THE SYNTHESIS OF O-METHYL-N-ACETYL-L-TYROSINE

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ABSTRACT

An efficient method of preparing N-acetyl-L-tyrosine methyl ester is described. This compound has been converted to the optically pure O-methyl derivative by reaction with diazomethane. The saponification of N-acetyl-L-tyrosine methyl ester is a more convenient rout to N-acetyl-L-tyrosine than the direct acetylation of L-tyrosine. N-acetyl-L-tyrosine can be converted to optically pure O-methyl-N-acetyl-L-tyrosine via the Williamson synthesis. The preparation of several other derivatives of L-tyrosine is also described.

INTRODUCTION

The ultimate goal of the research project to which the author of this thesis has made a modest contribution is to determine the nature of the active site of the proteolytic enzyme, alpha-chymotrypsin. The method of investigation is a study of the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of substrates of known structure. Certain derivatives of O-methyl-N-acetyl-L-tyrosine (L-2-acetamido-3-(4'-methoxyphenyl)propanoic acid) would be interesting substrates for reasons which will be discussed below.

The fundamental mathematical formulation of the reaction kinetics is that originated by Michaelis and Menten (1) and elaborated by Haldane (2). Their basic assumption is that the enzyme, E, unites reversibly with the substrate, S, to form an activated complex, ES, which can then decompose into the original enzyme and the product or products of reaction, P. The symbols to be used in the development of the Michaelis-Menten equation are defined as follows:

$[E_0]$ = the initial molar concentration of the enzyme, before any combination with the substrate has occurred

$[E]$ = the molar concentration of free enzyme

$[S]$ = the molar concentration of free substrate

$[ES]$ = the molar concentration of the complex

If one neglects any inhibition of the reaction resulting from a combination of the enzyme with the reaction product or products, the reaction can be formulated in the following way:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1)$$

A steady state is introduced with the assumption that [ES] is essentially constant. This will be a good approximation if $k_3 \ll k_2$, that is, if the decomposition of the complex is the rate determining step.

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES] = 0 ; \qquad (2)$$

$$\frac{k_2 + k_3}{k_1} = \frac{[E][S]}{[ES]}$$

$$-\frac{d[S]}{dt} = k_1[E][S] - k_2[ES] = k_3[ES] \qquad (3)$$

A new constant, K_s, is defined as follows:

$$K_s = \frac{k_2 + k_3}{k_1} = \frac{[E][S]}{[ES]} \qquad (4)$$

It is evident from equation 1 that [E] can be expressed in the following way:

$$[E] = [E_0] - [ES] \qquad (5)$$

$$K_s = \frac{([E_0] - [ES])[S]}{[ES]} ; \quad [ES] = \frac{[E_0][S]}{K + [S]} \qquad (6)$$

This leads directly to the Michaelis-Menten equation:

$$-\frac{d[S]}{dt} = \frac{k_3[E_0][S]}{K + [S]} \qquad (7)$$

Equation 7 has been shown to be a reasonably accurate description of the kinetics of the alpha-chymotrypsin catalyzed hydrolysis of a large number of substrates, at least in the early stages of the reaction (3-18). It is evident that if k_3 increases or if K_s decreases, the rate of hydrolysis, $-d[S]/dt$, will increase.

Following the initial observation that the alpha-chymotrypsin catalyzed hydrolysis of N-nicotinyl-L-tyrosinamide proceeded more rapidly than that of the corresponding phenylalanine derivative (19), a systematic study of the reaction kinetics provided the data tabulated below: (The units of K_s and k_3 are, respectively, M and M/min./mg. of protein-nitrogen/ml.)

SUBSTRATE	REFERENCE	$K \times 10^3$	$k_3 \times 10^3$
N-acetyl-L-phenylalaninamide	12, 15, 20	31±3	0.8±0.2
N-nicotinyl-L-phenylalaninamide	12, 15, 20	19±4	2.0±0.3
N-acetyl-L-tyrosinamide	4, 7, 8, 20	32±4	2.4±0.3
N-nicotinyl-L-tyrosinamide	4, 8, 10, 20	12±3	5.0±1.0

It is evident that k_3 is larger in the case of the tyrosine derivatives than in the case of the corresponding phenylalanine derivatives. An evaluation of the kinetic constants for the alpha-chymotrypsin catalyzed hydrolysis of the corresponding O-methyl tyrosine derivatives might shed some light on the reason for this difference.

There is reason to believe that this difference is a steric effect, that is results from the size of the phenolic hydroxyl group of the tyrosine. Kinetic studies involving N-acetyl-hexahydro-L-phenylalaninamide indicate that aromaticity of the ring is not important (20, 21); other studies indicate that the bonding of the enzyme to the substrate or inhibitor involves principally van der Waals forces rather than intermolecular hydrogen bond formation (11).

If the difference in rate constants is a steric effect, one might expect a still larger value for k_3 in kinetic studies involving O-methyl tyrosine derivatives. Furthermore, such a study would decisively prove whether or not the phenolic hydrogen is important.

For obvious reasons, a study of the para-alkyl homologues of phenylalanine would be extremely interesting; however, since these compounds could be obtained only by total synthesis, it seemed advisable to study the more accessible O-alkyl tyrosine derivatives first.

EXPERIMENTAL

I. Discussion:

This discussion is intended to preface the detailed account of the experimental work with a brief analysis of the problem and an interpretation of the more important experimental observations.

Since any reagent which alkylates a phenol will, with the possible exception of the tri-alkylphenylammonium hydroxides (22), also react with a free amino group, the blocking of the amino group necessarily precedes the alkylation of the phenolic hydroxyl group. Therefore, N-acetyl-L-tyrosine or some ester thereof is required as an intermediate in the synthesis of O-methyl-N-acetyl-L-tyrosine.

The direct acetylation of L-tyrosine without racemization can be accomplished only by a Schotten-Baumann reaction, in which a solution of tyrosine in excess aqueous sodium hydroxide is treated with a smaller excess of some acetylating agent, such as acetic anhydride. By this method, du Vigneaud prepared optically pure N-acetyl-L-tyrosine in reasonably good yield (23), but the isolation of the product from the reaction mixture is inconvenient.

N-acetyl-L-tyrosine is most conveniently prepared by saponification of N-acetyl-L-tyrosine methyl ester, which is very readily prepared by the following method:

By a new method of esterification, involving the use of thionyl chloride and methanol (24), tyrosine is conveniently and efficiently converted to the methyl ester hydrochloride, from which the ester is liberated with the stoichiometric amount of aqueous sodium hydroxide. The addition of acetic anhydride to a solution of the ester in aqueous acetic acid results in the rapid precipitation of N-acetyl-L-tyrosine methyl ester, in excellent yield. The only mechanisms of racemization likely to be operative at the temperature and pH of the reaction mixture, i. e., formation of the mixed anhydride and the azlactone, are manifestly impossible in the case of an ester. Furthermore, no di-acetylation is observed, probably because the O-acylation of a phenol is a much slower reaction than the N-acylation of an amine, and the N-acetyl compound precipitates from solution as rapidly as it forms.

The O-methylation of the methyl-N-acetyl-L-tyrosinate by application of the Williamson synthesis was attempted. Treatment of a methanolic solution of methyl-N-acetyl-L-tyrosinate with sodium methylate and methyl iodide gave a high yield of racemic O-methyl-N-acetyl-tyrosine methyl ester. The probable mechanism of racemization is abstraction of the proton from the alpha carbon atom by the methylate ion. Since the acidity of the alpha hydrogen is largely attributable to the adjacent carbomethoxy

group, racemization may be avoided by the substitution of N-acetyl-L-tyrosine for the methyl ester, as will be discussed more fully in the next paragraph. In addition to racemization, one might expect a certain amount of alkylation of the alpha carbon atom of the O-methyl-N-acetyl-D,L-tyrosine methyl ester, but the analysis of the product proved that this had not occurred.

The reaction of an aqueous solution of di-sodium N-acetyl-L-tyrosinate, prepared by saponification of the methyl ester with aqueous sodium hydroxide, with an equimolar quantity of methyl iodide, which was brought into solution with methanol, yielded about sixty per-cent of the theoretical quantity of optically pure O-methyl-N-acetyl-L-tyrosine. The success of this procedure probably depends on the fact that the phenoxide ion, with its more highly concentrated charge, participates in SN_2 type displacements much more readily than the carboxyl ion; therefore, if di-sodium acetyl tyrosinate and methyl iodide are present in equimolar amounts, only the phenolic function will be methylated.

Diazomethane converts N-acetyl-L-tyrosine methyl ester to the O-methyl derivative in approximately fifty per-cent yield, provided that ether is excluded from the reaction solvent. Although the use of diazomethane obviates

the saponification of the ester and subsequent re-esterification of the O-methyl derivative, it would seem that this procedure would be preferable to that described in the preceding paragraph only if it led to a nearly quantitative yield.

II. The Preparation of Compounds:

A. N-Acetyl-L-Tyrosine:

This compound was prepared by the Schotten-Baumann acetylation described by du Vigneaud (23). Since this type of acetylation necessitates the use of a rather large excess of acetic anhydride, a large amount of acetic acid is liberated with the N-acetyl-L-tyrosine when the reaction mixture is acidified. Since even small amounts of acetic acid prevent the crystallization of N-acetyl-L-tyrosine, the first attempts to prepare this compound failed. The acetic acid can be removed by repeatedly evaporating the acid solution to dryness under vacuum and redissolving the residue in distilled water, or, more conveniently, by lyophilization. A typical preparation of the compound is described below:

L-tyrosine (45 g., 0.248 mole) was dissolved in 2.15 N sodium hydroxide (119 ml., 0.256 equivalents) and 90 ml. of water. This solution was cooled in an ice bath, and 2.15 N sodium hydroxide (558 ml., 1.20 equivalents) and acetic anhydride (60.0 ml., 64.8 g., 0.635 mole) were added in eight equal portions, thorough mixing of the reactants being maintained by use of a vibro-stirrer. The addition of the sodium hydroxide preceded that of the acetic anhydride because the latter reagent causes racemization of the N-acetyl-L-tyrosine, except in strongly

alkaline solution. The pH of the alkaline solution, which had been permitted to stand for one half hour to insure complete hydrolysis of the acetic anhydride, was adjusted to 1.8±0.1 by the addition of 6 N sulfuric acid. Lyophilization of the acid solution yielded a white, powdery residue, from which the N-acetyl-L-tyrosine was extracted with about 500 ml. of acetone containing about 25 ml. of water to facilitate filtration. After the acetone had been removed from the filtrate under vacuum, the addition of about 40 ml. of water to the resulting syrup caused rapid crystallization. The total yield, after an additional quantity of crystalline material had been obtained by concentration of the mother liquor, was 36.8 g. of white crystals which melted at 142-148°. Recrystallization of this material from an equal weight of boiling water yielded 34.0 g. (61.5%) of white crystals having the following physical constants:

M.P. = 149-151°(corr.)

$[\alpha]_D^{25}$ = +46.5 (c = 1.86 g./100 ml. in H_2O)

These values are in fairly close agreement with those reported by du Vigneaud (23).

This method of preparation has been abandoned in favor of the saponification of the more conveniently prepared methyl N-acetyl-L-tyrosinate. See part D.

B. L-Tyrosine Methyl Ester:

L-tyrosine was converted to the methyl ester hydrochloride by the method of Brenner and Huber (24), which has proved to be much more convenient than the traditional Fischer method. The ester is conveniently precipitated from a cold, aqueous solution of the hydrochloride with the stoichiometric amount of standardized 4 N sodium hydroxide. Although sodium hydroxide catalyzes the rapid conversion of the ester to the diketopiperazine, this reaction has never been observed when the procedure described below was rigorously followed. It is necessary to use no more than the stoichiometric amount of sodium hydroxide and to wash the precipitated ester thoroughly with cold, distilled water. A typical preparation is described below:

Redistilled thionyl chloride (60.8 ml., 104.8 g., 0.88 mole) was added dropwise to mechanically stirred anhydrous methanol (260 ml., 205 g., 6.4 moles), the temperature of which was not permitted to exceed 0°. L-tyrosine (145 g., 0.80 mole) was then added, and the temperature of the reaction mixture was slowly increased to 55°, at which temperature the reaction began. After the reaction mixture had been maintained at 55-60° for 18 hours, the fact that sulfur dioxide was no longer evolved proved that the reaction had gone to completion. The excess methanol was removed under vacuum, and the

residue was redissolved in about 400 ml. of anhydrous methanol, most of which was then removed by distillation at atmospheric pressure in order to eliminate the hydrogen chloride and sulfur dioxide from the product. After the remainder of the methanol had been removed under vacuum, the residue was stored over sodium hydroxide in a vacuum desiccator for about 12 hours. The crude methyl ester hydrochloride was dissolved in about three times its weight of cold water and treated with the stoichiometric amount of 4 N sodium hydroxide (based on the assumption of quantitative conversion of the tyrosine to the methyl ester hydrochloride). The precipitated ester was collected by suction filtration, washed with three 200 ml. portions of cold water, and dried in a vacuum desiccator over sulfuric acid. The yield was 150 g. of tan crystals which melted at 132-135°. In many instances, the crude ester can be used without further purification. In this case, it was purified in the following way:

The crude ester was dissolved in about 1.5 liters of dry methanol which had been warmed to a temperature of 40°, and the insoluble material was removed by filtration. The filtrate was treated with Norite, again filtered, concentrated to a volume of 200 ml. under vacuum, and stored under refrigeration for 2 hours. The crystals were collected on a sintered glass funnel and

washed with a small amount of cold methanol. The yield consisted of 122.4 g. (78.1%) of white crystals which melted at 135-137°(corr.), and exhibited the following specific rotation:

$$[\alpha]_D^{25} = +27.1 \quad (c = 2 \text{ g./100 ml. in methanol})$$

C. Esterification of N-Acetyl-L-Tyrosine:

N-acetyl-L-tyrosine (10 g., 0.0494 mole) was esterified with thionyl chloride and methanol, using the technique described above. After the excess methanol had been removed under vacuum, the addition of sodium bicarbonate solution caused the rapid crystallization of the syrup. The yield was 9.5 g. (90%) of white crystals which melted at 108-112°(corr.).

The acetylation of L-tyrosine methyl ester is a much more convenient route to this compound.

D. Acetylation of L-Tyrosine Methyl Ester:

The only acetylation of methyl-L-tyrosinate described in the literature (25) involves the application of a very inconvenient technique devised by E. Fischer (26). It has been reported that the compound forms a hydrate, the exact composition of which has not been determined, which melts at 118-120°, and that the anhydrous material melts at 136-137° (25), both of which observations have been confirmed in this laboratory. The following specific rotation is

reported (25):

$[\alpha]_D^{20}$ = +29.7 (c = 0.41 g./100 ml. in methanol)

Two samples of methyl-N-acetyl-L-tyrosinate prepared in this laboratory by two different methods had the following specific rotations:

$[\alpha]_D^{25}$ = +23±1 (c = 0.523 g./100 ml. in methanol)

$[\alpha]_D^{25}$ = +24.6 (c = 4 g./100 ml. in methanol)

The optical purity of this material was established by its conversion to O-methyl-N-acetyl-L-tyrosine and O-methyl-N-acetyl-L-tyrosine methyl ester, both of which derivatives had specific rotations almost exactly equal to the values recorded in the literature.

The various methods of acetylation attempted are given below:

1. Attempted Acetylation by the Curtius Procedure:

Curtius reported the preparation of hippuric acid by refluxing a suspension of glycine in benzene with benzoyl chloride (27). This method was further investigated by Franzen, who reported, among other things, the preparation of ethyl hippurate from glycine ethyl ester hydrochloride and benzoyl chloride in quantitative yield by the same method (28). The application of this method to the preparation of O, N-diacetyl-L-tyrosine methyl ester from methyl L-tyrosinate was unsuccessfully attempted in the

following way:

A suspension of L-tyrosine methyl ester (10 g., 0.0512 mole) in 300 ml. of benzene was heated under reflux with acetyl chloride (10.7 ml., 11.8 g., 0.15 mole) for about 60 hours, at which time all of the solid material had dissolved. Removal of the benzene under vacuum yielded about 15 g. of a pale yellow oil, which could not be crystallized.

An attempt to convert L-tyrosine to O,N-diacetyl-L-tyrosine was also unsuccessful.

2. Schotten-Baumann Acetylation:

A suspension of L-tyrosine methyl ester (15 g., 0.0768 mole) in 300 ml. of diethyl ether was poured over a saturated aqueous solution of potassium bicarbonate (35.2 g., 0.35 mole) and cooled to 0°. While thorough mixing of the reactants was maintained by use of a vibro-stirrer, acetic anhydride (18.1 ml., 19.6 g., 0.192 mole) was added in four equal portions at 5 minute intervals. The insoluble reaction product, after it had been dried in a vacuum desiccator, consisted of 12.4 g. of white crystals which melted at 100-115°. An additional 2.9 g. of product was obtained by evaporation of the ether. The aqueous layer was evaporated to dryness under vacuum, but no significant amount of organic material could be extracted from the residue with acetone. The crude product (15.3 g.)

was shaken with ice-cold 0.2 N hydrochloric acid to extract any starting material which might have been present, and the insoluble residue was immeadiately collected on a sintered glass funnel, washed with cold water, and dried in a vacuum d desiccator. The yield consisted of 12.6 g. (69%) of white crystals which melted at 125-131°.

When larger relative amounts of potassium bicarbonate and acetic anhydride were used, a smaller yield of a product with a lower melting po point was obtained. No yield was obtained when the potassium bicarbonate was replaced with potassium carbonate or sodium hydroxide.

Although methyl-N-acetyl-L-tyrosinate can be prepared in this way, the following two procedures are more efficient.

3. The Use of Acetic Anhydride as a Reaction Solvent:

This method is convenient and gives an excellent yield, but the product is contaminated with a certain amount of the di-acetyl compound. The presence of the latter compound is not objectionable in many of the reactions in which methyl N-acetyl-L-tyrosinate is used as an intermediate. A typical experiment is described below:

L-tyrosine methyl ester (5.0 g., 0.0256 mole),

which had been recrystallized from methanol, was dissolved in acetic anhydride (10.0 ml., 10.8 g., 0.106 mole). The temperature of the reaction mixture immediately rose to about 70° and was maintained at 60-70° for 45 minutes. The reaction mixture was then poured into 100 ml. of ice water, and the product, which crystallized rapidly, was collected on a sintered glass funnel, washed with cold water, and dried under high vacuum at 100° for 1 hour. The yield consisted of 5.66 g. (94.4%) of white crystals which melted at 132-136°. After the product had been freed of the di-acetyl compound by recrystallization from chloroform and again dried under high vacuum at 100°, it consisted of 5.64 g. (94.1%) of white crystals which melted at 136.5-137.5°(corr.) and had the following specific rotation:

$$[\alpha]_D^{25} = +23 \pm 1 \quad (c = 0.523 \text{ g./100 ml. in methanol})$$

This procedure may be repeated successfully on a much larger scale: the acetylation of 100 g. of L-tyrosine methyl ester at one time is not difficult.

4. Acetylation in Aqueous Acetic Acid:

This is the most efficient procedure; furthermore, none of the di-acetyl compound is formed. A typical preparation is described below:

L-tyrosine methyl ester hydrochloride, prepared

from 100 g. of L-tyrosine, was converted to the ester by treatment with aqueous sodium hydroxide, according to the procedure described in part B. The moist, precipitated ester was immediately dissolved in 1080 g. of 10% aqueous acetic acid, and the insoluble material was removed by filtration. The filtrate was cooled to a temperature of about 5°, and acetic anhydride (78.5 ml., 84.4 g., 0.827 mole) was added. After the reaction mixture had been allowed to stand at a temperature of 5° for 1 hour, the N-acetyl-L-tyrosine methyl ester, which had precipitated rapidly from the solution, was collected on a sintered glass funnel, washed with cold water, and dried under high vacuum at 100° for 5 hours. The product was then dissolved in absolute methanol, and the solution was treated with Norite and filtered. After the methanol had been removed under vacuum, the resulting syrup crystallized rapidly, without the addition of any solvent, when seeded with a few crystals of the ester. The yield consisted of 105 g. (80% based on the tyrosine) of slightly off-white crystals which melted at 135-136°(corr.). The product may be recrystallized from ethyl acetate, using 2.9 ml. of solvent per gram of product, but the yield is thereby reduced to 70%. The physical constants of the recrystal-

lyzed product are given below:

M. P. = 136.5-137.5° (corr.)

$[\alpha]_D^{25}$ = +24.6 (c = 4 g./100 ml. in methanol)

E. O-Methylation of N-Acetyl-L-Tyrosine and of N-Acetyl-L-Tyrosine Methyl Ester:

The phenolic function of N-acetyl-L-tyrosine has been methylated by the reaction of N-acetyl-L-tyrosine with dimethyl sulfate in aqueous alkali (29, 30), with methyl iodide and silver oxide (31), and with diazomethane (32). All of these procedures have been tried, and the best of them, that involving the use of dimethyl sulfate in aqueous alkali, is not entirely satisfactory. Several new techniques have been devised.

The contamination of the yield with starting material is readily detected by use of the Folin-Denis reagent (33), which is an aqueous solution of phosphomolybdic acid, sodium tungstate, and phosphoric acid. The formation of molybdenum blue in basic solution constitutes an extremely sensitive test for phenol, provided that no other reducing agent is present.

The O-methylation of N-acetyl-L-tyrosine or of methyl N-acetyl-L-tyrosinate was attempted by the use of the following procedures:

1. Reaction of N-Acetyl-L-Tyrosine with Dimethyl Sulfate and Aqueous Sodium Hydroxide:

The procedure is that of Karrer (29):

Dimethyl sulfate (10 g., 7.4 ml., 0.079 mole) was added in small portions, over a period of 45 minutes, to a solution of N-acetyl-L-tyrosine (10 g., 0.0448 mole) in 18.4 g. of 30% aqueous sodium hydroxide. Thorough mixing of the reactants was insured by the use of a vibro-stirrer. After completion of the reaction, acidification of the reaction mixture caused the separation of an oil, which rapidly crystallized. The yield was 5.1 g. (48%) of white crystals which melted at 134-140° and gave a positive reaction with the Folin-Denis reagent. After the product had been recrystallized from 3 times its weight of boiling water twice, it consisted of 3.51 g. (33%) of white crystals which melted at 149-151°(corr.) and still gave a positive Folin-Denis reaction. When the removal of the starting material by passage of a methanolic solution of the product through a Norite column was attempted, only a negligible amount of crystalline material could be recovered.

2. Reaction of N-Acetyl-L-Tyrosine with Silver Oxide and Methyl Iodide:

The method is that of Synge (31):

A solution of N-acetyl-L-tyrosine (2.0 g., 0.00896 mole) in 12 ml. of dry acetone was heated under reflux for two hours with freshly precipitated silver oxide (12 g., 0.0513 mole) and methyl iodide (12 ml., 27.4 g., 0.193 mole). The silver salts were removed by filtration, and the filtrate was concentrated to a thick syrup under vacuum. The syrup was redissolved in 12 ml. of dry acetone, and the procedure just described was repeated. The syrup which was obtained the second time was dissolved in about 50 ml. of chloroform, and the chloroform solution was shaken with 50 ml. of 1% aqueous sodium hydroxide at a temperature of 0° in order to extract whatever starting material was present. After the chloroform solution had been washed twice with cold water, the chloroform was removed under vacuum. The syrup rapidly crystallized, yielding 1.2 g. (53.4%) of white crystals which melted at 102-104°(corr.) and gave a negative reaction with the Folin-Denis reagent.

In view of the relatively low yield, the slowness of the reaction, and the inconvenient manipulation, the expense of this procedure is not justified.

3. Reaction of Methyl N-Acetyl-L-Tyrosinate with Sodium and Dimethyl Sulfate:

A solution of methyl N-acetyl-L-tyrosinate (5.0 g.,

0.0211 mole) in 100 ml. of anhydrous tetrahydrofuran was heated under reflux with sodium wire (0.485 g., 0.0211 mole) until the latter had disintegrated. Dimethyl sulfate (2.68 g., 0.0211 mole) was added, the reaction mixture was heated under reflux for 24 hours, and the insoluble material was removed by filtration. Removal of the tetrahydrofuran from the filtrate under vacuum yielded 2.4 g. of white crystals which melted at 102-114°, gave a positive reaction with the Folin-Denis reagent, and appeared to be mainly starting material.

4. Reaction of N-Acetyl-L-Tyrosine with Sodium Methylate and Dimethyl Sulfate:

N-acetyl-L-tyrosine (10 g., 0.0552 mole) was dissolved in a methanolic solution of sodium methylate, which had been prepared by the reaction of sodium (2.8 g., 0.122 mole) with 150 ml. of absolute methanol. Dimethyl sulfate (15.3 g., 0.122 mole) was added, and the solution was heated under reflux for about 12 hours. The methanol was removed under vacuum, and the residue was washed with dilute sodium bicarbonate solution. The yield consisted of 6.4 g. of white crystals which melted at 71-73° and gave a positive reaction with the Folin-Denis reagent. Recrystallization from aqueous methanol did not change the melting point significantly.

5. Reaction of Methyl N-Acetyl-L-Tyrosinate with Methyl Iodide and Sodium Methylate:

Thoroughly dried methyl N-acetyl-L-tyrosinate (50 g., 0.211 mole) was dissolved in a methanolic solution of sodium methylate, which had been prepared by the reaction of sodium (4.86 g., 0.211 mole) with 250 ml. of absolute methanol. Methyl iodide (15.5 ml., 35.5 g., 0.25 mole) was added, and the solution was heated under reflux for 1 hour. Sodium methylate solution, prepared by dissolving sodium (4.86 g., 0.211 mole) in 125 ml. of absolute methanol, and methyl iodide (15.5 g., 35.5 g., 0.25 mole) were then added, and the solution was heated under reflux for one hour. Methyl iodide (15.5 ml., 35.5 g., 0.25 mole) was again added, and the solution was heated under reflux for 12 hours. The methanol was removed under vacuum, and the addition of 100 ml. of water to the resulting syrup caused rapid crystallization. The yield consisted of 51.3 g. of yellow crystals which melted at 75-90° and gave a weakly positive reaction with the Folin-Denis reagent. The crude yield was dissolved in 500 ml. of reagent grade chloroform, the insoluble matter was removed by filtration, and the chloroform solution was shaken vigorously with 500 ml. of 1% aqueous sodium hydroxide at a temperature of 0°. The chloroform layer was

separated, washed twice with cold water, and the chloroform was removed under vacuum. The addition of water caused the syrup to crystallize rapidly. The yield consisted of 41.6 g. (78.7%) of white crystals which melted at 98-103° and did not react with the Folin-Denis reagent. Recrystallization of the product from 4160 ml. of di-isopropyl ether (100 ml. of ether/g. of product) yielded 35.6 g. (67.5%) of white crystals which melted at 103-104° (corr.). A second recrystallization from this solvent yielded 32.2 g. (61%) of white crystals which melted at 103.5-104.8°(corr.). When an attempt was made to determine the specific rotation, it was found that the product had racemized completely.

Analysis:	$-OCH_3$
Required:	24.701%
Found:	24.62 %

A portion of the ester was converted to the hydrazide in approximately 90% yield by slowly adding an ethanolic solution of the ester to a 4 mole excess of hydrazine in boiling ethanol. This derivative melted at 173.1-173.2°(corr.).

An attempted enzymatic resolution of the O-methyl-N-acetyl-D,L-tyrosine methyl ester with alpha-chymotrypsin, a method which had been found

suitable for the resolution of N-acetyl-D,L-tyrosine methyl ester (7), failed completely, probably because of the large amount of organic solvent needed to bring the ester into solution. Solvent systems containing water and methanol and water and tetrahydrofuran were tried. Resolution by means of the stereo-specific papain catalyzed synthesis of the toluidide, a method which has been applied successfully to the resolution of N-acetyl-D,L-phenylalanine (34), was partially successful.

O-methyl-N-acetyl-D,L-tyrosine was prepared in 94% yield by saponification of the methyl ester with methanolic sodium hydroxide, removal of the methanol under vacuum, and acidification of an aqueous solution of the sodium salt. The compound melted at 152-155°(corr.).

Pulverized papain (1.23 g.) was stirred with 6.15 ml. of water at a temperature of 5° for three hours. The suspension was centrifuged, and the supernatent was used without further purification. O-methyl-N-acetyl-D,L-tyrosine (7.16 g., 0.0302 mole), p-toluidine (3.24 g., 0.0302 mole), and L-cysteine hydrochloride (0.368 g.) were dissolved in 210 ml. of a sulution which was 0.5 M in acetic acid and 0.5 M in sodium acetate, and the papain solution was added. This solution, which had a pH of 4.7±0.1,

was then incubated at a temperature of 40° for 6 days. The solution was then cooled to a temperature of 10° , and the toluidide was collected by filtration. The yield consisted of 1.65 g. (42%) of brown crystals which melted at 207-208°(corr.). The crude product was dissolved in hot ethanol, treated with Norite, filtered, and the filtrate was allowed to crystallize. The yield was 0.8 g. (20.8%) of silky white needles which melted at 211-212°(corr.). The specific rotation is given below:

$[\alpha]_D^{25}$ = +36.8 (c = 2g./100 ml. in pyridine)

The mother liquor from which the crude toluidide had been obtained was concentrated to about one half its original volume, and the pH of the solution was adjusted to 2.0±0.1 by the addition of hydrochloric acid. The crystalline precipitate, which weighed 3.58 g., was dissolved in hot, aqueous methanol and treated with Norite. Two more recrystallizations from aqueous methanol yielded 1.60 g. (44%) of white crystals which melted at 154.5-156.0°(corr.). The specific rotation of this meterial was almost equal to 0, a very slight positive rotation having been observed.

6. Reaction of Methyl N-Acetyl-L-Tyrosinate with Ethyl Iodide and Sodium Methylate:

Methyl N-acetyl-L-tyrosinate (60 g., 0.254 mole)

was converted to the O-ethyl derivative by the same technique described in the preceding section, an equimolar amount of ethyl iodide having been substituted for the methyl iodide. The yield, which had been freed of starting material by treatment of a chloroform solution with dilute aqueous sodium hydroxide, consisted of 53.1 g. (79.3%) of white crystals which melted at 122-125°(corr.). Three recrystallizations from di-isopropyl ether, using 160 ml. of ether per g. of solute, yielded 40.1 g. (59.8%) of white needles which melted at 125.5-127.5°(corr.).

Analysis:	C	H
Required:	63.38%	7.22%
Found:	63.72%	7.45%

$[\alpha]_D^{25}$ = +7.27 (c = 4g./100 ml. in methanol)

The low specific rotation indicates that extensive racemization had occurred.

7. Reaction of N-Acetyl-L-Tyrosine with Sodium Hydroxide and Methyl Iodide:

N-acetyl-L-tyrosine methyl ester (10 g., 0.0423 mole) was dissolved in 4.98 N aqueous sodium hydroxide (16.92 ml., 0.0843 equivalent), and the solution was allowed to stand at room temperature for 2 hours. Methyl iodide (7.93 g,, 3.46 ml., 0.0560 mole) and methanol (50 ml.) were added, and the solution was heated under reflux for 5 hours. The reaction solution

was concentrated to dryness under vacuum, the sodium iodide was extracted from the residue with 160 ml. of dry acetone, and the residue was redissolved in water. The solution was again concentrated to dryness under vacuum to remove the acetone. The residue was redissolved in about 20 ml. of water, and the pH of the solution was adjusted to 2.0±0.1 by the addition of hydrochloric acid. A yield of 6.7 g. (67%) of white crystals which gave a positive reaction with the Folin-Denis reagent was obtained. Recrystallization from 20 ml. of boiling water yielded 5.4 g. (54%) of white crystals which melted at 148-150°(corr.) and gave a weakly positive reaction with the Folin-Denis reagent.

$[\alpha]_D^{25}$ = +58.0 (c = 2 g./100 ml. in methanol)

This specific rotation agrees closely with that reported in the literature (31).

A sample of O-methyl-N-acetyl-L-tyrosine, which had been prepared in a previous experiment in essentially the same way, was quantitatively converted to the methyl ester with diazomethane. This latter derivative melted at 106-107°(corr.). and had the following specific rotations:

$[\alpha]_D^{25}$ = +28.4 (c = 4 g./100 ml. in methanol)

$[\alpha]_D^{20}$ = +27.2 (c = 4 g./100 ml. in 95% ethanol)

The melting point and the specific rotation in

ethanol are in close agreement with the values reported in the literature (31).

The use of a large excess of methyl iodide and sodium hydroxide does not increase the yield and makes the isolation of the product more difficult. It is necessary to extract the sodium iodide from the sodium salt of O-methyl-N-acetyl-L-tyrosine because O-methyl-N-acetyl-L-tyrosine will not crystallize in the presence of a large amount of sodium iodide.

8. O-Methylation with Diazomethane:

The reaction of a methanolic solution of N-acetyl-L-tyrosine with an excess of diazomethane in ether, prepared according to the directions in Organic Synthesis (35), yielded only the methyl ester. When the latter, in methanolic solution, was again treated with an excess of etheral diazomethane, an impure product which melted at 75-104° and gave a positive reaction with the Folin-Denis reagent was obtained. The preparation of O-methyl-N-acetyl-L-tyrosine by this method has been reported in the literature (32).

When an etheral solution of diazomethane, prepared from nitrosomethylurea and aqueous potassium hydroxide (35), was added to a solution of N-acetyl-L-tyrosine in anhydrous tetrahydrofuran, no visible

reaction occurred. Neither picric acid nor fluoboric acid were effective catalysts; boron trifluoride etherate catalyzed the decomposition of the diazomethane, but not the methylation of the phenol. It was observed that diazomethane rapidly converts methyl N-acetyl-L-tyrosinate to the O-methyl derivative in 50% yield if ether is excluded from the solvent system.

One hundred twenty ml. of a 50% aqueous solution of potassium hydroxide in a 1 liter flask was covered with about 300 ml. of n-pentane, and nitrosomethylurea (41.2 g., 0.40 mole) was added. The reaction flask was immediately connected to a condenser set for distillation. While the reaction flask was maintained at a temperature of 50-60°, the diazomethane was collected in about 100 ml. of toluene which was cooled in an acetone-dry ice bath. The diazomethane solution was decanted from the ice crystals into a solution of methyl N-acetyl-L-tyrosinate (28.2 g., 0.119 mole) in 200 ml. of absolute methanol. The solution was permitted to stand at room temperature for 48 hours after the evolution of nitrogen had ceased, and the excess diazomethane was destroyed with acetic acid. The solvent was removed under vacuum, and the residue was dissolved in 100 ml. of chloroform. After the chloroform solution had been

filtered, shaken with 200 ml. of 0.5% aqueous sodium hydroxide at a temperature of 0°, and washed twice with cold water, the chloroform was removed under vacuum to yield 17.0 g. (57.0%) of slightly yellow crystals which melted at 100-101°(corr.) and did not react with the Folin-Denis reagent. Recrystallization from 1000 ml. of di-isopropyl ether (55.8 ml. of ether/g. of solute) yielded 14.8 g. (49.6%) of crystals which melted at 104-105°(corr.). A second recrystallization from di-isopropyl ether yielded 13.4 g. ((44.9%) of colorless rods which melted at 105-108° (corr.). The pure L isomer is seen to be about twice as soluble in di-isopropyl ether as the racemate, which requires about 100 ml. of ether/g. of solute.

The application of a technique developed by Nierenstein for the methylation of the acetates of certain polyhydric phenols (36) resulted in a smaller yield of a less pure product.

A solution of O,N-diacetyl-L-tyrosine methyl ester (8.00 g., 0.0287 mole) in 20 ml. of methanol was treated with a 50% excess of a dry etheral solution of diazomethane, prepared in the usual manner (35). The addition of anhydrous, redistilled piperidine (2.44 g., 2.84 ml., 0.0287 mole) caused rapid evolution of nitrogen. After the solution had been allowed to stand at room temperature for about 12

hours, the excess diazomethane was destroyed with glacial acetic acid, and the solution was concentrated to about one half of its original volume under vacuum. The solution was then stirred into 200 ml. of ice cold water, and the rapidly precipitated crystals were collected. The yield consisted of 4.86 g. of white crystals which melted at 98-115° and gave a positive reaction with the Folin-Denis reagent.

Although methyl N-acetyl-L-tyrosinate can be converted to the O-methyl derivative with diazomethane, it would seem that the yield is not sufficiently high to justify the use of this reagent.

F. Miscellaneous Derivatives of L-Tyrosine:

The following tyrosine derivatives were also prepared for use as substrates or intermediates:

1. O,N-Diacetyl-L-Tyrosine Methyl Ester:

A solution of N-acetyl-L-tyrosine methyl ester (10 g., 0.0422 mole) and acetic anhydride (4.6 g., 4.3 ml., 0.045 mole) in 20 ml. of pyridine was maintained at a temperature of 100° for 3 hours. After the solution had been permitted to cool to room temperature, it was poured into 100 ml. of benzene, and enough low-boiling petroleum ether (30-60°) was added to make the solution turbid. As the product

slowly crystallized, 300 ml. of low boiling petroleum ether was added in small portions. The yield, after it had been collected on a sintered glass funnel and dried over paraffin shavings in a vacuum desiccator, consisted of 10.0 g. (84.9%) of yellow crystals which melted at 102-104°(corr.). Recrystallization from 1200 ml. of di-isopropyl ether (120 ml. of ether/g. of solute) yielded 9.2 g. (78%) of white needles which melted at 105-107°(corr.) and had the specific rotation given below:

$[\alpha]_D^{25}$ = +19.3 (c = 4.14 g./100 ml. in methanol)

2. L-Tyrosinamide:

A solution of L-tyrosine methyl ester (10 g., 0.0512 mole) in 300 ml. of absolute methanol was saturated with dry ammonia (47 g. of ammonia was absorbed), and the solution was permitted to stand at room temperature for 2 weeks. The methanol and excess ammonia were removed under vacuum, and the residue was redissolved in about 250 ml. of absolute methanol, boiled with Norite, and filtered. The filtrate was concentrated to dryness under vacuum, and the residue was recrystallized from 70 ml. of 1:1 ethanol-ethyl acetate to yield 4.80 g. (52%) of white crystals which melted at 155-156°(corr.) and had the specific rotation given below:

$[\alpha]_D^{25}$

3. L-Tyrosinehydroxamide:

A solution of sodium methylate, prepared by dissolving sodium (11.75 g., 0.512 mole) in 50 ml. of absolute methanol, was added to a solution of hydroxylamine hydrochloride (35.5 g., 0.512 mole) in 300 ml. of absolute methanol, the sodium chloride was removed by filtration, and the absence of sodium methylate in the filtrate was established by testing it with pH paper. L-tyrosine methyl ester (10 g., 0.0512 mole) was dissolved in the filtrate, and after this solution had been permitted to stand at room temperature for 2 weeks, the white crystals which had separated during this time were collected on a sintered glass funnel, washed with cold methanol, and dried in a vacuum desiccator. The yield was 8.40 g. (83.6%) of white crystals which melted at 160.0-162.5° (corr.) and had the specific rotation given below:

$[\alpha]_D^{25}$

4. L-Tyrosinehydrazide:

A solution of L-tyrosine methyl ester (10 g., 0.0512 mole) in 400 ml. of absolute ethanol was added dropwise to a refluxing, mechanically stirred solution of hydrazine (16.4 g., 16.2 ml., 0.512 mole) in 100 ml. of absolute ethanol. The reaction mixture was heated under reflux for 8 hours after all of the ester had been added, and was then concentrated to

a volume of about 100 ml. under vacuum. The reaction mixture was cooled to a temperature of 5 , and the hydrazide was collected on a sintered glass funnel. The yield consisted of 9.35 g. (93.5%) of white needles which melted at 191-194°(corr.). Two recrystallizations from large volumes of absolute ethanol yielded 6.38 g. (63.8%) of white needles which melted at 196-198°(corr.) and had the specific rotation given below:

$[\alpha]_D^{25}$

5. N-Acetyl-L-Tyrosinehydrazide:

A solution of N-acetyl-L-tyrosine methyl ester (15.0 g., 0.0634 mole) in 250 ml. of absolute ethanol was added dropwise to a mechanically stirred, refluxing solution of hydrazine (10.2 g., 10.2 ml., 0.317 mole) in 100 ml of absolute ethanol. The reaction mixture was heated under reflux for three hours after all of the ester had been added. After the reaction mixture had been allowed to cool to room temperature, the hydrazide was collected on a sintered glass funnel. The yield consisted of 14.3 g. (95.4%) of white needles which melted at 234-235°(corr.).

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