Characterization of reactions between water soluble trialkylphosphines and thiol alkylating reagents: Implications for protein conjugation reactions

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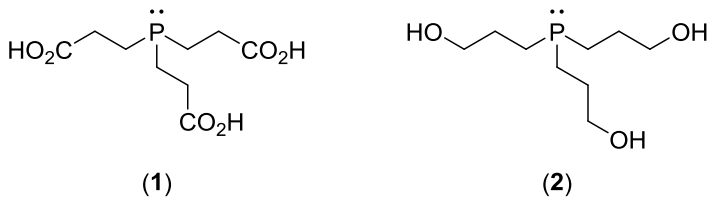
ABSTRACT

Water soluble trialkylphosphines such as tris(carboxyethyl)phosphine (TCEP) and trishydroxypropyl phosphine (THPP) are effective agents for reducing disulfide bonds in proteins and are increasingly becoming the reagents of choice for bioconjugation strategies which modify cysteine (thiol containing) amino acids. These reducing agents are often considered as being chemically compatible with Michael acceptors such as maleimides and, as such, are often not removed prior to performing protein conjugation reactions. Here we demonstrate the rapid and irreversible reaction of both TCEP and THPP with derivatives of the commonly employed thiol alkylating groups, maleimide and vinyl sulphone. Mechanistic investigations revealed distinct differences between the reactions of TCEP and THPP with maleimide, leading to the production of either non-productive ylenes or succidimidyl derivatives, respectively. Importantly, we also demonstrate the incorporation of non-productive ylenes formed between maleimide and TCEP into the Pneumococcal capsular polysaccharide Pn6b following strategies employed towards the production of conjugate vaccines.

INTRODUCTION

Bioconjugation reactions involving the modification of cysteine sulfhydryl groups have been used successfully to produce a wide variety of medicinally useful derivatives including PEGylated protein therapeutics, conjugate vaccines and, more recently, antibody drug conjugates (ADC's).[1-7] At pH's commonly employed for these conjugations (pH 7-8), it is predominantly the deprotonated sulfhydryl group, the thiolate anion, that is responsible for the highly nucleophilic characteristics of cysteine.[8] However, there is a strong tendency for cysteinyl thiols to oxidise to disulfides under aqueous conditions, or in some circumstances, to higher oxidation states resulting in sulfenic or sulfinic acids.[9] Unfortunately, these oxidised states of cysteine render the sulfur atom inert towards commonly used Michael acceptors such as maleimides and vinylsulfones.[10] A prerequisite therefore for many bioconjugation strategies based on conjugate addition by a thiol, first involves reduction of the cysteinyl sulphur to the sulfhydryl group prior to performing the conjugation step. In particular, the reduction of antibody interchain disulfide bridges is essential to enable attachment of cytotoxic payloads in the synthesis of ADC's such as brentuximab vedotin (Adcetris™).[11]

Water soluble alkylphosphines have established themselves as popular reducing reagents for effecting the liberation of peptidyl thiols, as they provide numerous advantages over the traditional thiol based reagents such as dithiothreitol (DTT), β-mercaptoethanol (BME) and β-mercaptoethylamine (2-MEA).[12-14] The phosphorus atom of commercial water-soluble alkylphosphines have pKa's of 7-8 which is a common pH range for performing bioconjugations and as such, the trialkylphosphines are more effective nucleophiles than thiol-based reducing agents to effect reduction within this pH range.[15] Furthermore, disulfide reduction utilising alkylphosphines are irreversible and driven by phosphorus-oxygen bond formation, unlike the reversible mechanism of disulfide reduction observed with thiol-containing reducing agents.[15] The two most commonly used water soluble alkylphosphines are tris-(2-carboxyethyl)phosphine (TCEP) **1** and tris-(3-hydroxypropyl)phosphine (THPP) **2 (Figure 1)**, with both reagents being effectively odourless and relatively stable towards oxygen dependant oxidation over a useful pH range for bioconjugation reactions.[15-17]



(**1**) (**2**)

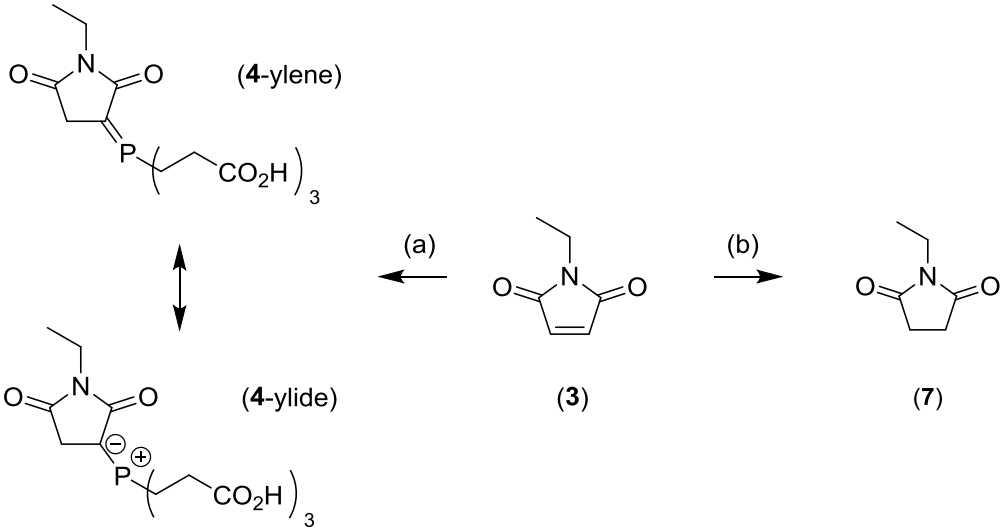
Figure 1. Structures of the trialkyl phosphine reducing agents TCEP (**1**) and THPP (**2**).

In addition to the favourable physico-chemical properties of TCEP and THPP, these reagents are also considered generally to provide methodological advantages over thiol based reducing agents, with reports in the literature that TCEP is unreactive towards maleimide and numerous examples of maleimide-based conjugations being performed in the presence of TCEP.[18-24] Contrary to this however, a number of groups have reported decreased yields of conjugation when using maleimide in the presence of TCEP, suggesting possible reaction between the two reagents.[25-28] Recently, Sánchez *et al.* have reported the observation of a phosphonium-ion adduct between TCEP and maleimide on the basis of mass spectrometry, though this adduct was not fully characterised.[28]

In this study we demonstrate that the water soluble phosphine reducing agents TCEP and THPP both react rapidly with maleimides and phenyl vinyl sulfone under conditions typically employed for bioconjugation reactions. We have performed full chemical characterisation on the reduction products formed, as well as investigated the mechanism of these reactions. In all cases, phosphines were found to reduce maleimides and vinylsulfones to species no longer capable of undergoing Michael addition with cysteinyl thiols, resulting in reduced yields of conjugation. Finally, we have evaluated the potential impact that the irreversible reaction between maleimide and phosphine reducing agents may have on common conjugation methods and demonstrate the potential for phosphine adducts to be incorporated within products of conjugation reactions which utilise components functionalised with multiple maleimides.

RESULTS AND DISCUSSION

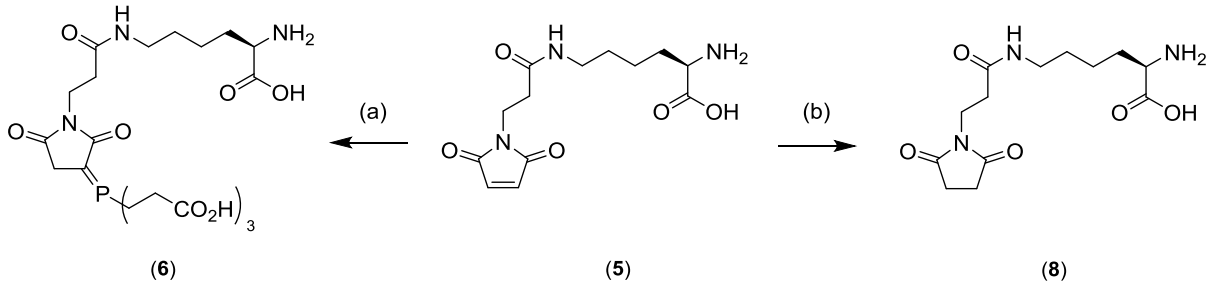
Reaction of trialkylphosphines with maleimides. Initial investigations into a reaction between TCEP and maleimide were performed under conditions commonly employed in protein conjugations utilising *N*-ethyl maleimide (**3**) as a model substrate, as it was considered that this simple structure may facilitate the characterisation of any adducts formed. Treatment of TCEP (**1**) with a solution of *N*-ethyl maleimide (**3**) (pH 7, phosphate buffer) at room temperature resulted in the rapid formation of the TCEP-maleimide adduct **4**, which was isolated in 73% yield (Scheme 1). This adduct can be represented in either the ylene or ylide resonance forms. ^{1}H- and ^{13}C- NMR spectroscopy allowed us to judge which representation is closer to the structure of 4. However, IR analysis showed a significant absorption at 1229 cm^{-1} which is indicative of a P=C bond (usual range 1180-1230 cm^{-1}), suggesting that the ylene is a better description of the structure.



Scheme 1. Reaction of *N*-ethyl maleimide (**3**) with TCEP (**1**) and THPP (**2**). (a) **1** (0.9 equiv.), THF/ 0.1M sodium phosphate (10 % v/v), pH 7, RT, 1 h. (b) **2** (1.0 equiv.), THF/ 0.1M sodium phosphate (10 % v/v), pH 7, RT, 1 h.

To demonstrate that the observed reaction was not simply a consequence of the reactive nature of the model substrate (**3**), the *N*-lysine maleimide derivative (**5**) was also treated with TCEP (**1**) under reaction conditions used previously. Here the ylene (**6**) was isolated in 70% yield, with IR analysis again confirming the structure as closer to the ylene (Scheme 2). The results observed here are consistent with previous observations that TCEP indeed reacts with maleimide to generate a phosphorus-containing adduct; however, it is

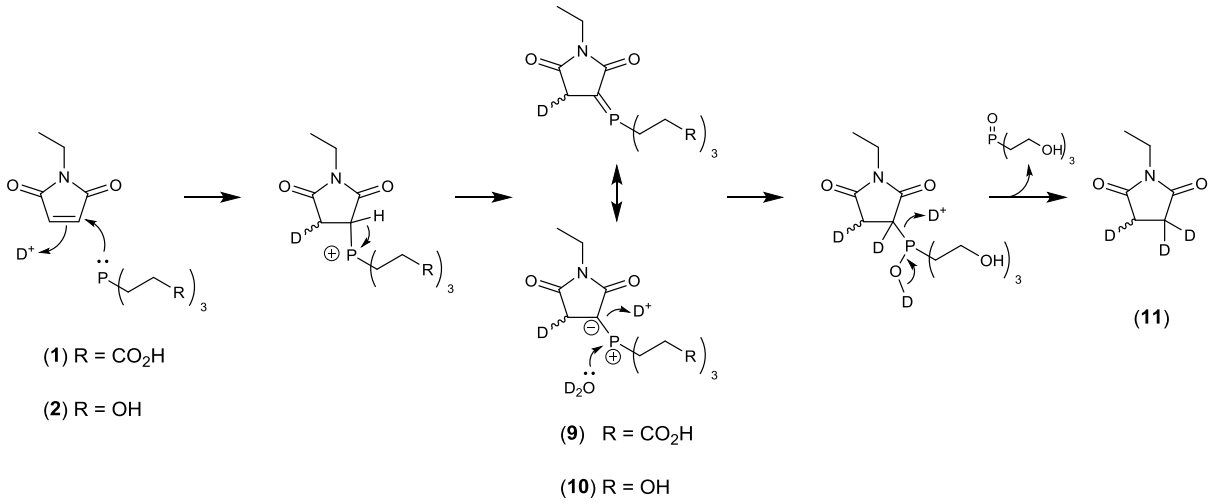
proposed here that this adduct is closer in structure to the neutral ylene rather than the phosphonium ylide proposed previously.[27-29]



Scheme 2. Reaction of lysine derivatized maleimide (**5**) with TCEP (**1**) and THPP (**2**). (a) **1** (0.9 equiv.), THF/ 0.1M sodium phosphate (10 % v/v), pH 7, RT, 1 h. (b) **2** (0.9 equiv.), THF/ 0.1M sodium phosphate (10 % v/v), pH 7, RT, 1 h.

Having characterised the products of reactions between TCEP (**1**) and the maleimide derivatives (**3**) and (**5**), it was then of interest to evaluate the generality of this reaction using other water soluble trialkyl phosphines. As such, the reactivity of the trihydroxyalkyl phosphine THPP (**2**) was investigated towards the same maleimide substrates. Interestingly, treatment of THPP with (**3**) under conditions used previously resulted in saturation of the maleimide group to give *N*-ethyl succinimide (**7**) in 61% yield (Scheme 1). Similarly, treatment of (**5**) with THPP resulted in formation of the succinimide (**8**) in 59% yield, rather than a phosphorus-based ylene as observed previously with TCEP. The reduction of maleimide to succinimide by a trialkylphosphine is not without precedent however, as Pal *et. al.* have reported previously that reduction of maleimides can be effected using triphenylphosphine in refluxing methanol.[30]

In light of the unexpected difference in products observed between the reactions of maleimides with TCEP and THPP, it was of interest to investigate mechanisms through which both reactions were operating. Repeating the reaction of TCEP (**1**) with *N*-ethyl maleimide (**3**) in deuterated phosphate buffer resulted in the formation of the ylene (**9**), where ^{1}H NMR identified the incorporation of a single deuterium atom at C-4. Likely, this reaction is proceeding through nucleophilic attack of the phosphorus atom at C-3 of maleimide, followed by double bond cleavage and deuteration at C-4, as shown in Scheme 3. Deprotonation of the intermediate phosphonium ion (at C-3) then generates (**9**).



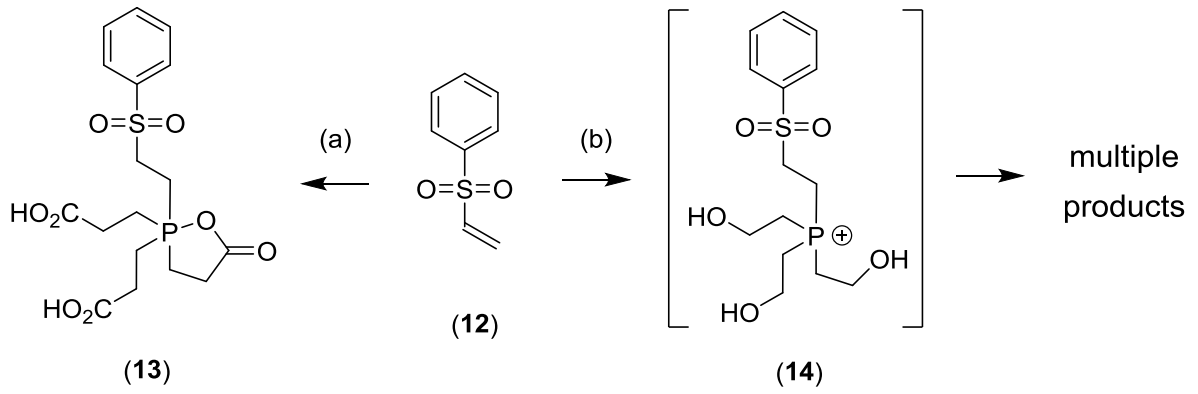
Scheme 3. Proposed mechanism for reaction of *N*-ethyl maleimide with trialkyl phosphines TCEP **1** (0.9 equiv.) or THPP **2** (0.9 equiv.) in THF/ 0.1 M deuterated phosphate buffer (10% v/v, pH 7) at room temperature for 1 hour.

When the reaction of *N*-ethyl maleimide with THPP was repeated in deuterated phosphate buffer, ^{1}H NMR analysis revealed that three deuterium atoms were incorporated into the succinimide product. This suggests that the initial reaction of THPP with maleimide follows a similar mechanism to that of TCEP through transient formation of the phosphine intermediate (**10**), which is subsequently hydrolysed to the tri-deuterated succinimide (**11**) as shown in Scheme 3. As such, the different products observed from the reaction of maleimides with either TCEP or THPP arises from the relative susceptibility of the respective phosphine adducts (**9** and **10**) towards hydrolysis, rather than the reactions proceeding through a different mechanism. Given that the pKa's of the phosphorus atoms of TCEP and THPP are very similar (7.7 and 7.2 respectively),[17] it is unlikely the observed difference in stability between (**9**) and (**10**) is solely a result of inductive differences between the carboxyl and hydroxyl sidechains. One possibility is that compound **10** is more ylide-like in character, making the phosphorus atom more susceptible to nucleophilic attack by water.

Reaction of trialkylphosphines with phenyl vinyl sulfone. Vinyl sulfones are an alternative class of thiol alkylating reagents to maleimides which have been used in a variety of bioconjugation reactions.[31,32] Like maleimides, vinyl sulfones are Michael acceptors and

react with a similar range of nucleophiles, so it was of interest to investigate the reactivity of a vinyl sulfone towards trialkyl phosphines. Treatment of phenyl vinyl sulfone (**12**) with TCEP in phosphate buffer (pH=7) under similar conditions to those employed previously on maleimides resulted in formation of a TCEP-sulfone adduct which was isolated in 84% yield following purification (Scheme 4). NMR spectra (^{1}H and ^{13}C) of the product confirms the presence of two protons on carbon-2 of the ethyl sulfone, indicating the phosphorus atom is attached through a single bond. The carbonyl region of the IR spectrum shows two distinct absorption bands at 1600-1721 cm^{-1} which is consistent with cyclic oxaphospholanes reported previously.[33] On this basis, we propose the product likely exists as compound **13**.

When considering the reasons behind the different products from reaction of TCEP with maleimide or vinyl sulfone, it is likely that the acidity of the proton on the carbon atom undergoing nucleophilic attack by phosphorus plays a key role in determining the products formed. In the case of the compound **12**, the protons on carbon-2 of the ethyl sulfone are not abstracted and so the initial phosphonium-ion formed is now stabilised through intereaction with a sidechain carboxylate.



Scheme 4. Reaction of phenyl vinyl sulfone (**12**) with TCEP (**1**) and THPP (**2**). (a) **1** (0.9 equiv.), THF/ 0.1M sodium phosphate (20 % v/v), pH 7, RT, 1 h. (b) **2** (0.9 equiv.), THF/ 0.1M sodium phosphate (10 % v/v), pH 7, RT, 1 h.

In contrast to TCEP, the reaction of THPP with phenyl vinyl sulfone **12** under similar conditions resulted in rapid consumption of the vinyl group to produce a complex mixture of aromatic containing species that could not be purified or characterised. Here, it is likely that the phosphorus atom of THPP undergoes addition to the vinyl group of **12** in a similar manner to TCEP, however the phosphonium-ion species generated (**14**) is unable to be stabilised

through either loss of a proton to form an ylide, or from participation of a sidechain group to form a cyclic oxaphospholane. Instead, breakdown of **14** occurs through several unidentified pathways.

Implications for the reaction between maleimide and trialkylphosphines on bioconjugations strategies. It has been demonstrated here that the trialkyl phosphines TCEP and THPP react rapidly with the Michael acceptor maleimide under commonly employed bioconjugation conditions. To understand the impact these reactions may have on conjugation strategies it is first necessary to consider the nature of the products formed. For the reduction of maleimide by THPP, it is clear that the succinimide products are incapable of undergoing subsequent addition to a cysteinyl thiol, so this reaction will result in consumption of alkylating reagent. For the case of the TCEP reaction with maleimide however, the impact on bioconjugation is not immediately obvious as the ylene product formed could itself undergo nucleophilic attack by thiols. As such, we sought to investigate the ability of the ylene **4** to undergo reaction with thiols. Solutions of the ylene **4** were generated *in situ* by reaction of TCEP with *N*-ethyl maleimide and subsequently incubated overnight with a solution of the cysteine containing peptide glutathione in phosphate buffer. Furthermore, the incubations were performed over a range of pH's (4, 7 and 8) to investigate any influence that protonation state of the side-chain carboxylate may have on ylene stability. The reactions were monitored by ^{31}P NMR or HRMS for loss of the ylene and formation of any product of conjugation. Under all conditions, no change was observed in the ylene **4** after 24 hours, suggesting that this adduct is indeed a 'dead-end' product and inert towards reaction with cysteinyl thiols. It is worthy of mention, however, that Sánchez *et al.* report a phosphonium-ion adduct formed between TCEP and maleimide was converted to a succinimide product.[28] Unfortunately the reaction conditions necessary to effect this conversion are not reported.

The effect of performing protein PEGylations with maleimide in the presence of trialkylphosphines. As the reaction of TCEP with maleimide results in formation of a non-productive ylene and hence, loss of active alkylating reagent, the impact on bioconjugation will be dictated by the relative rates of reactions between maleimide with either the cysteinyl thiol or the phosphine reducing agent. These observations are consistent with a previous

report by Tyagarajan *et al*. where the yield of conjugation of maleimide containing fluorescent dyes onto cysteine containing proteins was reduced when performing the reactions in the presence of TCEP.[26] It was of interest to us to expand on these observations and investigate the impact of performing maleimide based protein PEGylation reactions in the presence of TCEP.

Here, yeast enolase (47 kDa) was used as a model protein as it contains a single cysteine residue (Cys248) and no disulfide bridges, so that the role of TCEP is not complicated by any competing disulfide reductions. The yeast enolase was first denatured (8M urea) to expose the buried cysteine residue and then incubated with varying amounts of reducing agent (TCEP or THPP) prior to treatment with an equimolar amount of 2 kDa PEG-maleimide. The degree of conjugation was then evaluated by resolving the proteins using SDS-PAGE and visualising the change in mass by staining with Coomassie blue solution (Figure 2).

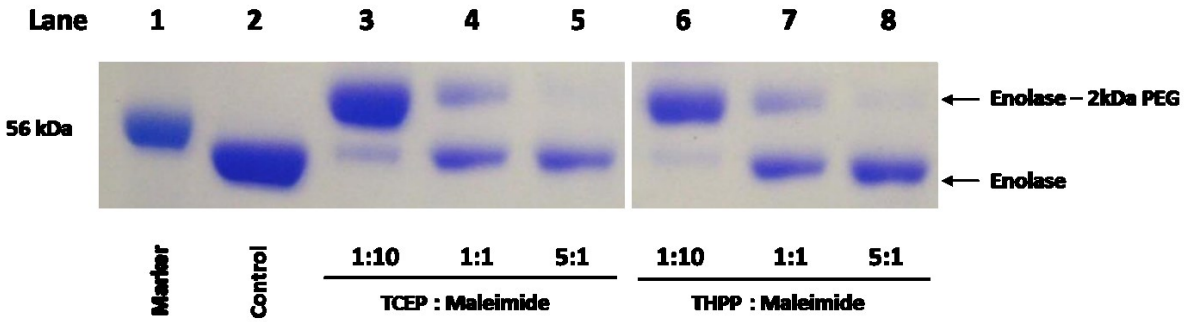


Figure 2. SDS-PAGE analysis showing degree of PEGylation of yeast enolase protein in the presence of varying ratios of phosphine reducing agent to 2kDa PEG-maleimide (stained using Coomassie blue solution). All conjugation experiments were performed in Tris.HCl buffer (pH 7.2) at 37°C for 18 hours using a 1:1 ratio of enolase to maleimide reagent. Lane 1: Marker protein (56kDa); Lane 2: enolase protein (control); Lanes 3-5: enolase treated with TCEP and maleimide-2kDa PEG in ratios of 1:10, 1:1 and 5:1; Lanes 6-8: enolase treated with THPP and maleimide-2kDa PEG in ratios of 1:10, 1:1 and 5:1.

It was observed that performing the PEGylation of denatured yeast enolase in the presence of excess maleimide resulted in good levels of protein PEGylation (Figure 2, lanes 3 and 6). However, repeating the reaction in the presence of even just equimolar amounts of TCEP (Figure 2, lane 4) or THPP (Figure 2, lane 7), resulted in significant amounts of non-PEGylated yeast enolase remaining. These results indicate that both TCEP and THPP react with maleimide at approximately the same rate as the addition of maleimide to the cysteinyl thiol. When the ratios of reducing agent to maleimide were increased to 5:1 (Figure 2, lane 5 for

TCEP and lane 8 for THPP), PEGylation of enolase was almost entirely abolished. These results suggest that even residual amounts of phosphine reducing agent present in protein PEGylation reactions are likely to result in measurable loss of conjugation yield and, as such, these reagents need to be removed prior to the introduction of maleimide.

Incorporation of phosphine adducts into conjugation components containing multiple maleimides. It has been demonstrated here that TCEP reacts rapidly with maleimide to form ylenes which are remarkably stable towards nucleophilic attack over an appreciable pH range. For all of the conjugation reagents investigated thus far, which contain a single maleimide group, this reaction will result in consumption of alkylating agent and may reduce yields of conjugation. However, it was of also of interest to consider the reaction of TCEP with conjugation components containing multiple maleimide groups, such as those employed in the production of conjugate vaccines.[34,35] For components containing multiple maleimides, potential may exist for its hetero-functionalisation by both the intended thiol nucleophile as well as any residual TCEP present during the conjugation reaction. Here, the antigenic polysaccharide Pn6B from the outer protective capsule of *Streptococcus pneumoniae* was chosen as model substrate to investigate the reaction of TCEP with conjugation components containing multiple maleimides. Pn6B (from ATCC) is a 0.9-1.5 MDa polysaccharide with a repeat unit of (→2-α-D-Galactopyranose-(1→3)-α-D-Glucopyranose-(1→3)-α-L-rhamnopyranose-(1→4)-D-ribitol-5-phosphate→) (Figure 3a). Maleimide was introduced into a sample of Pn6B by treatment with N-(5-isocyanatopentyl)maleimide to give **15** (Figure 3b) where the degree of maleimide functionalisation was determined to be 4%, based on ^{1}H NMR analysis. Analysis of the ^{31}P NMR spectrum of Pn6B-maleimide **15** revealed a single peak at δ = -0.08 ppm, characteristic of the repeat unit phosphodiester backbone (Figure 3c).

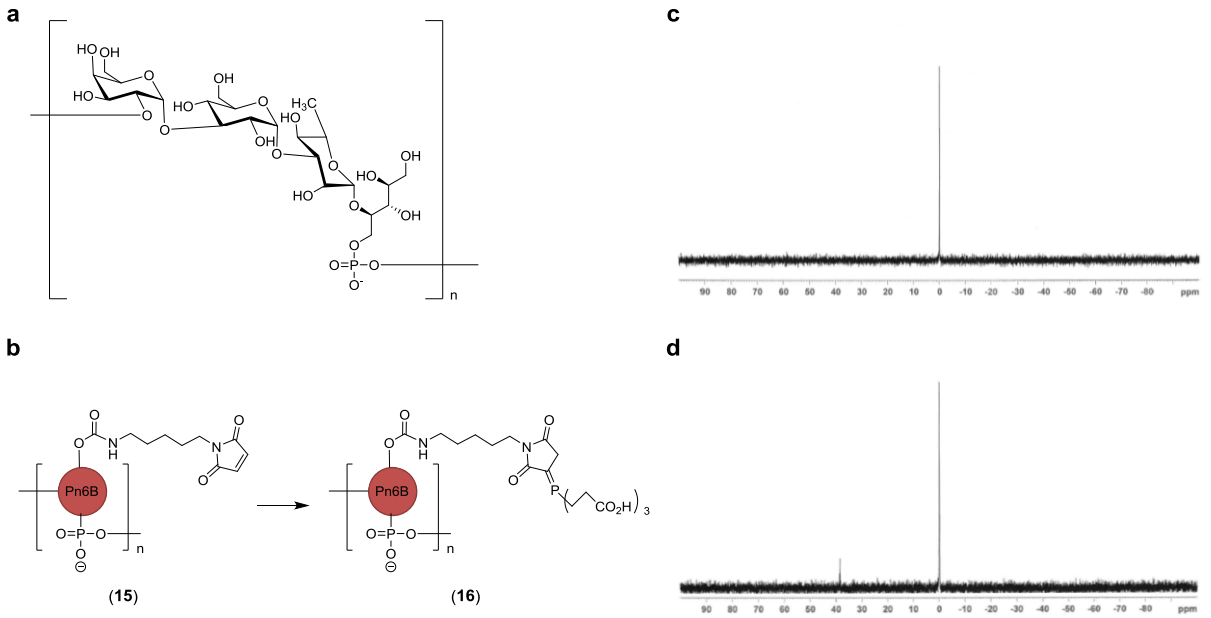


Figure 3. Incorporation of ylene adducts into maleimide labelled antigen Pn6B. a) Chemical structure of repeat unit for capsular polysaccharide Pn6B. b) Reaction of maleimide labelled Pn6B (**15**) with TCEP to give the ylene **16**. c) ^{31}P NMR spectrum of **15**. d) ^{31}P NMR spectrum of **16**.

Compound (**15**) was treated with an aqueous solution of TCEP for 1 hour at room temperature, then purified extensively by gel filtration chromatography and subsequently lyophilised. ^{31}P NMR analysis of the product showed two phosphorus signals, one corresponding to the repeat unit phosphodiester at δ = -0.08 ppm and the other characteristic of a maleimide-TCEP ylene (**16**) at δ = 34 ppm (Figure 3d). This result demonstrates that conjugation components activated with multiple maleimides, such as capsular polysaccharides used in conjugate vaccine production, are susceptible to ylene incorporation through reaction with TCEP.

Conclusions

Trialkylphosphines such as TCEP and THPP are popular as effective disulfide reducing agents in bioconjugation procedures. However, we have demonstrated here that both of these reducing agents are reactive towards commonly used Michael acceptors such as maleimide and vinyl sulfone. TCEP and THPP have been shown to react with maleimide to produce different products. TCEP reacts with maleimide to produce a stable ylene adduct that is resistant to nucleophilic attack by thiols. THPP reduces maleimide to succinimide which

could significantly reduce yields during the bioconjugation process. TCEP reacts with phenyl vinyl sulfone to generate a penta co-ordinate phosphorus product, while THPP reacts with the same sulfone to produce a complex mixture of products. These reactions will likely lower yields of bioconjugate production and may also complicate purification protocols. It is clearly evident that both the reducing agents need to be completely removed from bioconjugations before introducing the alkylating reagent to the reaction.

Experimental Procedures

Reaction of TCEP with N-ethyl maleimide:

Synthesis of *N-ethyl-3-(tris(carboxyethyl)phosphorylidene)pyrrolidine-2,5-dione* (**4**)

N-ethyl maleimide **3** (10.0 mg, 0.08 mmol) and TCEP **1** (0.9 eq., 20.6 mg, 0.072 mmol) were dissolved in a mixture of THF (1 mL) and argon purged aqueous sodium phosphate (0.1 M, pH = 7.0, 9 mL) and stirred under argon at room temperature for 1 hour. The solution was then concentrated *in vacuo* to 3 mL then loaded onto a C-18 column for purification (100 % H_2O → 20 % MeCN/H_2O) to yield **4** as a white sticky solid (19.7 mg, 73 %). ^{1}H-NMR (D_2O) 400 MHz: δ = 1.05 (t, 3H, C*H*$_3$, *J* = 7.2 Hz), 2.73-2.84 (m, 12H, 3 x C*H*$_2$C*H*$_2$CO), 3.09-3.26 (m, 2H, CC*H*$_2$CO), 3.49 (q, 2H, NC*H*$_2$, *J* = 7.2 Hz). ^{13}C NMR, D_2O, 125 MHz: δ 11.54 (*C*H_3), 14.34 (d, 3C, P*C*H_2, *J* = 49.9 Hz), 25.82 (d, 3C, 3 x CH_2*C*H_2CO_2H, *J* = 3.5 Hz), 29.39 (C*C*H_2CO), 33.51 (m, CH_2*C*CO), 35.05 (N*C*H_2), 172.95 (C*C*O, *J* = 3.0 Hz), 174.16 (d, 3C, 3 x CH_2*C*O_2H, *J* = 11.9 Hz), 175.97 (d, CH_2*C*ON, *J* = 8.8 Hz). ^{31}P NMR, D_2O, 162 MHz: δ 39.2. HRMS: Expected for $C_{15}H_{21}N_1O_8P_1$ (M-H$^+$) = m/z 374.1005. Found: m/z 374.1029. Infrared (KBr): 3442, 1700 cm^{-1}. HPLC (retention time: 12.81 mins., purity: 93 %), column: Phenomenex Luna-C18 (250 x 4.60 mm), gradient: (0.7 mL/min), 100 % water containing 0.1 % TFA → 100 % MeCN over 16 minutes, detection at 225 nm.

Reaction of THPP with N-ethyl maleimide:

Synthesis of *N*-ethyl succinimide (**7**)

A solution of THPP **2** (0.9 eq., 29.9 mg, 0.144 mmol) was prepared in THF (1 mL) and argon purged aqueous sodium phosphate (0.1 M, pH = 7.0, 9 ml). N-ethyl maleimide **3** (20.0 mg, 0.160 mmol) was added slowly to the rapidly stirring solution of THPP. The reaction was

left to stir for 30 minutes at room temperature. A further 0.1 eq. of THPP was added and left to stir for an additional 30 minutes. The reaction was diluted with 25 mL of diethyl ether and extracted with water (30 mL). The aqueous layer was extracted with diethyl ether (2 x 30 mL). The organic extraction layers were combined and dried using $MgSO_4$. The mixture was filtered and the organic solution was concentrated (550 mm Hg, 25 °C). The crude was purified by silica gel chromatography ($CH_2Cl_2 \rightarrow$ 2 % acetone/CH_2Cl_2) to yield **7** as a clear oil (12.4 mg, 61 %). Spectral data consistent with literature.[36] ^{1}H NMR ($CDCl_3$) 400 MHz: δ = 1.07 (t, 3H, CH_2CH_3, *J* = 7.2 Hz), 2.61 (s, 4H, 2 x COC*H*), 3.46 (q, 2H, NCH_2CH_3, *J* = 7.2 Hz). ^{13}C-NMR ($CDCl_3$) 100 MHz: δ = 12.8 (CH_2CH_3), 28.0 (2 x CO*C*H), 33.5 (NCH_2CH_3), 177.0 (2 x N*C*OCH). HRMS: Expected for $C_6H_9N_1Na_1O_2$ ($M+Na^+$) = *m/z* 150.0525. Found: *m/z* 150.0531.

Reaction of TCEP with the phenyl vinyl sulfone:

Synthesis *of 3,3'-{5-oxo-2-[2-(phenylsulfonyl)ethyl]-1,2 λ^5-oxaphospholane-2,2-diyl}dipropanoic acid* (**13**)

Phenyl vinyl sulfone **12** (10.0 mg, 0.059 mmol) and TCEP **1** (0.9 eq., 15.3 mg, 0.054 mmol) was dissolved in THF (2 mL) and argon purged aqueous sodium phosphate (0.1 M, pH = 7.0, 8 ml) and stirred under argon at room temperature for 1 hour. The reaction was concentrated *in vacuo* to 3 mL and then loaded onto a C-18 column for purification (100 % $H_2O \rightarrow$ 40 % MeCN/H_2O) to yield **13** as a white solid (18.8 mg, 84 %). ^{1}H-NMR (D_2O) 400 MHz: δ = 2.54-2.63 (m, 12H, 3 x CH_2CH_2CO), 2.66-2.74 (m, 2H, SCH_2CH_2), 3.72 (m, 2H, SCH_2CH_2), 7.67-7.95 (m, 5H, Ar-*H*). ^{13}C-NMR (D_2O) 100 MHz: δ 13.5 (d, PCH_2CH_2, *J* = 49.3 Hz), 14.6 (d, 3 x $CH_2CH_2CO_2$, *J* = 50.1 Hz), 26.5 (d, 3 x $CH_2CH_2CO_2$, *J* = 3.8 Hz), 47.2 (SCH_2CH_2), 128.09 & 130.0 (4C, *ortho* & *meta* Ar-*C*), 135.45 (*ipso* -*C* & *para* Ar-*C*), 175.3 (d, 3 x CH_2CO_2H, *J* = 12.7 Hz). ^{31}P-NMR (D_2O) 162 MHz: δ = 37.7. HRMS: Expected for $C_{17}H_{22}O_8P_1S_1$ ($M-H^+$) = *m/z* 417.0778. Found: *m/z* 417.0797. Melting point: 115 °C. Infrared (KBr): 3427, 2925, 1721, 1600, 1419, 1153 cm^{-1}. HPLC (retention time: 12.43 mins, purity: 97 %), column: Phenomenex Luna-C18 (250 x 4.60 mm), gradient: (0.7 mL/min) 10 % MeCN in water (containing 0.1 % TFA) $\rightarrow$ 100 % MeCN over 20 minutes, detection at 280 nm.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Detailed synthesis and analytical data of molecules, experimental methods and analytical spectra.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

TCEP, tris(carboxyethyl)phosphine; THPP, trishydroxypropyl phosphine; ADC, antibody-drug conjugate; DTT, dithiothreitol; BME, β-mercaptoethanol; BMA, β-mercaptoethylamine; IR, infrared; Pn6B, capsular polysaccharide 6B from *Streptococcus pneumoniae*.

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