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Preferred Hydrolysis of Methyl D-Pyroglutamates (5-Carbomethoxy-2-Pyrrolidones) by α-Chymotrypsin[1]

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Methyl D-pyroglutamate, D-**III-A**, is hydrolyzed by α-chymotrypsin, and the L-enantiomer is not. Methyl α-methylpyroglutamate, **III-B**, and methyl α-benzylpyroglutamate, **III-C**, are hydrolyzed by α-chymotrypsin with D-preference based on the glutamic structure, L-preference based on the alanine and phenylalanine structures. γ-Carbomethoxy-γ-valerolactone, **IV**, is hydrolyzed without stereoselectivity. Kinetics of hydrolysis of the substrates are reported. Modes of association of the substrates with the active site and the relevance of these results to the reactive orientation of the α-acylamido and hydrolyzing groups of noncyclized substrates are discussed.

INTRODUCTION

Studies of structure–reactivity relations in hydrolysis by α-chymotrypsin of derivatives of β-aryl-α-amino acids and compounds related to them have led to inferences about the dimensions of the active site, the interactions between enzyme and substrate which effect reactivity and stereoselectivity, and the reactive orientation and conformation of the substrate in the active site (*1–6*). X-Ray diffraction studies, following amino acid sequence determination (*7*), have led to detailed descriptions of enzyme–inhibitor and enzyme–substrate interactions (*8–10*). The aryl binding site, *ar*, is a pocket with residues 189–194 on one side, 214–220 on the other; the aromatic group in the binding of formyl L-tryptophane is proposed to be between the peptide bonds of Ser 190–Cys 191, Cys 191–Met 192, and Trp 215–Gly-216. The α-acylamido group has its N–H directed to C=O of Ser 214, indicated *am*, and its C=O transoid, toward the γ-CH_2 of Met-192; the α-H is directed toward the α-H of Met 192, *h*. The hydrolysing group is directed toward the essential –O–H–N⩽ of Ser 195 and His 57, *n* (*8–10*).

Cyclized hydroaromatic ester substrates with fused naphthalene nuclei have led to mapping of the side-chain binding cleft and to inferences as to the conformation of the ester group (*4, 11*). Comparison of acetamido, acetoxy, and hydroxy-substituted esters and diesters has indicated that hydrogen-bonding of the acetamido substituent at *am* helps fix the orientation of the substrate and leads to L-specificity, may not make binding to the enzyme more favorable, and may increase k_{cat} (*3, 12*). Very high reactivity and D-stereoselectivity in hydrolysis of both 1-keto-3-carbomethoxytetrahydroisoquinoline, **I-A** (*1, 13*), and methyl 3,4-dihydroisocoumarin-3-carboxylate, **II-A**

[1] This paper is dedicated to the memory of Professor Morris Kupchan. His death is a loss both to those who knew him and to those who might have benefited from continuation of his talented search for healing drugs.

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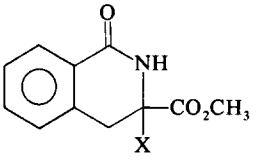
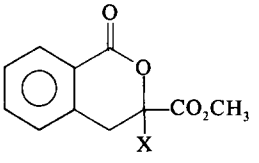
(*14*), indicated that fusion of the α-amide and α-ester substituents into the β-aryl group (i) restricted relative motion of the parts of the substrate and, in the D-enantiomers, fixed the carbomethoxy and aryl groups into the conformation which results in favorable binding at *ar* and high reactivity at *n*; and (ii) directed the lactam and lactone rings toward *h*, leaving *am* unused and unoccupied (*14*). The substantial enzymatic reactivity of the α-tetrasubstituted compounds, **I-B** and **II-B**, and the inertness of the α-methyl substituted acyclic analogs supported the latter conclusion (*15*). Studies of maleate and fumarate esters indicated that the hydrolyzing ester group in such cyclized substrates was equatorial during enzymic hydrolysis (*16*), an inference that is supported by X-ray studies (*17*).

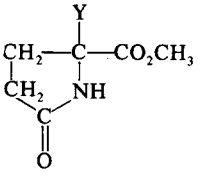


I-A, X = H
I-B, X = CH_3



II-A, X = H
II-B, X = CH_3

In these cyclized systems binding and orientation are dominated by the aryl group, and they are uninformative as to the relative positioning of the α-acylamido and hydrolyzing groups in a noncyclized substrate and thus also as to the N and C terminal parts of a protein substrate.



III-A, Y = H; **III-B**, Y = CH_3; **III-C**, Y = $CH_2C_6H_5$

The α-ester groups of diesters of *N*-acetyl L-aspartic and glutamic acids are hydrolyzed by α-chymotrypsin, as the β- and γ-ester groups apparently fill, less effectively, the role of the usual β-aryl group in binding at *ar* (*12*). Cyclization of a dimethyl glutamate leads to a pyroglutamate, a 5-carbomethoxy-2-pyrrolidone, **III**. The relative positions of the lactam and ester groups are fixed, binding need not be determined by an aryl group, and the effect of binding to the enzyme of an amide which is in this relation to the ester might be assessed. Effects of α-substituents might also be examined, since α-tetrasubstituted cyclized substrates **I-B** and **II-B** were hydrolyzed by α-chymotrypsin (*15*). Binding of the aryl group of **III-C** at *ar* would not in this case fix the lactam at *h* rigidly, as it does in **I**. Also study of γ-butyrolactones, related to **III** as **II** to **I**, might also be informative. We wish to report on such studies.

EXPERIMENTAL

D(—)-*Methyl Pyroglutamate* [D(—)-III-A]

A suspension of 6.0 g (0.04 mol) of D(+)-glutamic acid (Pierce) in 200 ml of methanol was saturated with hydrogen chloride, boiled for 5 hr, and concentrated. The

residue was brought to alkaline pH with 5% sodium bicarbonate and extracted with chloroform. The extract was dried and concentrated, and the residue was heated for 2 hr at 130°C to displace methanol and distilled: bp 115°C (0.1 mm); 1.8 g (0.013 mol), 32%; α_{obs} −0.59°; *c* 5.72, methanol; $[\alpha]_D^{27}$ −10.3°. The nmr spectrum in $CDCl_3$ displayed peaks (δ) 2.1–2.7 (m, 4H, CH_2CH_2), 3.75 (s, 3H, O–CH_3), 4.3 (m, 1H, CHCO), and 7.0–7.5 (s, 1H, NHCO). *Anal.* Calcd for $C_6H_9O_3N$: C, 50.35; H, 6.29; N, 9.79. Found: C, 49.62; H, 6.52; N, 10.03 (Galbraith).

L(+)-*Methyl Pyroglutamate*

This compound was prepared similarly: bp 105°C (0.035 mm); α_{obs} + 0.68°; *c* 6.16, methanol; $[\alpha]_D^{26}$ + 11°. The nmr spectrum was identical with that of the D-enantiomer. A solution (0.11 *M*) of the L-enantiomer, treated with 6.2×10^{-5} *M* α-chymotrypsin, consumed alkali only 25% more rapidly than in the absence of enzyme. On the basis that none of this was due to presence of the δ-enantiomer, the enzymatic hydrolysis of the L-enantiomer is estimated to be less than 1% as great as that of the D.

D(+)-*Pyroglutamic Acid*

D(+)-glutamic acid (20 g) was heated at 180–195°C for 45 min and poured into water, leading to the pyroglutamic acid: 8.3 g; mp 162°C; α_{obs} +0.52°; $[\alpha]_D^{25}$ +9.0°; lit (*18*) mp 162–163°C; $[\alpha]_D$ +10.7. The nmr spectrum in $(CD_3)_2SO$ displayed peaks (δ) 2.15 (m, 4H, CH_2CH_2), 4.05 (m, 1H, HC–C), and 7.9 (s, 1H, CO_2H).

DL-γ-*Carbomethoxy-*γ-*Valerolactone* (**IV**)

γ-Carboxy-γ-valerolactone (40 g, 0.28 mole) was prepared from levulinic acid (Eastman, 0.68 mol) via γ-cyano-γ-valerolactone following reported procedures (*19*). This was boiled for 2 hr in 600 ml of methanol saturated with HCl, and led to **IV** (22.6 g, 0.14 mol), bp 80–81°C (0.1 mm). The nmr spectrum in $CDCl_3$ displayed peaks (δ) 1.65 (s, 3H, CH_3C), 2.0–2.8 (m, 4H, CH_2CH_2), and 3.8 (s, 3H, $0CH_3$). *Anal.* Calcd. for $C_7H_{10}O_4$: C, 53.16; H, 6.33. Found: C, 53.86; H, 6.67 (Galbraith).

(i) A solution of 0.50 g (0.0032 mol) of **IV** and 0.2 g of α-chymotrypsin in 50 ml of 10% acetonitrile in water at pH 7.8 consumed 3.25 ml of 1 *N* NaOH in 17 hr, 100% reaction.

(ii) A solution of 11.3 g (0.072 mol) of **IV** and 0.4 g of α-chymotrypsin in 100 ml of H_2O and 15 ml of 0.1 *M* Na_2HPO_4 buffer was allowed to react at pH 7.8 until 37 ml of 1 *N* NaOH was consumed. It was lyophilized, and unhydrolyzed **IV** was extracted and distilled, bp 74–75°C (0.15 mm); ir and nmr spectra were identical with those of starting material, **IV**, and no optical rotation was observed; *c* 6, water and methanol. The residue was brought to pH2 and lyophilized, and γ-carboxy-γ-valerolactone was isolated. Its nmr spectrum was identical with that of the synthesized compound. It showed no optical rotation; *c* 6, water and methanol.

DL-*Methyl* α-*Methylpyroglutamate* (DL-**III-B**)

Ethyl levulinate (10 g, 0.07 mol, Matheson) was treated with 24 ml of 12% ammonium hydroxide, and a solution of 6 g of potassium cyanide and 5.6 g of ammonium chloride in 40 ml of water at 50–60°C overnight. This was added to 1 liter of concentrated hydrochloric acid and after 24 hr was boiled for 2 hr and taken to

dryness, leading to a residue containing DL-α-methylglutamic acid. This was esterified and cyclized as in the preparation of **III-A**, and crystallized from chloroform–hexane, DL-**III-B**; 3.5 g (0.02 mol), 30% yield; mp 58–59°C. The nmr spectrum in $CDCl_3$ displayed peaks (δ) 1.5 (s, 3H, C–CH_3), 1.7–2.7 (m, 4H, CH_2CH_2), 3.75 (s, 3H, O–CH_3) and 7.1 (b s, 0.9 H, NH). *Anal.* Calcd for $C_7H_{11}O_3N$: C, 53.51; H, 7.01; N, 8.92. Found: C, 53.67; H, 7.07; N, 8.90 (Galbraith).

(i) A solution of 2.6 g (0.0164 mol) of DL-**III-B** and 0.2 g of α-chymotrypsin in 250 ml of 2% aqueous acetonitrile, maintained at pH 7, consumed 11.5 ml of 1 *N* sodium hydroxide in 48 hr, 70% hydrolysis. Lyophilization and extraction with acetone led to unhydrolized **III-B**: α_{obs} +0.62°; *c* 6.0, methanol; $[\alpha]_D^{26}$ +10.3°. The residue was brought to pH 2, lyophilized, and extracted, leading to optically active α-methylpyroglutamic acid: α_{obs} −0.14°; *c* 5.6, methanol; $[\alpha]_D^{26}$ −2.45° (corr. −5.7°). Treatment with methanol saturated with HCl opened the lactone and esterified the carboxyls. Neutralization, concentration, and heating recyclized, leading to partially active **III-B**; α_{obs} −0.23°; *c* 6.9, methanol; $[\alpha]_D^{25}$ −3.3° (corr. −7.7°). The nmr spectra of the (+)- and (−)-esters were identical with that of the starting material.

(ii) A second similar run led to consumption of 1 *N* NaOH at an initial rate of 0.19 ml/min, 9.7 ml, 55% reaction in 21 hr, at which time hydrolysis was slow and the reaction was interrupted. Unhydrolyzed **III-B** was recovered: $[\alpha]_D^{25}$ +10.2°; *c* 6.1, methanol; the acidic hydrolysis product was recovered and had an nmr identical with that of α-methylpyroglutamic acid. The recovered ester and acid were each boiled in 2 *N* HCl for 2 hr and lyophilized. The ester led to (+)-α-methylglutamic acid; α_{obs} +0.86°; *c* 10.1, 3 *N* HCl; $[\alpha]_D^{25}$ + 8.5°; lit (*20*) $[\alpha]_D^{25}$ + 12.1° in 6 *N* HCl; the acid led to (−)-α-methylglutamic acid: $[\alpha]_D^{25}$ −4.6°; *c* 11.0, 3 *N* HCl; lit (*20*) −12.1° in 6 *N* HCl. The nmr spectra in CF_3CO_2H showed peaks (δ) 1.4 (s, 3H, CH_3), 1.8–2.6 (2 d, 4.1 H, CH_2CH_2), and 7.2 (b s, 1.9 H, NH_2). Optical rotatory dispersion measurements were made, 2% in 3 *N* HCl, 1-cm path, on a Cary 60 spectropolarimeter. Specific rotations of the (+)-compound were: +12.5°, 500 nm: +33.8°, 350 nm; and of the (−)-compound −6.0°, 500 nm; −19.3°, 350 nm; reported (*21*) values are for L-α-methylglutamic acid; +18.2°, 500 nm; +43.6°, 350 nm; for D-A-methylglutamic acid: −17.0°, 500 nm; −43.4°, 350 nm.

DL-*Methyl α-Benzylpyroglutamate* (DL-**III-C**)

DL-α-Benzylglutamic acid was prepared by reported procedures (*20*) from 35 g (0.30 mol) of benzyl cyanide and 80 g of diethyl succinate via ethyl δ-phenyl-δ-cyanolevulinate, bp 120°C (0.3 mm), and δ-phenyllevulinic acid. Treatment of the latter with sodium cyanide and ammonium chloride led to α-benzylglutamic acid; 18 g (0.076 mol), 25% yield; mp 215°C; lit (*20*) 214–215°C. This was esterified and cyclized to DL-**III-C**, bp 174–175°C (0.35 mm). The distillate crystallized on standing, mp 100–102°C, from chloroform–hexane, 1.2 g (0.0043 mol), 9% yield. The nmr spectrum in $CDCl_3$ displayed peaks (δ) 2.0–2.5 (m, 4H, CH_2CH_2), 3.04 (AB quartet, 2H, CH_2Ar), 3.7 (s, 3H, OCH_3), 6.35 (b s, 1H, NH), and 7.2 (m, 5H, Ar). *Anal.* Calcd for $C_{13}H_{15}NO_3$: C, 67.00; H, 6.44; N, 6.02. Found: C, 66.85; H, 6.40; N, 6.07 (Galbraith).

(i) A solution of 2.4 g (0.010 mol) of DL-**III-C** and 0.2 g of α-chymotrypsin in 250 ml of 2% aqueous acetonitrile consumed 5.2 ml of 0.1 *N* NaOH at pH 7 in 1 h. The reaction was interrupted, and the unhydrolyzed ester was extracted with ether; α_{obs}

+0.79°; *c* 3.90, benzene; $[\alpha]_D^{26}$ +20°. The aqueous phase was lyophilized, acidified, and extracted with acetone, leading to (−)-α-benzylpyroglutamic acid; mp 215–218°C, $[\alpha]_D^{25}$ −11° in 1 *N* NaOH. A portion of this was esterified in methanol saturated with HCl, leading to (−)-**III-C**: α_{obs} −0.70°; *c* 4.20, benzene $[\alpha]_D^{26}$ −17°. The nmr spectra of the (+)- and (−)-esters were identical with that of the DL-starting material.

(ii) A solution of 5.1 g (0.022) mol) of DL-**III-C** and 0.4 g of α-chymotrypsin in 250 ml of 2% aqueous acetonitrile consumed 10.7 ml of 1 *N* sodium hydroxide in 1 hr. The reaction was interrupted and (+)-**III-C** was extracted; α_{obs} +0.70°; *c* 5.00, benzene; $[\alpha]_D^{28}$ +14°. (−)-α-Benzylpyroglutamic acid was isolated as described above: mp 215–218°C; α_{obs} −0.05°; *c* 0.60, 1 *N* NaOH; $[\alpha]_D^{25}$ −8.0°. The nmr spectrum in DMSO displayed peaks (δ) 1.5–2.3 (m, 4H, CH_2CH_2), 3.03 (s, 2H, CH_2–Ar), 3.72 (s, 0.18 H, OCH_3 [6% unhydrolyzed ester]), 6.0–7.0 (1H, NH), 7.3 (s, 5H, Ar), and 8.04 (s, 1H, CO_2H). The (+)-ester and (−)-acid were each boiled in 2 *N* HCl for 2.5 hr and lyophilized. The residues were dissolved in water, filtered, and brought to pH 3.2, leading, from the (+)-ester, to (−)-α-benzylglutamic acid: mp 202–206°C, lit (*20*) mp 208°C; α_{obs} −0.0206°; *c* 2.08, 4 *N* HCl (Cary 60 polarimeter); $[\alpha]_D^{23}$ −0.99°, lit (*20*) $[\alpha]_D^{20}$ −1.60; and, from the (−)-acid, to (+)-α-benzylglutamic acid: mp 204–208°C, lit (*20*) mp 209°C; α_{obs} +0.0222°; *c* 2.59, 4 *N* HCl; $[\alpha]_D^{23}$ +0.86°, lit (*20*) $[\alpha]_D^{20}$ +1.65°. Rotatory dispersions of the (+)- and (−)-α-benzylglutamic acids, 0.02 g/ml in 4 *N* HCl, $l = 1$ mm, were examined in a Jasco J-20 automatic recording spectropolarimeter. The (+)- and (−)-compounds showed respectively negative and positive Cotton effects beginning at 240 nm. The nmr spectra of the (+)- and (−)-acids, isolated in this way, were identical with that of DL-α-benzylglutamic acid, prepared in the synthesis of DL-**III-C**.

Hydroxamidation with Glutamine Synthetase (Sheep brain, Sigma)

The procedure of Pamiljans et al. (*21*, *22*) was followed. Solution of components without enzyme was made up at 0°C and preincubated at 37°C for 1 min. Enzyme, 13 units, was added. The reaction was stopped after stated time intervals as described (*22*), and optical density at 535 nm was compared with that of standard γ-glutamylhydroxamate. When the reactions of 1 μmol of DL-glutamic acid and 1 μmol of DL-α-benzylglutamic acid were compared, the glutamic acid led to absorbance corresponding to 0.5 μmol of hydroxamate in 5 min, 1.05 μmol in 60 min, and 1.08 μmol in 80 min. The DL-α-benzylglutamic acid led to 0.16 μmol in 10 min, 0.42 μmol in 40 min, and 0.43 μmol in 160 min. A portion of the (−)-α-benzylpyroglutamic acid prepared in (i) above, $[\alpha]_D^{25}$ −11°, was hydrolyzed as in (ii) to (+)-α-benzylglutamic acid, and 1 μmol was treated with 13 units of glutamine synthetase along with L-glutamic acid and DL-α-benzylglutamic acid. The L-glutamic acid led to 0.55 μmol of hydroxamate in 5 min and 1.00 μmol in 40 min. The DL-α-benzylglutamic acid, as above, led to 0.17 μmol of hydroxamate in 10 min and 0.40 μmol in 120 min. The (+)-α-benzylglutamic acid, obtained via the chymotryptic hydrolysis, led to 0.14 μmol of hydroxamate after 80 and 120 min.

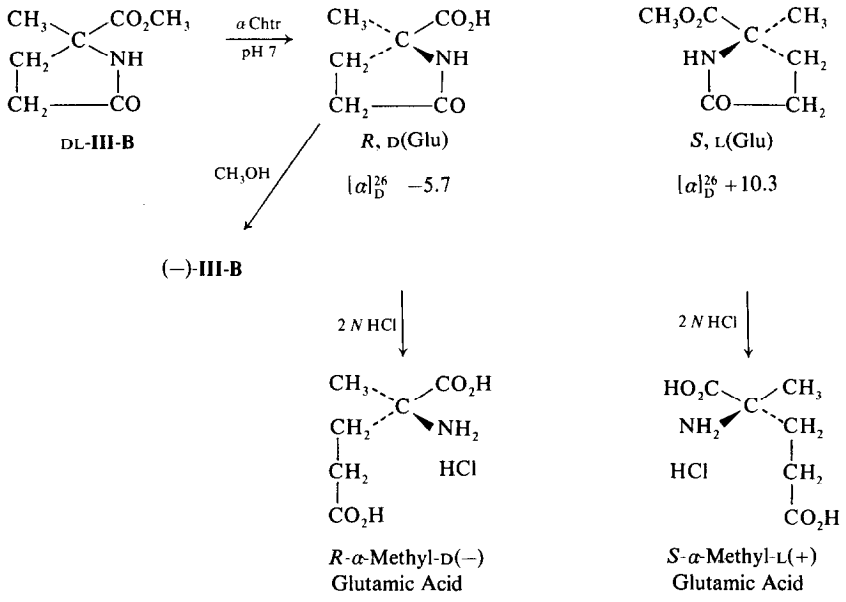
Kinetics

The kinetics of enzymic hydrolysis were studied in a Radiometer pH stat, Titrator TTTlC, Autoburette ABU 12, Titragraph SBR2C, calomel electrode K 401, and glass

electrode G202C, in a water-jacketed vessel, 25°C, pH 7.8. Acid was neutralized with 0.1 *N* NaOH. Runs were followed to ~10% reaction in 20 or 25 ml of 10% (v/v) acetonitrile (Eastman spectrograde), 0.1 *N* NaCl. Substrate was dissolved in acetonitrile. α-Chymotrypsin, Worthington, 3 × crystallized, salt-free, was dissolved in water and kept at 0°C; assay (*23*) indicated 80% activity. Rates were corrected for nonenzymatic hydrolysis and plots of inverse rate vs inverse substrate concentration were obtained. Slopes and intercepts were determined by least squares analysis, correlation 0.96–0.99. D-Methyl pyroglutamate and DL-γ-carbomethoxy-γ-valerolactone were studied at 2.6–13 m*M* substrate and 1 and 2 × 10^{-5} *M* enzyme. L-Methyl pyroglutamate was not hydrolyzed under these conditions. The D-enantiomer was hydrolyzed to completion. The DL-lactone was also hydrolyzed to completion. DL-Methyl α-benzylpyroglutamate was studied at 0.8–2.9 m*M* substrate and 4.5 × 10^{-6} *M* enzyme. DL-Methyl α-methylpyroglutamate was studied at 1–10 m*M* substrate and 2 × 10^{-5} *M* enzyme.

RESULTS

The substrates were prepared by modifications of standard procedures. D(−)- and L(+)-methylpyroglutamates, **III-A**, were prepared by cyclization, by heating, of the dimethyl glutamates. DL-γ-Carbomethoxy-γ-valerolactone, **IV**, was prepared from levulinic aid via the cyanohydrin and γ-carboxyl-γ-valerolactone. DL-Methyl α-methyl-pyroglutamate, **III-B**, was prepared from ethyl levulinate via Strecker synthesis, esterification, and cyclization. DL-Methyl β-benzylpyroglutamate, **III-C**, was prepared by condensation of benzyl cyanide and diethyl succinate to δ-phenyl-δ-cyanolevulinate, hydrolysis to δ-phenyllevulinic acid, and then to the product as for **III-B**.

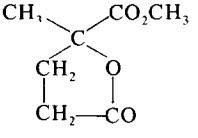


SCHEME I

The unsubstituted parent compound **III-A** showed D-specificity in hydrolysis by α-chymotrypsin. The L(+)-enantiomer was not hydrolyzed under the relatively low concentration conditions of the kinetic studies, and at 0.1 *M* substrate, 6.4×10^{-5} *M* enzyme, the reactivity was also very low. The methyl-D(−)-pyroglutamate was hydrolyzed, and solutions from the kinetic study were combined and run to completion in the pH stat. One equivalent of alkali was consumed, and the nmr spectrum of the isolated product was identical with that of synthesized D(+)-pyroglutamic acid.

The α-methyl derivative, **III-B**, also showed D-selectivity. Preparative hydrolysis of 0.07 *M* DL-**III-B** by 3×10^{-5} *M* enzyme slowed down after 21 hr, at about 50% hydrolysis, and led to optically active unhydrolyzed (+)-**III-B** and to active (−)-α-methylpyroglutamic acid, which was esterified to the enantiomeric (−)-**III-B** (Scheme I). Recovered unhydrolyzed (+)-**III-B** and (−)-α-methylglutamic acid were opened by acid hydrolysis. The ester led to (+)-α-methylglutamic acid hydrochloride and the acid to (−)-α-methylglutamic acid hydrochloride. It has been concluded that these correspond to (+)-α-methyl-L-glutamic acid and (−)-α-methyl-D-glutamic acid, respectively, on the basis of their behavior toward glutamine synthetase, D-glutamic acid cyclotransferase, and L-glutaminase (*21*). The hydrolysis by α-chymotrypsin occurred preferentially in the D-sense, if the substrate is viewed as a derivative of α-methyl-D-glutamic acid, and in the L-sense if it is viewed as derived from α-carbethoxyethyl-L-alanine. Rotation of the (+)-α-methylglutamic acid derived from the unhydrolyzed ester, +8.5°, compared with the literature value, +12.1° (*20*), indicated that D-**III-B** is six times as reactive as the L toward α-chymotrypsin, and that the value of $[\alpha]_D^{25}$ for D-**III-B** is +14.5°.

γ-Carbomethoxy-γ-valerolactone IV,



the lactone analog of lactam **III-B**, showed no evidence of stereoselectivity in its hydrolysis by α-chymotrypsin. Solutions from the kinetic runs hydrolyzed to completion. Hydrolysis of 0.7 *M* **IV** by 1.6×10^{-4} enzyme was interrupted at 50% reaction, and the unhydrolyzed ester and the γ-carboxy-γ-valerolactone were separated and found to be optically inactive.

Methyl-α-benzylpyroglutamate, **III-C**, showed stereoselectivity in its hydrolysis by α-chymotrypsin. Under the low concentration conditions of kinetic studies, hydrolysis of the DL-compound proceeded beyond 50% but at a decreased rate. Hydrolysis of DL-**III-C** on a preparative scale was more rapid than and similar to that of **III-B** (Scheme I). Hydrolysis of 0.04 *M* DL-**III-C** by 3.2×10^{-5} *M* α-chymotrypsin slowed down and was interrupted after 1 hr at 50% reaction. Unhydrolyzed (+)-**III-C**, $[\alpha]_D^{26}$ +20°, and (−)-α-benzylpyroglutamic acid, $[\alpha]_D^{25}$ −11°, were obtained, and the latter was reesterified to (−)-**III-C**, $[\alpha]_D^{26}$ −17°. Hydrolysis with 6.4×10^{-5} *M* enzyme appeared less stereoselective and led to (+)-**III-C**, $[\alpha]_D^{28}$ +14°, and (−)-α-benzylpyroglutamic acid, $[\alpha]_D^{25}$ −8.4°. In reactions analogous to those in Scheme I, these were opened: (+)-**III-C** to (−)-α-benzylglutamic acid, $[\alpha]_D^{23}$ −0.99; and (−)-α-benzylpyroglutamic acid to (+)-α-benzylglutamic acid, $[\alpha]_D^{23}$ +0.86°. Comparison with the reported value of 1.65° indicated 80 and 76% content of the (−)- and (+)-enantiomers, respectively, and

minimum values for specific rotation of **III-C** of 23° and of α-benzylpyroglutamic acid, 16°. The rotation of (+-**III-C** recovered unhydrolyzed by 3.2×10^{-5} *M* enzyme, +20°, indicates that in that run hydrolysis of (−)-**III-C** was 14 times as fast as that of the (+)-**III-C**.

The steric course of the hydrolysis of **III-C** by α-chymotrypsin was examined by hydroxamidation with glutamine synthetase (*21*, *22*). This enzyme converts glutamic acid and ammonia to glutamine, and glutamic acid and hydroxylamine to γ-glutamyl hydroxamate. Both L- and D-glutamic acid are substrates (*21*, *22*) with the L being more reactive (*24*). With α-methylglutamic acid the reaction is stereospecific and only the L-enantiomer is reactive (*22*). A sample of α-benzylpyroglutamic acid, $[\alpha]_D^{25}$ −11°, obtained from the hydrolysis by α-chymotrypsin was converted to (+)-α-benzylglutamic acid and this was subjected to hydroxyamidation, along with L-glutamic acid and DL-α-benzylglutamic acid. L-Glutamic acid led to 100% reaction, DL-α-benzylglutamic acid to 40%, and the (+)-α-benzylglutamic acid to 14% hydroxamidation. Forty percent reaction of the DL-compound indicates that α-benzylglutamic acid reacts stereospecifically with glutamine synthetase, presumably with *R*-α-benzyl-L-glutamic

TABLE 1

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ESTERS RELATED TO METHYL PYROGLUTAMATE

Substrate	k_{cat} (sec^{-1})	K_m (app) (m*M*)	k_{cat}/K_m (app) (M^{-1} sec^{-1})	$k_{(OH^-)}$[a] (M^{-1} sec^{-1})
D-**III-A**	0.18 ± 0.04	19 ± 5	9.6	16.0
DL-**IV**	0.062 ± 0.007	10 ± 2	6.2	13.0
DL-**III-B**	0.34 ± 0.69[b]	340 ± 70	1.0	6.0
DL-**III-C**	0.41 ± 0.09	6.5 ± 1.8	63.0	2.5

[a] Rate constant for reaction with hydroxide determined at pH 10.

[b] Intercept close to the origin.

acid the reactive enantiomer, by analogy with α-methylglutamic acid (*22*), and that the extent of hydroxamidation is ~80% under our conditions. On this basis, 14% hydroxamidation of the (+)-α-benzylglutamic acid corresponds to 17% content of the *R*,L(−)-enantiomer. Thus, reaction of α-chymotrypsin with DL-**III-C** led largely to hydrolysis of the *S*-α-benzyl-D(−)-pyroglutamate, which was in turn opened largely to *S*,D(+)-α-benzylglutamic, which was unreactive to glutamine synthetase. Hydrolysis of DL-**III-C** by α-chymotrypsin may also be considered to be, preferentially, that of an α-substituted L-phenylalanine. The specific rotation of the benzylpyroglutamic acid which was used, −11°, compared with the calculated value, 16°, indicates 16% content of the *R*,L-enantiomer, consistent with the observed extent of hydroxamination. The absolute configuration, established by anomalous X-ray dispersion, is *S*,D(+) (*25*), confirming the assignment.

Kinetic studies are summarized in Table 1. The enantiomers of Compound **IV** hydrolyze at similar rates, but those of **III-B** and **III-C** do not, and the observed kinetic constants for these are the averages for the two enantiomers. Reactivity of the $D_{(glu)}$ enantiomers of **III-B** and **III-C**, particularly the latter, are larger than indicated in the Table. Of the pyroglutamates, the α-benzyl compound **III-C** is the most reactive and the α-methyl compound, **III-B**, the least, with the difference due entirely to difference in

binding as reflected in K_m(app). Compound **III-B** binds very poorly. The unsubstituted parent compound D-**III-A** has intermediate reactivity, with k_{cat} half and K_m(app) three times the values for **III-C**. Compound **IV**, the oxygen analog of **III-B**, behaves very differently from the lactam, with much more favorable binding, less favorable k_{cat}, and higher reactivity.

DISCUSSION

Although the reactivities of these cyclized substrates may appear low, it should be noted that they are relatively high when compared with noncyclized analogs which have been studied. Methyl D-pyroglutamate, D-**III-A**, $k_{cat}/K_m = 9.6\ M^{-1}\ sec^{-1}$, is more reactive toward α-chymotrypsin than the closely related methyl L-*N*-acetylalaninate, $k_{cat}/K_m = 2\ M^{-1}\ sec^{-1}$ (*26*). Methyl α-benzylpyroglutamate, **III-C**, is far more reactive than the α-tetrasubstituted noncyclized analog, methyl *N*-acetyl-α-methylphenylalaninate, which is essentially inert (*27*). Consideration of other α-tetrasubstituted cyclic and acyclic compounds which we have studied (*15*) indicates that the acyclic analogs of **III-B** and **IV**, methyl *N*-acetyl-α-methylalaninate and methyl α-methyl-α-acetoxypropionate, may also be essentially inert. The cyclized compounds of this study are relatively reactive, and the steric course of their reactions merits interpretation.

The unsubstituted parent compound **III-A**, methyl pyroglutamate, unlike the α-substituted derivatives **III-B** and **III-C** is in the same configurational series whether considered as derived from the glutamic structure, as it was prepared, or from *N*-acetylalanine by linkage of the α- and acetyl methyl groups. Methyl L-*N*-acetylalaninate and dimethyl L-*N*-acetylglutamate are hydrolyzed by α-chymotrypsin, and the D-enantiomers are essentially inert (*12*, *16*). In the cyclized analog, **III-A**, the converse holds, and only the D-enantiomer is hydrolyzed by the enzyme. In all the noncyclized substrates which we have studied, an α-acetamido group has been sufficient to cause L-specificity, presumably by hydrogen bonding to C=O of Ser 214 (*8*), and other α-substituents have led to less stereoselectivity (*3*). The cyclic structure of the pyroglutamates makes the amide cisoid and fixes its orientation in relation to the hydrolyzing ester. That L-**III-A** is not hydrolyzed by the enzyme indicates that a reacting noncyclized L-substrate adopts a quite different relative orientation of the ester and α-amide groups. We had proposed (*28*), and X-ray studies (*8*) support, an orientation indicated schematically in Fig. 1. If the L-pyroglutamate should associate

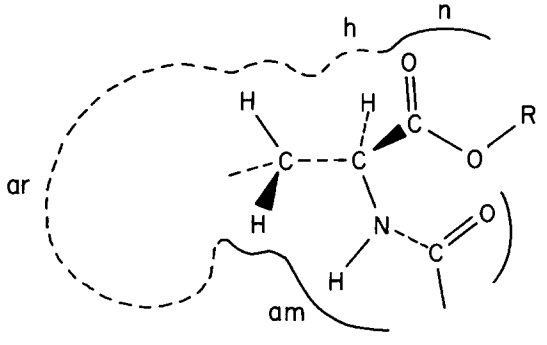


FIG. 1. Reacting orientation of L-acyclic substrate in active site.

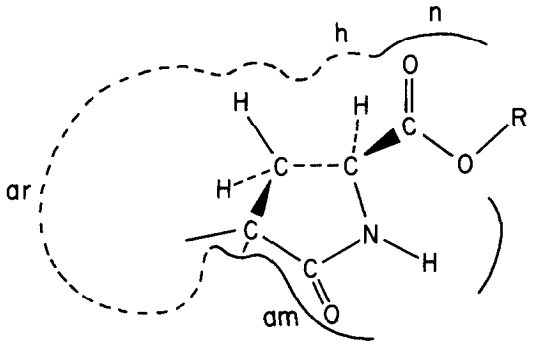


FIG. 2. Nonbinding fit of methyl L-pyroglutamate.

with the enzyme as in Fig. 2 (in an orientation apparently similar to that in Fig. 1), placing the hydrolyzing ester group necessarily at *n*, this would not allow the NH to hydrogen-bond to Ser 214 and would place the γ-CH_2 in a hindered position (*29*). The inertness of L-**III-A** supports the orientation of the α-amide and hydrolyzing ester groups of Fig. 1 as the reactive one.

Another orientation of L-**III-A** which places the hydrolyzing group at *n* may be indicated in Fig. 3. This would direct the polar NH to *H*, which is observed in the hydrolysis of D-**I** (*14*) and of D-α-hydroxy compounds (*30*); but, directing α-H toward *ar*, this orientation makes no use of this important binding site. The amide apparently finds a more favored position for its association with the enzyme, removing the ester group from *n*, and L-**III-A** does not hydrolyze. The α-substituted pyroglutamates **III-B** and **III-C** may have their $L_{(glu)}$ enantiomers fit into the active site as in Fig. 3, directing the substituents R, CH_3, and $CH_2C_6H_5$, respectively, toward *ar*. This may make use of this binding site and lead to reactivity. Comparison with Fig. 1 shows that these compounds correspond to derivatives of D-alanine and D-phenylalanine.

The D-enantiomer of **III-A** may fit into the active site as in Fig. 4, and this may allow NH to hydrogen-bond to Ser 214. The CH_2 is directed toward *h*; this is not a favorable interaction, but it is observed in hydrolysis of *S*-3-carboalkoxy-α-tetralones and *R* methyl 1,2,3,4-tetrahydro-2-naphthoate (*31*). The D-**III-A** hydrolyzes, and with L-**III-A** inert, D-specificity is observed. The α-substituted compounds **III-B** and **III-C** may have their $D_{(glu)}$ enantiomers also fit as in Fig. 4, again directing their respective CH_3 and

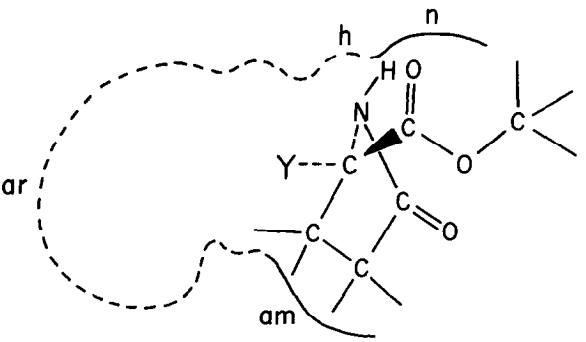


FIG. 3. Reacting orientation of $L_{(glu)}$-pyroglutamate in active site. Y = H, *S*,L-**III-A**; Y = CH_3, *S*,L-**III-B**; Y = $CH_2C_6H_5$, *R*,L-**III-C**.

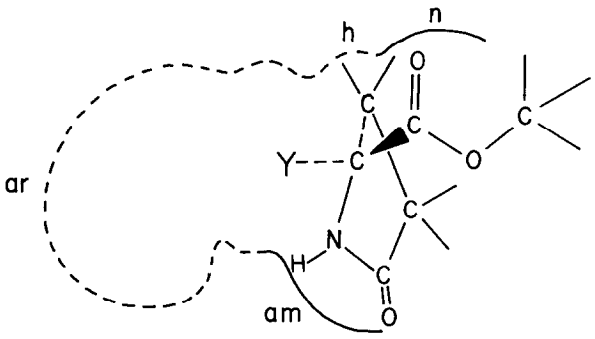


FIG. 4. Reacting orientation of $D_{(glu)}$-pyroglutamate in active site. Y = H, *R*,D-**III-A**; Y = CH_3, *R*,D-**III-B**; Y = $CH_2C_6H_5$, *S*,D-**III-C**.

$CH_2C_6H_5$ substituents toward *ar*. Compound **III-C** benefits from this substantially; it is difficult to account for the poor binding of **III-B**. The association is analogous to that in Fig. 1; these compounds correspond to L-alanine and L-phenylalanine derivatives, with the α-H replaced by the CH_2CH_2 bridge. The fit is more favorable than for the $L_{(glu)}$ enantiomers, possibly because of some hydrogen-bonding to Ser 214, and D-selectivity is observed by factors of about 6 and 14, respectively.

In the case of compound **IV**, the lactone analog of **III-B**, the $L_{(glu)}$ enantiomer may fit as in Fig 3, with —O— toward *h*, and the $D_{(glu)}$ enantiomer as in Fig. 4, with —O— directed toward Ser 214. Lacking an NH group, the L-enantiomer does not seek out a nonproductive binding mode as L-**III-A** may, and the $D_{(glu)}$ enantiomer of **IV** does not benefit from hydrogen-bonding at Ser 214. The L- and D-enantiomers of the lactone may react equally well via these associations leading to hydrolysis of both enantiomers and no stereoselectivity. α-Acyloxy and α-hydroxy substituents led to hydrolysis of both enantiomers of diethyl α-acetoxysuccinate and diethyl malate. The lactone ring in **IV** may be directed toward either *h* or *am* as was proposed for the hydroxyl of the malates (*32*). The lactam ring, as usual for α-NH, leads to greater stereoselectivity.

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