



Review Article

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Advancements in Protein Synthesis: Triazole Multi-Ligation and Cycloadditions for Pharmaceutical Applications

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To Cite This Article: Ghassan Shannan, Zeina S Malek and Nasser Thallaj*. Advancements in Protein Synthesis: Triazole Multi-Ligation and Cycloadditions for Pharmaceutical Applications. Am J Biomed Sci & Res. 2025 26(5) AJBSR.MS.ID.003481, DOI: 10.34297/AJBSR.2025.26.003481

Received: 📅 February 2, 2025; Published: 📅 April 24, 2025

Abstract

The synthesis of proteins remains a critical challenge in chemical biology, essential for understanding the intricate molecular mechanisms underlying biological processes. This article reviews innovative methodologies in protein synthesis, focusing on triazole multi-ligation and successive cycloadditions. These approaches facilitate the precise assembly of complex protein mimetics, thereby enhancing our understanding of protein structure and function. We discuss the advancements in Solid-Phase Peptide Synthesis (SPPS) and Native Chemical Ligation (NCL), which have significantly improved the efficiency and scalability of protein production. SPPS allows for the iterative assembly of peptides, while NCL enables the formation of native peptide bonds under mild conditions, overcoming the limitations of traditional recombinant methods. The integration of non-canonical amino acids and post-translational modifications in these techniques further expands the functional repertoire of synthesized proteins. Additionally, we explore mixed ligation strategies that leverage the compatibility of different ligation methods to streamline protein assembly.

The development of solid-support methods for successive ligation has expedited purification processes, allowing for the synthesis of larger protein constructs. Despite these advancements, challenges such as the stability of reactive intermediates and the specificity of ligation reactions persist, necessitating ongoing research. Future directions include the exploration of second-generation native chemical ligations and auxiliary-assisted methods, which aim to broaden the scope of amino acid utilization and enhance the versatility of chemical ligation techniques. By advancing these methodologies, we can improve our ability to produce functional protein analogues, facilitating deeper insights into protein interactions and biological functions. Ultimately, the evolution of these innovative strategies holds great potential for applications in biotechnology and medicine.

Keywords: Protein synthesis; Triazole multi-ligation; Cycloadditions; Solid-Phase Peptide Synthesis (SPPS); Native chemical ligation (NCL); Protein mimetics; Post-translational modifications

Introduction

The synthesis of proteins is a fundamental challenge in the field of chemical biology, driven by the necessity to understand the intricate molecular mechanisms that govern biological processes [1-5]. Proteins, composed of sequences of amino acids, perform a vast

array of functions within living organisms, ranging from catalysing biochemical reactions to providing structural support in cells [3-8]. As research in genomics and proteomics has advanced, the complexity of protein synthesis has become increasingly apparent [7-



12]. The human genome contains approximately 20,000 to 25,000 protein-coding genes, yet the functional diversity of the proteome far exceeds this number due to post-translational modifications and alternative splicing [13-19]. This variability complicates the task of elucidating the specific roles of individual proteins and their interactions within cellular systems [18-26].

To study protein function, researchers have predominantly relied on recombinant DNA technology, which allows for the expression of proteins in genetically modified cells. This method has revolutionized the field by enabling the production of large quantities of structurally defined proteins. However, it is not without its limitations [25-30]. The overexpression of certain proteins can lead to issues such as aggregation or insolubility, particularly for cytotoxic or membrane-associated proteins. Moreover, the reliance on cellular machinery restricts the introduction of specific post-translational modifications, which are crucial for the functional activity of many proteins [31-36]. While chemical modifications can be performed post-extraction, these processes often require complex methodologies and can result in low yields or undesired side products [36-40].

In contrast, chemical protein synthesis offers a promising alternative, allowing for precise control over the structure and modifications of polypeptides [41-42]. This approach enables the incorporation of non-canonical amino acids, post-translational modifications, and the synthesis of complex protein mimetics that are not readily achievable through biological methods. The ability to synthesize proteins chemically allows researchers to manipulate protein structures at an atomic level, facilitating detailed investigations into their biochemical properties and functions [43-49].

The advent of Solid-Phase Peptide Synthesis (SPPS), pioneered by Robert Merrifield, marked a significant milestone in the field of chemical protein synthesis. This technique simplifies the iterative assembly of peptides by anchoring the growing chain to an insoluble resin, allowing for straightforward purification and enhanced reaction efficiency [50].

Despite its revolutionary impact, SPPS is generally limited to peptides of approximately fifty amino acids in length [51]. To overcome this limitation, researchers have developed various strategies for the synthesis of larger proteins, including the condensation of fully or partially protected peptide fragments and the use of chemical ligation methods [50-51].

Chemical ligation techniques, such as Native Chemical Ligation (NCL), have gained widespread acceptance for their ability to join unprotected peptide segments in aqueous environments [52-55]. NCL relies on the unique reactivity of thioesters and N-terminal cysteine residues, enabling the formation of native peptide bonds under mild conditions.

However, this method is constrained by the requirement for cysteine at the ligation site and the inherent instability of thioesters in basic conditions [56-58]. To address these challenges, novel approaches, including second-generation NCL strategies and auxiliary-assisted ligation methods, have been developed to broaden the

applicability of chemical ligation [59-63].

Recent advancements in multi-ligation strategies, including the use of tandem and successive ligation techniques, have further enhanced the potential for synthesizing complex protein structures [64-69].

By employing a combination of compatible ligation methods, researchers can assemble larger protein constructs more efficiently. Additionally, the development of solid-support methods for successive ligation has shown promise in expediting purification processes and improving overall yields [70-77].

This article explores the innovative approaches in triazole multi-ligation and the development of new tools for synthesizing protein mimetics through successive cycloadditions [78]. By examining the principles and applications of these methodologies, we aim to highlight their significance in advancing the field of chemical protein synthesis and their potential to overcome the limitations of traditional methods [71-79].

Through a comprehensive understanding of these techniques, researchers can enhance their ability to produce functional protein analogues, thereby facilitating deeper insights into protein function and interaction in biological systems [77].

Chemical Protein Synthesis: A Challenge for Chemists

Genome and Proteome

A primary goal of biomedical research is to elucidate the molecular mechanisms underlying the diverse biological activities of proteins, as well as to predict and manipulate these activities. The complexity and significance of this endeavour have become increasingly apparent since the advent of genome sequencing. According to analyses conducted in 2004, the human genome is estimated to contain approximately 20,000 to 25,000 protein-coding genes [78].

However, this number does not fully account for the extensive variability present in the proteome, as functional proteins can undergo numerous modifications during synthesis, resulting in a much larger repertoire of expressed proteins than that suggested by gene studies.

Once the sequence of a protein is determined through genomic analysis, a significant challenge remains: ascertaining its biological function. At this juncture, the production of the protein becomes crucial for addressing this issue [79].

Recombinant Protein Synthesis

The majority of research into the mechanisms of protein action has utilized proteins produced via recombinant DNA technology, where proteins are expressed in cells that have been genetically modified. Since its inception, this method has transformed protein research by enabling the generation of substantial quantities of structurally defined proteins, while also facilitating systematic variations in protein sequences. Although expressing proteins in

competent cells has become a standard technique, it is not without limitations.

For instance, the overexpression of cytotoxic or poorly soluble proteins often presents challenges [80]. Furthermore, the reliance on the cellular machinery complicates the controlled introduction of post-translational modifications, which can be difficult or infeasible depending on the type of modification and the expression system employed [81].

Additionally, while it is possible to introduce post-translational modifications chemically or enzymatically after extraction and purification, this process remains complex. Recent advancements in the incorporation of non-proteinogenic amino acids, which possess unique properties such as fluorescence or specialized chemical reactivity, represent an exciting area of ongoing research [82].

Current methodologies include systematic residue replacement to alter the physicochemical properties of expressed proteins or the targeted introduction of non-canonical amino acids to facilitate the study of protein structure at the molecular level, both in vitro and in vivo [83].

Chemical Protein Synthesis

Chemical synthesis has emerged as a viable alternative for protein production, allowing for precise modifications of polypeptide structures to enable comprehensive investigations of their mechanisms of action. This approach permits the incorporation of:

- Amino acids not encoded by the genome
- Modifications to the protein backbone with atomic precision

sion

- Post-translational modifications (such as phosphorylation, glycosylation, and lipidation)
- Tools for spectroscopic characterization

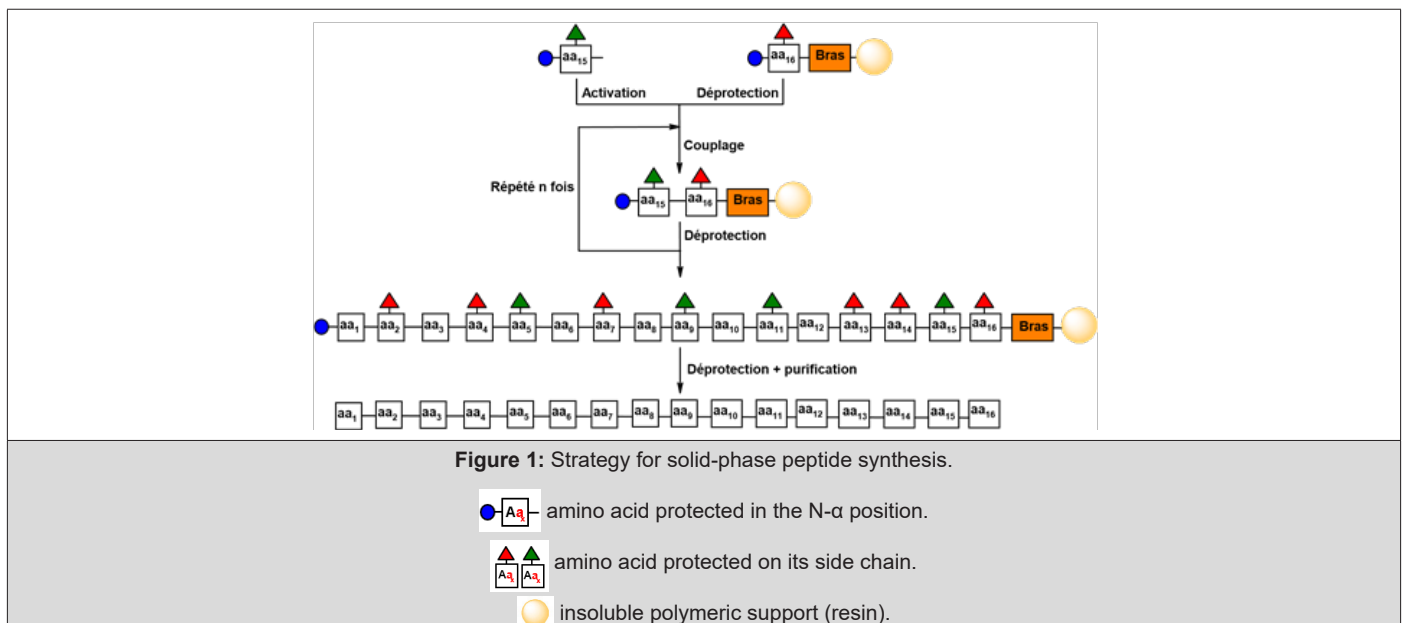
Given these advantages, total chemical synthesis of proteins has long posed a challenge for chemists. Emil Fischer, awarded the Nobel Prize in 1902, envisioned that "Physiological Chemistry" would enable the preparation of synthetic enzymes tailored for specific functions. This vision is steadily becoming a reality, thanks to remarkable advances in peptide synthesis achieved over the past century [84].

Chemical Synthesis of Proteins via Condensation of Protected Peptides

Solid-Phase Peptide Synthesis

Merrifield's Solid-Phase Peptide Synthesis (SPPS) has revolutionized the field of peptide synthesis, making it readily accessible. In SPPS, peptides are synthesized through a repetitive and iterative process rather than a convergent one. The innovative aspect of this method lies in the attachment of the peptide to an insoluble polymeric support, or "resin," at its C-terminal end.

This immobilization of one of the reactive partners allows for the straightforward separation of coupling or deprotection by-products from the growing peptide through simple filtration, thereby significantly expediting the synthesis process. Moreover, filtration enables the use of large excesses of amino acids for coupling, resulting in nearly quantitative yields (Figure1).



A significant advantage of Solid-Phase Peptide Synthesis (SPPS) is its repetitive nature, which allows for automation, thereby facilitating peptide synthesis in non-specialized laboratories. Despite

extensive optimization of the methodology introduced by Merrifield-including advancements in resins, coupling reagents, protecting groups, and synthesis arms-the maximum size of peptides

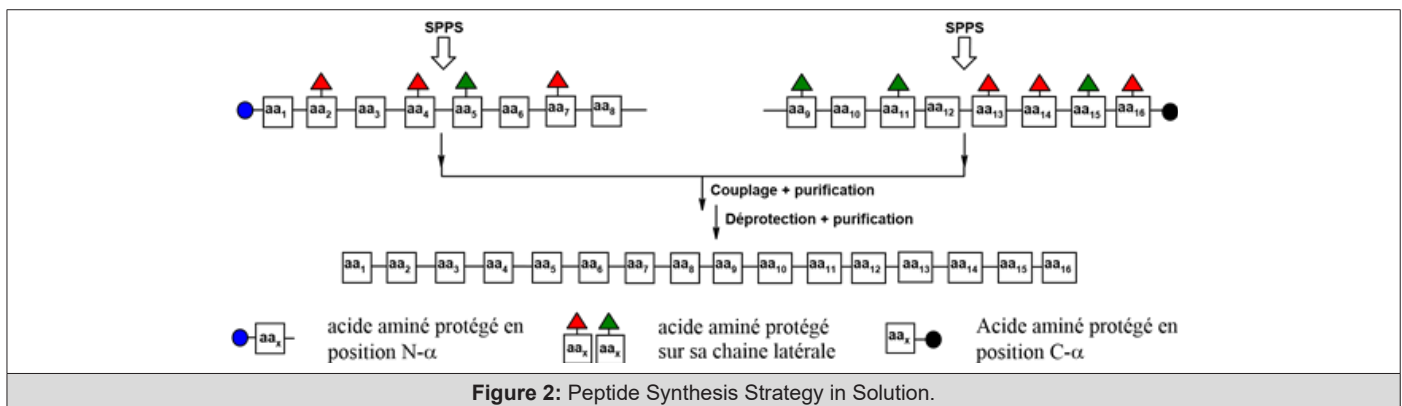
synthesized via this approach is generally limited to approximately fifty amino acids [85].

Protein Synthesis through the Condensation of Fully Protected Peptides

This strategy employs a convergent assembly technique that involves the condensation of two fully protected peptides synthesized by SPPS through the formation of an amide bond (Figure 2).

This method is particularly effective for producing proteins that exceed fifty residues or for generating high-purity therapeutic peptides [86].

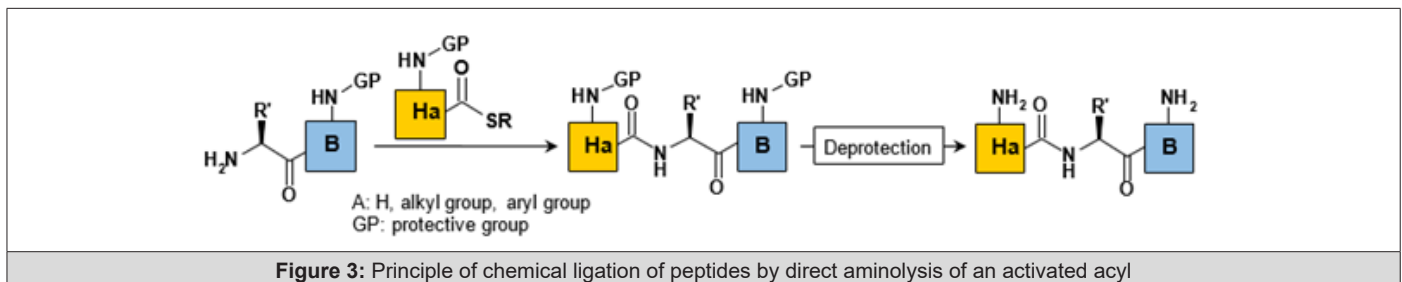
The coupling of protected peptides in solution presents several disadvantages, with the most significant being the limited solubility of these peptides in organic solvents, as well as the potential for epimerization at the α position of the activated amino acid [87].



Protein Synthesis through the Condensation of Partially Protected Peptides

This approach is pioneering in its use of partially protected

peptides for coupling and is the first to employ thiocarbonyls as activated acyl donor groups, enabling chemo selective couplings (Figure 3).



This strategy marks a significant advancement over previous methods, as it is compatible with solvents where unprotected peptides exhibit high solubility, such as water or DMF, and utilizes a weakly activated acyl donor to minimize epimerization issues. However, while highly chemo selective acylation offers benefits, it lacks regioselectivity, necessitating the protection of side chains on lysine residues, which reduces the peptide's solubility in water and extends coupling times to as long as 96 hours [88].

Despite these challenges, this method has experienced renewed interest following the foundational work of Blake and his collaborators, as well as Aimoto and Hojo. Initially constrained by the use of lipophilic amine protective groups that complicate cou-

pling in aqueous environments, this strategy is now being revisited and is the focus of ongoing research aimed at synthesizing peptides, glycopeptides, and glycoproteins.

Chemical Ligation of Peptides

Chemical peptide ligation involves the chemo selective and regioselective coupling of two fully unprotected peptides (Figure 4). Performing this coupling under such conditions allows for the avoidance of the primary disadvantages associated with convergent synthesis in organic solvents, including enhanced solubility of all peptide fragments and non-epimerizing coupling conditions.

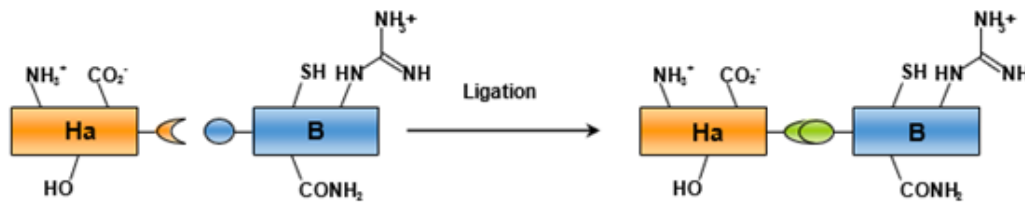


Figure 4: Principle of chemical ligation.

During chemical ligation of peptides, two peptide fragments are joined through the formation of a covalent bond between two reactive partners (e.g., an electrophilic aldehyde and a nucleophilic aminoxy group to yield an oxime) to create an amide bond mimic or a protein mimic. The primary challenge in developing an effective ligation method lies in identifying partners that can be readily incorporated into peptides via Solid-Phase Peptide Synthesis (SPPS) while maintaining sufficient chemo- and regioselective reactivity in aqueous environments, along with adequate stability for manipulation.

This challenge becomes significantly more complex when considering that, after the primary sequence of the protein is assembled through ligation, the resulting synthetic polymer must fold correctly to resemble the native protein and retain its biological activity. The quest for novel ligation methods remains an area of active research. Current investigations primarily focus on utilizing

pericyclic reactions (such as Diels-Alder and 1,3-dipole cycloadditions) or organometallic catalysis (including Suzuki couplings and cross-metathesis of hydro compatible olefins) to exploit the chemical stability of the partners and the exceptional chemo selectivity of the coupling reactions.

Among the reactions exhibiting these desirable characteristics, only a select few have been successfully employed for the total synthesis of proteins or bioactive structural analogs of proteins. We categorize these methods into two distinct types: native and pseudo-native ligations.

Native ligations

Native Chemical Ligation (NCL):

Native Chemical Ligation (NCL) involves the reaction between a thioester and an N-terminal cysteine residue (Figure 5).

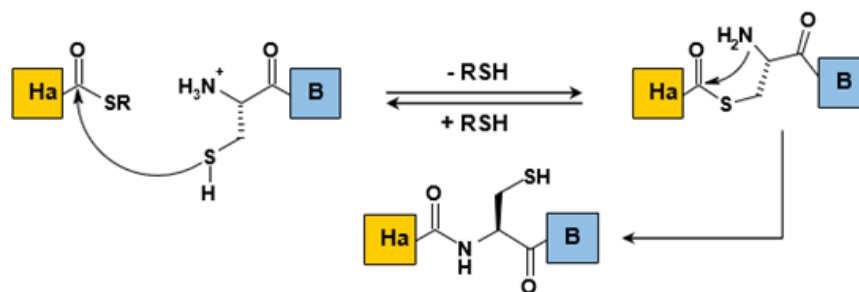


Figure 5: Principle of Native Chemical Ligation.

The initial step of this process is dynamic, featuring reversible trans-Thio esterification at the C-terminal end of the thioester fragment. This intermediate then undergoes spontaneous rearrangement through nucleophilic addition of the amine to the carbonyl, followed by elimination, resulting in the formation of a native peptide bond (Figure 5). The chemo selectivity of this reaction is attributed to trans-Thio esterification, which effectively positions the electrophilic carbonyl in close proximity to the nucleophilic amine, as well as the regioselective activation of the adjacent amine to the thioester at physiological pH (~ 7.4), where the amines on lysine side chains are primarily protonated. The coupling occurs without

epimerization, and its kinetics are influenced by the steric hindrance of the side chain of the amino acid carrying the thioester. The first demonstration of NCL was the synthesis of a 72-residue protein, human interleukin 8, conducted by Kent et al (Figure 6).

Native Chemical Ligation (NCL) is one of the most widely adopted techniques for native ligation. The efficacy of this method is underscored by nearly a thousand citations of its initial publication (source: ISI Web of Knowledge, 10/01/2010). NCL facilitates the synthesis of numerous proteins either through purely chemical means or via a hybrid approach that combines chemical and recombinant methods.

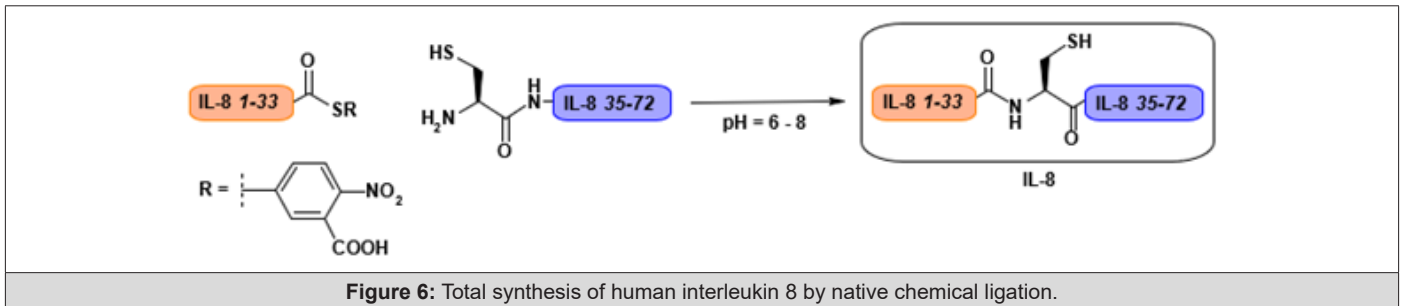


Figure 6: Total synthesis of human interleukin 8 by native chemical ligation.

Despite its advantages, NCL has notable limitations. Cysteines and thioesters exhibit high reactivity towards electrophiles and nucleophiles, respectively. Thioesters, in particular, are unstable and susceptible to hydrolysis at pH levels above neutral, which complicates their synthesis and handling. Currently, the predominant strategy for synthesizing thioester peptides is the Boc/tBu method; however, this approach is increasingly being abandoned in laboratories due to safety concerns associated with the use of anhydrous hydrofluoric acid. Additionally, thioesters are prone to instability in basic and nucleophilic environments, rendering their synthesis challenging when employing the Fmoc/tBu strategy. Consequently, no straightforward method exists for accessing these compounds. Lastly, the specificity of this reaction for N-terminal cysteines, which occur relatively infrequently (with an abundance of only 1.35%), constrains the range of proteins that can be synthesized using this technique.

“Second Generation” Native Chemical Ligations:

The methods characterized as second-generation Native Chem-

ical Ligations (NCLs) have been developed to address the primary limitation of traditional NCL, specifically the requirement for a cysteine at the ligation site. Among these approaches, desulfurization and the use of auxiliary reagents are the most prevalent strategies.

Both methods leverage the distinctive features of NCL, which involve the activation of the carboxylic acid at the C-terminal end as a thioester, alongside the regioselective activation of the amine near the thioester (Figure 5).

a. Desulfurization of the NL product:

Desulfurization of the cysteine utilized in NCL enables the coupling of peptide fragments to more abundant residues, such as alanine. This strategy has also been expanded to include residues like valine and phenylalanine. A significant drawback of this method is the necessity to protect thiol groups present in the peptide sequence (Figure 7).

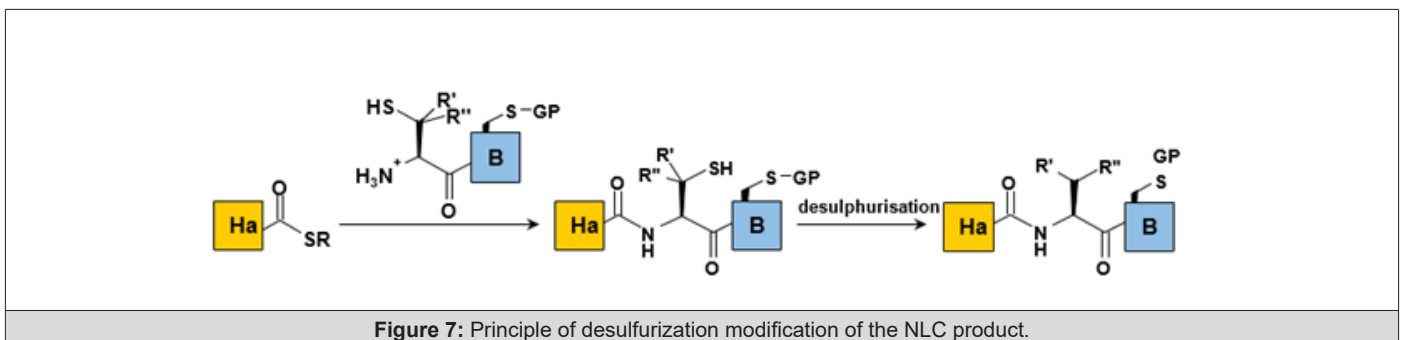


Figure 7: Principle of desulfurization modification of the NLC product.

Desulfurization employing transition metals presents several challenges, including the necessity for large quantities of metal reagents, the potential epimerization of secondary alcohols (such as threonine's), and the reduction of thioethers (such as methionine's). In contrast, radical desulfurization, developed by Danishefsky and collaborators, appears to be a milder approach; however, it still involves the delicate process of deprotecting any additional cysteine residues present in the protein.

b. Auxiliary-assisted native chemical ligation:

An alternative strategy to desulfurization involves the use of thioether auxiliaries, which can be selectively removed following the ligation process. The primary advantage of this approach is that it allows for ligation to occur with any amino acid residue, thereby expanding the versatility of the method (Figure 8).

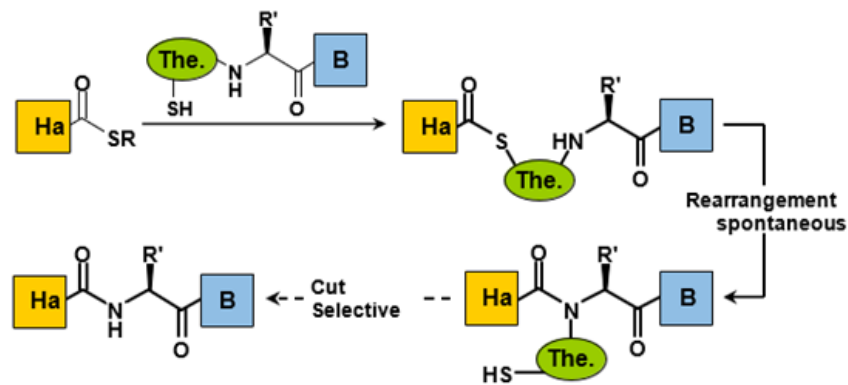


Figure 8: Principle of native chemical ligation assisted by auxiliaries.

The nature of the auxiliaries employed varies depending on their cleavage strategies. The most frequently utilized auxiliaries include acid-labile ones, such as those activated by Hydrofluoric Acid (HF) or Trifluoroacetic Acid (TFA), as well as photolabile types. However, challenges may arise during the removal of the auxiliary, and the substitution of the amine can impede the transfer of the final acyl group, increasing the likelihood of side reactions.

The native Staudinger ligation:

This method was independently discovered by the research teams of Bertozzi and Raines. The coupling mechanism relies on the reaction between a phosphine and an organic aside, resulting in the formation of an aza-yurea-like intermediate (Figure 9). This intermediate undergoes a nucleophilic attack by nitrogen on the carbonyl, ultimately yielding a native amide bond. This rearrange-

ment is particularly favoured when the phosphine is attached to a thioester at the C-terminal end.

The advantage of this method lies in its ability to perform ligation independently of the residue at the deconvolution point on the peptide. The chemo selectivity of a phosphine's attack on an aside is superior to that of previously discussed methods and is compatible with all functionalities present in peptides. However, this approach carries the inherent challenges associated with thioester synthesis, compounded by the presence of an oxidizable phosphine group. Furthermore, mechanistic studies and experimental findings indicate competition between water attacking the phosphonium species of azalure and amine attacking the carbonyl (Figure 9). To promote the latter reaction, the authors recommend using peptide concentrations around 100 mM, which poses challenges for practical peptide ligation.

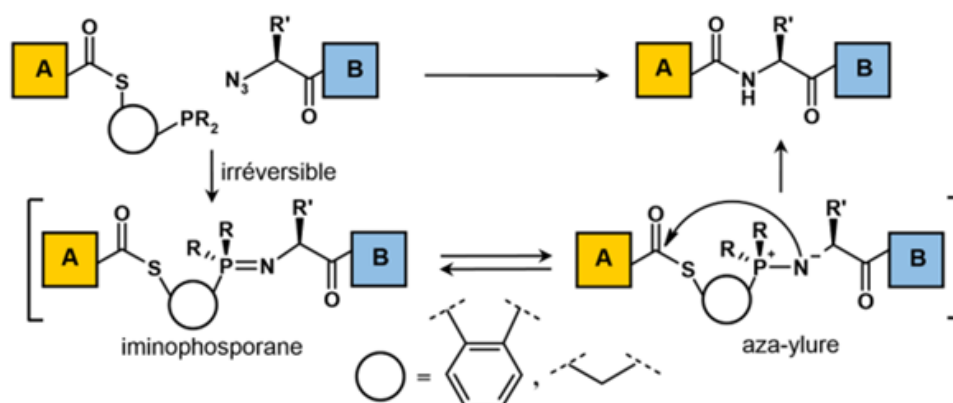


Figure 9: Principe de la ligation de Staudinger.

It is important to note that, despite its potential and promising results, this method has yet to be employed for the chemical synthesis of proteins via peptide ligation.

New Methods for Amide Bond Formation Compatible with Unprotected Peptide Ligation:

Despite the challenges associated with forming amide bonds

in aqueous conditions with chemo- and regioselectivity, innovative methods continue to emerge, although they have not yet been applied to the synthesis of functional proteins (Figure 10). One par-

ticularly noteworthy method involves the reaction of α -keto acids with hydroxylamine's, yielding an amide bond through a condensation reaction accompanied by decarboxylation.

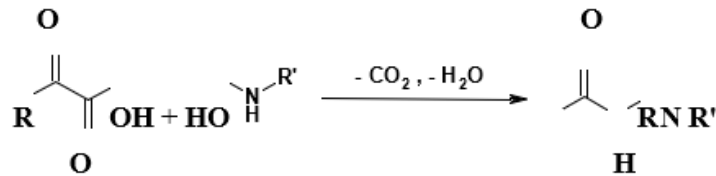


Figure 10: Novel amide formation methods compatible with the ligation of unprotected peptides.

Pseudo-Native Ligation Methods

Pseudo-native methods encompass a range of ligation techniques that facilitate the formation of amide bond mimics, including isosteres and bio isosteres.

Thioether Ligation

Thioether ligation capitalizes on the distinctive reactivity of the

cysteine residue side chain. The thiol group acts as a proficient nucleophile, enabling selective nucleophilic addition or substitution reactions with amines. This approach has been successfully employed in the synthesis of an analogue of HIV-1 protease (Figure 11). In this synthesis, the N-terminal fragment of the target protein, modified as a thioethyl amide at its C-terminus, reacts with the C-terminal fragment, which is functionalized at the N-terminus with a Bromo acetyl group.

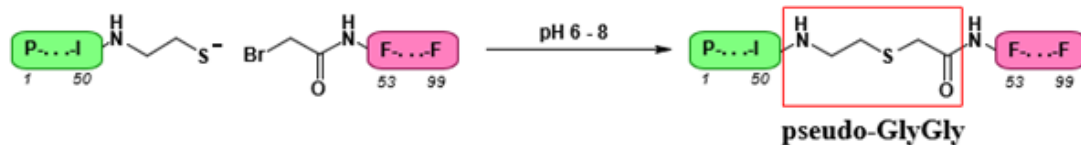


Figure 11: Example of thioether ligation, synthesis of an analogue of the HIV-1 protease by thioester ligation.

A significant limitation of this method is its lack of selectivity for a specific thiol within the peptide sequence. Consequently, it is applicable only when the native protein contains either a single cysteine residue or none at all.

Thioester Ligation

Thioester ligation is closely related to thioether ligation, as it

utilizes the same electrophilic partner. In this reaction, a Thio carboxylate undergoes nucleophilic substitution with a Bromo acetyl to form a thioester (Figure 12). This method can be classified as a pseudo-native technique because it generates an isostere of the amide bond. Notably, thioester ligation was the first technique successfully applied to fully unprotected peptides, resulting in the total synthesis of a functional analogue of HIV-1 protease (Figure 12).

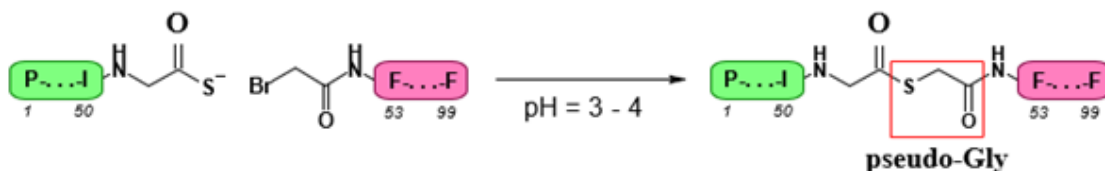


Figure 12: Synthesis of an analogue of the HIV-1 protease by thioester ligation.

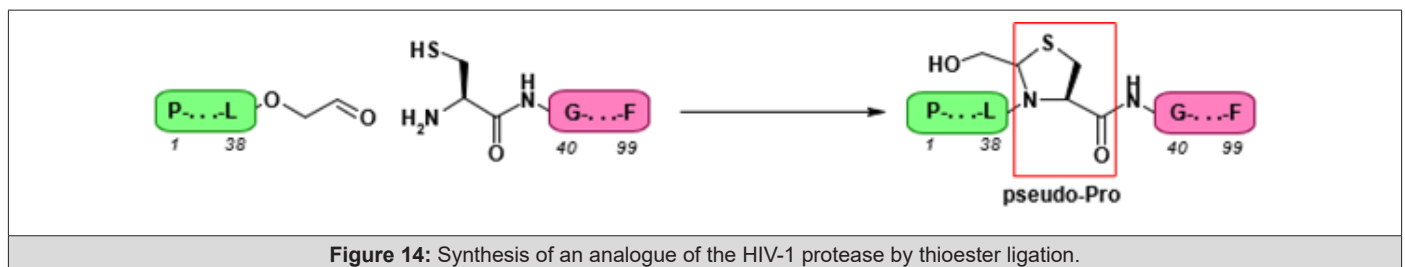
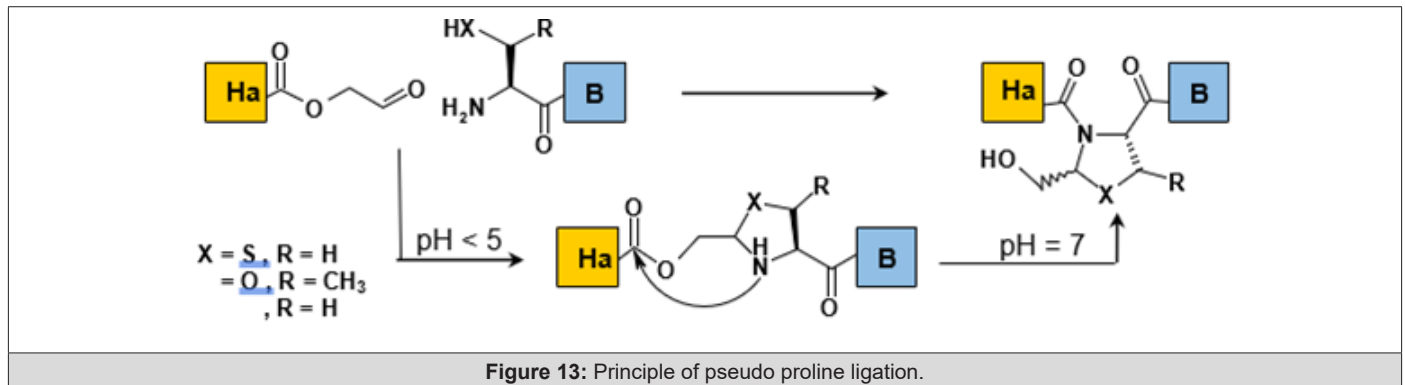
Despite these promising results, this method carries several disadvantages associated with thioether ligation. Additionally, it necessitates the use of D-bromo-acids or deconvolution specific to

glycyl residues (Figure 12). Furthermore, the thioester bond exhibits instability at pH levels above neutral, which restricts the handling and stability of the resulting ligation product.

Imine-Like Ligation: Thiaprolins and Oxaprolins

Developed by Liu and Tam, these ligation methods involve the condensation of an aldehyde with a β -aminothiol or a β -amino alcohol (Figure 13).

The aldehyde group reacts to form an imine, which is subsequently attacked by the thiol group to yield a thiazolidine (Figure 14). The resulting coupling product can undergo an acyl transfer from the oxygen to the nitrogen, quantitatively resulting in the formation of an amide bond (Figure 13).

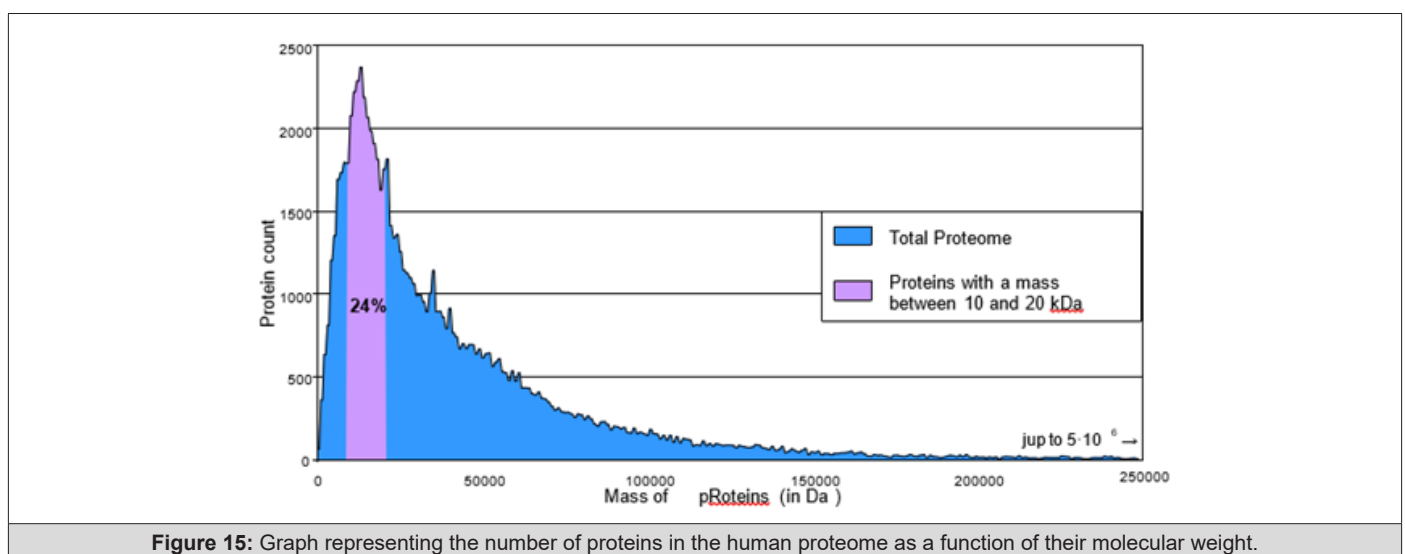


This ligation method exhibits excellent chemo selectivity; however, the high reactivity of aldehydes toward nucleophiles, particularly amines in lysine side chains, as well as the reactivity of N-terminal cysteines toward electrophiles, can present challenges.

Problem

The human proteome encompasses the complete set of proteins

expressed by the human genome. Figure 15 depicts the abundance of these proteins in relation to their size, highlighting that those accessible through solid-phase peptide synthesis (SPPS), which are less than 50 residues in length, represent only a small fraction of the proteome (approximately 5%). Furthermore, a single ligation of peptides containing fewer than 100 amino acids provides access to only about 15% of the human proteome (Figure 15).



To achieve proteins with a mass of approximately 20 kDa, it is essential to perform multiple successive ligations, thereby enabling a chemical synthesis method that could encompass about 38% of the human proteome. Multi-ligation of peptides significantly expands the potential biological targets for chemical peptide synthesis, as around 24% of the human proteome (depicted in purple in Figure 15) falls within the mass range of 10,000 to 20,000 Da-limits that are only accessible through chemical peptide multi-ligation. To date, only a few dozen proteins of this size have been synthesized

chemically.

Peptide Multi-Ligation Strategies

Two primary strategies have been identified to date:

- A mixed approach that employs two mutually compatible ligation methods (Figure 16).
- The repeated use of the same ligation method, involving the masking of a partner during the initial ligation (Figure 17).

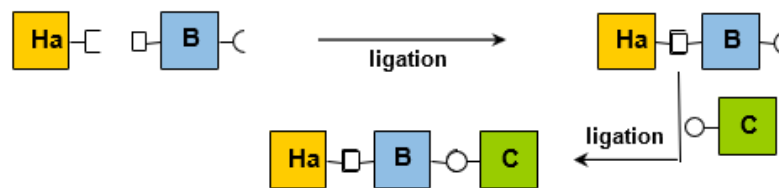


Figure 16: Principle of successive ligations using mixed ligation methods.

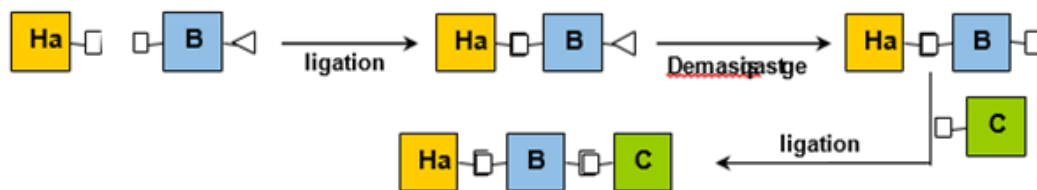


Figure 17: Principle of successive ligations by using protective groups.

Protein Synthesis by Mixed Ligation

A. Principle

This strategy leverages the mutual compatibility or partial compatibility of different ligation methods. It relies on the chemo selectivity of ligation reactions, enabling their simultaneous use (Figure 16). The effectiveness of this approach is exemplified by two cases

of tandem multi-ligation utilized for synthesizing proteins or protein analogues.

B. Mutually Compatible Ligation: Thioester and Oxime

A notable example of this strategy is the combined use of oxime and thioester ligation in the synthesis of the cMyc-Max protein analogue (Figure 18).

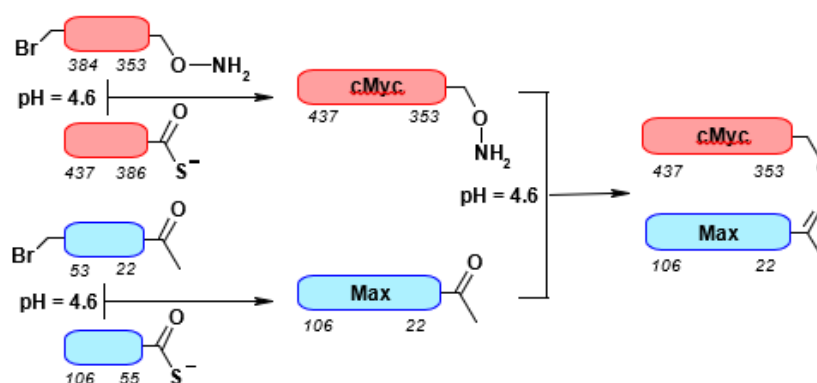


Figure 18: Combination of two ligation methods: thioester and oxime.

Kinetic control of thioesterification during the assembly of cMyc results in the preferential substitution of the bromoacetyl partner with thiocarboxylate, yielding the peptide-amino-oxy cMyc(353-437). Concurrently, a similar thioesterification generates the peptide-aldehyde Max(22-106). These two peptides are subsequently coupled through the condensation of the carbonyl fragment with the amino-oxy fragment, resulting in a functional analogue of the

target protein.

C. Partially Compatible Ligations: Association of Thioester and Pseudo proline Ligation

The combination of native ligation and pseudo proline ligation has been proposed as a strategy to enhance the efficiency of peptide synthesis (Figure 19).

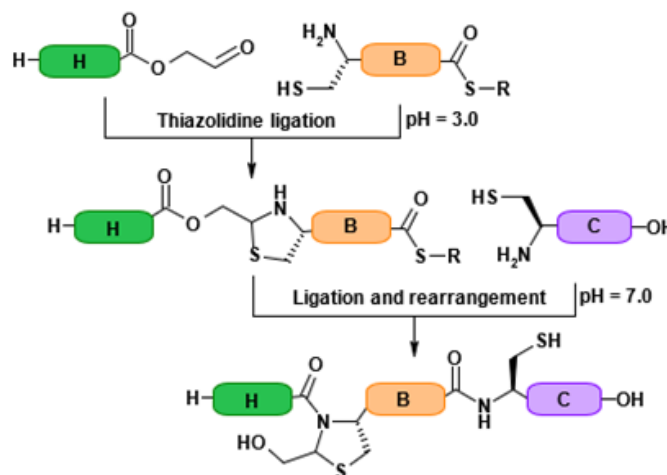


Figure 19: Combination of two ligation methods: NCL and pseudo proline according to Tam et al.

Although both pseudo proline and thioester ligations utilize the same nucleophilic partner, an N-terminal cysteine, the formation of a thiazolidine is favoured at pH 3. This prevents the cyclization of the B fragment (Figure 19). The resulting fragment AB is then subjected to native ligation conditions at neutral pH, allowing for condensation with fragment C. This process simultaneously facilitates the rearrangement of the thiazolidine into pseudo proline. It is crucial to emphasize that the order of fragment assembly and precise control of reaction conditions are essential, as both thiazolidine ligations and native chemical ligation share the same nucleophilic partner.

D. Advantages and Disadvantages of the Mixed Approach

The use of tandem ligations offers the significant advantage of streamlining the protein assembly process; however, it also accumulates the drawbacks associated with each individual technique. Additionally, since all ligation reactions involve nucleophilic addi-

tion or substitution on electrophilic partners, tandem ligation necessitates precise control of reaction conditions to prevent competition between the ligation reactions. Furthermore, the number of mutually compatible ligation techniques is quite limited, which restricts the overall number of ligations that can be performed using this strategy.

Synthesis by Masking a Ligation Partner

A more versatile strategy involves masking one of the ligation partners, followed by unmasking it to continue the protein assembly using the same ligation method (Figure 17).

A. Successive Native Chemical Ligations

The synthesis of crambine, which consists of 46 residues and poses challenges for linear solid-phase peptide synthesis (SPPS), serves as an excellent example of this approach through successive native chemical ligation (Figure 20).

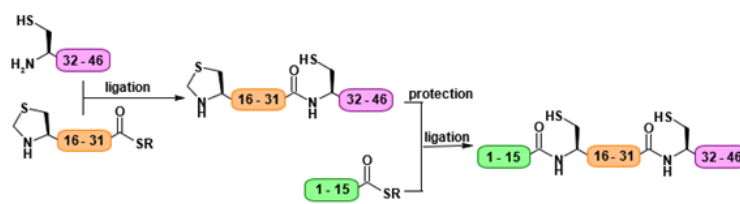
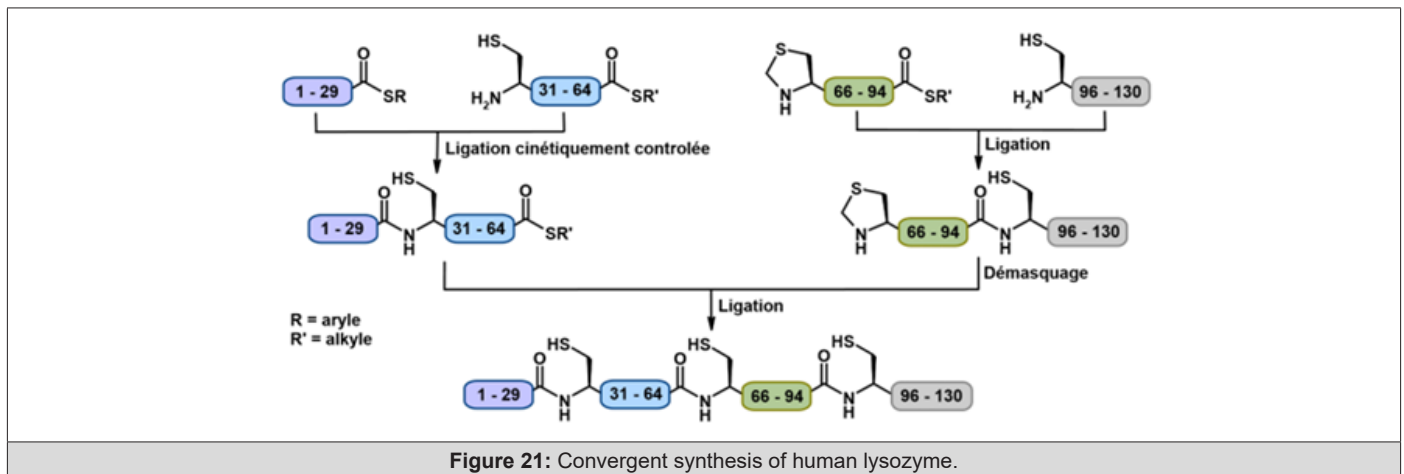


Figure 20: Synthesis of crambine by successive native chemical ligation according to Bang et al.^{Art. 66}

The initial ligation is carried out selectively by masking an N-terminal cysteine in the form of a thiazolidine. The removal of this protective group under mild and chemo selective conditions using methoxy amine hydrochloride allows for the regeneration of a new N-terminal cysteine, which can then be utilized in a subsequent ligation to construct crambine. The Ac_m protective group for cysteine has also been successfully employed in the synthesis of an EPO analogue (166 residues) through successive triple native ligation,

as well as in the synthesis of crambine using a similar strategy.

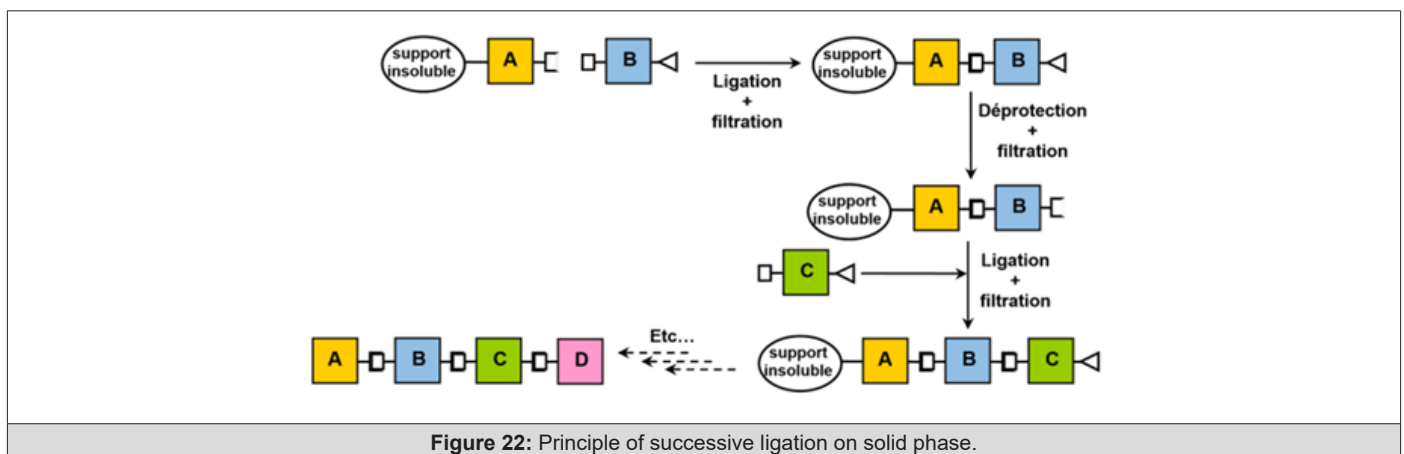
More recently, Kent and his collaborators have enhanced and expanded this multi-ligation approach by capitalizing on the differing reactivities of aryl and aliphatic thioesters. This advancement facilitated the synthesis of human lysozyme (130 residues, 8 cysteines, and 4 disulfide bridges) through Multi-Native Chemical Ligation (NCL) following a convergent strategy (Figure 21).



B. Successive Ligation on Solid Support

The successive or convergent ligation approach can be notably slow due to the need for purifying each synthetic intermediate and the challenges associated with manipulating the peptides. To address these issues, Kent and his collaborators proposed performing successive ligations on solid support. This method effectively combines the benefits of solid-phase synthesis with peptide ligation, providing an excellent strategy for synthesizing protein mimetics of up to 200 residues (Figure 22).

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The purification steps involved in ligation and deprotection are significantly expedited using this method (Figure 22). To date, this approach has been applied to only two ligation techniques: native chemical ligation (NCL) utilizing the thiazolidine protective group, and oxime ligation employing the allyloxy carbonyl protective group.

Conclusion

In summary, the advancements in protein synthesis methodologies, particularly through the innovative approaches of triazole multi-ligation and successive cycloadditions, represent a significant leap in the field of chemical biology. The ability to synthesize complex protein mimetics with precision not only enhances our under-

standing of protein structure and function but also opens new avenues for therapeutic and diagnostic applications.

The integration of chemical synthesis techniques, including Solid-Phase Peptide Synthesis (SPPS) and Native Chemical Ligation (NCL), has demonstrated the potential to produce larger and more intricate protein structures. These methods allow for the incorporation of non-canonical amino acids and post-translational modifications, thereby facilitating the design of proteins with tailored functionalities. The successful application of these techniques to synthesize proteins such as crambine and human lysozyme showcases their effectiveness in overcoming the limitations associated with traditional recombinant methods.

Additionally, the exploration of mixed ligation strategies, which leverage the compatibility of different ligation methods, has proven beneficial in streamlining the protein assembly process. By employing tandem and successive ligation techniques, researchers can efficiently construct protein analogues, significantly expanding the range of accessible biological targets.

The ability to manipulate reaction conditions and the order of fragment assembly plays a crucial role in optimizing yield and specificity, underscoring the importance of meticulous experimental design. Moreover, the introduction of solid-support methods for successive ligation has expedited purification processes and improved the overall efficiency of protein synthesis.

This advancement addresses the challenges of manipulating peptides and purifying intermediates, thus paving the way for the synthesis of larger protein mimetics, which are essential for a comprehensive understanding of biological systems. However, despite these promising developments, challenges remain. Issues related to the stability of reactive intermediates, such as thioesters, and the specificity of ligation reactions necessitate ongoing research.

The exploration of second-generation native chemical ligations and auxiliary-assisted methods represents a proactive approach to overcoming these limitations. These strategies not only broaden the scope of amino acid residues that can be utilized but also enhance the versatility and applicability of chemical ligation techniques.

As we advance our understanding of protein synthesis through these innovative methods, it is crucial to continue investigating the underlying chemical mechanisms and optimizing the conditions for ligation reactions. Future research should focus on developing new ligation strategies that maintain high chemo selectivity and regioselectivity in aqueous environments, as well as exploring the incorporation of additional functional groups that can facilitate more complex protein modifications. The field of chemical protein synthesis is evolving rapidly, driven by the need for precise and efficient methods to produce functional proteins and protein mimetics.

The innovative approaches discussed in this article not only enhance our capabilities in protein synthesis but also contribute to the broader understanding of protein functionality in biological systems. As these methodologies continue to evolve, they hold great

promise for advancing both fundamental research and practical applications in biotechnology and medicine. The ongoing exploration of multi-ligation techniques will undoubtedly play a pivotal role in shaping the future of protein science.

Acknowledgement

None.

Conflict of Interest

None.

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