Small µmol Scale Synthesis of a Labeled Antimicrobial Peptide using Biotage® Initiator+ Alstra™

Introduction

Labeled peptides are important tools to study molecular interactions[1] and are increasingly utilized with the growing number of peptide-based therapeutics.[2] Biological assays frequently require imaging agents to study drug molecules and fluorescent labels are often employed for this purpose, but can be expensive and thus present a cost barrier for synthesis. In an effort to demonstrate the cost savings by employing small µmol scale, automated solid phase peptide synthesis through robot liquid handling of reagents with accurate dispensing of small volumes, a 10-mer antimicrobial peptide[3] **1** was synthesized and labeled with 5(6)-carboxyfluorescein (FAM) at the *N*-terminus.

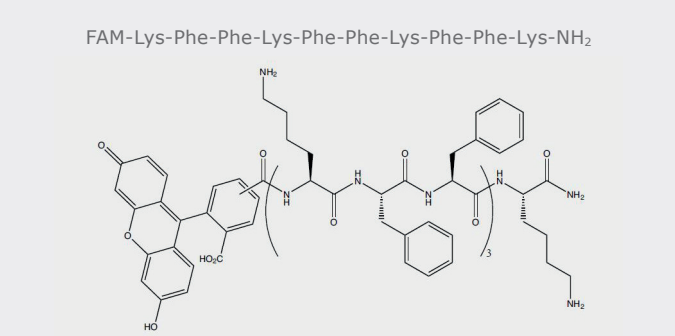


Figure 1. Sequence and structure of the fluorescently labeled antimicrobial peptide **1**.

Experimental

Materials

All materials were obtained from commercial suppliers; Fisher (*N,N*-diisopropylethylamine (DIPEA)), Biotage® (Rink Amide-ChemMatrix® resin), Sigma-Aldrich (5(6)-carboxyfluorescein, HPLC grade water, diisopropylcarbodiimide (DIC), 4-methyl-piperidine, *N*-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), triisopropylsilane (TIS) and dichloromethane (DCM)), Novabiochem (N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-amino acids (Fmoc aa), *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)), ChemPep (*N*-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU)), Anaspec (hydroxyben-zotriazole (HOBt)), and VWR (*N,N*-dimethylformamide (DMF), methanol (MeOH), acetonitrile)).

Peptide Synthesis and Analysis

The peptides [FAM-KFFKFFKFFK-NH_2 (MW = 1772.1) and KFFKFFKFFK-NH_2 (MW = 1412.8)] were prepared by Fmoc solid-phase peptide synthesis on a Biotage® Initiator+ Alstra™ microwave peptide synthesizer. The syntheses were carried out on Rink Amide-ChemMatrix® resin (loading 0.49 mmol/g) at 0.005, 0.006, 0.105 and 0.125 mmol scales. Micromole scale syntheses (5 and 6 µmol) were performed in a 5 mL reaction vessel and low mmol scale syntheses (0.105 and 0.125 mmol) were carried out in a 10 mL reaction vessel.

N^{α}-Fmoc deprotection was performed at room temperature (RT) in two stages by treating the resin with 4-methylpiperidine/DMF (1:4) for 3 min followed by 4-methylpiperidine/DMF (1:4) for 10 minutes. The resin was then washed with DMF (x 4). Peptide couplings were performed as described in Table 1 and washing steps were omitted after coupling steps.

Peptides were cleaved from the resin using a cocktail of TFA-TIS-H_2O (95:2.5:2.5) for 2 h at room temperature. The TFA was removed by evaporation with the Biotage® V-10 evaporation system and the resulting residue was washed with cold diethyl ether. Crude peptides were analyzed on an Agilent 1100 Infinity series HPLC-MS equipped with a Biotage® Resolux™ 120 C18 column (2.1 x 250 mm). The following solvent system was used: solvent A, water containing 0.1% TFA; solvent B, acetonitrile containing 0.1% TFA. The column was eluted using a linear gradient from 5–65% of solvent B over 60 min.

Synthesis ID	Peptide sequence	Scale (mmol)	Reagents	Conc. (M)	Vol. (µL)	Equiv.(eq.)	Coupling Conditions
A	FAM-$(KFF)_3K$- NH_2	0.005	Fmoc aa	0.2	100	4	75 °C, 5 min x1
			FAM	0.2	100	4	
			DIC	0.05	400	4	
			Oxyma	0.05	400	4	
B	$(KFF)_3K$- NH_2	0.005	Fmoc aa	0.2	125	5	75 °C, 5 min x1
			HBTU	0.2	123	4.9	
			HOBt	0.2	125	5	
			DIPEA	0.2	250	10	
C	$(KFF)_3K$- NH_2	0.005	Fmoc aa	0.2	125	5	75 °C, 5 min x2
			HBTU	0.2	123	4.9	
			HOBt	0.2	125	5	
			DIPEA	0.2	200	10	
D	FAM-$(KFF)_3K$- NH_2	0.006	Fmoc aa	0.2	150	5	Amino acid couplings: 75 °C, 5 min x2, FAM coupling: 75 °C, 5 min x1
			FAM	0.2	150	5	
			HATU	0.2	147	4.9	
			DIPEA	0.2	300	10	
E	$(KFF)_3K$- NH_2	0.125	Fmoc aa	0.5	1250	5	75 °C, 5 min x2
			HATU	0.5	1225	4.9	
			DIPEA	2	625	10	
F	FAM-$(KFF)_3K$- NH_2	0.005*	FAM	0.2	125	5	75 °C, 5 min x1
			DIC	0.2	125	5	
			Oxyma	0.2	125	5	
G†	FAM-$(KFF)_3K$- NH_2	0.105	Fmoc aa	0.4	1313	5	75 °C, 5 min x2
			HATU	0.4	1286	4.9	
			DIPEA	2	525	10	
			FAM	0.4	1313	5	75 °C, 5 min x1
			DIC	0.4	1313	5	
			Oxyma	0.4	1313	5	

* 0.005 mmol portion of peptide on resin from synthesis E was removed and to this was coupled FAM at the N-terminus.

† Fmoc-aa were coupled using HATU/DIPEA, FAM was coupled using DIC/oxyma.

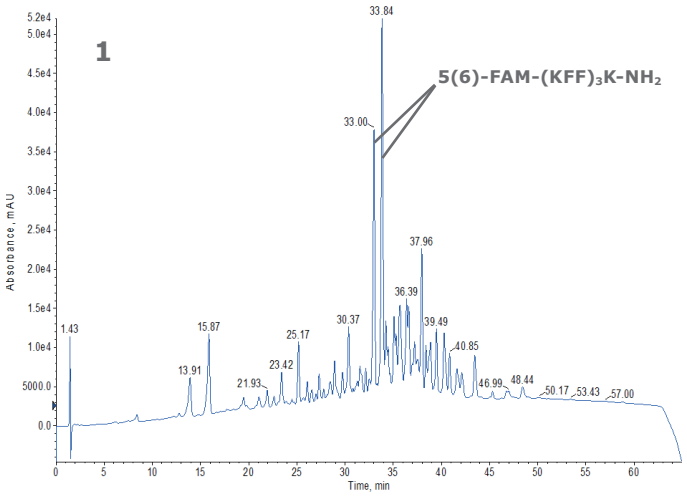
Table 1. Solid-phase peptide synthesis coupling conditions.

Results & Discussion

The desired peptide **1** has two peaks corresponding to each of the regioisomers of the fluorophore 5(6)-FAM. Small scale (5 µmol), fully automated synthesis of the labeled peptide using single couplings employing 4 eq. of Fmoc aa, DIC and oxyma (1:1:1) resulted in several peaks upon chromatographic analysis (Synthesis A, Chromatogram 1) due to the low final concentration of reactants.

The $(KFF)_3K$-NH_2 peptide was initially synthesized using HBTU/HOBt/DIPEA (4.9:5:10 eq.) with single couplings (Synthesis B, Chromatogram 2) and double couplings (Synthesis C, Chromatogram 3) resulting in one major peak that corresponded to the desired product. The double couplings provided a slightly cleaner chromatogram, thus double couplings were adopted for the subsequent syntheses of this particular peptide.

Since HATU recently became available at prices similar to HBTU, subsequent syntheses were carried out employing double couplings with HATU and DIPEA at 4.9 and 10 eq., respectively (Chromatogram 4).



Chromatogram 1. Labeled peptide **1** FAM-$(KFF)_3K$-NH_2, synthesized using conditions from Table 1 Synthesis A.

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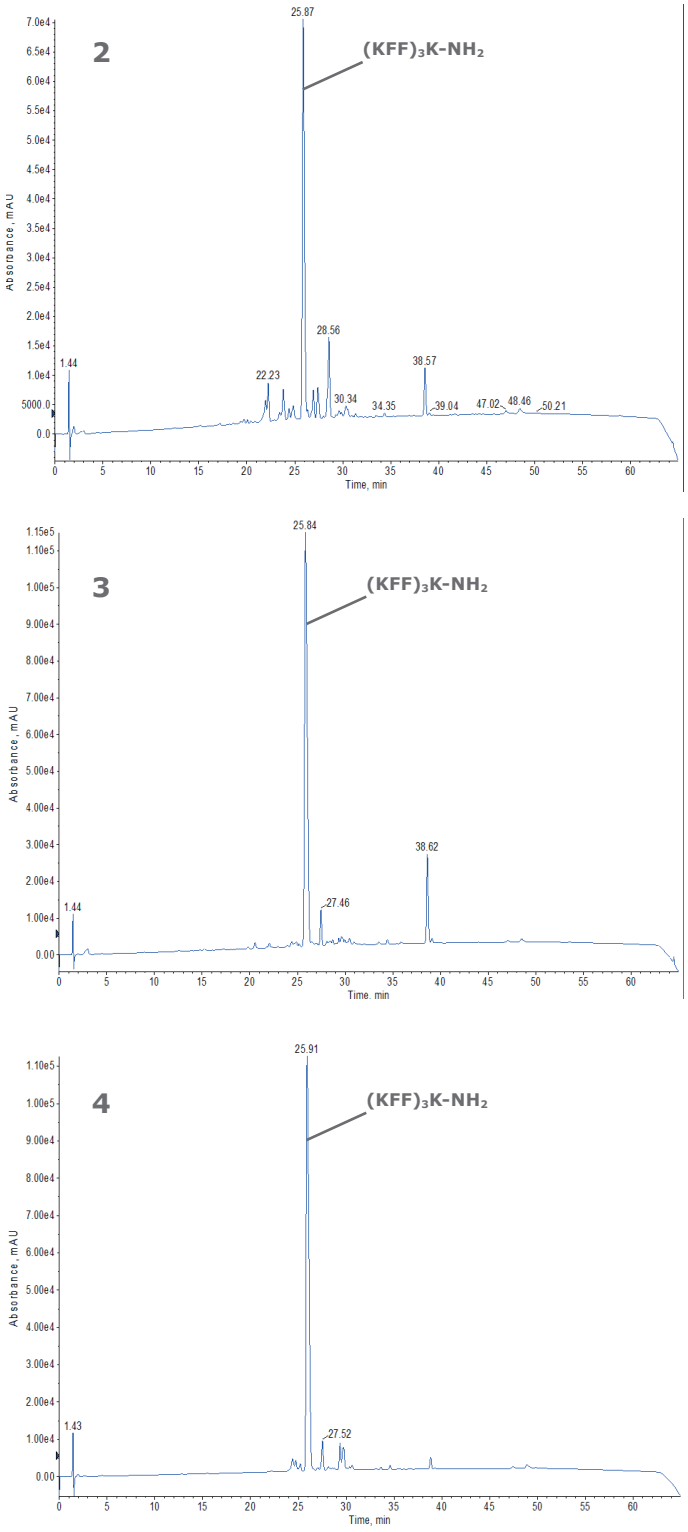
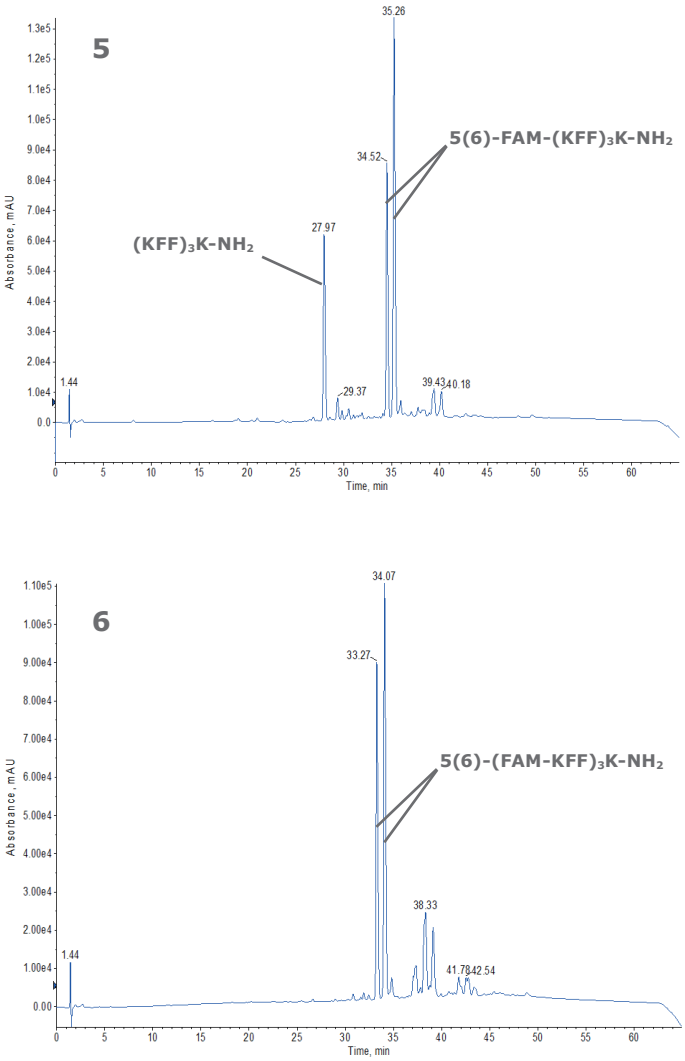


Chromatograms 2–4. Peptide $(KFF)_3K\text{-}NH_2$), synthesized using conditions from Table 1 entries B (2), C (3) and E (4).

A significant amount of starting material was observed in the 6 µmol scale synthesis of the labeled peptide when a single coupling of the fluorophore 5(6)-FAM was perfomed with HATU/DIPEA (Synthesis D, Chromatogram 5). In order to expedite the investigation of a variety of fluorophore coupling conditions, the $(KFF)_3K\text{-}NH_2$ peptide was synthesized at the 0.125 mmol scale (Synthesis E) then split to provide 5 mmol aliquots with which a single fluorophore coupling could be performed.



Chromatograms 5–6. Peptide **1** FAM-$(KFF)_3K\text{-}NH_2$, synthesized using conditions Synthesis D (5) and Synthesis F (6).

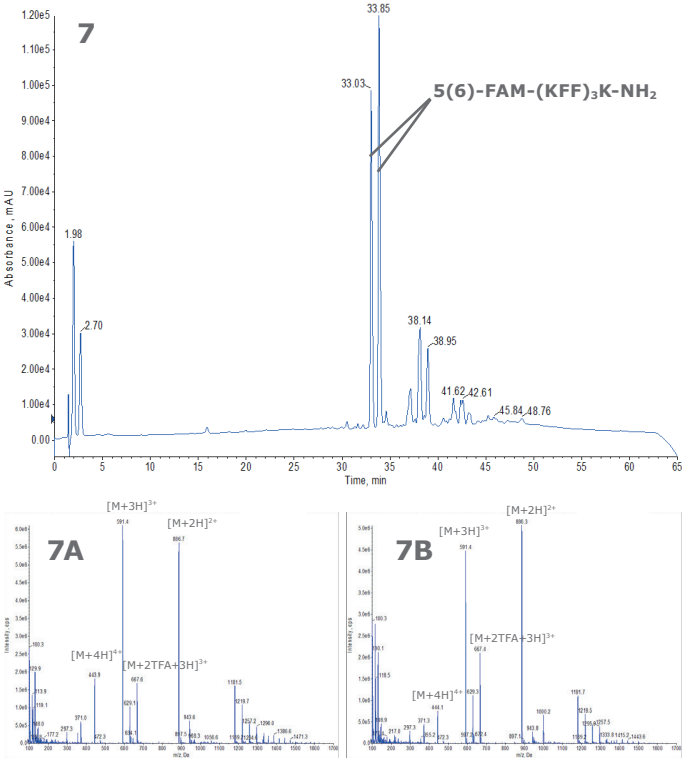
Although the synthesis employing DIC/oxyma resulted in several minor side products, it was noted that the majority of starting material was converted to labeled peptide **1** after a single coupling using 4 eq. of FAM (1:1 with DIC/oxyma) (Synthesis A, Chromatogram 1). Thus, DIC/oxyma was examined in the 5 µmol test condition. A single coupling of fluorescein using DIC/oxyma (1:1) at 5 eq. provided full conversion to the desired peptide (Synthesis F, Chromatogram 6).

		6 µmol		0.105 mmol	
Reagent	**List price /Unit**	**Mass/Volume required**	**Cost for synthesis (USD)**	**Mass/Volume required**	**Cost for synthesis (USD)**
DMF (Total to wash and dissolve reagents)	$561/4x4 L	690 mL	24.20	911.33 mL	31.95
NMP	$205/2L	N/A	N/A	6.223 mL	0.64
HATU	$160/100 g	0.231 g	0.37	3.341 g	5.35
DIPEA	$280/500 mL	0.212 mL	0.12	3.327 mL	1.86
DIC	$222/100 g	N/A	N/A	0.087 mL	0.19
Oxyma	$79/100 g	N/A	N/A	0.08 g	0.06
Fmoc-Lys(Boc)-OH	$182/100 g	0.108 g	0.20	1.495 g	2.72
Fmoc-Phe-OH	$60/100 g	0.147 g	0.09	2.253 g	1.35
5(6)-Carboxyfluorescein	$36.7/1 g	0.019 g	0.70	0.213 g	7.82
5-Carboxyfluorescein	$83.6/0.05 g	0.019 g	31.77	0.213 g	356.14
4-Methylpiperidine	$90.2/0.5 L	34 mL	6.13	47.7 mL	8.61
Rink Amide ChemMatrix™	$988/25 g	0.012 g	0.48	0.214 g	8.45
Total reagent cost with 5(6)-Carboxyfluorescein			**$32.29**		**$69**
Total reagent cost with 5-Carboxyfluorescein			**$63.36**		**$417.32**

Table 2. Cost of automated synthesis of FAM-$(KFF)_3K$-NH_2 at 6 µmol and 0.105 mmol scales.

After optimizing the conditions for the synthesis of the labeled peptide at the low micromole scale, a fully automated protocol that included the peptide synthesis as well as fluorophore labeling, was programmed and run at the 0.105 mmol scale (Synthesis G, Chromatogram 7).

Table 2 shows the cost of the synthesis of the 5(6)-FAM labeled peptide **1** at the 6 µmol scale ($32.29 USD) and at the 0.105 mmol scale ($69). While it is over twice the cost to synthesize this peptide at the larger scale using mixed regiosomeric FAM, the difference is ampilifed if one accounts for the synthesis of the peptide using a single regioisomer of FAM. At the 6 µmol scale, the synthesis using 5-carboxyfluorescein is $63.36 while synthesis of the same peptide at the 0.105 mmol scale is $417.32.



Chromatogram 7 and Mass spectra. Peptide **1** FAM-$(KFF)_3K$-NH_2, synthesized using conditions from Table 1 Synthesis G. Corresponding mass spectra t_R = 33.1 min (7A) and 33.9 min (7B).

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Conclusion

The ability to perform small scale peptide synthesis (from as low as 5 µmol) on Initiator+ Alstra saves on reagent costs when optimizing chemistry. Here we have demonstrated the synthesis of the labeled antimicrobial peptide **1** at low micromole scales (5 µmol) during the optimization process then scaled up (0.105 mmol) to obtain the desired product.

The cost of synthesizing the labeled peptide at the 6 µmol scale is approximately half the cost compared to the 0.105 mmol scale when using 5(6)-FAM and 6.5-fold less when using the single regioisomer 5-FAM. As such, the ability to perform low micromole scale syntheses of peptides during optimization of the synthetic conditions saves on reagent cost.

The completely flexible reagent setup on Initiator+ Alstra enabled the use of multiple coupling reagents and differing concentrations and equivalents of reagents to be used during a single fully automated synthesis of the labeled peptide **1**, and was easily adjusted and customized for the various synthesis scales performed. Initiator+ Alstra uses a robot liquid handler and digital syringe pumps which allows reagent volumes as low as 100 µl to be dispensed, thereby reducing costs and conserving expensive reagents, which lends itself to the small scale synthesis of peptides incorporating non-standard building blocks or other modifications.

References

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2. Kaspar, A. A; Reichert, J. M; *Drug Discov. Today*, **2013**, *18*, 807-817.
3. Vaara, M.; Porro, M.; *Antimicrob. Agents. Chemother.* **1996**, *40*, 1801-1805.

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