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Introduction to Chemical Ligation Reactions

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1.1 Introduction

Unraveling the molecular basis that controls the protein structure, function, folding dynamics, and interactions outstands as one of the most ambitious challenges of the post-genomic era. A complete understanding of protein properties will definitively put these biological macromolecules at the service of scientists, teaching about how to use nature's rules to design new proteins endowed with specific features and geared for specific functions. The ability to synthesize a protein by chemical route and selectively modify its primary structure provides a powerful tool to fulfill such requests. Until now, the DNA recombinant techniques have succeeded in preparing a wide number of proteins and protein mutants, allowing investigation of their properties and behavior. However, protein engineering approaches suffer some restrictions, mainly the genetic code barrier, which only tolerates the introduction of the natural amino acids and has been only in part overcome by the introduction of engineered tRNA/aminoacyl-tRNA synthetase (aaRS) [1, 2], and the poor control over site-specific protein modifications. For these reasons, novel stratagems are needed to make protein synthesis and site-selective modification a realistic goal. Nowadays, chemical synthesis is unanimously recognized as a key strategy in protein preparation and modification, which ensures open access to any site of any protein sequence with unique level of specificity, allowing a limitless and surgically precise modification of protein covalent structure [3, 4].

Chemical Ligation: Tools for Biomolecule Synthesis and Modification, First Edition.

Edited by Luca D. D'Andrea and Alessandra Romanelli.

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1.1.1 Chemical Synthesis of Proteins: From the Stepwise Synthesis to the Chemical Ligation Approach

Since the first peptide syntheses performed in solution by Emil Fisher in the early years of the eighteenth century, chemical synthesis appeared as the most powerful approach for preparing peptides and proteins. An important milestone in the development of chemical strategies for full-protein synthesis is represented by the introduction of an original synthetic method that revolutionized the chemical synthesis of peptides and is still in use, the stepwise solid-phase peptide synthesis (SPPS) [5] (Figure 1.1).

Essentially, in SPPS, the peptide is built up on a solid support, and each amino acid is used with its side chain and α -amino group masked by removable protecting groups. Peptide synthesis is performed in the C- to N-terminus direction and starts by anchoring the C-terminal amino acid of the target peptide sequence to an insoluble polymeric support through the covalent binding

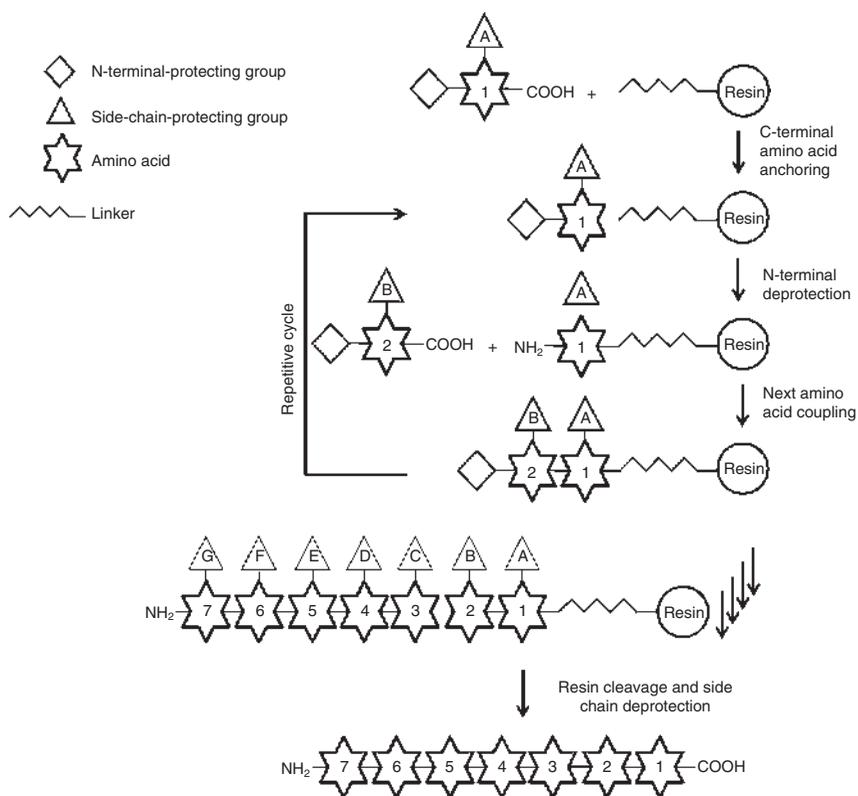


Figure 1.1 Schematic representation of the solid-phase peptide synthesis.

of its α -carboxyl group to a linker moiety. Repeated cycles of deprotection of the α -amino group of the resin-bound amino acid and coupling with the carboxyl-activated form of the next amino acid allow the assembly on the solid support of the full peptide with all the side chains of the amino acid residues properly protected. After the assembly of the full peptide, all side-chain-protecting groups are removed, and simultaneously, the covalent link to the polymer support is cleaved to give the fully unprotected peptide product in solution. As in SPPS, the nascent peptide chain remains bound to the resin for the entire synthesis, after each reaction step of the synthesis, a facile purification by filtration and washing with organic solvents can be performed, enabling the use of large excess of reactants and limiting side reactions. Consequently, reactions are rapid and near-quantitative, handling losses and side-product formation are strongly limited with respect to the classical synthesis in solution phase. As a result, the desired peptide is obtained in high yield and quality. Many impressive protein syntheses have been performed using SPPS in a stepwise manner, such as ribonuclease A (124 amino acids) [6], HIV-1 aspartyl protease (99 amino acids per chain) [7–10], and bovine pancreatic trypsin inhibitor and its analogs (58 amino acids) [11–13]. The ability to assemble a full protein in a linear and straightforward way by SPPS is, however, limited by the synthetic efficiency of each reaction step. Although each reaction step is almost quantitative, inevitable accumulation of by-products limits the ultimate size of high-purity polypeptides that can be effectively prepared by this way to chains of ~50 amino acids in length. In order to get beyond this limit and access longer polypeptide chains, SPPS was combined with the logic of the convergent synthesis (Figure 1.2).

Convergent synthesis consists of the sequential condensation of short protected peptide segments in organic solvent to obtain longer polypeptide chains. Human insulin protein (51 amino acids) and a series of analogs of insulin [14], the enzyme ribonuclease A (124 amino acids) [15], and a consensus lysozyme enzyme molecule (129 amino acids) [16] were some of the first full proteins chemically prepared by using convergent synthesis. Despite convergent synthesis allowed the preparation of many proteins, it faces laboriousness and arduousness. This strategy was hampered by low yield and technically demanding reaction steps for the preparation and purification of the protected segments and their condensation. The high level of skills required, the lack of chiral homogeneity in peptide bond formation, and the inability to manipulate fully protected peptides because of their low solubility led to the abandonment of the convergent synthesis in solution. The right answer came in the early 1990s, when a novel and revolutionary methodological advance, named “chemical ligation,” was proposed [17] (Figure 1.3).

The chemical ligation approach consists of preparation of a polypeptide chain by sequentially joining short and unprotected peptide segments. To this aim, chemical ligation exploits chemoselective reactions between two mutually

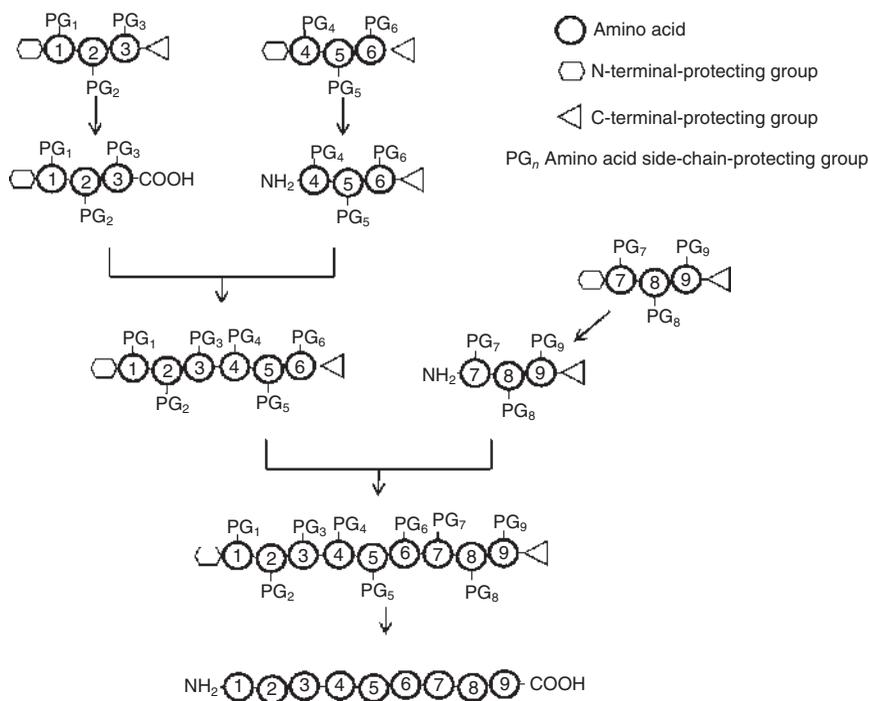


Figure 1.2 Chemical preparation of a polypeptide by convergent synthesis in solution phase.

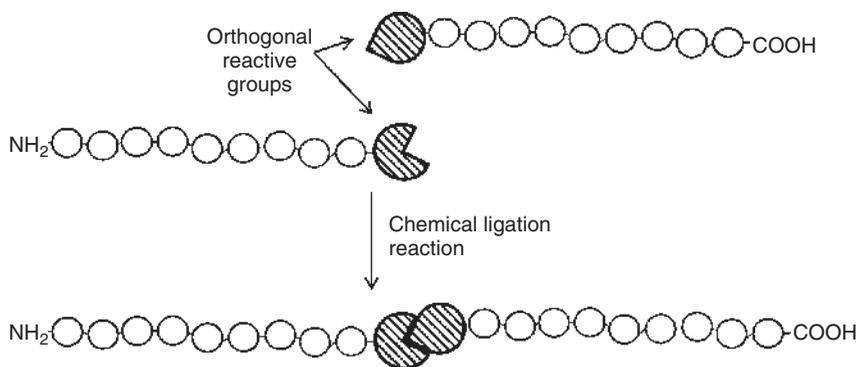


Figure 1.3 The chemical ligation general concept. A single polypeptide chain is obtained by covalently joining two peptide segments through the reaction of two mutually reactive functional groups. The type of covalent bond generated at the junction site depends on the reactive groups employed for the ligation reaction.

reactive functional groups placed at C- and N-terminus of two contiguous peptide segments, respectively. The functional groups react by forming a stable chemical bond without reacting with any other reactive group present in the peptides, thus permitting the use of peptide segments in their fully unprotected form. The use of unprotected peptides ensures ligation reactions to be performed under mild conditions and usually in aqueous buffers, thus overcoming many issues of convergent synthesis. The ingenious synthetic stratagem of chemical ligation allowed chemical protein synthesis and modification to become a reality. Synthetic peptide segments may harbor a plethora of possible chemical modifications allowing site-selective introduction of unnatural elements into proteins. Chemically synthesized proteins found widespread application in biochemistry, biotechnology, biophysics, and chemical biology, allowing the study and the characterization of many proteins and leading to a great number of scientific discoveries. In pharmacology, chemical synthesis of protein therapeutics offers the critical benefit of sample homogeneity and purity [18, 19]. Examples of the first proteins and unnatural protein analogs prepared by chemical synthesis include HIV-1 protease [17], a tethered dimer of HIV-1 protease [20], the covalent heterodimer of b/HLH/Z transcription factor cMyc-Max [21], a four α -helix template-assembled synthetic protein (TASP) molecule [22], and a folded β -sandwich fibronectin domain model [23].

1.1.2 Chemical Modification of Proteins: From Conventional Methods to Chemoselective Labeling by Chemical Ligation

The introduction of unnatural amino acids, biophysical probes such as fluorophores or isotopes, post-translational modifications (PTMs), or even backbone rearrangements into a protein sequence is fundamental to solve protein science issues with a molecular precision. Many insights were gained by combining protein engineering with site-specific modifications performed by the use of chemical reagents that selectively tag amino acid side chains. Conventional methods for protein derivatization exploit the reactivity of native or engineered Lys or Cys side chains and use molecular probes that react selectively with amino or thiol groups [24, 25]. However, these approaches are limited because of the presence of more than one reactive amino acid in the protein sequence, resulting in multiple labeling and heterogeneous protein preparations [26]. Specific reactions for protein labeling with fluorescent dyes have also been performed by introducing at protein N- or C-terminus an extra sequence recognized by specific reactants such as FAsH, CrAsH, or ReAsH (biarsenical derivatives of fluorescein and resorufin) [27–29]. Enzymatic approaches for protein labeling have also been described, such as the SNAP-tag, CLIP-tag, and Halo-tag technologies, which use enzymes that are able to recognize and label specific protein partner fused at the N- or C-terminus of the target [30–32]. These latter procedures are limited to N- or C-protein termini and

generally do not allow incorporation of multiple, different molecular probes into a protein. Similarly, recombinant expression of post-translationally modified proteins is not trivial. Proteins modified with PTMs are traditionally prepared by enzymatic treatments, which are often impossible to control precisely and not always proceed with high yields. Such approaches suffer from poor versatility, being often tailored on a specific protein target, and moreover, the incorporation of multiple, different types of molecular probes into discrete sites or regions of a protein is not possible. Recombinant biosynthesis using PTM machineries of the expression hosts often results in heterogeneous mixtures, especially in the case of glycosylated proteins [33]. Sample heterogeneity represents a strong limit for many scientific applications, in particular in the case of protein therapeutics. A significant portion of the post-translationally modified protein therapeutics, in particular glycoproteins, administered to patients are biosynthesized as complex mixture, with major drawbacks such as the irreproducible bioactivity from one batch to another [34]. For these reasons, even if, overall, the aforementioned protein modification approaches are original and sophisticated and led to great discoveries and innovations, more general approaches are needed to make site-selective modification of proteins a realistic goal. The great potential of protein chemical synthesis appeared as a straightforward way to incorporate any type and number of nonnative elements into proteins.

1.2 Chemical Ligation Chemistries

Chemical ligation methodology provides an excellent platform for preparing proteins and protein conjugates in high yield and good purity, which is currently a major frontier of the synthetic bioorganic chemistry. An ideal ligation chemistry should be highly selective, should be compatible with all the functional groups present in proteins, and should preferentially proceed under mild aqueous conditions. Considerable efforts led to the development of a portfolio of chemoselective ligation chemistries, satisfying the desire of harnessing several reactions that can be performed orthogonally to one another for the one-pot rapid assembly of a protein or a modified protein. Chemical ligation chemistries differ for the type of chemical bond introduced at the site of junction between the two reacting moieties, which in turn is correlated to the reactive groups employed for the ligation reaction. Although chemistries generating an amide bond are usually preferred, those leading to the formation of nonpeptide bond at the ligation site are also attractive approaches in synthetic protein research as far as the unnatural bond does not strongly affect the protein structure. Next section will dissect the major chemistries proposed as ligation strategies for protein synthesis, which, in their overall, ensure a complete flexibility in the manipulation of protein primary structure.

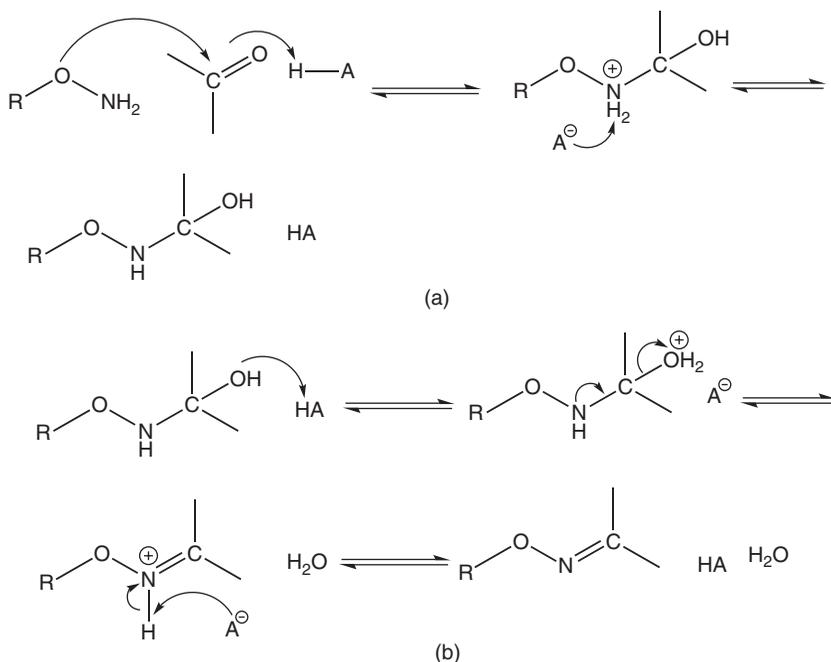
1.3 Imine Ligations

Imine ligations exploit the chemoselective reaction between amines and aldehydes or ketones to generate an imine-type bond. Imine ligations are among the firstly exploited ligation chemistries and have shown great utility in protein synthesis thanks to their high chemoselectivity in the presence of every natural amino acid and a variety of PTMs. Depending on the nature of the amine employed, imine ligations give rise to a different kind of imine bond at the ligation junction. For instance, the reaction of aldehydes or ketones with oxyamines generates an oxime bond, while the reaction of aldehydes or ketones with hydrazines leads to the formation of an hydrazone bond. The following subsections will describe in more detail the different types of imine ligations described in the literature.

1.3.1 Oxime Ligation

Oxime ligation refers to the highly chemoselective reaction between an aldehyde or a ketone and an aminoxy group to give an oxime bond (Scheme 1.1). Since the mid-1980s, the oxime ligation has found widespread use [35], not only for protein synthesis by peptide fragment assembly [36] but also for preparation of chemical microarrays [37], cyclic peptide libraries [38, 39], glycoprotein [40], protein–polymer conjugates [41, 42], peptide–oligonucleotide complexes [43], and for viral particle functionalization [44]. The oxime ligation is a particularly attractive chemistry since it is very efficient and chemoselective, taking place in aqueous solution under mild acidic conditions (usually pH around 5.0), and water is the only side product formed in this process. In acid medium, the first step of the oxime ligation leads to the formation of a carbinolamine from the nucleophilic addition of the oxyamine to the carbonyl compound (Scheme 1.1a). The second step is triggered by the protonation of the hydroxyl group, which causes the dehydration of the carbinolamine into the oxime (Scheme 1.1b) [45]. Oxime ligations are usually slow at physiological pH; maximum reaction rates are typically reached near the pK_a of the nucleophile while dropping off sharply at higher or lower pH values [46].

In general, in all types of imine ligations, and thus even in the oxime ligation, aldehydes are substantially more reactive than ketones, mainly because of steric effects, and aromatic carbonyls are more reactive than the aliphatic ones. Buré et al. compared the reactivity of C-terminal aldehyde and ketone peptides with aminoxy-containing peptides in acido-catalyzed oxime reactions. The results obtained confirmed that oxime ligation proceeds less smoothly in the case of peptide ketones compared to peptide aldehydes. The first step of the oximation reaction was the limiting step when ketone peptides are used, while the second step was the determining step in the case of peptide aldehydes [47]. The modest rate of oxime ligation reaction, especially observed when a



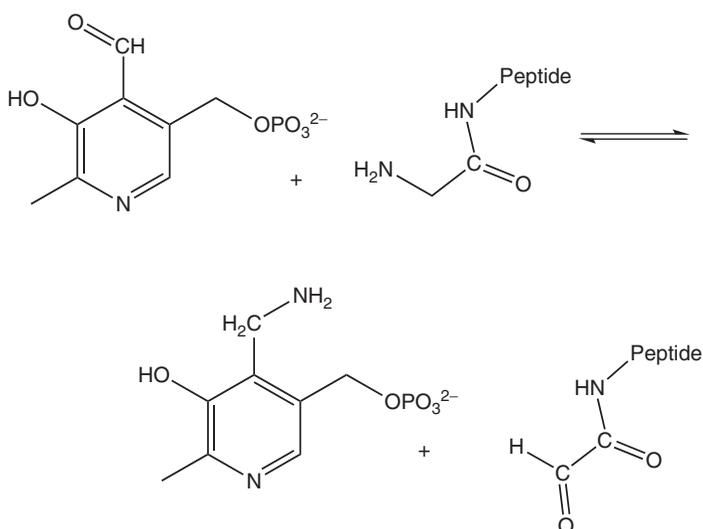
Scheme 1.1 The oxime ligation reaction.

ketone is used, can be improved by adding nucleophilic catalyst, such as aniline [48, 49], or the new-generation catalysts, such as *meta*- and *para*-phenylenediamine [50–52]. Catalysts are amines that form a reactive imine intermediate with the target carbonyl group. Use of catalysts allows oxime ligation to be performed faster even at neutral pH, as necessary for biomolecules not soluble or unstable under acidic pH and using lower reactant concentrations as needed for applications using cell extracts [53]. All the imine bonds are generally susceptible to hydrolysis. Among the imine-based ligation reactions, oxime ligation affords the most stable imine product. Despite the oxime bond being identified as the less hydrolysis-prone imine linkage, it remains thermodynamically unstable and undergoes hydrolysis at an appreciable extent in aqueous solution [54]. Although this is generally inconvenient when oxime ligation is used to synthesize proteins or protein bioconjugates, this feature may become a straightforward advantage in applications where covalent capture and controlled release are needed [35, 55]. For example, oxime chemistry was used to efficiently conjugate different peptide species to hyaluronic acid polymer. Peptides synthesized with an aminoxy N-terminus were reacted under slightly acidic aqueous conditions and without a catalyst with hyaluronic acid functionalized with aldehyde groups. The resulting oxime bond was found to

rapidly hydrolyze at pH 2, releasing the bound peptide molecules, but was stable at higher pH values. The strategy was applied to two different biologically active peptide species, a multiple sclerosis antigen and an ICAM-1 ligand, known to block immune cell stimulation [56]. Similarly, some quinone oxime molecules have been used as redox cleavable linkers [55].

Both functionalities required to perform oxime ligation, aldehydes and oxamines, can be straightforwardly introduced into peptides and proteins. In the case of aldehydes, several tools are available to incorporate such functionalities into peptides and proteins using chemical or enzymatic approaches. One of the firstly described strategy takes advantage of the extreme sensitivity to oxidation by periodate of 1,2-diols or 1,2-aminoalcohols, such as Ser and Thr residues, which permits the selective conversion of N-terminal Ser and Thr to an aldehyde group under mild aqueous conditions [57]. However, internal residues of Cys, Met, Trp, Tyr, and His can also be oxidized by periodate, thus requiring a careful control of the experimental conditions employed, such as pH, stoichiometry, and time of incubation. Moreover, the reaction must be quenched with excess of ethanolamine or ethylene glycol, and the aldehyde product must be immediately purified from the reaction mixture [58]. To overcome the limitation of periodate oxidation, some groups explored the use of protected aldehydes, such as the dimethylacetals, diisopropylthioacetals, or α,α' -diaminoacetic acid derivatives, which can be introduced in the last step of peptide synthesis and removed in solution after peptide cleavage from the resin [58]. N-terminal transamination as well offers a convenient way to install a reactive ketone or aldehyde functional group at a single location. Transamination reaction performed with pyridoxal-5'-phosphate offers a valuable tool for introducing such functionalities at the N-terminus of native proteins, as the reaction is carried out under very mild aqueous conditions (Scheme 1.2) [59].

This approach is, however, not without limitations. Many N-terminal amino acids provided high yields of the desired transaminated products, but other residues (His, Trp, Lys, and Pro) generated adducts with pyridoxal-5'-phosphate itself. Moreover, N-terminal Cys and Ser residues were observed to undergo β -elimination in addition to transamination, and the transamination product of N-terminal Gln was resistant to subsequent oxime formation attempts [60]. Another possible strategy uses α -amino aldehydes as starting materials present at the beginning of the peptide synthesis in a protected form. In this case, the aldehyde function will be located at peptide C-terminus [61]. Efficient solid-phase synthesis of C-terminal peptide aldehydes can be performed using the phenylacetamidomethyl (PAM) linker. Peptides synthesized on a PAM resin can be cleaved by aminolysis with aminoacetaldehyde-dimethyl acetal leading to a C-terminal aldehyde masked as an acetal [62]. In a subsequent work, the same group highlighted the influence of the nature of the polymeric support in aldehyde peptide synthesis and suggested the use of the poly(ethylene glycol)-poly(acrylamide) (PEGA) resin, which, among the solid

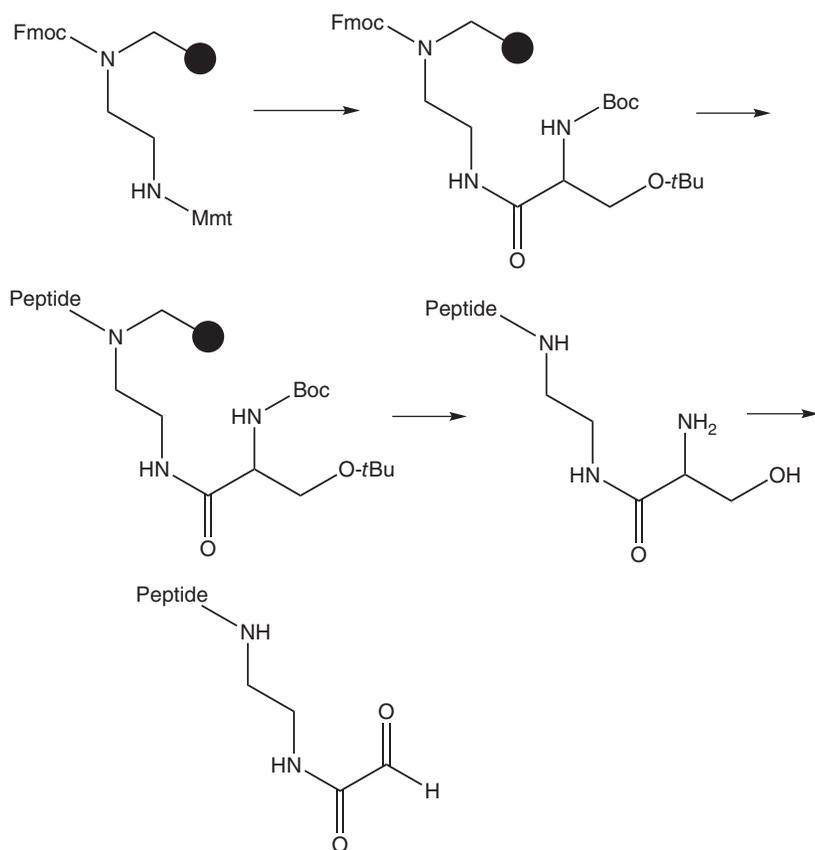


Scheme 1.2 Transamination with pyridoxal phosphate.

supports tested, was proved to afford the best cleavage yield [63]. The NovaTag™ resin, designed to perform the dual peptide labeling at N- and C-termini for fluorescence resonance energy transfer (FRET) studies, can also be used to introduce an aldehyde or a ketone moiety at the C-terminus of a peptide sequence [36, 47] (Scheme 1.3).

Enzymatic approaches to site-specifically introduce aldehyde function into peptides and proteins have also been described [64, 65], such as the approach using the enzyme protein farnesyltransferase (PFTase). PFTase catalyzes the transfer of a farnesyl isoprenoid group from farnesyl diphosphate to the sulfur atom of a cysteine residue located in the consensus sequence CAAX. The enzyme PFTase also accepts analogs of FPP containing an aldehyde group, which can thus be incorporated into a protein or a peptide by appending the CAAX-box sequence to its N- or C-terminus [66, 67]. Similarly, formylglycine-generating enzyme (FGE) converts cysteine within the motif CxPxR to the aldehyde-bearing residue formylglycine (FGly). This short motif can be installed within proteins or peptides for site-specific labeling with aminoxy probes or peptide segments [68]. Enzymatic approaches are, however, limited by the need of specific amino acid sequences, restricting their application to the targeting of terminal regions.

The aminoxy group is often introduced into a peptide by coupling of a protected NH—O function, the *N*-Boc-2-(aminoxy)acetic acid. However, this method is hampered by *N*-overacylation of the NH—O function. To circumvent this limitation, recently, the aminoxyacetic acid moiety has been successfully



Scheme 1.3 Synthesis of aldehyde and ketone peptides using NovaTag™ resin.

protected with a 1-ethoxyethylidene group. Subsequent deprotection under mild acidic conditions gave the corresponding pure aminoxyolated peptide [69, 70]. The aminoxy group can also be incorporated at the C-terminus of a recombinant protein using intein technology [71]. Yi et al. demonstrated how a bis(oxyamine) molecule can react with a C-terminal thioester protein obtained from intein thiolysis (see Section 1.6.2). One oxyamine group of the bifunctional molecule directly reacts with the thioester moiety to form a hydroxamic acid bond to the protein. The second oxyamine group would still be available for a subsequent ligation reaction with a ketone- or aldehyde-functionalized probe (Figure 1.4).

Conveniently, the oxyamino group could be introduced at the protein C-terminus under mild conditions (phosphate buffer pH 7.5, on ice), and the modified protein can subsequently undergo fast and chemoselective oxime ligation with high yield. The strategy was used to perform a one-pot dual-color labeling

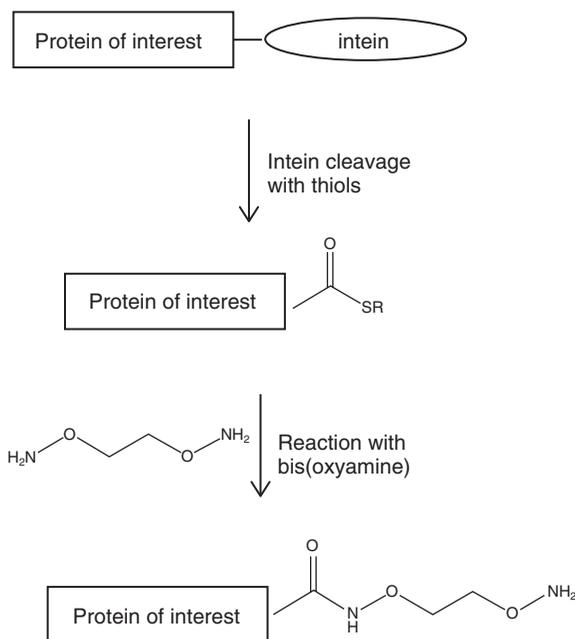


Figure 1.4 Derivatization of the C-terminus of a recombinant protein with an oxyamine functional group using intein chemistry.

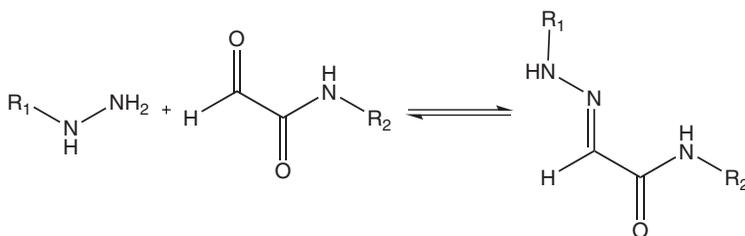
of the protein target Rab7, suitable for FRET studies. The protein was equipped with an N-terminal Cys residue and a C-terminal oxyamine and concurrently reacted with thioester-coumarin and keto-fluorescein [72]. More recently, the same approach using intein-mediated incorporation of bis(oxyamine) at protein C-terminus was employed to substitute Rab C-terminal domain with a polyethyleneglycol linker [41]. Another example applying a native chemical ligation (NCL) logic to oxime ligation was proposed by Delmas group. They elaborated an efficient streamlined approach for the preparation of polypeptides performing sequential oxime ligation reactions directly in solid phase, a concept initially applied to NCL [73]. In this example, the aminoxy function was temporarily protected with Trt or Alloc groups. The oxime-containing full polypeptides were obtained in high purity, highlighting the efficiency of solid-phase ligation approach. A dual oxime-/intein-mediated ligation reaction has also been proposed for the synthesis of macrocyclic organo-peptide hybrids (MOrPHs) [74]. In this case, a bifunctional oxyamino/1,3-amino-thiol was used to mediate the cyclization of a recombinantly expressed target peptide sequence harboring an N-terminal *p*-acetyl-phenylalanine and a C-terminal thioester group, the former genetically encoded through amber codon suppression and the latter obtained by intein thiolysis. This synthetic methodology

has been successively extended to the synthesis of bicyclic organo-peptide hybrids, in which peptide macrocyclization by means of a bifunctional oxyamine/1,3-amino-thiol synthetic precursor was followed by intramolecular disulfide formation between the synthetic precursor-borne thiol and a cysteine embedded in the peptide sequence [75].

1.3.2 Hydrazone Ligation

A hydrazone bond derives from the reaction of a hydrazine with an aldehyde. The reaction proceeds through the formation of a carbinolamine, which dehydrates into a hydrazone (Scheme 1.4).

At acid pH, the hydronium-ion-catalyzed formation of the carbinolamine appears as the rate-limiting step. At intermediate pH (pH 4–6), the rate-limiting step is an uncatalyzed or solvent-catalyzed addition of the free nucleophile to the carbonyl group. At neutral pH, the rate-limiting step is the acid-catalyzed breakdown of the tetrahedral intermediate carbinolamine to eliminate water, giving the hydrazone [45, 46, 58]. Hydrazone reactions are better performed at slightly acid pH (pH near 5). However, at near neutral pH, hydrazines are more nucleophile and thus more reactive than oxyamines, offering an attractive alternative to oxime ligation for bioconjugation in native conditions. Hydrazone ligation suffers, however, some drawbacks with respect to oxime ligation, principally referring to slower reaction kinetics, which require a large excess of the hydrazine reagent to achieve good yields, and to a greater susceptibility to hydrolysis of the hydrazone bond. For all these reasons, despite its high chemoselectivity, the use of hydrazone ligation has been strongly limited. Alkylhydrazones are more prone to hydrolysis under aqueous conditions compared to acylhydrazones [54]; thus, hydrazine derivatives such as hydrazides and semicarbazides are usually preferred in bioconjugation reactions with aldehydes and ketones because they gave the more stable acylhydrazones. However, hydrazide and semicarbazide reagents suffer a slower reaction kinetic because of the presence of electron-withdrawing groups adjacent to the nucleophile hydrazine moiety [76]. Similarly to oxime ligation, hydrazone ligation may also be catalyzed at neutral pH by the addition of aromatic amines to the



Scheme 1.4 The hydrazone ligation reaction.

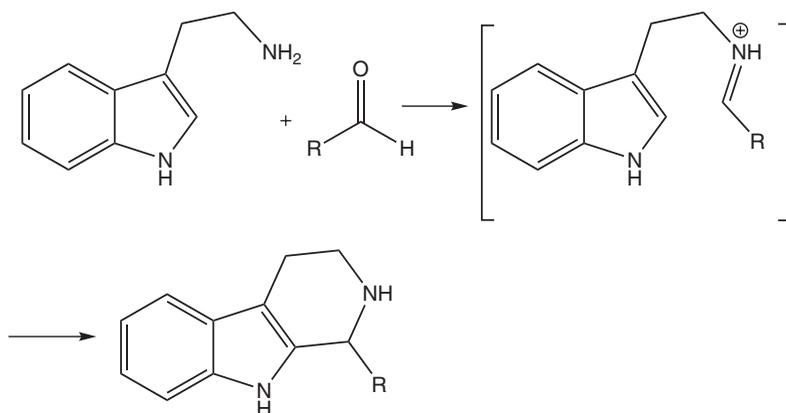
reaction mixture, such as aniline [49, 77], extending its application to biological systems [78, 79]. However, high concentrations of the catalyst are necessary (e.g., 100 mM aniline), which may be incompatible with the native structure of certain proteins and is toxic to cells during applications in living systems. Recently, two possible solutions were reported. Firstly, the use of 4-aminophenylalanine as a catalyst for hydrazone ligation was investigated, demonstrating that the molecule mostly retains the catalytic efficacy of aniline but, due to its more hydrophilic zwitterionic nature, is less detrimental to the native structure of the protein. As a proof of concept, tubulin was modified with the acid 3-formyltyrosine, which was attached to the protein carboxy terminus through the use of the enzyme tubulin tyrosine ligase [80], and conjugated with a hydrazine-containing dye in the presence of the catalyst 4-aminophenylalanine in aqueous buffer at neutral pH. Moreover, as the formation of the imine intermediate between the aldehyde and the catalyst is an exothermic reaction [81], in the same work, Blanden et al. demonstrated that the overall rate of the hydrazone reaction may be increased at low temperature (0°C), extending the application to biomolecular targets that are stable at low temperatures. Recently, *meta*- and *para*-phenyldiamine were also proposed to catalyze both hydrazone and oxime ligation reactions on native proteins, as the molecules do not present deleterious effects on protein structure and activity [51]. Crisalli and Kool suggested anthranilic acid derivatives (in particular, 5-methoxyanthranilic acid), diaminobenzoic acids (in particular 3,5-diaminobenzoic acid) [82], and phosphonates (in particular, 2-aminobenzenephosphonic acid) [83] as a new class of efficient catalysts for hydrazone and oxime ligation, which speed up the reaction at neutral pH over the traditional aniline-catalyzed reaction. Anthranilic acid is an anabolyte serving as an intermediate in the biosynthesis of tryptophan, and thus, it is likely that anthranilic acid and related derivatives will be considerably less toxic than aniline for *in vivo* applications. A second solution to the problem was reported by Wang and Canary [84]. They described the use of the enzyme cofactor pyridoxal-5'-phosphate as the source of aldehyde function in hydrazone formation. The uncatalyzed reaction between pyridoxal-5'-phosphate and 2-hydrazinopyridine showed comparable kinetic constants to that observed for the reaction catalyzed by excess of aniline between 2-hydrazinopyridine and 4-formyl benzoic acid. A completely different approach, aimed at speeding up hydrazone and oxime ligation at neutral pH, refers to the identification of structural features of the reactants that accelerate the rate of the ligation reaction without the need of catalysts. Such approaches have been applied to hydrazone ligation by Kool et al. [85, 86] and allowed to conclude that a careful choice of carbonyl and hydrazine substrate allows hydrazone ligation to be performed with a satisfactory rate at biological pH even in the absence of a catalyst. These authors performed a kinetic study of hydrazone ligation reaction in aqueous buffer at pH 7.4, comparing the hydrazone formation rate observed by reacting a range of carbonyl compounds

with a standard hydrazine (phenylhydrazine) [85]. They tested aldehydes and ketones, both aryl- and alkyl-substituted, confirming that aldehydes are more reactive than ketones and observed that, among all tested substrates, at pH 7.4, simple alkyl aldehydes are the most reactive species. Moreover, at pH 7.4, they observed a general trend favoring electron-deficient aldehydes, despite the fact that it has long been thought that electron-withdrawing groups can increase reactivity of aldehydes in hydrazone formation [87, 88]. In addition, they observed that acid/base groups *ortho*-substituted with respect to the carbonyl carbon in aldehydes speed up hydrazone formation, as reported several decades ago, although a satisfactory explanation for the effect was never discussed earlier [83, 89]. Kool et al. proposed that the acid group near the reactive center might donate a proton at the transition state, acting as an intramolecular catalyst in the reaction. Indeed, pyridoxal, which has an *ortho*-hydroxyl group, efficiently reacts to form an hydrazone bond, as previously described [84]. In a complementary work, an investigation of the best-performing hydrazines in phosphate buffer at pH 7.4 was performed, and structures that undergo especially rapid reactions were identified [86]. From this study, two hydrazines containing acid/base groups appeared to react significantly more rapidly: *ortho*-carboxy-phenylhydrazine (OCPH) and 2-(dimethylamino)ethylhydrazine (DMAEH).

An interesting application requiring the use of both hydrazone and oxime ligations takes advantage of the reversible nature of the hydrazone bond. Aldehyde proteins may be covalently immobilized on a solid support by incubation with hydrazide–agarose beads. Immobilization on the solid resin by hydrazone bonds was shown to be reversible via transoximization reaction, allowing protein elution through incubation with alkoxyamines. Using a conveniently functionalized alkoxyamine to elute proteins bound to the solid support, such as a fluorescently labeled or PEGylated alkoxyamine, results in the release of pure protein harboring the desired site-specific covalent modifications. This hydrazone–oxime exchange reaction was employed to produce C-terminal PEGylated glucose-dependent insulinotropic polypeptide, a modification that is able to prolong the circulating half-life of the protein showing potential therapeutic activity against diabetes [90]. Rashidian et al. demonstrated that the procedure allows isolation and labeling of an enzymatically functionalized aldehyde protein directly from crude *Escherichia coli* extract [53, 67]. The amine catalysts *meta*- and *para*-phenyldiamine were proposed as efficient catalysts for the protein labeling by hydrazone–oxime exchange reaction [50, 51].

1.3.3 Pictet–Spengler Ligation

The Pictet–Spengler reaction [91] exploits the bioorthogonal reaction between an aldehyde and a tryptamine molecule to form an intermediate iminium ion;

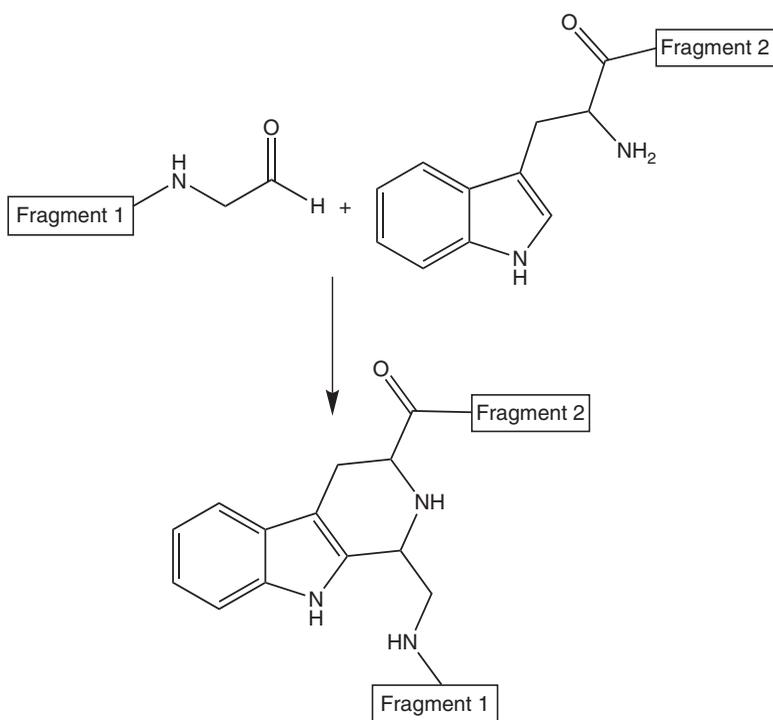


Scheme 1.5 The Pictet–Spengler reaction.

this intermediate ion undergoes ring closure through the formation of a new C–C bond (Scheme 1.5).

Tryptophan and tryptophan analogs react with aldehydes to give a tetrahydro- β -carboline through the Pictet–Spengler reaction. The reaction was applied to peptide ligation using peptide segments harboring an aldehyde at the C-terminus and a tryptophan residue at the N-terminus [92] (Scheme 1.6).

The pioneering example of Pictet–Spengler ligation reaction between two unprotected peptides was carried out in glacial acetic acid and proceeded with a high yield (90% after 12 h). The ligation afforded the formation of a stereoisomeric mixture due to the formation of a new chiral carbon in the final tetrahydro- β -carboline. The tetrahydro- β -carboline moiety incorporated into the peptide may affect the structure of the polypeptide chain by inducing β -turn and imposing the definite position of the peptide arms [93]. The use of Pictet–Spengler ligation for protein derivatization was reported by Sasaki et al., who applied the method to the labeling of the N-terminus of horse heart myoglobin using tryptamine analogs [94]. However, the reaction appeared to be considerably slow under protein compatible conditions and mild acid pH, requiring high concentration of the tryptamine analog to achieve good yield of derivatized protein. By performing kinetic studies of the Pictet–Spengler reaction, the Bertozzi group identified the limiting step in the formation of the iminium ion and modified the triptamine molecule in order to increase the reaction rate [95]. They designed a new indole molecule showing a 4–5 order of magnitude faster reaction rate with aldehydes and ketones with respect to triptamine. In order to increase the rate of iminium ion formation, in the new indole, the aliphatic amine of tryptamine was replaced with an aminoxy moiety. Additionally, the aminoxy substituent was moved to the 2-position of the indole, allowing the more nucleophilic 3-position to engage in electrophilic

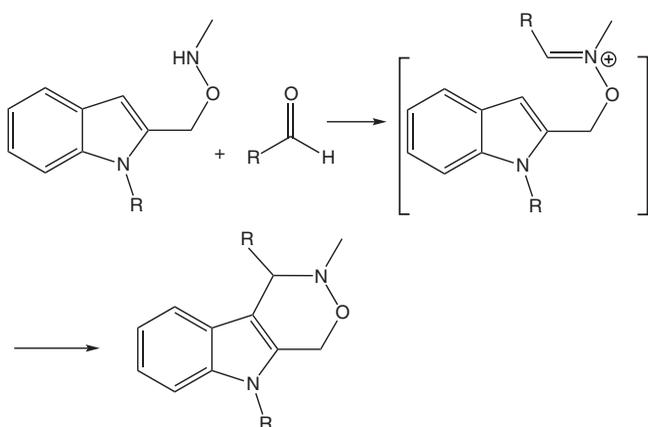


Scheme 1.6 The Pictet–Spengler ligation reaction in protein synthesis.

substitution. Finally, the aminoxy functionality was methylated to provide a reactive oxyiminium ion intermediate that would facilitate rapid C–C bond formation via intramolecular electrophilic substitution (Scheme 1.7).

As a proof of concept, the new indole molecule was used to label a series of aldehyde-functionalized proteins, demonstrating that in this case the reaction proceeded conveniently fast. The great advantage of Pictet–Spengler ligation with respect to the other imine ligations refers to the stability of the conjugate for a long period of time. The imine bonds generated from oxime and hydrazone ligations are susceptible to hydrolysis, limiting their application in cases in which long-term stability is required. Pictet–Spengler ligation overcomes this drawback, offering a means to generate hydrolytically stable conjugates with a reasonable reaction rate.

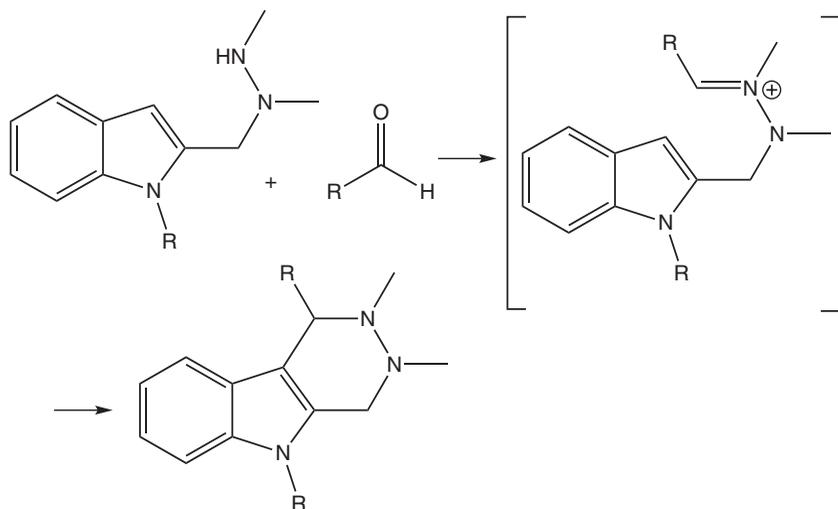
The Pictet–Spengler ligation is performed under mild acidic conditions (pH < 5.0) rather than at the preferable neutral pH, which should be preferentially compatible with the majority of native proteins and molecular probes. In order to extend the Pictet–Spengler ligation even to applications requiring pH around neutrality, another indole derivative was obtained by substituting the aminoxy nucleophile with an alkylhydrazine moiety [76]. Indeed, in contrast



Scheme 1.7 The Pictet–Spengler reaction mechanism with the optimized indole.

to aminoxy groups, hydrazines preserve their nucleophilicity at pH near neutrality. The designed indole would react at near neutral pH with aldehydes and ketones, giving a hydrazone intermediate ion, which intramolecularly rearranges into an azacarboline (Scheme 1.8).

The ligation reaction performed using the designed hydrazino-indole was demonstrated to overreact at pH 6.0 with aldehyde protein derivatives, giving conjugates stable to hydrolysis. The reaction of hydrazino-Pictet–Spengler ligation shows the advantages over the oxime and hydrazone ligation of being



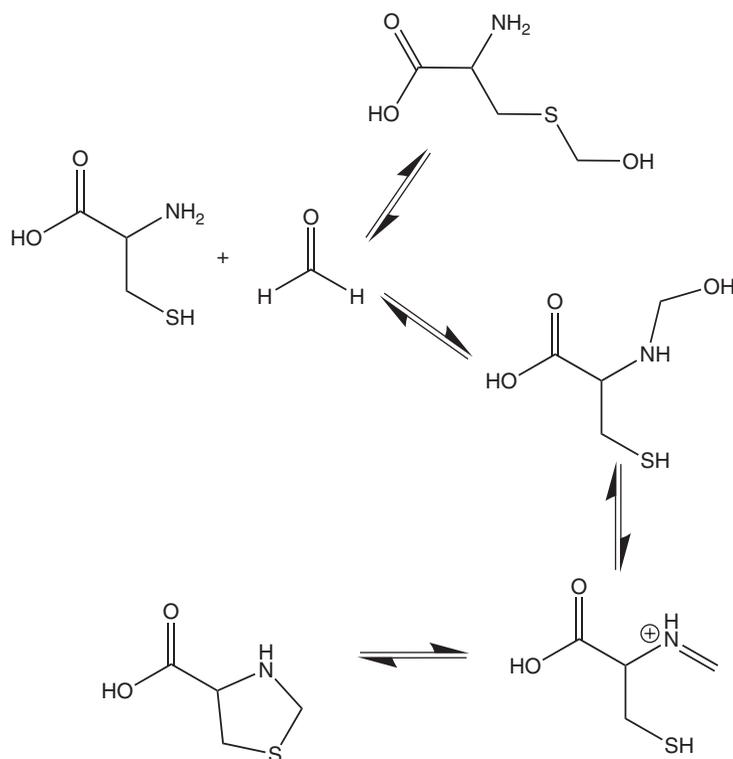
Scheme 1.8 Hydrazino-Pictet–Spengler ligation reaction.

outperforming at near neutral pH and of giving a stable-to-hydrolysis product. Recently, hydrazino-Pictet–Spengler ligation was employed to generate a panel of site-specifically conjugated antibody–drug complexes, differing in the conjugation site and linker composition [96, 97]. In the latter cited examples, hydrazino-Pictet–Spengler provided a uniquely stable linkage with respect to the other ligation chemistries available and used to generate such molecular complexes.

1.3.4 Thiazolidine Ligation

The reaction between an aldehyde and the mercaptamine moiety of the cysteine to give a thiazolidine was described many decades ago [98]. The main reaction pathway proceeds through the formation of a carbinolamine whose dehydration gives an iminium intermediate. Subsequent intramolecular cyclization of iminium intermediate brings the thiazolidine product (Scheme 1.9).

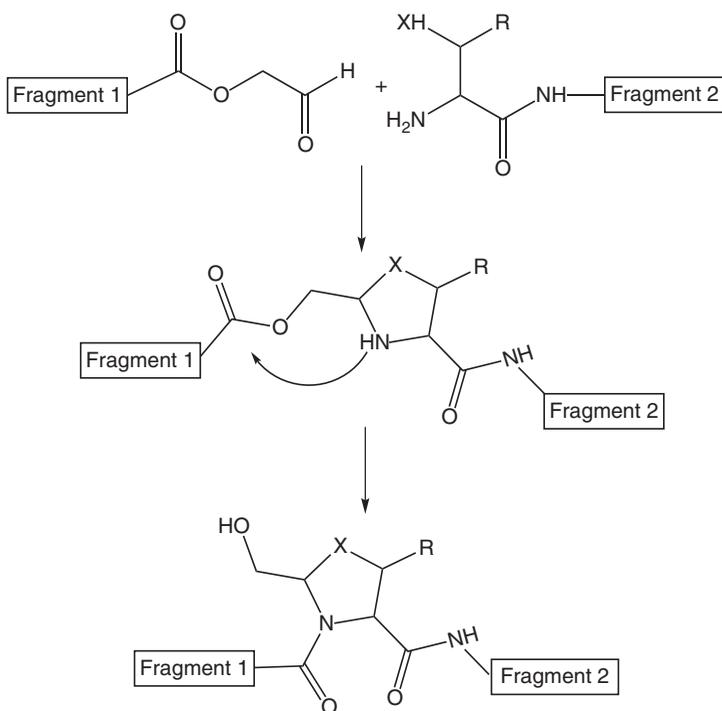
The formation of the carbinolamine appears as the limiting step of the reaction at acidic pH, while at basic pH, the limiting step is the dehydration of the



Scheme 1.9 Thiazolidine formation from the reaction between formaldehyde and cysteine.

carbinolamine. Thiazolidine ring formation proceeds in water solution, better under acidic conditions (at pH 4–6) [58]. The thiazolidine ligation was firstly applied as a chemical ligation strategy to assemble a polypeptide chain through the junction in sequence of two peptide fragments in 1994 by Tam and coworkers [99–101]. They introduced a simple alkyl aldehyde at the C-terminus of one peptide and reacted such fragments with a second peptide bearing a Cys (or Thr or Ser) as N-terminal amino acid. The reaction led to the formation of a thiazolidine (or an oxazolidine) five-membered ring at the junction site between the two fragments. In other imine ligations, such as oxime and hydrazone ligations, the condensation reaction products are imines that are often unstable and hydrolyzable in aqueous solution. In this case, a ring system stable to hydrolysis is formed. In their seminal work, Liu and Tam introduced the alkyl aldehyde function to the C-terminus of one peptide component via an ester linkage. In this way, an amide bond could then be obtained through an intramolecular O-to-N acyl transfer after ligation (Scheme 1.10).

Using this shrewdness, peptide segments can be ligated together through a native peptide bond, specifically a pseudoproline linkage as it resembles the



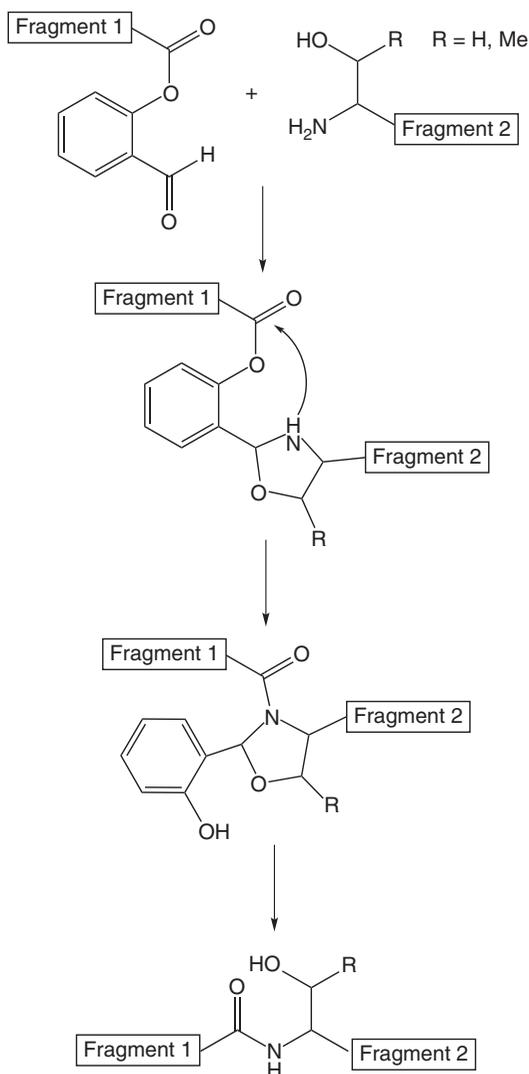
Scheme 1.10 General reaction scheme of pseudoproline ligation strategy.

proline structure. The potential of the conceived synthetic scheme was demonstrated by the preparation of a 50-residue epidermal growth factor-like peptide. Tam's group extended thiazolidine ligation to other targets and applications, such as the assembling of peptide dendrimers [102–104], the total synthesis of HIV-1 protease inhibitor [105], the ligation of a membrane-permeable sequence to a biologically active peptide [106], and to the end-to-side chain peptide cyclization using peptide precursors carrying both an N-terminal Cys and a C-terminal aldehyde on a Lys side chain [107]. Other examples reported the use of thiazolidine ligation to attach an active peptide on a peptide scaffold [108] and for the site-specific labeling of proteins [109, 110]. A more recent application of the thiazolidine ligation was reported by Lee and Zuckermann [111]. They explored how peptoid point mutations affected ribonuclease A (RNaseA) structure and function by introducing such backbone modification into the N-terminal catalytic region of the RNaseA, corresponding to the residues 1–20 and called S-peptide. A truncated version of the RNaseA lacking the N-terminal S-peptide and exhibiting an N-terminal Cys residue was prepared by recombinant means and conjugated by thiazolidine ligation to a series of C-terminal aldehyde synthetic S-peptides bearing the peptoid substitution in different positions. The thiazolidine ligation was selected as ligation tool and was preferred to the wider diffused NCL strategy because the latter method required the preparation of a peptide–peptoid C-terminal thioester, which turned out to be difficult and incompatible with the peptoid synthesis conditions [111].

1.4 Serine/Threonine Ligation (STL)

A traceless version of the pseudoproline ligation proposed by Tam's group was recently reported by Li et al. [112] and became an effective methodology for the convergent chemical synthesis of peptides and proteins. They proposed the use of an aromatic aldehyde ester, in particular the salicylaldehyde (SAL) ester, in place of the glycolaldehyde ester proposed by Tam for the pseudoproline ligation. C-terminal SAL ester peptides could selectively react with N-terminal amino-alcohol function of Ser and Thr generating a pseudoproline linkage that could rearrange under acid conditions to give a natural peptide bond (Scheme 1.11).

Since Ser and Thr residues are abundantly present in protein sequences, enabling the efficient and traceless serine/threonine ligation (STL) appears a very attractive tool in protein chemical synthesis. In a preliminary work, Li and coworkers tested the ability of Fmoc-protected amino-acid SAL ester derivatives to react with Ser and Thr. The reaction proceeded smoothly even when using SAL derivatives of β -branched amino acids, rapidly giving the desired *N,O*-benzylidene acetal intermediate in high yields. The reaction was



Scheme 1.11 Mechanism of serine/threonine ligation reaction.

carried out in pyridine acetate buffer, and after evaporation of the solvent, the crude mixture was treated with TFA/H₂O/TIPS to afford the removal of the acetal group, thus generating a native peptide linkage. The feasibility of the ligation strategy was confirmed using two short unprotected model peptides that were successfully joined through a peptide bond [112]. No epimerization at the junction site was observed under the STL conditions

[113]. Moreover, internal unprotected Lys residues were demonstrated to not adversely impact the efficiency of the ligation using N-terminal Ser/Thr peptides in which Lys was present at various positions [114]. Additionally, the reactivity of all the 20 proteinogenic amino acids at the C-terminus of the peptide SAL ester under STL conditions was systematically evaluated. Notably, all the amino acids, except Asp, Glu, and Lys, appeared to be compatible with STL when placed at the C-terminus of the SAL ester peptide, although showing different reactivities [114]. Different methods were developed to afford the synthesis of C-terminal SAL ester peptides by the use of both Fmoc- and Boc-SPPS. In the case of Fmoc chemistry, two methods were reported so far. Due to the lability of the O-SAL ester to piperidine, the reactive handle should be introduced at the peptide C-terminus after full peptide segment assembling. For C-terminal Gly and Pro, showing low propensity to epimerization, SAL ester has been obtained by direct coupling with a salicylaldehyde dimethyl acetal [113]. A more general approach, compatible with any type of C-terminal amino acid, takes advantage of the *N*-acyl-benzimidazolone (Nbz) resin previously reported by Dawson for Fmoc-based synthesis of thioester peptides [115] and uses an on-resin phenolysis of the peptide. After Fmoc-SPPS, the resin-bound peptide Nbz is cleaved from the resin by incubation with salicylaldehyde dimethyl acetal in the presence of Na_2CO_3 and then treated with TFA to give the desired unprotected C-terminal SAL ester peptide [113]. For the synthesis of SAL ester peptides via Boc chemistry, a 2'-hydroxyl cinnamate modified amino methyl resin was developed and used to bind the first amino acid through an ester bond. After full peptide assembling, an ozone treatment cleaves the alkene double bond of the cinnamate linker, releasing the desired SAL ester derivative in high yield [116, 117]. This Boc-chemistry-based latter method, however, is not compatible with Met, Trp, and Cys residues.

Several synthesis of peptides and proteins by STL have been reported. In a first effort, two peptides of therapeutic importance were synthesized by STL, namely the ovine corticoliberin (oCRH, 41 amino acids) and the parathyroid hormone (PTH, marketed as Forteo, 34 amino acids), by ligating two synthetic fragments, one bearing a C-terminal SAL ester while the other an N-terminal Thr or a Ser [117]. Both oCRH and Forteo peptides showed a Ser residue as first amino acid. Thus, to avoid SAL ester peptide cyclization, the oCRH and Forteo amino-terminus was protected with an azide or with a 4-methylsulfinylbenzyloxycarbonyl (Msz) group. In both cases, ligation proceeded smoothly and in high yields. STL reaction also afforded the synthesis of the 44-amino-acid human growth hormone-releasing hormone (hGH-RH) devising a three-segment C-to-N ligation scheme [118]. The synthetic strategy required the N-terminal Ser of the middle fragment to be protected with the Msz group, which is stable to the experimental condition of the Fmoc solid-phase synthesis of the peptide

salicylaldehyde esters but readily removable by reductive acidolysis for the subsequent ligation step.

The applicability of the STL method to the assembly of protein targets of biological interest was validated by performing the synthesis of the human erythrocyte enzyme acylphosphatase. The human erythrocyte acylphosphatase is composed of 98 amino acid residues and is acetylated at its N-terminus. Because it incorporates a number of well-distributed Ser and Thr residues, but no Cys residues, this enzyme represented an attractive platform for the application of Ser/Thr ligation in place of NCL [113]. The protein was assembled by sequentially ligating three segments of 45, 23, and 29 amino acids, respectively. The synthetic full acylphosphatase enzyme exhibited the expected hydrolytic activity. STL may also serve the synthesis of post-translationally modified proteins, as demonstrated through the preparation of MUC1 glycopeptide, a 80-mer protein target composed of a 20-amino-acid-long repeating unit carrying an O-glycosylation. A C-terminal SAL ester derivative of the glycosylated repeating unit was prepared and used to synthesize the full protein via three iterated ligation steps [119].

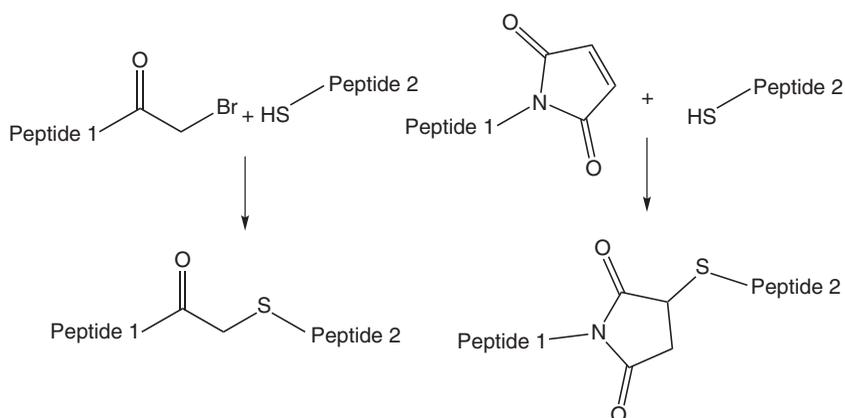
The STL method has also been recently applied to the site-specific N-terminal protein PEGylation [120] and to the synthesis of peptoid-peptide/protein hybrid structures [121]. In particular, STL was used to ligate an *N*-(methoxyethyl) glycine trimer peptoid oligomer to the N-terminus of the bioactive peptide Forteo. Additionally, a peptoid-containing peptide mimicking the amino acids 1–20 (S-peptide) of RNase A was ligated to a truncated form of the protein starting with Ser.

STL has also been successfully used to prepare cyclic peptides, such as cyclo-montanin B [122], yunnanin C [123], mahafacyclin B [117], daptomycin [124].

1.5 Thioether Ligation

The reaction of a thiol functionalized peptide with a bromo/chloro-acetylated or maleimide-substituted peptide led to ligation of the two fragments via the formation of a thioether linkage, which is a good surrogate of the peptide bond (Scheme 1.12).

The reaction occurs quickly under mild aqueous conditions and pH around neutrality. Thioether linkages have been used to generate cyclic peptide analogs and linear oligomers [125], in the synthesis of a platelet-derived integrin α IIb β 3 mimetic molecule containing two C-termini [126], for joining two recombinant protein fragments [127], HIV-1 protease [128] and transthyretin [129] analog. Thioether ligation has recently been used to synthesize MAP (multiple antigenic peptide) dendrimers by linking several copies of the peptide epitope onto the α - and ϵ -positions of a Lys core, either in solution or in the solid phase [130].

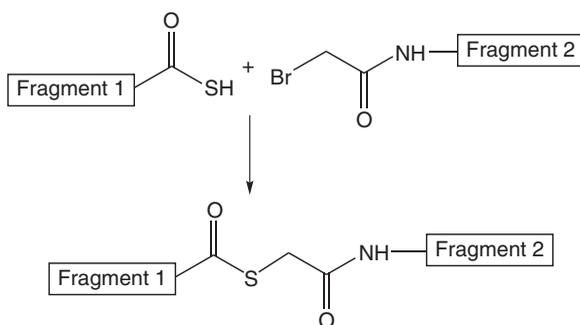


Scheme 1.12 The thioether ligation reaction.

1.6 Thioester Ligation

One of the oldest ligation chemistries yielding a nonnative bond at the junction site was developed by Schnolzer and Kent [17] and involved a nucleophilic substitution reaction between a thiocarboxylate peptide (a C-terminal thioacid) and an acyl halide (an N-terminal bromoacetyl-peptide) (Scheme 1.13).

Reaction proceeded in aqueous solution at mild acid pH to give a thioester-linked polypeptide product in which the thioester linkage may reasonably be considered a valuable mimic of the peptide bond. Schnolzer and Kent applied the thioester ligation reaction to the preparation of fully synthetic HIV-1 protease analog by joining two unprotected peptide fragments. Although the amide that joins residues 51 and 52 of HIV-1 protease was replaced by a thioester bond, the fully synthetic polypeptide was able to fold into an active



Scheme 1.13 The thioester ligation reaction.

molecule. However, the drawback of the thioester ligation refers to the hydrolyzable nature of the thioester bond under basic conditions, which limited its widespread application. The concept of thioester ligation was soon refined in the Kent laboratory so that a native peptide bond could be obtained rather than an unnatural thioester. The new strategy was named “NCL” and doubtless became the most powerful tool applied in the chemical total synthesis of proteins.

1.6.1 Native Chemical Ligation (NCL)

In 1994, Kent and coworkers introduced a breakthrough chemistry for the chemoselective ligation of unprotected peptides, called NCL [131]. The NCL immediately appeared as an important milestone in the total chemical synthesis of proteins and, 20 years after its first application, continues to play a pivotal role, and at present, it is the most widely used methodology for the chemical synthesis of proteins. To date, hundreds of proteins have been synthesized and modified using NCL [4]. The mechanism of NCL is simple and straightforward (Figure 1.5), leading to the formation of a single polypeptide chain bearing a native peptide bond at the ligation site by reacting two peptide segments, one containing a carboxy-terminal α -thioester group and the other an N-terminal 1,2-amino-thiol, generally a Cys residue. The reaction proceeds through the formation of a thioester intermediate, similarly to the product obtained from the thioester ligation reaction previously described in this chapter. In NCL, however, this thioester-linked adduct undergoes a spontaneous rearrangement via intramolecular S-to-N acyl shift, affording the desired native amide-linked product. While the formation of the thioester intermediate is a reversible step, the intramolecular nucleophilic attack forming the amide bond at the ligation site is irreversible, so that the overall reaction equilibrium is shifted toward the formation of the amide-linked polypeptide product [131, 132].

The reaction requires mild conditions and may be conveniently performed in aqueous solution at neutral pH. Denaturing agents, such as guanidinium hydrochloride (GdnHCl), can be used to increase the concentration of peptide reactants, resulting in higher ligation yields. Thus, after ligation, a refolding step may be required to obtain a functional protein. Similarly, an oxidation step is needed if the protein contains native disulfides, as NCL is usually performed in the presence of externally added thiols.

A remarkable feature of NCL is that ligation is orthogonal with respect to all the other functional groups present in a protein and selectively occurs between thioester and 1,2-amino-thiol even in the presence of internal Cys residues [131, 133]. No protecting groups are necessary for any of the side chains normally found in natural amino acids. Another notable feature of NCL refers to its high regioselectivity, as no racemization has been observed at the ligation site [134]. The efficiency of NCL reaction is strictly dependent on the identity

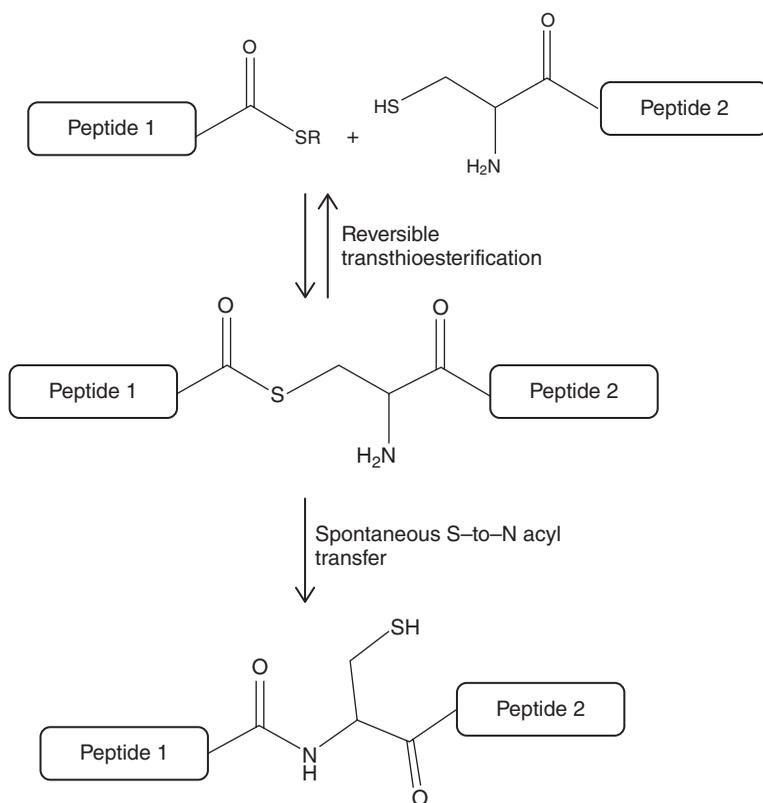


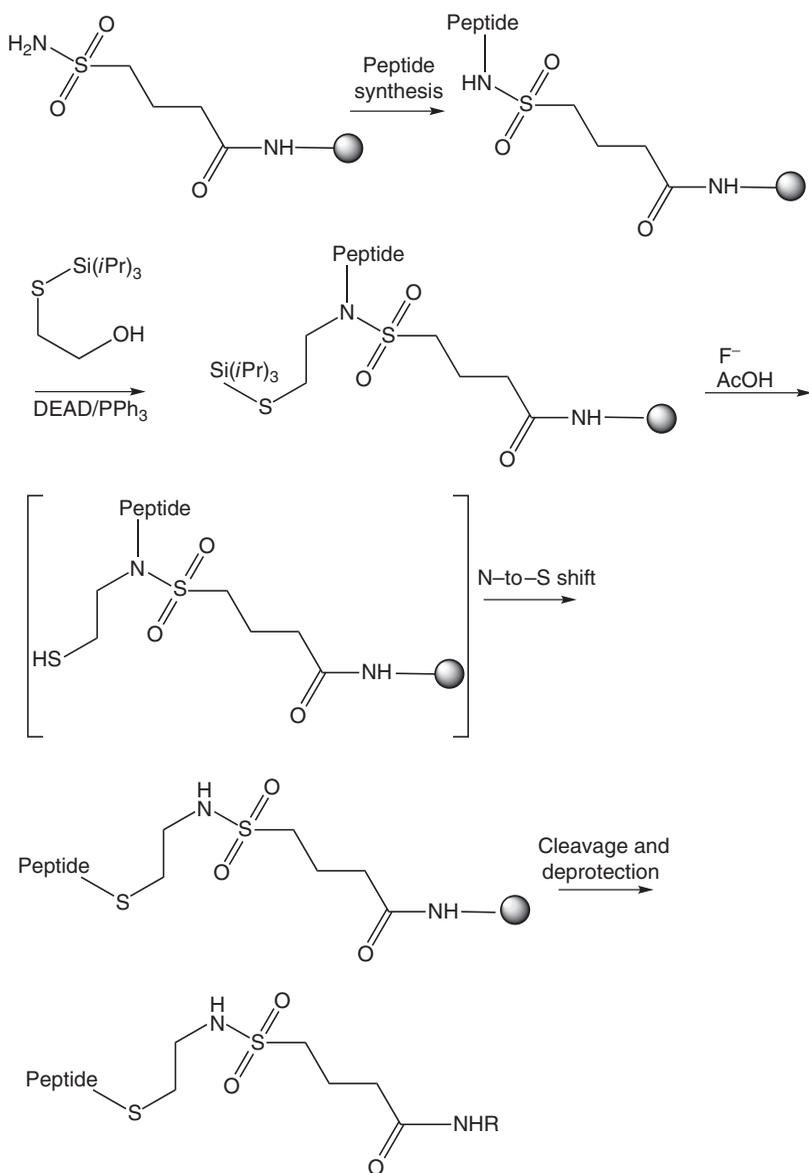
Figure 1.5 The native chemical ligation reaction mechanism.

of the C-terminal amino acid of the thioester peptide, and reactive preferences should be taken into account when selecting the ligation junction. Ligation occurs faster at less sterically hindered amino acids and slowly at the more hindered ones. In fact, Gly is considered as the best-performing amino acid, while Thr, Val, and Ile thioesters are reported to react slowly [132]. Ligation at Pro thioesters is generally not recommended [135], although recently, a solution to access NCL at Pro-Xaa sites has been reported [136]. Asn, Gln, Asp, and Glu are less favorable because of the formation of significant amounts of β -linked and γ -linked by-products [137, 138].

The yield and rate of NCL are also heavily related to the nature of the thioester moiety involved in the reaction. Thioalkyl-ester peptides are much less reactive than thioaryl-ester peptides in NCL. Their strong different reactivity in NCL reactions has been harnessed by Kent and coworkers to perform the fully convergent synthesis of proteins from multiple peptide segments by kinetically controlled ligation (KCL) [139]. In general, however,

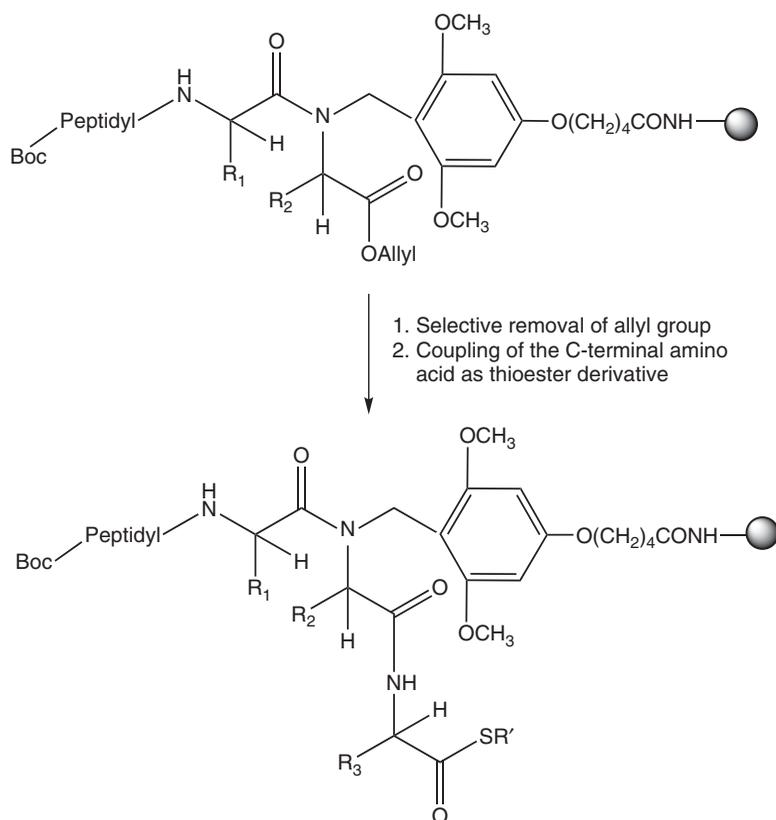
peptide α -thioalkyl-esters are more stable than α -thioaryl-esters and thus are preferred because of their easy handling and stability to long-term storage. For that reason, exogenous thiols are usually added to NCL reactions to convert *in situ* a less reactive and more stable thioester into a more reactive one. The addition of a thiol excess to the ligation mixture not only increases the reaction rate and yield by generating new thioesters but also counteracts thioester hydrolysis and prevents oxidation of the N-terminal Cys. The traditional thiol catalysts used for NCL are a benzyl mercaptan/thiophenol mix and the nonmalodorous and water-soluble thiol 2-mercaptoethanesulfonate sodium salt (MESNa). Johnson and Kent thoroughly investigated the reactivity of a range of commercially available thiols in NCL reaction and selected another excellent water-soluble aryl thiol additive, the mercaptophenylacetic acid (MPAA), currently reported as the “gold” NCL catalyst. MPAA does not have the unpleasant odor characterizing the majority of thiol additives and allows ligation to proceed more rapidly and with higher yield than with the traditionally more employed thiol additives, MESNa, thiophenol, and benzylmercaptan. Additionally, Kent and coworkers recently demonstrated that using the improved thioaryl catalyst MPAA, NCL can also be performed at Gln-Cys and Asn-Cys junction sites without the formation of β -linked and γ -linked side products and at the Glu-Cys site with a minimal level of by-product formation, thus extending the applicability of NCL also to such sites [140]. More recently, Payne and coworkers suggested the use of the efficient and volatile thiol catalyst 2,2,2-trifluoroethanethiol (TFET, bp 35–37°C), which can be easily removed from the reaction mixture following the ligation when necessary, for example, in cases in which MPAA cannot be used as it coelutes with the NCL product during HPLC purification or to allow *in situ* one-pot desulfurization reactions [141].

One of the greatest challenges of NCL refers to the ability of preparing C-terminal thioester peptides. Peptide thioesters may be effectively prepared by Boc-SPPS, using thioester linkers. However, the Fmoc chemistry is more commonly used compared to the Boc chemistry, as the Fmoc method does not use hydrofluoric acid (HF), which requires special precaution and apparatus for its handling. Additionally, the extremely strong acid conditions used for Boc-SPPS are not compatible with many peptide modifications introduced during synthesis, such as glycosylation and phosphorylation. However, the preparation of peptide thioesters by Fmoc chemistry is not trivial due to the use of the nucleophile piperidine for Fmoc removal that is incompatible with the use of the thioester linkers. Despite this latter issue, several strategies for the synthesis of thioester peptides compatible with Fmoc chemistry have been proposed [142–144]. The firstly described approach suggests the use of thioester-compatible deprotection cocktails for Fmoc removal [145, 146]; the synthesis of the peptide on an acid-labile resin, such as a Cl-trityl resin, in order to cleave the peptide chain in a fully protected form and introduce the thioester in solution



Scheme 1.14 Preparation of thioester peptides using the Kenner safety-catch system.

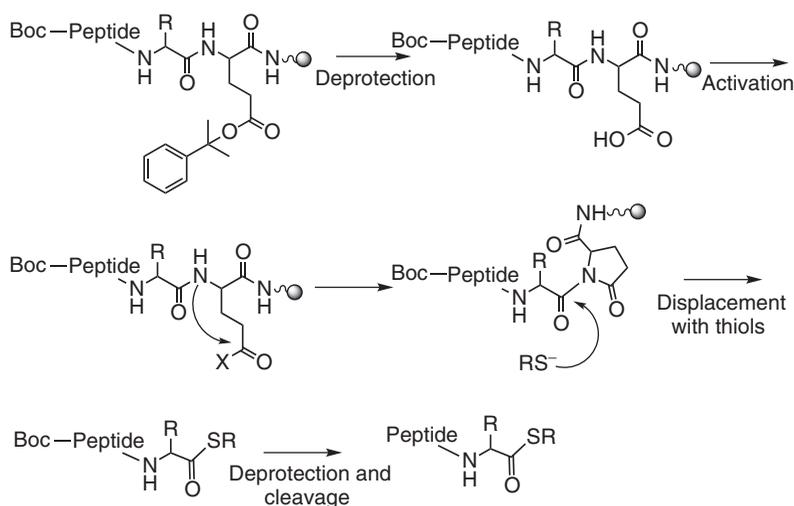
by thiol coupling to the peptide carboxy terminus [147, 148]; the safety-catch system [149] (Scheme 1.14), which uses the thiol labile Kenner's sulfonamide linker; the backbone amide linker (BAL) (Scheme 1.15) [150] and the backbone proglutamyl imide (Scheme 1.16) [151] strategies.



Scheme 1.15 Preparation of thioester peptides using the backbone amide linker (BAL).

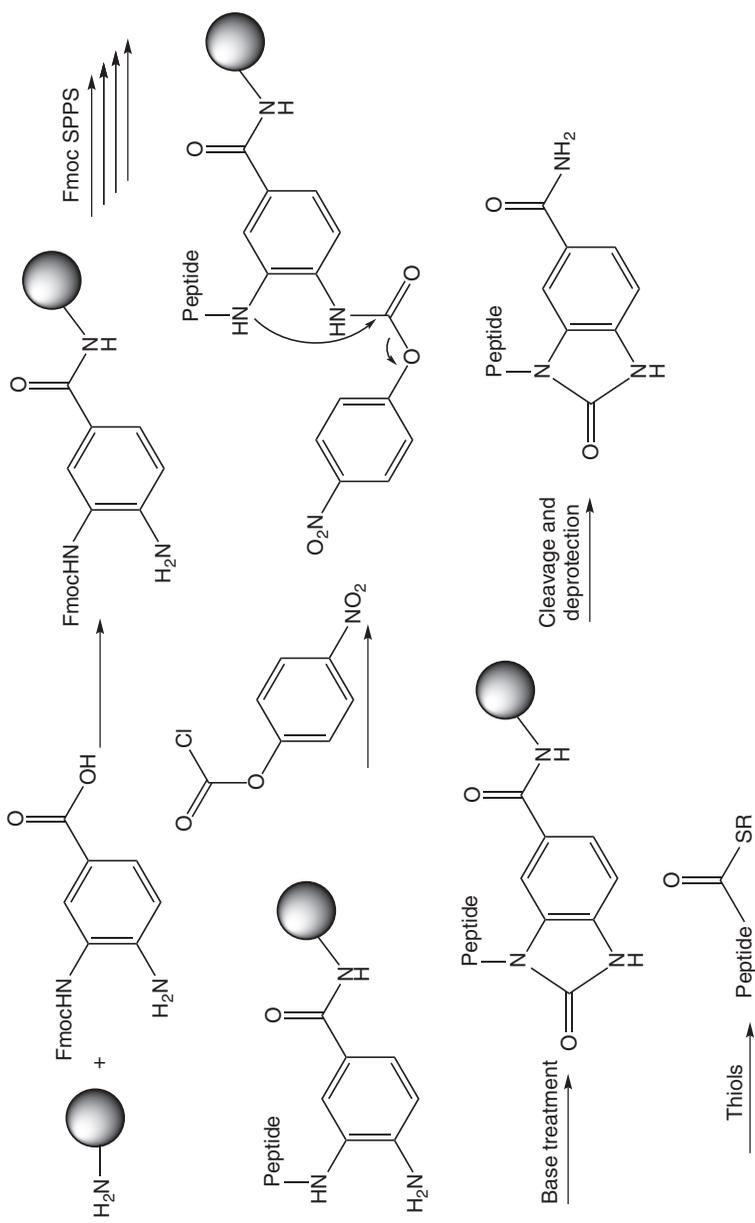
Many other convenient approaches, compatible with Fmoc chemistry, have been conceived, which ingeniously overcome the problems associated with thioester lability by temporary masking the thioester function with a more stable and readily synthesizable group. Notably, in this context, Blanco-Canosa and Dawson introduced a really efficient strategy for the routine production of peptide thioesters [115] (Scheme 1.17).

The strategy envisages the use of *o*-aminoanilide function as a surrogate of the C-terminal thioester group. Following chain elongation, the peptide C-terminal *o*-aminoanilide group is transformed into an *N*-acylurea derivative by on-resin treatment with *p*-nitrophenylchloroformate. After cleavage and deprotection, a fully unprotected *N*-acyl benzimidazolinone peptide is obtained that can undergo thiolysis in a neutral aqueous buffer to generate a peptide thioester for use in NCL. Importantly, this mechanism leads to a minimal (less than 2%) epimerization of the C-terminal amino acid. Conveniently, the

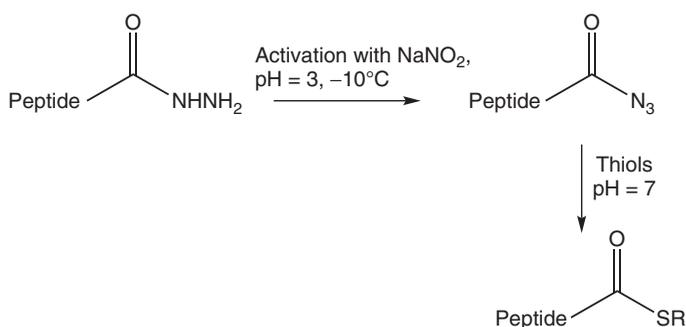


Scheme 1.16 Preparation of thioester peptides through a pyroglutamyl imide backbone activation.

o-aminoanilide-functionalized resin useful for the preparation of *N*-acyl benzimidazolinone peptides is commercially available, prompting the wide application of this approach in protein synthesis by NCL [152–155] (to cite only few of them). The synthesis of glycine-rich thioester peptides using the approach described by Dawson and coworkers may lead to the formation of diacylated products that cannot be converted into *N*-acylurea peptides. The Ottensen group extended the applicability of the synthetic approach also to Gly-rich sequences using the orthogonal allyloxycarbonyl (Alloc) protection of a single 3,4-diaminobenzoic acid (Dbz) amine [156]. Very recently, Blanco-Canosa et al. suggested a new *N*-acylurea linker bearing an *o*-amino(methyl) aniline (MeDbz) moiety that resulted efficiently in the synthesis of glycine-rich peptide sequences [157]. The activation of the *o*-aminoanilide was performed on the resin-bound fully protected peptide due to the incompatibility of *p*-nitrophenylchloroformate with nucleophilic groups present on unprotected peptides. Very recently, a new protocol that allows the activation of the *o*-aminoanilide peptide in aqueous solution by treatment with $NaNO_2$ has been described, thus allowing the convergent synthesis of proteins by NCL using the readily synthesizable peptide *o*-aminoanilides as crypto-thioester peptides [158]. Besides *o*-aminoanilide group, hydrazide function has been devised as thioester surrogate [159]. *C*-terminal hydrazide peptides can be readily prepared through standard Fmoc chemistry [160] and can be cleanly and rapidly converted into *C*-terminal thioester peptides in an epimerization-free manner by an oxidation step with $NaNO_2$, which affords an intermediate



Scheme 1.17 Preparation of thioester peptides through a C-terminal N-acylurea.



Scheme 1.18 Conversion of a hydrazide peptide into a thioester peptide.

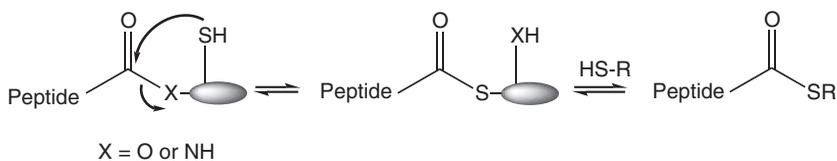
peptide acyl azide, followed by the addition of a thiol additive, such as MPAA (Scheme 1.18).

This process is compatible with all the 20 proteinogenic amino acids at the C-terminus, except Gln, Asn, and Asp because of intramolecular cyclization of their own side chains [159]. Peptide hydrazides have been used as crypto-thioesters in the convergent synthesis of proteins by sequential ligation of multiple peptide segments [152, 161–163].

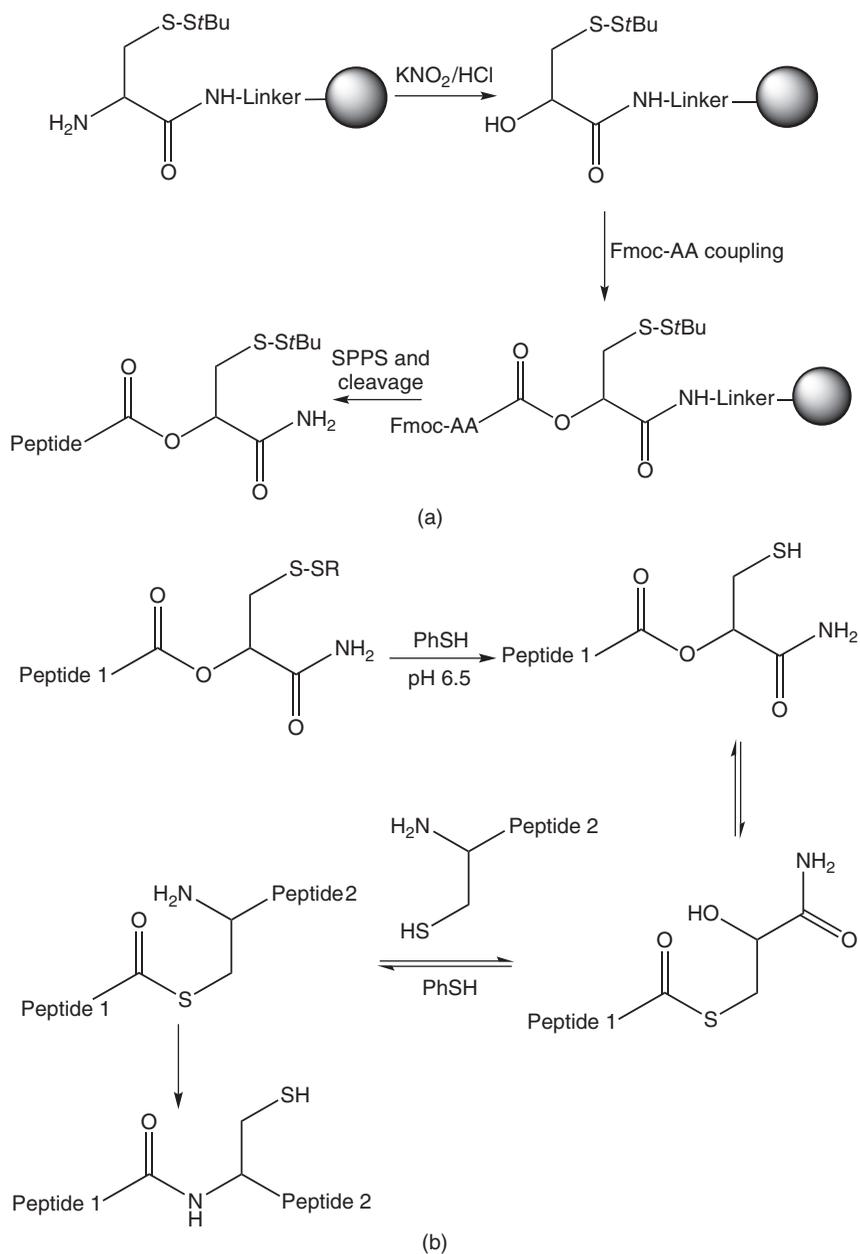
Many other useful approaches for Fmoc synthesis of thioester peptides devise intramolecular O-to-S or N-to-S acyl shift reactions to unmask a latent thioester functionality after its synthesis by Fmoc-SPPS. These systems exploit the ability of esters or amides featuring a thiol group to rearrange into a thioester intermediate, which can be exchanged by an external thiol to give a stable thioester (Scheme 1.19).

The O-to-S acyl shift methodology was firstly applied by Botti et al. to thioester peptide synthesis (Scheme 1.20) [164].

In their strategy, a peptide was synthesized and cleaved from the resin as C-terminal α -carboxyester of the 3-*tert*-butyldisulfanyl-2-hydroxypropionamide (Scheme 1.20a). They observed that such carboxyester peptide bearing a free mercaptan in the β position may undergo an *in situ* intramolecular O-to-S acyl shift, leading to the formation of its thioester isomer. In the same year, Danishefsky and coworkers also devised an O-to-S acyl shift reaction to prepare thioester from C-terminal-*ortho*-mercapto-phenolic ester peptides [165].



Scheme 1.19 Thioester synthesis by O-to-S or N-to-S acyl shift.



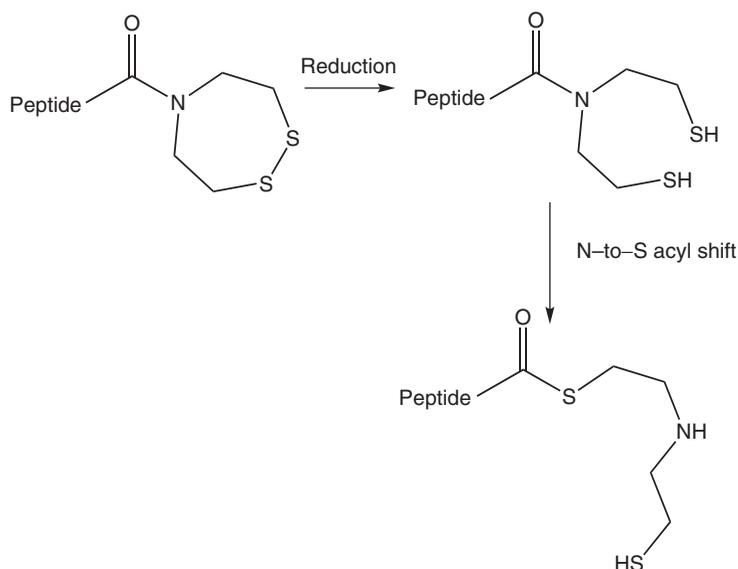
Scheme 1.20 (a) Preparation of peptide carboxy-ester using a rink amide PEGA resin and (b) native chemical ligation through *in situ* O-to-S acyl shift.

These seminal works inspired many subsequent applications of the O-to-S acyl shift as a route to synthesize thioester peptides from esters (Scheme 1.20b) [166–171]. A limitation of such approaches refers to low thioester yield due to the partial ester hydrolysis, despite many efforts aimed at minimizing the hydrolysis and maximizing the thioester formation by selecting more stable esters [167] and optimal O-to-S acyl shift conditions [170].

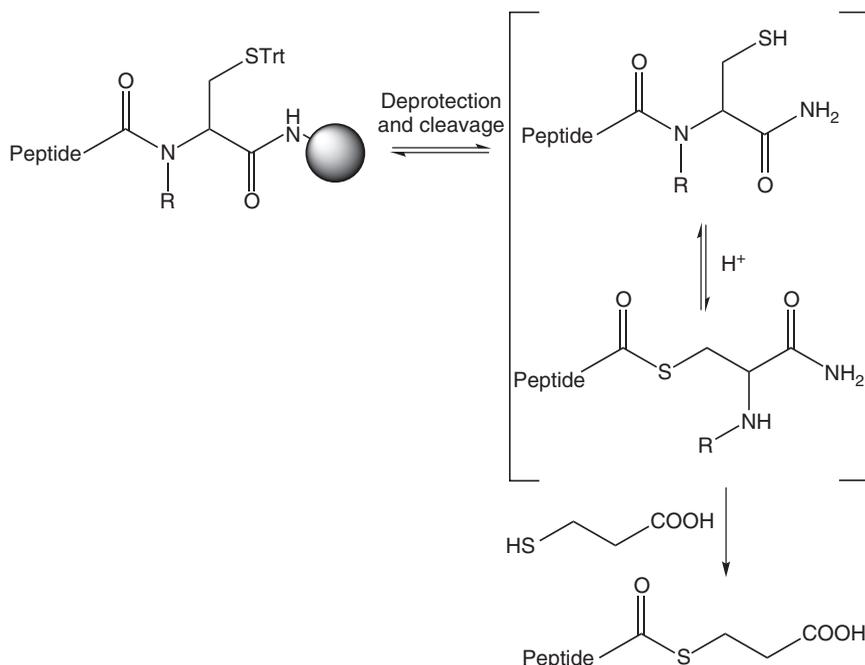
The intramolecular N-to-S acyl shift, which uses N-to-S migratory devices to obtain a thioester group from a relatively inert amide bond, has also been exploited to prepare C-terminal thioester peptide (Scheme 1.19).

A noteworthy chemical tool serving thioester peptide preparation by intramolecular N-to-S acyl shift is the bis(2-sulfanylethyl)amido (SEA) group [172, 173] (Scheme 1.21), whose application has been facilitated by the recent description of a practical experimental procedure for the synthesis of SEA polystyrene resin [174] and which has been used for the chemical synthesis of several functional proteins, such as the Kringle 1 domain of hepatocyte growth factor (HGF) in its native [175] or biotinylated [176] form, the heparin-binding N domain of HGF [177] and large polypeptides on a water-compatible solid support [178].

Another notable application of the N-to-S acyl shift mechanism for the preparation of thioester peptides is the use of C-terminal *N*-alkyl cysteines. *N*-Alkyl cysteines have been successfully proposed and applied as N-to-S migratory devices to allow thioester peptide preparation in acceptable yields and epimerization ratio [179, 180] (Scheme 1.22).



Scheme 1.21 Thioester peptides synthesis by the SEA methodology.

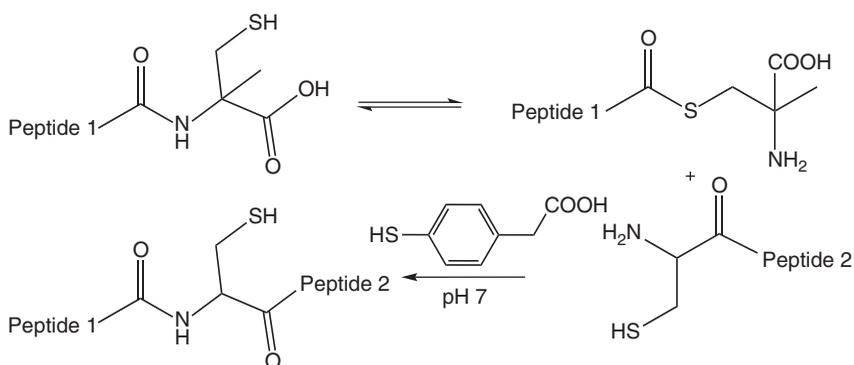


Scheme 1.22 Post-SPPS peptide thioesterification by N-to-S acyl transfer using N-alkyl cysteine.

To avoid the difficulties involved in acylating sterically hindered secondary amines and the propensity of N-substituted amino acids for diketopiperazine formation, Offer and coworkers explored the N-to-S acyl transfer properties of α -methylcysteine [181]. They showed that α -methylcysteine placed at peptide C-terminus may successfully serve as thioester precursor (Scheme 1.23).

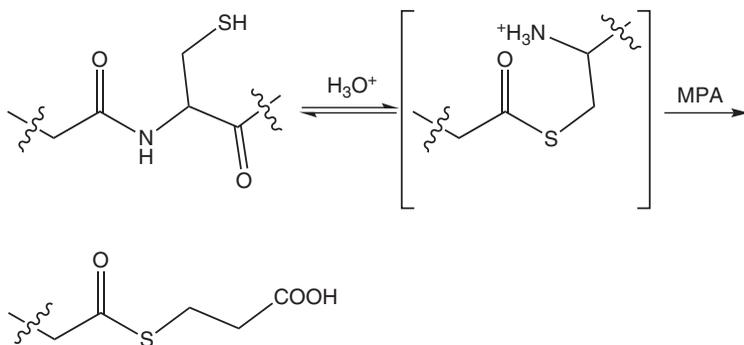
The synthesis of C-terminal α -methylcysteine peptide is simple and fully compatible with Fmoc peptide synthesis. Thus, this approach appears as a powerful new technology for chemical ligation because of its simplicity. Macmillan and coworkers reported that N-to-S acyl transfer and thioester exchange may also occur at cysteine residues in peptides using strong conditions of high temperature and acidic pH [182–184]. In particular, they observed thioesterification reaction in peptide and protein samples at GC, CC, and HC junctions in the presence of 3-mercaptopropionic acid (MPA) and high temperature, suggesting a retro-NCL reaction mechanism (Scheme 1.24).

The utility of the reaction for the preparation of thioester fragments in total protein synthesis was validated by Macmillan's group applying the strategy to the assembling of biologically active proteins, such as human β -defensin 3 (hBD3) [185] and the iron-regulatory peptide hepcidin [186].



Scheme 1.23 *In situ* thioester formation by N-to-S acyl transfer mediated by α -methylcysteine.

A drawback of NCL relies on the requirement of a Cys residue at the ligation site, which is a severe limitation considering that Cys is not so abundant in proteins. To overcome this limitation, many chemistries have been developed in order to extend the applicability of NCL to non-Cys sites. These chemistries use Cys analogs that, after NCL, can be selectively removed or converted into another amino acid. The most useful of these chemistries refers to the application of desulfurization reactions, a concept firstly devised by Yan and Dawson to perform NCL also at X-Ala sites [187]. Cys desulfurization to Ala may be accomplished in the final polypeptide product by reduction with Raney Nickel method [188] or by a milder, metal- and radical-free method requiring the water-soluble initiator VA-044 (2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride), in the presence of TCEP (tris(2-carboxyethyl) phosphine) and *t*-BuSH (*tert*-butyl mercaptan) [189] or glutathione [190]. Importantly, this latter method is compatible with the presence of Met



Scheme 1.24 Thioester synthesis through the proposed retro-NCL.

residues, a wide variety of functional groups, such as PTMs and biotin, and protecting groups as acetamidomethyl (Acm) and thiazolidine (Thz). The desulfurization approach has been generalized also to other β - and γ -thiol amino acid analogs, expanding the number of amino acids that could be obtained following desulfurization also to Phe [191], Val [190, 192], Thr [193], Lys [194–196], Leu [197, 198], Pro [199], Gln [200], Arg [201], Asp [202, 203], Glu [204], and Trp [205] (Figure 1.6).

In addition, ligation at X-Met site is possible, exploiting the latent thiol moiety of Met by the use of homocysteine; after ligation of the N-terminal homocysteine peptide with the C-terminal thioester moiety, S-methylation with excess of *p*-nitrobenzenesulfonate yields the native Met at the ligation site [206]. A limit of desulfurization is that it acts uniformly throughout all the protein, also reducing other Cys residues eventually present in the polypeptide chain. In this regard, Dawson group proposed the use of selenocysteine-mediated chemical ligation in combination with a protocol for the conversion of this amino acid to alanine, which preserves the native Cys residues present in the sequence without the need for side-chain protection [207]. The chemistry has also been extended to γ -selenoproline [208] and β -selenophenylalanine [209]. An efficient protocol for converting selenocysteine into serine has also been described and enables ligations to be performed at Ser [210]. A completely different kind of approach to ligation at non-Cys sites relies on the use of thiol-containing N(α) auxiliaries that mimic the presence of a Cys residue at the N-terminal of the peptide and that can be selectively removed from the newly formed amide bond after the ligation reaction [211]. The requirement for a Cys residue has also been overcome by devising a similar ligation chemistry, the direct aminolysis, between a C-terminal peptide thioester and an N-terminal amine group, without restriction on the nature of the first amino acid [212]. However, the method, in contrast to NCL, suffers great limitations, which prevented its exploitation, such as severe epimerization of the C-terminal thioester residue and thioester hydrolysis and low reaction rate and yield. Some efforts have been made to set better conditions for direct aminolysis, limiting the racemization and hydrolysis issues [213, 214]. However, some drawbacks continue to limit the application of direct aminolysis, that is, the lack of chemoselectivity in the presence of other functional groups, such as amine group of lysine residues, which requires selective protection, lower reaction rate with respect to NCL and reaction yields strongly variable depending on the nature of the amino acids at the junction site. Recent developments have shown that some N-terminal amino acids present enhanced reaction rate with C-terminal thioester peptides, such as phosphoserine, phosphothreonine (phosphate-assisted ligation) [215], glutamic and aspartic acid [216], fueling the interest toward this cys-free ligation strategy.

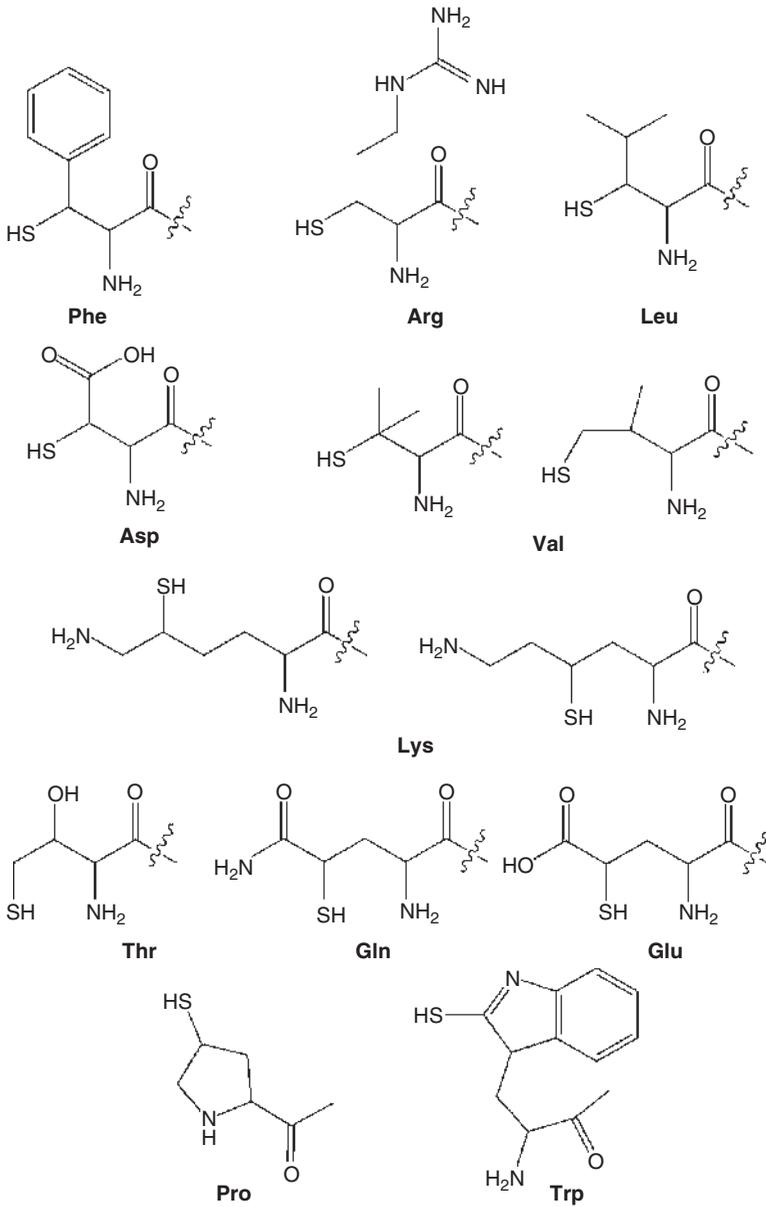


Figure 1.6 Amino acid analogs used in native chemical ligation–desulfurization synthetic strategies.

1.6.2 Expressed Protein Ligation (EPL)

The scope and application of the NCL have been significantly broadened by the introduction of a recombinant version of the strategy, named expressed protein ligation (EPL) [217, 218], in which one or both the C-terminal thioester peptide and the N-terminal cysteinyl peptide are prepared by recombinant DNA technology. After the introduction of NCL, small- to medium-sized proteins were readily synthesized, while the preparation of larger proteins through total synthesis still remained a challenge. EPL offered a practical solution to this size problem by combining the advantages of peptide chemistry with the easy and size-limitless recombinant protein expression. Thus, EPL expanded the access of chemical ligation approaches even to very large proteins [219].

Despite its recent introduction, EPL allowed the engineering of a plethora of protein targets. Site-specific chemical modifications with unnatural amino acids [220], PTMs [217, 221], biophysical probes [222–227], and topological rearrangements [228, 229] have been successfully realized by EPL. To achieve C-terminal thioester or N-terminal Cys containing protein segments, EPL exploits a class of proteins called inteins that are able to catalyze protein splicing. Protein splicing is a multistep process by which an intein mediates its self-excision from a polypeptide chain and concomitantly ligates its two flanking sequences, called exteins [230] (Figure 1.7). Protein splicing proceeds

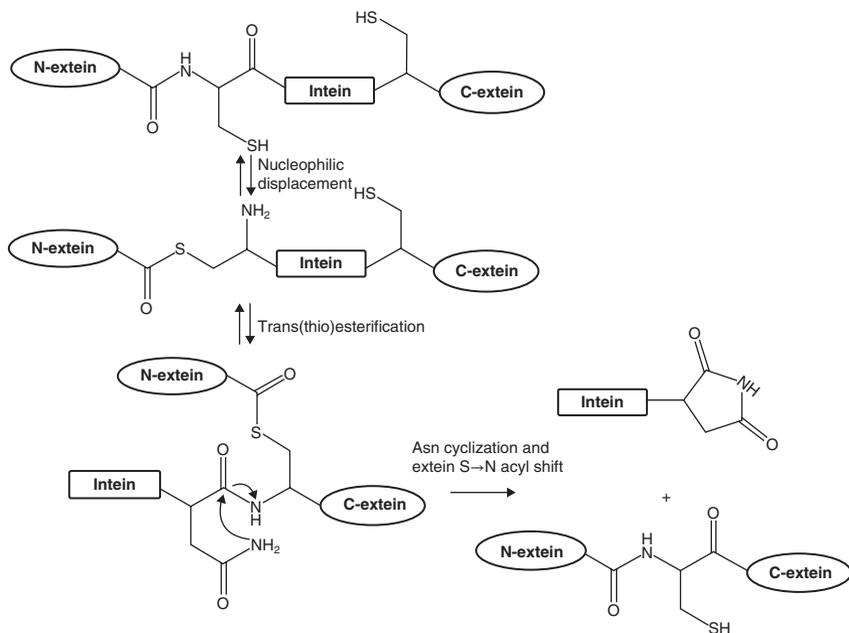


Figure 1.7 The mechanism of intein splicing.

through an N-to-S (or N-to-O) acyl shift in which the N-extein is transferred to the side chain -SH or -OH group of a Cys/Ser residue located at N-terminus of the intein. The N-extein is then transferred through a trans(thio)esterification reaction to a second Cys/Ser residue, this time located at the N-terminus of the C-extein. A cyclization reaction involving a conserved Asn residue at the intein C-terminus leads to intein excision and to the formation of a new peptide bond between the two exteins, giving the mature polypeptide chain.

Genetically engineered inteins have been envisioned as a powerful tool for preparing recombinant C-terminal thioester or N-terminal cysteinyl polypeptides required to perform EPL. In particular, mutation of the conserved intein C-terminal Asn residue to Ala blocks the splicing process to the thioester intermediate, which can be conveniently cleaved off by addition of thiols to obtain the N-extein as C-terminal thioester protein (Figure 1.8a).

The thiol MESNa is often employed to mediate intein thiolysis. Besides thiols, other nucleophiles may be used to induce intein splicing; for example, hydrazines have been employed to generate recombinant protein fragments bearing a C-terminal hydrazide function, which works as a crypto-thioester in the total synthesis of proteins [159]. An alternative chemical thioesterification reaction of a polypeptide prepared from *E. coli* expression was reported by Kajihara et al. [231]. In this strategy, the polypeptide is cyanylated on the thiol group of a Cys residue by treatment with 1-cyano-4-dimethylaminopyridium tetrafluoroborate (CDAP) reagent. The polypeptide is then cleaved at the S-cyanylated Cys in the presence of hydrazine giving a C-terminal hydrazinopeptide, which can be converted into the corresponding C-terminal thioester. However, the strategy requires that no other cys residues are present within the target polypeptide, limiting its applicability. To prepare amino-terminal Cys protein fragments, inteins have been mutated in order to induce the cleavage at the C-terminal splice junction, between the intein and a C-extein starting with Cys, through pH and temperature changes (Figure 1.8b) [230]. Several *E. coli* expression vectors are commercially available that allow the recombinant expression of fusion constructs with engineered inteins, such as the *Mycobacterium xenopi* DNA gyrase A (Mxe GyrA) intein [232], which is the most widely used intein for thioester protein preparation [233], and *Synechocystis* sp. PCC6803 DnaB helicase (Ssp DnaB) intein [234], mainly used instead for amino-terminal Cys protein release.

While the preparation of thioester protein is mainly restricted to the use of the protein-splicing technology, the introduction of N-terminal Cys residue into a recombinant polypeptide may also be accomplished without the use of the inteins, for example, by introducing a Cys downstream the initiating Met residue [235] or using site-directed proteolysis to unmask a cryptic N-terminal Cys [236–239]. Recently, an intein-free approach to the preparation of C-terminal thioester proteins has also been reported [240]. This alternative approach exploits the incorporation of oxoester moiety into the backbone of a

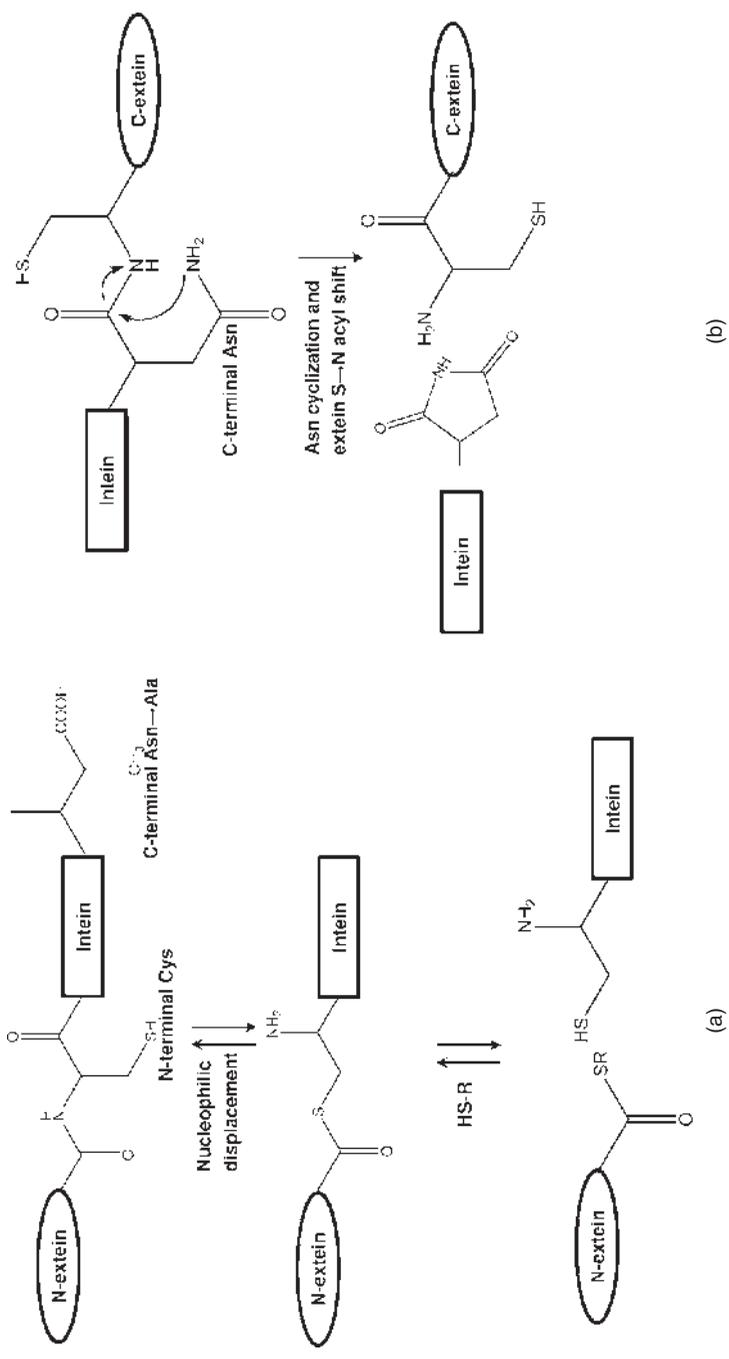


Figure 1.8 Preparation of recombinant α -thioester proteins (a) or N-terminal cysteinyl-proteins (b) using engineered inteins.

protein target via genetic code expansion technology. The oxoester function may be converted by hydrazinolysis into an α -hydrazide group, which in turn may easily be transformed into a thioester-reactive handle.

1.6.2.1 Protein trans-Splicing (PTS)

Besides EPL, another process based on the use of intein technology named protein trans-splicing (PTS) has been devised as chemical ligation and modification tool [241]. Protein trans-splicing is based on the use of a particular class of inteins, which are split into halves (Int^{N} and Int^{C}) and, upon mixing, are able to reassemble noncovalently into a functional splicing element, leading to the conjunction through a native peptide bond of the two polypeptide chains linked to each piece of the split intein (Figure 1.9).

Protein synthesis by PTS shows several potential advantages over NCL or EPL. To cite some, no synthesis and handling of the highly reactive thioester protein fragments are required and the reaction may be performed under very mild conditions, without thiol catalysts or denaturing agents. Furthermore, the reaction usually proceeds efficiently also at lower concentrations of the two split intein fragments, providing an extremely powerful platform for chemical modification of proteins both *in vitro* and *in vivo*. However, insolubility of fusion proteins with many split inteins has been often observed due to the exposure of hydrophobic patches, which represents a strong limitation to the use of PTS [242]. Another strong limitation of PTS is that often inteins placed outside of their native host proteins, and thus between two unnatural exteins, became unable to splice efficiently. This happens because inteins require the presence of specific amino acids in the extein regions directly flanking the intein. For instance, inteins require the first amino acid of the C-terminal extein to bear a nucleophile in its side chain, such as Ser, Thr, or Cys, as the amino acid in this position is directly involved in the intein-splicing reaction mechanism. Moreover, inteins may require the presence of several amino acid residues of the native exteins in the intein-flanking regions, which are not directly involved in the splicing process but contribute to stabilize the intermediate structure or drive intein catalysis. For these reasons, the general applicability of the system is strongly limited. However, to overcome this issue, inteins showing a higher flexibility with respect to the amino acid composition of their flanking region have been selected by sequential directed evolution [243].

Split inteins have been examined to identify the possible site of splitting along the sequence. Such investigations showed that many inteins may be split at several sites, and conveniently, in some of them, such as Mxe GyrA, Ssp DnaB, and Ssp GyrB inteins, the splitting site can be shifted very close to intein termini [244–247], rendering N-terminal or C-terminal split intein fragments so short that the preparation of these segments and their exteins can be accomplished by SPPS, expanding the repertoire of possible chemical modifications that can be introduced into a protein using PTS.

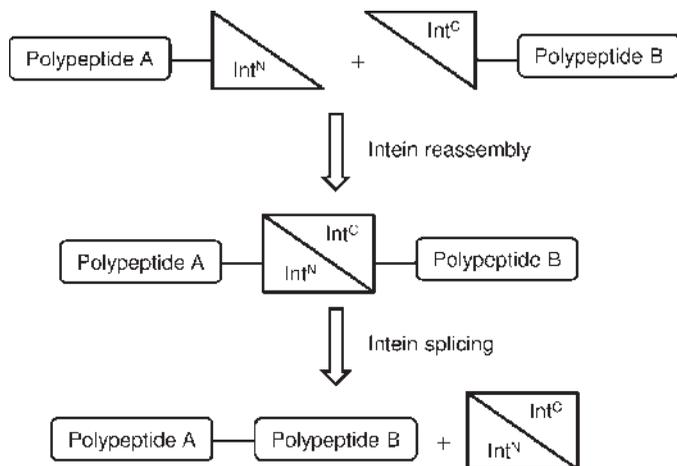


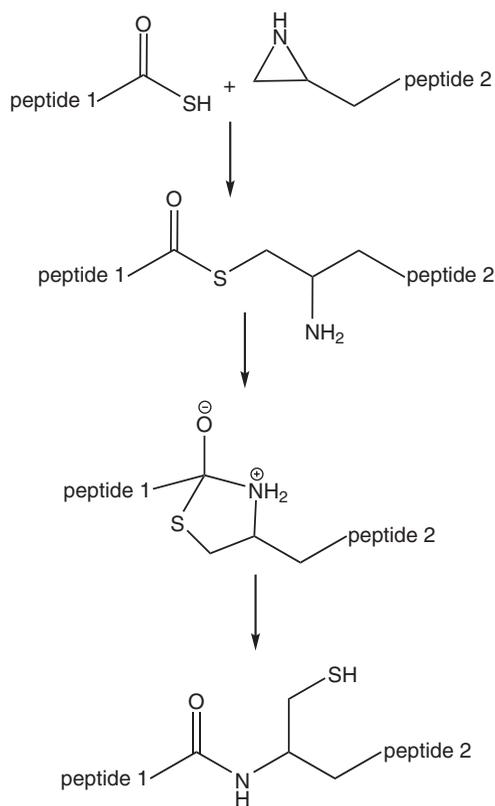
Figure 1.9 Schematic mechanism of protein trans-splicing. Int^{N} and Int^{C} are the halves, N- and C-terminal, respectively, of a split intein.

1.6.3 Thioacid-Mediated Ligation Strategies

Several chemical ligation chemistries share the use of a C-terminal peptide-thiocarboxylate as building block. Recently, new convenient methods have been reported for the preparation of peptide and protein thioacids by chemical [248–251] or biological [251–253] means, mostly based on a simple hydrothiolysis reaction of a thioester precursor. This makes thioacid peptides and even proteins easily available, hence encouraging the use of thioacid-based ligation methods.

Cysteine–aziridine ligation, also referred to as peptidomimetic ligation, is one example of thioacid-dependent ligation chemistry. The reaction enables the incorporation of a reduced amide bond as a peptidomimetic linkage at the site of ligation [254] by coupling of a C-terminal thioacid peptide with an N-terminal aziridine peptide (Scheme 1.25).

The thioacid function promptly reacts with aziridine via a ring-opening reaction, which leads to the formation of a thioester bond between the two peptide segments. Subsequently, an S-to-N acyl shift occurs giving the final product, which harbors an amide peptidomimetic bond at the junction site. Assem et al. prepared a series of peptide aziridines [254] via reductive amination between aziridine aldehyde dimers and peptides [255], while thio-amino acids were prepared through activation of the carboxylic acid with carbonyldiimidazole followed by addition of sodium sulfide. Depending on the substituents present in the aziridine aldehyde reagent used to synthesize the aziridine peptide, this reaction may enable the incorporation of reduced cysteine, reduced substituted cysteine, and, in combination with a desulfurization step, also reduced phenylalanine and reduced alanine.

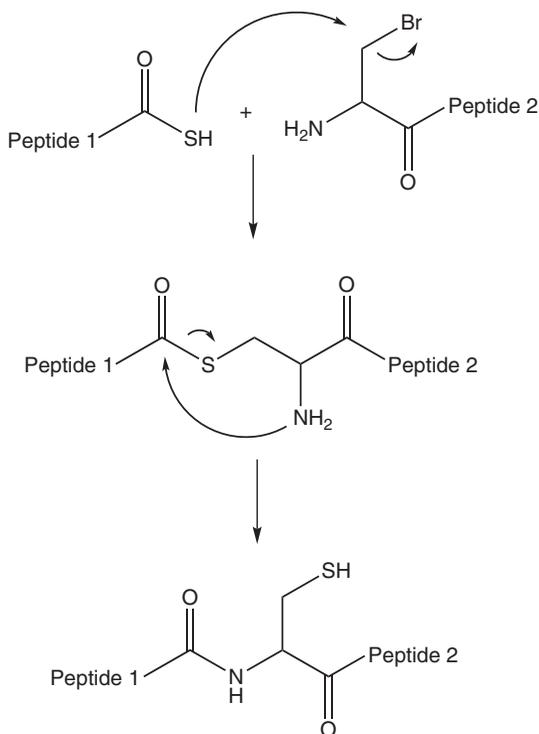


Scheme 1.25 The peptidomimetic ligation reaction.

A similar ligation approach is the cysteine–bromoalanine ligation. In this case, the C-terminal thioacid peptide reacts with an N-terminal halogenoalanine, often a bromoalanine (BrAla) peptide. BrAla is readily S-alkylated by a thioacid to form a covalent thioester that will rearrange rapidly to give the cysteine at the ligation site [256] (Scheme 1.26).

An analog synthetic method, giving thioester-linked polypeptides as final products, refers to the reaction of a C-terminal peptide-thiocarboxylate with an N α -bromoacetyl-peptide, as reported by Schnolzer and Kent to prepare a backbone engineered human immunodeficiency virus-1 (HIV-1) protease [17]. In the cysteine–perthioester ligation strategy, the C-terminal thiocarboxylic peptide is coupled to a peptide segment bearing an N-terminal persulfide handle (Scheme 1.27).

Liu et al. applied this method to the preparation of a 32-mer peptide analog of HIV-1 protease [257]. This method proceeds via the formation of a six-membered ring intermediate with a disulfide linkage that undergoes an



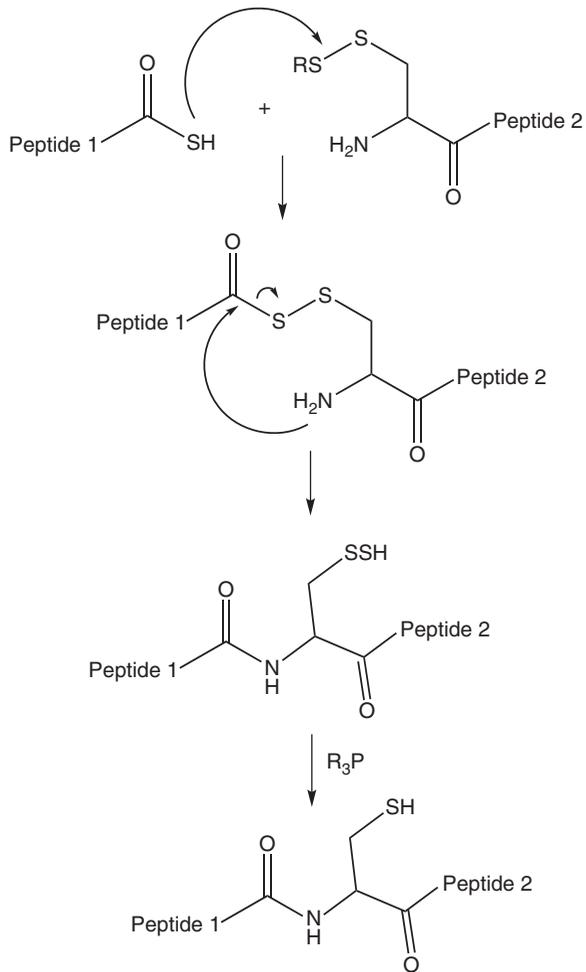
Scheme 1.26 The cysteine bromoalanine ligation.

intramolecular *S,N*-acyl transfer to result in a peptide product containing a hydrodisulfide group ($-SSH$). Reduction of the hydrodisulfide gives a cysteine residue at the ligation site.

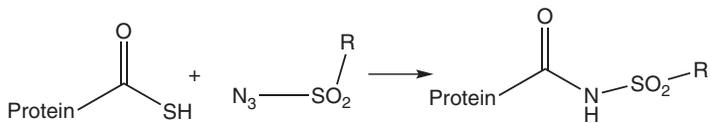
The thioacid functionality has also been shown to react with an azido moiety forming an amide linkage [258] (Scheme 1.28).

This thioacid/azide amidation reaction has been employed to specifically label the C-terminus of recombinant ubiquitin using an azide carrying a biofunctional tag [253]. More recently, the thioacid capture ligation (TCL) strategy (Scheme 1.29) has been reported to be a valuable tool for the chemical synthesis of proteins and post-translationally modified proteins by performing the synthesis of site-specifically modified histone H3 proteins [259].

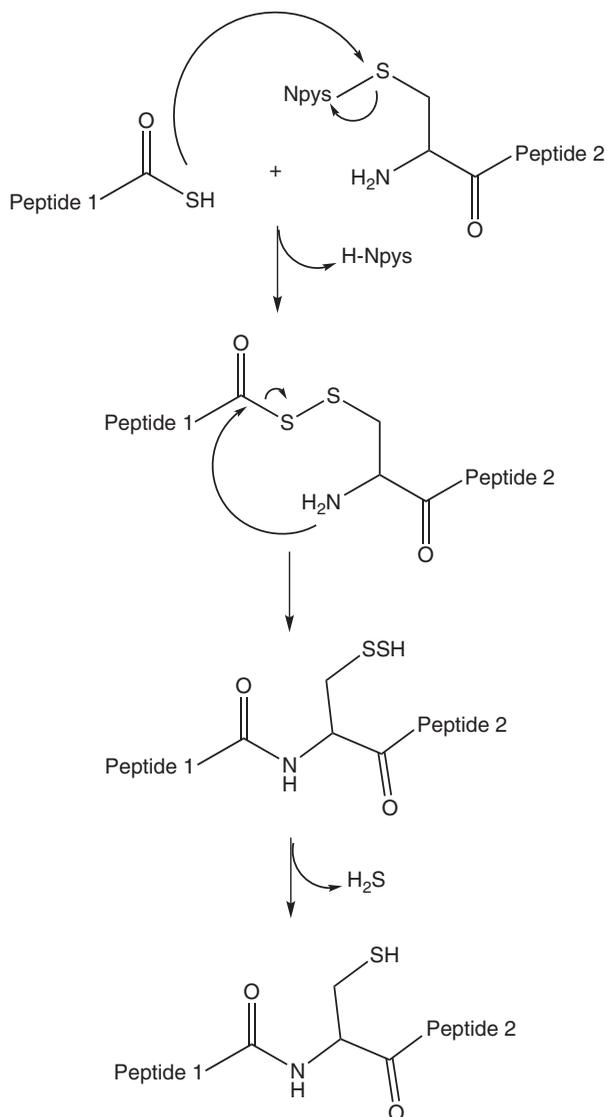
Key to this ligation method is a highly efficient capture reaction between a C-terminal thioacid of one peptide and an activated 5-nitro-2-pyridinesulfonyl (Npys)-S disulfide on the N-terminal Cys of another peptide. The reaction proceeds through the initial formation of an acyl disulfide intermediate, which then undergoes rapid intramolecular acylation to generate a peptide bond.



Scheme 1.27 The cysteine perthioester ligation reaction.



Scheme 1.28 The thioacid azide amidation reaction.



Scheme 1.29 Thioacid capture ligation.

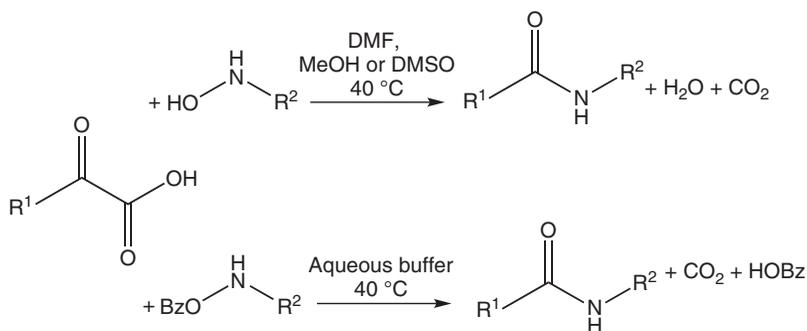
A simple reduction reaction with a reducing agent affords the final product with a native Cys residue at the ligation site. In another reported ligation chemistry, C-terminal thioacid peptides were reacted with amines to generate amide bond in the presence of the Sanger (2,4-dinitrofluorobenzene) or Mukaiyama (2-chloro-1-methylpyridinium iodide) reagent. This reagent generates highly

reactive thioesters *in situ* from the thioacids, which then react with the amine to form peptide bonds [260].

1.7 α -Ketoacid-Hydroxylamine (KAHA) Ligation

KAHA ligation was reported by the Bode group as a chemoselective way to couple two unprotected peptides, one bearing a C-terminal α -ketoacid functional group and the other an N-terminal hydroxylamine giving an amide bond at the ligation site [261]. Depending on the nature of the hydroxylamine moiety employed, KAHA ligation reaction exhibits two prototypical variants, named type I and type II ligations. The former uses *O*-unsubstituted hydroxylamines, while the latter employs *O*-substituted hydroxylamines (Scheme 1.30).

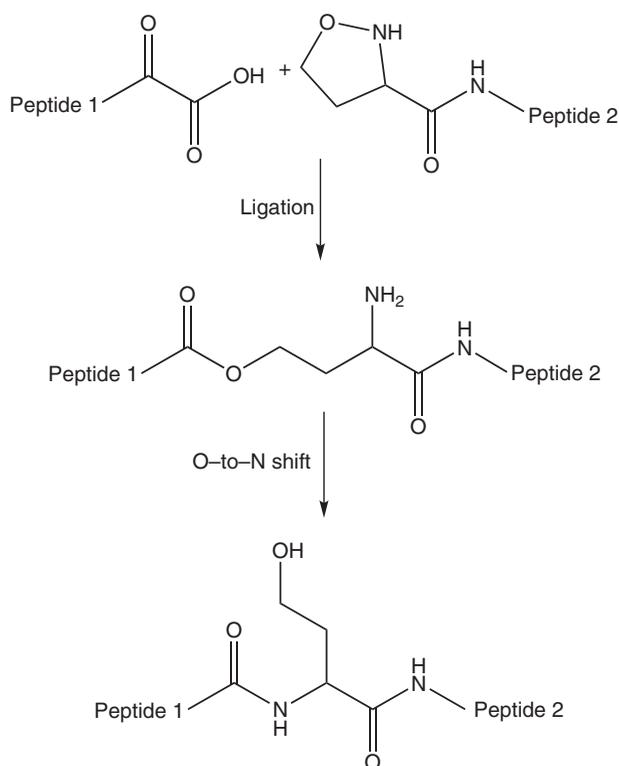
Both reactions yield an amide bond at the ligation junction, but type I ligations are performed in organic solvents, such as DMF, DMSO, or MeOH, and are slower compared to type II ligations, which are instead performed in aqueous media. The Bode group elucidated the mechanism of type I ligations [262], demonstrating that *O*-unsubstituted hydroxylamines react with α -ketoacids following a very complex reaction pathway, which involves the formation of many intermediates, including the formation of a nitron from a hemiaminal through a dehydration reaction. This reaction step may justify why water is detrimental to type I KAHA ligation. However, the complexity of the type I KAHA ligation is responsible of the high chemoselectivity of the reaction. The precise mechanism followed by type II ligations remains an open question but presumably does not proceed via a nitron intermediate and thus is suited for ligation in aqueous solution. Despite some small peptides, such as GLP-1, being efficiently synthesized by type I KAHA ligation [263], incompatibility with the aqueous conditions generally required for peptide fragment solubilization and handling limited its application. Instead, type



Scheme 1.30 Type I and type II KAHA ligation.

II KAHA ligation had greater success, in particular after the introduction of 5-oxaproline as an *O*-substituted hydroxylamine sufficiently reactive in ligation reaction with α -ketoacid peptides, stable under both SPPS and ligation reaction aqueous conditions [264]. The use of 5-oxaproline marked a significant advance in the KAHA ligation as it allowed to overcome many problems correlated to the use of *N*-hydroxyamino acids, which were often unstable under synthesis and ligation itself conditions. The Bode group proposed both the synthetic strategy to prepare enantiopure (*S*)-*N*-Boc-5-oxaproline [264] or (*S*)-*N*-Fmoc-5-oxaproline monomers [265] and the synthesis by SPPS of C-terminal α -ketoacid peptides using a cyanosulfur-ylide-based linker and oxidation of the pure sulfur-ylide peptide with potassium peroxymonosulfate [266]. As the oxidation reaction of the cyanosulfur-ylide to obtain the corresponding α -ketoacid is not compatible with peptide segments containing Cys, Met, or Trp, as an alternative, they proposed an additional protocol compatible with all amino acid side chains, which used a protected form of leucine α -ketoacid for obtaining the corresponding enantiopure peptide α -ketoacid directly upon cleavage from the resin, avoiding post-SPPS manipulations [267]. The authors focused on the preparation of Fmoc-leucine α -ketoacid because of its wide distribution into proteins, but the application of this strategy is, however, limited to the preparation of peptide fragments carrying a leucine as C-terminal amino acid. KAHA ligations between α -ketoacid and 5-oxaproline (Scheme 1.31) are usually carried out using peptide segments at the concentration of 10–20 mM in aqueous solutions, such as NMP/H₂O or DMSO/H₂O, in the presence of 0.1 M oxalic acid (pH 1–2).

While not necessary, oxalic acid has a beneficial effect for both peptide solubilization and ligation. The formation of the ligation product can be observed within a few minutes but reached maximum conversion after 6–8 h. Mechanistic studies showed that the primary products of KAHA ligation with 5-oxaproline are esters (depsipeptides), which readily rearrange to the amide-linked final product in basic buffers, such as 0.2 M NH₄HCO₃, at pH 9.5 for 1 h. The 5-oxaproline is converted into homoserine after ligation and *O*-to-*N* acyl shift. Although homoserine may serve as surrogate for threonine, serine, methionine, aspartic acid, and asparagine [264, 268], the presence of an unnatural amino acid may have an effect on protein structure and function. The KAHA ligation was applied to the synthesis of several proteins, such as Pup [264], CspA [264], UFM1 [265], SUMO2, and SUMO3 [267], and to the Heme Protein Nitrophorin 4 [269]. Very recently, an oxazetidine amino acid incorporated at the *N*-terminus of a peptide segment has been used in serine-forming ligations with peptide α -ketoacids, overcoming the limitation of the formation of a noncanonical homoserine residue at the ligation site. Additionally, in this case, the reaction proceeded at mild temperature and required lower reactant concentrations [270]. Some of the aforementioned protein syntheses were carried out



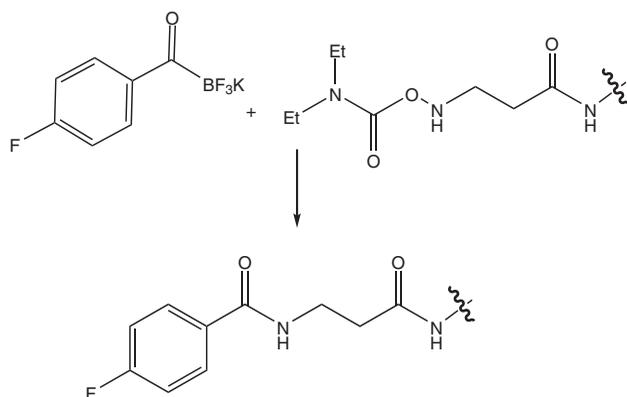
Scheme 1.31 KAHA ligation with 5-oxaproline.

using a sequential approach consisting of multiple KAHA ligations. To this aim, 5-oxaproline was temporarily masked with an $N\alpha$ -Fmoc group, which was removed at the occurrence by basic treatment (5% Et_2NH in DMSO at 15°C for 5–10 min) [265].

1.7.1 Acyltrifluoroborates and Hydroxylamines Ligation

The Bode group reported another amide-forming ligation reaction that uses potassium acyltrifluoroborates (KATs) as surrogates of α -ketoacids and *O*-carbamoylhydroxylamines [271, 272] (Scheme 1.32).

KAT ligation showed excellent chemoselectivity, rapid amide formation, and high stability under aqueous conditions. The applicability of the method was demonstrated using KAT ligation for the conjugation of unprotected peptides to polymers and dyes. Further efforts are needed to develop synthetic methods useful in introducing KAT ligation reactive moiety at C- and N-termini of two peptides [268].

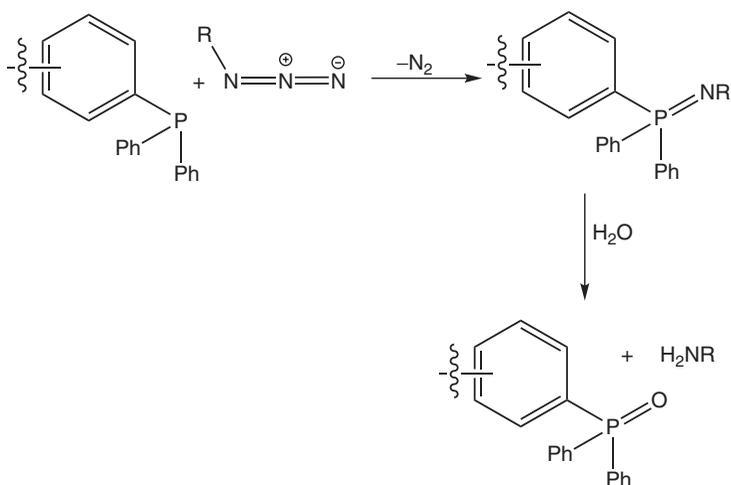


Scheme 1.32 KAT ligation reaction.

1.8 Staudinger Ligation

The reaction of a phosphine with an azide was described by Staudinger and Meyer in 1919 [273] and proceeds through the formation of an iminophosphorane, which, in the presence of water, spontaneously hydrolyzes to yield a primary amine and the corresponding phosphine oxide (Scheme 1.33).

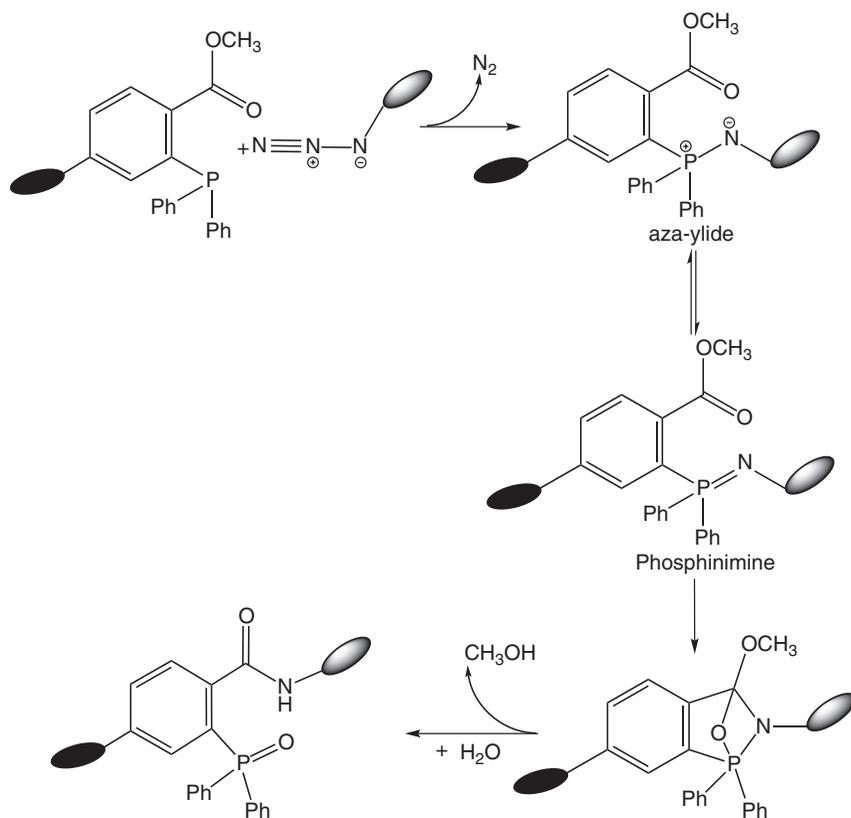
Saxon and Bertozzi firstly proposed the original use of the Staudinger reaction as bioconjugation tool [274]. In their pioneering application to the modification of cell-surface glycans, Saxon and Bertozzi proposed the use of



Scheme 1.33 The Staudinger reaction.

triphenylphosphines *ortho*-substituted with an electrophilic reactive group, such as a methyl ester group. In this case, the iminophosphorane intermediate undergoes a cyclization reaction to form an oxazaphosphetane, which, in water solution, ultimately hydrolyzes to afford a stable amide bond (Scheme 1.34).

Mechanistic studies performed by NMR spectroscopy using ^{31}P allowed the identification of the aza-ylide and the oxaphosphetane as intermediates in the ligation reaction and allowed the identification of the main parameters that affect the rate and yield of the Staudinger ligation, such as the electronic properties of both the phosphine and the azide and solvent polarity [275, 276]. The reaction requires mild aqueous conditions and is rapid, high yielding, and chemoselective, and thus immediately appeared as a promising tool in chemical biology. However, the drawback of the nontraceless Staudinger ligation refers to the incorporation of a phosphine oxide group in the final product, which



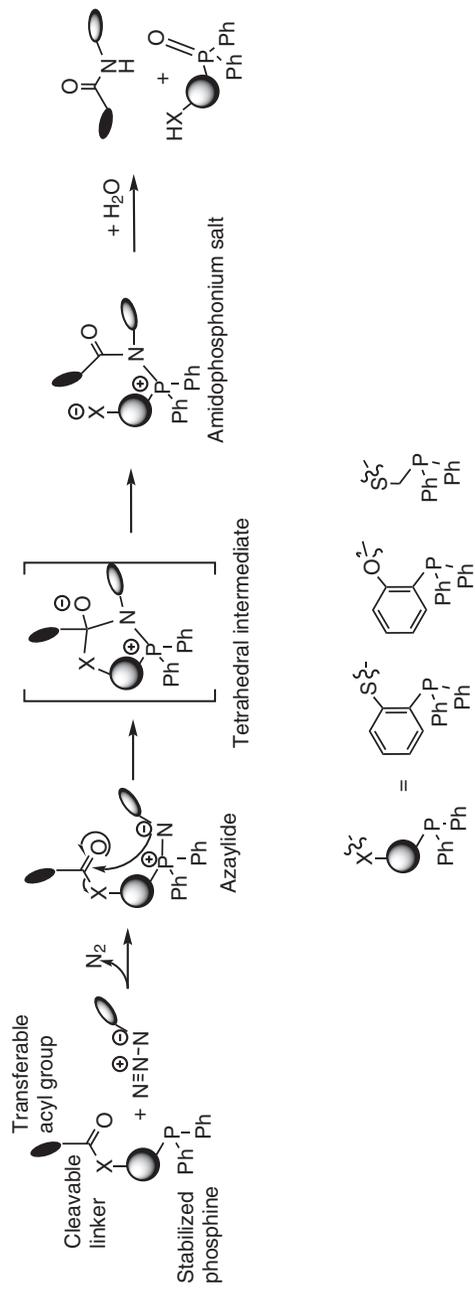
Scheme 1.34 The nontraceless Staudinger ligation reaction.

remains as a “scar” testifying the reaction performed. This limitation was soon overcome by the introduction of a “traceless” approach, developed independently by the Bertozzi [277] and Raines [278] groups. They refined the procedure to yield a final amide product that does not carry the phosphine oxide group (Scheme 1.35).

This is accomplished by reacting the azide with a phosphino(thio)ester derivative, in particular, alkyl-diphenyl-substituted phosphines such as diphenylphosphino-methane thioesters, which are the most often used reagents for effecting the traceless Staudinger ligation [279]. In the case of the traceless Staudinger ligation, after the formation of the aza-ylide, the nucleophilic nitrogen of this intermediate attacks the carbonyl group of the phosphino(thio) ester, cleaving the linkage with the phosphonium group and giving an amidophosphonium salt. Hydrolysis of this latter adduct produces an amide bond product and liberates a phosphine oxide. This modification to the Staudinger ligation allowed this chemistry to become a useful methodology for the convergent chemical synthesis of proteins and for protein bioconjugation [280–283], finding widespread application in the modification, labeling [284–287], and immobilization [288] of DNA and proteins even within a living animal [289].

Raines and coworkers firstly suggested and validated the use of Staudinger ligation as peptide ligation method [290]. In their proof-of-concept experiment, phosphino-thioester amino acid derivatives were reacted with azido amino acids to form dipeptides. The reaction proceeded smoothly and with a very high yield. The power of the traceless Staudinger ligation in chemical protein synthesis was definitely confirmed by Raines and coworkers, who adopted this chemistry to accomplish the total synthesis of a fully functional ribonuclease A (RNase A, 124 amino acids) [291]. The full protein was afforded by linking three peptide fragments. The middle fragment of RNase A (amino acids 110–111), harboring a N-terminal Cys, was synthesized as C-terminal phosphino-thioester by using a Kenner-type safety-catch resin, while the C-terminal fragment of RNase A (amino acids 112–124) was synthesized by standard Fmoc chemistry by coupling of α -azido glycine at the peptide N-terminus to introduce the azide-reactive handle. The two peptide fragments were ligated on the resin by the traceless Staudinger ligation in DMF/H₂O mixture. After cleavage from the resin and deprotection, the ligation product (amino acids 110–124 of the RNase A) was purified and reacted with the N-terminal RNase A fragment (1–109)-thioester by NCL, affording the final full-length RNase A.

Traceless Staudinger ligations are typically performed in organic solvents or organic/aqueous mixtures due to the scarce solubility of phosphinothioesters in water buffers at neutral pH. However, the ability to effect the traceless Staudinger ligation even in aqueous buffers could expand the scope of traceless Staudinger ligations even to native proteins or living cells. In this regard, the Raines group firstly reported the use of a water-soluble phosphinothiol, the bis(*p*-dimethylaminoethyl)



Scheme 1.35 The traceless Staudinger ligation reaction.

phosphino-methanethiol. They used such reagents to install a phosphino-thioester reactive handle at the C-terminus of a recombinant protein, specifically the RNase A, which was selected as model. RNase A was expressed as Mxe GyrA intein fusion construct, and a C-terminal phosphino-thioester derivative was obtained by incubating the fusion protein with the water-soluble phosphino-thiol. The reaction yielded a protein useful for Staudinger ligation with azide-containing peptides or molecule, thus integrating the traceless Staudinger ligation with EPL [292]. Water-soluble phosphinothiols were furthermore screened by Raines, and the reagent bis(*m*-*N,N*-dimethylaminomethylphenyl)phosphinomethanethiol, which carries *N,N*-dimethylamino group in *meta* rather than in *para* position and bears methylene rather than an ethylene linker, was found to be superior in its ability to efficiently mediate traceless Staudinger ligations in water at pH around neutrality [293]. Watzke et al. [288] combined intein-mediated EPL and Staudinger ligation to immobilize a protein onto a solid surface, developing a strategy for the generation of protein microarrays. C-terminal thioester Ras protein was obtained by intein splicing and was reacted by EPL with the cysteine handle of a multivalent ligand harboring an azide group. Azide function introduced at protein C-terminus was then employed to trigger covalent anchoring of the protein to a phosphane-functionalized glass surface.

The traceless Staudinger ligation has also been used for peptide head-to-tail cyclization by employing an intramolecular version of the traceless Staudinger ligation. The efforts of Hackenberger and coworkers in this scope are noteworthy [294, 295]. They used the borane group to preserve phosphine against oxidation in peptides harboring both the reactive moieties needed to perform the Staudinger ligation and developed two efficient methods for the synthesis of cyclic peptides employing Staudinger ligation on fully protected or unprotected peptide phosphinothioesters using a basic- or an acidic-mediated borane deprotection, respectively. An additional interesting application of the traceless Staudinger ligation was reported by Kim et al. [296], who developed a solid-phase Staudinger ligation strategy for the preparation of small peptides. To this aim, a core-shell-type resin was functionalized with a phosphinothiol functional group on which a model dipeptide was elongated. As a proof of concept, a Staudinger ligation reaction was directly performed on the solid resin using a series of azide-functionalized amino acids, obtained by diazo transfer. On-resin Staudinger reaction afforded the formation of the model tripeptides in quantitative yield.

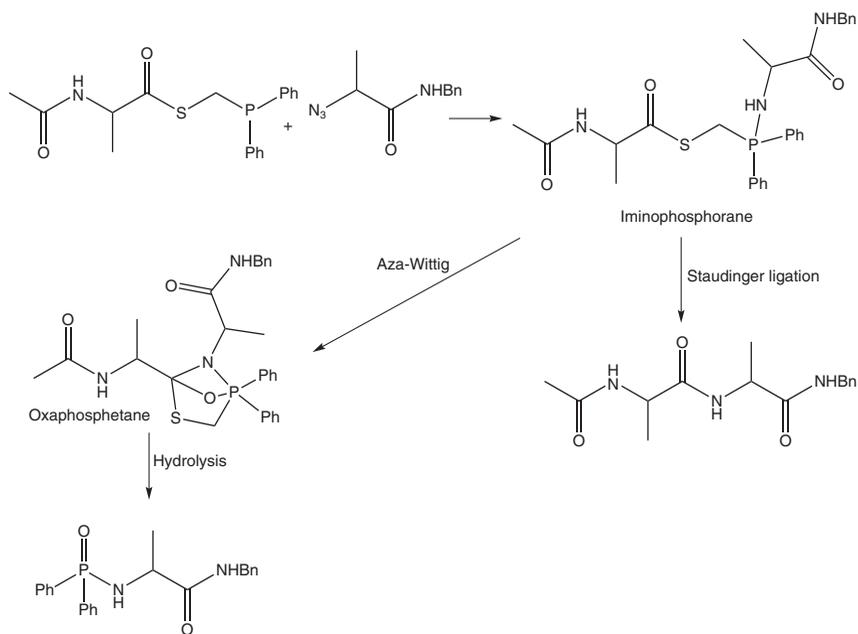
In the first reported examples of traceless Staudinger reaction, the ligation was usually performed at amino acid junctions at which at least one of the two residues was a Gly. However, the C-terminal phosphino-thioester and the N-terminal azide reactive moieties may be theoretically installed at any amino acid site, thus offering a great advantage over NCL, which instead necessitates a Cys residue or analog at the junction between the two peptides to be ligated. Although the traceless Staudinger ligation of peptides is independent from the presence of glycine residues at the ligation site, the yields may be substantially

lower at nonglycine junctions [279, 297, 298]. In the case of nonglycine residues, a competing aza-Wittig reaction leads to the parallel formation of a phosphonamide by-product (Scheme 1.36) [282].

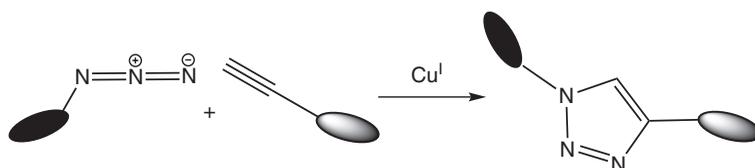
However, the reaction can be improved also at non-Gly sites by increasing the electron density on the phosphorous atom by adding functional groups on the phenyl substituents of (diphenylphosphino)methanethiol or by decreasing the polarity of the solvent [297]. Steric effects play a significant role in determining the rate and yield of traceless Staudinger ligations. Raines and coworkers tested differentially substituted phenyl moieties and found that variation in the steric effects of substituents was responsible for driving the reaction toward the aza-Wittig or the Staudinger ligation products [299]. Thus, Staudinger ligation may be extended to the chemoselective coupling of peptides containing amino acids other than glycine at the ligation site by optimizing the reaction conditions and the employed reactants.

1.9 Azide–Alkyne Cycloaddition

The 1,3-dipolar cycloaddition reaction of an azide with an alkyne yielding a 1,2,3-triazole was described by Huisgen in 1967 [300], but in recent years, it



Scheme 1.36 The Staudinger ligation at a non-Gly site involving the formation of a phosphonamide.



Scheme 1.37 General Cu-catalyzed azide–alkyne cycloaddition reaction.

has gained considerable utility as a bioconjugation and chemical ligation tool, in particular, after the discovery by Meldal [301] and Sharpless [302] groups that the reaction could be efficiently improved by the addition of copper(I) (Scheme 1.37).

Addition of copper catalyst (Cu-catalyzed azide–alkyne cycloaddition, CuAAC) allowed for gaining seven orders of magnitude in the reaction rate over the uncatalyzed azide–alkyne cycloaddition, eliminating the need for elevated temperatures and long reaction time. Moreover, while the uncatalyzed Huisgen cycloaddition does not ensure good regioselectivity, usually affording mixtures of the 1,4- and 1,5-disubstituted 1,2,3-triazole regioisomers [303], CuAAC affords the 1,4-isomer exclusively. CuAAC offers many advantages, such as being orthogonal and regioselective, high yielding, very fast, and easy to perform. Due to these qualities, CuAAC is generally referred to as “click” chemistry. CuAAC may be efficiently performed under mild conditions both in organic solvents and in water solutions. The compatibility with aqueous conditions paved the way to countless and outstanding applications. The two simple required reactants, an azide and an alkyne, are stable in a wide range of synthetic reaction conditions and selectively react, giving rise to a chemically inert triazole linkage. CuAAC reaction is commonly performed under aqueous conditions using CuSO_4 as a copper source and a reducing agent, such as sodium ascorbate. CuSO_4 provides Cu(II) , and the addition of a reducing agent in a large excess ensures the supply of Cu(I) at a high concentration at all times during the reaction. The presence of the reducing agent renders the reaction much less susceptible to oxygen, and there is no need for inert gas conditions. Other frequently used copper sources are CuI or CuBr , which directly provide Cu(I) . However, while using CuSO_4 , the catalytically active Cu(I) species is directly generated by reduction with ascorbate and immediately forms the reactive intermediate Cu-acetylides, CuI and CuBr salts require an amine base, such as *N,N*-diisopropylethylamine (DIPEA) [302], or high temperature to form the Cu-acetylide complexes [304]. CuAAC is generally recognized as one of the most efficient reactions described. However, some expedients may be taken into account to maximize the performance even in that few cases in which this ligation reaction is unsuccessful, for example, when the alkyne and azide reactive groups are attached to substrates of wide size, such as large proteins, in which very dilute reaction conditions may cause unproductivity. For

example, regulation of solvent composition and temperature or the use of microwaves or ultrasounds may drive the reaction toward product formation [305]. Additionally, in order to protect the copper(I) ions from disproportionation to Cu(0) and from reoxidation to Cu(II) by air and to enhance its catalytic activity, many molecules have been screened as suitable copper ligands. In particular, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) [306] was supposed to act as a tetradentate ligand that blocks all coordination sites at the metal so that no oxidant can attack at the Cu(I) ion. Other closely related molecules, such as tris(heteroarylmethyl)amine ligands, turned out to be potent additive for protecting the Cu(I) ions with regard to oxidation and disproportionation [307]. The use of phosphorus additives in combination with Cu(II) salts has also been reported to accelerate CuAAC reactions in aqueous media [308]. To ensure a successful outcome of the reaction, both alkyne and azide should be soluble in the solvent and under the conditions of choice. The reactivity is also related to the nature of the alkyne and azide substrates involved in the reaction. α -Carbonyl-alkynes are more reactive than alkyl-alkynes, while the aromatic alkynes are similar or marginally less reactive [304, 309]. All the azides are commonly well reactive. Only carboxylazides and sulfonylazides may show substrate instability, and allylic azides are subjected to 1,3-sigmatropic rearrangements [304, 310, 311]. Bis-azides have been demonstrated to ensure a rate enhancement during the formation of the second triazole linkage, and a sensitive rate enhancement was also observed in the case in which multiple azide groups are exposed on a polymeric scaffold, such as a calixarene [312]. The exposition of multiple alkyne functions does not show the same effect, and, moreover, high density of alkyne may cause suppression of triazole formation [304, 312].

The mechanistic details describing the role of Cu in azide–alkyne cycloaddition reaction remain a controversial matter. In 2005, Sharpless and Fokin group firstly proposed a mechanism in which the alkyne deprotonates and coordinates one Cu⁺ in the Cu-acetylide transition state and suggested that the rate enhancement of CuAAC with respect to the uncatalyzed cycloaddition was due to the lowering of the transition state energy of 11 kcal mol⁻¹. In their model mechanism, one Cu⁺ coordinated with the acetylene π -electrons [313]. Many of the successive mechanistic interpretations are based on these results proposed by Sharpless, who predicted unprecedented reactivity and intermediates of CuAAC via a computational study [307, 314, 315]. However, additional observations highlighted that it is probably incorrect that one Cu atom should be involved in the formation of the transition state of the reaction [315, 316]. Meldal and Tornøe highlighted that approximately 35 structures of the Cu(I)-acetylene complexes are reported in the Cambridge Crystal Database, and, in more than 90% of all Cu(I)-alkyne complexes, each C–C-triple bond coordinates with three Cu atoms. This may suggest that this type of coordination appears to be the energetically most favored coordination number of the acetylide [304, 317].

The 1,4-substituted 1,2,3-triazole can be considered as a valuable mimetic of the peptide bond exhibiting a three-dimensional planar structure, a high dipolar moment of $\sim 5\text{D}$ similar to that of the peptide bond ($\sim 4\text{D}$), a character of H-bond donor/acceptor, a distance between the one and four substituents, and a conformational restriction similar to that of peptide backbone [317–321]. For that reason, triazole-backbone-modified polypeptide chain, in which triazole replaces one or more of the peptide bonds, should preserve the structure and function of its native counterpart, thus prompting the use of CuAAC as a chemical ligation tool useful for the synthesis of peptide mimetics and proteins. The use of CuAAC as ligation chemistry requires unprotected peptide fragments bearing azide or alkyne functions at their C- and N-termini. These building blocks can be easily synthesized [322, 323] and introduced into peptide fragments in solid phase using standard peptide chemistry. For instance, N-terminal azido groups can be introduced by azidation of amino-free amino acid [324, 325], while C-terminal alkyne may be prepared by functionalization of the resin by reductive amination of propargylamine [150, 326]. Delmas group reported the first example of the use of the CuAAC for the sequential ligation of unprotected peptide fragments into a bioactive triazole-bond containing protein [327]. As a proof of concept, they applied a multiple-ligation approach to chemically synthesize human cystatin A. A silyl-protecting group [328, 329], the triisopropylsilyl (TIPS) [330, 331], was used for temporary masking of the terminal alkyne function in the multiple-ligation scheme. TIPS is fully compatible with the conditions of fluorenylmethoxycarbonyl (Fmoc) SPPS, and deprotection procedure requires a mild treatment with tetra-*n*-butylammonium fluoride (TBAF) in buffered aqueous solution. The synthetic cystatin A, harboring two triazole bonds in the backbone, preserved its biological activity, thus promoting CuAAC as a good protein chemical ligation method. Tolbert group also applied CuAAC to the semisynthesis of proteins [332]. They combined NCL and CuAAC to obtain an N-terminally linked protein G homodimer and an homotrimer. In these cases, the resultant triazoles served as a linker rather than a mimetic of the peptide bond. The N-terminal Cys of the protein G was modified by performing NCL with thioester derivatives of an azide or an alkyne group. Then, CuAAC afforded the head-to-head linked dimer of the protein G. In addition to the directly coupling of azide and alkyne modified protein G, a homodimer was prepared by reacting diazide linker with N-terminal alkyne-functionalized protein G. Similarly, an homotrimer was obtained by reacting a trialkyne linker with azide protein G. In the Kuger laboratory, CuAAC click chemistry was applied to the synthesis of hemoglobin (Hb) bis-tetramers [333–335]. Ubiquitin (Ub) dimers were also prepared by CuAAC [336, 337]. In this case, azidohomoalanine (Aha) or propargyl-lysine (Plk) was incorporated into recombinant Ub by extended genetic code technology. Plk-Ub and Aha-Ub were then successfully joined together to form Ub dimers using copper sulfate as the source of

catalyst, tris(2-carboxyethyl)phosphine (TCEP) as the reducing agent, and TBTA as the copper-stabilizing agent.

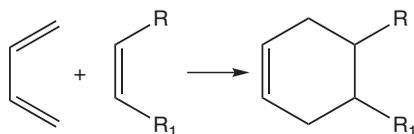
CuAAC has also been employed to prepare several triazole-containing peptide mimetics [338]. Unlike peptide bond, the triazole is stable against proteolytic degradation, providing an excellent tool to increase the *in vivo* half-life and stability of bioactive peptide compounds [317,318,338–340]. Additionally, the 1,2,3-triazole ring may serve as β -turn mimic [341].

1.10 Diels–Alder Ligation

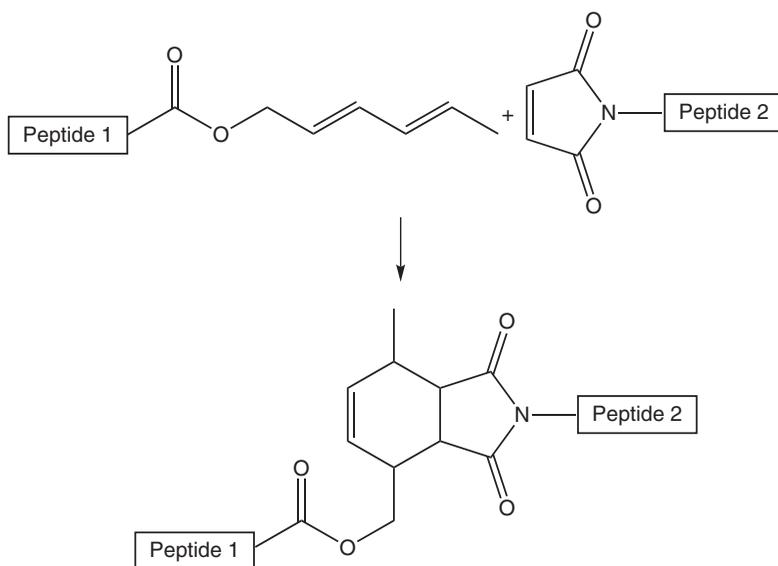
The Diels–Alder reaction [342] is a [4 + 2] cycloaddition in which a diene reacts with a dienophile to form two new carbon bonds in a six-membered ring (Scheme 1.38).

Diels–Alder reaction may be performed in aqueous buffer at pH value around neutrality and requires mild conditions. Thus, the reaction appeared a promising tool for bioconjugation and chemical ligation (Scheme 1.39).

Maleimide is generally used as the dienophile in a Diels–Alder reaction, allowing to take advantage of the plethora of commercially available maleimide derivative probes for protein and peptide labeling. The installation of the diene function poses instead particular challenges due to its acid sensitivity, which precludes the diene incorporation via standard SPPS. A straightforward strategy for the solid-phase synthesis of the dienyl ester peptides useful in Diels–Alder ligation employs a sulfonamide linker resin. After peptide synthesis, the linker is activated and treated with a dienol to simultaneously install a dienyl ester handle at the C-terminus of the peptide and cleave it from the resin. Side-chain-protecting groups are then removed in solution. Waldmann and coworkers adopted such a synthetic approach to prepare peptides harboring a C-terminal dienyl function, which were reacted with peptide segments containing an N-terminal maleimide group introduced by standard Fmoc chemistry [343, 344]. Diels–Alder ligation between two peptide segments was performed in aqueous solution at room temperature by mixing equal amounts of the dienyl and the dienophile peptides, affording the ligation cycloadduct product at high yield and thus revealing the applicability of Diels–Alder reaction as chemical ligation tool. It is noteworthy that



Scheme 1.38 The Diels–Alder reaction between a diene and a dienophile.

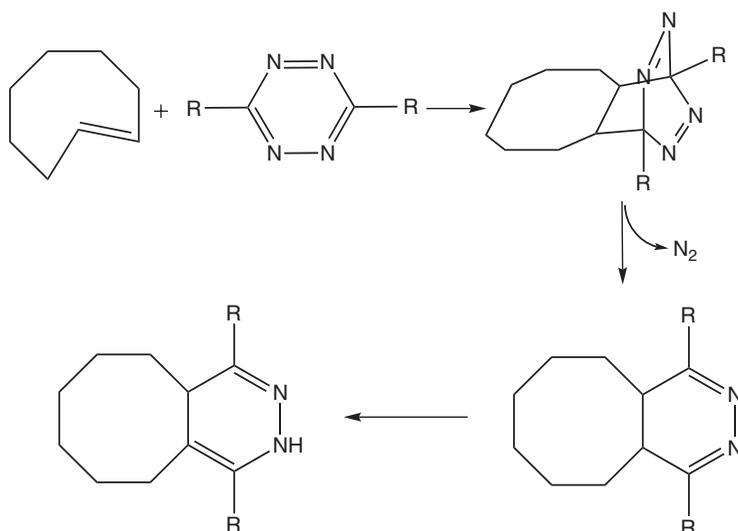


Scheme 1.39 Ligation of two peptides using Diels–Alder reaction.

Diels–Alder reaction may result in the formation of *endo*- or *exo*-isomers. A relevant troublesome of the chemistry is the Michael addition reaction, which may take place between the side chain of Cys residues and maleimide, leading to the formation of undesired side products. This collateral reaction may be prevented by using Cys protecting groups, which persist on the peptides during ligation and are removed after the reaction was completed. Waldmann et al. also applied Diels–Alder ligation to the site-specific C-terminal labeling of proteins [343, 344]. In this case, they combined the application of EPL and Diels–Alder reaction in a multistep ligation scheme. C-terminal thioester Rab protein was prepared by recombinant means using an engineered intein as fusion partner and was reacted by EPL with a synthetic peptide carrying an N-terminal Cys and a C-terminal dienyl function. EPL afforded the introduction of the dienyl function and also of an additional cysteine residue, which was added to the other three native cysteine residues of Rab. To avoid undesired coupling of cys mercapto group with maleimide derivatives in the subsequent ligation step, the accessible cysteine residues were temporary protected as disulfides by treatment with Ellmann’s reagent. Dienyl-ester protein was then reacted with a maleimide peptide or probe in buffer at pH 6.0, leading to various ligation yields depending on the excess of maleimide employed. Finally, treatment with a reducing agent, such as dithiothreitol (DTT), allowed to quench the exceeding maleimide-reagent and to deprotect cys residues.

Recently, the inverse electron-demand Diels–Alder reaction between *s*-tetrazine and *trans*-cyclooctene has been reported [345]. During this retro-[4 + 2] cycloaddition, tetrazine acts as a diene to produce the dihydropyrazine cycloadduct with N₂ as the only by-product (Scheme 1.40).

The utility of this reaction as protein modification tool was demonstrated by thioredoxin (Trx) functionalization. A *trans*-cyclooctene derivative carrying a maleimide handle was first conjugated to Trx through a Michael addition on a solved exposed Cys residue. Then a ligation reaction with *s*-tetrazine was performed, affording a 100% conversion in just 5 min [345]. Because of its very fast kinetics and ability to proceed in high yield in organic solvents, water, cell media, or cell lysate, the tetrazine ligation may be particularly useful in cases where rapid reactions are essential for tracking of fast biological events as well as for labeling of biomolecules of low abundance [346]. Hilderbrand and coworkers demonstrated the suitability of this chemistry for *in vivo* applications by using a tetrazine-derived fluorescent probe to label both in serum and in live cells a monoclonal antibody modified with a norbornene and raised against Her2/neu receptors [347]. The Braun group applied the inverse electron-demand Diels–Alder to the synthesis of TMZ-BioShuttle, a molecular vehicle used to target tumor cells with the anticancer molecule temozolomide (TMZ). Tetrazine derivative of TMZ was site-specifically ligated to a nuclear localization signal peptide harboring the dienophile function on an N-terminal Lys residue. The signal peptide was in turn conjugated through its C-terminal Cys



Scheme 1.40 Inverse electron-demand Diels–Alder reaction of tetrazine and *trans*-cyclooctene.

to a cell penetrating peptide via a disulfide bond formation. TMZ ligation to the peptidic components facilitates the cell membrane translocation as well as the targeting to subcellular compartments.

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