TITLE: Identification of N-terminally diversified GLP-1R agonists using saturation mutagenesis and chemical design

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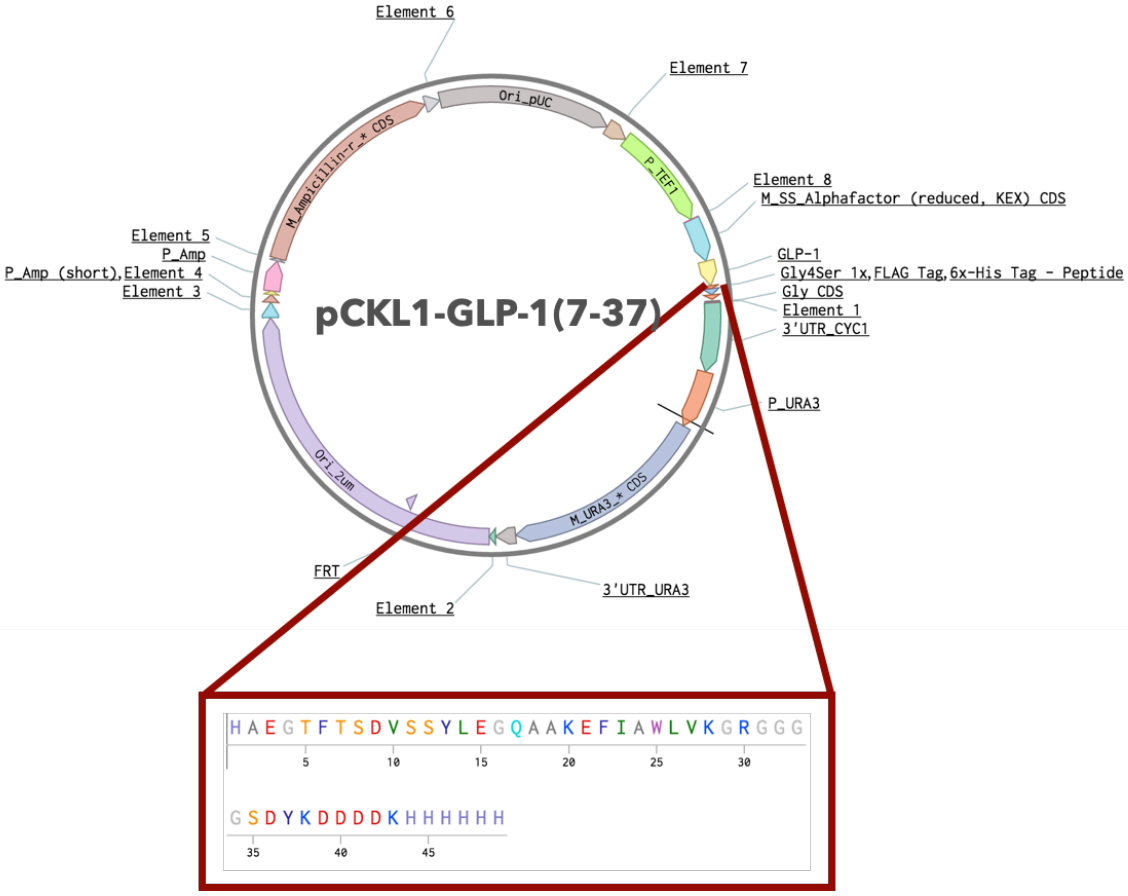
1 Table of Contents

2 Plasmid maps and primer sequences

2.1 pCKL1-GLP-1-Flag-His Vector Map and Insert Sequence

GLP-1 sequence obtained from Marathe et al[1].



2.2 pCKL1-GLP-1 Cloning and Mutagenesis Primers

2.2.1 XhoI-GLP-1-F

Cloning primer for incorporating GLP-1 into the pCKL1 vector, with capital letters indicating sequence overlap with GLP-1 peptide

```
5' atctctcgag aaaagaCATG CTGAAGGTAC TTTTACTTCT GATG 3'
```

2.2.2 BamHI-GLP-1-R

Cloning primer for incorporating GLP-1 into the pCKL1 vector, with capital letters indicating sequence overlap with His tag on the GLP-1 insert

```
5' gatcggatccctactaATGATGGTGGTGGTGATGc 3'
```

2.2.3 Nterm-5aa-GLP-1 F

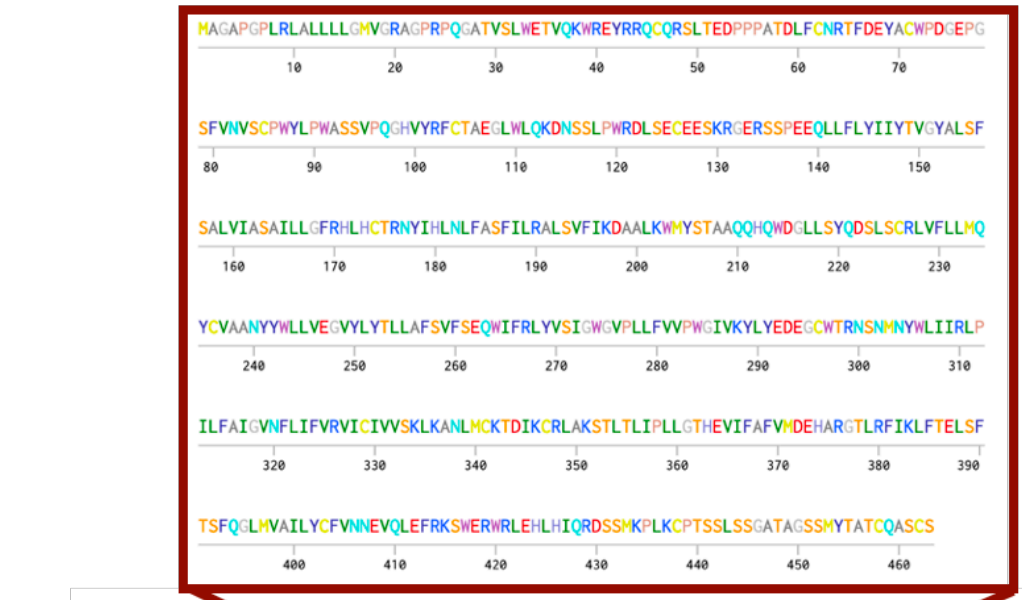
A) Codon randomized saturation mutagenesis primer (X01-05 = codon randomized positions, see table 2.2.3B below)

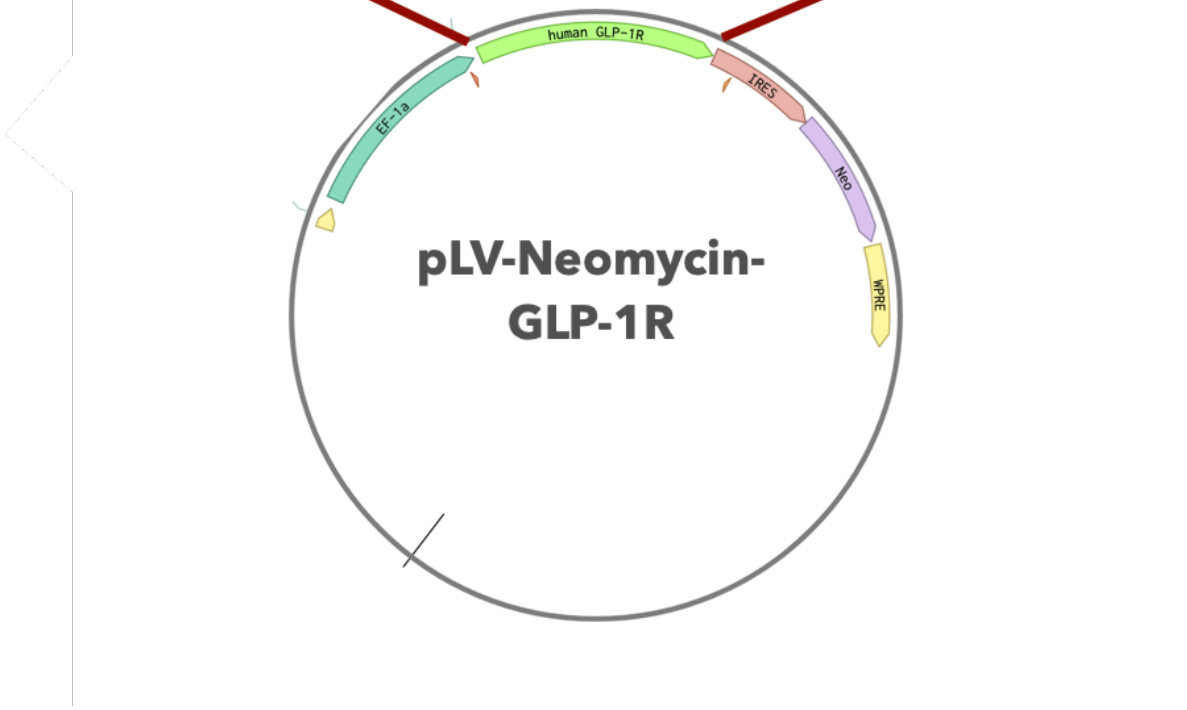
5' GGGTATCTCTCGAGAAAAGA **X01 X02 X03 X04 X05** TTTACTTCTGATGTTTCTTC 3'

B) Table from ELLA Biotech of the trimer phosphoramidites randomly incorporated into positions X01-05 in the saturation mutagenesis primer above, the percentage chance of incorporation according to mix parameters and the corresponding amino acid encoded.

		%	Amino Acid
Lys	AAA	5.26	**K**
Asn	AAC	5.26	**N**
Thr	ACC	5.26	**T**
Ile	ATC	5.26	**I**
Met	ATG	5.26	**M**
Gln	CAG	5.26	**Q**
His	CAT	5.26	**H**
Pro	CCA	5.26	**P**
Arg	CGT	5.26	**R**
Leu	CTG	5.26	**L**
Glu	GAA	5.26	**E**
Asp	GAT	5.26	**D**
Ala	GCA	5.26	**A**
Gly	GGT	5.26	**G**
Val	GTT	5.26	**V**
Tyr	TAC	5.26	**Y**
Ser	TCT	5.26	**S**
Cys	TGC	0	**C**
Trp	TGG	5.26	**W**
Phe	TTC	5.26	**F**
	Sum	99.94	

2.3 pLV-Neomycin-GLP-1R Vector Map & Insert Sequence





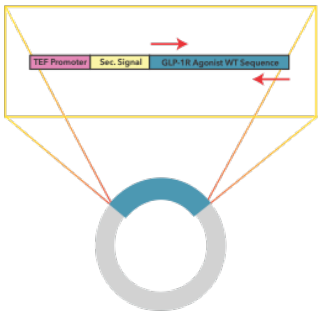
3 Peptide Screening Supplemental Methods and Data

3.1 Randomly sequenced saturation mutagenesis library peptide sequences before screening

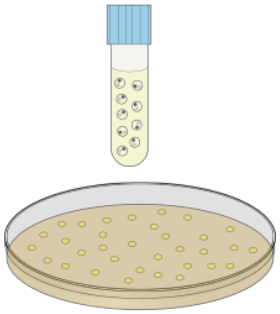
TABLE 3.1
DSHSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
DQIVVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
YRYVLFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
AFSAAFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
EPKMMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
TLAFMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
MQGYPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
DRRSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
EPMDLFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
DGMIPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
VWEHVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
VSHPTFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
LGIYAFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
WISGPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
RAHQYFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
DRRSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
GDYKVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH

3.2 Saturation mutagenesis screening analysis method

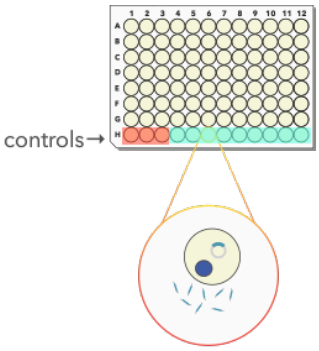
3.2.1 Detailed workflow for saturation mutagenesis screen



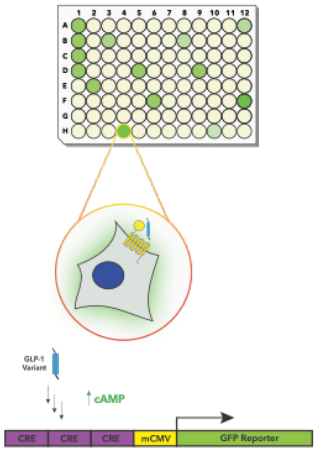
1 Use a mutagenic PCR to **diversify** the GLP1 peptide sequence.



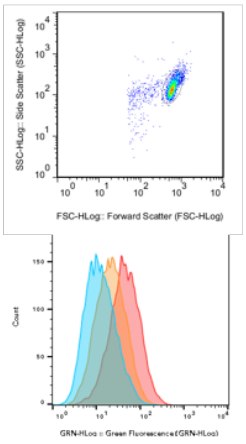
2 Transform the mutated sequences into yeast secretion strain and **grow individual clones into colonies.**



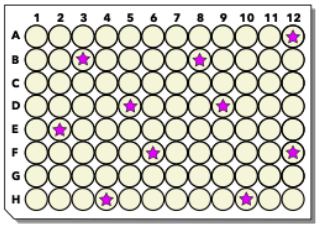
3 Inoculate colonies into a 96-deep-well culture plate so that each individual clone can **secrete its peptide variant.**



4 Treat CHO-K1 CRE-GFP-GLP1R reporter cells that **fluoresce green upon GLP-1R activation** with peptide-conditioned yeast media.

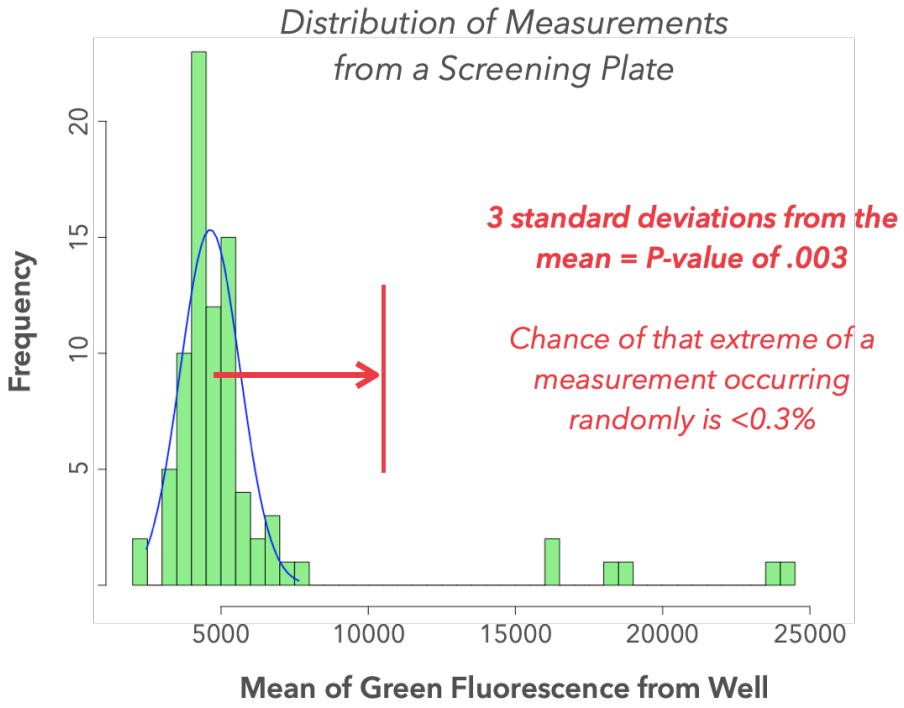


5 Analyze each well by **automated flow cytometry** to measure the fluorescence levels of the reporter cells in each well.



6 **Select wells that are hits** and propagate corresponding yeast clone for follow-up, sequencing, and/or additional rounds of mutagenesis.

3.2.2 Distribution of fluorescence among representative screening plate wells and hit cut-off



Since most variants from a given plate in the randomized mutagenesis library had very little or no activity, the majority of measurements clustered at low reporter cell fluorescence values when represented as a histogram. Since these values are normally distributed, we used the mean and standard deviation of the sample to set a cut off (z-score) above which we were confident a measurement was high enough to distinguish itself as a "hit." We chose 3 standard deviations above the sample mean as the cut off (indicated with a red line) such that there would be a probability of <.3% that a hit above this threshold happened by chance.

3.2.3 Demonstration of a representative saturation mutagenesis screening plate before and after hit cut-off was applied

A) Mean of green fluorescence for each well on a screening plate (plate 12).

	1	2	3	4	5	6	7	8	9	10	11	12
A	3818	4024	4691	5763	4442	4692	4895	6276	4464	4053	5283	4431
B	4009	3398	5379	4262	3572	18700	3298	5116	24300	5215	5530	4553
C	3931	3044	4206	4211	5531	3492	4867	6886	4762	23700	5671	4590
D	5447	2471	4918	7460	4348	3627	3636	4113	5253	6047	4944	4682
E	4246	3542	4469	3884	2498	4302	4201	4488	5188	5325	6873	7634
F	4371	3524	5226	4029	3614	3609	6811	5197	18500	4973	16500	5375
G	4179	3096	4540	4001	4030	16200	4067	5205	4292	5213	5233	5129
H	20300	6628	3748	19500	25000	25500	24300	25200	28300	24900	23300	24100

B) Number of standard deviations of each well from the plate's mean fluorescence

Panel A and B show the same screening plate and library members in rows A-G. Row H contains controls as follows: 1) treatment with a high concentration (1 µM) of pure GLP-1; 2) a low concentration (1 nM) of pure GLP-1; 3) PBS; 4-6) three wells of secreted GLP-1 starting from three separate transformed yeast colonies; 7-9) three wells of secreted exendin-4; and 10-12) three wells of oxyntomodulin. Panel A shows raw fluorescence of reporter cells in the well highlighted in increasingly saturated shades of green relative to their value. Panel B shows those fluorescence values converted to the number of standard deviations they are from the mean, highlighting the wells that surpassed the statistical threshold of 3 standard deviations from the mean in red.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-0.531	-0.432	-0.114	0.398	-0.233	-0.113	-0.016	0.643	-0.222	-0.418	0.169	-0.238
B	-0.439	-0.731	0.215	-0.319	-0.648	6.573	-0.779	0.089	9.246	0.136	0.287	-0.180
C	-0.477	-0.900	-0.345	-0.343	0.287	-0.686	-0.030	0.934	-0.080	8.960	0.354	-0.162
D	0.247	-1.174	-0.006	1.208	-0.278	-0.622	-0.617	-0.390	0.154	0.533	0.007	-0.118
E	-0.326	-0.662	-0.220	-0.499	-1.161	-0.300	-0.348	-0.211	0.123	0.189	0.928	1.291
F	-0.267	-0.671	0.141	-0.430	-0.628	-0.630	0.898	0.128	6.478	0.021	5.523	0.213
G	-0.358	-0.875	-0.186	-0.443	-0.429	5.380	-0.412	0.131	-0.304	0.135	0.145	0.095
H	7.337	0.811	-0.564	6.955	9.580	9.819	9.246	9.676	11.155	9.532	8.769	9.151

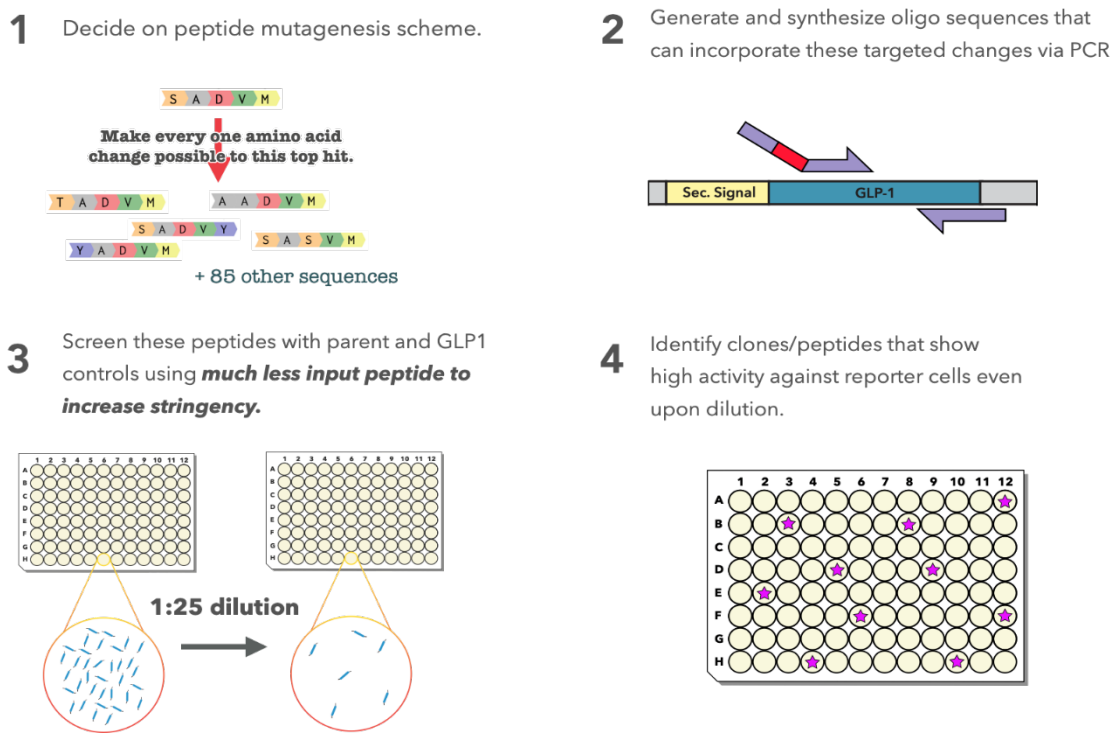
3.3 **Sequencing results sampled from saturation mutagenesis library plates.**

TABLE 3.3-1: Sequenced positives	
Well ID	Peptide Sequence
P01D12	HTDQMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P01G4	YVDHEFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P04D2	LFDSEFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P07E8	SADVMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P08A8	VPDWMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09A8	SVDHFFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09C2	SPEVEFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09C6	DMDWVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09E11	HTDPWFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09G8	TLAFMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P10D11	HTEHMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P10E11	NVDATFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P10G6	QVDQFFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P11C11	LADDDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P11G03	RNHAIFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P12B06	FSDFDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P12B09	NAEMEFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P12F09	LVDNDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P12F11	STDWTFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14C09	FADHLFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14C10	PEYADFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P15B04	LGVMMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P15C12	QLDWDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P16F11	LWDLDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P18C09	HVDYDFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P19F06	QPDSVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21G09	RFVESFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH

TABLE 3.3-2: Sequenced negatives	
Well ID	Peptide Sequence
P09B08	DSHSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09C08	DQIVVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09D08	YRYVLFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09E08	AFSAAFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09F08	EPKMMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P09G08	TLAFMFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14A10	MQGYPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14B10	DRRSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14D10	EPMDLFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14E10	DGMIPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14F10	VWEHVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P14G10	VSHPTFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21A09	LGIYAFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21B09	WISGPFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21C09	RAHQYFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21D09	DRRSRFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH
P21E09	GDYKVFTSDVSSYLEGQAAKEFIAWLVKGRGGGGSDYKDDDDKHHHHHH

3.4 P7E8 screen analysis details

3.4.1 Screening workflow for P7E8 screen



3.4.2 Representative screening plate from P7E8 screen

1:25 diluted peptide

Low — High

= top 10% of variant fluorescence

	1	2	3	4	5	6	7	8	9	10	11	12
A	2415	3685	1713	1720	1776	1838	1833	2531	4124		15200	1911
B	1803	4034	1702	1786	1765	1794	1857	1807	1901	8694	3263	2064
C	1898	3073	1818	1709	1870	2508	3779	1909	4454	2016	1942	2735
D	1674	2984	1774	2597	1861	1826	1950	1816	2020	2082	1927	1996
E	2189	1864	1719	1979	2176	2074	1813	1791	2221	2006	1997	1901
F	1783	1721	1869	1660	1793	1813	4332	2028	1884	2028	1954	2161
G	1734	1622	1884	4078	1890	1821	1733	1739	6705	1911	1940	2002
H	1678	1754	1776	1894	2137	1787	19000	19700	1986	1975	2313	2012

SADAM

SADMM

Controls: GLP-1 OXM P7E8

4 Peptide Synthesis Materials and General Methods

4.1 Reagents and solvents.

All reagents were purchased and used as received. Fmoc-Ala-OHxH_2O, Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp-(O*t*-Bu)-OH; Fmoc-Cys(Trt)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Glu(O*t*-Bu)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Met-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Ser(But)-OH; Fmoc-Thr(*t*-Bu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(*t*-Bu)-OH; and Fmoc-Val-OH, were purchased from the Novabiochem-line from Sigma Millipore; Fmoc-4-Nitro-L-Phenylalanine (≥99.0%) was purchased from ChemImpex; Fmoc-O-Benzylphospho-L-Serine (≥97.0%) was purchased from ChemImpex; Fmoc-L-cysteic acid (>98%) was purchased from Anaspec; O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, ≥97.0%) and (7-Azabenzotriazol-1-yloxy)tripyrrolidinophospho-nium hexa-fluorophosphate (PyAOP, ≥97.0%) were purchased from P3 Biosystems. Omnisolv grade *N*,*N*-dimethylformamide (DMF, HPLC grade) was purchased from EMD Millipore (DX1726-1). 4-(Dimethylamino)pyridine (DMAP, ≥99.0%) was purchased from Sigma-Aldrich. N-N'-Diisopropylcarbodiimide (DIC, 99.5%) was purchased from Chem-Impex. AldraAmine trapping agents (for 1000 – 4000 mL DMF, catalog number Z511706), Diisopropylethylamine (DIEA; 99.5%, biotech grade, catalog number 387649), piperidine (ACS reagent, ≥99.0%), Tetrafluoroethylene (TFE, extra pure, ≥99.8%) was purchased from Acros. Trifluoroacetic acid (HPLC grade, ≥99.0%), triisopropylsilane (≥98.0%), acetonitrile (HPLC grade), formic acid (FA, ≥95.0%), and dimethyl sulfoxide (DMSO, HPLC grade, ≥99.7%). H-Rink Amide (0.49 mmol/g) and HMPB ChemMatrix polyethylene glycol (0.44 mmol/g loading) resin were purchased from PCAS Biomatrix. Water was deionized using a Milli-Q Reference water purification system (Millipore). Nylon 0.22 μm syringe filters were TISCH brand SPEC17984.

4.2 Automated flow peptide synthesis (AFPS)

All peptides were either fully or partially synthesized on the "Amidator" or "Peptidator," automated-flow peptide synthesizers built in the Pentelute lab. The "Amidator" and "Peptidator" are similar to the previously published AFPS system[2]. Reported peptides were synthesized during the published synthesis protocol optimization process[3]. Unless otherwise noted, the parameters used for peptide synthesis are summarized in Table 4.2.1.

Parameter	Conditions
Temperature	90 °C reactor; amino acids were activated in 90 °C heated loop
Coupling step	0.40 M amino acid stocks in amine-free DMF 0.38 M activator stock in amine-free DMF DIEA (99.7%) as coupling base C, D, E, F, G, H, I, K, L, M, P, W, & Y were coupled with HATU (8 pump strokes) S & A were coupled with HATU (21 pump strokes) N, Q, R, V, & T were coupled with PyAOP (21 pump strokes)
Flow rate	40 mL/min of amine-free DMF
Deprotection Step	40% piperidine in amine-free DMF with 2% formic acid (13 pump strokes)
Washing Step	Amine-free DMF (40 pump strokes)

Table 4.2.1. AFPS conditions for peptides synthesized.

4.3 **Amine-free DMF preparation procedure**

One AldraAmine trapping agent was added per 4 L bottle of DMF at least 24 hours before use on the AFPS.

4.4 **Manual coupling procedure**

Unless otherwise noted, manual couplings were performed on a 0.04 mmol scale using 100 mg of HMPB resin (0.44 mmol/g loading). The resin was contained in a 6 mL fritted syringe on a manifold during coupling and subsequent deprotection.

For pre-coupling of the first C-terminus residue to HMPB resin, Fmoc-protected amino acid (0.44 mmol) was dissolved in DMF (3 mL). DIC (0.22 mmol) was added followed by DMAP in DMF (0.20 M solution, 4.4 μmol). The solution was mixed and then added to the resin. Coupling proceeded overnight, and the resin was rinsed with DMF (3×6 mL) the following morning. The first position C-terminus residues were deprotected on the AFPS system.

Three of the N-terminus positions were manually coupled to GLP-1 derivatives containing a non-canonical substitution at the N-terminal third position. To couple the non-canonical residues, a solution containing Fmoc-protected amino acid (0.22 mmol) and HATU (0.19 mmol) in amine-free DMF was prepared. DIEA (0.63 mmol) was added and the solution was mixed for 1 minute before it was added to the resin for coupling. To couple the canonical residues, a solution containing Fmoc-protected amino acid (0.26 mmol) and HATU (0.25 mmol) in amine-free DMF was prepared. DIEA (0.76 mmol) was added, the solution was mixed then added to the resin for coupling.

Both canonical and non-canonical amino acids were coupled for 40 minutes at room temperature, unless otherwise noted. After coupling, the resin was rinsed with amine-free DMF (3×6 mL). Both canonical and non-canonical residues were then deprotected with 20% (v/v) piperidine in amine-free DMF (5 mL) solution for 10 min (2 x). After the last deprotection step, resin was washed with amine-free DMF (3×6 mL) followed by DCM (3×6 mL) and dried under reduced pressure overnight prior to cleavage.

4.5 **Cleavage Protocol**

For each peptide, half of the resin was cleaved in a 50 mL propylene conical tube. Cleavage solution (94% TFA, 1% TIPS, 2.5% EDT, 2.5% water) was added to each tube until the resin was completely submerged (~3 mL). Peptides were cleaved for two hours at room temperature.

After cleavage, samples were rinsed with ice-cold diethyl ether (3×35 mL). Samples were centrifuged between each ether rinse and the supernatant was discarded after each centrifugation. Residual ether was evaporated, and the peptide was reconstituted in 70% acetonitrile in water with 0.1% TFA. The peptide solution was filtered through a Nylon 0.22 μm syringe filter, frozen with liquid nitrogen, and lyophilized.

4.6 **Liquid chromatography–mass spectrometry (LCMS)**

After lyophilization, a small amount of peptide was dissolved in 70% acetonitrile in water with 0.1% TFA. The sample was diluted to a concentration of ~0.1 mg/mL for LCMS analysis. LCMS chromatograms and associated high resolution mass spectra were acquired using an Agilent 6520

Accurate-Mass Q-TOF LCMS (abbreviated as 6520) or an Agilent 6550 iFunnel Q-TOF LCMS system (abbreviated as either 6550-1 or 6550-2). Solvent compositions used in the LCMS are 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LCMS methods were used:

- ***1-91% B over 9 min, Zorbax C3 column (6520)***
  LC conditions: Zorbax 300SB-C3 column: 2.1×150 mm, 5 μm, column temperature: 40 °C, gradient: 0-2 min 1% B, 2–11 min 1–91% B, 11–12 min 91–95% B; flow rate: 0.8 mL/min. A final 3-min hold was performed at a flow rate of 0.8 mL/min. The total method time was 15 min. MS is on from 4 to 12 min.
  MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300–3000 *m*/*z*.
- ***1-61% B over 33 min, Phenomenex Jupiter C4 column (6550-1)***
  LC conditions: Phenomenex Jupiter C4 column: 1.0×150 mm, 5 μm, column temperature: 40 °C, gradient: 0–2 min 1% B, 2-30 min 1–91% B, 30–34 min 61–90% B; flow rate: 0.1 mL/min. A final 4-min hold was performed at a flow rate of 0.1 mL/min. The total method time was 38 min. MS is on from 4 to 30 min.
  MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 100–1700 *m*/*z.*
- ***1-91% B over 20 min, Phenomenex Jupiter C4 column (6550-1)***
  LC conditions: Phenomenex Jupiter C4 column: 1.0×150 mm, 5 μm, column temperature: 40 °C, gradient: 0–2 min 1% B, 2-18 min 1–91% B, 18–21 min 91% B; flow rate: 0.1 mL/min. A final 4-min hold was performed at a flow rate of 0.1 mL/min. The total method time was 25 min. MS is on from 4 to 18 min.
  MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 100–1700 *m*/*z.*
- ***1-91% B over 10 min, Zorbax C3 column (6550-2)***
  LC conditions: Zorbax 300SB-C3 column: 2.1×150 mm, 5 μm, column temperature: 40 °C, gradient: 0–2 min 1% B, 2–12 min 1–91% B, 12–13 min 91% B; flow rate: 0.5 mL/min. A final 2-min hold was performed at a flow rate of 0.5 mL/min. The total method time was 15 min. MS is on from 4 to 12 min.
  MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300–3000 *m*/*z*.

Data were processed using Agilent MassHunter Workstation Qualitative Analysis Version B.06.00 with BioConfirm Software.

4.7 **Analytical high-performance liquid chromatography (HPLC)**

Analytical HPLC was used to determine purity of each crude and purified peptide. Lyophilized peptide powder was reconstituted in 70% acetonitrile in water with 0.1% TFA to a concentration of approximately 1.0 mg/mL. The samples were analyzed on Agilent Technologies 1200 Series, which was computer-controlled through Agilent ChemStation software.

Analytical HPLC spectra were recorded on an analytical Agilent Zorbax 300SB-C3 column (2.1 mm×150 mm, 5-μm particle size). A linear gradient of acetonitrile with a 0.08% TFA additive

(solvent B) in water with a 0.1% TFA additive (solvent A) was used. After a 3-min hold, gradients of 1% B per minute ramped up over 60 min at a flow rate of 0.4 mL/min. Gradient started at 5% B (annotated as "5–65% B over 60 min"). A final 3-min hold was performed. The total method time was 66 min. Purities were obtained by manually integrating all signals appearing in the 5–60 minute range at 214 nm wavelength.

4.8 Mass-directed reversed-phase high-performance liquid chromatography (RP-HPLC purification

RP-HPLC purification: the lyophilized peptide sample was dissolved in the gradient starting concentration (e.g. 5% acetonitrile in water with 0.1% TFA). All samples were filtered with a Nylon 0.22 μm syringe filter prior to purification. For all HPLC purifications, a gradient of acetonitrile with 1 % TFA additive (solvent B) and water with 0.1% TFA additive (solvent A) was used. Samples were purified on either a semipreparative Agilent Zorbax 300SB-C3 column (9.4 mm×250 mm, 5-μm particle size) at a flow rate of 4 mL/min or a preparative Zorbax 300SB-C3 column (21.2 mm×250 mm, 7μm particle size) at a flow rate of 20 mL/min. Fraction purity was assessed by assaying suspected clean fractions on Q-TOF LCMS and analyzing the total ion count and mass spectrum data. The cleanest fractions, as determined by Q-TOF LCMS, were pooled, frozen with liquid nitrogen, and lyophilized. Column and purification method are noted next to each sample.

4.9 Solid Phase Extraction (SPE)

Lyophilized peptide was dissolved in the starting conditions (1% acetonitrile in water with 0.1% TFA). Supelclean™ LC-18 SPE Tube was first conditioned with methanol (3 x 6 mL). The column was then equilibrated with three column volumes (3 x 6 mL) of starting conditions. Next, peptide was loaded onto the column and washed three times with the starting conditions. Peptide was then eluted in 50% acetonitrile in water with 0.1% TFA. Eluted peptides were collected, frozen, and lyophilized.

4.10 Yield Determination

The weights for the peptidyl resin after synthesis, crude, and HPLC-purified lyophilized peptide powders were measured using an analytical scale (XS205DU Analytical Balance, Mettler-Toledo). Crude yield and yield after purification are calculated similarly to previously published calculations (ref. 3). The yield calculation accounts for the uncleaved portion of resin by using the extrapolated values for crude mass and mass after purification. The extrapolated crude mass and extrapolated mass after purification are calculated by multiplying the respective mass by the ratio of total mass of peptidyl resin to mass of peptidyl resin cleaved.

Peptide	Number of residues coupled on AFPS	Total number of residues	Crude Purity	Purification Type	Purity after Purification
Wild-type (SN1717)[a]	29	30	61%	HPLC	99%
P4D2[a]	30	31	73%	SPE	77%
P7E8[a]	30	31	63%	SPE	71%
P14C09[a]	30	31	64%	SPE	61%
P14C10[a]	30	31	73%	SPE	75%
P21G09[a]	30	31	61%	SPE	70%
P7E8 V4A [a]	30	31	65%	SPE	72%
P7E8 V4M[a]	30	31	66%	SPE	71%
P7E8 D3E[a]	30	31	49%	HPLC	97%
P7E8 D3N[a]	30	31	54%	SPE	62%
GLP-1-**NPA**[a,b]	27	31	66%	HPLC	94%
GLP-1-**PS**[a,b]	27	31	53%	HPLC	96%
GLP-1-**CYA**[a,b]	27	31	52%	HPLC	99%

[a] C-terminal amino acid was manually coupled.
[b] Three N-terminal amino acids were manually coupled.

5 Analytical Data: Peptides

5.1 GLP-1 Derivatives containing canonical amino acids

5.1.1 P4D2

A) Synthesis Data for P4D2

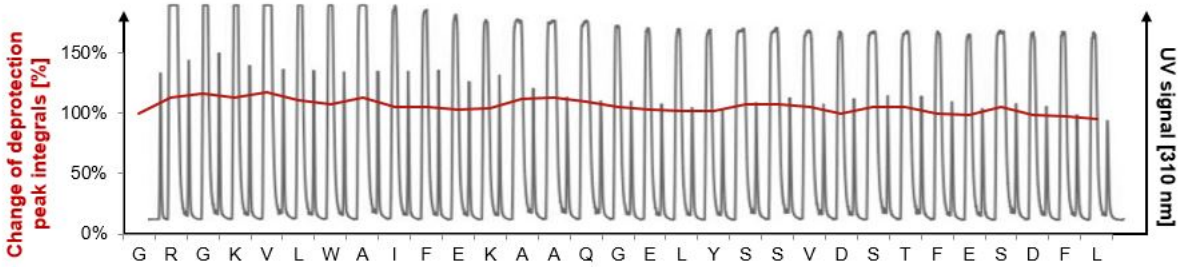
Sequence: LFDSEFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Synthesis method: All residues were coupled on the Amidator

Synthesis UV trace from AFPS:

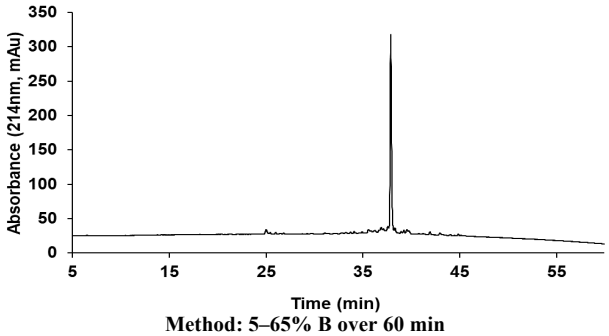


B) Cleavage and analytical Data for crude P4D2

Crude Purity: 73%

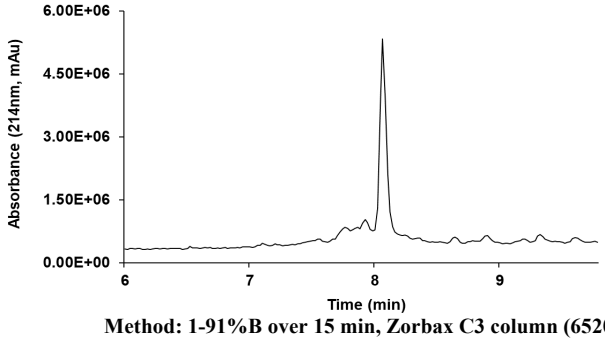
Crude Yield: 22%

Analytical HPLC:



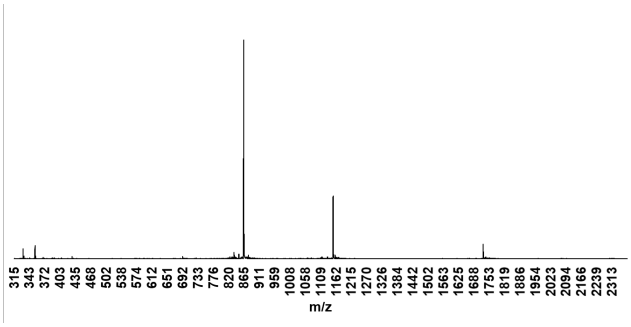
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

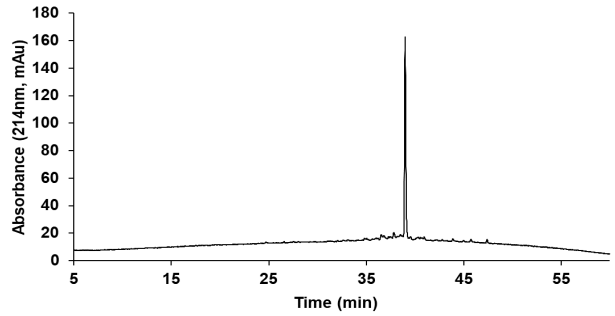
MS data from LC-MS:



C) SPE analytical data for P4D2

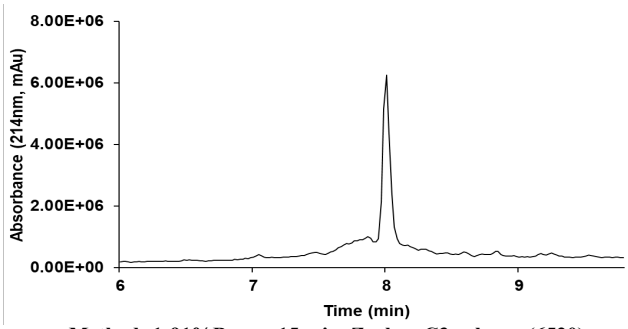
Purity after SPE: 77%

Analytical HPLC:



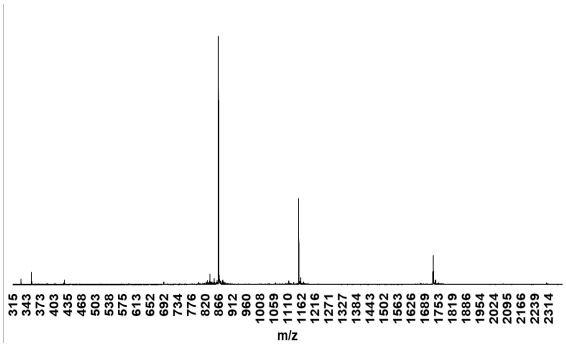
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3449.70
Obs.: 3449.87

5.1.2 P7E8

A) Synthesis Data for P7E8

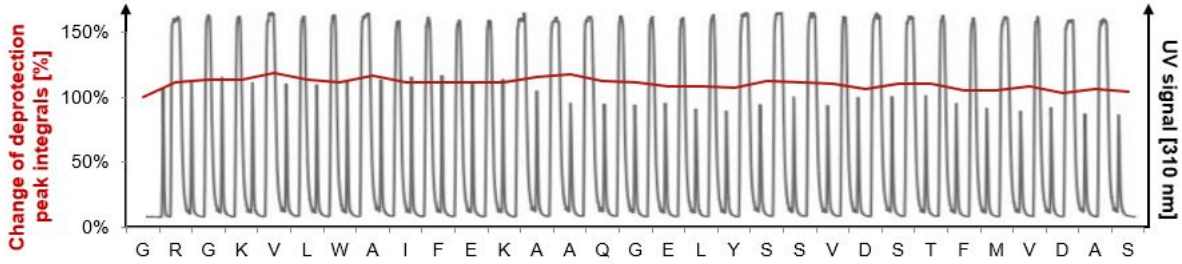
Sequence: SADVMFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Synthesis Method: All residues were coupled on the Amidator

Synthesis UV trace from AFPS:

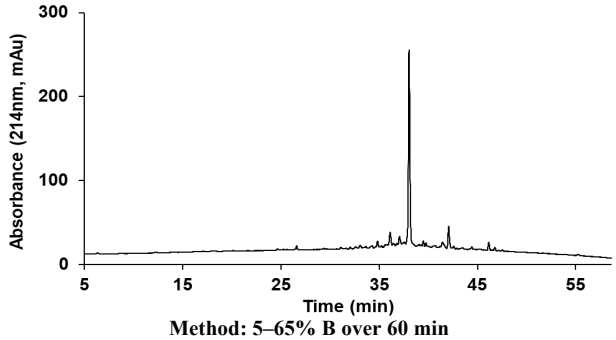


B) Cleavage and analytical data for crude P7E8

Crude Purity: 63%

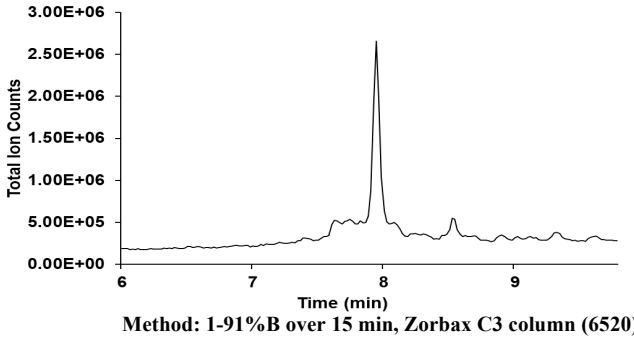
Crude Yield: 15%

Analytical HPLC:



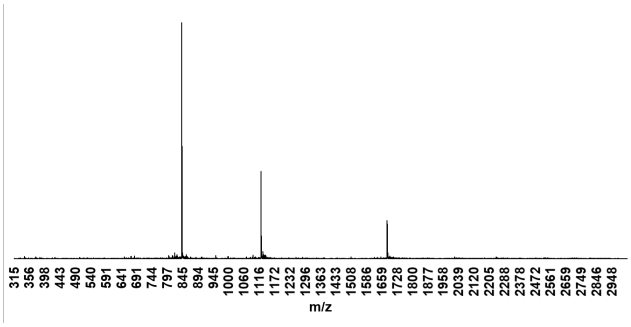
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

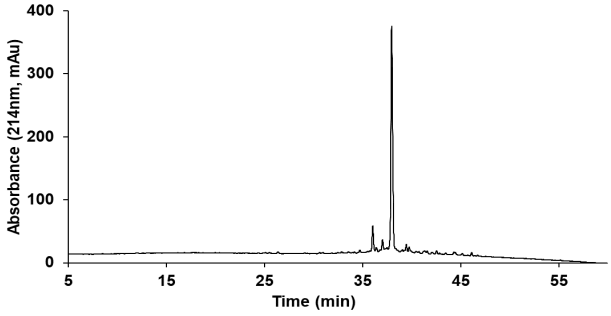
MS data from LC-MS:



C) SPE analytical data for P7E8

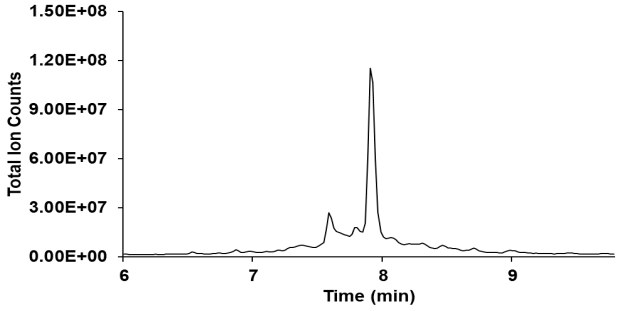
Purity after SPE: 71%

Analytical HPLC:



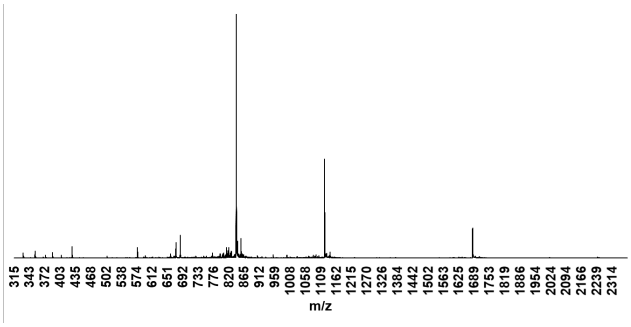
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3361.65
Obs.: 3361.83

5.1.3 P14C09

A) Synthesis Data for P14C09

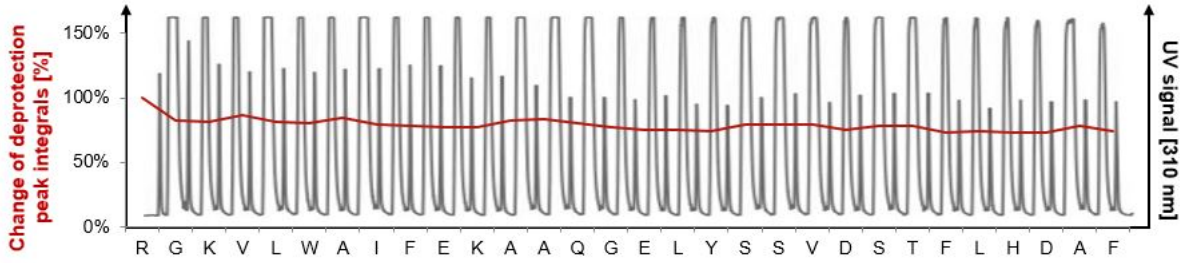
Sequence: FADHLFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Synthesis Method: All residues were coupled on the Amidator

Synthesis UV trace from AFPS:

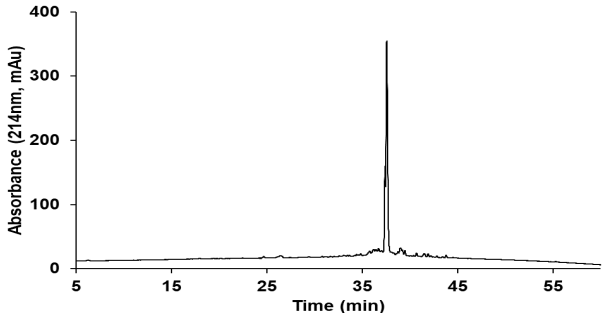


B) Cleavage and analytical Data for crude P14C09

Crude Purity: 64%

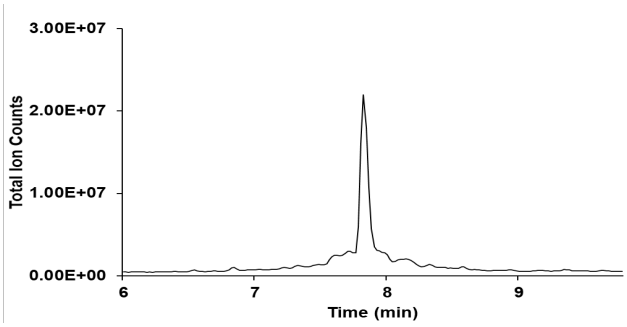
Crude Yield: 25%

Analytical HPLC:



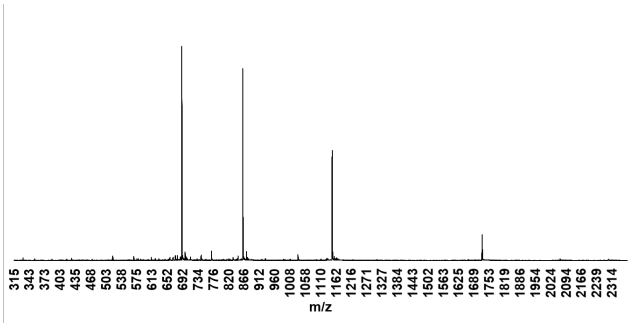
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

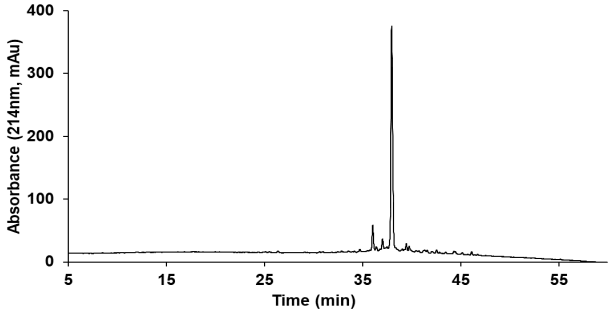
MS data from LC-MS:



C) SPE analytical data for P14C09

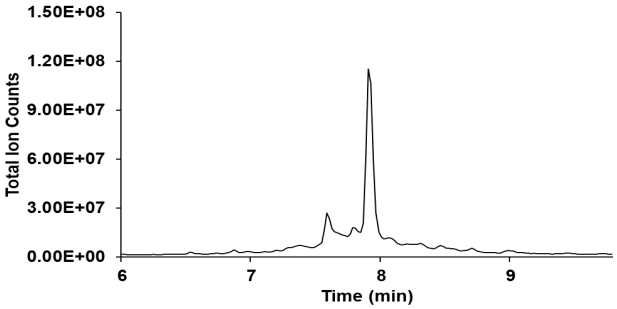
Purity after SPE: 61%

Analytical HPLC:



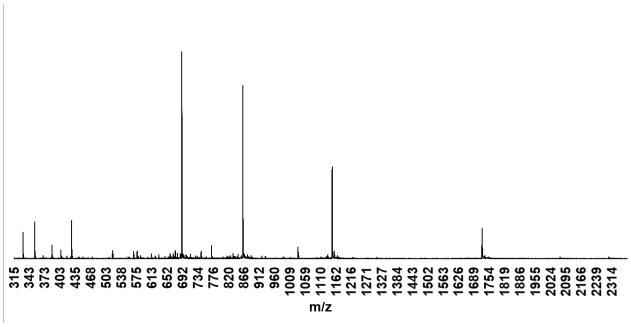
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3441.73
Obs.: 3441.91

5.1.4 P14C10

A) Synthesis Data for P14C10

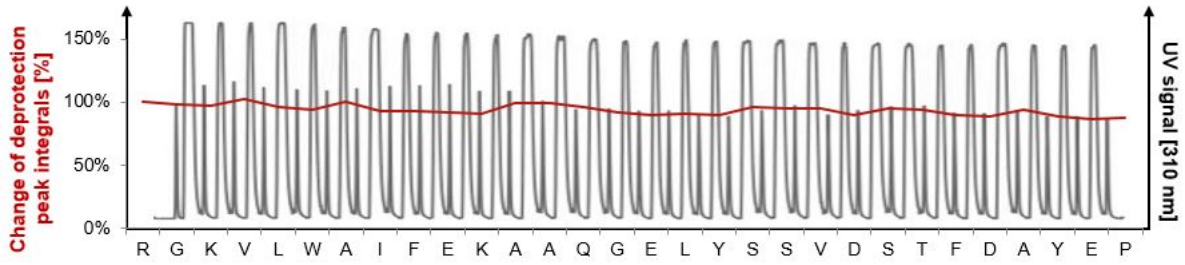
Sequence: PEYADFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.1 h

Synthesis Method: All residues were coupled on the Amidator

Synthesis UV trace from AFPS:

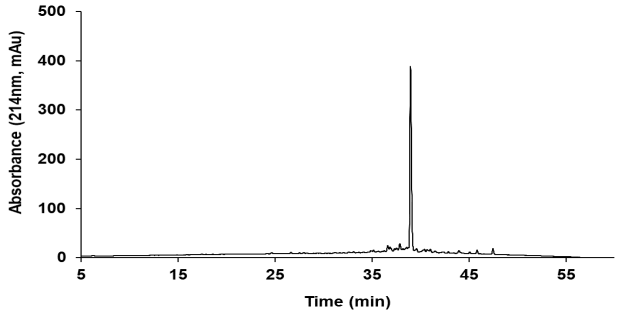


B) Cleavage and analytical Data for crude P14C10

Crude Purity: 73%

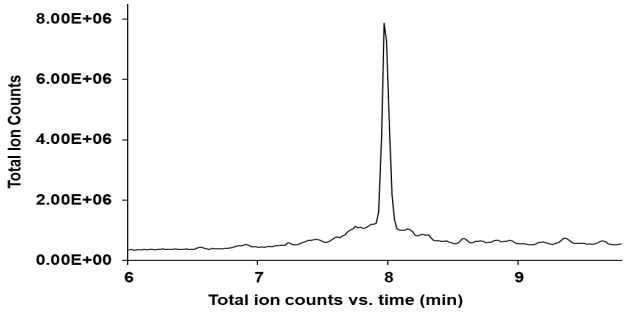
Crude Yield: 27%

Analytical HPLC:



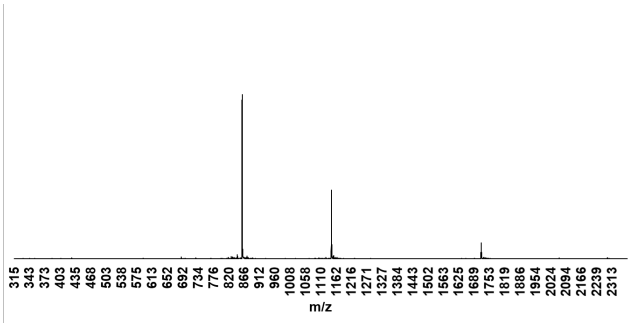
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

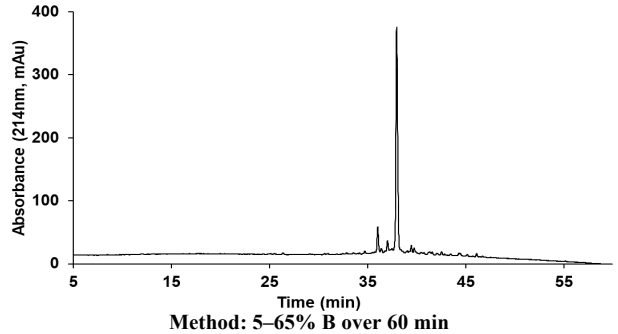
MS data from LC-MS:



C) SPE analytical data for P14C10

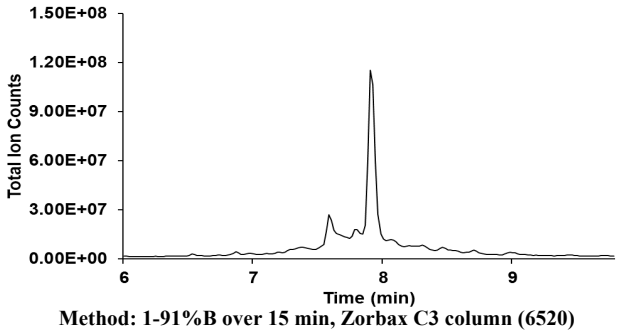
Purity after SPE: 75%

Analytical HPLC:



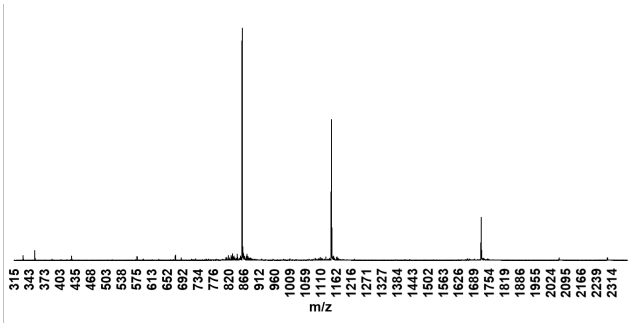
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3443.67
Obs.: 3443.83

5.1.5 P21G09

A) Synthesis Data for P21G09

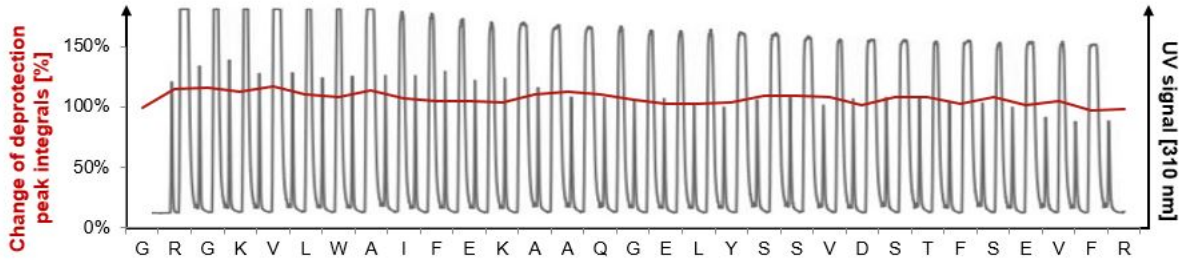
Sequence: RFVESFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.2 h

Synthesis Method: All resiudes were coupled on the Amidator

Synthesis UV trace from AFPS:

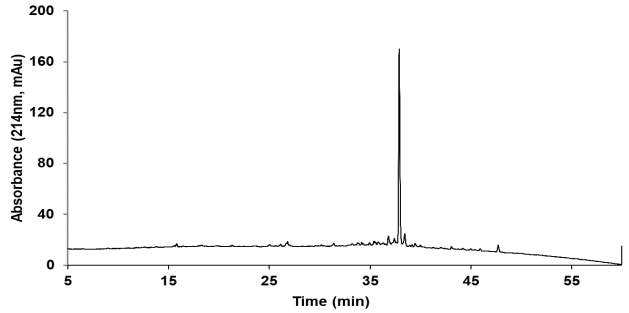


B) Cleavage and analytical Data for crude P21G09

Crude Purity: 61%

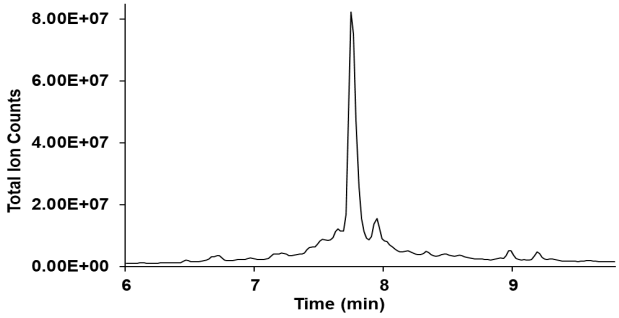
Crude Yield: 22%

Analytical HPLC:



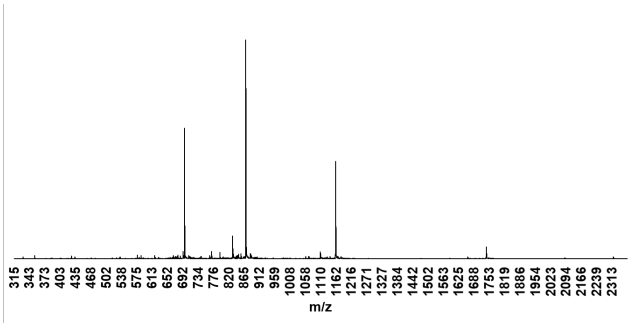
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

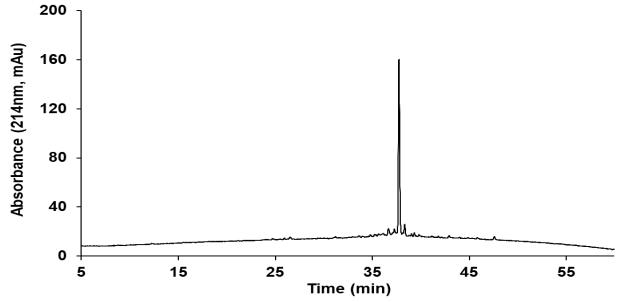
MS data from LC-MS:



C) SPE analytical data for P21G09

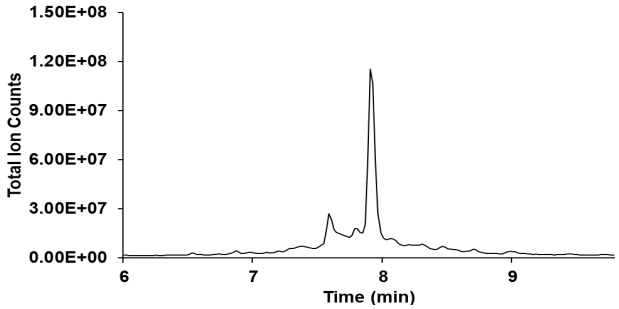
Purity after SPE: 70%

Analytical HPLC:



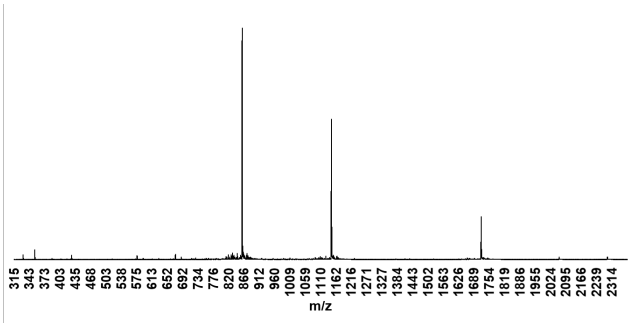
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3476.76
Obs.: 3476.93

5.1.6 P7E8 V4A

A) Synthesis Data for P7E8 V4A

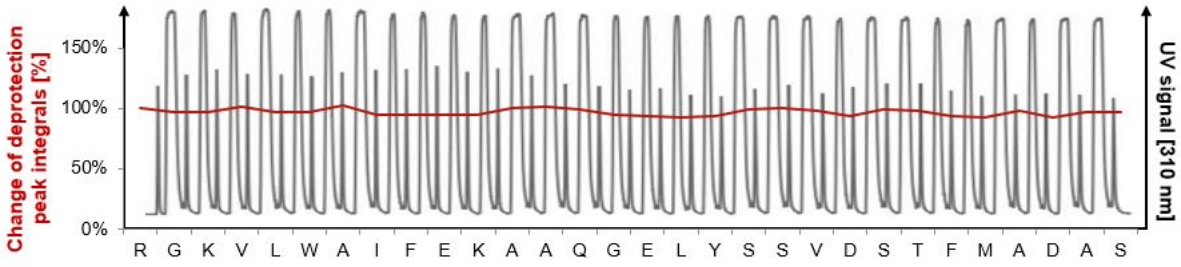
Sequence: SADAMFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Instrument: Amidator

Synthesis UV trace from AFPS:

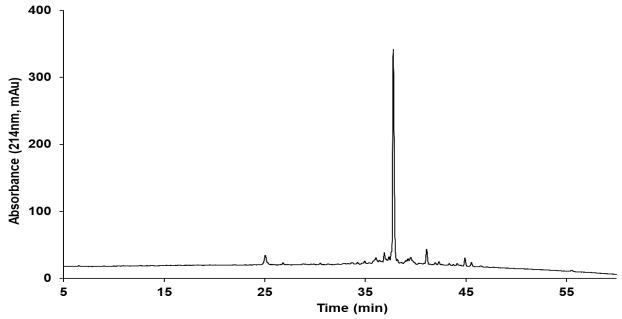


B) Cleavage and analytical Data for crude P7E8 V4A

Crude Purity: 65%

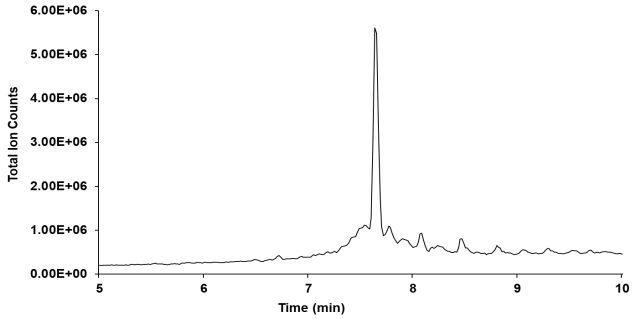
Crude Yield: 18%

Analytical HPLC:



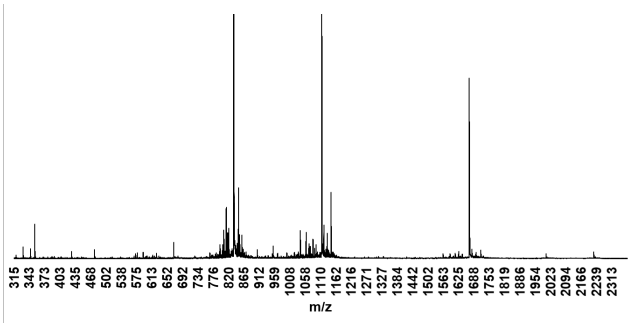
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

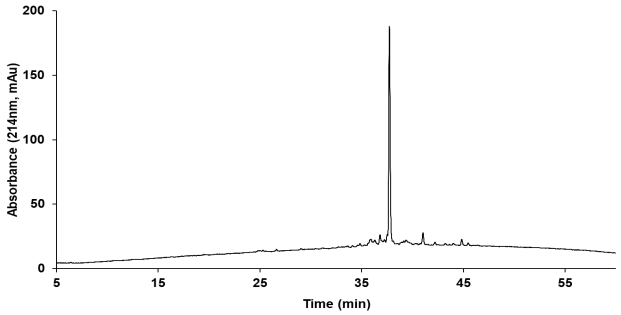
MS data from LC-MS:



C) SPE analytical data for P7E8 V4A

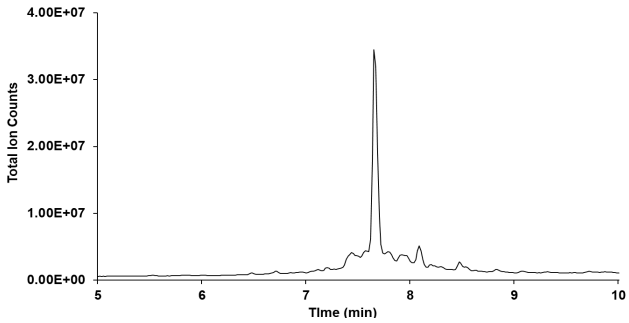
Purity after SPE: 72%

Analytical HPLC:



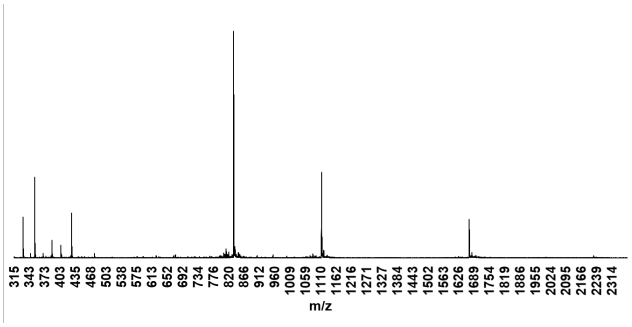
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3333.62
Obs.: 3333.75

5.1.7 P7E8 V4M

A) Synthesis Data for P7E8 V4M

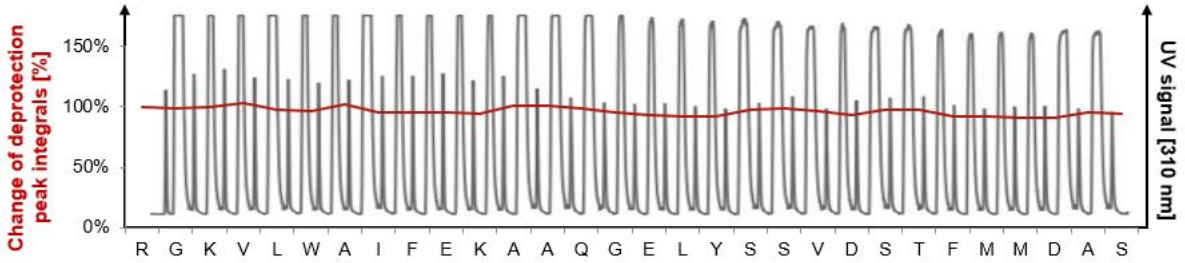
Sequence: SADMMFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.4 h

Instrument: Amidator

Synthesis UV trace from AFPS:

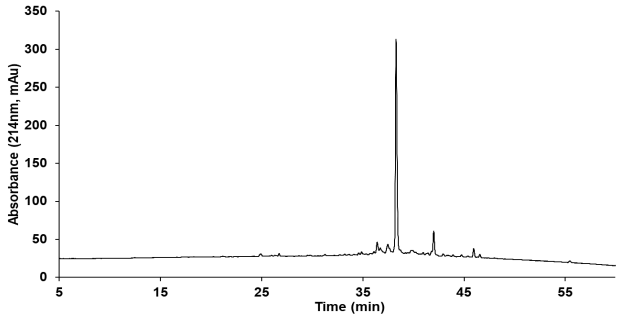


B) Cleavage and analytical Data for crude P7E8 V4M

Crude Purity: 66%

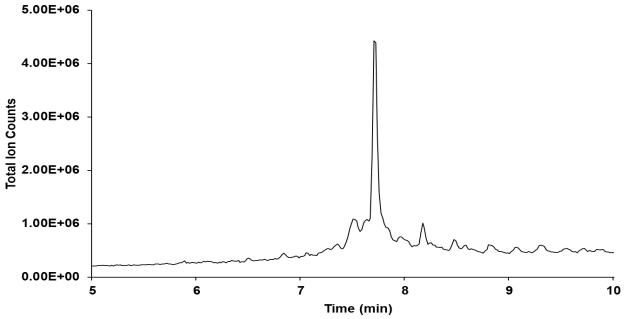
Crude Yield: 14%

Analytical HPLC:



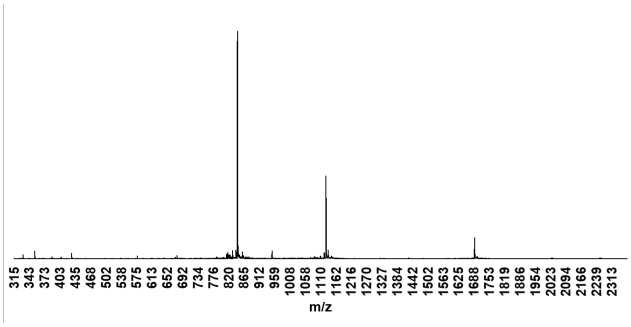
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

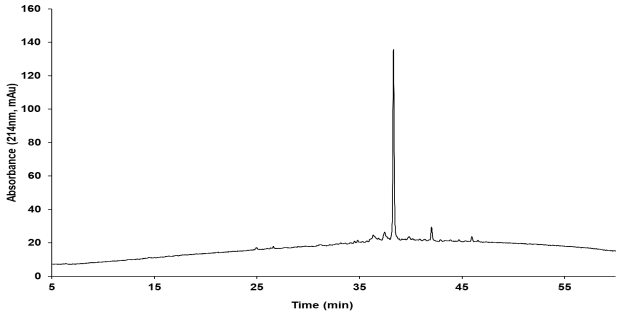
MS data from LC-MS:



C) SPE analytical data for P7E8 V4M

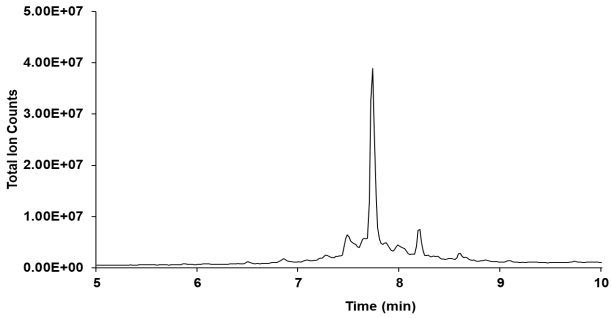
Purity after SPE: 72%

Analytical HPLC:



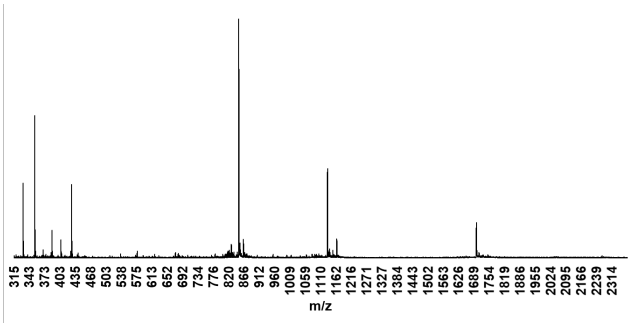
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc.: 3393.64
Obs.: 3393.63

5.1.8 P7E8 D3E

A) Synthesis Data for P7E8 D3E

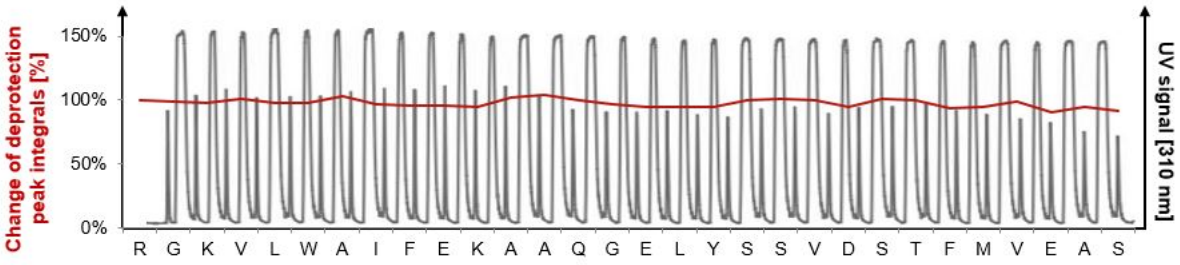
Sequence: SAEVMFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Instrument: Amidator

Synthesis UV trace from AFPS:

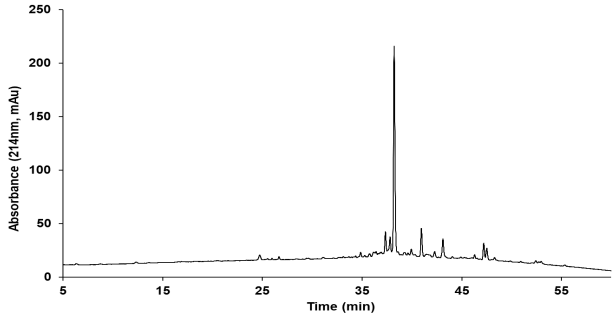


B) Cleavage and analytical Data for crude P7E8 D3E

Crude Purity: 49%

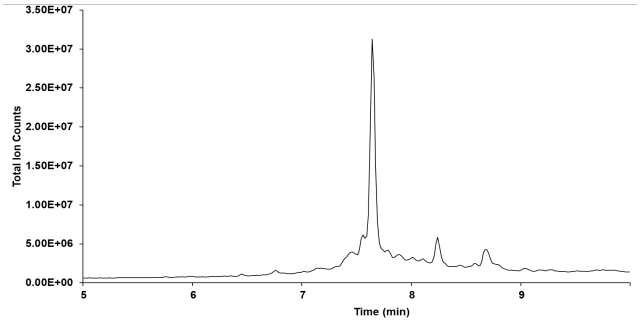
Crude Yield: 15%

Analytical HPLC:



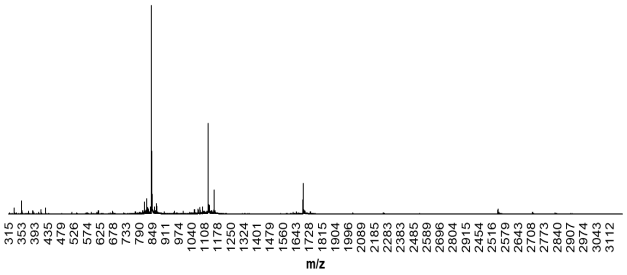
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



C) HPLC purification and analytical data for P7E8 D3E

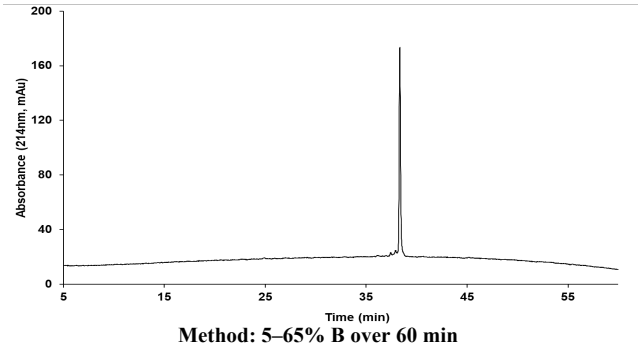
HPLC Purification: Purified on a preparative Zorbax 300SB-C3 column (gradient: 20-50%B over 60 min)

Purity after HPLC: 97%

Yield after HPLC: 1%

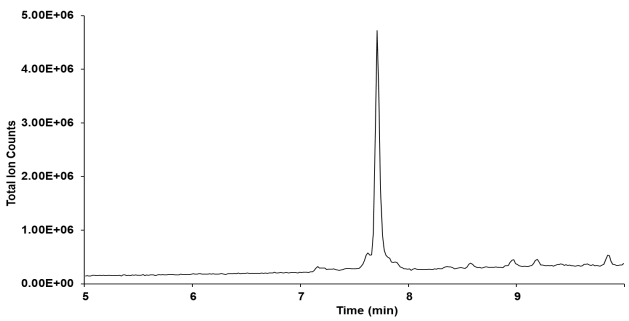
Isolated Material*: 0.6 mg (full cleavage: 1.0 mg)

Analytical HPLC:



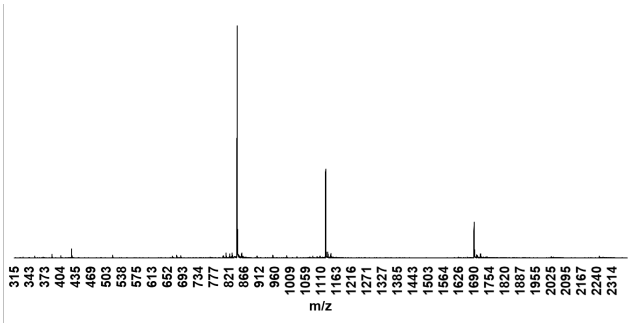
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc. mass: 3375.67
Obs. mass: 3375.80

5.1.9 P7E8 D3N

A) Synthesis Data for P7E8 D3N

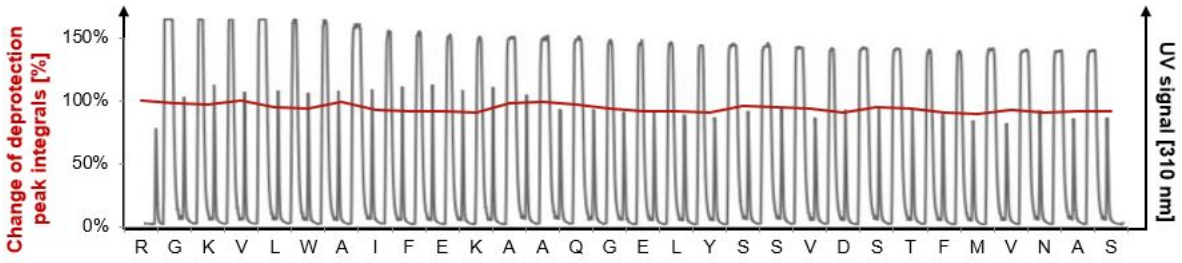
Sequence: SANVMFTSDV SSYLEGQAAK EFIAWLVKGR G (31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time: 1.3 h

Synthesis Method: All residues coupled on Amidator

Synthesis UV trace from AFPS:

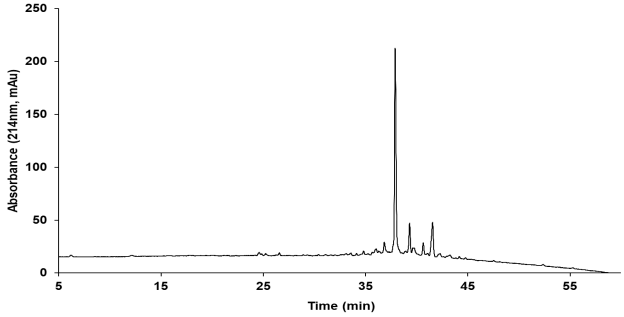


B) Cleavage and analytical Data for crude P7E8 D3N

Crude Purity: 54%

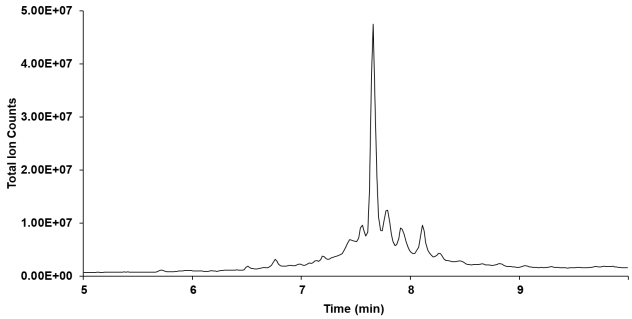
Crude Yield: 17%

Analytical HPLC:



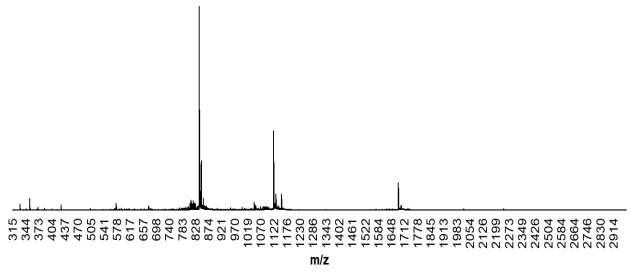
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

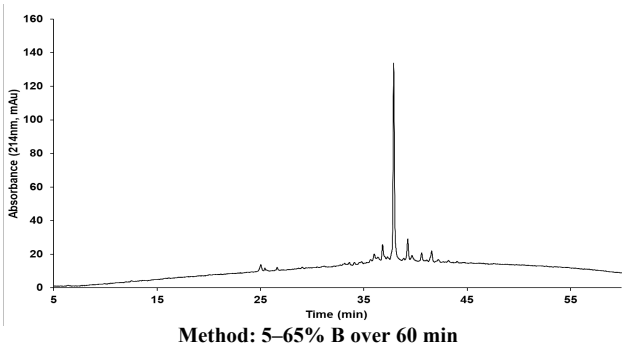
MS data from LC-MS:



C) SPE analytical data for P7E8 D3N

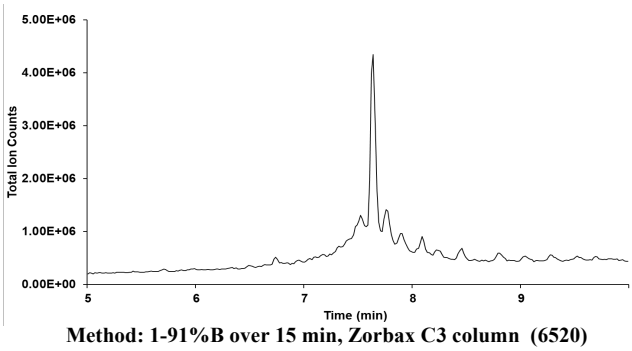
Purity after SPE: 62%

Analytical HPLC:



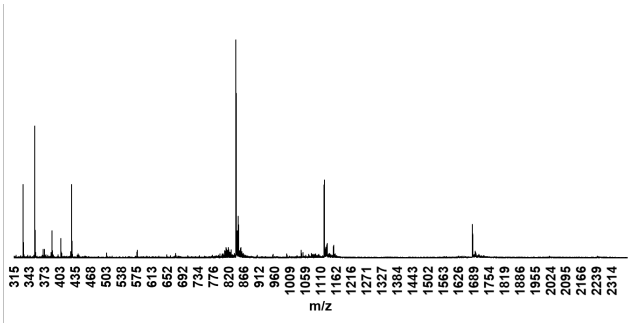
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6520)

MS data from LC-MS:



Calc. mass: 3360.67
Obs. mass: 3360.80

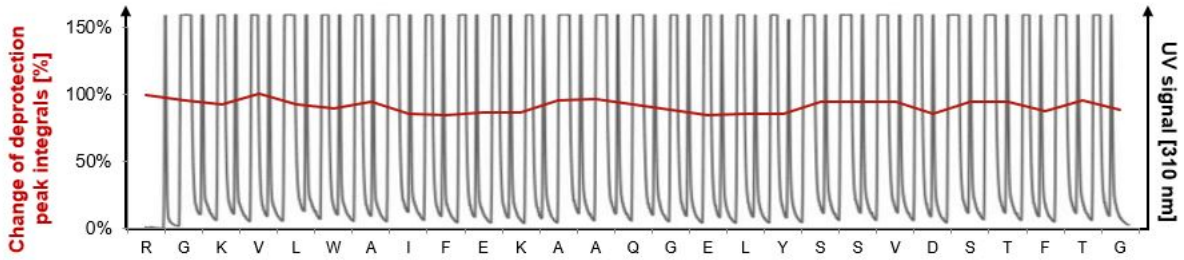
5.2 GLP-1 Derivatives containing a non-canonical substitution

5.2.1 GLP-1-NPA (Nitrophenylalanine (NPA))

A) Synthesis Data for GLP-1-NPA

Sequence:	HAXGTFTSDV SSYLEGQAAK EFIAWLVKGR G (X = Nitro-phenylalanine) (31 AA)
Resin:	100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage
Synthesis time (residues 1-28):	1.1 h
Synthesis Method:	Amino acids 1-28 were coupled on Peptidator. HAX residues were coupled manually.

Synthesis UV trace from AFPS (residues 1-28):

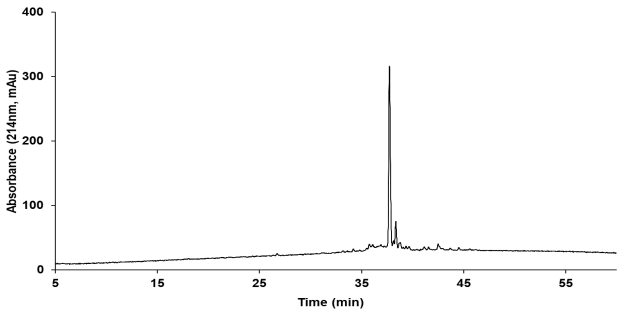


B) Cleavage and analytical Data for crude GLP-1-NPA

Crude Purity: 66%

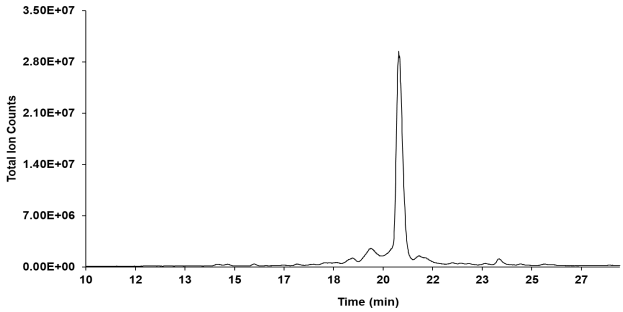
Crude Yield: 28%

Analytical HPLC:



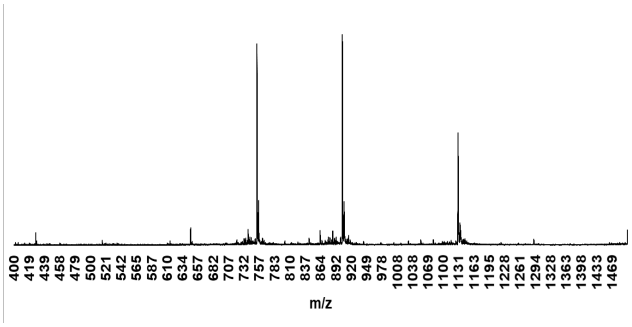
Method: 5–65% B over 60 min

LC-MS:



Method: 1-61%B over 34 min, Jupiter C4 column

MS data from LC-MS:



C) HPLC purification and analytical data for GLP-1-NPA

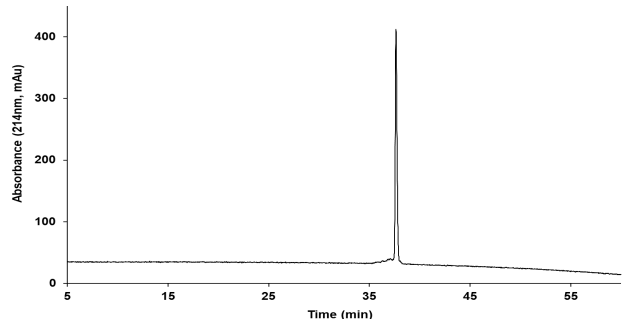
HPLC purification: Purified on a preparative Zorbax 300SB-C3 column (gradient: 5-32%B with 0.9%B per min; 33-53%B with 0.2%B per min; 52-65%B with 1%B per min)

Purity after HPLC: 94%

Yield after HPLC: 3%

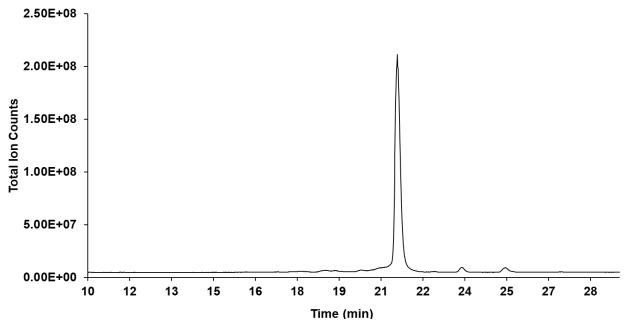
Isolated Material*: 1.7 mg (full cleavage: 5.0 mg)

Analytical HPLC:



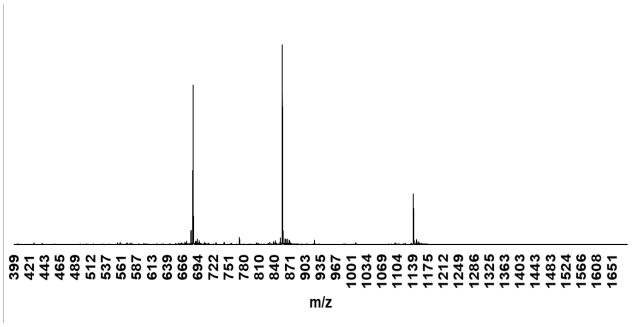
Method: 5–65% B over 60 min

LC-MS:



Method: 1-61%B over 34 min, Jupiter C4 column

MS data from LC-MS:



Calc. mass: 3416.69
Obs. mass: 3416.80

5.2.2 GLP-1-PS (Phosphoserine (PS))

A) Synthesis Data for GLP-1-PS

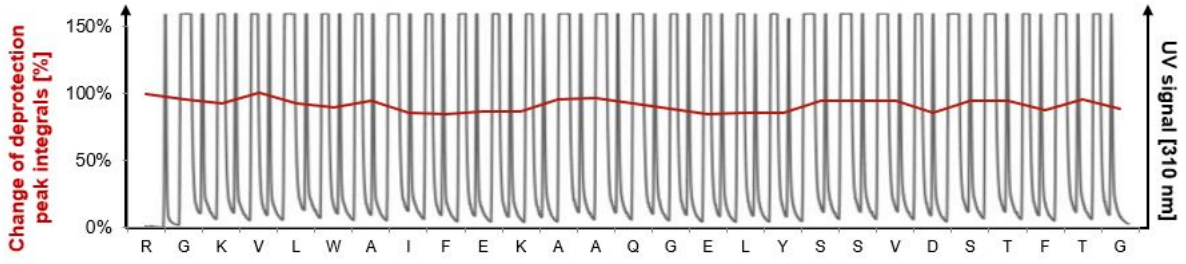
Sequence: HAXGTFTSDV SSYLEGQAAK EFIAWLVKGR G (X = Phosphoserine)
(31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time (residues 1-28): 1.1 h

Synthesis Method: Amino acids 1-28 were coupled on Peptidator. HAX residues were coupled manually.

Synthesis UV trace from AFPS (residues 1-28):

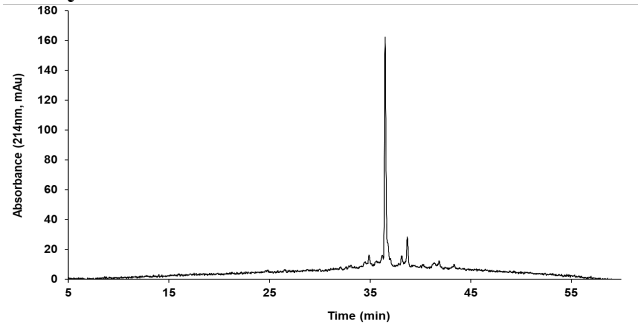


B) Cleavage and analytical Data for crude GLP-1-PS

Crude Purity: 53%

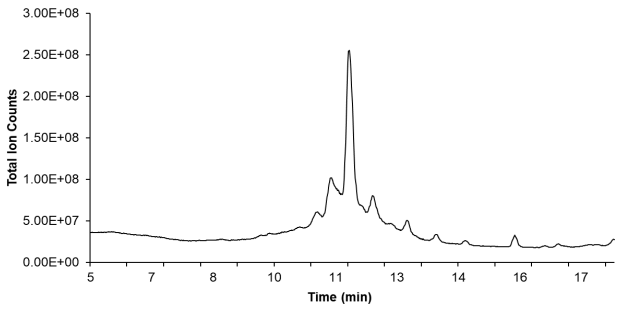
Crude Yield: 19%

Analytical HPLC:



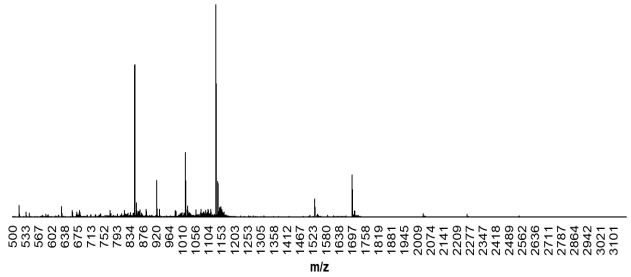
Method: 5-65%B over 60 min

LC-MS:



Method: 1-91%B over 21 min, Jupiter C4 column (6550-1)

MS data from LC-MS:



C) HPLC purification and analytical data for GLP-1-PS

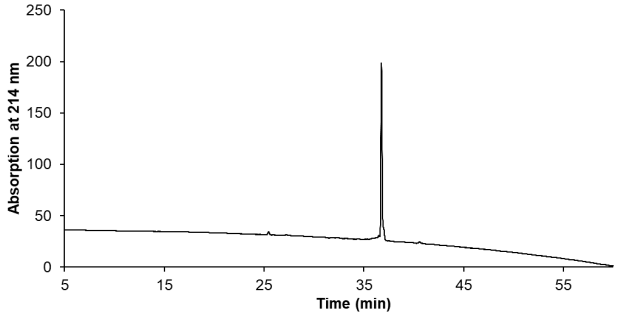
HPLC Purification: Purified on a semi-preparative Zorbax 300SB-C3 column (gradient: 5-28%B with 1%B per min; 28-48%B with 0.2%B per min; 48-65%B with 1.1%B per min)

Purity after HPLC: 96%

Yield after HPLC: 6%

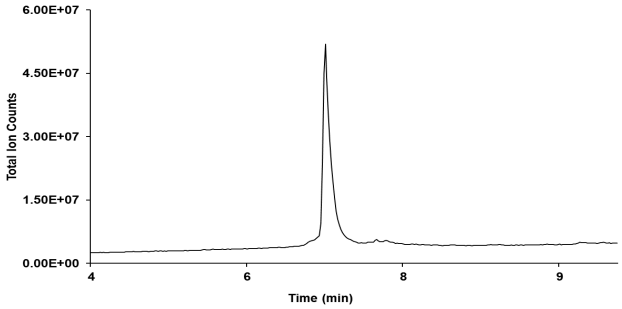
Isolated Material*: 4.0 mg (full cleavage: 10 mg)

Analytical HPLC:



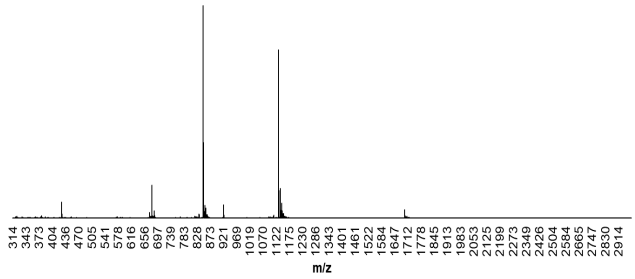
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column (6550-2)

MS data from LC-MS:



Calc. mass: 3391.62
Obs. mass: 3391.61

5.2.3 GLP-1-CYA (Cysteic Acid (CYA)) variant

A) Synthesis Data for GLP-1-CYA

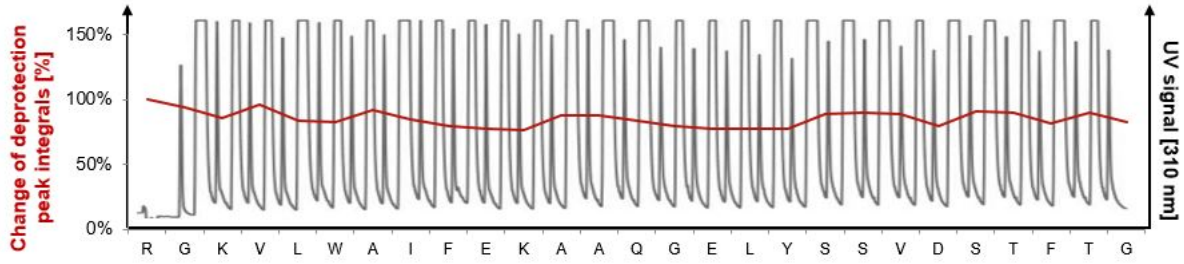
Sequence: HAXGTFTSDV SSYLEGQAAK EFIAWLVKGR G (X = Cysteic acid)
(31 AA)

Resin: 100 mg of HMPB ChemMatrix® (0.44 mmol/g) pre-coupled to Glycine, yielding the C-terminal carboxylic acid after cleavage

Synthesis time (residues 1-28): 1.1 h

Synthesis Method: Amino acids 1-28 were coupled on Peptidator. HAX residues were coupled manually. Cysteic acid was coupled with 50% DMSO co-solvent and coupling time was extended to 1.5 hours.

Synthesis UV trace from AFPS (residues) 1-28):

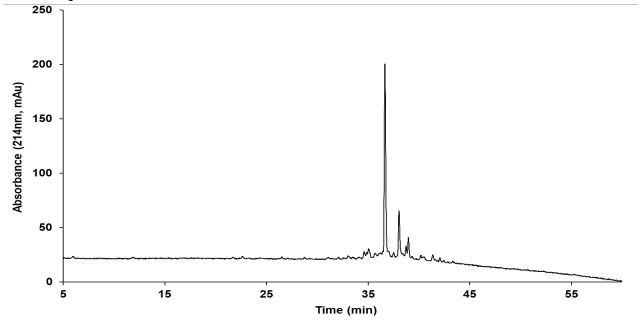


B) Cleavage and analytical Data for crude GLP-1-CYA

Crude Purity: 52%

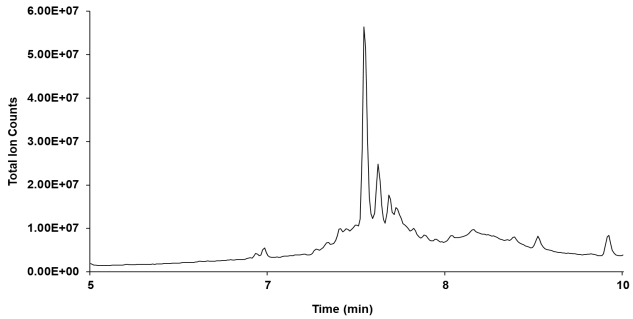
Crude Yield: 14%

Analytical HPLC:



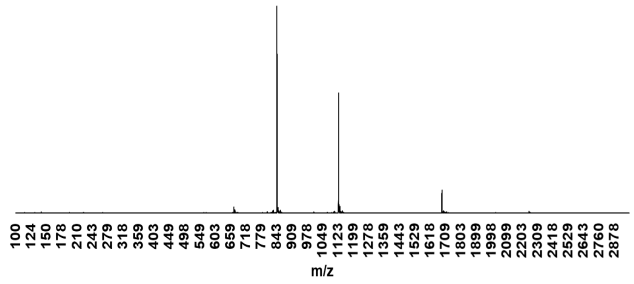
Method: 5–65% B over 60 min

LC-MS:



Method: 1-91%B over 15 min, Zorbax C3 column

MS data from LC-MS:



C) HPLC purification and analytical data for GLP-1-CYA

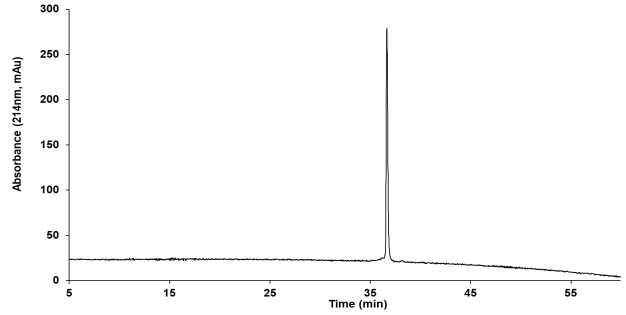
Preparative HPLC: Purified on a preparative Zorbax 300SB-C3 column (gradient: 5-32%B with 0.9%B per min; 32-52%B with 0.2%B per min; 52-65%B with 1%B per min)

Purity after HPLC: 99%

Yield after HPLC: 4%

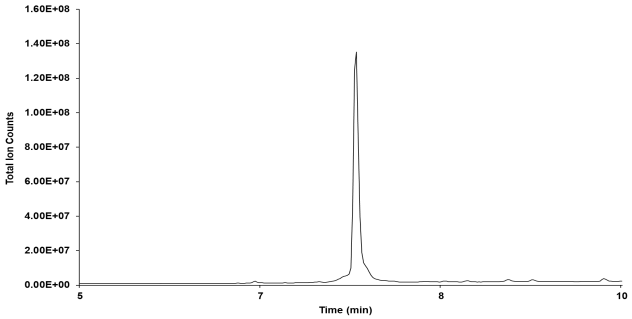
Isolated Material*: 1.8 mg (full cleavage: 7.0 mg)

Analytical HPLC:



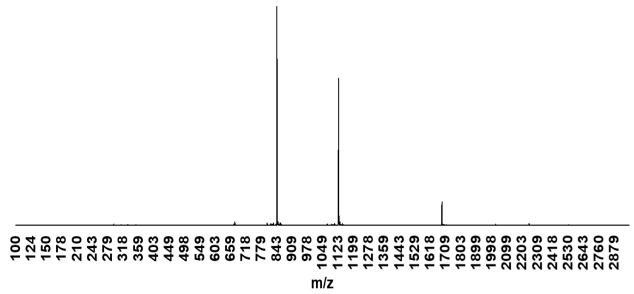
Method: 5–65% B over 60 min

LC-MS:



Method: 1-61%B over 34 min, Jupiter C4 column

MS data from LC-MS:



Calc. mass: 3375.78
Obs. mass: 3375.60

* Isolated material from partial cleavage of the resin; extrapolated value for full cleavage is given in parentheses

6 Rosetta Run Command & Options

Flags:

```
# standard inputs
-s [input pdb]
-remodel:blueprint [input blueprint]
-jd2:no_output
-overwrite
-ex1
-ex2
-num_trajectory [number of trials to run]
-save_top [number of outputs to save]
# skip stage 1 (centroid fragment sampling)
-bypass_fragments
-bypass_closure
# include stage 3 (relax designed residues and neighbors)
-remodel:use_pose_relax
-find_neighbors
# for NCAA
-extra_res_fa CYA.params
# optional
-remodel:dr_cycles 3
-no_optH false
```

**Bp

```
…
380 R .
381 K .
382 S .
383 W .
384 E .
385 R .
386 W .
387 R .
388 H H PIKAA H
389 A H PIKAA A
390 E H EMPTY NC CYA
391 G H PIKAA G
392 T H PIKAA T
393 F .
394 T .
395 S .
396 D .
397 V .
398 S .
399 S .
400 Y .
…
```

Command:

```
<path_to_Rosetta>/main/source/bin/remodel.default.linuxgccrelease @Flags
```

**Residues 1-380 and 401-418 are unmodified (i.e. '.'). in the blueprint (bp) file.

7 Literature

1. Marathe, C. S., Rayner, C. K., Jones, K. L. & Horowitz, M. Glucagon-like peptides 1 and 2 in health and disease: A review. *Peptides* **44**, 75–86 (2013).
2. Mijalis, A. J. *et al.* A fully automated flow-based approach for accelerated peptide synthesis. *Nat. Chem. Biol.* **13**, 464–466 (2017).
3. Hartrampf, N. *et al.* Synthesis of proteins by automated flow chemistry. *Science* **368**, 980–987 (2020).