

## Chemical synthesis of palmitoylated histone H4

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Dedicated to Prof. Horst Kunz on the occasion of his 80th anniversary

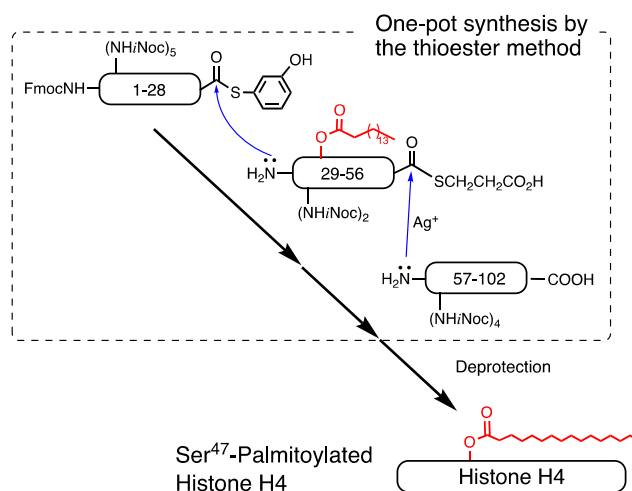
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### Abstract

Palmitoylation is one of the post-translational modifications of proteins. Recently, palmitoyl modification was found in histone H4, one of the components of the nucleosome core. To analyze this unusual modification in the nuclear protein, we chemically synthesized Ser<sup>47</sup>-palmitoylated histone H4. The entire sequence of H4 was divided into three segments and each was prepared by Fmoc-solid-phase peptide synthesis (SPPS). The palmitoyl group was introduced as a preformed palmitoyl Ser during SPPS or introduced at the end of SPPS by selective deprotection of the hydroxy group of Ser<sup>47</sup>, followed by palmitoylation. After three segments were condensed in one-pot by the thioester method, the desired Ser<sup>47</sup>-palmitoylated H4 was obtained.



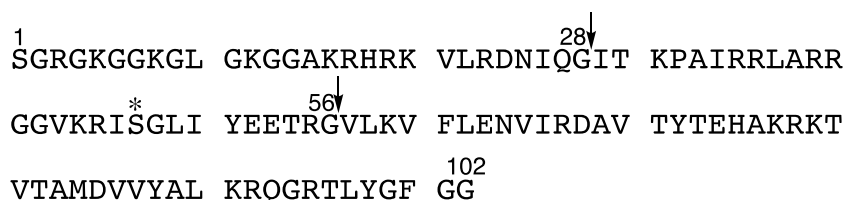
**Keywords:** Palmitoylation, histone H4, thioester method, segment condensation

## Introduction

Post-translational modification of proteins refers to the changes in the chemical structure of amino acid residues, and can modulate the structure, charge, localization, degradation and interactions of the proteins. For the analysis of the functions, multiple methods to assay the protein modifications have been developed over the past decades. According to the development of the methods, post-translational modifications of nuclear proteins, some of the core histones (H3 and H4), have come to be well known to epigenetically regulate gene expression.<sup>1,2,3</sup> To directly understand the function of a histone with specific modification(s), histones with modifications (methylation, acetylation, ubiquitination) on the specific target lysine residue were chemically synthesized.<sup>4,5</sup> By using synthetic histones, the functional interactions between the histone modifications and other epigenetic regulations, i.e., DNA modification, have been characterized.<sup>6,7</sup>

Protein lipidation is also an important post-translational modification, and the modification has been reported to increase its affinity to membranes and subsequent interaction with other proteins for cell signaling.<sup>8,9</sup> Among the lipid modifications, palmitoylation is the most abundant in human proteins (<https://www.labome.com/method/Protein-Modification.html>). In addition to proteins engaged in cell signaling, the palmitoylation of nuclear protein, histone H4 at Ser<sup>47</sup>, has been recently reported,<sup>10</sup> and the enzymes to erase the modification have been reported by using a peptide with the palmitoylated analogue.<sup>11</sup> However, the function of the lipidation is still not certain.

There are many reports on the chemical synthesis and semi-synthesis of lipidated peptides and proteins. For example, the N-myristoylated HIV-1 matrix protein p17,<sup>12</sup> N-palmitoylated sonic hedgehog protein<sup>13</sup> and O-stearoylated Autophagosomal Marker Protein LC3-II<sup>14</sup> were chemically synthesized by the native chemical ligation (NCL) method.<sup>15</sup> On the other hand, the S-palmitoylated and S-farnesylated Ras protein was prepared by semi-synthesis, in which the N-terminal large polypeptide thioester was expressed in *E. coli* and ligated with the C-terminal short S-palmitoylated and S-farnesylated peptide.<sup>16,17</sup> The same method was also used to obtain the geranylgeranylated Rab GTPase<sup>18</sup> and N-palmitoylated prion protein.<sup>19</sup> From the synthetic point of view, N-acylation can be easily performed at any step during the solid-phase peptide synthesis (SPPS) by the prevalent 9-fluorenylmethoxycarbonyl (Fmoc) method. Selective S-palmitoylation, farnesylation and geranylgeranylation can be achieved even after the free polypeptide is obtained due to the high nucleophilicity of the thiol group. On the other hand, the introduction of a fatty acid to the side chain hydroxy group during the peptide chain elongation has not yet been achieved. The site-specific O-acylation at the side chain hydroxy group of the free polypeptide chain is difficult, as the polypeptide usually has many hydroxy amino acids. We have now examined several strategies to synthesize the palmitoylated peptide and applied it to the total synthesis of the histone H4 palmitoylated at Ser<sup>47</sup> (Figure 1) using the thioester segment condensation method.<sup>20</sup>



**Figure 1.** Amino acid sequence of histone H4. The arrows indicate the sites of segment coupling by the thioester method. The asterisked serine retains the palmitoyl group at its side chain hydroxy group.

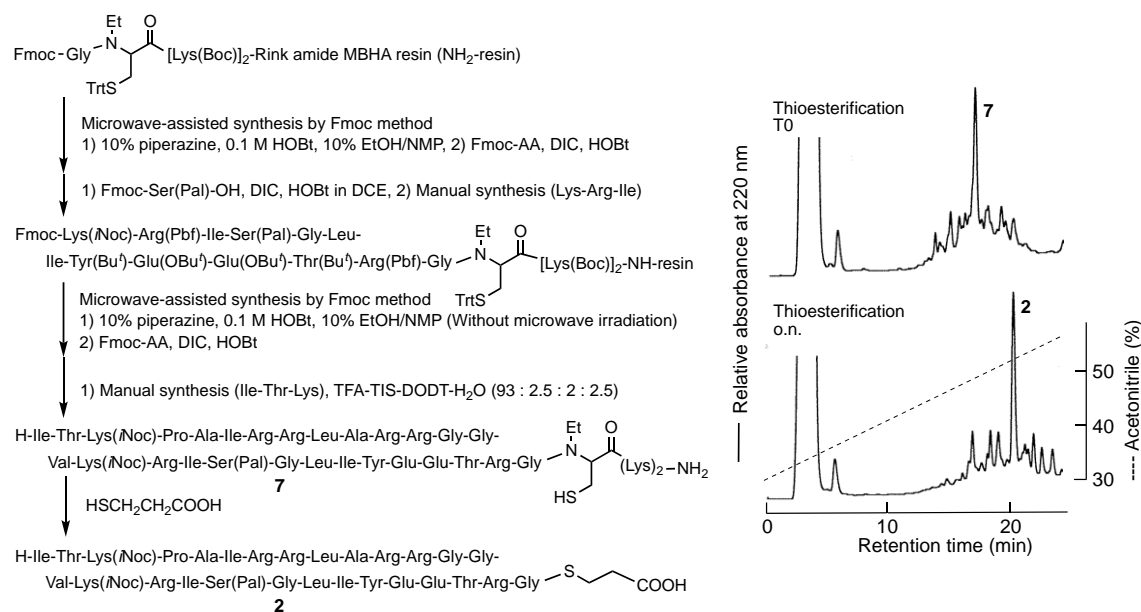
## Results and Discussion

Non-palmitoylated histone H4 has been chemically synthesized using the NCL method by several groups, including ourselves.<sup>21,22,23</sup> In this palmitoylated H4 synthesis, the thioester method, which uses the direct aminolysis reaction of the amino- and thiol-protected peptide thioester by the other segment,<sup>20</sup> was used to condense the peptide segments. According to Liu's report,<sup>21</sup> the middle region of H4 has a high hydrophobicity. Thus, we postulated that the thioester method, which uses an organic solvent for coupling, is advantageous to condense the hydrophobic segments, especially retaining the palmitoyl group. The segment coupling was designed to be performed at the C-terminus of Gly<sup>28,56</sup>, in order to avoid the potential danger of racemization of the C-terminus during the condensation reaction as shown in Figure 1. Thus, three segments, which correspond to H4(1-28) **1**, H4(29-56) **2**, H4(57-102) **3**, were prepared. We previously developed the efficient one-pot three segment condensation by the thioester method<sup>24</sup> using the reactivity difference between the aryl and alkyl thioesters, which was applied to the synthesis of histone H4 in this study. Following the procedure, segment **1** was prepared as a reactive aryl thioester, whereas segment **2** was an alkyl thioester, which can be activated by silver ions. For the protection of the side chain amino group during the ligation reaction, a hydrophilic and acid/base stable 4-pyridylmethoxycarbonyl (*i*Noc) group<sup>25</sup> was used.

First, we examined the synthetic route for the middle segment **2**, which has a palmitoyl group at Ser<sup>47</sup>. Considering the possibility that the palmitoyl ester is labile to piperidine used for the Fmoc group removal, the synthesis of segment **2** was first attempted by the *t*-butoxycarbonyl (Boc) method as the method does not use a nucleophilic base during the chain elongation (Figure S1). To the 4-methylbenzhydramine (MBHA)-resin, two Arg residues were introduced as a hydrophilic linker using Boc-Arg(Ts)-OH by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIEA). Boc-Gly-SCH<sub>2</sub>CH<sub>2</sub>COOH was then introduced by *N,N'*-diisopropylcarbodiimide (DIC) and ethyl cyano(hydroxyimino)acetate (Oxyma). The obtained resin was subjected to an automated synthesis by the Applied Biosystems 433A using the FastBoc protocol. At Ser<sup>47</sup>, preformed Boc-Ser(Pal)-OH was manually introduced by DIC/Oxyma. The Lys residues were also manually introduced using Boc-Lys(*i*Noc)-OH by HBTU and DIEA. After completion of the peptide chain assembly, the protected peptide resin was treated with anhydrous HF and the obtained crude peptide was analyzed. The results showed that the peptide did not contain the palmitoyl group. To prove that the palmitoyl ester is labile under the HF treatment conditions, a model peptide, the Boc-Lys(ClZ)-Lys(ClZ)-Leu-Ser(Pal)-Gly-Gly-OCH<sub>2</sub>-Pam resin, was synthesized by the Boc method and treated with 10% Ac<sub>2</sub>O, 5% DIEA for 20 min. If the ester linkage of the palmitoyl group is partially cleaved during the repetitive trifluoroacetic acid (TFA) treatment, the free hydroxy group should be acetylated by the Ac<sub>2</sub>O treatment. The analysis of the crude peptide obtained by the HF treatment of the resin showed that the major product was H-Lys-Lys-Leu-Ser-Gly-Gly-OH. A small amount of H-Lys-Lys-Leu-Ser(Pal)-Gly-Gly-OH was also observed, but H-Lys-Lys-Leu-Ser(Ac)-Gly-Gly-OH was not observed. Based on this result, the HF treatment is the main cause of the palmitoyl ester cleavage.

We next examined the use of the Fmoc method. However, the synthesis of the peptide thioester by the Fmoc method is difficult as the piperidine used for the Fmoc group removal decomposes the thioester linkage during the chain elongation.<sup>26</sup> We developed an efficient post-synthetic thioesterification method using the *N*-alkylcysteine (NAC) as the N-to-S acyl shift device,<sup>27</sup> which was applied for the synthesis of segment **2**. The synthetic route is shown in Figure S2. Fmoc-Gly-(Et)Cys(Trt)-OH as the NAC was first introduced to the dilysyl resin and the obtained resin was subjected to the automated microwave-assisted synthesis by Liberty Blue (CEM) following the standard protocol. Ser<sup>47</sup> was manually introduced using preformed Fmoc-Ser(Pal)-OH by the DIC – 1-hydroxybenzotriazole (HOBt) method. The manual synthesis was continued at room temperature

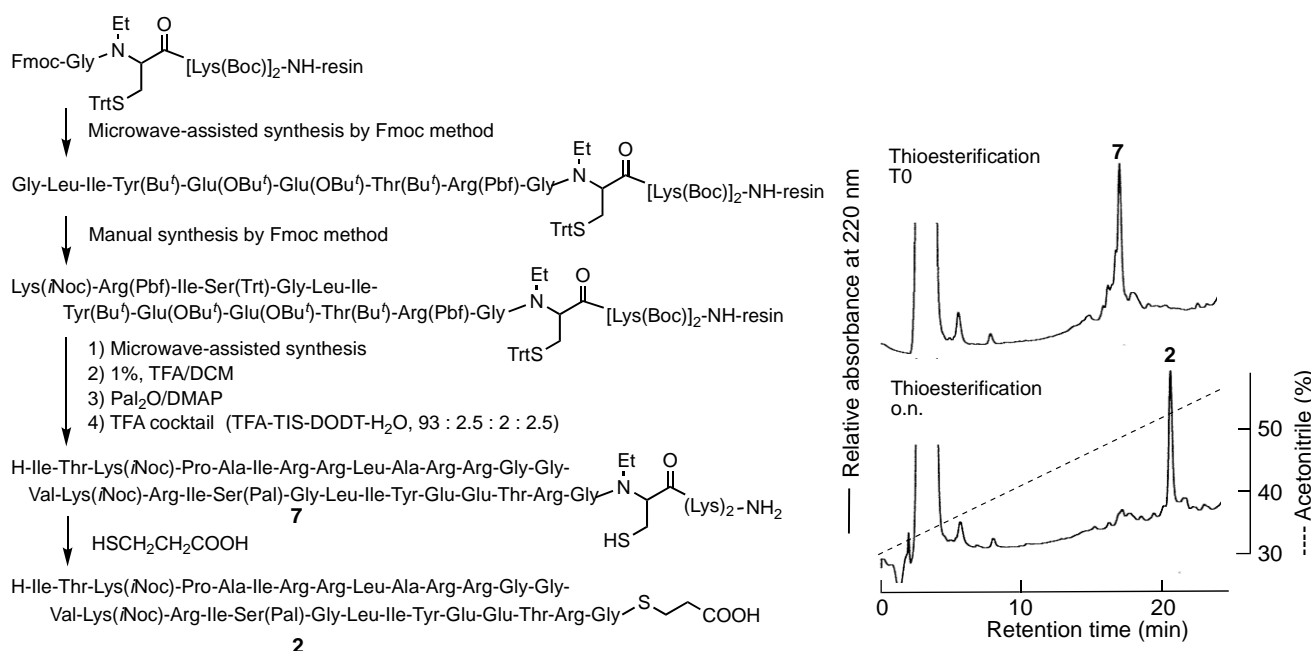
till Lys<sup>44</sup> was introduced. The analysis of the resin sampling showed that the O-palmitoyl group was stable during this manual chain elongation. To examine the applicability of the microwave synthesis to the O-palmitoylated peptide, a part of the obtained resin was subjected to the automated microwave synthesis following the standard protocol to obtain the (Pro<sup>32</sup>-Gly<sup>56</sup>)-(Et)Cys(Trt)-[Lys(Boc)<sub>2</sub>]-NH-resin. The mass analysis of the resin sample showed that the desired peptide with the palmitoyl group was obtained, however, the purity is low (See HPLC profile of Figure S2 a)), which might be due to the partial decomposition of the palmitoyl ester during the Fmoc group removal at high temperature. To reduce the decomposition, the synthesis was again performed as shown in Figure S2 b) and Figure 2, in which the Fmoc group removal was performed without microwave irradiation. As shown in the HPLC profile of Figure 2, the desired peptide was successfully obtained. After thioesterification by 3-mercaptopropionic acid, peptide **2** was obtained in 5.8% yield. The O-palmitoyl group was stable under the thioesterification conditions.



**Figure 2.** Synthetic route of palmitoylated peptide thioester **2**. The HPLC profile on the right shows the progress of the thioesterification by 3-mercaptopropionic acid at the NAC moiety. Elution conditions: column, Cosmosil 5C18 AR-II (4.6 x 150 mm, Nacalai Tesque, Kyoto) at the flow rate of 1 mL min<sup>-1</sup>; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA; temperature, 50 °C.

We also examined the post-chain assembly palmitoylation as shown in Figure 3. Starting from the NAC-resin, the peptide chain was elongated by the microwave synthesizer using the standard protocol, except for the Lys residues and Arg<sup>46</sup>-Ser<sup>47</sup>, which was manually introduced. Ser<sup>47</sup> was introduced using Fmoc-Ser(Trt)-OH. After the completion of the peptide chain assembly, the obtained resin was treated with 1% TFA / dichloromethane (DCM) to selectively remove the O-Trt group on Ser<sup>47</sup>. The resin was then treated with palmitic anhydride in the presence of 4-dimethylaminopyridine (DMAP) to introduce the palmitoyl group on Ser<sup>47</sup>. After the terminal Fmoc group was removed, the resin was treated with TFA to perform the peptide deprotection and resin detachment. The obtained crude peptide was then treated with 3-mercaptopropionic acid to perform the thioesterification. The S-Trt group at the NAC moiety would also be partially removed by the 1% TFA/DCM treatment and S-palmitoylated. However, during the thioesterification step, it would be cleaved and the free -SH group at the NAC moiety would be regenerated. As a result, we could successfully

obtain the desired palmitoylated peptide thioester **2** in 7.9% yield. This method is more convenient than the above method as the standard microwave protocol can be directly used.



**Figure 3.** Synthetic route of palmitoylated peptide thioester **2**. The HPLC profile on the right shows the progress of the thioesterification by 3-mercaptopropionic acid at the NAC moiety. Elution conditions: column, Cosmosil 5C18 AR-II (4.6 x 150 mm, Nacalai Tesque, Kyoto) at the flow rate of 1 mL min<sup>-1</sup>; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA; temperature, 50 °C.

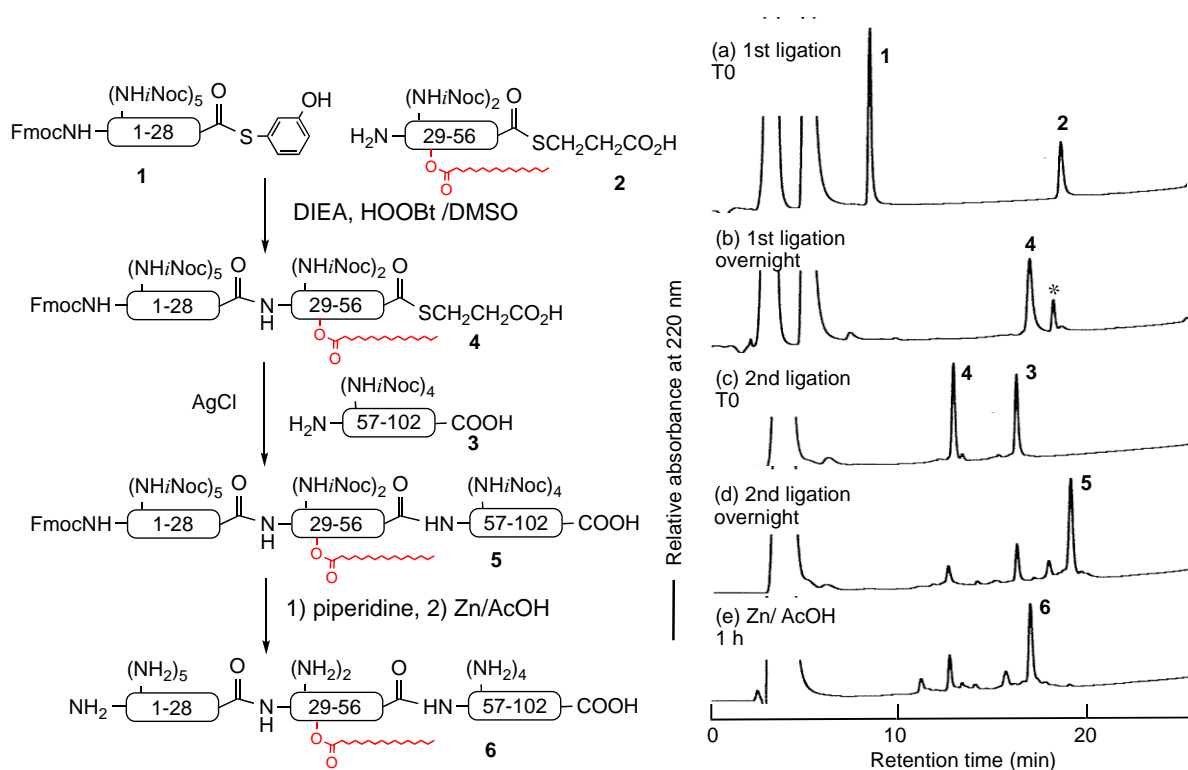
The synthesis of segment **1** was performed by the microwave-assisted Fmoc method starting from the NAC resin. After deprotection and thioesterification by 3-hydroxythiophenol, the desired aryl thioester was obtained. Segment **3** was also synthesized by the standard microwave-assisted Fmoc protocol starting from the Fmoc-Gly-CLEAR acid-resin.

Three segments were then condensed by the one-pot procedure shown in Figure 4. The peptide aryl thioester **1** and palmitoylated peptide alkyl thioester **2** were dissolved in dimethylsulfoxide (DMSO) containing 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HOObt), then DIEA was added to initiate the reaction. The reaction efficiently proceeded to give the peptide alkyl thioester **4**. Without purification, peptide **3** and silver ions were added to the mixture. The second reaction also efficiently proceeded to give the palmitoylated polypeptide **5**. The Fmoc group removal by piperidine and *i*Noc group removal by Zn in acetic acid treatment efficiently proceeded to give the desired palmitoylated histone H4 **6** in 20% overall yield through these reactions. Thus, we could successfully establish an efficient route for the O-palmitoylated protein.

## Conclusions

The O-palmitoyl group was unstable under the HF treatment conditions. On the other hand, the microwave-assisted Fmoc synthesis was successful, if the Fmoc deprotection was performed at room temperature. Post-chain assembly palmitoylation method was also efficient to obtain the desired palmitoylated peptide

thioester. The sequential one-pot ligation reaction by the thioester method allowed the efficient production of the Ser<sup>47</sup>-palmitoylated-histone H4. The functional study of the palmitoylated H4 is currently underway.



**Figure 4.** Synthetic route of Ser<sup>47</sup>-palmitoylated histone H4. HPLC profiles show the progress of the reaction. The asterisked peak in b) is derived from non-peptidic component. Elution conditions of a) and b): column, Cosmosil 5C18 AR-II (4.6 x 150 mm, Nacalai Tesque, Kyoto) at the flow rate of 1 mL min<sup>-1</sup>; eluent, A, 0.1% TFA, B, acetonitrile containing 0.1% TFA; temperature, 50 °C; gradient, 30% B to 70% B in 20 min. Elution conditions of c) to e): column, YMC Pack Protein RP (4.6 x 150 mm, YMC, Kyoto) at the flow rate of 1 mL min<sup>-1</sup>. Other conditions are the same.

## Experimental Section

**General.** Fmoc-(Et)Cys(Trt)-OH and Fmoc-Lys(*i*Noc)-OH were prepared by previously described methods.<sup>27,24</sup> The NMR spectra were recorded using an AV400 spectrometer (Bruker, MA). The chemical shifts are expressed in ppm downfield from the signal for the internal Me<sub>4</sub>Si in deuterated solvents. The ESI mass spectra were recorded using a LCQ DECA XP Plus (Thermo Fisher, MA). The microwave-assisted peptide synthesis was performed using Liberty Blue (CEM, NC) at 0.1 mmol scale. The standard protocol used the following conditions: 1) 10% piperazine, 0.1 M HOBt in 10% EtOH/NMP: 75 °C (15 sec), 90 °C (50 sec), 2) Fmoc-AA, DIC, HOBt in DMF: 75 °C (15 sec), 90 °C (110 sec). The Boc synthesis was performed by a 433A peptide synthesizer (Applied Biosystems) using the FastBoc protocol with modifications: 1) neat TFA 2min, 2) Boc-AA, HBTU, DIEA 12 min. The amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo) after hydrolysis with 6 M HCl at 180 °C for 25 min in an evacuated sealed tube. The content of the peptides in the powders was estimated based on the amino acid analysis.

### Peptide synthesis

**Peptide 1.** Fmoc-Rink amide MBHA-resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamidonorleucyl-4-methylbenzylhydrid-resin: Fmoc-NH-resin, 0.59 g, 0.2 mmol, 0.34 mmol/g) was treated with 20% piperidine in DCM (1 and 2 min) and 20% piperidine in DMF (2 min). After washing with DMF x4, the Fmoc-Lys(Boc)-OH (0.19 g, 0.40 mmol) activated by 0.45 M HBTU/DMF (0.87 mL, 0.39 mmol) and DIEA (0.14 mL, 0.81 mmol), was added and reacted for 12 min. The cycle was repeated to introduce another Lys residue. Fmoc-(Et)Cys(Trt)-OH (0.18 g, 0.3 mmol) was activated by DIC (62  $\mu$ L, 0.4 mmol) in the presence of HOBt (54 mg, 0.40 mmol) in 1,2-dichloroethane (DCE, 1.5 mL) and added to the resin, and the mixture was reacted for 30 min. After the Fmoc group removal, DMF solution of Fmoc-Gly-OH (0.30 g, 1.0 mmol), HATU (0.36 g, 0.95 mmol) and DIEA (0.31 mL, 1.8 mmol) was added to the resin and the mixture was stirred overnight to obtain the Fmoc-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin. Half of the resin (0.1 mmol) was removed and subjected to the automated synthesis by Liberty Blue using the standard protocol to obtain the Fmoc-Val-Leu-Arg(Pbf)-Asp(OBu<sup>t</sup>)-Asn(Trt)-Ile-Gln(Trt)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin. Using half of the obtained resin (0.05 mmol), peptide chain elongation was manually carried out using 0.2 mmol Fmoc amino acid activated by HBTU and DIEA. Lysine residues were introduced using Fmoc-Lys(*i*Noc)-OH (0.1 mmol) by HBTU-DIEA. After completion of the peptide chain assembly, the Fmoc-Ser(Bu<sup>t</sup>)-Gly-Arg(Pbf)-Gly-Lys(*i*Noc)-Gly-Gly-Lys(*i*Noc)-Gly-Leu-Gly-Lys(*i*Noc)-Gly-Gly-Ala-Lys(*i*Noc)-Arg(Pbf)-His(Trt)-Arg(Pbf)-Lys(*i*Noc)-Val-Leu-Arg(Pbf)-Asp(OBu<sup>t</sup>)-Asn(Trt)-Ile-Gln(Trt)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin was obtained (0.35 g). The resin (70 mg) was treated with 2.5% triisopropylsilane (TIS), 2.5% distilled water, and 2% 3,6-dioxa-1,8-octanedithiol (DODT) in TFA (0.7 mL) for 2 h. After filtration and concentration, the peptide was precipitated from ether and dried. The peptide was dissolved in 30% aq MeCN containing 5% AcOH, 5% *m*-hydroxythiophenol, 6 M urea (1.5 mL) and stored overnight at 37 °C. After 4 ether extractions, the solution was loaded on an HPLC column and the fraction containing the major peak was collected. Yield: 5.5 mg (1.4  $\mu$ mol, 14%). ESI mass, found: *m/z* 974.9, 1299.3 calcd for [M+4H]<sup>4+</sup>: 974.6, [M+3H]<sup>3+</sup>: 1299.1. Amino acid analysis: Asp<sub>2.00(2)</sub>Ser<sub>0.80(1)</sub>Glu<sub>1.07(1)</sub>Gly<sub>8.89(9)</sub>-Ala<sub>0.91(1)</sub>Val<sub>0.95(1)</sub>Ile<sub>0.93(1)</sub>Leu<sub>(2)</sub>Lys<sub>4.94(5)</sub>His<sub>1.01(1)</sub>Arg<sub>3.90(4)</sub>.

**Peptide 2. (1) 1st trial (Boc method).** The Fmoc-MBHA-resin (0.21 g, 0.10 mmol, 0.47 mmol/g) was treated with 20% piperidine in DCM (1 and 2 min) and 20% piperidine in DMF (2 min). After washing with DMF x4, Boc-Arg(Ts)-OH (0.21 g, 0.50 mmol), activated by 0.45 M HBTU/DMF (1.0 mL) and DIEA (87  $\mu$ L, 0.50 mmol), was added and reacted for 15 min. After washing with DMF, the resin was washed with TFA x2 and removal of the Boc group was performed with fresh TFA for 2 min. After washing with DMF, Boc-Arg(Ts)-OH (0.21 g, 0.50 mmol) and 0.45 M HBTU/DMF (1.0 mL) were reacted in the presence of DIEA (0.12 mL, 0.70 mmol). After 15 min, the resin was washed with DMF and the Boc group removal was performed in a similar manner. The resin was successively washed with 5% DIEA in DMF x2, DMF x5, DCM x3 and DCE x1. Boc-Gly-SCH<sub>2</sub>CH<sub>2</sub>COOH (79 mg, 0.3 mmol) was activated by Oxyma (64 mg, 0.45 mmol) and DIC (70  $\mu$ L, 0.45 mmol) and reacted overnight with the resin. The obtained Boc-Gly-SCH<sub>2</sub>CH<sub>2</sub>CO-[Arg(Ts)]<sub>2</sub>-NH-resin was subjected to automated synthesis by ABI 433A to obtain the Boc-Gly-Leu-Ile-Tyr(BrZ)-Glu(OBn)-Glu(OBn)-Thr(Bn)-Arg(Ts)-Gly-SCH<sub>2</sub>CH<sub>2</sub>CO-Arg(Ts)-Arg(Ts)-NH-resin. After the Boc group was removed in the same manner as described above, the resin was washed with 5% DIEA/DMF (x2), followed by DMF and DCM, Boc-Ser(Pal)-OH (0.13 g, 0.30 mmol), Oxyma (64 mg, 0.45 mmol), DIC (70  $\mu$ L, 0.45 mmol) in DCE was added and reacted for 2 h. After washing with DMF, Boc-Ile-OH, Boc-Arg(Ts)-OH and Boc-Lys(*i*Noc)-OH were manually introduced by HBTU/DMF and DIEA in a similar manner. The obtained resin was then subjected to the automated synthesis to obtain the Boc-Pro-Ala-Ile-Arg(Ts)-Arg(Ts)-Leu-Ala-Arg(Ts)-Arg(Ts)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Ts)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(BrZ)-Glu(OBn)-Glu(OBn)-Thr(Bn)-Arg(Ts)-Gly-SCH<sub>2</sub>CH<sub>2</sub>CO-Arg(Ts)-Arg(Ts)-NH-resin. Boc-Lys(*i*Noc)-OH, Boc-Thr(Bn)-OH and Boc-Ile-OH were then manually introduced to obtain the Boc-Ile-Thr(Bn)-Lys(*i*Noc)-Pro-Ala-Ile-Arg(Ts)-Arg(Ts)-Leu-Ala-Arg(Ts)-Arg(Ts)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Ts)-Ile-Ser(Pal)-Gly-Leu-

Ile-Tyr(BrZ)-Glu(OBn)-Glu(OBn)-Thr(Bn)-Arg(Ts)-Gly-SCH<sub>2</sub>CH<sub>2</sub>CO-Arg(Ts)-Arg(Ts)-NH-resin. A small amount of the resin was deprotected by 10% anisole in HF for 1.5 h at 0 °C and the obtained peptide was analyzed by HPLC. Peptide **2'**, which does not have a palmitoyl group at Ser<sup>47</sup> was obtained as the major product (Figure S1). ESI mass, found: *m/z* 768.9, 960.6, 1280.3 calcd for [M+5H]<sup>5+</sup>: 768.5, [M+4H]<sup>4+</sup>: 960.4, [M+3H]<sup>3+</sup>: 1280.2.

**(2) 2nd trial (Microwave-assisted Fmoc method by the standard protocol).** Half of the Fmoc-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin (0.1 mmol) obtained in the synthesis of peptide **1** was subjected to the microwave-assisted automated synthesis by Liberty Blue (CEM) at 0.1 mmol scale and Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin was obtained. Two-thirds of the resin (0.067 mmol) was reacted with Fmoc-Ser(Pal)-OH (75 mg, 0.13 mmol), HOBt (26 mg, 0.19 mmol) and DIC (30 μL, 0.19 mmol) in DCE (1.0 mL) for 30 min. Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(*i*Noc)-OH were also manually introduced by HBTU and DIEA to obtain the Fmoc-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin. One-eleventh of the resin was used for further chain elongation by Liberty Blue using the standard microwave protocol and the Fmoc-Pro-Ala-Ile-Arg(Pbf)-Arg(Pbf)-Leu-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin was obtained. A small amount of the resin was deprotected by 2.5% TIS, 2.5% DW, and 2% DODT in TFA and analyzed by HPLC. Any further chain elongation was abandoned.

**(3) 3rd trial (Microwave-assisted Fmoc method by the modified protocol).** Five-elevenths of the Fmoc-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin synthesized in the 2nd trial was subjected to the chain elongation by Liberty Blue. The standard protocol was modified so that the Fmoc deprotection was made by treating the resin with the same deprotection cocktail at room temperature for 1 min followed by 2-min treatment with the new cocktail. The obtained Fmoc-Pro-Ala-Ile-Arg(Pbf)-Arg(Pbf)-Leu-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin was further subjected to the manual synthesis to obtain the H-Ile-Thr-Lys(*i*Noc)-Pro-Ala-Ile-Arg(Pbf)-Arg(Pbf)-Leu-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Pal)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin (211 mg). A part of the resin (101 mg) was treated with 2.5% TIS, 2.5% distilled water and 2% DODT in TFA (1.2 mL) for 1.5 h. After filtration and concentration by a nitrogen stream, the residue was precipitated with ether and dried in vacuo. The residue was dissolved in 30% aq MeCN containing 6 M urea and 5% 3-mercaptopropionic acid (3 mL) and kept at 37 °C overnight. The product was purified by HPLC to obtain the peptide **2**. Yield: 3.2 mg (0.84 μmol, 5.8%). ESI mass, found: *m/z* 942.4, 1255.9 calcd for [M+4H]<sup>4+</sup>: 942.1, [M+3H]<sup>3+</sup>: 1255.9. Amino acid analysis: Thr<sub>1.61(2)</sub> Ser<sub>0.86(1)</sub> Glu<sub>2.12(2)</sub> Pro<sub>1.63(1)</sub> Gly<sub>4.08(4)</sub> Ala<sub>2.03(2)</sub> Val<sub>0.95(1)</sub> Ile<sub>3.66(4)</sub> Leu<sub>(2)</sub> Tyr<sub>1.00(1)</sub> Lys<sub>2.11(2)</sub> Arg<sub>6.20(6)</sub>.

**(4) 4<sup>th</sup> trial (on-resin palmitoylation method).** To one-third of the H-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin (0.033 mmol) synthesized in the second trial, Fmoc-Ser(Trt)-OH (0.14 g, 0.25 mmol) was manually introduced using 0.45 M HBTU/DMF (0.50 mL) and DIEA (87 μL, 0.5 mmol) for 12 min. Fmoc-Ile-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Lys(*i*Noc)-OH were also manually introduced by HBTU and DIEA to obtain the Fmoc-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Trt)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin. The entire resin was subjected to the automated synthesis by Liberty Blue, except for the three terminal amino acid residues, which were manually introduced, to obtain the H-Ile-Thr(Bu<sup>t</sup>)-Lys(*i*Noc)-Pro-Ala-Ile-Arg(Pbf)-Arg(Pbf)-Leu-Ala-Arg(Pbf)-Arg(Pbf)-Gly-Gly-Val-Lys(*i*Noc)-Arg(Pbf)-Ile-Ser(Trt)-Gly-Leu-Ile-Tyr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Arg(Pbf)-Gly-(Et)Cys(Trt)-[Lys(Boc)]<sub>2</sub>-NH-resin. Two-fifths of the resin was treated with 1% TFA in DCM 10 times. Palmitic acid (PalOH, 0.26 g, 1.0 mmol) and DIC (77 μL, 0.5 mmol) were dissolved in DCM (1 mL) and added to



the resin. DMAP (12 mg, 0.1 mmol) in 0.1 mL DMF was further added and the mixture was vortexed at room temperature for 6 h. After the resin was treated with 20% piperidine in DMF for 1 and 2 min, followed by successive washing with DMF, DCM, diethyl ether, the resin was dried to obtain 0.13 g of the peptide resin. The entire resin was treated with 2.5% TIS, distilled water, and 2% DODT in TFA (1.5 mL) for 1.5 h. After filtration and concentration by nitrogen stream, the residue was precipitated with ether and dried *in vacuo*. The residue was dissolved in 30% aq MeCN containing 6 M urea and 5% 3-mercaptopropionic acid (3 mL) and kept at 37 °C overnight. The product was purified by HPLC to obtain the peptide **2**. Yield: 4.1 mg (1.1 μmol, 7.9%). ESI mass, found:  $m/z$  942.6, 1256.1 calcd for  $[M+4H]^{4+}$ : 942.1,  $[M+3H]^{3+}$ : 1255.9. Amino acid analysis: Thr<sub>1.63(2)</sub>Ser<sub>0.85(1)</sub>Glu<sub>2.16(2)</sub>Pro<sub>1.26(1)</sub>Gly<sub>4.11(4)</sub>Ala<sub>2.07(2)</sub>Val<sub>0.97(1)</sub>Ile<sub>3.69(4)</sub>Leu<sub>(2)</sub>Tyr<sub>0.96(1)</sub>Lys<sub>2.05(2)</sub>Arg<sub>5.93(6)</sub>.

**Peptide 3.** Starting from the Fmoc-Gly-CLEAR-acid resin (270 mg, 0.10 mmol, 0.37 mmol/g), the peptide chain was elongated by Liberty Blue, except for the Lys residue, which was manually introduced using Fmoc-Lys(*i*Noc)-OH by HBTU and DIEA. Arg<sup>79</sup>, Leu<sup>58</sup> and Val<sup>57</sup> were also manually introduced using HBTU and DIEA. After completion of the peptide chain assembly, the H-Val-Leu-Lys(*i*Noc)-Val-Phe-Leu-Glu(OBu<sup>t</sup>)-Asn(Trt)-Val-Ile-Arg(Pbf)-Asp(OBu<sup>t</sup>)-Ala-Val-Thr(Bu<sup>t</sup>)-Tyr(Bu<sup>t</sup>)-Thr(Bu<sup>t</sup>)-Glu(OBu<sup>t</sup>)-His(Trt)-Ala-Lys(*i*Noc)-Arg(Pbf)-Lys(*i*Noc)-Thr(Bu<sup>t</sup>)-Val-Thr(Bu<sup>t</sup>)-Ala-Met-Asp(OBu<sup>t</sup>)-Val-Val-Tyr(Bu<sup>t</sup>)-Ala-Leu-Lys(*i*Noc)-Arg(Pbf)-Gln(Trt)-Gly-Arg(Pbf)-Thr(Bu<sup>t</sup>)-Leu-Tyr(Bu<sup>t</sup>)-Gly-Phe-Gly-Gly-CLEAR-acid resin (910 mg) was obtained. The 114 mg resin was treated with 2.5% TIS, 2.5% distilled water, and 1% 1,2-ethanedithiol in TFA (1.5 mL) for 2 h. After the TFA was removed by a nitrogen stream, the product was precipitate by ether and washed twice with ether. After dried *in vacuo*, the crude peptide was purified by HPLC to obtain the peptide **3**. Yield: 0.71 mg (0.12 μmol, 0.98%). ESI mass, found:  $m/z$  1153.1, 1440.4 calcd for  $[M+5H]^{5+}$ : 1152.3,  $[M+4H]^{4+}$ : 1440.2. Amino acid analysis: Asp<sub>3.14(3)</sub>Thr<sub>4.32(5)</sub>Glu<sub>3.26(3)</sub>Gly<sub>3.80(4)</sub>Ala<sub>4.21(4)</sub>Val<sub>6.30(7)</sub>Met<sub>0.90(1)</sub>Ile<sub>0.63(1)</sub>Leu<sub>(4)</sub>Tyr<sub>2.89(3)</sub>Phe<sub>1.90(2)</sub>Lys<sub>4.09(4)</sub>Tyr<sub>1.03(1)</sub>Arg<sub>4.17(4)</sub>.

### Synthesis of Ser<sup>47</sup>-palmitoylated histone H4 **6** by the thioester method

The aryl thioester **1** (88 nmol) and alkyl thioester **2** (73 nmol) were dissolved in DMSO (20 μL) containing HOObt (0.43 mg, 2.6 μmol) and DIEA (0.31 μL, 1.8 μmol) and stored overnight at room temperature in the dark. The peptide **3** (73 nmol), HOObt (0.43 mg, 2.6 μmol) and DIEA (0.31 μL, 1.8 μmol) in DMSO (20 μL) were added and a small portion of AgCl was added to initiate the second reaction. After an overnight reaction, AgCl was removed and DTT (0.15 mg, 1.0 μmol) was added. Piperidine (3.0 μL) was added and the solution was stored for 1 h at room temperature. The product was precipitated by ether and washed 3 times with the same solvent. The residue was dissolved in 50% aq MeCN and desalted by gel filtration (G3000PWXL, Tosoh, Tokyo) at the flow rate of 0.5 mL/min using 50% aq MeCN containing 0.1% TFA and lyophilized. The powder was dissolved in 50% AcOH, 15% MPA in distilled water containing 6 M guanidine hydrochloride (2.4 mL) and Zn powder (50 mg) were added and vortexed for 1 h. After filtration, the product was isolated by HPLC to give the palmitoylated histone H4 **6**. Yield: 0.16 mg (14 nmol, 20% based on peptide **3**). ESI mass, found:  $m/z$  766.4, 820.9, 883.9, 957.6, 1044.3, 1148.7, 1276.3, 1435.7, 1640.7 calcd for  $[M+15H]^{15+}$ : 766.0,  $[M+14H]^{14+}$ :  $[M+13H]^{13+}$ : 883.7,  $[M+12H]^{12+}$ : 957.2,  $[M+11H]^{11+}$ : 1044.2,  $[M+10H]^{10+}$ : 1148.5,  $[M+9H]^{9+}$ : 1276.0,  $[M+8H]^{8+}$ : 1435.3,  $[M+7H]^{7+}$ : 1640.2. Amino acid analysis: Asp<sub>5.05(5)</sub>Thr<sub>5.96(7)</sub>Ser<sub>1.91(2)</sub>Glu<sub>6.36(6)</sub>Pro<sub>1.19(1)</sub>Gly<sub>17.06(17)</sub>Ala<sub>7.27(7)</sub>Val<sub>7.96(9)</sub>Met<sub>0.59(1)</sub>Ile<sub>5.64(6)</sub>Leu<sub>(8)</sub>Tyr<sub>4.04(4)</sub>Phe<sub>2.32(2)</sub>Lys<sub>10.69(11)</sub>Tyr<sub>1.99(2)</sub>Arg<sub>13.67(14)</sub>.

**Boc-Ser(Pal)-OH.** Boc-Ser-OAll (0.49 g, 2.0 mmol) and Pal-OH (0.62 g, 2.4 mmol) were dissolved in DCM (6.0 mL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.54 g, 2.8 mmol) and DMAP (59 mg, 0.48 mmol) were added at room temperature. After one hour, the solvent was removed *in vacuo* and the residue was dissolved in ethyl acetate, successively washed with 0.5 M KHSO<sub>4</sub>, aq NaHCO<sub>3</sub>, brine and dried over Na<sub>2</sub>SO<sub>4</sub>. After filtration, the solvent was evaporated to give Boc-Ser(Pal)-OAll. Without further purification, the entire product was dissolved in THF (6.0 mL), then phenylsilane (0.37 mL, 0.3 mmol) and

$\text{Pd}(\text{PPh}_3)_4$  (10 mg) were added under nitrogen atmosphere. After the solution was stored for 30 min, the mixture was concentrated in vacuo and the residue was purified by silica gel column chromatography using chloroform-methanol (20:1) containing 1% acetic acid to obtain Boc-Ser(Pal)-OH (0.71 g, 1.6 mmol, 80%).  $[\alpha]_D^{+22.9}$  ( $\text{CHCl}_3$ , c 1.0). Anal. calcd for  $\text{C}_{24}\text{H}_{45}\text{NO}_6$ : C, 64.98; H, 10.22; N, 3.16. Found: C, 65.22; H, 10.37; N, 3.20.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.10 (d, 1H,  $J = 8.4$  Hz, -NH), 4.34 (dd, 1H,  $J=4.1, 10.8$  Hz,  $\beta\text{H}$ ), 4.28 (m, 1H,  $\alpha\text{H}$ ), 4.10 (dd, 1H,  $J=7.2, 10.7$  Hz,  $\beta\text{H}$ ), 2.26 (brt, 2H,  $J=7.3$  Hz,  $-\text{COCH}_2-$ ), 1.50 (m, 2H,  $-\text{COCH}_2\text{CH}_2-$ ), 1.39 (s, 9H,  $\text{C}(\text{CH}_3)_3$ ), 1.24 (brs, 24H,  $-\text{CH}_2-$ ), 0.85 (t, 3H,  $J = 6.6$  Hz,  $\text{CH}_3$ ).

**Fmoc-Ser(Pal)-OH.** Palmitic anhydride ( $\text{Pal}_2\text{O}$ ) was prepared by mixing Pal-OH (1.4 g, 5.4 mmol), DIC (0.42 mL, 2.7 mmol) in DCM (10 mL) for 10 min and the solvent was removed by evaporation. Fmoc-Ser-OAll (500 mg, 1.4 mmol) was dissolved in TFA (4 mL) and added to  $\text{Pal}_2\text{O}$ . After 15 min, the solvent was evaporated and the residue was dissolved in  $\text{CHCl}_3$ , washed with aq  $\text{NaHCO}_3$ , and brine then dried by  $\text{Na}_2\text{SO}_4$ . After evaporation, the residue was purified by silica gel chromatography using toluene-ethyl acetate (20:1), followed by chloroform-methanol (50:1) to obtain Fmoc-Ser(Pal)-OAll (0.68 g, 1.1 mmol, 80%).

Fmoc-Ser(Pal)-OAll (0.61 g, 1.0 mmol) was dissolved in THF (2.5 mL), then phenylsilane (0.14 mL, 1.1 mmol) was added.  $\text{Pd}(\text{PPh}_3)_4$  (5.0 mg) was added under a  $\text{N}_2$  stream and the mixture was stored for 1 h. After evaporation, the residue was dissolved in  $\text{CHCl}_3$  and purified by silica gel column chromatography using chloroform-methanol (50:1) containing 1% AcOH. The product was further purified by Bio-Beads SX-3 in toluene-ethyl acetate (3:1) to give Fmoc-Ser(Pal)-OH (0.55 g, 0.98 mmol, 98%).  $[\alpha]_D^{+11.2}$  ( $\text{CHCl}_3$ , c 0.98). Anal. calcd for  $\text{C}_{34}\text{H}_{47}\text{NO}_6$ : C, 72.18; H, 8.37; N, 2.48. Found: C, 72.17; H, 8.52; N, 2.45.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  7.87 (d, 2H,  $J = 7.5$  Hz, -Ar), 7.81 (d, 1H,  $J = 8.2$  Hz, -NH), 7.72 (d, 2H,  $J = 7.5$  Hz, -Ar), 7.40 (t, 2H,  $J = 7.2$  Hz, -Ar), 7.31 (t, 2H,  $J = 7.4$  Hz, -Ar), 4.41-4.35 (m, 2H,  $\alpha, \beta\text{H}$ ), 4.31-4.29 (m, 2H, Fmoc- $\text{CH}_2$ ), 4.26-4.18 (m, 2H, Fmoc-CH,  $\beta\text{H}$ ), 2.25 (t, 2H,  $J=6.9$  Hz,  $-\text{COCH}_2-$ ), 1.48 (m, 2H,  $-\text{COCH}_2\text{CH}_2-$ ), 1.26-1.16 (m, 24H,  $-\text{CH}_2-$ ), 0.83 (t, 3H,  $J=6.8$  Hz,  $\text{CH}_3$ ).

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## Supplementary Material

Trial synthetic routes of segment **2** by the Boc method (Figure S1) and by the Fmoc method using the standard and modified microwave method (Figure S2).

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