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# Chemical Synthesis of Native S-palmitoylated Membrane Proteins through Reversible Backbone Modification Assisted Ser/Thr Ligation

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**Abstract:** Preparation of native S-palmitoylated (S-palm) membrane proteins represents one of the unsolved challenges in chemical protein synthesis. Herein, we reported the first chemical synthesis of S-palm membrane proteins by reversible backbone modification assisted Ser/Thr Ligation (RBM<sup>GABA</sup>-assisted STL). This method involves two critical steps: (1) Synthesis of S-palm peptides by a new  $\gamma$ -aminobutyric acid group-based RBM (RBM<sup>GABA</sup>) strategy, (2) Ligation of the S-palm RBM-modified peptides to give the desired S-palm product by STL. The utility of RBM<sup>GABA</sup>-assisted STL method was demonstrated by the synthesis of rabbit S-palm sarcolipin (SLN) and the S-palm matrix-2 (M2) ion channel. The synthesis of S-palm membrane proteins highlights the importance of developing non-NCL methods for chemical protein synthesis.

## Introduction

Chemical protein synthesis provides access to preparation of custom-made proteins, such as site-specifically post-translationally modified proteins and mirror-image proteins for their biochemical, biophysical and pharmaceutical studies.<sup>1</sup> Native chemical ligation (NCL), in which a C-terminal peptide thioester reacts with an N-terminal Cys peptide to form a native amide linkage, is the most popular method for chemical protein synthesis.<sup>2</sup> NCL requires an N-terminal Cys to mediate ligation but the natural abundance of Cys is low (~1.8%). To expand the scope of NCL, a series of advanced versions of NCL have been developed for the ligation at non-Cys residues, such as Ala, Lys, Val, Ser and Thr.<sup>3</sup> On the other hand, some non-NCL methods<sup>4</sup> have also been developed, such as serine/threonine ligation (STL)<sup>4b,4c</sup> and  $\alpha$ -keto acid-hydroxylamine (KAHA) ligation<sup>4d,4e</sup>, to enable peptide ligation at non-Cys sites. These non-NCL methods have been used to

synthesize a number of proteins<sup>5</sup> (e.g. betatrophin, interleukin 25, and HMGA1a) which, however, can in principle also be obtained by NCL-based methods. Examples remain to be demonstrated where the non-NCL methods must be used to accomplish the chemical synthesis of certain practically important proteins, and one such case would be S-palmitoylated proteins.

S-palmitoylation refers to the covalent modification of palmitic acyl onto a Cys residue of protein substrates via a thioester bond. This modification is abundant in hydrophobic membrane proteins, and plays key roles in many biological processes such as, protein localization, signal transduction, immune response, and cell apoptosis.<sup>6</sup> Dysfunction of S-palmitoylation can cause neurological diseases, immune diseases or cancers. For example, depalmitoylation of melanocortin-1 receptor (MC1R) stimulates melanogenesis.<sup>7</sup> S-palmitoylation on programmed death ligand-1 (PD-L1) promotes tumor growth.<sup>8</sup> Structure-function relationships of S-palmitoylated membrane proteins need to be studied to decipher the role of S-palmitoylation and facilitate drug discovery.<sup>9</sup> Additionally, S-palmitoylated membrane protein probes are requisite tools for screening of substrate-specific depalmitoylase inhibitors.<sup>6a,10</sup> In this context, it is important to produce homogeneous samples of S-palmitoylated membrane proteins in workable quantities. Recent studies showed that the commonly used solid-phase peptide synthesis (SPPS) can only afford short S-palmitoylated peptides.<sup>11</sup> On the other hand, although conjugation methods (e.g. maleimidocaproyl conjugation, Diels-Alder ligation) were used to generate some S-palmitoylated protein analogues, the products contained non-native structural motifs.<sup>12</sup> To date, there has been no study on the preparation of native S-palmitoylated membrane proteins by chemical ligation methods.<sup>13</sup>

Herein, we reported the first chemical synthesis of native S-palmitoylated membrane proteins using a newly developed removable backbone modification assisted serine/threonine ligation (RBM<sup>GABA</sup>-assisted STL) strategy. This work demonstrates the importance of integrating different modern techniques to overcome the increasingly more difficult challenges in chemical protein synthesis. A critical problem encountered in our study is the incompatibility between the previous RBM method and S-palmitoylation as well as STL ligation. To meet this challenge, a new auto-cyclization  $\gamma$ -aminobutyric acid (GABA)-based RBM (RBM<sup>GABA</sup>) strategy was developed and found to be critical to the success of RBM<sup>GABA</sup>-assisted STL ligation (**Scheme 1**). The effectiveness of RBM<sup>GABA</sup>-assisted STL was examined through the synthesis of two practically important native S-palmitoylated membrane proteins, namely, rabbit S-palmitoylated sarcolipin (S-palm SLN) and S-palmitoylated matrix-2 (S-palm M2) ion channel from Influenza A virus. Thus, our work provides examples where non-NCL methods play an irreplaceable role in the chemical synthesis of some important protein targets.

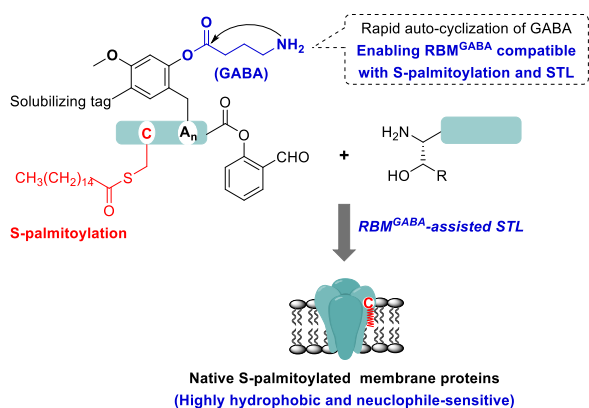
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**Scheme 1.** The RBM<sup>GABA</sup>-assisted STL strategy for chemical synthesis of native S-palmitoylated membrane proteins. Parts of the membrane protein are represented by blue frames. A<sub>n</sub> is any primary amino acid of the membrane protein sequence. R represents H or CH<sub>3</sub> group.

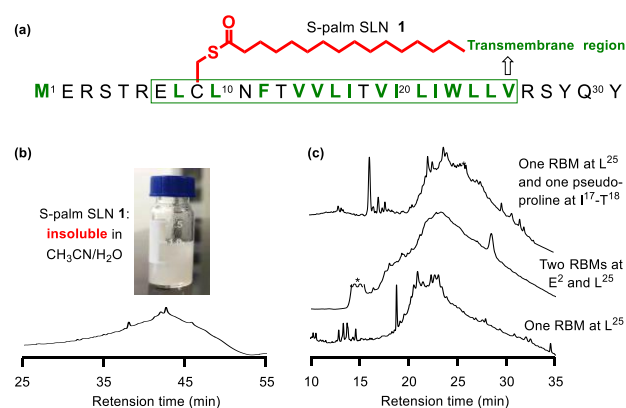
## Results and Discussion

### Chemical synthesis of S-palm SLN by Fmoc SPPS

Our work started with the synthesis of S-palm SLN, a 31-residue single transmembrane protein, for its biochemical and biophysical studies (Fig. 1a).<sup>14</sup> SLN regulates Ca<sup>2+</sup> transport activity of SERCA1a to control the intracellular calcium homeostasis and muscle contraction/relaxation. Dysfunction of SLN results in non-shivering thermogenesis and heart failure and therefore, SLN is considered as a potential drug target.<sup>15</sup> N- and C-terminus of SLN are located within the cytosol and the lumen respectively.<sup>16</sup> Recently, native rabbit SLN was found to be S-palmitoylated on Cys9 and it was suggested that S-palmitoylation/depalmitoylation of SLN might regulate SERCA1a activity.<sup>17</sup> To understand the detailed regulatory mechanism of S-palm SLN on SERCA1a activity at a molecular level, the S-palm SLN sample is required to have available isoforms from the same species and control of the stoichiometry during catalysis.<sup>18</sup>

To prepare the full-length S-palm SLN 1, we initially employed direct Fmoc solid-phase peptide synthesis (SPPS). In the SPPS, the 4-monomethoxytrityl (Mmt) protected Cys9 was installed into the peptide. After peptide assembly, the Mmt group in the resin-bound peptide precursor was selectively deprotected by treating with 2% trifluoroacetic acid (TFA) cocktails to give a free thiol.<sup>19</sup> The released thiol then reacted with palmitic anhydride to generate S-palm SLN 1. Unfortunately, the crude peptide 1 was found to be insoluble and no target product was detected on the reverse-phase high-performance liquid chromatography (RP-HPLC) profile (Fig. 1b). We also examined other previously developed strategies<sup>20</sup> (such as NMP/DMF co-solvents, microwave, pseudoproline, O-acyl isopeptide and attachment of solubilizing tags into peptide side chains) to improve the synthetic efficiency. However, no significant improvement was observed on RP-HPLC (Supplementary Fig. S1), presumably due to the strong hydrophobicity of 1.

One effective solution to improve the solubility of very poorly-soluble proteins is our removable backbone modification (RBM) strategy which enables installation of RBM groups into backbone amides and increases the solubility by both addition of solubilizing tag and disruption of aggregation to effectively facilitate the chemical synthesis of a number of membrane proteins.<sup>21</sup> Using the RBM strategy, we synthesized the S-palm SLN(L<sup>25,RBM</sup>,C<sup>9,Palm</sup>) 2



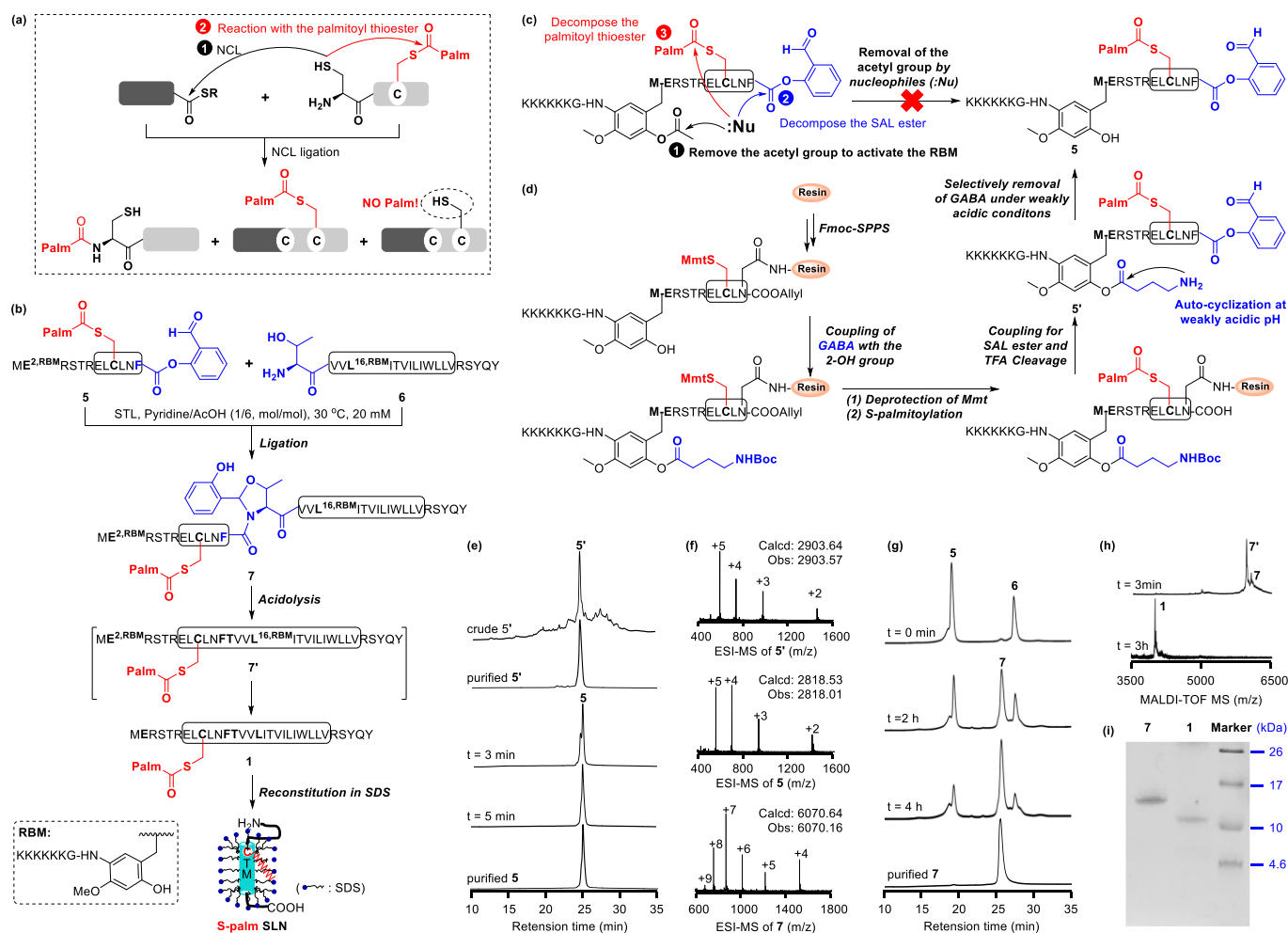
**Figure 1.** The synthesis of the full-length S-palm SLN by Fmoc SPPS. (a) Amino acid sequence of S-palm SLN. The hydrophobic residues are colored in green. (b) Solubility and HPLC profile of the crude native S-palm SLN 1 in 50% CH<sub>3</sub>CN/H<sub>2</sub>O. (c) HPLC traces of crude RBM-modified S-palm SLN samples synthesized by different methods. The non-peptide peak was highlighted with \*.

with a RBM tag at Leu25 using the reported protocol.<sup>22</sup> The crude RBM-modified peptide was found to be well soluble, but its crude RP-HPLC profile was too complex to identify any target peptide (Fig. 1c). To improve the purity of crude S-palm SLN, we tried many synthetic protocols (for example, NMP/DMF co-solvents, increasing the temperature, microwave, pseudoproline, changing the RBM's installing site and installing two RBM tags) during the peptide assembly, but there was no obvious improvement (Fig. 1c and Supplementary Fig. S3). These results indicated that the desired full-length RBM-modified S-palm SLN cannot be directly obtained by Fmoc SPPS. This may be attributed to the fact that SLN contains lots of sterically hindered and hydrophobic amino acids, which makes coupling/deprotection steps incomplete during peptide assembly. In addition, S-palmitoylation further increases the synthetic complexity.<sup>11</sup>

Overall, our attempts to synthesize S-Palm SLN showed that this family of proteins is extraordinarily difficult to produce and therefore, posing an interesting and practically important challenge for modern chemical protein synthesis.

### Development of the RBM<sup>GABA</sup>-assisted STL method for the chemical synthesis of S-palm SLN

After failure of direct synthesis of S-palm SLN by Fmoc SPPS, we turned to peptide ligation. However, the previous work<sup>23</sup> and our model NCL ligation of S-palmitoylated peptides (Supplementary Fig. S11–S13) showed that the S-palmitoylated peptide contains a reactive S-palmitoyl thioester group which is labile towards the N-terminal Cys of peptides in NCL, making the most popular NCL incompatible with S-palmitoylation (Fig. 2a). Therefore, the non-NCL ligation methods must be used for the synthesis of S-palm SLN, among which we chose STL ligation. In STL, an N-terminal Ser/Thr peptide and a C-terminal salicylaldehyde (SAL) ester peptide can undergo a chemoselective capture and a subsequent O-to-N acyl transfer to afford an N,O-benzylidene acetal-linked intermediate, which can be quantitatively converted to a native amide bond upon acidolysis.<sup>4c</sup> We hypothesized that the SAL ester and Ser/Thr groups would be compatible with S-palmitoylation. To implement the idea, S-palm SLN was divided into two peptides, a S-palmitoylated SAL ester peptide SLN(1–12,C<sup>9,Palm</sup>)-SAL 3 and an N-terminal Thr peptide



**Figure 2.** Chemical synthesis of S-palm SLN by RBM<sup>GABA</sup>-based STL method. (a) The incompatibility of S-palmitoylation with NCL. (b) The RBM<sup>GABA</sup>-based STL method for the synthesis of 1. (c) The incompatibility of S-palmitoylation with the previous RBM strategy. (d) The synthesis of RBM-modified S-palmitoylated 5 by the new RBM<sup>GABA</sup> strategy. (e) HPLC traces of the crude S-palmitoylated peptide 5' and the removal of GABA group of 5' to obtain 5. (f) ESI-MS data of S-palmitoylated peptides 5', 5 and the ligation product 7. (g) HPLC traces of STL between 5 and 6 to afford 7. (h) MALDI-TOF MS traces of the cleavage of RBM tags of 7 to give 1. (i) Tricine-SDS-PAGE of the cleavage of RBM tags of 7 to give 1.

SLN(13-31) 4. We tried to prepare peptide 3 and 4 through Fmoc SPPS. However, even though peptide 3 could be detected by analytical RP-HPLC, its isolated yield (<1%) was very low. Even worse, the other peptide 4 could not be detected in the crude peptide that was a complex mixture of insoluble materials.

To solve the problem, we proposed to use the strategies of incorporation of a solubilizing tag into peptide side chains or C-/N-terminus<sup>20</sup>, such as Lys-based Ddae-type tag<sup>20b</sup> and Cys-based Trt-/Phacm-type tag<sup>20c,20d</sup>. However, most of these strategies cannot be employed for the synthesis of S-palm SLN due to the lack of amino acid sites to install tags (no additional Cys and Lys) or the incompatibility of S-palmitoylation due to the use of nucleophilic conditions (e.g. high pH, heavy metals and thiol reagents) in the final tag's removal step. Therefore, we decided to use the RBM strategy and install RBM tags into the S-palmitoylated SAL ester peptide SLN(1-12, E<sup>2, RBM</sup>, C<sup>9, Palm</sup>)-SAL 5 and Thr peptide SLN(13-31, L<sup>16, RBM</sup>) 6 at Glu2 and Leu16, respectively (Fig. 2b). The acetylated peptide precursor SLN(1-12, E<sup>2, RBM-OAc</sup>, C<sup>9, Palm</sup>)-SAL 5'' was obtained smoothly, but we soon realized that the activation of RBM through our previous de-acylation strategy could not be accomplished due to the presence of both S-palmitoyl thioester and SAL ester groups (Fig. 2c). Thus, a critical challenge for the development of RBM-assisted STL is how to overcome the

compatibility problem between the RBM method and S-palmitoylation as well as STL.

To develop an effective method of RBM-assisted STL, we screened different designs by using intramolecular cyclization for deprotection. For example, we tested our previously used N-methyl-N-[2-(methylamino)ethyl]carbamoyl group, which was reported to undergo auto-cyclization at weakly alkaline pH.<sup>21a</sup> However, the test results showed that the deprotection conditions of this group caused serious hydrolysis of S-palmitoylation (Supplementary Fig. S14). After several trials, we turned to a previous finding of the remarkable self-cyclization property of the amino carboxyl ester structures under weakly acidic reaction conditions<sup>24</sup>. Thus the  $\gamma$ -aminobutyric acid (GABA) group was developed to mask the 2-OH group in RBM to ensure its stability towards TFA used in SPPS (Supplementary Fig. S16-S19). As shown in Fig. 2d, GABA was coupled with the phenolic hydroxyl group to give the phenolic ester on RBM. Subsequent S-palmitoylation and coupling of SAL ester were successful. More importantly, after TFA cleavage, the target peptide SLN(1-12, E<sup>2, RBM-OGABA</sup>, C<sup>9, Palm</sup>)-SAL 5' was obtained as a main peak from the RP-HPLC (Fig. 2e). 5' was characterized by ESI-MS and purified by semi-preparative RP-HPLC using the water-acetonitrile mobile phase with an isolated yield of 11 %.

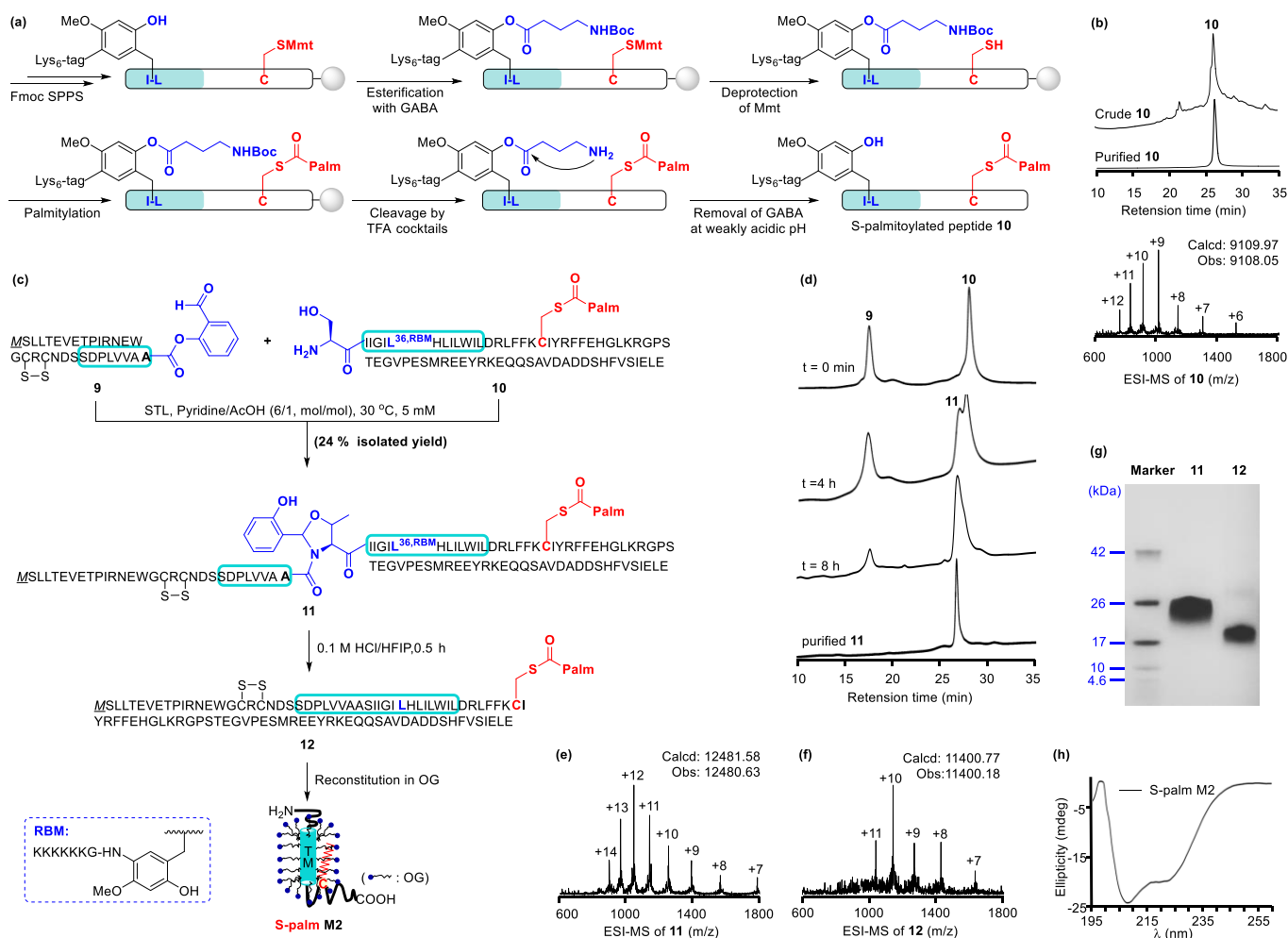
Next, we tested the critical step of RBM activation by the removal of the acyl group at its 2-OH site. Thus, purified **5'** was dissolved into pyridine/AcOH (7/6, mol/mol) conditions that was needed for carrying out STL. The RP-HPLC traces showed that the auto-cyclization was smooth under the weakly acidic conditions of STL and could be completed within 5 min to give the target peptide **5** in a 77% isolated yield (Fig. 2e). The results showed that the GABA-based RBM strategy was fully compatible with S-palmitoyl and SAL ester groups and could be activated in the special reaction conditions of STL (namely pyridine/AcOH) (Supplementary Fig. S20-23). Furthermore, using the same RBM<sup>GABA</sup> strategy, the other RBM-modified peptide **6** was also easily obtained with a 21% isolated yield (Supplementary Fig. S27).

The STL ligation of **5** (11.2 mg) and **6** (13.0 mg) was then carried out in 0.2 mL of pyridine/acetic acid buffer solution (1/6, mol/mol) at 30 °C. Analytical HPLC traces showed that the reaction took place smoothly within 4 h to give the *N,O*-benzylidene acetal intermediate **7** (Fig. 2g). The product **7** was purified by RP-HPLC (8.5 mg, 35% isolated yield) and characterized by ESI-MS (Fig. 2f). Finally, the purified peptide **7** was dissolved in 0.1 M HCl/hexafluoro-isopropanol (HFIP) containing 1% TIPS at room temperature, an optimized cleavage condition (Supplementary Fig. S29), to remove the *N,O*-benzylidene acetal and two RBM tags.<sup>25</sup> The final product **1** could not be monitored by RP-HPLC because of its strong hydrophobicity. Nonetheless, the matrix-

assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) traces showed that the *N,O*-benzylidene acetal group of **7** was removed to afford **7'** within 5 min and the two RBM tags were quantitatively cleaved within 3 h (Fig. 2h). The removal of the backbone modification groups was also characterized through the tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) analysis, which gave an expected single band shift before and after acidolysis treatment (Fig. 2i). After that, the diluted HCl solution was concentrated by N<sub>2</sub> blowing, and then precipitated by cold diethyl ether to afford the final S-palm SLN **1** with a 55% yield. The synthetic S-palm SLN was refolded in 10 mM Tris-HCl, pH 7.5, 4% (w/v) sodium dodecyl sulfate (SDS) at 25 °C and the circular dichroism (CD) spectrum showed two minima at 208 and 222 nm, indicating the  $\alpha$ -helical structuration (Supplementary Fig. S30).

### Chemical synthesis of S-palm M2 by RBM<sup>GABA</sup>-assisted STL

To further demonstrate the utility of the RBM<sup>GABA</sup>-based STL strategy, we synthesized S-palmitoylated M2 ion channel from influenza A virus. The 97-residue M2 can form homotetramers to transport protons across the viral envelope. It plays critical roles during the virus entry, replication and assembly of infectious virus particles. M2's functions are regulated by several posttranslational



**Figure 3.** Chemical synthesis and characterizations of the Cys50-palmitoylated M2 by the RBM<sup>GABA</sup>-assisted STL method. (a) The synthesis of S-palmitoylated **10** by RBM<sup>GABA</sup> strategy. M indicates a replacement of Met with Nle. (b) HPLC traces of crude and purified peptide **10** (up) and ESI-MS of **10** (down). (c) The synthetic route by RBM<sup>GABA</sup>-assisted STL. (d) HPLC traces of ligation of **9** and **10**. (e) ESI-MS of **11**. (f) ESI-MS of **12**. (g) Tricine-SDS-PAGE of **11** and **12**. (h) CD analysis of synthetic S-palm M2 in 50 mM n-octyl-β-D-glucopyranoside (OG) vesicles.

modifications, such as phosphorylation, ubiquitination and S-palmitoylation.<sup>26</sup> Recently, phosphorylated M2 has been chemically synthesized to investigate the role of phosphorylation on M2's functions.<sup>21a</sup> The synthesis of S-palmitoylated M2 would also provide unique research tools for the study of the role of M2's S-palmitoylation, whereas the reported NCL-based method for phosphorylated M2 cannot be used to prepare S-palmitoylated M2 owing to the incompatibility of NCL with S-palmitoylation.

The RBM<sup>GABA</sup>-assisted STL method was applied to the preparation of Cys50-palmitoylated M2 (S-palm M2). As shown in **Fig. 3c**, S-palm M2 was divided into two segments, M2(1-30)-SAL **9** and M2(31-97, L<sup>36,RBM</sup>, C<sup>50,palm</sup>) **10**. According to the previous work<sup>5c</sup>, peptide **9** was prepared by using Fmoc SPPS, in which Met was mutated to Norleucine to avoid the possible oxidation during the peptide synthesis.<sup>5e</sup> On the other hand, the segment M2(31-97, C<sup>50,palm</sup>) without RBM modification could not be prepared by Fmoc SPPS owing to poor handling property (**Supplementary Fig. S32**). When the previous acetyl-based RBM method was used, the RBM-modified peptide **10** could not be obtained because of the decomposition of S-palmitoyl groups during the removal of acetyl groups. When the new RBM<sup>GABA</sup> strategy was used, peptide **10** was obtained in 15% isolated yield (**Fig. 3a**). The purified peptide **10** was well soluble and characterized by RP-HPLC and ESI-MS (**Fig. 3b**). The two segments were subjected to STL ligation in pyridine/AcOH to produce the *N,O*-benzylidene acetal intermediate **11** in 24% isolated yield (**Fig. 3d**). The ligation intermediate **11** was well soluble and could be readily purified by RP-HPLC and verified by ESI-MS (**Fig. 3e**). Finally, the purified **11** powder was dissolved into 0.1 M HCl/TFIP containing 1% TIPS at room temperature to remove the backbone modifications. The solution was concentrated by N<sub>2</sub> blowing, and then precipitated with chilled diethyl ether to afford the target S-palm M2 **12**, which was successfully characterized by ESI-MS and Tricine-SDS-PAGE analyses, both indicating that the desired product was obtained (**Fig. 3f, 4g**). In addition, the CD spectrum of S-palm M2 in the presence of 50mM *n*-octyl- $\beta$ -D-glucopyranoside (OG) showed characteristic negative bands at 208 and 222 nm (**Fig. 3h**), suggesting the folded S-palm M2 with  $\alpha$ -helical structures.

## Conclusion

In summary, in the present study we show that preparation of S-palmitoylated membrane proteins poses an important, but very challenging problem for modern chemical protein synthesis. To meet the challenge, we developed a RBM<sup>GABA</sup>-assisted STL ligation method, which exploits the solubilizing effect of RBM groups and the compatibility of STL ligation with S-palmitoylation. Key to the success of the new method is the development of a new  $\gamma$ -aminobutyric acid (GABA) group in RBM, whose rapid auto-cyclization property enables its selective activation and removal in the presence of S-palmitoylation under the STL ligation conditions (pyridine/AcOH). The effectiveness of RBM<sup>GABA</sup>-assisted STL strategy was demonstrated by the successful synthesis of two S-palmitoylated membrane proteins, namely, sarcolipin and M2. Access to native S-palmitoylated proteins paves a way to their biochemistry, biophysics, and pharmaceuticals studies. Finally, the synthesis of S-palmitoylated membrane proteins provides examples to show the importance of developing non-NCL methods for chemical protein synthesis.

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** Chemical protein synthesis • Serine/threonine ligation • Removable backbone modification • S-palmitoylation • Membrane proteins

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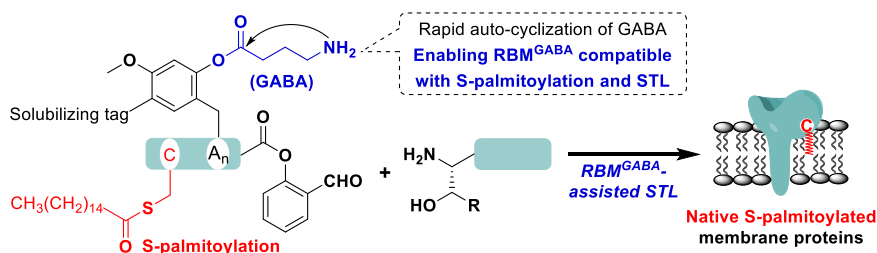
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## Chemical Protein Synthesis

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### Chemical Synthesis of Native S-palmitoylated Membrane Proteins through Reversible Backbone Modification Assisted Ser/Thr Ligation



RBM<sup>GABA</sup>-assisted STL strategy (removable backbone modification-assisted serine/threonine ligation) was developed for the chemical synthesis of native S-palmitoylated membrane proteins. The successful synthesis of S-palmitoylated membrane proteins (e.g. S-palmitoylated sarcosolipin and M2) for the first time demonstrated the importance of non-NCL methods in chemical protein synthesis.