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Peptide Synthesis

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In organic chemistry, peptide synthesis is the production of peptides, which are organic compounds in which multiple amino acids are linked via amide bonds which are also known as peptide bonds. The biological process of producing long peptides (proteins) is known as protein biosynthesis.

Chemistry

Peptides are synthesized by coupling the carboxyl group or C-terminus of one amino acid to the amino group or N-terminus of another. Due to the possibility of unintended reactions, protecting groups are usually necessary. Chemical peptide synthesis starts at the C-terminal end of the peptide and ends at the N-terminus. This is the opposite of protein biosynthesis, which starts at the N-terminal end.

Liquid-phase synthesis

Liquid-phase peptide synthesis is a classical approach to peptide synthesis. It has been replaced in most labs by solid-phase synthesis. However, it retains usefulness in large-scale production of peptides for industrial purposes.

Solid-phase synthesis

Solid-phase peptide synthesis (SPPS), pioneered by Robert Bruce Merrifield,[1] caused a paradigm shift within the peptide synthesis community, and it is now the standard method for synthesizing peptides and proteins in the lab. SPPS allows for the synthesis of natural peptides which are difficult to express in bacteria, the incorporation of unnatural amino acids, peptide/protein backbone modification, and the synthesis of D-proteins, which consist of D-amino acids.

Small porous beads are treated with functional units ('linkers') on which peptide chains can be built. The peptide will remain covalently attached to the bead until cleaved from it by a reagent such as anhydrous hydrogen fluoride or trifluoroacetic acid. The peptide is thus 'immobilized' on the solid-phase and can be retained during a filtration

Saarth
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process while liquid-phase reagents and by-products of synthesis are flushed away.

The general principle of SPPS is one of repeated cycles of deprotection-wash-coupling-wash. The free N-terminal amine of a solid-phase attached peptide is coupled (see below) to a single N-protected amino acid unit. This unit is then deprotected, revealing a new N-terminal amine to which a further amino acid may be attached. The superiority of this technique partially lies in the ability to perform wash cycles after each reaction, removing excess reagent with all of the growing peptide of interest remaining covalently attached to the insoluble resin.

The overwhelmingly important consideration is to generate extremely high yield in each step. For example, if each coupling step were to have 99% yield, a 26-amino acid peptide would be synthesized in 77% final yield (assuming 100% yield in each deprotection); if each step were 95%, it would be synthesized in 25% yield. Thus each amino acid is added in major excess (2~10x) and coupling amino acids together is highly optimized by a series of well-characterized agents.

There are two majorly used forms of SPPS – Fmoc and Boc. Unlike ribosome protein synthesis, solid-phase peptide synthesis proceeds in a C-terminal to N-terminal fashion. The N-termini of amino acid monomers is protected by either of these two groups and added onto a deprotected amino acid chain.

Automated synthesizers are available for both techniques, though many research groups continue to perform SPPS manually.

SPPS is limited by yields, and typically peptides and proteins in the range of 70 amino acids are pushing the limits of synthetic accessibility.[citation needed] Synthetic difficulty also is sequence dependent; typically amyloid peptides and proteins are difficult to make. Longer lengths can be accessed by using native chemical ligation to couple two peptides together with quantitative yields.

Since its introduction over 40 years ago, SPPS has been significantly optimized. First, the resins themselves have been optimized.[2] Furthermore, the 'linkers' between the C-terminal amino acid and polystyrene resin have improved attachment and cleavage to the point of mostly quantitative yields.[3][4][5] The evolution of side chain protecting

Saarth
E-Journal of Research
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groups has limited the frequency of unwanted side reactions. In addition, the evolution of new activating groups on the carboxyl group of the incoming amino acid have improved coupling and decreased epimerization. Finally, the process itself has been optimized. In Merrifield's initial report, the deprotection of the a-amino group resulted in the formation of a peptide-resin salt, which required neutralization with base prior to coupling. The time between neutralization of the amino group and coupling of the next amino acid allowed for aggregation of peptides, primarily through the formation of secondary structures, and adversely affected coupling. The Kent group showed that concomitant neutralization of the a-amino group and coupling of the next amino acid led to improved coupling.[6] Each of these improvements has helped SPPS become the robust technique that it is today.

BOP SPPS

The use of BOP reagent was first described by Castro et al. in 1975.

Solid supports

The name solid support implies that reactions are carried out on the surface of the support, but this is not the case. Reactions also occur within these particles, and thus the term "solid support" better describes the insolubility of the polymer. The physical properties of the solid support, and the applications to which it can be utilized, vary with the material from which the support is constructed, the amount of cross-linking, as well as the linker and handle being used. Most scientists in the field believe that supports should have the minimum amount of cross-linking to confer stability. This should result in a well-solvated system where solid-phase peptide synthesis can be carried out. Nonetheless, the characteristics of an efficient solid support include:[7]

It must be physically stable and permit the rapid filtration of liquids, such as excess reagents

It must be inert to all reagents and solvents used during SPPS

It must swell extensively in the solvents used to allow for penetration of the reagents

It must allow for the attachment of the first amino acid

There are four primary types of solid supports:[7]

Saarth
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Gel-type supports: These are highly solvated polymers with an equal distribution of functional groups. This type of support is the most common, and includes:

Polystyrene: Styrene cross-linked with 1–2% divinylbenzene

Polyacrylamide: A hydrophilic alternative to polystyrene

Polyethylene glycol (PEG): PEG-Polystyrene (PEG-PS) is more stable than polystyrene and spaces the site of synthesis from the polymer backbone

PEG-based supports: Composed of a PEG-polypropylene glycol network or PEG with polyamide or polystyrene

Surface-type supports: Many materials have been developed for surface functionalization, including controlled pore glass, cellulose fibers, and highly cross-linked polystyrene.

Composites: Gel-type polymers supported by rigid matrices.

Polystyrene resin

Polystyrene resin is a versatile resin and it is quite useful in multi-well, automated peptide synthesis, due to its minimal swelling in dichloromethane. The initial support used by R. Bruce Merrifield was polysytrene cross-linked with 2% divinylbenzene. This support is sometimes referred to as the 'Merrifield resin.' This produces a hydrophobic bead that is solvated by nonpolar solvent such as dichloromethane. Since then, new resins have been developed with the following advantages:

Enhanced swelling or rigidity (a property of mechanical strength)

Chemical inertness

Highly cross-linked (50%) polystyrene has been developed that possesses the features of increased mechanical stability, better filtration of reagents and solvents, and rapid reaction kinetics.

Polyamide resin

Polyamide resin is also a useful and versatile resin. It seems to swell much more than polystyrene, in which case it may not be suitable for some automated synthesizers, if the wells are too small.

Saarth
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PEG hybrid polystyrene resin

An example of this type of resin is the Tentagel resin. The base resin is polystyrene onto which is attached long chains (Mw ca. 3000 Da) of polyethylene glycol (PEG; also known as polyethylene oxide). Synthesis is carried out on the distal end of the PEG spacer making it suited for long and difficult peptides. In addition it is also attractive for the synthesis of combinatorial Peptide libraries and on resin screening experiments. It does not expand much during synthesis making it a preferred resin for robotic peptide synthesis.

PEG-based resin

ChemMatrix(R) is a new type of resin which is based on PEG that is crosslinked. ChemMatrix(R) has claimed a high chemical and thermal stability (is compatible with Microwave synthesis) and has shown higher degrees of swellings in acetonitrile, dichloromethane, DMF, N-methylpyrrolidone, TFA and water compared to the polystyrene-based resins. ChemMatrix has shown significant improvements to the synthesis of hydrophobic sequences. ChemMatrix may be useful for the synthesis of difficult and long peptides.

Improvements to solid supports used for peptide synthesis enhance their ability to withstand the repeated use of TFA during the deprotection step of SPPS.[8] Furthermore, different resins allow for different functional groups at the C-terminus. The oxymethylphenyl cetamidomethyl(PAM) resin results in the conventional C-terminal carboxylic acid. On the other hand, the paramethylbenzhydrylamine (pMBHA) resin yields a C-terminal amide, which is useful in mimicking the interior of a protein.

Along with the development of Fmoc SPPS, different resins have also been created to be removed by TFA. Similar to the Boc strategy, two primary resins are used, based on whether a C-terminal carboxylic acid or amide is desired. The Wang resin is the most commonly used resin for peptides with C-terminal carboxylic acids.[9] If a C-terminal amide is desired, the Rink amide resin is used.

Protecting groups

Amino acids have reactive moieties at the N- and C-termini, which facilitates amino acid coupling during synthesis. Many amino acids also

Saarth
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have reactive side chain functional groups, which can interact with free termini or other side chain groups during synthesis and peptide elongation and negatively influence yield and purity. To facilitate proper amino acid synthesis with minimal side chain reactivity, chemical groups have been developed to bind to specific amino acid functional groups and block, or protect, the functional group from nonspecific reactions. These protecting groups, while vast in nature, can be separated into three groups, as follows:

N-terminal protecting groups

C-terminal protecting groups (mostly used in liquid-phase synthesis)

Side chain protecting groups

Purified, individual amino acids are reacted with these protecting groups prior to synthesis and then selectively removed during specific steps of peptide synthesis.

N-terminal protecting groups

Amino acids are added in excess to ensure complete coupling during each synthesis step, and without N-terminal protection, polymerization of unprotected amino acids could occur, resulting in low peptide yield or synthesis failure. N-terminal protection requires an additional step of removing the protecting group, termed deportation, prior to the coupling step, creating a repeating design flow as follows:

Protecting group is removed from the trailing amino acids in a deportation reaction

Deportation reagents are washed away to provide a clean coupling environment

Protected amino acids dissolved in a solvent such as dimethylformamide (DMF) combined with coupling reagents are pumped through the synthesis column

Coupling reagents are washed away to provide clean deportation environment

Currently, two protecting groups (t-Boc, Fmoc) are commonly used in solid-phase peptide synthesis. Their liability is caused by the carbamate group which readily releases CO2 for an irreversible decoupling step.

Saarth
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t-Boc protecting group

The original method for the synthesis of proteins relied on (tert-butyloxycarbonyl or more simply "Boc") to temporarily protect the a-amino group. In this method, the Boc group is covalently bound to the amino group to suppress its nucleophilicity. The C-terminal amino acid is covalently linked to the resin through a linker. Next, the Boc group is removed with acid, such as trifluoroacetic acid (TFA). This forms a positively charged amino group (in the presence of excess TFA; note image on the right illustrates neutral amino group), which is neutralized (via in-situ or non-in-situ methods) and coupled to the incoming activated amino acid.[6] Reactions are driven to completion by the use of excess (two- to four-fold) activated amino acid. After each deprotection and coupling step, a wash with dimethylformamide (DMF) is performed to remove excess reagents, allowing for high yields (~99%) during each cycle.[7]

t-Boc protecting strategies retain usefulness in reducing peptide aggregation during synthesis. t-Boc groups can be added to amino acids with t-Boc anhydride and a suitable base. Some researchers prefer Boc SPPS for complex syntheses. In addition, when synthesizing nonnatural peptide analogs, which are base-sensitive (such as depsipeptides), the t-Boc protecting group is necessary, because Fmoc SPPS uses a base to deprotect the a-amino group.

Permanent side-chain protecting groups are typically benzyl or benzyl-based groups. Final removal of the peptide from the linkage occurs simultaneously with side-chain deportation with anhydrous hydrogen fluoride via hydrolytic cleavage. The final product is a fluoride salt which is relatively easy to solubilize. Importantly, scavengers such as cresol are added to the HF in order to prevent reactive t-butyl cations from generating undesired products. In fact, the use of harsh hydrogen fluoride may degrade some peptides, which was the premise for the development of a milder, base-labile method of SPPS—namely, the Fmoc method.

Fmoc protecting group

The capacity for anhydrous hydrogen fluoride to degrade proteins during the final cleavage conditions led to a new a-amino protecting group based on 9-fluorenylmethyloxycarbonyl (Fmoc). The Fmoc method

Saarth
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allows for a milder deportation scheme. This method utilizes a base, usually piperidine (20–50%) in DMF in order to remove the Fmoc group to expose the a-amino group for reaction with an incoming activated amino acid.[7] Unlike the acid used to deprotect the a-amino group in Boc methods, Fmoc SPPS uses a base, and thus the exposed amine is neutral. Therefore, no neutralization of the peptide-resin is required, but the lack of electrostatic repulsions between the peptides can lead to increased aggregation. Because the liberated fluorenyl group is a chromophore, deprotection by Fmoc can be monitored by UV absorbance of the runoff, a strategy which is employed in automated synthesizers.

The advantage of Fmoc is that it is cleaved under very mild basic conditions (e.g. piper dine), but stable under acidic conditions, although this has not always held true in certain synthetic sequences. This allows mild acid-labile protecting groups that are stable under basic conditions, such as Boc and benzyl groups, to be used on the side-chains of amino acid residues of the target peptide. This orthogonal protecting group strategy is common in organic synthesis. Fmoc is preferred over BOC due to ease of cleavage; however it is less atom-economical, as the fluorenyl group is much larger than the tert-butyl group. Accordingly, prices for Fmoc amino acids were high until the large-scale piloting of one of the first synthesized peptide drugs, enfuvirtide, began in the 1990s, when market demand adjusted the relative prices of the two sets of amino acids.

Semipermanent side chain protecting groups are t-butyl-based, and final cleavage of the protein from the resin and removal of permanent protecting groups is performed with TFA in the presence of scavengers. Water and triisopropylsilane (TIPS) present in a 1:1 ratio are often used as scavengers. Thus, the Fmoc method is orthogonal in two directions: deprotection of any a-amino group, deprotection of side groups and final cleavage from the resin occur by independent mechanisms. The resulting final product is a TFA salt, which is more difficult to solubilize than the fluoride salts generated in Boc SPPS. This method is thus milder than the Boc method because the deprotection/cleavage-from-resin steps occur with different conditions rather than with different reaction rates.

Saarth
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