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Synthesis and Characterization of 2′-Azidoaminopterin as a Potential Photoaffinity Label for Folate-Utilizing Enzymes[1]

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A photoreactive analog of aminopterin, 2′-azidoaminopterin (VI), was synthesized and evaluated as a potential inhibitor and photoaffinity label of folate-utilizing enzymes. The compound was tightly bound to dihydrofolate reductase (DHFR) from *Escherichia coli* (MB 1428) with K_i equal to 3×10^{-11} *M* and to the enzyme from mouse (S-180) cells with K_i approximately equal to 2×10^{-10} *M*. Dissociation constants measured by equilibrium dialysis using radioactive 2′-azidoaminopterin gave a value of $K_D = 3.2 \times 10^{-9}$ *M* for the bacterial enzyme. The presence of NADPH enhanced the affinity by more than an order of magnitude. Azidoaminopterin is also an inhibitor of thymidylate synthetase from *Lactobacillus casei*, competitive with methylene–tetrahydrofolate (K_i 7×10^{-7} *M*). Photolysis of the radioactive inhibitor in complex with DHFR from *E. coli* led to approximately 3% covalent incorporation of label into protein. The greater part of this attachment was nonspecific as shown by the lack of protection in the presence of methotrexate. Thymidylate synthetase from *L. casei* was not significantly inactivated upon photolysis in the presence of the inhibitor and deoxyuridylate. Model studies showed that photoreaction of the inhibitor led to covalent linkages with thiol, lysyl amino groups, and the hydroxyl groups of alcohols. Azidoaminopterin may be useful in labeling other enzymes of folate metabolism, although a minor photoproduct reacts nonspecifically with many proteins. The antifolate can be photoconjugated to polylysine as well as to proteins. The polylysine conjugates inhibit DHFR. Difference spectrum analysis of the photoproducts from the irradiation of the DHFR I complex indicates that water reacts efficiently with the enzyme-bound nitrene and must therefore have access to at least part of the bound *p*-aminobenzoyl group. This analysis suggests that azide analogs of protein ligands may be useful as reporter groups in probing the hydrophobicity of binding sites.

Enzymes catalyzing the reactions of folic acid and its derivatives have been the subjects of considerable structural analysis (*1–3*) because of both their mechanistic interest and their importance in chemotherapy (*4, 5*). For a number of reasons these proteins are excellent candidates for further examination by photoactivated covalent modification utilizing folate analogs that contain nitrene or carbene precursor groups. Products of such photoaffinity labeling are more readily analyzed when the protein sequence is already established (*6*) and provide an important

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comparison in solution with data obtained by spectroscopic methods (*7, 8*) and in the crystal state through X-ray diffraction (*9*). Multiple targets at an active site may be simultaneously modified, including some of the more inert amino acids (*10*). These and other special advantages of photoaffinity labeling have been reviewed (*11, 12*). The ability to convert reversibly bound antifolates into irreversible inhibitors may be of particular importance (*13, 14*). Recently efficient photoinactivation of a methotrexate transport system has been reported using a compound of moderate affinity (*15*). More specific reagents may also prove useful in similar applications.

The folic acid structure is sufficiently large to allow maintenance of tight binding even after considerable chemical alteration. This behavior is especially true for some of the very high-affinity antifolates. The *p*-aminobenzoyl (PABA) moiety is an appropriate site for the introduction of photoreactive groups since this part of the molecule appears to be bound in a hydrophobic crevice (*16, 17*), where nonproductive scavenging of reactive intermediates by water may be minimized. Substitutions in the PABA ring are generally well tolerated with respect to enzyme binding (*18*) and should be in sufficient proximity to the biochemically reacting bonds of folate derivatives to allow the tagging and identification of catalytically active amino acid residues.

For the above reasons we have synthesized 2′-azidoaminopterin and characterized it as a potential photoaffinity label for folate utilizing enzymes and folate binding proteins. Its interaction with dihydrofolate reductase (DHFR) was examined. We find that, although tightly bound to both the bacterial and mammalian enzyme, this compound does not photolabel the active site to a significant degree. Spectral changes occurring upon photolysis indicate that water competes successfully for the enzyme bound nitrene in this case. Model studies indicate, however, that a 2-azido-*p*-aminobenzoyl amide of this type may be useful in photolabeling the active sites of other folate enzymes. It also provides a means to photoconjugate an antifolate, through the PABA moiety, to a variety of nucleophilic polymers.

EXPERIMENTAL

DEAE-cellulose (DE-52) was purchased from Whatman. Sephadex and Sepharose 4B were from Pharmacia Fine Chemicals. Hydroxylapatite (Type C) was a product of Clarkson Chemical Company. NADPH, aminopterin, methotrexate (MTX), and l-glutamic acid dimethyl ester–HCl were from Sigma Chemical Company. 3,4-[^{3}H]-l-glutamic acid was purchased from New England Nuclear. Folic acid was a product of Nutritional Biochemicals. 2-Nitro-4-aminobenzoic acid was obtained from Chemical Procurements Laboratories, College Point, New York. *Escherichia coli* strains MB 1428 (MTX resistant) and MB 3746 (trimethoprim resistant) which overproduce DHFR were donated by Dr. M. Poe of Merck Institute. Dihydrofolate reductase from *E. coli* MB 1428 was purified to homogeneity according to the method of Poe (*19*) as modified by Williams (*20*) using MTX affinity chromatography. Electrophoretically pure

DHFR from strain MB 3746 was prepared by the method of Poe *et al.* (*21*). Pure DHFR from MTX resistant mouse (S-180) cells (*22*) was a gift of Dr. R. E. Kellems. *Lactobacillus casei* thymidylate synthetase overproducer (*23*) was kindly supplied by Dr. B. Dunlap. Thymidylate synthetase from this organism was partially purified through the last ammonium sulfate step of Dunlap (*23*) to a specific activity of 0.1 IU/mg. 7,8-Dihydrofolic acid was prepared by the reduction of folic acid with dithionite according to Blakley (*24*). 6(*R, S*)-Tetrahydrofolic acid was synthesized by H_2 reduction of folic acid using the procedure of Scott (*25*) except that mercaptoethanol was omitted with strict maintenance of anaerobic conditions.

A Beckman DU optical system modified with a Gilford 250A amplifier was used to record absorbance measurements at fixed wavelengths. Dihydrofolate reductase activity was assayed by the method of Poe *et al.* (*19*) except that NADPH was added last to minimize hysteresis (*26*). Thymidylate synthetase activity was determined by a modification of the spectrophotometric procedure of Dunlap *et al.* (*23*).

"Continuous" uv–vis spectra were obtained using a Hewlett–Packard 8450 microprocessor-controlled parallel-detection spectrophotometer. This instrument measures OD at 200 separate wavelengths simultaneously within a few seconds. Spectra can be stored in the computer memory and be added or subtracted from other stored spectra. Nuclear magnetic resonance spectra were obtained using a 60-MHz Varian 360-A spectrometer. Infrared spectra were recorded on a Perkin–Elmer grating spectrophotometer. Radioactivity measurements were carried out in Bray's solution (*27*) in a Packard Model 3255 Tri-Carb scintillation counter with quenching estimated through addition of internal standards. Ultraviolet irradiation was carried out with Rayonet photoreactor lamps (254- or 300-nm maximum output) purchased from the Southern New England Ultraviolet Company. Light fluxes were calibrated by means of ferrioxalate actinometry (*28*).

Synthesis of N-FMOC-2-nitro-4-aminobenzoic acid (*I*). 0.614 g (3.37 mmol) of 2-nitro-*4*-aminobenzoic acid was dissolved in 30 ml of dioxane. To this solution was added 0.73 g (6.92 mmol) of Na_2CO_3 dissolved in 50 ml of water. To the above dark yellow mixture was added 0.97 g (3.74 mmol) of solid fluorenylmethoxycarbonyl chloride (FMOC-Cl). After a few minutes a precipitate formed. Addition of 20 ml water and 40 ml additional dioxane caused the precipitate to redissolve. An additional 0.97 g of FMOC–Cl was added in one portion to the stirring reaction which was left at room temperature for 4 hr and at 4°C overnight. The solvent was removed under vacuum and the residue taken up in 15 ml of ethyl acetate. This was washed with 1 *N* HCl, then with water, dried over anhydrous sodium sulfate, and evaporated at reduced pressure. The tan residue was washed by trituration with dichloromethane and filtered giving 1.23 g (90% yield based on 2-nitro-*p*-aminobenzoic acid) of I, a light yellow powder essentially pure by thin-layer chromatography (TLC) ($CHCl_3$: CH_3OH, 4: 1). NMR in d_6-acetone: aromatic (δ7.3–8.3 11H), Ch_2, CH (δ4.3–4.9 complex 3H). IR (KBr): carboxyl C=O 1727 cm^{-1}, amide C=O 1680 cm^{-1}, NO_2 1521 cm^{-1}.

Synthesis of 4N-FMOC-2,4-diaminobenzoic acid HCl(*II*). 2.09 g (12.0 mmoles) of sodium dithionite was freshly dissolved in 10 ml H_2O. A solution containing

0.74 g (1.8 mmol) of I and 1.34 ml of concentrated ammonium hydroxide (28% NH_3) in 20 ml H_2O was placed in a dropping funnel and added dropwise over 30 min to the stirring dithionite solution. The bright yellow color of the added solution was bleached almost instantly upon contact with the dithionite. After the addition was complete, stirring continued for 10 min after which 2.5 ml of 12 *N* HCl was added to acidify the mixture. A copious white precipitate formed and was collected by centrifugation. The pellet was washed twice with 1 *N* HCl and then twice with water. The final pellet was dried under vacuum giving 0.816 g (100% yield assuming $2H_2O$/mol) of II. This ninhydrin positive material became easily darkened in air and was not characterized further.

Synthesis of 2-azido-4N-FMOC-p-aminobenzoic acid (III). 0.704 g (1.58 mmol) of II (dihydrate) was stirred in a mixture of 50 ml dioxane and 10 ml of 0.4 *N* HCl. This was carried out on an ice–salt bath at 0.4°C. Over a 20-min period, a solution of 0.124 g (1.8 mmol) of sodium nitrite in 5 ml H_2O was added dropwise to the above suspension. Stirring was continued for one additional hour leading to complete dissolution of the suspended material. Concentrated HCl (0.36 ml) was then added to the solution. This was followed by the addition at once of a solution of 0.117 g (1.8 mmol) of NaN_3 in 3 ml of cold H_2O. Gas evolution began after 30 sec and the dark yellow solution became lighter. Stirring was continued for 30 min. Solvent was removed at reduced pressure and the residue was taken up in 15 ml of ethyl acetate. This was washed with 1 *N* HCl, dried over anhydrous Na_2SO_4, and concentrated under vacuum. Purification was carried out by silica-gel (70 g) column chromatography using 10% methanol in chloroform as eluent. A uv-absorbing photoreactive peak was concentrated under vacuum to give 0.4 g of III (58% yield), a pale yellow solid. NMR in $CDCl_3$: singlets (δ6.33 broad 1H), doublets (δ4.02 2H), triplets (4.48 1H), aromatics (7.1–7.9 12H). IR (KBr): azide 2140 cm^{-1}, C=O broad 1695 cm^{-1}. UV (ethanol): λ_{max} 255 nm, ϵ_M 45,000; λ_{max} 245 nm after 30-sec photolysis with 300-nm lamp $\Delta\epsilon_M$ 13,500 (at 245 nm).

Synthesis of N-[N'-FMOC-2-azido-4-aminobenzoyl]-l-glutamic acid dimethylester (IV). 0.072 g (0.2 mmol) of III was dissolved in 5 ml of dry benzene containing 0.4 ml thionyl chloride and 2 mg of dimethylformamide as catalyst. The mixture was refluxed for 3 hr and the solvent and thionyl chloride was then removed at reduced pressure, carefully excluding moisture. The residue was taken up in 10 ml of chloroform. This solution was stirred vigorously at room temperature in a two-phase reaction with 0.063 g (0.3 mmol) of l-glutamic acid dimethylester HCl dissolved in 10 ml of 1% aqueous $NaHCO_3$. After 4 hr the chloroform layer was separated and washed with equal volumes of 2% $NaHCO_3$, 1 *N* in HCl, and brine. Drying with Na_2SO_4 and evaporation gave a yellow oil which was purified by chromatography on a column containing 5 g dry silica gel (70–230 mesh) eluted with anhydrous ether. The desired product, 0.045 g (42% yield), was a yellow oil, obviously photoreactive when spotted and held under uv light. It gave one band on TLC (silica gel, ethyl acetate: ether 1 : 5; R_f 0.5) whose R_f was reduced to 0.3 upon brief pretreatment with pyrrolidine in ether. NMR ($CDCl_3$): singlets (δ3.62 3H, 3.74 3H), doublets (δ4.22 1H, 4.5 1H), multiplets (δ2.37 4H, 4.85 1H), aromatics (δ6.95–8.3 12H). UV (ethanol): λ_{max} 254 nm, ϵ_M 45,000, ϵ_M 23,200 at 248 nm after photolysis with a 300-nm lamp.

Synthesis of N-[2-azido-4-aminobenzoyl]-1-glutamic acid dimethyl ester (V). 45 mg of IV was dissolved in 0.5 ml of chloroform containing 0.02 ml of pyrrolidine. After 30 min the mixture was diluted with 10 ml additional chloroform and washed with 10 ml of 0.2 *M* sodium phosphate buffer, pH 7.0. The organic phase was dried over Na_2SO_4, concentrated to 0.5 ml, and streaked onto a 2-mm silica-gel plate (Merck) and eluted with ethyl acetate: ether 1 : 5 avoiding excessive light. One major photoreactive, ninhydrin positive band (R_f 0.3) was scraped off and eluted with ethyl acetate to give 20 mg (71% yield) of V, a yellow oil. Mass spectrum: Most characteristic ions of *N*-[*p*-amino-*o*-azido-benzoyl] glutamic acid dimethyl ester [*m/z* (relative intensity)]: 335 (13) M^+, 307 (60) $M—N_2$, 275 (7) $M—N_2—CH_3OH$, 248 (35) $M—N_2—CO_2CH_3$, 216 (78) *m/z* 248 CH_3OH, 188 (67) *m/z* 216 CO, 161 (88) *p*amino-*o*-azidobenzoyl. UV (ethanol) λ_{max} 253 nm, ϵ_M 21,900; λ_{max} 290 nm, ϵ_M 18,500; trough at 269 nm. Upon 300-nm irradiation peaks at 253 and 290 nm decrease ($\Delta\epsilon_M$ 12,000, $\Delta\epsilon_M$ 12,500) and a new peak appears at 344 nm (ϵ_M 6000).

Synthesis of radioactive V. The above product was prepared with tritium in the glutamyl group by an analogous procedure to that described above using one equivalent of [3,4-^{3}H]-1-glutamic acid dimethyl ester HCl. The latter was prepared by bubbling HCl through a solution of [3,4-^{3}H]-1-glutamic acid in dry methanol at 0°C overnight followed by removal of solvent and excess HCl.

Synthesis of 2'-azidoaminopterin VI. 6-Bromomethyl-4-aminopteridine hydrobromide · isopropanol was prepared according to the method of Piper and Montgomery (*29*) as modified by Boyle and Pfleiderer (*30*). Eight milligrams (20 μmol) of the above compound was dissolved in 0.5 ml of distilled dimethylacetamide (DMA) containing 2 mg of suspended anhydrous sodium acetate. The mixture was stirred at 25°C for 1 hr. Then, 7.0 mg (21 μmol) of VII dissolved in 0.5 ml of DMA was added and the sample was heated at 80°C for 1 hr. The sample was then diluted with 9 ml of 1 *N* aqueous NaOH and stirred vigorously for 30 min at room temperature; 2 ml of 4 *N* HCl was added and the mixture was then filtered. The filtrate was adjusted to pH 4.5 by the dropwise addition of 4 *N* HCl, cooled at 4°C overnight, and the resulting precipitate was collected by centrifugation. After being washed with water in the centrifuge tube and resedimented, the crude product was dissolved in 1 ml of 0.05 *N* ammonium hydroxide and applied to a column (1 × 4 cm) of DEAE-cellulose in the hydroxide form. Elution was carried out with 0.1 *M*-stepwise gradient of ammonium bicarbonate, pH 8.3, from 0 to 0.4 *M*. 2'-Azidoaminopterin eluted at 0.3–0.4 *M* salt. Removal of the buffer by lyophilization gave 4 mg (45 yield, 7 overall yield) of fluffy yellow powder. This was reprecipitated from ammoniacal solution by acidification to pH 4.5. NMR (Na^+ salt in D_2O): singlets (δ6.70 1H, 8.94 1H, broad 4.75 2H), doublets (δ6.77 1H, 7.86 1H), multiplets (δ2.18 4H, 4.15 1H). UV, pH 7.2 (Fig. 3): Peaks (257 nm, ϵ_M 30,500; 280 nm, ϵ_M 17,400; 373 nm, ϵ_M 6650); trough (338 nm, ϵ_M 5300). After photolysis with either 254- or 300-nm light the uv spectrum had maxima at 256 nm (ϵ_M 24,800) and 345 nm (ϵ_M 12,750), isosbestic point at 316 nm (ϵ_M 9650). Elemental analysis for $C_{19}H_{19}N_{11}O_5 \cdot 5H_2O$. Calcd: C, 40.60; H, 5.15; N, 27.21. Found: C, 41.36; H, 5.30; N, 26.85. Note: Multiple hydration is commonly found in aminopterin derivatives unless exhaustive drying with heat is carried out (*31*). Heating was omitted

here because of the potential lability of the azido group. When made with tritiated V the product had a specific activity of 2.7×10^6 dpm/μmol.

Synthesis of N[2-azidobenzoyl]-l-alanine ethyl ester (VII). 0.69 g (5 mmol) of anthranilic acid was dissolved in 100 ml of stirring 0.5 *N* HCl at 4°C. Sodium nitrite (0.35 g) was dissolved in 5 ml; H_2O was added at once. After 5 min, 0.5 g of solid sodium azide was added portionwise over a 2-min period with vigorous stirring. After being allowed to come to room temperature, the mixture was extracted twice with 50-ml portions of ether. The ether extracts were pooled, dried over Na_2SO_4, and concentrated. The residue was taken up in 1 $NaHCO_3$, filtered, reacidified with 2 *N* HCl, and extracted again as above. Evaporation gave 0.35 g of *o*-azidobenzoic acid. The latter was stirred for 4 hr in 10 ml dry benzene containing 1.0 ml thionyl chloride and 10 mg dimethyl formamide. The solvent was removed thoroughly under vacuum and the residue was reacted in a two-phase system (CH_2Cl_2: 1% $NaHCO_3$) vigorously stirred with excess (0.75 g) of l-alanine ethyl ester HCl. After 6 hr the organic phase was washed with 1 *N* HCl and then with 1% bicarbonate. Evaporation and recrystalization from hot hexane (use caution with azides!) gave 0.4 g (75 yield) of solid VII. NMR ($CDCl_3$): singlet (δ1.58 3H), triplet (δ1.32 3H), quartet (δ4.25 2H), multiplet (δ4.74 $1H_\alpha$), aromatics + NH (δ7–7.6 and 7.9–8.25 5H). IR (KBr): azide (2130 cm^{-1}). UV (ethanol): λ_{max} 253 nm. Photolysis abolishes all peaks.

Synthesis of N-[o-azido-p-aminobenzoyl]-l-alanine ethyl ester (VIII). The synthesis of VIII was carried out essentially as that of V except that l-Alanine ethyl ester HCl was used instead of dimethylglutamic acid HCl. The uv spectrum and photochemical behavior of VIII was identical with that of V.

Electron-impact mass spectra were recorded using a Finnigan gas chromatograph/mass spectrometer Model 3200 combined with a Model 6000 data system (6 ft × 2-mm glass column, 3 OV 1 on Gas Chrom Q 100/120, initial temperature 150°C, final temperature 290°C, 6°C min^{-1} increase, injector temperature 250°C, 70 eV). Direct-inlet mass spectra were obtained using a heated probe.

Analysis of photolysis products of o-azidobenzoyl alanine ethyl ester. Four products (I–IV) were separated by gas chromatography/mass spectrometry (Figs. 1A and B). The most characteristic ions in the mass spectra of compounds I–IV were the following: [*m/z* (relative intensity)]. Product I (3% total product yield): 252 (5) M^+; 193 (2) M-CO_2CH_3; 150 (5) M-NH—$CH(CH_3)$—CO_2CH_3; 123 (34) M-OCN—$CH(CH_3)$—CO_2CH_3; 122 (100) M-CO—NH—$CH(CH_3)$—CO_2CH_3; 108 (26) *m/z* 123 CH_3; 93 (40). Product II (61% total product yield): 266 (8) M^+; 221 (2) M-OEt; 193 (5) M-CO_2Et; 150 (11) M-NH—$CH(CH_3)$—CO_2Et; 123 (52) M-OCN—$CH(CH_3$—CO_2Et; 122 (100) M-CO—NH—$CH(CH_3)$—CO_2Et; 108 (35); 93 (44). Product III (*o*-aminobenzoyl alanine ethyl ester, 7% total product yield): 236 (16) M^+; 163 (14) M-CO_2Et; 120 (100) M-NH—$CH(CH_3)$—CO_2Et; 91 (26). Product IV (29% total product yield): 266 (19) M^+; 193 (78) M-CO_2Et; 150 (72) MNH—$CH(CH_3)$—CO_2Et; 122 (100) M-CO—NH—$CH(CH_3)$—CO_2Et; 120 (27); 91 (20).

Products of photolysis in benzene were analyzed as a mixture by direct introduction into the mass spectrometer. The most characteristic ions in the resulting spectrum were found to be [*m/z* (relative intensity): 234 (35) M^+; 161 (100) M-

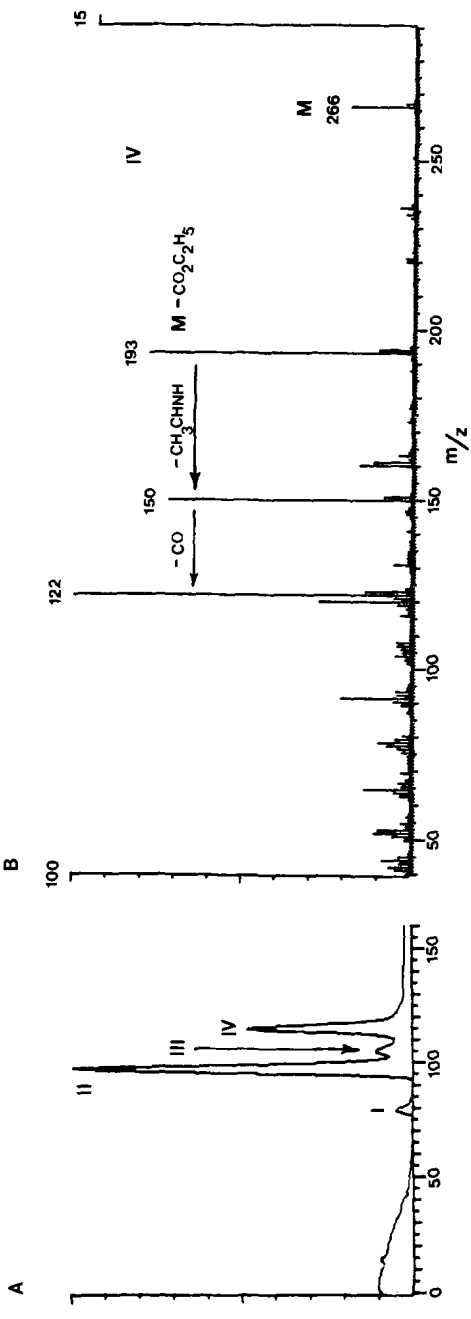


FIG. 1. Mass spectral analysis of the photoproducts of *N*-[*o*-azidobenzoyl]-l-alanine ethyl ester (10^{-3} *M*) irradiated in distilled methanol for 2 min at 2 cm from an RPR 3000-Å lamp. Solvent was removed immediately under vacuum, followed by dissolution in CH_2Cl_2 and reevaporation. (A) Gas chromatographic separation of photoproducts; (B) mass spectrum of product IV from (A).

CO_2Et. In addition, parent and fragment ions of *o*-aminobenzoyl alanine ethyl ester were found in this spectrum. Products of photolysis in diethyl ether were analyzed in the same way. The resulting spectrum also showed evidence of a product of molecular weight 234 and, to a smaller extent, of *o*-aminobenzoyl alanine ethyl ester.

Analysis of photolysis products of N-(p-amino-o-azidobenzoyl)-l-alanine ethyl ester. The most characteristic ions in the spectrum of *N*-(*p*-amino-*o*-azidobenzoyl) alanine ethyl ester were the following [*m/z* (relative intensity)]: 277 (9) M^+; 279 (37) M-N_2; 204 (5) M-CO_2Et; 176 (100) M-N_2—CO_2Et; 175 (83); 161 (66) M-NH—$CH(CH_3)$—CO_2Et. The most characteristic ions in the direct insertion spectrum of the photolysis products(s) of *N*-(*p*-amino-*o*-azidobenzoyl) alanine ethyl ester are the following [*m/z* (relative intensity)]: 281 (28) M^+; 208 (15) M-CO_2Et; 165 (100) M-NH—$CH(CH_3)$—CO_2Et; 137 (62) M-CONH—$CH(CH_3)$—CO_2Et; 105 (41) *m/z* 137 CH_3OH. In a third measurement, photolyzed and unphotolyzed material (as internal standard) were mixed in equimolar amounts and ion currents at the mass of the molecular ion of starting material (*m/z* 277) and that of methanol insertion product (*m/z* 281) were measured. After adjustment for specific molecular ion intensities, the amount of methanol insertion product was estimated to be approximately 40% of total product yield (*32*).

HAZARDOUS PROCEDURES

Low-molecular-weight azides are potentially explosive, especially when neat. These compounds should be handled in small amounts with proper shielding. Heat and light should be minimized to prevent detonation.

RESULTS AND DISCUSSION

A minimum requirement for a successful photoaffinity labeling by a nitrene or carbene precursor compound is that it does not internally rearrange to a much less active species upon photolysis (*11*). Since 2'-azidoaminopterin might or might not succeed in labeling a particular enzyme for various reasons and because its photoproducts are not easily analyzed, we carried out model studies on the photochemical behavior of simpler related 2-azido-benzoylamides in dilute solution. Concentrations greater than 10^{-3} *M* cannot be used because irrelevant bimolecular reactions then become dominant (*33*).

The products of the 300-nm photolysis of 1 m*M* *N*-(*o*-azidobenzoyl)-1-alanine ethyl ester in methanol were analyzed by gas chromatograph/mass spectrometry (gc/ms). The chromatographic separation resulted in four different product peaks (Fig. 1A), which were further examined by mass spectral analysis (Fig. 1B). Photolysis led to total conversion of the starting material to products, since ions characteristic of the starting material were not detected. One of the four products formed (7% of total product yield) was identified as *o*-aminobenzoyl alanine ethyl ester. The other three compounds (93% of total product yield) were formed by

covalent binding of methanol to the reactive nitrene or azirine (*34*) intermediate. Figure 1B shows that a major product (IV) has a mass spectrum consistent with loss of N_2 and fixation of methanol. The other major product (II) has also fixed methanol as indicated under Experimental, although the exact structures of these isomers have not been elucidated.

Photolysis in benzene or in diethyl ether resulted in a mixture of products. The major ions were consistent with isomer(s) of the nitrene intermediate as products, i.e., compounds formed by internal arrangement of the nitrene and not by covalent binding with the solvent (*m/z* 234, M^+; *m/z* 161, M-CO_2Et). A minor product in both cases was identified as *o*-aminobenzoyl alanine ethyl ester (*m/z* 236, M^+; *m/z* 163, M-CO_2Et), presumably formed by hydrogen abstraction from the solvent.

A *p*-amino substitution as occurs in aminopterin might deactivate a 2'-nitrene by electron donation to the ring. *N*-(*p*-Amino-*o*-azidobenzoyl) alanine ethyl ester undergoes uv spectral changes upon photolysis which are indistinguishable from those of 2'-azidoaminopterin, indicating that it is probably a good model for the latter compound. Its photolysis products were not amenable to analysis by gc/ms even after attempted derivatizations. The starting material and its photoproducts were therefore examined by direct insertion mass spectrometry. The electron impact direct insertion mass spectrum of *N* = (*p*-amino-*o*-azidobenzoyl) alanine ethyl ester photolyzed in methanol (Fig. 2) showed mainly product(s) of insertion of CH_3OH (*m/z* 281, M; *m/z* 208, M-*m/z* CO_2Et; 165, M-NH—CH(CH_3)—CO_2Et).

The starting material was almost totally converted to products, since ions characteristic of the azido compound were not found in the spectrum. In order to estimate the minimum percent yield of CH_3OH insertion product formed, an equimolar mixture of photolyzed and unphotolyzed material was prepared and analyzed by mass spectrometry (*32*). By using unphotolyzed material as internal standard, it was found that at least 40% of methanol insertion products (compounds with a molecular ion of *m/z* 281) were in the mixture of photolysis products. The true extent of solvent fixation may actually be much higher.

The above experiments indicate that enzyme-bound 2'-azido folates have the ability to covalently link nearby amino acid side chains with heteroatom groups of at least moderate reactivity (e.g., serine, threonine). In a largely apolar environment, however, nonproductive internal rearrangements and hydrogen abstractions would predominate.

The synthesis of 2'-azidoaminopterin involved the final condensation of the appropriate azido-PABA-glutamate diester with 6-bromomethyl-4-aminopteridine and subsequent deblocking (Scheme 1) as generally described by Piper and Montgomery (*29*). The purified product (VI) displayed an nmr spectrum identical to that of authentic aminopterin except for the aromatic region, as expected. Its photochemical behavior in aqueous buffer was essentially indistinguishable by uv spectral changes from that of the nonpteridine precursor compound (V). The uv spectrum of 2'-azidoaminopterin as a function of 300-nm irradiation is shown in Fig. 3 and indicates smooth and rapid photochemical decomposition of the azide with an isosbestic point at 316 nm. In dilute solution, photolysis was 90% complete in 20 sec under a light flux of 0.01 μEinstein/sec at 300 nm.

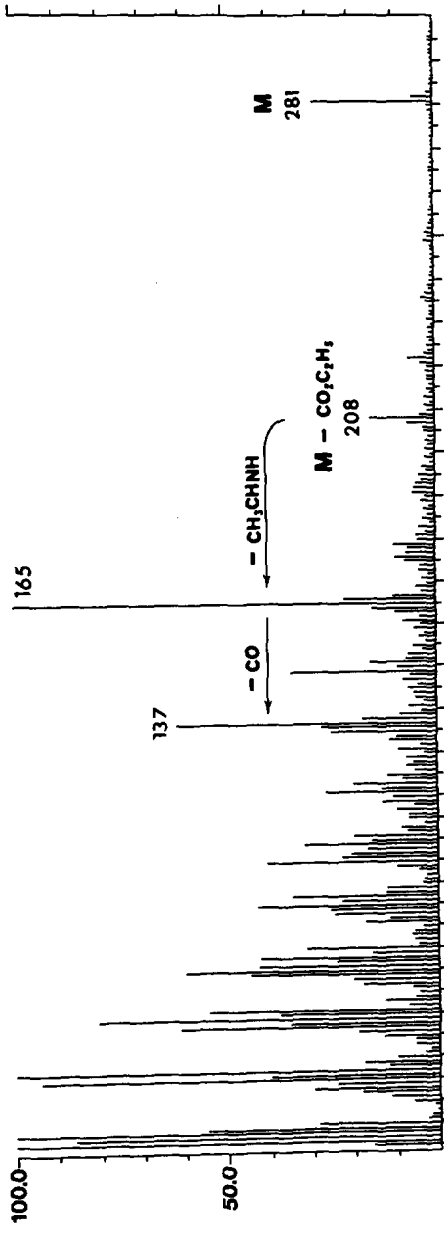
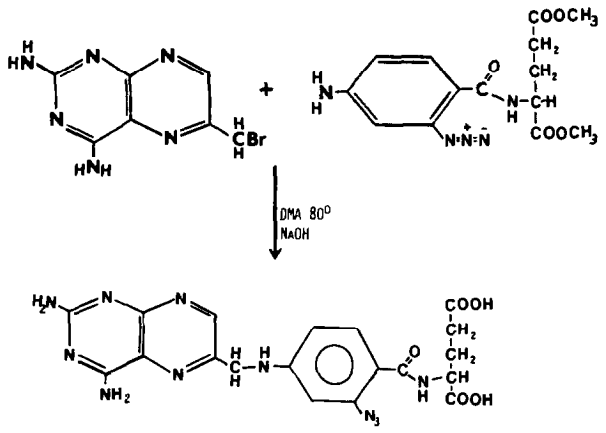


FIG. 2. Mass spectrum of the photoproducts of *N*-[*o*-azido-*p*-aminobenzoyl]-l-alanine ethyl ester. Irradiation conditions as in Fig. 1, but using direct probe mass spectrum.



The binding of this compound to DHFR from *E. coli* MB 1428 is illustrated in the spectrum shown in Fig. 4. In this wavelength region the enzyme itself contributed no significant absorption. The peak at 373 nm is due to the pteridine group of free azidoaminopterin and shifts to shorter wavelengths (λ_{max} 345 nm) upon addition of DHFR. This spectral change is very similar to that observed when aminop-

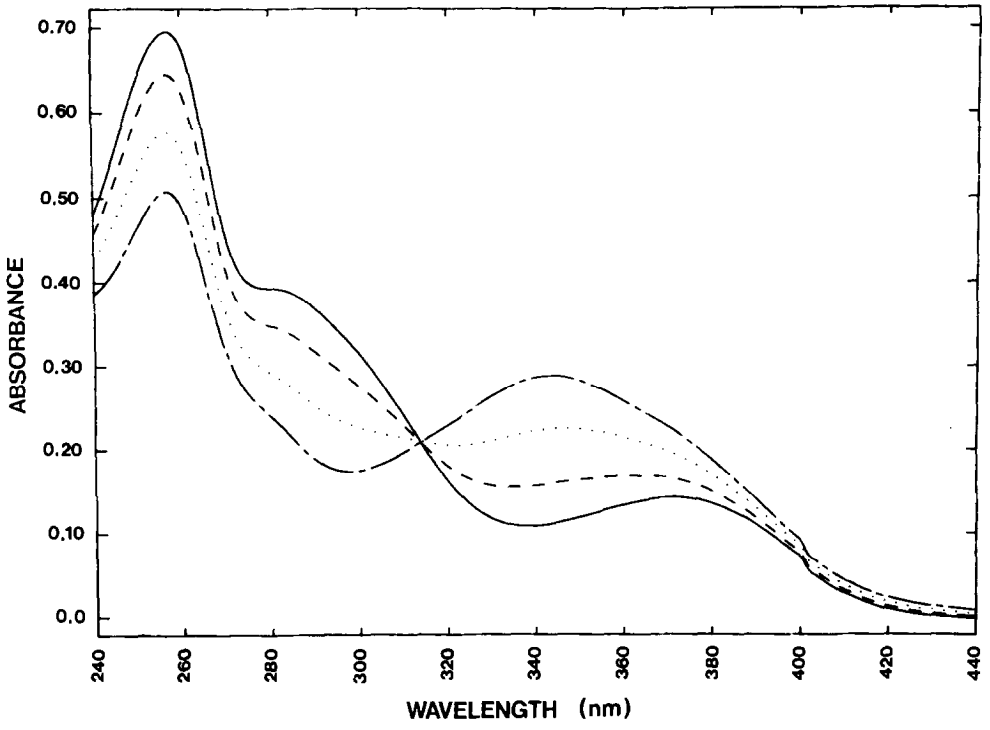


FIG. 3. Ultraviolet spectra of 2'-azidoaminopterin (44 μM) and its photoproducts in 0.05 *M* sodium phosphate, pH 7.2. —, No photolysis; ---, 5-sec photolysis in cuvette at 10 cm from RPR 3000-Å lamp; . . ., 10-sec total photolysis time; —-, 120-sec total photolysis time.

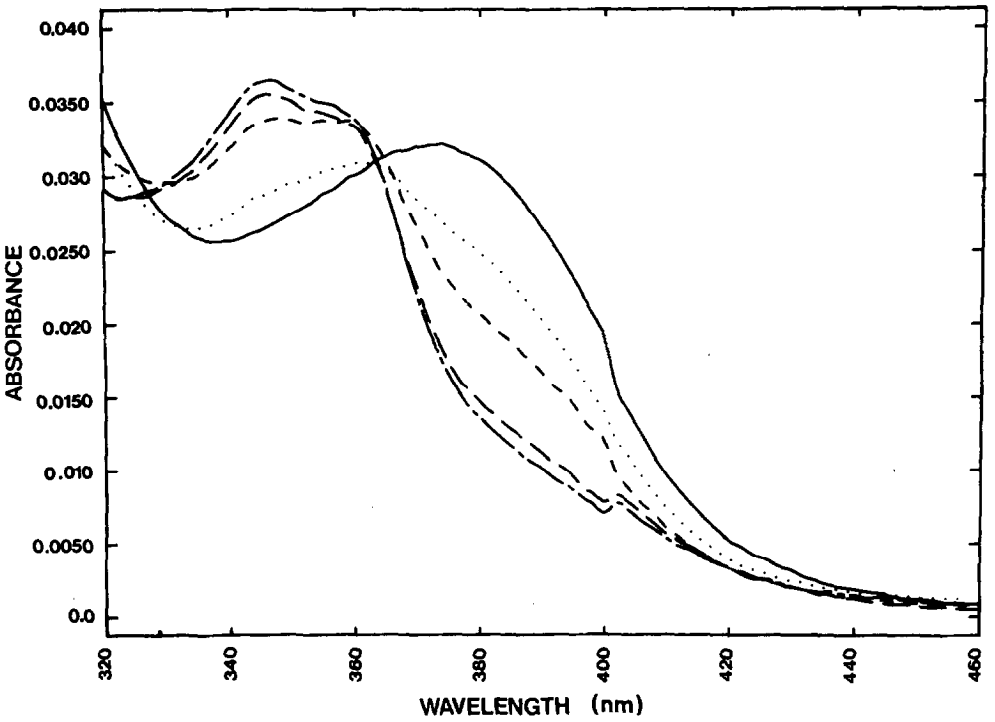


FIG. 4. Shift of 2'-azidoaminopterin uv spectrum upon binding to DHFR from MTX-resistant *E. coli* in 0.05 *M* sodium phosphate, pH 7.2. —, Spectrum of 5 μM inhibitor taken against buffer as reference. —·—, spectrum of 5 μM inhibitor with 7 μM DHFR present taken against identical concentration of enzyme in reference cell; . . ., 1.5 μM enzyme; ---, 2.9 μM enzyme; – –, 4.5 μM enzyme.

terin or MTX is mixed with the enzyme (*35*). At the lower limit of our ability to record spectra, enzyme and inhibitor concentrations each at 10^{-7} *M* still gave the characteristic shift, setting an upper limit for K_D of the E · I complex at 10^{-7} *M*. The same results are observed using DHFR from *E. coli* MB 3746 or with enzyme from mouse (S-180) cells. A similar shift was seen with 2'-azidoaminopterin which had previously been photolyzed in phosphate buffer indicating that the photoproduct(s) also was tightly bound by the enzyme. The dissociation constant for E · I (MB 1428) was measured by equilibrium dialysis (*36*) using radioactive azidoaminopterin. At concentrations of *E* and *I* of 10^{-6} *M* each, 98% of the radioactivity was found on the enzyme side of a dialysis apparatus at equilibrium. In 0.05 *M* sodium phosphate, pH 7.2, K_D was $3.2 \pm 0.4 \times 10^{-9}$ *M* for 2'-azidoaminopterin and $3.7 \pm 0.4 \times 10^{-9}$ *M* for its photoproduct(s). In the presence of 0.04 m*M* NADPH equilibrium binding was too tight to be measured accurately, but an upper limit for K_D could be set at 10^{-10} *M*. Kinetic measurements (Table 1) showed that azidoaminopterin is a powerful inhibitor of *E. coli* DHFR. It is competitive with dihydrofolate (data not shown). Using the published value of $K_m = 0.44$ μM (MB 1428) (*19*) for dihydrofolate and Cha's equation

$$I_{50} = K_i(1 + S/K_m) + \tfrac{1}{2}E_t \qquad [1]$$

TABLE 1

	Source of dihydrofolate reductase[a]		
	Escherichia coli		
	MB 1428	MB 3746	Mouse (S-180)
K_m (dihydrofolate)[b]	0.44×10^{-6}	1.6×10^{-6}	1×10^{-7}
K_i (azidoaminopterin)	3×10^{-11}	$16 \pm 5 \times 10^{-11}$	$9 \pm 3 \times 10^{-11}$
K_i (aminopterin)	$0.6 \pm 0.2 \times 10^{-11}$	$0.6 \pm 0.2 \times 10^{-11}$	—
K_D (azidoaminopterin)	$3.2 \pm 0.4 \times 10^{-9}$	$<10^{-8}$	$<10^{-8}$
K_D (photoproduct[s])	$3.7 \pm 0.4 \times 10^{-9}$	$<10^{-8}$	$<10^{-8}$
$\frac{K_m \text{ (dihydrofolate)}}{K_i \text{ (azidoaminopterin)}}$	14,700	10,000	1100

[a] All DHFR assays were carried out essentially under the conditions of Poe *et al.* (*19*), except that DHF was varied with and without inhibitor, and NADPH was added last.

[b] K_m values are taken to be those reported by Poe *et al.* (*19, 21*); K_D values were measured by equilibrium dialysis in 0.05 *M* sodium phosphate, pH 7.2; K_i values were calculated as described under Results and Discussion.

relating inhibition to K_i corrected for depletion of free inhibitor (*37*), we obtain $K_i = 3.0 \pm 0.2 \times 10^{-11}$ *M* for azidoaminopterin in 0.05 *M* Tris–HCl, 0.01 *M* KCl, pH 7.2. Values for the MB 3746 strain were comparable. Under identical conditions aminopterin bound only fivefold more tightly than the azido compound, with $K_i = 0.60 \pm 0.05 \times 10^{-11}$ *M*. Under similar assay conditions, K_i for mouse cell enzyme was found to be approximately 1×10^{-10} *M*. Kinetic, spectral, and thermodynamic measurements all indicate that 2'-azidoaminopterin is complexed tightly to DHRF from both bacterial and mammalian sources, binding constants being within an order of magnitude for those for aminopterin. Photoproduct(s) is also tightly bound.

Photolyses of the DHFR-[^{3}H]azidoaminopterin complexes were carried out in an attempt to label the enzyme covalently. As seen in Table 2, 3.5% of the theoretical amount of bound inhibitor could be fixed covalently to the enzyme. However, nearly the same amount of labeling occurred even when prephotolyzed inhibitor was used, suggesting that, at best, the labeling was due to "pseudophotoaffinity labeling" by a photoproduct. Increasing the time interval between prephotolysis of the inhibitor and mixing with enzyme from 1 to 10 min did not change the level of covalent binding, although increasing the *I*/*E* ratio tenfold did increase labeling nearly sevenfold. Most of this labeling, however, is probably nonspecific since 20 μM MTX did not reduce it significantly. NADPH did not affect the amount of label incorporated. Enzyme activity was not diminished (within experimental error) upon photolysis in the presence of inhibitor compared with controls where *E* and *I* were irradiated separately and then mixed only in the assay cuvette. Similar results were obtained using DHFR from *E. coli* MB 3746 or from mouse cells. We conclude, therefore, that although tightly bound to DHFR, 2'-azidoaminopterin leads to less than 0.5% photoaffinity labeling of this enzyme.

TABLE 2

ATTEMPTED PHOTOAFFINITY LABELING OF DHFR WITH 2'-AZIDOAMINOPTERIN[a]

Condition	Concentration of 2'-azidoaminopterin (μM)	Additions present during photolysis	Enzyme activity remaining	(mol)% ^3H bound after exhaustive dialysis
Control—no photolysis	4.0	—	100	0.2
Photolysis	4.0	—	>95	3.5 ± 0.2
Photolysis	4.0	20 μM NADPH	>95	3.5 ± 0.2
Photolysis	4.0	20 μM MTX	—	2.8 ± 0.2
Prephotolysis of I	4.0	—	>95	3.0 ± 0.2
Prephotolysis of I	40	—	—	20 ± 2
Same as 5 with $E + I$ incubated 20 min before denaturation	4.0	—	—	3.0 ± 0.2

[a] DHFR (2.5 μM) (*E. coli* MB 1428) was dissolved in 0.03 M sodium phosphate, pH 7.2, containing [^3H]azidoaminopterin to a final enzyme concentration of 4 μM. The solution was photolyzed at 300 nm in a quartz cuvette (approx 0.01 μEinstein/sec) for 30 sec. Where indicated, the inhibitor solution was made 6 N in quanidine HCl within 1 min of photolysis by the addition of the solid salt. Dialysis was carried out successively (12 hr) against 50 vol of 6 N guanidine HCl, then 50 vol of 8 M urea, and finally, 200 vol of 0.02 M phosphate buffer containing 0.1 sodium dodecylsulfate. Enzyme concentration in each sample was then estimated by reading its absorbance at 280 nm. An aliquot of each was counted.

The small amount of nonspecific labeling observed probably involves reaction of a minor and relatively stable photoproduct.

The tendency of 2'-azidoaminopterin to photoreact covalently with proteins in general was examined. Radioactive compound (10^{-5}–10^{-4} M) was photolyzed (95–98% to completion) in the presence of 0.5 to 3 mg/ml proteins including: ribonuclease (containing no Trp residues), insulin, trypsin, carbonic anhydrase (no SH or SS bonds), bovine serum albumin, and cytochrome *c*. In all cases fixed percentage (5–25%) of the total counts were covalently attached to protein although no prior associations could be demonstrated by equilibrium dialysis. When photolyses were carried out in the presence of 0.5 or 5 mM glutathione or, separately, with subsequent mixing of photoproducts and protein, the amount of covalent fixation was reduced (3–10%) but never abolished. By contrast, 1 mg/ml polylysine at pH 7.2 or 8.5 covalently fixed 80% of the 10^{-5} M azidoaminopterin photolyzed in its presence as determined by exhaustive dialysis. The yield of the latter reaction depends on the polylysine concentration up to 2 mg/ml and can be completely (98%) abolished by 0.5 mM glutathione. This outcome is also true for the photoattachment to urea or guanidine-denatured proteins. The inclusion of high concentrations ($\geq$10 mM) of amines, diamines, dipeptides, tetrapeptides, imidazole, glutamate, or *p*-aminobenzoate could not prevent the glutathione in-

sensitive photoattachment to proteins, which parallels the loss of the azide chromophore and is independent of the rate of uv irradiation.

We conclude that the photodecomposition of 2'-azidoaminopterin in water leads to approximately 90% production of a "normal" nitrene- or nitrenelike species which reacts preferentially with thiol and amino groups compared with water. This allows photoconjugation to proteins and polymers. The conjugate to polylysine (*n*-100 lysine, 5 aminopterin conjugated per polymer) was inhibitory to *E. coli* DHFR and competitive with DHF ($K_i \sim 10^{-9}$ *M*). A minor (10%) photoproduct also forms which does not react with nucleophiles in free solution but has the ability to covalently affix to native proteins. The nature of this reaction is unknown, although we may speculate that it is catalyzed by hydrogen bonding in the protein interior. The only method that we have found to minimize this reaction with a specific protein is by inclusion of an excess of a second scavenging protein, e.g., excess cytochrome *c* will protect ribonuclease from being labeled by prephotolyzed azidoaminopterin.

It was of interest to ascertain whether the failure to photolabel DHFR was due to rearrangement of the enzyme-bound nitrene in a hydrophobic crevice or to scavenging of the nitrene by water. Our mass spectral analysis had indicated that the photoproducts of *o*-azido-*p*-aminobenzoyl amides were highly dependent on the solvent used for photolysis. The substantial solvent effect on product distribution was also apparent in the uv difference spectra (azide minus photoproduct) obtained. Figure 5 shows the difference spectra recorded in phosphate buffer for 2'-azidoaminopterin which was photolyzed in 99% dioxane, 100% ethanol, or buffer. Since large perturbations in the 280- to 400-nm region (Fig. 5) are evident in moving from the nonhydroxylic solvent to progressively more hydroxylic ones, it is reasonable to assign the large $\Delta\epsilon$ at 345 nm to reaction(s) of the reactive intermediate with the O—H bond of the solvent. It should therefore be possible to examine the relative access of solvent to the 2'-azido group in the $E \cdot I$ complex by examining the difference spectra generated upon photolysis. Since the spectrum of the inhibitor is affected by enzyme binding, final absorption measurements were made in the denatured state in 6 *N* guanidine · HCl, although identical results were obtained without denaturation. Figure 6 shows that enzyme-bound 2'-azidoaminopterin gives photoproducts closely resembling those generated from the free compound in aqueous solution rather than in dioxane. Equivalent results are obtained in the presence or absence of NADPH. A similar result is found using the mouse enzyme. The intensity of the 345-nm minimum suggests that the hydrophobic cleft in which the PABA ring is bound (*16*) must be sufficiently shallow to allow access of water to the edge of the ring or must open rapidly relative to the half-life of the enzyme-bound nitrene, which is itself probably shorter than the association half-life of $E \cdot I$ (*33*). The azido-PABA ring appears to be bound with the azide group mainly facing outward from the cleft, since resonance Raman spectra suggest desolvation of the PABA carbonyl group in $E \cdot$ MTX (*35*). Even a large polymer such as polylysine can be photoattached to the 2' position and still allow rather tight binding to DHFR.

While the antifolate tested here is not useful for probing the structure of DHFR by direct photolabeling, the negative results obtained are still informative. The

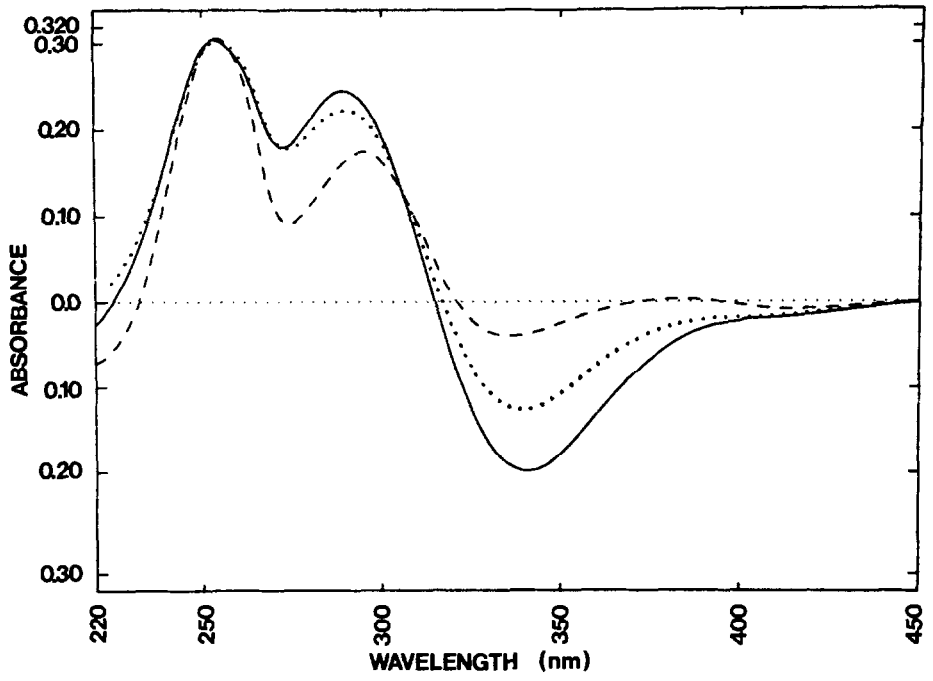


FIG. 5. Difference spectra of 2'-azidoaminopterin minus its photoproducts; 1.0 ml of 25.0 μM azidoaminopterin in dioxane, ethanol, or phosphate buffer was photolyzed for 60 sec at 2 cm from a 3000-Å lamp. Organic solvents were removed under vacuum and the residues dissolved in 1.0 ml of 0.05 *M* sodium phosphate buffer. Spectra were taken and subtracted from the spectrum of unphotolyzed compound in phosphate buffer. The difference spectra are shown for photolysis in buffer (—), 100% ethanol (. . .), and 99% dioxane (1% H_2O) (---).

lack of covalent modification is consistent with an absence of nucleophilic or hydroxylic amino acid side chains in the PABA binding region of DHFR as predicted by X-ray and nmr analysis (*16, 17*) since our mass spectral studies on model compounds indicated that such residues could react with the type of nitrene (or isomer thereof) which was presumably generated. Further, the spectral changes observed upon photolysis of $E \cdot I$ were consistent with a hydroxylic environment and show that the PABA ring is not so deeply buried in a hydrophobic region as to exclude water significantly from both edges. We know of no previous application of this type of spectral analysis using the photolysis of azide analogs to probe the hydrophobicity of a binding site. This technique may be generally useful, and it remains to be seen if a deep "dioxanelike" active site can be thus detected, using azido reporter groups.

Preliminary kinetic experiments with partially purified *L. casei* thymidylate synthetase were carried out in order to estimate the binding affinity of 2'-azidoaminopterin for this enzyme. Rate measurements as a function of 6(*R, S*)CH_2—H_4 folate concentration, plotted in double reciprocal form, extrapolated to a K_m value equal to 18.5 ± 2μM for the active enantiomer. This is in agreement with the value of 14.0 μM obtained by Daron and Aull (*38*). Inhibition by both aminopterin

and 2'-azidoaminopterin was competitive and gave K_i values of 6.8 and 7.6 $\times$ 10^{-6} M, respectively (*39*). The affinity of the photoreactive analog for thymidylate synthetase is high, making this enzyme a good candidate for further attempts at photoaffinity labeling. Photolysis of this enzyme in the presence of 10^{-5} M azidoaminopterin and 10^{-4} M dUMP, however, did not lead to significant inactivation (<5%) compared with separately irradiated controls. Staros *et al.* (*40*) have called attention to the susceptibility of aryl azides to reduction by thiols which would normally be present to maintain the above enzyme in its active form. 2'-Azidoaminopterin was incubated with 3 $\times$ 10^{-3} M dithiothreitol in 0.05 M Tris–HCl buffer, pH 8.5, at 25°. The azide chromophore slowly disappeared (256 and 292 nm) in a pseudo-first-order process ($t_{1/2}$ 21 min) without changes at 345 nm, presumably forming 2'-aminoaminopterin. This process should be 10-fold slower at pH 7.5 (*40*) and should not have interfered with the thymidylate synthetase experiments under the conditions employed (SH 1 mM), except possibly by intercepting an enzyme-bound nitrene more efficiently than water.

The azide derivative described may be useful in tagging folate binding sites where ROH or more reactive groups are in proximity. In many instances, as with DHFR this will not be the case. Covalent modification of more inert residues will

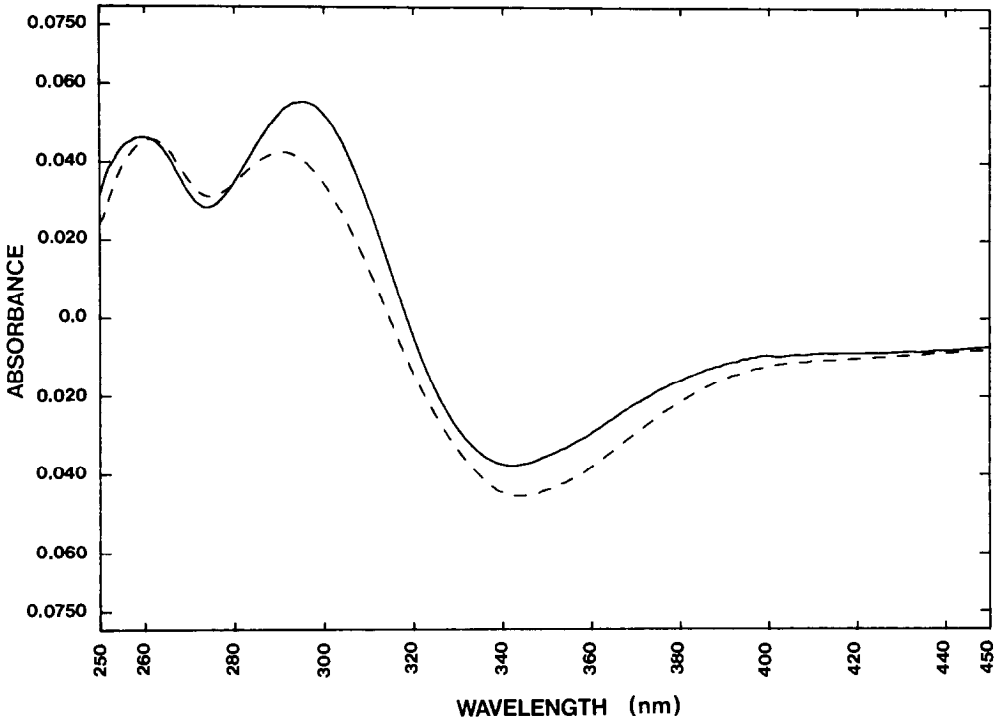


FIG. 6. Difference spectrum of 2'-azidoaminopterin (8 μM) and denatured DHFR (8 μM) taken against $E \cdot I$ complex photolyzed (at 12 μM), then denatured by addition of solid guanidine HCl to the same final concentration. Sample and reference cuvettes contained 0.05 M sodium phosphate, pH 7.2, and 6 N guanidine · HCl. —, Denaturation prior to photolysis; ---, denaturation after photolysis. NADPH (0.04 mM) was present throughout.

require more reactive nitrene, or preferably carbene precursor groups substituted into the PABA group, 2′-Azidoaminopterin, however, provides a means to photochemically conjugate aminopterin through its PABA moiety to nucleophilic compounds, including proteins and polymers and possibly to heterobifunctional reagents. Conjugation has previously been carried out through blocking of the glutamate carboxyl groups which may be essential to binding for some folate binding proteins (*41*).

ACKNOWLEDGMENT

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