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Synthesis and Characterization of Azidobenzphetamine Analogs 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 × 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.

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

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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 μM ethoxyresorufin and 5 μM 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 μM 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 (160,000*g* 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 $CDCl_3$. 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

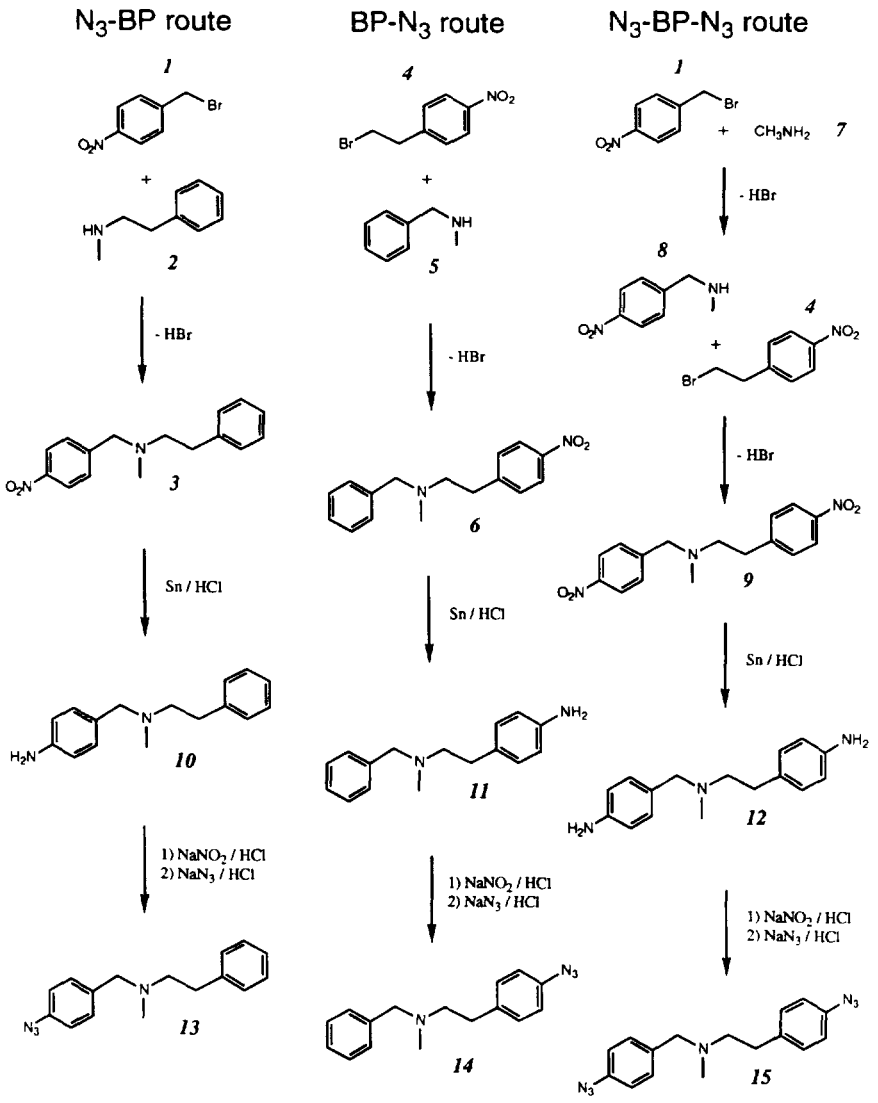

FIG. 1. The reaction routes for synthesis of *N*-(*p*-azidobenzyl)-*N*-methylphenethylamine (N_3–BP), *N*-benzyl-*N*-methyl-*p*-azidophenethylamine (BP–N_3), and disubstituted *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-azidophenethylamine (N_3–BP–N_3). Details are listed under Experimental Procedures.

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 K_2CO_3 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 K_2CO_3 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*-azidophenethyl-amine (**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 11 (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 M^{-1} 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 $C_{16}H_{19}N_4Cl$: C, 63.46; H, 6.32; N, 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 M^{-1} 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: CI(CH_4)-probe, *m/z* (relative intensity): 267 (M+1, 73%), 240 (65%), 134 (100%), 91 (17%). *Anal.* Calcd. for $C_{16}H_{18}N_4Cl$: 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 M^{-1} 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: CI(CH_4)-probe, *m/z* (relative intensity): 308 (M+1, 10%), 280

(20%), 252 (8%), 175 (100%), 132 (10%), 106 (10%) *Anal.* Calcd. for $C_{16}H_{18}N_7Cl$: C, 55.89; H, 5.28; N, 28.52; Cl, 1031. Found: C, 55.81; H, 5.48; N, 28.37; Cl, 10.42.

Thin-Layer Chromatography

During the synthesis the following solvent systems were used for individual compounds: methanol/acetone (3/2), **1**, **4** (R_f 0.85), **2** (R_f 0.15), **8** (R_f 0.30); benzene/ethylacetate (1/1), **1** (R_f 0.70), **3** (R_f 0.55), **4** (R_f 0.85), **6**, **9** (R_f 0.45); methanol, **3** (R_f 0.70), **10** (R_f 0.40), **11** (R_f 0.65), **12** (R_f 0.50); ethylacetate, **16** (R_f 0.65), **17** (R_f 0.80), **18** (R_f 0.55); ethylacetate/chloroform (1/2), **16** (R_f 0.35), **17** (R_f 0.30), **18** (R_f 0.40). Compounds resolved on TLC plates were visualized by exposure to uv light or I_2(g), or by a ninhydrin solution (*37*).

RESULTS AND DISCUSSION

Synthesis, Photolysis, and Properties of Azidodesmethylbenzphetamines

The synthetic routes, shown in Fig. 1, comprising 4–5 reaction steps, resulted in three azidodesmethylbenzphetamines: *N*-(*p*-azidobenzyl)-*N*-methyl-phenethylamine (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, as determined from elemental analyses.

These compounds showed an arylazide characteristic absorption maximum in the region 252–254 nm with high extinction coefficients: ε_{254} = 16,600 M^{-1} cm^{-1} for N_3–BP, ε_{252} = 14,900 M^{-1} cm^{-1} for BP–N_3, and ε_{253} = 27,800 M^{-1} cm^{-1} for N_3–BP–N_3 in methanol solutions. These values suggested a feasible photoactivation by the short-wavelength uv light.

All three azido-compounds were stabile as far as the azido-group is concerned (some oxidative degradation of a tertiary amine occurred under long-term exposure to aerobic environment). There was no apparent azido-group decomposition when they were exposed to "white" light (fluorescent tubes) for several hours. Even after irradiation by a strong 366-nm emitter for 10 min, only mild decay (15%) occurred. On the other hand, fast disappearance of azido-group absorption at 254 nm was observed when each of the azido-compounds was exposed to 254-nm light. To illustrate a typical time course of the photolysis at 254 nm, the decay of N_3–BP is depicted in Fig. 2. The disappearance of the azido-group absorption maximum exhibited first-order kinetics, hence the half-life of azido-compounds could be calculated; the half-life for both monoazidoderivatives was about 0.7 s, whereas the additional azido-group of N_3–BP–N_3 slowed the half-life for decay to almost 1 s. In the presence of microsomal suspensions (1 mg/ml) the rate of photolysis of each azido derivative was decreased due to absorption by protein and light scattering by microsomal particles. Within 15 s of the irradiation, however, less than 5% of the unreacted probes remained.

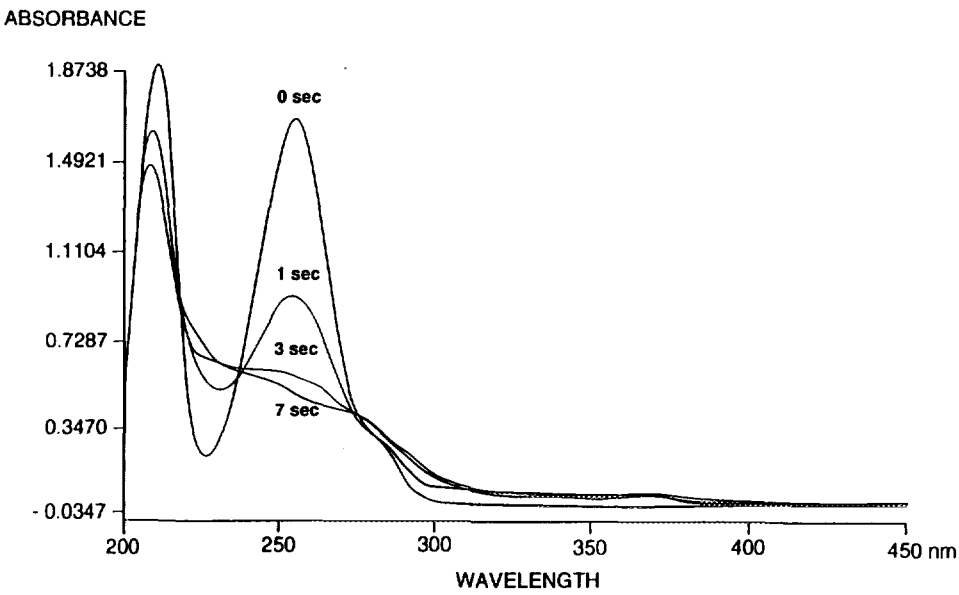

FIG. 2. The time course of N_3–BP photolysis. A methanol solution of N_3–BP (0.1 mM) was irradiated by uv light (100-W Hg Arc lamp) for 0, 1, 3, and 7 s, followed by absorption spectra recordings.

To examine the reactivity of photoactivated probes, photolytically generated nitrene from N_3–BP was reacted with a model compound—methanol. While a detailed photochemistry of the probe was not undertaken, the GC–MS analysis revealed *N*-methoxy-derivative as the major product, based on the derivative's fractionation pattern accounting for the majority of nitrene reaction with methanol. Other methanol derivatives observed were identified as the *N*-hydroxy- and nitro-derivatives. Thus, the results demonstrated a high reactivity for the photogenerated nitrene.

Cytochrome P450 Binding and Metabolism of Azidodesmethylbenzphetamines

Each of the three azido-compounds in the presence of either PB- or BNF-treated liver microsomes exhibited Type I substrate difference spectra with an absorption minimum at 421 nm and a maximum at 385 nm, which are the same as those for the parent compound—benzphetamine. Spectral parameters for the binding of each of the azido-compounds and benzphetamine are listed in Table 1. The spectral dissociation constants (K_S) of the compounds, which describe the affinity of cytochrome P450, are quite similar to those for benzphetamine.

In both PB- and BNF-treated rat liver microsomes, the binding of azido-compounds and benzphetamine followed a biphasic pattern. This can be attributed to the presence of two binding sites of a different affinity located in two individual enzymes or to secondary interactions. The same effect was observed when soluble P450 2B4 was titrated with desmethylbenzphetamine (*38*). Hence, the biphasic

TABLE I

Spectroscopic Data Characterizing Cytochrome P450 Interaction with Benzphetamine and Its Analogs

Substrate	Microsomes			
	PB-Ms		BNF-Ms	
	K_S (μM)	$A_{max} \times 10^3$ (nmol^{-1})	K_S (μM)	$A_{max} \times 10^3$ (nmol^{-1})
BP	10.13 ± 1.15	5.76 ± 0.34	3.05 ± 0.14	1.66 ± 0.02
	59.73 ± 4.16	14.14 ± 0.26	15.09 ± 3.77	1.93 ± 0.06
N_3–BP	9.76 ± 3.77	5.59 ± 0.91	3.24 ± 0.25	2.27 ± 0.05
	36.83 ± 4.40	12.36 ± 0.30	25.06 ± 1.86	3.50 ± 0.04
BP–N_3	11.61 ± 0.96	9.18 ± 0.41	2.08 ± 0.64	1.90 ± 0.12
	23.39 ± 2.03	13.14 ± 0.21	19.82 ± 2.80	3.19 ± 0.08
N_3–BP–N_3	5.01 ± 1.34	3.16 ± 0.33	1.73 ± 0.22	2.07 ± 0.06
	42.64 ± 4.77	8.20 ± 0.27	12.41 ± 3.87	3.13 ± 0.14

Note. The table contains two sets of values for each parameter because the binding of the compounds to cytochrome P450 is a biphasic process. Listed values are the means ± standard error.

pattern may be related to the nature of benzphetamine binding as well as the number of P450 isoenzymes involved in the interaction in microsomes.

Interestingly, K_S values obtained for all four benzphetamines tested in BNF-treated rat liver microsomes are comparable or several times lower than these values in PB-treated rat liver microsomes. Contrary to this observation, the extent of binding (represented by A_{max} values) of these compounds is much lower in BNF-treated liver microsomes than in PB-treated liver microsomes.

To study further the interaction of azido-compounds with P450, the metabolism of these compounds was examined. All azidodesmethylbenzphetamines were N-demethylated in the same manner as benzphetamine in microsomes from either treatment procedure. Kinetic data are summarized in Table 2. In agreement with the spectral parameters, azidobenzphetamines displayed an affinity for P450 which was comparable to that of benzphetamine. The K_m values of all benzphetamines for hepatic microsomes from BNF-treated rats were lower than those determined with microsomes from PB-teated rats. In addition, the maximum reaction rates (V) were, in some cases, more than seven times higher in microsomes from PB-treated rats than in microsomes from BNF-treated rats.

The results of spectroscopic and metabolic studies provide evidence that the introduction of an azido-group(s) into a desmethylbenzphetamine skeleton did not cause any significant changes of the P450 binding affinity, compared to benzphetamine.

Photolysis of P450 with Azidodesmethylbenzphetamines

The effect of uv-irradiation on P450 was monitored as a change in the ability of cytochrome P450 to produce a reduced CO complex. As the CO was produced

TABLE 2

Kinetic Data Describing the Metabolism of Benzphetamine and Its Analogs

	Microsomes			
	PB-Ms		BNF-Ms	
Substrate	K_m (μM)	V (nmol/min/mg)	K_m (μM)	V (nmol/min/mg)
BP	86.89 ± 20.30	7.39 ± 0.74	53.44 ± 1.68	1.21 ± 0.02
N_3–BP	115.08 ± 15.64	5.97 ± 0.36	51.62 ± 7.62	1.38 ± 0.08
BP–N_3	115.98 ± 19.46	6.18 ± 0.46	33.38 ± 3.20	0.81 ± 0.03
N_3–BP–N_3	99.54 ± 13.16	3.63 ± 0.21	31.53 ± 3.26	0.73 ± 0.03

Note. Listed values are the means ± standard error.

from CO_2 in the irradiated solution, reduced P450 CO complex was spectrophotometrically determined using an unreduced sample in the reference cuvette. In the absence of azidodesmethylbenzphetamines the P450 decay was linear over the monitored time period (Fig. 3). When azidocompounds were present in 30-times molar excess of P450 during the photolysis, the CO binding capacity of P450 dramatically decreased within 15 s. This finding is in agreement with the spectro-

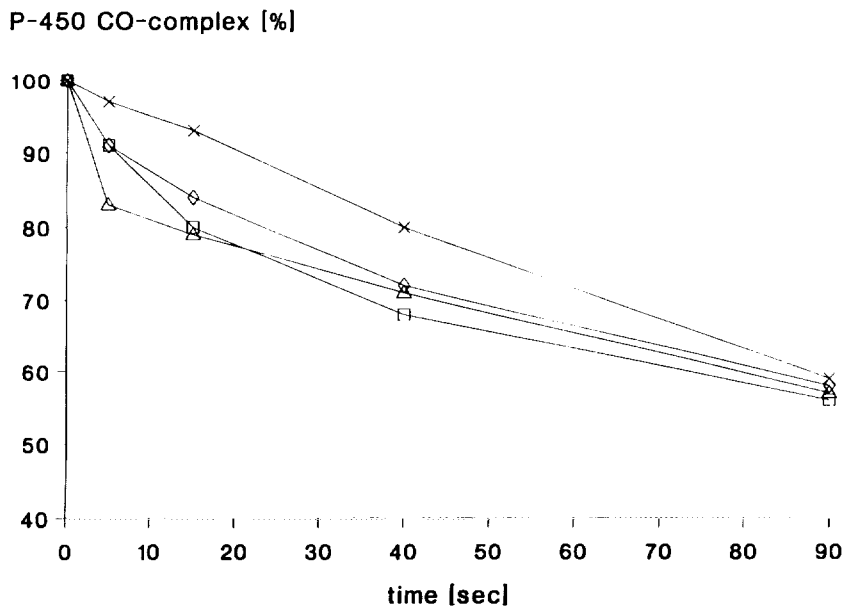

FIG. 3. The loss of cytochrome P450 CO-binding capacity by the photolysis. Microsomal suspension (1 mg/ml) was uv-irradiated in the absence (×) or presence of N_3–BP (◇), BP–N_3 (□), N_3–BP–N_3 (△) (0.1 mM) for 15 s, and subsequently, P450 concentrations were determined from reduced P450 CO-binding spectra. Experimental points are means of two determinations.

scopic observation that the photolysis of azidocompounds in microsomes was in large measure completed in 15 s (Fig. 3). It should be noted that products derived from azidobenzphetamines, quite aside from their role in P450 deactivation, were able to protect P450 by uv light absorption. This phenomenon was apparent mainly at photolysis time periods of 40–90 s where the slopes of lines for azido-compounds (see Fig. 3) become shallower compared to that of the control. As judged at the beginning of photolysis (0–5 s), when the rate of azido-compound photoactivation was high enough to allow one to neglect the protective effect of these compounds, diazidodesmethylbenzphetamine was more than twice as efficient in P450 deactivation than the monoazido-compounds.

In subsequent studies the inhibition of specific metabolic activities caused by photoactivated azido-compounds was measured. The pentoxyresorufin and ethoxyresorufin O-dealkylation assays were chosen as markers of P4502B1 activity in phenobarbital-treated microsomes and P4501A1 in BNF-treated microsomes, respectively. On the basis of CO-binding experiments and azido-compound determinations a 15-s photolysis time was used. That photolysis time allowed us to achieve nearly complete azido-compounds photoactivation and to minimize the P450 photodestruction (less than 5% as shown in Fig. 3). In order to avoid the interference of noncovalently bound photoactivated azido-compounds and CO on metabolic activities, the microsomes were recovered by ultracentrifugation after photolysis and resuspended in fresh medium. In order to test the efficacy of this wash procedure, CO was added externally to a microsomal suspension prior to the wash. After the wash, no inhibition of substrate hydroxylation actually could be detected in the CO-treated microsomes, compared with untreated, washed microsomes, thereby eliminating CO as an effective inhibitor in these experiments. The results of activity experiments with photoactivated azidobenzphetamine analogs are summarized in Fig. 4. Reference samples were irradiated in the presence of prephotolyzed azido-compounds. This approach assured a uniform protection of P450 in samples against uv light by desmethylbenzphetamine absorption and eliminated potential effects of residual desmethylbenzphetamine derivatives on metabolic assays.

The data of Fig. 4 demonstrate that the photoactivated probes inhibit cytochrome P4502B1- and 1A1-dependent catalytic activities in microsomes when compared to activities in samples irradiated with prephotolyzed probes. The extent of inhibition is similar in both kinds of microsomal samples, ranging from 13–23% for monoazido-derivatives to 30–32% for the diazido-compound. The higher inhibitory efficiency of the diazidoderivative is consistent with our P450 CO-binding experiments described above. This property of the disubstituted compound may be attributable to its crosslinking ability, which caused a stronger active center hindrance, and/or to the doubled probability of the compound's incorporation into the desired site. The azido analogs of benzphetamine inhibit microsomal P4501A1 and P4502B1 activities by the same percentage of total activity. The degree of inhibition is respectable rather than massive, though as a photoaffinity label this degree of inhibition is effective. The important feature here is that both 1A1 and 2B1 activities are inhibited, reflecting the same affinity seen

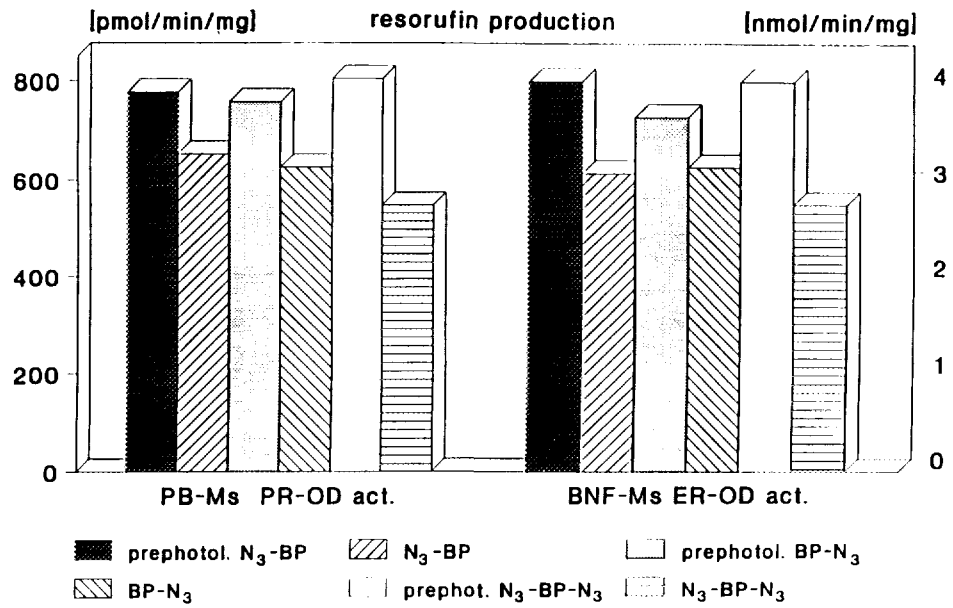

FIG. 4. Inhibition of pentoxy- (PR-) or ethoxyresorufin O-dealkylation (ER-OD) by photoactivated N_3–BP, BP–N_3, N_3–BP–N_3. Microsomal samples (1 mg/ml) with intact or prephotolyzed azido-compounds (0.1 mM) were photolyzed for 15 s, and then RP-OD and ER-OD activity was measured in phenobarbital- (PB-) and β-naphthoflavone-induced microsomes (BNF-Ms), respectively. Each bar represents the mean of two experimental values.

with benzphetamine, whereas the turnover of benzphetamine by these P450 enzymes is markedly different.

Moreover, among the samples only minor differences in reductase activities were caused by photoactivated azido-compounds (data not shown). Hence, these results suggested that the P450 activity inhibitions were directly related to covalent modification of the P4502B1 and 1A1.

Using the specific activity inhibition as a measure for the yield of azido-compound incorporation, these azidodesmethylbenzphetamines were more efficient than the average photoaffinity probe (*24*). Moreover, the extent of the P450 modification was most likely lowered by the water molecules (which are powerful nitrene scavengers) expected in the P450 active center (*39*).

In summary, three photolabile benzphetamine analogs were prepared. All three azido-benzphetamines bind to P450 with affinities comparable to that of benzphetamine. After photoactivation these azido-compounds interfered with P450 CO binding and significantly inhibited P450 specific catalytic activities in PB- and BNF-Ms. These results invite further use of azidodesmethylbenzphetamines as P450(s) photoaffinity probes. Applications of tritiated azidodesmethyl-benzphetamines now being undertaken will allow us to characterize further the interac-

tions of these azidoanalogs of benzphetamines with the various cytochromes P450.

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