Organic & Biomolecular Chemistry

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2013, **11**, 4405

The phenacyl group as an efficient thiol protecting group in a peptide condensation reaction by the thioester method†

Hidekazu Katayama*[a] and Hironobu Hojo*[a,b]

Received 2nd April 2013,
Accepted 9th May 2013

DOI: 10.1039/c3ob40644j

www.rsc.org/obc

One of the condensation methods for the preparation of long-chain peptides, the so-called thioester method requires protecting groups for amino and thiol groups for regioselective ligation. In this study, we demonstrated that the phenacyl (Pac) group acts as an efficient protecting group of cysteine side chains. We synthesized a cysteine derivative carrying the Pac group at the side chain sulfur atom, and Pac-containing peptides and peptide thioesters were synthesized using it by the ordinary 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis strategy. Pac-containing peptide segments could be condensed by the thioester method. After the condensation reaction, Pac groups could be removed by Zn/AcOH treatment. In addition, the azido group, which was used for the protection of lysine side chains, was simultaneously converted into an amino group, demonstrating that this protecting group scheme simplified the deprotecting reaction after the peptide condensation reaction to a single step.

Introduction

Peptide condensation methods enable protein chemical synthesis with long peptide chains. Especially, the native chemical ligation (NCL) method developed by Dawson *et al.* in 1994[1] is one of the most general methods for protein synthesis. Among the advantages of this method, no protecting group is required for forming a native peptide bond regioselectively; instead, the cysteine residue should be positioned at the ligation site. However, the synthesis of proteins often requires condensation at a non-Cys site. For overcoming this limitation, various approaches, such as a thiol group directly attached at the beta-carbon atom of an N-terminal amino acid/de-sulfurization strategy[2] and ligation auxiliary groups,[3] have been developed. However, specific amino acid derivatives are generally used in these methods, and complicated synthetic procedures are required for preparing those compounds. The thioester method, which is an alternative peptide condensation method developed by Hojo and Aimoto in 1991,[4] has an advantage that no specific residue is required at the condensation position. In the classical thioester method, C-terminal alkyl thioester functionality of the N-terminal segment is activated by silver ions, and allowed to condense with a free N-terminal amino group of the other segment in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOObt). Recently, we found that an aryl thioester could be directly activated by HOObt, and the condensation reaction proceeded without silver ions.[5]

In the thioester condensation method, protecting groups at Lys and Cys side chains are necessary to achieve regioselective condensation reaction. Until now, *tert*-butoxycarbonyl (Boc) and acetamidomethyl (Acm) groups have been generally used for protecting Lys and Cys residues, respectively. However, since the Boc group is labile in trifluoroacetic acid (TFA) solution which is usually used to cleave peptides from the solid support after 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS), the Boc group should be re-introduced into peptide segments after purification steps. In order to overcome this inconvenience, an alternative amino protecting group that is stable under the various conditions used in Fmoc-SPPS was required. Along this line, we have demonstrated that the azido group acted efficiently as an amino protecting group in the Fmoc-SPPS and peptide condensation reactions.[6] Using this azido-based strategy, we have achieved the total chemical synthesis of a glycoprotein.[7]

The azido group can be easily converted to an amino group by reduction using Zn powder in an acetic acid solution. On the other hand, the Acm group attached to Cys side chains is stable under reducing reaction conditions. Indeed, an additional deprotection step for cleaving the Acm group after the reduction is usually required in order to obtain

[a]*Department of Applied Biochemistry, School of Engineering, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan. E-mail: katay@tokai-u.jp, hojo@keyaki.cc.u-tokai.ac.jp; Tel: +81-463-50-2075*

[b]*Institute of Glycoscience, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan*

†Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ob40644j

unprotected polypeptides. In our previous study, we aimed to simplify the deprotection to a single step, and a novel protecting group, the *N*-methyl-phenacyloxycarbamidomethyl (Pocam) group, has been developed.[8] This protecting group can be removed by Zn reduction in an acetic acid solution simultaneously with the conversion of an azido group into an amino group. Using this protecting group, we have achieved the synthesis of several peptides by the thioester method. The Pocam group is, however, not completely stable under TFA acidic conditions, and careful manipulation was required to prepare Pocam-containing peptides.

It is widely known that the phenacyl (Pac) group is an efficient protecting group for carboxylic acid, and can be removed by reducing reaction with Zn powder under acidic conditions.[9] Recently, we found that the Pac group attached directly to a sulfur atom is also cleavable by Zn treatment in an acetic acid solution. In this paper, we demonstrate that the Pac group acts as an efficient thiol protecting group in the Fmoc-SPPS and peptide condensation reactions by the thioester method. Using this Pac-based strategy, we synthesized several model peptides as will be described below.

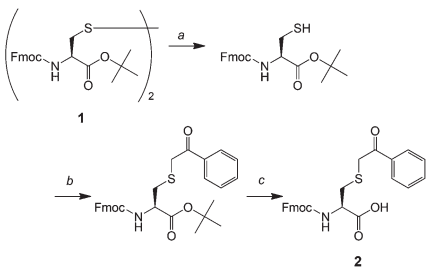
Results and discussion

Orthogonality of the Pac group with other known Cys protecting groups

A cysteine derivative carrying the Pac group at the side chain was synthesized at first. Starting from (Fmoc-Cys-OBu^t)$_2$ (**1**), *N*-Fmoc-*S*-Pac-cysteine [Fmoc-Cys(Pac)-OH] (**2**) was synthesized as shown in Scheme 1. The disulfide bond of **1** was reduced by treatment with Zn powder in an acetic acid solution, and then the Pac group was attached using phenacyl bromide in dichloromethane (DCM). Subsequently, the *tert*-butyl group was removed using TFA, giving the desired product **2** in 80% yield.

In order to investigate the orthogonality of the Pac group with other Cys protecting groups, a model peptide having three Cys residues with various protecting groups generally used for regioselective disulfide formation in peptide chemical synthesis was synthesized by Fmoc-SPPS. The peptide YCRVNGARYVRCCSRR was used as a model sequence. In the Fmoc-SPPS of the peptide *p*-methoxybenzyl (MeOBn), Acm and Pac groups were used for the protection of Cys side chains at positions 2, 12 and 13, respectively. Starting from Fmoc-Arg(Pbf)-Wang resin, the peptide chain was elongated manually by the ordinary Fmoc-SPPS strategy using *N*,*N*′-dicyclohexylcarbodiimide (DCC)–1-hydroxybenzotriazole (HOBt) as condensation reagents. After chain assembly, the crude peptide was cleaved from solid supports by treatment with a TFA cocktail, and purified by reversed-phase (RP)-HPLC, giving peptide **3**. However, during peptide chain assembly, the introduction efficiency of the amino acid residue at the N-terminal side of Cys(Pac) was low, and the isolated yield of **3** was low (9.4%). It may be partly due to the cyclization at Cys(Pac) by imine formation between the α-amino group and the carbonyl group of Pac. The imine structure is generally formed under weakly acidic conditions, and the condensation conditions are weakly acidic due to the acidity of HOBt. In order to keep the weakly basic conditions in the condensation reaction, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)–*N*,*N*-diisopropylethylamine (DIEA) was used as a condensation reagent instead of DCC–HOBt. As expected, the reaction efficiency was improved, and the desired peptide **3** was obtained by RP-HPLC purification in higher yield (19%) (Fig. 1).

The stability of the Pac group under the various deprotection conditions for Cys-protecting groups was examined. When peptide **3** was treated with Zn powder in an acetic acid aqueous solution, the Pac group was cleaved without the removal of the Acm or MeOBn group, and peptide **4** was obtained as a single peak in RP-HPLC (Fig. 2). On the other hand, when the MeOBn group of peptide **3** was removed by treatment with a 1 M trifluoromethanesulfonic acid (TfOH)–TFA solution, MeOBn-deprotected peptide **5** was obtained as the major product, although cleavage of the Pac group in part was observed as a minor peak in RP-HPLC analysis. When the Acm group was removed by treatment with silver nitrate, the desired Acm-deprotected product **7** was mainly obtained, although Pac-deprotected peptide **8** was also obtained as a minor product. These results indicated that the reducing



Scheme 1 Synthesis of Fmoc-Cys(Pac)-OH. Reaction conditions: (a) Zn powder in 50% AcOH–dichloromethane, RT, 2.5 h; (b) Pac-Br (1.1 eq.), DIEA (2 eq.) in dichloromethane, RT, 1 h; (c) 2% H_2O–TFA, RT, 1 h (80% yield in 3 steps).

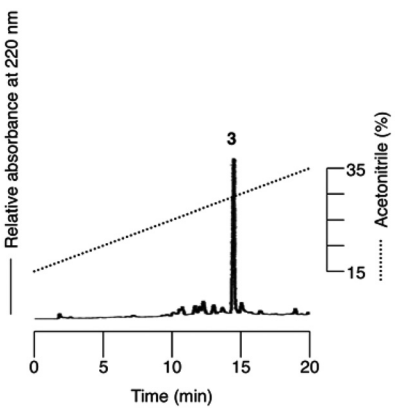


Fig. 1 RP-HPLC elution profiles of crude model peptide **3**. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}.

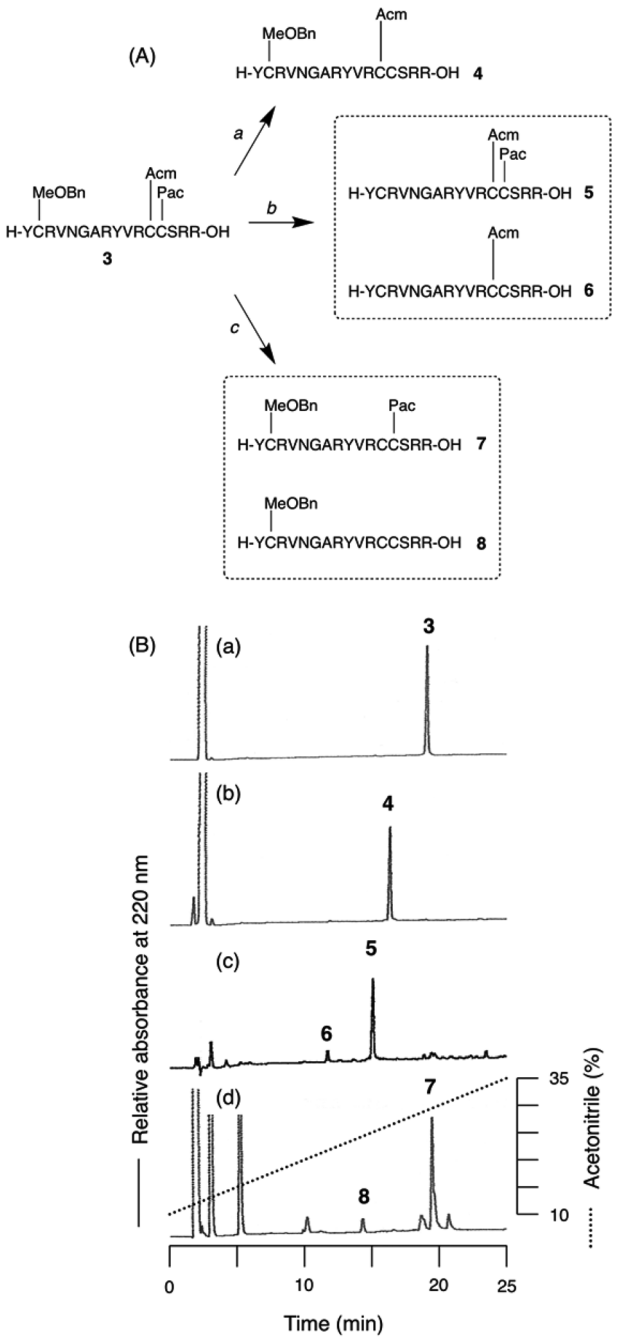


Fig. 2 (A) Deprotection conditions and structures of the obtained peptides. (a) Zn powder in 50% AcOH–H_2O, RT, 1 h; (b) TfOH–TFA–*m*-cresole–thioanisole (3/14/1/2), 0 °C, 20 min; $AgNO_3$ in 5% H_2O–0.1% DIEA–DMSO, RT, 2 h. (B) RP-HPLC elution profiles of model peptide **3** and crude samples after deprotection. (a) Purified peptide **3**; (b) the crude sample after Zn powder treatment under acidic conditions; (c) the crude sample after treatment with 1 M TfOH in TFA; (d) the crude sample after treatment with $AgNO_3$ in an aqueous DMSO solution. Numbers in the chromatograms indicate compounds shown in panel A. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}.

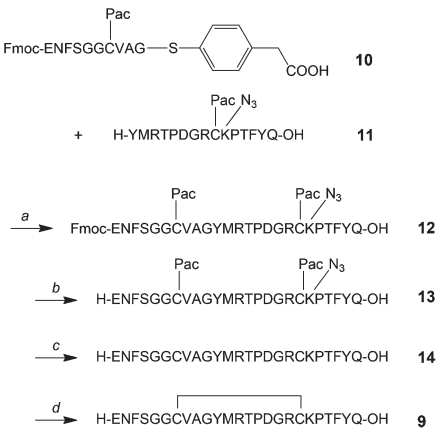
reaction using Zn/AcOH cleaved only the Pac group and did not affect the other Cys-protecting groups; on the other hand, the Pac group was slightly labile under strongly acidic conditions and silver ion treatment.

Synthesis of the growth-blocking peptide

In order to demonstrate the utility of the Pac group in the peptide condensation reaction by the thioester method, insect growth-blocking peptide (GBP) **9** from the armyworm, *Pseudaletia separata*, was used as a model sequence. GBP consists of 25 amino acid residues containing two Cys residues that form an intrachain disulfide bond.[10] The peptide chain was divided into two segments, (1–10) **10** and (11–25) **11**, and these were synthesized by the Fmoc-SPPS strategy and condensed by the thioester method as shown in Scheme 2.

The N-terminal segment **10** should have a thioester functionality at the C-terminus. To obtain the peptide thioester, we used the *N*-alkylcysteine (NAC)-assisted thioesterification method.[11] Fmoc-(Et)Cys(Trt)-OH was introduced into H-Arg(Pbf)-Arg(Pbf)-NH-resin by the DCC–HOBt method. After removal of the Fmoc group by piperidine treatment, Fmoc-Gly-OH was condensed using *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as a condensation reagent, and then the peptide chain was elongated manually by the DCC–HOBt method except for the amino acid residue next to Cys(Pac) which was introduced by the HBTU–DIEA method. After the peptide chain assembly, the crude peptide was cleaved from the resin by TFA cocktail treatment, and dissolved in a 50% aqueous acetonitrile solution containing 5% AcOH. 4-Mercaptophenylacetic acid (MPAA) was added to the solution at a concentration of 5%. The thioesterification reaction was almost complete within 20 h, giving the desired peptide thioester **10** in 5.3% yield. During thioesterification reaction using an excess amount of the thiol compound under weakly acidic conditions, no significant side reaction, such as thioketal formation at the carbonyl group of the Pac moiety, was observed. These results indicated that the Pac group was fully compatible with a NAC-assisted thioesterification reaction.

The C-terminal segment **11** was also synthesized by the ordinary Fmoc-SPPS strategy. Starting from Fmoc-Gln(Trt)-CLEAR acid resin, the peptide chain was elongated manually



Scheme 2 Synthesis of GBP. Reaction conditions: (a) 1% HOObt–1% DIEA–DMSO, RT, 4 h; (b) 10% piperidine, RT, 30 min; (c) Zn powder in 50% AcOH–H_2O, RT, 1 h (84% in 3 steps); (d) 10% DMSO–6 M guanidine–HCl–50 mM phosphate buffer (pH 7.0), RT, o/n (50%).

by the DCC–HOBt method or the HBTU–DIEA method, and the protected peptide resin corresponding to the GBP (11–25) sequence was obtained. A crude peptide was cleaved from the solid support by TFA cocktail treatment, and purified by RP-HPLC, giving the desired peptide segment **11** in 14% yield.

The two segments, **10** and **11**, were condensed by the silver ion-free thioester method. These were dissolved in anhydrous dimethyl sulfoxide (DMSO) containing 1% DIEA and 1% HOOBt, and the mixture was kept at room temperature. The reaction proceeded without significant side reaction, and was complete within 4 h. In the RP-HPLC analysis, a new peak corresponding to the desired product **12** appeared after the reaction (Fig. 3). The N-terminal Fmoc group of **12** was cleaved by adding piperidine at a concentration of 10%, giving peptide **13**. After precipitation of the crude peptide by addition of ether, Pac groups were removed by reduction using Zn powder in an AcOH aqueous solution. An azido group was simultaneously converted into an amino group by Zn reduction, and the desired linear GBP **14** was obtained in 84% yield. During deprotection steps, no significant side reaction was observed. These results clearly indicated that the Pac group acted efficiently as a protecting group of Cys side chains in the peptide condensation reaction by the thioester method. The disulfide bond was formed by oxidation in a phosphate buffer

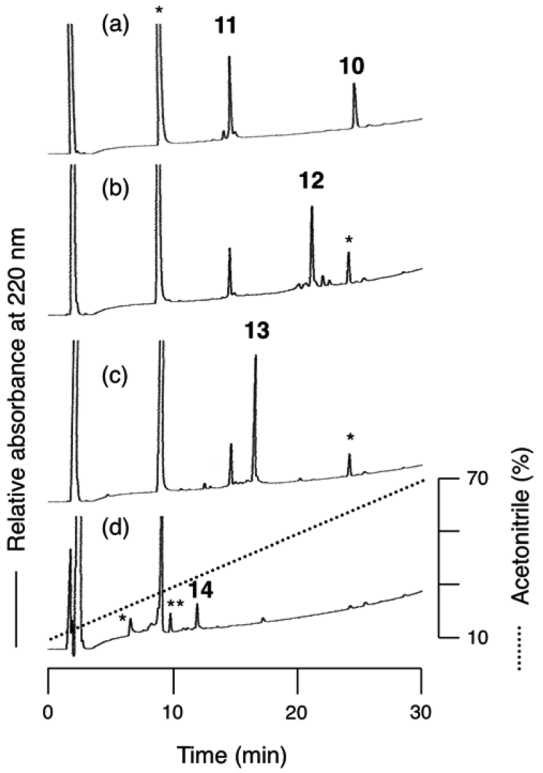


Fig. 3 RP-HPLC elution profiles in peptide condensation and deprotection of GBP. (a) Coupling reaction mixture of **10** and **11** (0 h). (b) At 4 hours after the coupling reaction. (c) Reaction mixture after piperidine treatment. (d) Reaction mixture after Zn/AcOH treatment. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}. *Non-peptidic components; ****11**-derived peptide.

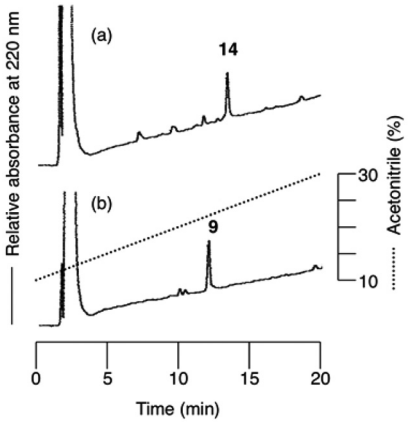
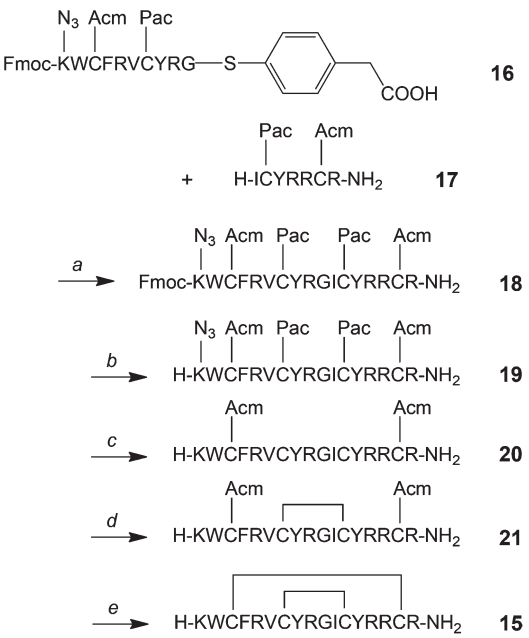


Fig. 4 RP-HPLC elution profiles in oxidation reaction of GBP. (a) 0 h. (b) 20 h. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}.

containing 10% DMSO, and GBP **9** was successfully obtained in 50% yield (Fig. 4).

Synthesis of tachyplesin

In order to investigate the applicability of the Pac group for regioselective disulfide formation in Cys-rich peptide synthesis, we tried to synthesize tachyplesin **15**, an antimicrobial peptide originally isolated from the hemocytes of the horseshoe crab *Tachypleus tridentatus*, as a model (Scheme 3). Tachyplesin consists of 17 amino acid residues including four Cys that form two intrachain disulfide bonds and an amidated



Scheme 3 Synthesis of tachyplesin. Reaction conditions: (a) 1% HOOBt–1% DIEA–DMSO, RT, 4 h; (b) 10% piperidine, RT, 30 min; (c) Zn powder in 50% AcOH–H_2O, RT, 1 h (30% in 3 steps); (d) 10% DMSO–6 M guanidine–HCl–50 mM phosphate buffer (pH 7.0), RT, o/n (59%); (e) I_2–HCl–CH_3OH–H_2O, RT, 60 min (70%).

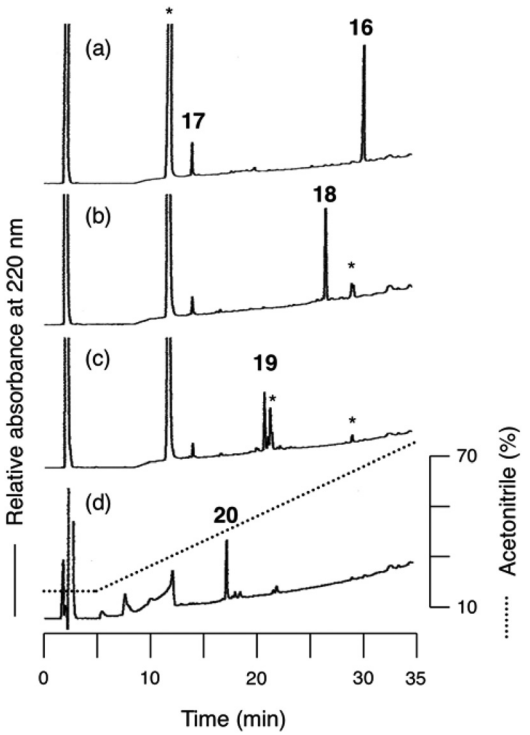


Fig. 5 RP-HPLC elution profiles in peptide condensation and deprotection of tachyplesin. (a) Coupling reaction mixture of **10** and **11** (0 h). (b) After overnight reaction. (c) Reaction mixture after piperidine treatment. (d) Reaction mixture after Zn/AcOH treatment. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}. *Non-peptidic components.

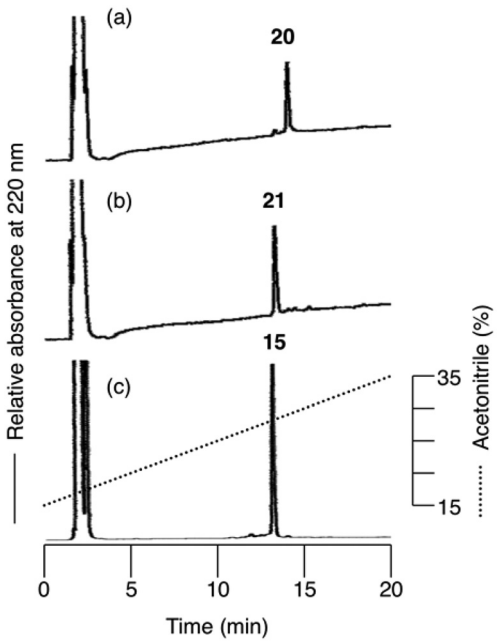


Fig. 6 RP-HPLC elution profiles in oxidation reaction of GBP. (a) 0 h. (b) 20 h. (c) I_2 oxidation. Column: Inertsil ODS-3 (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile at a flow rate of 1 mL min^{-1}.

C-terminus.[12] The tachyplesin sequence was divided into two segments, (1–10) **16** and (11–17) **17**, and these were separately synthesized by the ordinary Fmoc-SPPS and NAC-assisted thioesterification reactions.

Segments **16** and **17** were condensed by the thioester method (Fig. 5). These segments had Acm groups, and the Ag^+-free thioester method rather than the classical Ag^+-catalyzed condensation method facilitated efficient ligation without undesirable cleavage of the Acm groups. After the condensation reaction and the removal of the N-terminal Fmoc group, the Pac and azido groups were subsequently cleaved by treatment with Zn powder in an aqueous AcOH solution, giving the linear tachyplesin **20** in 30% yield. The first disulfide bond between Cys^7 and Cys^{12} was formed by oxidation in a phosphate buffer containing 10% DMSO, giving the desired product **21** in 59% yield (Fig. 6). Finally, the second disulfide bond between Cys^3 and Cys^{16} was formed by iodine oxidation, to give the final product **15** in 70% yield. All these results clearly indicated that the Pac group could be used for regioselective disulfide formation in Cys-rich peptide synthesis.

Conclusion

We demonstrated that the Pac group could be used as a Cys-protecting group in peptide chemistry. The Pac group was stable under various conditions used for the ordinary Fmoc-SPPS, and was cleavable with Zn reduction. Using this protecting group, we successfully synthesized GBP and tachyplesin by the thioester method. The azido group was simultaneously converted into an amino group by Zn-reduction, demonstrating that this Pac/azido-strategy simplifies the deprotection steps after the peptide condensation reaction to a single step. It is likely that this Pac-based strategy is applicable for synthesizing larger proteins with or without modifications. Along this line, we are currently trying to synthesize glycoproteins by this strategy.

Experimental

General

Fmoc-(Et)Cys(Trt)-OH[11] and Fmoc-Lys(N_3)-OH[6] were prepared by the previously described methods. Specific rotation values were determined with a Jasco P-2200 polarimeter (Jasco, Tokyo, Japan) at 20 ± 2 °C for solutions in $CHCl_3$. The NMR spectra were recorded by a Jeol AL-400 spectrometer (400 MHz in ^{1}H-NMR, JEOL, Tokyo, Japan) using $CDCl_3$ as a solvent. MALDI-TOF mass spectra were recorded using a Voyager-DE PRO spectrometer (Applied Biosystems, CA). The amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150 °C for 2 h in a vacuum-sealed tube.

N-(9-Fluorenylmethoxycarbonyl)-*S*-phenacyl-cysteine 2

(Fmoc-Cys-OBu^t)$_2$ (**1**, 400 mg, 0.50 mmol) was dissolved in 50% AcOH–dichloromethane (DCM) (4 mL), and Zn powder

(0.30 g) was added to the solution. After stirring at room temperature for 2.5 h, insoluble material was removed by filtration, and the solvent was evaporated. The residue was dissolved in EtOAc, washed with a saturated $NaHCO_3$ aqueous solution and brine, and dried over Na_2SO_4. After filtration and concentration, the crude material containing Fmoc-Cys-OBu^t was used in the following steps without further purification. The obtained sample was dissolved in DCM (2 mL), and phenacyl bromide (220 mg, 1.1 mmol) and DIEA (0.35 mL, 2.0 mmol) were added. After stirring at room temperature for 1 h, the solvent was removed under reduced pressure. The residue was dissolved in EtOAc, washed with 1 M HCl, H_2O and brine, and dried over Na_2SO_4. After filtration and concentration, the crude material containing Fmoc-Cys(Pac)-OBu^t was used in the next step without further purification. The obtained material was dissolved in 2% H_2O–TFA (10 mL), and stirred at room temperature for 1 h. After the solvent was removed under reduced pressure, the residue was chromatographed on silica gel with toluene–EtOAc–AcOH (50/50/1) to give Fmoc-Cys(Pac)-OH **2** (370 mg, 0.80 mmol, 80% in three steps). R_f 0.19 (toluene–EtOAc–AcOH, 50/50/1). $[\alpha]_D$ +14.1 (c, 1.0). ^{1}H-NMR: δ 7.93–7.22 (m, 13H, Ar), 6.05 (d, 1H, J = 8.0 Hz, NH), 4.66 (m, 1H, CαH), 4.36 (m, 2H, >CH–CH_2–O–), 4.20 (t, 1H, J = 7.0 Hz, >CH–CH$_2$–O–), 3.92 [s, 2H, C(=O)CH_2], 3.12–3.01 (m, 2H, CβH). ^{13}C-NMR: δ 195.0, 174.5, 156.1 (C=O), 143.7, 143.6, 141.2, 134.9, 133.7, 128.7, 127.7, 127.0, 125.1, 119.9 (Ar), 67.4 (>CH–CH$_2$–O–), 53.9 ($C\alpha$), 46.9 (>CH–CH$_2$–O–), 38.0 (C(=O)CH$_2$), 34.2 ($C\beta$). MALDI-TOF MS, found: m/z 484.2, calcd: 484.1 for $(M + Na)^+$.

Synthesis of model peptide 3 and deprotection

Fmoc-Arg(Pbf)-Wang resin (0.28 mmol g^{-1}, 179 mg, 50 μmol) was swelled in 1-methyl-2-pyrrolidinone (NMP) for 30 min, and was treated with 20% piperidine–NMP for 5 and 15 min. After washing with NMP, Fmoc-Arg(Pbf)-OBt, which was prepared by mixing Fmoc-Arg(Pbf)-OH (0.20 mmol), 1 M DCC–NMP (300 μL) and 1 M HOBt–NMP (300 μL) at room temperature for 30 min, was added and the reaction mixture was mixed with vortex at 50 °C for 1 h. The resin was washed with NMP and 50% DCM–CH_3OH, treated with 10% Ac_2O–5% DIEA–NMP for 5 min, and washed with NMP. The peptide chain was elongated in essentially the same manner as described above, except for Fmoc-Cys(Acm)-OH which was activated by HBTU (0.20 mmol) and DIEA (0.3 mmol) in NMP. After the chain assembly, H-Tyr(Bu^t)-Cys(MeOBn)-Arg(Pbf)-Val-Asn(Trt)-Gly-Ala-Arg(Pbf)-Tyr(Bu^t)-Val-Arg(Pbf)-Cys(Acm)-Cys(Pac)-Ser(Bu^t)-Arg(Pbf)-Arg(Pbf)-OCH$_2$-resin (304 mg) was obtained. A part of the resin (10 mg) was treated with the TFA cocktail (TFA–thioanisole–H_2O–phenol–triisopropylsilane, 82.5/5/5/5/2.5, 200 μL) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column (GL Science, Tokyo, Japan) with a linear gradient of acetonitrile containing 0.1% TFA to give model peptide **3** (310 nmol, 19% yield). MALDI-TOF mass, found: m/z 2270.7, calcd: 2271.1 for $(M + H)^+$. Amino acid analysis: $Asp_1Ser_{1.22}Gly_{1.08}Ala_{0.96}Cys_{0.19}Val_{1.75}Tyr_{1.76}Arg_{4.77}$.

For cleavage of the Pac group, peptide **3** (20 nmol) was dissolved in a 50% AcOH aqueous solution (100 μL), and an excess amount of Zn powder was added. After mixing with vortexing at room temperature for 1 h, the reaction mixture was analyzed by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA.

For cleavage of the MeOBn group, peptide **3** (20 nmol) was dissolved in a TfOH solution (TfOH–TFA–*m*-cresole–thioanisole, 3/14/1/2, 20 μL), and the mixture was kept at 0 °C for 20 min. The crude peptide was precipitated with diethyl ether, washed twice with ether, and dried under vacuum. The resulting material was analyzed by RP-HPLC.

For cleavage of the Acm group, peptide **3** (20 nmol) was dissolved in a 95% DMSO–0.1% DIEA aqueous solution (20 μL) containing $AgNO_3$ (0.20 μmol), and the mixture was kept at room temperature for 2 h. Then, the reaction mixture was analyzed by RP-HPLC.

Fmoc-[Cys(Pac)^7]-GBP (1–10)-SC$_6$H$_4$CH$_2$COOH 10

Fmoc-Rink amide MBHA resin (0.34 mmol g^{-1}, 147 mg, 50 μmol) was swelled in NMP for 30 min, and was treated with 20% piperidine–NMP for 5 and 15 min. After washing with NMP, Fmoc-Arg(Pbf)-OBt, which was prepared by mixing Fmoc-Arg(Pbf)-OH (0.20 mmol), 1 M DCC–NMP (300 μL) and 1 M HOBt–NMP (300 μL) at room temperature for 30 min, was added and the reaction mixture was mixed with vortexing at 50 °C for 1 h. The resin was washed with NMP and 50% DCM–CH_3OH, treated with 10% Ac_2O–5% DIEA–NMP for 5 min, and washed with NMP. Another Arg residue was introduced into the resin in the same manner, and the resin was washed with NMP. After the Fmoc group was removed by 20% piperidine–NMP treatment at room temperature for 5 and 15 min, the resin was washed with NMP. Fmoc-(Et)Cys(Trt)-OBt, which was prepared by mixing Fmoc-(Et)Cys(Trt)-OH (0.1 mmol), 1 M DCC–NMP (150 μL) and 1 M HOBt–NMP (150 μL) at room temperature for 30 min, was added to the resin, and the mixture was vortexed at 50 °C for 1 h. The resin was washed with NMP and 50% DCM–CH_3OH, treated with 10% Ac_2O–5% DIEA–NMP for 5 min, and washed with NMP. After the Fmoc group was removed by 20% piperidine–NMP treatment at room temperature for 5 and 15 min, the resin was washed with NMP. Fmoc-Gly-OH (0.50 mmol) and HATU (0.50 mmol) were dissolved in NMP (1 mL) containing DIEA (170 μL, 1.0 mmol), and the mixture was added to the resin. The reaction mixture was mixed with vortex at 50 °C for 1 h. After washing with NMP, the peptide chain was elongated by the ordinary Fmoc-based SPPS. The amino acids (0.20 mmol each) except for Gly just after Cys(Pac) were activated by mixing with 1 M DCC–NMP (0.30 mL) and 1 M HOBt–NMP (0.30 mL) at room temperature for 30 min, and the coupling reaction was carried out at 50 °C for 1 h. At the glycine position just after Cys(Pac), Fmoc-Gly-OH (0.2 mmol) was activated by mixing HBTU (0.2 mmol) and DIEA (0.3 mmol) in NMP (0.5 mL), and the coupling reaction was carried out at 50 °C for 1 h. After

elongation, Fmoc-Glu(OBu^t)-Asn(Trt)-Phe-Ser(Bu^t)-Gly-Gly-Cys-(Pac)-Val-Ala-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (247 mg) was obtained. A part of the resin (10 mg) was treated with a TFA cocktail (200 μL) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was dissolved in 50% CH_3CN–5% AcOH–H_2O (0.50 mL), and MPAA (25 mg) was added to the solution. After overnight reaction at room temperature, the crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide thioester **10** (110 nmol, 5.3% yield). MALDI-TOF mass, found: *m*/*z* 1452.6, calcd: 1452.5 for $(M + Na)^+$. Amino acid analysis: $Asp_{0.90}Ser_{0.74}Glu_{0.96}Gly_{2.87}Ala_1Val_{0.93}Phe_{0.93}$.

[Cys(Pac)^{19}, Lys(N$_3$)^{20}]-GBP (11–25) 11

Fmoc-Gln(Trt)-CLEAR acid resin (0.41 mmol g^{-1}, 122 mg, 50 μmoL) was swelled in NMP for 30 min, and treated with 20% piperidine–NMP for 5 and 15 min. After washing with NMP, the peptide chain was elongated by Fmoc-SPPS. The amino acids (0.20 mmol each) except for Arg just after Cys(Pac) were activated by mixing with 1 M DCC–NMP (300 μL) and 1 M HOBt–NMP (300 μL) at room temperature for 30 min, and the coupling reaction was carried out at 50 °C for 1 h. At the arginine position next to Cys(Pac) residue, Fmoc-Arg(Pbf)-OH (0.2 mmol) was activated by mixing HBTU (0.2 mmol) and DIEA (0.3 mmol) in NMP (0.5 mL), and the coupling reaction was carried out at 50 °C for 1 h. After elongation, H-Tyr(Bu^t)-Met-Arg(Pbf)-Thr(Bu^t)-Pro-Asp(OBu^t)-Gly-Arg(Pbf)-Cys(Pac)-Lys-(N$_3$)-Pro-Thr(Bu^t)-Phe-Tyr(Bu^t)-Gln(Trt)-OCH$_2$-resin (201 mg) was obtained. A part of the resin (10 mg) was treated with the TFA cocktail (200 μL) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **11** (360 nmol, 14% yield). MALDI-TOF mass, found: *m*/*z* 2006.9, calcd: 2006.9 for $(M + H)^+$. Amino acid analysis: $Asp_{0.91}Thr_{1.88}Glu_{1.08}Pro_{2.10}Gly_1Met_{0.72}Tyr_{1.74}Phe_{0.97}Lys_{0.13}Arg_{1.79}$.

Linear GBP 14

Peptides **10** (94 nmol) and **11** (140 nmol) were mixed and dissolved in DMSO (50 μL) containing 1% HOObt–1% DIEA, and incubated at room temperature for 4 h to give peptide **12**. Piperidine (5 μL) was then added to this solution and the reaction mixture was kept at room temperature for 30 min, yielding peptide **13**. The crude peptide was precipitated by addition of 20 times volume of diethyl ether, washed twice with ether and dried under vacuum. The precipitant was dissolved in 50% aqueous AcOH (1 mL), and an excess amount of Zn powder was added to the solution. The mixture was mixed with vortexing at room temperature for 1 h. After filtration to remove Zn powder, the desired product **14** was purified by RP-HPLC using an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA. The isolated yield of **14** was 84% (79 nmol). MALDI-TOF mass, found: *m*/*z* 2784.0, calcd: 2784.2 for $(M + H)^+$. Amino acid analysis: $Asp_{2.01}Thr_{1.87}Ser_{0.89}$-$Glu_{1.98}Pro_{2.60}Gly_{3.77}Ala_1Val_{0.95}Met_{0.74}Tyr_{2.38}Phe_{2.07}Lys_{0.93}Arg_{1.94}$.

GBP 9

Peptide **14** (40 nmol) was dissolved in 0.6 mL of 10% DMSO–100 mM phosphate buffer (pH 7.0), and the solution was kept at room temperature for 24 h. The crude peptide was separated by RP-HPLC using an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **9** (20 nmol, 50%). MALDI-TOF mass, found: *m*/*z* 2781.7, calcd: 2782.2 for $(M + H)^+$. Amino acid analysis: $Asp_{1.91}Thr_{1.89}Ser_{1.03}$-$Glu_{2.15}Pro_{1.74}Gly_{4.02}Ala_1Val_{0.92}Met_{0.54}Tyr_{1.97}Phe_{1.95}Lys_{0.94}Arg_{1.97}$.

Fmoc-[Lys(N$_3$)^1, Cys(Acm)^3, Cys(Pac)^7]-tachyplesin (1–10)-SC$_6$H$_4$CH$_2$COOH 16

Fmoc-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-Rink amide MBHA resin (50 μmol equivalent) was obtained in the same manner as described above. After washing with NMP, the peptide chain was elongated by the ordinary Fmoc-based SPPS. The amino acids (0.20 mmol each) except for Val just after Cys(Pac) were activated by mixing with 1 M DCC–NMP (300 μL) and 1 M HOBt–NMP (300 μL) at room temperature for 30 min, and the coupling reaction was carried out at 50 °C for 1 h. At the valine position next to Cys(Pac) residue, Fmoc-Val-OH (0.2 mmol) was activated by mixing HBTU (0.2 mmol) and DIEA (0.3 mmol) in NMP (0.5 mL), and the coupling reaction was carried out at 50 °C for 1 h. After elongation, Fmoc-Lys(N$_3$)-Trp(Boc)-Cys(Acm)-Phe-Arg(Pbf)-Val-Cys(Pac)-Tyr(Bu^t)-Arg(Pbf)-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (280 mg) was obtained. A part of the resin (10 mg) was treated with a TFA cocktail (200 μL) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was dissolved in 50% CH_3CN–5% AcOH–H_2O (0.4 mL), and MPAA (20 mg) was added to the solution. After the overnight reaction at room temperature, the crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide thioester **16** (112 nmol, 6.2% yield). MALDI-TOF mass, found: *m*/*z* 1904.8, calcd: 1904.8 for $(M + Na)^+$. Amino acid analysis: $Gly_{1.08}Val_{0.92}Tyr_{1.01}Lys_{0.34}Arg_2$.

[Cys(Pac)^{12}, Cys(Acm)^{16}]-tachyplesin (11–17)-NH$_2$ 17

Fmoc-Rink amide MBHA resin (0.34 mmol g^{-1}, 147 mg, 50 μmoL) was swelled in NMP for 30 min, and treated with 20% piperidine–NMP for 5 and 15 min. After washing with NMP, the peptide chain was elongated by Fmoc-SPPS. The amino acids (0.20 mmol each) except for Ile just after Cys(Pac) were activated by mixing with 1 M DCC–NMP (300 μL) and 1 M HOBt–NMP (300 μL) at room temperature for 30 min, and the coupling reaction was carried out at 50 °C for 1 h. At the isoleucine position next to Cys(Pac) residue, Fmoc-Ile-OH

(0.2 mmol) was activated by mixing HBTU (0.2 mmol) and DIEA (0.3 mmol) in NMP (0.5 mL), and the coupling reaction was carried out at 50 °C for 1 h. After elongation, H-Ile-Cys-(Pac)-Tyr(Bu^t)-Arg(Pbf)-Arg(Pbf)-Cys(Acm)-Arg(Pbf)-NH-resin (240 mg) was obtained. A part of the resin (10 mg) was treated with the TFA cocktail (200 μL) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **17** (390 nmol, 19% yield). MALDI-TOF mass, found: *m*/*z* 1157.5, calcd: 1157.6 for $(M + H)^+$. Amino acid analysis: $Cys_{0.51}Ile_{0.57}Tyr_{0.99}Arg_3$.

[Cys(Acm)^{3,16}, Cys(SH)^{7,12}]-tachyplesin 20

Peptides **16** (110 nmol) and **17** (160 nmol) were mixed and dissolved in DMSO (55 μL) containing 1% HOObt–1% DIEA, and incubated at room temperature overnight to give peptide **18**. Piperidine (6 μL) was then added to this solution and the reaction mixture was kept at room temperature for 30 min, yielding peptide **19**. The crude peptide was precipitated by addition of 20 times volume of diethyl ether, washed twice with ether and dried under vacuum. The precipitant was dissolved in 50% aqueous AcOH (1 mL), and an excess amount of Zn powder was added to the solution. The mixture was mixed with vortex at room temperature for 1 h. After filtration to remove Zn powder, the desired product **20** was purified by RP-HPLC using an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA. The isolated yield of **20** was 30% (32 nmol). MALDI-TOF mass, found: *m*/*z* 2409.0, calcd: 2409.2 for $(M + H)^+$. Amino acid analysis: $Gly_{1.09}Cys_{0.71}Val_{1.00}Ile_{0.87}Tyr_{1.95}Phe_{1.06}Lys_{1.00}Arg_5$.

[Cys(Acm)^{3,16}]-tachyplesin 21

Peptide **20** (32 nmol) was dissolved in 2 mL of 10% DMSO–100 mM phosphate buffer (pH 7.0), and the solution was kept at room temperature for 24 h. The crude peptide was separated by RP-HPLC using an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give peptide **21** (19 nmol, 59%). MALDI-TOF mass, found: *m*/*z* 2407.1, calcd: 2407.2 for $(M + H)^+$. Amino acid analysis: $Gly_{1.10}Cys_{0.69}Val_{0.93}Ile_{0.88}Tyr_{2.00}Phe_{1.03}Lys_{0.94}Arg_5$.

Tachyplesin 15

Peptide **21** (18 nmol) was dissolved in distilled water (150 μL), and the solution was added dropwise to CH_3OH (0.6 mL) containing 20 mM I_2–CH_3OH (20 μL) and 1 M HCl (36 μL) within 5 min with mixing. After the resulting solution was stirred for another 55 min at room temperature, the reaction was quenched by adding an ascorbic acid aqueous solution. The reaction mixture was separated by RP-HPLC on an Inertsil ODS-3 column with a linear gradient of acetonitrile containing 0.1% TFA to give the desired product **15** (13 nmol, 70% yield). MALDI-TOF mass, found: *m*/*z* 2262.8, calcd: 2263.1 for $(M + H)^+$. Amino acid analysis: $Gly_{1.02}Cys_{0.86}Val_{0.95}Ile_{0.84}Tyr_{2.07}Phe_{1.07}Lys_{1.09}Arg_5$.

Notes and references

1 P. E. Dawson, T. W. Muir, I. Clark-Lewis and S. B. H. Kent, *Science*, 1994, **266**, 776.

2 (*a*) L. Z. Yan and P. E. Dawson, *J. Am. Chem. Soc.*, 2001, **123**, 526; (*b*) D. Crich and A. Banerjee, *J. Am. Chem. Soc.*, 2007, **129**, 10064; (*c*) C. Haase, H. Rohde and O. Seitz, *Angew. Chem., Int. Ed.*, 2008, **47**, 6807; (*d*) J. Chen, Q. Wan, Y. Yuan, J. Zhu and S. J. Danishefsky, *Angew. Chem., Int. Ed.*, 2008, **47**, 8521; (*e*) K. S. A. Kumar, M. Haj-Yahya, D. Olschewski, H. A. Lashuel and A. Brik, *Angew. Chem., Int. Ed.*, 2009, **48**, 8090; (*f*) R. Yang, K. K. Pasunooti, F. Li, X.-W. Liu and C.-F. Liu, *J. Am. Chem. Soc.*, 2009, **131**, 13592; (*g*) Z. Harpaz, P. Siman, K. S. A. Kumar and A. Brik, *ChemBioChem*, 2010, **11**, 1232; (*h*) Z. Tan, S. Shang and S. J. Danishefsky, *Angew. Chem., Int. Ed.*, 2010, **49**, 9500; (*i*) J. Chen, P. Wang, J. Zhu, Q. Wan and S. J. Danishefsky, *Tetrahedron*, 2010, **66**, 2277; (*j*) S. Shang, Z. Tan, S. Dong and S. J. Danishefsky, *J. Am. Chem. Soc.*, 2011, **133**, 10784; (*k*) H. Ding, A. Shigenaga, K. Sato, K. Morishita and A. Otaka, *Org. Lett.*, 2011, **13**, 5588; (*l*) P. Siman, S. V. Karthikeyan and A. Brik, *Org. Lett.*, 2012, **14**, 1520.

3 (*a*) L. E. Canne, S. J. Bark and S. B. H. Kent, *J. Am. Chem. Soc.*, 1996, **118**, 5891; (*b*) D. W. Low, M. G. Hill, M. R. Carrasco, S. B. H. Kent and P. Botti, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 6554; (*c*) T. Kawakami, K. Akaji and S. Aimoto, *Org. Lett.*, 2001, **3**, 1403; (*d*) P. Botti, M. R. Carrasco and S. B. H. Kent, *Tetrahedron Lett.*, 2001, **42**, 1831; (*e*) J. Offer, C. N. C. Boddy and P. E. Dawson, *J. Am. Chem. Soc.*, 2002, **124**, 4642; (*f*) T. Kawakami and S. Aimoto, *Tetrahedron Lett.*, 2003, **44**, 6059; (*g*) D. Macmillan and D. W. Anderson, *Org. Lett.*, 2004, **6**, 4659; (*h*) B. Wu, J. Chen, J. D. Warren, G. Chen, Z. Hua and S. J. Danishefsky, *Angew. Chem.*, 2006, **118**, 4222, (*Angew. Chem., Int. Ed.*, 2006, **45**, 4116); (*i*) A. Brik, Y. Y. Yang, S. Ficht and C. H. Wong, *J. Am. Chem. Soc.*, 2006, **128**, 5626; (*j*) A. Saporito, D. Marasco, A. Chambery, P. Botti, S. M. Monti, C. Pedone and M. Ruvo, *Biopolymers*, 2006, **83**, 508; (*k*) B. L. Pentelute and S. B. H. Kent, *Org. Lett.*, 2007, **9**, 687; (*l*) Y.-Y. Yang, S. Ficht, A. Brik and C.-H. Wong, *J. Am. Chem. Soc.*, 2007, **129**, 7690; (*m*) Q. Wan and S. J. Danishefsky, *Angew. Chem.*, 2007, **119**, 9408, (*Angew. Chem., Int. Ed.*, 2007, **46**, 9248); (*n*) C. Chatterjee, R. K. McGinty, J.-P. Pellois and T. W. Muir, *Angew. Chem.*, 2007, **119**, 2872, (*Angew. Chem., Int. Ed.*, 2007, **46**, 2814); (*o*) R. Okamoto and Y. Kajihara, *Angew. Chem.*, 2008, **120**, 5482, (*Angew. Chem., Int. Ed.*, 2008, **47**, 5402); (*p*) H. Hojo, C. Ozawa, H. Katayama, A. Ueki, Y. Nakahara and Y. Nakahara, *Angew. Chem., Int. Ed.*, 2010, **49**, 5318.

4 (*a*) H. Hojo and S. Aimoto, *Bull. Chem. Soc. Jpn.*, 1991, **64**, 111; (*b*) S. Aimoto, *Biopolymers*, 1999, **51**, 247.

5 H. Hojo, Y. Murasawa, H. Katayama, T. Ohira, Y. Nakahara and Y. Nakahara, *Org. Biomol. Chem.*, 2008, **6**, 1808.
6 H. Katayama, H. Hojo, T. Ohira and Y. Nakahara, *Tetrahedron Lett.*, 2008, **49**, 5492.
7 H. Katayama, H. Hojo, I. Shimizu, Y. Nakahara and Y. Nakahara, *Org. Biomol. Chem.*, 2010, **8**, 1966.
8 H. Katayama, Y. Nakahara and H. Hojo, *Org. Biomol. Chem.*, 2011, **9**, 4653.
9 D. Hagiwara, M. Neya and M. Hashimoto, *Tetrahedron Lett.*, 1990, **31**, 6539.
10 (*a*) Y. Hayakawa, *J. Biol. Chem.*, 1990, **265**, 10813; (*b*) Y. Hayakawa, *J. Biol. Chem.*, 1991, **266**, 7982; (*c*) Y. Hayakawa and Y. Yasuhara, *Insect Biochem. Mol. Biol.*, 1993, **23**, 225.
11 H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 25; H. Hojo, Y. Onuma, Y. Akimoto, Y. Nakahara and Y. Nakahara, *Tetrahedron Lett.*, 2007, **48**, 1299.
12 T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao and Y. Shimonishi, *J. Biol. Chem.*, 1988, **263**, 16709.