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Robust Chemical Synthesis of Membrane Proteins through a General Method of Removable Backbone Modification

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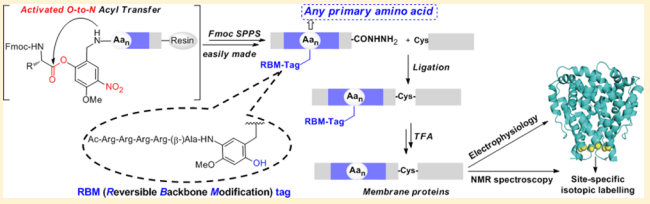
Supporting Information

ABSTRACT: Chemical protein synthesis can provide access to proteins with post-translational modifications or site-specific labelings. Although this technology is finding increasing applications in the studies of water-soluble globular proteins, chemical synthesis of membrane proteins remains elusive. In this report, a general and robust removable backbone modification (RBM) method is developed for the chemical synthesis of membrane proteins. This method uses an activated *O*-to-*N* acyl transfer auxiliary to install in the Fmoc solid-phase peptide synthesis process a RBM group with switchable reactivity toward trifluoroacetic acid. The method can be applied to versatile membrane proteins because the RBM group can be placed at any primary amino acid. With RBM, the membrane proteins and their segments behave almost as if they were water-soluble peptides and can be easily handled in the process of ligation, purification, and mass characterizations. After the full-length protein is assembled, the RBM group can be readily removed by trifluoroacetic acid. The efficiency and usefulness of the new method has been demonstrated by the successful synthesis of a two-transmembrane-domain protein (HCV p7 ion channel) with site-specific isotopic labeling and a four-transmembrane-domain protein (multidrug resistance transporter EmrE). This method enables practical synthesis of small- to medium-sized membrane proteins or membrane protein domains for biochemical and biophysical studies.



INTRODUCTION

Chemical protein synthesis may enable the preparation of proteins with predesigned structures at atomic precision, thus permitting the acquisition of otherwise difficult-to-obtain proteins bearing either post-translational modifications or site-specific labelings for advanced studies.[1] Thanks to the important work of many research groups, the technology for the synthesis of water-soluble globular proteins has been well-developed and applied to biochemical and pharmaceutical studies.[2−4] For instance, a number of glycoproteins have been made to study the effects of carbohydrates on their pharmaceutical potency.[3] Many modified histone proteins have been synthesized to investigate the chromatin regulatory mechanisms.[4] Notwithstanding these advances, the highly important class of membrane proteins such as multiple membrane-spanning ion channels and drug transporters remains elusive with regard to chemical synthesis.[5]

The difficulty of membrane protein synthesis stems from the hydrophobic nature of their membrane-spanning sequences, which causes severe handling troubles during the ligation and purification steps.[6] Several methods have been tested to overcome the problem, for instance, use of organic solvents or detergents,[7] attachment of a solubilizing tag at peptide terminals,[8] and introduction of an *O*-acyl isopeptide.[9] The range of membrane proteins accessible via the above methods still remains limited, and each synthesis involves time-consuming optimization. An important proposition by Kent et al. is that the poor handling properties of transmembrane peptides mainly comes from their high tendency to form aggregation-prone secondary structures (α helices or β sheets).[10] On the basis of this theory, we recently proposed to put a removable backbone modification (RBM) group in the transmembrane regions to disrupt their secondary structure formation (Scheme 1).[11] We were delighted to find that with RBM the transmembrane peptides behaved almost as if they were water-soluble peptides with regard to ligation, purification, and mass characterization. Thus, RBM may offer a practical solution to the problem of membrane protein synthesis. A limitation of our previous study is that the RBM can only be placed at Gly. This poses a critical question as how to develop a general method that allows the installation of RBM at other amino acids.

In this work we report a general and robust RBM method to overcome the above challenge. The present RBM can be placed at any primary amino acid so that it can be applied to the

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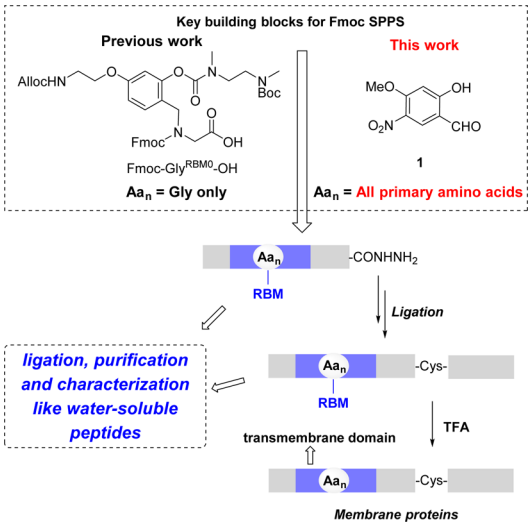
Scheme 1. New versus Previous RBM Methods



synthesis of membrane proteins without limitation regarding the sequence (Scheme 1a). The usefulness of the new RBM method has been demonstrated by the successful synthesis of a two-transmembrane-domain ion channel of hepatitis C virus (HCV) p7, which has site-specific isotopic labelings to enable the study of its binding interactions with ligands. Moreover, using the new method we have accomplished the synthesis of functionally active multidrug resistance efflux transporter EmrE from *Escherichia coli*, which represents the first total synthesis of a membrane protein containing four transmembrane helices. The method enables access to uncontaminated, labeled, or post-translationally modified membrane proteins or their domains. It is also applicable to the synthesis of globular proteins containing difficult hydrophobic segments.[12]

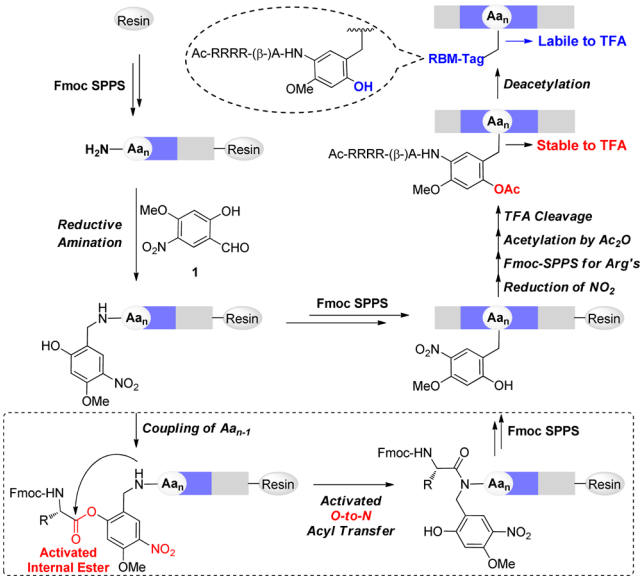
RESULTS AND DISCUSSION

Design and Performance with Model Peptides. The basic idea of the RBM strategy is the installation of polyarginine-tagged groups at amide backbone positions in the transmembrane domains. RBM can disrupt the formation of secondary structures (α helics or β sheet), thus avoiding poor solubility due to self-assembly and aggregation.[10,11] Besides, the attachment of positively charged Arg tags offers an additional driving force to improve the solubility in aqueous buffers.[8,11] A key demand of the RBM method is that the RBM group must stay stable on the transmembrane peptides during all the purification and ligation steps. Only after the entire protein sequence is obtained and purified, the RBM needs to be quantitatively removed, preferentially by trifluoroacetic acid (TFA), to afford the target native membrane protein that usually is well soluble in TFA.

In our previous work, we used a premade *N*-modified Gly (i.e., Fmoc-Gly^{RBM0}-OH, Scheme 1) to introduce RBM at Gly. This method cannot be applied to other amino acids because that will involve the peptide coupling of a sterically crowded secondary amine where the coupling efficiency can decrease to unacceptable levels. To expand the scope of the RBM strategy to versatile membrane proteins, we need to have a practical approach to incorporate the RBM group at general amino acids.

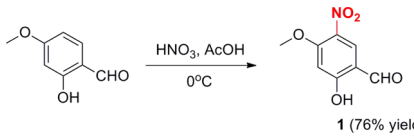
Our new strategy was inspired by the elegant method of activated *O*-to-*N* acyl transfer pioneered by Offer, Johnson, and Alewood.[13] In this method, the sterically crowded amide bond was constructed not through direct amide coupling, but instead, an activated phenyl ester was formed first, followed by a facile intramolecular *O*-to-*N* acyl transfer to produce the peptide backbone. We recognized that the NO_2 in the acyl transfer auxiliary not only enables the *O*-to-*N* acyl transfer but also can be reduced to NH_2 to allow installation of the Arg tag (Scheme 2). After a change of electronic property of the phenyl ring due to NO_2 reduction, the phenol OH acquires an interesting property. When the OH was acylated, the RBM group can well survive the TFA cleavage step. When the OH was free, the RBM group can be easily and quantitatively removed by using 95% TFA.

Scheme 2. Fmoc SPPS of the New RBM-Containing Peptides



To implement the above idea, we synthesized 4-methoxy-5-nitrosalicylaldehyde **1** from commercial materials (yield = 76%, Scheme 3).[15] With **1** in hand, we tested the synthesis of model peptide Ac-LAIK$^{RBM(1R)}$FERG-OH **2** (Figure 1a). We loaded Fmoc-Gly-OH onto 2-Cl-Trt-Cl resin and carried out standard Fmoc SPPS (solid-phase peptide synthesis) for the next four residues. Through a well-documented reductive amination protocol,[13] **1** was readily installed onto the α-NH_2 of Lys on solid phase. The attachment of the next amino acid (Ile) was accomplished through the formation of a phenyl ester first, followed by an automatic intramolecular *O*-to-*N* acyl transfer. Subsequent peptide synthesis still followed the standard Fmoc SPPS. After the full peptide was assembled, the NO_2 in the RBM was reduced by using $SnCl_2$ on solid phase.[16] The resulting NH_2 group was attached with an Arg tag through Fmoc SPPS. Next, the phenol OH of the RBM group was acetylated with acetic anhydride. Finally, TFA cleavage was

Scheme 3. Synthesis of Salicylaldehyde Derivative 1



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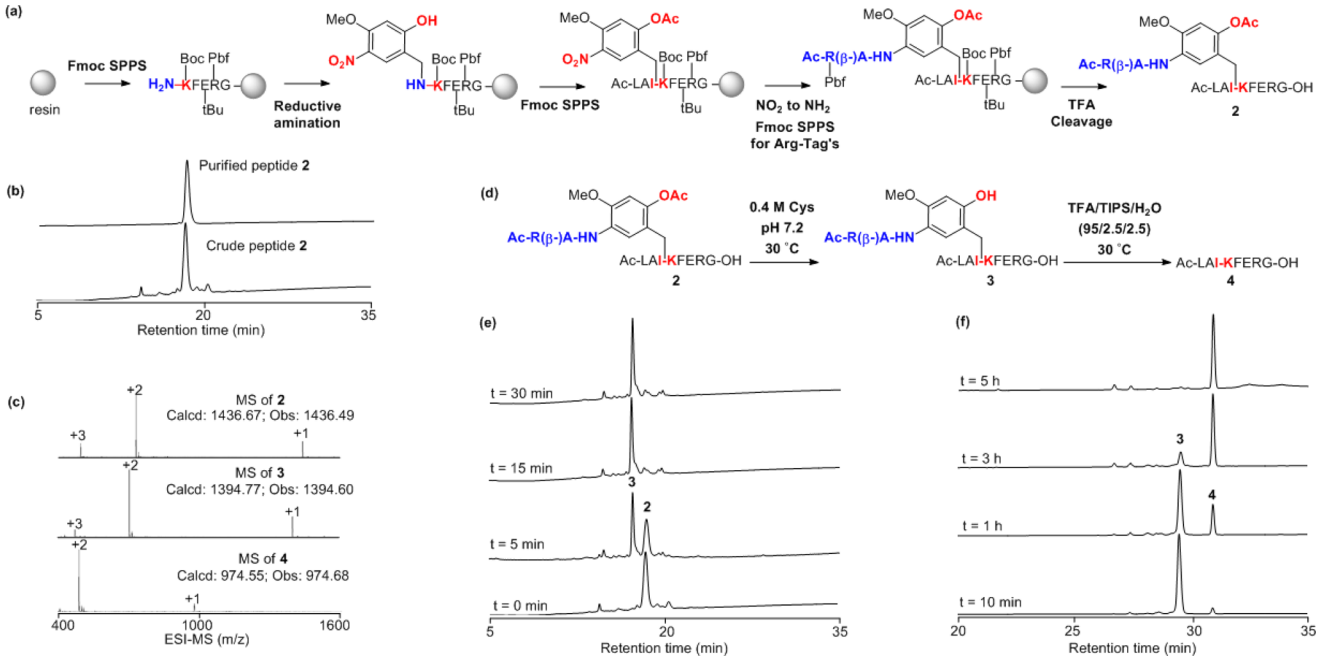

Figure 1. Model peptide. (a) Synthesis procedures for peptide **2**. (b) HPLC traces of the crude and purified **2**. (c) ESI-MS spectra of **2**–**4**. (d) The conversion of **2**–**4**. (e) HPLC traces for the deacetylation of **2** in neutral buffer (pH 7.2) at 30 °C. (f) HPLC traces for the cleavage of the RBM group from **3** in TFA cocktails at 30 °C.

carried out to generate the target peptide. As shown in Figure 1b,c, peptide **2** was obtained with 91% HPLC purity.

We next deacetylated the RBM group. Thus, crude peptide **2** was dissolved in the neutral aqueous buffer containing 0.4 M Cys (with 6 M Gn·HCl, 0.2 M phosphate salt, pH 7.2). This condition was previously found to be a mild approach to cleave phenyl esters.[14] The analytical HPLC traces showed a clean conversion of **2** to **3** within 30 min (Figure 1e). After the removal of the Ac protection, the RBM group of **3** can now be quantitatively removed by the TFA cocktails to give **4** within 5 h (Figure 1f).

To test the possibility of installing more than one RBM groups into a peptide, we synthesized another model peptide **5**. As shown in Figures S6 and S7, the introduction of two RBM groups into peptide **5** was successful. After TFA cleavage, the two Ac groups on the RBM groups were completely removed within 30 min to produce peptide **6**. The removal of the two RBM groups of **6** could be readily accomplished by a TFA treatment to give the final unmodified peptide **7** (38% isolated yield) within 5 h.

To investigate the scope of the method for different amino acids, we tested a number of peptides (**8**–**14** and **17**–**20**) where the RBM was placed between various two amino acids (Lys-Leu, Arg-Leu, Gln-Leu, Leu-Leu, Val-Leu, Ile-Leu, Thr-Leu, Ile-Lys, Ala-Ile, Cys-Ile, Phe-Ile, and Val-Ile). To save the experimental efforts in some of the tested systems, we put an Arg2 or Arg1 tag, or even no Arg. We paid particular attention to Leu, Phe, Ile, Val, and Ala because they make up 50% of residues in the transmembrane peptides.[17] As shown in Table 1 and Figure 2, most of the peptides were prepared with good efficiency (purity of crude peptides = 83–94%; isolated yields = 55–68%). Even for a highly sterically hindered site (Val-Ile), the RBM could be installed (entry 9, crude peptide purity = 39% and isolated yield = 15%).

Finally, to examine whether the acyl transfer may cause any epimerization, we synthesized Ac-LA$^{(D\text{-})}$**CI**$^{RBM(NO_2)}$KFERG-NH_2 **17** and Ac-LA$^{(L\text{-})}$**CI**$^{RBM(NO_2)}$KFERG-NH_2 **18**. HPLC analysis showed that the epimerization was less than 1.2% (Figure S18).

Table 1. Efficiency for Putting RBM at Different Amino Acids

entry	target peptides	purity %[a]	yield %[b]
1	Ac-LA**IK**$^{RBM(1R)}$FERG-OH **2**	91	53
2	Ac-AQ**KL**$^{RBM(1R)}$A**IK**$^{RBM(1R)}$FERG-OH **5**	83	44
3	Ac-A**VL**$^{RBM(NO_2)}$KFERG-NH_2 **8**	91	61
4	Ac-A**LL**$^{RBM(NO_2)}$KFERG-NH_2 **9**	92	55
5	Ac-A**IL**$^{RBM(NO_2)}$KFERG-NH_2 **10**	88	58
6	Ac-A**QL**$^{RBM(NO_2)}$KFERG-NH_2 **11**	93	63
7	Ac-A**RL**$^{RBM(NO_2)}$KFERG-NH_2 **12**	93	64
8	Ac-A**TL**$^{RBM(NO_2)}$KFERG-NH_2 **13**	90	57
9	Ac-LA**VI**$^{RBM(NO_2)}$KFERG-NH_2 **14**	39	15
10	Ac-WSWVY**FF**$^{RBM(2R)}$SFIII-$NHNH_2$ **16**	72	33
11	Ac-LA$_{(D\text{-})}$**CI**$^{RBM(NO_2)}$KFERG-NH_2 **17**	93	64
12	Ac-LA$_{(L\text{-})}$**CI**$^{RBM(NO_2)}$KFERG-NH_2 **18**	87	56
13	Ac-LA$_{(D\text{-})}$**FI**$^{RBM(NO_2)}$KFERG-NH_2 **19**	94	58
14	Ac-LA$_{(L\text{-})}$**FI**$^{RBM(NO_2)}$KFERG-NH_2 **20**	90	60

[a]The purity of crude peptides was determined according to the HPLC traces at 214 nm. [b]The isolated yield was determined by the weight of the purified product and the loading of the resin. The meanings of the symbols RBM(NO_2), RBM(1R), and RBM(2R) are denoted in Figure 2.

Application to Transmembrane Peptides. We next tested the new RBM method for the synthesis of realistic transmembrane peptides that are needed in biochemical and biophysical studies.[18] First, the transmembrane domain of the coat protein complex I (COPI) machinery protein p24 (p24-TM) can specifically interact with sphingomyelin to regulate COPI-dependent transport.[18c] Efficient synthesis of this peptide is needed for biophysical studies on the role of p24-TM in COPI-dependent transport. Previous work showed that

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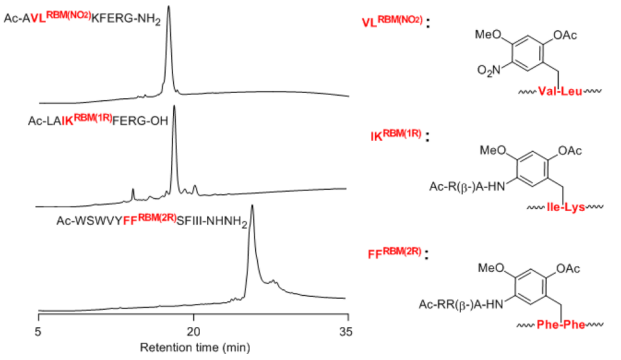

Figure 2. HPLC traces of the crude peptides with RBM groups.

p24-TM can easily form secondary structures and aggregate and has strong interactions with the reversed-phase HPLC columns. Its synthesis suffered from very poor yields (1.9%) even after trifluoroethanol was used as the mobile phase for HPLC purification.[19] In addition, p24-TM does not have Gly in its transmembrane region, so our previous Gly-only RBM method cannot be used.

By putting a RBM group at Leu10 with the new method, we successfully synthesized peptide p24-TM($L^{10,RBM}$) **21** (crude peptide purity = 65%) which behaves similar to a regular water-soluble peptide (Figure 3a). Peptide **21** can be easily purified

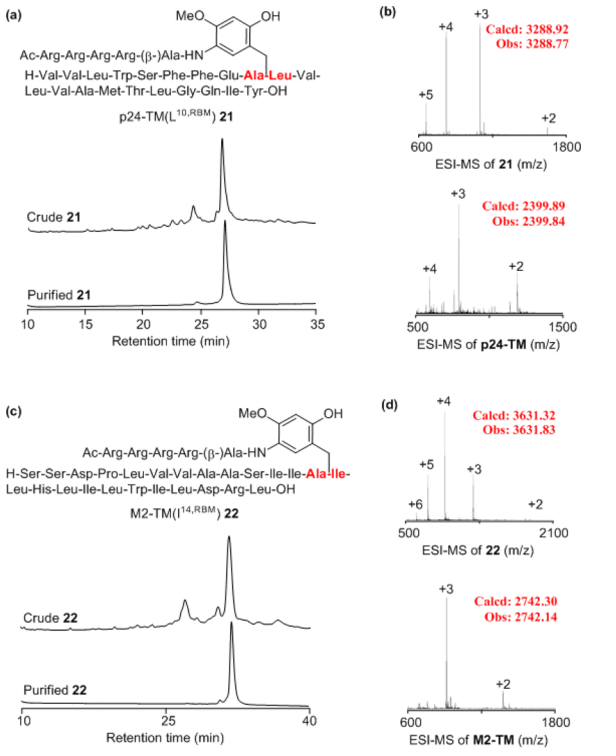

Figure 3. (a) HPLC traces of the crude and purified **21**. (b) ESI-MS of **21** and native p24-TM. (c) HPLC traces of the crude and purified **22**. (d) ESI-MS of **22** and native M2-TM.

with 24% isolated yield using standard mobile phase of water/acetonitrile. The HPLC traces of **21** show sharp and symmetric peaks, unlike the broad humps usually expected for hydrophobic peptides. Peptide **21** can also be well-characterized by electrospray ionization mass spectrometry (ESI-MS). The solubility of **21** was about 30 mg/mL in neutral buffer (6 M Gn·HCl, pH 7.2) according to NanoDrop A280 measurements. Treatment of purified **21** with TFA afforded native p24-TM as confirmed by ESI-MS. The solubility of native p24-TM was shown to be 0.4 mg/mL in the same neutral buffer. Thus, the solubility of **21** is nearly 80-fold higher than that of native p24-TM.

In addition to p24-TM, we tested the new RBM method with another important transmembrane peptide M2-TM from the proton channel of influenza A virus (Figure 3c). M2-related peptides have been intensively studied to understand their structures and the mechanism for inhibition of the M2 channel by amantadine.[20] Previous synthesis of M2-related peptides often suffers from the poor coupling near the center of the transmembrane domain and difficult purification due to low solubility.[21] By putting the new RBM group at Ile14, we can readily synthesize M2-TM($I^{14,RBM}$) **22** through Fmoc SPPS (crude peptide purity = 57%) and facile HPLC purification in 20% isolated yield. Subsequent TFA cleavage led to homogeneous native M2-TM that was successfully characterized by HPLC and ESI-MS (Figure S22). Again, M2-TM contains no Gly, so the present RBM method is necessary.

Total Synthesis of Wild-Type and Site-Specific ^{15}N-Labeled HCV p7 Ion Channel. The new RBM method can be used in combination with hydrazide-based native chemical ligation[1e] to synthesize small- to medium-sized membrane proteins. Such synthesis may provide otherwise difficult-to-obtain materials (for instance, precisely isotope-labeled membrane proteins) for advanced studies. To show an example, we chose the cation-specific ion channel p7 from HCV as the synthesis target. This 63-residue homohexameric membrane protein mediates H^+ intracellular conductance and is essential for viral replication, assembly, and release. p7 is an important candidate for therapeutic intervention.[22] However, the precise mechanism of p7 function remains unclear, thereby hindering the rational design of bespoke p7 inhibitors.[23] The unique ability of nuclear magnetic resonance (NMR) technology to elucidate the protein−drug binding sites and the conformation and dynamics of proteins makes it a powerful tool for studying membrane proteins.[24] However, the assignment of NMR signals is difficult for uniformly isotope-labeled membrane proteins.[8c,25] We expect that chemical protein synthesis will provide access to site-specifically isotopically labeled p7 for the detailed ion conduction mechanism analysis.

Torres et al. previously synthesized p7 by native chemical ligation of peptide segments p7(1−26) and p7(27−63). Unfortunately, the ligation suffered from the poor solubility of the reactants and the difficulty in purification of the peptide segments and thereby did not produce sufficient amount p7 for further study.[26] Here we have a much more efficient synthesis by using the new RBM method. As shown in Figure 4a, an Arg_4-tagged RBM was incorporated into p7(1−26,$H^{17,RBM}$)-$NHNH_2$ **23** and p7(27−63,$L^{53,RBM}$)-OH **24** at His17 and Leu53 located within each transmembrane region. With RBM, **23** and **24** were readily prepared through semiautomatic Fmoc SPPS.[27] Similar to water-soluble peptides, they were readily purified by HPLC using a water−acetonitrile gradient with isolated yields of 19 and 27% (Figures S23 and 24).

The improved handling properties of the transmembrane peptides allow their ligation under standard conditions. Thus, **23** (1.2 equiv) and **24** (1.0 equiv) were dissolved in 0.2 M phosphate buffer containing 6 M Gn·HCl. They were condensed using the in situ activation/thiolysis method of peptide hydrazides. The ligation went to completion within 5 h

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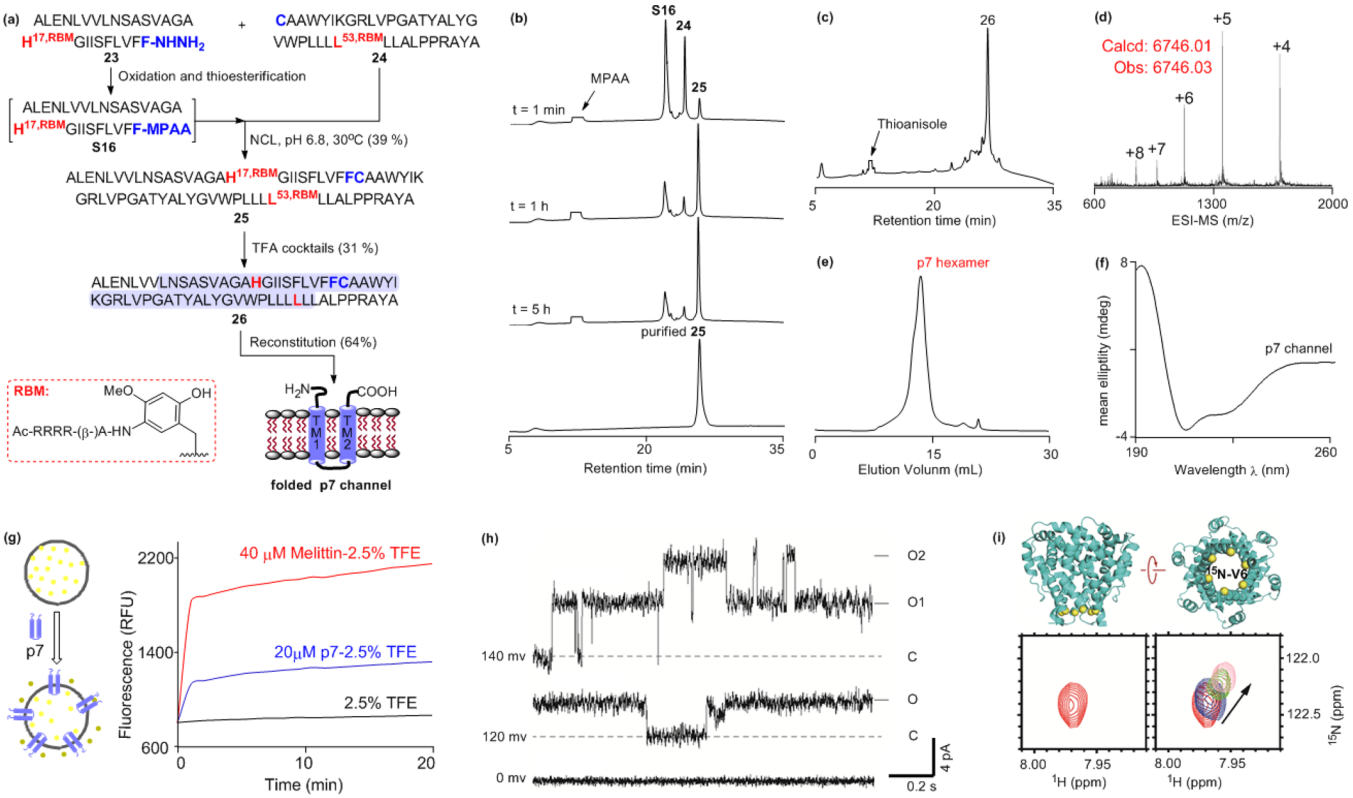

Figure 4. Chemical synthesis and characterizations of HCV p7 channels. (a) Synthesis route. (b) HPLC traces for the ligation between **23** and **24** (t = 1 min and 1 and 5 h) with a C4 column (4.6 mm × 150 mm) at 50 °C. (c) HPLC trace for the removal of RBM groups of **25** with a C4 column (4.6 mm × 150 mm) at 50 °C. (d) ESI-MS of native p7 **26**. (e) The purification of WT p7 **26** by gel-filtration chromatography on a Superdex 200 10/300 GL column. (f) CD analysis of reconstituted p7 (5 μM, in a 1 mm quartz cell) in DPC micelles. (g) p7-liposome-based fluorescent dye permeability assay. Small light yellow circles represent 5(6)-carboxyfluorescein (CF) at self-quenching concentrations, and the small dark yellow circles represent fluorescent CF being released from the liposomes (large gray circles) after p7 addition. (h) Single-channel currents of chemically synthesized p7 (1b) channels in POPC/POPG (3:1) lipid vesicles. Solid line: opening (O); dotted line: closure (C). (i) The reconstituted ^{15}N-labeled p7 in DPC micelles was titrated with 0 mM (rad), 1 mM (blue), 2 mM (green), and 4 mM (pink) rimantadine. Spectra were recorded on a Varian 700 MHz spectrometer at 303 K. The arrow indicates the movement of peak.

to provide full-length polypeptide **25** (Figure 4b). Peptide **25** was purified by semipreparative HPLC (39% isolated yield) and subsequently treated with TFA cocktails to remove the RMB groups within 5 h at 30 °C. The TFA solution was concentrated by N_2 blowing and precipitated by diethyl ether (Et_2O) to afford wild-type (WT) p7 **26** (Figure 4c). WT p7 **26** was purified by HPLC (26% isolated yield) and characterized by ESI-MS (Figure 4d).

The folding of p7 proceeded smoothly by the reconstitution of **26** into dodecylphosphocholine (DPC) vesicles and dialysis against the 2-(*N*-morpholine)-ethanesulfonic acid (MES) buffer. The folded p7 hexamer was purified by gel-filtration chromatography with 64% isolated yield (Figure 4e). Circular dichroism (CD) spectroscopy of the p7 hexamer exhibited the minimum absorption at 208 and 222 nm, indicating the formation of well-defined α-helical structure (Figure 4f).[28]

We used the liposome carboxyfulorescein (CF) release assay, which was well-established to characterize the ion channel activity, to measure the function of synthesized p7 channel.[29] As shown in Figure 4g, only when p7 hexamer correctly formed ion channels in the liposome could the enclosed CF fluorophore be released and detected. After addition of 20 μM synthesized p7 hexamer, an obvious release of CF from liposomes was induced. Addition of 40 μM melittin solution was conducted as a positive control (Figure 4g). Further study revealed that the p7 hexamer induced release of CF can be inhibited by some small molecules (e.g., amantadine) and was dose-dependent.[30] For the p7 channel concentration of 25 μM, the IC_{50} for rimantadine was ~35 μM (Figure S31).

The successful channel conductance measurement of synthesized p7 channel also indicated that p7 can form functional homomeric cational channels (Figure 4h). Single-channel currents of p7 channel were measured in planar lipid bilayer with symmetrical buffers in cis and trans chambers. Only at high voltages of +120 and +140 mV were obvious channel activities observed (Figure 4h). At lower voltages of +60 or +100 mV, the p7 channel showed no obvious activity, whereas the electronic noise changed with voltages (Figure S32). In addition to this typical open−close channel activity, a "burst activity" pattern of p7 with large current was observed in our experiment, similar to that in the previous report.[31] Results of single-channel recording demonstrated that chemically synthesized p7 can form a functional channel in planar lipid bilayer.

We then used NMR to reveal the binding sites of p7 with its inhibitor. ^{15}N-labeled Val6 was incorporated into peptide p7(1−26,$H^{17,RBM}$,V^{6}) **27**, which was prepared by the same way as peptide **23**. Subsequent ligation, purification, RBM removal, and folding afforded site-specific isotope-labeled p7 hexamer with 15% overall yield (Figure S33). NMR experiments were carried out at 30 °C for the detection of information on protein−drug binding sites. Four ^{1}H−^{15}N TROSY-HSQC spectra were recorded at rimantadine concentration of 0, 1, 2,

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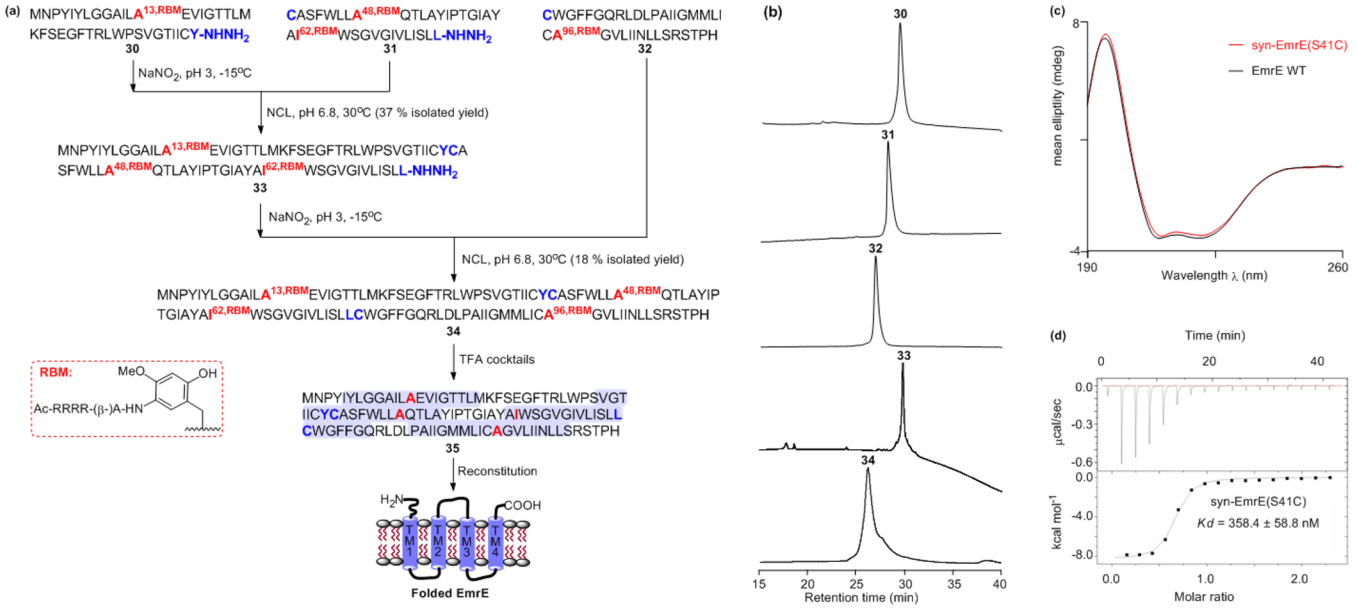

Figure 5. Chemical synthesis and characterizations of EmrE. (a) Synthesis route. (b) HPLC of the purified Arg-tagged EmrE peptides **30**−**32** and ligation products **33** and **34** with a C4 column (4.6 mm × 150 mm) at 50 °C. (c) CD analysis of reconstituted EmrE WT and syn-EmrE(S41C) (5 μM, in a 1 mm quartz cell) in DDM micelles. (d) ITC measurements of syn-EmrE(S41C) in DDM micelles.

and 4 mM, respectively. NMR data were processed using NMRPipe and analyzed by Sparky. The signal of $^{15}N-V^6$ on hexamer p7 moved significantly with the increase of the concentration of rimantadine (Figure 4i). The results reveal Val6 as a rimantadine binding site.

Total Synthesis of Four-Transmembrane Protein EmrE. Finally, we challenged the new RBM method with the total synthesis of a four-transmembrane-domain protein. Previous literature has examples for the total synthesis of membrane proteins with one (influenza A M2,[7a,11] HIV-1 Vpu,[31] and Tim-3 Ig),[9] two (a mechanosensitive channel,[7b] and functional domain of Kir5.1),[11] and three transmembrane domains (diacylglycerol kinase).[5a] Exploration is needed for the chemical synthesis of membrane proteins with more transmembrane domains.

For this purpose, we chose to synthesize *E. coli* integral membrane protein EmrE, a member of small multidrug resistance transporters. EmrE is a four-transmembrane-domain protein with 110 amino acids. It forms a homodimer in membrane environment to export a wide variety of cations such as tetraphenylphosphonium (TPP^+) and ethidium.[32] A great deal of controversies, such as the molecular recognition and transport mechanisms and the topology of the EmrE homodimer, remain to be elucidated.[33] A robust chemical synthesis may provide access to EmrE that is precisely modified for the advanced studies on the transport mechanism, the topology, and the role of key amino acids of EmrE.

The synthesis strategy for EmrE is depicted in Figure 5a. EmrE was divided into three segments: EmrE (1−40,$A^{13,RBM}$)-$NHNH_2$ **30**, EmrE (41−74,S41C,$A^{48,RBM}$,$I^{62,RBM}$)-$NHNH_2$ **31**, and EmrE (75−110,$A^{96,RBM}$) **32**. Ser^{41} was mutated to Cys as the ligation site to facilitate the protein synthesis. The Arg_4-tagged RBM groups were installed at Ala13, Ala48, Ile62, and Ala96 to improve the handling properties of the transmembrane peptides. The three peptide segments were smoothly synthesized by semiautomatic Fmoc SPPS. Well-behaved crude peptides were obtained that can be readily purified by HPLC with a water−acetonitrile gradient to afford **30**−**32** in isolated yields of 15, 17, and 12%.

The three segments were subjected to *N*-to-*C* sequential ligation. The first ligation of **30** (1.1 equiv) and **31** (1.0 equiv) at a final concentration of 1.5 mM was carried out at 30 °C. HPLC monitoring showed that this ligation reaction was completed to afford **33** within 2 h (Figure S33) in 37% isolated yield. The second ligation between **32** (2 equiv) and **33** (1.0 equiv) was conducted at pH 6.8 again at a final concentration of 1.5 mM. In this experiment, we observed precipitation of **33** when the hydrazide was oxidized at −15 °C. To overcome this problem, we added DPC into the ligation mixture. After 6 h, the desired product **34** was obtained (Figure S34). HPLC purification gave purified **34** in 18% isolated yield. Peptide **34** can also be readily characterized with ESI-MS (calcd 15 530.44 Da vs obs. 15 528.88 Da).

The purified **34** powder was dissolved into TFA cocktails for 5 h at 30 °C. After the TFA solution was concentrated by N_2 blowing and precipitated by Et_2O, we obtained final full-length peptide **35**. Unfortunately, **35** cannot be eluted from HPLC after many attempts with various columns and gradient systems. Furthermore, we cannot obtain the mass spectrum of **35** using either ESI-MS or matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) MS. These difficulties are typical of membrane protein synthesis previously experienced throughout the whole synthesis steps. Fortunately, with RBM we now need to handle the difficulty only after the target product is obtained. This means that we can use the standard biochemical methods to characterize and purify the final synthesized membrane proteins. In this case, we can characterize **35** through SDS-PAGE analysis, which gave a single band at the expected molecular weight of about 12 kDa in agreement with recombinant EmrE (Figure S41).

Full-length **35** was folded into its functional quaternary structure in detergent micelles by a procedure described previously.[34] Thus, **35** was dialysized against 0.1% (w/v) n-dodecyl-β-D-maltoside (DDM) buffer from 5% DDM and purified by gel-filtration chromatography with 57% yield

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(Figure S42). The CD spectrum of folded synthesized EmrE(S41C) showed minimum absorption at 208 and 222 nm. This observation is in good agreement with that of recombinant EmrE WT isolated from *E. coli*, suggesting correct folding of the synthesized protein (Figure 5c). Using isothermal titration calorimetry (ITC) measurements, we measured the binding constant of synthesized EmrE(S41C) with TPP^+ (K_d = 0.36 ± 0.06 μM). This value is slightly higher than the binding constant measured for the recombinant EmrE WT (K_d = 0.15 ± 0.03 μM) in DDM micelles (Figures 5d and S44).[34,35] The difference in the binding constants may be caused by Ser41 mutation to Cys41, hinting at the involvement of this residue in the protein−substrate interaction. Nonetheless, the substrate binding experiments indicated that the synthesized EmrE-(S41C) is functionally active.

CONCLUSIONS

The removable backbone modification method has been found to be a practical approach to solve the handling problems of membrane peptides. RBM not only disrupts the formation of aggregation-prone secondary structures but also introduces solubilizing tags into the system. Our previous version of RBM only allowed the modification at Gly and therefore had serious limitations in real-life applications. We now develop a generally applicable and easy-to-operate RBM method, which combines the technology of activated intramolecular *O*-to-*N* acyl transfer and the idea of amide modification group with switchable reactivity toward TFA. The new RBM method can be used for modification at versatile amino acids to solve the handling problems associated with membrane peptides. With the help of this robust RBM method, chemical synthesis of small- to medium-sized membrane proteins can be readily conducted with practical efficiency. Synthesis access to customer-designed membrane proteins with precise modifications paves a way to their advanced biochemistry and biophysics studies.

EXPERIMENTAL SECTION

Detailed experimental procedures for the synthesis of compounds, peptides, and proteins, their characterization data, and the electrophysiology experiments are given in the Supporting Information.

Synthesis of AA^{RMB}-Containing Peptides. The coupling of peptides was carried out using standard Fmoc SPPS procedure according to the predesigned sequence. Salicylaldehyde **1** was introduced onto the peptide at designated sites via reductive amination. The *N*,*N*′-diisopropylcarbodiimide/oxyma coupling protocol was employed for the condensation of the following amino acid with the secondary amine. Note that the amino group of the last amino acid of peptide assembly should be protected with Boc group to tolerate the following Fmoc SPPS for Arg tag. After the completion of chain assembly, the nitro group is reduced to amine by $SnCl_2$, and then Fmoc SPPS was continued for attaching the Arg tag. Finally, the 2-OH group was capped by Ac_2O/DIEA/DMF (1:1:8, v/v/v). A standard TFA cleavage was conducted to remove the side-chain protection groups and release the RMB-containing peptides.

Deacetylation of AA^{RMB}-Containing Peptides. The RBM-containing peptides were dissolved in the neutral aqueous buffer (0.4 M Cys, 6 M Gn·HCl, 0.2 M phosphate salt, pH 7.2) to remove the Ac group from the phenol. A complete conversion was achieved within 30 min at 30 °C.

Removal of the Backbone Modification Group. The RBM-containing peptides/proteins were dissolved in TFA cocktails for 5 h at 30 °C to remove quantitatively the backbone modification group. After the cleavage, the reaction mixture was concentrated and precipitated with Et_2O to yield the peptides without RBM.

Native Chemical Ligation of Peptide Hydrazides. Peptide hydrazides were dissolved into aqueous buffer (0.2 M phosphate, 6 M Gn·HCl, pH 3.0). The oxidation of peptide hydrazide to azide was carried out for 15 min at −15 °C. Subsequently, MPAA (4-mercaptophenylacetic acid, 100 equiv) was added to the reaction mixture to convert the peptide azide to the thioester. The following addition of the N-terminal Cys peptide (0.9−2 equiv) initiated native chemical ligation. The ligation reaction was carried out at pH 6.8 and monitored by analytical HPLC and ESI-MS. After completion of ligation, neutral tris(2-carboxyethyl) phosphine solution (TCEP, 0.1 M, 100 equiv) was added to reduce the reaction system.[1e]

Folding of the HCV p7 Channel. Synthesized p7 protein was dissolved in 6 M Gn·HCl buffer containing 5% DPC and refolded by dialysis against the MES buffer (25 mM MES, 5 mM dithiothreitol (DTT), pH 6.5). The p7 channel was purified by gel-filtration chromatography on a Superdex 200 10/300 GL column (GE healthcare) with buffer (25 mM MES, 100 mM NaCl, 5 mM DTT, pH 6.5). Fractions containing p7 oligomer were concentrated using an Amicon Ultra filter (3000 MWCO, Millipore) for the following functional measurements.

Single-Channel Conductance Measurement of p7 Channel. After reconstituting the p7 channel into planar lipid bilayer, single-channel recordings were carried out using Ionovation Compact (Osnabrück, Germany). The traces were recorded at various membrane potentials in 5 mM Tris-MOPS, pH 7.0 and 150 mM KCl both inside and outside. Working buffer (5 mM Tris, 5 mM MOPS, 150 mM KCl, pH 7.0) was present in both cis and trans polycarbonate chambers, which were separated by a TEFLON foil with 25 μm thickness and 50−100 μm aperture diameter. Two Ag/AgCl electrodes were bathed in working buffer. The planar lipid bilayer was formatted by painting the solution of POPC/POPG (3:1) in *n*-decane on the aperture, and formation was monitored by capacitance measurements. To insert protein into the planar lipid bilayer, the proteoliposomes were added to the cis chamber next to the bilayer after successful formation of a stable bilayer. Fusion of protein and planar lipid bilayer was detected through observation of channel conductance. Different dc voltages were used to measure channel conductance using an EPC-10 amplifier (HEKA Elektronik). Currents were measured with a 2 kHz low-pass filter at 10 kHz sampling rate. Data were analyzed using the clampfit software.

NMR Measurements of p7 Channel. ^{15}N-Labeled p7 was dissolved in DPC buffer (50 mM DPC, 25 mM MES, 100 mM NaCl, 5 mM DTT, 10% D_2O, pH 6.5). The sample concentration (monomer) for NMR measurements was 0.14 mM. All NMR experiments were carried out at 30 °C on a Varian 700 MHz spectrometer equipped with a triple-resonance cryo probe. Four $^1H-^{15}N$ TROSY-HSQC spectra were recorded at rimantadine concentrations of 0, 1, 2, and 4 mM. NMR data were processed using NMRPipe and analyzed by Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

Folding of EmrE(S41C). Synthesized EmrE(S41C) protein was dissolved in 6 M Gn·HCl buffer containing 5% DDM and refolded by dialysis against the 0.08% DDM buffer (25 mM MES, 5 mM DTT, pH 7.2). The folded protein was purified by gel-filtration chromatography on a Superdex 200 10/300 GL column with buffer (0.08% DDM buffer, 25 mM Tris, 100 mM NaCl, 5 mM MES, pH 7.2).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b00515.

Experimental details and compound characterizations. (PDF)

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Notes

The authors declare no competing financial interest.

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