Azide-specific labeling of biomolecules by Staudinger-Bertozzi ligation phosphine derivatives of fluorescent probes suitable for single-molecule fluorescence spectroscopy.

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AZIDE-SPECIFIC LABELLING OF BIOMOLECULES BY STAUDINGER-BERTOZZI LIGATION: PHOSPHINE DERIVATIVES OF FLUORESCENT PROBES SUITABLE FOR SINGLE-MOLECULE FLUORESCENCE SPECTROSCOPY

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Abstract

We describe the synthesis of phosphine derivatives of three fluorescent probes that have brightness and photostability suitable for single-molecule fluorescence spectroscopy and microscopy: Alexa488, Cy3B, and Alexa647. In addition, we describe procedures for use of these reagents in azide-specific, bioorthogonal labelling through use of the Staudinger-Bertozzi ligation and procedures for quantitation of labelling specificity and labelling efficiency. The reagents and procedures of this report enable chemoselective, site-selective labelling of azide-containing biomolecules for single-molecule fluorescence spectroscopy and microscopy.

1. INTRODUCTION

The Staudinger-Bertozzi ligation involves reaction between a first compound containing an azide moiety and a second compound containing a phosphine moiety and an adjacent methyl ester, and results in coupling of the compounds through an amide linkage (Saxon *et al*., 2000; Kiick *et al*., 2002; reviewed in Kohn *et al*., 2004; Sletten and Bertozzi, 2009). The reaction is bioorthogonal, since azides and phosphines are not present in natural biomolecules and since azides and phosphines do not react with moieties present in natural biomolecules. The reaction is biocompatible, since it proceeds in aqueous solution under mild conditions at moderate temperatures and moderate pH ranges. The reaction is efficient; yields of ≥90% routinely are achieved. The bioorthogonality, biocompatibility, and high efficiency of the Staudinger-Bertozzi ligation reaction render the reaction suitable for two applications: (i) biomolecule-specific labelling of engineered biomolecules containing azide moieties, and (ii) biomolecule-speifc, site-specific labelling of engineered biomolecules containing site-specifically incorporated azide moities. In published work, the reaction has been used for labelling of engineered azide-containing biomolecules *in vitro* with single proteins, *in vitro* with mixtures of proteins, *in vivo* in living cells, and *in vivo* in living organisms (Saxon *et al*., 2000; Kiick *et al*., 2002; Prescher *et al*., 2004).

Multiple strategies have been reported for incorporation of azides into biomolecules, providing potential targets for azide-specific, bioorthogonal labelling through use of the Staudinger-Betozzi ligation. For example, azides have been incorporated into carbohydrates and protein-

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linked carbohydrates by supplying cells with azide-functionalized carbohydrate precursors (Saxon *et al*., 2000; Saxon *et al*., 2002; Vocadlo *et al*., 2003; Hang *et al*., 2003; Prescher *et al*., 2004; Dube *et al*., 2006; Laughlin *et al*., 2006; Chang *et al*., 2007; Hangauer and Bertozzi, 2008); azides have been incorporated into proteins by supplying cells or organisms with azide-functionalized methionine (Kiick *et al*., 2002; Link *et al*., 2003; Link *et al*., 2004; Ngo *et al*., 2009); azides have been site-specifically incorporated into proteins *in vitro* by ligation with azide-functionalized farnesyl, lipoyl, or puromycin surrogates (Gauchet *et al*., 2006; Humenik *et al*., 2007; Baruah *et al*., 2008); and azides have been site-specifically incorporated into proteins *in vitro* and *in vivo* by use of unnatural-amino-acid mutagenesis (Krieg *et al*., 1986; Chin *et al*., 2002; Deiters *et al*., 2003; Tsao *et al*., 2005; Ohno *et al*., 2006; Nguyen *et al*., 2009).

Multiple phosphine derivatives suitable for azide-specific, bioorthogonal labelling through use of the Staudinger-Betozzi ligation have been reported, including phosphine derivatives of the affinity probe biotin and phosphine derivatives of the fluorescent probes fluorescein, coumarin, tetraethylrhodamine, and Cy5.5 (Saxon *et al*., 2000; Wang *et al*., 2003; Lemieux *et al*., 2003; Chang *et al*., 2007; Hangauer and Bertozzi, 2008).

Single-molecule fluorescence spectroscopy requires fluorescent probes that have exceptionally high brightness and exceptionally high photostability (fluorescent probes of greater brightness and photostability than fluorescein, coumarin, and tetraethylrhodamine (reviewed in Ha 2001; Kapanidis and Weiss, 2003; Roy *et al*., 2008). Single-molecule fluorescence resonance energy transfer (FRET) experiments further require pairs of fluorescent probes able to serve as efficient donor/acceptor, wherein the fluorescence emission spectrum of the donor overlaps the fluorescence excitation spectrum of the acceptor. In FRET experiments, the lengths and flexibilities of the linkers between biomolecule and fluorescent probes can significantly affect results; therefore, maximum flexibility in experimental design requires sets of reagents that yield different lengths and flexibilities of linkers between biomolecules and fluorescent probes.

Here we report the synthesis of phosphine derivatives of fluorescent probes that have brightness and photostability suitable for single-molecule fluorescence spectroscopy (Alexa488, Cy3B, and Alexa647; Panchuk-Voloshina *et al*., 1999; Cooper *et al*., 2004; Leung *et al*., 2005), that have spectral overlap suitable to serve as donor/acceptor pairs for FRET (Alexa488/Cy3B, Alexa488/Alexa647, and Cy3B/Alexa647), and that, in one case, yield alternatively a moderate-length, flexible biomolecule-probe linker or a longer, more flexible, biomolecule-probe linker (20 Å and 9 rotatable bonds vs. 24 Å and 12 rotatable bonds) (Figures. 1–3). In addition, we report procedures for application of these reagents in azide-specific, bioorthogonal labelling through use of the Staudinger-Bertozzi ligation and procedures for quantitation of labelling specificity and labelling efficiency. The reagents and procedures of this report enable chemoselective, site-selective labelling of azide-containing biomolecules for single-molecule spectroscopy.

2. MATERIALS AND METHODS

2.1. Materials

1-Methyl-diphenylphosphinoterephthalate (MDPT) was synthesized as in Kiick *et al*., 2002. Alexa Fluor 488 cadaverine, Alexa Fluor 647 cadaverine, Alexa Fluor 647 NHS ester, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), and N-hydroxysulfosuccinimide (NHSS) were purchased from Invitrogen (Carlsbad CA). Cy3B-NHS was purchased from GE Healthcare (Piscataway NJ). Mono-trityl-ethelenediamine (acetic acid salt) was purchased from Novabiochem (Madison WI). N-trityl-1,2-ethanediamine (hydrobromide salt), N,N′-trifluoroacetic acid (TFA), triethylamine (TEA), disopropylethylamine (DIPEA), and N,N′-

dimethylformamide (DMF) were purchased from Sigma-Aldrich (Milwaukee WI). Bio-Gel P30 was purchased from BioRad (Hercules, CA).

2.1. General methods

Reversed-phase HPLC was performed on a Hitachi L7100 instrument using Supelco Discover Bio C18 column (25cm X 10mm, 10um). All solutions used for HPLC were degassed. Flash chromatography was performed using silica gel (230–400 mesh, 60Å). MALDI-MS was performed on an Applied Biosystems MDS SCIEX 4800 instrument.

2.3. Synthesis of Alexa488-phosphine (Figure 1)

2.3.1. Alexa488-carboyl-pentylenediaminyl-phosphine (Alexa488-phosphine; II)—EDAC (4.2 mg; 21 μmol) in 50 μl degassed water and NHSS (4.2 mg; 16 μmol) in 50 μl degassed water were mixed, and MDPT (5.9 mg;; 15 μmol) in 50 μl DMF, was added. A precipitate was observed. Degassed water (50 μl) was added, followed by DMF (~200 μl), resulting in dissolution of the precipitate. **I** (Alexa Fluor 488 cadaverine; 1.0 mg; 1.5 μmol) in 50 μl DMF was added, followed by DIPEA (5.6 μl; 31 μmol), and the reaction mixture was incubated 3 h at 37°C. The product was purified by reversed-phase HPLC (solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile; gradient: 30 to 100% B in 30 min at 2 ml/min) and lyophilized. MS (MALDI): calculated, *m/z* 964.9 (MH^+); found 964.9.

2.4. Synthesis of Cy3B-phosphine (Figure 2)

2.4.1. Cy3B-carboyl-ethylenediaminyl-trityl (IV)—Mono-trityl-ethelenediamine (acetic acid salt; 23.5 mg; 65 μmol), **III** (Cy3B-NHS; 5.0 mg; 6.5 μmol), and TEA (60 μl; 430 umol) were added, in turn, to 200 μl anhydrous DMF, and the reaction mixture was incubated 1 h at room temperature. The product was purified by reversed-phase HPLC (solvent A: water; solvent B: 90% acetonitrile, 10% water; gradient 30 to 80% B in 30 min at 2 ml/min) and lyophilized. MS (MALDI): calculated, *m/z* 845.6 (MH^+); found, 845.6.

2.4.2 Cy3B-carboyl-ethylenediamine (V)—TFA (50 μl; 0.65 mmol) was added to **IV** (4.2 mg; 5.0 μmol) in 200 μl choloroform, and the reaction mixture was incubated at 1 h at room temperature. The product was purified by reversed-phase HPLC (solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile; gradient: 20 to 80% B in 30 min at 2 ml/min) and lyophilized. MS (MALDI): calculated, *m/z* 603.3 (MH^+); found, 603.3.

2.4.3. Cy3B-carboyl-ethylenediaminyl-phosphine (Cy3B-phosphine; VI)—EDAC (12.5 mg; 65 umol) in 50 μl DMF, NHSS (8.8 mg; 65 μmol) in 50 μl DMF, and MDPT (24 mg; 60 μmol) in 50 μl DMF were combined. **V** (2.4 mg; 4.0 μmol) in 50 μl DMF was added, followed by DIPEA (23 μl; 130 μmol), and the reaction mixture was incubated 3 h at 37°C. The product was purified by reversed-phase HPLC (solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile; gradient: 30 to 100% B in 30 min at 2 ml/min) and lyophilized. MS (MALDI): calculated, *m/z* 948.5 (MH^+); found, 948.5.

2.5. Synthesis of Alexa647-phosphine$^{20\ Å}$ (Figure 3A)

2.5.1. Alexa647-pentanoyl-ethylenediaminyl-trityl (VIII)—N-trityl-1,2-ethanediamine (hydrobromide salt) (23 mg; 60 μmol) was added, to **VII** (Alexa Fluor 647 NHS ester; 5.0 mg; 5.0 μmol) in 1 ml DMF. TEA (10.0 μl; 71 μmol) was added and the reaction mixture was incubated 30 min at room temperature. The reaction mixture was dried under vacuum, re-dissolved in 0.5 ml ethanol and 20 μl ammonium hydroxide. The product was isolated by flash chromatography and dried under vacuum.

2.5.2. Alexa647-pentanoyl-ethylenediamine (IX)—TFA (100 μl; 1.3 mmol) was added to **VIII** (5.0 mg; 4.4 μmol) in 200 μl choloroform, and the reaction mixture was incubated at 30 min at room temperature. The reaction mixture was dried under vacuum, and the product was purified using flash chromatography. MS (MALDI): calculated. *m/z* 901 (MH^+); found, 901.

2.5.3. Alexa647-pentanoyl-ethylenediaminyl-phosphine (Alexa647-phosphine$^{20\ Å}$; X)—EDAC (21 mg; 110 μmol) in 250 μl degassed water, NHSS (21 mg; 78 μmol) in 250 μl degassed water, **IX (**5.0 mg; 5.5 μmol) in 200 μl DMF and 50 μl degassed water, and MDPT (30 mg; 75 μmol) in 250 μl DMF were combined. A precipitate was observed. DMF (~700 μl) was added, resulting in dissolution of the precipitate. DIPEA (28 μl; 160 μmol) was added, and the mixture was incubated 3 h at 37°C. The product was purified by reversed-phase HPLC (solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile; gradient: 30 to 100% B in 30 min at 2 ml/min) and was dried under vacuum. MS (MALDI): calculated, *m/z* 1248.4 (MH^+); found, 1248.4.

2.6. Synthesis of Alexa647-phosphine$^{24\ Å}$ (Figure 3B)

2.6.1. Alexa647-pentanoyl-pentylenediaminyl-phosphine (Alexa647-phosphine$^{24\ Å}$; XII)—EDAC (21 mg; 110 μmol) in 250 μl degassed water, NHSS (21 mg; 78 μmol) in 250 μl degassed water, **XI** (Alexa Fluor 647 cadeverine; 5.0 mg; 5.5 μmol) in 200 μl DMF and 50 μl degassed water, and MDPT (30 mg; 75 μmol) in 250 μl DMF were combined. A precipitate was observed. DMF (~700 μl) was added, resulting in dissolution of the precipitate. DIPEA (28 μl; 160 μmol) was added, and the mixture was incubated 3 h at 37°C. The product was purified by reversed-phase HPLC (solvent A: 0.1% TFA in water; solvent B: 100% acetonitrile; gradient: 30 to 100% B in 30 min at 2 ml/min) and was dried under vacuum. MS (MALDI): calculated, *m/z* 1290.5 (MH^+); found, 1290.5.

2.7. Azide-specific labelling

Reaction mixtures (3 ml) contained 20 μM *P*-azide (derivative of protein *P* containing a single azide moiety) and 200 μM probe-phosphine (Alexa488-phosphine, Cy3B-phosphine, Alexa647-phosphine$^{20\ Å}$, or Alexa647-phosphine$^{24\ Å}$) in 50 mM Tris-HCl, pH 7.9, 6M guanidine-HCl, and 5% glycerol. Reaction mixtures were incubated 15 h at 37°C. Reaction mixtures then were applied to 10 ml columns of Bio-Gel P30 pre-equilibrated in 50 mM Tris-HCl, pH 7.9, 6 M guanidine-HCl, and 5% glycerol; columns were washed with 3 ml of the same buffer; and products were eluted in 3 ml of the same buffer.

2.8. Quantitation of labelling efficiency

The concentration of the product of the labelling reaction and the efficiency of labelling reaction are determined from UV/Vis-absorbance measurements and are calculated as:

$$\text{concentration of product} = [A_{280} - \epsilon_{F,280}(A_{max}/\epsilon_{F,max})]/\epsilon_{P,280}$$

$$\text{labelling efficiency} = 100\%[(A_{max}/\epsilon_{F,max})/(\text{concentration of product})]$$

where A_{280} is the measured absorbance at 280 nm, A_{max} is the measured absorbance at the long-wavelength absorbance maximum of fluorescent probe *F* (493 nm, 559 nm, and 652 nm for Alexa488, Cy3B, and Alexa647, respectively), $\epsilon_{P,280}$ is the molar extinction coefficient of protein *P* at 280 nm (calculated as in Gill and von Hippel, 1989), $\epsilon_{F,280}$ is the molar extinction coefficient of fluorescent probe *F* at 280 nm (8,030 M^{-1} cm^{-1}, 10,400 M^{-1} cm^{-1}, and 7,350 M^{-1} cm^{-1}, for Alexa488, Cy3B, and Alexa647, respectively), and $\epsilon_{F,max}$ is the extinction coefficient of fluorescent probe *F* at its long-wavelength absorbance maximum

($73,000\ M^{-1}\ cm^{-1}$ at 493 nm, $130,000\ M^{-1}\ cm^{-1}$ at 559 nm, and $245,000\ M^{-1}\ cm^{-1}$ at 652 nm for Alexa488, Cy3B, and Alexa647, respectively). Typical labelling efficiencies are ≥90%.

2.9. Quantitation of labelling specificity

The specificity of labelling is determined from the efficiencies of labelling (see preceding section) of (i) the product of the labelling reaction with *P*-azide and (ii) the product of a parallel labelling reaction with *P*. The specificity of labelling is calculated as:

$$\text{labelling specificity} = 100\%[1-[(\text{labelling efficiency with } P)-(\text{labelling efficiency with } P\text{-azide})]]$$

Alternatively, the specificity of labelling can be determined from fluorescence intensities at the emission maximum of fluorescent probe *F* (516 nm upon excitation at 493 nm, 570 upon excitation at 559 nm, or 672 nm upon excitation at 652 nm for Alexa488, Cy3B, and Alexa647, respectively) of (i) the product of the labelling reaction with *P*-azide and (ii) an equal concentration of the product of a parallel labelling reaction with *P*. In this case, the specificity of labelling is calculated as:

$$\text{labelling specificity} = 100\%[1-[(\text{fluorescence with } P)-(\text{fluorescence with } P\text{-azide})]]$$

Typical labelling specificities are ≥90%.

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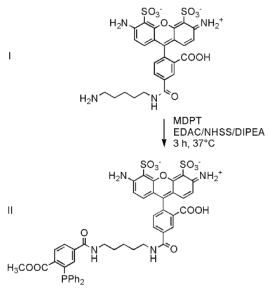

Figure 1. Synthesis of Alexa488-phosphine

Staudinger-Bertozzi ligation between **II** and an azide-containing biomolecule yields an ~18 Å linker between the biomolecule and the fluorophore (distance measured from first nitrogen atom of azide to fused ring system of fluorophore with fully extended conformation of linker).

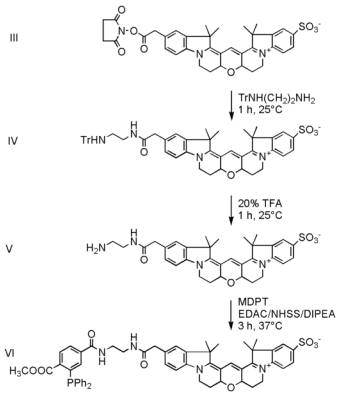

Figure 2. Synthesis of Cy3B-phosphine

Staudinger-Bertozzi ligation between **VI** and an azide-containing biomolecule yields an ~15 Å linker between the biomolecule and the fluorophore.

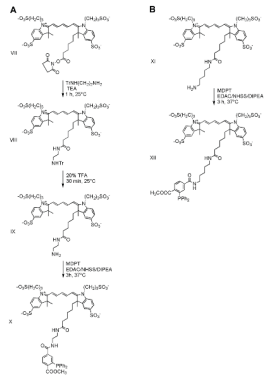

Figure 3. Synthesis of Alexa647-phosphine$^{20\ Å}$ (A) and Alexa647-phosphine$^{24\ Å}$ (B)
Staudinger-Bertozzi ligation between **IX** or **XII** and an azide-containing biomolecule yields, respectively, an ~20 Å or ~24 Å linker between the biomolecule and the fluorophore.