Copper(II) Lysinate and Pseudoproline Assistance in the Convergent Synthesis of the GLP-1 Receptor Agonists Liraglutide and Semaglutide

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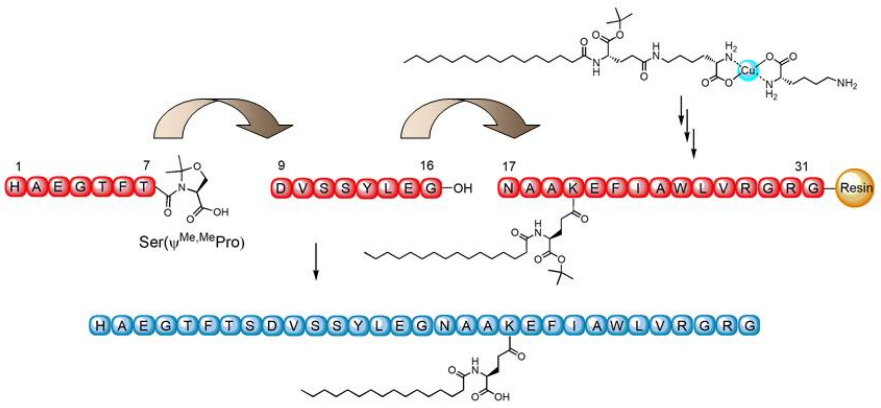
A growing interest in the peptides as active pharmaceutical ingredients (APIs) requires the development of efficient strategies for their preparation. These are particularly challenging in the case of long peptides with strong tendency to aggregation and folding. Here, we describe the pseudoproline-assisted, convergent synthesis of the GLP-1 receptor agonists Liraglutide and Semaglutide, which encompasses the stepwise condensation of three segments on the solid phase. The insertion of a pseudoproline residue at the site of the fragment coupling prevents epimerization of the C-terminal amino acid and allows obtaining these peptides with excellent purity and high yield. In addition, for the synthesis of the lipidated side chains we developed a novel approach that includes copper(II) lysinate intermediates and can be particularly suitable for the industrial preparation of both Liraglutide and Semaglutide and other peptides with similar branched structure.

KEYWORDS: Liraglutide, Semaglutide, solid-phase synthesis, pseudoproline, GLP-1, copper lysinate


1
7
9
16
17
31
H A E G T F T
D V S S Y L E G OH
N A A K E F I A W L V R G R G Resin
Ser(ψ^Me,MePro)
Cu
NH2
H A E G T F T S D V S S Y L E G N A A K E F I A W L V R G R G
OH

INTRODUCTION

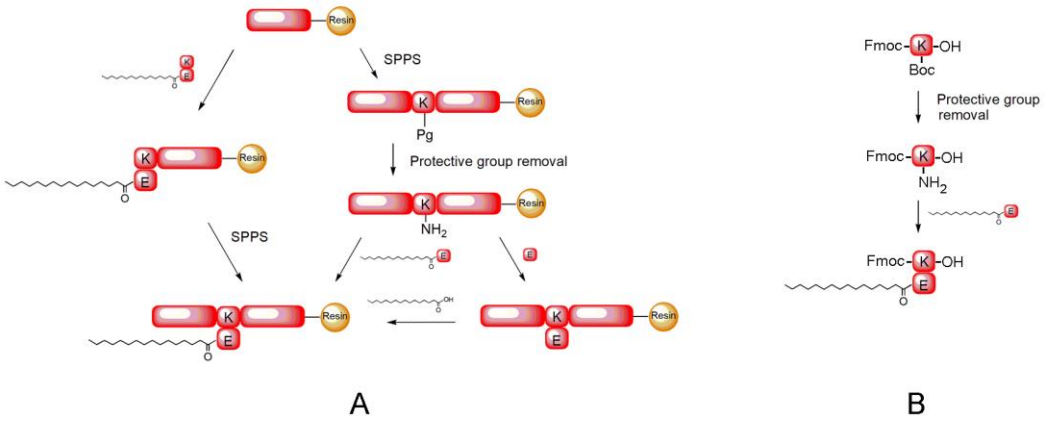
A significant growth of the number of peptide APIs during the last years was accompanied by the development and tightening of regulatory requirements to be satisfied for their release in the market.[1,2] The complexity of peptide molecules requires significant efforts for the identification of optimal strategies for their synthesis and purification, that at the same time must be appealing from an economical and industrial point of view. These strategies are particularly challenging in the case of peptides with modified or unnatural amino acids, as in the case of the synthesis of Degarelix that we recently faced.[3,4] The most convenient way for the chemical preparation of peptides with more than 15 amino acids is the solid-phase synthesis (SPPS) introduced decades ago by Merrifield.[5] However, inter- and intramolecular aggregation and peptide folding during the synthesis hamper significantly the deprotection and coupling steps, thus generating numerous side products and compromising the purity of the final peptides. This is the case of the GLP-1 receptor (GLP-1R) agonists Liraglutide and Semaglutide, which are widely used for the treatment of type II diabetes (Figure 1).[6,7]

Figure 1. Amino acid sequences of Liraglutide (top) and Semaglutide (bottom, U = Aib).

The unique therapeutic properties of Liraglutide are due to the presence of the fatty acid moiety that enhances peptide aggregation and albumin binding, thus favoring the increase of the peptide circulation time in the blood stream.[8] Semaglutide was designed to additionally enhance the GLP-1R affinity of the peptide and its stability against metabolic degradation, due to the presence of the non-coded α-aminoisobutyric acid (Aib) residue. Thus, only once-a-week injection is needed in this case to afford a potent pharmacological profile.

The branched structure with fatty acid modification and the peculiar amino acid sequence, which favor peptide folding and aggregation, make rather difficult the chemical syntheses of high-purity Liraglutide and Semaglutide. One of the strategies for the introduction of the ramification into Liraglutide, that was proposed by the inventor, was based on the acylation of Lys^{20} of the recombinant precursor peptide with N-palmitoyl glutamic acid γ-N-hydroxysuccinimido ester.[9,10] Numerous studies have been conducted during the last years aimed at identifying alternative strategies for Liraglutide API preparation. The research was focused on the use of efficient and selective coupling reagents and particular resin linkers, or on the introduction into the peptide sequence of pseudoprolines and depsipeptide intermediates or on the exploitation of chemo-enzymatic methods.[11-17] A number of chemical syntheses of Liraglutide were also described, where the lipophilic fragment is introduced after selective removal of the Mtt (4-methyltrityl), Alloc (allyloxycarbonyl) or Dde (1-[4,4-dimethyl-2,6-dioxocyclohex-1-ylidene]ethyl) protective groups upon completion of the stepwise assembly of the peptide main chain on the solid phase.[16-18] Semaglutide can be prepared in a similar way.[21-24]

Despite the number of approaches proposed so far, there remains a need for the development of more efficient strategies for the synthesis of high-purity Liraglutide and Semaglutide. An important issue to be addressed in the their solid-phase synthesis is the introduction of the lipophilic group on the Lys^{20} side chain. The creation of this branched structure can be achieved by direct insertion into the growing peptide chain of the lipidated intermediate dipeptide or by using an orthogonally protected lysine (Scheme 1, A).



Scheme 1. Possible strategies for introducing the lipidated side group on Lys^{20} of Liraglutide (Pg = protective group) (A) and the scheme for the preparation of palmitoylated intermediate dipeptide (B).

In the first case, an orthogonally protected lysine is required as a starting material in order to selectively form the peptide bond between the ε-amino group of lysine and the γ-carboxylic group of glutamic acid (Scheme 1, B).[25]

The use of copper(II) lysinate could noticeably simplify the preparation of this palmitoylated intermediate. Copper(II) complexes of trifunctional amino acids, such as Lys, Asp or Glu, can function as a temporary protection for selective operations on the side chain.[26,27] This method is frequently used for the large-scale manufacturing of protected amino acids as raw materials. Also, the procedures for storage and disposal of copper-containing waste are well established and in addition copper(II) salts have low toxicity. Here, we investigated the possibility of using copper(II) lysinate for the preparation of the intermediate lipidated building blocks to be exploited for the synthesis ofLiraglutide and Semaglutide.

Another important challenge in the lipopeptide synthesis is aggregation and folding occurring during the stepwise SPPS. Such events lead to the presence of many impurities in the crude product. In general, these impuritiesare truncated sequences or peptides lacking just one or two residues. The latter, are difficult to remove by means of high-performance liquid chromatography (HPLC) being very similar to the desired molecule.. From this point of view, the segment condensation approach can be a promising alternative. Usually, the choice of the condensation site depends on the presence of the achiral glycine or of amino acids resistant to epimerization during the coupling reaction (*e.g.*, proline). Indeed, the activation of the C-terminal carboxylic group of a peptide leads to fast oxazolone formation, which easily promotes the isomerization of the C-terminal amino acid.[28] As a consequence, the purification of the target peptide from the undesired peptide diastereoisomers is complicated by the small differences in the HPLC retention time.

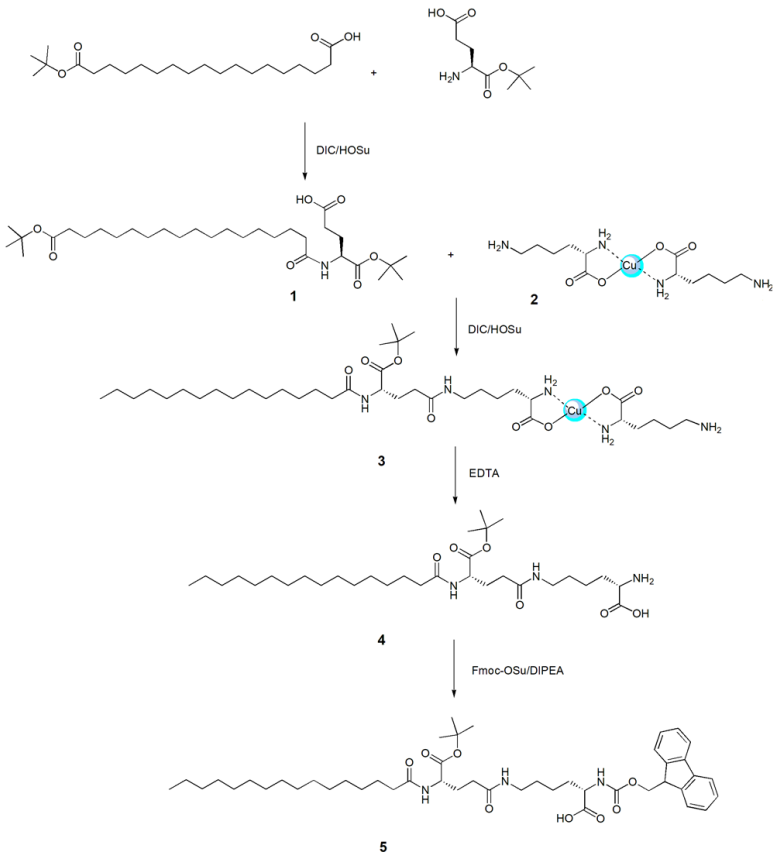
Recently, we investigated several approaches, where combined liquid/solid-phase syntheses were applied for Liraglutide preparation.[25] They consisted in the preparation of peptide segments on solid phase and their subsequent coupling in solution. However, these couplings were difficult to achieve because of the extremely low solubility of the protected segments in the commonly used organic solvents, likewise due to their strong tendency to aggregate. These features were particularly evident for the segment comprising the amino acids 1-16. A possible solution could be the use of three or more segments. However, in the amino acid sequence of Liraglutide segment 1-16 there is only one suitable splitting site (Gly at position four), which is too close to the N-terminus. Indeed, the removal of only four N-terminal amino acids can hardly improve the solubility of such a long fragment.

Therefore, in this study we propose a novel improved SPPS strategy that allows obtaining Liraglutide and Semaglutide API with high purity and yield.[29] It consists in the introduction at the site of the fragment condensation of the pseudoproline residue, which cannot racemize upon carboxylic group activation because the absence of the amide proton hampers the formation of the intermediate 2,4-dialkyl-5(4*H*)-oxazolone.[28] In addition, along with the side chain protective function for serine and threonine (oxazolidine cycle) and cysteine (thiazolidine cycle), pseudoprolines are powerful tool in SPPS for the suppression of peptide aggregation.[30-32] Surprisingly, very few works describe their use for segment couplings.[33,34] Indeed, in the case of Liraglutide and Semaglutide the insertion of the pseudoproline moiety noticeably enhanced the coupling efficiency, precisely because of the prevention of peptide aggregation during the fragment condensation. Thus, this approach can be particularly useful for the large-scale preparation of these GLP-1 analogues as API, as well as for the synthesis of other peptides of similar branched structure.

RESULTS AND DISCUSSION

Preparation of Liraglutide and Semaglutide side chain *via* copper(II) lysinate.

In order to find a suitable alternative to the previously described methods for the preparation of the lipidated intermediates and simplify their synthesis, we tested here a novel approach starting from copper(II) lysinate. In this case α-amino and carboxylic groups of lysine were protected in a single step through the formation of an internal complex between two molecules of Lys and a Cu^{2+} ion (Scheme 2).



Scheme 2. Synthesis of Fmoc-Lys(Pal-Glu-O*t*Bu)-OH *via* copper(II) lysinate (Liraglutide intermediate). For details see the Experimental part.

At the first step Pal-Glu-O*t*Bu (**1**) was prepared using partially protected glutamic acid and pre-activating its γ-carboxylic group by formaing the N-hydroxysuccinimide ester. This activated ester reacted without isolation with copper(II) lysinate (**2**), which, in turn, was easily formed by mixing lysine hydrochloride with copper(II) sulfate. In this case a mixture of mono-and diacylated complex can be formed due to the presence of two amino groups in copper lysinate (only monoacylation is shown in Scheme 2). In order to maintain all the reagents in solution and ensure the completion of the reaction, a water/acetone mixture was used. The compound (**3**) formed a blue precipitate, that turned white after addition of EDTA and disruption of the copper(II) complex to yield compound **(4)**. The target compound (**5**) was prepared by reacting **(4)** with Fmoc-OSu with excellent purity and yield, similar to those for the palmitoylated dipeptide synthesized from Fmoc-Lys-OH (Figure 2).[25]

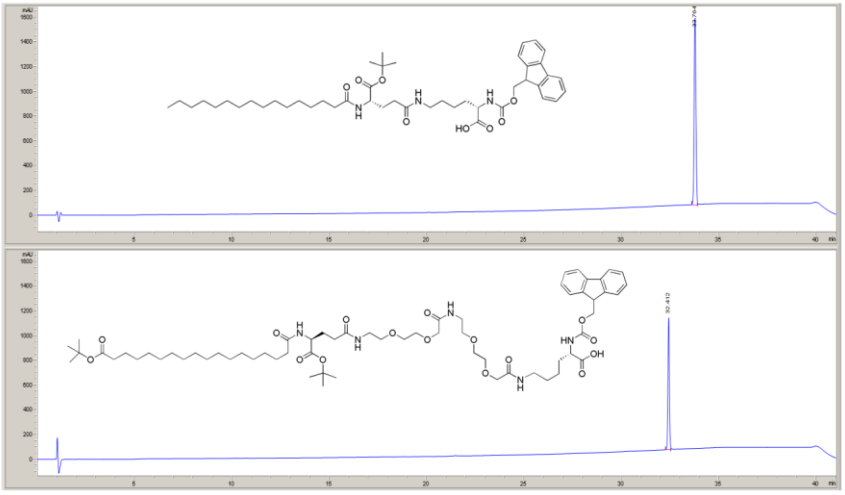
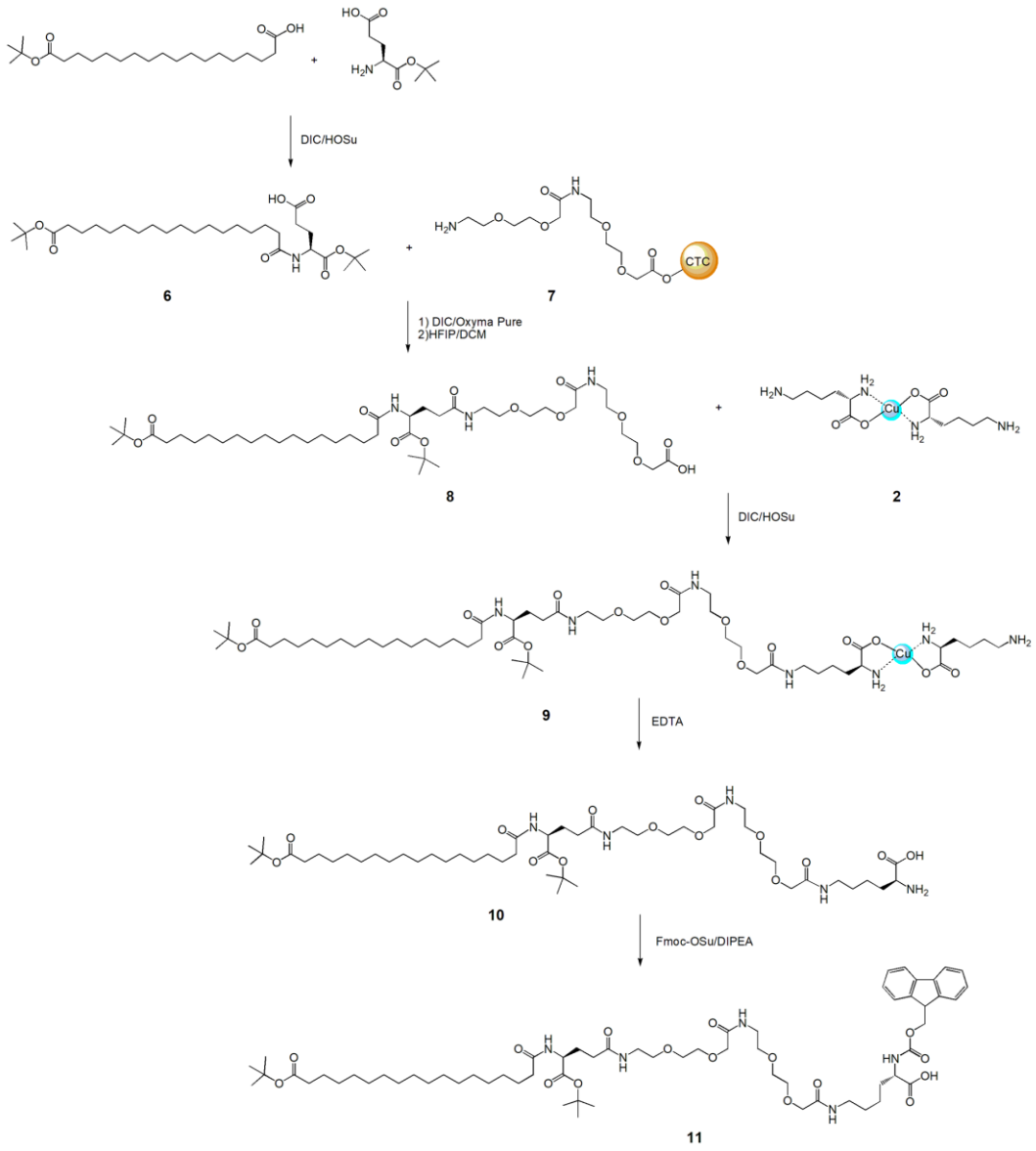


Figure 2. HPLC profile of Fmoc-Lys(Pal-Glu-O*t*Bu)-OH (top) and Fmoc-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH (bottom) prepared *via* copper(II) lysinate (Analytical method 1, see Experimental part).

Then, we studied the possibility of using the copper-mediated synthesis for the preparation of the lipidated building block of Semaglutide. In this case, a combined liquid/solid-phase approach was applied in order to minimize the intermediate purification steps and to facilitate the synthesis (Scheme 3).



Scheme 3. Synthesis of Fmoc-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH *via* copper(II) lysinate (for details see the Experimental part).

The acylated glutamic acid *tert*-butyl ester (**6**), obtained as described for the palmitoylated Liraglutide dipeptide, was coupled to the acid-labile resin loaded with 2-[2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (AEEA) in order to obtain the intermediate (**8**), that was used for the reaction with copper(II) lysinate. At variance from the Liraglutide dipeptide, in the case of the Semaglutide intermediate the decomposition of the copper complex **(9)** with EDTA led to the water-soluble compound (**10**). Therefore, the purification scheme was changed. The side products were precipitated by addition of acetonitrile and the target compound was extracted in an

acetonitrile/water mixture. After reaction with Fmoc-OSu and purification by "flash" chromatography the Semaglutide building block was obtained with high purity and yield 87% (Figure 2).

The use of the peptide intermediates synthesized *via* copper(II) lysinate for API preparation requires the analysis of possible residual Cu(II).Although not as toxic as many other heavy metals, Cu(II) has to be absent or present at low ppm levels.[35] For this reason, Fmoc-Lys(Pal-Glu-O*t*Bu)-OH and its intermediate were analyzed by inductively coupled plasma mass spectrometry (ICP-MS), to evaluate the quantity of copper salts remained. For the sake of comparison, the same dipeptide prepared without Cu(II) complex was used. The results of the ICP-MS analysis showed that the copper(II) content in the intermediate (**4**) is rather high (54.4 ppm), whereas after Fmoc attachment it is below the limit of quantification (0.5 ppm) (Table 1).

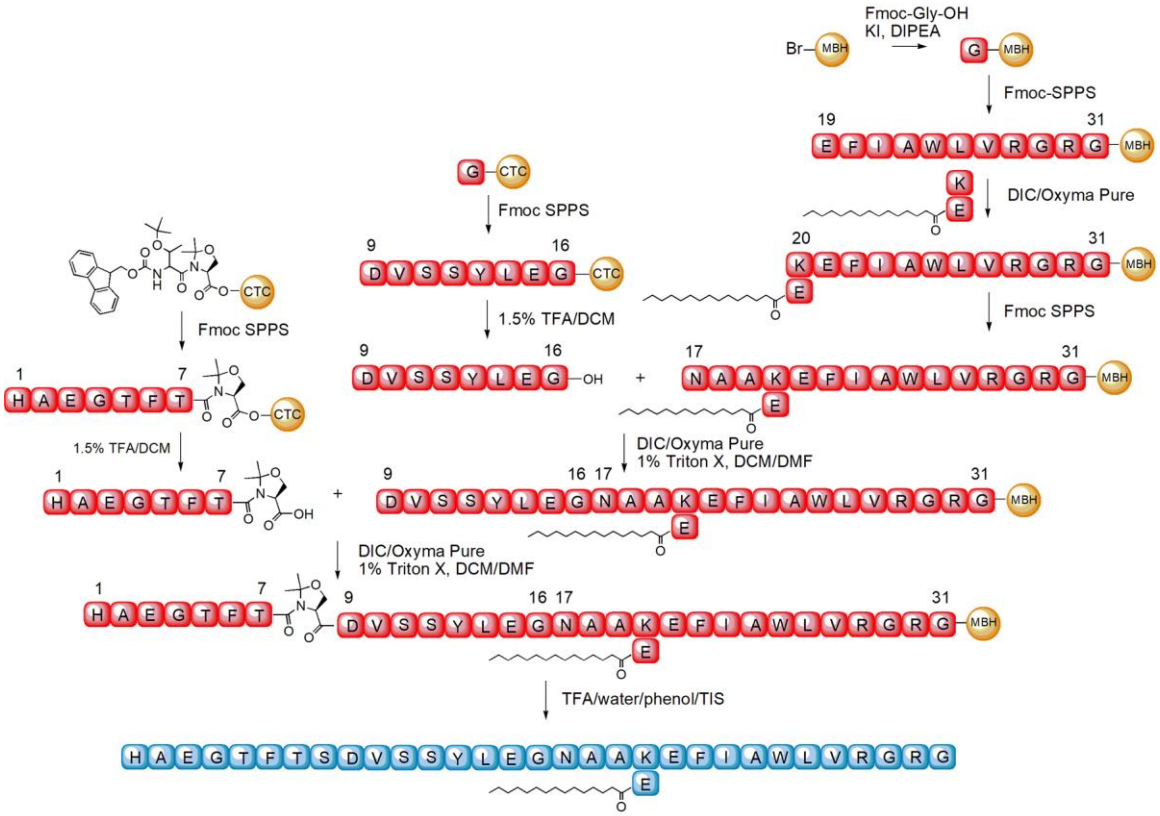
Table 1. ICP-MS analysis of Fmoc-Lys(Pal-Glu-O*t*Bu)-OH and its intermediate

Compound	Cu content, ppm
H-Lys(Pal-Glu-O*t*Bu)-OH (**4**)	54.4
Fmoc-Lys(Pal-Glu-O*t*Bu)-OH (**5**)	<0.5
Fmoc-Lys(Pal-Glu-O*t*Bu)-OH prepared without Cu complex	<0.5
Fmoc-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH (**11**)	<0.5

The same result was obtained for the Semaglutide building block prepared with copper(II) lysinate. Thus, the residual copper(II) salts can be successfully eliminated by extractions and recrystallizations during the last steps of the dipeptide synthesis without special purification procedures. These results show that the use of partially or completely deprotected amino acids allows obtaining the building blocks *via* copper(II) complex with a very good yield, high purity and a quality suitable for the API preparation.

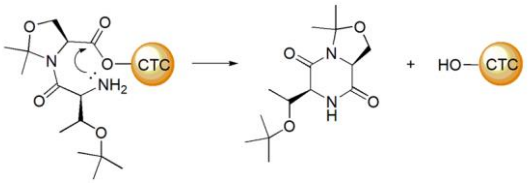
Solid-phase segment condensation approach for the synthesis of Liraglutide and Semaglutide

In order to avoid aggregation of the peptide segments and of the growing peptide chain and to ensure a high purity of the target peptide, we tested a pseudoproline-assisted condensation approach for the solid-phase synthesis of Liraglutide and Semaglutide (Scheme 4).



Scheme 4. Convergent synthesis of Liraglutide. Semaglutide was prepared in a similar way.

Liraglutide segment 17-31 was successfully obtained by stepwise Fmoc-SPPS on the 4-methylbenzhydryl bromide resin with direct insertion of the palmitoylated dipeptide into the amino acid sequence. In order to facilitate the attachment of the first amino acid by halide substitution the reaction was carried out in the presence of potassium iodide. The protected segments 1-8 and 9-16 were synthesized by loading on the 2-chloro trityl chloride resin (CTC) Fmoc-Thr(O*t*Bu)-Ser($\psi^{Me,Me}$pro)-OH and Fmoc-Gly-OH and subsequently performing a stepwise SPPS. Finally, cleavage form the resin was achieved with a 1.5% trifluoroacetic acid (TFA) solution in dichloromethane (see Experimental part and Figure S13). The preparation of peptides with a C-terminal pseudoproline residue linked to a resin with a trityl type linker is complicated because of the high propensity of pseudoproline dipeptides to intramolecularly cyclize to a diketopiperazine after Fmoc removal (Scheme 5).[36]



Scheme 5. Diketopiperazine formation from the pseudoproline dipeptides on CTC resin.

This is caused by the pseudoproline easy formation of the cis amide bond and the adoption of a type VI β-turn structure.[37] The vicinity of the amino group to the ester bond favors the intramolecular nucleophilic substitution with formation of the six-membered ring. Indeed, we found, that the use of the standard procedure of Fmoc removal from the dipeptide attached to the resin (*i. e.*, two cycles for 5 and 15 min with 20% piperidine in DMF) led to significant loss of the peptide and low yield of Liraglutide segment 1-8 (data not shown). A careful selection of the reaction conditions allows suppressing this side reaction. For example, in the abovementioned work of Heinlein et al.[38] it was shown that the use of piperidine/DBU/DMF mixture can help to prevent dipeptide detachment from the solid support. Nevertheless, the final yield of the peptide seagment even in this case was only 23%. We found that several fast treatments with a piperidine solution (3 × 3 min) are sufficient to remove Fmoc protective group completely with retention of the pseudoproline dipeptide on the solid support. In this way almost all the peptide remained intact and it was possible to obtain the protected Liraglutide seagment 1-8 with 93.6% purity and 80% yield.

Unexpectedly, we faced severe problems with the solubility of the protected segment 9-16, which formed gel-like solutions in the commonly used organic solvents (DMF, DCM, DMSO). Thus, the condensation with segment 17-31 immobilized on the solid support was very difficult. In order to find suitable conditions for the coupling reaction, we carried out a series of the experiments with following cleavage of the product from the resin and HPLC analysis (Table 2).

Table 2. Coupling reaction between Liraglutide fragments 9-16 and 17-31 in various conditions.

Solvent	Reagents (eq)	9-16, eq (respect to 17-31)	C_{9-16}, g/L	T, °C	Time, h	Conversion, %
DMF	PyBOP (1.5)/DIPEA (4)	1.5	50	40	96	30
	DIC (1.5)/Oxyma (1.5)	1.5	50	40	24	43
	DIC (1.5)/Oxyma (1.5)	1.5	25	40	24	33
	PyBOP (1.5)/DIPEA (1.5)	1.5	25	40	24	20
	DIC (1.5)/Oxyma (1.5)	1.5	25	40	48	66
	DIC (1.5)/Oxyma (1.5)	1.5	25	60	72	25
DMF/DCM (1:1)	DIC (1.5)/Oxyma(1.5)	1.5	25	RT	48	14
DMF/DCM (1:1)	DIC (1.5)/Oxyma (1.5)	1.5	25	40	48	42
DMF/DCM (1:1), 1% Triton X	DIC (1.5)/Oxyma (1.5)	1.5	90	40	3.5	>90

The comparison between PyBOP/DIPEA and OxymaPure/DIC showed a higher potency of the last activation mixture at different concentration of segment 9-16. Therefore it was chosen for the following tests. The increase of the reaction time, as well as the change of the solvent to the mixture of DCM/DMF, did not give satisfactory results. Heating in DCM/DMF allowed to increase the conversion (the ratio 9-31/17-31). However, a further increase of the temperature (in pure DMF) up to 60^{o} C led to a decrease of the yield, probably because of the decomposition of the activated ester. Finally, addition of the surfactant Triton X allowed us to bring the reaction almost to completion in 3.5 h, even at high concentration of segment 9-16 (Figure S14). After removal of the excess of reagents, the unreacted 17-31 was acetylated in order to facilitate the reverse-phase HPLC purification of the product.

Apparently, the reason of the difficult coupling can be ascribed not only to the low solubility of the segment 9-16, but also to the possible aggregation and folding on the solid support of the forming lipopeptide 9-31, which slows down the rate of the coupling reaction. The introduction into the system of a pseudoproline moiety with aggregate disrupting properties could facilitate

thesegment condensation. Indeed, the attachment of fragment 1-8 at the next step even at low concentration and without addition of surfactant led to a 76% conversion (Table 3).

Table 3. Coupling reaction between Liraglutide fragments 1-8 and 9-31.

Solvent	**Reagents (eq)**	**1-8, eq (respect to 9-31)**	**C_{1-8}, g/L**	**T, °C**	**Time, h**	**Conversion, %**
DMF	DIC (1.5)/Oxyma (1.5)	1.5	50	40	24	76
DMF/DCM (1:1), 1% Triton X	DIC (1.5)/Oxyma (1.5)	1.5	265	40	3.5	>90

The yield was further improved by using a 1% Triton X solution and increasing the concentration of the segment. At the end of the reaction the unreacted peptide chains were acetylated as in the previous case. Finally, the peptide was cleaved from the resin and completely deprotected on the side chains to give Liraglutide with an overall purity of 64% and an overall yield of 75% (Figure 3, B). The main impurities in this case are the acetylated fragments 17-31 and 9-31 with retention times 25.81 and 24.10 min, respectively.

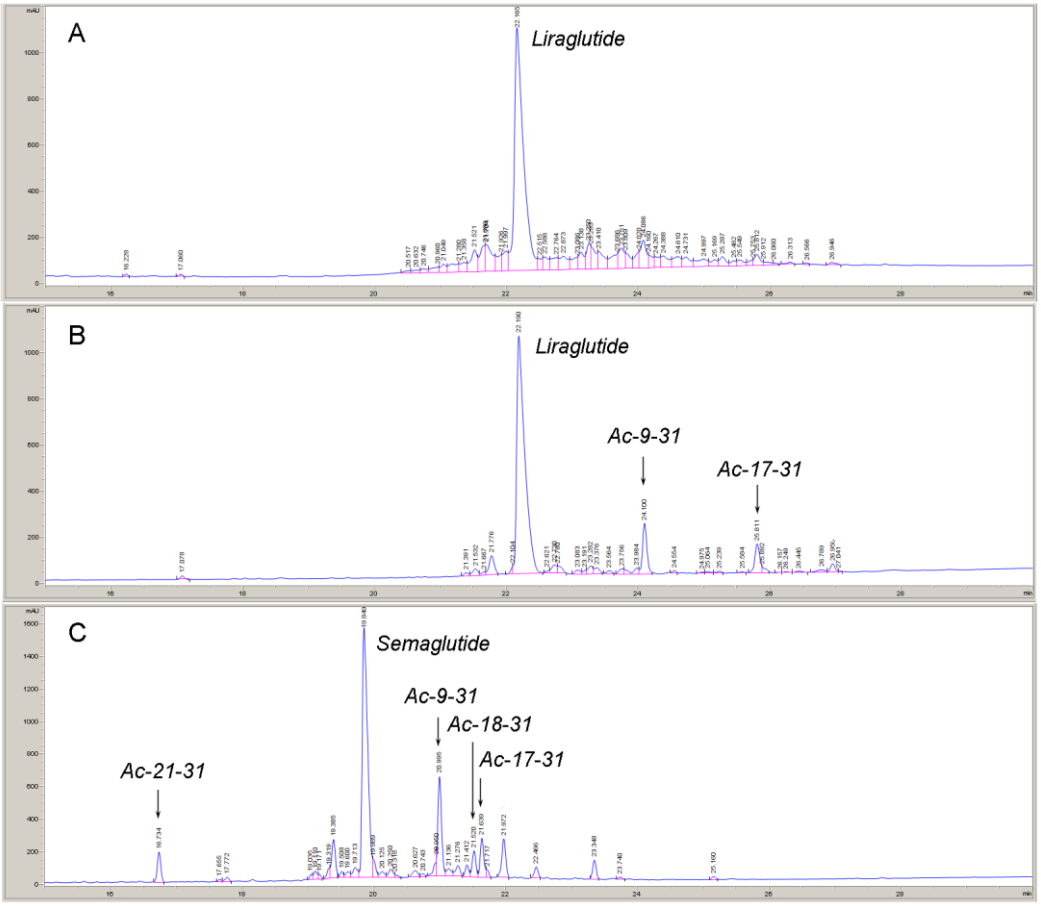


Figure 3. HPLC profiles of crude Liraglutide prepared by step-by-step SPPS (A) and crude Liraglutide and Semaglutide prepared by a convergent approach (B and C, respectively) (Analytical method 1, see Experimental part).

A comparison between the stepwise solid-phase synthesis of Liraglutide previously described by us[25] and the novel convergent approach shows clear advantages of the last method in terms of both peptide purity (36% *vs* 64%) and lower amount of close-eluting impurities (Figure 3, A and B; Tables S1 and S2).

The feasibility of this synthetic scheme was evaluated also for the preparation of Semaglutide by using Semaglutide side chain on Lys^{20} and replacing Ala^{2} with Aib. The same reaction conditions as for Liraglutide were applied. The synthesis of segment 1-8 was completed successfully with 65% yield and 94.2% HPLC purity. The insertion of the side chain, longer than that of Liraglutide, in segment 17-31 resulted in being more difficult: about 7% of peptide remained unreacted. However, the corresponding impurity after acetylation (Ac-21-31) had a HPLC retention time very different from the target peptide. The coupling of segments 9-16 and 17-31 was carried out successfully with high yield (about 95%) and purity 54.9%, whereas the attachment of the last octapeptide showed a

conversion of 85% after 24 hours (Figure 3, the corresponding acetylated segment 9-31 is seen at the retention time 21.00 min). As in the case of Liraglutide, the HPLC chromatogram of crude Semaglutide showed a low amount of close-eluting impurities around the main peak. Thus, the efficiency of the convergent approach for the preparation of Semaglutide can be confirmed also in this case, though some steps of the synthesis have to be further optimized.

CONCLUSIONS

In summary, here we reported a novel method for the preparation of the lipidated intermediates for the synthesis of the GLP-1 receptor agonists Liraglutide and Semaglutide. This innovative approach *via* copper(II) lysinate does not require orthogonally protected lysine as a starting material and can be particularly suitable for the industrial preparation of the peptides. In addition, we described the convergent approach for the solid-phase synthesis of GLP-1 receptor agonists Liraglutide and Semaglutide, which consists in the stepwise assembly of three peptide segments on the solid support. We showed that the insertion of a C-terminal pseudoproline residue at the site of the fragment condensation affords efficient coupling. The study of the coupling conditions allowed us to identify the parameters that give almost complete conversion to the target peptide with excellent purity and high yield. Thus, the approaches described here can be very useful not only for the synthesis of Liraglutide and Semaglutide, but also for the production of similar peptide APIs.

EXPERIMENTAL PART

Iris Biotech: N,N-dimetylformamide (DMF), dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), piperidine, MBH-Br resin, 2-chlorotrityl chloride resin (CTC). *Sigma Aldrich:* acetonitrile (MeCN) for mass spectrometry (MS) (>99,9%), TFA for MS (>99,9%), methyl *tert*-butyl ether (MTBE), triisopropylsilane (TIS), acetic anhydride, copper(II) sulfate pentahydrate, lysine hydrochloride, Fmoc-Aib-OH; *Carbosynth*: Ethyl (hydroxyimino)cyanoacetate (Oxyme Pure), N,N′-diisopropylcarbodiimide (DIC), 2-[2-[2-(Fmoc-amino)ethoxy]ethoxy]acetic acid (Fmoc-AEEA-OH), Octadecanedioic acid mono-tert-butyl ester; *GL Biochem:* Fmoc-Ala-OH (Fmoc, fluorenyl-9-methyloxycarbonyl), Fmoc-Asp(O*t*Bu)-OH, Fmoc-Arg(Pbf)-OH (Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Gln(Trt)-OH (Trt, trityl), Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Boc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH (Boc, *tert*-butyloxycarbonyl), Fmoc-Phe-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(N^{in}Boc)-OH, Fmoc-Val-OH, H-Glu-O*t*Bu.

HPLC and UPLC chromatography:

Analytical method 1. Agilent Technologies 1200; C18 Pore-shell column (4.6×100 mm). Eluent A, TFA/H_2O 0.1% *v/v*; eluent B: TFA/ACN 0.01% *v/v*; detection at 224 nm; gradient elution:

Time (min)	Eluent B
0	5
3	5
33	95
38	95
40	5
50	5

Analytical method 2. Agilent Technologies 1290; Water Cortex column, C18+ (4.6×150mm). Eluent A, TFA/H_2O 0.1% *v/v*; eluent B: ACN; detection at 224 nm; gradient elution:

Time (min)	Eluent B
0	45
5	45
70	75
75	75
76	45
85	45

Analytical method 3. Agilent Technologies 1290; Zorbax Eclipse Plus C8 column (4.6×150 mm). Eluent A KH_2PO_4 10 mM, pH adjusted to 6.00 with NaOH 1N; eluent B: acetonitrile; detection at 224 nm; gradient elution:

Time (min)	Eluent B
0	40
2	40
52	80
60	80
61	40
70	40

Analytical method 4. Agilent Technologies 1290; Water Cortex column, C18+ (4.6×150mm). Eluent A, TFA/H_2O 0.05% *v/v*; eluent B: H_2O:ACN:MeOH:TFA (150:50:800:0.6 *v/v*); detection at 224 nm; gradient elution:

Time (min)	Eluent B
0	80
30	95
35	100
37.5	100
38	80
43	80

Mass spectrometry analyses: mass spectra were acquired in the LC-MS mode on Agilent 6530 mass accuracy Q-ToF, operating in the positive mode.

NMR analysis: ^{1}H NMR spectra were recorded at 298 K in $CDCl_3$ or D_2O solution (Sigma Aldrich, peptide concentration 1.2 mM) using a Bruker AC spectrometer (200 MHz) and the TOPSPIN software package.

FT-IR absorption measurements were performed on a Perkin Elmer model 1720 X FT-IR spectrophotometer, nitrogen flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. The samples were finely grinded with KBr in order to obtain a homogeneous disk before the analyses.

ICP-MS analysis: inductively coupled plasma mass spectrometry analysis (ICP-MS) for the determination of the Cu content was carried out with an Agilent Technologies 7700x ICP-MS (Agilent Technologies International Japan, Ltd., Tokyo, Japan). ICP-MS instrument was equipped with an octopole collision cell operating in kinetic energy discrimination mode used for the removal of polyatomic interferences and argon-based interferences. The optimal performance was obtained by using the collision cell in He mode.[39] The ICP-MS was tuned daily using a tuning solution containing 10 μg/L ^{140}Ce, ^{7}Li, ^{205}Tl and ^{89}Y (Agilent Technologies, UK). The internal standard mixture (Agilent, 5183-4681) was added to the sample solution *via* a T-junction. Multi-element calibration standard IV-ICPMS-71A "Inorganic ventures" was used for Cu determination. Multielement standard solutions for calibration were prepared in acqua regia 5% by gravimetric serial dilution at twelve different concentrations (1 ng/L – 100 μg/L). The parameters of the calibration lines were obtained by using the Theil-Sen non-parametric regression technique. All regressions were linear with a determination coefficient (R^2) larger than 0.9999. In a typical

experiment about 10 mg of the sample were accurately weighed and digested using microwaves in 5 g of 69% solution of nitric acid accordingly to the following procedure: ramp temperature from room temperature to 200 °C in 5 min, then 200 °C for 5 min, pressure 400 PSI, power 300 W and stirring mode “medium”. Then the solutions were diluted with the same solvent used for calibrations and analyzed.

Synthesis of Pal-Glu-O*t*Bu

To a solution of palmitic acid (20.0 g, 0.0780 mol) in 100 mL of dichloromethane, a solution of HOSu (9.0 g, 0.0780 mol) in 100 mL of acetonitrile and DIC (12.2 mL, 0.0780 mol) were added. The reaction mixture was stirred overnight and the precipitate was filtered off. Then H-Glu-O*t*Bu (17.4 g, 0.0858 mol) and DIPEA (27.2 mL, 0.0156 mol) were added and the reaction mixture was stirred overnight. The solvent was evaporated and the oil was dissolved in 200 mL of ethyl acetate. The product was washed with 10% $KHSO_4$ (3 × 300 mL) and 300 mL of water, the solution was dried over $MgSO_4$ and the solvent was evaporated. The product was recrystallized twice from hot petroleum ether to yield 31.8 g (90%) of Pal-Glu-O*t*Bu with HPLC purity 94%.

Pal-Glu-O*t*Bu: $[M+H]^+_{calc}$ = 442.4, $[M+H]^+_{exp}$ = 386.3 (-*t*Bu)

^{1}H NMR (200MHz, $CDCl_3$): δ = 0.87 (m, 3H, CH_3), 1.25 (s, 24H, 12 CH_2), 1.47 (s, 9H, *t*Bu), 1.62 (m, 2H, CH_2), 2.05-1.70 (m, 2H, CH_2), 2.30-2.15 (m, 2H, CH_2), 2.50-2.35 (m, 2H, CH_2), 4.65-4.45 (m, 1H, CH), 6.25 (d, *J* = 8 Hz, 1H, NH).

FT-IR (KBr): ν_{max} 3376, 2909, 2842, 1722, 1707, 1649, 1522 cm^{-1}.

Synthesis of H-Lys(Pal-Glu-O*t*Bu)-OH

To a stirred solution of lysine hydrochloride (20.7 g, 0.1130 mol) in 100 mL of 2M $NaHCO_3$, a solution of copper (II) sulfate pentahydrate (14.2 g, 0.0567 mol) in 100 mL water was added portionwise in 1 h. The solution was stirred overnight and used for the following reaction after addition of $NaHCO_3$ (9.5 g, 0.0113 mol).

A solution of Pal-Glu-O*t*Bu (30.0 g, 0.0680 mol), HOSu (7.8 g, 0.0680 mol) and DIC (10.6 mL, 0.0680 mol) in acetone was stirred overnight. The precipitate was filtered off and the solution was added to the solution of copper(II) lysinate. The reaction mixture was stirred for 2 days. The precipitate was washed twice with 200 mL of ethyl acetate and three times with 200 mL of water. Then the precipitate was resuspended in 100 mL of water and EDTA (60.0 g, 0.2000 mol) was added. The reaction mixture was stirred overnight and the product was filtered off, washed three times with 200 mL of water and dried *in vacuo*. Yield: 30.2 g (78%), HPLC purity 89%.

H-Lys(Pal-Glu-O*t*Bu)-OH: $[M+H]^+_{calc}$ = 570.4, $[M+H]^+_{exp}$ = 570.5

^{1}H NMR (200MHz, CD_3OD): δ = 0.90 (m, 3H, CH_3), 1.29 (s, 24H, $12CH_2$), 1.47 (s, 9H, *t*Bu), 1.70-1.50 (m, 4H, $2CH_2$), 1.98-1.72 (m, 3H, CH, CH_2), 2.15-2.00 (m, 1H, CH), 2.35-2.15 (m, 4H, $2CH_2$), 3.20 (m, 2H, CH_2), 3.32 (m, 2H, CH_2), 3.54 (t, *J* = 6, 1H, CH), 4.35-4.15 (m, 1H, CH), 8.00 (t, *J* = 6 Hz, 1H, NH), 8.23 (d, *J* = 8 Hz, 1H, NH).

FT-IR (KBr): ν_{max} 3317, 2915, 2850, 1730, 1640, 1529, 1151 cm^{-1}.

Synthesis of Fmoc-Lys(Pal-Glu-O*t*Bu)-OH

Fmoc-OSu (17.8 g, 0.0527 mol) was dissolved in 200 mL of acetonitrile and added portionwise to the solution of H-Lys(Pal-Glu-O*t*Bu)-OH (30.0 g, 0.0527 mol) in 300 mL of the mixture water/methanol (*v*/*v* 1/1). pH of the reaction mixture was adjusted to 8 with DIPEA. After the completion of the reaction (HPLC control) the precipitate was filtered off and the organic solvent was evaporated. The suspension was diluted with 300 mL of 10% $NaHSO_4$ and the product was extracted twice with 200 mL of ethyl acetate. The organic phase was washed twice with 300 mL of 10% $NaHSO_4$, 300 mL of water, dried over $MgSO_4$ and evaporated. The product was crystallized from ethyl acetate. Yield: 35.5 g (85%) and HPLC purity >99%.

Fmoc-Lys(Pal-Glu-O*t*Bu)-OH: $[M+H]^+_{calc}$ = 792.5, $[M+H]^+_{exp}$ = 736.5 (-*t*Bu).

^{1}H NMR (200MHz, $CDCl_3$): δ = 0.87 (t, 3H, CH_3), 1.24 (m, 26H, $13CH_2$), 1.45 (s, 9H, *t*Bu), 1.70-1.48 (m, 4H, $2CH_2$), 1.98-1.70 (m, 3H, CH, CH_2), 2.15-2.00 (m, 1H, CH), 2.30-2.15 (m, 4H, $2CH_2$), 3.25 (m, 2H, CH_2), 3.75 (m, 1H, CH), 4.20 (m, 1H, CH), 4.40-4.25 (m, 3H, CH, CH_2), 5.77 (d, *J* = 8Hz, 1H, NH), 6.56 (d, *J* = 8Hz, 1H, NH), 6.76 (t, *J* = 6Hz, 1H, NH), 7.40-7.20 (m, 4H, 4CH), 7.59 (d, *J* = 8Hz, 2H, 2CH), 7.74 (d, *J* = 6Hz, 2H, 2CH).

FT-IR (KBr): ν_{max} 3331, 2923, 2850, 1730, 1685, 1640, 1529, 1159 cm^{-1}.

Synthesis of *t*BuOCO-$(CH_2)_{16}$-CO-Glu-O*t*Bu

To a solution of octadecanedioic acid mono-*tert*-butyl ester (0.90 g, 0.0024 mmol) in 10 mL of dichloromethane, a solution of HOSu (0.28 g, 0.0024 mmol) in 10 mL of acetonitrile and DIC (0.38 ml, 0.0024 mmol) were added. The reaction mixture was stirred overnight and the precipitate was filtered off. Then H-Glu-O*t*Bu (0.75 g, 0.0037 mmol) and DIPEA (1.28 mL, 0.0074 mmol) were added and the reaction mixture was stirred overnight. The solvent was evaporated and the oily residue was dissolved in 50 mL of ethyl acetate. The product was washed with 10% $KHSO_4$ (3 × 100 mL) and 100 mL of water, the solution was dried over $MgSO_4$ and the solvent was evaporated.

The product was purified by "flash chromatography" by eluting with $CHCl_3$ and then the mixtures $CHCl_3$/methanol 30:0.5 and 19.5:0.5 to give 1.13 g of *t*BuOCO-$(CH_2)_{16}$-CO-Glu-O*t*Bu (yield 85%) with HPLC purity 92.8%.

$C_{31}H_{57}NO_7$: $[M+Na]^+_{calc}$ = 578.4, $[M+Na]^+_{exp}$ = 578.4

^{1}H NMR (200MHz, $CDCl_3$): δ = 1.25 (m, 24H, 12 CH_2), 1.44 (s, 9H, *t*Bu), 1.47 (s, 9H, *t*Bu), 1.60 (m, 4H, 2CH_2), 1.91 (m, *J* = 8Hz, 1H, CH), 2.28-2.15 (m, 5H, CH, 2CH_2), 2.45-2.30 (m, 2H, CH_2), 4.52 (m, 1H, CH), 6.23 (d, *J* = 6Hz, 1H, NH).

FT-IR ($CDCl_3$): ν_{max} 3314, 2911, 2850, 1717, 1663, 1531 cm^{-1}.

Synthesis of *t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu

Fmoc-AEEA-OH (0.31 g, 0.8000 mmol) and DIPEA (0.42 ml, 0.3103 mmol) were dissolved in 5 mL of DCM and added to CTC resin (0.5 g, medium loading 1.6 mmol/g) swelled in DCM. The reaction mixture was stirred for 2 h and the resin was filtered and washed with DCM (3 × 5 mL). The unreacted sites of the resin were capped using 5 mL of the mixture DCM/DIPEA/methanol (*v/v/v* 17/2/1) and then with 10% solution of acetic anhydride in DCM. The loading of the resin was checked by UV adsorption measurement of the solution after Fmoc deprotection with 20% solution of piperidine in DMF and was found to be 1.48 mmol/g. The second Fmoc-AEEA-OH (0.62 g, 1.6000 mmol) was coupled in 90 min after activation with the mixture of DIC (1 eq) and Oxyma Pure (1 eq) during 3 min. The Fmoc deprotection was carried out using 20% solution of piperidine in DMF (2 × 5 mL, 10 min each) with following washing of the resin with DMF (4 × 10 mL). *t*BuOCO-$(CH_2)_{16}$-CO-Glu-O*t*Bu (1.5 eq) was activated by the mixture of DIC (1.5 eq) and Oxyma Pure (1.5 eq) and added to the resin. The reaction mixture was stirred overnight and unreacted amino groups were capped with 10% solution of acetic anhydride in DMF. The product was cleaved from the resin by treatment with 30% solution of HFIP in DCM (2 × 5 mL, 30 min each) and organic solvent was evaporated to give the oily residue. Yield: 0.58 g (92%), HPLC purity 83.6%.

$C_{43}H_{79}N_3O_{13}$: $[M+H]^+_{calc}$ = 846.6, $[M+H]^+_{exp}$ = 846.1

^{1}H NMR (200MHz, $CDCl_3$): δ = 1.31 (m, 24H, 12 CH_2), 1.50 (s, 9H, *t*Bu), 1.53 (s, 9H, *t*Bu), 1.65 (m, 4H, 2CH_2), 1.97 (q, *J* = 8Hz, 1H, CH), 2.45-2.15 (m, 7H, CH, 3CH_2), 3.80-3.48 (m, 16H, 8CH_2), 4.07 (s, 2H, CH_2), 4.20 (m, 3H, CH, CH_2), 6.65 (d, *J* = 8Hz, 1H, NH), 7.10 (t, *J* = 6Hz, 1H, NH), 7.45 (t, *J* = 6Hz, 1H, NH).

FT-IR (KBr): ν_{max} 3337, 2966, 2921, 2850, 1732, 1616, 1161 cm^{-1}.

Synthesis of H-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH

To a stirred solution of lysine hydrochloride (1.08 g, 5.9130 mmol) in 5 mL of 2M $NaHCO_3$, a solution of copper (II) sulfate pentahydrate (0.74 g, 2.9565 mmol) in 5 mL water was added portionwise in 1 h. The solution was stirred overnight and used for the following reaction after addition of $NaHCO_3$ (0.50 g, 5.9130 mmol).

A solution of *t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu (0.5 g, 0.5913 mmol), HOSu (68 mg, 0.5913 mmol) and DIC (92 µL, 0.5913 mmol) in acetone was stirred overnight. The precipitate was filtered off and the solution was added to the solution of copper(II) lysinate. The reaction mixture was stirred for 2 days. The precipitate was washed twice with 10 mL of ethyl acetate and three times with 10 mL of water. Then the precipitate was resuspended in 5 mL of water and EDTA (0.39 g, 1.3304 mmol) was added. The reaction mixture was stirred overnight, acetonitrile (30 mL) was added to the reaction mixture and the precipitate was filtered off. The precipitation was repeated twice and the solvent was evaporated to give the oily residue. The product was used without further purification. Yield: 0.48 g (83%), HPLC purity 92.6%.

$C_{49}H_{91}N_5O_{14}$: $[M+H]^+_{calc}$ = 974.7, $[M+H]^+_{exp}$ = 974.6

Synthesis of Fmoc-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH

Fmoc-OSu (0.17 g, 0.4930 mmol) was dissolved in 10 mL of acetonitrile and added portionwise to the solution of H-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH (0.48 g, 0.4930 mmol) in 10 mL of water. pH of the reaction mixture was adjusted to 8 with DIPEA. After the completion of the reaction (HPLC control) the organic solvent was evaporated. The suspension was diluted with 50 mL of 10% $NaHSO_4$ and the product was extracted twice with 30 mL of ethyl acetate. The organic phase was washed twice with 100 mL of 10% $NaHSO_4$, 100 mL of water, dried over $MgSO_4$ and evaporated. The product was purified by "flash" chromatography by eluting with $CHCl_3$ and then the mixtures $CHCl_3$/ethanol 20:1, 10:1 and 5:1 to give 0.51 g of Fmoc-Lys(*t*BuOCO-$(CH_2)_{16}$-CO-Glu(AEEA-AEEA)-O*t*Bu)-OH (yield 87%) and HPLC purity >99%.

$C_{64}H_{101}N_5O_{16}$: $[M+H]^+_{calc}$ = 1196.7, $[M+H]^+_{exp}$ = 1196.7

^{1}H NMR (200MHz, $CDCl_3$): δ = 1.30 (m, 28H, $14CH_2$), 1.50 (s, 11H, *t*Bu, CH_2), 1.51 (s, 9H, *t*Bu), 1.65 (m, 6H, $3CH_2$), 1.94 (m, 3H, CH, CH_2), 2.45-2.15 (m, 7H, CH, $3CH_2$), 3.37 (m, 2H, CH_2), 3.75-3.48 (m, 16H, $8CH_2$), 4.04 (s, 2H, CH_2), 4,06 (s, 2H, CH_2), 4.28 (t, *J* = 8Hz, 1H, CH), 4.45 (m, 4H, 2CH, CH_2), 5.86 (d, *J* = 8Hz, 1H, NH), 6.64 (d, *J* = 8Hz, 1H, NH), 7.02 (t, *J* = 6Hz, 1H, NH), 7.10 (t, *J* = 6Hz, 1H, NH), 7.55-7.30 (m, 4H, CH), 7.67 (d, *J* = 8Hz, 2H, CH), 7.82 (d, *J* = 8Hz, 2H, CH).

FT-IR ($CDCl_3$): ν_{max} 3305, 2911, 2842, 1740, 1655, 1531, 1153 cm^{-1}.

Synthesis of Liraglutide by step-by-step SPPS

The synthesis was carried out as described previously with an exception of carboxylic group activation method (Oxyma Pure/DIC instead of HBTU/DIPEA).[25]

Synthesis of Liraglutide by convergent approach

Step 1. Preparation of fragment 17-31.

H-Gln(Trt)^{17}-Ala-Ala-Lys(Pal-Glu-OtBu)-Glu-(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly^{31}-MBH resin

The synthesis of the peptide fragment 17-31 was carried out at room temperature by stepwise Fmoc SPPS using 100 g of MBH-Br resin (medium loading 1.6 mmol/g). After swelling of the resin in 0.8 L of DCM, Fmoc-Gly-OH (11.89 g, 0.25 eq), KI (39.84 g, 1.5 eq) and DIPEA (55.87 ml, 2 eq) in 0.5 L of DCM were added and the suspension was stirred for 15 h. Then the solvent was filtered off and the resin was washed with DCM (2 × 0.5 L) and treated with the solution of methanol (30 mL) and DIPEA (56 mL) in 0.4 L of DCM for 15 min. The remaining hydroxyl groups of the resin were capped by the treatment with the mixture of acetic anhydride (30 mL) and DIPEA (56 mL) in 0.41 L of DCM for 15 min and the resin was washed with DCM (2 × 0.5 L) and DMF (2 × 0.5 L). Fmoc deprotection was performed with 20% solution of piperidine in DMF (2 × 0.5 L, 10 min each) and the resin was washed with DMF (4 × 0.5 L). The substitution degree was checked by UV adsorption measurement of the solution collected after Fmoc deprotection and was found to be 0.53 mmol/g. Fmoc-protected amino acids (2-fold excess respect to the loading of the resin) were pre-activated with the mixture of DIC (2 eq) and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma Pure, 2 eq) during 3 min at 10ºC and consecutively coupled to the resin in 90 min. In the case of Arg, Val, Trp, Ala^{24}, Phe, Glu, Ala^{15} and Gln the coupling was repeated with 1 eq of acylating mixture. For the introduction of Fmoc-Lys(Pal-Glu-O*t*Bu)-OH into the peptide sequence 1.5 eq of palmitoylated dipeptide were used and the coupling was carried out in 6 h at 40ºC. At the end of each coupling the unreacted peptide chains were acylated with the solution of acetic anhydride (11 mL, 3 eq) in 0.49 L of DMF. The intermediate Fmoc deprotections were carried out using 20% solution of piperidine in DMF (2 × 0.5 L, 10 min each) with following washing of the resin with DMF (4 × 0.5 L). After the completion of the synthesis the resin was washed with DCM and dried. HPLC purity (small cleavage) 78.3%.

$C_{99}H_{161}N_{25}O_{23}$: $[M+H]^+_{calc}$ = 2069.2, $[M+H]^+_{exp}$ = 2069.0.

Step 2. Preparation of fragment 9-16

Fmoc-Asp(OtBu)^9-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly^{16}-OH

The synthesis of the peptide fragment was carried out by step-by-step SPPS using 2-chlorotrityl chloride resin (CTC resin) (117 g, medium loading 1.6 mmol/g). After the swelling of the resin using 0.94 L of DCM, Fmoc-Gly-OH and DIPEA (0.8 and 3 eq, respect to the loading of the resin) in 0.47 L of DCM were added. The reaction mixture was stirred for 1 hour and the solvent was filtered off. The unreacted sites of the resin were capped using the solution of methanol (23 mL) and DIPEA (98 mL) in 0.58 L of DCM in 15 min. Then the resin as treated with the solution of acetic anhydride (53 mL) and DIPEA (98 mL) in 0.55 L of DMF for 15 min and washed with DMF (3 × 0.7 L). The loading of the resin was checked by UV adsorption measurement of the solution after Fmoc deprotection and was found to be 1.08 mmol/g. Then Fmoc-Glu(O*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Val-OH and Fmoc-Asp(O*t*Bu)-OH (2-fold excess respect to the loading of the resin) were pre-activated by DIC (2 eq) and Oxyma Pure (2 eq) in 5 min at 10^{o}C and coupled consecutively to the resin in 90 min. In the case of the last three amino acids 3 eq of the coupling mixture was used. The intermediate Fmoc deprotections were carried out using 20% solution of piperidine in DMF (2 × 0.6 L, 10 min each) with following washing of the resin with DMF (4 × 0.6 L). After the completion of the synthesis the resin was washed with DCM and dried. The protected peptide was cleaved from the resin by the treatment with 0.7 L of 1.5% TFA in DCM for 2 min and the solution was filtered into 0.13 L of 10% solution of pyridine in methanol. The procedure was repeated 4 times. The combined solutions were concentrated to 30% of the volume. The protected octapeptide was precipitated by adding of 6 L of water, filtered, washed three times by water and dried. Yield: 173 g (90%), HPLC purity (small cleavage) 97.3%.

$C_{37}H_{56}N_8O_{16}$: $[M+H]^+_{calc}$ = 869.4, $[M+H]^+_{exp}$ = 869.3.

Step 3. Preparation of fragment 1-8

Boc-His(Trt)^1-Ala-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(ψ^{Me,Me}pro)^8-OH

The peptide fragment was prepared in a similar way as described in Step 2, using 350 g of CTC resin (medium loading 1.6 mmol/g). The attachment of Fmoc-Thr(*t*Bu)-Ser(ψ^{Me,Me}pro)^8-OH (0.8 eq) in presence of DIEA (3 eq) in DCM was carried out to give the loading 0.96 mmol/g. The first Fmoc deprotection was carried out using 20% solution of piperidine in DMF (3 × 2.1 L, 3 min each) with

following washing of the resin with DMF (4 × 2.1 L). Then Fmoc-Phe-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Ala-OH, and Boc-His(Trt)-OH (2-fold excess respect to the loading of the resin) were consecutively coupled to the resin in 90 min after pre-activation with DIC (2 eq) and Oxima Pure (2 eq) during 5 min. Boc-His(Trt)-OH was preactivated in 1 min at 0°C. For the Fmoc-deprotection of the amino acids two treatments with 20% solution of piperidine in DMF (10 min each) were performed. The protected octapeptide was cleaved from the resin using the same procedure as described previously. Yield: 376 g (80%), HPLC purity 93.6%.

$C_{75}H_{102}N_{10}O_{16}$: $[M+H]^+_{calc}$ = 1399.8, $[M+H]^+_{exp}$ = 1399.7.

Step 4. Condensation of fragments 17-31 and 9-16: preparation of fragment 9-31

H-Asp(OtBu)^{9}-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly-Gln(Trt)-Ala-Ala-Lys(Pal-Glu-OtBu)-Glu-(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly^{31}-MBH resin

Peptide fragment 17-31 obtained in Step 1 was swollen in 0.5 L of DMF/DCM mixture (*v/v* 1/1) containing 1% of Triton X and the organic solution was filtered off. Protected fragment 9-16 (123 g, 0.0900 mol, 1.7 eq) was dissolved in 1.3 L of DMF/DCM mixture (*v/v* 1/1) containing 1% of Triton X and pre-activated with Oxyma Pure (13 g, 0.0900 mol, 1.7 eq) and DIC (14 mL, 0.0900 mol) during 5 min. The coupling mixture was added to the resin and the reaction mixture was stirred for 3.5 h at 40°C. The resin was then washed with DMF (2 × 0.5 L) and the unreacted amino groups were capped with the solution of acetic anhydride (11 mL, 3 eq) in 0.49 L of DMF in 15 min. Lastly, Fmoc protective group was removed with 20% solution of piperidine in DMF containing 0.7M Oxyma Pure (2 × 0.5 L, 10 min each) with following washing of the resin with DMF (4 × 0.5 L). HPLC purity (small cleavage) 56.9%.

$C_{136}H_{215}N_{33}O_{38}$: $[M+H]^+_{calc}$ = 2919.6, $[M+2H]^{2+}_{exp}$ = 1460.2.

Step 5. Condensation of fragments 1-8 and 9-31: preparation of Liraglutide

Boc-His(Trt)^{1}-Ala-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(ψ^{Me,Me}pro)-Asp(OtBu)^{9}-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly-Gln(Trt)-Ala-Ala-Lys(Pal-Glu-OtBu)-Glu-(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly^{31}-MBH resin

Fmoc-deprotected peptidyl resin with fragment 9-31 obtained in Step 4 was swelled in 0.5 L of DMF/DCM mixture (*v/v* 1/1) containing 1% of Triton X and the organic solution was filtered off. Protected fragment 1-8 (119 g, 0.0850 mol, 1.6 eq) was dissolved in 0.45 L of DMF/DCM mixture (*v/v* 1/1) containing 1% of Triton X and pre-activated with Oxyma Pure (12 g, 0.0850 mol, 1.6 eq) and DIC (13 ml, 0.0850 mol, 1.6 eq) during 15 min. The coupling mixture was added to the resin

and the reaction mixture was stirred for 3.5 h at 40°C. The resin was then washed with DMF (2 × 0.5 L) and the unreacted amino groups were capped with the solution of acetic anhydride (11 mL, 3 eq) in 0.49 L of DMF in 15 min. Lastly, the resin was washed with DMF (2 × 0.5 L), DCM (3 × 0.5 L) and dried.

Step 6. Cleavage of Liraglutide

Dry peptidyl resin obtained in Step 5, was suspended in 1.3 L of the mixture TFA/water/phenol/TIS (v/v/v/v 88/5/5/2) and stirred for 1 h at 15°C and then 3 h at RT. Then the resin was filtered and washed with 0.3 L of the cleavage mixture. The solutions were collected and the crude peptide was precipitated in 5 L of MTBE. The precipitate was washed several times with MTBE and dried to get crude Liraglutide with overall yield 150 g (75%) and HPLC purity 64.1% (early eluting peak of the solvent DMSO not integrated).

$C_{172}H_{265}N_{43}O_{51}$: $[M+H]^{+}_{calc}$ = 3751.3; $[M+2H]^{2+}_{exp}$ = 1875.8, $[M+3H]^{3+}_{exp}$ = 1251.2.

Synthesis of Semaglutide by convergent approach

Step 1. Preparation of fragment 17-31.

H-Gln(Trt)^{17}-Ala-Ala-Lys([tBuOOC-(CH$_2$)$_{16}$-CO-γGlu-OtBu]-AEEA-AEEA)-Glu(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly^{31}-MBH-Resin

The synthesis of the peptide fragment was carried out at room temperature using 0.25 g of H-Gly-O-MBH resin (loading 1.1 mmol/g). The resin was swelled in 3 ml of DMF and used for stepwise Fmoc SPPS. Fmoc-protected amino acids (two-fold excess respect to the loading of the resin) were pre-activated with the mixture of DIC (2 eq) and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma Pure, 2 eq) during 3 min and consecutively coupled to the resin in 90 min. The couplings of Arg, Val, Trp, Ala^{24,19}, Phe, Glu and Gln were repeated with 1 eq of the coupling mixture for 60 min. The introduction of the lipidated side chain into the peptide sequence was carried out using 1.5 eq of Fmoc-Lys([*t*BuOOC-(CH$_2$)$_{16}$-CO-γGlu-O*t*Bu]-AEEA-AEEA)-OH, which was pre-activated with 1.5 eq of DIC and 1.5 eq Oxyma Pure and coupled in 6 h at 40°C. The completion of the coupling was monitored by ninhydrine test. At the end of each coupling the unreacted peptide chains were acetylated with 3 eq of acetic anhydride in DMF and washed with DMF (3 × 3 ml). The intermediate Fmoc deprotections were carried out using 20% solution of piperidine in DMF (2 × 3 ml, 10 min) with following washing of the resin with DMF (4 × 3 ml). For the last five amino acids a mixture of 20% of piperidine and 1.7 M Oxyma Pure was used for the Fmoc-deprotection. After the completion of the synthesis the resin was washed with DCM and dried. HPLC purity (small cleavage) 67.3%.

$C_{113}H_{185}N_{27}O_{31}$: $[M+H]^+_{calc}$ = 2417.4, $[M+H]^+_{exp}$ = 2417.8.

Step 2. Preparation of fragment 1-8

Boc-His(Trt)^1-Aib-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(ψ^{Me,Me}pro)^8-OH

The synthesis of the peptide fragment was carried using 0.5 g of CTC resin (medium loading 1.6 mmol/g). After swelling of the resin in 3 ml of DCM, a solution of Fmoc-Thr(*t*Bu)-Ser(ψ^{Me,Me}pro)-OH and DIEA (0.8 and 3 eq, respect to the loading of the resin) in 5 mL of DCM was added. The reaction mixture was stirred for 16 hours and the solvent was filtered off. The unreacted sites of the resin were capped using a solution of MeOH (3 eq) and DIEA (3 eq) in 5 ml of DCM for 15 minutes. After washing with DCM (2 × 5 mL) the resin was treated with a solution of acetic anhydride (3 eq) and DIEA (3 eq) in 5 mL of DCM for 15 minutes, washed with DMF (3 × 5 mL), DCM (3 × 5 mL) and dried. The loading of the resin was checked by UV adsorption measurement of the solution after Fmoc deprotection was found to be 1.3 mmol/g. Then Fmoc-Phe-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Ala-OH, and Boc-His(Trt)-OH (2 eq) were pre-activated by 2 eq of DIC and 2 eq of Oxyma Pure during 3 min and coupled consecutively to the resin in 90 min. In the case of His, Oxyma B was used and pre-activation time was reduced to 1 min at 0^{o}C. The intermediate Fmoc deprotections were carried out using 20% solution of piperidine in DMF (2 × 5 mL, 10 min) with following washing of the resin with DMF (4 × 5 mL). After the completion of the synthesis the resin was washed with DCM (2 × 5 mL) and dried. The protected peptide was cleaved from the resin by the treatment with 3 mL of 1.5% TFA in DCM for 2 min and the solution was filtered into 3 mL of 10% solution of pyridine in methanol. The procedure was repeated 4 times and the combined solutions were concentrated to 30% of the volume. The protected peptide was precipitated by adding of water in ice bath, filtered, washed several times by water and dried. Yield: 590 mg (65%). HPLC purity 94.2%.

$C_{76}H_{104}N_{10}O_{16}$: $[M+H]^+_{calc}$ = 1413.8, $[M+H]^+_{exp}$ = 1413.6.

Step 3. Condensation of fragments 17-31 and 9-16: preparation of fragment 9-31

H-Asp(OtBu)^9-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly-Gln(Trt)-Ala-Ala-Lys([tBuOOC-(CH$_2$)$_{16}$-CO-γGlu-OtBu]-AEEA-AEEA)-Glu-(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly^{31}-MBH Resin

Fmoc-deprotected resin with Semaglutide fragment 17-31 obtained in the step 1, was swelled in 2 mL of the mixture DMF/DCM (1/1 *v*/*v*) containing 1% of Triton-X at 40^{o}C. The solution of

protected peptide 9-16 (750 mg, 0.5500 mmol, 2 eq) in 7 ml of the mixture DMF/DCM (1/1 *v*/*v*) containing 1% of Triton-X was pre-activated with DIC (86 µL, 0.5500 mmol) and Oxyma Pure (78 mg, 0.5500 mmol) at 40°C during 15 min. Then the coupling mixture was added to the resin and the reaction mixture was stirred for 3.5 h. The completion of the coupling was monitored by the ninhydrine test. The unreacted peptide chains were acetylated with 3 eq of acetic anhydride in DMF and the peptide resin was washed with DMF (3 × 3 mL). At the end Fmoc protective group was removed with 20% solution of piperidine in DMF with 1.7M Oxyma Pure (2 × 3 mL, 10 min) with following washing of the resin with DMF (4 × 3 mL). HPLC purity (small cleavage) 54.9%.
$C_{150}H_{239}N_{35}O_{46}$: $[M+H]^{+}_{calc}$ = 3267.7, $[M+2H]^{2+}_{exp}$ = 1634.8.

Step 4. Condensation of fragments 1-8 and 9-31: preparation of Semaglutide
Boc-His(Trt)[1]-Aib-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(ψ^{Me,Me}pro)-Asp(OtBu)-Val-Ser(tBu)-Ser(tBu)-Tyr(tBu)-Leu-Glu(OtBu)-Gly-Gln(Trt)-Ala-Ala-Lys([tBuOOC-(CH$_2$)$_{16}$-CO-γGlu-OtBu]-AEEA-AEEA)-Glu-(OtBu)-Phe-Ile-Ala-Trp(N^{in}Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly[31]-Resin
Fmoc-deprotected resin with peptide Fragment 9-31 obtained in step 3 was swelled in 2 mL of the mixture DMF/DCM (1/1 *v*/*v*) containing 1% of Triton-X at 40°C. The solution of protected Semaglutide fragment 1-8 (780 mg, 0.5500 mmol, 2 eq) in 7 mL of the mixture DMF/DCM (1/1 *v*/*v*) containing 1% of Triton-X was pre-activated with DIC (86 µL, 0.5600 mmol) and Oxyma Pure (78 mg, 0.5500 mmol) at 40°C during 15 min. Then the coupling mixture was added to the resin and the reaction mixture was stirred for 3.5 h. The completion of the coupling was monitored by ninhydrine test. The unreacted peptide chains were acetylated with 3 eq of acetic anhydride in DMF and peptide resin was washed with DMF (3 × 3 mL), DCM (2 × 3 mL) and dried.

Step 5. Cleavage of Semaglutide
Dry peptidyl resin obtained in step 4, was suspended in 4 mL of the mixture TFA/water/phenol/TIS (88/5/5/2) and stirred for 1 h at 0°C and 3 h at RT. Then the resin was filtered and washed with 1 mL of TFA. The solutions were collected and the crude peptide was precipitated in 25 mL of MTBE. The precipitate was washed several times with MTBE and dried to get crude Semaglutide with overall yield 0.7 g (61%) and HPLC purity 42% (early eluting peak of the solvent DMSO not integrated).
$C_{187}H_{291}N_{45}O_{59}$: $[M+H]^{+}_{calc}$ = 4112.1; $[M+2H]^{2+}_{exp}$ = 2057.5, $[M+3H]^{3+}_{exp}$ = 1371.8.

ASSOCIATED CONTENT

Supporting information

HPLC profiles, NMR and FT-IR spectra of the intermediates used for the preparation of Liraglutide and Semaglutide building blocks *via* copper(II) lysinate. HPLC profiles of the protected fragment 1-8, deprotected fragments 9-16 and 17-31 of Liraglutide and Semaglutide, HPLC profiles and the tables with the percentage values of the impurities in the crude Liraglutide and Semaglutide.

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