CHEMICAL SYNTHESIS OF LABELLED INTERMEDIATES IN CYANOGENIC GLUCOSIDE BIOSYNTHESIS

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SUMMARY

Chemical syntheses of [UL-^{14}C]-labelled N-hydroxytyrosine, p-hydroxyphenylpyruvic acid oxime, p-hydroxyphenylacetaldoxime, and p-hydroxyphenylacetonitrile in high yields from L-[UL-^{14}C]-tyrosine are described. These syntheses involve initial conversion of L-tyrosine to p-hydroxyphenylpyruvic acid, which then is allowed to react with hydroxylamine to form p-hydroxyphenylpyruvic acid oxime. N-Hydroxytyrosine is obtained from the latter by reduction with sodium cyanoborohydride, and is oxidatively decarboxylated to p-hydroxyphenylacetaldoxime by treatment with ammonia. Finally, p-hydroxyphenylacetonitrile is obtained by dehydration of the aldoxime with thionylchloride. All synthesized compounds were identified by combined GLC/MS of their trimethylsilyl derivatives. The commercial availability of several specifically labelled ^{14}C, ^{2}H, and ^{3}H tyrosines and of ^{2}H and ^{3}H sodium cyanoborohydride makes the method equally useful for synthesis of various specifically labelled compounds.

KEY WORDS: [^{14}C]-*N*-hydroxytyrosine, [^{14}C]-*p*-hydroxyphenylacetaldoxime, cyanogenic glucoside intermediates.

INTRODUCTION

Sorghum seedlings contain the cyanogenic glucoside dhurrin, and a membrane bound (microsomal) enzyme system from this plant has been found to carry out all but the last step in the biosynthetic sequence which forms dhurrin. The intermediates invoked in this sequence are: tyrosine, *N*-hydroxytyrosine, *p*-hydroxyphenylacetaldoxime, *p*-hydroxyphenylacetonitrile, and *p*-hydroxybenzaldehyde, the last named compound being formed by nonenzymatic decomposition of *p*-hydroxy-

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mandelonitrile.[1,2] Studies on the biosynthesis of dhurrin have been hampered by the difficulties encountered in obtaining the labelled postulated intermediates. Attempts to prepare these by accumulation in enzymatic reaction mixtures were variable; tyrosine is efficiently converted to *p*-hydroxybenzaldehyde by the sorghum microsomes but the intermediates involved are either not observed or barely detectable. When prepared in the absence of mercaptoethanol, the sorghum microsomes accumulate *p*-hydroxyphenylacetaldoxime.[1] However, such microsomal preparations are unstable, often show no activity, and therefore do not constitute an efficient method by which this intermediate can be prepared. When ^{14}C-labelled tyrosine is fed to excised shoots of sorghum seedlings, it is extensively converted (e.g. 49 percent)[3] to dhurrin. Nevertheless, no radioactivity was detectable in any of the intermediates supposedly involved.[3] Efforts were therefore directed towards the chemical synthesis of these labelled intermediates. A method by which this can be achieved and the results obtained are presented in this communication.

MATERIALS AND METHODS

L-[UL-^{14}C]-tyrosine (spec. act. 420 mCi/mmole) was obtained from New England Nuclear. L-Amino acid oxidase (Type I, minimum activity 0.3 U/mg) and catalase (crystalline, 3000 U/mg) were obtained from Sigma Chemical Co. and Mann Research Laboratories, respectively. Sodium cyanoborohydride, $NaBH_3CN$, obtained from Alfa Products, Ventron Corporation, was purified by recrystallization of its dioxane complex.[4] Authentic unlabelled standards were purchased or synthesized as earlier described.[5] Aliquots of reaction mixtures were analyzed by combined gas chromatography/gas proportional counting (GLC/GPC) after freezing in liquid nitrogen, lyophilization to dryness and trimethylsilyl derivatization.[5] The synthesized compounds were identified by co-chromatography of their trimethylsilyl derivatives with authentic standards and finally by combined GLC/MS of the trimethylsilyl derivatives.[5]

[UL-^{14}C]-*p*-hydroxyphenylpyruvic acid

A solution of L-[UL-^{14}C]-tyrosine (250 µCi) in 1 N HCl was taken to dryness under nitrogen. The residue was dissolved in 7.50 ml 4 mM L-tyrosine (30 µmole)

and 2.0 ml 50 mM KH_2PO_4 (pH = 7.2) by heating (Figure 1,A). A solution of 5.0 mg L-amino acid oxidase plus 5.0 mg catalase in 1.0 ml 50 mM KH_2PO_4 (pH = 7.2) was added. After vigorous stirring for 60 min, the reaction was stopped by addition of 1.0 ml 10 N HCl. The protein precipitate formed was removed by centrifugation, washed with 1 ml 1 N HCl, and the combined supernatants taken to dryness by lyophilization. GLC/GPC analysis showed quantitative conversion of labelled tyrosine to *p*-hydroxyphenylpyruvic acid, the only labelled product which could be detected (Figure 1,B). Yield from tyrosine: 30 µmole (100%).

[UL-^{14}C]-*p*-hydroxyphenylpyruvic acid oxime

A lyophilized sample of [UL-^{14}C]-*p*-hydroxyphenylpyruvic acid (30 µmole) obtained as above was dissolved in 2.5 ml 50 mM KH_2PO_4 (pH = 7.2) and 2.0 ml H_2O. To this solution was added 8.33 mg NH_2OH:HCl (120 µmole) dissolved in 1 ml 50 mM KH_2PO_4 (pH = 7.2). After 1 min (Figure 1,C), the reaction mixture was washed with 2 ml ether, cooled to 0°C, and carefully acidified to pH = 4.0 in an ice bath. Radiochemically pure *p*-hydroxyphenylpyruvic acid oxime (GLC/GPC analysis) was obtained by extraction with 6 ml ether. Yield from tyrosine: 22 µmole (72%).

DL-[UL-^{14}C]-*N*-hydroxytyrosine

A lyophilized sample of [UL-^{14}C]-*p*-hydroxyphenylpyruvic acid (30 µmole) was allowed to react for 1 min with 120 µmole NH_2OH:HCl as described above (Figure 1,C), at which time 11.3 mg (180 µmole) $NaBH_3CN$ was added. The reaction mixture was adjusted to pH = 3.5 by slow addition of 1 N HCl and stirred for 1 hr. GLC/GPC analysis showed that nearly all ketoxime had been reduced to the *N*-hydroxyamino acid (Figure 1,D). Surplus $NaBH_3CN$ was destroyed by the addition of conc. HCl to pH = 1 and the reaction mixture lyophilized to dryness. The residue was dissolved in 4 ml 50 M KH_2PO_4 (pH = 7.2). The resulting solution had a pH of 5.5 and was applied to a strongly acidic cation exchange resin (Dowex 50W x 8, H^+-form, 200-400 mesh, 0.3 x 10 cm). After washing with 20 ml H_2O, *N*-hydroxytyrosine was eluted with 1 N pyridine. Fractions of 1 ml were collected in vials kept in dry ice and were immediately taken to dryness by lyophilization. *N*-Hydroxytyrosine eluted in fraction 5 and 6. GLC/GPC analysis showed that besides a 4% impurity

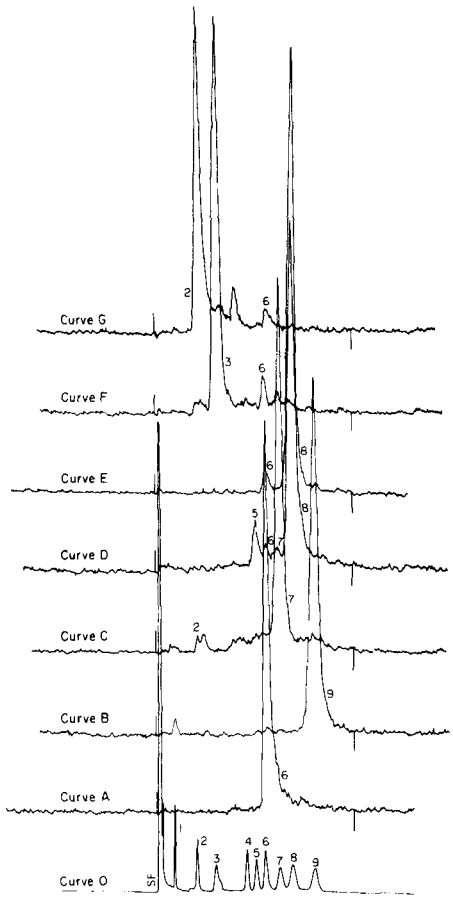

Figure 1. GLC/GPC analysis of reaction mixtures and products obtained. Curve O: GLC mass peaks of trimethylsilyl derivatized standards: 1: *p*-hydroxybenzaldehyde. 2: *p*-hydroxyphenylacetonitrile. 3: *p*-hydroxyphenylacetaldoxime. 4: tyramine. 5: *p*-hydroxyphenyllactic acid. 6: tyrosine. 7: *p*-hydroxyphenylpyruvic acid oxime. 8: *N*-hydroxytyrosine. 9: *p*-hydroxyphenylpyruvic acid. Curves A-G: Radiochromatograms of: A: L-[UL-^{14}C]-tyrosine. B: Enzymatic synthesis of [UL-^{14}C]-*p*-hydroxyphenylpyruvic acid. C: Chemical synthesis of [UL-^{14}C]-*p*-hydroxyphenylpyruvic acid oxime. D: Chemical synthesis of [UL-^{14}C]-*N*-hydroxytyrosine. E: Purified [UL-^{14}C]-*N*-hydroxytyrosine. F: Chemical synthesis of [UL-^{14}C]-*p*-hydroxyphenylacetaldoxime. G: Chemical synthesis of [UL-^{14}C]-*p*-hydroxyphenylacetonitrile. Column: 3% SP 2250 on 80/100-mesh Supelcoport. 6 ft. x 4 mm i.d. glass. Typical temperature program: 6 min at 130°C, temperature rise 10°C/min, 15 min at 170°C.

of tyrosine, no other labelled products were present in these fractions (Figure 1,E). Yield: 27 μmole (90% from tyrosine).

[UL-^{14}C]-*p*-hydroxyphenylacetaldoxime

A lyophilized sample of DL-[UL-^{14}C]-*N*-hydroxytyrosine (10 μmole) obtained as above was dissolved in 700 μl 1 N NH_3. After stirring for 2 hrs at room temperature, the reaction mixture was frozen in liquid nitrogen and lyophilized to dryness. GLC/GPC showed an 86% radiochemical yield of *p*-hydroxyphenylacetaldoxime (Figure 1,F). The residue was dissolved in EtOH and applied to a silica gel thin layer plate (Bakerflex 1B) which was developed in benzene: EtOAc (5:1).[1] The aldoxime spot (Rf = 0.18) was located on the plate by use of a radiochromatogram scanner (Packard Model 7201) and by comparison with an authentic standard. The area was then scraped off the TLC plate and the compound eluted with a total of 6 ml EtOH. The EtOH extracts were clarified by centrifugation and taken to dryness in a stream of nitrogen. Yield: 8.1 μmole (73% from tyrosine).

[UL-^{14}C]-*p*-hydroxyphenylacetonitrile

A lyophilized sample of [UL-^{14}C]-*p*-hydroxyphenylacetaldoxime (10 μmole) obtained as above was dissolved in 1.0 ml dried benzene in a 5 ml ampule, heated to 45°C, and a solution of 200 μl $SOCl_2$ in 800 μl benzene was slowly added.[6] The contents were refluxed for 5 min at 90°C and taken to dryness in a stream of nitrogen. GLC/GPC showed an 72% radiochemical yield of *p*-hydroxyphenylacetonitrile (Figure 1,G). The residue was purified by TLC as described above (RF = 0.45). Yield: 7.1 μmole (51% from tyrosine).

RESULTS AND DISCUSSION

All of the compounds synthesized here are stable when stored as lyophilized samples in a desiccator. However, when dissolved in aqueous media, *p*-hydroxyphenylpyruvic acid oxime undergoes a concerted decarboxylation/dehydration reaction to yield *p*-hydroxyphenylacetonitrile. This type of reaction for ketoximes has been reported earlier.[11] To minimize the decomposition, the *p*-hydroxyphenylpyruvic acid oxime sample used for *N*-hydroxytyrosine synthesis was not

prepurified (Figure 1,C) and after addition of $NaBH_3CN$, the pH of the reaction mixture was lowered to pH = 3.5 in order to increase the rate of reduction. A further decrease in pH results in partial hydrolysis of the ketoxime to *p*-hydroxyphenylpyruvic acid which subsequently is reduced to *p*-hydroxyphenyllactic acid. However, if these two acids are formed as by-products, they and *p*-hydroxyphenylacetonitrile all elute in the H_2O eluate during the following ion exchange purification step and are therefore easily separated from *N*-hydroxytyrosine (Figure 1,D and E). When dissolved in 1 N ammonia, *N*-hydroxytyrosine was found to undergo an oxidative decarboxylation to *p*-hydroxyphenylacetaldoxime. This type of reaction for *N*-hydroxyamino acids has not earlier been described. The rate of conversion is linear with time and the reaction is complete after 90 minutes. If the reaction is carried out in a nitrogen atmosphere, less than 10% of the *N*-hydroxytyrosine is converted to *p*-hydroxyphenylacetaldoxime. Thus molecular oxygen serves as the oxidant in the reaction. *N*-Hydroxytyrosine is more stable when dissolved at lower pH. When kept in a phosphate buffer at pH = 7.2, less than 5% of the *N*-hydroxytyrosine is converted to *p*-hydroxyphenylacetaldoxime in 90 min. *N*-Hydroxytyrosine is stable for several days when stored in concentrated hydrochloric acid. During the chemical synthesis of *N*-hydroxytyrosine, especially in those cases where very high specific radioactivity is wanted and therefore no carrier material is added, care should be taken only to use clean glassware. If impurities such as ferric ions are present, the *N*-hydroxytyrosine synthesized will be oxidized and *p*-hydroxyphenylacetaldoxime obtained as the final product.

The individual reactions here described were examined and optimized by extensive use of the combined gas chromatography/gas proportional counting analytical procedure described earlier.[5] Multiple attempts to design a thin layer chromatographic separation method involving *N*-hydroxytyrosine failed due to the instability of this compound. Besides the oxidative decarboxylation reaction described above, *N*-hydroxytyrosine has earlier been reported to disproportionate into tyrosine and *p*-hydroxyphenylacetaldoxime when refluxed under nitrogen.[12] When *N*-hydroxytyrosine was applied to cellulose thin layer chromatograms, the formation of these degradation products was also observed (Figure 2,A). The

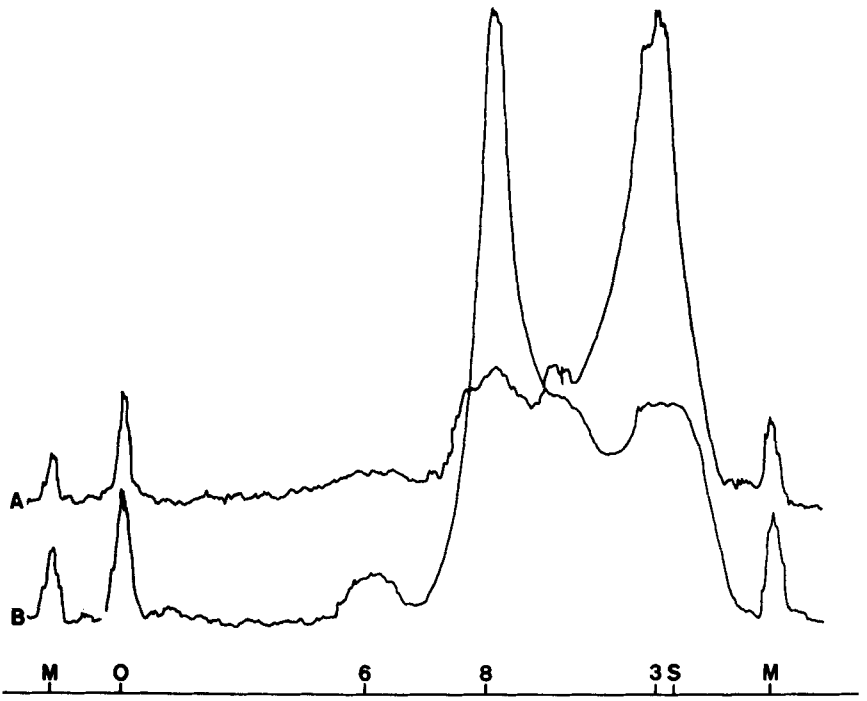

Figure 2. Thin layer chromatography of [UL-^{14}C]-*N*-hydroxytyrosine (Eastman 13254 Cellulose, 0.2 μCi (12 μg) of pure [UL-^{14}C]-*N*-hydroxytyrosine (Figure 1,E) were applied in each experiment). A: normal atmosphere. B: nitrogen atmosphere. Solvent system: isopropanol-formic acid-water (20:1:5). M: marker. O: origin. S: solvent front. For identification of standards see numbering on Figure 1.

decomposition of *N*-hydroxytyrosine could again be diminished by performing the thin layer chromatographic procedure in a nitrogen atmosphere by use of TLC plates pre-equilibrated under nitrogen (Figure 2,B). However, it was never possible to obtain more than approximately 10% yield when reisolating the *N*-hydroxytyrosine from the TLC plates. High voltage electrophoresis and high pressure liquid chromatography also proved unsuccessful as isolation and purification techniques. The spot on the TLC believed to be *N*-hydroxytyrosine produced a characteristic orange color when treated with ninhydrin. The discrepancies in

data earlier reported upon the color reaction of α-*N*-hydroxyamino acids with ninhydrin[13] is understandable in view of the extensive decomposition reactions which may have occurred during the different experimental conditions used.

The method described here for the chemical synthesis of various labelled intermediates involved in the biosynthesis of the cyanogenic glucoside dhurrin is advantageous for several reasons. First of all, the commercial availability of several specifically labelled ^{14}C, ^{2}H, and ^{3}H tyrosines and of ^{2}H and ^{3}H sodium cyanoborohydride makes the method equally useful for the chemical synthesis of a range of specifically labelled intermediates. Hitherto *p*-hydroxyphenylacetaldoxime had been synthesized by a pinacol-pinacolone type rearrangement of synephrine to give *p*-hydroxyphenylacetaldehyde[7] which then was allowed to react with hydroxylamine.[8] This method gives low yields and isotopically labelled synephrine is not commercially available. The method is therefore not easily applied for synthesis of the labelled compound. [1-^{14}C]-*p*-Hydroxyphenylacetaldoxime has been synthesized using *p*-hydroxybenzaldehyde and [^{14}C]-nitromethane as starting materials.[9] However, only aldoxime specifically labelled in C-1 can be synthesized by use of this procedure, and the yield (8%) is low compared to the 73% yield obtained by the procedure reported here. [1-^{14}C]-*p*-Hydroxyphenylacetonitrile has been reported synthesized from 4-benzyloxybenzylchloride and [^{14}C]-KCN.[10] Again this method restricts the choice of labelling to 1-^{14}C and only a 5% yield was obtained compared to 51% in the present study. By the use of other amino acids as starting materials, the procedure reported here should prove useful for the chemical synthesis of a whole range of isotopically labelled intermediates involved in the biosynthesis of different cyanogenic glucosides and glucosinolates.

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