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SYNTHESIS OF THE 3,5,3',5'-$^3H_4$-2,2'-DIPHENYLENE-IODONIUM CATION AND OF ITS 4-NITRO DERIVATIVE

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SUMMARY

The 3,5,3',5'-$^3H_4$-2,2'-diphenyleneiodonium cation [^{3}H]-**1** was synthesized in three steps from cold 2,2'-diaminodiphenyl. The nitration of [^{3}H]-**1** gave easily the 4-nitro derivative [^{3}H]-**2** These two compounds which contain four tritium labels were obtained with a high specific activity, and are proposed as tools to study the interaction of 2,2'-diphenyleneiodonium cations with redox enzymes, especially flavoproteins.

Keywords : Diphenyleneiodonium, DPI, synthesis, tritium labelling, enzyme inhibition.

INTRODUCTION

The aryliodonium cations are known to be inhibitors of flavoenzymes. The diphenyliodonium (IDP) and 2,2'-diphenyleneiodonium (DPI) **1** cations may react,

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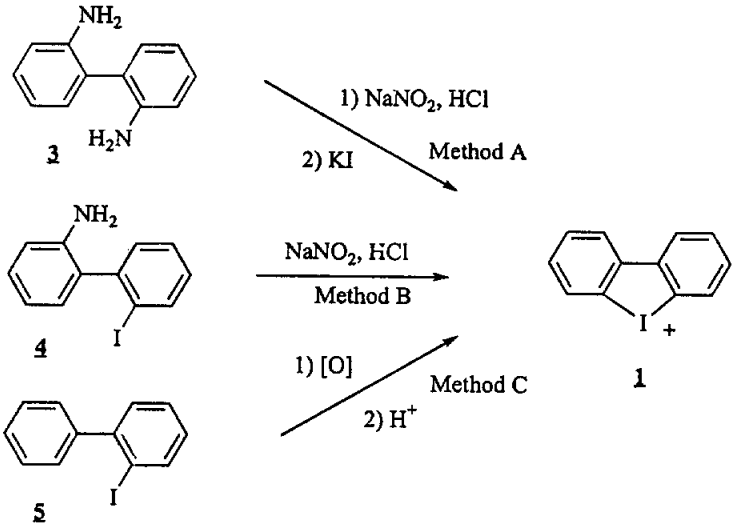
through a redox mechanism, with the isoalloxazine nucleus of FAD or FMN to give covalent adducts which have been isolated from the reduced free cofactors (1, 2) or from flavoenzymes (3, 4). The mechanism of inhibition of protoporphyrinogen oxidase by DPI **1** and some of its derivatives was shown to be of slow-binding type and was also expected to involve a reversible adduct to FAD (5). Not only flavinic coenzymes have been shown to interact with DPI **1** or IDP. Also PQQ gave with DPI **1** and other iodonium salts, non-covalent complexes which have been isolated from guinea-pigs neutrophils (6). As another example, in the neutrophil NADPH oxidase complex, a flavocytochrome b was shown to interact with DPI **1** or IDP not only through the flavin, but also through the heme b nucleus (7). A mechanism of inhibition was proposed (4, 8), that involves the one-electron oxidation of the reduced cofactor by the aryliodonium cation. The aryl radical thus formed can bind covalently either with the cofactor or with any aminoacid of the active site.

Some radiolabelled DPIs or IDPs have been synthesized. ^{125}I-DPI and ^{14}C-IDP were prepared by Gatley and Sherratt (9) and by Tew (4) respectively, but these reagents exhibit low specific activities. A tritium-labelled DPI was obtained by exposition for two weeks of cold DPI **1** to tritium gas (6). No indication about the specific activity obtained was mentioned. High specific activity is generally required to probe complexes between radiolabelled ligands and macromolecules. We describe here the synthesis of the 3,5,3',5'-$^{3}H_4$-2,2'-diphenyleneiodonium cation [^{3}H]-**1** obtained with a high level of specific activity. Because 4-nitro-DPI **2** was shown to be much more active than DPI in the inhibition of protoporphyrinogen oxidase (5), we also synthesized its tritiated derivative [^{3}H]-**2**.

RESULTS AND DISCUSSION

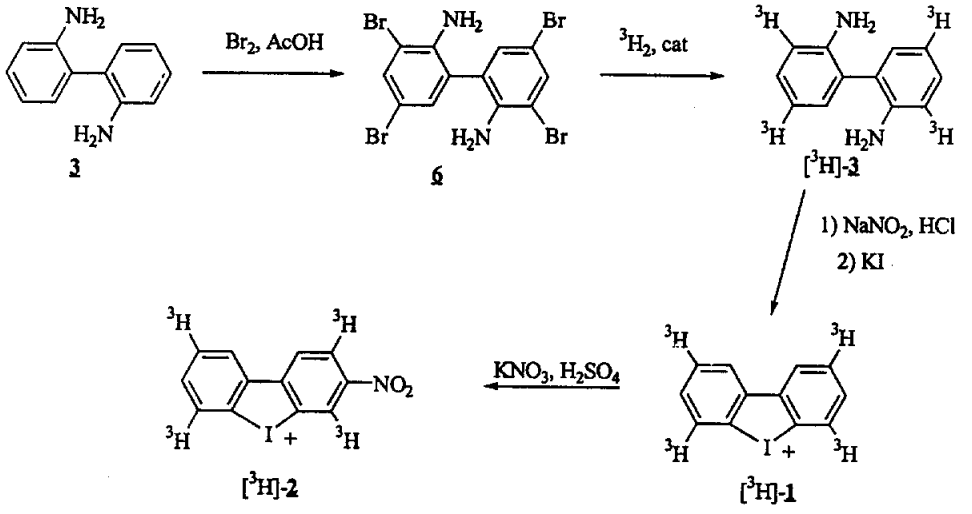
The DPI cation **1** can be obtained through three different ways (Scheme 1). The earlier method (Method A) used 2,2'-diaminodiphenyl **3** as the starting material, which upon diazotization with sodium nitrite in a hydrochloric solution followed by potassium iodide addition, gave a 56 % yield of DPI **1** (10). A similar reaction from 2-amino-2'-iododiphenyl **4** (Method B) allowed to prepare DPI **1** in an excellent yield (97%) (11). The third method (Method C) involved the peroxidation of 2-iododiphenyl **5** to an iodoso intermediate which cyclized in acidic solution (12).



Scheme 1

Synthesis of the 3,5,3',5'-$^3H_4$-2,2'-diphenyleneiodonium cation [^{3}H]-1.

We chose to use the Method A for the synthesis of the 3,5,3',5'-[^{3}H]-DPI, [^{3}H]-**1**, after considering the ease of preparation of the starting tritiated 2,2'-diaminodiphenyl [^{3}H]-**3** from which the target compound could be obtained in a one-pot radioactive preparation (Scheme 2).



Scheme 2

2,2'-diaminodiphenyl **3**, easily prepared by reduction of 2,2'-dinitrodiphenyl (13), was brominated by using bromine in acetic acid. The tetrabromo derivative **6** obtained was catalytically reduced by tritium gas to give the expected 3,5,3',5'-$^3H_4$-2,2'-diaminodiphenyl [^{3}H]-**3**. The specific activity of this compound was found to be 73 Ci.mmol^{-1}. The tritiated diamine [^{3}H]-**3** was treated at 0-5°C with sodium nitrite in a hydrochloric solution contained in a test tube. Addition of a large excess of potassium iodide led to a mixture of radiolabelled compounds as revealed by HPLC monitoring with radioactivity detection. We observed in the chromatogram a weak peak attributed to DPI [^{3}H]-**1**. In fact, most of this compound was adsorbed on the glass walls of the test tube. After neutralization of the solution, the adsorbed DPI [^{3}H]-**1** was recovered by rinsing with methanol, and the rinsing was added to the reaction solution before chromatography on an analytical HPLC column. The expected compound [^{3}H]-**1** was obtained with a 14 % radiochemical yield and more than 99% of radiochemical purity. The specific activity was found to be 60 Ci.mmol^{-1} (average value).

We tried to reproduce this synthesis with addition of cold 2-aminodiphenyl as a carrier. Additional by-products were then observed, which generated difficulties in the purification and we did not try to improve this procedure.

Synthesis of the 3,5,3',5'-$^3H_4$-4-nitro-2,2'-diphenyleneiodonium cation [^{3}H]-2.

According to Wasylewsky *et al* (13), 4-nitro-DPI **2** was easily obtainable by addition of 2,2'-diphenyleneiodonium (DPI **1**) nitrate in concentrated sulfuric acid at room temperature. Nitration of [^{3}H]-**1** was achieved similarly except that the source of nitrating agent was potassium nitrate (Scheme 2). The reaction was run in silylated glass, in order to prevent adsorption. 3,5,3',5'-$^3H_4$-4-nitro-DPI [^{3}H]-**2** was obtained chemically pure, as controlled by HPLC, with a 60% radiochemical yield. The specific activity of the product was found to be 59.7 Ci.mmol^{-1}.

EXPERIMENTAL

Radioactivity was determined with a Packard Tri-Carb® 2100TR liquid scintillation counter, using the Packard Ultima Gold™XR cocktail as the scintillation medium. HPLC experiments were performed on a Waters apparatus equipped with an U6K injector (2 mL sample loop) using Merck Lichrocart® 125-4 cartridges filled with Lichrospher®60 RP-Select B (5 μm) silica gel. Detection was obtained by measurement of UV absorption on a Waters 490 Multiwavelength programmable detector and by counting of the radioactivity on a Radiomatic Flo-One® 150-TR detector equipped with a solid cell for preparative experiments, and with a liquid cell where the column effluents were mixed with the Packard Flo-Scint™ III scintillation cocktail for analytical runs. The ^{1}H NMR of compound **6** was recorded on a JEOL GSX 270 WB spectrometer with tetramethylsilane as internal standard (δ in parts per million). UV spectra were recorded on a Unicam UV 2 spectrometer using 1cm length quartz cells.

2,2'-diamino-3,5,3',5'-tetrabromodiphenyl **6**: To a solution of 680 mg (3.69 mmol) of 2,2'-diaminodiphenyl **3** (14) in 15 mL of acetic acid, were added 5 g (31.29 mmol) of Br_2 dissolved in 1.3 g of acetic acid. The reaction mixture was left for 24 h at room temperature and was then flushed with nitrogen to remove HBr. The excess of bromine was reduced by addition of an aqueous solution of sodium sulfite. The precipitate was filtered and washed with water until we obtained a neutral filtrate. After drying under vacuum, the crude product was chromatographed through a short column of alumina, with petroleum ether/CH_2Cl_2 (7:3) as eluent. Recrystallization from CH_2Cl_2 left white crystals (858 mg, 46% yield); mp 166 °C [Litt. (15) 170°C] ^{1}H NMR (270 MHz) (DMSO-d_6) δ 7.60 (d, 2H, J = 0.7 Hz, 4 and 4'-H or 6 and 6'-H), 6.93 (d, 2H, J = 0.7 Hz, 4 and 4'-H or 6 and 6'-H), 4.57 (s, 4H, 2 NH_2).

3,5,3',5'-$^3H_4$-2,2'-diaminodiphenyl dihydrochloride [^{3}H]-**3**: This compound was prepared by Amersham, by catalytic reduction of the tetrabromo precursor by tritium

gas. It was delivered to us as the dihydrochloride in an ethanolic solution, after removal of the exchangeable tritium. The purification of a fraction of this crude product by HPLC, allowed us to obtain it with a radiochemical purity over 99%. The specific activity of the purified product was determined by counting aliquots of the solution and measuring the molar concentration by quantitative HPLC analysis (λ of UV detector at λ_{max} = 290 nm). It was found to be 73 Ci.mmol^{-1}.

3,5,3',5'-$^3H_4$-2,2'-diphenyleneiodium cation [^{3}H]-**1**: In a 8 mL Pyrex test tube, 0.5 mL of the solution (1.6 mCi) of the crude diamine salt was evaporated in a nitrogen stream. The residue was taken in 50 µL of a 10 M solution of hydrochloric acid and 500 µL of water, and the mixture was cooled to 0°C. Then 15 µL of a cold solution of sodium nitrite (12 mg) in 200 µL of water was slowly added. After stirring for 8 min, 20 µL of an aqueous solution of potassium iodide (310 mg.mL^{-1}) were rapidly poured, and the mixture was left at room temperature under stirring for 3 h. The solution was neutralized with K_2HPO_4,3H_2O (aqueous solution: 150 mg + 160 µL of water) and was taken, together with the rinsing of the test tube by 500 µL of methanol in a 2 mL plastic syringe and this mixture was submitted to HPLC separation. The column was eluted first with a solution of KH_2PO_4 0.01 M for 15 min, then with a mixture (50:50) of this buffer and methanol. The collected fractions of the eluent were analyzed by HPLC and the fractions of the expected product were gathered. The product was obtained with 9 to 14% radiochemical yield, according to preparations, and its radiochemical purity checked by HPLC was over 99%. The specific activity was determined by measuring UV absorbance of the solution at the maximum absorption of DPI **1** (264 nm - ε_{max} = 16500 L.mol^{-1}.cm^{-1}) and counting aliquots. An average value of 60 Ci.mmol^{-1} was obtained.

3,5,3',5'-$^3H_4$-4-nitro-2,2'-diphenyleneiodonium cation [^{3}H]-**2**: All manipulations were done in silylated test tubes. Silylation was obtained by filling the tubes with a solution of dichlorodimethysilane (1 g) in dichloromethane.

In a 8 mL Pyrex test tube, a solution containing 233 µCi of the tritiated DPI [^{3}H]-**1** was evaporated in a nitrogen stream. The residue was taken in 100 µL of

concentrated sulfuric acid and 2 mg of KNO_3 were added. The mixture was stirred at room temperature for 1,5 h. Then it was neutralized with a solution of $K_2HPO_4,3H_2O$ (1,3 g in 4 mL water). The resulting buffered solution obtained was injected in a Sep-pak® C-18 cartridge. After washing with 2.2 mL of water, the cartridge was eluted with four 2.2 mL fractions of different mixtures of a solution of KH_2PO_4 0.01 M in water and methanol (90:10, 80:20, 70:30 and 0:100). HPLC analyses of the eluted fractions showed that the first fraction contained a polar radioactive compound, expected to be tritiated water, and the next four fractions all contained the chemically pure expected product [^{3}H]-**2**. The radiochemical yield of the 4-nitro derivative was found to be 60.4 % with a radiochemical purity over 99%. Measuring the 4-nitro-DPI [^{3}H]-**2** concentration by quantitative HPLC analysis (λ of the detector at λ_{max} = 310 nm), and counting aliquots of the solution allowed us to calculate a specific activity of 50.8 Ci.mmol^{-1}.

CONCLUSION

The two tritiated 2,2'-diphenyleneiodonium cations, [^{3}H]-**1** and [^{3}H]-**2** are easily accessible from their tritiated precursor 2,2'-diaminodiphenyl [^{3}H]-**3**, and they retain high specific activities. They will be certainly useful tools to probe affine sites in redox enzymes, especially flavoproteins.

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