Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information for

Real-Time Monitoring of Solid-Phase Peptide Synthesis Using a Variable Bed Flow Reactor

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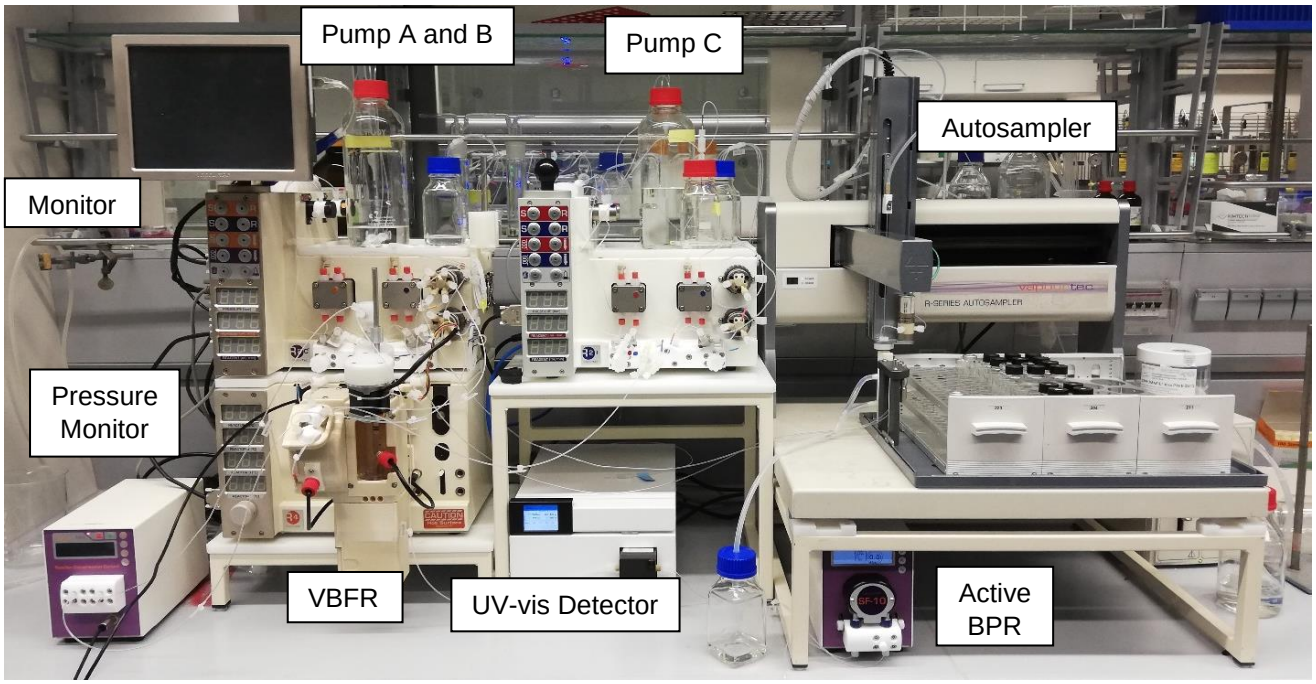
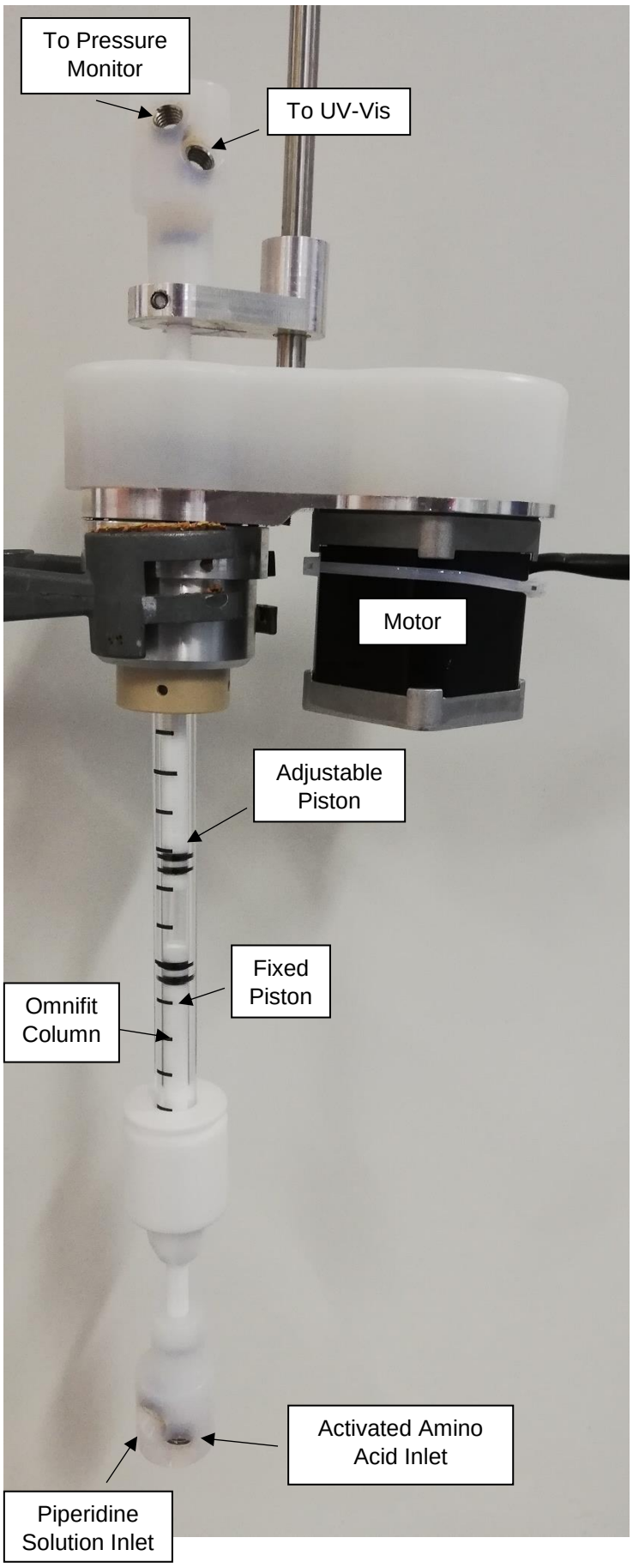
General Information

All reagents and solvents were acquired from commercial sources, unless stated otherwise. Fmoc amino acid building blocks and coupling reagents are from Iris Biotech GmbH. HPLC grade solvents were used. Resins were acquired from the following: MBHA-RAM and 2-Chlorotrityl chloride PS (Novabiochem®), Cl-TCP(Cl) ProTide Resin (CEM), TentaGel HL RAM (Rapp Polymere). (ESI-HRMS were performed with a Xevo G2-XS Q-Tof (Waters). Analytical HPLC was run on an Agilent instrument using an analytical column (Hydrosphere C18, 50x3.0mm.ID. S-3µm, 12nm, flow rate 0.5mL/min, 38 °C, gradient=5 to 70% B over 17 min, Solvent A=0.1% FA in water and solvent B=0.1% FA in acetonitrile, DAD=258 nm or 214 for FF03). UV-vis and VBFR data were processed using Origin software.

Additional Figures




To Pressure Monitor
To UV-Vis
Motor
Adjustable Piston
Fixed Piston
Omnifit Column
Activated Amino Acid Inlet
Piperidine Solution Inlet

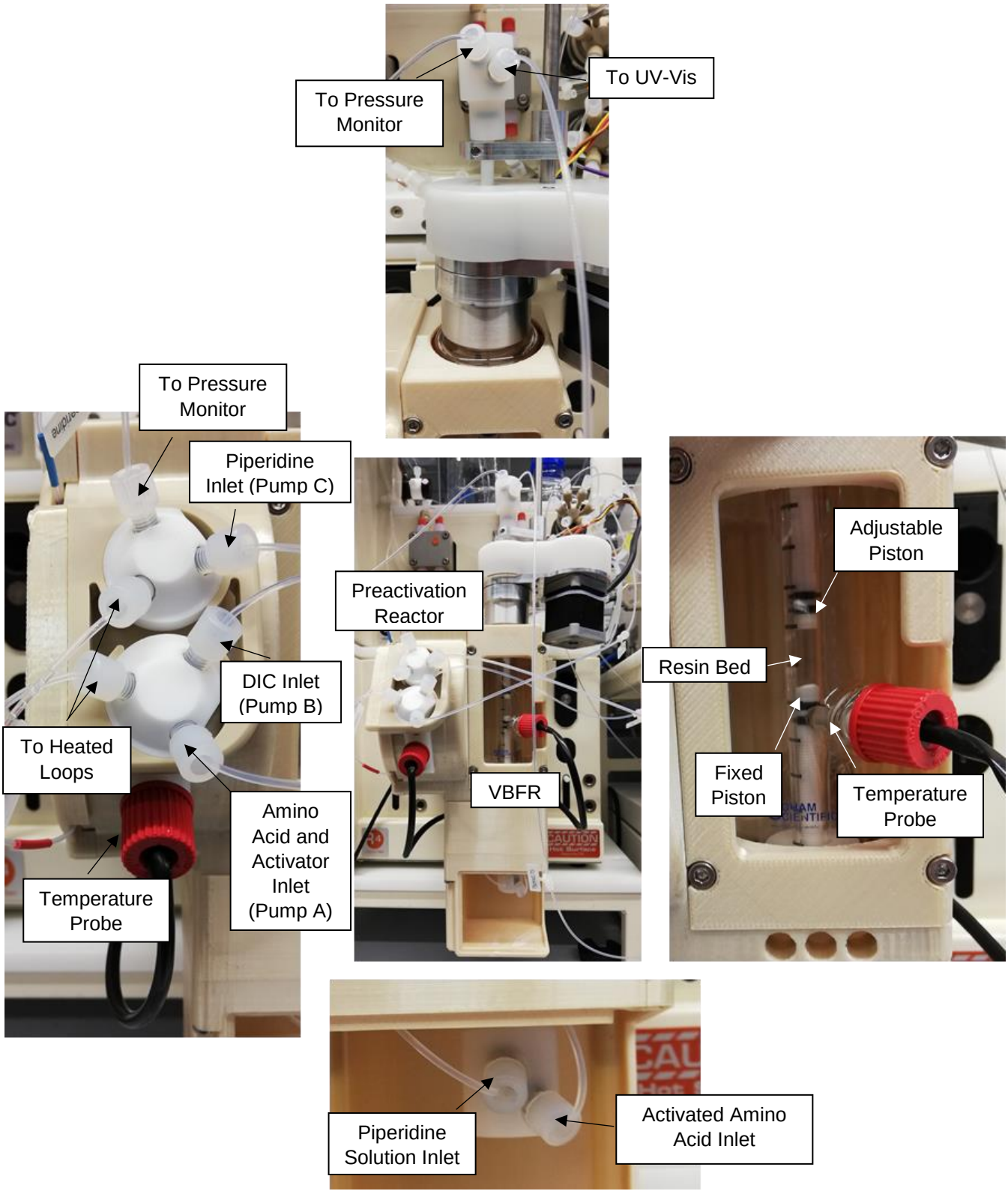


Figure S1. Automated VBFR-SPPS set-up.

Further details and diagrams of the VBFR can be found at Guthrie, D.; Nikbin, N.; Clarkson, A. Continuous Flow Reactor. International Application No.: PCT/EP2018/060354, April 23, 2017.

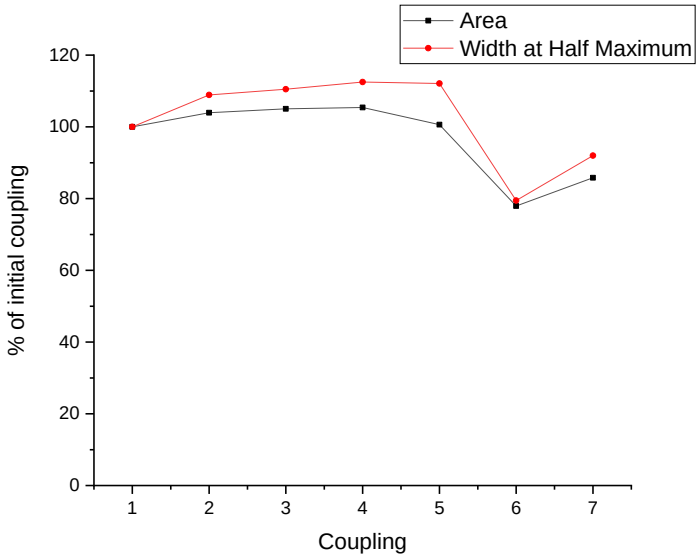
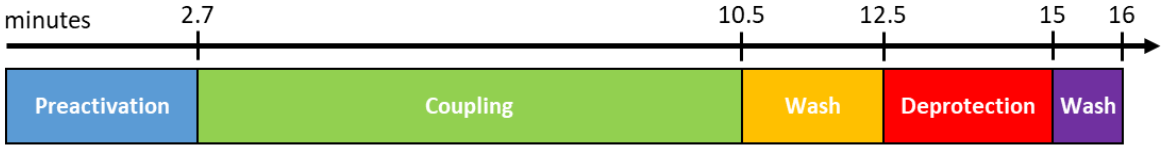


Figure S2. UV absorption data for the Fmoc deprotection during the AFLAFLA synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.

Table S1. Typical peptide coupling-deprotection cycle.



Preactivation/Coupling	Wash	Deprotection	Wash
Solution 1: 0.24 M Amino Acid and 0.24 M Activator in DMF **Solution 2:** 0.24 M DIC in DMF **Amount:** 2 mL of each solution **Flow Rate:** 0.7 mL/min **Temperature:** 80 °C	**Reagent:** DMF **Amount:** 4 mL **Flow Rate:** 8 mL/min **Temperature:** 80 °C	**Reagent:** 10% Piperidine in DMF **Amount:** 4 mL **Flow Rate:** 2.7 mL/min **Temperature:** 80 °C	**Reagent:** DMF **Amount:** 4 mL **Flow Rate:** 4 mL/min **Temperature:** 80 °C

Table S2. Synthesis of simple peptide sequence using different resins and activating reagents. Resin loading: MBHA-RAM = 0.59 mmol/g, TentaGel RAM = 0.39 mmol/g.

Sequence:	AFLAFLA	AFLAFLA	AFLAFLA
Resin:	MBHA-Rink Amide	MBHA-Rink Amide	TentaGel RAM
Activator (0.24 M in DMF):	HOBt	Oxyma Pure	HOBt
Carbodiimide (0.24 M in DMF):	DIC	DIC	DIC
Equiv. of Building Block:	4.0	4.0	6.0
Crude Yield:[a]	94%	90%	92%
HPLC Purity:	92%	88%	97%

[a]Crude yield corresponds to cleaved product without any purification.

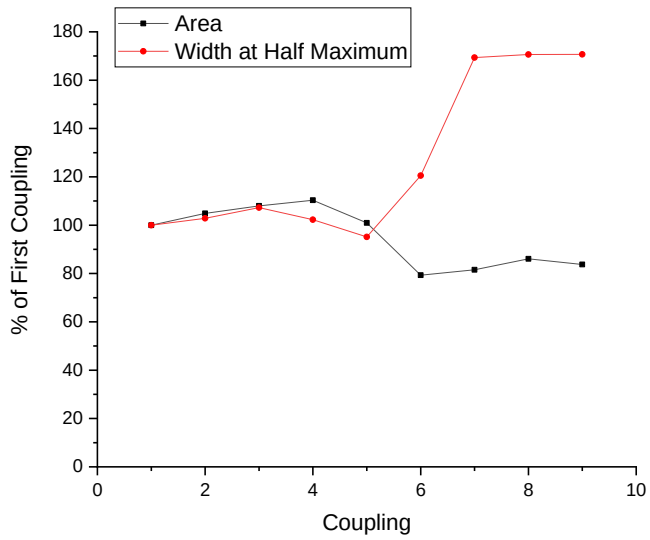


Figure S3. UV absorption data for the Fmoc deprotection during the oligio-alanine synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.

Sequence:	NFGAIL	FF03	TfR-Peptide
Resin:	MBHA-Rink Amide	Fmoc-Leu-2-Cl-trityl Protide	Fmoc-Pro-2-Cl-trityl PS
Activator (0.24 M in DMF):	HOBt	Oxyma Pure	Oxyma Pure
Carbodiimide (0.24 M in DMF):	DIC	DIC	DIC
Resin Loading (mmol/g):	0.59	0.19	0.18
Crude Yield:[a]	84%	<85%[b]	73%
HPLC Purity:	87%	91%	79%

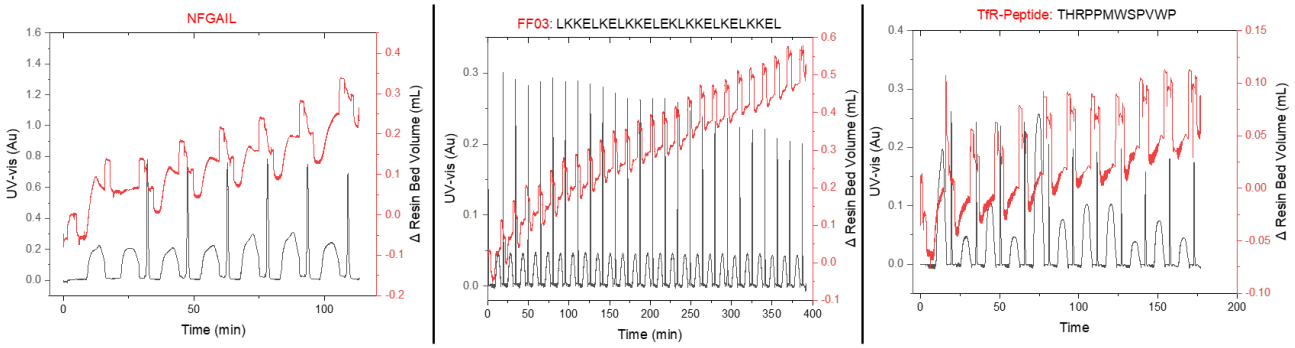


Figure S4. Synthesis of challenging peptide sequences (Δ resin bed volume = red, UV-vis = black) by automated VBFR-SPPS. Further UV-vis data analysis can be found in the Supplementary Information. [a]Crude yield corresponds to cleaved product without any purification. [b] Formation of lysine and glutamic acid salts proved difficult to determine an accurate yield. UV-vis measured at 360 nm.

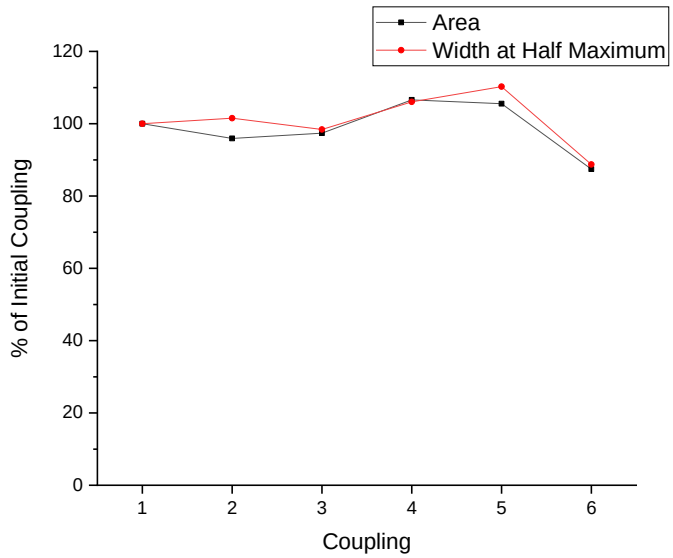


Figure S5. UV absorption data for the Fmoc deprotection during the NFGAIL synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.

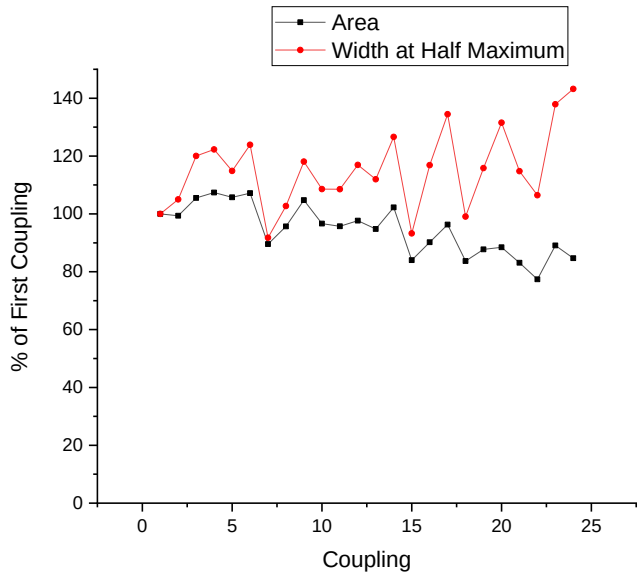


Figure S6. UV absorption data for the Fmoc deprotection during the FF03 synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.

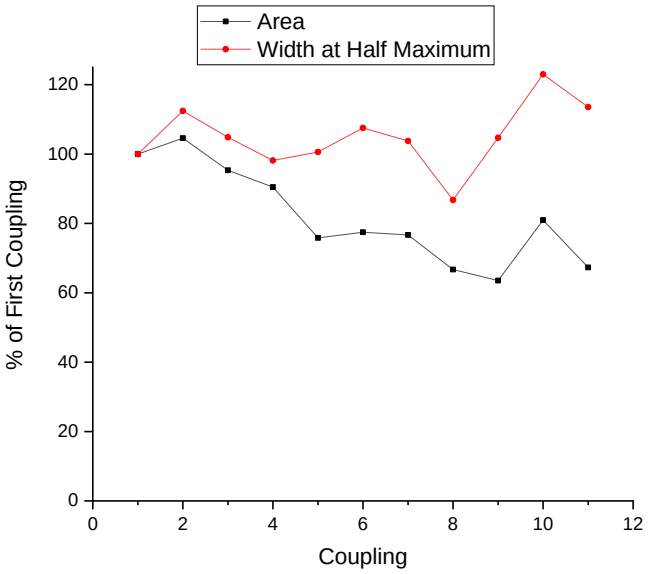
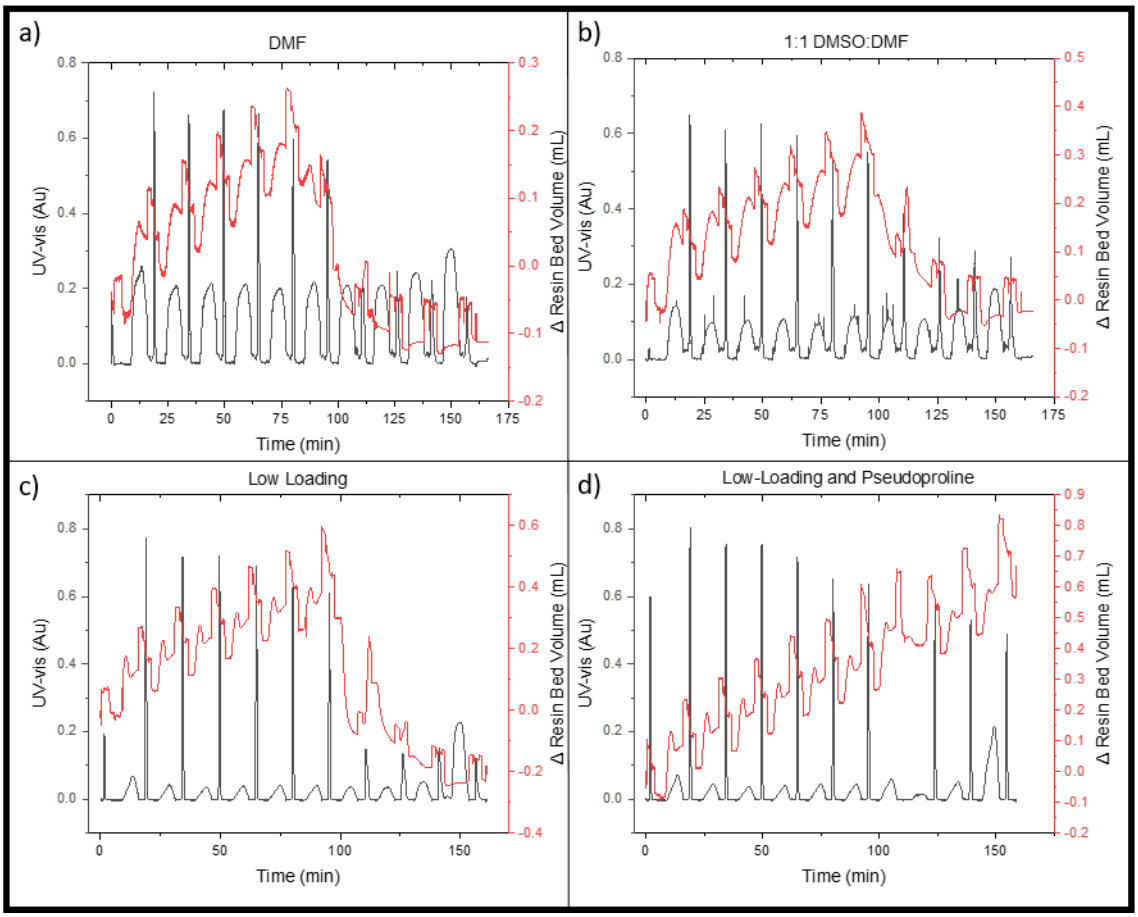


Figure S7. UV absorption data for the Fmoc deprotection during the TfR-peptide synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.



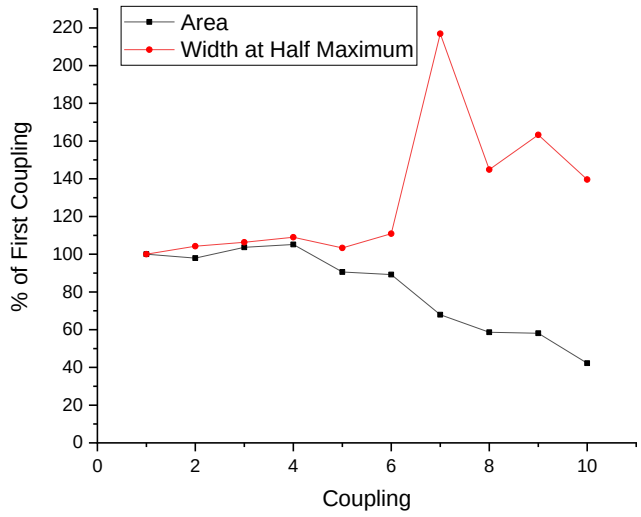


Figure S8. Top: Optimization of the JR10 peptide sequence utilizing VBFR. Bottom: UV absorption data for the Fmoc deprotection during the JR10 baseline synthesis. Each coupling step was analyzed for total area and width at the half-maximum height.

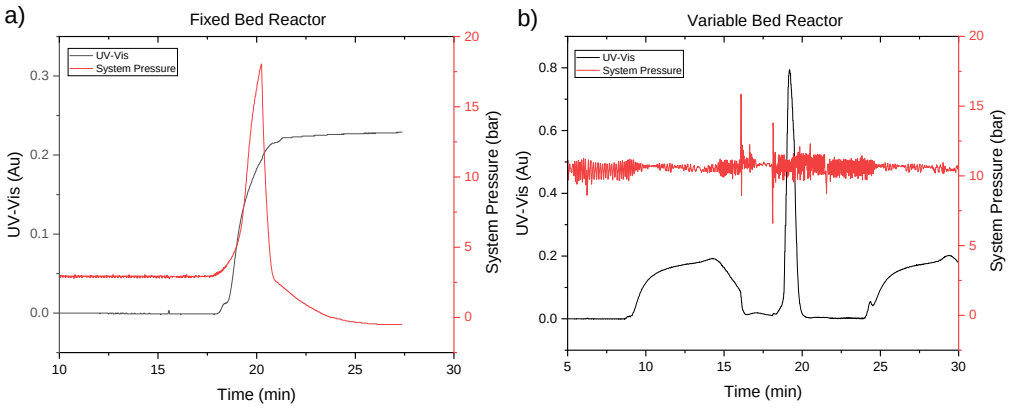


Figure S9. A comparative study of the internal pressure of the system as the peptide elongates. a) Use of a fixed bed reactor, the pressure exceeded the limits of the system (18 bar) during the first coupling, b) whereas the variable bed continuously adjusted to preserve a total system pressure of 10.5 bar. Omnifit® glass columns have a pressure limitation of 60 bar, which would be reached by a tripeptide in a fixed bed. (System Pressure = red, UV-vis = black). UV-vis measured at 360 nm.

General Synthetic Procedures

Peptide Synthesis

Resin Preparation: Desired resin (0.2 g) was loaded into the Omnifit column and the pistons were put into place. The VBFR was set to a resin bed height of 1.5 cm and resin was allowed to swell by flowing DMF from pumps A and B at a flow rate of 0.7 mL/min. After 10 min with the pumps still flowing, the resin was then heated to 80 °C for an additional 5 min. Once at temperature (80 °C) the VBFR was set to achieve a differential pressure of 8 bar to pack the resin. Pumps A and B continued to pass DMF (0.7 mL/min) over until synthesis began (no effect on synthesis).

Building Block Preparation: Stock amino acid solutions (0.24 M) in DMF were made in individual glass vials. The appropriate amount of activator (OxymaPure or HOBt) was then added to each amino acid solution. Solutions were filtered by PTFE syringe filters and transferred into test tubes placed in proper autosampler position. A stock solution of DIC (0.24 M) in DMF was also prepared in a glass vial and placed in the correct autosampler position.

Peptide Cleavage

After the synthesis of the peptide, the resin was transferred into a 10 mL fritted syringe and washed with DMF then CH_2Cl_2 and dried *in vacuo*. Peptides were then cleaved and deprotected by treatment with TFA:TIPS:H_2O* (95:2.5:2.5, 20 mL/g resin) at room temperature for 3 hours on an orbital shaker. The resin was filtered off and the filtrate was split into two 50 mL centrifuge tubes. Peptide was isolated by precipitation with cold diethyl ether (45 mL) followed by centrifugation (8 min at 5500 rpm). Washing with cold diethyl ether and centrifugation step repeated twice. The crude peptide was dried *in vacuo*, then dissolved in 20:80 acetonitrile:H_2O and analyzed by analytical HPLC (Agilent 1100).

*JR10 was cleaved using TFA:PhOH:H_2O:Thioanisole:EDT (90:2.5:2.5:2.5:2.5, 20 mL/g resin)

Manual Coupling of Initial Amino Acid

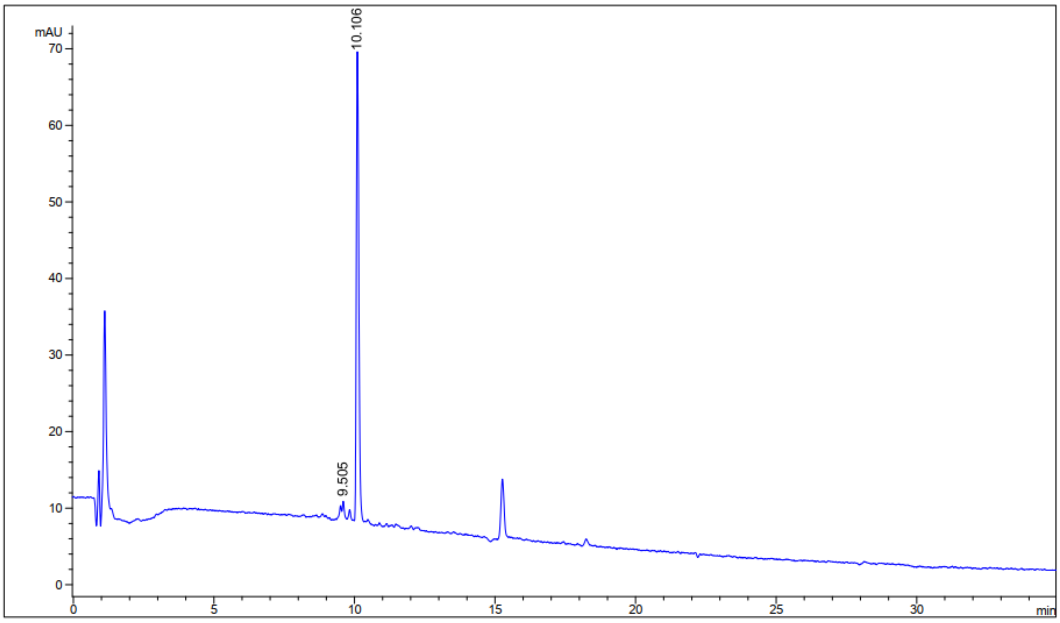
Into a 10 mL fritted syringe 2-Cl chloride resin (200 mg) and CH_2Cl_2 (5 mL) were added and allowed to shake at room temperature for 30 min. The desired amino acid (2 equiv. based on initial resin loading) and DIPEA (6 equiv.) were added to the syringe. The entire reaction was allowed to shake for 3 h on an orbital shaker. Resin was then filtered and washed in the syringe by CH_2Cl_2 (3 x 5 mL) and dried *in vacuo*. Following UV quantification of the initial amino acid loading, resin was then capped by a solution of CH_2Cl_2:MeOH:DIPEA (17:2:1) for 3 h at room temperature. Resin was then filtered and washed in the syringe by CH_2Cl_2 (3 x 5 mL) and dried *in vacuo*.

Compound Characterization

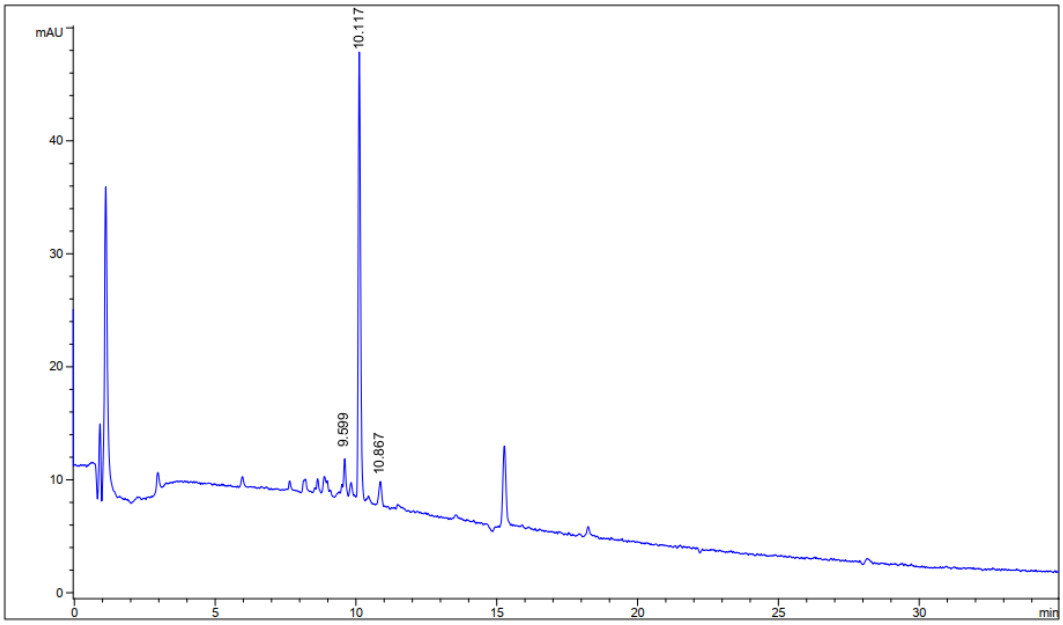
AFLAFLA

HRMS (ESI) calc. for $C_{39}H_{59}N_8O_7$, 751.4500; found: $[M+H]^+$, 751.4547.

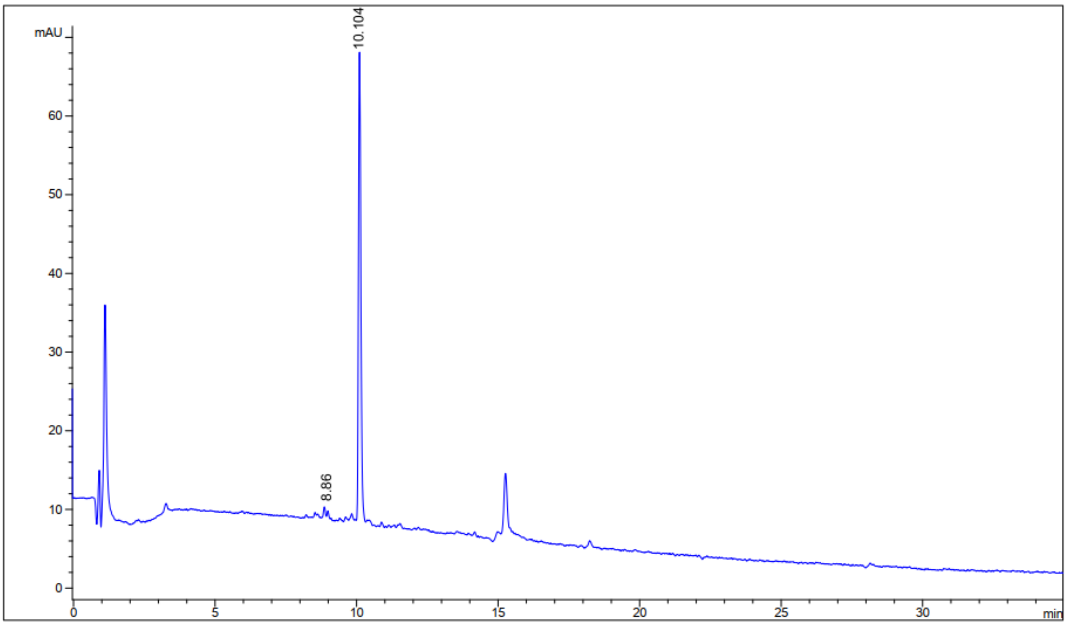
MBHA-HOBt



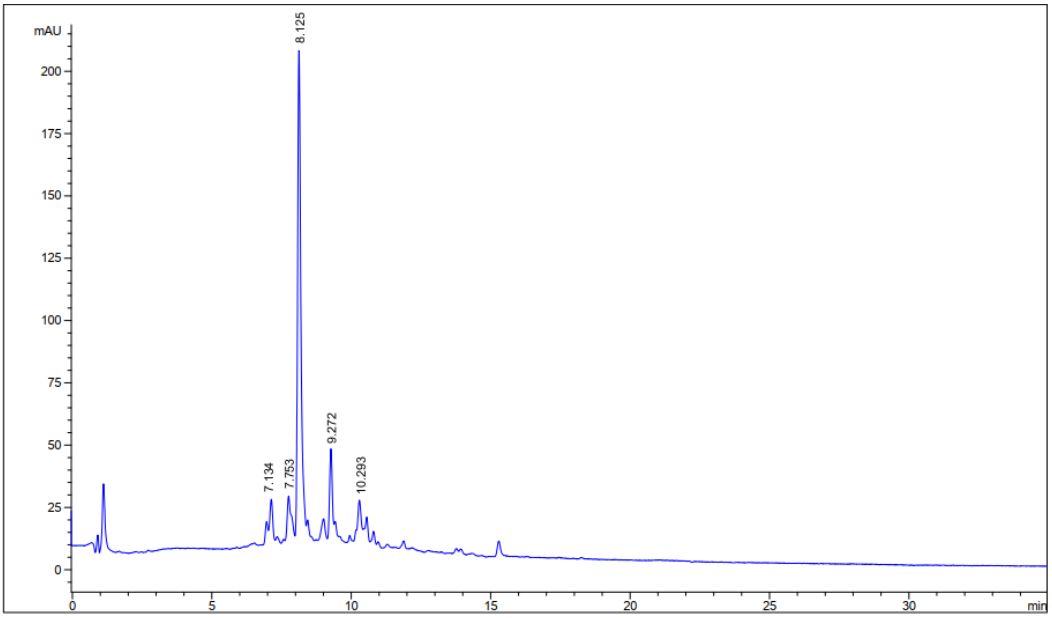
MHBA-OxymaPure



TentaGel

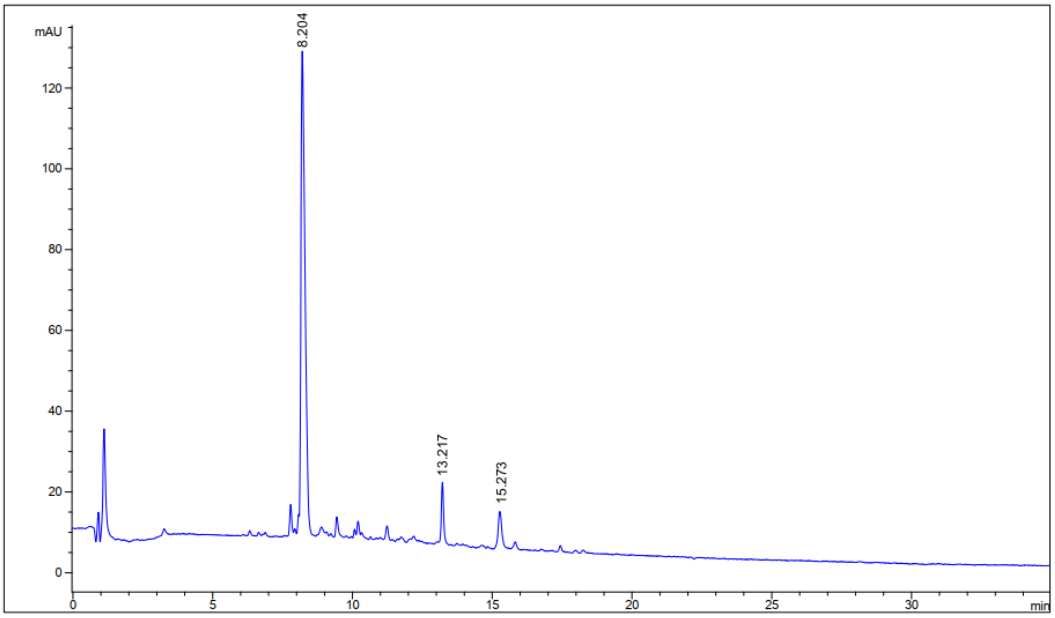


TfR



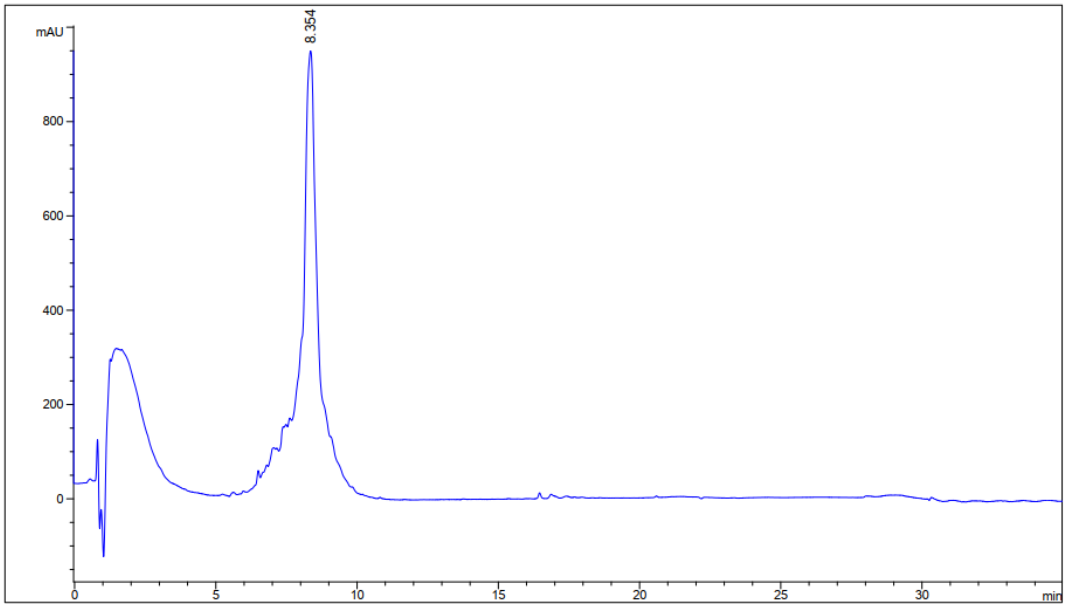
HRMS (ESI) calc. for $C_{71}H_{102}N_{19}O_{15}S$, 1492.7523; found: $[M+3H]^{3+}$, 497.5815.

NFGAIL

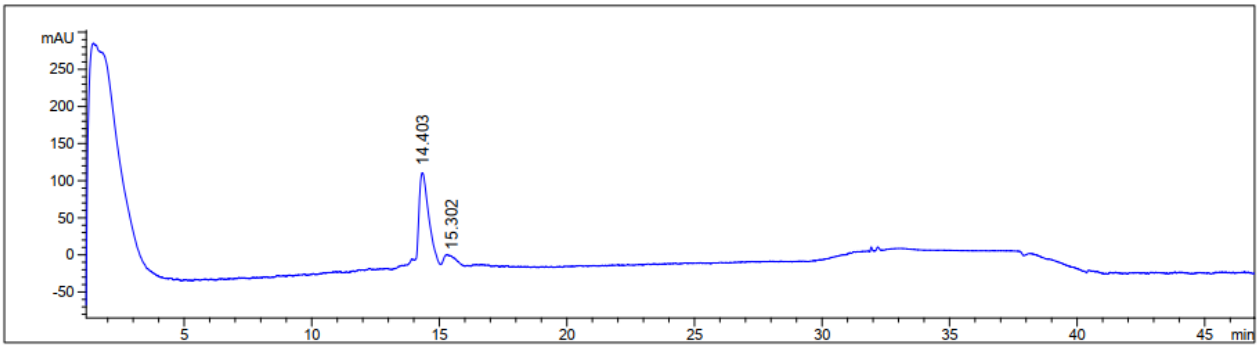


HRMS (ESI) calc. for $C_{30}H_{49}N_8O_7$, 633.3718; found: $[M+H]^+$, 633.3741.

FF03



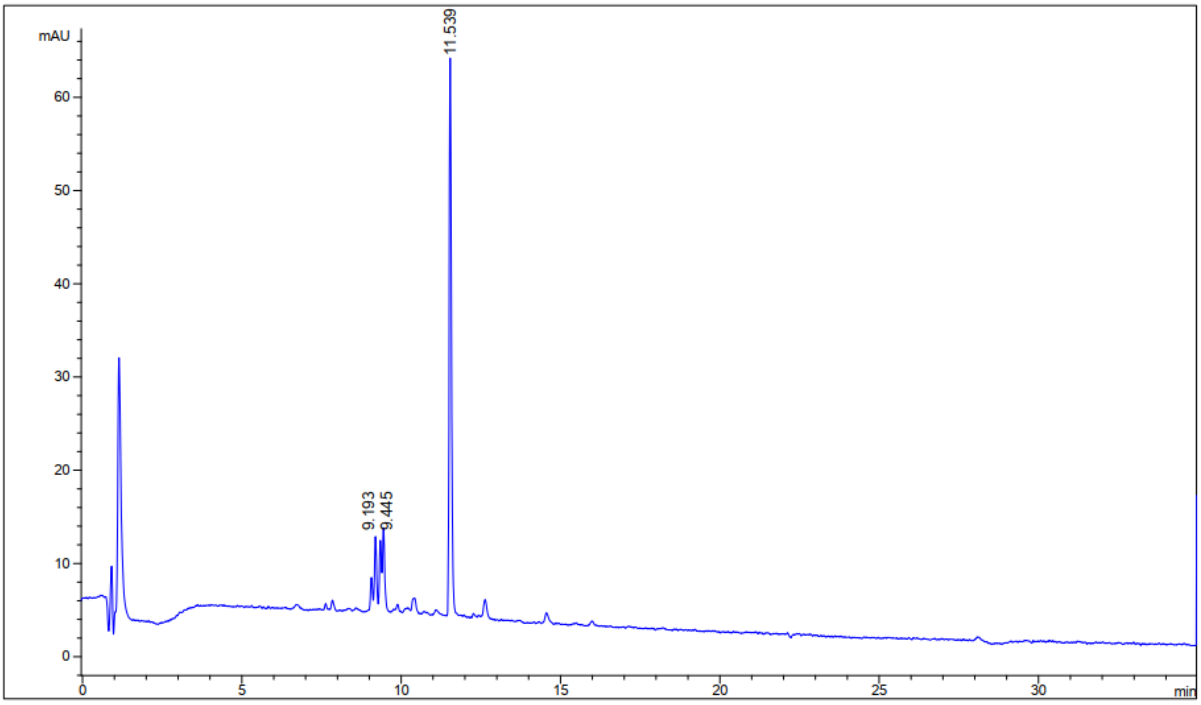
Gradient=5 to 70% B over 17 min



Gradient=5 to 40% B over 25 min

HRMS (ESI) calc. for $C_{149}H_{275}N_{37}O_{41}$, 3239.0571; found: $[M+4H]^{4+}$, 809.7687.

JR10



HRMS (ESI) calc. for $C_{58}H_{92}N_{12}O_{14}S$, 1212.6576; found: $[M+2H]^{2+}$, 606.3292.

Author Contributions

ETS and PHS designed the experiments that ETS performed. ETS and PHS wrote the manuscript. DG developed the continuous flow VBFR, MN undertook research to established initial reaction protocols and trained ETS to use the system. DG and MN loaned the prototype instrument for the duration of the research.