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# THE HYDROLYSIS OF ESTERS OF *N*-HIPPURYLGLYCINE AND *N*-PIVALOYLGLYCINE BY CARBOXYPEPTIDASE A

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## Summary

The kinetics of the hydrolysis of five esters of *N*-hippurylglycine ($C_6H_5CONHCH_2CONHCH_2CO_2CRR_1CO_2H$ (2)) and seven esters of *N*-pivaloylglycine ($((CH_3)_3CCONHCH_2CRR_1CO_2H$ (3)) by bovine pancreatic carboxypeptidase A (Peptidyl-L-amino-acidhydrolase, EC 3.4.12.2) have been studied at pH 7.5, 25°C and ionic strength 0.5. All *N*-hippurylglycine esters (2: R=H, $R_1$=H, $C_2H_5$, 4-$ClC_6H_4$, $C_6H_5CH_2$) display Michaelis-Menten kinetics up to at least 0.1 M substrate. The *N*-pivaloylglycine esters display either Michaelis-Menten kinetics (3: R=H, $R_1$=H, $C_2H_5$, $C_6H_5$), substrate activation (3: R=H, $R_1$=4-$ClC_6H_4$; R=$R_1$=$CH_3$) or substrate inhibition (3: R=H, $R_1=(CH_3)_2CHCH_2$, $C_6H_5CH_2$). Kinetic parameters have been evaluated for each ester and compared with those for the corresponding hippuric acid esters (1). The enzymic specificity is shown to be identical for the alcohol moieties of the esters 1, 2 and 3 and unrelated to the occurrence of substrate activation or inhibition phenomena. These latter phenomena are shown to be characteristic of the enzymic hydrolysis of *N*-acyl amino acid esters but unimportant for *N*-acyl dipeptide ester substrates.

## Introduction

Hippurate esters (1) have been used extensively as substrates in studies of the esterase activity of bovine pancreatic carboxypeptidase A (Peptidyl-L-amino-acidhydrolase, EC 3.4.12.2) [1—9]. The use of these esters for such studies has been complicated by substrate activation and substrate inhibition phenomena which complicate the kinetic analysis of the hydrolysis of these esters by this enzyme. Considerable progress has been made in mathematically describing these phenomena and in elucidating the reasons for individual esters displaying substrate activation or substrate inhibition [7—12]. However, some features of these phenomena are still not understood.

In an effort to gain further insight into substrate activation and inhibition of

the esterase activity of carboxypaptidase A, we decided to investigate the enzymic specificity for the alcohol moiety of two series of esters which earlier studies had indicated do not seem to be susceptible to substrate inhibition [10,12]. We report herein, steady-state kinetic analysis of the enzymic hydrolysis of five *N*-hippurylglycine esters (2) and seven *N*-pivaloylglycine esters (3) at pH 7.5. Our data indicate

$C_6H_5CONHCH_2CO_2CRR_1CO_2H$ (1)

$C_6H_5CONHCH_2CONHCH_2CO_2CRR_1CO_2H$ (2)

$(CH_3)_3CCONHCH_2CO_2CRR_1CO_2H$ (3)

the same enzymic specificity for the alcohol moiety of these two classes of esters and hippurate esters and provide further insight into the substrate activation and inhibition phenomena.

## Materials and Methods

*Synthesis of N-hippurylglycine esters (2).* *N*-Hippurylglycine was prepared by treatment of glycylglycine with benzoyl chloride in aqueous base by the method of Ingersoll and Babcock [13] and was recrystallized from methanol.

Each of the *N*-hippurylglycine esters was prepared by the following general route [14]:

*N*-Hippurylglycine (0.04 mol) was dissolved at room temperature in acetonitrile (150 ml) with the aid of a minimum amount of dimethyl formamide. Equimolar amounts of fresh dicyclohexylcarbodiimide and dry pyridine were slowly added. After stirring at room temperature for 1 h, the appropriate 2-hydroxycarboxylic acid (0.04 mol) was added to the milky solution, and stirring was continued overnight at room temperature. After removal of *N,N'*-dicyclohexyl urea by filtration, the solvents were removed on the rotary evaporator to give the crude esters as oils which crystallized upon standing [15]. Further recrystallizations gave analytically pure esters, which had melting points and PMR spectra as indicated in Table I.

*Synthesis of N-pivaloylglycine esters.* *N*-Pivaloylglycine was prepared from pivaloylchloride and glycine by the method of Ingersoll and Babcock [13], and recrystallized from diethyl ether/carbon tetrachloride (1 : 1, v/v).

*N*-Pivaloylglycyl chloride was synthesized by stirring *N*-pivaloylglycine with an equimolar amount of phosphorus pentachloride in acetyl chloride for 2 h at room temperature. The crude product separated as a white solid, was filtered off, and used immediately without further purification.

*O*-(*N*-Pivaloylglycyl)glycolic acid was prepared from *N*-pivaloylglycine and benzyl chloroacetate by the general method described previously [10]. All other esters were prepared via the pyridine-catalyzed esterification of *N*-pivaloyglycyl chloride with the appropriate 2-hydroxycarboxylic acid as described previously for other esters [7]. The crude products were recrystallized several times to give analytically pure esters. Table I contains melting points and PMR spectral data for each ester.

*Kinetic studies.* Preparation of enzyme and substrate stock solutions and all kinetic details are as previously described [8—10]. All kinetic data are for pH

TABLE I

CHARACTERIZATION OF *N*-HIPPURYLGLYCINE ESTERS (2) AND *N*-PIVALOYGLYCINE ESTERS (3)

All esters containing chiral carbon atoms are racemic mixtures.

| Ester | R | $n_1$ | Solvent * | m.p. (°C) | PMR spectrum ($CF_3CO_2H$) δ ppm. |
|---|---|---|---|---|---|
| 2 | H | H | $H_2O$ | 153—154 | 4.3—4.7 (4H,m), 4.96 (2H,s), 7.4—8.0 (7H,m) |
| 2 | H | $C_6H_5$ | $CH_3COCH_3/H_2O$ (1 : 1, v/v) | 175—176 | 4.4—4.7 (4H,m), 6.20 (1H,s), 7.3—8.0 (12H,m) |
| 2 | H | 4-$ClC_6H_4$ | $CH_3COCH_3/H_2O$ (1 : 1, v/v) | 179—180 | 4.4—4.7 (4H,m), 6.20 (1H,s), 7.4—8.0 (11H,m) |
| 2 | H | $C_6H_5CH_2$ | $CH_3COCH_3/H_2O$ (1 : 1, v/v) | 200—201 | 3.2—3.4 (2H,t), 4.2—4.6 (4H,m), 5.4—5.7 (1H,m), 7.3 (5H,s), 7.3—8.1 (7H,m) |
| 3 | H | H | $CHCl_3$ | 97—98 | 1.35 (9H,s), 4.30 (2H,d), 4.85 (2H,s), 7.35 (1H, broad) |
| 3 | $CH_3$ | $CH_3$ | $C_6H_6$ | 144—145 | 1.35 (9H,s), 1.75 (6H,s), 4.30 (2H,d), 7.4 (1H, broad) |
| 3 | H | $(CH_3)_2CHCH_2$ | $CHCl_3/CCl_4$ (1 : 1, v/v) | 113—114 | 1.05 (6H,d), 1.35 (9H,s), 1.8—2.0 (3H,m), 4.40 (2H,d), 5.40 (1H,t) 7.5 (1H, broad) |
| 3 | H | $C_6H_5$ | $CH_2Cl_2/C_6H_{14}$ (1 : 1, v/v) | 116—117 | 1.30 (6H,s), 4.40 (2H,d), 6.20 (1H,s), 7.45 (6H,s) |
| 3 | H | 4-$ClC_6H_4$ | $CH_3CO_2C_2H_5/CCl_4$ (1 : 1, v/v) | 134—135 | 1.35 (6H,s), 4.40 (2H,d), 6.20 (1H,s), 7.45 (5H,s) |
| 3 | H | $C_6H_5CH_2$ | $CHCl_3/CCl_4$ (1 : 1, v/v) | 110—111 | 1.35 (6H,s), 3.2—3.4 (2H,m), 4.35 (2H,d), 5.60 (1H,t), 7.3 (6H,s) |

* Recrystallization solvent.

7.5, 25°C and ionic strength 0.5. All calculations are in terms of the concentrations of the L-ester of racemic mixtures. It is well established that the D-esters are neither substrates nor competitive inhibitors for the hydrolysis of the L-esters by carboxypeptidase A [1,2,4,8,16].

## Results

*N-Hippurylglycine esters (2)*

For each of the *N*-hippurylglycine esters examined, the dependence of the initial velocity of enzymic hydrolysis upon substrate concentration was consistent with Michaelis-Menten kinetics up to at least 0.1 M substrate (pH 7.5, 25°C, ionic strength 0.5). The values of $k_{cat}$ and $K_m$ that were evaluated for each of these esters are collected in Table II. Further support for our use of racemic esters in these studies is found in the reasonable agreement (see Table II) between our data for the 3-phenyllactic acid derivative ($R=H, R_1=C_6H_5CH_2$) and the data of Auld and Holmquist [17] for the pure L-enantiomer (at ionic strength 1.0).

The enzymic hydrolysis of *O*-(*N*-hippurylglycyl)-3-phenyllactic acid was not inhibited by *N*-hippurylglycine at concentrations up to $I = 0.1$ M. Hippuric acid did inhibit the enzymic hydrolysis of this ester; however, a Dixon plot for this inhibitor was linear in $I^2$ rather than in $I$ ($I$ is the inhibitor concentration). Lineweaver-Burk plots in the presence of hippuric acid are linear and give the parameters: at $I = 30$ mM, $k_{cat}^{app} = (2.00 \pm 0.05) \cdot 10^4$ min$^{-1}$, $K_m^{app} = (1.4 \pm 0.1) \cdot 10^{-4}$ M; at $I = 50$ mM, $k_{cat}^{app} = (1.12 \pm 0.02) \cdot 10^4$ min$^{-1}$, $K_m^{app} = (1.5 \pm 0.1) \cdot 10^{-4}$ M. Comparison of these data with the uninhibited parameters for this ester in Table II indicates that this inhibitor is approximately non-competitive. Inhibition constants calculated assuming non-competitive inhibition give $K_i = 42$ mM at $I = 30$ mM, and $K_i = 24$ mM at $I = 50$ mM. However, these $K_i$ values can only be considered to be apparent inhibition constants in view of the clear indication from the Dixon plot that inhibition involves the binding of two inhibitor molecules per enzyme molecule.

TABLE II

PARAMETERS FOR THE HYDROLYSIS OF *N*-HIPPURYLGLYCINE ESTERS (2) BY CARBOXYPEPTIDASE A

At pH 7.5, 25°C, ionic strength 0.5.

| R | $R_1$ | $k_{cat}$(min$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (M$^{-1}$ · min$^{-1}$) |
|---|---|---|---|---|
| H | H | >800 | >100 | $8.6 \cdot 10^3$ |
| H | $C_2H_5$ * | $8.2 \cdot 10^3$ | 2.7 | $3.0 \cdot 10^6$ |
| H | $C_6H_5$ | $(2.01 \pm 0.02) \cdot 10^3$ | 2.27 ± 0.08 | $8.9 \cdot 10^5$ |
| H | 4-$ClC_6H_4$ | 12.7 ± 0.6 | 5.0 ± 0.1 | $2.5 \cdot 10^3$ |
| H | $C_6H_5CH_2$ | $(3.4 \pm 0.1) \cdot 10^4$ | 0.21 ± 0.02 | $1.6 \cdot 10^8$ |
| H | $C_6H_5CH_2$ ** | $3.0 \cdot 10^4$ | 0.33 | $9.0 \cdot 10^7$ |

* Data from ref. 10.

** Data from ref. 17 at pH 7.5, 25°C, ionic strength 1.0.

TABLE III

PARAMETERS FOR THE HYDROLYSIS OF *N*-PIVALOYLGLYCINE ESTERS (3) BY CARBOXYPEPTIDASE A

At pH 7.5, 25°C, ionic strength 0.5.

| R | $R_1$ | Kinetic form * | $k_2^{app}$ ($min^{-1}$) | $K_S^{app}$ (mM) | $k_2^{app}/K_S^{app}$ ($M^{-1} \cdot min^{-1}$) | $k_3$ ($min^{-1}$) | $K_{SS}^{app}$ (mM) |
|---|---|---|---|---|---|---|---|
| H | H | M.M. | >2000 | >100 | $1.6 \cdot 10^4$ | — | — |
| $CH_3$ | $CH_3$ | S.A. | 330 ± 40 | 4.4 ± 1.3 | $7.5 \cdot 10^4$ | $>1.1 \cdot 10^3$ | ** |
| H | $C_2H_5$ | M.M. | $(2.0 \pm 0.04) \cdot 10^4$ | 3.9 ± 0.2 | $5.2 \cdot 10^6$ | — | — |
| H | $(CH_3)_2CHCH_2$ | S.I. | $(1.41 \pm 0.03) \cdot 10^4$ | 1.16 ± 0.09 | $1.2 \cdot 10^7$ | $(2.3 \pm 0.2) \cdot 10^3$ | 5.1 ± 0.4 |
| H | $C_6H_5$ | M.M. | $(6.7 \pm 0.2) \cdot 10^3$ | 1.6 ± 0.1 | $4.2 \cdot 10^6$ | — | — |
| H | $4\text{-}ClC_6H_4$ | S.A. | 33 ± 2 | 8.1 ± 0.5 | $4.1 \cdot 10^3$ | >70 | ** |
| H | $C_6H_5CH_2$ | S.I. | $(2.69 \pm 0.04) \cdot 10^4$ | 0.30 ± 0.08 | $9.0 \cdot 10^7$ | $(5.6 \pm 0.5) \cdot 10^3$ | 44 ± 3 |

* S.A. = substrate activation; S.I. = substrate inhibition; M.M. = Michaelis-Menten kinetics.

** Not directly observable; probably greater than 100 mM.

*N-Pivaloylglycine esters (3)*

A variety of dependences of initial velocity of enzymic hydrolysis on substrate concentration was observed for *N*-pivaloylglycine esters. Lineweaver-Burk plots were linear over the range $10^{-4}$—$10^{-1}$ M in substrate concentration for the glycolic acid (3: R=$R_1$=H), 2-hydroxybutanoic acid (3: R=H, $R_1$=$C_2H_5$) and mandelic acid (3: R=H, $R_1$=$C_6H_5$) derivatives. Thus neither substrate activation nor substrate inhibition is observed for each of these esters. The values of $k_{cat}$ ($=k_2^{app}$) and $K_m$ ($=K_S^{app}$) that were determined for these esters are given in Table III.

Lineweaver-Burk plots (Fig. 1) for the 2-methyllactic acid (3: R=$R_1$=$CH_3$) and *p*-chloromandelic acid (3: R=H, $R_1$=4-$ClC_6H_4$) derivatives indicate substrate activation at substrate concentrations greater than about 10 mM. Below this concentration these plots are quite linear and the parameters given in Table III were obtained. For both of these esters the dependence of velocity on substrate concentration increases sharply in the range $S$=10—100 mM with no approach to limiting velocity being observed. Values of $k_3$ in Table III are the maximum velocities observed in this work.

Pronounced substrate inhibition was observed for the 2-hydroxyisocaproic acid (3: R=H, $R_1$=$(CH_3)_2CHCH_2$) and 3-phenyllactic acid (3: R=H, $R_1$=$C_6H_5CH_2$) derivatives (Fig. 2). The data in Fig. 2 were readily fitted to Eqn. 1 by the iteration technique described previously [18], and the parameters obtained are given in Table III.

$$\frac{v}{E} = \frac{k_2^{app}S + k_3S^2/K_{SS}^{app}}{K_S^{app} + S + S^2/K_{SS}^{app}} \qquad (1)$$

The curves in Fig. 2 were calculated using Eqn. 1 and the appropriate parameters from Table III, and give an acceptable fit to the experimental data.

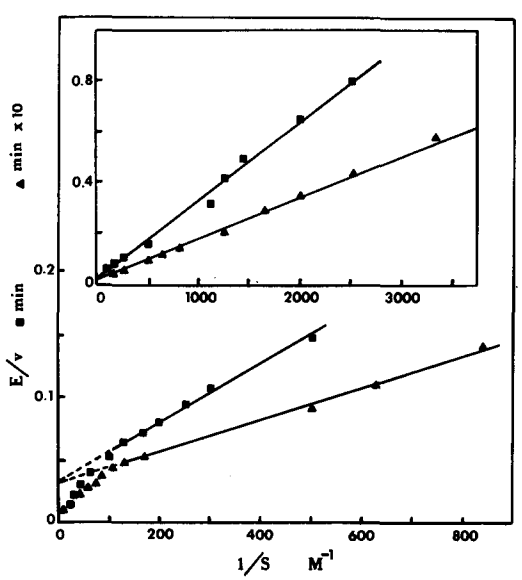


Fig. 1. Lineweaver-Burk plots for the enzymic hydrolysis of 3: R=$R_1$=$CH_3$ (▲) and 3: R=H, $R_1$=4-$ClC_6H_4$ (■) at pH 7.5, 25°C, ionic strength 0.5.

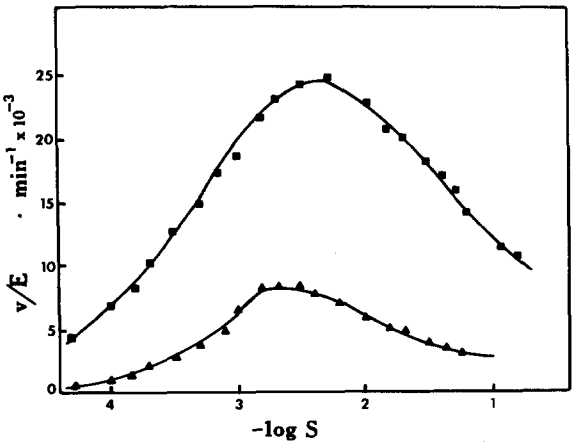


Fig. 2. Dependence of v/E on substrate concentration for the enzymic hydrolysis of 3: R=H, $R_1=(CH_3)_2CHCH_2$ (▲) and 3: R=H, $R_1=C_6H_5CH_2$ (■) at pH 7.5, 25°C, ionic strength 0.5. Curves drawn according to Eqn. 1 using the appropriate parameters from Table III.

## Discussion

In the hydrolysis of hippuric acid esters (1) by carboxypeptidase A, substrate activation is observed for the glycolic acid (R=$R_1$=H), 2-methyllactic acid (R=$R_1$=$CH_3$) and *p*-chloromandelic acid (R=H, $R_1$=4-$ClC_6H_4$) derivatives [7], while pronounced substrate inhibition occurs for the mandelic acid (R=H, $R_1=C_6H_5$) and 3-phenyllactic acid derivatives (R=H, $R_1=C_6H_5CH_2$) and also for a variety of aliphatic $R_1$ groups in 1: R=H [8]. In fact, for carboxypeptidase A there is no known hippuric acid ester substrate which displays Michaelis-Menten kinetics upon enzymic hydrolysis. In contrast, all the *N*-hippurylglycine esters in Table II display Michaelis-Menten kinetics when hydrolyzed by carboxypeptidase A; this is the case irrespective of whether the alcohol moiety leads to substrate activation or substrate inhibition in the hippurate ester series. Thus, both substrate activation and substrate inhibition appear to be properties of *N*-acyl amino acid ester substrates but not of ester substrates derived from *N*-acyl dipeptides. Similar conclusions have been reached earlier by other workers for substrate inhibition for both ester and peptide substrates of this enzyme [16,17,19].

It has been shown earlier [10—12] that substrate inhibition in the enzymic hydrolysis of hippuric acid esters is associated with a conformational change induced in the enzyme molecule upon the productive binding of the hippurate ester substrate molecule. This conformational change makes a binding site available for a second (inhibitory) substrate molecule. Other species such as the anions of hippuric acid ($K_i$ = 10 mM [11]) or *N*-hippurylglycine ($K_i$ = 8 mM [11]) may also bind at this inhibitory site and in so doing these species display uncompetitive inhibition of the enzymic hydrolysis of hippurate esters. The current observation of the inability of *N*-hippurylglycine to inhibit the enzymic hydrolysis of *N*-hippurylglycine esters strongly suggests that the conformational change which produces the inhibitory binding site with hippurate

ester substrates does not occur when *N*-hippurylglycine esters are used as substrates.

Also consistent with the above interpretation, is the fact that the hippuric acid anion does not display uncompetitive inhibition of the enzymic hydrolysis of esters of *N*-hippurylglycine, although this species is an uncompetitive inhibitor of the enzymic hydrolysis of both hippuric acid and *N*-pivaloylglycine esters [11,12]. The linear dependence of $E/v$ on $I^2$ observed for hippuric acid in Fig. 1 is similar to that previously observed for several other reversible inhibitors of this enzyme [11], and can be related to the formation of 1 : 2 enzyme-inhibitor complexes. A number of enzymic binding sites for carboxylate anions as reversible inhibitors have been kinetically identified [11,20—22].

The data in Table III for *N*-pivaloylglycine esters indicate that in all cases substrate activation or inhibition is shifted to higher substrate concentrations relative to the corresponding hippuric acid esters. In three cases this shift is sufficiently large that Michaelis-Menten kinetics are now observed up to 0.1 M substrate. For the corresponding hippuric acid esters, two of these esters display substrate inhibition (1: R=H, $R_1$=$C_2H_5$ or $C_6H_5$) and one displays substrate activation (1: R=$R_1$=H). Substrate activation is also sufficiently shifted to higher substrate concentrations that values of $k_{cat}$ ($=k_2^{app}$) and $K_m$ ($=K_S^{app}$) can be determined for the 2-methyllactic acid (3: R=$R_1$=$CH_3$) and *p*-chloromandelic acid (3: R=H, $R_1$=4-$ClC_6H_4$) derivatives of *N*-pivaloylglycine. For the corresponding hippuric acid esters substrate activation is too pronounced at even quite low substrate concentrations to allow the determination of these two parameters. For both of these *N*-pivaloylglycine esters it appears that the major factor leading to their slow enzymatic hydrolysis is a low $k_{cat}$ value rather than a large $K_m$ value. This interpretation assumes, of course, that $k_{cat}$ and $K_m$ are not significantly influenced by the presence of catalytically non-productive binding modes for these substrates, and is supported by a similar result for *O*-(*N*-hippurylglycyl)-*p*-chloromandelic acid (Table II). The current data also seem to indicate that an earlier suggestion [7] to the effect that the difference in reactivities of *O*-hippurylmandelic acid and *O*-hippuryl-*p*-chloromandelic acids is due to a dramatic increase in $K_m$ upon introduction of the chlorine substituent is probably incorrect.

For the two *N*-pivaloylglycine esters which display substrate inhibition, this phenomenon is also displaced to significantly higher substrate concentrations relative to the corresponding hippuric acid esters. For all hippurate esters which display substrate inhibition $K_{SS}^{app}$ is constant at 1.0 mM within experimental error [8]. The large variations seen in $K_{SS}^{app}$ in the *N*-pivaloylglycine series, from 5.1 mM for 3: R=H, $R_1$=$(CH_3)_2CHCH_2$ to >100 mM for 3: R=H, $R_1$=$C_2H_5$ or $C_6H_5$, suggest that a somewhat different mode of binding the inhibitory substrate molecule is involved in the *N*-pivaloylglycine series than in the hippuric acid series. It has been concluded previously [11,12] that a hydrophobic interaction between enzyme and substrate is mainly responsible for the binding of inhibitory substrate molecules. Significantly, for *N*-pivaloylglycine esters substrate inhibition is displayed only by the two esters having the largest $R_1$ side-chains. Thus binding of the *N*-pivaloylglycine esters, 3: R=H, $R_1$=$(CH_3)_2CHCH_2$ or $C_6H_5CH_2$, in the inhibitory site via a hydrophobic interaction of $R_1$ with the enzyme would readily explain why substrate inhibition is not seen when $R_1$ is

TABLE IV

COMPARISON OF SPECIFICITY OF CARBOXYPEPTIDASE A FOR HIPPURIC ACID (1), *N*-HIPPURYLGLYCINE (2) AND *N*-PIVALOYLGLYCINE (3) ESTERS

Based on data in Tables II and III and refs. 7 and 8.

| R | $R_1$ | $(k_{cat}/K_m)$ (2)/$(k_{cat}/Km)$ (1) | $(k_{cat}/K_m)$ (3)/$(k_{cat}/K_m)$ (1) |
|---|---|---|---|
| H | H | 0.14 | 0.26 |
| $CH_3$ | $CH_3$ | | 0.25 |
| H | $C_2H_5$ | 0.14 | 0.25 |
| H | $C_6H_5$ | 0.10 | 0.45 |
| H | 4-$ClC_6H_4$ | 0.25 | 0.40 |
| H | $C_6H_5CH_2$ | 0.73 | 0.42 |
| H | $(CH_3)_2CHCH_2$ | | 0.056 |

smaller and thus less hydrophobic (i.e. 3: R=H, $R_1=C_2H_5$ or $C_6H_5$). Such an hypothesis is also consistent with the difference in $K_{SS}^{app}$ values between hippuric acid esters and *N*-pivaloylglycine esters, and also accounts for the difference in alcohol moiety specificity for substrate inhibition in these two series of esters.

In Table IV the specificity constants $(k_{cat}/K_m)$ of carboxypeptidase A for the alcohol moieties of *N*-hippurylglycine esters (2) and *N*-pivaloylglycine esters (3) are compared with those for the corresponding hippuric acid esters (1). In both comparisons the specificity constant ratios vary less than 7-fold for individual variations in $k_{cat}/K_m$ of greater than $10^4$-fold for all three classes of ester. This apparent independence of the nature of the alcohol moiety for the specificity constant ratios for these ester pairs clearly indicates that productive binding of each of these three groups of esters involves identical interactions of the alcohol moiety with the enzyme. Thus despite the complex substrate activation and inhibition effects displayed by hippurate esters at high substrate concentrations, data obtained for these esters at low substrate concentrations can be confidently used to reflect normal productive ester binding.

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