VIP Radical Reactions

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Free-Radical-Based, Specific Desulfurization of Cysteine: A Powerful Advance in the Synthesis of Polypeptides and Glycopolypeptides**

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The total synthesis of complex glycoproteins bearing multiple oligosaccharide domains is a central focus of our research group. We are particularly interested in targeting, for de novo synthesis, specific glycoproteins which possess extraordinary biological activity. The sheer magnitude of the synthetic challenge is such that we often encounter the limits of current methodology. In this context, our glycoprotein synthetic program provides a unique imperative for developing new and more powerful capabilities.

A convergent strategy for glycoprotein synthesis entails the assembly of individual peptidyl substrates, each bearing a single carbohydrate domain. These subunits would then be merged, in an iterative fashion, to ultimately afford the homogeneous, multiply glycosylated peptide or protein target. The cysteine-based native chemical ligation (NCL) protocol developed by Kent and co-workers (Figure 1a),[1] following earlier feasibility demonstrations by Kemp et al.,[2] involves the merger of a C-terminal thioester

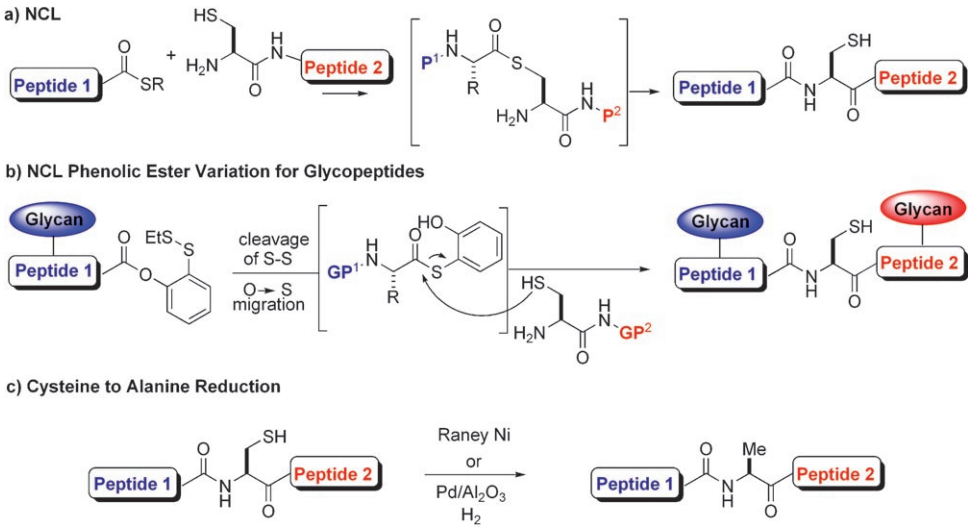

Figure 1. Native chemical ligation and its extensions.

and an N-terminal cysteine residue. NCL provided a major advance in peptide synthesis, and has served as a launching point for many further investigations, including our own.[3] Thus, as outlined in Figure 1b, we had developed a novel NCL variant which allows for the merger of glycopeptides, which bear either *O*- or *N*-linked glycan residues, through the use of a relatively inert C-terminal *ortho*-thiophenolic ester. This group harbors the latent thioester functionality required for cysteine-based NCL.[4] In light of the relative scarcity of cysteine residues in many naturally occurring proteins and glycoproteins, it would be valuable to be able to achieve comparable ligations at non-cysteine sites. As such, considerable effort has been addressed to the development of cysteine-free ligation methods. We recently disclosed two complementary non-cysteine-based ligation protocols which together provide a means by which to perform iterative NCL through kinetically controlled ligation.[5]

An alternative solution to the problem of cysteine dependence, first proposed by Yan and Dawson, took advantage of cysteine-based NCL and converted the erstwhile N-terminal cysteine residue into an alanine residue through the action of either Raney nickel or Pd/Al_2O_3 (Figure 1c).[6] In essence, Yan and Dawson had provided a means by which to use cysteine residues as surrogates for more abundant

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alanine residues. These NCL global desulfurization protocols[7] have since been employed in the synthesis of a number of cysteine-free proteins with some success. In the course of our own efforts to synthesize large, complex glycoprotein targets we examined the feasibility of such methods for the reduction of cysteine to alanine. In particular, we sought to determine whether such metal-based protocols are compatible with the extensive functionality present in our glycopeptide substrate systems. In this context, Pentelute and Kent recently reported that the Raney nickel method can effectively accommodate both methionine and the acetamidomethyl (Acm) functionality, which is commonly employed as a protecting group for cysteine.[8] However, a significant drawback of this method is that it requires large excesses of nickel.[9] Furthermore, the use of Raney nickel can cause epimerization of secondary alcohols[10] and the reduction of thiols, thioethers, and thioesters.[11] Meanwhile, although Wong and co-workers have found that another metal-based protocol, which utilizes Pd/Al_2O_3, is also able to accommodate both methionine and the Acm functionality,[12] we observed that the thiazolidine (Thz) moiety, which serves as an ideal masking group for N-terminal cysteine residues, is not stable under these conditions.[13] Thus, given these limitations, and given the complexity and level of functionalization of our glycopeptide constructs, we sought to develop a mild, nonmetal-based reduction method for cysteine. We would require that such a method be tolerant of a range of functional groups, including carbohydrate sectors, various amino acids (particularly methionine), and a range of sulfur-containing groups, such as Cys(Acm), biotin, Thz, and thioesters.

We began by taking note of a disclosure by Hoffmann et al. in 1956 which described a desulfurization reaction between mercaptan and trialkylphosphite derivatives under both thermal and photochemical conditions.[14] Soon thereafter, in a key advance, Walling and Rabinowitz put forth a proposed mechanistic sequence, wherein an alkylthiyl radical adds reversibly to phosphite, thereby generating an intermediate phosphoranyl radical.[15] Subsequent β scission was envisioned to provide an alkyl radical, and rapid hydrogen abstraction from the parent thiol would furnish the product alkane, thereby serving to propogate the chain (Scheme 1). In addition to this contribution to the mechanistic understanding of the reduction, Walling et al. extended the reaction to trialkylphosphines.[15b]

On the basis of these findings, Valencia and co-workers have developed a method by which cysteine can be reduced to alanine through the action of triethylphosphite and a borane radical initiator.[16] Our own efforts to apply such conditions to effect the reduction of model peptide substrates were met with very limited success. In addition to small amounts of the desired reduced adduct, we typically observed extensive side products. We suspected that the problems observed might be attributable to issues of phosphite solubility in aqueous solution.

Accordingly, we came to consider the possibility of utilizing trialkylphosphines to effect the desired cysteine reduction in peptide settings. In particular, we were drawn to tris(2-carboxyethyl)phosphine (TCEP), which has enjoyed wide use as a disulfide reducing agent in peptide and glycopeptide settings.[17,18] Many considerations served to identify TCEP as the phosphine source. Most importantly, its ability to tolerate a range of glycopeptide functionality is well-established. Furthermore, it is easily manipulated in air and reacts readily in aqueous solution over a wide pH range. We selected, as the radical initiator, the water-soluble 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044), which has a very low temperature of decomposition.[19]

In the event, peptide **1** (Fmoc-RYKDSG**C**AHPRG-OH) was exposed to TCEP, *t*BuSH, water, and VA-044 at room temperature. We were pleased to observe nearly quantitative conversion of the cysteine residue into alanine within 10 h, as determined by LCMS (Figure 2). Following purification,

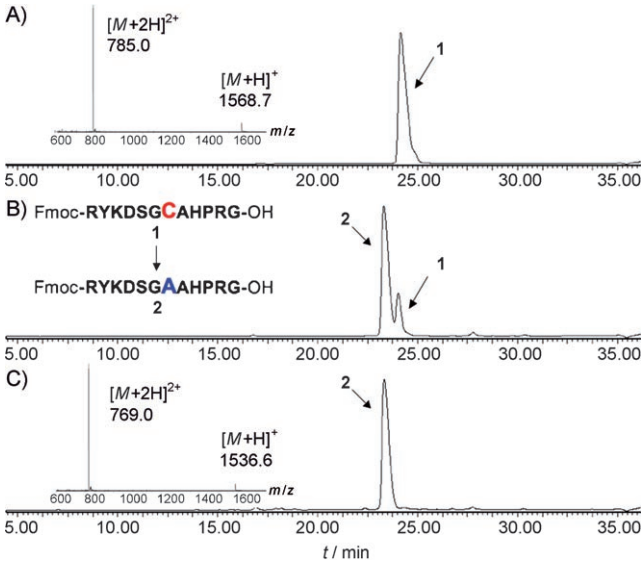
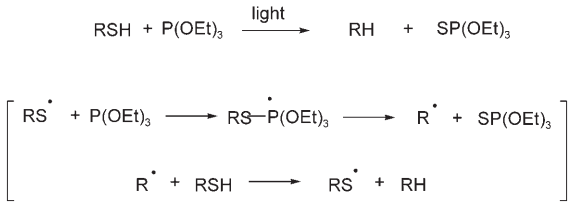


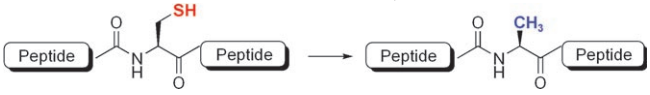
Figure 2. Model study for the selective free-radical desulfurization of Cys to Ala in water at RT. LCMS chromatograms of: A) cysteinyl peptide **1**; B) crude products observed 3 h after treatment with TCEP, *t*BuSH, VA-044 at RT; C) crude alanyl peptide **2** after 10 h. VA-044 = 2,2′-azobis-[2-(2-imidazolin-2-yl)propane]dihydrochloride. Fmoc = 9-fluorenylmethoxycarbonyl.



Scheme 1. Proposed mechanism of radical desulfurization reaction.

peptide **2** was isolated in 82% yield. We next prepared and evaluated a series of peptide substrates, incorporating a range of relevant functional groups (Table 1). Importantly, our reaction conditions were able to efficiently accommodate a variety of important functionalities, including methionine (entry 4), Cys(Acm) (entry 5), Thz (entry 6), and biotin (entry 7). To our knowledge, this TCEP-based method

Table 1: Free-radical-mediated transformation of Cys to Ala.



No.	Cysteinyl peptide	Alanyl peptide	Yield [%]
1	Fmoc-RYKDSG**C**AHPRG-OH (**1**)	Fmoc-RYKDSG**A**AHPRG-OH (**2**)	82[a]
2	H-LRHKDS**C**RWKITR-OH (**3**)	H-LRHKDS**A**RWKITR-OH (**4**)	81[b]
3	H-VETRFP**C**RNYEK-OH (**5**)	H-VETRFP**A**RNYEK-OH (**6**)	71[b]
4	H-RFDS**C**RPMHWR-OH (**7**)	H-RFDS**A**RPMHWR-OH (**8**)	74[b]
5	H-VRYT**C**KLSCys(AcM)WR-OH (**9**)	H-VRYT**A**KLSCys(AcM)WR-OH (**10**)	89[b]
6	Fmoc-Thz-YTRG**C**AKG-OH (**11**)	Fmoc-Thz-YTRG**A**AKG-OH (**12**)	75[c]
7	Biotin-KWRITN**C**EHR-OH (**13**)	Biotin-KWRITN**A**EHR-OH (**14**)	91[c]

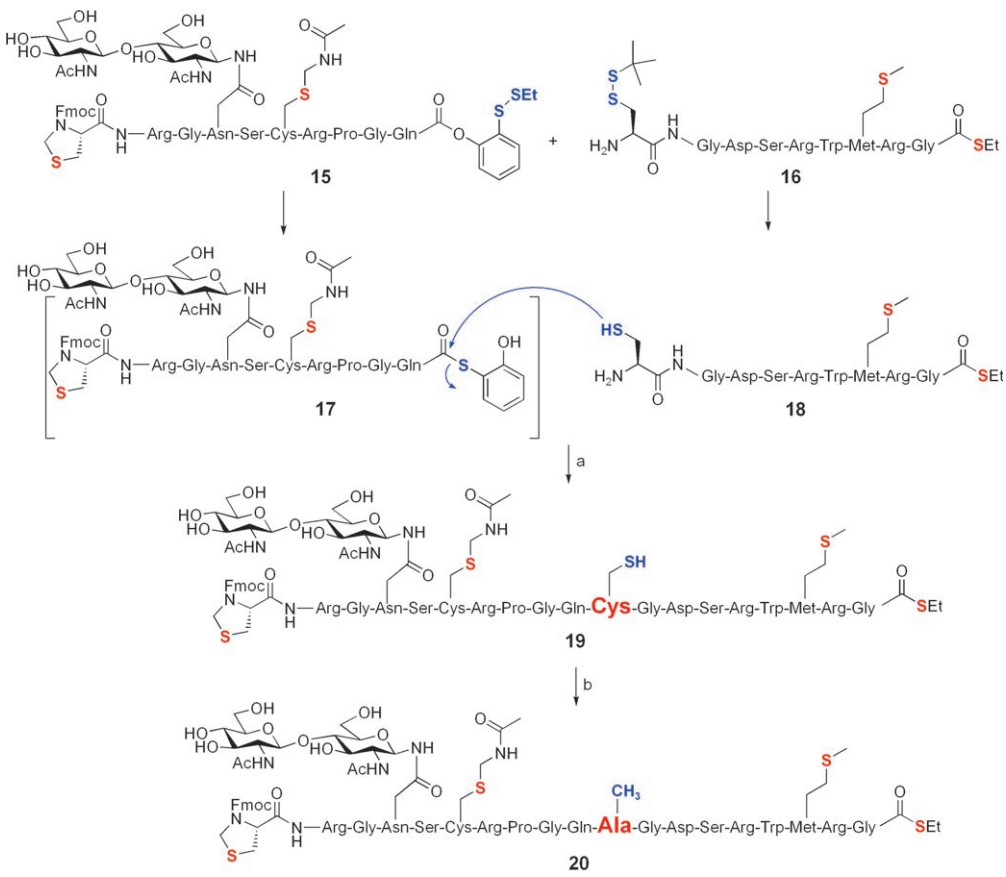
Reaction conditions: a) TCEP, *t*BuSH, VA-044 at RT; b) TCEP, *t*BuSH, VA-044, 37 °C; c) 6.0 M Gn·HCl, 0.2 M Na_2HPO_4, 0.19 mM TCEP·HCl buffer at pH 6.3, TCEP, *t*BuSH, VA-044, 37 °C. Thz = thiazolidine.

marks the mildest and most selective cysteine desulfurization protocol developed to date.

We next sought to probe the versatility of the reaction by combining kinetically controlled[20] glycopeptide–peptide ligation with cysteine reduction in a highly functionalized setting. The objective was to determine whether this newly developed protocol could be applied to the types of complex systems that we would encounter in the course of a typical glycoprotein synthesis. Thus, glycopeptide **15**, which has an N-terminal Thz group, an Acm-protected Cys residue, an *N*-linked glycan, and the requisite C-terminal *ortho*-thiophenolic ester, was prepared and coupled with peptide **16**, which possesses a methionine residue and a C-terminal thioester (Scheme 2). As shown, the merger proceeded smoothly, presumably through the thioester intermediate **17**, to provide ligated adduct **19** within 2 h. We were pleased to observe that the subsequent reduction of Cys to Ala occurred without incident under our established TCEP-mediated conditions, thereby providing intermediate **20** (Figure 3). Importantly, this glycopeptide adduct incorporates a C-terminal thioester as well as an N-terminal masked cysteine residue, and may thus be extended in either direction through recourse to standard ligation techniques.

Although our primary focus is on the development of methods to enable the synthesis of large glycoproteins, we were not insensitive to the possible applicability of this type of capability to other areas of peptide synthesis. In particular, the newly developed cysteine reduction protocol might have positive implications in the synthesis of cyclic peptides, which frequently do not possess any cysteine residues. In the event, we were able to successfully apply our mild, free-radical desulfurization method to the synthesis of the cyclic peptide crotogossamide (Scheme 3), which was isolated from the latex of *Croton gossypifolius*.[21] Thus, the linear peptide **21** was prepared by using Fmoc solid-phase peptide synthesis (Fmoc-SPPS) and standard thiol ester installation. Native chemical ligation provided cyclic peptide **22**, which incorporates an unnatural cysteine residue in place of the requisite alanine group. The key TCEP-mediated cysteine reduction occurred smoothly to provide the naturally occurring cyclic nonapeptide crotogossamide.[22]

Finally, in a related effort, we explored the ability of our free-radical desulfurization method to accomplish the reduction of an unnatural selenocysteine residue to an alanine



Scheme 2. Glycopeptide synthesis through kinetically controlled ligation followed by selective free-radical desulfurization. a) 6.0 M Gn·HCl, 0.2 M Na_2HPO_4, 0.19 mM TCEP·HCl buffer at pH 6.3, 67%; b) EtSH, *t*BuSH, TCEP and VA-044, 37 °C, 87%.

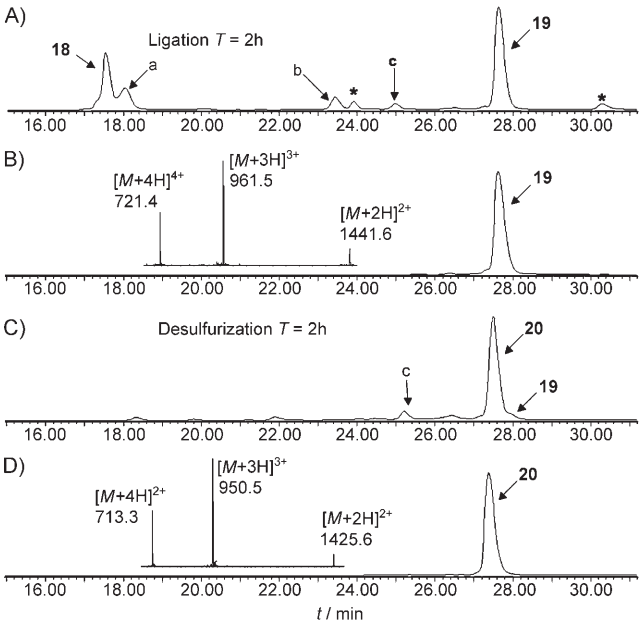
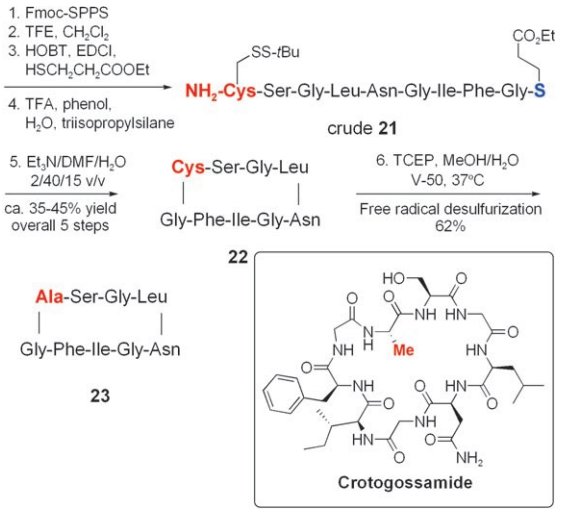
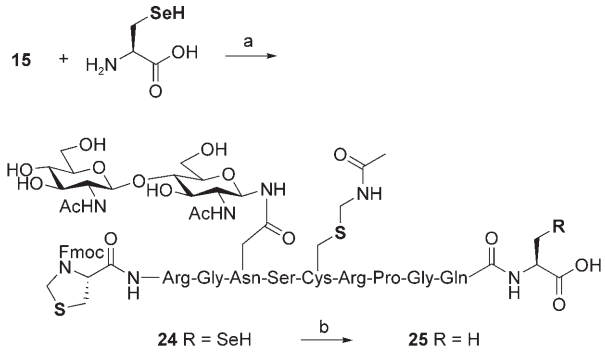


Figure 3. Glycopeptide synthesis through kinetically controlled ligation followed by selective free-radical desulfurization. LCMS chromatograms of: A) glycopeptide **15** and peptide **16** in the ligation after 2 h; peptide **18** with the observed mass $[M+2H]^{2+}=556.2$ and $[M+H]^{+}=1111.3$; peak a: 2-mercaptophenol; peak b: hydrolyzed glycopeptide **15** with the observed mass $[M+2H]^{2+}=895.0$; peak c: thiolactone with the observed mass $[M+3H]^{3+}=940.4$; the asterisks denote unidentified products. B) After HPLC purification, ligated product **19**. C) Crude products observed 2 h after treatment with TCEP, *t*BuSH, VA-044 at 37 °C. D) After HPLC purification, the newly formed alanyl compound **20** with the observed signals (*m*/*z*) at $[M+2H]^{2+}=1425.6$, $[M+3H]^{3+}=950.5$, and $[M+4H]^{4+}=713.3$.



Scheme 3. Synthesis of the cyclic peptide crotogossamide. TFE = trifluoroethanol, HOBT = 1-hydroxy-1*H*-benzotriazole, EDCI = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, TFA = trifluoroacetic acid.

residue.[23] Thus, glycopeptide **24**, prepared from **15** and L-selenocysteine (generated in situ from the reduction of L-selenocystine), as shown (Scheme 4), was subjected to our



Scheme 4. Conversion of selenocysteine into alanine by seleno-NCL. a) selenocysteine, 6.0 M Gn·HCl buffer (pH 6.3), TCEP, 1 h, RT; b) VA-044, 35 °C, TCEP, 4 h, 77% yield over two steps.

TCEP reduction protocol. We were pleased to observe that the desired transformation occurred readily to afford glycopeptide **25**, which incorporates an alanine residue in place of the selenocysteine group.

In summary, a mild and highly versatile free-radical[24] cysteine reduction method based on classical organic chemistry has been developed. This metal-free reduction protocol can accommodate a range of relevant functionality and will quite likely find broad application in complex peptide and glycopeptide settings.

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