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Building Peptidomimetics using Solid Phase Synthesis with 2-Chlorotrityl Chloride Resin

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ABSTRACT

Solid phase synthesis is a synthetic method commonly used for the construction of peptides. This approach is also useful with the synthesis of peptide-like organic molecules. There are numerous ways to synthetically construct peptidomimetic molecules, but solid phase synthesis provides an efficient way, over more traditional chemistry, when time for long synthesis is not available and smaller yields are desired for analysis. Many factors must be considered before constructing peptidomimetics on a 2-chlorotrityl polystyrene resin. The greatest advantage of this approach is the fact that it does not require lengthy purification columns after each step. After coupling the resin beads are washed with excess solvent, concentrated down and are then ready for the next coupling reaction. This approach can expedite the synthetic process and there are many techniques and recommendations to optimize the best yields. Unlike peptide synthesis, it is imperative to use anhydrous solvents in the synthetic construction. This review article will discuss the techniques and recommendations when using 2-chlorotrityl chloride resin to construct peptidomimetics when the C-terminus is a carboxylic acid.

1. Introduction

Peptidomimetic molecules provide a unique landscape to drug discovery and the increasing number of drug targets and therapeutic benefits to justify further investigation of this promising class of drugs. While solid phase synthesis has many advantages, including its utility for the synthesis of proteins, it is also very useful for constructing small organic molecules that mimic peptides. Traditional synthetic methods often employ various groups, such as trisilylchloride, to protect a carboxylic acid. This allows the acid to remain un-reactive while the rest of the molecule is constructed. The advantage of the latter group is higher yields and easier analysis of intermediates but it also has the disadvantage of lengthier purification methods. Solid phase synthesis instead anchors the C-terminus to an insoluble resin before successive couplings build the molecule. Using simple filtration, the resin is excessively washed with solvents after each coupling to elute by-products, excess reagents, soluble by-products, and solvents.

Bruce Merrifield is credited with the discovery and advancement of solid phase chemistry that is still used today [1]. The main advantage of using this chemistry to synthesize peptidomimetic molecules is that the construction of the molecule is faster and more efficient, due to the lack of lengthy purification steps after each reaction step. The polystyrene beads are selective and the purification only requires the beads to be washed after each step. 2-Chlorotrityl chloride resin (2-CTC) has selectivity for carboxylic acids and molecules are essentially built on the polystyrene resin one step at a time, similar to proteins [2, 3]. After the molecule is fully built, it is then clipped from the resin bead. Both solid phase and traditional methods yield the same end product, but solid phase synthesis is faster and more efficient for the construction of peptidomimetics. Synthetic strategies, advantages, disadvantages, yields, purification and effectiveness will be discussed in this review.

2. Experimental Methods

There are various strategies used to ensure resin coupling and there are many factors that must be considered prior to synthesis of molecules. Solid phase coupling requires proper agitation and for very small samples nitrogen gas may be funneled through a peptide vessel for 60 minutes to ensure complete coupling. For larger samples, the agitation required is achieved primarily through a vortex or shaker for 12-14 hours. It is important to note that 2-chlrotrityl chloride resin is highly hygroscopic and must be stored and handled under inert conditions and at a low temperatures [4]. Proper planning is required because the resin beads must be allowed to acclimate to room temperature for 1-2 hours prior to reaction setup. If the beads are not allowed to acclimate, then the resin beads will not swell properly and the reaction sites will not be properly exposed.

For manual synthesis, glass peptide vessels are preferred over plastic for synthesizing peptidomimetic organic molecules because of the corrosive nature of the solvents used in the peptidomimetic synthetic process. This ensures fewer byproducts and easier resin washes. Peptide vessels with glass frit are used for filtering and washing resin to limit subsequent transfer to another apparatus. Resin beads are washed to remove by-products and excess reagents. The porosity of the frit is an important consideration that must be considered, due to the availability of vessels come in various frit sizes. If the proper mesh size of the resin bead is not paired with the appropriate frit porosity, then the resin will get trapped in the frit during washes. If after several reactions solvents are not being properly filtered, this is a sign that the frit needs cleaning before further use of the vessel. The recommended procedure for cleaning the frit to dislodge contaminants is to wash the vessel with trifluoroacetic acid (TFA) and distilled water before heating the peptide vessel to 260 degrees Celsius for 5-6 hours. Allow the reaction vessel to cool to room temperature before a final flush with TFA through the frit to dislodge contaminants.

Mesh size determines the physical size of the resin beads upon swelling, i.e. the higher mesh has a smaller bead size [5, 6]. It is inversely related to the percent divinylbenzene (DVB) cross-links which allow for the reaction sites. 1-2% DVB as a cross-linking agent is commonly seen with resins. Fewer crosslinks (i.e. 1% DVB) swell 4-6 times its original volume in comparison to a 2% DVB that will swell only 2-4 times its original volume. A 1% DVB cross-linked resin offers adequate properties for stability and swelling properties so this has largely replaced the 2% DVB 2-CTC beads. Resins swell in solvents of low to medium polarity such as dimethylformamide (DMF), methylene chloride (DCM) or *N*-methyl pyrrolidone (NMP), and don't swell in protic solvents such as alcohol or water. When solubility is an issue, NMP is preferred over DCM. Swelling is important to reaction kinetics and is diffusion controlled. A more crosslinked resin (i.e. 2% DVB) will not swell as much and have a slower diffusion rate and longer reaction times, as compared to the 1% DVB resin.

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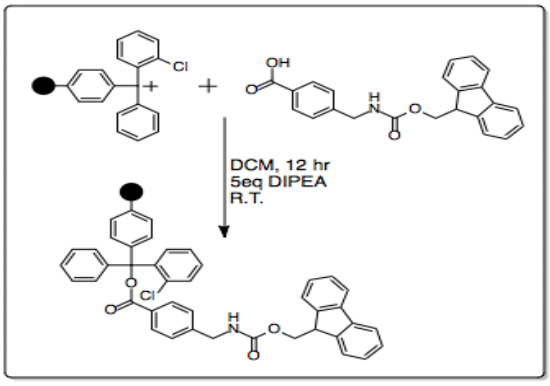
Reaction kinetics are faster with smaller beads due to the higher surface area to volume ratio. The 2-chlorotrityl chloride resin, (2-CTC), is commercially available in either a 100-200 or 200-400 mesh size. The 200-400 mesh is smaller (37-75 μm) compared to the 100-200 (75-150 μm). For peptidomimetic synthesis the larger mesh size was selected to decrease the likelihood of resin getting trapped in the frit, but it has the disadvantage of having fewer reaction sites available, and more resin will be required to optimize yields.

Proper resin swelling is important due to the fact that if the linker sites buried in the resin are not properly exposed during this period then the loading will be significantly decreased. For peptidomimetic synthesis it is important to use anhydrous solvents and the 2-CTC resin be allowed to swell for 30-40 minutes prior to coupling. For C-terminus carboxylic acid protection, 2-CTC is the ideal resin due to its selectivity for this functional group. It has an insoluble polystyrene X-linked divinylbenze core, is acid labile and is easy to work with. The more cross-linked the resin, the more reaction sites will be available. A 1% DVB cross-linked resin is adequate for peptidomimic synthesis. This ensures that active linker sites on the bead are exposed for loading. Steric hindrance of a molecule needs to be considered prior to choosing which degree of crosslinked resin is appropriate to use.

For peptidomimetic construction it is essential to maintain anhydrous conditions for optimal desired yields. This is not as important for protein construction. After the acid is coupled to the 2-CTC resin it must be ensured that the unreacted carbocation resin sites be covalently-methyl capped [7]. This is easily achieved by vortexing the resin with 2 equivalents of methanol, 1 equivalent of DIPEA and 20 equivalents of methylene chloride for one hour before washing with methylene chloride and DMF.

2.1 Amine Protection

Regardless whether peptidomimetics or peptides are being constructed, the amino end will first need to be protected prior to coupling to the resin. A common protecting group used for the amino end is either N-(9-fluorenylmethoxy carbonyloxy) succinimide (Fmoc) or t-butyloxycarbonyl (Boc) [8, 9]. The protecting group introduces a sterically hindered functionality to the amine that will increase the likelihood of preferential binding at the carboxy end. The biggest difference between the two is that the Boc protecting group is base sensitive whereas the Fmoc is stable under basic conditions. Depending on the coupling conditions the use of one usually has greater advantage over the other. For peptidomimetic construction Fmoc is used due to the requirement of basic coupling conditions (Scheme 1). 2-CTC resin also has the advantage of being stable in basic conditions since cleavage is only achieved in highly acidic conditions [10].

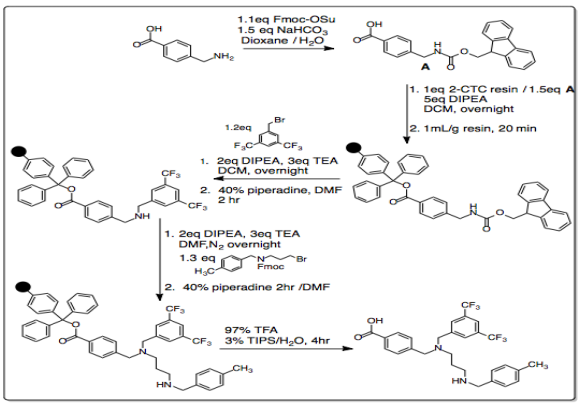


Scheme 1 Coupling to solid polystyrene 2-chlorotridyl chloride resin (2-CTC)

2.2 Cleavage from Resin and Analysis

There are numerous solvent cocktails used to cleave and expose the carboxy terminus from the 2-CTC resin bead but a common method of deprotection involves using a strong acid, such as trifluoroacetic acid (TFA). A 95% TFA, 2-3% triisopropylsilane (TIPS) and 2% water cocktail for 2-4 hours at room temperature is effective at clipping the 2-CTC resin bead from the peptidomimetic molecule. TIPS is used as a free radical scavenger because during the course of cleavage highly reactive cationic species can be generated and they have the ability to cause damage to the structure [11, 12]. Scavengers quench any reactive species that may be generated during exposure from this strong acid cleavage. Thioanisole or ethanedithiol are other scavengers that may also be used for this same purpose.

The Kaiser test is useful for the detection of amines [13]. Ninhydrin as an amine detector is useful in identifying primary or secondary amines. Primary amines are very sensitive to ninhydrin and a strong blue color will immediately result after heating a plate. A secondary amine is less sensitive to ninhydrin and a brown-red color will result. Bromocresol and malachite green are other chemical detectors that may be useful in the detection of a carboxylic acid and a green to yellow color will result.



Scheme 2 Solid phase synthetic route for construction of peptidomimetics

2.3 Nucleophilic Coupling

Anhydrous solvents are essential and overall yields will be severely decreased should these conditions not be maintained. Once the carboxylic acid is coupled to the resin the molecules are essentially built from the amine end, similar to protein construction. Bases such as DIPEA or TEA are recommended over stronger bases, such as sodium hydride, for deprotonating due to the Fmoc-protected amine. Stronger bases can cleave the Fmoc from the amine, leading to bis-alkylated by-products, and decreasing the desired yields of product.

2.4 Purification

Purification of intermediates with solid phase synthesis involves washing the resin beads with excess DCM and DMF instead of lengthy column chromatography required for more traditional chemical synthesis routes. After coupling is complete the 2-CTC resin is washed three times with DCM and DMF before drying and analyzing. The next coupling reaction will then be ready. Small samples may be clipped from the 2-CTC resin periodically and analyzed via NMR and mass spectrometry to characterize intermediates.

Once the final compound is released from the 2-CTC resin bead the product is concentrated down and lyophilized prior to reverse phase chromatography with HPLC. All final compounds are analyzed via mass spectroscopy and NMR before any in vitro analysis.

2.5 Chemistry

For peptidomimetic construction a 100-200 mesh size of 2-CTC resin is usually selected. Both 100-200 and 200-400 mesh sizes have been used but the smaller beads typically are lodged in the frit leading to lower yields. There are fewer reaction sites available with the 100-200 2-CTC mesh, which requires more resin [14].

Para amino benzoic acid is first Fmoc protected using 1.1 eq of Fmoc succinimide, 1.5 eq of $NaHCO_3$ in Dioxane/water overnight. The reaction is extracted with 15 mL of ethyl ether twice before the aqueous layer is adjusted to pH 2. [15, 9] The aqueous layer is then back extracted with ethyl acetate three times before the combined organic layers are combined, dried with magnesium sulfate, filtered and concentrated down for analysis. Fmoc is easily and completely deprotected using 40% piperadine in DMF for 2 hours.

Resin beads must be acclimated to room temperature for 1-2 hours before the initial coupling of A (Scheme 2) to the 2-CTC resin [16]. A 1.0 eq sample of resin, 1.5 eq of Fmoc acid, A, 5 eq of DIPEA in DCM are vortexed for 12 hours for complete coupling. Once completed the reaction is drained and washed with excess DCM, DMF and MeOH. The resin beads can then be prepared for the next coupling reaction and analysis and confirmation of complete coupling.

For the nucleophilic coupling reactions, a mixture of 1 eq resin, 2 eq DIPEA, 3 eq TEA in DCM is allowed to vortex for 12 hours. They were then washed 3 times with DCM and DMF before drying and analysis. IR, NMR and Mass spectroscopy analysis may be completed for confirmation of products.

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3. Conclusion

In conclusion it is advantageous to synthesize peptidomimetic molecules using solid phase synthesis routes instead of more traditional chemistry methods. There are advantages and disadvantages of solid phase construction, but its main advantage is elimination of lengthy purification steps after each coupling reaction. Though synthetic time to construct molecules is shortened, overall yields are much lower as compared to more traditional chemistry methods. Employing the practice of these various techniques discussed in this review can ensure higher yields and greater success when building peptidomimetics.

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