Tetrahedron Letters 58 (2017) 610–613

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Improvement of stability of phenacyloxycarbamidomethyl (Pocam) group, a cysteine protecting group removable with zinc reduction, under acidic conditions

Hidekazu Katayama *, Takuma Goto

Department of Applied Biochemistry, Faculty of Engineering, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan

ARTICLE INFO

Article history:
Received 10 November 2016
Revised 21 December 2016
Accepted 28 December 2016
Available online 31 December 2016

Keywords:
Pocam
Solid-phase peptide synthesis
Thioester method
Thiol protecting group

ABSTRACT

In order to improve the stability of phenacyloxycarbamidomethyl (Pocam) group, a cysteine protecting group removable with zinc reduction, under acidic conditions, various alkyl substituents on the nitrogen atom of Pocam group were examined. As a result, attachment of an electron-withdrawing group improved the stability, and 2,2,2-trifluoroethyl (Tfe) group was most effective among four substituents tested. Tfe-Pocam group could be used in solid-phase peptide synthesis and peptide condensation reactions, and it was also useful for regioselective disulfide formation reactions.

In the preparation of small peptides, solid-phase peptide synthesis (SPPS) developed by Merrifield[1] is widely used, whereas it is usually limited to a length of approximately less than 50 amino acid residues. To obtain long peptide chains chemically, peptide condensation methods, such as the native chemical ligation[2] and the thioester method,[3,4] are generally used.

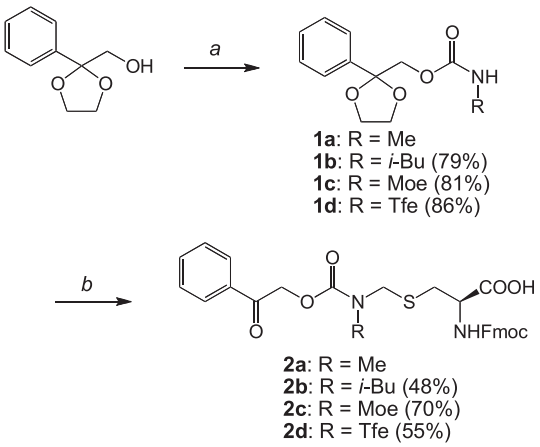
In the thioester method, to achieve the chemoselective condensation, protecting groups at Lys and Cys side chains are required. For the protection of Lys residue, *tert*-butoxycarbonyl (Boc) group was originally used.[4] However, Boc group was labile in trifluoroacetic acid (TFA) solution which was generally used for the final deprotection step of 9-fluorenylmethoxycarbonyl (Fmoc)-based SPPS, and it should be re-introduced to the peptide segments after the purification steps. In order to overcome this inconvenience, we have demonstrated that the azido group acted efficiently as an amino protecting group.[5] Azido group was stable under TFA acidic conditions, and could be converted into an amino group by the reduction with Zn powder in acetic acid (AcOH) solution. Using this strategy, we could synthesize a glycoprotein carrying both *N*-linked and *O*-linked glycans.[6] On the other hand, the deprotection conditions of azido group are different from those of acetamidomethyl (Acm) group which was used for protecting Cys side chains, and these protecting groups could not be removed in single step. In order to simplify the deprotection steps, we have developed Cys protecting groups, *N*-methyl-phenacyloxycarbamidomethyl (Me-Pocam)[7] and phenacyl (Pac),[8] which are cleavable with Zn/AcOH treatment, and applied them to the peptide condensation reactions by the thioester method. These protecting groups, however, have some disadvantages; Me-Pocam group was not completely stable under TFA acidic conditions, and careful manipulation was required.[7] Pac group was stable under acidic conditions, although the Pac-protected peptides could not be obtained in high yield by Fmoc-SPPS due to the undesirable side reaction on the carbonyl group of Pac moiety.[8] Therefore, a novel Cys protecting group which is stable under acidic conditions, does not cause significant side reactions and is cleavable with Zn reduction was desired.

Allyloxycarbonylaminomethyl (Allocam) group has been developed as a Cys protecting group.[9] Similar to Me-Pocam, Allocam group was labile under acidic conditions. On the other hand, it was also reported that the attachment of 2,3,5,6-tetrafluoro-4-(*N*'-piperidino)phenyl group, an electron-withdrawing group (EWG), to the nitrogen atom enhanced the stability under acidic conditions of Allocam group.[10] Based on these observations, we thought that the substitution of methyl group of Me-Pocam into EWG might enhance the stability of Pocam group. In this study, we synthesized several Cys(Pocam) derivatives, examined the stability of these protecting groups, and demonstrated that a Pocam group with EWG was quite useful for peptide synthesis and peptide condensation reactions by the thioester method.

We selected 2-methoxyethyl (Moe) and 2,2,2-trifluoroethyl (Tfe) groups as EWGs. In order to examine the effect of

* Corresponding author.
E-mail address: katay@tokai-u.jp (H. Katayama).

http://dx.doi.org/10.1016/j.tetlet.2016.12.081
0040-4039/



Scheme 1. Synthesis of Fmoc-Cys(Pocam)-OH derivatives. Reaction conditions: (a) 1. Disuccinimidyl carbonate, diisopropylethylamine (DIEA), DMAP, DMF; 2. R-NH_2, DIEA. (b) 1. Formalin, Na_2CO_3, H_2O/1,2-dimethoxyethane; 2. Cysteine hydrochloride, TFA; 3: Fmoc-OSu, DIEA, 1,2-dimethoxyethane.

electron-donated group on Pocam, we also tried to synthesize isobutyl (*i*-Bu) derivative other than Me-Pocam. The various Cys (Pocam) derivatives were synthesized essentially according to the method described previously (Scheme 1).[7] Equimolar amounts of 2-hydroxyacetophenone ethylene acetal and disuccinimidyl carbonate were mixed, and then alkylamine was added to the reaction mixture, giving phenacyl *N*-alkylcarbamate ethylene acetal **1**. The yields of Moe-, Tfe- and *i*-Bu-derivatives were 81%, 86% and 79%, respectively, and these values were comparable to that of Me-derivative (68%) reported previously.[7] The products **1** were then hydroxymethylated with formalin, and introduced to cysteine in neat TFA. Finally, the amino group of cysteine derivatives was protected by Fmoc group, giving Moe-, Tfe- and *i*-Bu-derivatized Fmoc-Cys(Pocam)-OH **2** in 70%, 55% and 48% yields, respectively.

In order to examine the stability of various Pocam groups under TFA acidic conditions, these Cys(Pocam) derivatives **2** were separately introduced to a peptide by Fmoc-SPPS, and the yields of Pocam-attached and Pocam-deprotected peptides were determined after deprotection step with TFA cocktail treatment and reversed-phase (RP)-HPLC purification. At first, we synthesized the C-terminal segment of insect growth-blocking peptide (GBP), GBP(11-25), as a model. GBP was originally isolated from the larval hemolymph of the host armyworm, *Pseudaletia separata*, whose development is halted in the last larval instar stage from parasitization by the parasitoid wasp, *Cotesia hariyai*.[11–13] Starting from Fmoc-Gln(Trt)-Wang resin, the peptide chain corresponding to the GBP sequence was elongated by the ordinary Fmoc-SPPS using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as condensation reagents. During SPPS, Fmoc-Lys(N_3)-OH and Fmoc-Cys(Pocam)-OH derivatives were used at Lys^{20} and Cys^{19} positions, respectively. After the chain assembly, crude peptides were cleaved from the solid support, and analyzed by RP-HPLC. As a result, no significant side reaction on any Pocam groups was observed during SPPS, and the desired peptides **3** were found on HPLC chromatograms (Fig. 1).

Pocam-deprotected peptide **4** was also observed on the all chromatograms, indicating that all of Pocam groups tested were cleaved at least in part with exposure to TFA. To evaluate the stability of Pocam groups in the TFA solution, Pocam-attached peptide **3** and Pocam-deprotected peptide **4** in each crude peptide were purified with RP-HPLC and quantified by amino acid analysis. The isolated yield of each peptide was summarized in Table 1. When *i*-Bu group was attached to Pocam group, the stability under TFA acidic conditions was decreased compared to Me-Pocam, and the

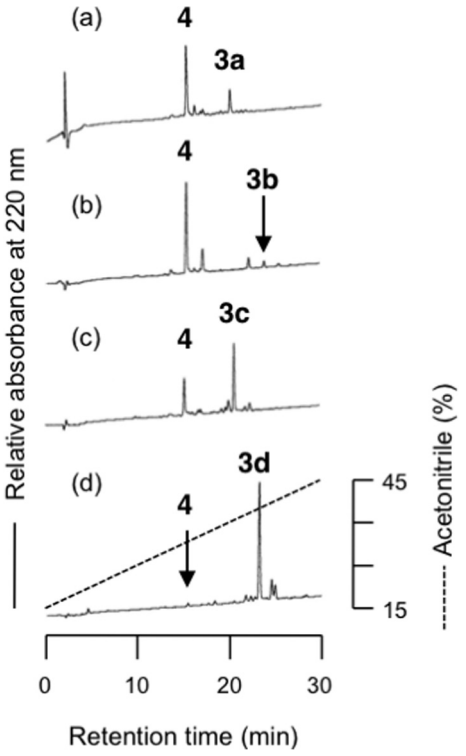


Figure 1. RP-HPLC elution profiles of GBP(11–25) segments containing various Cys (Pocam) derivatives after the TFA cocktail treatment at room temperature for 2 h. (a) Me-Pocam. (b) *i*-Bu-Pocam. (c) Moe-Pocam. (d) Tfe-Pocam. Column: Mightysil RP-18 GP (4.6ϕ × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL/min.

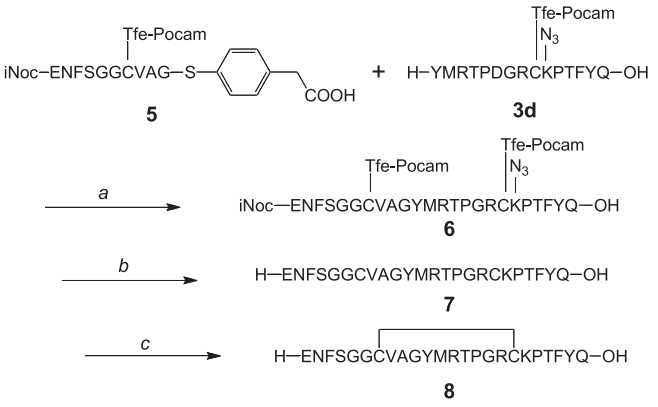
Table 1
The isolated yields of Pocam-attached (**3a-d**) and deprotected (**4**) peptides after TFA treatment.

Substituent	Protected (**3**) (%)	Deprotected (**4**) (%)
Methyl	7.8	35.0
Isobutyl	2.5	37.9
2-methoxyethyl	25.8	16.4
2,2,2-trifluoroethyl	44.9	1.4

Values mean isolated yield of each peptide quantified by amino acid analysis.

isolated yield of **3b** was quite low (2.5%). In contrast, the yields of Tfe- and Moe-Pocam peptides (**3c** and **3d**) were higher than that of Me-Pocam peptide **3a**, indicating that the substituent with EWG improved the stability of Pocam group. Especially, Tfe-Pocam group was almost completely stable in TFA solution, and only a trace amount of Pocam-deprotected peptide **4** was observed (Fig. 1d). These results were consistent with the observation on Allocam group reported previously.[10]

In order to examine the usefulness of Tfe-Pocam group on the peptide condensation reaction by the thioester method, we synthesized the *N*-terminal segment of GBP, GBP(1–10) thioester, and condensed it with the C-terminal segment prepared as described above. To obtain the peptide thioester, we used *N*-alkylcysteine (NAC)-assisted thioesterification reaction.[14] Fmoc-(Et)Cys(Trt)-OH, which was prepared by the previously described method,[14,15] was introduced to Rink-Amide MBHA resin. After the removal of Fmoc group, Fmoc-Gly-OH was condensed using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as a condensation reagent. Then, the peptide chain corresponding to the GBP sequence was elongated by the DCC-HOBt method. After the chain assembly, Fmoc group was removed with

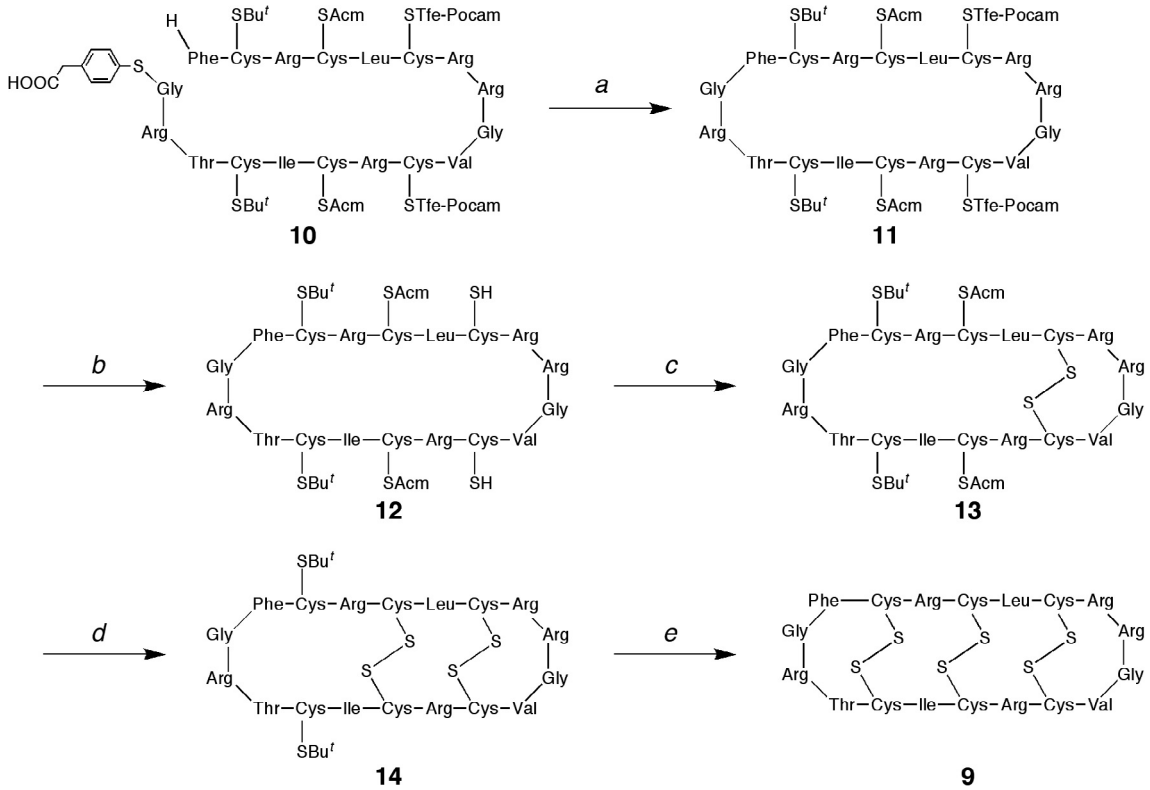


Scheme 2. Synthetic route for GBP **8**. (a) 1% HOObt/1% DIEA/DMSO, rt, 5 h. (b) Zn powder, 5% 2-mercaptoethanol/50% AcOH/H_2O, rt, 1 h. (c) 10% DMSO/50 mM phosphate buffer (pH 7.0), rt, 20 h.

piperidine, and the N-terminal amino group was protected by isonicotinyloxycarbonyl (iNoc) group, which has been shown to be cleavable with Zn reduction.[16,17] The crude peptide was cleaved from the resin with TFA cocktail, and the thioesterification was performed in 5% AcOH/50% acetonitrile aqueous solution containing 4-mercaptophenylacetic acid at a concentration of 2%. The reaction was almost complete within 48 h, and the desired peptide thioester **5** was obtained in 26% yield. Tfe-Pocam group was stable under the thioesterification reaction conditions, and it was likely that this protecting group was fully compatible with NAC-assisted thioesterification method.

The peptide segments **3d** and **5** were condensed by the Ag^+-free thioester method (Scheme 2).[18] The segments were dissolved in dimethyl sulfoxide (DMSO) containing 1% 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOObt) and 1% *N*,*N*-diisopropylethylamine (DIEA), and the mixture was gently mixed at room temperature. The N-terminal segment **5** was exhausted within 5 h, and an additional peak corresponding to the desired peptide **6** appeared in RP-HPLC chromatogram (see Supplementary Fig. S1). During the condensation reaction, no decomposition of Tfe-Pocam group was observed, and the reaction proceeded without significant side reactions. After the precipitation of crude peptides with diethyl ether, Tfe-Pocam group was removed by Zn powder treatment in 5% 2-mercaptoethanol (2-ME)/50% AcOH aqueous solution. Azido and iNoc groups were also removed simultaneously with Tfe-Pocam, and the linear GBP peptide **7** without protecting groups was obtained in 53% yield. Finally, a disulfide bond was formed between Cys^7-Cys^{19} by oxidation in a phosphate buffer containing 10% DMSO, giving GBP **8** in 66% yield (Supplementary Fig. S2). Thus, Tfe-Pocam group acted efficiently as a Cys-protecting group on Fmoc-SPPS and peptide condensation reactions.

The regioselective disulfide bond formation is the key step for chemical syntheses of disulfide-rich peptides, and several Cys protecting groups useful for such purposes have been newly developed also in last decade.[7,8,19–21] To demonstrate the utility of Tfe-Pocam group on the regioselective disulfide bond formation, we synthesized Rhesus θ-defensin-1 (RTD-1, **9**), a cyclic octadecapeptide with three disulfide bonds. RTD-1 has been identified in primate leukocytes with an antimicrobial activity, and was produced by the ligation of two truncated α-defensins.[22] The chemical synthesis of RTD-1 using native chemical ligation strategy and glutathione-mediated folding reactions has also been reported.[23] In this study, to form disulfide bonds m *tert*-butyl (Bu^t) groups for protecting Cys side chains other than Tfe-Pocam group. These protecting groups have been widely used for regioselective disulfide bond formations, and various Cys-rich peptides have been synthesized.[21,24]



Scheme 3. Synthetic route for RTD-1 **9**. (a) 1% HOObt/1% DIEA/DMSO, rt, 1 h. (b) Zn powder, 5% 2-mercaptoethanol/50% AcOH/H_2O, rt, 1 h. (c) 10% DMSO/50 mM phosphate buffer (pH 7.0), rt, 24 h. (d) I_2 in CH_3OH/H_2O containing HCl, rt, 60 min. (e) 5% DMSO/TFA, rt, 3 h.

To use the thioester method for the cyclization, we selected the Gly-Phe sequence as the condensation site (Scheme 3). As in the synthesis of peptide **5**, NAC residue was introduced to Rink-Amide MBHA resin, and then Fmoc-Gly-OH was condensed using HATU as a coupling reagent. The peptide chain corresponding to the RTD-1 sequence was elongated by the ordinary Fmoc-SPPS using DCC-HOBt as condensation reagents. During SPPS, Cys(Tfe-Pocam), Cys (Acm), and Cys(Bu^t) derivatives were used at Cys^6 and Cys^{11} Cys^4 and Cys^{13}, and Cys^2 and Cys^{15} positions, respectively. After the thioesterification with MPAA by the NAC-assisted method, the desired peptide thioester **10** was obtained in 5.3% yield.

To cyclize the peptide by the thioester method, the peptide thioester **10** was dissolved in DMSO containing 1% HOObt/1%DIEA at a low concentration. The cyclization reaction was almost complete within 1 h, and a new peak corresponding to the cyclic product **11** appeared on RP-HPLC chromatogram (Supplementary Fig. S3). After the cyclization, the crude peptide was precipitated with diethyl ether. The residue was then dissolved in 5% 2-ME/50% AcOH aqueous solution, and Tfe-Pocam groups were cleaved by mixing Zn powder, giving the reduced form of cyclic RTD-1 **12** in 80% yield.

The first disulfide bond was formed at Cys^6-Cys^{11} by DMSO-mediated oxidation, and the desired peptide **13** was obtained in 86% yield. The second disulfide bond was formed at Cys^4-Cys^{13} site by the iodine treatment, which oxidatively cleaved the Acm groups, giving the desired peptide **14** in 80% yield. Finally, Bu^t groups were oxidatively removed by the DMSO-TFA treatment, and the disulfide bond at Cys^2-Cys^{15} site was formed, giving RTD-1 **9** in 81% yield. Thus, Tfe-Pocam group was fully compatible with Acm and Bu^t groups widely used for the regioselective disulfide formation reactions, and was applicable to the synthesis of disulfide-rich cyclic peptide.

In conclusion, we demonstrated that EWG-attached Pocam group showed higher stability than Me-pocam, a prototype of Pocam group. Especially, Tfe-Pocam was most stable in Pocam derivatives tested. This protecting group was stable under various conditions used for the ordinary Fmoc-SPPS, and was cleavable with Zn reduction. It was also compatible with other Cys protecting groups, such as Acm and Bu^t groups, and we successfully synthesized GBP and RTD-1 by the thioester method. It is likely that this Tfe-Pocam-based strategy is applicable for synthesizing larger proteins with post-translational modifications. Along this line, we are currently trying to synthesize glycoproteins by this strategy.

Acknowledgments

We are grateful to Dr. Yoshimi Kanie and Prof. Osamu Kanie of Institute of Advanced Biosciences, Tokai University for the help on HRMS measuring. This work was supported in part by Research and Study Program of Tokai University Educational System General Research Organization.

A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.12.081.

References

1. Merrifield RB. *J Am Chem Soc*. 1963;85:2149–2154.
2. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. *Science*. 1994;266:776–779.
3. Hojo H, Aimoto S. *Bull Chem Soc Jpn*. 1991;64:111–117.
4. Aimoto S. *Biopolymers*. 1999;51:247–265.
5. Katayama H, Hojo H, Ohira T, Nakahara Y. *Tetrahedron Lett*. 2008;49:5492–5494.
6. Katayama H, Hojo H, Shimizu I, Nakahara Y, Nakahara Y. *Org Biomol Chem*. 2010;8:1966–1972.
7. Katayama H, Nakahara Y, Hojo H. *Org Biomol Chem*. 2011;9:4653–4661.
8. Katayama H, Hojo H. *Org Biomol Chem*. 2013;11:4405–4413.
9. Kimbonguila AM, Merzouk A, Guibé F, Loffet A. *Tetrahedron*. 1999;55:6931–6944.
10. Gomez-Martinez P, Kimbonguila AM, Guibé F. *Tetrahedron*. 1999;55:6945–6960.
11. Hayakawa Y. *J Biol Chem*. 1990;265:10813–10816.
12. Hayakawa Y. *J Biol Chem*. 1991;266:7982–7984.
13. Hayakawa Y, Yasuhara Y. *Insect Biochem Mol Biol*. 1993;23:225–231.
14. Hojo H, Onuma Y, Akimoto Y, Nakahara Y, Nakahara Y. *Tetrahedron Lett*. 2007;48:25–28.
15. Nakahara Y, Matsuo I, Ito Y, Ubagai R, Hojo H, Nakahara Y. *Tetrahedron Lett*. 2010;51:407–410.
16. Veber DF, Paleveda Jr WJ, Lee YC, Hirschmann R. *J Org Chem*. 1977;42:3286–3288.
17. Asahina Y, Kamitori S, Takao T, Nishi N, Hojo H. *Angew Chem Int Ed*. 2013;52:9733–9737.
18. Hojo H, Murasawa Y, Katayama H, Ohira T, Nakahara Y, Nakahara Y. *Org Biomol Chem*. 2008;6:1808–1813.
19. Shen F, Zhang ZP, Li JB, Lin Y, Liu L. *Org Lett*. 2011;13:568–571.
20. Karas JA, Scanlon DB, Forbes BE, et al. *Chem Eur J*. 2014;20:9549–9552.
21. Mochizuki M, Tsuda S, Tanimura K, Nishiuchi Y. *Org Lett*. 2015;17:2202–2205.
22. Tang Y-Q, Yuan J, Ösapay G, et al. *Science*. 1999;286:498–502.
23. Aboye TL, Li Y, Majumder S, Hao J, Shekhtman A, Camarero JA. *Bioorg Med Chem Lett*. 2012;22:2823–2826.
24. Cuthbertson A, Indrevoll B. *Org Lett*. 2003;5:2955–2957.