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Synthesis and resolution of o-hydroxy-DL-phenylalanine-2-^{14}C

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SUMMARY

o-Hydroxy-DL-phenylalanine-2-^{14}C was prepared by the condensation of o-methoxybenzyl chloride and ethyl acetamidocyanoacetate-2-^{14}C and subsequent hydrolysis. The racemate was resolved by the stereospecific action of chymotrypsin on the amino acid ethyl ester at pH 5.0 to give the two isomers in 26-30% yield.

For the purpose of metabolic studies, we have prepared *o*-hydroxy-D- and *o*-hydroxy-L-phenylalanine-2-^{14}C (hereafter referred to as *o*-tyrosine for convenience). DL-*o*-Tyrosine has previously been obtained in 20-25% yield by reaction of salicylaldehyde with hippuric acid [1], thiohydantoin [2], hydantoin [3], or acetylglycine [4], followed by hydrolysis, and reduction of the double bond. The availability of ethyl acetamidocyanoacetate-2-^{14}C and its utilization for the synthesis of DL-tyrosine-2-^{14}C [5] has prompted us to use the analogous method described in this paper for the preparation of DL-*o*-tyrosine-2-^{14}C. The reactions used are described in Scheme 1. *o*-Methoxybenzyl chloride was condensed with ethyl acetamidocyanoacetate-2-^{14}C in the presence of sodium ethoxide to give ethyl 2-(*o*-methoxybenzyl)-acetamidocyanoacetate-2-^{14}C which was hydrolysed and demethylated with hydrobromic acid without it being isolated. The small amount of glycine resulting from the hydrolysis of unreacted starting material was separated from the *o*-tyrosine by ion-exchange chromatography on Dowex 50 (H^+). Glycine-2-^{14}C was eluted from the resin with 1.5 N hydrochloric acid followed by 4 N hydrochloric acid to elute the *o*-tyrosine-2-^{14}C. The condensation product of the reaction was characterized by elementary analysis of a sample prepared from unlabelled starting material.

The resolution of DL-*o*-tyrosine-2-^{14}C was carried out by an enzymatic procedure devised for this particular purpose. This procedure takes advantage

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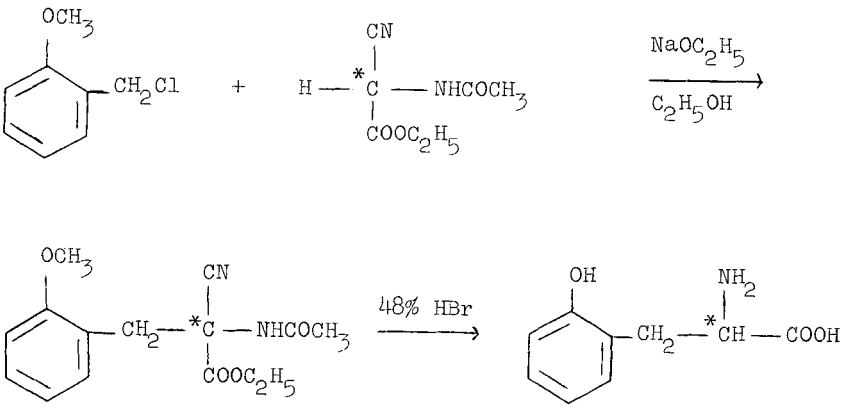
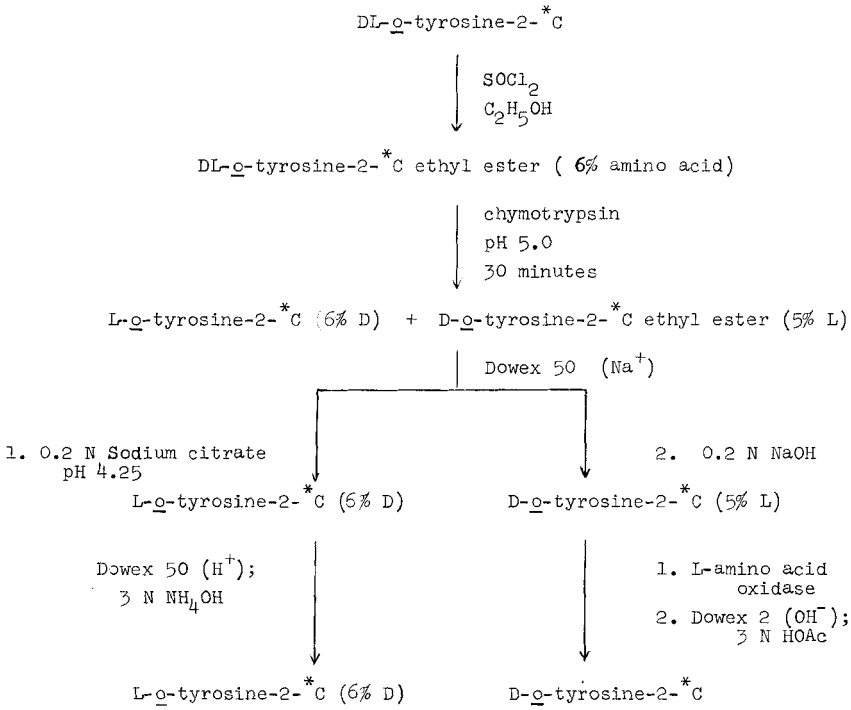
of the stereospecificity of the chymotrypsin-catalyzed hydrolysis of free aromatic amino acid esters at pH 5.0. A study of the kinetics and stereospecificity of this reaction will be reported elsewhere. The different steps in the resolution are described in Scheme 2. The uncrystallized amino acid hydrobromide was converted to the ethyl ester hydrohalide using thionyl chloride [6]. After repeated evaporation of the reaction mixture to remove all of the thionyl



Scheme 1



Scheme 2

chloride, the ester was incubated with chymotrypsin at pH 5.0. The L-*o*-tyrosine-2-^{14}C and the D-*o*-tyrosine-2-^{14}C ethyl ester were then separated by column chromatography on Dowex 50 (Na^+). The L-amino acid was eluted with pH 4.25 sodium citrate buffer, and the D-amino acid ester then eluted and saponified with sodium hydroxide. Both isomers were then desalted using ion-exchange resins.

Because the hydrolysis of DL-*o*-tyrosine ethyl ester by chymotrypsin under these conditions does not go to completion (about 5% of the L-ester remains), the resulting D-isomer was contaminated by about 5% of the L-isomer. This contamination in this case was completely removed by destruction of the L-isomer with L-amino acid oxidase [7].

The esterification of DL-*o*-tyrosine with thionyl chloride in ethanol as described is also incomplete. If the unreacted amino acid is not removed, the resulting L-isomer is contaminated with the D-isomer. The ester can be completely freed of this unreacted starting material by evaporating off the ethanol, adding aqueous sodium carbonate saturated with sodium chloride, and extracting the ester into ethyl acetate. However, this is accompanied by a considerable loss of ester which is either soluble in the aqueous phase or saponified during the manipulation. In order not to lose any material, we have by-passed this purification step with the result that the L-isomer obtained contained about 6% of the D-isomer.

EXPERIMENTAL.

Ethyl 2-(o-methoxybenzyl)-acetamidocyanoacetate.

Ethyl acetamidocyanoacetate (Mann Research Laboratories, New York; 681 mg; 4 mmole) was dissolved in 5 ml of ethanol containing 96 mg of sodium. *o*-Methoxybenzyl chloride [8] (b.p. 62-65° C/2 mm; 783 mg; 5 mmole) was then added, and the mixture was refluxed for 3 hours. Addition, with vigorous shaking, of 25 ml of water to the suspension at 0° C yielded 810 mg (70%) of the desired product, which had m.p. 142-143° C after recrystallization from ethanol-water.

Anal. Calcd. for $C_{15}H_{18}O_4N_2$: C, 62.0; H, 6.2; N, 9.65

Found : C, 62.4; H, 6.2; N, 9.6

DL-o-Tyrosine-2-^{14}C.

Ethyl acetamidocyanoacetate-2-^{14}C (New England Nuclear Corp., Boston; 13 mg; 500 μC), diluted with 327 mg of cold material was condensed with *o*-methoxybenzyl chloride in 2 ml of ethanol as described above. After refluxing for 3 hours, the cooled mixture was filtered, the ethanol was evaporated off under reduced pressure, and the residue was refluxed 5 hours in 10 ml of 48% hydrobromic acid. Excess HBr was removed by repeated evaporation to dryness. Analysis of an aliquot with an amino acid

analyzer indicated a yield of 55% of *o*-tyrosine based on ethyl acetamidocyanoacetate.

The pH of the solution was adjusted to 1.0, and the solution was placed on a 2 × 15 cm column of Dowex 50 (H^+; 100-200 mesh). The column was washed with 1.5 N HCl until all the glycine had emerged, and then 4.0 N HCl until the effluent was ninhydrin negative. The effluent was evaporated to dryness.

Resolution of DL-o-tyrosine-2-^{14}C.

To the above residue was added 2 ml of ethanol containing 0.1 ml of purified thionyl chloride, and the mixture was refluxed 1 hour. The reagents were evaporated off under reduced pressure and the esterification was repeated. After repeated evaporation to remove the thionyl chloride, the DL-*o*-tyrosine--2-^{14}C ethyl ester hydrohalide was dissolved in 10 ml of 0.25 M NaCl, the pH was adjusted to 5.0, and 10 mg of chymotrypsin in 0.25 M NaCl was added. The acid liberated by the hydrolysis of the L-isomer was neutralized during the digestion by the automatic addition of 0.2 M sodium hydroxide with an automatic titration system (Radiometer TTT1). After 30 minutes, the enzyme was precipitated by the addition of 20% sulfosalicylic acid to pH 1.0. The mixture was filtered, with 0.2 M sodium citrate, pH 2.2, being used for rinsing. The solution was then placed on a 1 × 10 cm column of Dowex 50 (100-200 mesh) previously equilibrated with 0.2 M sodium citrate, pH 4.25. The column was washed with 25 ml of the pH 2.2 buffer, and then the L-*o*-tyrosine--2-^{14}C was eluted with 75 ml of the pH 4.25 buffer. The D-*o*-tyrosine-2-^{14}C ethyl ester was then eluted from the column with 50 ml of 0.2 M NaOH and saponified by warming the solution at 45° C for 1 hour.

The L-*o*-tyrosine-2-^{14}C was desalted by passing the solution through a 2 × 10 cm column of Dowex 50 (H^+), and eluting with 3 N NH_4OH. Evaporation of the solution gave 54 mg, corresponding to a yield of 30% based on ethyl acetamidocyanoacetate.

The D-isomer contained about 5% of the L-isomer which was removed by destruction with L-amino acid oxidase. The alkaline column effluent containing the D-*o*-tyrosine-2-^{14}C was neutralized with HCl and adjusted to pH 8.5 with 0.1 M Tris buffer. Oxygen was bubbled through the solution which was incubated at 37° C in the presence of 2 mg of L-amino acid oxidase (*Crotalus adamanteus*) for 16 hours.

The solution was passed through a 2 × 10 cm column of Dowex 2 (OH^-) and the amino acid was eluted with 3 M acetic acid. Repeated evaporation of the effluent and trituration of the residue in ethanol gave 47 mg (26%).

Purity of the Products.

The L- and D-isomers prepared in this manner from unlabelled starting material, when chromatographed on a Beckman model 120B amino acid analyzer, gave constants of 20.0 and 18.6 respectively. These were both raised

to 21.6, the value obtained for a commercial sample of DL-*o*-tyrosine (Nutritional Biochemicals Corp.), after one recrystallization from water.

For verification of the radiochemical purity, each labelled isomer was chromatographed on the amino acid analyzer with the effluent stream directed through the flow-cell of a Nuclear-Chicago scintillation counter. The isomers were radiochemically pure when the columns were eluted with buffers of pH 3.28, 4.25, 5.28 and 10.5. The specific activities were 165 μC/mmole for both isomers.

Optical Purity of the Products.

The optical purity of the isomers prepared from unlabelled starting material exactly as described was established by a standard procedure using L- and D-amino acid oxidase [9]. Analysis by this method indicated that the D-isomer was greater than 99.0% optically pure; the L-isomer contained about 6% of the D-isomer.

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