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Selective Recognition of Protein Tetraserine Motifs with a Cell-Permeable, Pro-fluorescent Bis-boronic Acid

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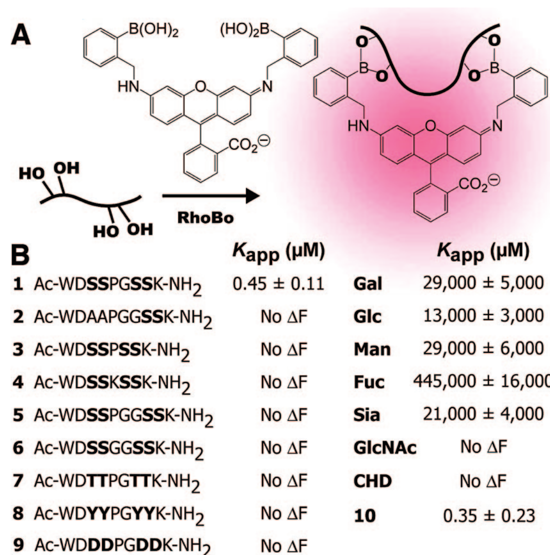
There is considerable interest in novel biomolecule imaging tools that avoid the use of fluorescent proteins.[1−3] One widely used class of such reagents are "pro-fluorescent" biarsenical dyes such as FlAsH,[4] ReAsH,[5] CrAsH,[6] and Cy3As.[7] These cell-permeable molecules selectively label recombinant proteins containing a linear[4] or split[8] tetracysteine motif via thiol−arsenic exchange reactions that convert the nonfluorescent 1,2-ethanedithiol (EDT)-bound forms of these dyes into highly fluorescent protein-bound complexes. Despite their utility, however, biarsenicals are plagued by high background signals and cytotoxicity[9,10] and can be challenging to apply in oxidizing cellular locales.[11,12] Nontoxic, redox-insensitive alternatives that combine the convenience and selectivity of a biarsenical with the brightness of a fluorescent protein would be a valuable addition to the cell biologist's "fluorescent toolbox".[2] Here we report that [(3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-3′,6′-diyl)bis(iminomethylene-2,1-phenylene)]bis-(9CI) (**RhoBo**, Figure 1A), a rhodamine-derived bisboronic acid described initially as a monosaccharide sensor,[13] can function as a cell-permeable, turn-on fluorescent sensor for tetraserine motifs in engineered proteins.

It has been known since 1953 that phenyl boronic acid condenses with polyols to form boronate esters[14,15] and since 1994 that the fluorescence of certain mono- and bis-boronic acid dyes increases upon esterification with simple sugars.[16,17] The equilibrium stabilities of these complexes are low, however, with K_d values in the mM concentration range. We hypothesized that bis-boronic acid dyes would form higher affinity complexes with proteins containing a linear tetraserine motif. **RhoBo** was chosen as the ideal molecule with which to investigate this hypothesis, as it benefits from a simple synthesis and low monosaccharide affinity and forms boronate esters that emit at wavelengths > 500 nm,[13] a useful range for experiments in live cells.

First, we asked whether **RhoBo** would form fluorescent complexes with peptides containing 2−4 serine residues separated by a variety of intervening sequences (Figures 1 and 2). Each peptide was incubated with **RhoBo** (17.1 μM) in buffer at 37 °C, and the fluorescence emission at 580 nm was monitored as a function of peptide concentration. Under these conditions, peptide **1**, containing the sequence Ser-Ser-Pro-Gly-Ser-Ser, formed the highest affinity complex with **RhoBo** (K_{app} = 452 ± 106 nM). Titrations with peptides containing two serines rather than four (**2**) or shorter (**3**, **4**) or longer (**5**) intervening sequences, or aspartate residues in place of serine (**9**), led to no detectable fluorescence change. Minimal fluorescence changes were observed with peptides containing threonine (**7**) or tyrosine (**8**) in place of serine. No fluorescence change was observed when **RhoBo** was incubated



	Peptide	K_{app} (μM)		K_{app} (μM)
1	Ac-WD**SS**PG**SS**K-NH$_2$	0.45 ± 0.11	Gal	29,000 ± 5,000
2	Ac-WDAAPGG**SS**K-NH$_2$	No ΔF	Glc	13,000 ± 3,000
3	Ac-WD**SS**P**SS**K-NH$_2$	No ΔF	Man	29,000 ± 6,000
4	Ac-WD**SS**K**SS**K-NH$_2$	No ΔF	Fuc	445,000 ± 16,000
5	Ac-WD**SS**PGG**SS**K-NH$_2$	No ΔF	Sia	21,000 ± 4,000
6	Ac-WD**SS**GG**SS**K-NH$_2$	No ΔF	GlcNAc	No ΔF
7	Ac-WD**TT**PG**TT**K-NH$_2$	No ΔF	CHD	No ΔF
8	Ac-WD**YY**PG**YY**K-NH$_2$	No ΔF	10	0.35 ± 0.23
9	Ac-WD**DD**PG**DD**K-NH$_2$	No ΔF		

Figure 1. (A) Scheme illustrating a likely mode of condensation between **RhoBo** and a compound containing four hydroxyl groups. (B) Apparent equilibrium dissociation constant (K_{app}) of complexes between **RhoBo** and the peptides and monosaccharides shown.

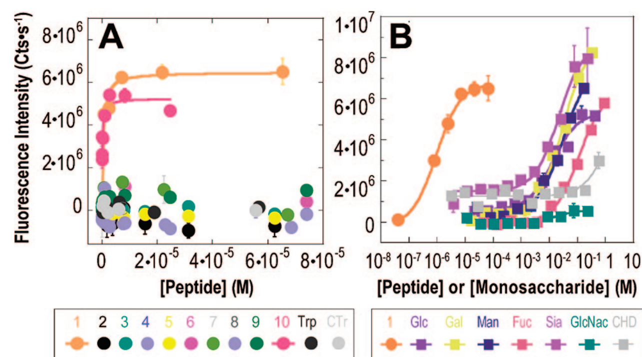


Figure 2. Changes in the fluorescence emission of **RhoBo** (17.1 μM) in the presence of (A) peptides **1**−**10** and (B) indicated monosaccharides. Reactions were incubated in 100 mM phosphate buffer (pH 7.4) supplemented with 10% DMSO (37 °C, 60 min) and the emission monitored at 580 nm. Each point represents the average of three or more independent trials ± the standard error.

with the serine proteases trypsin or chymotrypsin. Notably, the equilibrium stability and brightness of the RhoBo•**1** complex (3955.5 M^{-1} cm^{-1}) compares favorably with the complex formed between ReAsH-EDT$_2$ and the optimized tetracysteine sequence FLNCCPGC-CMEP.[18] **RhoBo** also formed a high affinity complex with a small well-folded protein, a derivative of the 36-aa pancreatic fold polypep-

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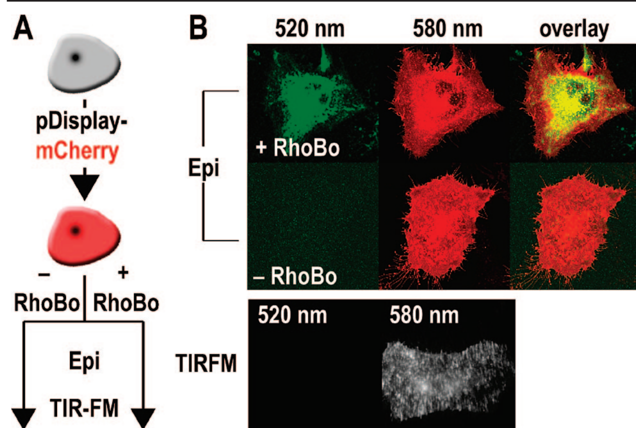

Figure 3. (A) Experimental strategy to evaluate the extent of cell surface labeling by **RhoBo**. HeLa cells were transfected with pDisplay-mCherry (emission maximum 580 nm), incubated in the presence or absence of RhoBo (1 μM) (emission maximum 520 nm), and (B) imaged using epifluorescent and TIRF microscopy.

tide aPP containing an N-terminal SSPGSS tag (**10**) (K_d = 347 ± 234 nM (Figure 2A)).

Next we examined the affinity of **RhoBo** for simple monosaccharides, including those prevalent in mammalian oligosaccharide frameworks,[19] to evaluate the extent to which these hydroxyl-rich functionalities might compete with protein tetraserine motifs. Although significant fluorescent changes were observed upon incubation of **RhoBo** with galactose (Gal), glucose (Glc), fucose (Fuc), mannose (Man), and sialic acid (Sia), these changes occurred at concentrations at least 10 000 times higher than required for peptide **1** (Figure 2B). Almost no fluorescence change was observed with *N*-acetylglucosamine (GlcNac) or with *cis*-1,2-cyclohexane diol (CHD). We also examined whether **RhoBo** would form fluorescent complexes with components of a 377-member mammalian glycan microarray (v.3.1; http://www.functionalglycomics.org/). None of the glycans on this array exhibited higher fluorescence than galactose when incubated with **RhoBo** (84 μM) (Table S-3).

To determine whether the **RhoBo•1** complex possessed the expected 1:1 stoichiometry, we performed a preparative-scale reaction at a concentration of each reactant that significantly exceeded K_{app} (140 μM), separated the products by HPLC, and determined their masses using MALDI-TOF mass spectrometry. Under these conditions, an equimolar mixture of **1** and **RhoBo** was converted into a single major product (71% yield) that possessed the expected 1:1 complex mass (expected m/z = 1696.18, found m/z = 1695.2; Table S-1). An isomeric product possessing the identical mass (16% yield) was also formed. These isomers possess identical quantum yields (φ) (0.89 ± 0.01) whose value compares well with the quantum yield of fluorescein (0.95)[20] and rhodamine 110 (0.91)[21] and exceeds that of peptide-bound FlAsH (0.5)[5] and **RhoBo** alone (0.3 ± 0.03).

Finally we evaluated whether **RhoBo** could circumnavigate the complex, saccharide-rich cell surface to image tetraserine-containing proteins in the cytosol. In contrast to the tetracysteine motif recognized by biarsenicals, which is absent from the human proteome,[22] more than 100 human proteins, including the highly abundant myosin heavy chain, contain an SSPGSS sequence. HeLa cells were first transfected with pDisplay-mCherry, to fluorescently label the plasma membrane, and then treated for 30 min with either 0 or 1 μM **RhoBo** before imaging using epifluorescence and total internal reflection fluorescence microscopy (TIRFM) (Figure 3A). The epifluorescence images show a significant signal at the outer plasma membrane at the mCherry emission maximum (580 nm), irrespective of RhoBo treatment. Only those cells treated with **RhoBo**, however, show a significant signal when **RhoBo** was excited specifically using 514 nm light (emission was monitored at 520 nm). In this case fluorescence is observed throughout the cell interior with maximal intensity in perinuclear regions and minimal intensity in the nucleus and outer plasma membrane (Figure 3B).[23] **RhoBo**-treated cells were then imaged using TIRFM, which revealed a significant signal at the mCherry emission maximum (580 nm) but not at the RhoBo emission maximum (520 nm). These images confirm the absence of even low levels of **RhoBo** emission within the outer 200 nm of the plasma membrane. It is of course possible that **RhoBo** is associated with constituents of the plasma membrane, but if so, then the complexes formed are not bright. The surprising observation that **RhoBo** does not fluoresce on the cell surface, coupled with its low toxicity (Figure S5), suggests that current efforts to identify more highly preferred tetraserine motifs will reveal **RhoBo** as a selective small-molecule tag for proteins on and within living cells.

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Supporting Information Available: Synthesis and spectroscopic characterization of RhoBO and RhOBO•1 and protocols for cell culture and fluorescence microscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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(23) We emphasize that the proteins or biomolecules labeled by **RhoBo** within the cell interior have not been identified.

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