**Electronic Supplementary Information (ESI)**

# Sustainable, cost-efficient manufacturing of therapeutic peptides using chemo-enzymatic peptide synthesis (CEPS)

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## 1. LC-HRMS analysis of crude H-22-39-$NH_2$, the original method.

Experimental conditions: column: Phenomenex Kinetex 2.6 µm 4.6 x 150 mm; column temperature: 30°C; injection volume: 5 uL; sampler temperature:10°C; MS mode: positive 50-3200; DAD: 220 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.8 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1%TFA in water, mobile phase B: 0.1 %TFA in 10% water/90 %MeCN. Gradient (Time(min), %B): 0, 10; 1, 10; 46, 40; 48, 90; 50, 90; 50.1, 10; 60,10.

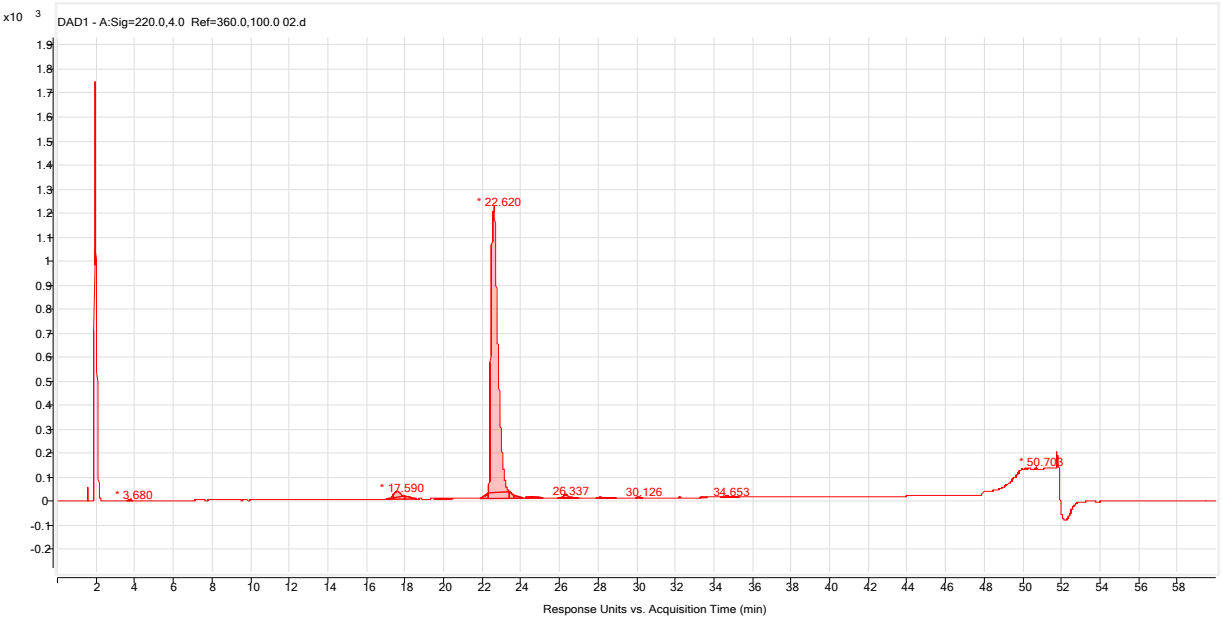



**Figure S1.** UV chromatogram overview.

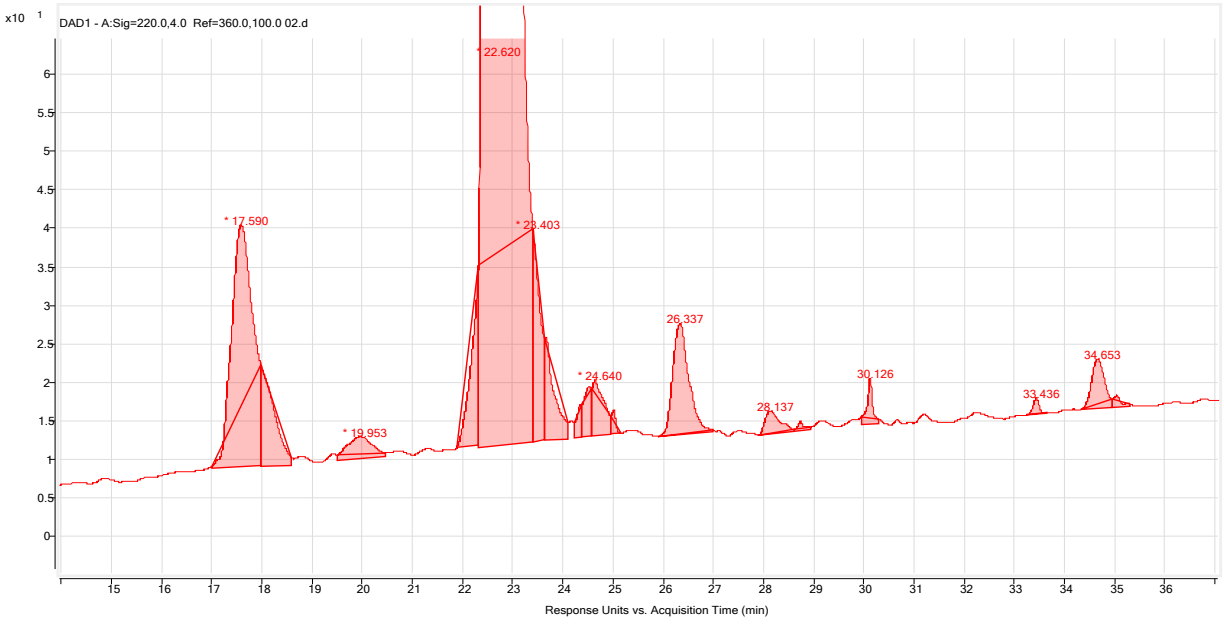



**Figure S2.** UV chromatogram zoom-in.

**Table S1.** Area% for integrated peaks and MS identities for H-22-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3,68 | 33,310 | | | 0,0000 | -1838,9315 | 0,163 | 0,10 | |
| 2 | 3,77 | 15,990 | | | 0,0000 | -1838,9315 | 0,167 | 0,05 | |
| 3 | 17,59 | 938,980 | 746,8815 | 2 | 1491,7484 | -347,1831 | 0,778 | 2,80 | Ac-(25-39)-NH2 |
| 4 | 17,98 | 235,840 | 811,4024 | 2 | 1620,7902 | -218,1413 | 0,795 | 0,70 | Ac-(24-39)-NH2 |
| 5 | 19,95 | 102,640 | | | 0,0000 | -1838,9315 | 0,882 | 0,31 | |
| 6 | 22,31 | 211,830 | | | | -1838,9315 | 0,986 | 0,63 | |
| **7** | **22,62** | **30675,060** | **920,5078** | **2** | **1839,0010** | **0,0695** | **1,000** | **91,57** | **Prod. H-(22-39)-NH2** |
| 8 | 23,40 | 266,020 | 876,9852 | 2 | 1751,9558 | -86,9757 | 1,035 | 0,79 | Des Ser |
| 9 | 23,66 | 174,930 | | | 0,0000 | -1838,9315 | 1,046 | 0,52 | |
| 10 | 24,37 | 28,060 | | | 0,0000 | -1838,9315 | 1,077 | 0,08 | |
| 11 | 24,52 | 64,660 | | | 0,0000 | -1838,9315 | 1,084 | 0,19 | |
| 12 | 24,64 | 113,690 | | | 0,0000 | -1838,9315 | 1,089 | 0,34 | |
| 13 | 25,01 | 17,880 | | | 0,0000 | -1838,9315 | 1,106 | 0,05 | |
| 14 | 26,34 | 322,270 | 867,9447 | 2 | 1733,8748 | -105,0567 | 1,164 | 0,96 | Ac-(23-39)-NH2 |
| 15 | 28,14 | 62,880 | | | 0,0000 | -1838,9315 | 1,244 | 0,19 | |
| 16 | 28,74 | 11,690 | | | 0,0000 | -1838,9315 | 1,270 | 0,03 | |
| 17 | 30,13 | 46,420 | | | 0,0000 | -1838,9315 | 1,332 | 0,14 | |
| 18 | 33,44 | 17,120 | | | 0,0000 | -1838,9315 | 1,478 | 0,05 | |
| 19 | 34,65 | 115,500 | | | 0,0000 | -1838,9315 | 1,532 | 0,34 | |
| 20 | 35,04 | 16,560 | | | 0,0000 | -1838,9315 | 1,549 | 0,05 | |
| 21 | 50,70 | 27,770 | | | 0,0000 | -1838,9315 | 2,242 | 0,08 | |

## 2. LC-HRMS analysis of crude H-22-39-$NH_2$, the revised method.

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume:1 uL; sampler temperature:5°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1%TFA in water, mobile phase B: 0.1 %TFA in MeCN. Gradient (Time(min), %B): 0, 5; 1, 5; 60, 30; 62, 90; 62.1, 5; 72, 5.

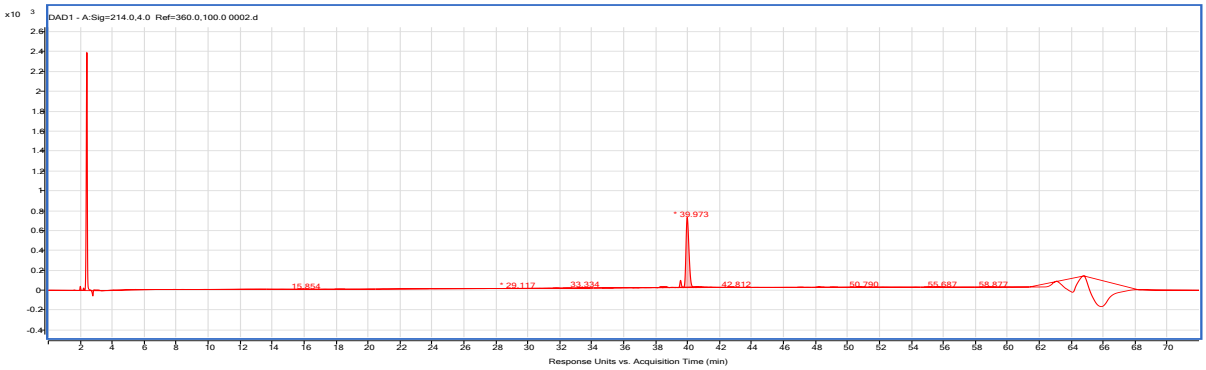



**Figure S3.** UV chromatogram overview.

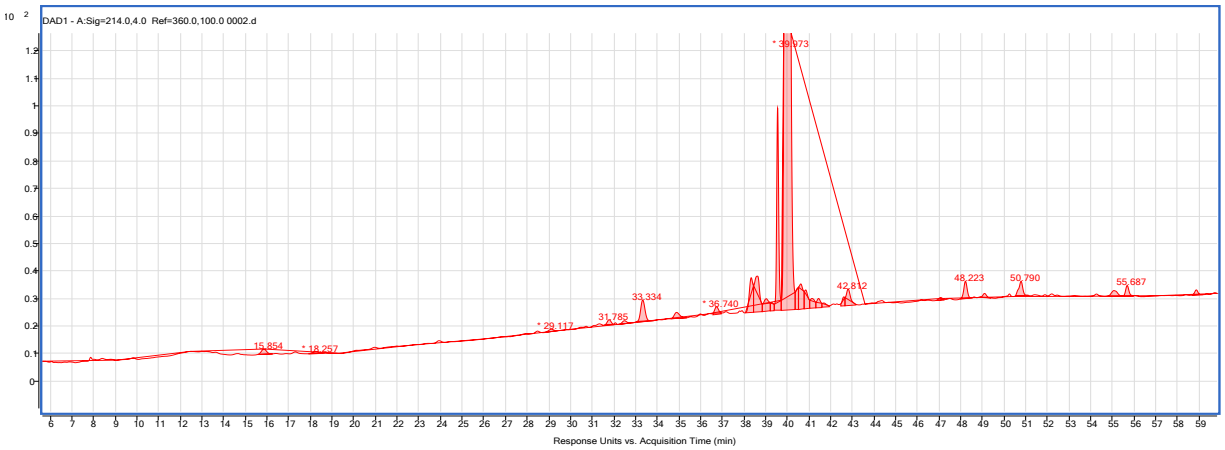


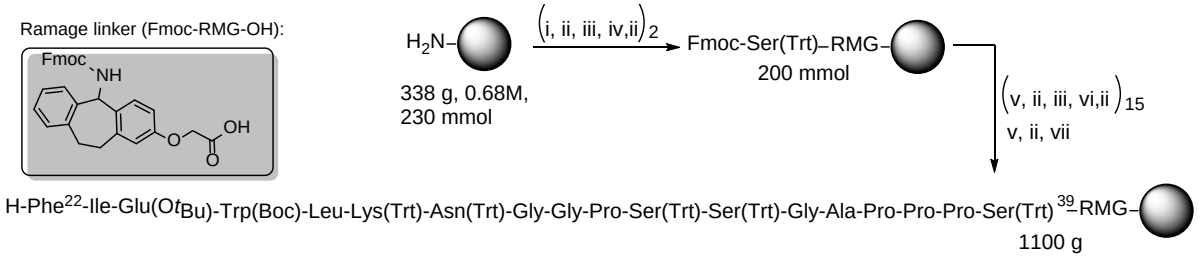

**Figure S4.** UV chromatogram zoom-in.

**Table S2.** Area% for integrated peaks and MS identities for H-22-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 15,85 | 31,680 | | | 0,0000 | -1838,9320 | 0,397 | 0,28 | |
| 2 | 18,26 | 14,990 | | | 0,0000 | -1838,9320 | 0,457 | 0,13 | |
| 3 | 29,12 | 13,910 | | | 0,0000 | -1838,9320 | 0,728 | 0,12 | |
| 4 | 31,79 | 23,800 | | | 0,0000 | -1838,9320 | 0,795 | 0,21 | |
| 5 | 32,49 | 15,430 | | | 0,0000 | -1838,9320 | 0,813 | 0,14 | |
| 6 | 33,33 | 111,600 | | | 0,0000 | -1838,9320 | 0,834 | 1,00 | |
| 7 | 34,90 | 38,420 | | | 0,0000 | -1838,9320 | 0,873 | 0,34 | |
| 8 | 36,74 | 36,890 | | | 0,0000 | -1838,9320 | 0,919 | 0,33 | |
| 9 | 38,34 | 151,670 | 920,4810 | 2 | 1838,9474 | 0,0154 | 0,959 | 1,35 | N to O acyl isomer |
| 10 | 38,63 | 223,700 | 920,4840 | 2 | 1838,9534 | 0,0214 | 0,966 | 2,00 | N to O acyl isomer |
| 11 | 39,03 | 81,050 | | | 0,0000 | -1838,9320 | 0,976 | 0,72 | |
| 12 | 39,30 | 29,090 | | | 0,0000 | -1838,9320 | 0,983 | 0,26 | |
| 13 | 39,56 | 483,380 | 823,4210 | 2 | 1644,8274 | -194,1046 | 0,990 | 4,31 | des Pro-Pro |
| **14** | **39,97** | **9178,530** | **920,5050** | **2** | **1838,9954** | **0,0634** | **1,000** | **81,89** | **Prod. H-(22-39)-NH2** |
| 15 | 40,52 | 65,380 | | | 0,0000 | -1838,9320 | 1,014 | 0,58 | |
| 16 | 40,63 | 123,900 | | | 0,0000 | -1838,9320 | 1,016 | 1,11 | |
| 17 | 40,85 | 79,480 | | | 0,0000 | -1838,9320 | 1,022 | 0,71 | |
| 18 | 41,15 | 51,130 | | | 0,0000 | -1838,9320 | 1,029 | 0,46 | |
| 19 | 41,45 | 42,940 | | | 0,0000 | -1838,9320 | 1,037 | 0,38 | |
| 20 | 41,69 | 20,600 | | | 0,0000 | -1838,9320 | 1,043 | 0,18 | |
| 21 | 42,61 | 27,500 | | | 0,0000 | -1838,9320 | 1,066 | 0,25 | |
| 22 | 42,81 | 87,900 | | | 0,0000 | -1838,9320 | 1,071 | 0,78 | |
| 23 | 47,08 | 12,420 | | | 0,0000 | -1838,9320 | 1,178 | 0,11 | |
| 24 | 48,22 | 70,270 | | | 0,0000 | -1838,9320 | 1,206 | 0,63 | |
| 25 | 49,11 | 18,000 | | | 0,0000 | -1838,9320 | 1,229 | 0,16 | |
| 26 | 50,79 | 71,520 | | | 0,0000 | -1838,9320 | 1,271 | 0,64 | |
| 27 | 55,08 | 40,850 | | | 0,0000 | -1838,9320 | 1,378 | 0,36 | |
| 28 | 55,69 | 43,570 | | | 0,0000 | -1838,9320 | 1,393 | 0,39 | |
| 29 | 58,88 | 19,380 | | | 0,0000 | -1838,9320 | 1,473 | 0,17 | |

## 3. Details of SPPS of H-22-39-RMG DEG AM resin (Scheme S1).

Piperidine (pip) was used as the base for all Fmoc removals and Oxyma + DIC[1] were used as coupling agents for all amino acid (AA) couplings. In the first step of the synthesis the Ramage (tricyclic amide) linker, known to provide better cleavage yields than the more common Knorr (Rink amide) linker,[2] was coupled to the base resin. To ensure suitable reaction kinetics throughout the SPPS the base resin was downloaded ~30% during the first two couplings (linker and Ser$^{39}$)[3] and the entire synthesis was carried out under mild conventional heating.[4] Furthermore, to mimimize the occurrence of cleavage related impurities encountered with Fmoc-Lys(Boc)-OH[5-6] and Fmoc-Ser(*t*-Bu)-OH[5-7] respectively, we used the trityl protected Fmoc-Lys(Trt)-OH and Fmoc-Ser(Trt)-OH counterparts instead. Pro$^{38}$, Pro$^{37}$ and Gly$^{30}$, Gly$^{29}$ couplings were carried out using the inexpensive Fmoc-Pro-Pro-OH and Fmoc-Gly-Gly-OH dipeptides, respectively. The progress of all couplings was followed by qualitative color tests[8-9] according to which Trp$^{25}$ to Phe$^{22}$ couplings were not complete after ~30 min and were recoupled. Upon completion of all couplings any remaining amino groups were capped by addition of acetic acid. The synthesis afforded ~1100 g of H-22-39 peptide resin and the yield determined by test TFA cleavages followed by HPLC quantifications was 75%. To obtain sufficient amounts of the fragment needed for the ensuing ligations up to 300 g portions of the peptide resin were cleaved using a TFA/TIS/DTT/$H_2O$ (92:4:3:1, v/v/w/v) cleavage cocktail for 2 h at rt, followed by precipitation of the crude material with cold ether.




**Scheme S1** SPPS of H-22-39 resin, reagents and conditions: i) Fmoc deprotection: 12.5% pip in DMF, 40 – 45 °C, 25 min; ii) DMF resin wash; iii) coupling: R-COOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 30 min; iv) capping: AcOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 15 min; v) Fmoc deprotection: 12.5% pip in DMF, 40 – 45 °C, 2 x 15 min; vi) capping: AcOH, 5 min; vii) isopropanol (*i*-PrOH ) wash and drying in vacuo.

It is worth noting that although the initial liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis revealed an excellent purity (~91%) of the crude amine fragment, upon conducting additional analytical development work a method was found in which the crude product was only ~82% pure. In fact, the new method disclosed a ~4% double Pro-Pro deletion (des PP) peak hidden underneath the main peak in the original LC spectrum (see Fig. S4), which illustrates the need for devoting adequate resources to the development of suitable analytical methods for fragments used in CEPS ligations. As the yield of the synthesis was deemed satisfactory and the des PP impurity, conceivably formed by piperidine induced diketopiperazine (DKP) formation during Fmoc removal steps,[10] does not constitute a critical impurity during downstream processing (DSP) of exenatide API, we used the amine fragment in the forthcoming ligations experiments without any attempts to further optimize the SPPS.

**Table S3**. AA, coupling reagent amounts and couplings times for $Pro^{38}$ – $Phe^{22}$ part of the SPPS

| AA nr | AA used | Coupling time (min) | MW | mmol AA | equiv AA | g AA | g Oxyma | Total mL DIC |
|---|---|---|---|---|---|---|---|---|
| $Pro^{38-37}$ | Fmoc-Pro-Pro-OH | 31 | 434,49 | 200,00 | **1,00** | 86,90 | 28,42 | 78,29 |
| $Pro^{36}$ | Fmoc-Pro-OH | 32 | 337,37 | 400,00 | **2,00** | 134,95 | 56,84 | 156,58 |
| $Ala^{35}$ | Fmoc-Ala-OH$xH_2O$ | 30 | 329,36 | 400,00 | **2,00** | 131,74 | 56,84 | 156,58 |
| $Gly^{34}$ | Fmoc-Gly-OH | 32 | 297,30 | 400,00 | **2,00** | 118,92 | 56,84 | 156,58 |
| $Ser^{33}$ | Fmoc-Ser(Trt)-OH | 35 | 569,66 | 400,00 | **2,00** | 239,86 | 56,84 | 156,58 |
| $Ser^{32}$ | Fmoc-Ser(Trt)-OH | 34 | 569,66 | 400,00 | **2,00** | 239,86 | 56,84 | 156,58 |
| $Pro^{31}$ | Fmoc-Pro-OH | 30 | 337,37 | 400,00 | **2,00** | 142,05 | 56,84 | 156,58 |
| $Gly^{30-29}$ | Fmoc-Gly-Gly-OH | 32 | 354,40 | 300,00 | **1,50** | 111,92 | 42,63 | 117,43 |
| $Asn^{28}$ | Fmoc-Asn(Trt)-OH | 32 | 596,67 | 400,00 | **2,00** | 251,23 | 56,84 | 156,58 |
| $Lys^{27}$ | Fmoc-Lys(Trt)-OH | 33 | 610,8 | 300,00 | **1,50** | 192,88 | 42,63 | 117,43 |
| $Leu^{26}$ | Fmoc-Leu-OH | 33 | 354,41 | 400,00 | **2,00** | 149,23 | 56,84 | 156,58 |
| $Trp^{25}$ | Fmoc-Trp(Boc)-OH | $33 + 21^1$ | 526,59 | 400,00 | **2,00** | 221,72 | 56,84 | 156,58 |
| $Glu^{24}$ | Fmoc-Glu(OtBu)-OH$xH_2O$ | $20 + 22^1$ | 443,49 | 400,00 | **2,00** | 186,73 | 56,84 | 156,58 |
| $Ile^{23}$ | Fmoc-Ile-OH | $20 + 22^1$ | 354,41 | 400,00 | **2,00** | 149,23 | 56,84 | 156,58 |
| $Phe^{22}$ | Fmoc-Phe-OH | $20 + 23^1$ | 387,45 | 400,00 | **2,00** | 163,14 | 56,84 | 156,58 |

[1]Recoupling. Same amounts of AAs and reagents as in the 1st couplings were used.

## 4. LC-HRMS analysis of loss of DKP during piperidine treatment of Fmoc-20-21-O-Cam-Leu-RMG DEG AM resin (Fig. 3).

100 mg of the resin was swollen in DMF, drained and exposed to 2 mL of mol sieves dried 20% (v/v) pip/DMF. The resulting slurry was shaken for 8 min at rt after which 50 μL of the supernate was taken out, diluted with 1.0 mL MeCN and LC-HRMS analysis of the reaction mixture was carried out. Experimental conditions: column: Waters CSH 1.7 μm 2.1 x 150 mm; column temperature: 50°C; injection volume: 1 μL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 220 nm; data rate: 5Hz; detector cell: standard cell 1uL; flow: 0.2 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1 % formic acid in water, mobile phase B: 0.10 % formic acid in 10% water/90 %MeCN. Gradient (Time(min), %B): 0, 10; 1, 10; 46, 40; 48, 90; 50, 90; 50.1, 10; 60,10.

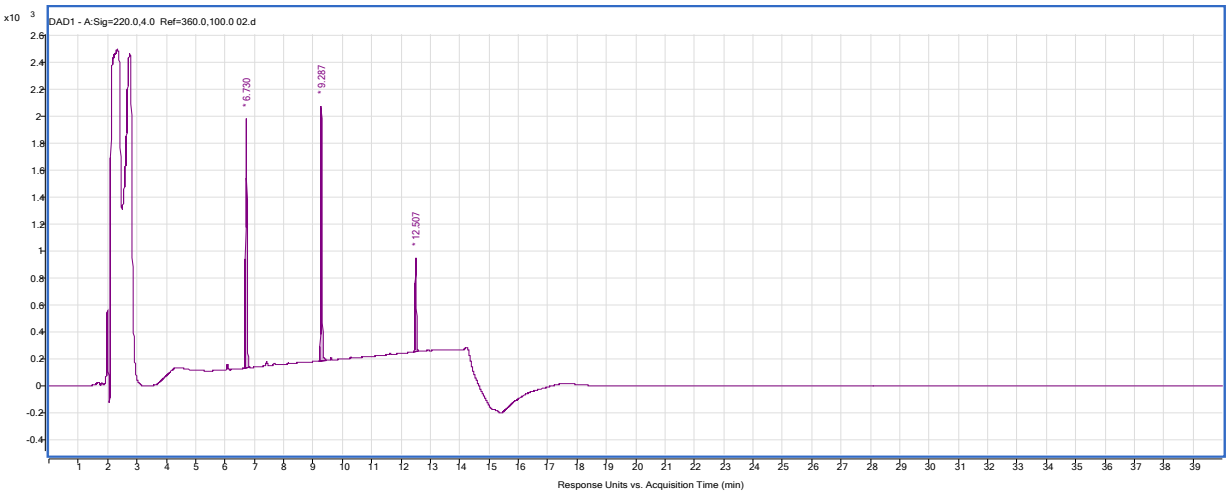



**Figure S5.** UV chromatogram overview.

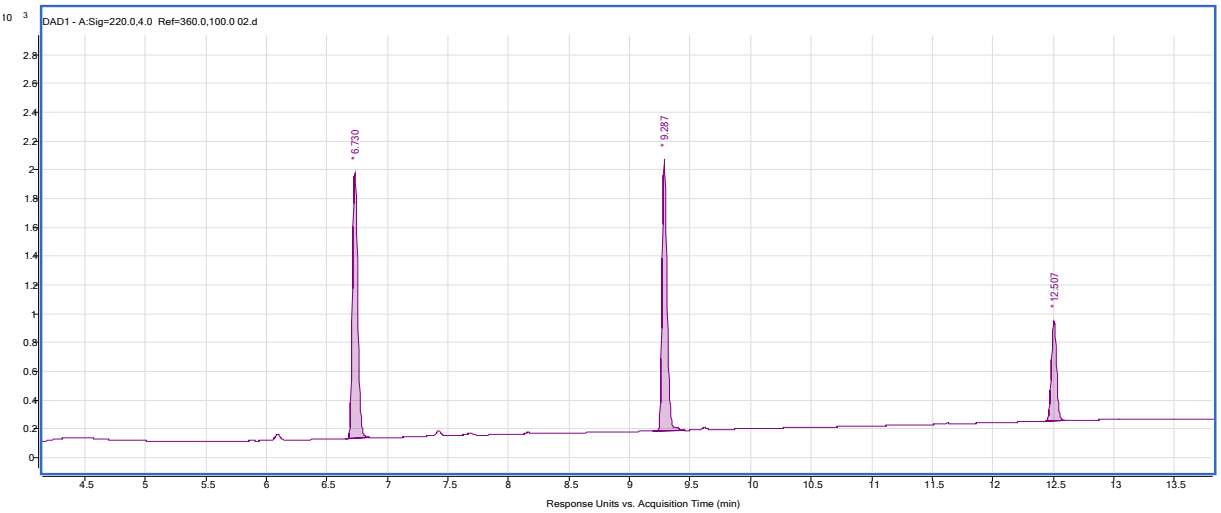



**Figure S6.** UV chromatogram zoom-in.

**Table S4.** Area% MS identities for integrated peaks.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6,73 | 5648,050 | 264,2624 | 1 | 263,2551 | -258,0149 | 0,725 | 42,83 | pip-DBF adduct m/z(M+H) 264,1752 |
| **2** | **9,29** | **5511,280** | **522,2749** | **1** | **521,2676** | **-0,0024** | **1,000** | **41,79** | **DKP (m/z(M+H) 522,2705)** |
| 3 | 12,51 | 2028,770 | 179,0898 | 1 | 178,0825 | -343,1875 | 1,347 | 15,38 | DBF m/z(M+H) 179,0861 |

## 5. Assessment of kinetics of loss of DKP during piperidine treatment of Fmoc-20-21-O-Cam-Leu-RMG DEG AM resin.

100 mg of the resin was swollen in DMF, drained and exposed to 2 mL of mol sieves dried 20% (v/v) pip/DMF. The resulting slurry was shaken at rt and 50 µL aliquots of the supernate were taken out over time, diluted with 1.0 mL MeCN and HPLC analyses of the reaction mixtures were carried out. The conversion of the DKP was determined by comparing the HPLC area of the DKP peak (mAu$^{x}$min) at a given timepoint vs the area of the peak upon complete removal of DKP from the resin. The $t_{1/2}$ for the loss of DKP was determined to be ~30 min (Fig. S7).

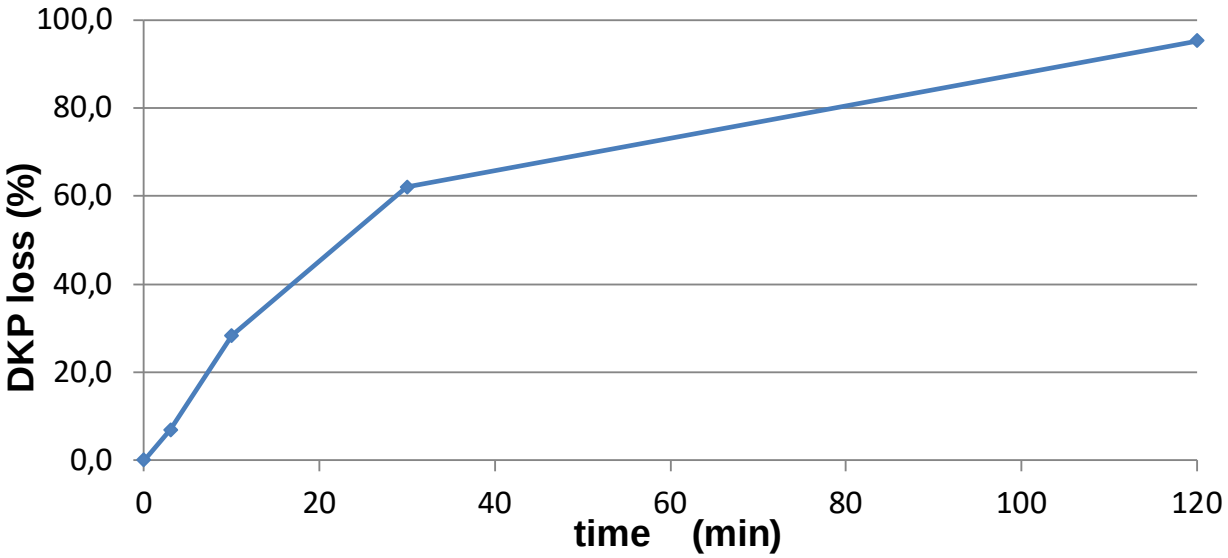
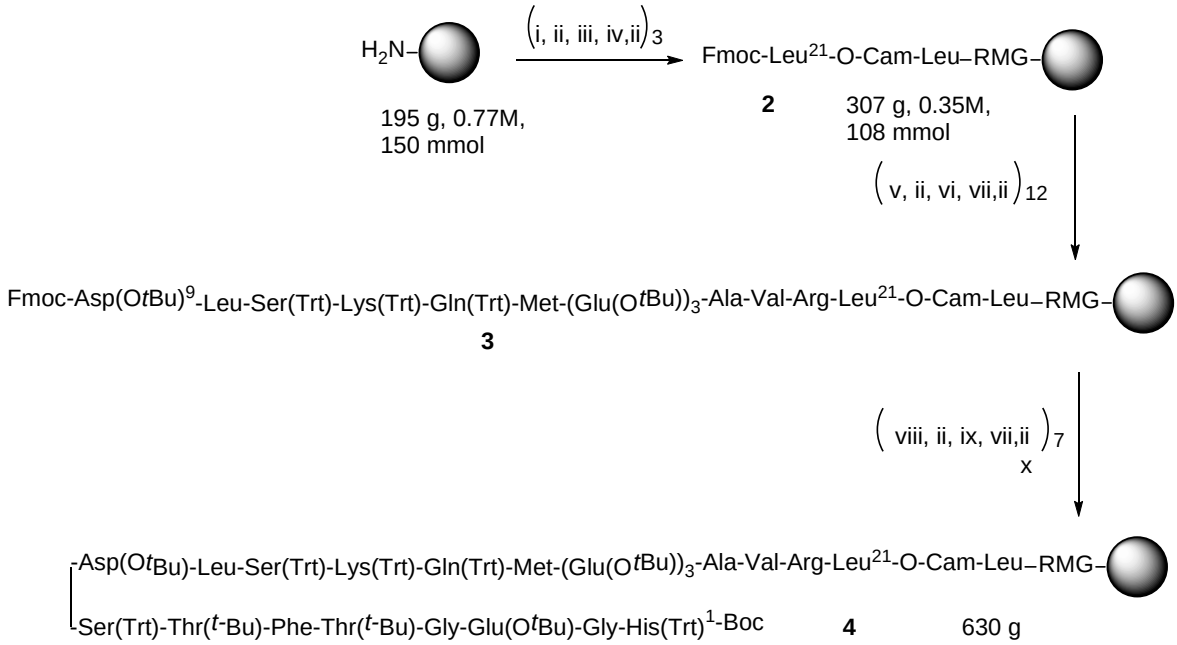



**Figure S7.** A schematic representation of removal of DKP from Fmoc-20-21-O-Cam Leu RMG DEG AM resin upon treatment with pip/DMF.

## 6. Details of SPPS of Boc-1-21-O-Cam-Leu-RMG DEG AM resin (Scheme 2).

First, ~108 mmol of the intermediate Fmoc-Leu$^{21}$-O-Cam-Leu-RMG resin **2** was prepared coupling the RMG linker to the base resin (150 mmol) followed by Fmoc deprotection. Next, as the laboratory scale CEPS process for exenatide utilized O-Cam-L as the linker[36] Fmoc-Leu-OH was coupled, followed by Fmoc deprotection and a coupling with Fmoc-Leu-O-Cam-OH[43] to furnish the resin **2** (Scheme S2). The resin loading was reduced ~30% over the three couplings in order to improve the reaction kinetics for the remainder of the SPPS and the yield of the synthesis of resin **2** based on the amount of Fmoc-Leu-OCam-OH used was ~98%. With resin **2** in hand we completed the synthesis of H-1-21-O-Cam-L fragment and in order to minimize the risk of peptide loss due to presence of the O-Cam ester we carried out the this SPPS under very mild conditions. At the same time, to prevent the formation of difficult to remove impurities caused by incomplete couplings and/or Fmoc deprotections we followed the conversion of all steps of the synthesis by suitable in-process control (IPC) tests. Thus, starting with the resin **2** the synthesis was carried out at rt using O-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)[11] in the presence of N-methylmorpholine (NMM) for AA couplings and pip for Fmoc deprotections (Scheme 2). Taking into the account the potential lability of the O-Cam moiety the Fmoc deprotection treatments were carried out under very mild conditions. Specifically, resin **3** was deprotected using 1% pip for 10 min + 11% pPip for 10 min, the DKP prone Fmoc-20-21-O-Cam resin was deprotected using 1.5% dry pip for 10 min + 11.5% dry pip for 5 min and the remaining deprotections up to Fmoc-9-21 resin **4** were done using 2 x (1% Pip for 10 min + 11% Pip for 5 min). According to IPC analyses carried out up to Asp$^{9}$ the conversion on all Fmoc deprotections was > 99%. Furthermore, as evidenced by the HPLC purity (85%) and yield (83%) for the Fmoc-9-21-O-Cam-Leu-$NH_2$ crude peptide the synthesis of intermediate resin **3** proceeded satisfactorily.




**Scheme S2** SPPS of Boc-1-21-O-Cam-Leu resin, reagents and conditions: i) Fmoc deprotection: 12.5% pip in DMF, 40 – 45 °C, 25 min; ii) DMF resin wash; iii) coupling: R-COOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 30 min; iv) capping: AcOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 15 min; v) Fmoc deprotection (rt): a) resin **2**: 1% pip/DMF for 10 min + 11% pip/DMF for 10 min, b) Fmoc-20-21-O-Cam resin: 1.5% dry pip/DMF for 10 min + 11.5% dry pip/DMF for 5 min c) remaining deprotections: 2 x 1% pip/DMF for 10 min + 11% pip/DMF for 5 min. vi) coupling (rt): R-COOH/TBTU/NMM (1:1:2), 15 min; vii) capping (from $Met^{15}$ onwards): $Ac_2O$, 5 min; viii) Fmoc deprotection: 20% pip in DMF, 30 – 40 °C, (until >99% conversion per IPC tests); ix) R-COOH/TBTU/NMM (1:1:2), 10 – 20 min; $Phe^6$ coupled 2 x, $Thr^5$ coupled 3 x, Boc-His(Trt)-Gly-OH used in final coupling x) *i*-PrOH wash and drying in vacuo.

Upon continuing the synthesis from resin **3** onwards we found that at rt the Fmoc deprotections did not go to completion despite increasing the pip concentration and/or prolonging the base treatments. In fact, several 20 min treatments with 20% pip at 30 – 40 °C were necessary to attain >99% conversion in these Fmoc deprotections (Scheme 3). Furthermore, $Phe^6$ and $Thr^5$ couplings had to be coupled twice and three times respectively in order to achieve a high conversion (negative ninhydrin[57] color test). The synthesis furnished 630 g of Boc-1-21 peptide resin and TFA cleavages of this material were carried out on up to 250 g scale using TFA/TIS/DTT/$H_2O$/$NH_4I$ (90:4:3:1:2, v,v,w,v,w) for 2 h which was essentially the same cleavage cocktail as was used for the amine fragment resin with the exception of ammonium iodide which was added to suppress $Met^{14}$ oxidation.[12]

**Table S5**. Conditions for Fmoc deprotections during SPPS of Boc-1-21-O-Cam-RMG DEG AM resin

| Deprotected resin | Fmoc deprotection | Fmoc deprotection time (min) | Pip concentration (% v/v) | Temperature (°C) | Fmoc deprotection conversion (%)[1] |
|---|---|---|---|---|---|
| Fmoc-Leu$^{21}$-O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Leu$^{21}$-O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 11 | 22 | >99,8 |
| Fmoc-Arg$^{20}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1,5 | 22 | n.d. |
| Fmoc-Arg$^{20}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11,5 | 22 | >99,8 |
| Fmoc-Val$^{19}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Val$^{19}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Val$^{19}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Val$^{19}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Ala$^{18}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Ala$^{18}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Ala$^{18}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Ala$^{18}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Glu$^{17}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{17}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Glu$^{17}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{17}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Glu$^{16}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{16}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Glu$^{16}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{16}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Glu$^{15}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{15}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Glu$^{15}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Glu$^{15}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Met$^{14}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Met$^{14}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Met$^{14}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Met$^{14}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Gln$^{13}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Gln$^{13}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Gln$^{13}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Gln$^{13}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Lys$^{12}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Lys$^{12}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Lys$^{12}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Lys$^{12}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Ser$^{11}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Ser$^{11}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Ser$^{11}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Ser$^{11}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Leu$^{10}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Leu$^{10}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 5 | 11 | 22 | n.d. |
| Fmoc-Leu$^{10}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 10 | 1 | 22 | n.d. |
| Fmoc-Leu$^{10}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 5 | 11 | 22 | >99,8 |
| Fmoc-Asp$^{9}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 30 - 35 | n.d. |
| Fmoc-Asp$^{9}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 30 - 35 | 99,9 |
| Fmoc-Ser$^{8}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 30 - 35 | n.d. |
| Fmoc-Ser$^{8}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 30 - 35 | 99,2 |
| Fmoc-Thr$^{7}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 30 - 35 | n.d. |
| Fmoc-Thr$^{7}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 30 - 35 | 78,6 |
| Fmoc-Thr$^{7}$-Leu$^{21}$ O-Cam-RMG DEG AM | 3$^{rd}$ | 20 | 20 | 30 - 35 | 86,9 |
| Fmoc-Thr$^{7}$-Leu$^{21}$ O-Cam-RMG DEG AM | 4$^{th}$ | 20 | 20 | 35 - 40 | 94,6 |
| Fmoc-Thr$^{7}$-Leu$^{21}$ O-Cam-RMG DEG AM | 5$^{th}$ | 20 | 20 | 35 - 40 | 98,8 |
| Fmoc-Phe$^{6}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 35 - 40 | n.d. |
| Fmoc-Phe$^{6}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 35 - 40 | 99,2 |
| Fmoc-Thr$^{5}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 35 - 40 | 96,7 |
| Fmoc-Thr$^{5}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 35 - 40 | 99,3 |
| Fmoc-Gly$^{4}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 35 - 40 | n.d. |
| Fmoc-Gly$^{4}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 35 - 40 | 99,2 |
| Fmoc-Glu$^{3}$-Leu$^{21}$ O-Cam-RMG DEG AM | 1$^{st}$ | 20 | 20 | 35 - 40 | n.d. |
| Fmoc-Glu$^{3}$-Leu$^{21}$ O-Cam-RMG DEG AM | 2$^{nd}$ | 20 | 20 | 35 - 40 | 99,4 |

[1]Determined by quantifying the Fmoc content on resin at the end of deprotection.

**Table S6**. Raw material amounts and coupling conditions during SPPS of Boc-1-21-O-Cam-RMG DEG AM resin

| AA nr | AA used | Coupling time (min) | Coupling temperature (°C) | MW | mmol AA | equiv AA | g AA | g TBTU | mL NMM |
|---|---|---|---|---|---|---|---|---|---|
| $Arg^{20}$ | Fmoc-Arg(Pbf)-OH | 15 | 22 | 648,77 | 200,00 | **1,9** | 129,75 | 64,22 | 44,00 |
| $Val^{19}$ | Fmoc-Val-OH | 15 | 22 | 339,39 | 200,00 | **1,9** | 67,88 | 64,22 | 44,00 |
| $Ala^{18}$ | Fmoc-Ala-OHx$H_2O$ | 15 | 22 | 329,36 | 200,00 | **1,9** | 65,87 | 64,22 | 44,00 |
| $Glu^{17}$ | Fmoc-Glu-OHx$H_2O$ | 15 | 22 | 425,50 | 200,00 | **1,9** | 85,10 | 64,22 | 44,00 |
| $Glu^{16}$ | Fmoc-Glu-OHx$H_2O$ | 15 | 22 | 425,50 | 200,00 | **1,9** | 89,58 | 64,22 | 44,00 |
| $Glu^{15}$ | Fmoc-Glu-OHx$H_2O$ | 15 | 22 | 425,50 | 200,00 | **1,9** | 89,58 | 64,22 | 44,00 |
| $Met^{14}$ | Fmoc-Met-OH | 15 | 22 | 371,45 | 200,00 | **1,9** | 78,20 | 64,22 | 44,00 |
| $Gln^{13}$ | Fmoc-Gln(Trt)-OH | 15 | 22 | 610,70 | 200,00 | **1,9** | 128,57 | 64,22 | 44,00 |
| $Lys^{12}$ | Fmoc-Lys(Trt)-OH | 15 | 22 | 610,74 | 200,00 | **1,9** | 128,58 | 64,22 | 44,00 |
| $Ser^{11}$ | Fmoc-Ser(*t*-Bu)-OH | 15 | 22 | 569,65 | 200,00 | **1,9** | 119,93 | 64,22 | 44,00 |
| $Leu^{10}$ | Fmoc-Leu-OH | 15 | 22 | 354,41 | 200,00 | **1,9** | 74,61 | 64,22 | 44,00 |
| $Asp^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 15 | 22 | 411,45 | 200,00 | **1,9** | 86,62 | 64,22 | 44,00 |
| $Ser^{8}$ | Fmoc-Ser(*t*-Bu)-OH | 13 | 30 - 40 | 569,65 | 200,00 | **1,9** | 119,93 | 64,22 | 44,00 |
| $Thr^{7}$ | Fmoc-Thr(*t*- Bu)-OH | 19 | 30 - 40 | 397,48 | 200,00 | **1,9** | 83,68 | 64,22 | 44,00 |
| $Phe^{6}$ | Fmoc-Phe-OH | $15 + 12^{1}$ | 30 - 40 | 387,45 | 200,00 | **1,9** | 81,57 | 64,22 | 44,00 |
| $Thr^{5}$ | Fmoc-Thr(*t*- Bu)-OH | $15 + 15^{1} + 11^{1}$ | 30 - 40 | 397,48 | 200,00 | **1,9** | 83,68 | 64,22 | 44,00 |
| $Gly^{4}$ | Fmoc-Gly-OH | 11 | 30 - 40 | 297,31 | 200,00 | **1,9** | 62,59 | 64,22 | 44,00 |
| $Glu^{3}$ | Fmoc-Glu-OHx$H_2O$ | 14 | 30 - 40 | 425,50 | 200,00 | **1,9** | 89,58 | 64,22 | 44,00 |
| $His^{1}$-$Gly^{2}$ | Boc-His(Trt)-Gly-OH | 20 | 30 - 40 | 554,65 | 200,00 | **1,9** | 116,77 | 64,22 | 44,00 |

[1]Recoupling. Same amounts of AAs and reagents as in the 1st couplings were used.

## 7. HPLC analysis of crude Fmoc-9-21-O-Cam-Leu-$NH_2$ obtained from TFA cleavage of intermediate Fmoc-9-21-O-Cam-Leu-RMG DEG AM resin (Scheme S2).

Experimental conditions: column: Waters XSelect CSH130 C18 2.5 µm 4.6x150mm; column temperature: 30°C; injection volume: 5 uL; sampler temperature:10°C; flow: 0.8 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 0; 40, 100; 54, 100; 55, 0; 62, 0.

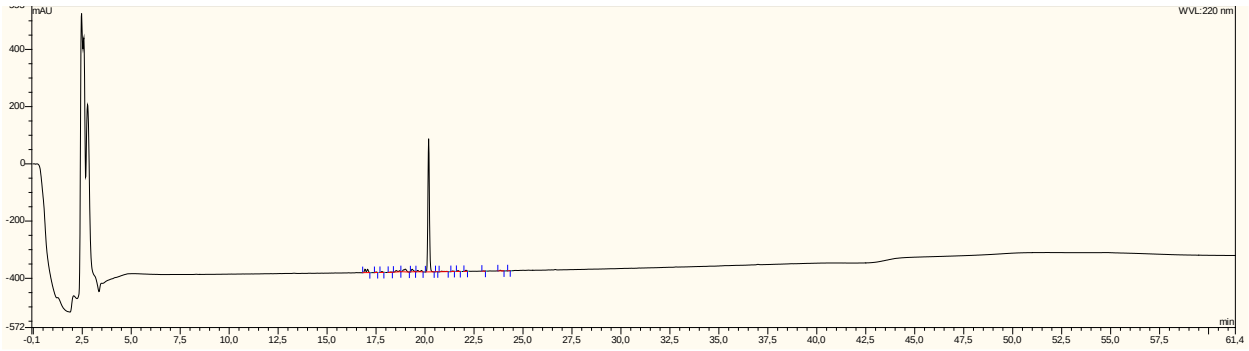



**Figure S8.** UV chromatogram overview.

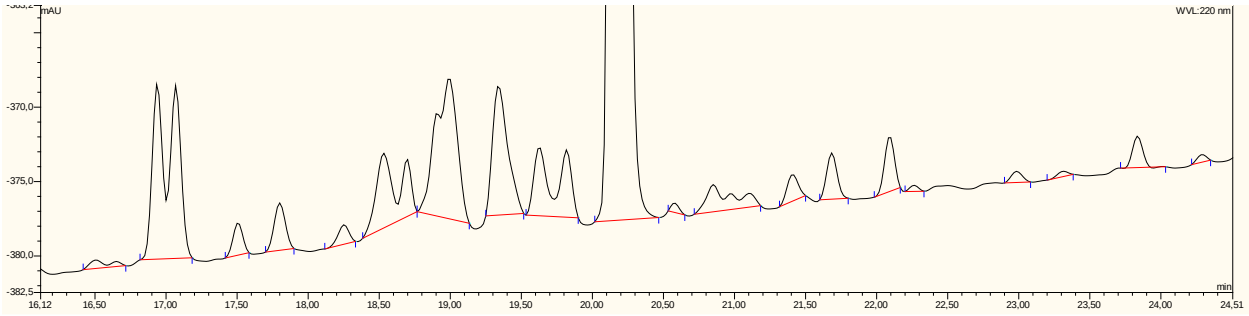



**Figure S9.** UV chromatogram zoom-in.

**Table S7.** Area% for integrated peaks.

| No | Ret. Time (min) | Area (mAu$^x$min) | Height (mAU) | Rel.Area (%) |
|---|---|---|---|---|
| 1 | 16,500 | 0,0846 | 0,565 | 0,17 |
| 2 | 16,933 | 2,0054 | 11,741 | 3,99 |
| 3 | 17,500 | 0,1701 | 2,148 | 0,34 |
| 4 | 17,800 | 0,2876 | 3,180 | 0,57 |
| 5 | 18,250 | 0,1160 | 1,311 | 0,23 |
| 6 | 18,533 | 0,9139 | 5,010 | 1,82 |
| 7 | 19,000 | 1,6690 | 9,383 | 3,32 |
| 8 | 19,333 | 1,0785 | 8,635 | 2,15 |
| 9 | 19,633 | 0,8864 | 4,547 | 1,76 |
| 10 | 20,183 | 41,5324 | 465,324 | 82,69 |
| 11 | 20,583 | 0,0400 | 0,625 | 0,08 |
| 12 | 20,850 | 0,3876 | 1,827 | 0,77 |
| 13 | 21,400 | 0,1621 | 1,782 | 0,32 |
| 14 | 21,683 | 0,2625 | 3,103 | 0,52 |
| 15 | 22,083 | 0,2965 | 3,637 | 0,59 |
| 16 | 22,267 | 0,0286 | 0,405 | 0,06 |
| 17 | 22,983 | 0,0675 | 0,746 | 0,13 |
| 18 | 23,300 | 0,0308 | 0,355 | 0,06 |
| 19 | 23,833 | 0,1706 | 2,105 | 0,34 |
| 20 | 24,283 | 0,0381 | 0,511 | 0,08 |
| Total: | | 50,2281 | 526,940 | 100,00 |

## 8. LC-HRMS analysis of crude H-1-21-O-Cam-Leu-$NH_2$ obtained from TFA cleavage of Boc-1-21-O-Cam-Leu-RMG DEG AM resin (Scheme S2).

Experimental conditions: column: Waters CSH 2.6 µm 4.6 x 150 mm; column temperature: 40°C; injection volume: 1 µL; sampler temperature: 5°C; MS mode: positive 50-3200; DAD: 220 nm; data rate: 2.5Hz; detector cell: standard cell 1uL; flow: 0.8 ml/min; jet weaver: v100 mixer; mobile

phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 5; 1, 5; 41, 95; 41.1, 5; 47, 5.

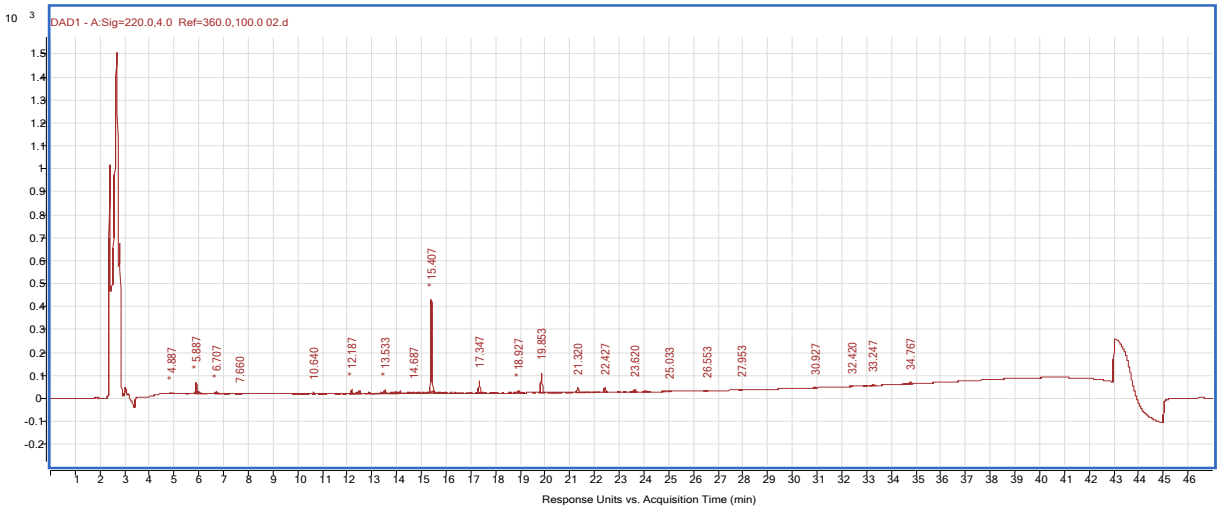



**Figure S10.** UV chromatogram overview.

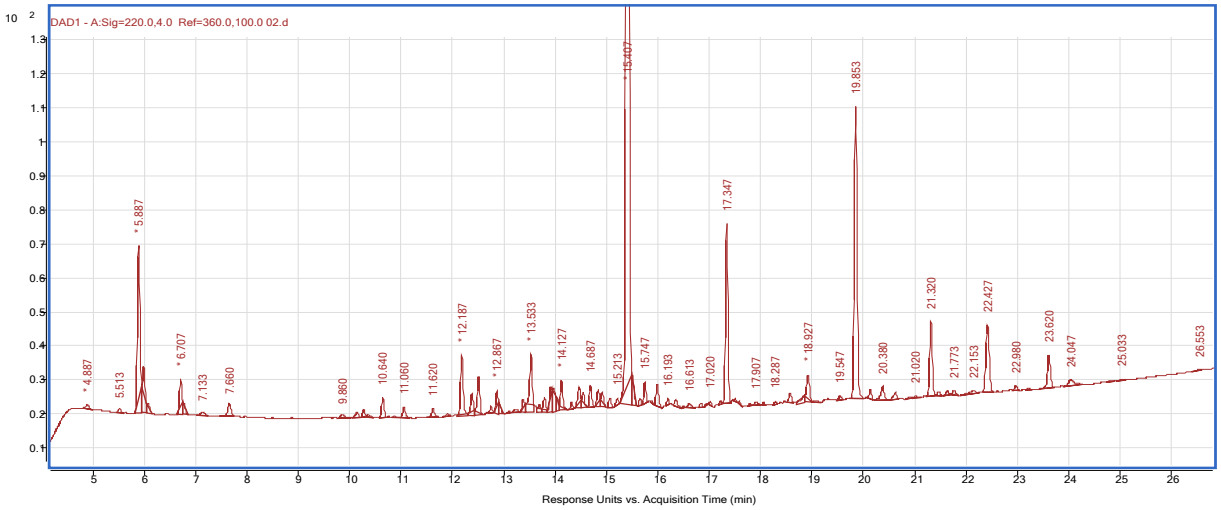



**Figure S11.** UV chromatogram zoom-in.

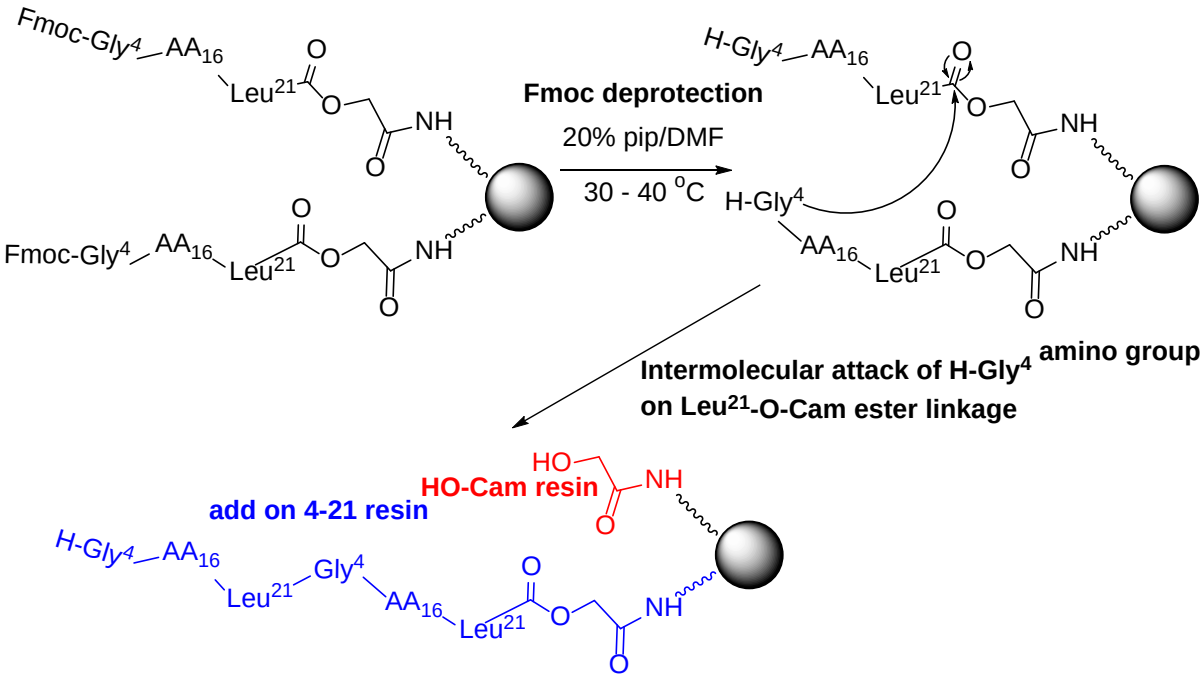
**Table S8.** Area% for integrated peaks and MS identities for H-1-21-O-Cam-Leu-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4,89 | 5,290 | | | 0,0000 | -2533,2120 | 0,317 | 0,15 | |
| 2 | 5,51 | 4,230 | | | 0,0000 | -2533,2120 | 0,358 | 0,12 | |
| 3 | 5,89 | 179,300 | 383,2114 | 1 | 382,2041 | -2151,0079 | 0,382 | 5,19 | H-(1-2)-O-Cam-Leu-NH2 |
| 4 | 5,99 | 47,770 | 512,2471 | 1 | 511,2398 | -2021,9722 | 0,389 | 1,38 | H-(1-3)-Cam-Leu-NH2 |
| 5 | 6,07 | 9,850 | | | 0,0000 | -2533,2120 | 0,394 | 0,29 | |
| 6 | 6,71 | 34,770 | | | 0,0000 | -2533,2120 | 0,435 | 1,01 | |
| 7 | 6,75 | 8,740 | | | 0,0000 | -2533,2120 | 0,438 | 0,25 | |
| 8 | 7,13 | 5,780 | | | 0,0000 | -2533,2120 | 0,463 | 0,17 | |
| 9 | 7,66 | 15,150 | | | 0,0000 | -2533,2120 | 0,497 | 0,44 | |
| 10 | 9,86 | 6,110 | | | 0,0000 | -2533,2120 | 0,640 | 0,18 | |
| 11 | 10,15 | 6,270 | | | 0,0000 | -2533,2120 | 0,659 | 0,18 | |
| 12 | 10,27 | 7,830 | | | 0,0000 | -2533,2120 | 0,667 | 0,23 | |
| 13 | 10,37 | 3,210 | | | 0,0000 | -2533,2120 | 0,673 | 0,09 | |
| 14 | 10,64 | 19,350 | | | 0,0000 | -2533,2120 | 0,691 | 0,56 | |
| 15 | 10,87 | 2,010 | | | 0,0000 | -2533,2120 | 0,705 | 0,06 | |
| 16 | 11,06 | 11,420 | | | 0,0000 | -2533,2120 | 0,718 | 0,33 | |
| 17 | 11,62 | 10,200 | | | 0,0000 | -2533,2120 | 0,754 | 0,30 | |
| 18 | 11,90 | 3,290 | | | 0,0000 | -2533,2120 | 0,772 | 0,10 | |
| 19 | 12,19 | 65,800 | | | 0,0000 | -2533,2120 | 0,791 | 1,90 | |
| 20 | 12,38 | 24,400 | | | 0,0000 | -2533,2120 | 0,804 | 0,71 | |
| 21 | 12,51 | 41,420 | 1047,9691 | 2 | 2093,9236 | -439,2884 | 0,812 | 1,20 | H-(1-19)-OH |
| 22 | 12,75 | 5,420 | | | 0,0000 | -2533,2120 | 0,828 | 0,16 | |
| 23 | 12,87 | 20,780 | | | 0,0000 | -2533,2120 | 0,835 | 0,60 | |
| 24 | 12,90 | 7,920 | | | 0,0000 | -2533,2120 | 0,837 | 0,23 | |
| 25 | 13,10 | 1,970 | | | 0,0000 | -2533,2120 | 0,850 | 0,06 | |
| 26 | 13,24 | 4,250 | | | 0,0000 | -2533,2120 | 0,859 | 0,12 | |
| 27 | 13,38 | 13,130 | | | 0,0000 | -2533,2120 | 0,868 | 0,38 | |
| 28 | 13,53 | 69,990 | 788,7102 | 3 | 2363,1088 | -170,1032 | 0,878 | 2,03 | (H-(1-21)-OH |
| 29 | 13,70 | 9,080 | | | 0,0000 | -2533,2120 | 0,889 | 0,26 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 30 | 13,79 | 15,220 | | | 0,0000 | -2533,2120 | 0,895 | 0,44 | |
| 31 | 13,91 | 28,440 | | | 0,0000 | -2533,2120 | 0,903 | 0,82 | |
| 32 | 13,97 | 26,450 | | | 0,0000 | -2533,2120 | 0,907 | 0,77 | |
| 33 | 14,02 | 11,020 | | | 0,0000 | -2533,2120 | 0,910 | 0,32 | |
| 34 | 14,13 | 27,920 | | | 0,0000 | -2533,2120 | 0,917 | 0,81 | |
| 35 | 14,32 | 6,010 | | | 0,0000 | -2533,2120 | 0,929 | 0,17 | |
| 36 | 14,47 | 23,570 | | | 0,0000 | -2533,2120 | 0,939 | 0,68 | |
| 37 | 14,54 | 15,110 | | | 0,0000 | -2533,2120 | 0,944 | 0,44 | |
| 38 | 14,69 | 20,920 | | | 0,0000 | -2533,2120 | 0,953 | 0,61 | |
| 39 | 14,84 | 17,950 | | | 0,0000 | -2533,2120 | 0,963 | 0,52 | |
| 40 | 14,92 | 14,500 | | | 0,0000 | -2533,2120 | 0,968 | 0,42 | |
| 41 | 15,06 | 9,400 | | | 0,0000 | -2533,2120 | 0,977 | 0,27 | |
| 42 | 15,21 | 7,040 | | | 0,0000 | -2533,2120 | 0,987 | 0,20 | |
| 43 | 15,41 | 1534,560 | 845,4161 | 3 | 2533,2265 | 0,0145 | 1,000 | 44,41 | Prod. H-(1-21)-O-Cam-Leu-NH2 |
| 44 | 15,49 | 23,140 | | | 0,0000 | -2533,2120 | 1,006 | 0,67 | |
| 45 | 15,65 | 5,600 | | | 0,0000 | -2533,2120 | 1,016 | 0,16 | |
| 46 | 15,75 | 17,520 | | | 0,0000 | -2533,2120 | 1,022 | 0,51 | |
| 47 | 15,87 | 1,670 | | | 0,0000 | -2533,2120 | 1,030 | 0,05 | |
| 48 | 15,99 | 22,320 | | | 0,0000 | -2533,2120 | 1,038 | 0,65 | |
| 49 | 16,19 | 4,820 | | | 0,0000 | -2533,2120 | 1,051 | 0,14 | |
| 50 | 16,35 | 7,630 | | | 0,0000 | -2533,2120 | 1,061 | 0,22 | |
| 51 | 16,49 | 1,710 | | | 0,0000 | -2533,2120 | 1,070 | 0,05 | |
| 52 | 16,61 | 5,400 | | | 0,0000 | -2533,2120 | 1,078 | 0,16 | |
| 53 | 16,84 | 3,730 | | | 0,0000 | -2533,2120 | 1,093 | 0,11 | |
| 54 | 16,95 | 1,910 | | | 0,0000 | -2533,2120 | 1,100 | 0,06 | |
| 55 | 17,02 | 2,680 | | | 0,0000 | -2533,2120 | 1,105 | 0,08 | |
| 56 | 17,22 | 2,960 | | | 0,0000 | -2533,2120 | 1,118 | 0,09 | |
| 57 | 17,35 | 170,400 | 1048,0244 | 2 | 2094,0342 | -439,1778 | 1,126 | 4,93 | Ac-(6-21)-O-Cam-Leu-NH2 |
| 58 | 17,51 | 1,700 | | | 0,0000 | -2533,2120 | 1,136 | 0,05 | |
| 59 | 17,58 | 1,790 | | | 0,0000 | -2533,2120 | 1,141 | 0,05 | |
| 60 | 17,79 | 2,050 | | | 0,0000 | -2533,2120 | 1,155 | 0,06 | |
| 61 | 17,91 | 4,520 | | | 0,0000 | -2533,2120 | 1,162 | 0,13 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 62 | 18,06 | 1,830 | | | 0,0000 | -2533,2120 | 1,172 | 0,05 | |
| 63 | 18,29 | 2,710 | | | 0,0000 | -2533,2120 | 1,187 | 0,08 | |
| 64 | 18,58 | 9,560 | | | 0,0000 | -2533,2120 | 1,206 | 0,28 | |
| 65 | 18,86 | 11,500 | | | 0,0000 | -2533,2120 | 1,224 | 0,33 | |
| 66 | 18,927 | 32,78 | | | 0,0000 | -2533,2120 | 1,228 | 0,95 | |
| 67 | 19,087 | 3,32 | | | 0,0000 | -2533,2120 | 1,239 | 0,1 | |
| 68 | 19,547 | 4,36 | | | 0,0000 | -2533,2120 | 1,269 | 0,13 | |
| 69 | 19,853 | 325,17 | 1519,4002 | 3 | 4555,1788 | 2021,9668 | 1,289 | 9,41 | add-on 4-21 |
| 70 | 20,14 | 7,95 | | | 0,0000 | -2533,2120 | 1,307 | 0,23 | |
| 71 | 20,38 | 21,46 | | | 0,0000 | -2533,2120 | 1,323 | 0,62 | |
| 72 | 20,627 | 9,14 | | | 0,0000 | -2533,2120 | 1,339 | 0,26 | |
| 73 | 20,92 | 1,72 | | | 0,0000 | -2533,2120 | 1,358 | 0,05 | |
| 74 | 21,02 | 2,79 | | | 0,0000 | -2533,2120 | 1,364 | 0,08 | |
| 75 | 21,32 | 77,33 | 1373,0104 | 3 | 4116,0094 | 1582,7974 | 1,384 | 2,24 | Ac-(6-21)-O-Cam-Leu-NH2 - add-on 4-21 |
| 76 | 21,467 | 4,39 | | | 0,0000 | -2533,2120 | 1,393 | 0,13 | |
| 77 | 21,64 | 5,09 | | | 0,0000 | -2533,2120 | 1,405 | 0,15 | |
| 78 | 21,773 | 5,67 | | | 0,0000 | -2533,2120 | 1,413 | 0,16 | |
| 79 | 22,153 | 8,95 | | | 0,0000 | -2533,2120 | 1,438 | 0,26 | |
| 80 | 22,427 | 91,2 | 1645,2968 | 4 | 6577,1581 | 4043,9461 | 1,456 | 2,64 | 2 x add-on 4-21 |
| 81 | 22,98 | 5,36 | | | 0,0000 | -2533,2120 | 1,492 | 0,15 | |
| 82 | 23,227 | 1,99 | | | 0,0000 | -2533,2120 | 1,508 | 0,06 | |
| 83 | 23,62 | 43,03 | | | 0,0000 | -2533,2120 | 1,533 | 1,25 | |
| 84 | 24,047 | 13,94 | | | 0,0000 | -2533,2120 | 1,561 | 0,4 | |
| 85 | 24,807 | 1,9 | | | 0,0000 | -2533,2120 | 1,610 | 0,06 | |
| 86 | 25,033 | 2,48 | | | 0,0000 | -2533,2120 | 1,625 | 0,07 | |
| 87 | 26,553 | 2,09 | | | 0,0000 | -2533,2120 | 1,723 | 0,06 | |
| 88 | 27,953 | 2,14 | | | 0,0000 | -2533,2120 | 1,814 | 0,06 | |
| 89 | 30,927 | 5,81 | | | 0,0000 | -2533,2120 | 2,007 | 0,17 | |
| 90 | 32,42 | 3,64 | | | 0,0000 | -2533,2120 | 2,104 | 0,11 | |
| 91 | 32,973 | 4,38 | | | 0,0000 | -2533,2120 | 2,140 | 0,13 | |
| 92 | 33,247 | 16,5 | | | 0,0000 | -2533,2120 | 2,158 | 0,48 | |
| 93 | 34,767 | 41,2 | | | 0,0000 | -2533,2120 | 2,257 | 1,19 | |

**9. Proposed mechanism of formation of the add-on 4-21 impurity during Fmoc deprotection of Fmoc-4-21-O-Cam-Leu-RMG DEG AM resin.**




**Scheme S3.** A schematic representation of the formation of add on 4-21 resin by an intermolecular attack of H-4-21-O-Cam-Leu-RMG DEG AM on the O-Cam moiety of another peptide strain.

**10. Kinetics of the omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$, original protocol (Fig. 4).**

Throughout the ligation 50 µL aliquots of the reaction mixture were taken out, quenched with 10% AcOH (1.0 mL) and analyzed by HPLC. When all H-1-21-O-Cam-Leu-$NH_2$ was consumed and no further conversion to the product could be observed the reaction was quenched with 10% AcOH. The S/H ratio was calculated by integrating the peak of H-1-39-$NH_2$ together with byproduct peaks formed by reactions between H-1-21-O-Cam-Leu-$NH_2$ and byproducts in the crude H-2-39-$NH_2$ (S) vs the peak of H-1-21-OH (hydrolyzed H-1-21-O-Cam-Leu-$NH_2$, H).

Experimental conditions: column: Waters XSelect CSH130 C18 2.5µm 4.6x150mm; column temperature: 45°C; injection volume: 5 µL; sampler temperature:10°C; flow: 1.0 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 1; 10, 100; 13, 100; 14, 100; 19, 1; 20, 1.

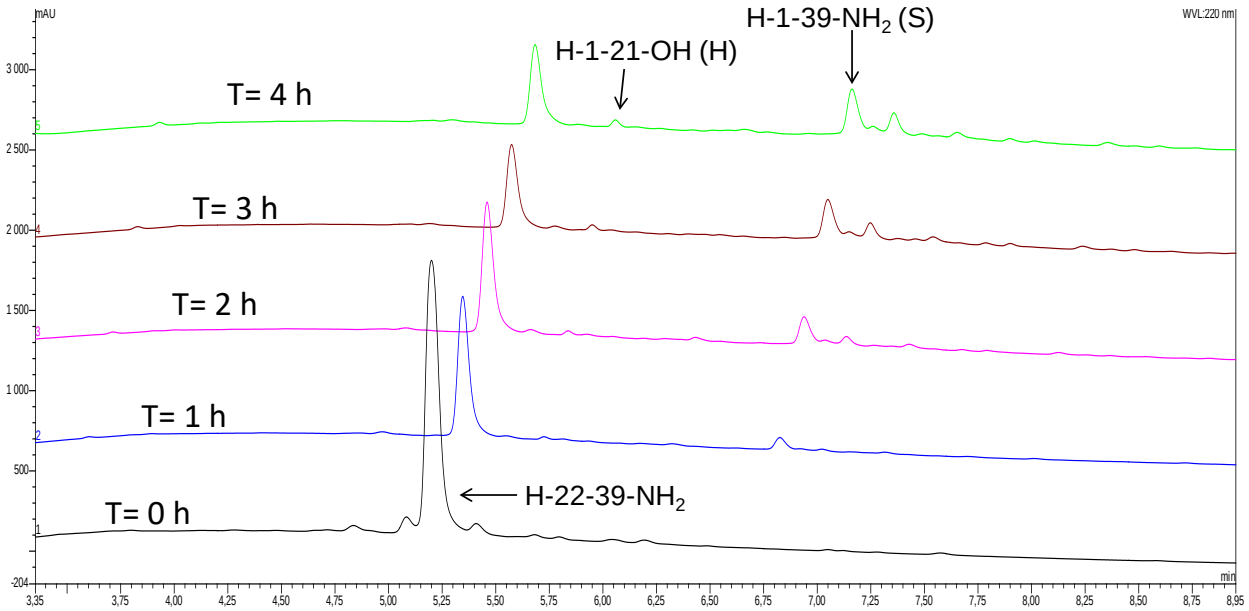



**Figure S12.** A schematic representation of the progress of Fig. 4 crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$ ligation.

**11. LC-HRMS of crude exenatide from omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$ (Fig. 4).**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 11; 1, 11; 5, 33; 50, 37; 54, 60; 56, 90; 58, 90; 58.1, 11; 70, 11.

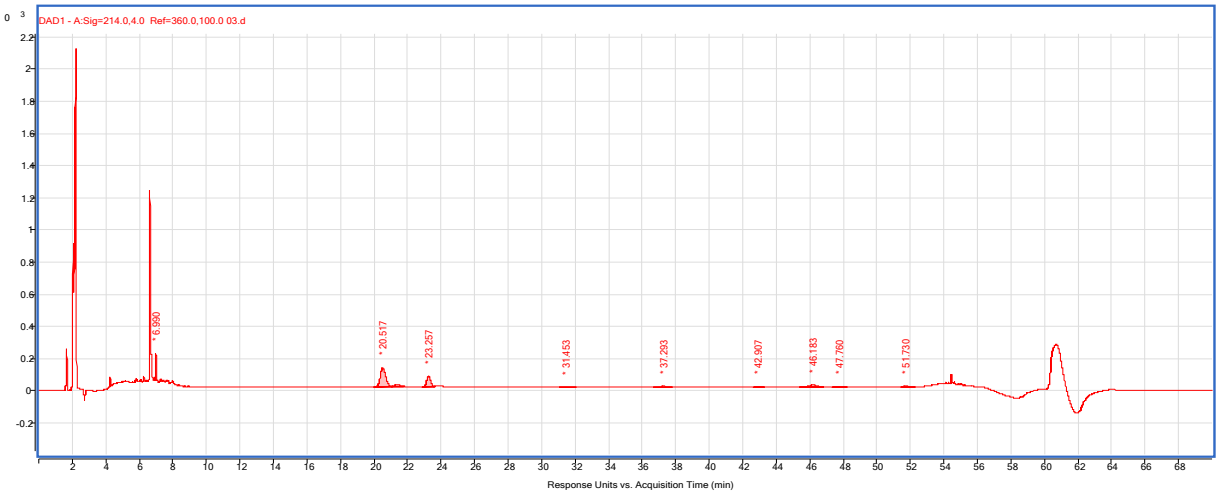



**Figure S13.** UV chromatogram overview.

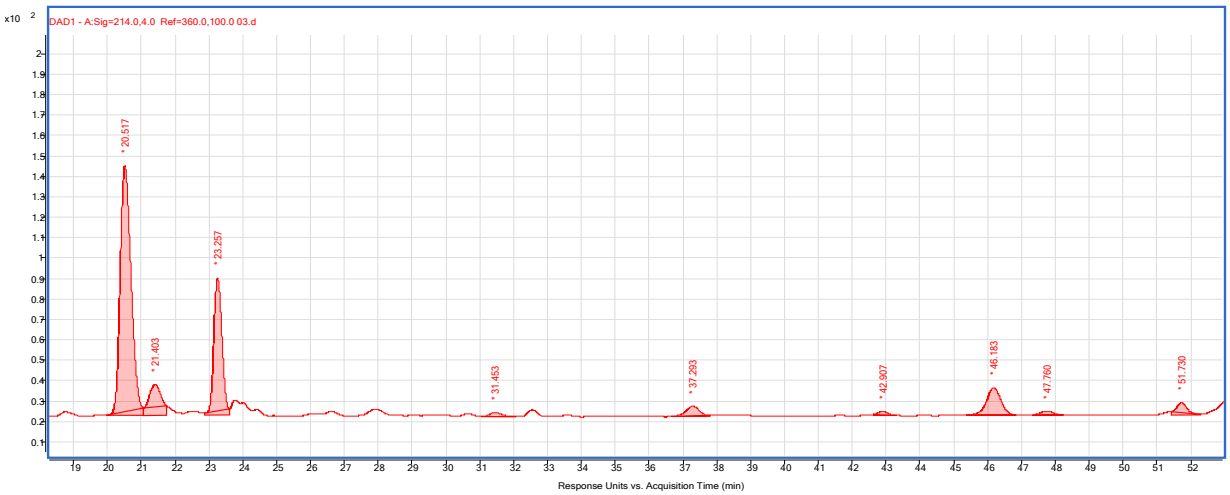



**Figure S14.** UV chromatogram zoom-in.

**Table S9.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 6,99 | 461,590 | 1182,5655 | 2 | 2363,1164 | -1820,9109 | 0,341 | 8,28 | H-(1-21)-OH |
| 2 | 20,52 | 2761,210 | 1395,6854 | 3 | 4184,0344 | 0,0071 | 1,000 | 49,52 | Prod. H-(1-39)-NH2 |
| 3 | 21,40 | 398,780 | 1396,0116 | 3 | 4185,0130 | 0,9857 | 1,043 | 7,15 | Deamidation |
| 4 | 23,26 | 1142,760 | 1396,0128 | 3 | 4185,0166 | 0,9893 | 1,134 | 20,49 | Deamidation |
| 5 | 31,45 | 48,560 | 1200,5953 | 3 | 3598,7641 | -585,2632 | 1,533 | 0,87 | n.d. |
| 6 | 37,29 | 137,950 | 1409,6986 | 3 | 4226,0740 | 42,0467 | 1,818 | 2,47 | Met to hCys(tBu) |
| 7 | 42,91 | 40,440 | 1410,0258 | 3 | 4227,0556 | 43,0283 | 2,091 | 0,73 | Deamidation + (Met to hCys(tBu) |
| 8 | 46,18 | 391,060 | 1249,2898 | 3 | 3744,8476 | -439,1797 | 2,251 | 7,01 | Ac-(6-39)-NH2 |
| 9 | 47,76 | 51,140 | 1249,6167 | 3 | 3745,8283 | -438,1990 | 2,328 | 0,92 | Deamidation + Ac-(6-39)-NH2 |
| 10 | 51,73 | 142,510 | 1249,6178 | 3 | 3745,8316 | -438,1957 | 2,521 | 2,56 | Deamidation + Ac-(6-39)-NH2 |

## 12. Structures of exenatide and its four possible deamidation impurities.

**Exenatide:**

H-HGEGTFTSDLSK**Q**$^{13}$MEEEAVRLFIEWLK**N**$^{28}$GGPSSGAPPP**S**$^{39}$**-NH$_2$**

**Glu$^{13}$ Exenatide:**

H-HGEGTFTSDLSK**E**$^{13}$MEEEAVRLFIEWLK**N**$^{28}$GGPSSGAPPP**S**$^{39}$**-NH$_2$**

**Asp$^{28}$ Exenatide:**

H-HGEGTFTSDLSK**Q**$^{13}$MEEEAVRLFIEWLK**D**$^{28}$**G**GPSSGAPPP**S**$^{39}$**-NH$_2$**

**β-Asp$^{28}$ Exenatide:**

H-HGEGTFTSDLSK**Q**$^{13}$MEEEAVRLFIEWLK**β**$\mathbf{D}^{28}$**G**GPSSGAPPP**S**$^{39}$**-NH$_2$**

**Ser-OH$^{39}$ Exenatide:**

H-HGEGTFTSDLSK**Q**$^{13}$MEEEAVRLFIEWLK**N**$^{28}$GGPSSGAPPP**S**$^{39}$**-OH**

**13. HPLC analytical system for separating exenatide and its four deamidation impurities.**

Experimental conditions: column: Waters XBridge 130 C18 3.5 µm, 4.6 x 150 mm; column temperature: 60°C; injection volume: 10 µL; sampler temperature:10°C; flow: 0.8 ml/min; mobile phase A: 10 mM $NH_4HCO_3$ pH 9.5, mobile phase B: 10/90 10 mM $NH_4HCO_3$ pH 9.5/MeCN. Gradient (Time(min), %B): 0, 26; 0.5, 26; 33, 37; 35, 90; 36, 90; 36.1, 26; 42, 26.

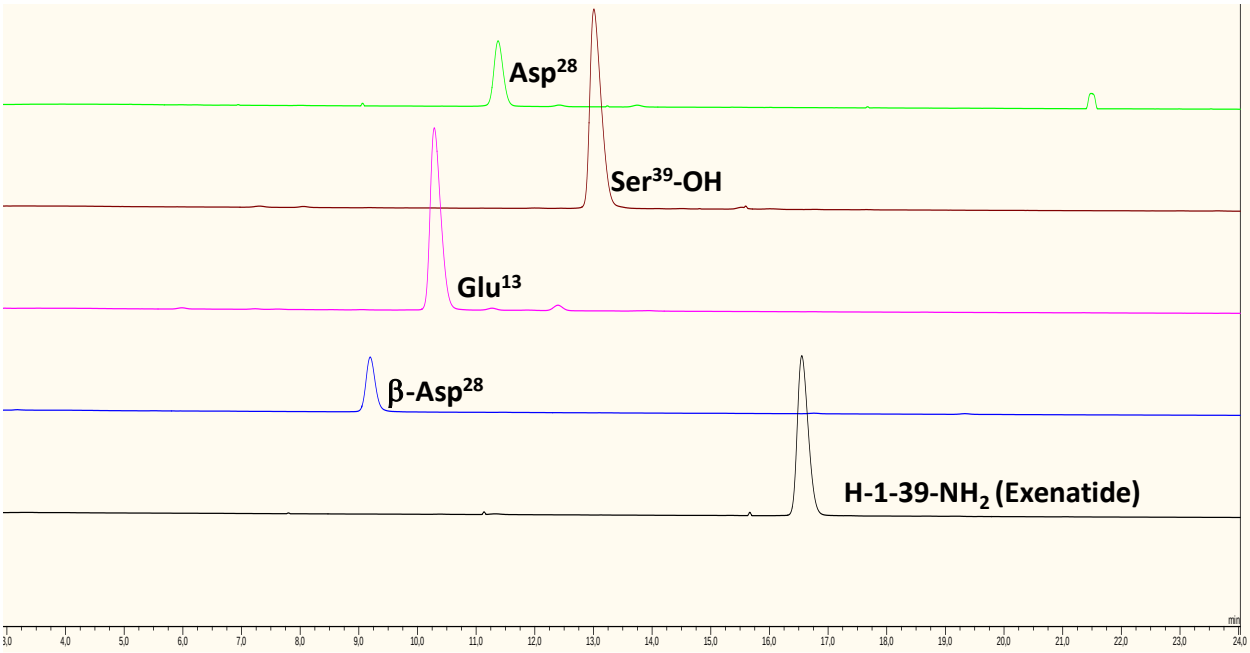



**Figure S15.** HPLC overlay of exenatide and its four deamidation impurities.

**14. Assessment of stability of exenatide API under different conditions.**

exenatide API was dissolved in a buffer solution (5 mg/mL) and the resulting reaction mixture was kept at a given temperature with stirring. 50 µL aliquots were taken out over time, quenched (1.0 mL 10% AcOH) and analyzed by HPLC using the method described above (section 13 of this ESI). The rate of breakdown of exenatide under given conditions was calculated by integrating the areas of peaks of the formed exenatide degradants vs the area of the peak of the remaining exenatide. Figure S16 shows an example of the assessment of exenatide stability whereas Figure S17 summarizes the effect of temperature and pH on stability of exenatide in 1M phosphate buffer.

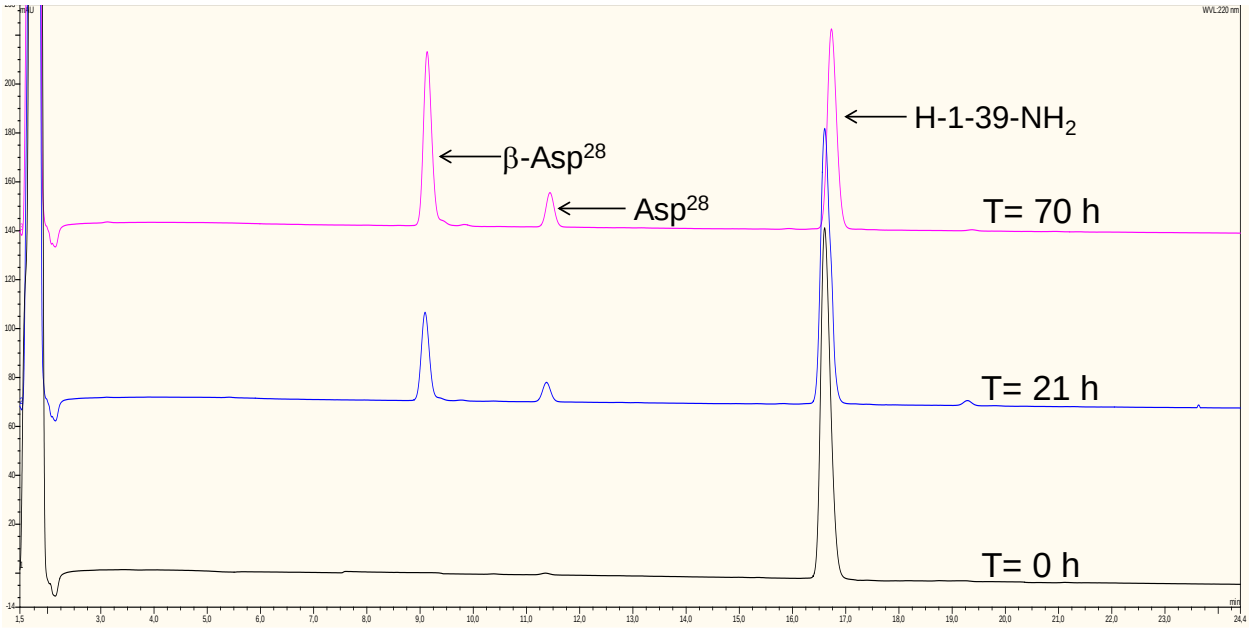



**Figure S16.** HPLC overlay of exenatide API in 1M phosphate buffer pH 9.2 at 37 °C over 70 h.

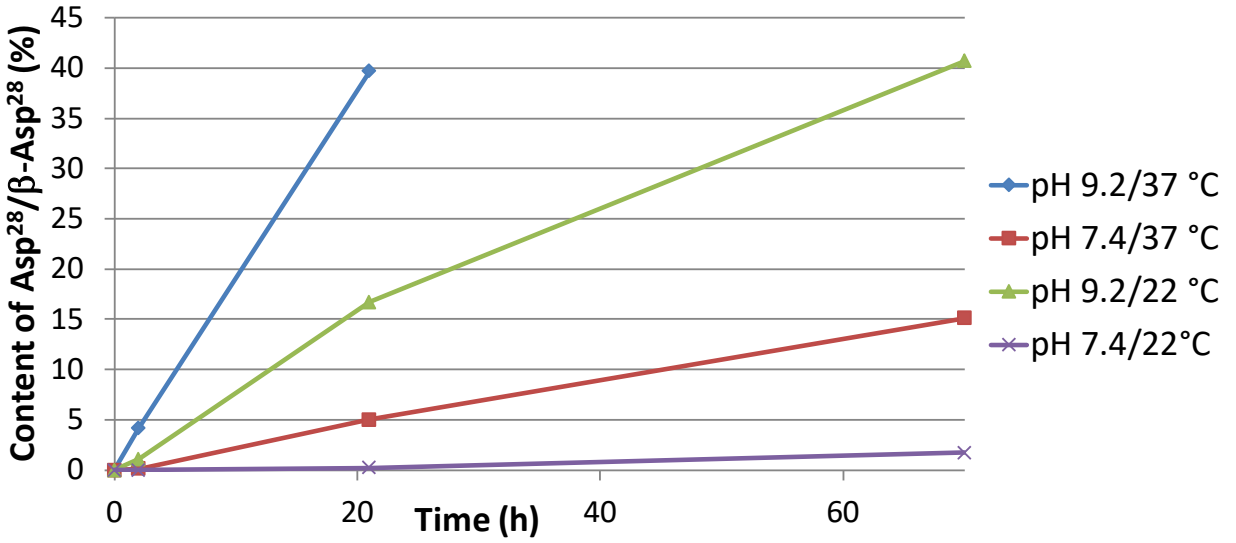



**Figure S17.** A schematic representation of effect of pH and temperature on stability of exenatide API in 1M phosphate buffer.

**15. Kinetics of the omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$, revised protocol (Fig. 6).**

Throughout the ligation 50 µL aliquots of the reaction mixture were taken out, quenched with 10% AcOH (1.0 mL) and analyzed by HPLC. When all H-1-21-O-Cam-Leu-$NH_2$ was consumed and no further conversion to the product could be observed the reaction was quenched with 10% AcOH. The S/H ratio was calculated by integrating the peak of H-1-39-$NH_2$ together with byproduct peaks

formed by reactions between H-1-21-O-Cam-Leu-$NH_2$ and byproducts in the crude H-22-39-$NH_2$ (S) vs the peak of H-1-21-OH (hydrolyzed H-1-21-O-Cam-Leu-$NH_2$, H).

Experimental conditions: column: Waters XSelect CSH130 C18 2.5µm 4.6x150mm; column temperature: 45°C; injection volume: 5 µL; sampler temperature:10°C; flow: 1.0 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 1; 10, 100; 13, 100; 14, 100; 19, 1; 20, 1.

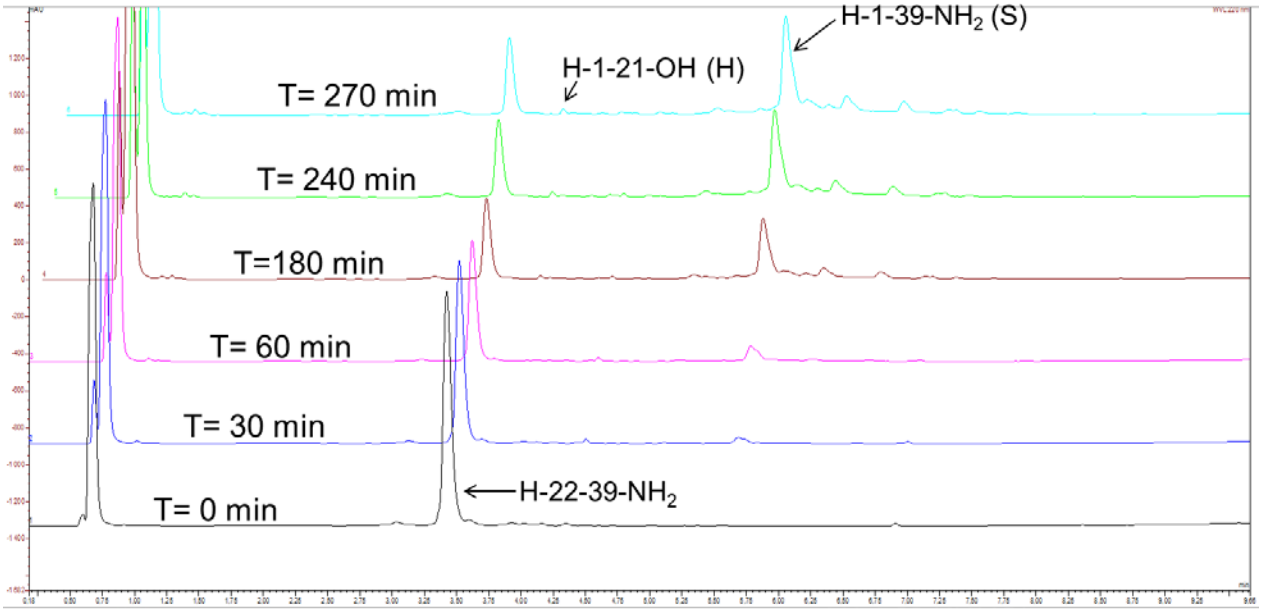



**Figure S18.** A schematic representation of the progress of Fig. 6 crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$ ligation.

**16. LC-HRMS of crude exenatide from omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-O-Cam-Leu-$NH_2$ (Fig. 6).**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 11; 1, 11; 5, 33; 50, 37; 54, 60; 56, 90; 58, 90; 58.1, 11; 70, 11.

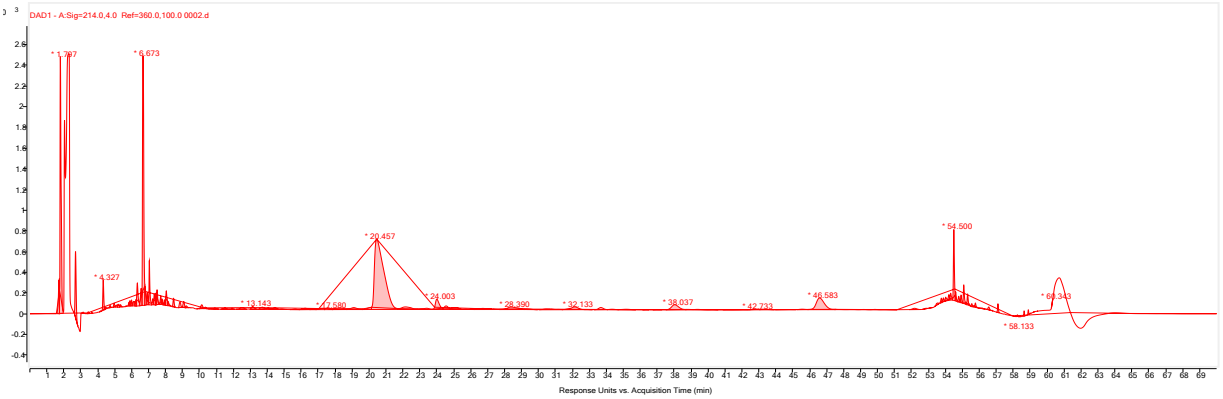



**Figure S19.** UV chromatogram overview.

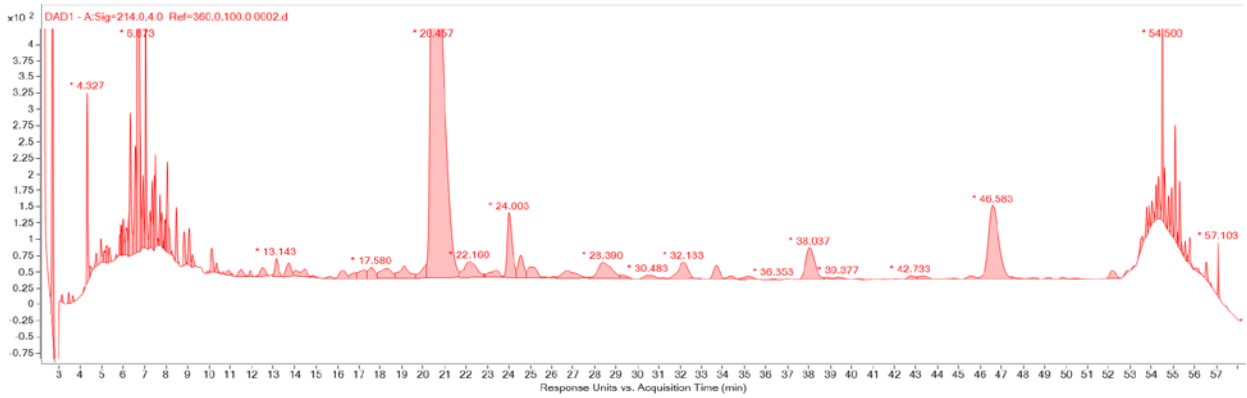



**Figure S20.** UV chromatogram zoom-in.

**Table S10.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1,71 | 1327,970 | | | 0,0000 | -4184,0273 | 0,084 | 1,44 | |
| 2 | 1,80 | 11129,600 | 920,5050 | 2 | 1841,0100 | -2343,0173 | 0,088 | 12,07 | H-(22-39)-NH2 |
| 3 | 2,69 | 2670,780 | | | 0,0000 | -4184,0273 | 0,131 | 2,90 | |
| 4 | 3,14 | 41,700 | | | 0,0000 | -4184,0273 | 0,153 | 0,05 | |
| 5 | 3,44 | 69,790 | | | 0,0000 | -4184,0273 | 0,168 | 0,08 | |
| 6 | 3,66 | 32,560 | | | 0,0000 | -4184,0273 | 0,178 | 0,04 | |
| 7 | 4,17 | 20,260 | | | 0,0000 | -4184,0273 | 0,203 | 0,02 | |
| 8 | 4,33 | 1117,110 | | | 0,0000 | -4184,0273 | 0,211 | 1,21 | |
| 9 | 4,47 | 82,460 | | | 0,0000 | -4184,0273 | 0,218 | 0,09 | |
| 10 | 4,73 | 100,390 | | | 0,0000 | -4184,0273 | 0,231 | 0,11 | |
| 11 | 4,97 | 172,320 | | | 0,0000 | -4184,0273 | 0,242 | 0,19 | |
| 12 | 5,11 | 113,250 | | | 0,0000 | -4184,0273 | 0,249 | 0,12 | |
| 13 | 5,24 | 165,620 | | | 0,0000 | -4184,0273 | 0,255 | 0,18 | |
| 14 | 5,34 | 119,980 | | | 0,0000 | -4184,0273 | 0,261 | 0,13 | |
| 15 | 5,67 | 18,360 | | | 0,0000 | -4184,0273 | 0,277 | 0,02 | |
| 16 | 5,84 | 93,350 | | | 0,0000 | -4184,0273 | 0,285 | 0,10 | |
| 17 | 5,88 | 129,310 | | | 0,0000 | -4184,0273 | 0,287 | 0,14 | |
| 18 | 5,95 | 133,480 | | | 0,0000 | -4184,0273 | 0,290 | 0,14 | |
| 19 | 5,99 | 213,400 | | | 0,0000 | -4184,0273 | 0,292 | 0,23 | |
| 20 | 6,12 | 280,830 | | | 0,0000 | -4184,0273 | 0,299 | 0,30 | |
| 21 | 6,28 | 214,360 | | | 0,0000 | -4184,0273 | 0,306 | 0,23 | |
| 22 | 6,34 | 1199,080 | | | 0,0000 | -4184,0273 | 0,309 | 1,30 | |
| 23 | 6,56 | 904,220 | | | 0,0000 | -4184,0273 | 0,320 | 0,98 | |
| 24 | 6,67 | 12619,470 | | | 0,0000 | -4184,0273 | 0,326 | 13,68 | |
| 25 | 6,78 | 718,570 | | | 0,0000 | -4184,0273 | 0,331 | 0,78 | |
| 26 | 6,93 | 448,430 | | | 0,0000 | -4184,0273 | 0,338 | 0,49 | |
| 27 | 7,05 | 1406,750 | 788,7140 | 3 | 2363,1202 | -1820,9071 | 0,344 | 1,53 | H-(1-21)-OH |
| 28 | 7,24 | 263,910 | | | 0,0000 | -4184,0273 | 0,353 | 0,29 | |
| 29 | 7,34 | 435,570 | | | 0,0000 | -4184,0273 | 0,358 | 0,47 | |
| 30 | 7,44 | 390,090 | | | 0,0000 | -4184,0273 | 0,363 | 0,42 | |
| 31 | 7,50 | 533,460 | | | 0,0000 | -4184,0273 | 0,366 | 0,58 | |
| 32 | 7,66 | 31,330 | | | 0,0000 | -4184,0273 | 0,374 | 0,03 | |
| 33 | 7,72 | 299,070 | | | 0,0000 | -4184,0273 | 0,376 | 0,32 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 34 | 7,80 | 174,490 | | | 0,0000 | -4184,0273 | 0,380 | 0,19 | |
| 35 | 7,90 | 31,520 | | | 0,0000 | -4184,0273 | 0,386 | 0,03 | |
| 36 | 7,93 | 127,810 | | | 0,0000 | -4184,0273 | 0,387 | 0,14 | |
| 37 | 8,01 | 151,820 | | | 0,0000 | -4184,0273 | 0,391 | 0,16 | |
| 38 | 8,05 | 645,110 | | | 0,0000 | -4184,0273 | 0,393 | 0,70 | |
| 39 | 8,13 | 165,410 | | | 0,0000 | -4184,0273 | 0,396 | 0,18 | |
| 40 | 8,27 | 23,470 | | | 0,0000 | -4184,0273 | 0,403 | 0,03 | |
| 41 | 8,47 | 381,270 | | | 0,0000 | -4184,0273 | 0,413 | 0,41 | |
| 42 | 8,85 | 315,480 | | | 0,0000 | -4184,0273 | 0,432 | 0,34 | |
| 43 | 9,08 | 329,550 | | | 0,0000 | -4184,0273 | 0,443 | 0,36 | |
| 44 | 9,22 | 117,650 | | | 0,0000 | -4184,0273 | 0,450 | 0,13 | |
| 45 | 9,947 | 28,09 | | | 0,0000 | -4184,0273 | 0,485 | 0,03 | |
| 46 | 10,137 | 294,32 | | | 0,0000 | -4184,0273 | 0,494 | 0,32 | |
| 47 | 10,35 | 88,48 | | | 0,0000 | -4184,0273 | 0,505 | 0,1 | |
| 48 | 10,433 | 7,29 | | | 0,0000 | -4184,0273 | 0,509 | 0,01 | |
| 49 | 10,66 | 23,53 | | | 0,0000 | -4184,0273 | 0,520 | 0,03 | |
| 50 | 10,9 | 84,75 | | | 0,0000 | -4184,0273 | 0,532 | 0,09 | |
| 51 | 11,08 | 14,63 | | | 0,0000 | -4184,0273 | 0,540 | 0,02 | |
| 52 | 11,503 | 172,92 | | | 0,0000 | -4184,0273 | 0,561 | 0,19 | |
| 53 | 11,923 | 81,4 | | | 0,0000 | -4184,0273 | 0,582 | 0,09 | |
| 54 | 12,487 | 231,02 | | | 0,0000 | -4184,0273 | 0,609 | 0,25 | |
| 55 | 13,143 | 312,09 | | | 0,0000 | -4184,0273 | 0,641 | 0,34 | |
| 56 | 13,713 | 324,57 | | | 0,0000 | -4184,0273 | 0,669 | 0,35 | |
| 57 | 14,457 | 332,41 | | | 0,0000 | -4184,0273 | 0,705 | 0,36 | |
| 58 | 14,857 | 31,73 | | | 0,0000 | -4184,0273 | 0,725 | 0,03 | |
| 59 | 15,65 | 59,8 | | | 0,0000 | -4184,0273 | 0,763 | 0,06 | |
| 60 | 16,24 | 288,02 | | | 0,0000 | -4184,0273 | 0,792 | 0,31 | |
| 61 | 16,88 | 160 | | | 0,0000 | -4184,0273 | 0,823 | 0,17 | |
| 62 | 17,243 | 338,09 | | | 0,0000 | -4184,0273 | 0,841 | 0,37 | |
| 63 | 17,58 | 368,45 | | | 0,0000 | -4184,0273 | 0,858 | 0,4 | |
| 64 | 18,27 | 576,7 | | | 0,0000 | -4184,0273 | 0,891 | 0,63 | |
| 65 | 19,107 | 600,4 | | | 0,0000 | -4184,0273 | 0,932 | 0,65 | |
| 66 | 20,147 | 346,78 | | | 0,0000 | -4184,0273 | 0,983 | 0,38 | |
| 67 | 20,457 | 26124,46 | 1395,684 | 3 | 4184,0302 | 0,0029 | 0,998 | 28,33 | Prod. H-(1-39)-NH2 |
| 68 | 22,16 | 999,15 | | | 0,0000 | -4184,0273 | 1,081 | 1,08 | |
| 69 | 23,407 | 353,69 | | | 0,0000 | -4184,0273 | 1,142 | 0,38 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 70 | 24,003 | 1591,57 | | | 0,0000 | -4184,0273 | 1,171 | 1,73 | |
| 71 | 24,55 | 590,12 | | | 0,0000 | -4184,0273 | 1,198 | 0,64 | |
| 72 | 25 | 496,24 | | | 0,0000 | -4184,0273 | 1,220 | 0,54 | |
| 73 | 26,077 | 56,47 | | | 0,0000 | -4184,0273 | 1,272 | 0,06 | |
| 74 | 26,717 | 498,78 | | | 0,0000 | -4184,0273 | 1,303 | 0,54 | |
| 75 | 28,39 | 1071,09 | | | 0,0000 | -4184,0273 | 1,385 | 1,16 | |
| 76 | 29,29 | 114,11 | | | 0,0000 | -4184,0273 | 1,429 | 0,12 | |
| 77 | 30,483 | 227,76 | | | 0,0000 | -4184,0273 | 1,487 | 0,25 | |
| 78 | 32,133 | 811,6 | | | 0,0000 | -4184,0273 | 1,567 | 0,88 | |
| 79 | 32,897 | 17,13 | | | 0,0000 | -4184,0273 | 1,605 | 0,02 | |
| 80 | 33,68 | 419,6 | | | 0,0000 | -4184,0273 | 1,643 | 0,45 | |
| 81 | 34,347 | 129,51 | | | 0,0000 | -4184,0273 | 1,675 | 0,14 | |
| 82 | 35,167 | 190,89 | | | 0,0000 | -4184,0273 | 1,715 | 0,21 | |
| 83 | 36,353 | 21,22 | | | 0,0000 | -4184,0273 | 1,773 | 0,02 | |
| 84 | 36,953 | 19,64 | | | 0,0000 | -4184,0273 | 1,803 | 0,02 | |
| 85 | 38,037 | 1437,89 | | | 0,0000 | -4184,0273 | 1,855 | 1,56 | |
| 86 | 39,377 | 228,6 | | | 0,0000 | -4184,0273 | 1,921 | 0,25 | |
| 87 | 42,053 | 21,79 | | | 0,0000 | -4184,0273 | 2,051 | 0,02 | |
| 88 | 42,733 | 129,86 | | | 0,0000 | -4184,0273 | 2,085 | 0,14 | |
| 89 | 43,327 | 146,06 | | | 0,0000 | -4184,0273 | 2,114 | 0,16 | |
| 90 | 44,76 | 30,76 | | | 0,0000 | -4184,0273 | 2,183 | 0,03 | |
| 91 | 45,55 | 113,95 | | | 0,0000 | -4184,0273 | 2,222 | 0,12 | |
| 92 | 46,583 | 3797,45 | 1249,2898 | 3 | 3747,8694 | -436,1579 | 2,272 | 4,12 | Ac-(6-39)-NH2 |
| 93 | 47,493 | 240,26 | | | 0,0000 | -4184,0273 | 2,317 | 0,26 | |
| 94 | 52,183 | 216,56 | | | 0,0000 | -4184,0273 | 2,546 | 0,23 | |
| 95 | 52,873 | 45,19 | | | 0,0000 | -4184,0273 | 2,579 | 0,05 | |
| 96 | 53,267 | 20,09 | | | 0,0000 | -4184,0273 | 2,598 | 0,02 | |
| 97 | 53,563 | 142,83 | | | 0,0000 | -4184,0273 | 2,613 | 0,15 | |
| 98 | 53,763 | 203,82 | | | 0,0000 | -4184,0273 | 2,623 | 0,22 | |
| 99 | 53,89 | 170 | | | 0,0000 | -4184,0273 | 2,629 | 0,18 | |
| 100 | 54,023 | 188,76 | | | 0,0000 | -4184,0273 | 2,635 | 0,2 | |
| 101 | 54,21 | 242,16 | | | 0,0000 | -4184,0273 | 2,644 | 0,26 | |
| 102 | 54,313 | 333,3 | | | 0,0000 | -4184,0273 | 2,649 | 0,36 | |
| 103 | 54,5 | 2690,51 | | | 0,0000 | -4184,0273 | 2,659 | 2,92 | |
| 104 | 54,61 | 299,98 | | | 0,0000 | -4184,0273 | 2,664 | 0,33 | |
| 105 | 54,79 | 242,81 | | | 0,0000 | -4184,0273 | 2,673 | 0,26 | |
| 106 | 54,927 | 307,46 | | | 0,0000 | -4184,0273 | 2,679 | 0,33 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 107 | 55,087 | 724,95 | 0,0000 | -4184,0273 | 2,687 | 0,79 |
| 108 | 55,21 | 60,44 | 0,0000 | -4184,0273 | 2,693 | 0,07 |
| 109 | 55,313 | 420,49 | 0,0000 | -4184,0273 | 2,698 | 0,46 |
| 110 | 55,387 | 33,97 | 0,0000 | -4184,0273 | 2,702 | 0,04 |
| 111 | 55,56 | 112,18 | 0,0000 | -4184,0273 | 2,710 | 0,12 |
| 112 | 55,693 | 34,3 | 0,0000 | -4184,0273 | 2,717 | 0,04 |
| 113 | 55,77 | 214,07 | 0,0000 | -4184,0273 | 2,720 | 0,23 |
| 114 | 56,043 | 71,08 | 0,0000 | -4184,0273 | 2,734 | 0,08 |
| 115 | 56,56 | 170,2 | 0,0000 | -4184,0273 | 2,759 | 0,18 |
| 116 | 56,753 | 12,56 | 0,0000 | -4184,0273 | 2,768 | 0,01 |
| 117 | 57,103 | 282,88 | 0,0000 | -4184,0273 | 2,786 | 0,31 |
| 118 | 58,133 | 6,34 | 0,0000 | -4184,0273 | 2,836 | 0,01 |
| 119 | 58,253 | 51,83 | 0,0000 | -4184,0273 | 2,842 | 0,06 |
| 120 | 58,39 | 23,83 | 0,0000 | -4184,0273 | 2,848 | 0,03 |
| 121 | 58,647 | 118,62 | 0,0000 | -4184,0273 | 2,861 | 0,13 |
| 122 | 58,893 | 119,92 | 0,0000 | -4184,0273 | 2,873 | 0,13 |
| 123 | 59,067 | 3,88 | 0,0000 | -4184,0273 | 2,881 | 0 |
| 124 | 59,307 | 11,4 | 0,0000 | -4184,0273 | 2,893 | 0,01 |
| 125 | 59,45 | 1,95 | 0,0000 | -4184,0273 | 2,900 | 0 |
| 126 | 60,343 | 41,72 | 0,0000 | -4184,0273 | 2,944 | 0,05 |

## 17. HPLC analysis of purified H-22-39-$NH_2$ fragment.

For the details of the purification of the fragment see the Materials & Methods section of the paper. Experimental conditions: column: Phenomenex Kinetex C18 2.6µm 4.6x50mm; column temperature: 50°C; injection volume: 1 µL; sampler temperature:10°C; flow: 2 ml/min; mobile phase A: 50 mM $KH_2PO_4$ pH 3.0, mobile phase B: MeCN. Gradient (Time(min), %B): 0, 15; 0.3, 15; 10, 20; 10.1, 60; 10.5, 60; 10.6, 15; 12, 15.

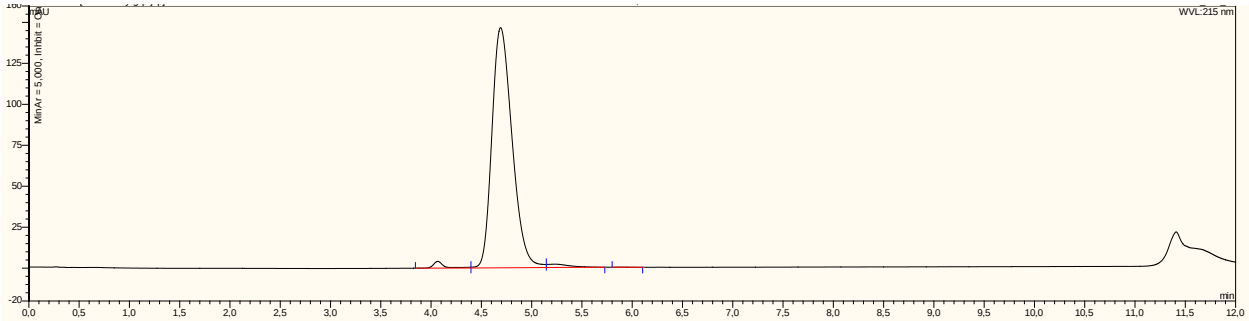



**Figure S21.** UV chromatogram overview.

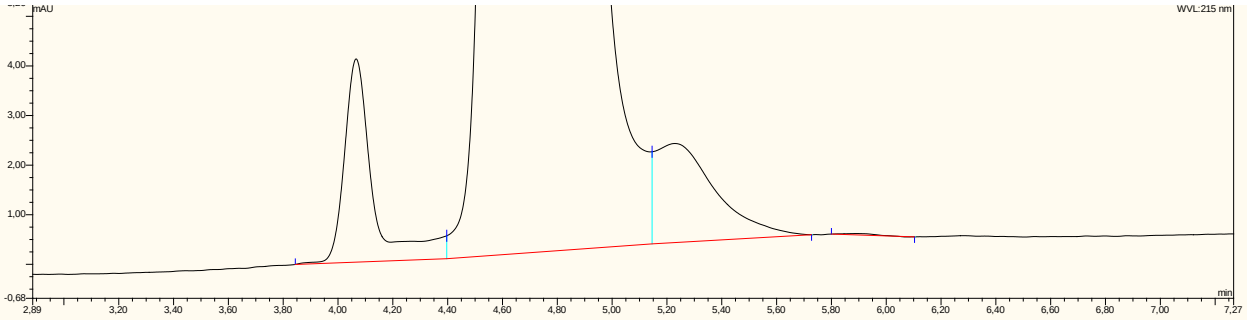



**Figure S22.** UV chromatogram zoom-in.

**Table S11.** Area% for integrated peaks.

| No | Ret. Time (min) | Area (mAu$^x$min) | Height (mAU) | Rel.Area (%) |
|---|---|---|---|---|
| 1 | 4,066 | 0,4949 | 4,096 | 1,41 |
| 2 | 4,691 | 34,0573 | 146,574 | 97,13 |
| 3 | 5,226 | 0,5086 | 1,998 | 1,45 |
| 4 | 5,923 | 0,0035 | 0,030 | 0,01 |
| Total: | | 35,0643 | 152,698 | 100,00 |

### 18. HPLC analysis of purified H-1-21-O-Cam-Leu-$NH_2$ fragment.

For the details of the purification of the fragment see the Materials & Methods section of the paper. Experimental conditions: column: Phenomenex Kinetex C18 2.6µm 4.6x50mm; column temperature: 30°C; injection volume: 5 µL; sampler temperature:10°C; flow: 1.5 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 5; 0.3, 5; 21, 95; 22, 95; 22.1, 5; 25, 5.

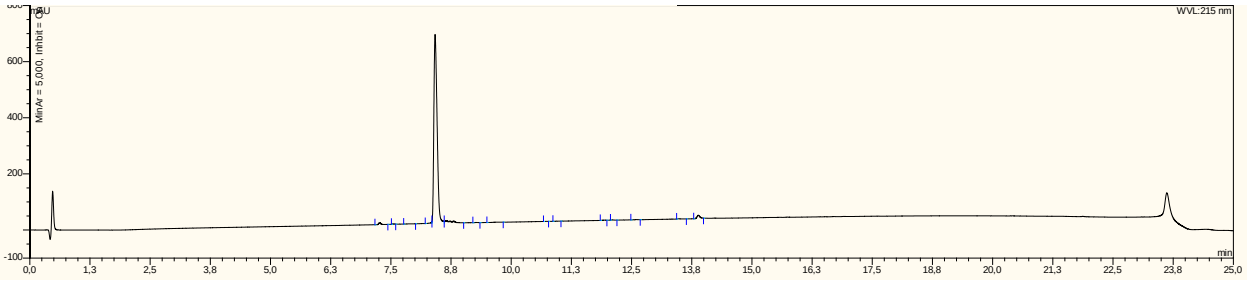



**Figure S23.** UV chromatogram overview.

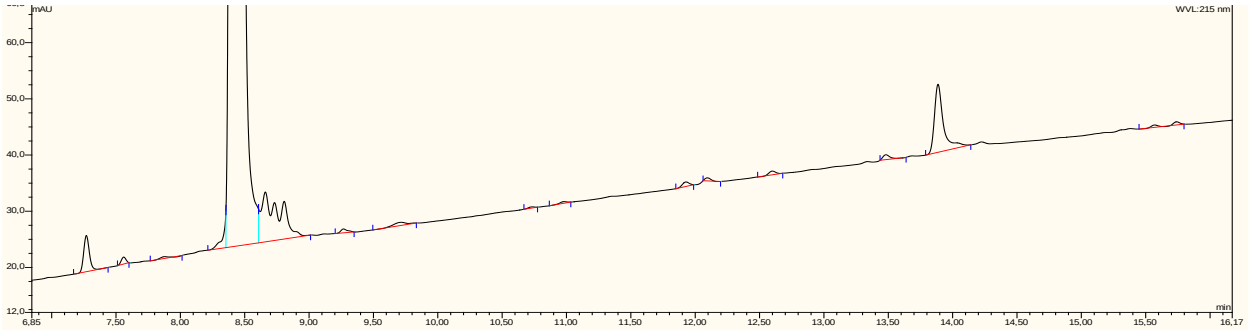



**Figure S24.** UV chromatogram zoom-in.

**Table S12.** Area% for integrated peaks.

| No | Ret. Time (min) | Area (mAu$^x$min) | Height (mAU) | Rel.Area (%) |
|---|---|---|---|---|
| 1 | 7,269 | 0,3254 | 6,418 | 0,59 |
| 2 | 7,558 | 0,0524 | 1,239 | 0,09 |
| 3 | 7,972 | 0,0169 | 0,081 | 0,03 |
| 4 | 8,354 | 0,1533 | 6,689 | 0,28 |
| 5 | 8,418 | 52,2867 | 673,055 | 94,00 |
| 6 | 8,660 | 1,4573 | 8,864 | 2,62 |
| 7 | 9,267 | 0,0419 | 0,692 | 0,08 |
| 8 | 9,703 | 0,0638 | 0,575 | 0,11 |
| 9 | 10,726 | 0,0117 | 0,218 | 0,02 |
| 10 | 10,973 | 0,0184 | 0,302 | 0,03 |
| 11 | 11,924 | 0,0490 | 0,804 | 0,09 |
| 12 | 12,095 | 0,0265 | 0,541 | 0,05 |
| 13 | 12,597 | 0,0399 | 0,667 | 0,07 |
| 14 | 13,480 | 0,0519 | 0,872 | 0,09 |
| 15 | 13,886 | 0,9762 | 12,093 | 1,76 |
| 16 | 15,738 | 0,0497 | 0,559 | 0,09 |
| Total: | | 55,6212 | 713,669 | 100,00 |

**19. Kinetics of the omniligase-1 catalyzed ligation of pure H-22-39-$NH_2$ + pure H-1-21-O-Cam-Leu-$NH_2$ (Fig. 7).**

Throughout the ligation 50 µL aliquots of the reaction mixture were taken out, quenched with 10% AcOH (1.0 mL) and analyzed by HPLC. When all H-1-21-O-Cam-Leu-$NH_2$ was consumed and no further conversion to the product could be observed the reaction was quenched with 10% AcOH. The S/H ratio was calculated by integrating the peak of H-1-39-$NH_2$ together with byproducts peaks formed by reactions between H-1-21-O-Cam-Leu-$NH_2$ and byproducts in the crude H-22-39-$NH_2$ (S) vs the peak of H-1-21-OH (hydrolyzed H-1-21-O-Cam-Leu-$NH_2$, H).

Experimental conditions: column: Waters XSelect CSH130 C18 2.5µm 4.6x150mm; column temperature: 45°C; injection volume: 5 µL; sampler temperature:10°C; flow: 1.0 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 1; 10, 100; 13, 100; 14, 100; 19, 1; 20, 1.

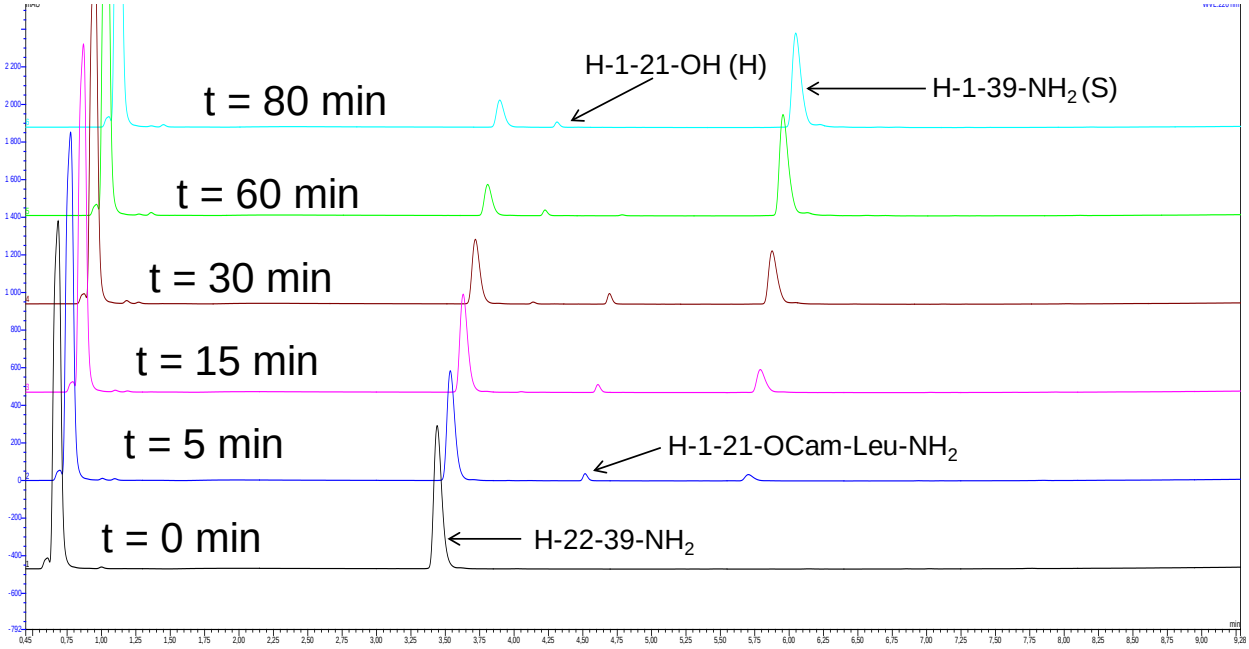



**Figure S25.** A schematic representation of the progress of Fig. 7 pure H-22-39-$NH_2$ + pure H-1-21-O-Cam-Leu-$NH_2$ ligation.

**20. LC-HRMS of crude exenatide from omniligase-1 catalyzed ligation of pure H-22-39-$NH_2$ + pure H-1-21-O-Cam-Leu-$NH_2$ (Fig. 7).**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v100 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 11; 1, 11; 5, 33; 50, 37; 54, 60; 56, 90; 58, 90; 58.1, 11; 70, 11.

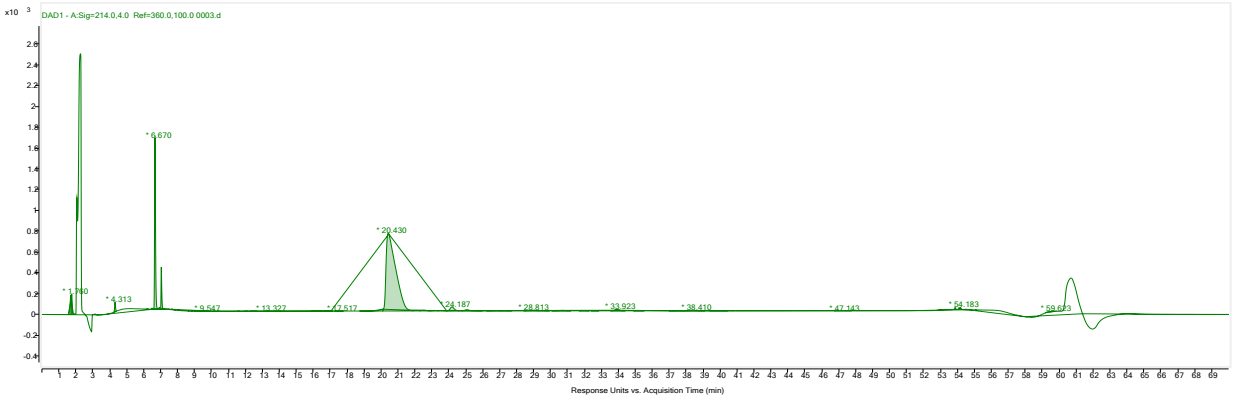



**Figure S26.** UV chromatogram overview.

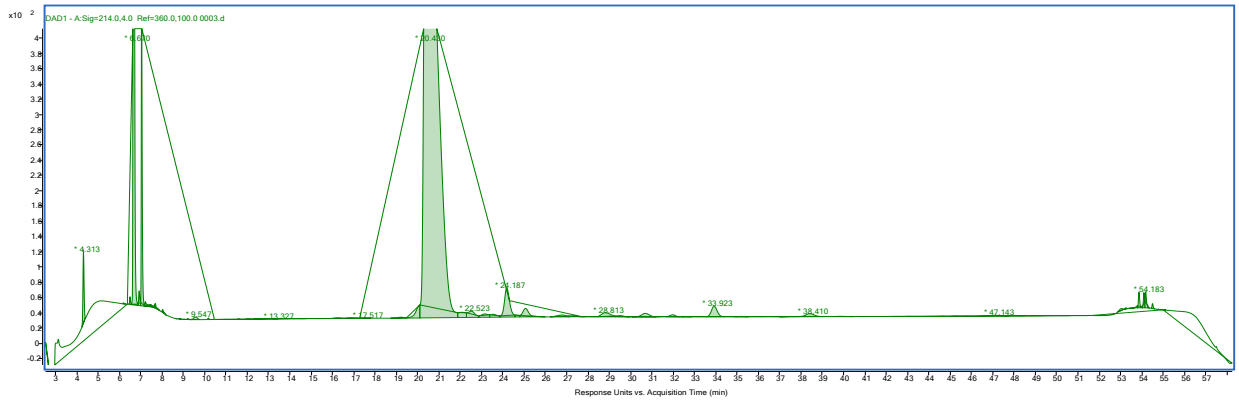



**Figure S27.** UV chromatogram zoom-in.

**Table S13.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1,70 | 713,700 | | | 0,0000 | -4184,0273 | 0,083 | 1,7 | |
| 2 | 1,76 | 666,790 | | | 0,0000 | -4184,0273 | 0,086 | 1,59 | |
| 3 | 4,31 | 329,760 | | | 0,0000 | -4184,0273 | 0,210 | 0,79 | |
| 4 | 6,19 | 4,540 | | | 0,0000 | -4184,0273 | 0,302 | 0,01 | |
| 5 | 6,50 | 26,450 | | | 0,0000 | -4184,0273 | 0,317 | 0,06 | |
| 6 | 6,67 | 6270,680 | 920,5060 | 2 | 1838,9974 | -2345,0299 | 0,325 | 14,94 | H-(22-39)-NH2 |
| 7 | 6,93 | 72,050 | | | 0,0000 | -4184,0273 | 0,338 | 0,17 | |
| 8 | 7,04 | 1168,700 | 788,7120 | 3 | 2363,1142 | -1820,9131 | 0,344 | 2,78 | H-(1-21)-OH |
| 9 | 7,22 | 24,270 | | | 0,0000 | -4184,0273 | 0,352 | 0,06 | |
| 10 | 7,32 | 10,910 | | | 0,0000 | -4184,0273 | 0,357 | 0,03 | |
| 11 | 7,42 | 9,870 | | | 0,0000 | -4184,0273 | 0,362 | 0,02 | |
| 12 | 7,48 | 7,920 | | | 0,0000 | -4184,0273 | 0,365 | 0,02 | |
| 13 | 7,51 | 6,230 | | | 0,0000 | -4184,0273 | 0,366 | 0,01 | |
| 14 | 7,63 | 7,640 | | | 0,0000 | -4184,0273 | 0,372 | 0,02 | |
| 15 | 7,68 | 21,780 | | | 0,0000 | -4184,0273 | 0,375 | 0,05 | |
| 16 | 7,80 | 3,970 | | | 0,0000 | -4184,0273 | 0,380 | 0,01 | |
| 17 | 7,85 | 1,320 | | | 0,0000 | -4184,0273 | 0,383 | 0,00 | |
| 18 | 8,03 | 12,720 | | | 0,0000 | -4184,0273 | 0,392 | 0,03 | |
| 19 | 8,07 | 6,840 | | | 0,0000 | -4184,0273 | 0,394 | 0,02 | |
| 20 | 8,64 | 0,840 | | | 0,0000 | -4184,0273 | 0,422 | 0,00 | |
| 21 | 8,85 | 3,180 | | | 0,0000 | -4184,0273 | 0,432 | 0,01 | |
| 22 | 9,04 | 2,180 | | | 0,0000 | -4184,0273 | 0,441 | 0,01 | |
| 23 | 9,20 | 6,950 | | | 0,0000 | -4184,0273 | 0,449 | 0,02 | |
| 24 | 9,55 | 43,700 | | | 0,0000 | -4184,0273 | 0,466 | 0,10 | |
| 25 | 10,17 | 7,280 | | | 0,0000 | -4184,0273 | 0,496 | 0,02 | |
| 26 | 10,44 | 0,860 | | | 0,0000 | -4184,0273 | 0,509 | 0,00 | |
| 27 | 11,60 | 1,470 | | | 0,0000 | -4184,0273 | 0,566 | 0,00 | |
| 28 | 12,04 | 1,020 | | | 0,0000 | -4184,0273 | 0,587 | 0,00 | |
| 29 | 12,52 | 4,920 | | | 0,0000 | -4184,0273 | 0,611 | 0,01 | |
| 30 | 13,33 | 1,120 | | | 0,0000 | -4184,0273 | 0,650 | 0,00 | |
| 31 | 16,22 | 14,830 | | | 0,0000 | -4184,0273 | 0,791 | 0,04 | |
| 32 | 16,84 | 11,800 | | | 0,0000 | -4184,0273 | 0,821 | 0,03 | |
| 33 | 17,52 | 13,480 | | | 0,0000 | -4184,0273 | 0,854 | 0,03 | |
| 34 | 19,21 | 24,390 | | | 0,0000 | -4184,0273 | 0,937 | 0,06 | |
| 35 | 20,10 | 217,150 | | | 0,0000 | -4184,0273 | 0,981 | 0,52 | |
| 36 | 20,43 | 29457,600 | 1395,6850 | 3 | 4184,0332 | 0,0059 | 0,997 | 70,19 | Prod. H-(1-39)-NH2 |
| 37 | 22,08 | 161,070 | | | 0,0000 | -4184,0273 | 1,077 | 0,38 | |
| 38 | 22,52 | 195,570 | | | 0,0000 | -4184,0273 | 1,099 | 0,47 | |
| 39 | 23,15 | 104,590 | | | 0,0000 | -4184,0273 | 1,129 | 0,25 | |
| 40 | 23,55 | 87,340 | | | 0,0000 | -4184,0273 | 1,149 | 0,21 | |
| 41 | 24,19 | 656,000 | 1330,9810 | 3 | 3989,9212 | -194,1061 | 1,180 | 1,56 | Des Pro-Pro |
| 42 | 24,56 | 30,730 | | | 0,0000 | -4184,0273 | 1,198 | 0,07 | |
| 43 | 25,07 | 225,130 | | | 0,0000 | -4184,0273 | 1,223 | 0,54 | |
| 44 | 26,79 | 75,260 | | | 0,0000 | -4184,0273 | 1,307 | 0,18 | |
| 45 | 27,29 | 27,35 | | | 0,0000 | -4184,0273 | 1,331 | 0,07 | |
| 46 | 28,813 | 149,15 | | | 0,0000 | -4184,0273 | 1,406 | 0,36 | |
| 47 | 29,49 | 32,7 | | | 0,0000 | -4184,0273 | 1,439 | 0,08 | |
| 48 | 30,693 | 116,15 | | | 0,0000 | -4184,0273 | 1,497 | 0,28 | |
| 49 | 31,973 | 47,48 | | | 0,0000 | -4184,0273 | 1,560 | 0,11 | |
| 50 | 32,6 | 3,22 | | | 0,0000 | -4184,0273 | 1,590 | 0,01 | |
| 51 | 33,923 | 259,98 | | | 0,0000 | -4184,0273 | 1,655 | 0,62 | |
| 52 | 35,207 | 8,79 | | | 0,0000 | -4184,0273 | 1,717 | 0,02 | |
| 53 | 37,44 | 8,07 | | | 0,0000 | -4184,0273 | 1,826 | 0,02 | |
| 54 | 38,41 | 117,6 | | | 0,0000 | -4184,0273 | 1,874 | 0,28 | |
| 55 | 39,867 | 9,87 | | | 0,0000 | -4184,0273 | 1,945 | 0,02 | |
| 56 | 47,143 | 19,77 | | | 0,0000 | -4184,0273 | 2,300 | 0,05 | |
| 57 | 53,047 | 26,68 | | | 0,0000 | -4184,0273 | 2,588 | 0,06 | |
| 58 | 53,257 | 9,78 | | | 0,0000 | -4184,0273 | 2,598 | 0,02 | |
| 59 | 53,57 | 4,31 | | | 0,0000 | -4184,0273 | 2,613 | 0,01 | |
| 60 | 53,787 | 11,31 | | | 0,0000 | -4184,0273 | 2,624 | 0,03 | |
| 61 | 53,867 | 84,37 | | | 0,0000 | -4184,0273 | 2,628 | 0,2 | |
| 62 | 54,037 | 8,76 | | | 0,0000 | -4184,0273 | 2,636 | 0,02 | |
| 63 | 54,1 | 73,76 | | | 0,0000 | -4184,0273 | 2,639 | 0,18 | |
| 64 | 54,183 | 86,3 | | | 0,0000 | -4184,0273 | 2,643 | 0,21 | |
| 65 | 54,503 | 23,57 | | | 0,0000 | -4184,0273 | 2,659 | 0,06 | |
| 66 | 54,787 | 0,79 | | | 0,0000 | -4184,0273 | 2,673 | 0 | |
| 67 | 55,083 | 0,8 | | | 0,0000 | -4184,0273 | 2,687 | 0 | |
| 68 | 59,34 | 17,72 | | | 0,0000 | -4184,0273 | 2,895 | 0,04 | |
| 69 | 59,623 | 107,72 | | | 0,0000 | -4184,0273 | 2,908 | 0,26 | |

**21. Assessment of kinetics of loss of DKP during piperidine treatment of Fmoc-20-21-linker-AA resins.**

100 mg of the resin (Fmoc-20-21-O-Cam-Leu-RMG DEG AM or Fmoc-20-21-HMBA-Lys(Trt) MBH) was swollen in DMF, drained and exposed to 2 mL of mol sieves dried 10% (v/v) pip/DMF. The resulting slurry was shaken at 30 °C and 50 µL aliquots of the supernate were taken out over time, diluted with 1.0 mL MeCN and HPLC analyses of the reaction mixtures were carried out. The conversion of the DKP was determined by comparing the HPLC area of the DKP peak (mAu$^{x}$min) at a given timepoint vs the area of the peak upon complete removal of DKP from the resin. DKP loss after 1 h was ~54% for the O-Cam linker and ~12% for the HMBA linker.

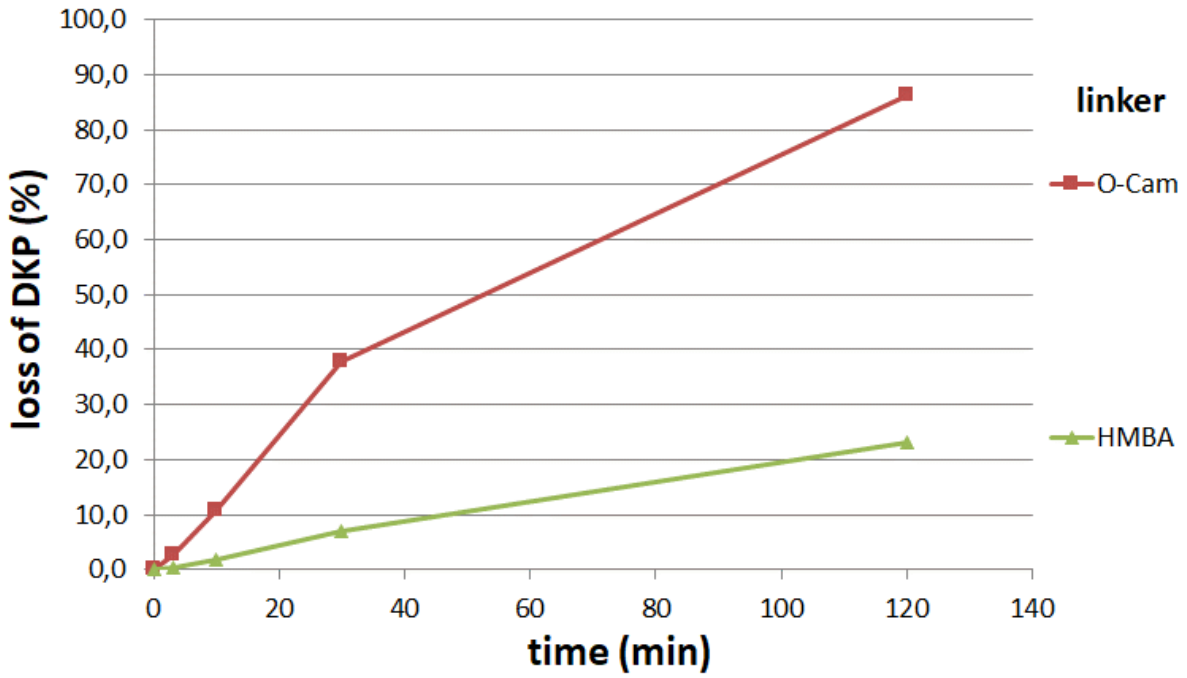
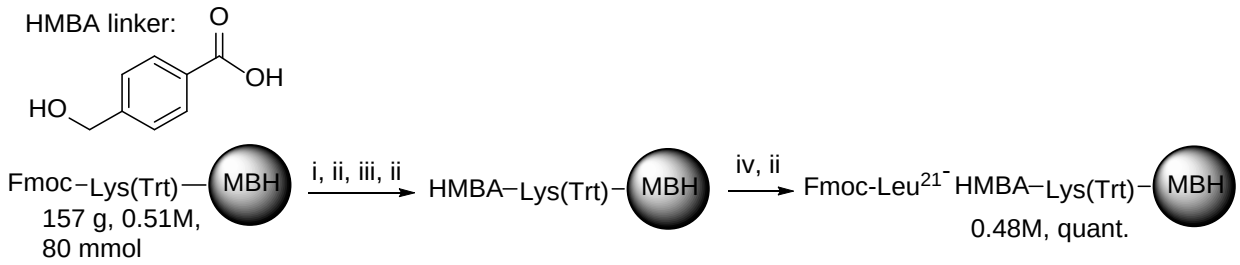



**Figure S28.** A schematic representation of loss of DKP for Fmoc-20-21-O-Cam Leu RMG DEG AM and Fmoc-20-21-HMBA-Lys(Trt) MBH resins upon treatment with pip/DMF. DKP loss after 1 h: O-Cam linker: ~54%; HMBA linker: ~12%.

**22. Details of SPPS of Boc-1-21-HMBA-K peptide resin**

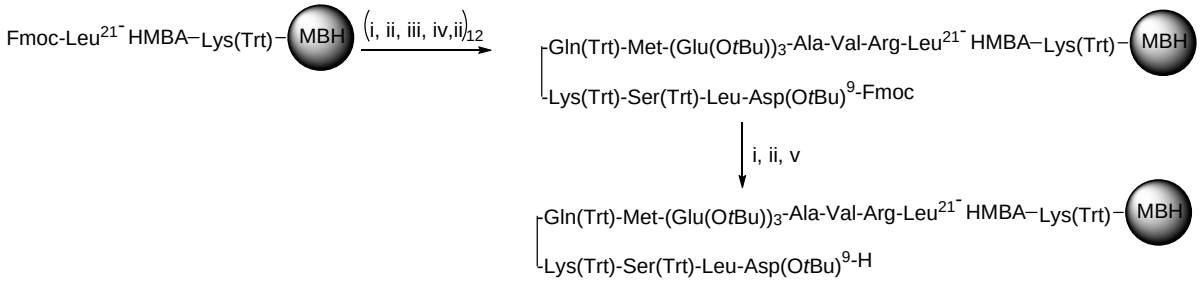
Regarding choice of the resin, MBH was used as the solid support for the synthesis of the H-1-21-HMBA-K fragment instead of the more common Wang resin,[13] as the latter is known to be susceptible to cause loss of peptide during peptide resin cleavages.[14] Furthermore, Lys was used as the C-terminal AA instead of Leu used in the synthesis of the O-Cam fragment to offset the more hydrophobic nature of the HMBA linker. The attachment of the HMBA linker followed by coupling of the Leu$^{21}$ residue is summarized in Scheme S4.

Thus, after removing the Fmoc group the resulting H-Lys(Trt)-MBH resin was treated with HMBA/Oxyma/DIC (1:2:2) for 2 h at 40 °C resulting in a complete conversion as judged by a Ninhydrin color test. It is worth noting that the content of double HMBA incorporation impurity (endo-HMBA) was <1% whereas examining the recently reported HMBA/Cl-HOBt/DIC (1:2:1.3), rt, 16 h conditions[15] resulted in ~11% endo-HMBA. Next, Leu[21] coupling was carried out for 16 h at rt using Fmoc-Leu-OH/DIC/Cl-HOBt/DMAP (1:1:1:0.2), which gave the Fmoc-Leu[21]-HMBA MBH resin in full conversion and with good chemoselectivity.



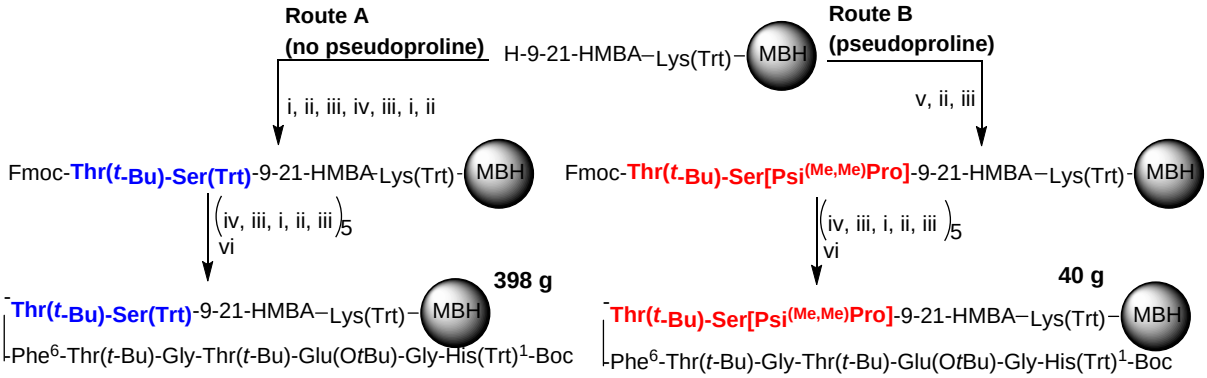

**Scheme S4** SPPS of Fmoc-Leu-HMBA-Lys(Trt) MBH resin, reagents and conditions: i) Fmoc deprotection: 10% pip in DMF, 30°C, 30 min; ii) DMF resin wash; iii) coupling: 2 equiv HMBA/Oxyma/DIC (1:2:2) in DMF, 40 °C, 2 h; iv) coupling: 4 equiv Fmoc-Leu-OH/Cl-HOBt/DIC/DMAP (1:1:1:0.2) in DMF, rt, 17 h.

Next, synthesis of the intermediate H-9-21 HMBA resin was carried out starting with Fmoc-21-HMBA resin and using mild conventional heating throughout with the exception of Fmoc deprotection of the Fmoc-20-21 HMBA resin which was done at 30 °C to minimize the peptide loss to DKP elimination (Scheme S5). The same protocol as for the synthesis of H-22-39-$NH_2$ fragment (Scheme S1) was followed and the synthesis furnished 335 g of intermediate H-9-21 -HMBA-Lys(Trt) MBH resin in 84% yield. HPLC purity of the H-9-21-HMBA-K fragment from a test TFA cleavage was 81%, which is comparable to the purity attained for the intermediate Fmoc-9-21-OCam-L-$NH_2$ fragment.




**Scheme S5** SPPS of H-9-21-Leu-HMBA-Lys(Trt) MBH resin, reagents and conditions: i) Fmoc deprotection: 10% pip in DMF, 40 – 45 °C, 2 x 15 min; ii) DMF resin wash; iii) coupling: R-COOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 30 min; iv) capping: AcOH, 5 min; v) *i*-PrOH wash and drying in vacuo.

From H-9-21-HMBA-K resin onwards the SPPS was carried out in two portions (1:9), one portion of the resin using Fmoc-Thr(tBu)-Ser(Psi$^{(Me,Me)}$Pro)-OH pseudoproline dipeptide for the Ser$^{8}$ and Thr$^{7}$ couplings (Scheme S6, route B) and the other portion using standard AA derivatives (Scheme 6, route A).




**Scheme S6** SPPS of Boc-1-21-Leu-HMBA-Lys(Trt) MBH resin, 90% of H-9-21-HMBA-K resin (80 mmol) used in route A, 10% in route B; reagents and conditions: i coupling: R-COOH/Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 30 min, Boc-His(Trt)-Gly-OH used in the final coupling; ii) capping: AcOH, 5 min; iii) DMF resin wash; iv) Fmoc deprotection: 10% pip in DMF, 40 – 45 °C, 2 x 15 min, deprotection of route A Fmoc-5-21-HMBA-K resin: 2x15+25 min; v) coupling: Fmoc-Thr(tBu)-Ser(Psi$^{(Me,Me)}$Pro)-OH /Oxyma/DIC (1:1:3) in DMF, 40 – 45 °C, 30 min; vi) *i*-PrOH wash and drying in vacuo.

As the aim was to develop a CEPS manufacturing process that would not only be greener than the conventional manufacturing approaches but also more cost-efficient, the plan was to employ the H-1-21-HMBA-K fragment prepared without the expensive pseudoproline dipeptide in large scale omniligase-1 catalyzed ligations. We therefore used 90% of the H-9-21-HMBA-K resin in the pseudoproline-free route (Scheme 6, route A), while the remaining 10% of the resin intermediate was employed in the route utilizing Fmoc-Thr(tBu)-Ser(Psi$^{(Me,Me)}$Pro)-OH pseudoproline for Ser$^{8}$ and Thr$^{7}$couplings as a means to obtain a sample of H-1-21-HMBA-K fragment as a reference (Scheme 6, route B).

**HPLC analysis of intermediate crude H-9-21-HMBA-K peptide (Scheme S5).**

For the details of the purification of the fragment see the Materials & Methods section of the paper. Experimental conditions: column: Waters XSelect CSH130 C18 2.5µm 4.6x150mm; column temperature: 30°C; injection volume: 5 µL; sampler temperature:10°C; flow: 0.8 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 0; 40, 100; 54, 100; 55, 0; 62, 0.

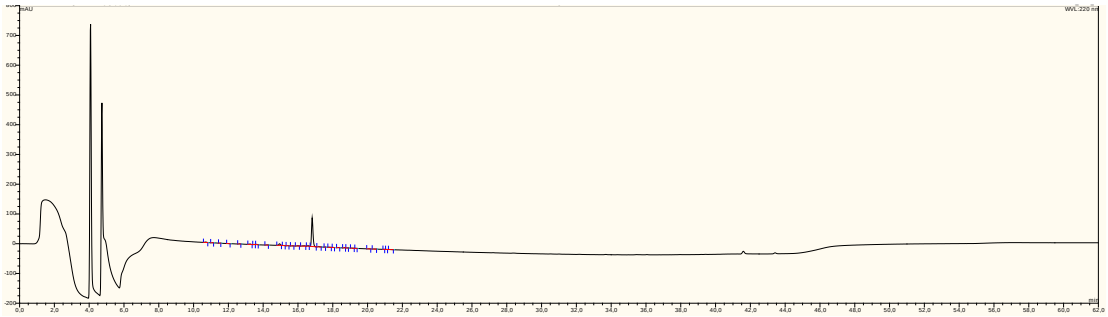


**Figure S29.** UV chromatogram overview.

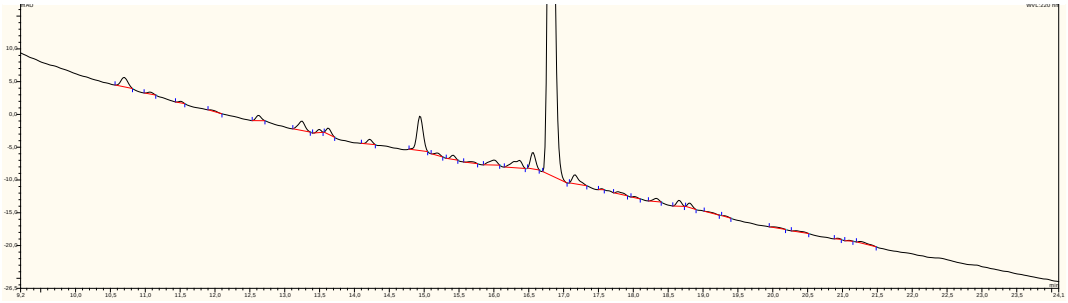


**Figure S30.** UV chromatogram zoom-in.

**Table S14.** Area% for integrated peaks.

| No | Ret. Time (min) | Area (mAu*min) | Height (mAU) | Rel.Area (%) |
|---|---|---|---|---|
| 1 | 10,700 | 0,1647 | 1,434 | 1,42 |
| 2 | 11,083 | 0,0256 | 0,293 | 0,22 |
| 3 | 11,517 | 0,0164 | 0,246 | 0,14 |
| 4 | 11,983 | 0,0152 | 0,141 | 0,13 |
| 5 | 12,617 | 0,0697 | 0,800 | 0,60 |
| 6 | 13,250 | 0,1608 | 1,421 | 1,39 |
| 7 | 13,500 | 0,0309 | 0,465 | 0,27 |
| 8 | 13,633 | 0,0721 | 0,914 | 0,62 |
| 9 | 14,217 | 0,0610 | 0,729 | 0,53 |
| 10 | 14,933 | 0,5271 | 5,212 | 4,55 |
| 11 | 15,200 | 0,0317 | 0,376 | 0,27 |
| 12 | 15,417 | 0,0477 | 0,638 | 0,41 |
| 13 | 15,700 | 0,0217 | 0,200 | 0,19 |
| 14 | 16,000 | 0,1005 | 0,754 | 0,87 |
| 15 | 16,367 | 0,1897 | 1,170 | 1,64 |
| 16 | 16,567 | 0,2094 | 2,482 | 1,81 |
| 17 | 16,817 | 9,3273 | 96,380 | 80,61 |
| 18 | 17,167 | 0,1533 | 1,363 | 1,33 |
| 19 | 17,550 | 0,0070 | 0,142 | 0,06 |
| 20 | 17,783 | 0,0394 | 0,307 | 0,34 |
| 21 | 18,033 | 0,0131 | 0,176 | 0,11 |
| 22 | 18,333 | 0,0446 | 0,489 | 0,39 |
| 23 | 18,650 | 0,0716 | 0,875 | 0,62 |
| 24 | 18,817 | 0,0526 | 0,673 | 0,45 |
| 25 | 19,150 | 0,0133 | 0,108 | 0,11 |
| 26 | 19,333 | 0,0095 | 0,118 | 0,08 |
| 27 | 20,067 | 0,0194 | 0,132 | 0,17 |
| 28 | 20,433 | 0,0244 | 0,142 | 0,21 |
| 29 | 20,933 | 0,0076 | 0,128 | 0,07 |
| 30 | 21,100 | 0,0060 | 0,100 | 0,05 |
| 31 | 21,283 | 0,0378 | 0,237 | 0,33 |
| Total: | | 11,5712 | 118,645 | 100,00 |

**23. LC-HRMS of crude H-1-21-HMBA-K from SPPS in Scheme S6, route A.**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v380 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 5; 1, 5; 41, 42; 50, 95; 52, 95; 52.1, 5; 66, 5.

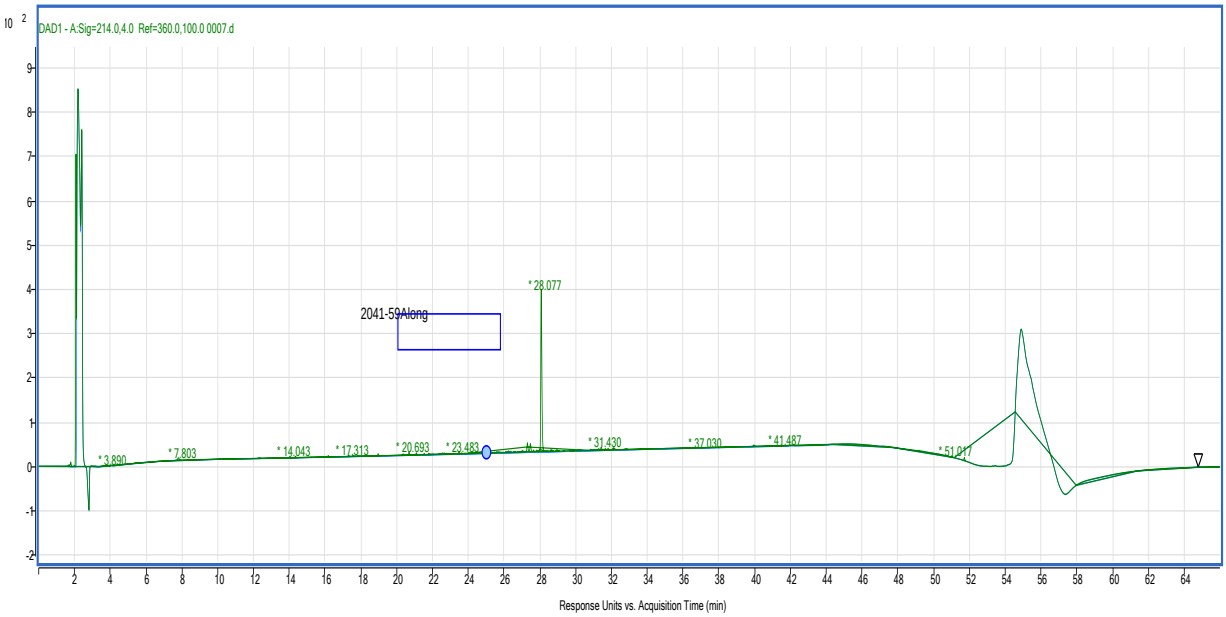



**Figure S31.** UV chromatogram overview.

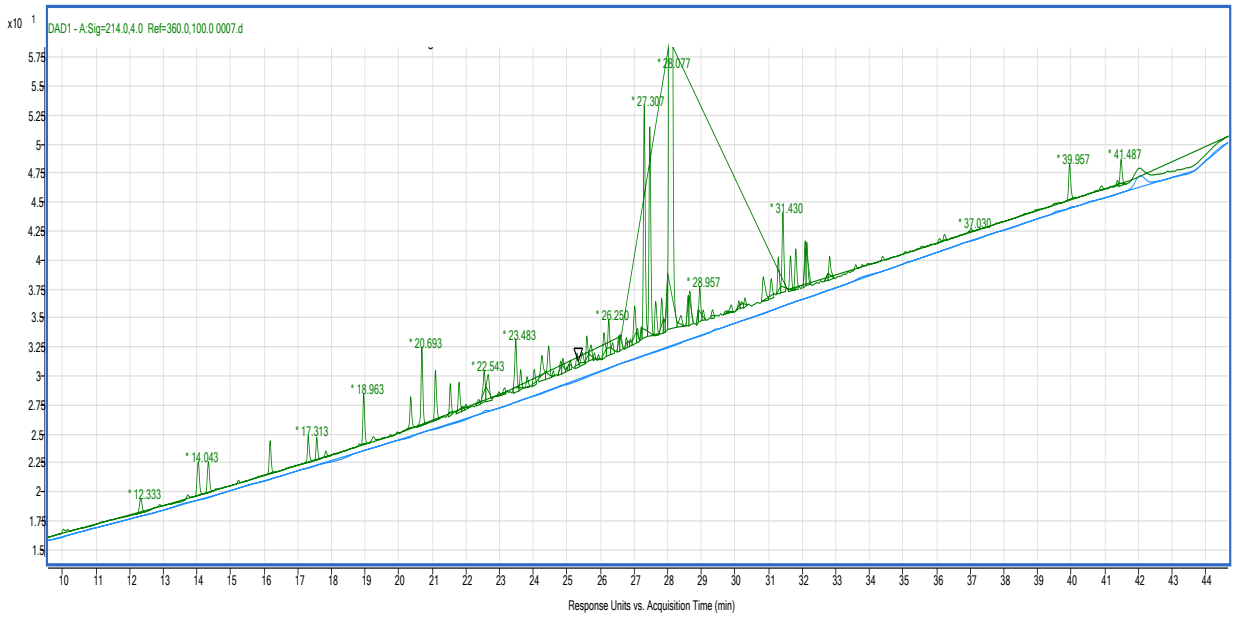



**Figure S32.** UV chromatogram zoom-in.

**Table S15.** Area% for integrated peaks and MS identities for H-1-21-HMBA-K and main byproducts.[1]

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3,89 | 4,390 | | | 0,0000 | -2625,2380 | 0,139 | 0,19 | |
| 2 | 5,47 | 6,700 | | | 0,0000 | -2625,2380 | 0,195 | 0,28 | |
| 3 | 7,80 | 34,610 | | | 0,0000 | -2625,2380 | 0,278 | 1,46 | |
| 4 | 12,33 | 6,630 | | | 0,0000 | -2625,2380 | 0,439 | 0,28 | |
| 5 | 14,04 | 14,020 | | | 0,0000 | -2625,2380 | 0,500 | 0,59 | |
| 6 | 14,35 | 12,090 | | | 0,0000 | -2625,2380 | 0,511 | 0,51 | |
| 7 | 16,18 | 10,850 | | | 0,0000 | -2625,2380 | 0,576 | 0,46 | |
| 8 | 17,313 | 8,49 | | | 0,0000 | -2625,2380 | 0,617 | 0,36 | |
| 9 | 17,567 | 6,84 | | | 0,0000 | -2625,2380 | 0,626 | 0,29 | |
| 10 | 17,84 | 2 | | | 0,0000 | -2625,2380 | 0,635 | 0,08 | |
| 11 | 18,963 | 15,75 | | | 0,0000 | -2625,2380 | 0,675 | 0,67 | |
| 12 | 20,36 | 10,14 | | | 0,0000 | -2625,2380 | 0,725 | 0,43 | |
| 13 | 20,693 | 25,48 | | | 0,0000 | -2625,2380 | 0,737 | 1,08 | |
| 14 | 21,097 | 16,42 | | | 0,0000 | -2625,2380 | 0,751 | 0,69 | |
| 15 | 21,537 | 9,72 | | | 0,0000 | -2625,2380 | 0,767 | 0,41 | |
| 16 | 21,797 | 10,69 | | | 0,0000 | -2625,2380 | 0,776 | 0,45 | |
| 17 | 21,92 | 1,61 | | | 0,0000 | -2625,2380 | 0,781 | 0,07 | |
| 18 | 22 | 1,65 | | | 0,0000 | -2625,2380 | 0,784 | 0,07 | |
| 19 | 22,383 | 2,18 | | | 0,0000 | -2625,2380 | 0,797 | 0,09 | |
| 20 | 22,543 | 12,61 | 575,293 | 2 | 1148,5714 | -1476,6666 | 0,803 | 0,53 | Ac-(15-23)-OH |
| 21 | 22,663 | 14,01 | 346,211 | 2 | 690,4074 | -1934,8306 | 0,807 | 0,59 | Ac-(19-23)-OH |
| 22 | 22,977 | 1,34 | | | 0,0000 | -2625,2380 | 0,818 | 0,06 | |
| 23 | 23,15 | 2,44 | | | 0,0000 | -2625,2380 | 0,825 | 0,1 | |
| 24 | 23,483 | 20,12 | 768,89 | 2 | 1535,7654 | -1089,4726 | 0,836 | 0,85 | (Ac-(12-23)-OH |
| 25 | 23,63 | 6,35 | | | 0,0000 | -2625,2380 | 0,842 | 0,27 | |
| 26 | 23,82 | 3,53 | | | 0,0000 | -2625,2380 | 0,848 | 0,15 | |
| 27 | 24,033 | 5,73 | | | 0,0000 | -2625,2380 | 0,856 | 0,24 | |
| 28 | 24,26 | 13,81 | 552,825 | 2 | 1103,6354 | -1521,6026 | 0,864 | 0,58 | DICU-(16-23)-OH |
| 29 | 24,463 | 14,42 | 918,771 | 3 | 2753,2912 | 128,0532 | 0,871 | 0,61 | Double Lys or Double Gln |
| 30 | 24,6 | 1,38 | | | 0,0000 | -2625,2380 | 0,876 | 0,06 | |
| 31 | 24,82 | 4,65 | | | 0,0000 | -2625,2380 | 0,884 | 0,2 | |
| 32 | 24,887 | 5,38 | | | 0,0000 | -2625,2380 | 0,886 | 0,23 | |
| 33 | 25,03 | 1,1 | | | 0,0000 | -2625,2380 | 0,891 | 0,05 | |
| 34 | 25,08 | 1,39 | | | 0,0000 | -2625,2380 | 0,893 | 0,06 | |
| 35 | 25,11 | 2,54 | | | 0,0000 | -2625,2380 | 0,894 | 0,11 | |
| 36 | 25,307 | 5,03 | | | 0,0000 | -2625,2380 | 0,901 | 0,21 | |
| 37 | 25,463 | 5,68 | | | 0,0000 | -2625,2380 | 0,907 | 0,24 | |
| 38 | 25,6 | 8,13 | | | 0,0000 | -2625,2380 | 0,912 | 0,34 | |
| 39 | 25,723 | 6,44 | | | 0,0000 | -2625,2380 | 0,916 | 0,27 | |
| 40 | 25,823 | 1,81 | | | 0,0000 | -2625,2380 | 0,920 | 0,08 | |
| 41 | 25,943 | 1,53 | | | 0,0000 | -2625,2380 | 0,924 | 0,06 | |
| 42 | 26,107 | 9,96 | | | 0,0000 | -2625,2380 | 0,930 | 0,42 | |
| 43 | 26,25 | 12,72 | 870,084 | 3 | 2607,2302 | -18,0078 | 0,935 | 0,54 | Asp to Aspartimide |
| 44 | 26,363 | 4,39 | | | 0,0000 | -2625,2380 | 0,939 | 0,19 | |
| 45 | 26,553 | 4,79 | | | 0,0000 | -2625,2380 | 0,946 | 0,2 | |
| 46 | 26,603 | 5,31 | | | 0,0000 | -2625,2380 | 0,948 | 0,22 | |
| 47 | 26,77 | 2,85 | | | 0,0000 | -2625,2380 | 0,953 | 0,12 | |
| 48 | 26,803 | 1,61 | | | 0,0000 | -2625,2380 | 0,955 | 0,07 | |
| 49 | 26,893 | 2,2 | | | 0,0000 | -2625,2380 | 0,958 | 0,09 | |
| 50 | 27,023 | 14,91 | | | 0,0000 | -2625,2380 | 0,962 | 0,63 | |
| 51 | 27,103 | 3,76 | | | 0,0000 | -2625,2380 | 0,965 | 0,16 | |
| 52 | 27,217 | 3,17 | | | 0,0000 | -2625,2380 | 0,969 | 0,13 | |
| 53 | 27,307 | 76,77 | 788,709 | 3 | 2363,1052 | -262,1328 | 0,973 | 3,25 | (H-(1-21)-OH (des HMBA-K) |
| 54 | 27,47 | 68,38 | 842,405 | 3 | 2524,1932 | -101,0448 | 0,978 | 2,89 | Des Thr |
| 55 | 27,647 | 12,49 | 388,263 | 2 | 774,5114 | -1850,7266 | 0,985 | 0,53 | DICU-(19-23)-OH |
| 56 | 27,823 | 11,61 | | | 0,0000 | -2625,2380 | 0,991 | 0,49 | |
| 57 | 27,893 | 3,63 | | | 0,0000 | -2625,2380 | 0,993 | 0,15 | |
| 58 | 28,003 | 11,32 | | | 0,0000 | -2625,2380 | 0,997 | 0,48 | |
| 59 | 28,077 | 1530,03 | 876,087 | 3 | 2625,2392 | 0,0012 | 1,000 | 64,73 | Product |
| 60 | 28,397 | 6,55 | | | 0,0000 | -2625,2380 | 1,011 | 0,28 | |
| 61 | 28,613 | 8,99 | | | 0,0000 | -2625,2380 | 1,019 | 0,38 | |
| 62 | 28,663 | 12,38 | 833,073 | 3 | 2496,1972 | -129,0408 | 1,021 | 0,52 | Des Glu |
| 63 | 28,913 | 2,37 | | | 0,0000 | -2625,2380 | 1,030 | 0,1 | |
| 64 | 28,957 | 11,13 | | | 0,0000 | -2625,2380 | 1,031 | 0,47 | |
| 65 | 29,06 | 3,33 | | | 0,0000 | -2625,2380 | 1,035 | 0,14 | |
| 66 | 29,337 | 3,06 | | | 0,0000 | -2625,2380 | 1,045 | 0,13 | |
| 67 | 29,763 | 1,61 | | | 0,0000 | -2625,2380 | 1,060 | 0,07 | |
| 68 | 29,89 | 3,14 | | | 0,0000 | -2625,2380 | 1,065 | 0,13 | |
| 69 | 30,12 | 2,32 | | | 0,0000 | -2625,2380 | 1,073 | 0,1 | |
| 70 | 30,203 | 2,56 | | | 0,0000 | -2625,2380 | 1,076 | 0,11 | |
| 71 | 30,303 | 2,83 | | | 0,0000 | -2625,2380 | 1,079 | 0,12 | |
| 72 | 30,847 | 12,71 | 847,07 | 3 | 2538,1882 | -87,0498 | 1,099 | 0,54 | Des Ser |
| 73 | 31,083 | 7,73 | | | 0,0000 | -2625,2380 | 1,107 | 0,33 | |
| 74 | 31,293 | 11,78 | 853,433 | 3 | 2557,2772 | -67,9608 | 1,115 | 0,5 | DICU-(3-23)-OH |
| 75 | 31,43 | 26,59 | 729,692 | 3 | 2186,0542 | -439,1838 | 1,119 | 1,12 | Ac-(6-23)-OH |
| 76 | 31,653 | 11,62 | | | 0,0000 | -2625,2380 | 1,127 | 0,49 | |
| 77 | 31,813 | 13,38 | 782,383 | 3 | 2344,1272 | -281,1108 | 1,133 | 0,57 | Ac-(4-23)-OH |
| 78 | 32,09 | 12,82 | 913,781 | 3 | 2738,3212 | 113,0832 | 1,143 | 0,54 | Double Leu |
| 79 | 32,143 | 13,21 | 920,763 | 3 | 2759,2672 | 134,0292 | 1,145 | 0,56 | Double HMBA |
| 80 | 32,773 | 2,05 | | | 0,0000 | -2625,2380 | 1,167 | 0,09 | |
| 81 | 32,82 | 9,17 | | | 0,0000 | -2625,2380 | 1,169 | 0,39 | |
| 82 | 36,087 | 1,42 | | | 0,0000 | -2625,2380 | 1,285 | 0,06 | |
| 83 | 36,233 | 2,16 | | | 0,0000 | -2625,2380 | 1,290 | 0,09 | |
| 84 | 37,03 | 1,08 | | | 0,0000 | -2625,2380 | 1,319 | 0,05 | |
| 85 | 39,957 | 13,2 | | | 0,0000 | -2625,2380 | 1,423 | 0,56 | |
| 86 | 40,907 | 1,88 | | | 0,0000 | -2625,2380 | 1,457 | 0,08 | |
| 87 | 41,377 | 1,66 | | | 0,0000 | -2625,2380 | 1,474 | 0,07 | |
| 88 | 41,487 | 10 | | | 0,0000 | -2625,2380 | 1,478 | 0,42 | |
| 89 | 51,017 | 9,5 | | | 0,0000 | -2625,2380 | 1,817 | 0,4 | |
| 90 | 51,72 | 22,11 | | | 0,0000 | -2625,2380 | 1,842 | 0,94 | |

[1] DICU-(X-23)-OH stands for an impurity in which DIC reacted with amino terminus of the growing peptide chain during coupling forming a diisopropylguanidinium truncation byproduct.

**24. LC-HRMS of crude H-1-21-HMBA-K from SPPS in Scheme 6, route B.**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v380 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 5; 1, 5; 41, 42; 50, 95; 52, 95; 52.1, 5; 66, 5.

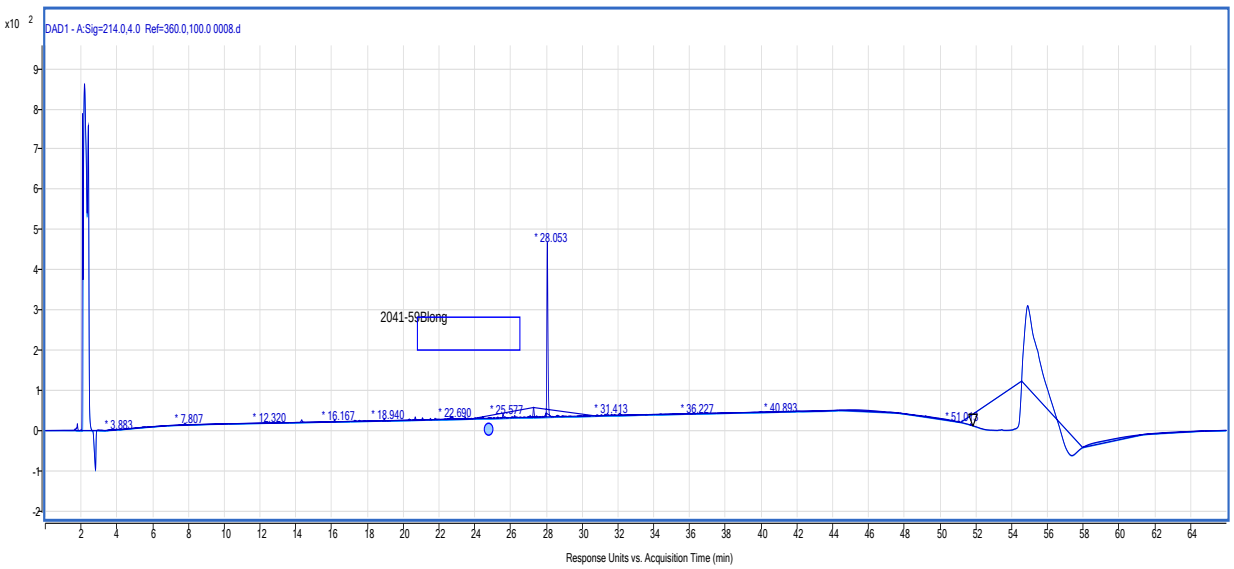



**Figure S33.** UV chromatogram overview.

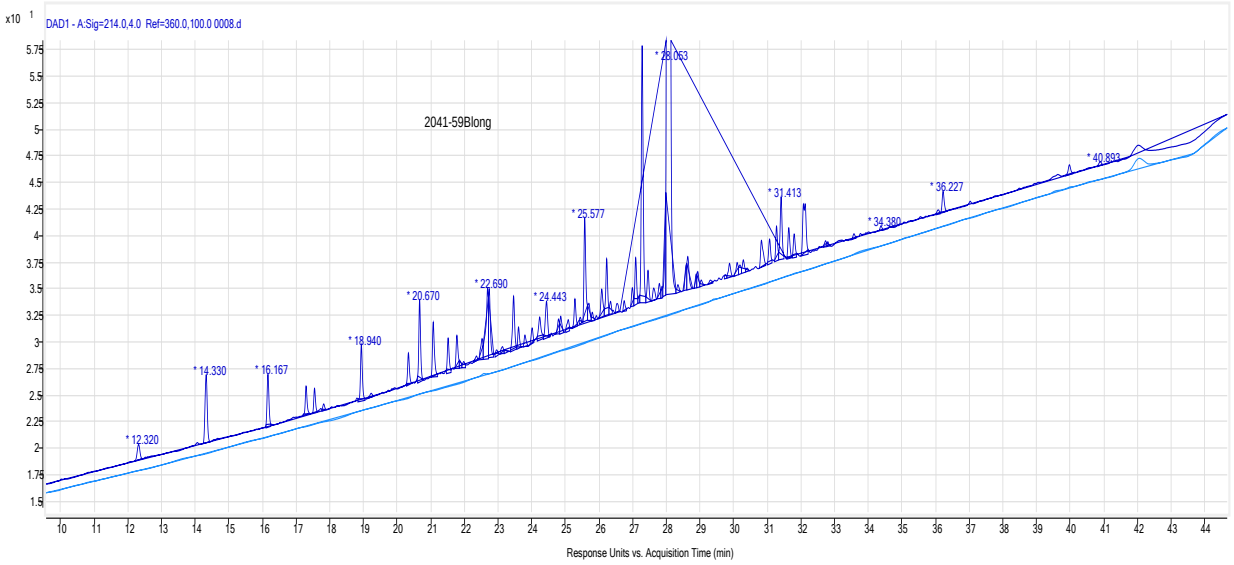


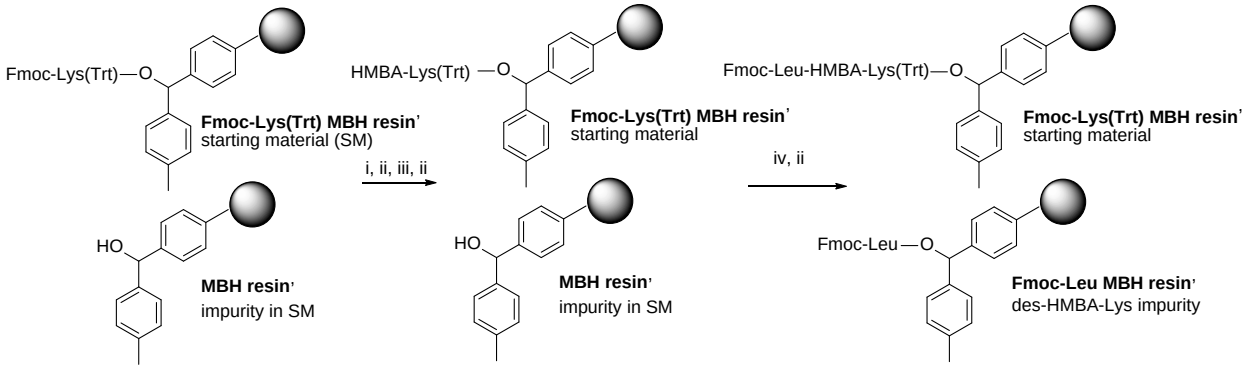

**Figure S34.** UV chromatogram zoom-in.

**Table S16.** Area% for integrated peaks and MS identities for H-1-21-HMBA-K and main byproducts.[2]

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % |
|---|---|---|---|---|---|---|---|---|
| 1 | 3,65 | 9,590 | | | 0,0000 | -2533,2120 | 0,130 | 0,35 |
| 2 | 3,88 | 8,410 | | | 0,0000 | -2533,2120 | 0,138 | 0,30 |
| 3 | 5,48 | 5,850 | | | 0,0000 | -2533,2120 | 0,195 | 0,21 |
| 4 | 6,62 | 2,150 | | | 0,0000 | -2533,2120 | 0,236 | 0,08 |
| 5 | 7,57 | 3,160 | | | 0,0000 | -2533,2120 | 0,270 | 0,11 |
| 6 | 7,81 | 29,930 | | | 0,0000 | -2533,2120 | 0,278 | 1,08 |
| 7 | 12,32 | 8,090 | | | 0,0000 | -2533,2120 | 0,439 | 0,29 |
| 8 | 14,33 | 29,37 | | | 0,0000 | -2533,2120 | 0,511 | 1,06 |
| 9 | 16,167 | 19,69 | | | 0,0000 | -2533,2120 | 0,576 | 0,71 |
| 10 | 17,297 | 10,15 | | | 0,0000 | -2533,2120 | 0,617 | 0,37 |
| 11 | 17,55 | 8,04 | | | 0,0000 | -2533,2120 | 0,626 | 0,29 |
| 12 | 17,823 | 2,66 | | | 0,0000 | -2533,2120 | 0,635 | 0,1 |
| 13 | 18,94 | 21,38 | | | 0,0000 | -2533,2120 | 0,675 | 0,77 |
| 14 | 19,233 | 2,01 | | | 0,0000 | -2533,2120 | 0,686 | 0,07 |
| 15 | 20,34 | 11,34 | | | 0,0000 | -2533,2120 | 0,725 | 0,41 |
| 16 | 20,67 | 28,64 | | | 0,0000 | -2533,2120 | 0,737 | 1,03 |
| 17 | 21,077 | 21,38 | | | 0,0000 | -2533,2120 | 0,751 | 0,77 |
| 18 | 21,52 | 12,67 | | | 0,0000 | -2533,2120 | 0,767 | 0,46 |
| 19 | 21,78 | 12,7 | | | 0,0000 | -2533,2120 | 0,776 | 0,46 |
| 20 | 21,85 | 2,6 | | | 0,0000 | -2533,2120 | 0,779 | 0,09 |
| 21 | 21,983 | 2,89 | | | 0,0000 | -2533,2120 | 0,784 | 0,1 |
| 22 | 22,36 | 2,05 | | | 0,0000 | -2533,2120 | 0,797 | 0,07 |
| 23 | 22,527 | 9,54 | | | 0,0000 | -2533,2120 | 0,803 | 0,34 |
| 24 | 22,69 | 35,38 | | | 0,0000 | -2533,2120 | 0,809 | 1,27 |
| 25 | 22,74 | 22,33 | | | 0,0000 | -2533,2120 | 0,811 | 0,8 |
| 26 | 22,957 | 2,58 | | | 0,0000 | -2533,2120 | 0,818 | 0,09 |
| 27 | 23,133 | 3,35 | | | 0,0000 | -2533,2120 | 0,825 | 0,12 |
| 28 | 23,213 | 1,61 | | | 0,0000 | -2533,2120 | 0,827 | 0,06 |
| 29 | 23,463 | 22,38 | | | 0,0000 | -2533,2120 | 0,836 | 0,81 |
| 30 | 23,61 | 7,23 | | | 0,0000 | -2533,2120 | 0,842 | 0,26 |
| 31 | 23,8 | 4,41 | | | 0,0000 | -2533,2120 | 0,848 | 0,16 |
| 32 | 24,013 | 5,74 | | | 0,0000 | -2533,2120 | 0,856 | 0,21 |
| 33 | 24,24 | 11,86 | | | 0,0000 | -2533,2120 | 0,864 | 0,43 |
| 34 | 24,443 | 16,75 | | | 0,0000 | -2533,2120 | 0,871 | 0,6 |
| 35 | 24,8 | 5,52 | | | 0,0000 | -2533,2120 | 0,884 | 0,2 |
| 36 | 24,867 | 6,59 | | | 0,0000 | -2533,2120 | 0,886 | 0,24 |
| 37 | 25,09 | 5,29 | | | 0,0000 | -2533,2120 | 0,894 | 0,19 |
| 38 | 25,287 | 9,96 | | | 0,0000 | -2533,2120 | 0,901 | 0,36 |
| 39 | 25,443 | 2,01 | | | 0,0000 | -2533,2120 | 0,907 | 0,07 |
| 40 | 25,577 | 37,39 | | | 0,0000 | -2533,2120 | 0,912 | 1,35 |
| 41 | 25,703 | 7,04 | | | 0,0000 | -2533,2120 | 0,916 | 0,25 |
| 42 | 25,803 | 3,02 | | | 0,0000 | -2533,2120 | 0,920 | 0,11 |
| 43 | 26,083 | 12,12 | | | 0,0000 | -2533,2120 | 0,930 | 0,44 |
| 44 | 26,23 | 20,74 | | | 0,0000 | -2533,2120 | 0,935 | 0,75 |
| 45 | 26,343 | 4,89 | | | 0,0000 | -2533,2120 | 0,939 | 0,18 |
| 46 | 26,54 | 3,7 | | | 0,0000 | -2533,2120 | 0,946 | 0,13 |
| 47 | 26,75 | 3,6 | | | 0,0000 | -2533,2120 | 0,954 | 0,13 |
| 48 | 26,993 | 6,35 | | | 0,0000 | -2533,2120 | 0,962 | 0,23 |
| 49 | 27,093 | 16,09 | | | 0,0000 | -2533,2120 | 0,966 | 0,58 |
| 50 | 27,213 | 2,05 | | | 0,0000 | -2533,2120 | 0,970 | 0,07 |
| 51 | 27,283 | 95,08 | | | 0,0000 | -2533,2120 | 0,973 | 3,42 |
| 52 | 27,46 | 11,97 | | | 0,0000 | -2533,2120 | 0,979 | 0,43 |
| 53 | 27,627 | 5,38 | | | 0,0000 | -2533,2120 | 0,985 | 0,19 |
| 54 | 27,797 | 5,68 | | | 0,0000 | -2533,2120 | 0,991 | 0,2 |
| 55 | 27,873 | 3,27 | | | 0,0000 | -2533,2120 | 0,994 | 0,12 |
| 56 | 27,987 | 19,16 | | | 0,0000 | -2533,2120 | 0,998 | 0,69 |
| **57** | **28,053** | **1869,63** | | | **0,0000** | **-2533,2120** | **1,000** | **67,34** |
| 58 | 28,347 | 3,6 | | | 0,0000 | -2533,2120 | 1,010 | 0,13 |
| 59 | 28,61 | 8,33 | | | 0,0000 | -2533,2120 | 1,020 | 0,3 |
| 60 | 28,64 | 13,46 | | | 0,0000 | -2533,2120 | 1,021 | 0,48 |
| 61 | 28,89 | 3,88 | | | 0,0000 | -2533,2120 | 1,030 | 0,14 |
| 62 | 28,937 | 5,47 | | | 0,0000 | -2533,2120 | 1,032 | 0,2 |
| 63 | 29,04 | 2,62 | | | 0,0000 | -2533,2120 | 1,035 | 0,09 |
| 64 | 29,317 | 1,95 | | | 0,0000 | -2533,2120 | 1,045 | 0,07 |
| 65 | 29,757 | 1,4 | | | 0,0000 | -2533,2120 | 1,061 | 0,05 |
| 66 | 29,88 | 6,41 | | | 0,0000 | -2533,2120 | 1,065 | 0,23 |
| 67 | 30,107 | 5,48 | | | 0,0000 | -2533,2120 | 1,073 | 0,2 |
| 68 | 30,19 | 3,8 | | | 0,0000 | -2533,2120 | 1,076 | 0,14 |
| 69 | 30,29 | 5,52 | | | 0,0000 | -2533,2120 | 1,080 | 0,2 |
| 70 | 30,403 | 1,41 | | | 0,0000 | -2533,2120 | 1,084 | 0,05 |
| 71 | 30,83 | 13,7 | | | 0,0000 | -2533,2120 | 1,099 | 0,49 |
| 72 | 31,07 | 9,77 | | | 0,0000 | -2533,2120 | 1,108 | 0,35 |
| 73 | 31,277 | 12,34 | | | 0,0000 | -2533,2120 | 1,115 | 0,44 |
| 74 | 31,413 | 24,33 | | | 0,0000 | -2533,2120 | 1,120 | 0,88 |
| 75 | 31,64 | 11,47 | | | 0,0000 | -2533,2120 | 1,128 | 0,41 |
| 76 | 31,8 | 8,87 | | | 0,0000 | -2533,2120 | 1,134 | 0,32 |
| 77 | 32,127 | 33,75 | | | 0,0000 | -2533,2120 | 1,145 | 1,22 |
| 78 | 32,733 | 2,53 | | | 0,0000 | -2533,2120 | 1,167 | 0,09 |
| 79 | 32,8 | 1,4 | | | 0,0000 | -2533,2120 | 1,169 | 0,05 |
| 80 | 33,58 | 1,93 | | | 0,0000 | -2533,2120 | 1,197 | 0,07 |
| 81 | 34,38 | 1,63 | | | 0,0000 | -2533,2120 | 1,226 | 0,06 |
| 82 | 36,083 | 2,14 | | | 0,0000 | -2533,2120 | 1,286 | 0,08 |
| 83 | 36,227 | 8,66 | | | 0,0000 | -2533,2120 | 1,291 | 0,31 |
| 84 | 39,98 | 5,13 | | | 0,0000 | -2533,2120 | 1,425 | 0,18 |
| 85 | 40,893 | 2,13 | | | 0,0000 | -2533,2120 | 1,458 | 0,08 |
| 86 | 51,013 | 7,2 | | | 0,0000 | -2533,2120 | 1,818 | 0,26 |
| 87 | 51,72 | 11,54 | | | 0,0000 | -2533,2120 | 1,844 | 0,42 |

[2] Impurity profile and peak identities were comparable to the Scheme 5, route A crude (see Section 23 in this ESI) with the exception of the des Thr impurity which was much smaller in the Scheme 5, route B, see overlay of the two crudes in Fig. 8).

**25. Mechanism of formation of the des HMBA-Lys impurity during SPPS of Boc-1-21-HMBA-Lys(Trt) MBH resin (Schemes S4 – S6).**




**Scheme S7.** A schematic representation of the proposed mechanism of formation of des HMBA-Lys impurity during SPPS of Boc-1-21-HMBA-Lys(Trt) MBH resin caused by the presence of MBH resin in the starting Fmoc-Lys(Trt) MBH resin. i) Fmoc deprotection: 10% pip in DMF, 30°C, 30 min; ii) DMF resin wash; iii) coupling: 2 equiv HMBA/Oxyma/DIC (1:2:2) in DMF, 40 °C, 2 h; iv) coupling: 4 equiv Fmoc-Leu-OH/Cl-HOBt/DIC/DMAP (1:1:1:0.2) in DMF, rt, 17 h.

**26. Automated SPPS of Boc-1-21-HMBA-Lys(Trt) MBH resin. Synthesis of H-1-21-HMBA-K with lower content of the des HMBA-K impurity.**

Using a batch of Fmoc-Lys(Trt)-MBH resin not employed in the large scale SPPS of Boc-1-21-HMBA-Lys(Trt) MBH resin detailed in Schemes S4 – S6 a batch of Fmoc-Leu-HMBA-Lys(Trt) MBH resin was prepared according to the protocol in Scheme S4. Next, 0.1 mmol of the Fmoc-Leu-HMBA-Lys(Trt) MBH resin thus prepared was used to synthesize Boc-1-21-HMBA-Lys(Trt) MBH resin on the PSW1100 automated synthesizer according to the protocols delineated in Schemes 4 and 5 (route A). The temperature for the synthesis was 50 °C throughout and all Fmoc deprotections were done for 3 x 15 min whereas all couplings were done for 2 x 20 min. A sample of the final resin was TFA cleaved and isolated by precipitation as described in the Materials & Methods section and the resulting crude H-1-21-HMBA-K was analyzed by LC-HRMS (see below). This analysis revealed that the content of the des HMBA-K impurity was 2.5% vs the product (1.8% vs 71.6%) while the content of des-HMBA-K in the crude material from the large scale H-1-21-HMBA-K SPPS (Schemes S4 – S6) was 5.0% (3.3% vs 64.8%), see section 23 of this ESI. The difference in the content of des HMBA-K in H-1-21-HMBA-K from two syntheses of Boc-1-21-HMBA-Lys(Trt) MBH resin employing different Fmoc-Lys(Trt) MBH resin starting

materials indicates that the des HMBA-K was conceivably formed according to the mechanism depicted in Scheme S7.

### 27. LC-HRMS of crude H-1-21-HMBA-K from section 20 automated SPPS.

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v380 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 5; 1, 5; 41, 42; 50, 95; 52, 95; 52.1, 5; 66, 5.

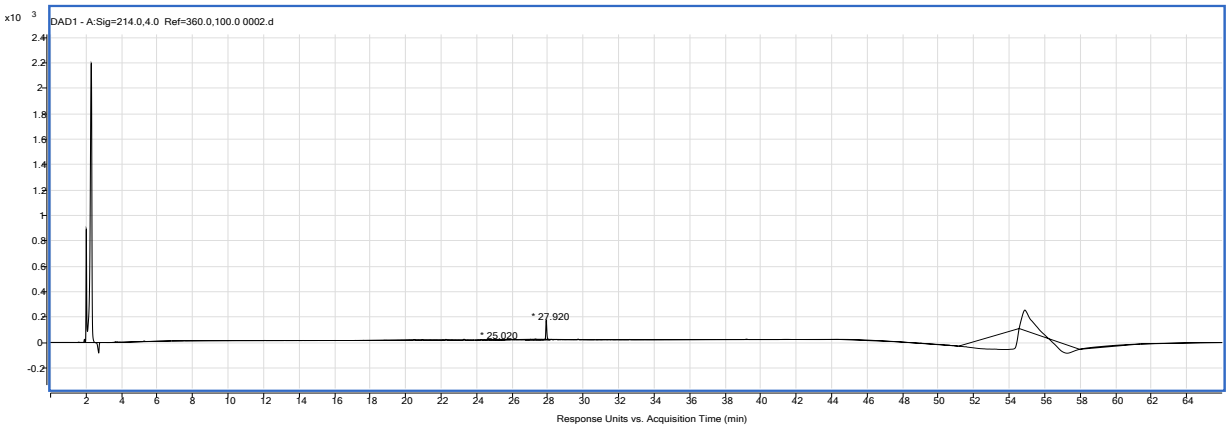



**Figure S35.** UV chromatogram overview.

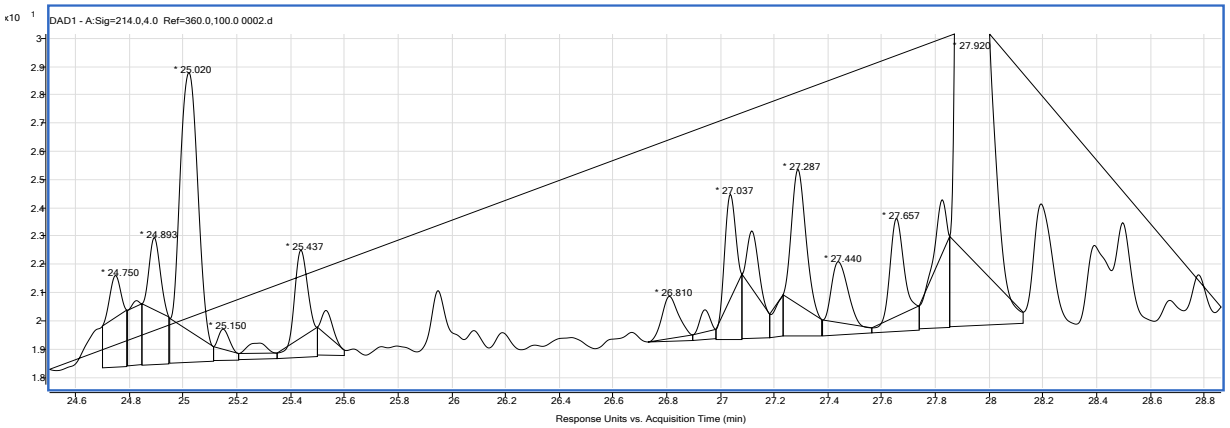



**Figure S36.** UV chromatogram zoom-in.

**Table S17.** Area% for integrated peaks and MS identities for H-1-21-HMBA-K and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 24,75 | 13,470 | | | 0,0000 | -2533,2120 | 0,886 | 1,58 | |
| 2 | 24,83 | 6,940 | | | 0,0000 | -2533,2120 | 0,889 | 0,82 | |
| 3 | 24,89 | 18,710 | 488,3060 | 2 | 974,5974 | -1558,6146 | 0,892 | 2,20 | DICU-(17-23)-OH |
| 4 | 25,02 | 50,840 | 640,8150 | 2 | 1279,6154 | -1253,5966 | 0,896 | 5,97 | Ac-(14-23)-OH |
| 5 | 25,15 | 3,900 | | | 0,0000 | -2533,2120 | 0,901 | 0,46 | |
| 6 | 25,28 | 3,500 | | | 0,0000 | -2533,2120 | 0,906 | 0,41 | |
| 7 | 25,44 | 15,200 | 876,4230 | 3 | 2626,2472 | 93,0352 | 0,911 | 1,79 | Deamidation |
| 8 | 25,53 | 5,740 | | | 0,0000 | -2533,2120 | 0,914 | 0,68 | |
| 9 | 26,81 | 7,350 | | | 0,0000 | -2533,2120 | 0,960 | 0,86 | |
| 10 | 26,94 | 3,360 | | | 0,0000 | -2533,2120 | 0,965 | 0,40 | |
| 11 | 27,04 | 17,800 | | | 0,0000 | -2533,2120 | 0,968 | 2,09 | |
| 12 | 27,12 | 15,190 | 788,7110 | 3 | 2363,1112 | -170,1008 | 0,971 | 1,78 | (H-(1-21)-OH (des HMBA-Lys) |
| 13 | 27,23 | 3,320 | 926,4590 | 2 | 1850,9034 | -682,3086 | 0,975 | 0,39 | Ac-(9-23)-OH |
| 14 | 27,29 | 26,200 | | | 0,0000 | -2533,2120 | 0,977 | 3,08 | |
| 15 | 27,44 | 12,920 | | | 0,0000 | -2533,2120 | 0,983 | 1,52 | |
| 16 | 27,66 | 17,120 | | | 0,0000 | -2533,2120 | 0,991 | 2,01 | |
| 17 | 27,83 | 18,820 | | | 0,0000 | -2533,2120 | 0,997 | 2,21 | |
| 18 | 27,92 | 610,500 | 876,4230 | 3 | 2626,2472 | 93,0352 | 1,000 | 71,75 | Product |

## 28. Kinetics of the omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-HMBA-K (Fig. 9).

Throughout the ligation 50 µL aliquots of the reaction mixture were taken out, quenched with 10% AcOH (1.0 mL) and analyzed by HPLC. When all H-1-21-HMBA-K was consumed and no further conversion to the product could be observed the reaction was quenched with 10% AcOH. The S/H ratio was calculated by integrating the peak of H-1-39-$NH_2$ together with byproducts peaks formed by reactions between H-1-21-HMBA-K and byproducts in the crude H-2-39-$NH_2$ (S) vs the peak of H-1-21-OH (hydrolyzed H-1-21-HMBA-K, H). The fact that the H-1-21-HMBA-K starting material contained ~5% of H-1-21-OH (des-HMBA-K, H) was taken into account when calculating the S/H ratio of the final crude product.

Experimental conditions: column: Waters XSelect CSH130 C18 2.5µm 4.6x150mm; column temperature: 45°C; injection volume: 5 µL; sampler temperature:10°C; flow: 1.0 ml/min; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.08 % TFA in 90% MeCN/10 %water. Gradient (Time(min), %B): 0, 1; 10, 100; 13, 100; 14, 100; 19, 1; 20, 1.

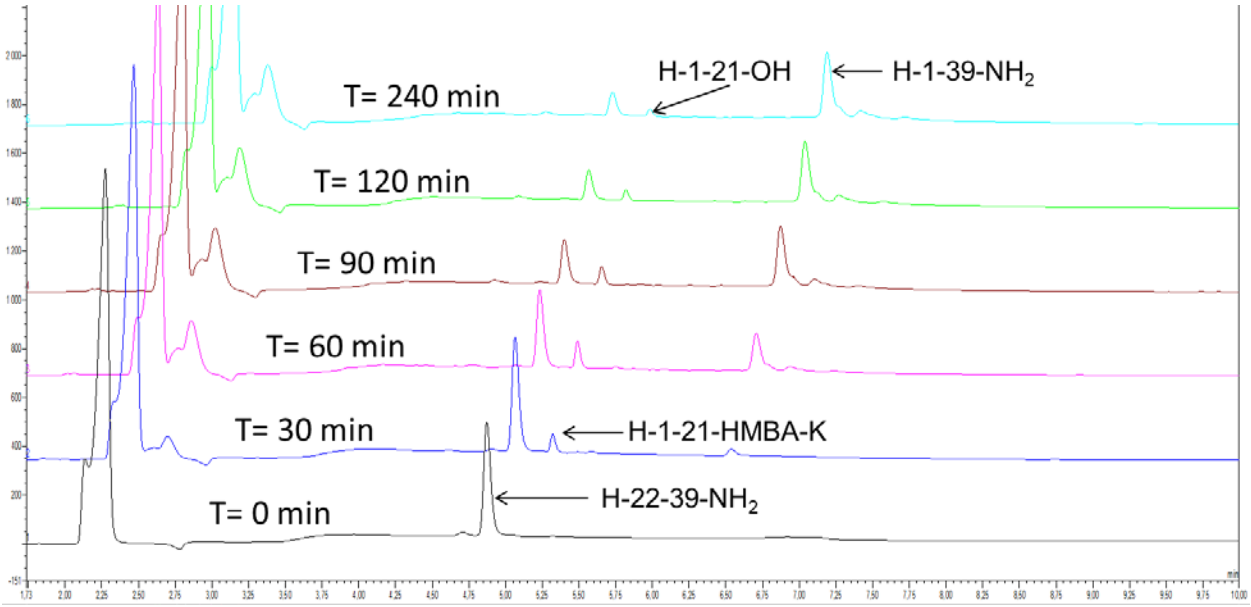



**Figure S37.** A schematic representation of the progress of Fig. 9 crude H-22-39-$NH_2$ + crude H-1-21-HMBA-K ligation.

**29. LC-HRMS of crude exenatide from omniligase-1 catalyzed ligation of crude H-22-39-$NH_2$ + crude H-1-21-HMBA-K (Fig. 9).**

Experimental conditions: column: Waters CSH 1.7 µm 2.1 x 150 mm; column temperature: 55°C; injection volume: 1 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 214 nm; data rate: 5Hz; detector cell: standard cell 1µL; flow: 0.2 ml/min; jet weaver: v380 mixer; mobile phase A: 0.1 % TFA in water, mobile phase B: 0.10 % TFA in MeCN. Gradient (Time(min), %B): 0, 11; 1, 11; 5, 33; 50, 39; 54, 60; 56, 90; 58, 90; 58.1, 11; 68, 11.

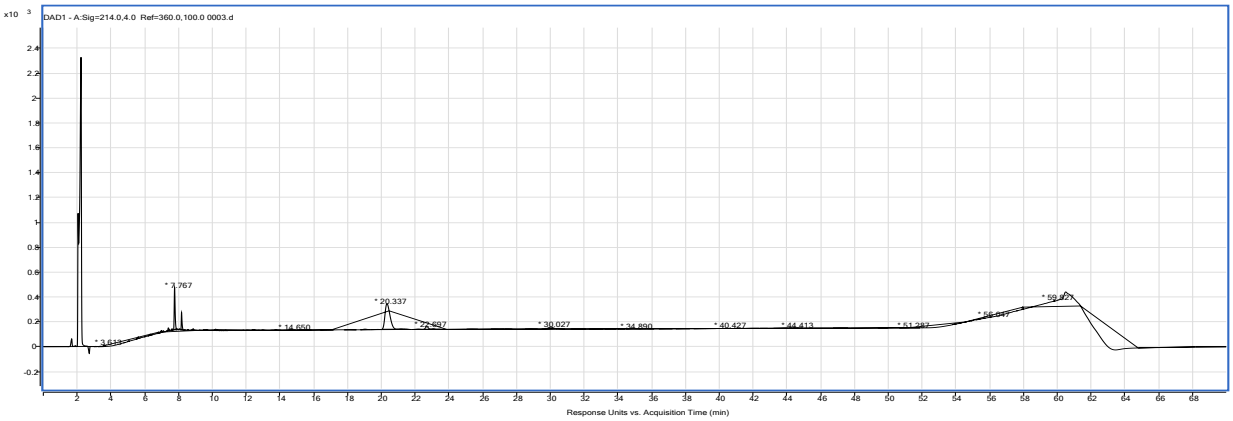



**Figure S38.** UV chromatogram overview.

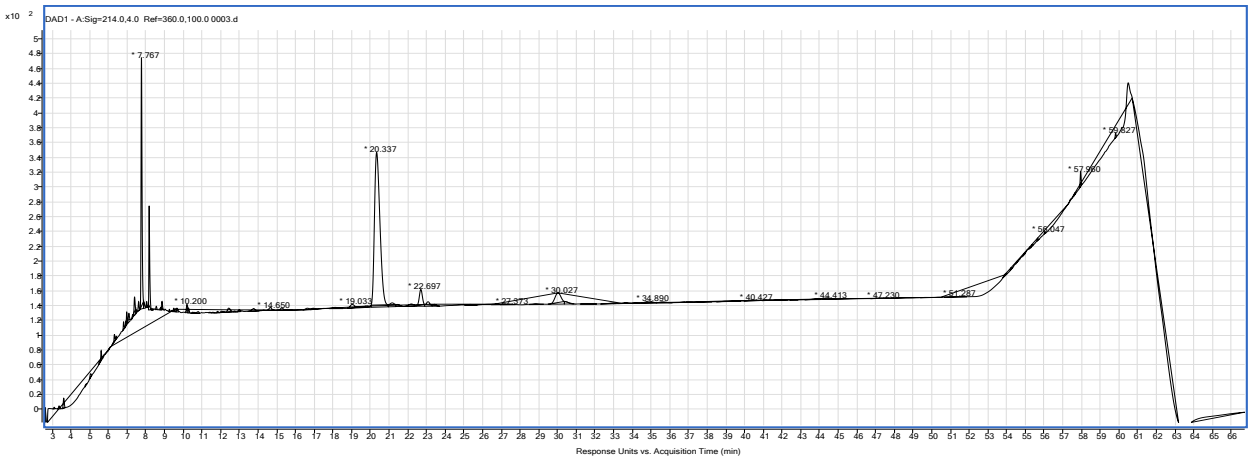



**Figure S39.** UV chromatogram zoom-in.

**Table S18.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3,10 | 7,210 | | | 0,0000 | -4184,0273 | 0,153 | 0,08 | |
| 2 | 3,36 | 18,320 | | | 0,0000 | -4184,0273 | 0,165 | 0,20 | |
| 3 | 3,613 | 52,24 | | | 0,0000 | -4184,0273 | 0,178 | 0,58 | |
| 4 | 4,86 | 6,490 | | | 0,0000 | -4184,0273 | 0,239 | 0,07 | |
| 5 | 5,05 | 20,530 | | | 0,0000 | -4184,0273 | 0,248 | 0,23 | |
| 6 | 5,53 | 12,360 | | | 0,0000 | -4184,0273 | 0,272 | 0,14 | |
| 7 | 5,61 | 36,020 | | | 0,0000 | -4184,0273 | 0,276 | 0,40 | |
| 8 | 6,057 | 5,25 | | | 0,0000 | -4184,0273 | 0,298 | 0,06 | |
| 9 | 6,277 | 5,44 | | | 0,0000 | -4184,0273 | 0,309 | 0,06 | |
| 10 | 6,32 | 29,47 | | | 0,0000 | -4184,0273 | 0,311 | 0,33 | |
| 11 | 6,42 | 8,45 | | | 0,0000 | -4184,0273 | 0,316 | 0,09 | |
| 12 | 6,813 | 29,86 | | | 0,0000 | -4184,0273 | 0,335 | 0,33 | |
| 13 | 6,933 | 14,59 | | | 0,0000 | -4184,0273 | 0,341 | 0,16 | |
| 14 | 6,98 | 49,14 | | | 0,0000 | -4184,0273 | 0,343 | 0,55 | |
| 15 | 7,097 | 52,45 | | | 0,0000 | -4184,0273 | 0,349 | 0,58 | |
| 16 | 7,343 | 17,61 | | | 0,0000 | -4184,0273 | 0,361 | 0,2 | |
| 17 | 7,397 | 97,78 | 811,403 | 2 | 1620,7914 | -2563,2359 | 0,364 | 1,09 | Ac-(24-39)-NH2 |
| 18 | 7,463 | 11 | | | 0,0000 | -4184,0273 | 0,367 | 0,12 | |
| 19 | 7,617 | 40,05 | | | 0,0000 | -4184,0273 | 0,375 | 0,44 | |
| 20 | 7,767 | 1268,58 | 920,506 | 2 | 1838,9974 | -2345,0299 | 0,382 | 14,08 | H-(22-39)-NH2 |
| 21 | 7,877 | 32,8 | | | 0,0000 | -4184,0273 | 0,387 | 0,36 | |
| 22 | 8,043 | 20,88 | | | 0,0000 | -4184,0273 | 0,395 | 0,23 | |
| 23 | 8,117 | 5,69 | | | 0,0000 | -4184,0273 | 0,399 | 0,06 | |
| 24 | 8,18 | 428,87 | 788,713 | 3 | 2363,1172 | -1820,9101 | 0,402 | 4,76 | H-(1-21)-OH |
| 25 | 8,34 | 8,23 | | | 0,0000 | -4184,0273 | 0,410 | 0,09 | |
| 26 | 8,56 | 14,72 | | | 0,0000 | -4184,0273 | 0,421 | 0,16 | |
| 27 | 8,797 | 11,93 | | | 0,0000 | -4184,0273 | 0,433 | 0,13 | |
| 28 | 8,867 | 31,74 | | | 0,0000 | -4184,0273 | 0,436 | 0,35 | |
| 29 | 9,26 | 7,21 | | | 0,0000 | -4184,0273 | 0,455 | 0,08 | |
| 30 | 9,493 | 16,09 | | | 0,0000 | -4184,0273 | 0,467 | 0,18 | |
| 31 | 9,573 | 15,23 | | | 0,0000 | -4184,0273 | 0,471 | 0,17 | |
| 32 | 9,66 | 28,34 | | | 0,0000 | -4184,0273 | 0,475 | 0,31 | |
| 33 | 10,2 | 48,73 | | | 0,0000 | -4184,0273 | 0,502 | 0,54 | |
| 34 | 10,283 | 25,99 | | | 0,0000 | -4184,0273 | 0,506 | 0,29 | |
| 35 | 10,51 | 6,69 | | | 0,0000 | -4184,0273 | 0,517 | 0,07 | |
| 36 | 10,6 | 7,28 | | | 0,0000 | -4184,0273 | 0,521 | 0,08 | |
| 37 | 10,797 | 18,24 | | | 0,0000 | -4184,0273 | 0,531 | 0,2 | |
| 38 | 11,43 | 5,09 | | | 0,0000 | -4184,0273 | 0,562 | 0,06 | |
| 39 | 11,577 | 6,43 | | | 0,0000 | -4184,0273 | 0,569 | 0,07 | |
| 40 | 11,837 | 11,7 | | | 0,0000 | -4184,0273 | 0,582 | 0,13 | |
| 41 | 12,053 | 13,28 | | | 0,0000 | -4184,0273 | 0,593 | 0,15 | |
| 42 | 12,137 | 5,03 | | | 0,0000 | -4184,0273 | 0,597 | 0,06 | |
| 43 | 12,433 | 54,38 | 1433,382 | 3 | 4297,1242 | 113,0969 | 0,611 | 0,6 | Double Ile/Leu |
| 44 | 12,743 | 5,42 | | | 0,0000 | -4184,0273 | 0,627 | 0,06 | |
| 45 | 12,987 | 17,8 | | | 0,0000 | -4184,0273 | 0,639 | 0,2 | |
| 46 | 13,76 | 47,37 | 845,466 | 3 | 2533,3762 | -1650,6511 | 0,677 | 0,53 | DICU-(17-39)-NH2 |
| 47 | 14,65 | 42,91 | | | 0,0000 | -4184,0273 | 0,720 | 0,48 | |
| 48 | 15,27 | 36,12 | | | 0,0000 | -4184,0273 | 0,751 | 0,4 | |
| 49 | 15,593 | 11,38 | | | 0,0000 | -4184,0273 | 0,767 | 0,13 | |
| 50 | 16,63 | 34,24 | | | 0,0000 | -4184,0273 | 0,818 | 0,38 | |
| 51 | 16,953 | 20,64 | | | 0,0000 | -4184,0273 | 0,834 | 0,23 | |
| 52 | 18,127 | 43,28 | | | 0,0000 | -4184,0273 | 0,891 | 0,48 | |
| 53 | 19,033 | 85,73 | 1362,002 | 3 | 4082,9842 | -101,0431 | 0,936 | 0,95 | Des Thr |
| 54 | 19,787 | 41,91 | | | 0,0000 | -4184,0273 | 0,973 | 0,47 | |
| 55 | 20,337 | 4297,26 | 1395,689 | 3 | 4184,0452 | 0,0179 | 1,000 | 47,71 | Product (H-1-39-NH2) |
| 56 | 21,167 | 93,73 | 903,465 | 3 | 2707,3732 | -1476,6541 | 1,041 | 1,04 | Ac-(15-39)-NH2 |
| 57 | 21,453 | 38,3 | | | 0,0000 | -4184,0273 | 1,055 | 0,43 | |
| 58 | 22,187 | 57,85 | | | 0,0000 | -4184,0273 | 1,091 | 0,64 | |
| 59 | 22,697 | 270,08 | 1330,977 | 3 | 3989,9092 | -194,1181 | 1,116 | 3 | Des Pro-Pro |
| 60 | 23,083 | 87,09 | 1339,659 | 3 | 4015,9552 | -168,0721 | 1,135 | 0,97 | Des Ala-Pro |
| 61 | 23,423 | 34,47 | | | 0,0000 | -4184,0273 | 1,152 | 0,38 | |
| 62 | 25,343 | 26,19 | | | 0,0000 | -4184,0273 | 1,246 | 0,29 | |
| 63 | 26,693 | 19,07 | | | 0,0000 | -4184,0273 | 1,313 | 0,21 | |
| 64 | 27,373 | 33,03 | | | 0,0000 | -4184,0273 | 1,346 | 0,37 | |
| 65 | 28,853 | 31,34 | | | 0,0000 | -4184,0273 | 1,419 | 0,35 | |
| 66 | 30,027 | 363,41 | 1427,678 | 3 | 4280,0122 | 95,9849 | 1,476 | 4,03 | Add on TFA |
| 67 | 30,373 | 44,61 | | | 0,0000 | -4184,0273 | 1,493 | 0,5 | |
| 68 | 31,443 | 16,02 | | | 0,0000 | -4184,0273 | 1,546 | 0,18 | |
| 69 | 32,927 | 32,09 | | | 0,0000 | -4184,0273 | 1,619 | 0,36 | |
| 70 | 33,657 | 22,4 | | | 0,0000 | -4184,0273 | 1,655 | 0,25 | |
| 71 | 34,183 | 8,3 | | | 0,0000 | -4184,0273 | 1,681 | 0,09 | |
| 72 | 34,89 | 73,33 | 1409,701 | 3 | 4226,0812 | 42,0539 | 1,716 | 0,81 | Met to hCys(tBu) |
| 73 | 35,623 | 37,08 | | | 0,0000 | -4184,0273 | 1,752 | 0,41 | |
| 74 | 36,9 | 13,45 | | | 0,0000 | -4184,0273 | 1,814 | 0,15 | |
| 75 | 37,883 | 15,45 | | | 0,0000 | -4184,0273 | 1,863 | 0,17 | |
| 76 | 39,177 | 8,57 | | | 0,0000 | -4184,0273 | 1,926 | 0,1 | |
| 77 | 39,51 | 9,54 | | | 0,0000 | -4184,0273 | 1,943 | 0,11 | |
| 78 | 40,427 | 25,16 | | | 0,0000 | -4184,0273 | 1,988 | 0,28 | |
| 79 | 41,283 | 8,26 | | | 0,0000 | -4184,0273 | 2,030 | 0,09 | |
| 80 | 41,97 | 34,27 | | | 0,0000 | -4184,0273 | 2,064 | 0,38 | |
| 81 | 42,46 | 7,34 | | | 0,0000 | -4184,0273 | 2,088 | 0,08 | |
| 82 | 43,93 | 29,93 | | | 0,0000 | -4184,0273 | 2,160 | 0,33 | |
| 83 | 44,413 | 55,03 | 1249,308 | 3 | 3744,9022 | -439,1251 | 2,184 | 0,61 | Ac-(6-39)-NH2 |
| 84 | 47,23 | 11,32 | | | 0,0000 | -4184,0273 | 2,322 | 0,13 | |
| 85 | 49,723 | 8,35 | | | 0,0000 | -4184,0273 | 2,445 | 0,09 | |
| 86 | 51,287 | 27,52 | | | 0,0000 | -4184,0273 | 2,522 | 0,31 | |
| 87 | 53,97 | 19,76 | | | 0,0000 | -4184,0273 | 2,654 | 0,22 | |
| 88 | 54,26 | 10,6 | | | 0,0000 | -4184,0273 | 2,668 | 0,12 | |
| 89 | 54,653 | 6,01 | | | 0,0000 | -4184,0273 | 2,687 | 0,07 | |
| 90 | 55,007 | 7,29 | | | 0,0000 | -4184,0273 | 2,705 | 0,08 | |
| 91 | 55,283 | 8,56 | | | 0,0000 | -4184,0273 | 2,718 | 0,09 | |
| 92 | 55,46 | 7,89 | | | 0,0000 | -4184,0273 | 2,727 | 0,09 | |
| 93 | 55,66 | 10,89 | | | 0,0000 | -4184,0273 | 2,737 | 0,12 | |
| 94 | 56,047 | 7,21 | | | 0,0000 | -4184,0273 | 2,756 | 0,08 | |
| 95 | 57,643 | 7,65 | | | 0,0000 | -4184,0273 | 2,834 | 0,08 | |
| 96 | 57,96 | 69,52 | 243,118 | 1 | 242,1107 | -3941,9166 | 2,850 | 0,77 | Trityl |
| 97 | 59,827 | 17,99 | | | 0,0000 | -4184,0273 | 2,942 | 0,2 | |

**30. LC-HRMS of commercial exenatide reference standard (Fig. 10).**

Experimental conditions: column: Waters Xbridge C18 3.5µm 4.6 x150 mm; column temperature: 60°C; injection volume: 10 µL; sampler temperature: 10°C; MS mode: positive 50-3200; DAD: 220 nm; data rate: 2.5Hz; detector cell: standard cell 1µL; flow: 0.8 ml/min; jet weaver: v380 mixer; mobile phase A: 10 mM $NH_4CO_3$ pH 9.5, mobile phase B: 10 % mobile phase A/90 % MeCN. Gradient (Time(min), %B): 0, 26; 0.5, 26; 33, 37; 35, 90; 36, 90; 36.1, 26; 42, 26.

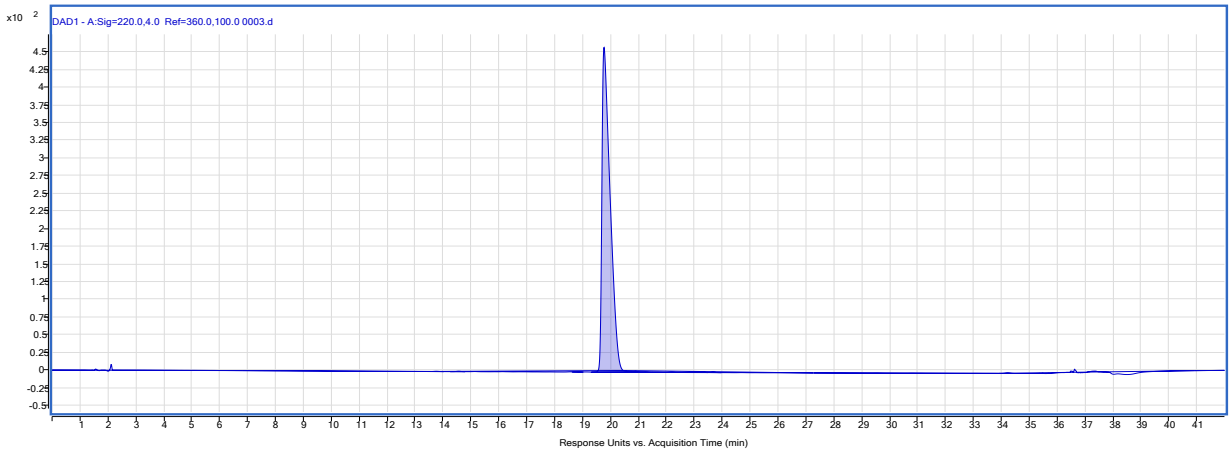



**Figure S40.** UV chromatogram overview.

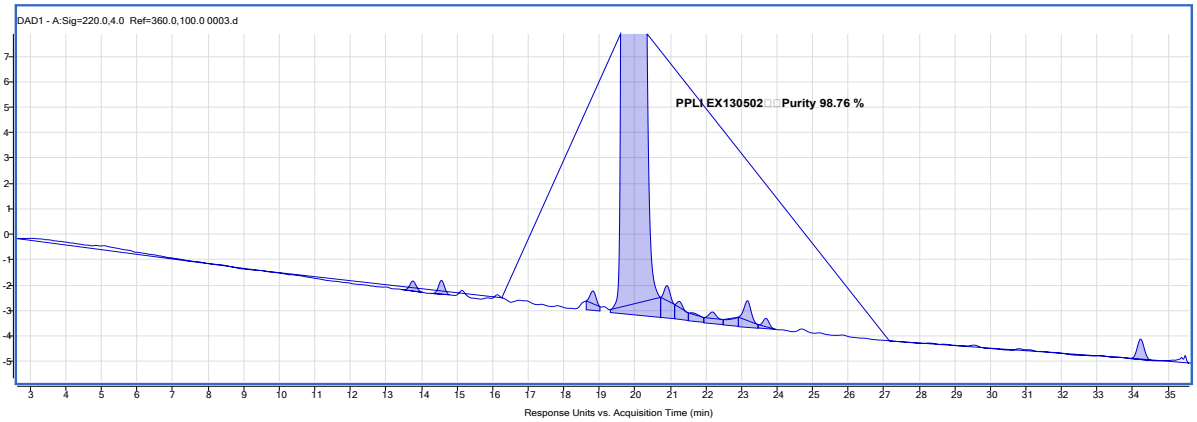



**Figure S41.** UV chromatogram zoom-in.

**Table S19.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | Identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 13,75 | 5,070 | 1047,2610 | 4 | 4185,0149 | 0,9876 | 0,695 | 0,05 | Deamidation |
| 2 | 14,56 | 6,410 | 1051,0130 | 4 | 4200,0229 | 15,9956 | 0,736 | 0,07 | Met to Met(O) or 2-Oxindole/5-HTTP |
| 3 | 18,82 | 11,750 | 1043,5100 | 4 | 4170,0109 | -14,0164 | 0,952 | 0,12 | Met to hCys |
| **4** | **19,77** | **9398,840** | **1395,6890** | **3** | **4184,0452** | **0,0179** | **1,000** | **98,76** | **Product (H-(1-39)-NH2)** |
| 5 | 20,90 | 22,480 | 1043,0170 | 4 | 4168,0389 | -15,9884 | 1,057 | 0,24 | Ser to Ala, (L,D) or Endo βAla des Ser |
| 6 | 21,25 | 12,100 | 1059,0150 | 4 | 4232,0309 | 48,0036 | 1,075 | 0,13 | hydroxy-NFK (Trp Ox) |
| 7 | 21,60 | 7,220 | 1057,5130 | 4 | 4226,0229 | 41,9956 | 1,093 | 0,08 | Met to hCys(tBu) or add on acetyl |
| 8 | 22,18 | 10,040 | 1057,5180 | 4 | 4226,0429 | 42,0156 | 1,122 | 0,11 | Met to hCys(tBu) or add on acetyl |
| 9 | 22,91 | 6,720 | 1042,7580 | 4 | 4167,0029 | -17,0244 | 1,159 | 0,07 | Asn to Aspartimide |
| 10 | 23,17 | 19,310 | 1042,7570 | 4 | 4166,9989 | -17,0284 | 1,172 | 0,20 | Asn to Aspartimide |
| 11 | 23,69 | 5,850 | 1061,0300 | 4 | 4240,0909 | 56,0636 | 1,198 | 0,06 | Add on tBu |
| 12 | 34,23 | 11,180 | 1032,5140 | 4 | 4126,0269 | -58,0004 | 1,731 | 0,12 | Endo βAla des Glu |

## 31. LC-HRMS of purified exenatide from H-1-21-O-Cam-Leu-$NH_2$+H-22-39-$NH_2$ c+c ligation (Fig. 10).

Experimental conditions: see section 30 of this ESI.

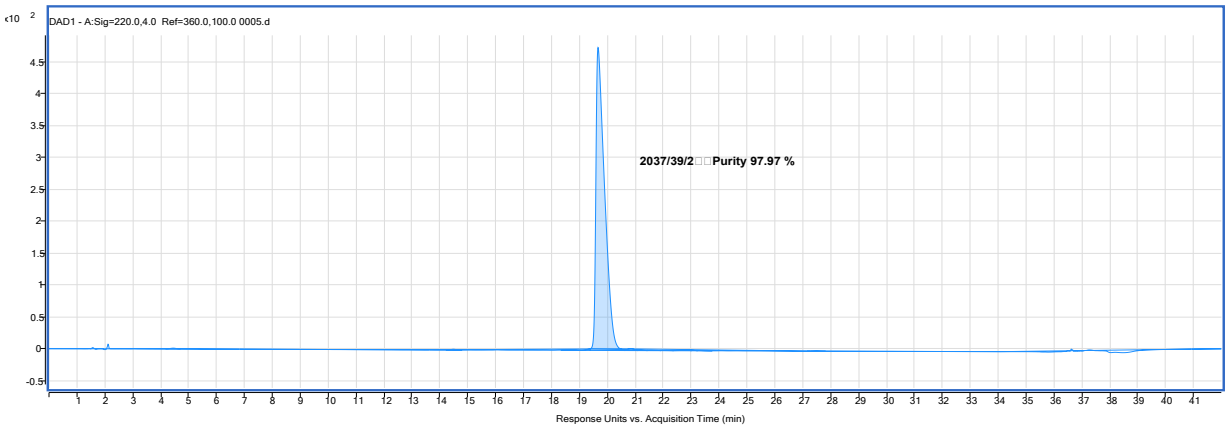



**Figure S42.** UV chromatogram overview.

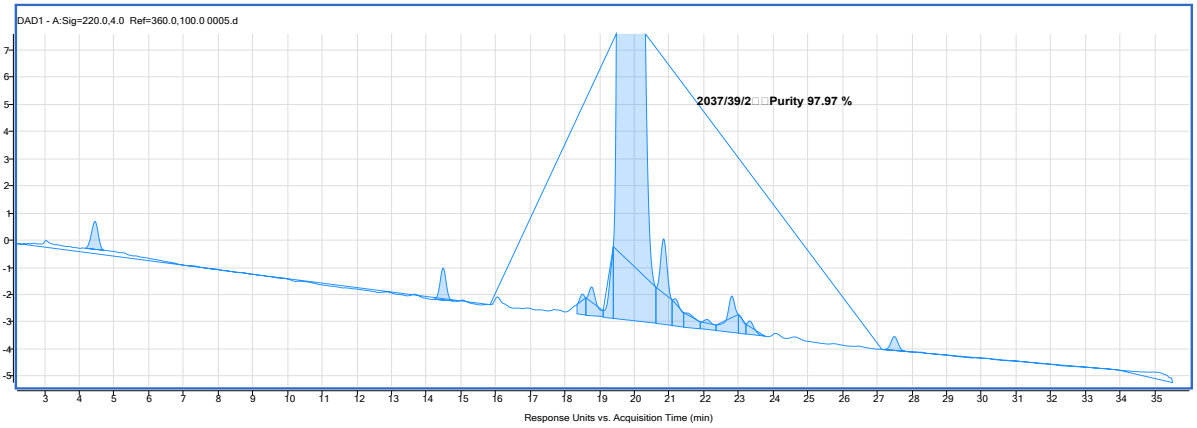



**Figure S43.** UV chromatogram zoom-in.

**Table S20.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4,45 | 13,990 | 501,7810 | 2 | 1001,5474 | -3182,4799 | 0,225 | 0,13 | H-(1-19)-OH/des -SCH3 |
| 2 | 14,49 | 14,400 | 1051,0140 | 4 | 4200,0269 | 15,9996 | 0,733 | 0,14 | Met to Met(O) or 2-Oxindole/5-HTTP |
| 3 | 18,50 | 9,360 | 1048,0130 | 4 | 4188,0229 | 3,9956 | 0,936 | 0,09 | Kynurenine |
| 4 | 18,77 | 18,370 | 1043,5120 | 4 | 4170,0189 | -14,0084 | 0,949 | 0,17 | Met to hCys |
| 5 | 19,39 | 19,070 | 1068,7770 | 4 | 4271,0789 | 87,0516 | 0,981 | 0,18 | Double Ser |
| **6** | **19,67** | **10320,940** | **1395,6910** | **3** | **4184,0512** | **0,0239** | **0,995** | **97,97** | **Prod. H-(1-39)-NH2** |
| 7 | 20,84 | 56,170 | 1043,0170 | 4 | 4168,0389 | -15,9884 | 1,054 | 0,53 | Ser to Ala, (L,D) or Endo βAla des Ser |
| 8 | 21,17 | 15,900 | 1050,5260 | 4 | 4198,0749 | 14,0476 | 1,071 | 0,15 | Endo βAla des Gly |
| 9 | 21,42 | 12,270 | 1057,5190 | 4 | 4226,0469 | 42,0196 | 1,083 | 0,12 | Met to hCys(tBu) or add on acetyl |
| 10 | 22,09 | 7,670 | 1061,0270 | 4 | 4240,0789 | 56,0516 | 1,117 | 0,07 | Add on tBu |
| 11 | 22,81 | 25,210 | 1075,2850 | 4 | 4297,1109 | 113,0836 | 1,154 | 0,24 | Double Ile/Leu |
| 12 | 23,00 | 6,650 | 1042,7500 | 4 | 4166,9709 | -17,0564 | 1,163 | 0,06 | Asn to Aspartimide |
| 13 | 23,33 | 7,120 | 1063,0230 | 4 | 4248,0629 | 64,0356 | 1,180 | 0,07 | [2Na +K] Asp to Aspartimide |
| 14 | 27,49 | 7,380 | 1146,5780 | 3 | 3436,7122 | -747,3151 | 1,390 | 0,07 | pyruvyl-(9-39)-NH2/RH-NH2 to R=O |

## 32. LC-HRMS of purified exenatide from H-1-21-O-Cam-Leu-$NH_2$+H-22-39-$NH_2$ p+p ligation (Fig. 10).

Experimental conditions: see section 30 of this ESI.

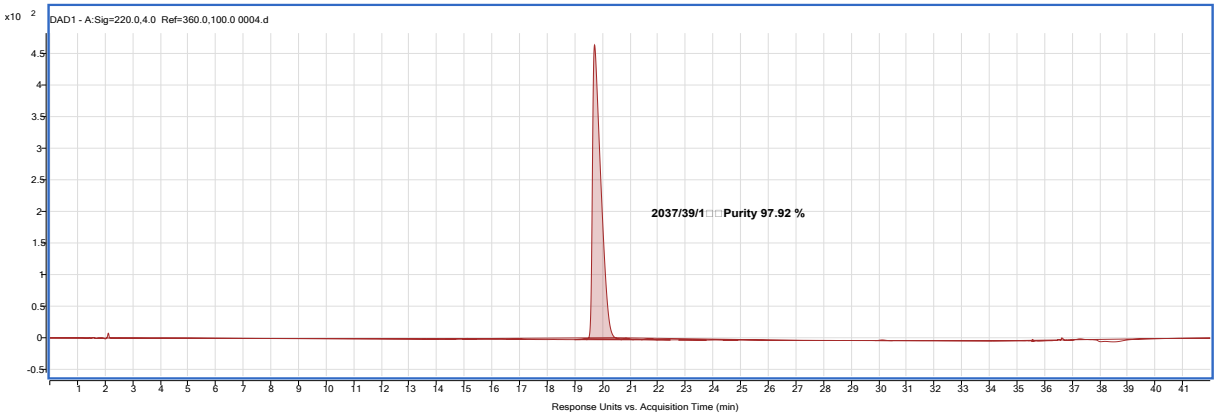



**Figure S44.** UV chromatogram overview.

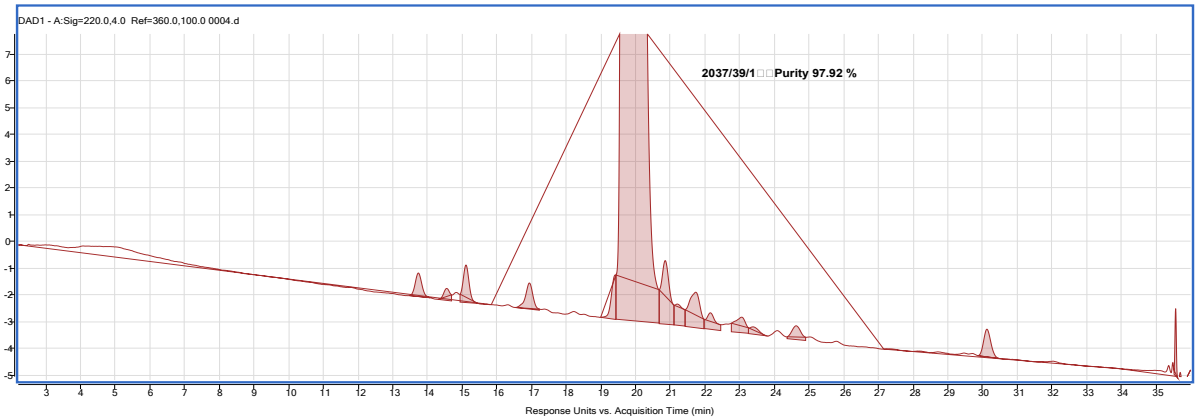



**Figure S45.** UV chromatogram zoom-in.

**Table S21.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 13,74 | 11,640 | 1047,2620 | 4 | 4185,0189 | 0,9916 | 0,695 | 0,12 | Deamidation |
| 2 | 14,55 | 5,610 | 1051,0130 | 4 | 4200,0229 | 15,9956 | 0,736 | 0,06 | Met to Met(O) or 2-Oxindole/5-HTTP |
| 3 | 15,11 | 17,310 | 1047,2610 | 4 | 4185,0149 | 0,9876 | 0,764 | 0,17 | Deamidation |
| 4 | 16,94 | 14,650 | 1079,2760 | 4 | 4313,0749 | 129,0476 | 0,857 | 0,15 | Double Glu |
| 5 | 19,41 | 14,890 | 1068,7720 | 4 | 4271,0589 | 87,0316 | 0,982 | 0,15 | Double Ser |
| **6** | **19,71** | **9873,440** | **1395,6880** | **3** | **4184,0422** | **0,0149** | **0,997** | **97,92** | **Product (H-(1-39)-NH2)** |
| 7 | 20,85 | 40,660 | 1043,0170 | 4 | 4168,0389 | -15,9884 | 1,055 | 0,40 | Ser to Ala, (L,D) or Endo βAla des Ser |
| 8 | 21,20 | 13,730 | 1025,2570 | 4 | 4096,9989 | -87,0284 | 1,072 | 0,14 | Des Ser |
| 9 | 21,73 | 30,790 | 1047,0170 | 4 | 4184,0389 | 0,0116 | 1,099 | 0,31 | Isomer |
| 10 | 22,15 | 11,450 | 1057,5170 | 4 | 4226,0389 | 42,0116 | 1,120 | 0,11 | Met to hCys(tBu) or add on acetyl |
| 11 | 23,05 | 12,780 | 1042,7580 | 4 | 4167,0029 | -17,0244 | 1,166 | 0,13 | Asn to Aspartimide |
| 12 | 23,39 | 4,970 | 1063,0220 | 4 | 4248,0589 | 64,0316 | 1,183 | 0,05 | [2Na +K] Asp to Aspartimide |
| 13 | 24,63 | 9,690 | 1042,5140 | 4 | 4166,0269 | -18,0004 | 1,246 | 0,10 | Asp to Aspartimide |
| 14 | 30,13 | 14,850 | 990,7560 | 4 | 3958,9949 | -225,0324 | 1,524 | 0,15 | Des Gly-Ala-Pro |
| 15 | 35,57 | 6,850 | 1061,0330 | 4 | 4240,1029 | 56,0756 | 1,799 | 0,07 | Add on tBu |

## 33. LC-HRMS of purified exenatide from H-1-21-HMBA-K+H-22-39-$NH_2$ c+c ligation (Fig. 10).

Experimental conditions: see section 30 of this ESI.

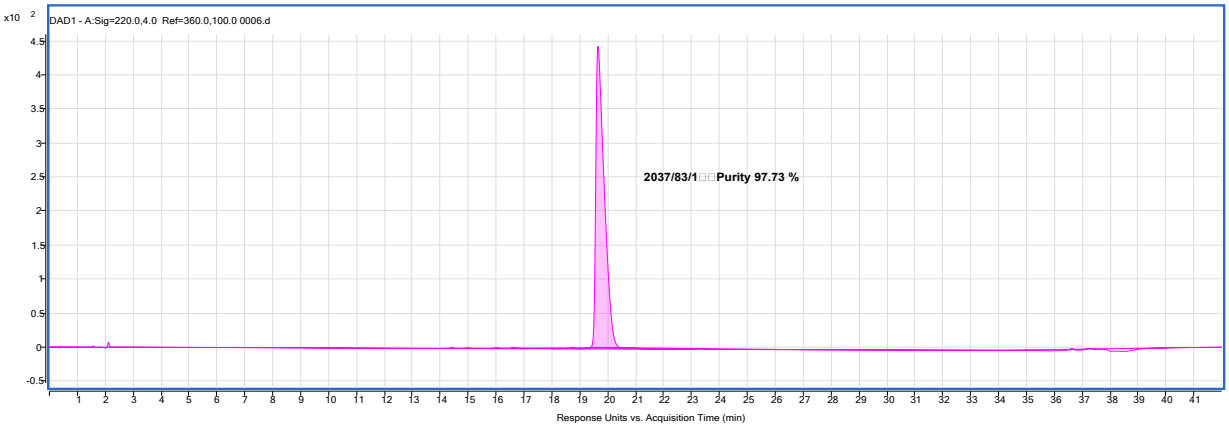



**Figure S46.** UV chromatogram overview.

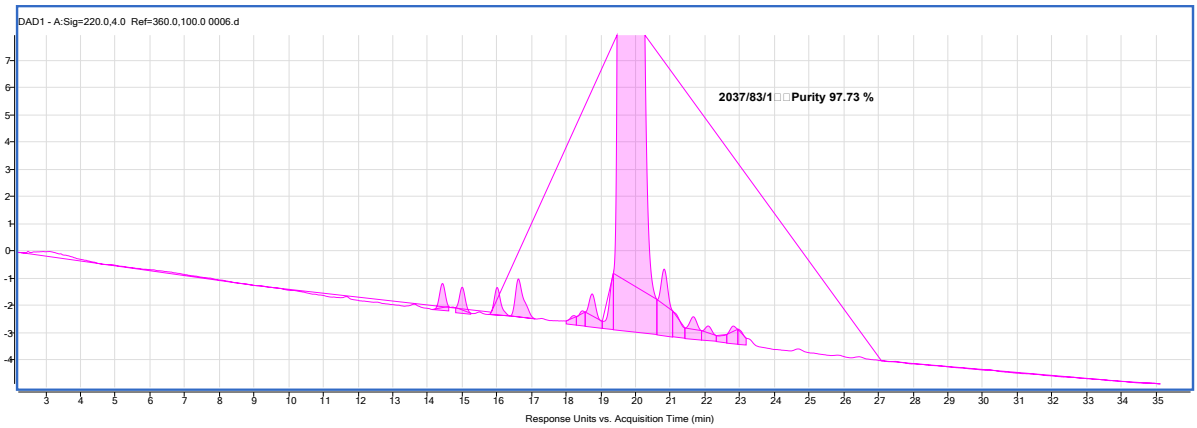



**Figure S47.** UV chromatogram zoom-in.

**Table S22.** Area% for integrated peaks and MS identities for H-1-39-$NH_2$ and main byproducts.

| Peak | Rt | Area | most(m+z)/z | z | decon. | diff | RRT | Area % | identity |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 14,43 | 12,160 | 1051,0140 | 4 | 4200,0269 | 15,9996 | 0,730 | 0,13 | Met to Met(O) or 2-Oxindole/5-HTTP |
| 2 | 15,00 | 12,280 | 1047,2600 | 4 | 4185,0109 | 0,9836 | 0,759 | 0,13 | Deamidation |
| 3 | 16,01 | 13,270 | 1014,2550 | 4 | 4052,9909 | -131,0364 | 0,810 | 0,14 | Des Met |
| 4 | 16,62 | 23,170 | 1047,2610 | 4 | 4185,0149 | 0,9876 | 0,841 | 0,24 | Deamidation |
| 5 | 18,22 | 4,420 | 1051,0150 | 4 | 4200,0309 | 16,0036 | 0,922 | 0,05 | Met to Met(O) or 2-Oxindole/5-HTTP |
| 6 | 18,47 | 6,970 | 1048,0090 | 4 | 4188,0069 | 3,9796 | 0,934 | 0,07 | Kynurenine |
| 7 | 18,75 | 21,220 | 1043,5110 | 4 | 4170,0149 | -14,0124 | 0,948 | 0,22 | Met to hCys |
| 8 | 19,36 | 16,990 | 855,2400 | 5 | 4271,1636 | 87,1363 | 0,979 | 0,18 | Double Ser |
| **9** | **19,65** | **9418,970** | **1395,6900** | **3** | **4184,0482** | **0,0209** | **0,994** | **97,73** | **Prod. H-(1-39)-NH2** |
| 10 | 20,82 | 46,730 | 1025,2570 | 4 | 4096,9989 | -87,0284 | 1,053 | 0,48 | Des Ser |
| 11 | 21,07 | 14,090 | 1004,9960 | 4 | 4015,9549 | -168,0724 | 1,066 | 0,15 | Des Ala-Pro |
| 12 | 21,67 | 16,730 | 999,9960 | 4 | 3995,9549 | -188,0724 | 1,096 | 0,17 | Des Thr-Ser |
| 13 | 22,09 | 10,060 | 849,0260 | 5 | 4240,0936 | 56,0663 | 1,117 | 0,10 | Add on tBu |
| 14 | 22,63 | 4,390 | 1042,7660 | 4 | 4167,0349 | -16,9924 | 1,145 | 0,05 | Asn to Aspartimide |
| 15 | 22,82 | 10,420 | 1075,2870 | 4 | 4297,1189 | 113,0916 | 1,154 | 0,11 | Double Ile/Leu |
| 16 | 22,95 | 6,100 | 1042,7590 | 4 | 4167,0069 | -17,0204 | 1,161 | 0,06 | Asn to Aspartimide |

## 34. Lyophilization details.

All three batches of exenatide API prepared herein as well as the purified H-22-39-$NH_2$ and H-1-21-O-Cam-Leu-$NH_2$ fragments respectively were isolated by lyophilization as follows: the product solution was placed in a tray of the lyophilizer, frozen to – 40 °C after which the main drying was performed at 15 °C at 0.2 mBar and the secondary drying was performed at 25 °C at 0.05 mB for 3 h. The total freeze drying cycle was 64 h.

## 35. Sustainability and cost assessment.

### 35.1. General

The cost and sustainability assessment for the six processes examined was carried out as follows: first, the amounts of starting materials, cost of starting materials, cost of waste disposal, cost of processing energy, $CO_2$ formed during manufacturing of starting materials, $CO_2$ formed during incineration of the waste and $CO_2$ formed by consumption of processing energy was determined for all six processes assuming that all these exenatide manufactures were carried out on 1 mol as the master scale. For schematic overviews of flow charts of all six manufacturing processes see Figs. S48 - S53 in sections 35.2 – 35.7 of this ESI, for full details see Tables S23 – S83 of this ESI. Next, the values thus obtained were recalculated for 1 kg exenatide API manufactured, taking into account that the six 1 mol manufactures each provided a different amount of the API. The assessment of sustainability and cost for the 6 manufacturing routes examined in Table 4 in the main article is thus based on 1 kg exenatide being the amount of API produced in each route.

With respect to the use of decimal separators in the ESI and in the main article. Commas were used as decimal separators in the tables in the ESI as all these tables were made in Excel. These ESI tables contain a large amount of data which was used to carry out calculations necessary to obtain the pertinent cost and sustainability results. These calculations were performed by using the formula functions of the program and as Excel does not recognize decimal points in calculations the use of commas as decimal separators was necessary. In the main article dots (decimal points) were used as decimal separators throughout as customary.

Cost of raw materials and waste disposal:

For estimated cost of raw materials actual and/or quoted prices from suppliers qualified as vendors for GMP manufacturing at PolyPeptide Group AB Sweden were used, see Tables S27, S36, S39, S48, S51, S56, S59, S67, S70, S78 and S81. These prices may vary depending on the

manufacturing location and/or vendor used. The cost of processing water as well as cost of inorganic salts, acid and bases used in the preparation of buffers for RPC purifications was insignificant and was not included.

To estimate the amount of solvent used during downstream processing of the crude exenatide fragments as well as exenatide API a value for a typical RPC purification of a pharmaceutical peptide carried out at PolyPeptide Group AB was used in which it was assumed that to perform one RPC purification step on a 1 kg of a crude peptide material requires ~1 $m^3$ of solvent which is $H_2O$/MeCN, 70:30.

Estimated price of waste disposal (incineration) at a waste treatment facility (Stena Recycling) utilized by PolyPeptide Group AB was 0,21 $EURkg^{-1}$.

Energy cost, consumption and carbon intensity (CI):

Cost of energy was based on the current price of electricity on the electric power grid in Sweden i.e.: 1 $kWh^{-1}$ equals ~0,066 Eur. Cost of energy for each process route was thus calculated as

Cost of processing energy ($EURkg^{-1}$ API) = $\sum$ of energy consumed x 0,066.

Estimates of energy consumed in manufacturing exenatide API were based on the values for a typical process for manufacturing of a therapeutic peptide in a GMP setting at PolyPeptide Group:

i) SPPS: it was assumed that heating 100 kg solvent from 22 to 50 ºC required 1,6 $kWh^{-1}$ and stirring required 4.5 $kWh^{-1}$, it was further assumed that a coupling cycle (Fmoc removal+washing+coupling+washing) for all rt SPPS processes lasted 6h, whereas a coupling cycle for an et SPPS process lasted 3h. The temperature of all et SPPS processes was assumed to be 50 ºC and the amount of the solvent in the reactor was assumed to be 50 $kgmol^{-1}$ throughout.

ii) TFA cleavage and precipitation: it was assumed that 12 kg total amount (2 kg for TFA cleavage, 2 x 5 kg for solvent precipitation) stirred for 1 h at 22 ºC: required 0,46 $kWh^{-1}$ ; for all cleavages/precipitations it was assumed that the cleavage was carried out for 2 h and the subsequent solvent precipitations were carried out for a total of 1 h. Furthermore, it was assumed that the amount of solvent used for cleavage & precipitation was the total amount throughout.

iii) enzymatic ligation: it was assumed that stirring 10 kg of solvent for 1 h at 22 ºC required 0,38 $kWh^{-1}$, it was also assumed that at the intended target scale (1 kg of exenatide API) all ligations were allowed to proceed for 3 h.

iv) RPC purification: it was assumed that passing 1 t of solvent through a RPC column at 22 ºC requires 4,5 $kWh^{-1}$.

v) lyophilization: it was assumed that lyophilizing 1 kg of a peptide fragment/peptide API at 20 $gkg^{-1}$ lyophilization concentration requires 35 $kWh^{-1}$.

The amount of cooling water consumed was negligible for all process steps of all process routes and was not included in the calculations herein.

The processing energy consumed was calculated for all six exenatide processes examined based on the energy consumption values for the individual process steps above see Tables S28, S29, S40, S41, S52, S53, S60, S61, S71, S72, S82 and S83 in this ESI for details.

For the conversion of the consumed energy to CI of energy consumption ($CI_{en}$) three contributors were considered based on previously reported values:[18]

i) electricity generated: 0,28307 kg $CO_2$ eq. $kWh^{-1}$ .

ii) electricity transmitted & distributed: 0,02413 kg $CO_2$ eq. $kWh^{-1}$.

iii) electricity used (benchmark processes I & II): 0,30720 kg $CO_2$ eq. $kWh^{-1}$; carbon intensity for electricity used on CEPS processes herein which used wind energy: 0,0 kg $CO_2$ eq. $kWh^{-1}$.[16]

$CI_{en}$ was therefore calculated as:

$CI_{en}$ = i) + ii) + iii)

Carbon intensity for all chemicals involved in exenatide processing ($CI_{chem}$): as for all six routes solvents constituted >90% of the mass of the utilized starting materials estimated CI of production of starting materials as well as estimated CI of waste disposal of all materials were based on assuming that the starting materials and the waste were the solvents used, excluding processing water used for DSP.

$CI_{production}$ for the production of raw materials: $CI_{production}$ for production of following solvents was used for the calculations herein: DMF, MeCN, MTBE, ether and heptane all of which were based on previously reported values.[17]

$CI_{process}$ for processing of exenatide: it was assumed that no loss of solvents and/or other materials occurred during the processing of the API and the impact of $CI_{process}$ was thus deemed as negligible and was not included in the calculations herein.

$CI_{waste}$ for waste disposal (incineration), theoretical CI:[18] it was assumed that under incineration all solvents underwent a perfect combustion i.e. each mol of C in the incinerated solvent produced one mol of $CO_2$ during incineration i.e.: 1.00 kg DMF produced 1.81 kg $CO_2$; 1.00 kg MeCN produced 2.15 kg $CO_2$; 1.00 kg MTBE produced 2.49 kg $CO_2$; 1.00 kg ether produced 2.37 kg $CO_2$ and 1.00 kg heptane produced 3.07 kg $CO_2$.

$CI_{chem}$ was therefore calculated as:

$CI_{chem} = CI_{production} + CI_{process}\,(0) + CI_{waste} = CI_{production} + CI_{waste}$

Total CI ($kg^{-1}$ API) for all six exenatide manufacturing routes was calculated as:

Total $CI_{chem} = CI_{chem} + CI_{en}$

**35.2. Conventional SPPS, benchmark 1[19]**

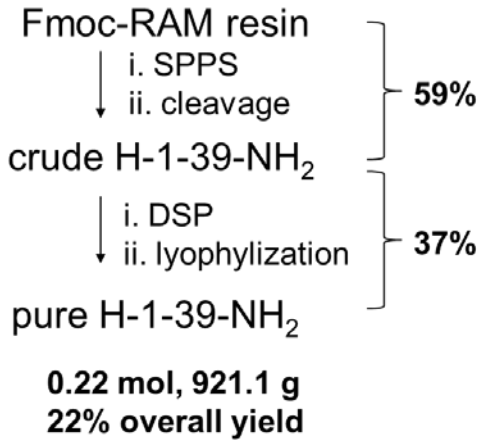



**Figure S48.** A schematic representation of the flow chart for a conventional SPPS exenatide manufacturing process.

**Table S23.** Overview of raw materials used in 1 mol SPPS of exenatide peptide resin, benchmark process 1.

| AA nr | AA | MW | **mol** | **equiv AA** | **kg AA** | **kg HOBt** | **kg DIC** | **kg pip** | **kg DMF[2]** |
|---|---|---|---|---|---|---|---|---|---|
| resin<br>linker | Fmoc-Rink amide resin, 0.24 mmol/g, 4,17 kg | n.a. | 1,00 | **n.a.** | n.a. | n.a. | n.a. | n.a. | n.a. |
| $Ser^{39}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 1,00 | **1,00** | **0,38** | 0,16 | 0,15 | 16,7 | 692,2 |
| $Pro^{38}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Pro^{37}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Pro^{36}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ala^{35}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 4,00 | **4,00** | **1,32** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gly^{34}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ser^{33}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ser^{32}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Pro^{31}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gly^{30}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gly^{29}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Asn^{28}$ | Fmoc-Asn(Trt)-OH | 596,67 | 4,00 | **4,00** | **2,39** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Lys^{27}$ | Fmoc-Lys(Boc)-OH | 468,54 | 4,00 | **4,00** | **1,87** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Leu^{26}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Trp^{25}$ | Fmoc-Trp(Boc)-OH | 526,59 | 4,00 | **4,00** | **2,11** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Glu^{24}$ | Fmoc-Glu(OtBu)-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ile^{23}$ | Fmoc-Ile-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Phe^{22}$ | Fmoc-Phe-OH | 387,45 | 4,00 | **4,00** | **1,55** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Leu^{21}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Arg^{20}$ | Fmoc-Arg(Pbf)-OH | 648,77 | 4,00 | **4,00** | **2,60** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Val^{19}$ | Fmoc-Val-OH | 339,39 | 4,00 | **4,00** | **1,36** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ala^{18}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 4,00 | **4,00** | **1,32** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Glu^{17}$ | Fmoc-Glu-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Glu^{16}$ | Fmoc-Glu-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Glu^{15}$ | Fmoc-Glu-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Met^{14}$ | Fmoc-Met-OH | 371,45 | 4,00 | **4,00** | **1,49** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gln^{13}$ | Fmoc-Gln(Trt)-OH | 610,70 | 4,00 | **4,00** | **2,44** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Lys^{12}$ | Fmoc-Lys(Boc)-OH | 468,54 | 4,00 | **4,00** | **1,87** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ser^{11}$ | Fmoc-Ser(*t*-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Leu^{10}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Asp^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 411,45 | 4,00 | **4,00** | **1,65** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Ser^{8}$ | Fmoc-Ser(*t*-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Thr^{7}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 4,00 | **4,00** | **1,59** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Phe^{6}$ | Fmoc-Phe-OH | 387,45 | 4,00 | **4,00** | **1,55** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Thr^{5}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 4,00 | **4,00** | **1,59** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gly^{4}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Glu^{3}$ | Fmoc-Glu-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,65 | 0,61 | 16,7 | 692,2 |
| $Gly^{2}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,65 | 0,61 | 16,7 | 692,2 |
| $His^{1}$ | Boc-His(Boc)-OH$^{x}$DCHA[1] | 355,39 | 4,00 | **4,00** | **1,42** | n.a.[1] | n.a. | 16,7 | 692,2 |
| $\sum$ | | | | | 60,51 | 24,16 | 22,56 | 651,30 | 26995,80 |

[1]HATU (4 mol, 1,52 kg), HOAT (4,8 moL, 0,65 kg) and TMP (8 mol, 0,97 kg) were used for the coupling; [2]in some steps of the synthesis DCM and NMP were used as well,as DMF accounts for >95% of solvent consumption DMF was considered as the sole solvent of the synthesis

**Table S24.** Summary of amounts of raw materials used in 1 mol SPPS of exenatide peptide resin, benchmark process 1.

| | |
|---|---|
| Scale (mol) | 1,00 |
| Weight resin (kg) | 4,17 |
| ∑ Weight AAs (kg) | 60,51 |
| ∑ Weight HOBt (kg) | 24,16 |
| ∑ Weight DIC (kg) | 22,56 |
| ∑ Weight His[1] coupling (kg) | 3,14 |
| ∑ Weight resin/AAs/coupling agents (kg) | 115,54 |
| ∑ Weight pip (kg) | 651,30 |
| ∑ starting materials (kg) | **766,84** |
| ∑ solvents (kg) | **26995,80** |
| peptide resin formed (kg) | 10,68 |

**Table S25.** Summary of amounts of raw materials used in 0,19 mol (reported) and 1 mol TFA cleavage of exenatide peptide resin, benchmark process 1.

| | | |
|---|---|---|
| Scale (mol) | 0,19 | 1,00 |
| TFA (kg) | 24,48 | 128,84 |
| TIS (kg) | 0,75 | 3,95 |
| $H_2O$ (kg) | 0,51 | 2,68 |
| EDT (kg) | 1,15 | 6,05 |
| PhSMe (kg) | 1,08 | 5,68 |
| PhOH (kg) | 0,21 | 1,11 |
| ∑ Reagents (kg) | 28,18 | 148,32 |
| solvents (ether, kg) | 579,00 | 3047,37 |

**Table S26.** Summary of amounts of raw materials used in DSP of crude exenatide, benchmark process 1.

| Step | kg peptide | kg solvent |
|---|---|---|
| API (1 RPC) | 0,92 | 861,12 |

**Table S27.** Summary of amounts and cost of raw materials used in the entire manufacturing process, benchmark process 1.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) | cost (%) |
|---|---|---|---|---|
| resin | 2800,0 | 4,2 | 11676,0 | 9,0 |
| HOBt | 62,0 | 24,2 | 1497,9 | 1,2 |
| piperidine | 10,6 | 651,3 | 6903,8 | 5,3 |
| std AA | 400,0 | 60,5 | 24204,0 | 18,7 |
| DIC | 240,0 | 22,6 | 5414,4 | 4,2 |
| DMF | 1,4 | 26995,8 | 37794,1 | 29,2 |
| HATU | 2700,0 | 1,5 | 4104,0 | 3,2 |
| HOAT | 2200,0 | 0,7 | 1430,0 | 1,1 |
| TMP | 370,0 | 1,0 | 358,9 | 0,3 |
| TFA | 20,0 | 128,8 | 2576,8 | 2,0 |
| TIS | 900,0 | 4,0 | 3555,0 | 2,7 |
| EDT | 50,0 | 6,1 | 302,5 | 0,2 |
| PhSMe | 100,0 | 5,7 | 568,0 | 0,4 |
| PhOH | 150,0 | 1,1 | 166,5 | 0,1 |
| Ether | 9,0 | 3047,4 | 27426,6 | 21,2 |
| MeCN | 6,0 | 258,3 | 1549,8 | 1,2 |
| $\sum$ | n.a. | n.a. | 129528,3 | 100,0 |
| $\sum$/1 kg API | | | 140791,7 | |

**Table S28.** Summary of total CI for organic solvents used in exenatide manufacturing benchmark process 1.

| **Total organic solvents** | weight (kg) | $CO_2$ formed during solvent production / kg kg$^{-1}$ | theoretical CI / kg kg$^{-1}$ | $CI_{\sum}$ / kg kg$^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 26995,8 | 1,75 | 1,81 | 3,56 | 96105,05 |
| Ether (cleavage) | 3047,4 | 1,08 | 2,37 | 3,45 | 10513,53 |
| MeCN (DSP) | 258,3 | 1,95 | 2,15 | 4,1 | 1059,03 |
| $\sum$ (kg $CO_2$) | | | | | 107677,61 |
| $\sum$(kg $CO_2$)kg$^{-1}$ API | | | | | 117040,88 |

**Table S29.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing benchmark process 1.

| Energy & CI | Process step | | | | ∑ | ∑ kg API$^{-1}$ |
|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | DSP[c] | lyophilization[d] | | |
| energy used (kWh$^{-1}$) | 526,55 | 365 | 3,9 | 32,2 | 927,65 | 1007,22 |
| cost of energy used (EUR) | 34,75 | 24,09 | 0,26 | 2,13 | 61,22 | 66,48 |
| CI for electricity generated (t $CO_2$) | 0,15 | 0,10 | 0,001 | 0,009 | 0,26 | 0,29 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,01 | 0,000 | 0,001 | 0,02 | 0,02 |
| CI for electricity used (t $CO_2$) | 0,16 | 0,11 | 0,001 | 0,010 | 0,28 | 0,31 |
| CI for electricity ∑ (t $CO_2$) | 0,32 | 0,22 | 0,002 | 0,020 | 0,57 | 0,62 |

[a]energy used=39 (AA cycles) x 6 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 4,5 (amount of energy needed to stir nominal amount of solvent for 1 h); [b]3176 (total amount of TFA+solvent in kg) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]861 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [d]0,921 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API).

**35.3. Lab scale CEPS (P+P), benchmark 2[20]**

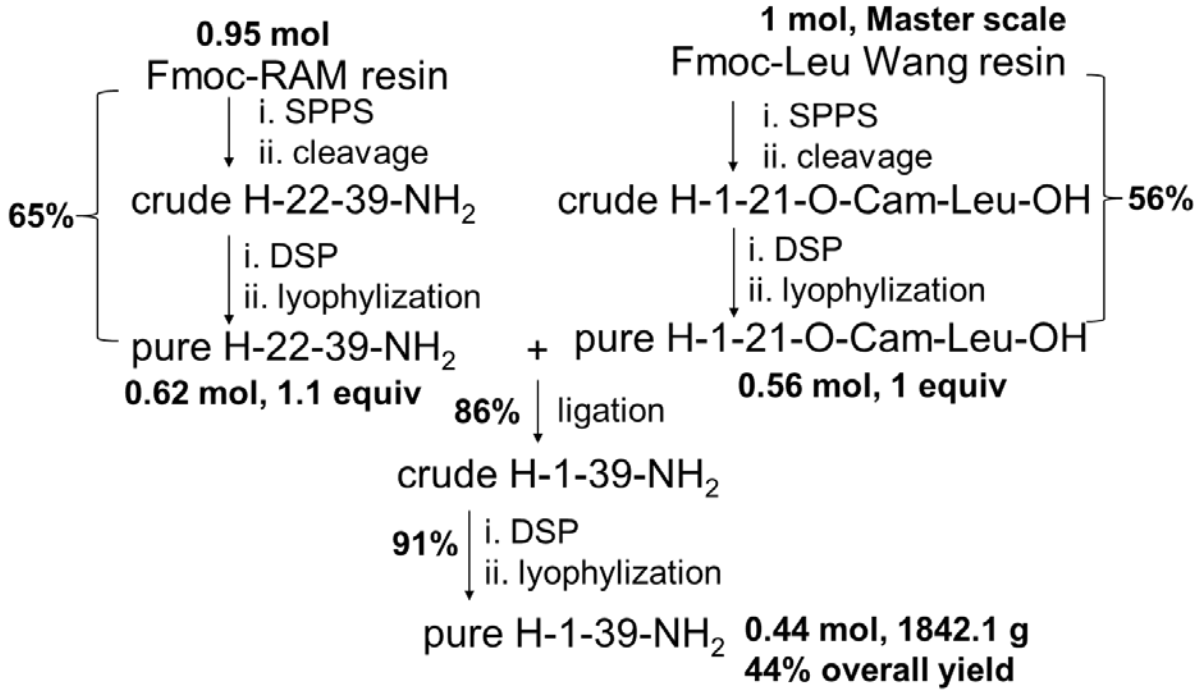



**Figure S49.** A schematic representation of the flow chart of a lab scale CEPS (P+P) exenatide manufacturing process.

**Table S30.** Overview of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, benchmark process 2.

| AA nr | AA | MW | **mol** | **equiv AA** | **kg AA** | **kg HBTU** | **kg HOBtxH$_2$O** | **kg DIEA** | kg pip | kg DMF |
|---|---|---|---|---|---|---|---|---|---|---|
| resin | Fmoc-Leu-Wang resin, 0.72 mmol/g, 1,388 kg | n.a. | 1,00 | **n.a.** | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| linker | iodoacetic acid | 185,96 | 4,00 | **4,00** | **0,74** | n.a. | n.a. | n.a. | 6,0 | 137,6 |
| Leu$^{21}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | n.a. | n.a. | 1,03 | 0,0 | 91,7 |
| Arg$^{20}$ | Fmoc-Arg(Pbf)-OH | 648,77 | 4,00 | **4,00** | **2,60** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Val$^{19}$ | Fmoc-Val-OH | 339,39 | 4,00 | **4,00** | **1,36** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Ala$^{18}$ | Fmoc-Ala-OHxH$_2$O | 329,36 | 4,00 | **4,00** | **1,32** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Glu$^{17}$ | Fmoc-Glu-OHxH$_2$O | 443,49 | 4,00 | **4,00** | **1,77** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Glu$^{16}$ | Fmoc-Glu-OHxH$_2$O | 443,49 | 4,00 | **4,00** | **1,77** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Glu$^{15}$ | Fmoc-Glu-OHxH$_2$O | 443,49 | 4,00 | **4,00** | **1,77** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Met$^{14}$ | Fmoc-Met-OH | 371,45 | 4,00 | **4,00** | **1,49** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Gln$^{13}$ | Fmoc-Gln(Trt)-OH | 610,70 | 4,00 | **4,00** | **2,44** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Lys$^{12}$ | Fmoc-Lys(Boc)-OH | 468,54 | 4,00 | **4,00** | **1,87** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Ser$^{11}$ | Fmoc-Ser(*t* -Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Leu$^{10}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Asp$^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 411,45 | 4,00 | **4,00** | **1,65** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Ser$^{8}$ | Fmoc-Ser(*t* -Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Thr$^{7}$ | Fmoc-Phe-Thr(Psime,Mepro)-OH | 528,6 | 4,00 | **4,00** | **2,11** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Phe$^{6}$ | | | | | | | | | | |
| Thr$^{5}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 4,00 | **4,00** | **1,59** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Gly$^{4}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Glu$^{3}$ | Fmoc-Glu-OHxH$_2$O | 443,49 | 4,00 | **4,00** | **1,77** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| Gly$^{2}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| His$^{1}$ | Boc-His(Trt)-OH | 497,57 | 4,00 | **4,00** | **1,99** | 0,57 | 0,61 | 1,03 | 6,0 | 137,6 |
| $\Sigma$ | | | | | 34,53 | 10,80 | 11,64 | 20,68 | 120,00 | 2843,70 |

**Table S31.** Overview of raw materials used in 1 mol SPPS of H-22-39-peptide fragment resin, benchmark process 2.

| AA nr | AA | MW | **mol** | **equiv AA** | **kg AA** | **kg HBTU** | **kg HOBtx$H_2O$** | **kg DIEA** | kg pip | kg DMF |
|---|---|---|---|---|---|---|---|---|---|---|
| resin<br>linker | Fmoc-Rink amide resin, 0.64 mmol/g, 1,563 kg | n.a. | 1,00 | **n.a.** | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| $Ser^{39}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 1,52 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Pro^{38}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Pro^{37}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Pro^{36}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Ala^{35}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 4,00 | **4,00** | **1,32** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Gly^{34}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Ser^{33}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Ser^{32}$ | Fmoc-Ser(t-Bu)-OH | 383,44 | 4,00 | **4,00** | **1,53** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Pro^{31}$ | Fmoc-Pro-OH | 337,37 | 4,00 | **4,00** | **1,35** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Gly^{30}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Gly^{29}$ | Fmoc-Gly-OH | 297,30 | 4,00 | **4,00** | **1,19** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Asn^{28}$ | Fmoc-Asn(Trt)-OH | 596,67 | 4,00 | **4,00** | **2,39** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Lys^{27}$ | Fmoc-Lys(Boc)-OH | 468,54 | 4,00 | **4,00** | **1,87** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Leu^{26}$ | Fmoc-Leu-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Trp^{25}$ | Fmoc-Trp(Boc)-OH | 526,59 | 4,00 | **4,00** | **2,11** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Glu^{24}$ | Fmoc-Glu(OtBu)-OHx$H_2O$ | 443,49 | 4,00 | **4,00** | **1,77** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Ile^{23}$ | Fmoc-Ile-OH | 354,41 | 4,00 | **4,00** | **1,42** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $Phe^{22}$ | Fmoc-Phe-OH | 387,45 | 4,00 | **4,00** | **1,55** | 0,57 | 0,61 | 1,03 | 6,7 | 154,6 |
| $\Sigma$ | | 1 | | | 27,41 | 11,18 | 11,03 | 18,61 | 120,60 | 2782,80 |

**Table S32.** Summary of amounts of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, benchmark process 2.

| | |
|---|---|
| Scale (mol) | 1,00 |
| Weight resin (kg) | 1,39 |
| ∑ Weight AAs (kg) | 34,53 |
| ∑ Weight HBTU (kg) | 10,80 |
| ∑ Weight HOBtx$H_2O$ (kg) | 11,64 |
| ∑ Weight DIEA (kg) | 20,68 |
| ∑ Weight resin/AAs/coupling agents (kg) | 80,04 |
| ∑ Weight pip (kg) | 120,00 |
| ∑ starting materials (kg) | **200,04** |
| ∑ solvents (kg) | **2843,70** |
| peptide resin formed (kg) | 5,04 |

**Table S33.** Summary of amounts of raw materials used in 1 mol (nominal) and 0,95 mol (actual) SPPS of H-22-39-peptide fragment resin, benchmark process 2.

| | | |
|---|---|---|
| Scale (mol) | 1,00 | 0,95 |
| Weight resin (kg) | 1,56 | 1,48 |
| ∑ Weight AAs (kg) | 27,41 | 26,04 |
| ∑ Weight HBTU (kg) | 11,18 | 10,62 |
| ∑ Weight HOBtx$H_2O$ (kg) | 11,03 | 10,48 |
| ∑ Weight DIEA (kg) | 18,61 | 17,68 |
| ∑ Weight AAs/coupling agents (kg) | 69,79 | 66,30 |
| ∑ Weight pip (kg) | 120,60 | 114,57 |
| ∑ starting materials (kg) | **190,39** | **180,87** |
| ∑ solvents (kg) | **2782,80** | **2643,66** |
| peptide resin formed (kg) | 3,84 | 3,65 |

**Table S34.** Summary of amounts of raw materials used in 0,72 mol (reported scaled-up protocol) and 1 mol TFA cleavage of Boc-1-21-O-Cam-Leu peptide fragment resin, benchmark process 2.

| | | |
|---|---|---|
| Scale (mol) | 0,72 | 1,00 |
| TFA (kg) | 21,09 | 29,29 |
| TIS (kg) | 0,27 | 0,38 |
| $H_2O$ (kg) | 0,38 | 0,53 |
| ∑ Reagents (kg) | 21,74 | 30,19 |
| solvents (MTBE/heptanes, kg) | 106,50 | 147,92 |

**Table S35.** Summary of amounts of raw materials used in 0,62 mol (reported scaled-up protocol) and 0,95 mol TFA cleavage of H-22-39 peptide fragment resin, benchmark process 2.

| Scale (mol) | 0,64 | 0,95 |
|---|---|---|
| TFA (kg) | 21,09 | 31,31 |
| TIS (kg) | 0,27 | 0,40 |
| $H_2O$ (kg) | 0,38 | 0,56 |
| $\sum$ Reagents (kg) | 21,74 | 32,27 |
| solvents (MTBE/heptanes, kg) | 106,50 | 158,09 |

**Table S36.** Summary of amounts and cost of raw materials used in the SPPS of H-22-39 fragment resin and TFA cleavage of H-22-39 fragment resin, benchmark process 2.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (Fmoc-Rink amide) | 2800,0 | 1,5 | 4144,0 |
| HOBtx$H_2O$ | 62,0 | 10,5 | 649,8 |
| piperidine | 10,6 | 114,6 | 1214,8 |
| std AA | 400,0 | 26,0 | 10400,0 |
| DIEA | 59,3 | 17,7 | 1048,4 |
| DMF | 1,4 | 2643,7 | 3701,2 |
| HBTU | 150,0 | 10,6 | 1590,0 |
| TFA | 20,0 | 31,3 | 626,0 |
| TIS | 900,0 | 0,4 | 360,0 |
| MTBE | 4,3 | 79,0 | 339,7 |
| heptanes | 2,1 | 79,0 | 165,9 |
| $\sum$ | n.a. | n.a. | 24239,7 |

**Table S37.** Summary of amounts of raw materials used in enzymatic ligation, 0,0021 mol (reported) and 0,56 mol, benchmark process 2.

| Scale (mol) | 0,0021 | 0,56 |
|---|---|---|
| Amine F (kg) | 0,0043 | 1,16 |
| O-Cam F (kg) | 0,0057 | 1,53 |
| Enzyme (g) | 0,0170 | 4,53 |
| Solvent (kg) | 0,2636 | 70,30 |

**Table S38.** Summary of amounts of raw materials used in DSP of crude exenatide, benchmark process 2.

| Step | kg peptide | kg solvent |
|---|---|---|
| Amine fragment (1 RPC) | 1,16 | 1085,76 |
| O-Cam fragment (1 RPC) | 1,53 | 1432,08 |
| API (1 RPC) | 1,84 | 1722,24 |
| ∑ | 4,53 | 4240,08 |

**Table S39.** Summary of amounts and cost of raw materials used in the entire process, benchmark process 2.[1]

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (Fmoc-Leu Wang) | 4004,0 | 1,4 | 5565,6 |
| HOBtx$H_2O$ | 62,0 | 11,6 | 721,7 |
| piperidine | 10,6 | 120,0 | 1272,0 |
| std AA | 400,0 | 32,4 | 12968,0 |
| Fmoc-Phe-Thr(Psime,Mepro)-OH | 9000,0 | 2,1 | 18990,0 |
| DIEA | 59,3 | 20,7 | 1227,5 |
| DMF | 1,4 | 2843,7 | 3981,2 |
| HBTU | 150,0 | 10,8 | 1620,0 |
| TFA | 20,0 | 29,3 | 586,0 |
| TIS | 900,0 | 0,5 | 477,0 |
| MTBE | 4,3 | 74,0 | 318,2 |
| heptanes | 2,1 | 74,0 | 155,4 |
| Omniligase (cost in EUR/g) | 750,0 | 0,0045 | 3,4 |
| MeCN | 6,0 | 1272,0 | 7632,0 |
| ∑+O-Cam fragment+ ligation+DSP | n.a. | n.a. | 55517,9 |
| ∑ amine fragment | n.a. | n.a. | 24239,7 |
| ∑ | | | 79757,6 |
| ∑/1 kg API | | | 43346,5 |

[1]Weight of Omniligase is in g.

**Table S40.** Summary of total CI for organic solvents used in exenatide manufacturing, benchmark process 2.

| **Total organic solvents** | weight (kg) | ng solvent pro | CI / kg kg $^{-1}$ | $CI_{\sum}$ / kg kg $^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 5487,4 | 1,75 | 1,81 | 3,56 | 19535,14 |
| MTBE (cleavage) | 153,0 | 1,75 | 2,49 | 4,24 | 648,72 |
| heptanes (cleavage) | 153,0 | 0,86 | 3,07 | 3,93 | 601,29 |
| MeCN (DSP) | 1272,0 | 1,95 | 2,15 | 4,1 | 5215,20 |
| $\sum$ | | | | | 26000,35 |
| $\sum$/kg API | | | | | 14130,63 |

**Table S41.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing benchmark process 2.

| Energy & CI | Process step | | | | | $\sum$ | $\sum$ kg API$^{-1}$ |
|---|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | ligation[c] | DSP[d] | lyophilization[e] | | |
| energy used (kWh$^{-1}$) | 527,9 | 42,2 | 9,7 | 19,1 | 64,47 | 663,37 | 360,14 |
| cost of energy used (EUR) | 34,84 | 2,79 | 0,640 | 1,26 | 4,26 | 43,78 | 23,77 |
| CI for electricity generated (t $CO_2$) | 0,15 | 0,01 | 0,003 | 0,005 | 0,018 | 0,19 | 0,10 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,00 | 0,000 | 0,000 | 0,002 | 0,02 | 0,01 |
| CI for electricity used (t $CO_2$) | 0,16 | 0,01 | 0,003 | 0,006 | 0,020 | 0,20 | 0,11 |
| CI for electricity $\sum$ (t $CO_2$) | 0,32 | 0,03 | 0,006 | 0,012 | 0,040 | 0,41 | 0,22 |

[a]energy used=[amine fragment: 18 (AA cycles) x 6 (duration of AA cycle in h) x 47,5 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 4,5 (amount of energy needed to stir nominal amount of solvent for 1 h)]+[O-Cam fragment: 22 (AA cycles) x 6 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 4,5 (amount of energy needed to stir nominal amount of solvent for 1 h)]; [b]367 (total amount of TFA+solvent in kg, amine+O-Cam fragments) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]70,3 (total amount of ligation solvent in kg) x 3 (total time ligation h)/10 (nominal amount of solvent in kg) x 0,38 (amount of energy needed to stir nominal amount of solvent for 1 h) [d]4240 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [e]1,842 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API).

**35.4. O-Cam fragment CEPS (C+C)**

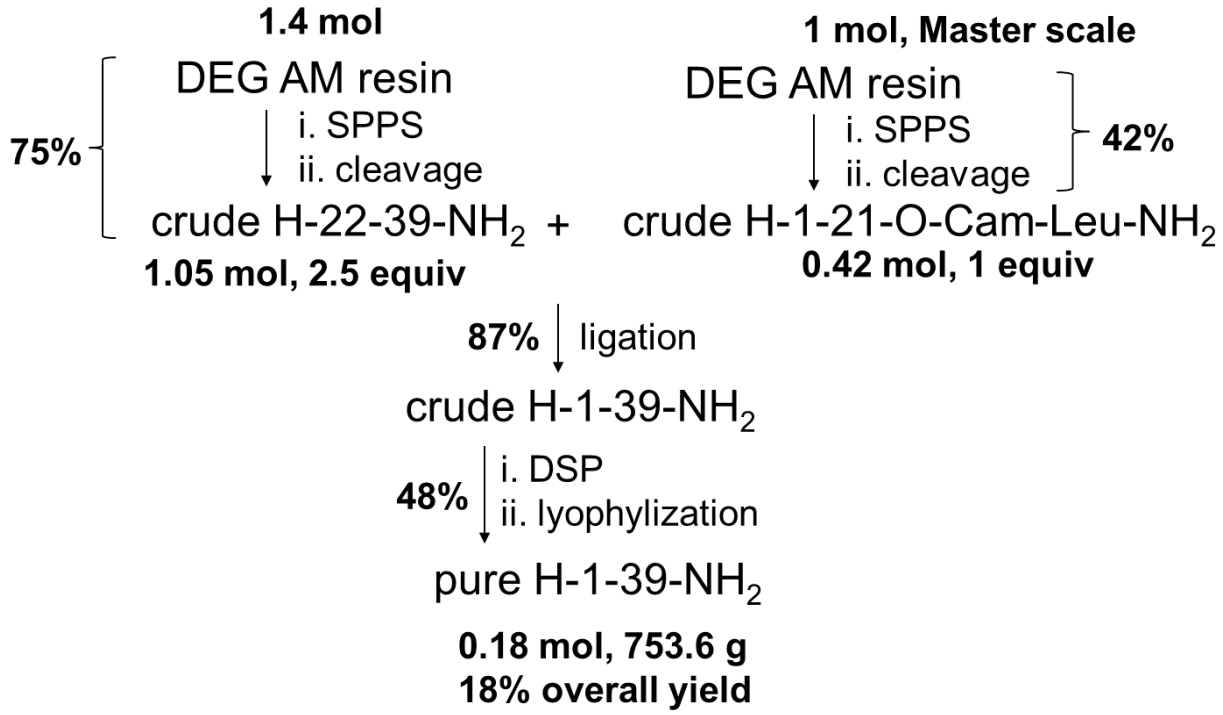



**Figure S50.** A schematic representation of the flow chart of the O-Cam fragment CEPS (C+C) exenatide manufacturing process herein.

**Table S42.** Overview of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| AA nr | AA | MW | **mol AA** | **equiv AA** | **kg AA** | **kg Oxyma** | **Total kg DIC** | **kg TBTU** | **kg NMM** | kg pip | kg DMF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| resin | DEG AM, 0.77 mmol/g, 1.77 kg | n.a. | 1,15 | **1,36** | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| linker | Fmoc-RMG-OH | 505,60 | 1,08 | **1,32** | 0,57 | 0,15 | 0,34 | n.a. | n.a. | 3,13 | 92,50 |
| Leu-linker | Fmoc-Leu-OH | 354,41 | 2,00 | **1,27** | 0,75 | 0,28 | 0,63 | n.a. | n.a. | 3,13 | 92,50 |
| $Leu^{21}$-O-Cam | Fmoc-Leu-O-Cam-OH | 411,45 | 1,00 | 1,00 | 0,43 | 0,14 | 0,32 | n.a. | n.a. | 3,13 | 92,50 |
| $Arg^{20}$ | Fmoc-Arg(Pbf)-OH | 434,49 | 1,90 | 1,90 | 0,87 | n.a. | n.a. | 0,61 | 0,38 | 1,38 | 80,00 |
| $Val^{19}$ | Fmoc-Val-OH | 337,37 | 1,90 | 1,90 | 0,67 | n.a. | n.a. | 0,61 | 0,38 | 1,44 | 80,00 |
| $Ala^{18}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 1,90 | 1,90 | 0,66 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Glu^{17}$ | Fmoc-Glu-OHx$H_2O$ | 297,30 | 1,90 | 1,90 | 0,59 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Glu^{16}$ | Fmoc-Glu-OHx$H_2O$ | 569,66 | 1,90 | 1,90 | 1,14 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Glu^{15}$ | Fmoc-Glu-OHx$H_2O$ | 569,66 | 1,90 | 1,90 | 1,14 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Met^{14}$ | Fmoc-Met-OH | 337,37 | 1,90 | 1,90 | 0,67 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Gln^{13}$ | Fmoc-Gln(Trt)-OH | 354,40 | 1,90 | 1,90 | 0,71 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Lys^{12}$ | Fmoc-Lys(Trt)-OH | 596,67 | 1,90 | 1,90 | 1,19 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Ser^{11}$ | Fmoc-Ser(*t*-Bu)-OH | 610,8 | 1,90 | 1,90 | 1,22 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Leu^{10}$ | Fmoc-Leu-OH | 354,41 | 1,90 | 1,90 | 0,71 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Asp^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 526,59 | 1,90 | 1,90 | 1,05 | n.a. | n.a. | 0,61 | 0,38 | 2,75 | 92,50 |
| $Ser^{8}$ | Fmoc-Ser(*t*-Bu)-OH | 443,49 | 1,90 | 1,90 | 0,89 | n.a. | n.a. | 0,61 | 0,38 | 5,00 | 92,50 |
| $Thr^{7}$ | Fmoc-Thr(*t*- Bu)-OH | 354,41 | 1,90 | 1,90 | 0,71 | n.a. | n.a. | 0,61 | 0,38 | 5,00 | 92,50 |
| $Phe^{6}$ | Fmoc-Phe-OH | 387,45 | 3,80 | 3,80 | 1,55 | n.a. | n.a. | 1,22 | 0,77 | 12,50 | 147,50 |
| $Thr^{5}$ | Fmoc-Thr(*t*- Bu)-OH | 387,45 | 5,70 | 5,70 | 2,32 | n.a. | n.a. | 1,83 | 1,15 | 5,00 | 127,50 |
| $Gly^{4}$ | Fmoc-Gly-OH | 387,45 | 1,90 | 1,90 | 0,77 | n.a. | n.a. | 0,61 | 0,38 | 5,00 | 92,50 |
| $Glu^{3}$ | Fmoc-Glu-OHx$H_2O$ | 387,45 | 1,90 | 1,90 | 0,77 | n.a. | n.a. | 0,61 | 0,38 | 5,00 | 92,50 |
| $His^{1}$-$Gly^{2}$ | Boc-His(Trt)-Gly-OH | 387,45 | 1,90 | 1,90 | 0,77 | n.a. | n.a. | 0,61 | 0,38 | 5,00 | 92,50 |
| $\sum$ | | | | | 20,19 | 0,58 | 1,29 | 13,42 | 8,46 | 82,21 | 2100,00 |

**Table S43.** Overview of raw materials used in 1 mol SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| AA nr | AA | MW | **mol AA** | **equiv AA** | **kg AA** | **kg Oxyma** | **Total kg DIC** | **kg pip** | **kg DMF** |
|---|---|---|---|---|---|---|---|---|---|
| resin | DEG AM, 0.68 mmol/g, 1.69 kg | n.a. | 1,15 | **1,15** | n.a. | n.a. | n.a. | n.a. | n.a. |
| linker | Fmoc-RMG-OH | 505,60 | 1,08 | **1,08** | 0,57 | 0,15 | 0,34 | 3,13 | 92,50 |
| $Ser^{39}$ | Fmoc-Ser(Trt)-OH | 569,66 | 1,00 | **1,00** | 0,60 | 0,14 | 0,32 | 3,13 | 92,50 |
| $Pro^{38-37}$ | Fmoc-Pro-Pro-OH | 434,49 | 1,00 | **1,00** | 0,43 | 0,14 | 0,32 | 3,13 | 92,50 |
| $Pro^{36}$ | Fmoc-Pro-OH | 337,37 | 2,00 | **2,00** | 0,67 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ala^{35}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 2,00 | **2,00** | 0,66 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Gly^{34}$ | Fmoc-Gly-OH | 297,30 | 2,00 | **2,00** | 0,59 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{33}$ | Fmoc-Ser(Trt)-OH | 569,66 | 2,00 | **2,00** | 1,20 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{32}$ | Fmoc-Ser(Trt)-OH | 569,66 | 2,00 | **2,00** | 1,20 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Pro^{31}$ | Fmoc-Pro-OH | 337,37 | 2,00 | **2,00** | 0,71 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Gly^{30-29}$ | Fmoc-Gly-Gly-OH | 354,40 | 1,50 | **1,50** | 0,56 | 0,21 | 0,47 | 3,13 | 92,50 |
| $Asn^{28}$ | Fmoc-Asn(Trt)-OH | 596,67 | 2,00 | **2,00** | 1,26 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Lys^{27}$ | Fmoc-Lys(Trt)-OH | 610,8 | 1,50 | **1,50** | 0,96 | 0,21 | 0,47 | 3,13 | 92,50 |
| $Leu^{26}$ | Fmoc-Leu-OH | 354,41 | 2,00 | **2,00** | 0,75 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Trp^{25}$ | Fmoc-Trp(Boc)-OH | 526,59 | 4,00 | 4,00 | 2,22 | 0,57 | 1,26 | 3,13 | 110,00 |
| $Glu^{24}$ | Fmoc-Glu(OtBu)-OHx$H_2O$ | 443,49 | 4,00 | 4,00 | 1,87 | 0,57 | 1,26 | 3,13 | 110,00 |
| $Ile^{23}$ | Fmoc-Ile-OH | 354,41 | 4,00 | 4,00 | 1,49 | 0,57 | 1,26 | 3,13 | 110,00 |
| $Phe^{22}$ | Fmoc-Phe-OH | 387,45 | 4,00 | 4,00 | 1,63 | 0,57 | 1,26 | 3,13 | 110,00 |
| $\Sigma$ | | | | | 17,38 | 5,41 | 12,01 | 53,21 | 1642,50 |

**Table S44.** Summary of amounts of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,00 |
| Weight resin (kg) | 1,77 |
| ∑ Weight AAs (kg) | 20,19 |
| ∑ Weight Oxyma (kg) | 0,58 |
| ∑ Weight DIC (kg) | 1,29 |
| ∑ Weight TBTU (kg) | 13,42 |
| ∑ Weight NMM (kg) | 8,46 |
| ∑ Weight resin/AAs/coupling agents (kg) | 45,71 |
| ∑ Weight pip (kg) | 82,21 |
| ∑ starting materials (kg) | **127,92** |
| ∑ solvents (kg) | **2100** |
| peptide resin formed (kg) | **5,73** |

**Table S45.** Summary of amounts of raw materials used in 1 mol (nominal) and 1,4 mol (actual) SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| | | |
|---|---|---|
| Scale (mol) | 1,00 | 1,40 |
| Weight resin (kg) | 1,69 | 2,37 |
| ∑ Weight AAs (kg) | 17,38 | 24,33 |
| ∑ Weight Oxyma (kg) | 5,41 | 7,57 |
| ∑ Weight DIC (kg) | 12,01 | 16,81 |
| ∑ Weight resin/AAs/coupling agents (kg) | 36,49 | 51,09 |
| ∑ Weight pip (kg) | 53,21 | 74,49 |
| ∑ starting materials (kg) | **89,7** | **125,58** |
| ∑ solvents (kg) | **1642,5** | **2299,50** |
| peptide resin formed (kg) | **5,5** | **7,70** |

**Table S46.** Summary of amounts of raw materials used in 1 mol TFA cleavage of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,00 |
| TFA (kg) | 38,00 |
| TIS (kg) | 0,84 |
| $H_2O$ (kg) | 0,30 |
| DTT (kg) | 0,86 |
| $NH_4I$ (kg) | 0,57 |
| $\sum$ Reagents (kg) | 40,57 |
| solvents (ether, kg) | 129,77 |

**Table S47.** Summary of amounts of raw materials used in 1,40 mol TFA cleavage of H-22-39 peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,40 |
| TFA (kg) | 60,28 |
| TIS (kg) | 1,12 |
| $H_2O$ (kg) | 0,38 |
| DTT (kg) | 1,15 |
| $\sum$ Reagents (kg) | 62,92 |
| solvents (ether, kg) | 271,09 |

**Table S48.** Summary of amounts and cost of raw materials used in the SPPS of H-22-39 fragment resin and TFA cleavage of H-22-39 fragment resin, O-Cam fragment CEPS (C+C) process.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 2,4 | 3081,0 |
| Oxyma | 85,0 | 7,6 | 643,5 |
| piperidine | 10,6 | 74,5 | 789,6 |
| std AA | 400,0 | 24,3 | 9732,0 |
| DIC | 240,0 | 16,8 | 4034,4 |
| DMF | 1,4 | 2299,5 | 3219,3 |
| TFA | 20,0 | 60,3 | 1205,6 |
| TIS | 900,0 | 1,1 | 1008,0 |
| DTT | 668,0 | 1,2 | 768,2 |
| Ether | 9,0 | 271,1 | 2439,9 |
| $\sum$ | n.a. | n.a. | 26921,4 |

**Table S49.** Summary of amounts of raw materials used in enzymatic ligation, O-Cam fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 0,42 |
| Amine F (kg) | 2,72 |
| O-Cam F (kg) | 3,94 |
| TCEPxHCl (kg) | 0,10 |
| Enzyme (g) | 3,90 |
| Solvent (kg) | 64,29 |

**Table S50.** Summary of amounts of raw materials used in DSP of crude exenatide, O-Cam fragment CEPS (C+C) process.

| Step | kg peptide | kg solvent |
|---|---|---|
| API (3 RPC) | 0,75 | 2106 |

**Table S51.** Summary of amounts and cost of raw materials used in the entire process, O-Cam fragment CEPS (C+C) process.[1]

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 1,8 | 2301,0 |
| Oxyma | 85,0 | 0,6 | 51,0 |
| piperidine | 10,6 | 82,2 | 871,4 |
| std AA | 400,0 | 19,0 | 7596,0 |
| dipeptide | 1500,0 | 1,2 | 1800,0 |
| DIC | 240,0 | 1,3 | 312,0 |
| NMM | 15,0 | 8,5 | 126,9 |
| DMF | 1,4 | 2100,0 | 2940,0 |
| TBTU | 150,0 | 13,4 | 2013,0 |
| TFA | 20,0 | 38,0 | 760,0 |
| TIS | 900,0 | 0,8 | 756,0 |
| DTT | 668,0 | 0,9 | 574,5 |
| $NH_4I$ | 450,0 | 0,6 | 256,5 |
| Ether | 9,0 | 129,8 | 1168,2 |
| $TCEP^xHCl$ | 582,0 | 0,1 | 58,2 |
| Omniligase (EUR/g) | 750,0 | 3,9 | 2925,0 |
| MeCN | 6,0 | 638,2 | 3829,2 |
| ∑+O-Cam fragment+ ligation+DSP | n.a. | n.a. | 28338,9 |
| ∑ amine fragment | n.a. | n.a. | 26921,4 |
| ∑ | n.a. | n.a. | 55260,3 |
| ∑/1 kg API | n.a. | n.a. | 73680,4 |

[1]Weight of Omniligase is in g.

**Table S52.** Summary of total CI for organic solvents used in exenatide manufacturing, O-Cam fragment CEPS (C+C) process.

| **Total organic solvents** | weight (kg) | $CO_2$ formed during solvent production / kg kg $^{-1}$ | CI / kg kg $^{-1}$ | $CI_{\sum}$ / kg kg $^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 4399,5 | 1,75 | 1,81 | 3,56 | 15662,22 |
| Ether (cleavage) | 400,9 | 1,08 | 2,37 | 3,45 | 1383,11 |
| MeCN (DSP+ligation) | 638,2 | 1,95 | 2,15 | 4,1 | 2616,62 |
| $\sum$ | | | | | 19661,95 |
| $\sum$/kg API | | | | | 26215,93 |

**Table S53.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing, O-Cam fragment CEPS (C+C) process.

| Energy & CI | Process step | | | | | ∑ | ∑ kg API$^{-1}$ |
|---|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | ligation[c] | DSP[d] | lyophilization[e] | | |
| energy used (kWh$^{-1}$) | 419,1 | 51,2 | 7,3 | 9,5 | 26,39 | 513,49 | 681,02 |
| cost of energy used (EUR) | 27,66 | 3,38 | 0,482 | 0,63 | 1,74 | 33,89 | 44,95 |
| CI for electricity generated (t $CO_2$) | 0,12 | 0,01 | 0,002 | 0,003 | 0,007 | 0,15 | 0,19 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,00 | 0,000 | 0,000 | 0,001 | 0,01 | 0,02 |
| CI for electricity used (t $CO_2$)[f] | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| CI for electricity ∑ (t $CO_2$) | 0,13 | 0,02 | 0,002 | 0,003 | 0,008 | 0,16 | 0,21 |

[a]energy used=[amine fragment: 17 (AA cycles) x 3 (duration of AA cycle in h) x 70 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]+[O-Cam fragment: 22 (AA cycles) x 3 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]; [b]445 (total amount of TFA+solvent in kg, amine+O-Cam fragments) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]64,3 (total amount of ligation solvent in kg) x 3 (total time ligation h)/10 (nominal amount of solvent in kg) x 0,38 (amount of energy needed to stir nominal amount of solvent for 1 h) [d]2106 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [e]0,754 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API); [f]wind energy was used in this exenatide API manufacture, the use of electricity was assumed to be $CO_2$ neutral.

**35.5. O-Cam fragment CEPS (P+P)**

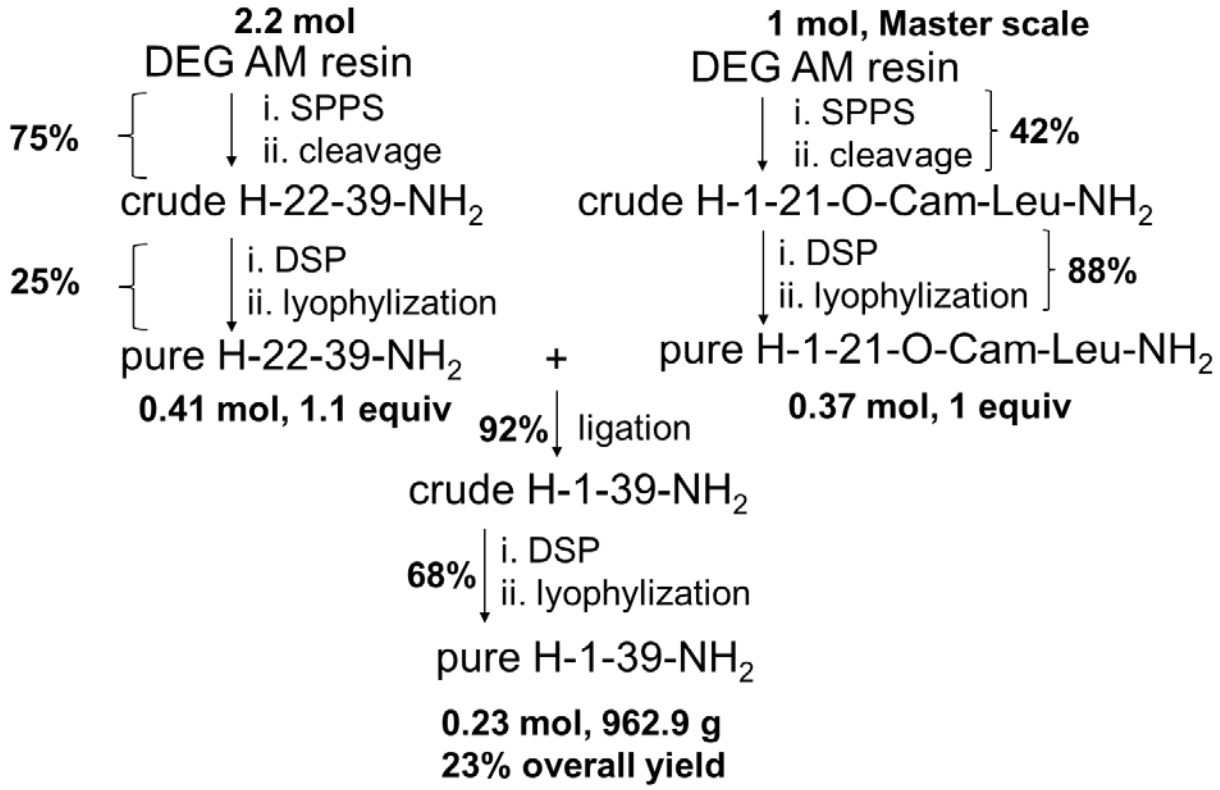



**Figure S51.** A schematic representation of the flow chart of the O-Cam fragment CEPS (P+P) exenatide manufacturing process herein.

For an overview of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (P+P) process see Table S42.

For an overview of raw materials used in 1 mol SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS (P+P) process see Table S43.

For a **s**ummary of amounts of raw materials used in 1 mol SPPS of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (P+P) process see Table S44.

**Table S54.** Summary of amounts of raw materials used in 1 mol (nominal) and 2,2 mol (actual) SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS (P+P) process.

| Scale (mol) | 1,00 | 2,20 |
|---|---|---|
| Weight resin (kg) | 1,69 | 3,72 |
| ∑ Weight AAs (kg) | 17,38 | 38,24 |
| ∑ Weight Oxyma (kg) | 5,41 | 11,90 |
| ∑ Weight DIC (kg) | 12,01 | 26,42 |
| ∑ Weight resin/AAs/coupling agents (kg) | 36,49 | 80,28 |
| ∑ Weight pip (kg) | 53,21 | 117,06 |
| ∑ starting materials (kg) | **89,7** | **197,34** |
| ∑ solvents (kg) | **1642,5** | **3613,50** |
| peptide resin formed (kg) | **5,5** | **12,10** |

For a summary of amounts of raw materials used in 1 mol TFA cleavage of Boc-1-21-O-Cam-Leu peptide fragment resin, O-Cam fragment CEPS (P+P) process see Table S46.

**Table S55.** Summary of amounts of raw materials used in 2,20 mol TFA cleavage of H-22-39 peptide fragment resin, O-Cam fragment CEPS (P+P) process.

| Scale (mol) | 2,20 |
|---|---|
| TFA (kg) | 94,72 |
| TIS (kg) | 1,76 |
| $H_2O$ (kg) | 0,60 |
| DTT (kg) | 1,80 |
| ∑ Reagents (kg) | 98,88 |
| solvents (ether, kg) | 426,00 |

**Table S56.** Summary of amounts and cost of raw materials used in the SPPS of H-22-39 fragment resin and TFA cleavage of H-22-39 fragment resin, O-Cam fragment CEPS (P+P) process.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 3,7 | 4841,6 |
| Oxyma | 85,0 | 11,9 | 1011,1 |
| piperidine | 10,6 | 117,1 | 1240,8 |
| std AA | 400,0 | 38,2 | 15293,1 |
| DIC | 240,0 | 26,4 | 6339,8 |
| DMF | 1,4 | 3613,5 | 5058,9 |
| TFA | 20,0 | 94,7 | 1894,5 |
| TIS | 900,0 | 1,8 | 1584,0 |
| DTT | 668,0 | 1,8 | 1207,2 |
| Ether | 9,0 | 426,0 | 3834,1 |
| $\sum$ | n.a. | n.a. | 42305,1 |

**Table S57.** Summary of amounts of raw materials used in enzymatic ligation, O-Cam fragment CEPS (P+P) process.

| | |
|---|---|
| Scale (mol) | 0,37 |
| Amine F (kg) | 0,81 |
| O-Cam F (kg) | 0,94 |
| TCEPxHCl (kg) | 0,07 |
| Enzyme (g) | 2,73 |
| Solvent (kg) | 45,00 |

**Table S58.** Summary of amounts of raw materials used in DSP of crude exenatide, O-Cam fragment CEPS (P+P) process.

| Step | kg peptide | kg solvent |
|---|---|---|
| Amine fragment (1 RPC) | 0,81 | 758,16 |
| O-Cam fragment (1 RPC) | 0,94 | 1759,68 |
| API (2 RPC) | 0,96 | 1797,12 |
| $\sum$ | 2,71 | 4314,96 |

**Table S59.** Summary of amounts and cost of raw materials used in the entire process, O-Cam fragment CEPS (P+P) process.[1]

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 1,8 | 2301,0 |
| Oxyma | 85,0 | 0,6 | 51,0 |
| piperidine | 10,6 | 82,2 | 871,4 |
| std AA | 400,0 | 19,0 | 7596,0 |
| dipeptide | 1500,0 | 1,2 | 1800,0 |
| DIC | 240,0 | 1,3 | 312,0 |
| NMM | 15,0 | 8,5 | 126,9 |
| DMF | 1,4 | 2100,0 | 2940,0 |
| TBTU | 150,0 | 13,4 | 2013,0 |
| TFA | 20,0 | 38,0 | 760,0 |
| TIS | 900,0 | 0,8 | 756,0 |
| DTT | 668,0 | 0,9 | 574,5 |
| $NH_4I$ | 450,0 | 0,6 | 256,5 |
| Ether | 9,0 | 129,8 | 1168,2 |
| $TCEP^xHCl$ | 582,0 | 0,1 | 40,7 |
| Omniligase (EUR/g) | 750,0 | 2,7 | 2047,5 |
| MeCN | 6,0 | 1299,0 | 7794,0 |
| ∑+O-Cam fragment+ ligation+DSP | n.a. | n.a. | 31408,7 |
| ∑ amine fragment | n.a. | n.a. | 42305,1 |
| ∑ | n.a. | n.a. | 73713,8 |
| ∑/1 kg API | n.a. | n.a. | 76785,3 |

[1]Weight of Omniligase is in g.

**Table S60.** Summary of total CI for organic solvents used in exenatide manufacturing, O-Cam fragment CEPS (P+P) process.

| **Total organic solvents** | weight (kg) | $CO_2$ formed during solvent production / kg kg $^{-1}$ | CI / kg kg $^{-1}$ | $CI_{\sum}$ / kg kg $^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 5713,5 | 1,75 | 1,81 | 3,56 | 20340,06 |
| Ether (cleavage) | 555,8 | 1,08 | 2,37 | 3,45 | 1917,51 |
| MeCN (DSP+ligation) | 1299,0 | 1,95 | 2,15 | 4,1 | 5325,90 |
| $\sum$ | | | | | 27583,47 |
| $\sum$/kg API | | | | | 28732,78 |

**Table S61.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing, O-Cam fragment CEPS (P+P) process.

| Energy & CI | Process step | | | | | $\sum$ | $\sum$ kg API$^{-1}$ |
|---|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | ligation[c] | DSP[d] | lyophilization[e] | | |
| energy used (kWh$^{-1}$) | 543,5 | 79,2 | 5,1 | 19,4 | 33,67 | 680,87 | 707,77 |
| cost of energy used (EUR) | 35,87 | 5,23 | 0,337 | 1,28 | 2,22 | 44,94 | 46,71 |
| CI for electricity generated (t $CO_2$) | 0,15 | 0,02 | 0,001 | 0,005 | 0,010 | 0,19 | 0,20 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,00 | 0,000 | 0,000 | 0,001 | 0,02 | 0,02 |
| CI for electricity used (t $CO_2$)[f] | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| CI for electricity $\sum$ (t $CO_2$) | 0,17 | 0,02 | 0,002 | 0,006 | 0,010 | 0,21 | 0,22 |

[a]energy used=[amine fragment: 17 (AA cycles) x 3 (duration of AA cycle in h) x 110 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]+[O-Cam fragment: 22 (AA cycles) x 3 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]; [b]689 (total amount of TFA+solvent in kg, amine+O-Cam fragments) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]45 (total amount of ligation solvent in kg) x 3 (total time ligation h)/10 (nominal amount of solvent in kg) x 0,38 (amount of energy needed to stir nominal amount of solvent for 1 h) [d]4315 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [e]0,962 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API); [f]wind energy was used in this exenatide API manufacture, the use of electricity was assumed to be $CO_2$ neutral.

**35.6. HMBA fragment CEPS (C+C)**

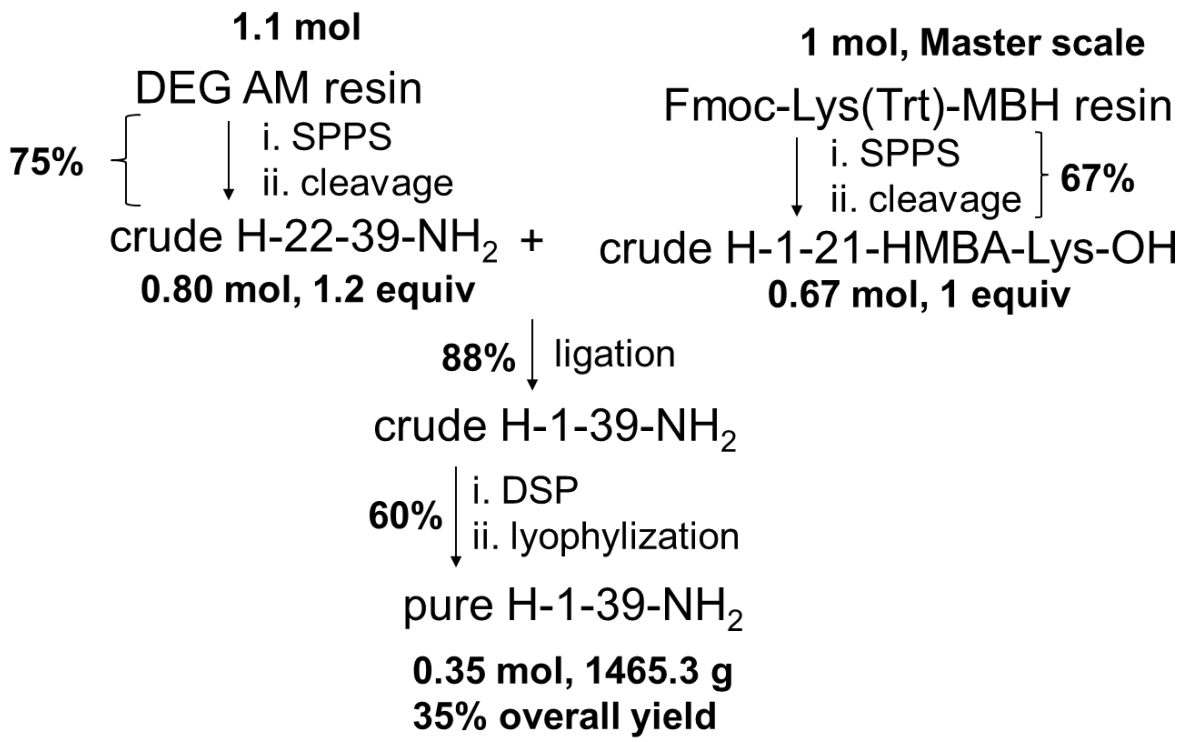



**Figure S52.** A schematic representation of the flow chart of the HMBA fragment CEPS (C+C) exenatide manufacturing process herein.

**Table S62.** Overview of raw materials used in 1 mol SPPS of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS (C+C) process.

| AA nr | AA | MW | mol AA | equiv AA | kg AA | kg Oxyma | Total kg DIC | kg pip | kg DMF |
|---|---|---|---|---|---|---|---|---|---|
| resin | Fmoc-K(Trt)-MBH resin, 0.52 mmol/g, 1.92 kg | n.a. | 1,00 | **1,00** | n.a. | n.a. | n.a. | n.a. | n.a. |
| linker | HMBA | 152,15 | 2,00 | **2,00** | 0,32 | 0,57 | 0,50 | 3,13 | 92,50 |
| $Leu^{21}$ | Fmoc-Leu-OH[1] | 354,41 | 4,00 | **4,00** | 1,49 | 0,68 | 0,50 | 0,00 | 17,50 |
| $Arg^{20}$ | Fmoc-Arg(Pbf)-OH | 648,77 | 2,00 | 2,00 | 1,37 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Val^{19}$ | Fmoc-Val-OH | 339,39 | 2,00 | 2,00 | 0,71 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ala^{18}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 2,00 | 2,00 | 0,69 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{17}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{16}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{15}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Met^{14}$ | Fmoc-Met-OH | 371,45 | 2,00 | 2,00 | 0,78 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Gln^{13}$ | Fmoc-Gln(Trt)-OH | 610,70 | 2,00 | 2,00 | 1,29 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Lys^{12}$ | Fmoc-Lys(Trt)-OH | 610,74 | 2,00 | 2,00 | 1,29 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{11}$ | Fmoc-Ser(Trt)-OH | 569,65 | 2,00 | 2,00 | 1,20 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Leu^{10}$ | Fmoc-Leu-OH | 354,41 | 2,00 | 2,00 | 0,75 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Asp^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 411,45 | 2,00 | 2,00 | 0,87 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{8}$ | Fmoc-Ser(Trt)-OH | 569,65 | 2,20 | 2,20 | 1,32 | 0,31 | 0,69 | 3,13 | 92,50 |
| $Thr^{7}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 2,20 | 2,20 | 0,92 | 0,31 | 0,69 | 3,13 | 92,50 |
| $Phe^{6}$ | Fmoc-Phe-OH | 387,45 | 2,20 | 2,20 | 0,90 | 0,31 | 0,69 | 3,13 | 92,50 |
| $Thr^{5}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 2,20 | 2,20 | 0,92 | 0,31 | 0,69 | 3,13 | 92,50 |
| $Gly^{4}$ | Fmoc-Gly-OH | 297,31 | 2,20 | 2,20 | 0,69 | 0,31 | 0,69 | 4,69 | 105,00 |
| $Glu^{3}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,20 | 2,20 | 0,99 | 0,31 | 0,69 | 3,13 | 92,50 |
| $His^{1}$-$Gly^{2}$ | Boc-His(Trt)-Gly-OH | 554,65 | 2,20 | 2,20 | 1,28 | 0,31 | 0,69 | 3,13 | 92,50 |
| ∑ | | | | | 20,45 | 6,85 | 13,44 | 64,16 | 1880,00 |

[1]Cl-HOBt used instead of Oxyma

For an overview of raw materials used in 1 mol SPPS of H-22-39-peptide fragment resin, HMBA fragment CEPS (C+C) process see Table S43.

**Table S63.** Summary of amounts of raw materials used in 1 mol SPPS of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,00 |
| Weight resin (kg) | 1,92 |
| ∑ Weight AAs (kg) | 20,45 |
| ∑ Weight Oxyma (kg) | 6,85 |
| ∑ Weight DIC (kg) | 13,44 |
| ∑ Weight resin/AAs/coupling agents (kg) | 42,66 |
| ∑ Weight pip (kg) | 64,16 |
| ∑ starting materials (kg) | **106,82** |
| ∑ solvents (kg) | **1880** |
| peptide resin formed (kg) | **5,53** |

**Table S64.** Summary of amounts of raw materials used in 1,10 mol SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,10 |
| Weight resin (kg) | 1,86 |
| ∑ Weight AAs (kg) | 19,12 |
| ∑ Weight Oxyma (kg) | 5,95 |
| ∑ Weight DIC (kg) | 13,21 |
| ∑ Weight resin/AAs/coupling agents (kg) | 40,14 |
| ∑ Weight pip (kg) | 58,53 |
| ∑ starting materials (kg) | **98,67** |
| ∑ solvents (kg) | **1806,75** |
| peptide resin formed (kg) | **6,05** |

**Table S65.** Summary of amounts of raw materials used in 1 mol TFA cleavage of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,00 |
| TFA (kg) | 27,75 |
| TIS (kg) | 0,61 |
| $H_2O$ (kg) | 0,21 |
| DTT (kg) | 0,63 |
| $NH_4I$ (kg) | 0,42 |
| ∑ Reagents (kg) | 29,61 |
| solvents (ether, kg) | 99,03 |

**Table S66.** Summary of amounts of raw materials used in 1,1 mol TFA cleavage of H-22-39 peptide fragment resin, HMBA fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,10 |
| TFA (kg) | 47,36 |
| TIS (kg) | 0,88 |
| $H_2O$ (kg) | 0,30 |
| DTT (kg) | 0,90 |
| ∑ Reagents (kg) | 49,44 |
| solvents (ether, kg) | 213,00 |

**Table S67.** Summary of amounts and cost of raw materials used in the SPPS of H-22-39 fragment resin and TFA cleavage of H-22-39 fragment resin, HMBA fragment CEPS (C+C) process.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 1,9 | 2420,8 |
| Oxyma | 85,0 | 5,9 | 505,6 |
| piperidine | 10,6 | 58,5 | 620,4 |
| std AA | 400,0 | 19,1 | 7646,6 |
| DIC | 240,0 | 13,2 | 3169,9 |
| DMF | 1,4 | 1806,8 | 2529,5 |
| TFA | 20,0 | 47,4 | 947,3 |
| TIS | 900,0 | 0,9 | 792,0 |
| DTT | 668,0 | 0,9 | 603,6 |
| Ether | 9,0 | 213,0 | 1917,1 |
| $\sum$ | n.a. | n.a. | 21152,6 |

**Table S68.** Summary of amounts of raw materials used in enzymatic ligation, HMBA fragment CEPS (C+C) process.

| | |
|---|---|
| Scale (mol) | 0,67 |
| Amine F (kg) | 2,08 |
| HMBA F (kg) | 3,11 |
| TCEPxHCl (kg) | 0,17 |
| Enzyme (g) | 8,93 |
| Solvent (kg) | 11,39 |

**Table S69.** Summary of amounts of raw materials used in DSP of crude exenatide, HMBA fragment CEPS (C+C) process.

| Step | kg peptide | kg solvent |
|---|---|---|
| API (2 RPC) | 1,47 | 2751,84 |

**Table S70.** Summary of amounts and cost of raw materials used in the entire process, HMBA fragment CEPS (C+C) process.[1]

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (Fmoc-K(Trt)-MBH) | 4000,0 | 1,9 | 7680,0 |
| Oxyma | 85,0 | 6,9 | 582,3 |
| piperidine | 10,6 | 64,2 | 680,1 |
| std AA | 400,0 | 19,2 | 7668,0 |
| dipeptide | 1500,0 | 1,3 | 1920,0 |
| DIC | 240,0 | 13,4 | 3225,6 |
| DMF | 1,4 | 1880,0 | 2632,0 |
| TFA | 20,0 | 27,8 | 555,0 |
| TIS | 900,0 | 0,6 | 549,0 |
| DTT | 668,0 | 0,6 | 420,8 |
| $NH_4I$ | 450,0 | 0,4 | 189,0 |
| Ether | 9,0 | 99,0 | 891,3 |
| $TCEP^xHCl$ | 582,0 | 0,2 | 98,9 |
| Omniligase (EUR/g) | 750,0 | 8,9 | 6697,5 |
| MeCN | 6,0 | 826,7 | 4960,1 |
| ∑+HMBA fragment+ ligation+DSP | n.a. | n.a. | 38749,6 |
| ∑ amine fragment | n.a. | n.a. | 21152,6 |
| ∑ | | | 59902,2 |
| ∑/1 kg API | | | 40749,8 |

[1]Weight of Omniligase is in g.

**Table S71.** Summary of total CI for organic solvents used in exenatide manufacturing, HMBA fragment CEPS (C+C) process.

| **Total organic solvents** | weight (kg) | $CO_2$ formed during solvent production / kg kg $^{-1}$ | CI / kg kg $^{-1}$ | $CI_{\sum}$ / kg kg $^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 3686,8 | 1,75 | 1,81 | 3,56 | 13125,01 |
| Ether (cleavage) | 312 | 1,08 | 2,37 | 3,45 | 1076,40 |
| MeCN (DSP+ligation) | 826,7 | 1,95 | 2,15 | 4,1 | 3389,47 |
| $\sum$ | | | | | 17590,88 |
| $\sum$/kg API | | | | | 11966,58 |

**Table S72.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing, HMBA fragment CEPS (C+C) process.

| Energy & CI | Process step | | | | | $\sum$ | $\sum$ kg API$^{-1}$ |
|---|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | ligation[c] | DSP[d] | lyophilization[e] | | |
| energy used (kWh$^{-1}$) | 363,3 | 44,5 | 1,3 | 12,4 | 51,3 | 472,80 | 322,73 |
| cost of energy used (EUR) | 23,98 | 2,94 | 0,086 | 0,82 | 3,39 | 31,20 | 21,30 |
| CI for electricity generated (t $CO_2$) | 0,10 | 0,01 | 0,000 | 0,004 | 0,015 | 0,13 | 0,09 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,00 | 0,000 | 0,000 | 0,001 | 0,01 | 0,01 |
| CI for electricity used (t $CO_2$)[f] | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| CI for electricity $\sum$ (t $CO_2$) | 0,11 | 0,01 | 0,000 | 0,004 | 0,016 | 0,15 | 0,10 |

[a]energy used=[amine fragment: 17 (AA cycles) x 3 (duration of AA cycle in h) x 55 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]+[HMBA fragment: 21 (AA cycles) x 3 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]; [b]387 (total amount of TFA+solvent in kg, amine+HMBA fragments) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]11,4 (total amount of ligation solvent in kg) x 3 (total time ligation h)/10 (nominal amount of solvent in kg) x 0,38 (amount of energy needed to stir nominal amount of solvent for 1 h) [d]2752 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [e]1,465 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API); [f]wind energy was used in this exenatide API manufacture, the use of electricity was assumed to

**35.7. HMBA fragment CEPS, pseudoproline (C+C)**

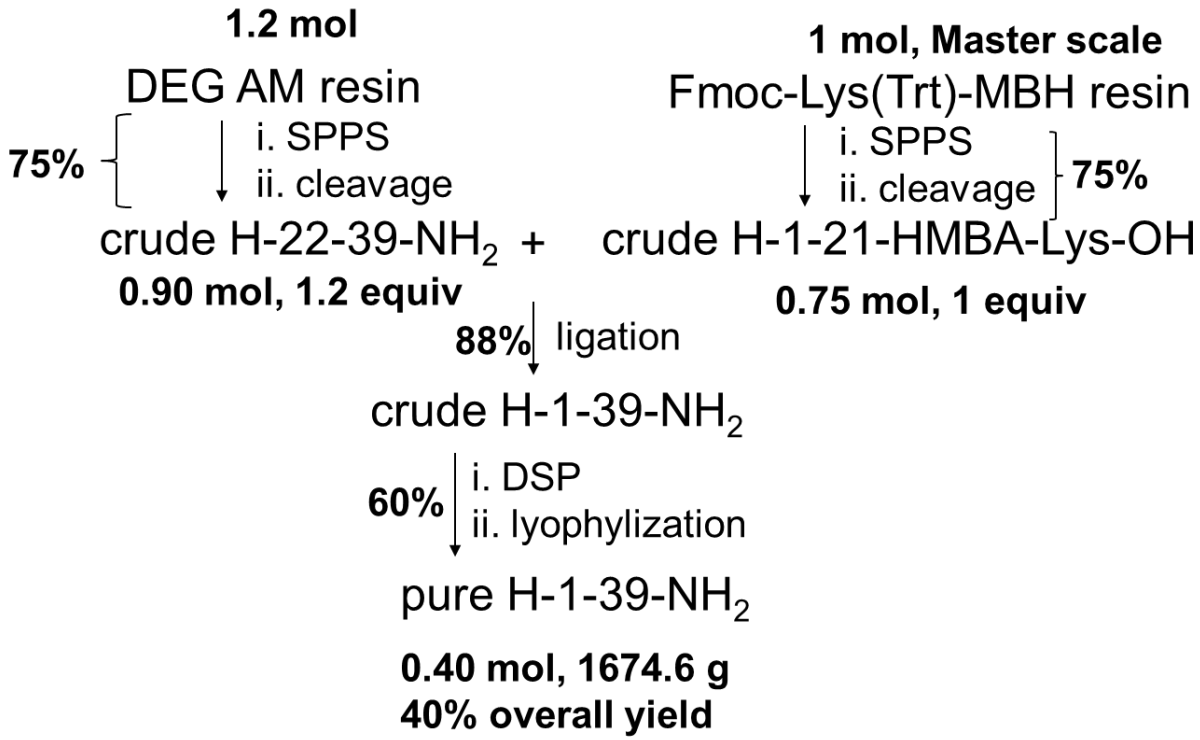



**Figure S53.** A schematic representation of the flow chart of A schematic representation of the flow chart of the HMBA fragment CEPS, pseudoproline (C+C) exenatide manufacturing process herein.

**Table S73.** Overview of raw materials used in 1 mol SPPS of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS pseudoproline (C+C) process.

| AA nr | AA | MW | mol AA | equiv AA | kg AA | kg Oxyma | Total kg DIC | kg pip | kg DMF |
|---|---|---|---|---|---|---|---|---|---|
| resin | Fmoc-K(Trt)-MBH resin, 0.52 mmol/g, 1.92 kg | n.a. | 1,00 | **1,00** | n.a. | n.a. | n.a. | n.a. | n.a. |
| linker | HMBA | 152,15 | 2,00 | **2,00** | 0,32 | 0,57 | 0,50 | 3,13 | 92,50 |
| $Leu^{21}$ | Fmoc-Leu-OH[1] | 354,41 | 4,00 | **4,00** | 1,49 | 0,68 | 0,50 | 0,00 | 17,50 |
| $Arg^{20}$ | Fmoc-Arg(Pbf)-OH | 648,77 | 2,00 | 2,00 | 1,37 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Val^{19}$ | Fmoc-Val-OH | 339,39 | 2,00 | 2,00 | 0,71 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ala^{18}$ | Fmoc-Ala-OHx$H_2O$ | 329,36 | 2,00 | 2,00 | 0,69 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{17}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{16}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{15}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Met^{14}$ | Fmoc-Met-OH | 371,45 | 2,00 | 2,00 | 0,78 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Gln^{13}$ | Fmoc-Gln(Trt)-OH | 610,70 | 2,00 | 2,00 | 1,29 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Lys^{12}$ | Fmoc-Lys(Trt)-OH | 610,74 | 2,00 | 2,00 | 1,29 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{11}$ | Fmoc-Ser(Trt)-OH | 569,65 | 2,00 | 2,00 | 1,20 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Leu^{10}$ | Fmoc-Leu-OH | 354,41 | 2,00 | 2,00 | 0,75 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Asp^{9}$ | Fmoc-Asp(O*t* Bu)-OH | 411,45 | 2,00 | 2,00 | 0,87 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Ser^{8}Thr^{7}$ | Fmoc-Thr(t-Bu)- Ser[Psi(Me,Me)Pro]-OH | 542,61 | 2,00 | 2,00 | 1,14 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Phe^{6}$ | Fmoc-Phe-OH | 387,45 | 2,00 | 2,00 | 0,82 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Thr^{5}$ | Fmoc-Thr(*t*- Bu)-OH | 397,48 | 2,00 | 2,00 | 0,84 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Gly^{4}$ | Fmoc-Gly-OH | 297,31 | 2,00 | 2,00 | 0,63 | 0,28 | 0,63 | 3,13 | 92,50 |
| $Glu^{3}$ | Fmoc-Glu-OHx$H_2O$ | 425,50 | 2,00 | 2,00 | 0,90 | 0,28 | 0,63 | 3,13 | 92,50 |
| $His^{1}$-$Gly^{2}$ | Boc-His(Trt)-Gly-OH | 554,65 | 2,00 | 2,00 | 1,17 | 0,28 | 0,63 | 3,13 | 92,50 |
| ∑ | | | | | 18,92 | 6,37 | 12,37 | 59,47 | 1775,00 |
| [1]Cl-HOBt used instead of Oxyma | | | | | | | | | |

For an overview of raw materials used in 1 mol SPPS of H-22-39-peptide fragment resin, HMBA fragment CEPS pseudoproline (C+C) process see Table S43.

**Table S74.** Summary of amounts of raw materials used in 1 mol SPPS of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS pseudoproline (C+C) process.

| | |
|---|---|
| Scale (mol) | 1,00 |
| Weight resin (kg) | 1,92 |
| ∑ Weight AAs (kg) | 18,92 |
| ∑ Weight Oxyma (kg) | 6,37 |
| ∑ Weight DIC (kg) | 12,37 |
| ∑ Weight resin/AAs/coupling agents (kg) | 39,58 |
| ∑ Weight pip (kg) | 59,47 |
| ∑ starting materials (kg) | **99,05** |
| ∑ solvents (kg) | **1775,00** |
| peptide resin formed (kg) | **5,00** |

**Table S75.** Summary of amounts of raw materials used in 1 mol (nominal) and 1,2 mol (actual) SPPS of H-22-39-peptide fragment resin, O-Cam fragment CEPS pseudoproline (C+C) process.

| Scale (mol) | 1,00 | 1,20 |
|---|---|---|
| Weight resin (kg) | 1,69 | 2,03 |
| ∑ Weight AAs (kg) | 17,38 | 20,86 |
| ∑ Weight Oxyma (kg) | 5,41 | 6,49 |
| ∑ Weight DIC (kg) | 12,01 | 14,41 |
| ∑ Weight resin/AAs/coupling agents (kg) | 36,49 | 43,79 |
| ∑ Weight pip (kg) | 53,21 | 63,85 |
| ∑ starting materials (kg) | **89,7** | **107,64** |
| ∑ solvents (kg) | **1642,5** | **1971,00** |
| peptide resin formed (kg) | **5,5** | **6,60** |

**Table S76.** Summary of amounts of raw materials used in 1 mol TFA cleavage of Boc-1-21-HMBA-K peptide fragment resin, HMBA fragment CEPS pseudoproline (C+C) process.

| Scale (mol) | 1,00 |
|---|---|
| TFA (kg) | 27,75 |
| TIS (kg) | 0,61 |
| $H_2O$ (kg) | 0,21 |
| DTT (kg) | 0,63 |
| $NH_4I$ (kg) | 0,42 |
| ∑ Reagents (kg) | 29,61 |
| solvents (ether, kg) | 99,03 |

**Table S77.** Summary of amounts of raw materials used in 1,2 mol TFA cleavage of H-22-39 peptide fragment resin, HMBA fragment CEPS pseudoproline (C+C) process.

| Scale (mol) | 1,20 |
|---|---|
| TFA (kg) | 51,67 |
| TIS (kg) | 0,96 |
| $H_2O$ (kg) | 0,33 |
| DTT (kg) | 0,98 |
| ∑ Reagents (kg) | 53,93 |
| solvents (ether, kg) | 232,36 |

**Table S78.** Summary of amounts and cost of raw materials used in the SPPS of H-22-39 fragment resin and TFA cleavage of H-22-39 fragment resin, HMBA fragment CEPS pseudoproline (C+C) process.

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (DEG AM) | 1300,0 | 2,0 | 2640,9 |
| Oxyma | 85,0 | 6,5 | 551,5 |
| piperidine | 10,6 | 63,8 | 676,8 |
| std AA | 400,0 | 20,9 | 8341,7 |
| DIC | 240,0 | 14,4 | 3458,1 |
| DMF | 1,4 | 1971,0 | 2759,4 |
| TFA | 20,0 | 51,7 | 1033,4 |
| TIS | 900,0 | 1,0 | 864,0 |
| DTT | 668,0 | 1,0 | 658,5 |
| Ether | 9,0 | 232,4 | 2091,3 |
| $\sum$ | n.a. | n.a. | 23075,5 |

**Table S79.** Summary of amounts of raw materials used in enzymatic ligation, HMBA fragment CEPS pseudoproline (C+C) process.

| | |
|---|---|
| Scale (mol) | 0,75 |
| Amine F (kg) | 2,33 |
| HMBA F (kg) | 3,48 |
| TCEPxHCl (kg) | 0,20 |
| Enzyme (g) | 10,00 |
| Solvent (kg) | 12,75 |

**Table S80.** Summary of amounts of raw materials used in DSP of crude exenatide, HMBA fragment CEPS pseudoproline (C+C) process.

| Step | kg peptide | kg solvent |
|---|---|---|
| API (2 RPC) | 1,67 | 3126,24 |

**Table S81.** Summary of amounts and cost of raw materials used in the entire process, HMBA fragment CEPS pseudoproline (C+C) process.[1]

| Raw material | cost (EUR/kg) | weight (kg) | cost (EUR) |
|---|---|---|---|
| resin (Fmoc-K(Trt)-MBH) | 4000,0 | 1,9 | 7680,0 |
| Oxyma | 85,0 | 6,4 | 541,5 |
| piperidine | 10,6 | 59,5 | 630,4 |
| std AA | 400,0 | 16,6 | 6644,0 |
| dipeptide | 1500,0 | 1,2 | 1755,0 |
| pseudoproline dipeptide | 9000,0 | 1,1 | 10260,0 |
| DIC | 240,0 | 12,4 | 2968,8 |
| DMF | 1,4 | 1775,0 | 2485,0 |
| TFA | 20,0 | 27,8 | 555,0 |
| TIS | 900,0 | 0,6 | 549,0 |
| DTT | 668,0 | 0,6 | 420,8 |
| $NH_4I$ | 450,0 | 0,4 | 189,0 |
| Ether | 9,0 | 99,0 | 891,3 |
| $TCEP^xHCl$ | 582,0 | 0,2 | 98,9 |
| Omniligase (EUR/g) | 750,0 | 10,0 | 7500,0 |
| MeCN | 6,0 | 939,1 | 5634,9 |
| ∑+HMBA fragment+ ligation+DSP | n.a. | n.a. | 48803,6 |
| ∑ amine fragment | n.a. | n.a. | 23075,5 |
| ∑ | | | 71879,1 |
| ∑/1 kg API | | | 43041,4 |

[1]Weight of Omniligase is in g.

**Table S82.** Summary of total CI for organic solvents used in exenatide manufacturing, HMBA fragment CEPS pseudoproline (C+C) process.

| **Total organic solvents** | weight (kg) | $CO_2$ formed during solvent production / kg $kg^{-1}$ | CI / kg $kg^{-1}$ | $CI_{\sum}$ / kg $kg^{-1}$ | $CI_{\sum}$ |
|---|---|---|---|---|---|
| DMF (SPPS) | 3746 | 1,75 | 1,81 | 3,56 | 13335,76 |
| Ether (cleavage) | 331,4 | 1,08 | 2,37 | 3,45 | 1143,33 |
| MeCN (DSP+ligation) | 939,1 | 1,95 | 2,15 | 4,1 | 3850,31 |
| $\sum$ | | | | | 18329,40 |
| $\sum$/kg API | | | | | 10975,69 |

**Table S83.** Summary of energy consumed, cost of energy and total CI for process energy in exenatide manufacturing, HMBA fragment CEPS pseudoproline (C+C) process.

| Energy & CI | Process step | | | | | $\sum$ | $\sum$ kg $API^{-1}$ |
|---|---|---|---|---|---|---|---|
| | SPPS[a] | cleavage/precipitation[b] | ligation[c] | DSP[d] | lyophilization[e] | | |
| energy used ($kWh^{-1}$) | 369,7 | 47,3 | 1,5 | 14,1 | 58,6 | 491,20 | 293,25 |
| cost of energy used (EUR) | 24,40 | 3,12 | 0,099 | 0,93 | 3,87 | 32,42 | 19,35 |
| CI for electricity generated (t $CO_2$) | 0,10 | 0,01 | 0,000 | 0,004 | 0,017 | 0,14 | 0,08 |
| CI for electricity transmitted & distributed (t $CO_2$) | 0,01 | 0,00 | 0,000 | 0,000 | 0,001 | 0,01 | 0,01 |
| CI for electricity used (t $CO_2$)[f] | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| CI for electricity $\sum$ (t $CO_2$) | 0,11 | 0,01 | 0,000 | 0,004 | 0,018 | 0,15 | 0,09 |

[a]energy used=[amine fragment: 17 (AA cycles) x 3 (duration of AA cycle in h) x 60 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]+[HMBA fragment: 20 (AA cycles) x 3 (duration of AA cycle in h) x 50 (amount of solvent in reactor in kg)/100 (nominal amount of solvent in reactor) x 6,1 (amount of energy needed to stir and heat nominal amount of solvent for 1 h)]; [b]411 (total amount of TFA+solvent in kg, amine+HMBA fragments) x 3 (total time of cleavage + precipitation in h) /12 (nominal amount of TFA+solvent in kg) x 0,46 (amount of energy needed to stir nominal amount of TFA+solvent for 1 h); [c]12,8 (total amount of ligation solvent in kg) x 3 (total time ligation h)/10 (nominal amount of solvent in kg) x 0,38 (amount of energy needed to stir nominal amount of solvent for 1 h) [d]3126 (amount of solvent used in DSP in kg)/1000 (nominal amount of solvent used in DSP in kg) x 4,5 (amount of energy needed to pass nominal amount of solvent through an RPC column); [e]1,675 (amount of API obtained in kg)/1,0 (nominal amount of API in kg) x 35 (amount of energy needed to lyophilize nominal amount of API); [f]wind energy was used in this exenatide API manufacture, the use of electricity was assumed to

### 35.8. Summary

The Table S84 below summarizes the results for the six manufacturing routes to exenatide API examined as delineated in sections 35.2 – 35.7 of this ESI. Specifically,

i) amounts of SPPS starting materials are summarized from Tables S23, S24, S30, S31, S32, S33, S42, S43, S44, S45, S54, S62, S63, S64, S73, S74 and S75.

ii) amounts of cleavage starting materials are summarized from Tables S25, S34, S35, S46, S47, S55, S65, S66, S76 and S77.

iii) amounts of ligation starting materials are summarized from Tables S37, S49, S57, S68 and S79.

iv) amounts of DSP starting materials are summarized from Tables S26, S38, S50, S58, S69 and S80.

v) overall yields and amounts of exenatide API obtained in 1 mol manufacture are summarized from Figs S48 – 53.

vi) cost of goods data are summarized from Tables S27, S36, S39, S48, S51, S56, S59, S67, S70, S78 and S81.

vii) cost of processing energy data and CI are summarized from Tables S28, S29, S40, S41, S52, S53, S60, S61, S71, S72, S82 and S83.

Furthermore, most important results from Table S84 (overall yield, total cost, cEF and total CI) are provided as excerpts in the main article, Table 4.

**Table S84.** Summary of amounts of reagents and solvents, cost of goods and processing energy consumed, overall yield, cEF and total CI for six process routes to exenatide API.

| **Process attributes** | | **Process routes** | | | | | |
|---|---|---|---|---|---|---|---|
| **Process stage** | **Materials used** | **Conventional SPPS, *benchmark 1*** | **Lab scale CEPS (P+P), *benchmark 2*** | **O-Cam fragment CEPS (C+C)** | **O-Cam fragment CEPS (P+P)** | **HMBA fragment CEPS (C+C)** | **HMBA fragment CEPS, pseudoproline (C+C)** |
| SPPS | reagents/reactants (kg) | 766,84 | 380,91 | 253,50 | 397,38 | 205,49 | 206,69 |
| | solvents (kg) | 26995,80 | 5487,36 | 4399,50 | 6456,88 | 3686,75 | 3746,00 |
| Cleavage | reagents/reactants (kg) | 148,32 | 62,46 | 103,49 | 129,07 | 79,05 | 83,54 |
| | solvents (kg) | 3047,37 | 306,01 | 400,86 | 573,92 | 312,03 | 331,39 |
| Ligation | reagents/reactants (kg) | n.a. | n.a. | 0,10 | 0,07 | 0,17 | 0,20 |
| | enzyme (g) | n.a. | 4,53 | 3,90 | 2,73 | 8,93 | 10,00 |
| | solvents (kg) | n.a. | 70,03 | 64,29 | 45,00 | 11,39 | 12,75 |
| DSP | solvents (kg) | 861,12 | 4240,08 | 2106,00 | 4314,96 | 2751,84 | 3126,24 |
| Overall | reagents/reactants (kg) | 915,16 | 443,37 | 357,09 | 526,52 | 284,71 | 290,43 |
| | enzyme (g) | n.a. | 4,53 | 3,90 | 2,73 | 8,93 | 10,00 |
| | solvents (kg) | 30043,17 | 10103,48 | 6970,65 | 11390,76 | 6762,01 | 7216,38 |
| **Overall yield (%)** | | **22** | **44** | **18** | **23** | **35** | **40** |
| exenatide API obtained (kg) | | 0,92 | 1,84 | 0,75 | 0,96 | 1,47 | 1,67 |
| ***Overall (per kg API)*** | ***reagents/reactants (kg)*** | ***993,66*** | ***240,96*** | ***476,12*** | ***548,46*** | ***193,68*** | ***173,91*** |
| | ***enzyme (g)*** | ***0,00*** | ***2,46*** | ***5,20*** | ***2,84*** | ***6,07*** | ***5,99*** |
| | ***solvents (kg)*** | ***32620,16*** | ***5491,02*** | ***9294,20*** | ***11865,38*** | ***4600,01*** | ***4321,19*** |
| **cEF** | | **33612,8** | **5731,0** | **9769,3** | **12412,8** | **4792,7** | **4494,1** |
| **Solvent contribution to cEF (%)** | | **97,0** | **95,8** | **95,1** | **95,6** | **96,0** | **96,2** |
| Cost of Goods (EURkg$^{-1}$ API) | | 140791,7 | 45178,9 | 73680,4 | 76785,3 | 40749,8 | 43041,4 |
| Cost of waste disposal (EURkg$^{-1}$ API) | | 7058,9 | 1203,7 | 2051,8 | 2606,9 | 1006,7 | 944,0 |
| Total Cost of Goods (EURkg$^{-1}$ API) | | 147850,6 | 46382,6 | 75732,2 | 79392,2 | 41756,5 | 43985,4 |
| Cost of processing energy (EURkg$^{-1}$ API) | | 66,5 | 23,8 | 45,0 | 44,9 | 21,3 | 19,4 |
| **Total cost (EURkg$^{-1}$ API)** | | **147917,1** | **46406,4** | **75777,2** | **79437,1** | **41777,8** | **44004,8** |
| **Solvent cost contribution to the total cost, 1mol scale (EUR)** | | **66770,5** | **16293,6** | **13596,6** | **20795,2** | **12930,0** | **13861,9** |
| **Solvent cost contribution to the total cost (EURkg$^{-1}$ API)** | | **72576,6** | **8855,2** | **18128,8** | **21661,7** | **8795,9** | **8300,5** |
| **Solvent cost contribution to the total cost (%)** | | **49,1** | **19,1** | **23,9** | **27,3** | **21,1** | **18,9** |
| Total CI for starting materials (kg $CO_2$kg$^{-1}$ API) | | 117040,9 | 14130,6 | 26215,9 | 28732,8 | 11966,6 | 10975,7 |
| Total CI for processing energy (kg $CO_2$kg$^{-1}$ API) | | 620 | 220 | 210 | 220 | 100 | 90 |
| **Total CI (kg $CO_2$kg$^{-1}$ API)** | | **117660,9** | **14350,6** | **26425,9** | **28952,8** | **12066,6** | **11065,7** |

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