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Chemical synthesis of mouse pro-opiomelanocortin(1–74) by azido-protected glycopeptide ligation *via* the thioester method

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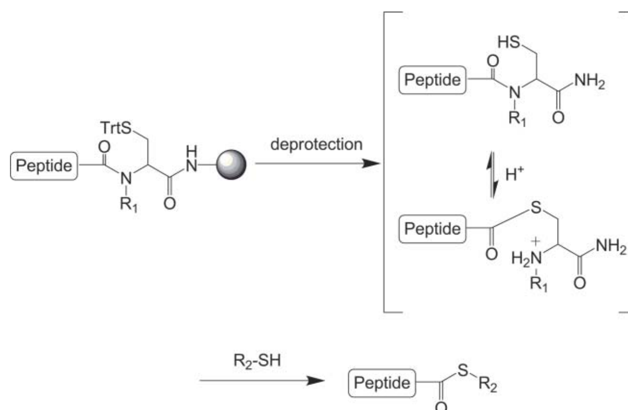
The thioester method is a peptide condensation reaction, which requires the protection of Lys side chains for chemoselective ligation. We recently found that the azido group could be used as an amino protecting group in the peptide condensation by the thioester method. In this study, we synthesized the glycosylated mouse pro-opiomelanocortin (1–74) by the thioester method. The N-terminal peptide thioester segment, whose Lys side chain was protected by an azido group, was prepared using a 9-fluorenylmethoxycarbonyl (Fmoc) strategy and an *N*-alkylcysteine (NAC)-assisted thioesterification reaction. The C-terminal azido-glycopeptide segment carrying *N*- and *O*-linked glycans was also prepared by the Fmoc chemistry and condensed with the N-terminal segment by the silver ion-free thioester method. These results showed that our azido-based strategy was fully compatible with the NAC-assisted method and glycoprotein synthesis.

Introduction

Solid phase peptide synthesis (SPPS) enables the easy preparation of peptides whose lengths are usually limited to less than 50 residues. To synthesize longer peptides, peptide condensation reactions, such as native chemical ligation,[1] traceless Staudinger ligation,[2] and the thioester method,[3] have been widely used. In all of these methods, peptide thioesters were used as key intermediates. Mainly accomplished by the *tert*-butoxycarbonyl (Boc) strategy, the preparation of peptide thioesters is difficult to achieve through the 9-fluorenylmethoxycarbonyl (Fmoc) method because of the lability of the thioester bond under the Fmoc removal conditions. Several post-SPPS thioesterification methods have been developed to produce peptide thioesters by the Fmoc chemistry.[4,5]

Recently, we developed a novel thioesterification reaction, in which *N*-alkylcysteine (NAC) was used as a thioesterification device.[4] First, NAC was introduced into a solid support, and then the peptide chain was elongated by the standard Fmoc strategy. After deprotection, the peptide carrying the NAC residue at the C-terminus was treated with a thiol compound in weakly acidic conditions, generating a peptide thioester by a thioester exchange reaction (Scheme 1). This method is fully compatible with the Fmoc–SPPS chemistry, and gave peptide and glycopeptide thioesters in good yields.[6–8]

In the thioester method, the C-terminal alkyl thioester group of one peptide segment is specifically activated by a silver ion, and condensed with the free N-terminal amino group of another peptide segment.[3] To achieve successful chemoselective condensation, the side chains of Lys and Cys residues in the segments should be protected. To date, Boc and acetamidomethyl (Acm) groups were generally used to protect amino and thiol groups, respectively. However, the Acm group was cleaved by the silver



Scheme 1 NAC-assisted thioesterification reaction.

ion under basic conditions, and careful manipulation of peptide segments was required during the Ag^+-mediated condensation reaction. Recently, we found that aryl thioesters such as 4-mercaptophenylacetic acid (MPAA) did not require silver ions for activation.[6]

The use of Boc groups on Lys side chains is also problematic. This protecting group is labile in the presence of trifluoroacetic acid (TFA), which is generally used to cleave peptides from the solid support. Therefore, it has to be reintroduced to the amino groups of the peptide segments just before the condensation reaction. To avoid this inconvenience, we recently developed a novel method using an azido-protected peptide.[7] The azido group is stable under acidic and basic conditions, and easily converted into an amino group by reduction. This azido-based method might be a powerful tool for protein synthesis, but it is not clear whether the azido-containing peptide thioester can be prepared by the NAC-assisted method, which uses excess amounts of thiol. It is also unclear whether the azido-based method is compatible with the classical thioester method, which uses silver ions to activate the thioester group.

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Mouse pro-opiomelanocortin (POMC) (1–74) is a glycoprotein consisting of 74 amino acid residues, and *N*- and *O*-linked glycans.[9] It is generated from a 31 kDa precursor POMC together with adenocorticotropic hormone (ACTH), β-lipotropin, β-endorphin, and melanocyte stimulating hormones (MSHs) by prohormone convertases (PC1 and PC2). POMC(1–74) also undergoes further processing at the Arg[49]-Lys[50] site to generate POMC(1–49) and γ_3-MSH.[10,11] Although POMC(1–74) is a precursor peptide of γ_3-MSH, it also shows biological activity; this fragment glycoprotein enhanced prolactin gene expression in cultured pituitary cells. The *N*-linked glycan at Asn[65] has been shown to be essential for conferring the biological activity.[9] It has also been suggested that *O*-glycosylation on Thr[45] prevents processing at the Arg[49]-Lys[50] site.[12] The chemical synthesis of a POMC(1–74) carrying homogenous glycans enables structure–activity relationship studies. In this study, we synthesized mouse POMC(1–74) through the thioester method to examine the compatibility of azidopeptide with the NAC-assisted thioesterification method. This seems to be the first report on the synthesis of glycosylated POMC.

Results and discussion

Stability of the azido group

To examine the stability of the azido group under the NAC-assisted thioesterification reaction conditions, an azidopeptide, H-Leu-Pro-Lys(N_3)-Phe-Met-Ile-Asp-Ala-NH_2 **1**,[7] was dissolved in a 5% 3-mercaptopropionic acid (MPA) solution, and the ratio between decomposed and intact peptides was measured by reversed-phase RP-HPLC. The peptide **2**, in which the azido group was converted to an amino group, increased in a time dependent manner (Fig. 1). However, more than 90% of the azido group remained after 2 d MPA treatment at room temperature. Therefore, azido-protected peptide may be used in the NAC method to prepare the POMC(1–37) thioester segment.

Synthesis of POMC(1–74)

The amino acid sequence of mouse POMC(1–74) is shown in Fig. 2 and the synthetic strategy is shown in Scheme 2. First, we synthesized the N-terminal peptide thioester segment (1–37). To enable RP-HPLC monitoring of the NAC-assisted thioesterification reaction, two Fmoc-Arg(Pbf)-OH were sequentially introduced to Rink Amide MBHA resin by the *N*,*N′*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method. Fmoc-(Et)Cys(Trt)-OH was subsequently introduced in the resulting resin by the same method. After Fmoc group removal, Fmoc-Gly-OH was introduced at 50 °C using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethylammonium hexafluorophosphate (HATU) as a condensation reagent. The Gly residue was introduced quantitatively by double coupling. Except for Fmoc-Lys(N_3)-OH, which was introduced manually by the DCC/HOBt method at Lys[25], the peptide chain was elongated by a peptide synthesizer using the Fmoc strategy. The crude peptide was cleaved from the resin with a TFA cocktail and dissolved in a mixture of 6 M urea and 2% MPAA or 5% MPA in aqueous acetonitrile. The conversion of the NAC-containing peptide into its corresponding thioester should make the peptide more hydrophobic because the thioester exchange reaction cleaves the

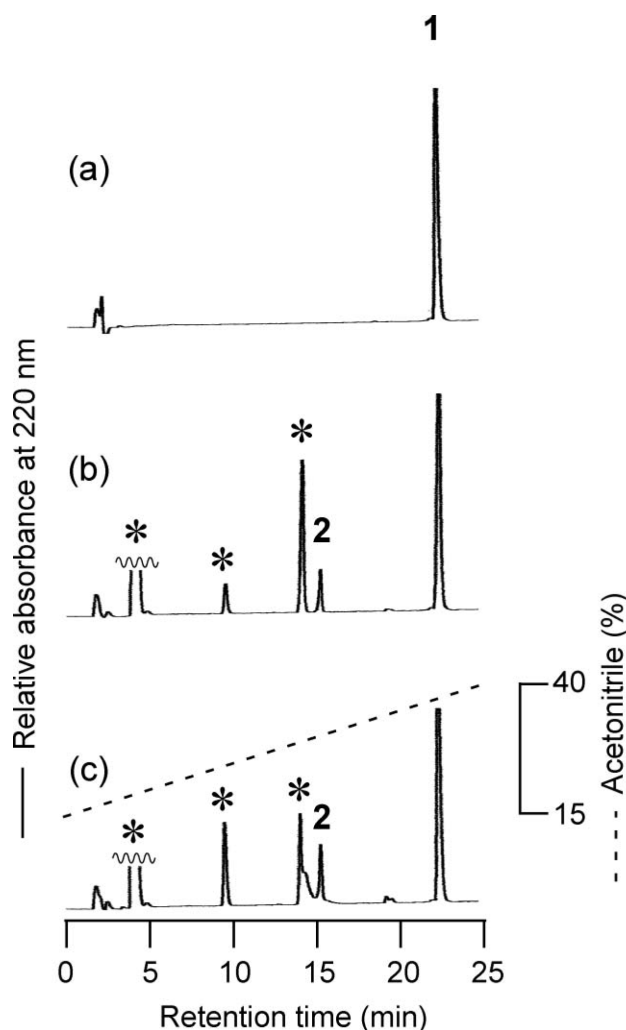

Fig. 1 RP-HPLC elution profiles of model azidopeptide **1** during the stability study. (a) Before treatment; (b) after a 1 d MPA treatment; (c) after a 2 d MPA treatment. The asterisks show nonpeptidic peaks.

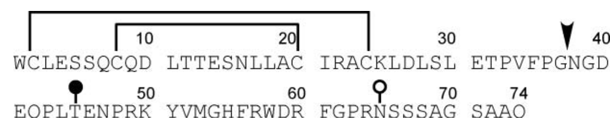

Fig. 2 Structure of mouse POMC(1–74). The arrowhead indicates the segment condensation site. Open and closed circles indicate *N*- and *O*-glycosylation sites, respectively. Solid lines represent disulfide bonds.

hydrophilic NAC-Arg-Arg-NH_2 part. Consequently, the retention time of the peptide thioester should be longer than that for the NAC-containing peptide on the RP-HPLC. In this experiment, a peak, which grew in a time-dependent manner, appeared after the NAC-containing peptide peak, consistent with peptide thioester formation. During the thioesterification reaction, the reduction of the azido group to an amino group was not observed in RP-HPLC and mass spectral analyses. The thioesterification reaction was almost complete within 1 d giving the desired peptide MPA thioester **3** and MPAA thioester **4**. These peptides were almost insoluble in aq. acetonitrile. By dissolving little by little in saturated guanidine hydrochloride solution, these peptides were isolated by

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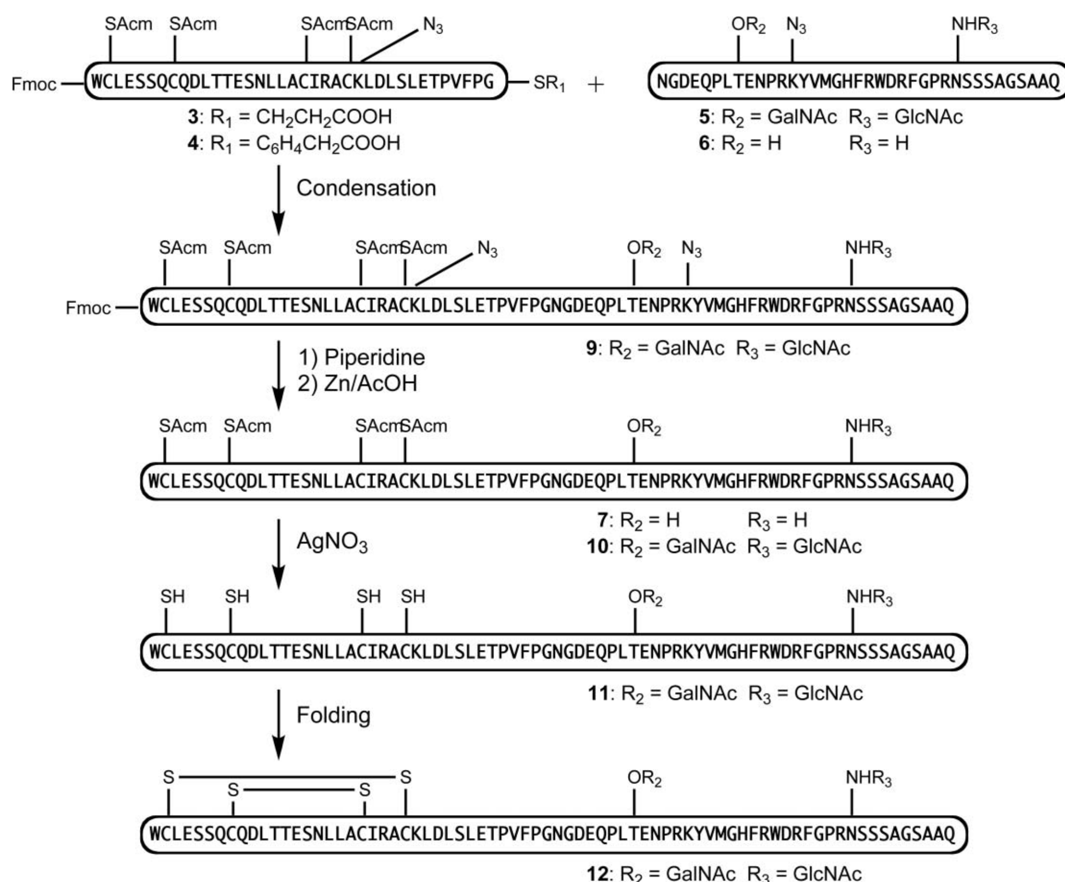

Scheme 2 Synthetic route for POMC(1–74).

RP-HPLC. The yields of **3** and **4** based on the amino group in the initial resin were 0.80% and 2.6%, respectively.

The C-terminal segment (38–74) **5** was also synthesized by the Fmoc chemistry. Starting from Fmoc-Gln(Trt)-CLEAR Acid resin, the peptide chain was elongated using the peptide synthesizer, except for residues Asn[45], Lys[50] and Thr[65], which were manually introduced by the DCC/HOBt method from Fmoc-Asn(GlcNAcBn$_3$)-OH, Fmoc-Lys(N$_3$)-OH and Fmoc-Thr(3-*O*-benzyl-4,6-*O*-benzylidene-GalNAc)-OH, respectively. The peptide was cleaved from the resin using a TFA cocktail, and the benzyl groups on the carbohydrate moieties were removed by further TFA/thioanisole treatment for 14 h at 30 °C. During this deprotecting reaction, no decomposition was observed for the azido group, indicating that the azido group is compatible with our benzyl-based glycopeptide synthesis method. The yield of the desired azido-glycopeptide **5** after RP-HPLC purification was 2.5% based on the Gln residue in the initial resin. Non-glycosylated C-terminal segment **6** was synthesized using the same approach as for **5**, except that Fmoc-Asn(Trt)-OH and Fmoc-Thr(Bu^t)-OH were used instead of glycosylated amino acid derivatives. The isolated yield of **6** after RP-HPLC purification was 3.3%.

We attempted to condense segments **3** and **6** through the Ag$^+$-mediated thioester method. However, about half of the peptide thioester **3** was hydrolyzed during the condensation reaction. The desired product **7** was obtained in 7.7% yield only after Fmoc and azido deprotection, whereas hydrolyzed by-product, Cys(Acm)[2,8,20,24]-POMC(1–37)-OH **8**, derived from peptide **3** was obtained in 9.7% yield. In this Ag$^+$-mediated reaction, the thioester bond was quickly activated by the silver ion, and the undesirable hydrolysis could not be suppressed.

Next, we tried to synthesize the glycosylated POMC(1–74) by a Ag$^+$-free thioester method. Segments **4** and **5** were mixed and dissolved in a solution of 3% 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (HOObt) and 2% *N*,*N*-diisopropylethylamine (DIEA) in dimethyl sulfoxide (DMSO). The reaction mixture was held overnight, yielding the partially protected glycopeptide **9** (Fig. 3). During the condensation reaction, little hydrolyzed peptide was observed by RP-HPLC analysis, suggesting that strict dehydration is not necessary in this Ag$^+$-free thioester method reaction. After the condensation, the Fmoc group on the N-terminal amino group was deprotected by adding piperidine. No decomposition was observed for the azido groups during condensation and piperidine treatment reactions. The azido groups were subsequently converted into amino groups to give peptide **10**. The total yield from **4** to **10** was 15%.

To generate the folded peptide, the Acm groups in **10** were removed with AgNO$_3$ in H$_2$O–DMSO first. The linear glycopeptide **11** was purified by gel filtration chromatography and placed in a redox buffer, which contained both reduced and oxidized forms of glutathione. This step allowed the disulfide bonds to form, giving the desired peptide carrying both *N*- and *O*-glycans **12** in 32% yield (Fig. 4). These results indicate that the azido-based thioester method is fully compatible with the NAC-assisted

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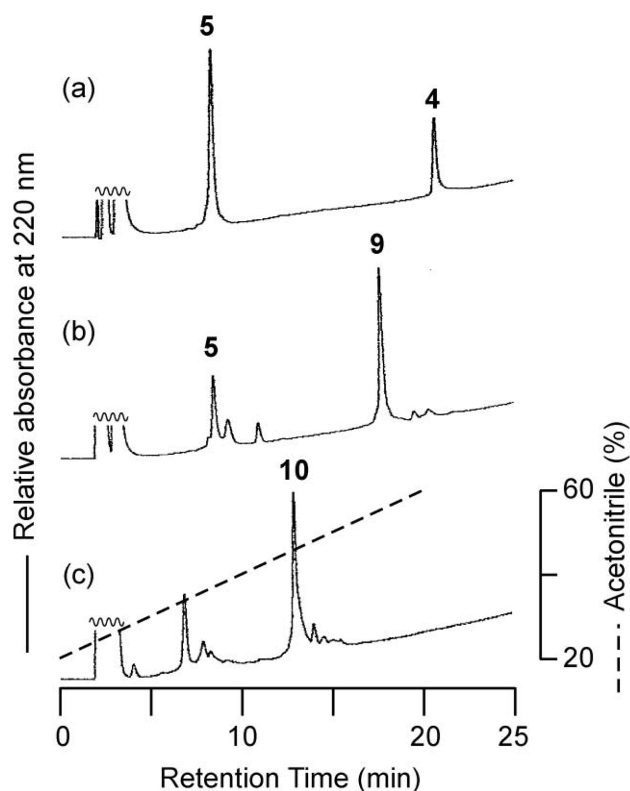

Fig. 3 RP-HPLC elution profiles obtained during peptide condensation and deprotection reactions. (a) Initial coupling reaction mixture of **4** and **5**. (b) Reaction mixture after the overnight reaction. (c) Reaction mixture after Fmoc deprotection and the azido reduction. RP-HPLC analysis was performed using a YMC-Pack PROTEIN-RP column (4.6ϕ × 150 mm, YMC, Japan) at a flow rate of 1 ml min^{-1}.

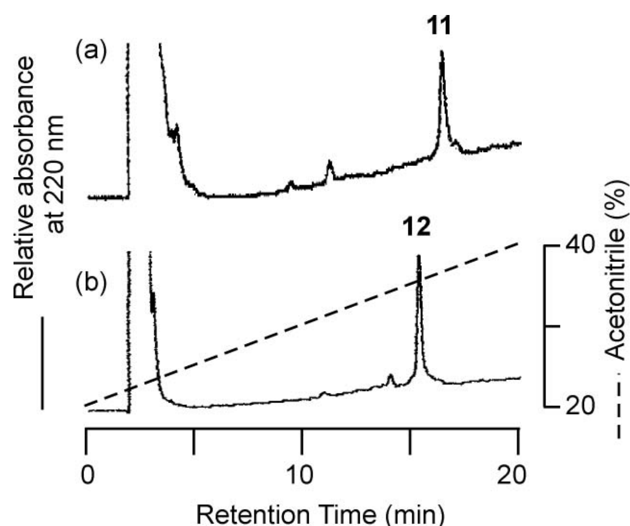

Fig. 4 RP-HPLC elution profiles obtained during the folding reaction of **11**. (a) 0 h; (b) 20 h. RP-HPLC analysis was performed using a YMC-Pack PROTEIN-RP column (4.6ϕ × 150 mm, YMC, Japan) at a flow rate of 1 ml min^{-1}.

thioesterification reaction and the glycopeptide synthesis by the Ag^{+}-free thioester method.

Table 1 Mass spectral data and sequences of the enzymatic digests

Enzyme	Peak No.	MS (observed)	Mass (calcd. for (M+H)$^+$)	Sequence	Position
Trypsin	1	932.8	933.5	WDRFGPR	58-64
	2	2841.1	2841.4	LDLSLETPVFPGN-GDEQPL***T***ENPR[a]	26-49
	3	2829.0	2829.0	WCLESSQCQD-LTTESNLLACIR	1-22
				ACK	23-25
Glu-C	1	868.0	868.4	WCLE	1-4
				ACK	23-25
	2	1997.6	1997.9	SSQCQDLTTE	5-14
				SNLLACIR	15-22

[a] ***T*** corresponds to the glycosylated Thr residue.

Mode of disulfide bonds

To determine the disulfide bond arrangement, the synthetic peptide **12** was digested with trypsin at 37 °C for 20 h, and the digests were separated by RP-HPLC. The mass spectral analysis of the digests indicated that one of them carried the two peptide chains, 1–22 and 23–25, connected by a disulfide bond. This digest was further treated with endoproteinase Glu-C, leading to two fragments carrying two peptide chains connected by a disulfide bond. The results of the mass spectral analyses of the digests are summarized in Table 1. These results clearly showed that the disulfide linkages were connected between Cys^{2}-Cys^{24} and Cys^{8}-Cys^{20}. This disulfide bond arrangement is the same as previously reported in the native POMC.[11]

Conclusion

In this study, we successfully synthesized the glycosylated mouse POMC(1–74) by the NAC-assisted thioesterification reaction and the azido-protected glycopeptide ligation *via* the thioester method. The results clearly indicated that our azido-based method was fully compatible with the NAC method and the glycoprotein synthesis by the thioester method, and that our method might be a powerful tool for synthesizing proteins and glycoproteins.

Experimental

General

Fmoc-(Et)Cys(Trt)-OH[4] and Fmoc-Lys(N_3)-OH[7] were prepared by a previously described method. H-Leu-Pro-Lys(N_3)-Phe-Met-Ile-Asp-Ala-NH_2 **1** was also prepared by the method described previously.[7] MALDI-TOF mass spectra were recorded using a Voyager-DE PRO spectrometer (Applied Biosystems, CA). 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 an vacuum-sealed tube.

Fmoc-[Cys(Acm)^{2,8,20,24}, Lys(N_3)^{25}]-POMC(1–37)-$SC_6H_4CH_2COOH$ 4

Fmoc-Rink amide MBHA resin (0.74 g, 0.25 mmol) was subjected to the automated synthesis using an Applied Biosystems 433A peptide synthesizer by the FastMoc protocol to give Arg(Pbf)-Arg(Pbf)-NH-resin. Fmoc-(Et)Cys(Trt)-OH (0.31 g, 0.50 mmol)

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was mixed with 1 M DCC/1-methyl-2-pyrrolidinone (NMP, 0.5 ml) and 1 M HOBt/NMP (0.5 ml) for 30 min at room temperature to produce Fmoc-(Et)Cys(Trt)-OBt, which was added to the resin. The reaction mixture was vortexed for 1 h at 50 °C. The resin was washed with NMP and treated with 20% piperidine/NMP for 5 and 15 min. After NMP washing, the resin was treated twice with Fmoc-Gly-OH (297 mg, 1.0 mmol), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N′*,*N′*-tetramethylammonium hexafluorophosphate (HATU, 380 mg, 1.0 mmol) and DIEA (2.0 mmol) dissolved in NMP (2.5 ml) for 1 h at 50 °C. The resin was returned to the synthesizer and the synthesis was continued using the FastMoc protocol. After Leu-Asp(OBu^t)-Leu-Ser(Bu^t)-Leu-Glu(OBu^t)-Thr(Bu^t)-Pro-Val-Phe-Pro-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin was obtained, Fmoc-Lys(N_3)-OH (200 mg, 0.50 mmol), HATU (190 mg, 0.50 mmol), and DIEA (0.17 ml, 1.0 mmol) in NMP were added, and the resulting mixture was vortexed at 50 °C for 1 h. The resin was washed with NMP and returned to the synthesizer. The synthesis was continued using the FastMoc protocol and Fmoc-Trp(Boc)-Cys(Acm)-Leu-Glu(OBu^t)-Ser(Bu^t)-Ser(Bu^t)-Gln(Trt)-Cys(Acm)-Gln(Trt)-Asp(OBu^t)-Leu-Thr(Bu^t)-Thr(Bu^t)-Glu(OBu^t)-Ser(Bu^t)-Asn(Trt)-Leu-Leu-Ala-Cys(Acm)-Ile-Arg(Pbf)-Ala-Cys(Acm)-Lys(N_3)-Leu-Asp(OBu^t)-Leu-Ser(Bu^t)-Leu-Glu(OBu^t)-Thr(Bu^t)-Pro-Val-Phe-Pro-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (1.6 g) was obtained. A portion of the resin (200 mg) was treated with TFA cocktail (TFA : thioanisole : phenol : H_2O : triisopropylsilane = 82.5 : 5 : 5 : 5 : 2.5, 4.0 ml) at room temperature for 2 h. After removing TFA under a nitrogen stream, the peptide was precipitated with ether. The precipitate was washed twice with ether, dried *in vacuo*, and dissolved in 50% aqueous acetonitrile. The crude peptide solution was filtered and lyophilized. The crude peptide obtained (41 mg) was dissolved in a mixture of 6 M urea and 2% 4-mercaptophenylacetic acid in 50% aqueous acetonitirile (2.0 ml) and the solution was maintained at 35 °C overnight. The reaction mixture was centrifuged and the supernatant was removed. HPLC analysis showed the absence of product in the supernatant. The precipitate was dissolved in a saturated guanidine hydrochloride solution and analyzed by HPLC, allowing the product to be detected. The solution was loaded on a RP-HPLC column (Mightysil 5C18, 10 × 250 mm). The fraction containing the product was isolated and lyophilized to give the desired product **4** (0.81 µmol, 2.6% based on the amino group in the initial resin). MALDI-TOF mass, found: *m*/*z* 4768.3 (average), calcd for $(M+H)^+$: 4769.5. Amino acid analysis: $Asp_{3.02}Thr_{2.65}Ser_{3.34}Glu_{4.32}Pro_{1.38}Gly_1Ala_{1.94}Val_{0.85}Ile_{0.84}Leu_{7.30}Phe_{1.11}Lys_{0.15}Arg_{0.85}$.

Fmoc-[Cys(Acm)^{2,8,20,24}, Lys(N_3)^{25}]-POMC(1–37)-SCH_2CH_2COOH 3

Fmoc-Trp(Boc)-Cys(Acm)-Leu-Glu(OBu^t)-Ser(Bu^t)-Ser(Bu^t)-Gln(Trt)-Cys(Acm)-Gln(Trt)-Asp(OBu^t)-Leu-Thr(Bu^t)-Thr(Bu^t)-Glu(OBu^t)-Ser(Bu^t)-Asn(Trt)-Leu-Leu-Ala-Cys(Acm)-Ile-Arg(Pbf)-Ala-Cys(Acm)-Lys(N_3)-Leu-Asp(OBu^t)-Leu-Ser(Bu^t)-Leu-Glu(OBu^t)-Thr(Bu^t)-Pro-Val-Phe-Pro-Gly-(Et)Cys(Trt)-Arg(Pbf)-Arg(Pbf)-NH-resin (500 mg) was treated with TFA cocktail (TFA : thioanisole : phenol : H_2O : triisopropylsilane = 82.5 : 5 : 5 : 5 : 2.5, 10 ml) at room temperature for 2 h. After removing TFA under a nitrogen stream, the peptide was precipitated with ether, washed twice with the same solvent, and dried *in vacuo*. The crude peptide was dissolved in a mixture of 6 M urea and 5% 3-mercaptopropionic acid in 50% aqueous acetonitirile (10 ml). The solution was filtered and maintained at room temperature overnight. The desired peptide thioester was purified by RP-HPLC on a column (Mightysil 5C18, 10 × 250 mm). The yield of the product **3** was 0.55 µmol (0.80% based on the amino group in the initial resin). MALDI-TOF mass, found: *m*/*z* 4703.6 (average), calcd for $(M+H)^+$: 4707.3. Amino acid analysis: $Asp_{2.52}Thr_{2.32}Ser_{3.04}Glu_{4.17}Pro_{2.22}Gly_{1.32}Ala_2Val_{0.86}Ile_{0.72}Leu_{6.17}Phe_{1.02}Lys_{0.56}Arg_{1.09}$.

Lys(N_3)^{50}-POMC(38–74) 6

Starting from Fmoc-Gln(Trt)-Wang Resin (0.44 g, 0.10 mmol), the peptide chain was elongated by the 433A synthesizer using the FastMoc protocol to obtain Tyr(Bu^t)-Val-Met-Gly-His(Trt)-Phe-Arg(Pbf)-Trp(Boc)-Asp(OBu^t)-Arg(Pbf)-Phe-Gly-Pro-Arg(Pbf)-Asn(Trt)-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Gly-Ser(Bu^t)-Ala-Ala-Gln(Trt)-Wang resin. The resin was removed from the synthesizer and added to Fmoc-Lys(N_3)-OBt, which was prepared by reacting Fmoc-Lys(N_3)-OH (0.2 mmol) with 1 M DCC/NMP (0.3 ml) and 1 M HOBt/NMP (0.3 ml) for 30 min at room temperature. The mixture was vortexed for 1 h at 50 °C, and the resin was washed with NMP. Half of the resin (50 µmol) was returned to the synthesizer. After the peptide chain assembly, Asn(Trt)-Gly-Asp(OBu^t)-Glu(OBu^t)-Gln(Trt)-Pro-Leu-Thr(Bu^t)-Glu(OBu^t)-Asn(Trt)-Pro-Arg(Pbf)-Lys(N_3)-Tyr(Bu^t)-Val-Met-Gly-His(Trt)-Phe-Arg(Pbf)-Trp(Boc)-Asp(OBu^t)-Arg(Pbf)-Phe-Gly-Pro-Arg(Pbf)-Asn(Trt)-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Gly-Ser(Bu^t)-Ala-Ala-Gln(Trt)-Wang resin (0.42 g) was obtained. A portion of the resin (0.10 g, 12 µmol) was treated with TFA cocktail (TFA : thioanisole : phenol : H_2O : triisopropylsilane = 82.5 : 5 : 5 : 5 : 2.5, 2.0 ml) at room temperature for 2 h. TFA was removed under a nitrogen stream and the peptide was precipitated with ether, washed twice with the same solvent, and dried *in vacuo*. The crude peptide was purified by RP-HPLC (Mightysil 5C18, 10 × 250 mm) using aqueous acetonitrile containing 0.1% TFA as an eluent to produce peptide **6** (0.39 µmol, 3.3% based on the Gln content in the initial resin).

[Asn(GlcNAc)^{65}, Lys(N_3)^{50}, Thr(GalNAc)^{45}]-POMC(38–74) 5

Starting from Fmoc-Gln(Trt)-CLEAR Acid Resin (0.24 g, 0.10 mmol), the peptide chain was elongated by the 433A synthesizer using the FastMoc protocol to obtain Fmoc-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Gly-Ser(Bu^t)-Ala-Ala-Gln(Trt)-CLEAR acid resin. The resin was removed from the synthesizer and half of the resin (50 µmol) was treated with 20% piperidine/NMP for 5 min. The reaction was repeated with a fresh reagent for 15 min. After NMP washing, the resin was mixed with Fmoc-Asn(GlcNAcBn$_3$)-OBt, which was prepared by reacting Fmoc-Asn(GlcNAcBn$_3$)-OH (62 mg, 75 µmol) with 1 M HOBt/NMP (0.1 ml) and 1 M DCC/NMP (0.1 ml) for 30 min at room temperature, and the mixture was vortexed for 1 h at 50 °C. Then, the resin was washed with NMP and treated with a mixture of 10% Ac_2O and 5% DIEA in NMP for 10 min. It was then returned to the synthesizer and the synthesis was continued.

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After Tyr(Bu^t)-Val-Met-Gly-His(Trt)-Phe-Arg(Pbf)-Trp(Boc)-Asp(OBu^t)-Arg(Pbf)-Phe-Gly-Pro-Arg(Pbf)-Asn($GlcNAcBn_3$)-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Gly-Ser(Bu^t)-Ala-Ala-Gln(Trt)-CLEAR acid resin was obtained, the resin was removed from the synthesizer and mixed with Fmoc-Lys(N_3)-OBt, which was prepared by reacting Fmoc-Lys(N_3)-OH (79 mg, 0.2 mmol) with 1 M HOBt/NMP (0.2 ml) and 1 M DCC/NMP (0.2 ml) for 30 min at room temperature, and the resulting mixture was vortexed for 1 h at 50 °C. The resin was again subjected to automated synthesis to add the Glu^{46}-Leu^{49} and produce the protected peptide resin corresponding to the Glu^{46}-Gln^{74} sequence. The resin was removed from the synthesizer and mixed with Fmoc-Thr(3-*O*-benzyl-4,6-*O*-benzylidene-GalNAc)-OBt [Fmoc-Thr(GalNAc*)-OBt], which was prepared by reacting Fmoc-Thr(GalNAc*)-OH (54 mg, 75 μmol) with 1 M HOBt/NMP (0.1 ml) and 1 M DCC/NMP (0.1 ml) for 30 min at room temperature, and the resulting mixture was vortexed for 1 h at 50 °C. The resin was washed with NMP and treated with 10% Ac_2O and 5% DIEA in NMP for 10 min. The resin was returned to the synthesizer and synthesis was continued to obtain Asn(Trt)-Gly-Asp(OBu^t)-Glu(OBu^t)-Gln(Trt)-Pro-Leu-Thr(GalNAc*)-Glu(OBu^t)-Asn(Trt)-Pro-Arg(Pbf)-Lys(N_3)-Tyr(Bu^t)-Val-Met-Gly-His(Trt)-Phe-Arg(Pbf)-Trp(Boc)-Asp(OBu^t)-Arg(Pbf)-Phe-Gly-Pro-Arg(Pbf)-Asn($GlcNAcBn_3$)-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Ala-Gly-Ser(Bu^t)-Ala-Ala-Gln(Trt)-CLEAR acid resin (0.35 g). A portion of the resin (41 mg, 5.5 μmol) was treated with TFA cocktail (TFA : thioanisole : phenol : H_2O : triisopropylsilane = 82.5 : 5 : 5 : 5 : 2.5, 2.5 ml) at room temperature for 1 h. TFA was removed under a nitrogen stream and the peptide was precipitated with ether and washed twice with ether. The crude peptide was dried *in vacuo* and dissolved in 50% aqueous acetonitrile. The solution was filtered and lyophilized. The crude peptide (15 mg) was treated with 20% thioanisole/TFA (1.5 ml) at 30 °C for 14 h. After TFA removal under a nitrogen stream, the product was precipitated with cold ether, washed twice with ether, and dried *in vacuo*. The crude peptide was isolated by RP-HPLC (column: Mightysil 5C18, 10 × 250 mm) using aqueous acetonitrile containing 0.1% TFA as an eluent to give peptide **5** (135 nmol, 2.5% based on the Gln content in the initial resin). MALDI-TOF mass, found: *m/z* 4597.3 (average), calcd for $(M+H)^+$: 4597.9. Amino acid analysis: $Asp_{4.62}Thr_{0.87}Ser_{3.79}Glu_{3.74}Pro_{2.90}Gly_4Ala_{3.15}Val_{1.43}Met_{1.30}Leu_{0.93}Tyr_{0.98}Phe_{2.09}Lys_{0.16}His_{1.01}Arg_{4.00}$.

$Cys(Acm)^{2,8,20,24}$-POMC(1–74) 7

Peptides **3** (50 nmol) and **6** (50 nmol) were dissolved in DMSO (20 μl) containing HOObt (1.0 mg) and DIEA (1 μl). The reaction was initiated by adding AgCl to the peptide solution, and the mixture was stirred at room temperature for 6 h. After AgCl was filtered off, piperidine (5 μl) was added and the solution was maintained at room temperature for 20 min. The crude mixture was precipitated with ether and dissolved in 50% aqueous acetic acid (0.20 ml). An excess amount of zinc powder was added to the solution and the mixture was stirred for 30 min at room temperature. After centrifugation, the supernatant was collected and loaded onto an HPLC column (YMC-Pack PROTEIN-RP 4.6 × 150 mm). The fraction containing the desired product was collected and lyophilized to obtain peptide **7** (3.9 nmol, 7.7%). MALDI-TOF mass, found: *m/z* 8514.8 (average), calcd for $(M+H)^+$: 8517.4. Amino acid analysis: $Asp_{6.92}Thr_{3.57}Ser_{5.55}Glu_{7.48}Pro_{5.39}Gly_5Ala_{4.53}Val_{1.86}Ile_{1.16}Leu_{9.10}Tyr_{1.00}Phe_{3.27}Lys_{1.40}His_{0.66}Arg_{4.24}$. The hydrolyzed peptide, $Cys(Acm)^{2,8,20,24}$-POMC(1–37)-OH **8** was also obtained after RP-HPLC purification in 9.7% yield. MALDI-TOF mass, found: *m/z* 4371.4 (average), calcd for $(M+H)^+$: 4371.0. Amino acid analysis: $Asp_{3.34}Thr_{2.28}Ser_{3.09}Glu_{4.12}Pro_{2.71}Gly_{1.92}Ala_{2.29}Val_{1.06}Ile_{0.90}Leu_7Phe_{1.77}Lys_{0.75}Arg_{1.80}$.

$[Asn(GlcNAc)^{65}, Cys(Acm)^{2,8,20,24}, Thr(GalNAc)^{45}]$-POMC(1–74) 10

Peptides **4** (130 nmol) and **5** (135 nmol) were dissolved in DMSO (25 μl) containing HOObt (0.64 mg, 3.9 μmol) and DIEA (0.45 μl, 2.6 μmol). After the solution was held at room temperature overnight, piperidine (1.3 μl) was added. The solution was maintained at room temperature for 1.5 h and ether was added to precipitate the product, which was then dissolved in 50% aqueous acetic acid (0.30 ml). Zinc dust (10 mg) was added to the solution and the mixture was stirred for 1 h at room temperature. After centrifugation, the supernatant was collected and loaded onto a YMC-Pack PROTEIN-RP HPLC column (10 × 250 mm) and the fraction containing the desired product was collected and lyophilized to give glycopeptide **10** (19 nmol, 15%). MALDI-TOF mass, found: *m/z* 8921.8 $(M+H)^+$ (average), calcd for $(M+H)^+$: 8923.9. Amino acid analysis: $Asp_{8.05}Thr_{3.73}Ser_{7.42}Glu_{9.35}Pro_{6.22}Gly_{5.82}Ala_5Val_{2.06}Met_{0.47}Ile_{0.93}Leu_{8.27}Tyr_{1.03}Phe_{3.15}Lys_{1.87}His_{1.07}Arg_{4.91}$.

$[Asn(GlcNAc)^{65}, Cys(SH)^{2,8,20,24}, Thr(GalNAc)^{45}]$-POMC(1–74) 11

Peptide **10** (19 nmol) was dissolved in 80% aqueous DMSO (25 μl) containing $AgNO_3$ (0.67 mg, 3.9 μmol) and DIEA (0.040 μl, 0.23 μmol). The solution was maintained at 50 °C for 2 h in the dark. DTT (1.9 mg, 12 μmol) was added to the solution and the mixture was acidified using 0.5 M HCl. The product was purified by gel filtration chromatography using a $G3000PW_{XL}$ column (7.5 × 300 mm, Tosoh) using 50% aqueous acetonitrile containing 0.1% TFA at a flow rate of 0.5 ml min^{-1} to produce glycopeptide **11** (quantitative). MALDI-TOF mass, found: *m/z* 8636.8 $(M+H)^+$ (average), calcd for $(M+H)^+$: 8639.6. Amino acid analysis: $Asp_{7.87}Thr_{3.55}Ser_{6.88}Glu_{8.83}Pro_{5.23}Gly_{5.28}Ala_5Val_{2.15}Met_{1.08}Ile_{0.86}Leu_{7.87}Tyr_{0.91}Phe_{2.89}Lys_{1.95}His_{0.82}Arg_{4.06}$.

$[Asn(GlcNAc)^{65}, Thr(GalNAc)^{45}]$-POMC(1–74) 12

Peptide **11** (21 nmol) was dissolved in 6 M guanidine hydrochloride (200 μl) and added dropwise to a mixture of 0.1 M NaCl, 9 mM glutathione (reduced form), 1 mM glutathione (oxidized form) and glycerol (2.0 ml) in 50 mM Tris buffer (pH 8.5, 8.0 ml) under gentle stirring. After the resultant solution was maintained at 5 °C overnight, the product was purified by RP-HPLC using a YMC-Pack PROTEIN-RP column (4.6 × 150 mm) to give peptide **12** (6.6 nmol, 32%). MALDI-TOF mass, found: *m/z* 8638.6 $(M+H)^+$ (average), calcd for $(M+H)^+$: 8635.6. Amino acid analysis: $Asp_{8.02}Thr_{3.66}Ser_{7.20}Glu_{9.47}Pro_{5.04}Gly_{5.62}Ala_5Val_{2.02}Met_{0.94}Ile_{0.88}Leu_{8.02}Tyr_{0.97}Phe_{2.93}Lys_{1.88}His_{1.00}Arg_{4.94}$.

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Enzymatic digestion

Peptide **12** (1 nmol) was dissolved in 50 mM HEPES buffer (pH 7.5, 200 μl) and TPCK-treated trypsin (Sigma) solution (1 mg ml^{-1}, 1 μl) was added. The mixture was maintained at 37 °C overnight. The digests were separated using a RP-HPLC column (Mightysil 5C18, 4.6 × 150 mm) with aqueous acetonitrile containing 0.1% TFA as an eluent.

The tryptic digest was dissolved in 50 mM HEPES buffer (pH 7.5, 100 μl) and endoproteinase Glu-C (Sigma) solution (1 mg ml^{-1}, 0.5 μl) was added. The mixture was kept at 37 °C overnight, and the digests were separated using a RP-HPLC column (Mightysil 5C18, 4.6 × 150 mm) with aqueous acetonitrile containing 0.1% TFA as an eluent.

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