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Improved solid phase synthesis of peptide carboxyamidomethyl (Cam) esters for enzymatic segment condensation

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ABSTRACT

Peptide C-terminal carboxyamidomethyl (Cam-)esters are pivotal building blocks for enzymatic segment condensation and their yield and purity are crucial for the overall efficiency of this strategy. Although a few methods for their preparation have been disclosed, the solid phase synthesis of peptide C-terminal Cam-esters is not straightforward. Herein, we describe two novel method types for their synthesis in high yield and good purity. The first type is based on the coupling of hydroxyl protected glycolic acid to a solid support, followed by ester synthesis using an N-protected amino acid and dicyclohexyl carbodiimide with catalytic 4-dimethylaminopyridine. The second type is based on the synthesis of amino acid carboxymethyl ester building blocks, which are coupled to the solid support using standard coupling reagents and procedures. The latter procedure is easily implemented in peptide synthesizer protocols and applicable to all standard Fmoc-protected amino acid building blocks.

Introduction

Solid phase peptide synthesis (SPPS) is the most commonly used method for the synthesis of medium-sized to long peptides (15–50 amino acids (AAs)) in academia as well as in industry.[1] The main advantages of SPPS are that it is relatively fast and easy to automate; the main disadvantages are that the product can only be purified at the very end of the multistep synthesis[2] and that the outcome of the coupling reactions is often uncertain, especially for longer peptides (i.e., >10 AAs). For instance, if a 39-mer peptide such as exenatide is synthesized with an average SPPS yield of 98% per step, the crude yield of the peptide product would only be 21% and it would have to be separated from a large number of different impurities. Often, multiple different preparative HPLC purifications are needed, resulting in very substantial yield loss and huge consumption of organic solvents. It is generally accepted that SPPS is applicable to peptides of up to 50 AAs length,[3] although in many cases smaller peptides can also pose a huge challenge.[4] For difficult or very long peptides, it is more convenient and economical to use a segment condensation approach.[5] Short peptide segments can be synthesized by SPPS in high yield and purity and then condensed to form the desired product, e.g., using a 13 + 13 + 13 strategy for the synthesis of the 39-mer peptide exenatide. By this approach, the final products are generally easier to purify and the overall yield is higher. Unfortunately, chemical fragment condensation is not always feasible due to racemization of the C-terminal amino acid residue of the N-terminal segment, as well as the often low solubility of fully protected peptides.[6] Racemization-free chemical ligation techniques for unprotected peptides are known, such as native chemical ligation[7] and KAHA (*α-ketoacid-hydroxylamine amide*) ligation,[8] however, both methods are sequence dependent and therefore not generally applicable.

Another option for the racemization-free segment condensation of unprotected peptides is the use of enzymes.[9] Ligases from Nature, such as sortases[10] or butelase,[11] have been described, but the efficient ligation relies on specific recognition sequences. More advantageous is the use of proteases designed by genetic engineering, for instance subtiligase,[12] which has been applied to peptide segment condensation, head-to-tail cyclization, and peptide-to-protein conjugation. Unfortunately, this enzyme retained significant hydrolytic activity resulting in low ligation yields. Recently, we published the discovery of peptiligase,[13] a genetically engineered enzyme designed for peptide segment condensation, which is capable of ligating peptide segments with extremely high efficiency. Peptiligase was further improved by enzyme engineering with respect to substrate scope, activity, and ligation efficiency and was used for the gram scale synthesis of pharmaceutical peptides such as exenatide as well as for head-to-tail peptide cyclizations.[14] One enzyme variant with an extremely broad substrate scope, called omniligase, is currently commercially available.

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In general, using the proteases described above, the first peptide segment bearing a C-terminal activated ester is ligated to the free N-terminus of a second peptide segment. After the enzymatic ligation reaction the ester moiety is cleaved in the form of an alcohol (Fig. 1A). Virtually any (primary or secondary) ester or thioester can be used, but the SPPS of peptides bearing a C-terminal ester is not usually straightforward and often requires complex protocols.[15] One of the most efficient and commonly used esters for protease catalyzed segment condensation is the carboxyamidomethyl or Cam-ester (Fig. 1B).[16] The Cam-ester represents an isostere of a natural peptide substrate (mimicking glycine, Fig. 1C) and binds to the enzyme with high affinity, thereby increasing the overall enzymatic ligation rate. The Cam-ester can be synthesized by SPPS using standard amide resins, e.g., a Sieber, Rink or Ramage linker.[17] Preferably, the Cam-ester is elongated with an extra amino acid or amino acid amide, e.g., Cam-Leu-NH_2 or Cam-Leu-OH (Fig. 1D and E, respectively). These elongated Cam-esters are not only more stable to basic conditions, but they also display higher affinity to proteases. Elongation with an amino acid (Fig. 1E) has the additional advantage that cheap acid resins such as the Wang or 2-chlorotritylchloride (CTC) resin can be used for their synthesis.

The first paper describing the SPPS of peptide Cam-esters relied on the coupling of bromoacetic acid to the resin followed by Cam-ester formation via nucleophilic substitution using Fmoc-AA-OH cesium salts at elevated temperatures.[18] We improved this method by using iodoacetic acid and performing the ester formation with Fmoc-AA-OH diisopropylethylamine (DIPEA) salts.[17] However, the long reaction times (24 h) at elevated temperature (50 °C) were not compatible with (i) most peptide synthesizers, (ii) sensitive AAs such as Met, Trp, Tyr, Cys, and His (causing alkylation and racemization) and (iii) the CTC resin (premature cleavage was observed). Furthermore, any trace of water (many Fmoc-AAs are hydrates) led to hydrolysis of the iodide or newly formed Cam-ester bond, resulting in yield loss and undesired by-products. In this paper, we describe several alternative methods for the SPPS of peptide Cam-esters having shorter reaction times and using lower temperatures. This entails less by-products or yield loss and these protocols can be carried out in virtually any automated peptide synthesizer.

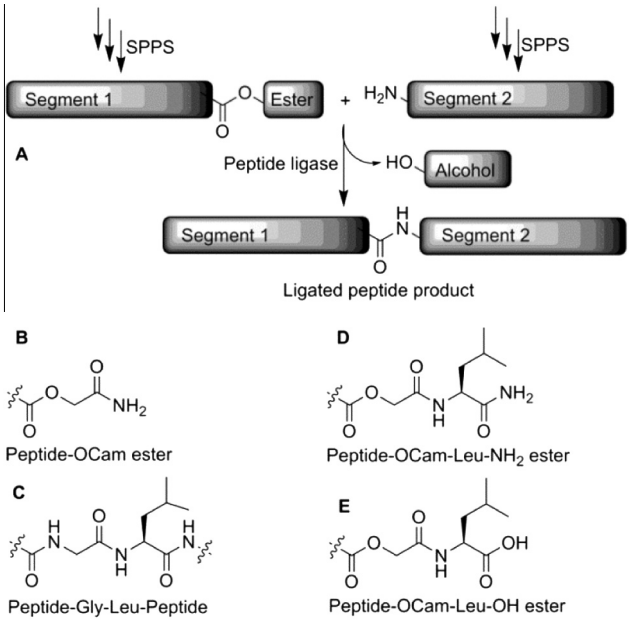


Figure 1. (A) Chemo-enzymatic peptide synthesis: a peptide C-terminal ester is ligated to a peptide bearing a N-terminal amine and the ester functionality is cleaved in the form of an alcohol. (B) Peptide C-terminal Cam-ester. (C) Peptide with Gly-Leu sequence. (D) Peptide with C-terminal Cam-Leu-NH_2 ester. (E) Peptide with C-terminal Cam-Leu-OH ester.

Results

To avoid the elevated temperatures used for the SPPS of peptide Cam-esters, we reasoned that ester synthesis based on coupling an Fmoc-AA-OH with glycolic acid would be the most straightforward. SPPS protocols for ester synthesis are well known (as also used for loading the first amino acid to a Wang resin), such as the *N*,*N*′-dicyclohexylcarbodiimide/4-dimethylaminopyridine (DCC/DMAP) or symmetric anhydride method. Although these methods possess the risk of partial racemization,[2] fortunately, proteases like peptiligase do not recognize D-AAs at the C-terminal position of the N-terminal fragment and the small amount of the diastereoisomeric by-product do not end up in the final ligation product.

Following a literature procedure,[19] we synthesized trityl (Trt) protected glycolic acid (Trt-OCH_2COOH), as a Cam-ester building block, in an overall yield of 88% and an HPLC purity of 99%. After coupling Trt-OCH_2COOH to a H-Leu-Wang resin, the Trt protecting group could be removed under mildly acidic conditions (2% trifluoroacetic acid (TFA) in CH_2Cl_2) followed by Cam-ester synthesis using Fmoc-AA-OH (4 equiv) in the presence of DCC/DMAP (45 min, double coupling, Fig. 2A).

This method was successfully performed for all 20 proteinogenic AAs. In most cases the ester synthesis was quantitative based on resin bound glycolic acid. Only with challenging AAs such as Val and Ile was the coupling efficiency decreased to 85%. Consequently,

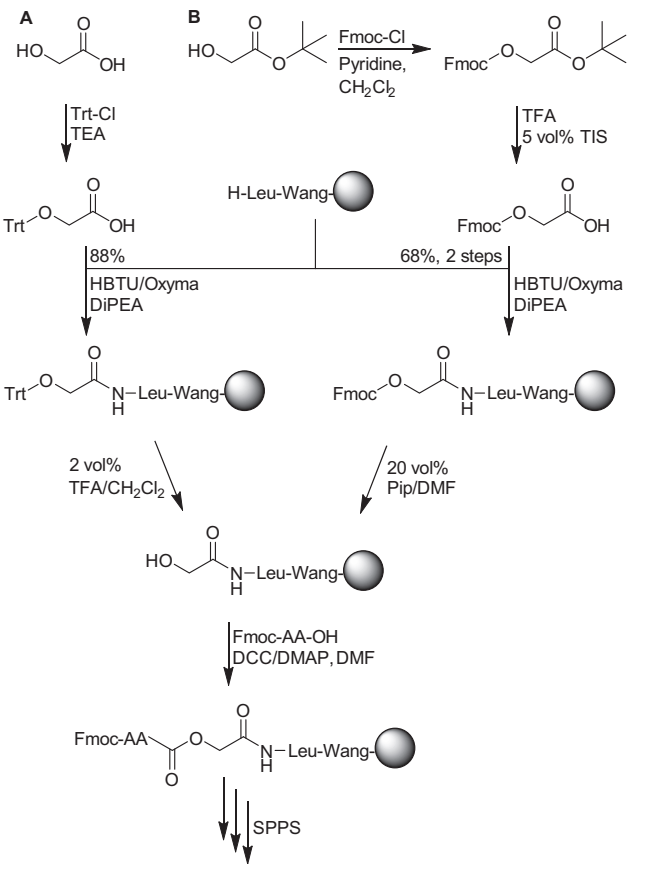


Figure 2. (A) Peptide Cam-Leu-OH ester synthesis using Trt protected glycolic acid. (B) Peptide Cam-Leu-OH ester synthesis using Fmoc protected glycolic acid. Trt-Cl = trityl chloride, Fmoc-Cl = 9-fluorenylmethyloxycarbonyl chloride, TEA = triethylamine, TIS = triisopropyl silane, TFA = trifluoroacetic acid, HBTU = (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Oxyma = ethyl (hydroxyimino)cyanoacetate, Pip = piperidine, DMF = *N*,*N*-dimethylformamide.

it is advised to cap the remaining free alcohol functionalities with a benzoyl group using benzoic anhydride and pyridine to avoid side-reactions.[2]

Unfortunately, the Trt-OCH_2COOH method is not compatible with the acid sensitive CTC resin. Therefore, we also adopted the protocol of Kodadek and co-workers[20] to synthesize Fmoc-protected glycolic acid (Fmoc-OCH_2COOH) in an overall yield of 68% and an HPLC purity of 98% (Fig. 2B). Although the synthesis of Fmoc-OCH_2COOH requires *tert*-butyl ester protection/deprotection steps, the method is compatible with all commonly used SPPS resins and it is easier to perform the consecutive Cam-ester synthesis in a peptide synthesizer without the use of corrosive TFA.

Alternatively, unprotected glycolic acid was coupled to a H-Leu-Rink resin, resulting in a resin bound glycolic acid oligomer (Fig. 3). After alkaline hydrolysis of the ester bonds (1 N NaOH in DMF), the Cam-ester could be synthesized in the manner described above. Even though this method is relatively cheap, it is not compatible with some acid resins, such as the Wang resin.

Besides loading glycolic acid onto the resin followed by ester synthesis with an Fmoc-AA-OH, the synthesis could also be performed in a different order. We reasoned that for a strategy compatible with all resins and peptide synthesizers, it would be easier to first assemble the Fmoc-AA-OCH_2COOH building blocks and couple them to a resin or H-AA-resin using the standard coupling reagents as used for subsequent AA elongation steps (Fig. 4A).

Recently, commercially available Fmoc-AA-benzotriazole derivatives have been described as good starting materials for the synthesis of Fmoc-AA-esters in the presence of DMAP.[21] Using glycolic acid *tert*-butyl ester ($HOCH_2COOtBu$), we were able to synthesize the corresponding Fmoc-AA-$OCH_2COOtBu$ derivatives in near quantitative yields. An aqueous washing step and simple silica filtration proved sufficient to remove the benzotriazole and any residual Fmoc-AA-OH hydrolytic by-product. After *tert*-butyl ester deprotection using 50 vol% TFA in CH_2Cl_2 and concentration in vacuo, the desired Fmoc-AA-OCH_2COOH building blocks were obtained. Optionally the *tert*-butyl esters could be crystallized prior to *tert*-butyl ester deprotection, e.g., using CH_2Cl_2/hexane. The use of the Fmoc-AA-OCH_2COOH derivatives in the SPPS of peptide C-terminal Cam-esters proved to be successful. This method is applicable to 8 (Ala, Gly, Ile, Leu, Met, Phe, Pro, Val) out of the 20 standard Fmoc-AAs, but not for those containing acid labile side-chain protecting groups. We investigated the use of benzyl-ester protected glycolic acid followed by cleavage by hydrogenation (data not shown). This method is feasible although partial deprotection of the Fmoc-group is a risk during hydrogenation, resulting in a troublesome purification.

Finally, we investigated the use of the hyper acid labile CTC resin for synthesis of the Fmoc-AA-OCH_2COOH building blocks (Fig. 4B). First, unprotected glycolic acid was coupled to the CTC resin using DIPEA. Although coupling of both the acid and the alcohol

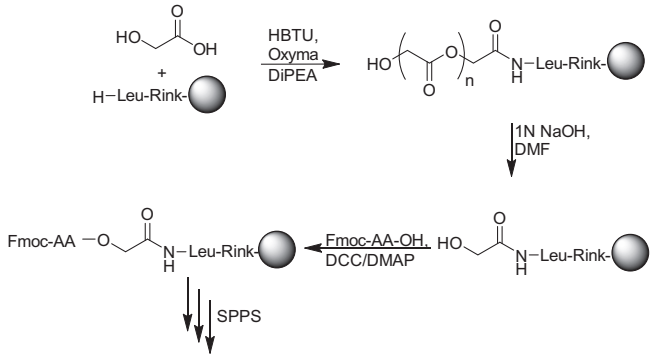
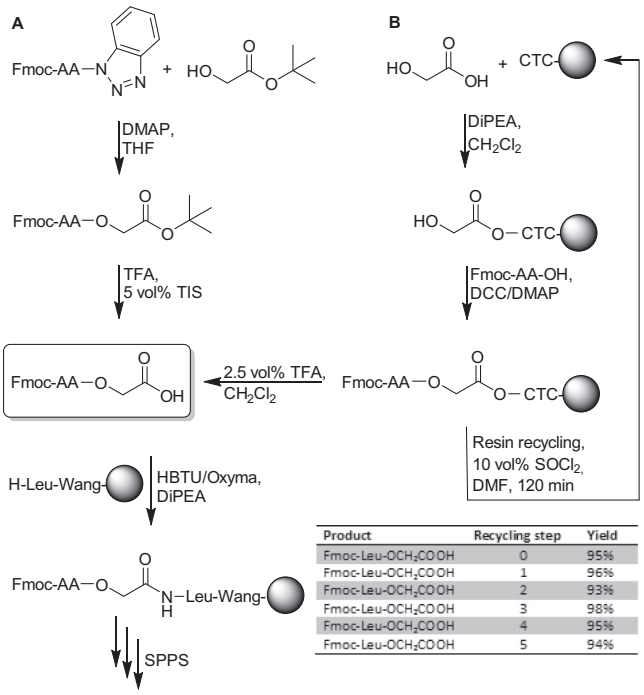


Figure 3. Synthesis of peptide C-terminal Cam-Leu-NH_2 esters using a Rink resin and glycolic acid oligomerization followed by hydrolysis of the ester bonds.



Product	Recycling step	Yield
Fmoc-Leu-OCH_2COOH	0	95%
Fmoc-Leu-OCH_2COOH	1	96%
Fmoc-Leu-OCH_2COOH	2	93%
Fmoc-Leu-OCH_2COOH	3	98%
Fmoc-Leu-OCH_2COOH	4	95%
Fmoc-Leu-OCH_2COOH	5	94%

Figure 4. Synthesis of peptide C-terminal Cam-Leu-OH esters using Fmoc-AA-OCH_2COOH building blocks. (A) Starting from Fmoc-AA-benzotriazole starting materials or (B) starting from a CTC resin.

functionality is theoretically possible, we only observed the desired coupling of the acid. This was proven by the coupling of benzoic anhydride, mildly acidic cleavage, concentration in vacuo and NMR analysis. Subsequently, the different Fmoc-AAs were coupled to the alcohol functionality using DCC/DMAP (45 min, double coupling). After mildly acidic cleavage using 2% TFA in CH_2Cl_2, washing with water (3×) and brine (1×) and concentration in vacuo, all 20 proteinogenic Fmoc-AA-OCH_2COOH building blocks were obtained in over 90% yield and over 95% purity. Moreover, the CTC resin could be reactivated using literature procedures[22] and used multiple times without any yield loss after 5 cycles (Fig. 4). The enantiomeric purity of all Fmoc-AA-OCH_2COOH building blocks was determined (C.A.T. GmbH & Co, see ESI) and proved to be over 99% except for His, Met, and Ser (97.3%, 98.7%, and 98.7%, respectively). Optionally these building blocks could be crystallized to remove any D-enantiomer. To show the applicability of the Fmoc-AA-OCH_2COOH building blocks made via the CTC resin method, a peptide library was synthesized with 20 different amino acids at the C-terminal position Ac-Asp-Phe-Ser-Lys-Xxx-OCam-Leu-OH. Except for Cys, all peptides were obtained in good yield (>75%) and excellent purity (>95%, see ESI). An enzymatic coupling reaction was performed using Ac-Asp-Phe-Ser-Lys-Leu-OCam-Leu-OH and H-Ala-Leu-Arg-NH_2. The coupling reaction was performed using Omniligase-1, a peptide ligase discovered by EnzyPep, which is commercially available from Iris Biotech GmbH. Full conversion of the peptide Cam-ester was achieved after 60 min to give the product (Ac-Asp-Phe-Ser-Lys-Leu-Ala-Leu-Arg-NH_2, 94 area%) and the hydrolyzed Cam-ester side-product (Ac-Asp-Phe-Ser-Lys-Leu-OH, 6 area%), showing the efficiency of peptide Cam-esters for enzymatic ligation reactions (see ESI).

Conclusions

Peptide Cam-esters are key building blocks for enzymatic segment condensation and their efficient synthesis is crucial for the overall peptide product purity and yield. We have developed several strategies for the efficient SPPS of peptide Cam-esters.

The first two methods are based on the coupling of protected glycolic acid, i.e., Trt-OCH_2COOH or Fmoc-OCH_2COOH, followed by deprotection of the alcohol functionality and Cam-ester synthesis with an Fmoc-AA-OH and DCC/DMAP. Using these two methods, peptide Cam-esters can be synthesized in high yield and purity. For use in peptide synthesizers, an even more convenient (and probably cheaper) method is based on the synthesis of Fmoc-AA-OCH_2COOH building blocks that can be coupled to the resin using standard coupling reagents and conditions. Two strategies were developed for the synthesis of these building blocks and the one using a CTC resin proved to be the most simple and broadly applicable.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.06.132.

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