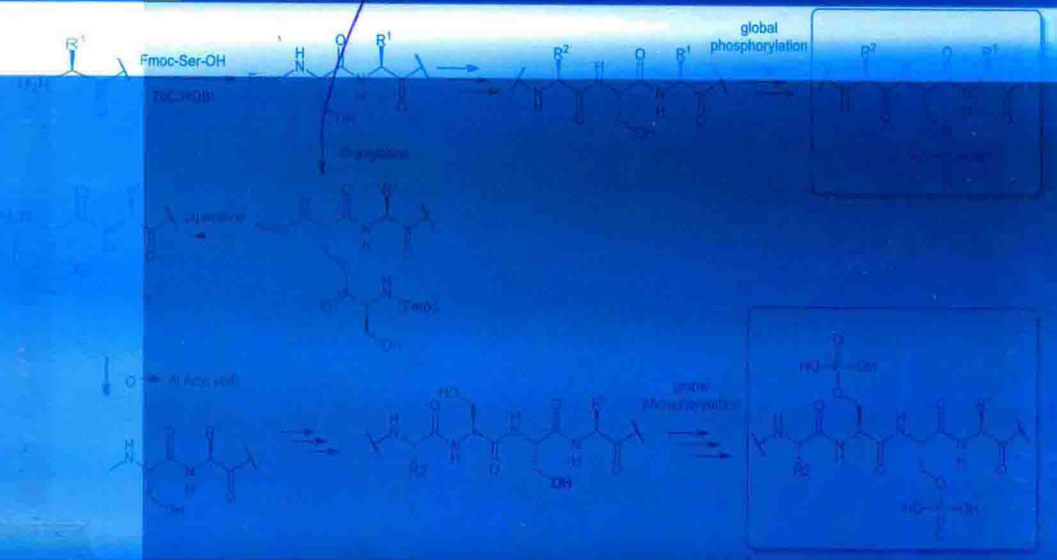
Yi Yang

多肽合成副反应

Side Reactions in Peptide Synthesis



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内 容 简 介

本书是作者在十多年多肽合成第一手经验的基础之上，结合大量相关文献完成的。全书系统地介绍了多肽合成中最常见的副反应，其产生的机理，以及相应的解决方案。其中很多副反应的产生是在GMP生产条件下被发现并加以研究的，其形成机理与生产工艺的开发紧密相关。多肽杂质的形成对于多肽类API的GMP生产具有非常关键的影响，因此检测和分析多肽杂质对成功的API工业生产至关重要。而掌握多肽副反应产生的机理、分析手段及相应的优化方案，则是整个多肽API工艺开发和生产环节中的核心要素。本书可供学术界与工业界相关人员参考使用。

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Dedication

Dedicated to my wife, Dan Liu and my son, Qinqin Yang.

Preface

Thanks to their superior properties in terms of high selectivity, enhanced efficacy, and appreciable safety, peptide/peptidomimetic APIs are playing increasingly important roles in the domains of pharmaceuticals and biotech industries by means of hormones, neurotransmitters, growth factors, ion-channel ligands, anti-infectives, and so forth. Simulated by the ever-improving performances of diverse peptide therapeutics, more and more intentions are therefore legitimate to be emphasized on the peptide design and synthesis, endeavored jointly by academic and industrial efforts.

Some inherently specific properties of peptide synthesis relative to those of conventional small molecules could cause rather complicated impurity profiles. Moreover, challenges originated from the impurity formation could be intensified by the fact that certain, if not all, peptide productions lack the intermediary purifying effects in the upstream process prior to chromatographic purification treatment. Needless to mention, the impacts of scaling effects on peptide impurity formation are sometimes tricky to be elucidated. All these intrinsic challenges legitimate the necessity to pay considerable attention to the side reactions that occur in peptide synthesis.

As a first step, the impurity profile of the subject peptide API is supposed to be thoroughly scrutinized, particularly in peptide cGMP production to identify the criticalities of each single impurity against the predefined specifications. The impurities, which are potentially critical to quality or business should be emphasized and paid with peculiar attentions. It is subsequently pertinent to correlate the formation of these critical impurities with the corresponding side reactions and make efforts to elucidate the mechanism of the identified side reactions. Solutions to tackle the relevant side reactions could be designed based on the solid in-depth understanding of the origins and attributes of these side reactions. Different reaction strategies and/or process parameters are supposed to be investigated in order to fit the designed models to the purpose of the impurity suppression. By this means, the critical impurities encountered in peptide production could be diminished or eliminated at the upstream process and alleviate the stress on the purification steps. Hence, the quality of the final peptide API is assured and the process performance could be enhanced accordingly.

It is implied from the aforementioned process optimization procedure that an insight into the peptide side reactions is of crucial importance to the success

of peptide API manufacturing. I have written this book to address the most frequent side reactions in peptide synthesis on the basis of a plethora of side reactions encountered in my 10 years of commitment to peptide synthesis research and peptide API production (which is simultaneously unfortunate and fortunate). The side reactions are classified in accordance with their extrinsic properties. Understandably, these categorizations are not absolutely orthogonal and indeed some independence exists. In each chapter of the book, the phenomenon of the side reactions is elucidated. The mechanism of each side reaction is either described or tentatively proposed for further discussion. Finally, diverse possible solutions are suggested in order to tackle the referred side reactions. Abundant literature references are listed for extensive reading.

The systematically organized knowledge behind a plethora of peptide-related side reactions could be sort of help for the colleagues who work in this area, no matter whether they are academic or production oriented. It is especially meaningful for the cGMP production in which a single out-of-specification impurity could ruin the whole production. Detection and analysis of the impurities in peptide synthesis, as well as their corresponding solution is therefore highly accentuated. How to find out the clues from a complicated impurity profile could understandably decide the outcomes of the peptide production. Hopefully this book could be of some help to treat the problems raised by peptide impurities, particularly in the realm of peptide API production and could ultimately assist us to fight relevant diseases.

I wish to express my thanks to Bielefeld University, Lonza AG, Ferring Pharmaceuticals, and GenScript Inc., the former three in particular, that provided me the outstanding platforms to explore the wonderland of peptide. I am grateful to Prof. Dr. Norbert Sewald and Prof. Fernando Albericio. The former introduced me to the peptide realm as a mentor and the latter gave me a lot of instructions in my career. I also appreciate the efforts made by Malik Leila and Jörgen Sjögren for their reviews of the manuscript. Fabrizio Badalassi (my boss) offered me tremendous support during the preparation of the book. Last but not the least, I am obliged to my wife Dan Liu, since her support bestows me all the strength to pursue my dream.

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Chapter 1

Peptide Fragmentation/Deletion Side Reactions

Due to the inherent attributes of certain peptide individuals they could undergo a variety of fragmentation processes during synthesis, purification or even storage. Fragmentation could selectively address peptides with characteristic sequences like *N*-terminal *N*-Ac-*N*-alkyl moiety, *N*-acyl-*N*-alkyl-Aib-Xaa- bond, -Asp-Pro-, *N*-terminal His-Pro-Xaa- moiety, *C*-terminal *N*-Me-Xaa, *N*-terminal FITC, thioamide bond and guanidinyl group on Arg side chain, etc. Moreover, utilization of isodipeptide Boc-Ser/Thr(Fmoc-Xaa)-OH as the building block for peptide synthesis could result in the formation of des-Ser/Thr impurity. On top of these specific cases DKP formation could also affect general peptide assembly that leads to the deletion of affected dipeptide moiety from the parental peptide sequence. The occurrence of these fragmentation/deletion side reactions on peptide materials could decrease the manufacturing yield, cause challenges for the down-stream peptide purification, and affect peptide stability upon processing and/or storage. Phenomenon and mechanism of common fragmentation/deletion in peptide synthesis are described in this chapter. Corresponding solutions to minimize these side reactions are proposed.

1.1 ACIDOLYSIS OF PEPTIDES CONTAINING *N*-Ac-*N*-alkyl-Xaa MOTIF

Peptides with a motif of *N*-Ac-*N*-alkyl-Xaa sequence at the *N*-terminus have the distinctively high propensity to suffer from an acidolysis side reaction at the step of acid-mediated peptide cleavage from resin and side chain global deprotection. The *N*-terminal *N*-Ac-*N*-alkyl-Xaa unit might be split from the parental peptide as a 5-member ring derivative, leading to the formation of des-*N*-Ac-*N*-alkyl-Xaa truncated side product.

This kind of side reaction has been detected in the process of the preparation of a series of Arodyn peptides ((acetylated Dyn A) Arodyn 1, 2, 3, 4).[1] It was reasoned that the synthesis of Arodyn 2 resulted in the acidolytic cleavage of *N*-terminal motif *N*-Ac-*N*-Me-Phe during the TFA-mediated global deprotection step (Table 1.1).

The proposed mechanism of the subjected acidolysis side reaction is indicated in Fig. 1.1. It is reasoned in the corresponding investigation that the

TABLE 1.1 Sequences of Arodyn 1, Arodyn 2, Arodyn 3, Arodyn 4 Peptides

Dyn A(1-11)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2
Arodyn 1	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2
Arodyn 2	Ac-*N*-Me-Phe-Phe-Trp-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2
Arodyn 3	CH_3OCO-*N*-Me-Phe-Phe-Trp-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2
Arodyn 4	*N*-Me-Phe-Phe-Trp-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

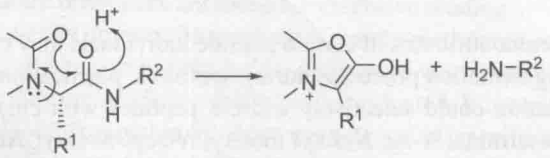

FIGURE 1.1 Proposed mechanism of the acidolytic cleavage of *N*-Ac-*N*-alkyl-Xaa from parental peptide.

occurrence of this side reaction is subject to the actual conditions under which the peptide global deprotection is conducted. It is verified that if the referred reaction is processed at 4°C in the absence of any scavengers the acidolysis of *N*-terminal *N*-Ac-*N*-alkyl-Xaa could be significantly suppressed. No similar impurities with deletion sequences have been detected in the process of Dyn A(1-11) or Arodyn 1 synthesis. The preparation of Arodyn 4 that is devoid of acetyl moiety on its *N*-terminus does not suffer from the concerned acidolysis side reaction upon TFA treatment, accounting for the involvement of the acetyl functional group in the process of *N*-Ac-*N*-alkyl-Xaa acidolysis. Significant *N*-terminus acidolysis side reaction has been invoked in the synthesis of Arodyn 2 in which Ac-*N*-Me-Phe is located on the *N*-terminus compared with the Ac-Phe motif from Arodyn1. This phenomenon is attributed to the presence of *N*-alkyl amino acid residue that favors the advantageous peptide secondary structure facilitating the acidolytic fragmentation of the *N*-terminal residue. In case the *N*-terminal acetyl is replaced by more electron-withdrawing group methyl carbamate, as is the case for Arodyn 3, the subjected acidolysis side reaction on the peptide *N*-terminus would be basically circumvented due to the decrease of the nucleophilicity of the carbonyl oxygen from the methyl carbamate that initiates the ring closure in the acidolytic fragmentation process. It could therefore be deduced from the aforementioned phenomenon that the acidolysis of peptide *N*-terminal *N*-Ac-*N*-alkyl-Xaa motif is induced by the acetyl oxygen nucleophilic attack on the amide bond between the subjected *N*-Ac-*N*-alkyl-Xaa and the neighboring amino acid at its *C*-terminus, facilitated by the advantageous local structure in that the ratio of *cis*-amide bond is significantly increased by the presence of an *N*-alkyl-amino acid residue. Under such

circumstances the *N*-acetyl group serves as a nucleophile that initiates the ring closure, and subsequent acidolytic fragmentation of the *N*-Ac-*N*-alkyl-Xaa unit.

1.2 Des-Ser/Thr IMPURITIES INDUCED BY *O*-acyl ISODIPEPTIDE Boc-Ser/Thr(Fmoc-Xaa)-OH AS BUILDING BLOCK FOR PEPTIDE SYNTHESIS

O-acyl isodipeptide derivatives have already found widespread application as effective building blocks in peptide synthesis, particularly for difficult peptide assemblies that are hardly quantitatively realized by the conventional stepwise coupling methods. This methodology takes advantage of the inherent feature of the base-induced reversible intramolecular acyl *O*→*N* shift that involves the ester bond from the Ser/Thr side chain and the α-amino group on the peptide backbone (Fig. 1.2).

The incorporation of the isodipeptide unit into the peptide sequence is intended to disrupt the adverse secondary structure of the subjected peptide that impedes the smooth coupling of the forthcoming amino acid to the elongating peptide chains, particularly for the "difficult couplings." Peptide secondary structures are basically induced and reinforced by diverse molecular interactions such as hydrogen bond, Van der Waals force, hydrophobic interaction, ionic bond, and so forth. The establishment of peptide secondary structure might considerably reduce the flexibility of the affected peptide chains that consequently adversely interferes with the subsequent amino acid couplings during peptide synthesis. This phenomenon is basically regarded as one of the major causes for the nonquantitative amino acid couplings occurred in peptide synthesis that accounts for the generation of peptide impurities with deletion sequences.

O-acyl isodipeptide building blocks[2–4] are utilized in an effort to address this inherent problem in peptide synthesis. The existence of -Xaa-Ser- or -Xaa-Thr- unit in the target peptide sequence is the prerequisite for the employment of

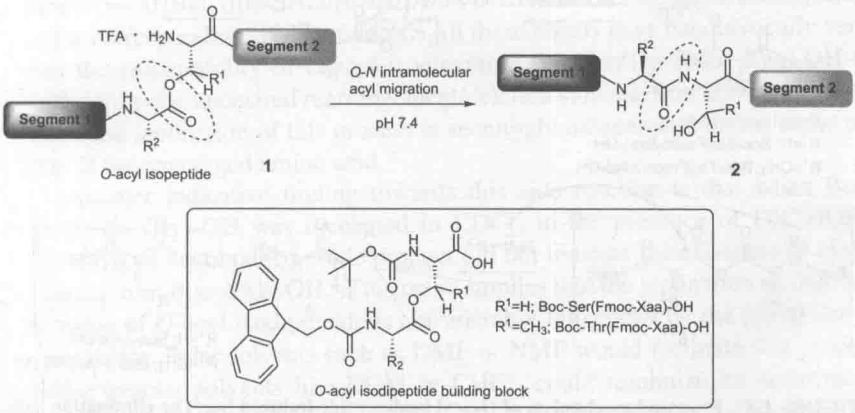

FIGURE 1.2 Peptide preparation via *O*-acyl isopeptide strategy.

O-acyl isodipeptide strategy. The subjected isodipeptide unit is incorporated in the manner of Boc-Ser/Thr(Fmoc-Xaa)-OH building block into the target peptide chains, functioning as the synthon for the natural -Xaa-Ser/Thr- counterpart. The intermediary product containing *O*-acyl isodipeptide structure is depicted as compound **1** in Fig. 1.2. The backbone carboxyl group of the -Xaa- unit is chemically linked with the hydroxyl side chain from Ser/Thr by means of an ester bond (highlighted in a dotted circle). The introduction of *O*-acyl isodipeptide moiety could manifestly disrupt the local peptide secondary structure. The solubility and liquid chromatographic properties of the peptide precursor **1** containing *O*-acyl motif are normally superior to those of its interchangeable *N*-acyl counterpart **2**. These outstanding features of *O*-acyl isopeptide could tremendously facilitate the otherwise challenging chromatographic purification. The purified *O*-acyl isopeptide **1** will be subsequently addressed to the base-catalyzed acyl *O*→*N* shift process that regenerates the natural form of the peptide amide bond via a five-member ring intermediate. The disadvantageous peptide secondary structure that impedes the smooth amino acid coupling is circumvented by this means, significantly facilitating the effective chemical preparation of the target peptide product.

In spite of the successful utility of *O*-acyl isodipeptide strategy manifested in the challenging peptide preparation such as β-amyloid 1-42,[5] it has been detected that this methodology could potentially induce side reactions such as β-elimination which leads to the formation of des-Ser/Thr impurities. The possible mechanism of this side reaction is originated from the formation of active ester Boc-Ser/Thr(Fmoc-Xaa)-OBt **4** derived from the carboxylate activation of its precursor *O*-acyl isodipeptide Boc-Ser/Thr(Fmoc-Xaa)-OH **3** (as depicted in Fig. 1.3). The lifespan of the activated derivative **4** in the reaction system is directly correlated to the kinetics of the subjected acylation reaction. If the referred reaction is proceeding

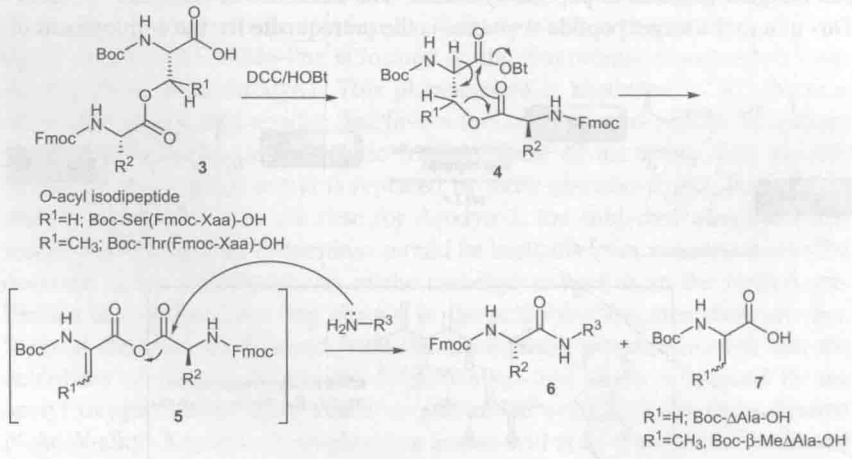

FIGURE 1.3 Proposed mechanism of *O*-acyl isodipeptide induced Ser/Thr elimination side reaction.

sluggishly, Boc-Ser/Thr(Fmoc-Xaa)-OBt **4** will be afforded with sufficient time to deviate from the target intermolecular condensation reaction and undergo intramolecular rearrangement by means of β-elimination, giving rise to the formation of the mixed anhydride 5 from Fmoc-Xaa-OH and Boc-(β-Me)ΔAla-OH, as indicated in Fig. 1.3. As a consequence, the unacylated peptide chain could possibly function with **5** at its two reactive sites, but the anhydride carbonyl at Fmoc-Xaa side is preferred due to the fact that the unsaturated (β-Me)ΔAla side chain unavoidably attenuates the electrophilicity of anhydride carbonyl on the Boc-(β-Me)ΔAla side. The unit of (β-Me)ΔAla is, therefore, excluded from the product structure as Boc-(β-Me)ΔAla-OH **7** upon the nucleophilic attack of the peptide N^{α} on the mixed anhydride **5**, giving rise to the formation of des-Ser/Thr impurity **6**.

In order to verify the proposed mechanism of *O*-acyl isodipeptide-induced deletion side reaction, Boc-Ser(Fmoc-Gly)-OH isodipeptide was incubated in NMP in the presence of DCC (2 equiv.)/HOBt (2 equiv.) for 2 h before 2.2 equiv. benzylamine was charged into the reaction system.[5] The obtained product was analyzed by MS and analytical RP-HPLC, and no Boc-Ser(Fmoc-Gly)-NHBzl was detected while large amount of Fmoc-Gly-NHBzl as well as Boc-ΔAla-NHBzl were located instead. As a matter of fact, the abundance of Fmoc-Gly-NHBzl side-product in the crude material is as high as 80%. In another experiment *O*-acyl isodipeptide Boc-Ser(Fmoc-Gly)-OH was subject to the activation process by 2 equiv. DIC/2 equiv. HOBt in DMF-d_7 for 2 h, ^{1}H-NMR analysis of the obtained product detected 2 types of olefin hydrogen signal which were assigned to E/Z isomers. This result combined with the corresponding MS and RP-HPLC analysis explicitly indicates that Boc-Ser(Fmoc-Gly)-OH has almost been quantitatively converted to the mixed anhydride composed of Fmoc-Gly-OH and Boc-ΔAla-OH within 2 h upon activation by DIC/HOBt.

Moreover, Boc-Ser(Fmoc-Ile)-OH, Boc-Thr(Fmoc-Gly)-OH and Boc-Thr(Fmoc-Ile)-OH were subject to DIC (2 equiv.)/HOBt (2 equiv.) activation in DMF for 2 h, respectively, before 2 equiv. benzylamine was charged into the reaction system to entrap the activated species. Abundant Fmoc-Gly-NHBzl, Fmoc-Ile-NHBzl, Boc-ΔAla/β-MeΔAla-NHBzl were detected as a consequence in the corresponding crude products.[5] All these results have unequivocally verified the susceptibility of *O*-acyl isodipeptide Boc-Ser/Thr(Fmoc-Xaa)-OH to suffer from the undesired rearrangement/deletion side reaction upon activation, while the inclination of this process is seemingly independent on the steric effect of the concerned amino acid.

Another indicative finding towards this side reaction is that when Boc-Ser(Fmoc-Gly)-OH was incubated in $CDCl_3$ in the presence of DIC/HOBt, ^{1}H-NMR of the obtained crude product did not indicate the existence of olefin signals from Boc-ΔAla-OH.[5] This result implies that the inclination of this side reaction of *O*-acyl isodipeptide is considerably influenced by the properties of the solvent. Polar solvents such as DMF or NMP would facilitate this process while unpolar solvents like DCM or $CHCl_3$ could minimize its occurrence. In light of this finding, it is advisable to utilize the unpolar solvents for the