

Synthesis and Characterization of Azidobenzphetamine Analogues of the Cytochrome P450 Substrate Benzphetamine

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Benzphetamine, an amphetamine with sympathomimetic stimulant activity in the central nervous system, is a substrate for cytochromes P450 with highest metabolic turnover being catalyzed by cytochrome P4502B1. We synthesized three photolabile azido-compound analogs, *N*-(*p*-azidobenzyl)-*N*-methylphenethylamine (N_3 -BP), *N*-benzyl-*N*-methyl-*p*-azidophenethylamine (BP- N_3), and disubstituted *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-azidophenethylamine (N_3 -BP- N_3), in overall yields of 34, 38, and 10%, respectively. From the comparison of spectral dissociation constants (K_s) of the azido-compounds (the K_s values for which range from 2.3 to 4.2×10^{-5} mol/liter) with the K_s value for the P450 substrate benzphetamine of 6.0×10^{-5} mol/liter, it is clear that the introduction of azido-group(s) into a desmethylbenzphetamine skeleton did not significantly change the cytochrome P450 active site binding affinity in phenobarbital induced microsomes. Similarly, there is almost no difference in K_m (values 1.0 – 1.2×10^{-4} mol/liter) for *N*-demethylation of these photolabile compounds and benzphetamine ($K_m = 0.9 \times 10^{-5}$ mol/liter). All three azido-compounds are extremely photolabile under irradiation at 254 nm (half-life about 1 s). Photolysis of N_3 -BP in methanol, revealing a *N*-methoxy compound as a major product (85%) of a nitrene reaction, demonstrates a high reactivity of these compounds after photoactivation. Photoactivated azidodesmethylbenzphetamines produced clear inhibition of cytochrome P4502B1 and 1A1 specific catalytic activities in corresponding microsomal samples, compared to activities in samples irradiated with prephotolyzed probes. Pentoxyresorufin and ethoxyresorufin *O*-dealkylase activities were inhibited from 13 to 32%, depending on the compound used. © 1994 Academic Press, Inc.

INTRODUCTION

Cytochromes P450 and the Use of Functional Probes to Detect Structural Features

Cytochromes P450 (P450) represent a superfamily of hemoproteins acting as terminal oxidases of an enzymatic system metabolizing foreign compounds and endogenous substrates (1–3). These enzymes have been found in a great variety of organisms including bacteria, fungi, plants, and animals (4). Even in a single tissue there are many distinct P450 isoforms differing from one another in their substrate specificities. Moreover, levels of some P450s expressed in a cell change in response to different factors, such as an administration of drugs or other exogenous compounds (5). Cytochrome P450 protein levels also change as a function of age, sex, or pathological conditions (5).

Despite the fact that P450 was discovered more than three decades ago, only the

three-dimensional structures for the soluble bacterial forms P450_{cam} and P450BM-3 are known. The crystallizations of P450_{cam} and P450BM-3 have provided significant insights into P450 tertiary structure (6–8). However, the P450s of higher organisms are not soluble but integral membrane proteins, which may explain some of the difficulties encountered in the examination of these P450s by means of X-ray crystallography. Nevertheless, numerous indirect methods have been used to characterize the structure and function of cytochromes P450.

It was shown that there is some significant sequence homology of P450_{cam} and mammalian P450s, which suggests similar architecture within a P450 enzyme superfamily (9, 10). One goal of cytochrome P450 structural studies is to characterize the features of P450 involved in substrate recognition and binding in the P450 active center. This is essential to understanding structure–function relationships with respect to metabolic changes of P450 substrates. For this purpose a combination of methods using models based on sequence alignments with P450_{cam} and site-directed mutagenesis of suspected active center amino acids is currently used (11–14). Among chemical modification methods, which include the applications of suicide substrates (15) or affinity labels (16), photoaffinity labeling approaches have assumed a more important role in the study of the P450 active center (17–23). Photoaffinity labeling, based on principles of affinity labeling, uses photolabile derivatives of substrates (or inhibitors)—photoaffinity probes—to identify active center amino acids (19, 24, 25). Recently, two photoaffinity probes, azidobiphenyl for P4501A2 (22) and methyltrienolone for P45011B1 (23), have been successfully used to identify peptides with substrate binding sites.

Fitness of Benzphetamine as a Functional Probe

Benzphetamine is an amphetamine with pronounced sympathomimetic stimulant activity in the central nervous system (26). Benzphetamine shows fair to good turnover numbers with many P450s (27). Benzphetamine shows very high activity with cytochrome P4502B1 and is often used as a diagnostic for the presence of P4502B1 (28). Moreover, benzphetamine is an important and almost unique probe for substrate binding features in that it binds to P4501A1 and P4502B1 with essentially equal affinity, yet the turnover number of 2B1 for benzphetamine is reported to be above 100 whereas the turnover number of P4501A1 is reported as 4 (27). Characterization of the interactions' kinetic values and the inhibitory capacity of a series of analogs would help to elucidate features important in binding.

The particular analogs we characterize in this paper elucidate features of the binding sites of P4502B1 and 1A1 which are common to both, supporting the postulate of shared tertiary structure. These characteristics and their consequences are described in this paper.

EXPERIMENTAL PROCEDURES

Materials

Liver microsomes (Ms) were prepared by differential centrifugation from male rats (50–75 g) treated intraperitoneally with phenobarbital (PB) (29) or β -naphthoflavone (BNF) (30) and stored at -70°C until used. Cytochrome P450

content was 3.1 and 2.0 nmol P450/mg protein in PB-microsomes and BNF-microsomes, respectively. Benzphetamine was a gift from The Upjohn Co. (Kalamazoo, MI). Resorufin, pentoxyresorufin, ethoxyresorufin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and β -naphthoflavone were purchased from Sigma Chemical Co. (St. Louis, MO), NADPH was from Boehringer (Mannheim, Germany), phenobarbital sodium salt was from J. T. Baker, Inc. (Phillipsburg, NJ), and silica gel TLC plates (254 nm fluorescent) were from Whatman Inc. (Clifton, NJ). Silica gel (200–400 mesh) and chemicals for preparation of benzphetamine analogs were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used were of analytical grade or better.

Enzyme Assays

N-demethylation of benzphetamine and its azido-analogs was determined based on formaldehyde production (31, 32). Into the reaction mixture containing microsomes diluted to 1 mg protein per milliliter, various amounts of substrate were added from stock water solutions to obtain final substrate concentrations of 0–0.4 mM. The reaction was supported by a NADPH-regenerating system consisting of 1 mM glucose-6-phosphate, 0.2 IU glucose-6-phosphate dehydrogenase per milliliter and 0.05 mM NADPH. The reaction was initiated by the addition of NADPH and proceeded for 10 min at 37°C. From the double-reciprocal plot of formaldehyde production vs substrate concentration the K_m and V values were determined.

The metabolism of ethoxy- and pentoxyresorufin in BNF- and PB-treated liver microsomes, respectively, was monitored by following the time course of resorufin production (33). Microsomes were diluted with 0.1 M Tris/Cl, 0.01 M MgCl, pH 7.8, buffer as follows: BNF-microsomes to 0.01 mg/ml and PB-microsomes to 0.08 mg of protein per milliliter. After a 3-min preincubation in the presence of the NADPH-regenerating system, the reaction was initiated by the addition of stock DMSO substrate solutions (less than 1%, by vol) to final substrate concentrations of 2 μ M ethoxyresorufin and 5 μ M pentoxyresorufin. Reactions were monitored on a Perkin Elmer LS-5 fluorescence spectrophotometer at 37°C for 10 min.

Substrate Difference Spectra

To estimate the spectral dissociation constants (K_s) (34), which describe affinities of P450 for azidoderivatives and benzphetamine, microsomes were diluted with 0.1 M potassium phosphate buffer, pH 7.2, containing 20% (v/v) glycerol, to 3.3 μ M P450 concentration. Diluted microsomes were divided into two cuvettes (1-cm pathlength, 3 ml) and P450 in the sample cuvette was titrated with a water solution of substrate over a concentration range of 0–0.8 mM. To eliminate concentration differences between cuvette contents, the same volume of water was added to the reference cuvette with each addition of substrate to the sample cuvette. Spectra were taken by scanning from 500 to 340 nm. Values of K_s were calculated from double reciprocal plots of concentration vs absorbance change.

Photolysis Experiments

Sample irradiations were carried out in quartz cuvettes (1-cm pathlength, 3 ml) at room temperature using a photolyzer consisting of a 100-W mercury arc lamp,

an ellipsoidal reflector, a dichroic mirror (reflecting desired wavelength light around 260 nm), a water filter (9 cm length), a shutter, and a cuvette holder with a magnetic stirrer. All optical equipment was purchased from Oriel Corp. (Stratford, CT). In control experiments, an emitter of 366-nm light (Black-Ray Lamp, B-100A, Ultra-Violet Products, Inc., 288 W) was used.

Azido-compounds in 0.1 mM methanol solutions were irradiated for 0, 1, 3, and 7 s, followed by spectral determinations. The half-lives were estimated after linearization of the decay curves for each of the analogues. Similar experiments were done with microsomes (PB-treated liver microsomes were diluted to a protein concentration of 1 mg/ml) in the presence of 0.1 mM azido-compounds. The light exposure was for 0, 5, 15, 40, and 90 s. In these experiments, concentrations of both azido-compounds and P450 were measured at all time points.

For the determination of the effect of photoactivated probes on P450 metabolic activities, microsomes were diluted to a protein concentration of 1 mg/ml with 0.1 M potassium phosphate buffer, pH 7.2. The samples were photolyzed for 15 s in the absence or presence of 0.1 mM azidobenzphetamine analogs, or these derivatives were inactivated by a 15-s photolysis in the buffer. Immediately after irradiation, a solution of reduced glutathione (GSH) was added to a final concentration 10 mM. Microsomes were recovered from the reaction mixture by ultracentrifugation (160,000g for 20 min at 5°C), and subsequently the pellets were resuspended to the original sample volume with 0.1 M potassium phosphate buffer, pH 7.2, containing 10 mM GSH for alkoxyresorufin metabolism assays.

Analytical Methods

Protein concentration of the samples was determined by the BCA (bicinchoninic acid) method (35), using bovine serum albumin as a standard. P450 content was measured, based on a reduced cytochrome P450 complex with CO (36). Cytochrome P450 reductase activity was quantified by its ability to transfer electrons from NADPH to cytochrome c (29). Azidoderivatives as well as products of *N*-(*p*-azidobenzyl)-*N*-methylphenethylamine photolysis (15 s) in methanol were identified by means of mass spectroscopy on Finnigan Mat INCOS 50. For photoproduct analysis the apparatus was connected with GC Varian 3400 (DB-1 column, 30 m, 1.5°C/min temperature gradient 100–300°C). NMR spectra were recorded with General Electric 300-MHz apparatus (QE 300), with samples dissolved in CDCl₃. Azido-compounds were analyzed on a Perkin Elmer 229B ir spectrometer. All absolute spectra were taken over specified wavelengths on Hewlett Packard 8452 diode array spectrophotometer with sample solution in the sample cuvette and solvent in the reference cuvette. A Beckman Acta MIV spectrophotometer was used for difference spectra.

Synthesis of Azidodesmethylbenzphetamines

The preparation of three azidoderivatives of desmethylbenzphetamine is schematically summarized in Fig. 1. In each reaction step the products were identified by NMR spectra.

N-(*p*-Nitrobenzyl)-*N*-methylphenethylamine. To a 50-ml suspension of 5.2 ml

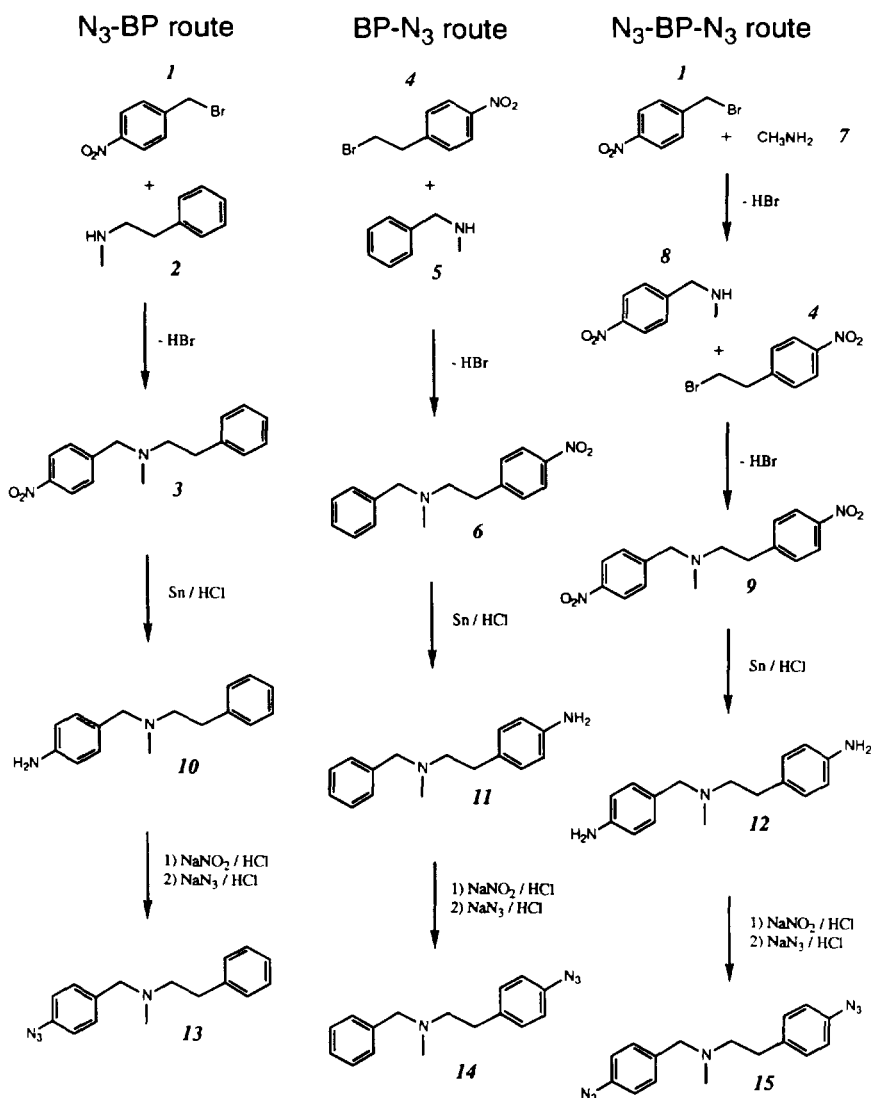


FIG. 1. The reaction routes for synthesis of *N*-(*p*-azidobenzyl)-*N*-methylphenethylamine (N₃-BP), *N*-benzyl-*N*-methyl-*p*-azidophenethylamine (BP-N₃), and disubstituted *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-azidophenethylamine (N₃-BP-N₃). Details are listed under Experimental Procedures.

of compound 2 (36 mmol) in methanol/benzene (11) containing 4.9 g potassium carbonate, a benzene solution (35 ml) of 7.7 g of compound 1 was added dropwise over 1 h with continuous stirring. The reaction mixture was stirred for an additional 20 h and then filtered through Whatman 5 filter paper to remove any inorganic compounds. Solvents from the filtrate were evaporated under reduced pressure. To the resultant residue 40 ml of 1 M HCl and 20 ml benzene were added. At

this point a three-layered system was formed. The resulting hydrochloride of *N*-(*p*-nitrobenzyl)-*N*-methyl-phenethylamine (**3**) was recovered as the densest (the lowest) layer and used without any further purification.

N-Benzyl-*N*-methyl-*p*-nitrophenethylamine. A reaction mixture (95 ml) containing 9.2 g *p*-nitrophenethylbromide (**4**) (40 mmol), 5.2 ml *N*-benzyl-methylamine (**5**) (40 mmol), and 5.5 g K₂CO₃ in ethanol/benzene (13) was refluxed for 24 h with another addition of 2 g of compound **4** after the first 12 h. By filtration through glass wool any inorganic material was removed. Solvents were evaporated from the filtrate under reduced pressure and subsequently 25 ml benzene and 46 ml 1 M HCl were added. The resultant suspension of light yellow crystals was washed in a separatory funnel with benzene (3 × 30 ml). The crystals of *N*-benzyl-*N*-methyl-*p*-nitrophenethylamine (**6**) were recovered by filtration through a sintered glass funnel and washed with benzene.

N-(*p*-Nitrobenzyl)-*N*-methyl-*p*-nitrophenethylamine. To prepare a precursor (*p*-nitro-*N*-methylbenzylamine (**8**)) for the synthesis of *N*-(*p*-nitrobenzyl)-*N*-methyl-*p*-nitrophenethylamine (**9**), alkylation of methylamine hydrochloride (**7**) by *p*-nitrobenzylbromide was undertaken. Methylamine hydrochloride (7.7 g) and potassium hydroxide (7.5 g) were dissolved in 260 ml ethanol. While stirring, 7.5 g *p*-nitrobenzylbromide in ethanol/benzene (12) solution were added dropwise over 2 h. The reaction mixture was stirred overnight at room temperature. After filtration through a sintered glass funnel, solvents were evaporated under reduced pressure and the residue was dissolved in benzene (50 ml). In a separatory funnel the benzene solution was washed with water and the product, *p*-nitro-*N*-methylbenzylamine (**8**), was recovered in 56% yield as a dense yellowish oil from the water layer after the solution was adjusted to basic pH with 12 M NaOH.

A reaction mixture containing 6.3 g *p*-nitro-*N*-methylbenzylamine (**8**) (38 mmol), 8.7 g *p*-nitrophenethylbromide (**4**), and 5.2 g K₂CO₃ in 90 ml ethanol/benzene (12) was refluxed for 24 h with an additional 3 g of compound **4** added after the first 12 h. The reaction mixture was filtered through a sintered glass funnel and solvents were evaporated under reduced pressure. To the residue 50 ml of 1 M HCl and 25 ml benzene were added. At this point a three-layered system was formed. The resulting hydrochloride of *N*-(*p*-nitrobenzyl)-*N*-methyl-*p*-nitrophenethylamine (**9**) was recovered as the densest (the lowest) layer.

Reduction of Nitro-Compounds

For the reduction of **3**, **6**, and **9** to their corresponding amines [*N*-(*p*-aminobenzyl)-*N*-methylphenethylamine (**10**), *N*-benzyl-*N*-methyl-*p*-aminophenethylamine (**11**), and *N*-(*p*-aminobenzyl)-*N*-methyl-*p*-aminophenethylamine (**12**)], a Sn/HCl procedure was employed. Typically, the solution of nitro-compound (20–30 mmol) in ethanol (200–250 ml) or acetone/ethanol (13) for compound **12** was slowly added to the mixture of concentrated HCl (100 ml) and granulated tin (25–35 g, 20 mesh). The reduction was completed in 30–90 min at a temperature maintained between 25 and 35°C using an ice-bath. After adjustment of the pH of reaction mixture with 12 M sodium hydroxide, the resulting aminoderivative was extracted into benzene. The solvent was evaporated from the pooled extracts

under reduced pressure and the residue was converted to the hydrochloride (light yellow crystals).

Azido-Compound Preparation

The conversion of nitro-compounds **10**, **11**, and **12** to the final *N*-(*p*-azidobenzyl)-*N*-methylphenethylamine (**13**), *N*-benzyl-*N*-methyl-*p*-azidophenethylamine (**14**), and *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-azidophenethylamine (**15**) proceeded through their diazoderivatives without separation of the intermediates. In 1 M HCl (1 liter) hydrochlorides of amino-compounds **10–12** (15–20 mmol) were dissolved and cooled to -5°C . While this solution was stirred continuously, a water solution (200 ml) of sodium nitrite was added dropwise to a molar ratio of 1:1 (nitrite/amino-group). The mixture was stirred for 1 h at -5°C . A solution (100 ml) of sodium azide (1.5 times molar excess of the original amino-groups) was added and the reaction mixture was stirred at 0°C for 1 h. The final products **13–15** were extracted into chloroform after adjustment of the reaction mixture to basic pH with 12 M sodium hydroxide and then purified on a silica gel column (2.2×25 cm or 4.2×40 cm) equilibrated with ethylacetate/chloroform (23). The ethylacetate/chloroform solvent system was used to elute fractions from the column. Collected fractions (15 ml) were pooled according to TLC analysis for the product and the solvent was subsequently evaporated. The pure compounds were converted into their hydrochloride forms and desiccated for 24 h under vacuum, in the presence of sodium hydroxide, to yield light yellow crystals. The yields of pure products after column chromatography were $>80\%$.

N-(*p*-Azidobenzyl)-*N*-methylphenethylamine. The overall yield based on elemental analysis of the pure **13** was 34%. Spectroscopic data: uv: absorption maxima (methanol) 210, 254 nm (ϵ 16,600 $\text{M}^{-1} \text{cm}^{-1}$), shoulders 280, 288 nm; ir: KBr pellet, 2110 cm^{-1} (strong); ^1H NMR: 300 MHz, δ (CDCl_3) 7.25 (7H,m), 7.00 (2H,d), 3.55 (2H,s), 2.85 and 2.62 (4H,AB pattern) and 2.25 (3H,s); mass spectra: EI-probe, m/z (relative intensity): 266 (M^+ , 0.4%), 239 (0.7%), 175 (95%), 132 (43%), 104 (100%), 91 (17%), 77 (44%). Anal. Calcd. for $\text{C}_{16}\text{H}_{19}\text{N}_4\text{Cl}$: C, 63.46; H, 6.32; N, 18.50; Cl, 11.71. Found: C, 63.30; H, 6.26; N, 18.34; Cl, 11.78.

N-Benzyl-*N*-methyl-*p*-azidophenethylamine. The overall yield based on elemental analysis of the pure **14** was 38%. Spectroscopic data: uv: absorption maxima (methanol) 210, 252 nm (ϵ 14,900 $\text{M}^{-1} \text{cm}^{-1}$), shoulders 281, 289 nm; ir: chloroform, 2110 cm^{-1} (strong); ^1H NMR: 300 MHz, δ (CDCl_3) 7.25 (7H,m), 7.00 (2H,d), 3.55 (2H,s), 2.80 and 2.60 (4H,AB pattern) and 2.30 (3H,s); mass spectra: $\text{Cl}(\text{CH}_4)$ -probe, m/z (relative intensity): 267 ($\text{M}+1$, 73%), 240 (65%), 134 (100%), 91 (17%). Anal. Calcd. for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{Cl}$: C, 63.46; H, 6.32; N, 18.50; Cl, 11.76. Found: C, 63.44; H, 6.30; N, 18.43; A, 11.93.

N-(*p*-Azidobenzyl)-*N*-methyl-*p*-azidophenethylamine. The overall yield based on elemental analysis of the pure **15** was 10%. Spectroscopic data: uv: absorption maxima (methanol) 210, 253 nm (ϵ 27,800 $\text{M}^{-1} \text{cm}^{-1}$), shoulders 280, 289 nm; ir: chloroform, 2110 cm^{-1} (strong); ^1H NMR: 300 MHz, δ (CDCl_3) 7.25 and 7.18 (2H,d), 6.95 (4H,d), 3.55 (2H,s), 2.80 and 2.60 (4H,AB pattern) and 2.25 (3H,s); mass spectra: $\text{Cl}(\text{CH}_4)$ -probe, m/z (relative intensity): 308 ($\text{M}+1$, 10%), 280