Cysteine-free Intramolecular Ligation of *N*-Sulfanylethylanilide Peptide Using 4-Mercaptobenzylphosphonic Acid: Synthesis of Cyclic Peptide, Trichamide

Keisuke Aihara[a]
Tsubasa Inokuma[a]
Takahisa Jichu[a]
Zhenjian Lin[b]
Feixue Fu[c]
Kosuke Yamaoka[a]
Akira Shigenaga[a]
David A. Hutchins[c]
Eric W. Schmidt[b]
Akira Otaka*[,a]

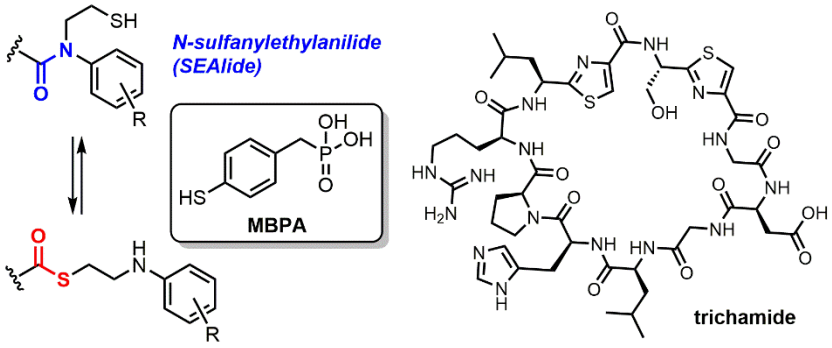
[a] Institute of Biomedical Sciences and Graduate School of Pharmaceutical Sciences, Tokushima University, Shomachi, Tokushima 770-8505, Japan.

[b] Department of Medicinal Chemistry, University of Utah, Salt Lake City UT 84112 USA

[c] Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA

* indicates the main/corresponding author.

E-mail: aotaka@tokushima-u.ac.jp.



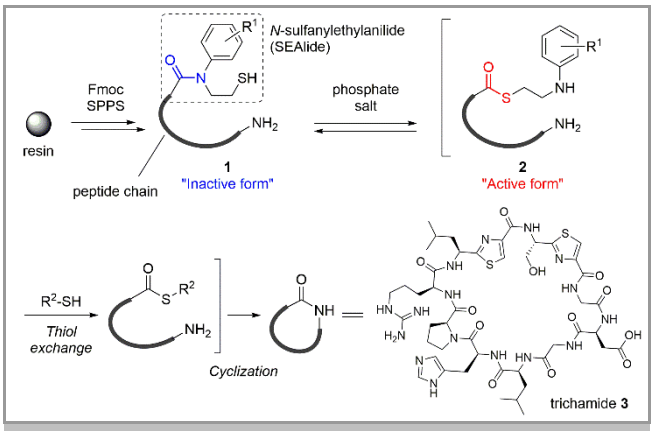
Received:
Accepted:
Published online:
DOI:

Abstract An *N*-sulfanylethylanilide (SEAlide)-based ligation was developed for the preparation of trichamide, a thiazole-containing cyclic peptide isolated from bloom-forming cyanobacterium *Trichodesmium erythraeum*. In this cysteine-free ligation, 4-mercaptobenzylphosphonic acid (MBPA) functions as a dual promoter both for the N–S acyl-transfer-mediated activation of the SEAlide unit and for subsequent ligation. Furthermore, we established a high-yielding route to enantiomerically pure thiazole amino acids using a one-pot Hantzsch process.

Key words

A wide variety of cyclic peptides and depsipeptides have been discovered from natural sources, constituting a potential chemical space for drug seeds or leads.[1] Additionally, by aiding in the arrangement of pharmacophore residues, structural simulation has provided a rational approach to develop cyclic peptide-based drugs.[2] Because of the importance of cyclization, much effort has focused on developing methods for the formation of the indispensable lactam or lactone linkage.[3] Among a vast number of cyclization protocols, the use of thioester unit is receiving increasing attention due to its chemoselective reactivity.[4] For example, native chemical ligation (NCL) is the reaction of a thioester with an N-terminal cysteine residue in aqueous solution.[5] While widely used, standard NCL offers a limited ligation site (Xaa–Cys: Xaa = amino acid) without using desulfurization of unnatural amino acids bearing mercapto group for the synthesis of cyclic peptides.[6,7] A cysteine-free ligation was developed using acidic amino acids such as aspartic or glutamic acid in aqueous *N*-methylpyrrolidone (NMP), where the carboxylate anion intramolecularly interacts with the amine unit facilitating direct aminolysis with thioesters.[8] The requirement of NMP for cysteine-free ligation is attributable to the suppression of thioester hydrolysis during an aminolysis that is slow in comparison to NCL.

We have developed an alternative approach to cysteine-free ligation using *N*-sulfanylethylanilide (SEAlide) peptide **1** as a crypto-thioester, with successful application to protein chemical synthesis using a one-pot/N–to–C-directive sequential NCL protocol.[9] In the presence of phosphate salts, the SEAlide peptide likely undergoes NCL through N–S acyl-transfer-mediated formation of thioester **2** to afford ligated material, whereas the amide-type SEAlide remains largely intact in the absence of phosphate salts.



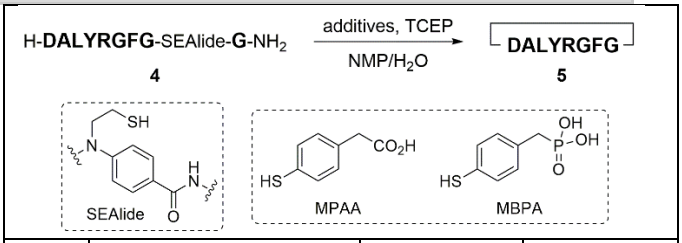
Scheme 1 Synthetic Strategy of Trichamide Using SEAlide Peptide

Recently, trichamide (**3**) possessing thiazole amino acids was isolated from the globally-occurring, bloom-forming cyanobacterium, *Trichodesmium erythraeum*.[10] The biological significance of **3** has yet to be elucidated, despite its potential presence in high amounts in oceanic blooms. Moreover, **3** was identified by genome mining and mass spectrometry, and its small quantity in culture made further structural evidence highly challenging. This naturally occurring cyclic peptide has Gly–Asp sequence in the molecule, which could probably be constructed by the cysteine-free ligation mentioned above. Although we did not know whether an acidic residue was required at the N-terminus, this provided a conservative starting point for assessing the potential of our method. In this context, we here examined the applicability of the SEAlide peptide to the cysteine-free ligation for the preparation of trichamide (Scheme 1).

First, we synthesized model SEAlide peptide **4** by Fmoc solid-phase peptide synthesis (SPPS) to examine the feasibility of a SEAlide-mediated cysteine-free ligation for the construction of the cyclic structure. The cysteine-free ligation was conducted in NMP/H_2O (4/1 (v/v)) in the presence of 15 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as an inhibitor of oxidation of thiols at pH 7.6, 37 °C. The use of 4-mercaptophenyl acetic acid (MPAA) for the cyclization of **4** resulted in low conversion yield (Table 1, entry 1). Our previous investigation on the SEAlide-mediated NCL reaction in aqueous solution indicated that phosphate salts accelerate the NCL through facilitation of the N–S acyl transfer of the SEAlide unit.[9] Thus, we added phosphate salts (20 mM) in NMP/H_2O to promote the cyclization. However, no significant improvement was observed (Table 1, entry 2).

One potential explanation for such results is that high concentration of phosphate salts cannot be used in the mixed solvent consisting of NMP/H_2O (4/1 (v/v)) due to the sparingly soluble nature of the phosphate in NMP. Instead of phosphate salts, addition of benzylphosphonic acid in the presence of MPAA dramatically improved the conversion yield (Table 1, entry 3). Here, benzylphosphonic acid probably works as a more promising promoter for the N–S acyl transfer in the NMP/H_2O system. Therefore, we next examined the usefulness of 4-mercaptobenzylphosphonic acid (MBPA) bearing thiol and phosphonic acid units in the molecule.[11] Further improvement of the conversion was observed, and desired cyclized product **5** was obtained from the SEAlide precursors **4** in reasonable isolated yields (Table 1, entry 4). Under employed reaction conditions (concentration of peptide: 5.0 x 10^{-4} M), some peaks derived from components of ligation buffer were observed by HPLC analysis; however, no oligomerized product was detected.

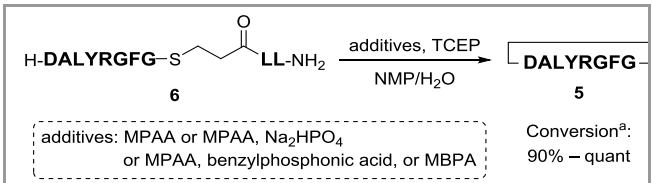
Table 1 Intramolecular Ligation of SEAlide Peptide Using Various Additives



entry	additive	conversion[a] (%)	yield[b] (%)
1	MPAA	24	
2	MPAA, Na_2HPO_4	19	
3	MPAA, benzylphosphonic acid	79	
4	MBPA	82	65

[a]Conversion was determined by HPLC separation and peak areas (See Supporting Information.). [b]Isolated yield after HPLC purification. Reaction conditions: peptides (0.5 mM), NMP/H_2O (4/1 (v/v)) containing 20 mM additives and 15 mM TCEP·HCl, final pH 7.6, 37 °C, 72 h. During the cyclization of **4**, aryl thioester intermediates were detected.

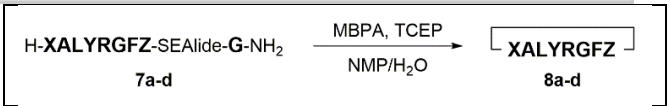
Benzylphosphonic acid derivatives promote N–S acyl transfer-mediated activation of SEAlide in aqueous solution. To elucidate its role in NMP/H_2O, we synthesized alkyl thioester peptide **6**, which would not require N–S acyl transfer prior to cysteine-free ligation. Using the conditions listed in Table 1, **6** was cyclized in nearly quantitative conversion yield (Scheme 2 and see supporting information). These results indicate that benzylphosphonic acid derivatives promote N–S acyl transfer in both aqueous and NMP/H_2O solvents.



[a]Conversion was determined by HPLC separation and peak areas (See Supporting Information.).

Scheme 2 Intramolecular Ligation of Thioester Fragment with Various Additives

Table 2 Intramolecular Ligation of SEAlide Peptide with Different N-/C-Terminal Amino Acids



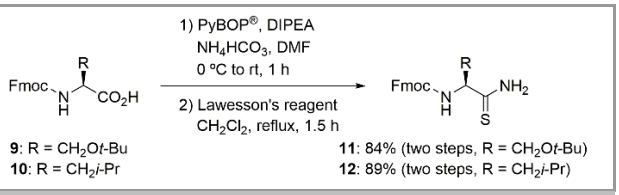
entry	peptide			Conversion[a] (%)
		X	Z	
1	**7a**	Glu	Gly	81
2	**7b**	Phe	Gly	74
3	**7c**	Asp	Ala	82

4	**7d**	Gly	Ala	86

[a]Conversion was determined by HPLC separation and peak areas (See Supporting Information.). Reaction conditions: peptides (0.5 mM), NMP/H_2O (4/1 (v/v)) containing 20 mM MBPA and 15 mM TCEP·HCl, final pH 7.6, 37 °C, 48 h.

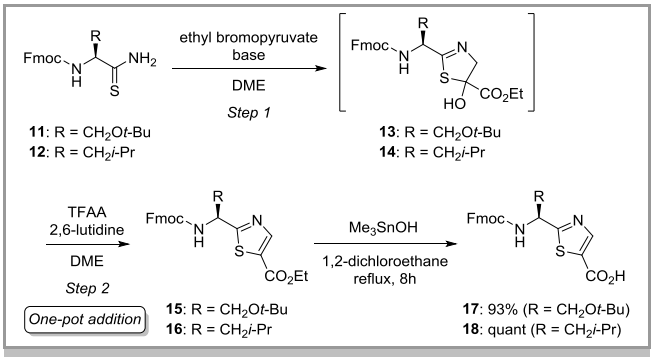
To confirm the general applicability of the SEAlide-mediated cysteine-free ligation to synthesis of cyclic peptides, non-acidic amino acids (Phe and Gly) as the amine component were subjected to the ligation protocol. Even at the non-acidic sites, cysteine-free ligation proceeded efficiently to yield cyclized materials (Table 2, entries 1 vs 2; entries 3 vs 4), although about 10% epimerization was observed at Ala adjacent to the thioester (Table 2, entries 3 and 4) (see supporting information).

Having established a synthetic platform using the SEAlide-based cysteine-free ligation for trichamide, we next turned our attention to the synthesis of two Fmoc-protected thiazole amino acids requisite for a preparation of trichamide (Schemes 3 and 4). Amidation of commercially available Fmoc amino acids **9** and **10** with ammonium hydrogen carbonate in the presence of (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP®) and diisopropylethylamine (DIPEA) in DMF, followed by thioamidation with Lawesson's reagent in CH_2Cl_2, afforded thioamides **11** and **12** in 84% and 89% yields over two steps, respectively.



Scheme 3 Synthesis of Thioamides

Next, the resulting thioamides **11** and **12** were subjected to the Hantzsch thiazole synthesis in accordance with procedures described in the literature.[12] Ethyl bromopyruvate was reacted with thioamides **11** and **12** in the presence of excess amount of $KHCO_3$ at –40 °C. After being stirred at 0 °C for 12 h, the reaction mixture was filtered, and the filtrate was concentrated. The generated hydroxythiazolines **13** and **14** were dehydrated by the action of trifluoroacetic anhydride (TFAA) and 2,6-lutidine to give thiazole units **15** and **16** in 79% isolated yields, respectively (Table 3, entries 1 and 2). Chiral high-performance liquid chromatography (HPLC) analysis indicated that the synthesis of **15** starting from serine was accompanied by partial loss of optical purity (Table 3, entry 1). Since the serine derivative tends to epimerize during the synthetic process,[13] serine-derived thioamide **11** was subjected to experiments examining epimerization. Generally, the first step of conventional Hantzsch thiazole synthesis uses an excess amount of inorganic base such as $KHCO_3$ (11 eq.) in 1,2-dimethoxyethane (DME) for the formation of hydroxythiazoline followed by filtration and subsequent concentration of the solvent.[12] Therefore, we investigated the use of organic bases. Reaction of **11** with ethyl bromopyruvate in the presence of DIPEA (3 eq.) in DME for 30 min at room temperature followed by direct addition of TFAA (2.5 eq.)/2,6-lutidine (3 eq.) into the reaction mixture with additional stirring for 30 min at 0 °C gave desired thiazole **15** in 8% isolated yield and 66% ee. Replacement of DIPEA with pyridine as a milder base allowed for the overall reaction to proceed smoothly, and the desired **15** was obtained in 88% isolated yield and 88% ee (Table 3, entries 3 and 4). Furthermore, the use of more hindered pyridine-type base (2,6-lutidine) in the first step enabled optically pure **15** (>99% ee) to be obtained in high chemical yield (94%) (Table 3, entry 5). These optimized reaction conditions were successfully applied to the preparation of highly optically pure leucine-derived thiazole **16** in highly chemical yield (Table 3, entry 6). Note that the optimized one-pot procedure for the Hantzsch thiazole synthesis is a practical and reliable method for the synthesis of enantiomerically pure thiazole derivatives from commercially available Fmoc-protected amino acids. During the course of our research, Yamaguchi and co-workers independently reported a similar one-pot procedure for preparation of thiazoles.[14]

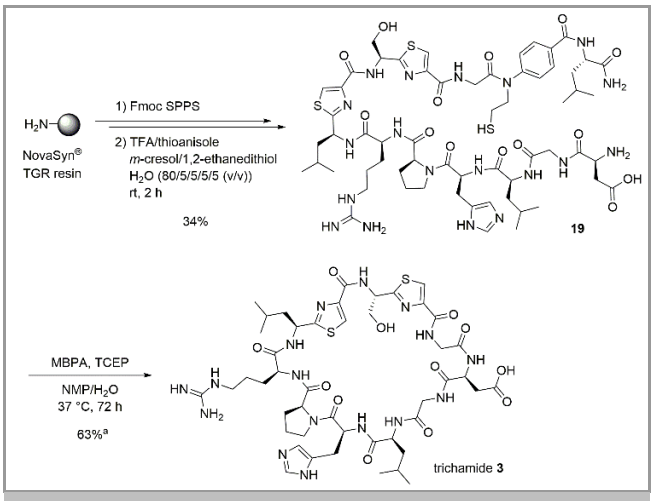


Scheme 4 Synthesis of Thiazole Building Blocks

Table 3 Optimization of One-pot Hantzsch Thiazole Synthesis

entry	substrate	base (Step 1)	temp (°C)/*t* (h)		Yield[a] (%)	ee[b] (%)
			Step 1	Step 2		
1[c]	**11**	$KHCO_3$	–40→0/12	–40/12	79	80
2[c]	**12**	$KHCO_3$	–40→0/12	–40/12	80	95
3[d]	**11**	DIPEA	rt/0.5	0/0.5	8	66
4[d]	**11**	Pyridine	rt/0.5	0/0.5	88	88
5[d]	**11**	2,6-lutidine	rt/0.5	0/0.5	93	>99
6[d]	**12**	2,6-lutidine	rt/0.5	0/0.5	98	>99

Reaction conditions in step 1: $KHCO_3$ (11 eq.), other bases (3 eq.). Reaction conditions in step 2: TFAA (entries 1 and 2: 9 eq., entries 3–6: 3 eq.), 2,6-lutidine (entries 1 and 2: 4 eq., entries 3–6: 3 eq.). [a]Isolated yield. [b]Determined by chiral HPLC analysis. [c]After a formation of **17** or **18**, reaction mixture was filtered and evaporated. [d]Reaction mixture was used for step 2 directly.



Scheme 5 Synthesis of Trichamide

Finally, the resulting ethyl esters **15** and **16** were hydrolyzed by refluxing in 1,2-dichloroethane in the presence of Me_3SnOH[15] for 8 h to yield the requisite Fmoc-protected thiazole amino acids **17** and **18** in 94% and quantitative yields, respectively. With required intermediates in hand, Fmoc-based preparation of trichamide (**3**) was attempted (Scheme 5). On the Fmoc-glycyl-*N*-sulfanylethylaniline linker-incorporated–Leu–NovaSyn® TGR resin, thiazole amino acids and other Fmoc amino acids were incorporated with the aid of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU)/ DIPEA and *N*,*N*′-diisopropylcarbodiimide (DIPCDI)/ 1-hydroxybenzotriazole monohydrate ($HOBt{\cdot}H_2O$), respectively. After completion of the linear peptide synthesis, treatment of the resin with TFA/thioanisole/*m*-cresol/1,2-ethanedithiol/H_2O (80/5/5/5/5 (v/v)) for 2 h at room temperature followed by HPLC purification gave amide-type linear SEAlide peptide **19** in 34% isolated yield. Following the conditions obtained from model experiments, intramolecular ligation was achieved in NMP/H_2O (4/1 (v/v)) in the presence of 20 mM MBPA and 15 mM TCEP at pH 7.6, 37 °C (concentration of peptide: 5.0×10^{-4} M).[16] Reaction proceeded smoothly over 72 h to yield desired trichamide **3** in 63% isolated yield after HPLC purification as shown in Figure 1. HPLC-monitoring of the SEAlide-based cysteine free ligation clearly indicated that amide-type linear SEAlide peptide **19** was gradually converted to trichamide (**3**) through linear MBPA thioester intermediate **20** without side reactions.

The structure of trichamide (**3**) was previously determined only by genomics and MS analysis. To provide definitive evidence for the structure of **3**, we obtained extracts of *T. erythraeum* and compared them with the synthetic compound. Two strains were selected for analysis: ISM101, from which trichamide was previously identified, and GBR. Trichamide (**3**) was only produced by ISM101, and only at lower temperatures. The presence or absence of phosphate did not matter to trichamide production (see supporting information). When produced, the yield of trichamide was ~5–10 μg per liter of culture broth, as estimated by comparison with synthetic **3**. Given the slow growth of the bacteria and the low abundance in culture, synthesis provides a practical means of structure elucidation.

Using HPLC- and UPLC-MS, synthetic **3** co-eluted with **3** from *T. erythraeum* under all conditions attempted (see supporting information). To further verify that the natural and synthetic compounds were likely identical, we subjected the compounds to the same Fourier transform MS protocol used in the initial identification of **3**. In comparing the resulting MS^2 spectra, fragment ions were identical. Thus, the synthesis provides strong evidence supporting the proposed structure of the natural product. In addition, using the synthetic material NMR chemical shift data was provided for **3** for the first time.

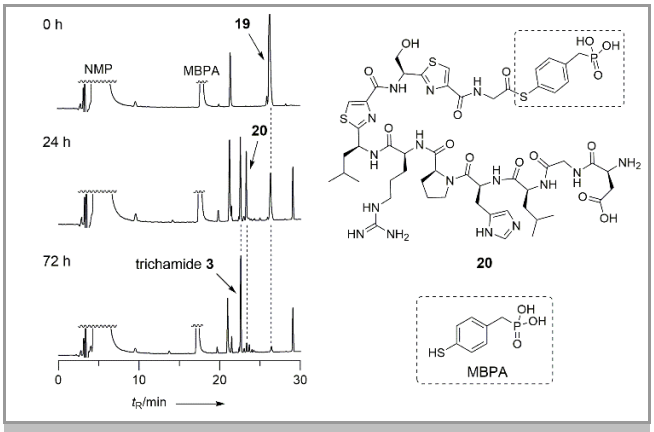


Figure 1 HPLC monitoring of the cysteine-free ligation for the synthesis of trichamide. HPLC conditions are described in the supporting information.

In conclusion, we achieved the chemical synthesis of trichamide using a cysteine-free ligation. One key to the success was development of a SEAlide-based cysteine-free ligation that featured the use of MBPA as dual promoter for the N–S acyl transfer and subsequent ligation as phosphate salts and MPAA, respectively. Further examination of the applicability of this ligation at various amino acids in the C- or N-termini is still ongoing. Another key is improvement of synthetic protocols for enantiomerically pure Fmoc-protected thiazole amino acids where one-pot Hantzsch procedure was developed.

Funding Information

This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI). K.A. is grateful for a JSPS fellowship (14J07568). T.J. is grateful for SUNBOR SCHOLARSHIP.

Acknowledgement

We thank K. Parawar and J. A. Maschek (University of Utah) for high-resolution MS analyses.

Supporting Information

YES (this text will be updated with links prior to publication)

Primary Data

NO (this text will be deleted prior to publication)

References and Notes

(1) Tapeinou, A.; Matsoukas, M.-T.; Simal, C.; Tselios, T. *Biopolymers* **2015**, *10*, 453.
(2) (a) Hruby, V. J. *Nat. Rev. Drug Discov.* **2002**, *1*, 847. (b) Tsomaia, N. *Eur. J. Med. Chem.* **2015**, *94*, 459. (c) Pelay-gimeno, M.; Glas, A.; Koch, O.; Grossmann, T. N. *Angew. Chem., Int. Ed.* **2015**, *54*, 2.
(3) (a) Lambert, J. N.; Mitchell, J. P.; Roberts, K. D. *J. Chem. Soc. Perkin Trans. 1* **2001**, 471. (b) Parenty, A.; Moreau, X.; Campagne, J. M. *Chem. Rev.* **2006**, *106*, 911. (c) White, C. J.; Yudin, A. K. *Nat. Chem.* **2011**, *3*, 509.
(4) (a) Zhang, L.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 4375. (b) Zhang, L.; Tam, J. P. *J. Am. Chem. Soc.* **1999**, *121*, 3311. (c) Li, Y.; Yongye, A.; Giulianotti, M.; Martinez-Mayorga, K.; Yu, Y.; Houghten, R. A. *J. Comb. Chem.* **2009**, *11*, 1066. (d) Li, Y.; Giulionatti, M.; Houghten, R. A. *Org. Lett.* **2010**, *12*, 2250. (e) Agrigento, P.; Albericio, F.; Chamoin, S.; Dacquignies, I.; Koc, H.; Eberle, M. *Org. Lett.* **2014**, *16*, 3922.
(5) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776. (b) Sun, Y.; Lu, G.; Tam, J. P. *Org. Lett.* **2001**, *3*, 1681. (c) Clark, R. J.; Craik, D. J. *Biopolymers* **2010**, *94*, 414. (d) Boll, E.; Dheur, J.; Drobecq, H.; Melnyk, O. *Org. Lett.* **2012**, *14*, 2222. (e) Taichi, M.; Hemu, X.; Qiu, Y.; Tam, J. P. *Org. Lett.* **2013**, *15*, 2620. (f) Wang, J.-X.; Fang, G.-M.; Qu, D.-L.; Yu, M.; Hong, Z.-Y.; Liu, L. *Angew. Chem., Int. Ed.* **2015**, *54*, 2194.
(6) (a) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526. (b) Zheng, J. S.; Tang, S.; Guo, Y.; Chang, H. N.; Liu, L. *ChemBioChem* **2012**, *13*, 542.
(7) (a) Malins, L. R.; Payne, R. J. *Aust. J. Chem.* **2015**, *68*, 521. (b) Rohde, H.; Seitz, O. *Biopolymers* **2010**, *94*, 551.
(8) (a) Payne, R. J.; Ficht, S.; Greenberg, W. A.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2008**, *47*, 4411. (b) Thomas, G. L.; Hsieh, Y. S. Y.; Chun, C. K. Y.; Cai, Z.-L.; Reimers, J. R.; Payne, R. J. *Org. Lett.* **2011**, *13*, 4770.
(9) Sato, K.; Shigenaga, A.; Tsuji, K.; Tsuda, S.; Sumikawa, Y.; Sakamoto, K.; Otaka, A. *ChemBioChem* **2011**, *12*, 1840.
(10) Sudek, S.; Haygood, M. G.; Youssef, D. T. A.; Schmidt, E. W. *Appl. Environ. Microbiol.* **2006**, *72*, 4382.
(11) (a) Cowper, B.; Sze, T. M.; Premdjee, B.; Bongat White, A. F.; Hacking, A.; Macmillan, D. *Chem. Commun.* **2015**, *51*, 3208. (b) Eto, M.; Naruse, N.; Morimoto, K.; Yamaoka, K.; Sato, K.; Tsuji, K.; Inokuma, T.; Shigenaga, A.; Otaka, A. *Org. Lett.* **2016**, *18*, 4416.
(12) (a) Just-Baringo, X.; Albericio, F.; Álvarez, M. *Curr. Top. Med. Chem.* **2014**, *14*, 1244. (b) Bruno, P.; Peña, S.; Just-Baringo, X.; Albericio, F.; Álvarez, M. *Org. Lett.* **2011**, *13*, 4648. (c) Pan, C.-M.; Lin, C.-C.; Kim, S. J.; Sellers, R. P.; McAlpine, S. R. *Tetrahedron Lett.* **2012**, *53*, 4065. (d) Wahyudi, H.; Tantisantisom, W.; Liu, X.; Ramsey, D. M.; Singh, E. K.; McAlpine, S. R. *J. Org. Chem.* **2012**, *77*, 10596.
(13) (a) Crawhall, J. C.; Elliott, D. F. *Biochem. J.* **1951**, *48*, 237. (b) Demarchi, B.; Collins, M.; Bergström, E.; Dowle, A.; Penkman, K.; Thomas-Oates, J.; Wilson, J. *Anal. Chem.* **2013**, *85*, 5835.
(14) Amaike, K.; Itami, K.; Yamaguchi, J. *Chem. Eur. J.* **2016**, *22*, 4384. Applicability of reaction conditions (50 °C) mentioned in the literature to racemization-prone thioamides remains unclear at present.
(15) Nicolaou, K. C.; Estrada, A. A.; Zak, M.; Lee, S. H.; Safina, B. S. *Angew. Chem., Int. Ed.* **2005**, *44*, 1378.
(16) SEAlide peptide **19** (5.2 mg, 3.0 μmol) was dissolved in 6.0 mL of degassed ligation buffer [NMP/H_2O = 4/1 (v/v) containing 20 mM MBPA, 15 mM TCEP·HCl, final pH 7.6], and the reaction mixture was incubated at 37 °C. After 72 h, the mixture was diluted with 0.1% aq. TFA and the product was purified by preparative HPLC to give trichamide **3** as a white lyophilized powder (2.5 mg, 1.9 μmol, 63%). **Preparative HPLC conditions**: Cosmosil 5C_{18}-AR-II preparative column (20 × 250 mm, flow rate 10.0 mL/min) with a linear gradient of solvent B/solvent A (20/80–30/70 over 30 min). **Analytical HPLC conditions**: Cosmosil 5C_{18}-AR-II analytical column (4.6 × 250 mm, flow rate 1.0 mL/min) with a linear gradient of solvent B/solvent A (10/90–55/45 over 30 min (curve 7 of the Waters 600E)), retention time: 22.4 min. **HRMS** (ESI-TOF): *m/z* calcd for $C_{46}H_{66}N_{16}O_{12}S_2$ ([M + H]$^+$): 1099.4566, found for 1099.4569.