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Chemical Reagents in Photoaffinity Labeling

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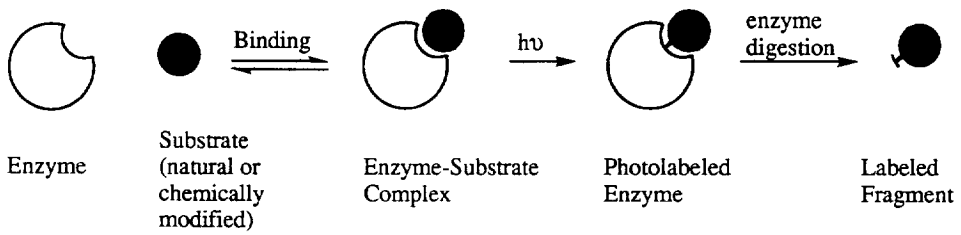
I. Background

A number of techniques have been employed to locate active sites and binding sites for enzyme-substrate complexes. These techniques include X-ray crystallography of the complex, spectroscopic analysis, protein modification to determine critical amino acids involved in binding, affinity labeling, and photoaffinity labeling. This review will address the photoaffinity labeling procedure from the perspective of an organic chemist.

The broad topic of photoaffinity labeling was effectively reviewed by Bayley in 1983.[1] His monograph is the fundamental textbook for those interested in this field. Numerous reviews on narrower aspects of photoaffinity labeling have also been published.[2] Since several important steps are involved in photoaffinity labeling and each one can be a result of specialization within a different area, the topic can be scrutinized from many angles.

This tool is used primarily to increase our understanding of a reversible enzyme-substrate complex as a result of covalent bonding between the enzyme and substrate (see Scheme 1). Thus, one can evaluate the procedure from a biochemical viewpoint by surveying the enzymes, membranes,[3] protein structures,[4] neural receptors,[5] and RNA or DNA structures [6] that have been successfully labeled.

Scheme 1



Photoaffinity labeling typically requires chemical modification of the substrate so that irradiation of the resultant complex will produce a reactive intermediate that will bond covalently to the enzyme. A critical element of photoaffinity labeling is synthesis of the modified substrate, and numerous reports in the literature deal exclusively with this aspect.[7] Usually the substrates have radioactivity incorporated to allow tracking of the tagged binding site. Synthetic methods for introduction of radiolabels[8] and choice of labeling reagent are also important aspects of this technique. On occasion, the substrate may have inherent photoreactivity that allows covalent bond formation in the absence of substrate modification. This type of photoaffinity labeling involving "native" substrates is sometimes termed crosslinking and will also be included in this review.[9]

Photolysis of the modified substrate generates an intermediate whose behavior ought to be understood. A review of reactive intermediates that have been exploited in photoaffinity labeling would need to include known physical data such as lifetimes, excited state characteristics (e.g. singlet or triplet), and reactivities (radicaloid, electrophilic, or nucleophilic). The most important data would be the reaction pathways that typify the various intermediates.

Finally, the covalently labeled enzyme obtained from photoaffinity labeling can be examined by a variety of methods. Such techniques include spectroscopic analysis,[10] fluorophore procedures,[11] visualization by staining,[12] partial and complete hydrolysis followed by fragment analysis (MS,[13] HPLC,[14] etc.[15]), and the most common approach of labeling with radioactive probes.[16]

This review will not attempt to summarize the synthesis of modified or radioactive substrates for photoaffinity labeling nor will it address the techniques for binding analysis. Hopefully, the references that have been provided above will be an adequate starting point for those interested in these aspects of photoaffinity labeling. This review will attempt to describe all the photoactive groups that have been applied to the technique of photoaffinity labeling.

II. Reactive Intermediates

Not all photoaffinity labeling involves substrates that have been chemically modified to become photoactive. Numerous substrates are inherently photoactive, thus do not require functionalization (e.g. sulfides, enones, dienones, and halogenated compounds). The literature also records many examples of crosslinking by use of native substrates. Applications include single amino acids, peptide chains, and nucleotides. Each of these "photoreagents" will be discussed in this section based on its reactivity.

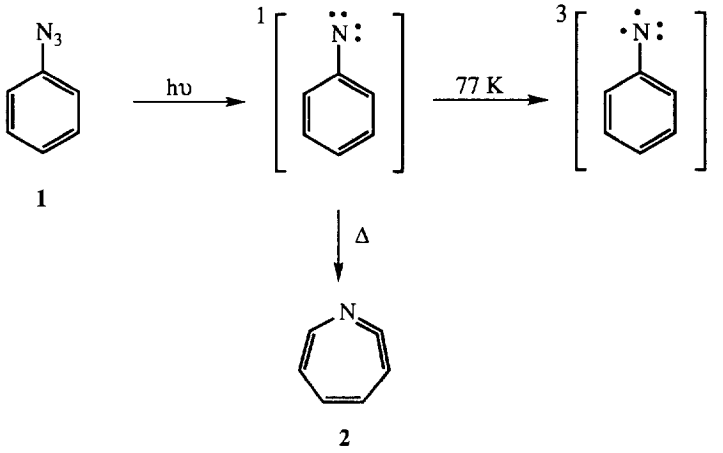
A. *Nitrenes*

Phenyl azides. Photoaffinity labeling with a nitrene intermediate from an aryl azide has been used more than any other procedure. Therefore, the photochemistry of phenyl azide and its subsequent chemistry have been studied in great detail. In spite of the frequent use of azides, it has been pointed out recently that this moiety "rarely gives stoichiometric labeling."[17] In fact, yields of azide photolabeling are generally less than 30%.[18] The few reported exceptions probably result from unusually tight binding[19] or from yields being based on the extent of enzyme inactivation.[20]

One of the most sensible approaches to understanding the effectiveness of a photoprobe has been described by Schwartz.[21] In that report, the relative photoreactivity for aryl azide was found to vary over 10^6 depending on the amino acid that was available for labeling. Cysteine showed the highest reactivity and glycine was essentially non-reactive to the nitrene intermediate. The implications from that work are obvious. If the reactive intermediate generated by photolysis has a long enough lifetime to allow mobility, then its bonding site will reflect the preference of reactant rather than initial substrate location.

Low cross-linking yields in aryl azide applications can result for several reasons. The first, and most probable complication, arises from chemistry that follows irradiation of the aryl azide. Schuster and Platz[22] have recently published a comprehensive review on the photochemistry of phenyl azide. They show that irradiation produces singlet nitrene, which intersystem crosses to the ground state triplet (see Scheme 2). However, the energy barrier for conversion of the singlet to triplet is sufficiently high that at low temperatures, 77 K for example, chemistry from the singlet species can be observed. In general, a singlet aryl nitrene undergoes ring expansion to a ketenimine azepine (e.g. **2**). This compound effectively reacts with available nucleophiles, although its long lifetime and stability are not ideal for rapid reaction.

Scheme 2



1

2

The triplet nitrene is expected to behave like a diradical. The triplet is the ground state of the nitrene and has been termed "totally useless in photoaffinity labeling,"[23] although radicals produced in a confined environment can either recombine or effect useful binding by hydrogen atom abstraction then radical coupling as will be discussed below.

A second possible reason for low crosslinking yields with aryl azides is instability of resultant photoproducts. Direct analysis of photoaffinity-labeled enzymes, which usually requires less hardiness in the new covalent bond than hydrolysis procedures, can be complicated by the alteration of gross enzyme structure after irradiation (especially at 254 nm). Degradation techniques (e.g. Edman degradation) may result in hydrolysis of the photoprobe from the binding site, a net loss of photoaffinity labeling.[18b] For example, amide bonds formed from nitrene reactions are likely to be cleaved by hydrolytic analysis. A thorough examination of photoproduct stability from nitrene reaction with various amino acids,[24] sugars, and nucleotides is lacking.

A related problem may be instability of the probe (radioactive, fluorescent, or staining agent) upon photolysis, an outcome that can lower the measured incorporation of label. This photo-instability should certainly be a concern for use of iodine-125 as a radioactive probe. The iodo-substituted aryl azide can suffer heavy-atom enhanced intersystem crossing to give the less preferred triplet nitrene. More importantly, the iodo aryl azide can undergo aryl-halogen bond cleavage[22] as expected and produce a more mobile radioactive probe (i.e. iodine atom).

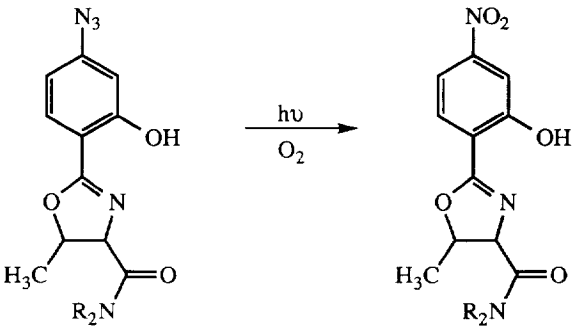
A third complication with simple phenyl azides is the wavelength of irradiation typically used to excite the chromophore. Most of the work has been carried out at 254 nm, a wavelength that can substantially damage biological systems.

Finally, one issue frequently ignored is the stability of the azide group. For example, dithiothreitol and mercaptoethanol are known to be effective reducing agents of the azide, and there is substantial loss of photoreactivity in their presence.[25]

Researchers have evaluated many substituents on the aryl azide that have potential for improving the effectiveness of photolabeling. Nitro, imino, and acyl groups improve the electrophilicity of the resultant

ketenimine (2), red-shift the absorption frequency, and boost the molar absorptivity. All three of these factors make for a better photolabel. Thus, applications that place the photolabeling substrate in nucleophilic environments may be well suited for these aryl azides.[26] Use of the imino group has not been extensively studied,[27] although photooxidation of an azido group has been reported[27a] and is shown in Scheme 3. Since oxygen is rarely excluded from labeling studies, this type of azide deactivation has serious implications.

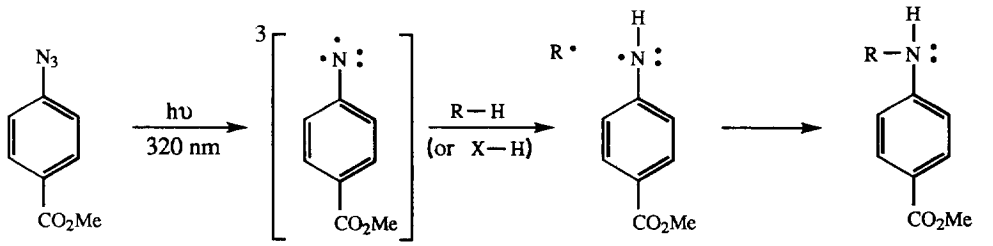
Scheme 3



Interesting comparisons have been found for nitro and for acyl substituted aryl azides. Photoaffinity labeling results from meta and para nitro phenyl azides indicate that meta nitro is more effective than para.[28] An ortho nitro phenyl azide has been synthesized,[29] but the literature is silent about its photochemistry. Photochemical studies on meta nitrophenyl azide indicate that the major pathway at room temperature is formation of singlet nitrene that ring expands (see Scheme 2) rather than undergoing bond insertion with solvent.[30] Support for the ketenimine pathway from this type of azide is found in the report that (4-azido-2-nitrophenyl)sulfenyl chloride covalently bonds to Trp-93 of soybean trypsin inhibitor (the photoproduct was not characterized).[31] If a less nucleophilic amino acid had bonded, then triplet chemistry or singlet bond insertion would have been implicated.

Because of the ease with which acyl groups can be attached to substrates, in addition to the advantages mentioned above, most of the aryl azides used recently have an acyl group.[12,26b,c,32] Earlier work on the photochemistry of para (see Scheme 4) and meta carbomethoxy phenyl azide demonstrated that: 1) the triplet nitrene is formed (based on EPR studies at low temperature) and, 2) the meta azide is more than 10 times less

Scheme 4

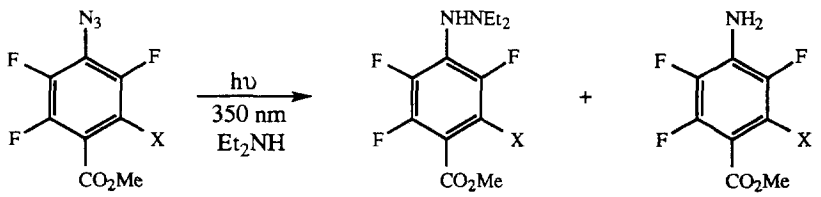


3 4

photoreactive than the para.[33] These results do not favor the desired direct bond insertion, which is likely to be the most universally applicable labeling pathway. In a recent application, a para carboalkoxy phenyl azide derivative of retinal was found to photolabel bacteriorhodopsin with only 7% efficiency.[34] Degradation of the labeled bacteriorhodopsin indicated that labeling occurred primarily at Asn-176 and Arg-175. Both amino acids are relatively non-nucleophilic and lend support to the triplet pathway indicated in Scheme 4.

Recently, considerable interest has been shown for use of fluorinated phenyl azide analogues. Early work on pentafluorophenylazide[35] indicates that both the singlet and triplet nitrene from this precursor can insert into C-H bonds at room temperature. In cyclopentane, 28% insertion occurred to give cyclopentyl substituted aniline. Results[36] from carbomethoxy tetrafluorophenylazide (**5**, X = F, see Scheme 5) irradiation in the presence of a nucleophile such as diethylamine confirm that bond insertion to give the hydrazine **6** (X = F) is a major pathway (60% yield). The mono iodide (**5**, X = I) was much less efficient at insertion with diethylamine, producing **6** (X = I, 24%) and also **7** (X = I, 57%) via a radical pathway similar to that shown in Scheme 4. Whereas the trifluoro analogue (**5**, X = H) gave essentially the same result as the carbomethoxy tetrafluoride, a 63% yield of hydrazine **6** (X = H). The iodo compound (**5**, X = I) is clearly less likely to undergo insertion, presumably due to enhanced intersystem crossing to the diradicaloid triplet nitrene.[37]

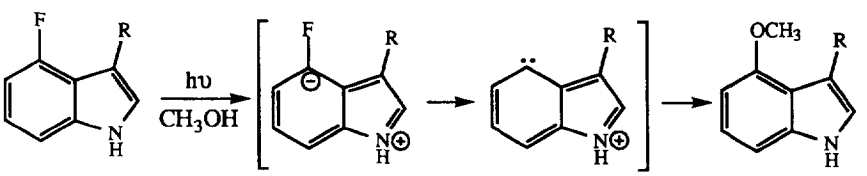
Scheme 5



5 **6** **7**

Products derived from irradiation of the nitrotrifluorophenylazide **5** (X = NO_2) also implicate triplet photochemistry (71% of **7**, X = NO_2). The triplet nitrene may still be useful since diffusion is limited, and coupling of the radicals derived from hydrogen atom abstraction could dominate.[38] Triplet chemistry is also observed with methanol solvent,[39] a behavior that has serious implications for photoaffinity labeling in hydroxy rich regions. Ortho substitution with electron donating groups such as amines or vinylogous amines could result in defluorination upon irradiation (see Scheme 6).[40] This alternative photochemical pathway may not be problematic since it generates a reactive carbene intermediate (vide infra).

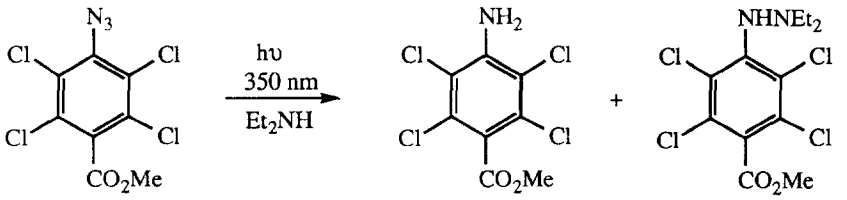
Scheme 6



The number and placement of the fluoro groups in the simple phenyl azide have been investigated.[41] Fluorines ortho to the azide significantly slow ring expansion from the initial singlet nitrene. In fact, the nitrene from 2,6-difluorophenylazide ring expands slowly enough to allow insertion of the singlet nitrene into the carbon hydrogen bond of benzene solvent.

Use of the synthetically more accessible chloro analogues has been evaluated.[42] Photolysis of carbomethoxy tetrachlorophenylazide **8** (see Scheme 7) gave approximately half as much N-H bond insertion as did the tetrafluoro compound (**5**, X = F). The tetrachloride differs from the non-halogenated azide, which gives no insertion and only ring expanded ketenimine followed by nucleophilic attack. A dichlorophenylazide was evaluated for photoaffinity labeling, but unfortunately it did not show sufficient binding affinity to allow comparison to the difluoride.

Scheme 7

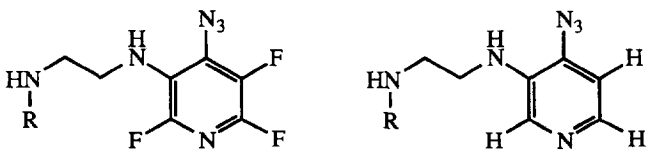


8 **9** **10**

Since use of fluorophenylazides is a recent development in the field, a number of comparisons have been performed with the more traditional non-halogenated azides. Irradiation of an acyl tetrafluorophenylazide in toluene after freeze-pump-thaw cycles (one of the few reports where oxygen was rigorously precluded) gave a 32% yield of benzyl tetrafluoroaniline compared to about 10% for the hydro analogue.[43] In an application with the enzyme ATPase, the tetrafluorophenylazide was found to undergo photochemical cross-linking with 22% incorporation compared to only 8% for the non-fluorinated species.[44] In contrast a recent study of tetrafluorophenylazide on taxol derivatives found that the fluorinated species is a less active substrate compared to the non-fluorinated azide.[45]

A disconcerting result was obtained in the irradiation of the trifluoropyridinylazide **11** (see Scheme 8) which gave less specific covalent bond formation than the hydro azide **12**.[46] An obvious goal for the fluoro substituted applications is for the halide to have little or no affect on binding location.

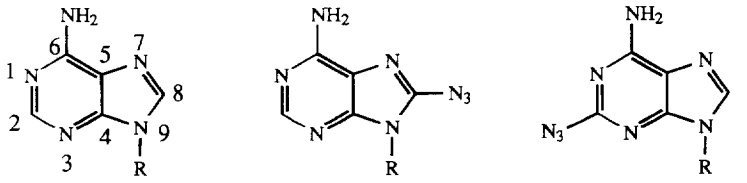
Scheme 8



11 **12**

Purine and pyrimidine azides. Adenosinyl azides comprise a class of nitrene precursors that has seen extensive application. The photoreactivity of the 8-N_3 and 2-N_3 adenosines (see Scheme 9) has been exploited for covalent bond formation in a variety of studies. Azides of other purine and pyrimidine bases have also been

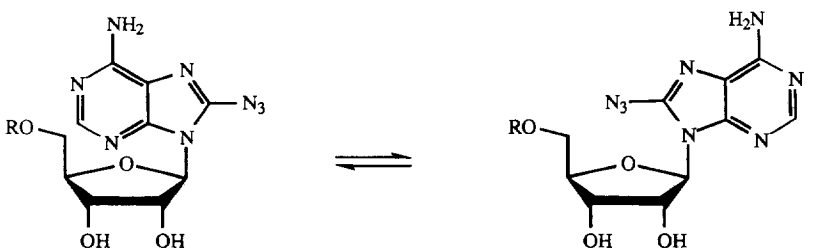
Scheme 9



Adenosine 8-Azidoadenosine 2-Azidoadenosine
R = sugar or phosphosugar

employed. Use of 8-N_3 ATP for photoaffinity labeling was described as early as 1975.[47] One of the first observations with the 8-azido species was that the preferred conformation shifts from anti to syn as a result of the substitutent at the 8 position (see Scheme 10, R = triphosphate).[48] Although the energy barrier between the rotamers is small, there is evidence to indicate that change in conformational preference alters the binding ability.[49]

Scheme 10



Syn Anti

The 8-N_3 ATP (or ADP) does not have a significant absorption above 300 nm. Thus, most of the studies with this photoprobe have been carried out at 254 nm. One recent investigation related to this azide reported a 23% reduction in enzyme activity after two minutes irradiation at 254 nm in the absence of the photoprobe.[50] This indicates the potential for complications due to the use of the higher energy wavelength.

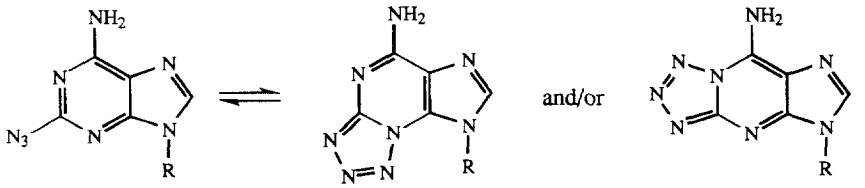
There are a number of examples of very efficient incorporation of the 8-azidoadenosine triphosphate. Julin and Lehman[51] reported a 50% incorporation into the recBCD enzyme of Escherichia coli. Powers-Lee and Corina[52] reported incorporation into carbamoyl phosphate synthetase after irradiation through pyrex (>280 nm). Palczewski and Kochman[53] found that fructose-1,6-bisphosphate aldolase labeling with 8-N_3 ATP gave incorporation primarily at Thr-265 with approximately 45% efficiency and also provided evidence that there is little rotational mobility of the purine in the ATP binding site of this particular aldolase.

The 8-azido c-AMP has been used successfully to label protein kinase and was found to attach covalently to Tyr-196 and Tyr-381.[54] The 8-azido ATP has also been found to bond with Lys-480 of Na, K-ATPase,[55] the Thr-532 or Thr-533 of Ca-ATPase,[56] and Tyr-345 of mitochondrial ATPase.[57] The measured photoincorporation of 8-azidoadenosine can be low as a result of lability of the photoadduct.[58]

The photochemical pathway for this particular azide (regardless of the sugar unit) has not been thoroughly described. It is not known, for example, whether the nitrene undergoes ring expansion followed by nucleophilic attack (by tyrosine, threonine, or lysine) or whether the initial singlet nitrene has electrophilic or nucleophilic character. There is no evidence of a long-lived reactive intermediate.[59]

Applications of photoaffinity labeling with 2-azidoadenosine can also be found in the literature. Several issues are relevant in the use of this reagent. First, the 2-azido compound has been shown to exist in a 1:1 mixture of the tetrazole(s) **13** and/or **14**, as shown in Scheme 11 (R = phosphosugar),[48,60] although, this equilibrium may not interfere with the photochemistry if irradiation drives the tetrazole to the azide as claimed.[49a] The binding efficiency and selectivity of the tetrazole may be altered considerably from that of the azide or the unsubstituted adenosine.[61] Another issue that may be relevant in the use of 2-azidoadenosine is the restricted rotation about the glycosidic C-N bond imposed by the azide substituent at C-2. Unlike the 8-azido, the 2-azido is reportedly forced into the anti, or "normal," conformation.[62]

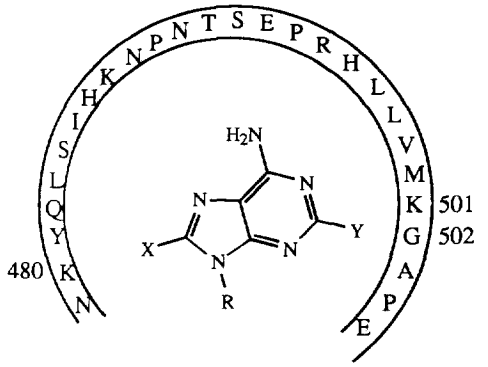
Scheme 11



13 **14**

Comparing the results from the 8-azido and the 2-azidopurine can be informative. In one study, incorporation of the 8-azidoadenosine into tRNA blocked all receptor activity prior to photolysis, whereas the same incorporation of 2-azidoadenosine retained activity and underwent photoaffinity labeling with 11% efficiency.[49a] In another interesting comparison, 8-N_3 ATP gave only 2-3% photoincorporation with skeletal myosin, whereas 2-N_3 ATP underwent 30% cross-linking and was found covalently bound to Trp-130.[49c] A complete study with the two azides of photoaffinity labeling in Na, K-ATPase resulted in the proposed binding sites shown in Scheme 12.[55] This elegant work is impressive because of the total picture that can be drawn. The binding site illustrated in Scheme 12 represents the code letters for the known amino acid[63] sequence from positions 479 to 505 for the ATPase. In addition, the report that 2-azidoadenosine covalently binds to Gly-502 is unique.[61] Controlled nitrene insertions into C-H bonds are relatively rare. Most C-H insertions from nitrenes are the result of long-lived triplet excited states. The mechanistic details for the photochemistry of the 2-azido compounds (Scheme 11, R = sugar or phosphosugar) have not been reported. One could envision triplet nitrene undergoing hydrogen atom abstraction followed by radical coupling to give the glycine-labeled product.

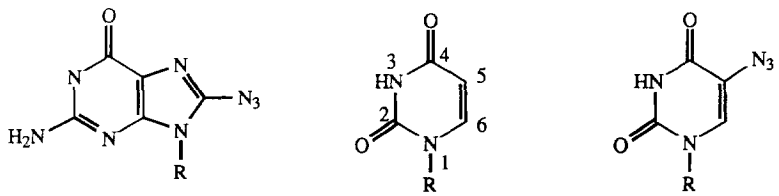
Scheme 12



15 X = N_3 Y = H
16 X = H Y = N_3

Another azidopurine that has been used successfully for photolabeling is 8-azidoguanosine, **17** (R = ribo triphosphate).[7d,64] The azidopyrimidine that has found the most application is 5-azidouridine, **18** (see Scheme 13).[65] This last azide has recently been used to label the following enzymes: glucouranosyl transferase,[66] glucosyltransferase,[67] subunits of β-glucan synthase,[68] sucrose phosphate synthase,[69] and DNA polymerase.[25] RNA polymerase has also been studied with the base azidouridine.[70] This enzyme is found to be labeled by 5-N_3 uridine 5'-triphosphate (UTP) differently than by 8-N_3 ATP, and this result is attributed to either: 1) different binding sites for RNA elongation or 2) different azide orientation within the same binding site for the two RNA

Scheme 13



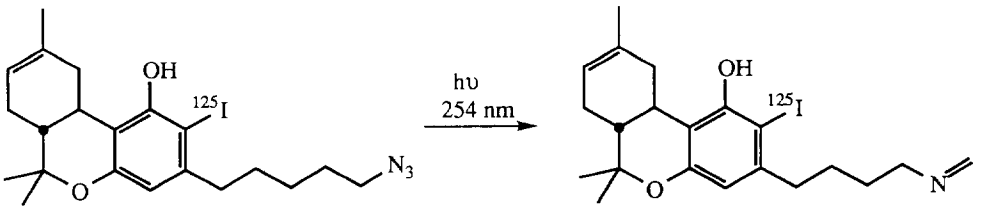
17 **18**

building units. While minimal information is available concerning the photochemistry of uridine **18**; it is known that the covalent linkages formed upon irradiation are relatively labile.[69]

Alkyl azides. Alkyl azides are of limited use due to their relative instability, but a few cases have been reported. For example, the alkyl azide analogue of tetrahydrocannibinol (**19**) shown in Scheme 14 undergoes photoaffinity labeling, albeit with very low selectivity.[71] The explanation offered to account for numerous labeled enzyme fragments focusses on the possibility of multiple binding sites. There was no mention of the lifetime of the reactive intermediate that results from irradiation of an alkyl azide. An azido substituted vitamin D_3 has been reported to photolabel a mixture of proteins with 3% efficiency.[72] Recent work on acyl-CoA demonstrates that 12-azido oleoyl-CoA labels the same oxidase protein as does 12-(arylazido) dodecanoyl-CoA.[73] Irradiation of

alkyl azides has been shown to give products resulting primarily from Wolff-type rearrangement.[74] Therefore, any applications of photolabeling with alkyl azides most likely result from nucleophilic attack on a long-lived imine species such as imine **20**.

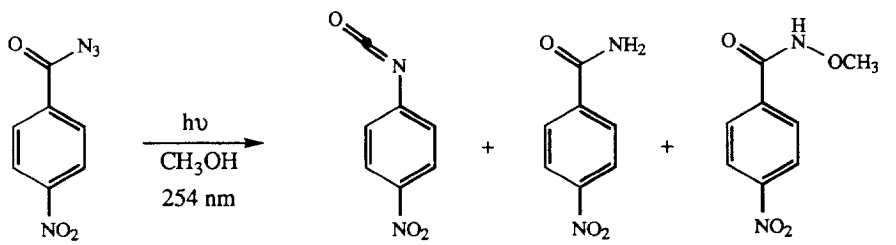
Scheme 14



19 **20**

Miscellaneous azides. Acyl azides have been evaluated for their effectiveness in photoaffinity labeling. In one of the earliest studies, p-nitrobenzoyl azide (**21**) was reported to undergo Wolff rearrangement (30-40%) to give isocyanate **22**, reduction of the azide to give amide **23**, and singlet nitrene trapping with alcohol (or alkane) solvent to give the substituted amide **24** (see Scheme 15).[75] That work was carried out with 254 nm light. In a more recent investigation of acyl azides by Schuster,[76] favorable labeling results were obtained with irradiation at longer wavelength. Unfortunately, m-acetylbenzoyl azide which has considerable potential in photoaffinity labeling has a very low ε value (20) at 350 nm. This reagent should be useful in non-nucleophilic binding sites, although it is unstable in the presence of cysteine. The m-acyl group presumably assists intersystem crossing and avoids the Wolff rearrangement pathway. Consistent with this assumption, recent work on acyl azides indicates that triplet sensitization also precludes the photoformation of isocyanate.[77]

Scheme 15



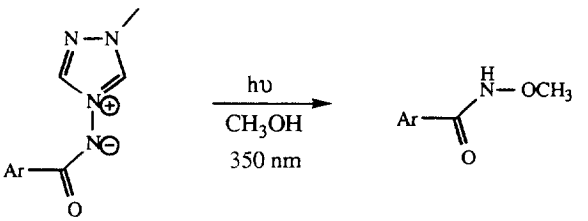
21 **22** **23** **24**

The photochemistry of naphthoyl azide has also been reported.[28] The singlet excited state of the azide gives isocyanate (50%) via the Wolff rearrangement as well as singlet nitrene, which inserts into C-H bonds. The isocyanate is termed "disasterous to photoaffinity labeling" due to its long lifetime coupled with its ability to bond covalently to nucleophiles. In a recent application, the acyl azide was found to bond irreversibly to an estrogen

receptor in the absence of light, then to be released from the bonding upon irradiation at >315 nm.[78]

Aroyl nitrenes can also be produced by irradiation of triazole amide ylides (**25**). Application of this class of compounds has not been reported, although the published photochemical behavior of the biphenyloyl nitrene precursor is promising.[79] No rearrangement pathways have been observed with these reagents. Only bond insertion products such as alkoxyamide **26** were detected (Scheme 16).

Scheme 16

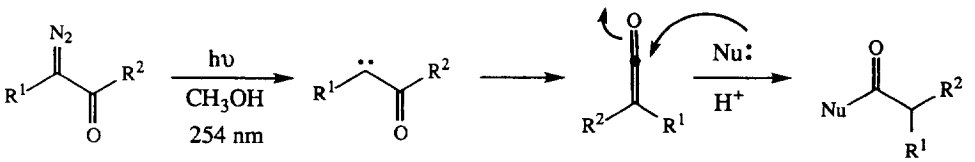


25 **26**

B. *Carbenes*

Diazo group. Westheimer used the diazo moiety as the first example of photoaffinity analysis.[80] The photochemical behavior of diazo ketones **27** is known and frequently the major outcome is Wolff rearrangement (**28** → **29**, see Scheme 17).[11c,81] The derived ketene intermediate **29** is relatively stable and can react selectively with nucleophiles — a dangerous combination for accurate photolabeling. The presence of water, for example, complicates the experiment because it reacts with the ketene to give carboxylic acid **30** (Nu = OH).

Scheme 17



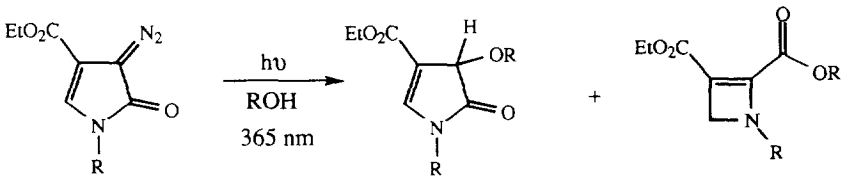
27 **28** **29** **30**

Several insect hormones have been studied through photoaffinity labeling by diazo ketone groups. Winder and Roberts reported a 2% incorporation of farnesyl diazoacetate (**27**, R^1 = H, R^2 = *trans-trans*-farnesyl) into a juvenile hormone after photolysis at 254 nm.[82] However, the incorporation continued to increase after irradiation was stopped; this is strong evidence for the persistence of the less reactive ketene intermediate. In a related report, a diazoketone-substituted peptide produced a ketene that reacted nearly quantitatively with a nucleophile and the resultant acid derivative was not stable to hydrolysis.[83] Furthermore, no carbene insertion was observed. In an

early application of diazo group labeling, lysine was found to form a covalent bond with an irradiated diazoketone.[84]

An interesting decarboxylation reaction (shown in Scheme 18) was recently reported[85] for the substituted 3-diazopyrrolidinone **31**. This reagent may be useful for photoaffinity labeling if the pathway to the ring-contracted product (cyclobutenamine **33**) avoids long-lived intermediates.

Scheme 18



31 **32** 32% **33** 28%

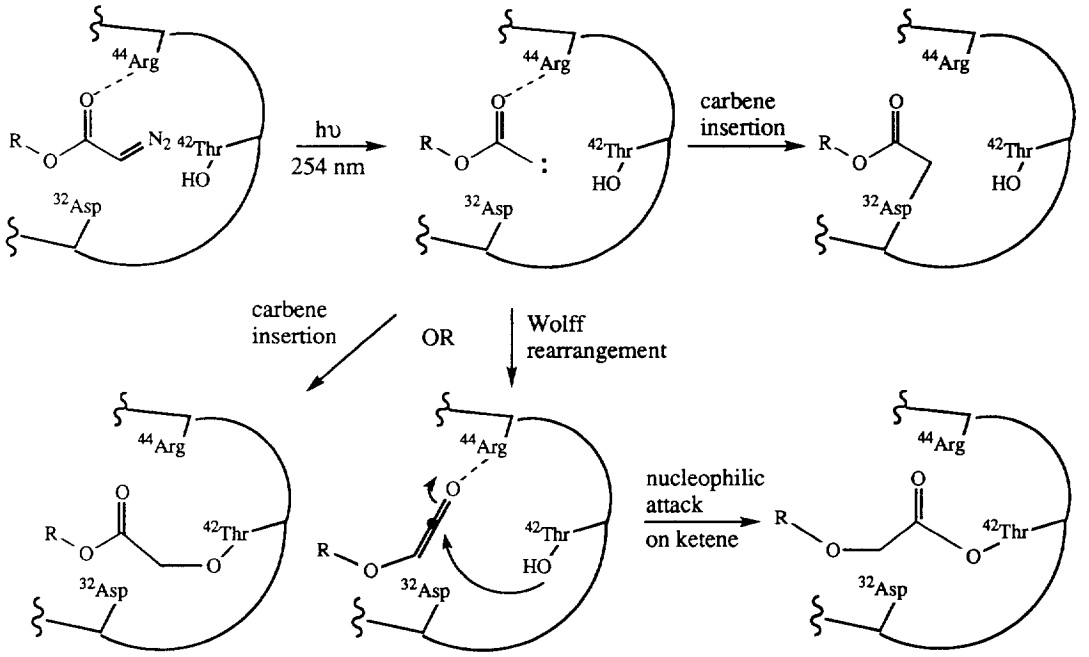
Considerable work with insect juvenile hormone binding proteins has been published by Prestwich and co-workers. The diazo group, because of its small size and relative ease of introduction, is quite often preferred for photoaffinity labeling with alkyl chain substrates. A recent publication describing the use of diazo ketones as the photoactive group demonstrated the importance of chain length and location of the photogenerated carbene on the alkyl chain within the enzyme.[86] If the diazo group is located at point A on an alkyl chain but point A is adjacent to non-reactive groups in the enzyme binding site, then long-lived Wolff rearrangement photoproducts can intervene and ultimately give incorrect information about the binding site location of point A.

In another recent example, Prestwich has shown a likely mechanism for bonding to Thr and Asp by an irradiated diazoacetate (see Scheme 19).[87] In this case the relatively weak nucleophile Asp was presumed to bind via carbene C-H insertion, whereas the Thr hydroxy group either reacts with the carbene or adds to the long-lived ketene.

Several studies have involved diazoacetate substituted retinal.[88] The photolabeling efficiency for this substrate with bacteriorhodopsin is approximately 25%, and the labeling site has been identified as Thr-121 and Gly-122. This is one of the few examples of carbene labeling on a glycine unit.

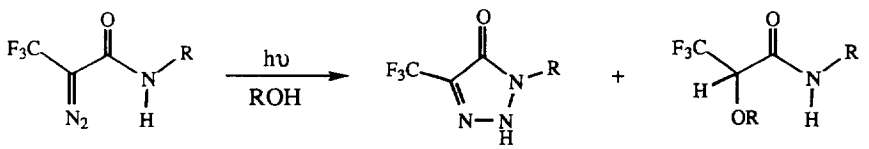
Researchers have noted that diazo compounds substituted with electron withdrawing groups have more carbenoid character and are less likely to undergo the α shift process. For example, Westheimer reported successful C-H photoinsertion of sulfonate-substituted diazo esters, with no competing Wolff rearrangement.[89] But the strong electron-withdrawing trifluoromethyl group was even more effective. The non-Wolff photochemical behavior of the 3,3,3-trifluoro-2-diazopropanoyl moiety (**27**, $R^1 = CF_3$, see Scheme 17) was first reported by Westheimer.[90] Although this reagent underwent reduction when dithiothreitol and 2-mercaptoethanol were used as protein protective agents, thioglycolate was used without adverse effect on the diazo group.[91] In the same study, cysteine was found to cause minimal reduction.

Scheme 19



Attempted photoaffinity labeling with the 3,3,3-trifluoro-2-diazopropanamide group (**34**) resulted in intramolecular cyclization to give the triazolone **35** as shown in Scheme 20 — a general problem with the α-diazo amide moiety.[92] Evidently a thioester linkage precludes the cyclization pathway. Thus, the thio 3,3,3-trifluoro-2-diazopropanoyl ester (**34**, SAr in place of NHR) underwent O-H insertion into methanol,[93] but no C-H insertion was detected for irradiation in toluene.

Scheme 20

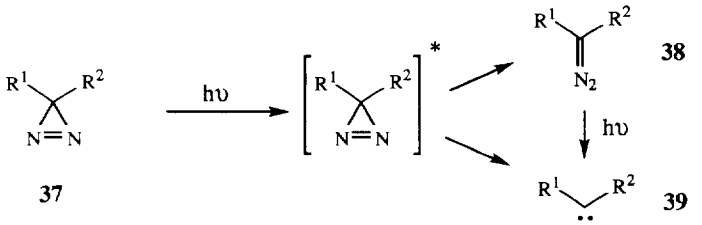


34 **35** **36**

A few published examples demonstrate the utility for photolabeling with a diazo group that is not alpha to a carbonyl (or vinylogous carbonyl). For example, Schuster has reported the photoinsertion of diazofluorene into O-H, S-H, and C-H bonds.[94]

Diazirines. Diazirines were first proposed as potential reagents for photolabeling by Smith and Knowles in 1975.[95] Irradiation of a substituted diazirine (i.e. **37**) has been shown to give carbene **39** and the corresponding diazo compound **38**, which is a longer lived species than **37** at the wavelengths typically used for the three membered ring (360 nm). Although the diazo product usually accounts for > 30% of the photochemical pathway, it can be a carbene precursor also as was described earlier. The interrelationships of diazirines, diazo compounds, and carbenes can be complex and are further complicated by singlet and triplet considerations.

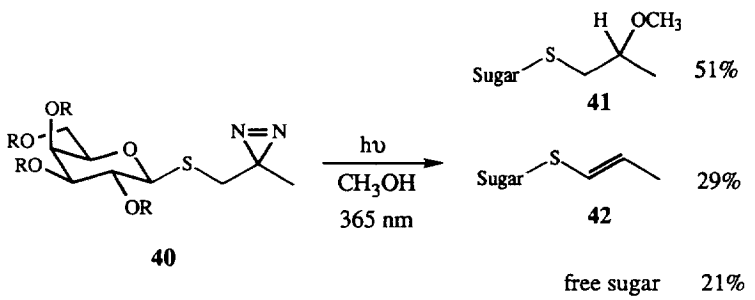
Scheme 21



The diazirine unit is small, non-bulky, and lipophilic. It has a chromophore that extends significantly into the 300 nm range. Thus, many applications of photo cross-linking have been reported for this moiety. Recent examples include labeling of a fatty acid binding protein with diazirinyl substituted sodium octadecanoates,[96] which undergo O-H bond insertion in methanol to the extent of 9-13%. Incorporation into the protein was much less efficient and gave only 1-3% labeling.

Lehmann examined a number of diazirine photolabeling applications and found that the diazirine location on the carbon chain significantly affects the photoreactivity within the binding site.[97] This finding implies that the carbene intermediate is not long-lived enough to discriminate among particular nucleophiles within the binding cavity. Other diazirinyl alkyl chains studied recently (see Scheme 22) have included thiopropyl (**40**) and thiobutyl groups. The resultant carbenes gave the solvent insertion product **41** in addition to 25-30% hydrogen shift to produce thiopropene **42**[98] (or thiobutene[99]) and free sugar from sulfur cleavage. In a labeling study of the maltose binding protein, the aziridinyl substituted thiobutyl system was found to photolabel Asn-12.[100] The corresponding thiopropyl moiety was not as effective due to C-S bond cleavage (shown in photochemical

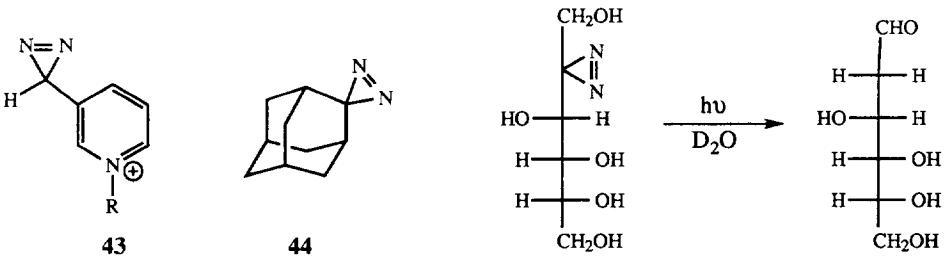
Scheme 22



studies in methanol). Photolabeling of other lipophilic sites by use of aziridinyl steroids has been examined, but the photolabeling proved to be inefficient.[101]

A few studies with miscellaneous substituted diazirines are reported in the literature. A diazirine analogue (**43**, R = spin-labeled adenine dinucleotide) of nicotinic acid photoadds to lactate dehydrogenase with an impressive 17% efficiency.[10b] Other aryl diazirines have been used in different systems and photolabel with lower efficiency.[102] Adamantyl diazirine (**44**) photoincorporated into Cytochrome P450 at a 5-8% level; however, 80% of this caged carbene was captured by water. In that particular study the water capture was informative; but typically, reaction with water is undesired in photoaffinity labeling. Intramolecular side-reactions (other than rearrangement) can also complicate the labeling procedure. Lehmann recently reported[103] that irradiation of diazirinyl hexitol **45** in D_2O results in "oxidation" of the adjacent 1° OH to give **46** with no D incorporation. In this class of diazirines, he reported that all photoproducts arise from intramolecular carbene chemistry.

Scheme 23



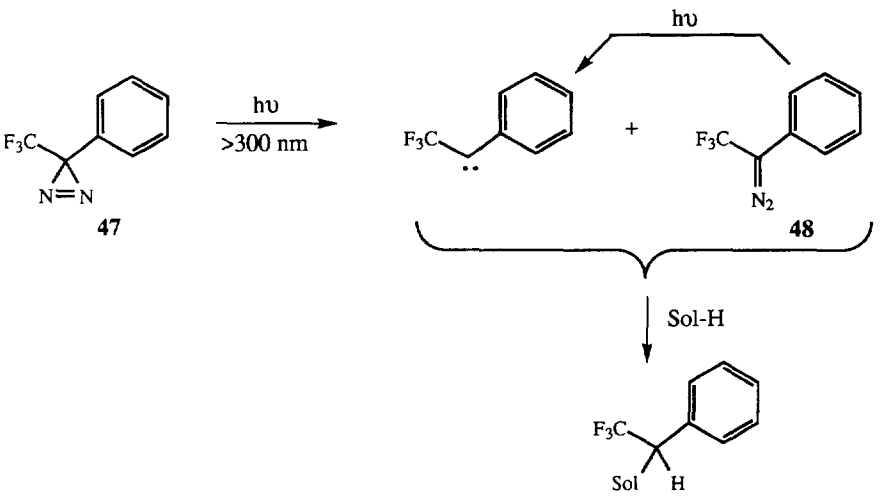
43 **44** **45** **46**

Most of the recent work with diazirines exploits the photochemical reactivity of the trifluoroethyldiazirinephenyl group (**47**), first introduced by Brunner (see Scheme 24).[104] This compound formed >30% diazo intermediate (**48**) upon irradiation. The carbene derived from **47** (or from **48**) gave approximately 50% C-H insertion when photolyzed in cyclohexane (**49**, Sol = C_6H_{11}) or 95% O-H insertion in methanol (**49**, Sol = OCH_3). Insertion into O-H is very efficient and is reported to occur from the singlet carbene,[105] but there is no indication in the literature whether the C-H insertion products found in cyclohexane arise from the singlet or triplet carbene. However, since no radical coupling byproducts were observed,[106] one can reasonably assume that the singlet carbene is the reactive intermediate in this case as well.

Substituents on the aryl ring of **47** have also been examined to a limited extent. A para alkoxy group has been used to link the photoprobe moiety to the substrate, and these reagents (**50**, Ar = p-C_6H_4OR) insert efficiently into the C-H bond of cyclohexane or the O-H bond of alcohol solvents.[107]

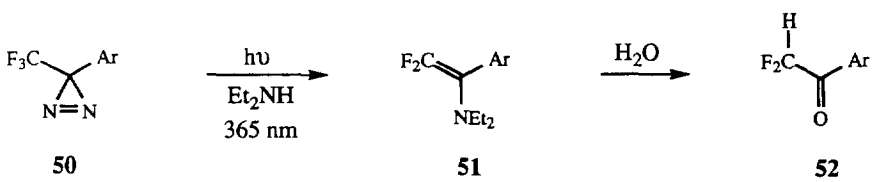
Several complications are associated with use of the trifluoroethyldiazirinyl group. First, the triplet carbene can react with molecular oxygen to give the corresponding ketone.[105] Second, the diazo intermediate, which appears as a significant (>30%) pathway may be photoreactive, but not at the wavelength typically used for the diazirine.[108] Thus, the relatively long life of the diazo compound coupled with its instability to acid could easily result in reactions outside the substrate binding site and consequent false labeling information. Furthermore, Platz and Watt have shown that amine groups react with this particular type of carbene (**50**, Ar = p-

Scheme 24



$C_6H_4CH_3$, see Scheme 25) via initial N-H insertion, followed by loss of HF to give **51**, and hydrolysis to the corresponding ketone (**52**, Ar = p-$C_6H_4CH_3$).[46,106] This pathway represents a net loss of the labeling substrate. Finally, ambient light can also be a significant problem for these very photolabile diazirines.[109]

Scheme 25



In spite of these potential pitfalls, a few significant advantages are associated with this class of diazirines. First, the non-destructive wavelength (>300 nm) absorbed by the reactive chromophore is clearly a selling point. Second, this diazirine is stable with respect to thiol groups.[110] The trifluoroethyldiazirinyl moiety is also stable in mild acidic or basic environments.[111]

Photolabeling of dihydropyridine receptor enzyme with the trifluoromethyldiazirinyl group (**50**, Ar = p-$C_6H_4C(O)NHR$) gave an 8% incorporation.[112] A similar trifluoromethyldiazirinyl benzoyl group (**50**, Ar = p-$C_6H_4C(O)OR$) gave 2% covalent bonding with cytochrome bc1.[113] Another application with the photoactive benzoyl group (**50**, Ar = p-$C_6H_4C(O)NHR$) attached to thymidine dimers gave 15% efficient photolabeling of the dimer binding site of endonuclease by covalent bonding to a Tyr or Thr amino acid.[114]

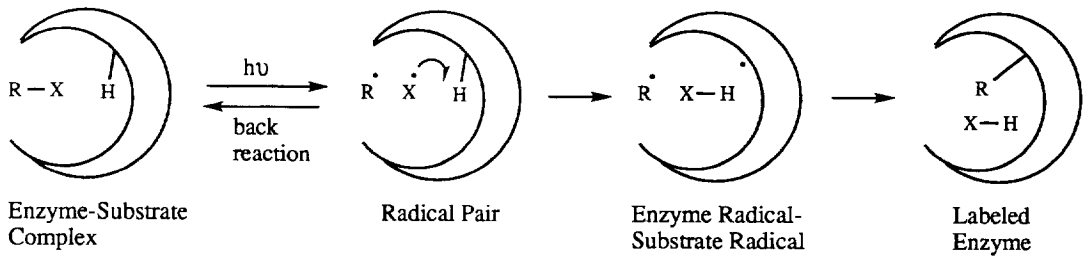
The para(trifluoroethyldiazirinyl)phenoxy moiety (**50**, Ar = p-C_6H_4OR) attached to the 22-amino acid peptide chain of conotoxin resulted in a 2% photolabeling of the electroplax sodium channel.[115] The cross-linking resulted from bonding of the probe to Lys-8. Despite the lower photoreactivity of the meta(trifluoroethyldiazirinyl)phenoxy group (**50**, Ar = m-C_6H_4OR) toward alcohols[107] this meta phenoxy reagent when attached to penicillin analogues has been useful in photolabeling penicillin synthase enzymes. A recent study [116] found an impressive 58% incorporation of this photolabel, and Cys-255 was indicated as the most nucleophilic group available in the binding site.

C. *Radicals*

In general, radical intermediates are ideally suited for photoaffinity labeling. They are known to abstract hydrogen atoms from virtually any available site (see Scheme 26), are more reactive with C-H bonds than are nitrenes, and have less propensity for intramolecular rearrangement than carbenes. But various free radical reactions can minimize the effectiveness of cross-linking including intramolecular hydrogen abstraction (which would leave the substrate unbound) and back reaction between the initial radical pair. Single electron transfer to generate long-lived charged species, can either minimize effectiveness or be conducive to covalent bonding between nucleophilic and electrophilic sites.

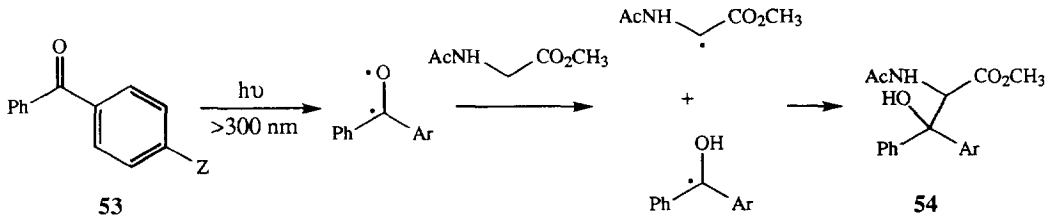
Free radical reactions of photoprobes have been useful in numerous situations, and applications most often touted are those that involve poor nucleophilic environments. Radicals are more dependable than carbenes or nitrenes in their reactions with C-H bonds. A photogenerated radical can hydrogen atom abstract, then non-diffusing radicals can couple to give products that are similar to those obtained by insertion. An additional attractive feature of this class of reagents is their low reactivity toward water and other common solvents.[117]

Scheme 26



Benzophenone. A benzophenone unit is commonly employed for this type of photoaffinity labeling. The first indication that this moiety has a propensity for C-H abstractions was reported by Pitts.[118] Later, Breslow suggested attachment of benzophenone groups for intramolecular functionalization[119] and early studies by Galardy of **53** (Z = H) with glycine indicated that the relatively long-lived benzophenone triplet diradical selectively abstracts an α-hydrogen from amino acid derivatives.[120] The accepted mechanism for photolabeling is shown in Scheme 27.[121] However, one can not rule out charge-transfer between the excited state of the carbonyl and a nearby nitrogen. The ultimate coupled product (**54**) is the same regardless of the mechanistic details.

Scheme 27



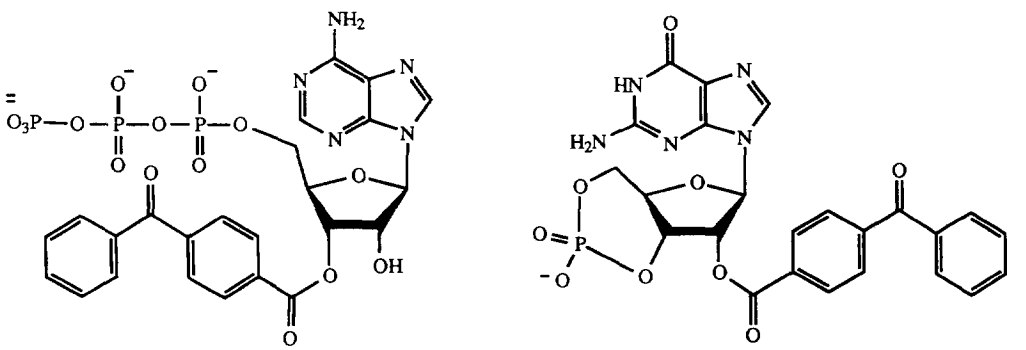
53 54

Prestwich has reviewed the utility of benzophenone as a photoprobe.[122] He reports a reactivity order for H abstraction by the benzophenone triplet diradical as: α to nitrogen > α to sulfur > methine > allylic > methylene > methyl. The relative reactivity ranges were not indicated. However, it is not likely that selectivity problems would arise since in the absence of an abstractable hydrogen in its immediate vicinity the triplet excited state reverts to ground state (i.e. regenerates photoprobe). The rate of H abstraction is on the order of 10^5 $dm^3 mol^{-1} s^{-1}$, and the triplet lifetime in the absence of easily abstracted hydrogen atoms is 300 ns.[123] These features tend to preclude false labeling that would result from diffusion of long-lived reactive intermediates prior to covalent attachment.

Benzophenone (Bz) substituted amino acids are stable under typical peptide synthesis conditions. Thus, several reports of photolabeling with Bz-peptides can be found in the literature. High efficiency covalent bonding is typical in these situations. For example, efficiency of: 70% on substance P receptors of rat membrane;[124] 50% cross-linking of chloroplast coupling factor;[125] 15% covalently bonded rat membrane;[126] labeling of Met-29 or Gly-30 of the β subunit receptor site when included in position 8 of human luteinizing hormone;[127] and an interesting example of double photolabeling by use of a disubstituted peptide to cross-link two sites of farnesyl protein transferase.[128]

Considerable work by Coleman on 3'-O-(4-benzoyl)benzoyl-ATP (**55**, see Scheme 28) has been published.[129] This compound photoadds to smooth muscle myosin with 32% efficiency. The labeling site is

Scheme 28

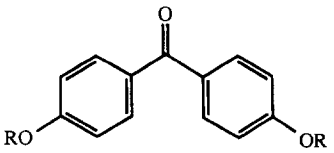


55 56

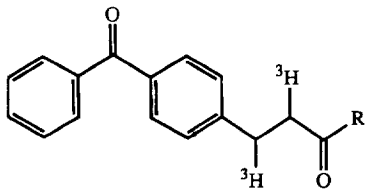
identified as Pro-324.[130] The same compound photolabels ATPase in the β subunit to give 80% inhibition.[131] It also has been used to label the erythrocyte plasma membrane.[132] A recent study with 2'-O-(4-benzoyl)benzoyl-cGMP (**56**, see Scheme 28) gave potent inhibition (but less than 1% labeling) of phosphodiesterase upon irradiation.[133] Attachment of the benzophenone moiety to tRNA results in covalent bonding at two selective sites of the corresponding DNA after irradiation.[134]

Substrates that are more lipophilic than nucleotides have also found application with benzophenone photolabeling. Very efficient labeling (~90%) was obtained in the phospholipid bilayer with 4,4'-dialkoxy long chain substituted benzophenone (**57**, Scheme 29).[135] Prestwich found 4-benzoyldihydrocinnamoyl (**58**) useful as a photoprobe on a variety of substrates. For example, photolabeling of inositol receptors with tritiated benzophenone-linked inositol (**58**, R = $NHCH_2CH_2CH_2O$inositol) gives 50-60% incorporation efficiency.[136] Horwitz has explored benzophenone-substituted derivatives of taxol (**58**, R = taxol) in an attempt to better understand the taxol binding site.[137]

Scheme 29



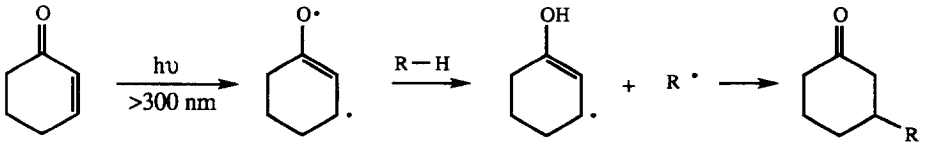
57



58

Enones. Enones have photoreactivity paralleling that of benzophenone. A few applications of enone photolabeling can be found in the literature, and most of these involve steroidal derivatives. A mechanistic analysis indicates that the triplet excited state of the enone (e.g. **59**, Scheme 30) abstracts available hydrogen atoms followed by radical coupling to give **60** prior to diffusion.[138] As with benzophenone, the enone triplet excited state will revert to ground state in the absence of abstractable hydrogens . But unlike benzophenone, the enone has a higher propensity for 2+2 cycloaddition with alkenes or with other enones. The cycloaddition pathway prevails when proximal thymidine groups are available.

Scheme 30

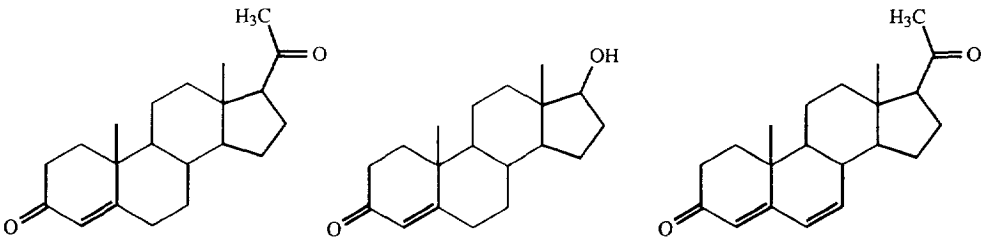


59 **60**

Photoaffinity labeling with unmodified steroid enones should be an accurate labeling procedure since the binding site is not altered by unnatural photoreactive groups. Irradiation of progesterone (**61**, Scheme 31) results

in covalent bonding of a glycoprotein from human leukemic lymphoblastic cell membrane.[139] Testosterone (**62**) photoadds to the steroid reductase protein with 10% efficiency[140] and is found to label Met-139 of the steroid binding site in sex hormone binding globulin.[138] The unsaturated progesterone **63** has been used to label a steroid binding globulin with high efficiency (60-80%), and Trp-371 was identified as the site of binding.[141]

Scheme 31

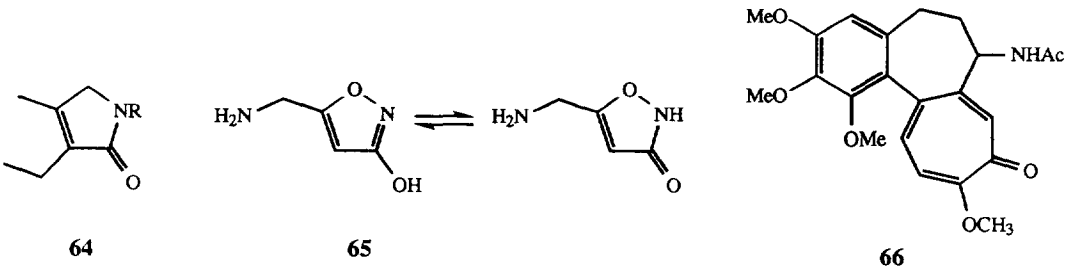


61 **62** **63**

Photocycloaddition with available alkene bonds is a pathway that has been exploited with enone reagents. Psoralen binding sites have been studied by initial irradiation of the enone functional group in psoralen at 390 nm to give a monoadduct with DNA. When this is followed by irradiation at 360 nm, a second photocycloaddition occurs with the matching RNA .[142] Other examples of psoralen cross-linking with strands of DNA or RNA have given impressive cross-linking efficiencies (95%) in the cases of thymidine and uridine.[143]

The literature reports several native substrates that possess inherent photoreactivity and are effective at photolabeling without needing chemical modification. Some of these cases involve enone moieties, and they likely attach covalently at the enone location. For example, the enone of pyrrolidone **64** (Scheme 32) is probably responsible for photolabeling of the sulfonylurea receptor.[144] Photolabeling with muscimol (**65**) may be a result of it having sufficient enone character in a tautomeric form. Incorporation of this last substrate into bovine γ-aminobutyric acid receptor α-subunit occurs with only 1% efficiency, and binding was determined to be with Phe.[145] Native colchicine (**66**) has also been used to photolabel b-tubulin. The photoactivity of colchicine may be due to its enone moiety, although there is little evidence for intersystem crossing to the triplet species, which is the typical pathway for enones.[146]

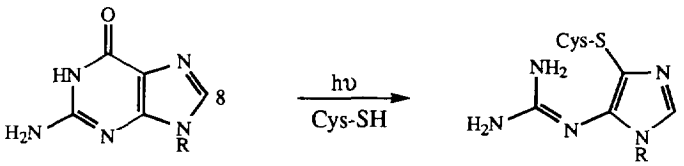
Scheme 32



64 **65** **66**

Photochemical reactivity of the thymidine enone group results in 1-3% incorporation by direct labeling of human c-myc protein.[147] For cytosine there is less consensus about the photochemical pathway, but in 2-propanol its mode of addition mirrors that of an enone.[148] Thus, it is not surprising that cytidine diphosphate (CDP) photolabels the ribonucleotide reductase[149] or that CDP-choline photoadds to cholinephosphotransferase from rat liver microsomes.[150] Recent work on guanosine (**67**) indicates that its photolabeling action gives cross-linking with Cys but not at the 8-position of guanosine as might have been expected based on the reported photolysis of guanosine in alcohol (see Scheme 33).[151] The proposed product **68** is based on spectral data, and its formation can be explained by initial enone excitation.

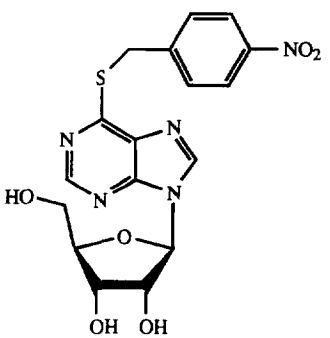
Scheme 33



67 **68**

Sulfur radicals. Sulfur compounds have been used in photoaffinity labeling. Nitrobenzylmercaptopurine riboside (NBMPR, see Scheme 34) undergoes efficient incorporation with the membrane nucleoside transport enzyme.[152] The reactive pathway results from initial benzyl-sulfide photocleavage (see Scheme 35).[153] In the

Scheme 34

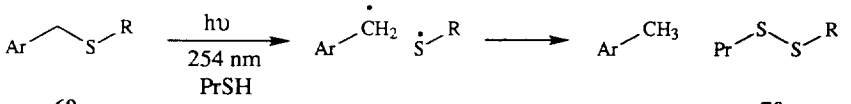


NBMPR

absence of abstractable hydrogens the sulfur and benzyl radical pair should combine to regenerate the probe molecule thereby avoiding side reactions and improving the labeling selectivity. Otherwise, the sulfur radical couples with the radical formed in the binding site (see earlier Scheme 26). The disulfide **70** derived from solvent radicals has been isolated and characterized as the major primary photoproduct from NBMPR irradiation. A study of substituents on the benzyl ring established that the para-nitro group is not necessary for the benzyl-sulfide

cleavage, although it has the advantage of extending the chromophore significantly above 300 nm.[154] A related compound, benzylthioimidate, has been reported to be a photolabeling agent.[155] Presumably, it is photoactive via the same benzyl-sulfide bond cleavage mechanism.

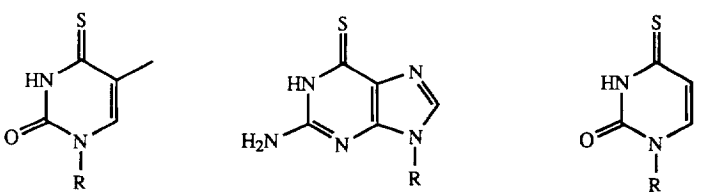
Scheme 35



69 **70**

Other studies of sulfur photochemistry include thiothymidine (**71**, see Scheme 36), thioguanosine (**72**),[156] and thiouridine (**73**).[157] Moderate photolabeling efficiencies (5-40%) are typical for a thione group substituted in an oligonucleotide and comlexed with its binding partner tRNA or protein. At least for thiothymidine, the photoaddition parallels the enone behavior of thymidine (i.e. dimerization). These thio derivatives behave quite differently when irradiated in the presence of oxygen.[157] Sulfur oxidation occurs and is followed by nucleophilic displacement, which may have practical applications in photoaffinity labeling.

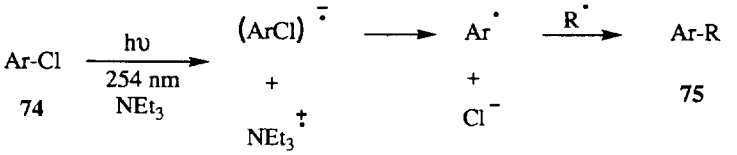
Scheme 36



71 **72** **73**

Halogenated substrates. Notable examples of photoaffinity labeling by use of halogenated substrates are described in the literature. Dioxin (2,3,7,8-tetrachlorodibenzodioxane) and related brominated analogues photoreact with binding sites in aromatic hydrocarbon receptor with 30-40% efficiency.[158] These high efficiencies are not observed with dioxin derivatives bearing photoactivatable groups such as the azide. The chemistry in these reactions presumably involves photo-cleavage of aromatic halogen bonds to form radicals, presumably via electron transfer from adjacent amines or sulfides (see Scheme 37). Solution

Scheme 37

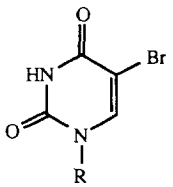


74 **75**

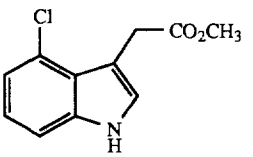
photochemistry on polyhalogenated aromatic compounds (**74**) has been reported[159] and is consistent with this assumption; but there is no direct evidence for formation of radical coupled products (e.g. Ar-R) in photoaffinity labeling experiments.

5-Bromouridine (**75**, R = deoxyribose, see Scheme 38) has been employed for photoaffinity labeling.[160] Irradiation of this compound at 254 nm for 90 s results in 28-37% crosslinking with paired DNA. The mechanism presumably involves initial radical cleavage of the carbon-halogen bond followed by H-atom abstraction from the C2' position of the opposing strand. Similar photochemistry is observed for 4-chloroindole, (**76**) which photocleaves to generate a radical that abstracts solvent hydrogen atoms, as shown by isotope studies.[40] Amiloride (**77**) is a chloro substituted dihydropyrazine that photolabels the hydrogen ion-sodium antiport protein with 15% incorporation.[161] The photochemical pathway likely involves initial halogen cleavage in this case also.

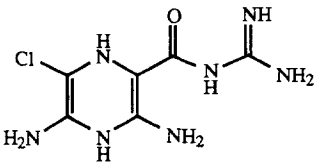
Scheme 38



75



76

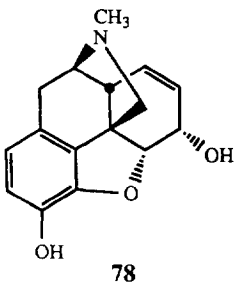


77

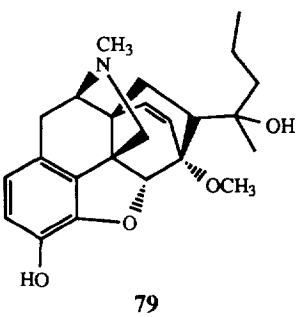
Interestingly, the most common application of halogenated photolabeling may occur inadvertently during use of iodo substituted aryl azides. Many studies have exploited the radioactivity of I-125 and the synthetic ease of its introduction onto aryl azides. As was indicated earlier, the aryl-iodide bond is not stable with respect to irradiation at 254 nm.

Miscellaneous radical reactions. Several non-modified substrates with aromatic groups have been effectively photoincorporated into binding sites. Irradiation of the opiates etorphine (**78**, see Scheme 39) and morphine (**79**) generate radicals that label brain opioid receptor with yields up to 86%.[162] The mechanism of photolabeling is not well established, although it is known that peptide opioids do not photolabel. The diethyl

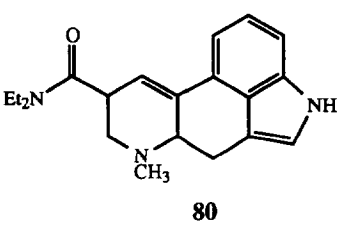
Scheme 39



78



79



80

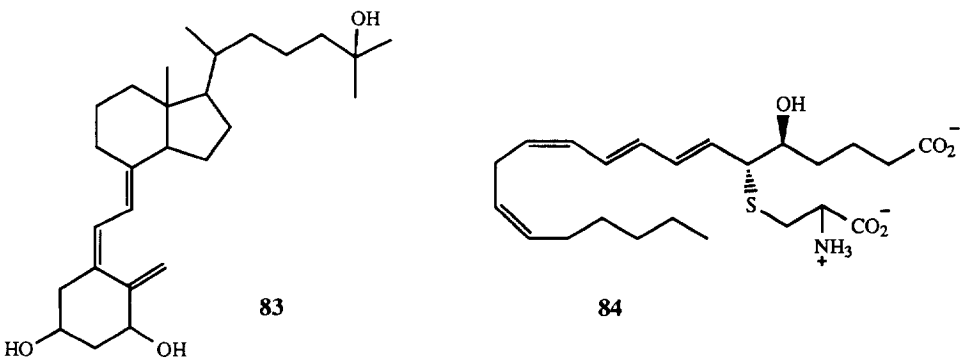
amide of lysergic acid (LSD, **80**) gives low incorporation into the serotonin receptor, and there is evidence that photoaddition occurs at the styrenyl double bond.[163] A logical pathway in an enzyme environment is initial excitation of the styrene moiety (**81**, see Scheme 40) followed by single electron transfer from a nearby amine. After protonation of the radical anion, radical coupling could give the covalently labeled substrate **82**.[164]

Scheme 40

81 **82**

Dihydroxy vitamin D_3 (**83**, see Scheme 41) has been studied in direct labeling experiments. On irradiation the triene inactivates the vitamin D receptor protein to the extent of 80%.[165] The triene chromophore is similar to that in leukotriene **84**, which also undergoes photolabeling.[166] Recent work on trienes and other polyenes implicates a triplet radicaloid intermediate and a singlet zwitterionic pathway, which are both available from the initial excited state. Solvent polarity (or enzyme environment) can alter the preferred path taken by the polyene.[167] Although the details of the photolabeling reaction have not been ascertained, the leukotriene at least has no triene moiety after irradiation.

Scheme 41



83 **84**

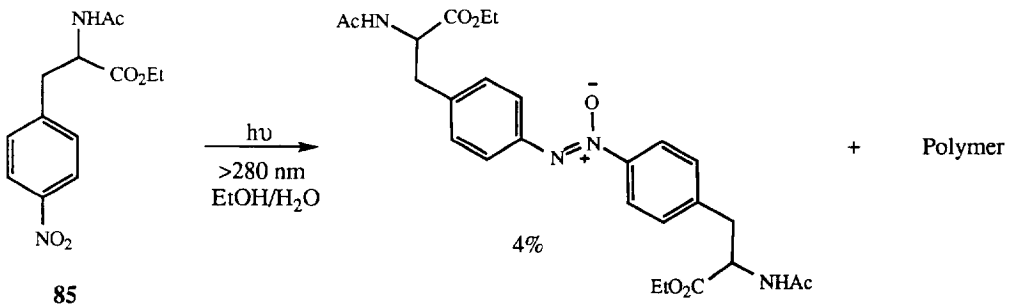
Photolabeling through irradiation of native peptides has found limited success. This approach is dependent on UV absorption by amino acid groups under conditions where other biological systems will also be excited and undergo photochemistry (e.g. thymine-thymine dimerization). The chemistry responsible for crosslinking with peptide chains is not known, although aromatic and thiol groups (phenylalanine, tyrosine, tryptophan, and cystein) have been implicated.[168] Although this class of photoaffinity labels was included earlier under the heading of free radical intermediates, there may be alternative mechanistic explanations for the observed chemistry.

Examples of crosslinking peptides include glucagon, bungarotoxin, and vasopressin. Glucagon, a 29 amino acid peptide, photoinserts into the glucagon receptor with ~1% efficiency.[169] The atrial natriuretic polypeptide gives 7.5% crosslinking at 365 nm. Oxygen and lipids are necessary for labeling, and a radical mechanism is assumed even though the radical scavenger dibutylhydroxytoluene (BHT) had no effect on photolabeling.[170] Bungarotoxin, a 74 amino acid neurotoxin, gave less than 5% covalent bonding with an 8 kilodalton protein when irradiated at 254 nm.[171] Vasopressin, a nine amino acid peptide antidiuretic, photolabels the human platelet receptor with much higher efficiency (~50%) than most of the direct irradiations, perhaps because the study was performed with 365 nm light.[172]

D. *Carbon Electrophiles*

Nitrobenzene. Substituted nitrobenzenes represent one of the more common photoaffinity labeling probes. These compounds have a triplet lifetime on the order of 10^{-7} to 10^{-9} s and are not likely to migrate away from the binding site before reacting.[173] Nitrobenzenes with no leaving group (-OR, -X) on the ring (e.g. **85**), however, are not very reliable as photolabeling reagents, although they have potential as DNA cleaving agents.[174] A common photochemical outcome is formation of polymeric material via a radical pathway (see Scheme 42).[174a]

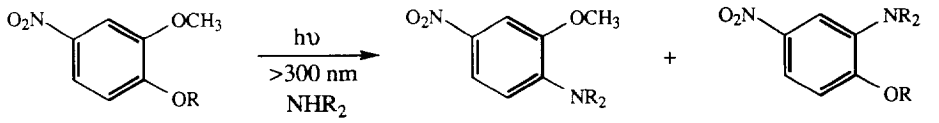
Scheme 42



85

Marquet and Moreno-Mañas have thoroughly studied photosubstitution of dimethoxynitrobenzene by amines. They describe the photoreaction with amine nucleophiles to give amino nitrobenzenes as a type of nucleophilic aromatic substitution.[175] Based on their work, it is apparent that the alkoxy group meta to nitro is preferentially displaced when methylamine or butylamine is the nucleophile, and this outcome is consistent with earlier findings.[176] Unfortunately, the para alkoxy group is selectively replaced by morpholine. Thus, if a nitrobenzene moiety (i.e. **86**, see Scheme 43) is used for labeling, then irradiation may give covalent bonding at the para site (**87**) or alternatively at the meta site (**88**) with substrate still attached. A significant drawback is that thiols, which react with the excited state nitrobenzene 20 times faster than do amines, quench the excited state and do not result in the formation of thioethers.[173] Further work is needed with regard to the thiol photoproducts that are formed.

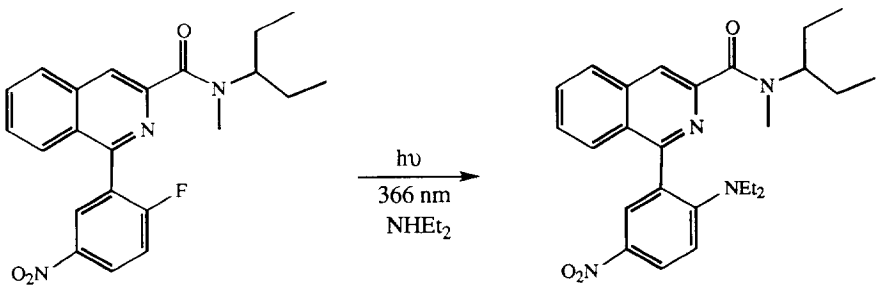
Scheme 43



86 **87** **88**

Nucleophilic displacement of fluoride from the para position of nitrobenzene is possible, and a para-fluoronitrophenyl isoquinoline (PK 14105) has been used to photolabel agarose with 50% incorporation,[177] to label a cardiac membrane with 80% efficiency,[178] and to label a variety of benzodiazepine acceptors with good efficiency.[179] In an excellent analysis of the photochemical behavior of PK 14105 (**89**), Doble et al.[178] verified nucleophilic aromatic substitution as the reaction pathway (see Scheme 44) by identification of the photoproduct **90**. A related fluoro compound, flunitrazepam, has also shown photolabeling ability. It likely reacts via nucleophilic attack to displace a fluoride or an amide group.[180]

Scheme 44

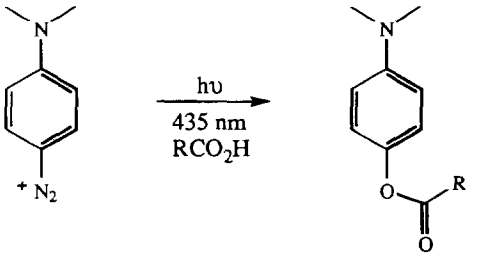


89 **90**

Diazonium salts. Aryldiazonium salts have been used to photolabel sites that bind cationic groups such as acetylcholine. Hirth has described the photochemistry of benzene diazonium ion in aqueous environment, which produces phenol, presumably via water capture of the intermediate carbocation.[181] The lifetime of the diazonium species in water is less than 500 ps.[182] Other weak nucleophiles have also been reported to photoadd to para-dimethylaminophenyldiazonium ion (**91**). For example, carboxylic acids (see Scheme 45) give aryl esters (**92**) and amide nitrogens give aryl amides.[183] This diazonium salt photolabels a nicotinic receptor with 60% efficiency; and subsequent enzyme digestion allowed identification of Cys-192 and Cys-193 as the labeled amino acids.[184] The same reagent covalently binds to Tyr-93 of the acetylcholine receptor.[185]

An N-substituted para-amidophenyldiazonium ion has been useful to probe polyamine binding location on DNA. In this study the diazonium chromophore was attached to the polyamine of interest. Irradiation of the modified polyamine substrate-DNA complex resulted in DNA cleavage.[186] The mechanism of photoreaction for this particular diazonium species has not been reported.

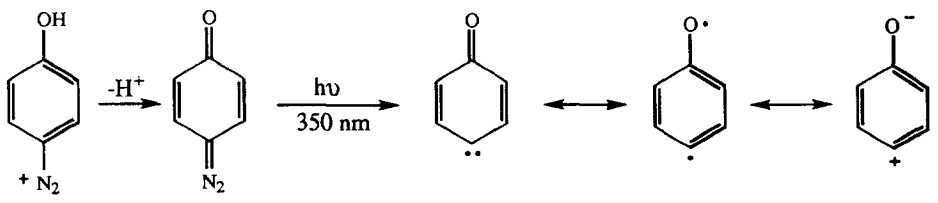
Scheme 45



91 **92**

An interesting variation on aryldiazonium ion photoreagents is the para-hydroxy analogue which can form **93** (see Scheme 46) under basic conditions. Loss of N_2 from **93** generates a species that can behave as a carbene (singlet or triplet), a radical (diradical resonance structure of the triplet), or a carbocation (dipolar ion resonance structure). Goeldner and Hirth have explored the photochemical behavior of these compounds.[187] A reported application of labeling with acetyl cholinesterase[188] indicates that this diazo/diazonium dichotomy is useful. Photolabeling of γ-aminobutyric acid (GABA) receptor gave 35% inhibition by use of a substituted diazocyclohexadienone similar to **93**; and the reaction pathway was described as carbenoid on the basis of observed insertion into the O-H bond of methanol,[189] although a carbocation intermediate should not be excluded since it would also give the alkoxyphenol product.

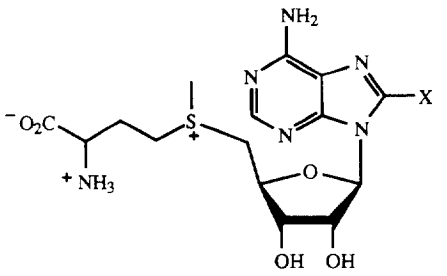
Scheme 46



93

Sulfonium. The literature records several examples of S-adenosylmethionine (AdoMet) photolabeling (see Scheme 47).[190] This natural substrate has been used primarily in crosslinking of methyltransferases. No mechanism is reported for the photochemical process, although there is reason to believe that it involves the sulfonium moiety. Earlier work compared the native AMP and the AdoMet photolabeling.[191] The photoreactivity of these two reagents was sufficiently different to lead to the conclusion that AdoMet probably does not react at any adenosine atom. In the case of 8-azido-S-adenosylmethionine (**95**), potential for labeling due to photochemical cleavage at the sulfur rather than nitrene formation upon irradiation has been suggested.[58] Sulfonium salts are known to undergo photoheterolysis to generate cationic intermediates via direct bond cleavage or via single electron transfer.[192]

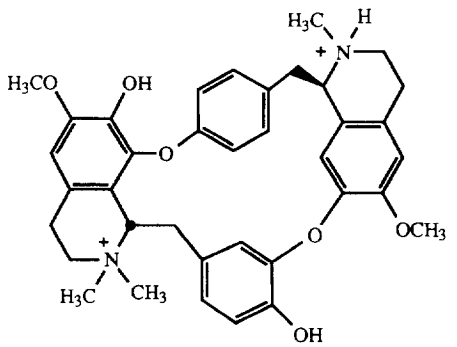
Scheme 47



94 X = H
95 X = N_3

Ammonium. Tubocurarine (**96**, see Scheme 48) has been used to label acetylcholine receptor subunits but with very low efficiency (0.1-0.4%).[193] The photoactive group has not been identified, although it is likely that photochemical cleavage of a benzyl-ammonium bond gives carbocation and, perhaps, radical intermediates.[192,194]

Scheme 48



96

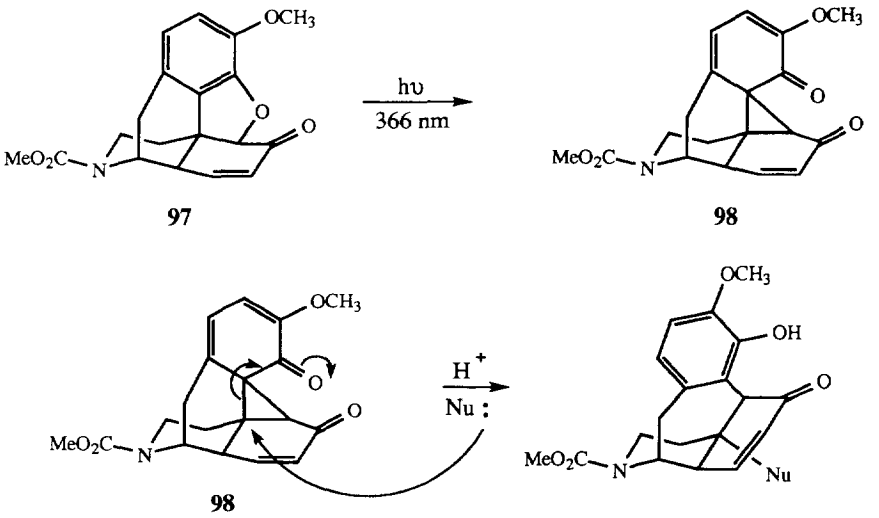
Phosphonium. Photolabeling of the acetylcholine receptor by methyltriphenylphosphonium salts with an efficiency of 1-2% has been reported.[195] The mechanism of labeling is not known, however carbocations from initial heterolytic photocleavage are likely involved.

Miscellaneous cationic reactions. Irradiation of codeinone (**97**, see Scheme 49) results in covalent bond formation with nucleophilic species.[196] Irradiation in benzene gives no reaction. The mechanism proposed for the capture by nucleophiles involves the spirocyclopropyl ketone intermediate **98**, which can become aromatic upon nucleophilic addition as shown. It isn't clear that the chromophoric group in **97** has potential as a general labeling moiety.

III. Comparisons

To obtain accurate information about binding sites in enzymes from a photoaffinity labeling experiment, it is imperative that: 1) the covalent bond occurs at the binding site; 2) the efficiency is sufficiently high to warrant conclusions about the binding locale, especially if the wavelength of excitation (e.g. 254 nm) can alter the

Scheme 49



macromolecular system independent of substrate; and 3) any chemical modification of the substrate so it can serve as a photolabel will have no impact on the binding site.

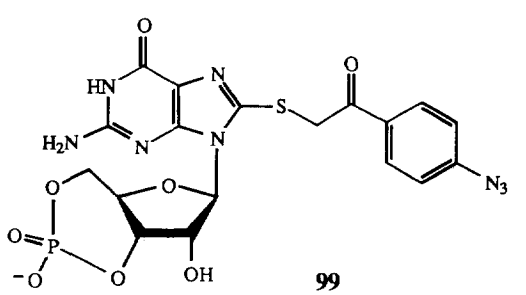
Any attempt to compare the merits of various reagents or functional groups is likely to give mixed signals. Owing to the vast differences in the types of intermediates the photoactivated species can produce (e.g. nitrene, carbene, radical, carbocation), one could get superior labeling with an inefficient reagent as a result of the make-up of the binding site. In spite of this, comparisons have been made and are worth noting.

A. *Azides.*

Several types of aryl azides have been useful for photoaffinity labeling. A tetrafluorinated aryl azide was found to be more effective than the corresponding protio aryl azide in labeling the phencyclidine receptor.[52,1] But another study of the aryl azide and the tetrafluoroaryl azide found that there was no significant difference. In an similar study, labeling of the estrogen receptor with a modified non-steroidal substrate gave similar inactivation regardless of the fluoro substitution.[35b]

The para-azido aryl (**99**, see Scheme 50) is more successful than 8-azido cGMP (**17**, R = ribose cyclo-3',5'- monophosphate, see Scheme 13) for photolabeling of retinal rod channel. The lack of labeling by

Scheme 50



99

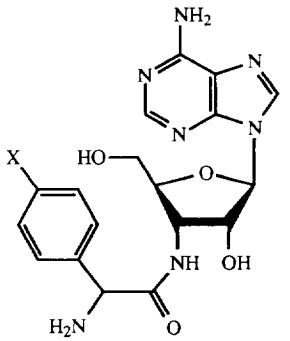
azidopurine **17** might be explained by differences in binding rates between the two substrates.[18a]

B. *Azides vs others.*

Since azides are the most popular reagents for photolabeling but have several drawbacks (vide supra), most comparisons are between the azide group and alternative choices.

If the natural substrate is photoactive there should be obvious advantages to its use for photoaffinity labeling. A few comparisons have been made between such direct irradiations and use of analogous aryl azides. For example, the labeling results with puromycin (**100**, see Scheme 51) and p-azidopuromycin (**101**) are reported as being the same. This finding implies that the azide is not involved in the covalent bond formation.[198] A summary of RNA-protein labeling studies that involved "direct" versus "aryl azide" methodology has also been published.[199]

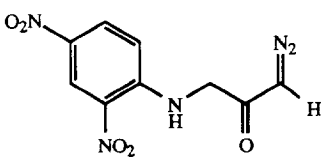
Scheme 51



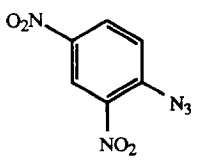
100 X = OCH_3
101 X = N_3

Azides have been compared to diazoketones in a number of studies. A complete analysis has been published for labeling enzymes, RNA, or DNA with para-azidoaniline, para-azidobenzoyl, diazoketone, or vinylogous diazoketone moieties. For each system studied, superior labeling efficiency was observed with the para-azidobenzoyl group.[200] Consistent with this work, recent comparisons of simple aryl azides to diazoketones report that non-acyl phenyl azides are less effective.[93,201] In one of the earliest contrasts, the diazoketone **102** (see Scheme 52) was found to label the same position of protein 460 as did the phenyl azide **103**. Both reagents became covalently linked to Lys-54.[84] Of course, efficiency of binding for the modified reagents may be the major issue in performance of the photoaffinity group.[202]

Scheme 52



102



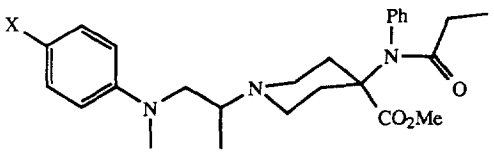
103

Comparisons between azides and diazirines have been published. The trifluoromethyldiazirinyl benzoyl unit reportedly gives a higher labeling efficiency than the para-azidobenzoyl moiety based on inactivation of the MSH receptor.[203] Another factor in the contrast between the carbene and nitrene insertions is that the nitrene-derived products are less stable to acidic hydrolysis, and this aspect may be problematic in subsequent binding-site analysis. Not all studies favor use of a diazirine. Early work on lipids and gramicidin A found that aryl azides and aryl diazirines both photolabel tryptophan, but the azide was more efficient presumably due to the longer lifetime of nitrenes over carbenes.[204]

A report of aryl azide, diazo ketone, and diazirine in the deactivation of the estrogen receptor indicates that diazirine is the most efficient at photo-inactivation of this particular enzyme.[98] The authors point out that "the attachment efficiency of a photoaffinity labeling agent is rarely equal to its photoinactivation efficiency, since many processes other than covalent attachment may contribute to the loss of exchangeable sites." If identification of the binding site is desired, then the attachment efficiency is more important than the inactivation efficiency.

There are two recent comparisons between aryl azide and aryl diazonium reagents. The size of these photoactive groups is similar, but their usable wavelengths of excitation differ significantly. In a study of photoactivatable peptidoleukotriene analogs, the aryl diazonium derivative gave only one photoproduct whereas the aryl azide analogue gave multiple products that indicate alkene reactivity.[205] This complication undoubtedly arises from the 254 nm wavelength needed to excite the azide compared to the less destructive >300 nm needed for excitation of the diazonium species. In an earlier study by Goeldner and Hirth, the azide of carfentanil (**104**, see Scheme 53) was found to be much more effective than the diazonium derivative (**105**).[206] In fact the latter compound proved completely ineffective at photolabeling.

Scheme 53



104 $X = N_3$

105 $X = N_2^+$

One of the first contrasts between azide, diazonium, and nitrophenyl in the photolabeling of pepsin found the azide to be the most efficient. The nitrophenyl case suffered from the length of time needed for photoreaction and the resultant enzymatic damage that took place.[207]

Finally, a comparison between azides and radical reagents (e.g. benzophenones or enones) is available for a few systems. The frenzied interest in taxol has prompted study of several labeling reagents in order to determine the tubulin binding site. A recent report indicates that an azide photolabels more efficiently and more selectively than does a benzophenone.[137a] In a very early paper, an aryl azide was shown to have nearly the same photobinding efficiency on albumin as did benzophenone. A slightly more effective reagent in this study contained the 4-azido-2-nitrobenzoyl moiety.[118] A tetrafluorophenylazide and an enone photoaffinity labeling group have been compared. The fluoroazide did not undergo labeling or C-H bond insertion.[208]

It is often fruitful to combine labeling information derived from different photoactive groups. For example, the trifluoromethylphenyldiazirine moiety attached to the ribose unit of GTP and the phenyl azide moiety

attached to the γ-phosphate of GTP allowed identification of different eukaryotic initiation factor (eIF-2) subunits as binding sites for the two portions of GTP.[209]

IV. Conclusions

Photoaffinity labeling can provide significant information about binding sites in a wide range of supramolecular systems. Knowledge of the relevant photochemical mechanisms is extremely important in order to understand labeling efficacy and selectivity. Researchers have pointed out: "as long as the mechanisms of photoaffinity labeling are not known, one can only speculate"[137] concerning the selectivity of labeling.

This review has not intended to dismiss any approach or to discourage the use of any particular reagent that has been discussed. The advantages and disadvantages should be carefully weighed in each application, since many variables play a role in the efficiency and selectivity of photolabeling. Clearly, there is room for much improvement, both in the types of reagents and in understanding the photochemistry of those that have proven to be useful.

That the field of photoaffinity labeling is immense and is growing rapidly are testaments to the utility of this analytical procedure. Although this Report did not include all applications of photoaffinity labeling, it did attempt to present all classes of reagents that have been used with emphasis on the most recent applications.

V. Acknowledgements

I appreciate the initial work by Dr. Anton Jensen and the literature search by Roger Flick. Proof-reading by Susan Ward, Cara Bradford, Professor Fred Lewis and Professor Gary Schuster is also acknowledged. Editorial advice from Professor Alex Nickon is greatly appreciated. This review is dedicated to Melissa.

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