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A Guide to Fluorescent Probes and Labeling Technologies

11th Edition (2010)

CHAPTER 5

Crosslinking and Photoactivatable Reagents

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CHAPTER 5

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5.1 Introduction to Crosslinking and Photoactivatable Reagents

Chemical and Photoreactive Crosslinkers

Bifunctional "crosslinking" reagents contain two reactive groups, thereby providing a means of covalently linking two target groups. The reactive groups in a chemical crosslinking reagent (Section 5.2) typically belong to the classes of functional groups—including succinimidyl esters, maleimides and iodoacetamides—described in Chapter 1 and Chapter 2.

In contrast, one of the reactive groups in each of our photoreactive crosslinking reagents (Section 5.3) requires light activation before reacting with a target group. Crosslinking of a biopolymer (such as an antibody, enzyme, avidin or nucleic acid) to a low molecular weight molecule (such as a drug, toxin, peptide or oligonucleotide) or to another biopolymer yields a stable heteroconjugate. This bioconjugate can serve as a detection reagent in a wide variety of research and diagnostic assays or as an immunogen designed to elicit antibody production. Crosslinking reagents are also useful for probing the spatial relationships and interactions within and between biomolecules.

In homobifunctional crosslinking reagents (Section 5.2), the reactive groups are identical. These reagents couple like functional groups—typically two thiols, two amines, two acids or two alcohols—and are predominantly used to form intramolecular crosslinks or to prepare polymers from monomers. When used to conjugate two different biomolecules, for example an enzyme to an antibody, these relatively nonspecific reagents tend to yield high molecular weight aggregates.

In heterobifunctional crosslinking reagents (Section 5.2, Table 5.1), the reactive groups have dissimilar chemistry, allowing the formation of crosslinks between unlike functional groups (Figure 5.2.2). As with homobifunctional crosslinking reagents, heterobifunctional crosslinking reagents can still form multiple intermolecular crosslinks to yield high molecular weight aggregates, but conjugations that use these reagents can be more easily controlled so as to optimize the stoichiometry of the target molecules. Thus, heterobifunctional crosslinking reagents are very useful for preparing conjugates between two different biomolecules.

The photoreactive crosslinking reagents (Section 5.3) are a special subset of the heterobifunctional crosslinking reagents. Upon UV illumination, these reagents react with nucleophiles or form C–H insertion products (Figure 5.1.1, Figure 5.1.2, Figure 5.1.3).

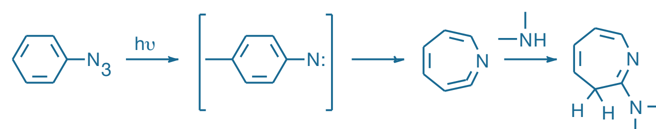

Figure 5.1.1 Photoreactive crosslinking reaction of a simple aryl azide.

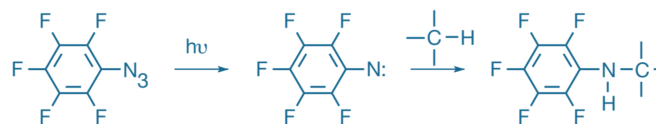

Figure 5.1.2 Photoreactive crosslinking reaction of a fluorinated aryl azide.

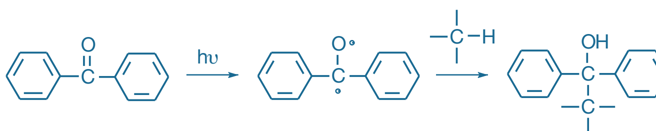

Figure 5.1.3 Photoreactive crosslinking reaction of a benzophenone derivative.

Table 5.1 Molecular Probes® heterobifunctional crosslinkers.

Cat. No.	Crosslinker	Reactivity				
		Thiol (R–SH)	Amine (R–NH$_2$)	Azide * (R–N$_3$)	Alkyne *	Photoreactive †
S1553	succinimidyl acetylthioacetate (SATA) ‡	●	●			
S1534	succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC) ‡	●	●			
S1531	succinimidyl 3-(2-pyridyldithio)propionate (SPDP)	●	●			
E2247	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) §		●			
P6317	*N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide (PEAS; AET) ‡	●				●
A2522	4-azido-2,3,5,6-tetrafluorobenzoic acid, succinimidyl ester (ATFB, SE)		●			●
B1508	benzophenone-4-maleimide	●				●
B1526	benzophenone-4-isothiocyanate		●			●
B1577	4-benzoylbenzoic acid, succinimidyl ester		●			●
I10188	iodoacetamide azide *	●			●	
I10189	iodoacetamide alkyne *	●		●		
A10280	azido (PEO)$_4$ propionic acid, succinimidyl ester *		●		●	
A10279	alkyne, succinimidyl ester *		●	●		

***** Azides react with alkynes via the copper-catalyzed azide–alkyne cycloaddition reaction, as discussed in Section 3.1 (where these products are also described). **†** Reacts nonspecifically with available sites upon UV illumination. **‡** These reagents contain cryptic thiols that are exposed by disulfide reduction (SPDP or PEAS) or deacetylation (SATA) and can be subsequently disulfide-coupled to other thiolated molecules or thiolether-coupled to maleimides or iodoacetamides. **§** EDAC couples amines to carboyxlic acids.

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An additional variation is the "zero-length" crosslinking reagent—a reagent that forms a chemical bond between two groups without itself being incorporated into the product (Figure 5.1.4). The water-soluble carbodiimide EDAC (E2247, Section 5.2), which is used to couple carboxylic acids to amines, is an example of a zero-length crosslinking reagent.

A noncovalent interaction between two molecules that has very slow dissociation kinetics can also function as a crosslink. For example, reactive derivatives of phospholipids can be used to link the liposomes or cell membranes in which they are incorporated to antibodies or enzymes. Biotinylation and haptenylation reagents (Chapter 4) can also be thought of as heterobifunctional crosslinking reagents because they comprise a chemically reactive group as well as a biotin or hapten moiety that binds with high affinity to avidin or an anti-hapten antibody, respectively. Similarly, avidin, streptavidin, NeutrAvidin™ biotin-binding protein and CaptAvidin™ biotin-binding protein (Section 7.6) can tightly bind up to four molecules of a biotinylated target.

REFERENCES

1. ACS Chem Biol (2009) 4:409; **2**. Nat Methods (2007) 4:619; **3**. Nature (1984) 310:74; **4**. Biotechniques (1993) 15:848; **5**. Anal Chem (2003) 75:1387.

Photoactivatable (Caged) Probes

In addition to the photoreactive crosslinking reagents that are briefly mentioned above and described in detail in Section 5.3, we prepare photoactivatable probes. Flash photolysis of photoactivatable or "caged" probes provides a means of controlling the release—both spatially and temporally—of biologically active products or other reagents of interest.[1,2] The chemical caging process may also confer membrane permeability on the caged ligand, as is the case for caged cAMP[3] and caged luciferin.[4] Our selection of caged nucleotides, chelators, second messengers and neurotransmitters has tremendous potential for use with both live cells and isolated proteins (Section 5.3).

We prepare caged versions of biologically active molecules, as well as caged fluorescent dyes that are essentially nonfluorescent until after photolysis. These caged fluorophores have proven useful for photoactivation of fluorescence (PAF) experiments, which are analogous to fluorescence recovery after photobleaching (FRAP) experiments except that the fluorophore is activated upon illumination rather than bleached. Measuring the fluorescent signal of the photoactivated fluorophore against a dark background is intrinsically more sensitive than measuring a dark photobleached region against a bright field.[5]

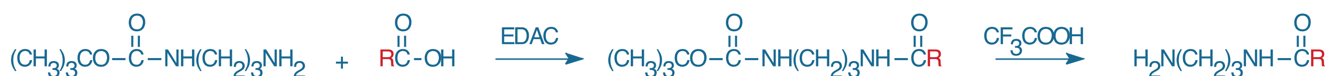

Figure 5.1.4 Conversion of a carboxylic acid group into an aliphatic amine. The activated carboxylic acid is derivatized with a half-protected aliphatic diamine (mono-*N*-(*t*-BOC)-propylenediamine, M6248), usually in an organic solvent, followed by removal of the *t*-BOC–protecting group with trifluoroacetic acid.

5.2 Chemical Crosslinking Reagents

The most common schemes for forming a well-defined heteroconjugate require the indirect coupling of an amine group on one biomolecule to a thiol group on a second biomolecule, usually by a two- or three-step reaction sequence. The high reactivity of thiols (Chapter 2) and—with the exception of a few proteins such as β-galactosidase—their relative rarity in most biomolecules make thiol groups ideal targets for controlled chemical crosslinking. If neither molecule contains a thiol group, then one or more can be introduced using one of several thiolation methods. The thiol-containing biomolecule is then reacted with an amine-containing biomolecule using a heterobifunctional crosslinking reagent such as one of those described in Amine–Thiol Crosslinking, below.

Thiolation of Biomolecules

Introducing Thiol Groups into Biomolecules

Several methods are available for introducing thiols into biomolecules, including the reduction of intrinsic disulfides, as well as the conversion of amine or carboxylic acid groups to thiol groups:

- Disulfide crosslinks of cystines in proteins can be reduced to cysteine residues by dithiothreitol[1] (DTT, D1532) or tris-(2-carboxyethyl)phosphine (TCEP, T2556; Figure 5.2.1). However, reduction may result in loss of protein activity or specificity. Excess DTT must be carefully removed under conditions that prevent reformation of the disulfide,[2] whereas excess TCEP usually does not need to be removed before carrying out the crosslinking reaction. TCEP is also more stable at higher pH values and at higher temperatures than is the air-sensitive DTT reagent.[3]
- Amines can be indirectly thiolated by reaction with succinimidyl acetylthioacetate[4] (SATA, S1553), followed by removal of the acetyl group with 50 mM hydroxylamine or hydrazine at near-neutral pH (Figure 5.2.2). This reagent is most useful when disulfides are essential for activity, as is the case for some peptide toxins.
- Amines can be indirectly thiolated by reaction with succinimidyl 3-(2-pyridyldithio)propionate[5] (SPDP, S1531), followed by reduction of the 3-(2-pyridyldithio)propionyl conjugate with DTT or TCEP (Figure 5.2.3). Reduction releases the 2-pyridinethione chromophore, which can be used to determine the degree of thiolation.
- Thiols can be incorporated at carboxylic acid groups by an EDAC-mediated reaction with cystamine, followed by reduction of the disulfide with DTT or TCEP;[6,7] see Amine–Carboxylic Acid Crosslinking below.
- Tryptophan residues in thiol-free proteins can be oxidized to mercaptotryptophan residues, which can then be modified by iodoacetamides or maleimides.[8–10]

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Our preferred reagent combination for protein thiolation is SPDP/DTT or SPDP/TCEP.[11] We use SPDP to prepare a reactive R-phycoerythrin derivative (P806, Section 6.4), providing researchers with the optimal number of pyridyldisulfide groups for crosslinking the phycobiliprotein to thiolated antibodies, enzymes and other biomolecules through disulfide linkages.[12] More commonly, the pyridyldisulfide groups are first reduced to thiols, which are then reacted with maleimide- or iodoacetamide-derivatized proteins (Figure 5.2.3). SPDP can also be used to thiolate oligonucleotides [13] and—like all of the thiolation reagents in this section—to introduce the highly reactive thiol group into peptides, onto cell surfaces or onto affinity matrices for subsequent reaction with fluorescent, enzyme-coupled or other thiol-reactive reagents (Chapter 2). In addition, because the 3-(2-pyridyldithio)propionyl conjugate releases the 2-pyridinethione chromophore upon reduction, SPDP is useful for quantitating the number of reactive amines in an affinity matrix.[14]

Measuring Thiolation of Biomolecules

To ensure success in forming heterocrosslinks, it is important to know that a molecule has the proper degree of thiolation. We generally find that two to three thiol residues per protein are optimal. Following removal of excess reagents, the degree of thiolation in proteins or other molecules thiolated with SPDP can be directly determined by measuring release of the 2-pyridinethione chromophore [5] (EC ~8000 $cm^{-1}M^{-1}$ at 343 nm).

Alternatively, the degree of thiolation and presence of residual thiols in a solution can be assessed using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent; D8451), which stoichiometrically yields the 5-mercapto-2-nitrobenzoic acid chromophore (EC ~13,600 $cm^{-1}M^{-1}$ at 410 nm) upon reaction with a thiol group.[15,16] DTNB can also be used to quantitate residual phosphines in aqueous solutions, including TCEP; [17] in this case, two molecules of 5-mercapto-2-nitrobenzoic acid are formed per reaction with one molecule of a phosphine.

Measure-iT™ Thiol Assay Kit

The Measure-iT™ Thiol Assay Kit (M30550) provides easy and accurate quantitation of thiol. The kit supplies concentrated assay reagent, dilution buffer, and concentrated thiol standard. The assay has

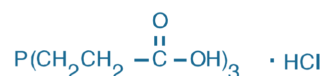

Figure 5.2.1 Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP, T2556).

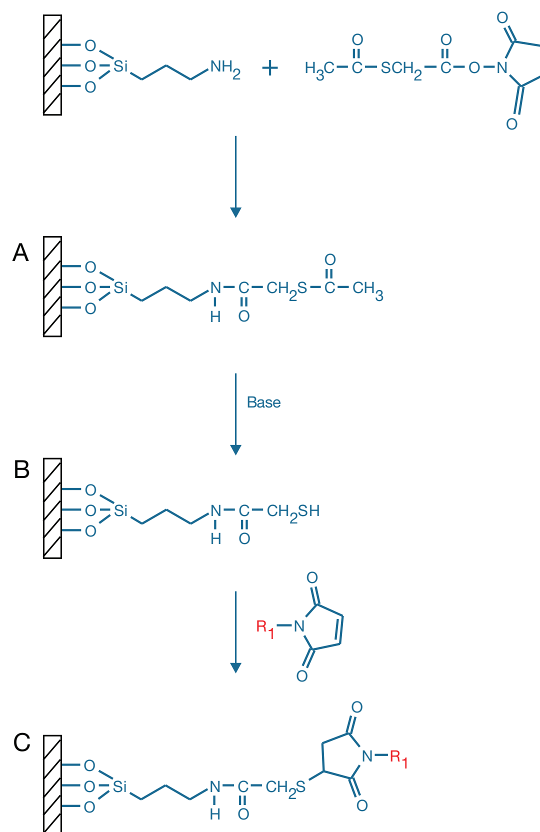

Figure 5.2.2 Schematic illustration of the heterobifunctional crosslinker succinimidyl acetylthioacetate (SATA, S1553): **A**) attachment to an aminosilane-modified surface, **B**) deprotection with base and **C**) reaction with a thiol-reactive biomolecule.

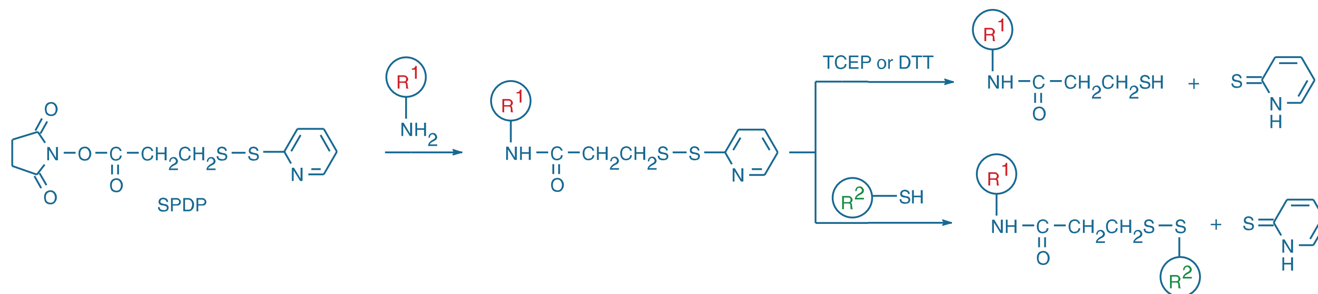

Figure 5.2.3 SPDP derivatization reactions. SPDP (S1531) reacts with an amine-containing biomolecule at pH 7 to 9, yielding a pyridyldithiopropionyl mixed disulfide. The mixed disulfide can then be reacted with a reducing agent such as DTT (D1532) or TCEP (T2556) to yield a 3-mercaptopropionyl conjugate or with a thiol-containing biomolecule to form a disulfide-linked tandem conjugate. Either reaction can be quantitated by measuring the amount of 2-pyridinethione chromophore released during the reaction.

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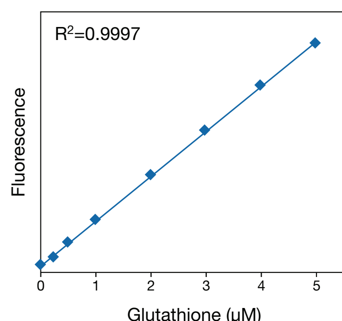

Figure 5.2.4 Linearity and sensitivity of the Measure-iT™ thiol assay. Triplicate 10 µL samples of glutathione were assayed using the Measure-iT™ Thiol Assay Kit (M30550). Fluorescence was measured using excitation/emission of 490/520 nm and plotted versus glutathione concentration. The variation (CV) of replicate samples was <2%.

a linear range of 0.05–5 µM thiol (Figure 5.2.4), making it up to 400 times more sensitive than colorimetric methods based on DTNB (Ellman's reagent).

Each Measure-iT™ Thiol Assay Kit contains:

- Measure-iT™ thiol quantitation reagent (100X concentrate in 1,2-propanediol)
- Measure-iT™ thiol quantitation buffer (50 mM potassium phosphate buffer)
- Measure-iT™ thiol quantitation standard (reduced glutathione)
- Detailed protocols

Simply dilute the reagent 1:100, load 100 µL into the wells of a microplate, add 1–10 µL sample volumes, mix, then read the fluorescence. Maximum fluorescence signal is attained within 5 minutes and is stable for at least 1 hour. The assay is performed at room temperature, and common contaminants are well tolerated in the assay. The Measure-iT™ Thiol Assay Kit provides sufficient materials for 500 assays, based on a 100 µL assay volume in a 96-well microplate format; this thiol assay can also be adapted for use in cuvettes or 384-well microplates.

Thiol and Sulfide Quantitation Kit

Ultrasensitive colorimetric quantitation of both protein and nonprotein thiols can also be achieved using the Thiol and Sulfide Quantitation Kit (T6060). In this assay, which is based on a method reported by Singh,[18,19] thiols reduce a disulfide-inhibited derivative of papain, stoichiometrically releasing the active enzyme. Activity of the enzyme is then measured using the chromogenic papain substrate L-BAPNA via spectrophotometric detection of *p*-nitroaniline release at 412 nm (Figure 5.2.5). Although thiols can also be quantitated using DTNB (Ellman's reagent), the enzymatic amplification step in this quantitation kit enables researchers to detect as little as 0.2 nanomoles of a thiol—a sensitivity that is about 100-fold better than that achieved with DTNB.[20,21] Thiols in proteins and potentially in other high molecular weight molecules can be detected indirectly by incorporating the disulfide cystamine into the solution. Cystamine undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain. Thiols that are alkylated by maleimides, iodoacetamides and other reagents are excluded from detection and can therefore be assayed subtractively.[22]

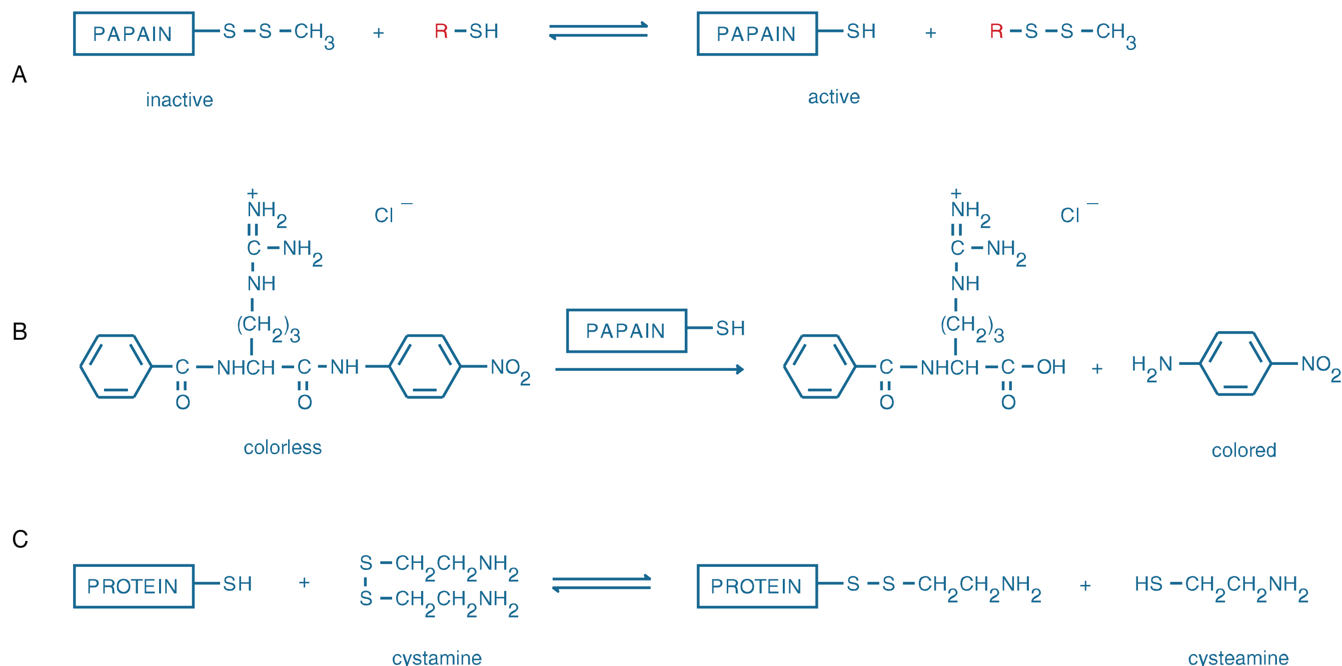

Figure 5.2.5 Chemical basis for thiol detection using the Thiol and Sulfide Quantitation Kit (T6060): **A**) the inactive disulfide derivative of papain, papain–$SSCH_3$, is activated in the presence of thiols; **B**) active papain cleaves the substrate L-BAPNA, releasing the *p*-nitroaniline chromophore; **C**) protein thiols, often poorly accessible, exchange with cystamine to generate 2-mercaptoethylamine (cysteamine), which is easily detected.

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The Thiol and Sulfide Quantitation Kit contains:

- Papain–$SSCH_3$, the disulfide-inhibited papain derivative
- L-BAPNA, a chromogenic papain substrate
- DTNB (Ellman's reagent), for calibrating the assay
- Cystamine
- L-Cysteine, a thiol standard
- Buffer
- Detailed protocols for measuring thiols, inorganic sulfides and maleimides

Sufficient reagents are provided for approximately 50 assays using 1 mL assay volumes and standard cuvettes or 250 assays using a microplate format.

Thiol–Thiol Crosslinking

Oxidation

Thiol residues in close proximity can be oxidized to disulfides by either an intra- or intermolecular reaction. In many circumstances, however, this oxidation reaction is reversible and difficult to control.

Fluorescent Thiol–Thiol Crosslinkers

Dibromobimane (bBBr, D1379; Figure 5.2.6) is an interesting crosslinking reagent for proteins because it is unlikely to fluoresce until both of its alkylating groups have reacted.[23] It has been used to crosslink thiols in myosin,[24] actin,[25] hemoglobin,[26] *Escherichia coli* lactose permease[27] and mitochondrial ATPase.[28] It has also been shown to intramolecularly crosslink thiols in a complex of nebulin and calmodulin.[29] In addition, dibromobimane has been used to probe for the proximity of dual-cysteine mutagenesis sites in ArsA ATPase[30] and P-glycoprotein.[31–33] Dibromobimane, a stimulator of the ATPase activity of a cysteine-free P-glycoprotein, was used with cysteine-scanning mutagenesis to identify amino acid residues important for function.[34]

In addition to dibromobimane, we offer the thiol-reactive homobifunctional crosslinker bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (B10621), which is derived from a relatively rigid rhodamine dye (Figure 5.2.7). This crosslinker is similar to a thiol-reactive rhodamine-based crosslinking reagent that was used to label regulatory light-chains of chicken gizzard myosin for fluorescence polarization experiments.[35] Researchers have attached bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine to the kinesin motor domain and determined the orientation of kinesin bound to microtubules in the presence of a nonhydrolyzable ATP analog by fluorescence polarization microscopy.[36,37] Images of single molecules of chicken calmodulin crosslinked between two engineered cysteines by bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine have been used to generate comparisons of experimental and theoretical super-resolution point-spread functions[38] (PSF).

Amine–Amine Crosslinking

The scientific literature contains numerous references to reagents that form crosslinks between amines of biopolymers. Homobifunctional amine crosslinkers include glutaraldehyde, bis(imido esters), bis(succinimidyl esters), diisocyanates and diacid chlorides.[39] These reagents, however, tend to yield high molecular weight aggregates, making them unsuitable for reproducibly preparing well-defined conjugates between two different amine-containing biomolecules. For example, glutaraldehyde is still used by some companies and research laboratories to couple horseradish peroxidase, which has only six lysine residues,[40] to proteins with a larger number of lysine residues. Unfortunately, this practice can result in variable molecular weights and batch-to-batch inconsistency.

Well-defined conjugates between two amine-containing molecules are more reliably prepared by thiolating one or more amines on one of the biomolecules and converting one or more amines on the second biomolecule to a thiol-reactive functional group such as a maleimide or iodoacetamide, as described below in Amine–Thiol Crosslinking. For example, we prepare our horseradish peroxidase conjugates (Section 7.2, Section 7.6) using SPDP- and SMCC-mediated reactions (Figure 5.2.3, Figure 5.2.8).

Direct amine–amine crosslinking routinely occurs during fixation of proteins, cells and tissues with formaldehyde or glutaraldehyde. These common aldehyde-based fixatives are also used to crosslink amine and hydrazine derivatives to proteins and other amine-containing polymers. For example, lucifer yellow CH (L453, Section 14.3) is nonspecifically conjugated to surrounding biomolecules by aldehyde-based fixatives in order to preserve the dye's staining pattern during subsequent tissue manipulations.[41] Also, biotin hydrazides (Section 4.2) have been directly coupled to nucleic acids with glutaraldehyde,[42,43] a reaction that is potentially useful for conjugating fluorescent hydrazides and hydroxylamines to DNA.

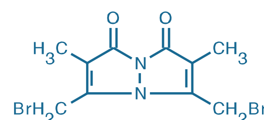

Figure 5.2.6 Dibromobimane (bBBr, D1379).

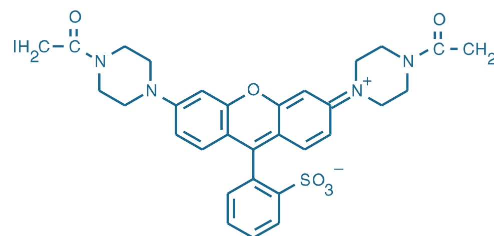

Figure 5.2.7 bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (B10621).

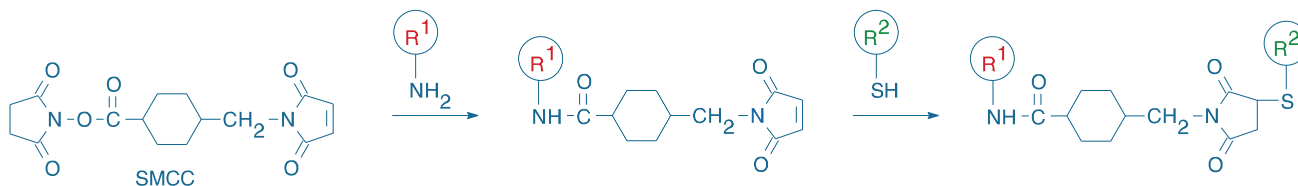

Figure 5.2.8 Two-step reaction sequence for crosslinking biomolecules using the heterobifunctional crosslinker SMCC (S1534).

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Amine–Thiol Crosslinking

Indirect crosslinking of the amines in one biomolecule to the thiols in a second biomolecule is the predominant method for forming a heteroconjugate. If one of the biomolecules does not already contain one or more thiol groups, it is necessary to introduce them using one of the thiolation procedures described above in Thiolation of Biomolecules. Thiol-reactive groups such as maleimides are typically introduced into the second biomolecule by modifying a one or more of its amines with a heterobifunctional crosslinker containing both a succinimidyl ester and a maleimide. The maleimide-modified biomolecule is then reacted with the thiol-containing biomolecule to form a stable thioether crosslink (Figure 5.2.8). Chromatographic methods are usually employed to separate the higher molecular weight heteroconjugate from the unconjugated biomolecules.

Introducing Maleimides at Amines

Succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate [44] (SMCC, S1534) is our reagent of choice for introducing thiol-reactive groups at amine sites because of the superior chemical stability of its maleimide and its ease of use[45] (Figure 5.2.8).

Introducing Disulfides at Amines

Our preferred method for preparing heteroconjugates employs the thiolation reagent SPDP (S1531). The pyridyldisulfide intermediate that is initially formed by reaction of SPDP with amines can form an unsymmetrical disulfide through reaction with a second thiol-containing molecule [5,12] (Figure 5.2.3). The thiol-containing target can be a molecule such as β-galactosidase that contains intrinsic thiols or a molecule in which thiols have been introduced using one of the thiolation procedures described above in Thiolation of Biomolecules. In either case, it is essential that all reducing agents, such as DTT and TCEP, are absent. The heteroconjugate's disulfide bond is about as stable and resistant to reduction as disulfides found in proteins; it can be reduced with DTT or TCEP to generate two thiol-containing biomolecules.

Protein–Protein Crosslinking Kit

Our Protein–Protein Crosslinking Kit (P6305) provides all of the reagents and purification media required to perform three protein–protein conjugations in which neither protein contains thiol residues. The chemistry used to thiolate the amines of one of the proteins with SPDP and to convert the amines of the second protein to thiol-reactive maleimides with SMCC is shown in Figure 5.2.3 and Figure 5.2.8, respectively. Included in the kit are:

- SPDP, for thiolating amines
- SMCC, for converting amines to thiol-reactive maleimides
- TCEP, for reducing the pyridyldisulfide intermediate
- *N*-ethylmaleimide (NEM), for capping residual thiols
- Six reaction tubes, each containing a magnetic stir bar
- Spin columns plus collection tubes
- Dimethylsulfoxide (DMSO)
- Detailed crosslinking protocols

The Protein–Protein Crosslinking Kit was designed to prepare and purify protein–protein conjugates; however, it can be readily modified for generating peptide–protein or enzyme–nucleic acid conjugates or for conjugating biomolecules to affinity matrices.

We have considerable experience in preparing protein–protein conjugates and will apply this expertise to a researcher's particular application through our custom synthesis service. We provide custom conjugation services on an exclusive or nondisclosure basis when requested. For more information or a quote, please go to www.invitrogen.com/handbook/custom/bioconjugates.

Assaying Maleimide- and Iodoacetamide-Modified Biomolecules

The potential instability of maleimide derivatives and the photosensitivity of iodoacetamide derivatives may make it advisable to assay the modified biomolecule for thiol reactivity before conjugation with a thiol-containing biomolecule. SAMSA fluorescein (A685, Figure 5.2.9), which is currently our only fluorescent reagent that can generate a free thiol group, was designed for assaying whether or not a biomolecule is adequately labeled with a heterobifunctional maleimide or iodoacetamide crosslinker. Brief treatment of SAMSA fluorescein with NaOH at pH 10 liberates a free thiol. By adding base-treated SAMSA fluorescein to a small aliquot of the crosslinker-modified biomolecule, the researcher can check to see whether the biomolecule has been sufficiently labeled before proceeding to the next step. The degree of modification can be approximated from either the absorbance or the fluorescence of the conjugate following quick purification on a gel-filtration column.

Alternatively, thiol reactivity of the modified biomolecule can be assayed using the reagents provided in our Thiol and Sulfide Quantitation Kit (T6060), a product that is described above.[19,22] Once unconjugated reagents have been removed, a small aliquot of the maleimide- or iodoacetamide-modified biomolecule can be reacted with excess cysteine. Thiol-reactive groups can then be quantitated by determining the amount of cysteine consumed in this reaction with the Thiol and Sulfide Quantitation Kit.

Amine–Carboxylic Acid Crosslinking

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, E2247) can react with biomolecules to form "zero-length" crosslinks, usually within a molecule or between subunits of a protein complex. In this chemistry, the crosslinking reagent is not incorporated into the final product. The water-soluble carbodiimide EDAC crosslinks a specific amine and carboxylic acid between subunits of allophycocyanin, thereby stabilizing its assembly; [46] we use EDAC to stabilize allophycocyanin in its allophycocyanin conjugates (Section 6.4). EDAC has also been

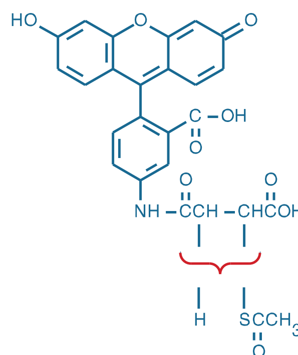

Figure 5.2.9 5-((2-(and-3)-*S*-(acetylmercapto)succinoyl)amino)fluorescein (SAMSA fluorescein, A685)

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used to form intramolecular crosslinks in myosin subfragment-1,[47] intermolecular crosslinks in actomyosin,[48] intersubunit crosslinks of chloroplast subunits[49] and DNA–protein crosslinks.[50] Addition of *N*-hydroxysuccinimide or *N*-hydroxysulfosuccinimide (NHSS, H2249) is reported to enhance the yield of carbodiimide-mediated conjugations,[51] indicating the *in situ* formation of a succinimidyl ester–activated protein (Figure 5.2.10). EDAC has been reported to be impermeant to cell membranes,[52] which should permit selective surface labeling of cellular carboxylic acids with fluorescent amines.

Reaction of carboxylic acids with cystamine ($H_2NCH_2CH_2S–SCH_2CH_2NH_2$) and EDAC followed by reduction with DTT results in thiolation at carboxylic acids.[7] This indirect route to amine–carboxylic acid coupling is particularly suited to acidic proteins with few amines, carbohydrate polymers,[6] heparin, poly(glutamic acid) and synthetic polymers lacking amines. The thiolated biomolecules can also be reacted with any of the probes described in Chapter 2.

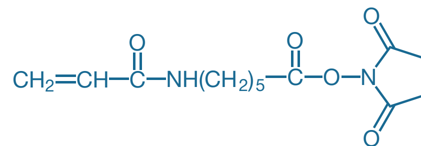

Figure 5.2.11 6-((acryloyl)amino)hexanoic acid, succinimidyl ester (acryloyl-X, SE, A20770).

Crosslinking Amines to Acrylamide Polymers

The succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (acryloyl-X, SE; A20770; Figure 5.2.11) reacts with amines of proteins, amine-modified nucleic acids and other biomolecules to yield acrylamides that can be copolymerized into polyacrylamide matrices or onto surfaces, such as in microarrays and in biosensors. For example, streptavidin acrylamide (S21379, Section 7.6) copolymerizes with acrylamide on polymeric surfaces to create a uniform monolayer of the immobilized protein. The immobilized streptavidin can then bind biotinylated ligands, including biotinylated hybridization probes, enzymes, antibodies and drugs.[53]

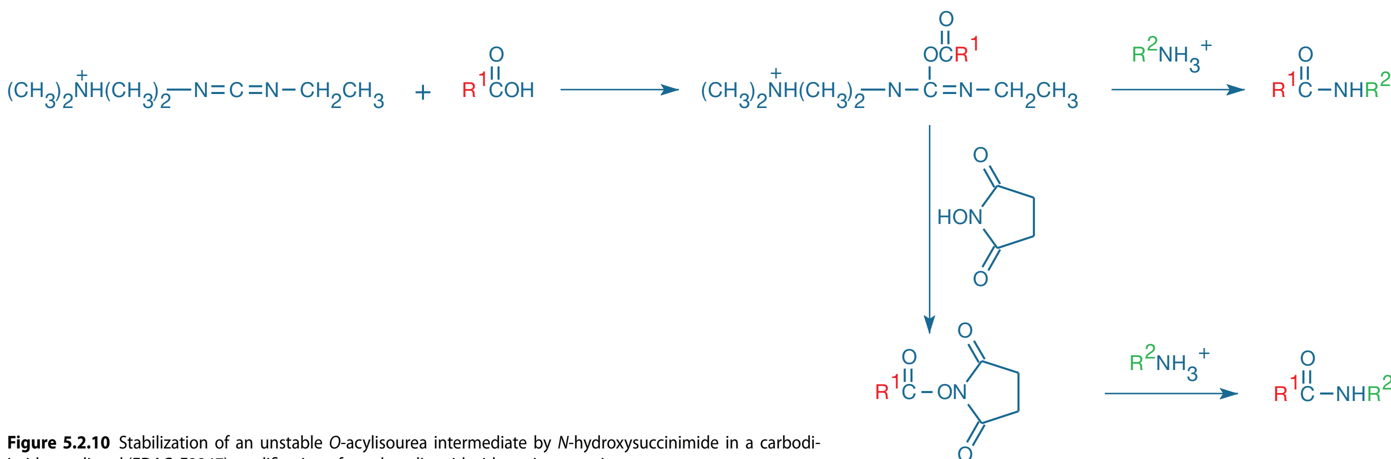

Figure 5.2.10 Stabilization of an unstable *O*-acylisourea intermediate by *N*-hydroxysuccinimide in a carbodiimide-mediated (EDAC, E2247) modification of a carboxylic acid with a primary amine.

REFERENCES

1. Bioconjug Chem (2001) 12:421; **2**. Methods Enzymol (1987) 143:246; **3**. Anal Biochem (2004) 325:137; **4**. Anal Biochem (1983) 132:68; **5**. Biochem J (1978) 173:723; **6**. Methods Md Biol (2008) 418:209; **7**. Biochim Biophys Acta (1990) 1038:382; **8**. Biochim Biophys Acta (1988) 971:307; **9**. Biochim Biophys Acta (1988) 971:298; **10**. J Biol Chem (1980) 255:10884; **11**. Methods Mol Biol (1995) 45:235; **12**. J Cell Biol (1982) 93:981; **13**. Nucleic Acids Res (1989) 17:4404; **14**. J Biochem Biophys Methods (1986) 12:349; **15**. Methods Enzymol (1994) 233:380; **16**. Methods Enzymol (1983) 91:49; **17**. Anal Biochem (1994) 220:5; **18**. Anal Biochem (1998) 265:8; **19**. Anal Biochem (1993) 213:49; **20**. Environ Sci Technol (2008) 42:8127; **21**. Anal Chem (2007) 79:1411; **22**. Bioconjug Chem (1994) 5:348; **23**. Anal Biochem (1995) 225:174; **24**. Proc Natl Acad Sci U S A (2000) 97:1461; **25**. J Mol Biol (2000) 299:421; **26**. Biochim Biophys Acta (1980) 622:201; **27**. Proc Natl Acad Sci U S A (1996) 93:10123; **28**. FEBS Lett (1982) 150:207; **29**. Biochemistry (2001) 40:7903; **30**. J Biol Chem (1996) 271:24465; **31**. J Biol Chem (2000) 275:39272; **32**. J Biol Chem (1999) 274:35388; **33**. Kidney Int (1997) 51:1797; **34**. J Biol Chem (1997) 272:31945; **35**. Bioconjug Chem (1998) 9:160; **36**. Nat Struct Biol (2001) 8:540; **37**. Biophys J (2001) 81:2851; **38**. Nat Methods (2010) 7:377; **39**. Methods Enzymol (1989) 172:584; **40**. Eur J Biochem (1979) 96:483; **41**. Nature (1981) 292:17; **42**. Nucleic Acids Res (1989) 17:4899; **43**. Chem Pharm Bull (Tokyo) (1989) 37:1831; **44**. Eur J Biochem (1979) 101:395; **45**. Anal Biochem (1991) 198:75; **46**. Cytometry (1987) 8:91; **47**. Biochemistry (1994) 33:6867; **48**. Biophys J (1995) 68:35S; **49**. Biochim Biophys Acta (1992) 1101:97; **50**. J Mol Biol (1978) 123:149; **51**. Anal Biochem (1986) 156:220; **52**. J Biol Chem (2000) 275:977; **53**. Anal Biochem (2000) 282:200.

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DATA TABLE 5.2 CHEMICAL CROSSLINKING REAGENTS

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A685	521.50	F,D,L	pH >6, DMF	491	78,000	515	pH 9	
A20770	282.30	F,D,L	DMSO	<300		none		
B10621	840.47	F,D,L	DMSO	549	88,000	575	MeOH	1
D1379	350.01	L	DMF, MeCN	391	6100	see Notes	MeOH	2
D1532	154.24	D	H_2O	<300		none		
D8451	396.35	D	pH >6	324	18,000	none	pH 8	3
E2247	191.70	F,D	H_2O	<300		none		
H2249	217.13	D	H_2O	<300		none		
S1531	312.36	F,D	DMF, MeCN	282	4700	none	MeOH	4
S1534	334.33	F,D	DMF, MeCN	<300		none		
S1553	231.22	F,D	DMF, MeCN	<300		none		
T2556	286.65	D	pH >5	<300		none		

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.
2. Bimanes are almost nonfluorescent until reacted with thiols. For monobromobimane conjugated to glutathione, Abs = 394 nm, Em = 490 nm (QY ~0.1–0.3) in pH 8 buffer. (Methods Enzymol (1987) 143:76, Methods Enzymol (1995) 251:133)
3. D8451 reaction product with thiols has Abs = 410 nm (EC = 14,000 $cm^{-1}M^{-1}$). (Methods Enzymol (1994) 233:380)
4. After conjugation of S1531 the degree of substitution can be determined by measuring the amount of 2-pyridinethione formed by treatment with DTT (D1532) or TCEP (T2556) from its absorbance at 343 nm (EC = 8000 $cm^{-1}M^{-1}$). (Biochem J (1978) 173:723)

PRODUCT LIST 5.2 CHEMICAL CROSSLINKING REAGENTS

Cat. No.	Product	Quantity
A685	5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino)fluorescein (SAMSA fluorescein) *mixed isomers*	25 mg
A20770	6-((acryloyl)amino)hexanoic acid, succinimidyl ester (acryloyl-X, SE)	5 mg
B10621	bis-((N-iodoacetyl)piperazinyl)sulfonerhodamine	5 mg
D1379	dibromobimane (bBBr)	25 mg
D8451	5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent)	10 g
D1532	dithiothreitol (DTT)	1 g
E2247	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDAC)	100 mg
H2249	N-hydroxysulfosuccinimide, sodium salt (NHSS)	100 mg
M30550	Measure-iT™ Thiol Assay Kit *500 assays*	1 kit
P6305	Protein–Protein Crosslinking Kit *3 conjugations*	1 kit
S1553	succinimidyl acetylthioacetate (SATA)	100 mg
S1534	succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC)	100 mg
S1531	succinimidyl 3-(2-pyridyldithio)propionate (SPDP)	100 mg
T6060	Thiol and Sulfide Quantitation Kit *50–250 assays*	1 kit
T2556	tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)	1 g

5.3 Photoactivatable Reagents, Including Photoreactive Crosslinkers and Caged Probes

This section describes two types of photoactivatable probes: products that form short-lived, high-energy intermediates that can chemically couple to nearby residues, and "caged" probes that are designed to be biologically inactive until UV light–mediated photolysis releases a natural product. Photolysis of each of these photoactivatable probes can be accomplished with high spatial and temporal resolution, releasing active probe at the site of interest.

Nonfluorescent Photoreactive Crosslinking Reagents

In contrast to chemical crosslinking reagents (Section 5.2), which are often used to prepare bioconjugates, photoreactive crosslinking reagents are important tools for determining the proximity of two sites. These probes can be employed to define relationships between two reactive groups that are on a single protein, on a ligand and its receptor or on separate biomolecules within an assembly. In the latter case, photoreactive crosslinking reagents can potentially reveal interactions among proteins, nucleic acids and membranes in live cells. The general scheme for defining spatial relationships usually involves photoreactive crosslinking reagents that contain a chemically reactive group as well as a photoreactive group. These crosslinkers are first chemically reacted with one molecule, for example a receptor ligand, and then this modified molecule is coupled to a second molecule, for example the ligand's receptor, using UV illumination. Depending on the reactive properties of the chemical and photoreactive groups, these crosslinkers can be used to couple like or unlike functional groups.

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We offer three types of photoreactive reagents for covalent labeling:

- Simple aryl azides that upon illumination (usually at <360 nm) generate reactive intermediates that form bonds with nucleophilic groups (Figure 5.3.1)
- Fluorinated aryl azides that upon UV photolysis generate reactive nitrenes, thereby producing more C–H insertion products than the simple aryl azides (Figure 5.3.2)
- Benzophenone derivatives that can be repeatedly excited at <360 nm until they generate covalent adducts, without loss of reactivity (Figure 5.3.3)

Simple Aryl Azide Crosslinker

The "transferable" aryl azide *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide (PEAS; AET; P6317; Figure 5.3.4) is a unique reagent for assessing protein–protein or protein–nucleic acid interactions. This aryl azide undergoes disulfide–thiol interchange of its pyridyldisulfide groups with the thiol groups of biomolecules to form mixed disulfides in the same way as SPDP [1] (S1531, Section 5.2). UV photolysis induces covalent crosslinking to residues or biomolecules adjacent to the crosslinker. The mixed disulfide can then be cleaved with DTT or TCEP (D1532, T2556; Section 5.2). If the phenolic PEAS reagent is radioiodinated before the coupling and photolysis steps, then only the resulting target biomolecule will be radioactive at the conclusion of the reaction.

Fluorinated Aryl Azides: True Nitrene-Generating Reagents

Although the simple aryl azides may be initially photolyzed to electron-deficient aryl nitrenes, it has been shown that these rapidly ring-expand to form dehydroazepines—molecules that tend to react with nucleophiles rather than form C–H insertion products.[2,3] In contrast, Keana and Cai have shown that the photolysis products of the fluorinated aryl azides are clearly aryl nitrenes [4] and undergo characteristic nitrene reactions such as C–H bond insertion with high efficiency. Moreover, conjugates prepared from the amine-reactive succinimidyl ester of 4-azido-2,3,5,6-tetrafluorobenzoic acid (ATFB, SE; A2522) may have quantum yields for formation of photocrosslinked products that are superior to those of the nonfluorinated aryl azides. An important application of the succinimidyl ester of ATFB is the photofunctionalization of polymer surfaces [5,6] (Figure 5.3.5).

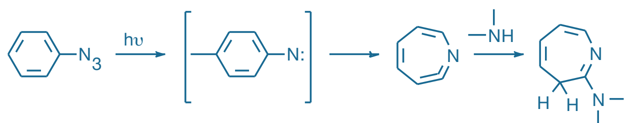

Figure 5.3.1 Photoreactive crosslinking reaction of a simple aryl azide.

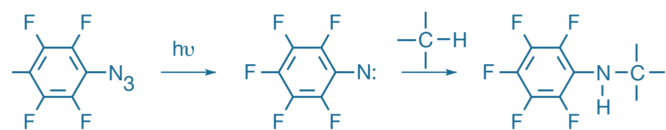

Figure 5.3.2 Photoreactive crosslinking reaction of a fluorinated aryl azide.

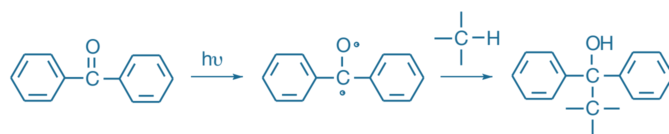

Figure 5.3.3 Photoreactive crosslinking reaction of a benzophenone derivative.

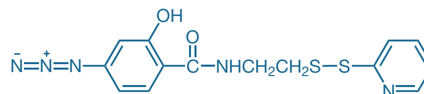

Figure 5.3.4 *N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide (PEAS; AET, P6317).

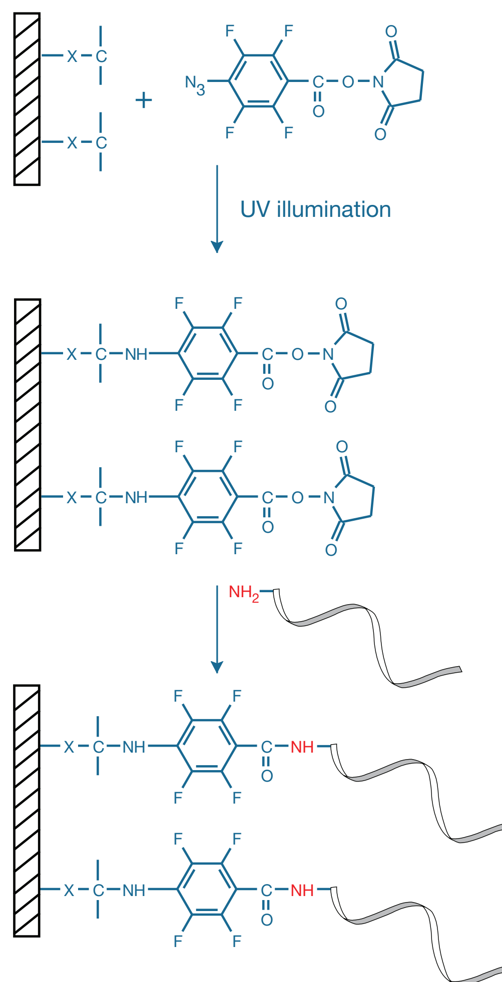

Figure 5.3.5 Schematic showing attachment of an amine-modified oligonucleotide to a surface using the photoreactive crosslinking reagent 4-azido-2,3,5,6-tetrafluorobenzoic acid, succinimidyl ester (ATFB, SE; A2522).

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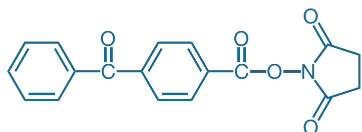


Figure 5.3.6 4-benzoylbenzoic acid, succinimidyl ester (B1577).

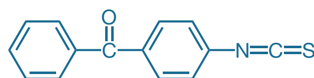


Figure 5.3.7 Benzophenone-4-isothiocyanate (B1526).

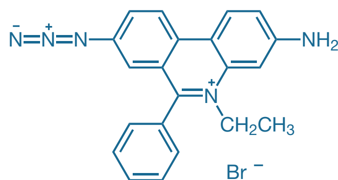
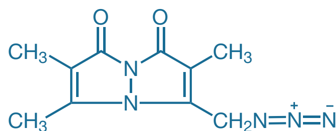


Figure 5.3.8 Ethidium monoazide bromide (EMA, E1374).



Figutre 5.3.9 Bimane azide (B30600).

Benzophenone-Based Photoreactive Reagents

Benzophenones generally have higher crosslinking yields than the aryl azide photoreactive reagents.[7] Benzophenone maleimide (B1508) has been used for efficient irreversible protein crosslinking of actin,[8] calmodulin,[9,10] myosin,[11,12] tropomyosin,[13] troponin,[14–17] ATP synthase[18,19] and other proteins. The succinimidyl ester of 4-benzoylbenzoic acid (B1577, Figure 5.3.6) and benzophenone isothiocyanate (B1526, Figure 5.3.7) have proven useful for synthesizing photoreactive peptides[20–23] and oligonucleotides.[24,25] A benzophenone-labeled ATP probe (BzBzATP, B22358) is described in Other Photoreactive Reagents below.

Other Photoreactive Reagents

Ethidium Monoazide for Photoreactive Fluorescent Labeling of Nucleic Acids

Ethidium monoazide (E1374, Figure 5.3.8) can be photolyzed in the presence of DNA or RNA to yield fluorescently labeled nucleic acids, both in solution and in cells.[26–29] The efficiency of the irreversible photolytic coupling of ethidium monoazide, which intercalates into nucleic acids like ethidium bromide, is unusually high[30] (>40%). The membrane-impermeant ethidium monoazide is reported to label only those cells with compromised membranes and can therefore serve as a fixable cell viability probe. This property, allied to the blocking of transcription caused by photoreaction of ethidium monoazide with DNA, provides a method for suppressing PCR amplification of dead-cell DNA.[31,32] Similarly, multiphoton-targeted photochemistry of vertebrate cells labeled with ethidium monoazide was used to selectively inactivate gene expression.[33] A mixed population of live and dead cells labeled with ethidium monoazide retains its staining pattern after aldehyde-based fixation, thereby reducing the investigator's exposure to potentially pathogenic cells during cell viability analysis.[34,35]

Bimane Azide for Photoaffinity Labeling of Proteins

Bimane azide (B30600, Figure 5.3.9) is a small blue-fluorescent photoreactive alkyl azide (excitation/emission maxima ~375/458 nm) for photoaffinity labeling of proteins. This reactive fluorophore's small size may reduce the likelihood that the label will interfere with the function of the biomolecule, an important advantage for site-selective probes.

Photoreactive ATP Derivative for Labeling Nucleotide-Binding Proteins

Functional ion channels can be assembled from both homomeric and heteromeric combinations of the seven P2X purinergic receptor subunits so far identified ($P2X_{1–7}$). Due to the lack of specific agonists or antagonists for P2X receptors, it is difficult to determine which receptor subtypes mediate particular cellular responses. We offer one of the most potent and widely used P2X receptor agonists,[36–39] BzBzATP (2′-(or 3′-)*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate, B22358; Figure 5.3.10). BzBzATP also has more general applications for site-directed irreversible modification of nucleotide-binding proteins via photoaffinity labeling.[40,41]

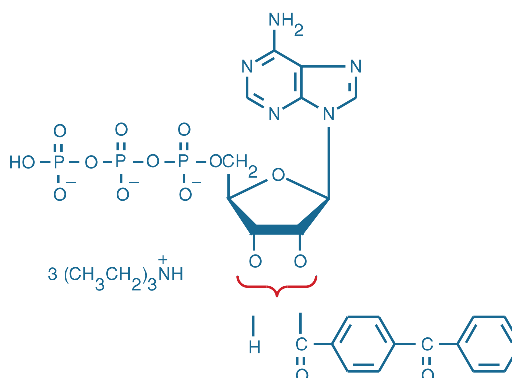


Figure 5.3.10 2′-(or-3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate, tris(triethylammonium) salt (BzBzATP, B22358).

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Caged Probes and Their Photolysis

Flash photolysis of photoactivatable or "caged" probes provides a means of controlling the release—both spatially and temporally—of biologically active products or other reagents of interest.[42–48] The chemical caging process may also confer membrane permeability on the caged ligand, as is the case for caged cAMP[49] and caged luciferin.[50] Our extensive selection of caged nucleotides, second messengers (Figure 5.3.11), chelators and neurotransmitters has tremendous potential for use with both live cells and isolated proteins. These caged probes provide researchers with important tools for delivering physiological stimuli by naturally active biomolecules with spatial and temporal precision that far exceeds that of microinjection or perfusion. A recent review by Ellis-Davies describes the optical and chemical properties of many of our caged compounds, as well as of several common caging groups.[51]

Caging Groups

The caging moiety (Table 5.2) is designed to *maximally* interfere with the binding or activity of the molecule. It is detached in microseconds to milliseconds by flash photolysis at ≤360 nm, resulting in a pulse (concentration jump) of active product. Uncaging can easily be accomplished with UV illumination in a fluorescence microscope or with a UV laser or UV flashlamp. Low-cost light-emitting diodes[52] (LED) and 405 nm violet diode lasers[53] are providing increased access to experimentation using caged compounds. The effects of photolytic release are frequently monitored either with fluorescent probes that measure calcium, pH, other ions or membrane potential, or with electrophysiological techniques.

Most of the caged reagents described in the literature have been derivatives of *o*-nitrobenzylic compounds. The nitrobenzyl group is synthetically incorporated into the biologically active molecule by linkage to a heteroatom (usually *O, S* or *N*) as an ether, thioether, ester (including phosphate or thiophosphate esters), amine or similar functional group. Both the structure of the nitrobenzylic compound and the atom to which it is attached affect the efficiency and wavelength required for uncaging. We currently use six different photolabile protecting groups in our caged probes.[44] Their properties are summarized in Table 5.2.

- Probes caged with our α-carboxy-2-nitrobenzyl (CNB) caging group generally have the most advantageous properties. These include good water solubility, very fast uncaging rates in the microsecond range, high photolysis quantum yields (from 0.2–0.4) and biologically inert photolytic by-products. Although the absorption maximum of the CNB caging group is near 260 nm, its absorption spectrum tails out to approximately 360 nm, allowing successful photolysis using light with wavelengths ≤360 nm.
- The 1-(2-nitrophenyl)ethyl (NPE) caging group has properties similar to those of CNB and can also be photolyzed at ≤360 nm.
- As compared with CNB and NPE, the 4,5-dimethoxy-2-nitrobenzyl (DMNB) and 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) caging groups have longer-wavelength absorption (absorption maximum ~355 nm) and therefore absorb 340–360 nm light more efficiently. However, photolysis rates and quantum yields of DMNB- and DMNPE-caged probes are generally lower than those obtained for CNB-caged probes.
- The 5-carboxymethoxy-2-nitrobenzyl (CMNB) caging group provides an absorption maximum of intermediate wavelength (absorption maximum ~310 nm), while imparting significant water solubility to the caged probe. Its photolysis rate and quantum yield are intermediate between those of CNB- and DMNB-caged probes.
- The nitrophenyl (NP) caging group is available on the caged calcium reagent NP-EGTA (N6802), a photolabile Ca^{2+} chelator that can be used to rapidly deliver a pulse of Ca^{2+} upon illumination with ultraviolet light, with a high photolysis quantum yield of 0.23.

Experiments utilizing probes caged with any of the above caging groups, except the CNB caging group, may require the addition of dithiothreitol (DTT, D1532; Section 2.1). This reducing reagent prevents the potentially cytotoxic reaction between amines and the 2-nitrosobenzoyl photolytic by-products.[54]

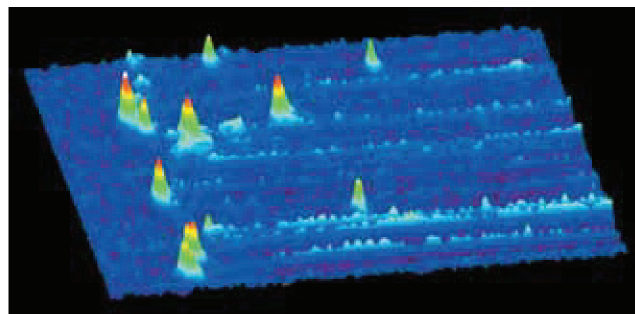

Figure 5.3.11 Confocal linescan image of calcium "puffs" in a *Xenopus* oocyte. Oregon Green® 488 BAPTA-1 (O6806) was used as the calcium indicator and Ca^{2+} liberation was evoked by flash photolysis of NPE-caged Ins 1,4,5-P_3 (I23580). Image contributed by Ian Parker and Nick Callamaras, University of California at Irvine.

Table 5.2 Properties of six different caging groups.

Caging Group	Uncaging Rate *	Photolysis Quantum Yield *	Inertness of Photolysis By-product	Confers Water Solubility	Long-Wavelength Absorption (≥360 nm)
CNB	++++	+++++	+++++	+++++	++
NPE	+++	+++	+++	+	++
DMNPE	+++	+++	+++	+	+++++
DMNB	+++	+++	++	+	+++++
CMNB	+++	+++	+	++++	+++
NP	+++	+++	+	+	++

+++++ = Optimal response. + = Poor response. * Both the structure of the nitrobenzyl moiety and the atom to which it is attached have some effect on the efficiency and wavelength required for uncaging.

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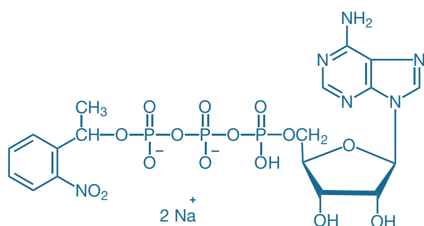

Figure 5.3.12 Adenosine 5′-triphosphate, P^3-(1-(2-nitrophenyl)ethyl) ester, disodium salt (NPE-caged ATP, A1048).

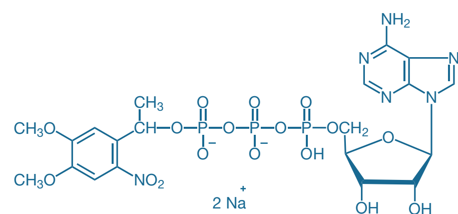

Figure 5.3.13 Adenosine 5′-triphosphate, P^3-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ester, disodium salt (DMNPE-caged ATP, A1049).

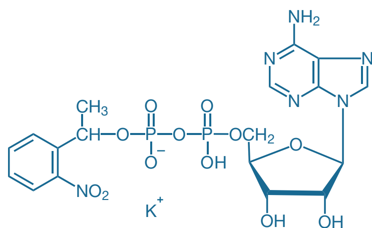

Figure 5.3.14 Adenosine 5′-diphosphate, P^2-(1-(2-nitrophenyl)ethyl) ester, monopotassium salt (NPE-caged ADP, A7056).

Caged Nucleotides

Photoactivatable nucleotides and phosphates have contributed significantly to our understanding of cytoskeleton dynamics, signal transduction pathways and other critical cellular processes.[46] Some of our caged nucleotides are available with a choice of caging group:

- Caged ATP (A1048, Figure 5.3.12; A1049, Figure 5.3.13), which has been shown to release ATP in skinned muscle fibers,[55] sarcoplasmic reticulum vesicles,[56] submitochondrial particles[57] and membrane fragments containing Na^+/K^+-ATPase[58]
- Caged ADP (A7056, Figure 5.3.14), which has been used to investigate the molecular basis of contraction of skeletal muscle fibers,[59,60] as well as transport by an ADP/ATP carrier[61]
- Caged cAMP (D1037, Figure 5.3.15), which is cell-permeant and rapidly photolyzed to cAMP[62]
- Caged inositol 1,4,5-triphosphate[63–67] (I23580, Figure 5.3.16) and caged cADP-ribose[68,69] (C7074, Figure 5.3.17), which are important probes for second messenger studies (Section 17.2)

NPE-caged Ins 1,4,5-P_3 can be used to generate rapid and precisely controlled release of Ins 1,4,5-P_3 in intact cells (Figure 5.3.11) and is widely employed in studies of Ins 1,4,5-P_3–mediated second messenger pathways.[63] Our NPE-caged Ins 1,4,5-P_3 (I23580) is a mixture of the physiologically inert, singly esterified P^4 and P^5 esters (Figure 5.3.16) and does not contain the somewhat physiologically active P^1 ester. NPE-caged Ins 1,4,5-P_3 exhibits essentially no biological activity prior to photolytic release of the biologically active Ins 1,4,5-P_3 (I3716, Section 17.2).

Cyclic ADP-ribose (cADP-ribose) is a potent intracellular Ca^{2+}–mobilizing agent that functions as a second messenger in an Ins 1,4,5-P_3–independent pathway.[70–75] Our NPE-caged cADP-ribose (C7074, Figure 5.3.17) induces Ca^{2+} mobilization in sea urchin egg homogenates only after photolysis, and this Ca^{2+} release is inhibited by the specific cADP-ribose antagonist 8-amino-cADP-ribose[69] (A7621, Section 17.2). Furthermore, when microinjected into live sea urchin eggs, NPE-caged cADP-ribose was shown to mobilize Ca^{2+} and activate cortical exocytosis after illumination with a mercury-arc lamp.[69]

Caged Ca^{2+} Reagents: NP-EGTA and DMNP-EDTA

Caged ions and caged chelators can be used to influence the ionic composition of both solutions and cells, particularly for ions such as Ca^{2+} that are present at low concentrations under normal physiological conditions. Developed by Ellis-Davies and Kaplan,[76] nitrophenyl EGTA (NP-EGTA) is a photolabile Ca^{2+} chelator that exhibits a high selectivity for Ca^{2+} ions, a dramatic increase in its K_d for Ca^{2+} upon illumination (from 80 nM to 1 mM) and a high photolysis quantum yield (0.23). NP-EGTA's affinity for Ca^{2+} *decreases* ~12,500-fold upon photolysis. Furthermore, its K_d for Mg^{2+} of 9 mM makes NP-EGTA essentially insensitive to

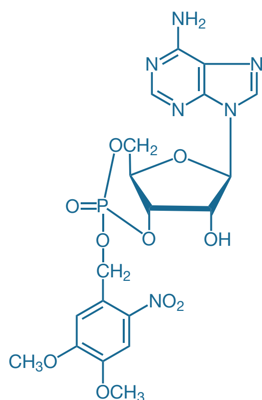

Figure 5.3.15 4,5-dimethoxy-2-nitrobenzyl adenosine 3′,5′-cyclicmonophosphate (DMNB-caged cAMP, D1037).

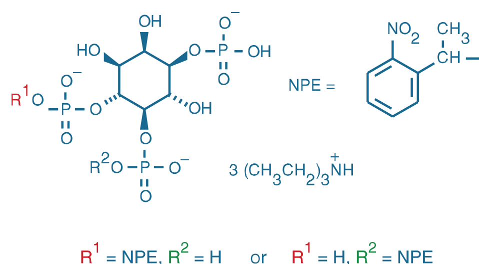

Figure 5.3.16 D-myo-inositol 1,4,5-triphosphate, $P_{4(5)}$-(1-(2-nitrophenyl)ethyl) ester, tris(triethylammonium) salt (NPE-caged Ins 1,4,5-P_3, I23580).

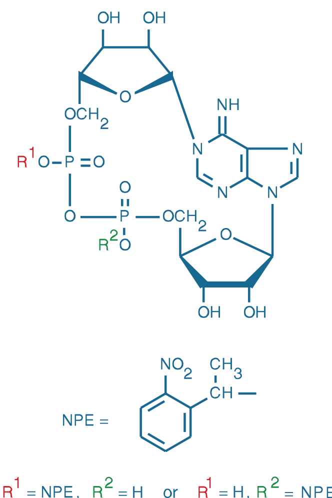

Figure 5.3.17 Cyclic adenosine 5′-diphosphate ribose, 1-(1-(2-nitrophenyl)ethyl) ester (NPE-caged cADP-ribose, C7074).

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physiological Mg^{2+} concentrations. We exclusively offer the tetrapotassium salt (N6802) and the acetoxymethyl (AM) ester (N6803) of NP-EGTA. The NP-EGTA salt can be complexed with Ca^{2+} to generate a caged Ca^{2+} reagent that will rapidly deliver Ca^{2+} upon photolysis [67,77,78] (Figure 5.3.18). The cell-permeant AM ester of NP-EGTA does not bind Ca^{2+} unless its AM ester groups are removed. This AM ester can serve as a photolabile chelator in cells because, once converted to NP-EGTA by intracellular esterases, it will bind free Ca^{2+} until photolyzed with UV light.

The first caged Ca^{2+} reagent described by Kaplan and Ellis-Davies was 1-(4,5-dimethoxy-2-nitrophenyl) EDTA (DMNP-EDTA, D6814), which they named DM-Nitrophen™ [79,80] (now a trademark of Calbiochem-Novabiochem Corp.). Because its structure more resembles that of EDTA than EGTA, we named it as a caged EDTA derivative (Figure 5.3.19). Upon illumination, DMNP-EDTA's affinity for Ca^{2+} *decreases* ~600,000-fold and its K_d for Ca^{2+} rises from 5 nM to 3 mM. Thus, photolysis of DMNP-EDTA complexed with Ca^{2+} results in a pulse of free Ca^{2+}. DMNP-EDTA has a stronger absorbance at longer wavelengths than does NP-EGTA (Figure 5.3.20), which facilitates uncaging. Furthermore, DMNP-EDTA has significantly higher affinity for Mg^{2+} (K_d = 2.5 µM) [79] than does NP-EGTA (K_d = 9 mM),[76] making it a potentially useful caged Mg^{2+} reagent. Two reviews by Ellis-Davies discuss the uses and limitations of DMNP-EDTA.[51,81]

Diazo-2: A Photoactivatable Ca^{2+} Knockdown Reagent

In contrast to NP-EGTA and DMNP-EDTA, diazo-2 (D3034) is a photoactivatable Ca^{2+} scavenger. Diazo-2 (Figure 5.3.21), which was introduced by Adams, Kao and Tsien,[82,83] is a relatively weak chelator (K_d for Ca^{2+} = 2.2 µM). Following flash photolysis at ~360 nm, however, cytosolic free Ca^{2+} rapidly binds to the diazo-2 photolysis product, which has a high affinity for Ca^{2+} (K_d = 73 nM). Intracellular loading of NP-EGTA, DMNP-EDTA and diazo-2 is best accomplished by patch pipette infusion with the carboxylate salt form of the caged compound added to the internal pipette solution at 1–10 mM. These reagents are increasingly being applied *in vivo* for controlled intervention in calcium-regulated fundamental processes in neurobiology [84] and developmental biology.[85]

Caged Amino Acid Neurotransmitters

Once activated, caged amino acid neurotransmitters rapidly initiate neurotransmitter action (Figure 5.3.22), providing tools for kinetic studies of receptor binding or channel opening.[42,46] We offer caged carbamylcholine [86–94] (*N*-(CNB-caged) carbachol, C13654, Figure 5.3.23) and caged γ-aminobutyric acid [67,95–97] (*O*-(CNB-caged) GABA, A7110, Figure 5.3.24), as well as two caged versions of L-glutamic acid [96,98–104] (C7122, G7055), all of which are biologically inactive before photolysis.[44]

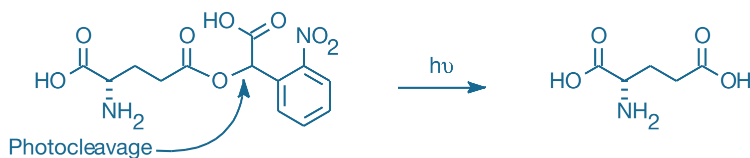

Figure 5.3.22 CNB-caged L-glutamic acid (G7055). The CNB-caging group is rapidly photocleaved with UV light to release L-glutamic acid.

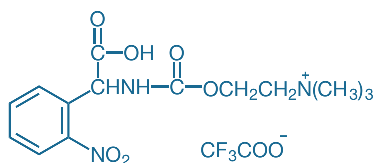

Figure 5.3.23 *N*-(CNB-caged) carbachol (N-(α-carboxy-2-nitrobenzyl)carbamylcholine, trifluoroacetic acid salt, C13654)

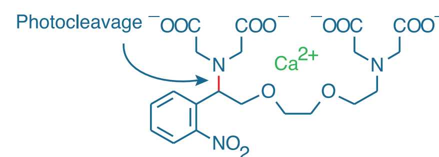

Figure 5.3.18 NP-EGTA (N6802) complexed with Ca^{2+}. Upon illumination, this complex is cleaved to yield free Ca^{2+} and two iminodiacetic acid photoproducts. The affinity of the photoproducts for Ca^{2+} is ~12,500-fold lower than that of NP-EGTA.

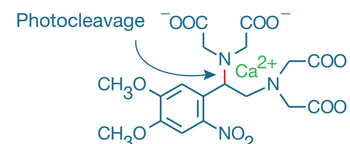

Figure 5.3.19 DMNP-EDTA (D6814) complexed with Ca^{2+}. Upon illumination, this complex is cleaved to yield free Ca^{2+} and two iminodiacetic acid photoproducts. The affinity of the photoproducts for Ca^{2+} is ~600,000-fold lower than that of DMNP-EDTA.

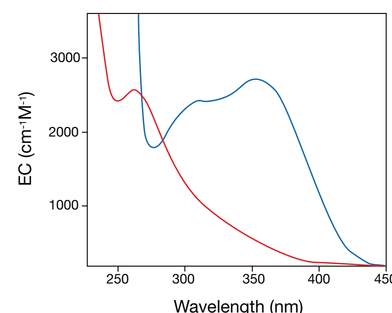

Figure 5.3.20 Spectral comparison of equimolar concentrations of the caged Ca^{2+} reagents NP-EGTA (N6802, red line) and DMNP-EDTA (D6814, blue line), illustrating the optimal wavelengths for photolysis and subsequent release of Ca^{2+} from these chelators. Spectra were taken in 100 mM KCl and 30 mM MOPS buffer containing 39.8 µM free Ca^{2+} at pH 7.2.

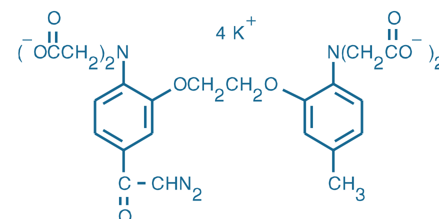

Figure 5.3.21 Diazo-2, tetrapotassium salt (D3034).

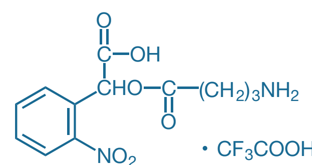

Figure 5.3.24 γ-aminobutyric acid, α-carboxy-2-nitrobenzyl ester, trifluoroacetic acid salt (O-(CNB-caged) GABA, A7110).

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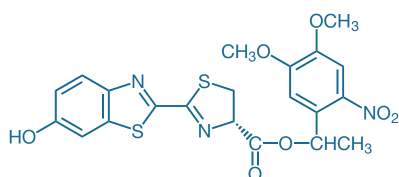

Figure 5.3.25 D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE-caged luciferin, L7085).

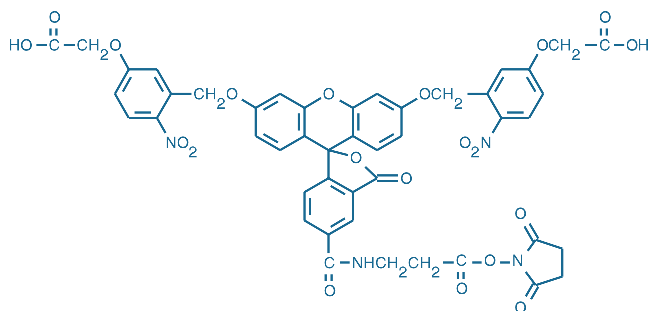

Figure 5.3.26 5-carboxyfluorescein-bis-(5-carboxymethoxy-2-nitrobenzyl) ether, β-alanine-carboxamide, succinimidyl ester (CMNB-caged carboxyfluorescein, SE, C20050).

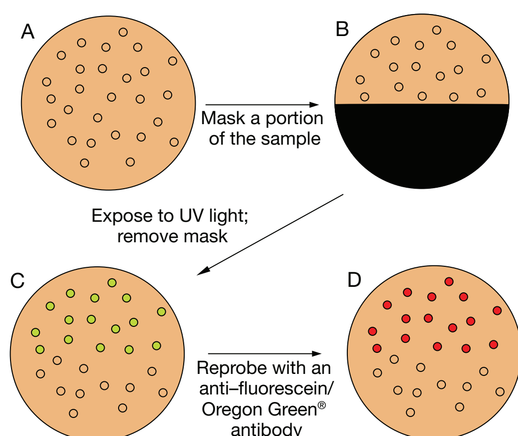

Figure 5.3.27 Schematic representation of photoactivated fluorescence combined with sample masking. Initially, no fluorescence is observed from samples stained with a CMNB-caged fluorescein-labeled secondary detection reagent (**A**). The desired mask is then placed over the sample (**B**), after which the sample is exposed to UV light. The mask is then removed; fluorescein molecules present in the unmasked portion of the sample are uncaged by the UV light and fluoresce brightly when viewed with the appropriate filters (**C**). Uncaged fluorescein may now also serve as a hapten for further signal amplification using our anti–fluorescein/Oregon Green® antibody. For example, probing with the anti–fluorescein/Oregon Green® antibody followed by staining with the Alexa Fluor® 594 goat anti–mouse IgG antibody can be used to change the color of the uncaged probe to red fluorescent (**D**).

Caged Luciferin

Luciferase produces light by the ATP-dependent oxidation of luciferin. The 560 nm chemiluminescence from this reaction peaks within seconds, with light output that is proportional to luciferase activity or ATP concentrations. DMNPE-caged luciferin (L7085, Figure 5.3.25) readily crosses cell membranes, allowing more efficient delivery of luciferin into intact cells.[50] Once the caged luciferin is inside the cell, active luciferin can be released either instantaneously by a flash of UV light, or continuously by the action of endogenous intracellular esterases found in many cell types.

Caged Fluorescent Dyes

Photoactivatable fluorescent dyes, which are generally colorless and nonfluorescent until photolyzed with UV light,[51] are particularly useful for investigating cell lineage[105] and for spatiotemporal interrogation of fluid flows.[106–108] In addition to CMNB-caged fluorescein[107] (F7103), we prepare the succinimidyl ester of CMNB-caged carboxyfluorescein (C20050, Figure 5.3.26), which can be used to attach the caged fluorophore to primary amine groups of a variety of biomolecules. CMNB-caged carboxyfluorescein succinimdyl ester is a key starting material in the preparation of probes for super-resolution photoactivation microscopy.[109,110] Furthermore, caged fluorescein probes are immunochemically cryptic; i.e., the probe is immunoreactive with anti–fluorescein/Oregon Green® antibodies (Section 7.4) after but not before photoactivation (Figure 5.3.27).

Kit for Caging Carboxylic Acids

Using organic synthesis methods, researchers can cage a diverse array of molecules. One of the preferred caging groups is the 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE) ester. Because the diazoethane precursor to DMNPE esters is unstable, we offer a kit (D2516) for the generation of 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane and the subsequent preparation of DMNPE esters. This kit includes:

- 25 mg of the hydrazone precursor
- MnO_2 for oxidation
- Celite® for filtration of the reaction mixture
- Detailed protocols for caging carboxylic acids

A wide range of compounds containing a weak oxy acid (with a pK_a between 3 and 7), including carboxylic acids, phenols and phosphates, should react with the diazoethane to form the DMNPE-caged analogs[54] (Figure 5.3.28).

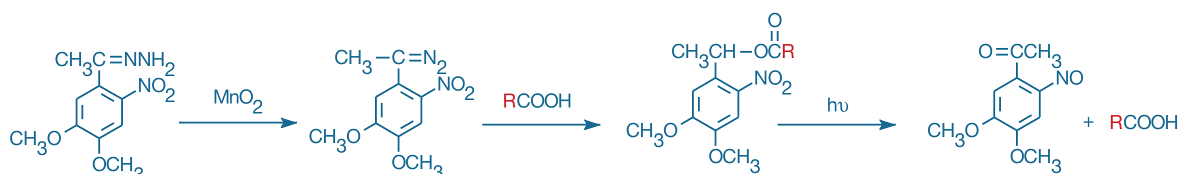

Figure 5.3.28 Caging of a carboxylic acid using the hydrazone precursor of DMNPE, a reagent that is provided in the 1-(4,5-Dimethoxy-2-nitrophenyl)diazoethane Generation Kit (D2516).

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DATA TABLE 5.3 PHOTOACTIVATABLE REAGENTS, INCLUDING PHOTOREACTIVE CROSSLINKERS AND CAGED PROBES

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A1048	700.30	FF,D,LL	H_2O	259	18,000	none	MeOH	1, 2, 3
A1049	760.35	FF,D,LL	H_2O	351	4400	none	H_2O	1, 2
A2522	322.17	F,D,LL	DMF	273	23,000	none	EtOH	3
A7056	614.44	FF,D,LL	H_2O	259	15,000	none	MeOH	1, 2, 3
A7110	396.28	F,D,LL	H_2O	262	4500	none	pH 7	2, 3
B1508	277.28	F,D	DMF, MeCN	260	17,000	none	MeOH	3, 4
B1526	239.29	F,DD	DMF, MeCN	300	26,000	none	MeOH	3
B1577	323.30	F,D	DMF, MeCN	256	27,000	none	MeOH	3
B22358	1018.97	FF,L	H_2O	260	27,000	none	pH 7	3, 5, 6, 7
B30600	233.23	F,D,L	DMSO	375	6000	458	MeOH	
C7074	690.45	FF,D,LL	H_2O	259	16,000	none	H_2O	2, 3
C7122	326.26	F,D,LL	H_2O	266	4800	none	pH 7	2, 3
C13654	439.34	F,D,LL	H_2O	264	4200	none	H_2O	2, 3
C20050	962.79	F,D,LL	DMSO	289	9500	none	MeOH	2, 8
D1037	524.38	F,D,LL	DMSO	338	6100	none	MeOH	1, 2
D3034	710.86	F,D,LL	pH >6	369	18,000	none	pH 7.2	2, 9
D6814	473.39	D,LL	DMSO	348	4200	none	pH 7.2	2, 10
E1374	420.31	F,LL	DMF, EtOH	462	5400	625	pH 7	11
F7103	826.81	FF,D,LL	H_2O, DMSO	333	15,000	none	DMSO	2, 8, 12
G7055	440.29	F,D,LL	H_2O, DMSO	262	5100	none	pH 7	2, 3
I23580	872.82	FF,D,LL	H_2O	264	4200	none	H_2O	2, 3, 13
L7085	489.52	FF,D,LL	DMSO, DMF	334	22,000	none	MeOH	2, 14
N6802	653.81	FF,D,LL	pH >6	260	3500	none	pH 7.2	2, 3, 15
N6803	789.70	FF,D,LL	DMSO	250	4200	none	MeCN	16, 17
P6317	347.41	F,D,LL	DMSO	271	24,000	none	MeOH	18

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Caged nucleotide esters are free of contaminating free nucleotides when initially prepared. However, some decomposition may occur during storage.
2. All photoactivatable probes are sensitive to light. They should be protected from illumination except when photolysis is intended.
3. This compound has weaker visible absorption at >300 nm but no discernible absorption peaks in this region.
4. Spectral data of the 2-mercaptoethanol adduct.
5. The molecular weight (MW) of this product is approximate because the degree of hydration and/or salt form has not been conclusively established.
6. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
7. This product can be activated by long-wavelength ultraviolet light (>300 nm) for photoaffinity labeling of proteins.
8. This product is colorless and nonfluorescent until it is activated by ultraviolet photolysis. Photoactivation generates a fluorescein derivative with spectral characteristics similar to C1359.

continued on next page

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DATA TABLE 5.3 PHOTOACTIVATABLE REAGENTS, INCLUDING PHOTOREACTIVE CROSSLINKERS AND CAGED PROBES—*continued*

9. The Ca^{2+} dissociation constant of diazo-2 is 2200 nM before photolysis and 73 nM after ultraviolet photolysis. The absorption spectrum of the photolysis product is similar to that of B1204. (J Am Chem Soc (1989) 111:7957)
10. $K_d(Ca^{2+})$ increases from 5 nM to 3 mM after ultraviolet photolysis. K_d values determined in 130 mM KCl, 10 mM HEPES, pH 7.1. (Proc Natl Acad Sci U S A (1988) 85:6571)
11. E1374 spectral data are for the free dye. Fluorescence is weak, but intensity increases ~15-fold on binding to DNA. After photocrosslinking to DNA, Abs = 504 nm (EC ~4000 $cm^{-1}M^{-1}$), Em = 600 nm. (Nucleic Acids Res (1978) 5:4891, Biochemistry (1980) 19:3221)
12. Unstable in water. Use immediately.
13. Ultraviolet photolysis of I23580 generates I3716 (Section 17.2).
14. L7085 is converted to bioluminescent luciferin (L2911, Section 10.6) upon ultraviolet photoactivation.
15. K_d (Ca^{2+}) increases from 80 nM to 1 mM after ultraviolet photolysis. K_d values determined in 100 mM KCl, 40 mM HEPES, pH 7.2. (Proc Natl Acad Sci U S A (1994) 91:187)
16. This product is intrinsically a liquid or an oil at room temperature.
17. N6803 is converted to N6802 via hydrolysis of its acetoxymethyl ester (AM) groups.
18. The absorption spectrum of P6317 includes an additional shoulder at 306 nm (EC = 10,000 $cm^{-1}M^{-1}$).

PRODUCT LIST 5.3 PHOTOACTIVATABLE REAGENTS, INCLUDING PHOTOREACTIVE CROSSLINKERS AND CAGED PROBES

Cat. No.	Product	Quantity
A7056	adenosine 5′-diphosphate, P^2-(1-(2-nitrophenyl)ethyl) ester, monopotassium salt (NPE-caged ADP)	5 mg
A1048	adenosine 5′-triphosphate, P^3-(1-(2-nitrophenyl)ethyl) ester, disodium salt (NPE-caged ATP)	5 mg
A1049	adenosine 5′-triphosphate, P^3-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ester, disodium salt (DMNPE-caged ATP)	5 mg
A7110	γ-aminobutyric acid, α-carboxy-2-nitrobenzyl ester, trifluoroacetic acid salt (*O*-(CNB-caged) GABA)	5 mg
A2522	4-azido-2,3,5,6-tetrafluorobenzoic acid, succinimidyl ester (ATFB, SE)	25 mg
B1526	benzophenone-4-isothiocyanate	100 mg
B1508	benzophenone-4-maleimide	100 mg
B1577	4-benzoylbenzoic acid, succinimidyl ester	100 mg
B22358	2′-(or-3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate, tris(triethylammonium) salt (BzBzATP) *5 mM in buffer*	2 mL
B30600	bimane azide	5 mg
C20050	5-carboxyfluorescein-bis-(5-carboxymethoxy-2-nitrobenzyl) ether, β-alanine-carboxamide, succinimidyl ester (CMNB-caged carboxyfluorescein, SE)	1 mg
C13654	*N*-(CNB-caged) carbachol (*N*-(α-carboxy-2-nitrobenzyl)carbamylcholine, trifluoroacetic acid salt)	5 mg
C7122	*N*-(CNB-caged) L-glutamic acid (*N*-(α-carboxy-2-nitrobenzyl)-L-glutamic acid)	5 mg
C7074	cyclic adenosine 5′-diphosphate ribose, 1-(1-(2-nitrophenyl)ethyl) ester (NPE-caged cADP-ribose) *mixed isomers*	50 µg
D3034	diazo-2, tetrapotassium salt *cell impermeant*	1 mg
D6814	1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-*N,N,N′,N′*-tetraacetic acid (DMNP-EDTA) *cell impermeant*	5 mg
D1037	4,5-dimethoxy-2-nitrobenzyl adenosine 3′,5′-cyclicmonophosphate (DMNB-caged cAMP)	5 mg
D2516	1-(4,5-Dimethoxy-2-nitrophenyl)diazoethane Generation Kit	1 kit
E1374	ethidium monoazide bromide (EMA)	5 mg
F7103	fluorescein bis-(5-carboxymethoxy-2-nitrobenzyl) ether, dipotassium salt (CMNB-caged fluorescein)	5 mg
G7055	L-glutamic acid, γ-(α-carboxy-2-nitrobenzyl) ester, trifluoroacetic acid salt (γ-(CNB-caged) L-glutamic acid)	5 mg
I23580	D-*myo*-inositol 1,4,5-triphosphate, $P_{4(5)}$-(1-(2-nitrophenyl)ethyl) ester, tris(triethylammonium) salt (NPE-caged Ins 1,4,5-P_3)	25 µg
L7085	D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester (DMNPE-caged luciferin)	5 mg
N6803	*o*-nitrophenyl EGTA, AM (NP-EGTA, AM) *cell permeant* *special packaging*	20 x 50 µg
N6802	*o*-nitrophenyl EGTA, tetrapotassium salt (NP-EGTA) *cell impermeant*	1 mg
P6317	*N*-((2-pyridyldithio)ethyl)-4-azidosalicylamide (PEAS; AET)	10 mg

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