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Interaction of α-Chymotrypsin with Several α-Methyl-α-Acylamino Acid Methyl Esters*

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Methyl (+)- and (−)-*N*-acetyl-α-methylphenylalaninate, methyl (+)- and (−)-α-*N*-acetyl-α-methyltyrosinate, and methyl (±)- and (−)-*N*-acetyl-α-methyl-β-(2-naphthyl)-alaninate have been evaluated as competitive inhibitors of the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide and as substrates of this enzyme. Replacement of the α-hydrogen atom of representative enantiomorphic α-*N*-acyl aromatic α-amino acid esters by a methyl group changes the substration or inhibition constants by less than an order of magnitude but for substrates decreases the rate of formation of products by a factor of about 10^5. These results have been explained on the basis of steric interference by the α-alkyl group with attack of the potentially hydrolyzable carboalkoxy group by electro- or nucleophilic groups present at the active site of the enzyme.

Hein and Niemann (1961) proposed that α-*N*-acylated aromatic α-amino acid esters such as methyl α-*N*-acetyl-L-phenylalaninate and L-tyrosinate are substrates approximating the $S^{3E}_{R_2R_3}$ limit type, a designation for trifunctional ester-type substrates where orientation at the active site of the enzyme is achieved through interaction of R_1, the α-acylamino moiety, R_2, the α-amino acid side-chain, and COR_3, the reactive carboalkoxy group, with their respective complementary loci, ρ_1, ρ_2, and ρ_3, but where the enzyme-substrate dissociation constants are determined by the degree to which R_2 interacts with ρ_2 and COR_3 with ρ_3.

The orientation of an $S^{3E}_{R_2R_3}$ limit type substrate with R_1 of adequate size and structure to achieve its stereochemical role at the active site is represented schematically in Figure 1, as is that of its inhibitory D-antipode. This representation implies that the rate of formation of products from the D-antipode is suppressed by several orders of magnitude because R_1, in the position occupied by the α-hydrogen atom of the L-antipode, not only is incapable of assuming its critical orienting role, which for the L-antipode is associated with a high rate of formation of products, but in addition sterically limits attack on the carbonyl group of COR_3 of the D-antipode by an electro- or nucleophilic group of the site that has ready access to the same group in the L-antipode. These two effects, augmenting each other, result in a decrease in the rate of formation of products from the D-antipode of several orders of magnitude and a substantial relative stereospecificity in favor of the L-antipode. Absolute specificity for the L-antipode would demand the absence of a COR_3-ρ_3 interaction for the D-antipode, an unreasonable situation for an en-

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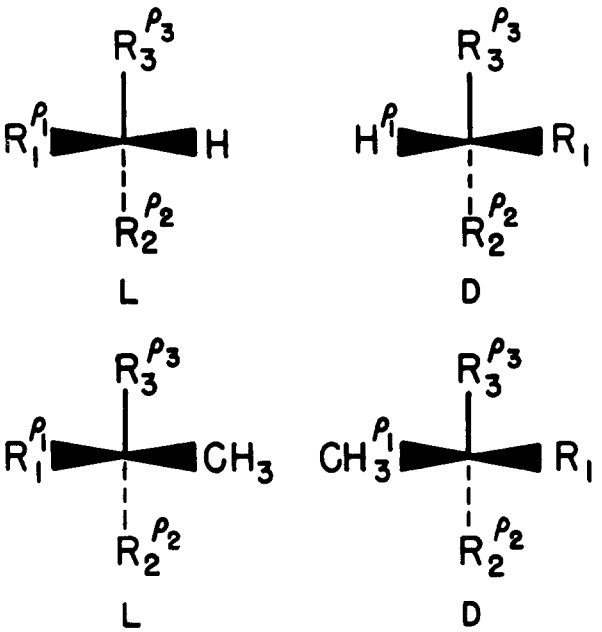

FIG. 1.—Disposition of α-*N*-acyl-α-amino acid esters of the $S^{3E}_{R_2R_3}$ limit type and their α-methyl derivatives at the active site of α-chymotrypsin.

antiomorphic pair if the L-antipode is a substrate of the $S^{3E}_{R_2R_3}$ limit type.

The preceding interpretation predicts an enzyme-inhibitor dissociation constant for the D-antipode within an order of magnitude of the enzyme-substrate dissociation constant of the L-antipode, even though the rate of formation of reaction products from the latter complex may be several orders of magnitude greater than from the former. The enzyme-inhibitor dissociation constants for methyl α-*N*-acetyl-D-phenylalaninate and ethyl α-*N*-acetyl-D-tyrosinate are $2.2 \pm 0.4 \times 10^{-3}$ M and $5.0 \pm 1.0 \times 10^{-3}$ M respectively (Foster and Niemann, 1955). The observed substration constants for the corresponding L-antipodes are 1.8×10^{-3} M and 0.7×10^{-3} M respectively (Bender and Glasson, 1960; Cunningham and Brown, 1956). The enzyme-substrate dissociation constants for these two substrates, and particularly the latter, may be greater than the observed substration constants, but probably not much greater (Spencer and Sturtevant, 1959; Hein and Niemann, 1961).

The argument developed above predicts that replacement of the α-hydrogen atom of a substrate approximating the $S^{3E}_{R_2R_3}$ limit type, or that of its D-antipode, by an alkyl group, *cf.* Figure 1, should result in a change of less then one order of magnitude in the dissociation constants of the complexes formed from the enzyme and either antipode. However, if the α-alkyl group is sterically equivalent to a minimally effective R_1 group, the rate of formation of products from the α-alkyl–substituted L-antipode should be substantially less than that observed for the unsubstituted compound. In this communication we wish to describe results obtained with the antipodes of methyl α-*N*-acetyl-α-methylphenylalaninate, -tyrosinate, and -β-(2-naphthyl)alaninate, which were evaluated as substrates of α-chymotrypsin and as competitive inhibitors of the hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide in the presence of this enzyme.

EXPERIMENTAL[1]

DL-α-*Methylphenylalanine.*—Reaction of phenylacetone with potassium cyanide and ammonium chloride followed by hydrolysis of the amino nitrile (Manning, 1954; Stein *et al.*, 1955; Steiger, 1955) gave 74–90% of the amino acid hydrochloride, m.p. 237°. A methanolic solution of the hydrochloride was saturated with ammonia, the precipitated ammonium chloride removed, and the solution evaporated to give the free acid, m.p. 297°. Stein *et al.* (1955) report a m.p. 241–243° for the hydrochloride and 294.5–295° for the amino acid.

N-*Acetyl*-DL-α-*Methylphenylalanine.*—Acetylation of the amino acid with acetyl chloride and aqueous sodium hydroxide (Schotten, 1884; Baumann, 1886) gave 39% of product, m.p. 196.5–197.5°. Acetylation with acetic anhydride and pyridine (Neuberger, 1938) gave 58–65% of product, m.p. 195–197°.

Anal. Calcd. for $C_{12}H_{15}O_3N$ (221): C, 65.1; H, 6.8; N, 6.3. Found: C, 65.0; H, 6.9; N, 6.3.

(+)- *and* (−)-*N*-*Acetyl*-α-*Methylphenylalanine.*—(A) Attempted resolution with Acylase I (Greenstein, 1957). To an aqueous suspension of 7 g of *N*-acetyl-DL-α-methylphenylalanine in 300 ml of water was added sufficient lithium hydroxide to bring the *p*H to 7.0; 0.2 g of Armour Acylase I, lot No. 945-40, was introduced and the solution maintained at 38°. At various time intervals 0.4-ml aliquots were withdrawn and analyzed for amino-nitrogen by the Van Slyke procedure (Archibald, 1957). The extent of hydrolysis was only 7.4% after 62 hours.

(B) Resolution with (+)-α-methylphenethylammonium sulfate. To an aqueous-ethanol solution containing equivalent amounts of (+)-α-methylphenethylammonium sulfate and *N*-acetyl-DL-α-methylphenylalanine was added one equivalent of 4 N aqueous sodium hydroxide. The precipitated sodium sulfate was removed, and the filtrate was concentrated until turbid and then stored at 4°. The crystalline precipitate was collected and fractionally recrystallized from aqueous-ethanol to give a series of dextrorotatory fractions, maximum $[\alpha]_D$ 17.9, minimum $[\alpha]_D$ 8.5°. Each fraction was dissolved in aqueous 4 N sodium hydroxide and the solutions (*p*H > 10) were extracted with ethyl ether, the aqueous phases acidified with concentrated hydrochloric acid, and the precipitated acylated amino acids collected. The end-fractions gave products $[\alpha]_D = 85.7°$ and $[\alpha]_D = -15°$ (*c*, 1% in water) respectively.

(C) Resolution with (+)- and (−)-α-phenethyl-

[1] All melting points are corrected. All optical rotations were determined at 25 ± 3° and have an uncertainty of ±2–3%.

amine. DL-α-Phenethylamine was resolved with D-tartaric acid (Theilacher and Winkler, 1954). The addition of one equivalent of (−)-α-phenethylamine in water, a mixture of isopropyl alcohol and isopropyl ether, ethyl acetate, or acetone, to one equivalent of the acylated DL-amino acid in the same solvent gave crystalline diastereoisomeric salts which were decomposed as described above to give acids of $[\alpha]_D$ −57°, −58°, −81°, and −85.6° (*c*, 0.3% in water) respectively. The acid $[\alpha]_D$ −85.6° (*c*, 0.3% in water), $[\alpha]_D$ −74.3° (*c*, 1% in methanol) was obtained in 55% yield. Reaction of (+)-α-phenethylamine with the acylated DL-amino acid in acetone, followed by decomposition of the crystalline salt, gave the other antipode of the acid, $[\alpha]_D$ 74.4° (*c*, 1% in methanol).

Methyl N-Acetyl-DL-α-methylphenylalaninate.—A suspension of 5 g of DL-α-methylphenylalanine in 100 ml of absolute methanol was cooled to 0–5° and saturated with hydrogen chloride. The solution was heated under refluxing conditions for 16 hours, the solvent removed *in vacuo*, the residual ester hydrochloride neutralized with cold aqueous potassium carbonate, and the solution stored at 4°. The stout prismatic crystals were collected and taken up in hot acetone, and the solution was filtered and the filtrate freed of solvent to give 4.8 g of crude ester. To a solution of 4 g of the latter product in 60 ml of 10% aqueous-acetone was added, in small portions, 6.3 g of acetic anhydride and sufficient aqueous sodium hydroxide to maintain the reaction mixture above *p*H 8 during addition of the first 4.2 g of anhydride. Addition of the remainder of the anhydride led to precipitation of the acetylated ester, which was recrystallized from aqueous ethanol to give 1.3 g of methyl *N*-acetyl-DL-α-methylphenylalaninate, m.p. 118.1–119°.

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235): C, 66.4; H, 7.3; N, 6.0. Found: C, 66.2, H, 7.2; N, 5.8.

An aqueous solution of the above ester and α-chymotrypsin was maintained at *p*H 8 and 25° for several hours. No significant enzymatic hydrolysis was observed.

Methyl (−)-N-Acetyl-α-methylphenylalaninate.—Esterification of (−)-*N*-acetyl-α-methylphenylalanine, $[\alpha]_D$ = −74.3 (*c*, 1% in methanol), by the method of Brenner and Huber (1953) gave the crude ester, which was recrystallized from water to give methyl (−)-*N*-acetyl-α-methylphenylalaninate, m.p. 82.0–82.5°, $[\alpha]_D$ = −82.4° (*c*, 1% in methanol).

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235): C, 66.4; H, 7.3; N, 6.0. Found: C, 66.0; H, 7.3; N, 6.1.

Methyl (+)-N-Acetyl-α-methylphenylalaninate.—Esterification of (+)-*N*-acetyl-α-methylphenylalanine, $[\alpha]_D$ = 74.4° (*c*, 1% in methanol), as described for the (−)-antipode, gave methyl (+)-*N*-acetyl-α-methylphenylalaninate, m.p. 79–80°, $[\alpha]_D$ = 82.9° (*c*, 1% in methanol).

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235): C, 66.4; H, 7.3; N, 6.0. Found: C, 66.0; H, 7.2; N, 6.0.

p-*Methoxybenzyl Methyl Ketone.*—Condensation of anisaldehyde with nitroethane in the presence of *n*-butylamine, as described by Hoover and Haas (1947), gave 19–33% of *p*-methoxyphenyl-2-nitropropene, m.p. 43.5–45.5°. Reduction of this intermediate with iron and hydrochloric acid (Hoover and Haas, 1947) gave 45–47% of *p*-methoxybenzyl methyl ketone, b.p. 121-124° at 5–6 mm, n_D^{25} = 1.5228.

DL-α-*Methyltyrosine.*—Reaction of *p*-methoxybenzyl methyl ketone with potassium cyanide and ammonium chloride followed by hydrolysis of the amino nitrile, essentially as described for the synthesis of the phenylalanine analogue, gave the crude *O*-methyl-α-methyl amino acid hydrochloride, m.p. > 320°, in 93% yield. A solution of 12.6 g of the crude hydrochloride in 50 ml of 31% hydrogen bromide in glacial acetic acid, contained in a pressure bottle, was heated in a boiling water bath for 1 hour, the reaction mixture was cooled and evaporated to dryness *in vacuo*, the residue was diluted with 300 ml of water, and the solution was adjusted to *p*H 6 with aqueous sodium hydroxide to precipitate the halide-free amino acid, m.p. 312–314°, with decomposition, in 60% yield.

α-N-*Acetyl-DL-α-methyltyrosine.*—(A) Acetylation of DL-α-methyltyrosine with acetic anhydride and pyridine, as described by Neuberger (1938), gave *O,N*-diacetyl-DL-α-methyltyrosine, m.p. 222–223°, neutral equivalent found 280 ± 5, calculated 279. A solution of the preceding intermediate in 2 N aqueous sodium hydroxide was allowed to stand at room temperature for 30 minutes, the solution was acidfied to *p*H 2, and the precipitate was collected, washed, and recrystallized from water to give α-*N*-acetyl-DL-α-methyltyrosine, m.p. 220.0–220.5°, neutral equivalent found 238 ± 4, calculated, 237.

(B) Acetylation of DL-α-methyltyrosine by the Schotten-Baumann procedure (Schotten, 1884; Baumann, 1886) gave 45% of α-*N*-acetyl-DL-α-methyltyrosine, m.p. 216.5–218°, after recrystallization of the crude product from aqueous dioxane.

(−)-α-N-*Acetyl-α-methyltyrosine.*—To a solution of 1 g of α-*N*-acetyl-DL-α-methyltyrosine in 100 ml of acetone was added 0.75 equivalents of (−)-α-phenethylamine. The precipitated salt was collected and the filtrate evaporated to dryness. Both fractions were dissolved in 4 N aqueous sodium hydroxide, the solutions were extracted three times with ethyl ether, and the aqueous phases were acidified to *p*H 2 with concentrated hydrochloric acid. The precipitated acids were collected, washed with water, and dried, and their rotations were determined. The fractionation was repeated until products of constant rotation were obtained. Six successive fractionations of 20 g of the DL-acid gave 0.3 g of (−)-α-*N*-acetyl-α-methyltyrosine $[\alpha]_D$ −61.1° (*c*, 1–2% in methanol).

(+)-α-N-*Acetyl-α-methyltyrosine.*—Fractionation of 1 g of α-*N*-acetyl-DL-α-methyltyrosine with (+)-α-phenethylamine gave 0.2 g of (+)-α-*N*-acetyl-α-methyltyrosine, $[\alpha]_D$ 61.0° (*c*, 1–2% in methanol).

Methyl α-N-*Acetyl-DL-α-methyltyrosinate.*—An ice-cold solution of 1.37 g of α-*N*-acetyl-DL-α-

methyltyrosine in 20 ml of absolute methanol was saturated with hydrogen chloride. The reaction mixture, after standing at room temperature for 4 days, was chilled, again saturated with hydrogen chloride, and allowed to stand overnight. The mixture was evaporated to dryness *in vacuo*, and the residue, freed of hydrogen chloride by vacuum dessication over solid sodium hydroxide, was dissolved in a small amount of methanol. Water was added to give, after storage at 4°, 0.45 g of crude ester. Recrystallization from aqueous-ethanol gave methyl α-*N*-acetyl-DL-α-methyltyrosinate, m.p. 121-122°.

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): N, 5.6. Found: N, 5.3.

Methyl (−)-α-N-*Acetyl-α-methyltyrosinate.*—Esterification of (−)-α-*N*-acetyl-α-methyltyrosine, as described for the phenylalanine analogue, gave methyl (−)-α-*N*-acetyl-α-methyltyrosinate, m.p. 149–150°, $[\alpha]_D$ −78.9° (*c*, 1% in methanol), after recrystallization from water.

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.1; H, 6.8; N, 5.5.

Methyl (+)-α-N-*Acetyl-α-methyltyrosinate.*—Esterification of (+)-α-*N*-acetyl-α-methyltyrosine, as described for the (−)-antipode, gave methyl (+)-α-*N*-acetyl-α-methyltyrosinate, $[\alpha]_D$ 77.9° (*c*, 1% in methanol).

2-Naphthylacetone.—This ketone was prepared from 98 g of 2-naphthaldehyde, 60 ml of nitroethane, and 22 ml of *n*-butylamine by the method of Heinzelmann (1951). The intermediate nitroalkene, obtained in 90% yield, was reduced with iron and hydrochloric acid, the reaction mixture was steam distilled, the 15 liters of distillate were extracted with toluene and then three times with ether, the combined extracts were freed of solvent *in vacuo*, and the residue was fractionally distilled to give a mid-fraction, b.p. 103–118° at 0.45–0.55 mm, in 33% yield. This product gave a colorless semicarbazone, m.p. 188–190°, a yellow picrate, m.p. 75–78°, and an orange 2,4-dinitrophenylhydrazone m.p. 270–275°. Newman and Mangham (1949) report a semicarbazone, m.p. 183–184°, and a picrate, m.p. 79–80°. Wilds *et al.* (1946) report a 2,4-dinitrophenylhydrazone, m.p. 172–173°. It is likely that an observed value of 272–273° was reported as 172–173°.

DL-α-*Methyl-β-(2-naphthyl)alanine.*—The procedure used previously for the synthesis of DL-α-methylphenylalanine and DL-α-methyltyrosine from the corresponding ketones gave a 66% yield of chloride-free DL-α-methyl-β-(2-naphthyl)alanine, m.p. 266-271°, from 2-naphthylacetone.

N-*Acetyl*-DL-α-*methyl-β-(2-naphthyl)-alanine.*—Acetylation of the amino acid with acetic anhydride and pyridine gave a crystalline product, long needles, m.p. 216–217.5°, after recrystallization from water.

Anal. Calcd. for $C_{16}H_{17}O_3N$ (271): C, 70.8; H, 6.3, N, 5.2. Found: C, 70.6; H, 6.4; N, 5.4.

(+)- *and* (−)-N-*Acetyl-α-methyl-β-(2-naphthyl)-alanine.*—The DL-compound was resolved with (+)- and (−)-α-phenethylamine, as described for the analogous α-methyltyrosine derivative, to give after five fractional precipitations (−)-*N*-acetyl-α-methyl-β-(2-naphthyl)alanine, $[\alpha]_D$ −160° (*c*, 1% in methanol). The (+) enantiomer, $[\alpha]_D$ 140° (*c*, 1% in methanol), was only partially resolved after seven fractional precipitations.

Methyl N-*Acetyl*-DL-α-*methyl-β-(2-naphthyl)alaninate.*—Esterification of the DL-acid by the procedure of Brenner and Huber (1953) gave the ester, m.p. 127–128°, in good yield.

Anal. Calcd. for $C_{17}H_{19}O_3N$ (285); C, 71.6; H, 6.7; N, 4.9. Found: C, 71 5; H, 6.7; N, 5.1.

Methyl (−)-N-*Acetyl-α-methyl-β-(2-naphthyl)-alaninate.*—Esterification of the (−) acid, as described for the DL-mixture, gave the ester, m.p. 127–129°, $[\alpha]_D$ −206° (*c*, 0.5% in methanol).

Sodium α-N-*Acetyl*-L-*tyrosinhydroxamate.*—This compound, m.p. 189.7–190.0°, with decomposition, $[\alpha]_D$ 34.7° (*c*, 4% in 0.2 N aqueous hydrochloric acid), was prepared as described by Kurtz (1960), who reported as m.p. 190.5–191.0°, with decomposition, and an $[\alpha]_D$ 35° (*c*, 5% in N aqueous hydrochloric acid).

α-*Chymotrypsin.*—An Armour preparation of salt-free bovine α-chymotrypsin, lot No. 797207, was analyzed for nitrogen by the procedure of Redemann (1939) and found to contain 14.6 ± 0.2% nitrogen.

Kinetic Studies.—Stock solutions of 0.05 M α-*N*-acetyl-L-tyrosinhydroxamide, 0.05 M sodium chloride, 1.65 M sodium chloride, 1.4 mg α-chymotrypsin per ml, and the inhibitors were prepared with carbon dioxide–free water. The substrate stock solution contained 7.8 ml of 0.6 N aqueous hydrochloric acid per 10 ml, to form the acid from the sodium salt used in its preparation. Reaction mixtures were prepared from 1 ml of inhibitor solution; *n* ml of substrate stock solution, with *n* = 1, 2, 3, 5, and 7; 7 − *n* ml 0.05 M sodium chloride; 1 ml of 1.65 M sodium chloride; and 1 ml of enzyme solution, which was added later. The reaction mixture (lacking enzyme and contained in an automatic recording *p*H-stat) and a portion of the enzyme stock solution were separately adjusted to *p*H 7.60, the reaction mixture with standardized aqueous sodium hydroxide and the enzyme stock solution with 1.0 N aqueous sodium hydroxide, just prior to the addition of the adjusted enzyme stock solution to the reaction mixture. In this way a tracing of the amount of acid produced by the enzyme-catalyzed reaction at *p*H 7.60 and 25.0° was obtained with a minimum amount of "hunting" by the *p*H-stat during the initial minute of reaction. This procedure is essentially that described, in greater detail, by Abrash (1961) and is similar to that developed by Kurtz (1960) for use of the *p*H-stat in those cases where one of the reaction products is a buffer.

Results

Evaluation of methyl (+)- and (−)-α-*N*-acetyl α-methylphenylalaninate, (+)- and (−)-α-*N*-

TABLE I

ENZYME-INHIBITOR DISSOCIATION CONSTANTS OF α-CHYMOTRYPSIN AND SEVERAL *N*-ACETYL-α-METHYL-α-AMINO ACID ESTERS[a]

α-Amino Acid Derivative[b]	[*I*][c]	*a'*/*a*[d]	$K_I \pm \sigma K_I$[c,e]	$K_I \pm \sigma K_I$[c,f]	K_I[c,g]
(−)-Ac-α-Me-Phe Me	1.182	1.1718	6.9 ± 6.0		
	0.788	1.0693	11.4 ± 23.0	8.2 ± 13	7
(+)-Ac-α-Me-Phe Me	1.071	1.1658	6.5 ± 5.8		
	0.714	1.0387	18.4 ± 66.7	8.7 ± 29	7
(−)-Ac-α-Me-Tyr Me	1.208	1.1231	9.8 ± 10.6		
	0.818	1.0742	11.0 ± 17.8	10.3 ± 14	10
(+)-Ac-α-Me-Tyr Me	0.651	1.4353	1.5 ± 0.6		
	0.434	1.2202	2.0 ± 1.2	1.7 ± 0.8	2
(−)-Ac-α-Me-Nap Me	0.0421	1.1331	0.32 ± 0.29		
	0.0280	1.1136	0.25 ± 0.26	0.28 ± 0.28	0.3
(±)-Ac-α-Me-Nap Me	0.0298	1.3680	0.081 ± 0.037		
	0.0199	1.2343	0.085 ± 0.054	0.082 ± 0.04	0.08
(+)-Ac-α-Me-Nap Me				0.048 ± 0.13[h]	0.05

[a] Evaluated against α-*N*-acetyl-L-tyrosinhydroxamide in aqueous solutions at 25.0°, *p*H 7.60 and 0.2 M with respect to sodium chloride with [*E*] = 0.02 mg protein-nitrogen per ml and [*S*] = 5 to 35 × 10^{-3} M. [b] Ac = α-*N*-acetyl, Phe = phenylalanine, Tyr = tyrosine, Nap = β-(2-naphthyl)alanine, α-Me = α-methyl, Me = methyl ester. [c] In units of 10^{-3} M. [d] Ratio of apparent intercept of inhibited reaction *a'*, *cf.* equation (1), to apparent intercept for reaction in absence of inhibitor, *a* = 1.4218 min. mg protein-nitrogen per ml. [e] Standard deviation, $\sigma K_I = K_I(2\sigma_b/b + \sigma a/a + \sigma a'/a')(a'/a/[(a'/a) - 1])$, where *b* is the common apparent slope. [f] Weighted in proportion to per cent inhibition, *i.e.*, $w_i = [(a_i'/a) - 1]/(\Sigma_i[(a_i'/a) - 1])$. [g] Preferred value recognizing greater accuracy inherent in evaluation at higher inhibitor concentrations. [h] Estimated from the two preceding values, see text.

acetyl-α-methyltyrosinate, and (±) and (−)-α-*N*-acetyl-α-methyl-β-(2-naphthyl)alaninate as competitive inhibitors of the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide was conducted in aqueous solutions at 25.0°, *p*H 7.60, 0.2 M with respect to sodium chloride. The enzyme concentration was 0.02 mg protein-nitrogen per ml, and the substrate concentrations were 5 to 35 × 10^{-3} M. Two inhibitor concentrations were used, which for each ester were approximately one tenth and one fifteenth of their saturated solutions. A total of 155 experiments were performed, 37 in the absence of inhibitor to evaluate kinetic constants for the uninhibited reaction, and 19 or 20 for each inhibitor. Values for initial velocities were obtained from the automatic pH-stat recorder traces by the orthogonal polynomial procedure of Booman and Niemann (1956) which had been programmed for a Datatron 220 computer (Abrash *et al.*, 1960). The enzyme and substrate blanks were insignificant. Since hydroxylamine, in aqueous solutions at 25.0°, *p*H 7.60 and 0.2 M with respect to sodium chloride, is protonated to an extent of 2.5%, the initial velocities were corrected for the buffering action of liberated hydroxylamine by the factor 1.025. The data were then fitted by a reiterative least-squares procedure, using the Datatron 220 computer (Abrash *et al.*, 1960), to equation (1):

$$[S][E]/v = (K_S(1 + [I]/K_I))/k_3 + [S]/k_3 \quad (1)$$

Values of K_I, the enzyme-inhibitor dissociation constant, were obtained from the ratios of the apparent intercepts for the inhibited and noninhibited reactions. These results are summarized in Table I.

Manning (1954) observed that methyl α-*N*-acetyl-DL-α-methylphenylalaninate and methyl-α-*N*-acetyl-*O*-methyl-DL-α-methyltyrosinate were not hydrolyzed by α-chymotrypsin under conditions where the α-hydrogen analogues were effective substrates. In order to determine whether any of the compounds evaluated as competitive inhibitors of the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinhydroxamide were substrates of this enzyme they were examined for possible substrate activity in aqueous solutions at 25.0°, *p*H 7.90 and 0.2 M with respect to sodium chloride, at an enzyme concentration substantially greater than that used in the inhibition studies. These results are given in Table II.

TABLE II

N-ACETYL-α-METHYL-α-AMINO ACID ESTERS AS SUBSTRATES OF α-CHYMOTRYPSIN[a]

α-Amino Acid Derivative[b]	[*S*][c]	*v*[d]	v_{base}[e]	k_3[f]
(−)-Ac-α-Me-Phe Me	13.1	0.80	0.08	9
(+)-Ac-α-Me-Phe Me	10.1	0.63	0.04	8
(−)-Ac-α-Me-Tyr Me	4.43	1.09	<0.02	25
(−)-Ac-α-Me-Nap Me	0.25	13.4 ± 0.2[g]	<0.02	196
(±)-Ac-α-Me-Nap Me	0.36	0.85	<0.02	7
Enzyme blank	0	0.85		

[a] In aqueous solutions at 25.0°, *p*H 7.90 and 0.2 M with respect to sodium chloride, with [*E*] = 0.145 mg protein-nitrogen per ml. [b] Ac = α-*N*-acetyl, Phe = phenylalanine, Tyr = tyrosine, Nap = β-(2-naphthyl)alanine, α-Me = α-methyl, Me = methyl ester. [c] In units of 10^{-3} M. [d] In units of 10^{-6} M/min. [e] Base-catalyzed hydrolysis in units of 10^{-6} M/min. [f] In units of 10^{-6} M/min./mg protein-nitrogen per ml and calculated from values of *v* and K_I, *cf.* Table I, ignoring enzyme blank reaction. Values of k_3 are for upper limits. [g] Calculated from a second-degree polynomial.

Since only methyl (±) and (−) *N*-acetyl-α-methyl-β-(2-naphthyl)alaninate were available the

value of K_I for the (+) enantiomer was estimated from equation (2),

$$v/[E] = \{k_3[S_1] + (k_6[S_2]K_{S_1}/K_{S_2})\}/\{K_{S_1}[1 + ([S_2]/K_{S_2}) + ([I]/K_I)] + [S_1]\} \quad (2)$$

which may be derived for the case of two competitive substrates and one competitive inhibitor, *i.e.*, equations (3), (4), and (5)

$$E + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_3} E + P \quad (3)$$

$$E + S_2 \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_2 \xrightarrow{k_6} E + P \quad (4)$$

$$E + I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EI \quad (5)$$

where $K_{S_1} = (k_2 - k_3)/k_1$, $K_{S_2} = (k_5 + k_6)/k_4$ and $K_I = k_8/k_7$. The symbols S_1, S_2, and I refer to α-*N*-acetyl-L-tyrosinhydroxamide and to methyl (−)- and (+)-*N*-acetyl-α-methyl-β-(2-naphthyl)-alaninate respectively. With $[S_2] = [I] = [(\pm)\text{-ester}]/2$, the data given in Table II indicate that the second term of equation (2) can contribute to the total velocity only to an extent of 0.1–0.7%, and consequently the contribution of this term was ignored in estimating the magnitude of K_I for the (+) enantiomer.

Discussion

The large uncertainties in the values of the enzyme-inhibitor dissociation constants given in Table I are clearly the result of their evaluation under conditions where the observed percentage inhibition, 100 $[(a'/a) - 1]$, was relatively small, which, in turn, was dictated by the limited solubilities of these compounds in water. The inhibitor stock solutions were essentially saturated. We believe that the preferred values of K_I, which are rounded values of those determined at the highest inhibitor concentration, are reliable as to order of magntiude and are preferable to values of lesser variability that might have been obtained by use of a mixed solvent system. We considered, and rejected, the latter alternative because of the uncertainty inherent in the interpretation of kinetic studies conducted in mixed solvent systems.

From the data given in Table II it is seen that only methyl (−)-*N*-acetyl-α-methyl-β-(2-naphthyl) alaninate is hydrolyzed with a velocity substantially greater than that of the enzyme blank reaction. However, it is known that the enzyme blank reaction is suppressed by added substrate or inhibitor (Martin and Niemann, 1957), and substantially so when the added compounds have low K_S or K_I values (Wolf, 1959). We may therefore ignore the enzyme blank reaction and calculate values of k_3 from the initial velocities. These values, given in Table II, are estimates of the probable maximum magnitudes of k_3 for the various compounds and are reliable only as to orders of magnitude.

The values of k_3 listed in Table II demonstrate that methyl (−)-*N*-acetyl-α-methyl-β-(2-naphthyl) alaninate is a substantially better substrate than its (+) enantiomer and that methyl (−)-α-*N*-acetyl-α-methyltyrosinate is also capable of functioning as a substrate, albeit a very poor one. We therefore suggest, as a working hypothesis, that the (−) enantiomers of the above compounds belong to the L- or "natural" series of α-amino acids. The upper limits of k_3 for methyl (−)- and (+)-*N*-acetyl-α-methylphenylalaninate are too close to the limits of detectability to permit a decision as to which of these compounds can function as substrates.

Comparison of the optical rotations of the α-methyl derivatives and their α-hydrogen analogues, *cf.* Table III, indicates that the (−) enantiomers of the α-methyl derivatives possess a common configuration, a conclusion generally consistent with their behavior, and that of the (+) enantiomers, as substrates or inhibitors of α-chymotrypsin. However, the ambiguity inherent in the use of optical rotation data for determination of the absolute configuration of α-alkyl-α-amino acids, or their derivatives (Winitz *et al.*, 1955), places the entire burden of provisional assignment of the L, or *S* (Cahn *et al.*, 1956), configuration to the (−) enantiomers on the enzymatic studies, particularly those with methyl (±)- and (−)-*N*-acetyl-α-methyl-β-(2-naphthyl)alaninate, where it is assumed that hydrolysis proceeds with the usual, but not invariable (Hein *et al.*, 1960), antipodal specificity in favor of the L-enantiomer.

Table III

Specific Rotations and Kinetic Constants of Several Aromatic α-Amino Acid and α-Methyl-α-Amino Acid Derivatives

α-Amino Acid Derivative[a]	$[\alpha]D^b$	K_S or K_I[c]	k_3[d]
Ac-L-Phe	21.0		
Ac-L-Phe Me	19.5[e]	1.8[f]	0.92[f]
Ac-D-Phe Me	−19.5[e]	2.2[g]	
(−)-Ac-α-Me-Phe	−74		
(−)-Ac-α-Me-Phe Me	−82	7	$<10^{-5}$
(+)-Ac-α-Me-Phe	74		
(+)-Ac-α-Me-Phe Me	83	7	$<10^{-5}$
Ac-L-Tyr	51.5		
Ac-L-Tyr Et	24.6[h,i]	0.7[i]	2.9[i]
Ac-D-Tyr Et	−24.6[h,i]	5.0[g]	
(−)-Ac-α-Me-Tyr	−61		
(−)-Ac-α-Me-Tyr Me	−79	10	2.5×10^{-5}
(+)-Ac-α-Me-Tyr	61		
(+)-Ac-α-Me-Tyr Me	78	2	
(−)-Ac-α-Me-Nap	−160		
(−)-Ac-α-Me-Nap Me	−206	0.3	2×10^{-4}
(+)-Ac-α-Me-Nap Me		0.05	

[a] Ac = α-*N*-acetyl, Phe = phenylalanine, Tyr = tyrosine, Nap = β-(2-naphthyl)alanine, α-Me = α-methyl, Me = methyl ester, Et = ethyl ester. [b] Determined in methanol unless otherwise noted. [c] In units of 10^{-3} M. [d] In units of M/min./mg protein-nitrogen per ml. [e] Huang *et al.* (1952). [f] Bender and Glasson (1960). [g] Foster and Niemann (1955). [h] Hogness and Niemann (1953). [i] Determined in ethanol. [j] Cunningham and Brown (1956).

The data summarized in Table III demonstrate that replacement of the α-hydrogen atom of several representative enantiomorphic α-*N*-acylated aro-

matic α-amino acid esters by a methyl group leads to changes in values of K_S or K_I of an order of magnitude or less. Thus, the extent to which either enantiomer can form an enzyme-substrate or inhibitor complex is influenced to only a minor degree by the preceding structural alteration. In contrast, the same structural modification profoundly influences the ability of the enzyme-substrate complex to yield products, the rates of their formation being decreased by a factor of *ca.* 10^5.

The argument developed earlier predicted values of K_I and K_S for the D- and L- enantiomers of the α-methyl derivatives within an order of magnitude of those of their α-hydrogen analogues. In addition it proposed that the rates of formation of products from the α-methyl substituted L-enantiomers would be substantially less than for the unsubstituted compounds if the α-methyl group were sterically equivalent to the α-acylamino moiety when either group assumed the position in the enzyme-substrate complex normally occupied by the α-hydrogen atom of the unsubstituted L-enantiomers.

The results of the present study provide support for the validity of the foregoing proposal, and it may be asserted that, for a substrate of the $S^{3E}_{R_2R_3}$ limit type, replacement of the α-hydrogen atom by a substituent of the size of a methyl group, or larger, will depress the rate of formation of products by a factor of *ca.* 10^5 even though the value of the enzyme-substrate dissociation constant remains within an order of magnitude of that of the unsubstituted analogue.

The experiments with methyl (−)-*N*-acetyl-α-methyl - β - (2 - naphthyl)alaninate suggest that methyl *N*-acetyl-L-β-(2-naphthyl)alaninate would be a particularly effective substrate of α-chymotrypsin and would be hydrolyzed at a rate faster than any hitherto evaluated α-*N*-acetyl-L-α-amino acid methyl ester. Although the compound would be relatively insoluble in water, its estimated K_S value of *ca.* 10^{-4} M and k_3 value of *ca.* 20 M/minute/mg protein-nitrogen per ml should permit evaluation of its kinetic constants in this solvent system.

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