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Novel diphenylmethyl-Derived Amide Protecting Group for Efficient Liquid-Phase Peptide Synthesis: AJIPHASE

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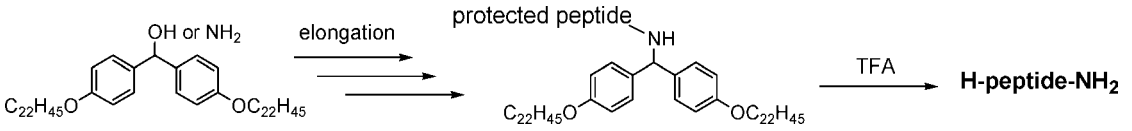
ABSTRACT



An efficient method for the synthesis of peptides bearing an amide at the C-terminal is described. This method involves the attachment of a C-terminal protecting group bearing long aliphatic chains, followed by the repetition of simple reaction and precipitation steps with the combined advantages of liquid-phase peptide synthesis (LPPS) and solid-phase peptide synthesis (SPPS). Using this method, a hydrophobic peptide was successfully synthesized in good yield and high purity, which cannot be obtained satisfactorily by SPPS.

Development of peptide drugs has increasingly expanded in recent years, and the need for peptide synthesis has increased accordingly.[1] The peptide synthesis methods are roughly divided into liquid-phase peptide synthesis (LPPS) and solid-phase peptide synthesis (SPPS) techniques, with much of the focus on SPPS, in which a simple workup procedure is possible. On the other hand, LPPS furnishes high-purity peptides and is economically efficient.[2] However, by using LPPS, it has been traditionally difficult to synthesize hydrophilic, hydrophobic, and long-chain peptides, mainly because of solubility issues and the time-consuming nature of its process development, which requires the establishment of workup procedures for each intermediate peptide.

However, several efficient LPPS methods have been developed to overcome these disadvantages.[3−7] Tamaki et al. reported an efficient LPPS protocol with a benzyl alcohol derivative bearing three long alkoxy chains **1**.[8] Chiba[9] and Sunazuka[10] also prepared peptides by a similar protocol (Figure 1). Separately, we have developed and reported[11] a novel fluorene-derived protecting group **2** that facilitates the efficient elongation of peptide chains and involved only simple repeated reaction and precipitation steps. This approach combines the advantages of both SPPS and LPPS. The fluorene-type protecting group exhibits an effect similar to that of the chlorotrityl linker used in SPPS, namely the avoidance of diketopiperazine formation. The selective detachment of this C-terminal protecting group with the protecting groups of the side chain intact leads to production of protected peptide acids that are useful for the convergent synthesis of long-chain peptides.

On the other hand, peptide drugs and peptide drug candidates are composed of various sequences and structures, and no efficient method has yet been discovered for the synthesis of these numerous types of peptides.

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In particular, C-terminal amide peptides have been developed with the aim of improving both their stability and activity,[12] and this type of peptide accounts for a large number of the current peptide drugs.[1] Therefore, an efficient method for the synthesis of amide peptides is required. Peptides with an amide at the C-terminus can be synthesized by an SPPS-based Fmoc strategy using the 4-methylbenzhydrylamine (MBHA) linker, the 5-[3,5-dimethoxy-4-(Fmoc-aminomethyl)phenoxypentanoic acid (PAL) linker, and the Rink amide resin, with detachment of the resin using TFA.[13] However, a C-terminal protecting group for efficient synthesis of peptide amides by LPPS has not yet been developed. Such a C-terminal protecting group must be able to not only form a stable bond with the peptide but also be detached by TFA in the final deprotection step.

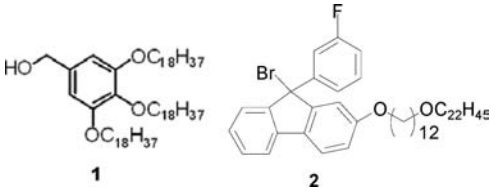
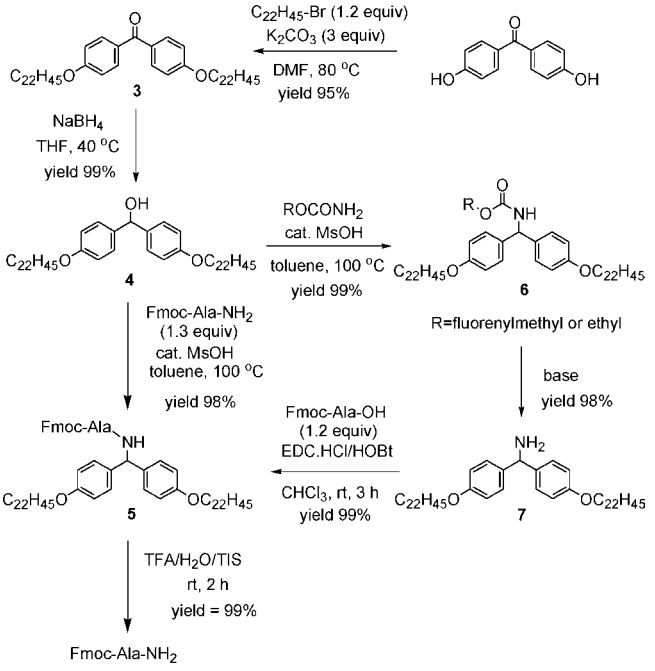


Figure 1. Benzyl alcohol derivative **1** and fluorene-derived protecting group compounds **2**.

In this study, we report the development of a diphenylmethyl-derived protecting group at the C-terminal and demonstrate its use in the efficient synthesis of various types of terminal amide peptides by an Fmoc strategy with a simple workup procedure.

A high-yield synthetic process for the targeted protecting group was accomplished (Scheme 1). First, the hydroxy groups of bis(4-hydroxyphenyl)methanone were treated with docosylbromide and K_2CO_3 in DMF to form ketone **3**. Second, the ketone was reduced using $NaBH_4$ to obtain bis[4-(docosyloxy)phenyl]methyl alcohol (Dpm-OH, **4**) in quantitative yield. This Dpm-OH type C-terminal protecting group **4** was then quantitatively coupled with Fmoc-Ala-NH_2 as the first amino acid using a catalytic amount of methanesulfonic acid to successfully afford **5**, suggesting that the compound **4** is very useful. However, in this Fmoc/tBu strategy, the acid-labile protecting groups of the side chain of Fmoc-AA-NH_2, such as Fmoc-Lys(Boc)-NH_2 and Fmoc-Asp(OtBu)-NH_2, could be partially lost during the loading to the Dpm-OH type protecting group because this loading step was performed under slightly acidic conditions. Therefore, the possible routes for the synthesis of amine **7** from alcohol **4** were investigated. Compound **4** was coupled with Fmoc-NH_2 or ethyl carbamate under high-temperature conditions with a catalytic amount of methanesulfonic acid to obtain intermediate **6** in quantitative yield. Subsequently, the Fmoc or ethoxycarbonyl group was deprotected under basic conditions to furnish the target molecule bis[4-(docosyloxy)phenyl]methylamine (**7**) (Dpm-NH_2).

Scheme 1. Synthesis, Loading of Fmoc-amino Acid, And Cleavage for Dpm-OH and Dpm-NH_2-Type Protecting Groups



Coupling of the Dpm-NH_2-type protecting group with Fmoc-Ala-OH using the typical coupling reagent system EDC·HCl/HOBt was then confirmed, and the coupling product **5** was precipitated from polar solvents such as MeOH or MeCN. As described above, the Dpm-OH-type protecting group **4** can be synthesized in only two steps from available reagents and coupled with Fmoc-AA-NH_2. On the other hand, the Dpm-NH_2-type protecting group **7** can be coupled with Fmoc-AA-OH with typical coupling reagents under mild conditions, although two additional steps are required. Both compounds are widely available for use as a C-terminal protecting group in the efficient synthesis of peptides. The Dpm-type protecting group was, as expected, readily removed using TFA, and therefore, the Dpm-type compound can be used as a protecting group for the C-terminal. These results imply that the protecting groups **4** and **7** are applicable as a protecting group at the carboxamide-containing side chain of amino acid residues, such as Asp/Glu and Asn/Gln. Thus, we confirmed that the Dpm-type protecting group can be applied to peptide amide synthesis as part of an efficient LPPS method involving the repetition of only simple reaction and precipitation operations. Furthermore, Dpm-type C-terminal protecting groups that can couple with Fmoc-AA-NH_2 or Fmoc-AA-OH were prepared.

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Using compound **7**, the synthesis of the peptide eptifibatide, which is a platelet aggregation inhibitor, was attempted employing this efficient LPPS method. Because eptifibatide is one of the best selling peptide drugs on the market, it would be beneficial if this peptide could be synthesized by our LPPS method.

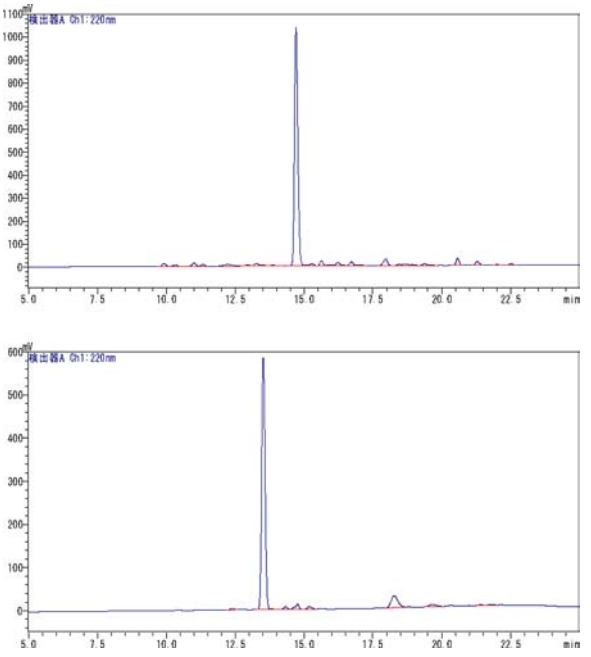


Figure 2. HPLC analysis. Top: crude peptide **9** after final deprotection. Bottom: crude peptide eptifibatide (**10**) after S to S disulfide bridge formation.

First, **7** was dissolved in $CHCl_3$ and coupled with Fmoc-Cys(Trt)-OH as the first amino acid using EDC·HCl/HOBt. The coupling reaction proceeded well with a negligible amount of racemization, and isolation was achieved by precipitation with MeOH. Subsequently, the loaded Fmoc-Cys(Trt)-OH compound was dissolved again in $CHCl_3$, the Fmoc group was deprotected using DBU/Et_2NH, and the product was precipitated with MeCN. These steps were repeated for the successful synthesis of the protected 7-mer peptide eptifibatide **8** in good yield (86%) from **7** (Scheme 2). The fully protected eptifibatide was then exposed to the standard cleavage cocktail (TFA/H_2O/triisopropylsilane = 95:2.5:2.5), and the crude linear eptifibatide **9** was obtained in high yield and in high purity without any closely related impurities that could affect the HPLC purification step (Figure 2). The crude peptide **9** was then smoothly cyclized at the disulfide bond between the mercaptopropionic acid (Mpa) and cysteine (Cys) residues under basic aqueous conditions, followed by purification via reversed-phase HPLC. As a result, eptifibatide (**10**) was successfully obtained in high purity. The quality of synthesized peptide **10** was confirmed with the comparison of NMR data to the authentic compound which was purchased from 2A PharmaChem Co.

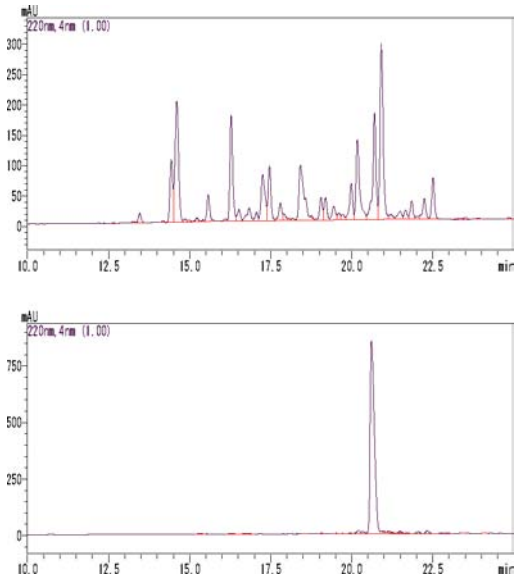


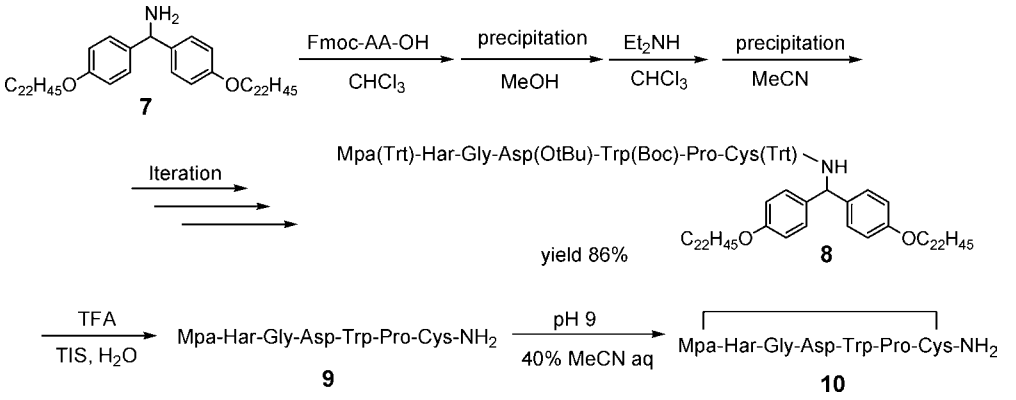
Figure 3. HPLC analysis. Top: crude peptide **11** (AAQVLISELAIA-NH_2) synthesized by SPPS after removal from the resin. Bottom: crude peptide **11** synthesized via the efficient LPPS using the Dpm-type C-terminal protecting group **7**.

It is commonly known that it is difficult to synthesize hydrophobic sequences of peptides employing the SPPS approach. Therefore, the synthesis of a hydrophobic peptide **11** (AAQVLISELAIA-NH_2) containing leucine (Leu), valine (Val), and others as a model peptide by both SPPS and LPPS was attempted using the Dpm-type protecting group **7** to compare the two methods. In the SPPS method, the FastMoc protocol was employed with preloaded Ala at the C-terminus of a Rink amide resin (Watanabe Chem. Ind. Ltd.) and 4 equiv of Fmoc-AA-OH for double coupling. The target peptide was not obtained as the main product, and some deletion peptides were observed because of incomplete reactions (Figure 3).

In contrast to the SPPS case, all of the coupling reactions were completed using only 1.1−1.5 equiv of Fmoc-AA-OH employing our efficient LPPS method. After the final deprotection, in which all the protecting groups of side chains and the Dpm-type C-terminal protecting group were removed, the crude peptide was successfully afforded in excellent purity (Figure 3). The yield of the total synthesis was 83% over 24 steps, indicating a 98.4% average yield for each amino acid elongation. The advantage of this peptide synthesis method can be easily recognized based on a comparison of the HPLC spectra for the SPPS synthesis and the new efficient LPPS method.

The reactivity of the coupling and Fmoc deprotection reactions is remarkably reduced during the synthesis of hydrophobic peptides via SPPS; thus, each reaction does not go to completion, and deletion peptides are generated during the elongation of the peptide sequence. To complete the reactions, heating, large excess of reagents, and extending reaction times are required. Unfortunately, side

Scheme 2. Elongation of the Eptifibatide Chain Using the Dpm-NH_2-Type Protecting Group



reactions, such as racemization and side-chain reactions, can occur as a result.

On the other hand, in the case of the conventional solution-phase approach with Bzl groups, the use of tBu as the protecting group at the C-terminus results in the decreased solubility of the protected peptide in the reaction solvents as the peptide chain is elongated, particularly for hydrophobic peptides.

However, in the newly developed LPPS method reported here, the long-chain character of the Dpm-type protecting group promotes the solubility of the growing peptide in chloroform. In fact, the solubility is increased, and complete dissolution can be accomplished with slight heating to only 30−35 °C. Therefore, by employing the efficient LPPS method using a Dpm-type protecting group, peptide chain elongation is possible. In addition, they can be obtained in very high yield and high purity because the reactivity of each reaction is favorable and side products are not generated.

Importantly, the bond between the Dpm-type protecting group and the peptide chain is not cleaved under the reaction or workup conditions. However, the Dpm-type C-terminal protecting group is easily cleaved under the standard final deprotection conditions (TFA) used in the Fmoc strategy. This behavior is another reason for obtaining target peptides in high yield and high purity.

Based on an investigation of the efficient LPPS, we developed Dpm-derived C-terminal protecting groups **4** and **7** that are useful for efficient LPPS. These molecules were synthesized from benzophenone derivatives in excellent yield and showed sufficient loading efficiency with both Fmoc-AA-OH and Fmoc-AA-NH_2.The bond between the peptide and the Dpm-type protecting group is stable during the elongation of the peptide sequence. Using the Dpm-type protecting groups **4** and **7**, the peptide chain can be elongated by simple repeated reaction and precipitation steps, and the crude peptide can be obtained using standard final deprotection conditions with TFA. Good solubility during a peptide-chain elongation reaction can be achieved by anchoring a protecting group bearing a long alkoxy chain at the C-terminus, and the byproducts can be readily removed by precipitation. In addition, we demonstrated that a hydrophobic peptide can be synthesized by this efficient method in higher yield and higher purity than that obtained with SPPS. As a result, this easy-to-implement and efficient LPPS protocol (AJIPHASE) was successfully extended to the synthesis of peptide amides, which account for the majority of current peptide drugs, and for which a sufficient LPPS method has not yet been developed to date. The synthesis of bioactive peptides and long-sequence peptides using the AJIPHASE protocol is in progress and will be reported elsewhere.

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Supporting Information Available. Synthetic procedure of diphenylmethyl-derived protecting groups, general procedure for peptide analysis and synthesis using the Dpm-type protecting group support compound **7**. Comparison and examination of model compounds for protecting group. This material is available free of charge via the Internet at http://pubs.acs.org

The authors declare no competing financial interest.