Towards peptide libraries: HPLC-based flow peptide synthesis and combinatorial chemistry

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Introduction

Solid-phase peptide synthesis (SPPS) is one of the most widely used methods to produce peptide drug molecules, but in its current form it poses a significant environmental burden and faces several efficiency limitations. The synthesis of so-called “difficult sequences” is particularly challenging, since under the synthesis conditions the growing polymer chains aggregate in an undesirable manner to form β-pleated sheets, which impairs the predictability and efficiency of the process. The recent emergence of more efficient and tightly controlled synthesis methods creates an opportunity to optimize the peptide synthesis process to be faster, cheaper and more environmentally friendly.
With a focus on keeping costs as low as possible and making amino acid coupling increasingly environmentally friendly, we are continuously improving our applied methods. We call this approach Smart Peptide Chemistry in Flow, or SPF for short. This strategy considers the size of the environmental footprint, as well as the synthesis time, reagent volume, and solvent type, all of which are optimized simultaneously. In our previous articles, we have shown that flow chemistry can significantly reduce solvent waste.

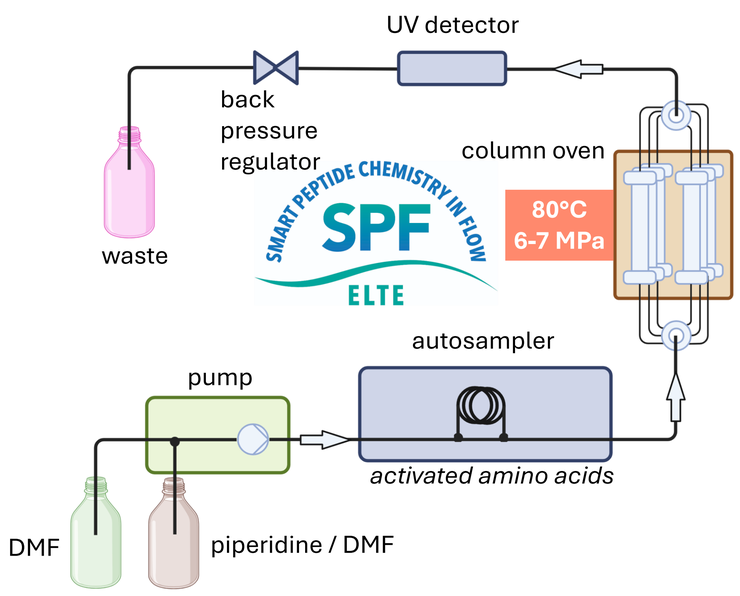
Goal

In this new project, we are interested in whether it is possible to produce **multiple peptides simultaneously in the same synthesis time** by trying to attach multiple amino acids to a given site in the sequence. If we succeed in producing multiple peptides, is this method suitable for producing peptide derivatives that **we can then purify**? Additional questions arise when trying to attach multiple amino acids at once, such as how much they "compete" for the given site.

Aim

Our next research direction is to investigate the efficiency of coupling reactions under flow conditions. Our goal is to analyze the different reaction rates of individual activated reagents in amino acid mixtures. Different amino acid derivatives participate in coupling reactions in different ways, so we need to understand this thoroughly to design effective experiments.
As a long-term goal, we are investigating the possibility of creating peptide libraries that can be used to study the simultaneous synthesis of large numbers of molecules in a single pot.

Schematic diagram of our system





Our system consists of **standard JASCO HPLC modules**, a PU4180 pump with RCY-4000 unit, AS4150L autosampler, UV4075 UV detector, CO4060 column oven. It is a commercially available flow peptide instrument (HPPS-4000, METALON Ltd., Hungary). The ChromNAV2 software ensures a fully automated process.

Residence time:

coupling:	3.30 min. (1.00 ml activated amino acid / NMP)
washing #1:	4.70 min. (1.41 ml DMF)
Fmoc-cleavage:	1.40 min. (1.05 ml 20% piperidine / DMF)
washing #2:	2.60 min. (2.43 ml DMF)
Cycle time:	**12 min.**

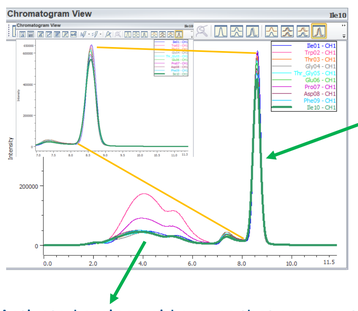
Synthesis of10-mer peptide in resin: less than 2.5 hours

We selected two sequences: one easy and one difficult to synthesize:

fkp1:
H-IFDPETGTWI-NH_2

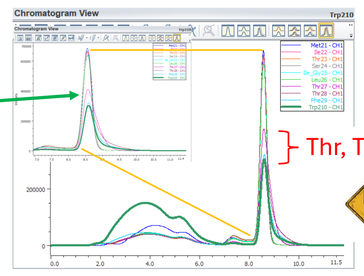
JR10:
H-WFTTLISTIM-NH_2

UV spectra of amino acids coupling



FMOC cleavage

UV spectra of amino acids coupling



Thr, Thr, Phe, Trp

DANGER ZONE



Activated amino acid excess that came out of the column.

Note: At the peak of Fmoc cleavage, it can be seen that after the first Thr coupling, the free amino group becomes difficult to access and the efficiency of coupling the remaining amino acids is greatly reduced.

In the case of the **easy sequence**, we were interested in:
- at what position the coupling of multiple amino acids could be applied so that it could be purified;
- whether the individual amino acids compete with glycine, that is, they couple better or worse if we inject them at the same time.

In the case of the difficult sequence, we will investigate how glycine competes with the original amino acid as we approach the DANGER ZONE and and how this influences overall synthesis.

fkp1 IFDPETGTWI	JR10 WFTTLISTIM
$\frac{A}{G}$FDPETGTWI	WFTTL$\frac{I}{G}$STIM
Ac-$\frac{A}{G}$FDPETGTWI	WFTT$\frac{L}{G}$ISTIM
IFDPE$\frac{A}{G}$GTWI	WFT$\frac{T}{G}$LISTIM
IFDPE$\frac{R}{G}$GTWI	all peptides have an amidated C-terminus
IFDPE$\frac{T}{G}$GTWI	
IFDPE$\frac{I}{G}$GTWI	
IFDPE$\frac{V}{G}$GTWI	

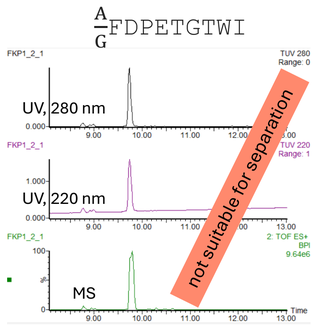
Synthesis:

150 mg of TentaGel resin with a capacity of 0.24 mmol/g
Coupling: AA/OxymaPure/DIC – 3/3/6
Cleavage: 95%TFA/2.5% TIS/2.5% H_2O

Results

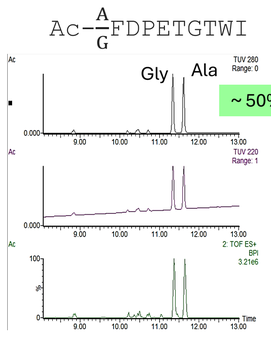
The cleaved products of syntheses were analyzed by UPLC-MS on an ACQUITY UPLCR BEH T3 1.8µm column (Waters, 100 mm × 2.1 mm, 100 A) using a gradient elution consisting of 0.1% TFA in water (eluent A) and 0.1% TFA in MeCN (eluent B) with a gradient from 25% to 65% B over 8 min and the column temperature set at 45 ◦C. The flow rate was set to 0.20 mL/min, and the absorbance was detected at λ = 220 and 280 nm. MS: Waters ACQUITY RDa Detector and on UPLC: Waters ACQUITY UPLC H-Class PLUS System using UNIFI 1.9.13.9 (Waters Corporation, Milford, UK)

$\frac{A}{G}$FDPETGTWI

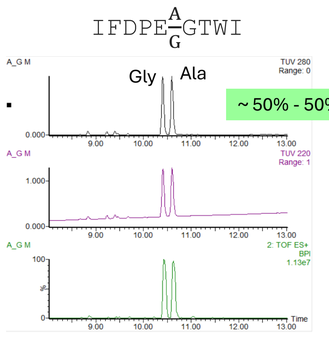


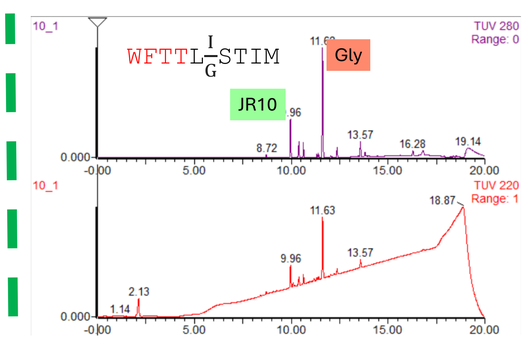
+Ac

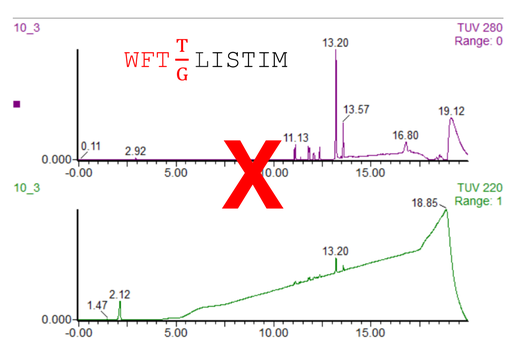
Ac-$\frac{A}{G}$FDPETGTWI

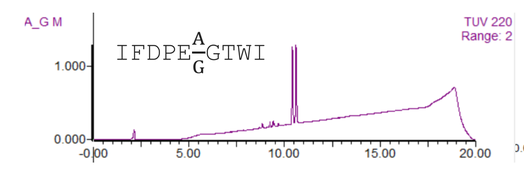


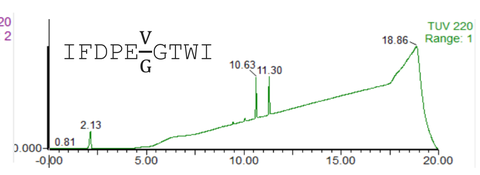
IFDPE$\frac{A}{G}$GTWI

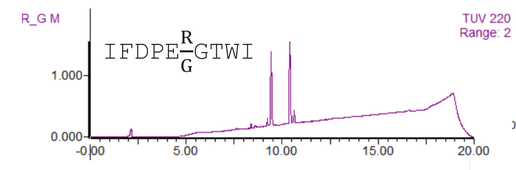


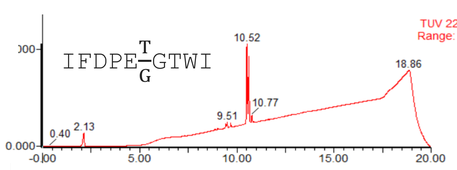


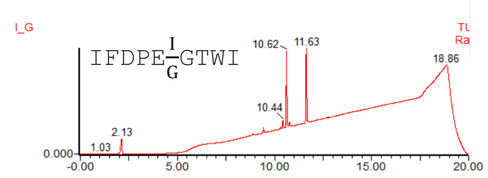




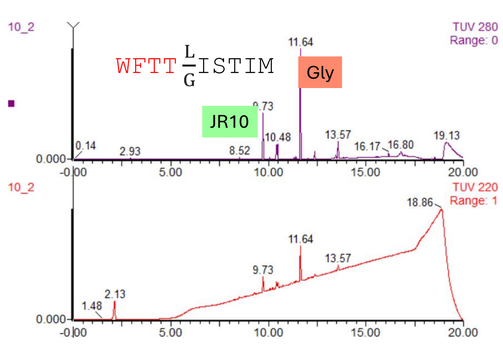








Based on the results of the easy sequence, we can say that if we want to produce several peptides during one synthesis time, then the successful production may be position-dependent. This mainly affects the purification step. It may happen that the applied chromatography method after cleavage is not suitable for separation. In our case, in the 5th position of the **fkp1** peptide, there seems to be no "competition" between glycine and the amino acids tested so far.



In the case of the difficult sequence, the result was not 50-50%, but the glycine-containing peptide was formed in a higher proportion. In the section most exposed to aggregation, we were unable to synthesize any detectable amount of peptide. Neither JR10 nor any glycine-containing molecule.

During synthesis, if one amino acid reacts at a higher rate than the other, then more of that amino acid remains and could be used to synthesize another peptide by attaching a modular column.

Modular column assembly in use, designed by Kristóf.



ReactChemEng **2024**, *9*: 58
OPRD **2023**, *27*: 1053
IJMS **2024**, *25*: 4150
OPRD **2021**, *25*: 182
ChemEurJ **2025**, *31*: e02335

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