(Worthington Biochemicals Corp., Lot No. 6082-3, 12% water, 2 mg in 1 ml of water, 0.35×10^{-5} *M*) was added. The pH was restored to 7.2, the consumption of 0.10 *N* NaOH was followed, and the corrected initial zero-order enzymic rates were determined. Data are summarized in Table I.

Kinetics of hydrolysis of D-(+)-, L-(−)-, and DL-diethyl malate by α-chymotrypsin (0.15×10^{-5} *M*) were followed as described above for the acetoxysuccinates. Corrections for the nonenzymic hydrolysis, 2–6%, were applied, and corrected enzymic rates at the several concentrations are summarized in Table II.

Stereospecificity in Hydrolysis by α-Chymotrypsin of Esters of α,α-Disubstituted Acetic and β,β-Disubstituted Propionic Acids[1]

Saul G. Cohen, Richard M. Schultz, and Sandra Y. Weinstein

Contribution from the Department of Chemistry, Brandeis University, Waltham, Massachusetts 02154. *Received June 29, 1966*

Abstract: Ethyl α-phenyl-α-acetamidoacetate is hydrolyzed by α-chymotrypsin with L specificity, $k_{cat} = 0.89$ sec^{-1}, $K_{m,app} = 0.0033$ *M*. Ethyl α-phenyl-α-benzamidoacetate is also hydrolyzed with L specificity. Ethyl mandelate is hydrolyzed without stereospecificity. The D specificity in hydrolyses of ethyl β-phenyl-β-acetamidopropionate and of ethyl β-phenyl-β-hydroxypropionate is accounted for in terms of the reactive conformation of the substrates and the correspondence of D-β and L-α substituents.

In the two preceding papers[2,3] the conformations of reacting substrates and the modes of their association with α-chymotrypsin have been described. α-Substituted, β-substituted, and α,β-disubstituted derivatives of ethyl propionate have been considered as model small molecule substrates. In hydrolyses proceeding with normal L stereospecificity, the α substituent associates at the α-acylamido site, am, the β substituent at the β-aryl site, ar, and the α hydrogen at a site of restricted volume, h. The D enantiomorphs may react under the following circumstances: (1) when there is no β substituent and the α substituent prefers to associate at the ar site, as in ethyl α-benzoyloxypropionate,[4] $CH_3CH(OCOC_6H_5)CO_2C_2H_5$; (2) when the α and β substituents may each associate at either the am or ar sites, as in diethyl α-acetoxysuccinate[3] and in effect in diethyl β-acetoxyglutarate;[5] (3) when the α substituent may associate at either the am or h site, as in ethyl β-phenyl-α-hydroxypropionate,[6,7] ethyl β-phenyl-α-chloropropionate,[6,7] and diethyl α-hydroxysuccinate.[3] This view of substrate–enzyme associations, combined with the transoid conformation of β substituent and hydrolyzing ester or amide group, and the location of the β substituent, directed into the enzyme, seems to be generally useful for α,β-disubstituted propionates and to have predictive value. It may also account for the D specificity of the cyclic substrate 1-keto-3-carbomethoxytetrahydroisoquinoline[8] which in effect falls into classes 1 and 3, above.

We have previously reported[9] that two β,β-disubstituted esters, ethyl β-phenyl-β-hydroxypropionate and ethyl β-phenyl-β-acetamidopropionate, are hydrolyzed slowly by α-chymotrypsin with remarkable D specificity. As to the corresponding α,β-disubstituted compounds, ethyl α-acetamido-β-phenylpropionate, the β-phenylalanine derivative,[10] shows essentially complete L specificity, and ethyl α-hydroxy-β-phenylpropionate also shows more rapid hydrolysis of the L enantiomorph. It was not clear whether the D specificity in the hydrolysis of the β,β-disubstituted compounds arose because the hydroxyl and acetamido substituents were β to the hydrolyzing ester group or because they were α to the important aryl substituent. In the β-substituted diethyl glutarates, the β-hydroxy[11] and β-acetamido[12] derivatives both hydrolyze with L specificity. In these compounds the hydroxyl and acetamido groups, while β to the hydrolyzing ester group, are also β to the second carbethoxyl, which associates at the ar site. In the latter sense the hydroxyl and acetamido groups could and did occupy the α am site and led to normal L specificity.

It seemed of interest to reexamine, with the procedures elaborated in the previous article, the D specificity of hydrolysis by α-chymotrypsin of β,β-disubstituted propionates, and to study the nature of the stereospecificity of hydrolysis of corresponding α,α-disubstituted acetates.

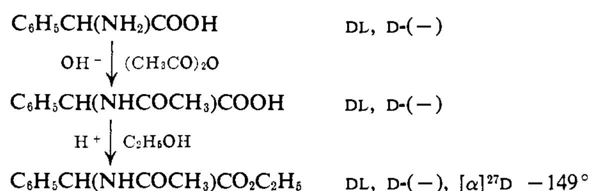
Results

Ethyl DL-α-phenyl-α-acetamidoacetate and the D-(−) enantiomorph were prepared from the commercially available DL- and D-(−)-phenylglycines by

(1) We are pleased to acknowledge generous support of this work by the Division of Research Grants, National Institutes of Health, GM-04584. This is paper XIII on specificity in the reactions of α-chymotrypsin.

(2) S. G. Cohen, L. H. Klee, and S. Y. Weinstein, *J. Am. Chem. Soc.*, **88**, 5302 (1966).
(3) S. G. Cohen, Z. Neuwirth, and S. Y. Weinstein, *ibid.*, **88**, 5306 (1966).
(4) S. G. Cohen and S. Y. Weinstein, *ibid.*, **86**, 5326 (1964).
(5) S. G. Cohen, J. Crossley, E. Khedouri, R. Zand, and L. H. Klee, *ibid.*, **85**, 1685 (1963).
(6) J. E. Snoke and H. Neurath, *Arch. Biochem. Biophys.*, **21**, 351 (1949).
(7) H. Neurath and G. Schwert, *Chem. Rev.*, **46**, 69 (1950).
(8) G. E. Hein and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1341 (1961).
(9) S. G. Cohen and S. Y. Weinstein, *J. Am. Chem. Soc.*, **86**, 725 (1964).
(10) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).
(11) S. G. Cohen and E. Khedouri, *J. Am. Chem. Soc.*, **83**, 4228 (1961).
(12) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 1093 (1961).

acetylation to the α-phenyl-α-acetamidoacetic acids, followed by esterification.



$C_6H_5CH(NH_2)COOH$ DL, D-(−)

$\downarrow$ OH^-, $(CH_3CO)_2O$

$C_6H_5CH(NHCOCH_3)COOH$ DL, D-(−)

$\downarrow$ H^+, C_2H_5OH

$C_6H_5CH(NHCOCH_3)CO_2C_2H_5$ DL, D-(−), $[\alpha]^{27}D$ −149°

L-(+)-Phenylglycine is not commercially available. L-(+)-α-Phenyl-α-acetamidoacetic acid was prepared by treatment of ethyl DL-α-phenyl-α-acetamidoacetate with α-chymotrypsin. Treatment for 2.2 hr at pH 7.8 of 1.28 g (5.78 mmoles) of the DL ester with 2.5 mg of the enzyme/ml led to hydrolysis of the L enantiomorph and to isolation in high yields of the unhydrolyzed ethyl D-(−)-α-phenyl-α-acetamidoacetate and the L-(+)-α-phenyl-α-acetamidoacetic acid, both in high optical purity. The L-(+) acid was converted to the L-(+) ester, $[\alpha]^{27}D$ +151°.

The kinetics of hydrolysis of ethyl L-(+)-α-phenyl-α-acetamidoacetate by α-chymotrypsin were studied under nitrogen in a pH-Stat at pH 7.8. The nonenzymic rate was less than 1% as great as the enzymic rate. The initial zero-order rates at varying substrate concentrations are given in Table I. A double reciprocal plot was linear. A least-squares treatment of the data led to k_{cat} = 0.89 sec^{-1}, $K_{m,app}$ = 0.0033 M, $k_{cat}/K_{m,app}$ = 270. The DL ester was studied similarly, k_{cat} = 0.72 sec^{-1}, $K_{m,app}$ = 0.0045 M. The D enantiomorph was not appreciably hydrolyzed when examined alone under these conditions.

Table I. Rates of Hydrolysis of Ethyl L-(+)-α-Phenyl-α-Acetamidoacetate by 0.15 mg of α-Chymotrypsin/ml, 25.5°, pH 7.8, 0.1 M NaCl

[S] × 10^3, M	$V \times 10^6$, $M\ sec^{-1}$
2.36	2.15
2.71	2.29
3.05	2.56
3.62	2.58
4.11	2.79
4.57	2.98
5.25	3.11
6.15	3.55

Some experiments were also carried out on ethyl α-phenyl-α-benzamidoacetate, $C_6H_5CH(NHCOC_6H_5)CO_2C_2H_5$. The DL and D-(−) esters were prepared from the DL- and D-(−)-phenylglycines by treatment with benzoyl chloride in alkali, leading to the DL- and D-(−)-α-phenyl-α-benzamidoacetic acids, followed by conversion to the esters. Treatment for 2.3 hr at pH 7.8 in 3:1 dioxane–water of 0.204 g (0.72 mmole) of the DL ester with 5 mg of α-chymotrypsin/ml led to 80% hydrolysis of one enantiomorph. The unreacted ester was isolated and had an appropriately high rotation, indicating that the L-(+) enantiomorph had hydrolyzed. The hydrolysis product, α-phenyl-α-benzamidoacetic acid, contained about 90% L enantiomorph. The benzamido ester is quite insoluble in water and in the dioxane–water, and kinetic studies were not made. The suspension in 0.1 M NaCl appeared to be hydrolyzed by α-chymotrypsin about one-tenth as fast as a solution of the acetamido ester. A suspension in 4:1 water–dioxane was hydrolyzed somewhat more rapidly than in the absence of dioxane.

The DL-, L-(+)-, and D-(−)-ethyl esters of mandelic acid, $C_6H_5CH(OH)CO_2C_2H_5$, were prepared and the action of α-chymotrypsin on them was studied. Hydrolysis of 1.00 g (5.55 mmoles) of the DL ester by 4 mg of α-chymotrypsin/ml at pH 7.8 was 36% complete in 3.75 hr and nonstereospecific. Neither the recovered ester nor the mandelic acid hydrolysis product showed optical rotation beyond the experimental error. This was confirmed in kinetic studies at pH 7.8 on the L-(+) and D-(−) enantiomorphs. The nonenzymic rates were about 4% as great as the enzymic rates. The corrected initial zero-order rates at the several substrate concentrations are given in Table II. Double reciprocal plots[13] were linear. Least-squares treatment of the data led to L-(+), k_{cat} = 0.13 sec^{-1}, $K_{m,app}$ = 0.021 M, $k_{cat}/K_{m,app}$ = 6 M^{-1} sec^{-1}; D-(−), k_{cat} = 0.09 sec^{-1}, $K_{m,app}$ = 0.013 M, $k_{cat}/K_{m,app}$ = 7 M^{-1} sec^{-1}.

Table II. Rates of Hydrolysis of L-(+)- and D-(−)-Ethyl Mandelates by 0.75 mg of α-Chymotrypsin/ml, 25.5°, pH 7.8, 0.1 M NaCl

	[S] × 10^3, M	$V \times 10^6$, $M\ sec^{-1}$
L-(+)	7.39	0.96
	7.67	0.96
	9.01	1.07
	9.64	1.18
	10.2	1.18
	11.5	1.29
D-(−)	6.42	0.85
	7.62	0.98
	8.47	1.03
	9.18	1.09
	10.1	1.12
	11.0	1.17
	11.95	1.24

Diethyl α-hydroxymalonate, $HOCH(CO_2C_2H_5)_2$, had previously been prepared and found to hydrolyze nonstereospecifically.[11] The kinetics of hydrolysis of this compound by 0.5 mg of α-chymotrypsin/ml were studied at 25°, pH 7.2, 0.1 M NaCl. The nonenzymic rate was substantial, 9–12% as great as the enzymic rate. The concentrations of substrate and corresponding corrected initial zero-order enzymic hydrolysis rates were as follows: 7.27 × 10^{-3} M, 5.4 × 10^{-7} M sec^{-1}; 8.42 × 10^{-3} M, 5.8 × 10^{-7} M sec^{-1}; 9.50 × 10^{-3} M, 6.6 × 10^{-7} M sec^{-1}; 11.5 × 10^{-3} M, 7.9 × 10^{-7} M sec^{-1}; 14.2 × 10^{-3} M, 9.2 × 10^{-7} M sec^{-1}; 17.2 × 10^{-3} M, 11.4 × 10^{-7} M sec^{-1}. A double reciprocal plot was linear and from it kinetic parameters were calculated, k_{cat} = 0.42 sec^{-1}, $K_{m,app}$ = 0.11 M. The kinetics of the stereospecific hydrolysis of diethyl α-acetamidomalonate had been determined previously.[14] The kinetic constants for these compounds are summarized in Table III.

Discussion

Ethyl α-phenyl-α-acetamidoacetate showed essentially complete L specificity in its hydrolysis by α-chymo-

(13) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **57**, 658 (1934).
(14) S. G. Cohen and J. Crossley, *ibid.*, **86**, 4999 (1964).

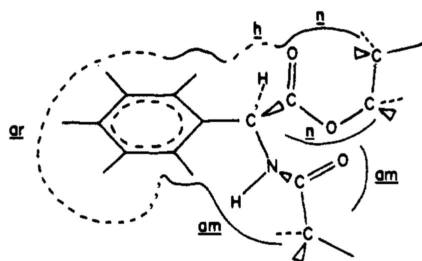
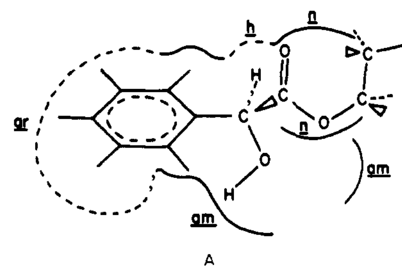


Figure 1. Association of ethyl L-α-phenyl-α-acetamidoacetate.



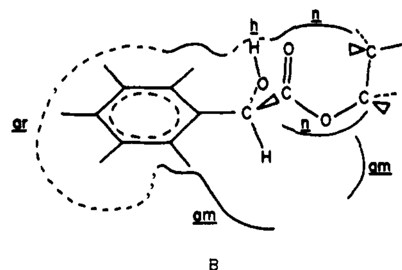


Figure 2. A, association of ethyl L-mandelate; B, association of ethyl D-mandelate.

trypsin, and hydrolysis of the L component of the DL mixture led to the L acid and recovered D ester. It appears that with the acetamido and phenyl groups both on the α-carbon a relatively normal association takes place, α-acetamido at am, carbethoxyl at n, α-hydrogen at h, and the phenyl spanning to ar, despite the absence of one methylene group as compared with the normal propionate substrates (Figure 1). The kinetics reveal quite favorable binding, low $K_{m,app}$, indicating that the α-phenyl may associate well at ar; the small kinetic constant, k_{cat}, may indicate unfavorable access to the nucleophilic site or ineffective organization of that site by the acetamido group due to absence of the β-methylene group.

Table III. Kinetic Constants for Hydrolysis by α-Chymotrypsin of Esters of α,α-Disubstituted Acetic Acids, $R,R'CHCO_2C_2H_5$

R	R′	k_{cat}, sec^{-1}	$K_{m,app}$, M	$k_{cat}/K_{m,app}$, M^{-1} sec^{-1}
C_6H_5	$NHCOCH_3$ (L+)	0.89	0.0033	270[a]
C_6H_5	OH (L+)	0.13	0.021	6[b]
C_6H_5	OH (D−)	0.09	0.013	7[b]
$CO_2C_2H_5$	$NHCOCH_3$	0.71	0.031	23[c]
$CO_2C_2H_5$	OH	0.42	0.11	4[d]

[a] Stereospecific hydrolysis, 0.15 mg of enzyme/ml, 25.5°, pH 7.8, 0.1 M NaCl. [b] Nonstereospecific hydrolysis, 0.75 mg of enzyme/ml, 25.5°, pH 7.8, 0.1 M NaCl. [c] Stereospecific hydrolysis, 0.5 mg of enzyme/ml, 25.0°, pH 7.2, 0.1 M NaCl. [d] Nonstereospecific hydrolysis, 0.5 mg of enzyme/ml, 25.0°, pH 7.2, 0.1 M NaCl.

The analogous symmetric compound diethyl α-acetamidomalonate, in which the α-phenyl is replaced by the second carbethoxyl group, had previously been found to be hydrolyzed stereospecifically by α-chymotrypsin, apparently in the L sense, with association like that indicated in Figure 1. The kinetic constants k_{cat} were similar, but the second carbethoxyl group led to substantially less favorable binding than the phenyl group. Less favorable binding due to a β-carbethoxyl group was seen in comparison of the aspartate and β-phenylalanine derivatives.

Ethyl α-phenyl-α-benzamidoacetate also hydrolyzed with high L specificity both in water and in water–dioxane, but low solubility made kinetic studies impractical. This compound had been examined previously and reported to be extremely unreactive.[15] The reactivity is sufficient to allow determination, under our conditions, of the L stereospecificity.

The stereospecific hydrolyses of the α-acylamido-α-phenylacetates, α-acetamidomalonate, α-acetoxymalonate,[5] and β-phenyl-α-acetoxypropionate[4] indicate that the nonlinear or branched substituents, phenyl and carbethoxyl, strongly prefer to locate at the ar site and not at the am site when they are directly attached to the α-carbon. The carbethoxyl and acyl groups may associate at am when they are separated from the α-carbon by methylene[3] or oxygen.[4]

Stereospecificity in hydrolysis of α,α-disubstituted esters was lost when the α-acetamido group was replaced by hydroxyl. Ethyl mandelate, $C_6H_5CH(OH)$-$CO_2C_2H_5$, was hydrolyzed by α-chymotrypsin without stereospecificity both in a run under preparative conditions on the DL compound and in kinetic studies on the separate enantiomorphs. While the kinetic and binding constants of the two enantiomorphs showed slight differences, the reactivities were essentially the same and substantially lower than that of the L-acetamido compound. The absence of stereospecificity may be accounted for in terms of associations with the enzyme analogous to those of diethyl malate[3] and ethyl lactate. The phenyl and carbethoxyl groups associate as usual at ar and n, respectively, while the hydroxyl lies at am for hydrolysis of the L enantiomorph and at h for hydrolysis of the D. (See Figure 2.)

The malonate and phenylacetate derivatives again showed similar stereochemistry. Diethyl α-hydroxymalonate had previously been shown to hydrolyze without stereospecificity and it may associate with the enzyme in the two ways indicated for the mandelate. The malonate showed a substantial kinetic constant, k_{cat}, probably reflecting the activating effect of the the two electronegative α substituents, and again its binding was less favorable by an order of magnitude than those of the corresponding phenyl compounds.

The α-acetamido and α-hydroxyphenylacetates showed "normal" stereochemistry in terms of our previous considerations, L specificity for the α-acetamido compound, and little or no specificity for the α-hydroxy compounds. The quite different outcome, D specificity of the β-acetamido and β-hydroxy-β-

(15) S. Kaufman and H. Neurath, *Arch. Biochem. Biophys.*, **21**, 437 (1949).

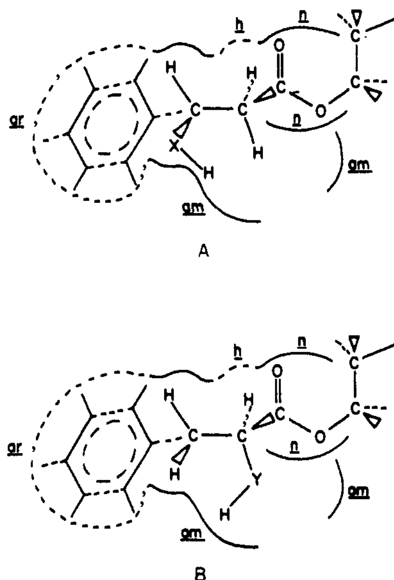


Figure 3. A, association of D-β-XH-β-phenylpropionate; B, association of L-α-YH-β-phenylpropionate.

phenylpropionates, may be considered similarly in terms of the reactive conformation of the substrates and the interactions of the substituents with parts of the active site of the enzyme. We assume that the active conformation is the same as that in the unsubstituted and α-substituted β-phenylpropionates and succinates,[2,3] and has the β-phenyl and hydrolyzing carbethoxyl groups transoid. This is indicated for the β-D configuration and compared with that of the α-L compounds in Figure 3.

Models indicate that the D-β-X substituent, OH or $NHCOCH_3$, occupies a position gauche, and thus close to that of an L-α-Y substituent, while an L-β substituent would of necessity occupy a position transoid and distant from that occupied by the L-α substituent in the reactive conformation. The H atom of a D-β-OH or a D-β-acetamido may approach as close as 1.4 A to a position in the am area which the corresponding atom of an L-α substituent may occupy. An L-β substituent is not only far more distant, ~4 A, but is separated from the am area by the D-β-hydrogen atom. Also an L-β substituent may possibly encounter steric hindrance.[16] The D-β substituent, hydroxyl or acetamido, corresponds much more closely to an L-α substituent than does an L-β substituent. The D-β substituent may possibly make some use of association by hydrogen bonding at the am site. This relationship appears to account for the D specificity of β-hydroxy and β-acetamido-β-phenylpropionates. A similar correspondence of a D-β substituent and an L-α substituent has been noted in enzymic reactions of D-β-glutamine and L-glutamine.[17]

Experimental Section

Melting points are uncorrected. Elementary analyses were by Swartzkopf. α-Chymotrypsin was from Worthington Biochemical Corp., three times recrystallized, salt free.

(16) H. I. Abrash and C. Niemann, *Biochemistry*, **2**, 947 (1963).
(17) E. Khedouri and A. Meister, *J. Biol. Chem.*, **240**, 3357 (1965).

D-(−)-α-Phenylglycine was obtained from Aldrich Chemical Co.: α_{obsd} −7.93°, 5.23% in 1 *N* HCl; $[\alpha]^{31}D$ −152° (lit.[18] $[\alpha]^{20}D$ −157.8°).

DL-α-Phenylglycine was from Aldrich Chemical Co.

L-(+)-Mandelic acid was from Aldrich Chemical Co.: mp 133–134°; α_{obsd} +2.62°, 1.75% in water; $[\alpha]^{30}D$ +150° (lit.[19] mp 133.8°, $[\alpha]^{20}D$ +156.2°, 3.2% in water).

D-(−)-Mandelic acid was from Aldrich Chemical Co.: mp 134°; α_{obsd} −2.22°, 1.49% in water; $[\alpha]^{30}D$ −149° (lit.[20] mp 133.5°; $[\alpha]^{20}D$ −156.9°, 2% in water).

DL-Mandelic acid was from Eastman Kodak Co.: mp 118–119° (lit.[21] mp 118–119°).

DL-α-Phenyl-α-acetamidoacetic Acid. DL-α-Phenylglycine (6.04 g, 0.040 mole) was suspended in 10 ml of water, maintained at 10–15°, brought into solution by addition of 6.7 ml of 6 *N* sodium hydroxide, and treated with 10.2 g (0.100 mole) of acetic anhydride and 33.3 ml of 6 *N* sodium hydroxide, added in eight equal portions. The solution was brought to pH 2–3 by addition of 6 *N* sulfuric acid with α-phenyl-α-acetamidoacetic acid precipitating, 6.05 g (0.031 mole), 79% yield, mp 199° (lit.[22] 198.5°).

D-(−)-α-Phenyl-α-acetamidoacetic acid was prepared in the same way from D-(−)-α-phenylglycine: mp 187° (lit.[23] 191°); α_{obsd} −0.95°, 0.460% in water; $[\alpha]^{27}D$ −206° (lit.[23] −197.4°); α_{obsd} −1.10°, 0.570% in 1 *N* NaOH; $[\alpha]^{28}D$ −193°.

Anal. Calcd for $C_{10}H_{11}NO_3$: C, 62.16; H, 5.74; N, 7.25. Found: C, 62.21; H, 5.88; N, 7.09.

Ethyl DL-α-Phenyl-α-acetamidoacetate. DL-α-Phenyl-α-acetamidoacetic acid, 2.5 g (0.013 mole), was heated under reflux overnight in 60 ml of ethanol containing 5 drops of sulfuric acid. The solution was diluted with water, made alkaline with 5% sodium bicarbonate, and extracted with methylene chloride. The extract was dried and concentrated, and the residue was treated with hexane, leading to ethyl α-phenyl-α-acetamidoacetate: 2.21 g (0.010 mole), 77% yield; mp 64.5–65° (lit.[24] 65–66°).

Ethyl D-(−)-α-phenyl-α-acetamidoacetate was prepared in the same way from D-(−)-α-phenyl-α-acetamidoacetic acid: mp 88–89° (lit.[24] 69–70°); α_{obsd} −2.32°, 1.56% in ethanol; $[\alpha]^{27}D$ −149° (lit.[24] −139°).

Anal. Calcd for $C_{12}H_{15}O_3N$: C, 65.21; H, 6.83; N, 6.33. Found: C, 65.21; H, 7.01; N, 6.09.

Treatment of Ethyl DL-α-Phenyl-α-acetamidoacetate with α-Chymotrypsin. A solution of 0.051 g of α-chymotrypsin in 5 ml of 0.1 *N* NaCl was added to a stirred suspension of 1.28 g (5.78 mmoles) of ethyl DL-α-phenyl-α-acetamidoacetate in 15 ml of 0.1 *N* NaCl, and the hydrolysis was followed, at 25.5°, in a pH-Stat at pH 7.8 by addition of 1 *N* NaOH from an automatic buret. After 130 min, 2.74 ml of NaOH had been added, corresponding to 95% hydrolysis of one enantiomorph, the reaction then proceeding very slowly. Sodium bicarbonate solution was added, and the solution was extracted with ether. The extract was dried and concentrated, and the residue was treated with hexane leading to **ethyl D-(−)-α-phenyl-α-acetamidoacetate**: 0.545 g (2.46 mmoles), 83% yield; mp 87°; α_{obsd} −1.98°, 1.46% in ethanol; $[\alpha]^{32}D$ −136°. The infrared spectrum in chloroform was identical with that of the synthesized optically inactive compound, above.

The aqueous solution was brought to pH 2 with 6 *N* H_2SO_4 and lyophilized, and the residue was extracted with acetone, leading to **L-(+)-α-phenyl-α-acetamidoacetic acid**: 0.300 g (1.56 mmoles), 57% yield; mp 194–195°; α_{obsd} 0.52°, 0.272% in 1 *N* NaOH; $[\alpha]^{27}D$ +191°. The infrared spectrum was identical with that of the D enantiomorph.

Ethyl L-(+)-α-phenyl-α-acetamidoacetate was prepared by treatment of 0.300 g (1.56 mmoles) of the L acid with 25 ml of ethanol containing 4 drops of sulfuric acid as described above for the DL compound: 0.197 g (0.89 mmole), 57% yield; mp 89°; α_{obsd} +1.05°, 0.695% in ethanol; $[\alpha]^{27}D$ 151°.

Anal. Found: C, 65.14; H, 6.80; N, 5.92.

DL-α-Phenyl-α-benzamidoacetic Acid. DL-α-Phenylglycine (1.51 g, 0.01 mole) was dissolved in 30 ml of 1 *N* NaOH, cooled to 0°,

(18) E. Fischer and O. Weichhold, *Chem. Ber.*, **41**, 1286 (1908).
(19) E. Rimbach and H. Horstein, *Z. Physik. Chem.* (Leipzig), **88**, 273 (1914).
(20) H. F. Manske and T. B. Johnson, *J. Am. Chem. Soc.*, **51**, 1908 (1929).
(21) P. Walden, *Chem. Ber.*, **29**, 1700 (1896).
(22) S. Searles and G. Cvejanovich, *J. Am. Chem. Soc.*, **72**, 3200 (1950).
(23) O. Neubauer and O. Warburg, *Z. Physiol. Chem.*, **70**, 1 (1910).
(24) C. S. Marvel and W. A. Noyes, *J. Am. Chem. Soc.*, **42**, 2264 (1920).

and treated with 1.7 ml (0.01 mole) of benzoyl chloride. The solution was acidified, leading to DL-α-phenyl-α-benzamidoacetic acid: 1.60 g (0.0063 mole), 63% yield; mp 177.5° (lit.[15] 175.5°).

D-(−)-α-Phenyl-α-benzamidoacetic acid was prepared in the same way from D-(−)-α-phenylglycine: mp 186–187° (lit.[25] 187–188°); α_{obsd} −1.18°, 1.40% in 1 *N* NaOH; $[\alpha]^{33}D$ −84.3° (lit.[25] $[\alpha]^{15}D$ −84.1°).

Anal. Calcd for $C_{15}H_{13}O_3N$: C, 70.58; H, 5.13; N, 5.49. Found: C, 70.81; H, 5.25; N, 5.39.

Ethyl DL-α-phenyl-α-benzamidoacetate was prepared from 2.50 g (0.098 mole) of the benzamido acid as described for the acetamido ester, in 90% yield, mp 89° (lit.[15] 84°).

Ethyl D-(−)-α-phenyl-α-benzamidoacetate was prepared similarly from the D-(−) benzamido acid: mp 108–108.5°; α_{obsd} −2.05°, 2.23% in ethanol; $[\alpha]^{32}D$ −92.0°.

Anal. Calcd for $C_{17}H_{17}O_3N$: C, 72.07, H, 6.05; N, 4.94. Found: C, 71.90; H, 6.18; N, 5.26.

Treatment of Ethyl DL-α-Phenyl-α-benzamidoacetate with α-Chymotrypsin. A solution of 0.102 g of α-chymotrypsin in 5 ml of water was added to a stirred suspension of 0.204 g (0.72 mmole) of ethyl DL-α-phenyl-α-benzamidoacetate in 15 ml of 0.1 *N* NaCl and 5 ml of dioxane. The hydrolysis was followed at pH 7.8 in a pH-Stat, and the reaction was interrupted after 140 min, when 0.288 ml of 1 *N* NaOH was consumed, corresponding to hydrolysis of 80% of one enantiomorph. The products were isolated as described above for the acetamido compounds: unreacted ethyl α-phenyl-α-benzamidoacetate: 0.071 g (0.25 mmole), 60% yield; mp 102–103°; α_{obsd} −0.63°, 0.85% in ethanol; $[\alpha]^{27}D$ −74°; and L-(+)-α-phenyl-α-benzamidoacetic acid: 0.043 g (0.17 mmole), 58% yield; mp 192°; α_{obsd} +0.37°, 0.517% in 1 *N* NaOH; $[\alpha]^{28}D$ +72°.

A suspension of 0.0212 g (0.075 mmole) of ethyl DL-α-phenyl-α-benzamidoacetate in 15 ml of 0.1 *N* NaCl was allowed to react in a pH-Stat at 25.5° at pH 7.8, the nonenzymic hydrolysis proceeding at a rate of 2×10^{-5} mmole min^{-1}. A solution of 0.0206 g of α-chymotrypsin in 0.1 *N* NaCl was added, 0.1024 ml of 0.1 *N* NaOH being consumed in 60 min, corresponding to 24% hydrolysis of one enantiomorph by the enzyme, 1.5×10^{-4} mmole min^{-1}.

A similar study was carried out at pH 7.8 on 0.0507 g (0.18 mmole) of the ester in 11 ml of 0.1 *N* NaCl and 4 ml of dioxane. The nonenzymic rate was determined, 1×10^{-4} mmole min^{-1}. A solution of 0.0204 g of α-chymotrypsin in 5 ml of 0.1 *N* NaCl was added, 0.544 ml of 0.1 *N* NaOH being consumed in 60 min corresponding to 52% hydrolysis of one enantiomorph by the enzyme, 8×10^{-4} mmole min^{-1}.

Ethyl L-(+)-Mandelate. L-(+)-Mandelic acid (2.03 g, 0.013 mole) was boiled for 4.5 hr in 100 ml of ethanol containing 5 drops of sulfuric acid. The solution was diluted, treated with 5% sodium bicarbonate, and extracted with methylene chloride, leading to ethyl L-(+)-mandelate: 2.2 g (0.012 mole), 92% yield; bp 109° (2 mm); α_{obsd} +3.63°, 2.68% in chloroform; $[\alpha]^{28}D$ +136° (lit.[26] bp 106–107° (4 mm); $[\alpha]^{25}D$ +136.6° in chloroform). **Ethyl D-(−)-mandelate** was similarly prepared: bp 109° (2.4 mm); α_{obsd} −3.53°, 2.61% in chloroform; $[\alpha]^{26}D$ −136° (lit.[26] bp 103–105° (2 mm); $[\alpha]^{25}D$ −136.6° in chloroform).

Ethyl DL-mandelate was similarly prepared: bp 104° (1.5 mm) (lit.[27] 155–156° (35 mm)).

Treatment of Ethyl Mandelate with α-Chymotrypsin. A suspension of 1.00 g (5.55 mmoles) of ethyl DL-mandelate in 15 ml of 0.1 *M* NaCl was treated with a solution of 0.080 g of α-chymotrypsin in 5 ml of 0.1 *M* NaCl under nitrogen at 25° in a pH-Stat at pH 7.8. The reaction was stopped after 3.75 hr, 1.99 ml of 1 *N* NaOH having been consumed, 36% reaction. Unreacted ester was extracted with ether, 0.63 g, 98% recovery. Its infrared spectrum was identical with that of the starting material. It showed no optical activity beyond experimental error, α_{obsd} −0.03°, 2.82% in chloroform. The aqueous reaction solution was acidified and lyophilized, and the residue was extracted with acetone, leading to DL-mandelic acid: 0.101 g (0.67 mmole), 33% yield; mp 115–116.5°; mmp 117.5–118.5°; α_{obsd} +0.01°, 1.25% in water, within experimental error.

(25) S. Minovici, *Bull. Soc. Chim. Romania*, 2, 8 (1920); *Chem. Abstr.*, **14**, 3228 (1920).

(26) W. Bonner and C. Hurd, *J. Am. Chem. Soc.*, **73**, 4293 (1951).

(27) J. Berson and M. Greenbaum, *ibid.*, **81**, 6458 (1959).

Stepwise Synthesis of Oligodeoxyribonucleotides on an Insoluble Polymer Support[1,2]

Robert L. Letsinger and V. Mahadevan

Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received August 5, 1966*

Abstract: Procedures are described for synthesizing oligonucleotide derivatives (*e.g.*, TrdCpTpTpT) on an insoluble support polymer.

The comprehensive studies by Khorana and his co-workers have provided elegant and effective methods for the chemical synthesis of a wide variety of oligonucleotides.[3] Nevertheless, the stepwise synthesis of relatively long chain polynucleotides remains a formidable task. Some time ago it occurred to us that the labor involved in repetitive step syntheses of this type might be materially reduced if the syntheses were carried out on an insoluble polymer support. In the initial step a nucleoside would be joined covalently to the support. Nucleotide units would subsequently be added stepwise to this nucleoside, and in the final reaction the covalent bond joining the oligonucleotide chain to the support would be broken and the oligonucleotide eluted. This technique would enable one to separate the products in the building stages from the solvents, excess reagents, and soluble by-products simply by filtration, thus avoiding numerous time-consuming isolation steps. In testing this idea we first developed an insoluble, functionalized polymer and

(1) Part III, Nucleotide Chemistry. A preliminary account of some of this work was published in *J. Am. Chem. Soc.*, **87**, 3526 (1965).

(2) This research was supported by the Division of General Medical Sciences, National Institutes of Health, Grant 10265.

(3) For leading references see: (a) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965); (b) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964); (c) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (d) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958). Since completion of the present paper a procedure for synthesizing oligonucleotides on a soluble polymer support has been described by H. Hayatsu and H. G. Khorana, *ibid.*, **88**, 3182 (1966).