cal arm of the immune system, as well as the evolution of antibodies and the role they may play in human diseases.[9,10] Several reviews have discussed the chemistry of the different reactive oxygen species and their lipid peroxidation products.[11–16]

The importance of the nitric oxide radical (abbreviated NO) and other reactive oxygen species as biological messengers has been increasingly recognized during the last several years.[17–22] Section 19.3 is devoted to our probes for promoting, inhibiting or detecting nitric oxide production in a variety of experimental systems.

References

1. Prog Neurobiol 62, 633 (2000); **2.** Annu Rev Biochem 64, 97 (1995); **3.** Methods Enzymol 234, (Complete Volume) (1994); **4.** Methods Enzymol 233, (Complete Volume) (1994); **5.** Methods Enzymol 186, (Complete Volume) (1990); **6.** Methods Enzymol 105, (Complete Volume) (1984); **7.** J Biol Chem 268, 18502 (1993); **8.** Pharmacol Ther 53, 375 (1992); **9.** Science 293, 1806 (2001); **10.** Proc Natl Acad Sci U S A 97, 10930 (2000); **11.** Free Radic Biol Med 30, 463 (2001); **12.** Clin Chem 41, 1819 (1995); **13.** Free Radic Biol Med 19, 77 (1995); **14.** Free Radic Biol Med 18, 1033 (1995); **15.** Free Radic Biol Med 18, 775 (1995); **16.** Biochimie 76, 355 (1994); **17.** Curr Biol 7, R376 (1997); **18.** J Med Chem 38, 4343 (1995); **19.** Annu Rev Biochem 63, 175 (1994); **20.** Cell 78, 919 (1994); **21.** Am J Physiol 262, G379 (1992); **22.** Science 257, 494 (1992).

19.2 Generating and Detecting Reactive Oxygen Species

Molecular Probes prepares an assortment of probes for the generation of reactive oxygen species (ROS) — singlet oxygen, hydroxyl radicals, superoxide, hydroperoxides and peroxides (Table 19.1) — as well as for their fluorometric detection in solution. Although there are no equilibrium sensors that continuously monitor the level of reactive oxygen species, this section discusses a number of probes that trap or otherwise react with singlet oxygen, hydroxyl radicals or superoxide. The optical or electron spin properties of the resulting products are in some way a measure of the presence or quantity of the reactive oxygen species and, in some cases, can measure the kinetics and location of their formation.

Generating Singlet Oxygen

Singlet oxygen is responsible for much of the physiological damage caused by reactive oxygen species, including nucleic acid modification through selective reaction with deoxyguanosine.[1] The lifetime of singlet oxygen is sufficiently long (4.4 microseconds in water[2]) to permit significant diffusion in cells and tissues.[3] In the laboratory, singlet oxygen is usually generated in one of three ways: photochemically from dioxygen ($^3O_2$) using a photosensitizing dye;[4] chemically, either by thermal decomposition of a peroxide or dioxetane; or by microwave discharge through an oxygen stream. Singlet oxygen can be detected by its characteristic weak chemiluminescence at 1268 nm[5] or at 634 and 703 nm.[6]

Hypocrellins and Hypericin

Among the most efficient reagents for generating singlet oxygen are the photosensitizers hypocrellin A (H-7515, Figure 18.10), hypocrellin B (H-7516, Figure 18.11) and hypericin (H-7476, Figure 18.9). These heat-stable dyes exhibit quantum yields for singlet oxygen generation in excess of 0.7, as well as high photostability, making them important agents for both anticancer and antiviral therapy.[7–13] Hypocrellins are also specific and potent inhibitors of protein kinase C[14,15] (Section 18.3), whereas hypericin is an effective inhibitor of both protein kinase C and tyrosine protein kinase[16] with antiretroviral activity.[17]

Because their chemical reactivities are well characterized,[18,19] hypocrellins and hypericin are amenable to conjugation to a variety of primary and secondary detection reagents. Not only do these photosensitizing dyes efficiently oxidize diaminobenzidine (DAB) to form an insoluble, electron-dense DAB oxidation product (see

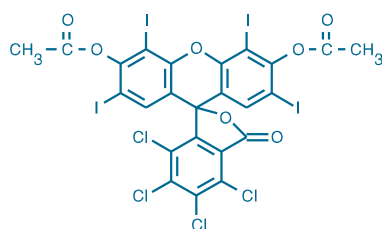

Figure 19.1 R-14000 rose bengal diacetate.

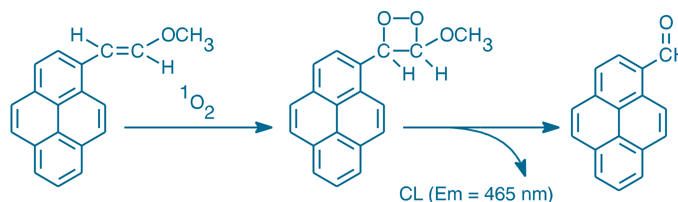

Figure 19.2 Reaction of *trans*-1-(2′-methoxyvinyl)pyrene (M-7913) with singlet oxygen ($^1O_2$), yielding a dioxetane intermediate that generates chemiluminescence (CL) upon decomposition to 1-pyrenecarboxaldehyde.

Fluorescent Probes for Photoconversion of Diaminobenzidine Reagents in Section 1.5), but they exhibit modest fluorescence quantum yields and broad UV and visible spectra. Thus, hypocrellin- and hypericin-labeled detection reagents are compatible with fluorescence, light or electron microscopy applications.[20]

Halogenated Fluoresceins and Rhodamines

Halogenated derivatives of fluorescein dyes are known to be effective photosensitizers and singlet oxygen generators.[21] Bioconjugates of eosin and erythrosin fluorophores can be prepared using the reactive derivatives described in Chapter 1, Chapter 2 and Chapter 3. The practical use of eosin and other conjugates to improve ultrastructural resolution through singlet oxygen generation is described in Fluorescent Probes for Photoconversion of Diaminobenzidine Reagents (see Section 1.5). The generation of singlet oxygen can be targeted to the mitochondria by several cationic dyes,[22] such as tetrabromorhodamine 123 (T-7539), which has a quantum yield for singlet oxygen generation of 0.65–0.7.[22,23]

Rose Bengal Diacetate

Rose bengal diacetate (R-14000) is an efficient, cell-permeant generator of singlet oxygen.[24] It is an iodinated xanthene derivative that has been chemically modified by the introduction of acetate groups (Figure 19.1). These modifications inactivate both its fluorescence and photosensitization properties, while increasing its ability to cross cell membranes. Once inside a live cell, esterases remove the acetate groups and restore rose bengal to its native structure. This intracellular localization allows rose bengal diacetate to be a very effective photosensitizer.

Merocyanine 540

Photolysis of merocyanine 540 (M-24571) produces both singlet oxygen and other reactive oxygen species, including oxygen radicals.[25–28] Merocyanine 540 is commonly used as a photosensitizer in photodynamic therapy.[10,29–37]

Detecting Singlet Oxygen

trans-1-(2′-Methoxyvinyl)pyrene (M-7913) can be used to detect picomole quantities of singlet oxygen in chemical and biological systems (Figure 19.2), making this compound perhaps the most sensitive singlet oxygen probe currently available.[38–40] Furthermore, this highly selective chemiluminescent probe does not react with other activated oxygen species such as hydroxyl radical, superoxide or hydrogen peroxide.

Generating Hydroxyl and Superoxide Radicals

Hydroxyl and superoxide radicals have been implicated in a number of pathological conditions, including ischemia, reperfusion and aging. The superoxide anion (Table 19.1) may also play a role in regulating normal vascular function. The hydroxyl radical is a very reactive oxygen species that has a lifetime of about 2 nanoseconds in aqueous solution and a radius of diffusion of about 20 Å. Thus, it induces peroxidation only when it is generated in close proximity to its target. The hydroxyl radical can be derived from superoxide in a reaction catalyzed by Fe^{2+} or other transition metals, as well as by the effect of ionizing radiation on dioxygen. Superoxide is most effectively generated from a hypoxanthine–xanthine oxidase generating system.[41–43] In one cell-based assay for hydroxyl radical formation, D-phenylalanine is specifically converted to D-tyrosine.[44] In phagocytic cells, H_2O_2 also produces *O,O′*-dityrosine, an oxidative crosslink product of appropriately situated tyrosine residues, which is formed through the intermediacy of phenoxyl radicals.[45]

Malachite Green

Malachite green is a nonfluorescent photosensitizer that absorbs at long wavelengths (~630 nm). Its photosensitizing action can be targeted to particular cellular sites by conjugating malachite green isothiocyanate (M-689, Figure 1.83) to specific antibodies.[46,47] Enzymes and other proteins within ~10 Å of the binding site of the malachite green–labeled antibody can then be selectively destroyed upon irradiation with long-wavelength light. Studies by Jay and colleagues have demonstrated that this photo-induced destruction of enzymes in the immediate vicinity of the chromophore is apparently the result of localized production of hydroxyl radicals, which have short lifetimes that limit their diffusion from the site of their generation.[48]

1,10-Phenanthroline Iodoacetamide

Conjugation of the iodoacetamide of 1,10-phenanthroline (P-6879) to thiol-containing ligands confers the metal-binding properties of this important complexing agent on the ligand. For example, the covalent copper–phenanthroline complex of oligonucleotides or nucleic acid–binding molecules in combination with hydrogen peroxide acts as a chemical nuclease to selectively cleave DNA or RNA.[49–54] Hydroxyl radicals or other reactive oxygen species appear to be involved in this cleavage.[55,56]

Detecting Hydroxyl and Superoxide Radicals

Fluorogenic Spin Traps

Hydroxyl radicals have usually been detected after reaction with spin traps. We offer TEMPO-9-AC (A-7923) and proxyl fluorescamine [57–60] (C-7924), two fluorogenic probes for detecting hydroxyl radicals and superoxide. Each of these molecules contains a nitroxide moiety that effectively quenches its fluorescence. However, once TEMPO-9-AC or proxyl fluorescamine traps a hydroxyl radical or superoxide, its fluorescence is restored and the radical's electron spin resonance signal is destroyed, making these probes useful for detecting radicals either by fluorescence or by electron spin resonance spectroscopy. Proxyl fluorescamine can also be used to detect the methyl radicals that are formed by the reaction of hydroxyl radicals with DMSO.[60] Radical-specific scavengers — such as the superoxide-specific *p*-benzoquinone and superoxide dismutase [61] or the hydroxyl radical–specific mannitol and dimethylsulfoxide (DMSO) [57,62,63] — can be used to identify the detected species.

Chemiluminescent and Chromogenic Reagents for Detecting Superoxide

In the absence of apoaequorin, the luminophore coelenterazine (C-2944) produces chemiluminescence in response to superoxide generation in phorbol ester– or chemotactic peptide–stimulated neutrophils.[64] Unlike luminol, coelenterazine exhibits luminescence that does not depend on the activity of cell-derived myeloperoxidase and is not inhibited by azide.[64]

In addition to coelenterazine, we offer MCLA (M-23800) for detecting superoxide. MCLA and coelenterazine are superior alternatives to lucigenin (L-6868) for this application because lucigenin can reportedly sensitize superoxide production, leading to false-positive results.[65–69] An additional advantage of MCLA is that its pH optimum for luminescence generation is closer to the physiological near-neutral range than are the pH optima of luminol and lucigenin.[70]

Lucigenin (L-6868) exhibits chemiluminescence that is reported to be sensitive to the superoxide anion.[71–75] Lucigenin has been employed to investigate superoxide generation in spermatozoa,[76] L929 cells,[77] chondrocytes contained within the matrix of living cartilage tissue,[78] and mitochondria of alveolar macrophages.[75] It has also been used to examine the role of the superoxide anion in reoxygenation injury in isolated rat hepatocytes.[79,80] We have purified our lucigenin to remove a blue-fluorescent impurity that is found in some commercial samples.

Nitro blue tetrazolium salt (NBT, N-6495; Table 19.2) and other tetrazolium salts are chromogenic probes useful for superoxide determination.[81,82] These probes are also widely used for detecting redox potential of cells for viability, proliferation and cytotoxicity assays; see below for more information.

Detecting Hydrogen Peroxide, Hydroperoxides and Peroxyl Radicals

In peroxisomes, H_2O_2 is produced by several enzymes that use molecular oxygen to oxidize organic compounds. This H_2O_2 is then utilized by catalase to oxidize other substrates, including phenols, formic acid, formaldehyde and alcohol. In liver and kidney cells, these oxidation reactions are important for detoxifying a variety of compounds in the bloodstream.[83–86] However, H_2O_2 also plays a role in neurodegenerative and other disorders through induction of apoptosis,[87] oxidation of glutathione, modification of intracellular Ca^{2+} levels and mitochondrial potential and induction of DNA strand breaks.[88] In addition, H_2O_2 is released from cells during hypoxia.[89]

Peroxidation of unsaturated lipids plays an important role in cell membrane properties,[90] signal transduction pathways,[91,92] apoptosis and the deterioration of foods and other biological compounds.[93] Lipid oxidation may also be responsible for aging, as well as pathological processes such as drug-induced phototoxicity and atherosclerosis.[94] Lipid hydroperoxides have been reported to accumulate in oxidatively stressed individuals, including HIV-infected patients.[95] To directly assess the extent of lipid peroxidation, researchers either measure the amount of lipid hydroperoxides directly or detect the presence of secondary reaction products[96–98] (e.g., 4-hydroxy-2-nonenal or malonaldehyde; see below).

Peroxyl radicals are formed by the decomposition of hydroperoxides, including lipid hydroperoxides. Experimentally, peroxyl radicals are generated from hydroperoxides such as cumene hydroperoxide or from compounds such as 2,2′-azobis(2-amidinopropane). Free radical–mediated damage in cells is often the result of lipid peroxidation. A problem with investigating the link between lipid peroxidation and diseases such as atherosclerosis, diabetes and Parkinson's has been the lack of suitable methods to detect the relationship between lipid peroxidation and the onset of such diseases.[99]

Table 19.2 Tetrazolium salts for detecting redox potential in living cells and tissues.

Cat #	Tetrazolium Salt	Color of Formazan	Water Solubility of Formazan	Applications
M-6494 (MTT)	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	purple	no	• Superoxide generation by fumarate reductase[1] and nitric oxide synthase[2] • Mitochondrial dehydrogenase activity[3] • Cell viability and proliferation[4–9] • Neuronal cell death[10] • Platelet activation[11] • Tumor cell adhesion[12] and invasion[13] • Multidrug resistance[14] • *In vitro* toxicity testing[15–17]
N-6495 (NBT)	Nitro blue tetrazolium chloride	deep blue	no	• Superoxide generation by xanthine oxidase[18] • Neutrophil oxidative metabolism[19,20] • NADPH diaphorase activity[21–23] • Succinic dehydrogenase histochemistry[24]
X-6493 (XTT)	2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide	orange	yes	• Antifungal susceptibility[25] • Drug sensitivity of cells[26] • Parasitic nematode viability[27] • Tumor cell cytotoxicity[28]

1. J Biol Chem 270, 19767 (1995); **2.** J Biol Chem 269, 12589 (1994); **3.** Cytometry 13, 532 (1992); **4.** Biotechniques 25, 622 (1998); **5.** J Immunol Methods 168, 253 (1994); **6.** Anal Biochem 214, 190 (1993); **7.** J Immunol Methods 164, 149 (1993); **8.** Anal Biochem 205, 8 (1992); **9.** J Immunol Methods 89, 271 (1986); **10.** J Cell Biol 128, 201 (1995); **11.** J Immunol Methods 159, 253 (1993); **12.** J Immunol Methods 164, 255 (1993); **13.** Cancer Res 54, 3620 (1994); **14.** Leuk Res 16, 1165 (1992); **15.** Biosci Biotechnol Biochem 56, 1472 (1992); **16.** J Immunol Methods 144, 141 (1991); **17.** J Immunol Methods 131, 165 (1990); **18.** J Reprod Fertil 97, 441 (1993); **19.** Clin Chim Acta 221, 197 (1993); **20.** J Leukoc Biol 53, 404 (1993); **21.** Neurosci Lett 155, 61 (1993); **22.** Proc Natl Acad Sci U S A 88, 7797 (1991); **23.** Proc Natl Acad Sci U S A 88, 2811 (1991); **24.** Histochemistry 76, 381 (1982); **25.** Antimicrob Agents Chemother 36, 1619 (1992); **26.** Cancer Res 48, 4827 (1988); **27.** Parasitology 107, 175 (1993); **28.** J Immunol Methods 147, 153 (1992).

Amplex Red Reagent

In the presence of horseradish peroxidase (HRP), the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine, A-12222, A-22177) reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin (Figure 10.50). The Amplex Red reagent has greater stability, yields less background and produces a red-fluorescent product that is more readily detected than the similar reduced methylene blue derivatives commonly used for colorimetric determination of lipid peroxides in plasma, sera, cell extracts and a variety of membrane systems.[33,100,101] Using the Amplex Red reagent in conjunction with HRP, we have found that release of hydrogen peroxide to the medium by as few as 2000 phorbol ester–stimulated neutrophils can be detected in a fluorescence microplate reader. The Amplex Red reagent has been used to detect H_2O_2 release from human keratinocytes after UV-B radiation [102] and from human leukocytes after phorbol myristate acetate treatment [103] and from proliferating U937 cells.[104] Using the Amplex Red reagent, researchers have discovered that antibodies can convert molecular oxygen to H_2O_2, which may be important in understanding a new chemical arm of the immune system, as well as the evolution of antibodies and the role they may play in human diseases.[105,106]

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit

Our Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188), which is described in greater detail in Section 10.5, includes the reagents required for analysis of *either* H_2O_2 in solutions or peroxidase activity in an ultrasensitive, one-step assay that can be measured using either fluorescence or absorbance. The Amplex Red peroxidase substrate can detect the presence of active peroxidases and the release of H_2O_2 from biological samples, including cells and cell extracts [103,107–109] and is also useful for detecting H_2O_2 that is produced as a product of enzyme-coupled reactions.[110,111] The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit contains:

- The Amplex Red reagent
- Horseradish peroxide (HRP)
- H_2O_2 for use as a positive control
- Concentrated reaction buffer
- A detailed protocol

Each kit provides a sufficient amount of reagent for approximately 500 assays using a reaction volume of 100 µL per assay.

Several additional kits that utilize our Amplex Red peroxidase substrate to detect H_2O_2 in coupled enzymatic reactions are described in Section 10.5.

Amplex Red Xanthine/Xanthine Oxidase Assay Kit

Xanthine oxidase plays a key role in the production of free radicals, including superoxide in the body. In healthy individuals, xanthine oxidase is present in appreciable amounts only in the liver and the jejunum. However, in various liver disorders the enzyme is released into circulation. Therefore, determination of serum xanthine oxidase level serves as a sensitive indicator of acute liver damage such as jaundice.[112] Previously, researchers have used chemiluminescence [42,113] or absorbance [114] to monitor xanthine oxidase activity. Using the Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182), one can detect xanthine oxidase activity in a purified system at levels as low as 0.1 mU/mL by fluorescence (Figure 19.3). The kit can also detect as little as 200 nM hypoxanthine or xanthine (Figure 19.4).

Xanthine oxidase catalyzes the oxidation of hypoxanthine or xanthine to uric acid and superoxide. In the reaction mixture, the superoxide spontaneously degrades to hydrogen peroxide, which in the presence of horseradish peroxidase reacts stoichiometrically with the Amplex Red reagent to generate the red-fluorescent oxidation product resorufin. Resorufin has absorption and emission maxima at approximately 563 and 587 nm, respectively (Figure 10.5), and because the extinction coefficient is high (54,000 $cm^{-1}M^{-1}$), the assay can be performed either fluorometrically or spectrophotometrically.

The Amplex Red Xanthine/Xanthine Oxidase Assay Kit (A-22182) contains:

- The Amplex Red reagent
- Dimethylsulfoxide (solvent for the Amplex Red reagent)
- Horseradish peroxidase (HRP)
- Hydrogen peroxide
- Concentrated reaction buffer
- Xanthine oxidase from buttermilk
- A hypoxanthine solution
- A xanthine solution
- A detailed protocol

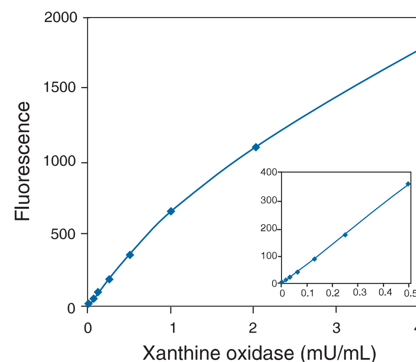

Figure 19.3 Detection of xanthine oxidase using the Amplex Red reagent–based assay (A-22182). Each reaction contained 50 µM Amplex Red reagent, 0.2 U/mL horseradish peroxidase, 0.1 mM hypoxanthine and the indicated amount of xanthine oxidase in 1× reaction buffer. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and detection at 590 ± 17.5 nm. A background of 65 fluorescence units was subtracted from each data point. The inset shows the assay's sensitivity and linearity at low enzyme concentrations.

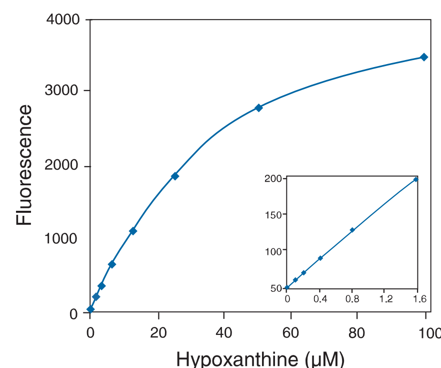

Figure 19.4 Detection of hypoxanthine using the Amplex Red reagent–based assay (A-22182). Each reaction contained 50 µM Amplex Red reagent, 0.2 U/mL horseradish peroxidase, 20 mU/mL xanthine oxidase and the indicated amount of hypoxanthine in 1× reaction buffer. Reactions were incubated at 37°C. After 30 minutes, fluorescence was measured in a fluorescence microplate reader using excitation at 530 ± 12.5 nm and detection at 590 ± 17.5 nm. A background of 54 fluorescence units was subtracted from each data point. The inset shows the assay's sensitivity and linearity at low enzyme concentrations.

Amplex Red reagent–based assays for numerous enzymes and analytes are described in Section 10.5. A particularly important advantage of these assays is their use of natural *substrates, rather than synthetic pseudosubstrates, in the assays.*

Each kit provides sufficient reagents for ~400 assays using either a fluorescence or absorbance microplate reader and reaction volumes of 100 µL per assay.

cis-Parinaric Acid

Fluorescence quenching of the fatty acid analog *cis*-parinaric acid (P-1901) has been used in several lipid peroxidation assays,[99,115–119] including quantitative determinations in living cells.[120–125] In a study investigating the membrane antioxidant properties of the *bcl-2* proto-oncogene product, researchers used *cis*-parinaric acid to detect lipid hydroperoxides together with 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (C-2938) to detect cytosolic reactive oxygen molecules.[126] Parinaric acid's extensive unsaturation (Figure 13.11) makes it quite susceptible to oxidation if not rigorously protected from air.[127] Consequently, we offer *cis*-parinaric acid specially packaged in 10 ampules sealed under argon, each containing 10 mg (P-1901). During experiments, we advise handling parinaric acid samples under inert gas and preparing solutions using degassed buffers and solvents. Parinaric acid is also somewhat photolabile and undergoes photodimerization when exposed to intense illumination, resulting in loss of fluorescence.[128]

Diphenyl-1-Pyrenylphosphine

The direct fluorometric detection of hydroperoxides in lipids, serum, tissues and foodstuffs[129,130] is now possible using the reagent diphenyl-1-pyrenylphosphine (DPPP, D-7894). DPPP is essentially nonfluorescent until oxidized to a phosphine oxide by peroxides. DPPP has previously been used to detect picomole levels of hydroperoxides by HPLC.[131–134] Its solubility in lipids makes DPPP quite useful for detecting hydroperoxides in the membranes of living cells[135] and in low-density lipoprotein particles,[136] as well as for studying superoxide dismutase (SOD), which catalyzes the conversion of superoxide to hydrogen peroxide.

BODIPY Dyes: Peroxyl Radical Scavengers

A unique assay for peroxyl radicals uses some of our BODIPY dyes, including the BODIPY 581/591 fatty acid[90] (D-3861) and the BODIPY 665/676 dye (B-3932) to measure the antioxidant activity in an organic lipid environment or in a liposomal medium.[90,137,138] This assay is based on the loss or shift of the dye's fluorescence as the result of its interaction with peroxyl radicals, and on the retention of the fluorescent signal in the presence of antioxidants that intercept these free radicals. It has been proposed that this assay is suitable for a fluorescence-based microplate reader or flow cytometer to examine the effect of lipid peroxidation on a cell-by-cell basis.[138]

The oxidation sensitivity of the conjugated double bonds of the BODIPY 581/591 fatty acid (Figure 19.5) has also been exploited in a ratiometric assay of lipid oxidation in live cells that is essentially independent of uneven dye loading, cell thickness and compartmentalization.[139] In this assay, the cell membranes of rat-1 fibroblasts and myocardial cells were labeled with the BODIPY 581/591 fatty acid, and then cumene hydroperoxide was added to induce oxidation. Upon oxidation, the BODIPY 581/591 fluorophore exhibited a shift in its fluorescence from red to green, which was observed using confocal laser-scanning microscopy. As compared with arachidonic acid, the fluorescence of this BODIPY probe was reportedly twice as sensitive to oxidation.

Peroxyl radicals have also been detected in erythrocyte and red blood cell membranes using BODIPY FL EDA[117] (D-2390, Section 3.3), a water-soluble BODIPY dye, or BODIPY FL hexadecanoic acid (D-3821, Section 13.2). BODIPY FL hexadecanoic acid exhibits the red shift common to the fluorescence of lipophilic BODIPY dyes when they are concentrated (Figure 13.6), permitting ratiometric measurements of hydroxyl radical production and allowing the onset of lipid peroxidation in live cells to be monitored.[117]

Other Scavengers for Peroxyl Radicals

The fluorescence of several other probes is lost following interaction with peroxyl radicals. Lipophilic fluorescein dyes such as hexadecanoylaminofluorescein[99] (H-110, Section 13.5) and fluorescein-labeled phosphatidylethanolamine (F-362, Section 13.2) have been or potentially are useful for detecting peroxyl radical formation in membranes and in solution. Phycobiliproteins such as B-phycoerythrin,[140–145] R-phycoerythrin[99,143,146,147] and allophycocyanin[148] (P-800, P-801, A-803, A-819; Section 6.4) may be similarly useful. R-phycoerythrin has been used to detect and measure total plasma antioxidant capacity, including peroxyl radicals.[143,145,147,149]

Luminol

Although luminol (L-8455) is not useful for detecting superoxide in living cells,[73] it is commonly employed to detect peroxidase- or metal ion–mediated oxidative events.[150–152] Used alone, luminol can detect oxidative events in cells rich in peroxidases, including granulocytes[153–156] and spermatozoa.[76] This probe has also been used in conjunction with horseradish peroxidase (HRP) to investigate reoxygenation injury in rat hepatocytes.[79,157] In these experiments, it is thought that the primary species being detected is hydrogen peroxide. In addition, luminol has been employed to detect peroxynitrite,[158–160] a molecule thought to be generated in a variety of pathological conditions.[62] Phospholipid hydroperoxides have been determined directly by chemiluminescence-detected HPLC with luminol[161–163] (L-8455) or a combination of luminol and cytochrome *c*.[164] This chemiluminescent probe has also been employed as a chemical sensor for dioxygen and nitrogen dioxide. It has been reported that luminol's chemiluminescence response to oxidative species may be competitively inhibited by biomolecules containing sulfhydryl and thioether groups.[165,166]

Detecting 4-Hydroxy-2-Nonenal

Formation of 4-hydroxy-2-nonenal from linoleic acid is a major cause of lipid peroxidation–induced liver toxicity. Several reagents for the direct fluorometric detection of aldehydes are described in Section 3.2. The modification of 4-hydroxy-2-nonenal or malonaldehyde with a fluorescent or chromophoric hydrazine reagent coupled with the separation and detection of the reaction product by a chromatography-based technique has rarely been reported but appears to be one of the most promising approaches for detecting these lipid peroxidation products.

Assaying Oxidative Activity in Live Cells with Leuco Dyes

Assaying oxidative activity in living cells with fluorogenic, chemiluminescent or chromogenic probes is complicated by the

possibility of having multiple forms of reactive oxygen in the same cell. In addition, the nitric oxide radical (Section 19.3) may produce the same changes in the optical properties of the probe as do other reactive oxygen molecules. Blocking agents and enzyme inhibitors can sometimes help to sort out the species responsible for the probe's optical response. Quantitative analysis is also difficult because of: 1) the high intracellular concentration of glutathione, which can form thiyl or sulfinyl radicals or otherwise trap or reduce oxygen species;[41] 2) the variable concentration of metals, which can either catalyze or inhibit radical reactions; and 3) the presence of other free radical–quenching agents such as spermine.[167]

Fluorescein, rhodamine and various other dyes can be chemically reduced to colorless, nonfluorescent leuco dyes. These "dihydro" derivatives are readily oxidized back to the parent dye by some reactive oxygen species and thus can serve as fluorogenic probes for detecting oxidative activity in cells and tissues;[168–170] however, their oxidation may not easily discriminate between the various reactive oxygen species. It has been reported that dihydroethidium, dichlorodihydrofluorescein (H_2DCF) and dihydrorhodamine 123 react with intracellular hydrogen peroxide in a reaction mediated by peroxidase, cytochrome *c* or Fe^{2+}.[170–173] The leuco dyes also serve as fluorogenic substrates for peroxidase enzymes (Section 10.5). All of these reagents are slowly oxidized by air back to the parent fluorescent dyes, and in some cases light appears to accelerate their oxidation.

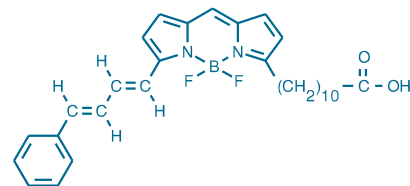

Figure 19.5 D-3861 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid.

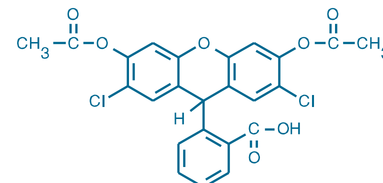

Figure 19.6 D-399 2′,7′-dichlorodihydrofluorescein diacetate.

Dichlorodihydrofluorescein Diacetate and Its Analogs

Dichlorodihydrofluorescein diacetate (H_2DCFDA, D-399; Figure 19.6), also known as dichlorofluores*cin* diacetate, is commonly used to detect the generation of reactive oxygen intermediates in neutrophils and macrophages.[174–178] Cell-permeant H_2DCFDA may also be extremely useful for assessing the overall oxidative stress in toxicological phenomena.[169,170] A review by Tsuchiya and colleagues outlined methods for visualizing the generation of oxidative species in whole animals. For example, they suggest using propidium iodide (P-1304, P-3566; FluoroPure Grade, P-21493; Section 8.1) with H_2DCFDA to simultaneously monitor oxidant production and cell injury.[179] H_2DCFDA has been used to visualize oxidative changes in carbon tetrachloride–perfused rat liver[180] and in venular endothelium during neutrophil activation,[181] as well as to examine the effect of ischemia and reperfusion in lung and heart tissue.[182,183] Using H_2DCFDA, researchers characterized hypoxia-dependent peroxide production in *Saccharomyces cerevisiae* as a possible model for ischemic tissue destruction.[184] A variety of toxicological phenomena in cultured cells have also been investigated with H_2DCFDA, including:

- Amyloid β protein–mediated increases in hydrogen peroxide in PC12 cells[185]
- Effects of calcium antagonists on oxidative metabolism in dissociated rat cerebellar and cortical neurons[186]
- Methamphetamine-induced oxidative stress in dopaminergic neurons[187]
- Effect of lipopolysaccharides on the level of oxygen metabolites in rat liver Kupffer cells[188]
- Nephrotoxin-induced oxidative stress in isolated proximal tubular cells[189]
- Nickel-induced increases in oxidant levels in Chinese hamster ovary (CHO) cells[190]
- Effect of transforming growth factor–β1 on the overall oxidized state of mouse osteoblastic cells[191]

In neutrophils, H_2DCFDA has proven useful for flow cytometric analysis of nitric oxide, forming a product that has spectral properties identical to those produced when it reacts with hydrogen peroxide.[192] In this study, H_2DCFDA's reaction with nitric oxide was blocked by adding the arginine analog N^G-methyl-L-arginine (L-NMMA, M-7898; Section 19.3) to the cell suspension.[192] H_2DCF is not directly oxidized by either superoxide or free hydroxyl radical in aqueous solution.[170] However, it has been reported that 2′,7′-dichlorofluorescein — the oxidation product of H_2DCF — can be further oxidized to a phenoxyl radical in a horseradish peroxidase–catalyzed reaction, and this reaction may complicate the interpretation of results obtained with this probe in cells undergoing oxidative stress.[193]

TECHNICAL NOTE

Detecting Oxidative Activity in Live Cells

Reactive oxygen species (ROS) are formed in cells in response to stimulation and may play a role in signal transduction. The principal probes that have been used to detect ROS in cells have been chemically reduced forms of the xanthenes (fluoresceins and rhodamines) or ethidium, which are colorless and nonfluorescent until oxidized back to the fluorophore by ROS. The precise ROS that mediates the oxidation can be difficult to ascertain. Dichlorodihydrofluorescein diacetate (D-399), dihydrorhodamine 123 (D-632) and dihydroethidium (D-1168) have been the standard dyes for these assays. However, we recommend the chloromethyl derivative of dichlorodihydrofluorescein diacetate (C-6827) because it is better retained in cells before and after stimulation. We also recommend evaluation of our Fc OxyBURST technology for applications that involve following phagocytosis. Reactive OxyBURST reagents permit conjugate of ROS-sensitive probes to other biomolecules to follow their uptake and oxidation by phagocytic cells.

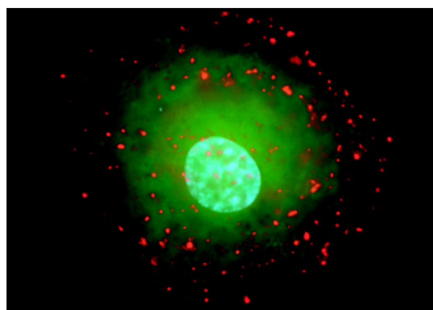

Figure 19.7 Bovine pulmonary artery endothelial cells (BPAEC) were initially stained with the reactive oxygen species (ROS) indicator, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (C-2938). After a 30-minute incubation, the cells were washed and then incubated simultaneously with FM 5-95 (T-23360) and Hoechst 33342 (H-1399, H-3570, H-21492) in phosphate-buffered saline (PBS) for an additional five minutes before washing and mounting in PBS. The red-fluorescent FM 5-95 appears to stain both the plasma membrane and early endosomes; the green-fluorescent, oxidized carboxydichlorofluorescein localizes to the cytoplasm; and the blue-fluorescent Hoechst 33342 dye stains the nucleus.

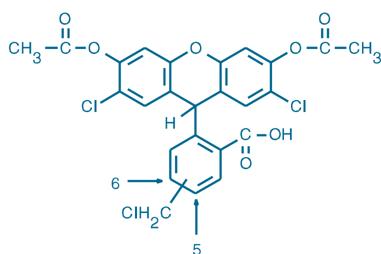

Figure 19.8 C-6827 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester.

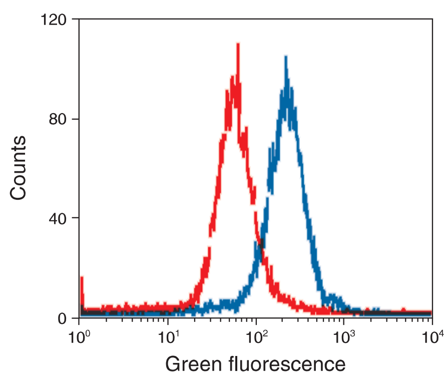

Figure 19.9 An oxidative burst was detected by flow cytometry of cells labeled with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, C-6827). Jurkat cells were incubated with 100 nM CM-H_2DCFDA. The cells were washed and resuspended in either phosphate-buffered saline (PBS, red) or PBS with 0.03% H_2O_2 (blue). The samples were analyzed on a flow cytometer equipped with a 488 nm argon-ion laser and a 525 ± 10 nm bandpass emission filter.

Improved Versions of H_2DCFDA

Intracellular oxidation of H_2DCF tends to be accompanied by leakage of the product, 2′,7′-dichlorofluorescein,[194] which may make quantitation or detection of slow oxidation difficult. To enhance retention of the fluorescent product, Molecular Probes offers the carboxylated H_2DCFDA analog (carboxy-H_2DCFDA, C-400), which has two negative charges at physiological pH, and its di(acetoxymethyl ester) (C-2938, Figure 15.79, Figure 19.7), which should more easily pass through membranes during cell loading. Upon oxidation and cleavage of the acetate and ester groups by intracellular esterases, both analogs form carboxydichlorofluorescein (C-368, Section 14.3), with additional negative charges that should impede its leakage out of the cell. Carboxy-H_2DCFDA (C-400) has been used to assess the oxidative process in isolated perfused rat heart tissue [183] and in transfected *cos*-1 cells expressing native or mutagenized prostaglandin endoperoxide H synthase.[195] Its di(acetoxymethyl ester) (C-2938) has been employed to investigate the role of the *bcl-2* proto-oncogene product in preventing apoptosis through its antioxidant properties.[126] Another probe for oxidative bursts and reactive oxygen species — 5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA, C-13293) — exhibits improved photostability when compared to other fluorescein derivatives in common use.

Molecular Probes has also developed 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester [196] (CM-H_2DCFDA, C-6827; Figure 19.8, Figure 19.9) — a chloromethyl derivative of H_2DCFDA that should exhibit much better retention in live cells. As with our other chloromethyl derivatives (see the description of our CellTracker probes in Section 14.2), we believe that CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long-term studies. CM-H_2DCFDA has been used to:

- Measure intracellular reactive oxygen species in cardiac myocytes [197]
- Detect insulin-stimulated production of H_2O_2 in insulin-sensitive hepatoma and adipose cells [198]
- Detect arsenic-induced production of oxyradicals by confocal laser-scanning microscopy [199]
- Explore the parallel induction of reactive oxygen species and Ca^{2+} transients in ouabain-treated myocytes [197,200]

The diacetate derivatives of the dichlorodihydrofluoresceins are quite stable. When used for intracellular applications, the acetates are cleaved by endogenous esterases, releasing the corresponding dichlorodihydrofluorescein derivative. If, however, these nonfluorescent diacetate derivatives are used for *in vitro* assays, they must first be hydrolyzed with mild base to form the colorless probe.

Dihydrocalcein AM

Calcein AM (C-1430, C-3099, C-3100; Section 15.2, Figure 15.2) is extremely useful as a probe for the study of cell viability, adhesion, multidrug resistance, chemotaxis and other processes. We have combined the superior retention of calcein — the hydrolytic product of calcein AM in viable cells — and the oxidation sensitivity of dihydrofluoresceins to yield the probe dihydrocalcein AM (D-23805, Figure 15.4). Dihydrocalcein AM is freely permeant to cell membranes and is oxidized to green-fluorescent calcein, which has superior retention properties in cells that have intact membranes (Figure 15.3). Dihydrocalcein AM is provided specially packaged as a set of 20 vials, each containing 50 µg of the probe.

OxyBURST Green Reagents

Molecular Probes' Fc OxyBURST Green assay reagent (F-2902) was developed in collaboration with Elizabeth Simons of Boston University to monitor the oxidative burst in phagocytic cells using fluorescence instrumentation. The Fc OxyBURST Green assay reagent comprises bovine serum albumin (BSA) that has been covalently linked to dichlorodihydrofluorescein (H_2DCF) and then complexed with purified rabbit polyclonal anti-BSA antibodies. When these immune complexes bind to Fc receptors, the nonfluorescent H_2DCF molecules are internalized within the phagovacuole and subsequently

oxidized to green-fluorescent dichlorofluorescein (DCF, Figure 16.1; Figure 16.2). Unlike dichlorodihydrofluorescein diacetate (H_2DCFDA), the Fc OxyBURST Green assay reagent does not require intracellular esterases for activation, making this reagent particularly suitable for detecting the oxidative burst in cells with low esterase activity such as monocytes.[201] The Fc OxyBURST Green assay reagent reportedly produces >8 times more fluorescence than does H_2DCFDA at 60 seconds and >20 times more at 15 minutes following internalization of the immune complex.[202]

OxyBURST Green H_2HFF BSA (O-13291) is a sensitive fluorogenic reagent for detecting extracellular release of oxidative products in a spectrofluorometer or a fluorescence microscope (Figure 16.4). This reagent comprises BSA that has been covalently linked to dihydro-2′,4,5,6,7,7′-hexafluorofluorescein (H_2HFF), a reduced dye with improved stability. Unlike our Fc OxyBURST Green assay reagent, OxyBURST Green H_2HFF BSA is not complexed with IgG. OxyBURST Green H_2HFF BSA provides up to 1000-fold greater sensitivity than conventional methods based on spectrophotometric detection of superoxide dismutase–inhibitable reduction of cytochrome *c* [203,204] and allows researchers to take advantage of the sample stirring and temperature control capabilities available in many spectrofluorometers. Because OxyBURST Green H_2HFF BSA is a protein conjugate, it is superior to low molecular weight probes such as dihydrotetramethylrosamine and dihydrorhodamine 123, which are cell permeant and therefore do not exclusively detect extracellular oxidants.

Amine-Reactive OxyBURST Green Reagents

As an alternative to our Fc OxyBURST Green assay reagent and OxyBURST Green H_2HFF BSA, Molecular Probes offers amine-reactive OxyBURST Green H_2DCFDA succinimidyl ester (2′,7′-dichlorodihydrofluorescein diacetate, SE; D-2935; Figure 16.6), which can be used to prepare oxidation-sensitive conjugates of a wide variety of biomolecules and particles, including antibodies, antigens, peptides, proteins, dextrans, bacteria, yeast and polystyrene microspheres.[202,205] Following conjugation to amines, the two acetates of OxyBURST Green H_2DCFDA can be removed by treatment with hydroxylamine at neutral pH to yield the dihydrofluorescein conjugate. The OxyBURST Green H_2DCFDA conjugates are nonfluorescent until they are oxidized to the corresponding fluorescein derivatives. Thus, like our Fc OxyBURST Green assay reagent, they provide a means of detecting the oxidative burst in phagocytic cells. In one application, OxyBURST Green H_2DCFDA succinimidyl ester was conjugated to an antibody that binds specifically to YAC tumor cells. YAC cells opsonized with this customized OxyBURST reagent were then used in a fluorescence microscopy study to show that Fc receptor–activated neutrophils appear to deliver reactive oxygen species to the surface of their target cells.[205]

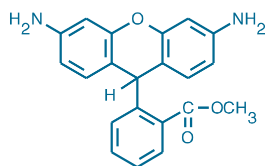

Figure 19.10 D-632 dihydrorhodamine 123.

Dihydrorhodamine 123

Dihydrorhodamine 123 (D-632, D-23806; Figure 19.10, Figure 19.11) is the uncharged and nonfluorescent reduction product of the mitochondrion-selective dye rhodamine 123 (R-302; FluoroPure Grade, R-22420; Section 12.2). This leuco dye passively diffuses across most cell membranes where it is oxidized to cationic rhodamine 123 (Figure 12.23), which localizes in the mitochondria. Like H_2DCF, dihydrorhodamine 123 does not directly detect superoxide,[172] but rather reacts with hydrogen peroxide in the presence of peroxidase,[172] cytochrome *c* or Fe^{2+}.[173] However, dihydrorhodamine 123 also reacts with peroxynitrite,[206,207] the anion formed when nitric oxide reacts with superoxide.[208,209] Peroxynitrite, which may play a role in many pathological conditions,[62,207] has been shown to react with sulfhydryl groups,[210] DNA [211] and membrane phospholipids,[212] as well as with tyrosine [213] and other phenolic compounds.[214]

Dihydrorhodamine 123 has been used to investigate reactive oxygen intermediates produced by human and murine phagocytes,[215] activated rat mast cells [216] and cultured endothelial cells.[173] It has also been employed to study the role of the CD14 cell-surface marker in H_2O_2 production by human monocytes.[217] In addition, dihydrorhodamine 123 has been used with the Fura Red calcium indicator (F-3020, F-3021; Section 20.3) to simultaneously measure oxidative bursts and Ca^{2+} fluxes in monocytes and granulocytes.[218] Dihydrorhodamine 123 is reportedly a more sensitive probe than H_2DCFDA for detecting granulocyte respiratory bursts.[219–221]

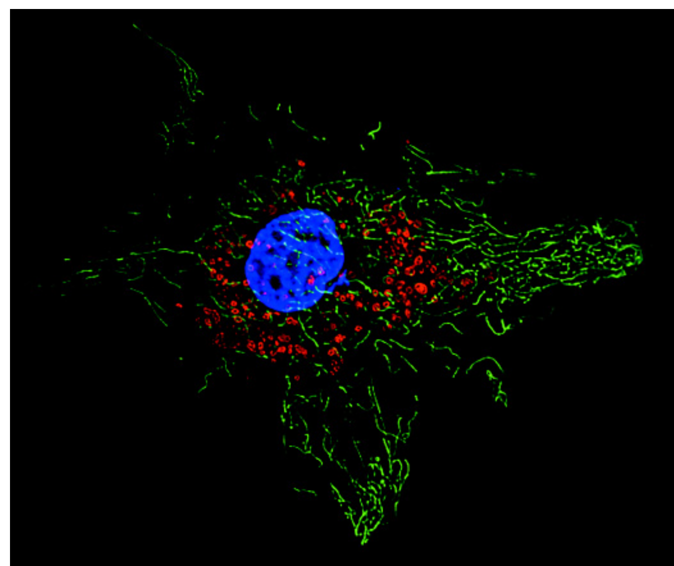

Figure 19.11 Live bovine pulmonary artery endothelial cells (BPAEC) were first stained with LysoTracker Red DND-99 (L-7528). Then, a solution of dihydrorhodamine 123 (D-632, D-23806) and Hoechst 33258 (H-1398, H-3569, H-21491) was added and allowed to incubate with the cells for an additional 10 minutes before the cells were subsequently washed and visualized. The green-fluorescent oxidation product (rhodamine 123, R-302) localized primarily to the mitochondria. The red-fluorescent LysoTracker Red DND-99 stain accumulated in the lysosomes and the blue-fluorescent Hoechst 33258 dye stained the nuclei. The image was acquired with filters appropriate for DAPI, fluorescein and the Texas Red dye and deconvolved using Huygens software (Scientific Volume Imaging, www.svi.nl).

Dihydrorhodamine 123 is available as a 10 mg vial (D-632) or as a stabilized 5 mM solution in DMSO (D-23806). Because of the susceptibility of dihydrorhodamine 123 to air oxidation, the DMSO solution is recommended when only small quantities are to be used at a time.

A Longer-Wavelength Reduced Rhodamine

Intracellular oxidation of dihydrorhodamine 6G (D-633) yields rhodamine 6G (R-634), which localizes in the mitochondria of living cells (Section 12.2). This cationic oxidation product has longer-wavelength spectra than those of dihydrorhodamine 123, making it especially useful for multicolor applications and in some autofluorescent cells and tissues. Dihydrorhodamine 6G has been used in the study of chloride conductance and mutations of the cystic fibrosis transmembrane conductance regulator.[222]

Reduced MitoTracker Probes

Two of our MitoTracker probes — MitoTracker Orange CM-H_2TMRos (M-7511, Figure 12.10) and MitoTracker Red CM-H_2XRos (M-7513, Figure 12.11) — are chemically reactive reduced rosamines. Unlike MitoTracker Orange CMTMRos and MitoTracker Red CMXRos (M-7510, M-7512; Section 12.2), the reduced versions of these probes do not fluoresce until they enter an actively respiring cell, where they are oxidized by reactive oxygen species to the fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. Both MitoTracker Orange CMTMRos and the reduced MitoTracker Orange CM-H_2TMRos have been used to investigate the metabolic state of *Pneumocystis carinii* mitochondria.[223]

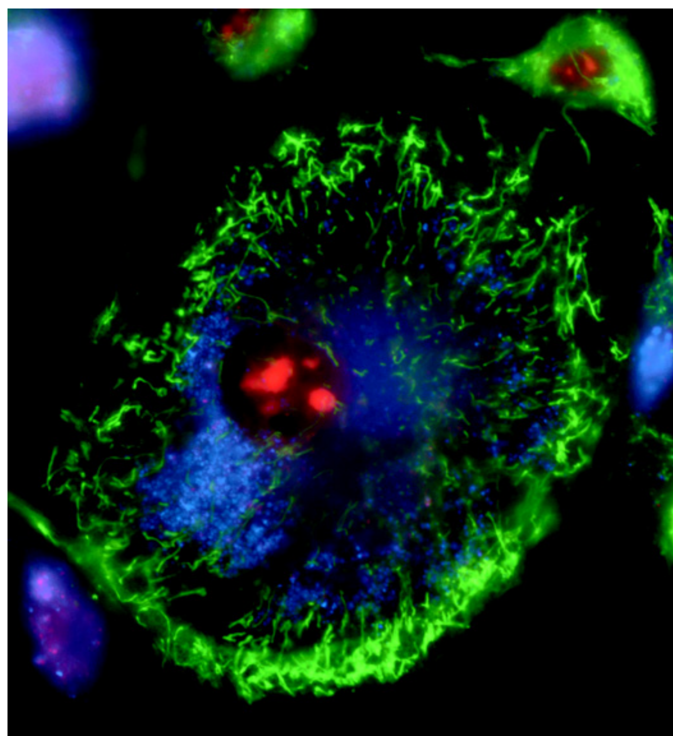

Figure 19.12 Live bovine pulmonary artery endothelial cells (BPAEC) were incubated with the cell-permeant, weakly blue-fluorescent dihydroethidium (D-1168, D-11347, D-23107) and the green-fluorescent mitochondrial stain, MitoTracker Green FM (M-7514). Upon oxidation, red-fluorescent ethidium accumulated in the nucleus.

Dihydroethidium (Hydroethidine)

Although dihydroethidium (Figure 15.20), which is also called hydroethidine, is commonly used to analyze respiratory burst in phagocytes,[224–226] it has been reported that this probe undergoes significant oxidation in resting leukocytes, possibly through the uncoupling of mitochondrial oxidative phosphorylation.[171] Cytosolic dihydroethidium exhibits blue fluorescence; however, once this probe is oxidized to ethidium (E-1305, Section 8.1, Figure 8.12), it intercalates within the cell's DNA, staining its nucleus a bright fluorescent red[227–229] (Figure 19.12). The mechanism of dihydroethidium's interaction with lysosomes and DNA has been described.[230] Dihydroethidium has been used for many purposes, including:

- Detecting multidrug-resistant cancer cells[231] (Section 15.6)
- Following phagocytosis and oxidative bursts by phagocytic blood cells[224,225,232,233]
- Investigating spermatozoal viability[234,235]
- Quantitating killer cell–target cell conjugates by flow cytometry methods[236–238]

Dihydroethidium (hydroethidine) is available in a 25 mg vial (D-1168), as a stabilized 5 mM solution in DMSO (D-23107) or specially packaged in 10 vials of 1 mg each (D-11347); the stabilized DMSO solution or special packaging is recommended when small quantities of the dye will be used over a long period of time.

RedoxSensor Red CC-1 Stain

RedoxSensor Red CC-1 stain (R-14060) is a unique probe whose fluorescence localization appears to be based on a cell's cytosolic redox potential. Scientists at Molecular Probes have found that RedoxSensor Red CC-1 stain passively enters live cells.[239] Once inside, the nonfluorescent probe is either oxidized in the cytosol to a red-fluorescent product (excitation/emission maxima ~540/600 nm), which then accumulates in the mitochondria, or the probe is transported to the lysosomes, where it is oxidized. The differential distribution of the oxidized product between mitochondria and lysosomes appears to depend on the oxidation–reduction (redox) potential of the cytosol. In proliferating cells, mitochondrial staining predominates, whereas contact-inhibited cells primarily exhibit lysosomal staining. The best method we have found to quantitate the distribution of the oxidized product is to use the mitochondrion-selective MitoTracker Green FM stain (M-7514, Section 12.2) in conjunction with the RedoxSensor Red CC-1 stain.

Tetrazolium Salts — Chromogenic Redox Indicators

Tetrazolium salts — especially MTT (M-6494) — are widely used for detecting the redox potential of cells for viability, proliferation and cytotoxicity assays. Upon reduction, these water-soluble colorless compounds form uncharged, brightly colored formazans. Several of the formazans precipitate out of solution and are useful for histochemical localization of the site of reduction or, after solubilization in organic solvent, for quantitation by standard spectrophotometric techniques. The extremely water-soluble formazan product of XTT (X-6493) does not require solubilization prior to quantitation. Many of these salts are re-

duced by specific components of the electron transport chain and may be useful for determining the site of action of specific toxins.[240] Selected applications of the tetrazolium salts are listed in Table 19.2. Our Vybrant MTT Cell Proliferation Assay Kit (V-13154, Section 15.4) provides a means of counting metabolically active cells; the Vybrant MTT assay can detect from 2000 to 250,000 cells, depending on the cell type and conditions. See also Section 15.2 for applications of tetrazolium salts in cells.

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Consequently the product's name, molecular weight and chemical structure have been changed to reflect presence of this residue. This change does not affect the use of this material for its intended purposes and the product is in every way identical to material previously provided by Molecular Probes, which is the exclusive supplier of this material. **197.** J Biol Chem 274, 19323 (1999); **198.** J Biol Chem 276, 21938 (2001); **199.** Proc Natl Acad Sci U S A 98, 1643 (2001); **200.** J Biol Chem 275, 27838 (2000); **201.** J Leukoc Biol 43, 304 (1988); **202.** J Immunol Methods 130, 223 (1990); **203.** J Biol Chem 255, 1874 (1980); **204.** J Clin Invest 61, 1081 (1978); **205.** J Cell Physiol 156, 428 (1993); **206.** Biochemistry 34, 3544 (1995); **207.** Free Radic Biol Med 16, 149 (1994); **208.** Free Radic Res Commun 18, 195 (1993); **209.** Free Radic

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Data Table — 19.2 Generating and Detecting Reactive Oxygen Species

Cat #	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A-7923	376.48	F,D,L	DMSO	358	11,000	424	MeOH	1
A-12222	257.25	FF,D,A	DMSO	280	6,000	none	pH 8	2
B-3932	448.32	F,L	DMSO, $CHCl_3$	665	161,000	676	MeOH	
C-400	531.30	F,D	DMSO, EtOH	290	5,600	none	MeCN	3
C-2938	675.43	F,D,AA	DMSO	291	5,700	none	MeOH	3
C-2944	423.47	FF,D,LL,AA	MeOH	429	7,500	see Notes	pH 7	4, 5, 6
C-6827	577.80	F,D,AA	DMSO	287	9,100	none	MeOH	3
C-7924	487.62	F,D,L	DMSO, H_2O	385	5,800	485	pH 7	1
C-13293	498.39	F,D	DMSO, EtOH	290	5,500	none	MeCN	7
D-399	487.29	F,D	DMSO, EtOH	258	11,000	none	MeOH	3
D-632	346.38	F,D,L,AA	DMF, DMSO	289	7,100	none	MeOH	8, 9
D-633	444.57	F,D,L,AA	DMF, DMSO	296	11,000	none	MeOH	8, 9
D-1168	315.42	FF,L,AA	DMF, DMSO	355	14,000	see Notes	MeCN	8, 10
D-2935	584.37	F,D,AA	DMF	258	11,000	none	MeOH	3
D-3861	504.43	F,L	DMSO	582	140,000	591	MeOH	11
D-7894	386.43	F,D,LL	MeCN	358	29,000	none	MeOH	12
D-11347	315.42	FF,L,AA	DMF, DMSO	355	14,000	see Notes	MeCN	8, 10
D-23107	315.42	FF,D,L,AA	DMSO	355	14,000	see Notes	MeCN	10, 13
D-23805	1068.95	F,D	DMSO	285	5,800	none	MeCN	14
D-23806	346.38	F,D,L,AA	DMSO	289	7,100	none	MeOH	9, 13
F-2902	see Notes	RR,L,AA	H_2O	<300		none		15, 16, 17
H-7476	504.45	F,D,L	DMSO, DMF	591	37,000	594	EtOH	
H-7515	546.53	F,D,L	DMSO	463	24,000	600	MeOH	18
H-7516	528.51	F,D,L	DMSO	459	23,000	616	MeOH	18
L-6868	510.50	L	H_2O	455	7,400	505	H_2O	19, 20
L-8455	177.16	D,L	DMF	355	7,500	411	MeOH	20
M-689	485.98	F,DD,L	DMF, DMSO	629	75,000	none	MeCN	21
M-6494	414.32	D,L	H_2O, DMSO	375	8,300	none	MeOH	22, 23
M-7511	392.93	F,D,L,AA	DMSO	235	57,000	none	MeOH	8, 9
M-7513	497.08	F,D,L,AA	DMSO	245	45,000	none	MeOH	8, 9
M-7913	258.32	F,L	DMF, DMSO	352	30,000	401	MeOH	24
M-23800	291.74	FF,D,LL,AA	DMSO	430	8,400	546	MeOH	25
M-24571	569.67	D,L	DMSO, EtOH	555	143,000	578	MeOH	
N-6495	817.65	D,L	H_2O, DMSO	256	64,000	none	MeOH	22
P-800	~240,000	RR,L	see Notes	546	2,410,000	575	pH 7	26
P-801	~240,000	RR,L	see Notes	565	1,960,000	578	pH 7	26
P-1901	276.42	FF,LL,AA	EtOH	303	76,000	416	MeOH	27
P-6879	363.16	F,D,L	DMSO	270	28,000	none	$CHCl_3$	28
R-14000	1057.75	F,D	DMSO	313	9,700	none	MeOH	29
R-14060	434.41	F,D,L,AA	DMSO	239	52,000	none	MeOH	8, 30
T-7539	740.87	F,D,L	MeOH, DMF	524	91,000	550	MeOH	
X-6493	674.53	D	H_2O, DMSO	286	15,000	none	MeOH	31

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

Notes

1. Fluorescence of A-7923 and C-7924 is weak. Reaction of the nitroxide moiety with superoxide or hydroxyl radicals results in increased fluorescence without a spectral shift (Anal Biochem 212, 85 (1993)).
2. Peroxidase-catalyzed reaction of A-12222 with H_2O_2 produces fluorescent resorufin R-363 (Section 10.1).
3. Dihydrofluorescein diacetates are colorless and nonfluorescent until both the acetates are hydrolyzed and the products are subsequently oxidized to fluorescein derivatives. The materials contain less than 0.1% of oxidized derivative when initially prepared. The end products from C-400, C-2938, C-6827, D-399 and D-2935 are 2′,7′-dichlorofluorescein derivatives with spectra similar to C-368 (Section 21.3).
4. C-2944 emits chemiluminescence (Em = 466 nm) on oxidation by superoxide (Anal Biochem 206, 273 (1992)).
5. Do NOT dissolve in DMSO.
6. Aqueous solutions of coelenterazine (>1 mM) can be prepared in pH 7 buffer containing 50 mM 2-hydroxypropyl-β-cyclodextrin (Biosci Biotechnol Biochem 61, 1219 (1997)).
7. Difluorodihydrofluorescein diacetates are colorless and nonfluorescent. Acetate hydrolysis and subsequent oxidation generate a fluorescent difluorofluorescein with spectra similar to O-6146 (Section 1.5).

8. This compound is susceptible to oxidation, especially in solution. Store solutions under argon or nitrogen. Oxidation appears to be catalyzed by illumination.
9. These compounds are essentially colorless and nonfluorescent until oxidized. Oxidation products (in parentheses) are as follows: D-632 and D-23806 (R-302; Section 12.2); D-633 (R-634; Section 12.2); M-7511 (M-7510; Section 12.2); M-7513 (M-7512; Section 12.2).
10. Dihydroethidium has blue fluorescence (Em ~420 nm) until oxidized to ethidium E-1305 (Section 8.1). The reduced dye does not bind to nucleic acids (FEBS Lett 26, 169 (1972)).
11. Oxidation of the polyunsaturated butadienyl portion of the BODIPY 581/591 dye results in a shift of the fluorescence emission peak from ~590 nm to ~510 nm (Methods Enzymol 319, 603 (2000); FEBS Lett 453, 278 (1999)).
12. Oxidation product is strongly fluorescent. Em = 379 nm. Oxidation occurs rapidly in solution when illuminated.
13. This product is supplied as a ready-made solution in DMSO with sodium borohydride added to inhibit oxidation.
14. D-23805 is colorless and nonfluorescent until the AM ester groups are hydrolyzed and the resulting leuco dye is subsequently oxidized. The final product is calcein (C-481, Section 14.3).
15. This product is supplied as a ready-made solution in the solvent indicated under **Soluble**.
16. F-2902 is essentially colorless and nonfluorescent until oxidized. A small amount (~5%) of oxidized material is normal and acceptable for the product as supplied. The oxidation product is fluorescent (Abs = 495 nm, Em = 524 nm) (J Immunol Methods 130, 223 (1990)).
17. This product consists of a dye–bovine serum albumin conjugate (MW ~66,000) complexed with IgG in a ratio of approximately 1:4 mol:mol (BSA:IgG).
18. H-7515 and H-7516 have weaker absorption peaks at longer wavelengths: Abs = 581 nm (EC = 12,000 $cm^{-1}M^{-1}$) for H-7515, Abs = 580 nm (EC = 9000 $cm^{-1}M^{-1}$) for H-7516. Their quantum yields for singlet oxygen production (0.83 for H-7515, 0.76 for H-7516) are essentially independent of the irradiation wavelength between 430 and 580 nm (J Photochem Photobiol A 64, 273 (1992)).
19. L-6868 has much stronger absorption at shorter wavelengths (Abs = 368 nm (EC = 36,000 $cm^{-1}M^{-1}$)).
20. This compound emits chemiluminescence upon oxidation in basic aqueous solutions. Emission peaks are at 425 nm (L-8455) and 470 nm (L-6868).
21. Isothiocyanates are unstable in water and should not be stored in aqueous solution.
22. Enzymatic reduction products are water-insoluble formazans with Abs = 505 nm (M-6494) and 605 nm (N-6495) after solubilization in DMSO or DMF. See literature sources for further information (Histochemistry 76, 381 (1982); Prog Histochem Cytochem 9, 1 (1976)).
23. M-6494 also has Abs = 242 nm (EC = 21,000 $cm^{-1}M^{-1}$) in MeOH.
24. Generates chemiluminescence (Em = 465 nm in 0.1 M SDS) upon reaction with $^1O_2$ (J Am Chem Soc 108, 4498 (1986)).
25. Generates chemiluminescence (Em = 455 nm) upon reaction with superoxide.
26. Phycobiliproteins are packaged as suspensions in 60% ammonium sulfate, pH 7.0. Store refrigerated at 4°C but DO NOT FREEZE.
27. *cis*-Parinaric acid is readily oxidized to nonfluorescent products. Use under N_2 or Ar except when oxidation is intended. Stock solutions should be prepared in deoxygenated solvents. *cis*-Parinaric acid is appreciably fluorescent in lipid environments and organic solvents but is nonfluorescent in water.
28. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.
29. Acetate hydrolysis of R-14000 yields rose bengal (Abs = 556 nm (EC = 104,000 $cm^{-1}M^{-1}$) Em = 572 nm in MeOH) (Photochem Photobiol 66, 374 (1997)).
30. R-14060 is colorless and nonfluorescent until oxidized. The spectral characteristics of the oxidation product (2,3,4,5,6-pentafluorotetramethylrosamine) are similar to those of T-639 (Section 12.2).
31. Enzymatic reduction product is a water-soluble formazan, Abs = 475 nm.

Product List — 19.2 Generating and Detecting Reactive Oxygen Species

Cat #	Product Name	Unit Size
A-7923	4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl, free radical (TEMPO-9-AC)	5 mg
A-22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A-22188	Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit *500 assays*	1 kit
A-12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A-22182	Amplex® Red Xanthine/Xanthine Oxidase Assay Kit *400 assays*	1 kit
B-3932	(*E*,*E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY® 665/676)	5 mg
C-400	5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) *mixed isomers*	25 mg
C-2938	6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)	5 mg
C-13293	5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) *mixed isomers*	5 mg
C-7924	5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one, potassium salt (proxyl fluorescamine)	5 mg
C-6827	5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) *mixed isomers* *special packaging*	20 x 50 µg
C-2944	coelenterazine	250 µg
D-399	2′,7′-dichlorodihydrofluorescein diacetate (2′,7′-dichlorofluorescin diacetate; H_2DCFDA)	100 mg
D-2935	2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2DCFDA, SE)	5 mg
D-3861	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY® 581/591 C_{11})	1 mg
D-23805	dihydrocalcein, AM *special packaging*	20 x 50 µg
D-1168	dihydroethidium (hydroethidine)	25 mg
D-11347	dihydroethidium (hydroethidine) *special packaging*	10 x 1 mg
D-23107	dihydroethidium (hydroethidine) *5 mM stabilized solution in DMSO*	1 mL
D-632	dihydrorhodamine 123	10 mg
D-23806	dihydrorhodamine 123 *5 mM stabilized solution in DMSO*	1 mL
D-633	dihydrorhodamine 6G	25 mg
D-7894	diphenyl-1-pyrenylphosphine (DPPP)	5 mg
F-2902	Fc OxyBURST® Green assay reagent *25 assays* *3 mg/mL*	500 µL
H-7476	hypericin	1 mg
H-7515	hypocrellin A	1 mg
H-7516	hypocrellin B	1 mg
L-6868	lucigenin (bis-*N*-methylacridinium nitrate) *high purity*	10 mg
L-8455	luminol (3-aminophthalhydrazide)	25 g
M-689	malachite green isothiocyanate	10 mg

Product List — 19.2 Generating and Detecting Reactive Oxygen Species — continued

Cat #	Product Name	Unit Size
M-24571	merocyanine 540	25 mg
M-7913	*trans*-1-(2′-methoxyvinyl)pyrene	1 mg
M-23800	2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, hydrochloride (MCLA)	5 mg
M-7511	MitoTracker® Orange CM-H_2TMRos *special packaging*	20 x 50 µg
M-7513	MitoTracker® Red CM-H_2XRos *special packaging*	20 x 50 µg
M-6494	MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	1 g
N-6495	nitro blue tetrazolium chloride (NBT)	1 g
O-13291	OxyBURST® Green H_2HFF BSA *special packaging*	5 x 1 mg
P-1901	*cis*-parinaric acid *special packaging*	10 x 10 mg
P-6879	*N*-(1,10-phenanthrolin-5-yl)iodoacetamide	5 mg
P-800	B-phycoerythrin *4 mg/mL*	0.5 mL
P-801	R-phycoerythrin *4 mg/mL*	0.5 mL
R-14060	RedoxSensor™ Red CC-1 *special packaging*	10 x 50 µg
R-14000	rose bengal diacetate	5 mg
T-7539	2′,4′,5′,7′-tetrabromorhodamine 123 bromide	5 mg
X-6493	XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide)	100 mg

19.3 Probes for Nitric Oxide Research

Nitric oxide (NO), the molecule that makes the firefly glow,[1,2] plays a critical role as a molecular mediator of a variety of physiological processes, including blood-pressure regulation and neurotransmission.[3–8] In endothelial cells, as well as neurons and astrocytes, NO is synthesized from L-arginine in a reaction catalyzed by nitric-oxide synthase (NOS) (Figure 19.13). NO that diffuses into smooth muscle cells binds to the heme group of guanylate cyclase. Because free NO is a transient species with a half-life of about five seconds, many investigations of this gaseous molecule have relied largely on studies of NOS. Preparing NO solutions and detecting NO in experimental systems require special precautions to achieve reproducibility.[9] NO can also complex with superoxide to form the strong oxidant, peroxynitrite anion[10] ($ONOO^-$, Table 19.1), which may be a major cytotoxic agent produced during inflammation, sepsis and ischemia/reperfusion.[11] Activated macrophage and neutrophils produce nitric oxide and superoxide, and thus peroxynitrite anion, at similar rates.[12] NO generators are also reported to produce an accumulation of chelatable Zn^{2+} in hippocampal neuronal perikarya, as determined with some of our Zn^{2+} indicators[13] (Section 20.7, Table 20.6).

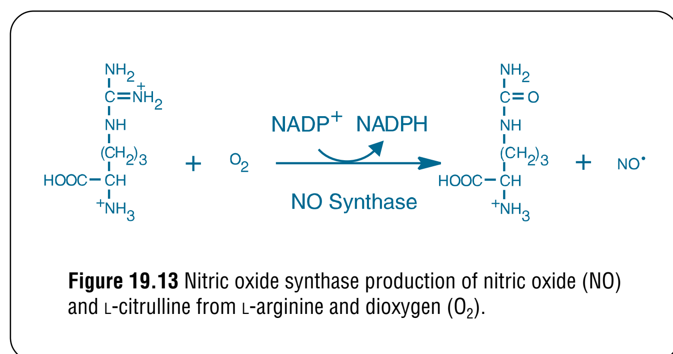

Figure 19.13 Nitric oxide synthase production of nitric oxide (NO) and L-citrulline from L-arginine and dioxygen (O_2).

Spontaneous Nitric Oxide Donors and Antagonist

DEANO and Spermine NONOate

The DEANO (diethylamine nitric oxide, D-7915) and spermine NONOate (S-7916) solids provide a means of preparing aqueous NO solutions.[14] When these solids are dissolved in buffer, cell culture medium or blood, they dissociate to form two molecules of NO and one molecule of the corresponding amine[15] (Figure 19.14). The delivery of NO can be easily controlled by preparing moderately basic solutions of these NONOates and then lowering the pH to initiate NO generation.

DEANO has a half-life of about two minutes in pH 7.4 phosphate buffer at 37°C, releasing two molecules of NO and one molecule of diethylamine.[14–17] DEANO therefore allows more sustained delivery of NO than is possible using NO-saturated aqueous solutions (Figure 19.15). The data in Figure 19.15 are from a study of cytochrome P-450 inhibition by NO in which DEANO was used in conjunction with fluorogenic microsomal dealkylase substrates[18] (R-441, R-352; Section 10.6). DEANO has also been used as a NO donor to stimulate ADP-ribosylation of various proteins.[19]

With a half-life of 39 minutes at 37°C in pH 7.4 buffer, spermine NONOate releases NO much more slowly than does DEANO, making it suitable for whole animal infusions and experiments with long incubations.[15] Also, spermine, the by-product of spermine NONOate dissociation, is less likely to be deleterious in living systems and may also demonstrate biological activity of its own.[20] Both DEANO and spermine NONOate are reported to inhibit *in vitro* proliferation of A375 melanoma cells.[21]

SNAP and SIN-1

Molecular Probes also offers the NO donors SNAP (*S*-nitroso-*N*-acetylpenicillamine, N-7892, N-7927) and SIN-1 (3-morpholinosydnonimine, hydrochloride; M-7891, M-7926), which spontaneously release NO (and superoxide in the case of SIN-1) under