Absolute Steric Course of Hydrolysis by α-Chymotrypsin. Esters of α-Benzylsuccinic, α-Methyl-β-phenylpropionic, and α-Methylsuccinic Acids

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Abstract: The active site of α-chymotrypsin is described in terms of parts complementary to parts of a natural substrate, L-methyl N-acetylphenylalaninate, L-MAPhe, ar, a cavity or fold in which the β-aryl group provides primary binding; a site of small volume, h, contiguous with ar, into which the α-H fits; a surface site, am, at which the α-acylamido group associates by hydrogen bonding; and a surface site, n, at which the ester group is hydrolyzed. DL-Diethyl α-benzylsuccinate, $C_6H_5CH_2CH(CH_2CO_2C_2H_5)CO_2C_2H_5$ (II), is hydrolyzed stereospecifically by α-chymotrypsin, leading to (*R*)-(+)-β-ethyl α-benzylsuccinate and to (*S*)-(−)-II. The common convention indicates hydrolysis of the D enantiomer. Effective association of (+)-II places the phenyl group at ar and the carbethoxymethylene group at am, in the same sense as association of L-MAPhe, with carbethoxymethylene replacing acetamido, and, in this view, hydrolysis occurs in the L sense. DL-Ethyl α-methyl-β-phenylpropionate (IV) is also hydrolyzed stereospecifically, leading to L-(+)-α-methyl-β-phenylpropionic acid, and to D-(−)-IV. Effective association of (+)-IV places the phenyl group at ar and the α-methyl group at am. The α-methyl group, unlike α-chloro or α-hydroxyl, may not fit at h. DL-Dimethyl α-methylsuccinate (VI) is hydrolyzed with partial stereospecificity, the L-(−) enantiomer hydrolyzing about six times more rapidly than the D-(+). Association of L-(−)-VI with carbethoxymethylene at ar and methyl at am is favored over association of D-(+)-VI with carbethoxymethylene at am and methyl directed toward ar. α-Carbethoxymethylene and α-acetoxy groups associate similarly with α-chymotrypsin; the hydrolysis of D-α-acyloxypropionates corresponds to that of L-α-methylsuccinates. These studies lead to descriptions of modes of enzyme–substrate association, to optically active products of varied structure, to assignment of absolute configurations, and to understanding of the significance of L and D configurations in these enzymic reactions.

Consideration of the hydrolysis by α-chymotrypsin of substrates of varied structure has led to the proposal[1-3] that substrates associate with the active site of the enzyme in a particular conformation, as indicated in Figure 1A for L-methyl N-acetylphenylalaninate (L-MAPhe). The β-phenyl group is directed into a cavity or fold in the enzyme, to the ar site; it is primarily responsible for the binding of the substrate to the enzyme, leads to favorable binding constants, K_m, and contributes to favorable reaction constant, k_{cat}.[4,5] The carbethoxyl group is at the hydrolytic site, n, and the α-N-acyl group is at a hydrogen-bonding site, am. These two groups correspond respectively to the C-terminal and N-terminal peptide chains of large molecule substrates and they lie at sites on the surface of the enzyme. The α-N-acetyl group increases k_{cat} but need not lead to more favorable[6] binding. The α-H points toward the interior at a domain h of restricted volume, contiguous with ar, and substitution of a substantially larger group for it may decrease reactivity[5] greatly. The active site comprises parts of the enzyme complementary in position and form to the indicated parts of the natural substrate, L-MAPhe.

Stereospecific hydrolysis results when one enantiomer of a substrate may associate, as indicated in Figure 1, and the other enantiomer cannot fit into the active site effectively. Nonstereospecific hydrolysis may occur when both enantiomers can fit into the site in ways which place the group to be hydrolyzed at the nucleophilic site n. Studies of the structure of compounds which are hydrolyzed by the enzyme, and of the observed reactivity and presence or absence of stereospecificity, indicate the nature of the substrate groups which may fit into the parts of the active site and may lead to inferences about the site itself.

In the association of MAPhe with α-chymotrypsin, binding of the aryl group at ar is dominant, and the ester group must be at n if hydrolysis is to occur. For the D enantiomer to hydrolyze, the acetamido group would have to fit in the restricted site h; it is too large, and stereospecific hydrolysis of the L enantiomer occurs. Similarly, with ethyl α-acetoxy-β-phenylalaninate, EAoPhe, association as indicated in Figure 1B places the acetoxy group at am; this group also is too large to fit at h, and hydrolysis of only the L enantiomer results.[7] The acetoxyl group fits at am but does not associate firmly, as the acetamido group does by hydrogen bonding. Thus it does not restrict the substrate effectively[8] and EAoPhe is hydrolyzed by α-chymotrypsin stereospecifically but slowly.[7]

The ester group, —C(=O)—OR, may, like the aryl group, also fit at ar. This is most apparent in the rapid stereospecific hydrolysis of L-ethyl N-acetylaspartate[9] by α-chymotrypsin, $k_{cat} = 22\ sec^{-1}$, $K_m = 0.023\ M$. The mode of association is indicated in Figure 2A.

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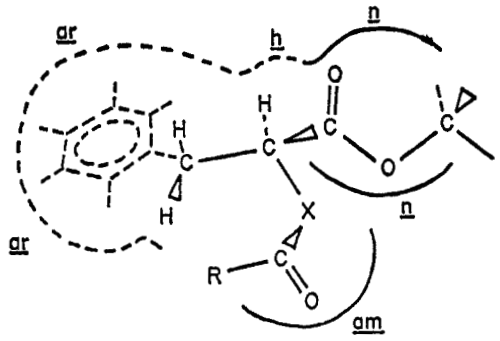

Figure 1. Association of substrates with α-chymotrypsin: (A) X = NH, L-(+)-methyl N-acetyl-β-phenylalaninate, MAPhe; (B) X = O, L-(−)-ethyl α-acetoxy-β-phenylpropionate, EAoPhe; (C) X = CH_2, (*R*)-(+)-diethyl α-benzylsuccinate.

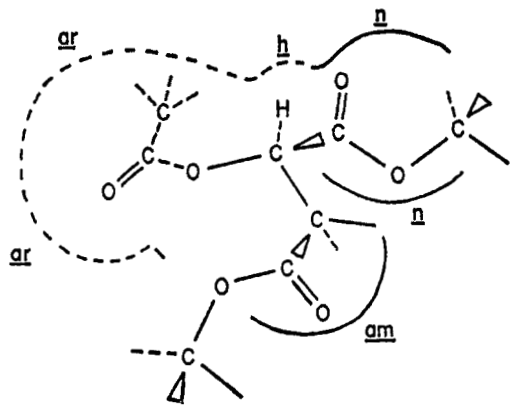

Figure 3. Association of D-(+)-diethyl α-acetoxysuccinate with α-chymotrypsin.

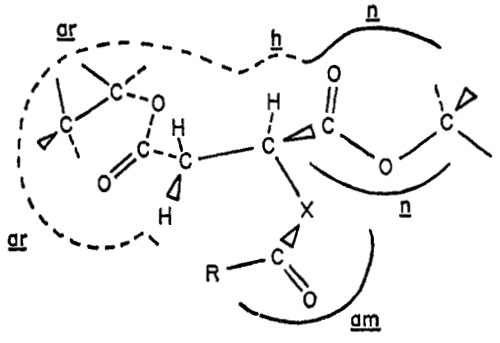

Figure 2. Association of substrates with α-chymotrypsin: (A) X = NH, L-(−)-diethyl N-acetylaspartate; (B) X = O, L-(−)-diethyl α-acetoxysuccinate.

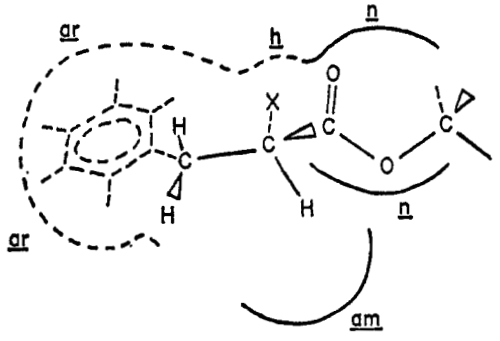

Figure 4. Association of esters of D-α-substituted β-phenylpropionates with α-chymotrypsin.

The second ester group increases markedly the reactivity of esters of homologous dibasic acids,[10] their α-acetamido derivatives,[10] and esters of β-substituted glutaric acids,[11] all as compared with the corresponding derivatives of the monobasic acids.

Although a second ester group may well have this effect by fitting at ar, it does not bind at ar as effectively as the aryl group, and less favorable K_m is observed than when the β-aryl group is present. Further evidence of this is seen in diethyl α-acetoxysuccinate, both enantiomers of which are hydrolyzed by α-chymotrypsin.[2] The L enantiomer may fit into the enzyme as indicated in Figure 2B. Since stereospecific hydrolysis of EAoPhe indicates that the acetoxyl group may not fit at h, it appears that the D enantiomer of the acetoxysuccinate places the acetoxyl group at ar and the carbethoxymethylene group at am, as indicated in Figure 3. That the acetoxyl group may fit at ar is also indicated by the inversion of stereospecificity observed in hydrolysis of D-ethyl α-acetoxypropionate,[12] in which the acetoxyl group lies at ar and the simple methyl group lies at am.

The β-aryl group prefers strongly to associate at ar, and the carbethoxymethylene group may be directed toward either ar or am. It was desirable to examine these inferences by a study of a compound which contains both a β-aryl group and a carbethoxymethylene group, diethyl α-benzylsuccinate, $C_6H_5CH_2CH(CH_2CO_2C_2H_5)CO_2C_2H_5$ (II), and this will be described below.

It was desirable also to consider substituents other than H, which may fit in the h position, and the nature of that part of the active site. A D enantiomer of an α-substituted β-phenylpropionate may be hydrolyzed by α-chymotrypsin if the α substituent fits a h and the α-H at am, making no effective use of the am site, as indicated in Figure 4. The L enantiomer has the α-substituent directed toward am during its hydrolysis as in Figure 1. While this may not occur with large α substituents, such as acylamido, acyloxy, and carbethoxy, it appears to occur with the α-chloro and α-hydroxy substituents. Methyl α-chloro-β-phenylpropionate may hydrolyze with no stereospecificity.[6] Both enantiomers of esters of α-hydroxy-β-phenylpropionic acid hydrolyze, the L more rapidly than the D,[6,7] and ethyl α-hydroxy-α-phenylacetate is hydrolyzed without stereospecificity.[3] It appears to us that D enantiomers of cyclized substrates also place the α substituent at h.[8] Questions as to steric and polar effects on interaction at h indicated that stereospecificity in hydrolysis of ethyl α-methyl-β-phenylpropionate, $C_6H_5CH_2CH(CH_3)CO_2C_2H_5$ (IV), by α-chymotrypsin might be examined, and this study of the α-methyl substituent will be described below.

Since the carbalkoxymethylene group may be directed toward am or ar, the simple α-methyl substituent may also be directed toward these two sites. Whether it may fit at h would be revealed in the study of IV. A comparison of the methyl and the carbalkoxymethylene groups would be available from study of dimethyl α-

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methylsuccinate, $CH_3O_2CCH_2CH(CH_3)CO_2CH_3$ (VI), and this too will be described.

Experimental Section

Benzylsuccinic Acid (I). Diethyl α-benzylmalonate (75 g, 0.30 mol, Matheson Coleman and Bell) and 51 g (0.30 mol) of ethyl α-bromoacetate were added in succession to a solution of 7.5 g (0.31 g-atom) of sodium in 100 ml of absolute ethanol; the solution was heated on the water bath for 12 hr. The solution was concentrated, and the residue was made slightly acidic with 3 *N* sulfuric acid and extracted with ether, leading to 8 g of crude triethyl 1-phenylpropane-2,2,3-tricarboxylate. This tricarboxylic ester (93 g, 0.28 mol) was boiled for 4 hr with 70 g of KOH in 280 ml of ethanol, diluted with 180 ml of water, acidified with 400 ml of 6 *N* HCl, and extracted with ether, leading to crude 1-phenylpropane-2,2,3-tricarboxylic acid. This was heated in three portions at 165–170° for 1.5 hr, leading to crude α-benzylsuccinic acid (I), 34 g (0.16 mol), 53% yield over-all, mp 160–161° after crystallization from water (lit.[13] mp 160–161°).

Diethyl α-Benzylsuccinate (II). A solution of 9.7 g (0.046 mol) of α-benzylsuccinic acid in 25 ml of absolute ethanol was boiled for 1.5 hr in a stream of dry HCl. The solution was poured into water, and the ester was extracted with ether and washed with water, sodium bicarbonate, and water, and distilled, bp 129° (0.7–0.8 mm), 7.5 g (0.028 mol), 62% yield, $n^{24}D$ 1.4875 (lit.[14] bp 169–171° (5 mm), $n^{20}D$ 1.4904).

Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.14; H, 7.54. (All elementary analyses were by Schwarzkopf Laboratories.)

Hydrolysis of Diethyl α-Benzylsuccinate (II) by α-Chymotrypsin. A suspension of 0.981 g (3.71 mmol) of DL-II in 20 ml of water containing 0.107 g of α-chymotrypsin in a pH-Stat at pH 7.2, 27°, consumed 1.87 ml of 1 *N* NaOH in 18 hr, and the hydrolysis then stopped, 50% reaction. The mixture was extracted with ether, and the aqueous solution was brought to pH 2 with 5% HCl and lyophilized. The residue was extracted with dry ether, leading to 0.393 g (1.66 mmol), 90% yield of (+)-β-**ethyl α-benzylsuccinate,** (+)-III, mp 41–42° from ether–pentane, α(obsd) +0.286° (*c* 2.9, $CHCl_3$), $[\alpha]^{28}D$ +10.0°.

Anal. Calcd for $C_{13}H_{16}O_4$: C, 66.08; H, 6.83. Found: C, 66.17; H, 7.07.

The original ether extract was dried and concentrated, leading to 0.431 g (1.64 mmol), 88% yield, of (−)-diethyl α-benzylsuccinate, (−)-II, α(obsd) −0.44° (*c* 2.5, $CHCl_3$), $[\alpha]^{27.5}D$ −17.6°, infrared spectrum identical with that of DL-II.

Anal. Found: C, 67.72; H, 7.65.

(−)-α-Benzylsuccinic Acid ((−)-I). (−)-II, above (0.0906 g, 0.343, mmol) was stirred with 1.0 ml of 1 *N* NaOH and 1.0 ml of ethanol for 24 hr at room temperature. The mixture was extracted with ether, and the aqueous solution was acidified with 2 *N* HCl and extracted with ether, leading to 0.0607 g (0.29 mmol), 85% yield, of (−)-I, mp 159–161°, α(obsd) −0.154° (*c* 1.5, ethanol), $[\alpha]^{26}D$ −10.3°, α(obsd) −0.398° (*c* 1.5, ethyl acetate), $[\alpha]^{26}D$ −26° (lit.[15] mp 164.5° $[\alpha]^{25}D$ −27° (*c* 2, ethyl acetate)).

Anal. Calcd for $C_{11}H_{12}O_4$: C, 63.45; H, 5.81. Found: C, 63.46; H, 5.99.

(+)-α-Benzylsuccinic Acid ((+)-I). (+)-β-Ethyl α-benzylsuccinate, above (0.0858 g, 0.363 mmol), was hydrolyzed as described above, leading to 0.0674 g (0.325 mmol), 90% yield, of (+)-I, mp 162–163° from water, α(obsd) +0.399° (*c* 1.5, ethyl acetate), $[\alpha]^{25}D$ +27°.

Anal. Found: C, 63.63; H, 5.95.

Synthesis of β-Ethyl α-Benzylsuccinate (DL-III). A solution of 21.2 g (0.20 mol) of benzaldehyde and 34.8 g (0.20 mol) of diethyl succinate was added to 17 g (0.25 mol) of sodium ethylate in 120 ml of ethanol, boiled for 3 hr, and concentrated. The residue was dissolved in 900 ml of water, washed with ether, acidified, and extracted with ether, leading to α-**ethyl α-benzalsuccinate,** 36 g (0.15 mol), 75% yield. This unsaturated monoester (8.3 g, 0.035 mol) was treated with 71 ml of 1 *N* NaOH and 71 ml of ethanol for 24 hr at room temperature. The solution was extracted with ether, acidified, and extracted with ether, leading to 6.55 g (0.032 mol), 90% yield, of isomeric **benzalsuccinic acids,** mp 172–174° from water–ethanol (lit. mp 185–188°[16] and 192°[17]), and 149–151° for the separate isomers. This acid (1.00 g, 4.85 mmol) was boiled with 15 ml of acetic anhydride for 1 hr and concentrated, leading to 0.65 g (3.5 mmol), 72% yield, of **benzalsuccinic anhydride,** mp 165–167.5° (lit. mp 166–168.5°[17] and 164–166°[18]) and 138–140° for the separate isomers. This anhydride (0.535 g, 2.87 mmol) was boiled with 3 ml of ethanol for 3 hr and concentrated. The residue was taken up in 2% $NaHCO_3$, filtered, acidified, and extracted with ether, leading to 0.365 g (1.56 mmol), 55% yield of β-**ethyl α-benzalsuccinate,** mp 74–75° from petroleum ether (bp 30–60°) (lit.[19] mp 77–78°). This unsaturated monoethyl ester (0.353 g, 1.51 mmol) was hydrogenated over 0.1 g of 10% palladium on carbon in ethanol, leading to 0.275 g (1.16 mmol), 77% yield of β-**ethyl α-benzylsuccinate** (DL-III), mp 38–39° from petroleum ether (lit.[19] mp 41–42.5°). The infrared spectra in chloroform and carbon tetrachloride were identical with those of the (+)-III obtained by enzymic hydrolysis of DL-II.

Anal. Calcd for $C_{13}H_{16}O_4$: C, 66.08; H, 6.83. Found: C, 66.42; H, 6.90.

Ethyl α-Methyl-β-phenylpropionate (IV). α-Methylcinnamic acid (30 g, 0.19 mol, mp 77–79°, Aldrich Chemical Co.) was boiled for 5 hr in 120 ml of anhydrous ethanol containing three drops of H_2SO_4. The solution was washed with bicarbonate and extracted with ether, leading to ethyl α-methylcinnamate, 9 g (0.047 mol), 25% yield, bp 126–130° (4–5 mm) (lit.[20] bp 130–140° (5–6 mm)). This ester, 7.25 g (0.038 mmol), was hydrogenated over 10% palladium on charcoal in 10 ml of ethanol, leading to IV, 6.82 g, 93% yield, bp 90° (4.5 mm) (lit.[21] bp 130–131° (12 mm)).

Anal. Calcd for $C_{12}H_{16}O_2$: C, 74.97; H, 8.39. Found: C, 74.34; H, 8.42.

Hydrolysis of Ethyl α-Methyl-β-phenylpropionate (IV) by α-Chymotrypsin. i. A suspension of 1.049 g (5.45 mmol) of DL-IV in 20 ml of water containing 0.1095 g of α-chymotrypsin in a pH-Stat at pH 7.2, 27.5°, consumed 1.223 ml of 1 *N* NaOH in 41 hr, 22.5% reaction. The mixture was extracted with ether, leading to 0.643 g, 80% recovery of partially optically active unhydrolyzed ester, with infrared spectrum identical with that of the racemic starting material, α(obsd) −0.20° (*c* 2.5, $CHCl_3$), apparent $[\alpha]^{26.5}D$ −8.1°, indicating $[\alpha]^{26.5}D$ −28° for optically pure ester. The aqueous solution was brought to pH 2 and lyophilized, and the residue was extracted with ether leading to L-(+)-α-**methyl-β-phenylpropionic acid (V),** 0.167 g (1.02 mmol), 83% recovery of the hydrolysis product, α(obsd) +0.295° (*c* 1.12, $CHCl_3$), $[\alpha]^{27}D$ +26.3° (lit.[22] +27.7° in $CHCl_3$).

Anal. Calcd for $C_{10}H_{12}O_2$: C, 73.14; H, 7.37. Found: C, 73.04; H, 7.69.

The recovered partially optically active ester, 0.377 g (1.96 mmol), was suspended in 20 ml of water containing 0.082 g of α-chymotrypsin in the pH-Stat at pH 7.2. Alkali was consumed, 0.512 ml of 1 *N* NaOH, 26.2% reaction, in 25.5 hr, and the reaction then stopped, total enzymic hydrolysis 48.7%, 97.4% of one enantiomer. The mixture was worked up as described above, leading to 0.224 g (1.17 mmol), 80% recovery, of D-(−)-IV, with infrared spectrum identical with that of the starting material, and to 0.0620 g (0.38 mmol) of L-(+)-V, 74% recovery, of the hydrolysis product, with infrared spectrum identical with that of the previous product. The recovered ester had α(obsd) −1.38° (*c* 5.2, ethanol), $[\alpha]^{28}D$ 26.6° (lit.[23] $[\alpha]^{21}D$ 29.2° in ethanol). The recovered acid had α(obsd) +0.362°, 1.35% in $CHCl_3$, $[\alpha]^{27.5}D$ +26.9°.

ii. A suspension of 0.844 g (4.39 mmol) of DL-IV in 185 ml of water containing 0.121 g of α-chymotrypsin in a pH-Stat at pH 7.2 consumed 1.37 ml of 1 *N* NaOH in 20 hr, 31.2% reaction. More α-chymotrypsin (0.118 g in 5 ml of H_2O) was added, and 0.684 ml of 1 *N* NaOH was consumed in 23 hr, 47% total reaction, 94% of one enantiomer; the reaction then stopped. The mixture was worked up as described above leading to 0.295 g (1.54 mmol), 70% recovery, of D-(−)-IV and 0.273 g (1.67 mmol), 76% yield, of L-(+)-V, with infrared spectra identical with those of previous samples.

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The ester had α(obsd) $-1.72°$ (*c* 5, ethanol), $[\alpha]^{27.5}D$ $-34.3°$; the acid had α(obsd) $+0.471°$ (*c* 1.89, $CHCl_3$), $[\alpha]^{26}D$ $+24.8°$.

Hydrolysis of Dimethyl α-Methylsuccinate (VI) by α-Chymotrypsin. A suspension of 0.853 g (5.33 mmol) of VI (K and K Co.) in 20 ml of water containing 0.0854 g of α-chymotrypsin, in a pH-Stat at pH 7.2, consumed 2.58 ml of 1 *N* NaOH, 48.4% reaction, in 28 hr. The mixture was extracted with ether, and the extract was dried and concentrated, leading to 0.318 g, 72% recovery, of optically active unhydrolyzed VI, with infrared spectrum identical with that of the starting material, α(obsd) $+0.128°$ (*c* 4.1, $CHCl_3$), $[\alpha]^{29}D$ $+3.12°$.

Anal. Calcd for $C_7H_{12}O_4$: C, 52.49; H, 7.55. Found: C, 52.19; H, 7.61.

The aqueous solution was brought to pH 2 and lyophilized, and the residue was extracted with ether, leading to an oil, 0.235 g (1.61 mmol), 62% yield of optically active crude monoester, β-methyl α-methylsuccinate (VII), α(obsd) $-0.26°$ (*c* 3.9, $CHCl_3$), $[\alpha]^{30}D$ $-6.6°$.

Anal. Calcd for $C_6H_{10}O_4$: C, 49.31; H, 6.90. Found: C, 48.00; H, 7.22.

This monoester (0.124 g, 0.85 mmol) was treated with 1.8 ml of 1 *N* NaOH in 1.8 ml of ethanol overnight at room temperature. The solution was extracted with ether, brought to pH 2 with 2 *N* HCl, saturated with NaCl, and extracted with ether, leading to partially active (−)-α-methylsuccinic acid (VIII), 0.065 g (0.49 mmol), 58% yield, mp 101–102.5°, α(obsd) $-0.190°$ (*c* 1.75, ethanol), $[\alpha]^{27}D$ $-10.9°$ (lit.[24] mp 111–113°, $[\alpha]^{24.2}D$ $-15.0°$ (*c* 1.89, ethanol)).

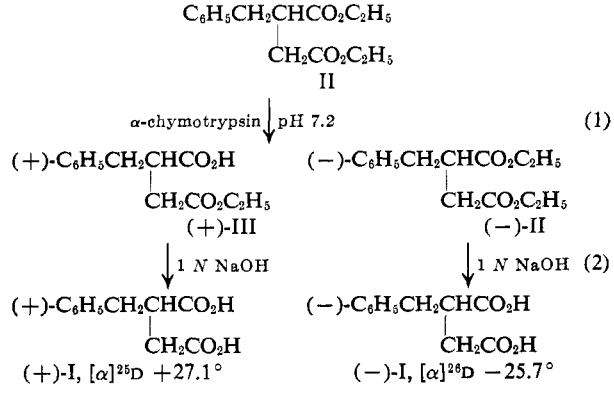
Anal. Calcd for $C_5H_8O_4$: C, 45.45; H, 6.10. Found: C, 45.13; H, 6.23.

The recovered unhydrolyzed (+)-diester (0.124 g, 0.77 mmol) was treated with 1.6 ml of 1 *N* NaOH and 1.6 ml of ethanol overnight at room temperature. The solution was diluted with water and extracted with ether, and the aqueous layer was brought to pH 2, saturated with NaCl, and extracted with ether, leading to partially active (+)-α-methylsuccinic acid (VIII), 0.093 g (0.70 mmol), 92% yield, mp 100–102°, α(obsd) $+0.166°$ (*c* 1.40, ethanol), $[\alpha]^{27}D$ $+11.90°$ (lit.[24] mp 113–115°, $[\alpha]^{25.5}D$ $+15.5°$, in ethanol).

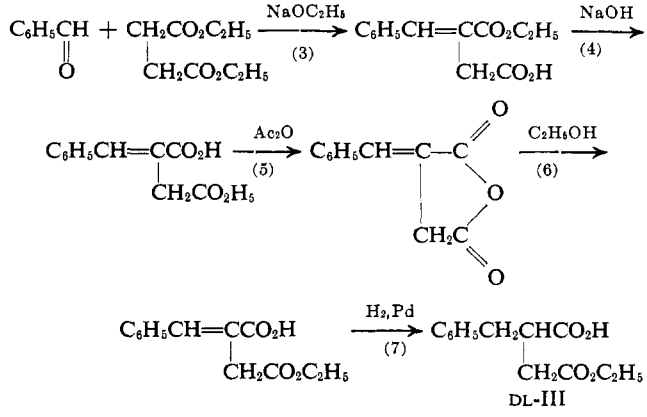
Anal. Found: C, 44.87; H, 6.01.

Results

Diethyl α-benzylsuccinate, $C_6H_5CH_2CH(CH_2CO_2C_2H_5)CO_2C_2H_5$ (II), was prepared by condensation of diethyl α-benzylmalonate with ethyl α-bromoacetate, leading to triethyl 1-phenylpropane-2,2,3-tricarboxylate, saponification of the triester, and decarboxylation to form benzylsuccinic acid (I), followed by esterification. A suspension of 3.71 mmol of DL-II in 20 ml of water containing 5 mg/ml (0.107 g) of α-chymotrypsin consumed alkali to 50% hydrolysis, 100% hydrolysis of one ester group of one enantiomorph, in 18 hr, and the reaction then stopped. Optically active products were obtained in *ca.* 90% yield, the half-ester, (+)-β-ethyl α-benzylsuccinate (III), and unhydrolyzed II, (−)-diethyl α-benzylsuccinate. The (+) half-ester III was hydrolyzed to (+)-benzylsuccinic acid (I), $[\alpha]^{25}D$ $+27.1$, and the (−) diester was hydrolyzed to (−)-I, $[\alpha]^{26}D$ $-25.7°$. The reactions are indicated in eq 1 and 2.



That the enzymic hydrolysis led to the β-monoester (+)-III is indicated by independent synthesis of DL-III, by a series of reactions 3–7. Stobbe condensation of



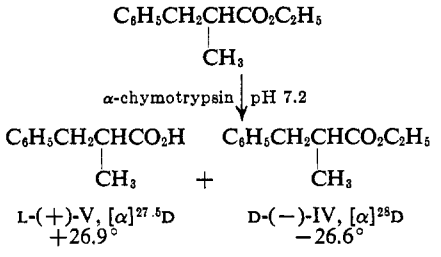
benzaldehyde with diethyl succinate led to *cis*- and *trans*-α-ethyl α-benzalsuccinates.[17] The half-esters were saponified to the diacids,[16,17] the diacids were converted to the anhydrides,[18] and treatment with ethanol led to the β-ethyl α-benzalsuccinates.[19] This material had λ_{max} in methanol at 263 mμ, which was shifted to 258 mμ when alkali was added, a shift characteristic of the α,β-unsaturated acid structure. α-Methylcinnamic acid similarly showed λ_{max} at 268 mμ, which was shifted to 263 mμ on addition of alkali. The α-ethyl α-benzalsuccinate formed in the Stobbe condensation showed λ_{max} at 268 mμ and no shift on addition of alkali, confirming that the ester, and not the free carboxyl group, was in conjugation with the styryl group.

Hydrogenation of the unsaturated β-monoethyl ester led to β-ethyl α-benzylsuccinate,[19] DL-III, eq 7, the infrared spectrum of which was identical with that of (+)-III formed by the enzymic hydrolysis of DL-II. Hydrogenation of the unsaturated α-monoethyl ester, formed initially in the Stobbe condensation, led to the isomeric compound, α-ethyl α-benzylsuccinate, the infrared spectrum of which in CCl_4 differed slightly from that of (+)-III, showing small absorption peaks at 5.6 and 8.9 μ which were absent in the β ester.

Ethyl α-methyl-β-phenylpropionate, $C_6H_5CH_2CH(CH_3)CO_2C_2H_5$ (IV), was found to be hydrolyzed by α-chymotrypsin with high stereospecificity in the L sense. The compound was prepared from α-methylcinnamic acid by esterification, followed by hydrogenation. A suspension of 5.45 mmol of DL-IV in 20 ml of water containing 5 mg/ml (0.110 g) of α-chymotrypsin consumed only 1.22 mmol of alkali in 41 hr. The acid produced, L-(+)-α-methyl-β-phenylpropionic acid (V),[25] $[\alpha]^{27}D$ $+26.3°$, had high optical purity,[24] and the rotation of the recovered unhydrolyzed IV also indicated high stereospecificity in the hydrolysis. The recovered partially active ester was treated again with α-chymotrypsin, and the hydrolysis proceeded to 97% hydrolysis of one enantiomorph and stopped. The unhydrolyzed ester was recovered in high yield, D-(−)-IV, $[\alpha]^{28}D$ $-26.6°$ (lit.[24] $[\alpha]^{21}D$ $-29.2°$); the hydrolysis product L-(+)-V was also recovered in high yield, $[\alpha]^{27.5}D$ $+26.9°$ (lit.[23] $[\alpha]D$ $+27.7°$).

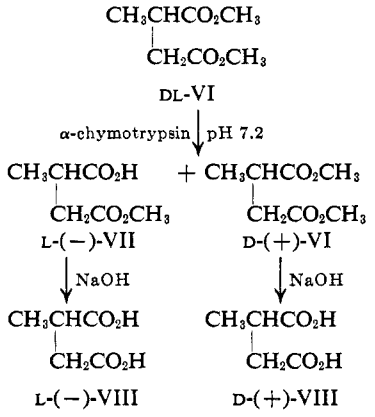
(24) E. J. Eisenbraun and S. M. McElvain, *J. Am. Chem. Soc.*, 77, 3383 (1955).

(25) A. W. Schrecker, *J. Org. Chem.*, 22, 33 (1957).



(8)

Dimethyl α-methylsuccinate, $CH_3C(CH_2CO_2CH_3)HCO_2CH_3$ (VI), was hydrolyzed by α-chymotrypsin with considerable but somewhat less, stereospecificity (eq 9). A suspension of 5.33 mmol of DL-VI in 20 ml of water containing 4.2 mg/ml of α-chymotrypsin consumed 2.58 mmol of alkali in 28 hr, and the reaction was then proceeding very slowly. Unhydrolyzed VI was isolated and found to be dextrorotatory. It was hydrolyzed with base, eq 10, leading to (+)-α-methylsuccinic acid (VIII), $[\alpha]^{27}D$ +11.9°. This may be compared with the reported[24] value, $[\alpha]^{25.5}D$ +15.5°, indicating about 88% (+) enantiomorph, 12% (−). The product of the enzymic hydrolysis, presumably the monoester, β-methyl α-methylsuccinate (VII), was levorotatory, and further hydrolysis of it with alkali led to (−)-α-methylsuccinic acid, $[\alpha]^{27}D$ −10.9°, indicating the presence of 85% (−) enantiomorph, 15% (+).



(9)

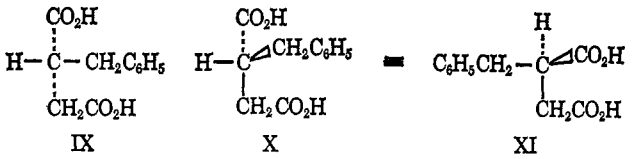
(10)

Discussion

Diethyl α-benzylsuccinate (II), ethyl α-methyl-β-phenylpropionate (IV), and dimethyl α-methylsuccinate (VI) have low solubility in water and are hydrolyzed slowly by α-chymotrypsin. High concentrations of enzyme were used, and suspensions of the substrates were hydrolyzed. Study of the kinetics of these reactions in solution is in progress. The stereochemical results allow further inferences to be drawn about interaction of parts of substrates with areas of the active site.

The products of the enzymic hydrolysis of DL-II, (+)-β-ethyl α-benzylsuccinate (III), and (−)-diethyl α-benzylsuccinate (II), led to (+)- and (−)-α-benzylsuccinic acids, respectively, $[\alpha]^{25}D$ +27° and $[\alpha]^{25}D$ −26° (eq 1 and 2). These rotations compare favorably with that reported[15] for resolved α-benzylsuccinic in the same solvent, ethyl acetate, $[\alpha]^{25}D$ −27°, indicating high optical purity in the products and a high degree of stereospecificity in the enzymic hydrolysis. A synthesis of DL-β-ethyl-α-benzylsuccinate (III), following procedures in the literature (eq 3–7), and comparison of infrared spectra, confirmed that the more hindered α-carbethoxyl group was hydrolyzed by the enzyme. Natural substrates are derivatives of α-amino acids, and other α substituents may fit into the am position (Figures 1, 2, and 3), favoring hydrolysis of the α-substituted ester group as compared with carbethoxymethylene groups. This is also seen in hydrolysis by α-chymotrypsin of α-ester groups of diesters of N-acetyl-aspartic,[9] malic[2] and α-acetoxysuccinic acids.[2]

The absolute configuration of benzylsuccinic acid has been indicated as follows. (−)-Benzylsuccinic acid and (+)-2-thienylsuccinic acid form a quas-iracemate and have opposite configurations.[26] (−)-2-Thienylsuccinic acid is hydrogenated to (−)-*n*-pentylsuccinic acid.[27] The method of quasi-racemates indicates that the lower *n*-alkylsuccinic acids, the α-mercaptosuccinic acids, and malic acids of the same sign have the same configurations.[28,29] (+)-Malic acid has been related to D-(+)-glyceraldehyde. (+)-Half-ester III, formed by the enzymic hydrolysis of DL-II, led to (+)-benzylsuccinic acid, and this would indicate that the D enantiomer of II had hydrolyzed. This is a D enantiomer when considered as a derivative of succinic acid with an α-benzyl substituent.[30,31] It may be described by the Fischer projection IX and by the three-dimensional representations X and XI. It is designated *R* by the sequence rule.[32] As a derivative of succinic acid (+)-benzylsuccinic acid is an analog of D-aspartic acid, with the benzyl group replacing the amino group. As a



(*R*)-(+)-α-benzylsuccinic acid

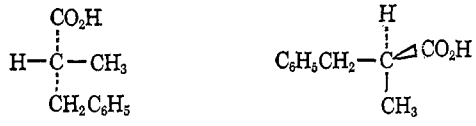
derivative of β-phenylpropionic acid, (+)-benzylsuccinic acid is an analog of L-β-phenylalanine, with the carbethoxymethylene group replacing the amino group. Formula XI and Figures 1A and 1C indicate that (+)-α-benzylsuccinic acid and its (+) diester may associate with the active site of α-chymotrypsin exactly like L-MAPhe. In this view the stereospecific hydrolysis has proceeded in the L sense, as anticipated. The use of D and L is confusing, since the assignment in the literature is based on the different, entirely justified, convention. The assignment of configuration based on the method of quasi-racemates appears valid. It is confirmed by the configuration which we would assign on the basis of the stereospecific hydrolysis by α-chymotrypsin and an association like that of L-MAPhe, Figure 1. This is predicated on association of the β-phenyl group at ar as dominant, the carbethoxymethylene group fitting at am, and the hydrolyzing ester group at n.

Diethyl α-benzylsuccinate (II) and ethyl α-acetoxy-β-phenylpropionate (EAoPhe, Figures 1B and 1C) are similarly affected by α-chymotrypsin because of the near

(26) A. Fredga and O. Palm, *Arkiv Kemi Mineral. Geol.*, **26A** [26], 9 (1949); *Chem. Abstr.*, **43**, 6611 (1949).
(27) A. Fredga, *Arkiv Kemi*, **6**, 277 (1953).
(28) M. Matell, *ibid.*, **5**, 17 (1953).
(29) A. Fredga, *Arkiv Kemi Mineral. Geol.*, **14B** [27], 8 (1941); *ibid.*, **15B** [23], 1 (1942); *Tetrahedron*, **8**, 126 (1960).
(30) A. Fredga, J. P. Jennings, W. Klyne, P. M. Scopes, B. Sjöberg, and S. Sjöberg, *J. Chem. Soc.*, 3928 (1965).
(31) R. P. Linstead, J. C. Lunt, and B. C. L. Weedon, *ibid.*, 3333 (1950).
(32) R. S. Cahn, C. K. Ingold, and V. Prelog, *Experientia*, **12**, 81 (1956).

equivalence of the interactions of the carbethoxymethylene and acetoxyl groups with the enzyme. Association is dominated by the phenyl group at ar. The α-carbethoxymethylene group of II and the α-acetoxy group of EAoPhe may lie at am and lead to slow stereospecific hydrolysis of (*R*)-(+)-II and L-(−)-EAoPhe. The carbethoxymethylene and acetoxy groups are too large to lie at h, the phenyl group precludes their being at ar, and the other enantiomers are not hydrolyzed. In the absence of the phenyl group, as in diethyl α-acetoxysuccinate, the carbethoxymethylene and acetoxy groups may each lie at either ar or am, and both enantiomers are then hydrolyzed.

Ethyl α-methyl-β-phenylpropionate (IV), with the simple methyl group as the α substituent, appeared to be hydrolyzed by α-chymotrypsin considerably more slowly than was compound II but, like II, with high stereospecificity. The hydrolysis of DL-IV led to (+)-α-methyl-β-phenylpropionic acid (V) and to (−) ester IV, both with specific rotations quite similar to the reported values.[22,23] The absolute configuration of the acid V has been established by (i) conversion of D-phenylalanine to (+)-α-methyl-β-phenylethylamine[33] and (ii) conversion of (−)-α-methyl-β-phenylpropionic acid (V) to (−)-α-methyl-β-phenylethylamine.[25] In the first case the carboxyl group was converted to a methyl group, in the second, to an amino group. Thus (−) acid V has the D configuration and the L enantiomer had been hydrolyzed. The acid D-(−)-V may be described by the Fischer projection XII, and the acid L-(+)-V, by the three-dimensional representation



XII (D-(−)-V)

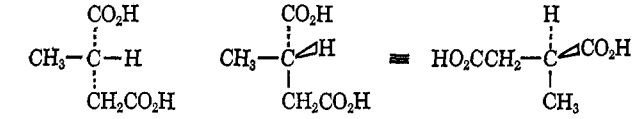
XIII (L-(+)-V)

XIII. Comparison of formula XIII and Figure 1 shows that L-(+)-IV fits into the enzyme like the L-phenylalaninate and (*R*)-(+)-II, with the α-methyl of IV lying at the am site. It may be that the methyl group at am provides less restriction of the substrate and contributes less to reactivity than does the carbethoxymethylene group of II, or the lower reactivity of IV may arise simply from lower solubility.

The failure of D-(−)-IV to be hydrolyzed by α-chymotrypsin indicates that the α-CH_3 group may not enter the h site. The methyl group has a somewhat larger van der Waals radius,[34] 2.0 Å, than that of chlorine, 1.8 Å, and is somewhat larger than the hydroxyl group, both of which may fit at h, as indicated by enzymic hydrolysis of D enantiomers of esters of α-chloro- and α-hydroxy-β-phenylpropionic acids.[6,7] It is not clear whether the difference is due to the size of the methyl group or the polar character of the chlorine and hydroxyl. Study of the larger halogens, mercaptan, and cyano groups as α substituents may prove informative. The nature of the h site and the groups it may accept is of particular interest since we believe it is involved in the hydrolysis of cyclized substrates.[8] The h site is contiguous with and may, in effect, be a part of the primary binding site ar.

(33) P. Karrer and K. Erhardt, *Helv. Chim. Acta*, 34, 2202 (1951).
(34) L. Pauling, "Nature of the Chemical Bond," 2d ed, Cornell University Press, Ithaca, N. Y., 1940, p 189.

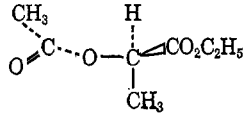
Dimethyl α-methylsuccinate (VI), with only the less specifically orienting methyl and carbomethoxymethylene groups, was hydrolyzed with partial stereospecificity. The products, after hydrolysis of one ester group, were (+) unhydrolyzed VI and the (−) half-ester, which were hydrolyzed with alkali to (+)- and (−)-α-methylsuccinic acids (VIII), respectively. The values of the rotations of the latter indicated that (−)-VI was hydrolyzed by α-chymotrypsin about six times as rapidly as (+)-VI from the DL mixture. As indicated above in the consideration of the absolute configuration of α-benzylsuccinic acid, (−)-α-methylsuccinic acid (VIII) and the diester (−)-VI are related to L-glyceraldehyde and may be described by Fischer projection XIV and the three-dimensional representations XV and XVI. Comparison of formula XVI and Figure 2 indi-



XIV (L-(−)-VIII) XV XVI

cates that the favored association of diester VI with α-chymotrypsin is similar to that of esters of L-N-acetylaspartic and L-α-acetoxysuccinic acids. In ester VI both the methyl and carbalkoxymethylene groups may associate at ar and am, but neither may associate readily at h. (The methyl group, more properly, may be directed toward ar.) Association of the ester group at the primary binding site ar, placing the methyl group at am, is the more favorable, leading to the observed L preference, and the alternate mode, with methyl at ar and carbalkoxymethylene at am, leads to the slower hydrolysis of the D enantiomer.

We havere marked on the similarity of the carbalkoxymethylene, $-CH_2CO_2R$, and acetoxy, $-OCOR$, groups in their associations with the α-chymotrypsin (i) in the stereospecific hydrolyses of diethyl α-benzylsuccinate and ethyl α-acetoxy-β-phenylpropionate, in which these groups lie at am, and (ii) in the nonstereospecific hydrolysis of diethyl α-acetoxysuccinate, in which these two groups lie either at am or ar. A further comparison can be made of these groups, present essentially alone, in the methylsuccinate VI and in ethyl α-acetoxypropionate, $CH_3CH(OCOCH_3)CO_2C_2H_5$. Orientation of the latter in the same mode as indicated for the former in formula XVI would lead to formula XVII and to Figure 5. Preferred orientation of



XVII, D-(+)-ethyl α-acetoxypropionate

the acetoxyl group toward ar, similar to that of the carbalkoxymethylene group in VI, brings the hydrolyzing ester group to n, in the D enantiomer with methyl at am, α-H at h, and in the L enantiomer with methyl at h, α-H at am. The methyl group, as indicated by the stereospecific hydrolysis of IV, may not be placed at h, and the D enantiomer would have the preferred configuration for hydrolysis by α-chymotrypsin. This is so, and ethyl α-acetoxypropionate was the first simple noncyclized compound to show preferred hydrolysis of its D enantiomer.[12]

Table I. Absolute Configurations. Hydrolysis by α-Chymotrypsin

No.	Compound	Rotation	Config	Ref
1	$C_2H_5O_2CCH(NHCOCH_3)CO_2^-$, (H)[a]	(+), (+)	*R*, L	36
2	$C_2H_5O_2CCH(OCOCH_3)CO_2^-$ [b]	(+)	*R*, L	12
3	$C_2H_5O_2CCH_2CH(NHCOCH_3)CH_2CO_2H$	(+)	*R*, L	37
4	$CH_3O_2CCH_2CH(OH)CH_2CO_2^-$, (H)[a]	(−), (−)	*R*, L	38
5	$C_2H_5O_2CCH_2CH(OH)CH_2CO_2^-$, (H)[a]	(−), (−)	*R*, L	38
6	$CH_3O_2CCH_2CH(OCOCH_3)CH_2CO_2H$	(+)	*R*, L	38
7	$C_6H_5CH(NHCOCH_3)CH_2CO_2^-$ (H), (C_2H_5)[c]	(−), (−), (−)	*S*, D	39, 40
8	$C_6H_5CH(OH)CH_2CO_2H$, (C_2H_5)[d]	(−), (−)	*S*, D	39, 40
9	$C_6H_5CH(NHCOCH_3)CO_2H$, (C_2H_5)[d]	(+), (+)	*S*, L	3
10	$C_6H_5CH(NHCO_6H_5)CO_2H$, (C_2H_5)[d]	(+), (+)	*S*, L	3
11	$C_6H_5CH_2CH(OCOCH_3)CO_2H$, ($C_2H_5$)[d]	(−), (−)	*S*, L	7
12	$C_6H_5CH_2CH(OH)CO_2H$, (C_2H_5)[d]	(−), (−)	*S*, L	7
13	$C_6H_5CH_2CH(CH_2CO_2C_2H_5)CO_2H$, ($C_2H_5$)[d]	(+), (+)	*R*, D	*e*
14	$C_6H_5CH_2CH(CH_3)CO_2H$, (C_2H_5)[d]	(+), (+)	*S*, L	*e*
15	$C_2H_5O_2CCH_2CH(CH_3)CO_2H$, ($C_2H_5$)[d]	(−), (−)	*S*, L	*e*
16	$C_2H_5O_2CCH_2CH(NHCOCH_3)CO_2H$, ($C_2H_5$)[d]	(+), (−)	*S*, L	9
17	$CH_3CH(OCOC_6H_5)CO_2H$, (C_2H_5)[d]	(−), (−)	*R*, D	7
18	$CH_3CH(OCOCH_3)CO_2H$, (C_2H_5)[d]	(+), (+)	*R*, D	12

[a] The anion, the free acid, and the signs of their rotations under the conditions indicated in the reference. [b] The anion in solution. [c] The anion, the free acid, and the ester. [d] The free acid and the ester. [e] This work.

The L enantiomer was essentially inert. Hydrolysis of the related ethyl α-benzoyloxypropionate, Figure 5C, also showed preferred hydrolysis of the D enantiomer.[7]

While the α-acetoxy and carbethoxymethylene groups may fit at either am or ar, in the presence of the benzyl groups they lie only at am and the aryl group fits at ar, leading in effect to L specificity. If there is a second aryl site[35] the acetoxyl and carbethoxymethylene groups might associate at ar, and the phenyl group at the second aryl site, allowing the D enantiomers to be hydrolyzed. This is not observed with esters of α-benzylsuccinic and α-acetoxy-β-phenylpropionic acids. It appears that convincing evidence for a second aryl site has not yet been found in experiments with substrates.

The primary purpose of these studies remains to explore the topography and associative properties of the active site of α-chymotrypsin and the factors that influence enzyme–substrate associations. Some incidental benefits accrue from this work. Stereospecific hydrolysis has been observed for a number of esters which are only formally related to the natural substrates, forming optically active materials, usually in high yield and in high optical purity. The experience acquired in fitting substrates properly into the diagram of the active site allows assignment of absolute configurations to many of the products of these reactions with a measure of confidence. This leads to an understanding of the relevance of L and D configurations to these enzymic reactions and predictions of steric course and relative reactivity. The absolute configurations of optically active compounds formed in these studies are indicated in Table I.[36-40]

Assignment of the *R* configuration to the malonates (compounds **1** and **2**, Table I) is based on the acetamido and acetoxy groups lying at am, the ester group at ar. This corresponds to a derivative of L-N-acetylalanine, with the unhydrolyzed ester group replacing the methyl group of alanine. Assignment of the *R* configuration to the β-acetamido- and β-hydroxyglutarates (compounds **3–5**) is also made by placing the acetamido and hydroxy groups at am. The L configuration of the monomethyl β-hydroxyglutarate was assigned also by stereospecific chemical correlation with 3-hydroxypentanoic acid.[41] In our work the optically active β-acetoxyglutarate (compound **6**) was not obtained enzymically but from the active monomethyl β-hydroxyglutarate. The optically active α-substituted malonate and β-substituted glutarate monoesters are formed from the nondissymmetric diesters. The glutarate diesters are converted completely to the single enantiomer of the monoesters.

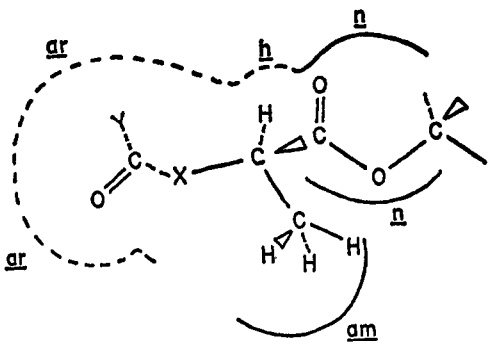


Figure 5. Association of substrates with α-chymotrypsin: (A) X = CH_2, Y = CH_3O, L-(−)-dimethyl α-methylsuccinate; (B) X = O, Y = CH_3, D-(+)-ethyl α-acetoxypropionate; (C) X = O, Y = C_6H_5, D-(−)-ethyl α-benzoyloxypropionate.

The *S* configuration of compounds **7** and **8** directs the β-acetamido and β-hydroxy groups toward the L-α-am site.[3] This corresponds to a D configuration of the β-carbon as indicated by correlations described in a previous publication.[40] Assignment of the *S* configuration to compounds **9–12** is based on the acetamido, benzamido, acetoxy, and hydroxy groups lying at am, the β-phenyl group at ar, Figure 1. Their L configurations were known previously. The *R* and *S* notations of

(35) B. F. Erlanger, *Proc. Natl. Acad. Sci. U. S.*, **58**, 703 (1967).
(36) S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.*, **82**, 6038 (1960).
(37) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 1093 (1961).
(38) S. G. Cohen and E. Khedouri, *ibid.*, **83**, 4228 (1961).
(39) S. G. Cohen, Y. Sprinzak, and E. Khedouri, *ibid.*, **83**, 4225 (1961).
(40) S. G. Cohen and S. Y. Weinstein, *ibid.*, **86**, 725 (1964).
(41) K. Serck-Hanssen, *Arkiv Kemi*, **10**, 135 (1956).

compounds **13**, **14**, **15**, **17**, and **18** are indicated by the modes of association of Figures 1 and 5, that of compound **16** in Figure 2. The D and L assignments of compound **13**, **14**, and **15** have been discussed above; the D assignment of compounds **17** and **18** and the L assignment of compound **16** were known previously.

The ideas developed in these studies about enzyme–substrate associations will be applied to a number of cyclized compounds in a forthcoming article.

Acknowledgment. We are pleased to acknowledge generous support of this work by the National Institutes of General Medical Sciences, GM-04584.

The Oxidative Cleavage of Amines by Aqueous Bromine at 25°

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Abstract: Primary, secondary, and tertiary amines are oxidatively cleaved by aqueous bromine at pH 5–7. An alkyl group is removed and the amine with one less alkyl group is produced. Secondary alkyl groups appear as ketones. The fate of primary alkyl groups depends on the type of amine being oxidized. The propyl group appears as propionaldehyde from tripropylamine, as propionic and pyruvic acids from dipropylamine, and exclusively propionic acid from propylamine. All of these results pertain to runs in which the amine is in excess. The selectivity is low between two alkyl groups, and on this basis the mechanism appears to be loss of proton plus an electron pair (Westheimer mechanism) and thus resembles the oxidative cleavage of ethers. However, the data are not conclusive. The oxidation of tertiary amines by triaryl carbonium ions is briefly examined.

The oxidative cleavage of tertiary amines by halogens has been periodically investigated. Wilstätter and Iglauer[1] were concerned with demethylating N-methyl alkaloids and found that tropidine, tropane, and N-methylpiperidine were demethylated by aqueous chlorine. Aqueous chlorine also cleaved trimethylamine with the formation of N-chlorodimethylamine.[2]

Meisenheimer examined the action of aqueous chlorine and bromine on trimethylamine and triethylamine and recognized that the alkyl group appeared as the aldehyde and that the aldehyde was formed by hydrolysis of an intermediate.[3] The work reported herein contributes only a modest addition to the mechanistic picture given by Meisenheimer. However, in the interim, a free-radical mechanism has been championed,[4] and the cleavage has been conducted under anhydrous conditions[5] and with N-bromosuccinimide,[6,7] all of which overshadow the fact that excellent yields can be achieved under simple aqueous conditions at 25° with ordinary bromine.

Two further studies on oxidative cleavage have been reported. The kinetics of the trimethylamine–aqueous chlorine reaction were examined[8] and the action of aqueous chlorine on $CH_3N(CH_2CH_2Cl)_2$ was studied.[9] Also the history of N-haloamines is germane. The N-chloro- and N,N-dichloroamines are well known. It appears that with aqueous chlorine the various rates and equilibria are such as to allow the isolation of the N-chloroamines.[8,10] The use of these N-chloroamines in converting aromatics to dialkylamino aromatics in 80–100% sulfuric acids[10,11] indicates that they are not readily converted to amines in strong aqueous acids. The reports on N-bromoamines are more limited. Though they have been well characterized from the interaction of amines and bromine in benzene and ether,[12] it is not clear whether they can be isolated from reaction in aqueous solution. An old report claims the isolation of $(CH_3)_2NBr$ in excess alkali in the cold[13] and a recent spectroscopic study is fragmentary.[14]

Products and Yields from Tertiary Amines. The stoichiometry of the oxidative cleavage of tertiary amines by aqueous Br_2 at pH 5 (acetate buffer) is exemplified by the following equation.

$$(CH_3CH_2CH_2)_3NH^+ + Br_2 + 2CH_3CO_2^- \longrightarrow CH_3CH_2CHO\ (90\%) + (CH_3CH_2CH_2)_2NH_2^+ + 2Br^- + 2CH_3CO_2H \quad (1)$$

It is surprising that propionaldehyde is produced because in similar oxidations of ethers and alcohols by aqueous bromine,[15] the intermediate aldehydes could not be detected during the course of the reaction. The formation of propionaldehyde in 90% yield becomes even more surprising in view of comparable rates of

(1) R. Wilstätter and F. Iglauer, *Ber.*, **33**, 1636 (1900).
(2) A. Hantzsch and H. Graf, *ibid.*, **38**, 2156 (1905).
(3) J. Meisenheimer, *ibid.*, **46**, 1148 (1913).
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