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# Characterisation of a Novel Growth Hormone Variant Comprising a Thioether Link between Cys182 and Cys189

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*A novel variant of recombinant human growth hormone (r-hGH), isolated from biopharmaceutical preparations produced in* E. coli*, was identified and characterised. This variant contains a nonreducible thioether bridge near the C terminus between Cys182 and Cys189 and was characterised using various analytical techniques. As previous work by Cunningham and Wells (1993) highlighted the involvement of several residues in this part of the sequence in the binding and affinity of the molecule to its receptor, the presence of this modified intramolecular link may have important implications with regard to the biological behaviour of the molecule. Furthermore, as the conversion of a disulfide into a thioether was previously reported for a therapeutic monoclonal antibody (Tous et al., 2005), this may imply that disulfide bridges located in this part of the molecule have a generic susceptibility to thioether formation. This in turn is relevant to the biopharmaceutical industry for monitoring the integrity of disulfide bridges near the protein C terminus. The present study exhibits a state of the art physicochemical investigation for the unequivocal elucidation of a novel structure involving peptide mapping with mass spectrometry and de novo peptide sequencing. Changes in the higher order structure of the molecule were highlighted by near UV circular dichroism and molecular modelling.*

## Introduction

Human growth hormone (hGH) is a nonglycosylated protein of 191 amino acid residues, containing two intrachain disulfide bridges. The first disulfide bridge lies between Cys53 and Cys165, whereas the second one is near the C terminus of the molecule and connects Cys182 and Cys189 (see Figure 1). The protein is recombinantly expressed in both bacteria[1] and mammalian cells and preparations are therapeutically used for various growth and metabolic disorders.[2,3]

Heterogeneity in biopharmaceutical products may arise during both the production process and the shelf life of the product resulting in the presence of various forms of post-translational modifications or degradation products. To understand the potential impact of such modifications on the bioactivity and safety of a biopharmaceutical, their identification and characterisation are particularly important. Furthermore, detailed characterisation supports the development of a suitable manufacturing process and helps to assess how process changes affect the identity, quality, and purity of a drug product and its biological activity.[4]

One analytical technique proposed in the European Pharmacopoeia for the assessment of product identity of hGH preparations is peptide mapping. This procedure requires that the profile of the chromatogram obtained with the test solution corresponds to that obtained with the reference solution. As a reference method, this procedure was employed in the analysis of different *E. coli* hGH samples. One of the examined samples revealed a variation in the chromatographic profile compared with that of the reference solution and was therefore further analysed.

The present article describes the identification and characterisation of a thioether structure in a commercial hGH product. This uncommon sulfur linkage was confirmed employing various analytical techniques and approaches.

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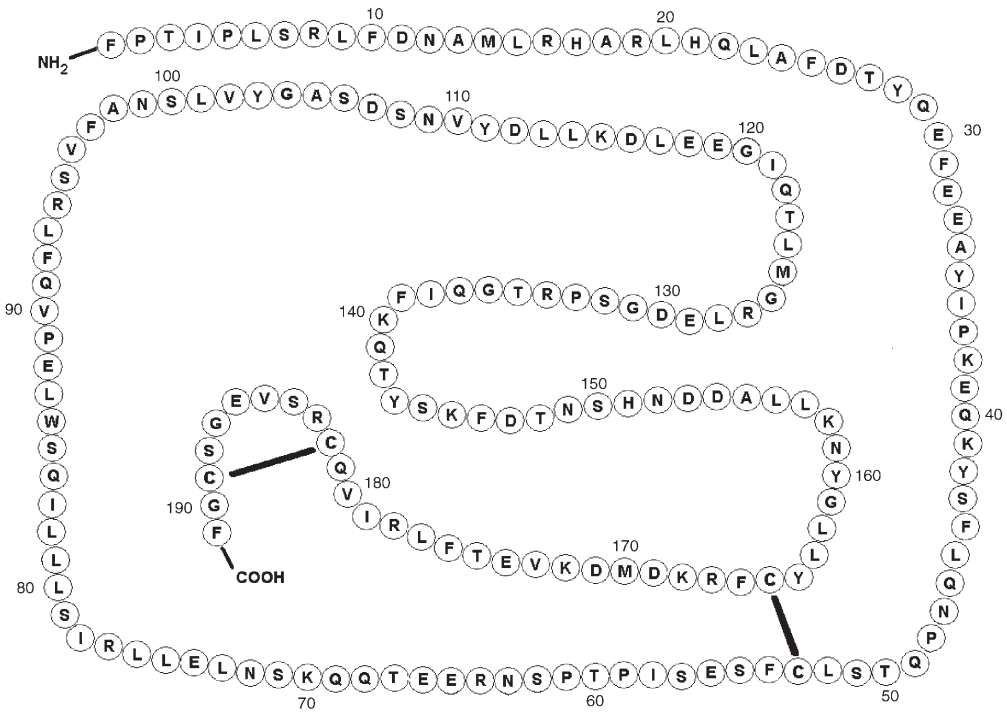


**Figure 1.** Primary structure of human growth hormone including the disulfide bridge pairing.

## Results and Discussion

### Whole molecule ESI-MS (undigested samples)

Scouting experiments by ESI-MS on the whole molecule, with the aim to assess the purity of the samples, were performed employing various commercially available r-hGH preparations from *E. coli*. Whereas the international standard NIBSC 98/574 yielded a single peak (Figure 2, panel A), one of the products exhibited an additional peak at minus 32 Da with respect to the theoretical mass of 22125.07 Da for r-hGH (Figure 2, panel B). Based on the ion intensity, the relative proportion of the additional species was estimated to be about 30% of the sample. This estimation is assuming that the ionisation efficacy of this supposedly modified molecule is unchanged. This estimated percentage was later confirmed by integrating the UV trace of the corresponding peaks (indicated in Figure 5), that is, the expected and the extra C-terminal peptide, of the analytical peptide mapping. The presence of this additional peak was confirmed in different batches of the same product. To identify the nature of this observation, the following investigation was performed.

### LC-ESI-MS peptide mapping with in-source fragmentation

As the unexpected minus 32 Da species detected in the *E. coli* sample may potentially arise from an amino acid modification, both the standard and the sample under investigation were subjected to tryptic digestion followed by LC-ESI-MS analysis to compare the individual peptides obtained after digestion with regard to their molecular mass and their structure. The on-line ESI-MS analyses were performed using two different scan acquisition programs. The first one aimed at obtaining a suitable ionisation for all peptides yielding their respective molecular mass. The second one was optimised to induce in-source fragmentation to obtain structural information. Figure 3 shows the RP-HPLC ESI-MS chromatograms of the tryptic digests. In the case of the international standard (panel A), all the tryptic peptides were detected with their expected molecular mass. In contrast to that, and similar to the initial evidence obtained by the whole molecule-ES-MS analysis, the chromatogram of the *E. coli* sample under investigation exhibited one additional peak (Figure 3, panel B), which provided another indication of the presence of a modified species. This additional peak was detected with a molecular mass of 1368.16 Da, which corresponds to a mass difference of minus 32 Da with respect to the expected mass of 1400.33 for peptide 179–183 + 184–191 indicating that the unknown species can be attributed to a modification of this peptide. This conclusion was further underlined by a comparison of the in-source fragmentation spectra of the unknown peak and that of peptide 179–183 + 184–191 (see Figure 4) which showed that the pattern of the former related well to that of the latter allowing for the minus 32 Da mass difference.

### Collection of peaks for identification and characterisation purposes

Similar to the LC-ES-MS peptide mapping results, the analytical peptide mapping described in the European Pharmacopoeia, confirmed the presence of one extra peak, see Figure 5. Here, although relative peak intensity may vary, additional peaks are indicative of the presence of either a modification of the native molecule or a contaminant.

In this analysis, the extra peak was well resolved from neighbouring peaks and eluted in a zone of the chromatogram, in which no interference was seen from partially digested products. Therefore, this peptide mapping was employed to collect, from several chromatographic runs, sufficient amounts of the peaks corresponding to the expected (intact) and the extra (modified) C-terminal peptide to be used for further characterisation (N-terminal and MS-MS). From the sample under investigation both the extra peak (−32 Da, eluting at about 21.9 min) and the expected peak of peptide 179–183 + 184–191 (about 24.1 min) were submitted to further analysis, in particular N-terminal analysis, ESI-MS-MS, and MALDI-TOF.

  

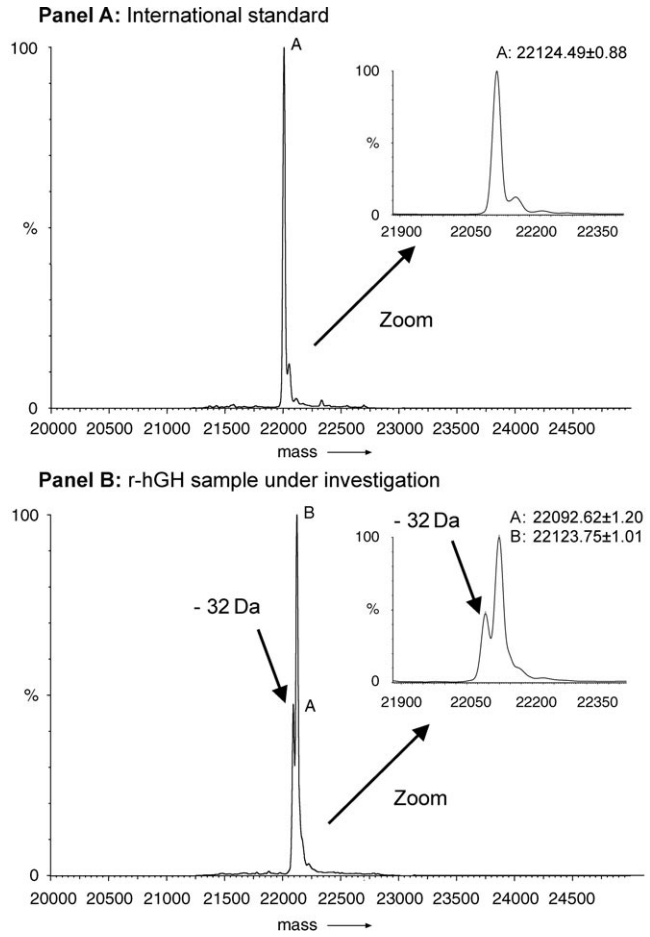


**Figure 2.** ESI-MS whole molecule spectra of r-hGH. Panel A: minor peaks at higher molecular mass with respect to the main peak, can be attributed to adduct formations with salts and excipients. Panel B: The molecule under investigation exhibits an additional peak at −32 Da with respect to the main peak. Both deconvoluted spectra were obtained by processing the corresponding ions in the range of *m/z* 600–2400.

N-terminal analysis of the collected (expected) peptide peak of peptide 179–183 + 184–191 (see inset in Figure 5) confirmed its correct primary structure (see Table 1). Analysis of this sample also yielded the typical signal corresponding to the presence of a disulfide bridge (Cys182–Cys189). For the extra peak the same primary structure as peptide 179–183 + 184–191 was found, however, in contrast to the latter, the extra peak did not yield the disulfide bridge-related signal.

**Table 1.** Primary structure by N-terminal analysis for collected peptide peaks.

| Cycle | Expected peak (pep. 179–183 + 184–191) | Extra peak (−32 Da) |
|---|---|---|
| 1 | Ile, Ser | Ile, Ser |
| 2 | Val, Val | Val, Val |
| 3 | Gln, Glu | Gln, Glu |
| 4 | X, Gly | X, Gly |
| 5 | Arg, Ser | Arg, Ser |
| 6 | Cys-Cys | No signal |
| 7 | Gly | Gly |

These findings confirmed that the extra peak arose from a modification of peptide 179–183 + 184–191 and furthermore that the modification is associated with the disulfide linkage. Given the mass difference of −32 Da, which corresponds to a loss of a sulfur, this modification can be interpreted as a linkage by a thioether rather than a disulfide.

### RP-HPLC ESI-MS peptide mapping under reducing and non-reducing conditions

To further investigate the nature of the linkage in the modified peptide, peptide mapping was performed both under reducing and nonreducing conditions. It could be anticipated that under reducing conditions the expected peak of peptide 179–183 + 184–191 (containing the disulfide) disappears whereas the extra peak (peptide containing the nonreducible thioether) maintains its retention time. This was indeed confirmed by the RP-HPLC analysis which substantiated that the modified peptide, in contrast to the expected, disulfide-containing one, retains its retention time upon reduction (data not shown). Thus, additional evidence was obtained that the modification is attributable to a thioether linkage.

### ESI-MS-MS analysis of collected peaks

Further analysis of the modified peptide was attempted by performing MS/MS fragmentation on the $(M+2H)2+$ *m/z* ion.[5] However, only incomplete fragmentation before the cysteine residues could be observed (results not shown). Although this confirmed again the sequence of the peptide, it yielded no additional structural information. The incomplete fragmentation may be attributable to an insufficient energy to break the disulfide or thioether bridge respectively.

### MALDI-TOF-MS analysis of collected peaks

As ESI-MS ionisation and CID fragmentation was insufficient to extensively fragment the collected peptides, the samples were further analysed by MALDI-MS to attempt a more efficient fragmentation by employing a higher laser energy. Figure 6 shows the spectra obtained for both the disulfide containing and the modified peptide. It can be seen that for both peptides, one fragment was obtained. In the case of the expected peak (panel A), the obtained fragment confirmed the opening of the disulfide bridge. On the other hand, the fragment obtained from the opening of the modified linkage (panel B) provided clear evidence that within peptide 179–183 cysteine 182 was transformed into dehydroalanine allowing for the −32 Da mass difference compared with correct sequence.

### ESI-MS-MS analysis of collected peaks by nanospray Q-TOF

To identify unequivocally this modification, additional MS/MS experiments were performed on Q-TOF by nanospray injection on the collected peptides (that is, with and without the modification). Fragmentations were performed in daughter mode.[5] Compared to the previous MS/MS approach (see above), this

  

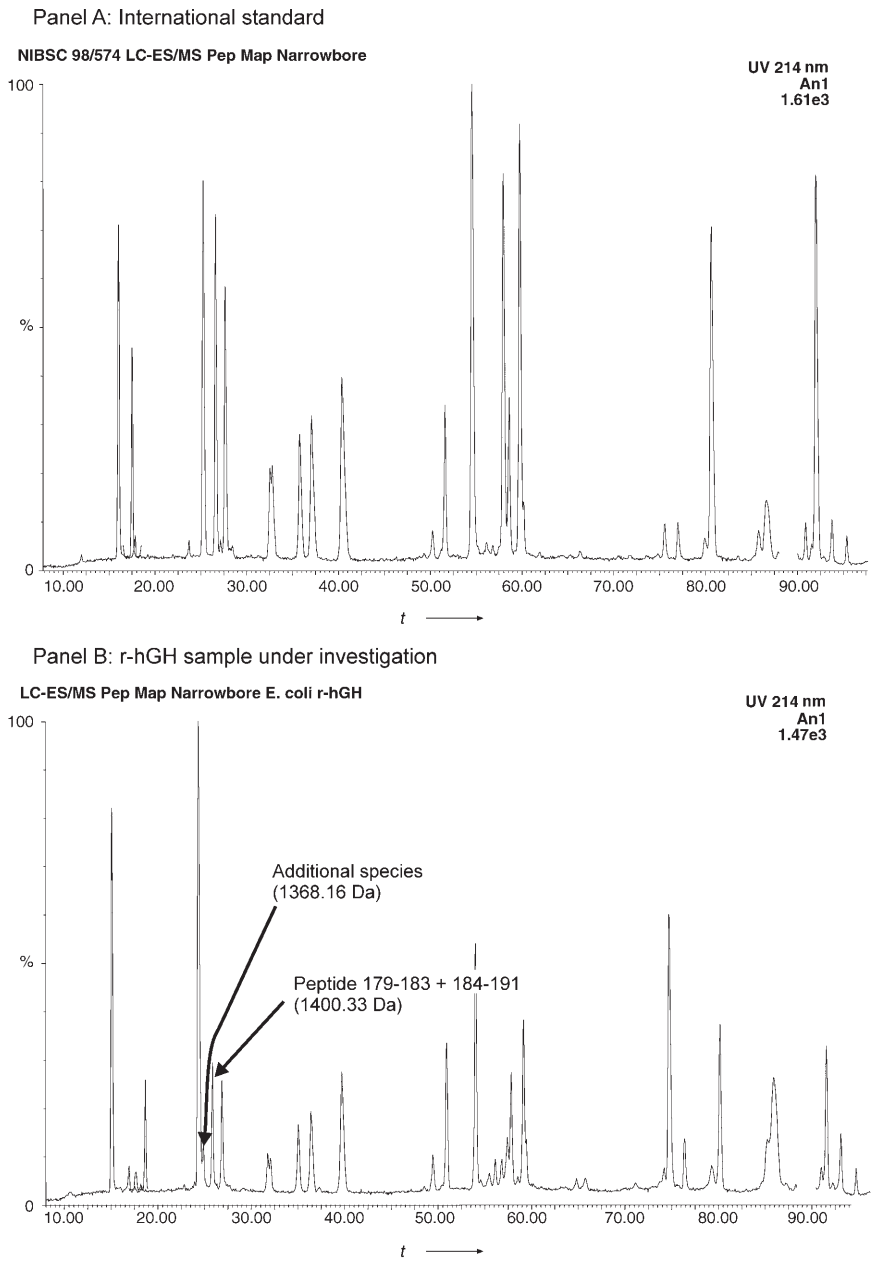


**Figure 3.** RP-HPLC ESI-MS chromatograms of tryptic digests of r-hGH.

experiment resulted in a complete fragmentation of the peptide (Figure 7) allowing the identification of the modification. Indeed, a specific fragment of the disulfide bridge (e5 + S) is detected on the MS/MS spectrum of the nonmodified peptide (panel A) and not for the modified one (−32 Da), panel B. As all the other fragments are detected for both peptides, the presence of the e5 + S fragments confirms that the modification is located at the level of the disulfide bridge and that the mass difference of −32 Da is due to the absence of a sulfur ion in the bridge of the modified peptide. Therefore, this analysis finally provided the unambiguous proof of the proposed modification whereas the initial experiments (ES-MS, peptide mapping, MALDI-TOF, and MS-MS on triple quadrupole) were only indicative thereof.

**Higher order structure analysis**

To investigate the potential impact of the thioether linkage on the tertiary structure of the protein, the *E. coli* sample under investigation (about 30% of the material carrying the thioether linkage) was subjected to near UV CD analysis and compared with other hGH preparations from both *E. coli* and mammalian cells (see Figure 8). It can be seen that the thioether containing the sample exhibited a significant deviation from the other spectra in the region of 250–270 nm. The CD signals in this region arise from phenylalanine residues and therefore the change in the spectrum indicates a modification in the environment of one or more phenylalanine residues. As solvent conditions may also influence the CD signal in this spectral range, the influences of excipients used for these products were investigated. Excipients of the samples under investigation included glycine, mannitol, and lactose. By performing spiking experiments it was ascertained that they did not influence the spectra in this wavelength range (data not shown).

As can be seen from the primary structure (Figure 1), there are two phenylalanine residues (176 and 191) in the C-terminal part of the protein that may be affected by the modified linkage. It should be noted here that a clearly different near UV profile was obtained with a sample containing about 30% of the variant and 70% of the native form. Thus it can be expected that the profile of the pure variant form would deviate even more distinctly from that of the native form.

Previous work published by Cunningham and Wells revealed that residues in the vicinity of the two C-terminal cysteine residues (Cys182 and Cys189) are involved in the contact with the receptor molecule.[6] Furthermore, the authors demonstrated that site-directed mutagenesis of various residues surrounding Cys182 and Cys189 may negatively impact on the affinity of the molecule to its receptor.

As can be seen from a comparison of different published X-ray structures of hGH (Figure 9), the C terminus is one of the

  

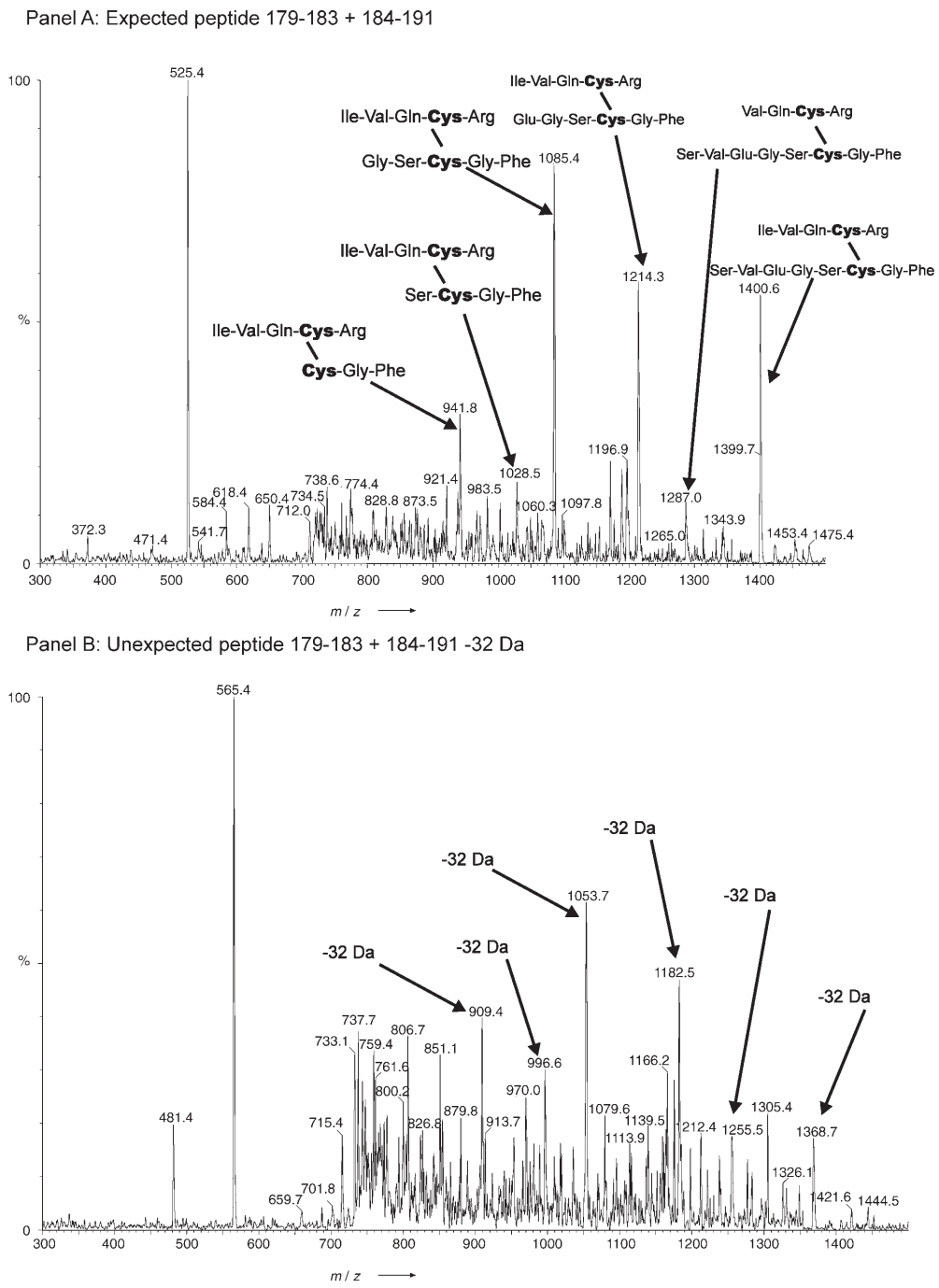


**Figure 4.** In-source fragmentation ESI-MS spectra of the *E. coli* r-hGH sample of peptide peaks obtained by tryptic mapping.

most mobile regions of the molecule. A modification of the disulfide bridge to a thioether linkage, as illustrated by molecular modelling in Figure 10, may result in an altered spatial arrangement of the C terminus. Furthermore, the shortened intramolecule linkage may reduce the mobility of this region. Both effects may impact on the interaction of hGH with its receptor.

## Conclusions

A contaminating species was detected in an r-hGH preparation exhibiting a mass difference of minus 32 Da. Analysis by peptide mapping coupled with MS analysis and N-terminal sequencing confirmed the localisation of this modification in the two C-terminal peptides of the protein, which are connected by a disulfide bridge. It was further demonstrated that the modification within the two peptides is associated with the disulfide link and that, in contrast to the native form, the modified one contains a nonreducible link. The collected evidence leads to the conclusion that the described variant form arose from the formation of a thioether bond between Cys182 and Cys189.

Spectroscopic analysis revealed that the described modification impacts on the tertiary structure of the protein. Molecular modelling yielded further evidence that the thioether linkage may indeed alter the spatial arrangement of the C terminus. This in turn may negatively affect the receptor binding properties of the protein and thus compromise its biological activity. Further studies will be designed to verify the impact on the receptor binding and the biological behaviour of the protein.

Previously, Andersson et al. and Canova-Davis described trisulfide variants of r-hGH.[7,8] More recently, Tous et al. reported the occurrence of a nonreducible thioether bridge between the light and heavy chains of different IgG1 monoclonal antibodies.[9] However, the present article is to our knowledge the first description of a thioether-containing hGH variant. Here, similar to the findings reported by Tous et al.,[9] the thioether modification was identified near the C terminus of the respective protein. This may imply that disulfide bridges located in this part of the molecule have a generic susceptibility to thioether formation. Consequently, this observation may be relevant to the biopharmaceutical industry wishing to monitor the integrity of disulfide bridges near the protein C terminus.

  

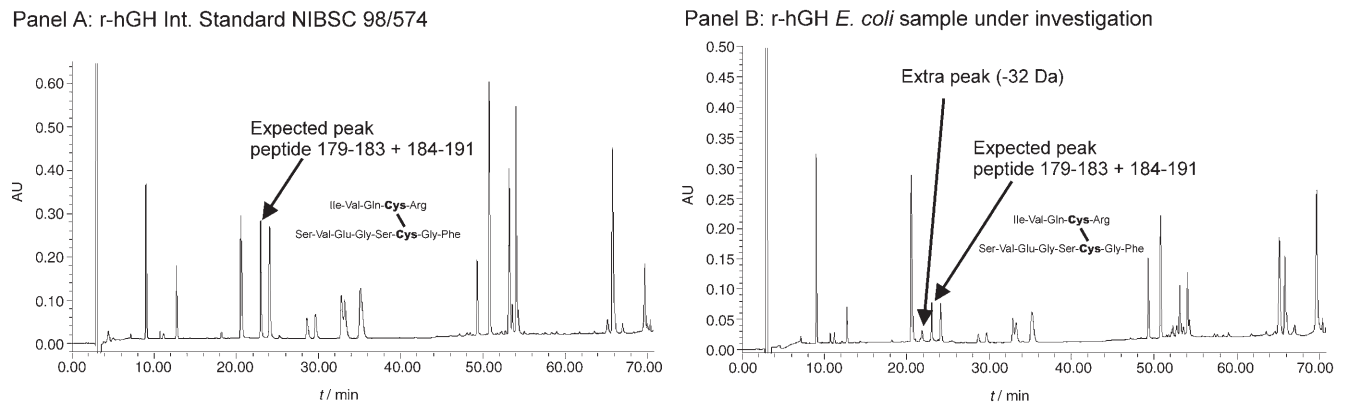


**Figure 5.** Peak collection for peptide identification and further characterisation. Collected peptide peaks of the chromatographic separation of the tryptic digest are indicated in the respective graph.

The structure of the thioether linkage resembles that of lanthionine, a monosulfide analogue of cysteine. Lanthionine was first described by Horn et al. who isolated the molecule from sodium-carbonate-treated wool.[10] The elimination mechanism of one of the sulfur atoms of the disulfide bond of the cysteine, leading to the formation of a thioether, was described by Earland and Raven.[11] Lanthionines are also commonly found in lantibiotics, a group of antimicrobial peptides that contains also several other modified amino acids.[12,13] Given the fact that lanthionine was first isolated from sodium-carbonate-treated wool, it may be hypothesised that the formation of this variant may be favoured by alkaline pH and elevated temperatures. Its formation may therefore potentially arise from conditions encountered during the upstream and downstream processing of the product.

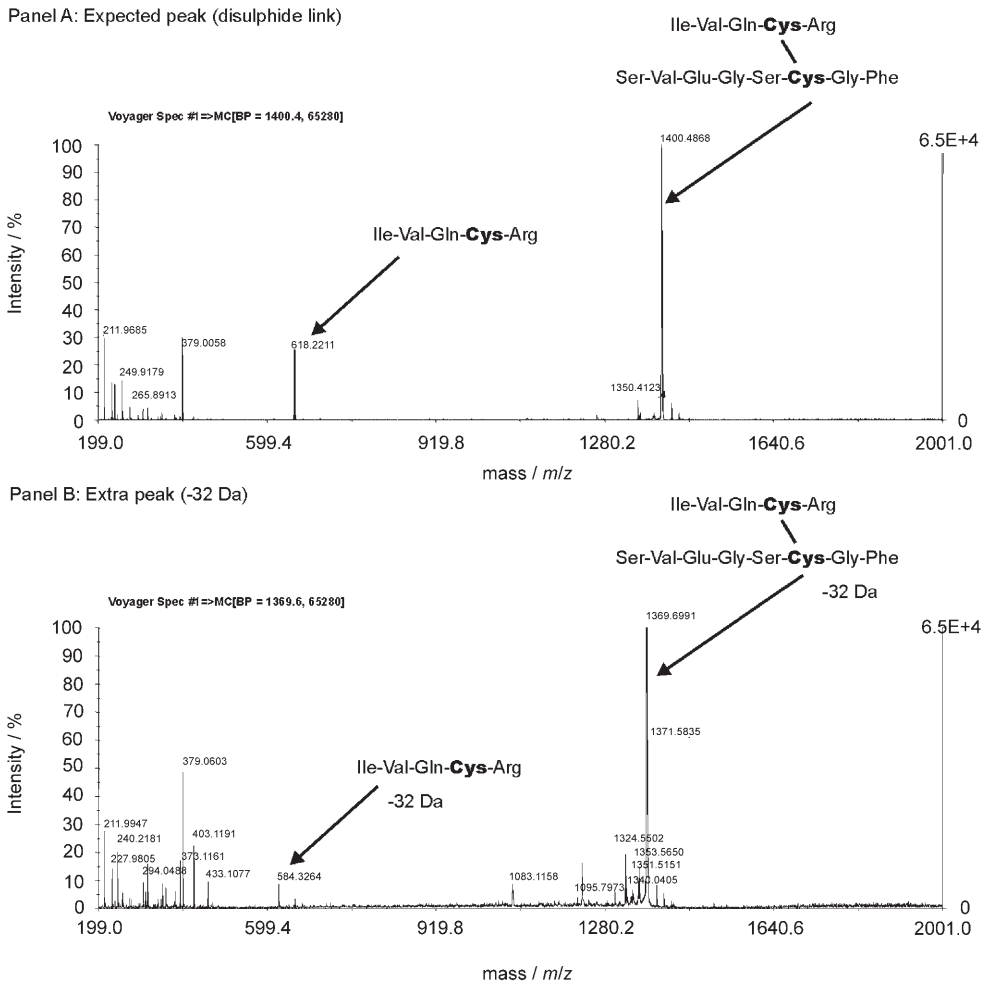


**Figure 6.** MALDI-TOF-MS of peptide peaks obtained by tryptic peptide mapping of the *E. coli* r-hGH sample under investigation.

## Experimental Section

**Materials**. The protein under investigation was a commercially available, recombinant GH product expressed in *E. coli*. Analytical results were compared to those of the hGH International standard (recombinant DNA-derived human growth hormone, 2nd International standard, NIBSC code: 98/574) which was analysed in parallel as a reference for the ES-MS (whole molecule), LC-MS peptide mapping, circular dichroism (CD), and analytical peptide mapping (EU-Ph). For the higher order structure comparison by near UV CD, three additional commercially available GH products were used, two from E. coli and one from mammalian expression (two different batches).

**ESI-MS whole molecule analysis of undigested samples**. ESI-MS analysis was performed on a Quattro II mass spectrometer (Quattro II, Waters, Manchester, UK) by

  

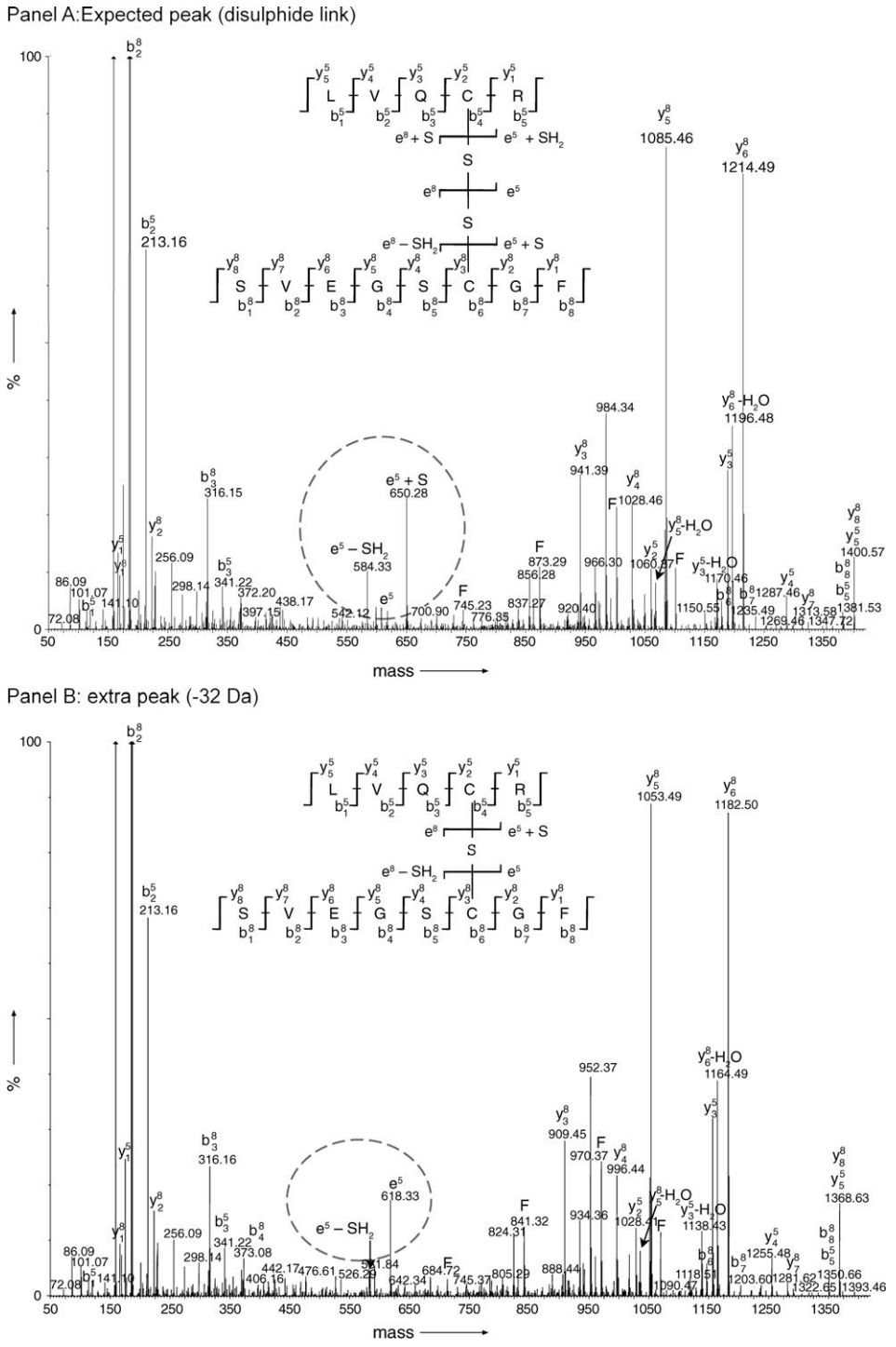


**Figure 7.** Daughter ESI-MS-MS spectra of the nonmodified peptide (panel A) and the thioether-containing peptide (panel B) obtained by tryptic peptide mapping of *E. coli* r-hGH sample.

direct injection of the sample diluted to 0.2 mg mL$^{-1}$ with mobile phase constituted of 50% $H_2O$-$CH_3CN$ containing 1% of $CH_3COOH$. The capillary was set to 3500 V and cone voltage was ramped from 30 V to 90 V in scale *m/z* of 600 to 2400. The MS data were collected with a scan time of 3.2 sec and an interscan time of 0.10 sec. The mass spectrometer was calibrated with Myoglobin in the *m/z* range 600 to 2400.

**RP-HPLC ESI-MS peptide mapping**. Lyophilised samples were reconstituted with pure water at a final concentration of 1 mg mL$^{-1}$. Trypsin (Trypsin Modified Sequencing Grade Roche code 11-418-025-001) was added at an enzyme substrate ratio (E/S) of 1:67 and the mixture was incubated for 4 h at 37 °C. In samples that were not immediately analysed thereafter, enzymatic activity was stopped by freezing and storage at −20 °C. The peptide mixture was analysed by LC-ESI-MS on a Waters Alliance HPLC (Waters, Milford, USA). Conventional separation was performed on a 250× 2.1 mm 5 mm 100 A column C18 (RP Merck Supersphere narrow bore RP 18 100 column) eluting with a linear gradient from 0.1% aqueous TFA acid to 99.9% acetonitrile/0.1% TFA over 95 min at 200 μL min$^{-1}$. HPLC was performed using a Waters Alliance system, equipped with a binary solvent delivery system, an autosampler, and a tunable UV detector (UV detector mod. UV/VIS 20 Micromass Waters). To divide the flow rate, a post UV detector split was set, so that in source the flow rate was 100 μL min$^{-1}$.

Mass spectrometry was performed using a Micromass Triple quadrupole (Quattro II, Waters, Manchester, UK) operating in positive ion electrospray mode. The nebulisation gas was set at 20 L h$^{-1}$ at, the drying gas at 350 L h$^{-1}$, and with the source temperature at 100 °C. The capillary was set to 3500 V and two different scan functions were used; the first aimed to measure the molecular mass of each peptide (cone voltage 35 V), and the second one was optimised to allow in source fragmentation (cone voltage ramp 30 V to 90 V) to obtain preliminary information on fragments of each peptide. The MS data were collected between 400 *m/z* and 2000 *m/z* with a scan time of 3.2 s and an interscan time of 0.10 s for the first scan function, and between 200 *m/z* and 1500 *m/z* with a scan time of 3.2 s and a interscan time of 0.10 s for the second scan function. The mass spectrometer was calibrated with a PEG mixture in the *m/z* range 80 to 2000.

**Analytical HPLC peptide mapping**. Lyophilised samples were reconstituted with pure water at a final concentration of 1 mg mL$^{-1}$, and trypsin (Trypsin Modified Sequencing Grade Roche code 11-

  

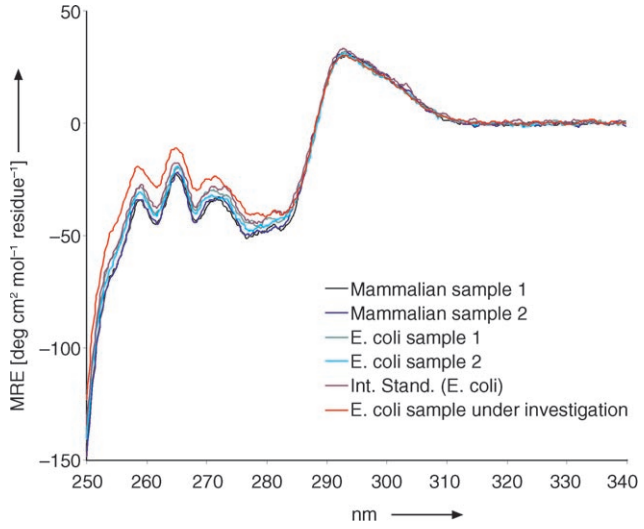


**Figure 8.** Near UV CD spectra of hGH samples expressed in *E. coli* and mammalian cells.

418-025-001) was added at an enzyme substrate ratio (E/S) of 1:67 and the mixture was incubated for 4 h at 37 °C. Here, water was used for sample reconstitution instead of buffer (for example, 50 mM TRIS-HCl pH 7.5) as it was shown in previous experiments that this minimised the presence of some partial digested peptides while maintaining complete sequence coverage (data not shown). For samples not immediately analysed thereafter, the enzymatic activity was stopped by freezing and storage at −20 °C. The peptide mixture was analysed on a C8 column (Vydac code 208TP54) connected to the HPLC system (Mod. 2695 Alliance Waters) equipped with an UV detector (Mod. 2487 Waters). The HPLC system was controlled by the Empower software (Waters). The employed analytical conditions were as reported in the EU Ph monograph.

**DTT reduction of the peptide mixture**. About 100 μg of peptide mixture were treated with 20 mg mL$^{-1}$ of DTT in TRIS-HCl 1 M pH 8.3 under a nitrogen atmosphere for 1 h at 37 °C. After incubation the peptide mixture was analysed by LC-MS.

**Collection of peaks for characterisation purposes**. The peaks of interest were collected employing the HPLC peptide mapping method (EU Ph). Each peak was dried in a speed vacuum system (Savant mod.PD111 V) and then stored at −80 °C prior to further analyses.

**N-terminal sequence analysis of collected peaks**. Sequence analysis was performed on the Procise 494 microsequencing system (Applied Biosystems). The method mainly consists in testing samples by the Edman analysis using commercial equipment based on a pulsed liquid technique. This technique allows sequencing of samples on a glass fibre filter using a delivery of TFA as a volume controlled pulse.

**ESI-MS analysis of collected peaks (Micro Quattro Mass Spectrometer)**. The ESI-MS-MS analysis was performed on a Micro Quattro mass spectrometer (Waters, Manchester, UK) by direct injection of the samples diluted with mobile phase of 50% $H_2O$-$CH_3CN$ containing 1% of HCOOH. The capillary and cone voltages were set to 3500 V and 40 V respectively. The acquisitions were performed on the $(M+2H)2+$ molecular ion of each peak with Argon as collision gas and employing a collision energy at 27 eV. The MS-MS data were collected between 80 *m/z* and 2000 *m/z* with a scan time of

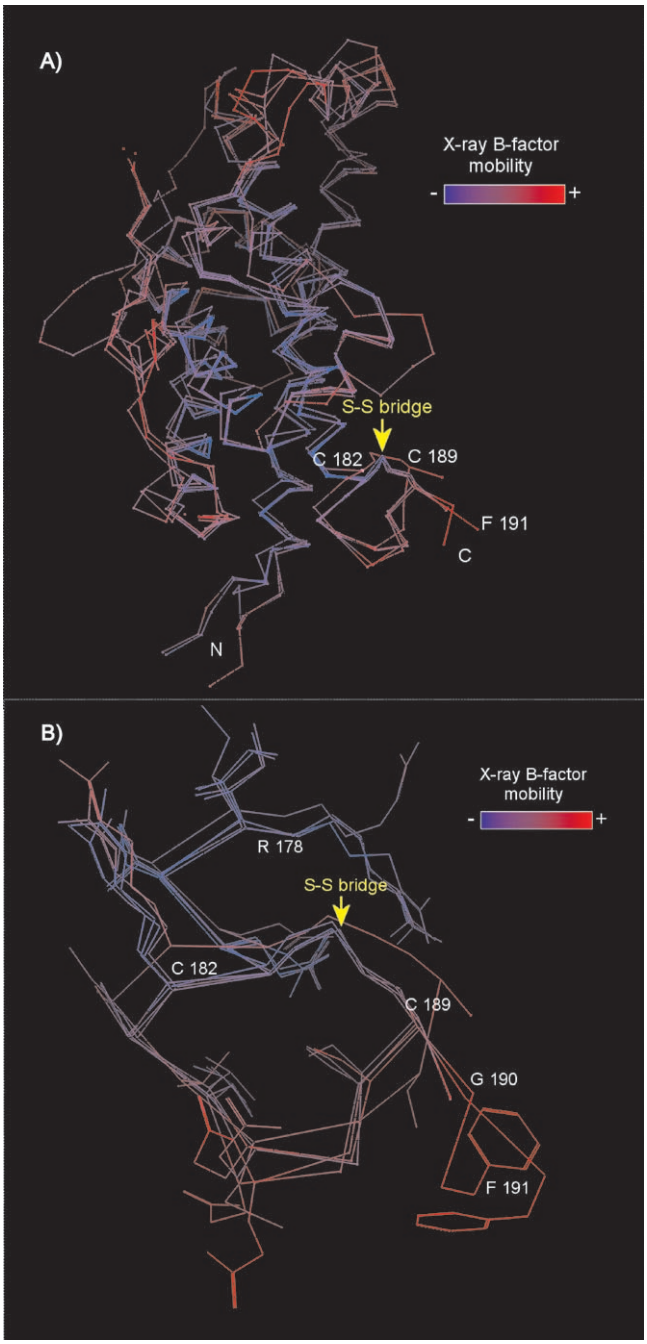


**Figure 9.** $C_\alpha$ trace representation of five superimposed X-ray structures of hGH (PDB 1a22, 1axi, 1hwg, 3hhr, 1hgu). Panel A: the structures are coloured according to the crystallographic B-factor. Atoms coloured red and blue have a high and low mobility, respectively. The hGH C terminus is one of the most mobile regions of the protein. Panel B: C-terminal regions of hGH showing residue side-chain conformations. Phe191, the last residue, cannot be seen in the electronic density of three of five structures because of its high mobility. Protein structure superimposition and molecular modelling were performed with the program MOE.[14]

1 s and an interscan time of 0.10 s. The mass spectrometer MS1 and MS2 were calibrated with a PEG mixture in the *m/z* range 80 to 2000.

**NanoESI-MS of collected peaks (Q-TOF Mass Spectrometer)**. The MS and MS/MS mass measurements were performed with a Q-TOF

  

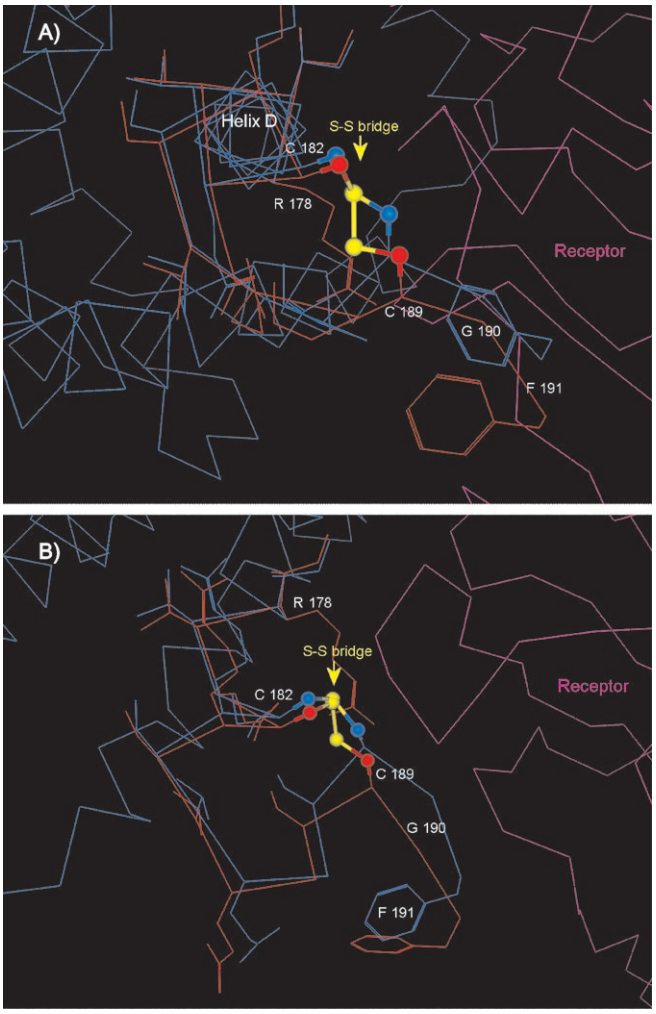


**Figure 10.** 90-degree views of the C-terminal regions of the wild type (red) and modified (blue) hGH. Atoms that belong to the disulfide or thioether bridge are displayed with balls and sticks, with sulfur atoms coloured yellow. The wild type corresponds to X-ray structure 1a22 with a normal C182–C189 disulfide bridge, whereas the modified version is a model with only one sulfur atom in the bridge. The hGH receptor is shown in magenta. Protein structure superimposition and molecular modelling were performed with the program MOE.[14]

2 hybrid quadrupole/time-of-flight mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray ion source. The instrument consists of a nanoelectrospray ionisation source, a quadrupole mass filter operating as a variable bandpass device, a hexapole collision cell, and an orthogonal acceleration time-of-flight (TOF) mass analyzer. The TOF mass analyzer is used to acquire data both in MS and MS/MS modes. The off-line nanospray was performed using a borosilicate spray capillary (Protana, Odensee, Denmark). The capillary was washed two times with 50% acetonitrile before loading 5 μL of sample into the capillary. The nanospray capillary voltage was set to 1500 volts with a cone voltage at 40 volts and the source temperature at 90 °C. The precursor ion was isolated in a mass window of 4 Da. The collision voltage was increased from 10 to 40 volts to induce fragmentation. Deconvolution of the MS/MS spectra was performed by MaxEnt 3. The interpretation of the spectra was done manually.

**MALDI-TOF-MS analysis of collected peaks**. This analysis was performed with a Voyager De Pro (Applied Biosystem) on the mixture 1:1 of sample and matrix (a-Cyano-4-Hydroxycinnamic acid). The scan was run in reflector mode with a laser intensity of 2400 with a laser rep rate of 3 Hz. The mass spectrometer was calibrated with a dedicated mixture provided by the manufacturer in the *m/z* range 80 to 6000.

**Tertiary structure analysis by Circular Dichroism**. The CD spectra were collected on a Jasco-810 Spectropolarimeter at room temperature. Measurement parameters for the collection of near UV spectra were as follows: wavelength range 340–250 nm, data pitch 0.2 nm, bandwidth 1 nm, response 4 seconds, scanning speed 10 nm min$^{-1}$, three accumulations, path length 10 mm. All spectra were corrected using a blank of water containing the corresponding amount of excipients (if known). Using water, the samples were diluted to approximately 1.2 mg mL$^{-1}$ for the near-UV measurements and for the far-UV measurements to approximately 0.18 mg mL$^{-1}$. Protein concentrations were verified by measuring UV absorption at 280 nm and concentrations were calculated using an extinction coefficient of 0.72 for a 1 mg mL$^{-1}$ solution. The results of all the CD measurements are expressed as mean residue ellipticity ($[\theta]_\lambda$) in deg cm$^{-2}$ dmol$^{-1}$ at a given wavelength $\lambda$ (nm) using the relation $[\theta]_\lambda = \theta_\lambda M_0/10cl$, where $\theta_\lambda$ is the observed ellipticity in millidegrees at wavelength $\lambda$, $M_0$ is the mean residue weight of the protein, $c$ is the protein concentration (mg mL$^{-1}$), and $l$ is the path length (cm). It should be noted that each observed $\theta_\lambda$ of the protein was corrected for the contribution of the solvent.

## Acknowledgements

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**Keywords:** bioactivity · growth hormone · immunogenicity · post-translational modification · protein structure · thioether

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