

Synthesis of Fluorescently Labeled NDP- α -MSH on the *Prelude*[®]

INTRODUCTION

The peptide hormone α -melanocyte stimulating hormone, or α -MSH, stimulates melanogenesis and plays a role in appetite, energy balance, and sexual function (1). The analogue NDP- α -MSH, also known as Melanotan-I, MT-I, or Afamelanotide, contains a norleucine substitution at position 4 and a *D*-phenylalanine in position 7 (Figure 1), which increase the potency and stability of the peptide (2). Formulations of this molecule are currently in clinical trials for a number of applications, and one, originally designated CUV1647 and now with the proprietary name Scenesse, is currently approved in Italy for use in the treatment of erythropoietic protoporphyria (EPP).

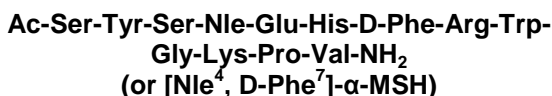


Figure 1: NDP- α -MSH sequence.

In addition to use as a therapeutic, NDP- α -MSH is an important tool compound for study of the melanocortin receptors, and a radiolabeled analogue is frequently used to perform binding assays at these receptors. Recently a europium-tagged analogue has been used for fluorescence-based binding assays (3). Fluorescein-labeled NDP- α -MSH might be useful for the development of a complementary assay method or other imaging studies. Furthermore, a variety of *in vitro* and *in vivo* experiments are facilitated by the introduction of these kinds of labels, including fluorescence microscopy for the anatomical localization of receptors (4) and visualization of peptide-receptor internalization (5), binding assays based on fluorescence polarization (6), and protease activity assays using fluorogenic substrates (7). Fluorescent probes are being explored for *in vivo* diagnostic imaging (8) and even as surgical aids (9).

The purpose of this application was to demonstrate a fully automated synthesis and labeling of the N-terminus of NDP- α -MSH with 5(6)-carboxyfluorescein (Figure 2) using a fully automated method and **Single-Shot**[™] deliveries on the **Prelude**[®] peptide synthesizer.

Fluorescein-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂

Figure 2: Fluorescein-labeled NDP- α -MSH

Conveniently, carboxyfluorescein can be introduced using standard coupling methods. The fluorophore has an excitation maximum around 494 nm and emission maximum around 518 nm in aqueous solution.

The **Prelude**[®]'s **Single-Shot**[™] delivery feature and extra amino acid bottle positions make it perfect for adding expensive special monomers to a peptide sequence or performing post-translational modifications such as fluorescent labeling. The **Prelude**[®]'s **Single-Shot**[™] delivery feature can deliver the entire contents of the special 10 mL **Single-Shot**[™] amino acid vial to the reaction vessel of your choice without priming or wasting a drop.

METHOD:

Peptide Synthesis: The peptide was synthesized on the **Prelude**[®] peptide synthesizer at 40 μ mol scale using Fmoc-Rink-MBHA resin (Subst. = 0.33 mmol/g). Deprotection was performed with 20% piperidine for 2 x 5 min. Washes: DMF 6 x 30 sec between deprotection and coupling, and between coupling and deprotection; 1 x 30 sec between couplings. Coupling was performed using 1.0 mL: 0.5 mL: 0.5 mL 0.2 M AA/0.4 M HCTU/0.8 M NMM (1:1:2 final ratio, 5x excess relative to loading) in DMF for 2 x 20 min except for the Fmoc-Nle, Fmoc-D-Phe, and 5(6)-carboxyfluorescein, added as the last residue, which were coupled for 1 x 30 min using the **Single-Shot**[™] delivery feature. Care was taken to minimize exposure of the fluorescein label to light, before, during, and after addition to the peptide. Cleavage was performed using 95/2.5/2.5 TFA/water/TIS for 2 hours. The resulting crude peptide was precipitated in cold diethyl ether and analyzed.

Analysis: Crude peptides (after precipitation) were dissolved in water and analyzed on a Varian ProStar HPLC using a C18, 180 Å, 5 μ m, 250 x 4.6 mm column (Agilent Polaris), 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/DISCUSSION:

After synthesis of des-acetyl NDP-α-MSH, and prior to labeling, a small portion of the peptide-resin was cleaved under standard conditions. A single major peak was observed in the HPLC trace, which integrated to 74% purity (Figure 3). The correct mass was also observed in the LC-MS (data not shown).

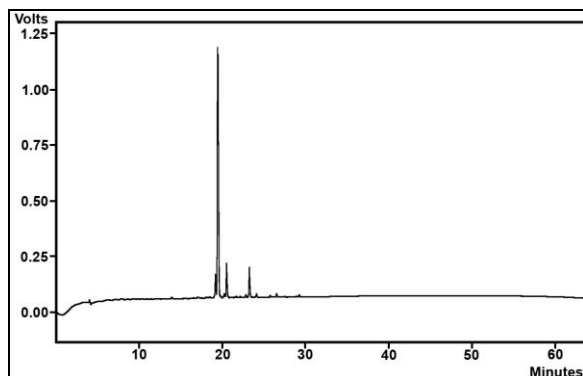


Figure 3: HPLC trace of des-acetyl NDP-α-MSH

5(6)-Carboxyfluorescein was added to the peptide at the N-terminus using standard HCTU coupling. This reagent is a mixture of the 5-carboxyfluorescein and 6-carboxyfluorescein isomers, although pure isomers could also have been used. The use of the isomeric mixture means two peaks with similar retention times should be observed by HPLC. Care was taken to minimize exposure of the label to light at any point during the synthesis or analysis, as fluorescein is known to photobleach.

After labeling, the expected closely-eluting major peaks were observed in the HPLC trace; when

combined these peaks integrate to 80% purity (Figure 4). The correct mass was detected in the LC-MS as well (data not shown).

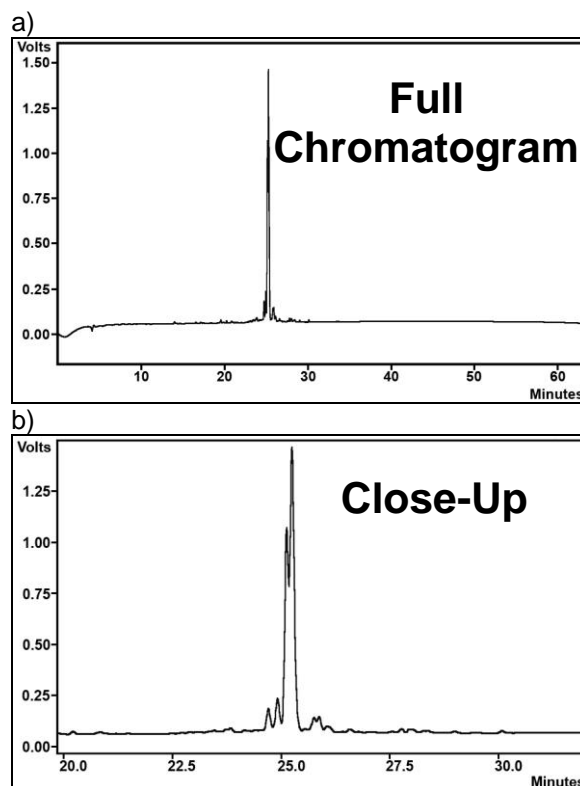


Figure 4: HPLC trace of 5(6)-Fluoresceinyl-NDP-α-MSH. a) Full chromatogram; b) Close-up of major peaks.

CONCLUSION:

We have successfully synthesized fluorescein-labeled NDP-α-MSH on the **Prelude**[®] peptide synthesizer. The fluorescent label and two non-standard amino acids were conveniently added in a fully automated fashion using the **Single-Shot**[™] delivery feature.

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