Organic LETTERS

Letter

pubs.acs.org/OrgLett

Access to Large Cyclic Peptides by a One-Pot Two-Peptide Segment Ligation/Cyclization Process

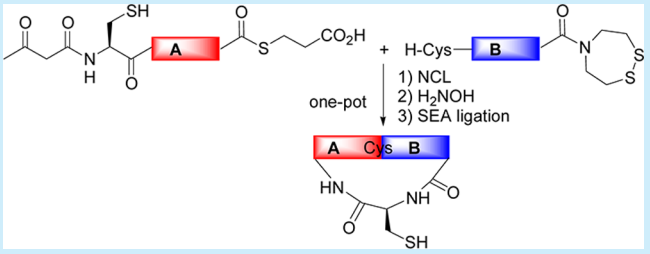
Emmanuelle Boll,[†] Jean-Philippe Ebran,[†] Hervé Drobecq,[†] Ouafâa El-Mahdi,[‡] Laurent Raibaut,[†] Nathalie Ollivier,[†] and Oleg Melnyk*[,†]

[†]UMR CNRS 8161, Université de Lille, Pasteur Institute of Lille 59021 Lille, France

[‡]Université Sidi Mohamed Ben Abdellah, Fes 30000, Morocco

Supporting Information

ABSTRACT: The use of the *N*-acetoacetyl protecting group for N-terminal cysteine residue enabled creation of an efficient and mild one-pot native chemical ligation/SEA ligation sequence giving access to large cyclic peptides.

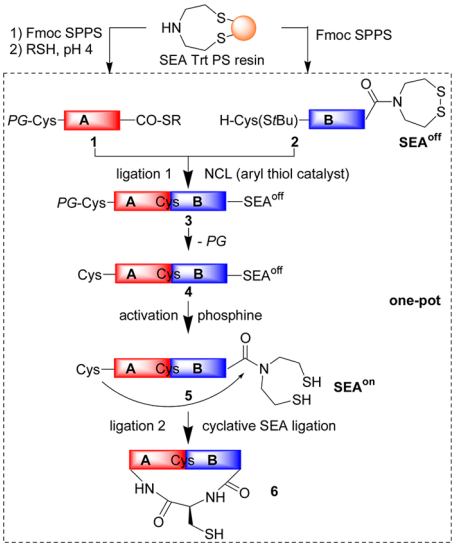


The modest in vivo stability, potency, or selectivity of some polypeptides can be significantly improved by restraining the conformational flexibility of the peptide backbone by cyclization.[1−9] Today, this modification constitutes an important tool for optimizing peptide or protein structures toward desired biological properties.[10] Various reactions have been explored for peptide macrocyclization.[11] Native chemical ligation (NCL),[12] that is the reaction of a C-terminal peptide thioester with an N-terminal cysteinyl (Cys) peptide, is frequently used for synthesizing peptides or proteins featuring a head-to-tail cyclized backbone.[13−15] Methods reminiscent of the NCL reaction also met considerable success in the field.[16−21] Expressed protein ligation (EPL), which relies on the use of recombinant C-terminal thioester polypeptides, is another powerful tool for accessing cyclic peptide scaffolds.[22−24] Cyclative EPL can be performed in purified media[3−5,25] or within cells.[24,26,27] Finally, cyclic peptides or proteins can also be produced using intein-mediated protein trans-splicing.[28,29]

The total synthesis of large cyclic peptides (>40 amino acids) is often faced with the difficulty of obtaining the linear Cys peptidyl thioester precursor in acceptable yield and purity. One potential solution to this problem is to start from shorter peptide thioester segments and to use the NCL reaction both for the assembly of the linear precursor and for the cyclization step. In particular, the assembly of two peptide thioester segments should already facilitate access to large cyclic peptides considering the power of several Boc[30,31] or Fmoc-SPPS[32−43] methods for C-terminal peptide thioester synthesis. Of course, this strategy can be applied only if the peptide contains at least two Cys residues. This is the case for several families of disulfide-rich cyclic peptides such as cyclotides[44] or backbone-cyclized venom toxins,[9] which are of potential therapeutic interest.

We therefore examined the potential of the sequential NCL/cyclative bis(2-sulfanylethyl)amido (SEA) ligation[45,46] process depicted in Scheme 1 for accessing large cyclic peptide scaffolds.

Scheme 1. One-Pot Sequential NCL/Cyclative SEA Ligation Process Gives Access to Large Cyclic Peptides



This strategy relies on the compatibility of the *N*-acylperhydro-1,2,5-dithiazepine functionality (SEA^{off} group)[47] with the NCL reaction[32,48−50] and also on the temporary protection of the N-terminal Cys of segment A to avoid its cyclization or oligomerization during the NCL reaction. One important goal was to design a one-pot process for saving time and yield (dotted square, Scheme 1). Therefore, the conditions used for removing the Cys protecting group (PG) between the two ligations steps

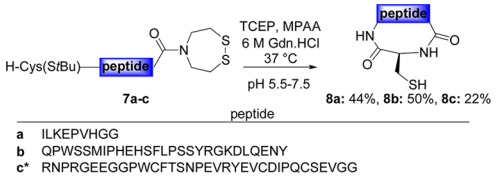
Received: November 19, 2014
Published: December 15, 2014

   dx.doi.org/10.1021/ol503359w | *Org. Lett.* 2015, 17, 130−133

had to be mild, efficient, and compatible with the second SEA intramolecular ligation reaction.

Since the synthesis of head-to-tail backbone cyclized peptides by intramolecular SEA ligation has not been reported before, we examined as a preliminary step the interest of Cys peptidyl SEA^{off} peptides **7a−c** for accessing cyclic peptides (Scheme 2).

Scheme 2. Intramolecular SEA Ligation Gives Access to Cyclic Peptides[a]



[a]For **7c**, internal cysteine thiols are protected by *tert*-butylsulfenyl groups.

Intramolecular SEA ligation was found to proceed efficiently in the pH range 5.5−7.5 in the presence of tris(2-carboxyethyl)-phosphine (TCEP, 200 mM) and 4-mercaptophenylacetic acid (MPAA, 200 mM). The cyclic structure of peptides **8a−c** was demonstrated by alkylating the Cys residues and analyzing the peptides formed by trypsin cleavage (see the Supporting Information).

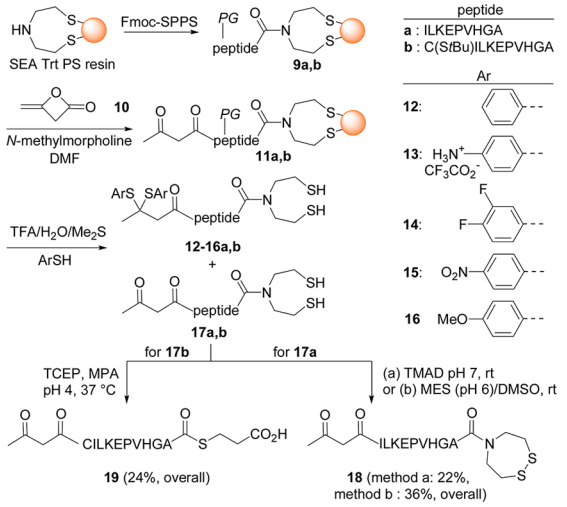
The SEA thioester surrogate is thus complementary to the hydrazide[19,35] method for the head-to-tail cyclization of peptides. Advantageously, the exchange of the SEA group by MPAA and Cys thiols as well as the NCL reaction proceed in a single step at mildly acidic or neutral pH, while other *N,S*-acyl shift systems such as *N*-butyl-*N*-(2-sulfanylethyl)amides[21] require two separate steps at pH 3 and pH 7, respectively.

These results set the stage for choosing the appropriate protecting group for N-terminal Cys residue of thioester segment **1** (Scheme 1). In a first approach, we examined the interest of thiazolidine protecting group (Thz, PG = CH_2) which constitutes a useful tool for protein total synthesis using NCL.[51] Thz can be removed at pH 4 by treatment with an excess of *O*-methylhydroxylamine (0.1 M). Unfortunately, significant hydrolysis of the Thz residue was observed in the mildly acidic conditions (pH 4) used for converting SEA peptides into peptide thioesters derived from 3-mercaptopropionic acid (MPA), thereby precluding the use of this useful reaction for accessing peptide thioesters of type **1** (Scheme 1, PG = CH_2).[33,36] Degradation of Thz residue in acidic conditions has also been mentioned by Liu and co-workers during the conversion of peptide hydrazides into peptide thioesters.[52]

In the search for an alternative protecting group for N-terminal cysteine, we drew our attention to the *N*-acetoacetyl group (AcA), which was introduced by D'Angeli and co-workers about 40 years ago (Scheme 1, PG = CH_3COCH_2CO-).[53] Contrary to Thz protection, the AcA group can be removed with nearly stoichiometric amounts of hydroxylamine.[54] This is because removal of AcA is driven by an intramolecular and irreversible amide bond-cleavage reaction, whereas reversal of the Thz group is an equilibrated reaction that needs an excess of *O*-methylhydroxylamine to be displaced toward the free Cys peptide. So far, the use of AcA group has been limited to a few peptide syntheses in solution[55] or as a protein modifier.[56,57] Moreover, no solid-phase approaches to the synthesis of AcA peptides and applications in chemoselective ligation chemistry have been reported so far.

The Fmoc-SPPS of *N*-acetoacetyl SEA^{off} peptides was undertaken as shown in Scheme 3. Peptidyl resins **9a,b** were

Scheme 3. SPPS of *N*-Acetoacetyl SEA^{off} Peptides



assembled on SEA trityl polystyrene resin using Fmoc-SPPS as described elsewhere.[33,46] The free amino group was acylated successfully using diketene **10** in the presence of *N*-methylmorpholine in DMF. The use of acetoacetyl *N*-succinimidyl ester was equally satisfactory (the isolated yield for model peptide AcA-ALKEPVHGA-NH_2 was 41%). We next examined the cleavage and deprotection step which proved to be challenging. Indeed, preliminary studies showed that triisopropylsilane (TIS) could not be used in the TFA cleavage cocktail due to the significant reduction of the ketone group into the corresponding secondary alcohol. The cleavage and deprotection step was optimized using AcA peptidyl resin **11a**. The presence of thiophenol in the cleavage cocktail yielded significant amounts of the dithioacetal product **12a** (ratio **17a**/**12a**: 1/1 as determined by HPLC using light scattering detection), while the absence of thiophenol in the cleavage cocktail resulted in poor yields, showing the critical role played by thiophenol in scavenging trityl carbocations formed within the PS resin. In the search for a thiol scavenger which could act as a good scavenger while minimizing dithioacetal side-product formation, we screened a series of thiophenols (Scheme 3). The introduction of an electron-withdrawing group on the arylthiol such as in the trifluoroacetate salt of *p*-aminothiophenol resulted in the almost suppression of dithioacetal **13a** formation (**17a**/**13a**: 32/1). However, the trifluoroacetate salt derived from *p*-aminothiophenol precipitated with the peptide in diethyl ether and complicated its purification. Interestingly, 3,4-difluorothiophenol yielded peptide **17a** as a major product (**17a**/**14a**: 59/1, 77% crude) and remained soluble during the peptide precipitation step in diethyl ether. *p*-Nitrothiophenol was less efficient in reducing dithioacetal product **15a** formation (**17a**/**15a**: 8/1) and in scavenging the trityl PS resin since the yield was significantly less than for the other arylthiols examined in this study. In contrast, the introduction of an electron donating group on the arylthiol such as in *p*-methoxythiophenol yielded dithioacetal **16a** as the major product (**17a**/**16a**: 1/5) and behaved as a good trityl PS resin scavenger.

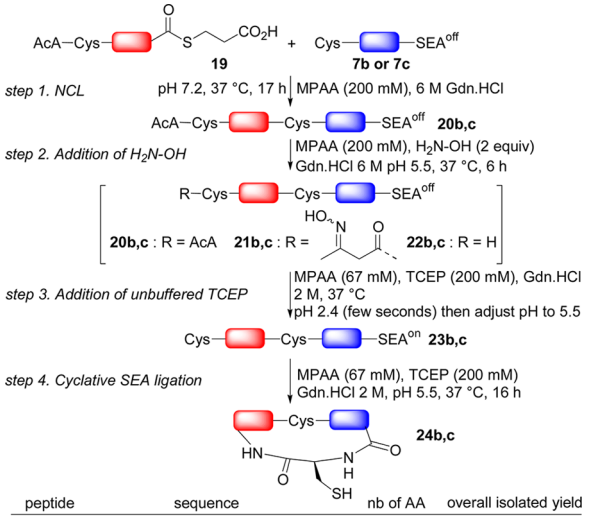
Considering the good results obtained with 3,4-difluorothiophenol, this reagent was selected as a scavenger for the cleavage and deprotection step. The crude SEA^{on} peptide **17a** was further

dx.doi.org/10.1021/ol503359w | *Org. Lett.* 2015, 17, 130−133

oxidized into the SEA^{off} derivative **18** and purified by HPLC. The best results were obtained using a mixture of 2-(*N*-morpholino)-ethanesulfonic acid (MES) pH 6 buffer and DMSO as the oxidant (36% overall yield starting from SEA PS resin). Advantageously, the crude SEA^{on} peptide **17b** could be converted directly into C-terminal peptide thioester **19** in the presence of MPA (5% by vol) and TCEP (200 mM) at pH 4 (24% overall), without detectable degradation of the *N*-acetoacetyl moiety.

The successful synthesis of peptide **19** set the stage for the assembly of large cyclic peptides as shown in Scheme 4. The first

Scheme 4. One-Pot Synthesis of Cyclic Peptides 24a,b



peptide	sequence	nb of AA	overall isolated yield
24b	CILKEPVHGACQPWSSMIPHEHSFLP SSYRGKDLQENY	38	34%
24c	CILKEPVHGACRNPRGEEGGPWCFT SNPEVRYEVCDIPQCSEVGG	45	20%

ligation step was performed in the presence of MPAA (200 mM) in 6 M Gdn·HCl phosphate buffer (pH 7.2, 3 mM for each peptide, step 1 in Scheme 4, Figure 1A). These conditions ensure an efficient NCL reaction while the SEA^{off} group remains unaffected and behaves as a latent thioester group. Figure 1A shows the formation of **20b**−MPAA mixed disulfides mostly during the HPLC sample preparation (extraction of MPAA with diethyl ether after acidification). Next, 2 equiv of hydroxylamine hydrochloride (6 mM) was added and the pH adjusted to 5.5 to trigger the removal of *N*-acetoacetyl group. Unfortunately and as shown in Figure 1B, deprotection was only partial after 6 h, and increasing the reaction time led to no improvement. Experiments with model peptide AcA-FILQEPVFG-NH_2 (1 mM) confirmed that removal of AcA group with hydroxylamine (5 mM) was highly incomplete in the pH range 5−6 but proceeded efficiently in 50% aqueous acetic acid as reported by Bello and co-workers (complete after ∼2 h, pH_{app} ∼ 1.5).[54] No deprotection was observed with hydroxylamine in 0.05% aqueous TFA (pH ∼ 1.8, peptide 1 mM, H_2NOH 5 mM).

Taken together, these data suggest a general acid catalysis of the cleavage reaction, which proceeds substantially faster in 50% aqueous acetic acid. Unfortunately, the addition of the required volumes of acetic acid for removing AcA group would complicate considerably the second SEA ligation step of the one-pot process, which proceeds preferably at pH 5.5 in the presence of TCEP. At this stage, we reasoned that the addition of *unbuffered* TCEP to a final concentration of 0.2 M could decrease significantly the pH of the reaction mixture, while increasing the total carboxylic acid

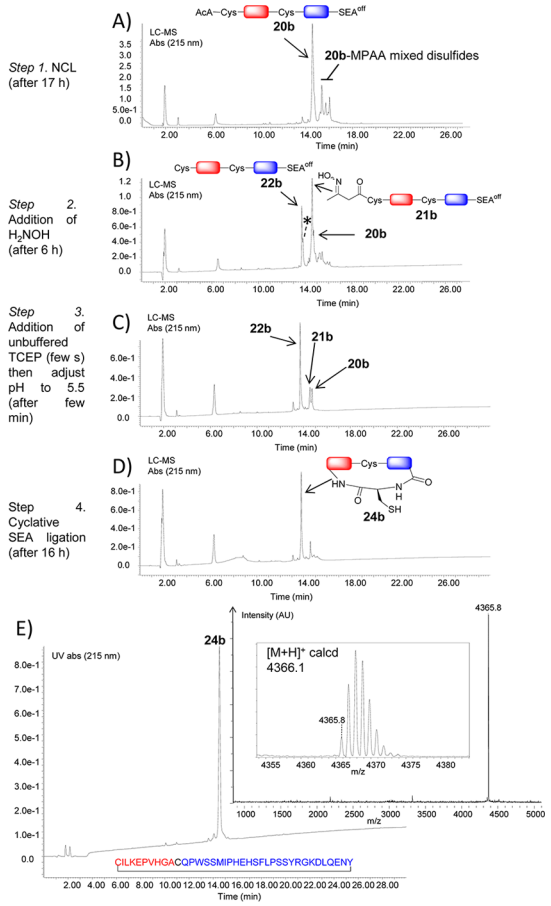


Figure 1. One-pot synthesis of cyclic peptide **24b**. (A−D) HPLC monitoring of the one-pot process. During step 2, peptide **22b** is partially converted into the corresponding cyclic disulfide involving the two cysteines (asterisk); The peaks eluting at 2 and 6 min are nonpeptidic; (E) HPLC and MALDI-TOF analysis of purified peptide **24b**.

concentration to 0.667 M (MPAA + TCEP), and thus allow to mimic the acidic aqueous acetic acid environment needed for AcA removal. Gratifyingly, this proved to be the case as shown in Figure 1C as most of the AcA group was removed in a few seconds after the addition of TCEP. The pH of the reaction mixture was adjusted to 5.5 to trigger the intramolecular SEA ligation and the formation of the target cyclic peptide **24b** (Figure 1D). No side products due to the presence of hydroxylamine were observed. Figure 1D highlights the efficiency of the one-pot process leading to the formation of **24b**, which was isolated successfully by HPLC (34% overall, Figure 1E). This sequence of reactions was repeated with peptides **19** and **7c** with similar efficacy and yielded the 45 amino acids cyclic peptide **24c** with 20% overall yield after HPLC purification. Both cyclic peptides **24b,c** were alkylated with iodoacetamide and subjected to proteolytic digestion by trypsin. The tryptic digests were analyzed by MALDI-TOF mass spectrometry. The peptide fragments formed in these experiments were all in accord with the proposed cyclic peptide structures (see the Supporting Information).

In conclusion, the *N*-acetoacetyl protecting group is an interesting alternative to thiazolidine protection for N-terminal cysteine residue in peptides. It survives the cleavage and deprotection step in TFA if triisopropylsilane is avoided in the cleavage cocktail and if thiophenol is substituted by 3,4-

dx.doi.org/10.1021/ol503359w | *Org. Lett.* 2015, 17, 130−133

difluorothiophenol as a scavenger. The *N*-acetoacetyl group is stable during the NCL reaction and is removed with only 2 equiv of hydroxylamine in the presence of TCEP, which acts as a powerful catalyst. The *N*-acetoacetyl group enabled the design of an efficient and mild one-pot sequential NCL/cyclative SEA ligation process which gives access to large backbone cyclized peptides. The *N*-acetoacetyl group can have potentially many other useful applications such as the design of traceless linkers for the purification of peptides by covalent capture or for the solid phase synthesis of proteins.[58] These novel developments will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Procedures and characterization for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: oleg.melnyk@ibl.cnrs.fr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank CNRS, Université de Lille, SIRIC OncoLille, Région Nord Pas de Calais, and the European Community for financial support. We thank also the CSB facility for technical help and Guy Lippens for performing some NMR experiments.

■ REFERENCES

(1) Aumailley, M.; Gurrath, M.; Muller, G.; Calvete, J.; Timpl, R.; Kessler, H. *FEBS Lett.* **1991**, *291*, 50.
(2) Sawyer, T. K.; Hruby, V. J.; Darman, P. S.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1751.
(3) Camarero, J. A.; Fushman, D.; Sato, S.; Giriat, I.; Cowburn, D.; Raleigh, D. P.; Muir, T. W. *J. Mol. Biol.* **2001**, *308*, 1045.
(4) Iwai, H.; Pluckthun, A. *FEBS Lett.* **1999**, *459*, 166.
(5) Camarero, J. A.; Muir, T. W. *J. Am. Chem. Soc.* **1999**, *121*, 5597.
(6) Tudan, C.; Willick, G. E.; Chahal, S.; Arab, L.; Law, P.; Salari, H.; Merzouk, A. *J. Med. Chem.* **2002**, *45*, 2024.
(7) Xu, S.; Li, H.; Shao, X.; Fan, C.; Ericksen, B.; Liu, J.; Chi, C.; Wang, C. *J. Med. Chem.* **2012**, *55*, 6881.
(8) Oren, Z.; Shai, Y. *Biochemistry* **2000**, *39*, 6103.
(9) Clark, R. J.; Craik, D. J. *Methods Enzymol.* **2012**, *503*, 57.
(10) Cheneval, O.; Schroeder, C. I.; Durek, T.; Walsh, P.; Huang, Y. H.; Liras, S.; Price, D. A.; Craik, D. J. *J. Org. Chem.* **2014**, *79*, 5538.
(11) White, C. J.; Yudin, A. K. *Nat. Chem.* **2011**, *3*, 509.
(12) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994**, *266*, 776.
(13) Clark, R. J.; Craik, D. J. *Biopolymers* **2010**, *94*, 414.
(14) Camarero, J. A.; Pavel, J.; Muir, T. W. *Angew. Chem., Int. Ed.* **1998**, *37*, 347.
(15) Camarero, J. A.; Muir, T. W. *Chem. Commun.* **1997**, 1369.
(16) Shao, Y.; Lu, W.; Kent, S. B. H. *Tetrahedron Lett.* **1998**, *39*, 3911.
(17) Quaderer, R.; Hilvert, D. *Chem. Commun. (Cambridge)* **2002**, 2620.
(18) Tchertchian, S.; Hartley, O.; Botti, P. *J. Org. Chem.* **2004**, *69*, 9208.
(19) Zheng, J.-S.; Tang, S.; Guo, Y.; Chang, H.-N.; Liu, L. *ChemBioChem* **2012**, *13*, 542.
(20) Boll, E.; Dheur, J.; Drobecq, H.; Melnyk, O. *Org. Lett.* **2012**, *14*, 2222.
(21) Taichi, M.; Hemu, X.; Qiu, Y.; Tam, J. P. *Org. Lett.* **2013**, *15*, 2620.
(22) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705.
(23) Evans, T. C.; Benner, J.; Xu, M.-Q. *Protein Sci.* **1998**, *7*, 2256.
(24) Borra, R.; Camarero, J. A. *Biopolymers* **2013**, *100*, 502.
(25) Kimura, R. H.; Tran, A. T.; Camarero, J. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 973.
(26) Camarero, J. A.; Fushman, D.; Cowburn, D.; Muir, T. W. *Bioorg. Med. Chem.* **2001**, *9*, 2479.
(27) Camarero, J. A.; Kimura, R. H.; Woo, Y. H.; Shekhtman, A.; Cantor, J. *ChemBioChem* **2007**, *8*, 1363.
(28) Scott, C. P.; Abel-Santos, E.; Wall, M.; Wahnon, D. C.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13638.
(29) Tavassoli, A.; Benkovic, S. J. *Nat. Protocols* **2007**, *2*, 1126.
(30) Canne, L. E.; Walker, S. M.; Kent, S. B. H. *Tetrahedron Lett.* **1995**, *36*, 1217.
(31) Hojo, H.; Aimoto, S. *Bull. Soc. Chem. Jpn.* **1991**, *64*, 111.
(32) Boll, E.; Drobecq, H.; Ollivier, N.; Raibaut, L.; Desmet, R.; Vicogne, J.; Melnyk, O. *Chem. Sci.* **2014**, *5*, 2017.
(33) Ollivier, N.; Raibaut, L.; Blanpain, A.; Desmet, R.; Dheur, J.; Mhidia, R.; Boll, E.; Drobecq, H.; Pira, S. L.; Melnyk, O. *J. Pept. Sci.* **2013**, *20*, 92.
(34) Zheng, J. S.; Chang, H. N.; Wang, F. L.; Liu, L. *J. Am. Chem. Soc.* **2011**, *133*, 11080.
(35) 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.
(36) Dheur, J.; Ollivier, N.; Vallin, A.; Melnyk, O. *J. Org. Chem.* **2011**, *76*, 3194.
(37) Raz, R.; Rademann, J. *Org. Lett.* **2011**, *13*, 1606.
(38) Mende, F.; Beisswenger, M.; Seitz, O. *J. Am. Chem. Soc.* **2010**, *132*, 11110.
(39) Tofteng, A. P.; Sørensen, K. K.; Conde-Frieboes, K. W.; Hoeg-Jensen, T.; Jensen, K. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 7411.
(40) Blanco-Canosa, J. B.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2008**, *47*, 6851.
(41) Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5*, 2951.
(42) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684.
(43) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369.
(44) Wang, C. K.; Gruber, C. W.; Cemazar, M.; Siatskas, C.; Tagore, P.; Payne, N.; Sun, G.; Wang, S.; Bernard, C. C.; Craik, D. J. *ACS Chem. Biol.* **2014**, *9*, 156.
(45) Hou, W.; Zhang, X.; Li, F.; Liu, C. F. *Org. Lett.* **2011**, *13*, 386.
(46) Ollivier, N.; Dheur, J.; Mhidia, R.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2010**, *12*, 5238.
(47) Melnyk, O.; Agouridas, V. *e-EROS Encycl. Reagents Org. Synth.* **2014**, DOI: 10.1002/9780470842898.rn01723.
(48) 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.
(49) Raibaut, L.; Vicogne, J.; Leclercq, B.; Drobecq, H.; Desmet, R.; Melnyk, O. *Bioorg. Med. Chem.* **2013**, *21*, 3486.
(50) Raibaut, L.; Adihou, H.; Desmet, R.; Delmas, A. F.; Aucagne, V.; Melnyk, O. *Chem. Sci.* **2013**, *4*, 4061.
(51) Bang, D.; Kent, S. B. *Angew. Chem., Int. Ed.* **2004**, *43*, 2534.
(52) Fang, G.-M.; Wang, J.-X.; Liu, L. *Angew. Chem., Int. Ed.* **2012**, *51*, 10347.
(53) D'Angeli, F.; Filira, F.; Scoffone, E. *Tetrahedron Lett.* **1965**, *6*, 605.
(54) Di Bello, C.; Filira, F.; Giormani, V.; D'Angeli, F. *J. Chem. Soc. C* **1969**, 350.
(55) Clemens, R. J. *Chem. Rev.* **1986**, *86*, 241.
(56) Marzotto, A.; Pajetta, P.; Scoffone, E. *Biochem. Biophys. Res. Commun.* **1967**, *26*, 517.
(57) Marzotto, A.; Pajetta, P.; Galzigna, L.; Scoffone, E. *Biochim. Biophys. Acta* **1968**, *154*, 450.
(58) Raibaut, L.; El Mahdi, O.; Melnyk, O. *Top. Curr. Chem.* **2014**, *10*, 1007/128−2014−609.