JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Trifluoroethanethiol: An Additive for Efficient One-Pot Peptide Ligation–Desulfurization Chemistry

Robert E. Thompson,[†] Xuyu Liu,[†] Noelia Alonso-García,[‡] Pedro José Barbosa Pereira,[‡] Katrina A. Jolliffe,[†] and Richard J. Payne*[,†]

[†]School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia

[‡]IBMC—Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal

Supporting Information

ABSTRACT: Native chemical ligation followed by desulfurization is a powerful strategy for the assembly of proteins. Here we describe the development of a high-yielding, one-pot ligation–desulfurization protocol that uses trifluoroethanethiol (TFET) as a novel thiol additive. The synthetic utility of this TFET-enabled methodology is demonstrated by the efficient multi-step one-pot syntheses of two tick-derived proteins, chimadanin and madanin-1, without the need for any intermediary purification.

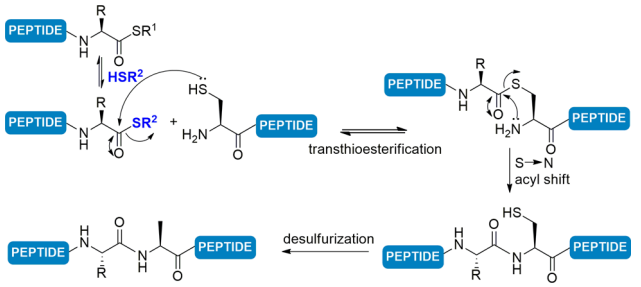
Chemical synthesis of proteins provides a means by which key structural and functional information on a given target can be elucidated.[1] Over the past two decades considerable advances in this area have been possible owing to the development of the venerable native chemical ligation method.[2] This transformation involves the chemoselective reaction between a peptide containing a C-terminal thioester and a peptide bearing an N-terminal Cys residue to afford a native peptide bond (Scheme 1). Due to the ease of preparation and stability to long-term storage, alkyl thioesters are often employed in ligation chemistry. However, this functionality is relatively inert in the ligation reaction, necessitating the inclusion of a thiol additive to generate a more reactive peptide thioester as the acyl donor. A transthioesterification then occurs between the side chain of the Cys residue and the newly formed thioester moiety, followed by an irreversible intramolecular S→N acyl transfer to form a native peptide bond.

Ligation technology has benefited greatly from the introduction of exogenous thiol additives to improve reaction rates via the *in situ* generation of reactive peptide thioesters. In a thorough study by Johnson and Kent, the relative reactivity of a range of commercially available thiols was investigated.[3] Aryl thiols with $pK_a > 6$ were shown to afford optimal ligation rates due to two key reactive properties: (1) the ability to rapidly exchange with alkyl thioesters to generate aryl thioesters and (2) excellent leaving group ability upon reaction with the N-terminal Cys residue. From this study the water-soluble aryl thiol additive mercaptophenylacetic acid (MPAA, $pK_a = 6.6$) was selected as an excellent additive that facilitated more rapid ligations than two other traditionally employed thiol additives, the water-soluble alkyl thiol mercaptoethanethiolate sodium salt (MESNa, $pK_a = 9.2$) and the sparingly water-soluble thiophenol ($pK_a = 6.6$).

A significant advancement in ligation methodology was the development of desulfurization chemistry which transforms Cys residues to Ala following the ligation event.[4] This methodology sparked interest in the use of the native chemical ligation concept at a variety of unnatural mercapto- and seleno-amino acids that can subsequently be converted to native amino acids by desulfurization or deselenization.[5] While desulfurization of Cys to Ala can be effected through the use of catalytic hydrogenation,[4a] radical desulfurization[4b] is the most widely employed method and has been used in the synthesis of a number of complex protein targets.[6] Given the high-yielding nature of desulfurization chemistry, the union of this transformation with efficient ligation chemistry into a one-pot procedure would represent a powerful addition to the toolbox of methods for use in chemical protein synthesis. Unfortunately, the necessity of aryl thiol additives in the ligation reaction prohibits this capability due to the inherent radical quenching activity of these species.[7] As such, products from ligation reactions require tedious purification and lyophilization before the purified materials are submitted to desulfurization. Solutions to this problem have been sought, including the use of MESNa which, despite significantly slower ligation rates, does not interfere with desulfurization chemistry.[6g] Alternatively, methods to remove aryl thiols from the reaction mixture have been employed, including extensive liquid/liquid extraction of aryl thiols such as thiophenol, or solid-phase extraction procedures.[5q] Recently, Brik and co-workers employed a synthetic bifunctional aryl thiol catalyst that could be captured with an aldehyde-derived solid-supported reagent prior to the desulfurization reactions.[8]

In an effort to streamline the two highly efficient reactions into a straightforward and operationally simple one-pot protocol, we sought to identify a novel thiol additive capable of facilitating

Scheme 1. Native Chemical Ligation–Desulfurization



Received: March 20, 2014

© XXXX American Chemical Society

dx.doi.org/10.1021/ja502806r | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

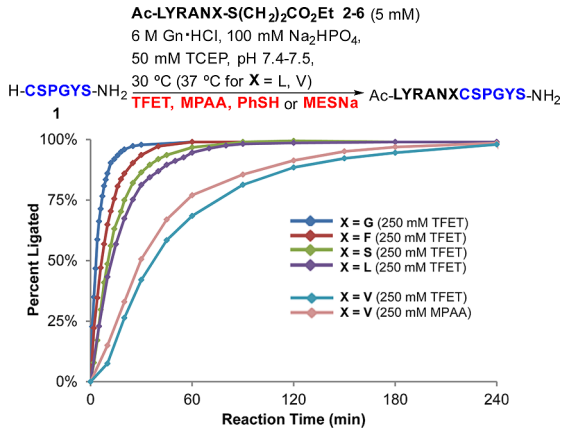


Figure 1. Kinetics for native chemical ligation reactions between peptide **1** and peptide thioesters Ac-**LYRANX**-S$(CH_2)_2CO_2$Et (**2**–**6**; for **2**, X = V; **3**, X = G; **4**, X = F; **5**, X = S; **6**, X = L). The percent ligated was calculated at each time point (mean of three independent experiments) by integrating the areas under the peaks of analytical HPLC chromatograms at λ = 280 nm. See SI for additional data and analysis.

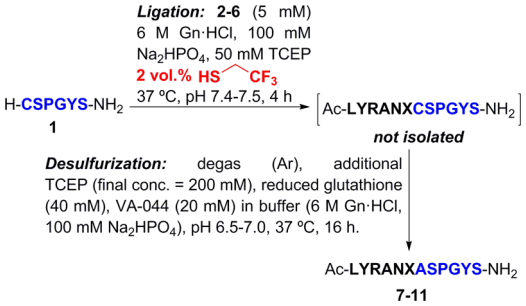
rapid ligation without disrupting the subsequent radical desulfurization. Here we show that 2,2,2-trifluoroethanethiol (TFET) is an efficient thiol catalyst and, importantly, as an alkyl thiol permits *in situ* one-pot desulfurization reactions. To demonstrate the utility of TFET, we undertook the synthesis of two small tick-derived proteins, chimadanin and madanin-1, via one-pot ligation–desulfurization of three peptide fragments either in the C→N-terminal direction or through kinetically controlled ligation chemistry[9] in the N→C direction, respectively.

TFET (pK_a = 7.30) has been shown to be similar to thiophenol in its propensity to participate in thiol–thioester exchange.[10] Owing to the comparatively low pK_a of TFET compared with other alkyl thiols, we envisaged that it would afford exchanged thioesters with acyl-donor capabilities similar to those of activated aryl thioesters. Furthermore, the fact that TFET is relatively volatile (bp 35–37 °C) permits facile removal following the ligation if necessary (unlike MPAA which can co-elute with products during HPLC purification).

We began investigating the use of TFET as an additive in native chemical ligation by comparing it with the commonly employed aryl thiol additives thiophenol and MPAA and the alkyl thiol additive MESNa, the most effective alkyl thiol catalyst currently known. To this end, we studied a challenging model ligation between model peptide **1** and peptide thioester **2**, bearing a sterically hindered C-terminal Val residue, one of the slowest sites for native chemical ligation.[11] Reactions were carried out in parallel in ligation buffer comprising 6 M Gn·HCl, 100 mM Na_2HPO_4 buffer at pH 7.4–7.5 in the presence of 250 mM TFET, PhSH, MPAA, or MESNa as thiol additives. Gratifyingly, ligation reactions carried out in the presence of TFET were significantly more rapid than those employing PhSH and MESNa (see Supporting Information (SI) for data). The reaction reached completion within 4 h, comparable to the same transformation employing MPAA, the current gold standard additive (Figure 1).

Having demonstrated that TFET was an efficient thiol additive for ligation at a valyl-thioester, we next investigated the rate of reaction between **1** and peptide thioesters **3**–**6** bearing a range of C-terminal residues, representative of practical ligation junctions (Figure 1). Gratifyingly, reaction rates between **1** and **3**–**6** all

Table 1. One-Pot Ligation–Desulfurization Reactions between Peptide 1 and Thioesters 2–6 Using TFET as a Thiol Additive



entry	thioester	product	yield (%)
1	Ac-**LYRANV**-SR (**2**)	**7**	84[a] (62)[b]
2	Ac-**LYRANG**-SR (**3**)	**8**	82[a] (66)[b]
3	Ac-**LYRANF**-SR (**4**)	**9**	80[a] (70)[b]
4	Ac-**LYRANS**-SR (**5**)	**10**	88[a] (68)[b]
5	Ac-**LYRANL**-SR (**6**)	**11**	85[a] (66)[b]

[a]Yields over two steps determined by analytical HPLC (see SI for weighed isolated yields). [b]Isolated yields following HPLC purification determined by optical density at λ = 280 nm; R = $(CH_2)_2CO_2$Et.

proved to be rapid (Figure 1). Specifically, reaction of **1** with peptide thioester **3** containing a C-terminal Gly reached completion in 20 min, while reaction with thioester **6** bearing a sterically encumbered Leu residue reached completion in 90 min.

An important development in the field of peptide ligation chemistry was the application of peptide hydrazides[12] as masked peptide thioesters.[13] Importantly, we show that TFET is capable of exchanging with acyl hydrazides bearing both C-terminal Gly and Val residues, leading to rapid formation of the corresponding TFET thioesters that were competent in ligation reactions with model peptide **1** (see SI for details).

With the knowledge that TFET could promote extremely rapid and high-yielding peptide ligation reactions, we next investigated its ultimate utility in the context of one-pot ligation–desulfurization chemistry (Table 1). Ligation reactions between peptide **1** and peptide thioesters **2**–**6** in the presence of 2 vol% (250 mM) TFET and 50 mM TCEP (Table 1) were left to proceed for 4 h, the time at which the slowest ligation (at Val thioester **2**) was complete (Figure 1). At this stage, the ligation product was not isolated, but rather the reaction mixture was thoroughly degassed by sparging with argon in preparation for the *in situ* radical desulfurization (see SI for full experimental details). This also led to the removal of the vast majority of the dissolved TFET owing to its volatility. At this point additional TCEP was added to the degassed solution to generate a final concentration of 200 mM, together with the radical initiator VA-044 (20 mM) and reduced glutathione[5b] (40 mM) as a H-atom source. Reactions were incubated at 37 °C for 16 h to ensure complete desulfurization. Importantly, all one-pot ligation–desulfurization reactions proceeded smoothly under these conditions (80–88% yield as judged by analytical HPLC, see Figure 2 for crude analytical data for the reaction between peptide **1** and peptide thioester **4** and the SI for other raw data). Following HPLC purification, the native peptide products were isolated in good yields (62–70%, see Table 1) over the two steps, highlighting the efficiency of the one-pot procedure (Table 1). It is important to note that while TFET was removed prior to desulfurization during the degassing step, we also show that

dx.doi.org/10.1021/ja502806r | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

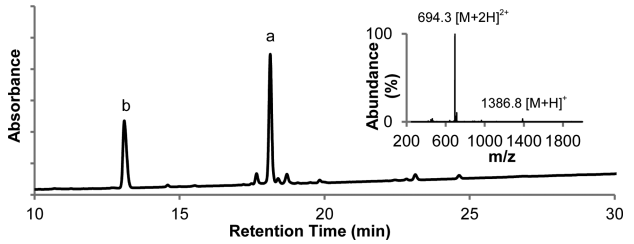
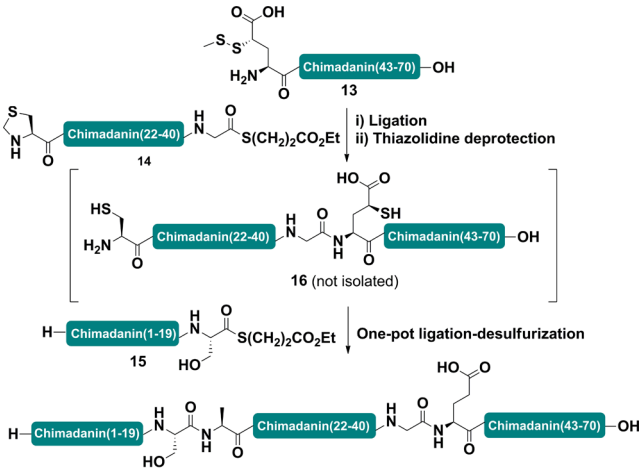


Figure 2. Crude analytical HPLC-MS for one-pot ligation–desulfurization reaction between **1** and **4** (λ = 230 nm). Peak a = **9**; peak b = S=P$(CH_2)_2CO_2H$.

Scheme 2. One-Pot Synthesis of Chimadanin (12) Using TFET[a]

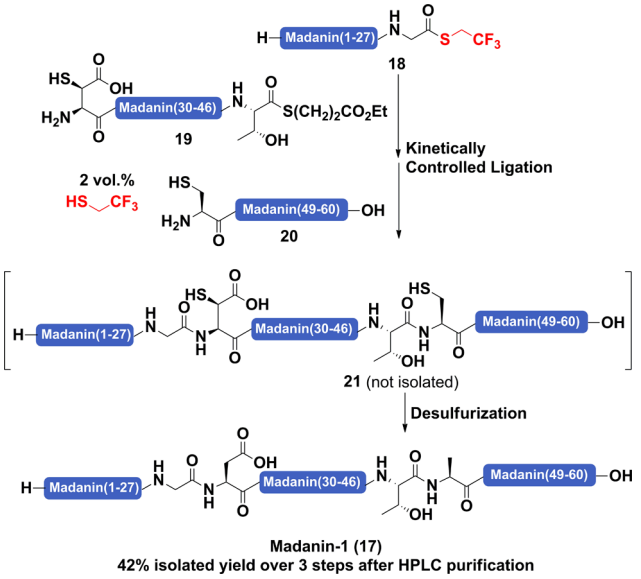


[a](i) Ligation: **14** (1.0 equiv) and **13** (1.2 equiv) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP), pH 6.8, 2.5 mM with respect to **14**, 2 vol% TFET, 30 °C, 2 h. (ii) Thiazolidine deprotection: 0.2 M methoxyamine (to pH 4.2), 30 °C, 3 h. One-pot ligation–desulfurization: for ligation, pH adjusted to 7.0, addition of **15** (1.3 equiv, 3.0 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 25 mM TCEP), pH 6.8, TFET (2 vol%), 1.0 mM with respect to **16**, 30 °C, 18 h; for desulfurization, adjusted to 500 mM TCEP and 40 mM reduced glutathione, Ar sparge, pH adjusted to 6.2, solid VA-044 (20 mM final concn), 37 °C, 5 h.

TFET does not have a detrimental effect on the desulfurization rate when present in solution (see SI for details).

Having demonstrated the utility of TFET as an additive for efficient and operationally simple one-pot ligation–desulfurization reactions, we were next interested in extending the scope of the methodology to the practical synthesis of some small protein targets. Our first target protein was the 70 amino acid thrombin inhibitory protein chimadanin (**12**, Scheme 2) produced by the hard tick *Haemaphysalis longicornis* to facilitate the hematophagous activity of the organism.[14] We envisaged the synthesis of the protein via the assembly of three fragments in the C→N direction. Specifically, we proposed using a γ-thiol Glu ligation[5q] followed by a native chemical ligation–desulfurization at Cys that would proceed with concomitant desulfurization of the γ-thiol auxiliary on the Glu residue to generate the native protein. Importantly, this proposed one-pot strategy would abolish intermediary purification steps, thus limiting the exposure of the sensitive γ-thiol moiety to acidic HPLC buffers, which leads to thiolactamization and peptide cleavage.[5q,15] The

Scheme 3. Synthesis of Madanin-1 (17) via a One-Pot Kinetically Controlled Ligation–Desulfurization with TFET[a]



[a]Kinetically controlled ligation: **18** (1.2 equiv), **19** (1.0 equiv, 5 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP), pH 7.4–7.5, 37 °C, 1 h, then addition of **20** (1.8 equiv), TFET (2 vol%), 37 °C, 12 h. Desulfurization: Ar sparge, adjust to TCEP (200 mM), reduced glutathione (40 mM), VA-044 (20 mM) in buffer (6 M Gn·HCl, 100 mM Na_2HPO_4), 2.5 mM final concn with respect to **21**, pH 6.5, 37 °C, 16 h.

synthesis began with the preparation of the requisite fragments via Fmoc-strategy SPPS, including chimadanin(42-70) (**13**) possessing an N-terminal γ-thiol Glu residue, chimadanin(21-41) (**14**) bearing an N-terminal thiazolidine and a C-terminal thioester functionality, and chimadanin(1-20) thioester **15** (see SI). Peptide **13** (1.2 equiv) bearing an N-terminal γ-thiol Glu residue was first ligated with peptide thioester **14** (1.0 equiv) in the presence of TFET. Following completion of the ligation (as judged by HPLC-MS), the reaction mixture was treated with methoxyamine at pH 4.2 to unmask an N-terminal Cys residue and afford intermediate **16**. Rather than purifying the intermediate, the pH of the reaction mixture was adjusted to 6.8 before addition of the N-terminal chimadanin fragment, peptide thioester **15**, and TFET. Ligation of **15** and **16** was again monitored by HPLC-MS; upon completion, the reaction was degassed before treatment with additional TCEP, reduced glutathione, and VA-044 to effect global desulfurization affording the native protein. Gratifyingly, chimadanin was isolated in 35% yield over the one-pot, four-step sequence following a single HPLC purification step (ca. 77% average yield per step).

To further probe the limits of one-pot ligation–desulfurization reactions employing the TFET additive, we next investigated the potential of combining kinetically controlled ligation chemistry with our one-pot methodology to assemble the 60 amino acid protein madanin-1 (**17**, Scheme 3), a Cys-free competitive thrombin inhibitor also produced by the hard tick *H. longicornis*.[16] The use of a kinetically controlled ligation sequence would enable the rapid assembly of multiple madanin-1 peptide segments in the N→C direction without intermediate purification steps through appropriate reactivity tuning of the requisite peptide thioesters.[9] With a view to future analogue generation, we were interested in assembling the protein via three short segments: madanin-1(1-28) (**18**) as a preformed

dx.doi.org/10.1021/ja502806r | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

TFET-thioester, madanin-1(29-47) (**19**) bearing an N-terminal β-thiol Asp residue and an unreactive C-terminal alkyl thioester, and madanin-1(48-60) (**20**) possessing an N-terminal Cys residue (Scheme 3 and SI). Peptide thioester **18**, activated as the preformed TFET-thioester, was first ligated with peptide thioester **19** bearing an N-terminal β-SH Asp^{5r} moiety and a C-terminal Thr residue. Following completion of the ligation after 1 h (as judged by HPLC-MS), peptide **20** was added in combination with 2 vol% TFET to activate the alkyl thioester and facilitate a second ligation reaction. Following completion of the second ligation (12 h), the product **21** was not isolated but rather subjected to *in situ* desulfurization of both the Cys and β-thiol Asp residues to afford the native protein madanin-1 (**17**) in an excellent 42% yield over the three steps. To our knowledge, this represents the first report of a one-pot kinetically controlled ligation−desulfurization reaction and clearly highlights the utility of TFET in the context of chemical protein synthesis. Importantly, the *in vitro* inhibitory activities of chimadanin (**12**, IC_{50} = 788 nM) and madanin-1 (**17**, IC_{50} = 1590 nM) against the amidolytic activity of thrombin were shown to be similar to that reported for recombinant madanin-1,[16b] thus confirming that the synthetic proteins possessed the expected thrombin-inhibiting activity (see SI).

In summary, we demonstrate that the alkyl thiol TFET can be successfully employed as an additive in native chemical ligation to facilitate ligations with rates comparable to those obtained with the gold standard additive, MPAA. More importantly, TFET can be used in ligation−desulfurization chemistry without the need for intermediate purification or removal/capture from the reaction mixture. We highlight the utility of TFET as an additive for one-pot ligation−desulfurization reactions both on model peptide systems and in the assembly of multiple peptide fragments to access proteins. Specifically, we used the additive for the efficient assembly of the tick-derived thrombin inhibitory proteins chimadanin and madanin-1 through C→N assembly and kinetically controlled approaches, respectively. Given the efficiency and simplicity of ligations employing TFET (a commercially available and affordable reagent), we anticipate that it will find widespread use in the chemical synthesis of proteins and post-translationally modified proteins, greatly improving the efficiency of the processes and reducing handling and purification of intermediates.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

richard.payne@sydney.edu.au

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the Australian Research Council for research funding (Discovery Project 120100194).

■ REFERENCES

(1) (a) Davis, B. G. *Chem. Rev.* **2002**, *102*, 579. (b) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923. (c) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338. (d) Gamblin, D. P.; Scanlan, E. M.; Davis, B. G. *Chem. Rev.* **2009**, *109*, 131. (e) Payne, R. J.; Wong, C. H. *Chem. Commun.* **2010**, *46*, 21. (f) Unverzagt, C.; Kajihara, Y. *Chem. Soc. Rev.* **2013**, *42*, 4408. (g) Wang, L.-X.; Amin, M. N. *Chem. Biol.* **2014**, *21*, 51.

(2) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.

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

(4) (a) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526. (b) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248.

(5) (a) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064. (b) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807. (c) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521. (d) Yang, R. L.; Pasunooti, K. K.; Li, F. P.; Liu, X. W.; Liu, C. F. *J. Am. Chem. Soc.* **2009**, *131*, 13592. (e) Kumar, K. S. A.; Haj-Yahya, M.; Olschewski, D.; Lashuel, H. A.; Brik, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 8090. (f) Harpaz, Z.; Siman, P.; Kumar, K. S. A.; Brik, A. *ChemBioChem* **2010**, *11*, 1232. (g) Tan, Z. P.; Shang, S. Y.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2010**, *49*, 9500. (h) Chen, J.; Wang, P.; Zhu, J. L.; Wan, Q.; Danishefsky, S. J. *Tetrahedron* **2010**, *66*, 2277. (i) Shang, S. Y.; Tan, Z. P.; Dong, S. W.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2011**, *133*, 10784. (j) Ding, H.; Shigenaga, A.; Sato, K.; Morishita, K.; Otaka, A. *Org. Lett.* **2011**, *13*, 5588. (k) Siman, P.; Karthikeyan, S. V.; Brik, A. *Org. Lett.* **2012**, *14*, 1520. (l) Malins, L. R.; Cergol, K. M.; Payne, R. J. *ChemBioChem* **2013**, *14*, 559. (m) Malins, L. R.; Cergol, K. M.; Payne, R. J. *Chem. Sci.* **2014**, *5*, 260. (n) Malins, L. R.; Payne, R. J. *Org. Lett.* **2012**, *14*, 3142. (o) Metanis, N.; Keinan, E.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2010**, *49*, 7049. (p) Townsend, S. D.; Tan, Z.; Dong, S.; Shang, S.; Brailsford, J. A.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 3912. (q) Cergol, K. M.; Thompson, R. E.; Malins, L. R.; Turner, P.; Payne, R. J. *Org. Lett.* **2014**, *16*, 290. (r) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 9723.

(6) (a) Brailsford, J. A.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 7196. (b) Wilkinson, B. L.; Stone, R. S.; Capicciotti, C. J.; Thaysen-Andersen, M.; Matthews, J. M.; Packer, N. H.; Ben, R. N.; Payne, R. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 3606. (c) Wang, P.; Dong, S.; Brailsford, J. A.; Iyer, K.; Townsend, S. D.; Zhang, Q.; Hendrickson, R. C.; Shieh, J.; Moore, M. A. S.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2012**, *51*, 11576. (d) Murakami, M.; Okamoto, R.; Izumi, M.; Kajihara, Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 3567. (e) Sakamoto, I.; Tezuka, K.; Fukae, K.; Ishii, K.; Taduru, K.; Maeda, M.; Ouchi, M.; Yoshida, K.; Nambu, Y.; Igarashi, J.; Hayashi, N.; Tsuji, T.; Kajihara, Y. *J. Am. Chem. Soc.* **2012**, *134*, 5428. (f) Liu, S. H.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2012**, *51*, 993. (g) Siman, P.; Blatt, O.; Moyal, T.; Danieli, T.; Lebendiker, M.; Lashuel, H. A.; Friedler, A.; Brik, A. *ChemBioChem* **2011**, *12*, 1097. (h) Wang, P.; Dong, S.; Shieh, J.-H.; Peguero, E.; Hendrickson, R.; Moore, M. A. S.; Danishefsky, S. J. *Science* **2013**, *342*, 1357.

(7) Rohde, H.; Schmalisch, J.; Harpaz, Z.; Diezmann, F.; Seitz, O. *ChemBioChem* **2011**, *12*, 1396.

(8) Moyal, T.; Hemantha, H. P.; Siman, P.; Refua, M.; Brik, A. *Chem. Sci.* **2013**, *4*, 2496.

(9) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem., Int. Ed.* **2006**, *45*, 3985.

(10) Hupe, D. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1977**, *99*, 451.

(11) Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10068.

(12) Fang, G.-M.; Li, Y.-M.; Shen, F.; Huang, Y.-C.; Li, J.-B.; Lin, Y.; Cui, H.-K.; Liu, L. *Angew. Chem., Int. Ed.* **2011**, *50*, 7645.

(13) Zheng, J. S.; Tang, S.; Qi, Y. K.; Wang, Z. P.; Liu, L. *Nat. Protoc.* **2013**, *8*, 2483.

(14) Nakajima, C.; Imamura, S.; Konnai, S.; Yamada, S.; Nishikado, H.; Ohashi, K.; Onuma, M. *J. Vet. Med. Sci.* **2006**, *68*, 447.

(15) Tam, J. P.; Yu, Q. T. *Biopolymers* **1998**, *46*, 319.

(16) (a) Iwanaga, S.; Okada, M.; Isawa, H.; Morita, A.; Yuda, M.; Chinzei, Y. *Eur. J. Biochem.* **2003**, *270*, 1926. (b) Figueiredo, A. C.; de Sanctis, D.; Pereira, P. J. B. *PLoS One* **2013**, *8*, No. e71866.