Application Note 69: Automated CF-SPPS and evaluation of GLP-1 peptide

Produced by Vapourtec

Abstract

This application note illustrates the capabilities of a Vapourtec RS-400 system, equipped with a novel flow reactor, the Variable Bed Flow Reactor, for continuous flow Solid-Phase Peptide Synthesis. Glucagon like peptide (GLP-1), a 30-mer peptide used to treat diabetes, was chosen to show the advantages of the Variable Bed Flow Reactor for solid-phase peptide synthesis regardless of the resin core used. By keeping the solid media continuously packed, the contact surface between the resin's active sites and the reagents pumped through is optimised. The use of the Variable Bed Flow Reactor not only reduces the amount of solvent required, but it also ensures a more efficient synthesis.

The packing density and overall protocol allows a final crude purity of the 30-mer peptide, GLP-1 up to 82 % in under 5 hours.

For more details, please contact:
Vapourtec Application Support
application.support@vapourtec.com or call:
+44 (0) 1284 728659

Background

Proteins are produced by ribosomes controlled by the codons of RNA in living cells for specific target functions within the living being [1]. Small proteins, peptides (typically <100-mer) have seen accelerated growth as potential API's in the last decade [2].

Biological expression is one of the synthetic ways to produce proteins, although it is a long process and their chemical composition is limited [3]. Thus, chemical syntheses, inherently more flexible [4], have been an essential part of research to produce proteins, using fast, pure, and less tedious procedures, with the highest possible yield.

Peptides can be synthesised using different chemical routes, liquid phase and solid phase [5]; carried out in both batch and flow [6]. Earlier studies highlighted the advantages of flow automated SPPS using a fixed bed flow reactor for the resin [4,7–9].

Select advantages of flow synthesis over batch methods are in-line spectroscopic monitoring, efficient mixing and precise control over the physical parameters limiting side-reactions.

Since early 2017, Vapourtec has worked towards the development of a controlled packed bed flow reactor which would accommodate resin growth during the synthesis, as well as significantly reducing mechanical damage to the resin caused by stirring in batch.

The effectiveness of coupling and deprotection steps are improved. Inline data is also generated by the reactor, allowing instant decisions to be made to adapt the synthesis, for example, by double coupling, to improve the peptides quality and yield during the synthesis.

Vapourtec recently launched its Variable Bed Flow Reactor (VBFR) which monitors in real time the packing density of the solid support and adjusts itself to maintain a constant packed bed. The change in volume of the packed bed is monitored with a resolution of 0.5 µl. Figure 1 shows the reactor layout of the VBFR, which fits, as any other Vapourtec reactor, into the R-Series.

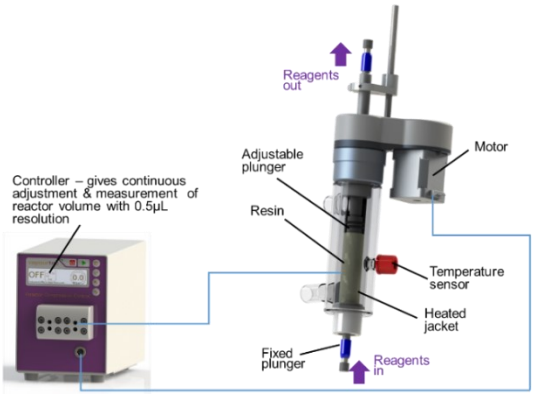

Figure 1 - Schematic diagram of the components of the VBFR

The outcome of several scientific projects have already been published featuring the VBFR for peptide and oligosaccharide syntheses [10,11]. In this work, the impact of different solid support media and the use of the VBFR on a peptide's synthesis are evaluated.

GLP-1 is a 30-mer hormone with a broad pharmacological interest [12], as it stimulates the secretion of insulin and has also cardio and neuroprotective effects. Adaptations of GLP-1 are administrated for the treatment of type-2 diabetes. Like many peptides, aggregation, histidine racemisation and aspartamide formation occur during its synthesis.

Two commonly used resins with different swelling properties were evaluated on the synthesis of GLP-1, Polystyrene and Polyethylene glycol core resins. ChemMatrix resin is often used for GLP-1 synthesis, as it possesses superior swelling properties than "standard" resins, aiding diffusion of reagents through the resin beads, keeping the peptide solvated. ChemMatrix is often used for complex peptides [13] as it is composed of only Polyethylene glycol (PEG) chains (Figure 2).

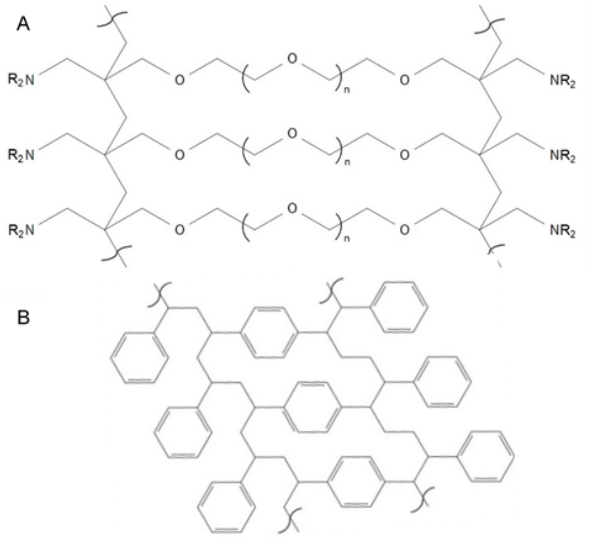

Figure 2 - A: ChemMatrix PEG based resins matrix. B: Polystyrene resin matrix

The distinct arrangements with gauche interaction between polarized bonds shown by vicinal carbon-oxygen bonds result in a relatively amphiphilic resin compared to other resins [1415]. The ether bonds form a resin-matrix chemically and mechanically stable in comparison to Polystyrene based resin matrices that only possess weaker, hydrocarbon based, interactions.

Due to the nature of the matrices, different packing density are required. Vapourtec's SPPS protocol was adapted for both types of resin to ensure the synthesis of challenging peptides like GLP-1, provides high crude purity and yield.

Setup

The CF-SPPS system consisted of a RS-400 flow chemistry system equipped with a VBFR, as shown in Figure 3.

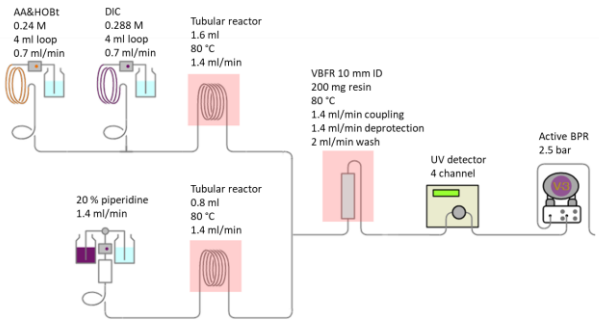

Figure 3 - Schematic of the R-Series used for GLP-1

The primary R_2C+ pump module was configured to deliver coupling reagents using the sample loop automatically loaded via an autosampler. The secondary R2C+ pump module was configured to deliver deprotection solution from a bottle.

The VBFR was set in a R4 reactor heater module. A dual core reactor is used to keep both deprotection and coupling reactors in one reactor cartridge. Amino acid solutions are activated in a 1.6 ml reactor volume, whereas piperidine solution is pre- heated in a 0.8 ml reactor volume. Both outputs are connected to the bottom of the VBFR reactor.

System pressure was maintained throughout the experiments using a SF-10 pump as active BPR.

PTFE frits ensured the resin was kept in the VBFR. To provide optimum transition of the reagents throughout the VBFR, Vapourtec developed diffuser plates which ensure uniform flow through the frits.

Reagents

All materials were purchased from commercial suppliers. **Fluorochem:** Fluorenylmethyloxycarbonyl (Fmoc) Amino acids, N,N-Di-isopropylcarbodiimide (DIC), 1-Hydroxybenzotriazole hydrate (HOBt). **Gyros Protein Technologies:** High purity amino acids. **Fisher scientific**: N,N Dimethylformamide (DMF), ACROS Organics, >99 %. **Sigma Aldrich**: Low Loading Rink Amide AM resin (100-200 mesh, 0.27 mmol/g), H-Rink Amide ChemMatrix resin (0.4 mmol/g loading), Piperidine ReagentPlus 99 %, Formic acid, Dichloromethane (DCM), Methanol (MeOH), Trifluoroacetic acid (TFA) Reagentplus 99 %. Commercially available amino acids showed a disparity of purities, a table with purities is available in the supporting information.

System Parameters

Reagent preparation: A solution of 20 % piperidine and 2 % formic acid (in v/v) in DMF was used for deprotection.

0.24 M stock Fmoc-amino acid solutions in DMF were prepared unless otherwise stated. The appropriate amount of HOBt was then added to each amino acid solution. Solutions were filtered with a 0.25 µm PTFE syringe filters. A 0.288 M stock solution of DIC (1.2 eq (to amino acid)) in DMF was prepared separately, unless otherwise stated. Both amino acid and DIC solutions were placed in an autosampler, which was used to load the R_2C+ sample loops automatically.

The approach for the synthesis of GLP-1 was to use same amount of resin for both ChemMatrix and Low Loading Rink Amide AM resin, resulting in a slight discrepancy of amino acid excess (8 and 12 fold excess respectively). This excess level was used to ensure full coupling post aggregation.

Resin: Low Loading Rink amide AM resin, 0.27 mmol/g, ChemMatrix resin, 0.4 mmol/g

System solvent: DMF

Reaction protocol for CF-SPPS using a Vapourtec RS-400

For a scale of 0.08-0.11 mmol (200 mg resin, 10 mm bore column reactor).

VBFR-SPPS: a Vapourtec Dual-Core™ PFA tube reactor and a VBFR reactor were used. The VBFR was loaded with 200 mg of resin of choice. The resin was set to swell by flowing DMF from pumps A and B at a total flow rate of 1.4 ml/min. After 5 min, the resin was then heated to 80 °C for an additional 20 min before

synthesis began. The temperature was maintained at 80 °C throughout the experiments, unless otherwise stated.

System pressure: 2.5 bar

CF-SPPS protocol A

Deprotection step

- Preheating loop: 0.8 ml reactor volume
- Temperature: 80 °C
- 8 ml deprotection solution at 1.4 ml/min
- Wash: 5 ml of DMF at 2 ml/min

Coupling step

- Activation reactor: 1.6 ml reactor volume
- Temperature: 80 °C
- Combined 8 ml coupling solution at 1.4 ml/min
  - 4 ml of Fmoc-amino acid and HOBt via channel A
  - 4 ml DIC via channel B
- Wash: 4 ml at 2 ml/min

CF-SPPS protocol B

Deprotection step

- Preheating loop: 0.8 ml reactor volume
- Temperature: 80 °C
- 5 ml deprotection solution at 4 ml/min
- Wash: 10 ml of DMF at 8 ml/min

Coupling step

- Activation reactor: 0.9 ml reactor volume
- Temperature: 80 °C
- Combined 4 ml coupling solution at 1.6 ml/min
  - 3.2 ml of 0.3 M Fmoc-amino acid and 1.5 eq /0.45M of HOBt via channel A
  - 1 ml of 3 M DIC via channel B
- Wash: 3 ml at 8 ml/min

Final Cleavage and Analysis

After the synthesis of the peptide, the resins were removed from the VBFR, washed 3 times with 20 ml, DMF, DCM, MeOH. The peptides were then cleaved for 1.5 hours with TFA:H_2O:TIPS (95:2.5:2.5, 15 ml/g resin) at room temperature. The resins were filtered off and the peptide isolated by evaporating TFA, from which a white precipitate was obtained by addition of cold diethyl ether (20 ml). Crude samples were then dissolved in acetonitrile:H_2O (1:1 v/v) and analysed by HPLC. {Agilent 1220; Eclipse XDB-C18 5 µm column (4.6 mm x 150 mm, flow rate = 2 ml/min) heated at 40 °C}. The following solvent 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 % to 60 % solvent B over 20 min. Mass analysis was carried out by ESI-MS (Advion expression CMS).

Results and Discussion

Figure 4 shows a typical deprotection/coupling cycle for CF-SPPS. As piperidine deprotects Fmoc-bound to resin, the VBFR adjusts its volume to counteract the loss of Fmoc (orange line).

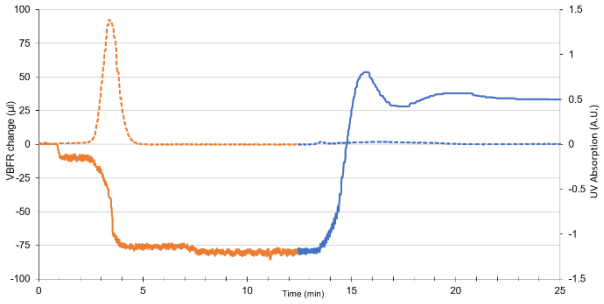

Figure 4 - Deprotection (orange) and coupling cycle (blue) (dotted line: Fmoc UV absorption at 370 nm; solid line: VBFR change in volume)

A UV signal corresponding to loss of Fmoc group is recorded at 370 nm. When coupling starts, as the amino ester reacts onto the resin, the reactor volume increases to accommodate the newly elongated peptide-bound to resin (blue line).

Effect of packing density

As previously described, both resins have different physicochemical properties, which make them behave differently under compression. Rink amide AM resin is a PS core resin, whereas Rink Amide ChemMatrix is composed of 100 % PEG chains. During early studies, it was found that different packing densities are required for different substrates. Rink amide AM resin performs better when a high packing density is applied, whereas ChemMatrix resin, as it swells more, requires a lower packing density for optimum performance.

Although not evaluated for this application work, a polystyrene core resin with higher proportion of PEG spacers could require an intermediate packing density [16].

Difference on aggregation effects with AM and ChemMatrix resin

The use of the VBFR does not only improve the final crude purity by packing the resin beads continuously throughout the synthesis, but it also provides valuable data of the actual peptide growth. In the case of GLP-1, as it aggregates, it can be easily determined on what residue the aggregation event occurred. Figure 7 shows the synthesis of GLP-1 on ChemMatrix, revealing it aggregated after the 10th deprotection (post Glutamic acid deprotection).

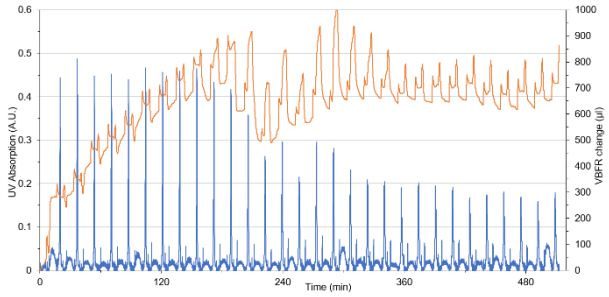

Figure 7 - VBFR volume change (orange) and UV (blue) data over time for the synthesis of GLP-1 on ChemMatrix resin

After the aggregation occurred, it appears the resin partially recovered. The UV trace seems to have decrease in height peak after the event. When the absolute Fmoc deprotections areas were calculated, there was little difference post aggregation, as the deprotection UV peaks widens.

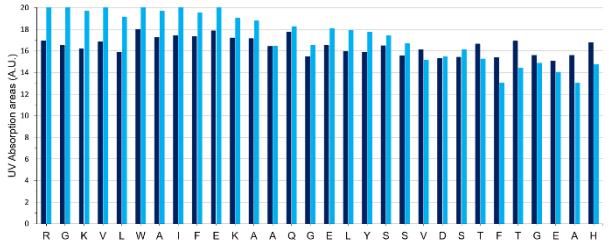

Figure 8 - Absolute Fmoc deprotection areas throughout the synthesis, light blue AM resin, dark blue ChemMatrix resin

The significant change in peak height post aggregation, but similar absolute areas, as figure 8 shows, would indicate the deprotection rate slowed down, making it more difficult to deprotect, and therefore taking more time to complete.

Due to different lipophilicity of both resins, the aggregation event occurred at different stages of the synthesis. While the aggregation occurred during the 10th deprotection when using ChemMatrix resin; for AM resin it occurred during the 9th coupling.

Figure 9 shows both syntheses, noting the total volume gain for ChemMatrix was four times the volume gain of AM resin. As expected, better purity was achieved with ChemMatrix resin, 74.0 % (GLP1.b) (see Table 1), compared to 64.9 % (GLP1.a) with AM resin. In these syntheses at 80 °C, Histidine racemisation can account for up to 30 % of isomer generation. There are different solutions to this problem, such as lowering coupling temperature followed by a double coupling; as well as using a Boc protected Histidine. Although the reduction on isomerisation was beyond the scope of this application note, it will be explored and covered in another application note

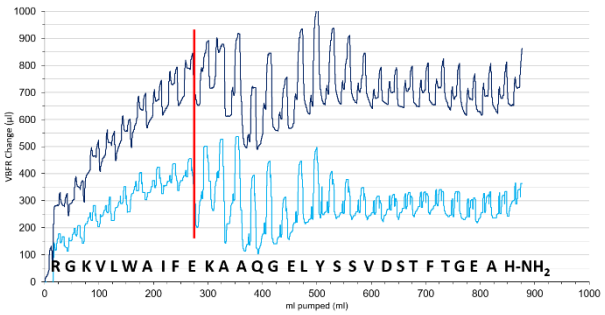

Figure 9 - Difference in VBFR volume change for AM (light blue) and ChemMatrix resin (dark blue) over ml pumped

Effect of temperature and reagent quality on final purity

Vapourtec's protocol A was developed at 80 °C and using standard Fmoc-amino acids, with purities lower than 98 %. Table 1 compiles various GLP-1 syntheses under different conditions using ChemMatrix resin (unless otherwise stated), along with the yield and purity of the cleaved peptide. GLP-1 was synthesised at different temperatures, while 70 °C seemed to have improved purity to 76 % (GLP1.g), 60 °C generated more deletions (GLP1.f) and 90 °C caused an increment in arginine

deletion (GLP1.h) than when using the standard conditions.

Table 1: Yield and Purities of different runs, the resin used was ChemMatrix except for GLP1.a

Entries	Theoretical mass (g)	Yield	GLP-1 isomers Purity	Run
GLP1.a	0.109	50 %	64.9 %	Rink Amide AM resin 80 °C protocol A
GLP1.b	0.127	66 %	74.0 %	ChemMatrix resin 80 °C protocol A
GLP1.c	0.116	56 %	71.4 %	Fixed volume reactor ChemMatrix resin 80 °C protocol A
GLP1.d	0.127	74 %	74.0 %	ChemMatrix resin 80 °C protocol A 1.5 eq HOBt
GLP1.e	0.116	62 %	74.9 %	ChemMatrix resin 80 °C protocol A 99.9 % AA pure
GLP1.f	0.093	n/a	68.4 %	ChemMatrix resin 60 °C protocol A
GLP1.g	0.127	65 %	75.6 %	ChemMatrix resin 70 °C protocol A
GLP1.h	0.141	60 %	43.9 % (11.6 % Arg deletion)	ChemMatrix resin 90 °C protocol A
GLP1.i	0.122	71 %	82 %	ChemMatrix resin 80 °C protocol B

The effect of excess HOBt was evaluated on GLP1.d, which was synthesised with 1.5 eq of HOBt, the HPLC baseline seemed cleaner. We obtained 74 % yield and 74 % crude purity in 8 hours.

High purity Fmoc-amino acids (99.9 %) were used for the synthesis of GLP1.e. The aim was to evaluate the effect of these high purity building blocks. Neither the overall purity, nor the impurity profile were detectably different than when using 97 % Fmoc-amino acids.

Based on the evaluated synthetic conditions, an optimised protocol (protocol B) was developed, aiming to increase wash efficiency, minimise solvent usage and improve overall cycle yield and time. The concentration of amino acid and DIC stock solutions was increased, wash volumes and flow rates were optimised, leading to an improved final purity in almost half of the time (GLP1.i). Figure 10 shows the volume change of the resin through the synthesis of GLP-1 using ChemMatrix resin. With the optimised synthesis (GLP1.i, 9 min cycle time), the aggregation events still occurred after the 10th deprotection (post Glutamic acid deprotection), and the solid media behaved in the same way as previously recorded.

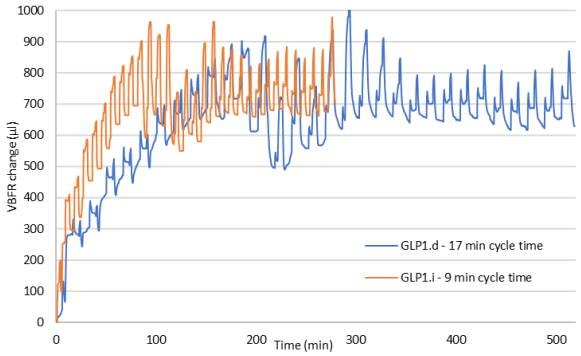

Figure 10 - Difference in VBFR volume change between protocol A and protocol B

Conclusions

Flow chemistry has proven multiple advantages in manual handling, rate of reactions and conversion over any conventional batch SPPS route. GLP-1 has been successfully synthesised with the VBFR in under 5 hours, using less than a litre of DMF and with HOBt & DIC activation, providing a final crude purity of over 82 % and yield of 71%.

This application note demonstrates the technological advantage of using a Vapourtec RS-400 equipped with a VBFR for CF-SPPS. By synthesising GLP-1 we have compared the type of resin matrix on final peptide purity and effect on aggregation events. Controlling the packing density of the resin throughout the synthesis appears to be crucial. Although the VBFR appears to be highly advantageous in the synthesis of difficult sequences. The use of the VBFR will highlight aggregation events, as well as difficult couplings throughout the synthesis. This valuable data will light the path to process scientists should they need to scale up that specific sequence.

Acknowledgments

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Supporting information

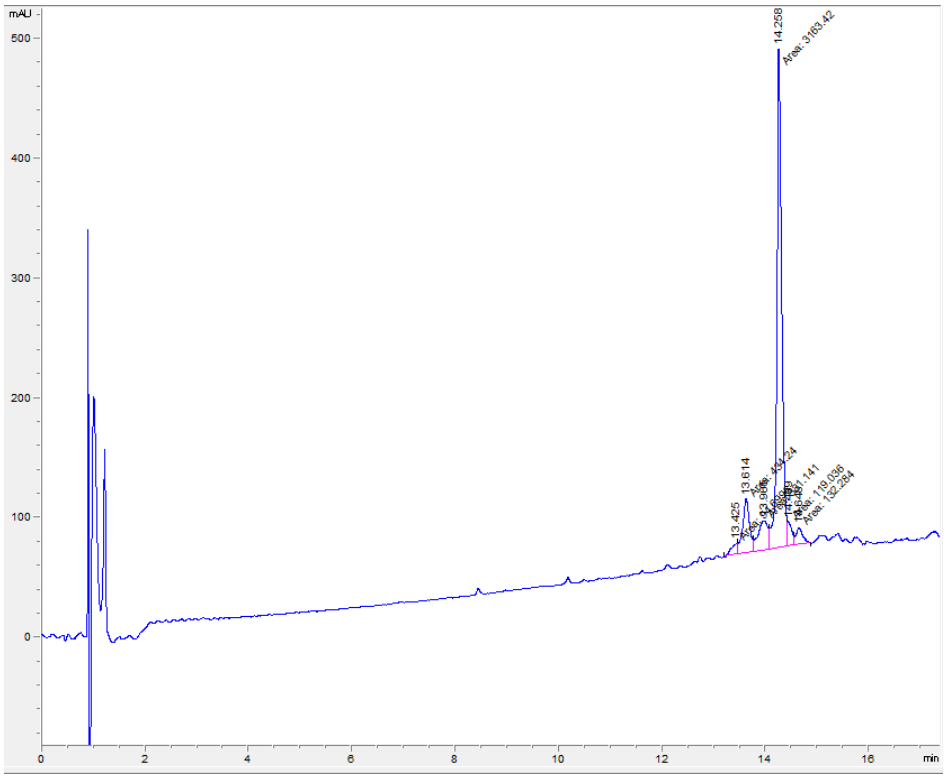

Figure S1 – HPLC chromatogram of GLP1.d

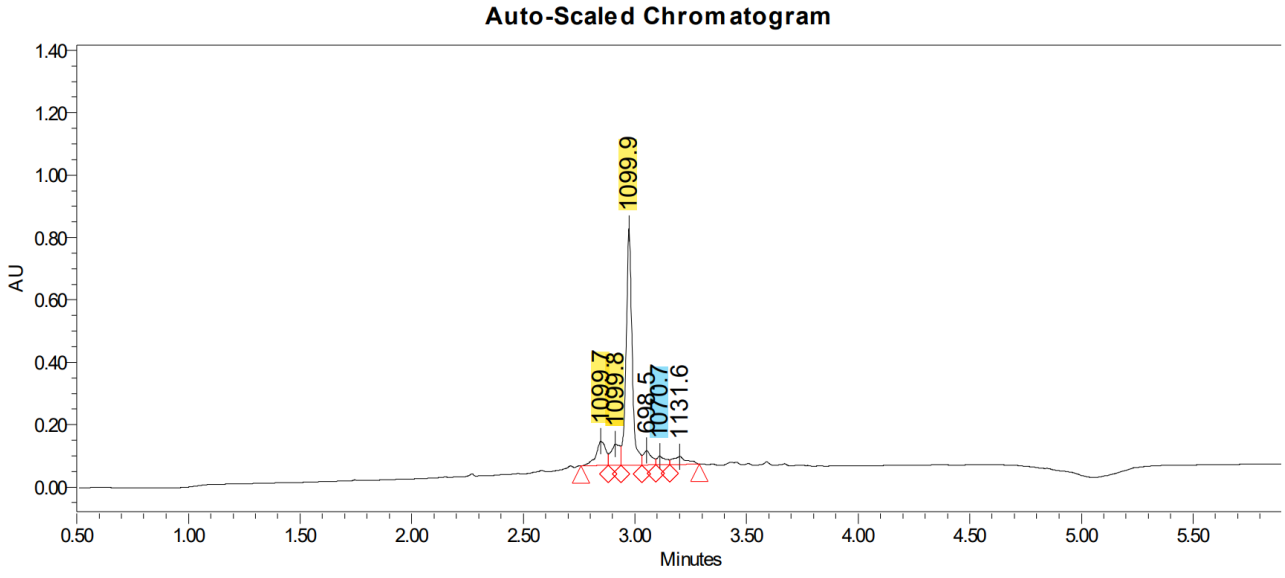

Figure S2 - GLP1.b LC-MS chromatogram. Yellow (GLP-1 and isomers), blue (Ser deletion)

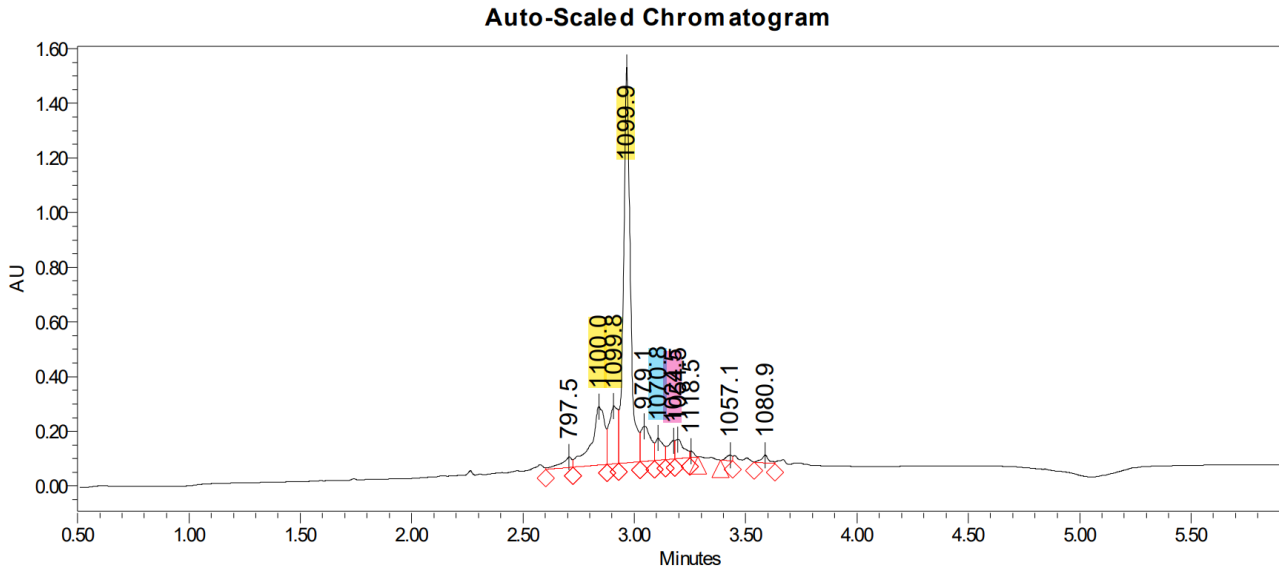

Figure S3 – GLP1.c LC-MS chromatogram. Yellow (GLP-1 and isomers), blue (Ser deletion), purple (His deletion)

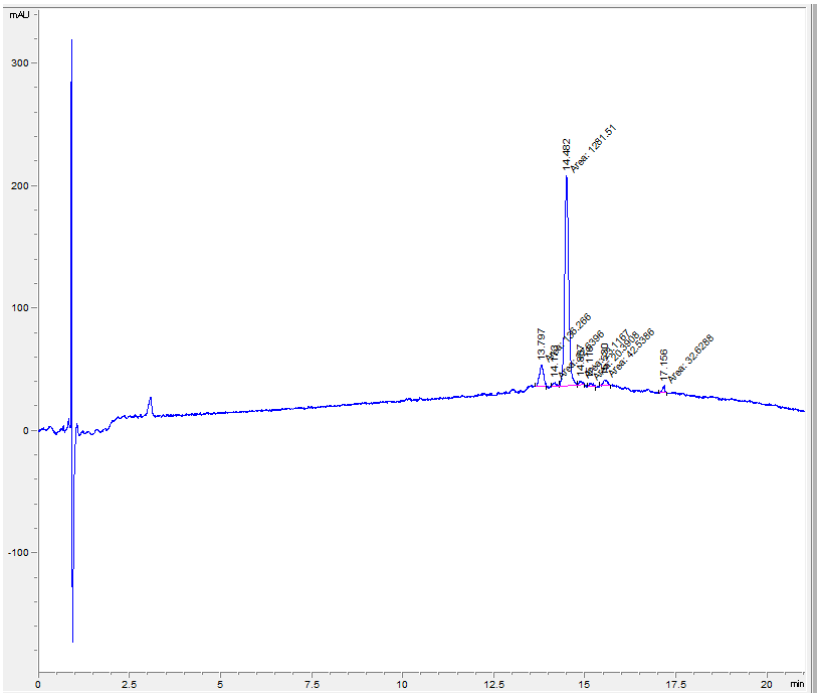

Figure S4 – HPLC chromatogram of GLP1.i

Table S1 – Aminoacid purities from supplier

Amino acids	Supplier purity	Amino acids	Supplier purity
Fmoc-Arg(Pbf)-OH	96 %	Fmoc-Phe-OH	99 %
Fmoc-Gly-OH	98 %	Fmoc-Glu(OtBu)-OH	98 %
Fmoc-Lys(Boc)-OH	98 %	Fmoc-Gln(Trt)-OH	99 %
Fmoc Val OH	99 %	Fmoc-Tyr(tBu)-OH	98 %
Fmoc-Leu-OH	98 %	Fmoc-Ser(tBu)-OH	98 %
Fmoc-Trp(Boc)-OH	95 %	Fmoc-Asp(OtBu)-OH	98 %
Fmoc-Ala-OH	98 %	Fmoc-Thr(tBu)-OH	97 %
Fmoc-Ile-OH	98 %	Fmoc-His(Trt)-OH	98 %