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N-Sulfanylethylaminooxybutyramide (SEAoxy): A Crypto-Thioester Compatible with Fmoc Solid-Phase Peptide Synthesis

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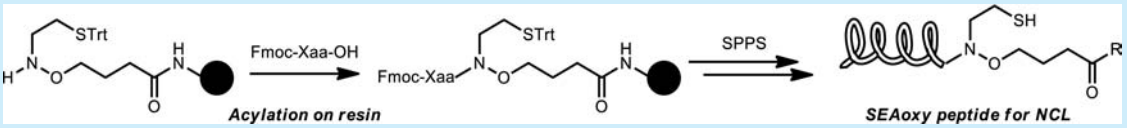
Supporting Information



ABSTRACT: An *N*-sulfanylethylaminooxybutyramide (SEAoxy) has been developed as a novel thioester equivalent for native chemical ligation. SEAoxy peptide was straightforwardly synthesized by conventional Fmoc solid-phase peptide synthesis without a problem. Moreover, SEAoxy peptide could be directly applied to native chemical ligation owing to the intramolecular *N*-to-*S* acyl shift that releases the peptide-thioester *in situ*. This methodology was successfully applied to the synthesis of two bioactive peptides.

Native chemical ligation (NCL) between N-terminal Cys-peptides and peptide-thioesters plays a significant role in the chemical synthesis of proteins.[1] One of the key components of NCL is peptide-thioester or its equivalent. However, peptide-thioesters are prone to be decomposed under the basic conditions employed in conventional Fmoc solid-phase peptide synthesis (SPPS). Even in cases where specially designed mild-base mixtures are applied, significant epimerization at the thioesterified residue occurs.[2] Thus, ever since Kent et al. reported NCL using peptide-thioesters prepared by Boc SPPS, many Fmoc-SPPS-compatible equivalents for peptide-thioester (so-called crypto-thioester) have been developed.[3−5] For example, we have previously reported that the *N*-sulfanyl-ethylanilide (SEAlide) peptide is a versatile crypto-thioester for NCL.[6] Base-treatment-compatible SEAlide peptides (*N*-acyl aniline derivatives) can be synthesized by Fmoc SPPS like other *N*-acyl anilines. Conversely, under phosphate-buffered neutral conditions, intramolecular *N*-to-*S* acyl shift occurs to afford the peptide-thioester. The SEAlide method has been successfully applied to the synthesis of bioactive proteins such as monoglycosylated GM2 ganglioside activator protein[7] and CXCL-14.[8] However, difficulties with the *N*-acylation of SEAlide units hamper their broad application. The poor nucleophilicity of the *N*-alkyl aniline amino group makes direct SPPS of SEAlide peptides difficult. Therefore, Fmoc-Xaa-SEAlide units synthesized by phosphoryl chloride- or thionyl chloride-activating methods[9] with sodium anilide derivatives in solution have been applied to Fmoc SPPS (Figure 1a). To overcome this disadvantage, here we report the novel surrogate *N*-sulfanyl-ethylaminooxybutyramide (SEAoxy) that enables straightforward Fmoc SPPS of thioester equivalents.

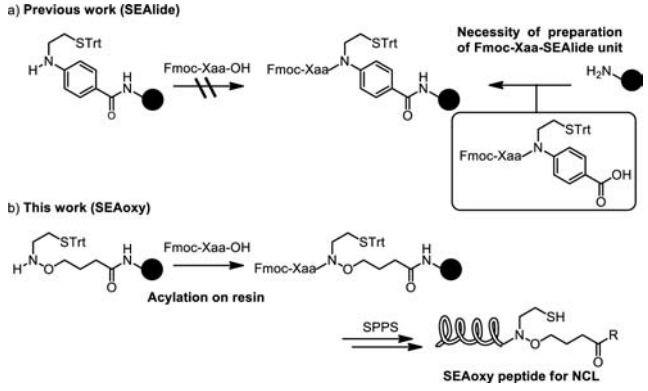


Figure 1. Preparation of peptide thioester equivalents. (a) Previous work (SEAlide), (b) this work (SEAoxy).

Generally, in Cys-containing peptides, *N*-to-*S* acyl shift from the amide to the thioester does not occur under NCL conditions.[4i]The atypical facile *N*-to-*S* acyl shift of the SEAlide structure is partially due to the relatively low basicity (high acidity of the conjugate acid) of the amino group in the aniline structure [the pK_a values of the conjugate acids are *N*-methylaniline, 4.85, and sarcosine (*N*-methyl glycine), 10.01].[10] It is widely accepted that the stronger the conjugate acid of the leaving group is, the better the leaving group is. Unfortunately, because nucleophilicity is generally in proportion to basicity, poorly basic compounds that enable *N*-to-*S* acyl shift are difficult to apply in *N*-acylation reactions. Meanwhile, an atom with an adjacent

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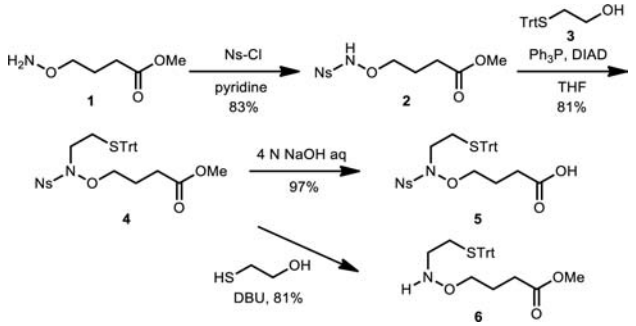
atom with lone pair electrons exhibits relatively high nucleophilicity,[11a] but this effect does not directly affect the basicity of a molecule (i.e., the so-called α effect). For example, highly nucleophilic *N,O*-dimethylhydroxylamine has exceptionally low basicity (pK_a of its conjugate acid: 4.75).[10] As a result, *N,O*-dimethylhydroxylamine exhibits similar basicity to that of *N*-alkylaniline, but shows sufficient nucleophilicity to be acylated with active esters.[11] Taking these factors into account, we devised the idea that *N*-sulfanylethylaminooxybutyramide (SEAoxy) would be a novel crypto-thioester that can be synthesized by straightforward Fmoc SPPS via direct on-resin coupling between Fmoc-Xaa and the SEAoxy unit (Figure 1b). Kent et al. have previously reported an N^{α}-2-mercaptoethyloxy structure as an auxiliary for non-Cys NCL; however, after the ligation reaction, the *S*-acyl isopeptide intermediate was sometimes observed.[11d] This result suggests that such structures exist in an equilibrium between the *S*-acyl and *N*-acyl forms, which also motivated us to undertake this study.

The SEAoxy units for Fmoc SPPS were synthesized as shown in Scheme 1. The aminooxy group of known compound **1**[12] was

Scheme 1. Synthesis of SEAoxy Derivatives



protected with a 2-nitrobenzenesulfonyl (Ns) group[13] to afford compound **2**. The subsequent Mitsunobu reaction with trityl (Trt)-protected compound **3** gave compound **4**. The final deprotection of the methyl ester gave carboxylic acid **5**, which enabled attachment of the SEAoxy structure to resins. In an additional reaction, the Ns group of **4** was removed to give compound **6** which was used for the investigation of the efficiency of the acylation of SEAoxy.

Initially, the reactivity of the NH group of SEAoxy unit **6** or SEAlide unit **7** with an active ester was compared using solution-phase chemistry. The coupling reaction between each unit (**6** or **7**) and Fmoc-Ala (5 equiv) was conducted using a combination of *N,N′*-diisopropylcarbodiimide (DIC) and Oxyma Pure.[14] HPLC analyses (Figure 2) showed that SEAoxy unit **6** was fully acylated with Fmoc-Ala within 3 h. By contrast, acylation of SEAlide unit **7** was not observed even after 20 h. These results indicate that the SEAoxy unit has higher nucleophilicity compared to that of the SEAlide unit and might be suitable for direct on-resin introduction of Fmoc-Xaa, unlike the SEAlide unit.

Next, we evaluated the compatibility of the SEAoxy structure using various model peptides (H-Leu-Tyr-Arg-Ala-Asn-Xaa-SEAoxy-Phe-NH_2, **8a**–**f**) as shown in Scheme 2. Initially, SEAoxy unit **5** was introduced on a Phe-Rink amide resin **9** by the conventional DIC/Oxyma Pure method to yield the Ns-protected resin **10**. The Ns group of **10** was removed by mercaptoethanol/1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to liberate the free aminooxy group.[15] Then, Fmoc-Xaa (Gly, Ala,

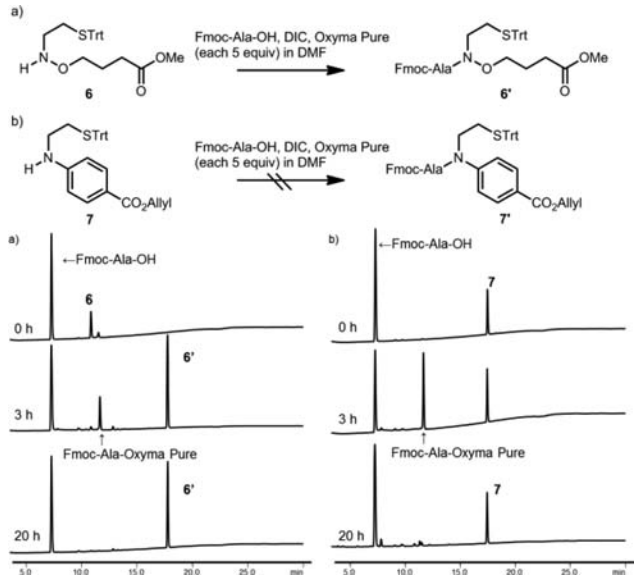
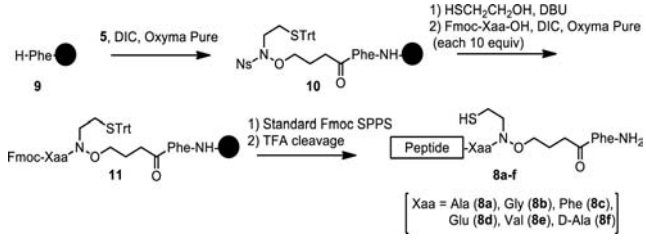


Figure 2. HPLC profiles (220 nm) of the coupling of Fmoc-Ala with (a) SEAoxy unit **6**, or (b) SEAlide unit **7**.

Scheme 2. Syntheses of SEAoxy Peptides



Phe, Glu, Val, or D-Ala, each 10 equiv) was coupled with the aminooxy group on the resin using the conventional DIC/Oxyma Pure method. With the Fmoc-protected resins **11** thus obtained, the subsequent peptide assemblies were carried out by standard Fmoc SPPS. The resulting resins were treated with a TFA cocktail to give the corresponding model SEAoxy peptides **8a**–**f** (Xaa = Ala (**8a**), Gly (**8b**), Phe (**8c**), Glu (**8d**), Val (**8e**), and D-Ala (**8f**)). In all cases, reasonable amounts of free SEAoxy peptides (25–49% yields) were obtained after HPLC purification. Moreover, only 0.2–0.3% epimerization during the preparation of SEAoxy peptide **8a** was detected, as evaluated using the authentic corresponding D-Ala epimer **8f** (see Supporting Information). These positive results indicate that the SEAoxy unit is compatible with both on-resin acylation with Fmoc-Xaa and the subsequent Fmoc SPPS. Furthermore, the stability of the SEAoxy peptide against base treatment during Fmoc SPPS was investigated. The *S*-Trt-protected[16] SEAoxy peptide diastereomers [H-Leu-Tyr-Arg-Ala-Asn-L-Ala-SEAoxy-(Trt)-Phe-NH_2 (**8a′**) and H-Leu-Tyr-Arg-Ala-Asn-D-Ala-SEAoxy(Trt)-Phe-NH_2 (**8f′**)] prepared from **8a** and **8f**, respectively, were treated with 20% piperidine/DMF for 24 h (see Supporting Information). In these model experiments, degradation and epimerization were not detected. These outcomes clearly indicate that the SEAoxy unit is applicable to Fmoc SPPS without problems.

Then, in order to investigate whether SEAoxy peptides function as crypto-thioesters, we evaluated NCL reactions using SEAoxy peptides **8a**–**f** or SEAlide peptide **12** (H-Leu-Tyr-Arg-Ala-Asn-Gly-SEAlide-Phe-NH_2) with a model Cys-peptide **13**

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(H-Cys-Ser-Pro-Gly-Tyr-Ser-NH_2). As shown in Figure 3, the SEAoxy-based NCL reactions were almost complete within 24 h,

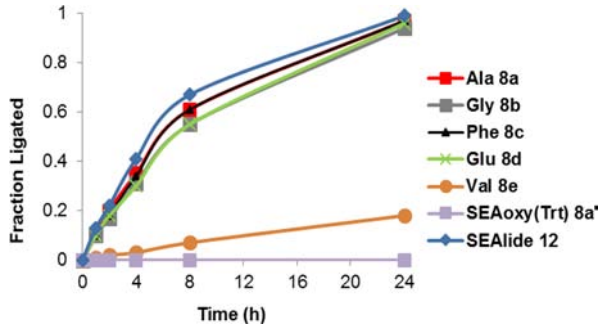

Figure 3. Time progress of NCL between model thioester equivalents and Cys-peptide **13**. The fraction ligated was calculated using the areas under the peaks of HPLC at λ = 280 nm.

except for the reaction at the Val site. Generally, the Val site is known as one of the most difficult sites for conventional NCL because Val is a bulky amino acid. Moreover, only 1% epimerization occurred at the Ala site during the NCL reaction (see Supporting Information). These results are similar to those using SEAlide peptides reported previously by our group,[9] indicating that the NCL efficiencies of SEAlide and SEAoxy are comparable. In addition, Trt-protected SEAoxy peptide **8a′** did not react with Cys-peptide **13** at all. This result suggests that an intramolecular *N*-to-*S* acyl shift takes place during SEAoxy-based NCL.

As a special characteristic of the SEAlide peptide, it is known that *N*-to-*S* acyl shift to afford the peptide-thioester takes place in the presence of phosphate[6b] but not in the absence of phosphate. Owing to this unique characteristic, SEAlide peptides have been successfully applied to N-to-C one-pot kinetically controlled ligation.[6b,7,8] In this context, we confirmed whether the SEAoxy peptide has a similar characteristic. Unfortunately, SEAoxy peptide **8b** underwent NCL in both phosphate and phosphate-free HEPPS buffer (see Supporting Information). We cannot currently explain the reason for this difference, but it confirms that SEAlide exhibits unique characteristics in NCL chemistry.

Finally, we applied SEAoxy-based NCL for the synthesis of human brain natriuretic peptide (BNP)-32[17] and protoxin-I (ProTx-I)[18] as model bioactive peptides. In the case of the synthesis of BNP-32 (Figure 4), the peptide-SEAoxy **14** and Cys-peptide **15** were reacted under conventional NCL conditions (6 M Gn·HCl, 200 mM Na_2HPO_4, 200 mM MPAA, 100 mM TCEP, pH 7.4, 37 °C). The NCL reaction at the Gly site was achieved after 48 h to afford BNP-32 2SH form **16** in 79% yield after HPLC purification. The subsequent oxidation using I_2 smoothly converted **16** to BNP-32 (**17**) (73% yield). We also investigated whether the SEAoxy unit can be adopted for thiol-additive-free NCL[19] to synthesize the Cys-rich model peptide ProTx-I (Figure 5). NCL at the Thr site between N-fragment **18** and C-fragment **19** was attempted without any thiol additives. The ligation was successfully accomplished to afford the reduced ProTx-I (**20**) within 48 h. The subsequent one-pot oxidative folding reaction by diluting the ligation mixture with 1 M NH_4OAc buffer (pH 7.8) containing 1 M Gn·HCl in the presence of reduced and oxidized gluthathione (GSH/GSSG) gave ProTx-I (**21**) in 48% yield (two steps) after HPLC purification. Although the ligation using SEAoxy peptide was slightly slow, the yield was similar to that previously reported by

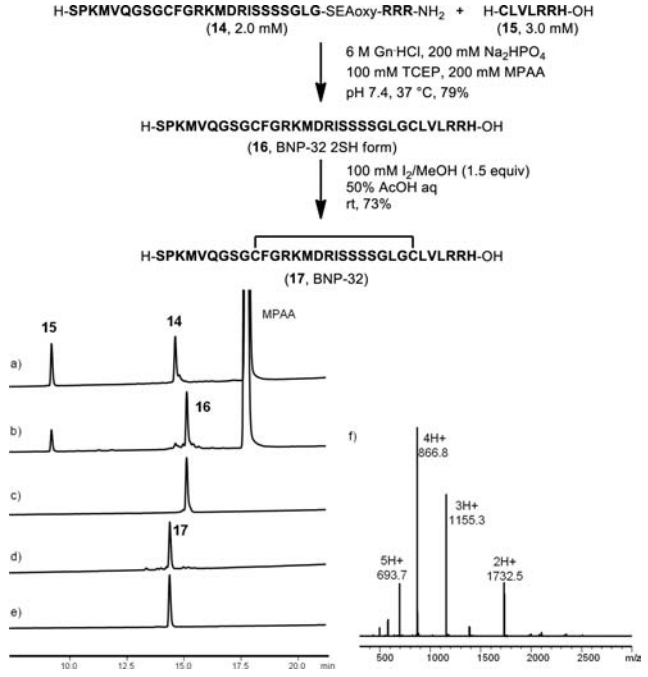

Figure 4. HPLC profiles (220 nm) and ESI-MS of the synthesis of BNP-32 (**17**): (a) NCL between **14** and **15** (t < 3 min), (b) NCL (t = 48 h), (c) purified product **16**, (d) oxidation of **16** (t = 1 min), (e) purified product **17**, (f) ESI-MS of **17** (calcd 3462.7; obsd 3463.1).

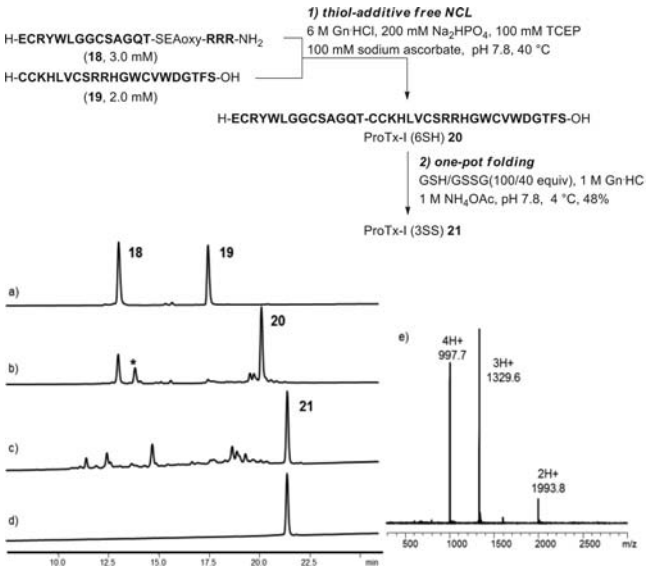

Figure 5. HPLC profiles (220 nm) and ESI-MS of the synthesis of ProTx-I (**21**): (a) NCL between **18** and **19** (t < 3 min), (b) NCL (t = 48 h), (c) folding reaction (t = 24 h), (d) purified product **21**, (e) ESI-MS of **21** (calcd 3985.7; obsd 3986.3). * hydrolysate of **18**.

our group using a peptide-alkylthioester,[19a] suggesting that the efficiency of NCL using the SEAoxy peptide is comparable to that using a peptide-alkylthioester.

In summary, based on our experience with the SEAlide peptide, we successfully developed the SEAoxy unit as a novel thioester equivalent for NCL. We confirmed that this surrogate is fully compatible with straightforward Fmoc SPPS and works as a crypto-thioester under standard NCL conditions. Using this method, BNP-32 and ProTx-I were synthesized. Based on these results, we conclude that the efficiency of SEAoxy-based NCL is

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comparable to those using many previously reported elegant thioester equivalents. SEAoxy-based NCL worked regardless of the presence or absence of phosphate, unlike SEAlide-based NCL. Thus, skillful combination of peptide-thioesters, SEAoxy peptides, SEAlide peptides, and other peptide-thioester equivalents in NCL will help effective protein preparation in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03055.

Detailed experimental procedures, characterization, spectroscopic and chromatographic data (PDF)

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Notes

The authors declare no competing financial interest.

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