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Hydrolysis of D-(−)-Ethyl α-Benzoyloxypropionate and L-(−)-Ethyl α-Acetoxy-β-phenylpropionate by α-Chymotrypsin[1]

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Hydrolysis of DL-ethyl α-benzoyloxypropionate by α-chymotrypsin leads to D-(−)-α-benzoyloxypropionic acid and to L-(+)-ethyl α-benzoyloxypropionate. Hydrolysis of DL-ethyl α-acetoxy β-phenylpropionate is more rapid and leads to L-(−)-α-acetoxy β-phenylpropionic acid and to D-(+)-ethyl α-acetoxy β-phenylpropionate. The kinetic parameters are: D-(−)-ethyl α-benzoyloxypropionate, $K_m = 0.0058$ M, $k_3 = 0.021$ sec.$^{-1}$; DL-, $K_m = 0.0067$ M, $k_3 = 0.014$ sec.$^{-1}$; L-(+)-, $K_m = 0.016$ M, $k_3 = 0.003$ sec.$^{-1}$; L-(−)-ethyl α-acetoxy-β-phenylpropionate, $K_m = 0.023$ M, $k_3 = 0.60$ sec.$^{-1}$; DL-, $K_m = 0.027$ M, $k_3 = 0.37$ sec.$^{-1}$; the D-(+)-compound was inert. A brief study was carried out on L-(−)- and D-(+)-ethyl α-hydroxy-β-phenylpropionate. Ethyl α-benzoyloxypropionate, lacking a β-aryl group and having enhanced aryl property at the usual acylamido position, shows marked D-specificity. Its reactivity toward α-chymotrypsin is similar to that of methyl β-phenylpropionate and D-methyl α-benzamidopropionate. The study of ethyl α-acetoxy-β-phenylpropionate shows that competition between a β-aryl and an α-acyloxy group for the β-aryl site on the enzyme is dominated by the β-aryl group, leading to high L-specificity. Reactivity of the L-α-acetoxy-β-phenylpropionate is greater than that of the β-phenylpropionate and less than that of the L-α-hydroxy-β-phenylpropionate.

Introduction

Stereospecificity of reactions of α-chymotrypsin has been described in terms of sites on the enzyme complementary to the four groups which are oriented tetrahedrally about the α-carbon atom of substrates which are derivatives of α-substituted carboxylic acids.[2-6] The substrate groups may fit into the corresponding sites or associate with them, with varying effects. (1) The *β-aryl* (or α-benzyl) substituent, characteristic of "natural" substrates of α-chymotrypsin,[7] leads to positive association[2] and favorable K_m and k_3. Non-aromatic β-substituents which may lead to favorable K_m are cyclohexyl,[8] isopropyl,[9] and carbethoxy,[10] the latter leading to favorable reactivity of dicarboxylic esters and their α-acetamido derivatives.[11] (2) The *α-acylamido* group may fit into its site and associate by hydrogen bonding[6,12]; N-methylation removes reactivity.[2,10] Its presence in a substrate leads to stereospecificity[2,13] and to favorable k_3, but may make little contribution to K_m.[11,12] This interaction may involve distortion and activation of the enzyme, thus not leading to more favorable K_m. (3) The *α-hydrogen* may fit into a site of restricted size,[2,5,6,13] and appears essential, compounds with quaternary α-carbons not being hydrolyzed readily by α-chymotrypsin.[2,13] (4) The *hydrolyzing* group, ester or amide, may associate at the reaction site proper, where a serine hydroxyl[14] may attack the carbonyl carbon. Esters are more readily attacked by α-chymotrypsin than are amides, showing more favorable K_m and k_3.[2]

These indicated modes of association of substrate with enzyme are not unique in detail.[15] D-Enantiomorphs may bind well,[16] the β-aryl and α-hydrogen presumably at their normal sites, leaving the acylamido and hydrolyzing groups at the hydrolytic and acylamido sites, respectively.[6] Reaction, therefore, does not occur and competitive inhibition[16] results from this nonproductive binding. Ethyl α-acetoxypropionate, lacking a β-aryl substituent, showed inversion of the normal stereospecificity,[3,6] more rapid hydrolysis of the D-enantiomorph than the L-, behaving in this respect quite differently from the nitrogen analog, ethyl N-acetylalaninate. It appears that the α-acetoxy group, lacking an amide hydrogen for hydrogen bonding, in the absence of a β-aryl substituent in this substrate has the acyl group associate preferentially at the β-aryl site.[6] If the α-hydrogen is maintained at its normal, restricted site, the D-enantiomorph delivers the carbethoxyl group to the hydrolytic site, while the L- does not, leading to firm nonproductive binding of the L-enantiomorph,[6] $K_m = 0.0017$ M, $k_3 = 0.00036$ sec.$^{-1}$, and to more rapid hydrolysis of the D- at concentrations above 3×10^{-3} M, $K_m = 0.18$ M, $k_3 = 0.020$ sec.$^{-1}$.

The cyclic compound, 1-keto-3-carbomethoxytetrahydroisoquinoline, had previously shown inversion of normal stereospecificity in hydrolysis by α-chymotrypsin,[2,4,17] the D-enantiomorph hydrolyzing quite rapidly and far more rapidly than the L-. In this compound the moiety that can be considered to be the β-aryl group is also part of the α-acylamido group. As such, it may force the acylamido group toward the β-aryl site, and, following the argument given above, the D-enantiomorph may then deliver the carbethoxyl group to the hydrolytic site.[2] Similar influences were then seen in acyclic substrates lacking the β-aryl group and with aryl properties at the acylamido group. N-Benzoylalanine methyl ester showed diminished stereochemical preference[18] for the L-antipode as compared with N-acetylalanine ester, in hydrolysis by α-chymotrypsin. With N-picolinylalanine methyl ester inversion of stereospecificity was observed,[18] K_0 being essentially equal for the L- and D-antipodes, 0.018 and

(1) We are pleased to acknowledge generous support of this work by the Division of Research Grants, National Institutes of Health, GM04584.

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0.017 M, respectively, and k_0 being greater for the D-antipode, 0.165 sec.$^{-1}$ as compared with 0.070 sec.$^{-1}$ for the L-.

In the meantime we have continued our study of α-acyloxy compounds and wish to report on two sets, the ethyl α-benzoyloxypropionates, $CH_3CH(OCOC_6H_5)CO_2C_2H_5$, and the α-acetoxy-β-phenylpropionates, $C_6H_5CH_2CH(OCOCH_3)CO_2C_2H_5$. The former, lacking a β-aryl group and having enhanced aryl property at the acyloxy site, might show enhanced D-stereospecificity as compared with the previously studied acetoxy compound. The latter would indicate outcome of competition between the α-acetoxy and the β-aryl groups for the aryl site on the enzyme.

Results

L-(+)- and D-(−)-ethyl α-benzoyloxypropionates, $[\alpha]^{22}D$ ±24.4°, neat, 18.3° in chloroform, were prepared by (1) conversion of zinc L-lactate and calcium D-lactate to L-(−)-ethyl lactate and D-(+)-ethyl lactate by a modification of a procedure in the literature[19] and (2) treatment of these esters with benzoyl chloride and pyridine. The DL-ester was prepared by treatment of ethyl lactate with benzoyl chloride and pyridine.

L-(−)- and D-(+)-ethyl α-acetoxy-β-phenylpropionates, $[\alpha]^{23}D$ ±8.6° in chloroform, were prepared by (1) conversion of L-(−)- and D-(+)-β-phenylalanine to L-(−)- and D-(+)-α-hydroxy-β-phenylpropionic acids, (2) thence to L-(−)- and D-(+)-ethyl α-hydroxy-β-phenylpropionates, and (3) to the acetoxy esters. DL-Ethyl α-acetoxy-β-phenylpropionate was prepared by (1) conversion of DL-β-phenylalanine to the hydroxy acid, (2) thence to the acetoxy acid, and (3) to the acetoxy ester.

A suspension of 1.8 g. of DL-ethyl α-benzoyloxypropionate was subjected to hydrolysis by 0.20 g. of α-chymotrypsin in a pH stat, pH 7.8. Hydrolysis was slow, 50% reaction after 46 hr., at which time the reaction was still proceeding. Extraction of the mixture led to recovery of the unhydrolyzed ester in high yield. It was optically active, $[\alpha]^{22}D$ + 10.5° in chloroform, indicating fourfold excess of L-(+)-compound in the recovered ester and that the D-(−)-ester was hydrolyzed at least four times as fast as its antipode by the enzyme under these conditions. The optically active D-(−)-α-benzoyloxypropionic acid was isolated from the extracted reaction solution, $[\alpha]^{22}D$ −19.7°.

The kinetics of hydrolysis of D-(−)-, L-(+)-, and DL-ethyl α-benzoyloxypropionates by α-chymotrypsin (2 mg./ml.) was studied in 4:1 water–ethanol at pH 7.8. The D-(−)- and DL-esters were hydrolyzed effectively by the enzyme and small corrections due to nonenzymatic hydrolysis were determined and applied. The L-(+)-ester was not hydrolyzed rapidly by the enzyme and the nonenzymatic corrections were large. Corrected initial pseudo-zero-order rates are given in Table I.

Linear double reciprocal plots[20] ($1/V$ *vs.* $1/S$) were obtained and the kinetic parameters K_m and k_3 were calculated by least-squares analysis: D(−)-, K_m = 0.0058 M, k_3 = 0.021 sec.$^{-1}$; DL-, K_m = 0.0067, k_3 = 0.014; L-(+)-, K_m = 0.016, k_3 = 0.003.

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TABLE I

RATES OF HYDROLYSIS OF D-(−)-, L-(+)-, AND DL-ETHYL α-BENZOYLOXYPROPIONATES BY α-CHYMOTRYPSIN, 2 MG./ML., IN 4:1 WATER–ETHANOL, 0.055 M NaCl, pH 7.8, 25°

Ester	10^3S, M	10^6V, M sec.$^{-1}$
D-(−)-	2.33	45.8
	3.84	60.0
	4.84	73.3
	5.81	80.7
	6.65	85.0
DL-	2.06	29.1
	3.29	37.5
	4.26	40.0
	6.60	49.3
L-(+)-	2.50	3.3
	3.29	3.3
	4.42	5.0
	5.45	6.7
	6.75	6.7

A suspension of 0.52 g. of DL-ethyl α-acetoxy-β-phenylpropionate was subjected to hydrolysis by 0.20 g. of α-chymotrypsin in a pH stat, 50% hydrolysis occurring in 1 hr., at which time the reaction was proceeding at about one-third the initial rate. The unhydrolyzed ester was separated and showed a very low rotation, while the acid product isolated from the hydrolysate showed a slightly larger rotation. Both ester and acid showed plain rotatory dispersion curves in chloroform. The ester showed low positive rotation above and high negative rotation below 385 mμ. The acid showed low negative rotation above and high positive rotation below 322 mμ. The dispersion curves indicated some stereospecificity, and the kinetics of hydrolysis of the L-(−)-, D-(+)-, and DL-materials by 0.5 mg./ml. of α-chymotrypsin were examined in 4:1 water–ethanol at pH 7.8. The L- and DL- materials were readily studied under these conditions, while the D-compound was unreactive and was not studied in detail. Corrected initial pseudo-zero-order rates of hydrolysis of the L- and DL-materials are given in Table II.

TABLE II

RATES OF HYDROLYSIS OF L-(−)- AND DL-ETHYL α-ACETOXY-β-PHENYLPROPIONATES BY α-CHYMOTRYPSIN, 0.5 MG./ML., IN 4:1 WATER–ETHANOL, 0.055 M NaCl, pH 7.8, 25°

Ester	10^3S, M	10^7V, M sec.$^{-1}$
L-(−)-	1.75	7.9
	2.31	8.9
	3.48	13.0
	3.49	15.9
	3.48	13.9
	3.49	13.9
	4.65	19.7
	5.25	21.0
DL-	2.27	5.3
	2.27	5.3
	3.41	7.4
	3.41	7.8
	4.51	9.6

Linear double reciprocal plots were obtained and the kinetic parameters were calculated for the L-(−)-compound by least-squares analysis, L-(−)-, K_m = 0.023 M, k_3 = 0.60 sec.$^{-1}$, and for the DL-material, graphically, K_m = 0.027 M, k_3 = 0.37 sec.$^{-1}$.

A brief study was also made of the kinetics of hydrolysis of L-(−)- and D-(+)-ethyl α-hydroxy-β-

TABLE III

RATES OF HYDROLYSIS OF L-(−)- AND D-(+)-ETHYL α-HYDROXY-β-PHENYLPROPIONATES BY α-CHYMOTRYPSIN, 0.1 MG./ML. AND 0.2 MG./ML., RESPECTIVELY, IN 4:1 WATER–ETHANOL, 0.075 *M* NaCl, pH 7.8, 25°

Ester	10^4S, *M*	10^7V, *M* sec.$^{-1}$
L-	2.38	11.0
	3.56	15.5
	4.75	20.3
D-	2.66	1.50
	3.98	1.92
	3.98	1.92
	5.20	2.50

phenylpropionate in 4:1 water–ethanol, 0.075 *M* NaCl, in the presence of 0.1 mg./ml. and 0.2 mg./ml. of α-chymotrypsin, respectively. The pseudo-zero-order rates are given in Table III. Linear double reciprocal plots were obtained and the kinetic parameters, K_m and k_3, were determined graphically: L-(−)-, K_m = 0.0027 *M*, k_3 = 4.1 sec.$^{-1}$; D-(+)-, K_m = 0.0062, k_3 = 0.069.

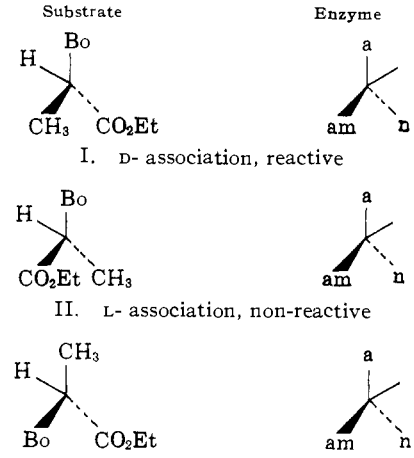
Discussion

Ethyl α-benzoyloxypropionate showed marked inversion of normal stereospecificity in its hydrolysis by α-chymotrypsin, the D-enantiomorph being hydrolyzed about 15 times as rapidly as the L- in the kinetic experiments in 4:1 water–ethanol, 0.055 *M* NaCl. Similarly the DL-material showed preferential hydrolysis of the D-material, perhaps to a lesser extent, in water containing phosphate buffer. The more rapid hydrolysis of the D-enantiomorph reflected both a more favorable K_m, 0.0058 *M vs.* 0.016 *M* for the L-, and a greater k_3, 0.021 sec.$^{-1}$ *vs.* 0.003 sec.$^{-1}$ for the L-, k_3/K_m D- = 3.6, L- = 0.2. Kinetic constants for the DL- material, K_m = 0.0067 *M*, k_3 = 0.013 sec.$^{-1}$, intermediate between the values of the D- and L-enantiomorphs, reflect concomitant hydrolysis of both isomers.

The benzoyloxy group (Bo) associates with the enzyme effectively at the aryl site (a), the α-hydrogen may fit into its normal position, and the D-enantiomorph delivers the carbethoxyl group to the nucleophilic site (n). This favors[2] K_m, and hydrolysis results (association I, Scheme I). The L-enantiomorph, associating analogously, delivers the carbethoxyl group to the acylamido site, leading to less favorable K_m and no reaction (association II). The L-enantiomorph hydrolyzes only when the benzoyloxy group associates with the acylamido site, a less favorable mode, but which is, in fact, that of the normal L-specific type (association III). The D-α-benzoyloxy compound hydrolyzes more rapidly than the L- and also more rapidly than D-ethyl α-acetoxypropionate, K_m = 0.18 *M*, k_3 = 0.020 sec.$^{-1}$, which had been more reactive than the L-acetoxy compound. The greater reactivity of the benzoyloxy compound results from more favorable K_m, although it was examined in 20% ethanol, while the acetoxy compound had been studied in water.

The benzoyloxy group behaving and associating like the β-aryl but occupying the acylamido orientation in the substrate leads to D-specificity. Association I of D-ethyl α-benzoyloxypropionate is much like that of ethyl β-phenylpropionate, or more properly would be like that of L-α-methyl-β-phenylpropionate. The latter has not been studied, but the kinetic constants,[21]

SCHEME I

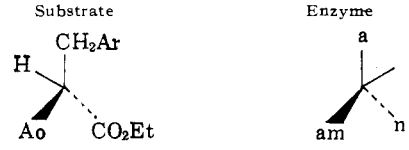


for hydrolysis of methyl β-phenylpropionate under conditions similar to those of the present work, are K_m = 0.0039 *M*, k_3 = 0.013 sec.$^{-1}$, k_3/K_m = 3.3, indicating that very similar reactivity results from the similar enzymatic associations of these structurally different materials.

D-Methyl N-benzoylalaninate, the nitrogen analog of the D-α-benzoyloxypropionate, may also hydrolyze by an association related to I, the benzamido group associating at the β-aryl site. At this site there is no opportunity for hydrogen bonding, the benzamido group behaves like benzoyloxy, and the reactivity of D-methyl N-benzoylalaninate,[4] in aqueous solution, is similar to what we find for the D-benzoyloxy compound, K_m = 0.0033 *M*, k_3 = 0.011 sec.$^{-1}$, k_3/K_m = 3.3. L-Methyl N-benzoylalaninate[4] hydrolyzes by an association related to III, K_m = 0.0098 *M*, k_3 = 0.26 sec.$^{-1}$, k_3/K_m = 26. At its normal site the acylamido group leads to higher reactivity due to increased k_3; K_m is essentially the same as that of the corresponding benzoyloxy compound.

Ethyl α-acetoxy-β-phenylpropionate, the oxygen analog of ethyl N-acetyl-β-phenylalaninate, showed high L-stereospecificity in the kinetic runs, L-(−)-, K_m = 0.023 *M*, k_3 = 0.60 sec.$^{-1}$. The D-compound was quite inert, and, in the DL-material, retarded hydrolysis of the L-. Although the acetoxy group, Ao, may associate with the aryl site and invert the stereospecificity in hydrolysis of ethyl α-acetoxypropionate,[6] when a β-phenyl group, Ar, is also present, its association at the aryl site dominates; the acetoxyl fits into the acylamido site and L-specificity follows by association IV (Scheme II).

SCHEME II



IV. L-ethyl-α-acetoxy-β-phenylpropionate, reactive

Comparison with methyl β-phenylpropionate,[21] K_m = 0.0039 *M*, k_3 = 0.013 sec.$^{-1}$, indicates that the acetoxy group, fitting into the acylamido site, but without the capability of hydrogen bonding, leads to less favorable K_m and more favorable k_3, and about an order of mag-

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nitude greater over-all reactivity. The acetoxy group may lead to the activating distortion without the compensating hydrogen bond of the nitrogen analog, L-methyl N-acetyl-β-phenylalaninate. The latter, of course, shows most favorable[8] K_m, 0.0013 M, and k_3, 53 sec.$^{-1}$, and very high reactivity. Comparison of the acetoxy compound with L-methyl α-hydroxy-β-phenylpropionate, which had been studied in the presence of phosphate buffer,[21] indicated that the hydroxyl group, which is smaller and may hydrogen bond, led to more favorable K_m, 0.010 M, and k_3, 1.0 sec.$^{-1}$, and greater over-all reactivity. The hydroxy compound, however, showed less stereospecificity than the acetoxy compound. Our brief study of the kinetics of hydrolysis by α-chymotrypsin of L- and D-ethyl α-hydroxy-β-phenylpropionates under the conditions of the remainder of our experiments leads to kinetic constants different from those reported for the methyl esters,[21] but comparison with the acetoxy compound leads to the same general conclusions.

Experimental

L-(+)-Ethyl α-Benzoyloxypropionate.—A solution of 15 g. (0.054 mole, Mann Research Laboratories) of L-zinc lactate dihydrate, 21 g. (0.11 mole) of toluenesulfonic acid hydrate, 32 g. of absolute ethanol, and 41 g. of 2,2-diethoxypropane stood for 2 days at room temperature, was then brought to pH 6 by addition of 38 g. of potassium carbonate, filtered, and concentrated. The residue was extracted with 450 ml. of ether, the ether was concentrated, and the product was distilled, **L-(−)-ethyl lactate**, 7.4 g. (0.063 mole), 58% yield, b.p. 53° (12 mm.), lit.[22] b.p. 50° (10 mm.), $n^{22}D$ 1.4114, lit.[23] $n^{25}D$ 1.4110, α_{obsd} −10.59°, 1 dm., neat, $[\alpha]^{21}D$ −10.29, lit.[24] $[\alpha]^{18}D$ −10.98. L-(−)-Ethyl lactate (6.0 g., 0.051 mole) was added slowly with stirring to 8.6 g. (0.062 mole) of benzoyl chloride and 4.9 g. (0.062 mole) of pyridine. The mixture was heated at 140° for 15 min., allowed to stand overnight, poured into 150 ml. of water, and extracted with ether. The extract was washed well with 10% sulfuric acid, water, 10% sodium carbonate, and water, dried, concentrated, and distilled, L-(+)-ethyl α-benzoyloxypropionate, 6.6 g. (0.030 mole), 58% yield, b.p. 108–110° (<1 mm.), $n^{22.5}D$ 1.4941, lit.[22] b.p. 153° (12 mm.), $n^{15}D$ 1.4973, α_{obsd} + 0.45°, c 2.46 in chloroform, $[\alpha]^{21}D$ +18.35°, α_{obsd} +27.07°, 1 dm., neat, $[\alpha]^{22}D$ +24.4, lit.[22] $[\alpha]^{20}D$ +24.6.

Anal. Calcd. for $C_{12}H_{14}O_4$: C, 64.85; H, 6.34. Found: C, 65.01; H, 6.39.

D-(−)-Ethyl α-Benzoyloxypropionate.—A solution of 15 g. (0.051 mole) of D-calcium lactate·$4H_2O$, 19 g. (0.10 mole) of toluenesulfonic acid hydrate, 54 g. of ethanol, and 33 g. of 2,2-diethoxypropane stood for 2 days and was worked up as in preparation of the L-compound, leading to **D-(+)-ethyl lactate**, 4.16 g. (0.035 mole), 35% yield, b.p. 54–55° (13 mm.), $n^{22.5}D$ 1.4111, lit.[22] b.p. 50° (10 mm.), $n^{13}D$ 1.4157, α_{obsd} +11.09, 1 dm., $[\alpha]^{22}D$ +10.71°, lit.[22] $[\alpha]^{23}D$ +11.29°. D-(+)-Ethyl lactate (4.0 g., 0.034 mole), 3.2 g. (0.041 mole) of pyridine, and 5.7 g. (0.041 mole) of benzoyl chloride, treated as described above, led to the product, 4.2 g. (0.019 mole), 56% yield, b.p. 100–102° (<1 mm.), $n^{22}D$ 1.4945, lit.[22] b.p. 149° (10 mm.), $n^{15}D$ 1.4973, α_{obsd} −27.04°, 1 dm., $[\alpha]^{22}D$ −24.4°, lit.[22] $[\alpha]^{20}D$ −24.6°.

Anal. Found: C, 64.70; H, 6.30.

DL-Ethyl α-Benzoyloxypropionate.—Ethyl lactate (15 g., 0.13 mole) was added to 22 g. (0.15 mole) of benzoyl chloride and 12 g. (0.15 mole) of pyridine. The mixture was heated under reflux for 0.5 hr., allowed to stand overnight, poured into water, and extracted with ether. The extract was washed with 10% sulfuric acid, 10% sodium carbonate, and water, dried, concentrated, and distilled, leading to the product, 18.4 g. (0.083 mole), 65% yield, b.p. 115–116° (<1 mm.), $n^{25}D$ 1.4927, lit.[25] b.p. 150° (12 mm.), $n^{15}D$ 1.4973.

Anal. Found: C, 64.62; H, 6.35 (by A. Bernhardt).

Infrared spectra of the DL-, D-(−)-, and L-(+)-esters in chloroform were identical, showing absorption bands (μ): 2.8 (w), 3.4 (w), 5.7–5.8 (s), 6.2, 6.25 (w), 6.9 (m), 7.2 (w), 7.4 (m), 7.6 (m), 7.85 (s), 8.45 (w), 8.9 (s), 9.1 (s), 9.3 (w), 9.7 (w), and 11.7 (w).

L-(−)-Ethyl α-Acetoxy-β-phenylpropionate.—L-(−)-β-Phenylalanine (5.0 g., 0.030 mole, Cal. Biochem., $[\alpha]D$ −33.5° (c 2 in H_2O)) was suspended in chloroform and treated with 3 ml. (0.036 mole) of concentrated hydrochloric acid, leading to L-phenylalanine hydrochloride. This hydrochloride (2.0 g., 0.010 mole), was dissolved in 30 cc. of 5% sulfuric acid and stirred with 1.5 g. (0.02 mole) of sodium nitrite in 8 ml. of water for 3 hr. at 0°.[26] The solution was extracted with ether, the ether was dried and concentrated, and the residue was treated with a little benzene, leading to **L-(−)-α-hydroxy-β-phenylpropionic acid**, 0.66 g., 0.0040 mole, 40% yield, m.p. 126–127°, α_{obsd} −1.04°, c 3.78 in acetone, $[\alpha]^{25}D$ −27.8°, lit.[27] m.p. 125–126°, $[\alpha]^{25.5}D$ −28.1°, (c 1.13 in acetone). The infrared spectrum showed absorption bands (μ): 2.8 (w), 3.3 (m), 3.8 (w), 5.8 (s), 6.2 (w), 6.7 (w), 6.9 (w), 7.5 (w), 8.4 (s), 8.7 (w), 9.2 (s), 9.4 (w), 9.7 (w), 10.8 (w), and 11.4 (w).

This acid (2.24 g., 0.0136 mole) was boiled in 50 ml. of ethanol and 0.5 g. of p-toluenesulfonic acid for 6 hr. and concentrated. The residue was dissolved in ether, and the extract was washed with water, sodium bicarbonate, dilute sulfuric acid, and water, dried, concentrated, and distilled, leading to **L-(−)-ethyl α-hydroxy-β-phenylpropionate**, b.p. 72° (0.2 mm.), m.p. 46–47°, α_{obsd} −0.95, c 4.33 in benzene, $[\alpha]^{24}D$ −22.6°, lit.[28] b.p. 159–160° (26 mm.), m.p. 46–47°, $[\alpha]^{13}D$ −22.6° in benzene. The infrared spectrum in chloroform showed absorption bands (μ): 2.8 (w), 3.35 (w), 5.75 (s), 6.2 (w), 6.7 (w), 6.85–7.05 (w), 7.3 (w), 8.4 (s), 9.1 (s), 9.7 (w), and 11.5 (w).

This ester (1.0 g., 0.0052 mole) was boiled for 3 hr. in 15 ml. of freshly distilled acetic anhydride, concentrated, and distilled, leading to **L-(—)-ethyl α-acetoxy-β-phenylpropionate**, 0.92 g. (0.0039 mole), 75% yield, b.p. 85–87° (0.2 mm.), $n^{21}D$ 1.4883, α_{obsd} −0.58°, c 6.65 in chloroform, $[\alpha]^{23}D$ −8.7°. The infrared spectrum in chloroform showed bands (μ): 2.8 (w), 3.35 (m), 4.1 (w), 5.1 (w), 5.3 (w), 5.7 (s), 6.2 (w), 6.7 (m), 6.9 (m), 7.3 (s), 7.4 (w), 7.8 (s), 8.4 (s), 9.3 (s), 9.7 (s), 10.6–10.8 (w), 11.0 (w), and 11.6 (w).

Anal. Calcd. for $C_{13}H_{16}O_4$: C, 66.10; H, 6.79. Found: C, 66.74; H, 6.97 (by Schwarzkopf).

D-(+)-Ethyl α-Acetoxy-β-phenylpropionate.—D-(+)-**α-Hydroxy-β-phenylpropionic acid** was prepared from D-(+)-phenylalanine, as described for the L-enantiomorph, m.p. 126–127°, α_{obsd} +1.12° c 3.76 in acetone, $[\alpha]^{25}D$ +32.8°. **D-(+)-Ethyl α-hydroxy-β-phenylpropionate** was prepared from this hydroxy acid as described for the L-enantiomorph, b.p. 66–68° (0.2 mm.), m.p. 46–47°, $[\alpha]^{23}D$ +23.2° in benzene, lit.[28] b.p. 152–154° (26 mm.), m.p. 46–47°, $[\alpha]^{17.5}D$ +22.5°. **D-(+)-Ethyl α-acetoxy-β-phenylpropionate** was prepared from this hydroxy ester as described for the L-enantiomorph, b.p. 85° (0.2 mm.), $n^{23.5}D$ 1.4876, α_{obsd} +0.60°, c 6.97 in chloroform, $[\alpha]^{22}D$ +8.6°. Infrared spectra of the D-(+)-hydroxy acid, -hydroxy ester, and -acetoxy ester were identical with those of the respective L-enantiomorphs.

Anal. Found: C, 66.67; H, 7.09.

DL-Ethyl α-Acetoxy-β-phenylpropionate.—DL-β-Phenylalanine (8.0 g., 0.048 mole) was dissolved in 208 ml. of 0.52 N HCl, cooled to 0°, and stirred with 9.8 g. (0.064 mole) of silver nitrite for 5 hr.[27] The mixture was filtered, the filtrate was extracted with ten 200-ml. portions of ether, and the extract was dried and concentrated, leading to an oil, **DL-α-hydroxy-β-phenylpropionic acid**, 5.8 g. (0.034 mole), 74% yield. This compound was heated with 2.76 g. (0.035 mole) of acetyl chloride leading to the acetoxy acid. The acetoxy acid, 4.9 g. (0.024 mole) in dry ether, was added slowly to a solution of diazoethane in dry ether, prepared as described previously[6,29] from 10 g. (0.068 mole) of N-nitrosoethylurethan. The solution was allowed to stand for 0.5 hr., dried over $MgSO_4$, allowed to stand overnight, filtered, evaporated, and distilled, leading to **DL-ethyl α-acetoxy-β-phenylpropionate**, b.p. 90° (0.1 mm.), $n^{23}D$ 1.4883. The infrared spectrum showed absorption bands (μ): 3.3 (w), 5.7 (s),

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6.2 (w), 6.65 (w), 6.9 (w), 7.2 (m), 7.5–8.4 (m), 9.2 (m), 9.75 (m), and 10.8 (w).

Anal. Found: C, 65.82; H, 6.77.

Hydrolysis of DL-Ethyl α-Benzoyloxypropionate by α-Chymotrypsin.—The ester, 1.80 g. (8.04 mmoles), suspended in 15 ml. of 0.1 *M* sodium chloride under nitrogen at 25°, was brought to pH 7.8 in a pH stat. Hydrolysis in the absence of enzyme was very slow. α-Chymotrypsin (0.201 g., Worthington Biochemical Corp., salt-free, three times recrystallized) was added, the system was brought back to pH 7.8, and the enzymatic hydrolysis was followed. It was slow, and 5 ml. of 0.1 *M* phosphate buffer was added after 3 hr. Uptake of 1 *N* NaOH was 3.97 ml. in 46 hr., 50% reaction, the hydrolysis then proceeding at about one quarter of the initial rate. The suspension was extracted with ether, the extract was washed with water, dried, and concentrated, leading to a residue, 0.79 g., 88% yield. This was distilled, leading to ethyl α-benzoyloxypropionate, 0.48 g. (2.2 mmoles), 53% yield, b.p. 93–95° (<1 mm.), α_{obsd} +0.55°, *c* 5.26 in $CHCl_3$, $[\alpha]^{22}D$ +10.45°. The infrared spectrum in chloroform was identical with that of the starting ester. The water layer, after extraction, was brought to pH 2 with concentrated sulfuric acid and lyophilized. The residue was extracted with acetone and the acetone was evaporated, leaving a crude residue, 1.12 g. This was extracted with benzene, and the benzene was concentrated, leading to α-benzoyloxypropionic acid, m.p. 82–83°, from benzene–petroleum ether, 0.30 g. (1.54 mmoles), 38% yield, α_{obsd} −0.97°, *c* 4.93 in chloroform, $[\alpha]^{22}D$ −19.7°.

Anal. Calcd. for $C_{10}H_{10}O_4$: C, 61.9; H, 5.6. Found: C, 61.8; H, 5.2 (by C. Fitz).

Kinetics of hydrolysis of D-(−)-, L-(+)-, and DL-ethyl α-benzoyloxypropionates by α-chymotrypsin were carried out in 4:1 water–ethanol. The substrate was dissolved in 11 ml. of 0.1 *M* NaCl and 4 ml. of ethanol with magnetic stirring under nitrogen in a pH stat. The rate of nonenzymatic hydrolysis was determined as pH was maintained at 7.8, 0.1 *N* NaOH being delivered from an automatic pipette. α-Chymotrypsin, 0.040 g. in 5 ml. of water, was added, the pH was readjusted, and the initial pseudo-zero-order rates of enzymatic hydrolysis were determined. Corrections were applied in each experiment for the nonenzymatic rates, 4–7% for the D-(−)-ester, 1–3% for the DL-ester. The rates of nonenzymatic hydrolysis of the L-(+)-ester were from one-half to as great as the enzymatic rates.

Hydrolysis of DL-Ethyl α-Acetoxy-β-phenylpropionate by α-Chymotrypsin.—A suspension of 0.516 g. (2.32 mmoles) of the ester in a solution of 0.205 g. of α-chymotrypsin in 20 ml. of 0.1 *M* NaCl was allowed to react at pH 7.8 in a pH stat at 25° under nitrogen. After 1 hr. consumption of alkali was proceeding at about one-third its initial rate, 1.21 ml. of 1 *N* NaOH having been consumed, 52% reaction. The suspension was extracted with ether, and the extract was dried and concentrated, leading to 0.1 g. of the ester, α_{obsd} +0.06, 5% in chloroform. The extracted aqueous layer was brought to pH 2 with hydrochloric acid and lyophilized. The residue was extracted with acetone, leading to 0.3 g. of crude α-acetoxy-β-phenylpropionic acid, α_{obsd} −0.3°, 11% in chloroform. Infrared spectra of the isolated ester and acid were identical with those of the synthesized inactive materials.

Kinetics of hydrolysis of L- and DL-ethyl α-acetoxy-β-phenylpropionate by 0.5 mg./ml. of α-chymotrypsin were studied in 4:1 water–ethanol, as described for the hydrolysis of ethyl α-benzoyloxypropionate. Corrections were applied in each experiment for the nonenzymatic rates, which were 1–3% of the enzymatic rates for L-(−)-compound, 4–7% for the DL-material.

Elemental analyses were by Schwarzkopf Microanalytical Laboratories except where otherwise indicated.

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COMMUNICATIONS TO THE EDITOR

An Identity of the Rates of Deacylation of Nonionic Acyl-α-chymotrypsins and Acyl-trypsins[1]

Sir:

The enzymes α-chymotrypsin and trypsin appear to catalyze the nucleophilic reactions of carboxylic acid derivatives by identical mechanisms. Both reactions follow eq. 1[2] where ES is the enzyme-substrate complex, ES′ is the acyl-enzyme intermediate, P_1 is the

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+ P_1$$

leaving group of the substrate, and P_2 is the carboxylic acid. Acyl-enzyme intermediates have been isolated from reactions catalyzed by both enzymes[3] and have been characterized as serine esters for both enzymes.[4] Both acyl-enzymes are partitioned by methanol or water in identical fashion.[5] The effect of deuterium oxide is identical for reactions catalyzed by both enzymes.[5] The individual acylation (k_2) and deacylation (k_3) steps of both enzymes may be characterized as nucleophilic reactions. Both chymotrypsin and trypsin acylations are dependent on two groups, one with a pK_a of 7 and the other with a pK_a of 9 or 10. Both deacylations are dependent on one group with a pK_a of 7 (Fig. 1). The ubiquitous group of pK_a 7 is presumably the imidazole group of a histidine moiety.[6] Thus all major mechanistic criteria point to a parallelism between these enzymes.

α-Chymotrypsin and trypsin show differences in binding constants. α-Chymotrypsin binds substances such as indole and N-acetyl-L- (or D-) tryptophan ethyl ester about 15-fold more effectively than does trypsin. Further, trypsin binds substances containing cationic groups such as benzylammonium ion and benzoyl-L-argininamide 5- to 22-fold more effectively than does α-chymotrypsin. Thus the binding constants define the specificity usually associated with α-chymotrypsin and trypsin.

Although very little data on acylation rate constants exist, a considerable amount of data on deacylation rate

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