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ORIGINAL ARTICLE

Biosynthesis of [1-^{15}N] L-tryptophan from ^{15}N labeled anthranilic acid by fermentation of *Candida utilis* mutant

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Abstract A new method for synthesizing the labeled L-tryptophan is described in this work. L-Tryptophan, labeled with 98% ^{15}N at position 1 was synthesized from the labeled anthranilic acid using *Candida utilis* mutants. The conversion ratio of ^{15}N of 50% was achieved. The labeled anthranilic acid was synthesized by [^{15}N] phthalimide that was prepared by 99.34% [^{15}N] urea and phthalic anhydride in *ortho*-xylene medium at 140°C and under atmospheric pressure.

Keywords [1-^{15}N] L-Tryptophan · [^{15}N] Anthranilic acid · *Candida utilis* mutant

Introduction

In recent years, [^{15}N] L-tryptophan has provided great help for studying L-tryptophan metabolism mechanism (Rapparini et al. 1999) and the structures of tryptophan-N-glucoside and violacein by determining kinetic isotope effects (Diem et al. 2000; Ruhul Momen and Hoshino 2000). Until now, there are two ways for synthesizing ^{15}N labeled L-tryptophan (1) ^{15}N labeled L-tryptophan is obtained from 3-indolylpyruvic acid and [^{15}N] ammonium nitrate by a chemical synthesis method (Rulin 1986); (2) The ^{15}N labeled indoles can be converted to the correspondingly labeled L-tryptophan using *E.coli* (Berg et al. 1988; Unkefer et al. 1991). However, both for [^{15}N] L-tryptophan require a multistep reaction, and the conversion of ^{15}N is very low.

This paper reports a new method for the fermentative synthesis of [1-^{15}N] L-tryptophan by using the labeled anthranilic acid as a precursor, obtaining a high conversion ratio of ^{15}N. [^{15}N] Anthranilic acid is synthesized from [^{15}N] phthalimide through the Hofmann reaction and [^{15}N] phthalimide is synthesized from [^{15}N] urea via the same chemical route as reported before (Murray and Williams 1958; Ott 1981). However, the experimental conditions in this work are different from that by Murray and Williams (1958) and Ott (1981). The reaction is conducted in the *ortho*-xylene medium at 140°C and under atmospheric pressure. The optically pure L-tryptophan is synthesized from the enriched anthranilic acid by *Candida utilis* mutants. The synthetic scheme of [1-^{15}N] L-tryptophan is shown in Fig. 1.

Material and methods

Materials

The wild type strain *C. utilis AS2.566* was purchased from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). The *Candida* cells were muted and selected with diethyl sulfate and ultraviolet radiation, and then were treated with 5-methyl-DL-tryptophan. [^{15}N] Urea was provided by the Shanghai Research Institute of Chemical Industry, China.

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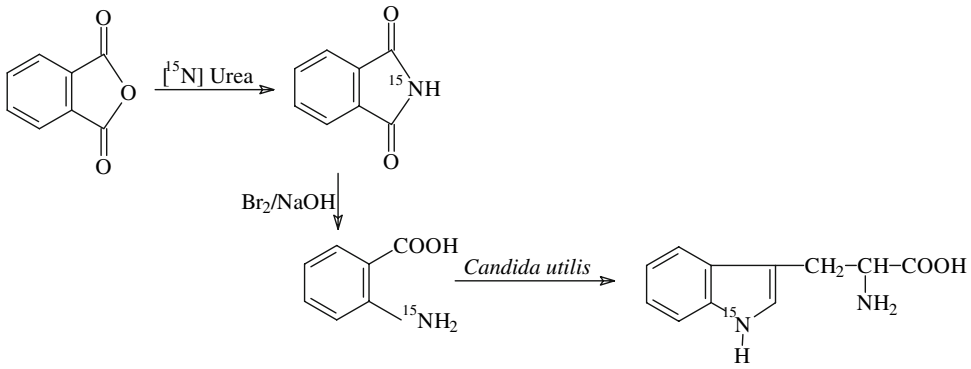

Fig. 1 The synthetic scheme of [1-^{15}N] L-tryptophan

Analysis methods

The labeled L-tryptophan, anthranilic acid and phthalimide were determined using a HITACHI L-7100 HPLC with a Diamonsil C18 column (Dikma Technologies, US). For the assay of the labeled anthranilic acid and phthalimide, the mobile phase was a 30:70 v/v mixture, containing aqueous solution of 75% methanol and 1.8% glacial acetic acid, the detector wavelength was 254 nm, and the flow rate was 1 ml/min. For the determination of the labeled L-tryptophan, gradient elution was used in the conditions of 0–10 min 75–40% B, 10–13 min 40–5% B, 13–17 min 5–75% B (solvent A was 75% methanol aqueous solution, solvent B was the mixture of 0.05 mol/l NaAc and HAc aqueous solution.). The detector wavelength was 360 nm and the flow rate was set to 1 ml/min.

Synthesis of [^{15}N] phthalimide

A mixture of 11.1 g of phthalic anhydride, 1.5 g of 99.34% [^{15}N] urea and 17.8 ml of *ortho*-xylene was placed in a 250 ml three-necked flask equipped with a reflux condenser and a thermometer. The mixture was then slowly heated to 140°C when it was swirled slowly. After maintaining at this temperature for 90 min, it was cooled down to room temperature, filtered and dried in vacuum. The solvent was reclaimed from the mixture for reuse. As a result, 9.53 g of the mixture of the labeled phthalimide and phthalic anhydride was obtained.

The mixture reacted with 8.7 ml of ethanol in the above apparatus at 78°C for 90 min, and after cooling down to room temperature, the newly formed mixture was filtered, and dried in vacuum. The yield of ^{15}N labeled phthalimide (99% ^{15}N) was 3.63 g (99%). MS (*m*/*z*): 148 (parent peak), 104, 76, 50. NMR (δ): 7.85–7.86, multiplet, 4H; 7.57–7.59, multiplet, 1H; taken in C_5D_5N.

Synthesis of [^{15}N] anthranilic acid

A quantity of 1.27 ml of bromine was dissolved in 73 ml of 20% sodium hydroxide solution and the mixture was cooled in an ice-salt bath until the temperature was about 0°C. Around 3.63 g of finely powdered [^{15}N] phthalimid was gradually added into the cold, alkaline sodium hypobromite solution, and the mixture was swirled vigorously. After the added [^{15}N] phthalimid was dissolved, the solution with light brown color was further cooled to −5°C. About 2.6 g of sodium hydroxide was then added and the mixture was swirled vigorously for 30 min. The mixture was heated to 70°C in a steam bath and maintained at that temperature for 5 min. Then, 0.9 ml of 36% sodium bisulfate solution was added to the reaction mixture and the mixture was filtered. The mixture was cooled to room temperature and concentrated HCl was added slowly until the reaction mixture was just slightly basic. The anthranilic acid was precipitated by slowly adding glacial acetic acid. Crystals were then filtered and washed with small portions of cold water until the odor of acetic acid was no longer detectable. The solid was dried in vacuum. Finally, 2.3 g (99%) of [^{15}N] anthranilic acid (98.63% ^{15}N) was obtained. MS (*m*/*z*): 138 (parent peak), 120, 92, and 65. NMR (δ): 6.559–6.586, multiplet, 5H; 7.220–7.248, multiplet, 4H; 6.727–6.746, multiplet, 3H; 7.803–7.819, multiplet, 6H; taken in CD_3OD.

Synthesis of [1-^{15}N] L-tryptophan

Candida mutants were inoculated into the seed medium containing 5% glucose, 0.5% $(NH_4)_2SO_4$, 1% corn hydrolysate, 0.05% $MgSO_4$, 0.15% K_2HPO_3 and 1.5% $CaCO_3$ in deionized water with pH adjusted to 6.8. The cultures were grown at 30°C and 150 rpm for 18 h, 3 ml was inoculated into 30 ml of the fermentation medium containing 6% glucose, 1% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.15% K_2HPO_3, 2% $CaCO_3$ and 0.05% [^{15}N] anthranilic acid in deionized

water with pH adjusted to 6.8. The fermentation was carried out at 30°C and 150 rpm for 144 h. Then, 1.5 g/l labeled anthranilic acid dissolved in ethanol (50%), 50 g/l glucose and 2.1 g/l ammonium sulfate were fed into the fermentation medium in a batch way at intervals of 12 h after 36 h of fermentation.

The cells and $CaCO_3$ were removed by centrifugation at 15,000*g* for 15 min. The resulting supernatant was adjusted to pH 4.5 with oxalic acid. By using a strongly acidic cationic exchange resin, the labeled L-tryptophan could be separated from the other components of the supernatant. The column was eluted with a 2% NH_4OH solution. The tryptophan fractions were collected and concentrated to dry. Colored matter dissolved in chloroform was removed by filtration. Furthermore, the residue was dissolved in water. The solution was decolored by the D3520 resin. The tryptophan fractions were collected and concentrated to dry. The residue dissolved in 60% hot ethanol at 70°C. About 0.87 g (98.9%) of labeled L-tryptophan (98% ^{15}N) was obtained after refrigeration and freeze–drying. MS (*m*/*z*): 205 (parent peak), 131, 103, and 77. NMR (δ): 3.887–3.420, multiplet, 1H; 3.202–3.241, multiplet, 2H; taken in D_2O.

Results and discussion

The results of these experiments show that L-tryptophan was labeled at the position 1 with 98% ^{15}N enrichment, almost no dilution of the label substance occurred. This indicates that the effect of ammonium sulfate on tryptophan enrichment is insignificant. The synthesis of [1-^{15}N] tryptophan with [^{15}N] anthranilic acid resulted in 62% conversion of ^{15}N.

Candida mutants can be used for overproducing the labeled L-typtophan on the gram scale from the labeled anthranilic acid. The conversion ratio of the ^{15}N atoms amount from the labeled urea into tryptophan reaches about 50%, which is nearly twice more than the ratios for the indoles conversion method and the chemical synthesis method, reported in the literature (Berg et al. 1988; Unkefer et al. 1991; Rulin 1986). As a result, 3.075 mol of [^{15}N] urea can be converted to 1 mol of [1-^{15}N] L-tryptophan by this work. In fact, considerable amounts of [1-^{15}N] L-tryptophan have been synthesized using this method in our lab. In conclusion, the synthesis scheme of the labeled L-tryptophan proposed here is simpler and more economical than previous methods.

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