Synthesis of a Poly-Alanine Peptide Using Different Deprotection Reagents and the IntelliSynth UV-Monitoring and Feedback Control System on the *Tribute*™

INTRODUCTION

The IntelliSynth™ UV-monitoring and Feedback Control System is a powerful tool that can be used to determine difficult cycles during a synthesis on the ***Tribute***™ peptide synthesizer. It works by monitoring the extent of Fmoc removal during the deprotection reaction by measuring the concentration of removed Fmoc in the deprotection solution at 301 nm. It can then use this data to control the deprotection time and number of repeats until the deprotection reaction is complete, and also extend the coupling time based on the total deprotection time.

The IntelliSynth™ system is the only UV-monitoring system on the market today that can monitor ***during*** the deprotection reaction. Other systems work by transfering a portion of the deprotection fluid to an external UV-monitor as it is being sent to waste (**Figure 1**). This only allows them to take one reading ***after*** the deprotection reaction is over and requires extra rinsing of the UV-monitor lines and calibration prior to each synthesis. The IntelliSynth™ system does not use an external UV-monitor. Instead, the patent-pending IntelliSynth™ system is composed of a 1 3/8" x 1 3/8" x 1 ¾" (3.5 cm x 3.5 cm x 4.5 cm) housing containing the UV light source and detector which mount directly onto the tubing just 2" below the reactor. During the deprotection reaction, the Tribute software pushes the fluid down the 2" to the UV light source and detector, measures the absorbance, then pushes the fluid back up into the reactor (**Figure 2**). In this way, the IntelliSynth™ system can take a measurement every 20 seconds during the deprotection reaction. When it detects no significant absorbance change in multiple consecutive measurements, it automatically stops the deprotection reaction. It will then repeat the deprotection reaction until the total absorbance for a given deprotection reaction falls below a set threshold. In this way, the IntelliSynth™ system ensures that the deprotection stops only when the chemistry has gone as far as it can go.

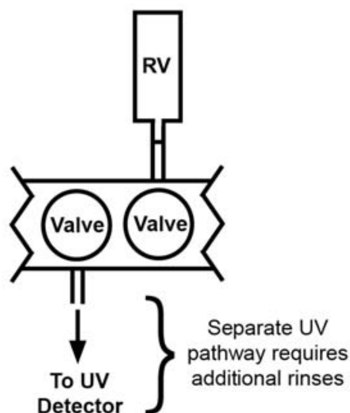

Figure 1: Diagram of other UV-monitoring systems that work by sampling the reaction fluid after the deprotection reaction while it is on its way to waste. The separate UV pathway requires additional rinses.

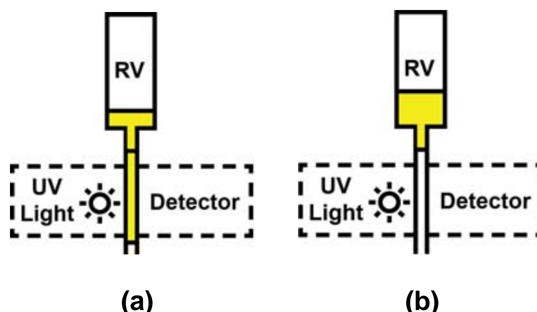

Figure 2: Diagram of PTI's IntelliSynth UV-monitoring system. The light source and detector are located just below the reactor. During the deprotection reaction, the reaction fluid is momentarily pushed down 2" to where the light source and detector are located (a). This allows a measurement to be taken. Then the fluid is pushed back up into the reactor (b). This process is repeated every 20 seconds during the deprotection reaction.

Typically, difficult deprotections occur when a peptide aggregates and obscures the reaction site. Difficult deprotections are indicated in the UV trace by higher numbers of repeats for the

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deprotection reaction, and higher peak heights (excluding the first peak) for a given cycle.
Peptides containing poly-alanine tracts have been associated with several human diseases and malformations [1] and have also been used to form model beta sheet systems for studying Alzheimer's disease [2,3]. Due to their high propensity to aggregate after the fifth residue, these sequences are extremely difficult to synthesize by conventional Fmoc solid phase peptide synthesis.

Over the years, several techniques have been explored to improve the synthesis of poly-alanines by overcoming aggregation. Incorporating proline into the sequence was found to disrupt aggregation until an additional six alanine residues were added [4]. Various alanine monomers have also been developed to disrupt aggregation including Tmob-Ala and Hmb-Ala [5-6]. Other methods used to improve the synthesis of poly-alanines include using microwave irradiation [6] and different deprotection reagents [7-8]. In particular, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) is a more efficient base than piperidine, and has been found to increase the amount of Fmoc removal from highly aggregated sequences when used with piperidine [7-8].

The purpose of this application note is to demonstrate the ability of the IntelliSynth system to push the chemistry as far as it can go. In this application note, the model poly-alanine peptide, $(Ala)_{10}$Lys-OH, was synthesized using conventional Fmoc solid phase peptide synthesis. Lysine was added to the C-terminus in order to improve the aqueous solubility of the final peptide [2]. Deprotection was performed with either 20% piperidine in DMF or 2% DBU/20% piperidine in DMF, and the syntheses were run under the deprotection with feedback mode. In this mode, the deprotection step is UV-monitored, then the data is used to control the individual deprotection times and number of repeats for each cycle. The coupling time was not controlled in this particular application, and was set to 10 minutes for each cycle. The effect of the two different deprotection reagents on the UV trace pattern and crude purity of the final peptide were studied.

Sequence: AAAAAAAAAAK-OH

METHOD

Materials: Fmoc-Lys(Boc) HMP resin was purchased from Nuros. Amino acids, HCTU and solvents were provided by Protein Technologies, Inc. (Tucson, AZ) and cleavage reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Peptide Synthesis: Syntheses were performed at the 20 μmol scale on a ***Tribute***™ peptide synthesizer from Protein Technologies, Inc. (Tucson, AZ) using a 10-fold excess of Fmoc amino acids (200 mM) with respect to the Fmoc-Lys(Boc)-HMP resin (0.48 mmol/g loading). Deprotection was performed using 20% piperidine in DMF or 2% DBU/20% piperidine in DMF with a minimum time of 30 seconds using UV-monitoring in the deprotection with feedback mode. Couplings were performed for 10 minutes using 1:1:2 amino acid/HCTU/NMM in DMF. Cleavage was performed by treating the resin with 95:2:2:1 TFA/anisole/water/TIS for 2 hours.

Analysis: Crude peptides were precipitated with anhydrous ether, air-dried overnight, dissolved in 1,1,1,3,3,3-Hexafluoro 2-propanol (HFIP) and analyzed using a Varian ProStar HPLC, equipped with a C18, 300 Å, 5 μm, 50 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 was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. Detection was at 214 nm. Mass spectrometry was used to verify the structure of the peptides using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry on a Voyager-DE™ BioSpectrometry Workstation from PerSeptive Biosystems or at the University of Arizona mass spectrometry facility (data not shown).

RESULTS

$(Ala)_{10}$Lys-OH was successfully synthesized on the Tribute peptide synthesizer using UV-monitoring in the deprotection with feedback mode, and using either 20% piperidine or 2% DBU/20% piperidine in DMF as the deprotectant. In this mode, the Tribute uses the UV data to extend the deprotection times and number of repeats in each cycle. When 20%

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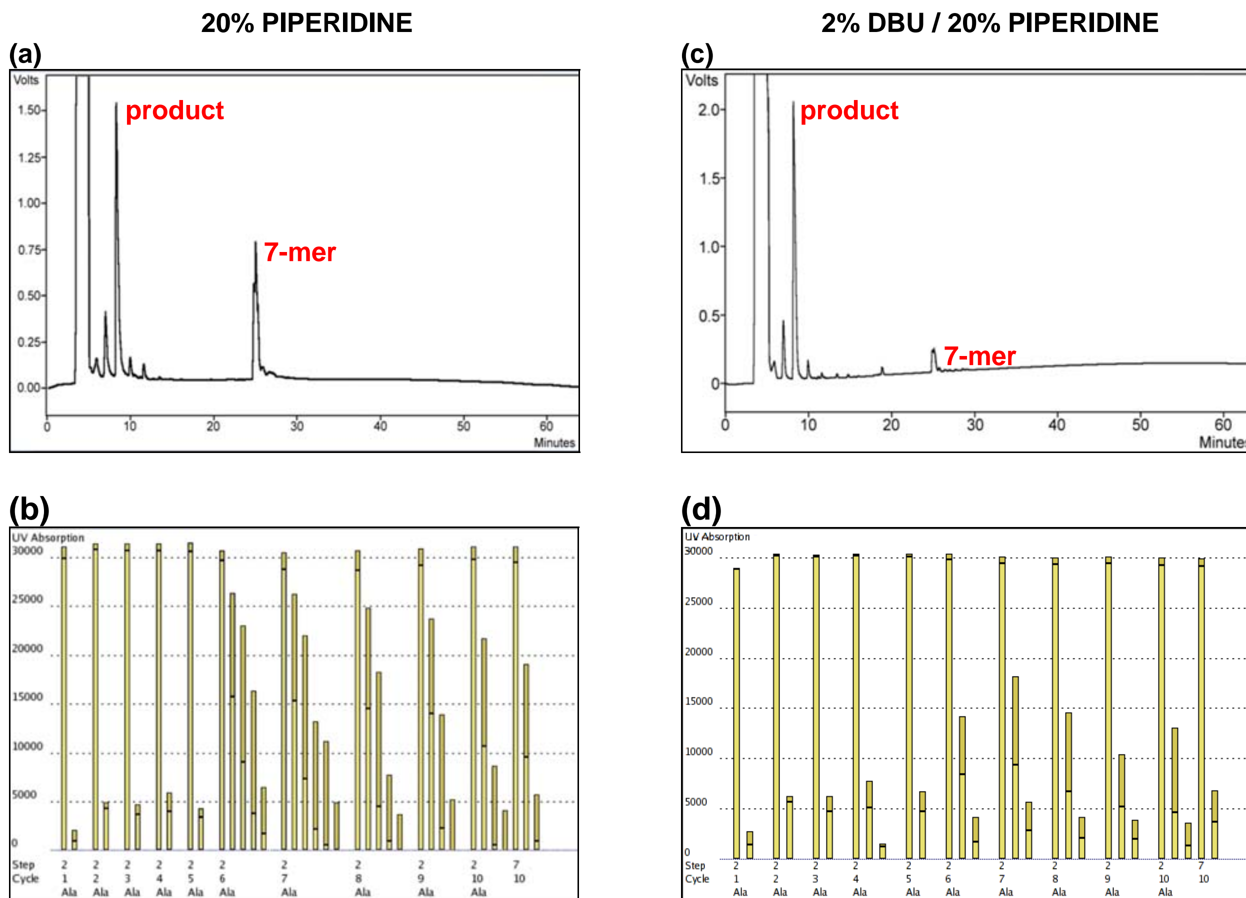

Figure 3: HPLC and UV results for $(Ala)_{10}$Lys-OH run using 20% piperidine or 2% DBU/20% piperidine in DMF for the deprotection reaction. (a) HPLC and (b) UV data for 20% piperidine and (c) HPLC and (d) UV data for 2% DBU/20% piperidine are shown. Oversized prepeak in HPLC graph is HFIP. 7-mer is Fmoc-$(Ala)_6$Lys-OH.

piperidine alone was used, there was a significant amount of the Fmoc-protected 7-mer impurity, Fmoc-$(Ala)_6$Lys-OH, present in the final crude product (**Figure 3(a)**). This indicates that the 20% piperidine solution was unable to completely remove all the Fmoc groups during the 7th cycle. The UV trace for the synthesis shows that the number of deprotection repeats significantly increased starting at cycle 6 with the highest number of repeats occurring during the 7th cycle. This indicates it was the most difficult to deprotect, which is consistent with the HPLC results (**Figure 3(b)**). It is also consistent with the known phenomenon of aggregation occurring after the 5th alanine in a poly-alanine sequence.

When 2% DBU was added to the 20% piperidine solution, the amount of 7-mer impurity significantly decreased (**Figure 3(c)**). This indicates that the DBU was able to remove more of the Fmoc group than piperidine alone. The UV trace also indicates that the deprotections were more difficult starting at cycle 6, with the most difficult deprotection occurring at cycle 7 (**Figure 3(d)**). This was indicated by the height of the peak for the second repeat. The higher the second peak, the more Fmoc was left to be removed after the first repeat. The second peak was highest during the 7th cycle, echoing the results of the 20% piperidine run, however, the Fmoc was removed much more easily when 2% DBU was added, since no more than 3 repeats were required for any cycle.

Under both conditions, the UV trace indicated that the deprotections became difficult after the 5th alanine, with the 7th cycle being the most difficult to deprotect (**Figure 3(b) & (d)**).

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CONCLUSION

A difficult poly-alanine peptide, $(Ala)_{10}$Lys-OH was successfully synthesized on the Tribute peptide synthesizer using UV-monitoring and either 20% piperidine or 2% DBU/20% piperidine as the deprotectant. In each case, the deprotection with feedback mode on the Tribute produced the best peptide that the chemistry was capable of producing. Difficult deprotections were indicated by higher numbers of repeats and higher peak heights (excluding the first peak) for a cycle. 2% DBU/20% piperidine was found to be more efficient than piperidine alone at removing the Fmoc group and resulted in a purer crude peptide and fewer repeats during the deprotection step.

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