Basic Concepts of using Solid Phase Synthesis to Build Small Organic Molecules using 2-Chlorotrityl Chloride Resin

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Abstract

Solid Phase Synthesis (SPS) is a chemical strategy that was developed and refined by Bruce Merrifield in the early 1960s and later led to a Nobel Prize in 1984. This discovery paved the way for chemists to be able to construct proteins with high yields, less time-consuming purification and much faster synthetic routes. The strategy utilizes an insoluble solid polystyrene cross-linked support resin, 2-chlorotrityl-chloride (2-CCR), to form an ester linkage with an acid so proteins or small molecules can be built from the N terminal one amino acid at a time. This same chemistry can be employed to construct peptidomimetics and small non-peptide molecules. This chemistry is especially useful for building molecules that require temporary protection of a carboxylic acid during the synthetic route. This article will provide the basic concepts and considerations when practicing SPS for small non-peptide molecule construction. Significant considerations in employing this chemistry include: resin selection, swelling of resin, coupling agents, solvents, mechanism, loading of resin, nucleophilic substitution, and cleavage from resin support, amine protecting groups, general reaction techniques as well as purification of final product.

Keywords: 2-Chlorotrityl chloride resin; Solid phase synthesis; Small molecules and solid phase; Solid phase techniques

Introduction

Peptide synthesis before the work of Bruce Merrifield was performed in solution and required time-consuming purification after each intermediate step. Attaching an Fmoc protected amino acid to an insoluble support, 2-CCR, allows for the temporary protection of the carboxylic acid while the molecule is coupled with additional building blocks. After the reaction is completed, the resin must be washed with excess solvents, allowing un-reacted building blocks and reaction by-products to be rinsed away. Synthesizing small non-peptide molecules using SPS is an efficient synthetic method with relatively short reaction and completion times in comparison to solution phase reactions [1-3]. This review is focused on using solid phase chemistry to build small peptidomimetic organic molecules. The techniques used in small molecule construction are different from the techniques used when synthesizing proteins (Scheme 1).

Helpful Techniques and Recommendations

A vortex apparatus is required to achieve the desired vibrational agitation necessary for complete coupling [1]. It is vortexed from 6-24 hours to drive reactions to completion. 2-CCR is a commonly used resin in solid phase synthesis for carboxylic acids. This resin is extremely moisture sensitive so it is highly recommended to store in desiccators under nitrogen gas. It is imperative that the resin acclimates to room temperature before opening and weighing desired resin. If this is not done then it will affect the ability of the resin to swell properly when solvent is added. When resin is removed from storage at low temperature it may take hours to reach room temperature so proper planning is required.

Glass and disposable peptide reaction vessels are commercially available. Glass peptide vessels are preferred when building small molecules because some of the solvents are corrosive to most disposable peptide vessels. Selecting the proper frit porosity for the vessel is critical because it is imperative to choose one that is smaller than the size of the resin bead to avoid getting trapped in the frit. For example, a 200-400 mesh was used with a C or D frit size to minimize any 2-CCR obstructing the frit. If after several reactions solvents are having difficulty filtering through the frit it is a sign that the frit needs cleaning [4]. The recommended procedure for cleaning a frit is to wash with trifluoroacetic acid (TFA), dichloromethane (DCM) and then double distilled water before being heated to 260°C (500°F) for 4 hours.

After the reaction vessel is removed from the oven, allow it to cool to room temperature before a final flushing of contaminants. For small molecule construction it is important that the glassware apparatus is dry and that only anhydrous solvents are used for reactions to ensure increased yields.

Resin Selection

Most commonly used resins are insoluble polystyrene x-linked divinylbenzene (DVB) beads. Generally smaller beads have faster reactions because of increased surface area to volume ratios [1]. Certain resins have a linker attached to the bead that helps with reactivity and selectivity for specific functional groups. The degree of cross-linking affects the swelling of the respective bead. A 1% DVB cross-linked resin swells 2-4 times the original volume in DCM while 2% DVB cross-linked resin swells 4-6 times the original volume in DCM. An increase in loading capacity corresponds to an increase in swelling and smaller resin beads [1,5]. When resin is swollen, active linker sites hidden inside resin are exposed increasing the amount of reagent that can be added to the resin [1]. A 1% cross-linked resin is adequate for optimal swelling [6]. The number of cross linking is important with respect to the number of reaction sites available for loading. Based on the cross-linking, some beads swell more than others but the more DVB cross-linking the greater the reaction sites. For protein construction it is desired to have fewer cross-links because the protein is being synthesized inside the bead and steric hindrance may become an issue. For small molecule construction, it is less important because sterics are not likely to be a concern due to the overall size of the molecules [7].
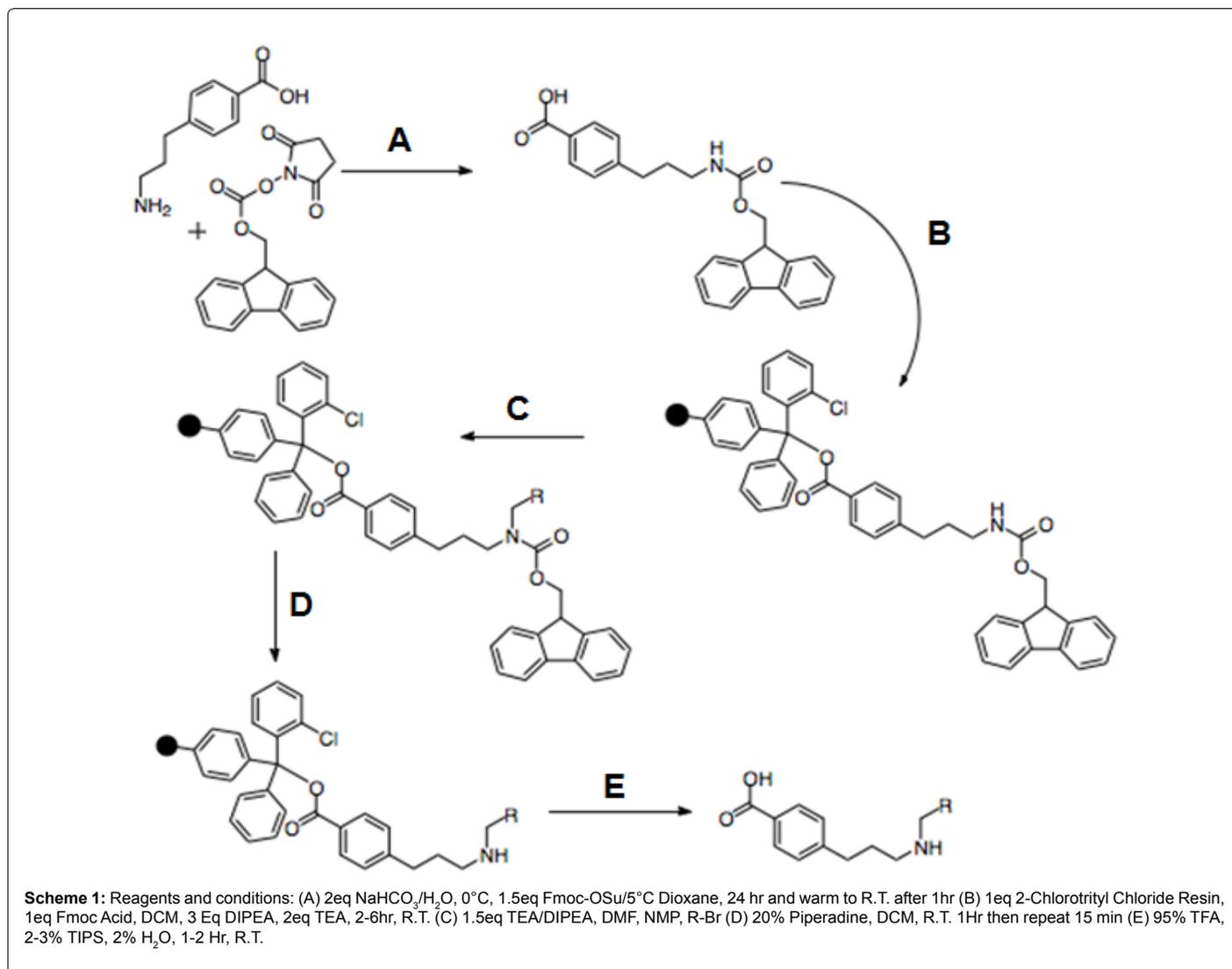
The selection of a particular insoluble resin is important as each resin has specific stereochemistry and affinities for different attachable functional groups [4]. Specific resins are cleaved in a variety of ways but

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Scheme 1: Reagents and conditions: (A) 2eq NaHCO$_3$/H$_2$O, 0°C, 1.5eq Fmoc-OSu/5°C Dioxane, 24 hr and warm to R.T. after 1hr (B) 1eq 2-Chlorotrityl Chloride Resin, 1eq Fmoc Acid, DCM, 3 Eq DIPEA, 2eq TEA, 2-6hr, R.T. (C) 1.5eq TEA/DIPEA, DMF, NMP, R-Br (D) 20% Piperadine, DCM, R.T. 1Hr then repeat 15 min (E) 95% TFA, 2-3% TIPS, 2% H$_2$O, 1-2 Hr, R.T.

if the chemistry requires use of bases for coupling then an acid labile resin is desirable [8-10]. This way it is assured the resin continues to protect the carboxylic acid during the construction of the molecule without fear that the resin will be removed from the acid in the process. 2-CCR is useful when the desired molecule requires temporary protection of a carboxylic acid [11].

A consideration when selecting which mesh size of 2-CCR to use is the porosity of the frit in the reaction vessel. Mesh size is inversely related to the percent DVB cross-links which allows for the number of reaction sites. For the 2-CCR there is only a 100-200 or a 200-400 mesh selection commercially available. The 100-200-mesh resin will be larger in size but will also have fewer reaction sites available. This is recommended for peptide synthesis but will require more resin because there are fewer reaction sites. The 200-400 mesh is smaller, has greater reaction sites and is recommended for small molecule construction. A factor mesh size plays is in reaction time. For the 200-400 mesh the mmol per gram for loading is larger so this allows for greater loading and less resin will be required. A higher mesh size corresponds to a faster reaction time because of increased surface area [6]. Resins swell in solvents of low to medium polarity such as dimethylformamide (DMF) or DCM, but do not swell well in protic solvents such as alcohol or water [6]. If the resin does not swell properly the linker sites buried in the resin will not be exposed decreasing the amount of carboxylic acid that can be loaded. To maximize the number of carboxylic acid substrate loaded to resin, the 2-CCR must be swollen 30 minutes in DCM prior to initiating coupling of the first amino acid. Prior to coupling the building block may require addition of DMF or N-Methyl-2-pyrrolidone(NMP) to help with solubility because it may be insoluble in DCM.

There are different techniques for loading an amino acid to the resin. The following is a method used for 2-CCR. First, dissolve and add one equivalent of Fmoc protected amino acid in DCM, (10 ml/g of resin), 3 equivalents of N,N-Diisopropylethylamine (DIPEA) and 2 equivalents of triethylamine (TEA) to the reaction vessel and let react for 5 minutes. After 5 minutes add an additional 1.5 equivalent of DIPEA. Next, add this mixture collectively to the pre-swollen resin and vortex for 1-2 hours [12]. After the amino acid is loaded to the resin the un-reacted sites must be end-capped with HPLC grade methanol (MeOH) (0.8mL per gram of resin) immediately to ensure that future reactions do not react at those unloaded sites. If methanol capping is not done the next amino acid will have the ability to add to a free reaction site on the resin and this will cause a mixture of final products. The process of methanol capping is fairly simple and involves a mixture of DCM, MeOH, and DIPEA in a 80:15:5 ratio (10 mL/g resin) added to the resin [1]. DCM

is added because MeOH will not properly swell the resin and will not expose all of the linker sites [1,13]. After the MeOH is used to end-cap the resin (15 minutes), the resin must be washed to remove any excess MeOH and DIPEA before being re-swollen and coupled with the next building block.

Amine Protection

Amino acid building blocks that have both a carboxylic acid as well as a primary amine require the amine group be protected prior to loading to the resin. This is important because addition of asterically-hindered protecting group, such as Fmoc, to the nitrogen will decrease the likelihood of the amine attaching to the resin. The two most commonly used amine-protecting groups are 9-fluorenylmethyloxy carbonyl (Fmoc) or t-butyloxycarbonyl (Boc). The N-(9-fluorenylmethoxy carbonyloxy)succinimide (Fmoc-OSu) is preferred over the fluoroenylmethyloxycarbonyl chloride (Fmoc-Cl) as the yields increase due to the enhanced stability of a leaving group in Fmoc-OSu.

A common procedure for attaching an Fmoc to an amine is to first dissolve the amino acid in water before the addition of 2 equivalents of sodium bicarbonate and then stir in an ice bath at 0°C (32°F). In a separate beaker dissolve 1.5 equivalents of Fmoc-OSu or Fmoc-Cl in cooled p-dioxane before slowly adding to the deprotonated amino acid solution over one hour. The reaction should be vigorously stirred and warmed to room temperature overnight. If using Fmoc-OSu, the addition of some acetone may be needed, due to lower solubility of the Fmoc in p-dioxane [14]. To extract the Fmoc-amino acid, double distilled purified water is added to the beaker and then extracted twice with ethyl acetate (EtOAc). Treat the organic layer with a saturated sodium bicarbonate solution twice. Next, combine the aqueous layers and then acidify with concentrated HCl to a pH of 1-2 and then extract the organic layer with EtOAc three times. Combine all organic layers and dry over magnesium sulfate. Using chromatography the Fmoc-amino acid can then be purified with silica gel chromatography [5,15]. The Fmoc is base-sensitive so this group can easily be cleaved using 20% piperidine in DMF (10 mL/g resin) and gently vortex for 30 minutes [16]. This step is usually repeated twice with the second duration only lasting 15 minutes to ensure full Fmoc cleavage. After cleavage the resin is washed with DCM, DMF and NMP twice before a final wash with DCM and MeOH. Fmoc removal can be monitored via UV spectroscopy to ensure completion [5,15].

The Boc amine protection group is acid labile and there may be times where the Boc serves as a more appropriateamine-protecting group in comparison to Fmoc [17]. Specifically, when the addition of Boc is used to protect an amine attached to a base labile resin. First dissolve the primary amine in enough MeOH to make it soluble and then add 2.5 eq of triethylamine (TEA). The reaction should be allowed to stir for 10 minutes before heating to 55°C for 30 minutes. Slowly 1.6 eq of Di-tert-butyl dicarbonate (Boc$_2$O) was added and the reaction was allowed to go for 16 hours. Boc is acid labile and vulnerable to acid cleavage. A common procedure used to cleave the Boc off the amine while on a solid phase resin is to add a solution of 1:1 TFA in DCM (10 mL/1g resin) and vortex at room temperature for one hour [8,9]. The amine will form a TFA salt and must be washed with 3 sequential steps of 1:1 DCM and DIPEA (10 mL/1 g resin) to prepare the deprotected amine product for the next addition step. If using 2-chlorotrityl-chloride resin this will also cleave it form the resin as well as the amine. It is recommended to use a base labile resin if one needs to remove the Boc for further alkylation prior to deprotection from a resin.

Basic Techniques

Solvent washing

In SPS the resin is washed and filtered from reaction byproducts and is considered the purification step. Excess solvents are filtered through the resin in an ordered sequence of DCM (10 mL/g resin), DMF (5 mL/g resin), NMP (1 mL/g resin) and MeOH (1 mL/g resin) three times [18]. It may help to use a steel scoopula to agitate the mixture as the vacuum is applied to wash the resin. After the wash is completed the resin is washed one final time with DCM (10 mL per gram resin) and MeOH (1 mL/g resin) and dried under vacuum. MeOH helps to decrease the size of the resin bead and aids with evaporation to dry the resin prior to the next step.The resin will need to be re-swollen in DCM before any further coupling reactions proceed [1].

Nucleophilic Substitution

Since solid phase is not being used in this case to create peptides but rather peptidomimetic organic molecules, different methods are practiced when using this chemistry. Once the carboxylic acid molecule is attached to the resin and the amine is N-protected then S$_N$2 displacement can help assist with building the molecule. It is imperative that all glassware and solvents are dry and anhydrous to ensure greater yields. Bases such as DIPEA, TEA and sodium hydride (NaH) have been used withdeprotonation for various S$_N$2 displacement reactions. Due to the strength of sodium hydride base it is important to note that it has a potential to deprotect the Fmoc group. Sometimes this is done with the intention of generating a primary amine before reductive animation with an aldehyde while other times it does not cause any harm to the molecule because low concentrations of NaH are used.

Cleavage from the Resin

The cleavage cocktail used to remove the resin from the final compound may vary slightly but a common mixture uses 95% trifluoroacetic acid (TFA) with 2-3% triisopropylsilane (TIPS) and 2% water. One important consideration when selecting the cleavage cocktail is whether to add a scavenger, such as TIPS [17]. The reason scavengers are often added is because during the course of cleavage, highly reactive cationic species can be generated that can cause damage to the structure. The purpose of a scavenger is to quench any reactive species that may be generated during exposure from TFA cleavage. Other scavengers that can be used besides TIPS are thioanisole or ethanedithiol (EDT) [18].

Detectors

Ninhydrin is a useful chemical to detect primary or secondary amines. Primary amines are the most sensitive to ninhydrin and a strong blue color will immediately result if present [18]. The secondary amine is less sensitive to ninhydrin so a less intense brown-red color will be displayed indicatingpresence of a secondary amine [19]. To facilitate this test with solid phase, place a small sample of resin on TLC paper and then spray with ninhydrin before gently heating with a heat gun. The ninhydrin test is useful in solid phase synthesis to confirm successful addition of the amino acid to the N-terminal. However, bromocresol and malachite green are other chemical detectors that can be used to detect carboxylic acids. Both of these detectors will transition from green to yellow if a carboxylic acid is present [20].

Purification

Purification of final product should first be initiated by lyophilization prior to Reverse Phase Chromatography (RPC). Compounds that have greater differences in their R$_f$ values will be easier to purify but C18

functionalized silica is required for optimal purification. RPC takes advantage of hydrophobic interactions between the stationary phase and the mobile phase for purification. Polar impurities elute faster than the hydrophobic ones. Therefore, reverse phase chromatography, combined with appropriate mobile phase polar to non-polar ratio, is most useful to desalt the compound and helppurify enantiomers. Hydrophobic molecules will elute from the column slower than hydrophilic ones so appropriate buffer solutions may need to be adjusted to extract those compounds from the functionalized silica column. A greater organic to polar solvent buffer solution may need to be used. The ability of RPC to tolerate both isocratic solvent systems and gradient solvent systems is important in purification of compounds synthesized via SPS due to presence of small amount of mostly polar impurities [21,22]. Typical solvents used in reverse phase purification processes are aqueous acetonitrile or methanol solutions.

Conclusion

Solid phase chemistry has advantages with protein construction, but it also is a valuable tool for the construction of small non-peptide molecules or peptidomimetics. This type of chemistry allows the chemist to accomplish their synthesis much more quickly compared to solution phase chemistry, which requires long purification methods after each intermediate step. Understanding the basic techniques discussed in this review should allowchemists to synthesize molecules with this method. Once the reaction is completed, reaction grade or ACS solvents can be used for washing the resin. The most important steps in the synthesis are the loading as well as cleaving from the resin. If poor yields are attained it is likely due to an error in either of those two steps so they should be investigated first. Numerous chemicals, apparatus and analytical techniques are used to confirm products between reaction steps. At any intermediate step throughout the synthetic procedure a small sample of resin may be cleaved from insoluble support so that NMR analysis can be conducted. Commonly used practices use chemical detectors, spectrophotometric analysis, IR, NMR and mass increases to help further clarify if reactions were successful. Once the molecule is removed from the resin and purified via HPLC the molecule will be fully analyzed by NMR to confirm the exact structure. Solid phase chemistry can be used as another tool to enhance small molecule construction.

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