Gram-scale preparation of C-terminal modified enkephalin analogues by typical liquid-phase peptide synthesis

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Abbreviations:

BBB, blood-brain barrier; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazole-1-yloxy)-tris(dimethylamino)-phosphonium hexafluorophosphate; DMF, *N,N*-dimethylformamide; Dmt, 2,6-dimethyltyrosine; EtOAc, ethyl acetate; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HR MS, high resolution mass spectroscopy; MeOH, methanol; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; RP-HPLC, reverse phase high performance liquid chromatography; SAR, structure-activity relationships; SM, starting material; TFA, trifluoroacetic acid; TLC. thin layer chromatography; UV, ultraviolet

Abstract

This protocol describes the gram-scale liquid-phase peptide synthesis of C-terminal modified enkephalin analogues. The C-terminus of enkephalin was modified by a moiety of fentanyl, *N*-phenyl-*N*-(piperidin-4-yl)-propionamide to acquire synergistic effects with mixed opioid receptor activities and to optimize metabolic stability and blood brain barrier penetration with lipophilic moiety substitution. Lead compounds showed high analgesic efficacy in nerve injured animal models with strong binding affinity at mu and delta opioid receptors. The preparation of large quantity of analogues were done by liquid phase peptide synthesis established for long period in the laboratory. Obviously, liquid phase synthesis even with long cycle time and purification step is ideal for the large-scale synthesis of peptides owing to cost effectiveness and easy detection of impurity profile compared to solid phase synthesis. Here the author shows a robotic liquid phase synthesis involving Bop-assisted coupling and Boc-deprotection for the gram-scale synthesis of C-terminal modified enkephalin analogues, which establishes a way to produce peptide drugs cost-effectively.

Introduction

Enkephalin is one of three endogenous opioid peptides that exhibit strong analgesic effects through mu and delta opioid receptors and has two forms, Met-enkephalin and Leu-enkephalin, which contain a Met and a Leu residue in the 5^{th} position, respectively. [1] Despite their high potency, an important role in combating pain, and numerous structure-activity relationship (SAR) studies, enkephalins are still limited as a clinically viable drug because of low bioavailability: low blood-brain barrier penetration and low metabolic stability. [2-9] In an effort to increase the opioid potency and change the overall physicochemical properties, a series of enkephalin analogues were designed and synthesized in which a *N*-phenyl-*N*-piperidin-4-yl propionamide (Ppp) moiety, a part of the fentanyl structure, was attached to the C-terminus (Fig. 1). [10-12] The design was to acquire potential synergistic effects between mu and delta opioid receptors, and to increase lipophilicity, which is a key factor for the BBB penetration, by adding the fentanyl moiety. As the results, highly lipophilic (aLogP: 3 ~ 4) enkephalin analogues were obtained, and **LYS739** was a lead compound to show enhanced opioid potency (IC_{50} = 0.26 and 0.37 nM in the GPI and MVD assay, respectively) at mu and delta opioid receptors. [11] For further bioassays including in vivo tests using pain animal models, the lead compound was synthesized in a gram scale by a robotic liquid phase synthesis involving Bop-assisted coupling and Boc-deprotection as described in detail (scheme 1). [13]

Materials

Reagents

Boc-Gly-OH (aapptec, catalog #ABG101)
Boc-Dmt-OH (Small Molecules, cat #18-4933)
Boc-DNle-OH (Bachem, cat #4007563)
Boc-Phe(F)-OH (aapptec, catalog #UBF121)
(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, aapptec, cat # CXZ001)
Diethylether (EM, cat # EX0190)
1-hydroxy benzotriazole (HOBt, aapptec, cat # CXZ010)
Ethyl acetate (EtOAc, Sigma, cat #270989)
Methanol (MeOH, J. T. Baker, cat# JT9093)
N-methylmorphorine (NMM, Aldrich, cat # M56557)
N,N-dimethyl formamide (DMF, EM, cat # DX1730)
N-phenyl-*N*-(4-piperidinyl)propionamide (Alfa Aesar, cat # H27522-03)
Trifluoroaceticacid (TFA, Aldrich, cat # T6508)
Na_2SO_4 anhydrous (Aldrich, cat # 106637)
Brine solution
5% citric acid solution
5% $NaHCO_3$ solution
Ninhydrin TLC stain solution

Apparatus and equipment

150 mL buchner funnel (Chemglass)
24/40 TS neck round bottomed flasks (Chemglass, 50, 100 & 500 mL)
250 mL Elenmeyer flask (Pyrex)
Filter papers (Whatmann)
100 mL Graduated cylinder
24/40 TS Glass stoppers (Chemglass)
Clamps
Jacks
500 mL separatory funnel (Pyrex)
Stirr bars (Fisher)
TLC plates (Silicycle, cat# TLGR10011B723)
Polypropylene conical centrifuge tubes (BD Falcon15 & 50 mL)
Thermometer (-20 to 110 ^{o}C)
Disposable syringes (1 mL& 5 mL)
600 mL Dewar ice bath (Chemglass)
Magnetic stir/hot plate (Corning)
Rotary evaporator (Buchi)

Vacuum pump (Fisher)
Lyophilizer (Labconco)
HPLC (HP1100)
Analytical C_{18} column (Microsorb-MV, 5 μm, 4.6 x 250 mm)
Preparative-C_{18} column (Vydac218TP, 15-20 μm, 10 x 250 mm)
MS (FAB-MS or MALDI-TOF)

Prodedure

Coupling

1. Add 1.40 g (6.0 mmol) of *N*-phenyl-*N*-(piperidin-4-yl)-propionamide to a 100 mL round bottom flask and dissolve with 30 mL of DMF with a stir bar on a stirrer.
2. Cool the round bottom flask down to 0 ^{o}C in an ice-bath for 10 min.
3. Add 1.86 g (6.6 mmol) of Boc-Phe(F)-OH, 2.92 g (6.6 mmol) of BOP and 0.92 g (6.6 mmol) of HOBt.
4. Add 1.86 mL (13.2 mmol) of NMM dropwise using a syringe for 5 min.
5. Stir for additional 15 min.
6. Remove the ice bath and allow stirring at room temperature (20-25 ^{o}C) for 3 hours.
7. Verify the coupling by TLC under UV and after ninhydrin stain (Fig. 1a). Dip the TLC plate into the ninhydrin stain and heat with a heat gun until brownish color is shown.
   - ❖ **Critical step** Completion of coupling must be confirmed by TLC observing the absence of starting materal (SM)-1.

Workup

8. Concentrate the reaction mixture with a rotavapor.
9. Dilute the concentrated reaction mixture with 100 mL of EtOAc
10. Transfer to a 500 mL separatory funnel.
11. Add 100 mL of 5% $NaHCO_3$ solution and shake vigorously during washing.
    - ❖ **Caution** Vent the separatory funnel several times during shaking
12. Leave the funnel until complete separation of two layers: longer than 30 min
13. Drain lower aqueous layer to a 500 mL Erlenmeyer flask.
14. Repeat the washing steps 11-13 2 more times.
15. Repeat the washing steps 11-13 with 100 mL of 5% citric acid solution 2 times.
16. Repeat the washing steps 11-13 with 100 mL of brine solution.
17. Repeat the washing steps 11-13 with 100 mL of water.
18. Pour the organic layer to a 250 mL Erlenmeyer flask.
19. Add anhydrous Na_2SO_4 (30-40 g) until free flowing to remove residual water.
20. Filter off Na_2SO_4 using a filter paper and collect the filtrate in a 500 mL round-bottom flask.
21. Concentrate the filtrate with a rotary evaporator

Deprotection of Boc-protecting group

22. Add 20 mL of TFA slowly to the residual oil from step 21 at 0 ^{o}C.
23. Stir for 30 min.
24. Confirm deprotection by TLC (Figure 2a) observing the absence of SM **1** both under UV and after ninhydrin stain.

Workup

25. After verification of deprotection, remove the TFA with a rotary evaporator.
26. Co-evaporate the concentrated mixture in 50 mL of toluene 2 times
    - ❖ **Critical step** This is to remove residual TFA, which can interrupt next coupling step.
27. Precipitate the mixture with 100 mL of diethyl ether.
28. Filter the whiter powder and wash with 100 mL of diethyl ether.
29. Collect white powder and dried under vacuum: 2.76 g (yield 90%)

Chain-elongations

30. Add 2.76 g (5.4 mmol) of compound **2** to a 100 mL round bottom flask and dissolve with 50 mL of DMF with a stir bar on a stirrer.
31. Cool the round bottom flask down to 0 ^{o}C in an ice-bath for 10 min.
32. Add 1.05 g (6.0 mmol) of Boc-Gly-OH, 2.65 g (6.0 mmol) of BOP and 0.81 g (6.0 mmol) of HOBt.
33. Add 1.6 mL (12 mmol) of NMM dropwise using a syringe for 5 min.
34. Repeat the coupling steps 5-21 and deprotection steps 22-29 to obtain 2.90 g (yield 94%) of compound **4** as white powder (Fig. 2b).
35. Add 2.90 g (5.1 mmol) of compound **4** to a 100 mL round bottom flask and dissolve with 50 mL of DMF with a stir bar on a stirrer.
36. Cool the round bottom flask down to 0 ^{o}C in an ice-bath for 10 min.
37. Add 1.30 g (5.6 mmol) of Boc-Gly-OH, 2.48 g (5.6 mmol) of BOP and 0.76 g (1.1 mmol) of HOBt.
38. Add 1.5 mL (11.2 mmol) of NMM dropwise using a syringe for 5 min.
39. Repeat the coupling steps 5-21 and deprotection steps 22-29 to obtain 3.12 g (yield 90%) of compound **6** as white powder (Fig. 2c).
40. Add 3.12 g (4.6 mmol) of compound **6** to a 100 mL round bottom flask and dissolve with 50 mL of DMF with a stir bar on a stirrer.
41. Cool the round bottom flask down to 0 ^{o}C in an ice-bath for 10 min.
42. Add 1.55 g (5.0 mmol) of Boc-Dmt-OH, 2.22 g (5.0 mmol) of BOP and 0.68 g (5.0 mmol) of HOBt.
43. Add 1.4 mL (10 mmol) of NMM dropwise using a syringe for 5 min.
44. Repeat the coupling steps 5-21 and deprotection steps 22-29 to obtain 3.01 g (yield 76%) of crude compound **8** as white powder (Fig. 2d).

Sample preparation for RP-HPLC

45. Add 3.01 g of crude compound **8** in a 1 dram glass vial and dissolve with 2 mL of MeOH
46. Filter the solution using a nonsterile 13 mm puradic syringe filter (PVDF, 0.45 µm pore size, PVDF).

Analysis of crude product by RP-HPLC

47. Analyze 20 µL of the solution of crude **8** by RP-HPLC using the gradient solution (10-90% of B within 40 mins) and semi-preparative column.
48. The crude product shows around 80% purity on the RP-HPLC. (Figure 3)

Purification of crude product by RP-HPLC

49. Purify the crude product by RP-HPLC using the gradient solution determined by the analysis result as described in Table 3.

Collection of pure product

50. Combine all the fractions of pure product.
51. Remove acetonitrile by rotary evaporation.
52. Transfer the concentrated solution to 50 mL VWR polypropylene centrifuge tubes.
53. Freeze the solution in a deep freezer (-75 ^{o}C) for 2 hours.
54. Lyophilize the solution overnight.
55. The final product is a white powder.

Validation of pure product

56. Assess the product by mass spectrometry and RP-HPLC as described in Table 3.
57. The product should be a single peak with > 95% purity on analytical RP-HPLC (Fig. 4) and showed correct molecular mass (Fig. 5) on mass spectrometry.

Analytical data

For **LYS739**: HPLC (Fig. 4); ^{1}H-NMR & HRMS [11]

Discussion

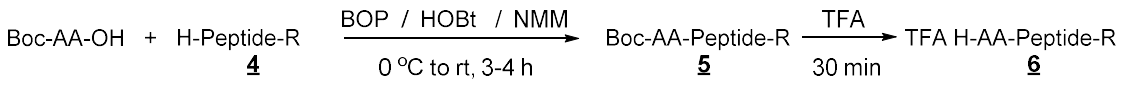
This describes a robotic liquid phase peptide synthesis for large scale production of peptides and involves efficient work-up and trituration which allow purification step simple and cost-effective. Key feature of this protocol is stoichiometric coupling reaction optimized on the basis of the reagent's chemical and physical property so that product can be isolated pure and used for

next step devoid of time consuming purification procedure. This is because after coupling reaction is done by consuming all (1.0 equiv) of a free amine group contained peptide or amino acid, slightly excess amount (1.1 equiv) of the starting materials, Boc-protected amino acids, Bop, and HOBt, can be simply removed during work-up by basic, acidic, and neutral solutions in sequence. The efficient work-up process get rid of excess of starting materials in sequence and afford pure product after simple trituration using diethyl ether or a typical organic solvent. Another key feature of this protocol is the Boc group deprotection using 100% TFA, followed by two times co-evaporation of reaction mixture with toluene to remove extra TFA inside which can cause a problem in next step coupling due to its acidic character. The co-evaporation allows the reaction mixture to be optimized for the next coupling step. It is well agreed that liquid phase synthesis is a better option for large-scale peptide synthesis thanks to the cost effective process. However, one confronts time-consuming process development to optimize every work-up process and reaction condition to obtain pure intermediates and target peptide. Purification step has been a major issue for large scale synthesis of peptide, and our typical purification process depending on the stoichiometric coupling reaction and the efficient work-up can aid the process development to render advantage of cost saving. It is concluded that through more than 10 years studies, each step has been well polished to apply for all the liquid phase peptide synthesis for gram-scale production.

Conflict of Interest

There is no conflict of interest declared.

Table 1. Typical procedure of liquid phase peptide synthesis



used	1.30 g	2.90 g	2.48 g	0.76 g	1.5 mL		50 mL	3.47 g
FW (g)	231[a]	568[b]	442	135	101[c]	668[d]		682[e]
mmol	0.56	0.51	0.56	0.56	1.12			0.51
equiv	1.1	1.0	1.1	1.1	2.2			1.0

[a]Boc-DNle-OH; [b]TFA·H-Gly-Phe(F)-Ppp; [c]d=0.782 (g/mL); [d]Boc-DNle-Gly-Phe(F)-Ppp; [e]TFA H-DNle-Gly-Phe(F)-Ppp

Table 2: Stepwise synthesis of **LYS739**

step	reaction condition	temp	time	product	yield (g)	yield (%)
1	Boc-Phe(F)-OH(1.1 equiv, 1.86 g), Ppp (1 equiv, 1.4 g), BOP (1.1 equiv, 2.92 g), HOBt (1.1 equiv, 0.92 g), NMM(2.2 equiv, 1.86 mL), DMF 30 mL	0 °C to rt	3 h	**1**		
2	TFA	0 °C	30 min	**2**	2.76	90
3	Boc-Gly-OH(1.1 equiv, 1.05 g), **2** (1 equiv, 2.76 g), BOP (1.1 equiv, 2.65 g), HOBt (1.1 equiv, 0.81 g), NMM(2.2 equiv, 1.6 mL), DMF 30 mL	0 °C to rt	3 h	**3**		
4	TFA	0 °C	30 min	**4**	2.90	94
5	Boc-DNle-OH(1.1 equiv, 1.30 g), **3** (1 equiv, 2.90 g), BOP (1.1 equiv, 2.48 g), HOBt (1.1 equiv, 0.76 g), NMM(2.2 equiv, 1.5 mL), DMF 30 mL	0 °C to rt	3.5 h	**5**		
6	TFA	0 °C	30 min	**6**	3.12	90
7	Boc-Dmt-OH(1.1 equiv, 1.55 g), **4** (1 equiv, 3.12 g), BOP (1.1 equiv, 2.22 g), HOBt (1.1 equiv, 0.68 g), NMM(2.2 equiv, 1.4 mL), DMF 30 mL	0 °C to rt	3 h	**7**		
8	TFA	0 °C	30 min	**LYS739**	3.01	76

[a]Used for the next reaction after precipitation

Table 3: Purification and analysis of **LYS739** using RP-HPLC

	Analytical HPLC	Semi-preparative HPLC
Column	C-18, Microsorb-MV, 5 µm, 4.6 mm x 250 mm	C-18, Vydac218TP, 15-20 µm, 10 mm x 250 mm
Solvents	Solution A: water containing 0.1% TFA Solution B: acetonitrile	
Gradient	10 – 90 % of solution B in 40 min	20-60% of solution B in 15 min
Flow rate	1 mL/min	3 mL/min
Wavelength	230 nm	230 nm

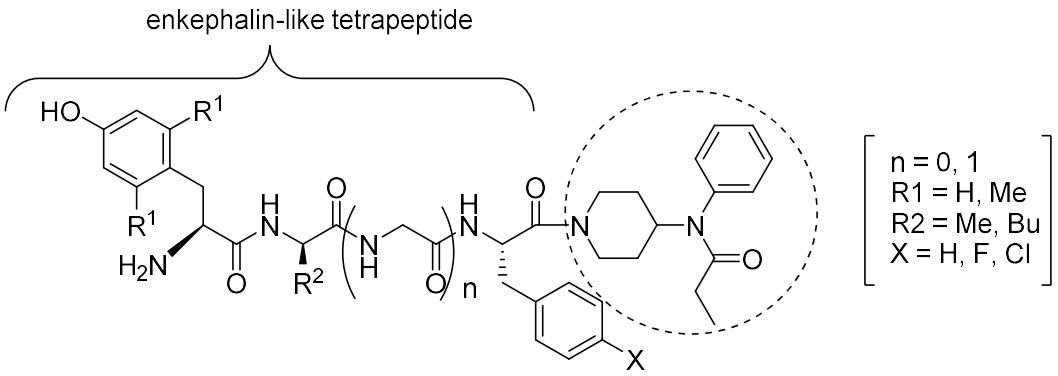


Figure 1. Design of C-terminal modified encephalin analogues

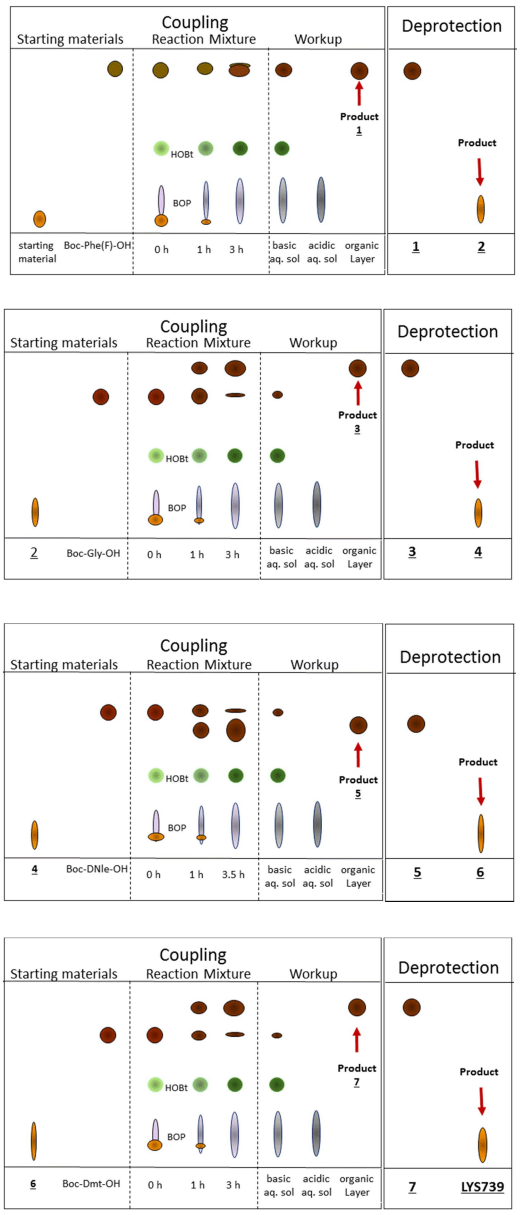


Figure 2. TLC of chain elongations in $CHCl_3$:MeOH:AcOH (90:10:5). Starting material: *N*-phenyl-*N*-(piperidin-4-yl)-propionamide; **1**: Boc-Phe(F)-Ppp; **2**: TFA H-Phe(F)-Ppp; **3**: Boc-Gly-Phe(F)-Ppp; **4**: TFA H-Gly-Phe(F)-Ppp; **5**: Boc-DNle-Gly-Phe(F)-Ppp; **6**: TFA H-DNle-Gly-Phe(F)-Ppp; **7**: Boc-Dmt-DNle-Gly-Phe(F)-Ppp; **LYS739**: TFA H -Dmt-DNle-Gly-Phe(F)-Ppp

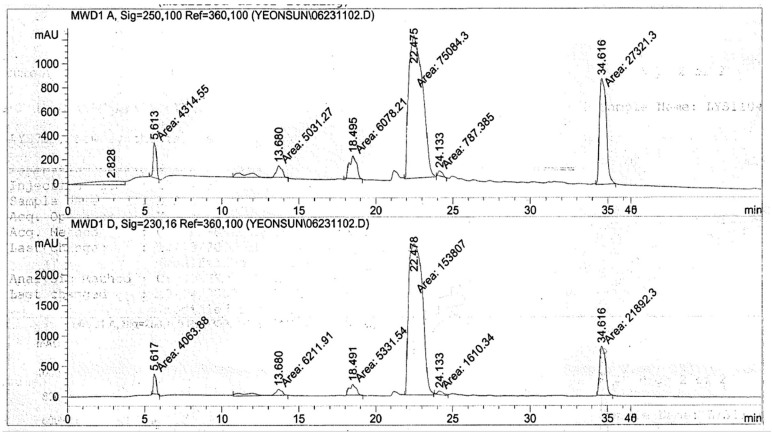


Figure 3. HPLC-profile for crude product **LYS739**. Analysis was run on semi-preparative HPLC conditions described in Table 3.

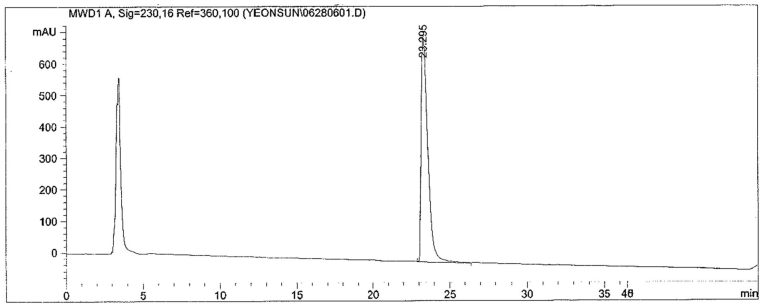
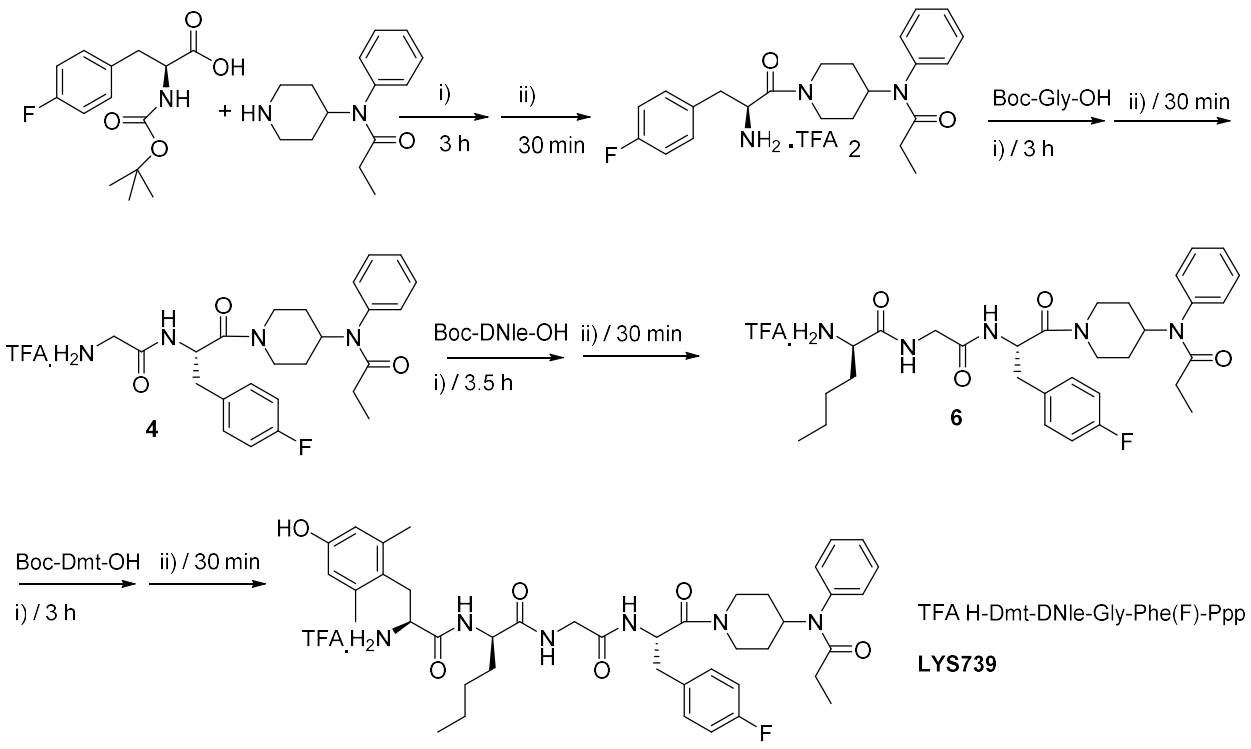


Figure 4. Evaluation of **LYS739**. Analytical RP-HPLC profile shows single peak at 23. 5 min, which was run as described in Table 3.



Scheme 1. Stepwise Synthesis of compound **LYS739**: i) BOP/HOBt/NMM (1.1 equiv/1.1 equiv/2.2 equiv), DMF, 0 ^{o}C to rt; ii) TFA, 0 ^{o}C

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