Rhodamine 110–Based Proteinase Substrates

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Bisamide substrate of rhodamine 110, lyophilized powder	See product label *	• ≤–20°C • Desiccate • Protect solutions from light	When stored as directed, product is stable for at least 6 months.

* The molecular weight is indicated on the product label.

Approximate Fluorescence Excitation and Emission, in nm: 498/521

Introduction

Molecular Probes' bisamide derivatives of rhodamine 110 are sensitive and selective substrates for assaying proteinases in solution or inside living cells. Originally developed by Walter F. Mangel and colleagues, these fluorogenic substrates contain an amino acid or peptide covalently linked to each of rhodamine 110's amino groups (Figure 1).[1,2] Upon enzymatic cleavage, the nonfluorescent bisamide substrate is converted first to the fluorescent monoamide and then to rhodamine 110, with a further increase in fluorescence (Figure 2). Both enzymatic products exhibit spectral properties similar to those of fluorescein, with peak excitation and emission wavelengths of 498 nm and 521 nm, respectively. Thus, proteinase assays that employ these substrates are compatible with flow cytometers and other argon laser–based instrumentation. Moreover, unlike fluorescein, the fluorescence intensity of the monoamide and rhodamine 110 is constant from pH 3–9.

The bis-(benzyloxycarbonyl-L-arginine amide) derivative of rhodamine 110 (bis-(CBZ-Arg)-R110, R6501) is a general substrate for serine proteinases. With several enzymes, this substrate has been shown to be 50- to 300-fold more sensitive than the analogous coumarin-based substrate.[2] The increased sensitivity of rhodamine 110–based assays can be attributed both to the greater fluorescence of the enzymatic product and to the enhanced reactivity of the cleavage site. The tripeptide derivative bis-(CBZ-Ile-Pro-Arg)-rhodamine 110 (known as BZiPAR, R6505) has also proven to be an excellent substrate for serine proteinases. Because this substrate allows direct and continuous monitoring of enzyme turnover, it was used by Leytus and co-workers to determine individual kinetic constants of fast-acting, irreversible trypsin inhibitors.[3]

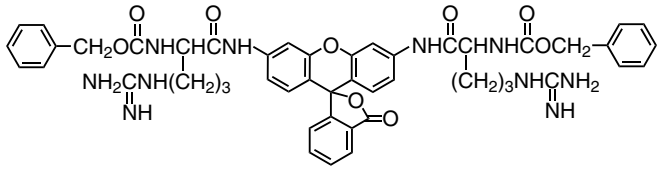

Figure 1. Chemical structure of the bis-(CBZ-L-arginine amide) derivative of rhodamine 110 (R6501).

This strategy has led to the development of several peptide derivatives of rhodamine 110 (Table 2) that can be used to detect specific proteinase activity *in vitro* and *in vivo*. Reyftmann and colleagues have shown that BZiPAR enters intact cells and acts as a substrate for lysosomal proteinases that are released in response to porphyrin photosensitization.[4] The bis-(CBZ-Phe-Arg) derivative of rhodamine 110 (R6502) has been employed for flow cytometric measurement of the cysteine proteinases cathepsin B and L in human monocytes and rat macrophages.[5-7] In similar experiments, the bis-(CBZ-Ala-Ala) derivative of rhodamine 110 (R6504) was used to measure the activity of the lysosomal serine proteinase elastase.[6]

Rhodamine 110 can be derivatized with other peptides to create any number of proteinase-specific substrates. For example, rhodamine 110 has been derivatized with the consensus sequence for a human adenovirus proteinase and then employed to identify cofactors required for virion proteinase activity.[8] Molecular Probes also offers rhodamine 110 derivatives which serve as effective substrates for various caspases.[9] These products are described in more detail in our product information sheet entitled *Rhodamine 110-Based Caspase Substrates*, mp22120.

Table 2 lists the rhodamine 110–based substrates that we currently have available, along with the proteinases for which they were designed. Our Custom and Bulk Sales Department will be pleased to respond to your inquiries about other rhodamine 110–based proteinase substrates to meet your specific research needs.

Our quality analysis includes HPLC and for many substrates, spectrophotometric quantitation of the rhodamine 110 released by complete enzymatic hydrolysis. To ensure consistency between different lots of these products, we have designed our packaging protocol so that each vial contains the specified amount of anhydrous bis-amide–rhodamine 110. In addition, the product may also contain a variable amount of water of hydration and a small amount of inorganic salts that do not affect its use. For several substrates, the lot-specific weight purity is indicated on the product label. For these products, the total amount of material in the container is:

$$\text{Total material (mg)} = \left[\frac{(\text{substrate (mg)} \times 100)}{\text{weight purity (\%)}} \right]$$

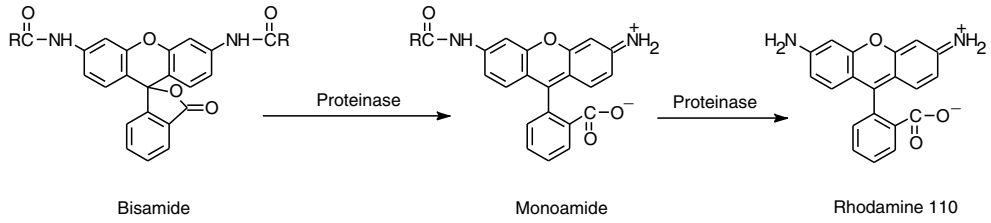

Figure 2. Upon proteinase cleavage, the nonfluorescent bisamide derivative of rhodamine 110 is converted first to the fluorescent monoamide and then to rhodamine 110, with a further increase in fluorescence.

Table 2. Rhodamine 110–based bis-peptide substrates.

Cat #	Proteinase Substrates *	Enzymes
R6504	$(CBZ\text{-}Ala\text{-}Ala)_2$-R110	elastase [2]
R6506	$(CBZ\text{-}Ala\text{-}Ala\text{-}Ala\text{-}Ala)_2$-R110	elastase [3]
R6508	$(CBZ\text{-}Ala\text{-}Arg)_2$-R110	trypsin [4]
R6501	(CBZ-Arg)2-R110	trypsin [4,5]
R6505	$(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-R110	trypsin [6,7]
R6502	$(CBZ\text{-}Phe\text{-}Arg)_2$-R110	plasmin, cathepsin L [1,8,9]
R22124	(p-tosyl-Gly-Pro-$Arg)_2$-R110	thrombin[12]

* SC = succinoyl; CBZ = benzyloxycarbonyl; *p*-tosyl = toulenesulfonyl; R110 = rhodamine 110; **1.** Biochemistry 38, 13906 (1999); **2.** Biol Chem Hoppe-Seyler 373, 547 (1992); **3.** Anal Chem 65, 2352 (1993); **4.** Biochem J 215, 253 (1983); **5.** Biochem J 209, 299 (1983); **6.** Biochim Biophys Acta 788, 74 (1984); **7.** Photochem Photobiol 44, 461 (1986); **8.** Glia 7, 183 (1993); **9.** Biol Chem Hoppe-Seyler 373, 433 (1992); **10.** J Biol Chem 274, 17484 (1999); **11.** Exp Cell Res 250, 203 (1999); **12.** Biomed Instrum Technol 30, 245 (1996).

Guidelines for Use

Materials Required but Not Provided

- DMSO or DMF
- Buffer such as Tris or HEPES, pH 7.5
- EDTA
- Ethanol or detergents for cell lysis

Preparing the Rhodamine 110 Stock Solutions

A 5–10 mM stock solution may be prepared in high-quality, anhydrous dimethylsulfoxide (DMSO) or dimethylformamide (DMF). This solution can be stored desiccated at 2–6°C or below for at least six months.

In Vitro Assay of Proteinase Activity

In vitro proteinase assays with the bisamide derivatives of rhodamine 110 are typically performed at 22°C in 10 mM Tris or HEPES buffer, pH 7.5; 15% (v/v) ethanol [1,2] or detergents used to promote cell lysis are sometimes present in the assay buffer. The substrate stock solution in DMSO or DMF is diluted into the assay buffer just prior to initiating the reaction by addition of the enzyme preparation or cell lysate. If reaction conditions are chosen such that less than 15% of the substrate is hydrolyzed, then the increase in fluorescence is due solely to the production of the monoamide derivative of rhodamine 110.[2] Thus, under these conditions, the interpretation of kinetic data is not complicated by the bifunctionality of the bisamide substrate.

Intracellular Proteinase Assays

In preparation for intracellular proteinase assays with the rhodamine 110–based substrates, cells should be sedimented, resuspended in HEPES-buffered saline (HBS; 5 mM HEPES, 0.15 M NaCl, pH 7.35) containing 2 mM EDTA (HBS-EDTA) to a density of approximately $0.5–1 \times 10^7$ cells/mL and stored at 4°C for no longer than two hours.[5,6] Just prior to adding substrate, the cell suspension is diluted 100-fold in HBS-EDTA and incubated for approximately 20 minutes in the presence of 10 µM substrate. If desired, dead cells can then be identified by briefly counterstaining the sample with propidium iodide or other cell-impermeant nucleic acid dye before analysis by flow cytometry or fluorescence microscopy.

Spectral Properties The peak excitation and emission wavelengths of rhodamine 110 are 498 nm and 521 nm, respectively. Rhodamine 110 is reported to have an extinction coefficient of 81,000 $cm^{-1}M^{-1}$ at 498 nm, with a quantum yield of 0.91.[2] The monoamide, CBZ-(L-arginine amide)-rhodamine 110, has an extinction coefficient of about 23,500 $cm^{-1}M^{-1}$ at 492 nm, with a quantum yield of 0.29.[2]

References

1. Biochem J 215, 253 (1983); **2.** Biochem J 209, 299 (1983); **3.** Biochim Biophys Acta 788, 74 (1984); **4.** Photochem Photobiol 44, 461 (1986); **5.** Glia 7, 183 (1993); **6.** Biol Chem Hoppe-Seyler 373, 547 (1992); **7.** Biol Chem Hoppe-Seyler 373, 433 (1992); **8.** Nature 361, 274 (1993); **9.** Biochemistry 38, 13906 (1999).

Product List **Current prices may be obtained from our website or from our Customer Service Department.**

Cat #	Product Name	Unit Size
R22124	rhodamine 110, bis-(p-tosyl-L-glycyl-L-prolyl-L-arginine amide)	2 mg
R6479	rhodamine 110 (R110) *reference standard*	25 mg
R6501	rhodamine 110, bis-(CBZ-L-arginine amide), dihydrochloride (BZAR)	5 mg
R6502	rhodamine 110, bis-(CBZ-L-phenylalanyl-L-arginine amide), dihydrochloride	5 mg
R6504	rhodamine 110, bis-(CBZ-L-alanyl-L-alanine amide)	5 mg
R6505	rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride (BZiPAR)	5 mg
R6506	rhodamine 110, bis-(CBZ-L-alanyl-L-alanyl-L-alanyl-L-alanine amide)	5 mg
R6507	rhodamine 110, bis-(CBZ-L-prolyl-L-arginine amide), dihydrochloride	5 mg
R6508	rhodamine 110, bis-(CBZ-L-alanyl-L-arginine amide), dihydrochloride	5 mg
R6513	rhodamine 110, bis-(t-BOC-L-leucyl-L-methionine amide)	5 mg
R6560	rhodamine 110, 4-(chloromethyl)benzoyl amide, CBZ-L-arginine amide, hydrochloride (CMB-R110, CBZ-Arg)	1 mg
R6577	rhodamine 110, bis-(L-phenylalanine amide), di(trifluoroacetic acid) salt	5 mg

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