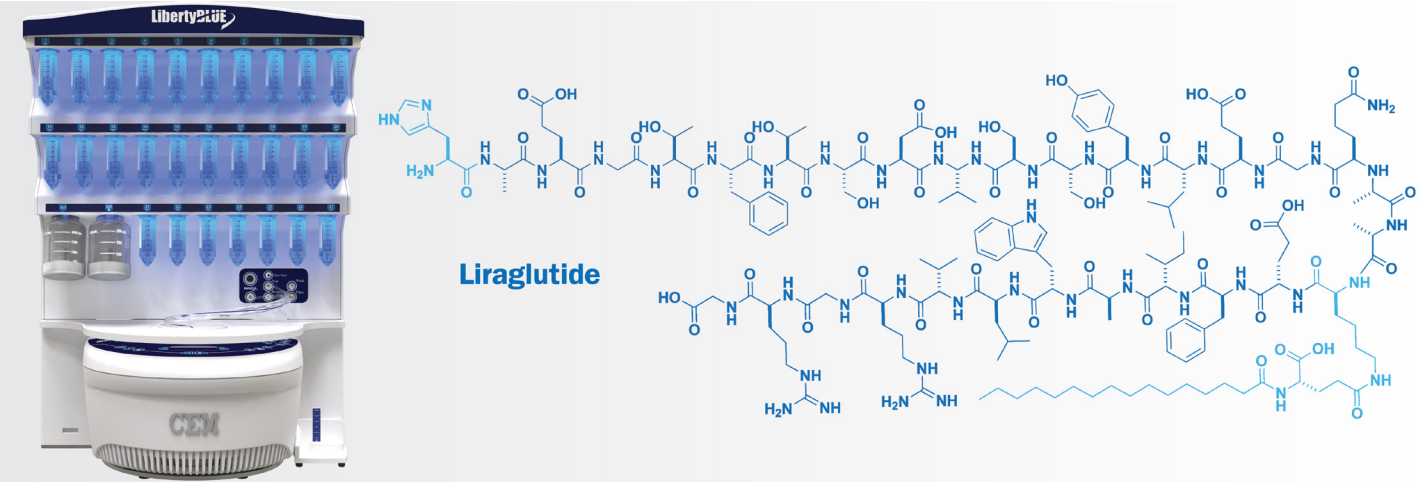
Application of Fmoc-His(Boc)-OH in Fmoc-based SPPS



Histidine Epimerization

Enantiomeric purity greatly affects a peptide's biological activity; therefore, avoiding increased amounts of D-isomer is important.[1] Histidine is particularly prone to epimerization during activation of the coupling process in solid phase peptide synthesis (SPPS). Histidine's susceptibility to epimerization (**Figure 1**) is an intramolecular side reaction attributed to the lone pair electrons on the imidazole N^{π}, which are in close proximity to the acidic alpha-carbon hydrogen. When the amino acid is activated, the lone pair electrons on **1** are sufficiently basic enough for deprotonation thus forming an achiral ester enolate, **2**.[2] At this point, there is not a thermodynamically preferred pathway for conversion to the L- or D-isomer, **3**. The likelihood of epimerization increases when the reaction site is aggregated and histidine remains in an activated state for longer periods.

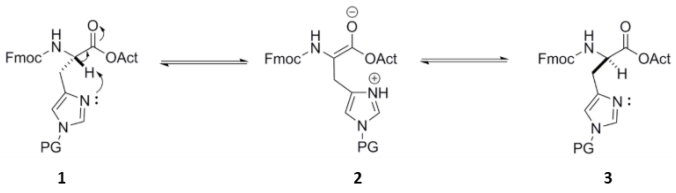


Figure 1: Mechanistic explanation for high levels of epimerization of Fmoc-His(PG)-OH during activation.

Histidine Side-Chain Protection

Protection of the imidazole (**Figure 2**) typically occurs through use of the trityl (Trt) group at the N^{τ} position, **4**. The Trt bulk and electron withdrawing capabilities suppress side reactions such as N-acylation on the ring but only offers minor help when suppressing epimerization. Other side-chain protecting groups, specifically those which offer N^{π} protection as is the case for Fmoc-His(π-Mbom)-OH (**5**), were developed to suppress epimerization by blocking accessibility to the alpha-hydrogen. A downside to these derivatives is their inherent high cost and low bulk availability due to the multi-step synthesis strategy that requires orthogonal protection of the N^{α} site while attaching the Mbom group.[3,4,5,6] In addition, extra scavenging reagents are required during peptide cleavage to suppress hydroxymethlation from occurring on newly uprotected amino functionality groups within the peptide.

Herein, Fmoc-His(Boc)-OH (**6**) is demonstrated to be a valuable substitute for incorporation of histidine during Fmoc SPPS because it possess high temperature stability towards epimerization, lower cost, and greater bulk availability than any other commercially available derivative.

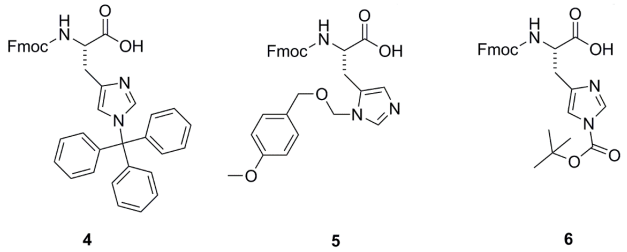


Figure 2: His derivatives for Fmoc-SPPS: Fmoc-His(Trt)-OH (4), Fmoc-His(π-Mbom)-OH (5), and Fmoc-His(Boc)-OH (6).

Fmoc-His(Boc)-OH Benefits

Fmoc-His(Boc)-OH is commercially available in bulk quantities as both a free acid and a cyclohexylamine (CHA) salt. In the salt form, extraction is required to remove the CHA group. Since this is a tedious process, our study focused on applications of the free acid. Previous reports indicate His(Boc) has a lower tendency to epimerize compared to His(Trt).[7] This phenomenon can be attributed to the greater electron withdrawing characteristic of the urethane group pulling electron density away from the π-electrons, decreasing basicity.

Discussion

A feasibility study using Liraglutide and $^{1\text{-}42}$Beta Amyloid evaluated the -Boc group's suppressive effect on epimerization and the stability on the side-chain during microwave (MW) assisted SPPS. Peptides were prepared using *HE*-SPPS conditions featuring a 1 min 90 °C deprotection and 2 min 90 °C coupling using DIC and Oxyma Pure.[8] DIC/Oxyma Pure activation gives superior results compared to uronium based activation strategies in terms of coupling efficiency and epimerization suppression. The latter is due to the acidic environment inherent to carbodiimide activation.[9,10] Histidine incorporation at room temperature or slightly elevated conditions such as 50 °C further reduces D-isomer formation, however such conditions are still problematic with His(Trt). The following coupling conditions with His(Trt) and His(Boc) were compared using two common protocols: (1) 10 min 50 °C and (2) 2 min 90 °C. Last, we studied stability in solution to determine applicability to high throughput automation on the Liberty Blue™ HT12.

Synthesis of Liraglutide

Liraglutide possesses an N-terminal histidine, which is difficult to couple to the peptide chain and thus, benefits from microwave heating to enhance acylation. Coupling histidine at 50 °C for 10 min using trityl protection resulted in elevated formation of the D-isomer (6.8%) as shown in **Table 1**. Under the same conditions, Fmoc-His(Boc)-OH significantly reduced epimerization to only 0.18%. Fmoc-His(Boc)-OH performed well at 90 °C as well with an observed epimerization level of 0.81% as compared to greater than 16% with His(Trt). Both Fmoc-His(Trt)-OH and Fmoc-His(Boc)-OH afforded the target peptide in comparable crude purity (**Figure 3**). Fmoc-His(π-Mbom)-OH afforded similar results in terms of both purity and D-His relative to Fmoc-His(Boc)-OH.

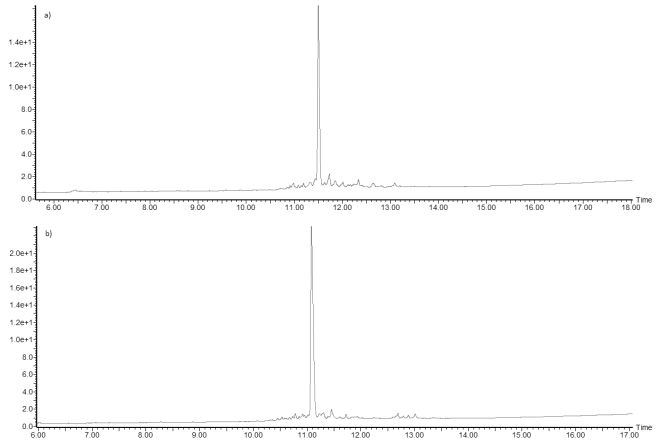


Figure 3: Liraglutide UPLC chromatograms using (a) Fmoc-His(Trt)-OH or (b) Fmoc-His(Boc)-OH. His coupling conditions = 50 °C, 10 min. Total synthesis time = 2 hr 55 min.

Table 1: D-isomer formation of Histidine in Liraglutide under different coupling conditions.

#	His Derivative	Coupling Conditions	D-Isomer (%)
1	His(Trt)	50 °C, 10 min	6.85
2	His(Trt)	90 °C, 2 min	15.6
3	His(Boc)	50 °C, 10 min	0.18
4	His(Boc)	90 °C, 2 min	0.81
5	His(π-Mbom)	90 °C, 2 min	0.85

Synthesis of $^{1\text{-}42}$Beta Amyloid

Previous reports indicate the N^{τ}-Boc side chain group to be unstable during long piperidine treatments.[11] $^{1\text{-}42}$Beta Amyloid synthesis, which contains three histidine residues, was synthesized to test –Boc lability during high temperature deprotection. $^{1\text{-}42}$Beta Amyloid synthesis is a notoriously difficult sequence to synthesize requiring special coupling reagents, and even under strenuous conditions, purity is often too low to analyze and purify.[12] Unlike conventional synthesis, *HE*-SPPS results in very high crude purity without optimization. His(Trt) and His(Boc) were compared by coupling at 50 °C for 10 min and 90 °C for 2 min respectively. His(Boc) reduced the total synthesis time from 4 hr 24 min to 3 hr 58 min and resulted in decreased epimerization from 2.88% to 1.29% D-isomer (**Table 2**.). UPLC analysis showed the two syntheses afforded target in comparable crude purity (**Figure 4**).

Table 2: Epimerization of His(Trt) and His(Boc) in BA.

#	His Derivative	Coupling Conditions	Synthesis Time	D-Isomer (%)
1	His(Trt)	50 °C, 10 min	4 hr 24 min	2.88
2	His(Boc)	90 °C, 2 min	3 hr 58 min	1.29

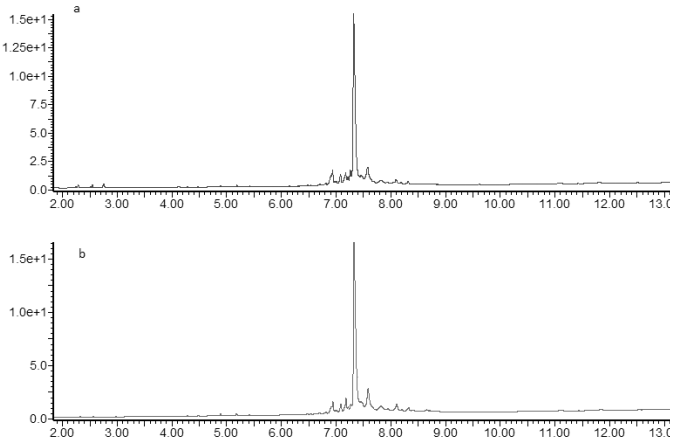


Figure 4: UPLC chromatogram of 1-42 Beta Amyloid using (a) His(Trt) and (b) His(Boc).

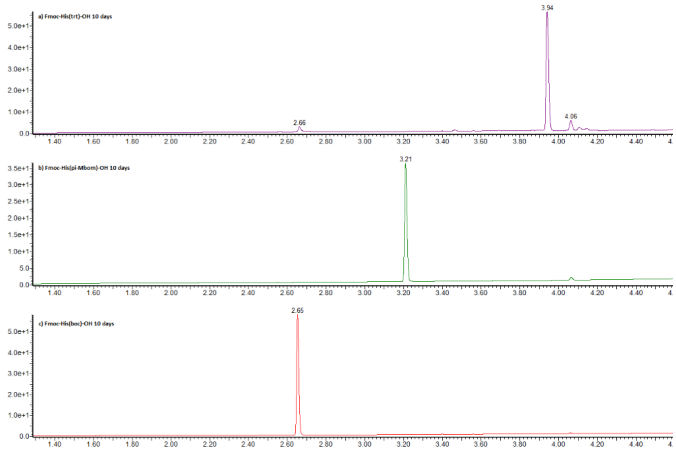


Figure 6. UPLC analysis of His derivatives in DMF (0.2 M) after 10 days; (a) = Fmoc-His(Trt)-OH (b) = Fmoc-His(π-Mbom)-OH (c) = Fmoc-His(Boc)-OH

Stability in Solution

Applications in automated high-throughput SPPS require substrates to stay dissolved in solution for up to 10 days. Typically, reactants such as histidine are limited to only a 5 day solution longevity due to discoloration and precipitation via degradation/loss of protecting groups. In this study, His solutions (DMF, 0.2 M) were stored under atmospheric conditions for 10 days (**Figure 5**). All samples dissolved quickly to yield colorless solutions. Fmoc-His(Trt)-OH discoloration occurred in as little as 24 hours and intensified over a 10 day period. The Fmoc-His(π-Mbom)-OH solution was slightly yellow after 10 days while the Fmoc-His(Boc)-OH solution remained colorless for the duration of the study. UPLC analysis demonstrated Fmoc-His(Boc)-OH and Fmoc-His(π-Mbom)-OH retained >99% purity. As expected based on intense discoloration, several impurities in the Fmoc-His(Trt)-OH sample formed during the 10 day study (**Figure 6**). Qualification of the impurities using mass spectroscopy was unsuccessful.

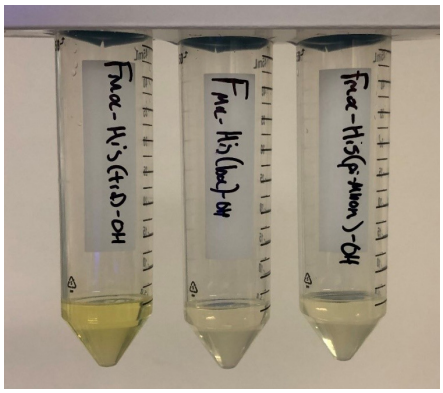


Figure 5: Stability in solution color test between different His derivatives.

Conclusion

The aforementioned data demonstrates that His(Boc) is a powerful His derivative that can be effectively coupled at 90 °C, providing favorable crude purity while both decreasing coupling time and dramatically reducing epimerization. Fmoc-His(Boc)-OH is widely available as compared to other epimerization suppressing N^{π} protected derivatives while maintaining comparable synthetic performance. In summary, the key benefits of Fmoc-His(Boc)-OH are:

- Commercial bulk availability and competitive price relative to Fmoc-His(Trt)-OH
- Low epimerization levels at elevated temperatures; coupling temperatures of 50 °C and below make Fmoc-His(Boc)-OH applicable for use in the synthesis of active pharmaceutical ingredients and avoiding complex coupling reagents and conditions.[13]
- Excellent solution stability; comparable to Fmoc-His(π-Mbom)-OH and greater than Fmoc-His(Trt)-OH.

Materials and Methods

Reagents

The following Fmoc amino acids and resins were obtained from CEM Corporation (Matthews, NC) and contain the indicated side chain protecting groups: Ala, Arg(Pbf), Asn(Trt), Asp(OMpe), Gln(Trt), Gly, His(Boc), His(Trt), Ile, Leu, Lys(Boc), Lys(palmitoyl-Glu-OtBu), Phe, Pro, Ser(tBu), Tyr(tBu), Val. Rink Amide ProTide™ LL, Cl-MPA ProTide™ LL, and Fmoc-Gly Wang PS LL resin were also obtained from CEM Corporation. Diisopropylcarbodiimide (DIC), piperidine, trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol (DODT), and triisopropylsilane (TIS) were obtained

from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-Dimethylformamide (DMF), anhydrous diethyl ether (Et_2O), acetic acid, HPLC grade water, and acetonitrile were obtained from VWR (West Chester, PA). LC-MS grade water (H_2O) and LC-MS grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA). D-isomer determined with chiral GC-MS (C.A.T. GmbH).

Peptide Synthesis: Liraglutide

The peptide was synthesized on a 0.10 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.313 g Fmoc Gly Wang PS LL resin (0.32 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[10] Cleavage was performed using the CEM Razor™ high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated with Et_2O and lyophilized overnight.

Peptide Synthesis: $^{1\text{-}42}$Beta Amyloid

The peptide was synthesized on a 0.10 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.512 g Cl-MPA ProTide resin[14] (0.19 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[10] Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated with Et_2O and lyophilized overnight.

Stability Study

Histidine solutions (0.2 M in DMF, 5 mL total) were prepared in 50 mL centrifuge tubes and sealed. The solutions remained in a lab setting at room temperature for 10 days. Samples were prepared for UPLC-MS analysis by diluting 10 µL of His solution in 5 mL of 50/50 (v/v) acetonitrile and water. Injection volume was adjusted until an absorbance of 35 – 55 au was achieved.

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