our data showed that salicylic acid was generated in the hydrolysis of As-Arg-OH by CPB after the hydrolysis of As-Arg-OH to Sa-Arg-OH. It is thought that Sa-Arg-OH is a substrate for CPB but As-Arg-OH is not. It is likely that the existence of the acetyl group in As-Arg-OH gives rise to the unfavorable effects in the hydrolysis of this compound by CPB.

Possible hydrolytic pathways of As-Arg-OEt in the presence of Tp and CPB are shown in Fig. 3. As-Arg-OH was generated by the hydrolysis of the ester bond of As-Arg-OEt by Tp, while Sa-Arg-OEt was produced by nonenzymatic hydrolysis of acetoxyl group of As-Arg-OEt. Then, Sa-Arg-OH was formed due either to the CPB-catalyzed and nonenzymatic hydrolysis of As-Arg-OH or to the hydrolysis of Sa-Arg-OEt by Tp. Salicylic acid was finally released in the hydrolysis of Sa-Arg-OH by CPB. As-Arg-OEt is therefore a prodrug for salicylic acid, but not for aspirin, since the acetoxyl group of As-Arg-OH was hydrolyzed by CPB before aspirin was released.

TABLE 2

Hydrolysis of As-Arg-OH and Sa-Arg-OH by carboxypeptidase B at pH 7.65 at 37 °C

Compounds	Salicylic acid releasing rate (mM h^{-1})
As-Arg-OH	0.0923 ± 0.014
Sa-Arg-OH	1.92 ± 0.15

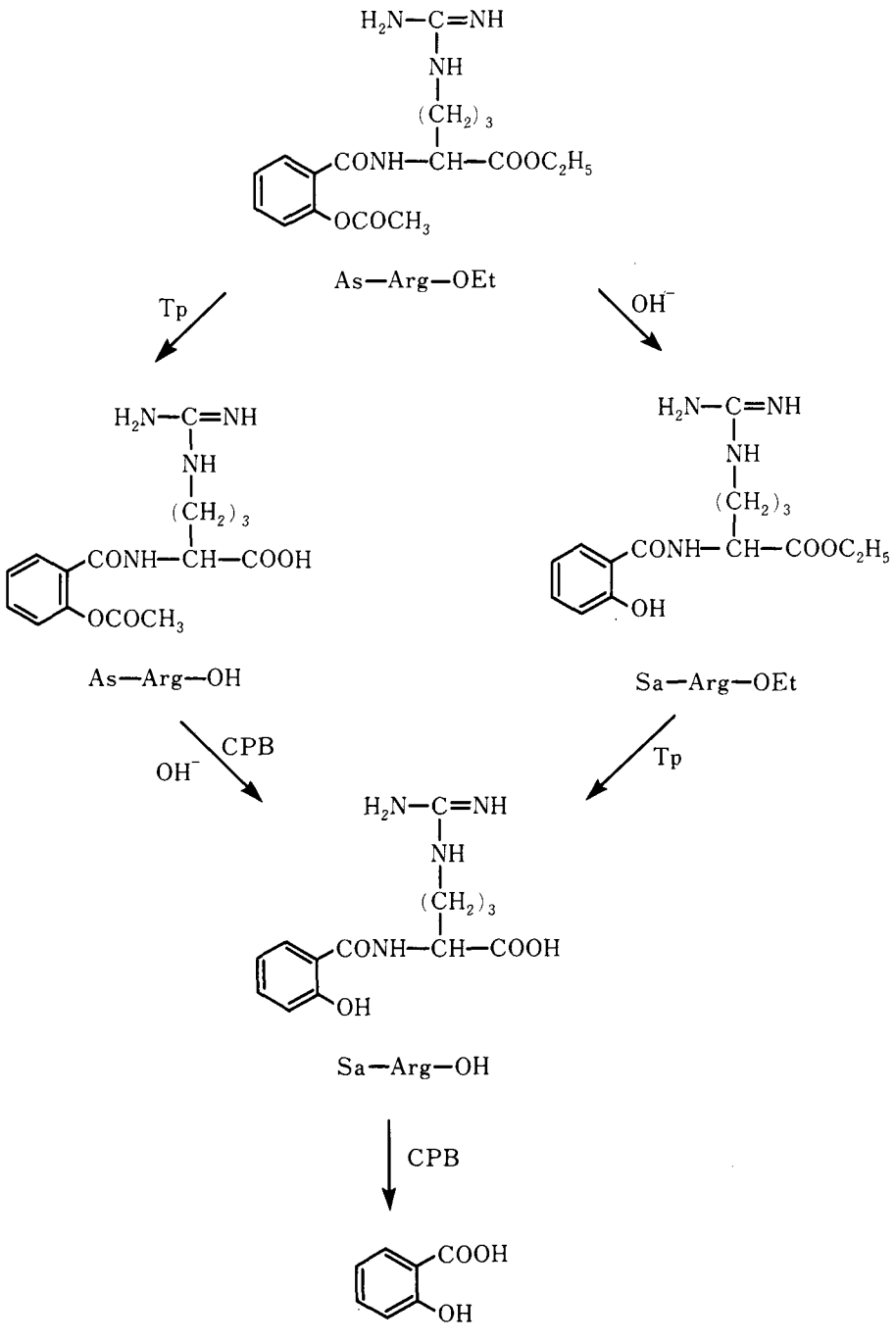

Fig. 3. Possible hydrolytic pathways of As-Arg-OEt in the presence of trypsin (Tp) and carboxypeptidase B (CPB).

Hydrolysis of As-GPA-OH by CPB

The hydrolysis of As-GPA-OH by CPB was also examined by the same method as that of As-Arg-OH. The HPLC chromatogram of a mixture of equimolar concentration (2.70×10^{-4} M) of aspirin, salicylic acid, Sa-GPA-OH (5.7 min), As-GPA-OH (5.9 min), Sa-GPA-OEt (9.2 min) and As-GPA-OEt (10 min) was almost the same as that of the Arg derivatives (data not shown). In the hydrolysis of As-GPA-OH by CPB, neither salicylic acid nor aspirin was detected at all and Sa-GPA-OH was a final product. This result indicates that As-GPA-OH and Sa-GPA-OH are not hydrolyzed by CPB. It is likely that phenylguanidine in the side chain of GPA is not as preferable to the active center of CPB as alkylguanidine is in that of Arg. Our results show that alkylguanidine in the side chain of basic amino acid is more available than phenylguanidine in the CPB-mediated release of drugs from their prodrugs.

Inhibition of CPB by Sa-GPA-OH

As Sa-GPA-OH is not hydrolyzed by CPB, its effects on the CPB-catalyzed hydrolysis of a specific substrate, Hip-Arg, were examined. As can be seen in Fig. 4, all Lineweaver-Burk plots meet at a single point on the $1/V$ axis. Thus, a pattern indicating competitive inhibition was obtained. This compound was found to be a weak competitive inhibitor of CPB with a K_i value of $(1.98 \pm 0.11) \times 10^{-3}$ M. The K_i value for Sa-GPA-OH was 50 times larger than that for Bz-Arg-OH reported previously (Wolff et al., 1962). These results indicate that Sa-GPA-OH is not preferable to Bz-Arg-OH with respect to the binding ability to the binding site of CPB due to the existence of the hydroxyl group or the benzene ring in this derivative.

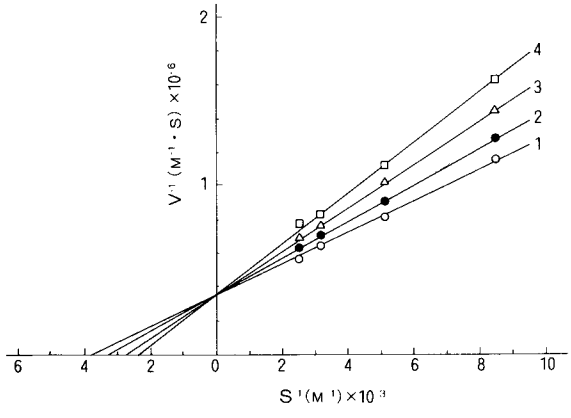

Fig. 4. Lineweaver-Burk plots for inhibition of CPB-catalyzed hydrolysis of Hip-Arg by Sa-GPA-OH. The concentrations of Sa-GPA-OH were (1) 0 mM, (2) 0.41 mM, (3) 0.82 mM, and (4) 1.23 mM. The initial rates were determined at pH 7.65 and 25 °C, in 0.025 M Tris-HCl buffer containing 0.1 M NaCl.

The study described above indicates that Sa-Arg-OH was hydrolyzed by CPB, while Sa-GPA-OH was not. We have synthesized several Arg derivatives of the non-steroidal anti-inflammatory carboxylic acids and found that parent drugs were released at the different hydrolytic rate in the CPB-mediated hydrolysis of these compounds. Therefore, our research can hopefully be utilized to control the release of the parent drugs from their prodrugs by the action of enzymes.

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