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Solid-Phase Peptide Synthesis

N,*O*-Benzylidene Acetal Dipeptides (NBDs) Enable the Synthesis of Difficult Peptides via a Kinked Backbone Strategy

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Abstract: Proteins with highly hydrophobic regions or aggregation-prone sequences are typically difficult targets for chemical synthesis at the current stage, as obtaining such type of peptides via solid-phase peptide synthesis requires sophisticated operations. Herein, we report *N*,*O*-benzylidene acetal dipeptides (NBDs) as robust and effective building blocks to allow the direct synthesis of difficult peptides and proteins via a kinked backbone strategy. The effectiveness and easy accessibility of NBDs have been well demonstrated in our chemical syntheses of various challenging peptides and proteins, including chemokine, therapeutic hormones, histone, and glycosylated erythropoietin.

The past decades have witnessed a dramatic increase in studies aimed at deciphering the roles of posttranslational modifications (PTMs) on proteins in regulated biological processes,[1,2] such as acylation and methylation for histone epigenetic regulation,[3–5] glycosylation for controlling protein stability and biofunction,[6–10] phosphorylation for nerve disease-related protein aggregation[11–13] and enzyme activities,[14] as well as lipidation for regulating protein secretion and cell apoptosis.[15,16] These findings highlight the indispensable effects of PTMs in protein biofunctions. Unfortunately, progress in this field has been hampered by the limited access to homogeneous proteins with site-specific PTMs due to the inherent limitations of current recombinant protein expression technology.[17] On the other hand, chemical protein synthesis, which allows for site-specific installation of modified amino acid residues, has become a powerful tool to address homogeneity issues and has permitted further studies on the relationship between PTMs and protein function.[18,19]

To chemically synthesize proteins with customized either natural or unnatural modifications, peptide chemical ligation is the key strategy for convergent protein chemical synthesis. Over the past decades, the development of ligation technologies, including native chemical ligation (NCL),[20] Ser/Thr ligation (STL),[21,22] Cys/Pen ligation (CPL),[23] α-ketoacid-hydroxylamine ligation (KAHA)[24] and diselenide-selenoester ligation (DSL),[25] in combination with late-stage desulfurization reactions,[26,27] has enabled the joining of unprotected peptide segments at different amino acid sites and led to effective synthesis of a variety of homogeneous proteins. Moreover, the invention of expressed protein ligation,[28] on-resin ligation[29] and one-pot sequential ligation[30] has further expanded the application scope and accelerated protein synthesis process. Despite these achievements, chemical ligation of unprotected peptides bearing highly hydrophobic regions remains a challenging mission. To overcome this obstacle, various innovative strategies, such as ligation embedding aggregation disruptor,[31] solubilizing tags installation,[32–36] removable backbone modification[37] and tunable backbone modification,[38] have been developed to alter the hydrophobic nature by adding hydrophilic tags and enable solubilizing peptides in the ligation solvent.

However, there is a widespread unsolved problem of "difficult peptide" synthesis during solid-phase peptide synthesis (SPPS).[39–43] These side chain protected peptides are prone to aggregate on resin, causing serious peptide deletion/truncation. In 1995, Mutter introduced pseudoproline dipeptides (*ψpro*),[44] Johnson introduced 2-hydroxy-4-methoxybenzyl (Hmb) protecting group[45] and in 2004, Kiso introduced *O*-acyl isopeptide[46] respectively to inhibit hydrogen-bonded interchain association and disrupt peptide backbone conformation. Although above strategies are widely employed for disrupting peptide aggregation during SPPS in academia, their applications in industries still remain limited nowadays, probably because the syntheses of those building blocks are not very cost-effective.

Herein, we report the NBDs as effective and convenient building blocks for chemical syntheses of difficult peptides or proteins via a kinked backbone strategy (Figure 1). Briefly, the insertion of highly twisted moieties during the SPPS process prevents the peptide aggregation on resin by kinking the Ser/Thr side chain with the amide buried in the main chain. These new building blocks allowed us to achieve the syntheses of several previously inaccessible peptides/proteins directly via SPPS, and help avoid the aspartimide

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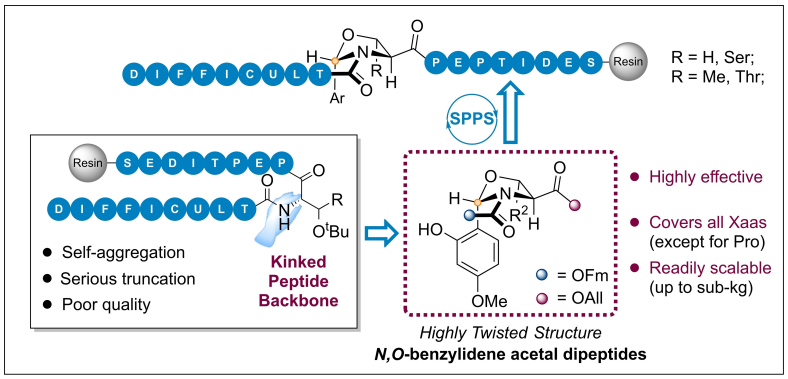

Figure 1. Design of the *N,O*-benzylidene acetal dipeptides.

formation problem in the synthesis of glycoprotein bearing *N*-glycans.

The idea of NBDs stemmed from our long-term pursuit of controlling peptide chain behaviors to avoid aggregation and truncation. Previously, we found that the *N,O*/*S*-benzylidene acetal intermediate after STL and CPL could effectively prevent peptide aggregation due to self-assembling[31] and could be used to introduce solubilizing tags[38] as the ligation embedding aggregation disruptor, and tunable backbone modification, respectively. Thus, we envisioned that the installation of a pre-formed NBD building block might kink the peptide conformation and facilitate "difficult peptide" synthesis on resin for SPPS. To primarily validate our hypothesis, extensive NMR studies were performed, and the highly twisted structures of NBDs were confirmed (Figure S7–S8). One set of t*rans* (major) and *cis* (minor) rotamers were elucidated with *S*-configuration stereochemistry at the position of *N,O*-benzylidene acetal carbon.[47] Such dramatic conformation of NBDs suggested that they could interfere the interaction of peptide backbone after their installation to the growing peptide during SPPS.

To examine the substrate scope of NBDs, firstly, the salicylaldehyde ester of each amino acid **1**–**19** (except for Pro) was prepared by hexafluorophosphate azabenzotriazole tetramethyl uranium (HATU)-mediated condensation between protected Fmoc-amino acid and 4-methoxysalicylaldehye (Figure 2A). Then, precursor **1**–**19** reacted with a trifluoroacetic acid (TFA) salt of Ser **20** or Thr allyl ester **21** under 5 % pyridine-acetic acid buffer in dichloromethane (DCM) for few hours to afford NBDs **22a**–**40**. Following this synthetic Scheme, 26 representatives of Xaa-Ser/Thr NBDs were facilely prepared in gram scale with 70–95 % yields, showing impressive compatibility to amino acid building blocks with various types of side chains. Even for Cys and His with delicate protecting groups, related NBDs **31** and **34** could be easily obtained. Notably, the NBD synthesis could be further scaled up to a sub-kg level, where we successfully achieved 143.6-gram preparation of Thr-Thr NBD **40** with only one purification step (85 %). The excellent tolerance of functional groups and the scalability in NBDs syntheses ensure its efficiency in production and wide applications, which will be crucial for investigating and manufacturing therapeutic peptide/protein with difficult sequence.

Then we started to develop a practical NBD-incorporated SPPS (NBD-SPPS), evaluating their effectiveness and stability during peptide elongation (Figure 2B). First, the allyl group of NBD **26** was selectively removed within 30 min by a catalytic amount of $Pd(PPh_3)_4$ and stoichiometric 1,3-dimethylbarbituric acid (DBBA) in DMF, generating NBDs with free carboxylic acids. Next, without any purification, the crude mixture of **26** after deallylation was activated by HATU and the NBD was directly coupled to the resin-bound peptide prepared by SPPS. Followed by the standard Fmoc-SPPS, the desired peptide **41** was constructed with insertion of an NBD peptide, which was pleasingly confirmed by LC–MS analysis of protected crude peptide **41b** from soft cleavage of **41** by hexafluoroisopropanol(HFIP)/DCM. A representative 16-mer peptide example **41b** with Ile-Thr NBD clearly showed that no decomposition of NBD was found in LC–MS analysis during the long-time process of SPPS, even including multiple overnight couplings (Figure 2C, Figure S4.1–6). Meanwhile, the acid-labile *N,O*-benzylidene acetal in the NBD was cleaved upon global deprotection of **41** by TFA cocktail, affording peptide **41a** as well as salicylaldehyde (Figure 2C). Similar results were also observed with substitution of Ile-Thr NBD by Leu-Ser and Val-Thr ones (**42**&**43**, Figure S4.2–4.3). Those results indicated a remarkable compatibility of NBD structures with routine Fmoc-SPPS containing amino-acid coupling and Fmoc-deprotection. The product with native peptide linkage could be subsequently restored under common conditions of global deprotection. Impressively, the one-pot deallylation-activation method rendered a convenient and robust way to introduce the twisted NBD structure in SPPS, simplifying the operation procedure and broadening its potential applications in challenging target synthesis.

To evaluate the effectiveness of NBD, we selected several short sequences, all of which were known as difficult peptides, including PD-1 N-terminus (41–52) **44**,[31] programmed death-ligand 1 C-terminus (PD–L1, 121–132) **45**,[48] Interleukin-2 C-terminus (IL2, 125–133) **46**,[49,50] peptide derived from influenza virus hemagglutinin (HA) **47**[51] and from human growth hormone (hGH) **48**[52] (Figure 3).

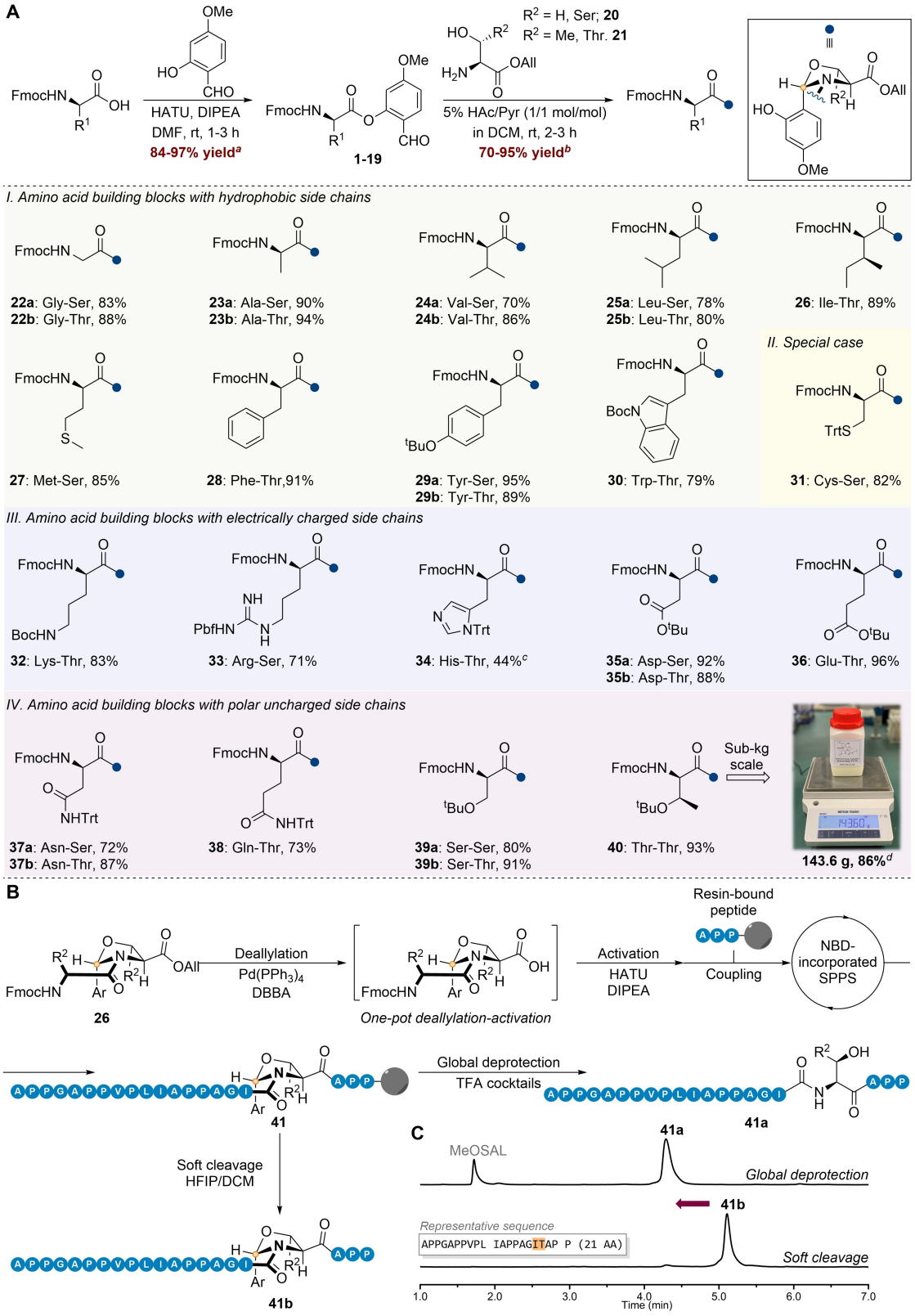

Figure 2. Synthesis and application of *N*,*O*-benzylidene acetal dipeptides (NBDs). A. Scope and limitation of NBDs preparation. [a&b]Except for His NBD; [c]Partial decomposition of side chain protection and serious tailing during purification; [d]Prepared by one-pot salicylaldehyde ester formation and Ser/Thr ligation. B. Scheme of NBD-incorporated SPPS via convenient one-pot deallylation and activation method. C. LC traces of the selected peptide cleaved from resin with/without protecting groups. NBD position was highlighted in peptide sequence. MeOSAL: 4-Methoxysalicylaldehyde. For more examples, see Figure S4.1–4.3.

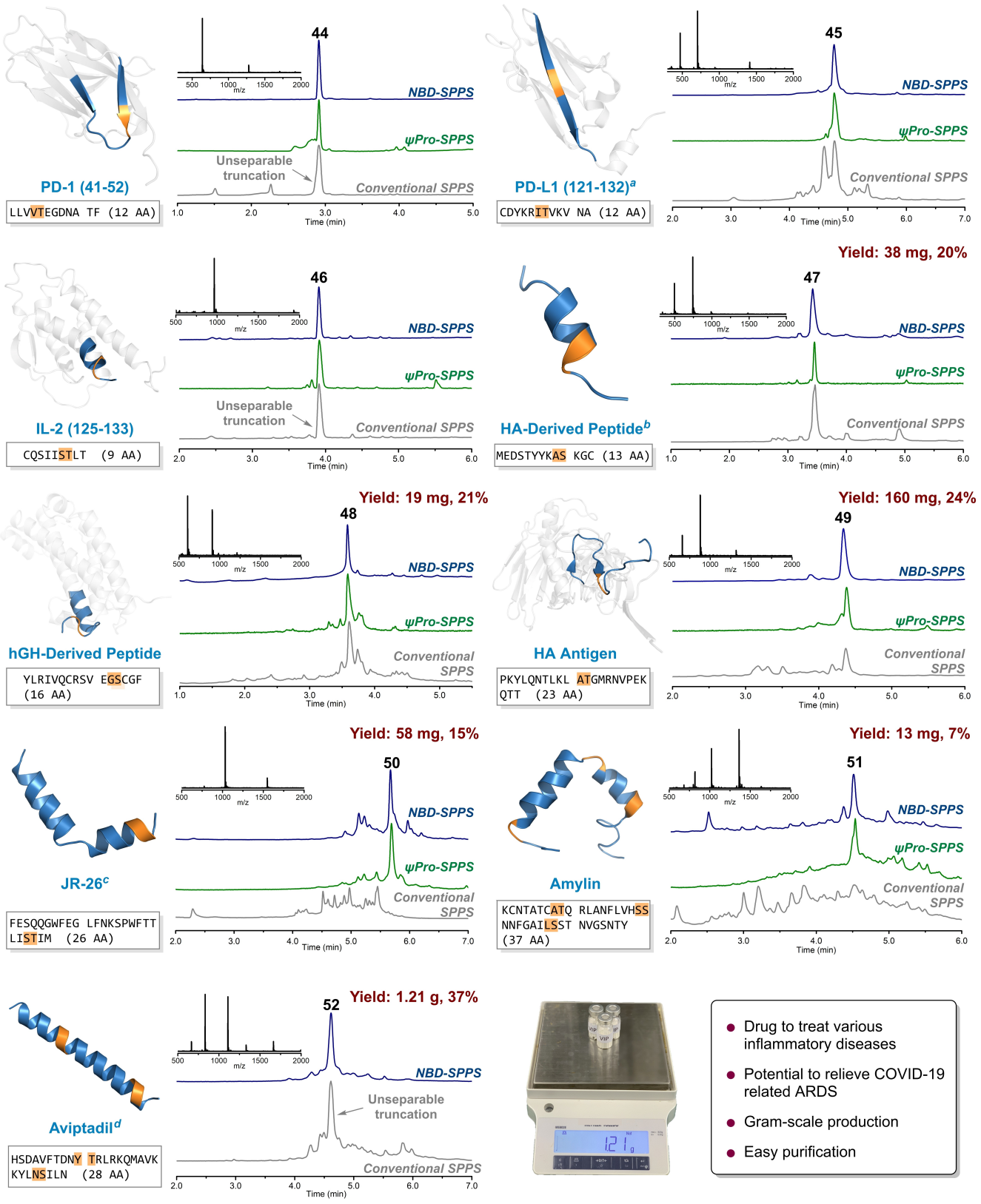

Figure 3. Comparison of conventional SPPS, *ψpro*-SPPS and NBD-SPPS in syntheses of difficult peptides. The insertion of NBDs was highlighted in peptide sequences. [a]A121C mutation for potential chemical ligation; [b,c] and [d]Conformations were predicted by AlphaFold.[64] Protein structure was present in transparent white as reference of synthesized difficult peptides (2M2D, 5C3T, 1IRL, 3HHR, 1EO8 and 2I86).

Owing to abundant β-sheets structures in the immunoglobulin-like (Ig-like) domain of PD-1, its peptide fragments are highly prone to aggregate during SPPS so that modifications for peptide backbone interference were necessary during the chemical synthesis of PD-1.[31] Through introduction of Val–Thr NBDs, the crude **44** from NBD-SPPS exhibited higher purity compared to that obtained by conventional SPPS where truncation of Leu occurred (Figure S4.4-4). Similarly, satisfactory quality was observed in the synthesis of PD–L1 C-terminus **45** with insertion of one Ile–Thr NBD, while serious truncation was present without using NBD. These results provide solid evidence that NBDs can act as useful reagents for the effective synthesis of difficult peptides with β-sheet structures, especially in the Ig-like domain of other immune cell receptors such as PD-1,[53] PD–L1,[54] CTLA-4[55] and TIGIT.[56] Interestingly, when *ψpro* dipeptides were employed in the synthesis of these two difficult sequences, they showed the similar results with NBDs in preventing aggregation.

In addition, the challenging sequence **46** with α-helix structures was successfully constructed as well by solely applying Ser-Thr NBD. Without the introduction of NBD in SPPS, the peptide suffered from serious truncation issues reflected by MS analysis (Figure S4.6-3), though its UV spectrum was similar to that from NBD-SPPS. In synthesis of another α-helix case **47**, introducing an Ala–Ser NBD also improved the overall quality of the crude product obtained from NBD-SPPS compared to that from the standard SPPS. Meanwhile, we selected a "SPPS-inaccessible" 16-mer peptide **48** derived from hGH with therapeutic interest, which was a highly aggregation-prone peptide and even required NCL for its synthesis.[57] As shown in Figure 3, the insertion of the Gly-Ser NBD rendered the construction of **48** smoothly. The successful synthesis of these demanding sequences through NBD-SPPS demonstrated the feasibility of utilizing NBDs in handling difficult short peptides. In comparison with *ψpro* during the synthesis of **46** and **48**, NBDs exhibited better results than *ψpro* ones, indicating the powerfulness of NBDs in difficult peptide synthesis.

Furthermore, SPPS of medium-size difficult peptides (20–40 mer) was performed with or without NBDs. The first case is peptide **49**, a model antigen from HA, which could be applied for anti-virus vaccine development.[51] The installation of Ala-Thr NBD or *ψpro* dipeptide afforded significantly purer **49** than that from the conventional SPPS. The advantage of NBD-SPPS was particularly evident in the synthesis of Jung-Redemann 26-mer peptide **50**.[58] In the absence of Ser-Thr NBD or *ψpro* dipeptide, no desired product could be observed, while 15 % isolated yield of the corresponding peptide was obtained from NBD-SPPS. These results emphasized the efficiency of both NBDs and *ψpro* dipeptides in synthesizing aggregated prone peptides. Next, a frustrating outcome was obtained in the standard SPPS of Amylin **51**, a glycemic-regulating peptide hormone. This target posed more challenges as there was significant amino-acid deletion occurring during its synthesis. However, by sequentially installing Leu-Ser, Ser-Ser and Ala-Thr NBDs, the full length of Amylin **51** was successfully synthesized with impressive purity and isolated yield. On the other hand, *ψpro* dipeptides could provide the desired product with slight truncations (Figure S4.11-4). Finally, we envisioned that our powerful NBD-SPPS could be further adopted for gram-scale preparation of therapeutic peptides. For this purpose, the synthesis of Aviptadil (VIP) **52** was evaluated. VIP is a peptide drug currently used in clinical practice to treat various inflammatory diseases and has the potential to relieve acute respiratory distress syndrome (ARDS) caused by COVID-19. Fortunately, 1.21 g of **52** was successfully prepared in 37 % yield by simply employing two NBDs in NBD-SPPS. By contrast, fine-tuning and tedious HPLC conditions were required for the conventional SPPS of **52**, otherwise it would easily co-elute with truncated side products.

Encouraged by results of NBD-SPPS in synthesizing difficult peptides, we turned to investigate the performance of NBD in the direct synthesis of small proteins with highly demanding sequences (Figure 4A). To this end, protein **53**, which is the linear form of the plant-based storage protein Crambin (1–46), would be an ideal case to start with. It was so prone to aggregation that direct SPPS of **53** was impossible and it had to be prepared by utilizing NCL between multiple fragments.[59] Indeed, our attempt to synthesize it by the standard SPPS failed with tremendous residue deletion. Delightfully, through introducing only one Ala-Thr NBD, the linear form of Crambin **53** was directly assembled with SPPS. Apparently, the NBD insertion greatly suppressed the interaction of polypeptide chain and thus improved the quality of the final synthetic protein, enabling its HPLC purification in 27 % yield. Unexpectedly, when *ψpro* dipeptide-SPPS was employed, **53** was obtained in poor quality since significant truncations were found during the synthesis (Figure S4.13-4). Then **53** was subjected to a refolding procedure to provide folded Crambin **53a** in 45 % yield (Figure S4.13-7).

Subsequently, the synthesis of a natural HIV-suppressive factor RANTES (1–68) **54**, also known as chemokine ligand 5, was performed. It has been proven that the synthesis of **54** was extremely difficult and required the combination of *ψpro* dipeptides and the expensive ChemMatrix resin.[60] Remarkably, by coupling four NBDs, RANTES could be smoothly constructed even with the conventional polystyrene resin. Moreover, the quality of crude peptide from NBD-SPPS fulfilled the requirement of HPLC purification and afforded 98 mg of **54** in 19 % yield. On the other hand, the failure to synthesize **54** using SPPS without NBDs undoubtedly emphasized the key role of NBDs in preventing peptide aggregation regardless of polymer-support types. Moreover, when employing equal amounts of *ψpro* dipeptides at the identical sites for its synthesis on the polystyrene resin, the final product encountered with inseparable impurities issues, which was consistent with the reported case (Figure S4.14-4).[60]

Next, we continued to explore the synthesis of Ubiquitin (1–76) **55** using NBD-SPPS. Although the direct SPPS of **55** was previously reported,[61] its strategy was complicated where *ψpro* dipeptides and HMB building blocks were both required in the synthesis. However, in our trial, only three NBDs were sufficient to provide 50 mg of **55** with excellent

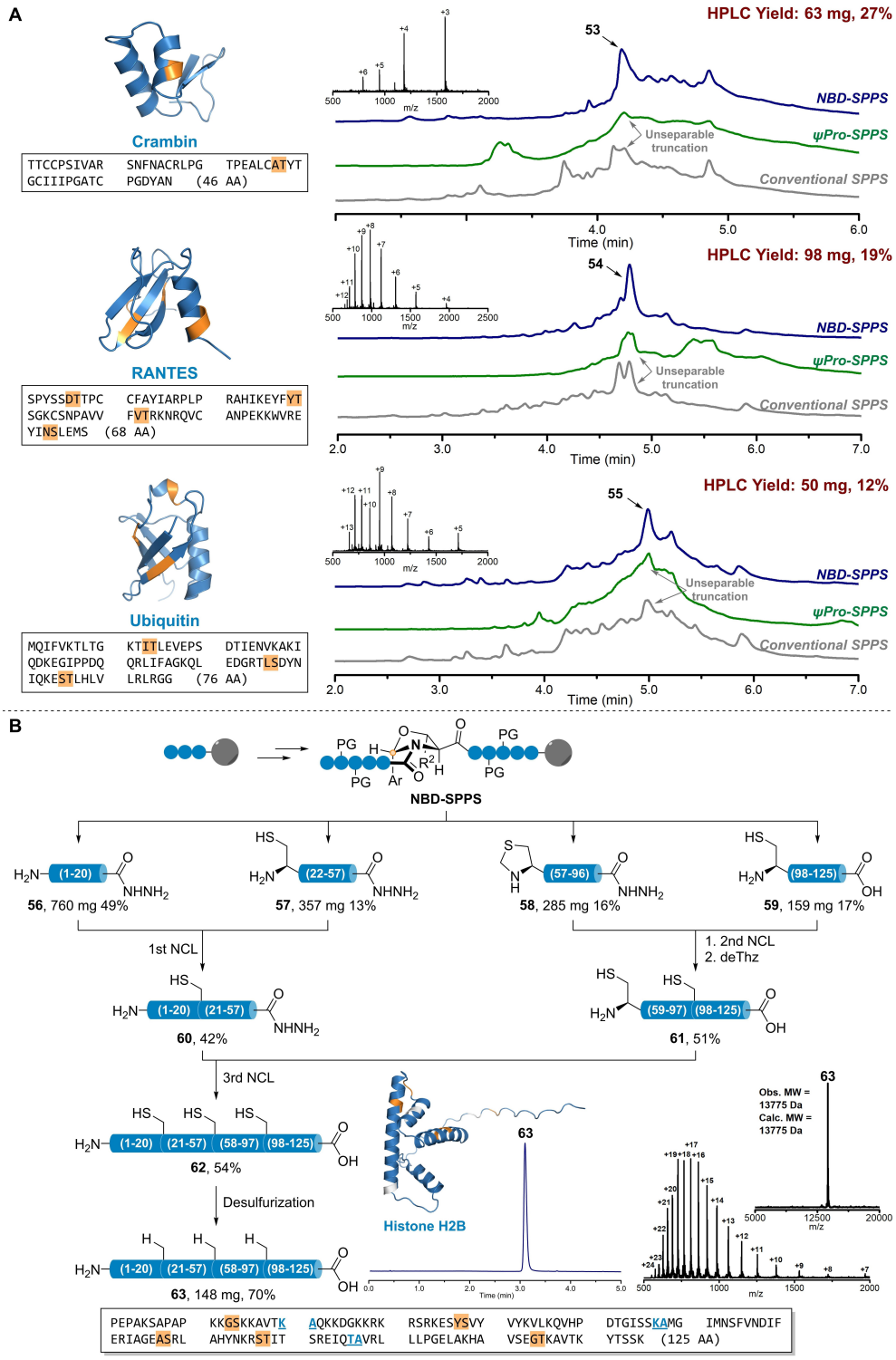

Figure 4. NBD-SPPS for chemical protein synthesis. A. Synthesis of Crambin (1CCM), RANTES (1RTN) and Ubiquitin (1D3Z) through direct NBD-SPPS. Crude LC–MS traces were present; B. Synthesis of H2B by combination of NBD-SPPS and NCL. LC–MS spectra of purified H2B were present. NBDs position and NCL sites (if any) were highlighted as orange and blue/white respectively in sequences and protein structures. Conformation of H2B was predicted by AlphaFold.[64]

quality in 12 % yield after HPLC purification. As a control, **55** with poor purity was obtained from the conventional SPPS due to the inseparable deletion side-products. Nevertheless, when NBDs were replaced by *ψpro* dipeptides, **55** was obtained with slightly truncated impurities, highlighting the importance of HMB during the synthesis (Figure S4.15-4).

These results are very promising to showcase the generality of NBDs. We further combined the NBD-SPPS with NCL-desulfurization for the large-scale synthesis of proteins with longer sequence (Figure 4B). Histone H2B is a structural protein that helps organize eukaryotic DNA and its PTM regulates transcription, replication and repair activities of DNA. Chemical synthesis of H2B with homogeneous PTMs for either investigation of fundamental chemical biology process or development of novel epigenetic drug is of great interest.[62] The synthesis was previously reported by Brik using NCL to join four peptide fragments together in a convergent way.[63] As stated by the author, the usage of combination of multiple reported approaches was essential for all the H2B peptide fragments preparation. This dilemma is believed as a major obstacle for the large-scale synthesis of therapeutic peptides and proteins. By applying the NBD reagents, several hundred milligrams of each fragment could be robustly and conveniently produced in high yield (159–760 mg, 13 %–49 %). It should be noted that without NBDs, all H2B peptide fragments suffered from serious truncation as described in the literature, which led to inseparable impurities and rendered the next-stage NCL an impossible task. With these fragments in hand, a convergent synthetic scheme was performed as below. Firstly, the first NCL between **56** and **57** based on $NaNO_2$ activation was performed to generate **60** in 42 % isolated yield. Then **58** was activated by acetylacetone to give rise to the corresponding peptide thioester, followed by the second NCL with **59** and Thz deprotection to allow the formation of **61** in 51 % yield. Afterwards, the third NCL between **60** and **61** provided the ligated product **62** in 54 % yield. After desulfurization, 148 mg of native H2B protein **63** was produced in 70 % HPLC yield with satisfactory characterization (Figure 4B). Overall, the effectiveness and robustness of NBDs were well supported in the above unprecedentedly large-scale preparation of histone protein by chemical synthesis.

With success of synthesizing various difficult peptides/proteins via the NBD-SPPS, we further extended this strategy to the chemical synthesis of *N*-linked glycoproteins. Typically, a conservative –Asn–Xaa–Ser/Thr– sequence is present in *N*-glycosylated proteins where the Asn is the glycosylation site. We hypothesized that the adoption of Xaa–Ser/Thr NBD in such sequence may prevent the aspartimide formation during SPPS, which is an unavoidable problem in the Lansbury-aspartylation-mediated glycoprotein synthesis. Thus, we planned to synthesize the Erythropoietin (EPO) with homogeneous GlcNAclyations to evaluate whether NBD could be a new tool for the synthesis of N-linked glycoproteins (Figure 5A).

To further extend the application scope of the NBD-SPPS, we also developed the on-resin in situ insertion strategy to install NBDs. Basically, an Fmoc-amino acid salicylaldehyde ester reacted with the N-terminal unprotected Ser/Thr residue of resin-bound peptide **64a**–**68a** under HAc/Pyr buffer to generate **64b**–**68b** with NBD on resin. All fragments with one or multiple NBDs in this EPO synthesis were readily prepared by such in situ NBD insertion method. For glycosylated fragments, the Asp building block with side chain allylation was coupled to resin-bound peptide bearing NBD structures. After elongation of peptide chain, no aspartimide was detected. Then, on-resin deallylation and Lansbury aspartylation were performed to obtain site-specific GlcNAclyations on the protected resin-bound peptide **64f**–**66f**. To our delight, **64**, **65** and **66** were all successfully prepared in good quality, implying that NBDs could effectively prohibit aspartimide formation during the synthesis of glycoprotein fragments. As for the other fragments without glycosylation, routine NBD-SPPS from **64b**–**68b** smoothly afforded **67** and **68** in good yield. Notably, omitting the Ile-119-Ser-120 and Tyr-156-Thr-157 NBDs of **67** and **68** respectively during SPPS hardly produced pure materials.

With five fragments in hand, we started to proceed the total synthesis of glycosylated EPO in a sequential C-to-N manner. Firstly, the peptide thioester of **67** from acetylacetone activation reacted with **68** as the first NCL to form the ligated product, which was then subjected to Thz opening to afford the desired **69** in 41 % yield after HPLC purification. Through the similar procedure, the second NCL was performed between activated **66** and **69**, followed by deThz to generate **70** in 33 % isolated yield. After the 3rd NCL between the peptide thioester of **65** and **70**, the ligated segment was desulfurized followed by deAcm to generate **73** in 51 % HPLC yield. In the 4th NCL, **73** was ligated with **64** thioester to provide the EPO linear form **74** in a reasonable yield. Finally, we performed a refolding process to generate the well-folded EPO **75** with homogeneous GlcNAclyations, which was confirmed by UPLC-MS and circular dichroism (CD) spectra (Figure 5B–5D). In this synthesis, our NBD strategy offers several advantages, including synthetic convenience, inexpensive materials, and no requirements of tedious synthetic work.

In conclusion, we have designed and developed *N*,*O*-benzylidene acetal dipeptides (NBDs) as easy-to-handle and highly effective building blocks to enable syntheses of difficult peptides and proteins. This strategy allows direct synthesis of a variety of previously inaccessible peptides and proteins, including glycemic-regulation hormone Amylin, Crambin, Ubiquitin, and potent leukocyte chemokine RANTES. Meanwhile, we have demonstrated NBD's viability in suppressing aspartimide formation during the synthesis of the N-glycosylated EPO, supporting the use of NBDs as a powerful strategy for the synthesis of N-linked glycoproteins.

The wide structure scope (all Xaa residues except proline are covered), scalability, and cost-effectiveness (with a sub-kg synthesis of Thr-Thr NBD demonstrated in a cost-effective manner) of Xaa-Ser/Thr NBDs allowed for the high yield and unprecedented construction of gram-scale inflammatory drug human vasoactive intestinal peptide (VIP) and hundreds of milligram scale histone 2B protein, which will be crucial for the downstream biological studies and development of novel therapeutics. We believe that NBDs, as powerful tools to enable the solid-phase peptide synthesis of difficult sequences, will be appealing for both academic research and industrial applications.

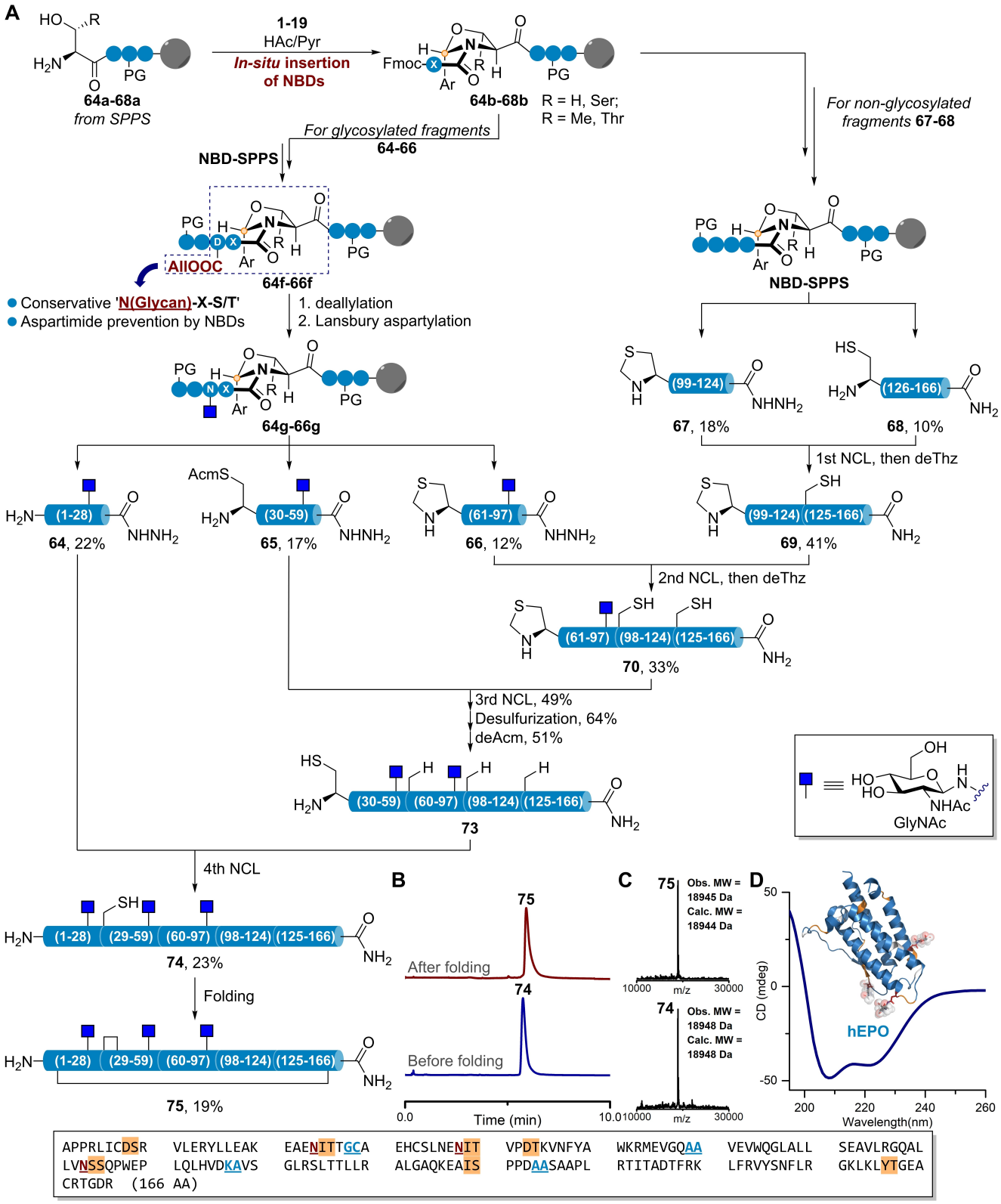

Figure 5. Chemical synthesis of EPO. A. Scheme of on-resin NBDs installation and the following NCL to assemble EPO (1–166); B. UV spectra of EPO before and after folding; C. Deconvolution spectra of folded and unfolded EPO; D. CD spectrum of folded EPO. The 3D structure was calculated in silico based on AlphaFold[64] and GlyProt.[65]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Chemical Protein Synthesis · Difficult Peptide · Dipeptide Reagent · Hydrophobic Peptide · Solid-Phase Peptide Synthesis

[1] W. Li, F. Li, X. Zhang, H.-K. Lin, C. Xu, *Signal Transduction Targeted Ther.* **2021**, *6*, 422.
[2] L. Chen, A. Kashina, *Front. Cell Dev. Biol.* **2021**, *9*, 719590.
[3] E. R. Gibney, C. M. Nolan, *Heredity* **2010**, *105*, 4–13.
[4] L. A. Farrelly, R. E. Thompson, S. Zhao, A. E. Lepack, Y. Lyu, N. V. Bhanu, B. Zhang, Y. Loh, A. Ramakrishnan, K. C. Vadodaria, K. J. Heard, G. Erikson, T. Nakadai, R. M. Bastle, B. J. Lukasak, H. Zebroski, N. Alenina, M. Bader, O. Berton, R. G. Roeder, H. Molina, F. H. Gage, L. Shen, B. A. Garcia, H. Li, T. W. Muir, I. Maze, *Nature* **2019**, *567*, 535–539.
[5] G. P. Dann, G. P. Liszczak, J. D. Bagert, M. M. Muller, U. T. T. Nguyen, F. Wojcik, Z. Z. Brown, J. Bos, T. Panchenko, R. Pihl, S. B. Pollock, K. L. Diehl, C. D. Allis, T. W. Muir, *Nature* **2017**, *548*, 607–611.
[6] K. T. Schjoldager, Y. Narimatsu, H. J. Joshi, H. Clause, *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 729–749.
[7] P. Wang, S. Dong, J. Shieh, E. Peguero, R. Hendrickson, M. A. S. Moore, S. J. Danishefsky, *Science* **2013**, *342*, 1357–1360.
[8] M. Murakami, T. Kiuchi, M. Nishihara, K. Tezuka, R. Okamoto, M. Izumi, Y. Kajihara, *Sci. Adv.* **2016**, *2*, e1500678.
[9] Y. Maki, R. Okamoto, M. Izumi, Y. Kajihara, *J. Am. Chem. Soc.* **2020**, *142*, 20671–20679.
[10] H. Li, J. Zhang, C. An, S. Dong, *J. Am. Chem. Soc.* **2021**, *143*, 2846–2856.
[11] J. M. Lee, H. M. Hammaren, M. M. Savitski, S. H. Baek, *Nat. Commun.* **2023**, *14*, 201.
[12] N. P. Marotta, Y. H. Lin, Y. E. Lewis, M. R. Ambroso, B. W. Zaro, M. T. Roth, D. B. Arnold, R. Langen, M. R. Pratt, *Nat. Chem.* **2015**, *7*, 913–920.
[13] D. Ellmer, M. Brehs, M. Haj-Yahya, H. A. Lashuel, C. F. W. Becker, *Angew. Chem. Int. Ed.* **2019**, *58*, 1616–1620.
[14] F. Ardito, M. Giuliani, D. Perrone, G. Troiano, L. L. Muzio, *Int. J. Mol. Med.* **2017**, *40*, 271–280.
[15] H. Jiang, X. Zhang, X. Chen, P. Aramsangtienchai, Z. Tong, H. Lin, *Chem. Rev.* **2018**, *118*, 919–988.
[16] C. C. Hanna, J. Kriegesmann, L. J. Dowman, C. F. W. Becker, R. J. Payne, *Angew. Chem. Int. Ed.* **2022**, *61*, e202111266.
[17] H. Dorai, S. Ganguly, *Curr. Opin. Biotechnol.* **2014**, *30*, 198–204.
[18] Y. Tan, H. Wu, T. Wei, X. Li, *J. Am. Chem. Soc.* **2020**, *142*, 20288–20298.
[19] O. Harel, M. Jbara, *Angew. Chem. Int. Ed.* **2023**, *62*, e202217716.
[20] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776–779.
[21] Y. Zhang, C. Xu, H. Lam, C. Lee, X. Li, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 6657–6662.
[22] H. Liu, X. Li, *Acc. Chem. Res.* **2018**, *51*, 1643–1655.
[23] Y. Tan, J. Li, K. Jin, J. Liu, Z. Chen, J. Yang, X. Li, *Angew. Chem. Int. Ed.* **2020**, *59*, 12741–12745.
[24] J. W. Bode, *Acc. Chem. Res.* **2017**, *50*, 2104–2115.
[25] N. J. Mitchell, L. R. Malins, X. Liu, R. E. Thompson, B. Chan, L. Radom, R. J. Payne, *J. Am. Chem. Soc.* **2015**, *137*, 14011–14014.
[26] V. Agouridas, O. E. Mahdi, V. Diemer, M. Cargoet, J.-C. M. Monbaliu, O. Melnyk, *Chem. Rev.* **2019**, *119*, 7328–7443.
[27] Z. Sun, W. Ma, Y. Cao, T. Wei, X. Mo, H. Y. Chow, Y. Tan, C. H. P. Cheung, J. Liu, H. K. Lee, E. C. M. Tse, H. Liu, X. Li, *Chem* **2022**, *8*, 2542–2557.
[28] T. W. Muir, D. Sondhi, P. A. Cole, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6705–6710.
[29] V. Agouridas, V. Diemer, O. Melnyk, *Curr. Opin. Chem. Biol.* **2020**, *58*, 1–9.
[30] C. Zuo, B. Zhang, B. Yan, J. Zheng, *Org. Biomol. Chem.* **2019**, *17*, 727–744.
[31] H. Wu, T. Wei, W. L. Ngai, H. Zhou, X. Li, *J. Am. Chem. Soc.* **2022**, *144*, 14748–14757.
[32] M. Paradís-Bas, J. Tulla-Puche, F. Albericio, *Chem. Soc. Rev.* **2016**, *45*, 631–654.
[33] J. Liu, T. Wei, Y. Tan, H. Liu, X. Li, *Chem. Sci.* **2022**, *13*, 1367–1374.
[34] S. Tsuda, M. Mochizuki, H. Ishiba, K. Yoshizawa-Kumagaye, H. Nishio, S. Oishi, T. Yoshiya, *Angew. Chem. Int. Ed.* **2018**, *57*, 2105–2109.
[35] M. T. Jacobsen, M. E. Petersen, X. Ye, M. Galibert, G. H. Lorimer, V. Aucagne, M. S. Kay, *J. Am. Chem. Soc.* **2016**, *138*, 11775–11782.
[36] D. Zhao, X. Fan, H. Hao, H. Zhang, Y. Guo, *Curr. Org. Chem.* **2019**, *23*, 2–13.
[37] J. B. Li, S. Tang, J. S. Zheng, C. L. Tian, L. Liu, *Acc. Chem. Res.* **2017**, *50*, 1143–1153.
[38] H. Wu, Y. Tan, W. L. Ngai, X. Li, *Chem. Sci.* **2023**, *14*, 1582–1589.
[39] A. K. Tickler, J. D. Wade, *Curr. Protoc. Protein Sci.* **2007**, *50*, 18.8.1–18.8.6.
[40] R. C. D. Milton, S. C. F. Milton, P. A. Adams, *J. Am. Chem. Soc.* **1990**, *112*, 6039–6046.
[41] V. Krchnak, Z. Flegelova, J. Vagner, *Int. J. Pept. Protein Res.* **1993**, *42*, 450–454.
[42] S. B. H. Kent, *Annu. Rev. Biochem.* **1988**, *57*, 957–989.
[43] C. Hyde, T. Johnson, R. C. Sheppard, *J. Chem. Soc. Chem. Commun.* **1992**, 11573–1575.
[44] T. Haack, M. Mutter, *Tetrahedron Lett.* **1992**, *33*, 1589–1592.
[45] T. Johnson, M. Quibell, D. Owen, R. C. Sheppard, *J. Chem. Soc. Chem. Commun.* **1993**, 369–372.
[46] Y. Sohma, M. Sasaki, Y. Hayashi, T. Kimura, Y. Kiso, *Chem. Commun.* **2004**, 124–125.
[47] X. Li, H. Y. Lam, Y. Zhang, C. K. Chan, *Org. Lett.* **2010**, *12*, 1724–1727.
[48] H. Chang, B. Liu, Y. Qi, Y. Zhou, Y. Chen, K. Pan, W. Li, X. Zhou, W. Ma, C. Fu, Y. Qi, L. Liu, Y. Gao, *Angew. Chem. Int. Ed.* **2015**, *54*, 11760–11764.

[49] Y. Asahina, S. Komiya, A. Ohagi, R. Fujimoto, H. Tamagaki, K. Nak-agawa, T. Sato, S. Akira, T. Takao, A. Ishii, Y. Nakahara, H. Hojo, *Angew. Chem. Int. Ed.* **2015**, *54*, 8226–8230.
[50] C. E. Murar, M. Ninomiya, S. Shimura, U. Karakus, O. Boyman, J. W. Bode, *Angew. Chem. Int. Ed.* **2020**, *59*, 8425–8429.
[51] W. R. Sampson, H. Patsiouras, N. J. Ede, *J. Pept. Sci.* **1999**, *5*, 403–409.
[52] S. R. Manne, A. Chakraborty, K. Rustler, T. Bruckdorfer, B. G. D. L. Torre, F. Albericio, *ACS Omega* **2022**, *7*, 28487–28492.
[53] A. J. Korman, S. C. Garrett-Thomson, N. Lonberg, *Nat. Rev. Drug Discovery* **2022**, *21*, 509–528.
[54] D. M. Pardoll, *Nat. Rev. Cancer* **2012**, *12*, 252–264.
[55] X. Du, F. Tang, M. Liu, J. Su, Y. Zhang, W. Wu, M. Devenport, C. A. Lazarski, P. Zhang, X. Wang, P. Ye, C. Wang, E. Hwang, T. Zhu, T. Xu, P. Zheng, Y. Liu, *Cell Res.* **2018**, *28*, 416–432.
[56] Q. Zhang, J. Bi, X. Zheng, Y. Chen, H. Wang, W. Wu, Z. Wang, Q. Wu, H. Peng, H. Wei, R. Sun, Z. Tian, *Nat. Immunol.* **2018**, *19*, 723–732.
[57] J. Spengler, J. B. Blanco-Canosa, L. Forni, F. Albericio, *Org. Lett.* **2018**, *20*, 4306–4309.
[58] L. A. Carpino, E. Krause, C. D. Sferdean, M. Schumann, H. Fabian, M. Bienert, M. Beyermann, *Tetrahedron Lett.* **2004**, *45*, 7519–7523.
[59] D. Bang, N. Chopra, S. B. H. Kent, *J. Am. Chem. Soc.* **2004**, *126*, 1377–1383.
[60] F. García-Martín, P. White, R. Steinauer, S. Cote, J. Tulla-Puche, F. Albericio, *Pept. Sci.* **2006**, *84*, 566–575.
[61] F. El Oualid, R. Merkx, R. Ekkebus, D. S. Hameed, J. J. Smit, A. D. Jong, H. Hilkmann, T. K. Sixma, H. Ovaa, *Angew. Chem. Int. Ed.* **2010**, *49*, 10149–10153.
[62] S. Nadal, R. Raj, S. Mohammed, B. G. Davis, *Curr. Opin. Chem. Biol.* **2018**, *45*, 35–47.
[63] P. Siman, S. V. Karthikeyan, M. Nikolov, W. Fischle, A. Brik, *Angew. Chem. Int. Ed.* **2013**, *52*, 8059–8063.
[64] J. Jumper, R. Evans, A. Prtzel, T. Green, M. Figurnov, O. Ronneberger, K. Tunyasuvunakool, R. Bates, A. Zidek, A. Potapenko, C. Meyer, S. A. A. Kohl, A. J. Ballard, A. Cowie, B. Romera-Paredes, S. Nikolov, R. Jain, J. Adler, T. Back, S. Petersen, D. Reiman, E. Clancy, M. Zielinski, M. Steinegger, M. Pacholska, T. Berghammer, S. Bodenstein, D. Silver, O. Vinyals, A. W. Senior, K. Kavukcuoglu, P. Kohli, D. Hassabis, *Nature* **2021**, *596*, 583–589.
[65] A. Bohne-Lang, C.-W. von der Lieth, *Nucleic Acids Res.* **2005**, *33*, W214–W219.

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