Organic LETTERS

Letter

pubs.acs.org/OrgLett

Development of an Anilide-Type Scaffold for the Thioester Precursor *N*-Sulfanylethylcoumarinyl Amide

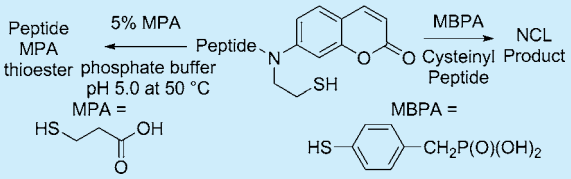
Mitsuhiro Eto,[†,§] Naoto Naruse,[†,§] Kyohei Morimoto,[†] Kosuke Yamaoka,[†] Kohei Sato,[†,∥] Kohei Tsuji,[†,⊥] Tsubasa Inokuma,[†] Akira Shigenaga,[*,†,‡] and Akira Otaka[*,†]

[†]Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University, Tokushima 770-8505, Japan

[‡]PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan

Supporting Information

ABSTRACT: *N*-Sulfanylethylcoumarinyl amide (SECmide) peptide, which was initially developed for use in the fluorescence-guided detection of promoters of N−S acyl transfer, was successfully applied to a facile and side reaction-free protocol for N−S acyl-transfer-mediated synthesis of peptide thioesters. Additionally, 4-mercapto-benzylphosphonic acid (MBPA) was proven to be a useful catalyst for the SECmide or *N*-sulfanylethylanilide (SEAlide)-mediated NCL reaction.



Native chemical ligation (NCL) featuring the use of peptide thioesters and N-terminal cysteinyl peptides has paved the way for easy chemical access to small size proteins consisting of up to about 100 residues.[1] The NCL-mediated synthesis of proteins over 150 residues has also been accomplished with a C-to-N- or N-to-C-directed sequential protocol using more than two peptide fragments.[2] To avoid byproducts resulting from intramolecular NCL of the middle fragment in sequential NCL, special consideration must be given to the molecular form of middle fragments used in the second NCL step.

In the C-to-N-directed protocol, the middle fragment has an N- and/or S-protected N-terminal cysteine residue,[3] whereas in the N-to-C-directed protocol, the middle fragment has an N-terminal cysteinyl thioester in which the thioester (or thioester equivalent) possesses tunable reactivity toward the cysteine residue.[4] Because the N−S acyl-transfer-mediated preparation of thioester is compatible with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry,[5] we developed *N*-sulfanylethylanilide (SEAlide) peptide as a crypto thioester and applied it successfully to one-pot/N-to-C-directed sequential NCL (Figure 1).[6] In this protocol, N-terminal cysteinyl SEAlide peptide **1** was used as middle fragment. In the absence of phosphate salts, the SEAlide moiety remained essentially intact and inactive in the first NCL, whereas addition of phosphate salts enabled the SEAlide moiety to function as thioester, thereby yielding ligated product in one-pot manner. Here, phosphate salts acted as a promoter of N−S acyl transfer. Therefore, we sought a more effective alternative promoter of N−S acyl transfer using a fluorescence-guided approach involving 7-sulfanylethyl-7-aminocoumarine scaffold **2**, anticipating that **2** behaves in a manner similar to that of SEAlide in the presence of acyl-transfer promoters (Figure 2).[7]

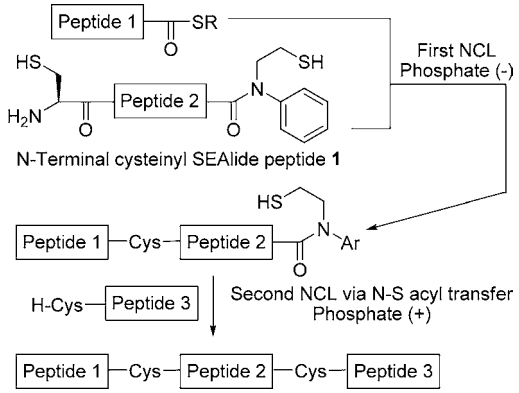


Figure 1. One-pot/N-to-C-directed sequential NCL using SEAlide peptide.

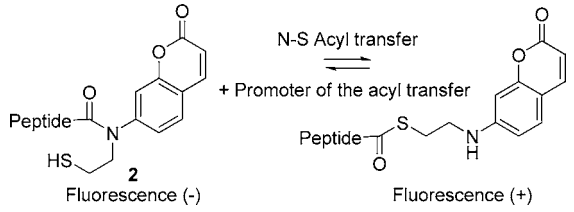


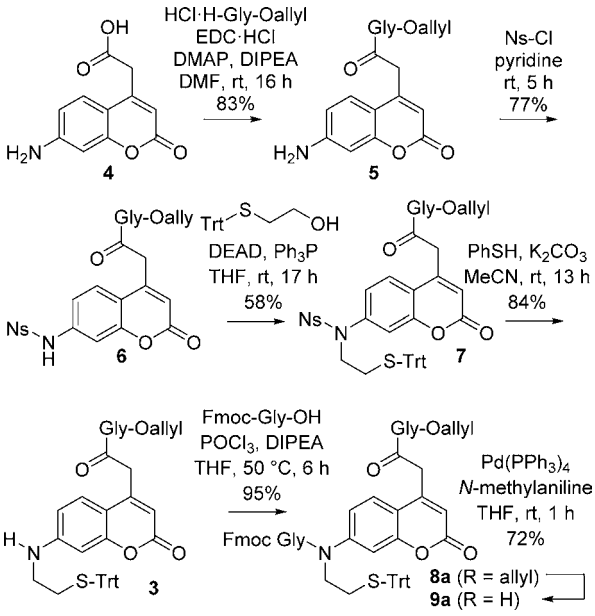
Figure 2. Fluorescence-guided detection of promoters of N−S acyl transfer.

Synthesis of 7-aminocoumarin-type linker **3** was performed according to Scheme 1. Starting from the known compound **4**, condensation with H-Gly-Oally in the presence of 1-ethyl-3-(3-

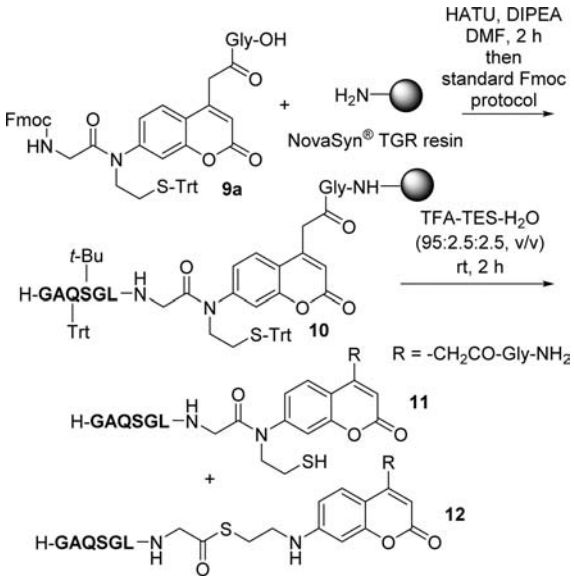
Received: July 26, 2016
Published: August 16, 2016

DOI: 10.1021/acs.orglett.6b02207
Org. Lett. 2016, 18, 4416−4419

Scheme 1



Scheme 2



(dimethylamino)propyl)carbodiimide hydrochloride (EDC·HCl), diisopropylethylamine (DIPEA), and 4-dimethylamino pyridine (DMAP) in dimethylformamide (DMF) at ambient temperature for 16 h gave compound **5** in 83% isolated yield. Subsequent incorporation of an *o*-nitrobenzenesulfonyl (Ns) group with NsCl in pyridine proceeded efficiently to yield the desired material **6** in 77% isolated yield.[5] A Mitsunobu reaction of **6** with triphenylmethylsulfanylethyl alcohol in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine (Ph_3P) at ambient temperature for 17 h gave the sulfanyl-unit-incorporated compound **7** in 58% isolated yield. Then the Ns group was removed by thiophenol/K_2CO_3 in MeCN at ambient temperature overnight to afford the desired fluorescence 7-aminocoumarine-type linker **3** in 84% isolated yield.

Next, attachment of Fmoc amino acids to the linker **3** was examined. Although the initially attempted coupling of Fmoc-Gly-Cl with **3** in the presence of NaH did not proceed efficiently as in the case for the *N*-sulfanylethyl aniline linker of the SEAlide, another option (which was the use of $POCl_3$ and DIPEA in THF for coupling of Fmoc-Gly-OH) yielded the desired *N*-Fmoc-glycyl-*N*-sulfanylethylaminocoumarin **8a** in 95% isolated yield. Encouraged by this result, we applied the above $POCl_3$-mediated conditions to the coupling of 19 other proteogenic amino acids.[7] As shown in Table SI-1 in the Supporting Information, condensations in from moderate to good yields were achieved at 50 °C within 20 h, except for Asp(O-*t*-Bu) and Pro. Because the use of the thioester derived from Asp[8] or Pro[9] has had limited success in NCL, further examination of Asp or Pro was not attempted. Efficient removal of the allyl group was achieved by the action of $Pd(Ph_3P)_4$ (0.1 equiv) in the presence of *N*-methylaniline in THF at room temperature for 1 h to afford the acidic compounds **9a** in good yields. Then the model peptide resin H-**GAQ**(Trt)**S**(*t*-Bu)**GLG**-linker-resin **10** was constructed on Rink-amide-type NovaSyn TGR resin. Coupling of **9a** with the NovaSyn TGR resin using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) in the presence of DIPEA in DMF followed by standard Fmoc SPPS protocols afforded the protected resin **10** (Scheme 2). Treatment of **10** with trifluoroacetic acid (TFA)−triethylsilane (TES)−H_2O (95:2.5:2.5, v/v) at room temperature for 2 h gave the *N*-peptidyl-*N*-sulfanylethylcoumarinyl amide **11**. Here, we refer to *N*-sulfanylethylcoumarinyl amide peptide as SECmide peptide. High-performance liquid chromatography (HPLC) analysis of the resulting materials indicated that the crude sample consisted of amide-type SECmide **11** and corresponding thioester-type SECmide **12** in a ratio of about 14:1 (Figure SI-1). Prolonged acidic deprotection resulted in no significant change of the product ratio.

Purification of the mixture by HPLC with acidic eluents containing TFA undoubtedly gave pure amide-type peptide **11**; however, regeneration of thioester-type peptide **12** was observed after removal of acidic solvent by lyophilization. Therefore, HPLC isolation of homogeneous amide-type SECmide peptide needs to be performed under neutral conditions. Actually, the use of a 10 mM aqueous ammonium acetate (NH_4OAc) (pH 6.7)−MeCN system as an eluent followed by lyophilization gave the homogeneous material.[8] Of note is that incubation of the resulting homogeneous amide-type SECmide peptide **11** in 0.1% TFA-containing aqueous solution resulted in a mixture of the amide **11** and thioester **12**, with the ratio of **12** to **11** gradually increasing over time until an equilibrium ratio of **11**:**12** = 1.0:0.7 was reached in about 24 h (Figure SI-2).

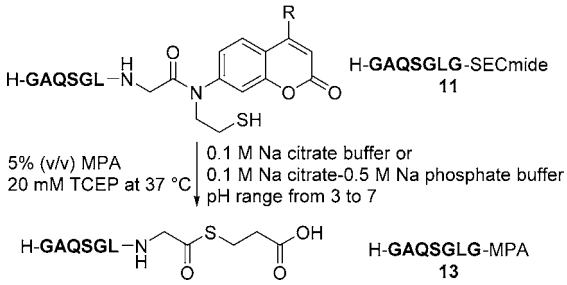
Although the amide-type SEAlide peptide is also converted to the corresponding thioesters under acidic conditions, harsh acidic treatment with 4 M HCl in DMF for several hours is required.[6a] Such treatment was not ideal due to accompanying side reactions including racemization of the C-terminal amino acid. On the other hand, N−S acyl transfer of the amide-type SECmide under less acidic conditions (0.1% TFA) was observed, which was not the case for the SEAlide peptide. This unexpected observation prompted us to investigate whether the SECmide peptide could be completely converted to its thioester form under mild acidic conditions without accompanying side products before performing the fluorescence-guided search of promoters for the N−S acyl transfer.

In terms of the N−S acyl transfer under mild acidic conditions, Hojo and co-workers reported that the *N*-peptidyl-

DOI: 10.1021/acs.orglett.6b02207
Org. Lett. 2016, 18, 4416−4419

N-ethylcysteine was cleanly converted to the corresponding thioester by treatment of 3-mercaptopropionic acid (MPA) in H_2O at pH 1.0 within 12 h−3 days.[10] Bis(2-sulfanylethyl)amido peptides also underwent the transthioesterfication with MPA under milder acidic conditions at pH 4.0.[11] Thus, we examined the MPA-assisted conversion of the amide-type SECmide **11**. Although the attempted reaction of **11** in 5% MPA in H_2O undoubtedly proceeded to afford the desired MPA thioester **13**, completion of the reaction required 5 days. Therefore, we examined possible enhancement of the conversion rate by varying reaction pH in the presence or absence of phosphate salts. Reactions were carried out in the presence of 5% MPA and 20 mM TCEP in 0.1 M sodium citrate buffer or in 0.1 M sodium citrate−0.5 M sodium phosphate buffer over a pH range from 3−7. The lack of the intermediary thioester-type SECmide **12** formation during the reactions indicated that the rate-determining step was the N−S acyl transfer, and since a large excess equivalent of MPA was used, the disappearance of the amide-type SECmide **11** showed a pseudo-first-order dependence on the concentration of **11**. The kinetic data are summarized in Table 1.

Table 1. Optimization of MPA-thioester 13 Synthesis under Mild Acidic Conditions



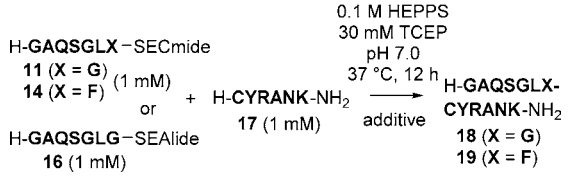
entry	phosphate salts	pH	half-life of **11** $t_{1/2}$ (h)
1	−	7.0	8.6
2	−	6.0	6.7
3	−	5.0	1.9
4	−	4.5	1.2
5	−	4.0	1.4
6	−	3.5	2.6
7	−	3.0	4.4
8	+	7.0	0.78
9	+	6.0	0.57
10	+	5.0	0.47
11	+	4.0	0.57

The reaction was obviously dependent on pH in the presence or absence of phosphate salts. Maximum reaction rates with or without phosphate salts were obtained under slightly acidic conditions as follows: at pH 4.5 without phosphate; at pH 5.0 with phosphate. Additionally, the presence of phosphate salts greatly enhanced the N−S acyl transfer of the SECmide peptide as was the case with the SEAlide peptide. Actually, conversion of the amide-type SECmide **11** to the thioester **13** with 5% MPA in 0.5 M phosphate in the presence of 50 mM ascorbate and 20 mM TCEP at pH 5.0 went to completion within 4 h at 50 °C to afford the desired MPA thioester **13** in 68% isolated yield (Figure SI-3). The phenylalanyl SECmide peptide, H-**GAQSGLF**-SECmide **14**, was also almost completely converted to the corresponding MPA thioester, H-**GAQSGLF**-MPA **15**, under conditions mentioned above within 8 h at 50 °C (half-life of **14** $t_{1/2}$ = 1.8 h, Figure SI-4). Furthermore, no detectable amount of epimerization occurred during the conversion of **14** to **15**. These results indicated that the SECmide peptides are sufficiently applicable to the N−S acyl-transfer-mediated synthesis of peptide thioesters (Figure SI-5).

Next, we attempted an extensive search for promoters of N−S acyl transfer, with the amide-type SECmide peptide **11** subjected to NCL conditions including a large excess amount of cysteine in the presence of a wide variety of additives. The time course of the fluorescence intensity of the NCL mixture including 0.3 M additive in the presence of 40 mM MPAA[12] and 30 mM TCEP at pH 5, 6, or 7 at 37 °C was measured using a plate reader (λ_{ex} = 373 nm; λ_{em} = 465 nm). The amount of **11** remaining was estimated based on relative fluorescence intensity. Because there was a 200-fold excess of cysteine in the reaction mixture, the N−S acyl transfer of **11** (which was the rate-determining step) showed first-order dependence on the concentration of **11**, and the half-lives of **11** in the presence of various additives were determined. As shown in Figure SI-6, no additives were much superior to phosphate salts. However, phosphoric- or phosphonic-containing substances were also effective promoters albeit to a lesser extent. Among such substances, 4-mercaptobenzylphosphonic acid (MBPA)[13] had thiol and phosphonic acid moieties that might enable dual promotion of NCL involving SECmide or SEAlide peptides.

We then evaluated the applicability of the SECmide peptide versus SEAlide peptide to NCL using MBPA or phosphate salts as the promoter. Model SECmide or SEAlide peptide **11**, **14**, or **16** was subjected to ligation with the N-terminal cysteinyl peptide, H-**CYRANK**-NH_2 **17**, under various reaction conditions to examine the feasibility of direct involvement of the SECmide peptide in NCL. Results are summarized in Table 2

Table 2. Effect of Additives on the NCL of SECmide or SEAlide Peptide with N-Terminal Cysteinyl Peptide



entry	peptide	additive[a]	fraction ligated[b]
1	**11**	MPAA	0.11
2	**11**	MBPA	0.83
3	**11**	MPAA + Na phosphate	0.67
4	**14**	MPAA	0.03
5	**14**	MBPA	0.44
6	**14**	MPAA + Na phosphate	0.33
7[c]	**14**	MPAA	0.05
8[c]	**14**	MBPA	0.77
9[c]	**14**	MPAA + Na phosphate	0.51
10	**16**	MPAA	0.13
11	**16**	MBPA	0.86
12	**16**	MPAA + Na phosphate	0.61

[a]40 mM of additive(s) was (were) added. [b]The fraction ligated was determined by HPLC separation and the fraction integrated by spectrophotometry. The ligated peptide **18** (or **19**) (integ 18 (or integ 19)) detected at 220 nm is expressed as a fraction of the total (unreacted **17** (integ 17) plus integ 18 (or integ 19)). [c]Reactions were conducted at 50 °C.

DOI: 10.1021/acs.orglett.6b02207
Org. Lett. 2016, 18, 4416−4419

(progress of reactions including racemization check was shown in Figure SI-7). As expected, the SECmide peptide **11** was proven to show NCL reactivity in an efficacy similar to that observed for the SEAlide peptide **16** (entries 2 or 3 vs 11 or 12). Of note is the fact that MBPA acted as a dual-functional promoter, greatly enhancing the NCL of the SECmide and SEAlide peptides as well as phosphate salts + MPAA system. Sterically hindered phenylalanyl SECmide **14** could be also used in direct NCL with **17** in the presence of MBPA, even though an elevated temperature was required (50 °C) (entries 5 vs 8).

Finally, stability of the anilide linkage in the SECmide during piperidine treatment was confirmed by exposure of the protected peptide resin, H-**H**(Trt)**S**(*t*-Bu)**FAG**-SECmide-(Trt)-**L**-aminomethyl ChemMatrix resin **20**, in 20% piperidine, and then detemination of the ratio of Phe to Leu by amino acid analysis. The ratio indicated that the anilide linkage remained intact even after 20 h of treatment with piperidine.

In conclusion, the SECmide peptide, initially developed for the fluorescence-guided screening of promoters for N−S acyl transfer, has shown its utility in N−S acyl-transfer-mediated synthesis of thioesters under mild acidic conditions without accompanying side reactions. In addition to phosphate salts, MBPA possessing thiol and phosphonic acid moieties was shown to be a dually functional promoter for SEAlide (or SECmide)-mediated NCL. Further uses of SECmide peptide and/or MBPA are now under investigation in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02207.

General procedures and additional HPLC, NMR, and MS data (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: shigenaga.akira@tokushima-u.ac.jp.
*E-mail: aotaka@tokushima-u.ac.jp.

Present Addresses

[‖](K.S.) Department of Applied Chemistry and Biochemical Engineering, and Green Energy Research Division, Shizuoka University, Hamamatsu, Shizuoka 432-8561, Japan.
[⊥](K.T.) Chemical Biology Laboratory, National Cancer Institute National Institutes of Health, Frederick, MD 21702.

Author Contributions

[§]M.E. and N.N. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) and PRESTO, JST.

■ DEDICATION

This paper is dedicated to Professor Nobutaka Fujii on the occasion of his retirement from Kyoto University.

■ REFERENCES

(1) (a) Dawson, P.; Muir, T.; Clark-Lewis, I.; Kent, S. *Science* **1994**, *266*, 776−779. (b) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960. (c) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338−351.

(2) (a) Aihara, K.; Yamaoka, K.; Naruse, N.; Inokuma, T.; Shigenaga, A.; Otaka, A. *Org. Lett.* **2016**, *18*, 596−599. (b) Raibaut, L.; Ollivier, N.; Melnyk, O. *Chem. Soc. Rev.* **2012**, *41*, 7001−7015. (d) Lee, J. Y.; Bang, D. *Biopolymers* **2010**, *94*, 441−447. (e) Bang, D.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534−2538.

(3) (a) Boerema, D. J.; Tereshko, V. A.; Kent, S. B. H. *Biopolymers* **2008**, *90*, 278−286. (b) Li, J.; Li, Y.; He, Q.; Li, Y.; Li, H.; Liu, L. *Org. Biomol. Chem.* **2014**, *12*, 5435−5441. (c) Pan, M.; He, Y.; Wen, M.; Wu, F.; Sun, D.; Li, S.; Zhang, L.; Li, Y.; Tian, C. *Chem. Commun.* **2014**, *50*, 5837−5839. (d) Tang, S.; Si, Y.-Y.; Wang, Z.-P.; Mei, K.-R.; Chen, X.; Cheng, J.-Y.; Zheng, J.-S.; Liu, L. *Angew. Chem., Int. Ed.* **2015**, *54*, 5713−5717. (e) Jbara, M.; Maity, S. M.; Seenaiah, M.; Brik, A. *J. Am. Chem. Soc.* **2016**, *138*, 5069−5075.

(4) (a) Torbeev, V. Y.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2007**, *46*, 1667−1670. (b) Ollivier, N.; Vicogne, J.; Vallin, A.; Drobecq, H.; Desmet, R.; El Mahdi, O. E.; Leclercq, B.; Goormachtigh, G.; Fafeur, V.; Melnyk, O. *Angew. Chem., Int. Ed.* **2012**, *51*, 209−213. (c) Thompson, R. E.; Liu, X.; Alonso-García, N.; Pereira, P. J. B.; Jolliffe, K. A.; Payne, R. J. *J. Am. Chem. Soc.* **2014**, *136*, 8161−8464. (f) Raibaut, L.; Drobecq, H.; Melnyk, O. *Org. Lett.* **2015**, *17*, 3636−3639.

(5) (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) Zheng, J. S.; Chang, H. N.; Wang, F. L.; Liu, L. *J. Am. Chem. Soc.* **2011**, *133*, 11080−11083. (d) Zheng, J.-S.; Chen, X.; Tang, S.; Chang, H.-N.; Wang, F.-L.; Zuo, C. *Org. Lett.* **2014**, *16*, 4908−4911. (e) Burlina, F.; Papageorgiou, G.; Morris, C.; White, P. D.; Offer, J. *Chem. Sci.* **2014**, *5*, 766−770.

(6) (a) Tsuda, S.; Shigenaga, A.; Bando, K.; Otaka, A. *Org. Lett.* **2009**, *11*, 823−826. (b) Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Sumikawa, Y.; Sakamoto, K.; Otaka, A. *ChemBioChem* **2011**, *12*, 1840−1844. (c) Sato, K.; Shigenaga, A.; Kitakaze, K.; Sakamoto, K.; Tsuji, D.; Itoh, K.; Otaka, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 7855−7859. (d) Otaka, A.; Sato, K.; Shigenaga, A.; Chemical Synthesis of Proteins Using N-Sulfanylethylanilide Peptides, Based on N−S Acyl Transfer Chemistry. In *Protein Ligation and Total Synthesis II*; Liu, L., Eds.; Topics in Current Chemistry; Springer International Publishing, 2015; Vol. *363*, pp 33−56.

(7) Maly, D. J.; Leonetti, F.; Backes, B. J.; Dauber, D. S.; Harris, J. L.; Craik, C. S.; Ellman, J. A. *J. Org. Chem.* **2002**, *67*, 910−915.

(8) Sakamoto, K.; Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Hibino, H.; Nishiuchi, Y.; Otaka, A. *J. Org. Chem.* **2012**, *77*, 6948−6958.

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

(10) (a) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25−28. (b) Asahina, Y.; Kamitori, S.; Takao, T.; Nishi, N.; Hojo, H. *Angew. Chem., Int. Ed.* **2013**, *52*, 9733−9737.

(11) Dheur, J.; Ollivier, N.; Vallin, A.; Melnyk, O. *J. Org. Chem.* **2011**, *76*, 3194−3202.

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

(13) (a) Gurbhele-Tupkar, M. C.; Perez, L. R.; Silva, Y.; Lees, W. J. *Bioorg. Med. Chem.* **2008**, *16*, 2579−2590. (b) Cowper, B.; Sze, T. M.; Premdjee, B.; Bongat White, A. F.; Hacking, A.; Macmillan, D. *Chem. Commun.* **2015**, *51*, 3208−3210.

DOI: 10.1021/acs.orglett.6b02207
Org. Lett. 2016, 18, 4416−4419