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Tris(3-hydroxypropyl)phosphine (THPP): A mild, air-stable reagent for the rapid, reductive cleavage of small-molecule disulfides

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ABSTRACT

Tris(3-hydroxypropyl)phosphine (THPP) is demonstrated to be a versatile, water-soluble and air-stable reducing agent, allowing for the rapid, irreversible reductive cleavage of disulfide bonds in both aqueous and buffered aqueous–organic media. The reagent shows exceptional stability at biological pH under which condition it permits the rapid reduction of a wide range of differentially functionalized small-molecule disulfides.

The reduction of symmetrical or non-symmetrical disulfides to the corresponding thiols is a process of considerable importance in both chemistry and biochemistry. Reagents that are used for the reduction of disulfide bonds may additionally serve as antioxidants, preventing oxidative disulfide bond forming reactions from occurring, a process that is restrictive in the handling of naturally occurring thiols and proteins. This problem is particularly challenging in the handling of cysteine derivatives and proteins at and around physiological pH (7.0–8.5).[1,2]

A variety of reducing agents and methodologies are available for the chemical reduction of disulfides to monomeric thiols.[3–9] Many of the methods that are successful for the chemical process employ harsh hydride and/or dissolving metal conditions, for example, zinc metal in hydrochloric acid, and are generally not applicable to biological applications. Thiols such as dithiothreitol (DTT) (Scheme 1), mercaptoethanol, 3-mercaptopropionate and glutathione have been employed during protein purification/isolation allowing disulfide reduction, and/or thiol protection under mild chemoselective conditions.[2,10] The reagent DTT, or Cleland's reagent,[1] is most often used as a protective reagent in biochemical applications, however its ease of oxidation is a significant limiting factor. Aqueous solutions of DTT show limited stability, particularly at biological pHs and slightly above (pH 7.5–8.5).[2,11] The half-life of DTT and the other thiol-based reagents mentioned are quite similar, varying from about 10 h at pH 7.5 to less than 1 h at pH 8.5.[2] This factor necessitates the use of an excess of the thiol reagent to reduce disulfides (to drive the equilibrium shown in Scheme 1 to the right) or the continued addition of aliquots of DTT over time in order to protect sensitive thiols.

Triaryl[4] and trialkylphosphine-containing compounds are also known to reduce disulfides, including protein disulfide bonds, and have been employed at preparative scales for this purpose.[12] Trialkylphosphines such as tributylphosphine have been shown to selectively reduce disulfide bonds and are compatible with other functional groups commonly found in proteins.[13,14] Nonetheless, the use of trialkylphosphines is hindered by low solubility in water and aerial oxidation of the phosphine. The water-soluble reagent tris-(2-carboxyethyl)phosphine (TCEP, Scheme 2) was introduced by Whitesides and co-workers[11] 25 years ago but has found limited application,[15a,b] perhaps due to its low-stability at biological pH (optimal working range pH 4–5) and cost. While the search for novel reagents continues,[15c] we were attracted to the tri-substituted phosphine tris(hydroxypropyl)phosphine (THPP, Scheme 2), as an alternative water soluble reducing/protective reagent. THPP has mainly been investigated as a ligand in view of its water solubility and coordination chemistry.[15d]

While the protective effect of THPP during the isolation of two air-sensitive reductases is known,[2b–e] no systematic comparative study on its ability to reduce small-molecule disulfides has been reported. THPP is readily available through radical-mediated addition of phosphine to allyl alcohol. A single report on the air stability of THPP in the desired pH range[15d] drew our attention

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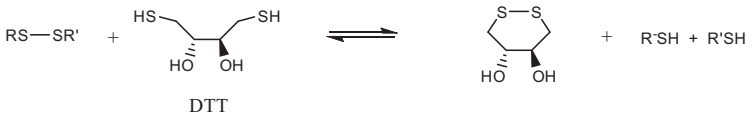
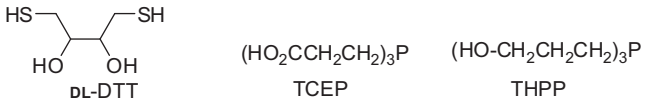


Scheme 1. Reduction of disulfide bridge or thiol protection mediated by the standard reagent DTT.[1]



Scheme 2. Water soluble trialkylphosphines TCEP and THPP used in this study in comparison to the standard reagent DL-DTT.

and, in conjunction with the Whitesides reagent, prompted the present comparative investigation into the air-stability and reducing ability of THPP directly with TCEP and DTT in the reduction of small-molecule disulfides. The results of this analysis and investigation into the scope of small-molecule disulfide reduction mediated by THPP are reported herein.

THPP was found to be soluble in water in all proportions, giving rise to an odorless solution. The oxidative stability of aqueous solutions of DTT, TCEP and THPP were compared at pH 4.50 (citrate based buffer) and 8.00 [tris-(hydroxymethyl)-aminomethane-$CaCl_2$ based buffer] through the direct monitoring of aqueous solutions of each by NMR analysis. The NMR spectra of THPP and TCEP are complicated by the formation of the protonated phosphines around pH 6–7. At pH 4.50 the phosphines are fully protonated while at pH 8.00 the free base of each is clearly observed. Hence the ^{31}P NMR of THPP at pH 4.50 shows the phosphorus resonance at δ +15.8 ppm, corresponding to the protonated form (pK_a reported at 7.2).[15a] At pH $\geqslant$ 7, the ^{31}P-resonance for the free base is observed at −30 ppm, while the corresponding phosphine oxide is observed at +60 ppm. The ^{31}P NMR of TCEP at pH 4.50 similarly demonstrated a resonance at δ +17 ppm corresponding to the protonated TCEP species, a resonance at −26 ppm at pH $\geqslant$ 7, corresponding to TCEP free base and the oxide of TCEP at +56 ppm. In case of DTT, progress of the auto-oxidation was conveniently followed by ^{1}H NMR. The methylene multiplet resonance for DTT was observed at δ 2.47 ppm, clearly distinguished from the corresponding peak for the cyclic disulfide centered at δ 2.41 ppm.

The results of these analyses are reported in Figures 1 and 2. At pH 4.50 (Fig. 1) DTT demonstrated considerable auto-oxidation after 2–3 days while both of the phosphines proved to be relatively stable, most likely being protected in their salt forms. More importantly, at physiological pH (Fig. 2) aqueous solutions of THPP were observed to be significantly more stable than that of TCEP or DTT. The half-life of DTT at pH 8.0 under these conditions was observed to be approximately 4 h, in accord with literature values.[2] While the relative stability of TCEP relative to DTT has been remarked upon before,[15b] literature data on the relative stability of TCEP[11,15a,b] and THPP[15d] are difficult to directly compare as they have been performed at differing pH's in various buffers. The direct comparison reported here (Fig. 2) shows the exceptional stability of THPP under physiological conditions, suffering only 10% oxidation under these conditions over 72 h.

In order to gauge the relative reducing power of TCEP and THPP, the 2-hydroxyethyl disulfide **1b** was prepared and experiments were performed involving the conversion of this compound to the corresponding thiol **2b** (Scheme 3). The reduction of **1b** proved to be very fast at room temperature using THPP in comparison to TCEP or DTT. The reduction of **1b** using 1.1 equiv of THPP, TCEP and DTT was followed in three separate side by side experiments at pH 8.0 buffer. After 15 min, the reduction with THPP was complete (82% isolated yield) while TCEP provided 25% and DTT 30% isolated yields under these identical conditions.

In order to quantify the relative rates of reduction of **1b** using TCEP and THPP, direct competition experiments were performed with a 1.0:1.0 solution of TCEP and THPP in aqueous citrate buffer at pH 4.5 and 8.0. The addition of 0.67 equiv of the disulfide **1b** resulted in its rapid reduction to **2b**. Analysis of the NMR spectra showed formation of the phosphine oxides of THPP and TCEP in the ratio of 1.00:1.25. Repetition of the same procedure using Tris-buffer at pH 8.0 showed formation of phosphine oxides of THPP and TCEP in the ration 1.50:1.00 indicating a reversal in the relative rates of reduction and showing THPP to be the superior reducing reagent at biologically relevant pH. Similarly, the reduction of the peptide-like disulfide **1a** was shown to proceed 3.91 times faster with THPP relative to TCEP at pH 8.0 buffer.

The scope of the use of THPP, TCP and DTT in reducing a group of selected disulfides was next investigated, the results of which are collected in Table 1. The disulfides **1a**–**g** were prepared following literature methods.[16,17] Structures were chosen to cover a range of aryl, heterocyclic, benzylic and aliphatic disulfide functionalities. As such, the polarity and solubility of these starting

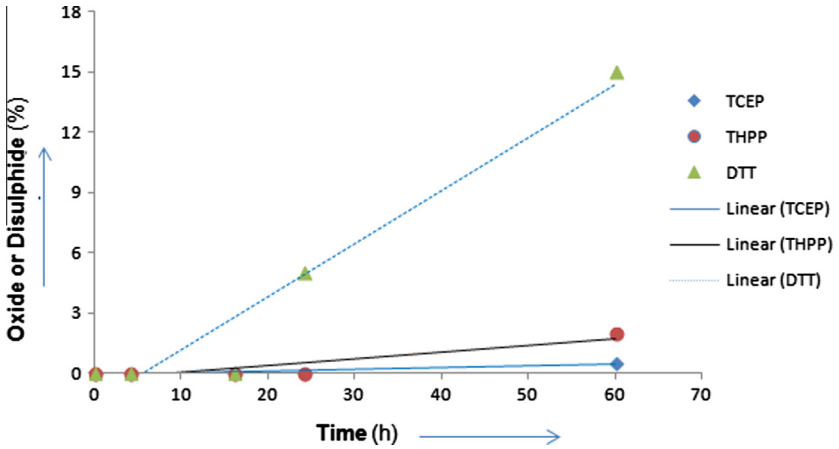


Figure 1. Relative stability of aq solutions of DTT, TCEP and THPP to room temperature auto-oxidation at pH 4.50.

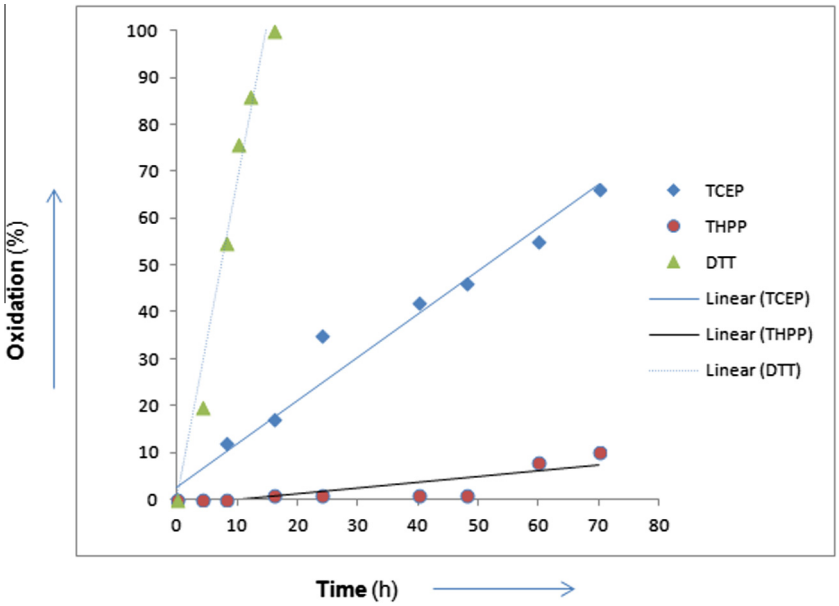
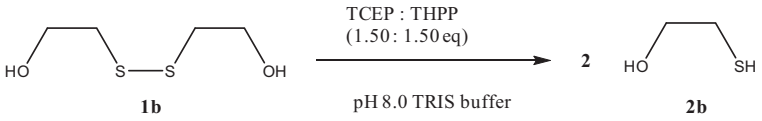
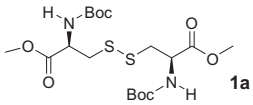
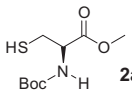
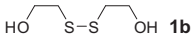
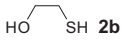
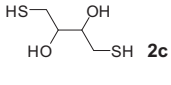
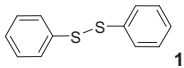
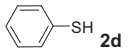
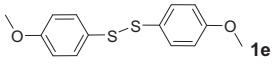
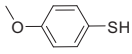
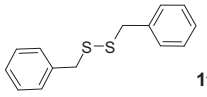
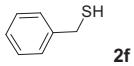
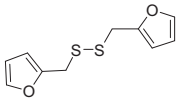
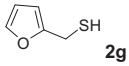


Figure 2. Relative stability of aq solutions of DTT, TCEP and THPP to room-temperature auto-oxidation at pH 8.0.

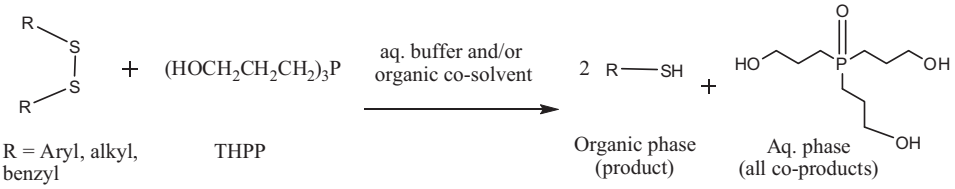


Scheme 3. Reduction of 2-hydroxyethyl disulfide **1b** using TCEP and/or THPP.

Table 1
Reductive cleavage of disulfides **1a–g** to form thiols **2a–g** via Scheme 4[a,b]

Entry	Disulfide, **1a–1g**	Thiol, **2a–2g**	Reducing agent		
			THPP	TCEP	DTT
1	 **1a**	 **2a**	99[c]	77[c]	35[c], 85[d]
2	 **1b**	 **2b**	82[e]	25[e]	30[e]
3	 **1c**	 **2c**	94[f]	90[f]	–
4	 **1d**	 **2d**	0[b], 93[g]	0[b], 58[g]	0[b], <5[g]
5	 **1e**	 **2e**	0[b], 97[g]	0[b], 46[g]	0[b], 63[g]
6	 **1f**	 **2f**	0[b], 92[g]	0[b], 23[g]	0[b], <5[g]
7	 **1g**	 **2g**	0[b], 94[h]	0[b], 5[h], 0[i]	0[b], <5[h]

[a] Isolated yields after column chromatography.
[b] Unless otherwise mentioned, the reactions are performed with 1.10 equiv of reducing agent dissolved in 1 mL of aq buffer (pH 8.0) at room temp for 30 min. In the case of TCEP mediated reactions, aqueous solutions were internally adjusted to pH 8, before addition of the disulfide.
[c] Acetonitrile (ACN) (0.50 mL) was used as a co-solvent to dissolve the L-cystine based disulfide.
[d] 2.20 equiv of DTT was used.
[e] Reaction was performed for 15 min at room temp in aqueous buffer only.
[f] Based on NMR analysis.
[g] Tetrahydrofuran (THF) (0.50 mL) was added as co-solvent.
[h] THF (1.0 mL) was added as co-solvent.
[i] THF or ACN was added as solvent.



Scheme 4. Reduction of various disulfides using THPP and simple green-chemical work-up processing.

materials varies significantly. Nonetheless, by performing the reaction in buffered aqueous media alone and/or with the addition of quantities of water miscible organic solvents (MeOH, ACN or THF), successful disulfide reduction could be achieved in all cases investigated. As can be seen (Table 1) THPP proved to be a highly effective disulfide reducing agent, capable of rapidly reducing a wide range of substrates to the corresponding thiols in comparison with the standard reagents TCEP and DTT (see Supplemental experimental procedures).

Finally, the overall process using THPP is attractive from a green-chemistry perspective (Scheme 4). Both THPP and its oxide are fully water soluble and the disulfide reductions shown (Table 1) require only a slight excess (1.10 equiv) of THPP to achieve full conversion. Reaction work-up (with the exception of entry 3) involves simple organic/aqueous partition to separate the product thiol from all inorganic impurities (buffer, phosphine oxide) and permitted chromatography-free isolation of the products.

In conclusion, THPP is a readily available,[18] fully water-soluble phosphine, aqueous solutions of which are odorless and easy to handle in open laboratory conditions. The reagents shows high stability to oxidation in comparison to previously employed reagents DTT and TCEP. THPP was shown to rapidly and irreversibly reduce a range of functionalized polar disulfides to the corresponding thiols in aqueous buffered solutions and to also work effectively with addition of organic solvents to reduce non-polar disulfides. The disulfide reduction process is atom-economical and allows for the clean separation of organic thiols without chromatography. The extended stability of buffered aqueous solutions of THPP at and around physiological pH is noteworthy and the reagent should also find application as a preventative antioxidant in handling small-molecule thiols as well as biological materials containing air-sensitive thiol functionality.[2]

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.027.

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18. The material, tris(3-hydroxypropyl)phosphine (THPP) was obtained from Cytec Industries Inc. and has been used without further purification.