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Native Chemical Ligation−Photodesulfurization in Flow

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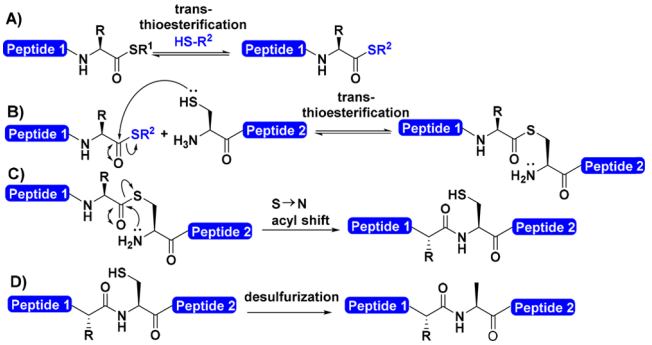
Supporting Information

ABSTRACT: Native chemical ligation (NCL) combined with desulfurization chemistry has revolutionized the way in which large polypeptides and proteins are accessed by chemical synthesis. Herein, we outline the use of flow chemistry for the ligation-based assembly of polypeptides. We also describe the development of a novel photodesulfurization transformation that, when coupled with flow NCL, enables efficient access to native polypeptides on time scales up to 2 orders of magnitude faster than current batch NCL−desulfurization methods. The power of the new ligation−photodesulfurization flow platform is showcased through the rapid synthesis of the 36 residue clinically approved HIV entry inhibitor enfuvirtide and the peptide diagnostic agent somatorelin.

Peptides and proteins are ubiquitous molecules in living systems and generally exhibit exquisite selectivity for their targets, a property that has led to renewed interest in polypeptides as therapeutics. These "biologics" have been reported to have twice the approval rate of small molecule therapeutics[1] and currently make up 10% of approved drugs.[2] As a result of this renaissance in polypeptide-based therapeutics, it is not surprising that attention has turned to the development of methods to efficiently access these biomolecules. While solid phase peptide synthesis (SPPS) represents the most efficient avenue for generating peptides via chemical synthesis,[3] there is a significant limitation on the size of targets that can be produced *en bloc*. This limitation of SPPS was largely addressed through the development of native chemical ligation (NCL), a transformative technology that enables convergent and chemoselective fusion of unprotected peptide fragments.[4] NCL is performed between a peptide containing an N-terminal cysteine (Cys) residue and a peptide functionalized as a C-terminal thioester, and usually requires a suitable thiol additive to generate a reactive thioester (SR^2) that accelerates the rate-limiting trans-thioesterification step (Scheme 1).[5]

An important advance to the seminal methodology was the development of metal-based desulfurization[6] and later a milder radical-based protocol[7] that facilitate the conversion of the least abundant proteinogenic amino acid Cys to an alanine (Ala) residue at the ligation junction (Scheme 1). The subsequent development of thiolated amino acids (as Cys surrogates) has served to expand the number of targets accessible via NCL technology.[8] A further innovation has been the development of one-pot ligation−desulfurization chemistry using thiol additives,[5b,9] e.g. trifluoroethanethiol (TFET).[5b] These additives enhance the rate of the NCL reaction but, unlike traditionally used aryl thiols, do not interfere with subsequent radical desulfurization chemistry which can therefore be performed without intermediary purification.

Scheme 1. Native Chemical Ligation−Desulfurization: (A) Trans-thioesterification with a Thiol Additive; (B) Trans-thioesterification between Two Reacting Peptide Fragments; (C) Intramolecular S → N Acyl Shift of Thioester Intermediate; (D) Desulfurization of Cys to Ala at the Ligation Junction



Given our long-standing interest in the development and exploitation of ligation technologies for accessing peptides and proteins, we envisaged a novel flow chemistry platform to facilitate rapid and automated polypeptide assembly *via* NCL. We hypothesized that a flow manifold may improve the efficiency of the ligation−desulfurization transformations through automation and enhanced mixing,[10] features that have been capitalized on for SPPS but not for peptide ligation chemistry.[11] Herein, we report the development of an efficient flow-based NCL methodology. We also describe a photochemical transformation that enables traceless desulfurization within minutes in flow. We show that these two methods can be combined *via* "in-line" ligation−desulfurization for the rapid and efficient assembly of peptide targets. The power of this novel flow platform is demonstrated through the synthesis of two clinically used polypeptides.

Our investigations began with the development and optimization of a model NCL reaction in flow (Scheme 2A). We initially performed a flow ligation whereby a stream of H-CSPGYS-NH_2 **1** (10 mM) in aqueous ligation buffer (6 M Gdn·HCl, 0.1 M HEPES, pH 7.4−7.5) containing TCEP (50 mM) and the alkyl thiol TFET[5b] (500 mM) was mixed through a T-piece with a stream of peptide thioester Ac-LYRANX-$S(CH_2)_2CO_2Et$ (**2**: X = A, **3**: X = V, 12 mM) and TCEP (50 mM) in ligation buffer (pH 7.1−7.2) at equal flow rates using

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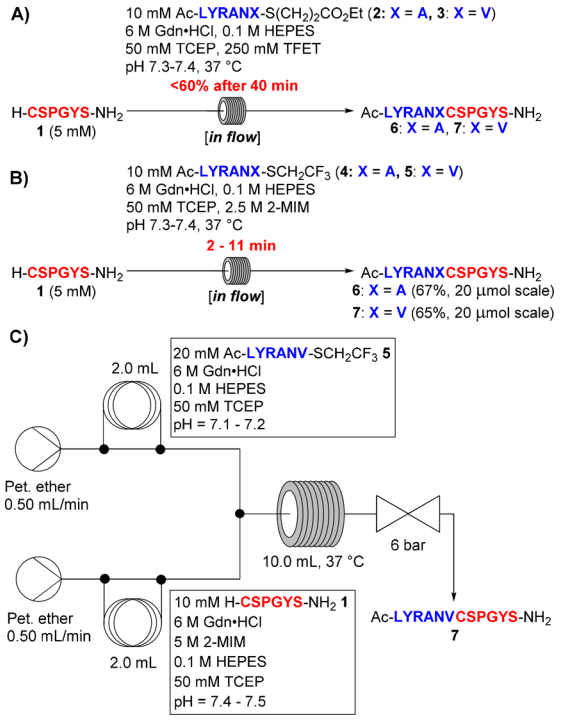
Scheme 2. (A) Flow NCL between Peptide 1 and Thioesters 2 and 3; (B) Flow NCL between Model Peptide 1 and Trifluoroethyl Thioesters 4 and 5; (C) Schematic Diagram of the Optimized NCL Flow System for the Reaction between Peptide 1 and Peptide Thioester 5



petroleum ether as an immiscible system solvent (see Scheme S2 for flow setup). The resultant reaction stream entered a polytetrafluoroethylene (PTFE) coil reactor at 37 °C for a variety of residence times. Unfortunately, this led to slow and incomplete ligation (<60% after 40 min) due to axial diffusion of TFET into the system solvent. The use of other common thiol additives, namely 4-mercaptophenylacetic acid (MPAA) and MESNa, led to incomplete ligation (Figure S1). An alternative NCL flow strategy was devised whereby preformed trifluoroethyl thioesters Ac-LYRANX-SCH_2CF_3 (**4**: X = A, **5**: X = V) were ligated with **1** using 2-methylimidazole (2-MIM)[12] as an additive to enhance the rate of ligation (Scheme 2B). Using the same flow setup above, these conditions led to complete ligation between peptide **1** and thioester **4** in 2 min, and with thioester **5** in 11 min (see Scheme 2B and 2C, Figure S2 for additional data). Having optimized the flow method we next compared the reaction rates to batch ligations between **1** and thioester **3** with TFET as an additive,[5b] and between **1** and preactivated trifluoroethyl thioester **5** (Figure 1A). The optimized flow procedure was substantially faster than both of these batch reactions (4–50-fold), the rate difference being more pronounced on a larger scale (20 μmol) despite active mixing (Figure 1A). As expected, the reaction end points of flow ligations did not change upon scale-up (see Figure 1B for crude UPLC trace of the ligation between **1** and **5**, after quenching with 3 vol % hydrazine) and led to excellent isolated yields upon direct HPLC purification of the flow eluent (18 mg, 67% for **6** and 18 mg, 65% for **7**).

Having established an optimized protocol for NCL in flow, we next investigated whether desulfurization could be carried out under a similar manifold. Initial attempts at performing VA-044-promoted radical desulfurization[7a] in flow proved unsuccessful with inefficient and incomplete desulfurization observed (ca.

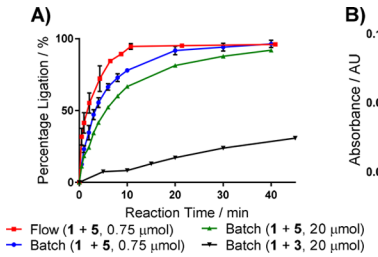
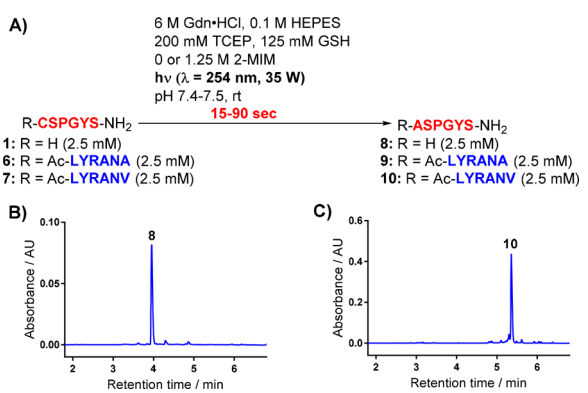


Figure 1. (A) Reaction of peptide **1** and peptide trifluoroethyl thioester **5** with 2-MIM as an additive (2.5 M) to generate **7** on a 0.75 μmol scale in batch (blue) and flow (red), on a 20 μmol scale in batch (green), and reaction of **1** and **3** to generate **7** using TFET as an exogenous additive on a 20 μmol scale in batch (black). (B) UPLC of the crude eluent from the optimized flow ligation between **1** and **5**. (gradient: 0 to 28%B over 5 min, λ = 214 nm). * Peak corresponds to the acyl hydrazide derivative of **5** following quenching with hydrazine; # peak corresponds to hydrolyzed **5**.

28% completion after 1 h for desulfurization of **1**, Figure S3, Scheme S4). We therefore sought an alternative method to facilitate the desulfurization of ligation products in flow. Recently, Guo and co-workers reported the use of a ruthenium photocatalyst, together with a phosphine and a hydrogen atom donor, for the conversion of Cys to Ala.[13] However, the kinetics of the desulfurization process (5–16 h for reaction completion) were deemed too slow for implementation of this method into our flow platform. Based on early work by Hoffmann and co-workers for the desulfurization of mercaptans[14] and, more recently, by Ge et al. for the desulfurization of deoxyglycosides,[15] we instead considered the use of UV irradiation (in the absence of an additional catalyst) to photochemically initiate the desulfurization reaction in flow. To this end, a flow system was assembled where a solution of peptide **1** (5 mM in ligation buffer with 2.5 M 2-MIM and 50 mM TCEP, pH 7.3–7.4, to mimic the NCL flow eluent) was mixed through a T-piece with 250 mM L-glutathione (GSH) as a hydrogen atom source[16] and 350 mM TCEP in ligation buffer (pH 7.4–7.5) with or without VA-044. The reagent stream was directed into a polyfluoroalkoxy (PFA) coil inside a Rayonet RPR-100 photoreactor and irradiated at multiple wavelengths (Scheme 3A, Schemes S5–S7, Figures S4, S5). In the absence of VA-044, clean conversion of **1** to the desulfurized product **8** was observed in only 15 s upon photoirradiation at λ = 254 nm (35 W, rt), which served as our

Scheme 3. (A) Novel Peptide Photodesulfurization; UPLC Chromatograms for the Crude Photodesulfurization Reaction of (B) 1 to 8 (0 to 15%B over 5 min, λ = 214 nm) and (C) 7 to 10 (0 to 28%B over 5 min, λ = 214 nm)



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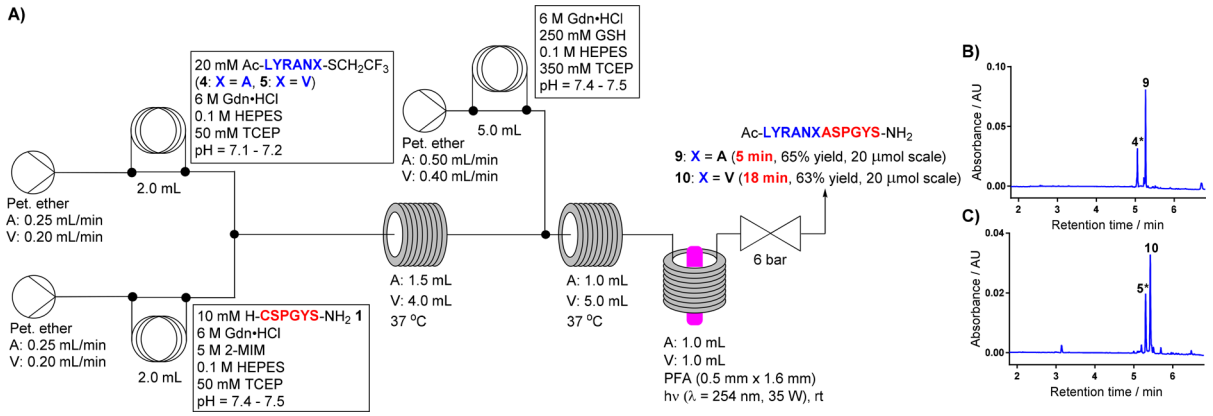
Scheme 4. (A) Setup for the Ligation−Photodesulfurization Procedure in Flow; Crude UPLC Trace (Gradient: 0 to 28%B over 5 min, λ = 280 nm) of the Ligation−Photodesulfurization Procedure Performed between 1 and (B) Alanine Thioester 4 To Generate 9 (Total Reaction Time 5 min) and (C) Valine Thioester 5 To Generate 10 (Total Reaction Time 18 min) (* Peak Corresponds to the GSH Thioester of 4 and 5)



optimal conditions in the reaction screen (Scheme 3A and 3B). We also submitted products **6** and **7** from the earlier flow NCL reactions to these photodesulfurization conditions that cleanly generated **9** and **10** in 60−90 s (Scheme 3C). The reaction was also chemoselective in the presence of potentially reactive side chains, e.g. methionine and side chain protected Cys residues (Schemes S12−S14). Finally, we showed that peptides bearing penicillamine[16,17] and β-thiol-aspartate[18] residues could be photodesulfurized cleanly in <1 min to afford native valine and aspartate residues, respectively (Schemes S15 and S16). It should be noted that prolonged exposure of batch reactions to UV irradiation led to product degradation. This highlights both the efficiency and homogeneity of the photoirradiation under a flow manifold.

Having optimized the ligation and photodesulfurization reactions in flow, we next combined these into an "in-line" ligation−photodesulfurization flow platform. A 20 μmol flow experiment was performed whereby a solution of **1** (10 mM in ligation buffer with 5 M 2-MIM and 50 mM TCEP) was mixed at a T-piece with thioester **4** or **5** (20 mM in ligation buffer with 50 mM TCEP, Scheme 4A). After passing through a PTFE coil reactor at 37 °C (**4**: 3 min, **5**: 11 min), the product was mixed at a second T-piece with a stream of 250 mM GSH and 350 mM TCEP in ligation buffer and directed to a second PTFE reactor at 37 °C (**4**: 1 min, **5**: 6 min) to thiolyze any product thioesters generated by trans-thioesterification between the internal Cys of the ligation product **6** or **7** and excess thioester **4** or **5** (Figure S6, Schemes S8−S11). It should be noted that product thioester species accrue here due to the absence of external thiol additives that would normally be added to facilitate transthioesterification to regenerate a reactive thioester under typical batch NCL conditions. Finally, the reaction mixture was photoirradiated (λ = 254 nm) for 1 min to facilitate photodesulfurization (see Scheme 4B and 4C and Figures S7 and S8 for additional data). Following HPLC purification, the desired products were isolated in excellent yields (**9**: 17 mg, 65%, **10**: 17 mg, 63%). The peptides were also generated on rapid time scales; a total reaction time of 5 min for **9** and 18 min for **10** was required, the latter representing one of the most challenging ligation junctions (Val thioester).

To make a valid rate comparison, we next performed the novel ligation−photodesulfurization as a one-pot procedure in batch. Notably, we observed over an order of magnitude increase in the reaction time when using preactivated thioesters (**4**: 2.6 h, **5**: 3.2 h, Figure S9, Scheme S20) in batch compared to flow. Furthermore, the flow NCL-photodesulfurization process was 2 orders of magnitude faster compared to a traditional ligation−desulfurization reaction in batch[5b] (with thioesters **2** or **3** and TFET as an additive), while affording comparable isolated yields (see Figure S10, Schemes S18 and S19).

Having demonstrated the efficiency and rate acceleration of the ligation−photodesulfurization flow manifold for model peptides, we next investigated more challenging polypeptide targets. As a target for synthesis, we first investigated the clinically approved 36-residue peptide HIV entry inhibitor enfuvirtide that is produced commercially by the condensation of three protected fragments in organic solvent.[19] We envisaged a flow-based assembly whereby peptide **11** (enfuvirtide 1−18) bearing a C-terminal lysine, derivatized as a trifluoroethyl thioester, could be reacted with peptide **12** (enfuvirtide 19−36) bearing an N-terminal β-thiol-asparagine residue[20] (Scheme 5A). Both peptides were made using Fmoc-SPPS (SI). Thioester **11** (2.0 equiv, 10 mM final concentration) was then ligated with β-thiol-asparagine peptide **12** (1.0 equiv, 5 mM final concentration) in the presence of 2.5 M 2-MIM and 50 mM TCEP at 37 °C (pH 6.8). After 32 min the reaction mixture was mixed at a T-piece with a solution containing 250 mM GSH and 800 mM TCEP in ligation buffer (pH 7.4−7.5) and directed into a PTFE coil reactor at 37 °C for thiolysis (1 min). The reaction stream was then passed through a PFA coil with UV photoirradiation (λ = 254 nm, 35 W, 3 min) to effect desulfurization and afford the native polypeptide (see Scheme 5B). The total reaction time was 36 min, and enfuvirtide **13** was isolated in 40% yield (9.7 mg) from a single HPLC purification (see Scheme 5C−5E). This result demonstrates the utility of the flow methodology in complex target assembly, as well as the compatibility with thiolated amino acids.

We next investigated a second target, the 44-residue peptide somatorelin, used as a diagnostic agent for growth hormone deficiency (Scheme 6A).[21] Peptide **14** (somatorelin 1−18), bearing a C-terminal thioester, and peptide **15** (somatorelin 19−44), containing an N-terminal Cys residue, were prepared using Fmoc-SPPS (see SI). Peptide **15** (115 mg, 30 μmol, 5 mM final concentration) was then ligated with peptide **14** (149 mg, 60 μmol, 10 mM final concentration) in flow (11 min). Following thiolysis (1 min) the stream was directed into a PFA coil and photoirradiated (λ = 254 nm, 35 W, 3 min). After a total reaction

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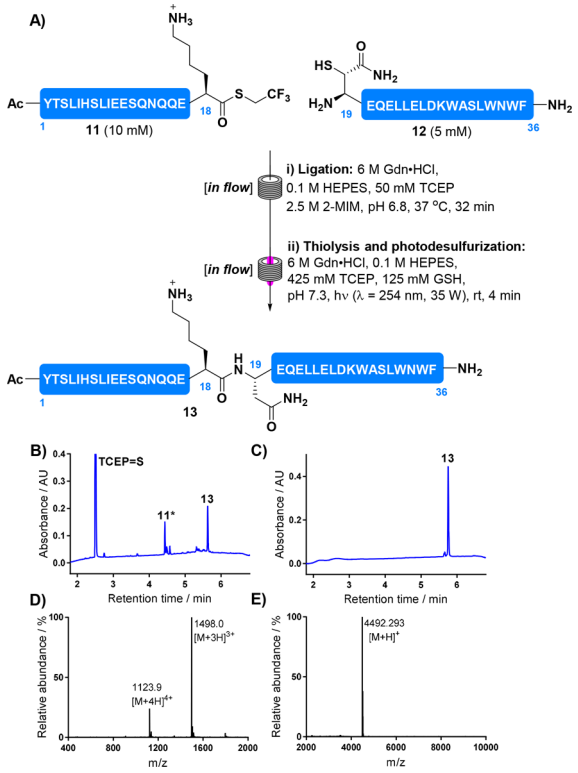
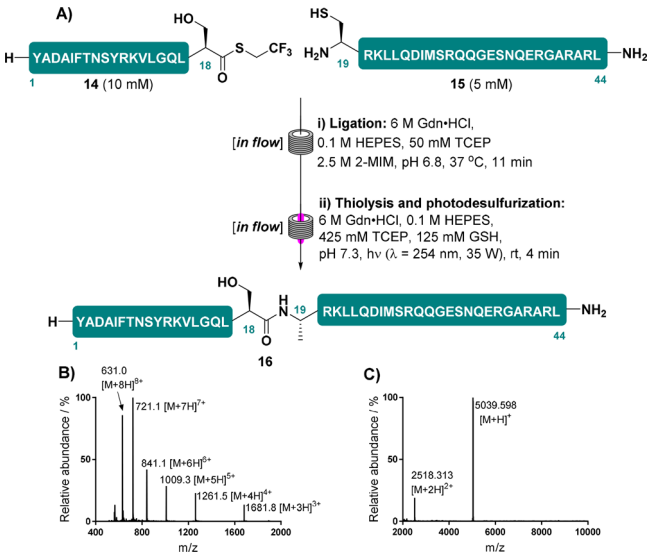
Scheme 5. (A) Synthesis of Enfuvirtide *via* NCL–Photodesulfurization in Flow; (B) UPLC Trace of the Crude Reaction Mixture after in-Line Flow NCL–Photodesulfurization (Gradient: 0 to 70%B over 5 min, λ = 214 nm; * Peak Corresponds to GSH Thioester of 11); (C) Analytical UPLC Trace of Purified Enfuvirtide 13 (Gradient: 0 to 70%B over 5 min, λ = 214 nm); (D) ESI-MS of Synthetic Enfuvirtide 13 (MS Data Were Collected over the Entire UV Peak in the UPLC-MS Chromatogram); (E) MALDI-TOF of Enfuvirtide 13[a]



[a]See Figure S11 and Schemes S22–S23 for additional details.

Scheme 6. (A) Synthesis of Somatorelin *via* NCL–Photodesulfurization in Flow; (B) ESI-MS of Synthetic Somatorelin 16 (MS Data Were Collected over the Entire UV Peak in the UPLC-MS Chromatogram); (C) MALDI-TOF MS of Synthetic Somatorelin 16[a]



[a]See Figure S12 and Schemes S24–S25 for additional details.

time of 15 min, HPLC purification provided somatorelin **16** in excellent yield (57%, 103 mg, see Scheme 6B, 6C and Scheme S25 for analytical data).

In summary, we describe here the first flow platform for polypeptide assembly using NCL. We demonstrate an improved rate of ligation using our flow protocol compared to traditional batch chemistry, particularly at sterically hindered junctions. We also describe a novel photodesulfurization transformation for the rapid and efficient conversion of Cys to Ala in peptides. We show that combining these two reactions into an in-line flow-based ligation–photodesulfurization process enables access to polypeptide targets much faster than traditional batch chemistry and can now be extended to larger scale production of polypeptides under a continuous flow regime. The power of the flow ligation–photodesulfurization platform was illustrated by the synthesis of the HIV drug enfuvirtide and the diagnostic agent somatorelin. Given the enabling nature of this technology, we anticipate its application to the scalable synthesis of proteins and biologics as well as extension to other ligation reactions, e.g. diselenide-selenoester ligation chemistry[22] in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b03115.

Experimental, reaction, rate and NMR data, Figures S1–S58 and Schemes S1–S25 (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Hay, M.; Thomas, D. W.; Craighead, J. L.; Economides, C.; Rosenthal, J. *Nat. Biotechnol.* **2014**, *32*, 40.

(2) (a) Fosgerau, K.; Hoffmann, T. *Drug Discovery Today* **2015**, *20*, 122. (b) Usmani, S. S.; Bedi, G.; Samuel, J. S.; Singh, S.; Kalra, S.; Kumar, P.; Ahuja, A. A.; Sharma, M.; Gautam, A.; Raghava, G. P. *PLoS One* **2017**, *12*, e0181748.

(3) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. (b) Kent, S. B. *Annu. Rev. Biochem.* **1988**, *57*, 957.

(4) (a) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776. (b) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338.

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

(6) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526.

DOI: 10.1021/jacs.8b03115
J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

(7) (a) Wan, Q.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2007**, *46*, 9248. (b) Jin, K.; Li, T.; Chow, H. Y.; Liu, H.; Li, X. *Angew. Chem., Int. Ed.* **2017**, *56*, 14607.
(8) (a) Dawson, P. E. *Isr. J. Chem.* **2011**, *51*, 862. (b) Kulkarni, S. S.; Sayers, J.; Premdjee, B.; Payne, R. J. *Nat. Rev. Chem.* **2018**, *2*, 122. (c) Malins, L. R.; Payne, R. J. *Aust. J. Chem.* **2015**, *68*, 521. (d) Malins, L. R.; Payne, R. J. *Curr. Opin. Chem. Biol.* **2014**, *22*, 70. (e) Malins, L. R.; Payne, R. J. *Top. Curr. Chem.* **2014**, *362*, 27. (f) Bondalapati, S.; Jbara, M.; Brik, A. *Nat. Chem.* **2016**, *8*, 407.
(9) Huang, Y. C.; Chen, C. C.; Gao, S.; Wang, Y. H.; Xiao, H.; Wang, F.; Tian, C. L.; Li, Y. M. *Chem. - Eur. J.* **2016**, *22*, 7623.
(10) (a) Pastre, J. C.; Browne, D. L.; Ley, S. V. *Chem. Soc. Rev.* **2013**, *42*, 8849. (b) Plutschack, M. B.; Pieber, B.; Gilmore, K.; Seeberger, P. H. *Chem. Rev.* **2017**, *117*, 11796.
(11) Mijalis, A. J.; Thomas, D. A.; Simon, M. D.; Adamo, A.; Beaumont, R.; Jensen, K. F.; Pentelute, B. L. *Nat. Chem. Biol.* **2017**, *13*, 464.
(12) Sakamoto, K.; Tsuda, S.; Mochizuki, M.; Nohara, Y.; Nishio, H.; Yoshiya, T. *Chem. - Eur. J.* **2016**, *22*, 17940.
(13) Gao, X.-F.; Du, J.-J.; Liu, Z.; Guo, J. *Org. Lett.* **2016**, *18*, 1166.
(14) Hoffmann, F. W.; Ess, R. J.; Simmons, T. C.; Hanzel, R. S. *J. Am. Chem. Soc.* **1956**, *78*, 6414.
(15) Ge, J.-T.; Li, Y.-Y.; Tian, J.; Liao, R.-Z.; Dong, H. *J. Org. Chem.* **2017**, *82*, 7008.
(16) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem., Int. Ed.* **2008**, *47*, 6807.
(17) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 8521.
(18) Thompson, R. E.; Chan, B.; Radom, L.; Jolliffe, K. A.; Payne, R. J. *Angew. Chem., Int. Ed.* **2013**, *52*, 9723.
(19) Bray, B. L. *Nat. Rev. Drug Discovery* **2003**, *2*, 587.
(20) Sayers, J.; Thompson, R. E.; Perry, K. J.; Malins, L. R.; Payne, R. J. *Org. Lett.* **2015**, *17*, 4902.
(21) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisky, M. *Drug Discovery Today* **2010**, *15*, 40.
(22) (a) Mitchell, N. J.; Malins, L. R.; Liu, X.; Thompson, R. E.; Chan, B.; Radom, L.; Payne, R. J. *J. Am. Chem. Soc.* **2015**, *137*, 14011. (b) Mitchell, N. J.; Sayers, J.; Kulkarni, S. S.; Clayton, D.; Goldys, A. M.; Ripoll-Rozada, J.; Pereira, P. J. B.; Chan, B.; Radom, L.; Payne, R. J. *Chem.* **2017**, *2*, 703.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX