Bioorganic & Medicinal Chemistry 20 (2012) 554–570

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Diazirine based photoaffinity labeling

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ARTICLE INFO

Article history:
Available online 29 June 2011

Keywords:
Diazirine
Photoaffinity labeling
Photochemistry
Chemical proteomics
Protein profiling

ABSTRACT

Diazirines are among the smallest photoreactive groups that form a reactive carbene upon light irradiation. This feature has been widely utilized in photoaffinity labeling to study ligand–receptor, ligand–enzyme and protein–protein interactions, and in the isolation and identification of unknown proteins. This review summarizes recent advances in the use of diazirines in photoaffinity labeling.

1. Introduction

The study of interactions between ligands and macromolecules is key to advance our understanding of the chemistry of life. The concept of photoaffinity labeling (PAL) was introduced by Frank Westheimer in the early 1960s,[1] and since then it has revolutionized research in the life sciences. The ability to introduce targeted covalent ligands (e.g., fluorophores, crosslinkers, isotopic labels, spin labels, etc.) to biological systems has dramatically enhanced our capacity to ask and resolve fundamental questions, and to design new ligands for proteins that play a role in pathological processes. In recent years various kinds of possible interactions have been targeted successfully, such as protein–ligand (drug, inhibitor); protein–protein; protein–nucleic acid; and protein–cofactor.[2] In the process of photoaffinity labeling a ligand is covalently modified with a photoreactive group (PG), which upon irradiation generates a reactive species that covalently binds the ligand to its target macromolecules. This modification can then, for instance, be used to investigate ligand–receptor interactions; to identify the location of an enzyme inhibitor; to isolate and identify unknown enzymes or receptors; or to identify amino acid residues at protein–protein or protein–lipid interfaces. After covalent modification the desired protein is then usually purified by chromatography and characterized using spectroscopic methods.

Three major types of PGs are commonly used in PAL: benzophenones (**1**), arylazides (**2**) and diazirines (**3**) (Fig. 1). Benzophenones generate reactive triplet carbonyl states upon irradiation. One disadvantage is that benzophenones sometimes need a long period of irradiation. On the other hand the advantage in using

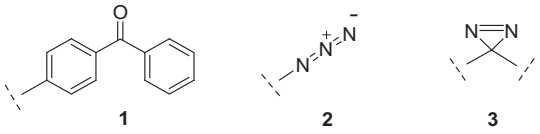

Figure 1. Three types of PGs: benzophenones (**1**), arylazides (**2**) and diazirines (**3**).

benzophenones is a long wavelength of irradiation and inertness to the solvent. However, the long irradiation period bears a risk for non-specific labeling.[3] Its wide use has revealed a good labeling potential—a few examples will be discussed further on. Arylazides are easily prepared, however the short wavelengths at which they are excited may damage biological macromolecules. Besides the desired carbenes among the products of the irradiation are nitrenes.[4] The latter decrease photolabeling yields and cause non-specific labeling while the rearranged keteimines[5] may leave the desired labeling site. Several studies compared between probes containing azides and diazirines and the latter appeared to be more stable.[6,7]

One potential pitfall in the use of PGs is that such a modification may significantly alter those features of the ligand that are essential for its biological activity. Especially in the case of small molecules, when probing the interaction with a protein, the photophore should be as small as possible in order to generate a suitable mimic of the original ligand. Of all commonly used PGs in PAL the smallest ones are diazirines. Upon irradiation, diazirines generate reactive carbenes which are bivalent carbon intermediates containing two non-bonding orbitals with two options of division of two electrons between them: singlet, two spin-paired electrons, and triplet spins of the electrons are parallel (Fig. 2).[8]

The singlet state can be stabilized by substituents that donate electrons to the empty p orbital.[9] Aliphatic diazirines, for example,

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doi:10.1016/j.bmc.2011.06.066

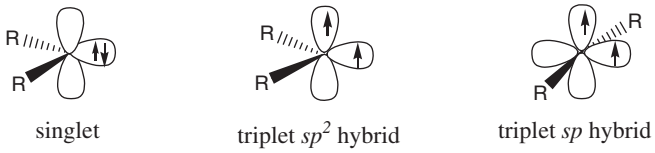


Figure 2. Representation of the electronic states of the carbene.

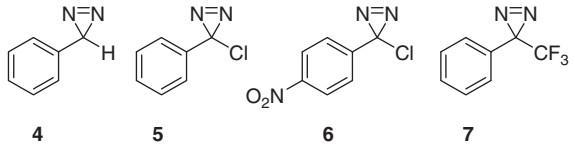


Figure 3. Aryldiazirines **4**–**7**.

isomerize to a diazomethane upon photolysis and consequently produce the singlet state carbine,[10] although 3*H*-diazirine might give a triplet state upon photoactivation.[11] Phenyldiazirine (**4**, Fig. 3) and phenylchlorodiazirine (**5**, Fig. 3) produce the singlet phenylcarbene,[12] while *p*-nitrophenylchlorocarbene (**6**, Fig. 3)[13] and trifluorophenylcarbene[14,15] have a triplet ground state (**7**, Fig. 3).

The most significant feature of carbenes is their ability to rapidly form a covalent bond with the nearest target molecule—through C–C, C–H, O–H and X–H (X = heteroatom) insertions. In addition to its small size, further advantages of the diazirine group are its stability at room temperature; its relative stability to nucleophiles (such as thiols), and both acidic and alkaline conditions; its absorption at longer-wavelengths (350–355 nm), reducing damage to the targeted biological system; and its short lifetime upon irradiation and high subsequent reactivity.[7] Nevertheless, despite these many advantages, there are reports in which diazirines failed to label proteins where other photophores proved successful. In an early study by Smith and Knowles (1973) aryldiazirine derivatives were suggested as potential photoaffinity probes.[16] Since singlet aryldiazirine undergoes photoizomerization, one of the possible products of the irradiation besides the singlet state carbene is a long-lived linear diazo compound. To overcome this process an alternative carbene precursor would be trifluoromethylaryldiazirines.[17] Seventy two percent of the probes described in the current review contain a trifluoromethylaryldiazirine moiety, which shows favorable photochemical properties and excellent chemical stability. Furthermore, a great deal of work has been invested in improving its synthesis. In cases where there are spatial limitations, a good compromise is to use an aliphatic diazirine, which will serve both as a good mimic of the ligand or inhibitor as well as a successful photoaffinity label.

In general, the yields of photolabeling are low since carbenes are quenched quickly by reaction with water molecules.[18] However, this feature is actually an advantage since it minimizes unspecific labeling; only those ligand molecules that are bound tightly by their host receptor/enzyme will react covalently with the protein, whereas unbound ligand molecules will react with water before being able to undergo non-specific reactions with other proteins.

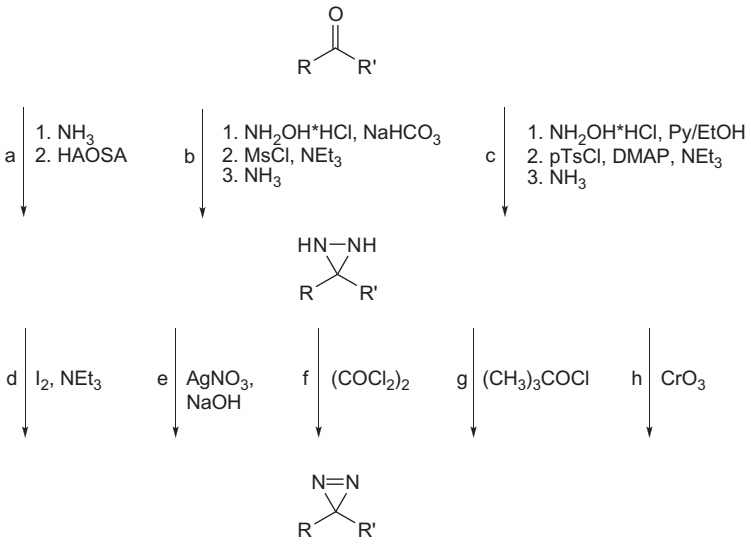
As there is a strong increase in the number of studies in which the diazirine group is used in photoaffinity labeling, the current review looks at recent advances in the use of this particular photophore. Six years ago Blencowe and Hayes published a comprehensive review on the use of diazirines in photoaffinity labeling.[19] The current review thus has a focus on diazirine based labeling methods (including a summary of synthetic methods to prepare diazirines) within the past 6 years. While most of the described studies bear no particular relationship to one another—apart from their use of diazirinyl photoactivity labels—we decided to divide the studies into groups according to the particular ligand or protein of interest. Thus, we will describe the use of diazirine in PAL of receptors, enzymes, DNA and RNA; its use in the development of drugs; and its use in the study of ligand–membrane, lipid–protein and protein–protein interactions.

2. Synthetic methods to prepare diazirines

Several synthetic methods to prepare diazirines have been described. The main strategy is to start from a ketone, modify it to a diaziridine group (Table 1, step 1) followed by oxidation to a diazirine (Table 1, step 2). The most commonly used diazirine in PAL is 3-aryl-3-trifluoromethyl-3*H*-diazirine. The diaziridine precursors are prepared from the corresponding α,α,α-trifluoroacetophenone via oximation and tosylation using sequential treatment with hydroxylamine, tosyl chloride and ammonia. The oximation step is performed by reaction of the ketone with hydroxylammonium chloride at 60–85 °C,[20,21] usually in pyridine and ethanol (though other bases, such as NaOH[22] or $NaHCO_3$ (Scheme 1, b),[23] are used as well). This is usually followed by tosylation (or mesylation) in the presence of pyridine, dimethylaminopyridine or triethylamine (Scheme 1).[24] Upon treatment of the tosyl (or mesyl) oxime with ammonia the diaziridine is formed (Scheme 1, b, c).[20,21,24,25] Kass and Broadus found that addition of the Lewis acid catalyst ytterbium (III) trifluoromethanesulfonate aids in activating the tosyl oxime.[26] A different route to prepare the diaziridine is by direct reaction of a ketone with ammonia and an aminating reagent such as chloramines or hydroxylamine-*O*-sulfonic acid (Scheme 1, a).[27] Finally, the desired diazirine is prepared by oxidation of the corresponding diaziridine with a variety of oxidants: chromic acid (Jones oxidation) in acetone (Scheme 1, h)[28]; iodine in the presence of NEt_3 in methanol[20] or dichloromethane (Scheme 1, d)[27]; silver oxide (Scheme 1, e)[29]; oxalyl chloride (Scheme 1, f)[24] or $(CH_3)_3COCl$ (Scheme 1, g).[25] Less used is electrochemical oxidation of the diaziridine on a Pt/Ti anode.[30] The diazirine can also be prepared by heating the tosyl oxime in the presence of ammonia at 50 °C for 2.5 h, though it

Table 1
Summary of reported diazirine syntheses; reagents and conditions

Step to diaziridine (step 1)	Step to diazirine (step 2)	Ref.
1. NH_3, 0–10 °C, MeOH 2. HAOSA, −12 °C 3. +7 °C O/N	Jones oxidation in acetone	28
1. $NH_2OH{\cdot}HCl$, Py/EtOH, 60 °C 2. *p*TsCl, DMAP, NEt_3, DCM, 0 °C to rt 3. $NH_{3(l)}$, DCM, −78 °C to rt	I_2, NEt_3, MeOH, rt	20
1. $NH_2OH{\cdot}HCl$, Py/EtOH, 85 °C 2. TsCl, Py, 85 °C 3. NH_3, EtOH, −78 °C to rt	I_2, NEt_3, MeOH, 0 °C to rt	21
1. $NH_{3(l)}$, −35 °C 2. HAOSA, −78 °C, MeOH	I_2,, NEt_3, DCM, rt	27
1. $NH_2OH{\cdot}HCl$, Py/EtOH, 60 °C 2. *p*TsCl, DMAP, NEt_3, DCM, 0 °C to rt 3. $NH_{3(l)}$, DCM, −78 °C to rt	$(COCl_2)_2$/DMSO/DCM, −78 °C	24
1. $NH_{3(l)}$, MeOH, −78 °C, 10 min 2. HAOSA, −78 °C, 2 h	Electrochemical oxidation on Pt/Ti anode	30
1. $NH_{3(l)}$, −35 °C, 3 h 2. HAOSA, −60 °C, MeOH, 3 h	$AgNO_3$ (in H_2O), DCM, NaOH	29
1. $NH_2OH{\cdot}HCl$, $NaHCO_3$, H_2O 2. MsCl, NEt_3, DCM, 0 °C 3. NH_3, ether, −25 °C, 3 d	$(CH_3)_3COCl$, ether, 0 °C	23



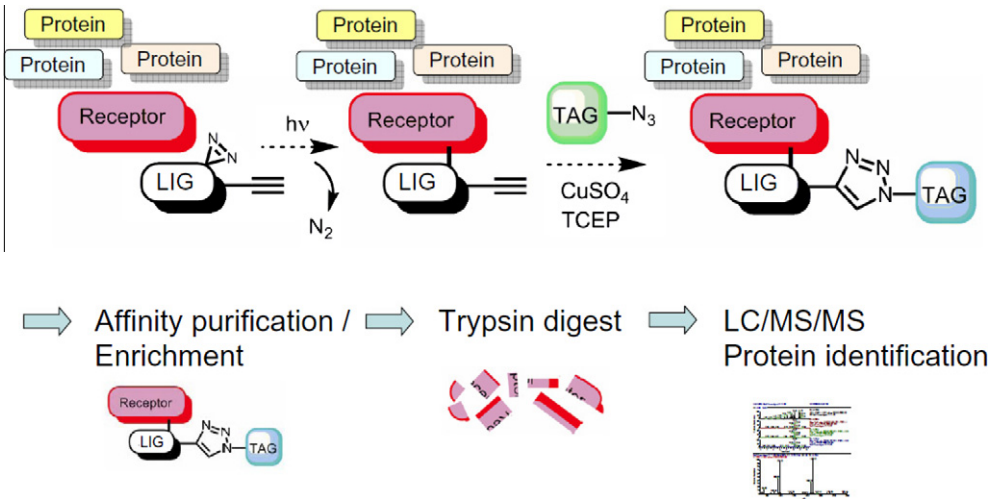
Scheme 1. Diazirine syntheses. HAOSA: hydroxylamine-*O*-sulfonic acid; MsCl: mesyl chloride; Py: pyridine; *p*TsCl: *p*-tosyl chloride; DMAP: *N*,*N*-dimethyl-4-aminopyridine.

should be noted that the tosyl oxime has to be stable at this temperature.[26]

3. Receptor labeling

Diazirines are widely used to study receptor–ligand interactions, or to identify unknown receptors. Upon ligand attachment, a receptor may or may not change its conformation, which can lead to its activation or deactivation. The receptor may have more than one binding site, and by analyzing peptides obtained through digestion (often by trypsin) of the receptor after ligand binding followed by irradiation, the binding site(s) can be identified. In recent years significant advances were made in the development of methodologies to identify and profile enzymes and enzyme families, spearheaded by the groups of Cravatt and Bogyo. They advanced the field of activity based protein profiling, in which biomimetic probes directly monitor the functional state of large protein families, by providing a complete profile of the active protein present in a given proteome. Such activity based probes (ABPs) can generally be defined as reagents that meet the following criteria[31]: (a) they react directly with a broad range of proteins from a particular class in complex proteomes; (b) they react with such proteins in a manner that correlates with their activities; (c) they display minimal cross-reactivity with other classes of proteins; (d) they possess either a tag, or a handle that will react specifically with a tag, for rapid detection and isolation of the proteins. One of the most widely used tags is biotin, due to its ability to be specifically identified with use of avidin beads. The tag might also be a fluorescent moiety allowing for protein identification by visualizing the tagged bands on the gel. Also, radioactive labeling, by iodine-125 for instance, has been developed and proved very successful. If the handle is a functional group that can react with an appropriate partner in a highly specific bioorthogonal (or 'click') reaction, then this modification can be used to isolate and enrich the modified protein from the cell lysate, using a strategy shown schematically in Scheme 2.

ABPs have been employed successfully to characterize many enzyme classes, including serine,[32–34] cysteine,[35,36] aspartyl[37] and



Scheme 2. Labeling of unknown receptors with use of activatable bifunctional probes. Addition of two minimally perturbing moieties to a ligand (LIG) will allow for specific binding to a targeted receptor. Irradiation of the diazirine, as an example for the photoreactive group, with UV light (360 nm) will result in a covalent bond between the probe and the receptor. A highly specific 'click' reaction—for example, the copper(I) catalyzed 1,3-dipolar azide–alkyne cycloaddition—with a tag (or fluorophore) will then allow for affinity purification and identification of the receptor, after digestion and LC/MS/MS analysis.

metallohydrolases,[38] kinases,[39] glycosidases,[40] histone deacetylases[41] and oxidoreductases.[42] By introducing a photoreactive group to the ABP, proteins that do not contain a reactive nucleophile in their binding site, such as most receptors, can be targeted. The following section focuses on the use of diazirines in receptor labeling.

3.1. Plant hormones

The brassinosteroid insensitive 1 (BRI1) protein is known to have an efficient role in the recognition and signaling of a class of plant steroid hormones called brassinosteroids (BRs). These hormones are involved in a wide variety of important regulatory processes, such as cell elongation and vascular differentiation. A BR binding site, however, has for long been elusive. In a breakthrough study Kinoshita et al. demonstrated that BRI1 binds directly to physiologically active brassinosteroids, and the authors defined a new binding domain for steroid hormones.[43] A photoreactive analog of castasterone (**8**, Fig. 4), a precursor of brassinolide, was examined for its ability to bind its suspected receptor, BRI1, fused with a green fluorescent protein.

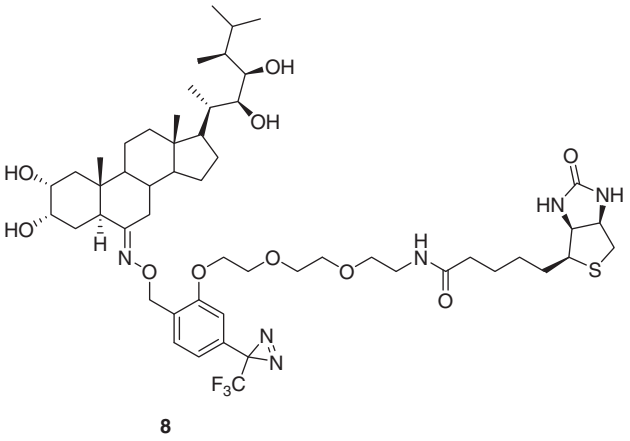


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Figure 4. Photoreactive analog of castasterone.

After the successful identification of this brassinosteroid receptor,[43] a similar PAL strategy was applied to other plant hormones. Jasmonate (JA) plays an important role in plant defense. The interaction between jasmonate and a cytosolic F-box protein, called coronatine insensitive 1 (COI1), was tested using the PAL probe PACOR (**9**, Fig. 5), which is an analog of coronatine. Coronatine acts as a molecular mimic of the Ile-conjugated form of JA (JA-Ile)—the form that promotes physical interaction of COI1 with a negative regulator of JA signaling. In this study, Yan et al. showed that PACOR physically binds to COI1, and together with genetic evidence these experiments prove that COI1 is a receptor for JA.[44] In a follow up study, the authors examined two similar ABPs (**10**–**11**, Fig. 5), in order to assess their ability to mimic PACOR. The very first PACOR (**9**, Fig. 5), however, exhibited the strongest binding, supporting its use as a tool for elucidating the interaction between COI1 and JA.[25]

Nakamura et al. described the synthesis of a new photoaffinity label to target a membrane target protein (MTJG) in motor cells of the large tropical tree *Albizia saman*, using a benzophenone derivative of jasmonate glucoside.[45] A recent paper describes advantages of biaryl-linked molecular probes, including the diazirine based probe **12** (Fig. 6), in order to develop a general technique to label low abundant membrane proteins. Probe **12** provided superior labeling efficiency over the other probes.[46]

In a more recent study, the same group prepared PAL analogs of potassium isolespedezate, a bioactive substance that affects leaf opening of the leguminous plant *Cassia mimosoides L.* Diazirine probes **13** and **14** (Fig. 7) were examined for their ability to label and bind the cells efficiently. Probe **13** failed to label any specific proteins, while probe **14** was successful in labeling two unknown proteins (180 kDa and 210 kDa). Nevertheless the benzophenone probe **15** (Fig. 7) appeared to show better labeling results and the follow-up study was based on this probe.[47]

Recently the MetE (5-methyl-tetrahydropteroyl-triglutamate homocysteine methyl-transferase) was isolated and identified as a cytosolic target protein of potassium isolespedezate. In this

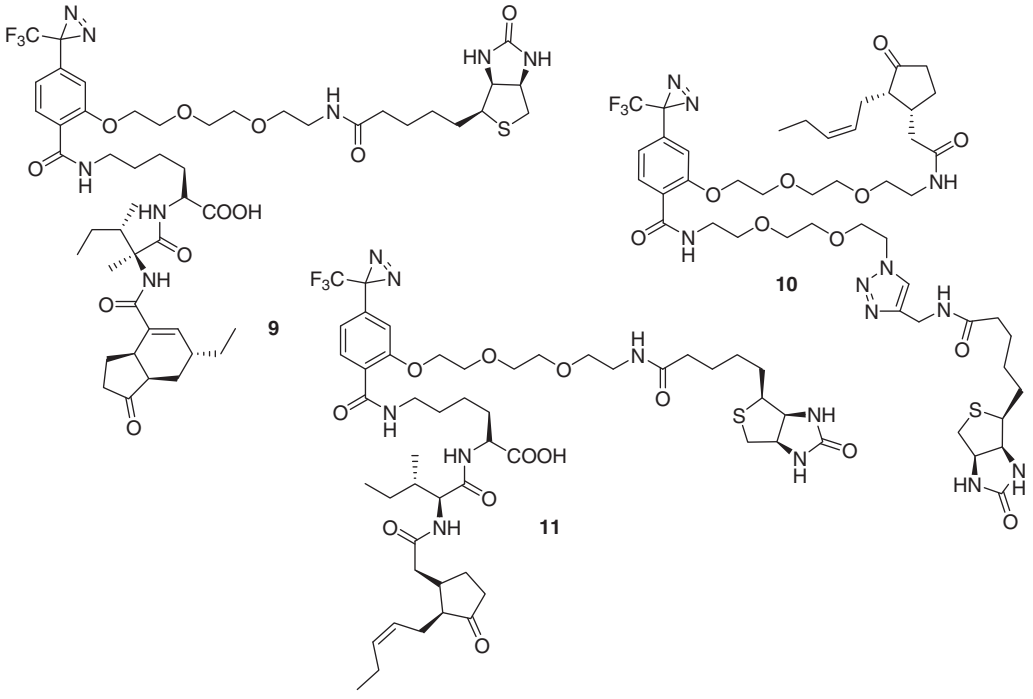

Figure 5. Activity based probes of jasmonate.

particular study the iodoacetamide probe (**16**, Fig. 7) was successful in the labeling over the diazirine probe (**17**, Fig. 7).[48]

3.2. Interkingdom signaling

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that uses *N*-(3-oxododecanoyl) homoserine lactone (OdDHL) as a primary quorum sensing molecule. A growing number of reports indicate that homoserine lactone autoinducers can influence gene expression in eukaryotic cells.[27] This effect is highly specific for OdDHL, and small changes in its structure, such as shortening or elongation of the lipid chain resulted in a marked decrease in activity. Thus in order to identify the putative receptor of OdDHL in eukaryotic cells using a dual tagging strategy (Scheme 2) Dubinsky et al. prepared an analog containing a diazirine group and an alkyne moiety. This diazirine analog, **18** (Fig. 8), was shown to be a good mimic of the native OdDHL in bone marrow derived macrophages and in the fungus *Candida albicans*. Labeling experiments showed that under the cross-linking conditions a significant fraction of a heterogolously expressed OdDHL receptor, LasR, was labeled covalently and specifically by the diazirine probe.[27]

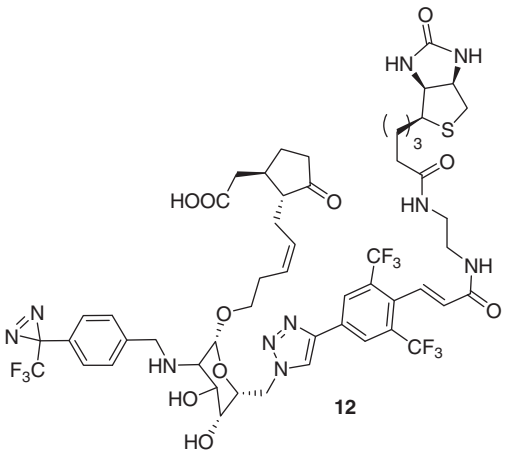

Figure 6. Photoaffinity probe **12**, based on jasmonate glucoside.

4. Drug development

Photoaffinity labeling has recently been applied in the development of several drugs and drug targets (e.g., epilepsy, pain relief, microbial infections, and bone disease).

4.1. Neurochemistry

Diazirine probe **19** (Fig. 9) was based on the recently approved drug lacosamide (Vimpat), which selectively enhances the slow inactivation of voltage gated sodium channels. The diazirine analog (**19**, Fig. 9) of this drug was found to be more effective than the parent drug in preventing epileptic seizures. However, no covalent linking of the probe to the receptor was achieved, probably due to photodegradation of the (trifluoromethyl)diazirine moiety.[49]

In another example of PAL to study neuroactive receptors, Balas et al. reported the synthesis of photoactivatable anandamide probes, to target cannabinoid receptors; diazirine probes **20** and **21** (Fig. 10) appeared too stable when irradiated, while arylazide probe **22** (Fig. 10) was successfully used to label the cannabinoid receptors.[50]

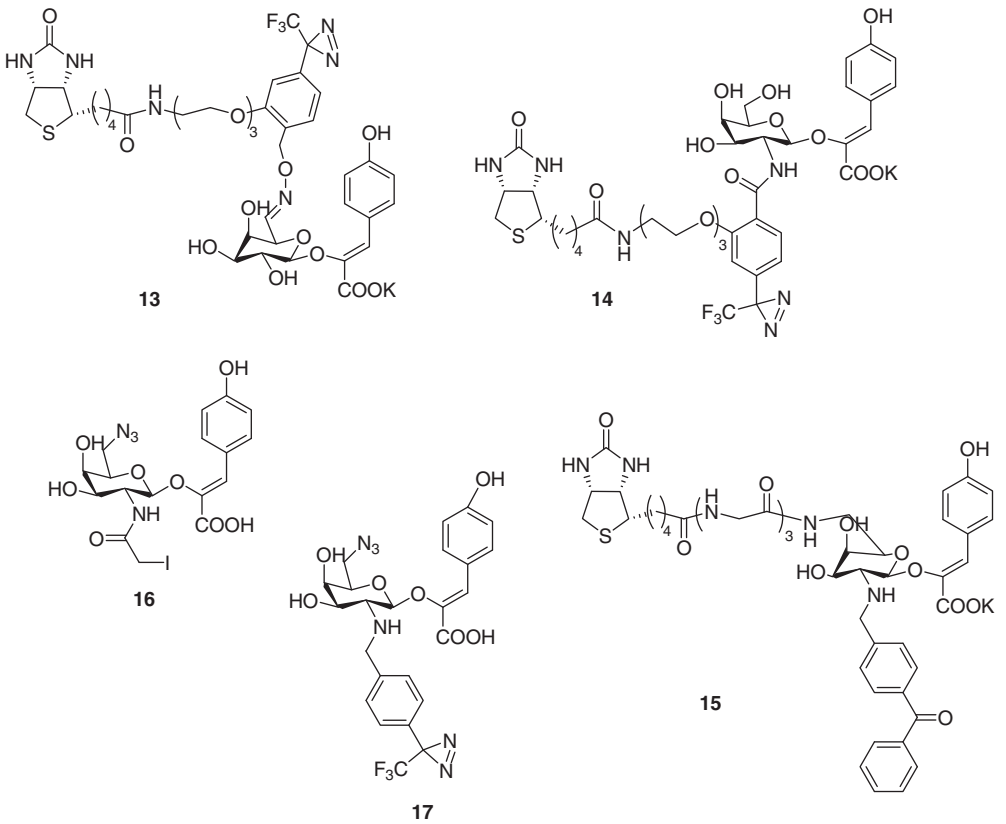

Figure 7. PAL analogs of potassium isolespedezate.

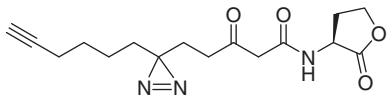

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Figure 8. OdDHL based photoaffinity analog.

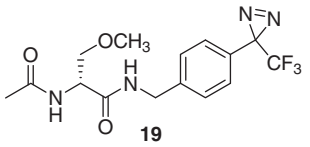

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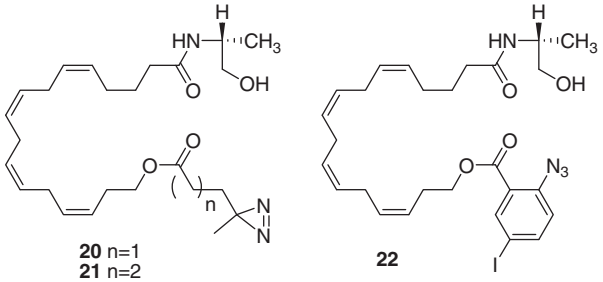
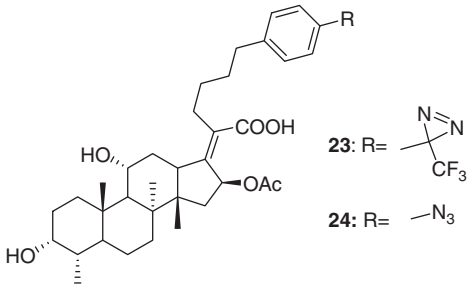
Figure 9. Lacosamide based diazirine probe.



20 n=1
21 n=2

22

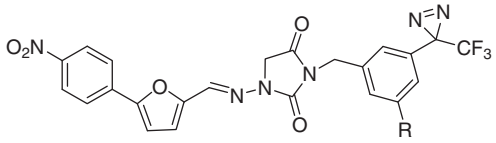
Figure 10. Anandamide based photoaffinity labels.



23: R=

24: R=

Figure 11. Fusidic acid based photoaffinity labels.



25: R=I **26:** R=I^{125} **27:** R=CH_2N_3 **28:** R= CCH

Figure 12. Dantrolene based photoaffinity labels.

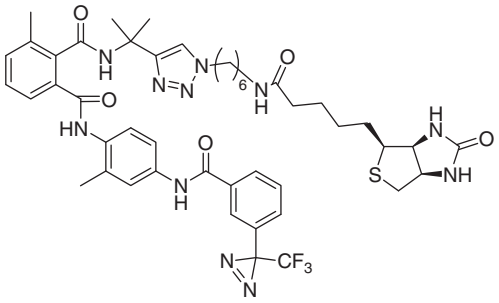


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Figure 13. Fubendiamide diazirine based photoaffinity label.

4.2. Antibiotics

In another study where arylazide and diazirine probes were compared, Riber et al. prepared fusidic acid photoaffinity labels. Fusidic acid inhibits *Staphylococcus aureus* protein synthesis by interference with its elongation factor G (EF-G) receptor. Despite potent observed bioactivity of the fusidic acid diazirine analog (**23**, Fig. 11), the labeling of the EF-G was successful only for the arylazide probe (**24**, Fig. 11).[51]

4.3. Calcium signaling

Dantrolene is a known muscle relaxant in clinical use. It is the primary treatment to relieve malignant hyperthermia, a rare, but life-threatening complication that can occur during anesthesia due to abnormal Ca^{2+} release. Dantrolene is a nonselective inhibitor for both, physiological Ca^{2+} release (PCR) and Ca^{2+}-induced Ca^{2+} release. Both these processes are mediated by a ryanodine receptor in skeletal muscle cells that is responsible for the control of Ca^{2+} concentrations. Diazirine probes **25**–**28** (Fig. 12) showed selective inhibition of calcium release and labeled a specific protein, the identification of which is currently under investigation.[52]

A different calcium release channel that was investigated with the use of a diazirine photoaffinity label, is RyR, responsible for Ca^{2+} release in insect muscle microsomal membranes. This process is considered to be affected by flubenediamide a potent insecticide. Photoaffinity labeling of cloned silkworm RyR (sRyR), by a flubendiamide diazirine derivative (**29**, Fig. 13), revealed that flubendiamide is mainly incorporated into the transmembrane domain, indicating its importance in establishing a mechanism of action for this insecticide.[53]

In yet another example of the use of diazirine labels in studying Ca^{2+} signaling disorders, Lee et al. prepared photolabeled analogs of tanshinones. These natural products, that are isolated from the traditional Chinese medicinal herb, *Salvia Miltiorrhiza Bunge* (or Danshen roots), have been shown to inhibit osteoclast differentiation. Diazirine probes **30** and **31** (Fig. 14) were shown to block the RANKL/RANK (receptor activator of nuclear factor kappa-B ligand/receptor activator of nuclear factor kappa-B) signaling pathway, elucidating a potential mechanism of action for the tanshinones. The probes were also found to be potent inhibitors of RANKL-induced osteoclastogenesis.[54]

Another natural product used in Chinese medicine is paeoniflorin, isolated from *Paeonia lactiflora* Pall roots. This compound has been shown to affect calcium signaling and was found to have antithrombin and anticonvulsive effects, and its protein target was proposed to be the adenosine A1 receptor. A trifunctional photoaffinity probe (**32**, Fig. 15), containing a biotin moiety as a handle for purification, was synthesized in order to label this putative receptor. Preliminary experiments suggest that **32** indeed is able to label the adenosine A1 receptor specifically, and therefore provides a potential tool for purification and identification of the target protein, as well as for elucidation of its binding site.[55]

4.4. Anti-cancer agents

Ispinesib is a potent anti-tumor agent, which acts by inhibiting the kinesin spindle protein (KSP), resulting in arrest of mitosis, and

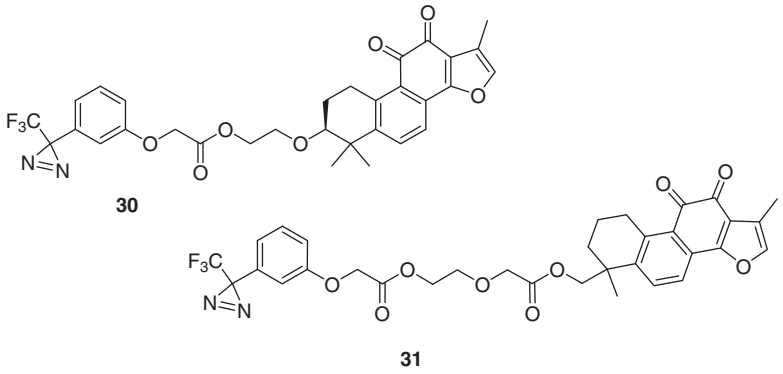


Figure 14. Diazirinyl analogs of tanshinones as photoaffinity labels

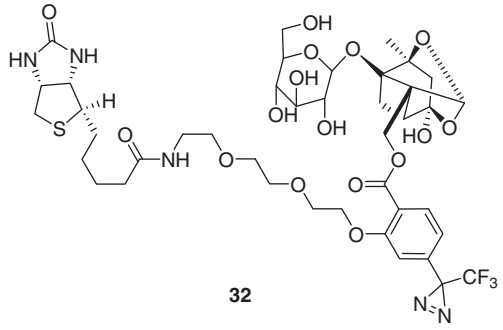


Figure 15. Paeoniflorin based photoaffinity label.

its efficacy is tested currently in phase 2 clinical trials. One of the main challenges in developing anti-cancer drugs is to overcome the formation of resistant cells. An important study by Luo et al. described the synthesis and evaluation of new biphenyl urea inhibitors of KSP that are active against ispinesib-resistant cell lines. Using a diazirine-based inhibitor (**33**, Fig. 16), in addition to site directed mutagenesis, the authors discovered that the novel inhibitors compete with ATP binding via a novel allosteric mechanism.[56]

4.5. Anesthetics

There is strong interest in the use of small photoaffinity labels in the investigation and development of anesthetics, which induce a reversible loss of sensation. Although much is known about the use of anesthetics, in most cases the main interaction between the drug and its target receptor is still unknown. Usually there is low affinity between the anesthetic and the target receptor.[57] And as most anesthetics are small and volatile molecules, the use of diazirines to affect minimal structural modifications is advantageous. In an important study, Eckenhoff et al. showed that the diazirine analog of the known anesthetic isoflurane (**34**, Fig. 17) (azi-isoflurane, **35**, Fig. 17), attaches to the crystallographically defined binding sites of isoflurane.[58] This promising reagent is currently used for identification of other molecular targets of isoflurane.

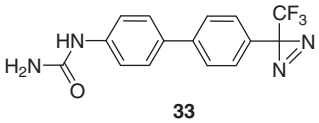


Figure 16. Diazirine based biphenyl urea inhibitors of KSP.

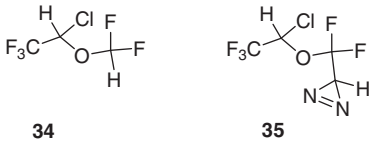


Figure 17. Isoflurane (**34**) and its diazirine analog (**35**).

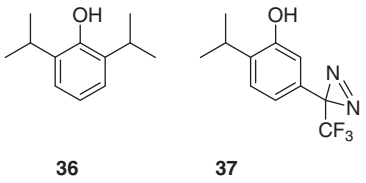


Figure 18. Propofol (**36**) and its diazirine analog (**37**).

A different general anesthetic agent whose mechanism of action is not fully understood, is propofol (**36**, Fig. 18),[59] which is administered intravenously. The interaction between propofol with the gamma aminobutyric acid A ($GABA_A$) receptor was assessed using photoaffinity labeling, in order to determine its binding site. Diazirine analog of propofol (*m*-azipropofol (**37**, Fig. 18)) was irradiated in the presence of the $GABA_A$ receptor, followed by digestion and LC/MS/MS sequencing, revealing that the analog's adducts were located at a previously identified propofol binding cavity.

A related anesthetic agent is etomidate (**38**, Fig. 19),[60] which, similar to propofol has been proposed to act through interaction with the GABA receptor. Early studies by Husain et al. focused on photoreactive analogs of etomidate with various photoreactive groups at the ethoxy end of this probe (e.g., **39**, **40**, Fig. 19).[61,62] It was found that benzophenone, trifluoromethyldiazirinyl and 3-azibutoxy analogs are all potent general anaesthetics in addition to being effective and selective photolabels.[61,63] In a recent published paper,[64] Husain et al. reported an additional photoreactive analog of etomidate, TFD–etomidate (**41**, Fig. 19), with a trifluoromethyldiazirine moiety attached to the phenyl ring. While **41** induced the same effect as etomidate, it appeared to have a different binding site. It is important to note that the incorporated photoactivating groups do not significantly alter the binding affinity of the ligand to its receptor or its functionality, compared with the native ligand.

The metabotropic $GABA_B$ receptor, like the $GABA_A$ receptor, is a key regulator of the central nervous system. The mechanism that regulates its oligomerization at the plasma membrane is not known yet.[65] The trifunctional fluorescent photoaffinity label (**42**,

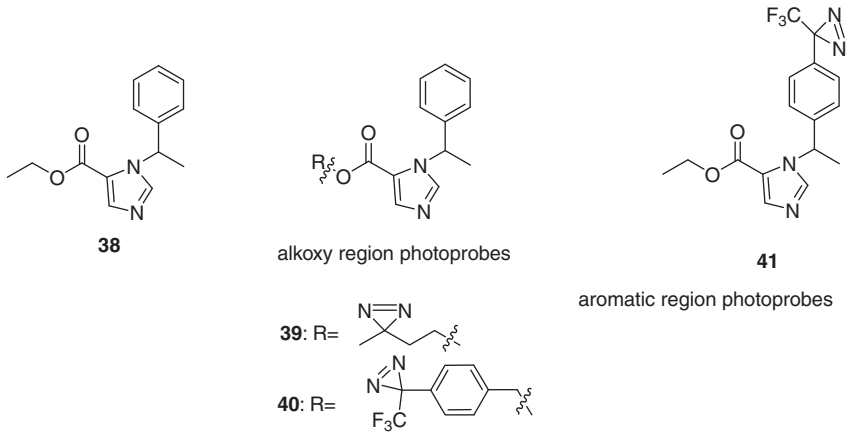


Figure 19. Etomidate (**38**) and its diazirine based photoaffinity probes (**39**–**41**).

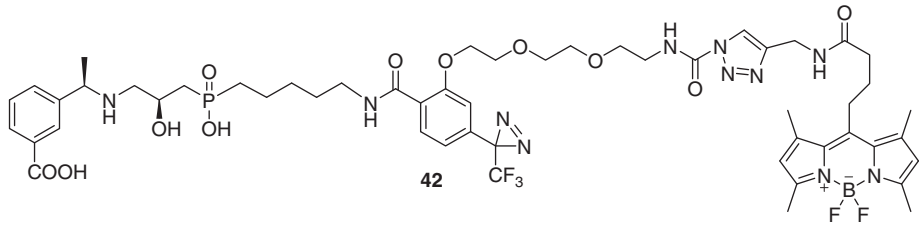


Figure 20. Photoaffinity label **42**, based on a $GABA_B$ receptor antagonist.

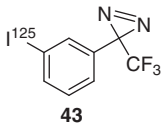


Figure 21. Nicotinic acetylcholine receptor photoaffinity label **43**.

Fig. 20) that was used in a study by Li et al. is based on a selective high affinity antagonist of the receptor. The $GABA_B$ receptor was successfully labeled in living cells suggesting that diazirine probe **42** may be used successfully in direct studies of $GABA_B$ receptor dynamics in living cells.

The nicotinic acetylcholine receptor (nAChR) is a member of the Cys-loop ligand gated ion channel superfamily (LGICs).[66] Due to its abundance and key role in neurochemistry, it is the most studied and best understood member of this enzyme family. After binding of the receptor by its ligand, the ion channel switches from a closed to an open state.[67] Receptor activation is terminated by either removal of the ligand or by desensitization.[68] 3-(trifluoromethy1)-3-(m-[^{125}I]iodophenyl)diazirine (TID) was one of the first trifluoromethyl diazirine based photoaffinity labels to be synthesized and applied (**43**, Fig. 21).[69] TID is a hydrophobic probe, which is used in studying the binding sites or states (open/desensitized) of nAChR and other membrane proteins. Advances in understanding which residues interact with this agonist were achieved by Addona et al., after introduction of an improved method of time-resolved photolabeling, using small amounts (nmol) of nAChR and short ligand exposure (1 ms).[70]

While the structure of the receptor is already known, the conformational change during ligand binding is not yet fully characterized. Recent studies by Arevalo et al. employed TID with time-resolved photoaffinity labeling in order to examine agonist induced conformational changes.[71] The authors discovered that the structure of the extracellular end of the transmembrane domain of the δ-subunit changes when the channel opens. A follow-up study by Yamodo et al. aimed to determine the phase of desensitization in which the former structural change is reversed and to search for similar changes in other subunits.[72] The results revealed changes in three distinct domains of nAChR in which the structures of the closed, open and fast desensitized states are all different, indicating the confirmation of the hypothesis about existence of separate gates for channel opening and desensitization

Leukotrienes are lipid mediators involved in numerous diseases. A key step in the biosynthesis of leukotrienes from arachidonic acid is mediated by 5-lipoxygenase. Kumar and Young described the synthesis of a tri-functional, activity based probe (**44**, Fig. 22) that is based on a known 5-lipoxygenase inhibitor (**45**, Fig. 22), in order to use in a dual tagging strategy (Scheme 2).[24,73] More recently the same authors introduced a tritium label into this probe, resulting in photolabel **46** (Fig. 22). Using this radiolabel the interaction between **46** and the labeled protein was studied.[73]

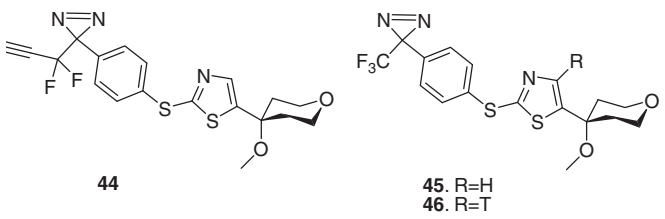


Figure 22. Diazirine probes **44**–**46**, based on 5-lipoxygenase inhibitors.

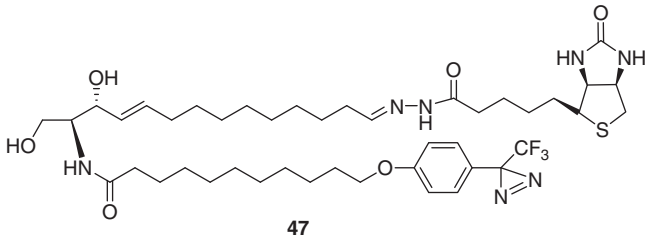


Figure 23. Diazirine based bifunctional photoaffinity label **47**.

5. Lipid–protein interactions

After purification and reconstitution of the *Torpedo* nAChR, Blanton and co-workers published a PAL study of the 5-HT_3 receptor, which is, like the nAChR, a LGICs.[74] The 5-HT_{3A} subunit has functional significance in both, the central and the peripheral nervous system. Furthermore, the 5-HT_{3A}R M4 helix was found to be exposed to a lipid area. Since there is 22–30% homology between the 5-HT_{3A} and nAChR subunits, the same photoaffinity probe that was used to study nAChR, TID (**43**, Fig. 21), was also used to study the lipid surface that 5-HT_{3A} is exposed to.[75,76] Interestingly, the lipid surface surrounding the *Torpedo* nAChR is equivalent in structure to the surface that flanks the 5-HT_{3A}R M4 helix.[74]

Due to their high abundance (both intracellular and membranous), and their physiological relevance, sphingolipids have been investigated extensively. Several diazirine-based photoaffinity labels were reported by Hashimoto and Hatanaka.[77,78] The authors performed biological experiments with this glycosphingolipid, revaeling a lack of recognition by sphingolipid ceramide N-deacylase. On the other hand, the ceramide diazirine derivative (**47**, Fig. 23) showed more promise as a substrate for the enzyme.[74]

6. Diazirinyl amino acids

In 2005, Thiele and co–workers first introduced diazirine containing amino acids. These methionine/leucine/isoleucine analogs (**48**, **49**, **50**, Fig. 24, designated photo ile, leu, met, resp.) contain a diazirine group positioned such that the corresponding tRNA partially recognizes and incorporates them into a protein, functionally replacing the natural amino acids.[79]

One of the uses of photo-met was reported by Kent and co-workers, who incorporated the photoactive amino acid into the trypsin inhibitor type II (EETI-II) of the squash *Ecballium elaterium* using Boc chemistry solid phase peptide synthesis.[80] Similarly,

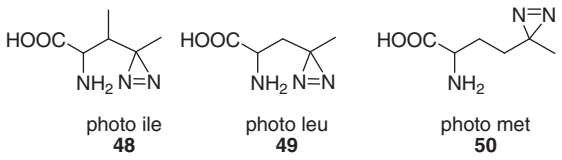


Figure 24. Photoactive amino acids **48**–**50**.

Muir and co-workers introduced photo-met into the semi-synthetic MH2 domain of the Smad2 signaling protein (which is involved in embryogenesis), by an expressed protein ligation technique. The MH2–MH2 interaction was captured, showing that this technique can be applied to the study of protein–protein interactions.[81]

MacKinnon et al. incorporated photo-leucine into the fungal cyclodepsipeptide which inhibits the expression of the vascular cell adhesion molecule (VCAM). A heterotrimeric membrane protein, the Sec61 complex, was assumed to be the peptide's target. The labeling analysis revealed that Sec61α is in fact the target of the inhibitor.[82]

Jiao et al. reported the acylation of lysine with a trifluoromethyldiazirine moiety, and then introduced photo-lys into penetratin. Penetratin is a cationic membranotropic peptide, a prototype of a cell-penetrating peptide which enters eukaryotic cells and exerts antimicrobial activity.[83]

Another lysine based diazirine amino acid (**51**, Fig. 25) was recently synthesized by Chou et al., showing that the unnatural amino acid was incorporated by tRNA into bacterial and mammalian proteins. Furthermore, efficient cross-linking was demonstrated on a test model protein.[84]

As the most widely used diazirine derivative is based on the 3-aryl-3-trifluoromethyl-3*H*-diazirine moiety, modification of phenylalanine with a trifluoromethyl diazirine group (**52**, Fig. 25, photo-phe) was a highly pursued synthetic target. Several synthetic challenges were met upon alkylating the aromatic diazirine, but the development of a straightforward synthesis of this phenylalanine analog by Hatanaka and co-workers provided an answer to these problems. The photo-phe was incorporated into the calmodulin binding peptide (CBP) by automated peptide synthesis, and CBP was shown to specifically label calmodulin upon irradiation of the CBP-calmodulin complex.[85]

A different application of photo-phe was described in a study on the mechanism of action of human angiotensin II (AngII), a hormone that is involved in the regulation of blood pressure. Fillion et al. presented a new stereospecific convergent synthesis procedure to prepare diazirine and benzophenone analogs of AngII, which were then used to investigate the interaction of AngII and its target, the AngII type 1 receptor. Using a mutant AngII type 2 model receptor of human origin, the authors investigated Met-selectivity and reactivity of a carbene-generating compound compared to the widely used biradical ketone-generating *p*-benzoyl-L-phenylalanine probe. It appears that a diazirine probe is not Met-selective and use of the benzophenone probe will allow a better determination of ligand–receptor binding points.[86]

The use of photo-phe was subsequently expanded to site specific incorporation into *Escherichia coli*, using non-natural amino acid incorporation, and Schultz and co-workers presented this incorporation with high efficiency and fidelity.[87]

A different use of photo-phe was reported by Masuda et al., who targeted the sweet taste receptor with diazirinyl analogs of D-photo-phe derivatives **52** and **53** (Fig. 25), that were synthesized and tested for their ability to activate the human sweet taste receptor. Both analogs were active, and even displayed stronger activity than D-tryptophan and D-phenylalanine, indicating that they can

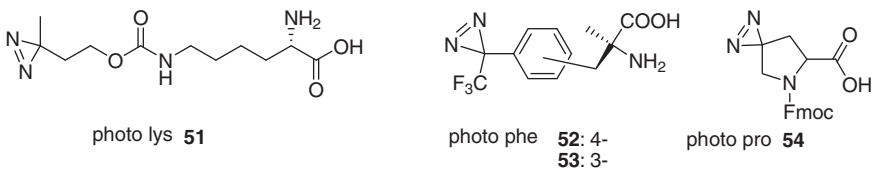


Figure 25. Photoactive amino acids **51**–**54**.

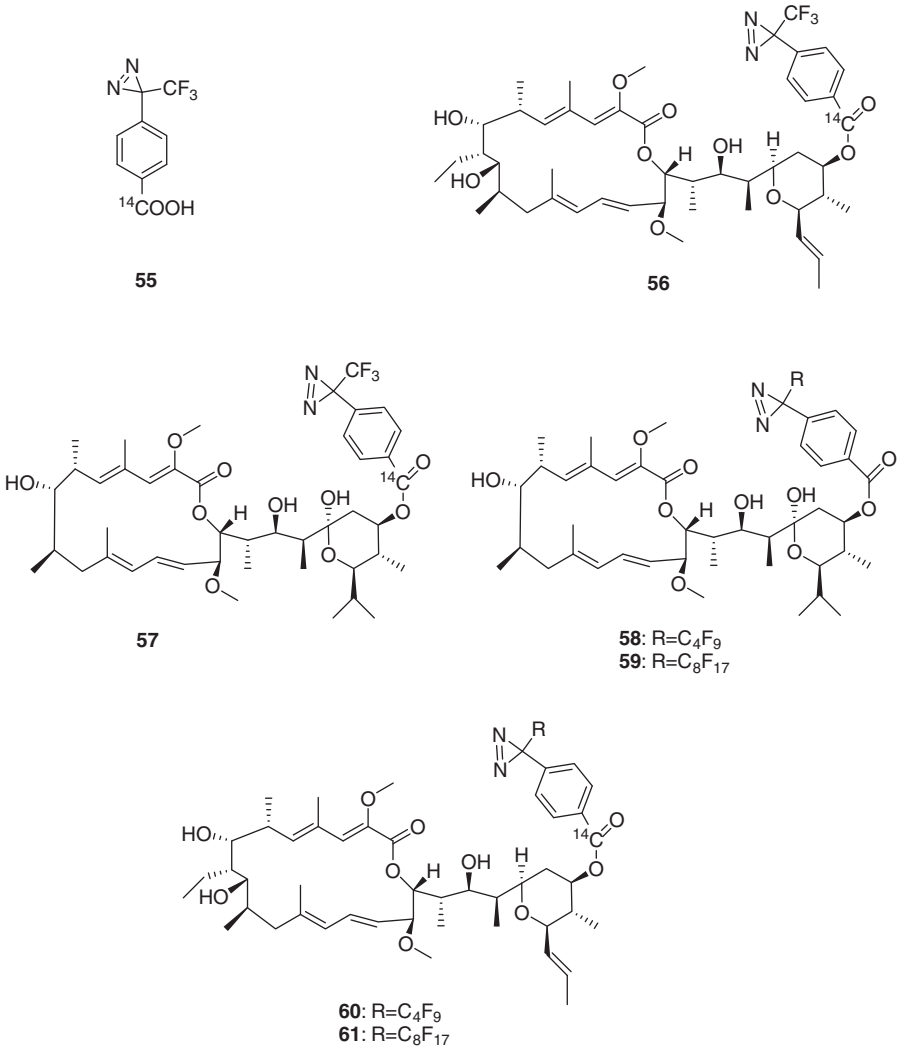


Figure 26. Diazirine photoaffinity label **55**; 21-deoxyconcanolide A analogs **56**, **60**–**61**; bafilomycin A_1 analogs **57**–**59**.

be used successfully as photoaffinity labels to probe the binding site of the sweet taste receptor.[88]

Robinson and co-workers reported the synthesis of a diaziryl analog of proline (**54**, Fig. 25, photo-pro) in order to probe the antibiotic effect of a new family of cyclic peptide analogs of the antimicrobial agent protegrin I.[89] In a breakthrough study, the authors not only identified a new cyclic peptide that is highly active against *P. aeruginosa*, but through photolabeling studies with the incorporated photo-pro analog, they furthermore established that the cyclic peptide binds to the outer membrane protein LptD.

Oberfeld et al. modified cysteine with diazirine and benzophenone photolabels to investigate the mechanism of action of ATPase in *E. coli*. The photolabels were attached to engineered cystein residues of the central cavity of the membrane-embedded c ring of the ATP synthase. Upon irradiation and following analysis of the cross-linked products, the authors identified the presence of phospholipids in the inner lumen of the c ring of the enzyme.[90]

7. Photoaffinity labeling of enzymes

The V-type ATPase is an important molecular proton transporting machine and pH regulator in almost all eukaryotic cells. Among the specific inhibitors of this enzyme are 21-deoxyconcanolide A and bafilomycin A_1. However, their binding site/s are still unknown. Bender et al. reported the synthesis of the small diazirine radiolabeled probe **55** (Fig. 26) as a tool to label carboxylic acids through previous conversion of the carboxy moiety to a hydroxymethyl group.[91] The authors also present an attachment (in form of **55**) to 21-deoxyconcanolide A and bafilomycin A_1 to produce photoaffinity analogs of these inhibitors which include the radiolabel ^{14}C as the tracer (**56**, **57**, Fig. 26). Probes **56** and **57** showed high inhibitory activities which makes them good potential probes for PAL in the future.

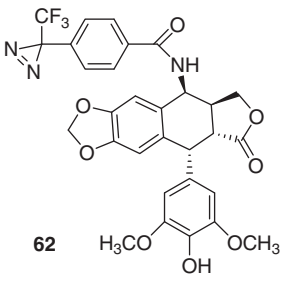


Figure 27. The diazirine analog of etoposide.

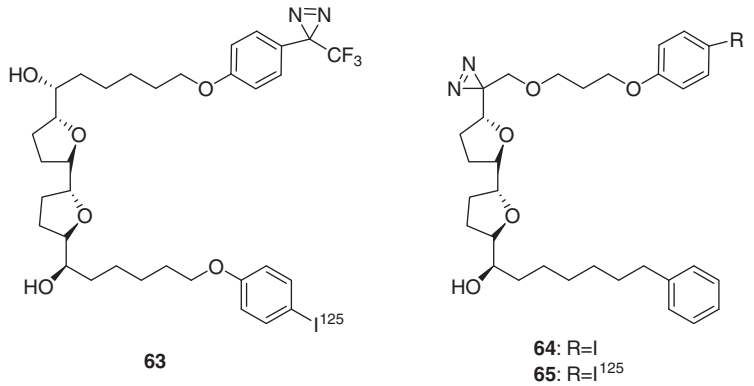


Figure 28. Acetogenin based diazirine analogs.

Very recently Burkard et al. introduced additional nonradioactive bafilomycin A_1 and 21-deoxyconcanolide A photoaffinity analogs containing a diazirine and a perfluoroalkyl moiety in order to use a new method of fluorous chromatography and fluorous solid phase extraction (**58**–**61**, Fig. 26).[20] The bafilomycin A_1 analogs **58** and **59** showed low inhibitory activities compared to 21-deoxyconcanolide A analogs **60** and **61**, which appear promising as inhibitors in the PAL studies.

Chin and Deiters, and co-workers, introduced a new approach to incorporate unnatural amino acids such as photo-lys (**51**, Fig. 25) via the pyrrolysyl-tRNA synthetase/tRNA pair into human superoxide dismutase expressed in yeast. Their report demonstrates the power of this technique to generate proteins with site-specific modifications that can be used to reveal important protein–protein interactions.[92]

Etoposide is a widely used anti-cancer drug that binds topoisomerase II. The details of the binding site of this drug at its receptor, however, are unknown. Chee et al. prepared a diazirine analog of etoposide, and they showed that this photoaffinity label binds topoisomerase II with similar affinity. Their data indicate that this photolabel (**62**, Fig. 27) is a promising probe for revealing the binding site of etoposide in topoisomerases.[93]

Bovine heart mitochondrial NADH-ubiquinone oxidoreductase (complex I) catalyzes the oxidation of NADH by ubiquinone.[94] Murai et al. developed a photoaffinity labeling strategy, using an analog of the native enzyme's inhibitor acetogenin [^{125}I]TDA (**63**, Fig. 28),[95] to investigate labeling at the ND1 subunit in the membrane domain of the oxidoreductase.[96] The authors probed the terminal electron transfer step of complex I, through synthesis and evaluation of a new type of inhibitors. Δlac-acetogenins (without a lactone ring) and recently reported photolabel containing Δlac-acetogenins revealed that the binding site of acetogenin is different to that of Δlac-acetogenin. Using several Δlac-acetogenin-diazirine probes (**64**, **65**, Fig. 28) the authors concluded that photo-cross-linked residues are located at two different sites, site A and site B in the third matrix-side loop connecting the fifth and sixth transmembrane helices in the ND1subunit. Site B may contribute to the binding or the access path of ubiquinone.[94–96] Unlike in most studies that describe the use of diazirines in PAL, in this case the natural and modified inhibitors show different inhibitory effects and likely occupy different binding sites.

7.1. Kinases

The most potent and selective cGMP-dependent protein kinase (PKG) inhibitor is DT-2. In order to better understand the molecular nature of DT-2 (a poly-cationic (lys-arg) 21-mer) inhibition, several photoaffinity probes based on structural features of DT-2 were synthesized by Pinkse et al. The photoreactive groups which were introduced are analogs of phenylalanine (4-benzoyl-L-phenylalanine (Bz) and 4′-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)-L-phenylalanine (Tmd)). Out of nine diazirine based peptides only one (W64-[Phe(Tmd^{14})] displayed cross-linking results. In contrast, all six benzoyl-based peptides showed cross-linking. Taken together, the results using benzoyl and diazirine based peptides show that it is still unclear where DT-2 exactly binds PKG, however, the presented data contain a series of novel and significant clues about the molecular mechanism of DT-2 inhibition.[97]

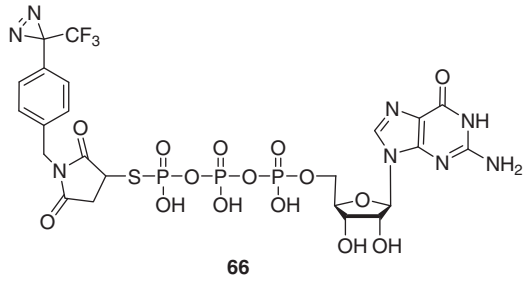


Figure 29. Diazirinyl GTP-based photoaffinity label **66**.

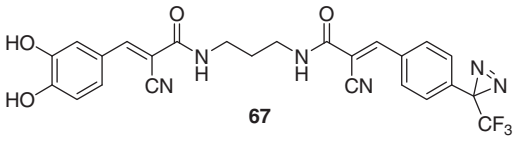


Figure 30. Diazirine based photoaffinity label **67**, based on GTPase inhibitor Bis-T.

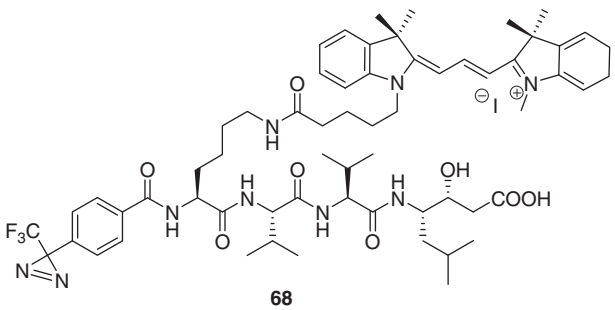


Figure 31. Diazirine based ABP **68**, used to profile aspartic proteases.

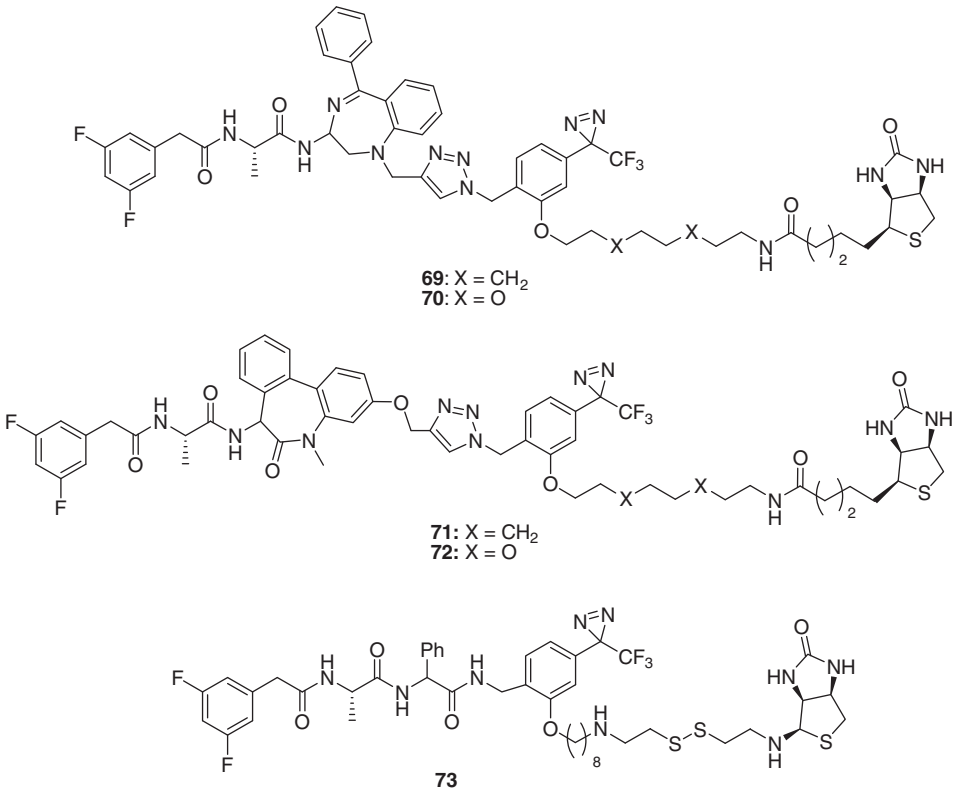


Figure 32. Diazirinyl photoaffinity labels (**69**–**73**) based on γ-secretase inhibitors.

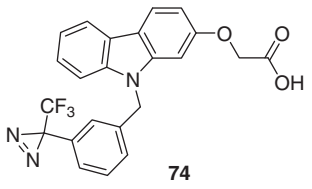


Figure 33. Fenofibrate based diazirine probe **74**.

In another elegant study by Hatanaka and co-workers, diazirine probes were used in the study of GTP-binding proteins. The authors synthesized a GTP-based photoactive molecule (**66**, Fig. 29) and used a known GTP-binding probe for photolabeling experiments. Experiments were performed on the model GTP-binding protein, H-Ras (part of a family of small GTPases). Using this experimental protocol the authors successfully isolated and identified the enzyme.[98]

In a related study on the mechanism of action of dynamin I, a member of a superfamily of large GTPases, photoaffinity labels based on the known inhibitor Bis-tyrphostin (Bis-T) were prepared. Odell et al. showed that diazirine (**67**, Fig. 30) and corresponding azide analogs possess dynamin I inhibitory activity,[99] even though subsequent studies to determine the binding pocket of this enzyme were not conclusive.

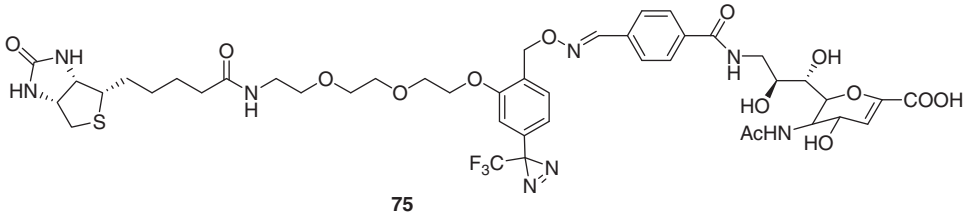


Figure 34. Diazirine photoaffinity label **75**, based on the sialidase inhibitor DANA.

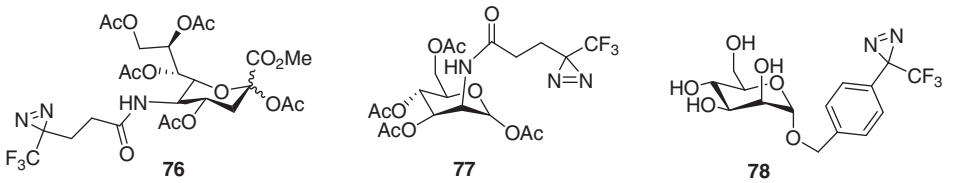


Figure 35. Diazirine based photoaffinity labels based on sialic acid (**76**), *N*-acetylmannosamine (**77**) and mannose (**78**).

7.2. Aspartic proteases

Due to their involvement in many human diseases aspartic proteases are an important class of enzymes. Yao and co-workers describe an ABPP strategy to selectively label aspartic proteases. Probe **68** (Fig. 31) showed high selectivity and succeeded in labeling aspartic proteases in complex proteome samples.[100]

In a series of elegant studies by the groups of Tomita, Fukuyama and Iwatsubo, γ-secretase, which is one of the aspartic proteases that is thought to be involved in the generation of Alzheimer's disease, was targeted with diaziryl photoaffinity labels. Three caprolactam-type dipeptidic γ-secretase inhibitors (GSI) are known: compound E (CE), LY411575 (DBZ) and (*N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-(*S*)-phenylglycine *tert*-butyl ester (DAPT). The secretase is a mulitmeric membrane protein complex, consisting of, amongst other molecules, preseniline (PS) 1 and an intramembrane cleaving aspartic protease named signal peptide peptidase (SPP). Several diazirine based (CE based-**69**, **70**, Fig. 32; and DBZ based-**71**, **72**, Fig. 32) and benzophenone based probes were found to specifically bind to the N-terminal fragment of PS1 and SPP, providing evidence that dipeptidic GSIs directly target SPP.[101] The DAPT based benzophenone probe revealed that this inhibitor binds the C-terminal fragment of SPP[102] and the diazirine probe **73** (Fig. 32) showed similar inhibitory and labeling activity as the benzophenone probe.[103]

A related study by Baumann et al. showed that the activity of a parent compound is not retained in every case of modification by a diazirine moiety. The authors found that a diaziryl photoaffinity label (**74**, Fig. 33) based on the non-steroidal anti-inflammatory drug (fenofibrate), a known γ-secretase modulator, was inactive.[104]

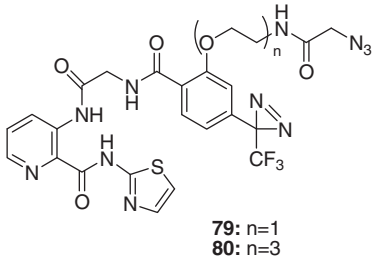


Figure 36. Diazirine probes (**79**, **80**) based on a type I methionine aminopeptidase inhibitor.

7.3. Sialidases

Significant efforts have been made to identify and characterize sialidase, the enzyme that catalyzes the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins, and glycolipids. The isolation of mammalian sialidases, deficiency of which has been implicated in lysosomal storage disease, has been hampered by its low solubility and membrane association. Kannappan et al. report the preparation of a diazirine analog (**75**, Fig. 34) of the known inhibitor DANA from the starfish *Asterina pectinifera*, and showed successful labeling and identification of the starfish sialidase,[105] indicating it as a promising probe to identify human homologs.

Glycoproteins that contain sialic acids are important for cell–cell communication and immunological recognition. Several research groups used metabolic oligosaccharide engineering to incorporate the sialic acid analogue **76** (Fig. 35) and *N*-acetylmannosamine (a metabolic precursor of sialic acid) analogue **77** (Fig. 35) into cell-surface glycoproteins/glycoconjugates[106] in order to identify and characterize the glycoconjugate binding partners.[107] Similarly,

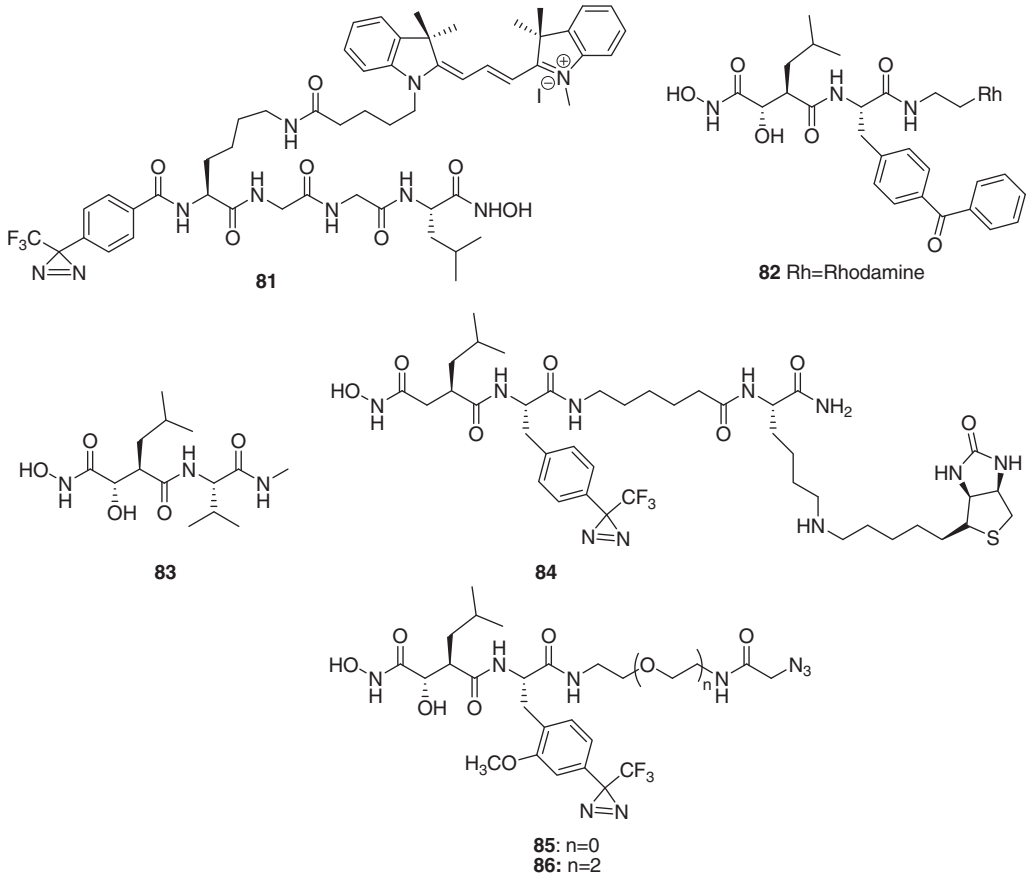


Figure 37. Diazirine and cyanine based dye **81**, containing an MMP inhibitor motif; the MMP inhibitor marimastat (**83**) and its photoaffinity analogs (**82**, **84**–**86**).

a diazirine-based mannose probe was synthesized in order to investigate the binding site of the α-mannose-specific adhesion protein of *E. coli*, FimH. The experiments were done with the soluble probe **78** (Fig. 35). [108]

A trifunctional photoaffinity label was used by Qiu et al., applying diazirine analogs (**79**, **80**, Fig. 36) of a known inhibitor of type I methionine aminopeptidase. This enzyme facilitates the removal of N-terminal (initiator) methionine residues from nascent polypeptides in a nonprocessive manner. The trifunctional probe contained an affinity group, a photoreactive group and an azide (for tagging purposes), enabling labeling of overexpressed methionine aminopeptidase in whole cell lysates of different bacteria.[109]

7.4. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent proteases that play important roles in various tumor processes. Progress in revealing the mechanism of action of these enzymes may accelerate the development of potent MMP inhibitors. Several studies were performed to elucidate unknown MMPs and profile MMP activity. In most cases the probes contain a zinc-chelating hydroxamate, a photoreactive group (benzophenone or diazirine) and a reporter tag (rhodamine, cyanine dye, biotin) or an azide (or the alkyne) group in the case of the tag free approach. Chan et al. reported an ability of a diazirine based probe **81** (Fig. 37) to label the MMP-9 enzyme from a crude yeast extract with a higher sensitivity over a benzophenone based probe.[110] Independently, Cravatt and co-workers succeeded in labeling of MMPs (including MMP-9 and MMP-2) by rhodamine–benzophenone containing probe **82** (Fig. 37), which is based on a hydroxamate inhibitor, marimastat (**83**, Fig. 37).[38] Previous attempts by the same group to use a tag-free approach showed no significant difference between the labeling of MMP-2 by the probes that contained rhodamine and benzophenone and the probes without the reporter tag.[111]

Leeuwenburgh et al. suggested probe **84** as an ABP for MMPs and additional metalloproteinases, ADAMs, which contain an MMP domain.[112] The diazirine and the biotin containing probe revealed to be highly potent inhibitors of both, MMP-12 and ADAM-17. The labeling experiment also revealed binding to ADAM-10, which shows great potential of the probe in activity based labeling experiments. Qiu et al. designed additional marimastat analogs containing an azide (allowing application of the tag-free approach) and the diazirine moiety (**85**–**86**, Fig. 37).[113] Two probes efficiently labeled the purified MMP-2 catalytic domain and a secretive MMP-2 protein in B16F10 cell culture medium, while probe **86** was more sensitive, showing an advantage of the longer linker probe in the click chemistry experiments.

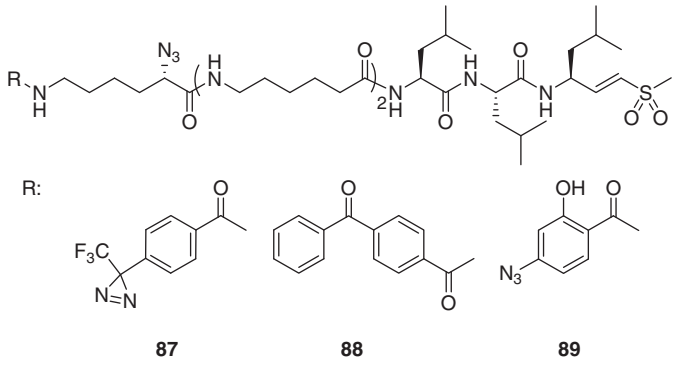


Figure 38. Photoaffinity labels (**87**–**89**) as ABP probes based on proteosome inhibitors.

7.5. Proteasome profiling

Overkleeft and co-workers established that extended hydrophobic peptide vinyl sulfones are potent proteasome inhibitors.[114,115] In an elegant new study by the same group, Geurink et al. synthesized tri-functional activity based probes based on the peptide vinyl sulfone core structure, in order to investigate interactions between the inhibitor and its binding subunits. The authors identified two subunits (β5 and β6) of the 20S proteasome bound to the probe.[116] Interestingly, of the three photoaffinity labels used (arylazide, benzophenone and diazirine) only the arylazide containing probe efficiently captured proteasome subunits. It is possible that the

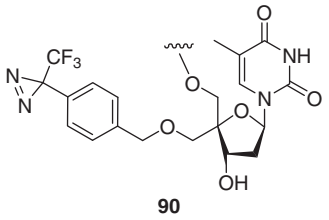


Figure 39. A diazirine photolabel attached to the sugar moiety of thymidine.

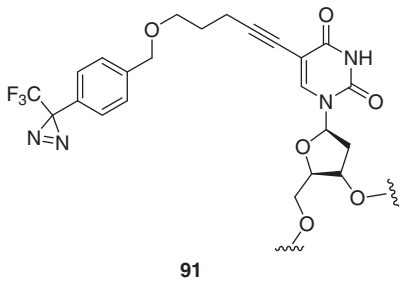


Figure 40. A diazirine photolabel attached to the thymine moiety of thymidine.

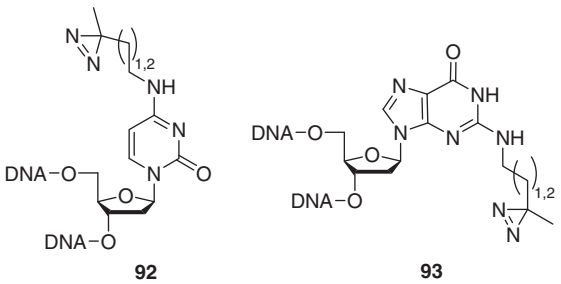


Figure 41. Diazirine containing DNA through modification of cytosine (**92**) and guanine (**93**).

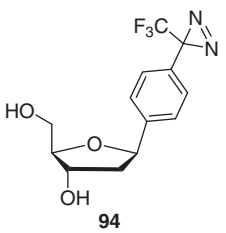
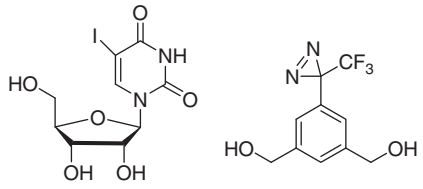


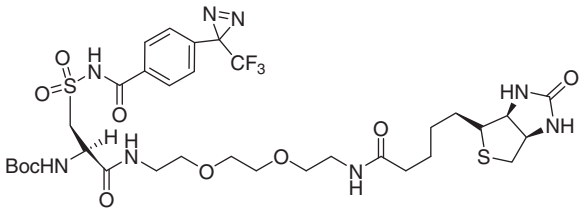
Figure 42. Diazirine based nucleoside analog **94**.

diazirine (**87**, Fig. 38) and benzophenone (**88**, Fig. 38) based probes did not give the desired result, compared to the arylazide probe (**89**, Fig. 38), because compared to nitrenes, carbenes and biradicals have a relatively short half-life in aqueous media. This could have prevented the reactive intermediates to react with the proteasome subunit before reacting with water.



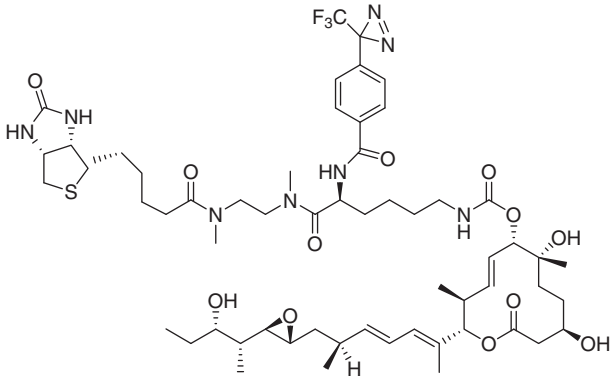
95 **96**

Figure 43. Functional diazirine based photolabels that were incorporated into siRNA.



97

Figure 44. Biotinylated diazirine based acidic α-amino acid mimic **97**.



98

Figure 45. Diazirinyl photoaffinity label based on pladienolide (**98**).

8. Photoaffinity labeling of nucleic acids

While most of the studies in which diazirines are used focus on small molecules, some of the recent reports also show its use in macromolecules, such as DNA. Liebmann et al. reported the labeling of DNA polymerase β by introduction of a diazirine moiety into the DNA polymer. The modification on the sugar moiety of the incorporated thymidine (**90**, Fig. 39) permitted an investigation of the interactions in the minor groove of the helix.[117]

Winnacker et al. performed a labeling study of two DNA repair enzymes (Rad14-yeast homologue of the human XPA protein and Fpg/MuM-DNA glycosilase from *Lactococcus lactis*), through modification of the target DNA. In contrast with the previous study, here the diazirine modification of the nucleotide was on the thymine moiety (**91**, Fig. 40).[118]

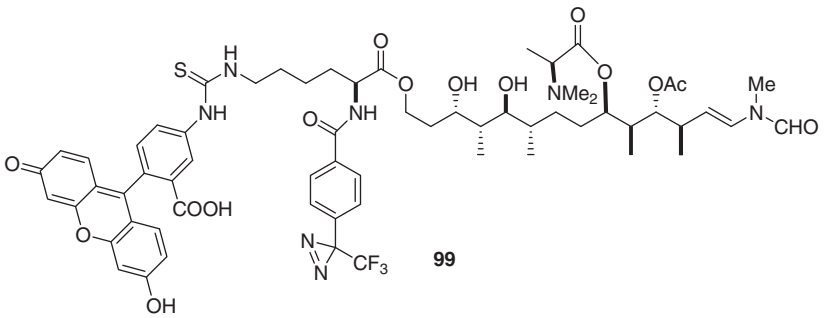
In addition to thymine (T), also cytosine (C, **92**, Fig. 41) and guanine (G, **93**, Fig. 41) were modified with the diazirine moiety in order to investigate DNA–protein interactions.[119] Cross-links with two different proteins were observed when DNA probes were irradiated and the resulting DNA–protein complexes were isolated and characterized.

In order to examine interstrand DNA–DNA interactions, Qiu et al. incorporated a diazirine based nucleoside analog (**94**, Fig. 42) into DNA by solid phase oligonucleotide synthesis. The photolabeled DNA was examined for its potential to crosslink a complementary strand. Their findings revealed interstrand crosslinking in dsDNA upon near UV irradiation. This nucleoside analog may find broad applications in future research of interactions between nucleic acids.[120]

Small interfering RNAs (siRNA) are small RNA duplexes that are involved in sequence-specific posttranscriptional gene silencing. The RNA-induced silencing complex (RISC) with an argonaute protein positioned at the catalytic center of the RISC was studied by Ueno and co-workers, using photolabeled siRNA.[121] The synthesis of siRNA carrying a photoactivatable diazirine moiety in the 3′ overhang region was achieved, and compared to a 5-iodouridine RNA derivative (**95**, Fig. 43). The diazirine probe (**96**, Fig. 43) allowed much more sensitive detection and might be a useful tool to investigate the mechanism of RISC assembly of argonaute proteins.

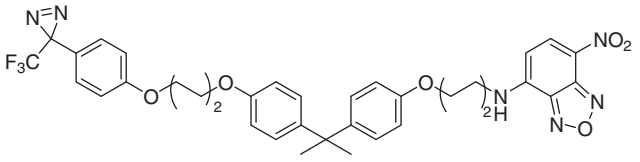
8.1. Other targets

Hatanaka and co-workers showed broad use of a diazirine analog as a photoreactive mimic of α-amino acids bearing an acidic residue. Incorporation of an N-acylsulfonamide moiety with an acidic proton resulted in a photoprobe that could be cleaved upon treatment with mild base. Irradiation yielded specific photolabeling of glutamyl endopeptidases, L-glutamate dehydrogenase, glutamic oxalacetic transaminase, and L-glutamine synthetase, all enzymes that exhibit high affinity toward acidic α-amino acids (**97**, Fig. 44).[122]



99

Figure 46. Diazirinyl photoaffinity label based on aplyronine (**99**).



100

Figure 47. Diazirinyl photoaffinity label based on bisphenol A (**100**).

In another use of photoaffinity labeling to establish the target protein of an anti-cancer agent—the tumor arresting macrolide pladienolide, a diazirine and biotin containing photoaffinity probe (**98**, Fig. 45) was prepared and evaluated. In this important study, Kotake et al. identified a 140 kDa protein named splicing factor SF3b, establishing this protein as a potential anti-tumor drug target.[123]

A different macrolide, Aplyronine A, was studied by Kuroda et al., by synthesizing the side chain of this macrolide with diazirine and fluorescein moieties (**99**, Fig. 46). Photolabeling experiments showed attachment of the probe to actin, indicating that actin interacts with the apolyronine A side chain.[124]

Bisphenol A (BpA) is widely used in industry in the preparation of polycarbonate and epoxy resins. While the compound was reported to induce developmental abnormalities in the neuronal systems of humans and other animals, it is still widely used, due to a lack of alternative reagents to prepare resins. As BpA was found to inhibit the hypoxic expression of erythropoietin, several photoaffinity probes were prepared by Maezawa et al. to investigate the mechanism behind BpA toxicity. Diazirinyl derivative **100** (Fig. 47) displayed superior inhibitory activity over two other diazirine probes.[125]

9. Conclusions

There is a growing interest in the use of diazirine as a photoaffinity label to characterize, identify and profile proteins and protein families. In most cases where diazirines are compared to other photoreactive groups, the diazirine analogs show advantageous properties, which, combined with advances in synthetic strategies to prepare diazirines, account for its increased use in photoaffinity labeling studies. Given the success and progress in the field of activity based protein profiling in recent years, most likely the use of diazirines in labeling and identifying unknown receptors and receptor families will further rise.

Acknowledgments

We thank the European Research Council (Starting Grant 240356, M.M.M.) and the Human Frontier Science Program (Young Investigator Grant RGY0072/2007, M.M.M. and B.P.K.) for financial support. We thank Dr. Antonia Delago for many helpful suggestions.

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