P-B desulfurization: an enabling method for protein chemical synthesis and site-specific deuteration

Kang Jin, Tianlu Li, Hoi Yee Chow, Han Liu and Xuechen Li*

Abstract: Cysteine-mediated native chemical ligation has provided a powerful tool for protein chemical synthesis. Herein, we report an unprecedented mild system (TCEP-NaBH$_4$ or TCEP-LiBEt$_3$H) for chemoselective peptide desulfurization to achieve effective protein synthesis via the native chemical ligation-desulfurization approach. This method, termed *P-B* desulfurization, features usage of common reagents, simplicity of operation, robustness, high yielding, clean conversion and versatile functionality compatibility with complex peptides/proteins. In addition, this method allows for incorporating deuterium into the peptides after cysteine desulfurization when running the reaction in the D$_2$O buffer. Moreover, this method enables clean desulfurization for peptides carrying post-translational modifications, such as phosphorylation and crotonylation. The effectiveness of this method has been demonstrated in the synthesis of cyclic peptides *Dichotomin C* and *E*, and synthetic proteins including ubiquitin, γ-synuclein and histone H2A.

Protein chemical synthesis[1] and modification[2] provide important tools to produce chemical probes to study the function of proteins[3, 4] and develop therapeutic biologics[5]. However, due to the presence of diverse functionalities of amino acid side chains in a peptide/protein sequence, development of effective chemical methods to directly manipulate these biomacromolecules is challenging, for which mildness, robustness and chemoselectivity are essential but difficult to achieve. Chemoselective peptide ligations[6-11], provide effective tools to chemically synthesize proteins that are difficult to produce by any biologically controlled methods, such as all D-proteins[12, 13] and proteins carrying site-specific stoichiometric post-translational modification(s)[14-16] or unnatural side chains[17]. Of great importance, native chemical ligation (NCL) developed by Kent and co-workers represents a powerful and chemoselective reaction to merge two unprotected peptide fragments with N-terminal cysteine and C-terminal thioester respectively.[6, 7] To overcome the limitation of NCL that cysteine residue is required at the convergent connection site, Dawson and co-workers developed the Pd/Al$_2$O$_3$ and Raney nickel mediated desulfurization of the ligation products that converted cysteine into alanine and expanded cysteine-based peptide ligation repertoire to alanine sites.[18] In this regard,

chemoselective peptide desulfurization that is highly compatible with diverse functional amino acid side chains and avoids or minimizes the use of metal reagents is of great value. To this end, a metal-free radical condition with TCEP (tris(2-carboxyethyl)phosphine)/VA-044/*t*-BuSH developed by Danishefsky and co-worker has dramatically advanced the NCL-desulfurization strategy in protein chemical synthesis.[19] Along with this discovery, various mercapto-amino acids as the cysteine surrogates have been synthesized and used in protein synthesis in the recent decade, which significantly enhanced the power of the NCL-desulfurization strategy.[20-22] Although other efforts have been dedicated to develop alternative peptide desulfurization conditions, such as photoreaction-based conditions,[23-25] these methods still rely on usage of metal complex or other initiators, which likely causes adsorption problems in complex protein synthesis.

Herein, we report an unprecedented mild desulfurization condition with inexpensive TCEP-NaBH$_4$ or TCEP-LiBEt$_3$H, which allows for chemoselective desulfurization and deuteration of peptides/proteins.

Our exploration started with a serendipitous observation in our lab that TCEP-NaBH$_4$ affected a thiol compound. Thus, we started to investigate whether such a condition could be optimized and applied in the native chemical ligation-desulfurization strategy for protein chemical synthesis. In this event, peptide **1** H-A**C**V**C**VRPRVMG-OH carrying two cysteine residues was chosen as the model substrate and subjected to various conditions (Figure 1a and Table S1). To enable general utility of reactions on protein chemical synthesis, the solvent for condition screening was limited to aqueous solutions. Either TCEP or NaBH$_4$ alone could not affect the peptide at all in the PBS buffer at pH 7 (Figure 1a and Table S1, entries 1 and 2). Addition of NaBH$_4$ to the peptide solution followed by TCEP did not produce any desulfurized peptide product either (Table S1, entry 3), while the addition of NaBH$_4$ to the premixed solution of the peptide **1** and TCEP realized the peptide desulfurization successfully to provide **2** with an 80% conversion after 6 hours at room temperature (Figure 1a, Table S1, entry 4). Moreover, the addition of the solution of premixed TCEP and NaBH$_4$ (1:1 mole ratio) to the solution of peptide **1** further improved the reaction and produced the desulfurized peptide **2** with more than 95% conversion after 6 hours (Figure 1a, Table S1, entry 5).

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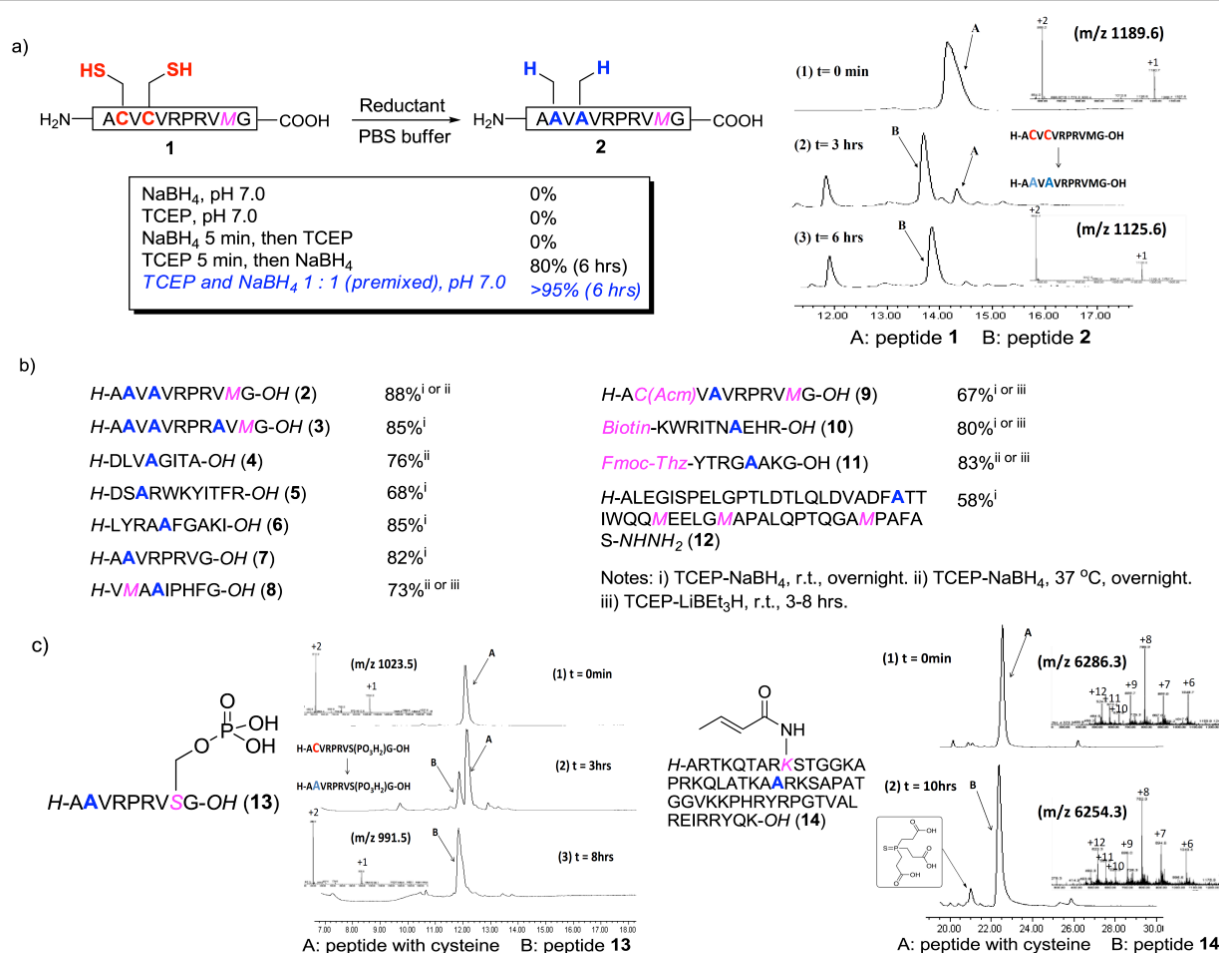
Figure 1. Condition optimization and substrate scope of the peptide desulfurization. a) Selected examples of reaction conditions for the desulfurization of the model peptide **1**. b) Substrate scope of the desulfurization. c) Desulfurization of peptides carrying post-translational modifications.

Next, we varied the ratio of TCEP-NaBH$_4$. Neither TCEP nor NaBH$_4$ in excess was good for the reaction, resulting in much slower reactions (Table S1, entries 6 and 7). The reaction proceeded optimally in the solution with a pH of 6–7. The acidic condition at pH 5 slowed down the desulfurization reaction (Table S1, entry 9), while the basic condition at pH of 8–9 caused low yields accompanied by formation of side products (Table S1, entries 10 and 11). Elevating temperature to 37 ^{o}C could expedite the reaction with a conversion of > 95% within 3 hours (Table S1, entry 12).

Other boron reagents, including NaBH(OAc)$_3$, NaBH$_3$CN, and B(OH)$_3$ in combination with TCEP could also participate in the desulfurization, however in inferior efficiency to NaBH$_4$ (Table S1, entries 13–17). Surprisingly, the peptide desulfurization with LiBEt$_3$H (1.0 M in THF)-TCEP condition proceeded even faster than that of TCEP-NaBH$_4$, with a > 95% conversion within 3 hours at room temperature (Table S1, entry 18). Thus, the conditions TCEP-NaBH$_4$ and TCEP-LiBEt$_3$H were chosen for further investigations.

Next, we explored the scope and robustness of TCEP-NaBH$_4$ and TCEP-LiBEt$_3$H system for desulfurization of various peptide substrates. As illustrated in Figure 1b, all amino acids were compatible with this desulfurization condition, affording the desired peptide desulfurization products **2–11** in 67–88% yields after HPLC purification (> 90% conversion in LC-MS monitoring). Up to three cysteine residues could be desulfurized simultaneously with high efficiency. More importantly, sulfur-containing moieties other than cysteine, such as methionine (**2**, **3** and **8**), biotin (**10**), cysteine side chain protected with Acm group (**9**), and N-terminal cysteine protected with Fmoc and Thz (**11**) were untouched under this desulfurization condition. Peptide hydrazide of **12** (granulocyte colony-stimulating factor121-172)[26] was also compatible with the desulfurization condition with 58% isolated yield after HPLC purification. The results showed that TCEP-NaBH$_4$ or TCEP-LiBEt$_3$H system could be used not only in the global desulfurization of peptides, but also in the desulfurization of intermediates/precursors for sequential NCL.

For more challenging cases, we also tested the effectiveness of our condition on the peptides carrying post-translational modifications (PTMs). The preparation of homogeneous peptides/proteins with site-specific PTMs showcases the state of the art of the protein chemical synthesis. To our delight, as shown in Figure 1c, the results were very encouraging. The peptide **13** with phosphorylation on serine

side chain was generated smoothly in 8 hours via TCEP-NaBH$_4$ mediated desulfurization in good yield, and no hydrolysis of phosphate and β-elimination was observed. In the case of peptide **14** (histone H3 1-56) with crotonylation on lysine side chain, the desulfurization was completed in 10 hours without detectable conjugate addition or reduction of the labile crotonyl group.

Having demonstrated the potential of this new desulfurization condition, we tried to understand the mechanism of this transformation. It has been previously shown that borohydrides could participate in reductive radical chain reactions to act as a hydrogen source, such as photoreduction of halides.[27] However, all these reactions required either a radical initiator or photo-irradiation. As a comparison, darkness and extreme anaerobic reaction using de-gassed solvents did not diminish the effectiveness of our peptide desulfurization condition. In addition, the desulfurization product could be obtained when B(OH)$_3$, of which without B-H or B-Et bonds, was used as boron source, albeit in a lower yield. More importantly, one deuterium was incorporated site-specifically at the alanine residue in **16** and **18** during the desulfurization of peptides **15** and **17** when the reactions were conducted in the D$_2$O buffer (Figure 2a), while no deuteration was observed when TECP-NaBD$_4$ was used in H$_2$O. On the contrary, incorporation of deuterium was not observed when the TCEP/VA-044/ *t*-BuSH condition was used in the D$_2$O buffer. These results unambiguously indicated the solvent but not the borohydride species served as the hydrogen source in the desulfurization. Indeed, this method provides a new approach to produce site-specifically deuterated peptides.
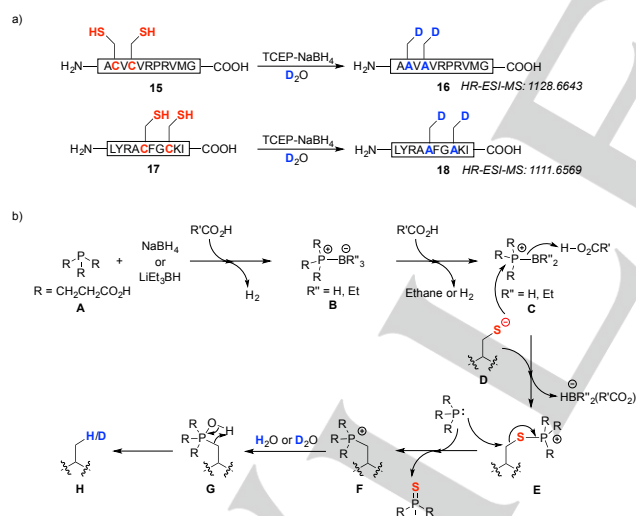
Figure 2b, premixed phosphine **A** (e.g. TCEP) and NaBH$_4$ or LiBEt$_3$H reacted in water to generate phosphane-borane complex **B** with gas bubbles evolved (H$_2$ or ethane).[29] Further protonolysis of the B-H and B-Et bonds provided positively charged complex **C**, which reacted with the thiol anion species **D** of the cysteine residue to give the P-S species **E** together with P-B bond cleavage. In the next step, the carbon atom of the C-S bond was attacked by another molecule of phosphine to give phosphonium salt **F** and phosphine sulfide. The salt **F** underwent alkaline hydrolysis[30] to produce the desulfurized product **H** via intermediate **G**, in which the proton from water (or deuterium from D$_2$O) transferred to the carbon center. As the evidence for this mechanism, both phosphine oxide and phosphine sulfide derived from TCEP were observed in the desulfurization process.

To further validate the applicability of our desulfurization method, we investigated the total synthesis of cyclic peptide natural products *Dichotomin C* **21** and *Dichotomin E* **24**. These peptides were isolated from *Stellaria dichotoma* with growth inhibiting activities in the preliminary testing.[31] As shown in Figure 3a and 3c, the linear hexapeptide hydrazide **19** and pentapeptide hyrazide **22** were assembled on hydrazine resin via Fmoc SPPS. After the generation of thioesters following the procedure developed by Liu and co-workers,[32-34] the cyclic peptides **20** and **23** bearing cysteine were obtained after intramolecular native chemical ligation. Upon treatment with TCEP-NaBH$_4$, the natural products *Dichitomin C* and *E* were obtained in 77% and 71% yields respectively after HPLC purification.
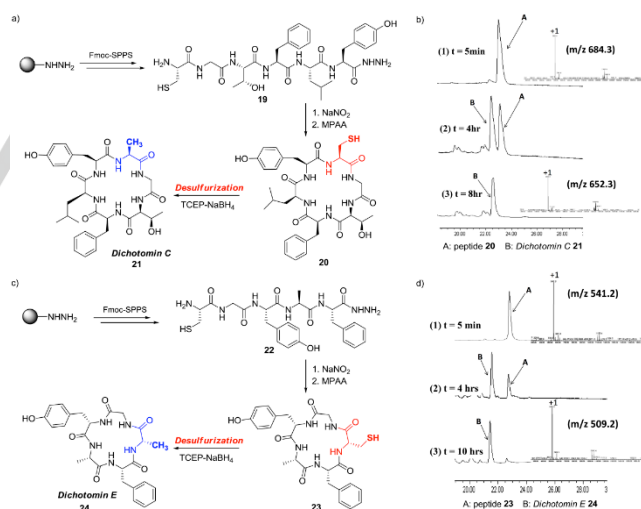

Figure 2. a) Site-specific deuteration of peptides via TCEP-NaBH$_4$ desulfurization. b) Proposed mechanism of this reaction.

At this stage, although the possibility of a radical reaction-based desulfurization could not be completely ruled out,[28] we proposed an alternative mechanistic pathway. As shown in

Figure 3. Total synthesis of *Dichotomin C* and *E* via NCL and TCEP-NaBH$_4$ mediated desulfurization. a) Synthetic route of *Dichotomin C*. b) LC trace of the desulfurization of peptide **20**. c) Synthetic route of *Dichotomin E*. d) LC trace of the desulfurization of peptide **23**.
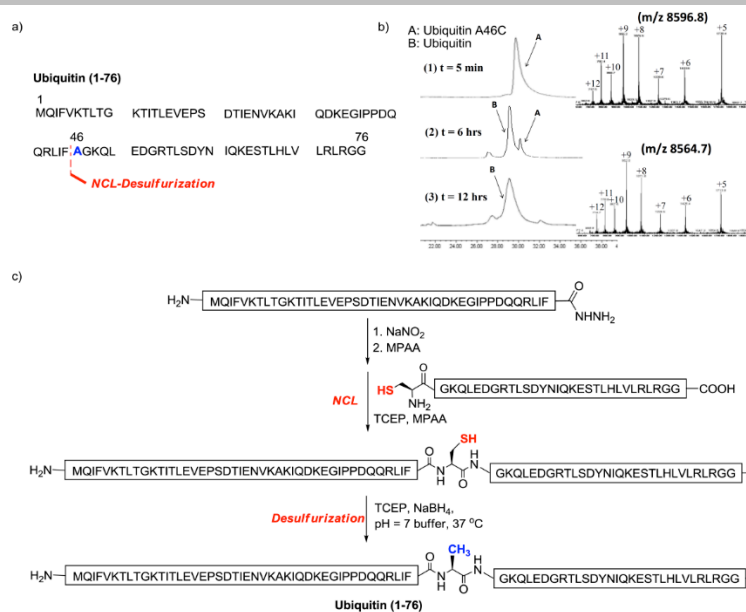
Figure 4. Total synthesis of ubiquitin via NCL-desulfurization. a) Sequence of ubiquitin and ligation site. b) LC trace of the desulfurization and MS analysis. c) Synthetic route towards ubiquitin.
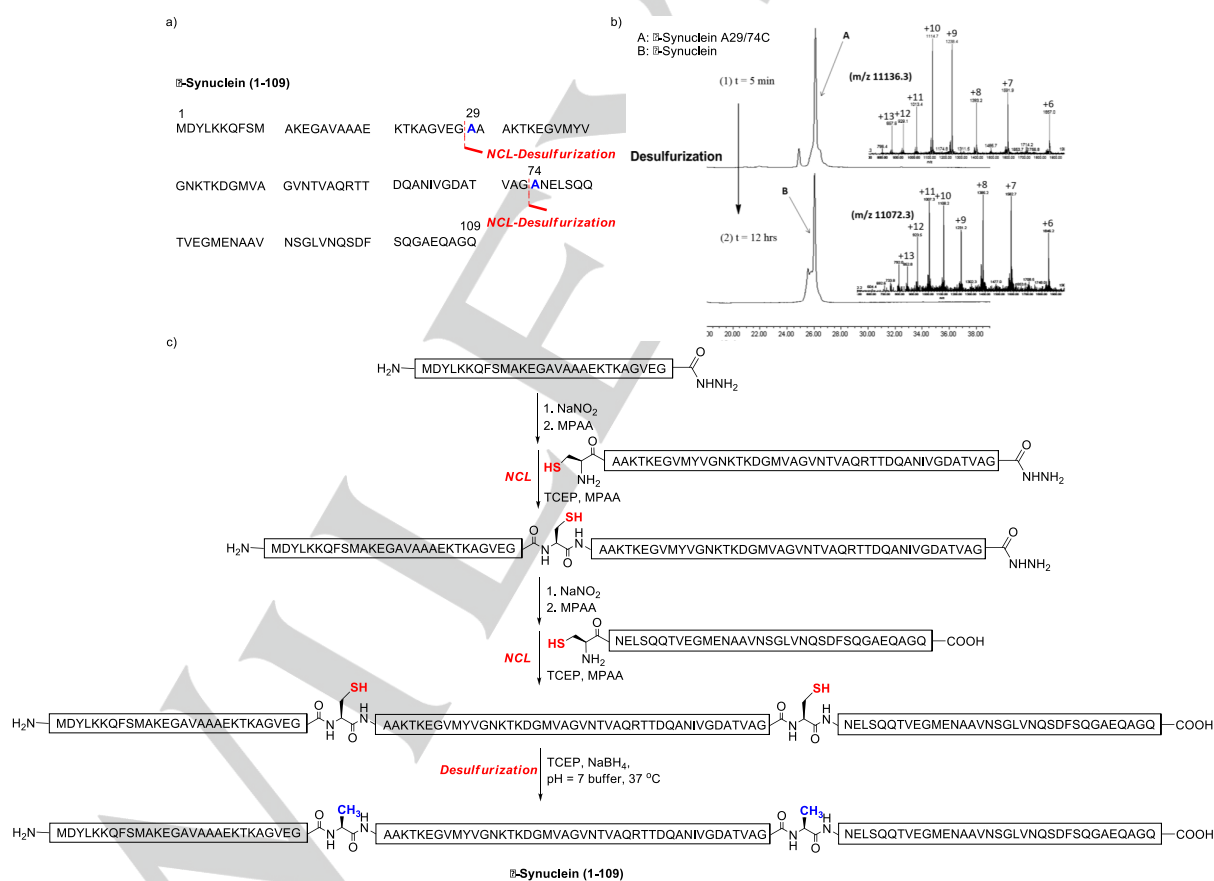

Figure 5. Total synthesis of γ-synuclein via NCL-desulfurization. a) Sequence of γ-synuclein and ligation sites. b) LC trace of the desulfurization and MS analysis of the products. c) Synthetic route towards γ-synuclein.

Finally, we further demonstrated the effectiveness of this newly developed desulfurization condition in protein chemical synthesis. Small protein ubiquitin (76 residues),[35] which plays key roles in proteasome-dependent protein degradation[36] and signalling[37], was first chosen to showcase this desulfurization method. As shown in Figure 4, the ubiquitin was disconnected into peptide fragment (1–45) and fragment (46–76), while the Ala46 was replaced by Cys46 to facilitate the native chemical ligation. The peptide fragment (1–45) thioester was prepared via peptide hydrazide method using Fmoc-SPPS.[33] After the NCL with fragment (46–76), the obtained product was treated with the TCEP-NaBH$_4$ (overnight at 37 ^{o}C) condition and the TCEP-LiBEt$_3$H condition (overnight at room temperature) to smoothly convert Cys46 to Ala46. The native ubiquitin was isolated in 72% yield after HPLC purification.

Encouraged by this success, we next went forward to synthesize two other proteins with larger size, namely γ-synuclein (109 residues)[38, 39] and histone H2A (129 residues)[40]. Each protein was assembled via two native chemical ligations of three peptide fragments at the alanine sites which were replaced by cysteine residues. In the case of γ-synuclein, the thioester of peptide fragments 1–28 and 29–73 were prepared via hydrazide method using Fmoc-SPPS,[33] and the full length double sites mutated protein (A29C and A74C) was obtained via two sequential NCLs. In the final desulfurization step, the mutant was treated with the TCEP-NaBH4 overnight at 37 ^{o}C or TCEP-LiBEt$_3$H for 12 hours at room temperature, and γ-synuclein was obtained in 65% yield after HPLC purification (Figure 5). In a similar manner, the full-length double sites mutated histone protein H2A (A48C and A87C) which was generated via two sequential NCLs, was treated with TCEP-LiBEt$_3$H for 12 hours at room temperature for desulfurization. The native H2A protein was isolated in 61% yield after HPLC purification (see Supporting Information for details).

In summary, we have developed an inexpensive, mild and versatile chemoselective peptide desulfurization method, termed *P-B* desulfurization, using TCEP-NaBH$_4$ or TCEP-LiBEt$_3$H system. The reaction very likely proceeds via *in situ* generated phosphane-borane complex. This method features simplicity of operation, robustness, high yielding, clean conversion, and versatile functionality compatibility with protein chemical synthesis. It is noteworthy that this condition could incorporate deuterium selectively at the desulfurization site when running in D$_2$O, thus providing a new approach to produce site-specifically deuterated peptides. We have combined this protocol with native chemical ligation for chemical total synthesis of several proteins via NCL-desulfurization. We believe such a method could become an important supplement to the repertoire of protein chemical synthesis and modifications.

ACKNOWLEDGMENT

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Layout 2:

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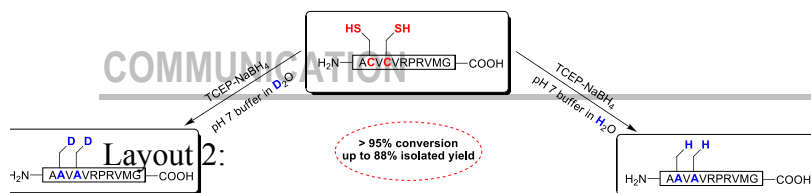
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