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Kinetically Controlled Chemoselective Cyclization Simplifies the Access to Cyclic and Branched Peptides

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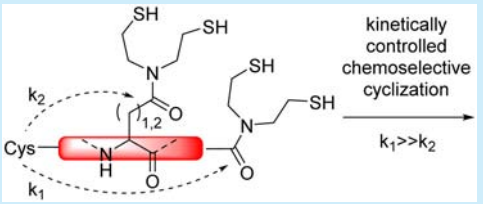
Supporting Information

ABSTRACT: A bis(2-sulfanylethyl)amido group reacts significantly faster with cysteinyl peptides when installed on the C-terminal end of a peptide in comparison with the side-chain of Asp and Glu. This property enabled the design of a kinetically controlled chemoselective peptide cyclization reaction, giving straightforward access to cyclic and branched peptides in one pot.



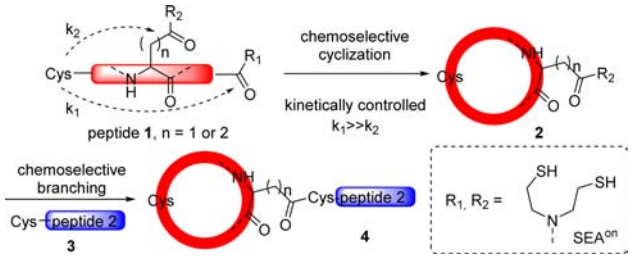
The modest in vivo stability of polypeptides is a well-known limitation for their development as therapeutics. In contrast, the incorporation of bioactive peptidic motifs within unusual peptidic scaffolds is a way to achieve in vivo half-lives that are compatible with medical applications. One often studied modification is peptide cyclization,[1] which besides improving the stability of peptides in biological fluids, can also stabilize the peptide conformation,[2,3] selectivity,[4,5] and potency.[6−8] In particular, polycyclic peptides stabilized by backbone cyclization and multiple disulfide bridges such as cyclotides have promising applications in drug design.[9−11] Another class of cyclic peptides that have attracted a lot of attention these recent years is bicyclic peptides, which are produced by tethering linear peptides through the reaction of cysteine (Cys) thiols with tris(bromomethyl)benzene.[12,13]

The properties of peptides can also be modulated by incorporating branched motifs.[14−16] Some natural peptides produced by nonribosomal synthesis such as lasso peptides[17] combine the cyclic and branched modifications. Some lasso peptides have antibacterial properties and were used as scaffolds for presenting integrin-binding motifs.[18] Very recently, Lassomycin was identified as a novel lasso peptide lacking the family's characteristic knot and having a potent and selective bactericidal activity against *M. tuberculosis*.[19]

The native chemical ligation (NCL)[20] and related methodologies,[21] which consists in the reaction of a C-terminal peptide thioester with an N-terminal cysteinyl peptide, is a popular method for producing backbone-cyclized peptides or proteins.[22−24] It is less frequently used for producing branched peptides due to the difficulty in installing a thioester group on an amino acid side-chain using the Fmoc solid-phase peptide synthesis (SPPS).[25−27] A recent study addressed the synthesis of cyclic and branched peptides (CBs) by the NCL reaction of cyclic peptides containing an alkylthioester handle with Cys peptides.[26] The access to the cyclic thioester peptide segment required setup of a sophisticated solid-phase approach which involved the manipulation of orthogonal protecting groups, an on-resin peptide macrolactamization, a sulfonamide linker alkylation and thiolysis step to produce the thioester and finally a peptide deprotection in TFA.

Since the NCL reaction allows the synthesis of cyclic or branched peptides, setting up two sequential NCLs or related reactions might in principle give access to CBs in a straightforward manner (Scheme 1). This strategy implies to address the selectivity of amide bond formation during the peptide cyclization step. One solution to this challenging problem would be to perform a kinetically controlled cyclization reaction, if one thioester function or thioester surrogate can be reacted significantly faster than the other.

Scheme 1. SEA Kinetically Controlled Cyclization



Kinetically controlled ligations (KCLs) were successfully used for the synthesis of linear polypeptides using either thioesters,[28] thioester surrogates,[29] or selenoester surrogates.[30,31]

However, the KCL approach was not extended yet to the preparation of CBs probably due to the difficulty in installing fast- and slow-reacting thioester functionalities such as alkyl and arylthioesters in the same polypeptide.

Given the potential of CBs for drug design, we sought to develop a simple and efficient access to these scaffolds. We

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show here that the bis(2-sulfanylethyl)amido[32] (SEA^{on}) chemoselective cyclization/branching strategy shown in Scheme 1 (R_1, R_2 = $-N(CH_2CH_2SH)_2$) gives access to CBs in a convergent and modular approach. The first ligation is an unprecedented kinetically controlled cyclization (KCC), which results in the formation of a head-to-tail cyclic peptide equipped with a SEA group on an aspartic or glutamic acid side-chain. The pendant SEA functionality is used in the second step to branch Cys peptide 2 on the cyclic peptide scaffold. Importantly, we show that the kinetically controlled strategy shown in Scheme 1 was performed in one pot, thereby saving time and yield.

The SEA group belongs to the growing family of thioester surrogates relying on an initial intramolecular *N*,*S*-acyl shift.[33−35] Previous studies showed that the SEA group, when positioned on the side-chain of an Asp or Glu residue, is less reactive toward Cys peptides than a C-terminal SEA group.[36] In order to have a better insight into this difference of reactivity, we examined the intermolecular ligation of Asp(SEA^{off}) and Glu(SEA^{off}) peptides **5a** and **5b**, respectively, or C-terminal SEA^{off} peptides **6a,c** with model Cys peptide **7** (CILKEPVHGV-NH_2, Figure 1). Note that SEA^{off} denotes the

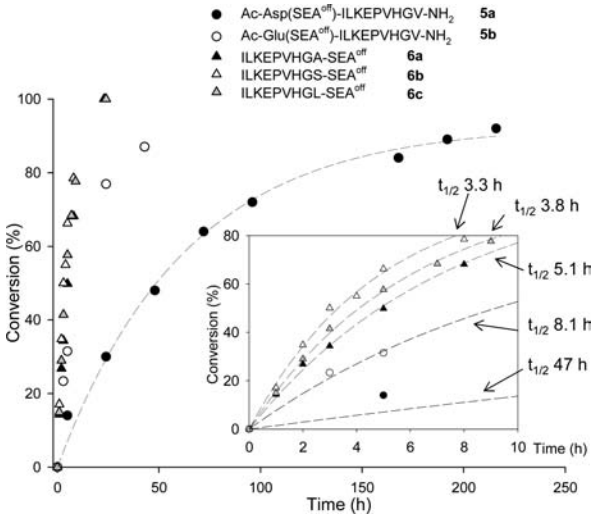
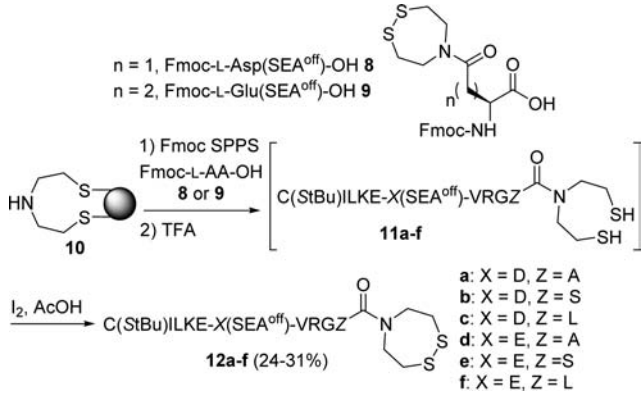


Figure 1. Time course of the ligation of model peptides **5a,b** and **6a,c** (7 mM) with CILKEPVHGV-NH_2 **7** (10.5 mM) in 6 M Gdn·HCl 0.1 M pH 7.2 sodium phosphate buffer and 37 °C (TCEP 200 mM, MPAA 200 mM, nitrogen atmosphere). The dashed lines correspond to the fitting curves (pseudo-first-order kinetic law).

SEA cyclic disulfide which is reduced in situ by tris(carboxyethyl)phosphine (TCEP) during the ligation.[37−39] The kinetics were nicely fitted to a pseudo-first-order kinetic law, from which the half-lifes ($t_{1/2}$) of the ligations could be determined (Figure 1, insert). The $t_{1/2}$ for Asp(SEA^{off}) peptide **5a** was >9-fold those obtained for C-terminal SEA^{off} peptides **6a,c**, while the $t_{1/2}$ for Glu(SEA^{off}) peptide **5b** was only 1.6−2.4-fold.

These data established the following order of reactivity: C-terminal SEA^{off} **6a,c** ≫ Glu(SEA^{off}) **5b** ≫ Asp(SEA^{off}) **5a**. The higher reactivity of the C-terminal SEA^{off} functionality relative to Asp/Glu(SEA^{off}) one might be due to the electron-withdrawing effect of the α-amino group in the former, which is expected to increase the electrophilicity of the α-carbonyl. This difference in reactivity encouraged us to examine the feasibility of the kinetically controlled cyclization reaction. To this end, peptides **12a−f** were produced by Fmoc SPPS starting from SEA polystyrene resin **10** (Scheme 2).[32,40] Asp(SEA^{off})

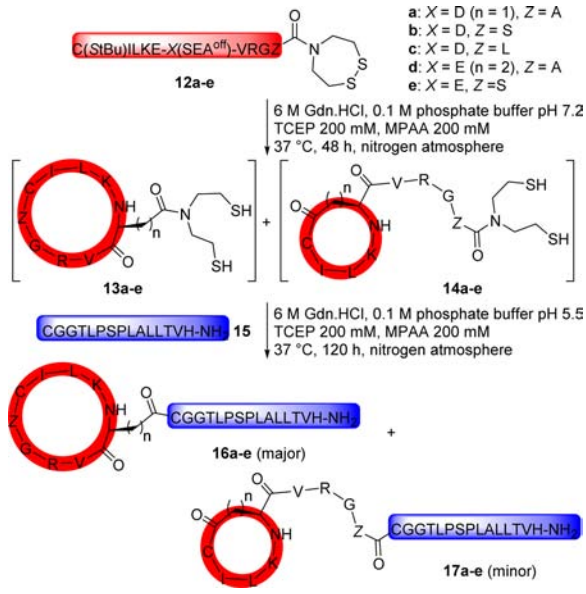
Scheme 2. SPPS of SEA Peptides



and Glu(SEA^{off}) residues were introduced using Fmoc-protected derivatives derivatives **8** and **9**, as described elsewhere.[36] The peptidyl resin was deprotected in TFA to furnish the crude C-terminal SEA^{on} peptides **11a−f** which were oxidized into the SEA^{off} derivatives **12a−f** prior to the HPLC purification step.

We first started with the cyclization of peptide **12a** (7 mM) at pH 7.2 in the presence of MPAA (200 mM) and TCEP (200 mM), as shown in Scheme 3. Cyclization proceeded in ∼48 h,

Scheme 3. One-Pot Kinetically Controlled Cyclization/Branching Process



as determined by LC-MS. Unfortunately, the cyclic intermediates **13a** and **14a** co-eluted by HPLC, thereby precluding any quantification of the regioselectivity of the cyclization process at this stage. Therefore, Cys peptide **15** (1.5 equiv) was added to the mixture to trigger the second ligation step which was carried out at pH 5.5. (Ligation of Asp(SEA) or Glu(SEA) functionalities proceeds significantly faster at pH 5.5 than at neutral pH, see ref 36.) Hopefully, branched and cyclic peptides **16a** and **17a** could be separated by HPLC. The HPLC analysis of the crude mixture after the two ligation steps is shown in

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Figure 2A. Importantly, this analysis revealed that peptide **16a** was by far the major product formed in the reaction and thus

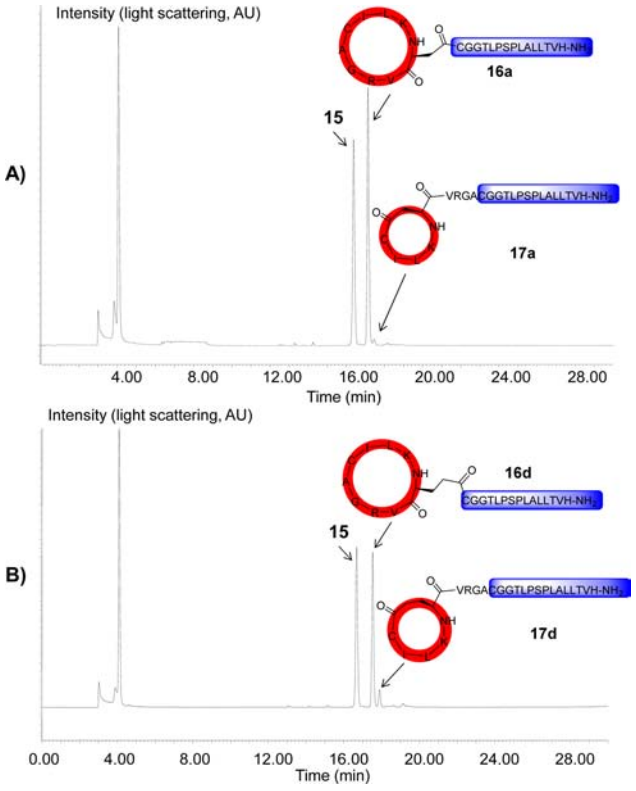

Figure 2. LC-MS analysis of the crude one-pot cyclization/branching process leading to the formation of peptide **16a** (A) or **16d** (B). Peptide **12a** or **12d** (7 mM in 6 M Gdn·HCl 0.1 M pH 7.2 sodium phosphate buffer, TCEP 200 mM, MPAA 200 mM) was cyclized at 37 °C for 48 h in an nitrogen atmosphere. Then peptide **15** (10.5 mM final concentration, 1.5 equiv) was added at the pH adjusted to 5.5. The reaction was stirred at 37 °C for 120 h. The aliquots were acidified with diluted TFA, extracted with diethyl ether to remove MPAA, and then analyzed by LC-MS.

that the KCC process favored backbone cyclization (ratio **16a**/**17a** > 30, entry 1 of Table 1).

Peptide **16a** was isolated by HPLC with a 32% yield overall for the two steps. Its cyclic and branched structure was demonstrated by thorough proteomic analysis following alkylation of the Cys thiols with iodoacetamide and digestion with trypsin (see Supporting Information (SI)). A similar yield and selectivity was obtained with cysteine as the ligating partner

Table 1. Synthesis of Cyclic and Branched Peptides 16a–e,g[a]

entry	SEA peptide	Cys peptide	ratio **16**/**17**[b]	product	yield (%)[c]
1	**12a**	**15**	>30	**16a**	32
2	**12a**	Cys-OH	>30	**16g**	27
3	**12b**	**15**	>30	**16b**	32
4	**12c**	**15**	>30	**16c**	28
5	**12d**	**15**	9	**16d**	28
6	**12e**	**15**	>30	**16e**	12

[a]See the legend of Figure 2 for the experimental conditions. [b]Ratio of peak areas obtained by LC-MS (light scattering detection). [c]Peptides were purified by RP-HPLC. [d]The lower yield in this case is probably due to the small scale of synthesis.

for the second ligation step (peptide **16g**, 27%, entry 2, Table 1). Furthermore, peptide **16g** was analyzed by NMR spectroscopy to definitely establish its cyclic and branched structure (Figure 3, see SI for a full interpretation of the NMR spectra).

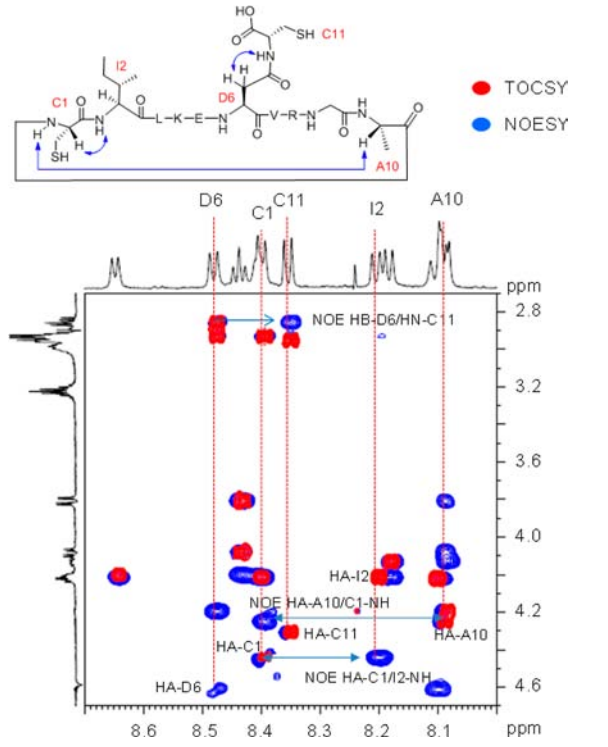

Figure 3. TOCSY (in red) and NOESY (in blue) NMR spectra for peptide **16g** (H_2O/D_2O: 9/1 by vol, 298 K).

In particular, the NOESY spectrum showed that the Cys residue involved in the backbone cyclization step, i.e., Cys1, was in-between Ala10 and Ile2, while the Cys residue introduced during the second ligation step was linked to Asp6.

The other CBs **16b–e** listed in Table 1 were produced similarly. Importantly, CBs **16b–e** and analytical samples of the minor CBs produced in the one-pot cyclization/ligation process, i.e., peptides **17a–e,g**, were all subjected to proteomic analysis to ascertain their structures and the selectivities for backbone cyclization reported in Table 1 (see SI).

Interestingly, comparing entries 1 and 5 of Table 1 reveals that the preference of the KCC process for backbone cyclization was greater for Asp(SEA^{off}) peptide **12a** (Z = Ala, entry 1, Table 1) than for Glu(SEA^{off}) analog **12d** (Z = Ala, entry 5, Table 1), in accord with the observation that intermolecular SEA ligation proceeds significantly faster with Glu(SEA^{off}) peptides than with Asp(SEA^{off}) analogs (compare peptides **5a** and **5b** in Figure 1). The difference in the selectivity for backbone cyclization is clearly visible by comparing Figure 2A (ratio **16a**/**17a** > 30) and Figure 2B (ratio **16d**/**17d** = 9).

Note that this difference in selectivity was not observed for the cyclization of peptides **12b** and **12e** (entries 3 and 6 respectively, Table 1) which are both equipped with a C-terminal Ser residue. This is probably due to the higher reactivity of C-terminal Ser(SEA^{off}) functionality in comparison with the C-terminal Ala(SEA^{off}) one (compare peptides **6a** and **6b** in Figure 1), which favors the backbone cyclization process to a point where the selectivity becomes >30 for both peptides.

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Importantly, the selectivity of the KCC process was good and of synthetic value for all the examined cases.

In conclusion, an N-terminal cysteinyl peptide featuring an internal Asp(SEA^{off}) or Glu(SEA^{off}) residue and a C-terminal SEA^{off} functionality can be cyclized selectively upon activation of the two SEA^{off} groups by reduction. Backbone cyclization is preferred due to the higher reactivity of the C-terminal SEA group in comparison with the Asp/Glu(SEA^{off}) functionalities. The kinetically controlled cyclization process results in the formation of a pendant SEA group which can be ligated in a second step with a Cys peptide to produce cyclic and branched peptides in one pot. Besides the synthesis of CBs, this method can potentially have other useful applications such as the one-pot synthesis of cyclic peptides equipped with a label or chelating moiety.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01847.

Experimental procedures and characterization data for all compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Craik, D. J. *Science* **2006**, *311*, 1563−1564.
(2) Camarero, J. A.; Fushman, D.; Sato, S.; Giriat, I.; Cowburn, D.; Raleigh, D. P.; Muir, T. W. *J. Mol. Biol.* **2001**, *308*, 1045−1062.
(3) Iwai, H.; Pluckthun, A. *FEBS Lett.* **1999**, *459*, 166−172.
(4) Aumailley, M.; Gurrath, M.; Muller, G.; Calvete, J.; Timpl, R.; Kessler, H. *FEBS Lett.* **1991**, *291*, 50−54.
(5) Oren, Z.; Shai, Y. *Biochemistry* **2000**, *39*, 6103−6114.
(6) Sawyer, T. K.; Hruby, V. J.; Darman, P. S.; Hadley, M. E. *Proc. Natl. Acad. Sci. U. S. A.* **1982**, *79*, 1751−1755.
(7) Tudan, C.; Willick, G. E.; Chahal, S.; Arab, L.; Law, P.; Salari, H.; Merzouk, A. *J. Med. Chem.* **2002**, *45*, 2024−2031.
(8) Xu, S.; Li, H.; Shao, X.; Fan, C.; Ericksen, B.; Liu, J.; Chi, C.; Wang, C. *J. Med. Chem.* **2012**, *55*, 6881−6887.
(9) Schroeder, C. I.; Swedberg, J. E.; Craik, D. J. *Curr. Protein Pept. Sci.* **2013**, *14*, 532−542.
(10) Clark, R. J.; Craik, D. J. *Methods Enzymol.* **2012**, *503*, 57−74.
(11) Cui, H.-K.; Guo, Y.; He, Y.; Wang, F.-L.; Chang, H.-N.; Wang, Y.-J.; Wu, F.-M.; Tian, C.-L.; Liu, L. *Angew. Chem., Int. Ed.* **2013**, *52*, 9558−9562.
(12) Timmerman, P.; Beld, J.; Puijk, W. C.; Meloen, R. H. *ChemBioChem* **2005**, *6*, 821−824.
(13) Heinis, C.; Rutherford, T.; Freund, S.; Winter, G. *Nat. Chem. Biol.* **2009**, *5*, 502−507.
(14) Pini, A.; Falciani, C.; Bracci, L. *Curr. Protein Pept. Sci.* **2008**, *9*, 468−477.
(15) Falciani, C.; Lozzi, L.; Pini, A.; Corti, F.; Fabbrini, M.; Bernini, A.; Lelli, B.; Niccolai, N.; Bracci, L. *Chem. Biol. Drug Des.* **2007**, *69*, 216−221.
(16) Lakatos, A.; Gyurcsik, B.; Nagy, N. V.; Csendes, Z.; Weber, E.; Fulop, L.; Kiss, T. *Dalton Trans.* **2012**, *41*, 1713−1726.
(17) Wilson, K.-A.; Kalkum, M.; Ottesen, J.; Yuzenkova, J.; Chait, B. T.; Landick, R.; Muir, T.; Severinov, K.; Darst, S. A. *J. Am. Chem. Soc.* **2003**, *125*, 12475−12483.
(18) Hegemann, J. D.; De Simone, M.; Zimmermann, M.; Knappe, T. A.; Xie, X.; Di Leva, F. S.; Marinelli, L.; Novellino, E.; Zahler, S.; Kessler, H.; Marahiel, M. A. *J. Med. Chem.* **2014**, *57*, 5829−5834.
(19) Gavrish, E.; Sit, C. S.; Cao, S.; Kandror, O.; Spoering, A.; Peoples, A.; Ling, L.; Fetterman, A.; Hughes, D.; Bissell, A.; Torrey, H.; Akopian, T.; Mueller, A.; Epstein, S.; Goldberg, A.; Clardy, J.; Lewis, K. *Chem. Biol.* **2014**, *21*, 509−518.
(20) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776−779.
(21) 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*, 7645−7649.
(22) Shao, Y.; Lu, W.; Kent, S. B. H. *Tetrahedron Lett.* **1998**, *39*, 3911−3914.
(23) Camarero, J. A.; Muir, T. W. *Chem. Commun.* **1997**, 1369−1370.
(24) Zheng, J. S.; Tang, S.; Guo, Y.; Chang, H. N.; Liu, L. *ChemBioChem* **2012**, *13*, 542−546.
(25) Dolphin, G. T. *J. Am. Chem. Soc.* **2006**, *128*, 7287−7290.
(26) van de Langemheen, H.; Brouwer, A. J.; Kemmink, J.; Kruijtzer, J. A. W.; Liskamp, R. M. J. *J. Org. Chem.* **2012**, *77*, 10058−10064.
(27) Lu, J.; Tian, X.-B.; Huang, W. *Chin. Chem. Lett.* **2015**, *26*, 946−950.
(28) Bang, D.; Pentelute, B. L.; Kent, S. B. *Angew. Chem., Int. Ed.* **2006**, *45*, 3985−3988.
(29) Zheng, J. S.; Cui, H. K.; Fang, G. M.; Xi, W. X.; Liu, L. *ChemBioChem* **2010**, *11*, 511−515.
(30) Raibaut, L.; Drobecq, H.; Melnyk, O. *Org. Lett.* **2015**, *17*, 3636−3639.
(31) Raibaut, L.; Cargoet, M.; Ollivier, N.; Chang, Y. M.; Drobecq, H.; Boll, E.; Desmet, R.; Monbaliu, J.-C. M.; Melnyk, O. *Chem. Sci.* **2016**, *7*, 2657−2665.
(32) Ollivier, N.; Dheur, J.; Mhidia, R.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2010**, *12*, 5238−5241.
(33) Zheng, J. S.; Chang, H. N.; Wang, F. L.; Liu, L. *J. Am. Chem. Soc.* **2011**, *133*, 11080−11083.
(34) Melnyk, O.; Agouridas, V. *Curr. Opin. Chem. Biol.* **2014**, *22*, 137−145.
(35) Zheng, J.-S.; Tang, S.; Huang, Y.-C.; Liu, L. *Acc. Chem. Res.* **2013**, *46*, 2475−2484.
(36) Boll, E.; Dheur, J.; Drobecq, H.; Melnyk, O. *Org. Lett.* **2012**, *14*, 2222−2225.
(37) Ollivier, N.; Vicogne, J.; Vallin, A.; Drobecq, H.; Desmet, R.; El-Mahdi, O.; Leclercq, B.; Goormachtigh, G.; Fafeur, V.; Melnyk, O. *Angew. Chem., Int. Ed.* **2012**, *51*, 209−213.
(38) Boll, E.; Drobecq, H.; Ollivier, N.; Blanpain, A.; Raibaut, L.; Desmet, R.; Vicogne, J.; Melnyk, O. *Nat. Protoc.* **2015**, *10*, 269−292.
(39) Drobecq, H.; Boll, E.; Senechal, M.; Desmet, R.; Saliou, J. M.; Lacapere, J. J.; Mougel, A.; Vicogne, J.; Melnyk, O. *Bioconjugate Chem.* **2016**, *27*, 1540−1546.
(40) Ollivier, N.; Raibaut, L.; Blanpain, A.; Desmet, R.; Dheur, J.; Mhidia, R.; Boll, E.; Drobecq, H.; Pira, S. L.; Melnyk, O. *J. Pept. Sci.* **2014**, *20*, 92−97.

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