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p-Nitrophenyl 3-Diazopyruvate and Diazopyruvamides, a New Family of Photoactivatable Cross-Linking Bioprobes†

Val S. Goodfellow, Marc Settineri, and Richard G. Lawton*

Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

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ABSTRACT: *p*-Nitrophenyl 3-diazopyruvate (DAPpNP) has been developed as a heterobifunctional cross-linking agent for synthesis of photoaffinity probes and photoactivatable cross-linking agents that are nucleophile specific. *p*-Nitrophenyl chloroglyoxylate is formed in high yield from oxalyl chloride and *p*-nitrophenol. Subsequent reaction with diazomethane produces DAPpNP in 50–60% overall yield. DAPpNP acylates primary and secondary amines to form 3-diazopyruvamides in high yields. 3-Diazopyruvamide derivatives have been formed from a wide variety of amines including aromatic amines, amino acids, and peptides. 3-Diazopyruvamides undergo photolysis and Wolff rearrangement at 300 nm to produce a ketene amide, which efficiently acylates nucleophilic species to form malonic acid amide derivatives. A family of photoactivatable 3-diazopyruvamide cross-linking agents was synthesized from amino acids. A cleavable, thiol-specific photoactivatable cross-linking agent was synthesized from cystamine. These reagents were caused to react with rabbit muscle aldolase to form mainly dimeric cross-linked species.

The techniques of photoaffinity labeling and the introduction of photogenerated cross-links in biological structures and aggregates are powerful methods for determining a number of characteristics of biochemical systems. These characteristics include the location and nature of the active site of enzymes, the receptors for hormones and drugs, the relationships of proteins within aggregate structures, and the study of protein–protein interactions involved in immunochemical interactions (Bayley & Knowles, 1977; Bayley, 1983; Chowdry & Westheimer, 1979; Tometsko & Richards, 1980). The development of new classes of photoactivatable reagents aids the elucidation of the structure of biological molecules by providing probes that can be fine tuned to react efficiently with a given biologically active target. Nevertheless, the number and variety of photosensitive functional groups for incorporation in these reagents is quite limited, and of these only the nitroaryl azides seem to have much popularity with the biochemical community (Westheimer, 1980; Bayley & Staros, 1984). The

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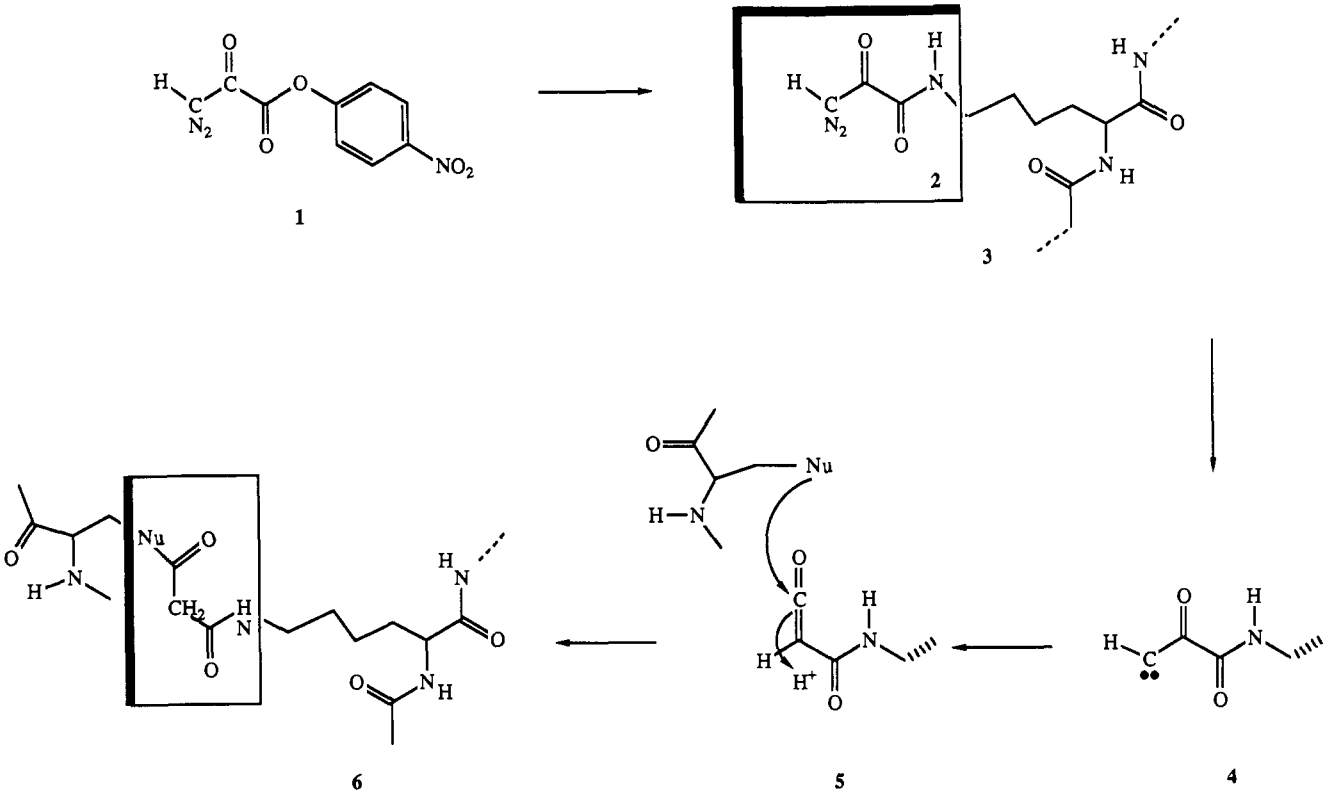
Scheme I



well-known and popular photoactivatable groups such as nitroaryl azides have characteristics that are often not compatible with many biological photoprobe experiments, and further, their photochemistry and subsequent reaction chemistry with proteins are uncertain (Liang & Schuster, 1986a,b, 1987).

We wish to introduce the diazopyruvoyl (DAP) function (**2**) as a useful photochemically activatable cross-linking function. It is a logical chemical extension of the diazoacetyl function first used by Westheimer (Chowdhry et al., 1976) in enzyme active site modification, and it provides a new family of complementary probes to existing photoaffinity labels and cross-linking agents. The addition of the extra keto function imparts a number of features that make the new group an excellent general photoactivatable probe. We have found its use to be quite efficient (Welsh, Treisman, Sweet, Lawton, and Redmond, unpublished results). The active ester, *p*-nitrophenyl 3-diazopyruvate (DAPpNP, diazopyruvoyl *p*-nitrophenyl ester, **1**), is conveniently synthesized in two steps. By use of the active ester (**1**), the diazopyruvoyl function (DAP, **2**) can be attached easily to a variety of structures, providing derivatives that are stable to most biological conditions but can be readily photorearranged to an active acylating function (Scheme I).

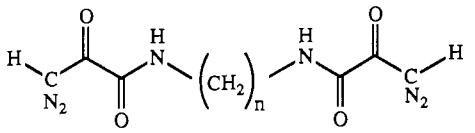
The diazopyruvoyl (DAP) group (**2**) is small in size yet highly dipolar, so that attachment to a protein residue such as a lysine amino group affords substitution (**3**) which may not perturb the protein environment or conformation; it has a UV chromophore with absorption maxima at 305 and 265 nm so that photolysis in biological systems is efficient. The photolysis generates, through carbene formation (**3** → **4**) and Wolff rearrangement (**4** → **5**), a highly reactive ketene amide (**5**) that can interact with a neighboring nucleophile, either intra- or intermolecularly (in the case of protein aggregates), to afford a 3-carbon cross-link bridge of a malonic residue (**6**). Of practical advantage is the fact that in contrast to most photoprobes the absorption of the activatable function, diazoketocarbonyl group (**2**), is completely lost as the photodecomposition occurs. The product malonic residue **6** is transparent in the UV at these wavelengths resulting in complete photoreaction.

The diazopyruvoyl function (**2**) is stable enough that it can be attached to polyfunctional molecules to create homobifunctional or heterobifunctional reagents. Other functional groups can be manipulated in the presence of the diazopyruvoyl group to allow the convenient synthesis of a variety probes. The spectrum of reactive functional groups within this small molecule allows the easy conversion to other useful derivatives. Thus, *p*-nitrophenyl 3-diazopyruvate (DAPpNP, **1**) and its protein or peptide diazopyruvoylamide derivatives (**3**) not only are new heterobifunctional cross-linking agents but also serve as precursors for other new classes of cross-linking agents and affinity-type cross-linking probes such as bis(diazopyruvoylamide)s (**7**), extended diazopyruvoylamides (**8**), chloropyruvoylamides (**9**), and amino acid and peptide derivatives (**10**, Chart I).

We have used the probe in the cross-linking of proteins by the attachment of the diazopyruvoyl group to protein lysine amino groups of calmodulin followed by calcium-activated association of the modified protein with adenylate cyclase and have shown cross-linking to this enzyme, calmodulin antibody (Welsh, Treisman, Sweet, Lawton, and Redmond, unpublished results), and other cell proteins. A bifunctional photoactive probe and a heterobifunctional probe have also shown photo-cross-linking behavior.

Recent research in the development of photoactivatable reagents has focused on functional groups that upon photolysis generate reactive intermediates such as carbenes (Richards, & Brunner, 1980; Brunner et al., 1980; Peters & Richards, 1977), nitrenes (Kauer et al., 1986), or other radical-like species (Kerr & O'Grady, 1967). It is believed these intermediates react with unactivated carbon–carbon or carbon–hydrogen bonds within a specific protein domain to produce "indiscriminate" labeling or cross-linking of target protein residues (Jakoby & Wilchek, 1977; Glazer et al., 1975).

Chart I



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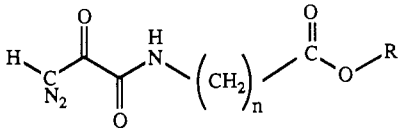

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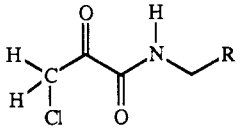

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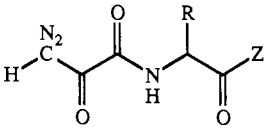


10

Currently, aryl azides and (trifluoromethyl)aryldiazirines are popular reagents. Although many of these photoreactive functional groups do show ability to insert into carbon–hydrogen bonds under ideal conditions, almost all reactions performed in the presence of nucleophiles result in preferential trapping of the nucleophile by the inherently electrophilic reactive intermediates (Bayley, 1983). Unfortunately, it is rare that the chemical links produced are identified. Even methylene carbene, a highly indiscriminate and reactive photointermediate, reacts 22 times faster by trapping the nucleophilic hydroxyl of methanol than by carbon–hydrogen insertion reactions (Kerr & O'Grady, 1967).

Photoaffinity reagents have been designed by using the 2-diazo-3,3,3-trifluoropropionyl group, and these reagents appear to have the ability to undergo the desired insertion. Photolysis of ethyl 2-diazo-3,3,3-trifluoroprionate in methanol gave only about 15% Wolff rearrangement, and 85% of the methyl ether formed by nucleophilic trapping of the carbene. Complete characterization of the insertion products in proteins and peptides remains to be achieved (Chowdry & Westheimer, 1979; Chowdry et al., 1976).

In a direct comparison of the ability of aryl azide (nitrene) and [(trifluoromethyl)phenyl]diazirine (carbene) derived photoaffinity reagents to label gramicidin A in liposomes, the nitrene-producing reagents were found to be 4 times more efficient in labeling the peptide, even though it has been repeatedly demonstrated that simple arylnitrenes do not undergo intermolecular carbon–hydrogen bond insertion (Richards & Brunner, 1980). Actual protein photo-cross-linking efficacy can only be determined in diverse biological samples that provide a competition between hydrophilic and hydrophobic environments in the domain where the carbene or nitrene is photogenerated. Our conclusion is that many of the reagents that are classed as indiscriminately inserting into C–H bonds probably link mostly to sites that are nucleophilic, perhaps in some situations even linking to protein backbone amides. Others have come to similar conclusions (Liang & Schuster, 1986a,b, 1987).

In many biological studies the mechanism, mode, and efficiency of the linking process may not be of immediate concern. The only significant question being asked is whether the photoreagent links to a neighboring structure. In the example of the DAP group, either cross-linking through a ketocarbene by insertion or cross-linking by Wolff rearrangement to the ketene amide and capture of a nucleophile results in the bridging process. It was important to understand the chemistry of the photoprocess of diazopyruvamides and define it as either alkylation (insertion) or acylation (rearrangement and acylation) so that in protein systems the subsequent processing of the linked proteins and identification of the linked residues could be made. The diazopyruvoyl group complements previous photoreagent groups in many ways.

Experimental Procedures

General. Reagents were purchased from Aldrich or Sigma. *p*-Toluenesulfonate salts of amino acid esters were prepared by refluxing the corresponding amino acid in dry methanol with 1.5 equiv of *p*-TsOH and precipitating with ether (Bodanszky, 1984). Melting points were determined in open glass capillary tubes with a Thomas-Hoover Uni-Melt melting point apparatus and are uncorrected. Infrared spectra were obtained on Perkin-Elmer 457 or Nicolet 60-SX spectrophotometers. Ultraviolet spectra were recorded on a Cary 219 spectrophotometer using standard 1-cm cells. Proton NMR were recorded at 60 MHz by using a Varian T-60A spectrometer; 300-MHz (300.133) spectra were obtained on a Bruker AM-300 instrument, and 360-MHz (360.132) spectra were obtained on a Bruker WM-360 spectrometer. Broadband proton-decoupled carbon-13 NMR spectra were measured at 75.0 MHz on a Bruker AM-300 spectrometer. Mass spectra were obtained on a Finnigan 4021 GCMS/DS system with direct probe sample introduction or as effluent from a gas chromatograph. Ammonia was used as reagent gas for chemical ionization spectra as specified. GC–MS column parameters are reported with the specific mass spectral data. Masses are reported as mass per unit charge values (m/z). Elemental analyses were performed by Spang Microanalytical Laboratories, Eagle Harbor, MI. Ultraviolet radiation was provided by a Rayonet reactor equipped with an array of 14 Rayonet RPR-3000 angstrom bulbs. Flash chromatography was accomplished by using EM grade 0.040–0.063-μm silica gel. Analytical thin-layer chromatography was carried out on Merck silica gel 60 glass backed, fluorescent indicator plates. Plates were visualized with UV light and iodine, ceric ammonium molybdate stain, or ninhydrin. The following solvents were purified or dried by distillation from the reagents indicated: chloroform, dichloromethane, and toluene from phosphorus pentoxide; benzene, acetonitrile, and dimethyl sulfoxide from calcium hydride; pyridine from potassium hydroxide; methanol and ethanol from magnesium; tetrahydrofuran from sodium benzophenone ketyl; and dimethylformamide from phthalic anhydride. Solvents for column chromatography were distilled or of HPLC grade. All other solvents were of ACS reagent grade.

Electrophoresis samples were made up in SDS–PAGE sample buffer (Laemmli, 1970) and added to slab gels with and without mercaptoethanol depending upon the experiment. The samples were run on 8% SDS–polyacrylamide gels, stained

Table I

compound	mp (°C)	reaction time (h)	solvent	yield (%)
DAP-*N*-*tert*-butylamide (**13**), S	47–48	30	$CHCl_3$	59
DAP-*p*-toluamide (**14**)	183–184	25	$CHCl_3$	85
DAP-tryptamine (**15**), S	132–133	25	$CHCl_3$	85
DAP-benzylamine (**16**)	95–95.5	18	$CHCl_3$	93
DAP-*N*-methyl-*p*-toluamide (**17**), S	119–120	18	CH_3CN	98
DAP-*N*-(6-hydroxyhexyl)amide (**18**)	90–91	3	$CHCl_3$	73
DAP-*N'*-(*t*-BOC)-1,6-diaminohexane (**19**), S	104–105	8	$CHCl_3$	85
DAP-*N'*-(TFA)-1,6-diaminohexane (**20**), S	123–124	8	CH_3CN	76
bis(DAP)-cystamine (**21**)	170–171 (dec)	18	$CHCl_3$/TEA	69
bis(DAP)-1,6-diaminohexane (**22**)	199–200	24	$CHCl_3$	94
bis(DAP)-1,6-diaminohexane (**22**)	199–200	24	CH_3CN/H_2O	51
DAP-proline methyl ester (**23**), S	oil	8	$CHCl_3$/TEA	50
DAP-sarcosine methyl ester (**24**)	oil	18	$CHCl_3$/TEA	78
DAP-serine ethyl ester (**25**), S	55–56	18	$CHCl_3$	69
DAP-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (**26**), S	dec	24	DMF/DMAP	74
DAP-phenylalanine methyl ester (**27**), S	93–94	15	$CHCl_3$	84
DAP-tryptophan methyl ester (**28**)	foam	4	$CHCl_3$	83
DAP-tyrosineamide (**29**), S	195–196 (dec)	18	$CHCl_3$	87
DAP-Phe-L-Ala OEt (**31**), S	130–131	12	pyridine	48
methyl DAP-6-aminohexanoate (**33**)	79–79.5	8	$CHCl_3$	91
N,N'-bis(DAP)-L-cystine dimethyl ester (**49**), S	85–87	22	CH_3CN/pyrid	61
N,N'-bis(DAP)-1,12-diaminododecane (**50**), S	153–156	6	ether	97

with Coomassie blue, and destained. The electrophoresis apparatus of the research laboratories of Dr. Michael Welsh, Department of Anatomy and Cell Molecular Biology, University of Michigan Medical School, Ann Arbor, and the technical help of T. Redmond is appreciated.

The experimental procedures and analytical and physical data for compounds in Table I followed with an S can be found in the supplemental experimental section along with the proton and ^{13}C NMR and mass spectral data for compounds.

p-Nitrophenyl 3-Diazopyruvate (*DAPpNP*, **1**). Dry, recrystallized *p*-nitrophenol (4.63 g, 0.0330 mmol) was added to freshly distilled oxalyl chloride (30 mL) with good stirring. The reaction was refluxed (64 °C) for 16 h in an atmosphere of nitrogen. The majority of excess oxalyl chloride was distilled at atmospheric pressure, and then the residual was removed in vacuo. The residue was dissolved in dry ether and filtered from the trace of precipitated diester, and the solvent was removed from the filtrate at reduced pressure. The white ester acid chloride (**12**) was again dissolved in ether and then concentrated to dryness. Traces of solvent were removed by drying in vacuo (0.05 Torr) for 6 h. The ester is very sensitive to traces of water and should be used immediately. The ester acid chloride was supsended in dry ether, filtered, and evaporated to approximately 15-mL volume immediately prior to the addition to the diazomethane solution: mp 95–100 °C; ^1H NMR ($CDCl_3$, 60 MHz) δ 7.80 (d, J = 8.5 Hz, 2 H), 8.40 (d, J = 8.5 Hz); IR ($CHCl_3$) 1785 m, 1755, 1620, 1600, 1520 (cm^{-1}); MS (*m/z*) 229 (M^+, 1.6%), 231 ($M^+ + 2$, 0.48%), 65 (27%), 63 (ClCO, 100%).

A dry ether solution of diazomethane was prepared according to the method of Arndt (1943) from 30.82 g of Diazald in 150 mL of ether and 136 mL of ethylene glycol monomethyl ether added to a solution of 8.69 g potassium hydroxide, 15 mL of H_2O, 25 mL of ethylene glycol monomethyl ether, and 15 mL of ether at a temperature between 70 and 75 °C. This distillate was dried twice for 30 min over KOH at 0 °C and then for 2 h over sodium metal at 0 °C.

The acid chloride active ester suspended in ca. 15 mL of dry ether was then added to the ethereal diazomethane solution at 0 °C, with vigorous stirring, over a period of about 3 min. After cessation of nitrogen evolution, the reaction mixture was filtered under nitrogen blanket to yield 3.35 g of a white solid: mp 121–121.5 °C. Removal of most of the ether at the water aspirator and chilling with dry ice yielded a second crop of 0.50 g: mp 119–121 °C; total yield 3.85 g (50%); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.45 (s, 0.08 H), 6.33 (s, 0.92 H), 7.39 (d, J = 9.2 Hz, 2 H), 8.32 (d, J = 9.2 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 174.64 (6.5%), 158.05 (6.8%), 154.68 (14%), 146.16 (10%), 125.32 (100%), 122.04 (99%), 57.61 (13%); IR (KBr) 3090.6, 2147.7, 1758.0, 1616.9, 1354.0 cm^{-1}; MS (*m/z*) 236 (0.76%), 139 (8.35%), 133 (5.06%), 69 (100%). Anal. Calcd for $C_9H_5N_3O_5$: C, 45.93; H, 2.13. Found: C, 46.00; H, 2.24.

Phenyl 3-Diazopyruvate (**11**). To an ether solution of excess diazomethane [prepared from *N*-(nitrosomethyl)urea; see Arndt (1943), footnote 3] cooled to 0 °C was added dropwise with stirring over 3–4 min 1.62 g (8.8 mmol) of phenyloxalyl chloride (Simon & Seyferth, 1958) dissolved in 25 mL of ether. There was rapid evolution of nitrogen during the addition, and the yellow color of the diazomethane was discharged. Near the end of the addition of the diazopyruvate may precipitate from solution. (Caution! Though we have never experienced problems, diazomethane has been observed to detonate from contact with edges of crystals.) After the addition, the excess diazomethane and ether were removed under a stream of nitrogen, and the remaining solid was washed in cold ether and dried in vacuo. Recrystallization of the solid from carbon tetrachloride affords light yellow needles: mp 92–93 °C; yield 1.40–1.45 g (80–86%); R_f (1:1 hexane/ethyl acetate) 0.68; ^1H NMR ($CDCl_3$, 360 MHz) δ 7.43 (m, 2 H), 7.28 (m, 1 H), 7.18 (m, 2 H), 6.28 (s, 1 H); IR (KBr) 3340, 3150, 2990, 2140 w, 2110, 1680, 1625, 1530, 1360 cm^{-1}; ^{13}C NMR ($CDCl_3$, 360 MHz) δ 175.9, 158.80, 150.2, 129.6, 126.6, 121.0, 57.8. Anal. Calcd for $C_9H_6N_2O_3$: C, 56.85; H, 3.18; N, 14.73. Found: C, 56.67; H, 3.14; N, 14.62.

General Procedures for DAP-amide Synthesis. A list of the 3-diazopyruvamides prepared from diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) and their mp, solvent, added bases, yield, and reaction times are given in Table I. Except for the representative examples of compounds **13**, **14**, and photo-cross-linkers, proton NMR, ^{13}C NMR and mass spectral data are collected in the supplemental experimental material.

Method A. In oven-dried glassware, under nitrogen, the appropriate amine was dissolved in the solvent to give approximately 0.5–1.5 M solutions and treated with 1 equiv of DAPpNP (**1**), with vigorous stirring. The reactions were stirred in flasks protected from light by foil. Care was taken not to expose the mixtures to intense light sources or diffuse

lighting for long periods of time, but the compounds are quite stable and do not require low-light conditions for normal laboratory manipulations. After the stated reaction time (Table I), the reaction mixture was diluted with, or taken up in, 20 mL of chloroform and washed with equal volumes of 10% citric acid, saturated sodium bicarbonate (four times), and brine. The chloroform solutions were then dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product obtained was often homogeneous except for extremely minor traces of *p*-nitrophenol as detected by TLC and were usually suitable for use.

Method B was identical with method A, except the hydrochloride or *p*-toluenesulfonate salts were treated with 1 equiv of the base identified in Table I prior to the addition of DAPpNP (**1**).

N-tert-Butyl-3-diazopyruvamide (**13**). Reaction of diazopyruvoyl *p*-nitrophenyl ester (**1**) (0.600 g, 2.55 mmol) with tert-butylamine in $CHCl_3$ according to method A and recrystallization from $CHCl_3$/petroleum ether provided 0.257 g (59.5%) of white microcrystalline solid: mp 47–48 °C; ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.52 (s, 9 H), 6.60 (s, 1 H), 7.15 (br, 1 H); IR (KBr) 3340, 3150, 2990, 2140 w, 2110, 1680, 1625, 1530, 1360 cm^{-1}; ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 182.70 (4.5%), 159.10 (4.5%), 53.99 (12.7%), 51.40 (9.3%), 28.28 (100.0%). High-resolution MS calcd for $C_7H_{11}NO_2$ (loss of molecular nitrogen): 169.0851. Found: 169.0852.

N-(p-Tolyl)-3-diazopyruvamide (**14**). *p*-Toluidine was reacted with diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) (0.950 g, 4.04 mmol) in CH_2Cl_2 according to method A. The resulting yellow solid was recrystallized from $CHCl_3$/petroleum ether to yield 0.700 g of a yellow microcrystalline solid: mp 183 °C. A second crop was obtained, 0.08 g: mp 181–182 °C; total yield 95.1%; r_f = 0.69 (6:4 hexane/acetone); ^{1}H NMR ($CDCl_3$, 60 MHz) δ 2.17 (s, 3 H), 6.42 (s, 1 H), 7.07 (d, J = 9 Hz), 7.51 (d, J = 9 Hz), 7.8 (br, 1 H); IR (KBr) 3300, 3190, 2105, 1685, 1630, 1590, 1535, 1380 cm^{-1}; MS (m/z) 203 (M^+, 41.3%), 134 (21.0%), 106 (100.0%), 69 (31.8%). Anal. Calcd for $C_{10}H_{11}N_3O_2$: C, 59.11; H, 4.46. Found: C, 59.20; H, 4.53.

N-Benzyl-3-diazopyruvamide (**16**). Benzyl amine (0.2300 g, 2.14 mmol) and 0.1924 g (0.8187 mmol) of DAPpNP were reacted in $CHCl_3$ according to method B. Recrystallization from ether yielded 0.1120 g of a white microcrystalline solid: mp 95.5 °C. A second crop of 0.0431 g (mp 94–95 °C) was also obtained. Total yield 0.1551 g (93.2%). ^{1}H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR ($CDCl_3$, 75.5 MHz), IR (KBr), and MS (m/z) are in the supplementary material. Anal. Calcd for $C_{10}H_9N_3O_2$: C, 59.11; H, 4.46. Found: C, 59.12; H, 4.52.

N-(3-Diazopyruvoyl)-6-aminohexanol (**18**). Reaction of diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) (0.300 g, 1.27 mmol) with 6-aminohexanol in $CHCl_3$ according to method A produced 0.244 g (89.7%) of a homogeneous white microcrystalline solid: mp 90–91 °C. Recrystallization from $CHCl_3$/cyclohexane provided 0.200 g (73.8%) of an analytical sample: mp 90–91 °C; R_f = 0.19 ($CHCl_3$/MeOH 9:1). ^{1}H NMR ($CDCl_3$, 60 MHz) and IR (KBr) are in the supplementary material. Anal. Calcd for $C_9H_{15}N_3O_3$: C, 50.70; H, 7.10. Found: C, 50.87; H, 7.28.

N,N'-Bis(3-diazopyruvoyl)-2,2'-dithiobis(ethylamine) (**21**). Cystamine dihydrochloride in $CHCl_3$ was treated with diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) (1.00 g, 4.25 mmol) according to method B. After 18 h, the solvent was removed in vacuo, and the solid residue was transferred to a coarse fritted filter and washed well with water, 10% citric acid, saturated $NaHCO_3$, again with water, and cold ether and allowed to air dry. The crude dark brown product was dissolved in hot acetone, filtered, and allowed to crystallize. This yielded 0.500 g (68%) of a yellow microcrystalline solid: mp 170–171 °C dec. ^{1}H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR ($CDCl_3$, 75.5 MHz), IR (KBr), and MS (m/z) are in the supplementary material. Anal. Calcd for $C_{10}H_{12}N_6O_4S_2$: C, 34.88; H, 3.51. Found: C, 34.95; H, 3.62.

N,N'-Bis(3-diazopyruvoyl)-1,6-diaminohexane (**22**). 1,6-Hexanediamine (0.1112 g, 0.9574 mmol) was suspended in 3 mL of dry $CHCl_3$ and treated with DAPpNP (0.4501 g, 1.915 mmol). The reaction stirred under nitrogen, protected from light, for 24 h. The solvent was removed (in vacuo), and the residue was suspended in 10% $NaHCO_3$. The product was collected by filtration and washed with 10% $NaHCO_3$ until the filtrate was colorless. The residue was washed with water, followed by cold ether, and then dried over P_2O_5. The resulting light yellow solid (0.3005 g) was recrystallized from ethyl acetate to give 0.1900 g (64.6%) of a white microcrystalline solid: mp 172 ° dec. A second crop of 0.0880 g (29.9%, 94.5% total) was obtained: mp 171–173 dec. ^{1}H NMR ($CDCl_3$, 300 MHz), IR (KBr), and MS (m/z) are in the supplementary material. Anal. Calcd for $C_{12}H_1N_6O_4$: C, 46.74; H, 5.23. Found: C, 46.72; H, 5.26.

Competitive Reaction of DAPpNP with Amine and Water. N,N'-Bis(3-diazopyruvoyl)-1,6-diaminohexane (**22**). 1,6-Hexanediamine (1.16 g, 10.0 mmol) was dissolved in 10 mL of ether and treated with 1.12 g (11.0 mmol) of pivalic acid, dropwise with rapid stirring. The addition was complete within 15 min. A flocculent precipitate formed. This was collected and washed with ether and dried to give the monopivalate salt of 1,6-hexanediamine, as determined by NMR: mp 199–200 °C; yield 2.10 g (92.11%).

This amine salt (0.092 g, 0.43 mmol) was dissolved in 4 mL of water and treated dropwise with a 2-mL solution of DAPpNP (0.1 g, 0.43 mmol) in acetonitrile. After about three-fourths of the solution had been added, there was the formation of a large amount to flocculent precipitate. After stirring for 1 h the precipitate was collected and washed with dry ether. There was obtained 0.034 g (51% on the basis of DAPpNP) of the bis amide **22**: mp 172–173 °C. TLC and IR showed identity with the bis amide, and there appeared to be no other material. No basic material was found upon extraction of the aqueous acetonitrile reaction mixture.

N-(3-Diazopyruvoyl)-L-phenylalanine Methyl Ester (**27**). Phenylalanine methyl ester hydrochloride (2.6 mmol) and diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) (0.5983 g, 2.55 mmol) were caused to react in $CHCl_3$. Triethylamine (0.3080 g, 3.050 mmol) was added in 1 mL of $CHCl_3$ according to method B. Recrystallization from ether/petroleum ether resulted in 0.545 g of yellow microcrystalline solid (77.6%): mp 93 °C. A second crop (0.052 g, mp 90–93 °C) was also obtained: total yield 84.3%; R_f = 0.70 ($CHCl_3$/MeOH 97:3); $[\alpha]_D$ 59.77° ($CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$), IR (KBr), MS (m/z), and UV (EtOH) are in the supplementary material. Anal. Calcd for $C_{13}H_{13}N_3O_4$: C, 56.73; H, 4.60. Found: C, 56.59; H, 4.70.

N-(3-Diazopyruvoyl)sarcosine Methyl Ester (**24**). Sarcosine methyl ester *p*-toluenesulfonic acid salt was reacted in dry $CHCl_3$ with diazopyruvoyl *p*-nitrophenyl ester (DAPpNP) (1.50 g, 6.38 mmol) according to method B. Chromatography on silica gel (15 g), eluting with 95:5 $CHCl_3$/EtOAc provided 0.97 g (78%) of a clear oil: R_f = 0.20 ($CHCl_3$/EtOAc 9:1). ^{1}H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR (75.5 MHz, $CDCl_3$), UV (EtOH), and IR ($CHCl_3$) are in the supplementary ma-

terial. High-resolution MS calcd for $C_7H_9N_1O_4$ (loss of molecular nitrogen): 171.0532. Found: 171.0532.

N-(3-Diazopyruvoyl)-L-tryptophan Methyl Ester (**28**). DAPpNP (0.6482 g, 2.758 mmol) and tryptophan methyl ester hydrochloride were caused to react in chloroform according to method B. Column chromatography on silica gel, eluting with diethyl ether, provided 0.724 g (83.6%) of a white foam: R_f = 0.51 (Et_2O); $[\alpha]_D$ +47.9° (*c* 1, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR ($CDCl_3$, 75.5 MHz), IR (neat), and MS (*m/z*) are in the supplementary material. Anal. Calcd for $C_{15}H_{14}N_4O_4$: C, 57.32; H 4.49. Found: C, 57.47; H, 4.52.

Stability of N-(3-Diazopyruvoyl-L-phenylalanine Methyl Ester (**27**) *in Acid Solution.* *N*-(3-Diazopyruvoyl)-L-phenylalanine methyl ester (**27**) (0.100 g, 0.363 mmol) in 2 mL of THF was added dropwise to a solution of 5 mL of 1 N HCl and 5 mL of THF. This was stirred for 2 h at room temperature after which time the solvent was removed (in vacuo), at 30 °C on the rotovap, to yield a clear oil. This oil was dried overnight over P_2O_5 under high vacuum. A NMR spectrum of this material appeared to be a 50:50 mixture of the starting material and the chloro derivative. This mixture was dissolved in 5 mL of THF and treated with 1 mL of 6 N HCl. After 1.5 h, the THF was removed (in vacuo), and the resulting aqueous solution was treated with 5 mL of saturated NaCl solution and extracted with ether. The organic material was dried over Na_2SO_4 and concentrated to 0.1016 g (98%) of an oil that was indistinguishable by NMR from *N*-(3-chloropyruvoyl)-L-phenylalanine methyl ester (**32**).

N-(3-Chloropyruvoyl)-L-phenylalanine Methyl Ester (**32**). *N*-(3-Diazopyruvoyl)-L-phenylalanine methyl ester (**27**) (0.120 g, 0.436 mmol) was dissolved in 5 mL of acetic anhydride and treated dropwise with 0.75 mL of concentrated HCl with rapid stirring. After 1 h, 10 mL of H_2O was added, and the majority of solvent was removed on the rotovap at 35 °C. Saturated NaCl solution (10 mL) was added, and the mixture was extracted three times with 10 mL of $CHCl_3$. The colorless solution was dried over Na_2SO_4 and concentrated (in vacuo) to a colorless oil. This oil was purified by flash chromatography on silica gel, eluting with ether, to give 0.1190 (96.7%) of a clear oil: $[\alpha]_D$ 41.63° (*c* 2, $CHCl_3$); R_f = 0.65 ($CHCl_3$/MeOH 9:1). ^1H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR ($CDCl_3$, 75.5 MHz), IR (KBr), and MS (*m/z*) are in the supplementary material. Anal. Calcd for $C_{13}H_{14}NO_4Cl$: C, 55.04; H, 4.97. Found: C, 54.89; H, 4.89.

N-(3-Diazopyruvoyl)-6-aminohexanoic Acid Methyl Ester (**33**). DAPpNP (0.200 g, 0.851 mmol) and ε-aminocaproic acid methyl ester *p*-toluenesulfonic acid salt were caused to react in chloroform according to method B. After 4 h, normal workup provided 0.188 g (91.7%) of white microcrystalline solid: mp 79–79.5 °C; R_f = 0.76 (ether). The identity of this compound was confirmed by ^1H NMR ($CDCl_3$, 300 MHz), IR (KBr), and MS and by ultimate conversion to *p*-nitrophenyl ester **49**.

Photolysis of p-Toluidide **14** *in Methanol. Methyl N-p-Tolylmalonamide* (**34**). p-Toluidide (**14**) (0.500 g, mmol) was dissolved in 50 mL of dry methanol and photolyzed for 8 h in a quartz tube fitted with a calcium chloride drying tube. The solvent was removed in vacuo, and the residue was taken up in 10 mL of EtOAc and washed with equal volumes of saturated $NaHCO_3$ and brine. The solution was dried over Na_2SO_4 and concentrated in vacuo. Traces of solvent were removed by drying under high vacuum: mp dec; yield 0.0489 g (96.0%); R_f = 0.32 (97:3 $CHCl_3$/MeOH). None of the ketol ether (**35**) could be detected by TLC or NMR. ^1H NMR ($CDCl_3$, 60 MHz) δ 2.43 (s, 3 H), 3.62 (s, 2 H), 3.95 (s, 3 H), 7.35 (d, *J* = 9 Hz, 2 H), 7.75 (d, *J* = 9 Hz, 2 H), 8.3 (br, 1 H); IR ($CHCl_3$) 3340, 1735, 1680, 1600, 1515 cm^{-1}; MS (*m/z*) 207 (M^+, 35.1%), 176 (1.7%), 133 (10.9%), 107 (100.0%). High-resolution MS calcd for $C_{11}H_{13}N_1O_3$: 207.0895. Found: 207.0898.

N-(p-Tolyl)-3-methoxypyruvamide (**35**). N-(*p*-Tolyl)-3-diazopyruvamide (**14**) (0.120 g, 0.591 mmol) was suspended in 5 mL of dry MeOH. To this was added 0.5 mL of boron trifluoride etherate. After 36 h, 5 mL of $NaHCO_3$ was added, and the majority of the methanol was removed on the rotovap. The aqueous solution was extracted three times with 5 mL of $CHCl_3$. The extracts were dried over Na_2SO_4 and concentrated in vacuo to a white solid: yield 0.120 g (98%); mp 134 °C; ^1H NMR ($CDCl_3$, 60 MHz) δ 2.47 (s, 3 H), 3.69 (s, 3 H), 5.07 (s, 2 H), 7.50 (d, *J* = 9 Hz, 2 H), 7.92 (d, *J* = 9 Hz, 2 H), 9.12 (br, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 194.85 (9.7%), 156.66 (6.6%), 135.27 (14%), 133.41 (3.1%), 129.67 (100%), 119.90 (95%), 74.34 (45%), 59.45 (26%), 20.86 (24%); IR (KBr) 3340, 2940, 1738, 1670, 1598, 1200, 1130, 1050 cm^{-1}.

Photolysis of N-(p-Tolyl)-3-diazopyruvamide (**14**) *with Benzylamine. N-Benzyl-N'-(p-tolyl)malondiamide* (**36**). *N*-(*p*-Tolyl)-3-diazopyruvamide (**14**) (0.385 g, 0.190 mmol) and freshly distilled benzylamine (0.30 g, 0.280 mmol) were dissolved in 3 mL of dry THF in a small quartz tube fitted with a stir bar and a calcium chloride drying tube, and the mixture was photolyzed. After 17.5 h of irradiation, no starting material remained, and the solvent was removed in vacuo. The solid residue was dissolved in 10 mL of EtOAc and washed with equal volumes of saturated $NaHCO_3$ (three times), 10% citric acid (twice), and brine. The solution was dried over Na_2SO_4, and the solvent was removed in vacuo. The product was dried under high vacuum. This resulted in 0.051 g (96.2%) of a yellowish microcrystalline solid: mp 170–171.5 °C. Recrystallization from MeOH provided an analytical sample: mp 170–171.5 °C; ^1H NMR ($CDCl_3$, 60 MHz) δ 2.31 (s, 3 H), 3.22 (s, 2 H), 4.50 (d, *J* = 6 Hz, 2 H), 7.0–7.6 (m, 11 H); MS (*m/z*) 282 (M^+, 1.0%), 177 (19.1%), 106 (48.8%), 91 (100%). Anal. Calcd for $C_{17}H_{18}N_2$: C, 72.32; H, 6.43; N, 9.92. Found: C, 72.48; H, 6.42; N, 9.97.

Photolysis of N-Benzyl-3-diazopyruvamide (**16**) *in the Presence of Benzylamine. N,N'-Bis(benzylmalondiamide)* (**37**). *N*-Benzyl-3-diazopyruvamide (**16**) (0.110 g, 0.541 mmol) and freshly distilled benzylamine (0.064 g, 0.60 mmol) were dissolved in 3 mL of dry THF in a small quartz tube, flushed with dry nitrogen, and photolyzed at 27 °C for 12 h in the Rayonet reactor with vigorous stirring. The THF solution was removed in vacuo, and the resulting solid was dissolved in EtOAc (5 mL) and washed with equal volumes of 1 N HCl, saturated $NaHCO_3$ solution, and brine. After drying over Na_2SO_4 and removal of the solvent, the solid was dried under high vacuum. This resulted in 0.145 g (94.7%) of a white solid. Recrystallization from methanol provided an analytical sample. mp 141–141.5 °C [lit. 142 °C (Kutney & Still, 1980)]; R_f = 0.74 (9:1 $CHCl_3$/MeOH); ^1H NMR (DMSO-d_6, 300 MHz) δ 3.17 (s, 2 H), 4.29 (d, *J* = 5 Hz, 4 H), 7.21–7.33 (m, 10 H), 8.51 (br t, *J* = 5 Hz, 2 H); IR 3285, 3064, 1656, 1625, 1547 cm^{-1}; MS (*m/z*) 282 (M^+, 7.0%), 191 (1.2%), 106 (100%), 91 (60.7%).

Photolysis of N-tert-Butyl-3-diazopyruvamide (**13**) *in Methanol. Methyl N-tert-Butylmalonamide* (**38**). *N-tert*-Butyl-3-diazopyruvamide (**13**) (0.503 g, 0.298 mmol) was dissolved in 45 mL of dry distilled methanol and irradiated at 300 nm in a quartz tube at room temperature. After 12

h, none of the diazoketone was detectable by TLC (9:1 $CHCl_3$/EtOAc, R_f = 0.56). A 2-mL aliquot of the mixture was saved for GC–MS analysis. The remaining 43 mL was concentrated on the rotovap, and traces of solvent were removed by pumping on the residual yellow oil at high vacuum pressure: yield of **38**, 0.487 g (99% on the basis of the aliquot which was removed); R_f = 0.11 (9:1 $CHCl_3$/EtOAc), CAM stain showed very minor spots at R_f = 0.20 and 0.26; ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.43 (s, 9 H), 3.36 (s, 2 H), 3.88 (s, 3 H), 5.80 (br, 1 H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.17 (5.6%), 164 (6.7%), 52.26 (19.1%), 51.41 (10.1%), 41.73 (28.6%), 28.54 (100%); IR ($CHCl_3$) 3320, 2985, 1765, 1650, 1540 cm^{-1}. GC–MS column DB-5, 30-m capillary, 55 °C 2 min, 55–250 °C, at 15 °C/min; injector 200 °C: Peak 1 identified as *N-tert*-butyloxalic acid amide methyl ester, also observed with preparation of *N-tert*-butyl-3-methoxypyruvamide (**39**); retention time 8.5 min; area 1.2% of base peak; (*m/z*) 159 (M^+, 4.0%), 144 (43.7%), 100 (11.7%), 72 (8.7%), 59 (18.3%), 57 (100%) 17. Peak 2 (base peak) identified as methyl *N-tert*-butylmalonamide (**38**); retention time 9.4 min; area 100%; (*m/z*) 173 (M^+, 5.8%), 158 (23.1%), 142 (1.8%), 101 (11.6%), 58 (100%), 15. Peak 3 identified as *N,N'*-bis(*tert*-butyl)oxalic diamide; retention time 10.6 min; area 2.1% of base peak; (*m/z*) 200 (M^+, 3.4%), 185 (3.0%), 129 (14.6%), 102 (19.6%), 57 (100%).

N-tert-Butyl-3-methoxypyruvamide (**39**). A solution of *N-tert*-butyl-3-diazopyruvamide (**13**) (0.0436 g, 0.2579 mmol) was treated with 0.5 mL of boron trifluoride etherate and allowed to stir for 24 h. A 2-mL aliquot was removed for gas chromatographic analysis. The remaining portion was treated with 5 mL of saturated sodium bicarbonate solution, and the mixture was extracted with CH_2Cl_2. The solution was dried over Na_2SO_4 and concentrated to a clear oil: crude yield of **39** (on the basis of an aliquot removed) 0.0341 g (95.5%); R_f = 0.36 (9:1 $CHCl_3$/EtOAc); ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.43 (s, 9 H), 3.57 (s, 3 H), 4.91 (s, 2 H), 7.13 (br, 1 H); IR ($CHCl_3$) 3390, 2985, 1735 m, 1680, 1520 1450 cm^{-1}. GC–MS column DB-5, 30-m capillary, 40 °C 2 min, 40–250 °C, 15 °C/min; injector 200 °C: Peak 1 identified as *N-tert*-butyloxalic acid amide methyl ester; retention time 9.5 min; (*m/z*) 159 (M^+, 17.3%), 144 (47.7%), 100 (14.8%), 72 (9.3%), 59 (19.9%), 57 (100%). Peak 2 identified as *N-tert*-butyl-3-methoxypyruvamide (**39**); retention time 10.2 min; (*m/z*) 173 (M^+, 2.1%), 158 (0.59%), 100 (6.7%), 117 (22.2%), 100 (6.7%), 57 (100%), 45 (31.0%). Peak 3 identified as *N,N'*-bis(*tert*-butyl)oxalic diamide; retention time 11.7 min; (*m/z*) 200 (M^+, 5.0%), 185 (4.6%), 129 (18.2%), 102 (22.3%), 57 (100%).

Photolysis of N-(3-Diazopyruvoyl)sarcosine Methyl Ester (**24**). *N*-(3-Diazopyruvoyl)sarcosine methyl ester (**24**) (0.150 g, 0.754 mmol) was dissolved in 50 mL of dry methanol in a 50-mL quartz photolysis cell. The solution was flushed with nitrogen and photolyzed in the Rayonet reactor with rapid stirring for 24 h. The solvent was removed on the rotovap, and the residue was dissolved in 15 mL of dichloromethane, washed once with 10 mL of saturated $NaHCO_3$ and 10 mL of brine and dried over Na_2SO_4. The solvent was removed on the rotovap to yield 0.150 g (98.0%) of a clear yellow oil: R_f = 0.70 ($CHCl_3$); gas chromatography OV-17, column temperature 250 °C, retention time 6.6 min; ^{1}H NMR ($CDCl_3$, 60 MHz) δ 3.20 (s, 1 H, *N*-Me), 3.38 (s, 2 H, *N*-Me), 3.8 (s, 2 H, $COCH_2CO$), 4.0 (br s, 6 H, OMe), 4.40 (br s, 2 H, NCH_2CO); IR ($CHCl_3$) 2960, 1745, 1650 cm^{-1}. High-resolution MS calcd for $C_8H_{13}NO_5$: 203.0794. Found: 203.0792.

Photolysis of N-(3-Diazopyruvoyl)-6-aminohexanol (**18**). *Cyclic Dimer* **40**. *N*-(3-Diazopyruvoyl)-6-aminohexanol (**18**) (0.0591 g, 2.77 mmol) was dissolved in 25 mL of dry distilled THF and was added in small droplets via an infusion pump to 20 mL of dry THF in a quartz photolysis cell that was constantly irradiated with 300-nm bulbs. The end of the syringe needle was fitted with a conical foil shield to reduce exposure of the concentrated solution to UV radiation before addition to the dilute solution was achieved. The addition was complete after 26 h. Irradiation was continued for another 4 h, and then the reaction mixture was allowed to stand at room temperature for 8 h. The solvent was removed (in vacuo), and the residue was taken up in $CHCl_3$ and washed with equal volumes of saturated $NaHCO_3$ and saturated sodium chloride solution. Crude yield was 0.0513 g of a clear oil. Preparative thin-layer chromatography on silica gel, developing with 9:1 $CHCl_3$/MeOH, separated the cyclic dimer **40** from a number of minor side products: yield 0.0308 g (60.0%) of clear oil; R_f = 0.70 (9:1 $CHCl_3$/MeOH); ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.05–2.40 (m, 16 H), 3.17 (br s, 4 H), 3.18–4.43 (m, 8 H), 8.11 (br, 2 H); IR ($CHCl_3$) 3420, 2950, 1735, 1665 cm^{-1}; MS (*m/z*) 370 (M^+, 0.06%), 116 (8.9%), 99 (13.0%), 71 (100%), 55 (22.6%), 43 (41.1%). Anal. Calcd for $C_{18}H_{30}N_2O_6$: C, 58.36; H, 8.16; N, 7.56. Found: C, 58.41; H, 8.10; N, 7.68.

N-(3-Diazopyruvoyl)-L-tryptophan (**42**). *N*-(3-Diazopyruvoyl)-L-tryptophan methyl ester (**28**) (0.1000 g, 0.318 mmol) was dissolved in 4 mL of methanol and treated with 0.25 g of 50% NaOH solution in 1 mL of H_2O. The reaction stirred for 2 h at room temperature, after which time the majority of methanol was removed on the rotoevaporator. Ether (10 mL) was added, and the mixture was cooled to 0 °C. Ice-cold 20% acetic acid (10 mL) was used to acidify the hydrolysate. Combined ethereal extracts were washed with brine, dried, and concentrated to 0.0720 g (75.4%) of a yellow foam. This compound was dried for 12 h at 0.5 Torr and 45 °C over P_2O_5. R_f = 0.81 (EtOH). NMR analysis showed this crude compound was contaminated with a minor amount of acetic acid: NMR (300 MHz, $CDCl_3$) δ 2.09 (s, acetic acid impurity), 3.37 (d, J = 5.7 Hz), 4.86 (dt, J = 5.7 Hz, J = 8.1 Hz), 5.12 (s, 0.10 H), 6.28 (s, 0.9 H), 6.97 (d, J = 2.3 Hz, 1 H), 7.12 (ddd, 1 H), 7.18 (ddd, 1 H), 7.32 (d, J = 7.9 Hz, 1 H), 7.55 (d, J = 7.7 Hz, 1 H), 7.66 (d, J = 8.17 Hz, 1 H), 8.16 (br s, 1 H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 180.5 (0.4%), 175.31 (42%), 159.82 (40%), 136.15 (39%), 127.26 (35%), 123.04 (75%), 122.39 (100%), 119.82 (93%), 118.45 (89%), 111.31 (90%), 109.26 (43%), 55.04 (67%), 52.88 (61%), 27.4 (90%), 20.62 (40.1%); IR (KBr) 3389.6 br, 3062.1 br, 2126.7, 1739.3, 1684.5, 1623.8, 1526.2, 1373.1 cm^{-1}. CI–MS desorption (NH_3) 318 (M^+ + 18).

N-(3-Diazopyruvoyl)-6-aminohexanoic Acid (**43**). Methyl *N*-(3-diazopyruvoyl)-ϵ-aminocaproate (**33**) (0.1800 g, 0.7468 mmol) was suspended in 5 mL of methanol. This was treated with a solution of 0.59 g of 50% NaOH diluted with 3 mL of H_2O. The ester immediately dissolved, and the resulting deep yellow solution, stirred at room temperature for 1.5 h, was protected from light. H_2O (5 mL) was added, and the majority of methanol was removed on the rotovap. Ether (20 mL) was added, and this mixture was chilled to 0 °C. A solution of ice-cold 20% aqueous acetic acid (30 mL) was added to the hydrolysate with rapid stirring, resulting in a colorless clear solution. This solution was extracted three times with 20 mL of ether. The organic extracts were combined, washed with brine, and dried over Na_2SO_4 and then concentrated to a yellowish solid. The crude product was dried under

high vacuum over P_2O_5: yield 0.145 g (83.4%); mp 97–98 °C dec; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.38–1.5 (m, 2 H), 1.57–1.68 (m, 4 H), 2.09 (s, HOAc impurity), 2.35 (t, 7 Hz), 3.34 (dt, J = 7, 7 Hz, 2 H), 5.23 (s, 0.10 H), 6.44 (0.90 H), 7.42 (br, 1 H), 10.70 (br, 1 H); IR ($CHCl_3$) 3326.8, 3090.7, 2948.6, 2115.3, 1666.3, 1619.9, 1528.0, 1378.8 cm^{-1}; CI–MS (NH_3) (m/z) 245 (M^+ + 18), 228 (M^+ + 1). The analysis of this compound was confirmed by subsequent transformation to *p*-nitrophenyl ester **44** for which correct carbon–hydrogen analysis data were obtained.

p-Nitrophenyl N-(3-Diazopyruvoyl)-6-aminohexanoate (**44**). *N*-(3-Diazopyruvoyl)-ε-aminocaproic acid (**43**) (0.0984 g, 0.4082 mmol) and *p*-nitrophenol (0.0681 g, 0.489 mmol) were suspended in 3 mL of ethyl acetate. Dicyclohexylcarbodiimide (0.0842 g, 0.0984 mmol) was added in one portion and the reaction stirred and protected from light at room temperature. After 20 h, DCU was filtered from the reaction mixture and washed well with ethyl acetate. The filtrate was diluted to 20 mL and washed with 20 mL of 10% saturated $NaHCO_3$ and 20 mL of brine. The organic solution was concentrated to a yellow solid. The material was dissolved in 10 mL of $CHCl_3$ and washed four times (10 mL each) with saturated $NaHCO_3$ and once with 10 mL of brine, dried over Na_2SO_4, and concentrated to a solid. Recrystallization from ethanol provided a white microcrystalline solid: 0.1280 g (90.5%); mp 104 °C; R_f = 0.10 [$CHCl_3$, R_f = 0.85 (Et_2O)] ^{1}H NMR ($CDCl_3$, 300 MHz), ^{13}C NMR ($CDCl_3$, 75.5 MHz), IR (neat), and MS (m/z) are in the supplementary material. anal. Calcd for $C_{15}H_{16}N_4O_6$: C, 51.72; H, 4.63. Found: C, 51.77; H, 4.62.

N-(3-Diazopyruvoyl)-L-tryptophan p-Nitrophenyl Ester (**45**). *N*-(3-Diazopyruvoyl)-L-tryptophan (**42**) (0.0550 g, 0.1833 mmol) and *p*-nitrophenol (0.0331 g, 0.2382 mmol) were suspended in 2 mL of ethyl acetate, chilled to 0 °C, and treated with DCC (0.378 g, 0.1833 mmol). The reaction stirred, protected from light, for 3 h at 0 °C and then for 9 h at room temperature. The reaction mixture was chilled to 0 °C, and precipitated DCU was removed by filtration. The residue was washed well with cold ethyl acetate, and then the filtrate was evaporated. The residue was dissolved in 10 mL of $CHCl_3$ and washed four times with equal volumes of 10% $NaHCO_3$ and once with brine, and the solvent was removed on the rotoevaporator. The crude product was contaminated with DCU. Chromatography on silica gel eluting with $CHCl_3$ produced 0.0520 g (67.3%) of a yellow foam that would not crystallize. R_f = 0.29 ($CHCl_3$). If allowed to stand under room lighting at room temperature, this compound decomposed, but was stable if stored in the dark below 0 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.50 (m, 2 H), 5.09 (dt, J = 6.1, 7.7 Hz), 5.17 (s, 0.12 H), 6.33 (0.88 H), 6.94 (d, J = 9.2 Hz), 7.11 (m, 2 H), 7.23 (ddd, 1 H), 7.39 (d, J = 7.4 Hz, 1 H), 7.57 (d, J = 8 Hz, 1 H), 7.77 (br d, 7.7 Hz, 1 H), 8.16 (d, J = 9.2 Hz, 2 H), 8.30 (br s, 1 H); IR (KBr) 3392.4, 3384.1, 3125.6, 2933.8, 2122.9, 1768.0, 1687.8, 1629.7, 1523.1 cm^{-1}; MS (m/z) 421 (M^+, 1.70%), 139 (27.3%), 130 (100.0%), 69 (8.9%). High-resolution MS calcd for $C_{20}H_{15}N_5O_6$: 421.1022. Found: 421.1025.

Reagent Coupling and Photo-Cross-Linking Experiments with Aldolase. (*a*) *Cross-Linking of Aldolase with DAP-cystamine* (**21**). Crystalline aldolase (Sigma type X) was dissolved in 20 mM Tris-HCl buffer, and the enzyme concentration was determined spectrophotometrically (A_{280} = 0.91 mg/mL) (Huang & Richards, 1977). The concentration was found to be 0.89 mg/mL. Molecular weight for aldolase is assumed to be 160 000 for the tetramer, 40 000 for the monomer. A stock solution of cross-linker 34 (M_r = 344.374) was made up by using DMSO as the solvent. The concentration was 2.20 mg/mL.

Aliquots of 1.00 mL of stock aldolase solution were added to each tube, and then each sample was treated with the requisite amount of cross-linker, mixed well, and stored at 22 °C in the dark for 12 h. At the appropriate time the sample was transferred to a Pyrex culture dish so that the depth of the solution was less than 2 mm. The sample was then placed in the Rayonet reactor on a Pyrex beaker and photolyzed for 6 min. After photolysis, the solutions were transferred to a via and treated with 0.050 mL of a solution of 10 mg of *N*-ethylmaleimide in 1 mL of H_2O, incubated at room temperature for 3 h, and then frozen. Solutions were mixed with sample buffer and heated for 3 min in a water bath. In solutions 2–4 a precipitate was evident upon thawing. Track 5 was a sample not treated with NEM but with mercaptoethanol (0.05 mL).

The following aldolase stock solutions, in 33 mM phosphate buffers, were prepared and used in cross-linking experiments with active ester based cross-linkers. Crystalline aldolase (Sigma type X) was dissolved in pH 8.0 phosphate buffer, and the enzyme concentration was determined spectrophotometrically to be 0.91 mg/mL. Aldolase was dissolved in pH 7.0 phosphate buffer and the enzyme concentration was determined spectrophotometrically to be 0.88 mg/mL.

(*b*) *Cross-Linking of Aldolase with DAP-aminohexanoate pNP* (**44**). A stock solution (0.0148 g/mL) of cross-linker **44** (M_r = 348.325) was made up by using DMSO as the solvent. Aliquots of 1.00 mL of stock aldolase solution were added to each tube, and then each tube was treated with the requisite amount of cross-linker, mixed well, and stored at 22 °C in the dark for 6 h. At the appropriate time the sample was transferred to a Pyrex culture dish so that the depth of the solution was less than 2 mm. The sample was then placed in the Rayonet reactor on a Pyrex beaker and photolyzed for 6 min. After photolysis, the solutions were transferred to a vial and then frozen. Solutions for tracks 7 and 9–12 showed a precipitate upon thawing. Solutions were mixed with sample buffer and heated for 3 min in a water bath prior to application to the wells.

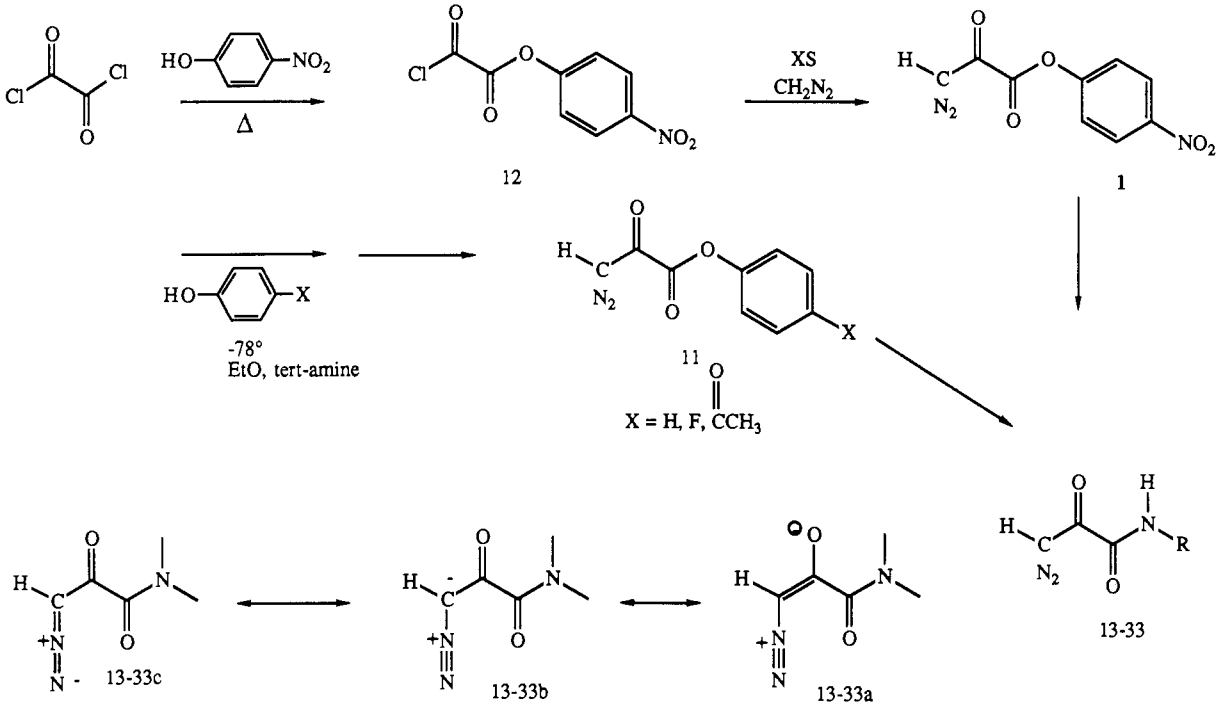
(*c*) *Cross-Linking of Aldolase with DAP-tryptophan pNP* (**45**). A stock solution (0.0179 g/mL) of cross-linker **45** (M_r = 421.369) was made up by using DMSO as the solvent. Samples were prepared and irradiated as for 6 min. Solutions for tracks 14, 16, and 17 showed a precipitate upon thawing. Again solutions were mixed with sample buffer and heated for 3 min in a water bath prior to application to the wells.

Results and Discussion

Synthesis of Phenyl and DAPpNP and Diazopyruvoylamides. Although Binder and Meier (1981) reported the reaction of chloroglyoxylate ester **12** with excess diazomethane, only minor constituents of the reaction mixture were characterized and DAPpNP (**1**) was not isolated. In our hands, when chloroglyoxylate **12** was added to concentrated ether solutions of diazomethane at 0 °C under anhydrous conditions, DAPpNP (**1**) was formed in good yields (50–60%). We also found chloroglyoxylate **12** could be produced in excellent yields by simply refluxing *p*-nitrophenol in oxalyl chloride, bp 80 °C (Scheme II).

Other diazopyruvoyl active esters can be synthesized through the condensation of oxalyl chloride with phenols in ether solvent by using pyridine as a base and at dry ice/acetone bath temperatures to give the half-acid chloride ester (Simon & Seyferth, 1958; Mueller & Lawton, 1979). Reaction of these

Scheme II



with diazomethane yields the corresponding diazopyruvoyl ester (**11**, X = H, F, acetyl, etc). In this way, a variety of diazopyruvoyl active esters may be synthesized [e.g., phenyl diazopyruvate (**11**)]. The less reactive esters are convenient for reaction with materials in aqueous or mixed aqueous solvents where the competition between hydrolysis and nucleophilic attack may not be favorable for attachment of the probe or where it is desirable to impart substrate-like character to transfer the acyl function to a specific protein domain.

DAPpNP (**1**) is a highly activated ester with a half-life for hydrolysis of 25.4 s at pH 7.0 in phosphate buffer (0.033 M). At pH ~4 the half-life for hydrolysis is on the order of 1 h. As expected, DAPpNP efficiently acylates a wide variety of amines to give 3-diazopyruvamides in excellent yields as illustrated in Table I. Addition of nucleophiles can be accomplished in a variety of solvents (acetonitrile, chloroform, acetone, dimethoxyethane, DMSO, and water) and may be monitored by the release of *p*-nitrophenolate anion by observing the change in absorbance at 412 nm. Competitive hydrolysis in the reaction of DAPpNP with amines in aqueous solution is not a crucial problem if the amine is sufficiently nucleophilic. For example, reaction of DAPpNP with 1,6-hexanediamine monopivalate salt in 1:2 acetonitrile/water afforded a 51% yield of the bis(diazopyruvoylamide) (**22**) within minutes. The corresponding phenyl ester (**11**) required 24 h, giving a 60% yield.

3-Diazopyruvamides show UV absorbances between 293 and 303 nm (ϵ = 5700–6200) that allow photolysis with light sources above 300 nm. The absorbances near 300 nm give a method for assaying the number of diazopyruvate groups attached to a macromolecule or for determining the rate of photolysis of probe molecules.

The proton NMR of the diazopyruvoylamide function is not exceptional, having a characteristic peak at about δ 6.3–6.60. Like the diazoketones, certain diazopyruvoylamides show two discrete peaks for the diazo methine hydrogen in a ratio of about 1:9, one at about δ 5.2, the other at δ 6.3. This is due to the two conformations and restricted rotation about the CO–CHN_2 bond (Sorriso, 1978; Kaplan & Meloy, 1966). The situation becomes more complicated in diazopyruvamides of secondary amines, where in some cases four different diazo methine hydrogens could be observed due to the restricted rotations about both the amide bond and the diazocarbonyl function. A good example is diazopyruvoylproline methyl ester (**23a–d**, Scheme III, Table I, and supplementary material), which shows proton resonances at δ 6.57 (100), 6.46 (62), 5.87 (4.8), and 5.77 (5.6) in DMSO-*d* and at δ 6.34, 6.26, and 5.18 in $CDCl_3$ (the peak at 5.77 in DMSO was obscured by the α proton in $CDCl_3$).

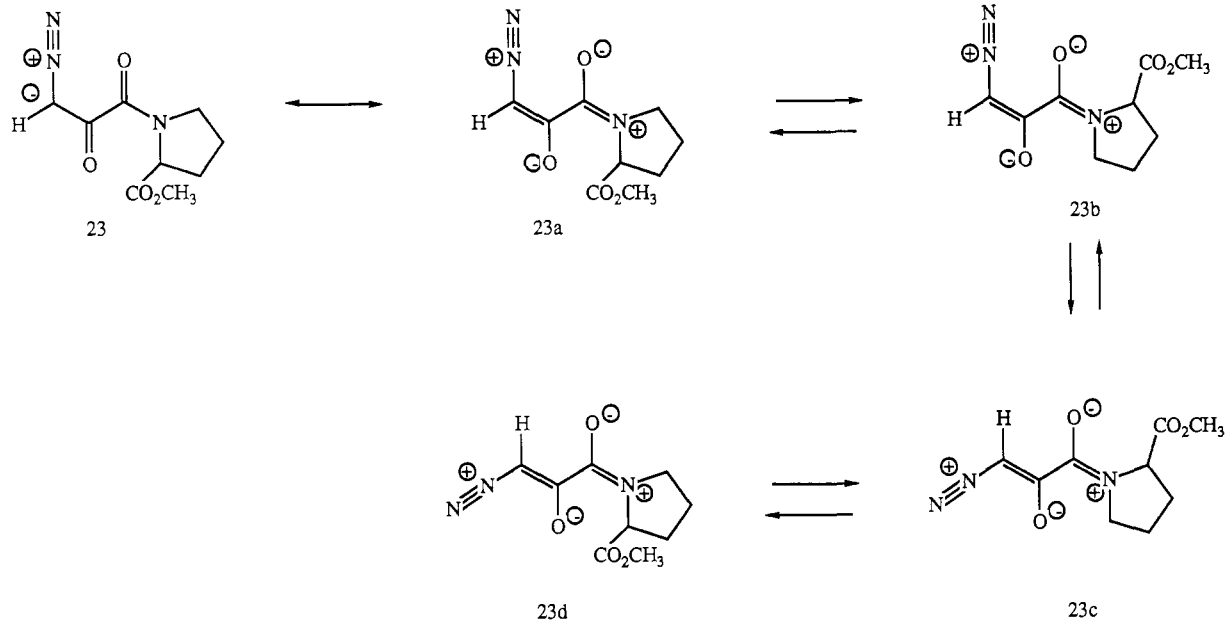
The IR shows the expected intense diazocarbonyl band at 2147 cm^{-1}, amide bands at 1680–1660 cm^{-1}, and ester bands at 1758 cm^{-1} for DAPpNP. The diazopyruvoylamides are usually crystalline materials.

Chemical Reactivity Pathways of 3-Diazopyruvamides. In addition to the photoinitiated activation, 3-diazopyruvamides can exhibit two alternative modes of reaction (Scheme IV). Decomposition in the presence of metals produces metal carbenoid species that may undergo 1,3 dipolar cycloadditions or insertion into Z–H bonds, where Z = N, O, S (3 → 46 → 47, Scheme IV), a process different from that observed when a simple carbene is created (Mueller & Lawton, 1979; Alonso et al., 1980; Wenkert, 1980).

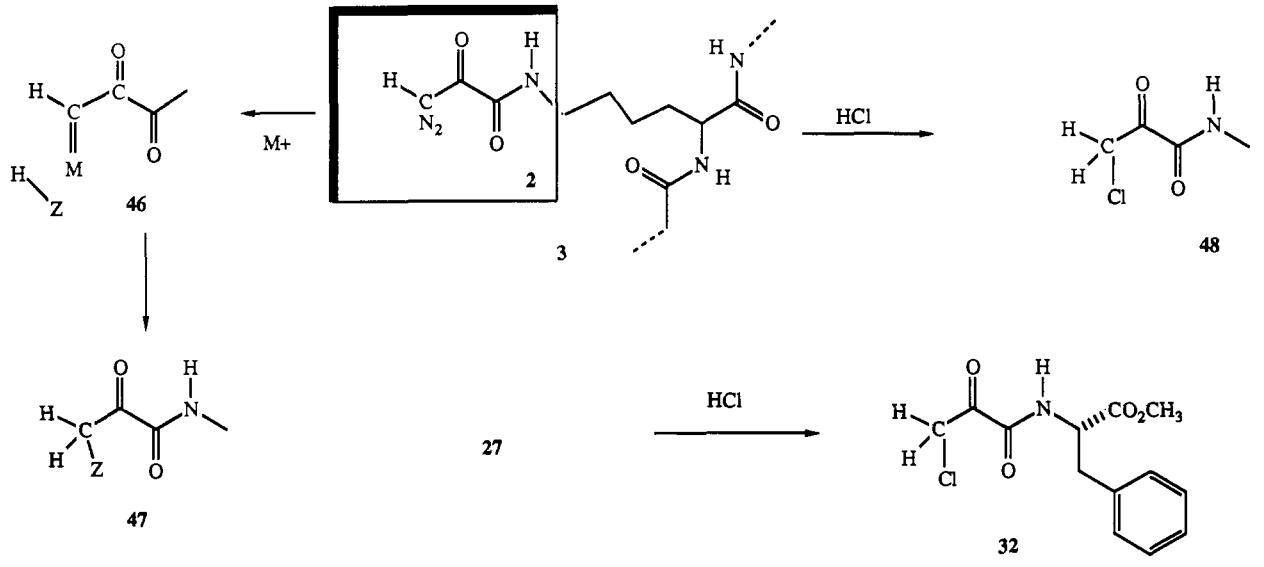
Lewis or Brønstead acids reacting with the DAP group can create alkyl diazonium intermediates, and subsequent attack by halogen nucleophiles produces 3-halopyruvamides. For example, reaction of diazopyruvoyl phenylalanine methyl ester (**27**) with HCl in THF or acetic anhydride affords the corresponding α-chloropyruvoyl derivative **32**. This path to N-substituted 3-halopyruvamide derivatives represents a simple approach to new families of potential affinity-labeling agents, enzyme inactivators, and cross-linking agents of the haloketone type [for example, Powers (1977)]. Their chemistry and biochemistry will be reported subsequently.

3-Diazopyruvoyl function is stable at fairly low pH values. The phenylalanine derivative **27** has a 2-h half-life in 0.5 M HCl in THF. Although this stability is exceptional compared to that of most of the DAP derivatives we have prepared, it demonstrates that diazoketoamides are not unduly reactive at low pH. Thus, diazoketones have the advantage of being much more stable to thiol reducing agents used as scavengers

Scheme III



Scheme IV



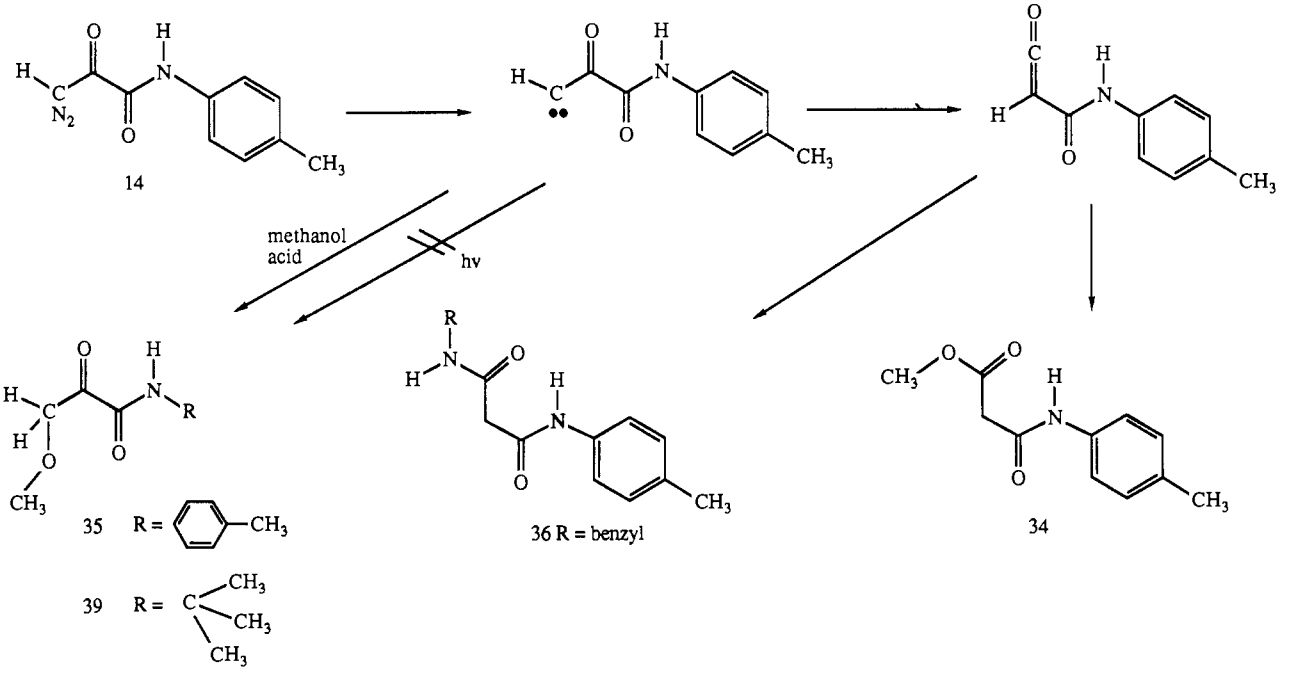
or protein reductants, in photoaffinity-labeling experiments, than the commonly utilized aryl azide photoreactive functional groups (Bayley, 1983).

Photochemistry of 3-Diazopyruvamides. Photolysis of *p*-toluidide (**14**, Scheme V) in dry methanol, irradiated at 300 nm in a quartz tube, resulted in nearly quantitative yield of the malonic ester **34**. We assume, on the basis of the photochemistry of other diazoketones (Schroeter, 1909; Staudinger & Hirzel, 1916; Gilman & Adams, 1929; Arndt & Eistert, 1935; Horner et al., 1951), that photolysis generates the carbene and then the ketene amide intermediate, which reacts rapidly with methanol to give the ester **34**. The pyruvamide ketol ether **35**, which is the expected product if a ketocarbene intermediate is trapped by methanol, was not detected by thin-layer chromatography, by GC–MS, or by NMR analysis. An authentic sample of ketol ether **35** showed a two-hydrogen singlet near 5 ppm that was not present in the spectrum of the crude product; consequently, the concentration of any insertion product must be below 2–3%, which is the limit of sensitivity for detection by NMR.

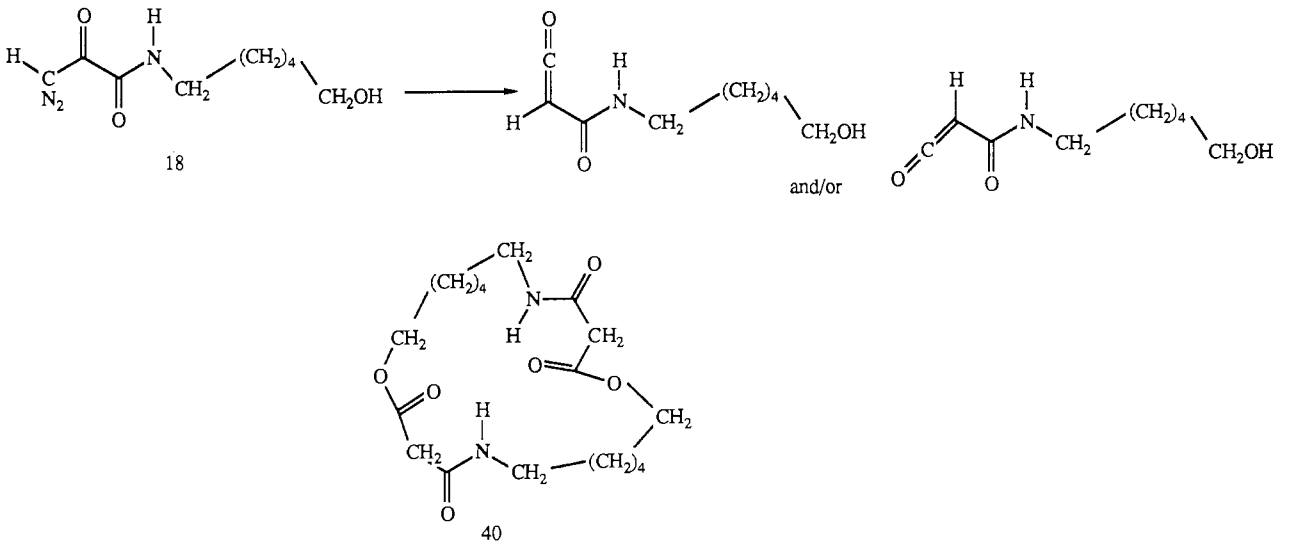
Photolysis of *N*-(3-diazopyruvoyl)sarcosine methyl ester (**24**), an example of a tertiary 3-diazopyruvamide of a non-aromatic amino acid, was carried out in methanol. The clear *N*-methyl signal in the NMR allowed the easy diagnostics of starting material and product during the course of the photolysis. The corresponding malonamide ester was formed by methanolysis of the intermediate Wolff rearrangement product, again in near-quantitative yield.

To apply 3-diazopyruvamides as discriminating photoactivatable affinity labels and cross-linking agents, their photochemical reactivity and trapping of the intermediate ketene amide with nucleophiles (amines) in solution would be of importance. Consequently, *p*-toluidide **14** was irradiated in a dilute THF solution containing 1.5 equiv of benzylamine. The expected malonamide **36** was obtained in almost quantitative yield. Similarly, when *N*-benzyl-3-diazopyruvamide (**16**) was photolyzed in THF in the presence of 2.6 equiv of benzylamine, the expect malonamide **37** was isolated in 95% yield. This demonstrated that the photolysis of 3-diazopyruvamides to yield reactive ketene amides is a general reaction and that the photoproduct ketene amides acylate amines smoothly to give malonamides in high yields. Even in mixed aqueous solvent environments, the competition of the amine nucleophiles versus water for the ketene amide is excellent.

Scheme V



Scheme VI

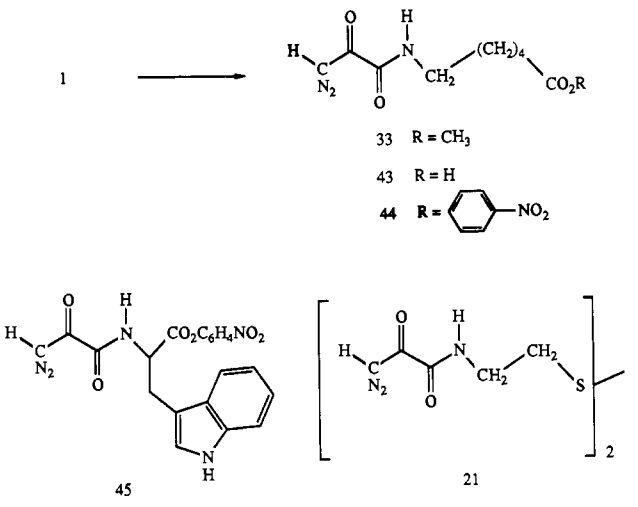


To demonstrate that significant but minor reaction products were not being ignored by analysis of the photolysis reaction mixtures by NMR, an investigation of the photolysis of *N-tert*-butyl-3-diazopyruvamide (**13**) was undertaken. When **13** was irradiated for 12 h at 300 nm in dry methanol, only the corresponding malonic ester **38** was detectable in the NMR spectrum of the crude product. Visualization of a thin-layer chromatogram with CAM stain showed two very minor spots, in addition to the ester. An authentic sample of the expected ketocarbene insertion product **39** was prepared by BF_3 etherate catalyzed decomposition of **13** in methanol. Compound **39** could not be detected in the crude photolysis product. The crude photolysis product was analyzed by HPLC monitoring at 254 nm and by GC–MS. Compound **39** was not detected in significant amounts. In addition to the expected product, only very minor peaks eluted from the gas chromatograph related to decomposition products and derivatives of oxalic acid amides.

Photolysis of the diazopyruvoyl derivative of 6-aminohexanol (**18**, Scheme VI) was accomplished under conditions of high dilution where the compound was added by syringe pump to a constantly irradiated solution (THF) over 26 h. The intention was to observe if this process might yield the monomeric macrocyclic lactone. However, only a cyclic dimer (**40**) was identified. We believe this result is because of the restricted conformation of the amide bond in the generated ketene amide intermediate. Closure by intramolecular interaction of the hydroxyl group with the ketene is unlikely (Scheme VI). Even under high dilution conditions, intermolecular addition to a hydroxyl on another molecule occurs with eventual closure of the dimeric macrocyclic ring. This same situation may prevail when DAP derivatives of proteins are photolyzed. The geometry of the intermediate ketene amide may prevent capture by closely neighboring residues on the peptide backbone allowing bridges to form between chains. The question of the stereochemistry of the photogenerated ketene amide (intermediate ketene C=O *Z* or *E* to the amide N) remains.

Synthetic Manipulations and Stability of 3-Diazopyruvamides. The 3-diazopyruvamides illustrated thus far have been

Scheme VII



limited to derivatives of simple amines, or amino acids and peptides, where the carboxylate function is masked as a ester or amide. The synthesis of the protected derivatives and small peptides is unexceptional. The ability to form 3-diazopyruvamide derivatives of free carboxylate compounds would allow the synthesis of photolabile amino acid derivatives for use as affinity labels and would also facilitate the development of a family of photoactivatable heterobifunctional cross-linking agents, where the diazopyruvamide and acylating function could be separated by chains of variable length.

Direct acylation of free α-amino acids using DAPpNP with added amine bases was unsuccessful. For example, if glycine was treated with 1 to 2 equiv of TEA and then reacted with DAPpNP, the reaction mixture rapidly turned dark, nitrogen evolved, and insoluble tar was formed. The same course of events occurred when the acylation of ϵ-*N*-(benzyloxycarbonyl)lysine or ϵ-aminocaproic acid was attempted. However, these derivatives were easily synthesized by the uneventful hydrolysis of an ester derivative to the *N*-(3-diazopyruvoyl) amino acids. With DAP-phenylalanine methyl ester **27**, in the presence of at least 2 equiv of hydroxide, the reaction was complete in 24 h. The solution was brought to neutrality, and the sodium salt of the 3-diazopyruvoyl amino acid was isolated. This salt was protonated with acetic acid and the carboxylic acid **41** isolated in 86.6% yield. Other amino acids behave in a similar fashion.

Synthesis of DAP Heterobifunctional Cross-Linking Agents. The simple conditions, high yield and straightforward synthesis of DAP amino acids, allow as well the formation of heterobifunctional cross-linkers such as **44** (Scheme VII). This compound is also of interest as a dipeptide pseudoisostere. The coupling of the *p*-toluenesulfonate salt of ϵ-aminocaproic acid methyl ester with DAPpNP in $CHCl_3$ in the presence of TEA resulted in the desired diazoamide **33** in 91.7% yield. The ester was rapidly hydrolyzed with 10 equiv of hydroxide in methanol, and the free carboxylic acid derivative **43** was isolated in 83.4% yield by acidification with acetic acid.

DCC coupling of **43** and *p*-nitrophenol, under standard conditions, produced the extended heterobifunctional cross-linking agent **44** in 90.5% yield. The same scheme was applied to the synthesis of a *N*-(3-diazopyruvoyl) α-amino acid *p*-nitrophenyl acylating agent from tryptophan methyl ester **45**. Thus, a family of heterobifunctional DAP cross-linking agents can be synthesized with variable lengths of linker arms and substituted with reporter groups that can be monitored by UV spectroscopy.

Cross-Linking of Aldolase with 3-Diazopyruvamide Cross-Linking Agents. To demonstrate the generality of the reactions observed in model systems, for 3-diazopyruvamide-based cross-linking agents, a series of prototypical bifunctional reagents was synthesized. All of these compounds are related by at least one 3-diazopyruvamide functional group, but differ in the span and architecture of the cross-linker arm and alkylating or acylating functional groups that form the initial attachment to the protein. Rabbit muscle aldolase was chosen as a model protein to survey and peruse cross-linking, since under proper conditions it exists as a tetramer with subunits of M_r = 40 000. Each subunit contains eight sulfhydryls, and a number of exposed amino groups, which were potential targets for cross-linking. Aldolase has served as a model in a number of initial studies of cross-linking agents (Huang & Richards, 1977).

Cross-Linking with N,N'-Bis(3-diazopyruvoyl)-2,2'-dithiobis(ethylamine) (**21**). *N,N'*-Bis(3-diazopyruvoyl)-2,2'-dithiobis(ethylamine) (**21**) was synthesized by conjugation of cystamine with DAPpNP in 68% yield. This compound was chosen as a simple model of a cleavable cross-linking agent containing the 3-diazopyruvamide functional group. Initial covalent attachment of the cross-linker to the protein should occur by disulfide interchange. If photolysis produces a short-lived reactive intermediate, the probability of forming measurable cross-links by reaction at both photoactivatable ends of the symmetrical reagent, which has not undergone disulfide interchange, is very low. If extensive cross-linking to provide multimeric structures was observed with **21**, it would indicate that nonspecific labeling by a long-lived intermediate was occurring (Scheme VIII).

Bis(diazopyruvamide) **21**, dissolved in DMSO, was incubated with aldolase, in pH 8.0 Tris buffer. The initial reaction was allowed to proceed for 12 h at room temperature, and then appropriate samples were irradiated. Control reactions were performed by irradiating an aldolase solution containing no cross-linker and by allowing a solution of aldolase and cross-linker to interact without irradiation. The samples were irradiated for 6 min. In a test reaction the disappearance of the diazo function in the reaction mixture was monitored by UV spectroscopy. The disappearance of the diazo group is initially very rapid and appeared to be 50% complete in less than 20 s, but slows with time. The reaction did not exhibit first-order kinetics. No further decrease at 299 nm was noted after 6 min of irradiation.

The photolyzed samples were treated with excess *N*-ethylmaleimide (NEM), a thiol-specific alkylating agent, to prevent cleavage of artificial cross-links by disulfide interchange reactions. One fraction was not treated with NEM, but with mercaptoethanol, to test the cleavability of the artificial disulfide cross-links. No reducing agents were added during the gel electrophoresis analysis.

In all of the cross-linking studies described here, the samples were heated at 100 °C, prior to application to the electrophoresis gels, but the samples were not desalted nor was the organic solvent removed by dialysis or gel filtration. Consequently, there are minor irregularities in the development of the gels. The reaction mixtures were not ultrafiltered or dialyzed since precipitates formed upon standing after photolysis. It was initially believed that these precipitates could have been moderately insoluble high molecular weight cross-linked products, which dissolved upon heating. The lack of major high molecular weight bands suggests that the precipitate was aggregate or so highly cross-linked that solution did not occur.

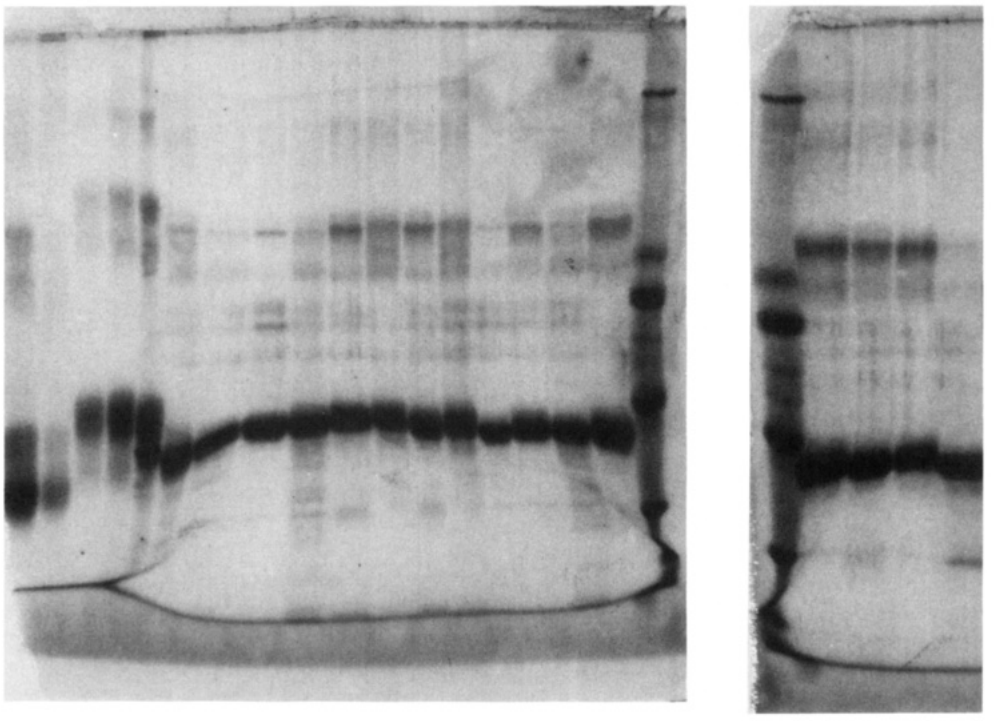
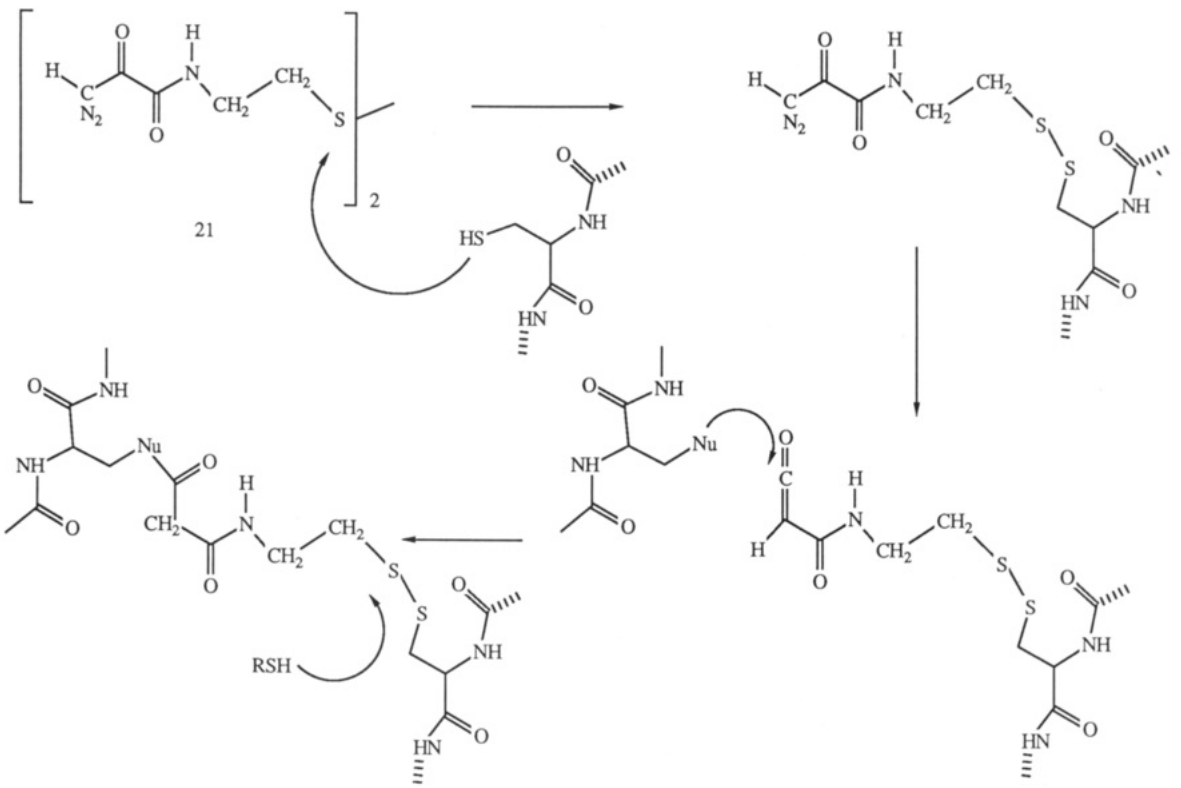


FIGURE 1: (Lanes 1–6) SDS–PAGE of samples of aldolase treated with DAP-cystamine (**21**). (Lane 1) 0.05 mL of cross-linker solution, no photolysis; (lane 2) no cross-linker, 6-min photolysis; (lane 3) 0.01 mL of cross-linker, 6-min photolysis; (lane 4) 0.025 mL of cross-linker, 6-min photolysis; (lane 5) 0.050 mL of cross-linker, 6-min photolysis; (lane 6) 0.050 mL of cross-linker, 6-min photolysis, reduced with mercaptoethanol. (Lanes 7–13) SDS–PAGE of samples of aldolase treated with DAP-aminocaproate *p*-nitrophenyl ester (**44**). (Lane 7) No cross-linker solution, no photolysis; (lane 8) 0.025 mL of cross-linker, no photolysis; (lane 9) no cross-linker, 6-min photolysis; (lane 10) 0.025 mL of cross-linker, 6-min photolysis, pH 7; (lane 11) 0.0250 mL of cross-linker, 6-min photolysis, pH 8; (lane 12) 0.0150 mL of cross-linker, 6-min photolysis, pH 7; (lane 13) 0.0150 mL of cross-linker, 6-min photolysis, pH 8. (Lanes 14–17) SDS–PAGE of samples of aldolase treated with DAP-tryptophan *p*-nitrophenyl ester (**45**). (Lane 14) No cross-linker solution, no photolysis, pH 8; (lane 15) 0.025 mL of cross-linker, no photolysis, pH 7; (lane 16), no cross-linker, 6-min photolysis, pH 7; (lane 17) 0.025 mL of cross-linker, 6-min photolysis, pH 8. (Lanes 18, 19) MW Markers. Ribonuclase, 14 000; lysozyme, 20 000; ovalbumin, 46 000; BSA, 66 000; phosphorylase, 97 000. (Lane 20) 0.0250 mL of cross-linker solution, 6-min photolysis, pH 7. (Lane 21) 0.0150 mL of cross-linker, 6-min photolysis, pH 8 (Lane 22) 0.0150 mL of cross-linker, 6-min photolysis, pH 7. (Lane 23) Aldolase.

Scheme VIII



An examination of the one-dimensional SDS–PAGE gels (Figure 1) shows a number of interesting features. A minor amount of cross-linked material was formed in the control reaction performed with cross-linker but without irradiation. Similar results have been observed with aldolase when reacted with unsymmetrical disulfide–dioxide reagents containing aryl azides and are presumed to result from disulfide interchange reactions to form an artificial zero-length disulfide cross-link, promoted by the cross-linking agent (Huang & Richards, 1977). Trace amounts of cross-linking were observed in the dark, with cross-linkers that did not contain disulfide linkages. Since 3-diazopyruvamides undergo thermolysis near 100 °C, the minor amounts of cross-linked product observed in the "dark" control reactions may have resulted from thermal Wolff rearrangements. The reaction in the dark may also have been the result of chemical decomposition of the diazoketone. Minor amounts of cross-linked materials are also observable in the control reaction that was irradiated at 300 nm in the absence of cross-linker.

As has been the case in previous studies of aldolase, cross-linking occurred in the presence of **21** to produce mainly dimeric structures, with perhaps some trimer. At the highest concentration of cross-linker used, minor amounts of the tetramer were detectable. The absence of large amounts of tetramer in cross-linking studies is due to the nonsymmetrical orientation of the four aldolase subunits that has been observed in X-ray structures of aldolase and in other cross-linking studies. The absence of large amounts of multimeric structures demonstrates that the lifetime of the reactive ketene intermediate is sufficiently short to prevent migration of a photoactivated protein, before nucleophilic trapping by the buffer occurs. Most importantly, cleavage of the cross-linked products occurred with mercaptoethanol, to give mainly monomeric aldolase subunits. Reagents patterned after the model-cleavable cross-linker **21**, therefore, should show great potential for use as cleavable cross-linking agents in two-dimensional electrophoresis cross-linking studies.

Cross-Linking with p-Nitrophenyl N-(3-Diazopyruvoyl)-6-aminohexanoate (**44**). *p*-Nitrophenyl *N*-(3-diazopyruvoyl)-6-aminohexanoate (**44**) was chosen as a simple model for 3-diazopyruvamide cross-linking agents containing active ester acylating functional groups that show specificity for amine functions in proteins.

Diazopyruvamide active ester **44**, dissolved in DMSO, was incubated with aldolase in pH 7.0 and 8.0 phosphate buffer. The initial reaction was allowed to proceed for 6 h at room temperature, and then appropriate samples were irradiated. Control reactions were performed by irradiating an aldolase solution containing no cross-linker and by allowing a solution of aldolase and cross-linker to interact without irradiation. The reaction samples were all reduced with mercaptoethanol prior to electrophoresis.

Cross-linking to form dimeric species was readily detectable in all photolysis reaction mixtures containing cross-linker **44**. Minor amounts of higher molecular weight molecules were also detected. A dependence on pH was noted for the dimeric cross-linked product. Reactions performed at pH 7.0 gave sharp bands corresponding to the dimeric species. The cross-linking reactions at pH 8.0 exhibited broader, more diffuse bands with portions of the band moving faster. Such behavior is usually attributed to intrachain cross-linking, which allows less unraveling of the protein on the gel. At the highest concentration of cross-linker at pH 8, an easily detectable amount of cross-linked tetramer was observed.

Cross-Linking of Aldolase with N-(3-Diazopyruvoyl)-L-tryptophan p-Nitrophenyl Ester (**45**). *N*-(3-Diazopyruvoyl)-L-tryptophan *p*-nitrophenyl ester (**45**) contains a short linker arm and a indole ring that can function as a spectrophotometric tag for the monitoring of the amount of cross-linker introduced in the first step of a cross-linking experiment or in cross-linked product. The tryptophan moiety might also be useful in fluorescence studies of the environment of the cross-linker after attachment to the protein has occurred. The reactions with the tryptophan active ester were performed in a similar manner to those for active ester **44**.

The cross-linked products formed were mainly dimers (M_r 80 000). Unlike the longer chained active ester **44**, the tryptophan active ester cross-linking agent **45** showed little dependence on pH or concentration for the production of interchain cross-linked species for the dimer band. The cross-linking pattern for all pH values and cross-linking concentrations were qualitatively very similar.

The model cross-linking reactions of aldolase have demonstrated that a number of 3-diazopyruvamide-based cross-linkers can be synthesized and that they undergo photoactivated conjugation reactions with proteins to label near neighbors in the tetrameric structures. The reagents tested were designed to produce different spans for cross-linked species as well as different reactive alkylating or acylating functional groups for the initial step of cross-linking. The disulfide reagent **21** presumably underwent disulfide exchange to produce reversible artificial disulfide cross-links. These prototypical reagents are currently being expanded to include species that are water soluble.

Conclusion

DAPpNP has been synthesized, and its reactivity with amine nucleophiles has been established. DAPpNP is a highly reactive acylating agent that reacts with many amines to yield diazopyruvoylamides in almost quantitative yield. 3-Diazopyruvamides undergo photolysis to produce a highly reactive ketene amide that is attacked by nucleophiles to produce malonic acid derivatives. In model reactions with amine and alcohols, no evidence was found for carbene insertion type products.

The 3-diazopyruvate group possesses many of the properties required of a superior photoactivatable probe. The function can be photolyzed above 300 nm. Photolysis efficiently produces one intermediate, which reacts with nucleophiles in a well-behaved manner. DAPpNP is easily synthesized, stable, and well characterized as an acylating agent. It is small in size and has been attached to large variety of biological molecules in simple synthetic pathways. The disadvantage of its small span can be modulated by the attachment of simple tethers. There is the disadvantage that the bridge formed in the cross-linking process is not stable to protein hydrolysis, making difficult the identification of the particular modified and cross-linked amino acid residues.

3-Diazopyruvamides are stable compounds that can be purified, if required, by chromatographic techniques. They are also stable to many synthetic transformations that might be encountered in the synthesis of photoaffinity labels and photoactivated cross-linking agents. A family of 3-diazopyruvamide cross-linking agents has been developed and found to cross-link aldolase. A cross-linking reagent with a disulfide linkage was found to form artificial reversible cross-links between aldolase subunits. Molecules derived from DAPpNP have already been demonstrated to be valuable tools for biochemists and cell biologists and exhibit potential for further biological applications as well as applications in organic syn-

thetic and medicinal chemistry (Pettit & Nelson, 1983, 1986; Earhart et al., 1982; Cantane et al., 1979).

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SUPPLEMENTARY MATERIAL AVAILABLE

Experimental procedures and analytical and physical data for compounds **15–33**, **41**, **44**, **49**, and **50** (9 pages). Ordering information is given on any current masthead page.

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