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Total chemical synthesis of a biologically active and homogeneous analog of Human Growth Hormone [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107}, Asp^{109}]hGH-NH$_2$ by sequential native chemical ligation

Javier Sueiras-Diaz *, Ying Zhang, Anastasia Velentza, Buyung Santoso, Suijin Yang

Ferring Research Institute, 4245 Sorrento Valley Blvd, San Diego, CA 92121, United States

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ABSTRACT

We report herein, for the first time, a sequential total chemical synthesis of the Human Growth Hormone analog [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH$_2$, composed of a 191 amino acid residue polypeptide chain containing two disulfide bonds and nine modifications in the natural sequence. Sequential native chemical ligation of three discrete segments of 52, 52 and 87 amino acid residues gave the target full-length polypeptide chain. Subsequent folding with concomitant formation of the native disulfide bonds afforded a correctly folded homogeneous analog which is biologically active.

Human Growth Hormone (hGH), also known as somatotropin,[1,2] is a 191-amino acid single chain polypeptide containing two disulfide linkages with a molecular weight of 22124.76. Crystallographic data indicate that the tertiary structure consists of four antiparallel alpha-helices[3] which are necessary for functional interaction with the GH receptor. hGH is synthesized, stored and secreted by somatotropic cells within the lateral wings of the anterior pituitary gland. Its main function is to spur growth in children and adolescents and also helps to regulate body composition, body fluids, muscle and bone growth, sugar and fat metabolism, and possibly heart function.[4]

A recombinant form of hGH called somatropin (generic name) or Genotropin (brand name), introduced in 1992, is used as a prescription drug to treat children's growth disorders and adult growth deficiency.[5,6] hGH therapy has been administered successfully for decades. However, one of the major drawbacks is its short plasma half-life, which is about 3.4 h if administered subcutaneously (SC) and 0.36 h if administered intravenously (IV).[7] This requires daily or thrice-weekly administration, which is inconvenient and potentially painful for the patients.[8] To overcome this, great efforts have been made to develop long-acting hGH preparations such as zinc complexes, microspheres formulations[9] and PEGylated analogues.[10]

In attempt to augment progression in the field we reasoned that a successful total chemical synthesis of hGH or its analogues would be highly desirable for establishing a base to develop a structure activity program, which is more diverse, requires shorter time than recombinant technologies and at the same time reaches our goals of making more stable and long acting molecules. It would be a relevant step forward, as it allows us to easily incorporate unnatural amino acids, post-translational modifications or labeling agents that are not present in the natural form of the hormone. To the best of our knowledge, a total chemical synthesis of hGH or any of its analogues has not been reported.

We chose as a prototype hGH molecule for the synthesis the analog [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH$_2$ **8**, where we replaced the three oxidation-prone methionines in the chain with the more robust amino acid norleucine and the C-terminal acid with the more enzymatically stable primary amide, with the goal of designing a more stable molecule. We also incorporated additional changes of interest to our exploratory investigations, Glu^{91} and also Glu^{29}, Gln^{74}, Asn^{107} and Asn^{109}. The last four substitutions are those reported by Li in 1972,[11] which differ from those in the definitive sequence of hGH reported by DeNoto and col. in 1981[12] after sequencing of the hGH gene had been accomplished. In total our prototype hGH analogue **8** features nine modifications with respect to the somatropin molecule as shown in Fig. 1.

Although synthetic peptides are routinely made using solid-phase peptide synthesis (SPPS),[13] a sequence of this magnitude

* Corresponding author.
E-mail address: javier.sueiras-diaz@ferring.com (J. Sueiras-Diaz).

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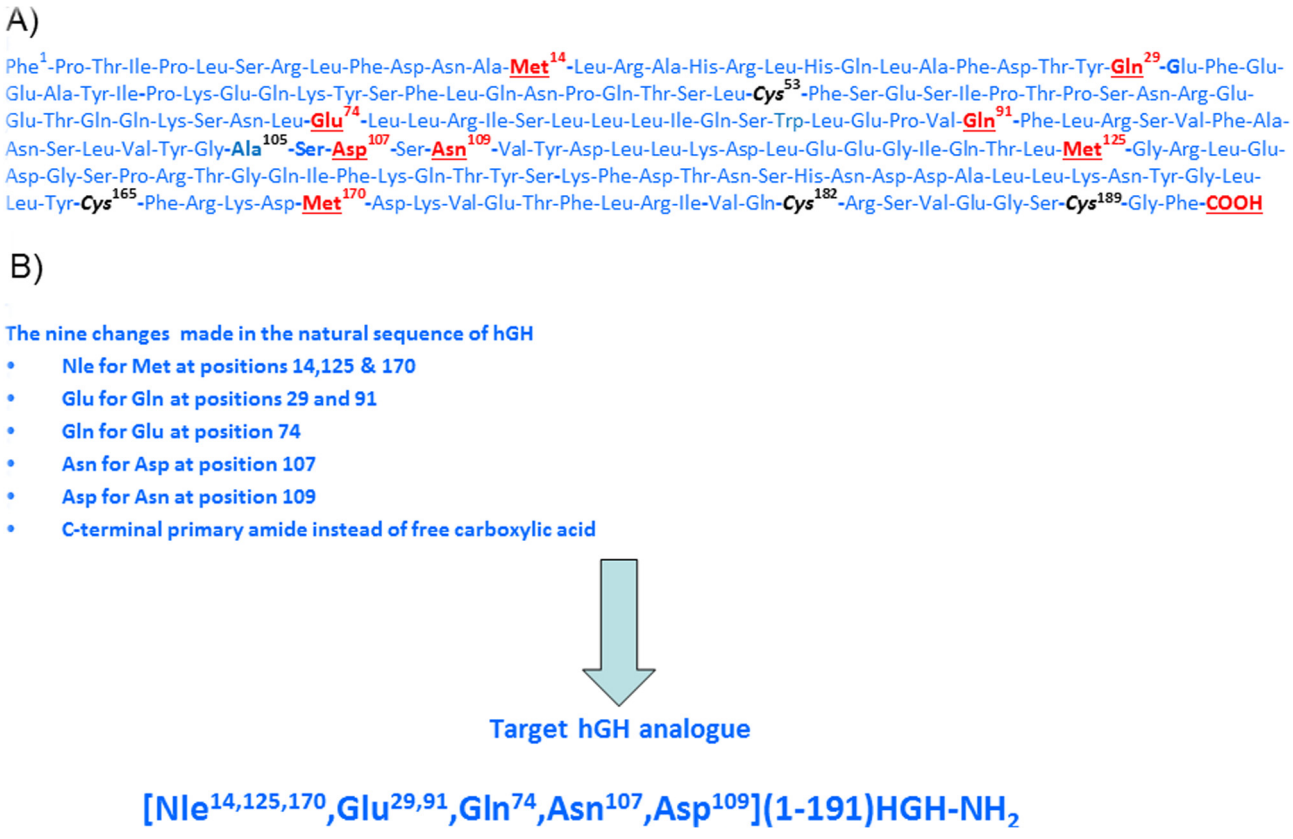


Fig. 1. A) Amino acid sequence of wild-type Human Growth Hormone, disulfide bonds are between (Cys^{53},Cys^{165}) and (Cys^{182},Cys^{189}). Highlighted in red the positions to be modified. B) The proposed target analogue to be synthesized showing the nine mutations of the natural sequence.

(191 residues) is beyond the capability of current SPPS. To access larger synthetic assemblies, chemoselective ligation techniques,[14–16] especially native chemical ligation (NCL),[17,18] are used to join peptide segments into larger constructs. This approach entails the assembly of individual peptidyl substrates and these substrates are then merged, in an iterative fashion, to ultimately afford the homogeneous protein target. The cysteine-based NCL protocol developed by Kent and coworkers,[19] which involves the merger of a C-terminal thioester and an N-terminal cysteine residue, remains the method of choice when attempting the ligation of unprotected peptides in aqueous media and provides a major advance in protein synthesis. In 1994, Interleukin 8 with a fully native backbone was convergently synthesized by NCL.[20] Since then various challenging bioactive proteins have been prepared using this technique, such as tetraubiquitin,[21] covalent HIV protease dimer,[22] glycosylated EPO,[23] the gamma-subunit of F-ATPase[24] and the 312-residue GroEL/ES-dependent protein,[25] that are inaccessible by conventional peptide synthesis.

Taking into account the inherent complexity of our target hGH analogue **8** and the positions of the cysteine sites in the sequence, we designed a linear sequential synthetic strategy based on the native chemical ligation method.

As NCL involves the chemoselective condensation of unprotected peptide segments at Xaa-Cys sites, our retrosynthetic analysis began by locating all Cys residues (potential ligation junctions) in **8**. One challenge of this chemical synthesis is the limited number and uneven distribution of the natural cysteine residues in the target 191 amino acid polypeptide chain. There are four Cys residues in the sequence as shown in Fig. 2. However, three of these, Cys^{165}, Cys^{182} and Cys^{189}, are too close to the C-terminus and could contribute only short fragments to the synthetic pathway. Only Cys^{53} could conveniently be used to ligate an N-terminal 52-amino acid fragment to the rest of the peptide chain. To expand the range of potential ligation junctions, we turned to the free radical-based desulfurization reaction[26,27] which can selectively convert unprotected Cys to Ala. In essence, it utilizes cysteine residues as surrogates for the more abundant alanine residues and allows one to substitute a Cys for a native Ala residue during peptide synthesis and ligation, and then converts the Cys back to the native Ala following assembly. Taking advantage of this method, we decided to introduce one additional junction site by replacing Ala^{105} by Cys^{105}, resulting in three segments overall, ranging in size from 50 to 90 residues. The three peptide fragments and key junctions are depicted in Fig. 2.

This approach enabled us to design a sequential coupling strategy using three segment unprotected peptides, (Phe^{1}-Leu ^{52})-MeNbz-Gly-NH_2 **1**, 52aa, [(Cys^{53}(Acm)-Gly^{104}]-MeNbz-Gly-NH_2 **2**, 52aa, and (Cys^{105}-Phe^{191})-NH_2 **3**, 87aa, by means of a conversion of cysteine^{105} into alanine after NCL, which gave the full-length 191-residue protein chain that folded efficiently to form an active analogue of hGH.

To perform the sequential ligation from the C-terminus to the N-terminus the four $Cys^{53,165,182,189}$ residues in the original peptide sequence were protected with the acetamidomethyl (Acm) group, which prevents cyclization/polymerization of peptides containing both an activated C-terminal peptide thioester precursor and a N-terminal Cys and also prevents Cys desulfurization. Selective desulfurization in the presence of protected Cys(Acm) residues enabled the preservation of the four native Cys residues.[28] The cysteine^{105} was introduced as the trityl derivative. Our strategy for the assembly of these three segments required five steps: two ligations, one desulfurization, one Acm removal, followed by cyclization and folding and their associated purifications as shown in the scheme of the synthesis in Fig. 3.

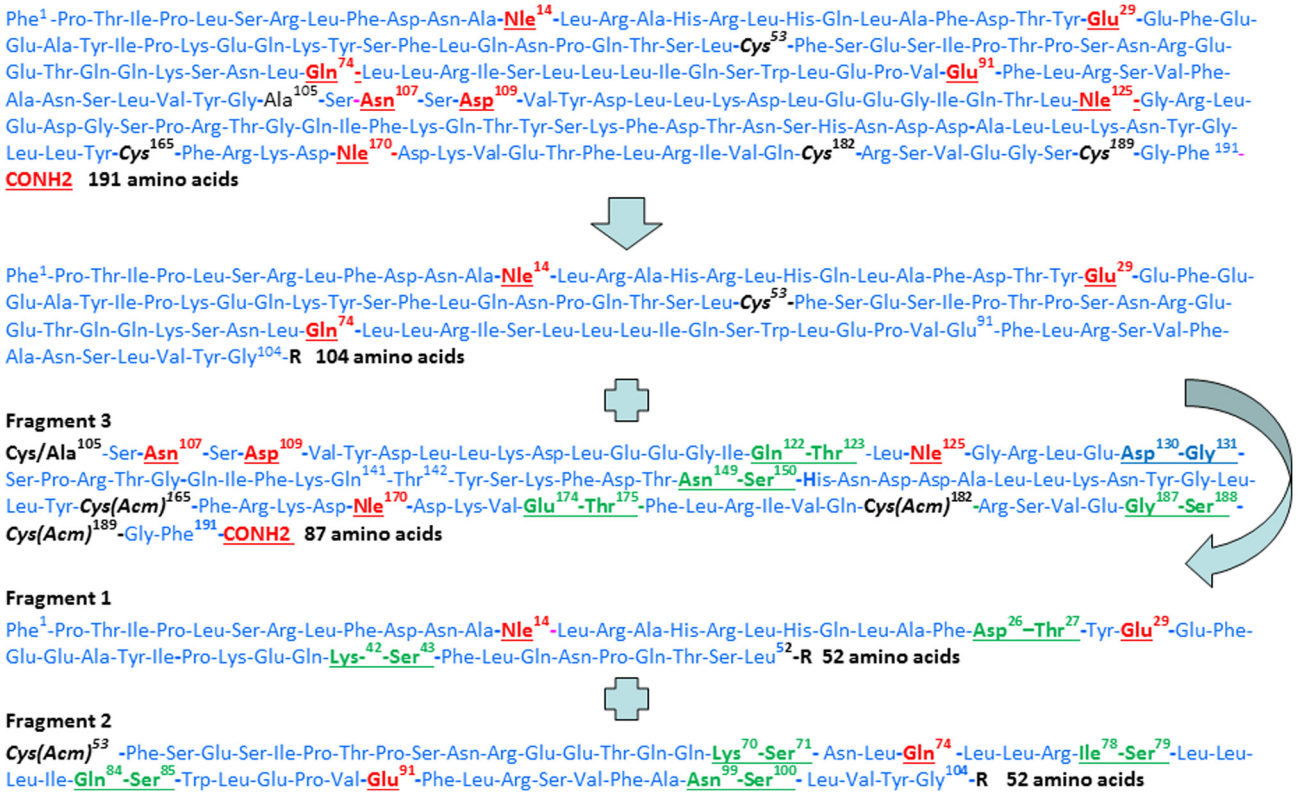


Fig. 2. Synthetic scheme: Deconvolution steps and the resultant fragments utilized to proceed with the synthesis of the target hGH analog [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH_2 **8**. Mutations and pseudoprolines positions are highlighted in red and green respectively. Cysteine positions are in bold-italics characters. R = MeNbz-Gly-NH_2.

To perform the NCL condensation of the segments, we used a recently developed method[29] that joins the peptide segments via native peptide bonds formed between a thioester peptide fragment generated *in situ* from a C-terminal *N*-acyl-*N*′-methylacylurea (MeNbz) derivative and a peptide with an N-terminal Cys. We selected this chemistry because of the convenient route to form peptide *N*-acylureas via Fmoc SPPS, the robustness of the NCL reaction and the ease of carrying out the protein assembly on a linear C- to N-fashion. Execution of this strategy began with the microwave-assisted solid phase synthesis and reversed-phase (RP)-HPLC purification of each segment. Fragments **1** and **2** were synthesized by applying the aforementioned protocol to a ChemMatrix Rink resin and an *ortho*-amino(methyl)aniline 3-[(Fmoc)amino]-4(aminomethyl)benzoic acid (Fmoc-MeDbz) linker. ChemMatrix resin was functionalized with a Rink linker, and in order to facilitate the introduction of the Fmoc-MeDbz-OH linker, a glycine spacer was first coupled to the resin. The 87-amino acid peptide amide, fragment **3**, was assembled in the same way, from a ChemMatrix resin with a Rink linker, using our microwave heating protocol.

The three segments contain numerous well-distributed Ser or Thr residues which offer plenty of oportunities to introduce oxazolidine dipeptides to improve the yield of the fragments and to avoid aggregation. Pseudoproline dipeptides[30] at positions 26–27, 42–43, 70–71, 78–79, 84–85, 99–100, 122–123, 141–142, 149–150, 174–175 and 187–188 as shown in Fig. 2, were interspaced at regular intervals where the sequence allowed them, at least six amino acids apart, and taking into consideration that the presence of native prolines or the Asp(OtBu)-(Dmb)Gly dipeptide could also contribute to prevent aggregation during the chain assembly. We kept in mind that should aggregation occur we had the option of incorporating additional pseudoprolines or change the location of some of them.

Fmoc deprotection was performed in three stages using 35% piperidine/*N*-methyl-2-pyrrolidone (NMP) containing 0.1 M 1-hydroxybenzotriazole (HOBt) at room temperature for 2 and 6 min, followed by another deprotection step for 4 min at 48 °C. Amino acid couplings were performed using 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and *N*,*N*-diisopropylethylamine (DIEA) or with *N*,*N*′-diisopropylcarbodiimide (DIC) and ethyl cyano(hydroxyimino)acetate (Oxyma) at several conditions as indicated in Table 1.

The target peptide **8** contains 11 aspartic acid residues which could be expected to form aspartimides during the Fmoc removal steps. The degree of aspartimide formation depends on the nature of the amino acid located at the C-terminus of the aspartic acid, with Gly, Ser, Thr, Asn and Asp known to be the most prone to this side reaction.[31,32] In order to minimize this side reaction at these specific sequences, present in fragments **1** and **3**, aspartates at positions 11, 147, 153 and 154 were side chain protected with the very effective 3-ethyl-3-pentyl (Epe) ester[33] and Fmoc deprotections were performed using HOBt as an additive to the piperidine solution. To avoid aspartimide formation at the very prone sequence Asp^{130}-Gly^{131}, these amino acids were introduced as the dipeptide Fmoc-Asp(OtBu)-(Dmb)Gly-OH carrying temporal protection of the amide bond with the trifluoroacetic acid (TFA)-labile 2,4 dimetoxybenzyl (Dmb) group.

Following chain assembly of fragments **1** and **2**, acylation with p-nitrophenyl chloroformate and cyclization under basic conditions afforded the corresponding *N*-acylurea protected peptide resins. Acidolytic cleavage of the resin and concomitant amino acid side chain deprotection afforded the unprotected mildly activated *N*-acylurea peptides **1** and **2** with a yield of 18% and 12%, respectively, after RP-HPLC purification. These peptide fragments were

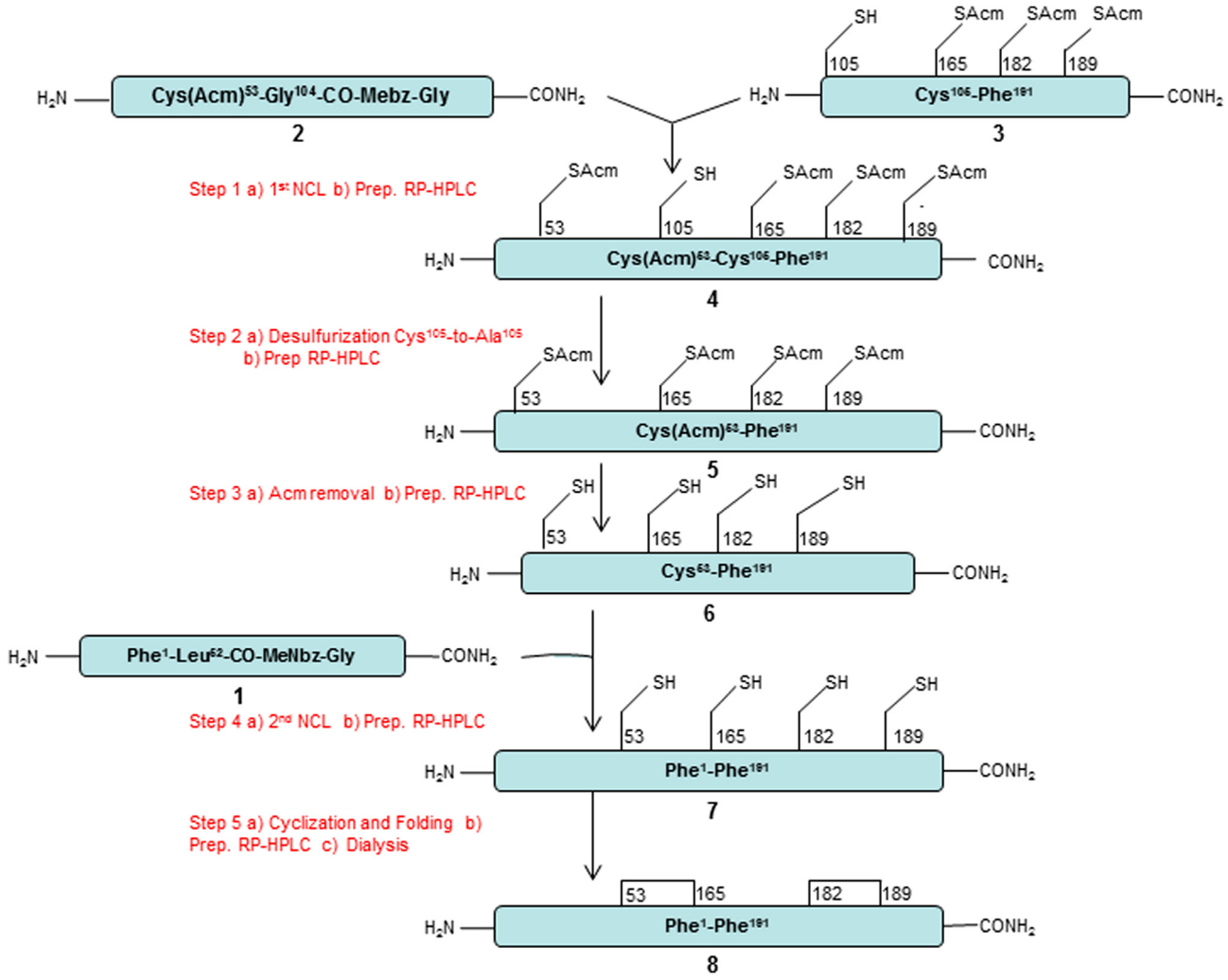


Fig. 3. The C-to-N native chemical ligation segment condensation strategy for the total chemical synthesis of [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH_2 **8**. Step 1): 0.2 M sodium phosphate buffer solution pH 6.8–7.0 containing 6 M guanidine-HCl, 35 mM tris(carboxyethyl)phosphine (TCEP) and 0.2 M mercaptophenyl acetic acid (MPAA); Step 2): 0.2 M sodium phosphate buffer solution at pH 7.0 containing 6 M guanidine-HCl, TCEP, sodium 2-mercaptoethanesulfonate(MESNA) and 2-2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044); Step 3): AgOAc in AcOH-water (1:1); Step 4): The conditions are the same as the 1st NCL; Step 5): a) **7** was dissolved in degassed solubilization buffer containing 6 M guanidine-HCl and 0.1 M Tris-HCl at pH 5.45 and refolded by diluting 25 fold in the refolding buffer containing 0.1 M Tris-HCl, 2 M guanidine-HCl, 0.4 M Arginine, 10 mM reduced glutathione and 2 M oxidized glutathione, pH 9.2–9.4, at room temperature overnight; c) Dialysis against four changes of water at pH 8.5–9.2 with ammonia for 36 h. and then concentrated to a small volume.

Table 1
Coupling conditions used in the microwave-assisted SPPS.

Amino acids	Coupling	Coupling reagent	Equiv.	Temp. (°C)	Time (min)
All except: His, Arg, Cys	Double	HATU 0.5 M/DIEA 2 M/NMP	8	60	8 each
His and Arg	Double	HATU 0.5 M/DIEA 2 M/NMP	8	(Room Temp. + 50)	(25 + 5) each
Cys	Double	DIC 1.0 M/NMP + Oxyma 0.5 M/NMP	8	Room Temp.	40 each
Pseudoprolines	Single	HATU 0.5 M/DIEA 2 M/NMP	8	60	10

utilized directly in the native chemical ligation reactions in the presence of a thiol catalyst.

Segment **3** was assembled by consecutive coupling of its 87 amino acids using microwave heating. Acidolytic cleavage of the resin and concomitant amino acid side chain deprotection provided the unprotected peptide amide **3** with a yield of 8% after RP-HPLC purification. Using these optimized synthesis conditions, the three segments were obtained in excellent purity (>95%) following purification. Full details of the preparation of these synthetic peptide segments are given in the Supplementary information.

With all the necessary building blocks at hand, the final assembly of the three individual peptide fragments was conducted in the C to N direction by first ligating the C-terminal segment **3** to the middle segment **2** as it is shown in Fig. 3. Fragment **3** (Cys^{105}-Phe^{191})-NH_2 was treated with fragment **2** (Cys(Acm)^{53}-Gly^{104})-MeNbz-Gly-NH_2 (1.2 equiv) under NCL-like ligation conditions, in the presence of thoroughly degassed 6 M guanidine-hydrochloride (GnHCl), 0.2 M sodium phosphate buffer at pH 6.8–7.0, 0.035 M Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 0.2 M mercaptophenyl acetic acid (MPAA). The progress of the reaction was monitored by analytical RP-LC coupled to

electrospray ionization mass spectrometry (ESI-MS) and a complete conversion was observed after overnight reaction to give the desired ligation fragment **4** (Cys(Acm)^{53}-Phe^{191})-NH_2 in 50% yield after RP-HPLC purification.

Following the ligation step, the non-native Cys^{105} in fragment **4** was converted to Ala^{105} using the mild metal-free desulfurization conditions of Danishefsky and Wan.[27] In this protocol, the water-soluble radical initiator 2-2′-azobis[2-92-imidazolin-2-yl)propane]dihydrochloride (VA-044) and TCEP were used to reduce the cysteine residue. The desulfurization was performed at room temperature and monitored by RP-HPLC–ESI-MS. The reaction was completed within 5–6 h, showing a mass decrease of 32 Da compared with the starting material. After HPLC purification, the desulfurized product Fragment **5** was isolated in 45% yield. Subsequently, the four Acm groups were cleaved with a silver acetate treatment in 1:1 acetic acid/H_2O[34] to reveal the native Cys^{53,165,183,189} residues confirmed by RP-HPLC–ESI-MS, followed by RP-HPLC purification to afford the peptide chain fragment **6** in 52% yield. LC–MS characterization of fragment **5** and **6** are shown in Supplementary Figs. S-5 and S-6 respectively.

The final NCL coupling took place between fragment **6** (Cys^{53}-Phe^{191})-NH_2 and fragment **1** (Phe^{1}-Leu^{52})-MeNbz-Gly-NH_2 under the same NCL-like ligation conditions as mentioned above. The progress of the reaction was followed by analytical RP-HPLC–ESI-MS and complete conversion was observed after overnight reaction to give, after RP-HPLC purification, the desired full length reduced hGH analogue polypeptide target sequence **7** (Phe^{1}-Phe ^{191})-NH_2 in moderate 32% yield (based on **6**). Electrospray ionization analysis of **7** is shown in Fig. 4.

After assembling the linear sequence, the final challenge in the synthesis was developing the disulfide oxidation and folding conditions to produce a bioactive protein. For this purpose we applied a chemical refolding dilution procedure[35] where a 0.085 μM protein solution of **7** in 6 M Gn-HCl dissolving buffer of high denaturant concentration and 0.1 M Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) pH 5.45, was diluted 25 fold to a 3.4 nM concentration by delivering it, drop by drop, into a large volume of reduced-oxidized glutathione (GSH-GSSG) based redox refolding buffer containing 0.1 M Tris-HCl, 2 M guanidine-HCl, 0.4 M Arginine, 10 mM GSH and 2 mM GSSG at pH 9.2. The disulfide bond formation was monitored by LC–TOF MS (Fig. 5) and was completed within 24 h. The formation of a main product at 5.21 min with MW 22070.80, the loss of four Daltons in mass, was consistent with the protein folding process that led to the concurrent formation of two intramolecular disulfide bonds. Furthermore, comparison of the ESI-MS of **7** (Fig. 4) and **8** (Fig. S-7) showed a shift in the MS charge state distribution to less protonated species and such change is well-known in the ESI-MS characterization of a folded protein.[36] In addition to the main product, we noticed the formation of two other small and more hydrophobic peaks, as displayed in the LC–TOF MS profile (Fig. 5). The major peak at 5.6 min. seems a mixture of glutathione adducts running together, from which we can distinguish two semi-oxidized glutathione thioether adducts: the more abundant one with four sulfures [Cys-SH, Cys-(S-glutathione), Cys-S-S-Cys] ($C_{1003}H_{1551}N_{265}O_{306}S_4$, Calculated MW 22346.23; Observed MW 22346.20) and the less abundant one with six sulfurs [Cys-S-S-(S-glutathione), Cys-(S-glutathione), Cys-S-S-Cys] or [Cys-S-(S-glutathione), Cys-S-(S-glutathione), Cys-S-S-Cys] ($C_{1013}H_{1566}N_{268}O_{312}S_6$, Calculated MW 22683.6; Observed MW 22683.40). The minor peak at 6.44 min. corresponds to a possible dimer (data not shown). After 24 hours the reaction was stopped by lowering the pH to 3 with 6 M HCl and the solution was purified by RP-HPLC to give 1 mg of lyophilized powder **8** in 25% yield.

After a five step process comprised of two native chemical ligations, desulfurization, deprotection of the acetamidomethyl (Acm) groups and cyclization and folding, the full length hGH analogue **8** was obtained in milligram scale with an overall yield of ~1%. We would like to remark that we did not attempt to modify the conditions of all the chemical reactions that we used to improve the yields. These reactions were first time attempts with no optimization, of which the only purpose was to quickly demonstrate that the synthetic approach we designed could reach the target compound, and therefore there is substantial room for improving the yield.

With the purpose of eliminating all the TFA from the synthetic lyophilized analog **8** to avoid further problems with incorrect conformation and its biological activity, compound **8** was dissolved in a 2 M Guanidine-HCl buffer plus 60 μL of 0.2 M NaOH, transferred to a 3 ml Slide-A-Lyzer dialysis cassette (3500 MWCO) and dialyzed against four changes (5 h, overnight, 9 h and overnight) of 2 L of water at pH 8.5 adjusted with ammonia. The dialyzed material was clarified by centrifugation and concentrated to a small volume (~400 μl) to continue its evaluation. To confirm the correct disulfide bond formation, a sample of dialyzed compound **8** was trypsinized and the reaction mixture was analyzed by LC–MS/MS. The resulting peptide fragments shown in Fig. 6 were consistent with the expected structure, including the two segments that were linked by disulphide bonds, i.e., T6–T16 and T19–T20.

The analytical data and biological activity of the synthetic hGH analog **8,** after RP-HPLC purification and dialysis, are shown in Fig. 7. RP-HPLC analysis gave a single major peak. The LC–TOF MS analysis showed after deconvolution a mass of 22070.80 Da which is in excellent agreement with the calculated average mass

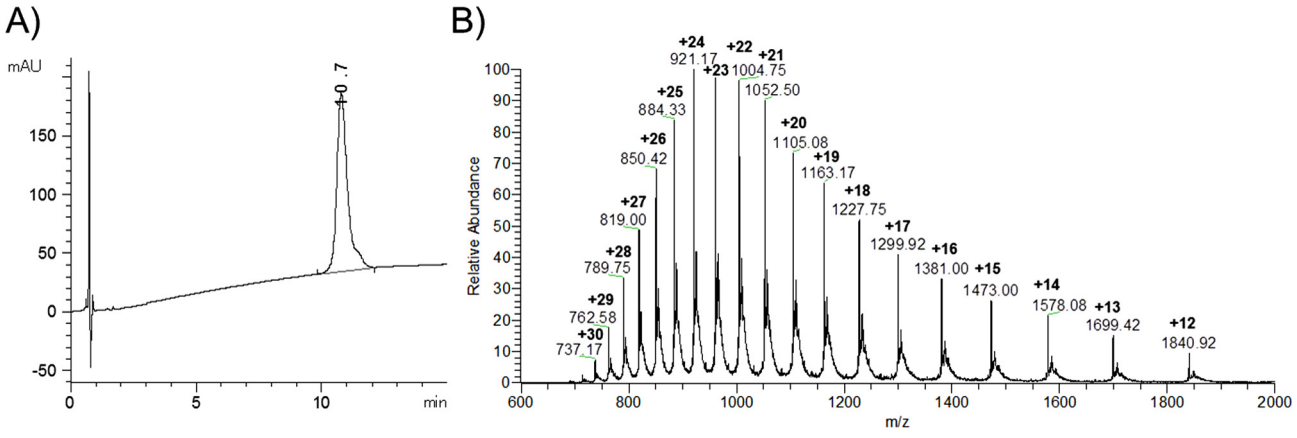


Fig. 4. Analytical HPLC profile and ESI-MS spectrum of the purified full-length polypeptide **7**.

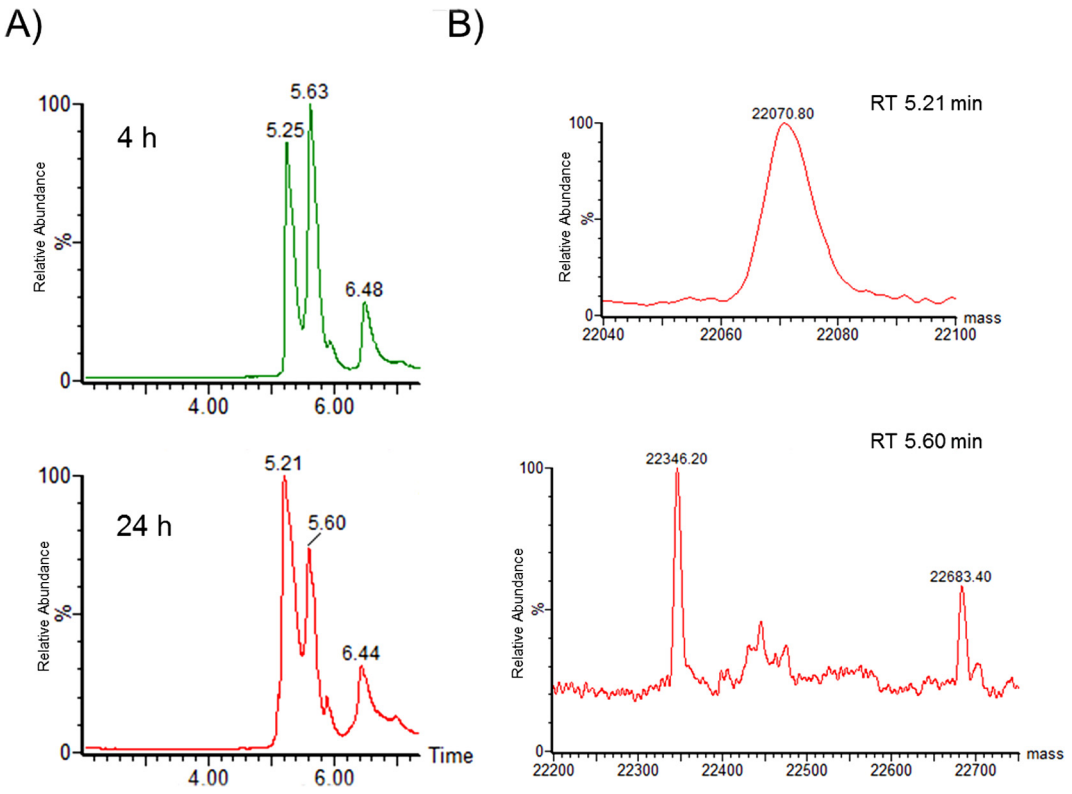
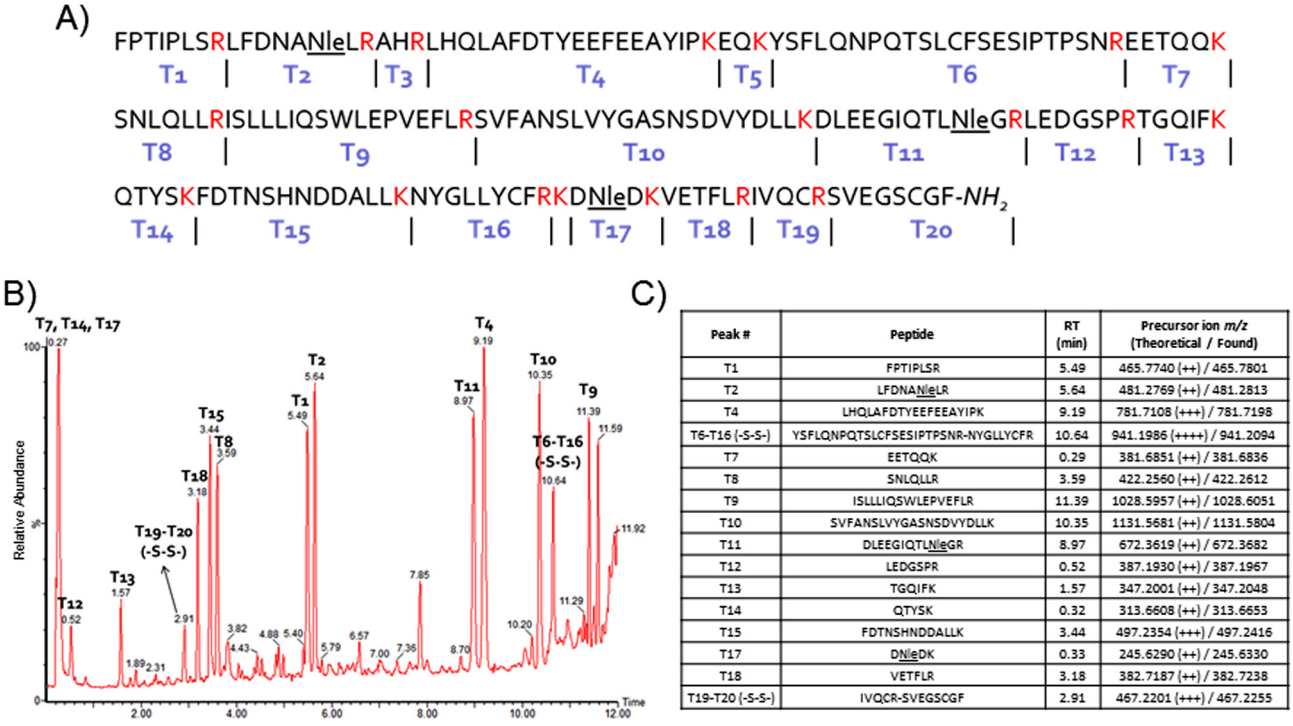


Fig. 5. Summarized data for the folding of hGH analog **7**. A) Total ion current (TIC) traces obtained by RP-HPLC–MS analysis for the time course study at 4 h (top) and at 24 h (bottom); B) Deconvoluted intact protein mass spectra for the oxidized product corresponding to the target compound **8** (peak at 5.21 min) after 24 h (top), and for the peak at 5.60 min containing the by-products after 24 h (bottom). The peak at 5.60 min contains a mixture of glutathione adducts running together from which we can distinguish two semi-oxidized glutathione thioether adducts: the more abundant one with four sulfures [Cys-SH, Cys-(S-glutathione), Cys-S-S-Cys] MW 22346.20 and the less abundant one with six sulfurs [Cys-S-S-(S-glutathione), Cys-(S-glutathione), Cys-S-S-Cys] or [Cys-S-(S-glutathione), Cys-S-(S-glutathione), Cys-S-S-Cys] MW 22683.40.



Peak #	Peptide	RT (min)	Precursor ion *m/z* (Theoretical / Found)
T1	FPTIPLSR	5.49	465.7740 (++) / 465.7801
T2	LFDNANleLR	5.64	481.2769 (++) / 481.2813
T4	LHQLAFDTYEEFEEAYIPK	9.19	781.7108 (+++) / 781.7198
T6-T16 (-S-S-)	YSFLQNPQTSLCFSESIPTPSNR-NYGLLYCFR	10.64	941.1986 (++++) / 941.2094
T7	EETQQK	0.29	381.6851 (++) / 381.6836
T8	SNLQLLR	3.59	422.2560 (++) / 422.2612
T9	ISLLLIQSWLEPVEFLR	11.39	1028.5957 (++) / 1028.6051
T10	SVFANSLVYGASNSDVYDLLK	10.35	1131.5681 (++) / 1131.5804
T11	DLEEGIQTLNleGR	8.97	672.3619 (++) / 672.3682
T12	LEDGSPR	0.52	387.1930 (++) / 387.1967
T13	TGQIFK	1.57	347.2001 (++) / 347.2048
T14	QTYSK	0.32	313.6608 (++) / 313.6653
T15	FDTNSHNDDALLK	3.44	497.2354 (+++) / 497.2416
T17	DNleDK	0.33	245.6290 (++) / 245.6330
T18	VETFLR	3.18	382.7187 (++) / 382.7238
T19-T20 (-S-S-)	IVQCR-SVEGSCGF	2.91	467.2201 (+++) / 467.2255

Fig. 6. RP-HPLC–(TOF)MS analysis of Tryptic peptides of the synthetic hGH analog **8**. A) Predicted tryptic peptides fragments; B) HPLC–MS chromatographic profile of the tryptic digest; C) *m/z* values of the observed peptide fragments.

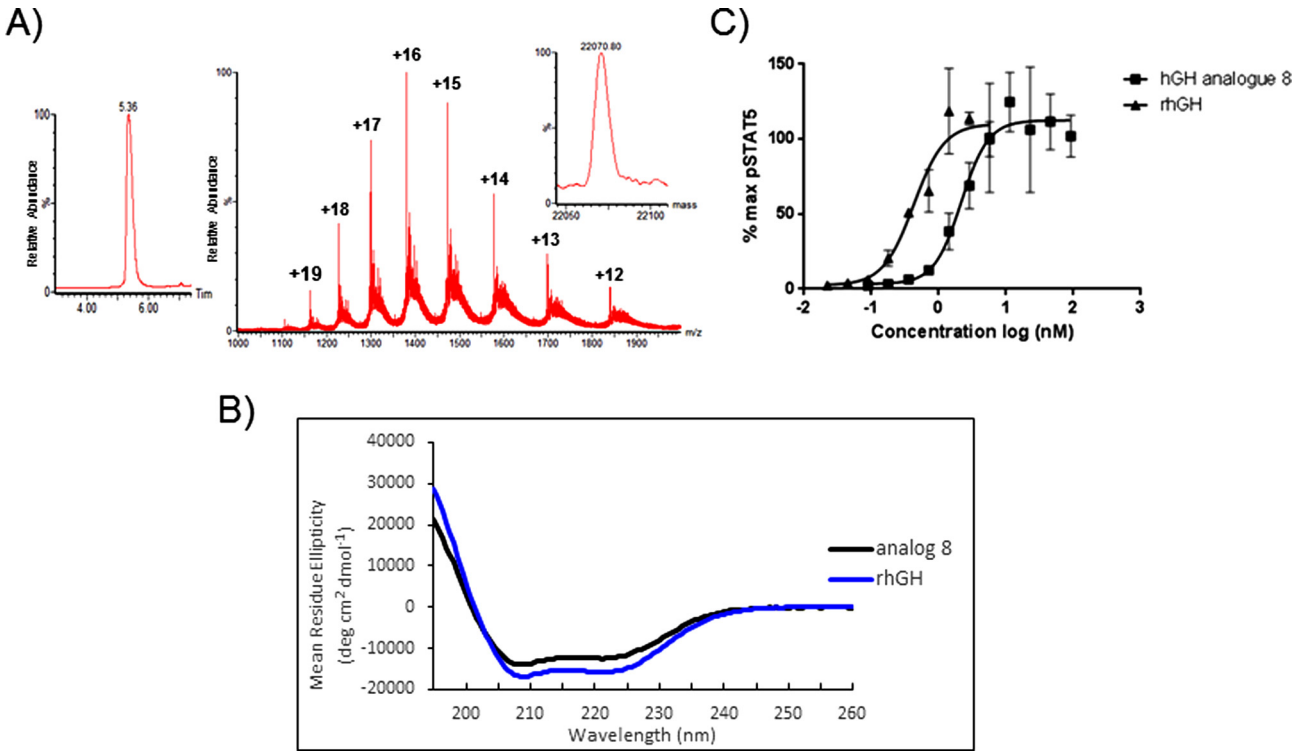


Fig. 7. Biophysical characterization of the folded synthetic hGH analog **8**. A) Analytical RP-HPLC–(TOF)MS analysis of the intact protein: TIC trace (left) and protein charge envelop (right). The insert displays the deconvoluted mass spectrum indicating the mass value obtained for the intact protein: calculated average mass: 22070.65 Da, found: 22070.80. B) CD spectrum of hGH analog **8** and rhGH in double-distilled water. C) STAT5 phosphorylation assay. The ability of the synthetic analog **8** to stimulate the GHR was assessed using cells that endogenously express the GH receptor. Analog **8** was fully efficacious with a calculated half maximal effective concentration (EC50) value of 2.2 nM (or 47.31 ng/mL), about five fold higher than the calculated EC50, 0.424 nM, for the rhGH tested in parallel. Data are expressed as percent of the average of the maximum signal of the control hGH.

of 22070.65 Da. The CD spectrum, presented a characteristic shape with a minimum around 208 nm and a shoulder around 222 nm, indicating highly helical secondary structure and closely similar to the CD spectra of recombinant hGH (rhGH). The *in vitro* biological activity of synthetic analog **8** [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH_2 was evaluated in a functional cell-based assay. The ability of the synthetic analogue **8** or rhGH to stimulate the GHR was assessed using a STAT5 phosphorylation assay in IM-9 cells, human lymphoblastoid B cells that endogenously express the GHR. Quantitative detection of phospho-STAT5 (phosphorylated on Tyr694/699) in IM-9 lysates was performed using the AlphaLISA SureFire Ultra p-STAT5 (Tyr694/699) assay (PerkinElmer). The calculated half-maximal effective concentration (EC50) value for the hGH analogue **8** was 2.2 nM (or 47.3 ng/mL), about five fold higher than the calculated EC50, 0.424 nM, for the (rhGH) tested in parallel. Analog **8** exhibits similar efficacy to rhGH in this assay, indicating that it is a fully agonist at the human GH receptor. This, together with the above data, suggested that the dialyzed compound **8** has the correct protein tertiary structure.

In conclusion, our strategy utilizing only three peptidic segments and two native chemical ligation steps was a simple design that afforded, only by synthetic means, a 191 amino acid bioactive analog of hGH. A key feature of the successful synthesis was the native chemical ligation at Xaa-Ala^{105} site which was enabled by the use of metal-free desulfurization. The combination of characterizations using HPLC, mass spectrometry, CD spectroscopy, tryptic fingerprint and a functional cell-based assay showed that this synthetic analog [Nle^{14,125,170},Glu^{29,91},Gln^{74},Asn^{107},Asp^{109}]hGH-NH_2 **8** had the correct sequence and disulfide bridge topology, was folded correctly and was biologically active. We have demonstrated as well that the nine modifications made to the native sequence of hGH have a minimal effect on the biological activity of the molecule and that the synthesized analogue was a fully active agonist at the hGH receptor.

We have shown, for the first time, that our prototype total chemical synthesis is able to afford a homogeneous bioactive analog of hGH which complements other recombinant and semi-synthetic approaches to the hGH protein production. We believe this work is an important contribution to the effective total synthesis of analogues of hGH and will provide a more flexible approach to the systematic preparation of derivatives of hGH with any desired modification on the polypeptide backbone.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2017.05.027.

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