In Situ Quenching of Trialkylphosphine Reducing Agents Using Water-Soluble PEG-Azides Improves Maleimide Conjugation to Proteins

Terrence Kantner, Bayan Alkhawaja, and Andrew G. Watts*

Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

ABSTRACT: Trialkylphosphines tris(2-carboxy-ethyl)-phosphine and tris(3-hydroxypropyl)-phosphine are popular reagents for the reduction of cysteine residues in bioconjugation reactions using maleimides. However, it has been demonstrated that these phosphines are reactive toward maleimide, necessitating their removal before the addition of the Michael acceptor. Here, a method using water-soluble PEG-azides is reported for the quenching of trialkylphosphines in situ, which is demonstrated to improve the level of maleimide conjugation to proteins.

INTRODUCTION

The water-soluble trialkylphosphines, tris(2-carboxy-ethyl)-phosphine (TCEP) and tris(3-hydroxypropyl)-phosphine (THPP), are effective reagents for the reduction of disulfides before performing protein-conjugation reactions using Michael acceptors such as maleimides (Figure 1a).1−5 Reactions such as these are important within the pharmaceutical industry for the manufacture of several types of products including antibody−drug conjugates, PEGylated proteins, and conjugate vaccines. Preference for the use of TCEP and THPP over traditional thiol-based reducing agents can be attributed to a number of practical advantages. TCEP and THPP are relatively stable toward aerial oxidation at pH values common for protein conjugations, as well as being nonvolatile and relatively odorless.6,7 Importantly, reduction of cysteinyl residues by these phosphines does not result in the formation of mixed disulfides, as is the case with traditional thiols such as dithiothreitol and 2-mercaptoethanol.7−10

Early reports on the use of TCEP in protein conjugation strategies suggested that this phosphine was compatible with maleimide and did not need to be removed before the addition of the Michael acceptor.2,11−13 A number of recent reports, however, have confirmed that TCEP and THPP do indeed react with maleimides to reduce conjugation yields significantly.8,14−17 Importantly, it has also been demonstrated that ylenes formed between maleimide and TCEP, such as 3, are remarkably stable under physiological conditions and can remain incorporated in the products of some protein conjugations (Figure 1b).16 As such, it is advantageous to remove the phosphine from the reaction before the addition of maleimide.

RESULTS AND DISCUSSION

A variety of methods are available for the removal of TCEP (e.g., dialysis, TCEP-immobilized resin, column chromatography); however, each has associated drawbacks.14 As an alternative, Henkel et al. have reported an elegant approach that uses 4-azidobenzoic acid (4-ABA) 4 to quench excess TCEP through a Staudinger reaction, thus circumventing the need for a purification step when using phosphines in maleimide-based bioconjugations (Figure 1c).17

In our hands, however, we have found the application of this method to be limited by the low aqueous solubility of 4, which necessitated increased reaction volumes, thereby reducing the substrate concentrations and reaction rates. Here, we describe the use of azide-modified polyethylene glycols as water-soluble reagents for the quenching of TCEP and THPP in situ to improve yields of protein conjugation reactions using maleimide.
values ranging from 25 mg/mL for the di-PEG 5, up to 130 mg/mL for the penta-PEG 8 (Table 1). By comparison, 4-ABA (4) was found to have a significantly lower mass solubility under similar conditions (Table 1). The solubility of 4 could be increased using different solvent systems, such as 50% aqueous methanol or Tris–HCl buffer at pH 8.0. However, solvent systems such as these are suboptimal for performing maleimide conjugation reactions with proteins.

The ability of azides 5–8 to quench TCEP and THPP by promoting oxidation was then evaluated using 31P NMR spectroscopy to quantify the rate of consumption of phosphine.

Table 1. Summary of the Mass and Molar Aqueous Solubilities Measured for PEG-Azides 5–8 and 4-ABA 4

<table>
<thead>
<tr>
<th>compound</th>
<th>solvent</th>
<th>solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.1 M Tris–HCl buffer (pH 7.0)</td>
<td>0.5 mg/mL, 3 mM</td>
</tr>
<tr>
<td>4</td>
<td>0.1 M Tris–HCl buffer (pH 8.0)</td>
<td>4.4 mg/mL, 27 mM</td>
</tr>
<tr>
<td>4</td>
<td>50% MeOH/water</td>
<td>1.5 mg/mL, 9 mM</td>
</tr>
<tr>
<td>5</td>
<td>0.1 M Tris–HCl buffer (pH 7.0)</td>
<td>25 mg/mL, 125 mM</td>
</tr>
<tr>
<td>6</td>
<td>0.1 M Tris–HCl buffer (pH 7.0)</td>
<td>60 mg/mL, 246 mM</td>
</tr>
<tr>
<td>7</td>
<td>0.1 M Tris–HCl buffer (pH 7.0)</td>
<td>88 mg/mL, 304 mM</td>
</tr>
<tr>
<td>8</td>
<td>0.1 M Tris–HCl buffer (pH 7.0)</td>
<td>130 mg/mL, 356 mM</td>
</tr>
</tbody>
</table>

Solutions of TCEP (25 mM) and PEG-azole (10 equiv) were monitored every 10 min (31P NMR), with all azides promoting rapid oxidation of TCEP. Azides 5–7 were found to have similar reactivities, with complete consumption of TCEP occurring after 50–60 min (Figure 2). The penta-PEG azide, 8, was found to react slightly more rapidly, with complete oxidation occurring after 40 min.

Figure 2. Oxidation rates of TCEP in the presence of PEG-azides 5–8 (250 mM) in a 0.1 M Tris–HCl buffer. Remaining TCEP (%) was determined using 31P NMR spectroscopy.
Figure 3. Fluorescent labeling of yeast enolase in the presence or absence of PEG-azide 7. (a) Chemical structure of the fluorescein-maleimide label 12. (b) Lanes 1–3: Yeast enolase (11 μM) in Tris-HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then treated with 12 (1 mM) for 18 h (37 °C). Lanes 4–6: Yeast enolase (11 μM) in Tris-HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 °C) before the addition of 12 (1 mM) for 18 h (37 °C). (c) Lanes 1–3: Yeast enolase (11 μM) in Tris-HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with THPP (1, 5, or 10 mM) for 45 min (RT) and then treated with 12 (1 mM) for 18 h (37 °C). Lanes 4–6: Yeast enolase (11 μM) in Tris-HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with THPP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 °C) before the addition of 12 (1 mM) for 18 h (37 °C). All protein samples were resolved by SDS-PAGE (4–12% gradient gel) and fluorescence visualized at 525 nm (Dark Reader).

oxidation of the phosphine occurring after only 40 min. Similarly, all of the azides (5–8) were found to promote the oxidation of THPP under similar conditions to those used for the oxidation of TCEP; however, oxidation occurred at much higher rates and complete consumption of THPP was observed in less than 5 min.

Having established that all of the PEG-azides, 5–8, can effectively promote rapid oxidation of TCEP and THPP under conditions suitable for performing maleimide-based protein conjugation reactions, it was necessary to consider potential side reactions that might occur from their use in situ in a conjugation mixture. In particular, the ability of alkyl azides to react with maleimide to form triazoles is reported in the literature, typically using organic solvents at elevated temperatures.18 To investigate the potential for PEG-azides to form triazoles with maleimide in an aqueous buffer at an ambient temperature, the tetra-PEG azide, 7, was treated with N-ethylmaleimide (9) for 2.5 days, which resulted in the formation of both the monotriazole (10) and ditriazole (11) derivatives isolated in 42 and 25% yields, respectively (Scheme 2). Despite this observation, it was considered that the cross-reaction with maleimide was unlikely to interfere with the use of PEG-azides in situ during protein conjugation strategies given the very slow rate of triazole formation (days) compared to typical rates of maleimide reaction with thiols (min–h).

The use of PEG-azides to improve the yield of bioconjugation reactions was then evaluated in applications in which maleimide is commonly employed as a Michael acceptor. The functionalization of proteins using maleimide-derivatized fluorescent labels is often used to enable visualization of proteins, with a number of these reagents, for example, N-(5-fluoresceinyl)maleimide (12), being commercially available. Tyagarajan et al. have demonstrated previously that the level of labeling of yeast enolase protein (which contains a single internal cysteine and requires denaturation for conjugation to occur) by maleimide-containing fluorescent dyes is significantly diminished when performing the conjugation reaction in the presence of TCEP.15 We sought to evaluate the impact that the use of PEG-azides in situ would have on the level of fluorescent labeling of yeast enolase (which contains a single internal cysteine and requires denaturation for conjugation to occur) by maleimide-containing fluorescent dyes is significantly diminished when performing the conjugation reaction in the presence of TCEP.15 We sought to evaluate the impact that the use of PEG-azides in situ would have on the level of fluorescent labeling of yeast enolase (which contains a single internal cysteine and requires denaturation for conjugation to occur) by maleimide-containing fluorescent dyes is significantly diminished when performing the conjugation reaction in the presence of TCEP.15

Scheme 2. Reaction of PEG-Azide 7 with N-Ethylmaleimide 9 (1.5 equiv) To Give Monotriazole 10 and Ditriazole 11, in 42 and 25% Yields, Respectively

Figure 2. Reaction of PEG-Azide 7 with N-Ethylmaleimide 9 (1.5 equiv) To Give Monotriazole 10 and Ditriazole 11, in 42 and 25% Yields, Respectively

5787 DOI: 10.1021/acsomega.7b01094
ACS Omega 2017, 2, 5783–5791
between the amount of TCEP used and the extent of fluorescent labeling, with all samples showing significant levels of fluorescence (Figure 3b, lanes 4–6). Similarly, when the above experiment was repeated using THPP as the reducing agent, fluorescent labeling was again seen to decrease significantly with increasing amounts of the phosphine in the absence of azide 7 (Figure 3c, lanes 1–3). In the presence of azide 7, however, the amount of THPP used did not appear to influence the extent of conjugation (Figure 3c, lanes 4–6), consistent with the results observed for TCEP-treated enolase. As such, the preincubation of TCEP- or THPP-treated enolase with PEG-azide 7 before maleimide addition was found to improve the level of fluorescent conjugation regardless of the amount of the phosphine used.

Another important application of maleimide for conjugation to proteins involves the attachment of large PEG polymers (kDa’s), a technique used widely within the pharmaceutical industry to improve the pharmacokinetic properties of protein therapeutics. Here, it was considered that investigating the use of azides 5–8 in situ in protein PEGylation reactions would also facilitate the quantification of any improvements to conjugation, as PEGylated protein products could be resolved on the basis of their mass (SDS-PAGE). In addition, the use of excess PEG-maleimide to protein (100 equiv) would identify whether any nonspecific conjugation was occurring, as multiple higher-molecular-weight products would be observed. Dena-

tured yeast enolase protein was reduced with varying amounts of TCEP (1, 5, and 10 equiv) and then incubated with 2 kDa PEG-maleimide. In parallel, samples of the TCEP-reduced enolase were treated with 11 μM in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) and then incubated with 2 kDa PEG-maleimide (1 mM) for 18 h (37 °C). Lanes 6–8: Yeast enolase (11 μM) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with THPP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 2 kDa PEG-maleimide (1 mM) for 18 h (37 °C). All protein samples were resolved by SDS-PAGE (4–12% gradient gel) and stained using Coomassie blue solution. PEGylated enolase (%) was determined by scanning each band (n = 3) using a LI-COR Odyssey CLx and calculating the ratio of PEGylated enolase/total enolase (Image Studio Lite).

**Figure 4.** Functionalization of yeast enolase with 2 kDa PEG-maleimide in the presence or absence of 7. (a) Lane 1: protein marker; lane 2: yeast enolase control; lanes 3–5: Yeast enolase (11 μM) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was incubated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then treated with 2 kDa PEG-maleimide (1 mM) for 18 h (37 °C). Lanes 6–8: Yeast enolase (11 μM) in Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) was treated with TCEP (1, 5, or 10 mM) for 45 min (RT) and then incubated with 7 (100 mM) for 1 h (37 °C) before the addition of 2 kDa PEG-maleimide (1 mM) for 18 h (37 °C). Similar results were also observed using THPP as the reducing agent.
Treatment of denatured enolase with 2 kDa PEG-maleimide immediately following reduction showed significant levels of unmodified protein (Figure 4b, lanes 3–5), whereas incubation of the reduced protein with 7 before the addition of maleimide resulted in high levels of PEGylation for all concentrations of THPP used (Figure 4b, lanes 6–8). Interestingly, the highest overall levels of protein PEGylation were observed using a 10:1 ratio of the phosphine (TCEP or THPP) when incubated with 7 before the addition of maleimide (Figure 4a, lane 8; and Figure 4b, lane 8). Conversely, the poorest levels of conjugation were seen when using this same 1:10 ratio of the phosphine in the absence of 7 (Figure 4a, lane 3; and Figure 4b, lane 3). This observation could result from more extensive reduction (more thiol present) as a consequence of the ability to use higher TCEP concentrations, given that all excess TCEP is now being removed through the use of PEG-azides.

**CONCLUSIONS**

In summary, we have shown here that PEG-azides 5–8 have suitable aqueous solubilities to enable their use in bioconjugation strategies employing maleimide and that all of these azides affect the rapid oxidation of both TCEP and THPP. Furthermore, it has been observed that the treatment of phosphate-reduced protein with PEG-azides such as 7 in situ, before the addition of the maleimide, allows for the use of high TCEP concentrations and leads to improved levels of conjugation using both small fluorescent probes and higher-molecular-weight PEGs.

**EXPERIMENTAL SECTION**

**Synthesis of 1,8-Diazido-3,6-dioxaoctane (5).** 4-Toluene sulfonyl chloride (3.3 g, 17.3 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (1.3 g) and triethylene glycol (1.0 g, 6.7 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N2 overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9-trioxaundecane-1,11-ditosylate as a colorless oil (2.0 g, 85%). NMR spectra were consistent with those reported. 21 1H NMR (400 MHz, CDCl3): δ 7.78 (d, J = 8 Hz, 4H), 7.34–7.31 (m, 4H), 4.14–4.10 (m, 4H), 3.68–3.63 (m, 4H), 3.51 (s, 4H), 2.43 (s, 6H). 13C NMR (100 MHz, CDCl3): δ 144.77, 132.90, 129.77, 127.81, 70.50, 69.26, 68.54, 21.51. HRMS (ESI): Expected for C22H30S2O9 (M + Na+) = m/z 525.1229. Found: m/z 525.1279.

Sodium azide (1.04 g, 16.0 mmol, 4 equiv) was added to a solution of 3,6,9-trioxaundecane-1,11-ditosylate (2.0 g, 4.0 mmol) in acetonitrile/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetonitrile, and the product was extracted using ethyl acetate (3 × 20 mL). The organic extract was then washed with saturated brine solution, dried over MgSO4, and then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give the diazide (6) as a colorless oil (0.80 g, 82%). NMR spectra were consistent with those reported. 21 1H NMR (400 MHz, CDCl3): δ 7.36–3.62 (m, 12H), 3.35 (t, J = 8 Hz, 4H). 13C NMR (100 MHz, CDCl3): δ 70.63, 70.62, 69.94, 50.59. HRMS (ESI): Expected for C21H32N6NaO3 (M + Na+) = m/z 267.1181. Found: m/z 267.1228.

**Synthesis of 1,11-Diazido-3,6,9-trioxaundecane (6).** 4-Toluene sulfonyl chloride (2.5 g, 13.4 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (1 g) and tetraethylene glycol (1.0 g, 5.2 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N2 overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9-trioxaundecane-1,11-ditosylate as a colorless oil (2.2 g, 85%). NMR spectra were consistent with those reported. 21 1H NMR (400 MHz, CDCl3): δ 7.74–7.71 (m, 4H), 7.30–7.72 (m, 4H), 4.11–4.08 (m, 4H), 3.62–3.60 (m, 4H), 3.56–3.43 (m, 8H), 2.38 (s, 6H). 13C NMR (100 MHz, CDCl3): δ 144.77, 132.90, 129.77, 127.81, 70.50, 69.26, 68.54, 21.51. HRMS (ESI): Expected for C23H32S2O9 (M + Na+) = m/z 526.1447. Found: m/z 526.1415.

Sodium azide (0.95 g, 14.6 mmol, 4 equiv) was added to a solution of 3,6,9,12-tetraoxatetradecane (7). 4-Toluene sulfonyl chloride (2.07 g, 10.9 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (0.8 g) and pentaethylene glycol (1.0 g, 4.2 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N2 overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9,12-tetraoxatetradecane-1,14-ditosylate as a colorless oil (2.0 g, 87%). NMR spectra were consistent with those reported. 21 1H NMR (400 MHz, CDCl3): δ 7.78 (d, 4H, J = 8 Hz), 7.33 (t, 4H, J = 8 Hz), 4.14 (t, 4H, J = 8 Hz), 3.68–3.65 (m, 4H), 3.60 (t, 12H, J = 8 Hz), 2.43 (s, 6H). 13C NMR (100 MHz, CDCl3): δ 144.71, 133.00, 129.94, 127.83, 70.62, 70.49, 69.81, 68.61, 21.55. HRMS (ESI): Expected for C23H32N6NaO3 (M + Na+) = m/z 547.1672. Found: m/z 547.1666.

Sodium azide (0.95 g, 14.6 mmol, 4 equiv) was added to a solution of 3,6,9,12-tetraoxatetradecane-1,14-ditosylate (2.0 g, 3.7 mmol) in acetonitrile/water (3:1, 24 mL), and the mixture was allowed to stir at 37 °C overnight. The mixture was then concentrated under reduced pressure to remove the acetonitrile, and the product was extracted using ethyl acetate (3 × 20 mL). The organic extract was then washed with saturated brine solution, dried over MgSO4, and then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9,12-tetraoxatetradecane as a colorless oil (2.0 g, 85%). NMR spectra were consistent with those reported. 21 1H NMR (400 MHz, CDCl3): δ 7.78 (d, 4H, J = 8 Hz), 7.33 (t, 4H, J = 8 Hz), 4.14 (t, 4H, J = 8 Hz), 3.68–3.65 (m, 4H), 3.60 (t, 12H, J = 8 Hz), 2.43 (s, 6H). 13C NMR (100 MHz, CDCl3): δ 144.71, 133.00, 129.94, 127.83, 70.62, 70.49, 69.81, 68.61, 21.55. HRMS (ESI): Expected for C23H32N6NaO3 (M + Na+) = m/z 579.2670. Found: m/z 579.2670.
50.64. HRMS (ESI): Expected for C_{20}H_{12}NaNO_{14} (M + Na^+) = m/z 311.1443. Found: m/z 311.1434.

**Synthesis of 1,17-Diazido-3,6,9,12,15-pentaoxaheptadecane (8).** 4-Toluenesulfonyl chloride (1.73 g, 9.1 mmol, 2.6 equiv) was added to a solution of anhydrous pyridine (0.76 g) and hexaethylene glycol (1.0 g, 3.5 mmol) in anhydrous DCM (10 mL), and the mixture was left to stir under N₂ overnight at room temperature. The solution was then concentrated in vacuo and was subjected to standard work-up (EtOAc). The resultant residue was then purified by silica gel chromatography (10 → 60% EtOAc/petroleum ether) to give 3,6,9,12,15-pentaoxaheptadecane-1,17-ditosylate as a colorless oil (1.70 g, 82%). NMR spectra were consistent with those reported. HRMS (ESI): Expected for C_{25}H_{33}O_{11}S_{2} (M + H^+) = m/z 561.2392. Found: m/z 561.2365. IR (thin film): 1714 cm⁻¹. HPLC: 5 → 100% MeCN in H₂O (0.5 mL/min) over 20 min; retention time = 13.53 min, purity = 99.4%. Detection at 254 nm.

**Rates of TCEP Oxidation by PEG-Azides (5–8).** Control.

The rate of background (aerial) oxidation of TCEP was initially evaluated. A solution of TCEP (100 mM) in Tris–HCl buffer (0.1 M, pH 7) was held at 37 °C, and the mixture was analyzed at 3 and 63 min using ³¹P NMR spectroscopy to quantify the levels of phosphine oxide present. No oxidation of TCEP was observed under these conditions (Figure S1).

**PEG-Azide-Promoted Oxidation of TCEP.** A solution of TCEP–HCl (14 mg, 50 μmol) and PEG-azide (500 μmol, 10 equiv) in 0.1 M Tris–HCl buffer (2 mL, pH 7) was held at 37 °C, and the solution was analyzed by ³¹P NMR spectroscopy at times 3, 13, 23, 33, 43, 53, and 63 min. The level of TCEP oxidation was calculated by comparison of integrals corresponding to the phosphine and phosphine oxide. It should be noted that PEG-azide S was only partially soluble under these conditions.

**PEG-Azide-Promoted Oxidation of THPP.** A solution of THPP (10 mg, 48 μmol) and PEG-azide 8 (40 mg, 120 μmol, 2.5 equiv) in 0.1 M Tris–HCl buffer (2 mL, pH 7) was held at 37 °C, and the solution was analyzed by ³¹P NMR spectroscopy at time 3 min. Complete conversion to the phosphine oxide was observed at this time as no signal corresponding to the remaining phosphine was present.

**Fluorescent Labeling of Yeast Enolase in the Absence/Presence of PEG-Azide (7).** Denaturation of Yeast Enolase Protein. A solution of yeast enolase (1 mg/mL) in deoxygenated Tris–HCl buffer (0.5 M, pH 7.2, 5 mM EDTA) containing 8 M urea was heated to 85 °C and held at this temperature for 15 min. The solution was allowed to cool to room temperature before use in subsequent experiments.

**Labeling of Yeast Enolase with Maleimide Fluorescein (11) Following Reduction with TCEP or THPP.** Aliquots (100 μL) of denatured yeast enolase (1 mg/mL, 11 μM) were treated with varying concentrations of TCEP or THPP (1–10 mM) and incubated for 45 min at 25 °C. Maleimide fluorescein 11 (1 mM) was subsequently added to the enolase solutions at each phosphine concentration and incubated at 37 °C for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 °C, 8 min). Aliquots (9 μL) of these solutions were then loaded into a precast gel (4–12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were first stained by Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution to confirm equal protein loading. Fluorescence was then visualized at 525 nm (Dark Reader).

**Labeling of Yeast Enolase with Maleimide Fluorescein (11) Following Quenching of TCEP or THPP with PEG-Azide (7).** Aliquots (100 μL) of denatured yeast enolase (1 mg/mL,
11 μM) were treated with varying concentrations of TCEP or THPP (1−10 mM) and incubated for 45 min at 25 °C. Samples were then treated with PEG-azide 7 (100 mM) and held for 1 hour at 37 °C. Maleimide-fluorescein (1 mM) was subsequently added to these solutions and incubated at 37 °C for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 °C, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4−12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution to confirm equal protein loading. Fluorescence was then visualized at 525 nm (Dark Reader).

PEGylation of Yeast Enolase in the Absence/Presence of Azide (7) Following Reduction by TCEP or THPP. Labeling of Yeast Enolase with 2 kDa-PEG Maleimide Following Reduction with TCEP or THPP. Aliquots (100 μL) of denatured yeast enolase (1 mg/mL, 11 μM) were treated with varying concentrations of TCEP or THPP (1−10 mM) and incubated for 45 min at 25 °C. 2 kDa-PEG maleimide (1 mM) was subsequently added to the enolase solutions at each phosphine concentration and incubated at 37 °C for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 °C, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4−12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution. The gel was then scanned using a LI-COR Odyssey CLx to quantify PEGylated enolase (Image Studio Lite).

Labeling of Yeast Enolase with 2 kDa-PEG Maleimide Following Quenching of TCEP or THPP with PEG-Azide (7). Aliquots (100 μL) of denatured yeast enolase (1 mg/mL, 11 μM) were treated with varying concentrations of TCEP or THPP (1−10 mM) and incubated for 45 min at 25 °C. Samples were then treated with PEG-azide 7 (100 mM) and held for 1 hour at 37 °C. 2 kDa-PEG maleimide (1 mM) was subsequently added to these solutions and incubated at 37 °C for 18 h. Samples (15 μL) were taken from each of the reactions and added to the Laemmli sample buffer (15 μL) and heated (85 °C, 8 min). Aliquots (9 μL) of these solutions were loaded into a precast gradient gel (4−12% Bis-Tris, Invitrogen) along with a protein ladder (EZ-Run, Fisher Scientific) and resolved by SDS Page electrophoresis [MOPS running buffer (Invitrogen), 180 V, 60 min]. The precast gels were stained by the Coomassie solution and destained using a water/ethanol/acetic acid (16:3:1) solution. The gel was then scanned using a LI-COR Odyssey CLx to quantify PEGylated enolase (Image Studio Lite).

ASSOCIATED CONTENT

Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01094.

Supplemental figures as described in the text; 1H NMR and 13C NMR spectra, HRMS, IR spectra, and HPLC chromatograms for compounds 10 and 11 (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: A.Watts@bath.ac.uk.

Notes
The authors declare no competing financial interest.

Data access statement: All data supporting this study are provided in the Results section of this paper or in the Supporting Information accompanying this paper.

ACKNOWLEDGMENTS

This work was supported by an MRC CiC grant (MC-PC-13058) to the University of Bath, a University Research Scholarship from the University of Bath (T.K.), and a PhD studentship from the University of Petra, Jordan (B.A.).

REFERENCES