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Detection of Thiol Functionality and Disulfide Bond Formation by Polyoxometalate

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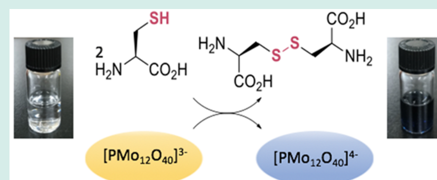
ABSTRACT: The detection of thiol functionality and intramolecular disulfide bond formation of peptides using the α-Keggin type polyoxometalate molybdenum−oxygen cluster ($H_3PMo_{12}O_{40}\cdot nH_2O$) is described. Our method entails the addition of this polyoxometalate to solutions of thiol, whereupon the color of the solution changes from colorless to deep blue. Reduction of the polyoxometalate from Mo(VI) to Mo(V) occurs with concomitant oxidation of the thiol functionality, to form disulfide bonds. To exemplify the utility this phenomenon, we accomplished the oxidation of glutathione, reduced linear oxytocin, bactenecin, and α-conotoxin SI; all of which proceeded smoothly and in good conversion in 24 h to less and were accomplished by a change in the color of the reaction solutions.



KEYWORDS: *polyoxometalate, thiol, disulfide bond formation, blue staining, detection of cysteine residue, cyclic peptides*

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Polyoxometalates (POMs) are a versatile class of negatively charged early transition metal−oxygen nanoclusters whose unique catalytic, electrochemical, magnetic, and luminescence properties have stimulated research in broad fields of science.[1,2] They can be categorized based on their chemical structures: Lindqvist-type POMs are iso-polyoxometalates; α-Keggin and Wells-Dawson POMs have tetrahedrally coordinated heteroatoms; and Anderson−Evans POMs incorporate an octahedral central atom. Modification of all types of POMs can be accomplished by replacement of their M=O moieties with other transition metals (ruthenium, rhodium, palladium, etc.). In addition, giant POMs with nanosized cavities can be obtained by the aggregation of simple clusters in water the presence of reducing reagents and templates and with control of pH.[3−5] POMs are of particular interest in the context of heterogeneous catalysis: for example, porous materials based on POMs have been recently studied as catalysts in asymmetric dihydroxylation of olefins[6] and the allylation of benzaldehyde with allyl tributyltin,[6] and Hill reported that the Keggin-type POM $[CuPW_{11}O_{39}]_5^-$ catalyzed the rapid chemo- and shape-selective oxidation of thiols to disulfides, and the rapid and sustained removal of toxic H_2S by the oxidation of S_8.[7] Therefore, POMs have huge potential in organic synthesis and the life sciences. The detection and measurement of free thiols (i.e., free cysteine, glutathione, and cysteine residues on proteins) is an essential task in the study of biological processes and events in many biological systems. 5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman's reagent)[8] has been used widely for the quantification of thiols, especially for assays of various enzymes including acetyl- and butyrylcholinesterase, the substrates of which release thiols through enzymatic reactions. However, other reagents have been used too: for example, 4,4′-bipyridyl disulfide[9] and 5-(2-aminoethyl)dithio-2-nitrobenzoic acid[10] have proved useful for the colorimetric analyses of thiols, and a wide variety of fluorescent probes for thiols are also available.[11,12] In addition, disulfide bonds play a key role in stabilizing protein structures, the disruption of which is strongly associated with a loss of protein function and activity.[13] Disulfide bond formation is therefore one of the most important steps in peptide/protein synthesis. Many methods for disulfide bond formation have been reported to date and include the use of oxidizing reagents such as dimethyl sulfoxide,[14] iodine,[15] silyl chloride-sulfoxide,[16] thallium trifluoroacetate,[17] silver trifluoromethansulfonate,[18] and 3-nitro-2-pyridinesulfenate (Npys).[19] Regioselective disulfide bond formation has also been accomplished in the synthesis of multidisulfide peptide/proteins, such as insulin, the defensins, and the cyclotides, by using these reagents in combination with product precursors bearing orthogonal thiol-protecting groups on the side chains of their cysteine residues. Herein, we describe our study of the POM (**1**) $H_3PMo_{12}O_{40}\cdot nH_2O$ and our discovery of its utility as a mild and self-indicating oxidizing reagent for thiols to give the corresponding disulfides. The utility was exemplified first using glutathione (**2**) and, then, in the synthesis of the disulfide-containing bioactive peptides, such as oxytocin (**3**),[20−22] bactenecin (**4**),[23] and α-conotoxin SI (**5**).[24,25]

First, we screened eight different POMs for their ability to oxidize thiols to the corresponding disulfide and the degree to which this change in redox state altered the color of the

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reaction solutions. Solutions to which the α-Keggin type POMs, $H_3PMo_{12}O_{40}\cdot nH_2O$ (**1**) and $(NH_4)_3PMo_{12}O_{40}\cdot nH_2O$ and Wells−Dawson-type tungsten−oxygen cluster $K_6P_2W_{18}O_{62}\cdot nH_2O$, were added all changed from colorless to deep blue. None of the reaction mixtures containing the other POMs changed in color. Therefore, the ability to oxidize the thiol functionality of cysteine in not general to all POMs (see Figure S1). Figure 1A depicts the absorbance of each reaction

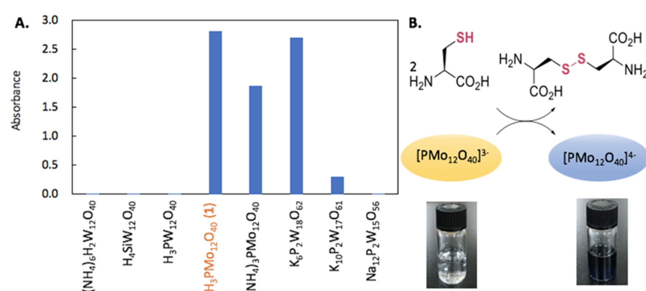

Figure 1. Absorbance of separate solutions of eight different POMs (10 mg/mL, 100 μL) and L-Cys (10 mg/mL, 500 μL) in water (after 2 h).

mixture at 700 nm; this wavelength was selected as it is associated with the intervalence charge transfer of M^V-O-M^{VI} to M^{VI}-O-M^V, which underlies the reduction of PMo_{12} and P_2W_{18}. For the solutions containing the POMs indicated, significant absorbance was observed, from which we inferred that oxidation had taken place. The new disulfide bonds formed between thiols of the cysteine side chains (Figure 1B) were also observed by ^1H NMR analysis (see Figure S2) and MS spectra (data not shown). On the basis of the very high absorbance with which it was associated, the POM (**1**), $H_3PMo_{12}O_{40}\cdot nH_2O$, was selected for further development as a thiol-detection and oxidizing agent (Figure 1).

The reactivity of solutions of all 20 proteinogenic amino acids with POM (**1**) was also studied. Only the solution of cysteine and the POM (**1**) changed being colorless to a deep blue solution; the other 19 solutions did not undergo an observable change at room temperature even over a time period of 1 week (Figure 2A, see Figure S3). Analogues

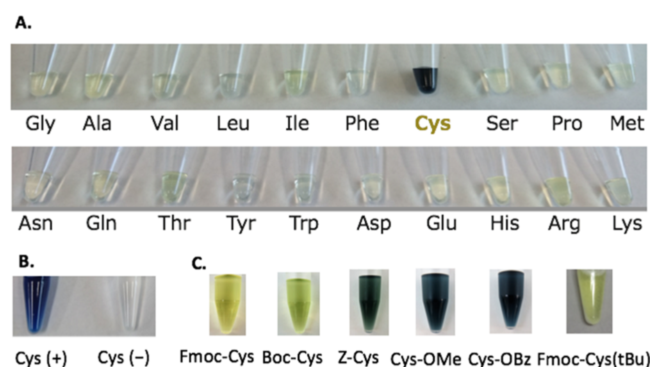

Figure 2. Detection of cysteine in solutions of different 20 proteinogenic amino acids. (a) Each microtube contains 10 mg/mL L-amino acid (100 μL) 10 min after addition of 10 mg/mL POM (**1**) (20 μL). (b) Cys (+): A mixture of 20 proteinogenic amino acids with POM (**1**). Cys (−): A mixture of 19 proteinogenic amino acids except for cysteine with POM (**1**). (c) treatment of protected cysteines with POM (**1**).

behavior was observed for solutions containing POM (**1**) with (i) all 20 proteinogenic amino acids and (ii) all proteinogenic amino acids except for cysteine (19 amino acids in total) (Figure 2B). We also treated a selection of protected cysteines with the POM (**1**); the solution of Fmoc-Cys and Boc-Cys turned pale yellow after 24 h, and those of Z-Cys, Cys-OMe, and Cys-OBz turned a similar blue color to that previously observed with the solution of Cys. These results suggest that the POM (**1**) could effectively recognize the peripheral Fmoc and Boc groups, presumably by virtue of its large size (nanoscale) (Figure 2C).

Next, we monitored how the absorbance of a solution of 2.5 mM POM (**1**) and 5 mM Cys changed over a period of 720 min, Figure 3A. The absorbance intensity was observed to

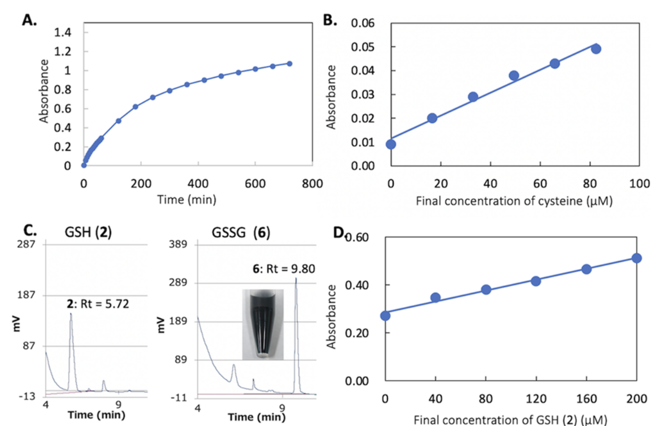

Figure 3. (A) Temporal changes in absorbance of reaction mixture (2.5 mM POM (**1**), 5 mM Cys). (B) Calibration curve of cysteine concentration (10 mM POM (**1**)). (C) HPLC charts of GSH (**2**) and GSSH (**6**). (D) Calibration curve of GSH (2) concentration (10 mM POM (**1**)). HPLC conditions: linear gradient starting from 10% to 90% MeCN in 0.1% aqueous TFA over 30 min at a rate of 1.0 mL/min and detection at 220 nm.

increase over the first 60 min, before stabilizing and reaching a maximum at around 720 min. In addition, plots of absorbance against cysteine concentration (from 1 to 80 μM) show a good correlation (Figure 3B). Finally, the detection limit of Cys was found to be about 1 μM (using 10 mM POM (**1**)), and the minimum concentration of POM (**1**) at room temperature was found to be about 1 mM (see Figure S4). Glutathione (**2**, reduced, GSH) is a cellular antioxidant thought to protect important cellular components from damage by reactive oxygen species and believed to be present in cells at a concentration of about 0.5−10 mM. Detection of GSH (**2**) is an important task in biological science to evaluate oxidative stress, apoptosis, and cell death. We sought to determine if POM (**1**) could detect GSH in cells. Ten millimolar solutions of POM (**1**) were treated with solutions of GSH (**2**) ranging in concentration from 6.5 to 200 μM for 24 h; the absorbance at 700 nm was found to be dependent on the concentration of GSH (**2**), and the initially colorless reaction mixture gradually turned blue. Furthermore, GSSG (**6**) (R_t = 9.80 min), the oxidation product of GSH (**2**) (R_t = 5.72 min), was detected by HPLC as a single peak. No trimeric products derived from GSH (**2**) were detected. Therefore, the quantitative analysis of GSH (**2**) can be accomplished using POM (**1**) oxidative methodology (Figure 3C and 3D). Other solvents were tested as well. POM (**1**) was most effective in water, perhaps because

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of its superior solubility in water compared to other solvents. However, POM (**1**)-mediated disulfide bond formation was also found to proceed in common organic solvents, such as DMSO, DMF, and THF (see Figure S5). To investigate the reactivity of POM (**1**) as an oxidant for intramolecular disulfide bond formation, we sought to determine to what extent it could oxidize the reduced linear peptide (**7**) to give oxytocin (**3**), a cyclic nonapeptide incorporating a disulfide bond. Peptide **7** was prepared by a conventional Fmoc-based solid phase peptide synthesis (Fmoc-SPPS) using a Rink amide resin. After the elongation of peptide chain, treatment with a TFA solution (TFA/TIPS/H_2O = 95:2.5:2.5) afforded crude peptide, which was purified with RP-HPLC to give the reduced linear peptide **7** in 10% yield. This was dissolved in DMSO and the solution treated with 10 mM POM (**1**) at room temperature. After 24 h, the reaction mixture has turned from yellow to deep green, suggesting POM (**1**) to have been reduced. Furthermore, a single peak (R_t = 13.79 min) corresponding to oxytocin (**3**) was observed by HPLC. Subsequently purification by preparative HPLC afforded oxytocin (**3**) in 90% yield. No oligomeric byproducts were observed by HPLC (Figure 4).

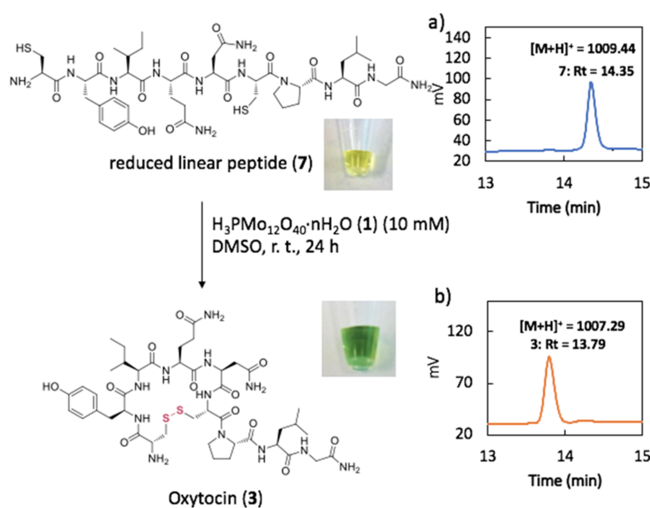

Figure 4. Intramolecular disulfide bond formation of reduced linear peptide (**7**) to give oxytocin (**3**). Synthetic scheme and HPLC profiles of oxytocin synthesis (a) before addition of POM (**1**) and (b) after 24 h. HPLC conditions: linear gradient starting from 10 to 50% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min, and detection at 220 nm.

The oxidation of **7** to give **3** was accomplished using three different oxidizing agents—POM (**1**), I_2, and air—and the results compared, Figure 5. Conversion was assessed by HPLC. Using air alone, disulfide bond formation was very slow; a conversion of only 12% was observed after 24 h, and most of the unreacted substrate was recovered. Using 0.1 M I_2/MeOH in 50% aqueous AcOH, the reaction was much faster, and after 24 h, the conversion was estimated to be 40%, although peaks corresponding to several byproducts were also observed, presumably arising from iodination of the side chains of Trp, Tyr, and His residues. However, using POM (**1**), conversion proceeded smoothly in a time-dependent manner, without observable byproducts, and reached 95% after 24 h. Therefore, POM (**1**) is concluded to be a very mild oxidizing agent well suited to the oxidation of peptides to give products

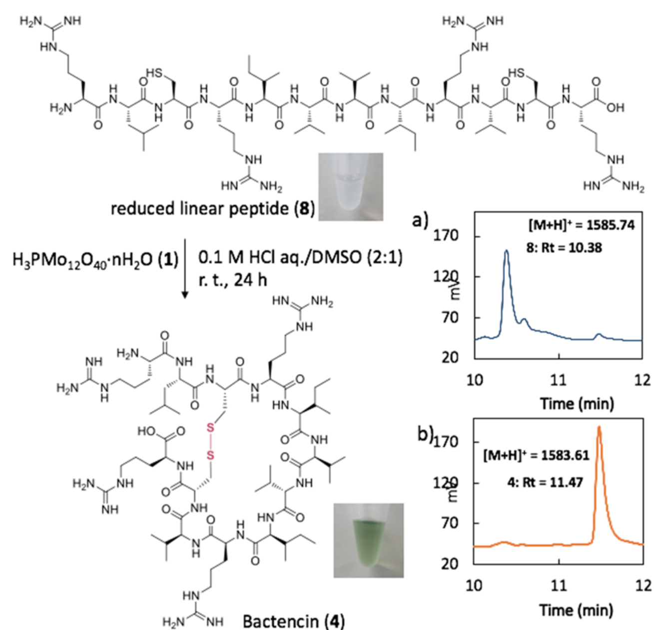

Figure 5. Intramolecular disulfide bond formation of reduced linear peptide **8** to give bactenecin (**4**). Synthetic scheme, pictures of reaction mixtures and HPLC profiles of bactenecin synthesis (a) before addition of POM (**1**) and (b) after 24 h. HPLC conditions: Linear gradient starting from 10% to 90% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min and detection at 220 nm.

incorporating a disulfide bond (see Figure S5). Next, we studied the oxidation by POM (**1**) of the linear peptide (**8**) to give bactenecin (**4**), a 12-mer peptide containing one disulfide bond between Cys^3 and Cys^{11}. Linear peptide **8** was prepared by Fmoc-SPPS on 2-CT resin in 10% yield after cleavage from the resin and HPLC purification. Treatment of linear peptide **8** with 10 mM POM (**1**) in 0.1 M HCl/DMSO (2:1), caused intramolecular disulfide bond formation to proceed gradually over 24 h, with a concurrent reduction in the area under the peak corresponding to the reduced linear peptide (**8**), Figure 6. The reaction mixture changed from colorless to a deep green over this time period. This reaction also proceeded using air but was very slow (Figure 5).

Finally, we attempted the synthesis of α-conotoxin SI (**5**), a tridecapeptide amide that binds selectivity to the muscle

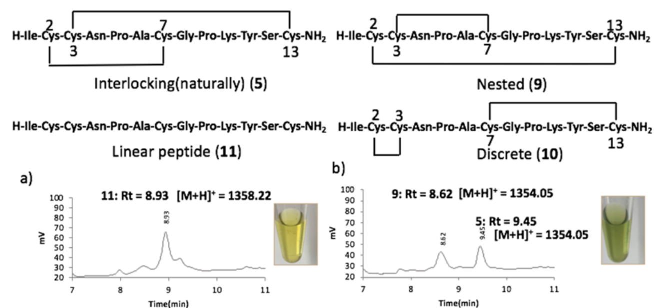

Figure 6. Chemical structure of reduced linear peptide **11**, naturally occurring “interlocking” (**5**), “nested” (**9**), and “discrete” (**10**) α-conotoxin SI (a) before addition of POM (**1**) and (b) after 24 h. HPLC conditions: Linear gradient starting from 10 to 90% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 1.0 mL/min, and detection at 220 nm.

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subtype of nicotinic acetylcholine receptors and that was originally isolated from marine cone snail venom.[26] Naturally occurring α-conotoxin SI (**5**) has two "interlocking" disulfide bridges connecting Cys^2−Cys^7 and Cys^3−Cys^{13}, but the two "mispaired" isomers **9** and **10** are also known, corresponding to "nested" α-conotoxin SI (**9**) (Cys^2−Cys^{13} and Cys^3−Cys^7) and "discrete" α-conotoxin SI (**10**) (Cys^2−Cys^3 and Cys^7−Cys^{13}). The synthesis of α-conotoxin SI (**5**) was attempted starting from linear α-conotoxin SI (**11**), which was assembled by Fmoc-SPPS on Rink amide resin (ESI-MS: m/z 1358.22). Following cleavage from resin and deprotection of all protecting groups by TFA/TIPS/H_2O, the linear tetrathiol (**11**) was oxidized successfully by stirring with POM (**1**) for 24 h at room temperature in 0.1 M HCl/DMSO (2:1). At a concentration of peptide (**11**) of 10 mM, two main peaks with m/z 1354.05 from ESI-MS spectra were detected after consumption of the linear peptide (**11**). These were confirmed to be the naturally occurring "interlocking" α-conotoxin SI (**5**) and "nested" isomer (**9**) in the literature.[26] In this case, the monomeric disulfide species were not detected. Although sequential disulfide formation using an orthogonal strategy is required to give high quality α-conotoxin SI (**5**), POM (**1**) is nevertheless well suited to disulfide bond formation in relatively large peptides bearing a multitude of functionality. Peptide **11** could also be oxidized using 0.1 M I_2/MeOH; the HPLC profile of the reaction mixture was similar that obtained using POM (**1**) (Figure 6).

POM (**1**), the α-Keggin type molybdenum−oxygen clusters, $H_3PMo_{12}O_{40}\cdot nH_2O$, has been validated as a versatile and mild oxidant of thiols to give disulfides, and because solutions of POM (**1**) change color upon its oxidation, a thiol detecting agent. It is noted that combination of detection and oxidation to thiol functionality specifically using mild reagent POM (**1**) is associated with chemical application including combinatorial science. In addition, syntheses of disulfide-containing bioactive peptides, such as GSSH (**6**), oxytocin (**3**), bactenecin (**4**), and α-conotoxin SI (**5**), were achieved using POM (**1**), all of which cleanly obtained in good conversions of about 24 h. POM (**1**) shows no cytotoxicity against mammalian cell lines and a variety of bacteria (data not shown), and therefore, biological application of this process are expected. Additional uses for a variety of POMs will be disclosed in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.0c00176.

> Each reaction tube of L-cysteine and POMs, ^{1}H-NMR spectra of L-cysteine and POM mixture, absorbance of each reaction tube of 20 proteinogenic amino acids and POMs, detection limit of cysteine, relationship of absorbance, relationship of absorbance and reaction temperature, absorbance of POM solutions, optimization of solvents for the oxidative disulfide bond formation, generation rates of oxytocin by respective methods, HPLC profile of three conotoxins, HPLC profile of conotoxicin SI derived from POM treatment, retention time of HPLC profile and ESI-MS analysis for preparation of three conotoxin SIs, and experimental details (PDF)

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Author Contributions

H.K. and S.Y. designed the research plan, H.Y., R.A., and I.N. performed the experiments and analyzed the data, and H.K. supervised the research. H.K. wrote the manuscript with assistance from S.Y.

Notes

The authors declare no competing financial interest.

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