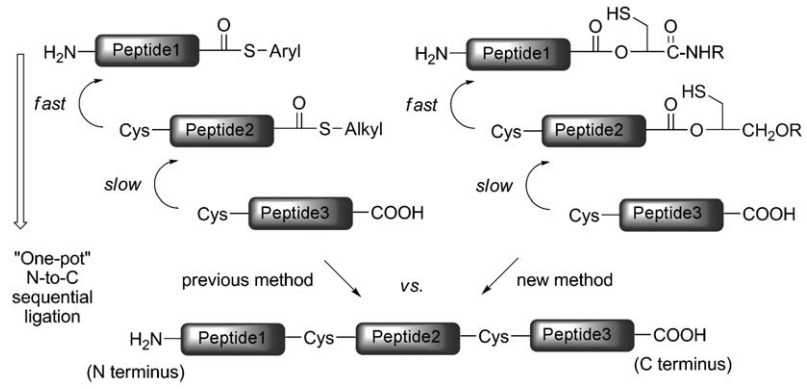
DOI: 10.1002/cbic.200900789

Chemical Protein Synthesis by Kinetically Controlled Ligation of Peptide O-Esters

Ji-Shen Zheng, Hong-Kui Cui, Ge-Min Fang, Wei-Xian Xi, and Lei Liu*[a]

Protein chemical synthesis can overcome potential limitations of protein expression and produce proteins with predesigned changes and modifications with atomic precision. The development of increasingly efficient and general methods for peptide ligation comprises a central objective.[1] One important challenge is to synthesize proteins by sequential ligation of three or more unprotected peptide segments. Earlier strategies focused on sequential ligations toward the N terminus from a C-terminal Cys peptide segment,[2] but the counterpart N-to-C sequential assembly of the peptide segments[3] was difficult, and this prevented fully convergent protein synthesis. To solve the problem Kent et al. recently invented kinetically controlled ligation (KCL; Scheme 1).[4] The success of KCL relies on the large



Scheme 1. Previous vs. new kinetically controlled ligation.

reactivity difference between a peptide-α-thioarylester and a peptide-α-thioalkylester. Despite this advance, difficulties still exist for the preparation of peptide thioesters[5] and more troublesome thioarylesters.[6] Here we describe a novel, practical approach for chemical protein synthesis through *kinetically controlled ligation of peptide O-esters*. The new method overcomes the difficulties in the preparation of ligation segments. The study also reveals interesting reactivity variations in the intramolecular acyl shift reaction. This study was inspired by the seminal work of Danishefsky,[7] Botti,[8] and others,[9] who pioneered peptide ligation through their use of the in situ O-to-S acyl shift.

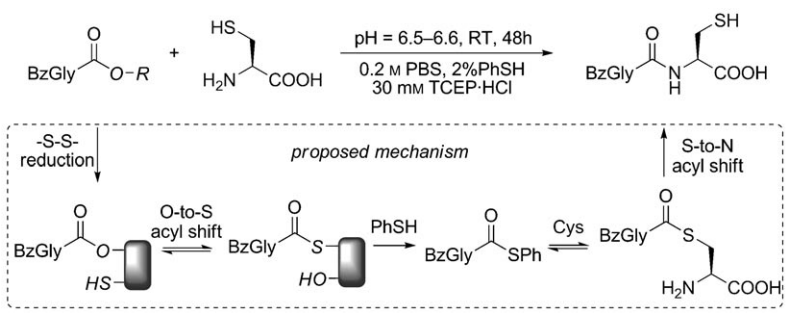
Our investigation began by examining model peptide O-ester **1** (Table 1).[7] It was found that **1** (as an O-arylester) is not suitable for Fmoc solid-phase peptide synthesis (SPPS) because it cannot survive the conditions used for Fmoc cleavage. Subsequently we focused on peptide O-alkylesters (**2**–**12**), which have structures that differ by the number of carbon atoms between O and S and by the position and nature of substitution. It was found that in cases in which the number of carbon atoms between O and S equals to two, the reactants (**4**–**9**) can be smoothly consumed. However, HPLC analysis indicated that except for **5** and **6**, all other peptide O-esters (**4**, **7**–**9**) were heavily hydrolyzed into peptide–CO_2H. Thus, the presence of a side chain adjacent to the oxygen atom is beneficial to the O-to-S acyl shift (which possibly proceeds through a five-membered ring intermediate), whereas the presence of a side chain adjacent to the sulfur atom does the opposite. In cases in which the number of carbon atoms between O and S increases to three, esters (**10**–**12**) show very low conversions after 48 h; this indicates that the corresponding O-to-S acyl shift (through a six-membered ring intermediate) is slow. Fusion of a phenyl ring into the linker (**2**) can dramatically accelerate the conversion, but in such cases only the hydrolyzed product was obtained. Finally, ester **3** (in which the number of carbon atoms between O and S equals to four) shows a modest conversion; however, one half of the product is the hydrolyzed peptide.

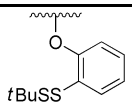
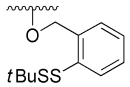
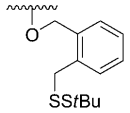
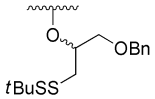
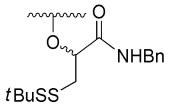
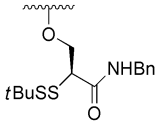
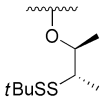
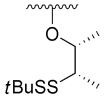
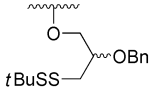
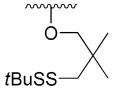
The above systematic examinations showed that only **5** and **6** are useful for the peptide ligation through in situ O-to-S acyl shift. Note that **6** (denoted as type I ester in the following discussion) was previously examined by Botti[8] and Muir.[9b,c] Compared to **6**, **5** (denoted as type II ester) exhibited an improved ligation yield (93% vs. 84%) due to less hydrolysis. More experiments showed that for the type II ester of Ala (Table 2), the ligation yield was 73% at pH 6.75 (entry 1). This value is also higher than the parallel value for the type I ester of Ala under the same conditions (62%, entry 13). Replacement of the ligation accelerator to MPAA[10] (entry 2) or change of pH (entries 3–4) did not improve the ligation. On the other hand, in cases in which the buffer was changed to imidazole (entry 11) less hydrolysis and a higher ligation yield (89%) were

[a] J.-S. Zheng, H.-K. Cui, G.-M. Fang, W.-X. Xi, Prof. L. Liu
Department of Chemistry
Key Laboratory of Bioorganic Phosphorus Chemistry
and Chemical Biology (Ministry of Education), Tsinghua University
Beijing 100084 (China)
Fax: (+86) 10-62771149
E-mail: lliu@mail.tsinghua.edu.cn

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900789.

Table 1. Performance of different peptide O-esters.[a]



	O–R	Conversion	Hydrolysis	Ligation
1		n.d.	n.d.	n.d.
2		100	100	0
3		69	31	38
4		100	90	10
5		99	6	93
6		100	16	84
7		99	87	12
8		97	86	11
9		85	79	6
10		11	3	8
11		10	2	8
12		14	2	12

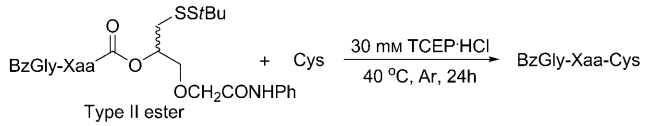
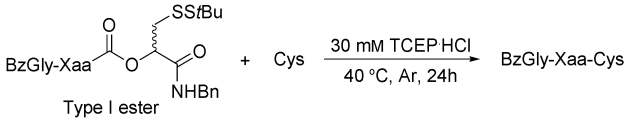
[a] All the values were measured by using HPLC. [b] n.d. not determined. [c] Diastereomers were difficult to separate and therefore were used as a mixture.

observed. The same behavior was also observed for the type I ester of Ala, in which the ligation yield increased to 78% in the imidazole buffer (entry 14). Finally, the esters of amino acids carrying more complex side chains (entry 6) could be successfully ligated, but the ligation yields were low for the most sterically hindered amino acids (entries 7–8).

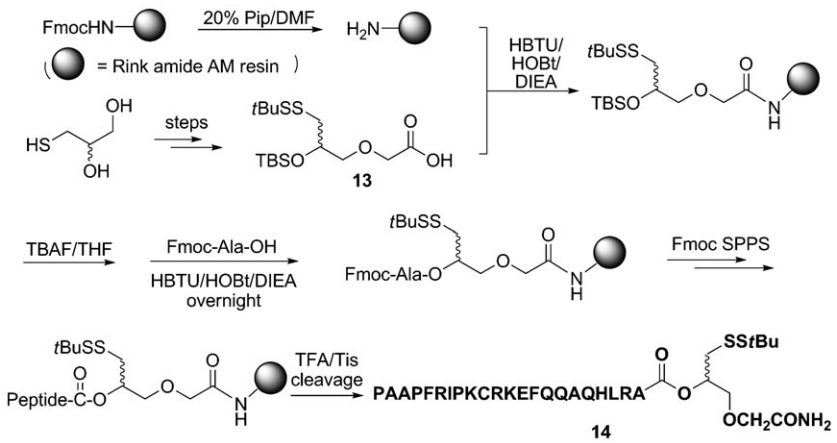
The above results show that the newly developed type II ester is better than the type I ester for peptide ligation. To demonstrate the utility of the type II ester in chemical protein synthesis, we chose the S4 small chain of napin (a plant antifungal defensive protein) as a model.[11] The synthesis started with the Fmoc protected Rink amide aminomethyl resin and compound **13**, which was prepared readily from 3-mercaptopropane-1,2-diol (Scheme 2). By using the standard Fmoc protocol the desired peptide ester (**14**) was successfully prepared. Subsequently **14** was reacted with the Cys-containing peptide segment **15** [Eq. (1)] to afford the desired product **16** smoothly in 80% yield. Compared to the standard native chemical ligation, the major improvement of the present approach is that all the peptide segments used in the ligation can be readily prepared through the Fmoc method under mild conditions.

Interestingly and importantly, it was found that the optimal temperature for the ligation of the type II ester of peptides is about 40 °C. At 40 °C the ligation between **5** and Cys is completed in about 6 h, but at lower temperatures the same reaction is much slower (Figure 1). By comparison, the ligation between **6** and Cys is a much faster reaction that is completed in about 2 h at 12–15 °C. Estimation of the initial rates revealed that the reactivity of **5** and **6** differs by about 30-

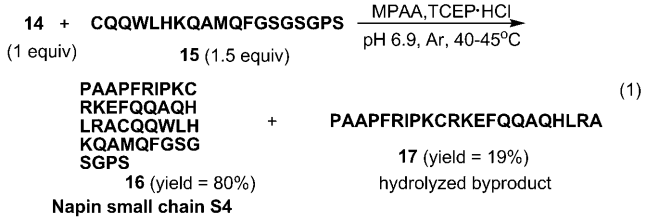
Table 2. Ligation of peptide O-esters with cysteine.[a]

	Xaa	Medium	pH	Yield [%][b] Ligation	Hydrolysis
					
1	Ala	PBS	6.75[c]	73	25
2	Ala	PBS	6.75[d]	68	30
3	Ala	PBS	6.50[c]	70	24
4	Ala	PBS	7.00[c]	67	24
5	Gly	PBS	6.75[c]	93	6
6	Leu	PBS	6.75[c]	63	35
7	Val	PBS	6.75[c]	18	25
8	Pro	PBS	6.75[c]	< 2	16
9	Ala	HEPES	6.75[c]	76	24
10	Ala	Tris	6.75[c]	75	19
11	Ala	Imi	6.75[c]	89	9
					
12	Gly	PBS	6.75[c]	84	16
13	Ala	PBS	6.75[c]	62	37
14	Ala	Imi	6.75[c]	78	20

PBS concentration was 0.2 M, HEPES concentration was 1.0 M, Tris concentration was 1.0 M, Imi concentration was 1.0 M. [a] Peptide O-ester (1.6 mM) was reacted with Cys (2.0 mM) in 6 M guanidine buffer containing 20% NMP. [b] HPLC yields. [c] Accelerator 2% PhSH. [d] Accelerator 30 mM MPAA.



Scheme 2. Solid-phase synthesis of the type II ester of a peptide.



fold. Such a large reactivity difference between **5** and **6** can be attributed to their structures. In **5** the linker connecting the O and S atoms carries an alkyl side chain, whereas in **6** the side chain is an acyl group. Because an acyl group is electron withdrawing whereas an alkyl group is electron donating, the ester group in **6** is expected to be much more electrophilic than that in **5**. Thus, the intramolecular O-to-S acyl shift in **6** is more favorable both kinetically and thermodynamically than that in **5**. The outcome is that **6** ligates with Cys much faster than **5**.

An important application of the large reactivity difference between the type I and II peptide O-esters is the possibility of carrying out KCL,[4] for the first time solely with peptide O-esters. Three peptide segments (peptide1-type I ester, Cys-peptide2-type II ester, and Cys-peptide3) can be sequentially ligated from the N to the C terminus (Scheme 1); this takes advantage of the dual reactivity of the middle segment (that is, a bifunctional Cys-peptide2-type II ester), which can be controlled so that it can firstly react with a peptide1-type I ester. To demonstrate the utility of the KCL of peptide O-esters, we report an effective chemical synthesis of [V15A]crambin. The same model protein was previously synthesized by Kent and coworkers.[2b,c] Briefly, three peptide segments were synthesized through Fmoc SPPS (Scheme 3). The left segment (**18**) was firstly treated with the middle segment (**19**) under native chemical ligation reaction conditions at 15 °C. After 1 h most of the reactants were consumed. Mass analysis indicated that the major product **21** is the ligated product of **18** and **19**, whereas the main byproducts (**22** and **23**) correspond to the hydrolyzed peptide from **18** and cyclized peptide from **19**. After 2 h the C-terminal peptide segment **20** was added into the reaction mixture. The ligation temperature was increased to 40 °C and the reaction was completed overnight. The full-length 46-residue polypep-

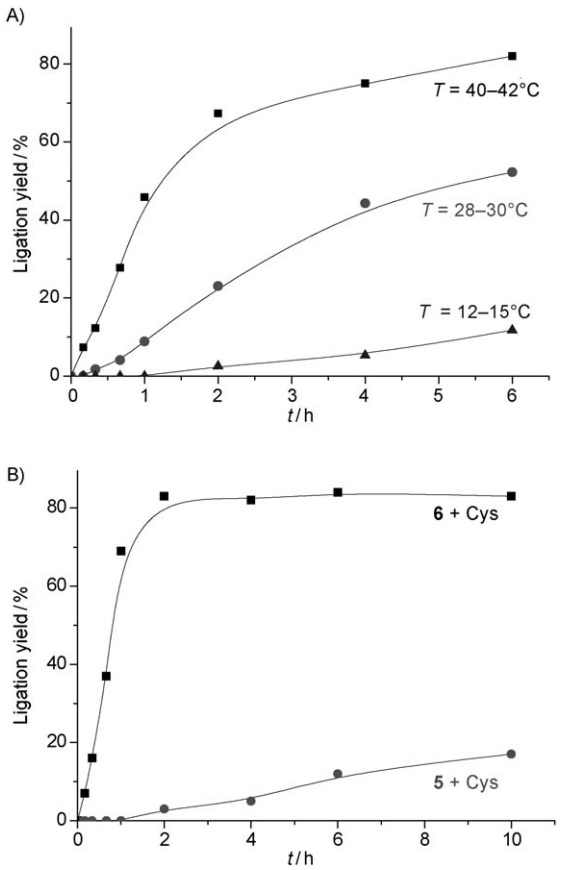


Figure 1. Rates for the ligation of peptide O-esters. A) The ligation between **5** (1.6 mM) and Cys (2.0 mM) at different temperatures. B) The ligation between **5** (1.6 mM) and Cys (2.0 mM) versus the ligation between **6** (1.6 mM) and Cys (2.0 mM) at 12–15 °C.

tide chain of [V15A]crambin was successfully obtained in 59% yield (HPLC yield).

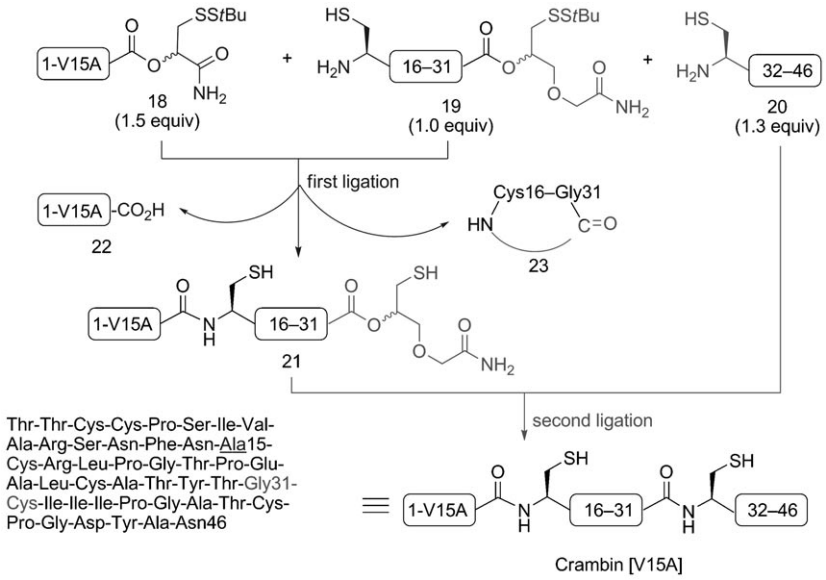
There are three advantages of the above "one-pot" KCL method: 1) First, starting from the three purified peptide segments, the one-pot synthesis can be completed to afford the desired product with good yields and with only one final purification step in two days; 2) Second, the peptide segments in the present KCL can be prepared through mild Fmoc methods, which might be useful for the synthesis of more difficult targets such as glycoproteins; 3) Third, the N-terminal segment in the present KCL can tolerate unprotected Cys residues (for example, **18** contains two unprotected Cys residues), whereas in the previous KCL an unprotected Cys residue in the left segment (as a peptide-αthioarylester) might easily form a less reactive thiolactone that will impede the KCL.[4b]

In summary, through systematic examinations we have confirmed one and identified a new peptide O-ester that can be used for peptide ligation through in situ O-to-S acyl shift. These peptide O-esters can be readily prepared through mild Fmoc solid-phase synthesis and thus we have extended the practical utility of native chemical ligation. Most importantly, a large reactivity difference was observed between the two peptide O-esters. This allows, for the first time, a "one-pot" N-to-C sequential condensation of peptide segments through kinetically controlled ligation with more readily accessible *peptide O-esters instead of peptide thioesters*.

Acknowledgements

This work was supported by NSFC (No. 20802040 and No. 20932006), and the Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant No. 200800030074).

Keywords: ligation · peptide O-esters · peptide thioesters · peptides · solid-phase synthesis



Scheme 3. Kinetically controlled ligation strategy for one-pot synthesis of crambin[V15A].

[1] a) P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923; b) B. L. Nilsson, M. B. Soellner, R. T. Raines, *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91; c) C. Haase, O. Seitz, *Angew. Chem.* **2008**, *120*, 1575; *Angew. Chem. Int. Ed.* **2008**, *47*, 1553; d) S. B. H. Kent, *Chem. Soc. Rev.* **2009**, *38*, 338.

[2] a) A. Brik, E. Keinan, P. E. Dawson, *J. Org. Chem.* **2000**, *65*, 3829; b) D. Bang, N. Chopra, S. B. H. Kent, *J. Am. Chem. Soc.* **2004**, *126*, 1377; c) D. Bang, S. B. H. Kent, *Angew. Chem.* **2004**, *116*, 2588; *Angew. Chem. Int. Ed.* **2004**, *43*, 2534; d) D. Bang, S. B. H. Kent, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 5014; e) S. Ingale, T. Buskas, G. J. Boons, *Org. Lett.* **2006**, *8*, 5785; f) E. C. B. Johnson, T. Durek, S. B. H. Kent, *Angew. Chem.* **2006**, *118*, 3361; *Angew. Chem. Int. Ed.* **2006**, *45*, 3283; g) E. C. B. Johnson, E. Malito, Y. Shen, D. Rich, W. J. Tang, S. B. H. Kent, *J. Am. Chem. Soc.* **2007**, *129*,

11480; h) R. K. McGinty, J. Kim, C. Chatterjee, R. G. Roeder, T. W. Muir, *Nature* **2008**, *453*, 812; i) B. L. Pentelute, Z. P. Gates, J. L. Dashnau, J. M. Vanderkooi, S. B. H. Kent, *J. Am. Chem. Soc.* **2008**, *130*, 9702.

[3] a) L. E. Canne, A. R. Ferred Amare, S. K. Burley, S. B. H. Kent, *J. Am. Chem. Soc.* **1995**, *117*, 2998; b) L. E. Canne, P. Botti, R. J. Simon, Y. J. Chen, E. A. Dennis, S. B. H. Kent, *J. Am. Chem. Soc.* **1999**, *121*, 8720; c) Z. W. Miao, J. P. Tam, *J. Am. Chem. Soc.* **2000**, *122*, 4253; d) J. P. Tam, Q. T. Yu, J. L. Yang, *J. Am. Chem. Soc.* **2001**, *123*, 2487.

[4] a) D. Bang, B. L. Pentelute, S. B. H. Kent, *Angew. Chem.* **2006**, *118*, 4089; *Angew. Chem. Int. Ed.* **2006**, *45*, 3985; b) T. Durek, V. Y. Torbeev, S. B. H. Kent, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4846; c) V. Y. Torbeev, S. B. H. Kent, *Angew. Chem.* **2007**, *119*, 1697; *Angew. Chem. Int. Ed.* **2007**, *46*, 1667; d) Q. Wan, S. J. Danishefsky, *Angew. Chem.* **2007**, *119*, 9408; *Angew. Chem. Int. Ed.* **2007**, *46*, 9248; e) D. Crich, I. Sharma, *Angew. Chem.* **2009**, *121*, 7727; *Angew. Chem. Int. Ed.* **2009**, *48*, 7591.

[5] a) H. Hojo, S. Aimoto, *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111; b) M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Res. Ther.* **2007**, *13*, 31; c) T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705; d) Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684; e) R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11369; f) A. Sewing, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3503; *Angew. Chem. Int. Ed.* **2001**, *40*, 3395; g) A. R. Mezo, R. P. Cheng, B. Imperiali, *J. Am. Chem. Soc.* **2001**, *123*, 3885; h) J. Brask, F. Albericio, K. J. Jensen, *Org. Lett.* **2003**, *5*, 2951; i) J. A. Camarero, B. J. Hackel, J. J. De Yoreo, A. R. Mitchell, *J. Org. Chem.* **2004**, *69*, 4145; j) C. Dose, O. Seitz, *Org. Lett.* **2005**, *7*, 4365; k) N. Ollivier, J. B. Behr, O. El-Mahdi, A. Blanpain, O. Melnyk, *Org. Lett.* **2005**, *7*, 2647; l) T. Kawakami, M. Sumida, K. Nakamura, T. Vorherr, S. Aimoto, *Tetrahedron Lett.* **2005**, *46*, 8805; m) S. Mezzato, M. Schaffrath, C. Unverzagt, *Angew. Chem.* **2005**, *117*, 1677; *Angew. Chem. Int. Ed.* **2005**, *44*, 1650; n) F. Nagaike, Y. Onuma, C. Kanazawa, H. Hojo, A. Ueki, Y. Nakahara, Y. Nakahara, *Org. Lett.* **2006**, *8*, 4465; o) T. Kawakami, S. Aimoto, *Tetrahedron Lett.* **2007**, *48*, 1903; p) F. Mende O. Seitz, *Angew. Chem.* **2007**, *119*, 4661; *Angew. Chem. Int. Ed.* **2007**, *46*, 4577; q) N. Yamamoto, Y. Tanabe, R. Okamoto, P. E. Dawson, Y. Kajihara, *J. Am. Chem. Soc.* **2008**, *130*, 501; r) S. Ficht, R. J. Payne, R. T. Guy, C. H. Wong, *Chem. Eur. J.* **2008**, *14*, 3620; s) D. Lelièvre, P. Barta, V. Aucagne, A. F. Delmas, *Tetrahedron Lett.* **2008**, *49*, 4016; t) T. Kawakami, S. Aimoto, *Tetrahedron* **2009**, *65*, 3871.

[6] a) D. Bang, B. L. Pentelute, Z. P. Gates, S. B. H. Kent, *Org. Lett.* **2006**, *8*, 1049; b) J. B. Blanco-Canosa, P. E. Dawson, *Angew. Chem.* **2008**, *120*, 6957; *Angew. Chem. Int. Ed.* **2008**, *47*, 6851; c) H. Hojo, Y. Murasawa, H. Katayama, T. Ohira, Y. Nakahara, Y. Nakahara, *Org. Biomol. Chem.* **2008**, *6*, 1808; d) A. P. Tofteng, K. Sørensen, K. W. Conde-Frieboes, T. Hoeg-Jensen, K. J. Jensen, *Angew. Chem.* **2009**, *121*, 7547; *Angew. Chem. Int. Ed.* **2009**, *48*, 7411.

[7] a) J. D. Warren, J. S. Miller, S. J. Keding, S. J. Danishefsky, *J. Am. Chem. Soc.* **2004**, *126*, 6576; b) G. Chen, J. D. Warren, J. H. Chen, B. Wu, Q. Wan, S. J. Danishefsky, *J. Am. Chem. Soc.* **2006**, *128*, 7460; c) B. Wu, J. H. Chen, J. D. Warren, G. Chen, Z. H. Hua, S. J. Danishefsky, *Angew. Chem.* **2006**, *118*, 4222; *Angew. Chem. Int. Ed.* **2006**, *45*, 4116; d) G. Chen, Q. Wan, Z. Tan, C. Kan, Z. Hua, K. Ranganathan, S. J. Danishefsky, *Angew. Chem.* **2007**, *19*, 7527; *Angew. Chem. Int. Ed.* **2007**, *46*, 7383; e) Q. Wan, J. Chen, Y. Yuan, S. J. Danishefsky, *J. Am. Chem. Soc.* **2008**, *130*, 15814; f) Z. Tan, S. Shang, T. Halkina, Y. Yuan, S. J. Danishefsky, *J. Am. Chem. Soc.* **2009**, *131*, 5424.

[8] P. Botti, S. Manganiello, H. Gaertner, *Org. Lett.* **2004**, *6*, 4861.

[9] a) A. P. Tofteng, K. J. Jensen, T. Hoeg-Jensen, *Tetrahedron Lett.* **2007**, *48*, 2105; b) E. A. George, R. P. Novick, T. W. Muir, *J. Am. Chem. Soc.* **2008**, *130*, 4914; c) K. P. Chiang, M. S. Jensen, R. K. McGinty, T. W. Muir, *ChemBioChem* **2009**, *10*, 2182.

[10] a) E. C. B. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640; b) E. Wieczerzak, R. Hamel Jr. , V. Chabot, V. Aimez, M. Grandbois, P. G. Charette, E. Escher, *Biopolymers* **2008**, *90*, 415.

[11] G. M. Neumann, R. Condron, I. Thomas, G. M. Polya, *Biochim. Biophys. Acta* **1996**, *1295*, 23.

Received: December 29, 2009
Published online on February 15, 2010

