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Highly Efficient Synthetic Methodology AJIPHASE® for One-pot Peptide Elongation Using an Fmoc Strategy in the Solution Phase

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Abstract: We report an efficient peptide synthesis methodology, AJIPHASE®, comprising repeated reactions and isolations by precipitation. This methodology utilizes an anchor molecule as a protecting group for the C-terminus with long-chain alkyl groups. For further improvement of this method, we developed a one-pot synthesis of a peptide sequence wherein the synthetic intermediates are worked up by solvent extraction instead of precipitation. A branched chain anchor molecule is used in the new process, significantly enhancing the solubility of a long peptide and operational efficiency compared with the previous method, which employs precipitation for isolation using a straight chain aliphatic group. Another prerequisite for this solvent extraction-based strategy was thiomalic acid and DBU in Fmoc deprotection, which facilitates removal of byproducts such as the fulvene adduct.

As the mainstream modalities of drug discovery shifts from small molecules to biological entities, 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 absolute 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 such as highly lipophilic and severely insoluble peptide intermediates that can lead to difficult work ups. In particular, handling of long-chain hydrophobic or hydrophilic peptides is problematic in process development by LPPS.[2]

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

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

In this work, our novel and widely applicable method was examined and reported for one-pot elongation of a peptide where only solvent extraction is used for the work-up procedure. In order to establish this method, the following two challenges need to be addressed; (i) keeping peptide intermediates fully soluble in the extraction solvent during elongation and (ii) completely removing byproducts by aqueous washing must be achieved as excessive Fmoc-amino acids, coupling reagents, deprotecting reagents, and other byproducts/residues can affect the subsequent reactions and generate impurities.

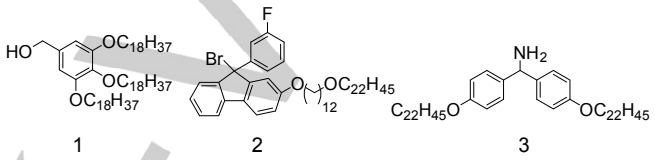

Figure 1. Chemical structures of anchor molecules **1**-**3**.

Considering the solubility, long or hydrophobic peptide intermediates bearing anchor molecule **1-3** are completely dissolved in halogenated solvents or THF with increased viscosity, thus leading to inefficiency for an extraction-based process.

We hypothesized that improved lipophilicity of the anchor molecule would improve solubility and decrease viscosity, and a branched phytyl group was tested for peptide intermediates. Phytol was hydrogenated on Pt/C and treated with HBr/H2SO4 to afford dihydrophytylbromide **4**.[14] The phytyl group (Phy) was introduced to benzyl- and diphenylmethan-type anchors via a previously reported method,[6, 9] and anchors **5** and **6** were obtained (Figure 2 and the Supporting Information).

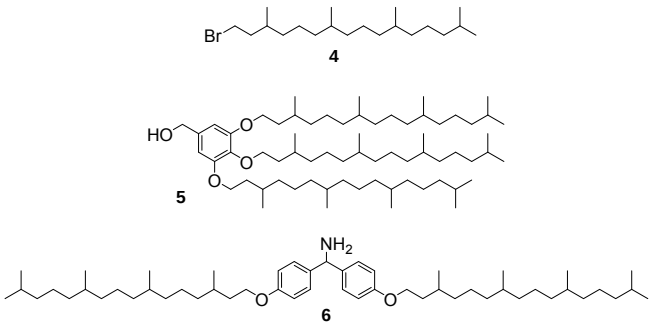

Figure 2. Chemical structures of branched chain anchor molecules and related molecules **4**-**6**.

Interestingly, solubility of these new branched-chain anchors **5** and **6** in various organic solvents improved significantly to perform efficient extraction-based work up compared with those of compounds **1** or **3** (Table 1). With these results, compounds **5** and **6** were chosen as the anchor

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molecules for examining the new work-up protocol. 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	No data	0.01	>50	No data

* cyclopentyl methyl ether

To confirm that the anchor molecule with a branched alkyl chain could enhance solubility of intermediate peptides during elongation leads to less problematic extraction, we examined the applicability of compound **6** to the synthesis of a hydrophobic peptide. This class of peptides is well known to be difficult to be synthesized in LPPS owing to 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-Glyl-Gly-Val with anchor molecules **1** or **5** at the C-terminus. During the coupling reaction for the 4th residue Fmoc–Val-OH, insoluble matter was observed in the reaction mixture for the intermediate peptide possessing compound **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).

Next, we studied the second challenge. After the coupling reaction, residual amino acids and amines, such as piperidine conventionally used in removal of Fmoc groups and in formation of fulvene adducts via dibenzofulven (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. In order to quench, solubilize, and remove 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** to convert to **8** (Table 2).

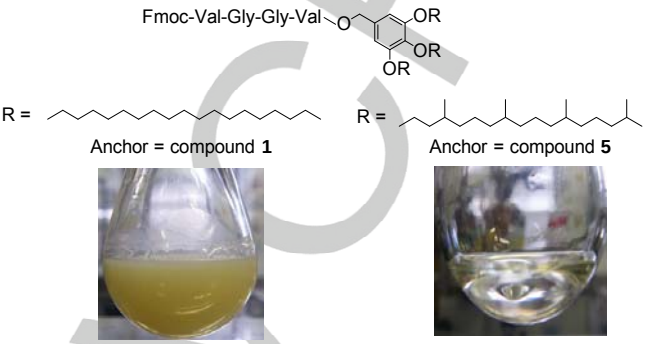
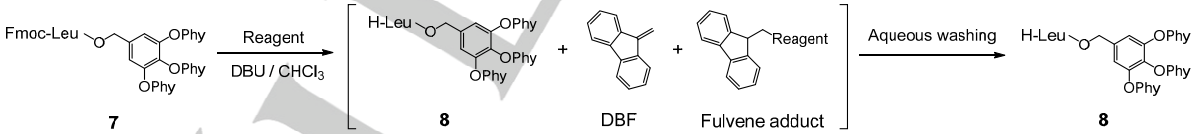


Figure 3. Reaction mixtures of the coupling reaction of Fmoc-Val-OH with H-Gly-Gly-Val-OAnchor in chloroform with compound **1** or **5** as the anchor compound, respectively.

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 diethylene triamine and piperidine, did not completely capture DBF; thus, a considerable amount of 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 phase separation was insufficient. We assumed that the acidic aqueous solution, used for removal of solubilized fulvene adducts and residual amines, led to formation of the emulsion during aqueous washing. This is presumably owing to protonation of the peptide N-terminus under acidic conditions, giving an amphiphilic form in combination with the long aliphatic chain of the anchor molecule that could behave as a surfactant.

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



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

Reagent	(eq)[a]	DBU (eq) [a]	Solvent	DBF/Fulvene adduct	Washing solvent	Layer separability[b]	Removal rate
Diethylene triamine	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[c]	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.	+	81%

[a] eq: equivalent, [b] "-" stands for phase separation is insufficient. "+" means sufficient phase separation (Supporting info.). [c] Mpa: mercaptoporpionic acid

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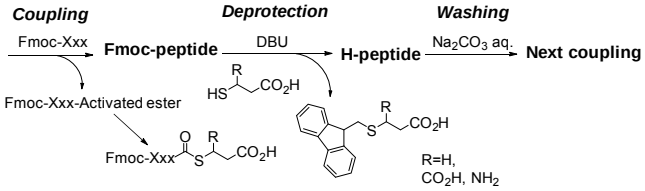
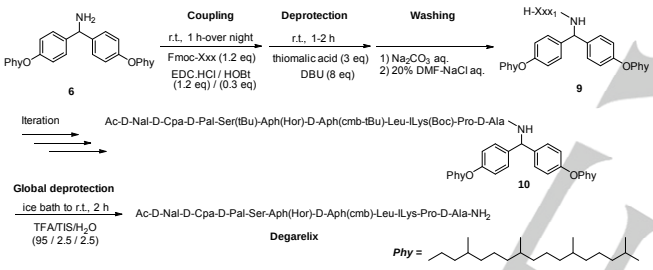
Accordingly, we then tried basic conditions for aqueous washing to keep the terminal amine group of the peptide deprotonated. To achieve this, mercaptopropionic acid (Mpa) with both carboxylic acid and nucleophilic thiol moieties was next examined in combination with DBU. Fmoc-Leu loaded on anchor molecule **5** was treated with Mpa/DBU, and the resulting solutions were washed with a basic aqueous solution of sodium carbonate, resulting in facile deprotection and formation of adducts of DBF. 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 to yield advantageous properties for transferring the corresponding fulvene adduct into the basic aqueous layer (Table 2).



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



Scheme 2. Synthetic scheme for Degarelix elongation.

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

We then tried LPPS using the newly developed anchor molecule with a branched phytyl group. The first actual peptide was the anti-oval cancer drug Degarelix[15] that possesses an amide in the C-terminus. Therefore, a diphenylmethan-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 eq) for coupling reactions with EDC.HCl/HOBt, and condensation of each residue proceeded smoothly. Fmoc deprotection was performed in the presence of 3 eq. of thiomalic acid and 8 eq. of DBU. Fmoc was promptly deprotected in 1-2 h from each residue to form a fulvene adduct with the scavenger. On washing with 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 problem. Successive concentration of the organic layer and addition of MeCN afforded fully protected peptide **10** with an anchor molecule at the C-terminus. The fully protected compound was then treated using standard global deprotection conditions (TFA:triisopropylsilan(TIS):H_2O = 95:2.5:2.5) to afford crude Degarelix in 89% purity (Scheme 2 and Figure 4).

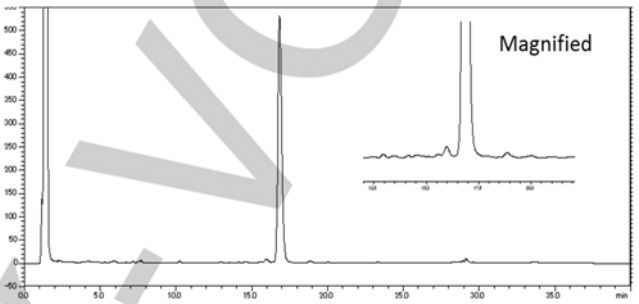
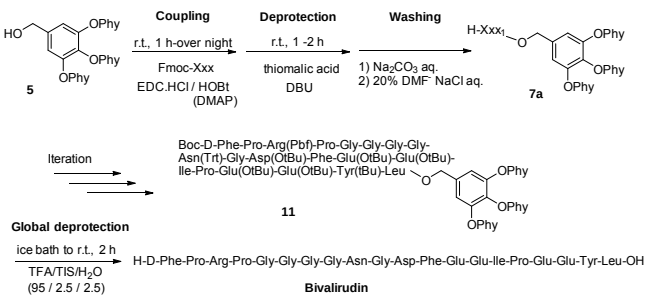


Figure 4. HPLC analysis of crude Degarelix; Zorbax Eclipse XDB-C18 4.6× 150 mm, 5 μm, 55 ° C, 1.2 mL/min, 17.6 mM Na_2HPO_4 aq. (pH 8.2)-MeCN, gradient 27% to 33% in 0-5 min, 33% to 37% in 5-25 min, 70% in 25-30 min.

We also tried to synthesize a peptide bearing a carboxylic terminus. Bivalirudin,[16] with 20 amino acid residues, was elongated using a similar protocol to Degarelix via a one-pot method using compound **5** as an anchor molecule and only solvent extraction as a work-up (Scheme 3). All reactions and work ups were performed smoothly. The fully protected 20 residue peptide was completely soluble in the organic solvent and phase separation went smoothly in each washing step. As with Degarelix, all synthesized intermediate peptides during elongation were simply washed and subjected to the next coupling reaction without isolation such as precipitation as formerly reported.[8, 9] Fully protected product **11** was obtained through the one-pot synthesis in 73% yield (based on **5**, Figure 5). The purity of the crude product from global deprotection was 84%.



Scheme 3. Synthetic scheme for Bivalirudin elongation.

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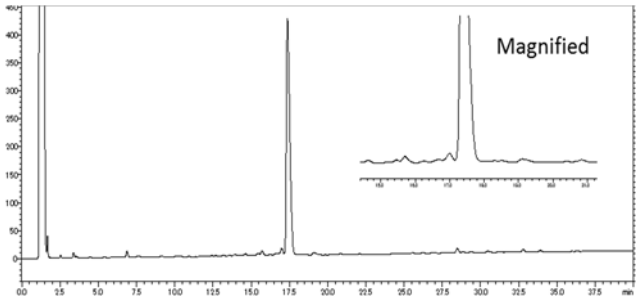

Figure 5. HPLC analysis of crude Bivalirudin; YMC-Pack ODS-A 4.6×150 mm, 5 μm, 40 ° C, 1.0 mL/min, 0.1% TFA in 5% THF-Water and 0.1% TFA in 5% THF-MeCN, gradient 20% to 40% in 0-25 min, 80% in 25-30 min.

Of note, peptide elongation can be performed in non-halogenated organic solvents such as CPME. Only a small portion of solvents were added at each elongation cycle, corresponding to increasing intermediate molecular weight, and the initial solvent stayed in the vessel throughout the synthesis. Therefore, solvent consumption is very low, approximately 1/10 compared with SPPS. This is an advantage of our AJIPHASE® method with **5** and **6** (Figure 6).

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

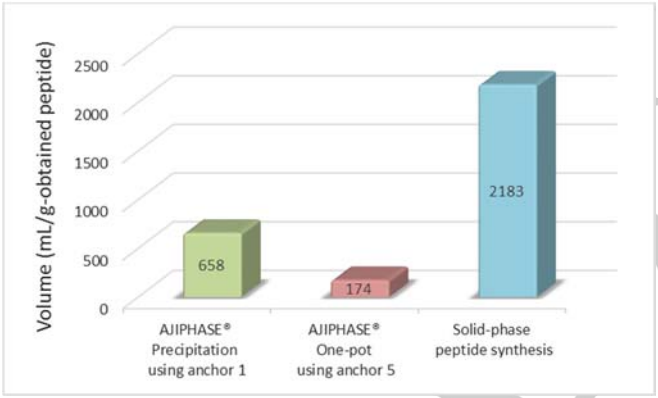

Figure 6. Solvent consumption of each peptide synthesis protocol in 20 mer peptide case.

In this work, successful development of a simple, efficient, and widely-applicable peptide synthesis protocol was reported using novel branched anchor compounds **5** or **6** in combination with a newly applied Fmoc deprotection system. The process afforded highly pure peptides, even for long or hydrophobic peptide, and is readily scaled. Evaporation of solvent and dehydration in the work up for following coupling steps are not necessary, and only a limited amount of solvent is added for each reaction. Thus, total solvent consumption is dramatically smaller, 1/12 that of the general protocol for SPPS. We are confident that these advantages can make the AJPHASE® protocol a significantly useful LPPS method even at a large scale.

Acknowledgements

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Keywords: AJIPHASE® • liquid-phase peptide synthesis • anchor molecule • one-pot peptide synthesis • Fmoc strategy

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AJIPHASE® one-pot peptide synthesis using solvent extraction without any isolation step during elongation. Highly efficient, High purity synthesis and significantly reduce solvent consumption.

Daisuke Takahashi, Tatsuji Inomata, Tatsuya Fukui*

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Highly efficient synthetic methodology AJIPHASE® for one-pot peptide elongation using an Fmoc strategy in the solution phase

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