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N→*S* Acyl-Transfer-Mediated Synthesis of Peptide Thioesters Using Anilide Derivatives

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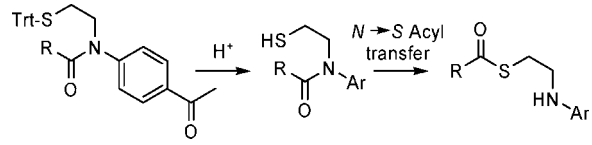
ABSTRACT



***N*→*S* acyl-transfer-mediated synthesis of peptide thioesters utilizing an *N*-aminoacyl-*N*-sulfanylethylaminobenzoic acid derivative has been examined. The developed synthetic methodology for peptide thioesters is compatible with Fmoc solid-phase peptide synthesis (SPPS).**

Peptide thioesters have played an indispensable role in native chemical ligation (NCL) for the chemical synthesis of peptides/proteins, whereby a thioester fragment chemoselectively reacts with the thiol group of an *N*-terminal cysteine fragment followed by *S*→*N* acyl transfer to give a ligated product.[1] Success in NCL-mediated protein synthesis partly depends on the accomplishment of preparing peptide thioesters. Boc solid-phase peptide synthesis (Boc SPPS) on a thioester-linked peptidyl resin has served as a standard protocol for the preparation of peptide thioesters[2] due to the instability of the thioester linkage to base treatment such as 20% piperidine/DMF used with Fmoc SPPS. However, the increasing widespread use of Fmoc SPPS in the preparation of various peptides including phosphorylated or glycosylated materials has prompted the development of a synthetic methodology for peptide thioesters via Fmoc protocols,[3] including the use of Kenner's safety catch linker[3d,e,i] or orthothioester.[3g] Alternatively, *N*→*S* acyl transfer is seen in naturally occurring thioester formations of the intein−extein system,[4,5] a feature which should have potential utility in the chemical synthesis of thioesters.

Aimoto reported *N*→*S* acyl-transfer-mediated protocols based on the side reaction observed in the acidolytic deprotection of thiol-auxiliary-containing peptides.[6−8] Moreover, we independently developed an acyl-transfer protocol using a cysteine-derived *N*-peptidyloxazolidinone **1**[8b] in

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which inhibition of delocalization of the amide nitrogen lone pair to the *exo*-carbonyl π^* orbital by the ring carbonyl weakens the peptide−oxazolidine linkage and thereby leads to the formation of peptide thioester **3** through nucleophilic involvement of the thiol group neighboring to the activated amide[9] (Figure 1). We synthesized a peptide thioester using

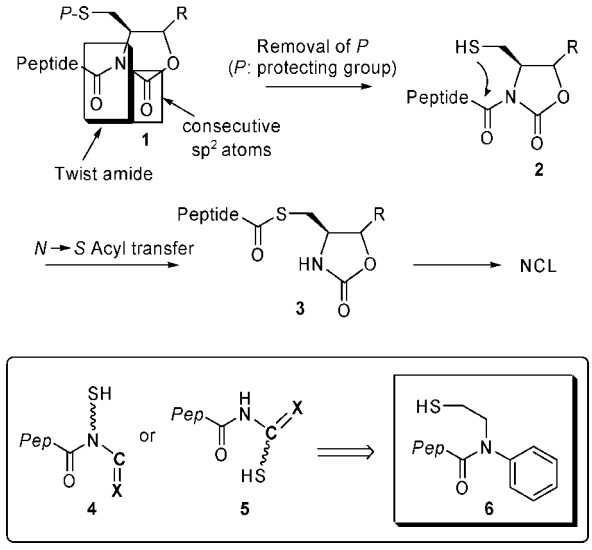
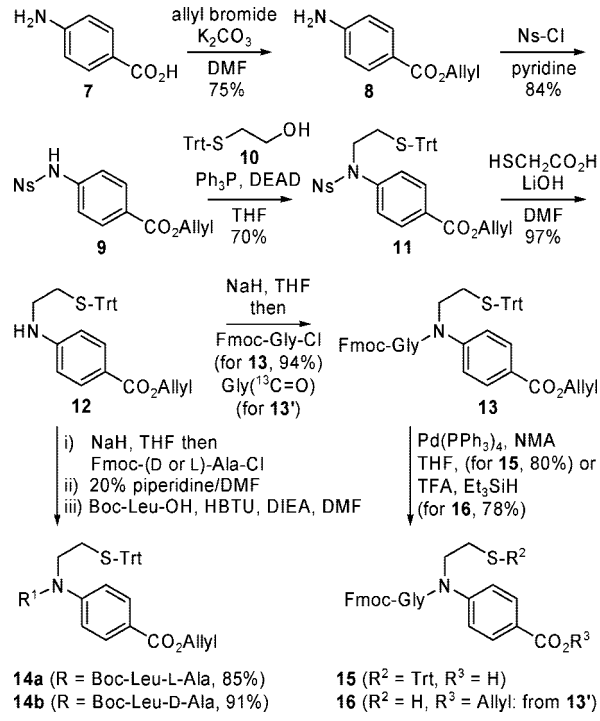


Figure 1. Formation of peptide thioester utilizing peptydyl oxazolidinone and general concept for *N*→*S* acyl-transfer-mediated synthesis of thioester.

the oxazolidinone system with Fmoc protocol; however, partial loss of the peptide from the acylated oxazolidinone and epimerization of the *C*-terminal amino acid during 20% piperidine treatment were observed. Therefore, we have sought a robust *N*→*S* acyl-transfer protocol for thioester synthesis compatible with Fmoc-chemistry. On the basis of the hypothesis that the presence of consecutive sp^2-atoms adjacent to an amide nitrogen (**4** or **5** in Figure 1) should induce nitrogen-pyramidalization and activate the amide bond, we investigated the applicability of anilide derivatives possessing thiol function at an appropriate position. Among the potential structural units, we selected 4-(2-sulfanylethylamino)benzene unit **6** as a core structure following preliminary examination.

Synthesis of requisite substrates **14**−**16** for evaluating the *N*→*S* acyl-transfer-mediated preparation of thioesters is shown in Scheme 1. Starting from 4-aminobenzoic acid **7**, the carboxy and amine units were protected by allyl and *o*-nitrobenzenesulfonyl (Ns) groups,[10] respectively. On the

Scheme 1



resulting Ns-protected ally ester **9**, a trityl (Trt)-protected thiol unit **10**[11] was introduced under Mitsunobu conditions[12] to give ethyl sulfide-modified compound **11**. The Ns group on **11** was removed by sulfanylacetic acid/LiOH in DMF to give *N*-ethyl sulfide aniline **12**. Reaction of **12** with Fmoc-L- or -D-Ala-Cl in the presence of NaH in THF[13] followed by Fmoc-removal and subsequent coupling of Boc-Leu-OH using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)[14]/diisopropylethylamine (DIEA) yielded dipeptide **14a** (L-Ala form) or **14b** (D-Ala form), respectively, required for examination of the susceptibility of the anilide derivatives to base treatment. Acylation of **12** with Fmoc-Gly-Cl (and ^{13}C-carbonyl derivative) gave anilides **13** (and **13′**). The allyl group on **13** was removed by treatment with $Pd(PPh_3)_4$/*N*-methylaniline (NMA)[15] to give the corresponding benzoic acid **15** which served as an indispensable intermediate for the *N*→*S* acyl-transfer-mediated preparation of peptide thioesters. For evaluation of the

(8) For synthesis of peptide thioesters using *N*→*S* acyl transfer except for ref 6, see: (a) Ollivier, N.; Behr, J.-B.; El-Mahdi, O.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2005**, *7*, 2647–2650. (b) Ohta, Y.; Itoh, S.; Shigenaga, A.; Shintaku, S.; Fujii, N.; Otaka, A. *Org. Lett.* **2006**, *8*, 467–470. (c) Nagaike, F.; Onuma, Y.; Kanazawa, C.; Hojo, H.; Ueki, A.; Nakahara, Y.; Nakahara, Y. *Org. Lett.* **2006**, *8*, 4465–4468. (d) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25–28. (e) Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **2007**, *48*, 1903–1905.

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(13) After coupling of Boc-Leu-OH to L- or D-Ala derivative, no epimerization was detected, which indicates that coupling of Fmoc-Ala-Cl with the sodium amide resulting from **12** and NaH does not induce an observable level of racemization. This coupling protocol can be used for other amino acids (Phe, Leu, Val, Ile), although application of the resulting coupling products to the *N*→*S* acyl transfer is underway.

(14) Dourtoglou, V.; Gross, B. *Synthesis* **1984**, 572–574.

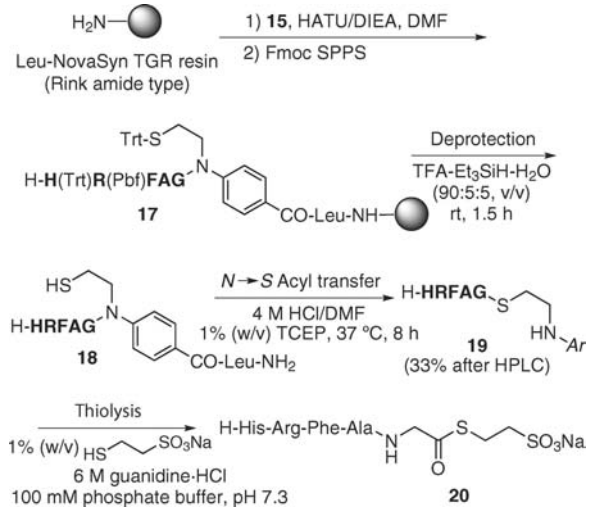
(15) Payne, R. J.; Ficht, S.; Tang, S.; Brik, A.; Yang, Y. Y.; Case, D, A.; Wong, C. H. *J. Am. Chem. Soc.* **2007**, *129*, 13527–13536.

N→*S* acyl transfer, compound **16** was obtained from **13′** by removal of Trt with TFA/Et_3SiH at room temperature for 30 min.[16]

First, the stability of the anilide linkage and the risk of epimerization of Ala in **14** under reaction conditions for Fmoc-removal were confirmed. Compound **14** remained unchanged after base treatment (20% piperidine/DMF) for 24 h at room temperature,[17] which indicated that anilide **15** could be applicable to Fmoc SPPS. Next, trends of the *N*→*S* acyl transfer were examined by exposure of **16** to acidic or basic conditions followed by HPLC/MS analyses. Reaction of **16** with 4 M HCl/dioxane in the presence of 1% (w/v) tris(carboxyethyl)phosphine (TCEP) at room temperature for 2 h was completed to yield the corresponding *S*-acyl compound. Furthermore, the observed trend in 4 M HCl/dioxane was reconfirmed by ^{13}C NMR measurements where the ^{13}C-signal at 167.8 ppm (amide carbonyl) was changed to that at 197.3 and 199.6 ppm (thioester carbonyl).[18] In contrast to the cysteine-derived peptidyl oxazolidinone, no *N*→*S* acyl transfer under basic conditions was observed.

Encouraged by these results, we next attempted to synthesize pentapeptide thioester **19** (H-His-Arg-Phe-Ala-Gly-SR) (Scheme 2). First, anilide **15** was introduced on Leu-

Scheme 2[a]

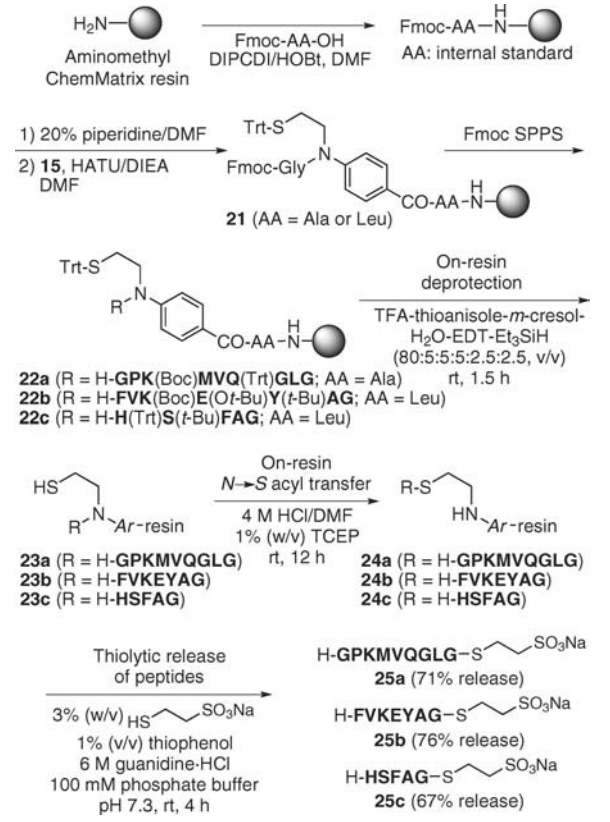


[a] Amino acids are represented by boldface one-letter codes. HATU = 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate. Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

incorporated NovaSyn TGR (Rink amide-type) resin with the aid of HATU[19]/DIEA, and peptide-chain elongation was then conducted using standard Fmoc SPPS. Deprotection of the completed resin **17** with TFA−Et_3SiH−H_2O (90:5:5, v/v) at room temperature for 90 min gave crude anilide peptides **18**[16] which were then subjected to the treatment with 4 M HCl/DMF[20] in the presence of 1% (w/v) TCEP for 8 h at 37 °C to achieve the *N*→*S* acyl transfer. After HPLC purification of the reaction mixture, peptide thioester **19** was obtained in 33% isolated yield based on the protected resin.[21] The resulting peptide **19** was proven to be thioester by the conversion to another thioester **20** via sodium sulfanylethanesulfonate treatment. Preliminary examination of the acyl transfer revealed that reaction with 4 M HCl/DMF with a powerful solubilizing performance allowed anilide peptide **18** to be efficiently converted to the corresponding thioester **19**; however, the use of organic solvent on deprotected peptides sometimes hampers the purification of peptides.

Therefore, we next examined an on-resin *N*→*S* acyl transfer followed by the thiolytic release of peptide thioesters using an aqueous solution (Scheme 3). Three model peptide

Scheme 3



resins **22** with an internal standard amino acid for amino acid analysis (AAA) were prepared using aminomethyl ChemMatrix resin.[22] The internal standard (Ala for **22a** and

(16) Little *N*→*S* acyl transfer occurred during short TFA treatment (at room temperature for ca. 30−90 min) for removal of side-chain protecting groups.

(17) Neither decomposition of the anilide linkage nor epimerization at the Ala residue was observed by HPLC analyses of reactions of **14** with 20% piperidine/DMF; see the Supporting Information.

(18) Nakamura, K.; Sumida, M.; Kawakami, T.; Vorherr, T.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **2006**, *79*, 1773–1780.

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(20) Compared to HCl/dioxane, HCl/DMF shows a powerful solubilizing performance for deprotected peptides.

(21) Generally, a reversed reaction is encountered in the *N*→*S* acyl transfer; however, little regeneration of the amides from the resulting thioesters is observed in our system, which is probably due to low nucleophilicity of the aniline nitrogen.

Leu for **22b** and **22c**) and Fmoc-glycyl anilide **15** were subsequently coupled on the resin with diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) and HATU/DIEA, respectively. Standard Fmoc SPPS on the resulting resins afforded model peptide resins **22**. Deprotection of each resin with TFA−thioanisole−*m*-cresol−H_2O−1,2-ethanedithiol (EDT)−Et_3SiH (80:5:5:5:2.5:2.5, v/v) at room temperature for 1.5 h gave the side-chain-deprotected peptide resin **23**. Each resulting peptide resin was treated with 4 M HCl/DMF−1% TCEP for 12 h at room temperature for the on-resin *N*→*S* acyl transfer and was subsequently subjected to a thiolytic release protocol using 3% (w/v) sodium sulfanylethanesulfonate in the presence of 2% (v/v) thiophenol in 6 M guanidine•HCl−100 mM phosphate buffer at pH 7.3. In all cases, the sequence of treatments mentioned above afforded peptide thioesters **25** with high purity in approximately 70% release yields (**25a**, 71%; **25b**, 76%; **25c**, 67%). Release yields of the thioesters were calculated based on the internal standard whereby resin samples after thiol treatment were hydrolyzed and the resulting hydrolysates were analyzed by AAA.

The use of Fmoc-glycyl aniline derivative **15** in Fmoc chemistry allowed peptide thioesters to be synthesized efficiently. Therefore, we next attempted to synthesize phosphoamino acid-containing peptide thioesters. On the internal standard incorporated resins **21** was constructed a protected peptide chain corresponding to RRVpSVAADG **26** using monobenzyl-protected phosphoamino acids.[23] The application of procedures identical to those used for the preparation of peptide thioesters **25** to the protected resin yielded phosphoamino acid-containing peptide thioester **26** in 67% release yield.

As mentioned in the experiment of treatment of **14** with 20% piperidine/DMF, no epimerization was observed during the Fmoc procedure; however, partial epimerization was detected on treatment of H-Phe-Ala-Ala-*Ar* peptide with 4 M HCl/DMF. Although the reason for the epimerization has yet to be disclosed, reaction with TFA suppressed the epimerization, although the *N*→*S* acyl transfer proceeded slowly.[24,25]

In conclusion, we present an *N*→*S* acyl-transfer-mediated synthesis of peptide thioesters using an *N*-substituted aniline linker. The use of the linker allowed peptide thioesters to be efficiently prepared by Fmoc-SPPS. Various applications of the linker to peptide/protein synthesis via thioesters are currently underway.

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

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(24) *N*→*S* acyl transfer with HCl/DMF proceeds at about two times the reaction rate compared with that with TFA; see the Supporting Information.

(25) Treatment of HPLC-purified thioester sample (Phe-Ala-L-Ala-thioester) with 4 M HCl/DMF at 37 °C for 4 h afforded a mixture of diastereomers (parent peptide (80%) and epimerized Phe-Ala-D-Ala-thioester (20%)).