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Protection Side Chain of Lysine by Host-Affinity Molecules during Solid Phase Peptide Synthesis in Aqueous Phase

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Abstract

A new approach to protecting the side chain of lysine during solid-phase peptide synthesis in an aqueous environment is explored. The objectives of this study are twofold: 1) to reduce the consumption of chemical solvents in peptide synthesis, and 2) to lower the costs associated with lysine side chain protection. In an effort to minimize the usage of organic solvents in amino acid side chain protection for peptide synthesis,18-crown-6 molecule (18C6) was introduced as protective agent for Lysine (Lys) side chain protection. 18C6 is utilized to protect ε-amino group of on side chain of Lys in peptide synthesis. 18C6 features cavity at its center. The 18C6 molecules exhibit a unique and distinctive host-guest affinity towards ε -amino group on side chain of Lys. 18C6 bind to Lys side chain through hydrogen bonds with ε-amino group of Lys, eliminating the need for chemical reactions in organic solvents to attach protective molecules to the side chain groups. This simplifies the side chain protection procedure significantly for Lys, reducing the consumption of organic solvents. Consequently, this procedure offers advantages for contributing to a greener planet by minimizing chemical reactions and organic solvent usage. Additionally, the cost of side chain protection of Lys is dramatically reduced.

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Introduction

This research work relates to the protection of side chains of amino acids during solid phase peptide synthesis. Particularly, this research work involves a method for creating side chain-protected Lys using host-guest affinity molecule during peptide synthesis. Using host-guest affinity molecules to protect side chains of Lys, offers advantages for contributing to a greener SPPS (Solid Phase Peptide Synthesis) by minimizing chemical reactions and organic solvent usage.

The protection of side chains of amino acids in peptide synthesis has been extensively studied. A document from ETH Zürich on protecting groups in peptide synthesis provides a comprehensive overview of various protective strategies for amino acids, such as lysine, during peptide synthesis. It covers common protecting groups, their mechanisms, and the conditions for their removal. This resource is particularly valuable for selecting and applying protecting groups effectively [1].

The article 'Next Generation Lysine Side Chain Protecting Groups,' published by Iris Biotech, discusses advanced protecting groups designed for the lysine side chain in peptide synthesis. These new protecting groups aim to improve stability, selectivity, and ease of removal compared to traditional methods [2].

The 2009 review by Albericio et al., published in *Chemical Reviews*, offers an extensive overview of peptide synthesis, with a particular focus on the use of protecting groups. The review highlights advancements in solid-phase peptide synthesis (SPPS) and the development of new strategies for protecting side chains and reactive groups. It also addresses challenges in peptide synthesis, such as racemization and side reactions, and discusses strategies to overcome these issues [3].

The interaction between 18-crown-6 ether and the ε-amino group of Lys side chain has been well established. 18-crown-6 molecules bind strongly to protonated Lys in proteins and peptides through three hydrogen bonds. As a crown ether, 18-crown-6 binds to various small cations, including positively charged amino acids and N-termini in small peptides, with a particular affinity for potassium cations.

The review article by Steed provides a comprehensive overview of the host-guest chemistry involving 18-crown-6 complexes and amino acids, including lysine. It discusses various aspects such as the structural features of the complexes, binding interactions, and potential applications [4].

A book edited by Gale provides an in-depth exploration of the specific interactions between 18-crown -6 and lysine within the broader context of macrocyclic chemistry, including fundamental principles, synthetic strategies, and emerging trends [5].

This research article by Pease, A.R., et al. explores the binding behavior of 18-crown-6 with amino acids, including Lys in aqueous solutions. It investigates the thermodynamics and kinetics of the host-guest interactions and discusses the implications for designing self-assembled receptor systems [6].

A research work published in 2000 focuses specifically on lysine-containing peptides, investigating the binding affinity and selectivity of 18-crown-6 through solution-phase and gas-phase experiments. This study sheds light on the factors influencing the molecular recognition process [7].

This research article by Zhang, Y., et al. explores the intricate interplay between 18-crown-6 and lysine in aqueous environments, focusing on noncovalent interactions such as cation-π, CH-π, and lone pair-π interactions. It provides insights into the molecular recognition mechanisms underlying their complexation [8].

The 2002 study by Ryan R. Julian et al. investigates the high proton affinity of aza-18-crown-6 ether and its implications for the molecular recognition of lysine in peptides by lariat crown ethers. The research highlights how the unique structure of aza-18-crown-6 ether enables selective interaction with lysine residues, potentially influencing peptide binding and recognition processes, particularly in mass spectrometry applications. This work provides valuable insights into designing selective molecular receptors for lysine-containing peptides [9].

The 2016 study published in *Analyst* explores the ubiquitin and its interaction with crown ethers using ion mobility-mass spectrometry (IM-MS). The research focuses on identifying specific sites on ubiquitin where crown ethers complex with Lys residues. These findings deepen the understanding of how crown ethers recognize and bind to specific amino acids in proteins, providing valuable insights into protein structure and function. This approach sheds light on protein-ligand interactions in the gas

phase [10].

The 2006 study published in the *Journal of the American Society for Mass Spectrometry* explores the interactions between crown ethers and peptides, with a focus on the recognition of Lys residues. The research employs mass spectrometry techniques to investigate how crown ethers selectively bind to Lys, offering insights that have significant implications for protein analysis and molecular recognition. This study enhances the understanding of the role crown ethers play in increasing the selectivity and sensitivity of mass spectrometric detection of peptides [11].

The 2010 study published in *Analytica Chimica Acta* investigates the extraction and separation of a lysine-rich protein using crown ethers in an aqueous two-phase system. The research demonstrates that crown ethers can form supramolecular complexes with lysine residues on proteins, facilitating selective extraction and separation. This method shows promise for efficiently purifying lysine-rich proteins, presenting a novel approach to protein separation and purification processes in biochemical applications [12].

These research articles collectively provide a deeper understanding of how 18-crown-6 molecules interact with lysine at the molecular level, elucidating their potential applications in supramolecular chemistry and biomolecular recognition.

In peptide synthesis, protecting the side chain of lysine (Lys) is crucial to prevent unwanted reactions at its ε-amino group. This protection is typically achieved by using specific protecting groups that temporarily block the reactive sites on Lys, allowing selective reactions at other functional groups within the peptide chain. Common protecting groups for the ε-amino group of Lys include Boc (tert-butyloxycarbonyl) and Fmoc (9-fluorenylmethyloxycarbonyl). These groups can be selectively removed under mild conditions to expose the reactive amino group of lys, enabling further peptide synthesis steps.

Lys has two amino groups: the α-amino group attached to the α-carbon, which is involved in peptide bond formation, and the ε-amino group on the side chain. During peptide synthesis, the ε-amino group must be protected to prevent it from participating in unwanted side reactions. Due to its high reactivity, effective protection of the ε-amino group is essential.

Traditionally, one of the side chain protection schemes used in peptide synthesis for Lys is the Alloc (allyloxycarbonyl) group. Alloc protects the ε-amino group of Lys by forming a stable bond with it. This protection is typically achieved by reacting Lys with allyl chloroformate in the presence of a base, such as triethylamine (TEA), in an organic solvent like dichloromethane (DCM) or tetrahydrofuran (THF). This reaction yields a Lys derivative with the Alloc group attached to the ε-amino group. After peptide synthesis is complete, the Alloc protecting group is removed to expose the free amino group of Lys, typically accomplished by treating with phenylsilane (PhSiH3)

The Boc (tert-butyloxycarbonyl) group is also used to protect the side chain of Lys . Boc is stable under the acidic conditions typically used for peptide coupling and can be removed by treatment with strong acids like trifluoroacetic acid (TFA). The removal of Boc requires strong acidic conditions, which can be detrimental to peptides that are sensitive to acids.

Attaching a protective chemical group to the amino group on the side chain of Lys presents a unique challenge. Lys has two amino groups: the ε-amino group on the side chain and the α-amino group adjacent to the carboxyl group. Both amino groups exhibit similar reactivity toward Fmoc and Alloc (or Boc). To selectively attach Fmoc to the α-amino group and Alloc (or Boc) to the ε-amino group

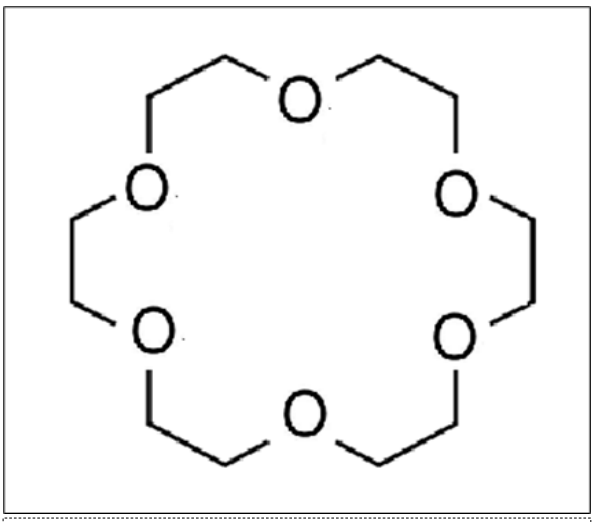

Figure 1. Molecule 18C6 (18-crown-6) structure

requires a series of tedious chemical reactions in organic solvents. This process is not environmentally friendly and can be quite costly.

Recent studies [2-5] have found on 18-Crown-6 (18C6) enhanced affinity for the side chains of Lys. 18C6 features a cavity structure, and its cavity specifically interact with the ε-amino group on amino acid side chains. Figure 1 illustrates the molecular structure of 18-Crown-6 (18C6).

We have developed a novel, environmentally friendly approach that streamlines the side chain protection of Lys in peptide synthesis. By utilizing 18-crown-6 (18C6) to protect the ε-amino group on the side chain of lysine, we simplify the process required for lysine side chain protection

In solid-phase peptide synthesis (SPPS), Fmoc-protected amino acids (where Fmoc stands for fluorenylmethoxycarbonyl) are commonly used for sequential peptide elongation. We have developed procedures for preparing Fmoc-Lys with side chains protected by 18-crown-6 (18C6), abbreviated as Fmoc-Lys(18C6), and for utilizing Fmoc-Lys(18C6) as building blocks in SPPS.

The 18-crown-6 (18C6) molecule binds to the ε-amino group on the side chain of Lys through hydrogen bonds, eliminating the need for chemical reactions to attach protective groups to ε-amino group on side chain groups of Lys. This significantly simplifies the side chain protection procedure and reduces the consumption of organic solvents. As a result, this method contributes to a greener planet by minimizing chemical reactions and organic solvent usage

Results and Discussion

Fmoc-AA, where the alpha amino groups of amino acids are protected by Fmoc, is one of the most widely used building blocks in solid-phase peptide synthesis. By leveraging the specific affinity of 18-crown-6 ether towards the ε-amino group on the side chain of lysine, we developed a procedure for synthesizing molecule of Fmoc-Lys with side chain protected by 18C6 denoted s Fmoc-Lys(18C6), Figure 2 shows molecule structure of Fmoc-Lys(18C6).

This Fmoc-Lys(18C6) molecule can serve as a building block in peptide synthesis, especially in solid-phase peptide synthesis when conducted in aqueous solution.

Experiment

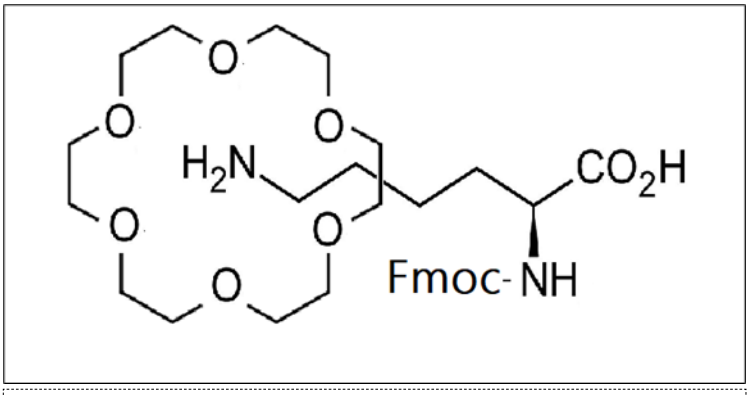

Figure 2. Fmoc-Lys(18C6) structure

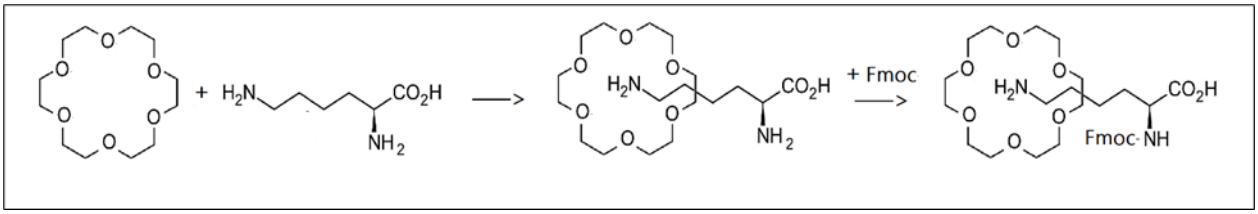

Figure 3. The chemical reaction for synthesis of Fmoc-Lys(18C6)

Preparing Fmoc-Lys(18C6)

First, Lys is incubated with 18C6 in an aqueous solution to ensure that 18C6 is attached to the ε-amino group on side chain groups of Lys. Subsequently, the Lys with attached 18C6 in the aqueous solution is added to a solution of Fmoc in DMSO (Dimethyl sulfoxide). This results in the formation of Fmoc-Lys (18C6) in the solution. Figure 3 demonstrates the chemical reaction for synthesis of Fmoc-Lys(18C6).

Procedure for synthesizing Fmoc-Lys with the side chain protected by the 18C6 molecule is outlined in the following steps for preparing Fmoc-Lys(18C6):

1. Dissolve Lys and 18C6 in water to create Solution A.
2. Dissolve Fmoc in DMSO to create Solution B. Ensure that the volume of DMSO is at least four times the volume of Solution A to prevent Fmoc precipitation before the reaction.
3. Add Solution A dropwise into Solution B. If the solution becomes cloudy, add more DMSO. Allow the reaction to proceed for 2 hours.
4. Fmoc-Lys(18C6). will form in the mixture.
5. To remove unreacted Lys and 18C6, add additional water to the mixture, causing precipitation. Lys and 18C6 are highly soluble in water, while Fmoc-Lys(18C6) is poorly soluble, leading to a precipitate mainly composed of Fmoc-Lys(18C6).
6. Filter out the precipitate, and the Fmoc-Lys(18C6) is ready for peptide synthesis.
7. After completing peptide synthesis, the Lys side chain can be deprotected by removing the 18C6 with K^+ or diamine.

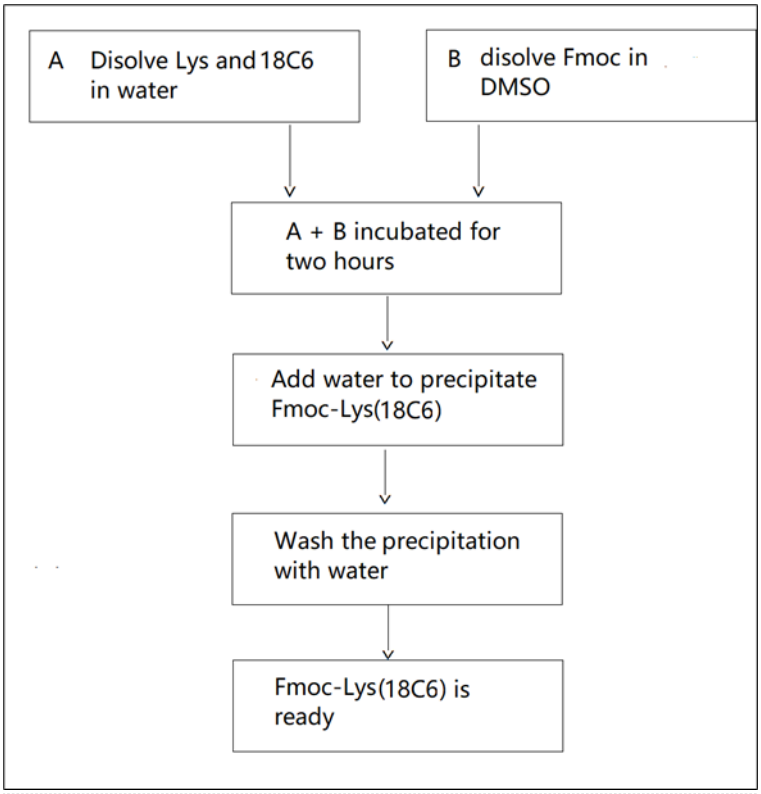

Figure 4. Flow chart for making Fmoc-Lys(8C6)

The flowchart for synthesizing Fmoc-Lys(18C6) is illustrated in Fig. 4.

Experiment of preparing Fmoc-Lys(18C6)

1. Dissolve approximately 1 gram of Lys and 1.8 grams of 18C6 in 20 ml of water. Incubate the solution for 12 hours to prepare Solution A. Note that 18C6 dissolves slowly in the aqueous phase. The molar ratio of Lys to 18C6 is approximately 1:1.2, with an excess of 18C6 relative to Lys. Based on our lab experiments, 18C6 increases the solubility of Lys in organic solvents, which is beneficial for its reaction with Fmoc.

2. Dissolve 1.7 grams of Fmoc in 80 ml of DMSO to create Solution B. The molar ratio of Lys to Fmoc is approximately 1.2:1.

3. Add Solution A dropwise into Solution B. If precipitation forms, add additional DMSO to ensure complete solubility of Fmoc in the reaction mixture. Allow the mixture to stand for 2 hours.

This mixture contains Fmoc-Lys(18C6), where the side chain of Fmoc-Lys is protected by an 18-crown -6 (18C6) ether molecule.

Purify Fmoc-Lys(18C6) based on its hydrophobic nature. While Fmoc-Lys(18C6) is poorly soluble in water, both Lys and 18C6 are soluble. The molar ratio of Lys(18C6) should be in excess relative to Fmoc to ensure complete reaction. This approach has two main advantages: 1) Cost reduction, as Fmoc is more expensive than lysine and 18C6, and 2) Simplified purification, since both Fmoc and Fmoc-Lys (18C6) are poorly soluble in aqueous solutions, while Lys and 18C6 are highly soluble, making it easier to separate them by adding water.

Add 200 ml of water to the mixture to induce precipitation. Lys and 18C6 are highly soluble in water, while Fmoc-Lys(18C6) is poorly soluble, resulting in the precipitation of Fmoc-Lys(18C6).

Filter the precipitate; the collected Fmoc-Lys(18C6) is ready for peptide synthesis. The yield is approximately 75%. The chemical reactions are depicted below:

Lys + 18C6 → Lys(18C6), by affinity of 18C6 toward amino group on side chain of Lys

Lys(18C6) + Fmoc → Fmoc-Lys(18C6), Since the amine on side chain of Lys is protected by 18C6, Fmoc only reacts with the alpha amine of Lys.

Scheme for Removing the 18C6 Protective Group from Lys

In an aqueous solution, potassium ions (K^+) can effectively remove the 18C6 protective group from the side chain of Lys. The strong affinity of K^+ for the cavity of 18C6 enables it to bind with 18C6 in solution, thereby facilitating the removal of the protective group.

Fmoc-Lys(18C6) + K^+ → Lys + K+18C6

In organic solvents, the protective group 18C6 can be removed by a diamine (such as ethylenediamine), which is soluble in organic solvents and can replace 18C6 on the ε-amino group of the Lys side chain.

Lys(18C6) + diamine → Lys + diamine (18C6)

Example Experiment: Synthesis of a Peptide Using Fmoc-Lys(18C6) as a Building Block,

Procedure for Synthesizing the Taurine-Lysine Dipeptide in Aqueous Solution:

1.Taurine Resin Preparation: Incubate 1 gram of Taurine with 5 grams of anion exchange resin in 20 mL of water for two hours. Wash thoroughly to remove any excess Taurine.

2. Dissolution of Fmoc-Lys(18C6): Using the method for dissolving hydrophobic molecules [13], dissolve 0.5 grams of Fmoc-Lys(18C6) in 4 mL of DMSO with 2 grams of Tween 80. Introduce this mixture into the Taurine-bound anion exchange resin in 20 mL of water.

3. Stabilization with 18C6: Add 0.1 grams of 18C6 to the solution to prevent the dissociation of 18C6 from Lys.

4. Coupling Reaction: Add the coupling agent EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) to the mixture.

5. pH Adjustment: Adjust the pH of the solution to approximately 4.5–5.0 and incubate for 60 minutes.

The dipeptide Taurine-Fmoc-Lys(18C6) is formed.

6. Washing: Wash away any excess EDC with a 10% Tween solution.

7. Deprotection: Remove the Fmoc protective group by adjusting the pH to 9.5–10.0.

8. Final Washing: Wash with a 10% Tween solution.

Result: The dipeptide Taurine-Lys(18C6) remains attached to the resin.

Deprotection of Protective Group 18C6

1. Introduce 2 ml of 0.5% KCl solution.

2. The reaction proceeds as follows: Taurine-Lys(18C6) + KCl → Taurine-Lys + K18C6. Potassium has a stronger affinity for 18C6 than the side chain of Lys, thereby displacing 18C6 from the Lys side chain. Using Potassium ion to remove 18C6 is better in aqueous solution.

3. To remove the dipeptide from the anion exchange resin, a 20% salt solution can be used.

Another way to remove 18C6:

1.Introduce 2 ml of a 0.5% ethylenediamine solution.

2.The reaction is as follows: Taurine-Lys(18C6) + diamine → Taurine-Lys + K18C6(diamine) The diamine has a higher affinity for 18C6 than the side chain of Lys, thereby displacing 18C6 from the Lys side chain. Using diamine to remove 18C6 is better in organic solvents.

3.Use 20% salt to remove the dipeptide from the anion exchange column.

Chemical reactions are depicted in here:

Taurine + Anion Exchange Resin → Resin-Taurine

(Binding through ionic bond in water)

Resin-Taurine + Fmoc-Lys(18) + EDC → Resin-Taurine-(Fmoc)-Lys(18C6)

(Coupling reaction facilitated by EDC)

Resin-Taurine-(Fmoc)-Lys(18C6) in pH 9.5–10 → Resin-Taurine-Lys(18C6) + Fmoc

(Deprotection of Fmoc at pH 9.5–10)

Resin-Taurine-Lys(18C6) + KCl → Resin-Taurine-Lys

(Removal of protective group 18C6 by potassium ion)

Resin-Taurine-Lys + 20% Salt → Resin + Taurine-Lys

(Removing of the peptide from the resin)

Conclusion

Fmoc-AA derivatives are among the most widely used building blocks in solid-phase peptide synthesis (SPPS). Traditionally, the side chain of Lysine (Lys) is protected using Boc or Alloc groups, with Fmoc-Lys(Boc) being a common choice. In this study, we developed a new approach to synthesize Fmoc-Lys(18C6), where the ε-amino group is protected by 18C6 and the α-amino group by Fmoc. Fmoc-Lys(18C6) can serve as a valuable building block in peptide synthesis, particularly advantageous for aqueous solutions.

The 18C6 molecule exhibits a unique host-guest interaction with the ε-amino group of the Lys side chain, binding through hydrogen bonds and eliminating the need for organic solvent-based chemical reactions to attach protective groups. This innovation significantly reduces the consumption of organic solvents. Furthermore, the specific affinity of 18C6 for the ε-amino group allows for a marked reduction in the number of chemical reactions required to protect both the α-amino group with Fmoc and the ε-amino group with 18C6. This method not only supports greener practices by minimizing chemical reactions and solvent usage but also substantially lowers the cost of Lys side chain protection.

In summary, this approach offers significant environmental and economic benefits by reducing the reliance on chemical reactions and organic solvents, while also lowering the cost associated with Lys side chain protection.

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