in an ice bath and treated with 15 drops of an 8 *N* chromic acid solution (CrO_3 (2.67 g) in concentrated sulfuric acid (2.3 ml) and water (4.0 ml), diluted to 10 ml with water). The solution was kept in the ice bath for 10 min (frequent swirling), then diluted to 100 ml with water containing a small amount of methanol, and extracted with chloroform. The chloroform layer was washed with water, dried over anhydrous sodium sulfate, filtered, and concentrated to give 66 mg of a solid, mp 177–182°. Recrystallization from benzene–Skellysolve B gave 47 mg of the ketone (**14**), mp 184–186°; $[\alpha]^{29}D$ −198° (*c* 0.88, $CHCl_3$); λ_{max}^{EtOH} end absorption at 212 mμ (ε 6050); $\lambda_{max}^{CHCl_3}$ 2.90, 5.66, 5.75, 6.03 μ.

Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.06; H, 7.57.

Dehydration of Desacetyldihydrodehydrogaillardin (14). a. Thionyl Chloride–Pyridine. A solution of **14** (50 mg) in pyridine (5 ml) was treated with thionyl chloride (2 ml) with stirring for 0.5 hr in an ice bath and then 1 hr at room temperature. The reaction mixture was diluted with water and extracted with chloroform. The chloroform layer was washed with dilute acid, saturated sodium bicarbonate solution, and water, dried over anhydrous sodium sulfate, filtered, and concentrated to give 32 mg of syrup which could not be crystallized. The infrared absorption spectrum was consistent with structure **13**; $\lambda_{max}^{CHCl_3}$ 5.62 (s), 5.90 (s), 6.18 (m), 6.02 (w) μ.

b. *p*-Toluenesulfonic Acid. A mixture of **14** (50 mg) and *p*-toluenesulfonic acid (20 mg) in benzene (10 ml) was refluxed for 25 min. The reaction mixture was diluted with water and extracted with chloroform. The chloroform layer was washed with saturated sodium bicarbonate solution and water, dried over anhydrous sodium sulfate, filtered, and concentrated to a syrup. This material was purified by preparative thick layer (1 mm) chromatography (Brinkmann silica gel HF; methanol–chloroform (2:98); bands detected by ultraviolet lamp). Extraction of band II with chloroform gave 40 mg of a homogeneous material which was crystallized from ether–Skellysolve B to give 24 mg of the dienone **15**, mp 146–147°; $[\alpha]^{29}D$ + 37° (*c* 0.683, $CHCl_3$); λ_{max}^{EtOH} 255 mμ (ε 16,600); $\lambda_{max}^{CHCl_3}$ 5.63 (s), 5.95 (s), 6.11 (m), 6.18 (s) μ.

Anal. Calcd for $C_{15}H_{18}O_3$: C, 73.14; H, 7.37. Found: C, 72.97; H, 7.46.

13-Methoxydesacetylgaillardin (5). A solution of gaillardin (**6**, 55 mg) in 20% aqueous methanol (5 ml) containing potassium carbonate (100 mg) was refluxed 1 hr and allowed to stand at room temperature overnight. The solution was concentrated, diluted with ice–water, acidified to pH 1 with dilute hydrochloric acid, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate, filtered, and evaporated to give 48 mg of syrup containing a mixture of **5** and **8**. Chromatography on Merck acid-washed alumina (5 g) with benzene–chloroform (25:75) gave 22 mg of **5**. Recrystallization from methylene chloride–Skellysolve B gave 10 mg of **5**, mp 151–152°; $\lambda_{max}^{CHCl_3}$ 2.90 (OH, strong), 5.65 μ (lactone carbonyl); mass spectrum *m/e* 296.

Anal. Calcd for $C_{16}H_{24}O_5$: C, 64.83; H, 8.16. Found: C, 65.04; H, 8.27.

Desacetylgaillardin (8). A solution of gaillardin (**6**, 97 mg) in 40% aqueous dioxane (15 ml) containing potassium hydroxide (150 mg) was allowed to stand for 1 day. This solution was concentrated, diluted with water, acidified, and extracted with methylene chloride. The methylene chloride layer was washed with saturated sodium bicarbonate solution and water, dried over anhydrous sodium sulfate, filtered, and evaporated to give 76 mg of syrup. Crystallization from benzene–Skellysolve B gave 20 mg of **8**, mp 148–150°. Chromatography of the mother liquor from crystallization on Merck acid-washed alumina (9 g) gave, on elution with benzene–chloroform (50:50), 35 mg of a syrup containing **8**. Crystallization from benzene–Skellysolve B gave an additional 22 mg of **8**, mp 149–151°; $[\alpha]^{29}D$ −38° (*c* 0.807, $CHCl_3$); λ_{max}^{EtOH} end absorption at 210 mμ (ε 14,600); $\lambda_{max}^{CHCl_3}$ 2.90 (OH strong), 5.66 μ (lactone carbonyl).

Anal. Calcd for $C_{15}H_{20}O_4$: C, 68.16; H, 7.63. Found: C, 68.12; H, 7.37.

Tetrahydrogaillardin (10). A solution of gaillardin (**6**, 87 mg) in ethyl acetate (20 ml) was hydrogenated in the presence of 10% palladium on charcoal (80 mg) at atmospheric pressure and room temperature. Uptake of hydrogen stopped after approximately 0.5 hr, at which time 2 mole equiv of hydrogen had been absorbed. The suspension was filtered and concentrated to give a syrup (85 mg). Chromatography on Merck acid-washed alumina (10 g) with benzene–chloroform (75:25) gave **10** (55 mg) which could not be crystallized.

Treatment of Gaillardin with Acetic Anhydride–Pyridine. A solution of gaillardin (**6**, 50 mg) in acetic anhydride (2 ml) and dry pyridine (4 ml) was allowed to stand at room temperature for 24 hr. Standard work-up led to recovery of starting material (40 mg).

Action of α-Chymotrypsin on the Diethyl Esters of Fumaric, Maleic, and Acetylenedicarboxylic Acids[1]

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Abstract: Diethyl maleate is not hydrolyzed, while diethyl fumarate is hydrolyzed by α-chymotrypsin, $k_{cat} = 0.28$ sec^{-1}, $K_{m,app} = 0.023$ *M*. The fumarate shows reactivity a little greater than that of diethyl succinate, and leads to monoethyl fumarate in high yield. Diethyl acetylenedicarboxylate is hydrolyzed by α-chymotrypsin with first-order kinetics, the enzyme becoming inactivated. It is concluded that common substrates for the enzyme, α,β-disubstituted propionates, hydrolyze in a conformation in which the β substituent and the hydrolyzing ester or amide group are transoid. The distance between the entrances to the aryl, ar, and nucleophilic, n, sites of the enzyme is ~3.8 A. The results indicate that the cyclic substrate D-1-keto-3-carbomethoxytetrahydroisoquinoline is hydrolyzed by the enzyme with the carbomethoxyl group in the equatorial conformation.

Small molecule substrates for α-chymotrypsin of varied structure have been studied and information has been obtained about the effects of substituents on the rates and stereospecificity of their enzymic hydrolyses.[2,3] A major objective has been to draw inferences about the size and geometry of the active area of the enzyme, about the interactions between

(1) We are pleased to acknowledge generous support of this work by the Division of Research Grants, National Institutes of Health, GM-04584. This constitutes article XI on the specificity of α-chymotrypsin; for the previous article in this series see S. G. Cohen and S. Y. Weinstein, *J. Am. Chem. Soc.*, **86**, 5326 (1964).

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sites of the active area and substituents of the substrate molecules, and about the effects of these interactions upon binding and reactivity. Reactive substrates for α-chymotrypsin, which are related to the point of attack by this enzyme on proteins,[4] are esters of L-N-acyl-β-phenylalanine, $C_6H_5CH_2CH(NHCOR)CO_2R'$. Compounds useful in these studies have been esters of α,β-disubstituted propionic acids, XCH_2CHYCO_2R, in which the α substituent Y has been acylamido,[2] aroylamido,[5] acetoxyl,[3,6] aroyloxyl,[7] hydroxyl,[8] and carbethoxymethylene,[9] and the β substituent X has been aryl,[4] alkyl,[10] carbethoxyl,[11] acyl,[3,6] and aroyl.[7] Implicit in such studies is the idea that the active area of the enzyme has several definitely articulated parts or sites, including the nucleophilic or hydrolyzing groups, a site at which the β substituent of the substrate normally associates, and another at which the α substituent interacts. Other parts are present, at which the other substituents, in these cases the three α,β,β-hydrogen atoms, find themselves, but these need not be considered for present purposes. The carbethoxyl group has been a useful β substituent and diethyl succinate[12] and its α-acetamido,[11] α-acetoxy,[13] and α-hydroxy[13] derivatives have been studied. The N-acetylaspartate is a reactive substrate for α-chymotrypsin.[11] However, the carbethoxy and acyloxy groups may act both as α and β substituents, interacting at the two corresponding enzymic sites and leading to several modes of binding each of which results in hydrolysis.[9,13]

In interpretation of such experiments the parts of the active area are thought to remain relatively firmly fixed with respect to each other, and the small molecule substrates are, conceptually, placed in the enzyme in various ways. This has led to consistent interpretation of the stereochemistry of the hydrolysis of symmetric molecules, the α-substituted malonate esters,[14] and the β-substituted glutarate esters;[9] of inversions of stereospecificity in hydrolysis of α-substituted propionates;[3,6] and of predominant absence of stereospecificity in the hydrolysis of the α-hydroxy and α-acetoxy derivatives of diethyl succinate.[13] These procedures, which now have predictive value, do not require a specific conformation of the small molecule substrates. Inferences which might be drawn about the reacting conformation would be useful both in giving information about the geometrical disposition of the sites of the active area of the enzyme and in facilitating interpretation of the reactions of new substrates. For this purpose we have studied the action of α-chymotrypsin on diethyl fumarate and diethyl maleate which, while related structurally to the α,β-disubstituted propionates, lack free rotation about the C_α–C_β bond and to this extent have diminished freedom of conformation as compared with the succinate and β-phenylpropionate derivatives. We will also describe some experiments with diethyl acetylenedicarboxylate.

The relatively rigid substrate which had been studied previously in Professor Niemann's laboratory,[15] 1-keto-3-carbomethoxytetrahydroisoquinoline, is a condensed cyclized analog of methyl N-benzoyl-β-phenylalaninate, in which the one aromatic ring acts as both the β substituent and as the N-aroyl group of the α substituent. This compound is most unusual, being highly reactive in the D configuration, a matter which we will discuss in a later article. It has been proposed that this molecule reacts enzymically with the carbomethoxyl group in the axial conformation.[2,16] The results of the present work may have some bearing on this.

Results

The kinetics of hydrolysis of diethyl fumarate by 0.5 mg of α-chymotrypsin/ml were followed in a pH-Stat at pH 7.2 in 0.075 *M* sodium chloride at 25° under nitrogen. The rate of nonenzymic hydrolysis was about 1% as great as that of the enzymic reaction. The concentrations of diethyl fumarate and the initial zero-order rates were as follows: 2.04×10^{-3} *M*, 4.68×10^{-7} *M* sec^{-1}; 3.08×10^{-3} *M*, 5.58×10^{-7} *M* sec^{-1}; 3.52×10^{-3} *M*, 7.44×10^{-7} *M* sec^{-1}; 3.63×10^{-3} *M*, 7.71×10^{-7} *M* sec^{-1}; 5.05×10^{-3} *M*, 10.5×10^{-7} *M* sec^{-1}; 6.15×10^{-3} *M*, 10.7×10^{-7} *M* sec^{-1}; 6.83×10^{-3} *M*, 14.1×10^{-7} *M* sec^{-1}. A double reciprocal plot of the data was linear.[17] The kinetic parameters were calculated by least-squares treatment of the data, $k_{cat} = 0.28$ sec^{-1}, $K_{m,app} = 0.023$ *M*, $k_{cat}/K_{m,app} = 12$ M^{-1} sec^{-1}.

In similar kinetic experiments the maleate at first also appeared to be hydrolyzed by α-chymotrypsin, but far less readily, $k_{cat}/K_{m,app} = 0.1$ M^{-1} sec^{-1}. However, this hydrolysis proved to be due to the presence of fumarate impurity, and in fact the maleate was not measurably hydrolyzed by α-chymotrypsin. In kinetic studies, zero-order rates of hydrolysis are determined over the initial few per cent of reaction, and under these conditions the maleate appeared to be reacting.

In a preparative run, hydrolysis of 1.09 g (6.3 mmoles) of diethyl fumarate at pH 7.2 by 0.119 g of α-chymotrypsin was complete for one ester group in 8 hr. Ethyl hydrogen fumarate was recovered in 77% yield, and was identical with an authentic sample. However, hydrolysis of 1.13 g (6.6 mmoles) of diethyl maleate by 0.120 g of α-chymotrypsin was only 12% complete after 10 hr, and the reaction had essentially stopped. Addition of more α-chymotrypsin led to no further reaction. The product of this small extent of hydrolysis was ethyl hydrogen fumarate, and not the maleate half ester. The recovered unhydrolyzed ester was pure diethyl maleate. Comparison of the infrared and nmr spectra of samples of diethyl maleate before and after treatment with the enzyme indicated that the commercially available, redistilled, maleate ester was contaminated with about 10% of fumarate. Furthermore, the recovered diethyl maleate appeared un-

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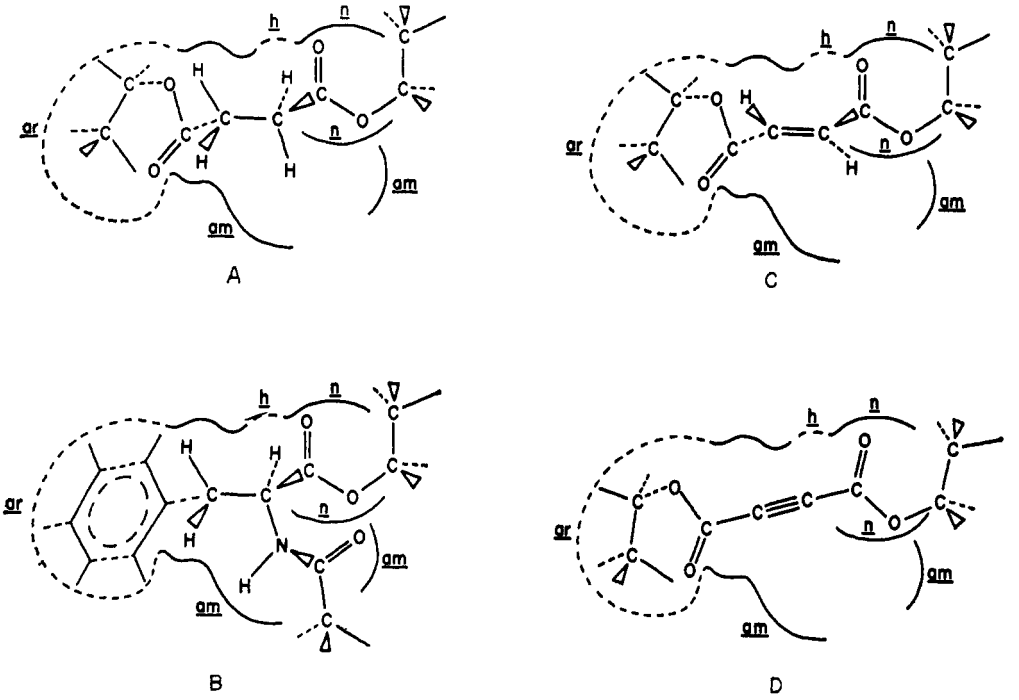

Figure 1. Association of ester substrates: A, diethyl succinate; B, ethyl L-N-acetyl-β-phenylalaninate; C, diethyl fumarate; D, diethyl acetylenedicarboxylate.

affected by α-chymotrypsin, hydrolyzing essentially no more rapidly in its presence than in its absence. Addition of diethyl fumarate to this system led to hydrolysis at a rate normal for the fumarate, the maleate apparently not binding well.

The linear diester, diethyl acetylenedicarboxylate, was also hydrolyzed by α-chymotrypsin. The kinetics of hydrolysis by 0.1 mg of enzyme/ml was followed at pH 7.2 in 0.10 *M* sodium chloride at 25° under nitrogen. The nonenzymic hydrolysis was substantial, 17% as great as the enzymic reaction. The latter was fairly rapid, but was first order in substrate over the range of concentration which could be studied in the medium. The concentrations of the diester and the corrected initial zero-order rates were as follows: $1.91 \times 10^{-3} M$, 3.0×10^{-7} *M* sec^{-1}; 2.32×10^{-3} *M*, 3.8×10^{-7} *M* sec^{-1}; 2.90×10^{-3} *M*, 5.1×10^{-7} *M* sec^{-1}; 3.82×10^{-3} *M*, 5.8×10^{-7} *M* sec^{-1}; 4.24×10^{-3} *M*, 6.6×10^{-7} *M* sec^{-1}; 5.24×10^{-3} *M*, 8.33×10^{-7} *M* sec^{-1}. The double reciprocal plot was linear and passed through the origin, and from the slope the second-order rate constant, $k_{cat}/K_{m,app}$, was obtained, 42 M^{-1} sec^{-1}. In a preparative run, treatment of 0.49 g (2.9 mmoles) of the diester with 0.103 g of α-chymotrypsin led to 24% hydrolysis of one ester group after 1.6 hr, the reaction slowing down markedly thereafter. The infrared spectrum indicated the product of hydrolysis to be monoethyl acetylenedicarboxylate. The acetylenic ester undergoes addition of nucleophiles to the unsaturation,[18] and such a reaction of the starting diester or the product monoester with the enzyme may have led to inhibition.

Discussion

The *cis* compound, diethyl maleate, was inert. The *trans* compound, diethyl fumarate, was hydrolyzed by α-chymotrypsin somewhat more effectively than the aliphatic analog diethyl succinate, which was comparable in reactivity to ethyl β-phenylpropionate. The fumarate showed a larger kinetic constant than the succinate, 0.28 sec^{-1}, as compared with 0.065 sec^{-1}, a less favorable binding constant, 0.023 *M*, as compared with 0.0087 *M*, and a slightly more favorable ratio of these constants, 12, as compared to 8 M^{-1} sec^{-1}. The larger kinetic constant may reflect the higher reactivity of the fumarate toward the nucleophilic reactant group of the enzyme, by analogy with its base-catalyzed hydrolysis which is more rapid than that of the succinate.[19] The less favorable binding constant may be reasonable in terms of the aliphatic C_α–C_β structure of natural substrates for this enzyme. In the hydrolysis of the acetylenic ester, the first-order kinetics indicate a still less favorable binding constant, and the larger value of the second-order constant, $k_{cat}/K_{m,app} = 42$ M^{-1} sec^{-1}, indicates a larger value for k_{cat}. These results may be accounted for similarly in terms of higher intrinsic reactivity[20] and still less favorable fit.

The second carbethoxyl group in the diester substrates corresponds to the aryl group in derivatives of β-phenylpropionate and associates with the enzyme at the aryl site, ar, of the active area.[11] The inference may be drawn that the succinate associates with the enzyme, like the fumarate, with its two carbethoxyl groups in a transoid conformation, and that the association of ethyl N-acetyl-L-β-phenylalaninate and other natural substrates with α-chymotrypsin also involves a conformation in which the β-aryl group and the hydrolyzing ester or amide group are transoid. This conformation is similar to that proposed by Hein and Niemann.[2] The aryl, ar, and nucleophilic, n, sites of the enzyme are complementary to these two functional groups (Figure 1).

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In these conformations the distance between the two carboxyl carbons in diethyl succinate and between the proximate phenyl carbon and the carboxyl carbon in the β-phenylalaninate is 3.84 A, and this presumably is the distance between the corresponding parts of the ar and n sites in the enzyme. The distance between the carboxyl carbons in diethyl fumarate is quite similar, 3.79 A; that in the acetylenedicarboxylate is slightly larger, 4.04 A. These unsaturated molecules span or fit between the ar and n sites with little difficulty, while they interact differently, presumably less well, with the intervening structural features of the enzyme. The ar and n sites of the enzyme are fixed rather rigidly and can accommodate the β substituent and the hydrolyzing group on the α-carbon when they are transoid. Small-molecule substrates may fit at these sites in various ways and in various conformations to allow hydrolysis of L or D enantiomorphs.[7,13] When rigid substrates are used, a β substituent *cis* to the hydrolyzing group does not associate at the ar site, and the enzyme may not adjust or become distorted to accept a *cis* β substituent.

In the cyclic substrate, D-1-keto-3-carbomethoxytetrahydroisoquinoline,[15] when the carbethoxyl group is axial the carboxyl carbon is cisoid to the phenyl carbon and only 3.17 A distant from it. This situation resembles that of the inactive maleate ester, in which the corresponding distance is 2.82 A. When the carbethoxyl is equatorial, the carboxyl carbon and phenyl carbon are transoid and ~3.8 A apart. The analogy with the fumarate indicates that the equatorial conformation may be the reactive one.

Experimental Section

Hydrolysis of Diethyl Fumarate by α-Chymotrypsin. Diethyl fumarate (Eastman Organic Chemicals; bp 114° (13 mm); 1.085 g, 6.30 mmoles) was suspended in 15 ml of water at pH 7.2 in a pH-Stat. The mixture consumed 1 *N* NaOH at a rate of 7×10^{-4} mmole/min. α-Chymotrypsin (Worthington Biochemical Corp., 0.119 g) was added and alkali was consumed at an initial rate of 0.018 mmole/min. After 8 hr consumption of alkali was at the rate of 6×10^{-4} mmole/min, 6.35 mmoles having been consumed. The solution was brought to pH 2.2 with hydrochloric acid and extracted with ether. The extract was dried and concentrated, leading to ethyl hydrogen fumarate; 0.70 g (4.9 mmoles), 77%; mp and mmp, with an authentic sample, 70°; infrared spectrum identical with that of a synthesized sample; neut equiv 143.8, calcd 144.1; $pK_a = 3.40$ (lit.[21] 3.40). A sample was synthesized by treatment of 32.7 g (0.34 mole) of maleic anhydride with 17 g (0.37 mole) of absolute ethanol under reflux for 14 hr. The viscous solution was filtered from precipitated fumaric acid, and the filtrate was dissolved in chloroform and extracted with sodium bicarbonate. The extract was acidified to pH 2.5 and filtered, and the filtrate was extracted with ether. The ether solution was dried and concentrated, leading to ethyl hydrogen fumarate: 6.0 g (0.042 mole), 12% yield; mp 69.5° (lit.[21] 69°).

Treatment of Diethyl Maleate with α-Chymotrypsin. Diethyl maleate (Eastman Organic Chemicals; bp 102° (10 mm); $n^{20}D$ 1.4396; 1.130 g, 6.56 mmoles) in 15 ml of water was treated with 0.120 g of α-chymotrypsin. The solution consumed 1.0 *N* alkali initially at a rate of 3.5×10^{-3} mmole/min, and after 80 min, 2.5×10^{-3} mmole/min, 4% reaction. After 10 hr the rate was 5×10^{-5} mmole/min and 0.78 mmole of alkali had been consumed, 12% reaction. The neutral solution was extracted with ether, leading to 0.980 g of diethyl maleate, 86% recovery; its infrared spectrum was identical with that of the starting material except for the absence of a shoulder at 12.9 μ, where diethyl fumarate has a strong band. The nmr spectrum (tetramethylsilane external standard) of the starting material showed 90% of the vinyl hydrogens at τ 4.05, corresponding to the *cis* diester, and 10% at τ 3.57, corresponding to this extent of content of the *trans* diester.

The extracted aqueous solution was brought to pH 2.5 with sulfuric acid and extracted with ether. The extract was dried and concentrated, leading to ethyl hydrogen fumarate, 0.056 g (0.42 mmole), 6.4% yield, mp 68–69°, not depressed on mixture with an authentic sample, infrared spectrum identical with that of an authentic sample.

The recovered diethyl maleate, 0.622 g, in 15 ml of water was treated with 0.121 g of α-chymotrypsin, consuming alkali at a rate of 1×10^{-4} mmole/min. Another sample, 0.092 g, in 20 ml of 0.1 *M* NaCl, consumed 0.1 *N* NaOH at a rate of 3×10^{-5} mmole/min alone and in the presence of 0.0135 g of α-chymotrypsin. When diethyl fumarate, 0.163 g (0.94 mmole), was added to this solution alkali was consumed at a rate of 4×10^{-3} mmole/min.

Diethyl acetylenedicarboxylate was prepared[22] by treatment of acetylenedicarboxylic acid (Aldrich) with ethanol and sulfuric acid: 66% yield; bp 46° (0.2 mm) (lit.[22] 60–62° (0.3 mm)). A suspension of 0.417 g (2.450 mmoles) of the diester in 20 ml of water, maintained at pH 10.7, consumed 4.89 mmoles of NaOH in 1.5 hr, the reaction then stopping. The nmr spectrum of the diester showed only ethyl protons. The infrared spectrum showed bands at 2.93 (w), 3.35 (m), 3.40 (w), 3.45 (w), 3.47 (w), 5.92 (s), 6.8 (m), 6.9 (m), 7.3 (m), 7.4 (s), 8.0 (s), 8.6 (w), 9.0 (w), 9.2 (m), 9.7 (s), 10.3 (w), 11.7 (m), 12.6 (w), 13.4 (m), and 14.8 (m) μ.

Treatment of Diethyl Acetylenedicarboxylate with α-Chymotrypsin. A suspension of 0.49 g (2.9 mmoles) of the diester in 20 ml of water containing 0.103 g of α-chymotrypsin was allowed to react in a pH-Stat at pH 7.2. The initial rate of consumption of 1 *N* NaOH, 0.0257 ml/min, fell to 0.0027 ml/min after 95 min, 0.696 ml being consumed, corresponding to 24% hydrolysis of the ester group. The suspension was extracted with ether, leading to unreacted starting diester, 0.22 g, 45% recovery; infrared spectrum identical with that of the starting material. The aqueous layer was dialyzed against 80 ml of water and lyophilized. The residue showed absorption bands (KBr pellet) at 2.95 (broad), 3.35 (m), 3.4 (m), 4.55 (w), 5.92 (s), 6.2 (s), 6.9 (w), 7.4 (s), 8.0 (s), 9.0 (m), 9.7 (m), 10.2 (m), 10.8 (w), 11.6 (m), 12.8 (m), 13.4 (m), 14.9 (m), and 19.5 (m) μ.

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