

## 1.1 Introduction to Amine Modification

Molecular Probes provides a full spectrum of fluorophores and haptens for labeling biopolymers and derivatizing low molecular weight molecules. Chapters 1–5 describe the chemical and spectral properties of the reactive reagents we offer, whereas the remainder of this *Handbook* is primarily devoted to our diverse collection of fluorescent probes and their applications in cell biology, immunology, biochemistry, biophysics, microbiology, molecular biology, genomics, proteomics and neuroscience.

### Common Applications for Amine-Reactive Probes

#### Labeling Biopolymers

Amine-reactive probes are widely used to modify proteins, peptides, ligands, synthetic oligonucleotides and other biomolecules. In contrast to our thiol-reactive reagents (Chapter 2), which frequently serve as probes of protein structure and function, amine-reactive dyes are most often used to prepare bioconjugates for immunochemistry, fluorescence *in situ* hybridization (FISH), cell tracing, receptor labeling and fluorescent analog cytochemistry.<sup>1</sup> In these applications, the stability of the chemical bond between the dye and biomolecule is particularly important because the conjugate is typically stored and used repeatedly over a relatively long period of time. Moreover, these conjugates are often subjected to rigorous hybridization and washing steps that demand a strong dye–biomolecule linkage.

Our selection of amine-reactive fluorophores for modifying biomolecules covers the entire visible and near-infrared spectrum (Table 1.1). An up-to-date bibliography is available on our Web site for most of our amine-reactive probes. Also available are other product-specific bibliographies, as well as keyword searches of the over 44,000 literature references in our extensive bibliography database. Chapter 1 discusses the properties of Molecular Probes' most important proprietary fluorophores, including our premier sets of Alexa Fluor dyes (Section 1.3) and BODIPY dyes (Section 1.4), our Oregon Green and Rhodamine Green dyes (Section 1.5), the red-fluorescent Rhodamine Red-X and Texas Red dyes (Section 1.6) and the UV light–excitable Cascade Blue, Cascade Yellow, Marina Blue, Pacific Blue and AMCA-X fluorophores (Section 1.7). Our essentially nonfluorescent QSY dyes (Section 1.6, Section 1.8) have strong visible absorption, making them excellent acceptors for fluorescence resonance energy transfer (FRET, see Section 1.3) applications.

#### Preparing the Optimal Bioconjugate

The preferred bioconjugate usually has a high fluorescence yield (or, in the case of a haptenylated conjugate, a suitable degree of labeling) yet retains the critical parameters of the unlabeled biomolecule, such as selective binding to a receptor or nucleic acid, activation or inhibition of a particular enzyme or the ability to incorporate into a biological membrane. Frequently, however, conjugates with the highest degree of labeling precipitate or bind nonspecifically. It may therefore be necessary to have a less-than-maximal fluorescence yield to preserve function or binding specificity. Although conjugating dyes to biomolecules is usually rather easy, preparing the *optimal* conjugate may require extensive experimentation. Thus, for the most critical assays, we recommend that researchers consider preparing and optimizing

their own conjugates. We offer a detailed protocol describing how to use several of our amine-reactive dyes for labeling biomolecules. The procedure is straightforward and requires no special equipment. Following conjugation, it is very important to remove as much unconjugated dye as possible, usually by gel filtration, dialysis, HPLC or a combination of these techniques. The presence of free dye, particularly if it remains chemically reactive, can greatly complicate subsequent experiments with the bioconjugate.

With the exception of the phycobiliproteins (Section 6.4, Table 6.2), fluorescent microspheres (Section 6.5, Table 6.7), Zenon One Labeling Kits (Section 7.2, Table 7.1) and ULYSIS Nucleic Acid Labeling Kits (Section 8.2, Table 8.7), virtually all the dyes used to prepare Molecular Probes' fluorescent bioconjugates are amine-reactive reagents and almost all are described in this chapter. We have also developed useful kit formats for labeling proteins with several of our most important dyes, or alternatively with biotin or DSB-X biotin. Table 1.2 and Section 1.2 include a complete description of these kits, including our Alexa Fluor and FluoReporter Protein Labeling Kits, as well as our new Zenon One Labeling Kits (Section 7.2) for the rapid and quantitative labeling of mouse IgG<sub>1</sub> antibodies.

Alternatively, Molecular Probes prepares custom fluorescent protein conjugates for research use; contact our Custom and Bulk Sales Department for more information. Conjugations with phycobiliproteins and fluorescent polystyrene microspheres require unique procedures that are described in Section 6.4 and Section 6.5, respectively.

Molecular Probes also has what are probably the best reagents and kits for labeling oligonucleotides and nucleic acids (see details in Section 8.2), including:

- ARES DNA Labeling Kits (Section 8.2, Table 8.8), which permit the indirect labeling of DNA with a wide variety of our amine-reactive dyes
- Alexa Fluor Oligonucleotide Amine Labeling Kits (Section 8.2, Table 8.9) for efficient labeling of 5'-amine-derivatized DNA or RNA oligonucleotides with our premiere dyes
- ULYSIS Nucleic Acid Labeling Kits (Section 8.2, Table 8.7), which make labeling of nucleic acids as easy as protein labeling
- ChromaTide UTP, ChromaTide OBEA-dCTP and ChromaTide dUTP nucleotides labeled with several of our best dyes or with biotin (Section 8.2; Table 8.6, Table 8.5), which can be incorporated into nucleic acids by a variety of enzymatic methods<sup>2–5</sup>

In addition, we offer amine-reactive versions of three of our SYBR dyes (Section 8.2), which can be conjugated to oligonucleotides, nucleic acids, peptides or proteins that interact with nucleic acids or affinity matrices. The SYBR dyes remain essentially nonfluorescent until complexed to nucleic acids.

#### Derivatizing Low Molecular Weight Molecules

Some amine-reactive probes described in this chapter are also important reagents for various bioanalytical applications, including amine quantitation, protein and nucleic acid sequencing and chromatographic and electrophoretic analysis of low molecular weight molecules. Reagents that are particularly useful for deriva-

tizing low molecular weight amines — including fluorescamine, *o*-phthalaldehyde, our ATTO-TAG reagents, NBD chloride and dansyl chloride — are discussed in Section 1.8. However, many of the reactive dyes described in Sections 1.2 to 1.7 can also be used as derivatization reagents; likewise, some of the derivatization reagents in Section 1.8 can be utilized for biomolecule conjugation.

## Reactivity of Amino Groups

The amine-reactive probes described in this chapter are mostly acylating reagents that form carboxamides, sulfonamides, ureas or thioureas upon reaction with amines. The kinetics of the reaction depends on the reactivity and concentration of both the acylating reagent and the amine. Of course, buffers that contain free amines such as Tris and glycine must be avoided when using *any* amine-reactive probe. Ammonium sulfate that has been used for protein precipitation must also be removed before performing dye conjugations. In addition, high concentrations of nucleophilic thiols should be avoided because they may react with the reagent to form an unstable intermediate that could consume the dye. Reagents for reductive alkylation of amines (Figure 3.21) are described in Chapter 2 and Chapter 3.

The most significant factors affecting an amine's reactivity are its class and its basicity. Virtually all proteins have lysine residues, and most have a free amine at the N-terminus. Aliphatic amines such as lysine's  $\epsilon$ -amino group are moderately basic and reactive with most acylating reagents. However, the concentration of the free base form of aliphatic amines below pH 8 is very low; thus, the kinetics of acylation reactions of amines by isothiocyanates, succinimidyl esters and other reagents are strongly pH dependent. A pH of 8.5 to 9.5 is usually optimal for modifying lysine residues. In contrast, the  $\alpha$ -amino group at a protein's N-terminus usually has a  $pK_a$  of  $\sim 7$ , so it can sometimes be selectively modified by reaction at near neutral pH. Furthermore, although amine acylation should usually be carried out above pH 8.5, the acylation reagents tend to degrade in the presence of water, with the rate increasing as the pH increases. Protein modification by succinimidyl esters can typically be done at pH 8.5,

whereas isothiocyanates usually require a pH  $>9$  for optimal conjugations; this high pH may be a factor when working with base-sensitive proteins.

Aromatic amines, which are uncommon in biomolecules, are very weak bases and thus unprotonated at pH 7. Modification of aromatic amines requires a highly reactive reagent, such as an isocyanate, isothiocyanate, sulfonyl chloride or acid halide, but can be done at any pH above  $\sim 4$ . A tyrosine residue (Section 3.1) can be selectively modified to form an *o*-aminotyrosine aromatic amine (Figure 3.3), which can then be reacted at a relatively low pH with certain amine-reactive probes.

In aqueous solution, acylating reagents are virtually unreactive with the amide group of peptide bonds and the side chain amides of glutamine and asparagine residues, the guanidinium group of arginine, the imidazolium group of histidine and the nonbasic amines, such as adenosine or guanosine, found in nucleotides and nucleic acids. The ULYSIS Kits described in Section 8.2 provide an alternative method for direct modification of guanosine residues in nucleic acids.

## Isothiocyanates

Molecular Probes does not sell any isocyanate ( $R-NCO$ ) reagents because they are very susceptible to deterioration during storage. However, some acyl azides (Section 3.1) are readily converted to isocyanates (Figure 3.7), which react with amines to form ureas. As an alternative to the unstable isocyanates, we offer a large selection of isothiocyanates ( $R-NCS$ ), which are moderately reactive but quite stable in water and most solvents. Isothiocyanates form thioureas upon reaction with amines (Figure 1.1). Although the thiourea product is reasonably stable, it has been reported that antibody conjugates prepared from fluorescent isothiocyanates deteriorate over time,<sup>6</sup> prompting us to use fluorescent succinimidyl esters and sulfonyl halides almost exclusively for synthesizing our bioconjugates. The thiourea formed by the reaction of fluorescein isothiocyanate (FITC) with amines is also susceptible to conversion to a guanidine by concentrated ammonia.<sup>7</sup> Despite the growing number of choices in amine-reactive fluorophores, fluorescein isothiocyanate and tetramethylrhoda-

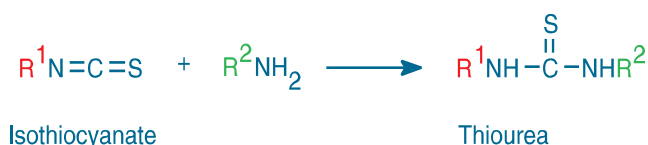


Figure 1.1 Reaction of a primary amine with an isothiocyanate.

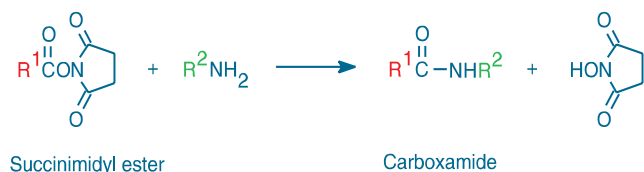


Figure 1.2 Reaction of a primary amine with a succinimidyl ester.



Figure 1.3 Reaction of a primary amine with an STP ester.



Figure 1.4 Reaction of a primary amine with a sulfonyl chloride.

mine isothiocyanate (TRITC) are still widely used reactive fluorescent dyes for preparing fluorescent antibody conjugates.

## Succinimidyl Esters and Carboxylic Acids

Succinimidyl esters are excellent reagents for amine modification because the amide bonds they form (Figure 1.2) are as stable as peptide bonds. Molecular Probes has available over 100 succinimidyl esters of fluorescent dyes and nonfluorescent molecules, most of which have been developed within our own laboratories. These reagents are generally stable during storage if well desiccated, and show good reactivity with aliphatic amines and very low reactivity with aromatic amines, alcohols, phenols (including tyrosine) and histidine. Succinimidyl esters will also react with thiols in organic solvents to form thioesters. If formed in a protein, a thioester may transfer the acyl moiety to a nearby amine. Succinimidyl ester hydrolysis can compete with conjugation, but this side reaction is usually slow below pH 9.

Some succinimidyl esters may not be compatible with a specific application because they can be quite insoluble in aqueous solution. To overcome this limitation, Molecular Probes also offers carboxylic acid derivatives of some of its fluorophores, which can be converted into sulfosuccinimidyl esters or STP esters. These sulfonated reagents have higher water solubility than simple succinimidyl esters and sometimes eliminate the need for organic solvents in the conjugation reaction. However, they are also more polar, which makes them less likely to react with buried amines in proteins or to penetrate cell membranes. Because of their combination of reactivity and polarity, sulfosuccinimidyl esters are not easily purified by chromatographic means and thus only a few are currently available from Molecular Probes. Sulfosuccinimidyl esters can generally be prepared *in situ* simply by dissolving the carboxylic acid dye in an amine-free buffer that contains *N*-hydroxysulfosuccinimide (NHSS, H-2249; Section 3.3) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, E-2247; Section 3.3). Addition of NHSS to the buffer has been shown to enhance the yield of carbodiimide-mediated conjugations<sup>8</sup> (Figure 3.23). STP esters (Figure 1.3) are prepared in the same way from 4-sulfo-2,3,5,6-tetrafluorophenol<sup>9</sup> (S-10490, Section 3.3), and we find them to be more readily purified by chromatography than their sulfosuccinimidyl ester counterparts. The carboxylic acids may also be useful for preparing acid chlorides and anhydrides, which, unlike succinimidyl esters, can be used to modify aromatic amines and alcohols.

### Searching for Information?

We invest considerable effort to make the information in this print version of the *Handbook* accessible and easy to use; however, the vast amount of information that is included here is much more readily accessed and searched at our Web site ([www.probes.com](http://www.probes.com)). Except for PDF files and a few other file formats, our Web site is completely searchable by keyword. Lists of relevant products can

## Sulfonyl Chlorides

Sulfonyl chlorides, including the dansyl, pyrene, Lissamine rhodamine B and Texas Red derivatives, are highly reactive. These reagents are quite unstable in water, especially at the higher pH required for reaction with aliphatic amines. For example, we have determined that dilute Texas Red sulfonyl chloride is totally hydrolyzed within 2–3 minutes in pH 8.3 aqueous solution at room temperature.<sup>10</sup> Protein modification by this reagent is best done at low temperature. Once conjugated, however, the sulfonamides that are formed (Figure 1.4) are extremely stable; they even survive complete protein hydrolysis (for example, dansyl end-group analysis<sup>11</sup>). Sulfonyl chlorides can also react with phenols (including tyrosine), aliphatic alcohols (including polysaccharides), thiols (such as cysteine) and imidazoles (such as histidine), but these reactions are not common in proteins or in aqueous solution. Sulfonyl chloride conjugates of thiols and imidazoles are generally unstable, and conjugates of aliphatic alcohols are subject to nucleophilic displacement.<sup>12</sup> Note that sulfonyl chlorides are unstable in dimethylsulfoxide (DMSO) and should never be used in that solvent.<sup>13</sup>

## Other Amine-Reactive Reagents

Aldehydes react with amines to form Schiff bases. Notable aldehyde-containing reagents include *o*-phthalaldehyde (OPA), naphthalenedicarboxaldehyde (NDA) and the 3-acylquinolinecarboxaldehyde (ATTO-TAG) reagents devised by Novotny and collaborators.<sup>14,15</sup> All of these reagents are useful for the sensitive quantitation of amines in solution, as well as by HPLC and capillary electrophoresis. In addition, certain arylating reagents such as NBD chloride, NBD fluoride and dichlorotriazines react with both amines and thiols, forming bonds with amines that are particularly stable.

## References

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be located using partial word searches (such as “maleimide”). Most of the footnoted references in the *Handbook* are linked to the full citation and the PubMed abstract. Chemical structures and full product information sheets are available for many of our products. If additional information is required, it can be obtained from our Technical Assistance Department or from our distributors.