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Internal Cysteine Accelerates Thioester-Based Peptide Ligation

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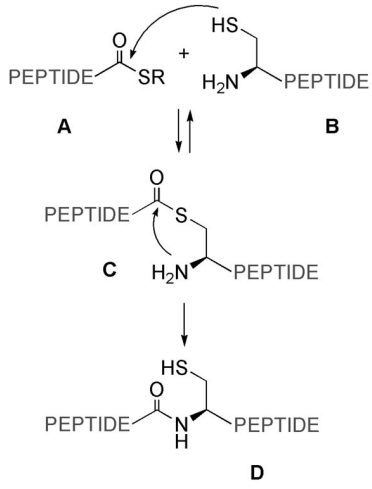
Thioester-mediated peptide coupling reactions are powerful tools in protein synthesis. The fragment coupling occurs extremely fast at ligation sites that contain an N-terminal cysteine residue. This native chemical ligation involves a capture step that affixes the acyl component in the immediate vicinity of the N-terminal amino group. The subsequent intramolecular *S,N*-acyl transfer step proceeds via an entropically favored five-membered ring intermediate. In this study we investigated whether sequence internal cysteine residues that lead to the formation of macrocyclic intermediates are also able to accelerate the rate of thioester-based fragment couplings. It was the aim to identify distance requirements that enable internal cysteine-mediated ligation reactions to proceed at synthetically useful rates. It was found that appropriately positioned cysteine residues can induce up to 25-fold rate enhancements compared to a cysteine-lacking control peptide. Highest ligation rates and yields were obtained when the internal thiol amino acid was incorporated at the fifth or sixth position from the N-terminus of the C-terminal coupling segment. The findings reveal a correlation between the size of the macrocyclic ring intermediate and the ease of the peptide ligation. Internal cysteine ligation may provide the opportunity to shift amino acid cysteine bonds that are difficult to access through native chemical ligation (such as Pro–Cys bonds) by 4–5 amino acids to the N-terminus.

Introduction

The recent developments in peptide chemistry have significantly extended the repertoire available for the total synthesis of proteins. Among the techniques employed, the native chemical ligation of unprotected fragments has proven exceptionally useful.[1] In this reaction a peptide thioester fragment **A** is allowed to react with a cysteine peptide fragment **B** (Scheme 1). The mercapto group of **B** initially replaces the thiol component of thioester **A** in a reversible manner. The formed thioester intermediate **C** reacts in a favoured 5-*exo*-trig ring closing reaction to a tetrahedral intermediate. The release of the cysteine mercapto function from the cyclic intermediate state establishes the native peptide bond in product **D**.



Scheme 1. Native chemical ligation. In the reversible initial step the cysteine replaces the thiol in thioester peptide **A** to form intermediate **C**. The subsequent S→N acyl transfer furnishes the peptide bond in **D**.

In its original form native chemical ligation is restricted to nucleophiles that have cysteine at the N-terminus. However, cysteine is a relatively rare amino acid (1.3% average content).[2] Various methods have been developed with the aim to extend the repertoire of native chemical ligation. For example, involved cysteine residues have been converted to alanine,[3] serine[4] and mimics of glutamine.[5] A homocysteine-ligation/methylation sequence has provided access to methionine-containing ligation sites.[6] Ligation was performed at β-mercapto-phenylalanine.[7] Subsequent desulfurization furnished phenylalanine. Penicillamine, the β-mercapto derivative of valine (6.6%), is commercially available and allows the synthesis of crowded leucine–valine conjunctions.[8] A γ-thiolated valine building block has been used as alternative precursor to valine.[9] Though useful in many cases, these methods have one drawback; the need for protection of non-participating cysteine residues.

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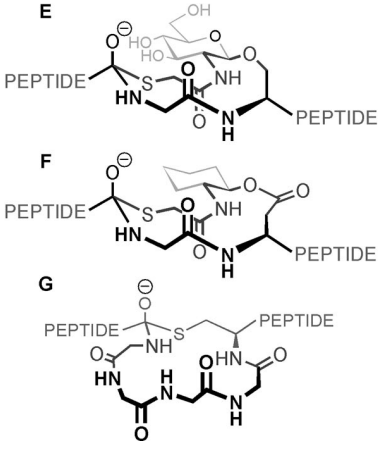
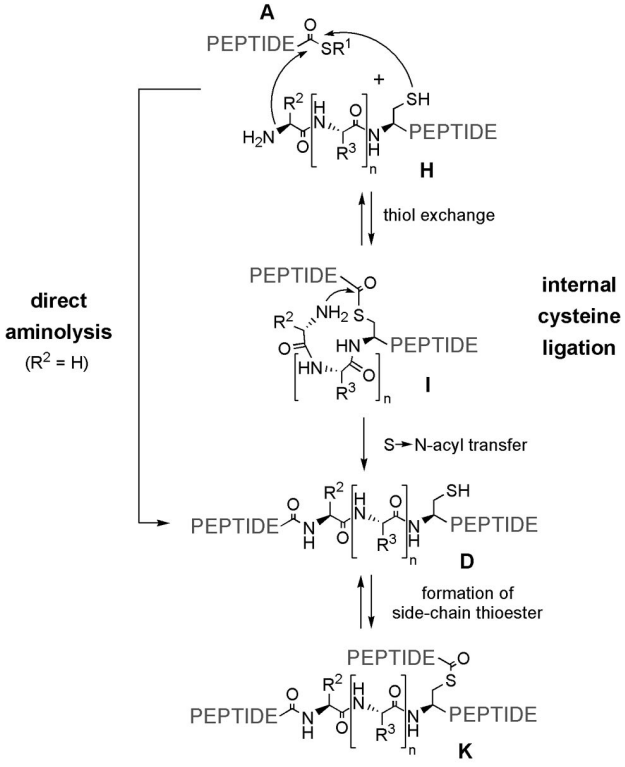
In the so-called extended native chemical ligation approach, auxiliary groups are attached to the N-terminus of the nucleophilic peptide fragment.[10] These groups provide a cysteine mimic that can be removed in a subsequent reaction to liberate the newly formed amide. Unfortunately, coupling reactions via *N*-linked auxiliaries proceed slowly because the commonly used scaffolds confer steric hindrance to an already weakly nucleophilic secondary amino group.

The above discussed native chemical ligation-like reactions involves a thiol-mediated capture step that affixed the acyl donor peptide in the immediate vicinity of the amino group. According to a generally accepted notion the subsequent S→N acyl shift reactions should proceed via entropically favored 5- and 6-membered ring intermediates in order to occur at useful rates. Recent contributions from the laboratories of Wong and Brik have challenged this paradigm.[11a,12] Wong and co-workers attached a removable thiol handle to *O*- and *N*-linked carbohydrates and observed remarkable ligation efficiencies when the ligation site included at least one glycine unit.[11b] The reaction involved primary amino groups, which couple more rapidly than secondary amino groups. Up to five amino acids can be inserted between the glycosylated residue and the N-terminal amino acid of the nucleophilic peptide fragment.[11c] It was proposed that the acyl transfer proceeded via 14- to 29-membered ring intermediates such as **E** (Scheme 2) and may, thus, become rate limiting.[11] The authors showed evidence that the sugar part critically affects the proximity of the N-terminal amine to the thioester.[11c] Brik and coworkers attached thiol-containing cyclohexane auxiliaries to amino acid side-chains such as serine or aspartic/glutamic acid.[12] In this case, the S→N acyl transfer had to proceed via 15- to 16-membered rings such as **F**.



Scheme 2. Ring intermediates formed during A) first-generation sugar-assisted ligation via an α-GlcNAc unit *O*-linked to serine; B) side chain-auxiliary ligation, in which 2-aminocyclohexanol is attached to an aspartic acid side chain via an ester linkage and C) internal cysteine ligation wherein a sequence internal cysteine presents the acyl residue to the terminal amino acid.

The remarkable results achieved in peptide ligations via internally appended thiol handles inspired us to explore whether internal cysteine residues likewise facilitate ligation reactions (see **G** in Schemes 2 and 3). In an internal cysteine ligation strategy no special building blocks would be required as a cysteine within a sequence would offer its side chain function for the initial thioester exchange. This approach would be useful when the C-terminal amino acid of the used peptide thioester hampers ligation. For example, proline thioesters are known to react reluctantly in native chemical ligations.[13] Thioesters of aspartic or glutamic acid or lysine are prone to side reactions and may form cyclic anhydrides[14] or lactams,[15] respectively. We herein report a systematic study of internal cysteine ligation. This investigation reveals a sequence-dependence and suggests optimal distances between the internal cysteine residue and the N-terminal amino group. It is demonstrated that internal cysteine residues can provide up to 25-fold acceleration of ligation rate compared to cysteine-lacking control peptides.



Scheme 3. Thioester-mediated peptide coupling via a) direct aminolysis and b) internal cysteine ligation. The latter proceeds through side-chain thioester intermediate **I** which undergoes a subsequent acyl transfer to form the ligation product **D**. If the formation of **D** proceeds fast, an *S*-acyl ligation product **K** can be observed.

Results and Discussion

The ligation partners were designed in analogy to published ligation reactions.[13] The known H-LYRAG-SR thioester **1** was allowed to react with glycinyl-peptides **2**–**6** (Table 1). In the latter, the number of glycine spacer units between the N-terminal glycine and the internal cysteine residue was successively increased from none to four amino

acids. The peptides were dissolved in a degassed buffer containing 6 M guanidine hydrochloride, 100 mM Na_2HPO_3 and 25 mM tris(carboxyethyl)phosphane (TCEP) at pH = 7.0 in 5 mM concentration. Thiophenol (5%) was added as sole additive to the reaction mixture. The course of the ligation reactions of the peptides **2** – **6** is illustrated by the representative HPLC-analysis of the coupling reaction between **1** and **4** (Figure 1, A/B). The HPLC trace after 48 h shows three main peaks, with the ligation product (e in Figure 1, B) as major peak, followed by peaks for the excess nucleophile (a) and the hydrolyzed thioester (b). The HPLC trace after 8 h is more informative and provides evidence of formed reaction intermediates such as the non-rearranged thioester (d in Figure 1, A) and a side chain thioesterified ligation product (f in Figure 1, A) that is amenable to hydrolysis. In this series, the N-terminal glycine peptides **5** and **6** exhibited the fastest ligation rates (Table 1). This suggested that internal cysteine-mediated ligation of glycinyl-peptides may proceed fastest with cysteine in Gly+4 and Gly+5 sites. The applied reaction conditions have been reported to enable chemoselective native chemical ligation reactions.[17] Nonetheless, the coupling of the thiol-lacking alanine-containing peptide **7** still occurred at a significant rate, which was only threefold lower than the ligation of the most reactive cysteine-containing peptides **4** and **5**. This implies that direct aminolysis of the thioester **7** partially contributes to product formation (see also Scheme 3). Of note, peptide **3**, which contained the Cys residue in Gly+2 position had a 0.6-fold lower reactivity than cysteine-free peptide **7**. It can be speculated that the medium sized 11-membered ring that occurs upon formation of the tetravalent intermediate is unfavorable for an intramolecular S→N acyl transfer. According to this notion the acyl donor segment would be trapped at the thioester intermediate stage, which would effectively decrease the concentration of thioesters available for direct aminolysis.

It was our aim to identify distance requirements that enable internal cysteine-mediated ligation reactions to proceed at synthetically useful rates. In Gly–Gly ligation the achievable rates were perturbed by the direct aminolysis reaction, which complicated the determination of effects induced by the internal cysteine residue. Thus, the steric demand of one ligation partner was increased to suppress interference by direct fragment fusion.

The peptides **8**–**14** were incubated with LYRAG-thioester **1** under conditions denoted above. The replacement of the N-terminal glycine in **7** with the alanine in **14** dramatically decreased the rate of direct aminolysis. Only 3.1% of the coupling product **41** were formed after 48 h. The peptide H-AGGGCRAEYS-NH_2 **11** ligated 25-fold faster than

Table 1. Peptides used in the internal cysteine-mediated ligation with thioester H-LYRAG-SR (**1**).

Nucleophilic peptide	Ligation product	Rate acceleration	Yield[a]/%
H-G_nCRAEYS-NH_2	H-LYRAGG_nCRAEYS-NH_2		
2, n = 1	**29**, n = 1	1.0[b]	25.2
3, n = 2	**30**, n = 2	1.0[b]	29.7
4, n = 3	**31**, n = 3	0.6[b]	18.0
5, n = 4	**32**, n = 4	3.0[b]	54.4
6, n = 5	**33**, n = 5	3.0[b]	56.4
7, H-G_3ARAEYS-NH_2	**34**, H-LYRAG$_4$ARAEYS-NH_2	/	42.8
H-AG$_n$CRAEYS-NH_2	H-LYRAGAG$_n$CRAEYS-NH_2		
8, n = 0	**35**, n = 0	1.4[b]	4.3
9, n = 1	**36**, n = 1	3.7[c]	6.5
10, n = 2	**37**, n = 2	6.3[c]	18.0
11, n = 3	**38**, n = 3	25.1[c]	49.2
12, n = 4	**39**, n = 4	9.2[c]	20.0
13, n = 5	**40**, n = 5	4.8[c]	9.1
14, H-AGGARAEYS-NH_2	**41**, H-LYRAGAG$_2$ARAEYS-NH_2	/	3.1
H-AG$_n$DCRAEYS-NH_2	H-LYRAGAG$_n$DCRAEYS-NH_2		
15, n = 1	**42**, n = 1	3.3[c]	11.5
16, n = 2	**43**, n = 2	18.0[c]	33.4
17, n = 3	**44**, n = 3	5.7[c]	15.5
18, n = 4	**45**, n = 4	5.3[c]	9.5
H-AG$_n$NCRAEYS-NH_2	H-LYRAGAG$_n$NCRAEYS-NH_2		
19, n = 1	**46**, n = 1	6.1[c]	18.4
20, n = 2	**47**, n = 2	21.0[c]	39.8
21, n = 3	**48**, n = 3	7.2[c]	15.6
22, n = 4	**49**, n = 4	3.9[c]	10.2
H-A$_a$G$_n$PCRAEYS-NH_2	H-LYRAGA$_a$G$_n$PCRAEYS-NH_2		
23, a = 0; n = 0	**50**, a = 0; n = 0	0.8[c]	3.4 (10.5)[d]
24, a = 1; n = 0	**51**, a = 1; n = 0	2.8[c]	8.5 (15.1)[d]
25, a = 1; n = 1	**52**, a = 1; n = 1	1.4[c]	6.0 (15.0)[d]
26, a = 1; n = 2	**53**, a = 1; n = 2	4.8[c]	12.8 (43.5)[d]
27, a = 1; n = 3	**54**, a = 1; n = 3	2.4[c]	3.7 (13.6)[d]
28, a = 1; n = 4	**55**, a = 1; n = 4	3.9[c]	8.8 (15.7)[d]

[a] Determined by HPLC analysis of aliquots withdrawn after 48 hours, conditions: see Figure 1. [b] Compared to cysteine-lacking control ^{H}GGGARAEYS^{NH2} (**7**). [c] Compared to cysteine-lacking control ^{H}AGGARAEYS^{NH2} (**14**). [d] 10 mM peptides, 100 mM Na_2HPO_4, pH 8.0, 35 °C, 72 hours.

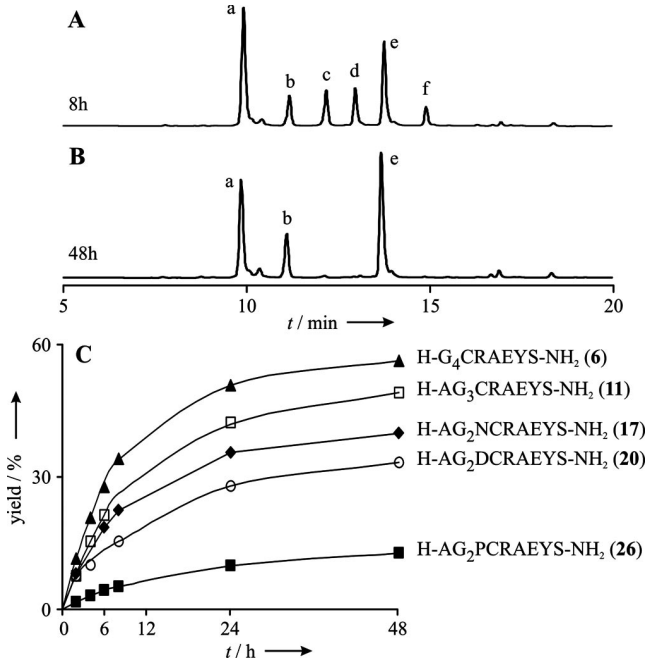


Figure 1. A) Ligation of **1** and **4** after 8 h and B) after 48 h. a: H-GGGCRAEYS-NH_2; b: thioester hydrolysis; c: H-LYRAG-$SCH_2CH_2CONHCH_2CONH_2$ (**1**); d: non-rearranged reaction product; e: ligation product H-LYRAGGGGCRAEYS-NH_2 (**31**); f: side chain thioester adduct of **31** and **1**. Conditons: 5 mM peptide concentrations, 6 M GnHCl, 100 mM Na_2HPO_4, 25 mM tris(carboxyethyl)phosphane (TCEP), pH = 7.0, 25 °C, 5% thiophenol. C) Kinetics of product formation of the ligations of the thioester H-LYRAG-$SCH_2CH_2CONHCH_2CONH_2$ (**1**) with the peptides **6**, **11**, **17**, **20** and **26**.

cysteine-lacking control **14**. This confirms a trend that was observed in ligation of glycinyl-peptides **2**–**7** where three spacer amino acids conferred highest ligation accelerations. The positive effect of internal cysteine residues was attenuated when the alanine terminus was positioned in Cys-3 (**10**) and Cys-5 (**12**) sites. Nevertheless, the internal cysteine still provided six- and ninefold rate acceleration, respectively. The other peptides, which offered internal cysteine residues at Cys-1 (**8**), Cys-2 (**9**) and Cys-6 (**13**) sites showed low ligation rates, which were too small to provide useful synthesis yields.

These results suggest that internal cysteine residues may significantly accelerate thioester-mediated ligation reactions when positioned 3–5 amino acids apart from the nucleophilic ligation site. Bearing this in mind, we turned our attention to a set of nucleophiles, in which asparagine, aspartic acid and proline were the N-terminal neighbours of cysteine. The Asn-Cys, Asp-Cys and Pro-Cys ligation sites would be difficult to access by conventional native chemical ligation.[14,15] Thus, the ligation sites were shifted to the N-termini by 2–5 amino acids. Among this series, peptides terminating in Cys-4 positions were found to provide the highest ligation accelerations. The peptides H-AGGDCRAEYS-NH_2 (**16**) and H-AGGNCRAEYS-NH_2 (**20**) reacted 18–20-fold faster than the cysteine-lacking peptide H-AGGARAEYS-NH_2 (**14**). The proline-containing peptides were less reactive. The peptide that terminated at a Cys-4 position was the most reactive nucleophile among the series. This suggests that 17-20 membered rings have the optimal size for the cyclic intermediate in the S,N-acyl transfer step of the internal cysteine ligation. The different reactivity of asparagine peptide **20**, aspartate peptide **16** and proline peptide **26** suggests that both steric and electronical properties of the amino acids next to the internal cysteine affect the ligations rates.

We decided to modify the ligation conditions to improve reactions of the proline-containing peptides. The optimization of native chemical ligation-like reactions has been reported previously.[8,13,17] For example, the use of organic co-solvents such as NMP and high pH has enabled facile ligation reactions.[11e] We chose comparatively mild ligation conditions and performed the ligation in a plain phosphate buffer (100 mM Na_2HPO_4) without guanidine hydrochloride and tris(carboxyethyl)phosphane (TCEP) with a pH-value adjusted to 8.0. The temperature was elevated to 35 °C and the concentration of the peptides was increased from 5 mM to 10 mM to increase the probability for effective collisions. Prior to the final estimation of the ligation yields a solution of tris(carboxyethyl)phosphane (250 mM) was added to the reaction solutions to ensure the absence of disulfides. HPLC-MS analysis (Figure 2) revealed two major pathways, ligation and competing thioester hydrolysis. The reactions proceeded smoothly at the optimized conditions furnishing up to 3.5-fold increases in ligation yields when compared to reactions performed at lower pH, temperature and concentration. In particular, ligation product **53** was formed at synthetically useful 44% yield with hydrolyzed thioester and starting material **26** as sole by-products. Again, the peptide **26**, which displayed the N-terminus at Cys-4 position, reacted faster than peptides **23**, **24**, **25**, **27** and **28**. This suggests that appropriately placed internal cysteine residues can accelerate ligation reactions regardless of the reaction conditions applied.

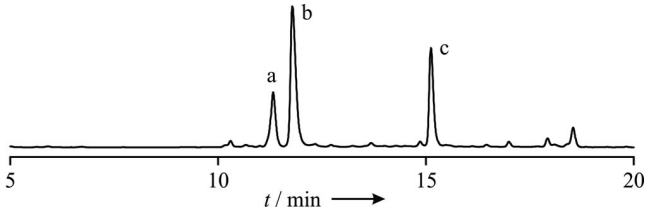


Figure 2. Ligation of **1** and **26** under optimized conditions after 72 h a: thioester hydrolysis; b: H-AGGPCRAEYS-NH_2 (**26**); c: ligation product H-LYRAGAGGPCRAEYS-NH_2 (**53**).

Conclusions

The work of Blake,[18] Aimoto[19] and Wong[16] has indicated the usefulness of thioester-mediated fragment couplings in peptide and protein synthesis. Dawson and Kent demonstrated extremely fast and chemoselective segment couplings in thioester-based ligation reactions that involved N-terminal cysteine.[1a] The investigation described here

suggests that internal cysteine residues can also confer rate accelerations. Appropriately positioned cysteine residues were found to induce up to 25-fold acceleration of ligation rates. Highest ligation rates and yields were obtained when the internal thiol amino acid was incorporated at the fifth or sixth position from the N-terminus of the C-terminal coupling segment. Cysteine-mediated ligations must proceed via the formation of cyclic intermediates. The observed distance dependence of internal cysteine-mediated ligations suggests that 17-membered peptide macrocycles are formed most readily. By contrast, peptide nucleophiles which contained cysteine at the second or third position showed only modest rate accelerations. This implies that 8- or 11-membered peptide rings are difficult to form. A similar behaviour is known from attempted syntheses of the 9- and 12-membered rings in cyclotri- and cyclotetrapeptides, which have to accommodate unfavourable strain.[20]

The findings of this investigation may have practical implications for the convergent total synthesis of peptides and proteins. The native chemical ligation will, in most cases, be the first choice. Thus, the selected ligation site will predominantly involve a thiol-containing amino acid at the N-terminal end of the nucleophilic coupling partner. However, there are a few amino acid-cysteine bonds such as Pro–Cys, Asp–Cys, Glu–Cys and Lys–Cys bonds, which are sometimes difficult to access by native chemical ligation. For these cases, one may envision to shift the ligation site such that less difficult peptide thioesters are used. These may be employed in direct aminolysis reactions, which proceed even in the absence of cysteine residues. The results obtained in this study showed that internal cysteine residues can significantly enhance ligation rates and provide synthetically useful yields. We showed that there is a correlation between the size of the macrocyclic ring formed during the S→N acyl transfer and the reaction rates. The present study was restricted to ring intermediates comprised of glycine and alanine. It is thus difficult to draw conclusions about the general applicability of internal cysteine ligation. One may assume that the achievable reaction rates will decrease upon introduction of more bulky substituents. However, the positive influence of appropriate cysteine residues may prevail. Future investigations will reveal the sequence context required for high rate accelerations in internal cysteine ligation reactions.

Experimental Section

General Procedure for Fragment Coupling of Peptides with Sequence-Internal Cysteines and C-Terminal Glycine Peptide Thioesters: The freeze-dried peptides were dissolved in 5 mM concentration in a degassed buffer containing 6 M guanidine hydrochloride, 100 mM NaH_2PO_4, 25 mM tris(carboxyethyl)phosphane at pH = 7.0. To accelerate the ligation thiophenol (5 vol.-%) was added as sole additive. The mixture was agitated at 25 °C. For the HPLC monitoring aliquots of the reaction mixture (5 μL) were dissolved in water (95 μL) containing 0.5% TFA. The resulting mixture (98 μL) was injected into a Merck–Hitachi Elite LaChrom chromatograph (column: Varian Polaris C18 A 5μ 250/4). As eluents water (containing 1% acetonitrile and 0.1% TFA) (A) and acetonitrile (containing 1% water and 0.1% TFA) (B) were used. A linear gradient (3% B – 30% B in 20 min at 55 °C) with a flow rate of 1 mL/min was employed to separate the reaction components. Detection was achieved with a UV/Vis detector (λ = 280 nm). Yields are calculated based upon the integration of the HPLC traces under consideration of the extinction coefficients. [ε_{280} (thioester) = ε_{280} (cysteine peptides) = 1280 $L\,mol^{-1}\,cm^{-1}$; ε_{280} (ligation products) = 2560 $L\,mol^{-1}\,cm^{-1}$].

Supporting Information (see footnote on the first page of this article): Details regarding materials and methods, synthesis protocols and analytical data of synthesized peptides, HPLC analysis and reaction time course of all ligation reactions.

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