Title: Peptide Synthesis Using Unprotected Amino Acids

Authors: Tao Liu†, Zejun Peng†, Junfeng Zhao*

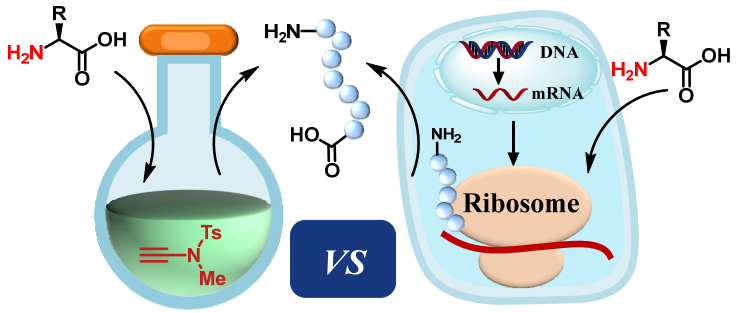
Affiliations:

Key Laboratory of Molecular Target & Clinical Pharmacology and the NMPA & State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences & The Fifth Affiliated Hospital; Guangzhou Medical University, No.1 Xinzao, Panyu District, Guangzhou, 511436, China

*Corresponding author. Email: zhaojf@gzhmu.edu.cn
†These authors contributed equally.

Abstract: The mandatory use of N^{α}-protecting group for amino acids, which invokes protection and deprotection operations and imposes a significant negative effect on environment sustainable development, constitutes the essential principle of peptide synthesis. Using unprotected amino acids for peptide synthesis represents an ideal and attractive greening strategy. However, this strategy has been unsuccessful for more than 60 years owing to the severe epimerization that occurs during N→C peptide chain elongation. The first general unprotected amino acid strategy was developed in this study by employing a ynamide coupling reagent, which successfully addressed the notorious epimerization issue. This step- and atom-economic strategy is not only effective for synthesizing dipeptides but also adaptable to longer peptides containing more than two amino acids. The synthetic application of this method was exemplified by successful syntheses of a range of peptide active pharmaceutical ingredients (APIs). It is foreseeable that this study will bring about disruptive technologies to both academia and industry.

One-Sentence Summary: Ynamide coupling reagent enables peptide synthesis using unprotected amino acids as building blocks.



Main Text:

Introduction

Peptides are chains of amino acids linked via amide bonds. They are of prime importance in the regulation and maintenance of all biological processes and play important roles in the fields of biology, chemistry, and medicine. Consequently, peptides are widely used in drug discovery, vaccines, dietary supplements, cosmetics, and material science. Owing to their remarkable potency, high specificity, low toxicity, and innovations in drug delivery and formulation, peptide therapeutics[1,2] have witnessed a rapid renaissance over the past three decades, with over 80 peptide drugs currently on the market. With an increasing number of peptide drugs, in particular the orally active ones with low bioavailability[3,4], reaching the market, the demand for peptide active pharmaceutical ingredients (APIs) increases dramatically. Despite the possibility of the preparation of natural peptides using biological methods, most peptide drugs are synthesized chemically because non-natural amino acids and chemical modifications are typically necessary to enhance the poor chemical and physical stabilities and short circulation times of natural peptides.

Owing to the bifunctional feature of amino acids, protection of the non-participating amino group of one amino acid and the carboxylic group of the other amino acid is mandatory to construct a target dipeptide. Theoretically, either the N- or C-terminal protecting group of the aforementioned dipeptide could be removed to incorporate the third N^{α}-protected or C-protected amino acid to execute peptide synthesis via the C→N[5] or N→C[6] elongation mode, respectively. However, in contrast to the ribosomal peptide synthesis that proceeds in the N→C direction, chemical peptide synthesis is required to be executed via the C→N elongation mode owing to severe racemization/epimerization that occurs during the N→C peptide elongation. The N→C elongation mode involves the iterative repetition of activation and aminolysis of peptide acids, the racemization/epimerization degree of which is usually 35–110 times higher than that of the corresponding carbamate-protected amino acids[7]. Consequently, a four-step cycle, including N^{α}-protection, activation, coupling, and deprotection, is required to incorporate a single amino acid into the growing peptide chain[8,9]. This essential principle of chemical peptide synthesis results in poor step- and atom-economy as both the introduction and removal of the N^{α}-protecting group require extra reagents, time, energy, and labor, which not only increases the production cost but also imposes significant negative effects on sustainable development. Solid-phase peptide synthesis (SPPS) exacerbates this situation because it requires large excesses of N^{α}-protected amino acids, coupling reagents, racemization suppressors, base additives, and deprotection reagents to ensure maximal conversion for each step. As a result, hundreds of atoms are wasted in constructing a single peptide bond, and metric tons of chemical waste is generated to produce each kilogram of peptide[10]. In the last two decades, the development of general sustainable methods for peptide bond formation has been identified as one of the top ten challenges in organic synthesis for twice[11,12]. Furthermore, current peptide synthesis relies heavily on legacy technologies and reagents developed in the 1950s-1980s, before the establishment of green chemistry principles. In addition, most of these technologies have inherent limitations, and only the innovative approaches to peptide-bond formation can address these problems.

Various efforts have been made towards developing atom-economic peptide synthesis strategies[13], among which the use of unprotected amino acids as building blocks has shown the most promise because it eliminates the need for protection and deprotection steps[14]. The significant amount of chemicals, time, energy, solvent, and labor needed and waste generated during the protection and deprotection steps could be eliminated. However, to avoid homocoupling and polymerization, the

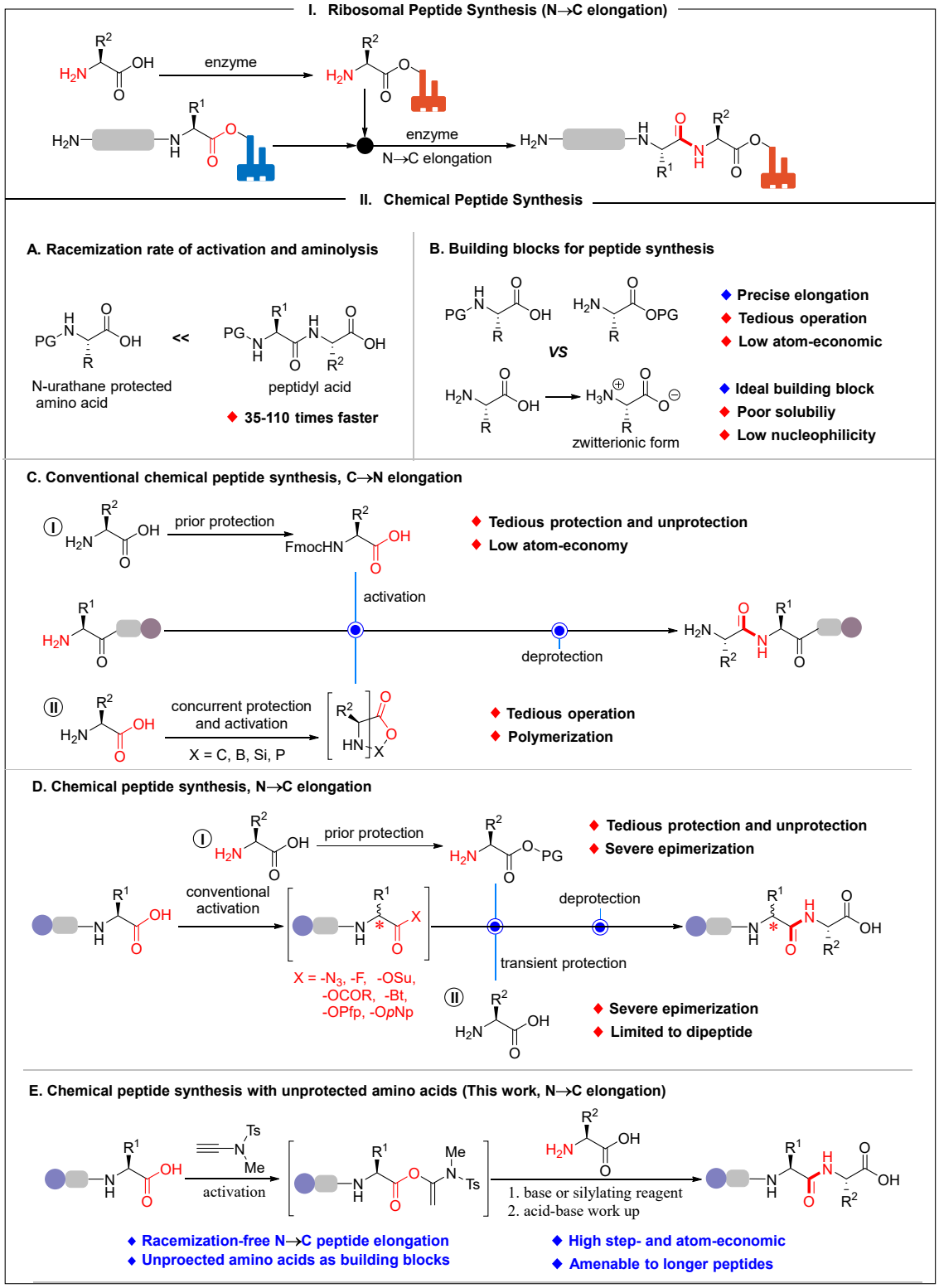


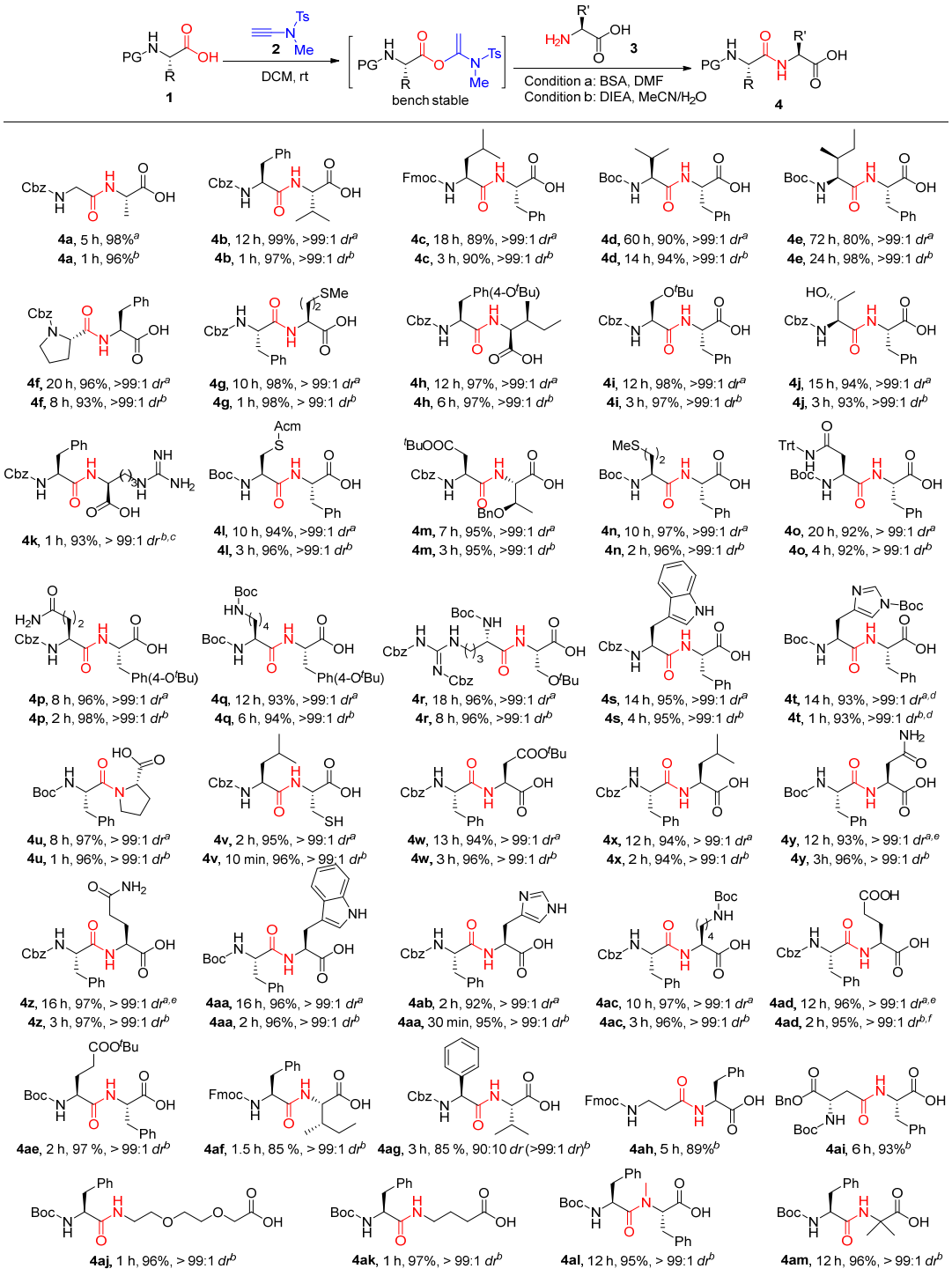
Fig. 1. Ribosomal and Chemical Peptide Synthesis

peptide synthesis with unprotected amino acids is required to follow the highly epimerization-prone N→C elongation mode[7]. Since a pioneering study conducted in the 1960s, various activated

amino acid derivatives such as acyl azides[15], acyl fluorides[16], N-hydroxysuccinimide esters[17], substituted phenol esters[18,19], N-(Z-α-aminoacyl)benzotriazoles [20], and mixed anhydrides[21,22] have been employed for reacting with unprotected amino acids. Unfortunately, these methods are adaptable to only dipeptides because of the severe epimerization that occurs in the case of longer peptides[23]. Although the micro-flow technology has expanded the use of the unprotected amino acid strategy to tripeptides and tetrapeptides with a low level of epimerization, it is far from practical application[24]. Meanwhile, the C→N peptide synthesis, which takes advantage of simultaneous protection–activation of unprotected amino acids, has also been explored. The *in situ* formed five-membered rings of unprotected amino acids such as N-carboxyanhydrides[25], oxazolidinones[26], cyclic borates[27], cyclic silyl derivatives[28], and cyclic phosphoric-amino acid mixed anhydride[29] not only protect the amino acids but also activate them. However, these strategies are highly prone to polymerization, which limits their application. Very recently, Yamamoto and his coworkers[30,31] developed a C→N unprotected amino acid peptide synthesis by employing a transient masking strategy with silylating reagents. However, this approach is currently limited to single peptide bond formations. The rapidly growing demand in the peptide therapeutic market requires cost-effective and environmentally benign peptide manufacturing. Herein, we report the first general peptide synthesis with unprotected amino acids to furnish the target peptides in a racemization/epimerization-free manner using an ynamide coupling reagent. In contrast to conventional unprotected amino acid strategies, which are limited to the synthesis of dipeptides, this strategy is also applicable to longer peptides containing more than two amino acid residues. A series of peptide APIs containing two to nine amino acids were efficiently prepared by employing this atom- and step-economic method.

Results and discussion

Recently, our group has reported that ynamides could be used as coupling reagents for amide bond formation[32,33]. Ynamide coupling reagents exhibit a remarkable superiority in suppressing the racemization of carboxylic acids containing an α-chiral center, thereby enabling a peptide API such as carfilzomi to be synthesized in an N→C elongation manner without any epimerization[34]. Most importantly, ynamide-mediated peptide-bond formation proceeds with an active vinyl ester as the stable intermediate, which offers great flexibility and opportunities for optimizing the aminolysis reaction conditions. We envisioned that a ynamide coupling reagent might be a promising tool for addressing the racemization/epimerization issue of N→C peptide synthesis using unprotected amino acids as the building blocks. Unprotected amino acids are typically in the zwitterionic form, which not only reduces the nucleophilicity of the amino group but also renders them insoluble in most organic solvents. Two *in situ* transient protection strategies including base additives[17,35] and silylating reagents[22,29,36] have been employed to address the solubility and nucleophilicity issues of unprotected amino acids. Silylating reagents and base additives destroy zwitterionic amino acids by transforming the carboxylic group into a silyl ester[29] and a salt, respectively, with the concurrent release of the free amino group with recovered nucleophilicity. A significant advantage of both strategies is that the transient protection proceeds spontaneously at room temperature, and the transiently protected amino acids can be used directly for aminolysis without purification. More importantly, no additional deprotection step is required to remove the transient protecting group because the carboxyl group was released simultaneously during the acid-base work-up, thus allowing transient protection, activation, aminolysis, and *in situ* deprotection to be performed in a one-pot manner. Unprotected H–Phe–OH and the active vinyl ester Boc-Ala-OY derived from Boc-Ala-OH and N-methylynetoluenesulfonamide (MYTsA)



Reaction conditions: **1** (0.5 mmol), **2** (0.55 mmol), DCM (3 mL), [a]**3** (1 mmol), BSA (1 mmol), DMF (5 mL); [b]**3** (0.75 mmol), DIEA (0.75 mmol), MeCN (4 mL), H_2O (2 mL); [c]Without additive; [d]Boc-His(Boc)-OH (1 mmol), **2** (0.5 mmol); [e]BSA (2 mmol), [f]1.5 mmol DIEA.

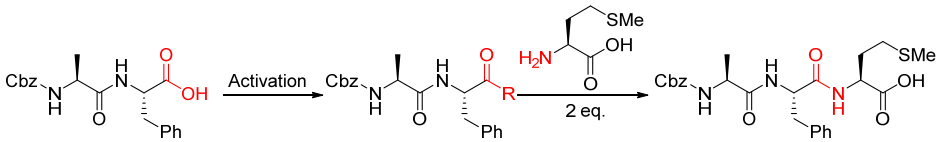
Fig. 2. Dipeptide syntheses using unprotected amino acids as the nucleophilic partner

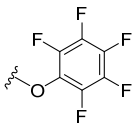
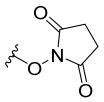
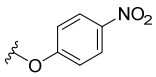
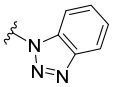
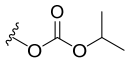
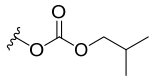
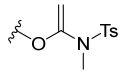
were employed as the model substrates to explore the aminolysis reaction conditions (for details, see Supporting Information). A preliminary study revealed that both silylating reagents and base additives were effective in our reaction system (for details, see Supporting Information). In terms of silylating reagents, bis(trimethylsilyl)acetamide (BSA), N-methyl-N-trimethylsilyl-acetamide (MSA), N-(trimethylsilyl)acetamide (TMSA), and 1,3-bis(trimethylsilyl)urea (BSU) were efficient additives for addressing the solubility and nucleophilicity issues of unprotected amino acids by spontaneously transforming them into N,O-bis(trimethylsilyl)amino acids or O-silylamino acids at room temperature. The target dipeptide was obtained in 95% yield without any detectable epimerization when a solution of unprotected H-Phe-OH and the cheap and readily available silylating reagent BSA was treated with an active vinyl ester of Boc-Ala-OH in DMF (Table S1, entry 4). The loading of BSA and the unprotected amino acid could be decreased to 2 equiv. while retaining a good reaction efficiency. As in the case of silylating reagents, treatment of zwitterionic unprotected amino acids with a base not only made them soluble but also released the nucleophilic amino group. N, N-Diisopropylethylamine (DIEA) was identified as the best choice for the base. Interestingly, a remarkable improvement in the reaction efficiency was observed when water was used as the co-solvent[33]. Finally, the mixture of acetonitrile and water in the 2:1 ratio proved to be the optimal media for the aminolysis reaction. The loading of unprotected amino acid and the DIEA additive could be decreased to 1.5 equiv. without a significant negative effect on the reaction. However, a further decrease in the loadings resulted in prolonged reaction times and lower yields. It should be noted that no detectable epimerization was observed for both silylating and base additive strategies. In addition, the transient protecting group, excess unprotected amino acid, silylating reagent, and base additive could be readily removed via an acid–base work-up to conveniently afford the target peptidyl acids.

With the two sets of optimized reaction conditions, we evaluated 20 proteinogenic amino acids for their ability to serve as electrophilic carboxyl and nucleophilic amino partners. The N^{α}-protected amino acids were activated by the ynamide coupling reagent MYTsA to produce active vinyl esters, which were used directly in the aminolysis step without further purification. In the case of the silylating reagent strategy, the mixture of the unprotected amino acid and silylating reagent BSA was stirred at room temperature for 1 h before it was mixed with the solution of the active vinyl ester in DMF and stirred until the active ester was completely consumed. Regarding the base additive strategy, a mixture of MeCN/H_2O (2:1) was used as the solvent for the aminolysis of the active vinyl ester. The mixture of DIEA and unprotected amino acids was stirred in MeCN/H_2O for a few minutes and then added to the solution of active vinyl ester. In both these strategies, an acid–base work-up after the aminolysis reaction afforded the target dipeptide acids in excellent yields. As shown in **Fig. 2**, all 20 proteinogenic amino acids could be used as both carboxyl and amino partners to provide the target dipeptide acids in excellent yields without any detectable racemization/epimerization in either reaction system. Even sterically demanding amino acids such as Val, Ile, Pro, and Aib were tolerated as amino partners to afford the target dipeptides in excellent yields, although longer reaction times were required when they were used as carboxyl partners. Despite side-chain functional groups such as -SH, -CO_2H, -NH_2, -OH of phenol, and NH of imidazole must be protected when they are present in the carboxyl partners, their protection (except for Tyr and Lys) was unnecessary when the corresponding amino acids were used as the amino partners. It was noted that the aminolysis reaction times were dramatically shortened when the side-chain unprotected Cys and His were used as the amino partners, which might be attributed to the side-chain assistance effect. N-methyl α-amino acids and non-natural amino acids (**Fig. 2**, **4ah-4am**) such as Aib, β-amino acids, and γ-amino acids were also compatible. Generally, both the

silylating reagent and base additive strategies provided comparable excellent results in terms of yield and chiral integrity retention, albeit longer aminolysis reaction times were required for the former. In addition, chromatography purification was not required for most of these dipeptide acids, except for a few oily products, because a simple acid–base work-up or recrystallization offered pure dipeptide acids, provided they were solid. It is worth noting that the one-pot transient protection, activation, aminolysis, and *in situ* deprotection allowed the preparation of dipeptide acids in one step, while at least three steps were required using traditional peptide synthesis strategies.

Table 1. Comparative Study of Hydrolysis and Epimerization During Tripeptide Synthesis[a]

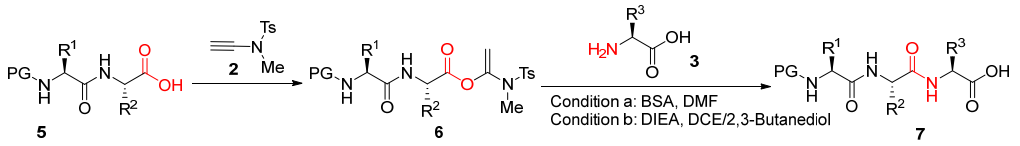


entry	R	additive	solvent	time	*dr* (**6**)[b]	*dr* (**7g**)[b]	yield (**5g**)[c]	yield (**7g**)[c]
1		DIEA (2 eq.)	MeCN/H_2O (2:1)	1 h	60:40	68:32	23%	69%
2		$NaHCO_3$ (2 eq.)	1, 4-Diox/H_2O (1:1)	2 h	60:40	80:20	34%	60%
3		$NaHCO_3$ (2 eq.)	1, 4-Diox/H_2O (1:1)	3 h	63:37	64:36	10%	84%
4		BSA (4 eq.)	DCM	9 h	63:37	65:35	1%	93%
5		TEA (2 eq.)	THF/H_2O (1:1)	1 h	73:27	68:32	9%	83%
6		TEA (2 eq.)	MeCN/H_2O (2:1)	4 h	--	65:35	10%	81%
7		TEA (3 eq.), $NaHCO_3$ (2 eq.)	MeCN/H_2O (2:1)	1 h	--	67:33	34%	59%
8		NMM (1 eq.), DIEA (2 eq.)	MeCN/H_2O (2:1)	1 h	--	68:32	7%	85%
9		DIEA (2 eq.)	DCM/2,3-Butanediol (1:1)	12 h	> 99:1	> 99:1	< 1%	92%
10		BSA (2 eq.)	DCM	16 h	> 99:1	> 99:1	< 1%	95%

[a]Reaction conditions: **6** (0.1 mmol), H-Met-OH (0.2 mmol); solvent (1 mL); [b]Determined by HPLC; [c]Isolated yield.

With the success of the dipeptide synthesis, we moved forward to the considerably more challenging tripeptide synthesis, which involved the activation and aminolysis of highly epimerization-prone dipeptide acids. As expected, severe epimerization—i.e., 85:15 and 87:13 *dr* for silylating and base additive strategies, respectively—was observed when the active vinyl ester of dipeptide Cbz-Ala-Leu-OH was treated with unprotected H-Phe-OH under either optimized dipeptide synthesis reaction system. This result was consistent with that of conventional

unprotected amino acid strategies and further confirmed that the α-chiral-center of peptide C-terminus has a greater tendency to epimerization than that of the N^{α}-carbomate-protected amino acids. Fortunately, our study revealed that the epimerization was occurred during the aminolysis step but not the activation step. This result is rather important because it is different from that of the conventional unprotected amino acid strategies, wherein epimerization was occurred not only in the aminolysis step but also activation step (Table 1). It was promising because the ynamide coupling reagent opened up an opportunity to address the notorious epimerization issue by optimizing the aminolysis reaction conditions. Next, extensive optimization was performed for both the base and silyl reagent strategies (Tables S3 and S4). The aminolysis of the dipeptide active ester was faster than that of the corresponding N^{α}-protected amino acid, indicating that the dipeptide active ester was more reactive than the carbomate protected α-amino acid. Therefore, we hypothesized that the epimerization would be alleviated if the aminolysis reactivity of the dipeptide active ester was attenuated. Our previous study suggested that the solvent polarity had a strong effect on the aminolysis rate [33]. To our delight, the epimerization of the silylating reagent strategy was significantly reduced when the aminolysis solvent DMF was replaced by the less polar acetonitrile, and the epimerization could even be completely suppressed by employing the less polar dichloroethane (DCE) as the aminolysis solvent (Table S3, entries 6-9). This feature offered an additional advantage because the preparations of active dipeptide vinyl ester and O-silylamino acids were both performed in DCE, implying that no solvent replacement was involved in the entire peptide bond formation process. In terms of the base additive strategy, we noted that the co-solvent water was the major contributor to the epimerization because of its accelerating effect (Table S4, entries 1-10) [33]. Remarkable suppression of epimerization was accomplished when the water cosolvent was replaced by a protonic solvent such as ethylene glycol. However, an esterification side reaction of ethylene glycol was observed. Further optimization suggested that the sterically hindered 2,3-butanediol was an optimal co-solvent for suppressing the esterification side reactions by exploiting its steric bulkiness. Finally, a 1:1 mixture of 2,3-butanediol and DCE or dichloromethane (DCM) was identified as the best solvent with DIEA as the base additive (Table S4, entry 18). To demonstrate the generality of these strategies, the feasibility of 20 proteinogenic amino acids serving as acyl and amino partners was evaluated under the optimized silylating (condition A) as well as base additive (condition B) reaction conditions (**Fig. 3**). To our delight, all 20 proteinogenic amino acids, including sterically demanding Val and Ile worked well as both coupling partners to provide the target tripeptides in excellent yields under both reaction systems. Despite low levels of epimerization (<3%) were observed for a few tripeptide substrates under the base additive reaction conditions, no epimerization was observed for any of the tripeptide substrates on using the silylating reagent strategy. Fortunately, the undesired minor epimer produced in the base additive strategy could be readily removed by simple recrystallization to obtain the target tripeptides with excellent stereochemistry (>99:1 *dr*). Although a two-step strategy was employed to study tripeptide synthesis in detail, all the tripeptides could be synthesized in a two-step, one-pot manner with comparable or better efficacy. As in dipeptide synthesis, the aminolysis rate was affected by the bulkiness of the acyl donor; therefore, longer reaction times were required for dipeptide acids with bulky Val and Ile C-terminus (**Fig. 3**, **7d** and **7e**). Furthermore, no additional deprotection step was required because the transient protecting group could be removed during the acid–base work-up. Generally, acidic aqueous treatment followed by simple recrystallization afforded pure target tripeptides with excellent chirality retention and yields provided the tripeptides were solid.



5 6 7

7a, 6 h, 97%[a]
7a, 1 h, 95%[b]

7b, 8 h, 92%, >99:1 *dr*[a]
7b, 6 h, 93%, 98:2 *dr*[b], >99:1 *dr*[c]

7c, 14 h, 95%, >99:1 *dr*[a]
7c, 14 h, 95%, >99:1 *dr*[b], >99:1 *dr*[c]

7d, 48 h, 92%, >99:1 *dr*[a]
7d, 40 h, 91%, 98:2 *dr*[b], >99:1 *dr*[c]

7e, 60 h, 94%, >99:1 *dr*[a]
7e, 72 h, 88%, >99:1 *dr*[b], >99:1 *dr*[c]

7f, 24 h, 95%, >99:1 *dr*[a]
7f, 4 h, 93 %, >99:1 *dr*[d], >99:1 *dr*[b]

7g, 8 h, 95%, >99:1 *dr*[a]
7g, 7 h, 93%, >99:1 *dr*[b], > 99:1 *dr*[c]

7h, 10 h, 93%, >99:1 *dr*[a]
7h, 23 h, 90%, >99:1 *dr*[b], >99:1 *dr*[c]

7i, 15 h, 95%, >99:1 *dr*[a]
7i, 12 h, 92% , 97:3 *dr*[b], >99:1 *dr*[c]

7j, 15 h, 94%, >99:1 *dr*[a]
7j, 12 h, 88%, >99:1 *dr*[b], >99:1 *dr*[c]

7k, 16 h, 94%, >99:1 *dr*[a]
7k, 20 h, 92% , >99:1 *dr*[b], >99:1 *dr*[c]

7l, 7 h, 95%, >99:1 *dr*[a]
7l, 7 h, 93%, 98:2 *dr*[b], >99:1 *dr*[c]

7m, 9 h, 95%, >99:1 *dr*[a]
7m, 6 h, 96%, >99:1 *dr*[b], >99:1 *dr*[c]

7n, 10 h, 98%, >99:1 *dr*[a]
7n, 7 h, 93%, >99:1 *dr*[b], >99:1 *dr*[c]

7o, 16 h, 97%, >99:1 *dr*[a]
7o, 6 h, 94%, 98:2 *dr*[b], >99:1 *dr*[c]

7p, 14 h, 89%, >99:1 *dr*[a]
7p, 8 h, 85%, >99:1 *dr*[b], >99:1 *dr*[c]

7q, 16 h, 94%, >99:1 *dr*[a]
7q, 23 h, 92%, 98:2 *dr*[b], >99:1 *dr*[c]

7r, 72 h, 93%, >99:1 *dr*[a]
7r, 72 h, 85%, >99:1 *dr*[b], > 99:1 *dr*[c]

7s, 14 h, 87% >99:1 *dr*[a]
7s, 8 h, 87%, 98:2 *dr*[b], >99:1 *dr*[c]

7t, 24 h, 92% >99:1 *dr*[a]
7t, 24 h, 89%, >99:1 *dr*[b], >99:1 *dr*[c]

7u, 16 h, 94%, >99:1 *dr*[a]
7u, 8 h, 95%, >99:1 *dr*[b], >99:1 *dr*[c]

7v, 16 h, 96%, > 99:1 *dr*[a]
7v, 12 h, 89%, 97: 3 *dr*[b], > 99:1 *dr*[c]

7w, 15 h, 94%, > 99:1 *dr*[a]
7w, 10 h, 89%, 97:3 *dr*[b], > 99:1 *dr*[c]

7x, 48 h, 95%, >99:1 *dr*[a]
7x, 5 h, 97%, >99:1 *dr*[b], >99:1 *dr*[c]

7y, 24 h, 97%, >99:1 *dr*[a]
7y, 6 h, 94%, 98:2 *dr*[b], > 99:1 *dr*[c]

7z, 36 h, 96%, >99:1 *dr*[a]
7z, 10 h, 91%, 97:3 *dr*[b], >99:1 *dr*[c]

7aa, 20 h, 93%, >99:1 *dr*[a]
7aa, 7 h, 95%, 99:1 *dr*[b], >99:1 *dr*[c]

7ab, 14 h, 95%, > 99:1 *dr*[a]
7ab, 8 h, 92%, 98:2 *dr*[b], > 99:1 *dr*[c]

7ac, 8 h, 96%, > 99:1 *dr*[a]
7ac, 8 h, 90%, 98:2 *dr*[b], > 99:1 *dr*[c]

7ad, 9 h, 92%, > 99:1 *dr*[a]
7ad, 15 h, 90%, > 99:1 *dr*[b], > 99:1 *dr*[c]

Reaction condition: [a]**6** (0.5 mmol), **3** (1 mmol), BSA (1 mmol), DCE (5 mL); [b]**6** (0.5 mmol), **3** (1 mmol), DIEA (1 mmol), DCM (3 mL), 2,3-butanediol (3 mL); [c]Purified by recrystallization.

Fig. 3. Preparation of tripeptides from dipeptide acids and unprotected amino acids

The success in the tripeptide syntheses was exciting because the notorious epimerization issue of peptidyl acids that plagued the unprotected amino acid strategy for six decades has been successfully addressed. To further confirm the robustness of this strategy, its application in the syntheses of longer peptides such as tetrapeptides, pentapeptides, and hexapeptides was investigated. As shown in **Fig. 4**, tripeptides **9a–9d** were readily prepared in excellent yields by employing the base-additive-assisted unprotected amino acid strategy. Then, tripeptides **9a–9d** were activated by the ynamide coupling reagent MYTsA and treated with unprotected H-Ser(*t*Bu)-OH, H-Phe-OH, H-Met-OH, and H-Glu(*t*Bu)-OH under the revised base additive conditions to provide the corresponding tetrapeptides in excellent yields (**Fig. 4**, **10a–10d**). It was interesting to note that further elongation of tetrapeptide **10d** could be easily realized by repeating the coupling cycle of activation and aminolysis with unprotected H-Phe-OH, H-Cys(Bn)-OH to obtain the target pentapeptide **11** and hexapeptide **12**, respectively, in excellent yields. Notably, no epimerization was observed during the entire syntheses of the tetrapeptides, pentapeptide, and hexapeptide. In addition, no chromatography purification was involved for the syntheses of these peptides because the acid–base work-up and a single recrystallization were sufficient to obtain the pure target peptide products. These results unambiguously demonstrated the potency of this strategy for the syntheses of longer peptides.

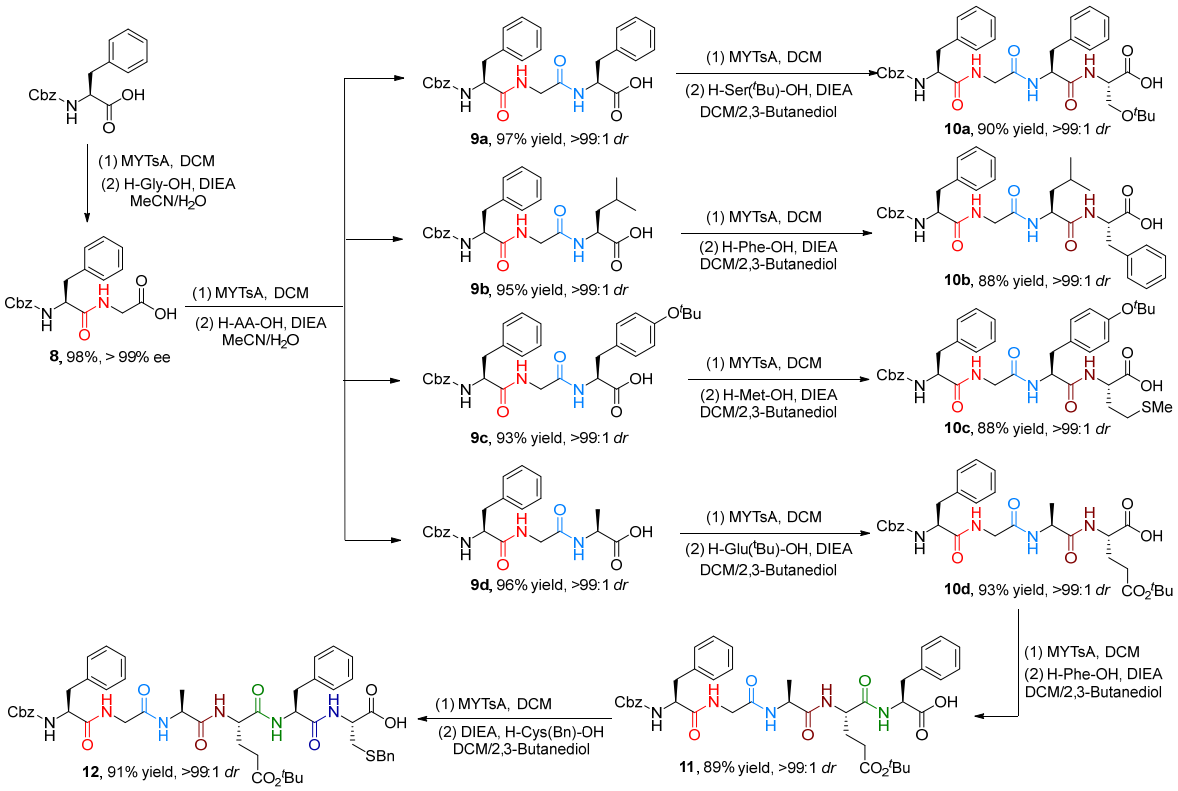


Fig. 4. Syntheses of tetrapeptide, pentapeptide, and hexapeptide using unprotected amino acids

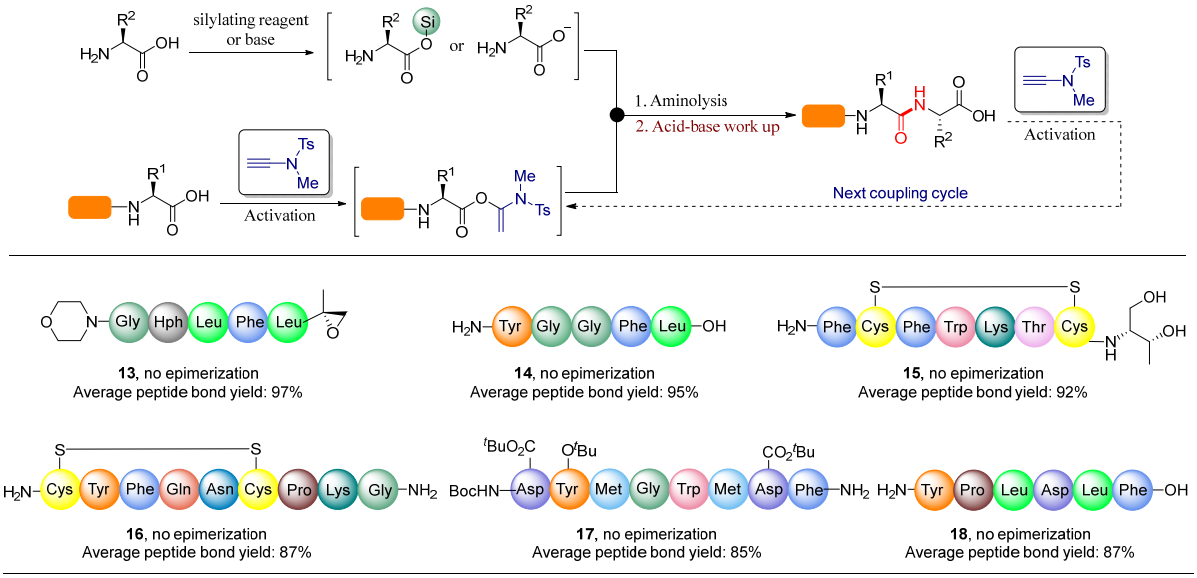


Fig. 5. Synthetic application of the unprotected amino acid strategy

The potential application of the unprotected amino acid strategy was further exemplified by the synthesis of a series of peptide APIs. As shown in **Fig. 5**, the base additive strategy was employed for the synthesis of carfilzomib **13**. After each peptide bond formation was completed, a simple acidic aqueous work-up followed by recrystallization provided the target peptides with a free carboxylic group at the C-terminus ready for the next coupling cycle. The four amide bonds (three peptide bonds) of carfilzomib were constructed concisely via only four coupling steps with an average yield of 96% for each peptide bond, whereas at least seven steps (four couplings and three deprotections) were required in conventional protected amino acid strategy with prior N^{α}-protected amino acids as the building blocks. Owing to the considerably shortened synthetic route and excellent efficacy of each step, the total yield of **13** was remarkably higher than that of the protection strategy (85% vs 68%, respectively) [34]. It should be noted that no column chromatography purification was required during the entire synthesis of **13** because the intermediates and final product were used directly or purified via recrystallization. For the syntheses of Leu-enkephalin **14**, octreotide **15**, vasopressin **16**, and cholecyxtokinin octapeptide **17**, the silylating reagent strategy was employed in the later stage owing to the poor solubility of longer peptides in the 1:1 mixture of 2,3-butanediol and DCM. Thus, the base additive strategy was employed only for the synthesis of short peptide intermediates. No detectable epimerization was observed for any peptide intermediate. The step-economy of the unprotected amino acid strategy was remarkable because the transient protection, activation, aminolysis, and *in situ* deprotection could be performed in one-pot to furnish the peptide with a carboxylic acid C-terminus ready for the next coupling cycle. Herein, although most peptide bonds were constructed in a two-step manner for studying each step carefully, they could be performed in a one-pot manner with comparable or higher yields. Taking vasopressin **16** as an example, 8 vs 24 steps were required for this unprotected amino acid strategy and conventional peptide synthesis strategy, respectively, for constructing the peptide chain. All the reactions were carried out using a normal solvent at room temperature although heating was beneficial for shortening the reaction time while retaining the excellent chiral integrity. In addition, no column chromatography purification was required for most peptide intermediates, thereby offering a convenient operation. As shown in **Fig. 5**, the average formation yield for each peptide bond was in the range of 85% to 97% in the one-

by-one stepwise elongation strategy for peptide API syntheses. Meanwhile, the gram-scale synthesis of hexapeptide Rnbiscolin-6 **18** was easily realized (**Fig. 6**) with a higher average peptide bond yield than that of small-scale synthesis (92% *vs* 87%, **Fig. 5** and **Fig. 6**). In addition, there was no difference between the coupling of L-Phe and D-Phe. The excellent efficacy of the gram-scale synthesis, mild reaction conditions, convenient operation, and user-friendly protocol illustrate its potential application in large-scale peptide synthesis.

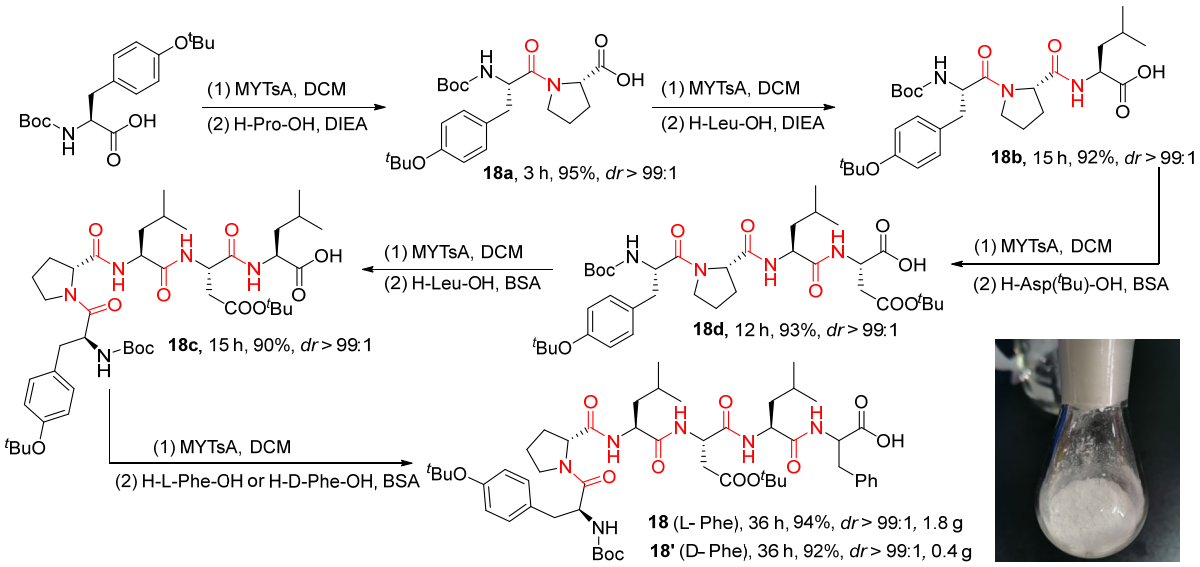


Fig. 6. Gram-scale synthesis of hexapeptide Rnbiscolin-6 (18)

In conclusion, we have developed the first general peptide synthesis strategy using unprotected amino acids as the building blocks. The key to the success is the superiority of the ynamide coupling reagent in suppressing racemization/epimerization, thus enabling the peptide elongation in the N→C manner, with excellent chiral integrity retention and yields. Two transient protection strategies involving the silylating reagent or base additive were employed to accomplish the coupling cycle of transient protection, activation, aminolysis, and *in situ* deprotection in a single reaction vessel. As a consequence, the number of operations, chemicals, energy, time, labor, and solvents required and the amount of chemical waste were significantly reduced compared with those in the case of the conventional peptide synthesis, and accordingly, the production cost was significantly reduced. In contrast to previous unprotected amino acid strategies limited to dipeptide synthesis, this method successfully addressed the epimerization encountered in the syntheses of longer peptides containing more than two amino acids. As shown in peptide APIs syntheses, two-thirds of the conventional peptide synthesis steps are eliminated by considering the one-pot transient protection, activation, aminolysis, and *in situ* deprotection as one step [14]. This study offers a cost-effective, convenient, and environmentally benign approach to oligopeptides, which can also serve as valuable building blocks for large peptides and even proteins. Owing to the advantages of the air- and moisture-compatible reaction conditions, room temperature, simple workup and purification procedures, and low time consumption, this strategy has made a great stride toward the greening of peptide synthesis. It is foreseeable that this study will bring about disruptive technologies by combining with "the third wave for preparation of peptide"[9] in both academia and industry.

Acknowledgments:

We thank Dr. Long Hu for his help in preparing this manuscript.

Funding:

The National Natural Science Foundation of China (22277015).

Author contributions:

T.L., Z.P., and J.Z. conceived the study, designed the experiments, and analyzed the data. T.L. and Z.P. optimized the reaction conditions and performed the experiments. All the authors co-wrote the manuscript.

Competing interests:

The authors declare that they have no competing interests.

Data and materials availability:

All the data are available in the main text or the supplementary materials.

References:

1 Bray, B. L. Large-scale manufacture of peptide therapeutics by chemical synthesis. *Nat. Rev. Drug Discov.* **2**, 587-593, doi:10.1038/nrd1133 (2003).

2 Muttenthaler, M., King, G. F., Adams, D. J. & Alewood, P. F. Trends in peptide drug discovery. *Nat. Rev. Drug Discov.* **20**, 309-325, doi:10.1038/s41573-020-00135-8 (2021).

3 Drucker, D. J. Advances in oral peptide therapeutics. *Nat. Rev. Drug Discov.* **19**, 277-289, doi:10.1038/s41573-019-0053-0 (2020).

4 Räder, A. F. B. *et al.* Orally Active Peptides: Is There a Magic Bullet? *Angew. Chem. Int. Ed.* **57**, 14414-14438, doi:https://doi.org/10.1002/anie.201807298 (2018).

5 Letsinger, R. L. & Kornet, M. J. Popcorn Polymer as a Support in Multistep Syntheses. *J. Am. Chem. Soc.* **85**, 3045-3046, doi:10.1021/ja00902a054 (1963).

6 Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **85**, 2149-2154, doi:10.1021/ja00897a025 (1963).

7 Kovacs, J., Holleran, E. M. & Hui, K. Y. Kinetic studies in peptide chemistry. Coupling, racemization and evaluation of methods useful for shortening coupling time. *J. Org. Chem.* **45**, 1060-1065, doi:10.1021/jo01294a029 (1980).

8 Hartrampf, N. *et al.* Synthesis of proteins by automated flow chemistry. *Science* **368**, 980-987, doi:10.1126/science.abb2491 (2020).

9 Sharma, A., Kumar, A., de la Torre, B. G. & Albericio, F. Liquid-Phase Peptide Synthesis (LPPS): A Third Wave for the Preparation of Peptides. *Chem. Rev.* **122**, 13516-13546, doi:10.1021/acs.chemrev.2c00132 (2022).

10 Isidro-Llobet, A. *et al.* Sustainability Challenges in Peptide Synthesis and Purification: From R&D to Production. *J. Org. Chem.* **84**, 4615-4628, doi:10.1021/acs.joc.8b03001 (2019).

11 Constable, D. J. C. *et al.* Key green chemistry research areas—a perspective from pharmaceutical manufacturers. *Green Chem.* **9**, 411-420, doi:10.1039/B703488C (2007).

12 Bryan, M. C. *et al.* Key Green Chemistry research areas from a pharmaceutical manufacturers' perspective revisited. *Green Chem.* **20**, 5082-5103, doi:10.1039/c8gc01276h (2018).

13 Pattabiraman, V. R. & Bode, J. W. Rethinking amide bond synthesis. *Nature* **480**, 471-479 (2011).

14 Young, I. S. & Baran, P. S. Protecting-group-free synthesis as an opportunity for invention. *Nat. Chem.* **1**, 193-205, doi:10.1038/nchem.216 (2009).
15 Hofmann, K., Thompson, T. A., Yajima, H., Schwartz, E. T. & Inouye, H. Studies on Polypeptides. XIV. The Synthesis of Peptides Related to the N-Terminus of α-MSH and of the Corticotropins1,2. *J. Am. Chem. Soc.* **82**, 3715-3721, doi:10.1021/ja01499a062 (1960).
16 Brown, Z. Z. & Schafmeister, C. E. Exploiting an Inherent Neighboring Group Effect of α-Amino Acids To Synthesize Extremely Hindered Dipeptides. *J. Am. Chem. Soc.* **130**, 14382-14383, doi:10.1021/ja806063k (2008).
17 Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. The Use of Esters of N-Hydroxysuccinimide in Peptide Synthesis. *J. Am. Chem. Soc.* **86**, 1839-1842, doi:10.1021/ja01063a037 (1964).
18 Gagnon, P., Huang, X., Therrien, E. & Keillor, J. W. Peptide coupling of unprotected amino acids through in situ p-nitrophenyl ester formation. *Tetrahedron Lett.* **43**, 7717-7719 (2002).
19 Meneses, C., Nicoll, S. L. & Trembleau, L. Multigram-scale synthesis of short peptides via a simplified repetitive solution-phase procedure. *J. Org. Chem.* **75**, 564-569, doi:10.1021/jo902116p (2010).
20 Katritzky, A. R., Suzuki, K. & Singh, S. K. Highly Diastereoselective Peptide Chain Extensions of Unprotected Amino Acids withN-(Z-α-Aminoacyl)benzotriazoles. *Synthesis* **2004**, 2645-2652, doi:10.1055/s-2004-831255 (2004).
21 Chemistry LettersNoguchi, T., Tehara, N., Uesugi, Y., Jung, S. & Imai, N. Convenient Peptide Synthesis without Protection of C-Terminals. *Chem. Lett.* **41**, 42-43, doi:10.1246/cl.2012.42 (2012).
22 Kurasaki, H. *et al.* Isostearyl Mixed Anhydrides for the Preparation of N-Methylated Peptides Using C-Terminally Unprotected N-Methylamino Acids. *Org. Lett.* **22**, 8039-8043, doi:10.1021/acs.orglett.0c02984 (2020).
23 Johansson, A., Åkerblom, E., Ersmark, K., Gunnar Lindeberg, A. & Hallberg, A. An Improved Procedure for N- to C-Directed (Inverse) Solid-Phase Peptide Synthesis. *J. Comb. Chem.* **2**, 496-507 (2000).
24 Fuse, S., Masuda, K., Otake, Y. & Nakamura, H. Peptide-Chain Elongation Using Unprotected Amino Acids in a Micro-Flow Reactor. *Chem. Eur. J.* **25**, 15091-15097, doi:10.1002/chem.201903531 (2019).
25 Leuchs, H. Ueber die Glycin-carbonsäure. *Berichte der deutschen chemischen Gesellschaft* **39**, 857-861, doi:https://doi.org/10.1002/cber.190603901133 (1906).
26 Spengler, J., Böttcher, C., Albericio, F. & Burger, K. Hexafluoroacetone as Protecting and Activating Reagent: New Routes to Amino, Hydroxy, and Mercapto Acids and Their Application for Peptide and Glyco- and Depsipeptide Modification. *Chem. Rev.* **106**, 4728-4746, doi:10.1021/cr0509962 (2006).
27 Sabatini, M. T., Boulton, L. T. & Sheppard, T. D. Borate esters: Simple catalysts for the sustainable synthesis of complex amides. *Sci Adv* **3**, e1701028, doi:10.1126/sciadv.1701028 (2017).
28 van Leeuwen, S. H., Quaedflieg, P. J. L. M., Broxterman, Q. B. & Liskamp, R. M. J. Synthesis of amides from unprotected amino acids by a simultaneous protection–activation strategy using dichlorodialkyl silanes. *Tetrahedron Lett.* **43**, 9203-9207, doi:https://doi.org/10.1016/S0040-4039(02)02275-X (2002).
29 Fu, H., Li, Z.-L., Zhao, Y.-F. & Tu, G.-Z. Oligomerization of N,O-Bis(trimethylsilyl)-α-amino Acids into Peptides Mediated by o-Phenylene Phosphorochloridate. *J. Am. Chem. Soc.* **121**, 291-295, doi:10.1021/ja980662c (1999).

30 Muramatsu, W. & Yamamoto, H. Peptide Bond Formation of Amino Acids by Transient Masking with Silylating Reagents. *J. Am. Chem. Soc.* **143**, 6792-6797, doi:10.1021/jacs.1c02600 (2021).
31 Hattori, T. & Yamamoto, H. Synthesis of Silacyclic Dipeptides: Peptide Elongation at Both N- and C-Termini of Dipeptide. *J. Am. Chem. Soc.* **144**, 1758-1765, doi:10.1021/jacs.1c11260 (2022).
32 Hu, L. *et al.* Ynamides as Racemization-Free Coupling Reagents for Amide and Peptide Synthesis. *J. Am. Chem. Soc.* **138**, 13135-13138, doi:10.1021/jacs.6b07230 (2016).
33 Xu, S. *et al.* Ynamide-Mediated Peptide Bond Formation: Mechanistic Study and Synthetic Applications. *Angew. Chem. Int. Ed.* **61**, e202212247, doi:https://doi.org/10.1002/anie.202212247 (2022).
34 Liu, T., Zhang, X., Peng, Z. & Zhao, J. Water-removable ynamide coupling reagent for racemization-free syntheses of peptides, amides, and esters. *Green. Chem.* **23**, 9916-9921, doi:10.1039/D1GC03498G (2021).
35 Isied, S. S. & Kuehn, C. G. Peptide formation in the presence of a metal ion protecting group. Pentaammine cobalt(III)-peptide complexes. *J. Am. Chem. Soc.* **100**, 6752-6754, doi:10.1021/ja00489a036 (1978).
36 Huang, Y. & Feng, W.-H. N,O-Bis(trimethylsilyl)acetamide/N-hydroxysuccinimide ester (BSA/NHS) as coupling agents for dipeptide synthesis. *Chin. Chem. Lett.* **27**, 357-360, doi:https://doi.org/10.1016/j.cclet.2015.11.012 (2016).