Recent extensions to native chemical ligation for the chemical synthesis of peptides and proteins

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Abstract.

Native chemical ligation continues to play a pivotal role in the synthesis of increasingly complex peptide and protein targets twenty years after its initial report. This opinion article will highlight a number of recent, powerful extensions of the technology that have expanded the scope of the reaction, accelerated ligation rates, enabled chemoselective post-ligation modifications, and streamlined the ligation of multiple peptide fragments. These advances have facilitated the synthesis of a number of impressive protein targets to date and hold great promise for the continued application of native chemical ligation for the detailed study of protein structure and function.

Introduction.

Proteins exhibit a diverse array of structure and function. The immense variety of functional roles played by these macromolecules results from the precise make-up of the polypeptide amino acid sequence, the consequent three-dimensional structure of the folded protein molecule, and finally, the potential for adornment with post-translational modifications. Thus, as a result of the multi-layered structural complexities of these important molecules, the total chemical synthesis of homogeneous, functional proteins represents a challenging facet of modern organic synthesis.

One of the most influential advancements in the chemical synthesis of peptides and proteins has been the development of chemoselective ligation protocols, in particular the discovery of native chemical ligation[1]. This reaction enables the condensation of two unprotected peptide fragments, in aqueous media and under mild reaction conditions, to generate a native amide linkage in an efficient and high-yielding manner. First reported in 1994 by Kent and coworkers, this methodology involves the reaction of a peptide containing a C-terminal thioester with a peptide bearing an N-terminal cysteine (Cys) residue (Figure 1). Mechanistically, the reaction proceeds *via* an initial transthioesterification between the thiol side-chain of Cys and the C-terminal acyl donor to generate an intermediate thioester-linked adduct, which rearranges through an intramolecular S-to-N acyl shift *via* a 5-membered ring intermediate to generate a native peptide bond. Since its inception, a number of advances to the initial ligation methodology have aimed to increase the scope of the reaction (particularly to address the requirement for an N-terminal Cys residue), accelerate the rate of ligation, and to facilitate iterative ligations and chemoselective post-ligation amino acid modifications. These advancements can be broadly distilled into three main research areas: 1) the development of new N-terminal Cys surrogates; 2) the development of new C-terminal acyl donors; and 3) the inclusion of various exogenous thiol additives to modulate the reactivity of the C-terminal thioester moiety[2] (see Figure 1). Over the years, there have been several comprehensive reviews of native chemical ligation and the application of the technology in

the chemical synthesis of peptides and proteins[3-9]. In addition, Dirksen and Dawson provided an insightful perspective on the expanding scope of ligation strategies, including native chemical ligation, in 2008[10]. On the 20th anniversary of the seminal report of the reaction, this opinion article serves to update the reader by highlighting a number of recent, powerful extensions to the native chemical ligation manifold in each of the three areas listed above as well as provide a commentary on current limitations and future challenges.

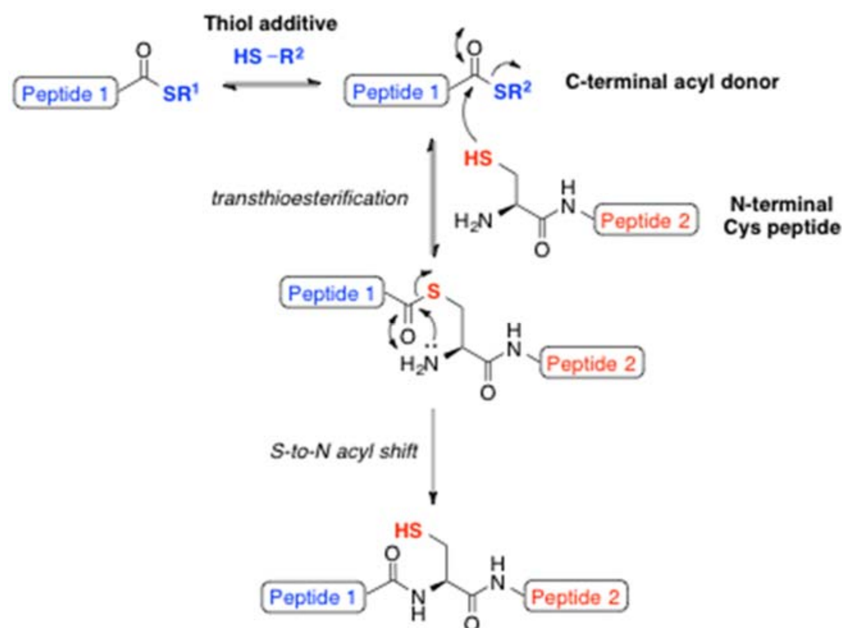


Figure 1. Mechanism of the native chemical ligation reaction.

1. Development of new N-terminal Cys surrogates.

Ligation-desulfurization chemistry.

Native chemical ligation traditionally relies on the presence of an appropriately placed Cys residue in a peptide or protein sequence in order to effectively disconnect the target. However, the relatively low abundance of Cys (1.1%) in naturally occurring proteins has prompted intense efforts to develop N-terminal Cys surrogates that can extend the applicability of the reaction, *via* a similar pathway to native chemical ligation, but that can be subsequently removed or manipulated to generate other proteinogenic amino acids at the ligation junction. In the early 2000s, there was a flourish of activity in the study of removable N-linked and side-chain ligation auxiliaries in the ligation-based assembly of peptides and proteins[10]. Challenges in expanding the scope and increasing the yields of these auxiliary-promoted ligations, however, have prompted the exploration of alternative approaches. These efforts have recently converged on the use of ligation-desulfurization chemistry[11,12], a concept first demonstrated by Yan and Dawson through reductive desulfurization of Cys following the ligation event to generate a native Ala [13••]. The development of a mild and metal-free radical desulfurization protocol employing the water-soluble radical initiator VA-044 in the presence of tris(2-carboxyethyl)phosphine (TCEP) and *t*-BuSH[14••] or reduced glutathione[15] has further fuelled the adoption of post-ligation desulfurization methodologies.

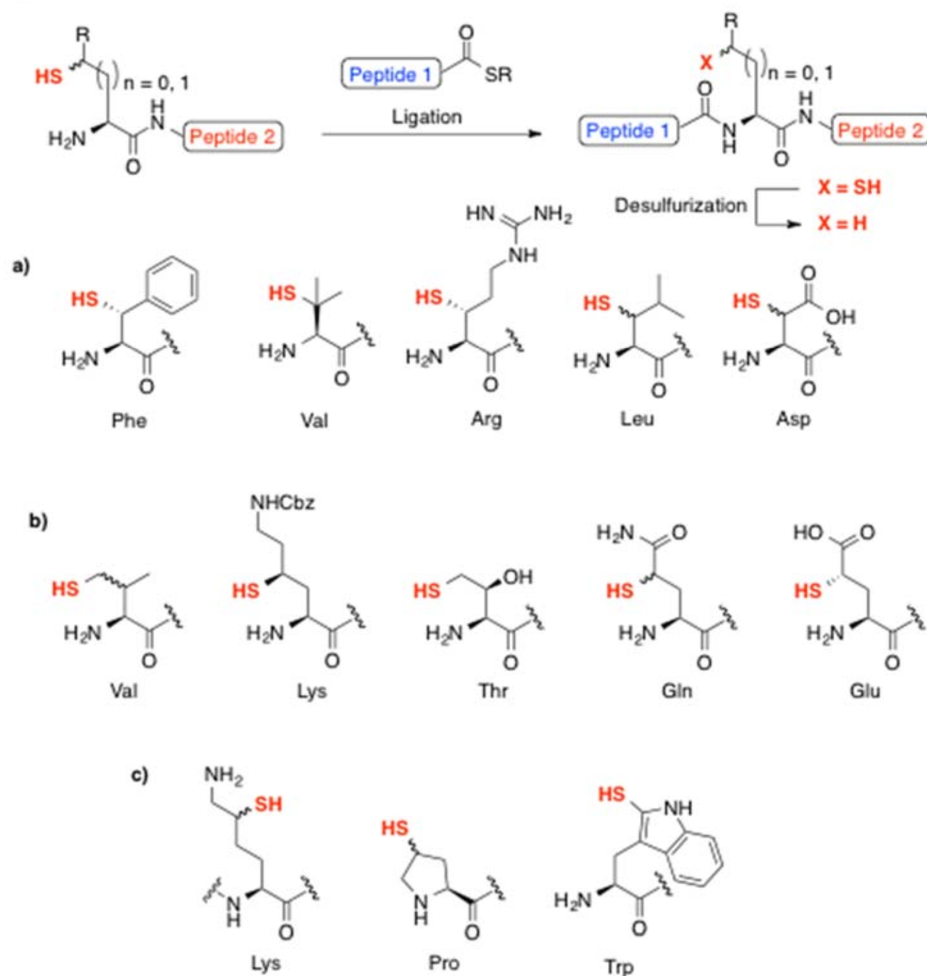
The concept of employing unnatural amino acid derivatives bearing suitably positioned thiol auxiliaries[13••] in ligation-desulfurization chemistry has recently come to fruition (Figure 2I) and represents a crucial extension to the native chemical ligation methodology. Beginning with the preparation of β-thiol phenylalanine (Phe)[16,17] and the application of this Phe derivative in peptide ligation followed by a post-ligation reductive desulfurization with nickel boride[17], a number of additional thiol-derived amino acids have been added to the ligation-desulfurization toolbox. These additions include access to post-desulfurization Xaa-Yaa ligation junctions where Yaa can be valine (Val)[15,18], lysine (Lys)[19-21], threonine (Thr)[22], leucine (Leu)[23,24], proline (Pro)[25,26], glutamine (Gln)[27], arginine (Arg)[28], aspartic acid (Asp)[29•,30], glutamic acid (Glu)[31] or tryptophan (Trp)[32] using suitable thiol-derived amino acid building blocks (Figure 2Ia-c). Importantly, thiolated amino acids tend to exhibit increased rates of reactivity and improved reaction scope relative to N-linked and side-chain appended auxiliaries, owing in part to the decreased steric bulk at the ligation junction (relative to N-linked auxiliaries) and the ability to proceed primarily through 5-membered (for β-thiol derivatives, Figure 2Ia) or 6-membered (for γ-thiol derivatives, Figure 2Ib) ring intermediates in the S-to-N acyl transfer step. Ligation-desulfurization chemistry using these thiol-derived building blocks has also been successfully employed in the synthesis of a number of complex peptide and protein targets, including the construction of human parathyroid hormone[33•], a mucin 1 (MUC1) glycopeptide oligomer[28] and to facilitate side-chain ubiquitination of α-synuclein[34].

On-going challenges in the development of ligation-desulfurization methodologies include the development of more practical routes to thiol-derived amino acid building blocks. With the exception of penicillamine[15] and γ-thioproline building blocks[25], which are commercially available, and the late-stage installation of a Trp thiol auxiliary onto unprotected peptides[32], the majority of synthetic approaches to thiol-derived amino acids require multiple synthetic steps. Challenging syntheses of the various β-, γ- and δ-thiol amino acids in suitably protected form currently present a barrier to the widespread adoption of these building blocks by the peptide and protein chemistry community.

Chemoselective ligation-deselenization and ligation-desulfurization chemistries.

One drawback of employing a post-ligation global desulfurization is that the chemistry in most cases is not chemoselective in the presence of other sulfhydryl moieties. As such, it is necessary to protect native Cys residues within the target sequence to avoid concomitant conversion to Ala upon treatment with reductive or radical desulfurization conditions. The development of side-chain ligation auxiliaries that can be chemoselectively manipulated in the presence of unprotected, non-ligation site Cys residues therefore represents an important area of on-going research. Towards this end, following a 2010 discovery by Dawson and coworkers that selenocysteine (Sec) residues can be chemoselectively deselenized in the presence of unprotected Cys residues (Figure 2II)[35••], there has been renewed interest in the use of Sec as a viable Cys ligation surrogate[36]. The key deselenization reaction takes place at room temperature in the presence of TCEP and dithiothreitol (DTT) and is thought to proceed through a similar mechanism to the phosphine-mediated radical desulfurization of Cys in the presence of a radical initiator [35••]. The selectivity of the deselenization may be attributed to the preferential formation of selenium-centered radicals over sulfur-centered radicals and the relatively weak carbon-selenium bond; however, detailed mechanistic studies have not yet been reported.

The observed chemoselectivity in the deselenization of Sec in the presence of Cys has catalyzed an interest in unnatural, selenol-derived amino acid derivatives for use in ligation-deselenization chemistry[35••]. To date, a γ-selenoproline[37] and a β-selenophenylalanine[38] derivative have been successfully employed in chemoselective deselenization reactions in the presence of unprotected thiols. Our laboratory has recently reported that β-thiol Asp residues can also be chemoselectively desulfurized in the presence of unprotected Cys residues upon treatment with TCEP and DTT at elevated temperatures[29•]. The utility of this unique reactivity was demonstrated through the synthesis of CXCR4(1-38), a chemokine receptor fragment bearing an internal Cys residue and two post-translational modifications, using a one-pot β-thiol Asp-mediated ligation followed by a chemoselective desulfurization reaction. In light of these recent discoveries, it is anticipated that the development of post-ligation, chemoselective manipulations of both thiol- and selenol-derived amino acids will be a subject of further exploration to increase the flexibility of ligation chemistry in the synthesis of proteins.



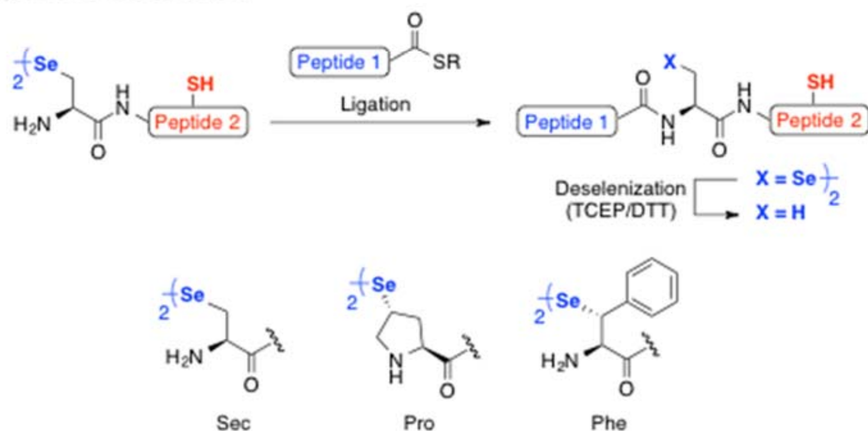


Figure 2. Cys surrogates used for native chemical ligation: (I) ligation-desulfurization chemistry employing (Ia) β-thiol amino acids, (Ib) γ-thiol amino acids and (Ic) other thiol-derived amino acids; (II) development of chemoselective ligation-deselenization chemistry at Sec and selenol-derived amino acids. NB: With the exception of 2-thiol Trp [32], the thiol and selenol amino acids are synthesized in protected form before incorporation into resin-bound peptides by standard solid-phase synthesis methods. The reader is referred to the original articles for the details of these protecting groups.

2. Development of new C-terminal acyl donors.

The efficiency of a given native chemical ligation reaction is heavily dependent on the nature of the C-terminal thioester in the acyl donor. A number of studies focused on varying this component of the reaction have led to improvements in ligation rate, synthetic accessibility of precursors, and the ability to perform iterative ligation reactions. The rate of ligation between N-terminal Cys residues and peptide thioesters at Xaa-Cys ligation sites is known to be heavily dependent on the steric and electronic properties of the C-terminal residue Xaa on the thioester component[39]. Sterically encumbered β-branched thioesters (e.g. Xaa = Ile, Thr and Val) substantially reduce the rate of ligation at these sites, and decreased electrophilicity combined with steric hindrance in the Xaa = Pro thioester (resulting from an orbital interaction with the carbonyl oxygen of the adjacent amide bond)[40] generally prohibits ligation at a Pro-Cys site. However, the recent report of rapid ligation at preformed peptide Pro selenoesters[41], which have superior leaving group ability compared to the corresponding thioester analogs, has now provided an innovative solution to accessing the otherwise intractable Pro-Cys ligation site.

While Boc chemistry *in situ* neutralization solid-phase peptide synthesis (SPPS) can be used for the efficient construction of peptide thioesters, a number of new methods that are compatible with the more widely practiced Fmoc chemistry SPPS have been developed to facilitate the efficient construction of C-terminal thioesters and acyl donors[42]. In particular, the use of *in situ* N-to-S acyl transfer strategies has garnered significant attention for the preparation of peptide thioesters and has been the subject of a recent review[43]. A noteworthy advance in this area has been the development of the bis(2-sulfanylethyl)amino (SEA) group[44,45] which has been employed as an on/off redox switch (Figure 3a) enabling one-pot iterative ligations in the N-to-C direction[46]. Dawson and coworkers have also reported an efficient Fmoc-SPPS approach to peptide *N*-acyl-benzimidazolinones (Nbz), which can be readily converted to peptide thioesters upon treatment with an exogenous aryl thiol (Figure 3b)[47]. This strategy begins with the synthesis of resin-bound peptide *o*-aminoanilides, which, following peptide elongation using standard Fmoc chemistry SPPS, undergo rapid cyclization to generate the resin-bound benzamidazolinone upon sequential treatment with *p*-nitrophenylchloroformate and a hindered base. Cleavage from the resin using a standard acidic cocktail generates the unprotected peptide-Nbz, poised for conversion to the corresponding thioester through the addition of a suitable thiol. Kent and coworkers have recently employed a glycopeptide-Nbz fragment for the total chemical synthesis of the chemokine protein CCL1, demonstrating the compatibility of the Nbz synthetic strategy with a complex, unprotected N-linked asialo-nonasaccharide[48•].

Another innovative approach to the facile preparation of peptide thioesters as well as an enabling technology for iterative ligations has been the development of peptide acyl hydrazides for use in the context of the native chemical ligation reaction[49••]. In 2011, Liu and coworkers reported a one-pot protocol for the ligation of peptide hydrazides to peptides bearing N-terminal Cys residues (Figure 3c)[49••]. In the first step, treatment of a fully deprotected peptide hydrazide with the oxidant $NaNO_2$ affords an intermediate peptide acyl azide in a chemoselective manner. The subsequent addition of an aryl thiol additive (e.g. 4-mercaptophenylacetic acid, MPAA) promotes *in situ* formation of the reactive thioester, which readily ligates with a peptide bearing an N-terminal Cys residue. Importantly, as conversion of the peptide hydrazide to the corresponding thioester first requires an "activation" step (the addition of the oxidant $NaNO_2$), the hydrazide effectively serves as a

masked thioester moiety, enabling careful, programmed control of the reactivity of bifunctional peptides bearing both an N-terminal Cys residue and a C-terminal hydrazide. As such, this methodology represents a powerful advance to the toolbox of ligation strategies, and has been used to enable iterative ligations in the N-to-C direction[49••] and, in combination with other ligation approaches, in the C-to-N[50] direction. Most recently, peptide hydrazides have been employed in the synthesis of the 140 amino acid protein α-synuclein using a four segment, iterative N-to-C ligation approach (Figure 3d)[51]. In addition, this methodology has been successfully applied to protein semi-synthesis using protein hydrazides obtained from recombinant expression[49••,52].

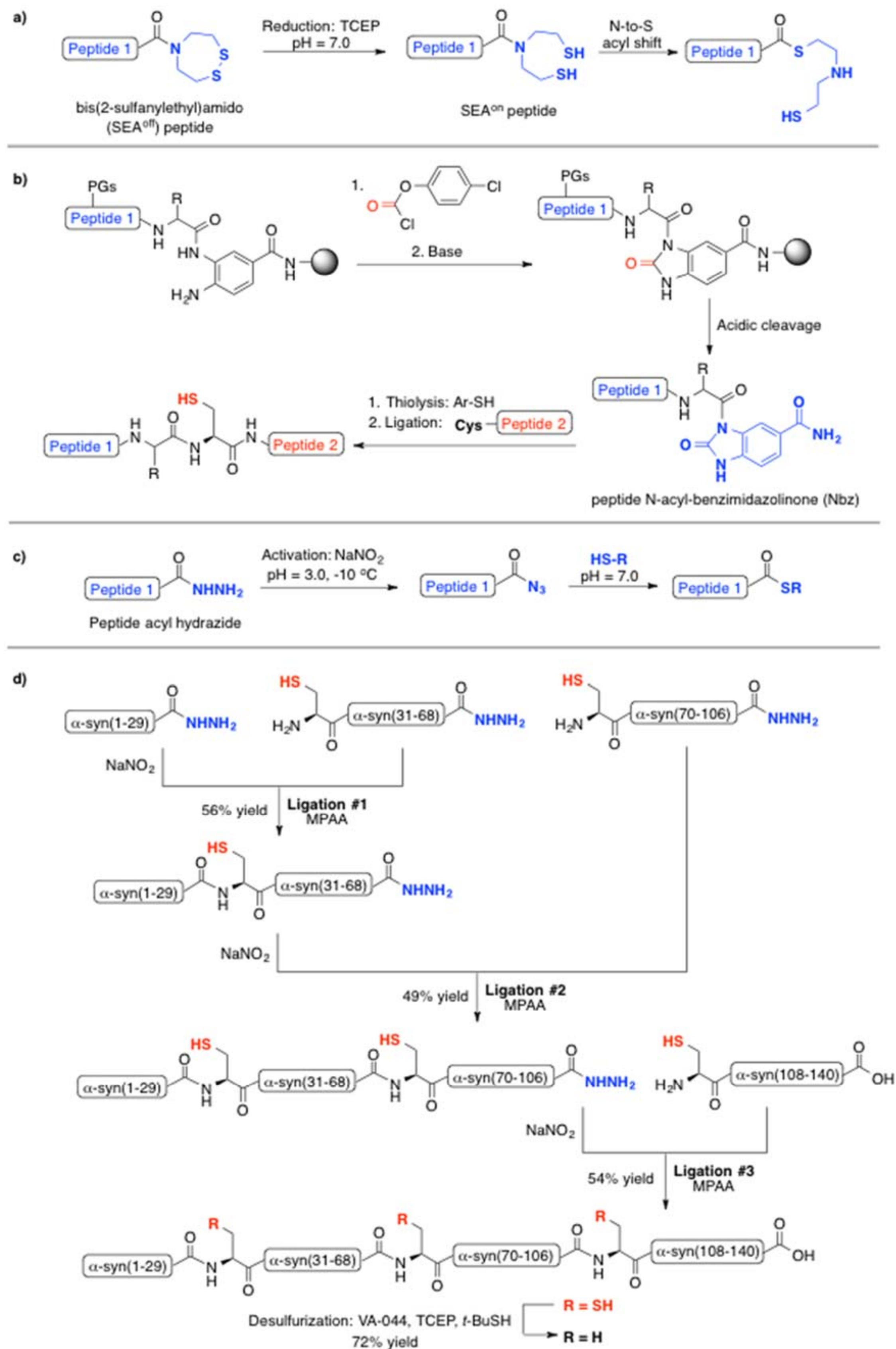


Figure 3. a) SEA$^{on/off}$ ligation methodology [46]; b) Synthesis of peptide *N*-acyl-benzimidazolinones [47]; c) Activation of peptide acyl hydrazides [49••]; d) Synthesis of α-synuclein [51].

3. Exploration of new thiol additives.

For operational convenience, preformed peptide thioesters are typically synthesized as alkyl thioesters, which are facile to prepare and amenable to long-term storage. However, alkyl thioesters as acyl donors in native chemical ligation are, in general, relatively unreactive in the absence of an added thiol catalyst. Treatment with excess exogenous thiol (typically an aryl thiol) serves to promote a thiol-exchange reaction with the C-terminal alkyl thioester, generating a more reactive aryl thioester *in situ* (see Figure 1) that promotes rapid ligation with peptides bearing N-terminal Cys residues or Cys surrogates. In this manner, thiol additives enable fine-tuning of thioester reactivity[2], and have been the source of considerable improvement in reaction scope and rate as well as the key inspiration for the development of kinetically-controlled ligation chemistry[53••]. Following a comprehensive screen of various thiol additives in 2006, Johnson and Kent identified the water-soluble, aryl thiol 4-mercaptophenylacetic acid (MPAA) as the ideal additive, giving enhanced rates of ligation relative to other commonly employed thiol additives[54].

Despite their enhanced reactivities, aryl thiol additives such as MPAA have hindered recent efforts to streamline native chemical ligation and radical desulfurization into an efficient one-pot protocol. The ability of aryl thiols to function as radical scavengers complicates radical desulfurization using a phosphine and a radical initiator (e.g. TCEP and VA-044), mandating complete removal of the aryl thiol in an intermediary purification step prior to carrying out a desulfurization reaction. A number of very recent developments have aimed to circumvent these limitations. Brik and coworkers have demonstrated the use of a bifunctional aryl thiol additive (Figure 4a) that can be efficiently captured using a resin-bound aldehyde prior to a radical desulfurization reaction [55]. Furthermore, trifluoroethanethiol (TFET) (Figure 4a) has recently emerged as a versatile, alkyl thiol additive that affords similar reactivity to the gold standard aryl thiol MPAA, but that does not interfere with subsequent desulfurization reactions[56•]. The utility of the TFET alkyl thiol additive was demonstrated through the total synthesis of two tick-derived proteins, madanin-1 and chimadanin, using iterative one-pot ligation-desulfurization approaches (Figure 4b). It is envisaged that the adoption of new thiol additives, together with the increased flexibility for iterative ligations offered by novel C-terminal acyl donor moieties as reagent equivalents to the thioester synthon (*vide supra*), will serve to greatly streamline the synthesis of complex protein targets from multiple peptide fragments.

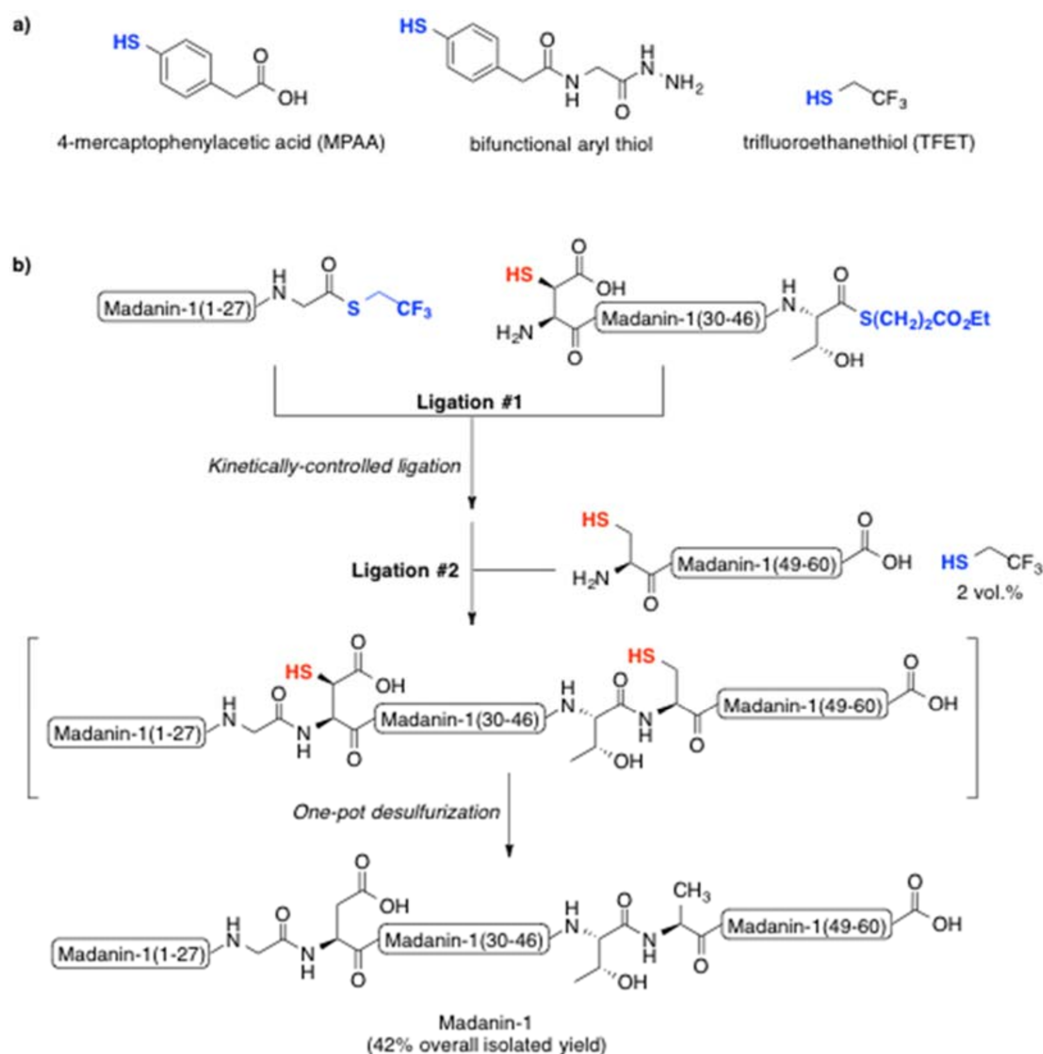


Figure 4. a) Thiol catalysts: MPAA [54], Brik's bifunctional aryl thiol [55] and trifluoroethanethiol (TFET) [56•]; b) Application of TFET in a one-pot kinetically-controlled ligation-desulfurization protocol to afford madanin-1 [56•].

Perspectives.

The combination of recent advances in the development of novel N-terminal Cys surrogates, C-terminal acyl donors and thiol additives has facilitated the synthesis of increasingly large and more complex protein targets. Indeed, application of native chemical ligation in combination with some of the technologies outlined here in the recent total chemical syntheses of a number of impressive targets, including a 304 amino acid tetraubiquitin protein by Brik and coworkers[57••], homogeneous human glycosyl interferon-β by Kajihara and coworkers[58•], and the N-glycosylated chemokine protein CCL1 by Kent and coworkers[48•,59] provides a powerful testament to the functional utility of these methodologies. As synthetic chemists continue to push the envelope of protein size and complexity, it is anticipated that improvements to existing ligation technologies will be necessary. In particular, rapid and selective ligation will need to take place under the high dilution conditions often required to solubilize large proteins, and novel techniques will be required to access difficult targets, including membrane-bound proteins, *via* chemical synthesis. To this end, the logic of native chemical ligation will no doubt continue to serve as a platform for discovery.

Acknowledgements.

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