Peptide Synthesis

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AJIPHASE®: A Highly Efficient Synthetic Method for One-Pot Peptide Elongation in the Solution Phase by an Fmoc Strategy

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Abstract: *We previously reported an efficient peptide synthesis method, AJIPHASE®, that comprises repeated reactions and isolations by precipitation. This method utilizes an anchor molecule with long-chain alkyl groups as a protecting group for the C-terminus. To further improve this method, we developed a one-pot synthesis of a peptide sequence wherein the synthetic intermediates were isolated by solvent extraction instead of precipitation. A branched-chain anchor molecule was used in the new process, significantly enhancing the solubility of long peptides and the operational efficiency compared with the previous method, which employed precipitation for isolation and a straight-chain aliphatic group. Another prerequisite for this solvent-extraction-based strategy was the use of thiomalic acid and DBU for Fmoc deprotection, which facilitates the removal of byproducts, such as the fulvene adduct.*

As the mainstream modalities of drug discovery shift from small molecules to biological entities, an increasing number of peptide-based drugs and drug candidates that are smaller than proteins have been developed in recent years.[1] This trend increases the requirement for improving solid- and liquid-phase peptide synthesis (SPPS and LPPS, respectively). SPPS is mainly used owing to its automation and simple operation. In contrast, LPPS is advantageous in terms of product purity, scalability, and cost. However, the physicochemical properties of intermediate peptides strongly depend on their sequence, and highly lipophilic and severely insoluble peptide intermediates can lead to difficult workups. In particular, the handling of long-chain hydrophobic or hydrophilic peptides is problematic in LPPS process development.[2]

To address this problem, some LPPS-based methods[3–7] have been reported, such as the methods using PEG, fluorous molecules, and anchor molecule **1**. We have also developed the AJIPHASE® method, a unique LPPS approach that uses "anchor" molecules[8,9] such as fluorene-based **2** or diphenylmethane-based **3** to elongate the sequence by repeated coupling/deprotection reactions and isolation by simple precipitation (Figure 1); however, this precipitation-based method requires halogenated solvents, and reducing their use is important from an economic and environmental perspective.

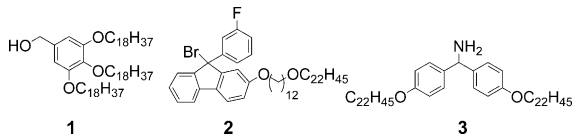

Figure 1. Chemical structures of anchor molecules **1**–**3**.

In addition, further improvement in terms of operational efficiency with simplified workup steps is required to perform industrial-scale syntheses on reasonable timescales. In fact, some attempts have been made for one-pot peptide synthesis where product isolation was replaced by solvent extraction, but the solubilities of peptide intermediates during extraction differ significantly depending on their sequences and lengths, limiting the usefulness of these strategies.[10–13]

Herein, a widely applicable method was examined and used for the one-pot elongation of a peptide where only solvent extraction was used for the workup procedure.[17] To establish this method, the following two challenges needed to be addressed: 1) The peptide intermediates must be fully soluble in the extraction solvent during elongation, and 2) the byproducts must be completely removed by aqueous washing as residual amounts of Fmoc-protected amino acids, coupling reagents, deprotection reagents, and other byproducts/residues can affect subsequent reactions and generate impurities.

Considering the solubility, long or hydrophobic peptide intermediates bearing anchor molecule **1**–**3** are completely dissolved in halogenated solvents or THF with increased viscosity, rendering extraction-based processes inefficient.

We hypothesized that increasing the lipophilicity of the anchor molecule would improve the solubility and decrease the viscosity, and a branched phytyl group was tested. Phytol was hydrogenated on Pt/C and treated with HBr/H_2SO_4 to afford dihydrophytylbromide **4** (Figure 2).[14] The phytyl

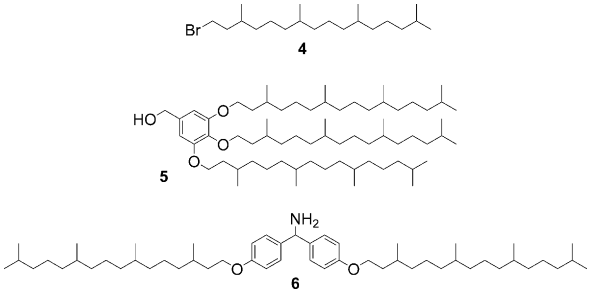

Figure 2. Chemical structures of the branched-chain anchor molecules and related molecules **4**–**6**.

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group (Phy) was introduced into benzyl- and diphenylmethane-type anchors according to a previously reported method,[6,9] and anchors **5** and **6** were obtained (Figure 2; see also the Supporting Information).

Interestingly, the solubilities of these new branched-chain anchors **5** and **6** in various organic solvents were significantly better than those of compounds **1** and **3**, and allowed for an efficient extraction-based workup (Table 1). Based on these results, compounds **5** and **6** were chosen as the anchor molecules for examining the new workup procedure. In addition, the anchor compounds are less soluble in polar solvents such as MeCN and DMF, which are occasionally used as solvents in peptide synthesis.

Table 1: Solubility of anchor molecules in extraction solvents [wt%].

	Straight chain		Branched chain	
Solvent	**1**	**3**	**5**	**6**
$CHCl_3$	9.9	0.8	>50	>50
EtOAc	0.2	0.2	>50	>25
CPME	4.5	0.01	>50	>50
toluene	–[a]	0.01	>50	–[a]

[a] No data. CPME = cyclopentyl methyl ether.

To confirm that the use of anchor molecules with a branched alkyl chain enhances the solubility of intermediate peptides during elongation and leads to less problematic extractions, we employed compound **6** in the synthesis of a hydrophobic peptide. This class of peptides is well-known to be difficult to be synthesized by LPPS owing to their low solubility in various types of solvents.

Fmoc-Val-OH, Fmoc-Gly-Gly-OH, and Fmoc-Val-OH were sequentially condensed to form the hydrophobic sequence Fmoc-Val-Gly-Gly-Val with anchor molecule **1** or **5** at the C-terminus. During the coupling reaction for the forth residue Fmoc-Val-OH, insoluble matter was observed in the reaction mixture for the intermediate peptide with anchor **1**. Synthesis with the branched-chain anchor **5** led to a completely homogeneous solution. This interesting result indicated that the newly developed anchor with a branched alkyl chain is useful for the synthesis of hydrophobic peptides whose intermediates may have low solubility (Figure 3).

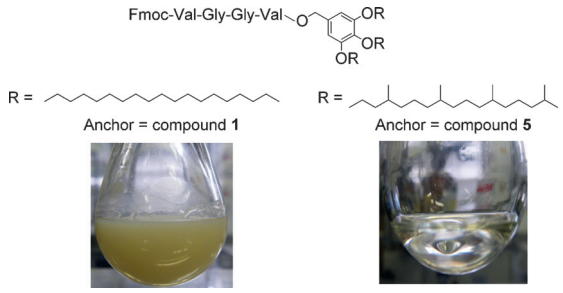


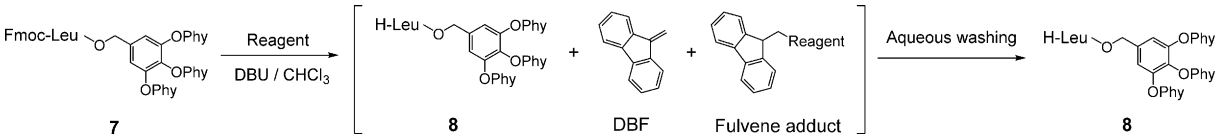
Figure 3. Reaction mixtures of the coupling reaction of Fmoc-Val-OH with H-Gly-Gly-Val-OAnchor in chloroform with compound **1** or **5** as the anchor compound, respectively.

Next, we studied the second challenge. After the coupling reaction, residual amino acids and amines, such as piperidine, which is conventionally used for the removal of Fmoc groups and in the formation of fulvene adducts via dibenzofulvene (DBF) derived from Fmoc groups, are a potential cause of undesirable side reactions. Therefore, it is essential to remove these byproducts before the next amino acid coupling reaction. To quench, solubilize, and remove the fulvene derivatives into an aqueous layer, some reagents were tested using Fmoc-Leu-OH loaded onto anchor **5** for Fmoc deprotection of Fmoc-Leu-OAnchor **7** into **8** (Table 2).

In the case of diethylenetriamine,[11] unfortunately, separation of the organic layer from the aqueous layer was troublesome. In fact, standard amine-based Fmoc removal reagents, including diethylenetriamine and piperidine, did not completely capture all DBF; thus a considerable amount of the fulvene adduct remained intact in the organic layer. When washed with acidified water to remove the base and its adduct byproducts of fulvene, the solution emulsified during the extraction, and the phase separation was insufficient. We surmise that the acidic aqueous solution that was used for the removal of solubilized fulvene adducts and residual amines led to the formation of the emulsion during aqueous washing. This is presumably due to the protonation of the peptide N-terminus under acidic conditions, which renders the peptide amphiphilic, in combination with the long aliphatic chain of the anchor molecule, which could act as a surfactant.

Accordingly, we then tried basic conditions for aqueous washing to keep the terminal amine group of the peptide

Table 2: Conversion into the fulvene adduct during Fmoc deprotection of Fmoc-Leu loaded onto **5** and the removal rate in the washing step.



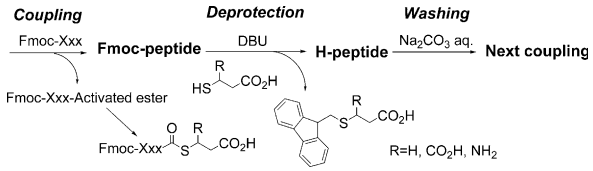
7 **8** DBF Fulvene adduct **8**

Reagent (equiv)	DBU [equiv]	Solvent	DBF/fulvene adduct	Washing solvent	Layer separability[a]	Removal rate [%]
diethylenetriamine (30)	0	$CHCl_3$	35:65	HCl aq.	–	48
piperidine (5)	3	$CHCl_3$	31:69	HCl aq.	–	2
piperidine (5)	3	CPME	8:92	HCl aq.	–	0
Mpa (3)	6	CPME	3:97	Na_2CO_3 aq.	+	100
Mpa (3)	6	$CHCl_3$	1:99	Na_2CO_3 aq.	+	30
thiomalic acid (3)	9	$CHCl_3$	1:99	Na_2CO_3 aq.	+	100
cysteine (3)	6	$CHCl_3$	2:98	Na_2CO_3 aq.	+	8

[a] "–": Insufficient phase separation; "+": sufficient phase separation. See the Supporting Information for details. Mpa = mercaptopropionic acid.

deprotonated. To achieve this, mercaptopropionic acid (Mpa), with both carboxylic acid and nucleophilic thiol moieties, was examined in combination with DBU. Fmoc-Leu loaded onto anchor molecule **5** was treated with Mpa/DBU, and the resulting solutions were washed with a basic aqueous solution of sodium carbonate, which resulted in facile deprotection and the formation of DBF adducts. In this extraction process, the fulvene adducts were smoothly removed into the basic aqueous layer with good phase separation. Cysteine also showed good results. Finally, thiomalic acid, with two carboxylic acid moieties, was tested and showed advantageous properties for transferring the corresponding fulvene adducts into the basic aqueous layer (Table 2).

Based on the examination above, when Fmoc deprotection is conducted with a compound possessing both thiol and carboxylate moieties, such as Mpa, thiomalic acid, or Cys, in the presence of DBU, excess active esters of the amino acids and dibenzofulvene are converted into acidic species bearing a carboxylic acid moiety, which are removed by simple washing with basic aqueous solution. Consequently, the next condensation can be performed without interference from these byproducts. These processes can be performed consecutively, and a practical method for one-pot peptide elongation without an isolation step has been devised (Scheme 1).



Scheme 1. Novel Fmoc deprotection system for one-pot peptide elongation.

We then studied LPPS with the newly developed anchor molecule with a branched phytyl group. The first actual peptide was the anti-ovary-cancer drug Degarelix,[15] which contains an amide at the C-terminus. Therefore, the diphenylmethane-type anchor molecule **6** was employed using chloroform as the solvent. Fmoc-protected natural and unnatural amino acids were used slightly in excess (1.1–1.3 equiv) for coupling reactions with EDC·HCl/HOBt, and condensation of each residue proceeded smoothly. Fmoc deprotection was performed in the presence of 3 equiv of thiomalic acid and 8 equiv of DBU. Fmoc was promptly removed in 1–2 h from each residue to form a fulvene adduct with the scavenger. Upon washing with an aqueous solution of sodium carbonate, the fulvene adduct was easily removed into the aqueous layer. The organic layer was subjected to the next coupling reaction without concentration or drying. No impurities derived from thiomalic acid or fulvene were observed in the subsequent coupling reaction. The fully protected full-length peptide demonstrated satisfactory solubility in the organic layer, and phase separation of the organic and aqueous layers was accomplished without any problems. Successive concentration of the organic layer and addition of MeCN afforded the fully protected peptide **10** with an anchor molecule at the C-terminus. The fully protected compound was then subjected to standard global deprotection conditions (TFA/triisopropylsilane (TIS)/H_2O = 95:2.5:2.5) to afford crude Degarelix in 89% purity (Scheme 2 and Figure 4).

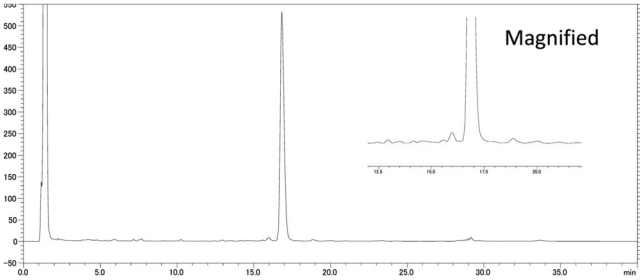
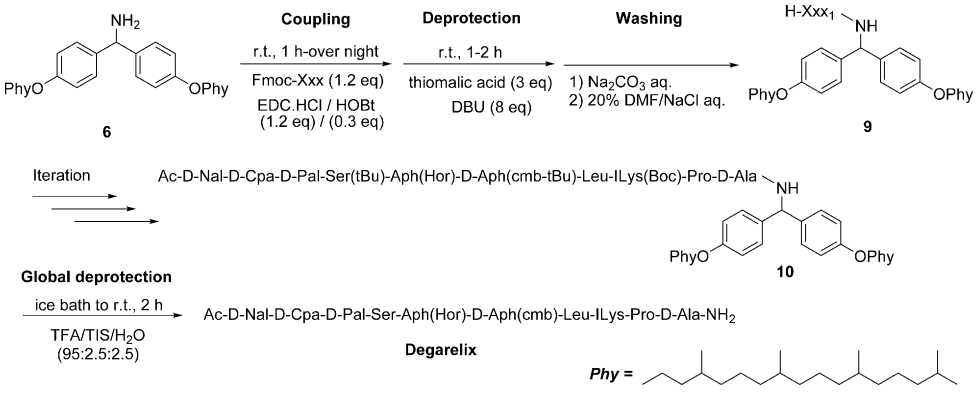
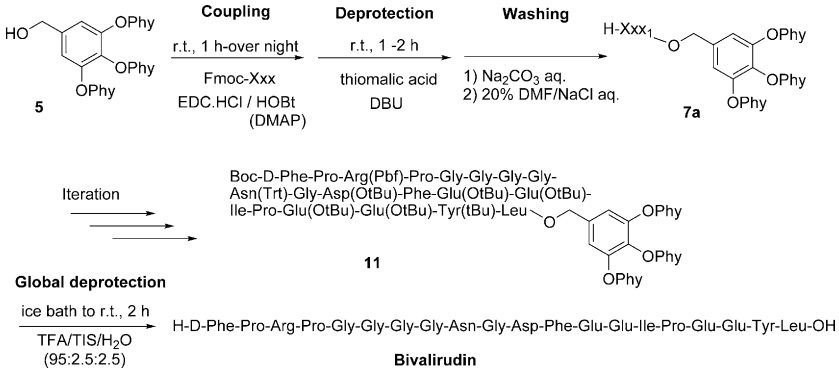


Figure 4. HPLC analysis of crude Degarelix. Zorbax Eclipse XDB-C18, 4.6×150 mm, 5 μm, 55 °C, 1.2 mL min^{-1}, 17.6 mM aq. Na_2HPO_4 (pH 8.2)/MeCN, gradient: 27–33% (0–5 min), 33–37% (5–25 min), 70% (25–30 min).

We also synthesized a peptide bearing a carboxylic acid terminus. Bivalirudin,[16] with 20 amino acid residues, was elongated using a similar method to that employed for Degarelix in a one-pot process using compound **5** as the anchor molecule and only solvent extraction for workup (Scheme 3). All reactions and workups proceeded smoothly. The fully protected 20 residue peptide was completely soluble in the organic solvent, and phase separation proceeded smoothly in each washing step. As with Degarelix, all intermediate peptides synthesized during elongation were simply washed and subjected to the next coupling reaction without isolation, for example, by precipitation as previously reported.[8,9] The fully protected product **11** was obtained in



Scheme 2. Synthetic scheme for Degarelix elongation.



Scheme 3. Synthetic scheme for Bivalirudin elongation.

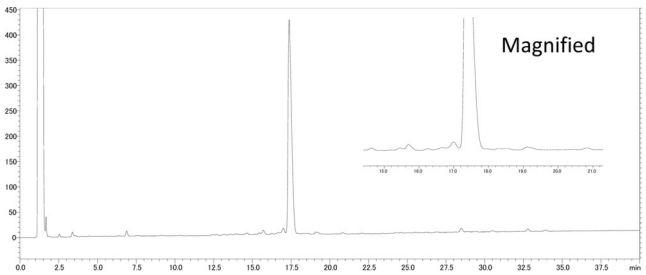


Figure 5. HPLC analysis of crude Bivalirudin. YMC-Pack ODS-A, 4.6×150 mm, 5 µm, 40 °C, 1.0 mL min^{-1}, 0.1 % TFA in 5 % THF/water and 0.1 % TFA in 5 % THF/MeCN, gradient: 20–40 % (0–25 min), 80 % (25–30 min).

73 % yield in this one-pot process (based on **5**; Figure 5). The purity of the crude product after global deprotection was 84 %.

It should be noted that peptide elongation can be performed in non-halogenated organic solvents such as cyclopentyl methyl ether (CPME). Only a small amount of solvent was added in each elongation cycle, corresponding to the increasing intermediate molecular weight, and the initial solvent remained in the vessel throughout the synthesis. Therefore, solvent consumption is very low, and amounts to approximately one tenth of that of SPPS. This is an advantage of our AJIPHASE® method with compounds **5** and **6** (Figure 6).

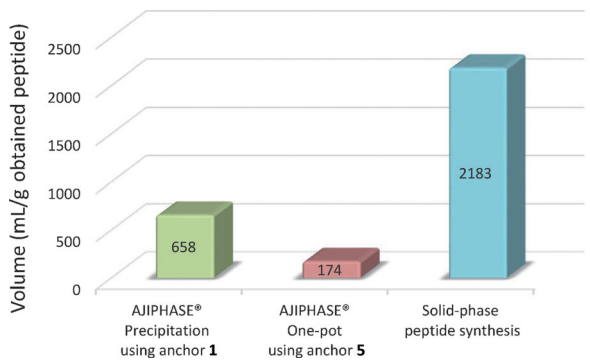


Figure 6. Solvent consumption of various peptide synthesis methods for 20-mer peptides.

Conventionally and conveniently, peptides have been prepared by solid-phase synthesis; however, an easy-to-handle and ready-to-scale-up LPPS method for the synthesis of long and/or hydrophilic peptides in improved purity and yield is still required.

In conclusion, we have developed a simple, efficient, and widely applicable peptide synthesis method based on the branched anchor compounds **5** or **6** in combination with a new Fmoc deprotection system. This process afforded pure peptides, even for long or hydrophobic peptides, and can be readily scaled up. Solvent evaporation and dehydration are not necessary during the workups for the following coupling steps, and only a limited amount of solvent is added in each reaction. Thus the total solvent consumption is significantly lower than for SPPS. We are confident that these advantages will render the AJPHASE approach a useful LPPS method even on large scales.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: AJIPHASE · liquid-phase peptide synthesis · peptides · protecting groups · solvent extraction

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