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Manufacturing and PEGylation of a Dual-Acting Peptide for Diabetes

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In the given manuscript we summarize our activities to develop a scalable synthesis for a PEGylated peptide, which undergoes development for metabolic disorders. The polypeptide contains a sequence of 31 natural amino acids and carries a site-specific PEGylation at the C-terminus. Initial synthetic studies revealed that the peptide moiety of the target molecule is not an ideal target for a linear solid-phase process, as we were confronted with "difficult sequences", which led to a low-yielding process. Subsequently, we developed a mixed-phase synthesis, which included production of fragments through solid-phase peptide synthesis, followed by an assembly of these fragments in solution. We were able to show that this new process delivered the desired peptide moiety in gram-scale with high purity. The overall yield was improved from 1 % for the sequential solid-phase process to 9 % for the fragment synthesis. Finally, a PEGylation process was installed to deliver the drug substance for preclinical and clinical testing.

Introduction

The advent of GLP-1 (glucagon-like peptide) agonists has provided a significant impetus for novel therapeutic concepts in the treatment of type 2 diabetes.[1] At Bayer Schering Pharma, we pursued a dual mechanism of action, which combined a GLP-1 agonist with the beneficial properties of a glucagon antagonist.[2] Accordingly, the active ingredient was designed as a chimeric peptide that combined structural elements of both native ligands (cf. Figure 1). Additionally, a site-specific 43 kD PEGylation was introduced at the C-terminus to increase the metabolic stability of the peptide and to reduce CNS-mediated nausea, which is a common adverse event associated with GLP-1 receptor agonists.

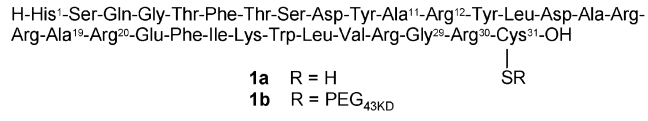

Figure 1. Structure of the hybrid peptide including structural elements from both glugacon (1–19) and GLP-1 (20–30) and a site-specific PEGylation at the C-terminus.

The synthesis of target molecule **1b** is divided into two stages, which include the peptide synthesis followed by PEGylation. Whereas the PEGylation more or less follows a standard protocol, the construction of the polypeptide backbone remains a challenge for synthetic chemistry. In particular, a clinical development program has to be accompanied by a robust and scalable production process covering a broad scale (from g to kg) with continuous output of the active ingredient in high purity. Our endeavour towards the development of a robust and scalable synthesis of **1b** is detailed in this manuscript.

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Results and Discussion

After its introduction by Merrifield in 1963,[3] solid-phase peptide synthesis (SPPS) has evolved as a powerful tool to rapidly access complex peptides.[4] For the supply of initial quantities of **1a**, a sequential SPPS protocol with the use of a standard Fmoc-*t*Bu procedure was applied to deliver peptidic backbone **1a** on a milligram-to-gram scale. However, after cleavage from the resin, the crude peptide was obtained in poor quality, and tedious chromatographic purification steps were required to obtain reasonable purity levels of ≥95 area-% by HPLC. The total yield even for an optimized sequential SPPS protocol did not exceed 1 %.

We speculated that the low performance of the sequential SPPS process was due to the specific amino acid sequence found in **1a**. Thus, the growing peptide chain tends to form aggregates during assembly, which leads to deteriorated swelling properties of the resin. As a consequence, the accessibility of the reagents during synthesis is reduced, which leads to low-yielding transformations. Indeed, in the course of the linear solid-phase synthesis, 15 coupling steps had to be repeated due to incomplete conversion, and the unreacted terminal amino groups had to be capped by acetylation. This showed that **1a** is not a trivial target for a standard solid-phase process and can be classified as a "difficult sequence".[5]

As a consequence, we embarked on a mixed-phase fragment synthesis, which is a well-accepted strategy to cope

with difficult sequences in SPPS.[6] Accordingly, the protected fragments are produced by SPPS and subsequently assembled in solution. The high convergence of such an approach adds value to a complex overall process, as the individual arms of the synthesis can be operated in parallel and the consumption of raw materials and reagents can be significantly reduced. More importantly, the risk to come across difficult sequences during SPPS is considerably reduced, because the fragments are smaller in size.[7] However, the overall risk to fail with this fragment strategy is shifted to the late stage of the synthesis, which addresses the solution-phase peptide synthesis. In particular, issues on solubility, isolation and purification protocols for the protected and most likely amorphous intermediates are difficult to predict in the planning phase.[8]

Retrosynthesis

A retrosynthetic analysis for a fragment approach was guided by the following considerations: (1) If possible, define fragments of similar size. (2) Avoid critical amino acids at the disconnections, which are known to undergo racemization or side reactions upon activation (e.g., Arg, Asp, Cys). (3) Avoid long sequences involving *S*-trityl protected Cys_{31}, which was found to undergo side reactions during the sequential SPPS protocol.[9] (4) Select lipophilic side-chain protecting groups to increase chances for good solubility in the later stage of the synthesis.

Eventually, we divided target molecule **1** into four fully protected fragments as displayed in Figure 2. Protected Arg-Cys dimer **2** was proposed in order to isolate the sensitive cysteine, whereas the remaining three building blocks **3**, **4** and **5** were selected for their C-terminal glycine or alanine units. From these amino acids we expected good coupling kinetics in the assembly of the subsequent fragments and a narrow side-product profile.

H-[30–31]-OtBu: H-Arg(Pbf)-Cys(Trt)-O*t*Bu
2

Fmoc-[20–29]-OH: Fmoc-Arg(Pbf)-Glu(O*t*Bu)-Phe-Ile-Lys(Boc)-Trp(Boc)-Leu-Val-Arg(Pbf)-Gly-OH
3

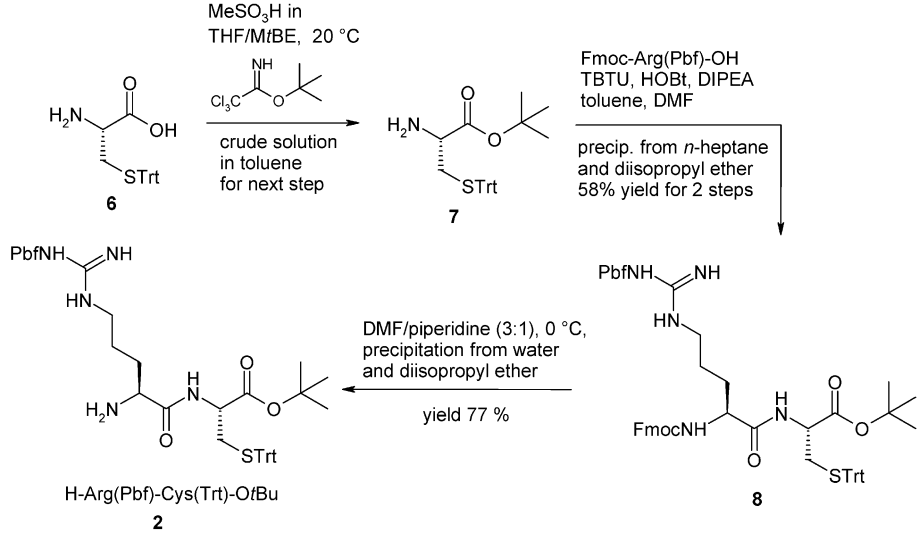
Fmoc-[12–19]-OH: Fmoc-Arg(Pbf)-Tyr(*t*Bu)-Leu-Asp(O*t*Bu)-Ala-Arg(Pbf)-Arg(Pbf)-Ala-OH
4

Boc-[1–11]-OH: Boc-His(Trt)-Ser(*t*Bu)-Gln(Trt)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Asp(O*t*Bu)-Tyr(*t*Bu)-Ala-OH
5

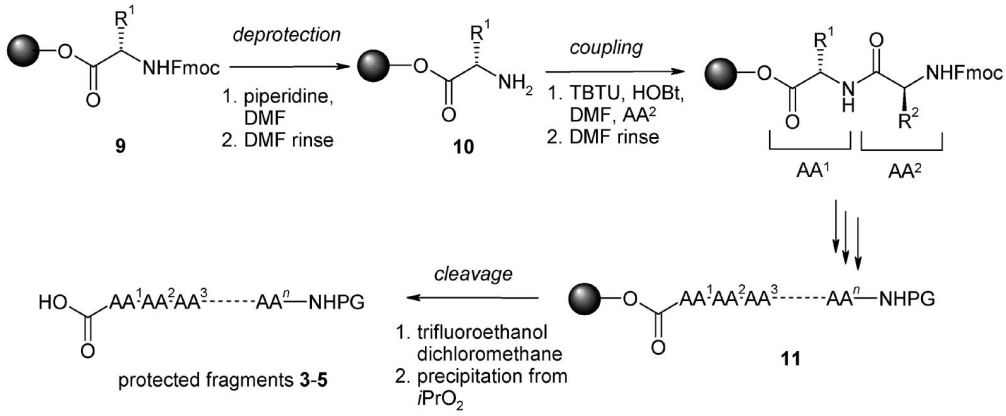
Figure 2. Protected fragments **2**–**5** resulting from a retrosynthetic disconnection between Gly^{29}-Arg^{30}, Ala^{19}-Arg^{20} and Ala^{11}-Arg^{12}. (Pbf: 2,2,4,6,7-pentamethyldihydrobenofuran-5-yl-sulfonyl, Fmoc: 9-fluorenylmethoxycarbonyl, Trt: trityl, *t*Bu: *tert*-butyl, Boc: *tert*-butoxycarbonyl).

Synthesis of the Fragments

For the synthesis of fragment **2**[10] we anticipated a short solution-phase sequence as outlined in Scheme 1. The introduction of the terminal *t*Bu ester towards **7** has been described for a variety of *N*-protected cysteine derivatives;[11] however, only one report details direct esterification commencing from H-Cys(Trt)-OH by using $HClO_4$ in *t*BuOAc.[12] From the scale-up perspective, we considered the use of $HClO_4$ as critical due to its explosive properties. Therefore, we introduced an alternative protocol for the direct conversion of **6** into **7** by employing *tert*-butyl trichloroacetimidate and methanesulfonic acid. After aqueous workup, ester **7** was isolated as a solution in toluene, and it was directly used in the subsequent coupling step with Fmoc-Arg(Pbf)-OH. Coupling was accomplished by a TBTU protocol, and protected dipeptide **8** was obtained with a purity of 92 area-% in 58% yield for both steps. Finally, the Fmoc group was removed with a cocktail of piperidine in DMF to give fragment **2**. This three-step process provides quick access to desired building block **2**, which did



Scheme 1. Solution-phase synthesis of fragment **2**. [MtBE: methyl *tert*-butyl ether, THF: tetrahydrofuran, TBTU: 2-(1*H*-benzotriazol-1-yl)-1,1,2,3-tetramethyluronium tetrafluoroborate, HOBt: 1-hydroxy-1*H*-benzotriazole, DIPEA: *N*,*N*-diisopropylethylamine, DMF: *N*,*N*-dimethylformamide].



Scheme 2. Solid-phase peptide synthesis (SPPS) of fragments **3**–**5** (AA: side-chain-protected amino acid; PG: protecting group; PG = Fmoc for **3** and **4**, PG = Boc for **5**).

not require elaborate chromatographic purification. Eventually, fragment **2** was obtained on a 50-g scale with HPLC purity of 93 area-% and a low level of cysteine epimerization (0.6%).

The remaining three fragments **3**–**5** were ideal targets for SPPS by using an Fmoc–*t*Bu protocol.[13] We selected chlorotrityl resin as the solid-phase carrier for a number of reasons:[14] (1) It allows selective and mild cleavage of side-chain protected fragments from the resin. (2) Racemization upon loading is minimized. (3) It has little tendency to form diketopiperazines. (4) It is available in multikilogram quantities.

The generic SPPS protocol, which was used for the production of fragments **3**–**5**, is outlined in Scheme 2. Resin loading was achieved upon addition of the first amino acid to the resin in the presence of diisopropylethylamine to yield functionalized resin **9**. After loading was complete, the resin was capped with MeOH to saturate the remaining active sites. In order to maximize space–time yield for fragment production, we used rather high resin loadings of 0.4–0.6 mmol g^{-1}.

Subsequently, Fmoc deprotection towards **10** was achieved by treatment of the resin with a 20% piperidine solution in DMF. Exhaustive rinsing with DMF to effect complete removal of the piperidine proved essential for a high-quality peptide.

Peptide coupling was accomplished by a TBTU/HOBt protocol by using diisopropylethylamine as the base. In our hands, the optimum concentration of running the coupling reactions was 10 mL of solvent per 1 g of resin. The excess amount of reagents can be reduced to 2 equiv. of the amino acid, 2 equiv. of the coupling reagent, 0.5 equiv. HOBt monohydrate and 2 equiv. of the base. We monitored the progress of the peptide coupling reactions by the on-beat "Kaiser test"[15] and found complete conversion after 1 h in all cases without the need to repeat the coupling steps.

There are a variety of cleavage conditions reported in the literature.[16] In our hands, a cleavage cocktail of trifluoroethanol/dichloromethane (1:4) worked reliably, and the conditions were mild enough to yield the fully protected fragments in good yields. Additionally, this cocktail avoided the common use of acetic acid or trifluoroacetic acid, which are difficult to remove without chromatography and which were found to interfere with the coupling reactions during the subsequent fragment condensations in solution. After cleavage, the crude products were isolated by precipitation from diisopropyl ether to give the amorphous products, which were stored at –20 °C.

Accordingly, the three fragments were produced on a 100–200-g scale to yield **3**, **4** and **5** in good purity after precipitation (cf. Table 1). Moreover, the rather high quality of the SPPS products enabled us to proceed with the fragment condensation without additional purification steps.

Table 1. Results for SPPS for fragments **3**–**5**.

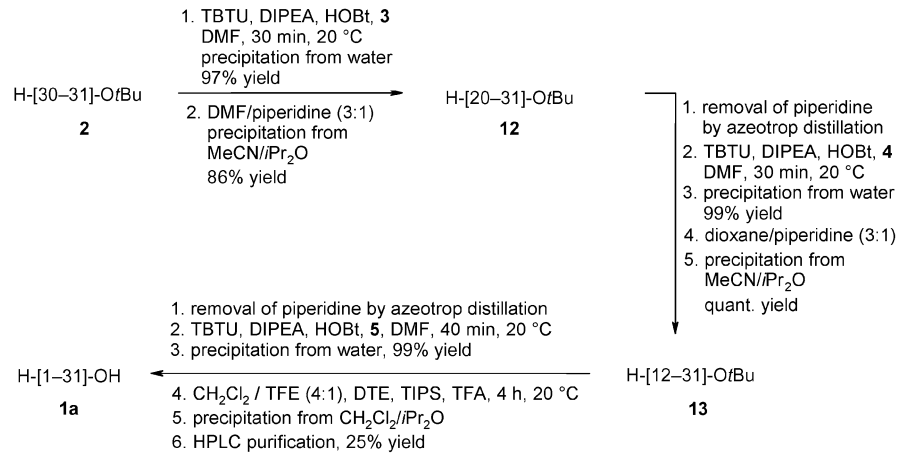
	Resin loading [mmol g^{-1}]	Yield [%]	Average yield per step [%]	Purity[a] [%]
Fragment **3**	0.4	67	98	93
Fragment **4**	0.6	82	99	93
Fragment **5**	0.5	52	97	93

[a] Area-% according to HPLC analysis.

Fragment Condensation

Condensation of the fragments in solution is displayed in Scheme 3. The chemical transformations were accomplished by standard procedures including TBTU-mediated peptide couplings and piperidine-induced Fmoc deprotection to give clean conversions for all steps. The stoichiometry of the fragments was optimized for each step to avoid tedious purification protocols for removal of an excess amount of the fragments.

To our delight, we observed good-to-excellent solubility properties for the fully protected intermediates throughout the sequence. However, as a drawback to this sequence, we experienced that the amorphous character of the reaction intermediates was a major obstacle for convenient isolation and purification procedures. In particular, reagents and side products, such as the piperidine dibenzofulvene adduct arising from Fmoc fragmentation, were difficult to remove during workup. Thus, for each step, we elaborated individual



Scheme 3. Solution-phase coupling of fragments **2**–**5** (MeCN: acetonitrile, TFE: trifluoroethanol, DTE: dithiothreitol, TIPS: triisopropylsilane).

precipitation protocols in order to isolate the amorphous products with a high level of purity as outlined in Scheme 3 and the Experimental Section. Additionally, residual traces of piperidine were removed by azeotropic distillation. This procedure operated under mild conditions (40–45 °C, 15 mbar) and no degradation of the protected peptide intermediates was observed.

The HPLC purity of the intermediates gradually decreased from 93 area-% for **2** to 79 area-% for fully protected peptide **1a** prior to global deprotection without chromatographic purification throughout the entire sequence (Figure 3). Eventually, TFA-mediated removal of the protecting groups led to a 20–30% loss of purity. The major impurity found in crude **1a** was assigned to a *t*Bu alkylation product.[17] Thus, we further optimized the cleavage conditions with a focus on cationic scavengers. Finally, a cocktail of triisopropylsilane and dithiothreitol worked best to curb the level of alkylated side products to ca. 10%.[16] Finally, crude peptide **1a** was isolated on gram scale with a purity of 55 area-% and subsequent HPLC purification yielded pure **1a** in not-yet fully optimized 25% yield.

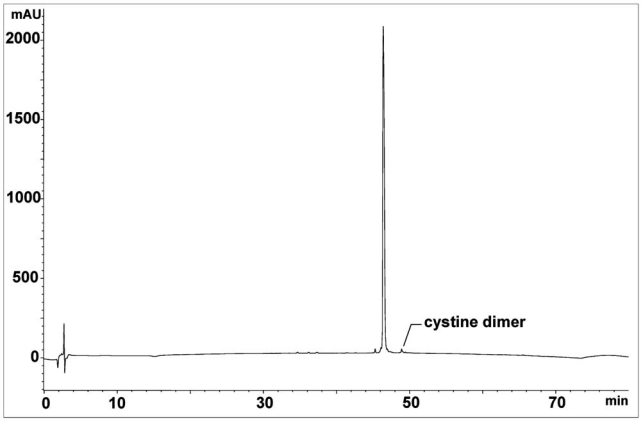


Figure 3. RP-18-HPLC chromatogram of target peptide **1a** (see Experimental Section for details).

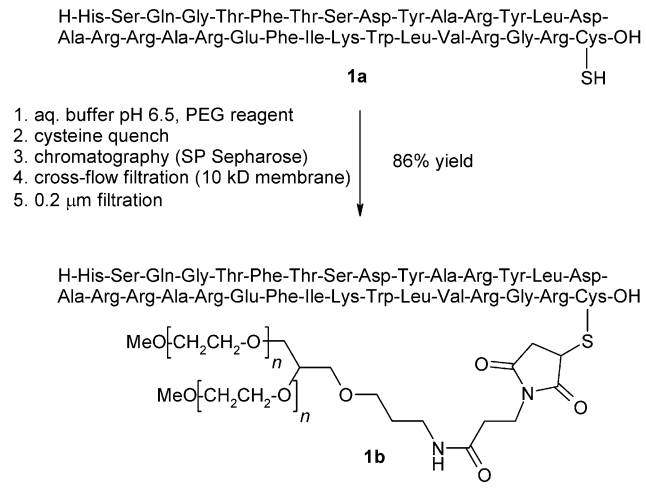
We conducted comprehensive analytical characterization of **1a**, including, HPLC, MS, N-terminal sequencing, amino acid analysis, SDS electrophoresis and capillary electrophoresis. Accordingly, the identity and amino acid sequence of **1a** was confirmed, the HPLC purity was determined to be 96% and no significant racemization had occurred during the synthesis. The main impurity arises from cysteine oxidation, which gives the cystine dimer upon extended storage periods.

In total, the fragment approach towards **1a** is a 68-step convergent synthesis with a remarkable overall yield of 9%, which translates into an average yield of 96% per step. In particular, the mixed-phase synthesis offers a significant reduction of raw materials and resource consumption. For instance, production of 1 g of target peptide **1a** required a total of 11 g of resin and 50 g of protected amino acids, whereas the initial SPPS protocol consumed 50 g of resin and 740 g of protected amino acids for the same result.

PEGylation

The current PEGylation process[18] is depicted in Scheme 4. Accordingly, peptide moiety **1a** was coupled with a branched 43kD PEG molecule by Michael addition of the cysteine sulfhydryl group to the PEG maleimide.

The PEGylation protocol operated in an aqueous buffer on a 4-L scale by using 4 g of the peptide and 40 g of the PEG reagent, which is used in slight excess. The reaction was complete after 30 min at 23 °C, and cysteine was added to quench the remaining PEG reagent. The crude reaction mixture was purified by ion-exchange chromatography by employing SP-sepharose FF as the stationary phase and an aqueous salt gradient to effect elution of the product. The pooled product fractions were concentrated by cross-flow filtration by employing a 10 kD Hydrosart membrane. Following sterile filtration (0.2 μm), the product was stored in flexible sterile bags as a buffered solution at –78 °C and defrosted prior to final formulation. Eventually, 2.3 g of PEGylated drug substance **1b** (Figure 4) was obtained per batch, which corresponds to a yield of 86%.[19]



Scheme 4. PEGylation process.

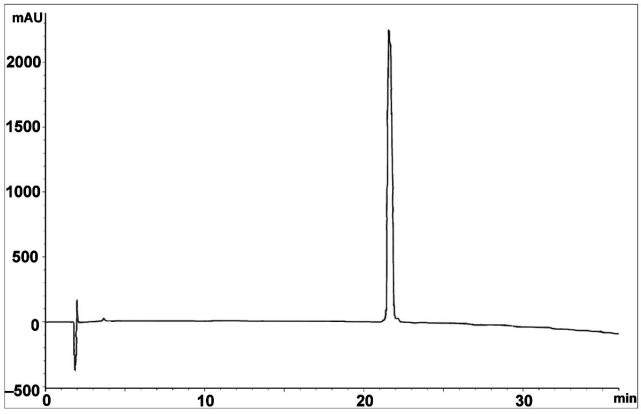


Figure 4. RP-18-HPLC chromatogram of PEGylated peptide **1b** (see Experimental Section for details).

Conclusions

In summary we identified a scalable synthetic route towards target peptide **1a**, which encompassed a mixed-phase fragment based synthesis. The salient features of the new process are: (1) It is a robust and convergent 68-step synthesis. (2) In comparison to the linear SPPS, the new protocol is a high-yielding process. (3) Workup and purification protocols are adapted to technically feasible processes. (4) The current route is the basis for the production of the peptide on technical scale.

Moreover, a PEGylation protocol was introduced, which delivered PEGylated peptide **1b** in good yields with continuous high purity.

Experimental Section

General: All SPPS reactions were performed by using custom-made devices with overhead stirring and porous filter plates at the bottom to allow efficient filtration.

Amino Acid Analysis: Amino acid analysis was performed by using an Eppendorf-Biotronik amino acid analyzer LC 3000 equipped with a UV detector and the data system Chromeleon version 6.8 from Dionex. A modified standard separation program with sodium acetate buffer was used. The amino acids were determined after post-column reaction with ninhydrin on an ion-exchange column filled with the ion-exchange resin BTC 2410 (4 μ material; 8 × 150 mm; Laborservice Onken). All chemicals used were of pro analysis or biochemical grade. Standard amino acid analysis (example): the peptide (100 μg) was hydrolyzed with HCl (6 M, 200 μL) in vacuo at 166 °C for 1 h. Subsequently, the HCl was evaporated, and the sample was diluted with sample buffer (pH 2.2, 400 μL). For amino acid analysis, 20 μL of the resulting solution were used. Homoarginine was employed as internal standard. Determination of cysteine: the peptide (100 μg) was dissolved in performic acid solution (formic acid/hydrogen peroxide, 9:1; 200 μL), which had been incubated at room temperature for 2 h prior to use. The protein was treated at 4 °C for 1 h. The performic acid solution was evaporated. The sample was then hydrolyzed with 6 M HCl and analyzed as described above. Homoarginine was used as internal standard. The released amino acids were identified and quantified with an external 4 nM amino acid standard.

N-Terminal Sequence Analysis: N-terminal sequence analyses were performed by using the gas–liquid–solid-phase protein sequencer ProciseTM from Applied Biosystems. The standard sequencer program fast normal was used. The detection of PTH-amino acids was performed on-line by using a RP-18-PTH-column (220 mm × 2 mm, 5 μ material) from Applied Biosytems. The PTH-amino acids were identified and quantified by a 40 pM standard of PTH-amino acids. The data were collected and integrated by using the sequencer data system 610A from Applied Biosystems. The peak heights of the PTH-amino acids from the first sequencer cycle were used for determination of PTH-His and PTH-Ser. N-terminal sequence analysis of peptide: the peptide (3 nmol) was applied onto a Biobrene® pretreated sequencer sheet. The peptide was sequenced over 32 cycles.

Molecular Weight Determination: Molecular weight determination was performed by using a LCT system from Waters Corporation equipped with the Masslynx processing software. Mathematical transformation of the multiply charged protonated molecular ions $[M + nH]^{n+}$ produced the molecular mass profile. The peptide Ala-Ser-Thr-Thr-Asn-(3,5-Di-iodo)Tyr-Thr was used for internal calibration. The instrument was run in the high-resolution mode. LC–MS was carried out by using a Finnigan LCQ Deca XP MAX in combination with a HP 1100 chromatography unit. High-resolution mass spectrometry was conducted with a LTQ-Orbitrap from Thermo Scientific.

Capillary Zone Electrophoresis: The peptide (~4 ng) dissolved in 0.1% TFA/30% ACN was hydrodynamically applied (1 s) onto the fused silica capillary. A 100-mM potassium phosphate buffer pH 3 was used as running buffer. The applied potential was 20 kV, and the column temperature was 30 °C. The electropherograms were measured at 210 nm. Capillary electrophoresis analyses were performed by using a system from Applied Biosystems model 270A-HT equipped with a data system from Perkin–Elmer and a variable UV wavelength detector. The fused-silica capillary from Applied Biosystems had an internal diameter of 50 μm, with a total length of 72 cm. All chemicals used were of pro analysis grade.

SDS-Electrophoresis: 5, 7.5, 10 and 20 μg of the peptide were dissolved in 20 μL sample buffer containing a reducing agent. The samples were incubated for 10 min at 70 °C and then applied onto a 12% Criterion XT gel and run for about 2 h. The gel was then

stained by using Coomassie Blue Brilliant R-250; 0.5–2 μg of the peptide were applied in case of silver staining. All chemicals used were of biochemical or pro analysis grade quality. The electrophoresis chamber, the power supply and the gels were from Bio-Rad. SDS-electrophoresis under nonreducing conditions was run as described above but without reducing agent.

NMR Spectroscopy: NMR spectra were recorded with a Bruker AC500 instrument. All spectra are calibrated against tetramethylsilane as an internal standard (δ = 0 ppm).

HPLC Chromatography

Method A: Column: Phenomenex Prodigy ODS3, column symmetry: 50 × 4.6 mm, 3 μm; T = 45 °C; eluent: A = 1 mL formic acid in 1 L of water, B = 1 mL formic acid in 1 L of acetonitrile, gradient: 0–10 min from 95 to 0% A, 10–20 min 100% B; flow rate: 1.2 mL min^{-1}; detection at 220 nm.

Method B: Column: Chiral AD-H, column symmetry: 250 × 4.6 mm, 5 μm; T = 45 °C; eluent: A = *n*-heptane + 0.2% trifluoroacetic acid, B = ethanol + 0.2% trifluoroacetic acid, 0–20 min isocratic at 90% A; flow rate: 1 mL min^{-1}; detection at 210 nm.

Method C: Column: ZORBAX 300SB-C3 (Agilent), column symmetry: 150 × 3.0 mm, 3.5 μm; T = 45 °C; eluent: A = water + 0.1% trifluoroacetic acid, B = tetrahydrofuran + 0.05% trifluoroacetic acid, gradient: 0–45 min from 90 to 0% A, 45–50 min 100% B; flow rate: 0.5 mL min^{-1}; detection at 220 nm.

Method D: Column: ZORBAX 300SB-C3 (Agilent), column symmetry: 150 × 3.0 mm, 3.5 μm; T = 45 °C; eluent: A = water + 0.1% trifluoroacetic acid, B = tetrahydrofuran + 0.05% trifluoroacetic acid, gradient: 0–15 min from 90% A to 100% B, 15–20 min 100% B; flow rate: 0.5 mL min^{-1}; detection at 220 nm.

Method E: Column: Phenomenex Prodigy ODS3, column symmetry: 150 × 3.0 mm, 3 μm; T = 60 °C; eluent: A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile, gradient: 0–10 min 23% B, 10–40 min from 23 to 38% B; 40–70 min from 38 to 100% B, 70–75 min 100% B; flow rate: 1.0 mL min^{-1}; detection at 220 nm.

Method F: Column: Zorbax-C18-column, column symmetry: 4.6 mm × 125 mm; 3.5 μm material; T = 40 °C; eluent: A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile, gradient: 0–10 min 0% B, 10–70 min from 0 to 60% B; 80 min 0% B; flow rate: 0.7 mL min^{-1}; detection at 192–300 nm.

Synthesis of Fragment H-[30–31]-O*t*Bu (2): To a solution of H-Cys(Trt)-OH (50.0 g, 138 mmol) in THF (500 mL) was added methylsulfonic acid (33.2 g, 345 mmol) under an inert gas atmosphere within 10 min. The temperature should not exceed 25 °C during addition. Subsequently, at 20 °C a solution of *tert*-butyl trichloroacetimidate (151 g, 690 mmol) in methyl *tert*-butyl ether (1.0 L) was added, and the mixture was stirred for 18 h at 20 °C. For workup, water (500 mL) was added, and the pH was adjusted to 9.0 by employing aqueous NaOH (1 M, 355 mL). The phases were separated, and the organic layer was washed with water (500 mL), dried with magnesium sulfate and concentrated in vacuo. The residue was suspended in toluene (500 mL), and the mixture was stirred at 20 °C for 30 min and then filtered. To the filtrate was added water (250 mL), and the pH was adjusted to 11 with aqueous NaOH (1 M). The layers were separated, and the toluene layer was washed with water (2 × 250 mL).[20] The resulting toluene solution of H-Cys(Trt)O*t*Bu (**7**) (86 area-%, method A) was diluted with DMF (125 mL). Subsequently, HOBt monohydrate (9.3 g, 69 mmol), *N*,*N*-diisopropylethylamine (53.5 g, 414 mmol), Fmoc-Arg(Pbf)-OH (53.8 g, 83 mmol) and TBTU (66.5 g, 207 mmol) were added at 20 °C, and the mixture was stirred for 30 min. HPLC analysis (method A) indicated 90% conversion. Therefore, additional Fmoc-Arg(Pbf)-OH (9.1 g, 14 mmol) was added, and the mixture was allowed to stir at 20 °C for an additional 30 min, after which the conversion rose to 97%. For workup, water (500 mL) was added, the layers were separated and the organic layer was washed with water (500 mL). Subsequently, the product solution was added dropwise to heptane (2.5 L), and the resulting suspension was stirred for 1 h at 20 °C prior to isolation. The mixture was filtered, washed with heptane (2 × 100 mL) and dried in vacuo to yield Fmoc-Arg(Pbf)-Cys(Trt)-O*t*Bu (**8**; 98 g) in a quality of 85 area-% (HPLC method A). For further purification, the crude product was redissolved in toluene (490 mL), and the resulting solution was added dropwise to diisopropyl ether (2.45 L). The resulting suspension was stirred at 20 °C for 1 h. The mixture was then filtered and washed with diisopropyl ether (2 × 100 mL). Finally, the product was dried in vacuo at 20 °C to yield **8** (84 g) with a purity of 92 area-% (method A). This corresponds to a yield of 58% for two steps commencing from H-Cys(Trt)-OH. The resulting Fmoc-Arg(Pbf)-Cys(Trt)-O*t*Bu (**8**; 84 g, 80 mmol) was dissolved in DMF/piperidine (3:1, 840 mL) at 0 °C. After 10 min the reaction mixture was slowly added to water (4.2 L) over a period of 3.75 h, and the mixture was stirred at 0 °C for an additional 30 min. The mixture was then filtered and washed with water (2 × 200 mL). Subsequently, it was redissolved in methyl *tert*-butyl ether, and the aqueous layer was separated. The organic layer was added to diisopropyl ether (2.1 L) over a period of 1.5 h. The mixture was stirred for 30 min at 20 °C, filtered and washed with diisopropyl ether (2 × 100 mL). The residue was dried in vacuo for 18 h to yield **2** (51 g, 77%). According to HPLC analysis (method C), the product was obtained in 93% purity containing 0.6% of the diastereomer H-Arg(Pbf)-D-Cys(Trt)-O*t*Bu. ^{1}H NMR (500 MHz, [D$_6$]-DMSO): δ = 1.30–1.65 (m, 4 H), 1.31 (s, 9 H), 1.40 (s, 6 H), 1.85 (br. m, 2 H), 1.98 (s, 3 H), 2.35–2.50 (m, 2 H), 2.42 (s, 3 H), 2.50 (s, 3 H), 2.95 (s, 2 H), 3.03 (m, 2 H), 3.11 (m, 1 H), 4.12 (m, 1 H), 6.40 (br. s, 1 H), 6.58 (br. s, 1 H), 7.22–7.35 (m, 15 H), 8.27 (br. s, 1 H) ppm. ^{13}C NMR (125 MHz, [D$_6$]DMSO): δ = 12.1, 17.2, 17.5, 18.9, 27.4 (3 C), 28.2 (2 C), 33.2, 42.4, 51.4, 53.9, 54.8, 66.0, 81.1, 86.2, 116.1, 124.2 (3 C), 126.7 (6 C), 128.0 (6 C), 128.9, 131.3, 137.1, 144.0 (3 C), 155.9, 157.3, 169.1, 174.9 ppm. HRMS (ESI+): calcd. for $C_{45}H_{57}O_6N_5S_2$ [M + H] 827.375; found 828.3814.

Synthesis of Fragment Fmoc-[20–29]-OH (3)

Resin Loading: At 20 °C, the chlorotrityl resin (100 g; loading: 1.56 mmol g^{-1}, source: Iris Biotech) was allowed to swell for 30 min in dichloromethane (800 mL). The mixture was filtered and fresh dichloromethane (800 mL), Fmoc-Gly-OH (14.8 g, 49.8 mmol) and diisopropylethylamine (DIPEA; 20.2 g 156.3 mmol) were added. The mixture was stirred for 2 h at 20 °C, filtered and then rinsed with dichloromethane (800 mL). Subsequently, the mixture was stirred with a solution of 5% *N*,*N*-diisopropyl ethylamine and 10% methanol (500 mL) in dichloromethane for 10 min. After filtration, this process was repeated allowing a reaction time of 20 min. Subsequently, the resin was washed with DMF (1 × 500 mL) and dichloromethane (3 × 500 mL) and dried in vacuo at 20 °C to yield the loaded resin (110 g). The loading was determined as follows:[21] a sample of the resin (ca. 10–20 mg) was incubated with a solution of 20% piperidine in DMF (50 mL) for at least 4 h. The absorbance of the supernatant was measured at 301 nm, and the loading was calculated according to the Lambert–Beer law with ε = 7800 L mol^{-1} cm^{-1}. In this example, the loading was 0.38 mmol g^{-1}.

Fmoc Deprotection: After resin loading and prior to the first deprotection, the resin was allowed to swell in DMF (500 mL) for 1 h.

In general, Fmoc deprotection was accomplished upon stirring of the resin in a 20% piperidine solution in DMF (1 L) for 10 min at 20 °C. The mixture was filtered, and the process was repeated with a fresh portion of the piperidine solution (1 L) for another 10 min. Finally, the mixture was filtered and rinsed with DMF (5 × 1 L). On a routine basis, the filtrates were tested on traces of piperidine by using the chloranil test. Accordingly, the filtrate (3 drops) was added to a solution of acetone (3 mL) containing three drops of a concentrated solution of chloranil (tetrachlorobenzochinone) in toluene. The blue colour indicated the presence of amine in the filtrate.

Coupling Reactions: To a suspension of the resin in DMF (1 L) was added the amino acid (83 mmol, 2 equiv.), HOBt (2.8 g, 21 mmol, 0.5 equiv.), *N*,*N*-diisopropylethylamine (10.8 g, 83 mmol, 2 equiv.) and TBTU (26.7 g, 83 mmol, 2 equiv.), and the mixture was stirred at 20 °C for 1 h. The reaction mixture was drained, and the resin was rinsed with DMF (3 × 1 L). The following amino acids were used for the synthesis of **3**: Fmoc-Arg(Pbf)-OH (2 × 54.0 g), Fmoc-Val-OH (28.2 g), Fmoc-Leu-OH (29.4 g), Fmoc-Trp(Boc)-OH (43.8 g), Fmoc-Lys(Boc)-OH (39.0 g), Fmoc-Ile-OH (29.4 g), Fmoc-Phe-OH (32.2 g), Fmoc-Glu(O*t*Bu)-OH (35.4 g). In situ analyses were conducted by using the Kaiser test: a small aliquot of the resin was thoroughly rinsed with ethanol. Subsequently, 2 drops of each of the following solution were added: 80% phenol in ethanol, 5% ninhydrin in ethanol and 2% of an aqueous 1 mM KCN solution in pyridine. The mixture was heated to 100 °C for 5 min, and the resin was washed with ethanol. A blue-coloured resin indicated the presence of amine, which corresponds to insufficient conversion. Alternatively, in selected examples the progress of the reactions was also monitored by HPLC. Thus, a sample of the resin was thoroughly rinsed and incubated three times, each with of a solution of 0.5% trifluoroacetic acid in dichloromethane for 10 min. The combined filtrates were diluted with acetonitrile and 3 μL were injected (HPLC method A). During the production of fragment Fmoc-[20–29]-OH (**3**), all coupling reactions were complete within 1 h, as indicated by the Kaiser test and HPLC analysis, respectively.

Cleavage from the Resin: The resin was rinsed with dichloromethane (1 L). Subsequently, it was stirred in a mixture of dichloromethane/trifluoroethanol (4:1, 1 L) for 1 h at 20 °C. This step was repeated allowing for an extended reaction time of 1.5 h. The resin was rinsed with dichloromethane (500 mL), and the filtrates were combined. The majority of the solvent was distilled off, leaving 285 g of a concentrated solution, which was slowly added to diisopropyl ether (2 L) at 20 °C. It was filtered off and dried in vacuo at 20 °C for 16 h to yield the desired fragment **3** (64 g) with a purity of 93 area-% (HPLC; method A). This corresponds to an overall yield of 67%. The molecular mass was determined by LC–MS (LC: method C, MS: ion trap ESI+): *m*/*z* = 2286 [M + H]$^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.21 (d, *J* = 7.0 Hz, 1 H), 7.94–8.09 (m, 5 H), 7.84–7.93 (m, 5 H), 7.63–7.77 (m, 4 H), 7.46–7.54 (m, 2 H), 7.37–7.44 (m, 2 H), 7.25–7.36 (m, 3 H), 7.07–7.25 (m, 6 H), 6.72–6.96 (m, 3 H), 6.69 (t, *J* = 4.9 Hz, 1 H), 6.45 (br. s., 3 H), 4.64–4.71 (m, 1 H), 4.54–4.62 (m, 1 H), 4.33–4.40 (m, 1 H), 4.10–4.32 (m, 8 H), 3.93–4.03 (m, 1 H), 3.64–3.77 (m, 2 H), 2.88–3.11 (m, 11 H), 2.80–2.87 (m, 2 H), 2.70–2.79 (m, 1 H), 2.50 (s, 3 H), 2.48 (s, 3 H), 2.43 (s, 3 H), 2.42 (s, 3 H), 2.10–2.18 (m, 2 H), 2.00 (s, 3 H), 1.99 (s, 3 H), 1.96–1.98 (m, 1 H), 1.75–1.85 (m, 1 H), 1.63–1.72 (m, 7 H), 1.61 (s, 9 H), 1.52–1.59 (m, 4 H), 1.42–1.50 (m, 6 H), 1.40 (s, 6 H), 1.38 (s, 6 H), 1.35 (s, 9 H), 1.33 (s, 9 H), 1.30 (br. s., 1 H), 1.12–1.26 (m, 3 H), 0.67–0.89 (m, 18 H) ppm. $[\alpha]_D^{20}$ = –9.1 (*c* = 0.41, DMSO).

Synthesis of Fragment Fmoc-[12–19]-OH (4)

Resin Loading: The loading of chlorotrityl resin (100 g) was accomplished as outlined for fragment **3** by using Fmoc-Ala-OH (24.9, 80.0 mmol). The loading was determined by UV (0.56 mmol g^{-1}).

SPPS: Fmoc deprotection, peptide coupling and cleavage from the resin were accomplished in analogy to the method described for **3**. The following amino acids were used: Fmoc-Arg(Pbf)-OH (3 × 81.7 g), Fmoc-Ala-OH (39.2 g), Fmoc-Asp(O*t*Bu)-OH (51.8 g), Fmoc-Leu-OH (44.5 g), Fmoc-Tyr(*t*Bu)-OH (57.8 g). In situ analyses (Kaiser test and HPLC) revealed that all reactions went to completion within the provided timeframe. Thus, no recoupling steps or acetyl capping was necessary. After cleavage from the resin and precipitation from diisopropyl ether, **4** (109 g, 51.6 mmol) was isolated with 93 area-% (method A) according to HPLC analysis. This corresponds to a yield of 82%. The molecular mass was determined by LC–MS (LC: method C, MS: ion trap ESI+): *m*/*z* = 1056 [(M+ 2 H)/2]$^+$. ^{1}H NMR (500 MHz, [D$_6$]-DMSO): δ = 8.22 (d, *J* = 7.6 Hz, 1 H), 8.01–8.05 (m, 2 H), 7.94–7.99 (m, 3 H), 7.91 (br. s., 2 H), 7.89 (s, 2 H), 7.77–7.81 (m, 1 H), 7.68–7.73 (m, 2 H), 7.45–7.49 (m, 1 H), 7.40 (s, 2 H), 7.31 (s, 2 H), 7.08 (s, 2 H), 7.01 (br. s., 1 H), 6.77 (d, *J* = 7.9 Hz, 2 H), 6.28–6.72 (m, 7 H), 4.48–4.63 (m, 2 H), 4.17–4.34 (m, 7 H), 4.05–4.15 (m, 1 H), 3.88–3.98 (m, 1 H), 2.91–3.08 (m, 13 H), 2.74–2.79 (m, 1 H), 2.64–2.72 (m, 1 H), 2.49–2.50 (m, 1 H), 2.48 (s, 9 H), 2.43 (s, 9 H), 2.00 (s, 9 H), 1.30–1.80 (m, 42 H), 1.25 (d, *J* = 7.0 Hz, 3 H), 1.17–1.18 (m, 3 H), 1.19 (s, 9 H), 0.83 (d, *J* = 6.4 Hz, 3 H), 0.79 (d, 3 H) ppm. $[\alpha]_D^{20}$ = –5.8 (*c* = 0.40, DMSO).

Synthesis of Fragment Boc-[1–11]-OH (5)

Resin Loading: The loading of chlorotrityl resin (200 g) was accomplished as outlined for fragment **3** by using Fmoc-Ala-OH (50 g, 160 mmol). The loading was determined via UV (0.51 mmol g^{-1}).

SPPS: Fmoc deprotection, peptide coupling and cleavage from the resin were accomplished in analogy to the method described for **3**. The following amino acids were used: Fmoc-Tyr(*t*Bu)-OH (119.3 g), Fmoc-Asp(O*t*Bu)-OH (99.4 g), Fmoc-Ser(*t*Bu)-OH (2 × 92.7 g), Fmoc-Thr(*t*Bu)-OH (2 × 96.2 g), Fmoc-Phe-OH (93.6 g), Fmoc-Gly-OH (71.9 g), Fmoc-Gln(Trt)-OH (147.6 g), Boc-His(Trt)-OH (120.4 g). In situ analyses (Kaiser test and HPLC) revealed that all reactions went to completion within the provided time frame. Thus, no recoupling steps or acetyl capping was necessary. After cleavage from the resin and precipitation from diisopropyl ether, Boc-[1–11]-OH (**5**; 135 g 63.3 mmol) was isolated with 93 area-% (method A) according to HPLC analysis. This corresponds to a yield of 52%. The molecular mass was determined by LC–MS (LC: method C, MS ion trap APES): *m*/*z* = 2132 [M + H]$^+$, 1068 [(M + 2 H)/2]$^+$, 1891 [(M – Trt) + H]$^+$. ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 8.48 (s, 1 H), 8.17 (d, *J* = 7.0 Hz, 2 H), 8.00 (d, *J* = 7.9 Hz, 1 H), 7.94 (d, *J* = 7.6 Hz, 1 H), 7.79–7.88 (m, 3 H), 7.75 (d, *J* = 7.0 Hz, 1 H), 7.65–7.72 (m, 2 H), 7.33–7.40 (m, 10 H), 7.19–7.27 (m, 11 H), 7.14–7.19 (m, 10 H), 7.12 (d, *J* = 8.5 Hz, 2 H), 7.03–7.08 (m, 6 H), 6.88 (d, *J* = 7.9 Hz, 1 H), 6.82 (d, *J* = 8.2 Hz, 2 H), 6.67 (s, 1 H), 4.71–4.81 (m, 1 H), 4.54–4.62 (m, 1 H), 4.46–4.53 (m, 1 H), 4.11–4.41 (m, 7 H), 3.90–3.95 (m, 1 H), 3.78–3.84 (m, 1 H), 3.75 (d, *J* = 4.6 Hz, 2 H), 3.49–3.56 (m, 2 H), 3.43 (ddd, *J* = 15.4, 9.5, 5.3 Hz, 2 H), 3.02–3.09 (m, 1 H), 2.99 (dd, *J* = 13.9, 4.1 Hz, 1 H), 2.81–2.92 (m, 2 H), 2.69–2.80 (m, 2 H), 2.59 (dd, *J* = 15.7, 5.3 Hz, 1 H), 2.40 (dd, *J* = 15.9, 7.9 Hz, 1 H), 2.32 (br. s., 2 H), 1.83–1.95 (m, 1 H), 1.65–1.78 (m, 1 H), 1.35 (s, 9 H), 1.30–1.33 (m, 9 H), 1.26–1.28 (m, 3 H), 1.25 (s, 9 H), 1.16 (s, 9 H), 1.10 (s, 9 H), 1.06 (s, 9 H), 1.03 (s, 9 H), 1.00 (d, *J* =

6.1 Hz, 3 H), 0.95 (d, J = 6.4 Hz, 3 H) ppm. $[\alpha]_D^{20}$ = +12.0 (c = 0.43, DMSO).

Assembly of the Fragments in Solution

Fmoc-[20–31]-O*t*Bu: From a solution of H-[30–31]-O*t*Bu (**2**; 29.9 g, 32.8 mmol, 95 area-%) in DMF (1 L) approx. 500 mL DMF was distilled off at 40–45 °C and 15 mbar. Fresh DMF (500 mL) was added, and the distillation protocol was repeated twice to ensure complete removal of trace amounts of piperidine. Subsequently, Fmoc-[20–29]-OH (**3**; 75.0 g 32.9 mmol, 93 area-%), HOBt monohydrate (2.22 g, 16.4 mmol), DIPEA (12.7 g, 98.4 mmol) and TBTU (21.1 g, 65.6 mmol) were added, and the mixture was stirred at 20 °C for 30 min. The reaction mixture was added to water (3.75 L), and the solution was stirred for 30 min at 20 °C. The mixture was filtered and rinsed with water (1 L) and finally dried at 25 °C in vacuo to give Fmoc-[20–31]-O*t*Bu (98.4 g, 97%). HPLC (method D): 92 area-%. The molecular mass was determined by LC–MS (LC: method C, MS: ion trap ESI+): m/z = 1549 [(M+ 2 H)/2]$^+$.

H-[20–31]-O*t*Bu (12): A solution of Fmoc-[20–31]-O*t*Bu (98.0 g, 31.65 mmol) in DMF/piperidine (3:1, 490 mL) was stirred at 20 °C for 1 h. To the reaction mixture was added acetonitrile (490 mL), and the resulting solution was poured within 10 min into diisopropyl ether (1.96 L). The solution was stirred at 20 °C for 1 h, filtered and washed with diisopropyl ether (3 × 250 mL). Finally, it was dried at 20 °C in vacuo to yield **12** (78.2 g, 86%). HPLC (method D): 83 area-%. LC–MS (LC: method C, MS: ion trap ESI+): m/z = 1437 [(M+ 2 H)/2]$^+$.

Fmoc-[12–31]-O*t*Bu: From a solution of H-[20–31]-O*t*Bu (**12**; 73.3 g, 25.5 mmol, 83 area-%) in DMF (1.1 L) approx. 550 mL DMF was distilled off at 40–45 °C and 15 mbar. Fresh DMF (550 mL) was added, and the distillation protocol was repeated twice to ensure complete removal of piperidine. Subsequently, Fmoc-[12–19]-OH (**4**; 56.5 g 26.8 mmol), HOBt monohydrate (1.72 g, 12.8 mmol), DIPEA (9.9 g, 76.5 mmol) and TBTU (16.4 g, 51 mmol) were added, and the mixture was stirred at 20 °C for 30 min. The reaction mixture was poured into water (1.83 L), and the solution was stirred for 30 min at 20 °C, filtered, washed with water (2 × 250 mL) and finally dried at 30 °C in vacuo to give Fmoc-[12–31]-O*t*Bu (126.0 g, 99%). HPLC (method D): 79 area-%. LC–MS (LC: method C, MS: ion trap ESI+): m/z = 1656 [(M+ 3 H)/3]$^+$.

H-[12–31]-O*t*Bu (13): To a suspension of Fmoc-[12–31]-O*t*Bu (125.7 g, 25.3 mmol, 79 area-%) in dioxane/piperidine (3:1, 629 mL) was added acetonitrile (629 mL) to give a clear solution after 5 min stirring at 20 °C. The solution was stirred at 20 °C for an additional 40 min. Then, the reaction mixture was poured into diisopropyl ether (4.71 L) within 5 min, and it was stirred at 20 °C for 30 min and at 0 °C for an additional 30 min. The residue was isolated by filtration by using a vacuum not below 100 mbar to avoid blockage of the frit. The product was rinsed with diisopropyl ether (3 × 330 mL). Finally, the product was dried at 30 °C in vacuo to obtain **13** (120.3 g, quant. yield). HPLC (method D): 77 area-%. The molecular mass was determined by MS (ion trap ESI+): m/z = 2372 [(M+ 2 H)/2]$^+$.

Boc-[1–31]-O*t*Bu: From a solution of H-[12–31]-O*t*Bu (**13**; 112.1 g, 23.6 mmol, 77 area-%) in DMF (1.68 L) approx. 560 mL DMF was distilled off at 40–45 °C and 15 mbar. Fresh DMF (560 mL) was added, and the distillation protocol was repeated twice. Subsequently, Boc-[1–11]-OH (**5**; 55.4 g, 26.0 mmol, 93 area-%), HOBt monohydrate (1.60 g, 11.8 mmol), DIPEA (9.15 g, 70.8 mmol) and TBTU (15.2 g, 47.2 mmol) was added, and the solution was stirred at 20 °C for 40 min. The reaction mixture was poured into water (3.73 L) and stirred for 30 min at 20 °C. The mixture was filtered, washed with water (3 × 200 mL) and finally dried at 30 °C in vacuo to give Boc-[1–31]-O*t*Bu (160.5 g, 99%). HPLC (method D): 79 area-%. MS (ion trap ESI+): m/z = 1595 [(M+ 3 H)/3]$^+$, 1715 [(M+ 4 H)/4]$^+$, 1372 [(M+ 5 H)/5]$^+$.

H-[1–31]-OH (1a): To a solution of Boc-[1–31]-O*t*Bu (159 g, 23.2 mmol) in of a mixture of dichloromethane/trifluoroethanol (4:1, 636 mL) was added dithiothreitol (239 g, 1549 mmol), triisopropylsilane (239 mL, 1166 mmol) and trifluoroacetic acid (1.35 L), and the mixture was stirred at 20 °C for 4 h. To the reaction mixture was added dichloromethane (636 mL), and the resulting solution was poured into cold diisopropyl ether (5.4 L) within 2 min. The solution was stirred at 5 °C for 1 h, filtered and rinsed with diisopropyl ether (3 × 500 mL). Finally, it was dried for 3 d at 20 °C in vacuo to yield crude peptide **1a** (126 g) with an HPLC purity of 55 area-% (method D). The purification protocol was demonstrated with an aliquot of crude **1a** (11.9 g). The crude material was dissolved in a mixture of acetonitrile/water + 0.1%TFA (30:70, 1125 mL). The resulting solution was purified by employing a Prodigy 10 µ ODSIII 100A (Phenomenex), particle size 10 µm, column dimension: 250 × 50 mm. A total of 45 injections each of 25-mL (264 mg of crude peptide) were used. The eluent consisted of A: water + 0.1% TFA and B: acetonitrile and a flow of 140 mL min^{-1} was applied. The following gradient was used: 0 min injection, 0.5–23.0 min A from 75 to 20%, 23.0 to 29.0 min A at 75%. The fractions were analyzed by using HPLC method D. Fractions with >95 area-% were pooled, acetonitrile was removed in vacuo and the residue was freeze dried to yield 2.05 g of the pure peptide. This corresponds to a yield of 25% for two steps commencing from **13**.

Amino Acid Analysis of 1a: The composition of **1a** matches the composition of the theoretical numbers very well by taking into account the limitations of the method (cf. Table 2).

Table 2. Amino acid analysis of **1a**.

Amino acid	Theory	Found for **1a**	D-Amino acid (%)[a]
Cys	1	0.99	1.60
Asp	2	2.00	0.20
Thr[b]	2	1.55	<0.10
allo-Thr	–	–	<0.10
Ser[b]	2	1.21	0.87
Glu	2	1.89	0.13
Gly	2	1.95	–
Ala	3	2.92	1.51
Val	1	0.96	<0.10
Ile	1	0.89	<0.10
allo-Ile	–	–	<0.10
Leu	2	1.74	<0.10
Tyr	2	1.89	0.69
Phe	2	2.01	<0.10
His	1	1.27	1.13
Lys	1	1.18	0.13
Arg	6	5.75	<0.10
Trp	1	1.00	0.10
Total	31	29.20	
Loss by hydrolysis (%)		5.84	
Amino acid content		80.73	

[a] Level of racemization determined at C.A.T. Analysentechnik Tübingen, Germany. [b] Low values found for Thr and Ser are explained by decomposition during hydrolysis; Cysteic acid was determined from performic acid oxidation.

Protein Sequence Analysis of 1a: Automated Edman degradation was performed. Total sequence analysis was carried out over 32 steps to assess identity with the theoretical sequence, to detect microheterogeneities, clipping and blockage of the peptide. One main sequence (AA 1–30) was detected which confirmed the primary structure of **1a** on the amino acid level. The PTH-Cys in position 31 gave no UV signal and could therefore not be detected. No microheterogeneity was detected.

Molecular Weight Determination of 1a: The molecular weight of **1a** was determined by ESI-MS measurement in the high-resolution mode with internal calibration with 3757.1 Da. This is in good agreement with the calculated monoisotopic mass of 3757.9 Da.

Reverse-Phase Chromatography of 1a (Method F): Compound **1a** eluted as a main peak at 46 min with a purity of 94 area-%. The cystine dimer was present at a level of ca. 1%.

Capillary Zone Electrophoresis: Compound **1a** eluted as one main peak with a purity of 93%.

SDS-Gel Electrophoresis: Compound **1a** ran under reducing conditions as a single band with an apparent molecular weight of about 4 kD. Only minor amounts of a dimeric structure were present, whereas other oligomeric forms were not found.

Synthesis of PEGylated Peptide 1b

PEGylation Buffer: A solution of sodium dihydrogen phosphate (7.9 g) in water (5.0 L) for injection was adjusted to pH 6.5 by using 1 M sodium hydroxide (11 mL).

Chromatography Buffer A: A solution of sodium acetate trihydrate (150 g) in water (55 L) for injection was adjusted to pH 4.5 by using 2 M acetic acid (750 mL).

Chromatography Buffer B: A solution of tris(hydroxymethyl)aminomethane (133 g) and sodium chloride (321 g) in water (55 L) for injection was adjusted to pH 9.5 by using 1.2 M hydrochloric acid (25 mL).

Washout Buffer: A solution of sodium acetate trihydrate (5.4 g) in water (2 L) for injection was adjusted to pH 5.0 by using 2 M acetic acid (9 mL). For all buffers endotoxins were determined prior to use.

PEGylation: All operations were carried out in a sterile environment. A 4-L glass reaction vessel was flooded with a 0.5 M aqueous solution of sodium hydroxide. It was allowed to stand overnight, and the vessel was drained and washed with water for injection until the pH was neutral. To a clear solution of PEGylation reagent (40.0 g; GL2–400 MA Sunbright from NOF, Japan) in PEGylation buffer (2 L) was added a solution of **1a** (4.0 g, 69% peptide content according to UV-absorbance) in PEGylation buffer (2 L) at 23 °C. The pH of the resulting solution was adjusted to 6.5 by using 1 M sodium hydroxide (4.5 mL). The mixture was stirred (250 rpm) for 30 min at 23 °C before cysteine (0.96 g) dissolved in PEGylation buffer (40 mL) was added. The reaction mixture was filtered through a Sartopore 2 filter (300 5441307H5–00-B), and the resulting solution (4.04 L) was submitted to subsequent chromatography in batches (2 × 2.02 L). The column was packed with 2.9–3.0 L of SP Sepharose Fast Flow, which was equilibrated by using ethanol (20% in water for injection). Prior to use, the column was flooded with 0.5 M sodium hydroxide and then washed with chromatography buffer A. The column was charged with the first portion of the reaction mixture, and it was washed with one column volume of chromatography buffer A and then a gradient towards 100% chromatography buffer B was initiated. The desired product eluted at a buffer A/buffer B ratio of approx. 60:40. The pH of the product fraction (8.0 L) was adjusted to 5.0 by using 1.2 M hydrochloric acid (11 mL). The solution was filtered through a Sartopore 2 filter (300 5441307H5–00-B) and stored at 2–8 °C for 2 d. The second batch of the reaction mixture was purified accordingly. Both product fractions were combined to give a total of 16.5 L of the purified product. The cross-flow filtration unit was equilibrated with 0.5 M sodium hydroxide and subsequently flushed with water for injection until the pH was neutral. The pooled product fractions from chromatography were concentrated to a volume of 1 L by using a Slice Hydrosart 10 kD membrane. Eventually, the cross-flow filtration unit was washed with the washout buffer (1 L) to yield a combined product phase of 2 L. The level of endotoxins was determined (<0.12 EE mL^{-1}), and it was filtered through a Sartopore 2 filter (300 5441307H5–00-B) into sterile bottles and finally stored at –20 °C.

The product concentration was determined by UV absorbance (c = 1.18 g L^{-1}). This translates into a yield of 2.36 g (86%) based on the peptide. HPLC (method E): 100 area-%.

N-Terminal Sequence: Compound **1b** (3 nmol) was applied onto a Biobrene® pretreated sequencer sheet. The conjugate was sequenced over 32 cycles. One main sequence (AA 1–30) was detected, which confirmed the primary structure of **1a** on the amino acid level.

SDS-Electrophoresis: The Coomassie blue gel was stained again by using the silver stain method to identify the free peptide. Only trace amounts of free peptide **1a** were found after silver staining, which indicates that the peptide was completely bound to the PEG moiety. PEG conjugate **1b** ran as a broad band at about 70 kD.

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