Development of a New Family of Thiol Specific Photoactivatable Cross-Linking Agents

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A new family of thiol specific photoactivatable cross-linking agents has been developed by the combination of the bromoacetyl function and the diazopyruvamide group. Various bromoacetyldiazopyruvoyl (bromoacetyl-DAP) derivatives have been synthesized and their reaction chemistry with thiols investigated and photochemistry explored. The trimodular bioprobe **17** design evolved from application of this chemistry. The trifunctionality of **17** is characterized by the presence of (1) a bromoacetyl function that modifies thiol groups through alkylation, (2) a diazopyruvoyl function which upon photolysis undergoes Wolff rearrangement generating a reactive ketene that can acylate nucleophiles, and (3) a phenolic function that acts as a characterization handle through radioiodination labeling via standard techniques. These bifunctional and trifunctional photoprobes typified by *N*-bromoacetyl-*N'*-(3-diazopyruvoyl)-*m*-phenylenediamine (**7a**) have been used to successfully cross-link a 14 aa peptide(Q) from the α_2-adrenergic receptor to purified G protein.

Introduction

Photoaffinity labeling and photoinitiated cross-linking are powerful techniques for use in biochemical systems. Some of the many attributes identified by using these techniques include the location and identification of active site residues of enzymes, detection of receptor sites for hormones and drugs, determination of the relationships of proteins within aggregate structures, and the resolution of protein–protein interactions involved in immunochemical systems.[1–4] The number and variety of photosensitive functional groups for incorporation in these reagent bioprobes is quite limited, and of these only the nitroaryl azides seem to have much widespread use with the biochemical community.[5,6] However, these popular nitroaryl azides have features that are often not compatible with many biological photoprobe experiments, and further, their photochemistry and subsequent reaction chemistry with proteins is uncertain.[7–11] We believe the development of alternative photoactivatable groups and reagents will aid the elucidation of the structure of biological molecules and relationships by providing an assortment of probes that can be fine-tuned to react efficiently with a given target.

Recent research in the development of photoactivatable reagents has focused on functional groups that upon photolysis generate reactive intermediates such as carbenes,[12–14] nitrenes,[15] or other radicallike species.[16] It is believed that these intermediates react with unactivated carbon–carbon or carbon–hydrogen bonds within a specific domain to produce "indiscriminate" labeling or cross-links to target protein residues.[17,18] Currently, aryl azides and (trifluoromethyl)aryldiazirines are the fashionable 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 an aqueous environment and in the presence of nucleophiles result in preferential capture of the inherently electrophilic intermediates by the nucleophile.[2] Unfortunately the chemical links produced in the biological system are rarely identified. This is understandable because in practice there is usually a complicated sequence of photochemical and chemical transformations superimposed upon the initial photochemical event. Aryl azides have a further disadvantage of not being able to withstand the presence of thiols. Many aromatic and aliphatic azides are readily reduced to the corresponding amines in the presence of the thiols necessary for the maintaining or providing the reducing conditions needed for protein modification.[26,27]

In 1989 we introduced the diazopyruvoyl (DAP) function **2** as a useful photochemically activatable cross-linking function.[19] It is a logical chemical extension of the diazoacetyl function first used by Westheimer[20] and it provides a new family of probes complementary 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. By use of the active ester, (DAP*p*NP, **1**) the diazopyruvoyl function can be attached to a variety of structures, providing derivatives that are stable toward most biological conditions but can be

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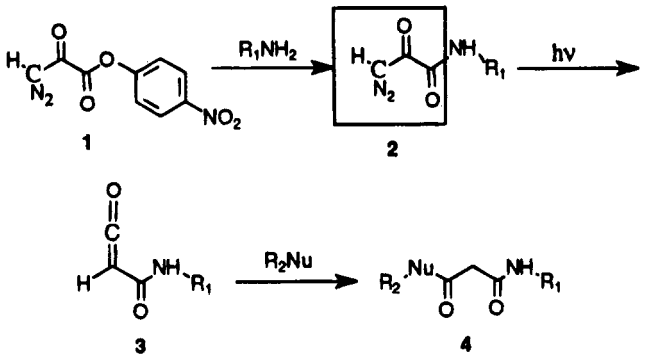
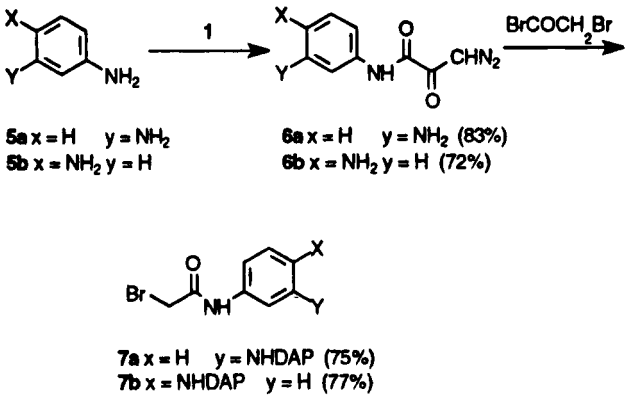
Scheme 1



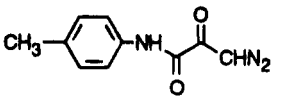
Scheme 2



readily photorearranged to an active acylating function **3** (Scheme 1). The photochemistry is very clean and simple and unlike nitroaryl azides, the UV transparent nature of the photoproducts results in complete transformation to the active acylating intermediate. This probe has been found to be quite efficient in the crosslinking of DAP modified Calmodulin with adenylate cyclase.[21] The diazopyruvoyl function is stable enough that it can be attached to polyfunctional molecules to create photoactivatable homobifunctional or heterobifunctional reagents. Other functional groups can be manipulated without perturbing the diazopyruvoyl group to allow the convenient synthesis of a variety of different photoactivatable probes. The presence of sulfhydryl groups in many protein structures encouraged the development and use of modification agents containing maleimide, α-halomethyl ketones, and disulfide functions.[22-25] We consider it to be of particular importance to develop a class of diazopyruvoyl photoactivatable crosslinking agents that would bind to thiol groups irreversibly, and simultaneously tolerate a third or fourth function. It is the focus of this paper to discuss the synthesis and chemistry of such agents.

Results and Discussion

DAP*p*NP (**1**) reacts, cleanly and in high yield, with primary and secondary amines to yield 3-diazopyruvamide derivatives. Diamines react easily with 2 equiv of DAP*p*NP (**1**) to form bis-DAP derivatives, such as the previously reported *N,N'*-bis(3-diazopyruvoyl)-1,6-diaminohexane.[19] Bis-DAP derivatives of *m*- or *p*-phenylenediamine are also easily formed but these have very poor solubility properties in organic and aqueous solvent systems and thus were not pursued. However, limiting DAP*p*NP (**1**) to 1 equiv leads to the high yield synthesis of the mono-DAP aromatic amine derivatives **6a** and **6b**. By taking advantage of the remaining amine functionality of **6a** and **6b**, a wide variety of bioprobes have evolved. In this work, we center on a series of α-bromo amide derivatives which permits the easy attachment to the thiol of cysteine residues in peptides and proteins or to the thiol functions that have been introduced into the system under study via thiolating agents such as 2-iminothiolane (Traut's Reagent), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), and *N*-succinimidyl *S*-acetylthioacetate (SATA). Thus, a thioacetyl function can be formed while the photoactivatable DAP group remains intact.



8

Before embarking on the development of the new class of bioprobes which modify thiols, it seemed prudent to confirm that the diazopyruvoyl functionality would remain intact in the presence of sulfhydryl groups. After incubation of *N*-(*p*-tolyl)-3-diazopyruvamide (**8**) with excess ethanethiol in chloroform or in ethanol in the presence of 1 equiv of base for 18 h, **8** was recovered in 70% yield, confirming that the diazopyruvoyl functionality survives the conditions necessary for protein conjugation. As indicated previously, this is in direct contrast with many aryl azides that are readily reduced to the corresponding aryl amines in the presence of thiols.[26,27]

The mono-DAP amine derivative **6a** or **6b** condensed with bromoacetyl bromide in the presence of *N*-methylmorpholine to yield the α-bromo amide derivatives **7a** and **7b** respectively (Scheme 2). These latter derivatives, prepared in this simple four-step synthesis, have a stability of several months, at 0°, in the absence of light. The *meta* derivative **7a** was found to have higher solubility properties than the *para* derivative **7b** in solvents such as aqueous acetone and aqueous acetonitrile. This enabled reactions with water soluble materials to be carried out with ease and thus **7a** became the compound of focus for the synthesis of further derivatives for biological studies. The bromoacetyl moiety of both **7a** and **7b** reacted smoothly and in good yield with a variety of thiols without effect on the DAP function (Table 1).

The bifunctional reagent **7a** has been employed to study interactions between purified G protein and the α_2-adrenergic receptor, using a 14 amino acid peptide (Q) synthesized from the distal third cytoplasmic loop of the receptor. In studies to be published elsewhere, the α-bromo amide derivative **7a** is shown to link efficiently with a C terminal cysteine residue attached to peptide Q. This tagged peptide interacts with a purified G

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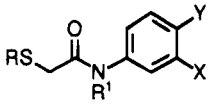
Table 1. Yields of the Thiol Acetyl Adducts Resulting from the Reactions of Bromoacetyl-DAP Derivatives with Various Thiols



product	R	R^1	X	Y	% yield[a]
9a	$CH_3C_6H_4$	H	NHDAP	H	73
9b	$CH_3C_6H_4$	H	H	NHDAP	92
18	$CH_3C_6H_4$	*o*-$HOC_6H_4CH_2$	NHDAP	H	77
19	CH_3CH_2	*o*-$HOC_6H_4CH_2$	NHDAP	H	92
20	CH_3CH_2	*p*-$HOC_6H_4CH_2$	NHDAP	H	79

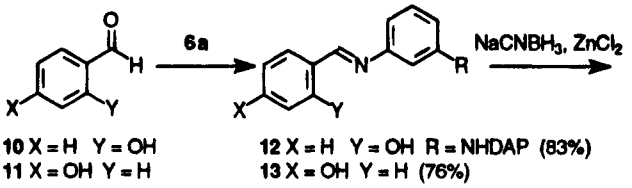
[a] Yields are of isolated pure products after crystallization.

protein complex and photochemically couples to it.[28-31] The requirement of another "handle" on the Q peptide, without changing the amino acid residues necessary for recognition, led to the addition of a third functional group on the photoprobe and the development of "trimodular" cross-linking agents. Included in the design are two alkylating sites, an alkylation site for attachment to the protein SH group, a latent but photoactivatable site that can form a cross-link in the biostructure, and a third moiety that can serve either as a recognition element or a characterization handle, such as a fluorescent or radioiodinated function. We thus undertook the synthesis of **16** and **17**. Phenolic functions were attached to provide molecules that could be readily radioiodinated by standard techniques, as attempts to iodinate **6a** under mild conditions failed.[32,33]

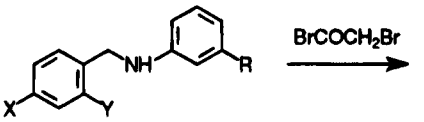
The diazopyruvoyl imine **12** was made by Schiff base condensation of the mono-DAP amine derivative **6a** with salicylaldehyde. Reduction of imine **12** with sodium cyanoborohydride and zinc chloride[34] provided the 2-hydroxybenzyl amine **14** in good yield. Compound **14** was treated with bromoacetyl bromide to afford the trimodular cross-linking agent **16** in 75% yield (Scheme 3). Analogous to the reactions of **7a** and **7b**, both **16** and **17** demonstrated their ability to act as valuable sulfhydryl specific modification agents by reacting with thiols under mild conditions, leaving the diazoketo amide functionality intact and providing the adducts in 70–90% yield (Table 1).

Previous work has demonstrated the efficiency of the diazopyruvoyl moiety as a photoactivatable bioprobe.[19,21] Upon photolysis of the DAP group, at 300 nm, **2** undergoes loss of nitrogen and Wolff rearrangement to generate the highly reactive ketene amide **3**. The ketene **3** can interact with solvent or with a neighboring nucleophile, either intra- or intermolecularly (in the case of protein aggregates), to afford a three-carbon malonic residue cross-link bridge **4** (Scheme 1). Photolysis of the thiol specific trimodular cross-linking agents **16** and **17**, and the thiol-coupled derivative **20**, in methanol, afforded the rearranged methyl ester product with isolated yields ranging from 10 to 70% (Scheme 4).

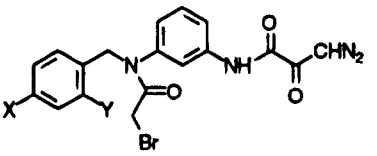
Scheme 3



10 X = H Y = OH
11 X = OH Y = H
12 X = H Y = OH R = NHDAP (83%)
13 X = OH Y = H (76%)

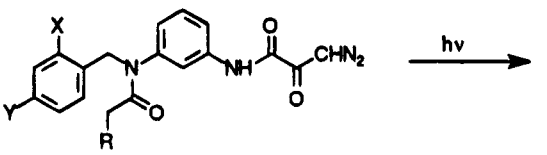


14 X = H Y = OH R = NHDAP (99%)
15 X = OH Y = H (98%)

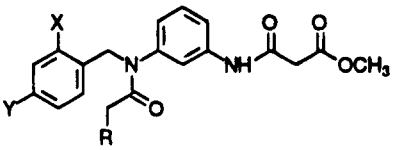


16 X = H Y = OH (75%)
17 X = OH Y = H (77%)

Scheme 4



16 X = OH Y = H R = Br
17 X = H Y = OH R = Br
20 X = H Y = OH R = SEt



21 X = OH Y = H R = Br (72%)
22 X = H Y = OH R = Br (19%)
23 X = H Y = OH R = SEt (10%)

The bifunctional and trimodular thiol specific photoactivatable cross-linking agents described above have been synthesized with ease and in high yield while maintaining the diazopyruvoyl function intact. These bioprobes have been shown to react cleanly with thiols, with no trace of decomposition of the photoactivatable moiety. Photolysis of the DAP derivatives in methanol yielded the expected methyl ester products. Thus, the chemistry and the feasibility of using this new family of thiol specific photoactivatable compounds as cross-linking agents has been clearly established.

Experimental Section

General. Reagents and most solvents were obtained from commercial suppliers and were used without further purification. THF was distilled from sodium benzophenone ketyl. Na_2SO_4 was used as the drying agent. Melting points were obtained in open capillary tubes and are not corrected. All solvents were removed by rotary evaporation unless otherwise stated. Mass spectra were obtained using electron impact (EI)

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ionization unless otherwise stated. Ammonia was used as reagent gas for ionization spectra as specified. Combustion analyses were performed by Spang Microanalytical Laboratories (Eagle Harbor, MI) or by the microanalytical facility operated by the University of Michigan. Preparative thin layer chromatography was performed on Kieselgel 60F 254 silica gel on 20 × 20 plates of 2 mm thickness. All reactions were performed at room temperature in dim light unless otherwise specified. Photolyses were carried out in a photochemical reactor with 300 nm lamps at ambient temperature under an atmosphere of nitrogen. DAP*p*NP and 3-diazopyruvamides show two discrete peaks for the diazomethine hydrogen by NMR. This is due to the two conformations and the restricted rotation about the CO–CHN_2 bond. For spectral characteristics and discussion of DAP*p*NP and 3-diazopyruvamides see ref 19.

***N*-(3-Aminophenyl)-3-diazopyruvamide (6a).** To a solution of *m*-phenylenediamine (0.89 g, 8.3 mmol), in EtOAc (15 mL), was added dropwise over a period of 0.5 h a solution of DAP*p*NP (1.0 g, 4.3 mmol) in EtOAc (25 mL). A yellow solution immediately formed. The reaction was stirred under nitrogen for 12 h. The resulting reaction mixture was washed with 10% $NaHCO_3$ (4 × 50 mL), water (2 × 50 mL), and brine (1 × 50 mL). The solution was dried and decolorized over anhydrous Na_2SO_4 and activated carbon. After filtration and evaporation to dryness, 0.73 g (83%) of a yellow solid was obtained, mp 151–152.5 °C. The crystallization solvent was EtOAc. ^1H NMR ($CDCl_3$, 300 MHz) δ 5.23 (s, 0.1H), 6.46 (s, 0.9H), 6.51 (d, 1H), 6.86 (d, 1H), 7.12 (t, 1H), 7.20 (s, 1H), 8.83 (br, 2H). ^{13}C NMR (acetone-d_6, 75 MHz) δ 55.30, 107.06, 109.89, 112.24, 130.27, 138.97, 149.96, 158.95, 183.04. IR (KBr) 3474, 3363, 3283, 3108, 2104, 1677, 1624, 1599, 1443, 846, 777, 554 cm^{-1}. MS (*m*/*z*) 204, 176, 149, 120, 105, 69, 41. HRMS calcd for $C_9H_8N_4O_2$ 204.0647, found 204.0647. Anal. Calcd for $C_9H_8N_4O_2$: C, 52.94; H, 3.95; N, 27.44. Found: C, 53.07; H, 4.09; N, 27.07.

***N*-(4-Aminophenyl)-3-diazopyruvamide (6b).** An analogous procedure and workup as for **6a** was followed. *p*-Phenylenediamine (0.46 g, 4.3 mmol) and DAP*p*NP (0.50 g, 2.1 mmol) were used with a reaction time of 24 h. A yellow solid was obtained, weighing 0.43 g (72%), mp 128–129.5 °C. The crystallization solvent was EtOAc. ^1H NMR ($CDCl_3$, 300 MHz) δ 5.21 (s, 0.1H), 6.46 (s, 0.9H), 6.66 (d, 2H), 7.44 (d, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 54.35, 115.46, 121.55, 127.79, 144.18, 156.92, 182.18. IR (KBr) 3391, 3340, 3303, 3132, 2137, 2103, 1679, 1633, 1594, 1544, 741, 703 cm^{-1}. MS (*m*/*z*) 204, 134, 107, 92, 69, 41. HRMS calcd for $C_9H_8N_4O_2$ 204.0647, found 204.0646. Anal. Calcd for $C_9H_8N_4O_2$: C, 52.94; H, 3.95; N, 27.44. Found: C, 53.06; H, 4.06; N, 27.24.

***N*-(Bromoacetyl)-*N*′-(3-diazopyruvoyl)-1,3-phenylenediamine (7a).** To a solution of **6a** (0.300 g, 1.47 mmol) and *N*-methylmorpholine (0.148 g, 1.47 mmol) in EtOAc (15 mL) cooled to 0 °C was added bromoacetyl bromide (0.297 g, 1.47 mmol) dissolved in EtOAc (5 mL). A white slurry formed immediately. The reaction was stirred, with ice cooling, for 0.5 h. The mixture was diluted with EtOAc (20 mL). The organic layer was washed with 10% citric acid followed by a 5% Na_2CO_3 and brine wash. The extract was concentrated to dryness. The solid residue was extracted with acetone which was then dried and concentrated under a stream of nitrogen. A cream colored solid was obtained, weighing 0.410 g (75%), mp 185 °C dec. The crystallization solvent was EtOAc/hexane. ^1H NMR (DMF-d_7, 360 MHz) δ 4.14 (s, 2H), 5.92 (s, 0.1H), 6.78 (s, 0.7H), 7.35 (t, 1H), 7.55 (d, 1H), 7.61 (d, 2H), 8.30 (s, 1H). ^{13}C NMR (DMF-d_7, 90 MHz) δ 30.75, 55.45, 112.26, 116.31, 116.80, 129.71, 138.91, 140.19, 159.62, 165.66, 182.50. IR (KBr) 3293, 3060, 2142, 2109, 1682, 1661, 1629, 1542, 1225, 559 cm^{-1}. MS (*m*/*z*) 324, 255, 228, 212, 189, 161, 121, 69, 43. HRMS calcd for $C_{11}H_9{}^{79}BrN_4O_3$ 323.9858, found 323.9850. Anal. Calcd for $C_{11}H_9BrN_4O_3$: C, 40.64; H, 2.79; N, 17.23. Found: C, 40.68; H, 2.73; N, 17.28.

***N*-(Bromoacetyl)-*N*′-(3-diazopyruvoyl)-1,4-phenylenediamine (7b).** An analogous procedure as for **7a** was followed. *N*-Methylmorpholine (0.10 g, 0.98 mmol), **6b** (0.20 g, 0.98 mmol) and bromoacetyl bromide (0.20 g, 0.98 mmol) were used with a reaction time of 0.5 h. A cream colored solid was obtained, 0.25 g (77%), mp 198 °C dec. The crystallization solvent was EtOAc/hexane. ^1H NMR (DMF-d_7, 360 MHz) δ 4.12 (s, 2H), 5.88 (s, 0.1H), 6.78 (s, 0.7H), 7.71 (d, 2H), 7.90 (d, 2H). ^{13}C NMR (DMF-d_7, 90 MHz) δ 30.74, 55.91, 120.18, 121.56, 134.39, 136.47, 159.34, 165.51, 182.63. IR (KBr) 3303, 3212, 3118, 3088, 2139, 2106, 1674, 1662, 1625, 1593, 1499, 848 cm^{-1}. MS (*m*/*z*) 323, 296, 256, 230, 120, 95, 69, 41. HRMS calcd for $C_{11}H_9{}^{79}BrN_4O_3$: 323.9858, found 323.9851. Anal. Calcd for $C_{11}H_9BrN_4O_3$: C, 40.64; H, 2.79; N, 17.23; Found: C, 40.66; H, 2.89; N, 17.15.

***N*-[(4-Tolylthio)acetyl]-*N*′-(3-diazopyruvoyl)-1,3-phenylenediamine (9a).** To a solution of **7a** (0.200 g, 0.620 mmol) and *N*-methylmorpholine (0.135 g, 1.23 mmol) in acetone (5 mL) and water (5 mL) was added a solution of thiocresol (0.150 g, 1.23 mmol) in acetone (2 mL). Within a few minutes a precipitate began to form. The reaction was stirred under nitrogen for 1.5 h. The acetone was evaporated and the residue was taken up in $CHCl_3$ (20 mL). The organic layer was washed with water and brine. The resulting organic phase was dried and concentrated. A white solid was obtained, 0.165 g (73%), mp 169–171 °C. The crystallization solvent was EtOAc. ^1H NMR ($CDCl_3$, 300 MHz) δ 2.31 (s, 3H), 3.73 (s, 2H), 5.28 (s, 0.1H), 6.46 (s, 0.9H), 7.14 (d, 2H), 7.30 (m, 4H), 7.45 (m, 1H), 7.91 (s, 1H), 8.66 (br, 1H), 8.99 (br, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 20.90, 39.48, 54.63, 111.45, 116.12, 116.66, 129.72, 129.81, 130.35, 130.55, 137.08, 137.68, 138.42, 157.75, 166.47, 181.50. IR (KBr) 3110, 3086, 2133, 2100, 1687, 1669, 1655, 1598, 1536, 1227, 686 cm^{-1}. MS (*m*/*z*) 368, 203, 189, 137, 123, 91, 77, 69, 42. Anal. Calcd for $C_{18}H_{16}N_4O_3S$: C, 58.68; H, 4.38; N, 15.21. Found: C, 58.37; H, 4.66; N, 15.29.

***N*-[(4-Tolylthio)acetyl]-*N*′-(3-diazopyruvoyl)-1,4-phenylenediamine (9b).** An analogous procedure as for **9a** was followed. *N*-methylmorpholine (0.063 g, 0.62 mmol), **7b** (0.20 g, 0.62 mmol) and thiocresol (0.077 g, 0.62 mmol) were used with a reaction time of 10 h. A tan solid was obtained, 0.21 g (92%), mp 190–192 °C. The crystallization solvent was $CHCl_3$. ^1H NMR ($CDCl_3$, 360 MHz) δ 2.31 (s, 3H), 3.72 (s, 2H), 5.22 (s, 0.1H), 6.45 (s, 0.6H), 7.14 (d, 2H), 7.24 (d, 2H), 7.50 (d, 2H), 7.58 (d, 2H), 8.60 (br, 1H), 8.93 (br, 1H). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 20.98, 39.03, 54.91, 120.45, 120.58, 129.23, 129.76, 130.29, 132.81, 134.52, 137.53, 157.33, 166.13, 181.50. IR (KBr) 3302, 3088, 2138, 2107, 1673, 1658, 1627, 1551, 1531, 699 cm^{-1}. MS (*m*/*z*) 368, 342, 298, 272, 189, 164, 137, 120, 91, 69, 42. HRMS calcd for $C_{18}H_{16}N_4O_3S$ 368.0943, found 368.0945.

***N*-(2-Hydroxybenzylidene)-*N*′-(3-diazopyruvoyl)-1,3-phenylenediamine (12).** To a solution of **6a** (0.300 g, 1.47 mmol) in EtOH (8 mL) was added salicylaldehyde (0.195 g, 1.60 mmol). Within 10 min an orange slurry formed. The reaction was stirred for 0.5 h. The slurry was diluted with ether and filtered. A yellow solid was obtained, 0.375 g (83%), mp 171–172.5 °C. The crystallization solvent was MeOH. ^1H NMR ($CDCl_3$, 300 MHz) δ 5.65 (s, 0.1H), 6.64 (s, 0.8H), 6.97 (t, 2H), 7.23 (d, 1H), 7.46 (m, 2H), 7.59 (d, 1H), 7.79 (d, 1H), 7.95 (s, 1H), 8.90 (s, 1H), 9.86 (br, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 55, 112, 117, 118, 118.5, 119, 130, 132, 133, 137, 149, 158, 161, 163, 181. IR (KBr) 3305, 3091, 2134, 2100,1677, 1619, 1595, 788, 683 cm^{-1}. MS (*m*/*z*) 308, 211, 196, 120, 93, 77, 69, 41. HRMS calcd for $C_{16}H_{12}N_4O_2$ 308.0909, found: 308.0915 Anal. Calcd for $C_{16}H_{12}N_4O_2$: C, 62.33; H, 3.92; N, 18.17 Found: C, 62.30; H, 3.62; N, 18.14.

***N*-(4-Hydroxybenzylidene)-*N*′-(3-diazopyruvoyl)-1,3-phenylenediamine (13).** To a solution of **6a** (0.500 g, 2.45 mmol) in EtOH (15 mL) was added 4-hydroxybenzaldehyde (0.299 g, 2.45 mmol). After 18 h, the volume was reduced by half. The precipitate was filtered and washed with ether. Crystallization from MeOH afforded 0.570 g (76%) of a yellow solid, mp 160–161.5 °C. ^1H NMR (acetone-d_6, 360 MHz) δ 5.63 (s, 0.1H), 6.63 (s, 0.8H), 6.97 (d, 2H), 7.02 (d, 1H), 7.37 (t, 1H), 7.70 (d, 1H), 7.77 (s, 1H), 7.84 (d, 2H), 8.45 (s, 1H), 9.16 (br, 1H), 9.75 (br, 1H). ^{13}C NMR (acetone-d_6, 90 MHz) δ 55.67, 113.50, 116.45, 117.84, 118.03, 129.23, 130.28, 131.60, 138.97, 154.07, 159.12, 160.88, 161.50, 182.45. IR (KBr) 3474, 3284, 3108, 2105, 1677, 1624, 1600, 846 cm^{-1}. MS (*m*/*z*) 308, 280,

251, 223, 211, 204, 196, 120, 93, 77, 69, 41. Calculated FW for $C_{16}H_{12}N_4O_3$ 308.0909.

***N*-(2-Hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (14).** To a slurry of **12** (0.500 g, 1.62 mmol) in MeOH (10 mL) was added sodium cyanoborohydride (0.102 g, 1.62 mmol) and zinc chloride (0.110 g, 0.81 mmol) dissolved in MeOH (5 mL). The reaction was stirred for 6 h. The slurry was filtered and the solid was washed with ether. A bright yellow solid was obtained, 0.500 g (99%), mp 170–173 °C. The crystallization solvent was $CHCl_3$. ^1H NMR (DMSO-d_6, 360 MHz) δ 4.16 (d, 2H), 6.05 (s, 1H), 6.37 (d, 1H), 6.71 (s, 1H), 6.81 (d, 1H), 7.02 (m, 3H), 7.15 (m, 2H), 9.45 (s, 1H). ^{13}C NMR (DMSO-d_6, 90 MHz) δ 41.39, 55.63, 104.21, 108.33, 109.06, 114.81, 118.72, 125.69, 127.44, 128.09, 128.95, 138.02, 149.30, 155.01, 158.51, 182.06. IR (KBr) 3408, 3366, 3306, 3103, 2123, 2093, 1690, 1642, 1609, 1594, 1376, 772 cm^{-1}. MS (m/z) 310, 282, 211, 204, 120, 107, 69, 41. HRMS calcd for $C_{16}H_{14}N_4O_3$ 310.1066, found: 310.1060. Anal. Calcd for $C_{16}H_{14}N_4O_3$: C, 61.93; H, 4.55; N, 18.06. Found: C, 61.81; H, 4.37; N, 17.87.

***N*-(4-Hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (15).** An analogous procedure as for **14** was followed. Sodium cyanoborohydride (0.10 g, 1.6 mmol), **13** (0.50 g, 1.6 mmol), and zinc chloride (0.11 g, 0.81 mmol) were used with a reaction time of 3 h. An orange solid was obtained, 0.49 g (98%), mp 173–175 °C. The crystallization solvent was EtOAc. ^1H NMR (acetone-d_6, 360 MHz) δ 2.93 (s, 1H), 4.21 (d, 2H), 5.57 (s, 0.1H), 6.45 (d, 1H), 6.59 (s, 0.7H), 6.78 (d, 2H), 7.06 (d, 2H), 7.22 (m, 3H), 8.20 (br, 1H), 9.39 (br, 1H). ^{13}C NMR (acetone-d_6, 90 MHz) δ 47.63, 55.37, 104.79, 109.11, 110.39, 115.94, 129.50, 130.04, 131.45, 138.96, 150.42, 157.19, 158.75, 182.80. IR (KBr) 3377, 3359, 3291, 2131, 2100, 1664, 1639, 1606, 1597 cm^{-1}. MS (m/z) 310, 204, 176, 107, 69. Calcd FW for $C_{16}H_{14}N_4O_3$: 310.1066. Anal. Calcd for $C_{16}H_{14}N_4O_3$: C, 61.93; H, 4.55; N, 18.06. Found: C, 61.98; H, 4.65; N, 18.11.

***N*-Bromoacetyl-*N*-(2-hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (16).** To a slurry of **14** (0.50 g, 1.6 mmol), in EtOAc (15 mL) at 0 °C, was added *N*-methylmorpholine (0.18 g, 1.73 mmol). Bromoacetyl bromide (0.35 g, 1.7 mmol) in EtOAc (5 mL) was added slowly to the stirred reaction mixture. The reaction was stirred at 0 °C for 45 min. The resulting solution was washed with 10% citric acid, 5% Na_2CO_3, and brine, dried, and concentrated to give 0.52 g (75%) of a cream colored solid, mp 171–172 °C. The crystallization solvent was $CHCl_3$/hexane. ^1H NMR ($CDCl_3$, 360 MHz) δ 3.67 (s, 2H), 4.79 (br, 2H), 5.26 (s, 0.1H), 6.44 (s, 0.7H), 6.61 (d, 1H), 6.63 (t, 1H), 6.88 (d, 1H), 6.99 (d, 1H), 7.22 (t, 1H), 7.44 (t, 1H), 7.65 (m, 2H), 9.01 (br, 1H), 9.10 (br, 1H). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 26.09, 51.49, 55.31, 118.00, 119.01, 119.55, 120.46, 120.97, 124.65, 130.55, 130.75, 131.75, 137.96, 141.29, 155.80, 157.73, 168.71, 180.83. IR (KBr) 3282, 3226, 3107, 2125, 2093, 1691, 1638, 1623, 1445, 810 cm^{-1}. MS (m/z) 432, 351, 323, 239, 203, 189, 107, 77, 69. HRMS calcd for $C_{18}H_{15}{}^{79}BrN_4O_4$ 430.0277, found: 430.0283. Anal. Calcd for $C_{18}H_{15}BrN_4O_4 \cdot {}^1/_6CHCl_3$: C, 48.03; H, 3.37; N, 12.31. Found: C, 47.87; H, 3.32; N, 12.07.

***N*-Bromoacetyl-*N*-(4-hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (17).** An analogous procedure as for **16** was followed. *N*-methylmorpholine (0.16 g, 1.6 mmol), **15** (0.50 g, 1.6 mmol), and bromoacetyl bromide (0.33 g, 1.6 mmol) were used with a reaction time of 1 h. Crystallization from $CHCl_3$ afforded 0.53 g (77%) of a cream colored solid, mp 160–162 °C. ^1H NMR (acetone-d_6, 360 MHz) δ 2.92 (s, 1H), 3.84 (s, 2H), 4.82 (s, 2H), 5.62 (s, 0.1H), 6.62 (s, 0.8H), 6.71 (d, 2H), 6.98 (d, 1H), 7.03 (d, 2H), 7.38 (t, 1H), 7.77 (s, 1H), 7.88 (d, 1H), 8.30 (s, 1H), 9.84 (br, 1H). ^{13}C NMR (acetone-d_6, 90 MHz) δ 28.57, 53.32, 55.75, 115.95, 120.72, 121.02, 125.44, 128.83, 130.68, 130.75, 139.38, 142.96, 157.64, 159.28, 166.37, 182.13. IR (KBr) 3303, 3266, 3117, 3033, 2138, 2105, 1688, 1641, 1613, 1592, 807 cm^{-1}. MS (CI m/z) 450 (M + NH_4), 432 (M + H), 422, 407, 325, 283, 218, 151, 124, 107. HRMS calcd for $C_{18}H_{15}{}^{79}BrN_4O_4$ 431.0355, found: 431.0376. Anal. Calcd for $C_{18}H_{15}BrN_4O_4$: C, 50.13; H, 3.51; N, 12.99. Found: C, 49.91; H, 3.63; N, 12.75.

***N*-[(4-Tolylthio)acetyl]-*N*-(2-hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (18).** An analogous procedure as for **9a** was followed. *N*-methylmorpholine (0.047 g, 0.46 mmol), **16** (0.20 g, 0.46 mmol), and thiocresol (0.060 g, 0.48 mmol) were used with a reaction time of 2.5 h. A white solid was obtained, 0.17 g (77%), mp 100–102 °C. The crystallization solvent was $CHCl_3$/hexane. ^1H NMR ($CDCl_3$, 360 MHz) δ 2.31 (s, 3H), 3.39 (s, 2H), 4.73 (br, 2H), 5.26 (s, 0.1H), 6.46 (s, 0.8H), 6.56 (d, 1H), 6.64 (m, 2H), 7.04 (m, 4H), 7.23 (m, 3H), 7.33 (t, 1H), 7.70 (s, 1H), 8.99 (br, 1H), 9.34 (br, 1H). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 21.01, 37.63, 51.08, 55.22, 117.94, 119.29, 120.01, 121.48, 124.88, 129.77, 130.31, 130.51, 131.71, 132.56, 137.65, 138.08, 141.64, 155.92, 157.62, 171.23, 180.88. IR (KBr) 3295, 3162, 3160, 3087, 2125, 2101, 1685, 1627, 1595, 1537 cm^{-1}. MS (m/z) 474, 378, 351, 309, 203, 137, 107. Calcd FW for $C_{25}H_{22}N_4O_4S$ 474.5392. Anal. Calcd for $C_{25}H_{22}N_4O_4S$: C, 63.28; H, 4.67; N, 11.81. Found: C, 63.18; H, 4.56; N, 11.76.

***N*-[(Ethylthio)acetyl]-*N*-(2-hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (19).** An analogous procedure as for **9a** was followed. *N*-methylmorpholine (0.069 g, 0.68 mmol), **16** (0.26 g, 0.61 mmol), and ethanethiol (0.042 g, 0.68 mmol) were used with a reaction time of 4 h. A pale yellow solid was obtained, 0.23 g (92%), mp 138–139 °C. The crystallization solvent was $CHCl_3$/hexane. ^1H NMR ($CDCl_3$, 360 MHz) δ 1.21 (t, 3H), 2.65 (q, 2H), 3.05 (s, 2H), 4.77 (br, 2H), 5.25 (s, 0.1H), 6.45 (s, 0.8H), 6.62 (d, 1H), 6.68 (t, 1H), 6.88 (d, 1H), 6.98 (d, 1H), 7.21 (t, 1H), 7.41 (t, 1H), 7.58 (s, 1H), 7.65 (d, 1H), 9.07 (br, 1H), 9.37 (br, 1H). ^{13}C NMR ($CDCl_3$, 90 MHz) δ 14.19, 26.64, 32.60, 51.10, 55.25, 117.87, 119.32, 119.43, 120.03, 121.47, 125.14, 130.34, 130.53, 131.72, 137.74, 141.88, 155.94, 157.65, 171.97, 180.93. IR (KBr) 3303, 3128, 3087, 2119, 1685, 1645, 1620, 1596 cm^{-1}. MS (m/z) 412, 352, 307, 256, 203, 189, 107, 75, 69. HRMS calcd for $C_{20}H_{20}N_4O_4S$ 412.1205, found: 412.1212. Anal. Calcd for $C_{20}H_{20}N_4O_4S \cdot {}^1/_{20}CHCl_3$: C, 57.55; H, 4.83; N, 13.43. Found: C, 57.66; H, 4.91; N, 13.27.

***N*-[(Ethylthio)acetyl]-*N*-(4-hydroxybenzyl)-*N'*-(3-diazopyruvoyl)-1,3-phenylenediamine (20).** An analogous procedure as for **9a** was followed. *N*-methylmorpholine (0.048 g, 0.47 mmol), **17** (0.20 g, 0.46 mmol), and ethanethiol (0.029 g, 0.47 mmol) were used with a reaction time of 15 h. A pale yellow solid was obtained, 0.15 g (79%), mp 149–151 °C. The crystallization solvent was EtOAc. ^1H NMR (acetone-d_6, 360 MHz) δ 1.13 (t, 3H), 2.58 (q, 2H), 2.99 (s, 1H), 3.14 (s, 2H), 4.81 (s, 2H), 5.62 (s, 0.1H), 6.61 (s, 0.8H), 6.71 (d, 2H), 6.94 (d, 1H), 7.04 (d, 2H), 7.36 (t, 1H), 7.74 (s, 1H), 7.84 (d, 1H), 8.29 (br, 1H). ^{13}C NMR (acetone-d_6, 90 MHz) δ 14.65, 26.71, 33.59, 52.79, 55.73, 115.88, 120.31, 121.23, 125.77, 129.30, 130.46, 130.62, 139.23, 143.62, 157.48, 159.23, 169.51, 182.16. IR (KBr) 3268, 3127, 3076, 2969, 2111, 1678, 1637, 1600, 1534, 700 cm^{-1}. MS (m/z) 412, 352, 282, 220, 203, 107, 75, 69. HRMS calcd for $C_{20}H_{20}N_4O_4S$ 412.1205, found: 412.1213. Anal. Calcd for $C_{20}H_{20}N_4O_4S$: C, 58.24; H, 4.89; N, 13.58. Found: C, 58.09; H, 4.95; N, 13.49.

Stability of *N*-(4-Tolyl)-3-diazopyruvamide (8) in Solutions of Thiol. To a solution of **8** (0.10 g, 0.49 mmol) in $CHCl_3$ (3 mL) was added ethanethiol (0.30 g, 4.9 mmol). The reaction was stirred under nitrogen for 18 h and concentrated. Solid compound **8** was recovered as indicated by the identical NMR spectra obtained for the solid before and after the incubation. Recovered **8** from above was dissolved in EtOH (3 mL), and ethanethiol (0.30 g, 4.9 mmol) and triethylamine (0.049 g, 0.49 mmol) were added to it. The reaction was stirred under nitrogen for 26 h. The solution was diluted with $CHCl_3$, washed with 10% HCl, 10% $NaHCO_3$, and brine, dried, and concentrated, 0.070 g (70%) of **8** was recovered.

General Procedure for the Photolysis of 3-Diazopyruvamides in Methanol. Solvent Trapping of the Intermediate Ketene. A solution of 3-diazopyruvamide (0.23 mmol) in methanol (50 mL) was photolyzed, at 300 nm, at ambient temperature, in a quartz tube, under an atmosphere of nitrogen for approximately 5 h. The reactions were monitored by TLC using 9/1 $CHCl_3$/MeOH. The resulting solution was reduced in volume (5 mL) and diluted with ethyl acetate (25 mL). This was then washed with 10% citric acid, 10% $NaHCO_3$, and brine, dried, and concentrated. The resulting

oil was chromatographed on a preparative thin layer chromatography plate, using 6/4 hexane/acetone as the developing solvent.

Photolysis Product 21. Isolated yield 72%. ^{1}H NMR (acetone-d_6, 360 MHz) δ 3.49 (s, 2H), 3.67 (s, 3H), 3.85 (s, 2H), 4.86 (s, 2H), 6.69 (t, 1H), 6.81 (d, 1H), 6.83 (d, 1H), 6.97 (t, 1H), 7.13 (t, 1H), 7.37 (t, 1H), 7.63 (s, 1H), 7.73 (d, 1H), 9.68 (br, 1H). ^{13}C NMR (acetone-d_6, 75 MHz) δ 27.84, 44.06, 50.38, 50.78, 117.41, 119.46, 120.08, 120.25, 123.06, 124.03, 129.93, 130.30, 130.82, 131.95, 141.11, 142.45, 156.58, 164.93, 168.78. IR (neat) 3295, 3029, 2960, 1750, 1679, 1644, 1609, 1236, 765 cm^{-1}. M/S (m/z) 434, 420, 355, 341, 328, 313, 208, 107, 77, 59. HRMS calcd for $C_{19}H_{19}{}^{79}BrN_2O_5$ 434.0477, found 434.0477.

Photolysis Product 22. Isolated yield 19%. ^{1}H NMR (acetone-d_6, 300 MHz) δ 3.49 (s, 2H), 3.67 (s, 3H), 3.81 (s, 2H), 4.79 (s, 2H), 6.74 (d, 2H), 6.86 (d, 1H), 7.03 (d, 2H), 7.31 (t, 1H), 7.53 (s, 1H), 7.70 (d, 1H), 8.31 (t, 1H). ^{13}C NMR (acetone-d_6, 75 MHz) δ 28.51, 44.10, 52.35, 53.31, 115.98, 119.87, 123.20, 124.37, 128.95, 130.57, 141.04, 142.93, 157.70, 164.89, 166.37, 168.83. IR (neat) 3300, 3022, 2952, 1735, 1644, 1595, 1215 cm^{-1}. M/S (CI m/z) 454 (M + NH_4), 437 (M), 421, 393, 355, 313, 208, 107. HRMS calcd for $C_{19}H_{19}{}^{79}BrN_2O_5$ + H 434.0477, found 434.0477.

Photolysis Product 23. Isolated yield 10%. ^{1}H NMR (acetone-d_6, 360 MHz) δ 1.14 (t, 3H), 2.58 (q, 2H), 3.46 (s, 2H), 3.57 (s, 2H), 3.67 (s, 3H), 4.78 (s, 2H), 6.73 (d, 2H), 6.86 (d, 1H), 7.03 (d, 2H), 7.30 (t, 1H), 7.47 (s, 1H), 7.66 (d, 1H), 9.56 (br, 1H). ^{13}C NMR (acetone-d_6, 75 MHz) δ 14.69, 26.79, 33.55, 44.12, 52.36, 52.82, 115.93, 119.48, 120.22, 124.75, 129.48, 130.43, 130.40 , 140.86, 143.66, 157.56, 164.82, 168.85, 169.52. IR (neat) 3353, 3022, 2966, 1743, 1644, 1609, 1560, 1222 cm^{-1}. M/S (m/z) 416, 356, 250, 208, 107, 77, 59. HRMS calcd for $C_{21}H_{24}N_2O_5S$ 416.1406, found 416.1408.

Supporting Information Available: ^{1}H- and ^{13}C-NMR spectra of compounds **9b**, **21**, **22**, and **23** (8 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS. Ordering information is given on any current masthead page.

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