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A Discovery-Oriented Approach to Solid-Phase Peptide Synthesis

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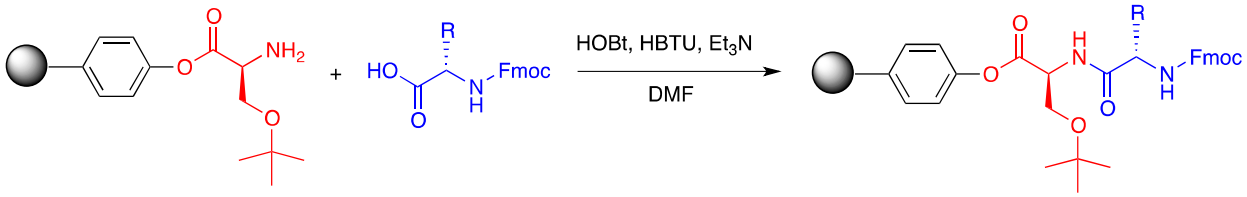
In this discovery-oriented laboratory experiment, students use solid-phase synthesis techniques to construct a dipeptide containing an unknown amino acid. Following synthesis and cleavage from the polymeric support, electrospray ionization mass spectrometry is employed in order to identify the unknown amino acid that was used in the peptide coupling. This experiment, performed in two three-hour laboratory periods, provides an opportunity for students to learn the technique of solid-phase peptide synthesis and to critically evaluate data that they generate in the laboratory.



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Laboratory Experiment

Solid-phase chemistry was first described in the chemical literature nearly 50 years ago and has revolutionized organic synthesis.[1] It is commonly used for the synthesis of peptides, oligonucleotides, carbohydrates, and a large variety of other organic compounds.[2-5] In solid-phase synthesis, the starting material is covalently linked to a solid, polymeric support and converted to the product through a series of step-wise chemical reactions. Intermediate products throughout the multi-step synthesis are attached to a physical handle (the polymer). In order to ensure that each individual reaction goes to completion, excess reagents are used. The use of a polymeric support allows for facile removal of unused reagents and obviates the need for column purification throughout the course of the synthesis. Once the product is synthesized on the polymeric support, it is cleaved from the resin and recovered from solution.

One of the most common applications of solid-phase chemistry is the synthesis of polypeptides.[2] The iterative fashion in which peptides are synthesized from single amino acids, the commercial availability of necessary reagents, and the high yielding nature of the couplings all make peptide synthesis ideally suited for this approach. While there are other synthetic strategies available for solid-phase peptide synthesis (SPPS), the Fmoc approach has proven the most versatile.[2, 6] In this method, the N-terminus of each amino acid to be incorporated into the growing peptide chain is protected by the base labile 9-fluorenylmethoxycarbonyl (Fmoc) group (Figure 1A). As a result, once the desired amino acid is attached, further coupling is prevented. Following formation of the peptide bond, the Fmoc group is removed under mildly basic conditions so that the next amino acid can be incorporated. Because several amino acid side chains would interfere with constructing the peptide, they too are protected. However, these side chains are protected with acid-labile groups that are only removed upon completion of the synthesis. Through a series of coupling reactions followed by deprotection of the Fmoc group, the peptide is constructed (Figure 1B). Once the full-length peptide has been synthesized, it can be cleaved from the resin and fully deprotected with trifluoroacetic acid.

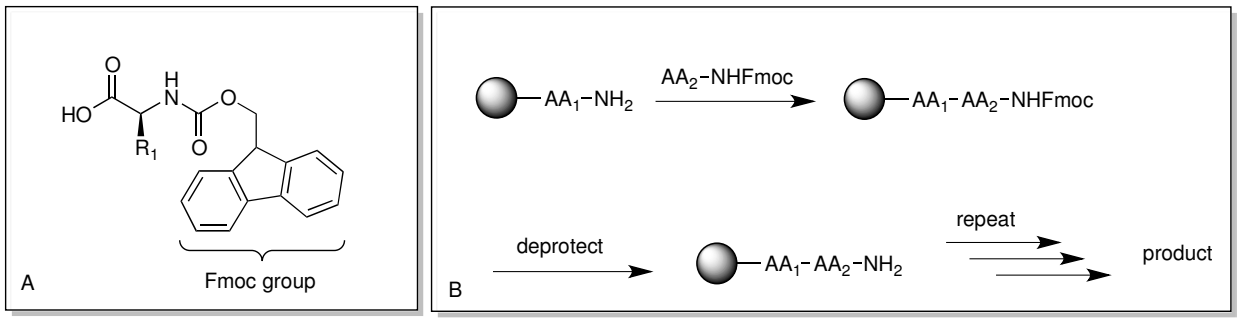
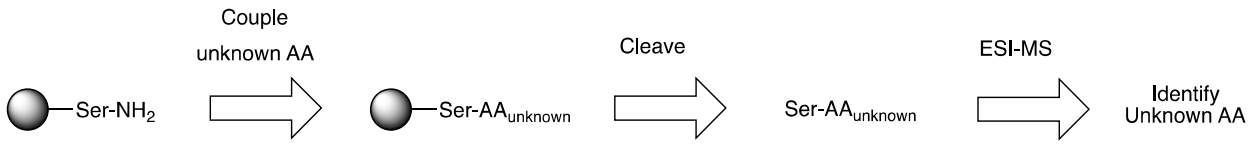


Figure 1: (A) An Fmoc protected L-amino acid (B) General procedure for constructing a peptide with solid phase synthesis. AA represents an amino acid.

Despite the importance of SPPS in the field of chemistry, few undergraduate students gain experience with this technique throughout their chemistry education. Although there have been some experiments reported, expanding the repertoire of available laboratory experiments will certainly help introduce more students to this exciting technique.[7-10] A two-week discovery-oriented laboratory experiment is described in which students synthesized a dipeptide with one unknown amino acid, cleaved it from a solid-support, and determined the structure of the resulting product by electrospray-ionization mass spectrometry (ESI-MS) (Scheme 1). This

approach illustrates several important synthetic concepts such as (i) solid phase synthesis, (ii) protection-deprotection concepts, and (iii) mass spectrometry. The goal of this experiment is not only to expose students to the technique of SPPS, but also to go a step further and ask them to apply their knowledge of mass spectrometry in order to determine the identity of the unknown that they used for the synthesis. The discovery-oriented approach, in which students critically evaluate data generated in the laboratory, sets this experiment apart from others described in this Journal.[7-10]

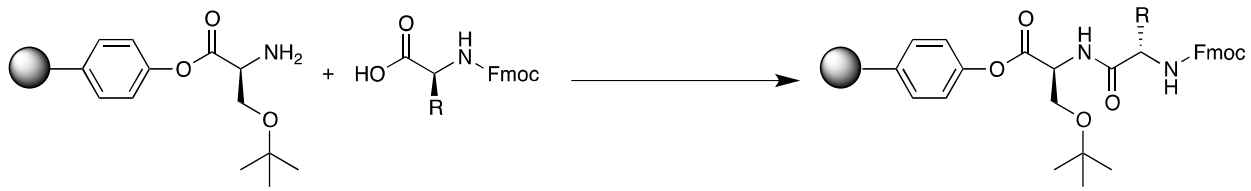


Scheme 1: Overview of experimental procedure.

The experiment described is suitable for second semester organic chemistry students as they learn methods for the formation of amide bonds or upper level biochemistry students as they learn about the primary structures of proteins. Students were required to apply basic concepts of electrospray ionization mass spectrometry as they analyzed the subtle differences between various amino acid unknowns. In addition to determining the structure of their product, students were asked the following questions as they analyzed their spectra. (i) Are there any amino acids that cannot be distinguished with this method? (ii) How would the data differ if the mass spectrometry were performed under basic conditions? (iii) If you were not told the identity of the first amino acid, would you be able to unambiguously determine the identity of the second?

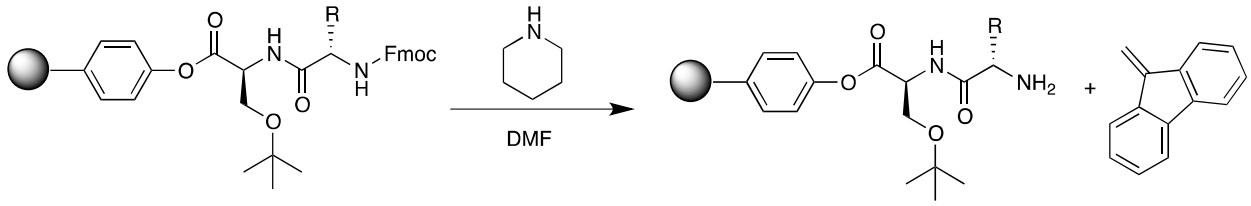
Experimental Overview:

The experiment was performed over two three-hour laboratory periods. Each group of students was provided a solid-phase reaction vessel containing 50 mg of pre-swelled CLEAR™ Acid resin (Cross-Linked Ethoxylate Acrylate Resin) with the first amino acid attached (Serine). Additionally, each group was assigned an unknown amino acid to couple to the serine residue that was already in place. In order for the reaction to proceed, the unknown amino acid was converted to an HOBt ester. This conversion was achieved by treatment of the amino acid with a solution of HBTU (O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate), HOBt (Hydroxybenzotriazole), and Et_3N in DMF (N,N-Dimethylformamide). This "coupling solution" was prepared in advance and used throughout the week for five laboratory sections that performed the experiment. Once the unknown amino acid was dissolved in the coupling solution, it was combined with the resin in the solid-phase reaction vessel and allowed to gently shake for 30 minutes (Scheme 2).



Scheme 2: Initial coupling step performed.
In order to monitor the progress of reactions in SPPS, the disappearance of the amino group is often tracked via a ninhydrin test.[2] If the coupling goes to completion, the primary amine in the starting material would be absent in the product, resulting in a negative ninhydrin test. However, due to the toxic and corrosive nature of the material required for the ninhydrin test, the reaction progress was not tested at this stage.

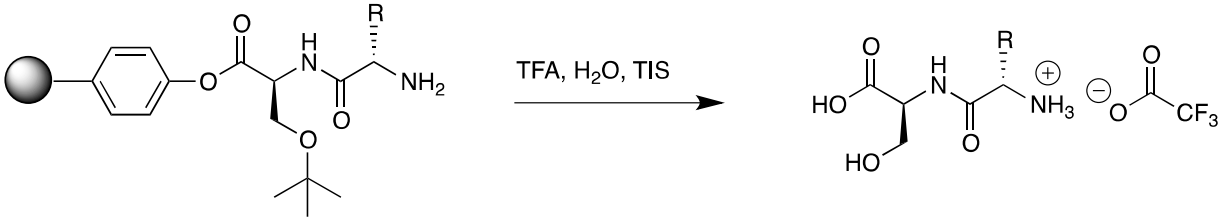
Once the amino acid was coupled to the resin, the base labile Fmoc group was removed by treatment with a solution of 20% piperidine in DMF (v/v) for 5 minutes (Scheme 3).



Scheme 3: Deprotection of the terminal Fmoc group with piperidine liberates the highly UV-active 9-methylene-fluorene.

At this point, the students tested to confirm that the previous coupling reaction was successful by spotting a small amount of the solution in the reaction vessel onto a Silica plate. The deprotection of the Fmoc group liberated a highly UV-active adduct which was easily observable under UV light. Students also placed a sample of the piperidine solution on the plate for comparison. Because the UV active adduct could only have come from the successful coupling of the amino acid in the previous step, this served as a check of the reaction without having to use the harsh chemicals of the ninhydrin test. While this did not ensure that the coupling in the previous step went to *completion*, it did provide evidence that some of the unknown amino acid had formed an amide bond to the serine. However, in the development of this laboratory procedure, numerous ninhydrin tests were performed to ensure completion in 30 minutes and this was found to be successful in all cases.

In the final stage of the synthesis, students cleaved the peptide from the resin by treatment with 0.5 mL of a solution containing 95% CF_3COOH (TFA), 2.5% H_2O, and 2.5% triisopropylsilane (TIS) for 1 h. The "cleavage cocktail" served to both remove the peptide from the solid support and to remove protecting groups present on the side chains of serine and the unknown amino acids (Scheme 4). In order to prepare students for the mass spectral analysis that they performed, each group was assigned a mass spectrometry worksheet to be completed during the one-hour cleavage reaction (see Supporting Information).



Scheme 4: Treatment of the peptide with trifluoroacetic acid results in its cleavage from the resin and removal of all side chain protecting groups.

Once the peptide was cleaved from the solid support and removed from the reaction vessel, it was recovered from the TFA solution by precipitation with 10 mL of cold diethyl ether followed by centrifugation. The pellet was washed with diethyl ether in order to remove trace TFA, allowed to dry in a fume hood, and dissolved in methanol for mass spectral analysis.

Electrospray ionization mass spectrometry analysis of the samples was performed by the instructor with an Agilent Series 1100 MSD in positive ion mode. The samples were injected without the use of a column. The solvent used for injection was 50% acetonitrile containing 0.1% formic acid and 50% water containing 0.1% formic acid and the detector was set to collect from 150 to 300 m/z.

Hazards

Goggles and gloves should be worn at all times in the laboratory. This experiment involves the use of strong acids and corrosive reagents. Diethyl ether is extremely flammable and should only be used in a fume hood. No open flames should be present during the course of this experiment. All students are required to perform a risk assessment by looking up the MSDS for all chemicals used throughout the experiment. While there is minimal waste produced, all chemicals should be disposed of in proper containers. Ether/TFA waste should be collected in a separate container from the DMF waste and no chemicals should be disposed down the drain. Liquid and vapor trifluoroacetic acid can cause severe burns and extreme caution should be taken with its use.

Typical Results

This experiment has been performed in sophomore organic chemistry with groups of two students where possible (60 students divided up into 32 groups over 5 days) and advanced biochemistry individually (9 students). In all cases, the expected mass was observed in the mass spectrum (representative student spectra can be found in Supporting Information). The correct unknown was identified by all 9 biochemistry students and by 27/32 organic groups. While the biochemistry students did not have any problems drawing the structure of the dipeptide product, the organic students often had difficulty. Many of the groups inadvertently drew the serine on the N-terminus and the unknown amino acid on the C-terminus.

The primary goal of the experiment, teaching students the technique of solid-phase peptide synthesis, was clearly achieved with all students obtaining a product with the correct mass. The second goal of the experiment involved critical evaluation of data generated in the laboratory. This too was largely achieved in that 36 out of 42 students/groups that performed the experiment correctly identified the unknown they were given. In the future, more emphasis will be placed on peptide structure when preparing the organic students for this laboratory experiment. Overall, however, the experiment was deemed a success in both contexts.

Associated Content

Supporting Information

Student handout, instructor notes, representative student data, mass spectrometry problem set, pre-lab assignment, and student report sheets. This material is available via the Internet at http://pubs.acs.org

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