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Cysteine-Derived *S*-Protected Oxazolidinones: Potential Chemical Devices for the Preparation of Peptide Thioesters

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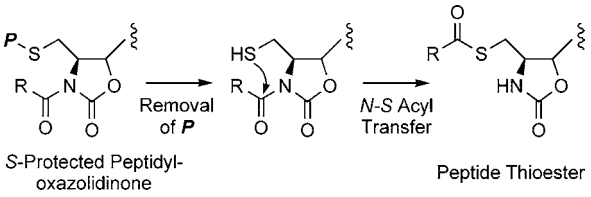
ABSTRACT



An *N*–*S* acyl-transfer-mediated preparation of peptide thioesters using the *S*-protected oxazolidinone derived from cysteine has been developed and applied to the synthesis of a 32-mer biologically active peptide by native chemical ligation protocols.

Intein-mediated protein splicing is a self-catalytic editing system of proteins in which an intervening sequence (termed "intein") is cleaved off from a protein precursor with simultaneous ligation between the flanking fragments (*N*- and *C*-exteins) to form a mature extein protein and the free intein.[1] These intein-mediated splicing systems have received increasing attention in the field of protein chemistry including purification,[2] labeling,[3] and semisynthesis of proteins.[4] In this splicing process, three consecutive acyl-transfer steps are involved.[5] The first *N*−*S* acyl transfer on the cysteine residue at intein *N*-terminus has been extensively applied to the biochemical preparation of protein (or peptide) thioesters. These have shown great utility in the preparation of a wide variety of proteins by native chemical ligation (NCL) procedures.[4,6] Therefore, we anticipated that mimicking the intein-mediated *N*−*S* acyl-transfer step by chemical means could provide an unprecedented alternative to reported chemical methods for the synthesis of peptide thioesters.[7] A recent elegant NMR study[8] on this acyl transfer revealed that "ground-state destabilization" by inducing nonplanarity in the scissile amide bond was responsible for activation of the

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amide leading to the *N*−*S* acyl transfer.[9] On the basis of this finding, we attempted to use an *N*-acyloxazolidinone derivative to prepare thioesters by a "ground-state destabilization" mechanism. In an acyloxazolidinone, the carbonyl in the oxazolidinone ring is likely to influence the $n_N-\pi^*_{CO}$ delocalization and to result in the activation (ground-state destabilization) of the *exo*-amide linkage. Actually, *N*-acyloxazolidinones have been documented to show active ester character which is regulated by distortion of the amide planarity.[10] Because neighboring thiol group participation in the cleavage of the acyloxazolidinone bond could facilitate the formation of thioesters,[11] an *S*-protected oxazolidinone derived from cysteine was selected as a chemical device for the preparation of peptide thioesters by an intein mimicking mechanism. In this study, we examined the applicability of cysteine-derived acyloxazolidinones to the *N*−*S* acyl shift-mediated synthesis of peptide thioesters with application to peptide synthesis using NCL.[12] Very recently, two groups have reported *N*−*S* acyl-transfer-mediated preparation of peptide thioesters using chemical devices other than the oxazolidinones.[13,14]

The use of *S*-protected oxazolidinones in the synthesis of peptide thioesters is conceptually outlined in Figure 1. A protected peptide chain is assembled on the *S*-protected oxazolidinone-type linker by solid-phase peptide synthesis (SPPS) followed by removal of protecting groups except for the *S*-protection on the oxazolidinone. Concomitant release of the peptidyloxazolidinone from the resin yields the *S*-protected peptide oxazolidinone as a precursor to the thioester. Removal of the *S*-protection and subsequent *N*−*S* acyl transfer affords the peptide thioester.

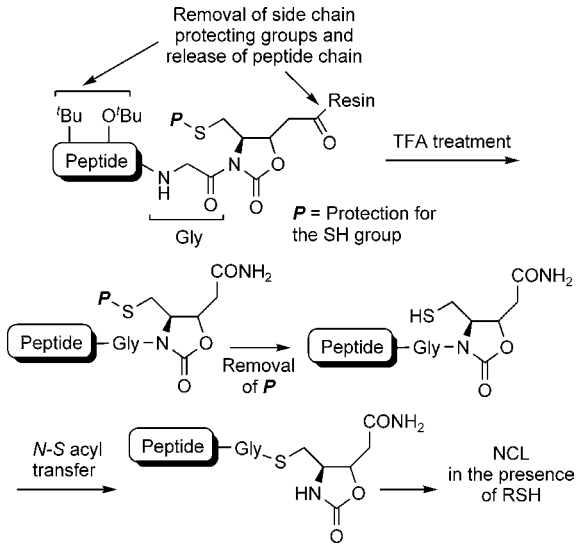
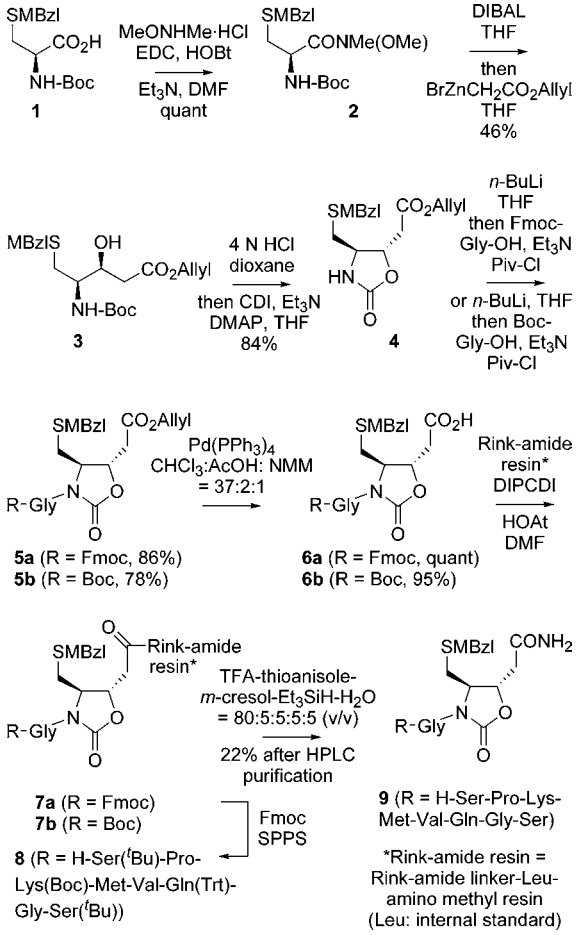


Figure 1. Concept of *N*−*S* acyl transfer-mediated synthesis of peptide thioesters using an *S*-protected oxazolidinone.

The *p*-methoxybenzyl (MBzl) group was selected as an oxazolidinone *S*-protecting group because it remains intact during standard Fmoc-based SPPS protocols (20% piperidine treatment for Fmoc-removal and trifluoroacetic acid (TFA) treatment for the final deprotection). Preparation of a protected oxazolidinone derivative suitable for attaching to an amino-functionalized resin and its use in the synthesis of the peptide thioester precursor are shown in Scheme 1.

Scheme 1



Starting from the protected cysteine derivative **1**, *syn*-1,2-amino alcohol derivative **3** was obtained exclusively by a sequence of reactions consisting of Weinreb-amidation using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),[15] diisobutylaluminum hydride (DIBAL) reduction, and Refor-

(7) For synthesis of peptide thioesters using Boc strategy, see: (a) Aimoto, S. *Biopolymer* **1999**, *51*, 247−265. (b) Hackeng, T. M.; Giffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10068−10073. For synthesis of peptide thioesters using the Fmoc strategy, see: (c) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11374. (d) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684−11689. (e) Sewing, A.; Hilvert, D. *Angew. Chem., Int. Ed.* **2001**, *40*, 3395−3396. (f) Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5*, 2951−2953.

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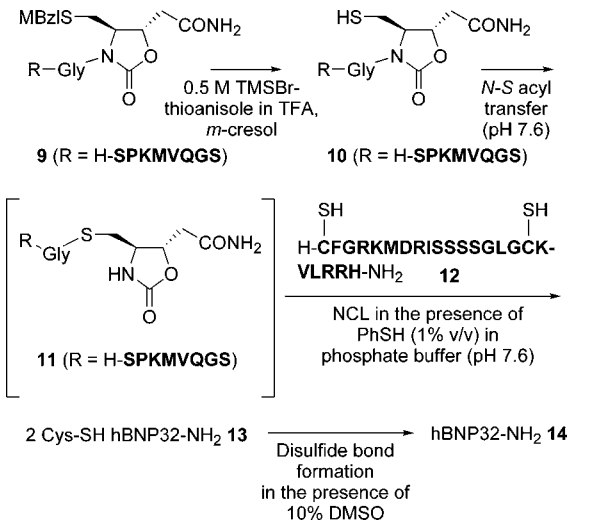
matsky reaction of allyl bromoacetate. Deprotection of the Boc group of **3** with 4 N HCl−dioxane followed by carbonyldiimidazole (CDI)/4-(dimethylamino)pyridine (DMAP)-mediated cyclization of the resulting deprotected compound afforded the *S*-MBzl oxazolidinone **4**. Acylation of lithiated **4** with Fmoc-Gly-OH in the presence of pivaloyl chloride (Piv-Cl) and Et_3N gave the Fmoc-glycyloxazolidinone **5a**. For attachment of the acyloxazolidinone to the resin, allyl ester on **5a** was quantitatively removed by treatment with $Pd(PPh_3)_4$ in $CHCl_3$/AcOH/*N*-methylmorpholine (NMM) (37:2:1, v/v).[16]

The resulting carboxylic acid **6a** can be coupled to an amino-functionalized resin using standard peptide coupling conditions. Before elongation of the peptide chain, the stability of the acyloxazolidinone linkage toward basic treatment needed for Fmoc-removal was examined. For this purpose, Rink amide linker-functionalized resin possessing an internal standard amino acid (Leu) for amino acid analysis following acid hydrolysis was prepared by successive coupling of Fmoc-Leu-OH and Fmoc-Rink linker[17] on the amino methyl resin. On this resin was coupled the Boc-glycyloxazolidinone **6b**, which was prepared by reactions identical to those used for **6a**. Treatment of the resulting resin **7b** with basic reagent systems followed by amino acid analysis after acid hydrolysis indicated that treatment with 20% piperidine in *N*,*N*-dimethylformamide (DMF) (standard Fmoc deprotection) partially induced the decomposition of the amide linkage (ca. 20% of the linkage was broken after 5 h treatment). On the other hand, the use of Aimoto's reagent mixture[18] consisting of 1-methylpyrrolidine-hexamethyleneimine-1-hydroxybenzotriazole (HOBt) (25% (v/v)−2% (v/v)−3% (w/v) in 1-methylpyrrolidin-2-one (NMP)-dimethyl sulfoxide (DMSO) (1:1)) proved to be compatible with the resin in the presence of the acyloxazolidinone linkage.[19] This basic reagent system has been successfully applied to the Fmoc-based synthesis of peptide thioesters without affecting thioester linkages. However, one potential limitation with the use of this procedure in the synthesis of thioesters is racemization of chiral thioester-linked *C*-terminal amino acids.[20] This is the case with aminoacylated oxazolidinones.[21] At this stage, since only Fmoc-glycyloxazolidinone such as **6a** can be applicable to the *N*−*S* acyl-transfer-mediated methodology, extensive efforts are in progress in our laboratory to develop deprotection conditions suitable for chiral *C*-terminal aminoacylated oxazolidinones.

Next, to evaluate the practical usefulness of **6a** in application to the NCL-mediated preparation of peptides, we undertook the synthesis of hBNP32-NH_2 **14**. The peptide chain assembly for the peptide thioester precursor fragment **9** (corresponding to hBNP32-NH_2 (1−9)) was conducted using the Fmoc-Rink linker-Leu resin, on which Fmoc-glycyloxazolidinone **6a** and Fmoc-amino acids were successively coupled with the aid of diisopropylcarbodiimide (DIPCDI)-HOBt (or 1-hydroxy-7-azabenzotriazole (HOAt)). Treatment with Aimoto's reagent cocktail (20 min reaction/each step) was utilized for the removal of Fmoc groups. Amino acid analysis of the hydrolysate resulting from the completed resin **8** revealed that the peptide chain assembly proceeded efficiently without significant decomposition of the acyloxazolidinone linkage.[22] Treatment of the completed resin **8** with TFA−thioanisole−*m*-cresol−Et_3SiH−H_2O (80:5:5:5:5, v/v) at room temperature for 2 h, followed by HPLC purification, gave the *S*-protected peptidyloxazolidinone **9** as a thioester precursor in 22% yield.

The resulting peptidyloxazolidinone was then subjected to the NCL-mediated synthesis of hBNP32-NH_2 (Scheme 2). HPLC-purified *S*-protected derivative **9** was treated with

Scheme 2



0.5 M trimethylsilyl bromide (TMSBr)−thioanisole (1:1) in TFA and *m*-cresol[23] at −10 °C for 1 h to afford the *S*-deprotected peptidyloxazolidinone **10**, which was then purified by HPLC. NCL of the purified **10** with the

(11) For nucleophilic involvement of hydroxy group leading to *N*−*O* acyl transfer, see: Bew, S. P.; Bull, S. D.; Davies, S. G.; Savory, E. D.; Watkin, D. J. *Tetrahedron* **2002**, *58*, 9387−9401 and references cited herein.

(12) A 32-mer disulfide-containing peptide, human brain natriuretic peptide derivative (amide form: hBNP32-NH_2 **14**), was selected as model synthetic peptide. For hBNP32, see: Kambayashi, Y.; Nakao, K.; Mukoyama, M.; Saito, Y.; Ogawa, Y.; Shiono, S.; Inoue, K.; Yoshida, M.; Imura, H. *FEBS Lett*. **1990**, *259*, 341−345.

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(19) Treatment of **7b** with Aimoto's reagent cocktail for 5 h induced ca. 3% cleavage of the linkage. See the Supporting Information.

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(21) Oxazolidinone derivative **4** was derivatized with Fmoc-L-Ala-OH. Then, treatment of the resulting Fmoc-L-Ala-linked derivative with the Aimoto's reagent cocktail afforded the mixture of H-L-Ala- or H−D-Ala-linked oxazolidinone derivative. Results of quantitative HPLC analysis of the reaction mixture are shown as a graph in the Supporting Information.

N-terminal cysteine peptide (hBNP32-NH_2 (10−32)) **12**, which was prepared by standard Fmoc-based peptide synthesis, proceeded efficiently in phosphate buffer (pH 7.6) containing 6 M guanidine·HCl in the presence of 1% (v/v) thiophenol to yield the ligated 2Cys-SH hBNP32-NH_2 **13**. At this step in the NCL protocol, the *N*-acylated compound **10** (retention time on HPLC analysis = 10.8 min) disappeared completely within 1 min and a new compound (putatively **11**) with the same *m*/*z* value as that of **10** eluted at 7.8 min (see the Supporting Information).[24] After dilution with phosphate buffer to three times volume, DMSO (10%, v/v) was added to effect disulfide bond formation.[25] HPLC purification of the crude product gave purified hBNP32-NH_2 **14** in 78% yield calculated from the NCL step.

Based on the reaction mechanism of intein-mediated formation of peptide thioesters, we have developed a method for *N*−*S* acyl-transfer-mediated synthesis of thioesters using acylated oxazolidinones derived from *S*-protected cysteine. Removal of peptidyloxazolidinone *S*-protection provides the corresponding peptide thioesters through *N*−*S* acyl transfer involving the thiol group in the adjacent activated acyl-oxazolidinone linkage. This synthetic protocol was successfully applied to the NCL-mediated synthesis of hBNP32-NH_2. The use of cysteine-derived oxazolidinones as chiral auxiliaries with application to the preparation of thioesters could provide a new access to chiral thioester derivatives, which could have potential utility in the synthesis of a wide variety of compounds.

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Supporting Information Available: Experimental procedures, NMR charts for key compounds, and HPLC charts of the analyses of *N*−*S* acyl-transfer-mediated synthesis of hBNP32-NH_2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) The decomposition of the acyloxazolidinone linkage is attributed to both base-induced cleavage of the activated bond (ca. 3% decomposition after 5 h treatment)[19] and diketopiperazine (DKP) formation. These two factors were probably responsible for the partial loss of peptide chain (Glu (in the peptide)/Leu (in the solid support) = 0.9:1.0). We have yet to prove to attribute the loss to the base-induced cleavage, DKP formation, or precision in amino acid analysis.

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(24) Removal of the thiol protection is needed for the coupling with *N*-terminal cysteine peptide. Subjection of *S*-protected peptidyloxazolidinone such as **9** to the NCL condition does not afford the ligated product.

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