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REVIEW ARTICLE

Synthetic strategies for polypeptides and proteins by chemical ligation

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Abstract This review focuses on chemical ligation methods for the preparation of oligopeptides and proteins. Chemical ligation is a practical and convenient methodology in peptide and protein synthesis. Longer peptides and proteins can be obtained with high yield in aqueous buffer solutions by coupling unprotected peptide segments even without activation by enzymes or further chemical agents. Several methods and protocols were developed in the past. The potential of the most important approaches of the thioester- and imine-ligation techniques is demonstrated by a broad spectrum of applications. In addition, special features and protocols such as the template-directed ligation, ligation with novel additives or solvent media, microwave-assisted ligation, and the achievements obtained with those are also highlighted herein.

Keywords Chemical ligation · Native chemical ligation · Thioester · Chemoselective synthesis · Protein synthesis

Introduction

Polypeptides and proteins are biomacromolecules consisting of amino acid residues which are linked together via peptide bonds between their respective carboxyl and amino groups. Proteins, in particular, serve as the major structural and functional components in numerous biological and physiological processes in living organisms. Concerning the length, a sequence containing 50–200 amino acids is often regarded as a small protein or protein domain in the context of a larger assembly. The structural organization of peptides and proteins, however, differs in that peptides generally possess a greater flexibility and, in turn, different structural and functional roles compared to proteins (Ratnaparkhi et al. 2011), though exceptions of highly structured and conformationally constrained peptides such as toxins stabilized by several disulfide bonds exist. Nevertheless, a great number of both biologically active peptides and proteins are nowadays used as drugs or diagnostic agents in pharmaceutical and medicinal applications (Vlieghe et al. 2010; Ratnaparkhi et al. 2011). This can be exemplified with diagnostic reagent γ-GT II (dipeptide glycylglycine used for diagnosis and/or prediction of osteoporosis) (Tanaka et al. 2011), Prialt® (SNX-111 or ziconotide, 25mer peptide N-type calcium channel blocker) (Wermeling 2005), Fuzeon® (enfuvirtide, 36mer antiretroviral peptide) (Greenberg and Cammack 2004), and Herceptin® (trastuzumab, a 1328mer monoclonal antibody protein approved for breast cancer) (Tan and Swain 2003).

Looking back on the 100 years of peptide chemistry, several synthetic methods and technologies for their production have been developed, among which the stepwise approach and the segment condensation approach are especially relevant (Kimmerlin and Seebach 2005; Chandrudu et al. 2013). The stepwise assembly, usually performed by solid-phase peptide synthesis (SPPS), employs a repetitive process resulting in the elongation of a polymer-bound peptide chain by successive additions of amino acids until the peptide of the desired sequence and length has been synthesized. The length of peptides prepared by SPPS is generally limited to approximately 50 (maximum may vary depending on peptide sequence) amino acid residues in order to

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get a high yield of end product. Several reports also demonstrated the successful preparation of 66mer (Böhm et al. 2012), 95mer (White et al. 2004), or even longer polypeptides (Fukuda et al. 1999), yet the overall yields are often unsatisfactory and may fluctuate in the extreme. In addition, the applicability of SPPS for such long peptides or miniproteins may not be appropriate depending on the individual sequence, i.e., specific features like aggregation potential and solvation capacity may influence the outcome of such a synthesis (Coin et al. 2007).

In the second approach, i.e., the segment/fragment condensation, the formation of amide (peptide) or other chemical bonds, e.g., peptide thioester, thiazolidine, and thiazolidine ester, can be achieved (Hojo and Aimoto 1991; Tam et al. 1995; Zhang et al. 1998; Tam et al. 1999). Ideally, the peptide fragments to be connected to a polypeptide chain should be protected in order to avoid side products. To overcome these difficulties, several segment ligation methods, which can generate either amide or non-amide bonds at the ligation sites, have been developed (Robey and Fields 1989; Dawson et al. 1994; Tam and Yu 1998; Bode et al. 2006). These peptides are functionalized with groups that react chemoselectively with only one group of the acceptor peptide preserving the integrity of unprotected side chains, i.e., using these ligation methods unprotected peptide segments can be coupled efficiently to form long peptide chains and even large proteins, usually in good yield (42–95 % total yield) and of high purity (Böhm et al. 2012; Raibaut et al. 2013; Bodapati et al. 2013). The methods range from thioester ligation to imine ligation (Tam et al. 1999, 2001), and several peptides and proteins such as cytochrome b562 (106 AAs) (Low et al. 2001), His-tagged interleukin-2 (133 AAs) (Tolbert et al. 2005), and insulin lispro (51 AAs) (Sohma and Kent 2009) were produced in this way.

In this review, the most frequently used ligation methodologies for peptide/protein synthesis are summarized in Table 1, and individual methods are further explained in Schemes as for example thioester ligation (Scheme 1), thioether ligation (Scheme 2), and imine ligation (Scheme 2). The enzyme-catalyzed ligation methods, which are still evolving, are not within the focus of this review. The interactions between the different nucleophilic or electrophilic groups at the C-terminus and N-terminus of the ligation segments have been fully elaborated in the past years (Liu and Tam 1994a; Dawson et al. 1994; Tam et al. 1999). Previous excellent reviews on the topic have focused on the concept of peptide ligation strategies, which are achieved through variable chemoselective capture procedures (Dawson and Kent 2000; Tam et al. 2001; Hackenberger and Schwarzer 2008). In addition, these reviews have largely been confined to peptide/protein ligation studies published prior to 2008. Herein, we, thus, survey the recent progress (2008–2015) of the applications of the different ligation methods for peptide/protein synthesis and also further development of these methods. The first section is dedicated to the basic types of chemical ligation methods reported thus far. Recent applications of these chemical ligation techniques on peptide/protein synthesis will be paid attention and, finally, various novel ameliorations and improvements of ligation methods using different molecular templates, new additive agents, or specific solvent media are included.

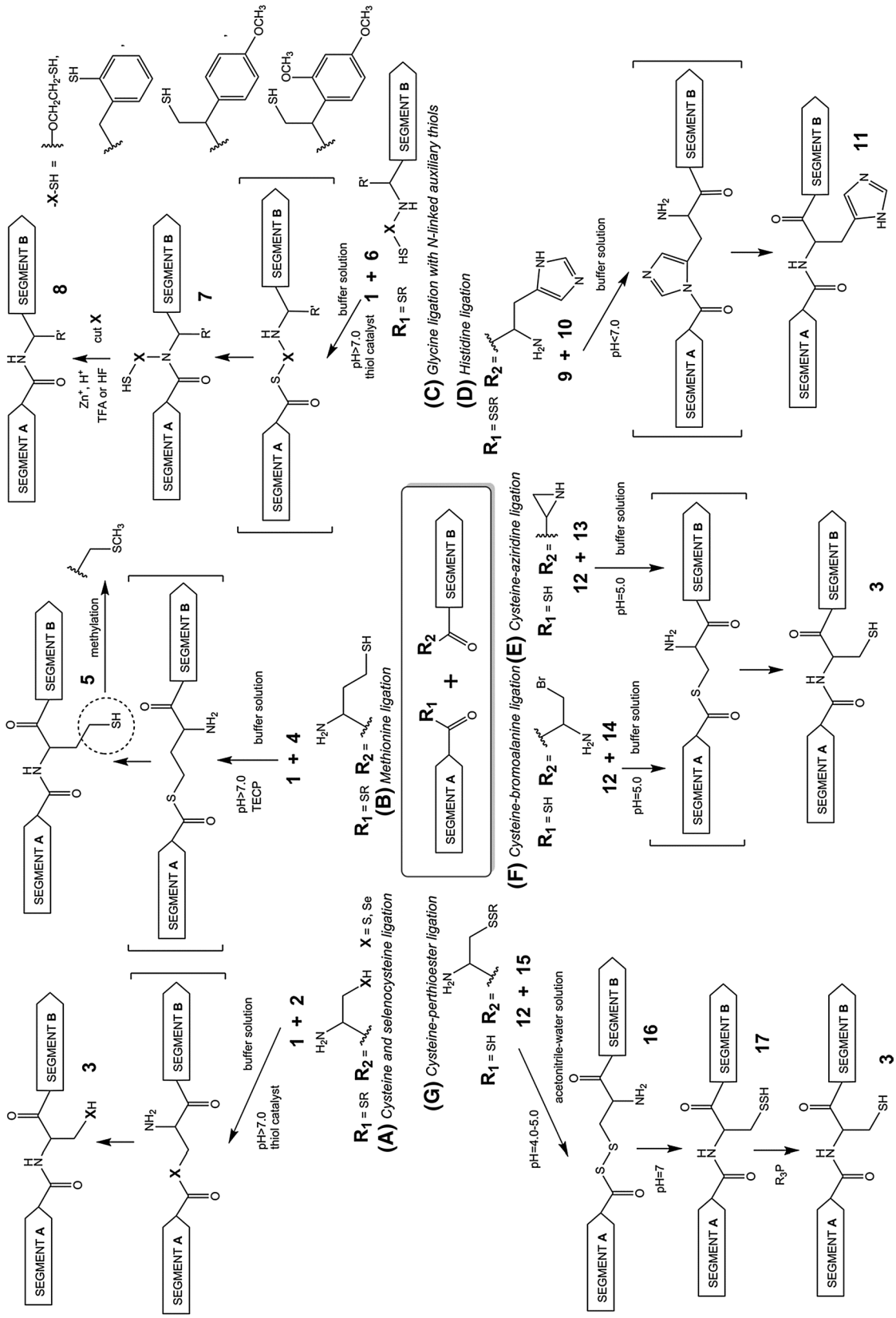
Thioester-based ligation methods

Theodor Wieland and coworkers first initiated the thioester-based chemistry in (Wieland et al. 1953). The reaction

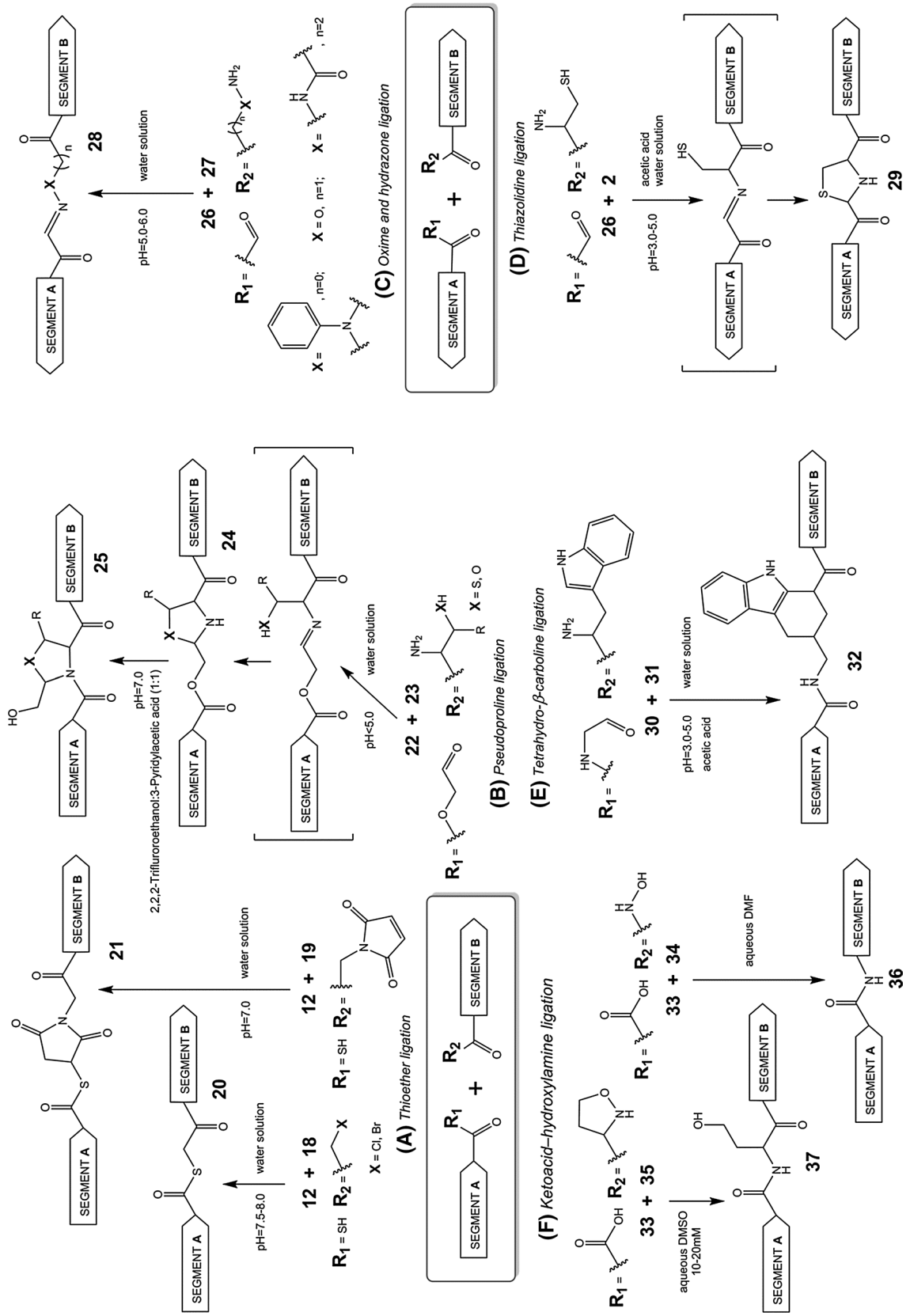
Table 1 Imine and thioester capture strategies for orthogonal ligation methods

Method	Segment	Product	Example[a]	Publ.[a]
Cysteine and selenocysteine ligation	**1 + 2**	**3**	Human interleukin 8 (IL-8)	1994
Methionine ligation	**1 + 4**	**5**	Parathyroid hormones	1998
Glycine ligation with N-linked auxiliary thiols	**1 + 6**	**7**	Gly12-brain natriuretic peptide$_{5–26}$, porcine	2007
Histidine ligation	**9 + 10**	**11**	–	–
Cysteine-aziridine ligation	**12 + 13**	**3**	–	–
Cysteine-bromoalanine ligation	**12 + 14**	**3**	–	–
Cysteine-perthioester ligation	**12 + 15**	**16**	K48-linked diubiquitin	2010
Thioether ligation	**12 + 18, 19**	**20, 21**	HIV-1 protease	1992
Pseudoproline ligation	**22 + 23**	**24**	Model 50-residue peptide	1994
Oxime and hydrazone ligation	**26 + 27**	**28**	–	–
Thiazolidine ligation	**26 + 2**	**29**	–	–
Tetrahydro-β-carboline ligation	**30 + 31**	**32**	–	–
Ketoacid–hydroxylamine ligation	**33 + 34, 35**	**36, 37**	–	–

[a] Selection is based on peptide product larger than 20 amino acids



Scheme 1 **a** Cysteine and selenocysteine ligation, **b** methionine ligation, **c** glycine ligation with N-linked auxiliary thiols, **d** histidine ligation, **e** cysteine-aziridine and cysteine-bromoalanine ligation, **f** cysteine-perthioester ligation



Scheme 2 **a** Thioether ligation, **b** pseudoproline ligation, **c** oxime and hydrazine ligation, **d** thiazolidine ligation, **e** tetrahydro-b-carboline ligation, **f** reverse imine ligation

mechanism of a thioester-based ligation can be usually understood as a two-step process. In the first step, an intermediate, such as a covalent thioester or perthioester, is formed by a thioester or its analogs and a thiol nucleophile. This process is normally reversible. After a rapid S–N acyl rearrangement step, the thiol bond of the intermediate is transformed into an amide bond spontaneously and irreversibly (Wieland et al. 1953; Dawson et al. 1994). After about 40 years, the thioester-based method is now being applied successfully in cysteine (Dawson et al. 1994), selenocysteine (Quaderer et al. 2001), methionine (Tam and Yu 1998), glycine (Canne et al. 1996), and histidine (Zhang and Tam 1997) ligation for e.g., producing unprotected peptides.

Cysteine and selenocysteine thioester ligation

In the early 1990s, the first convenient ligation method for peptide synthesis called "Native Chemical Ligation" (NCL) was introduced by Stephen Kent and his coworkers (Dawson et al. 1994). Cysteine ligation utilizes two unprotected peptide segments with the following functionalities: a C-terminal electrophile thioester **1** and an N-terminal nucleophile cysteine **2**. Just like for the common thiol-based ligation, these two peptide segments initially combine with each other via reversible transthioesterification, and a subsequent irreversible S–N acyl shift yields a "native" peptide bond as well as a cysteine residue at the ligation site of **3** (Dawson et al. 1994) (Scheme 1).

Cysteine ligations are usually required to proceed at neutral pH (pH 6.5–7.5) in aqueous phosphate buffer containing a chaotropic agent, e.g., guanidine hydrochloride or urea, by increasing solubility (Dawson et al. 1994, 1997). Furthermore, the addition of thiol additive(s) is necessary for cysteine ligation to keep the cysteine side chains in a reduced state and facilitate the initial transthioesterification step, such as benzyl mercaptan, thiophenol (Dawson et al. 1997; Johnson and Kent 2006) and presumably the most popular thiol accelerant 4-mercaptophenyl acetic acid (MPAA), the advantage of which is discussed below.

Meanwhile, application of NCL has already witnessed a long history and may be regarded as a classical peptide synthesis method (Schnolzer and Kent 1992; Kimmerlin and Seebach 2005). NCL at cysteine sites is one of the most convenient and popular methods for the synthesis of large peptides and small proteins, as already pointed out by other recent comprehensive reviews in related fields of peptide chemistry (Hackenberger and Schwarzer 2008; Raibaut et al. 2012; Thapa et al. 2014; Malins and Payne 2014). A huge number of molecules has been successfully synthesized by NCL or methods deriving therefrom, including the recently reported pore-forming antimicrobial protein caenopore-5 (82 AA) (Medini et al. 2015), the sialic acid-binding lectin siglec-7 (127 AA) (Izumi et al. 2014), human interleukin-6 glyoprotein (183 AA) (Reif et al. 2014), and polydiscamides B, C, D from the sponge *Ircinia sp.* (13 AA) (Santhakumar and Payne 2014).

While performing native ligation at -Glu-Cys- and -Asp-Cys- sites the formation of a significant high level of β- and γ-linked byproducts has been reported by Villain et al. (2003). Compared to the previous findings from Villain, the use of additive MPAA instead of classical additives like PhSH avoided the β- and γ- isomer formation occurring at the "-Glu-Cys-" site, while a rather high amount of β-linked byproduct was still formed in the ligation reaction at the "-Asp-Cys-" site (Dang et al. 2013). In addition to using LYRAXCFANF (X = Q, E, N, D) as model peptide, they applied MPAA for the synthesis of the toxin protein ShK (35 AA) with the aim to optimize reaction conditions. This approach increases the accessibility of -Glu-Cys- ligation sites in peptide synthesis. Similarly, it is also reported that -Pro-Cys- ligation is hardly performed in NCL (Hackeng et al. 1999). Recent improvements from Nakamura et al. (2014) showed that increasing the concentration of MPAA and the reaction temperature could significantly enhance the reaction rates.

In 2014, Van de Langemheen et al. (2014a, b) demonstrated for the first time ligation of three different cyclic peptides by introducing a thioester into the triazacyclophane (TAC) linker to yield a series of protein mimics of gp120. At the same time, the SAMS research group of the University of Strasbourg discovered that two fragments of corresponding linear peptide sequences containing thioester linkage can be exchanged by a dynamic and reversible NCL reaction even under mild conditions (Ruff et al. 2014). Indeed, these authors found that this process takes place at an N-methylated cysteine with addition of dithiothreitol making the method suitable for the exchange of peptide fragments or the release of bioactive molecules. Also, this application can be used to prepare random peptide libraries with the restriction that at least one N-methylated cysteine needs to be present for performing the chemoselective and reversible NCL at this special position.

Instead of the commonly used thioester at the C-terminal of the N-terminal segment various authors introduced alternative reactive sites, e.g., N-acylbenzimidazolinone (Nbz) for the preparation of several proteins such as the antimicrobial peptide lucifensin (40 AA) (Stanchev et al. 2014), histone H2B (125 AA), and cyclic protein lactocyclicin Q (61 AA) (Wang et al. 2015). This represents a novel modification of the conventional method possibly with future potential.

In addition, a third variant, NCL ligation using a C-terminal selenoester instead of the thioester was developed

by Durek and Alewood (2011). This specific method has been applied later to explore dynamic redox active peptide self-assembly (Rasale et al. 2014). Besides the NCL of peptide–peptide segments, the NCL between protein and ubiquitin, called "native chemical ubiquitination", has been introduced by Yang et al. (2014) as a follow-up procedure of the original perthioester ubiquitination (see also chapter 1.6).

Apart from cysteine residues at the N-terminal of the C-terminal fragment, selenocysteine residues were used for NCL, primarily because of the lower redox potential compared to cysteine (Moroder 2005). Selenocysteine is present in several natural enzymes, such as glutathione peroxidases (Epp et al. 1983), glycine reductases (Wagner et al. 1999), thioredoxin reductases (Mustacich and Powis 2000), methionine-R-sulfoxide reductase B1 (Lescure 1999), and selenophosphate synthetase 1 (Low et al. 1995). The selenol of selenocysteine, however, is also a strong nucleophilic group like the thiol group of cysteine (Wessjohann et al. 2007). Thus, in many cases, selenocysteine could act as a good surrogate of cysteine in NCL (Gieselman et al. 2001; Quaderer et al. 2001; Hondal 2009; Malins et al. 2014) (Scheme 1). Two advantages have been found in the application of selenocysteine-based ligation. First, NCL peptide products containing selenocysteine can be transformed into non-selenocysteine peptides by selective deselenization so that further peptide/protein synthesis can be carried out without cysteine protection (Metanis et al. 2010; Malins and Payne 2012). Second, some research groups reported that partial substitution of selenocysteine for cysteine residues in peptide/protein sequences supports optimization of oxidative folding, thus avoiding accumulation of unusable byproducts or intermediates that reduce folding efficiency (Craik 2012; Metanis and Hilvert 2012).

Subsequently to the work of Ruff et al. mentioned above (Ruff et al. 2014), it has been demonstrated that selenopeptides can be transamidated by N-terminal cysteinyl peptides in water using mild experimental conditions (pH 5.5). These conditions enable the reversibility of the peptide bond to selenocysteine, therefore, transamidation reactions are similar to those used for NCL indicating a covalent dynamic process. In addition, these conditions also catalyzed the metathesis of selenopeptides. These findings offer promising new tools for future work in the field (Ollivier et al. 2014; Melnyk and Agouridas 2014).

Nevertheless, an N-terminal cysteine or selenocysteine residue is always a prerequisite of native chemical ligation. Both cysteine and selenocysteine may be rare in particular natural proteins. In the next chapter, we, therefore, summarized several cysteine-free ligation methods available so far, which have been developed to overcome this limitation of NCL.

Methionine ligation

Methionine ligation, a thioester-based ligation method derived from NCL, involves an unprotected peptide segment bearing an N-terminal homocysteine **4** and another unprotected peptide segment containing a C-terminal thioester **1**. The initial stage of methionine ligation is nearly similar to the cysteine ligation described above. The ligated homocysteine of the product **5**, which has been generated by an S–N acyl transfer through a six-membered ring intermediate, is methylated to form a methionine residue at the ligation site in the subsequent step (Tam and Yu 1998) (Scheme 1).

Methionine ligation is usually performed under basic conditions although the methylation reaction of peptides could be carried out at neutral or acidic conditions (Naider and Becker 1997). A large excess of methyl 4-nitrobenzene sulfonate as methylating reagent is used in this respect in order to achieve an adequate methylation yield (Heinrikson 1971; Tam and Yu 1998). The reaction time, however, needs to be reduced in some cases to avoid an undesirable methylation of lysine amines or imidazole rings of histidine (Tam and Yu 1998). The methionine ligation has been successfully applied to the preparation of several small proteins, such as BRD7 bromodomain (Van de Vijver et al. 2012) and α-synuclein (Tanaka et al. 2013).

Additionally, the Dawson group has combined this methodology of methionine ligation with post-ligation desulfurization (Yan and Dawson 2001). As an example, they use a methionine ligation precursor homocysteine residue at the ligation site and desulfurize it into a 2-aminobutyric acid (Abu) residue by hydrogen and a series of metal catalysts, e.g., Pd/Al_2O_3, Pd/C, Nickel boride, or Raney Nickel. This approach has broadened the application of this ligation method to a larger field of peptides since desulfurization after ligation increases availability of many more amino acids compared to the original method. Therefore, the selection of the desired peptide/protein sequence is not limited to cysteine or methionine residues. It is also worth mentioning that the metal-free desulfurization to transform cysteine into alanine (Wan and Danishefsky 2007) is also possible and represents a further advantage of this method. Nevertheless, the usage of this desulfurization protocol with or without a metal catalyst is always depending on the sequence and the specific amino acids of the product (Siman et al. 2012; Haase et al. 2008).

Glycine ligation with N-linked auxiliary thiols

In 1996, Canne and coworkers first reported on an auxiliary (oxyalkyl)-mediated ligation method (Canne et al. 1996). In this method, a thiol-containing auxiliary, which is linked with the N-terminal amino group of the peptide **6**, reacts

with a C-terminal peptide thioester **1** to form a six-membered ring intermediate via thiol–thioester exchange. After an S–N acyl transfer, an N-substituted amide bond with the auxiliary of **7** is generated resulting in the linked C- and N-terminal segments (Offer 2010). The thiol-containing auxiliary group can be removed by treatment with Zn^{+}/H^{+}, TFA, or HF yielding a glycine residue in the final product sequence **8** (Shao et al. 1998; Botti et al. 2001; Offer et al. 2002; Li et al. 2010) (Scheme 1). In most cases, the intermediate of a glycine ligation can be isolated because it rearranges much slower than the intermediate of a cysteine ligation (Canne et al. 1996).

Apart from N-linked oxyalkyl, mercaptoethoxy, and mono- or dimethoxy substituted benzyl thiol auxiliaries (Shao et al. 1998; Offer and Dawson 2000; Low et al. 2001; Botti et al. 2001; Li et al. 2010), various photolabile auxiliary thiols have been designed for glycine ligation. These include, e.g., the 4,5-dimethoxy-2-mercaptobenzyl group and the 1-amino-1-phenyl ethane-2-thiol group, which can be removed easily by light illumination after reaction (Kawakami and Aimoto 2003; Marinzi et al. 2004; Campbell 2013). A method called sugar-assisted ligation (SAL), an extended methodology of glycine ligation, has been applied to oligopeptide productions since 2006 (Brik et al. 2006; Yang et al. 2007; Payne et al. 2007; Brik and Wong 2007; Zhong et al. 2009). Here, the N-linked glycosyl groups were used as auxiliaries to directly form glycopeptides and glycoproteins, such as diptericin (Yang et al. 2007), and the starting unit of the cancer-associated MUC1 glycoprotein (Payne et al. 2007). Glycoproteins and peptides have several biological functions. Therefore, the SAL group is showing promising new results with their syntheses. However, only a few approaches have been developed thus far and further studies are still needed for full exploitation.

Histidine ligation

The realization of a histidine ligation in high yields depends on the nucleophilicity of the N-terminal histidine of the C-terminal segment **10** (Scheme 1) for the thioester group at the C-terminal of segment **9**. This thioester group is activated by a suitable thiophilic promoter, e.g., aryl disulfide (Offord 1969) or Ellman's reagent (Ellman 1959), to mediate a chemoselective peptide ligation thereby enabling the combination of two peptide segments. A peptide bond in **11** is formed hereafter by a $N^{\text{imidazole}}$–N^{α} acyl transfer (Zhang and Tam 1997) (Scheme 1). The histidine ligation occurs under acidic conditions in aqueous buffer, and was already applied to produce several peptides containing histidine, including calcitonin and parathyroid hormone (PTH), both were reported to be formed in rather high yields of 60–75 % (Tam et al. 1999). These syntheses, however, have been performed a relatively long time ago. Since then no further examples have been reported, indicating that this ligation method seems less important than the other ligation methods.

Cysteine-aziridine and cysteine-bromoalanine ligation

Cysteine-aziridine ligation, also called "peptidomimetic ligation" (Assem et al. 2010), introduced in 2010, utilizes an N-terminal aziridine-2-carbonyl peptide segment **13** and a C-terminal thioacid segment **12** (Scheme 1). The N-terminal aziridine-2-carbonyl moiety acts as the electrophile, while the C-terminal thioacid is the nucleophile. This is diametrically opposed to the normal cysteine ligation employing a C-terminal electrophile and an N-terminal nucleophile. Thus, the cysteine-aziridine ligation can be regarded as a reverse thioester ligation. The thioacid groups react readily with aziridines via a ring-opening reaction to connect the two segments by a thioester linkage. Similarly to the common thioester ligation, an S–N acyl transfer occurs hereafter to generate the ligated product containing a native amide bond as in **3** (Assem et al. 2010) (Scheme 1).

Cysteine-aziridine ligation is usually performed under acidic conditions where the thioacid functionality **12** can open the azaridine ring with a nucleophilic attack. Alternative conditions and different combinations of solvents and catalysts have been used to enhance this ligation reaction. Examples are application of ethanol, tributylphosphine, or $Et_2PCH_2CH_2PEt_2$ in acetonitrile and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dimethoxyethane (Galonić et al. 2005; Assem et al. 2010). Interestingly, the addition of $Cu(OAc)_2 \times 3\ H_2O$ resulted in an aziridine-linked peptide instead of the product 3 when starting from the same segments. Fortunately, the aziridine linkage could be turned back to a native amide bond by a nucleophilic ring-opening reaction resulting in a peptide nucleophile adduct. This Cu(II)-mediated reaction, however, is not completely understood, but a critical role of Cu(II) in the aziridine-mediated coupling process as well as the reaction's compatibility with aqueous conditions was discussed (Dyer et al. 2011).

Furthermore, a photo-deprotection of aziridine has been reported by Dyer (2013) in the aziridine-mediated ligation and may find applications to total chemical synthesis of natural peptides. Recently, Jebrail et al. have applied the digital microfluidic (DMF) technology for the combinatorial synthesis of peptides by cysteine-aziridine ligation (Jebrail et al. 2012). Their study may lead to new methods for generating libraries of natural peptides. These methods show also a high potential for generating modified peptides, proteins, and peptidomimetics after the ligation process.

Another similar ligation approach, which was much earlier introduced, is the cysteine-halogenoalanine ligation.

Here, a thioacid is used as the C-terminal nucleophile and a halogenoalanine group, often a bromoalanine (BrAla), as the N-terminal electrophile (Scheme 1). In the cysteine-bromoalanine ligation, the thiolate group of the thioacid of an unprotected peptide (**12**) attacks the bromoalanine moiety of the other peptide segment **14** at an acidic pH of 5.0 (Scheme 1) (Tam et al. 1995). Bromoalanines allow conjugation of thiosugars (e.g. thiotrisaccharide) thus generating small glycopeptides, such as the *Shigella flexneri* serotype Y O-polysaccharide and its peptide mimetics with a sugar-peptide linkage (Hossany et al. 2009). Due to the high importance of glycopeptides and proteins, this approach may represent a useful tool in the future to access novel sugar-linked lead compounds.

Cysteine-perthioester ligation

The cysteine-perthioester ligation method is another reverse thioester ligation, involving the coupling of two unprotected peptide segments including a C-terminal thiocarboxylic acid **12** and an N-terminal perthioether **15**, respectively. This method proceeds via formation of a six-membered ring intermediate with a disulfide linkage that undergoes a reversible intramolecular *S*,*N*-acyl transfer to result in the peptide product **17** containing an hydrodisulfide group (–SSH) after a rapid irreversible rearrangement. The hydrodisulfide group is reduced afterwards to form a cysteine residue at the ligation site (Liu et al. 1996a) (Scheme 1).

The cysteine-perthioester ligation proceeds generally at low pH (pH ~4.0 to 5.0). An early application of this method was the preparation of a 32mer peptide analog of the 99mer HIV protease in a moderately acidic solution (25 % acetonoitrile in water containing 0.05 % of TFA, pH 2.0) (Liu et al. 1996a).

Other ligation methods

Thioether ligation

Thioether ligation encompasses an N-terminal halogen- or maleimide-substituted peptide **18/19** and a C-terminal segment **12** containing a sulfhydryl group, such as a cysteine peptide (Scheme 2) (Robey and Fields 1989; Beekman et al. 2001). In this method, a sulfide bridge is formed instead of a native peptide bond between two peptide segments. The formation of the thioester peptide generally occurred quickly under mild basic conditions (pH ~7.0 to 8.0). However, tris(2-carboxyethyl)phosphine (TCEP) is required to avoid simultaneous oxidation of the sulfhydryl groups (Burns et al. 1991). This method already has been widely applied to the modification of proteins, such as the HIV-1 protease analog LPGKWKPKMI-[NH–CH_2–CH_2–SH) (Englebretsen et al. 1995) and transthyretin (Wilce et al. 2001). Recently, a reverse thioether ligation method has been reported (Monsó et al. 2012). In this study, multiple antigenic peptides have been successfully produced either on the solid phase or in solution by joining the haloacetyl-actived peptide and the lysine core with N-terminal cysteine through a thioether bond to produce multiple antigenic peptide (MAP) dendrimers. Though interesting, this method can only be applied to a small selection of substances due to the generated thioether bond.

Imine ligation

Apart from the aforementioned methods, imine ligation has been introduced already two decades ago. In imine ligation, which involves an imine capture step that results from a peptide aldehyde reacting with an N-terminal amine on the other peptide segment, no natural amino acid residue is formed at the ligated site. Below, different types of imine ligation, i.e., oxime and hydrazine ligation as well as pseudoproline, thiazolidine, and tetrahydro-β-carboline ligation (Scheme 2), are mentioned because of the reduced use or even missing need of chemical additives and thus mild reaction conditions. Furthermore, this method enables the production of peptidomimetic structures or the introduction of new features into the natural peptide bond. In addition, like described for the thioester ligation above, there is only one example of reverse imine capture approach containing an amine electrophile **34** and a nucleophilic acetyl segment **32** (Tam et al. 1999). The reverse method has been successfully applied to form bicyclic lactams at the ligation site of **35**, yet no further examples are available so far.

Oxime and hydrazone ligation

In the first examples of imine ligation methods, i.e., oxime ligation and hydrazone ligation, the ligated product (e.g. **28**) remains in a stable state of the intermediate structure, e.g. an oxime bond or hydrazone bond (Scheme 2). Reactions of oxime and hydrazone ligation are generally carried out under weak acidic conditions (Gaertner et al. 1992; Tuchscherer 1993; Rose 1994; Shao and Tam 1995). As recently shown, oxime ligation can be enhanced using *m*-phenylenediamine (*m*PDA), aniline, or other appropriate amine derivatives (Dirksen et al. 2006; Rashidian et al. 2013). This method also has been applied to generate a fluorescence-labeled protein, i.e., ciliary neurotrophic factor (Rashidian et al. 2013). However, a major drawback of these methods is that the hydrazone or oxime products are susceptible to hydrolysis over time. Therefore, the hydrazino-Pictet-Spengler (HIPS) ligation has been introduced in 2013 with the aim to avoid the hydrolysis effect

of hydrazone or oxime products (Agarwal et al. 2013a, b). Here, aminooxy-functionalized indoles react with aldehydes to generate an oxyiminium ion intermediate, which readily rearranges into a stable ligation product. In contrast to the aforementioned method, this reaction can be performed under almost neutral conditions. A variant of the therapeutic monoclonal antibody Herceptin that contains a formylglycine FGly-α-HER2, a maltose-binding protein (FGly-MBP), and an N-terminally transaminated myoglobin were conjugated to a commercially available fluorophore, respectively (Tan and Swain 2003). In continuation, the same set of proteins could also be labeled with biotin using this method. It thus represents a useful approach to synthesize biotin- or fluorescence-labeled (or even other labels) proteins starting from aldehyde-tagged precursors and resulting in unnatural, hydrolytically stable bonds.

Pseudoproline, thiazolidine, and tetrahydro-β-carboline ligation

In the second type of imine ligation, the reaction stops in the step of the spontaneous cyclization yielding a heterocyclic structure, e.g., containing a pseudoproline bond **24**, a thiazolidine bond **29**, or a tetrahydro-β-carboline ring **32** (Scheme 2). While pseudoproline ligation reactions can be performed in both aqueous and non-aqueous media, e.g., in pyridine-acetic acid (1:1) (Liu and Tam 1994a, b; Liu et al. 1996b; Tam and Miao 1999), thiazolidine ligation and tetrahydro-β-carboline ligation require aqueous acidic conditions (pH ~3.0–5.0) (Shao and Tam 1995; Botti et al. 1996; Li et al. 2000). In all cases, only small (5–26 AA) model peptides were used to perform the respective reaction. Therefore, these methods represent, up to now, only exotic variants of ligation reactions lacking information on limiting factors and applicability for full exploitation for other suitable targets.

Ketoacid–hydroxylamine ligation

A mechanistically unique approach, the ketoacid–hydroxylamine (KAHA) amide-forming ligation, has been developed by Bode and coworkers for peptide synthesis first (Bode et al. 2006; Ju et al. 2008). This method involves two unprotected fragments, a C-terminal peptide α-ketoacid **33** of segment A reacts with an N-terminal peptide hydroxylamine **34** of segment B and thus requires no peptide thioester. A bioactive form of human glucagon-like peptide GLP-1 (7–36) has been prepared using KAHA ligation (Wu et al. 2011). After that, Bode et al. used an N-terminal 5-oxaproline **35** instead of an alkylhydroxylamine in the synthesis of ubiquitin-fold modifier 1 (UFM1) proteins (Ogunkoya et al. 2012), prokaryotic- ubiquitin-like protein (Pup), and probable cold shock protein A (cspA) (Pattabiraman et al. 2012), because the 5-oxaproline group is imported readily into peptides by solid-phase peptide synthesis and can effectively be protected by Fmoc group during the process.

There are, of course, several other types of ligation methods, such as Staudinger ligation, click ligation, and different kinds of enzyme-catalyzed reactions. However, these are not further described in this review, since focus herein is laid on the improvements and novel applications of the aforementioned ligation methods.

Recent improvements on thioester ligation methods

Over the past 10 years, several ligation methods based on the utilization of masked or crypto-thioesters have been developed. In one approach, for example, C-terminal hydrazide peptide precursors or peptide benzotriazole masking the thioester component were successfully used (Fang et al. 2011, 2012; Wang et al. 2015). Another approach represents the *O* to *S* acyl transfer, which up to now has only been applied to the synthesis of short model peptides (Botti et al. 2004; Warren et al. 2004). A further interesting strategy is the in situ self-activation of the N-terminal bis(2-sulfanylethyl)amino (SEA)-modified segment, which is discussed in more detail the next section.

Bis (2-sulfanylethyl) amino native peptide ligation

The bis(2-sulfanylethyl)amino (SEA) native peptide ligation, a new method via N to S acyl transfer, involves the reaction between an N-terminal peptide modified with a C-terminal bis(2-sulfanylethyl)amino (SEA) group and a cysteinyl or homocysteinyl C-terminal peptide segment. Here, the nucleophilic SEA thiol group has the ability to rearrange into thioesters through an in situ N–S acyl shift. (Ollivier et al. 2010). Compared with the common NCL, the advantages of SEA ligation are as follows: (a) SEA peptides can be prepared using standard Fmoc-SPPS conveniently (Hou et al. 2011; Sato et al. 2011; Zheng et al. 2011, 2013), and (b) the SEA group can be oxidized in a cyclic disulfide to protect itself from intermolecular reaction (SEA^{off}), while it can be also reduced later to continue SEA ligation (SEA^{on}) (Ollivier et al. 2010, 2012; Boll et al. 2012; Yang et al. 2012; Raibaut et al. 2013) (Fig. 1). After investigation and optimization of this recently introduced method on 19mer model peptides, the authors performed a two-step SEA ligation to finally result in the 97mer biotinylated hepatocyte growth factor peptide (AA 31-127) demonstrating the high potential of this method. After that, Boll and his coworkers have also successfully applied SEA ligation method to the synthesis

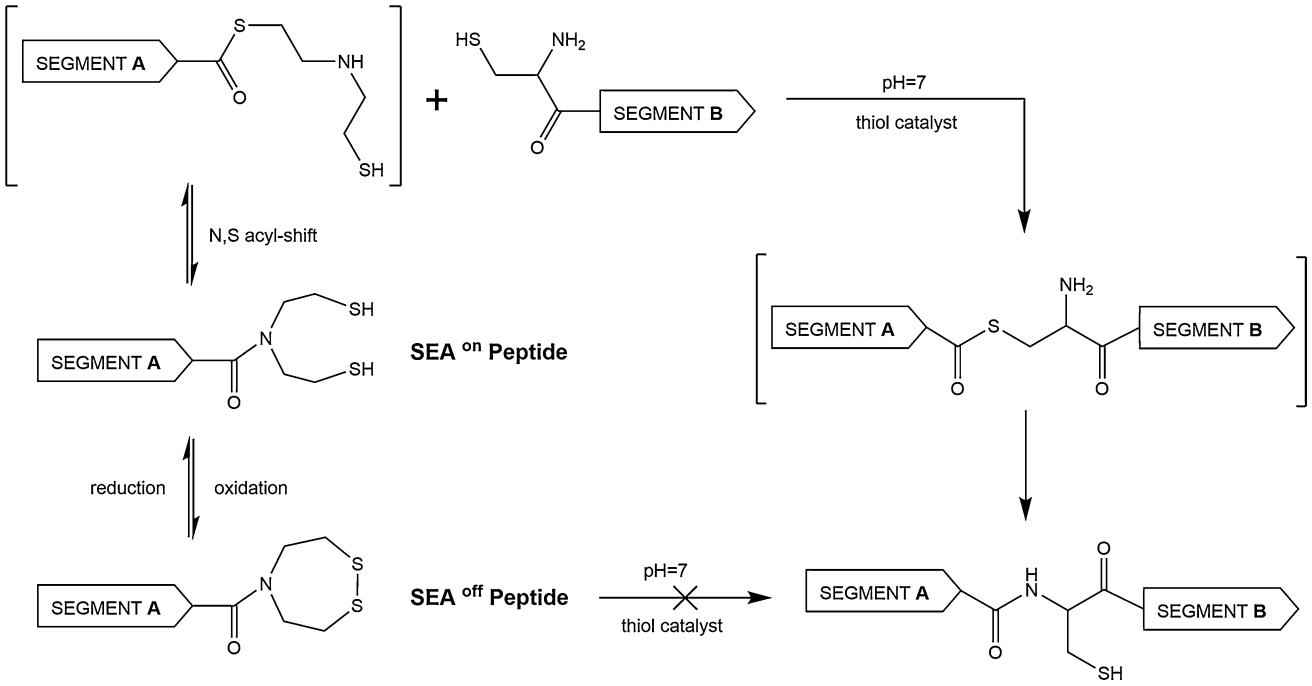


Fig. 1 Bis(2-sulfanylethyl)amido native peptide ligation (SEA ligation) and SEA$^{on/off}$ concept (modified from Ollivier et al. 2010, 2012; Boll et al. 2012; Raibaut et al. 2013)

of several head-to-tail cyclic peptides (38-71 AA) (Boll et al. 2014) and a small ubiquitin-like modifier protein–peptide conjugate (107 AA) (Boll et al. 2015). This ligation can easily be controlled using reducing agents (e.g., phosphine) after simple SPPS of theprecursors. This renders the method suitable for a broad range of applications in the future.

Template-directed ligation

Ligation reactions between two segments in solution can be promoted when performed in a template-directed fashion (Selvasekaran and Turnbull 1999; Liu et al. 2008; Goldmann et al. 2012). The usual function of templates is to align the reactive groups in close proximity to enhance the efficiency of ligation by increasing the effective concentration of the bound substrates or to promote the joining of the adjacent ends (Bruick et al. 1996). Except of oligonucleotide templates (Bruick et al. 1996), chemists have discovered and developed various different biomolecular templates to direct orthogonal ligation between peptides or a peptide and another large molecule. Meanwhile, research studies on DNA/PNA strand or DNA primer templates have increased very fast (Erben et al. 2011; Zhang et al. 2012; Roloff and Seitz 2013a, b; Vázquez and Seitz 2014; Kern and Seitz 2015), while those on protein templates (Maki et al. 2013), e.g., sortase A (SrtA) from *Staphylococcus aureus* (Steinhagen et al. 2013), started only recently.

In 2013, Lewandowski et al. (2013) have designed an ingenious molecular machine system for peptide synthesis by the NCL method. An initial peptide sequence containing a thiolate group was based on an annulus template such as a rotaxane, which was threaded onto a molecular axial template. The annulus template passed through the axis and the peptide on the ring picked up the amino acids along the axis sequentially through an iterative NCL reaction process. Finally, the prolonged peptide sequence can be cut from the annulus template by a dichloromethane–water mixture (Fig. 2). This method imitates the ribosome and synthesizes peptides by a step-by-step ligation method. However, one major disadvantage of this method is the accurate preparation of the template and thus, the synthesis efficiency combined with high costs possibly turns it unfavourable for broad application.

Application of novel additives and solvent systems in ligation reactions

Additives in peptide ligation chemistry, in general, play an important role in facilitating the main reaction, while inhibiting possible side reactions. 2-mercaptoethanesulfonate sodium salt, benzyl mercaptan, thiophenol, and 4-mercaptophenyl acetic acid (MPAA) were the most commonly used additives in NCL. Some of these thiol additives, however, possess a pungent odor and high toxicity. The thio additives can form mixed disulfides and even co-elute

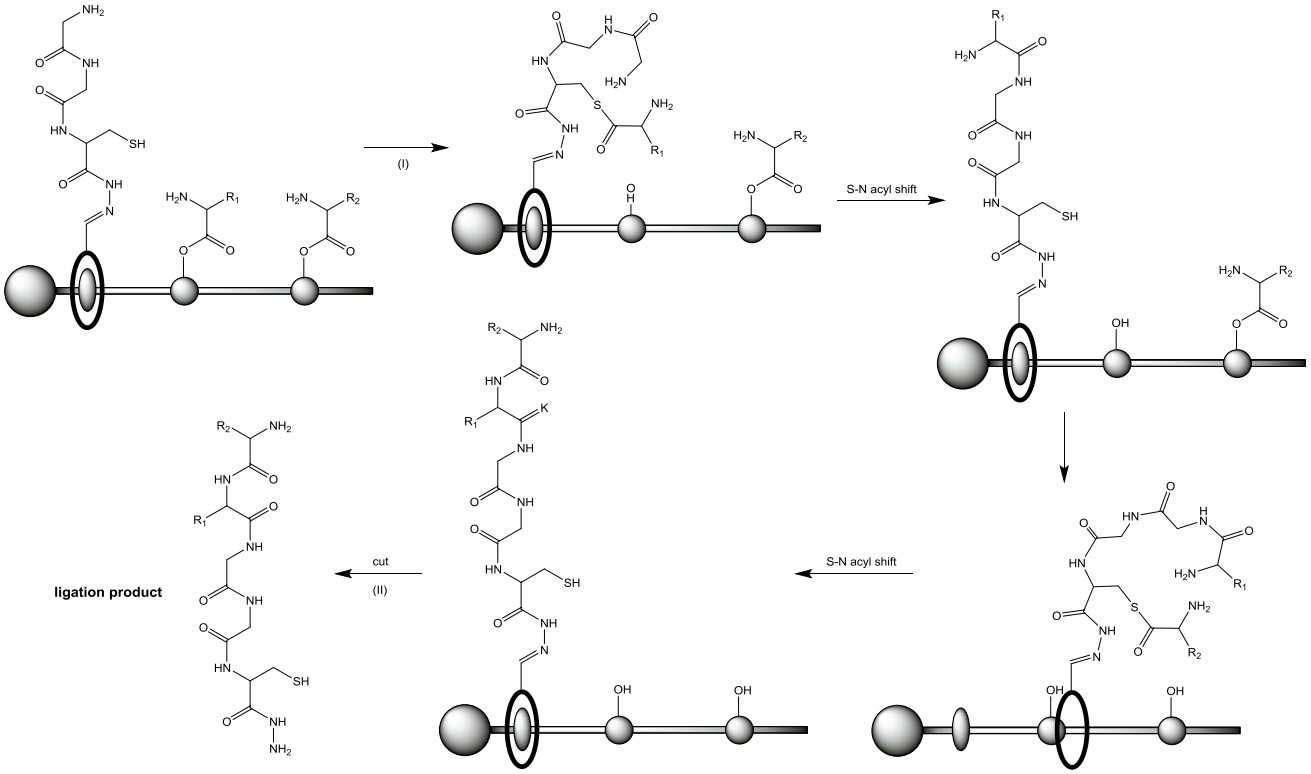


Fig. 2 Proposed mechanism for sequence-specific peptide synthesis by molecular machine. The product peptide is subsequently released from the macrocycle by hydrolysis. Reaction conditions: (I) $((CH_3)_2CH)_2NEt$, $(HO_2CCH_2CH_2)_3P$ in 3:1 acetonitrile:dimethylformamide, 60 °C, 36 h. (II) 30 % CF_3CO_2H in 3:1 dichloromethane: water, room temperature, 18 h (modified from Lewandowski et al. 2013)

with products during HPLC separation. Furthermore, thio additives have been reported to cause side reactions such as addition to multiple carbon–carbon bonds (Diezmann et al. 2010). Several novel thio additives, such as trifluoroethane thiol (TFET), Brik's bifunctional aryl thiol (Malins and Payne 2014) and 3/4-mercaptobenzyl sulfonates (3/4-MBSA) (Cowper et al. 2015) were introduced recently. Brik's bifunctional aryl thiol (Moyal et al. 2013) and TFET (Thompson et al. 2014) do not need to be removed from the reaction mixture before the desulfurization step. 3-MBSA-catalyzed NCL proceeds at a similar rate to MPAA, but is more stable. Its increased polarity and solubility may favor its selection as NCL catalyst in many instances (Cowper et al. 2015).

It has been published meanwhile that a triscarboxyethylenephosphine (TCEP)/ascorbate-containing buffer was successfully used in NCL. Sodium ascorbate, which is cheap, odorless, safe, and water-soluble, was herein applied as a substitute for thiol additives. In addition, it has been observed that the rates of the ligation reactions in the TCEP/ascorbate-buffer were similar to the ligation reactions in TCEP/thiophenol-containing buffer. Moreover, TCEP was added to avoid the formation of disulfides. At the same time, however, it can cause desulfurization of the product. It is interesting to note that this unwanted effect can be completely inhibited using sodium ascorbate (Rohde et al. 2011).

Besides the additives, the choice of the solvent is also an important factor in peptide chemistry. In general, most of the solution reactions involving longer peptides and proteins are performed in aqueous buffer systems. The low water solubility of hydrophobic peptides/proteins, however, represents a real disadvantage of the chemical synthesis under aqueous conditions. In addition, the self-aggregation effect of some hydrophobic peptides/proteins can also prevent the ligation reaction (Johnson and Kent 2007; Olschewski and Becker 2008). Several research groups have successfully performed ligations in various organic solvents, including toluene, dimethyl sulfoxide (DMSO), or dimethylformamide (DMF) (Dittmann et al. 2012; Sohma et al. 2011). Noteworthy, NCL reaction in DMF has been found to proceed chemoselectively without racemization (Dittmann et al. 2012).

Due to the different properties of the peptides present in a ligation mixture, the performance in an unfavorable medium may thus be a serious problem in the course of the reaction. Apart from conventional organic solvents room temperature ionic liquids (RTILs) received great attention

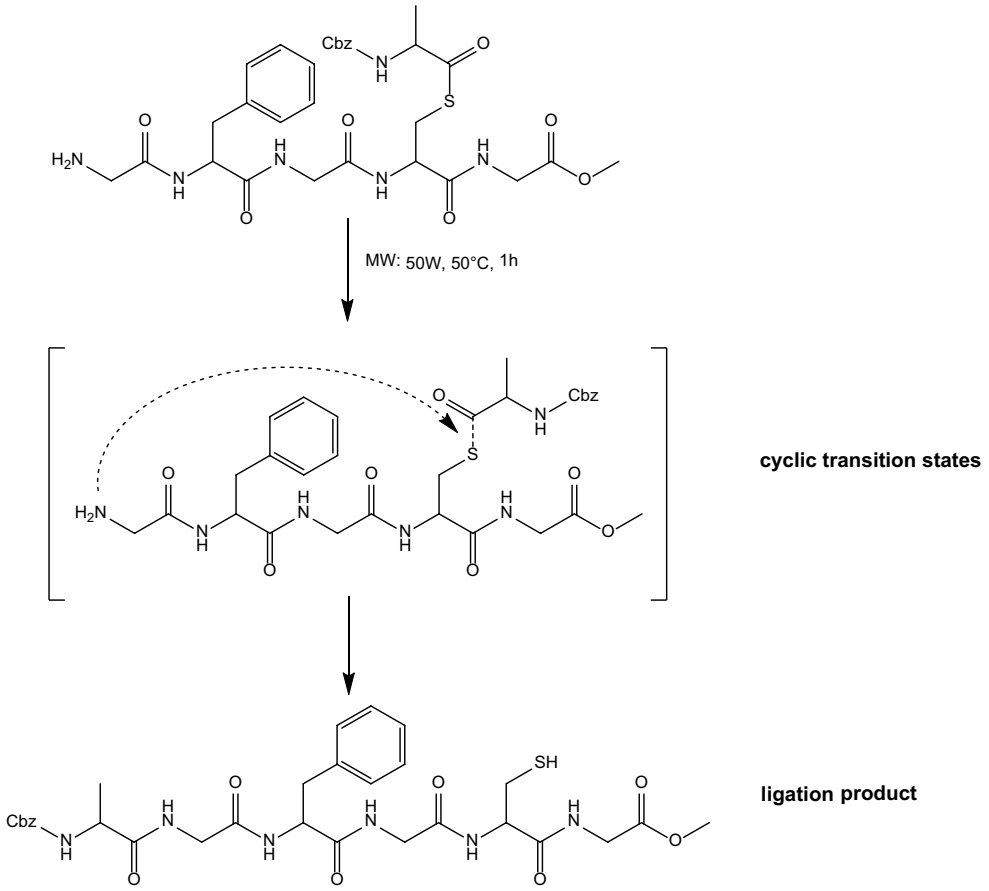


Fig. 3 Chemical ligation of S-acyl pentapeptide (modified from Hansen et al. 2011)

for chemical synthesis in the last decade (Hallett and Welton 2011). RTILs such as methylimidazolium triflate [*H*-EIM][TfO] (Galy et al. 2013) and 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) (Böhm et al. 2012; 2013; Kühl et al. 2014) were successfully applied as alternative solvents for peptide chemical reactions. Here, we could show that the NCL reaction of model peptides of the type LYRAXCRANK (X = G, A, F, D, L, Q, K) or even the 66mer oligopeptide tridegin can proceed efficiently even in the absence of additives in [C_2mim][OAc] and can be completed much faster than in buffer systems (Böhm et al. 2012; Kühl et al. 2014). However, it needs to be mentioned that several side products such as succinimide and oxidation products were detected for some of the model peptides (Kühl et al. 2014). Despite these problems, the use of ionic liquids for ligation reactions may offer new possibilities and promising applications in the future.

Microwave-accelerated ligation

In the past decade, microwave-assisted methods have been shown to be useful in organic syntheses (Lidström et al. 2001). Peptide synthesis was also performed in a kitchen microwave oven already in 1991 (Yu et al. 1992). The first attempts, however, were not a great advance due to the side reactions accelerated by MW heating and the heat sensitivity of the coupling reagents used. Nevertheless, numerous successful applications of the MW technology in SPPS were reported meanwhile (Erdélyi and Gogoll 2002; Bacsa and Kappe 2007; Palasek et al. 2007; Cemazar and Craik 2008; Galanis et al. 2009; Gunasekera et al. 2013). In contrast, there are only very few studies concerning the enhancement of chemical ligation reactions using microwave-assisted technologies so far. In 2011, the first examples of isopeptide ligations starting from *S*-acyl peptides containing non-terminal cysteine residues were reported (Hansen et al. 2011). In general, a five-membered ring is known to be a suitable ring size for acyl migration (Wieland et al. 1953). This study, however, indicated that the eleven- and fourteen-membered cyclic transition states in the intramolecular *S–N* acyl migration step are also formed under microwave radiation (Fig. 3). These chemical ligation experiments were carried out at 50 °C and 50 W for 1 h resulting in a pentapeptide (Hansen et al. 2011).

Recently, a novel low temperature microwave technique (LTMT) has been optimized for a series of heat-sensitive reactions, such as the synthesis of α-ketoamide and also protein hydrolysis (Tong et al. 2010). It is possible that LTMT could be applied for peptide chemical ligations in the future since most peptides and proteins are also heat-sensitive. Examples of MW-accelerated ligation reactions are currently rare, yet similar approaches as described above might turn out suitable for ligation methods and related applications perspectively.

Conclusions

In the last decades, the chemical ligation methods have been successfully applied in the production of long peptides and proteins. Ligation strategies are often based on a chemoselective capture step, which is connecting to peptide fragments covalently, followed by an intramolecular rearrangement that is forming a native amide bond. As an important advantage thioester ligation can be performed using several different educts such as cysteine-, selenocysteine-, homocysteine-, methionine-, glycine- and histidine-based C-terminal segments. Therefore, these methods found broad application in peptide and protein chemistry because of the opportunity to use different amino acids of the final sequence which corresponds to an extension of the possible options for the ligation site.

Nowadays, chemical ligation has been recognized even as a useful alternative to protein expression because of the limitations of the latter approach regarding posttranslational modifications or introduction of unnatural amino acids. This fact together with the developments in the field during the last decade demonstrates the future potential, yet ongoing challenges of native chemical ligation and its derivations.

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Conflict of interest The authors declare that they have no conflict of interest.

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