Simultaneous Fmoc and Boc Synthesis of a Contryphan Positional Scanning Library and the Conotoxin Prialt on the Overture™ Robotic Peptide Library Synthesizer

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ABSTRACT
The Overture™ (Figure 1) is the only robotic peptide library synthesizer that can perform Boc and Fmoc chemistry at the same time! In this work, the flexible Overture™ Library Design Tools are used to generate a focused positional scanning library of contryphans. This contryphan libraryis then synthesized using Fmoc chemistry, while the conotoxin drug, Prialt, is synthesized using Boc chemistry in the same synthesis. This has never been seen before on a peptide synthesizer.

INTRODUCTION
Snail venoms offer a rich source of biologically active peptides known generally as conopeptides, for study and potential therapeutic application [1,2]. One such peptide, the conotoxin MVIIA, has already been developed as the analgesic Prialt (Ziconotide). Originally isolated from the marine snail *Conus magus* [3] and developed as SNX-111 [4], Prialt acts as a potent and selective antagonist of N-type calcium channels and has been approved for the treatment of severe pain, particularly when refractory to morphine [5,6]. This ω-conotoxin contains 25 amino acid residues, including six cysteines which in the native peptide form three specific disulfide bonds [7] (Figure 2).

Figure 2: Prialt peptide sequence with three disulfide bridges

A related class of molecules is the contryphans [8-13], small conopeptides which have been suggested to represent potential ω-conotoxin mimetics [14]. Typically less than ten residues long, contryphans contain just one disulfide linkage in the final native peptide, and tend to have unusual post-translational modifications, including enzymatic epimerization of single residues to D-amino acids, and hydroxylation of prolines. Relatively little is known about the biological targets of most of these structures, but Contryphan-Vn has been shown to modulate calcium-dependent potassium channels [15]. With a molecular target for Contryphan-Vn elucidated, it may be possible to explore structure-activity relationships by screening analogue libraries for binding to this protein. Like standard peptide library generation tools, the Overture™'s Library Design Tools can be used to generate positional scanning libraries utilizing all 20 standard amino acids. However, only the Overture™ software has the added flexibility to generate focused scanning libraries using amino acids selected by the user. In this way, it is possible to generate positional scanning libraries containing only hydrophobic, basic, acidic, or even non-standard amino acids. In this work, the Overture™ software is used to generate a positional scanning library based on Contryphan-Vn, varying position 2 to include the acidic amino acids and their amides (D, E, N, Q) (Figure 3).

Figure 3: Positional scanning library based on Contryphan-Vn, where w = D-Trp. Substitutions are shown in red.

Figure 1: The Overture™ Robotic Peptide Library Synthesizer from Protein Technologies, Inc.

In addition, the Overture™ is the only commercially available peptide synthesizer on which Fmoc and Boc chemistry can be run simultaneously. Most routine syntheses today are run using Fmoc chemistry, however, certain peptides have been optimized to run with greater success using Boc chemistry. These peptides include thioesters for native chemical ligation applications, and the conotoxin, Prialt. The Overture™ is the only peptide synthesizer that can run different protocols simultaneously, including Fmoc and Boc chemistry. With its 96 reaction vessels, the Overture™ increases productivity by eliminating the need to perform two separate syntheses every time a different chemistry isrequired. In this poster, the positional scanning library of Contryphan-Vn was synthesized using Fmoc chemistry, while the conotoxin Prialt was synthesized using Boc chemistry all in the same synthesis on the Overture™.

EXPERIMENTAL
Fmoc Peptide Synthesis: The contryphan peptides were synthesized at the 20 µmol scale using Rink Amide MBHA resin (0.33 mmol/g) on the Overture™ Robotic Peptide Library Synthesizer (Protein Technologies, Inc.). *Deprotection:* 20% piperidine/DMF for 2 x 5 min. *Washes:* (1) After deprotection and second coupling: DMF 6 x 30 sec. (2) After first coupling: DMF 1 x 30 sec. *Coupling:* 1:1:4 0.05 M AA/0.05 M HCTU/0.2 M NMM in DMF (5x excess) for 2 x 10 min. Cleavage: 86.5/5/5/2.5/1 TFA/Phenol/water/EDT/TIS for 2 hours. The peptides were precipitated in ice-cold ether and dried overnight, then dissolved in water for analysis.

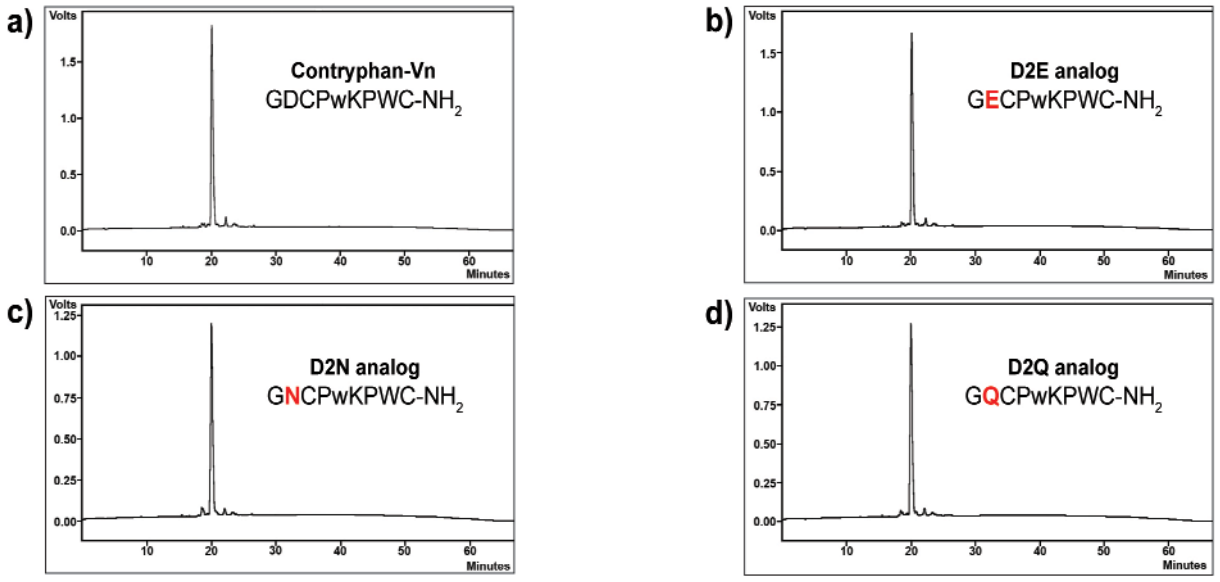

Figure 4: HPLC results for Contryphan-Vn positional scanning library crude peptides synthesized using Fmoc chemistry on the Overture™. Substitutions are shown in red.

Boc Peptide Synthesis: Prialt was synthesized at the 20 µmol scale using MBHA resin (0.89 mmol/g) on the Overture™ Robotic Peptide Library Synthesizer (Protein Technologies, Inc.). *Deprotection:* 99/1 TFA/EDT, 2 x 5 min. Neutralization of TFA resin: 10% DIEA/DCM 2 x 5 min. *Washes:* (1) After deprotection: DCM 3 x 30 sec, MeOH 3 x 30 sec, DCM 3 x 30 sec. (2) After neutralization: DCM 3 x 30 sec. MeOH 3 x 30 sec. DCM 3 x 30 sec. DMF 3 x 30 sec. Coupling: 1:1:40.05 M AA/0.05 M HCTU/0.2 M NMM in DMF (5x excess) for 2 x 10 min. *Cleavage:* Anhydrous HF with cresol and thiocresol as scavengers (at approximately 5% each of the total mixture), at 0°C for 2 h. The HF was removed by vacuum, and the crude peptide washed with cold diethyl ether. The peptide was dissolved in 10% acetic acid and lyophilized. Crude Prialt was dissolved in 25 mM phosphate buffer, pH 7.5, containing 50 mM of dithiothreitol (DTT), and allowed to sit overnight at room temperature to reduce the disulfide bonds prior to analysis.
Analysis: Crude peptides were analyzed on a Varian ProStar HPLC using a C18, 300 Å, 5 µm, 250 x 4.6 mm column (Varian Microsorb-MV), over 60 minutes with a flow rate of 1 mL/min, and using a gradient of 5-95% B, where Buffer A is 0.1% TFA in water, and Buffer B is 0.1% TFA in acetonitrile. Detection was at 214 nm. Mass analysis was performed on a Shimadzu LCMS-2020 Single-Quad mass spectrometer, equipped with a C18, 100 Å, 2.6 µm, 50 x 2.1 mm column (Phenomenex Kinetex), over 7 minutes with a flow rate of 1 mL/min and using a gradient of 5-50% B where Buffer A is 0.1% formic acid in water and Buffer B is 0.1% formic acid in acetonitrile.

RESULTS

A positional scanning library based on the Contryphan-Vn sequence was synthesized using Fmoc chemistry alongside the conotoxin peptide, Prialt, synthesized using Boc chemistry in the same synthesis on the Overture™. The HPLC results for the crude peptides are shown in Figures 4 & 5.

CONCLUSIONS

Using the Overture™ Robotic Peptide Library Synthesizer, we have successfully synthesized Prialt using Boc chemistry and a small positional scanning library based on Contryphan-Vn with Fmoc chemistry in the same synthesis. Unlike existing robotic platforms, the Overture™ is an extremely flexible platform for generating focused peptide libraries and running different chemistries at the same time.

ACKNOWLEDGEMENTS

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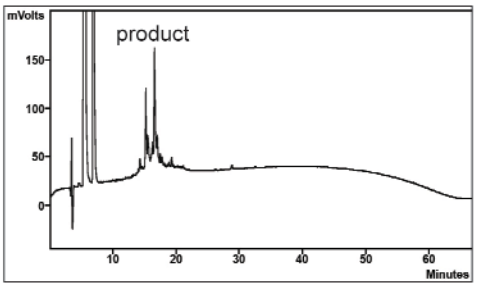

Figure 5: HPLC results for crude Prialt synthesized using Boc chemistry on the Overture™.The product elutes at 16.5 min. The peaks with RT < 9 min are due to DTT and background.

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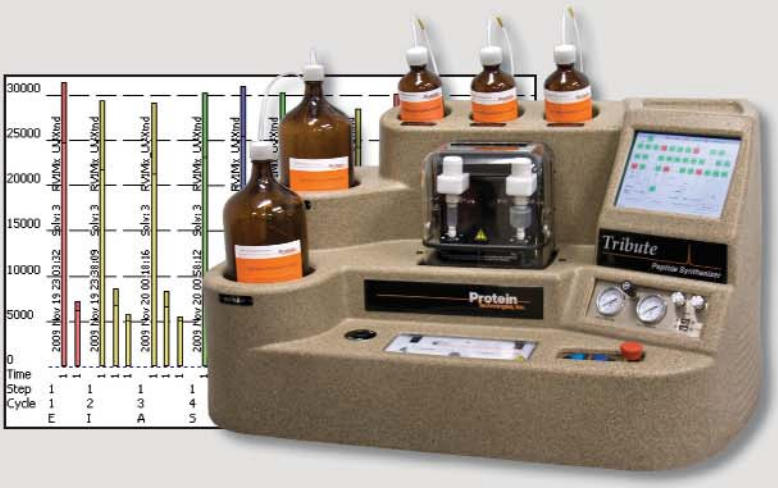

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