Advances in Peptide Synthesis

8

Gajanan K. Rathod, Rajkumar Misra, and Rahul Jain

Abstract

Peptide drug development has made significant progress over the last century. The discovery of solid-phase peptide synthesis has enabled chemists to synthesize various peptides with divergent sequence patterns. However, due to the increased demand for various peptide sequences in the modern pharmaceutical industry, there is always room for new methods to modify the existing methods to improve yield, purity, and synthesis time. The current century has witnessed a lot of progress in the field of peptide synthesis, including developments in new synthetic strategies, suitable selection of protecting groups, and introduction of efficient coupling reagents, as well as the development of automated peptide synthesizers. This chapter will give a summary of the recent reports on the most significant breakthroughs in peptide chemical synthesis in current years.

Keywords

Amino acids · Microwave · Peptides · Solid phase · Solution phase

8.1 Introduction

Proteins and peptides are ubiquitous in every living organism and play a crucial role in the development of life. They are responsible for a variety of physiological and biological activities that occur in the living system, including the transport of diverse ingredients across membranes and intercellular communication, and serve as the

G. K. Rathod · R. Misra · R. Jain (✉)
Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Mohali, Punjab, India
e-mail: rahuljain@niper.ac.in

P. P. Singh (ed.), *Recent Advances in Pharmaceutical Innovation and Research*,
https://doi.org/10.1007/978-981-99-2302-1_8

primary component of antibodies that protect the organisms. In addition, due to their inherent biocompatibility as well as high specificity, polypeptides and proteins are considered active pharmaceutical ingredients (API) in most of the modern pharmaceutical industries (Liu et al. 2012; Domalaon et al. 2016; Våbenø et al. 2015; Uhlig et al. 2014; Fosgerau and Hoffmann 2015). Polypeptides, which are composed of 20 natural amino acids have exceptional therapeutic and biological properties, including antioxidant, antibacterial, anti-inflammatory, anticancer, and anti-HIV activity. Due to the high biological potential of the peptides, more than 150 peptides have been approved as drugs and some of them are in clinical trials (Wang et al. 2022). Currently, the peptide therapeutics market is valued at approximately USD 28 billion and is expected to increase by more than 10% over the coming years (Henninot et al. 2018). Even though peptides and proteins have great therapeutic potential; however, poor bioavailability and proteolytic degradation by endogenous enzymes limit their in vivo application (Diao and Meibohm 2013). These shortcomings have been overcome by the incorporation of non-natural amino acids (Werner et al. 2016; Cheloha et al. 2016) or the introduction of other functionalities such as glycosylation, and PEGylation (Harris and Chess 2003), lipidation, or *N*-methylation (Chatterjee et al. 2013). Polypeptides and proteins are synthesized either chemically or biologically. The chemical synthesis of peptides can be accomplished by either solution phase or solid-phase synthesis and each methodology has its own set of benefits and drawbacks. While short peptides ($\leq$10–15 amino acids) can be chemically synthesized using the solution-phase fragmentation condensation strategy, the larger or longer peptides have been synthesized employing solid-phase methods. The foundation work by Bruce Merrifield, the development of solid-phase synthesis revolutionized peptide synthesis by simplifying the purification step involved with solution-phase peptide synthesis (Merrifield 1963). SPPS is now a popular choice for the production of longer and difficult peptide sequences; however, solution-phase synthesis can still be effective for large-scale production of a given peptide. The constant high demand for peptides in various disciplines stimulated the advancement of peptide synthesis to achieve high yield, high purity, and a shorter synthesis time. Over the last 20 years, several approaches have emerged for the development of peptide synthesis both in solid and solution phase methods. In this chapter, we'll cover all of the new advanced approaches.

8.2 Classical Approaches of Chemical Peptide Synthesis

Prior to Merrifield's discovery of solid-phase peptide synthesis, solution-phase synthesis was the only way to make the peptides. In this traditional approach, peptide synthesis is manifested using the fragment condensation method. Suitably protected amino acids are synthesized or commercially supplied and coupled together using coupling reagents (Tsuda and Okada 2010; Anderson 1960). Typically, the N-terminal group is protected by Boc, and the C-terminal group is converted to ester. The amide bond formation is accomplished with carbodiimide-based coupling

reagents such as DCC or EDC in DMF and HOBt used as the racemization suppressor. Finally, the N-terminal Boc and C-terminal ester are de-protected by strong acids like TFA and strong bases such as NaOH. The methodology can be very helpful for synthesizing short and ultra-short peptides but is not applicable for longer and complex peptide sequences (Verlander 2007).

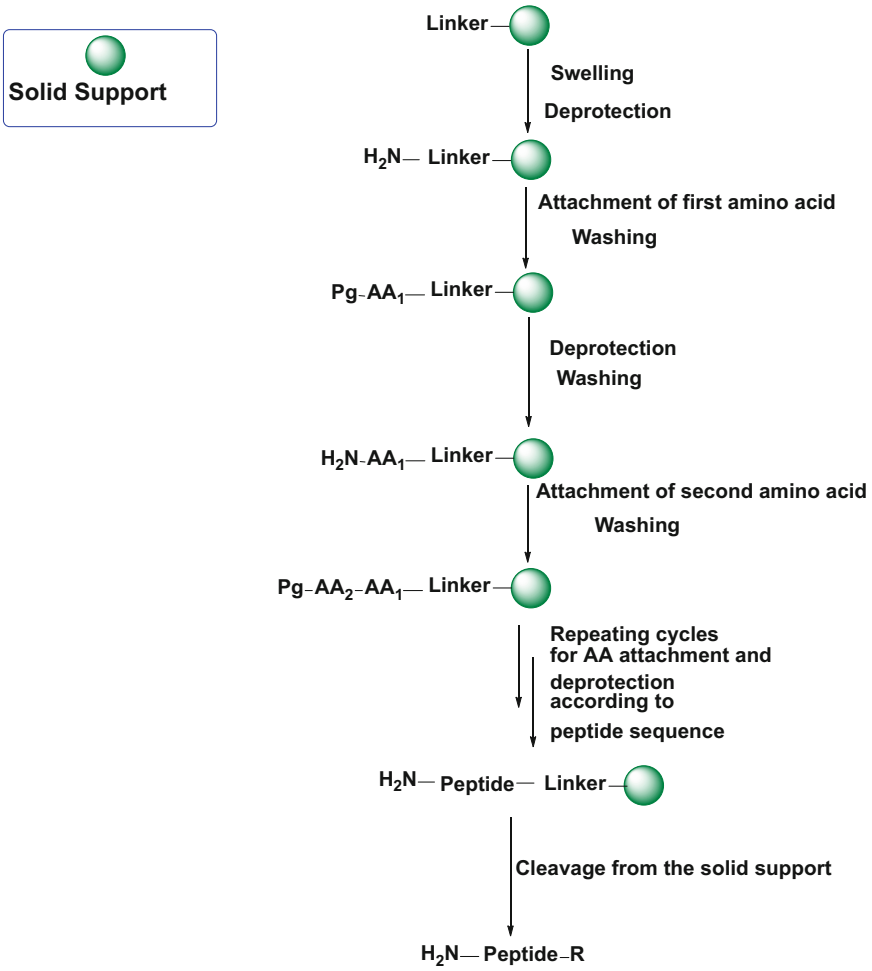
8.3 Solid-Phase Peptide Synthesis

Solid-phase synthesis is one of the most successful methods for synthesizing a wide range of peptides, from short to long sequences, and has gained widespread acceptance in the peptide industry. The method was first introduced by R. Bruce Merrifield in 1963 and 20 years later he got a Nobel Prize (Merrifield 1985).

The SPPS method revolutionizes peptide synthesis by simplifying the extensive and complicated purification steps involved with the solution phase synthesis. The use of reactive solid support allows for the sequential coupling of individual amino acids. After the amino acids have been attached, the unreacted amino acids and coupling reagents are removed by filtration and washing. Once the anchoring of individual amino acids of the desired peptide sequence is completed, the peptide is released from the solid support, and simultaneously the temporary side chain protective groups of amino acids are also removed under the same conditions. The principle of SPPS is shown in Scheme 8.1. Typically, the two most common N-terminal protected amino acids are used one is acid-labile Boc (Merrifield 1986) and the other is base-labile Fmoc (Fig. 8.1) (Carpino and Han 1970). The peptide synthesized by Boc-protected amino acid requires the use of extremely hazardous and toxic HF to cleave the peptide from the solid support (Muttenthaler et al. 2015) and thus is less often used than the Fmoc-strategy.

The Fmoc- strategy has been extensively applied to the synthesis of diverse peptide sequences since this protective group can be easily removed under mild conditions with secondary amines, usually 20% piperidine–DMF. Crosslinked polystyrene (PS)-based resins (Merrifield 1985) or copolymer of polyethylene glycol and polystyrene (PEG-PS)-based resins are routinely used in Fmoc SPPS (Barany et al. 1992; Rapp et al. 1988; Carpino et al. 1994). The swelling of resin is carried out in presence of solvents such as DMF and DCM which expose the reactive functional groups and make them available for amino acid coupling.

The side chains of the amino acids like Cys, Asn, Gln, His Asp, Glu, Lys, Ser, Tyr, and Arg need to be protected during the FMOC SPPS as they can react during the peptide synthesis. The commonly used protecting group for Glu, Asp, Ser, Thr, and Tyr is *tert*-butyl (*t*-Bu); 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg; and trityl (Trt) for Cys, Asn, Gln, and His. The coupling reaction between the free α-amino group and the Fmoc-protected α-carboxylic acid of the next amino acid is mediated by various coupling reagents. Carbodiimide-based coupling reagents such as DCC were used earlier in SPPS and later it is replaced by DIC as their urea is more soluble in organic solvent and can be removed simply by washing the resin (Fig. 8.2) (Carpino 1993).



Scheme 8.1 Principle of Solid-Phase Peptide Synthesis

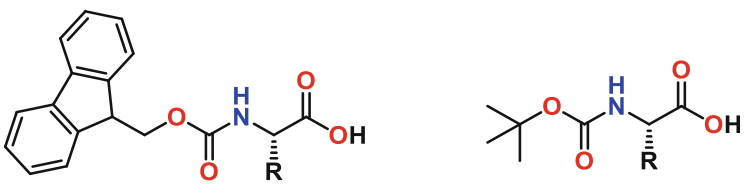


Fig. 8.1 Commonly used protected amino acid in solid-phase peptide synthesis

Benzotriazole derivatives such as HOBt and HOAt are generally utilized as additives to activate the C-terminal amino acids and as racemization suppressors during the coupling reaction (Fig. 8.3) (Carpino 1993).

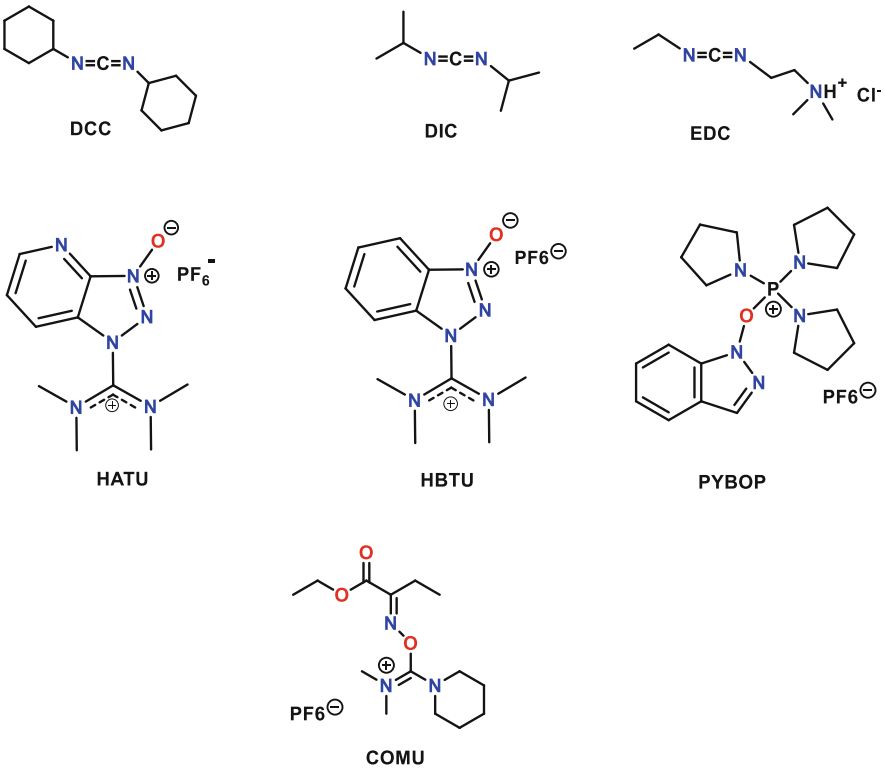


Fig. 8.2 Commonly used coupling reagents in solid and solution-phase peptide synthesis

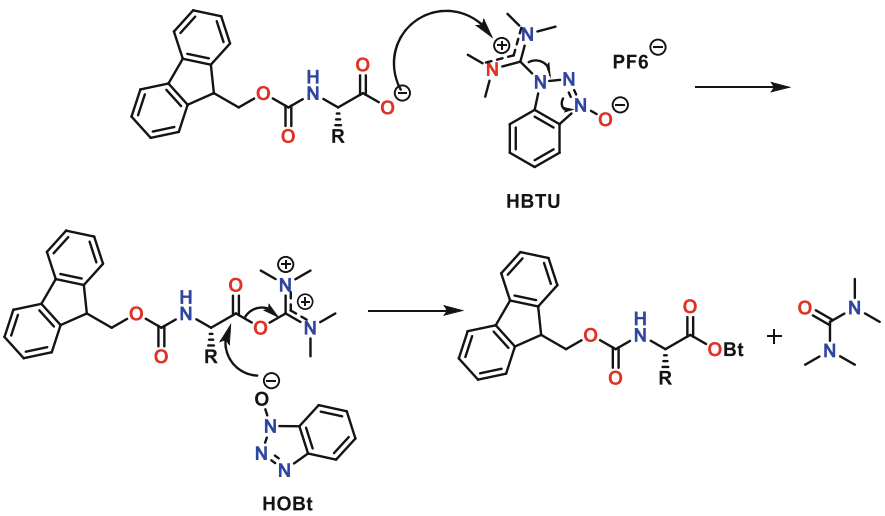


Fig. 8.3 Coupling reaction mediated by HBTU and HOBt

However, because of thermal instability and considering class 1 explosive materials limited their use. Recently Ethyl 2-cyano-2-(hydroxyimino) acetate (OxymaPure) appeared to be a safer and more efficient carbodiimide additive than HOBt and HOAt and demonstrated exceptional racemization suppressor and activating abilities in both manual and automated synthesis (Subirós-Funosas et al. 2009). In addition to additives, significant advancements in the coupling reagent have been achieved to decrease coupling time and minimize epimerization. The most significant breakthrough since the discovery of carbodiimide-based coupling reagents includes HBTU, HATU (Carpino et al. 2002), PyBOP (Coste et al. 1990), and the new COMU (Fig. 8.2) (El-Faham et al. 2009).

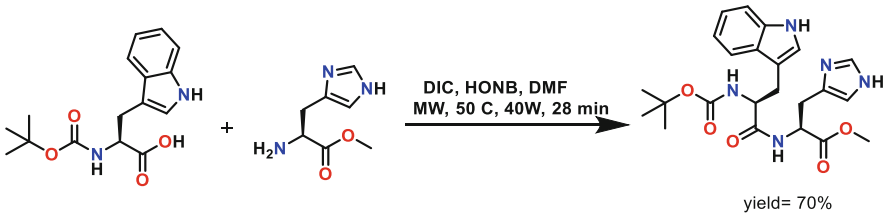
The evolution of solid-phase synthesis into large-scale peptide synthesis has permitted the fabrication of peptide-based "active medicinal ingredients." Thus, it is now possible to synthesize peptides of 30–50 amino acids on a kilogram scale within a very short period. This synthetic availability along with the strategies to increase the stability of peptides has led to the commercialization of a wide range of short to lengthy peptides, with more on the horizon.

Over the years solid-phase synthesis has been extensively refined to achieve superior outcomes, such as faster synthesis, higher yields, and greater purity. The developments of microwave-assisted automated peptides synthesizer, implementation of native chemical ligation (NCL), and flow-based approach revolutionize the SPPS. In addition, efforts have been made to make peptide synthesis more environmentally friendly. We will discuss all of them in the coming sections.

8.4 Microwave-Assisted Peptide Synthesis

8.4.1 Microwave-Assisted Solution-Phase Peptide Synthesis

Conventional solution-phase peptide synthesis requires a long reaction time for coupling, along with low yield, and purity of products, and often leads to enantiomerization of the final product. To overcome these problems earlier Babu and co-workers (Babu and Rao 2005) reported microwave-assisted solution-phase peptide synthesis; however, it has not been explored very well. This prompted many researchers to explore robust and efficient protocols with wide scope for peptide synthesis. Jain and co-workers reported the racemization-free protocol for the synthesis of peptides under microwave irradiation (Mahindra et al. 2012). They have demonstrated the synthesis of di- and tripeptide under microwave irradiation and after the screening of various coupling reagents, it was found that DIC/HONB-mediated coupling was best suited for the synthesis of dipeptide, whereas HATU/HOAt/DIEA was the best coupling combination for the synthesis of the tripeptide. The conventional synthesis of peptides was also carried out and as expected it produced a lower yield comparatively. The representative dipeptide Boc-Trp-His-OMe as shown in Scheme 8.2 was synthesized using the developed protocol with a 70% yield.

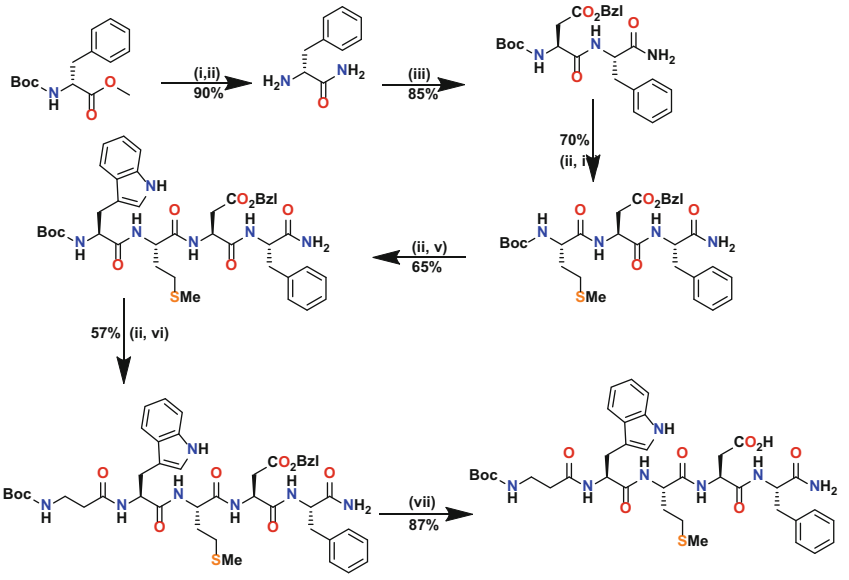


Scheme 8.2 Synthesis of Boc-Trp-His-OMe

In the quest to find an environmentally benign method for peptide synthesis, Jain and co-workers next reported microwave-assisted solution-phase peptide synthesis in neat water as a reaction medium (Mahindra et al. 2013). This was the first report of solution-phase peptide synthesis in water as a medium. The use of water as a reaction medium offers a great advantage as it is considered a greener solvent that is non-toxic, non-flammable, and reduces the generation of waste. Along with the use of a greener solvent, the use of this protocol produces the racemization-free peptide in a short reaction time and both protecting group BOC and Fmoc can be used. While optimizing reaction protocol various parameters were screened such as reaction time and temperature, solvent, and coupling reagents. Optimization studies lead to the use of a coupling combination of TBTU/HOBt in water and DIC/HONB in *tert*-Butyl methyl ether (TBME), DIEA as base produced the highest yield up to 90% at 60 °C in 30 min of microwave irradiation. Later the developed protocol was applied for the synthesis of pentapeptide pentagastrin in water as shown in Scheme 8.3. It is a synthetic analogue of endogenous gastrin and is used in diagnostic aid. The pentagastrin peptide was efficiently synthesized with high yield and purity.

8.4.2 Microwave-Assisted Solid-Phase Peptide Synthesis

In the conventional SPPS method, it is believed that the elongation of the peptide chain leads to an increase in steric hindrance and aggregation. Moreover, coupling of secondary amino acids such as proline or other sterically hindered amino acids decreases the reaction rates and leads to incomplete reaction. As a result, such synthesis required excess reagents and solvents *N, N*-dimethylformamide (DMF), and *N*-methyl-2-pyrrolidinone (NMP) for coupling and washing to remove impurities in addition to long reaction times that reduce the efficiency of SPPS. Moreover, an incomplete reaction could result in the formation of deleted sequence, which can be difficult to remove from the desired product (Jad et al. 2019; Collins et al. 2014). So, to improve the efficiency of the SPPS protocol researchers started using different energy sources one of them being microwave irradiation or employing automated peptide synthesizers or heating. In the microwave, heating energy is directly transferred to a solvent via the interaction of molecules in a solvent with an electromagnetic field. Based on data it is suggested that SPPS microwave



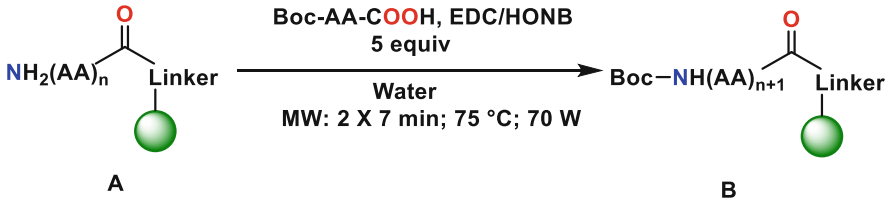
Reagents and conditions: (i) $HCONH_2$, NaOMe, 100 °C; (ii) 6N HCl, 15 min, 25 °C; (iii) Boc-Asp(Bzl)-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (iv) Boc-Met-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (v) Boc-Trp-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (vi) Boc-β-Ala-OH, TBTU, HOBt, DIEA, H_2O, MW, 60 °C; (vii) 10% Pd/C, $HCOONH_4$, CH_3OH, reflux

Scheme 8.3 Synthesis of Pentagastrin

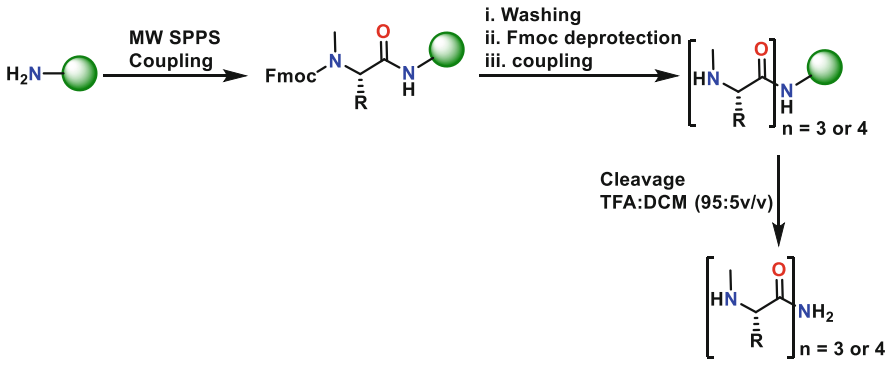
heating can improve the reaction time and yield of the synthesized products (Pedersen et al. 2012).

Gogoll and co-workers (Erdelyi and Gogoll 2002) reported the first-time use of microwave irradiation in SPPS. It has been known that the use of microwaves in organic synthesis improves the reaction yield and time. Gogoll et al. demonstrated the synthesis of sterically hindered peptides which have been difficult to access via SPPS at room temperature. The desired peptides can be obtained in a short reaction time of 1.5–20 min without racemization.

As we know SPPS involves excess use of highly toxic organic solvents for coupling, washing, and deprotection therefore to further improve the SPPS technique by using an environmentally benign solvent such as water is of high interest. Grøtli and co-workers (Galanis et al. 2009) reported the solid phase peptide synthesis using water as a solvent for coupling and deprotection. It involves the use of Boc-protected amino acid along with the combination of EDC/HONB as coupling reagents under microwave irradiation. This is the first report in which whole peptide synthesis has been carried out in the water. Moreover, peptides are obtained in high yield and purity without racemization (Scheme 8.4).



Scheme 8.4 Microwave-Assisted Solid-Phase Synthesis of Peptide in Water



Scheme 8.5 Microwave-Assisted Synthesis of *N*-Methylated Peptides

8.4.3 Synthesis of Difficult Peptides Using Microwave-Assisted SPPS

8.4.3.1 N^{α}-Methylated Peptides

Peptides are highly susceptible to proteolytic degradation; however, several approaches have been developed to improve their proteolytic stability. Among recent advances, the synthesis of N^{α}-methylated peptides has emerged as an effective strategy for improving their short half-life (Chatterjee et al. 2008). Angell and co-workers (Angell et al. 1994) reported the coupling of sterically hindered *N*-methylated amino acids under SPPS protocol using HOAt as coupling reagents. However, the coupling of *N*-methyl amino acids often results in low yields and requires excess coupling reagents along with a long reaction time. Microwave heating can be used to overcome these problems therefore Albericio and co-workers (Rodríguez et al. 2010) reported the efficient coupling of Fmoc-*N*-methyl amino acid under microwave irradiation in SPPS conditions using rink amide MBHA resin. The *N*-methyl-rich peptides were obtained in high yield and purity using a developed protocol. The short *N*-methylated peptides were synthesized using microwave irradiation at 35 °C for 20 min using DIC/HOAt as a coupling combination in DCM as solvent (Scheme 8.5).

8.4.3.2 β-Peptides

β-Amino acids oligomers (β-peptides) are an important class of bioactive peptides that possess high interest because of their demanding application in medicinal chemistry. The major drawbacks of α-peptides are the low proteolytic stability and possess high conformational freedom. These problems can be overcome by incorporating β-amino acids into bioactive peptides as they are proven to be effective in modulating structures and physicochemical properties (Cabrele et al. 2014). There are two types of β-amino acids often used as building blocks for bioactive peptides. Homologues of α-amino acids consist of an extra methylene group and the second one is β-amino acids based on a cycloalkane ring. The synthesis of β-peptides via conventional SPPS protocol often leads to low yield and difficult *N*-deprotection.

Gellman and co-workers reported (Murray and Gellman 2005) the microwave-assisted synthesis of β-peptides. The 14-helical hexa and deca-β-peptides were synthesized using *trans*-2-aminocyclohexane carboxylic acid (ACHC) via both conventional and microwave SPPS. It was identified that for the synthesis of this β-peptide under microwave irradiation, coupling at 60 °C for 2 min and *N*-deprotection at 50 °C, 4 min results in higher purity of 80% and 57% of hexa and deca-β-peptide respectively, which is much superior relative to conventional SPPS (Fig. 8.4).

In 2003 the completely automated microwave-assisted peptide synthesizer was introduced by CEM Corporation. Ultrafast automation of microwave-based Liberty Blue synthesizer can be used in the synthesis of longer peptides within a short reaction time with more than 90% reduction in solvent wastage. The single-mode microwave reactor Discover has been employed widely in the Liberty system. The system uses the amino acid stock solution and consists of reagent ports for washing, deprotection, activation, and cleavage (Collins and Collins 2003). It is also connected with a nitrogen cylinder, which maintains the inert atmosphere while performing coupling, deprotection, and washing steps. All procedures in liberty are

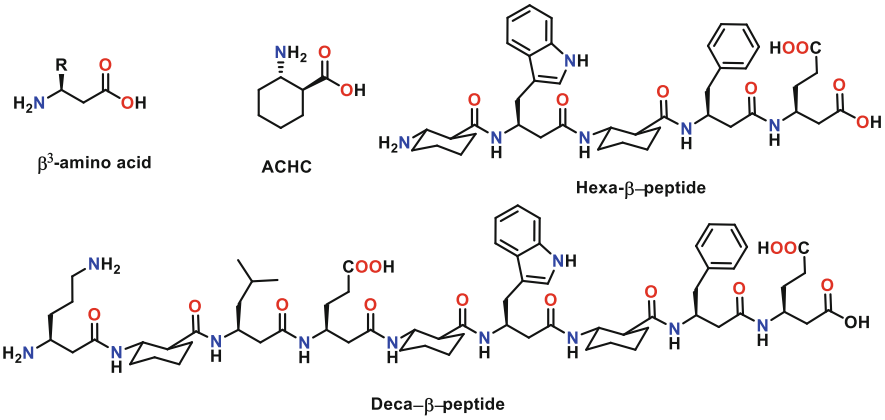


Fig. 8.4 β-Amino acids along with hexa and deca-beta-peptide

controlled by an external computer, the complete manual is available by Discover SPS.

8.5 Peptide Synthesis Using Ball Mill

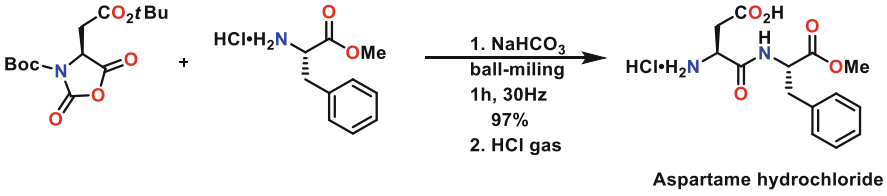
Despite well-established protocols for the production of peptides, they are associated with major problems that involve the use of a huge amount of highly volatile and toxic solvents mainly in SPPS. Therefore, exploration of a convenient and environmentally benign protocol is highly desirable. One of the attempts to avoid the use of toxic solvents is the use of a ball mill for the synthesis of peptides (Sato et al. 2006).

Lamaty and co-workers (Declerck et al. 2009) reported the solvent-free synthesis of peptides without epimerization using ball milling. The developed protocol involves the coupling of urethane-protected α-amino acid *N*-carboxyanhydride (UNCA) with amino acid ester in presence of base $NaHCO_3$. It was confirmed that for coupling reaction all the solids are necessary and the absence of one of the reagents results in failure of reaction. They have also demonstrated for the first-time synthesis of artificial sweetener aspartame in the absence of solvent using ball milling technology. Aspartame is a dipeptide composed of α-L-aspartyl-L-phenylalanine and its sweetening ability is 150 times greater than sucrose (Scheme 8.6).

Lamaty and co-workers (Bonnamour et al. 2013) in their further study have demonstrated the environmentally benign protocol liquid-assisted ball milling for the synthesis of short and long peptides such as tetra and pentapeptide on a gram scale with high yield. It involves the coupling of Boc-protected α-amino acid *N*-carboxyanhydrides or *N*-hydroxysuccinimide esters (Boc-AA-OSu) (Boc-AA-NCA or OSu) with amino acid ester in presence of $NaHCO_3$ and a small amount of ethyl acetate under ball milling. The developed protocol was used for the synthesis of pentapeptide leu-enkephalin in nine steps with a 46% of yield (Fig. 8.5).

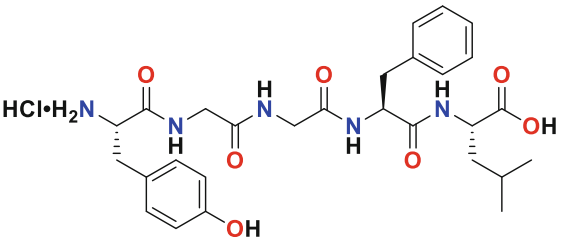
8.6 Native Chemical Ligation Strategy for Peptide Synthesis

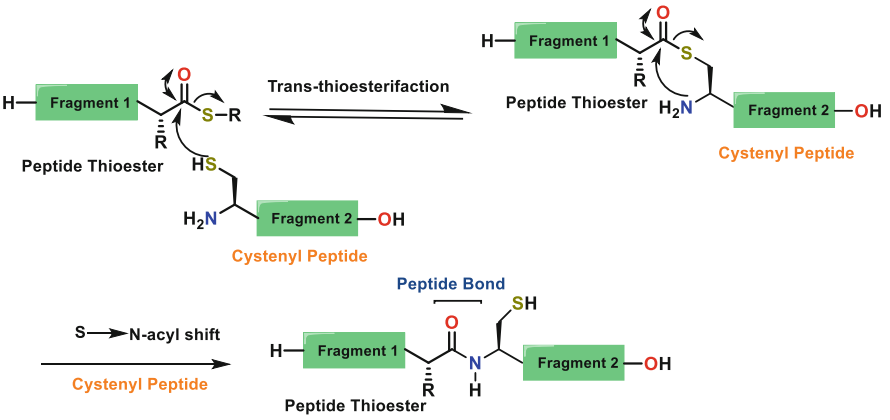
The native chemical ligation method is one of the most widely employed for the synthesis of larger polypeptides and proteins. Conventional SPPS is ineffective for the synthesis of larger polypeptides because of the increased aggregation and steric crowding caused by elongation of the peptide chain, which resulted in undesired side



Scheme 8.6 Synthesis of Aspartame Using Ball Milling Technology

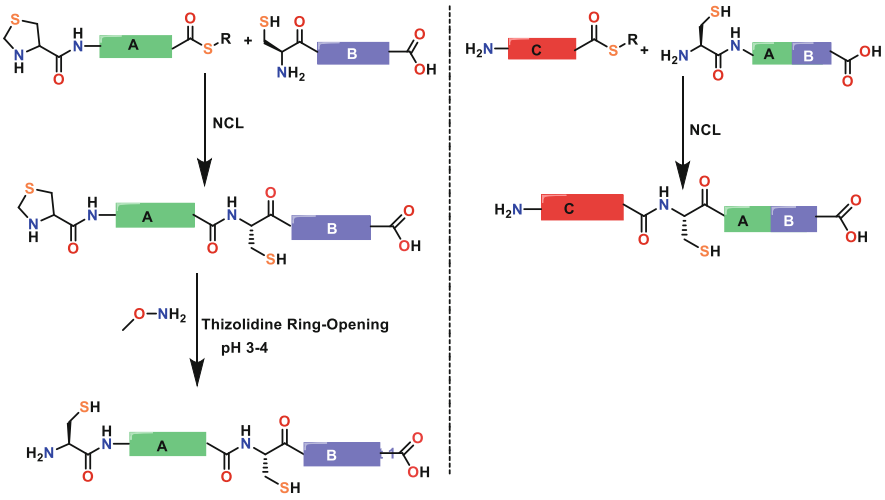
Fig. 8.5 Synthesis of leu-enkephalin using ball mill





Scheme 8.7 Mechanism of Native Chemical Ligation (NCL). Initially peptide thioester reacts with cysteinyl peptide fragments via an initial trans-thioesterification followed by an S-N acyl shift to produce a native peptide (amide) bond

products and epimerization. The invention of the native chemical ligation (NCL) technique has overcome these flaws, and using this strategy hundreds of protein targets have been synthesized to date. The principle of the strategy was first introduced by Kent and co-workers in the early 1990s (Dawson et al. 1994) which is further extrapolated and modified by Dawson (Dirksen and Dawson 2008) and others (Haase and Seitz 2008; Macmillan 2006; Bondalapati et al. 2016). The reaction involves the condensation of two unprotected peptide segments in aqueous conditions at neutral pH to form a native amide bond in a chemoselective and high-yielding manner. In general, C-terminal peptide thioesters react with another peptide containing an N-terminal Cys residue, and following the reversible transthioesterification reaction, an irreversible S-to-N acyl shift occurs through the five-membered ring intermediate that joins the two peptides via an amide bond (Scheme 8.7). The important key feature of the method is that the condensation of the two peptide fragments is carried out in aqueous conditions and physiological pH which helps the solubility of unprotected peptide and protein fragments. The success of the strategy is highlighted by the synthesis of HIV1 protease (Torbeev and Kent



Scheme 8.8 Coupling of more than two peptide fragments through ligation chemistry: The N-terminal Cys of one of the peptide fragments is protected as a thiazolidine (Thz) moiety which after the ligation is removed and coupled with another peptide thioester through native chemical ligation (NCL)

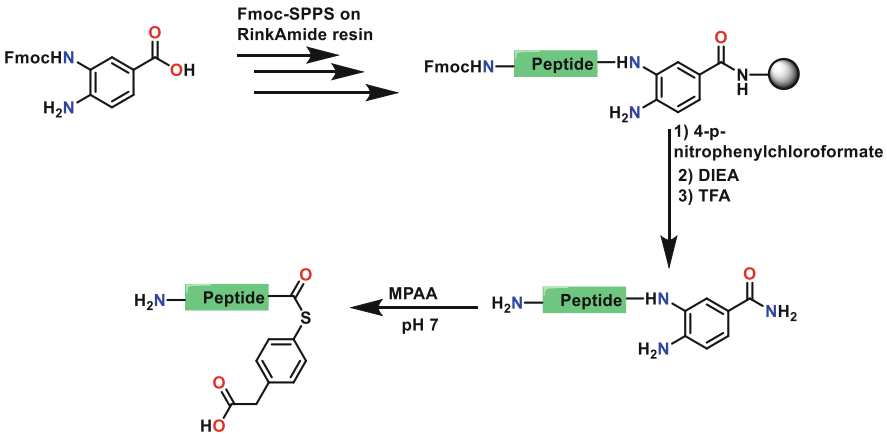
2007), D-Dpo4 enzyme (Jiang et al. 2017), and different head-to-tail cyclic peptides (Clark and Craik 2010).

The major drawback of the methodology is the requirement of a Cys residue which is not frequently found in protein. To address the issue, Dawson and co-workers (Yan and Dawson 2001) introduced β- or γ-thiol-substituted amino acids in the N-terminal position.

Similar to Cys, these amino acids have thiol functionality at the β- or γ-position, which is involved in the ligation akin to the original strategy and provides the peptide with an additional thiol group that is subsequently desulfurized to get the target protein. However, desulfurization is not chemoselective in the presence of other unprotected cysteines in the sequences. This limitation can be handled by the introduction of selenocysteine as well as selenoamino acids (Hondal et al. 2001; Quaderer et al. 2001; Gieselman et al. 2001). The main advantage of using selenoamino acids instead of thio amino acids for ligation chemistry is that chemoselective deselenization can be done under mild conditions without affecting exposed Cys residues.

Further, to couple more than two peptide segments, the N-terminal Cys residue of one peptide segment is protected by thiazolidine (Thz) (Bang and Kent 2004). Following successful ligation of the two peptide segments, the N-terminal Thz moiety was removed to generate a native Cys residue peptide, which was then used in another NCL to obtain the desired protein (Scheme 8.8).

The peptide thioester is an important reactive intermediate in native chemical ligation, and a substantial amount of work has been done on the development of an efficient synthetic pathway for peptide thioesters. Although (Boc)-SPPS has been



Scheme 8.9 Synthesis of Peptide Thioester through Fmoc-SPPS

used in the past (Schnölzer et al. 1992), however, in order to make it widely employed for protein synthesis, it must also be possible to prepare peptides using fluronylmethoxy carbonyl (Fmoc)-SPPS. This unmet requirement prompted the development of new synthetic ways for preparing the crucial intermediate using Fmoc strategies, which have shown to be extremely useful in protein synthesis (Zheng et al. 2013). Dawson and co-workers disclosed a strategy for preparing the peptide thioester in Fmoc SPPS. In this approach, 3,4-diaminobenzoic acid is initially attached to Rink amide resin (Blanco-Canosa and Dawson 2008). The first amino acid is then coupled into one of the amino groups of 3,4-diaminobenzoic acid, and the peptide chain is elongated. After completion of the whole sequence, the resin-bound peptide is treated with 4-nitro chloroformate under basic conditions to form *N*-acyl-benzimidazolinone (Nbz). Finally, the peptide is deprotected from the resin and reacted with aryl thiol to afford the corresponding thioester (Scheme 8.9). Interestingly, this method is applicable to all essential amino acids; however, the branching of Dbz functionality has been observed when a Gly residue is present at the C-terminus, which can be prevented by allyloxycarbonyl (alloc) protection of the linker's free amine (Mahto et al. 2011).

The native chemical ligation (NCL) has proven to be a highly successful method for the ligation of peptide segments. In combination with SPPS and NCL, it is now possible to synthesize larger proteins, which was very difficult in earlier days. In the future, it is expected that the native chemical ligation method will be intended to enable the synthesis of diverse complex protein targets for a variety of biological applications.

8.7 Automated Flow-Based Approach for Peptide Synthesis

The continuous shifting of the drug development from small molecules to peptides fuelled the demand for efficient automated peptide synthesizers which can synthesize peptides with high yield and purity within a very limited amount of time. Although numerous automated systems are available for Fmoc/Boc solid-phase peptide synthesis (SPPS), the complex setup, microwave heating and the requirement of a significant amount of excess reagent, and optimization of microwave heating limited their frequent use. In this context, the flow-based approach to peptide synthesis gains much attention in recent years (Simon et al. 2014; Mong et al. 2014). In the flow-based approach, the pre-heated reagents and solvents deliver to the solid support with a very high flow rate which increases the efficiency and speed of coupling cycles thereby reducing the time required for peptide synthesis and minimizing the requirement for excess reagents (Fig. 8.6). In addition, due to the very high coupling efficiency, it reduces the amount of waste generated during the synthesis.

Pentelute and co-workers made a significant breakthrough in the flow-based approach to peptide synthesis (Mijalis et al. 2017). They developed a fully automated, flow-based solid-phase polypeptide synthesis technology that allows for amide bond formation in 7 s, and the complete cycle for each amino acid is finished in 40 s. In addition, the presence of a UV–visible spectrometer enables the monitoring of Fmoc deprotection and gives information about the synthetic yields of each step. Further, the automated replacement of disposable reactors facilitates the quick shift from one peptide synthesis to the next. The efficiency of the technology is validated by synthesizing difficult sequences and longer peptides. For instance, the insulin B chain is synthesized in high purity and with greater yield compared to the synthesized by other existing methods. In traditional microwave-assisted peptide synthesis, epimerization of Cys and His residues is always a possibility; however, AFPS minimizes the chances of epimerization by increasing flow rate and thus shortening the residence time at high temperature for activated His and Cys monomers.

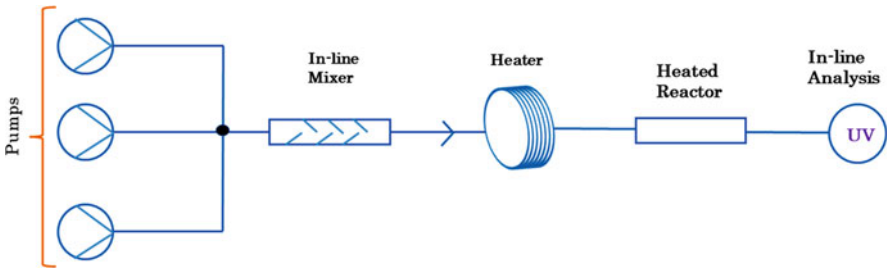


Fig. 8.6 Flow diagram of automated fast-flow peptide synthesizer described by Pentelute and Co-workers

In another interesting study (Hartrampf et al. 2020), Pentelute and co-workers described the proficiency of an automated fast-flow peptide synthesizer by synthesizing various protein chains that represent enzymes within hours. More intriguingly, compared to other conventional methods, AFPS produces better outcomes in terms of synthesis time, purity, and yields of the crude protein. Surprisingly, the physicochemical and enzymatic properties of the synthesized proteins are similar to those of the biologically expressed proteins. It is believed that shortly AFPS will show great potential for large-scale synthesis of peptides and proteins for therapeutic applications.

8.8 Enzymatic Approach for Peptide Synthesis

The enzymatic approach to peptide synthesis is one of the greener approaches for peptide synthesis. The chemical synthesis of peptides involves the use of vast amounts of toxic organic solvents which can be avoided using the chemoenzymatic method (Guzmán et al. 2007). In addition, there are no requirements for protected monomers and peptide synthesis can be carried out in a very selective and stereo-specific manner with high yield (Kumar and Bhalla 2005).

Proteolytic enzymes play a very important role in various biological systems and are ubiquitous in living organisms. It has a significant contribution to cell division, tissue dissociation, immune function, and protein recycling, among other important processes (Mótyán et al. 2013). Although the primary role of the proteolytic enzymes is the hydrolyzing proteins; however, they can also function in the reverse direction, thereby facilitating peptide bond formation.

Halling and co-workers reported protease catalyzed amide bond formation on the solid support (Ulijn et al. 2002). They explored PEGA1900 as a solid substrate for the attachment of amino acids. After the coupling of the first amino acid via Wang linker and followed by deprotection, the coupling next amino acid was performed in the presence of thermolysin. Finally, the peptide was cleaved from the resin and purified through HPLC (Fig. 8.7). The efficiency of the reaction was demonstrated by the formation of high yield and purity of the various dipeptides. Further, this methodology was explored for the formation of longer peptides where the yield varied from 3–55% for sequences containing up to six amino acids (Ulijn et al. 2003).

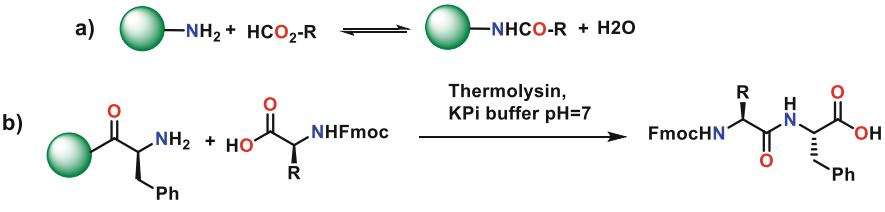


Fig. 8.7 (**a**) Amide bond formation/hydrolysis on solid substrate, (**b**) thermolysin catalyzed synthesis of dipeptides on PEGA190

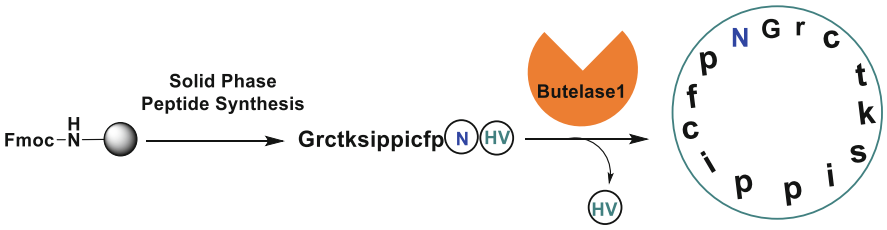


Fig. 8.8 Butelase catalyzed synthesis of cyclic dipeptide comprised of D-amino-acid

The cyclic peptide has great potential in peptide therapeutics due to its high biostability and bioavailability (Gibson and Lecci 2006). Inspired by the therapeutic import of the cyclic peptide, Guo and co-workers explored SrtA a class of transpeptidases found in gram-positive bacteria (Mazmanian et al. 1999) to form head-to-tail cyclization of a 19 residues peptide having a sorting signal of SrtA and a diglycine motif at its C- and N-termini, respectively (Wu et al. 2011). It is found that at 0.5 mM peptide and 40 mM SrtA concentration direct head-to-tail cyclization product is observed with an overall yield of 79%.

Tam and co-workers explored for the first time the Butelase-1 enzyme to catalyze cyclization of linear peptides composed of D-amino acid and Asn as well as Histidine and Valine (Fig. 8.8). The C-terminal Asn was used for the ligating side for butelase 1 and the dipeptide segment His-Val was used for substrate recognition. It has been found that butelase 1 efficiently catalyzes the cyclization of a variety of D-amino acid-containing linear peptide sequences (Nguyen et al. 2016).

Chemoenzymatic synthesis of peptides can be exploited as a surrogate for the traditional synthesis of the peptides due to their high yield and lesser use of hazardous chemicals. However, there are some limitations such as the difficulty in controlling the sequences and difficulty in synthesizing longer peptides which must be circumvented by engineering the protease and optimizing the reaction conditions.

8.9 Conclusion

As demonstrated in the chapter, recent advances in peptide synthesis enabled the synthesis of long and complex peptide sequences. In light of increasing interest in peptide therapeutics, the above-mentioned approaches and technologies will be of great assistance in the quick access to complex bioactive peptides with high yield and purity. The emergence of automated microwave peptide synthesizers and automated flow-based technology will play a central role in the future of synthesizing longer and more complex peptides. Peptide chemists in the twenty-first century are emphasizing the use of green, non-hazardous solvents and the improvement of atom economy in the protection/deprotection methods to make peptide synthesis more environmentally friendly. Moreover, the greener approaches must be widely used and applied to the various chemical alterations of peptides, such

as cyclization and *N*-methylation, before they can be of real worth for commercial uses.

Acknowledgment G.K.R. is thankful to the National Institute of Pharmaceutical Education and Research, S. A. S. Nagar for providing a Senior Research Fellowship. R.M. thanks the Department of Science and Technology, New Delhi for DST Faculty Inspire Fellowship (No. DST/INSPIRE/04/2020/002499).

References

Anderson GW (1960) New approaches to peptide synthesis. Ann N Y Acad Sci 88:676–688

Angell YM, García-Echeverría C, Rich DH (1994) Comparative studies of the coupling of N-methylated, sterically hindered amino acids during solid-phase peptide synthesis. Tetrahedron Lett 35:5981–5984

Babu VVS, Rao RVR (2005) Microwave irradiated high-speed solution synthesis of peptide acids employing Fmoc-amino acid pentafluorophenyl esters as coupling agents. Indian J Chem 44B:2328–2332

Bang D, Kent SB (2004) A one-pot total synthesis of crambin. Angew Chem Int Ed 43:2534–2538

Barany G, Albricio F, Biancalana S et al (1992) Biopolymer syntheses on novel polyethylene glycol-polystyrene (PEG-PS) graft supports. Minnesota University Minneapolis Department of Chemistry, Minneapolis, MN

Blanco-Canosa JB, Dawson PE (2008) An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. Angew Chem Int Ed 120:6957–6961

Bondalapati S, Jbara M, Brik A (2016) Expanding the chemical toolbox for the synthesis of large and uniquely modified proteins. Nat Chem 8:407–418

Bonnamour J, Métro TX, Martinez J et al (2013) Environmentally benign peptide synthesis using liquid-assisted ball-milling: application to the synthesis of Leu-enkephalin. Green Chem 15:1116–1120

Cabrele C, Martinek TA, Reiser O et al (2014) Peptides containing β-amino acid patterns: challenges and successes in medicinal chemistry. J Med Chem 57:9718–9739

Carpino LA (1993) 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. J Am Chem Soc 115:4397–4398

Carpino LA, Han GY (1970) 9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. J Am Chem Soc 92:5748–5749

Carpino LA, El-Faham A, Minor CA (1994) Advantageous applications of azabenzotriazole (triazolopyridine)-based coupling reagents to solid-phase peptide synthesis. J Chem Soc Chem Comm 2:201–203

Carpino LA, Imazumi H, El-Faham A et al (2002) The uronium/guanidinium peptide coupling reagents: finally, the true uronium salts. Angew Chem Int Ed 41:441–445

Chatterjee J, Gilon C, Hoffman A et al (2008) N-methylation of peptides: a new perspective in medicinal chemistry. Acc Chem Res 41:1331–1342

Chatterjee J, Rechenmacher F, Kessler H (2013) N-methylation of peptides and proteins: an important element for modulating biological functions. Angew Chem Int Ed 52:254–269

Cheloha RW, Watanabe T, Dean T (2016) Backbone modification of a parathyroid hormone receptor-1 antagonist/inverse agonist. ACS Chem Biol 1:2752–2762

Clark RJ, Craik DJ (2010) Invited review native chemical ligation applied to the synthesis and bioengineering of circular peptides and proteins. Peptide Sci 94:414–422

Collins JM, Collins MJ (2003) Biopolymers 71:267

Collins JM, Porter KA, Singh SK et al (2014) High-efficiency solid phase peptide synthesis (HE-SPPS). Org Lett 16:940–943

Coste J, Le-Nguyen D, Castro B (1990) PyBOP®: a new peptide coupling reagent devoid of toxic by-product. Tetrahedron Lett 31:205–208
Dawson PE, Muir TW, Clark-Lewis I et al (1994) Synthesis of proteins by native chemical ligation. Science 266:776–779
Declerck V, Nun P, Martinez J et al (2009) Solvent-free synthesis of peptides. Angew Chem Int Ed 48:9318–9321
Diao L, Meibohm B (2013) Pharmacokinetics and pharmacokinetic–pharmacodynamic correlations of therapeutic peptides. Clin Pharmacokinet 52:855–868
Dirksen A, Dawson PE (2008) Expanding the scope of chemoselective peptide ligations in chemical biology. Curr Opin Chem Biol 12:760–766
Domalaon R, Zhanel GG, Schweizer F (2016) Curr Top Med Chem 16:141–155
El-Faham A, Funosas RS, Prohens R et al (2009) COMU: a safer and more effective replacement for benzotriazole-based uronium coupling reagents. Chem Eur J 15:9404–9416
Erdelyi M, Gogoll A (2002) Rapid microwave-assisted solid phase peptide synthesis. Synthesis 11: 1592–1596
Fosgerau K, Hoffmann T (2015) Peptide therapeutics: current status and future directions. Drug Discov Today 20:122–128
Galanis AS, Albericio F, Grøtli M (2009) Solid-phase peptide synthesis in water using microwave-assisted heating. Org Lett 11:4488–4491
Gibson SE, Lecci C (2006) Amino acid derived macrocycles-an area driven by synthesis or application? Angew Chem Int Ed 45:1364–1377
Gieselman MD, Xie L, Van Der Donk WA (2001) Synthesis of a selenocysteine-containing peptide by native chemical ligation. Org Lett 3:1331–1334
Guzmán F, Barberis S, Illanes A (2007) Peptide synthesis: chemical or enzymatic. Electron J Biotechnol 10:279–314
Haase C, Seitz O (2008) Extending the scope of native chemical peptide coupling. Angew Chem Int Ed 47:1553–1556
Harris JM, Chess RB (2003) Effect of pegylation on pharmaceuticals. Nature Rev Drug Disc 2:214–221
Hartrampf N, Saebi A, Poskus M et al (2020) Synthesis of proteins by automated flow chemistry. Science 368:980–987
Henninot A, Collins JC, Nuss JM (2018) The current state of peptide drug discovery: back to the future? J Med Chem 61:1382–1414
Hondal RJ, Nilsson BL, Raines RT (2001) Selenocysteine in native chemical ligation and expressed protein ligation. J Am Chem Soc 123:5140–5141
Jad YE, Kumar A, El-Faham A et al (2019) Green transformation of solid-phase peptide synthesis. ACS Sustain Chem Eng 7:3671–3683
Jiang W, Zhang B, Fan C et al (2017) Mirror-image polymerase chain reaction. Cell Discov 3:1–7
Kumar D, Bhalla TC (2005) Microbial proteases in peptide synthesis: approaches and applications. Appl Microbiol Biotechnol 68:726–736
Liu Y, Hu Y, Liu T (2012) Recent advances in non-peptidomimetic dipeptidyl peptidase 4 inhibitors: medicinal chemistry and preclinical aspects. Curr Med Chem 19:3982–3999
Macmillan D (2006) Evolving strategies for protein synthesis converge on native chemical ligation. Angew Chem Int Ed 45:7668–7672
Mahindra A, Sharma KK, Jain R (2012) Rapid microwave-assisted solution-phase peptide synthesis. Tetrahedron Lett 53:6931–6935
Mahindra A, Nooney K, Uraon S et al (2013) Microwave-assisted solution phase peptide synthesis in neat water. RSC Adv 3:16810–16816
Mahto SK, Howard CJ, Shimko JC (2011) A reversible protection strategy to improve Fmoc-SPPS of peptide thioesters by the N-acylurea approach. Chembiochem 12:2488–2494
Mazmanian SK, Liu G, Ton-That H et al (1999) Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. Science 285:760–763

Merrifield RB (1963) Solid phase peptide synthesis I. the synthesis of a tetrapeptide. J Am Chem Soc 85:2149–2154
Merrifield RB (1985) Solid phase synthesis (Nobel lecture). Angew Chem Int Ed 24:799–810
Merrifield RB (1986) Solid phase synthesis. Science 232:341–347
Mijalis AJ, Thomas DA, Simon MD et al (2017) A fully automated flow-based approach for accelerated peptide synthesis. Nat Chem Biol 13:464–466
Mong SK, Vinogradov AA, Simon MD et al (2014) Rapid total synthesis of DARPin pE59 and barnase. Chembiochem 15:721–733
Mótyán JA, Tóth F, Tőzsér J (2013) Research applications of proteolytic enzymes in molecular biology. Biomol Ther 3:923–942
Murray JK, Gellman SH (2005) Application of microwave irradiation to the synthesis of 14-helical β-peptides. Org Lett 7:1517–1520
Muttenthaler M, Albericio F, Dawson PE (2015) Methods, setup and safe handling for anhydrous hydrogen fluoride cleavage in Boc solid-phase peptide synthesis. Nat Protoc 7:1067–1083
Nguyen GK, Hemu X, Quek JP (2016) Butelase-mediated macrocyclization of d-amino-acid-containing peptides. Angew Chem Int Ed 128:12994–12998
Pedersen SL, Tofteng AP, Malik L (2012) Microwave heating in solid-phase peptide synthesis. Chem Soc Rev 41:1826–1844
Quaderer R, Sewing A, Hilvert D (2001) Selenocysteine-mediated native chemical ligation. Helv Chim Acta 84:1197–1206
Rapp W, Zhang L, Habich R et al (1988) Polystyrene-polyoxyethylene graft copolymers for high-speed peptide synthesis, pp 199–201
Rodríguez H, Suarez M, Albericio F (2010) A convenient microwave-enhanced solid-phase synthesis of short chain N-methyl-rich peptides. J Pept Sci 16:136–140
Sato AK, Viswanathan M, Kent RB et al (2006) Therapeutic peptides: technological advances driving peptides into development. Curr Opin Biotechnol 17:638–642
Schnölzer M, Alewood P, Jones A et al (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis: rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 40: 180–193
Simon MD, Heider PL, Adamo A et al (2014) Rapid flow-based peptide synthesis. Chembiochem 15:713–720
Subirós-Funosas R, Prohens R, Barbas R et al (2009) Oxyma: an efficient additive for peptide synthesis to replace the benzotriazole-based HOBt and HOAt with a lower risk of explosion. Chem Eur J 15:9394–9403
Torbeev VY, Kent SB (2007) Convergent chemical synthesis and crystal structure of a 203 amino acid "covalent dimer" HIV-1 protease enzyme molecule. Angew Chem Int Ed 46:1667–1670
Tsuda Y, Okada Y (2010) Amino acids, peptides and proteins in organic chemistry. Wiley, Hoboken, NJ, pp 201–251
Uhlig T, Kyprianou T, Martinelli FG (2014) The emergence of peptides in the pharmaceutical business: from exploration to exploitation. EuPA Open Proteom 4:58–69
Ulijn RV, Baragaña B, Halling PJ et al (2002) Protease-catalyzed peptide synthesis on solid support. J Am Chem Soc 124:10988–10989
Ulijn RV, Bisek N, Halling PJ et al (2003) Understanding protease catalysed solid phase peptide synthesis. Org Biomol Chem 1:1277–1281
Våbenø J, Haug BE, Rosenkilde MM (2015) Progress toward rationally designed small-molecule peptide and peptidomimetic CXCR4 antagonists. Future Med Chem 7:1261–1283
Verlander M (2007) Industrial applications of solid-phase peptide synthesis–a status report. Int J Pept Res Ther 13:75–82
Wang L, Wang N, Zhang W et al (2022) Therapeutic peptides: current applications and future directions. Signal Transduct Target Ther 7:48
Werner HM, Cabalteja CC, Horne WS (2016) Peptide backbone composition and protease susceptibility: impact of modification type, position, and tandem substitution. Chembiochem 17:712–718

Wu Z, Guo X, Guo Z (2011) Sortase A-catalyzed peptide cyclization for the synthesis of macrocyclic peptides and glycopeptides. Chem Commun 47:9218–9220
Yan LZ, Dawson PE (2001) Synthesis of peptides and proteins without cysteine residues by native chemical ligation combined with desulfurization. J Am Chem Soc 123:526–533
Zheng JS, Tang S, Huang YC et al (2013) Development of new thioester equivalents for protein chemical synthesis. Acc Chem Res 46:2475–2484