

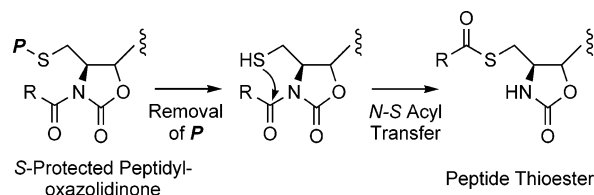
Cysteine-Derived S-Protected Oxazolidinones: Potential Chemical Devices for the Preparation of Peptide Thioesters

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Received November 14, 2005

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 study⁸ 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

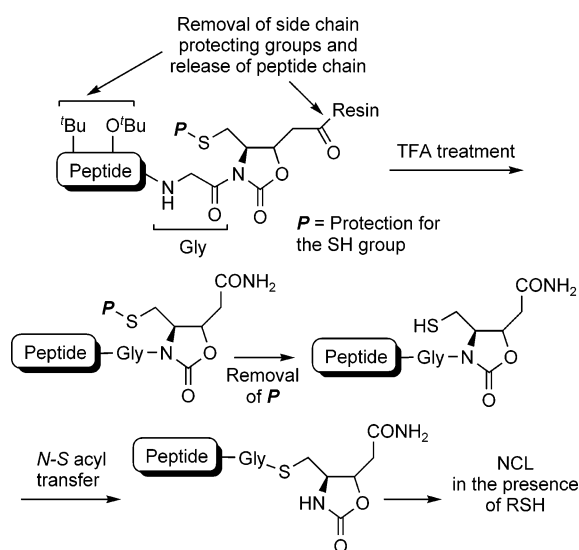
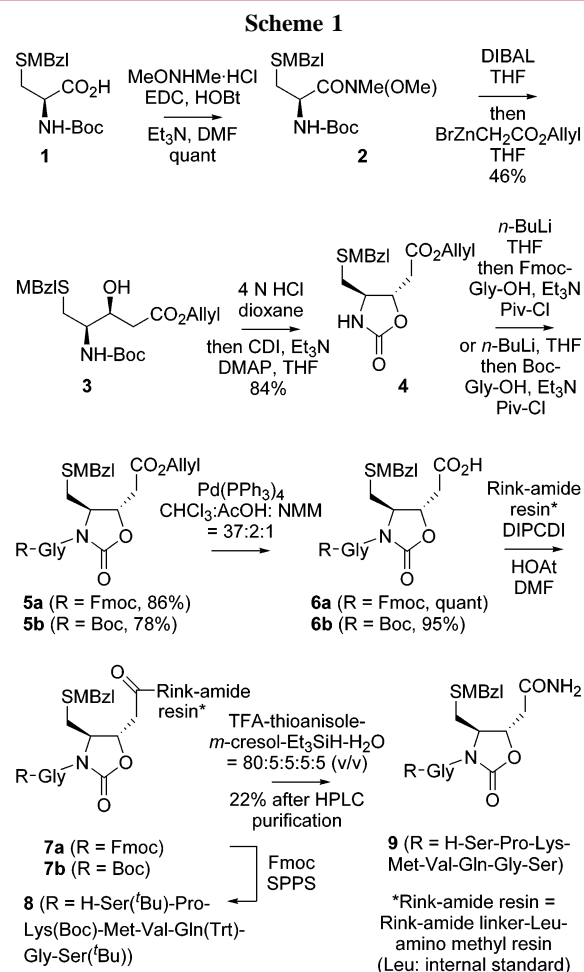


Figure 1. Concept of *N*–*S* acyl transfer-mediated synthesis of peptide thioesters using an *S*-protected oxazolidinone.

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 peptidylloxazolidinone 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.

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.



(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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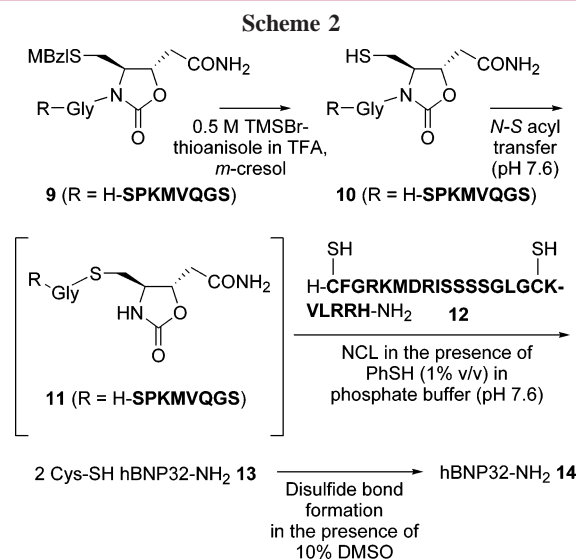
matsky reaction of allyl bromoacetate. Deprotection of the Boc group of **3** with 4 N HCl–dioxane followed by carbonyldiimidazole (CDI)/4-(dimethylamino)pyridine (DMAP)-mediated cyclization of the resulting deprotected compound afforded the *S*-MBzl oxazolidinone **4**. Acylation of lithiated **4** with Fmoc-Gly-OH in the presence of pivaloyl chloride (Piv-Cl) and Et₃N gave the Fmoc-glycyloxazolidinone **5a**. For attachment of the acyloxazolidinone to the resin, allyl ester on **5a** was quantitatively removed by treatment with Pd(PPh₃)₄ in CHCl₃/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 linker¹⁷ 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 mixture¹⁸ 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-NH₂ **14**. The peptide chain assembly for the peptide thioester precursor fragment **9** (corresponding to hBNP32-NH₂ (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–Et₃SiH–H₂O (80:5:5:5:5, v/v) at room temperature for 2 h, followed by HPLC purification, gave the *S*-protected peptidylloxazolidinone **9** as a thioester precursor in 22% yield.

The resulting peptidylloxazolidinone was then subjected to the NCL-mediated synthesis of hBNP32-NH₂ (Scheme 2). HPLC-purified *S*-protected derivative **9** was treated with



(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-NH₂ **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.

0.5 M trimethylsilyl bromide (TMSBr)–thioanisole (1:1) in TFA and *m*-cresol²³ at –10 °C for 1 h to afford the *S*-deprotected peptidylloxazolidinone **10**, which was then purified by HPLC. NCL of the purified **10** with the

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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-NH₂ (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-NH₂ **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-NH₂ **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

(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 peptidylloxazolidinone such as **9** to the NCL condition does not afford the ligated product.

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for *N*–*S* acyl-transfer-mediated synthesis of thioesters using acylated oxazolidinones derived from *S*-protected cysteine. Removal of peptidylloxazolidinone *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-NH₂. 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.

Acknowledgment. We thank Dr. Terrence R. Burke, Jr., NCI, NIH, for proofreading this manuscript. This research was supported in part by the 21st Century COE Program “Knowledge Information Infrastructure for Genome Science”, a Grand-in-Aid for Scientific Research (KAKENHI). A.O. is grateful for research grants from Sekisui Integrated Research and Suzuken Memorial Foundation.

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-NH₂. This material is available free of charge via the Internet at <http://pubs.acs.org>.

OL052755M