JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

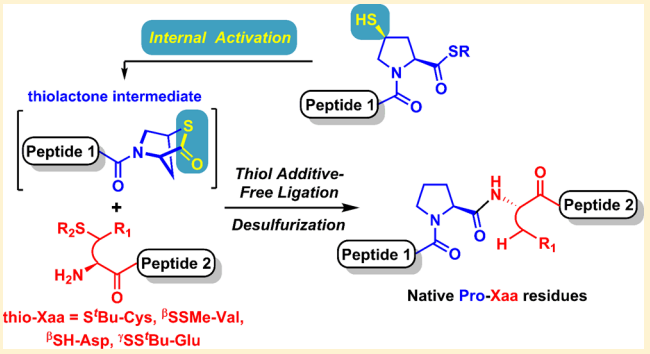
Internal Activation of Peptidyl Prolyl Thioesters in Native Chemical Ligation

Yue Gui,[†] Lingqi Qiu,[†] Yaohao Li,[†] Hongxing Li,[†] and Suwei Dong*[,†]

[†]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xueyuan Road, Haidian District, Beijing 100191, China

Supporting Information

ABSTRACT: Prolyl thioesters have shown significantly lower reactivities in native chemical ligation (NCL) in comparison to that of the alanyl thioester. This report describes a mild and efficient internal activation protocol of peptidyl prolyl thioesters in NCL without using any thiol-based additives, where the introduction of a 4-mercaptan substituent on the C-terminal proline significantly improves the reactivity of prolyl thioesters via the formation of a bicyclic thiolactone intermediate. The kinetic data indicate that the reaction rate is comparable to that of the reported data of alanyl thioesters, and the mechanistic studies suggest that the ligation of two peptide segments proceeds through an NCL-like pathway instead of a direct aminolysis, which ensures the chemoselectivity and compatibility of various amino acid side chains. This 4-mercaptoprolyl thioester-based protocol also allows an efficient one-pot ligation−desulfurization procedure. The utility of this method has been further demonstrated in the synthesis of a proline-rich region of Wilms tumor protein 1.



INTRODUCTION

Protein-based biomacromolecules have been broadly studied for their essential roles in organisms. Although biochemical methods are still predominantly used for protein production,[1] chemical peptide synthesis has shown its irreplaceability among several research areas, including D-protein-based mirror image phage display,[2] racemic protein crystallography,[3] and syntheses of homogeneously modified proteins, especially glycoproteins.[4] Over the past century, the requirements of diverse natural and unnatural peptides and derivatives in biochemical research and drug design have prompted the development of a series of new synthetic methods for polypeptides, including solid-phase peptide synthesis (SPPS) pioneered by Merrifield in the 1960s[5] as well as native chemical ligation (NCL) reported by Kent and coworkers in 1994 (Figure 1).[6] These advancements in turn accelerated the development of related research fields. As a milestone advance, in conjunction with the broadly scoped metal-free desulfurization (MFD),[7] NCL is not only one of the most powerful methods in the field of protein chemical synthesis, but it has inspired chemists seeking new chemical methods and tools to study biological problems. A number of ligation methods were thus invented, including auxiliary-based ligation methods,[8] Staudinger ligation,[9] α-ketoacid-hydroxylamine (KAHA) ligation,[10] seleno-amino-acids based ligation,[11] serine/threonine ligation (STL),[12] and peptide hydrazides-based ligation,[13] among others. However, nature always poses new challenges to chemists. For instance, the synthesis of highly diverse peptide sequences requires various possible ligation sites in the events where two peptide segments join together selectively. Under the conditions of metal-free desulfurization, the possible ligation sites have been largely expanded, and a number of thio-amino acids have been utilized as the N-termini in NCL,[14] even the secondary amine proline.[15]

For the requisite N-terminal peptide segment in NCL, numerous endeavors have been made to obtain stable and easy to handle peptidyl thioesters or surrogates without racemizing the corresponding C-terminal amino acid residues, particularly the fragments derived from the routinely applied Fmoc-SPPS. The developments of several epimerization-free protocols were thus investigated, including the usage of a stable alkyl thioester[6,16] and Dawson's resin,[17] as well as Liu's acyl

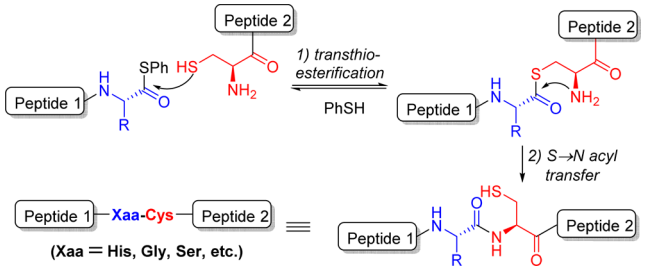


Figure 1. Native chemical ligation (NCL).

Received: February 2, 2016
Published: March 16, 2016

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

hydrazide-based protocol.[18] Notably, these procedures all require exogenous thiol additives, such as 4-mercaptophenylacetic acid (MPAA)[19] to accelerate the reaction rate and ensure satisfactory ligation efficiency. Application of MPAA expanded the scope of the C-termini of peptidyl thioesters to almost all native amino acids, including a number of previously problematic residues,[20] such as glutamate,[3b] valine,[14g] and isoleucine.[3c] However, the inherent radical quenching property of aryl thiols was incompatible with the radical-based desulfurization method, which led to a required operation of removing such species between the ligation and dethiylation steps, compromising the overall efficiency of the powerful ligation−desulfurization strategy. Chemical synthesis of large polypeptides and complex proteins in a more practical and efficient manner calls for solutions to address this issue.

To circumvent epimerization at the ligation sites when preparing the peptidyl thioesters, an alternative strategy is the activation of peptide fragments at their C-terminal proline or glycine sites.[21] Drawing inspiration from the direct condensation method for peptidyl fragments coupling, it would be ideal to conduct NCL at the proline or glycine sites because the corresponding thioesters (or other derivatives) could be prepared using common coupling reagents without the concern of epimerization and not require strictly controlled reaction conditions.[22] While glycinyl thioesters have been frequently used in protein synthesis,[4h,15b,23] prolyl thioesters have been suggested to be extremely unreactive under typical NCL conditions (Figure 2a),[24] which significantly limited the use of peptidyl prolyl thioesters in the chemical synthesis of polypeptides and proteins. Aiming to exploit the reactivity of C-terminal prolyl esters under NCL conditions, a number of research groups attempted to tackle this problem. For instance, Danishefsky et al. noticed that the *p*-nitrophenyl ester of proline was able to react with cysteinyl peptides in weak acidic buffers, in spite of the competing hydrolysis.[25] In 2011, Durek et al. reported that the use of prolyl selenoesters enabled rapid ligation with cysteine-containing peptides in the presence of a selenol catalyst under mild buffered conditions, accompanied by a competing side reaction that forms unreactive thioesters on unprotected nonligation site cysteines (Figure 2b).[26] More recently, the Otaka group developed a protocol where the prolyl thioesters were activated in suitable ligation conditions, which required the addition of MPAA (200 mM) and elevated temperature (50 °C) (Figure 2b).[27] Despite these advancements, a more effective and mild, thiol-additive-free proline ligation method would be desirable in the preparation and studies of various peptide sequences and proteins, including the ones containing proline-rich regions (PRRs).[28] Herein, we report the development of such an optimized method based on a design of proline-derived active intermediate.

RESULTS AND DISCUSSION

Synthesis Design. In 2011, on the basis of a systematic study, Kent et al. proposed that the n → π^* orbital interaction[29] and steric hindrance of the *N*-carbonyl of proline may reduce the electrophilicity of the prolyl thioester carbonyl, thus resulting in an extremely low reactivity (Figure 2a).[24] We envisioned that a heterobicyclo[2.2.1]septane structure **2** (Figure 2c) may preclude the *N*-carbonyl oxygen/thioester carbonyl n → π^* interaction and form a strained ring system, both of which may promote the intermolecular transthioesterification, and the subsequent irreversible S → *N* acyl transfer would afford a ligated peptide containing the thio-Pro-Cys segment. The

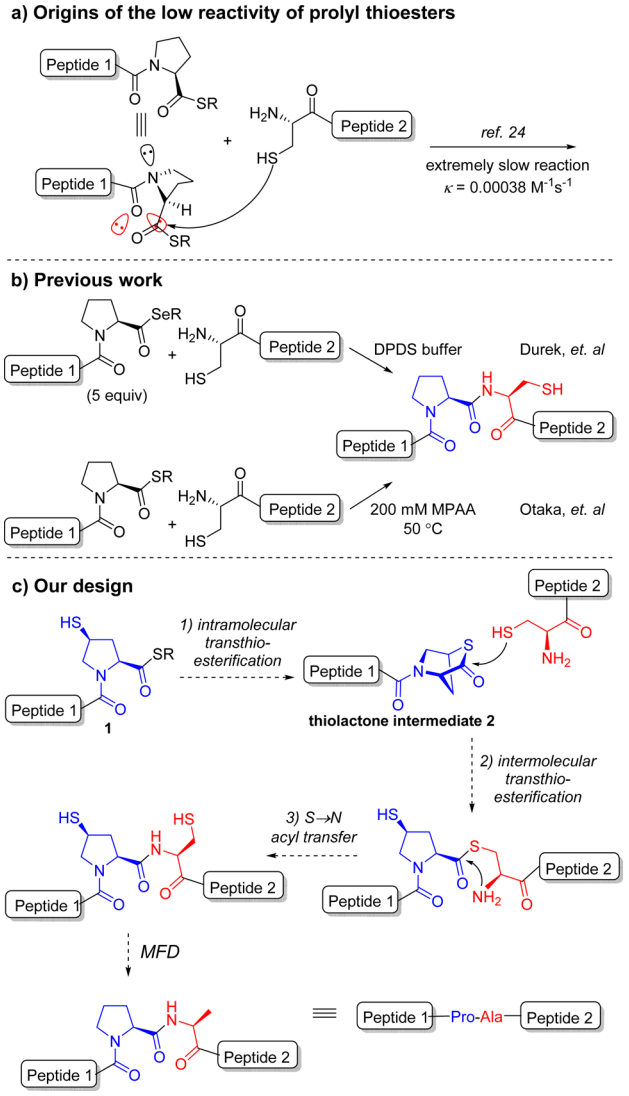


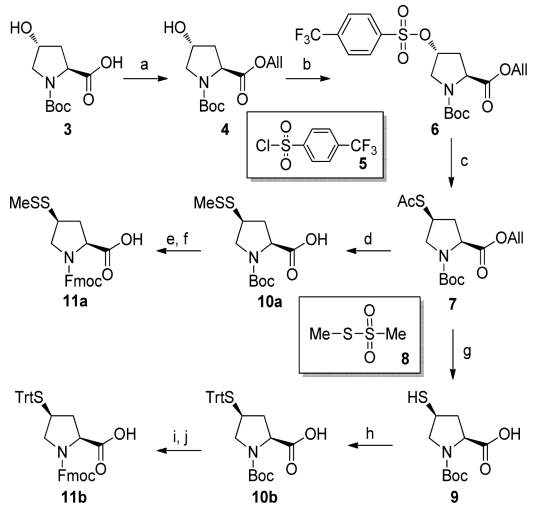
Figure 2. Prolyl thioesters vs thioprolyl thioesters in native chemical ligation.

resulting peptides may be converted to the native sequences with Pro-Ala residues using the desulfurization protocol. Considering the potential lability of **2** during the preparation of the corresponding peptides, thio-prolyl thioester **1** was designed as the precursor to **2**.

Synthesis of Unnatural Amino Acids and Peptide Segments. We initiated our investigation by synthesizing the thio-proline derivatives **11** (Scheme 1). Starting from the commercially available *N*-Boc-4-hydroxyl proline (**3**), selective allylation of the carboxylate afforded compound **4**,[30] followed by the activation of hydroxyl with 4-(trifluoromethyl)benzene-1-sulfonyl chloride (**5**)[31] to produce sulfonate **6**. The thio substituent was introduced in *cis*-configuration to the carboxylate using potassium thioacetate[32] to form compound **7**, ensuring the formation of the requisite cyclic bridge in the ligation reactions. Hydrolysis of the thioacetate and carboxylate was accomplished simultaneously using aqueous lithium hydroxide, followed by protection of the free thiol with methylthio group in the same reaction flask, affording carboxylic acid **10a**. The corresponding Fmoc derivative **11a** was generated from **10a** in two steps and was ready for use in Fmoc-based SPPS.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

Scheme 1. Synthesis of Thioproline Derivatives 11a and 11b[a]



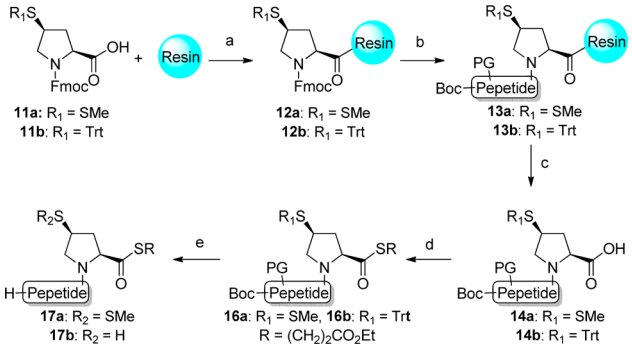
[a]Reaction conditions: (a) 3-Bromo-1-propene, DIEA, DMF, rt, 14 h, 93%. (b) **5**, TEA, DMAP, DCM, rt, 2 h, 95%. (c) KSAc, DMF, 40 °C, 3 h, 96%. (d) LiOH·H_2O, THF/H_2O (1:1), rt, 6 h; then **8**, 2 h, 71%. (e) DCM/TFA/TES (8:2:1), rt, 3 h. (f) FmocOSu, TEA, MeCN, rt, 6 h, 87% over two steps. (g) LiOH·H_2O, THF/H_2O (1:1), rt, 7 h, 99%. (h) TrtCl, DCM, rt, 19 h, 91%. (i) 4 M HCl in 1,4-dioxane, rt, 1 h. (j) FmocCl, TEA, DCM, rt, 15 h, 97% over two steps. DIEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TEA = triethylamine, DMAP = 4-dimethylaminopyridine, DCM = dichloromethane, THF = tetrahydrofuran, TFA = trifluoroacetic acid, TES = triethylsilane. Boc = *tert*-butyloxycarbonyl, All = propenyl, Trt = triphenylmethyl, Fmoc = 9-fluorenylmethoxycarbonyl.

In a similar manner, trityl-protected thio-proline derivative **11b** was synthesized, where carboxylic acid **9** with a free thiol group was isolated after hydrolysis of **7** and was further protected with a trityl group to provide **10b**. The final *N*-protecting-group manipulation afforded Fmoc amino acid **11b** in decent yield, thus providing an alternative 4-mercaptan-proline derivative for further evaluation of peptide synthesis and ligation conditions.

Although it was plausible to incorporate the thio-proline moiety via direct condensation with side-chain protected peptide fragment,[33] we decided to preload the proline derivatives on resin to take full advantage of the convenience of SPPS and to circumvent any possible racemization in the preparation of peptidyl thioesters. Accordingly, Fmoc-amino acids **11** were loaded on 2-chlorotrityl chloride resin in DCM in the presence of DIEA (Scheme 2). Resulting preloaded resins **12** were further employed in SPPS under Fmoc-based conditions,[34] followed by cleavage from the resin using a cocktail containing DCM/TFE/AcOH (3:1:1), which afforded peptide **14** as a peptidyl acid with N-terminal and side-chain protecting groups untouched. The C-terminal carboxylic acid was then coupled with ethyl 3-mercaptopropionate (**15**) to generate prolyl thioesters **16**.

In these coupling reactions, several activating reagents, including EDC (Table 1, entry 1), PyBOP (entry 2), and HATU (entry 3), were evaluated, and the HATU-mediated condition was found to be optimal.[35] After global deprotection using TFA/TIS/H_2O (95:2.5:2.5), purification by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) provided the desired peptidyl thioesters **17** with either a methylthio-protected thiol on the C-terminal proline (**17a**) or a free thiol in the case of peptide **17b**. In contrast, the east-side peptides containing Cys or thio-amino acid derivatives at the N-termini were synthesized following the Fmoc-based SPPS protocol and deprotection procedures.[36]

Scheme 2. Preparation of Peptidyl Thioprolyl Thioesters[a]



[a]Reaction conditions: (a) 2-Chlorotritylchloride resin, DIEA, DCM; (b) Fmoc-based SPPS; (c) DCM/TFE/AcOH (3:1:1); (d) Ethyl 3-mercaptopropionate (**15**), HATU, DIEA, DCM; (e) TFA/TIS/H_2O (95:2.5:2.5). TFE = trifluoroethanol, HATU = 1-[bis(dimethylamino)-methyl-ene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluoro-phosphate, TFA = trifluoroacetic acid, TIS = triisopropylsilane, PG = protecting groups.

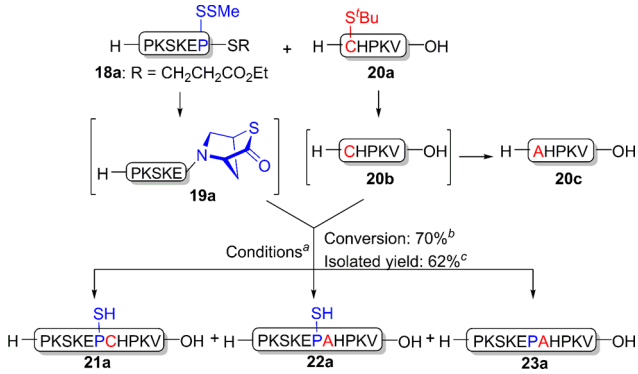
Table 1. Optimization of Thioester Formation

entry	reaction conditions	time (h)	conversion[a]
1	1.2 equiv EDC, DCM	3	<5%
2	1.2 equiv PyBOP, 1.1 equiv DIEA, DCM	24	>95%
3	1.2 equiv HATU, 2.0 equiv DIEA, DCM	1	>95%

[a]Estimated conversions based on calculations of the analytical HPLC integrations of free peptides obtained from the global deprotection of **14b** and **16b**. EDC = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, PyBOP = (benzotriazol-1-yloxy)-tripyrrolidino-phosphonium hexafluorophosphate.

Experimental Evaluation and Optimization. We chose two peptide segments **18a** and **20a** (Scheme 3) to investigate

Scheme 3. Ligation between 18a and 20a



[a]Reaction conditions: **18a** (3 mM), 1.0 equiv **20a**, 400 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl), room temperature, 8 h. [b]Estimated conversion based on calculations of the analytical HPLC integrations of peptides **20b** and **20c**, and products **21a**, **22a**, and **23a**. [c]Isolated yield after HPLC purification. TCEP = Tris(2-carboxyethyl)phosphine.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

suitable reaction conditions, where the ligated peptide product would resemble the sequence of a proline-rich region in cornifin-B protein.[37] With the requisite peptide segments in hand, we studied the ligation reaction under a typical NCL condition (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0, room temperature),[24] and the reaction progress was monitored using HPLC-MS. When equal amounts of peptides **18a** and **20a** were mixed in buffer, it was observed that a new peak formed almost immediately after the addition of buffer (Figure 3), and analysis

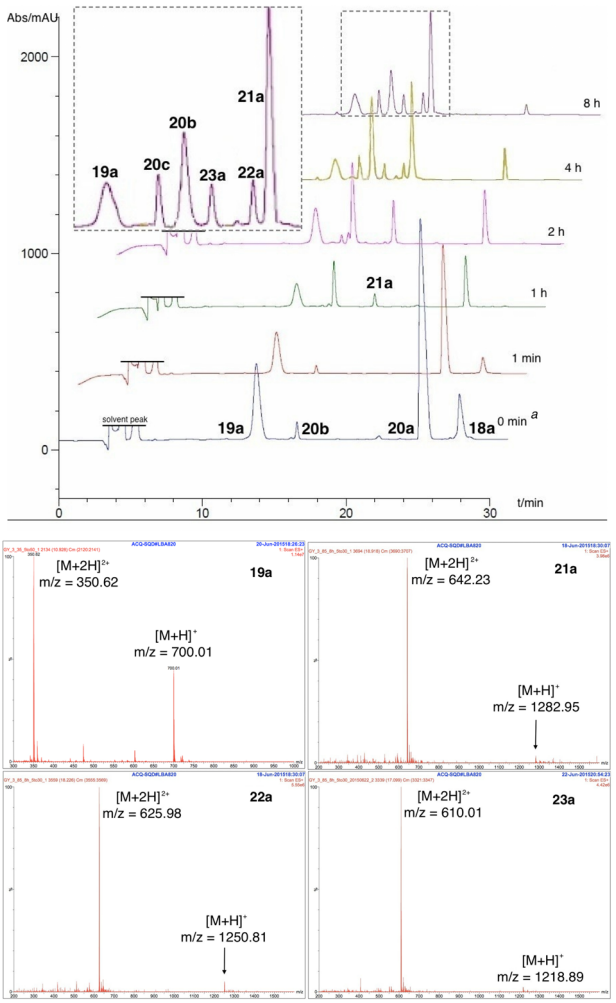


Figure 3. UV traces and ESI-MS data from HPLC-MS analysis of ligation between **18a** and **20a**. Reaction conditions: **18a** (3 mM), 1.0 equiv of **20a**, 400 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), room temperature. UV trace labeled a is from LC-MS analysis of the reaction quenched right after the addition of buffer.

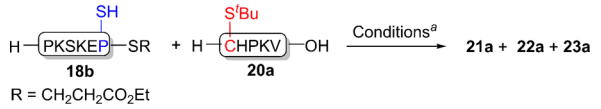
of the mass spectra suggested thiolactone-containing intermediate **19a** as we originally designed. This observation indicated that a rapid intramolecular transthioesterification favored the formation of such a bicyclic structure in the reaction buffer.[36]

As the reaction proceeded, the desired ligation product **21a** was observed, along with the formation of products **22a** and **23a**, which were desulfurized peptides as indicated by the mass spectra. On the basis of the integrations of UV signals of reactive starting peptide segment H−CHPKV−OH (**20b**), desulfurized east-side peptide H−AHPKV−OH (**20c**), and all ligation products (**21a**−**23a**), the conversion of this reaction after 8 h was calculated as ca. 70%. The desulfurized products may have resulted from a TCEP-promoted reaction involving a phosphoranyl radical-based mechanism,[7,38,39] which was supported by the fact that formation of side products was suppressed when using a phosphine-free DTT-buffer.[36,40] Our observation was also in accord with a previous report where prolonged ligation time led to desulfurized product under NCL conditions.[26] In our case, the extraneous thiol groups needed to be eventually removed to reveal the native amino acid residues; thus, desulfurization occurring in the ligation step was inconsequential. Regardless, all products could be either isolated separately using prep-HPLC or as a mixture subjected to the next step.

To probe the desulfurization process further, hoping to obtain the product with native Pro-Cys segment, we elucidated the structure of the monodesulfurized product by comparing its HPLC retention time against two authentic samples, H−PKSKEP(SH)AHPKV−OH (**s22a**) and H−PKSKEPCHPKV−OH (**s22a′**).[36] The co-injection experiments unambiguously assigned that under the reaction conditions the −SH group of Cys was reduced prior to the −SH on Pro, which suggested that it would be difficult to remove selectively the thiol on Pro and leave Cys untouched.

To improve the ligation efficiency, alternative peptide **18b** (Scheme 4) and several reaction conditions were evaluated.[36]

Scheme 4. Ligation between 18b and 20a



[a]Reaction conditions: refer to the Supporting Information.

The ligation between protection-free thioester **18b** and **20a** was found to be cleaner than that between **18a** and **20a**, leading to higher isolated yield of desired products (70%). We found that using a slight excess (1.2 equiv) of either starting material improved the conversion under neutral pH conditions.

Because desulfurization of starting peptide **20a** was also observed in the reaction, where the N-terminal Cys was converted to Ala to afford an unreactive material **20c**, we wondered whether this process was competing with the desired ligation and possibly diminishing the reaction conversion. To test this hypothesis, *tert*-butylthiol was added as a scavenger of the free radicals in the reaction to eliminate the dethiylations.[36] As a result, the formation of desulfurized products was significantly minimized (Figure 4), and the conversion was approximately the same as the one without the addition of *t*-BuSH. This finding suggests that formation of **2oc** may have no significant impact on the reaction conversion. On the basis of these results and in consideration of reaction scales in most cases of protein chemical synthesis, we conducted further experiments at the concentration of 3 mM for west-side peptidyl thioesters, and the reactions were stirred in pH 7.0 buffer at room temperature for 8 h.

Scope and Limitations. To evaluate the applicability of peptidyl thioproline thioester in reactions with peptides containing noncysteine N-termini, several sequences with previously reported N-terminal β- or γ-thio-containing amino acid derivatives were tested (Table 2). Peptide **20d** containing β-methyldisulfide-Val,[14q] a representative sterically hindered ligation site, reacted with **18b** to afford the ligation products in a combined conversion of 92% within 8 h (entry 2). Considering

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

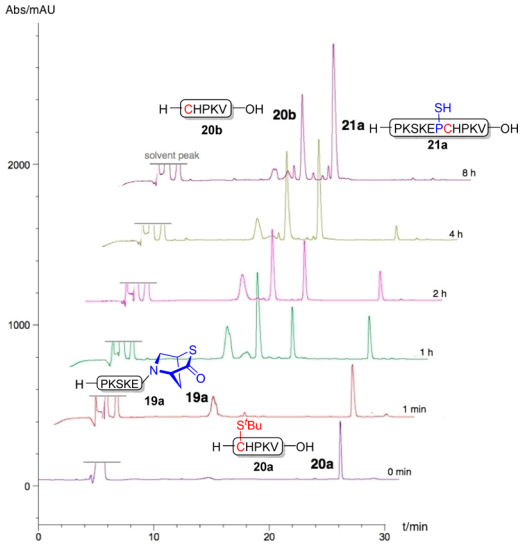


Figure 4. HPLC-MS traces of ligation between **18b** and **20a**. **18b** (3 mM), 1.2 equiv of **20a**, 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), 20 μL of *t*-BuSH, room temperature.

that the ligation of penicillamine usually requires more than 12 h,[14q] this result demonstrated the high reactivity of the thiolactone intermediate.

When peptides containing γ-thio-Glu[14e] (entry 3) and β-thio-Asp[14b] (entry 4) as the N-termini were subjected to the optimized conditions, the reactions proceeded smoothly and generated the ligated products in good yields. However, when we tested *trans*-4-thiolproline-containing peptide **20g**,[15a] hoping to conquer the challenging Pro-Pro ligation, neither the ligated thioester intermediate from intermolecular transthioesterification nor the final amide bond forming product was detected (entry 5). Further attempts under elevated temperature (60 °C) did not generate any desired product but rather promoted an intramolecular condensation. In this case, the free amino group at either the N-terminus or the side chain of the west-side peptide directly reacted with the thiolactone moiety, producing a cyclic peptide as indicated by mass spectrometry.[36] It was noticeable that this intramolecular aminolysis process was not observed at room temperature, which underscores the distinct reactivity of the bicyclic thiolactone structure in NCL conditions, in contrast to the observed aminolysis in THF reported by Brands et al.[41] The unsuccessful Pro-Pro ligation suggested that two prolines might be too strained to adopt a suitable conformation for the S → N acyl transfer and whether the transthioesterification would be affected was unclear thus far.

Further explorations of a number of sequences consisting of commonly used natural amino acids demonstrated the compatibility of diversed functional groups in the thiolactone-mediated ligation (entries 6−10). In particular, Lys, Ser, and Thr were found to be tolerated and no direct aminolysis or esterification was observed, which further underscored the chemoselectivity of this internal activation of prolyl thioesters and suggested an NCL-type process. Peptides containing *O*-mannosylated Ser (entry 6) or Acm-protected Cys (entry 10) were also proven to be compatible under the reaction conditions, suggesting potential applications of this strategy in the synthesis of cysteine-containing proteins or glycopeptides and glycoproteins.

To evaluate further this thioprolyl thioester-based method in different sequences, in particular the problematic ones in Otaka's studies,[27] two peptides **18g** (entry 11) and **18h** (entry 12) with Gly and Ser adjacent to prolyl thioester, respectively, were prepared. Although **18g** was found to be prone to forming an amino-acid-deleted byproduct in the reported procedure, under our standard conditions the ligation reaction with peptide **20i** generated the desired product in decent yield without any observed amino acid deletion. However, ligation of peptide **18h** afforded the product in only 37% isolated yield, similar to the previously reported result,[27] indicating that such poor yield was probably due to an unstable sequence under the buffered conditions.

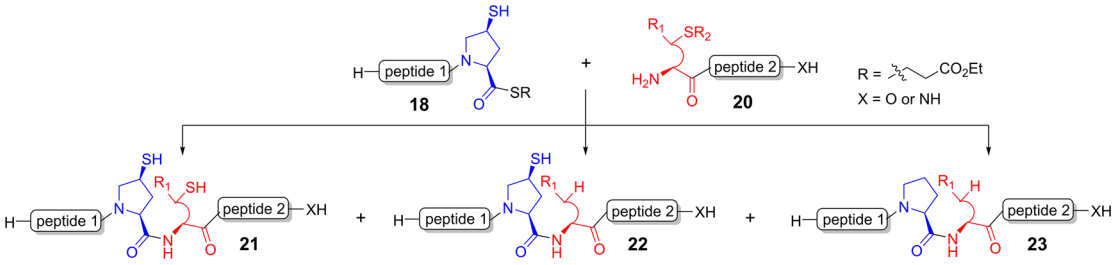
It is important to point out that the desulfurized products were observed in most of our examples, although the amount of dethiylation varied in different peptide sequences. Such desulfurization process during ligation was not substantial in previous studies,[14,27] presumably because the presence of radical quenching MPAA in the reactions. Even in the cases of desulfurization, the −SH groups on peptides were mostly isolated and exogenous thiols (e.g., *tert*-butylmercaptan) were required as hydrogen donors to accelerate radical propagation of the desulfurization. It could be possible that in our cases the −SH on proline may act as an internal hydrogen donor leading to an accelerated rate of radical-based desulfurization on the adjacent thioamino acids, particularly the sequences that better suit the conformational requirements for such intramolecular hydrogen delivery.

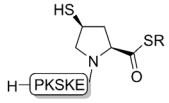
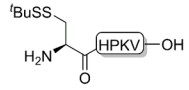
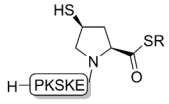
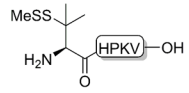
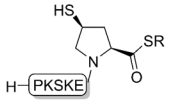
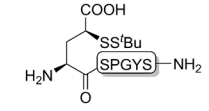
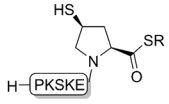
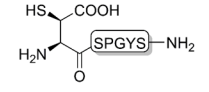
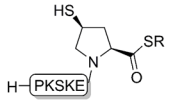
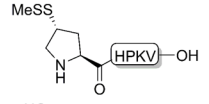
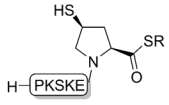
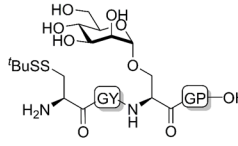
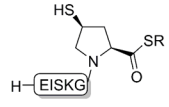
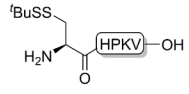
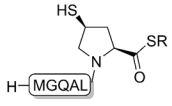
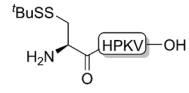
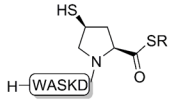
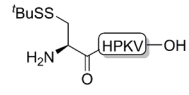
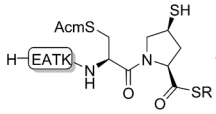
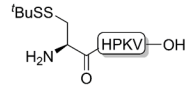
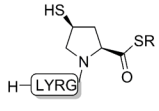
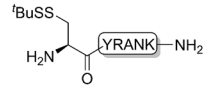
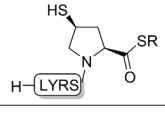
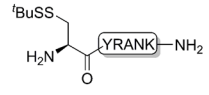
Desulfurization. The formation of native proline residues requires the removal of 4-mercaptan group on the ligation site prolines. Accordingly, Danishefsky's protocol was employed on the obtained ligation products (Table 3).[7] In the representative cases, the dethiylation proceeded efficiently to reveal the native Pro-Ala (entry 1), Pro-Val (entry 2), Pro-Glu (entry 3), and Pro-Asp (entry 4) residues in full conversion and excellent isolated yields. Furthermore, because the external activation using aryl thiol additives was not necessary in the thiolactone-mediated procedure, the ligation and desulfurization steps were able to be streamlined into a straightforward one-pot protocol. In the case of reaction between **18b** and **20a**, we found that eliminating the purification step after ligation and directly conducting desulfurization in the same reaction flask afforded product **23a** in 85% isolated yield which was more efficient in comparison to the stepwise procedure (ca. 80% of **23a** over two steps, cf. Table 3, entry 1). This improved yield using a one-pot operation versus a multistep reaction−purification procedure was in accord with previous studies.[42] The nonessential of radical quenching additives[19,43] eased the operation in our case, where after the ligation step only the addition of reagents for dethiylation under an argon atmosphere was required, thus improving the overall efficiency.[14e,44]

Mechanistic Studies. To probe further the reactions using 4-mercapto-prolyl thioester, we carried out mechanistic investigations through several control experiments (Table 4). First, the poor reactivity of normal prolyl thioesters was verified by conducting the reaction between peptidyl prolyl thioester **24a** and N-terminal cysteinyl peptide **20a** in a ligation buffer containing 30 mM MPAA (entry 1). No ligation product was observed, which was consistent with the results obtained by the Kent group.[24] When N-terminal alanyl peptide **20c** was subjected to the ligation with **18b**, the reaction did not afford any ligated peptide, which further confirmed that the thioprolyl thioesters react via an NCL-like process instead of direct

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

Table 2. Substrate Scope and Limitations[a]



Entry	West-side peptide		East-side peptide		Conversion[b] %	Yield[c] %	
						total	(**21**, **22**, **23**)
1		**18b**		**20a**	85	85	(23, 27, 35)
2		**18b**		**20d**	92	78	(28, 25, 35)
3		**18b**		**20e**	85	71	(14, 31, 26)
4		**18b**		**20f**	80	69	(69, 0, 0)
5		**18b**		**20g**	ND[d]	NA	NA
6[e]		**18b**		**20h**	72	66	(66, 0, 0)
7		**18c**		**20a**	84	82	(40, 23, 19)
8		**18d**		**20a**	78	52	(12, 18, 22)
9		**18e**		**20a**	93	90	(13, 41, 36)
10		**18f**		**20a**	77	75	(35, 25, 15)
11		**18g**		**20i**	97	78	(0, 0, 78)
12		**18h**		**20i**	96	37	(0, 0, 37)

[a]Reaction conditions: **18** (3 mM), 1.2 equiv **20**, 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), room temperature, 8 h. [b]Estimated conversion based on calculations of the analytical HPLC integrations of peptides **20** and the corresponding desulfurized peptide, and products **21**–**23**. [c]Isolated yield after HPLC purification. [d]Not detected on HPLC-MS. [e]20 μL of *t*-BuSH was added.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

Table 3. Metal-Free Desulfurization on Thioproline-Containing Peptides[a]

Entry	Ligation product	desulfurized product	conversion[b]	yield[c]
1	H–PKSKEPCHPKV–OH (SH) **21a**	H–PKSKEPAHPKV–OH **23a**	100%	94%
2	H–PKSKEPVHPKV–OH (HSSH) **21d**	H–PKSKEPVHPKV–OH **23d**	100%	95%
3	H–PKSKEPESPGYS–NH_2 (HSSH) **21e**	H–PKSKEPESPGYS–NH_2 **23e**	100%	92%
4	H–PKSKEPDSPGYS–NH_2 (HSSH) **21f**	H–PKSKEPDSPGYS–NH_2 **23f**	100%	92%

[a]Reaction conditions: **21** (3 mM), 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), 200 μL of 0.5 M bond-breaker TCEP solution (Pierce), 20.0 μL of 2-methyl-2-propanethiol, and 10.0 μL of radical initiator (0.1 M VA-044 in water), 37 °C, 1 h. [b]Estimated conversion based on calculations of the analytical HPLC integrations of peptides **21** and **23**. [c]Isolated yield after HPLC purification.

Table 4. Mechanistic Studies[a]

Entry	West-side peptide	East-side peptide	conversion %
1[b]	H–PKSKE– **24a** (–SR)	^{t}BuSS– **20a**	ND[c]
2	H–PKSKE– (SH) **18b** (–SR)	**20c**	ND
3	H–PKSKE– (SSMe) **24b** (–SR)	^{t}BuSS– **20a**	ND
4	H–PKSKE– (SMe) **24c** (–SR)	^{t}BuSS– **20a**	ND

[a]Reaction conditions: **24** or **18b** (3 mM), 1.0 equiv **20**, 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), room temperature, 8 h. [b]Reaction buffer contained 30 mM MPAA. [c]Not detected on HPLC-MS. R = $CH_2CH_2CO_2Et$.

aminolysis (entry 2). Moreover, the peptidyl thioester **24b** containing a *trans*-4-mercapto-substituted C-terminal proline was also found to be unreactive in the reaction conditions (entry 3). These experimental results, along with the kinetic data we obtained (Figure 5), clearly indicate the significantly improved reactivity of prolyl thioesters by the introduction of a *cis*-4-mercapto substituent.

Although the observed mass from HPLC-MS analysis provided evidence for thiolactone intermediate **19a** in the reaction, at this stage we could not rule out the possibility that the activation was originated from the conformational change of proline resulting from the 4-substitution.[45] To determine whether **19a** was the actual active intermediate, we prepared peptide **24c** containing a proline with 4-methylthio substituent, which would presumably introduce a similar conformational change as that in **18b** but could not form a bridged bicyclic

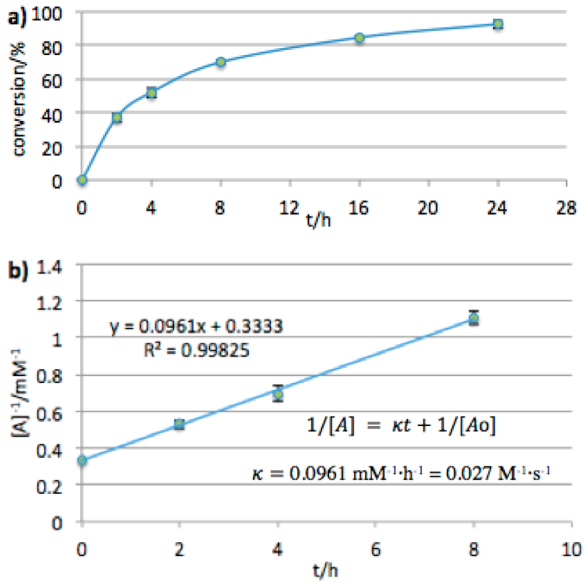
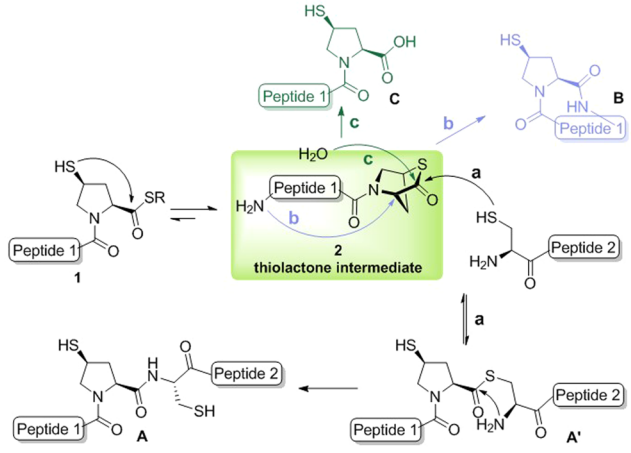


Figure 5. (a) Reaction conversion as a function of time for the reactions between **18b** and **20a**. (b) Reciprocal of concentration as a function of time for the reactions between **18b** and **20a**: [A], combined concentration of **20a** and **20b**; [A_0], initial concentration of **20a**. Data are the average of three replicates. The determined second order rate constant k = 0.0961 mM^{-1}·h^{-1} = 0.027 M^{-1}·s^{-1}, which is approximately on the same order of magnitude as the reported data of alanyl thioester (0.087 M^{-1}·s^{-1}).[24] Reaction conditions: **18b** (3 mM), 1.0 equiv of **20a**, 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), room temperature.

structure (Table 4, entry 4). From the HPLC-MS analysis of the reaction between **24c** and **20a** under our optimized conditions, we could not detect any ligation product, indicating that key bicyclic thiolactone **19a** is most likely the reactive intermediate, whereas the effect from the 4-substitution was not obvious in this case.

On the basis of all experimental results, a reaction mechanism was proposed as shown in Scheme 5. The equilibrium between thioprolyl thioester **1** and thiolactone intermediate **2** preferred

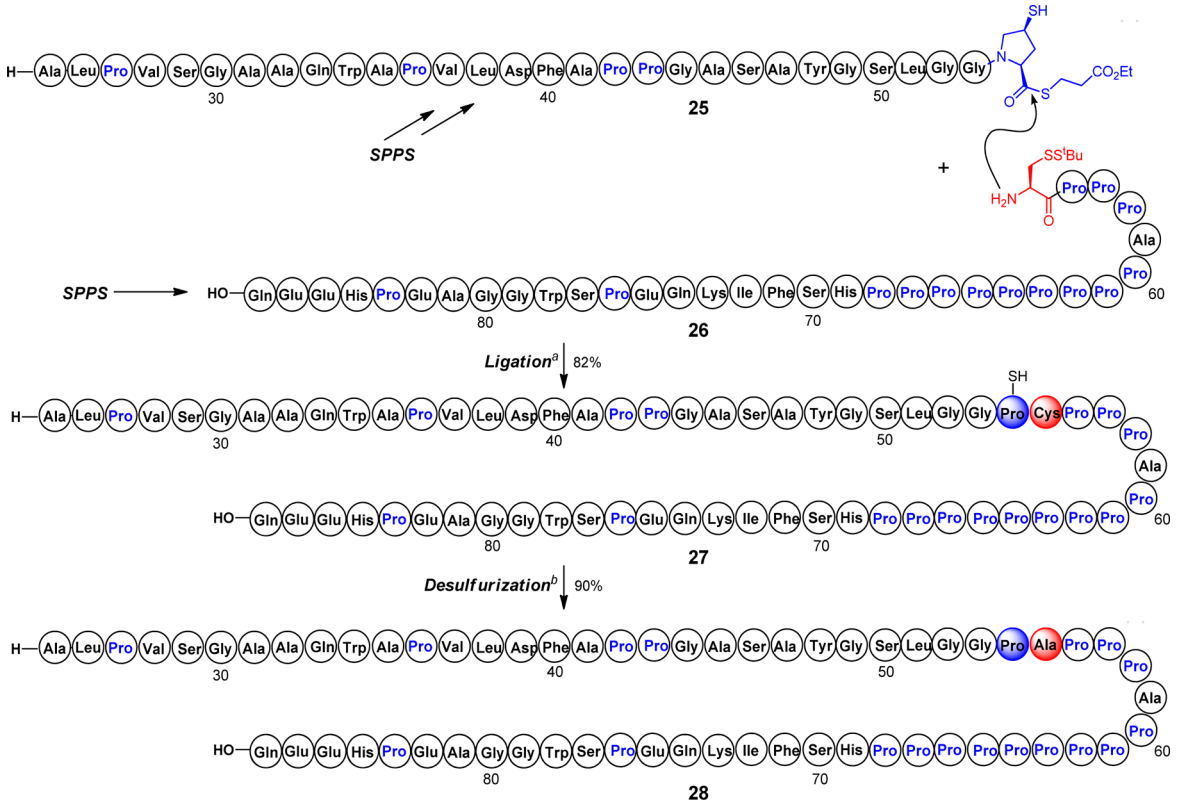
Scheme 5. Proposed Mechanism for the Thiolactone-Mediated Ligation Reaction[a]



[a]Pathway a: intermolecular transthioesterification with C-terminal peptide; pathway b: intramolecular aminolysis; and pathway c: hydrolysis of intermediate **2**.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890–4899

Scheme 6. Synthesis of PRR Ala_{25}-Gln_{87} (28) of WT1



[a]Reaction conditions: **25** (3 mM), 1.2 equiv **26**, 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), room temperature, 8 h. [b]Reaction conditions: 200 μL of NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 20 mM TCEP·HCl, pH 7.0), 200 μL of 0.5 M bond-breaker TCEP solution (Pierce), 20.0 μL of 2-methyl-2-propanethiol, and 10.0 μL of radical initiator (0.1 M VA-044 in water), 37 °C, 1 h.

the latter bicyclic structure in buffer,[46] which possessed a more electrophilic thioester carbonyl. At room temperature, pathway a was favored for peptides containing N-terminal cysteine or several other thio-amino acid analogs, where the reversible intermolecular trans-thioesterification followed by irreversible S → N acyl transfer led to the elongated peptide, similar to that in the original NCL. At the same time, the intramolecular aminolysis (pathway b) is suppressed under such conditions. The hydrolysis of **2** (pathway c) highly depends on the acidity of the reaction buffer, where increasing pH would promote such a process.[47] In the case of east-side peptide with an N-terminal *trans*-4-thiol-proline residue, although we did not observe the **A′**-type cross-linked thioester, the generation of **A′** could not be completely ruled out.[15a] Nevertheless, the resulting thioester could not proceed further to generate the corresponding Pro-Pro segment.

Synthetic Application. The *cis*-4-thiol-prolyl thioester-mediated ligation protocol was employed to synthesize a proline-rich region (PRR) of Wilms tumor protein 1 (WT1), in order to evaluate its applicability in the synthesis of proline-rich polypeptides (Scheme 6). WT1 is a zinc finger transcription factor that has an essential role in the development of urogenital system and regulates several reproductive genes.[48] It has also been reported that WT1 may have a potential role in luteinizing hormone β (LHβ) transcription in clonal mouse gonadotrope LβT2 cells.[49] However, the detailed function of WT1, as well as the proline-rich regions presented in this protein, has not been fully understood. The full length of WT1 contains 449 amino acids, where prolines make up approximately 10.5% of the protein. We chose one of the most proline-rich segments, Ala_{25}–Gln_{87}, as our synthetic target, which includes a stretch of 9 contiguous prolines and contains 19 prolines in total.

Retrosynthetically, the sequence could be dissected into a C-terminal thio-prolyl thioester Ala_{25}–$thioPro_{54}$ (**25**) and an N-terminal cysteinyl peptide Cys_{55}–Gln_{87} (**26**). Accordingly, segments **25** and **26** were prepared using the optimized protocols described above. Ligation reaction was conducted under the standard conditions to afford ligated peptide **27** in 82% isolated yield. After desulfurization, native segment **28** was obtained in 74% overall yield from **25**. Noticeably, our attempts to directly prepare this 63 amino acids sequence failed to afford any desired peptide under the same SPPS conditions,[36] which further underscores the synthetic difficulties inherent in proline-rich sequences.

CONCLUSIONS

Among all natural amino acids, glycine and proline have attracted significant attention in peptide synthesis because of their tolerance in various activation conditions without the concern of racemization.[21] In particular before the era of native chemical ligation, syntheses of peptides/proteins using a direct fragment condensation strategy were mostly conducted at a Gly or Pro site.[50] As we have shown in this work, the successful utilization of proline as the C-terminal reaction site in NCL offered convenience during the preparation of the corresponding thioester and at the same time took full advantage of the mild and highly chemoselective ligation conditions without side-chain protections. Ensured by the highly effective metal-free desulfurization protocol, the activation of otherwise less-reactive

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890–4899

prolyl thioester was accomplished using an easily removable internal mercaptan group, which allowed for ligation and desulfurization in the same flask with satisfactory overall efficiency. As demonstrated by synthesizing a proline-rich sequence, we believe this strategy will have further utility in the preparation of important proteins and glycoproteins, and would be complementary to the existing toolbox of chemical ligations. The once challenging and avoided proline site is now a synthetically useful or in some cases even more effective choice as the ligation site under the thiol-additive-free conditions. The strategy of utilizing intramolecular reactions and strained structures to accelerate desired transformations may be further applied to reactions of other difficult C-terminal amino acid residues, e.g., Val, Leu, Thr, etc., as well as possible developments of novel bio-orthogonal transformations. This strategy has been inspired not only by the logic of ligation−desulfurization,[7,51] and more importantly, also the rationale of tuning biolevel large molecules utilizing chemical principles and reactivities used on small molecules, which has also been the most valuable inspiration from NCL to the methodology developments in the field of peptide/protein synthesis, and will continue generating huge impacts on research at the chemistry−biology interface.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01202.

General experimental procedures, including spectroscopic and analytical data for reactions and new compounds. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*dongs@hsc.pku.edu.cn

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful for financial support from Peking University Health Science Center (BMU20130354), State Key Laboratory of Natural and Biomimetic Drugs, the National Recruitment Program of Global Youth Experts (1000 Plan), and the National Natural Science Foundation of China (21502005). We thank Dr. Yuan Wang, Weiqing Zhang, and Xulin Sun (Peking University) for spectroscopic assistance, Professor Qian Wan (Huazhong University of Science and Technology) and Dr. Neil Lajkiewicz (Incyte Corporation) for helpful discussions, and Yuankun Dao and Changdong He (Peking University) for the experimental assistance. This paper is dedicated to Professor Samuel J. Danishefsky on the occasion of his 80th birthday.

■ REFERENCES

(1) Selected reviews on commonly used protein expression systems: (a) Wegmuller, S.; Schmid, S. *Curr. Org. Chem.* **2014**, *18*, 1005−1019. (b) Mattanovich, D.; Branduardi, P.; Dato, L.; Gasser, B.; Sauer, M.; Porro, D. In *Recombinant Protein Production in Yeast*; Lorence, A., Ed.; Methods in Molecular Biology Series 824; Human Press: New York, 2012; pp 329−358. (c) Kost, T. A.; Condreay, J. P.; Jarvis, D. L. *Nat. Biotechnol.* **2005**, *23*, 567−575.

(2) (a) Schumacher, T. N. M.; Mayr, L. M.; Minor, D. L., Jr.; Milhollen, M. A.; Burgess, M. W.; Kim, P. S. *Science* **1996**, *271*, 1854−1857. (b) Wiesehan, K.; Willbold, D. *ChemBioChem* **2003**, *4*, 811−815. (c) Mandal, K.; Uppalapati, M.; Ault-Riché, D.; Kenney, J.; Lowitz, J.; Sidhu, S. S.; Kent, S. B. H. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 14779−14784.

(3) (a) Yeates, T. O.; Kent, S. B. H. *Annu. Rev. Biophys.* **2012**, *41*, 41−61. (b) Dang, B.; Kubota, T.; Mandal, K.; Bezanilla, F.; Kent, S. B. H. *J. Am. Chem. Soc.* **2013**, *135*, 11911−11919. (c) Okamoto, R.; Mandal, K.; Sawaya, M. R.; Kajihara, Y.; Yeates, T. O.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2014**, *53*, 5194−5198.

(4) (a) Wang, P.; Dong, S.; Brailsford, J. A.; Iyer, K.; Townsend, S. D.; Zhang, Q.; Hendrickson, R. C.; Shieh, J.; Moore, M. A. S.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 11576−11584. (b) Sakamoto, I.; Tezuka, K.; Fukae, K.; Ishii, K.; Taduru, K.; Maeda, M.; Ouchi, M.; Yoshida, K.; Nambu, Y.; Igarashi, J.; Hayashi, N.; Tsuji, T.; Kajihara, Y. *J. Am. Chem. Soc.* **2012**, *134*, 5428−5431. (c) Wang, P.; Dong, S.; Shieh, J.; Peguero, E.; Hendrickson, R.; Moore, M. A. S.; Danishefsky, S. J. *Science* **2013**, *342*, 1357−1360. (d) Sato, K.; Shigenaga, A.; Kitakaze, K.; Sakamoto, K.; Tsuji, D.; Itoh, K.; Otaka, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 7855−7859. (e) Hsieh, Y. S. Y.; Wijeyewickrema, L. C.; Wilkinson, B. L.; Pike, R. N.; Payne, R. J. *Angew. Chem., Int. Ed.* **2014**, *53*, 3947−3951. (f) Okamoto, R.; Mandal, K.; Ling, M.; Luster, A. D.; Kajihara, Y.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2014**, *53*, 5188−5193. (g) Reif, A.; Siebenhaar, S.; Tröster, A.; Schmälzlein, M.; Lechner, C.; Velisetty, P.; Gottwald, K.; Pöhner, C.; Boos, I.; Schubert, V.; Rose-John, S.; Unverzagt, C. *Angew. Chem., Int. Ed.* **2014**, *53*, 12125−12131. (h) Asahina, Y.; Komiya, S.; Ohagi, A.; Fujimoto, R.; Tamagaki, H.; Nakagawa, K.; Sato, T.; Akira, S.; Takao, T.; Ishii, A.; Nakahara, Y.; Hojo, H. *Angew. Chem., Int. Ed.* **2015**, *54*, 8226−8230. (i) Murakami, M.; Kiuchi, T.; Nishihara, M.; Tezuka, K.; Okamoto, R.; Izumi, M.; Kajihara, Y. *Sci. Adv.* **2016**, *2*, e1500678.

(5) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154. (b) Merrifield, R. B.; Stewart, J. M. *Nature* **1965**, *207*, 522−523.

(6) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.

(7) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248−9252.

(8) (a) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891−5896. (b) Offer, J.; Boddy, C. N.C.; Dawson, P. E. *J. Am. Chem. Soc.* **2002**, *124*, 4642−4646. (c) Wu, B.; Chen, J.; Warren, J. D.; Chen, G.; Hua, Z.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 4116−4125. (d) Brik, A.; Yang, Y.; Ficht, S.; Wong, C. *J. Am. Chem. Soc.* **2006**, *128*, 5626−5627. For a recent review, see (e) Malins, L. R.; Payne, R. J. In *Modern Extensions of Native Chemical Ligation for Chemical Protein Synthesis*; Liu, L., Ed.; Protein Ligation and Total Synthesis series I; Springer: New York, 2015; pp 36−46.

(9) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939−1941.

(10) Bode, J. W.; Fox, R. M.; Baucom, K. D. *Angew. Chem., Int. Ed.* **2006**, *45*, 1248−1252.

(11) (a) Gieselman, M. D.; Xie, L.; van der Donk, W. A. *Org. Lett.* **2001**, *3*, 1331−1334. (b) Metanis, N.; Keinan, E.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2010**, *49*, 7049−7053. (c) Townsend, S. D.; Tan, Z.; Dong, S.; Shang, S.; Brailsford, J. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 3912−3916. (d) Mitchell, N. J.; Malins, L. R.; Liu, X.; Thompson, R. E.; Chan, B.; Radom, L.; Payne, R. J. *J. Am. Chem. Soc.* **2015**, *137*, 14011−14014. (e) Malins, L.; Mitchell, N.; McGowan, S.; Payne, R. J. *Angew. Chem., Int. Ed.* **2015**, *54*, 12716−12721. (f) Malins, L. R.; Giltrap, A. M.; Dowman, L. J.; Payne, R. J. *Org. Lett.* **2015**, *17*, 2070−2073.

(12) (a) Li, X. C.; Lam, H. Y.; Zhang, Y. F.; Chan, C. K. *Org. Lett.* **2010**, *12*, 1724−1727. For recent review, see (b) Lee, C. L.; Li, X. *Curr. Opin. Chem. Biol.* **2014**, *22*, 108−114.

(13) Fang, G.; Li, Y.; Shen, F.; Huang, Y.; Li, J.; Lin, Y.; Cui, H.; Liu, L. *Angew. Chem., Int. Ed.* **2011**, *50*, 7645−7649.

(14) NCL with N-ternimal Arg: (a) Malins, L. R.; Cergol, K. M.; Payne, R. J. *ChemBioChem* **2013**, *14*, 559−563. Asp: (b) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 9723−9727. (c) Guan, X.; Drake, M. R.; Tan, Z. *Org. Lett.* **2013**, *15*, 6128−6131. Gln: (d) Siman, P.; Karthikeyan, S. V.; Brik, A. *Org. Lett.* **2012**, *14*, 1520−1523. Glu: (e) Cergol, K. M.; Thompson, R. E.; Malins, L. R.; Turner, P.; Payne, R. J. *Org. Lett.* **2014**, *16*, 290−293.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899

Leu: (f) Tan, Z. P.; Shang, S. Y.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2010**, *49*, 9500−9503. (g) Harpaz, Z.; Siman, P.; Kumar, K. S. A.; Brik, A. *ChemBioChem* **2010**, *11*, 1232−1235. Lys: (h) Yang, R. L.; Pasunooti, K. K.; Li, F. P.; Liu, X. W.; Liu, C. *J. Am. Chem. Soc.* **2009**, *131*, 13592−13593. (i) Ajish Kumar, K. S.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 8090−8094. (j) El Oualid, F.; Merkx, R.; Ekkebus, R.; Hameed, D. S.; Smit, J. J.; de Jong, A.; Hilkmann, H.; Sixma, T. K.; Ovaa, H. *Angew. Chem., Int. Ed.* **2010**, *49*, 10149−10153. (k) Merkx, R.; de Bruin, G.; Kruithof, A.; van den Bergh, T.; Snip, E.; Lutz, M.; El Oualid, F.; Ovaa, H. *Chem. Sci.* **2013**, *4*, 4494−4498. Phe: (l) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064−10065. (m) Malins, L. R.; Payne, R. J. *Org. Lett.* **2012**, *14*, 3142−3145. Thr: (n) Chen, J.; Wang, P.; Zhu, J. L.; Wan, Q.; Danishefsky, S. J. *Tetrahedron* **2010**, *66*, 2277−2283. Trp: (o) Malins, L.; Cergol, K.; Payne, R. *Chem. Sci.* **2014**, *5*, 260−266. Val: (p) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521−5824. (q) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807−6810.

(15) (a) Shang, S.; Tan, Z.; Dong, S.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2011**, *133*, 10784−10786. (b) Ding, H.; Shigenaga, A.; Sato, K.; Morishita, K.; Otaka, A. *Org. Lett.* **2011**, *13*, 5588−5591.

(16) Muir, T. W.; Dawson, P. E.; Kent, S. B. H. *Methods Enzymol.* **1997**, *289*, 266−298.

(17) Blanco-Canosa, J. B.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2008**, *47*, 6851−6855.

(18) Zheng, J.-S.; Tang, S.; Qi, Y.-K.; Wang, Z.-P.; Liu, L. *Nat. Protoc.* **2013**, *8*, 2483−2495.

(19) Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640−6646.

(20) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 10068−10073.

(21) Bodanszky, M. In *Peptide Synthesis: A Practical Textbook*; Springer-Verlag: Berlin Heidelberg, 1993; pp 117−128.

(22) Kajihara, Y.; Yoshihara, A.; Hirano, K.; Yamamoto, N. *Carbohydr. Res.* **2006**, *341*, 1333−1340.

(23) (a) Aussedat, B.; Fasching, B.; Johnston, E.; Sane, N.; Nagorny, P.; Danishefsky, S. J. *J. Am. Chem. Soc* **2012**, *134*, 3532−3541. (b) Fernańdez-Tejada, A.; Vadola, P. A.; Danishefsky, S. J. *J. Am. Chem. Soc* **2014**, *136*, 8450−8458.

(24) Pollock, S. B.; Kent, S. B. H. *Chem. Commun.* **2011**, *47*, 2342−2344.

(25) Wan, Q.; Chen, J.; Yuan, Y.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2008**, *130*, 15814−15816.

(26) Durek, T.; Alewood, P. F. *Angew. Chem., Int. Ed.* **2011**, *50*, 12042−12045.

(27) Nakamura, T.; Shigenaga, A.; Sato, K.; Tsuda, Y.; Sakamoto, K.; Otaka, A. *Chem. Commun.* **2014**, *50*, 58−60.

(28) (a) Bennick, A. *Mol. Cell. Biochem.* **1982**, *45*, 83−99. (b) Carregaro, F.; Stefanini, A. C. B.; Henrique, T.; Tajara, E. H. *Arch. Dermatol. Res.* **2013**, *305*, 857−866.

(29) (a) Hinderaker, M. P.; Raines, R. T. *Protein Sci.* **2003**, *12*, 1188−1194. (b) Hodges, J. A.; Raines, R. T. *Org. Lett.* **2006**, *8*, 4695−4697. (c) Choudhary, A.; Kamer, K. J.; Powner, M. W.; Sutherland, J. D.; Raines, R. T. *ACS Chem. Biol.* **2010**, *5*, 655−657.

(30) Shaffer, K. J.; Taylor, C. M. *Org. Lett.* **2006**, *8*, 3959−3962.

(31) (a) Yale, H. L.; Sowinski, F. *J. Org. Chem.* **1960**, *25*, 1824−1826. (b) Mangion, I. K.; Ruck, R. T.; Rivera, N.; Huffman, M. A.; Shevlin, M. *Org. Lett.* **2011**, *13*, 5480−5483.

(32) Mollica, A.; Paradisi, M. P.; Varani, K.; Spisani, S.; Lucente, G. *Bioorg. Med. Chem.* **2006**, *14*, 2253−2265.

(33) Kimura, T.; Takai, M.; Masui, Y.; Morikawa, T.; Sakakibara, S. *Biopolymers* **1981**, *20*, 1823−1832.

(34) For a recent review, see Jensen, K. J. In *Peptide Synthesis and Applications*; Jensen, K. J., Shelton, P. T., Pedersen, S. L., Eds.; Methods in Molecular Biology series no. 1047; Human Press: New York, 2013; pp 1−21.

(35) Joullie, M. M.; Lassen, K. M. *ARKIVOC* **2010**, *8*, 189−250.

(36) See the Supporting Information for complete procedures and details.

(37) (a) Kartasova, T.; van de Putte, P. *Mol. Cell. Biol.* **1988**, *8*, 2195−2203. (b) Marvin, K. W.; George, M. D.; Fujimoto, W.; Saunders, N. A.; Bernacki, S. H.; Jetten, A. M. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 11026−11030.

(38) (a) Hoffmann, F. W.; Ess, R. J.; Simmons, T. C.; Hanzel, R. S. *J. Am. Chem. Soc.* **1956**, *78*, 6414. (b) Walling, C.; Rabinowitz, R. *J. Am. Chem. Soc.* **1957**, *79*, 5326. (c) Walling, C.; Basedow, O. H.; Savas, E. S. *J. Am. Chem. Soc.* **1960**, *82*, 2181−2184.

(39) Wang, Z. X.; Rejtar, T.; Zhou, Z. S.; Karger, B. L. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 267−275.

(40) Cleland, W. W. *Biochemistry* **1964**, *3*, 480−482.

(41) Brands, K. M. J.; Marchesini, G.; Williams, J. M.; Dolling, U.-H.; Reider, P. L. *Tetrahedron Lett.* **1996**, *37*, 2919−2922.

(42) Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534−2538.

(43) Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325−4329.

(44) Several solutions to avoid the desulfurization inhibiting agent have also been investigated. For representative reports, see the following: The use of MESNa: (a) Siman, P.; Blatt, O.; Moyal, T.; Danieli, T.; Lebendiker, M.; Lashuel, H. A.; Friedler, A.; Brik, A. *ChemBioChem* **2011**, *12*, 1097−1104. The use of ascorbate: (b) Rohde, H.; Schmalisch, J.; Harpaz, Z.; Diezmann, F.; Seitz, O. *ChemBioChem* **2011**, *12*, 1396−1400. The use of bifunctional aryl thiol catalyst: (c) Moyal, T.; Hemantha, H. P.; Siman, P.; Refua, M.; Brik, A. *Chem. Sci.* **2013**, *4*, 2496−2501. The removal of aryl thiol: the use of TFET: (d) Thompson, R. E.; Liu, X.; Alonso-García, N.; Pereira, P. J. B.; Jolliffe, K. A.; Payne, R. J. *J. Am. Chem. Soc.* **2014**, *136*, 8161−8164. (e) Sayers, J.; Thompson, R. E.; Perry, K. J.; Malins, L. R.; Payne, R. J. *Org. Lett.* **2015**, *17*, 4902−4905. Additive-free TCEP-prompted ligation: (f) Tsuda, S.; Yoshiya, T.; Mochizuki, M.; Nishiuchi, Y. *Org. Lett.* **2015**, *17*, 1806−1809. Additive-free selenocysteine ligation: see ref 11d.

(45) Cadamuro, S. A.; Reichold, R.; Kusebauch, U.; Musiol, H.-J.; Renner, C.; Tavan, P.; Moroder, L. *Angew. Chem., Int. Ed.* **2008**, *47*, 2143−2146.

(46) Although we have not been able to isolate the intermediate **19a** and conduct full characterization, the data from a series of LC-MS experiments suggest that the bicycle-containing peptide is most likely the structure of this reactive species. See the Supporting Information for more details on the conducted experiments.

(47) Because the thiolactone intermediate was found to be much more reactive than the nonactivated prolyl thioester in our case, we presumed that the hydrolysis, if happened, would likely be mainly resulted from the thiolactone intermediate other than its precursor.

(48) (a) Gessler, M.; Poustka, A.; Cavenee, W.; Neve, R. L.; Orkin, S. H.; Bruns, G. A. P. *Nature* **1990**, *343*, 774−778. (b) Call, K. M.; Glaser, T.; Ito, C. Y.; Buckler, A. J.; Pelletier, J.; Haber, D. A.; Rose, E. A.; Kral, A.; Yeger, H.; Lewis, W. H.; Jones, C.; Housman, D. E. *Cell* **1990**, *60*, 509−520. (c) Drummond, I. A.; Madden, S. L.; Rohwer-Nutter, P.; Bell, G. I.; Sukhatme, V. P.; Rauscher, F. J., 3rd. *Science* **1992**, *257*, 674−678. (d) Barbolina, M. V.; Adley, B. P.; Shea, L. D.; Stack, M. S. *Cancer* **2008**, *112*, 1632−1641.

(49) Bagchi, D.; Andrade, J.; Shupnik, M. A. *PLoS One* **2015**, *10*, e0116825.

(50) (a) Clark-Lewis, I.; Aebersold, R.; Ziltener, H.; Schrader, J. W.; Hood, L.; Kent, S. B. H. *Science* **1986**, *231*, 134−139. (b) Shin, S. Y.; Kaburaki, Y.; Watanabe, M.; Munekata, E. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 404−408.

(51) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526−533.

DOI: 10.1021/jacs.6b01202
J. Am. Chem. Soc. 2016, 138, 4890−4899