Organic LETTERS

Letter

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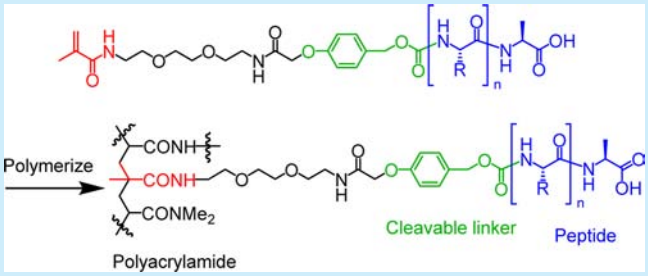
Purification of Synthetic Peptides Using a Catching Full-Length Sequence by Polymerization Approach

Mingcui Zhang, Durga Pokharel, and Shiyue Fang*

Department of Chemistry, Michigan Technological University, 1400 Townsend Drive, Houghton, Michigan 49931 United States

Supporting Information

ABSTRACT: During automated solid-phase peptide synthesis, failure sequences were capped with acetic anhydride. After synthesis, a polymerizable methacrylamide tag was attached to the full-length sequences. Peptide purification was then achieved by polymerizing the full-length sequences, washing away impurities, and cleaving the peptide product from the polymer.



Synthetic peptides have wide applications. For example, there are 65 therapeutic peptides on the market and around 270 peptides in clinical trials.[1] Peptides are mostly synthesized on a solid support by stepwise addition of amino-protected amino acid (aa) monomers. Each synthetic cycle accomplishes the addition of one aa. It usually consists of three steps, which are coupling, capping, and deprotection. Excess reagents and side products in each step are removed by washing. After synthesis, the product is cleaved from the support and fully deprotected. Besides the desired full-length peptide, the major impurities in the crude product include failure sequences and small molecules from side chain protecting groups. The latter can usually be removed by precipitation with cold diethyl ether from a trifluoroacetic acid (TFA) solution. However, the failure sequences have physical properties similar to those of the full-length peptide and cannot be removed in the process.

Currently, the first industrial choice for peptide purification is reversed-phase (RP) HPLC. However, it has limitations. For example, in the manufacture of enfuvirtide, which is a 36-aa peptide used to treat HIV infection, RP HPLC is the primary tool for purification. When a linear solid-phase synthesis method was used, the purity of the crude product was 30−40%, which is acceptable considering the length of the synthesis and the convenience of the procedure. Unfortunately, the product had to be purified by a complicated procedure involving difficult, low-concentration, low-throughput, and two-pass HPLC.[2] To ease purification, later a combined solid-phase and solution-phase synthesis method was developed, and the purity of product was improved to ∼75%. However, the purification was not much easier. The product was loaded in multiple portions onto a column for preparative HPLC.[2] In general, the disadvantages of HPLC for large scale purification include high capital costs on instrument and column, labor intensiveness, high energy demand for solvent evaporation, and inability to resolve peptides with multiple higher order structures. Most importantly, HPLC consumes large volumes of harmful solvents, which results in high waste to product ratios, usually more than 1,000. Other methods for peptide purification include fluorous affinity purification,[3] antigen−antibody affinity purification,[4] covalent capture with a solid matrix,[5] and lipophilic tag assisted chromatography.[6] All these methods are extensively studied but are still not ideal for large scale peptide drug purification.

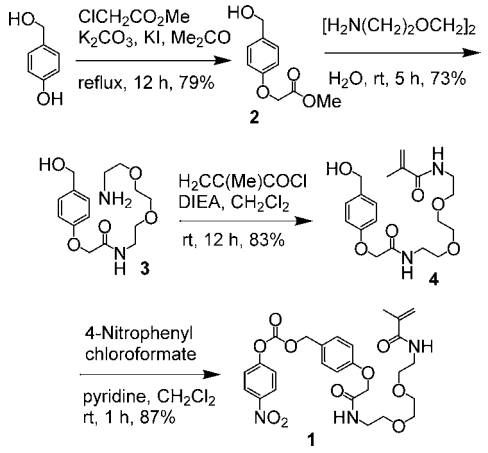
Recently, we reported a catching by polymerization strategy for purification of oligodeoxynucleotides (ODN).[7] In one method, at the end of automated synthesis, a polymerizable methacrylamide group is attached to the full-length sequence. ODN purification is then achieved by polymerizing the full-length sequences, washing away failure sequences and other impurities, and cleaving product from the polymer.[7a] The method avoided many drawbacks associated with chromatography and other known methods and could be used for large scale and high throughput ODN purification. Here we report our results on using the strategy for synthetic peptide purification.

To demonstrate feasibility, compound **1**, which contains an acid-labile linker and a reactive 4-nitrophenyl carbonate function, was designed. Because we planned to use the commercially available 2-Cl-trityl polystyrene resin as the solid support for peptide synthesis, the linker must be stable in 1% TFA, which are the conditions for cleaving peptides from the resin.[2,8] However, the linker must be readily cleavable under more acidic conditions to obtain good recovery yield in the purification process. These goals were achieved by careful tuning the electron density of the benzene ring in the linker. The molecule was also designed to be easy to synthesize. As shown in Scheme 1, 4-hydroxybenzyl alcohol was alkylated with methyl chloroacetate to give **2**,[9] which was reacted at room temperature with 2,2′-(ethylenedioxy)bis(ethylamine) to give

Received: November 26, 2013
Published: February 14, 2014

dx.doi.org/10.1021/ol403426u | Org. Lett. 2014, 16, 1290−1293

Scheme 1. Synthesis of Compound 1

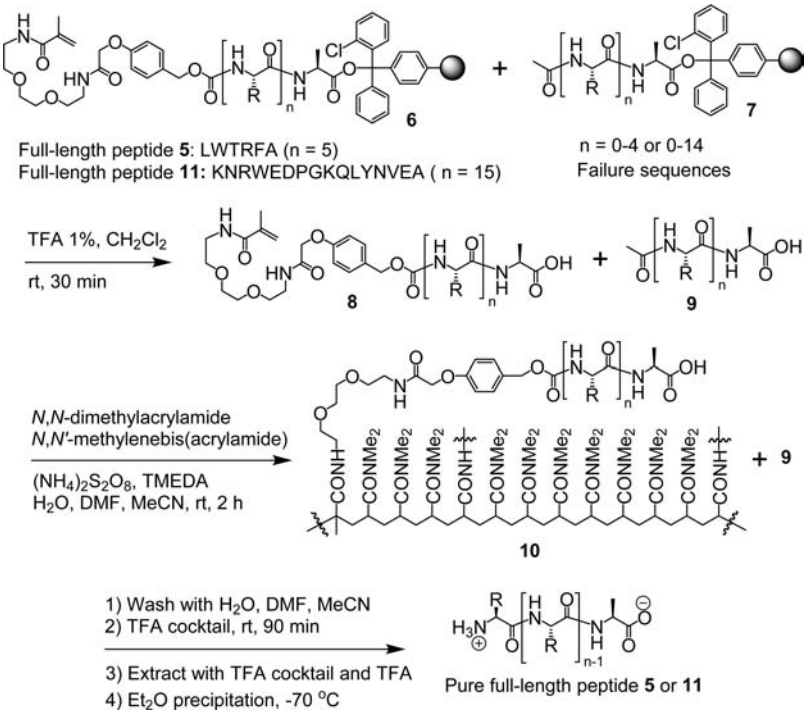


3.[10] Methacrylation of **3** gave compound **4**, which was converted to **1** by reacting with 4-nitrophenylchloroformate. The synthesis did not use any expensive and highly reactive reagents and is expected to be readily scalable.

Since peptide synthesis and purification are much more complicated than ODN, at the beginning of the project, we used the short 6-aa peptide **5** (LWTRFA) to demonstrate the feasibility of the concept. The 2-Cl-trityl polystyrene resin preloaded with Ala was selected as the solid support. The Fmoc-protected Phe, Arg(Pbf), Thr(*t*Bu), Trp(Boc), and Leu were used as aa monomers. Synthesis was carried out on an automated synthesizer. In the coupling steps, only 2.1 equiv of monomers was used, which was lower than the amounts usually suggested by manufacturers of peptide synthesizers. The lowered amount was expected to generate more failure sequences and to better show the effectiveness of the new purification method. After coupling, failure sequences were capped with excess acetic anhydride. The steps for removing the Fmoc protecting group were normal. After assembling the sequence, the full-length peptide was reacted with compound **1** in the presence of a base at room temperature overnight to give **6** (see Scheme 2). The failure sequences (**7**) could not react because they were capped with acetic anhydride. The crude product, which mainly contained **6** and **7**, were cleaved from the resin using 1% TFA to give full-length peptide **8** and failure sequences **9**. Under these conditions, the side chain protecting groups and the 4-hydroxybenzyl alcohol linker were stable.[2] The mixture was analyzed with RP HPLC (trace a, Figure 1). The full-length peptide appeared at 61 min. To generate a control HPLC profile, a portion of the crude was treated with a TFA cocktail containing 81.5% TFA, 1.0% triisopropylsilane (TIPS), 5.0% water, 2.5% ethane dithiol (EDT), 5.0% thioanisole, and 5.0% phenol to globally remove protecting groups and to cleave the methacrylamide tag. After Et_2O precipitation, the crude product was analyzed with RP HPLC (trace b). The fully deprotected full-length peptide (**5**) appeared at 23 min.

Another portion of the crude containing **8** and **9** (with side chain protecting groups and methacrylamide tag not removed) was subjected to polymerization under typical polyacrylamide gel formation conditions [*N*,*N*-dimethylacrylamide, *N*,*N*′-methylenebis(acrylamide), $(NH_4)_2S_2O_8$, TMEDA]. The full-length peptide **8** was incorporated into the polymer **10**. The failure sequences **9** and other impurities remained in solution (Scheme 2). The gel was washed extensively with solvents such as water, DMF, and MeCN to remove impurities. Full-length peptide was then cleaved from the gel and fully deprotected by treating with the TFA cocktail described above. At this stage, the peptide was still contaminated with small molecules from side chain protecting groups and the cocktail. Removing them was achieved by precipitation of peptide with cold Et_2O. The pure peptide **5** was analyzed with RP HPLC (trace c, Figure 1).

Scheme 2. Peptide Cleavage from Resin and Purification by Catching Full-Length Sequence by Polymerization



dx.doi.org/10.1021/ol403426u | *Org. Lett.* 2014, 16, 1290−1293

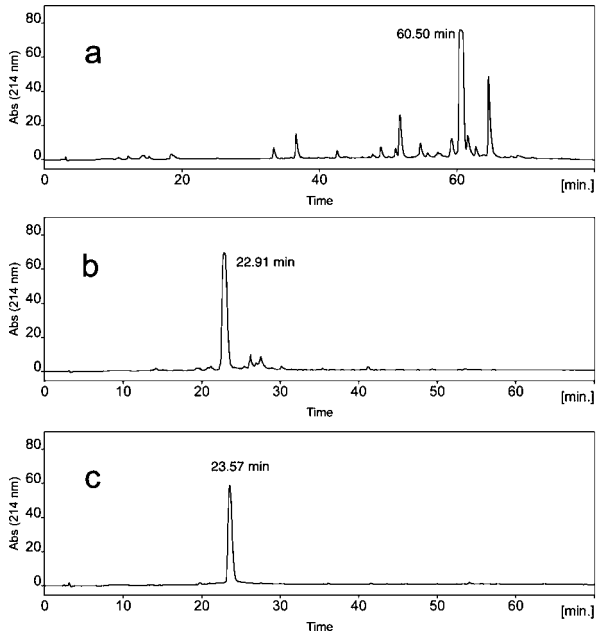


Figure 1. RP HPLC profiles of peptides. (a) Crude 6-aa peptide containing full-length sequences **8** and failure sequences **9**; the side chain protecting groups and the methacrylamide tag on **8** were on. (b) Crude peptide with side chain protecting groups and methacrylamide tag removed. (c) Peptide **5** purified using the catching full-length sequences by polymerization approach.

The purity of the peptide was more than 98%. The recovery yield of the purification process was estimated to be 66% by comparing the areas of the peaks at 23 min in traces b and c. The identity of the peptide was confirmed with ESI-MS and NMR (see Supporting Information).

To further demonstrate the effectiveness of the purification technique, the 16-aa peptide **11** (KNRWEDPGKQLYNVEA), which is a peptide derived from human C3d and carries the LYNVEA CR2 binding sequence,[11] was synthesized under similar conditions as described for **5**. Fmoc aa Glu(O*t*Bu), Val, Asn(Trt), Tyr(*t*Bu), Leu, Gln(Trt), Lys(Boc), Gly, Pro, Asp(O*t*Bu), Trp(Boc), and Arg(Pbf) were used as the monomers. To further increase the difficulty of the purification task, the amount of aa monomers was lowered to 1.5 equiv. The purification process was exactly the same. The RP HPLC profiles for the crude peptide with the methacrylamide tag and side chain protecting groups on, that with tag and protecting groups removed, and purified full-length peptide are included in Supporting Information. The purity of the peptide (**11**) is more than 96%. The recovery yield was estimated to be 73%. The identity of **11** was confirmed with ESI MS and NMR.

The catching by polymerization peptide purification method has significant advantages over other techniques. Compared with chromatographic methods such as HPLC and lipophilic tag assisted purification,[6] the method does not need any expensive instrument, column, or large volumes of harmful solvents. Compared with RP cartridge, fluorous, and antigen–antibody affinity purification methods,[3,4] the method does not require a disposable column. Compared with the methods using covalent capture,[5] the method does not need a functionalized solid matrix. Importantly, using the method, purification is achieved by simple manipulations such as shaking, washing, extraction, and precipitation. As a result, it is expected to be particularly useful for large scale peptide drug purification. Some drugs need to be purified for more than one time, and ideally, each time uses a different purification technique. The catching by polymerization method may be an excellent choice for initial removal of large quantities of failure sequences. This is predicted to be particularly useful for the purification of peptide sequences that have inherently low coupling yields in certain cycles. Furthermore, due to the ease of removal of failure sequences using the method, it is possible for drug manufacturers to reduce the equivalents of aa monomers and their activators during synthesis. The rebalance of reagent costs and purification costs will provide new opportunities to lower the overall expenses of drug production.

In conclusion, we have successfully developed a new method for peptide purification. The method used a concept that is completely different from any previously reported peptide purification techniques. It is easy to use and readily scalable and gives peptides with good purity and recovery yields. Adapting the method for purification of several peptide drugs such as Bivalirudin (**20** aa),[12] Ziconotide (**25** aa),[13]Thymalfasin (**28** aa),[14] Enfuvirtide (**36** aa),[15] and Pramlintide (**39** aa)[16] is underway.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compound characterization, and images of NMR spectra, ESI-MS, and HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: shifang@mtu.edu.

Notes

The authors declare the following competing financial interest(s): The authors are inventors of a provisional US patent filed by Michigan Technological University.

■ ACKNOWLEDGMENTS

Financial support from US NSF (CHE-0647129 and CHE-1111192), MTU Biotech Research Center, MUCI and MTU REF Technology Commercialization; the assistance from Mr. Dean W. Seppala (electronics) and Mr. Jerry L. Lutz (NMR); and an NSF equipment grant (CHE-9512445 and CH-1048655) are gratefully acknowledged.

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dx.doi.org/10.1021/ol403426u | *Org. Lett.* 2014, 16, 1290−1293