A Stable Diazo Photoaffinity Label with High Absorptivity and Effective Photoactivation Beyond 300 nm[1]

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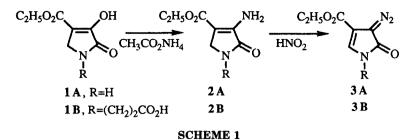*Carnegie Mellon University, Pittsburgh, Pennsylvania 15213*

The sulfosuccinimidyl active ester of 3-(3-carbethoxy-4-diazo-5-oxo-2-pyrrolin-1-yl)propanoic acid (DIAZOPY-SE) has been synthesized for use as a photoaffinity labeling reagent. This compound was obtained from commercial chemicals by a four-step synthesis requiring no complex procedures or special apparatus. The active ester efficiently derivatizes protein amino groups with the chromophore 3-carbethoxy-4-diazo-5-oxo-2-pyrroline (DIAZOPY, ϵ 8800 M^{-1} cm^{-1} at λ_{max} 330 nm), which on irradiation yielded products expected from formation of a reactive carbene intermediate. Brief irradiation of DIAZOPY in 2-propanol using wavelengths >300 nm for photolysis yielded mainly an isopropyl ether resulting from insertion of the carbene into the O–H bond of the alcohol. Formed concurrently and to a somewhat lesser extent was an isopropyl ester, resulting from a ring-contracting Wolff rearrangement of the carbene and subsequent reaction with isopropanol. Analogous products were produced by photolysis in 2-propanol of DIAZOPY-PA (for DIAZOPY propanoic acid), the carboxylic acid precursor of DIAZOPY-SE. Facile protein derivatization by DIAZOPY-SE was demonstrated using actin and sheep IgG. Actin labeled with DIAZOPY-SE and irradiated while in the F-actin (reversibly polymerized) form was crosslinked to yield a covalently-linked dimer, illustrating the potential of the reagent in photoaffinity applications. Advantages of DIAZOPY-SE as a photoaffinity labeling reagent include ease of synthesis, chemical and photostability, efficient photolysis at wavelengths >300 nm, and a capacity for crosslinking by carbene insertion processes. © 1991 Academic Press, Inc.

When carrying out investigations in biochemistry and molecular biology it often becomes important to estab-lish which molecules or portions thereof are closely bound to each other within the biological system. The possibility of utilizing for this purpose photoaffinity labeling with carbenes photolytically generated from diazo compounds was first proposed and tested by Singh, Thornton, and Westheimer (1) a number of years ago. In intervening years a number of improvements and extensions of the methodology have been introduced both by Westheimer and his associates (2) and by others (3,4), and useful results have been obtained, but better photolinking structures are still needed.

Among the chief shortcomings of previously described photoaffinity reagents of the diazo type have been a requirement for photolysis at biologically damaging short ultraviolet wavelengths, a low molar absorptivity at the photochemically effective wavelength, a marked susceptibility to acid-induced decomposition in solution, and a predominating Wolff rearrangement of the intermediate carbene, in which the carbene forms a ketene rather than immediately linking covalently to an associated structure by direct insertion into a proximate C–H, O–H, or N–H bond (3–5). Moreover, the synthesis of these compounds has often been laborious, costly and/or hazardous. The diazo compounds have, nevertheless, continued to attract attention because, as compared with the more frequently invoked aryl azide reagents, they hold greater promise of providing significant results. The original assumption that the aryl azides would link photochemically to adjacent molecules via direct insertion reactions through photodecomposition to aryl nitrenes now appears to have been realized only rarely and to very limited extent (4–7), although some improvement has been achieved recently by use of polyfluorinated derivatives (8). The present investigation represents an effort to reduce the shortcomings of the diazo alternative by constructing a photochemical linking reagent based on a readily accessible type of diazo-containing structure which has a combination of properties not shared by the diazo compounds previously employed in photoaffinity studies.

SCHEME 1

3-Carbethoxy-4-diazo-5-oxo-2-pyrrolines (**3**, Scheme 1, DIAZOPY)[2] have been produced by nitrous acid treatment of easily synthesized 3-amino-4-carbethoxy-2-oxo-3-pyrrolines (**2**) (9). As compared with other diazo compounds that have been studied as photoaffinity linking reagents, DIAZOPY congeners have a greater absorptivity at an advantageously long wavelength (λ_{max} = 330 nm., ϵ = 8,800 M^{-1} cm^{-1}) reminiscent of the nitroaryl azides. They have, moreover, been found to be unaffected by acetic acid at room temperature, stable in aqueous buffers over the range pH 4–9.5, and appreciably affected by laboratory lighting only after many hours of exposure. It was anticipated that the undesired Wolff rearrangement of the photochemically derived DIAZOPY carbenes (**4**, Scheme 2) would be relatively disfavored because of strain introduced by the required ring contraction to the 2,3-dihydroazetoketene (**5**). For these reasons it seemed worthwhile to explore the possibility of obtaining an improved photochemical linking reagent based on the DIAZOPY structure. The photochemistry and chemical stability of this type of diazo structure have therefore been examined, a congener which carries an active ester function for linking to other molecules has been synthesized, and protein derivatization and crosslinking experiments have been carried out to demonstrate the utility of the reagent obtained.

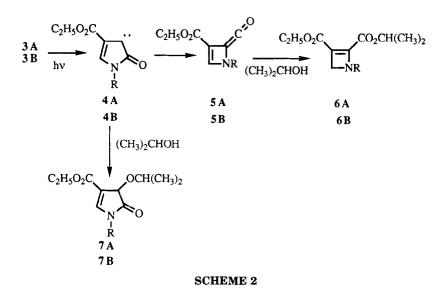## MATERIALS AND METHODS

General

All buffers and electrophoresis molecular weight standards were obtained from the Sigma Chemical Co. (St. Louis, MO). Except as noted, other reagents and solvents were from the Aldrich Chemical Co. (Milwaukee, MI). Microanalyses are by Atlantic Microlab, Inc.

(Norcross, GA). Spectra were determined with the following instruments: uv–vis spectra on a Hewlett–Packard 8452 diode array spectrophotometer, ir spectra on a Nicholet Model 5DXB FT-IR spectrophotometer, and NMR spectra on an IBM NR/300 FTNMR spectrometer. With NMR spectra the abbreviations used are the following: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad signal. Mass spectra were determined at the University of Pittsburgh Mass Spectrometry Laboratory under the direction of Dr. Kasi Somajajula.
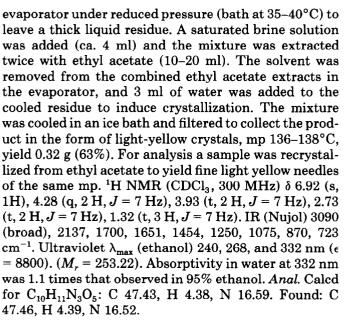
Synthesis of the Photoaffinity Label

3-(3-Amino-4-carbethoxy-2-oxo-3-pyrrolin-1-yl)propanoic acid (2B). A solution prepared from 3-(4-carbethoxy-3-hydroxy-2-oxo-3-pyrrolin-1-yl)propanoic acid (**1B**) (10) (2.43 g, 0.01 mol), glacial acetic acid (12.5 ml), and ammonium acetate (2.3 g, 0.03 mol) was stirred and heated under reflux for 3 h. The mixture was concentrated to a small volume and a small amount of water was added sufficient to dissolve suspended solids. The solution was brought to ca. pH 2 with concentrated hydrochloric acid, which was added cautiously to avoid exposure of the product to an excessive amount of acid. The precipitated pale-yellow crystalline product was collected by filtration and dried in a vacuum desiccator. The yield was 1.3 g (53%), mp 136–141°C. ^{1}H NMR (CDCl$_3$, 300 MHz) δ 5.62 (br, 2H), 4.26 (q, 2H, J = 7 Hz), 4.08 (s, 2H), 3.82 (t, 2H, J = 7 Hz), 2.73 (t, 2H, J = 7 Hz), 1.33 (t, 3H, J = 7 Hz). Ultraviolet (ethanol) λ_{max} = 298 nm. (M_r = 242.23). *Anal.* Calcd for $C_{10}H_{14}N_2O_5$: C 49.59, H 5.83, N 11.56. Found: C 49.67, H 5.83, N 11.48.
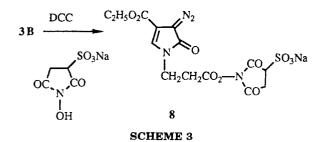
3-(3-Carbethoxy-4-diazo-5-oxo-2-pyrrolin-1-yl)propanoic acid (3B, DIAZOPY-PA). The procedure was a modification of the method of Madhav *et al.* (10). A solution of sodium nitrite (0.28 g, 0.004 mol) in water (2 ml) was added slowly to a stirred solution of 3-(3-amino-4-carbethoxy-2-oxo-3-pyrrolin-1-yl)propanoic acid (**2**) (0.48 g, 0.002 mol) dissolved in a mixture prepared from acetic acid (6 ml), water (1.5 ml), and concentrated hydrochloric acid (0.32 ml) which was cooled (kept at 0 to 5°C) during the addition and for 30 min thereafter. The solvents were removed in a rotary

SCHEME 2

evaporator under reduced pressure (bath at 35–40°C) to leave a thick liquid residue. A saturated brine solution was added (ca. 4 ml) and the mixture was extracted twice with ethyl acetate (10–20 ml). The solvent was removed from the combined ethyl acetate extracts in the evaporator, and 3 ml of water was added to the cooled residue to induce crystallization. The mixture was cooled in an ice bath and filtered to collect the product in the form of light-yellow crystals, mp 136–138°C, yield 0.32 g (63%). For analysis a sample was recrystallized from ethyl acetate to yield fine light yellow needles of the same mp. ^{1}H NMR (CDCl$_3$, 300 MHz) δ 6.92 (s, 1H), 4.28 (q, 2 H, J = 7 Hz), 3.93 (t, 2 H, J = 7 Hz), 2.73 (t, 2 H, J = 7 Hz), 1.32 (t, 3 H, J = 7 Hz). IR (Nujol) 3090 (broad), 2137, 1700, 1651, 1454, 1250, 1075, 870, 723 cm^{-1}. Ultraviolet λ_{max} (ethanol) 240, 268, and 332 nm (ϵ = 8800). (M_r = 253.22). Absorptivity in water at 332 nm was 1.1 times that observed in 95% ethanol. Anal. Calcd for C$_{10}$H$_{11}$N$_3$O$_5$: C 47.43, H 4.38, N 16.59. Found: C 47.46, H 4.39, N 16.52.

Sulfosuccinimidyl 3-(3-Carbethoxy-4-diazo-5-oxo-2-pyrrolin-1-yl)-propanoate (8, Scheme 3, DIAZOPY-SE). The diazo acid (3B, DIAZOPY-PA) (51 mg, 0.2 mmol) and N-hydroxysulfosuccinimide sodium salt

(Pierce Chemical Co., Rockford, IL) (43 mg, 0.2 mmol) were mixed with 1 ml of dry DMF. To the stirred suspension was added dicyclohexylcarbodiimide (DCC) (50 mg, 0.24 mmol) plus 0.2 ml of DMF, and stirring was continued overnight at room temperature. The mixture was centrifuged to remove dicyclohexylurea and the supernatant was diluted with 35 ml of ethyl acetate to precipitate the product, which was separated by centrifugation, washed with ethyl acetate, and dried in a vacuum desiccator. Yield: 32 mg (38%). (M_r = 452.33). The IR spectrum (Nujol) showed the expected bands at 2123 cm^{-1} (diazo group), 1825 cm^{-1} (succinimidyl ester), and 1792 cm^{-1} (succinimidyl ester).

Photolysis and Stability Experiments

Stability studies. DIAZOPY-PA (3B) was dissolved at a concentration of 30 μM in the following buffers: 10 mM sodium acetate, pH 4.5; 10 mM Mops-Na, pH 7.0; 10 mM sodium carbonate/bicarbonate, pH 9.5. Each solution was incubated in the dark, and a second portion of the Mops-buffered solution was kept in a sealed 50-ml Pyrex Erlenmeyer flask under ambient laboratory lighting (1 m distant from a 34-W fluorescent tube). Ultraviolet absorbance from 200–400 nm was monitored over time.

Irradiation conditions. Unless otherwise mentioned, irradiation was performed as follows: The reaction solution was placed in a Pyrex petri dish (3.7 cm diameter × 0.7 cm depth) and stirred with a magnetic stir bar. A 2-mm-thick Pyrex watch glass was placed atop the dish and the uv lamp used to irradiate the sample was placed directly on the watch glass. Absorbance of the Pyrex watch glass was 2.05 at 290 nm, 2.74 at 284 nm, and greater at shorter wavelengths. A Cole Parmer 6-W

SCHEME 3

9815 Series dual-wavelength lamp was used at the 365 nm setting. Spectra supplied by the manufacturer indicate that the light source and filters used in this lamp do not produce emission below 300 nm at the settings used. Large scale irradiation experiments performed for product isolation were carried out in larger Pyrex dishes. (4.7 cm diameter × 3.7 cm depth; 9.2 cm diam × 1.8 cm depth). Ultraviolet spectra indicated completion of the reaction after 55 min for 20 ml of 3.3×10^{-3} M DIAZOPY-PA (3B) in the deeper dish.

Isolation and characterization of irradiation products of DIAZOPY (3A). After irradiation, the isopropanol solution was evaporated to dryness at room temperature. The solid residue was dissolved in 1 ml acetonitrile per 20 mg DIAZOPY used in the reaction and then injected directly on the HPLC column. (250 × 4.6 mm C18, 10U column from the Alltech Co. (Houston, TX), 10 μl of sample per injection, 0.5 ml/min flow, eluate monitored at 230 nm, elute with isocratic acetonitrile). Two large peaks comprised most of the material detected during elution. These peaks were collected and evaporated to yield the pure reaction products on evaporation of solvent acetonitrile. In some cases the products were partially purified by preparative TLC on silica (Analtech (Newark, NJ) "taper plates," developed with 97.5:2.5 dichloromethane:methanol) followed by silica flash column chromatography (Aldrich, grade 60 Merck, 230–400 mesh, 60 Å, eluted with 97.5:2.5 dichloromethane:methanol).

The two major products from photolysis of DIAZOPY (see Scheme 2) were identified on the basis of their NMR and mass spectra. The first, now identified as the direct insertion product 7A, contained an oxygen-linked isopropyl group which showed the septet from the isopropyl methine proton centered at δ 3.90 ppm, the expected chemical shift for such a proton in an isopropyl ether. The two methyl groups of the isopropyl group appear in the NMR spectrum as two different doublets (centered at 1.21 and 1.24 ppm), showing that these methyl groups have a diastereotopic relationship and that the photolytic reaction has introduced a chiral center, as present in structure 7A. The two methylene protons of the ester ethoxy group give rise to a symmetrical multiplet centered at 4.32 ppm and resembling two closely spaced overlapping quartets, also reflecting a diastereotopic relationship. The downfield signal from the vinyl proton at the 2-pyrroline position (6.68 ppm) remained in the spectrum of this product, and there was a one-proton singlet at 5.68 ppm representing the proton at the 4-ring position as well as a broad one-proton signal at 6.23 ppm from the proton on the ring nitrogen and a triplet (1.35 ppm) from the methyl of the ethoxy group. The mass spectrum of this product showed an [M + H]$^+$ ion corresponding to structure 7A at *m/z* 214

and, as the base peak, an ion of exact mass 198.0791 and formula $[C_9H_{12}NO_4]^+$ (calcd *m/z* 198.0766) ([M-15]$^+$), which would result from the expected loss of CH_3 from the molecular ion derived from an isopropyl ether of structure 7A.

In the second photolysis product the isopropyl methine NMR septet was at δ 5.65 ppm, the chemical shift expected in an isopropyl ester, and only one doublet, (δ 1.35 ppm) from the isopropyl methyls was seen. The ester ethoxy group produced only the usual triplet (δ 1.33 ppm) and quartet (δ 4.27 ppm). The NMR spectrum gave no indication of a chiral center. There was no downfield signal corresponding to the vinyl proton of a 2-pyrroline, but there appeared instead a 2-proton signal (δ 4.05 ppm) attributable to the ring methylene of a compound of structure 6A, which would result from a ring-contracting Wolff rearrangement followed by 2-propanol addition to the intermediate ketene 5A and double bond migration into a position of maximum conjugation. There was a broad one-proton signal at δ 6.05 ppm from the proton on the ring nitrogen. Thus the NMR spectrum was in total agreement with structure 6A. From the mass spectrum an exact mass of 213.1025 was obtained for the molecular ion $[C_{10}H_{15}NO_4]^+$ (calcd *m/z* 213.1001) and the base peak, exact mass 171.0535, corresponded to a fragment $[C_7H_9NO_4]^+$ (calcd *m/z* 171.0532) resulting from elimination of propylene [M-42]$^+$ from an isopropyl ester via a McLafferty rearrangement. Thus, the mass spectrum confirmed the assignment of structure 6A to this second photolysis product.

Isolation and characterization of irradiation products of DIAZOPY-PA (3B). After irradiation, the isopropanol solution was evaporated to dryness at room temperature. The residual oil was dissolved in 10 mM sodium bicarbonate, pH 6.8 (1 ml per 20 mg of 3A used in the reaction), and then injected directly on the HPLC column described above (30 μl of sample per injection, 2 ml/min flow, eluate monitored at 240 nm, eluted with 0–20% acetonitrile in 10 mM sodium bicarbonate, pH 6.8 over 10 min). Two large peaks comprised most of the material detected during elution. These peaks were collected, chilled in an ice bath, and brought to pH 2–3 using 0.1 N HCl. Each was then frozen and lyophilized. The resulting residues were triturated with chloroform and the chloroform solution evaporated to yield the final products.

The products thus isolated were analogous to those formed from DIAZOPY (3A). The ether (7B) produced by carbene insertion into the OH bond of the solvent was most easily recognized by the retained downfield NMR singlet (δ 6.73 ppm) from the proton at the 2-position of the pyrroline ring and the newly introduced singlet (δ 5.70 ppm) from the proton at the 4-position. The ester (6B) formed by solvolysis of the ketene resulting

from Wolff rearrangement of the carbene was distinguished by the presence of the symmetrical NMR downfield isopropyl ester multiplet (centered at δ 5.64 ppm) and the singlet (δ 4.08 ppm) from the methylene of the 4-membered ring.

Yield of DIAZOPY-PA (3B) photolysis products. DIAZOPY-PA (3B), 14.5 mg, 0.057 mmol) in 2-propanol (20 ml) was irradiated for 65 min at 0–5°C using the conditions described above for large scale irradiation. The reaction mixture was evaporated at room temperature. The resulting solid was kept under vacuum for 9.5 h at room temperature and dissolved in 970 μl of deuterochloroform. Ethyl p-aminobenzoate (200 μl of a 0.167 M solution in deuterochloroform) was added, and a proton NMR spectrum of the solution was obtained. A proton relaxation time of 15 s was used to insure accurate integration. The yields of 7B and 6B were determined by comparing areas of appropriately chosen peaks in the NMR spectrum of the photolysis mixture with the areas of peaks from the aromatic ring protons of the ethyl p-aminobenzoate introduced following the photolysis as the internal quantitative NMR standard. The following peaks were used for quantitation: for 7B, 6.78 ppm, singlet, one proton (relative area 22.6) and 5.70 ppm, singlet, one proton (relative area 22.3); for 6B, 5.62 ppm, multiplet, one proton (relative area 20.6) and 4.09 ppm, singlet, two protons (relative area 38.8); for ethyl p-aminobenzoate, 7.86 ppm, doublet, two protons (relative area 86.5) and 6.65 ppm, doublet, two protons (relative area 80.8). The yields of the two photolysis products were determined to be 32% for the insertion product 7B and 28% for the Wolff rearrangement product 6B. Apart from the 60% total yield of these identified photolysis products the NMR spectrum of the photolysis mixture revealed the presence of smaller amounts of other nonvolatile materials apparently containing isopropoxy groups.

Protein Labeling and Photocrosslinking Experiments

IgG derivatization. Sheep γ globulin (Sigma G 9887, 99% electrophoretic purity, avg M_r = 155,000) (1 mg, 6.5 × 10^{-9} mol) was dissolved in 250 μl of 0.1 M sodium carbonate/bicarbonate, pH 9.6. DIAZOPY-SE (60 μg, 1.5 × 10^{-7} mol) in 1 μl of dry dimethylformamide was added with stirring. The solution was incubated at room temperature in the dark for 1.5 h, and then passed through a 6.5 × 1.5-cm column of Sephadex G50-150 fine (Pharmacia Co., Piscataway, NJ). The column was equilibrated and eluted with distilled water. Eluate absorbance was monitored at 220 and 350 nm. The protein fraction, which eluted in the void volume, was dialyzed against 1 mM sodium bicarbonate (12–15,000 M_r cut-off dialysis membrane, Spectrum Co., Los Angeles, CA). To

determine the moles of photoaffinity reagent incorporated per mole of IgG, the absorbance of a labeled IgG solution was measured at 328 and 280 nm. These values were used to determine the concentration of photoaffinity reagent and protein respectively. Absorbance of the dye at 280 nm was calculated as 0.2× the absorbance at 328 (based on relative dye ϵ values at 328 and 280), and subtracted out prior to calculating protein concentration. The molar extinction coefficient of the IgG was ϵ_{280} = 245,000, as given by the supplier (Sigma).

Actin crosslinking. Actin was purified as described by Spudich and Watt (12). All centrifugations were performed at 4°C and 200,000g for 1.5 h. Buffer A was 2 mM Tris–Cl, pH 8.0; 0.5 mM 2-mercaptoethanol; 0.005% Azide–Na; 0.2 mM $CaCl_2$; and 0.2 mM ATP. Labeling buffer was 2 mM Bicine–Na, pH 8.4; 0.2 mM ATP–Na; 0.2 mM $CaCl_2$. Actin (5 mg) was polymerized by incubation overnight at 4°C in 1 ml of buffer A containing 100 mM KCl and 2 mM $MgCl_2$. The solution was centrifuged, and the resulting pellet of filamentous actin was overlayed with 1 ml of labeling buffer and incubated at 4°C for 1 h. The pellet was suspended in the overlayed buffer and dialyzed against 450 ml of labeling buffer at 4°C to induce depolymerization. After residual polymeric material was removed by centrifugation, absorbance of the supernatant indicated a protein concentration of 3 mg/ml ($\epsilon_{1\%}$ at 290 nm = 6.2, (13)). The supernatant was split into 2 aliquots of equal volume. Each was made 100 mM in KCl and 2 mM in $MgCl_2$, and incubated 2 h at room temperature to induce polymerization. While vortexing vigorously, 5 μl of a 0.15 M solution of DIAZOPY-SE in dimethylformamide was added to 1 aliquot, and 5 μl of dimethylformamide to the other. The solutions were incubated at room temperature in the dark for 6.5 h, and then centrifuged. Each pellet of filamentous actin was overlayed with 1 ml of labeling buffer and incubated at 4°C for 30 min. The pellets were suspended in labeling buffer and dialyzed against 500 ml of labeling buffer overnight at 4°C to induce depolymerization. Residual polymeric material was removed by centrifugation and the supernatant was diluted with labeling buffer to a total volume of 2.2 ml. Absorbance values at 290 and 328 nm indicated that 0.8 mol of photoaffinity reagent had been incorporated per mole of actin monomer. No absorbance at 328 nm was observed in the aliquot from which DIAZOPY-SE (8) had been omitted. The solutions were made 100 mM in KCl and 2 mM in $MgCl_2$ and incubated 1.5 h in the dark to induce polymerization. Each solution was vortexed, irradiated for 3 min under the conditions described above, and then centrifuged. Each pellet of polymerized actin was overlayed with 0.5 ml SDS–PAGE sample buffer and boiled for 2.5 min. The resulting solutions were used directly for gel electrophoresis. SDS–PAGE

of actin was carried out in 8.75% gels using the method of Laemmli (14).

RESULTS AND DISCUSSION

Synthesis and photochemistry. The photochemical experiments on these compounds were begun with DIA-ZOPY (**3A**), one of the previously known members of the series. Solutions of this compound in 2-propanol were irradiated through Pyrex glass which excluded >99% of the energy from wavelengths below 300 nm. The resulting reaction products were separated by HPLC and shown to consist principally of two compounds which would result from addition of 2-propanol to an intermediate produced by loss of N_2. The proton NMR and mass spectra of these compounds showed that one was an isopropyl ether (**7A**), which would result from insertion of the carbene **4A**, the expected immediate photodecomposition product, into the O–H bond of 2-propanol. The other product was the isopropyl ester (**6A**) expected from reaction of 2-propanol with a ketene (**5A**), which could arise from the Wolff rearrangement of the carbene **4A**.

Upon the basis of these results a reagent expected to be useful in photoaffinity experiments (DIAZOPY-SE, **3B**) was produced by synthesizing a compound having a propanoic acid side chain in place of the hydrogen on the ring nitrogen of DIAZOPY (**3A**). The requisite first pyrrolidine intermediate for the synthesis (**1B**) was obtained from a previously described one-pot procedure in which β-alanine is added to ethyl acrylate and the adduct is condensed with ethyl oxalate with the aid of sodium ethoxide (10). It should be noted that commercially available radioactively labeled β-alanine and/or oxalate could be introduced at this stage to produce a radioactive reagent. Treatment of **1B** with ammonium acetate in refluxing acetic acid led to the aminopyrroline **2B**, which yielded the diazo compound DIAZOPY-PA (**3B**) upon treatment with nitrous acid. The synthesis required no special skills and no costly chemicals or equipment. Photolysis of DIAZOPY-PA in 2-propanol gave results paralleling those observed in the photolysis of DIAZOPY (**3A**). The NMR spectra of the principal products showed that they were the isopropyl ester **6B** and the isopropyl ether **7B**. Results of an NMR-based quantitative analysis of a photolysis product mixture showed that the yield of **7B** was at least 32%, that of **6B**, at least 28%. The minor products in the photolysis mixture appeared to have resulted from participation of the initial photolysis products in secondary reactions which probably would not occur to a significant extent under the very different conditions of a photoaffinity crosslinking experiment. Since the outcome of these preliminary experiments was favorable, DIAZOPY-PA has been coverted into DIAZOPY-SE (**8B**), a sulfosuccini-
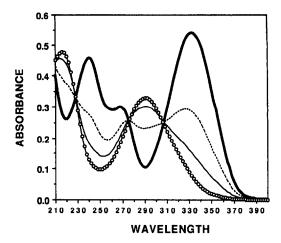

FIG. 1. Irradiation of compound **3B** in isopropanol. A 0.5 mM solution of compound **3B** in isopropanol was irradiated through Pyrex with a 6-W lamp (see Materials and Methods). Spectra taken at different times of irradiation are shown. The drop in absorbance at 328 nm indicated that photolysis was >95% complete at 120 s. —, 0 s; –––, 30 s; —, 60 s; O, 120 s.

midyl active ester (11), the derivative intended to serve as a candidate photoaffinity linking reagent.

Conditions for Use of the Reagent, Labeling and Crosslinking Properties

Tests of stability. The chemical stability of DIA-ZOPY-PA was tested by monitoring its uv absorbance spectrum in dilute aqueous solutions of pH 4.5, 7.0, and 9.5, in the dark. No change was observed after 41 h. When exposed to ambient laboratory lighting in a Pyrex test tube for 41 h, the pH 7.0 solution showed only a 14% drop in absorbance at 330 nm.

Rates of photolysis. The rate of photolysis was tested under irradiation conditions and label concentrations resembling those used for protein–protein or protein–ligand crosslinking. A 0.5 mM solution of DIAZOPY-PA in 2-propanol was irradiated through Pyrex with a 6 W hand-held uv lamp. (For protein of M_r 50,000, 0.5 mM = 25 mg/ml. More dilute label solutions should result in equal or shorter photolysis times.) Changes in the solution's uv spectrum induced by irradiation were >95% complete at 2 min. The uv spectrum of the product mixture was identical to that produced by irradiation of the more concentrated solutions used for isolation of reaction products. TLC indicated that the same two major products were produced in irradiation of the concentrated and dilute solutions. Irradiation-induced changes in the uv spectrum of DIAZOPY-PA can be seen in Fig. 1.

Protein labeling conditions. The attachment of DIA-ZOPY-SE to protein through reaction of its active ester function was demonstrated by derivatization of sheep

IgGs. Reaction of the protein at pH 9.6 for 1.5 h, followed by gel filtration and dialysis to remove unattached reagent, produced covalently labeled IgG showing both protein absorbance and the characteristic longer wavelength absorbance of the photoaffinity label. These absorbance values indicated that 6.0 mol photoaffinity groups had been attached per mole protein. This experiment also indicated the stability of the photoreactive moiety during attachment. Other workers have found a similar extent of labeling when reacting sheep IgG with succinimidyl esters of fluorescent dyes under similar conditions and using a similar excess of reactive reagent (15).

Protein photochemical crosslinking. The utility of DIAZOPY-SE in intermolecular crosslinking of proteins was also demonstrated. Actin was polymerized into filaments and then derivatized with DIAZOPY-SE. The actin was depolymerized, dialyzed, repolymerized, and collected by centrifugation to remove both unattached photoaffinity label and actin which had been rendered polymerization-incompetent. Ultraviolet spectra revealed that 0.8 mol photoaffinity group had been incorporated per mole actin. The difference in extent of derivatization found in this experiment and in the IgG labeling can be attributed to differences in the number and reactivity of actin and IgG lysines, and to the fact that the actin labeling reaction was carried out at lower pH, well below the solution pK_a of lysine's ϵ-amino group. Low pH was required to prevent actin denaturation. Labeling of actin lysines with other succinimidyl reagents at similar pH values has yielded similar levels of derivatization (Dr. Kenneth Giuliano, personal communication). A control sample of actin was treated identically, but introduction of DIAZOPY-SE was omitted from the derivatization procedure. The labeled and control samples of filamentous actin were irradiated and boiled in SDS. Electrophoresis showed the presence of higher molecular weight actin oligomers in the derivatized actin only, indicating that crosslinking was caused by the photoaffinity label and not by a different chemical or photochemical reaction of the actin (see Fig. 2). The relative mobility of the crosslinked actin and the molecular weight standards in Fig. 2 corresponded to somewhat higher than expected molecular weights for the actin dimers. However, other workers have observed actin covalent dimers with similar anomalously high molecular weights in gel electrophoresis. The molecular weight, as measured by comparison with electrophoretic standards, was found to depend on the sites of crosslinking (16).

SUMMARY

This initial investigation of photocrosslinking by means of a photoactivatable active ester (DIAZOPY-

FIG. 2. Polyacrylamide gel electrophoresis of actin crosslinked using the photoaffinity reagent, DIAZOPY-SE (**8**). Lanes are numbered consecutively from left to right. Lane 1 Molecular weight markers: carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase b (97,400), β-galactosidase (116,000). Lane 2 Actin was derivatized with the photoaffinity reagent, polymerized, and irradiated for 3 min through Pyrex (see Materials and Methods) using a 6-W lamp. The sample was then boiled in SDS-containing electrophoresis buffer and 12 μg was loaded on the gel. Lane 3 Actin was treated exactly as in lane 2, but the photoaffinity reagent was omitted during the derivatization reaction.

SE, **8**) of the diazopyrroline propionic acid DIAZOPY-PA support the belief that such active esters of DIAZOPY-PA will represent a promising addition to the rather limited list of reagents actually useful in such applications. By comparison with previously developed reagents based on a diazo photolinking group, these reagents will have a greater chemical and photostability in solution, will be more easily synthesized, and, because photolysis is induced at significantly longer wavelengths with higher absorption maxima, will undergo the required photolysis step with much less risk of radiation damage to the labeled specimen. As compared to those reagents containing an arylazide photolinking group, these reagents have the advantages of the much better established direct bond insertion capability of the diazo-based photolinkers, as well as more efficient photolysis under less damaging uv irradiation above 300 nm. Although there are nitroarylazides with absorption maxima well above 300 nm, their exposure to much shorter uv wavelengths is required for efficient photolysis (4,17).

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