Steric Hindrance in α-Chymotrypsin-catalyzed Reactions*

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The α-chymotrypsin-catalyzed hydrolysis of acetyl-DL-*tert*-leucine methyl ester and of acetyl-DL-β,β-dimethylphenylalanine methyl ester, in aqueous solutions at 25.0° and *p*H 7.90, could not be demonstrated. The rate of hydrolysis of α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester was estimated to be *ca.* 10^{-3} that of α-*N*-acetyl-L-tyrosine methyl ester. These results, and those obtained previously, demonstrate the presence of steric hindrance in α-chymotrypsin-catalyzed reactions and establish a point of similarity with comparable acid- or base-catalyzed reactions. With aliphatic α-amino acid derivatives steric hindrance arises when the α-hydrogen atom is replaced by a bulkier group or when β-branching occurs in the side chain. With aromatic α-amino acid derivatives an added steric factor is encountered in β-branching of the side chain or when the β-aryl group is substituted in the *o*-positions.

Previous studies conducted in these laboratories (Almond *et al.*, 1962; Waite and Niemann, 1962; Jones and Niemann, 1962) have shown that steric hindrance arising from the structure of the substrate molecule can be an important factor in determining the rate of its α-chymotrypsin-catalyzed hydrolysis. Thus, with substrates of the type $R_1'CONHCHR_2CO_2R_3'$, e.g., acetyl-L-phenylalanine methyl ester, where the

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]) \quad (1)$$

rate of hydrolysis is described by equation (1), replacement of the α-hydrogen atom by a methyl group leads to *ca.* a 10^5-fold decrease in the magnitude of the rate constant k_0. Since the change in the substration constant K_0 is less than an order of magnitude, the above effect has been explained on the basis of steric interference by the α-alkyl group with attack of the potentially hydrolyzable carboalkoxy group by electro- or nucleophilic groups present at the active site of the enzyme (Almond *et al.*, 1962).

Another steric effect has its origin in the structure of the component R_2. If one compares the behavior of acetyl-L-valine methyl ester, K_0 = 112 ± 12 mM, k_0 = 0.151 ± 0.007 sec^{-1} (Waite and Niemann, 1962), and acetyl-L-norvaline methyl ester, K_0 = 10.2 ± 0.9 mM, k_0 = 2.70 ± 0.17 sec^{-1} (Jones and Niemann, 1962), it is at once apparent that β-branching in the former substrate is associated with *ca.* an 11-fold increase in the magnitude of K_0 and *ca.* an 18-fold decrease in that of k_0.

It is known that aliphatic groups β to the carboxyl group decrease the rates of the acid-catalyzed esterification of carboxylic acids (Smith, 1939; Smith and Burn, 1944) and the acid- (Smith and Steele, 1941; Smith and Meyers, 1942) and base- (Levenson and Smith, 1939, 1940; Evans *et al.*, 1938) catalyzed hydrolysis of esters and amides (Cason and Wolfhagen, 1949). Detailed analysis (Taft, 1956) of these data indicates that the above are steric rather than electronic effects. Since such steric effects observed for a single β-substituent are greater than those for a single α-substituent and since γ-substituents exhibit noticeable steric effects only in the case of α,γ or β,γ-disubstitution, the above effects have been taken as evidence for a coiled structure for carboxyl derivatives in solution (Smith and McReynolds, 1939). Esterifications and hydrolyses, with the exception of those in concentrated sulfuric acid, have been shown to proceed through a tetrahedral transition state (Bender, 1951), probably by approach of reactant in a direction perpendicular to the plane of the carbonyl group. Atoms situated five bonds from the carbonyl oxygen atom could present large steric repulsions toward approach of reactants and the formation of tetrahedral intermediates, thus causing large decreases in rates of reactions that proceed by such a mechanism. This effect has been qualitatively formulated in Newman's "rule of six" (Newman, 1950).

Consideration of the variation in the magnitude of the kinetic constants associated with the α-chymotrypsin-catalyzed hydrolysis of substrates with increasing β-substitution could yield important information about the mechanism of enzyme action. If increasing β-substitution in a particular series of acylated α-amino acid esters causes a decrease in k_0 the α-amino acid side chain might be coiled in such a way as to lead to significant interaction between atoms in the six position and the carbonyl oxygen atom. Insensitivity of k_0 to β-substitution would suggest that the side chain in the enzyme-substrate complex is uncoiled or that the enzyme-substrate complex decomposition transition state does not resemble the transition states for the acid- or base-catalyzed reactions.

Interpretation of the effect of β-substitution on the magnitude of K_0 is inherently ambiguous because of uncertainty of the exact nature of this constant. If K_0 approximates a true equilibrium constant then K_0 will be independent of the energy barrier for formation and decomposition of the transition states. Any observed variation in K_0 with degree of β-substitution would be due to specific interactions between substrate and active site. If K_0 is not an equilibrium constant, the effect on the two transition states, for decomposition to reactants as well as to products, must be considered. Interpretation must then be based on a number of assumptions concerning the nature of the complex as well as its transition states of decomposition and conclusions based upon these assumptions would necessarily be questionable. The magnitude of K_0 can still be increased by internal steric hindrance due to repulsions between groups in the substrate, preventing proper orientation of the substrate at the active site, even if K_0 is not an equilibrium constant.

The kinetic constants obtained for acetyl-L-valine and acetyl-L-norvaline methyl ester, referred to above, suggest that both binding of substrate and subsquent attack of the carboalkoxy group are subject to steric hindrance arising from β-substitution. In order to

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obtain more information along these lines it was decided to examine the behavior of analogous derivatives of *tert*-leucine(α-amino-β,β-dimethyl-*n*-butyric acid), β,β-dimethylphenylalanine, and 2,6-dimethyltyrosine.

Experimental

All melting points are corrected and all analyses by Dr. A. Elek.

tert-Leucine(α-Amino-β,β-dimethyl-n-butyric Acid)

Pinacolone.—Rearrangement of pinacol hydrate as described by Hill and Flosdorf (1941) gave pinacolone, bp 100–102°, in 85% yield.

Trimethylpyruvic Acid.—To a solution of 63 g of potassium permanganate and 20 g of sodium hydroxide in 2 liters of water was added 14.5 g of pinacolone, the mixture was shaken in a tightly stoppered thick-walled bottle for 2 hours, a small amount of sodium sulfite was added to reduce any residual oxidant, and the mixture was filtered through a Celite pad. The residue was washed with warm dilute aqueous sodium hydroxide and the filtrate and washings were evaporated to dryness. The crystalline residue was dissolved in 300 ml of water and acidified with concentrated hydrochloric acid, and the mixture was extracted with five 50-ml portions of ethyl ether. The ethereal extracts were combined and dried over anhydrous sodium sulfate, and the solvent was evaporated to give a pale yellow liquid. This liquid was fractionally distilled to give 14.9 g (57%) of trimethylpyruvic acid, bp 51–52°/4 mm. Kjaer (1953) gives bp 73.5–75.0°/10 mm.

α-Oximino-β,β-dimethyl-n-butyric Acid.—To a solution of 13.9 g of anhydrous potassium carbonate in 40 ml of water was added 14.7 g of trimethylpyruvic acid followed by 11.7 g of hydroxylamine hydrochloride. The reaction mixture was allowed to stand at room temperature for 30 hours, after which it was acidified with concentrated hydrochloric acid and the crystalline precipitate was collected and dissolved in ethyl ether. The aqueous filtrate was extracted with five 50-ml portions of ethyl ether, the ethereal extracts and solution were combined and dried over anhydrous sodium sulfate, and the ether was removed by evaporation to dryness *in vacuo* at room temperature or below. The residue was dissolved in benzene, the solution was reduced in volume by evaporation *in vacuo* at room temperature or below, and the colorless crystals were collected, washed with hexane, and dried *in vacuo* to give 12.9 g (77%) of product, mp 81–85°. Analysis indicated that the product was a 2:1 mixture of the anhydrous and monohydrated forms of α-oximino-β,β-dimethyl-*n*-butyric acid.

Anal. Calcd. for $C_6H_{11}O_3N$ (145): C, 49.6; H, 7.7; N, 9.7. Calcd. for $C_6H_{13}O_4N$ (163): C, 44.2; H, 8.0; N, 8.6. Found: C, 47.7; H, 8.0; N, 9.3.

Richard (1910) reported that the monohydrate melts at 85° and Kjaer (1953) gave the melting point of the anhydrous form as 116–117°. Recrystallization of our product from a mixture of benzene and hexane gave the anhydrous material, long, colorless needles, mp 115–117°. If, as in other experiments, the ethereal solution of the oximino acid was freed of solvent by evaporation on a steam bath, a noncrystallizable oil, exhibiting an intense nitrile absorption in the infrared region, was obtained.

DL-*tert-Leucine.*—A mixture of 5.0 g of α-oximino-β,β-dimethyl-*n*-butyric acid and 5.0 g of zinc dust was refluxed in 250 ml of 50% acetic acid for 40 hours, after which time all the zinc dust had dissolved. The solution was cooled and saturated with hydrogen sulfide, the colorless precipitate was removed, and the filtrate was evaporated to dryness. The colorless solid was dissolved in 150 ml of warm water, the insoluble residue was removed, the filtrate, which gave a positive ninhydrin test, was evaporated to 20 ml, and 150 ml of acetone was added to the warm solution. A colorless precipitate formed. The mixture was warmed to *ca.* 50° and water was added dropwise until the precipitate had dissolved. The solution was then cooled, the product was allowed to crystallize, and the crystals were collected and dried to give 1.92 g of crude product. The mother liquor was evaporated to dryness and the residue recrystallized from aqueous-acetone to give an additional 0.60 g of crude amino acid. The two fractions were combined and recrystallized from aqueous-acetone to give DL-*tert*-leucine, large, irregular, hexagonal platelets, mp 275–280°, with decomp.

Anal. Calcd. for $C_6H_{13}O_2N$ (131): C, 54.9; H, 10.0; N, 10.7. Found: C, 55.3; H, 10.0; N, 10.7.

Acetyl-DL-tert-leucine.—To a cold solution of 0.50 g of DL-*tert*-leucine in 5 ml of 2 N aqueous sodium hydroxide was added 0.54 g of acetic anhydride, and the mixture was vigorously shaken for 2 minutes and then carefully acidified by the dropwise addition of concentrated hydrochloric acid. The precipitate that formed was collected and recrystallized from water to give 0.33 g (53%) of acetyl-DL-*tert*-leucine, mp 227–230°, with decomp.

Anal. Calcd. for $C_8H_{15}O_3N$ (173): C, 55.5; H, 8.7; N, 8.1. Found: C, 55.3; H, 8.6; N, 8.1.

Acetyl-DL-tert-leucine Methyl Ester.—Acetyl-DL-*tert*-leucine, 0.75 g, was dissolved in an ice-cold solution of 0.83 g of thionyl chloride in 4.0 ml of anhydrous methanol. The mixture was allowed to warm to room temperature and to stand overnight. The solution was then evaporated to a clear oil which soon crystallized. The crystalline residue was recrystallized from water to give 0.49 g (60%) of acetyl-DL-*tert*-leucine methyl ester, thick prisms, mp 110–111°.

Anal. Calcd. for $C_9H_{17}O_3N$ (187): C, 57.7; H, 9.2; N, 7.5. Found: C, 57.3; H, 9.0; N, 7.4.

β,β-Dimethylphenylalanine

4,6-Di-(α,α-dimethylbenzyl)pyrogallol.—Pyrogallol (38.4 g) was dissolved in 100 ml of glacial acetic acid containing 3 ml of concentrated sulfuric acid. This solution was added, with stirring, to a cold solution of 100 g of α,α-dimethylbenzyl alcohol (Grignard, 1901) in 100 ml of glacial acetic acid. The orange solution was allowed to stand overnight and was then poured into 700 ml of water. The precipitate was collected, dried, and recrystallized from hexane to give 90 g (83%) of 4,6-di-(α,α-dimethylbenzyl)pyrogallol, mp 121–123°. Jönsson (1954) reports mp 120–121°. The use of α-methylstyrene (Jönsson, 1954), instead of α,α-dimethylbenzyl alcohol, in the same molecular proportions, gave essentially the same results.

Air Oxidation of 4,6-Di-(α,α-dimethylbenzyl)pyrogallol.—4,6-Di-(α,α-dimethylbenzyl)pyrogallol (30 g) was dissolved in 900 ml of methanol containing 45 ml of 7 N aqueous sodium hydroxide. The solution rapidly turned a deep violet. Oxygen was bubbled through the solution for 2 hours, the solution turning cherry red and then yellow. It was then evaporated to *ca.* 300 ml, 750 ml of water was added, and the solution was acidified with concentrated hydrochloric acid and extracted with three 100-ml portions of ethyl ether. The combined ethereal extracts were in turn extracted with aqueous sodium bicarbonate and then with 5% aqueous sodium hydroxide. The bicarbonate extract was acidified and allowed to stand for several hours. The yellow crystalline precipitate which formed was

collected and dried. This material was washed with boiling hexane, leaving undissolved 15 g of 3,5-di-(α,α-dimethylbenzyl)coumalic acid, mp 147–148°, An additional 1.0 g of the acid crystallized from the hexane filtrate, giving a total yield of 52%. Recrystallization from boiling hexane gave the acid, mp 149.0–149.5°.

Anal. Calcd. for $C_{24}H_{24}O_4$ (376): C, 76.6; H, 6.4; Found: C, 76.6; H, 6.5. Molecular weight (Rast), 382. Neutralization equivalent 358; saponification equivalent 167; $pK_A' = 4.6$.

3,5-Di-(α,α-dimethylbenzyl)coumalic acid in methanol exhibits in addition to benzenoid absorption a strong band at λ_{max} 3030 A, ϵ_{max} 8,650. In 5% aqueous sodium hydroxide the band appears at 3050 A and disappears over a 12-hour period at room temperature. The acid in chloroform has a broad strong carbonyl absorption at 1725 cm^{-1} which may include a shoulder at *ca.* 1700 cm^{-1}. The compound gives neither a precipitate with 2,4-dinitrophenylhydrazine nor a color with ferric chloride. As will be shown later it is oxidized by alkaline permanganate to α-keto-β-phenylisovaleric acid.

If the time of oxidation was extended to 5 hours the lactone of 2,4-di-(α,α dimethylbenzyl)-4-hydroxycrotonic acid, mp 134–135° after recrystallization from aqueous acetone, precipitated from the reaction mixture.

Anal. Calcd. for $C_{23}H_{26}O_2$ (334): C, 82.6; H, 7.8; $C_{22}H_{24}O_2$ (320): C 82.5; H, 7.6. Found: C, 82.6; H, 7.7.

2,4-Di-(α,α-dimethylbenzyl)-4-hydroxycrotonic acid in chloroform exhibited an intense carbonyl absorption at 1755 cm^{-1}. However, no OH stretching absorption was discernible. The ultraviolet absorption spectrum of a methanol solution exhibited only phenyl group absorption. The compound was insoluble in water, aqueous sodium bicarbonate, 5% aqueous sodium hydroxide, and 85% phosphoric acid. It was soluble in ethyl ether and concentrated sulfuric acid. It gave no precipitate with 2,4-dinitrophenylhydrazine and no color with ferric chloride, and did not decolorize solutions of bromine in carbon tetrachloride or aqueous potassium permanganate. When it was refluxed in a mixture of 0.2 ml of 6 M sodium hydroxide and 1 ml of 0.5 N hydroxylamine hydrochloride in 95% ethanol for 5 hours and the mixture was acidified, the solution gave no color with ferric chloride. However, the infrared spectrum of the product showed that the carbonyl absorption was shifted to 1705 cm^{-1}, and an alcoholic stretch had appeared at 3500 cm^{-1}. Partition of this product between aqueous bicarbonate and ether did not change the infrared spectrum of either fraction.

Infrared analyses of the neutral fractions obtained from shorter oxidation periods showed that the lactone of 2,4-di-(α,α-dimethylbenzyl)-4-hydroxycrotonic acid was a major component of these fractions.

Several air oxidations were inadvertently conducted in methanol contaminated with lead salts. A description of such an experiment follows.

4,6-Di-(α,α-dimethylbenzyl)pyrogallol (50 g) was dissolved in 1.5 liter of contaminated methanol containing 75 ml of 7 N aqueous sodium hydroxide. Oxygen was bubbled through the solution for 4 hours, after which time the solution was still a deep cherry red and a pink precipitate had formed. The reaction mixture was allowed to stand overnight, the precipitate, consisting largely of lead salts, was removed, and the filtrate was evaporated to a yellow-green solid. This residue was dissolved in 500 ml of water and the solution was acidified with concentrated hydrochloric acid. The orange oil that separated was taken up in ethyl ether and the ethereal solution was extracted with aqueous sodium bicarbonate. The green aqueous phase was acidified to precipitate an orange oil. The orange, acidic oil was again taken up in ether, and the solution was dried over anhydrous sodium sulfate and evaporated to give an orange oil. Crystallization of this oil from benzene-hexane gave 16 g (39%) of 3,5-di-(α,α-dimethylbenzyl)2-ketohexene-4-dioic acid, soft colorless crystals, mp 116.5–118.0°.

Anal. Calcd. for $C_{24}H_{26}O_5$ (394): C, 73.1; H, 6.6. Found: C, 73.4; H, 7.0.

A mixed melting point of the above acid and that prepared by saponification of 3,5-di-(α,α-dimethylbenzyl)-coumalic acid showed no depression.

α-Oximino-β-phenylisovaleric Acid.—To 34.8 g of 3,5-di-(α,α-dimethylbenzyl)coumalic acid in 1150 ml of 1 N aqueous sodium hydroxide was added 50 g of potassium permanganate. After 48 hours the excess oxidant was destroyed by addition of 6 g of sodium sulfite, the mixture was filtered, and the precipitate was washed with 300 ml of hot, dilute aqueous sodium hydroxide. The filtrate was acidified and extracted three times with ethyl ether and the ethereal extract was dried over anhydrous sodium sulfate and evaporated to give an oily crystalline residue. This residue was taken up in 140 ml of 5% aqueous sodium hydroxide, the mixture was filtered, the filtrate was preserved, and the precipitate was washed with dilute aqueous sodium hydroxide. The precipitate was recrystallized from 90% ethanol and proved to be the lactone of 2,4-di-(α,α-dimethylbenzyl)-4-hydroxycrotonic acid. In a subsequent experiment, the manganese dioxide precipitate was continuously extracted with ether to give the lactone in 5% yield. Hydroxylamine hydrochloride (14 g), dissolved in the minimum amount of water, was added to the above filtrate. A precipitate appeared but redissolved on the addition of 20.5 g of sodium carbonate. The mixture, *p*H 8, was warmed on the steam bath for 2 hours, diluted with 250 ml of water, and acidified with concentrated hydrochloric acid. A colorless oil precipitated and crystallized, when cooled and seeded, to give 21.65 g of crude α-oximino-β-phenylisovaleric acid, mp 98–110°. This material was recrystallized from benzene-hexane to give 16.6 g of the acid, mp 119–121° with decomp. Jönsson (1954) gives a mp of 123.0–123.5°.

Anal. Calcd. for $C_{11}H_{13}O_3N$ (207): C, 63.8; H, 6.3; N, 6.8. Found: C, 63.8; H, 6.3; N, 6.9.

An additional 5.8 g of the acid, mp 120–122°, was obtained from mother liquors, bringing the total yield to 58%. Oxidation of 3,5-di-(α,α-dimethylbenzyl)2-ketohexene-4-dioic acid gave almost identical results.

DL-*β,β-Dimethylphenylalanine.*—To a mixture of 2% sodium amalgam and 75 ml of absolute ethanol, maintained at 45–55°, was added 5.18 g of α-oximino-β-phenylisovaleric acid. The reaction mixture was kept acid to bromcresol green by the periodic addition of 7 N ethanolic hydrogen chloride. After 1 hour the reaction mixture was carefully acidified with dilute aqueous hydrochloric acid, the solution was decanted, the residual mercury was washed with water, and the solution and washings were combined and evaporated until a crystalline precipitate began to form. Sufficient warm water was added to dissolve the precipitate, and the warm solution was saturated with hydrogen sulfide, boiled to coagulate the grey precipitate, and then filtered. The filtrate was adjusted to *p*H 6.5 with aqueous sodium carbonate and stored for 2 days at 4°. The crystalline amino acid, 1.2 g mp 225–230° with decomp, was collected. The mother liquor was evaporated to 50 ml to give an additional 1.0 g of product, mp 235–237° with decomp. Further reduction in the

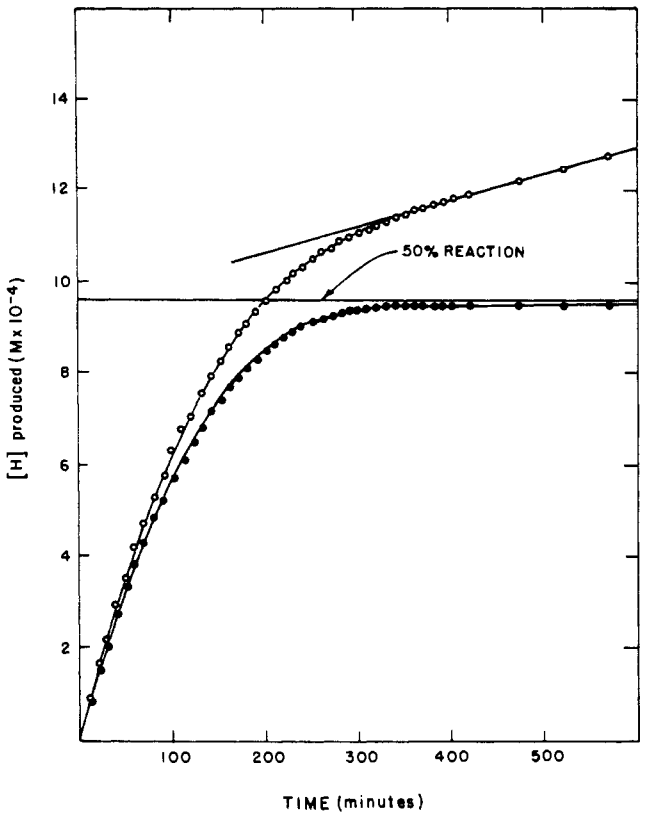


FIG. 1.—α-Chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-DL-2,6-dimethyltyrosine methyl ester in 25 (v/v) % aqueous-methanol at 25.0°, pH 7.90 and 0.30 M in sodium chloride. $[S]_0$ = 1.92 mM and [E] = 50.9 μM (based upon a molecular weight of 25,000 and a nitrogen content of 16.5%). Open circles uncorrected values; solid circles values corrected for nonenzyme-catalyzed hydrolysis of substrate.

volume of the residual mother liquor gave 0.2 g of amino acid, mp 225–228° with decomp, bringing the total yield to 2.4 g or 50%. The above fractions were combined and recrystallized from water to give DL-β,β-dimethylphenylalanine, mp 237–240° with decomp. Jönsson (1954) reports mp 240°.

Anal. Calcd. for $C_{11}H_{15}O_2N$ (193): C, 68.4; H, 7.8; N, 7.2. Found: C, 68.2; H, 7.8; N, 7.3.

Acetyl-DL-β,β-dimethylphenylalanine.—To an ice-cold solution of 8.9 g of the amino acid in 90 ml of 2 N aqueous sodium hydroxide was added 9.7 g of acetic anhydride and the mixture was shaken vigorously. The resulting clear solution was acidified to *p*H 2 with concentrated hydrochloric acid, and the crystalline precipitate was collected and recrystallized from water to give 9.9 g (92%) of acetyl-DL-β,β-dimethylphenylalanine, mp 183.5–185°. Jönsson (1954) reports mp 178–179°.

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235): C, 66.4; H, 7.3; N, 6.0. Found: C, 66.2; H, 7.2; N, 5.9.

Acetyl-DL-β,β-dimethylphenylalanine Methyl Ester.—To an ice-cold solution of 0.42 g of thionyl chloride in 2 ml of absolute methanol was added 0.5 g of acetyl-DL-β,β-dimethylphenylalanine. The solution was allowed to stand at room temperature for 36 hours and was then evaporated to a pale yellow oil. The oil was triturated with water to induce crystallization and the product was recrystallized from water to give 0.47 g (88%) of acetyl-DL-β,β-dimethylphenylalanine methyl ester, mp 80–82°.

Anal. Calcd. for $C_{14}H_{19}O_3N$ (249): C, 67.4; H, 7.8; N, 5.7. Found: C, 67.4; H, 7.7; N, 5.6.

DL-*2,6-Dimethyltyrosine*

O-Carbethoxy 3,5-dimethylphenol.—To a solution of 97.6 g of 3,5-dimethylphenol in 600 ml of benzene and 65 ml of pyridine was added dropwise and with stirring 100 g of ethyl chlorocarbonate. The reaction mixture was filtered, the filtrate was evaporated to an oily residue, and the residue was fractionally distilled to give 113 g (73%) of *O*-carbethoxy-3,5-dimethylphenol, bp 90–91°/1.5 mm.

O - Carbethoxy - 3,5 - dimethyl - 4 - chloromethylphenol.—A mixture of 113 g of *O*-carbethoxy-3,5-dimethylphenol, 120 ml of 37% aqueous formaldehyde, and 570 ml of concentrated hydrochloric acid was maintained at 50–60° and anhydrous hydrogen chloride was introduced into the reaction mixture for 3.5 hours. The cooled reaction mixture was taken up in chloroform, the chloroform phase was dried over anhydrous sodium sulfate, and the solvent was evaporated and the residue was fractionally distilled to give 98.5 g (71%) of *O*-carbethoxy-3,5-dimethyl-4-chloromethylphenol, bp 169–170°/6 mm, 145–146°/3 mm. Sommelet and Marzak (1934) give bp 151–153°/3 mm.

Anal. Calcd. for $C_{12}H_{15}O_3Cl$ (242.5): C, 59.4; H, 6.2; Cl, 14.6. Found: C, 59.4; H, 6.3; Cl, 14.5.

The nuclear magnetic resonance spectrum of the above compound in chloroform showed it to be the symmetrical isomer. When the reaction was conducted under more vigorous conditions (60–70° for 7 hours) 38% of 3,5-dimethyl-2,4,6-tri-chloromethylphenol, mp 143–147°, was obtained.

Anal. Calcd. for $C_{11}H_{13}OCl_3$ (267.5): C, 49.4; H, 4.8; Cl, 39.8. Found: C, 50.1; H, 5.2; Cl, 39.3.

Diethyl Acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate.—A solution of sodium ethoxide was prepared by adding 1.4 g of sodium to 200 ml of absolute ethanol. To this solution was added 13.0 g of diethyl acetamidomalonate and then after 10 minutes 15.2 g of *O*-carbethoxy-3,5-dimethyl-4-chloromethylphenol. The mixture was refluxed for 2 hours, cooled, and filtered, and the filtrate was evaporated *in vacuo* to a clear colorless oil which crystallized when triturated with warm 20% aqueous-acetone. The crude product (12.2 g, 58%) was recrystallized from benzene and then from water to give diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate, mp 156–157°.

Anal. Calcd. for $C_{18}H_{25}O_6N$ (351): C, 61.5; H, 7.2; N, 4.0. Found: C, 61.7; H, 6.9; N, 4.0.

DL-2,6-Dimethyltyrosine.—A mixture of 20.8 g of diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)-malonate and 90 ml of 48% hydrobromic acid was refluxed under an atmosphere of nitrogen for 3.5 hours. The reaction mixture was cooled, and the crystalline product was collected, washed with 25% hydrobromic acid, and dried to give 16.1 g of DL-2,6-dimethyltyrosine hydrobromide. The hygroscopic hydrobromide was dissolved in 200 ml of warm water, the solution was filtered, neutralized to *p*H 6.5 with aqueous sodium carbonate, and cooled, and the crystalline amino acid was collected and washed with acetone and then ethyl ether to give 5.1 g of product. An additional 1.2 g was obtained from the mother liquor. The two fractions were combined and recrystallized from water to give DL-2,6-dimethyltyrosine, mp 230–231° with decomp.

Anal. Calcd. for $C_{11}H_{15}O_3N$ (209): C, 63.1; H, 7.2; N, 6.7. Found: C, 62.9; H, 7.1; N, 6.5.

O-N-Diacetyl-DL-2,6-dimethyltyrosine.—To a solution of 0.5 g of DL-2,6-dimethyltyrosine in 5 ml of 2 N aqueous sodium hydroxide was added 0.5 ml of acetic anhydride, and the reaction mixture was shaken and then acidified with concentrated hydrochloric acid to give an orange oil that slowly crystallized. The crude product was decolorized with Norit and recrystallized from water to

give 0.4 g (54%) of *O,N*-diacetyl-DL-2,6 dimethyltyrosine. This preparation melted at *ca.* 120°, resolidified, and again melted at 174–175°. The product dried *in vacuo* over phosphorous pentoxide melted sharply at 174–175°. Analysis indicated that the product recrystallized from water was the monohydrate.

Anal. Calcd. for $C_{15}H_{21}O_6N$ (311): C, 57.9; H, 6.8; N, 4.5. Found: C, 57.7; H, 6.8; N, 4.3.

α-N-Acetyl-DL-2,6-dimethyltyrosine Methyl Ester.—To an ice-cold solution of 0.25 ml of thionyl chloride in 2 ml of anhydrous methanol was added 0.5 g of *O,N*-diacetyl-DL-2,6-dimethyltyrosine monohydrate, and the solution was allowed to warm to room temperature and stand for 36 hours. It was then evaporated to a brown oil which was triturated with water to form pale yellow crystals which were recrystallized from water to give 0.23 g (54%) of *α-N*-acetyl-DL-2,6-dimethyltyrosine methyl ester, mp 193.5–195°.

Anal. Calcd. for $C_{14}H_{19}O_4N$ (265): C, 63.4; H, 7.2; N, 5.3. Found: C, 63.5; H, 7.2; N, 5.3.

Enzyme Experiments.—The enzyme preparation employed in these studies was crystalline bovine salt-free α-chymotrypsin, Armour lot No. 283. All kinetic experiments were conducted with the aid of a *p*H-stat (Applewhite *et al.*, 1958). Other pertinent details are given in the text and in the legends of Table I and Figure 1.

TABLE I
α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF *α-N*-ACETYL-DL-2,6-DIMETHYLTYROSINE METHYL ESTER[a]

[S] (mM)	[E][b] (mM)	Solvent Aqueous-Acetone (v/v %)	v_0[c] Corrected[d]	v_0[c] Extrapolated[e]
2.02	0.269	20	81	73
2.02	0.259	15	109	102
2.02	0.250	10	146	142
		0		210[f]

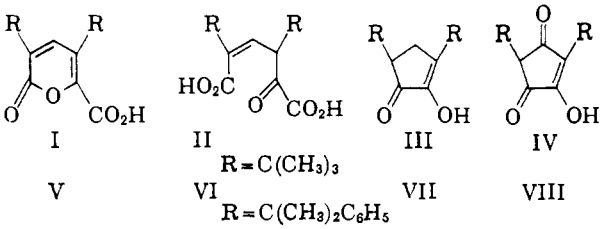
[a] At 25.0°, *p*H 7.90 and in solutions 0.20 M in sodium chloride. [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. [c] In units of M $min^{-1} \times 10^{-6}$. [d] Corrected for nonenzyme-catalyzed hydrolysis of substrate. [e] Linearly extrapolated to [E] = 0.2424 mM, i.e., 1 mg protein-nitrogen per ml. [f] Linearly extrapolated to zero acetone concentration.

RESULTS

tert-Leucine, prepared by the method of Knoop and Landmann (1914), was acetylated and esterified to give acetyl-DL-*tert*-leucine methyl ester. This compound was incapable of functioning as a substrate in aqueous systems at 25.0°, *p*H 7.90, 0.20 M in sodium chloride, 2.4×10^{-4} M in α-chymotrypsin, and 10^{-3} to 10^{-2} M in acylated α-amino acid ester. We may infer that the ratio k_0/K_0 for this compound is less than 0.01 M^{-1} sec^{-1}.

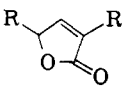
Jönsson (1954) described a successful synthesis of β,β-dimethylphenylalanine based upon the alkaline permanganate oxidation of 4,6-di-(α,α-dimethylbenzyl)-pyrogallol to α-keto-β-phenylisovaleric acid and reduction of the corresponding oximino acid. In our hands difficulty was encountered when it was found that the pyrogallol derivative could not be dissolved in 0.8 N aqueous sodium hydroxide.

Campbell (1951) found that air oxidation of 4,6-di-*tert*-butylpyrogallol in alkaline methanol gave 3,5-di-*tert*-butylcoumalic acid (I), 3,5-di-*tert*-butyl-2-ketohexene-4-dioic acid (II), 3,5-di-*tert*-butylcyclopentadione-1,2 (III), and 3,5-di-*tert*-butylcyclopentatrione (IV) in approximately equal amounts.



The difficulty encountered in the permanganate oxidation of 4,6-di-(α,α-dimethylbenzyl)pyrogallol and the low yields reported by Jönsson (1954) led us to attempt the air oxidation of this compound with the expectation that products analogous to those obtained by Campbell (1951) could be isolated and then cleanly oxidized to the desired α-keto-β-phenylisovaleric acid. 3,5-Di-(α,α-dimethylbenzyl)coumalic acid (V) and 3,5-di-(α,α-dimethylbenzyl)-2-ketohexene-4-dioic acid (VI) were considered to be the most desirable intermediates.

Oxygenation of an alkaline methanol solution of 4,6-di-(α,α-dimethylbenzyl)pyrogallol for 2 hours led to the isolation of 3,5-di-(α,α-dimethylbenzyl)coumalic acid in 52% yield. An increase in time of reaction to 5 hours gave, in addition, readily isolable amounts of the lactone of 2,4-di-(α,α-dimethylbenzyl) 4-hydroxycrotonic acid (IX). This substance was also formed, but in lesser amounts, when the reaction time was 2 hours.



IX, $R = C(CH_3)_2C_6H_5$

It was also found to be a minor product of the alkaline permanganate oxidation of 3,5-di-(α,α-dimethylbenzyl)-coumalic acid. In other experiments conducted in methanol contaminated with lead salts 3,5-di-(α,α-dimethylbenzyl)-2-ketohexene-4-dioic acid (VI) was isolated in 39% yield.

Oxidation of 3,5-di-(α,α-dimethylbenzyl)coumalic acid with alkaline permanganate gave α-keto-β-phenylisovaleric acid which was converted without isolation to α-oximino-β-phenylisovaleric acid. Reduction of the latter compound with sodium amalgam (Jönsson, 1954) gave DL-β,β-dimethylphenylalanine. The amino acid was acetylated and esterified to give acetyl-DL-β,β-dimethylphenylalanine methyl ester. This compound was also found to be incapable of functioning as a substrate when examined under the conditions described above for acetyl-DL-*tert*-leucine methyl ester. We may infer that the ratio k_0/K_0 for acetyl-DL-β,β-dimethylphenylalanine methyl ester is less than 0.01 M^{-1} sec^{-1}.

Chloromethylation of *O*-carbethoxy-3,5-dimethylphenol (Sommelet and Marzak, 1934) gave *O*-carbethoxy-3,5-dimethyl-4-chloromethylphenol. The latter compound was condensed with diethyl acetamidomalonate and the resulting diethyl acetamido-(2,6-dimethyl-4-hydroxybenzyl)malonate was hydrolyzed and decarboxylated to give DL-2,6-dimethyltyrosine. Acetylation of the amino acid gave *O,N*-diacetyl-DL-2,6-dimethyltyrosine, which was esterified and partially deacetylated to give *α-N*-acetyl-DL-2,6-dimethyltyrosine methyl ester.

α-N-Acetyl-DL-2,6-dimethyltyrosine methyl ester is relatively insoluble in water. Therefore, its hydrolysis by α-chymotrypsin was first examined in 25 v/v % aqueous-methanol at 25.0°, *p*H 7.90 and 0.30 M in sodium chloride. With $[S]_0 = 1.92$ mM and [E] = 5.09×10^{-5} M (based upon a molecular weight of

25,000 and a nitrogen content of 16.5%) the results depicted in Figure 1 were obtained. From these data it is evident that the above compound is a substrate of α-chymotrypsin and is hydrolyzed stereospecifically by this enzyme.

In a second experiment, conducted in 23 v/v % aqueous acetone, a levorotatory α-*N*-acetyl-2,6-dimethyltyrosine methyl ester, $[\alpha]_D$ − 17.8°, was isolated after 50% of the DL-mixture had been hydrolyzed. From these observations and those recorded earlier (Almond *et al.*, 1962) we may conclude that α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester is capable of functioning as a substrate of α-chymotrypsin. There is no indication that the D-antipode is hydrolyzed.

Comparison of the reactivity of α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester with that of α-*N*-acetyl-L-tyrosine methyl ester is difficult because of the absence of reliable data for the latter compound. However, as a first approximation we may extrapolate from the values obtained for acetyl-L-phenylalanine methyl ester (Jones and Niemann, 1963) and estimate that the constants k_0 and K_0 of equation (1) are of the order of magnitude of 10^2 sec^{-1} and 1 mM, respectively, for α-*N*-acetyl-L-tyrosine methyl ester (Peterson *et al.*, 1963).

Turning to the data given in Table I we estimate that for α-*N*-acetyl-DL-2,6-dimethyltyrosine methyl ester with [S] = 2.0 mM, [E] = 0.2424 mM, and in aqueous solutions, $d[P]/dt = 210 \times 10^{-6}$ M min^{-1}. For α-*N*-acetyl-L-tyrosine methyl ester with assumed values of $k_0 = 10^2$ sec^{-1} and K_0 = 1 mM, and with [S] = 1.0 mM and [E] = 0.2424 mM, application of equation (1) leads to a value of $d[P]/dt = 0.723$ M min^{-1}. This value implies that α-*N*-acetyl-L-tyrosine methyl ester is 3.4 × 10^3 times more reactive than α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester when the latter compound is hydrolyzed in the presence of an equimolar amount of its D-antipode. Since competitive inhibition by the D-antipode will lead to a decrease in rate of less than an order of magnitude, we may assert that replacement of the aromatic hydrogen atoms vicinal to the methylene group of α-*N*-acetyl-L-tyrosine methyl ester by methyl groups leads to *ca.* a 10^3-fold decrease in reactivity as a substrate of α-chymotrypsin.

Discussion

The inertness of carboxy derivatives of *tert*-leucine in nonenzyme-catalyzed reactions has been observed by Izumiya *et al.* (1953). Not only was difficulty encountered in the acid-catalyzed esterification of this amino acid, but the attempted ammonolysis of *tert*-leucine methyl ester, or of *N*-carbobenzyloxy-*tert*-leucine methyl ester, led only to recovery of starting materials.

The metabolic inertness of L-*tert*-leucine, which resulted in the earlier incorrect assignment of the absolute configuration of this amino acid (Abderhalden *et al.*, 1934; Greenstein and Winitz, 1961), might lead one to anticipate lack of reactivity for derivatives of *tert*-leucine in *in vitro* enzyme-catalyzed reactions. However, Izumiya *et al.* (1953) effected the resolution of DL-*tert*-leucinamide through hydrolysis of the L-antipode with hog renal amidase.

Our observation that acetyl-DL-*tert*-leucine methyl ester is hydrolyzed very slowly, if at all, by α-chymotrypsin supports the earlier conclusion (Waite and Niemann, 1962; Jones and Niemann, 1962) that steric hindrance arising from β-branching is a feature of reactions catalyzed by this enzyme. Thus, a point of similarity is established between the enzyme and acid- or base-catalyzed reactions, i.e., both are subject to steric hindrance arising from β-branching of the side chain.

Acetyl-L-phenylalanine methyl ester, K_0 = 1.25 mM, k_0 = 52.5 sec^{-1}, and $k_0/K_0 = 4.2 \times 10^4$ M^{-1} sec^{-1} (Jones and Niemann, 1963), is one of the more reactive substrates of α-chymotrypsin. The observation that acetyl-L-β,β-dimethylphenylalanine methyl ester is too unreactive to evaluate as a substrate of this enzyme serves to illustrate the magnitude of the steric effect that can arise from β-branching of the side chain. In this particular instance the decrease in reactivity associated with replacement of the two β-hydrogen atoms by methyl groups appears to be comparable in magnitude to that arising from replacement of the α-hydrogen atom by a methyl group (Almond *et al.*, 1962).

In earlier studies (Waite and Niemann, 1962; Jones and Niemann, 1962) the decrease in reactivity arising from β-branching was associated with an increase in values of K_0 and a decrease in those of k_0. The lack of reactivity of acetyl-DL-*tert*-leucine methyl ester and acetyl-DL-β,β-dimethylphenylalanine methyl ester has prevented us from obtaining similar information from studies with these compounds. The alternative of using them as inhibitors was contemplated. However, for acetyl-L-*tert*-leucine methyl ester it was decided that the value of K_I would be so large as to be indeterminable. Attempts to obtain a value of K_I for the experimentally more promising acetyl-L-β,β-dimethylphenylalanine methyl ester aborted when we were unable to effect a satisfactory resolution of the DL-acid.

In the absence of more quantitative information we can assert that the relative reactivities of the L-antipodes of the following α-*N*-acetyl methyl esters are in the order, phenylalanine >> alanine > valine >> β,β-dimethylphenylalanine (Jones and Niemann, 1962, 1963; Wolf and Niemann, 1963). Replacement of a β-hydrogen atom of the alanine derivative by a phenyl group causes a substantial increase in reactivity. The same replacement in the valine derivative leads to a marked decrease in reactivity. These observations raise the question whether some factor other than shielding of the carbonyl group of the hydrolyzable carbomethoxy component by the β-methyl groups is in part responsible for the very low order of reactivity of the β,β-dimethylphenylalanine derivative. Examination of scale models revealed that the β-methyl groups could interfere with the *o*-hydrogen atoms of the benzene nucleus and thus limit the number of conformations that could be assumed by the molecule. It is conceivable that this internal structural constraint could lead to a less than optimal orientation of the molecule when combined with the active site of the enzyme or to an unfavorable compression in the transition state.

To ascertain whether interaction between β-methyl groups and the benzenoid *o*-hydrogen atoms was a likely possibility it was decided to examine the inverse situation. Synthetic considerations led to the selection of α-*N*-acetyl-DL-2,6-dimethyltyrosine methyl ester as the model compound. As noted previously, only the L-antipode is hydrolyzed by α-chymotrypsin but at a rate estimated to be *ca.* 10^{-3} of that of α-*N*-acetyl-L-tyrosine methyl ester. It is unlikely that the slower rate of hydrolysis of α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester arises from shielding of the carbonyl group of the potentially hydrolyzable carbomethoxy component by the *o*-methyl groups. However, these groups should interact with the β-methylene hydrogen atoms to the same degree that the β-methyl groups interact with the benzenoid *o*-hydrogen atoms in the β,β-

dimethylphenylalanine derivative. Thus, the diminished rate of hydrolysis observed for α-*N*-acetyl-L-2,6-dimethyltyrosine methyl ester appears to have its origin in a conformational constraint arising from interaction of the *o*-methyl groups with the β-methylene hydrogen atoms. In acetyl-L-β,β-dimethylphenylalanine methyl ester the preceding effect is augmented by the more conventional type of steric hindrance associated with β-branching, thus leading to a very constrained and unreactive substrate.

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