A Synthesis of C^{14}-Labeled Pyridoxol[1,2]

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Pyridoxol hydrochloride labeled with C^{14} at the 5-hydroxymethyl group was synthesized. The carbon atom of the 5-hydroxymethyl group of pyridoxol hydrochloride was removed and reintroduced as C^{14} through the following sequence of reactions: CH_2OH, COOH, $COOCH_3$, $CONH_2$, NH_2, C*N, C*OOH, C^*H_2OH. As high as two-thirds of the specific activity of the C^{14}-labeled potassium cyanide could be introduced into pyridoxol hydrochloride. The chemical and radiochemical purity of the final product was almost 100% as was shown by column chromatography, paper chromatography (two solvent systems), paper electrophoresis, and isotope dilution assay.

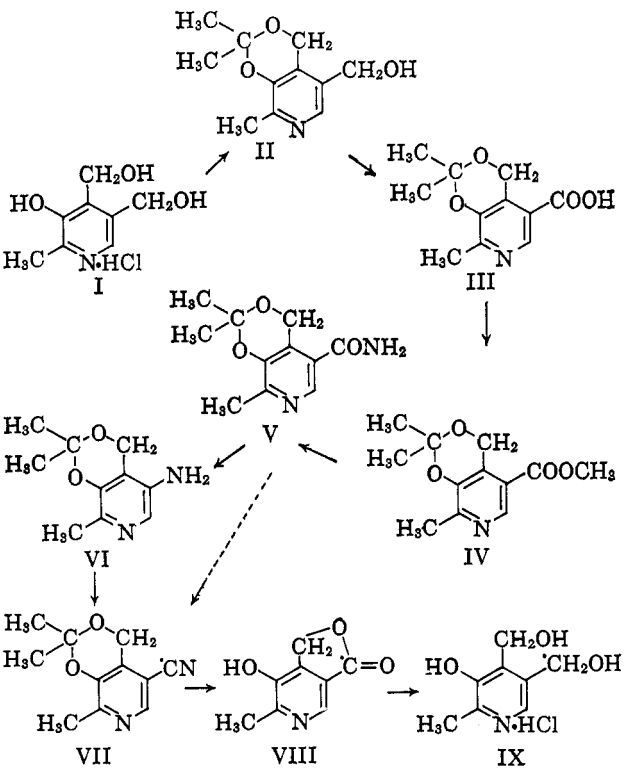
Pyridoxol labeled with C^{14} at carbon atom 2 of the pyridine ring has been used[3] to elucidate its catabolism in *Pseudomonas* SP. IA. However, until recently,[4] there had been no report concerning the synthesis of C^{14}-labeled pyridoxol. Our interest in the catabolism of vitamin B_6 in animals and its possible role in lipid metabolism called for a practical synthetic route to pyridoxol labeled with C^{14} of high specific activity.

In devising a synthetic route to a desired compound, it is advisable to choose a scheme in which, if possible, the label is introduced near the end of a sequence of reactions, so as to maintain as high an over-all yield based on the isotopic starting materials as possible. However, the common feature of the numerous[5] routes reported so far for the synthesis of pyridoxol is that all of the carbon atoms of its molecule were introduced from the first step of these processes. Attempts to introduce a carbon atom into such precursors so that the resulting compound could be transformed in one or two steps into pyridoxol have been unsuccessful.[6,7]

Pyridoxol labeled with C^{14} at the 5-hydroxymethyl group—the same compound we report here—has been synthesized recently by Al'Perovich[4] who followed the route of Harris and Folkers.[5] In this method the specific activity of the C^{14}-labeled sodium cyanide, which was introduced in the initial step, was subjected to a sixtyfold dilution with cold sodium cyanide in order to provide workable quantities of material. The yield based on the radioactivity of the C^{14}-labeled sodium cyanide was 2.5%; the radiochemical purity of the final product was not reported.

Two factors are very important in the use of labeled nutrients that are required by an organism in minute quantities: (1) radiochemical purity and (2) high specific activity. Both requirements are fulfilled in the scheme we followed for the synthesis of the C^{14}-labeled pyridoxol. The removal of one of the two hydroxymethyl groups of pyridoxol (I, Scheme I) and its reintroduction as C^{14} through the following sequence of reactions, CH_2OH, COOH, $COOCH_3$, $CONH_2$, NH_2, C*N, C*OOH, C^*H_2OH, seemed feasible. The 5-hydroxymethyl group was preferred over the 4-, mainly because the amino group at the 5-position of pyridine behaves as if it were on a benzene ring. Although 2-methyl-5-aminopyridine gave the corresponding cyanopyridine[8] by the Sandmeyer procedure, there is no report of the successful conversion of a 2- or 4-aminopyridine into a 2- or 4-cyanopyridine by any modification of the diazo procedure.[9]

SCHEME I



An ingenious method[10] has become available in which the phenolic hydroxyl and the 4-hydroxymethyl groups of pyridoxol are protected by the formation of a cyclic ketal with acetone, leaving the 5-hydroxymethyl group free for further reactions. The compound was called "isopropylidene pyridoxine" (II). Compound

(1) This work was presented before the Organic Chemistry Division at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., 1963.

(2) This compound is generally known as pyridoxine. However, according to the I.U.P.A.C. 1957 Definitive Rules for Nomenclature of the Vitamins [*J. Am. Chem. Soc.*, **82**, 5581 (1960)], the term *pyridoxine* has been extended to designate all naturally occurring pyridine derivatives with vitamin B_6 activity.

(3) V. W. Rodwell, B. E. Volcani, M. Ikawa, and E. E. Snell, *J. Biol. Chem.*, **233**, 1548 (1958).

(4) N. E. Al'perovich, *Metody Polucheniya i Izmeren. Radioaktivn. Preparatov, Sb. Statei*, 37 (1962); *Chem. Abstr.*, **59**, 1584 (1963).

(5) For example: S. A. Harris and K. Folkers, *J. Am. Chem. Soc.*, **61**, 1245 (1939); P. G. Stevens, *Chem. Abstr.*, **49**, 6315 (1955); A. Cohen, J. W. Haworth and E. G. Hughes, *J. Chem. Soc.*, 4374 (1952); N. Elming and N. Clauson-Kaas, *Acta Chem. Scand.*, **9**, 23 (1955); E. E. Harris, R. A. Firestone, K. Pfister, 3rd, R. R. Boettcher, F. J. Cross, R. B. Currie, M. Monaco, E. R. Peterson, and W. Reuter, *J. Org. Chem.*, **27**, 2705 (1962).

(6) L. A. Perez-Medina, R. P. Mariella, and S. M. McElvain, *J. Am. Chem. Soc.*, **69**, 2574 (1947).

(7) C. J. Argoudelis, Ph.D. Thesis, University of Illinois, 1961; C. J. Argoudelis and F. A. Kummerow, *J. Org. Chem.*, **26**, 3420 (1961).

(8) C. Rath and F. Schiffmann, *Ann.*, **487**, 127 (1931).

(9) H. S. Mosher, "Heterocyclic Compounds," Vol. I, R. C. Elderfield, Ed., John Wiley and Sons, Inc., New York, N. Y., 1950, p. 555.

(10) A. Cohen and E. G. Hughes, *J. Chem. Soc.*, 4384 (1952).

II was obtained in almost quantitative yield by the procedure of Korytnyk and Wiedeman.[11] The oxidation of II to III was accomplished with potassium permanganate in a weak alkaline solution. Although application of the Schmidt[12] reaction to III may yield the desired amino compound VI, only the lactone of 5-pyridoxic acid could be isolated in spite of the precautions which were taken to avoid extensive contact of the sulfuric acid (catalyst) with the compound. It is obvious that under the acidic conditions of the experiment the isopropylidene group was hydrolyzed, and the derived 5-pyridoxic acid formed the lactone instead of reacting with the hydrazoic acid. The methyl ester of III, compound IV, was prepared with diazomethane and ammonolysis of IV yielded the amide V.

Free or methylated phenolic hydroxyl groups present in aromatic amides facilitate[13] rearrangement in the Hofmann[14] reaction, but they also promote the halogenation of the ring, particularly by hypobromite.[15,16] This effect is minimized by the use of hypochlorite and a large excess of alkali, the rearrangement then being rapid enough to compete favorably with the side reaction of halogenation. Thus, veratric amide is converted by alkaline hypochlorite to 4-aminoveratrole in 80% yield.[17] Application of the Hofmann degradation to the amide V using sodium hypochlorite gave VI.

Diazotization is favorable under acidic conditions. The ketal VI, however, is easily hydrolyzed under these conditions, forming a phenol which will couple with the diazonium salt at neutral pH. When an amino group is going to be replaced by a cyano group using C^{14}-labeled potassium cyanide (reactant in limited quantity) the diazonium salt must be first neutralized to avoid losses in radioactivity due to the hydrogen cyanide formed. The replacement of the amino group of VI by a C^{14}-cyano group to give VII with 20% yield, based on the C^{14}-potassium cyanide used, was successful when the diazotization took place under weakly acidic conditions and the diazonium salt formed was neutralized with calcium carbonate. A brown radioactive by-product which was not identified was isolated from the reaction mixture. The structure of VII was verified by also preparing it from V with phosphorus pentoxide. Hydrolysis of VII with 1 *N* hydrochloric acid gave the lactone VIII. Lactone VIII has been reduced to pyridoxol with sodium amalgam and acetic acid.[18] We were able to reduce the lactone VIII to C^{14}-labeled pyridoxol with lithium aluminum hydride in anhydrous tetrahydrofuran.[19] When either ethyl ether or diethylene glycol dimethyl ether (diglyme) was used as solvent the yield was very small. The facile reduction of 5-pyridoxic acid lactone with lithium aluminum hydride in anhydrous tetrahydrofuran to pyridoxol can lead to an improved synthesis of vitamin B_6. Pyridoxol labeled with tritium at the 4- or 5-hydroxymethyl group by reduction of 4- or 5-pyridoxic acid lactone is under preparation.

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(18) S. A. Harris, *Chem. Abstr.*, **35**, 6741 (1941).

(19) This reduction is analogous to that of 4-pyridoxic acid lactone: J. H. Mowat and J. S. Webb, U. S. Patent 2,918,471; *Chem. Abstr.*, **54**, 8855*f* (1960).

Experimental[20]

5-(Hydroxymethyl)-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (Isopropylidenepyridoxine, II).[21]—This compound was synthesized by the method of Korytnyk and Wiedeman.[11]

5-(Carboxyl)-2,2,8-Trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (III).—A solution of 10.5 g. of II in 2 l. of 0.1% hot aqueous potassium hydroxide was cooled to 50° and 11.5 g. of potassium permanganate dissolved in 400 ml. of water was added dropwise with stirring over a 3 hr. period. Stirring was continued for an additional 7 hr. and the temperature throughout the whole experiment was maintained at 40–50°. The manganese dioxide was removed by filtration *in vacuo* while the solution was warm, the filtrate was poured into a porcelain dish and evaporated to dryness on a steam bath, the residue was dissolved in 50 ml. of water and filtered to remove additional manganese dioxide, and the filter cake was washed with water. The filtrate was acidified with hydrochloric acid (1:1) to pH 1 and the precipitated acid was filtered immediately and washed with water, yielding 6.32 g. (56%). A test with 2,6-dichloroquinonechlorimide[22] was negative which indicated that the phenolic hydroxy group was not free, and no color was given with ferrous sulfate solution[23] which indicated that the 8-methyl group was not oxidized. An analytical sample prepared by recrystallization from absolute ethanol had m.p. 219–220° dec. (lit.[24] m.p. 221–222° dec.); $\lambda_{max}^{acid\text{-}methanol}$ 302 (log ϵ 3.92) and 235 mμ (sh) (log ϵ 3.76); $\lambda_{max}^{base\text{-}methanol}$ 288 mμ (log ϵ 3.80).

Anal. Calcd. for $C_{11}H_{13}NO_4$: C, 59.18; H, 5.87; N, 6.27; neut. equiv., 223.2. Found: C, 59.52; H, 5.67; N, 6.02; neut. equiv., 221.

5-Carbomethoxy-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (IV).—A solution of 4 g. (0.018 mole) of recrystallized III in 200 ml. of boiling absolute ethanol was allowed to come to room temperature. To this solution was added with stirring an ethereal solution of diazomethane (0.036 mole) until the color of the alcoholic solution became very light red. About three-fourths of the diazomethane solution was consumed. The solution remained at room temperature overnight and was concentrated *in vacuo* to a small volume. An equal volume of water was added and the solution cooled in a refrigerator. By filtration 3.85 g. (90%) of IV was collected, m.p. 85–86°. An analytical sample prepared by recrystallization from 40% ethanol had m.p. 86–87° (lit.[24] m.p. 86.5–87°); $\lambda_{max}^{acid\text{-}methanol}$ 302 (log ϵ 3.92) and 235 mμ (sh) (log ϵ 3.77); $\lambda_{max}^{base\text{-}methanol}$ 296 (log ϵ 3.81) and 234 mμ (sh) (log ϵ 3.87).

Anal. Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 61.14; H, 6.14; N, 5.77.

5-Carbamyl-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (V).—Thirty milliliters of ethanol was added to a suspension of 4.62 g. of the methyl ester (IV) in 100 ml. of concentrated ammonium hydroxide. The suspension was saturated with gaseous ammonia and left at room temperature for 5 days. The solution was concentrated *in vacuo* until the first crystals appeared and was then placed in the refrigerator; 2.84 g. (81%) of the amide V was isolated by filtration. The amide was recrystallized from ethanol–water (1:3); m.p. 177–178° (lit.[24] m.p. 174–175°); $\lambda_{max}^{acid\text{-}methanol}$ 298 mμ (log ϵ 3.93); $\lambda_{max}^{base\text{-}methanol}$ 290 mμ (log ϵ 3.77).

Anal. Calcd. for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.61. Found: C, 59.71; H, 5.96; N, 12.69.

5-Amino-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (VI).—A cold solution of sodium hypochlorite (*ca.* 0.01 mole of chlorine)

(20) All melting points are corrected. Microanalyses were carried out by Clark Microanalytical Laboratories, Urbana, Ill. Ultraviolet absorption spectra were determined on a Model 11 M Cary recording spectrophotometer with the test compounds at *ca.* 0.002% in methanol solution, either 0.01 *N* in sodium hydroxide (base) or 0.01 *N* in hydrochloric acid (acid). Infrared spectra were determined in Nujol suspension with the aid of a Beckman Model IR-7 recording spectrophotometer. The radioactivity of aqueous solutions was measured with a Model 314 EX-2 Packard Tri-Carb liquid scintillation counting system.

(21) A. M Patterson, L. T. Capell, and D. F. Walker, "The Ring Index," American Chemical Society, Washington, D. C., 1960.

(22) H. D. Gibbs, *J. Biol. Chem.*, **72**, 649 (1927).

(23) H. Ley, C. Schwarte, and A. Münnich, *Ber.*, **57**, 349 (1924).

(24) W. Korytnyk, E. J. Kris, and R. P. Singh, *J. Org. Chem.*, **29**, 574 (1964); see also ref. 7.

was added to a suspension of 1.78 g. of V in 30 ml. of 10% aqueous potassium hydroxide. After 20 min. of stirring the amide dissolved, and the color of the solution became light orange. The solution was kept at room temperature for 30 min. and then heated on a steam bath for 40 min. When the solution reached room temperature, crystals started to separate out. After cooling, 0.65 g. of the amine VI was isolated by filtration. The filtrate was evaporated to dryness *in vacuo*, and the residue was extracted repeatedly with hot absolute ethanol. The combined ethanol extracts were evaporated to dryness and the residue was extracted with chloroform. Skellysolve C was added to the chloroform extract, and the precipitated compound was removed by filtration; total yield 0.95 g. (61%). Recrystallization from cyclohexane, after decolorization with activated carbon, gave white crystals of m.p. 160–161°; $\lambda_{max}^{acid\text{-}methanol}$ 323 (log ϵ 3.74), 233 (log ϵ 4.30), and 250 mμ (sh) (log ϵ 3.53); $\lambda_{max}^{base\text{-}methanol}$ 302 (log ϵ 3.73) and 235 mμ (sh) (log ϵ 3.92).

Anal. Calcd. for $C_{10}H_{14}N_2O_2$: C, 61.84; H, 7.26; N, 14.42. Found: C, 61.58; H, 7.09; N, 14.47.

5-Cyano-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine.—Equal amounts of phosphorus pentoxide and the amide V were mixed, and the mixture was sublimed for 24 hr. at 95° and 80-mm. Hg. pressure. The compound isolated from the cold finger had identical infrared and ultraviolet spectra as well as an identical melting point and mixture melting point with the compound obtained through the diazonium salt from the amine VI.

5-Cyano-C^{14}-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (VII).—A solution of 83 mg. (0.33 mmole) of cupric sulfate pentahydrate in 5 ml. of water was added to a 300 ml., round-bottomed, three-necked flask equipped with a dropping funnel and a gas trap which contained an aqueous solution of sodium hydroxide. While this solution was on a steam bath, a warm solution of 44 mg. (0.67 mmole) of potassium cyanide in 5 ml. of water was added to it. The white precipitate, which was formed immediately, was redissolved by adding a warm solution of 44 mg. (0.67 mmole) of C^{14}-labeled potassium cyanide in 2 ml. of water. The specific activity of the radioactive solution of potassium cyanide was determined in an aliquot by precipitating the cyanide with silver nitrate and oxidizing the silver cyanide to C^{14}-carbon dioxide which was measured with a vibrating reed electrometer.

The diazonium solution was made by adding a solution of 120 mg. (1.4 mmoles) of potassium nitrite in 4 ml. of water to a cold solution of 215 mg. (1.1 mmoles) of the amine VI in 10 ml. of water and 3 ml. of 1 *N* sulfuric acid. The diazonium salt was neutralized with solid calcium carbonate, filtered through a fritted glass funnel, and added slowly through the dropping funnel to the radioactive solution of potassium cyanide which had been removed from the steam bath. During the addition the solution was stirred with a magnetic stirrer and a low external nitrogen pressure was applied to the dropping funnel. The diazonium salt was kept at about 0° from its preparation until its addition to the radioactive solution. After the addition was completed the flask was left at room temperature and stirred for 45 min. Fifty milliliters of ether was added and stirring was continued for 15 min. The reaction mixture was filtered through a fritted-glass funnel. The brown radioactive filter cake[25] was washed with water and then with ether. The filtrate was extracted five times with 50 ml. of ether each time. The water layer, after it was extracted with ether, showed almost no ultraviolet absorption and the pH was approximately 7. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by sublimation. To avoid contamination of the sublimate, the sublimation apparatus consisted of a cold finger that had a right angle side arm which was equipped with a small flask. The flask with the residue in it was placed in an oil bath (95°) and the side arm was heated with an electrical wire coiled around it. The residue was sublimed for 24 hr. at 80-mm. Hg pressure. The sublimate deposited on the cold finger as colorless needles yielding 40 mg. (20%) based on C^{14}-potassium cyanide. The infrared spectrum showed a peak in the nitrile region (2230 cm.$^{-1}$) and a test with 2,6-dichloroquinonechlorimide was negative. A sample recrystallized from ethanol–water had m.p. 111–112°; $\lambda_{max}^{acid\text{-}methanol}$ 300 mμ (log ϵ 3.85); $\lambda_{max}^{base\text{-}methano}$ 295 (log ϵ 3.77) and 233 mμ (log ϵ 3.92). Specific activity of C^{14}-potassium cyanide solution was 48.2 μc./mmole. Specific activity of VII was 31.8 μc./mmole.

Anal. Calcd. for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.66; H, 5.84; N, 13.57.

5-Hydroxy-4-hydroxymethyl-6-methylnicotinic Acid γ-Lactone (5-Pyridoxic Acid Lactone).—Thirty milligrams of nonradioactive compound VII which had been obtained by diazotization of VI was hydrolyzed with 10 ml. of 1 *N* hydrochloric acid by heating on a steam bath for 4 hr. No color was given with 2,6-dichloroquinonechlorimide at the beginning of hydrolysis, but the test was positive after 15 min. When the solution reached room temperature, solid sodium acetate was added until turbid; the flask was placed in the refrigerator for 24 hr. By filtration and washing with water, 15 mg. of a very light yellow compound was isolated, m.p. 275–277° dec. (lit.[10] m.p. 282–283°). This compound did not depress the melting point of an authentic sample of the lactone of 5-pyridoxic acid, and the infrared and ultraviolet spectra were identical.

5-Hydroxy-4-hydroxymethyl-6-methylnicotinic-(carboxy-C^{14}) Acid γ-Lactone (5-C^{14}-Pyridoxic Acid Lactone, VIII).—To a 50-ml., round-bottomed flask, were added 40 mg. of compound VII and 10 ml. of 1 *N* hydrochloric acid. The flask was loosely stoppered and heated on a steam bath for 4 hr. The solution was evaporated to dryness *in vacuo*, and dried in a vacuum oven at 60° overnight.

3-Hydroxy-4-hydroxymethyl-5-hydroxymethyl-C^{14}-2-methylpyridine Hydrochloride (Pyridoxol-C^{14} Hydrochloride, IX).—Ten milliliters of anhydrous tetrahydrofuran and 40 mg. of powdered lithium aluminum hydride were added to the flask that contained the dry lactone. The suspension was refluxed on a steam bath for 3 hr. The flask was cooled and 20 ml. of water was added. The tetrahydrofuran was evaporated *in vacuo* at room temperature, the suspension was filtered through an "M" porosity fritted-glass funnel, and the filter cake was washed with 20 ml. of water. The filtrate was brought to pH 2 with dilute (1:1) hydrochloric acid, and then to pH 7.6 with aqueous (1:1) ammonium hydroxide. The small amount of aluminum hydroxide which precipitated was removed by filtration through an "F" porosity fritted-glass funnel. The filtrate was made alkaline with solid sodium hydroxide, applied to a 50-ml. Dowex-1-formate column (100-ml. buret wrapped with aluminum foil), and eluted with 60 ml. of water and then with 0.1 *M* formic acid. The radioactive fractions that contained the pyridoxol were combined, acidified with 1 ml. of 1 *N* HCl, and evaporated to dryness. The residue was recrystallized from absolute ethanol, yielding 28 mg. (70% based on VII), m.p. 208–209° dec. Mixture melting point with an authentic sample of pyridoxol hydrochloride was 208–209° dec. These two samples had identical infrared and ultraviolet spectra and R_f values in two paper chromatography solvent systems[3] and in electrophoresis.[26] The single radioactive spot which was obtained coincided with the ultraviolet fluorescence spot and the color spot given with 2,6-dichloroquinonechlorimide. Immediately before and after the fraction of C^{14}-pyridoxol two fractions of small radioactivity could be collected which were tentatively identified as "4-desoxypyridoxine" and "isopyridoxal," respectively. After the elution of these fractions, traces of the lactone of 5-pyridoxic acid and then 5-pyridoxic acid were eluted using the same solvent, 0.1 *M* formic acid. The conversion of 5-pyridoxic acid to its lactone during elution from the column has been reported.[27] Samples of C^{14}-pyridoxol hydrochloride (1 mc./mmole) dissolved in water (2 μc./ml.) and placed in a refrigerator for 1 year showed only one radioactive spot on paper chromatography.

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(25) The infrared spectrum of this compound showed a sharp strong peak at 2150 cm.$^{-1}$. The brown radioactive compound was dissolved in aqueous hydrochloric acid (1:1) and heated on a steam bath for 3 hr. The solution was neutralized with aqueous sodium hydroxide and applied to a Dowex-1-formate column. By elution with 0.1 *M* formic acid and evaporation of the solvent a radioactive yellow compound, not further identified, was obtained.

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