

Peptide Cyclization

Synthesis of Constrained Head-to-Tail Cyclic Tetrapeptides by an Imine-Induced Ring-Closing/Contraction Strategy**

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Head-to-tail cyclic peptides exhibit remarkable biological activities with extraordinary potency and oral-bioavailability owing to their compacted structures and high stability against enzymatic degradation and physical denaturation; thus, cyclic peptides have attracted a lot of attention in the pharmaceutical industry.^[1,2] In particular, cyclic peptides with small-sized rings have a drug-like scaffold that is ideal for therapeutic development.^[3] A range of cyclic tetrapeptides have been shown to exhibit a wide spectrum of biological activities, including the cytotoxic agents trapoxin and hirsutide, the opioid receptor-binding CJ-15208, the antiprotozoal apicidin, and the antifungal rhodopeptins.^[4]

The exploration of cyclic peptides for medicinal chemistry requires efficient synthetic methods.^[5,7] In principle, lactamization of linear peptides can be achieved by the standard dehydration reagent-mediated reactions^[6] for coupling peptides. However, peptide cyclization often proceeds more slowly than a regular intermolecular peptide coupling reaction and, therefore, is accompanied by the competing dimerization and oligomerization reactions.^[7] Because of the constrained geometry of the peptide backbone, small cyclic peptides (less than six amino acid residues) present a challenge for chemical peptide cyclization.^[7,8] Particularly, the synthesis of cyclic tetrapeptides with rigid 12-membered ring structures^[14] rely on: 1) the use of external conformational assistance^[9] and 2) the introduction of internal turn-inducing residues, such as glycine, proline, D-amino acids, tertiary amide,^[8a,10] or pseudoproline.^[11] Therefore, synthesis of tetrapeptides comprising any of the regular L-amino acids without a pre-organized structure is still considered a challenging, if not impossible, task.^[7,10] Herein, we describe the synthesis of cyclic tetrapeptides through an intramolecular serine/threonine ligation (Figure 1),^[12,13] which uses an imine-induced ring-closure contraction to decrease the activation energy barrier, thus enabling tetrapeptide cyclization.

We hypothesized that the formation of the head-to-tail imine (**2**) with a 16-membered ring structure would be relatively more favorable than that for a peptide with a 12-

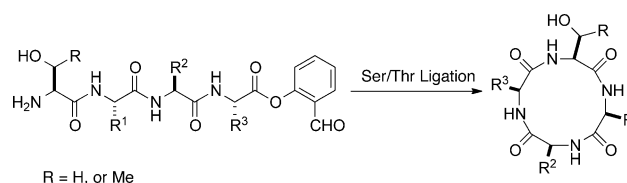
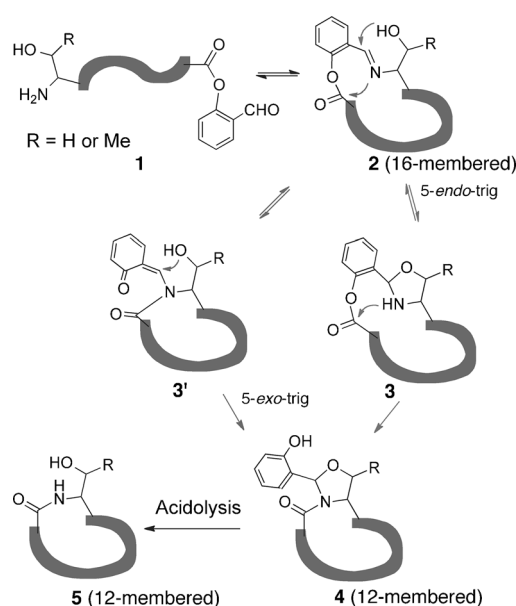


Figure 1. Synthesis of cyclic tetrapeptides. R¹⁻³ = amino acid side chains.



Scheme 1. Two possible pathways for the proposed tetrapeptide cyclization using serine and threonine ligation.

membered ring (Scheme 1). Subsequently, the intramolecular ring contraction by way of an O→N acyl transfer to afford the *N,O*-benzylidene acetal intermediate (**4**) through intermediate **3** or **3'** would be made possible by an increase in enthalpy, resulting from the amide bond formation. Then the resulting acetal group could be removed to form the cyclic tetrapeptide with a natural peptidic bond. We were also aware that the four bonds introduced are not flexible (i.e., the *ortho*-substituents on the benzene with a fixed angle), thereby likely not reducing the strain energy very much during the ring contraction step, which challenges our hypothesis.^[15]

Our first task was to devise an efficient strategy to prepare the requisite peptide salicylaldehyde (SAL) esters. Owing to the lability of the O-SAL ester during the process of Fmoc-based solid-phase peptide synthesis (SPPS), we previously introduced an Fmoc-SPPS of peptide SAL esters using on-

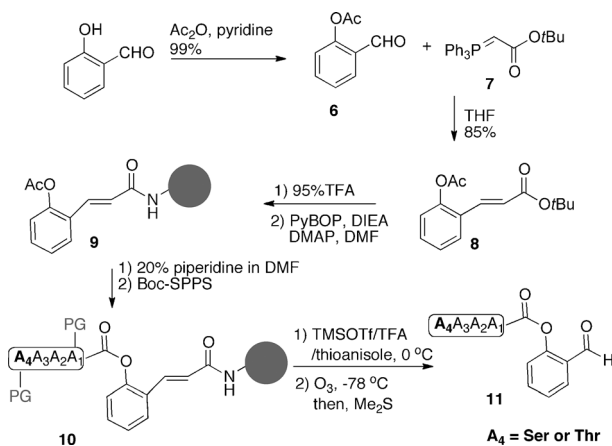
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resin phenolysis.^[12] Herein, we developed a Boc-SPPS of peptide SAL esters. Towards this goal, we elected to link the aldehyde group of salicylaldehyde to the resin through an alkene linker, which could be restored to the aldehyde group by ozonolysis (Scheme 2).



Scheme 2. Synthesis of peptide SAL esters using aminomethyl resin (sphere). PG = protecting group; THF = tetrahydrofuran; TFA = trifluoroacetic acid; PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; DIEA = *N,N*-diisopropylethylamine; DMAP = 4-dimethylaminopyridine; DMF = dimethylformamide; TMSOTf = trifluoromethanesulfonic acid trimethylsilylester.

To this end, the hydroxy group of salicylaldehyde was first protected with an acetate group affording **6**, followed by the Wittig reaction with **7** to form **8**. The liberated carboxylic acid upon TFA treatment was coupled to the aminomethyl resin with PyBOP, generating **9**. The phenol group, after removal of the acetate group, became amenable to Boc-SPPS. After the resin-bound peptide (**10**) was synthesized, TMSOTf/TFA/thioanisole was used to remove the side-chain protecting groups if present,^[17] while keeping the peptide intact on the resin. Lastly, the aldehyde was regenerated by ozonolysis and, at the same time, the peptide SAL ester (**11**) was released from the resin (Scheme 2). According to this method, we prepared and purified, by reverse-phase HPLC, a series of tetrapeptide SAL esters with an N-terminal serine or threonine in overall yields of 9–21 %, based on resin loading. The obtained tetrapeptide SAL esters were very stable and could be stored at room temperature without any decomposition. This strategy thus provides a reliable and facile solution to the preparation of peptide SAL esters. Admittedly, ozone is an extremely reactive oxidizing species, which caused oxidation of some amino acid residues. In our studies, we observed that Cys, Met, and Trp were affected by this oxidation.

To exclude the possibility of a pre-organized conformation induced by turn-inducing residues, our peptide sequences were randomly selected, while intentionally avoiding glycine, proline, or D-amino acids as the turn-inducing element. We realized that the ring strain inherent in head-to-tail peptide cyclization, in particular those of small sizes, would favor the cyclodimerization reaction.^[5a, 8a, 10a] We carefully analyzed the

crude cyclization mixture and tried to pinpoint the ratio of the monomeric product to the dimerized cyclic product.

The obtained peptide SAL esters were dissolved in a pyridine–acetic acid mixture (molar ratio 1:2) at a concentration of 1 mM. Gratifyingly, these peptide SAL esters cyclized smoothly and most of the cyclizations were complete within four hours. The desired monocyclization was observed as the major reaction product, with ratios of cyclomonomer to cyclodimer from 3:2 to 9:1. Notably, the cyclization of the sequence SYIA-SAL ester produced almost exclusively the cyclomonomer product (Table 1, entry 6). Following acid-

Table 1: Synthesis of a series of cyclic tetrapeptides.

Entry	Sequence	Mass [Da]	Monomer:Dimer ^[b]
1	cyclo-(SAAA) ^[a]	404	4:1
2	cyclo-(TALL)	398	7:3
3	cyclo-(TLLA)	398	9:1
4	cyclo-(SHIF)	484	3:2
5	cyclo-(TVVA)	370	9:1
6	cyclo-(SYIA)	434	99:1
7	cyclo-(SFIA)	418	9:1
8	cyclo-(TINA)	399	3:2
9	cyclo-(TKLA)	413	3:2

[a] The cyclized product with *N,O*-benzylidene acetal was isolated by reverse-phase HPLC. The cyclic product after acidolysis co-eluted with the solvent in HPLC. [b] The ratio was determined by analytical LC–MS.

olysis with TFA/H₂O, the cyclic tetrapeptides were purified by reverse-phase HPLC and characterized by NMR spectroscopy. In comparison, the conventional lactamization conditions (HATU, DIEA, DMF; DEPBT, DIEA, DMF; PyBOP, DIEA, DMF) to cyclize TLLA produced none or trace amounts of the desired cyclic peptides.

The unprotected amino acid residues, including tyrosine, histidine, asparagine, and lysine, did not cause any apparent side reactions during the cyclization. In particular, the direct aminolysis of peptide salicylaldehyde esters, a great concern of ours, by the N-terminus or the internal amino group (e.g. lysine) if present, did not occur. This serine/threonine-mediated cyclization involves two steps. The first step is the formation of the cyclic acetal intermediate (**4** in Scheme 1). During this step, if the unwanted head-to-side-chain or head-to-tail cyclization resulting from direct aminolysis were to occur, the resulting product would have a different molecular weight and physical properties from the head-to-tail *N,O*-benzylidene acetal linked cyclic product, thereby allowing for easy differentiation and separation. We carefully analyzed the crude reaction mixture by LC–MS and did not observe any such undesired products. The second step uses acidolysis to remove the *N,O*-benzylidene acetal group, affording the desired head-to-tail cyclic product with a natural peptidic bond.

Another important issue was epimerization at the cyclization site. Thus, we synthesized both a SYIA-SAL ester and the epimeric SYIA-SAL ester to study their cyclization products. Both cyclizations proceeded well, without epimerization at the C-terminal alanine site (Figure 2).

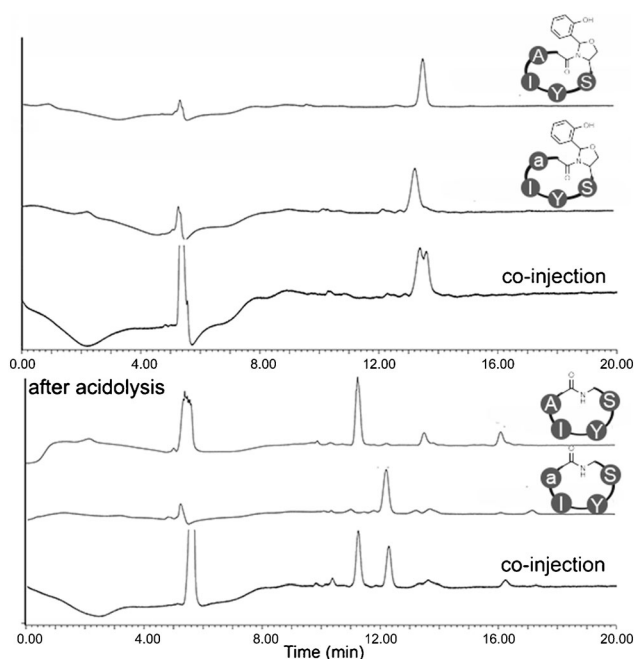


Figure 2. HPLC analysis of the crude cyclization mixture of linear peptides SYIA-SAL ester and SYIa-SAL ester. No epimerization at the cyclization site was observed.

To gain insight into the mechanism of cyclization of tetrapeptide-SAL esters as well as understand the enabling factors, we performed the molecular modeling studies to address two critical issues: 1) which pathway $2 \rightarrow 3 \rightarrow 4$ versus $2 \rightarrow 3' \rightarrow 4$ is favored, and 2) what is the factor that contributes to a lower energy barrier for the tetrapeptide cyclization using the intramolecular Ser/Thr ligation strategy.

As shown in Scheme 1, the *N,O*-benzylidene acetal intermediate (**4**) is chemoselectively generated between a salicylaldehyde and the N-terminal serine or threonine through imine capture followed by ring formation (5-*endo*-trig) by way of an anti-Baldwin cyclization^[16] of the α -hydroxy group of Ser/Thr, generating an sp^3 -hybridized N atom (as in **3**) to initiate acyl transfer. Alternatively, it is also likely that an sp^2 -hybridized N atom could induce acyl transfer forming **3'**, followed by a 5-*exo*-trig pro-Baldwin cyclization.^[16]

Table 2 summarizes the enthalpy changes (ΔH) and free energy changes (ΔG) of cyclization of the tetrapeptide SAAA-SAL ester using the serine ligation shown in

Table 2: Thermodynamic parameters for cyclization of tetrapeptides.^[a]

Species	$\Delta H^{[b]}$ [kcal mol ⁻¹]	$\Delta G^{[c]}$ [kcal mol ⁻¹]
2	1.0	-7.5
3	1.5	-5.3
3'	27.4	18.7
4	-2.3	-9.3
5	6.8	-2.8

[a] Change in enthalpy (ΔH) and free energy (ΔG) of the cyclization of tetrapeptide SAAA-SAL ester using serine ligation in acetic acid at 298.15 K and 1 atm. [b] $\Delta H(x) = H(x) + H(H_2O) - H(1)$.

[c] $\Delta G(x) = G(x) + G(H_2O) - G(1)$.

Scheme 1 (with R=H). Formation of the intermediate **2** through imine capture is thermodynamically favored (ΔG) over the reactant **1** and the product cyclo-(SAAA) by 7.5 kcal and 4.7 kcal mol⁻¹, respectively. Next, formation of oxazolidine intermediate **3** has the enthalpy and free energy changes of 1.5 kcal mol⁻¹ and -5.3 kcal mol⁻¹, respectively, over **1**. In contrast, the intermediate **3'** generated by the sp^2 -hybridized N atom induced acyl transfer is thermodynamically hindered with enthalpy and free energy changes of 27.4 kcal mol⁻¹ and 18.7 kcal mol⁻¹, respectively (Figure S29). Compared to **1**, the formation of intermediate **3** brings the nitrogen atom of the N-terminus closer and the carbon atom of the C-terminal carboxylic; their distances in **1** and **3** are 6.94 Å and 3.97 Å, respectively. This prepares it for the subsequent intramolecular ring contraction by way of an O \rightarrow N acyl transfer.

The resultant *N,O*-benzylidene acetal intermediate **4** has the lowest enthalpy and free energy of -2.3 kcal mol⁻¹ and -9.3 kcal mol⁻¹ over **1**, respectively. This ensures that the whole reaction stops at the stable intermediate **4** prior to acidolysis. Although, the ring size decreases from 16-member to the less-stable 12-member ring during the process of $3 \rightarrow 4$, the enthalpy gained from the amide bond formation compensates and surpasses the destabilization caused by the ring strain. Notably, the process $1 \rightarrow 4$ is thermodynamically favorable over the cyclization of tetrapeptides with turn-inducing residues (APAA, AA_{N-CH₃}AA, AaAA) or pentapeptide (AAAAA), with ΔG for these ranging from -5.1 kcal mol⁻¹ to -6.2 kcal mol⁻¹ (Table S1).

We estimated the activation energies for various cyclization processes using a previously implemented method (Figure S30).^[15a] The calculated activation energies for the imine capture (Figure 3a) and the intramolecular ring contraction (Figure 3b) are 7.0 kcal mol⁻¹ and 6.7 kcal mol⁻¹, respectively, which are similar to those for amide bond formation in the cyclization of the unsubstituted linear pentapeptide and its auxiliary-linked equivalents, with values 6.2–7.6 kcal mol⁻¹.^[15a] In contrast, the direct amide bond formation in the cyclization of both unsubstituted tetrapeptide SAAA (Figure 3c) and its SAL equivalent (Figure 3d) have sub-

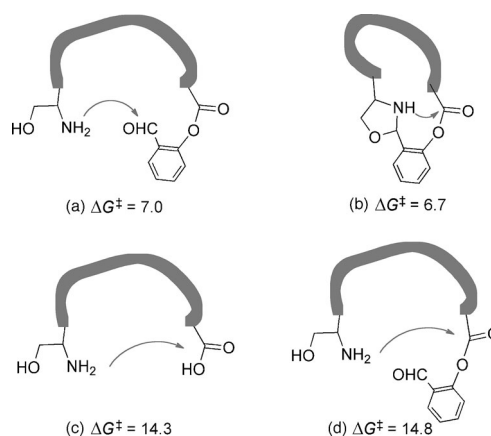


Figure 3. Estimated activation energies (kcal mol⁻¹) for cyclizations of (a) SAL ester tetrapeptide SAAA forming a Schiff base; (b) pseudoproline structure (**3**); (c) direct lactamization of tetrapeptide SAAA; (d) direct aminolysis of the tetrapeptide SAAA-SAL ester.

stantially higher values of 14.3 kcal mol⁻¹ and 14.8 kcal mol⁻¹, respectively. This is consistent with the previous observation that the head-to-tail tetrapeptide cyclization is difficult to realize using a conventional approach, and indicates that the high energy barrier in the cyclization can be bypassed by an imine-induced ring-closing/ring-contraction strategy using tetrapeptide SAL esters.

In conclusion, we demonstrated the applicability of salicylaldehyde ester-induced ligation at serine/threonine sites in cyclizing small peptides. We first developed an efficient approach to prepare the requisite peptide salicylaldehyde ester using Boc-SPPS. Then, several cyclic tetrapeptides, which did not contain any turn-inducing amino acid residue, were synthesized accordingly. On the basis of molecular-modeling investigations, we concluded that, compared to the conventional head-to-tail lactamization approach, intramolecular Ser/Thr ligation can bypass the high energy barrier, which is needed to overcome the disfavored backbone strain, by a quick and chemoselective ring-closing capture, followed by ring contraction using an O-to-N acyl transfer. Finally, after acidolysis, a head-to-tail amide-bonded cyclic tetrapeptide with natural peptidic bonds was synthesized. During the cyclization, epimerization at the ligating C-terminus was not observed. We will extend our studies to synthesis of cyclic peptides with different sizes using intramolecular Ser/Thr ligation.

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- [1] a) D. J. Craik, N. M. Allewell, *J. Biol. Chem.* **2012**, *287*, 26999; b) M. Katsara, T. Tselios, S. Deraos, G. Deraos, M. T. Matsoukas, E. Lazoura, J. Matsoukas, V. Apostolopoulos, *Curr. Med. Chem.* **2006**, *13*, 2221.
- [2] E. M. Driggers, S. P. Hale, J. Lee, N. K. Terrett, *Nat. Rev. Drug Discovery* **2008**, *7*, 608.
- [3] a) D. A. Horton, G. T. Bourne, M. L. Smythe, *Chem. Rev.* **2003**, *103*, 893; b) J. M. Beierle, W. S. Horne, J. H. van Maarseveen, B. Waser, J. C. Reubi, M. R. Ghadiri, *Angew. Chem.* **2009**, *121*, 4819; *Angew. Chem. Int. Ed.* **2009**, *48*, 4725.
- [4] a) M. Kijima, M. Yoshida, K. Sugita, S. Horinouchi, T. Beppu, *J. Biol. Chem.* **1993**, *268*, 22429; b) T. Saito, H. Hirai, Y. J. Kim, Y. Kojima, Y. Matsunaga, H. Nishida, T. Sakakibara, O. Suga, T. Sujaku, N. Kojima, *J. Antibiot.* **2002**, *55*, 847; c) H. Chiba, H. Agematu, K. Dobashi, T. Yoshioka, *J. Antibiot.* **1999**, *52*, 700;
- d) A. S. Norgren, F. Büttner, S. Prabpai, P. Kongsaree, P. I. Arvidsson, *J. Org. Chem.* **2006**, *71*, 6814.
- [5] For reviews, see: a) J. S. Davies, *J. Pept. Sci.* **2003**, *9*, 471; b) S. Jiang, Z. Li, K. Ding, P. P. Roller, *Curr. Org. Chem.* **2008**, *12*, 1502; c) S. R. Gracia, K. Gaus, N. Sewald, *Future Med. Chem.* **2009**, *1*, 1289; d) Y. Hamada, T. Shioiri, *Chem. Rev.* **2005**, *105*, 4441; e) J. P. Tam, C. T. T. Wong, *J. Biol. Chem.* **2012**, *287*, 27020; f) J. N. Lambert, J. P. Mitchell, K. D. Roberts, *J. Chem. Soc. Perkin Trans. 1* **2001**, 471.
- [6] A. El-Faham, F. Albericio, *Chem. Rev.* **2011**, *111*, 6557.
- [7] C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509.
- [8] a) M. El Haddadi, F. Cavelier, E. Vivies, A. Azmani, J. Verducci, J. Martinez, *J. Pept. Sci.* **2000**, *6*, 560; b) D. Skropeta, K. A. Jolliffe, P. Turner, *J. Org. Chem.* **2004**, *69*, 8804; c) J. Lécaillon, P. Gilles, G. Subra, J. Martinez, M. Amblard, *Tetrahedron Lett.* **2008**, *49*, 4674; d) J.-S. Zheng, S. Tang, Y. Guo, H.-N. Chang, L. Liu, *ChemBioChem* **2012**, *13*, 542; e) F. Cavelier-Frontin, S. Achmad, J. Verducci, R. Jacquier, G. Pèpe, *THEOCHEM* **1993**, *286*, 125; f) M. Malesevic, U. Strijowski, D. Bächle, N. Sewald, *J. Biotechnol.* **2004**, *112*, 73; g) A. Ehrlich, H.-U. Heyne, R. Winter, M. Beyermann, H. Haber, L. A. Carpino, M. Bienert, *J. Org. Chem.* **1996**, *61*, 8831.
- [9] a) D.-F. Tai, Y.-F. Lin, *Chem. Commun.* **2008**, 5598; b) K. Hass, W. Ponikvar, H. Nöth, W. Beck, *Angew. Chem.* **1998**, *110*, 1200; *Angew. Chem. Int. Ed.* **1998**, *37*, 1086.
- [10] a) U. Schmidt, J. Langner, *J. Peptide Res.* **1997**, *49*, 67; b) V. D. Bock, R. Perciancante, T. P. Jansen, H. Hiemstra, J. H. Van Maarseveen, *Org. Lett.* **2006**, *8*, 919; c) E. Cini, C. B. Botta, M. Rodriguez, M. Taddei, *Tetrahedron Lett.* **2009**, *50*, 7159; d) Y. Takeuchi, G. R. Marshall, *J. Am. Chem. Soc.* **1998**, *120*, 5363; e) J. Pastuszak, J. H. Gardner, J. Singh, D. H. Rich, *J. Org. Chem.* **1982**, *47*, 2982.
- [11] K. A. Fairweather, N. Sayyadi, I. J. Luck, J. K. Clegg, K. A. Jolliffe, *Org. Lett.* **2010**, *12*, 3136.
- [12] a) Y. Zhang, C. Xu, H. Y. Lam, C. L. Lee, X. Li, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 6657; b) X. Li, H. Y. Lam, Y. Zhang, C. K. Chan, *Org. Lett.* **2010**, *12*, 1724.
- [13] H. Y. Lam, Y. Zhang, H. Liu, J. Xu, C. T. Wong, C. Xu, X. Li, *J. Am. Chem. Soc.* **2013**, *135*, 6272.
- [14] H. Kawai, R. D. Jasensky, D. H. Rich, *J. Am. Chem. Soc.* **1983**, *105*, 4456.
- [15] Previous examples using an auxiliary-mediated ring-closure/ring-contraction strategy with significant epimerization: a) W. D. F. Meutermans, S. W. Golding, G. T. Bourne, L. P. Miranda, M. J. Dooley, P. F. Alewood, M. L. Smythe, *J. Am. Chem. Soc.* **1999**, *121*, 9790; b) W. D. F. Meutermans, G. T. Bourne, S. W. Golding, D. A. Horton, M. R. Campitelli, D. Craik, M. Scanlon, M. L. Smythe, *Org. Lett.* **2003**, *5*, 2711; c) J. P. A. Rutters, Y. Verdonk, R. de Vries, S. Ingemann, H. Hiemstra, V. Levacher, J. H. van Maarseveen, *Chem. Commun.* **2012**, *48*, 8084.
- [16] K. Gilmore, I. V. Alabugin, *Chem. Rev.* **2011**, *111*, 6513.
- [17] Arg(Tos) cannot be cleanly deprotected.