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Synthesis and Applications of Tritiated Affinity Probes

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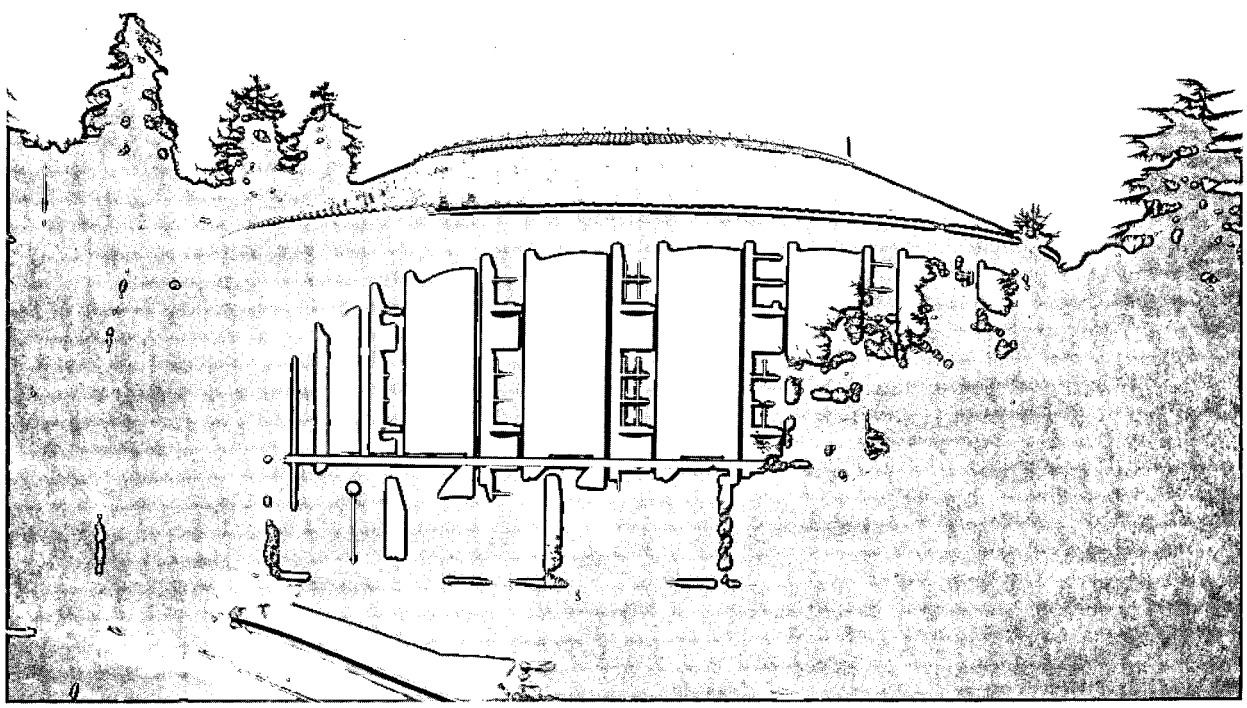

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Chapter 2

Synthesis and Applications of Tritiated Affinity Probes

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CONTENTS

I. INTRODUCTION

Affinity labeling by definition applies to all molecules possessing a site that binds another molecule with a certain degree of specificity and affinity. In biology the proteins are the obvious ligand-binding molecules, and in practice the affinity labeling techniques have involved primarily proteins. Therefore, affinity labeling is a technique for selective, covalent labeling of binding sites in macromolecules and the most significant contribution of affinity labeling methods to date have been in the elucidation of active site structures and in unraveling mechanistic details in the catalytic processes. The incorporation of the affinity label provides the necessary handle to draw out the proper peptide sequences from the labeled proteins and, with due care, lead to conclusions about the

amino acid sequences in or around the active sites.[1] The methodology is adequate to label receptors covalently with a sufficient degree of selectivity so that they can be easily detected. Affinity labeling technology, involves initially the reversible binding of an affinity probe, which is a ligand modified so as to contain a reactive functional group, within the binding site of the macromolecule, followed then by the covalent attachment of the affinity probe to the molecular constituents within or near the binding site. The selectivity of the labeling process derives from the selective interaction of the affinity probe within the binding site.[2]

Methods of affinity labeling are generally classified based on the type of reaction involved in the covalent attachment. Thus, probes that embody electrophilic alkylating or acylating agents are conveniently termed electrophilic affinity labeling agents, while those whose reaction requires photochemical activation to generate a reactive species are termed photoactivated affinity labeling agents or more simply photoaffinity labeling agents. One of the critical factors in covalent attachment is labeling efficiency and labeling selectivity. Therefore, in order to obtain the best results with this technique, the labeling process must be both efficient which means as much as possible of the receptor should become labeled-and selective-that is, as little as possible of the other non-receptor proteins should become labeled. Thus, affinity probes that are efficient and selective can be used in situations where receptor concentrations are low and where receptors are not in a purified state, without interference due to non-specific labeling, i.e., the labeling of other proteins. The ultimate test of an affinity probe is its application in intact systems, whole cells in culture or tissues in organ culture or even *in vivo* .[2]

The action of substrates (e.g. hormones) *in vivo* involves only reversible interaction between the substrate and the receptor. Thus, receptors covalently labeled by an affinity probe represent modified forms of receptor possibly altered in structure as well as function. The covalently linked ligand-receptor complex may be interpreted differently by the cell than the reversibly associated substrate-receptor complex. Thus, the affinity labeling method is a powerful and convenient tool as a useful model system.[2] It has been well documented that, one of the most convenient procedures to determine the efficiency and selectivity of the affinity labeling is the application of radiolabeled ligands, and in most cases, not only the question of covalent attachment to receptors can be resolved through studies with radiolabeled material, but also for impure preparations, the modified receptor can be followed during purification if a radio ligand was employed as the affinity label. Therefore, radio-labeling of such molecules which possess a photoactivatable functional group with high specific activity provide a technique of affinity labeling by which it will be possible to identify the molecular species responsible for the initial interaction with substrates *in vivo*.[3] In these studies the preferred choice of radioisotope based on considerations such as maximum specific activity, ease of monitoring and facility with which the label can be incorporated into organic compounds, has been tritium.

Since affinity labeling is a field of great interest, a short review chapter outlining the most recently used synthetic routes and the application of photoaffinity probes labelled with tritium seems timely. The scope of the present chapter is limited to the application of tritium labelled ligands for photoaffinity labeling of membranes and receptors for vitamins, steroids and insect hormones.

II. AFFINITY LABELING

A. Photoaffinity Labeling Experiments

An affinity labeling experiment is a general tool with great potential for specificity in studying binding sites. In these experiments, measurement of the attachment efficiency and selectivity of affinity probes available in radiolabeled form is straightforward: a receptor preparation is incubated with increasing concentrations of the agent, with or without an excess of unlabeled ligand for the receptor (to determine total and non-specific attachment, respectively). Periodically, aliquots are removed and the extent of covalent attachment is determined. With efficient selective agents, the latter can simply be effected by precipitation of the receptor preparation with a denaturant, such as trichloroacetic acid or a hot organic solvent; this will be enough to release and extract unattached agent, the covalently attached probe remaining with the precipitated protein fraction. The covalent attachment of tamoxifen aziridine to the rat uterine estrogen receptor proceeds quite rapidly at 25°C, reaching a maximum at 1 hour and is a good example.[2]

In these experiments nearly all of the receptor in the cytosol preparation (3n*M*) is covalently labeled after a one hour exposure to 16n*M* of [^{3}H]tamoxifen aziridine, which represents only one equivalent to the receptor, labels nearly 40% of the sites, indicating that nearly half of the reagent is reacting with the receptor.[4] In cases where the attachment efficiency and selectivity are lower or the receptor concentration or purity is lower, it may be necessary to employ a separation method such as polyacrylamide gel electrophoresis in order for the labeled receptor species to be observable over the background of nonspecific labeling.[4]

Photoaffinity labeling has been used successfully on numerous occasions to identify polypeptides that bind reagents in more or less complex mixtures. Eppler et al[5] illustrated the utility of photoaffinity labeling for characterizing receptors present in low concentrations in a very complex system. In their work, they have tried to find out the number of different cAMP-binding proteins, and their distribution, in nervous tissue from *aplysia californica,* a marine snail with which simple forms of learning have been studied.

In many cases it is of interest to know which part or parts of a polypeptide are labeled by photoaffinity labeling reagent. Kerlavage and Talor[6] labeled cAMP-binding subunit of pig heart protein kinase II with 8-azido-cAMP and obtained very good results. Brems et al[7] labeled prenyltransferase with the substrate analog o-[^{3}H]azidophenylethyl pyrophosphate and found that the site of modification was not restricted to a single amino acid. After cleavage of the labeled polypeptide with CNBr, 80% of the tritium was found in a 30-amino acid peptide, representing 4% w/w of each transferase polypeptide. When the location of the radiolabel was further investigated by amino acid analysis, peptide mapping after trypsin digestion, and Edman degradation, it was found to be distributed throughout the CNBr fragment. This suggested that the active site of prenyltransferase is formed from a single linear sequence of the polypeptide (which is not the case for enzymes). The orientation of the amino acid side-chains and the reactivity of the nitrene (and/or other photogenerated intermediates) was such that labeling occurred in several positions. However, it is impossible for the azido group to be in contact with so many residues in the active site simultaneously, and the result implies that the binding site or the reagent at the site is conformationally flexible. Therefore, in this case, one of the advantages of a photoaffinity reagent,

the generation of a highly reactive intermediate, led to technical difficulties in defining a binding site. It is suggested that to obviate this problem, less reactive species could be photogenerated, but then difficulties such as nonspecific labeling and pseudophotoaffinity labeling would become more serious. Actually, this photoaffinity labeling experiment yielded more information about the active site than could be obtained by chemical labeling, which usually only identifies a single amino acid residue.

While photoaffinity reagents have been used predominantly to identify receptors or their binding sites, they may also be used in studies of function. For example, ligand-binding sites may be irreversibly blocked with photochemical reagents. If a site is allosteric, activation or inactivation of biological activity will result. The fate of labeled receptors in living cells may also be conveniently studied with photoaffinity reagents.[3]

It has been shown that the dipeptide transport system of living *E. coli* cells is selectively and irreversibly inactivated by irradiation in the presence of glycyl-4-azido-2-nitrophenylalanine.[8] The fate of insulin receptors labeled with radioactive, photoactivatable insulin molecules has been studied by several groups. The receptors of adipocytes with B2-(2-nitro-4-azidophenylacetyl)des-Phe^{B1}-insulin were derivatized by Berhanu and colleagues.[9] At 37°C, the insulin-receptor complexes were taken into the cells and processed by proteolytic enzymes. In contrast, insulin covalently bound to receptors on lymphocytes was shed into the extracellular medium with no apparent change in molecular weight of the complex.[10]

There are some special requirements for photoaffinity reagents that are used in functional studies. First of all, a stable bond must be formed with the receptor in all experiments. Although it can not be always achieved, but in most cases a one-to-one correspondence between binding-site inactivation and covalent attachment of the reagent is desirable. Damage can occur to the receptor by photochemical reactions that do not culminate in covalent attachment. Photolysis products may bind tightly (but not covalently) to the receptor, and occasionally, covalent attachment of a bulky, flexible ligand occurs just outside the receptor site, resulting in a complex that will bind fresh ligand. These problems can lead to difficulties in interpreting the results of functional studies, and the need for reagents that undergo clean photochemical attachment at the center of a binding site is obvious.[11]

Nonspecific labeling occurs when the photoaffinity reagent becomes covalently attached to sites other than the ligand-binding site. These nonspecific labeling sites may be on the receptor or on completely different and irrelevant molecules. The worse cases of nonspecific labeling are seen when high concentrations of a reagent are used in order to obtain measurable extents of labeling of weak binding sites. In these experiments, the ratio of free to bound ligand is high and there is a real danger of macromolecules being labeled in bimolecular collisions. Nonspecific labeling will be particularly apparent where a highly reactive functional group is present on an irrelevant macromolecule.[12] In an unreactive binding site, the activated photoaffinity reagent might rearrange to a relatively unreactive species, and react elsewhere. The case where the activated reagent and the binding site have so little chemical affinity that the reagent is able to dissociate from and reassociate with the binding site several times before it reacts there or elsewhere is akin to the state of affairs in

conventional affinity labeling. Here many of the advantages of photoaffinity labeling are lost and the situation has been termed *pseudo*photoaffinity labeling.[3]

Nonspecific labeling is detected in protection experiments and prevented by scavengers. Nicolson et al[12] dramatically illustrated the use of 2-mercaptoethanol as a scavenger. *Escherichia coli* ribosomes were labeled with the inhibitor p-azido-[^{3}H]puromycin, and the most strongly labeled ribosomal component was polypeptide S18. In contrast, the labeling of S18 was insignificant in the presence of only 2m*M* 2-mercaptoethanol. In their report the authors presented convincing evidence that a particularly reactive cysteine on S18 (Cys 10) reacts with a long-lived intermediate generated from p-azidopuromycin either in solution or at a weak binding site, and that S18 is not part of the high-affinity puromycin-binding site.

As scavengers, thiols appear to be the reagents of choice, but they have three disadvantages as follows: First, all thiols react slowly with aryl azides, and the appropriate experiments must be done to ensure that a reduction in labeling is not due to destruction of the reagent. Second, thiols ionize at pH values 8-10, and the effect of such scavengers may be strongly pH dependent. Third, thiols can cleave disulfide bonds in proteins in the sample and perhaps alter ligand-binding properties or supramolecular organization.

The extent of labeling in a photoaffinity labeling experiment requires consideration. When the goal is solely to identify a receptor in a complex mixture of macromolecules, the extent of labeling of the receptor is unimportant, provided it is specific and measurable. The extent of labeling is important when the site of labeling within a polypeptide is to be determined.

A conventional chemical affinity labeling reagent is considered to be successful if it reacts quantitatively with its target.[3] For example, the chloromethyl ketone of N-tosyl-L-phenylalanine (TPCK) reacts stoichiometrically with a *His* residue at the active site of chymotrypsin. In the case of enzymes in particular, it is expected to find especially reactive nucleophiles at the active site, and reagents that react with them can be designed or discovered by trial and error. As it was mentioned before, many of the binding sites with which photoaffinity reagents reacted may be quite inert, and a fraction of the photogenerated intermediates will rearrange to unreactive species or diffuse from the site before reacting, while an electrophilic reagent would not react at all.

In practice, the extent of labeling in photoaffinity labeling experiments varies and several factors may reduce the efficiency of reaction. First, if the reagent is photolyzed relatively slowly, photochemical destruction of the binding site, cross-linking of macromolecules in the sample, or some other problems may occur before substantial photoaffinity labeling is achieved. Thus, it is emphasized that the photoactivatable reagents to be designed should be efficiently photolyzed at $\geq$300nm. Second, long-lived photogenerated intermediates may diffuse from the binding site and react elsewhere (preferably with a buffer component or a scavenger) and the need for reagents that form highly reactive species on photolysis is stressed. Third, the reagent may be poorly oriented for reaction at the binding site and this can happen when a bulky photoactivatable group is appended to a ligand to form a photoaffinity reagent. Of course, ligands must be modified in such a way that their activity is not destroyed, and this itself can result in the placement of photoactivatable groups at positions that are not essential for binding, in turn resulting in poor labeling efficiencies. Fourth, the formation of unstable covalent bonds between the ligand and the receptor that break soon after

photolysis or on denaturing the protein will result in a low extent of labeling. Finally, photolysis products formed during irradiation may bind more tightly to the receptor than the photoaffinity reagent, again reducing the labeling efficiency.

It is often possible to increase substantially the extent of labeling by repeating the labeling procedure on the same sample that has been washed free of noncovalently attached photolysis products. A second useful approach is to separate the labeled and unlabeled receptor molecules (e.g., by affinity chromatography). It is usually much easier to perform protein chemistry on a purified derivatized polypeptide than on a mixture containing unmodified material. These improvements to photoaffinity labeling techniques have little to do with azides and nitrenes themselves. Here again it is emphasized that these need to design reagents that have high values of ϕ (radiant flux) and ε (emissivity), that are not bulky, that yield only highly reactive intermediates on irradiation, and that give rise to stable covalent bonds between the receptor and the ligand.[3]

It is important that a stable bond be formed between a photogenerated reagent with a receptor. If a very unstable bond is formed, the radiolabeled reagent will be separated from the macromolecule soon after photolysis and the extent of labeling will be very low. More easily diagnosed is a loss of label during the cleavage of a labeled receptor subunit into fragments, and there are several cases in which severe problems have been reported.

Abercrombie et al[13] photoaffinity labeled a peptide binding site on neurophysin using NH_2-[^{3}H]MetTyr(4-N_3)Phe$CONH_2$. To aid the analysis, the labeled and unlabeled neurophysins were separated by affinity chromatography. Incidentally, the extent of labeling as judged by the incorporation of radiolabeled reagents was not equal to the extent to which the binding site was blocked, possibly, the reagent had damaged some sites without coupling to them covalently, a complication that has been often observed.

The derivatized polypeptide was oxidized with performic acid (to cleave disulfide bonds) and then digested with trypsin. HPLC revealed that a tryptic fragment present in digests of unmodified neurophysin was missing and that a new fragment had appeared. The new fragment had an amino acid analysis corresponding to residues 44-66 of neurophysin, except that it lacked Tyr 49. Further, the fragment was not radioactive. It was deduced that the reagent had labeled Tyr 49 but had been cleaved from neurophysin by the action of performic acid leaving a modified amino acid at position 49. Thus the site of attachment was found indirectly. In the same work it was discovered that the Tyr-reagent bond was unstable to acid hydrolysis or to the action of the reducing agent dithiothreitol. Both released the radioactive reagent and the Tyr residue was recovered unmodified. It is likely that the reagent coupled to the Tyr to form a 2-phenoxy-^{3}H-azepine. It was expected that this reagent to be unstable to hydrolysis, a reaction that might be catalyzed by nucleophiles such as sulfhydryls. The nature of the reaction with performic acid that destroys the Tyr remains to be defined.

Hoppe and colleagues[14] reported a second example of an unstable bond formed by a photochemical reagent. They labeled a subunit of the ATP synthase of *E. coli* with a photoreactive lipid, 1-palmitoyl-2-(2-azido-4-nitro)benzoyl-*sn*-glycero-3-[^{3}H]phosphatidylcholine. Subunit b was predominantly labeled, but when an attempt was made to further delineate the site of attachment within the subunit by CNBr cleavage, all the radiolabel was released as low molecular weight

material. After enzymatic cleavage and gel filtration, a radiolabeled peptide was recovered but most of the radiolabel had been released. Perhaps the adduct was unstable in the acidic eluant that was used (80% formic acid). When the remaining radioactivity was located within the proteolytic fragment by Edman degradation, it was found on a Cys residue. The authors speculated that the photogenerated arylnitrene reacted with the nucleophilic cysteine, forming a derivative with a labile S-N bond. These observations emphasise the need to learn more about the reactions of nitrenes and their rearrangement products with the functional groups that are present in biological macromolecules, and more about the stability of the products that are formed.[3]

B. Radioactive Labeling

The binding method is a useful screening procedure for prospective photochemical labels, but must be accompanied by radioactive labeling studies if it is to be of real value. Radioactivity gives the most dependable measure of the extent of labeling and in addition to the photoactivatable label, isotopically labeled molecules are required for a number of reasons. As the radioisotopic labels are the best choice for researchers involved in affinity labeling, the substitution of radioactive isotopes of atoms already in the molecules offer both minimum perturbation of the molecule and maximal sensitivity. The extremely short half-lives (<120 min) of the positron emitters ^{18}F, ^{11}C and ^{15}O despite the high specific activity (>10000 Ci/mmol) attainable make them unsuitable for this kind of work. The substitution of ^{125}I ($t_{1/2}$=60 days, >2000 Ci/mmol) into a number of photoaffinity probes has been reported in the literature[15], but increasing use of tritium as the radioisotope has recently been seen and this could be due to the ease of synthesis, ease of monitoring and high specific activity attainable with this radionuclide. The maximum specific activity is 29 Ci/mg-atom of tritium for this 12 year half-life, low energy β emitter. The major routes of interest are summarized as follows:

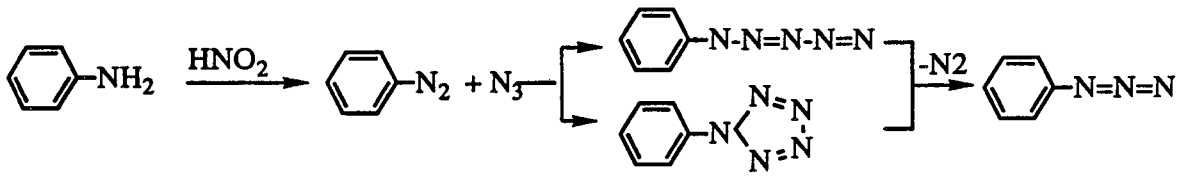
Tritium can be introduced by reductive tritiation with T_2 gas and an appropriate catalyst. This is the least expensive per curie and the most versatile method for obtaining products with high specific activity. A hydride-type reduction of an aldehyde or ketone with 60 Ci/mmol sodium borotritide provides a direct and selective triton introduction. Quenching of an organometallic with tritium oxide or use of labeled water to exchange enolizable hydrogens is common. Tritiodehalogenation and tritiodetosylation with tritide under SN_2 conditions can also be employed. The development of lithium triethylborotritide (super-tritide) as a stereo-selective and more nucleophilic single tritide labeling reagent is described in the literature,[16] but it requires a convenient commercial source of high specific activity lithium tritide. This reagent has recently been prepared in 100% isotopic abundance and used successfully for reduction of a number of organic functions at NTLF.[17] In each case, the location of the label can be confirmed directly by observing the Tritium-NMR spectra. With a 300 MHz spectrometer, as low as 1mCi can be readily observed in under 1 hr acquisition. The triton is the most sensitive NMR-active nucleus and has the advantage in this case of zero natural abundance; moreover, its couplings and chemical shifts are essentially identical to those of protons.

III COVALENT ATTACHING FUNCTIONS

A. Photoactivated Affinity Precursors

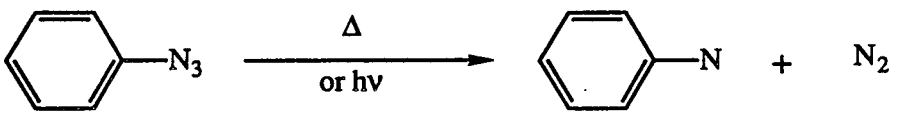
1. Azides and their Photochemistry

While the aliphatic azides are chemically stable, they do not have a chromophore that is readily accesible in the UV. Aromatic azides, on the other hand, have accessible chromophores, and upon irradiation decompose to species with capacity for covalent attachments to proteins. The most general and widely used method to prepare aryl azides is diazotization of the corresponding aniline followed by addition of sodium azide. The process is quick and is readily carried out in a test tube. There are two paths for the reaction: one is through a linear dipolar pentazadiene, from which the outer two of the erstwhile azide nitrogens are lost, and the other is through a pentazole, in the fragmentation of which (to nitrogen and aryl azide) some isotope scrambling may occur if isotopically labelled reactants are used.[18]



Alternatively, the diazonium salt may be converted to azide by other reactions which are rarely used anymore.[18]

By far the most widely used method for generating aryl nitrenes is thermolysis or photolysis of arylazides, which readily lose two of the three nitrogens as N_2. The detailed photochemical processes by which these occur are still not understood in detail; it is generally felt that photoinduced loss of nitrogen from the azide generates an aryl nitrene.



Photolysis is generally effected by near-UV radiation; even sunlight can be used. Use of a solvent is strongly recommended and the solvent should preferably be transparent to UV light. Tetrahydrofuran (THF) is a widely used solvent for photolysis. Nitrenes are electron-deficient species that can exist in either singlet or triplet states, with the latter as the ground state. The aryl nitrene undergoes either insertions as the singlet or hydrogen abstraction radical center coupling as the triplet.

It is quite likely that these courses of reaction are incorrect. Nevertheless, our lack of mechanistic understanding notwithstanding, aryl azides have been widely used in photoaffinity labeling studies for many years.[18]

The photochemistry of aryl azides is summarized in Figure 1. At least four reactive intermediates are involved: the singlet nitrene, the triplet nitrene, fully bonded electrophiles formed by rearrangements of the singlet (benzazirines and cycloheptatetraenes), and the anilinyl radical formed when the triplet nitrene abstracts a hydrogen atom from a donor.

The major reactions that these species undergo are with nucleophiles, with carbon-hydrogen bonds, and with oxygen. The formation of dimers, which is often an important reaction in the hands of photochemists, will be less important in some cases as most of the reagents are used at quite low concentrations, and in the case of photoaffinity labeling experiments, they are (ideally) bound to proteins at isolated sites.[3]

2. Diazocarbonyl Compounds and Diazirines

One of the first photoactivatable reagents to be used for the labeling of proteins were Diazocarbonyl compounds. The steroid-binding serum proteins α-fetoprotein and corticosteroid-binding globulin have been labeled with diazoketones and thyroid hormone-binding sites have been labeled by a diazoamide derivative of the hormone.[2] Although labeling has been very selective the efficiency of labeling in these cases has been low (10-15%).

It is believed that α-diazocarbonyl compounds which are formed after photochemical decomposition react as α-ketocarbenes. The latter in the singlet state would undergo insertion into a variety of bonds found in amino acid residues (or in the triplet state, by hydrogen abstraction, radical coupling). The efficiency of photoattachment of diazocarbonyl compounds is believed to be followed by two processes, Wolff rearrangement of the α-keto-carbene intermediate to an electrophile and intramolecular transfer of a β-hydrogen. The former rearrangement is suppressed (and the inherent reactivity of the carbene thereby enhanced) by electron-withdrawing substituents on the α-carbon; hence the use of carbethoxy-substituted (diazomalonyl), or sulfonyl, and trifluoromethyl-substituted diazocarbonyl systems.[2] Problems with β-hydrogen transfer are avoided by designing systems that have no β-hydrogens.

Although, simple unsubstituted diazo compounds are too unstable for utilization with photoaffinity labeling agents, the isomeric diaziridines are quite stable. Photolysis converts these functions, either directly or via the diazo form, into the carbene, with reactions and side reactions proceeding as with the ketocarbenes.[2] Despite the numerous cases in which diazocarbonyl and diaziridine functions have been utilized in photoaffinity labeling, it is not at all clear that optimally reactive structures for the ketocarbene intermediated have yet been defined.

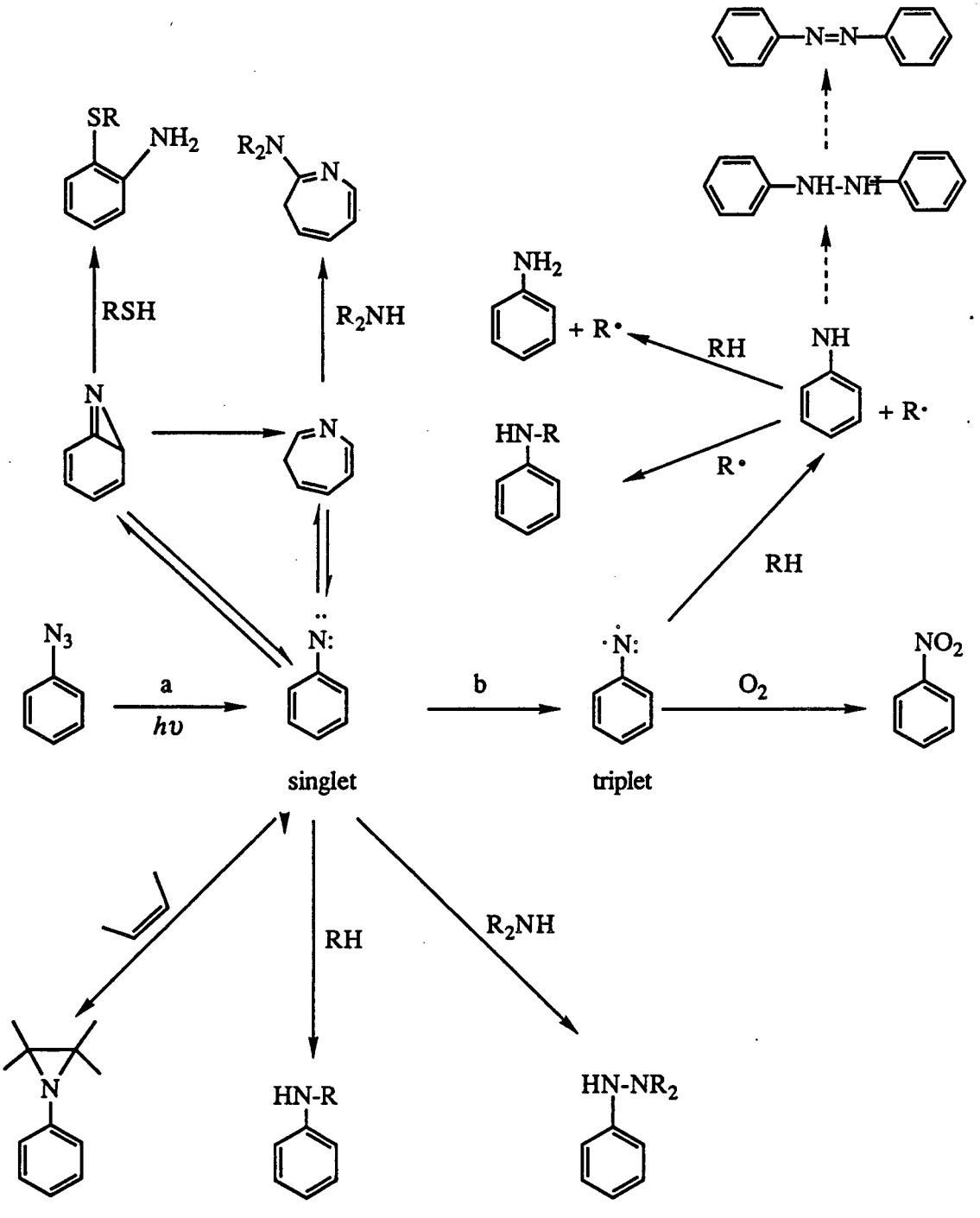


Figure 1. Photochemical reactions of aryl azides

3. Unsaturated Carbonyl Compounds and Other Functionalities

A promising approach to photoaffinity labeling is offered by the possibility of photoexciting the α,β-unsaturated ketone groups of natural or readily available synthetic steroids.[19] This procedure which eliminates the need for preparing reactive derivatives, has been recently used to label steroid-binding proteins and receptors. Two steroid hormone receptors and a steroid-binding protein, uteroglobin, have been successfully labeled by this procedure using the natural ligands, or routinely available synthetic analogues.[19] Unsaturated ketone functional groups have also been used with very considerable success in the labeling of progesterone, glucocorticoid, and androgen receptors and binding sites for ecdysone. It has proven particularly convenient that this unsaturated ketone is present as an enone function in the natural ligands for these receptors. Dienone and trienone functions are part of several commercially prepared synthetic ligands. While usually highly selective, photoaffinity labeling mediated by unsaturated ketones is generally inefficient (10%), photochemical insertion reactions of aromatic ketones have been utilized successfully in photoaffinity labeling studies with peptide hormones.[2]

Many other functional groups can be irradiated and transformed into a chemically reactive, electronically excited state or will undergo conversion into a reactive, electronically excited state. Iodoaromatics(thyroxine) and nitroanisoles are examples of some more unusual photoactive species of utility in receptor labeling. m-Nitroanisoles, as well as many other aromatic systems, can be excited to electronically activated states where some of the substituents are readily susceptible to displacement by nucleophiles. These processes may be applicable to protein labeling and receptor labeling.[2]

B. Electrophilic Affinity Functions

1. Halocarbonyl Groups

Chloromethylketone derived from N-tosyl-L-phenylalanine was one of the first affinity labels, which provided convincing evidence for the catalytic involvement of an imidazole side chain in proteolytic enzymes. Shaw[20] and his colleagues were the first group to design ligandlike α-haloketones as probes of the active sites of many different enzymes and nonenzymic proteins. α-Haloketones remain one of the more popular chemical classes of affinity labels.

Their principal advantages as affinity labels are that they are highly reactive and provide a means for introducing a radioactive marker, after the covalent modification has taken place, through reduction with sodium borotritride. Haloketones are more reactive as alkylating agents than are haloacetylated amines. Since haloketones are potentially reactive with most nucleophiles found in proteins, the chances are good that modification of some residue within the active site will occur, provided the reagent has an affinity for the active site. The opportunity to introduce conveniently and economically an isotopic label into the reagent moiety of the derivatized protein removes the necessity of synthesizing the labeled reagent.

In general, α-halo ketones, esters, and amides, and the related sulfonate esters of α-hydroxycarbonyl compounds have long been known to be excellent alkylating agents, and are highly efficient in their labeling of receptor in cell-free preparations, their high reactivity toward

sulfhydryl compounds lowers the efficiency and selectivity of their reaction in intact systems. α-Halocarbonyl and related systems are known to provide a large activation toward SN_2 displacement. These have been used as attaching functions in labeling the progesterone, androgens, glucocorticoid, and thyroid receptors as well as hormone-binding sites in enzymes and carrier proteins.[2]

2. Aziridines

Aziridines are relatively nonbasic, and since they are most reactive when protonated, (as the aziridinium ion), they may be particularly favorable agents to use in the selective labeling of binding sites containing an acidic function that might serve to protonate them selectively. This selective activation, coupled with a generally low inherent reactivity toward nucleophiles under physiological conditions, makes them appear promising in receptor labeling. Tamoxifen aziridine, a highly efficient and selective affinity label for the estrogen receptor, utilizes an aziridine attaching function.[4]

3. Oxiranes and other Functionalities

Oxiranes are similar to aziridines, being reactive on the basis of ring strain. Although their reactivity under neutral conditions is somewhat higher than that of the aziridines, they are activated by acids too. So far, this function has been used in labeling studies on steroid isomerase enzymes.[2]

There are many other organic functional groups with electrophilic activity,but they have not yet been successfully applied in electrophilic affinity labeling of receptors. α,β-Unsaturated carbonyl compounds, provided the β-terminus is not sterically hindered, are known to be good electrophiles (Michael acceptors for thiols) and have been used to label hormone binding sites on enzymes. A whole variety of acylating functions-active esters, imino esters-have been used in protein modification studies and in other affinity labeling studies. Mercurials, highly reactive toward sulfhydryl functions, have been utilized in some affinity labeling studies with the estrogen receptor.[2]

C. Carbenes *vs.* Nitrenes as Photoactivated Species

Since the photoaffinity labeling method depends upon the generation of a highly reactive species by irradiation of a chemically stable precursor, the criteria for an effective reagent are as follows:

1. The precursor should be readily synthesized, chemically stable (which for these purposes often means a half-life in aqueous solution of hours or longer), and susceptible to smooth photolysis at wavelengths long enough (or for times short enough) not to cause photooxidative or other irrelevant photochemical damage to the system.
2. The photochemically derived species should be highly reactive, of extremely short half-life, and not susceptible to intramolecular rearrangement to a much less reactive compound.

Aside from the generation of electronically excited states, irradiation can give rise to two general classes of species, each of which is produced by the homolytic cleavage of chemical bonds.

The absorption of ultraviolet or visible radiation may lead to fragmentation either at a single bond, resulting in the formation of two free radicals or a diradical, or at a double bond to carbon or nitrogen, which results in a carbene or a nitrene. All these approaches have been and are being used, even though the choice of reagent for particular systems has undoubtedly been based more on synthetic accessibility than on the desirability or effectiveness of the different reactive species.

In much of the work involving aryl azides, it has been explicitly or implicitly assumed that, once generated, the nitrene reacts rapidly and nonspecifically with its immediate environment including aliphatic residues. However, aryl nitrenes may in fact not react rapidly on a molecular time scale, many having half-lives in the millisecond time range and insertion of an aryl nitrene into an aliphatic C-H bond is likely to be one of the slowest processes. In addition, it has recently been recognized that aryl azides are rapidly reduced to amines by thiols at room temperature and physiological pH. This may represent a disadvantage since thiols are often added to buffer solutions used in biochemical studies.[21]

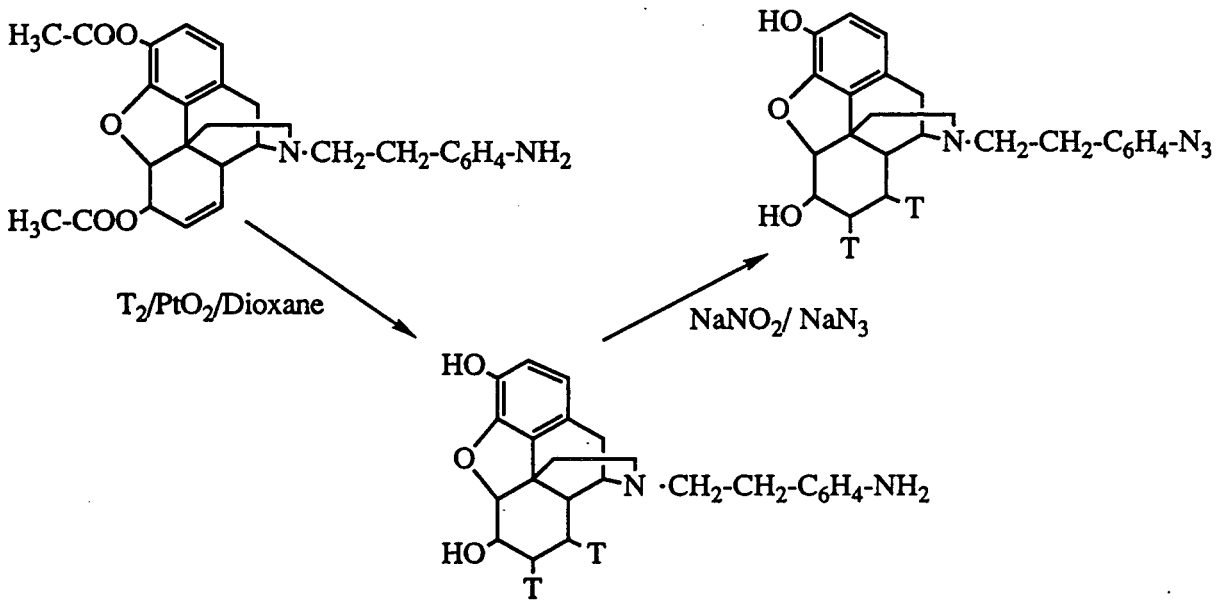
The work of Singh et al[22] on photochemical labeling with diazoacetates, showed that many carbenes suffered from marked competition between bimolecular insertion and internal rearrangement reactions. The rearrangement products, such as ketenes, were frequently very reactive and resulted in unintended labeling by paths unrelated to the original photochemical product. More recently, new carbene-generating reagents have been prepared. However, some problems still remain. Diazo-3,3,3-trifluoropropionates[23] suffer from the disadvantage that harsh conditions for photolysis are required. Potential advantages are offered by p-toluenesulfonyl diazoacetates[24] since the molar extinction coefficient at 370nm is approximately 10 times that for the long wavelength absorption of other diazoesters and this permits reasonably rapid photolysis in the near ultraviolet.

As precursors for carbenes, the photolabile diazirine group holds much promise since, unlike most of the diazoesters, diazirines are relatively stable in acid. In addition, they can be photolyzed very efficiently at wavelengths around 360 nm where most of the biological molecules do not absorb radiation. Bayley and Knowles[25] introduced ^{3}H,3-phenyldiazirine and spiro-(adamantane-2,2'-diazirine) as probes for the hydrophobic core of membranes and many of the advantages of carbenes over nitrenes were illustrated. One complication discovered so far derives from the fact that photolysis generates the linear diazoisomer as well as the carbene. Although these can be photolyzed at suitable wavelengths to generate the carbene, diazo compounds are in general highly sensitive to protonation and subsequent nucleophilic attack. Phenyldiazomethane, the isomer of ^{3}H,3-phenyldiazirine has indeed been used to alkylate carboxyl groups of carbonic anhydrase[26]. A transient accumulation of the diazoisomer during photolysis could therefore lead to photochemically irrelevant labeling.

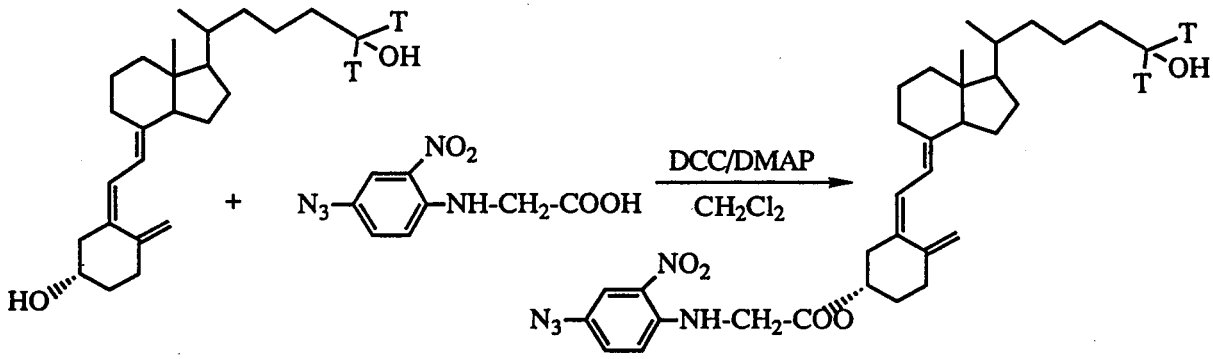
Regardless of the advantages and disadvantages of the nitrene and carbene precursors, many reseachers have used tritiated photoaffinity probes. The following represent the most recent studies in this area.

IV. ^3H PHOTOAFFINITY PROBES USING NITRENE PRECURSORS

Cooper and Rapoport[27] synthesized N-P-azidophenylethyl-7,8-dihydronormorphine and its [7,8-$^3H_2$] analogue from morphine. This material, a potential photoaffinity label with high specific activity and with opiate agonist activity comparable to morphine, will be useful for labeling of opiate receptors, either membrane bound or in solubilized form. The high specific activity tritium labeled analogue should make identification of receptors in low concentrations possible.



It is well recognized that the vitamin D binding protein (DBP) is important for the transport of vitamin D, 25-hydroxyvitamin D (25-OH-D), and its metabolites. In an attempt to better understand the molecular-binding properties of this ubiquitous protein, Ray et al[28] designed and synthesized a photoaffinity analogue of 25-OH-D_3 and its radiolabeled counterpart. This analogue, 25-hydroxyvitamin D_3 3β-[N-(4-azido-2-nitrophenyl)glycinate], was recognized by the rat DBP and was about 10 times less active than 25-OH-D_3 in terms of binding. Incubation of [^3H]25-OH-D_3 or [^3H]25-OH-D_3-ANG with rat DBP revealed that both compounds were specifically bound to a protein with a sedimentation coefficient of 4.1S. Each was displaced with a 500-fold excess of 25-OH-D_3. When [^3H]25-OH-D_3-ANG was exposed to UV radiation in the presence of rat DBP followed by the addition of a 500-fold excess of 25-OH-D_3, there was no displacement of tritium from the 4.1S peak. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiographic analysis of [^3H]25-OH-D_3-ANG exposed to UV radiation in the presence of rat DBP followed by the addition of a 500-fold excess of 25-OH-D_3 revealed one major band with a molecular weight of 52000. These results provided strong evidence that [^3H]25-OH-D_3-ANG was covalently linked to the rat DBP. This photoaffinity probe should provide a valuable tool for the analysis of the binding site on this transport protein.



p-Azido[^{3}H]puromycin [6-(dimethylamino)-9-[3'-deoxy-3'-[(p-azido-L-phenylalanyl) amino]-β-D-ribofuranosyl]purine] has been used to photoaffinity label the *Escherichia coli* ribosome. Initial studies with this compound, reported earlier[29] indicated a very diffuse labeling pattern with many proteins labeled to a significant extent . In their recent work, much of the previous apparent labeling is shown to arise from both light-independent non-covalent binding and light-independent incorporation of photolyzed p-azidopuromycin with ribosomal protein, and procedures are described for measuring true covalent photoincorporation. When these new procedures are used, p-azido-puromycin is shown to photoincorporate into ribosomal protein and RNA. The protein labeling pattern, as determined by both polyacrylamide gel electrophoresis and immunoprecipitation, is quite specific and is essentially unchanged whether 2537A or 3500 A lamps are used. The extent of photoincorporation into proteins falls in the order S18>L23>L18/22>L15>S7,S14. When β-mercaptoethanol is present during photolysis as a photoaffinity label scavenger, S18 and most other S protein labeling is not site specific but is due rather to its high chemical reactivity. The specific suppression of S18 labeling by pretreatment of the ribosomes with N-ethyl-maleimide supports this view. L23 labeling by p-azidopuromycin is azide dependent and proceeds by a mechanism which is most probably different from that responsible for photoincorporation of puromycin, yet under the appropriate conditions both of these compounds label L23 to the highest extent of any ribosomal protein. This constitutes strong evidence for the site specificity of L23 labeling.

Brems et al[30] previously presented evidence for the selective modification of the catalytic site of prenyltransferase by photoaffinity labeling with o-azidophenylethyl pyrophosphate . Recently, they reported the isolation and characterization of a CNBr fragment of 30 amino acid residues from the photoaffinity-labeled enzyme. This CNBr fragment contains over 80% of the total label attached to prenyltransferase as a result of photoaffinity labeling. Several lines of evidence indicate that a number of residues in this CNBr fragment have been modified. First, Edman degradation of the labeled peptide demonstrates that at least 16 of the 30 amino acids have been modified by the photoaffinity reagent. The two most extensively modified amino acids are a specific arginine and alanine. Second, two-dimensional chromatography of Pronase digestions of the labeled CNBr fragment indicates that at least 11 different products resulted from photoaffinity

labeling. Third, peptide maps of a trypsin digest of this CNBr fragment show that the attached affinity label is distributed among at least three of the resulting products of tryptic hydrolysis. Finally, comparison of amino acid analysis of this CNBr fragment with that of its counterpart isolated from native enzyme is consistent with the modification of a number of amino acids rather than a few by the photoaffinity labeling process.

Abercombie et al[32] investigated the chemical structure of the hormone binding region of the neurophysins by photoaffinity labeling with the photolabile tripeptide, L-[methyl-^{3}H]Met-L-Tyr-p-azido-L-Phe amide. Photolysis of the photoaffinity tripeptide in the presence of bovine neurophysin I and II and a human neurophysin II led to approximately equal extent of covalent incorporation of radioactivity into the proteins. Photolabeled bovine neurophysin II was fractionated into binding site derivatized protein and nonbinding site derivatized protein by affinity chromatography. The results of amino acid and radiolabel analysis of the hormone binding site blocked protein indicated that 1 mol of tripeptide was covalently incorporated/mol of protein. Tyrosine 49 was the only protein amino acid modified in the binding site photolabeling reaction as assessed by peptide mapping of the performic acid oxidized and trypsin-digested photolabeled protein using reverse phase high performance liquid chromatography. Modification of the single neurophysin tyrosine also was found by amino acid analysis of performic acid oxidized photolabeled bovine neurophysin II. The covalent bond formed in neurophysin upon photolysis was cleaved by either exhaustive acid hydrolysis or reduction-carboxymethylation without loss of the protein amino acid residues and by performic acid oxidation with loss of both protein and tripeptide tyrosine residues. These overall results indicate that tyrosine 49 is the probable site for specific covalent attachment of the photoaffinity tripeptide. Assuming that the tripeptide binding site is the high affinity hormone binding site reported for the neurophysins, this conclusion suggests that tryrosine 49 is close to or within this site.

Hoppe et al[14] as reported earlier purified ATP synthetase composed of a hydrophilic component F_1 and a membrane--integrated part F_o from *Escherichia coli* K12 which was labeled with the hydrophobic photoreactive label 1-palmitoyl 2-(2-azido-4-nitro)benzoyl *sn*-glycero-3-[^{3}H]phosphocholine in reconstituted proteoliposomes. The F_0-subunit b was predominantly labeled. A very low amount of label was detected on the other F_0-subunits a and c. The label in subunit b could be traced back by proteolytic digestion to the NH_2-terminal fragment 1 to 53 which contains the stretch of hydrophobic amino acid residues 1 to 32. By sequencing the intact protein, the distribution of label among the amino acids in this segment was determined. Cysteine 21 was predominantly labeled. Other labeled amino acids occurred at the NH_2-terminal (Asn-2) and at position 26 (tryptophan). Due to the restricted mobility of the label in the lipid bilayer, these residues are likely to be located in or close to the polar head of the lipid bilayer. These results have been compared with predictions for the arrangement of the polypeptide b derived from the hydrophobicity profile.

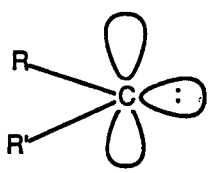
Finally, just recently Cooper et al[33] developed a method for tritiation of 2-azido-N(6)-benzyladenine (AZBA), a photoaffinity analog of the plant hormone N(6)-benzyladenine (BA). The

synthetic sequence involves condensation of 2,6-dichloropurine with m-iodobenzylamine to yield 2-chloro-N(6)-m-iodobenzyladenine, followed by selective hydrodeiodination with tritium gas over palladium catalyst in pyridine solution to give 2-chloro-N(6)-m-tritiobenzyladenine. The known conversion of this compound to AZBA enables preparation of the photoaffinity label compound with specific activity high enough to permit detection of cytokinin-binding proteins existing at low concentrations in plant tissues.

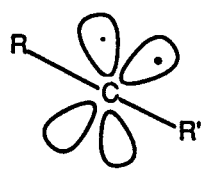
V. ^{3}H PHOTOAFFINITY PROBES USING CARBENE PRECURSORS

As was mentioned before, carbenes react very rapidly with a variety of chemical functions as follows: coordination to nucleophiles, addition to multiple bonds, insertion into single bonds and hydrogen abstraction. Carbenes can become unreactive species, through hydrogen migration if there is a hydrogen atom on the carbon atom adjacent to the carbene center. For this reason, the adjacent atom should not have hydrogen, as in the following precursors: α-Diazoketones, α-Diazoacetyl-, α-Diazomalonyl-, Trifluoromethyldiazoacetyl-, α-Diazobenzylphosphate, Aryldiazomethane and Aryldiazarines.[1]

The electronic configuration of carbenes has been studied in a series of substituted diphenylmethanes by low-temperature photolysis of the appropriate diazo compound. In these studies, the chemistry of methylenes, R-C-R', has been interpreted in terms of two different configurations: 1- a singlet state (A) in which the unshared electrons are paired in an sp^2 orbital, leaving an empty *p* orbital; or 2- a triplet state (B) in which the two unshared electrons are unpaired, each in a *p* orbital and in which the R-C-R' angle is more nearly linear than when the molecule is in configuration (A)[34]

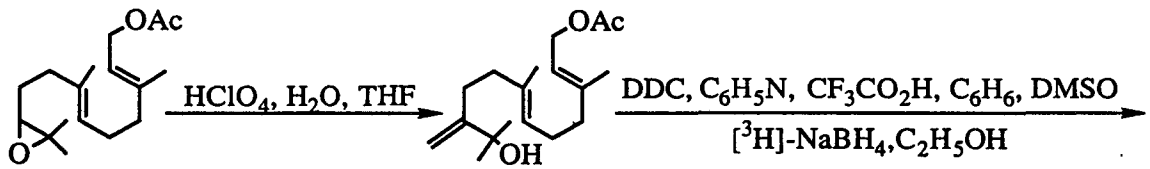


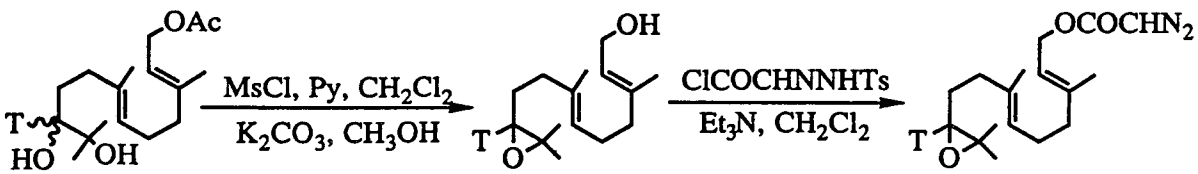
A



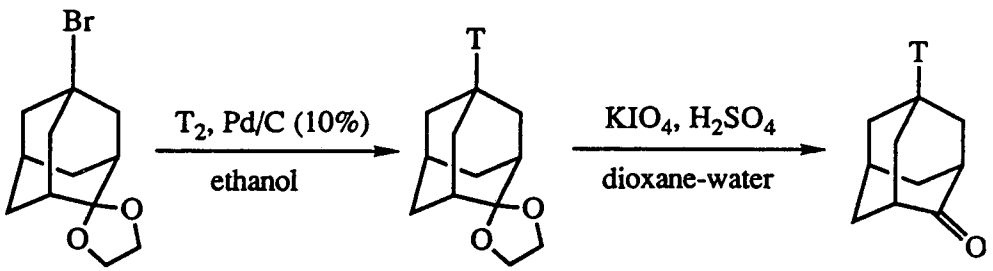
B

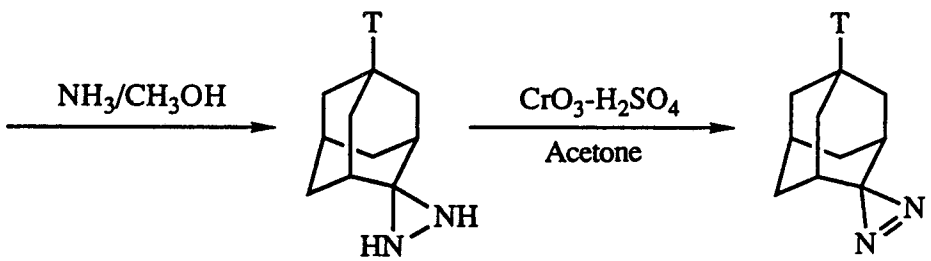
The interactions of insect juvenile hormones (JH) with proteins are critically important to tighter regulation, transport, and hormone action at a molecular level. Prestwich et al[35] have synthesized several JH analogs bearing photolabile diazocarbonyl groups as potential photoaffinity labels for JH binding proteins (JHBP). The most promising compound, 10, 11-epoxyfarnesyl diazoacetate (EFDA) competes with JH III for the JH binding site of JHBP from *Leucophaea hemolymph* and ovaries and from cultured *Drosophila* cells. Moreover, irradiation of protein solutions containing micromolar amounts of EFDA gave irreversible loss of [^{3}H]-JH III binding capacity with no change in binding affinity of the unlabelled protein. The protein could be protected against photoinactivation by the presence of equimolar JH III during irradiation. High specific activity [10-^{3}H]-EFDA was prepared and used to demonstrate specific, finite binding of EFDA to the JH III receptor site of the binding protein.





1-[^{3}H]Spiro[adamantane-4,4'-diazirine], a lipophilic, photoactivatable reagent designed to label those segments of intrinsic proteins that lie within the lipid bilayer of biological membranes, has been evaluated[36]. The reagent labels the intrinsic proteins of human erythrocyte membranes far more heavily than it labels the extrinsic proteins. The result, together with a detailed analysis of the label distribution in several well-characterized membrane proteins, demonstrates that labeling with adamantanediazirine is a convenient and rapid method both for distinguishing intrinsic from extrinsic membrane proteins and for locating within intrinsic proteins those amino acid residues that are in contact with the hydrocarbon core of the lipid bilayer.

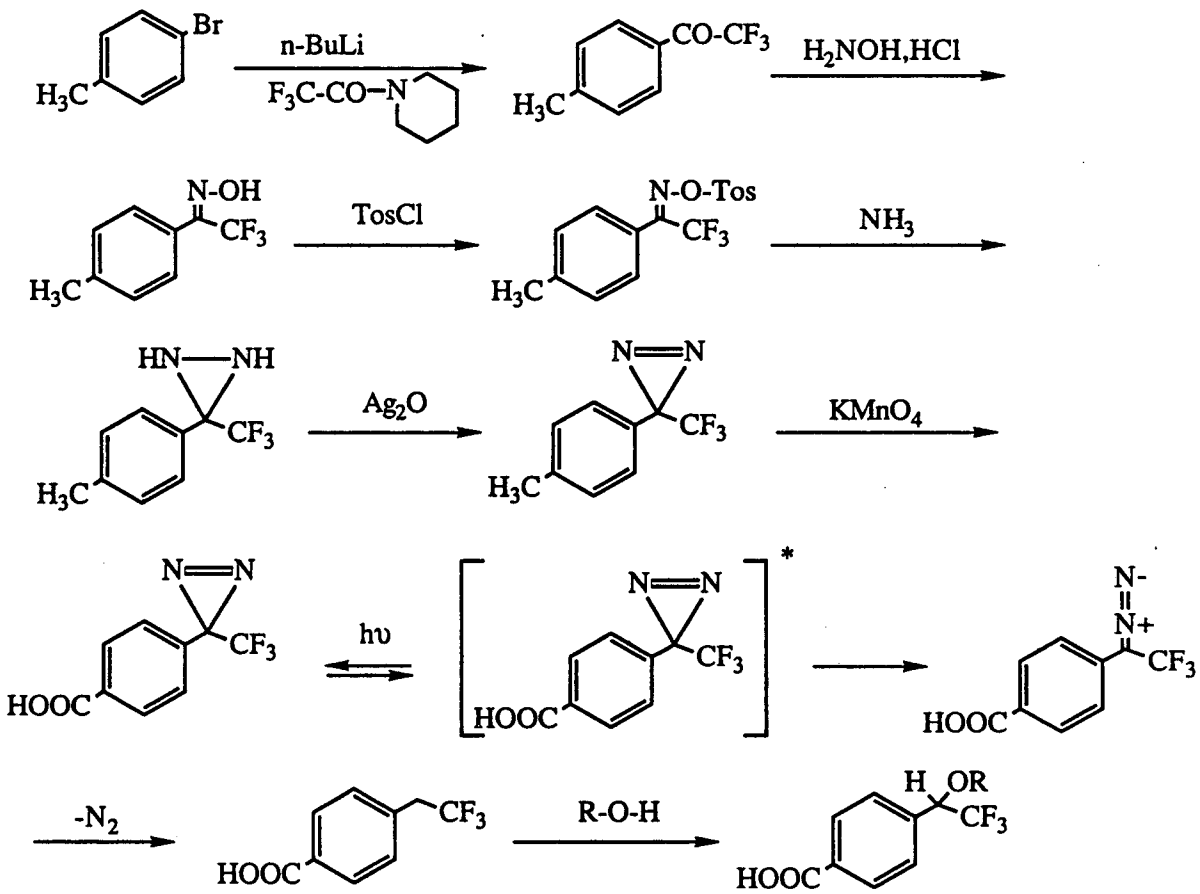




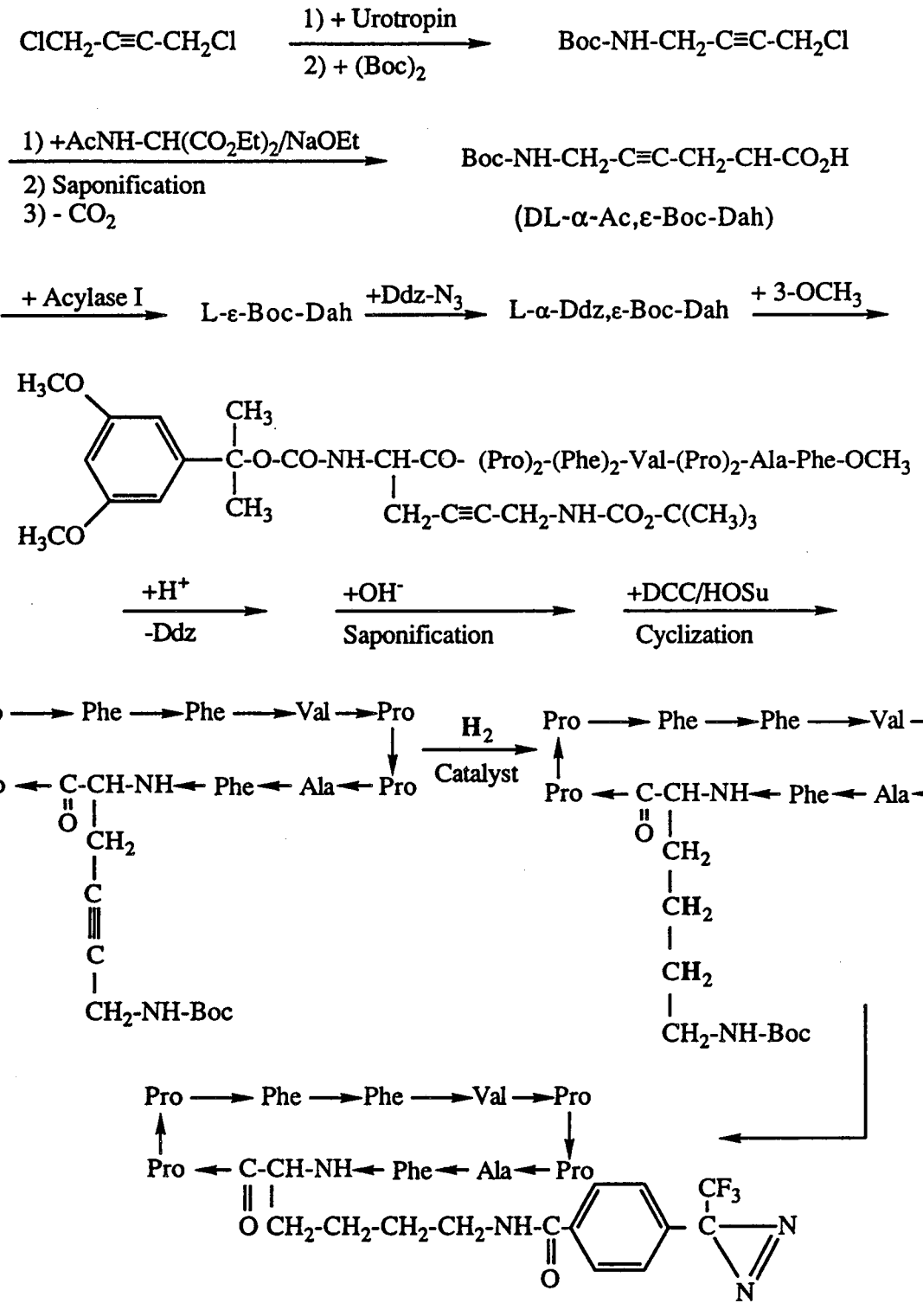
The synthesis and testing of several diazocarbonyl JH analogs (diazo JHA) which act as photoaffinity labels for insect juvenile hormone binding proteins are reported by Prestwich et al.[37] The best competitor, 10,11-epoxyfarnesyl diazoacetate, has been shown to irreversibly reduce [^{3}H]-JH III binding to both ovarian and hemolymph JHBP from *Leucophaea maderae* after irradiation at 254nm for 20 seconds. No loss of activity was observed after incubation of JHBP and diazo JHA without irradiation. Protection from photoinactivation by diazo JHA II was achieved by the presence of an equimolar amount of JH III during the photolysis. Photoaffinity labeled proteins show loss of binding capacity without alteration of the binding affinity. This is the

first example of the use of a photoaffinity label in the study of JH action on a molecular level, and may become a valuable tool in the elucidation of JH-receptor-chromatin interactions.

Nassal[38] has synthesized 4-(1,Azi-2,2,2-trifluoroethyl) benzoic acid, a highly photolabile carbene generating label readily excitable to biochemical agents, starting simply from 4-bromotoluene. In the first step Br was replaced by Li using *n*-butyllithium, then the organometallic compounds were converted into the trifluoroacetophenones with *N*-trifluoroacetylpiperidine. The azi moiety (diazirine) was prepared from the oximes *via* O-tosyloximes plus ammonia yielding the diaziridine and oxidation of the latter with Ag_2O. Oxidation by permanganate yields the title compound. On irradiation (λ>300nm) by elimination of N_2 with a half-life period of 22 s, the precursor generates the corresponding carbene. The diazirine as its *N*-hydroxysuccinimide ester , or using other methods of amide synthesis, can readily be coupled to amino functions of biochemically interesting agents thus forming photoaffinity labels.

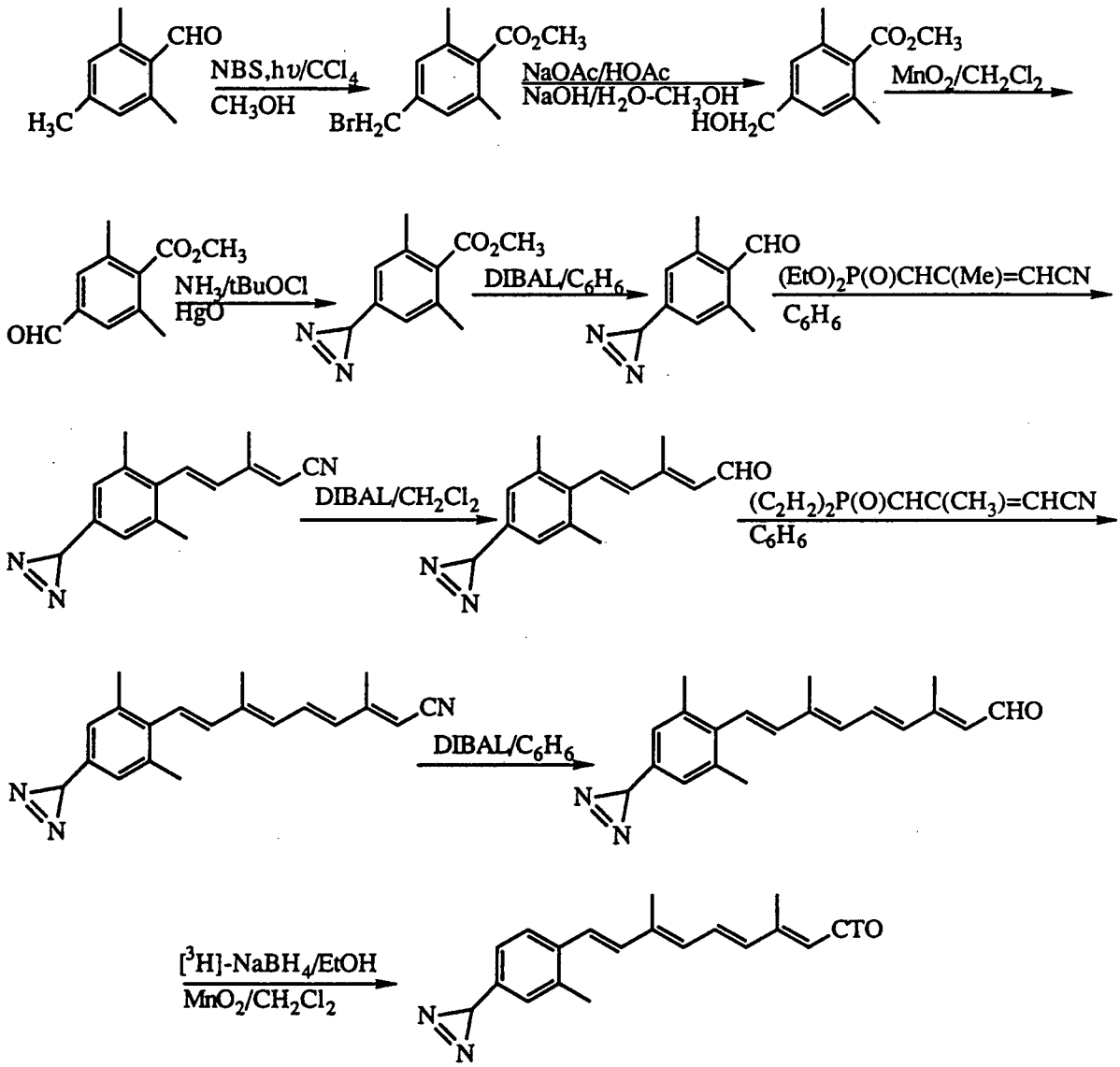


Nassal et al[39] also synthesized L-2,6-Diamino-4-hexynoic acid, protected at its 2-amino group by 1-(3,5-dimethoxyphenyl)-1-methylethoxycarbonyl (Ddz), and its 6-amino group by *tert*-butylozycarbonyl (Boc), which was then coupled to the nonapeptide ester H-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-OMe (3-OMe). The decapeptide so obtained, after deprotection of its terminals is cyclised by N,N'-dicyclohexylcarbodiimide/n-hydroxysuccinimide to yield the ε-Boc-protected 6-



analogue Boc-2 of antamanide. This, by catalytic hydrogenation yields [ε-Boc-Lys^6]antamanide (Boc-1) thus offering the possibility to introduce tritium into an antiphallotoxic active molecule. Acylation of the ε-amino group of [Lys^6]antamanide[1] by the previously described 4-(1-azi-2,2,2-trifluoroethyl)benzoic acid yields a derivative suitable for photoaffinity labeling.

Sonnewald and Seltzer[40] have reported that one of the routes to probe retinal's changing environment in halo-bacteria is to photoaffinity label its neighbors at particular stages of the photocycle for subsequent analysis. They have studied the synthesis, incorporation, and general photoaffinity labeling of one of the retinal's analogue. Photoaffinity labeling studies with retinal analogue 3,7-dimethyl-9-(4'-(3H diazirinyl)-2',6'-dimethylphenyl-2E, 4E, 6E, 8E-nonatetraenal · 1-^{3}H has been carried out to determine whether retinal bridges protein molecules in the purple membrane. The synthesis of the desired compound can be shown in the following scheme:



VI. SUMMARY

The purpose of this review chapter is to present an overview of the current status of affinity labeling with a general picture of the principles involved as well as the current and prospective applications of photoaffinity labeling techniques using tritium as the radio-tracer and carbene and nitrene precursors as photoactivatable moieties. The examples shown in this paper are a few of many illustrations covering this rapidly expanding field in the literature and the reader is referred to the most recent references therein.

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