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PREPARATION OF [^{3}H]-LABELLED BENZPHETAMINE PHOTOAFFINITY PROBES FOR CYTOCHROME P-450

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SUMMARY

We have synthesized two tritium labelled photolabile azido-α-desmethylbenzphetamine analogues: N-benzyl-N-methyl-p-azido-phenethyl-amine ([^{3}H]BP-N_3) and N-(p-azidobenzyl)-N-methyl-p-azido-phenethylamine ([^{3}H]N_3-BP-N_3). These compounds, photoaffinity probes for cytochrome P450 2B1, were prepared in a two step synthetic process. First, the iodinated aminoderivative precursors N-benzyl-N-methyl-4-amino-3,5-diiodophenethylamine and N-(4-amino-3,5-diiodobenzyl)-N-methyl-4-amino-3,5-diiodophenethylamine were subjected to reductive deiodination on a Pd/C catalyst using sodium [^{3}H]borohydride as an *in situ* generator of tritium gas. Second, resultant radiolabelled amino-compounds were converted into the final [^{3}H]BP-N_3 and [^{3}H]N_3-BP-N_3 compound through their diazoderivatives without separation of their intermediates.

Key Words: Cytochrome P-450, Photoaffinity probe, Azidobenzphetamine analogue, Catalytic dehydroiodination, [^{3}H]borohydride.

INTRODUCTION

Cytochromes P-450 (P-450) are hemoproteins acting as terminal oxidases of an enzymatic system metabolizing xenobiotics and endogenous substrates [1-3]. Recently, the photoaffinity labelling has been used to identify amino acid residues involved in the substrate binding sites of mammalian P-450s [4-8]. To study the active center of P-450 2B1 we synthesized and evaluated azido-group bearing photoaffinity probes [8]. These probes are photolabile analogues of

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benzphetamine, a specific substrate of P-450 2B1 [9]. Under UV-light irradiation, these photoaffinity probes are extremely photolabile, generating a highly reactive nitrene. Each of the probes interact specifically with P-450 2B1, and after photoactivation inhibit cytochrome P-450 2B1 mediated catalytic activities [8]. Herein, we report a convenient method of the synthesis of two tritium labelled azido-α-desmethylbenzphetamine photoaffinity probes.

RESULTS AND DISCUSSION

Precursors for synthesis of the radiolabelled photoaffinity probes, N-benzyl-N-methyl-p-aminophenethylamine (BP-NH_2) and disubstituted N-(p-aminobenzyl)-N-methyl-p-aminophenethylamine (NH_2-BP-NH_2), were prepared from their corresponding nitroderivatives. The nitroderivatives were synthesized by alkylation of the N-methylalkylbenzenes with bromoalkylarene. This process has previously been used in this laboratory to synthesize an α-desmethylbenzphetamine skeleton [8]. An alternative means for BP-NH_2 and NH_2-BP-NH_2 synthesis was done using dithiothreitol reduction of azidoderivatives.

The first step in synthesis of the photoaffinity probes, [^{3}H]BP-N_3 and [^{3}H]N_3-BP-N_3, was substitution of the BP-NH_2 and NH_2-BP-NH_2 benzene ring hydrogens with tritium. This required a two step process whereby benzene rings of BP-NH_2 and NH_2-BP-NH_2 were first iodinated using iodine monochloride in diluted acetic acid. Exchange of iodine with tritium on the benzene ring was done by low-pressure catalytic hydrogenation using tritiated sodium borohydride in ethanol solution (Figure 1). Decomposition of sodium borohydride with ethanol provided a source of hydrogen generated directly in the reaction mixture. An attempt to minimize the number of reaction steps with radiolabelled precursors was tried using an iodine substitution for hydrogen in the stage of azidoiododerivatives, however, dehydroiodination and azido-group reduction proceeded simultaneously regardless of the reaction conditions. Hence, an efficient hydrogenation resulting solely in the desired product was achievable only with aminoiododerivatives. The reaction pathway is schematically summarized in Figure 1.

The catalytic hydrogenation was optimized with non-radioactive sodium borohydride - the in situ generator of hydrogen. Using this process we prepared two radiolabelled photoaffinity

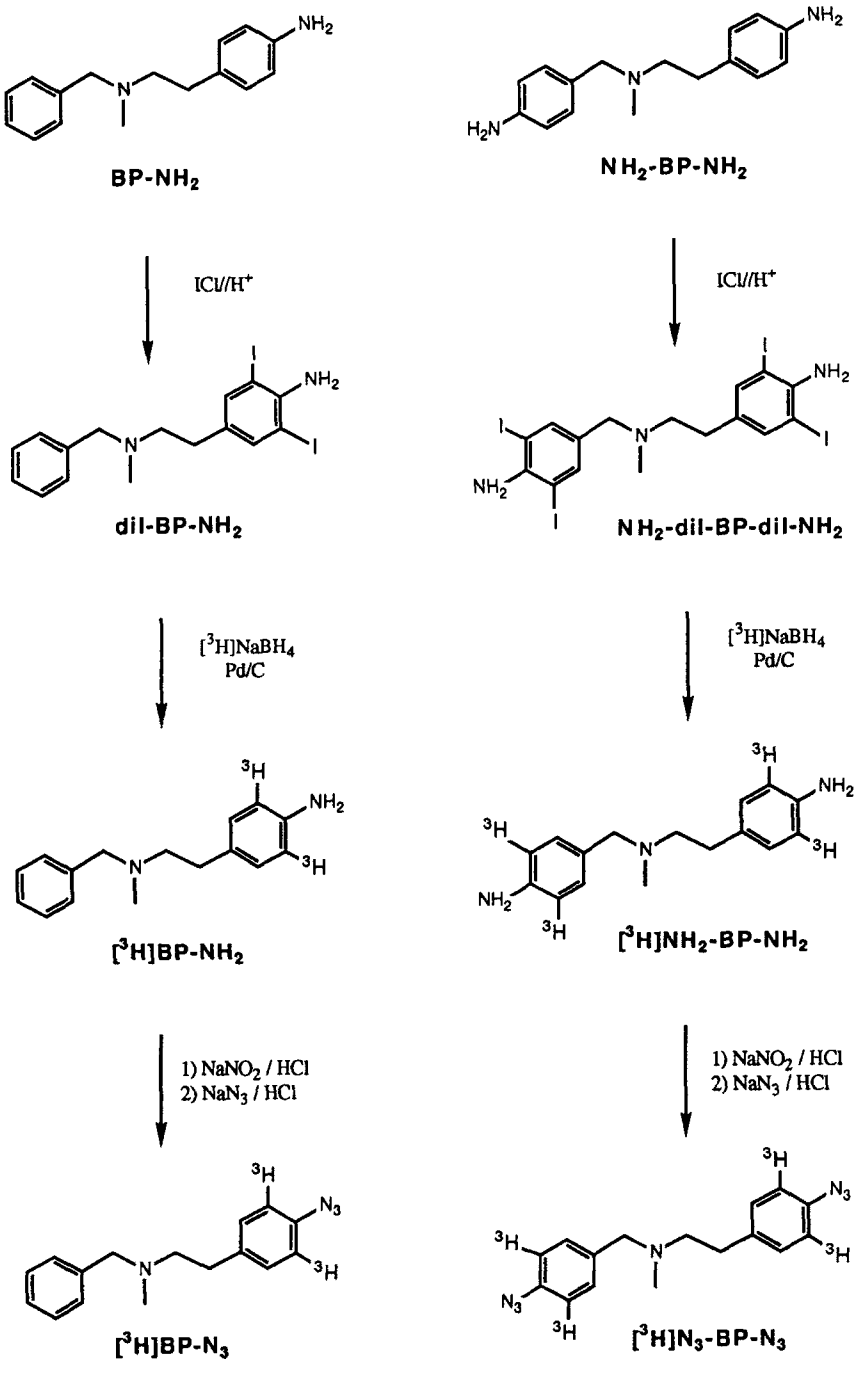

Figure 1. Scheme for synthesis of tritium labelled benzphetamine photoaffinity probes

probes of different specific activity. Tritiation of aminoiododerivatives was carried out with sodium [^{3}H]borohydride samples having specific activities of 10 Ci/mmol for N-benzyl-N-methyl-4-amino-3,5-diiodophenethylamine (diI-BP-NH_2) and 200 mCi/mmol for N-(4-amino-3,5-diiodobenzyl)-N-methyl-4-amino-3,5-diiodophenethylamine (NH_2-diI-BP-diI-NH_2). In both cases the same ratio of sodium [^{3}H]borohydride per iodine was used, however, with the first aminoiododerivative (diI-BP-NH_2) the reaction proceeded to about 50% completion. Therefore, an additional amount of non-radioactive sodium borohydride was necessary for complete iodine substitution. On the other hand, the tritiation of NH_2-diI-BP-diI-NH_2 was achieved with the original amount of sodium [^{3}H]borohydride added. Taken together, these results suggest that use of 1.3 and 2.3 times molar excess sodium borohydride over diI-BP-NH_2 and NH_2-diI-BP-diI-NH_2, respectively, was sufficient to complete the dehydroiodination.

The tritium labelled amino-α-desmethylbenzphetamine derivatives were next converted to their final products, [^{3}H]BP-N_3 and [^{3}H]N_3-BP-N_3, through diazotization followed by azidization (Figure 1). [^{3}H]BP-N_3 and [^{3}H]N_3-BP-N_3 were purified using a silica gel column and compared to elementary analyzed non-radiolabelled standards by thin layer chromatography [8]. The standards and radiolabelled compounds were prepared simultaneously, and were judged identical according to TLC analysis. The yield of both radiolabelled photoaffinity probes was determined based on their absorption spectra in methanol using a molar extinction coefficient of ε 14,900 $M^{-1}cm^{-1}$ for [^{3}H]BP-N_3 and ε 27,800 $M^{-1}cm^{-1}$ for [^{3}H]N_3-BP-N_3. After purification the overall yield was 29.5% and 36.7% for [^{3}H]BP-N_3 and [^{3}H]N_3-BP-N_3, respectively.

EXPERIMENTAL

Analytical methods

TLC was carried out using silica gel plates (Whatman) containing a 254 nm fluorescent indicator. Amines were visualized on TLC plates by spraying the plates with ninhydrin reagent [10]. Reaction intermediates and final products were analyzed by means of mass spectroscopy on a Finnigan Mat INCOS 50 and by FAB on a Kratos MS50TC using p-nitrobenzylalcohol as a matrix. UV-spectra were taken on a Hewlett Packard 8452 array spectrophotometer.

Radioactivity of the liquid samples and for the TLC plates was measured in a Beckman LS 5000 TD liquid scintillation counter using Cytoscint (ICN) cocktail solution.

Preparation of α-desmethylbenzphetamine aminoderivatives

Both precursors of radiolabelled photoaffinity probes, N-benzyl-N-methyl-p-aminophenethylamine (BP-NH_2) and disubstituted N-(p-aminobenzyl)-N-methyl-p-aminophenethylamine (NH_2-BP-NH_2), were prepared as we have described previously [8]. An alternative procedure can be done by dithiothreitol reduction of azidoderivatives as follows: N-benzyl-N-methyl-p-azidophenethylamine (BP-N_3) (500 mg), dithiothreitol (500 mg) and potassium carbonate (450 mg) were stirred in methanol (7 ml) at room temperature for 2 hours. The reaction mixture was then filtered through glass wool to separate insoluble material. Methanol was evaporated from the filtrate, and the resultant residue dissolved in chloroform (15 ml). The chloroform solution was washed five times with 1 mM NaOH (400 ml) and once with water. The solvent was evaporated under reduced pressure and the residue converted into a hydrochloride derivative (light yellow crystals) using HCl gas. A typical yield of aminoderivative was > 85%. The same procedure was utilized for the preparation of N-(p-azidobenzyl)-N-methyl-p-azidophenethylamine (N_3-BP-N_3) with the exception that a doubled amount of dithiothreitol (1 g) and methanol (14 ml) were used per mmol of the diazidoderivative (1 g).

Iodination of aminoderivatives

BP-NH_2 (182 mg) was dissolved in a mixture consisting of methanol (6 ml), acetic acid (15 ml), and water (9 ml). While stirring, 143 µl of ICl solution (8.7 M) in acetic acid was added to the solution and iodination was allowed to proceed for 3 hrs at 35°C. At the end of this period, an additional amount of the ICl solution (50 µl) was added and the reaction mixture was stirred under the same conditions until TLC analysis showed that conversion of remaining monoiododerivative to diiodinated compound was completed. The reaction was terminated by adjusting the pH to 8.5 with 12 M NaOH, and by adding of 0.1 M sodium bisulfite (30 ml).

Iodinated product was extracted into chloroform. Crystallization of a crude N-benzyl-N-methyl-4-amino-3,5-diiodophenethylamine (diI-BP-NH_2) was carried out using cold ethanol. The final product was identified by FAB, revealing a compound with mass of 493 (M+1). The yield of diI-BP-NH_2 was typically 70%.

N-(4-amino-3,5-diiodobenzyl)-N-methyl-4-amino-3,5-diiodophenethylamine (NH_2-diI-BP-diI-NH_2) was synthesized as described above, except its synthesis required use of twice the amount of ICl per mmol of the diaminocompound. The final product was characterized by FAB showing masses of 760 (M+1) for the major compound and 634 (M+1) for traces of a triiododerivative.

Reductive deiodination of diI-BP-NH_2 using $NaBH_4$

The conditions for catalytic hydrogenation of diI-BP-NH_2 were optimized by examining product formation in solvent systems containing methanol, ethanol, or ethylacetate, with various amounts of Pd/C (10%) and $NaBH_4$. The best results were obtained when diI-BP-NH_2 (21.9 mg) was dissolved in a mixture of ethylacetate and ethanol (1:1, 440 µl) and exposed to hydrogen generated from $NaBH_4$ (2.2 mg) in the presence of Pd/C (10 mg) as a catalyst. Solid reagents were placed in a Pierce reaction vial (1 ml) and the reaction was started by injecting the solvent mixture (ethylacetate:ethanol, 1:1), through a septum. The reaction mixture was stirred for 1 hour at room temperature. The catalyst was removed by filtration through glass wool and solvents evaporated with nitrogen. The residue, dissolved in chloroform, was washed with 1 mM NaOH. The solvent was evaporated with nitrogen to yield a colorless oil containing a pure BP-NH_2 (Rf 0.4) as judged from TLC (methanol with 2% ammonia). A FAB analysis revealed a single compound of mass 241 (M+1).

Preparation of [^{3}H]BP-NH_2

[^{3}H]BP-NH_2 was prepared by injecting a solution of diI-BP-NH_2, (37.8 mg/ml, 100 µl) in ethanol/ethylacetate (1:1), through a septum into a reaction vial (1 ml) that contained [^{3}H]$NaBH_4$ (100 mCi, 10 Ci/mmol, American Radiolabelled Chemicals) and Pd/C (1.5 mg). The reaction

mixture was stirred at room temperature for 1 hour. Analysis of the reaction mixture by TLC (ethylacetate with 2% ammonia) showed a remaining intermediate - the [^{3}H]monoiododerivative (Rf 0.40). Therefore, an additional aliquot of $NaBH_4$ (1 mg) was added and the reaction mixture was stirred for another hour. Following a second TLC (methanol with 2% ammonia) analysis, the reaction was judged completed since only a single radioactive spot (Rf 0.35) corresponding to the mobility of the BP-NH_2 standard was detected. The reaction mixture was filtered through glass wool and solvents evaporated with nitrogen. The residue was dissolved in chloroform and washed with 1 mM NaOH. Chloroform was removed by a nitrogen stream to yield a colorless oil that was used for synthesis of [^{3}H]BP-N_3 without further purification.

Preparation of [^{3}H]NH_2-BP-NH_2

The reaction scheme was the same as that described above for preparation of [^{3}H]NH_2-BP, however, additional $NaBH_4$ was not necessary to complete the reaction. [^{3}H]$NaBH_4$ (10 mCi, 200 mCi/mmol, American Radiolabelled Chemicals), Pd/C (10 mg) and NH_2-diI-BP-diI-NH_2 dissolved (17.5 mg/ml, 650 µl) in ethanol/ethylacetate (1:1) were stirred for 2 hours in a reaction vial (2 ml). The resultant compound, showed a single radioactive spot (Rf 0.30) of the same mobility as NH_2-BP-NH_2 standard on TLC (methanol with 2% ammonia). This compound was used without further purification.

Synthesis of [^{3}H]azidocompounds

The conversion of amino-compounds [^{3}H]BP-NH_2 and [^{3}H]NH_2-BP-NH_2 to the final radiolabelled N-benzyl-N-methyl-p-azidophenethylamine ([^{3}H]BP-N_3) and N-(p-azidobenzyl)-N-methyl-p-azidophenethylamine ([^{3}H]N_3-BP-N_3) proceeded through their diazoderivatives without separation of the intermediates as we described previously [8]. In order to estimate the amount of radiolabelled aminocompounds used for a reaction, 90% recovery of the reductive deiodination was assumed.

[^{3}H]BP-NH_2 was dissolved in 1M HCl (1 ml) and the solution cooled to -5°C. While stirring continuously, an aqueous solution (150 µl) of sodium nitrite (483 µg) was added for

15 min. The mixture was stirred for 1 hour at -5 - 0°C and then acetone (200 µl) was added to improve solubility of intermediate diazotitated products. The reaction was terminated by adding urea (8 mg) and stirring for 10 minutes. A solution (25 µl) of sodium azide (638 µg) was added to the reaction mixture and stirred for 30 min at 0°C. The solution was made alkaline by adding 12 M NaOH (100 µl), and the final product was extracted into chloroform and analyzed by TLC. Analysis of TCL plate radioactivity revealed a highly radioactive spot that comprised 54% of the radioactivity applied. This spot had the same Rf (0.65 for ethylacetate, 0.35 for ethylacetate/chloroform 2:3) as the BP-N_3 standard. The crude [^{3}H]BP-N_3 was purified on a silica gel column (7.5 ml, 200-400 mesh, Aldrich) equilibrated with ethylacetate/chloroform (2:3). This solvent system was also used to elute BP-N_3 from the column. Collected fractions (2.5 ml) were pooled according to TLC/radioactivity analysis, and the solvent was evaporated using nitrogen. The pure compound was converted into its hydrochloride salt and dissolved in methanol. Yield was calculated as 29.5% based on the absorption at 252 nm (ε 14,900 $M^{-1}cm^{-1}$). [^{3}H]BP-N_3 had a radiochemical purity of 99.7%, yield of 8.1 mCi, and specific radioactivity of 3.6 Ci/mmol. [^{3}H]BP-N_3 was stored as a 4.5 mM solution in methanol at -20°C.

[^{3}H]N_3-BP-N_3 was prepared using the procedure described for [^{3}H]BP-NH_2. To a solution of [^{3}H]NH_2-BP-NH_2 in 1M HCl (2 ml) cooled to -7°C an aqueous solution (500 µl) of sodium nitrite (1.93 mg) was added. The mixture was stirred for 1 hour at -5 - 0°C. The reaction was stopped with urea (8 mg). A solution (100 µl) of sodium azide (2.7 mg) was added and the reaction mixture stirred at 0°C for 40 min. The final product was extracted into chloroform after alkalization by 12M sodium hydroxide (200 µl). A counting of a TLC plate radioactivity revealed a spot comprising 74% of radioactivity applied. This spot showed Rf 0.55 (in ethylacetate) coincident with that of N_3-BP-N_3 standard. The crude [^{3}H]N_3-BP-N_3 was purified on a silica gel column (9 ml, 200-400 mesh, Aldrich) equilibrated and eluted with ethylacetate/chloroform (1:2). Fractions (1.5 ml) were pooled according to TLC/radioactivity analysis. The pure [^{3}H]N_3-BP-N_3 was converted into its hydrochloride salt and dissolved in methanol. The overall yield 36.7% of the pure product was determined spectrophotometrically

at 253nm (ε 27,800 $M^{-1}cm^{-1}$). The radiochemical yield of [^{3}H]N_3-BP-N_3 was 1.0 mCi, specific radioactivity 175 mCi/mmol. [^{3}H]N_3-BP-N_3 was stored as a 2 mM solution in methanol at -20°C.

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