



Prior disulfide bond-mediated Ser/Thr ligation†

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In this work, we developed a novel strategy, prior disulfide bond-mediated Ser/Thr ligation (PD-STL), for the chemical synthesis of peptides and proteins. This approach combines disulfide bond-forming chemistry with Ser/Thr ligation (STL), converting intermolecular STL into intramolecular STL to effectively proceed regardless of concentrations. We demonstrated the effectiveness of PD-STL under high dilution conditions, even for the relatively inert C-terminal proline at the ligation site. Additionally, we applied this method to synthesize the N-terminal cytoplasmic domain (2-104) of caveolin-1 and its Tyr14 phosphorylated form.

Introduction

Protein chemical synthesis *via* peptide ligation is a powerful and versatile approach for generating proteins with well-defined structures and properties for various applications in biotechnology, pharmaceutical research, and the study of protein function.^{1–3} This method involves the chemical synthesis of individual peptide segments through solid-phase peptide synthesis (SPPS), which are then ligated together to form the full-length protein. Chemical protein synthesis offers researchers precise control over the final protein structure, enabling the incorporation of specific modifications, such as post-translational modifications (PTM), unnatural amino acids, or site-specific labels, which can be difficult or impossible to achieve using traditional recombinant protein expression methods.^{4–9} In this regard, native chemical ligation (NCL),^{10–12} ketoacid-hydroxylamine (KAHA) ligation,^{13–15} serine/threonine ligation (STL)^{16–18} and their variants^{19,20} have been demonstrated as effective synthetic methods to prepare hundreds of long peptides and proteins over the past few decades. The successful synthesis of functional proteins containing more than 300 amino acid residues²¹ (*e.g.*, ubiquitin oligomers,^{22,23} ubiquitinated α -globin,²⁴ GroEL/ES-dependent protein DapA,²⁵ and mirror image DNA polymerases^{26–30}) showcases the increasing capability of chemical protein synthesis.

Despite the ambition and promising achievements to increase the size of synthetic proteins, the synthesis of a protein with more than 200 amino acids can still be very difficult in most cases, due to the following reasons: (1) proteins with large sizes typically contain “difficult peptide” fragments, which have poor solubility and high aggregation propensity in the ligation buffer;^{31,32} (2) ligation between two peptide fragments usually

needs to be conducted at a concentration in the millimolar range, which can be difficult to achieve when peptide fragments are of large size due to solubility issues; (3) the reactivity of the ligating groups at the termini is dramatically affected by the size of the fragment. Facilitating efficient ligation at sub-millimolar concentrations can be a way to avoid solubility and aggregation related adverse issues. As a bimolecular process, the rate of an intermolecular ligation is highly dependent on the substrate concentration. Thus, transforming intermolecular ligation into an intramolecular process by linking the N-terminal and C-terminal fragments together, both covalently and non-covalently, is a promising way to overcome the obstacle. In Kemp's pioneering work reported in 1981, a dibenzofuran-based template was developed to covalently link N-terminal and C-terminal fragments *via* phenolic ester and disulfide, respectively.^{33,34} In 1999, Dawson reported the conformationally assisted NCL.³⁵ The N-terminal and C-terminal ligation partners of the chymotrypsin inhibitor 2 protein folded into a 3D structure before ligation *via* a non-covalent interaction, where the ligating Cys40 and Asp39 thioester were located in proximity. Since then, several templated ligation strategies have been developed.^{36,37} Unfortunately, the peptide assembly assisted ligation³⁸ and RNA catalyzed ligation³⁹ are less general due to the critical sequence dependency. Meanwhile, difficult removal of peptide or PNA tags in NCL templated by coiled coil peptides,^{40,41} proteins⁴² or DNA,^{43–45} renders these methods more efficient for protein labelling rather than synthesis. As recent examples, Kay⁴⁶ and Liu⁴⁷ reported the templated NCL which was facilitated by introducing cycloalkyne/azide and split intein Cfa^N/Cfa^C pairs to the ligation partners through removable Lys modification and backbone Hmb installation, respectively (Fig. 1a). The click reaction and intein excision led to covalent prior capture formation, which allowed efficient NCL at low concentrations (2–50 μ M). Both methods have been successfully applied for the synthesis of the *E. coli* 50S ribosome subunit L32,⁴⁶ D-form extracellular domain of TIGIT,⁴⁷ and D-form Ig-like C2-type domain of tropomyosin kinase C.⁴⁷

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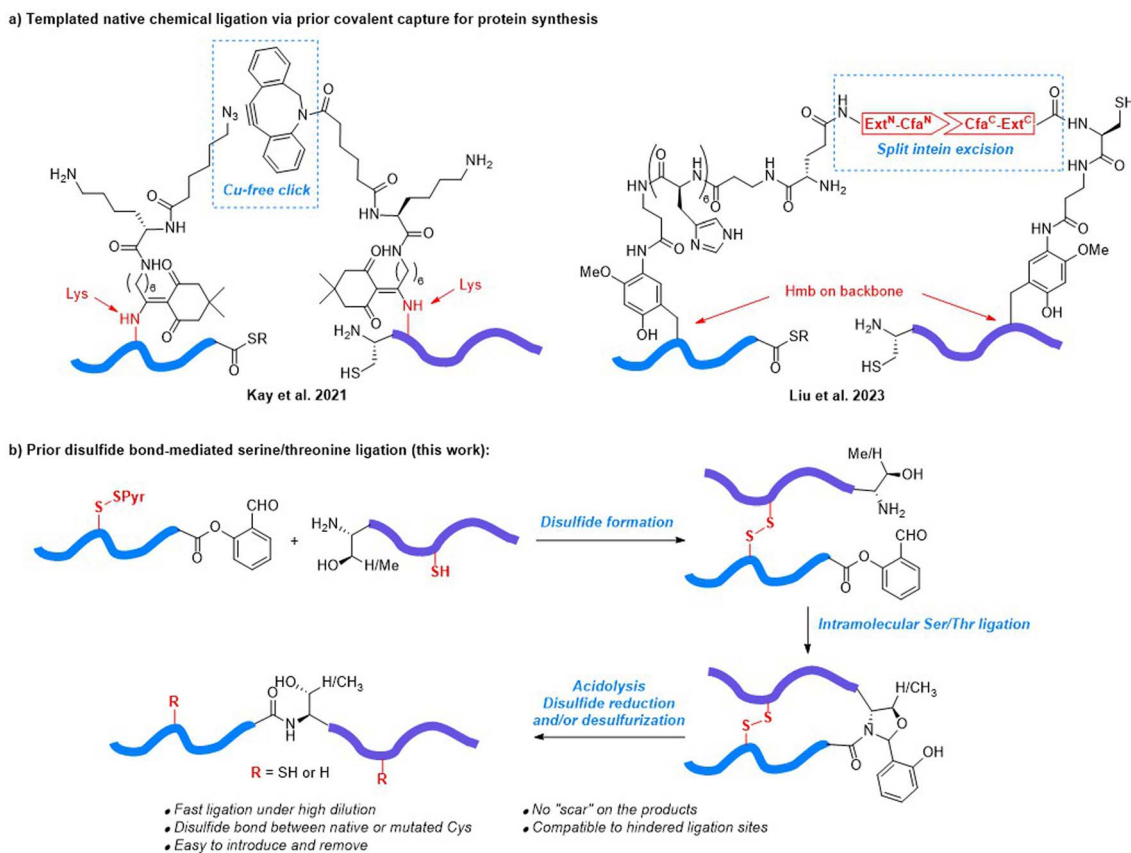


Fig. 1 Reported templated native chemical ligation strategies and the design of prior disulfide bond-mediated serine/threonine ligation. (a) Templated native chemical ligation via prior covalent linkage in protein synthesis. (b) Prior disulfide bond-mediated serine/threonine ligation (this work).

Disulfide is the native conformation restricting element in proteins and peptide hormones. The intrachain and interchain disulfide networks play key roles in the stabilization of secondary and tertiary structures. We hypothesized that the disulfide linkage between native or mutated Cys residues could be leveraged to facilitate prior capture-mediated STL at low concentrations, without the need for incorporating complex sidechain/backbone modifications. In this prior disulfide bond-mediated Ser/Thr ligation (PD-STL), a peptide C-terminal salicylaldehyde (SAL) ester and a peptide with an N-terminal serine or threonine residue, were first joined together by a disulfide linkage, followed by an intramolecular STL to form the ligated product (Fig. 1b). The non-reducing conditions of STL perfectly fit the demand for disulfide linkage, and the available disulfide formation⁴⁸ and desulfurization methods^{49–51} allow the disulfide template installation with site flexibility. Herein, we report the development of PD-STL and its application in the synthesis of the N-terminal cytoplasmic domain of caveolin-1 (Cav-1) and the Tyr14 phosphorylated form.

Results and discussion

As a simple but effective model to test the effect of PD-STL, we chose a peptide with a C-terminal proline for ligation. Peptides with C-terminal proline thioesters are known to be difficult to

ligate to N-terminal cysteine peptides by native chemical ligation.⁵² Moreover, Ser/Thr ligation barely proceeds with peptides carrying C-terminal proline SAL esters at low concentrations.⁵³ Thus, we started our model study using C-terminal proline SAL esters to demonstrate the rate-increasing effect of disulfide linkage. As a control experiment, peptides **1a** and **2a** were dissolved in pyridine/AcOH buffer to trigger the ligation process with a final concentration of 0.25 mM. As expected, it took a long time (3 days) to fully convert to the ligation intermediate, based on liquid chromatography-mass spectrometry (LC-MS) analysis (Table 1, entry 1). Increasing the ligation concentration to 10 mM led to faster conversion, but 6 hours were still needed for the reaction to be completed (Table 1, entry 3). Similar results were observed using peptide **2b** as the N-terminal Thr peptide, where the location of Cys was moved away from the ligation site (Table 1, entries 2 and 4).

Alternatively, peptide **1b** with a side chain of cysteine temporarily protected by *S*-pyridine (SPyr) was synthesized, where Cys(SPyr) functions as an effective precursor for hetero-disulfide bond formation.⁵⁴ As a result, peptides **1b** (1.0 equiv.) and **2a** (1.5 equiv.) efficiently formed a heterodimer *via* a disulfide bond when dissolved in MeOH containing 0.1% TFA with a concentration of 10 mM. Encouragingly, after the formation of the disulfide bond, the intramolecular STL proceeded smoothly at low concentration (0.25 mM), reaching completion within 12 minutes



Table 1 Rate improvement of STL with proline at the ligation site by introducing interchain disulfide linkage^{ab}

Entry	SAL esters	Thr peptides	Ligation conc.	Time to finish
1	Fmoc-GPARGP-OSAL ester 1a	TCLVGSQKAPSEVPTAGF 2a	0.25 mM	>3 days
2	Fmoc-GPARGP-OSAL ester 1a	TALVGSQKCPSEVPTAGF 2b	0.25 mM	>3 days
3	Fmoc-GPARGP-OSAL ester 1a	TCLVGSQKAPSEVPTAGF 2a	10 mM	6 h
4	Fmoc-GPARGP-OSAL ester 1a	TALVGSQKCPSEVPTAGF 2b	10 mM	3 h
5	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TCLVGSQKAPSEVPTAGF 2a	0.25 mM	<12 min
6	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGSQKCPSEVPTAGF 2b	0.25 mM	<78 min

^a For prior disulfide bond-mediated STL, peptides **1b** (1.0 equiv.) and **2a/2b** (1.5 equiv.) were dissolved in MeOH containing 0.1% TFA with a concentration of 10 mM (peptide SAL esters) to form disulfide. After removing 2-thiopyridone, the crude product together with excess peptides **2a/2b** was dissolved in pyridine/HOAc 1/1 v/v for STL. ^b For STL without disulfide bond capture, peptides **1a** (1.0 equiv.) and **2a/2b** were also dissolved in MeOH containing 0.1% TFA for 4 hours to mimic the prior capture cases, followed by STL in pyridine/HOAc 1/1 v/v buffer.

(Table 1, entry 5). No adverse effect caused by excess **2a** like the competing intermolecular STL was observed. In the case of the PD-STL of peptide **2b**, the ligation rate remained faster (<78 min) compared to that of the control experiments without prior disulfide capture (Table 1, entry 6). The accelerating effect was less significant, because a larger and more flexible cyclic structure than that in the **1b/2a** case was involved. This performance was in accordance with our former observations that STL generally showed good reaction kinetics in peptide cyclization.^{55–58}

Subsequently, we explored how the performance of PD-STL was influenced by the locations of the two designated Cys residues, *i.e.*, the size of the macrocycles formed in the ligation (Table 2). When the distance between Cys and the C-terminus of the peptide SAL ester was too small (Table 2, entries 2 and 3), the disulfide bond-assisted ligation was slow, which can be attributed to the strained reaction conformation. However, the ligation still proceeded more rapidly at 0.25 mM concentration than the STL without disulfide bond assistance. When the cysteine was located one amino acid further from the C-terminus of the peptide SAL ester (Table 2, entries 4–12), the ligation rate improved dramatically. The ligation was more sensitive to the location of the Cys residue in the peptide SAL ester, since **1d/2a** ligation was much faster than that in the **1c/2c**

case, though the same 17-membered ring was formed in the ligation intermediate. Furthermore, the disulfide bond-assisted STL rate was the highest when the distance between the two designated cysteines was 3 or 4 amino acids (forming 17-membered and 20-membered rings, respectively) (Table 2, entries 4 and 5). All disulfide bond-assisted ligations exhibited much higher rates than the standard STL in control experiments. PD-STL can successfully enhance ligation performance, though the Cys location can influence the ligation rate.

With the exciting results obtained, we applied the PD-STL to peptides with varied sequences to confirm its generality. As shown in Table 3, all PD-STL reactions were completed within 5 to 305 minutes at a low concentration of 0.25 mM. All those peptides resulted in rapid and high-yielding ligations (29–63% isolated yields) under high dilution conditions (0.25 mM), even for highly sterically hindered amino acids (*e.g.*, Ile, Val, and Pro, Table 3, entries 3–5 and 7) at the C-terminus, which typically exhibited lower ligation rates and yields in both NCL and STL. These results demonstrated the capability of PD-STL.

Synthesis of CAV-1 (2-104)

To showcase the effectiveness of this disulfide bond-assisted Ser/Thr ligation for chemical protein synthesis, we

Table 2 The impact of the site of cysteine on the rate of PD-STL^a

Entry	SAL esters	Thr peptides	No. of A.A. in between	Ligation time
1	Fmoc-GPARGP-OSAL ester 1a	TCLVGSQKAPSEVPTAGF 2a	—	>3 days
2	Fmoc-GPRGC(<i>SPyr</i>)P-OSAL ester 1c	TCLVGSQKAPSEVPTAGF 2a	2	>12 h
3	Fmoc-GPRGC(<i>SPyr</i>)P-OSAL ester 1c	TACVGSQKAPSEVPTAGF 2c	3	>12 h
4	Fmoc-GPRC(<i>SPyr</i>)GP-OSAL ester 1d	TCLVGSQKAPSEVPTAGF 2a	3	≤12 min
5	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TCLVGSQKAPSEVPTAGF 2a	4	≤12 min
6	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TACVGSQKAPSEVPTAGF 2c	5	≤63 min
7	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALCGSQKAPSEVPTAGF 2d	6	≤50 min
8	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGSQKAPSEVPTAGF 2e	7	~40 min
9	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGCQKAPSEVPTAGF 2f	8	≤65 min
10	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGSCKAPSEVPTAGF 2g	9	≤52 min
11	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGSQCAPSEVPTAGF 2h	10	≤75 min
12	Fmoc-GPC(<i>SPyr</i>)RGP-OSAL ester 1b	TALVGSQKCPSEVPTAGF 2b	11	≤78 min

^a The SAL esters **1b–1d** (1.0 equiv.) and peptides **2a–2h** (1.5 equiv.) were dissolved in MeOH containing 0.1% TFA with a concentration of 10 mM (SAL ester) to form disulfide. After removing 2-thiopyridone, the crude peptides together with excess peptides **2a–2h** were dissolved in pyridine/HOAc 1/1 v/v with a concentration of 0.25 mM for STL. The conversion was monitored at different time points by UPLC-MS.



Table 3 Disulfide-bond assisted ligation between different peptides^a

Entry	N-terminal peptide	C-terminal peptide	Ligation time	Isolated ^b yield
1	LEQLKC(<i>SPyr</i>)/GF-OSAL ester 1e	SGGCGLFDVVKG 2i	5 min	30%
2	FRKSGFC(<i>SPyr</i>)/GT-OSAL ester 1f	TCRSYFPGSTYTG 2j	23 min	50%
3	GMTHGLIC(<i>SPyr</i>)/GI-OSAL ester 1g	TGKCQRM 2k	50 min	29%
4	PTIPC(<i>SPyr</i>)/KARGI-OSAL ester 1h	TCHYIPRPKPR 2l	44 min	57%
5	IPAC(<i>SPyr</i>)/IAGV-OSAL ester 1i	TKKCFLGGLMKA 2m	88 min	52%
6	GRKSDC(<i>SPyr</i>)/FPA-OSAL ester 1j	SRKCFLI 2n	23 min	63%
7	GSKKPVIHYC(<i>SPyr</i>)/NRP-OSAL ester 1k	SGFCAFLKSPS 2o	305 min	49%

^a The SAL esters **1e–1k** (1.0 equiv.) and peptides **2i–2o** (1.3–2.0 equiv.) were dissolved in MeCN/H₂O 1/1 v/v containing 0.1% TFA with a concentration of 10 mM (SAL ester) to form disulfide. After lyophilization and 2-thiopyridone removal *via* ether wash, the crude peptides together with excess peptides **2i–2o** were dissolved in pyridine/HOAc 1/1 v/v with a concentration of 0.25 mM for STL. The conversion was monitored at different time points by UPLC-MS. ^b The ligation intermediate was treated with TFA/H₂O/TIPS 95/2.5/2.5 v/v/v for acidolysis and 20 mM TCEP in MeCN/H₂O 1/1 v/v for disulfide reduction, followed by HPLC purification.

synthesized the N-terminal cytoplasmic domain (2-104) of caveolin-1 (Cav-1). Cav-1 is a V-shaped membrane protein that plays multiple roles in cellular processes including signal transduction, metabolism, endocytosis (*via* caveola formation), and differentiation.^{59,60} Cav-1 is also involved in several pathogenic processes like kidney diseases and cancer.^{61–63} Cav-1 bears various post-translational modifications (acetylation, phosphorylation, palmitoylation, *etc.*),^{59,60} and the Tyr14 phosphorylation located in the N-terminal cytoplasmic domain has been implicated in numerous biological functions, including focal adhesion, cancer biology,^{64–66} endothelial cell signalling in sepsis-induced lung injury,⁶⁷ regulation of mechanotransduction,^{68–70} and insulin signaling.⁷¹ Chemical synthesis of Tyr14 phosphorylated or non-phosphorylated molecular probes will be important for biological studies. Full length Cav-1 bearing triple *S*-palmitoylations has been successfully synthesized by Hojo, *via* sequential thioester ligations in DMSO assisted by *i*Noc (4-pyridylmethoxycarbonyl) protected *O*-isopeptides and Lys residues.⁷² The chemical synthesis of a phosphorylated Cav-1 N-terminal cytoplasmic domain has not been reported.

Our synthetic plan is shown in Fig. 2. Since Cav-1 (2-104) does not contain a native Cys residue, we mutated Ala31 and Ala44 to Cys to facilitate disulfide linkage formation. Ala87 was also mutated to Cys to install a reducible solubilizing tag (RST).⁷³ An extra Lys residue was added to the C-terminus to install a biotin, which would be used for probe enrichment by streptavidin beads in future chemical biology study. For protein assembly, we devised an N-to-C sequential ligation process, which involved the Leu36–Ser37 juncture as the first ligation site (PD-STL) and the Gly77–Thr78 juncture as the second ligation site (conventional STL).

The peptide fragments **3a**, **4** and **7** were readily synthesized *via* the standard Fmoc-SPPS. The C-terminus of **4** was modified to the peptide SAL^{off} ester state⁷⁴ to avoid head-to-tail cyclization. As shown in Fig. 2a, fragments **3a** and **4** were dissolved in a 1 : 1 ratio in MeCN/H₂O (3/1, v/v, containing 0.1% TFA) at a concentration of 5 mM to form the disulfide linkage. During the disulfide formation, peptide fragments **3a** and **4** and product **5** showed good solubility in the solvent system we used. After achieving full conversion (16 h), the solvent was removed through freeze-drying to yield the crude disulfide-linked

intermediate **5**. As the precursor of PD-STL, **5a** demonstrated good solubility in the ligation buffer at a concentration of 1 mM (collidine/AcOH 1/2 mol/mol containing 50% DMSO), where hydrophilic fragment **c3a** acted as a solubilizing tag to improve the solubility of **4**. After stirring at 37 °C overnight, the PD-STL achieved near quantitative conversion, with negligible hydrolysis of the SAL ester observed (Fig. S84[†]). The ligation intermediate (*N*-peptidyl *N,O*-benzylidene acetal) was precipitated using diethyl ether and directly subjected to the acidolysis cocktail (TFA/H₂O/dimethylsulfide 95/5/5 v/v/v). After 30 minutes, pyruvic acid was added to facilitate the SAL^{off}-to-SAL^{on} switch, and the product **6a** was isolated in 34% yield by reversed-phase preparative HPLC. As a comparison, we also tested the conventional STL between peptides **3'** and **4'** without Ala-to-Cys mutation and prior disulfide capture (refer to the ESI for details[†]). After overnight reaction at a concentration of 5 mM, only a minimal amount of the ligation intermediate was detected (Fig. S80[†]), largely due to the poor solubility of peptide **4'**. When the concentration was reduced to 1 mM, even worse ligation performance was observed, with the formation of a tiny amount of ligation intermediate and numerous side products after overnight reaction (Fig. S81[†]). This result further demonstrates the rate-accelerating capability of the PD-STL strategy in the synthesis of poorly soluble peptides.

In Hojo's synthesis, two *O*-isopeptides were used to overcome the hydrophobicity of fragment Thr78–Ser104.⁷² In our case, since STL is conducted under non-reducing conditions, we applied our reducible solubilizing tag (RST) strategy⁷³ to incorporate a His₈ tag into this sequence at the Cys87 mutated from Ala *via* disulfide linkage. The RST modified fragment **7** was synthesized in 30% yield and subjected to the final STL with **6a**. In this step, through condition screening, we found that using 2-picoline instead of collidine in the ligation buffer gave much better results by suppressing the hydrolysis of **6a**. After overnight reaction at a concentration of 2.5 mM at 37 °C, the ligation intermediate was precipitated using diethyl ether. After acidolysis by TFA/H₂O/TIPS treatment, product **8a** overlapped with **7** as monitored by UPLC-MS (Fig. S87[†]), which hampered efficient separation. To tackle this problem, the ligation intermediate was purified by RP-HPLC with an isolated yield of 17% (Fig. 2B and S88[†]). After acidolysis for 30 minutes, the peptide **8a** was directly subjected to disulfide reduction and



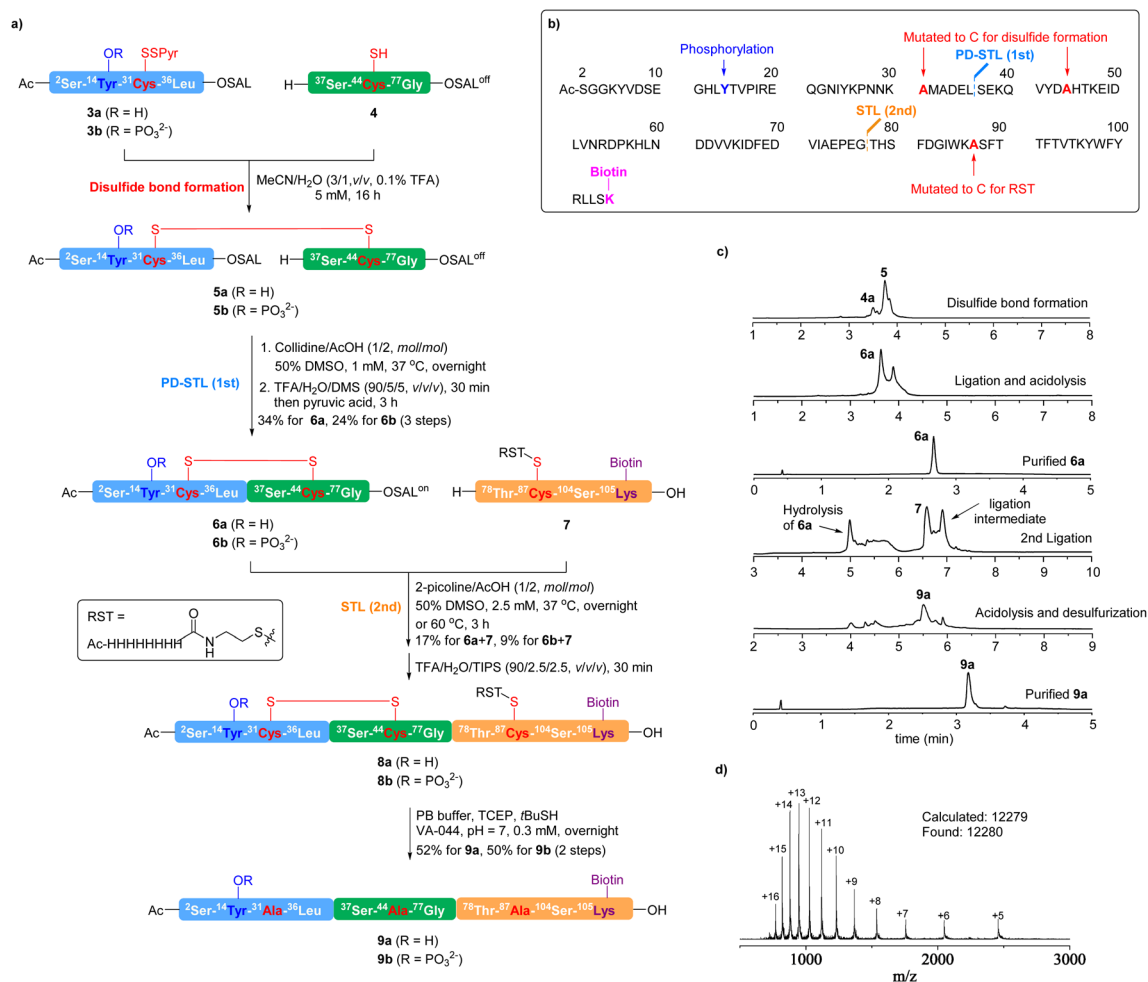


Fig. 2 Total chemical synthesis of caveolin-1 (2-104) and the Tyr14 phosphorylated form facilitated by PD-STL. (a) Synthesis of caveolin-1 (2-104) and the Y14 phosphorylated variant. (b) Sequence of Cav-1 (2-104) and the synthetic plan. (c) LC traces of steps in the synthesis of Cav-1 (2-104) **9a**. (d) ESI-MS of **9a**.

desulfurization in a phosphate buffer at pH 7 containing TCEP, *t*BuSH, and VA-044. After overnight reaction, the Cav-1 (2-104) product **9a** was isolated in 52% yield over two steps.

Following the same protocols, Tyr14 phosphorylated Cav-1 (2-104) was also synthesized, where the phosphorylated fragment **3b** was used instead of **3a**. The PD-STL between **3b** and **4** showed comparable performance to the former case, giving rise to **6b** in 24% yield. In contrast to the former case, the STL between **6b** and **7** was less effective in picoline/HOAc/DMSO buffer, leading to serious hydrolysis of the SAL ester and only a tiny amount of the ligation intermediate. To address this issue, a higher reaction temperature (60 °C) was employed, allowing **6b** to be consumed within 3 hours with an acceptable level of hydrolysis, affording the ligation intermediate in 9% isolated yield. After acidolysis, disulfide reduction and desulfurization, the product **9b** was isolated in 50% yield.

Conclusions

Prior capture-mediated ligation, in other words, converting intermolecular reactions into intramolecular reactions, can

effectively proceed regardless of the concentration, providing a promising strategy for large molecule synthesis. The key to this strategy is identifying an efficient capture reaction that can proceed at low concentrations and under a wide range of conditions. In this study, we integrated disulfide bond-forming chemistry with Ser/Thr ligation to develop prior disulfide bond-mediated Ser/Thr ligation (PD-STL). To this end, a peptide salicylaldehyde ester with a Cys-S-pyridine residue first reacts with another peptide featuring an N-terminal Ser/Thr and an internal Cys residue. A hetero-disulfide bond is readily formed, and the disulfide linkage is stable under non-reducing STL conditions. Subsequently, an intramolecular STL takes place. We demonstrated its effectiveness at low concentrations (*e.g.*, 0.25 mM) even for the C-terminal proline at the ligation site. Furthermore, we applied PD-STL for the syntheses of the N-terminal cytoplasmic domain (2-104) of caveolin-1 and its Tyr14 phosphorylated form. Compared with the reported templated native chemical ligation strategies which required peptide sidechain or backbone modification, PD-STL uses the native or mutated cysteine residues, thus simplifying the operation. We anticipate that this strategy will inspire the



development of prior capture-based peptide ligation for challenging protein synthesis.

Data availability

The data supporting this article ("Prior disulfide bond-mediated Ser/Thr ligation") have been included as part of the ESI.†

Author contributions

X. L. conceived and supervised the project. H. L. and H. Y. C. designed the methodology and model study. J. L. and P. S. conducted the peptide synthesis, peptide ligation and protein synthesis. X. L. and P. S. wrote the manuscript. All the authors reviewed the manuscript.

Conflicts of interest

There are no conflicts to declare.

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