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Acyl donors for native chemical ligation

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Native chemical ligation (NCL) has become one of the most important methods in chemical syntheses of proteins. Recently, in order to expand its scope, considerable effort has been devoted to tuning the C-terminal acyl donor thioesters used in NCL. This article reviews the recent advances in the design of C-terminal acyl donors, their precursors and surrogates, and highlights some noteworthy progress that may lead the future direction of protein chemical synthesis.

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Introduction

Proteins perform a vast array of critical functions within organisms, and their mutation or abnormal regulation is the pathogenic factor underlying many diseases. Investigation of the biochemical or biophysical mechanisms by which proteins function requires protein samples that are usually produced through recombinant technology; but not all proteins can be made recombinantly, such as those with site-specific, post-translational modifications (PTMs). The total synthesis and semi-synthesis [1] of proteins are practical solutions to this problem; and the process of native chemical ligation (NCL), invented by Kent *et al.* in 1994, has proven to be a robust approach for protein chemical synthesis [2••], enabling the generation of proteins in aqueous phase and under mild conditions. NCL entails the sequential transthioesterification and intramolecular S-to-N acyl shift of a peptide C-terminal thioester (acyl donor) and a peptide N-terminal cysteine, to give a native amide bond. Attempts to improve the efficiency and scope of NCL through development of the N-terminal cysteine substitutes and C-terminal acyl donors are ongoing [3–6] (see Figure 1). This article surveys the recent progress of acyl donors in NCL.

Developments of new C-terminal acyl donors

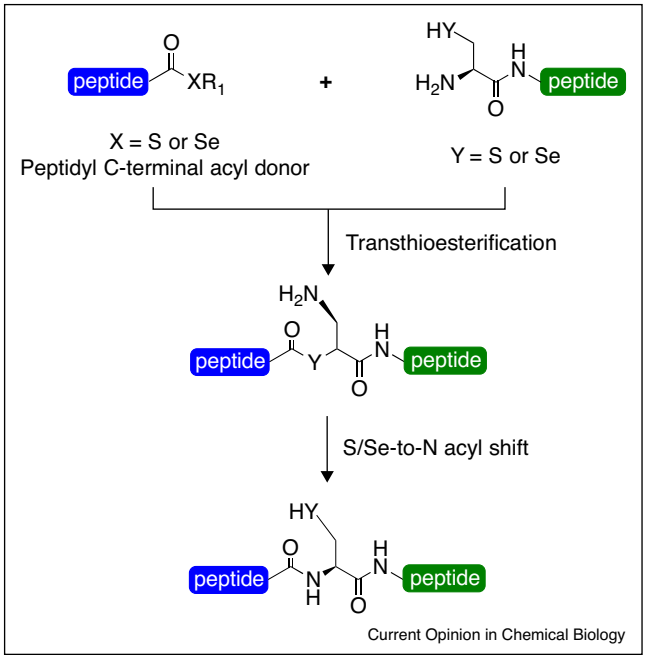
Peptide chemistry is heavily dependent on solid phase peptide synthesis (SPPS), a technique able to synthesize peptides of up to about 50 amino acids in length. However, the required C-terminal thioesters for NCL are not directly available through the widely used Fmoc-SPPS method (Fmoc = 9-fluorenylmethoxycarbonyl), due to the susceptibility of the thioester bond to the Fmoc deprotecting reagent piperidine [8,9]. In the first few years after NCL, enormous effort had been made to overcome this obstacle, including improved deprotecting reagents [10] with weaker nucleophilicity, thioesterification of partially protected peptides in solution [11], safety-catch linkers that can be transformed into a thioester prior to global deprotection [12], side-chain anchoring strategy in pursuing peptide thioester [13]. However, these methods were not efficient enough to be widely adopted. Accordingly, alternative C-terminal acyl donors or precursors suitable for NCL need to be developed. This review will first introduce two well-researched and applied acyl donors, *N*-acylurea (Nbz) and hydrazide, which in terms of mechanism are distinct from other acyl donors in this article. Then O-to-S acyl transfer will be described before N-to-S acyl transfer, for the latter is more extensively applicable and its being placed together with closely connected N-to-Se acyl transfer discussed in the last.

Peptide *N*-acylurea (Nbz)

Dawson and co-workers established an efficient Fmoc-SPPS synthetic protocol for the preparation of peptide thioesters using the 3,4-diaminobenzoic acid (Dbz) linker (see Figure 2a) [14••]. In this protocol, *o*-aminoanilide **1** is efficiently transformed into *N*-acyl-benzimidazolinone (Nbz) **2** by acylation with 4-nitrophenyl chloroformate and cyclization. This Nbz leaving group can tolerate the trifluoroacetic acid (TFA) cocktail used to deprotect the peptide and cleave it from the resin. Subsequently, at neutral pH and in the presence of aryl or alkyl thiols, peptide-Nbz **3** can undergo either rapid thiolysis to generate the stable peptide thioester **4**, or direct ligation with an additional N-terminal cysteine.

Some experiments showed that the other primary amine of Dbz (see structure **1**) could be susceptible to acylation, thereby preventing full conversion of the Dbz moiety into an Nbz moiety, especially in the synthesis of Gly-rich sequences; or long, challenging peptide segments [15]. In order to overcome this difficulty, Ottesen and co-workers

Figure 1



Mechanism of the NCL reaction [7••].

used reversible allyloxycarbonyl (Alloc) protection of this primary amine to eliminate the undesired side products, and developed a protected base resin that is compatible with common NCL conditions (see Figure 2b). Using this improved protocol, both a Gly-rich segment of histone H4 and a 44-residue peptide segment of histone H3 were synthesized [15].

In 2015, Dawson and co-workers introduced a second-generation *N*-acylurea (Nbz) linker, *o*-amino(methyl)aniline (MeDbz), to deal with the side acylation problem (see Figure 2c) [16•]. Compared with the first-generation Dbz linker, the methylated, second generation MeDbz amine resists acylation during SPPS, and is compatible with various coupling conditions, including those involving microwave irradiation and heating to 90°C. The resulting peptide-MeNbz (*N*-acyl-*N*′-methylurea) is still readily amenable to NCL with thiol additives. The utility of this new approach was exemplified in the successful synthesis of two cysteine-rich cyclotides of Kalata B1 and MCoTi-II [16•].

Peptide hydrazide

The peptide acyl hydrazide strategy, developed by our group [17••], is another method for preparing peptide thioesters. As depicted in Figure 2d, peptide hydrazide **5** can be easily and selectively oxidized into peptide azide **6** by $NaNO_2$ under aqueous acidic conditions. With thiol additives, peptide azide **6** will cleanly transform into peptide thioester **7** ready for NCL. Notably, all the aforementioned steps — oxidation, thioesterification, and NCL — can be done in one-pot. Also, the peptide hydrazide acyl donor precursor **5** can be readily prepared by either conventional Fmoc-SPPS or recombinant expression [17••]. This method has proved versatile and effective, and its utility exemplified in the chemical synthesis of many proteins such as crambin [18], histone [19], membrane protein [20], ubiquitin chains [21,22], cyclic protein lactocyclicin Q [23], chemokines [18,24], D-peptide inhibitor [25] and a functional mirror-image polymerase [26].

Thioester surrogate preparation by intramolecular acyl transfer

Methods based on an intramolecular O-to-S or N-to-S acyl shift are another solution to the problem of preparing C-terminal thioesters by conventional Fmoc-SPPS. In each case, a precursor peptide is prepared by Fmoc-SPPS and then transformed into the desired thioester.

Peptide α-thioester formation based on O-to-S acyl transfer

The Hmp (2-hydroxy-3-mercaptopropionic acid) mediated thioester generation, first demonstrated by Botti and co-workers [27••], is a typical example of an O-to-S acyl transfer. An S-*t*Bu protected Hmp linker, synthesized on resin, undergoes standard Fmoc-SPPS and cleavage followed by treatment with an arylthiol, whereupon it is readily transformed into a C-terminal thioester (see Figure 3a). Seeking to expand this technology beyond Rink-Amide-PEGA resin, Muir and co-workers proposed the solution-phase synthesis of protected Hmp followed by its coupling to any resin, thereby avoiding the low yields associated with insufficient resin swelling [28]. To further optimize this approach, Liu and co-workers introduced an S-trityl protected Hmp linker, synthesized by way of a 2-MP (2-methylpiperidine) based Fmoc chemistry protocol [29] (see Figure 3b). In addition to Hmp derivatives, mercaptoethyl esters [30], peptide O-esters [31,32] (the use of which involves a direct, intermolecular O-to-S acyl shift), and thiol bearing phenolic esters [33,34] can also mediate the O-to-S acyl transfer and yield the target peptide thioester after thiolysis. The susceptibility of the oxo-ester intermediate to nucleophilic attack and hydrolysis is the main drawback to this strategy.

Peptide α-thioester formation based on N-to-S acyl transfer

The feasibility of both C-terminal CPE (cysteinyl prolyl ester) [35,36] and C-terminal NAC (*N*-alkyl cysteine) [37–39] as thioester surrogates (see Figure 3c and d), was established by Hojo and co-workers, who demonstrated that both moieties were susceptible to thiolysis at pH values above 7.8 or between 4 and 6, respectively; and that the resulting thioester could be used to accomplish