SYNTHESIS OF 2,3,4-TRIHYDROXY-L-PHENYLALANINE FROM S-METHYL-L-CYSTEINE AND PYROGALLOL BY L-TYROSINE PHENOL-LYASE

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Summary

A trihydroxy derivative of phenylalanine was synthesized from S-methyl-L-cysteine and pyrogallol by the crystalline tyrosine phenol-lyase (L-tyrosine phenol-lyase (deaminating) EC 4. I. 99.2 formerly known as β-tyrosinase) from *Escherichia intermedia*. The product was isolated as its N-acetyl-triacetoxymethylester and identified as 2,3,4-trihydroxy-L-phenylalanine by the analyses of NMR, MS spectra and optical rotation.

Tyrosine phenol-lyase is a pyridoxal phosphate-dependent multifunctional enzyme which catalyzes α,β-elimination (equations i-iv) (1, 2), β-replacement (equations v, vi) (3, 4), racemization (equation vii) (5) and the reverse of α,β-eliminations (equations viii, ix) (6, 7), to form L-tyrosine or its derivatives from pyruvic acid, ammonia and phenols.

$$\text{L-Tyrosine} + H_2O \longrightarrow \text{phenol} + \text{pyruvic acid} + NH_3 \qquad \text{(i)}$$

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S-Methyl-L-cysteine + H_2O ⟶ methylmercaptan + pyruvic acid + NH_3 (ii)

L-Cysteine + H_2O ⟶ H_2S + pyruvic acid + NH_3 (iii)

L-Serine ⟶ pyruvic acid + NH_3 (iv)

L-Tyrosine + pyrocatechol ⟶ 3,4-dihydroxy-L-phenylalanine + phenol (v)

S-Methyl-L-cysteine + resorcinol ⟶ 2,4-dihydroxy-L-phenylalanine + phenol (vi)

L(D)-Alanine ⟶ D,L-alanine (vii)

Phenol + pyruvic acid + NH_3 ⟶ L-tyrosine + H_2O (viii)

Pyrocatechol + pyruvic acid + NH_3 ⟶ 3,4-dihydroxy-L-phenylalanine (ix)

The mechanism for these reactions has been studied in some detail (8), by adopting the general mechanism proposed for pyridoxal-dependent reactions (9, 10).

Microbial methods for preparing L-tyrosine and its related amino acids were developed using cells of *Erwinia herbicola* with high tyrosine phenol-lyase activity (11), that is, the production of L-tyrosine or L-DOPA from DL-serine and phenol or pyrocatechol (12), and the production of these aromatic amino acids from pyruvate, ammonia and phenol or its corresponding derivative (13).

During the course of further investigation on the catalytic properties of tyrosine phenol-lyase, we have studied the synthesis of trihydroxyphenylalanine through the β-replacement reaction between S-methyl-L-cysteine and trihydroxybenzenes. This paper deals with the synthesis of trihydroxy-L-phenylalanine from S-methyl-L-cysteine and pyrogallol.

Methods

The enzymatic synthesis of trihydroxyphenylalanine was carried out at 30°C for 2 hr in a reaction mixture containing 100 μmoles S-methyl-

L-cysteine, 40 µmoles of pyrogallol, 4 µmoles of pyridoxal phosphate, 250 µmoles of potassium phosphate buffer, pH 8.0, and 150 µg of crystalline L-tyrosine phenol-lyase in a total volume of 4 ml. Crystalline tyrosine phenol-lyase was prepared from cells of *E. intermedia* A-21 grown in a bouillon peptone medium supplemented with L-tyrosine, according to the methods of Kumagai *et al.* (1).

Results

Chromatographic examination of the incubation mixture on thin layer (Merck, Kieselgel GF_{254}, developed with butanol-acetic acid-water 4:2:1 (v/v)) and ion-exchange resin (Fig. 1) showed the presence of a

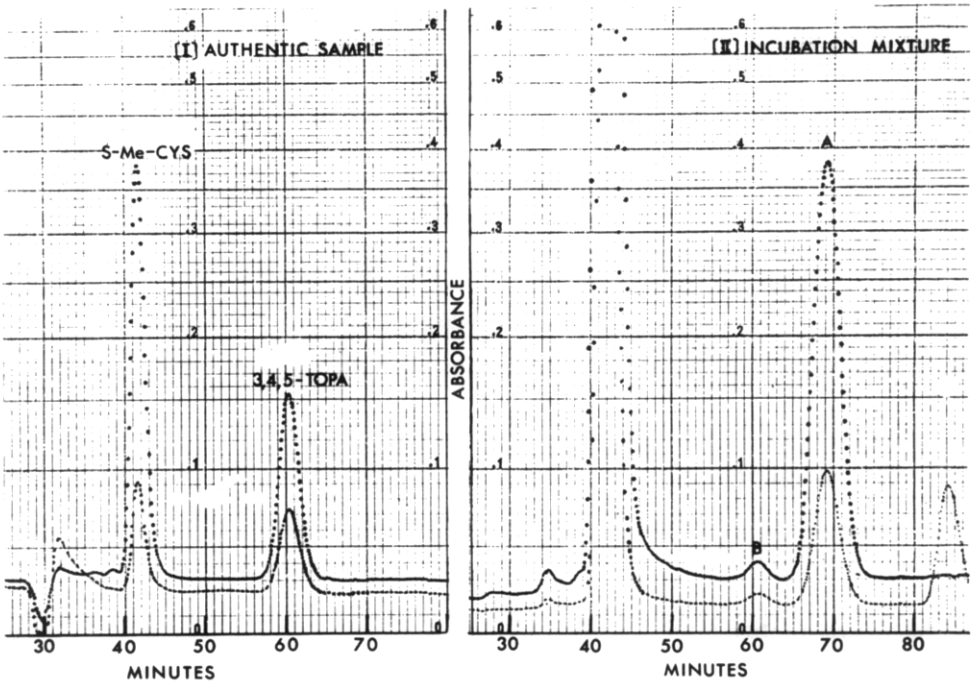

Fig. 1. Ion-exchange chromatography of an incubation mixture of S-methyl-L-cysteine and pyrogallol with tyrosine phenol-lyase. The chromatography was done with a Yanagimoto model LC-5S automatic amino acids analyzer. Samples were placed on a 70 x 0.9 cm column of Aminex A-4 resin and eluted with 0.2 N sodium citrate buffer, pH 4.25. Samples were (I), standard (0.1 µmole of S-methyl-L-cysteine and 3,4,5-trihydroxyphenylalanine); (II), incubation mixture (1-ml aliquot of a 4 ml mixture). 3,4,5-Trihydroxyphenylalanine was a kind gift from F. Hoffman-La Roche & Co. AG. Basel.

new ninhydrin-positive compound. In the amino acid analysis, beside the new main peak (Peak A), a small peak (Peak B) was observed at the same retention time with 3,4,5-trihydroxyphenylalanine. The amount of the compound (Peak A) was dependent on the reaction time and on the amount of enzyme used. With boiled enzyme, the synthesis of the compound was not observed.

When pyrogallol was replaced by hydroxyhydroquinone in the reaction mixture, a ninhydrin positive compound was observed at different retention time. With phloroglucinol, no new ninhydrin positive spot was detected.

The enzymatically synthesized trihydroxyphenylalanine from pyrogallol (Peak A in Fig. 1) was isolated from a large scale incubation mixture, as its N-acetyl-tri-O-acetoxy-methylester. The incubation was carried out with 40 times large scale reaction mixture of that described in the text. Under these conditions, approximately 0.5 mmoles of the compound were synthesized, calculated from the chart of automatic amino acid analysis using authentic 3,4,5-trihydroxyphenylalanine as the standard.

The deproteinized incubation mixture was applied to a Dowex 50-W x 8 column (12.5 x 2.4 cm, Na^+-form). The elution was carried out with 0.6 M NaCl solution containing 0.05 M sodium sulfite. The pH of the solution was adjusted by the addition of hydrochloric acid. The column was washed with one liter of the elution agent of pH 1.5 and then the product was eluted with that of pH 5.0. The elution of the product was followed by the ninhydrin reaction as well as by the examination with an automatic amino acid analyzer. Fractions containning the product were acidified with hydrochloric acid and evaporated to dryness at 50°C under reduced pressure. The solid obtained was then extracted with methanol containing hydrogen chloride to separate from contaminated inorganic salts, and evaporated again to dryness.

Remaining water was azeotropically removed with benzene. The residual solid was then treated with thionylchloride (2 ml) in methanol (10 ml) overnight at room temperature. After the evaporation of the mixture to dryness *in vacuo*, the obtained syrup was treated with acetic anhydride (10 ml) in pyridine (10 ml) overnight at room temperature. After removing acetic anhydride and pyridine by evaporation *in vacuo*, the reaction mixture was treated with 30% hydrogen peroxide in anhydrous acetic acid for one hour on the boiling water bath, to transform possibly remained methylated and acetylated S-methyl-L-cysteine into its sulphone, which was easily separated from the trihydroxyphenylalanine derivative by chromatography on a silicic acid column (Mallinckrodt, eluted with benzene-ethanol 9:1 (v/v)). Further purification of the obtained trihydroxyphenylalanine derivative was performed by a preparative thin layer chromatography (Merck, Kiesel gel GF_{254}, developed with benzene-ethanol 9:1 (v/v)).

The chemical structure of the purified derivative of the enzymatically synthesized product was assigned to N-acetyl-2,3,4-tri-O-acetoxy-L-phenylalanine methylester, based on the following spectral data. The UV spectrum of the compound showed a strong absorbance at 262 nm. The MS spectra of the derivative (Fig. 2) gave $[M + 1]^+$ ion peak at m/e 396 (consistent with the molecular formula + 1H, C_{18} H_{22} NO_9), and the

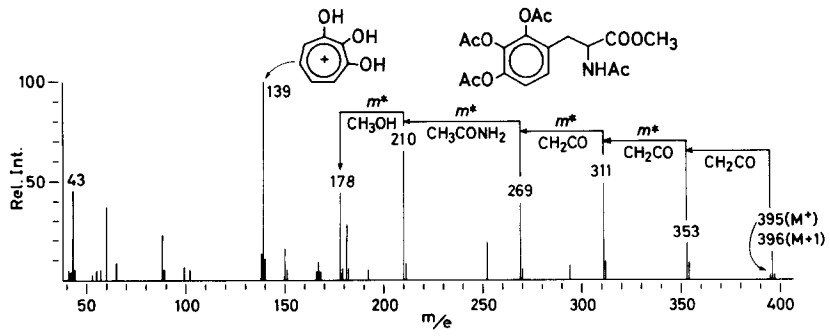

Fig. 2. MS spectrum of N-acetyl-2,3,4-tri-O-acetoxy-phenylalanine methylester synthesized by tyrosine phenol-lyase. The MS spectrum was taken with a Hitachi RMS-4, 70eV, direct inlet 120°C.

successive loss of three ketenes, acetamide and methanol from the molecular ion (M^+ 395 → m/e 353 → m/e 311 → m/e 269 → m/e 210 → m/e 178), and the appearance of m/e 139 (base peak), trihydroxytropylium cation, suggested the presence of triacetoxybenzyl moiety and N-acetyl and carbomethoxy group in the molecule. As shown in Table I, the NMR spectrum of the compound in CD_3OD also revealed N-acetyl (3H), carbomethoxy (3H) and three acetoxy (9H) signals. From the coupling constant (J_{AB} = 9.0 Hz *ortho* coupling) of the AB pattern in the lowest field, the acetoxy signals must be attributed to 2,3 and 4 position of the aromatic ring and consequently, the structure of the compound was unequivocally concluded to be N-acetyl-2,3,4-tri-O-acetoxy-phenylalanine methylester, considering the multiplet signals at δ3.00 (2H) and δ4.60 (1H) which were assigned to benzyl protons and α-proton of the amino acid respectively.

Table I. NMR data of N-acetyl-2,3,4-tri-O-acetylphenylalanine methylester synthesized by tyrosine phenol-lyase.

δ (assignments)
7.22, 7.12 (2H, AB, J = 9.0)
4.60 (1H, m*, $-\underline{CH}-\underline{N}-$)
3.70 (3H, s**, $-COO\underline{CH_3}$)
3.00 (2H, m, $-\underline{CH_2}-$ ph)
2.30, 2.25, 2.22 (each 3H, s, $-OCO\underline{CH_3}$)
1.90 (3H, s, $-\underline{N}-COCH_3$)

* m, multiplet
** s, singlet

Table I. The NMR spectrum was taken in CD_3OD with internal tetramethyl silane by a Hitachi R-24 Spectrometer.

The absolute configuration of the compound was assigned to L-series from the fact that the ORD spectra of this compound gave the same positive plain curve as that of the authentic N-acetyl-3,4-di-O-acetoxy-L-phenylalanine methylester.

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