

## References

1. Nucleic Acids Res 16, 2203 (1988); 2. Nucleic Acids Res 15, 4593 (1987); 3. Biochemistry 24, 692 (1985); 4. J Mol Biol 156, 113 (1982); 5. Anal Biochem 160, 376 (1987); 6. J Chromatogr B Biomed Appl 659, 227 (1994); 7. Eur J Biochem 168, 169 (1987); 8. Methods Enzymol 143, 246 (1987); 9. Anal Biochem 273, 73 (1999); 10. J Org Chem 56, 2648 (1991); 11. Anal Biochem 282, 161 (2000); 12. Anal Biochem 282, 89 (2000); 13. Biochemistry 25, 5036 (1986); 14. Biochemistry 25, 4887 (1986); 15. Biochemistry 20, 7021 (1981); 16. Biochemistry 22, 1208 (1983); 17. Biophys J 50, 75 (1986); 18. Biochemistry 15, 2863 (1976); 19. Bioconjug Chem 5, 348 (1994); 20. Anal Biochem 213, 49 (1993); 21. Biochem J 89, 296 (1963); 22. Methods Enzymol 233, 380 (1994); 23. Methods Enzymol 143, 44 (1987); 24. Methods Enzymol 91, 49 (1983); 25. Anal Biochem 138, 95 (1984).

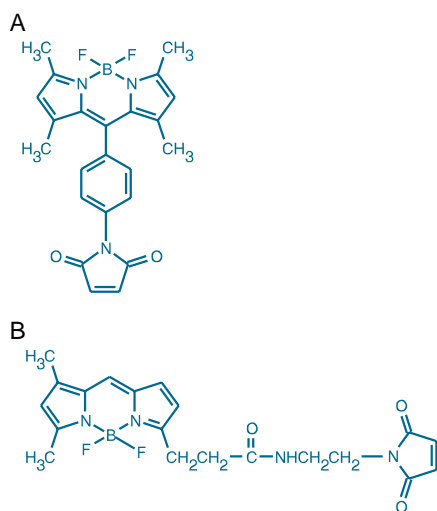
## Data Table — 2.1 Introduction to Thiol Detection and Modification

Cat #	MW	Storage	Soluble	Abs	Em
D-1532	154.24	D	H <sub>2</sub> O	<300	none
D-8451	396.35	D	pH >6	324	none
T-2556	286.65	D	pH >5	<300	none
T-6052	193.19	D	MeCN	<300	none

For definitions of the contents of this data table, see "How to Use This Book" on page viii.

## Product List — 2.1 Introduction to Thiol Detection and Modification

Cat #	Product Name	Unit Size
D-8451	5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent) .....	10 g
D-1532	dithiothreitol (DTT) .....	1 g
T-6060	Thiol and Sulfide Quantitation Kit *50–250 assays* .....	1 kit
T-2556	tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP) .....	1 g
T-6052	tris-(2-cyanoethyl)phosphine .....	1 g



**Figure 2.5** Comparison of the fluorophore orientation relative to the reactive moiety of two spectrally similar thiol-reactive BODIPY dyes, A) BODIPY 499/508 maleimide (D-20350) and B) BODIPY FL *N*-(2-aminoethyl)maleimide (B-10250).

## 2.2 Thiol-Reactive Probes Excited with Visible Light

The probes described in this section are those that have visible absorption maxima beyond 400 nm. Our thiol-reactive probes that have peak absorption below 400 nm are described in Section 2.3. Among all the thiol-reactive probes, the BODIPY, Alexa Fluor, fluorescein, Oregon Green, tetramethylrhodamine and Texas Red derivatives have the strongest absorptivity and highest fluorescence quantum yields. This combination of attributes makes these compounds the preferred reagents for preparing protein and low molecular weight ligand conjugates to study the structural properties, diffusion and interactions of proteins and ligands using techniques such as fluorescence recovery after photobleaching (FRAP), fluorescence polarization (FP), fluorescence correlation spectroscopy (FCS) or fluorescence resonance energy transfer (FRET, see Section 1.3). In this section and in Section 2.3, thiol-reactive reagents with similar spectra, rather than the same reactive group, are generally discussed together. The exception to this organization is the description of our reactive BODIPY and Alexa Fluor fluorophores, which are available in several choices of excitation and emission wavelengths; they are therefore each considered as groups. Table 2.1 summarizes the iodoacetamide and maleimide probes listed in this section.

### BODIPY Derivatives

Like their amine-reactive BODIPY counterparts (Section 1.4), BODIPY iodoacetamides, BODIPY maleimide and BODIPY methyl bromides yield thiol adducts with several important properties:

- High extinction coefficients ( $\epsilon > 60,000 \text{ cm}^{-1}\text{M}^{-1}$ )
- High fluorescence quantum yields, often approaching 1.0, even in water

- Narrow emission bandwidths (Figure 1.39)
- Good photostability
- Spectra that are relatively insensitive to solvent polarity and insensitive to the pH of the medium<sup>1</sup>
- Lack of ionic charge, which is especially useful when preparing membrane probes and cell-permeant reagents

### **BODIPY Maleimides, Iodoacetamides and Methyl Bromides**

Our selection of patented thiol-reactive BODIPY reagents includes a maleimide and two iodoacetamide derivatives of the fluorescein-like BODIPY FL fluorophore<sup>2-5</sup> (B-10250, D-2005, D-6003), BODIPY 507/545 iodoacetamide (D-6004), BODIPY 530/550 iodoacetamide<sup>5</sup> (D-2006), BODIPY TMR cadaverine iodoacetamide (D-6012), BODIPY 493/503 methyl bromide<sup>5</sup> (B-2103) and the very long-wavelength BODIPY 630/650 methyl bromide (B-22802). Two additional symmetric maleimidyphenyl BODIPY derivatives are available (D-20350, D-20351; Figure 2.5) that have emission

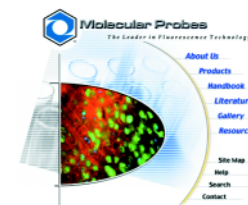
**Table 2.1** Thiol-reactive dyes with absorption maxima >400 nm.

Derivative	Abs *	Em *	Maleimide	Haloacetamide	Bromomethyl
Alexa Fluor 488	495	519	A-10254 †		
Alexa Fluor 532	528	552	A-10255		
Alexa Fluor 546	556	575	A-10258 †		
Alexa Fluor 568	578	603	A-20341 †		
Alexa Fluor 594	590	617	A-10256 †		
Alexa Fluor 555	555	565	A-20346		
Alexa Fluor 633	632	647	A-20342 †		
Alexa Fluor 647	650	665	A-20347		
Alexa Fluor 660	663	690	A-20343 †		
Alexa Fluor 680	679	702	A-20344 †		
BODIPY FL	505	513	B-10250 †	D-2005, D-6003 ‡	
BODIPY TMR	544	570		D-6012	
BODIPY 493/503	493	503			B-2103
BODIPY 499/508	499	508	D-20350		
BODIPY 507/545	508	543		D-6004	
BODIPY 530/550	534	554		D-2006	
BODIPY 577/618	577	618	D-20351		
BODIPY 630/650	625	640			B-22802
Eosin	524	544	E-118 §	E-99 §	
Fluorescein	494	518	F-150 §	I-3 §, I-15 **	B-1355 §
Lucifer yellow	426	531		L-1338	
NBD	478	541		I-9 ††, D-2004	
Oregon Green 488	496	524	O-6034 §	O-6010 †	
PyMPO	415	570	M-6026		
QSY 7	560	NA	Q-10257		
QSY 35	470	NA		Q-20348	
Rhodamine Red	570	590	R-6029 †		
Sulfonerhodamine	555	580		B-10621 ‡	
Tetramethylrhodamine	555	580	T-6027 §, T-6028 **	T-6006 §	
Texas Red	595	615	T-6008 †		T-6009 †

NA = not applicable.

\* Absorption (Abs) and emission (Em) maxima, in nm. † Mixture of 5- and 6-isomers. ‡ Bifunctional crosslinker.

§ 5-Isomer. \*\* 6-Isomer. †† Iodoacetate ester.



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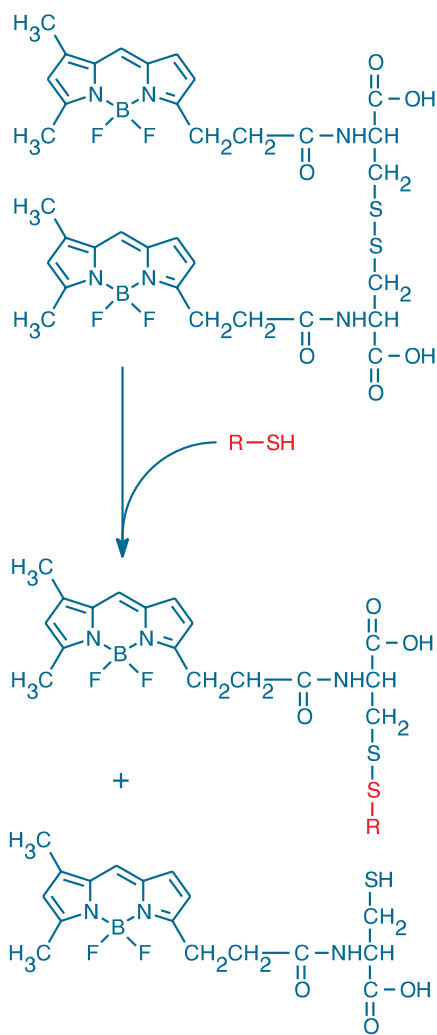
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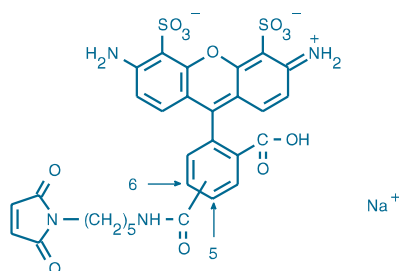
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**Figure 2.6** Reaction of intramolecularly quenched BODIPY FL L-cystine (B-20340) with a thiol, yielding two fluorescent products — a mixed disulfide labeled with the BODIPY FL dye and a BODIPY FL cysteine derivative.



**Figure 2.7** A-10254 Alexa Fluor 488 C<sub>5</sub> maleimide.

maxima near 508 nm and 618 nm, respectively, once conjugated to a thiol. These thiol-reactive BODIPY probes are suitable for labeling cysteine residues in proteins and thiolated oligonucleotides and for detecting thiol conjugates separated by HPLC and capillary electrophoresis using ultrasensitive laser-scanning techniques.<sup>1</sup> The BODIPY probes are chemically stable between about pH 3 and pH 10, although they are less stable to extremes of pH than are fluorescein derivatives.

### BODIPY FL L-Cystine

We have attached the BODIPY FL fluorophore to the amino groups of the disulfide-linked amino acid cysteine to create a reagent for reversible, thiol-specific labeling of proteins, thiolated oligonucleotides and cells.<sup>6</sup> BODIPY FL L-cystine (B-20340) is virtually nonfluorescent due to interactions between the two fluorophores; however, thiol-specific exchange to form a mixed disulfide results in significant enhancement of the green fluorescence (Figure 2.6).

## Alexa Fluor Derivatives

Molecular Probes' patented Alexa Fluor dyes (see The Alexa Fluor Dye Series — Peak Performance Across the Visible Spectrum in Section 1.3) set new standards for fluorescent dyes and the bioconjugates prepared from them. The Alexa Fluor dyes exhibit several unique features:

- Strong absorption, with extinction coefficients greater than 65,000 cm<sup>-1</sup>M<sup>-1</sup>
- More photostable than spectrally similar dyes, allowing more time for observation and image capture (Figure 1.10)
- pH-insensitive fluorescence between pH 4 and 10
- Superior fluorescence output per protein conjugate, surpassing that of any other spectrally similar fluorophore-labeled protein, including fluorescein, tetramethylrhodamine, Cy3, Cy5 and Texas Red conjugates

For labeling thiol groups, we offer Alexa Fluor 488 C<sub>5</sub> maleimide<sup>7-10</sup> (A-10254, Figure 2.7), Alexa Fluor 532 C<sub>5</sub> maleimide (A-10255), Alexa Fluor 546 C<sub>5</sub> maleimide<sup>7,11</sup> (A-10258), Alexa Fluor 555 C<sub>2</sub> maleimide (A-20346), Alexa Fluor 568 C<sub>5</sub> maleimide (A-20341), Alexa Fluor 594 C<sub>5</sub> maleimide<sup>12-14</sup> (A-10256) and Alexa Fluor 350 C<sub>5</sub> maleimide (A-20380, Section 2.3). The Alexa Fluor 633 C<sub>5</sub> maleimide (A-20342), Alexa Fluor 647 C<sub>2</sub> maleimide (A-20347), Alexa Fluor 660 C<sub>2</sub> maleimide (A-20343) and Alexa Fluor 680 C<sub>2</sub> maleimide (A-20344) are our longest-wavelength thiol-reactive dyes. The Alexa Fluor maleimides are expected to be particularly useful for labeling thiol-containing proteins on the surface of live cells, where their polarity should allow the sensitive detection of exposed thiols.<sup>8</sup> Subsequent to labeling, the Alexa Fluor protein conjugates can be electrophoretically separated and then detected without staining.

## Fluorescein Derivatives, Including Thiol-Reactive Oregon Green Dyes

### Fluorescein Derivatives

The excellent water solubility of the fluorescein iodoacetamide single isomers (I-3, I-15) and fluorescein-5-maleimide (F-150, Figure 2.8) at pH 7 makes it easy to prepare green-fluorescent thiol conjugates of biomolecules. Fluorescein maleimide and 5-iodoacetamidofluorescein (one of the five original products offered by Molecular Probes when it started business in 1975) have been the most extensively used visible wavelength-excitatable, thiol-reactive dyes for modification of proteins, nucleic acids and other biopolymers. Following conjugation to thiols, fluorescein-5-maleimide can be radioiodinated.<sup>15</sup> When compared with these iodoacetamide and maleimide derivatives, 5-(bromomethyl)fluorescein (B-1355, Figure 2.9) reacts more slowly with thiols of peptides, proteins and thiolated nucleic acids but forms stronger thioether bonds that are expected to remain stable under the conditions required for complete amino acid analysis. With the possible exception of our Alexa Fluor maleimides and the thiol-reactive BODIPY dyes described above, 5-(bromomethyl)fluorescein has the highest intrinsic detectability of all thiol-reactive probes, particularly for instrumentation that uses the 488 nm spectral line

of the argon-ion laser. Furthermore, its negative charges should make capillary electrophoretic separation of 5-bromomethylfluorescein adducts possible.

### Oregon Green Derivatives

One of our fluorescein substitutes — Oregon Green 488 (2',7'-difluorofluorescein, D-6145; Section 1.5) — has absorption and emission spectra that are a perfect match to those of fluorescein. In addition to the Oregon Green 488 isothiocyanate, carboxylic acid and succinimidyl ester derivatives (Section 1.5), we have synthesized the isomer mixture of Oregon Green 488 iodoacetamide (O-6010) and the single-isomer Oregon Green 488 maleimide (O-6034, Figure 2.10). These thiol-reactive probes yield conjugates that have several important advantages when directly compared with conjugates of fluorescein. These include:

- Greater photostability (Figure 1.42)
- A lower  $pK_a$  ( $pK_a$  of 4.8 for 2',7'-difluorofluorescein versus 6.4 for fluorescein) (Figure 1.11)
- Higher fluorescence and less quenching at comparable degrees of substitution (Figure 1.49)

### Eosin Derivatives

Although eosin iodoacetamide (E-99) and eosin maleimide (E-118) are much less fluorescent than the corresponding fluorescein derivatives, they are more phosphorescent and better photosensitizers.<sup>16</sup> With eosin's high quantum yield of 0.57 for singlet oxygen generation,<sup>17–19</sup> eosin conjugates can be used as effective photooxidizers of diaminobenzidine (DAB) in high-resolution electron microscopy studies.<sup>20,21</sup>

Currently, the principal application of eosin conjugates, including those prepared with the thiol-reactive derivatives in this chapter and the amine-reactive derivatives in Section 1.5, is to follow localized rotational motions in proteins, protein assemblies and proteins in membranes using phosphorescence anisotropy.<sup>22–24</sup> Eosin (excitation/emission maxima ~519/540 nm) derivatives efficiently absorb the fluorescence from fluorescein and other fluorophores such as the BODIPY FL, Alexa Fluor 488, Oregon Green 488, dansyl and coumarin dyes, making them good acceptors in fluorescence resonance energy transfer (FRET, see Section 1.3) techniques.<sup>25</sup> Although usually selectively reactive with thiols, eosin maleimide reportedly also reacts with a specific lysine residue of the band-3 protein in human erythrocytes, inhibiting anion exchange in these cells.<sup>26,27</sup>

### Rhodamine Derivatives, Including Thiol-Reactive Texas Red Dyes

#### Tetramethylrhodamine Derivatives

Tetramethylrhodamine iodoacetamide (TMRIA) and tetramethylrhodamine maleimide yield photostable, pH-insensitive, red-orange-fluorescent thiol conjugates.<sup>28,29</sup> However, the iodoacetamide and maleimide derivatives are difficult to prepare in pure form and different batches of our mixed-isomer products have contained variable mixtures of the 5- and 6-isomers. Apparently certain cytoskeletal proteins preferentially react with individual isomers, leading to complications in the interpretation of labeling results.<sup>30–33</sup> Consequently, we now prepare the 5-isomer of TMRIA (T-6006) and the 5-isomer (T-6027, Figure 2.11) and 6-isomer (T-6028, Figure 2.12) of tetramethylrhodamine maleimide. TMRIA is reported to predominantly label SH-1 (Cys-707) of the myosin heavy chain in skinned muscle fibers.<sup>34</sup> Literature references in our bibliography for the mixed isomers are combined with those for the single-isomer products.

#### A Rhodamine-Based Crosslinking Reagent

Our bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (B-10621) is derived from a relatively rigid rhodamine dye (Figure 5.6). This crosslinker, which 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 (FP) experiments,<sup>35</sup> may also be useful for proximity studies.

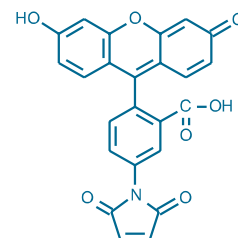


Figure 2.8 F-150 fluorescein-5-maleimide.

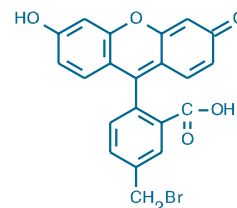


Figure 2.9 B-1355 5-(bromomethyl)fluorescein.

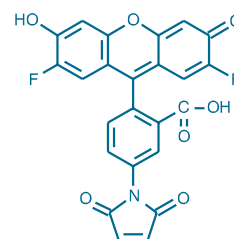


Figure 2.10 O-6034 Oregon Green 488 maleimide.

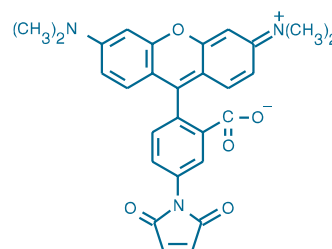


Figure 2.11 T-6027 tetramethylrhodamine-5-maleimide.

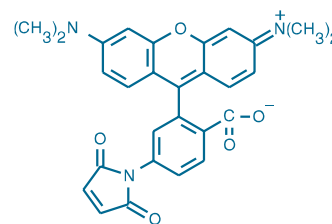


Figure 2.12 T-6028 tetramethylrhodamine-6-maleimide.

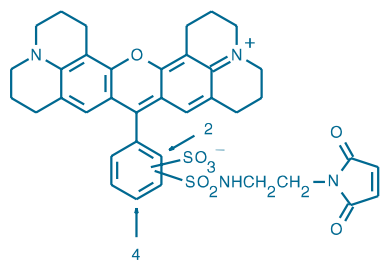


Figure 2.13 T-6008 Texas Red C<sub>2</sub> maleimide.

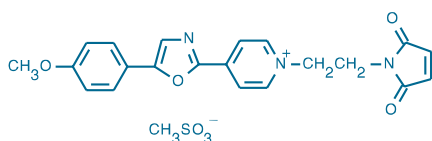


Figure 2.14 M-6026 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methane-sulfonate.

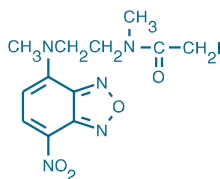


Figure 2.15 D-2004 *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine.



## Bioconjugate Techniques

*Bioconjugate Techniques* (B-7884), by G.T. Hermanson, is an essential guide that captures the entire field of bioconjugate chemistry in a single volume. The chemistry, reagents and applications for generating conjugated molecules are described in detail. In addition, this well-illustrated and highly referenced book provides easy-to-follow protocols for creating modified and crosslinked reagents that can be used for detecting, quantitating and analyzing biomolecules in research, diagnostic and clinical applications.

## Rhodamine Red Derivative

We offer a maleimide derivative of our Rhodamine Red fluorophore (R-6029), which is a preferred dye for excitation (Figure 7.21) by the 568 nm spectral line of the Ar–Kr mixed-gas laser used in some confocal laser-scanning microscopes. This maleimide is a mixture of two isomeric sulfonamides.

## Texas Red Derivatives

Conjugates of the bromoacetamide and maleimide derivatives of our Texas Red fluorophore (T-6009, T-6008) have very little spectral overlap with fluorescein or Alexa Fluor 488 conjugates, yet can be excited by the 568 nm spectral line of the Ar–Kr mixed-gas laser (Figure 1.69). Thus, Texas Red conjugates are useful as a second label in multicolor applications or as energy transfer acceptors from green-fluorescent dyes. Bromoacetamides are only slightly less reactive with thiols than are iodoacetamides. The Texas Red bromoacetamide and maleimide (Figure 2.13) derivatives are mixtures of the two isomeric sulfonamides.

## Pyridyloxazole Derivatives

The pyridyloxazole derivatives — including the thiol-reactive maleimide<sup>36,37</sup> (M-6026, Figure 2.14) and the amine-reactive succinimidyl ester (PyMPO, SE; S-6110) — fill the spectral gap between UV-excited dyes and the fluoresceins. These derivatives of the laser dye PyMPO exhibit absorption maxima near 415 nm and unusually high Stokes shifts, with emission at 560–580 nm.<sup>38</sup> These oxazole-based dyes exhibit environment-sensitive fluorescence spectra.

## Benzodiazole Derivatives, Including NBD Probes

### NBD Chloride and NBD Fluoride

Benz-2-oxa-1,3-diazoles (also called benzofurazans) are a diverse group of reactive dyes that include both nitrated derivatives of the NBD series and sulfonated analogs of the SBD series. NBD chloride (FluoroPure Grade, C-20260; Figure 3.2) and the more reactive NBD fluoride (F-486) are common reagents for amine modification (Section 1.8). However, they also react with thiols<sup>39–41</sup> and cysteine in several proteins<sup>42–46</sup> to yield thioethers. NBD conjugates of thiols usually have much shorter wavelength absorption and weaker fluorescence than do NBD conjugates of amines.<sup>41</sup> Selective modification of cysteines in the presence of reactive lysines and tyrosines is promoted by carrying out the reaction at pH < 7,<sup>47,48</sup> however, NBD conjugates of thiols are often unstable, resulting in time-dependent label migration to adjacent lysine residues.<sup>41,48</sup>

### SBD Probe

Thiol conjugates of the SBD probe 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide<sup>49,50</sup> (ABD-F, F-6053) are much more stable in aqueous solution than are the thiol conjugates prepared from NBD chloride or NBD fluoride.<sup>49</sup> ABD-F is nonfluorescent until reacted with thiols and therefore can be used to quantitate thiols in solution,<sup>51</sup> as well as thiols separated by HPLC<sup>52</sup> or TLC.<sup>53</sup> ABD-F labels the thiols of thionin only in the presence of the chelating agent EDTA, which removes the bound Zn<sup>2+</sup> ions.<sup>54</sup> ABD–cysteine conjugates are very stable to acid hydrolysis but labeling is partially reversed in basic solution containing dithiothreitol<sup>55,56</sup> (DTT, D-1532; Section 2.1). ABD-F can also be combined with tributylphosphine for the determination of disulfides in peptides and proteins.<sup>57–59</sup> As possible alternatives to the malodorous tributylphosphine, we recommend tris-(2-carboxyethyl)phosphine (TCEP, T-2556; Section 2.1) or the less polar tris-(2-cyanoethyl)phosphine (T-6052, Section 2.1). Due to its high polarity, TCEP selectively reduces only those disulfides that are located on protein or live-cell surfaces.

### IANBD Ester and IANBD Amide

When conjugating the NBD fluorophore to thiols located in hydrophobic sites of proteins, we recommend using the NBD iodoacetate ester (IANBD ester, I-9) or, preferably, the more hydrolytically stable NBD iodoacetamide<sup>60</sup> (IANBD amide, D-2004; Figure 2.15). These reactive reagents exhibit appreciable fluorescence only after reaction

with thiols that are buried or unsolvated, and this fluorescence is highly sensitive to changes in the solvation level of the fluorophore.

### Lucifer Yellow Iodoacetamide

Lucifer yellow CH is a well-known polar tracer for neurons (Section 14.3). Its iodoacetamide derivative (L-1338) similarly has high water solubility and visible absorption and emission similar to those of lucifer yellow CH (Figure 14.23). As with our Alexa Fluor maleimides (see above) and the stilbene iodoacetamide and maleimide (A-484, A-485; Section 2.3), a principal application of lucifer yellow iodoacetamide is the labeling of exposed thiols of proteins in solution, as well as in the outer membrane of live cells.<sup>61–63</sup> Lucifer yellow iodoacetamide has also been used as a fluorescence energy acceptor from stilbene iodoacetamide to follow subunit assembly of  $\alpha$ -crystallin.<sup>64</sup>

### Chromophoric Maleimides and Iodoacetamides

QSY 7 maleimide (Q-10257, Figure 2.16) is an essentially nonfluorescent, thiol-reactive diaryl rhodamine with an absorption spectrum similar to that of our QSY 7 succinimidyl ester (Q-10193, Section 1.7, Figure 1.66). The principal application of QSY 7 maleimide is expected to be as a quencher for fluorescent donor dyes in fluorescence resonance energy transfer (FRET, see Section 1.3) studies. Most FRET detection is based on the interaction between fluorescent donor and acceptor dyes. However, the use of nonfluorescent acceptor dyes avoids the background fluorescence that often results from direct (i.e., nonsensitized) excitation of the acceptor dye. The broad and strong absorption of the QSY 7 dye in the visible-light spectrum (absorption maximum  $\sim$ 560 nm,  $\epsilon \sim$ 90,000  $\text{cm}^{-1}\text{M}^{-1}$ , quantum yield in water  $<0.002$ ) yields extraordinarily efficient quenching of donors that have blue, green, orange or red fluorescence at relatively long spatial separations (Table 1.8). A QSY 7 amine derivative for modifying carboxylic acids (Q-10464) is described in Section 3.3. We also have prepared a QSY 7 derivative of  $\alpha$ -Fmoc lysine (Q-21930, Section 9.5) for automated synthesis of peptides that contain this important quencher.

QSY 35 iodoacetamide (Q-20348) is an essentially nonfluorescent thiol-reactive analog of the amine-reactive nitrobenzoxazole (NBD) dye. QSY 35 derivatives absorb maximally near 470 nm (Figure 1.66), making their conjugates excellent FRET acceptors from UV light-excited fluorescent dyes. The amine-reactive QSY 35 acetic acid succinimidyl ester (Q-20133) is described in Section 1.6. A QSY 35 aliphatic amine is also available (Q-20540, Section 3.3), as is a QSY 35 Fmoc-protected amino acid for automated peptide synthesis of fluorogenic protease substrates (Q-21931, Section 9.5).

The broad visible absorption of the chromophoric maleimide DABMI (D-1521) conjugates makes DABMI a useful nonfluorescent thiol-reactive acceptor for fluorescence resonance energy transfer studies.<sup>65,66</sup> This reagent is also used to derivatize thiols for HPLC detection.<sup>67,68</sup>

### Labeling Cell-Surface Thiols and Disulfides

Polar reagents for labeling cell-surface thiols of proteins include the stilbene iodoacetamide and maleimide<sup>69</sup> (A-484, A-485; Section 2.3), lucifer yellow iodoacetamide (L-1338), 5-iodoacetamidofluorescein (5-IAF, I-3), fluorescein-5-maleimide (F-150), and maleimidylpropionyl biocytin<sup>69</sup> (M-1602, Section 4.2). However, we now recommend the sulfonated rhodamine dyes Alexa Fluor 488 C<sub>5</sub> maleimide<sup>8</sup> (A-10254) and Alexa Fluor 594 C<sub>5</sub> maleimide (A-10256) as the preferred reagents. Their high water solubility, selectivity for thiols, strong absorption of visible light and high resistance to photobleaching should result in high fluorescence yields and significantly better detectability of their conjugates. Following electrophoretic separation, protein conjugates can be detected in gels by their visible fluorescence. Our antibodies to lucifer yellow, fluorescein (which strongly crossreacts with the Oregon Green fluorophore) and the Alexa Fluor 488 dye (Section 7.4, Table 7.13) may facilitate isolation and detection of proteins labeled with the thiol-reactive lucifer yellow, fluorescein (or Oregon Green) and Alexa

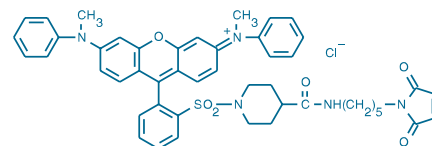


Figure 2.16 Q-10257 QSY 7 maleimide.

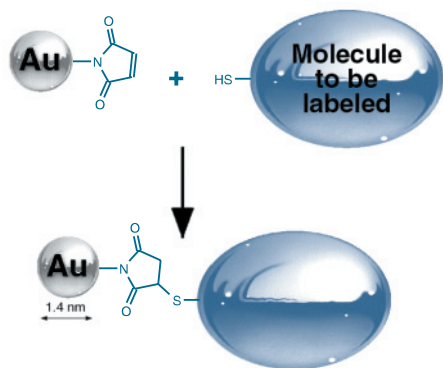
## TECHNICAL NOTE

### Applications of Thiol-Reactive Probes

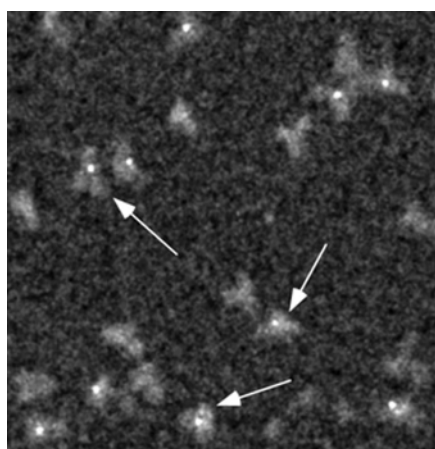
The relative infrequency, high chemical reactivity and availability of many thiol-reactive probes make protein thiols ideal targets for selective labeling of proteins. Furthermore, methods for site-directed mutagenesis make it easy to incorporate thiols at selected locations. Consequently, many biophysical studies of proteins and cells have utilized thiol-reactive probes to:

- Estimate distances within and between labeled sites using fluorescence resonance energy transfer (FRET)
- Detect particularly reactive thiols in proteins, which are often associated with ligand binding sites or “active sites” of enzymes
- Detect “buried” thiols in hydrophobic sites with environment-sensitive probes such as the NBD, Dapoxyl, acrylodan, badan, IAANS, MIANS probes
- Label cell-surface expressed thiol-containing proteins prior to their electrophoretic separation with the sulfonated thiol reactive probes, including the Alexa Fluor maleimides, lucifer yellow iodoacetamide, and stilbenedisulfonate maleimide and iodoacetamide
- Derivatize thiols for chromatographic analysis, in particular with monobromobimane
- Prepare single-site labeled fluorescent analogs of cytoskeletal proteins for microinjection

Numerous references to these applications are available at our Web site ([www.probes.com](http://www.probes.com)).



**Figure 2.17** Reaction of NANOGOLD monomaleimide (N-20345) with a thiol. Image courtesy of Nanoprobes, Inc.



**Figure 2.18** Scanning transmission electron microscope (STEM) image indicating that labeling with NANOGOLD monomaleimide (N-20345, arrows) occurs specifically at a hinge-thiol site on the IgG molecule. Image courtesy of Nanoprobes, Inc.

*Conjugates of NANOGOLD and Alexa Fluor FluoroNanogold with antibodies and streptavidin are described in Chapter 7. We maintain an extensive bibliography on the applications of our NANOGOLD products under catalog number N-24833 at our Web site ([www.probes.com/search](http://www.probes.com/search)).*

Fluor 488 derivatives, respectively, following blotting onto nitrocellulose membranes.

Disulfides in peptides and proteins can be reduced to thiols by a variety of reagents (Section 5.2). The three negative charges of the phosphine TCEP (T-2556, Section 2.1) render it membrane impermeant, which makes it particularly useful for selective reduction of disulfides on the cell's outer membrane. The less polar tris-(2-cyanoethyl)-phosphine (T-6052, Section 2.1) may be a useful complementary reagent for reducing intramembrane and intracellular disulfides. Unlike DTT (D-1532, Section 2.1), phosphines do not contain thiols and therefore may not need to be removed prior to thiol modification.

## NANOGOLD Monomaleimide

In collaboration with Nanoprobes, Inc., Molecular Probes offers NANOGOLD particles, small metal cluster complexes of gold particles for research applications in light or electron microscopy.<sup>70,71</sup> The NANOGOLD clusters are discrete chemical compounds, not gold colloids. NANOGOLD monomaleimide (N-20345) permits attachment of these very small (1.4 nm) yet uniformly sized gold particles to biomolecules in the same way that one reacts the maleimide of a dye with a biomolecule (Figure 2.17, Figure 2.18). The product, which is supplied as a set of five vials of a powder lyophilized from pH 7.5 HEPES buffer, is resuspended with the thiol-containing protein in deionized water at room temperature or below, then any excess NANOGOLD monomaleimide is removed by gel filtration and the conjugate is stored frozen.<sup>72,73</sup> 100 nmol of NANOGOLD monomaleimide is sufficient to label about 100 µg of a protein with a MW of 100,000. Excess reagent should not be stored, and the conjugation mixture must be free of thiols or amine-containing buffers. In addition to its many uses for light and electron microscopy, NANOGOLD monomaleimide has been shown to be an extremely efficient quencher for dyes in molecular beacons — probes that can be used for homogeneous fluorescence *in situ* hybridization assays<sup>74</sup> (Figure 8.101). NANOGOLD conjugates of antibodies and streptavidin are described in Section 7.3 and Section 7.6, respectively, along with reagents and methods for silver enhancement to amplify electron microscopy detection<sup>74</sup> (Section 8.5). We also supply NANOGOLD mono(sulfosuccinimidyl ester) (N-20130, Section 1.6).

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## Data Table — 2.2 Thiol-Reactive Probes Excited with Visible Light

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A-10254	720.66	F,DD,L	H <sub>2</sub> O, DMSO	493	72,000	516	pH 7	1
A-10255	812.88	F,DD,L	H <sub>2</sub> O, DMSO	528	78,000	552	MeOH	1
A-10256	908.97	F,DD,L	H <sub>2</sub> O, DMSO	588	96,000	612	pH 7	1
A-10258	1034.37	F,DD,L	H <sub>2</sub> O, DMSO	554	93,000	570	pH 7	1
A-20341	880.92	F,DD,L	H <sub>2</sub> O, DMSO	575	92,000	600	pH 7	1
A-20342	~1300	F,DD,L	H <sub>2</sub> O, DMSO	622	143,000	640	MeOH	1
A-20343	~900	F,DD,L	H <sub>2</sub> O, DMSO	668	112,000	697	MeOH	1
A-20344	~1000	F,DD,L	H <sub>2</sub> O, DMSO	684	175,000	714	MeOH	1
A-20346	~1250	F,DD,L	H <sub>2</sub> O, DMSO	556	158,000	572	MeOH	1
A-20347	~1300	F,DD,L	H <sub>2</sub> O, DMSO	651	265,000	671	MeOH	1
B-1355	425.23	F,D,L	pH >6, DMF	492	81,000	515	pH 9	2
B-2103	341.00	F,D,L	DMSO, MeCN	533	62,000	561	CHCl <sub>3</sub>	3, 4
B-10250	414.22	F,D,L	DMSO, MeCN	504	79,000	510	MeOH	4
B-10621	840.47	F,D,L	DMSO	549	88,000	575	MeOH	5
B-20340	788.44	F,D,L	DMSO	504	132,000	511	MeOH	6
B-22802	449.14	F,D,L	DMF, MeCN	658	73,000	678	CHCl <sub>3</sub>	7
C-20260	199.55	F,D,L	DMF, MeCN	336	9,800	none	MeOH	8, 9
D-1521	320.35	F,D,L	DMF, MeCN	419	34,000	none	MeOH	10
D-2004	419.18	F,D,L	DMF, DMSO	478	25,000	541	MeOH	5, 10
D-2005	502.11	F,D,L	DMSO, MeCN	504	81,000	511	MeOH	4, 5
D-2006	626.25	F,D,L	DMSO, MeCN	534	69,000	552	MeOH	4, 5
D-6003	417.00	F,D,L	DMSO, MeCN	502	76,000	510	MeOH	4, 5
D-6004	431.03	F,D,L	DMSO, MeCN	508	69,000	543	MeOH	4, 5
D-6012	650.31	F,D,L	DMSO, MeCN	544	63,000	570	MeOH	4, 5
D-20350	419.24	F,D,L	DMSO	499	88,000	508	MeOH	11
D-20351	575.38	F,D,L	DMSO	577	60,000	618	MeOH	11
E-99	830.84	F,D,L	pH >6, DMF	519	100,000	540	pH 9.5	1, 5, 12
E-118	742.95	F,D,L	pH >6, DMF	524	103,000	545	MeOH	1, 12
F-150	427.37	F,D,L	pH >6, DMF	492	83,000	515	pH 9	1, 2, 13
F-486	183.10	F,D,L	MeCN, CHCl <sub>3</sub>	328	8,000	none	MeOH	8
F-6053	217.17	F,D,L	DMF, DMSO	320	4,800	none	MeOH	14
I-3	515.26	F,D,L	pH >6, DMF	492	75,000	515	pH 9	1, 2, 5
I-9	406.14	F,D,L	DMF, MeCN	472	23,000	536	MeOH	5, 10
I-15	515.26	F,D,L	pH >6, DMF	492	81,000	516	pH 9	1, 2, 5
L-1338	659.51	F,D,L	H <sub>2</sub> O	426	11,000	531	pH 7	5
M-6026	471.48	F,D,L	DMSO	412	23,000	561	MeOH	15
O-6010	551.24	F,D,L	pH >6, DMF	491	68,000	516	pH 9	1, 5, 15
O-6034	463.35	F,D,L	pH >6, DMF	491	81,000	515	pH 9	1, 15
Q-10257	858.45	F,D,L	DMSO	560	92,000	none	MeOH	
Q-20348	453.20	F,D,L	DMSO	475	24,000	none	MeOH	5
R-6029	680.79	F,D,L	DMSO	560	119,000	580	MeOH	
T-6006	825.22	F,D,L	DMSO	543	87,000	567	MeOH	5
T-6008	728.83	F,D,L	DMSO	582	112,000	600	MeOH	
T-6009	811.80	F,D,L	DMSO	583	115,000	603	MeOH	
T-6027	481.51	F,D,L	DMSO	541	95,000	567	MeOH	
T-6028	481.51	F,D,L	DMSO	541	91,000	567	MeOH	

For definitions of the contents of this data table, see “How to Use This Book” on page viii.

### Notes

1. Aqueous stock solutions should be used within 24 hours; long-term storage is NOT recommended.
2. Absorption and fluorescence of fluorescein derivatives are pH dependent. Extinction coefficients and fluorescence quantum yields decrease markedly at pH <7.
3. B-2103 spectra are for the unreacted reagent. The thiol adduct has Abs = 493 nm, Em = 503 nm in MeOH.
4. The absorption and fluorescence spectra of BODIPY derivatives are relatively insensitive to the solvent.
5. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.
6. Fluorescence emission of B-20340 is relatively weak until the disulfide linkage between its two BODIPY FL fluorophores is reductively cleaved.
7. B-22802 spectral data are for the unreacted reagent. The thiol adduct has Abs = 629 nm in dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>).
8. Spectra of 2-mercaptoethanol adduct of NBD chloride in MeOH: Abs = 425 nm (EC = 13,000 cm<sup>-1</sup>M<sup>-1</sup>), Em = 520 nm. NBD fluoride yields the same derivatives as NBD chloride but is more reactive.
9. This product is specified to equal or exceed 98% analytical purity by HPLC.
10. Spectral data of the 2-mercaptoethanol adduct.
11. Spectral data are for the unreacted reagent and are essentially unchanged upon reaction with thiols.
12. Eosin and erythrosin derivatives also exhibit phosphorescence with an emission maximum at ~680 nm. The phosphorescence lifetime is ~1 millisecond for eosin and 0.5 milliseconds for erythrosin (Biochem J 183, 561 (1979); Spectroscopy 5, 20 (1990)). Fluorescence lifetimes (τ) are 1.4 nsec (QY = 0.2) for eosin and 0.1 nsec (QY = 0.02) for erythrosin (J Am Chem Soc 99, 4306 (1977)).
13. QY increases on reaction with thiols; Abs, EC and Em are essentially unchanged (Anal Biochem 295, 101 (2001)).
14. F-6053 reaction product with dimethylaminoethanethiol has Abs = 376 nm (EC ~8000 cm<sup>-1</sup>M<sup>-1</sup>), Em ~510 nm in MeOH.
15. Fluorescence emission spectrum shifts to shorter wavelengths in nonpolar solvents.
16. Absorption and fluorescence of Oregon Green 488 derivatives are pH dependent only in moderately acidic solutions (pH <5).

**Product List — 2.2 Thiol-Reactive Probes Excited with Visible Light**

Cat #	Product Name	Unit Size
A-10254	Alexa Fluor <sup>®</sup> 488 C <sub>5</sub> maleimide	1 mg
A-10255	Alexa Fluor <sup>®</sup> 532 C <sub>5</sub> maleimide	1 mg
A-10258	Alexa Fluor <sup>®</sup> 546 C <sub>5</sub> maleimide	1 mg
A-20346	Alexa Fluor <sup>®</sup> 555 C <sub>2</sub> maleimide	1 mg
A-20341	Alexa Fluor <sup>®</sup> 568 C <sub>5</sub> maleimide	1 mg
A-10256	Alexa Fluor <sup>®</sup> 594 C <sub>5</sub> maleimide	1 mg
A-20342	Alexa Fluor <sup>®</sup> 633 C <sub>5</sub> maleimide	1 mg
A-20347	Alexa Fluor <sup>®</sup> 647 C <sub>2</sub> maleimide	1 mg
A-20343	Alexa Fluor <sup>®</sup> 660 C <sub>2</sub> maleimide	1 mg
A-20344	Alexa Fluor <sup>®</sup> 680 C <sub>2</sub> maleimide	1 mg
B-10621	bis-(( <i>N</i> -iodoacetyl)piperazinyl)sulfonerhodamine	5 mg
B-10250	BODIPY <sup>®</sup> FL <i>N</i> -(2-aminoethyl)maleimide	5 mg
B-20340	BODIPY <sup>®</sup> FL L-cystine	1 mg
B-22802	8-bromomethyl-4,4-difluoro-3,5-bis-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY <sup>®</sup> 630/650 methyl bromide)	1 mg
B-2103	8-bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY <sup>®</sup> 493/503 methyl bromide)	5 mg
B-1355	5-(bromomethyl)fluorescein	10 mg
C-20260	4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride; 4-chloro-7-nitrobenzofurazan) *FluoroPure™ grade*	100 mg
D-20351	4,4-difluoro-3,5-bis(4-methoxyphenyl)-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY <sup>®</sup> 577/618 maleimide)	5 mg
D-2005	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)- <i>N'</i> -iodoacetylenediamine (BODIPY <sup>®</sup> FL IA)	5 mg
D-6003	<i>N</i> -(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl)iodoacetamide (BODIPY <sup>®</sup> FL C <sub>1</sub> -IA)	5 mg
D-6012	<i>N</i> -(5-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl)amino)pentyl)-iodoacetamide (BODIPY <sup>®</sup> TMR cadaverine IA)	5 mg
D-2006	<i>N</i> -(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)- <i>N'</i> -iodoacetylenediamine (BODIPY <sup>®</sup> 530/550 IA)	5 mg
D-6004	<i>N</i> -(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-2-yl)iodoacetamide (BODIPY <sup>®</sup> 507/545 IA)	5 mg
D-20350	4,4-difluoro-1,3,5,7-tetramethyl-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY <sup>®</sup> 499/508 maleimide)	5 mg
D-1521	4-dimethylaminophenylazophenyl-4'-maleimide (DABMI)	100 mg
D-2004	<i>N,N'</i> -dimethyl- <i>N</i> -(iodoacetyl)- <i>N'</i> -(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide)	25 mg
E-99	eosin-5-iodoacetamide	100 mg
E-118	eosin-5-maleimide	25 mg
F-150	fluorescein-5-maleimide	25 mg
F-6053	7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F)	10 mg
F-486	4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD fluoride; 4-fluoro-7-nitrobenzofurazan)	25 mg
I-3	5-iodoacetamidofluorescein (5-IAF)	100 mg
I-15	6-iodoacetamidofluorescein (6-IAF)	100 mg
I-9	<i>N</i> -((2-(iodoacetoxy)ethyl)- <i>N</i> -methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester)	100 mg
L-1338	lucifer yellow iodoacetamide, dipotassium salt	25 mg
M-6026	1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide)	5 mg
N-20345	NANOGOLD <sup>®</sup> monomaleimide *special packaging*	5 x 6 nmol
O-6010	Oregon Green <sup>®</sup> 488 iodoacetamide *mixed isomers*	5 mg
O-6034	Oregon Green <sup>®</sup> 488 maleimide	5 mg
Q-10257	QSY <sup>®</sup> 7 maleimide	5 mg
Q-20348	QSY <sup>®</sup> 35 iodoacetamide	5 mg
R-6029	Rhodamine Red™ C <sub>2</sub> maleimide	5 mg
T-6006	tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA) *single isomer*	5 mg
T-6027	tetramethylrhodamine-5-maleimide *single isomer*	5 mg
T-6028	tetramethylrhodamine-6-maleimide *single isomer*	5 mg
T-6008	Texas Red <sup>®</sup> C <sub>2</sub> maleimide	5 mg
T-6009	Texas Red <sup>®</sup> C <sub>5</sub> bromoacetamide	5 mg

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