SYNTHESIS OF MALEYLACETOACETATE-2-^{14}C, MALEYLACETONE-1-^{14}C AND HOMOGENTISIC ACID γ-LACTONE-α-^{14}C.[1]

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SUMMARY

Syntheses of the title compounds were accomplished by a sequence of reactions involving Friedel Crafts ^{14}C-acetylation of p-dimethoxybenzene, Willgerodt rearrangement of 2,5-dimethoxyacetophenone-carbonyl-^{14}C (I) to 2,5-dimethoxyphenylacetic acid-α^{14}C (II), followed by hydrolysis to homogentisic acid γ-lactone-α-^{14}C (III). Oxygenation of the lactone by an oxygenase from animal liver provided a solution of maleyacetoacetate-2-^{14}C (IV). Decarboxylation of this material leads to a solution of maleylacetone-1-^{14}C (V). Fumarylacetone-1-^{14}C (VI) can be obtained from V by silver ion-catalysed cis-trans isomerization.

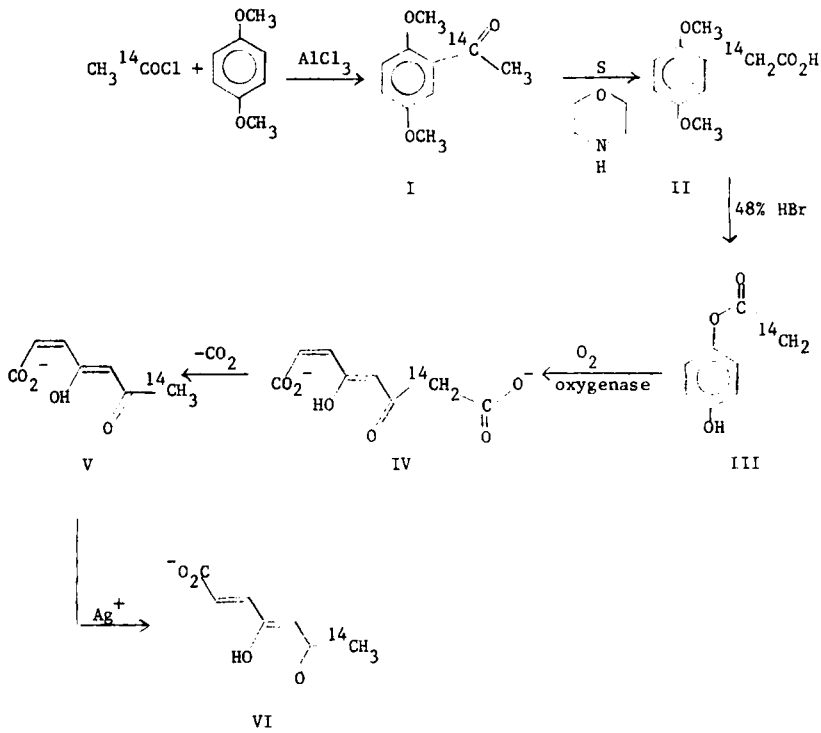
INTRODUCTION

Because of our interest in the mechanism of enzyme-catalyzed cis-trans isomerization of maleylacetoacetoacetate and maleylacetone to fumarylacetoacetate and fumarylacetone, respectively,[2,3] it became necessary to synthesize ^{14}C-labeled substrates. Substrates of relatively high specific activity were desired in order that

attachment of one molecule of substrate per 100 enzyme molecules of molecular weight 35,000 could be detected. Maleylacetone has been enzymically[4] and chemically[5] synthesized while maleylacetoacetate has only been synthesized through oxidation of homogentisic acid catalyzed by an animal liver oxygenase.[6] It seemed most reasonable to prepare both substrates by a mixed chemical and enzymic synthesis.

RESULTS AND DISCUSSION

The synthesis, outlined in Scheme 1, was carried out.



Scheme 1

Sodium acetate-1-^{14}C was prepared almost carrier free from carbon dioxide-^{14}C. It was dissolved in natural glacial acetic acid and used to acetylate p-dimethoxybenzene by the method of Shuford et al[7]. The procedure for the next two steps, the Willgerodt reaction of 2,5-dimethoxyacetophenone-carbonyl-^{14}C (I) to 2,5-dimethoxyphenylacetic acid-α-^{14}C (II) and subsequent hydrolysis to homogentisic acid lactone (III), is essentially the same as that described by Abbott and Smith,[8] except for isolation and purification of the lactone. Previous studies[9] suggested that the carbon skeleton would remain intact during Willgerodt reaction (I → II). That this also obtains in this system can be shown by the comparison of the specific molar activities for II and VI.

In this study is was found that guinea pig liver oxygenase could act directly upon the lactone (III) besides catalyzing the oxidation of its normal substrate, homogentisic acid. Thus the necessity of lactone hydrolysis was avoided.[10] An aqueous solution of sodium maleylaceto-acetate-2-^{14}C (IV) was obtained after precipitation of the oxygenase with metaphosphoric acid, centrifugation, and neutralization of the resulting solution. Maleylacetone-1-^{14}C (V) could be obtained in a similar way if the acidic solution, remaining after removal of denatured oxygenase, was lyophilized and the residue treated with absolute ethanol at ambient temperature.‡ Nmr analysis indicated some cis-trans isomerization of V resulted during decarboxylation.

Compounds II and III, obtained by chemical synthesis, are readily isolable products which could be analyzed and radioassayed. The quantities of IV and V, however, are limited by this method because they are enzymically derived and are relatively unstable.

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‡Crandall et al.[11] carried out decarboxylation on the neutral lyophilized maleylacetoacetate in absolute ethanol at 70°.

EXPERIMENTAL PART

Sodium Acetate-1-^{14}C.

Barium carbonate-^{14}C (25 mCi, specific activity 59.7 mCi/mmole, Amershame-Searle) was treated with sulfuric acid in a vacuum line and used to carbonate an excess of freshly prepared methyl magnesium iodide (2 mmoles in 12.5 ml of ether). The reaction was worked up by the standard steam distillation-titration with base-method.[13] From analysis of subsequent intermediates it can be calculated that the yield of sodium acetate-1-^{14}C was 90% if it is assumed that the specific activity of this product is 59.7 mCi/mmole (vide infra).

2-5-Dimethoxyacetophenone-carbonyl-^{14}C(I).

The above sodium acetate-1-^{14}C was dissolved in 3.0 ml of fresh natural glacial acetic acid and the mixture allowed to equilibrate for two hours. In a three necked flask, having a joint and stopcock on the bottom, 38 ml of carbon disulfide, 7.2 g of p-dimethoxybenzene (Eastman Kodak), and 12 g of phosphorus pentachloride were mixed. The sodium acetate-acetic acid-1-^{14}C mixture was then added dropwise with stirring and the mixture was allowed to stand for one hour before the contents were allowed to drip slowly into another flask containing 20 g of aluminum chloride in 23 ml of carbon disulfide. Addition took place with stirring over 30 min while the flask was cooled in ice. The contents were allowed to warm slowly and remain in the flask overnight. The supernatant was carefully added to a mixture of 25 ml concentrated hydrochloric acid and 225 g of ice. The ice-water-carbon disulfide mixture was then carefully returned to the flask to dissolve the solid residue. The layers were separated and the aqueous phase was extracted with three 50 ml-portions of carbon disulfide. The combined extracts were washed with two 50 ml-portions of 10% sodium hydroxide and then four 50 ml-portions of water (neutral pH). The carbon disulfide

solution was dried overnight over calcium chloride after which it was decanted and the solvent separated by distillation. The final amount of solvent was removed with a stream of nitrogen leaving 6.30 g of a red-brown oil. Analysis by nmr of a similar non-radioactive sample at this stage of the synthesis indicated it to be composed of 75% 2,5-dimethoxyacetophenone and 25% p-dimethoxybenzene. Further purification at this stage was not attempted.

2,5-Dimethoxyphenylacetic Acid-α-^{14}C (II).

To 6.30 g of the 2,5-dimethoxyacetophenone-carbonyl-^{14}C - p-dimethoxybenzene mixture was added 1.34 g of sulfur and 3.64 g of morpholine and the mixture refluxed for 7 hours. The subsequent work up was the same as that given by Abbott and Smith[8] except for a modification to remove p-dimethoxybenzene. The aqueous mother liquor (∿ 240 ml) from the second crystallization was made basic with potassium carbonate and extracted with three 100 ml-portions of ether. The ether was discarded; the aqueous layer was made acidic (pH 2) with 12 N HCl and extracted with three 80 ml-portions of ether. The ether extract was dried over sodium sulfate. The dried ether solution yielded about one ml of a yellow brown residue upon evaporation of the solvent. The oil solidified and was recrystallized from 30 ml of H_2O containing charcoal and cellite yielding crystals m.p. 119.6-121.2° Anal: Found C, 61.83%; H, 6.22%; Calc'd for $C_{10}H_{12}O_4$: C, 61.21%; H, 6.17%. Specific activity: 3.55 μCi/mgC.

The first batch of crystals (2.5 g, m.p. 118-122.5°) were recrystallized from 85 ml of water containing charcoal and cellite and these crystals and the second batch were combined to give a total of 1.667 g of 2,5-dimethoxyphenylacetic acid-α-^{14}C.

Homogentisic Acid γ-Lactone-α-^{14}C (III).

2,5-Dimethoxyphenylacetic acid-α-^{14}C (1.667 g) was mixed with 10.8 ml of colorless 48% hydrobromic acid. The mixture was refluxed for 4 hours

under nitrogen. The solvent was then evaporated in a slow stream of nitrogen. The residue was washed with 3.3 ml of chloroform and the dried residue was extracted for 3 days with 200 ml of benzene in a soxhlet extractor. The cooled benzene solution deposited light tan crystals which, after drying, weighed 0.894 g, m.p. 192.2-193.8° (dec), reported:[8] 188-189°. Anal. Found: C, 63.54, 63.81%; H, 4.09, 3.98%; calc'd for $C_8H_6O_3$: C, 64.00; H, 4.03%. Specific activity: 4.29 μCi/mgC; Theory (based on specific activity of precursor): 4.44 μCi/mgC.

Sodium Maleylacetoacetate-2-^{14}C (IV).

Homogentisate oxidase was obtained from fresh guinea pig livers by the method of Edwards and Knox.[6] Twenty ml of 0.025 M sodium bicarbonate was saturated with a 5% CO_2-95% oxygen mixture. To this was added 1.0 ml of a 0.03 M neutralized ascorbic acid solution and 12.3 g of the crude frozen oxygenase. After equilibration of the mixture with the 5% CO_2-95% oxygen gas phase, 29.5 mgs of homogentisic acid γ-lactone-α-^{14}C was added and stirring was continued under the carbon dioxide-oxygen atmosphere. Previous experiments in a different buffer indicated complete reaction after two hours. After 2.25 hours, stirring was stopped, the contents cooled, and 2.0 ml of 20% metaphosphoric acid was added to precipitate the protein and to free the solution of bicarbonate. The suspension was centrifuged at 0° and 33,000 x g for 15 min. The supernatant was adjusted to pH 7 and an aliquot, diluted 100-fold with 0.1 N sodium hydroxide, indicated a sharp symmetrical maximum at 327 nm (O.D. 0.455). The undiluted solution was lyophilized to yield a sodium maleylacetoacetate-2-^{14}C - phosphate salt mixture which was stored in the freezer for further use.

Maleylacetone-1-^{14}C (V).

Enzymic oxidation was carried out in a similar way and after protein precipitation the acidic supernatant from 59.2 mgs of lactone was lyophilized.

Fifty ml of absolute ethanol were added and the mixture agitated at ambient temperature for one hour. The mixture was filtered and the solvent evaporated on a rotary evaporator. The yellow oily residue was extracted with 30 ml of methylene chloride and the solution decanted. The solvent was evaporated and the white solid residue was taken up in 1 ml of chloroform and transferred to a volumetric flask. The chloroform was evaporated and the residue then dissolved in standard phosphate buffer for use in studies with maleylacetone cis-trans isomerase. At this stage uv examination of the aqueous solutions of a few parallel preparations indicated both maleylacetone and fumarylacetone to be present; the maleylacetone content varied between 70 and 40%.

In order to obtain the specific activity of the maleylacetone (and fumarylacetone) product another preparation was carried out in which the product was converted to fumarylacetone. Oxidation of 94 mgs of homogentisic acid γ-lactone-α-^{14}C was accomplished with 20 g of the crude liver oxygenase in the same buffer as described above. The suspension, which also contained 16 mgs of ascorbic acid, was stirred in the 5% CO_2-95% O_2 atmosphere for 6 hours whereupon it was cooled and an equal volume of absolute ethanol was added. After the mixture was centrifuged at 35,000 x g for 5 min the ethanol from the supernatant was evaporated at reduced pressures. The remaining solution was acidified to pH 2.35 with dilute nitric acid and the solution lyophilized. The residue was taken up in 6 ml of ethanol and 5 ml of 0.2 N silver nitrate (in 95% ethanol) was added. A solution of concentrated ammonia-95% ethanol (1:10) was added dropwise until the precipitate that developed upon addition of each drop, darkened.

The centrifuged residue was washed with a minimum amount of absolute ethanol and then dried over calcium sulfate in vacuo. The dried solid was extracted twice with 1.5 ml portions of 1 N nitric acid and the supernatant was extracted continuously with ether for 20 hrs. The ether

extract, dried over sodium sulfate, was evaporated in a stream of nitrogen and the residue sublimed at 60° and 100 μ to yield a white solid. The solid was dissolved in 0.01 M phosphate buffer (pH 7.4). One portion was used to obtain the activity by liquid scintillation counting and the other to determine its concentration from its uv spectrum. The spectrum indicated that some ascorbic acid was carried through the purification but their uv maxima are well separated. The activity of fumarylacetone determined this way was 446 μCi/mmole.

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