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C-Terminally PEGylated hGH-derivatives

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Abstract—A two-step strategy was used for the preparation of C-terminally PEGylated hGH-derivatives. In a first step a CPY-catalyzed transpeptidation was performed on hGH-Leu-Ala, introducing reaction handles, which were used in the second step for the ligation of PEG-moieties. Both oxime-ligation and copper(I) catalyzed [2+3]-cycloaddition reactions were used for the attachment of PEG-moieties. The biological data show a dependency of the potency of the hGH-derivatives on both size as well as shape of the PEG-group.
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1. Introduction

The use of human growth hormone (hGH) as a therapeutic protein is well established.[1] Daily injections are required for most patients, making the treatment quite inconvenient, especially when keeping in mind, that a lot of patients are children. Therefore, projects have been started to identify long-acting hGH-drugs which have to be injected much less frequently. One way to achieve this goal could be the use of a hGH-formulation from which the active protein is released slowly after subcutaneous injection.[2] Another way to approach this problem is to attach a polyethylene glycol (PEG) moiety covalently to hGH,[3] in order to increase its plasma half-life. The plasma-concentration of the hGH-PEG-derivative would thus be easily predicable and controllable over the entire period. The use of PEG for the modulation of pharmacokinetic parameters has been demonstrated for a number of proteins.[4,5] A drawback of PEGylation is, however, that the activity of the protein on its receptor may be altered,[6,7] because of the structural changes or at least changes of the surface to which the PEG-group is attached covalently. At the outset of the project, we speculated, whether it should be possible to identify PEGylated hGH-derivatives with the desired pharmacokinetic properties, yet with only negligible loss of activity on the receptor. The basic idea was to find areas of hGH, where a PEG-moiety could be attached site-specifically, without disturbance of its biological activity. Herein, the site-specific PEGylation of hGH analogues at their C-termini is being explored. The activity of these products has been studied on the hGH-receptor.

2. Results and Discussion

We followed a two-step strategy to attach a PEG-group at the C-terminus of hGH: during the first step a reaction handle was incorporated by a carboxypeptidase Y (CPY) catalyzed reaction, in the second step this reaction handle was used for the ligation of the PEG moiety. We assumed that a PEG derived molecule could not be used directly in an enzyme-mediated attachment reaction, because its high molecular weight would prevent its use in excess and at high molar concentrations.

CPY—EC 3.4.16.5[8,9]—is a serine protease, which usually removes the C-terminal amino acids from a protein,

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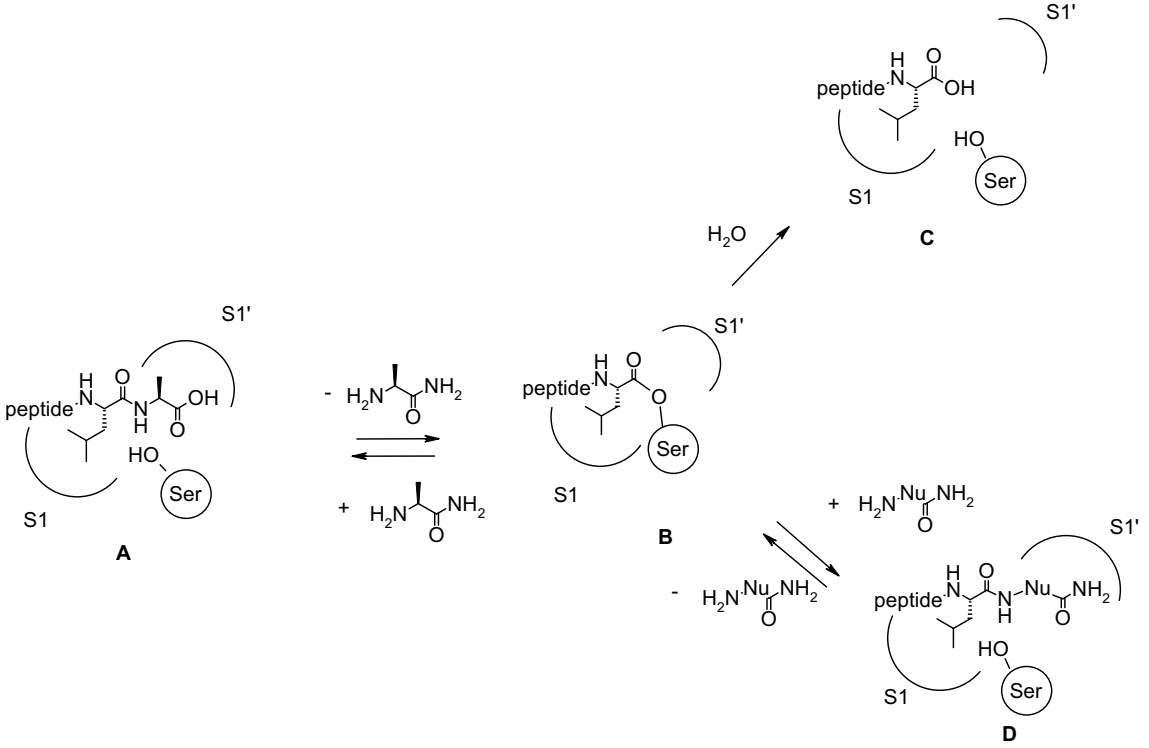
but which can also be used for the introduction of groups to the C-terminus of peptides. A simplified mechanism—shown in Scheme 1—may illustrate both the desired coupling reaction pathway and the formation of the possible by-products. Common for all serine proteases is the reaction of a peptide **A** to a serine–ester intermediate **B**. In the natural pathway this intermediate is hydrolyzed by water to form the hydrolyzed product **C**. In the presence of a suitable nucleophile, intermediate ester **B** may instead be cleaved by the nucleophile to form a product **D**, in which the nucleophile has formally replaced the C-terminal amino acid of the starting protein. Therefore, this reaction path has been called a transpeptidation reaction.[10–15] When performing these reactions in water, the solvent will always compete with the nucleophile, and the hydrolyzed product **C** will therefore always be considered as a possible byproduct. Another issue for the outcome of the transpeptidation reaction is the fact that the transpeptidated peptide **D** itself may be a substrate for the carboxypeptidase. CPY may cleave peptide **D** to the serine–ester **B**, which either can react back to peptide **D** or be hydrolyzed to product **C**. The hydrolyzed product **C** will not react back to the serine–ester **B**. This means that the enzyme reaction has to be kinetically controlled, otherwise the hydrolyzed product will be the main product. The practicability of the reaction would be enhanced significantly, if a nucleophile was found which reacts readily with the intermediate serine–ester **D**, but leads to a product, which is not a good substrate for the enzyme, or even not a substrate at all. In this case, the time-dependency on the yield of the product could be minimized.

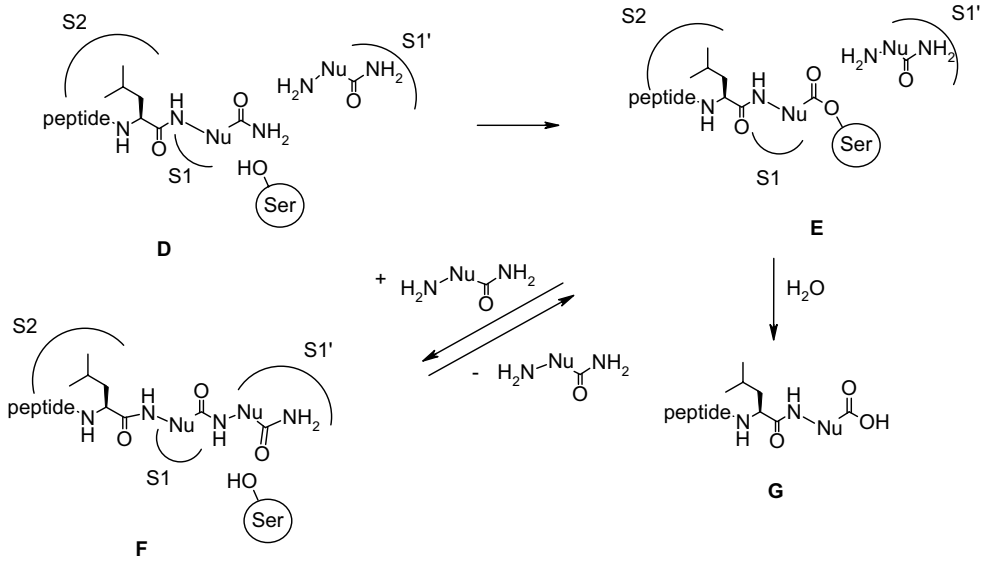
Besides its carboxypeptidase-activity, CPY also has some amidase-activity,[11] in which primary amides are hydrolyzed to the corresponding carboxylic acids via serine–esters. Thus cleavage of the transpeptidated product **D** by an amidase can give rise to the serine–ester **E** (Scheme 2) which then can be cleaved by either hydrolysis or by another nucleophile. The cleavage with a nucleophile leads to product **F** where two nucleophilic groups have been attached to the C-terminus of peptide **C**. We never observed the hydrolysis of a serine–ester, which was formed by the amidase activity of CPY.

In the first step of our PEGylation strategy for hGH, we wanted to utilize the transpeptidation reaction to introduce a reaction handle. The reaction handle could later be used in the second step, the ligation of the PEG-group. Therefore, we needed a nucleophile with a suitable functional group. The literature describes uncharged amino acid amides as possible nucleophiles for a CPY-catalyzed reaction.[10,15] For example ε-acylated lysine based nucleophiles had been used previously.[10] Following this example, we decided to prepare lysine-derivatives carrying suitable reaction handles at the ε-amino group. Two functional groups were chosen to be incorporated to the C-terminus of hGH as reaction handles: a ketone, which could be used in an oxime-forming ligation reaction, and an azide, to be used in an 1,2,3-triazole-forming ligation reaction.

The syntheses of the amino amides **11** and **12** proved to be difficult due to the polarity and accompanying water solubility of the final products and almost all intermediates containing a lysine amide fragment. Therefore, the synthesis had to be designed in a way, that no purification steps were performed on these compounds (Scheme 3). First, the active esters **4** and **5** were prepared from either the commercially available acid **2** or from acid



Scheme 1.

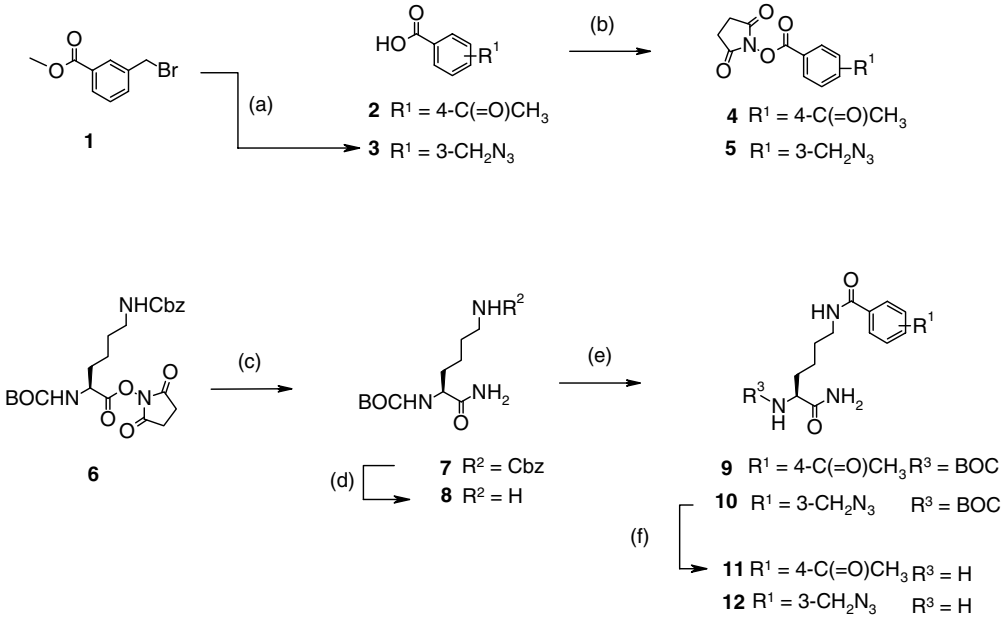


Scheme 2.

3, which could be obtained from ester **1** in two simple steps: exchange of the bromine with sodium azide and subsequent saponification of the ester.

Next, the BOC-protected derivative **8** was prepared straightforwardly in two steps from commercially available *N*-hydroxysuccinimide ester (NHS ester **6**) via amidation and subsequent hydrogenation of the obtained amide **7** in the presence of palladium on charcoal to remove the Cbz-protective group. The free amine was reacted with NHS esters **4** and **5** giving rise to amides **9** and **10**, respectively. Removal of the BOC group by trifluoroacetic acid (TFA) furnished amino amide **11** from amide **9**, whereas these conditions led to considerable degradation of the product, when applied to amide **10**. The amino amide **12** could be obtained in an acceptable purity by treatment of amide **10** with gaseous hydrogen chloride. Finally, amino amides **11** and **12** were purified by preparative HPLC.

As described in the literature, high yields of transpeptidated material can be obtained, when alanine is used as leaving amino acid at the C-terminus of a peptide. Therefore, the first model peptide for the studies on the CPY-catalyzed transpeptidation reaction had an alanine at its C-terminus. We thus used peptide repre-



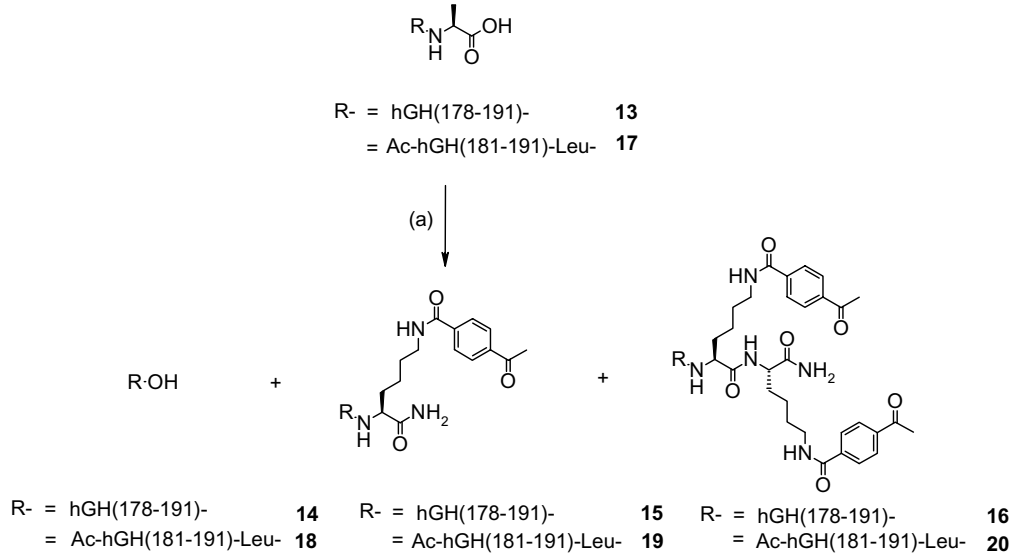
Scheme 3. Reagents: (a) i—NaN_3, DMF, ii—LiOH, H_2O/1,4-dioxane; (b) TSTU, NEt_3; (c) NH_3, H_2O, THF; (d) H_2,Pd/C; (e) **4** or **5**, respectively, DIPEA; (f) TFA, CH_2Cl_2 for **9**, gaseous HCl, EtOAc for **10**.

senting the C-terminal sequence of hGH extended with an alanine, hGH(178-191)ylalanine (**13**) as a first model-peptide (Scheme 4).

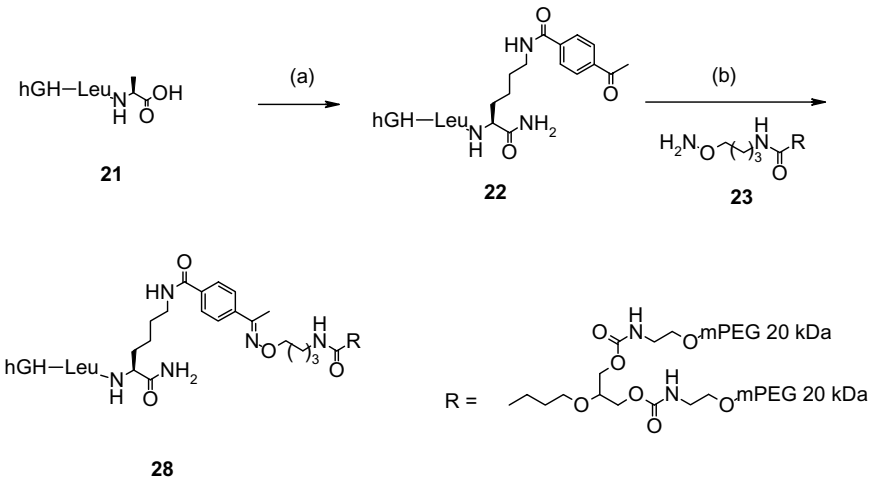
The reaction with the test-peptide was run in a HEPES-buffer (2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid buffer), containing EDTA (ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid), adjusted to pH 7.8. Concentrations of 1 mM for peptide **13**, a 200 mM for amino amide **11**, and a 640 U/ml for CPY were used. Analyses were run directly on the reaction mixture. The desired product **15** was formed with a HPLC-purity of 48% (Scheme 4). The reaction was rather slow and did not proceed cleanly. Besides the starting peptide **13** (16%), hydrolyzed product **14** was found in an amount of 30%. Even more problematic was the formation of significant amounts of the di-transpeptidated peptide **16**, in which two molecules of the nucleophile had been incorporated (6%) to the C-terminus of peptide **13**. Thus two carbonyl groups were present in this product. A loss of the full control over the site-specificity In the second step of our approach, the ligation reaction would be the effect of a presence of two carbonyl groups. This would be very serious in the full-length hGH due to expected problems with the separation of such di-transpeptidated material from mono-transpeptidated material. We decided to optimize the transpeptidation reaction with model-peptides until no di-transpeptidated material was found.

The reaction pathway to the undesired di-transpeptidated compound **16** is best explained by the amidase activity of CPY as described in Scheme 2. We hypothesized that a disulfide-bridge may have been responsible for the poor outcome of the first experiments. This disulfide bridge was present in our model peptide **13**, resembling the disulfide bridge in hGH between Cys182 and Cys189. The close proximity of the disulfide-bridge to the C-terminus may have imposed rigidity to the peptide during the enzyme-reaction and therefore hampered a proper positioning of substrate to the enzyme. Because the effect of the disulfide bridge should decrease with distance from the reaction site, the amidase reaction of the primary product **15** might be relatively favored compared to a transpeptidation reaction of starting peptide **13**. We thus speculated that an extension of the C-terminus by one additional amino acid might facilitate the transpeptidation reaction relative to the unwanted amidase derived reaction. Because it was well known that non-polar amino acids are beneficial for reactions catalyzed by CPY,[16] a leucine-residue was introduced at position P1. Keeping the concentration of the new model peptide **17** constant compared to our earlier experiments, we were now able to reduce not only the concentration of the nucleophile **11** to 100 mM but also to reduce the concentration of the enzyme more than ten fold to 40 U/ml. After almost complete consumption of the starting peptide in a reaction at pH 8, the product mixture comprised the desired product **19** in 85%, next to 10% hydrolyzed product **18** and 4% dihydrolyzed product (Scheme 4). More importantly no di-transpeptidated compound **20** was detected by HPLC or MS.

On the basis of these positive results, we assumed that hGH-Leu-Ala (**21**) would be a suitable substrate for the CPY-reaction. Protein **21** was produced in *Escherichia coli*. Protein **21** was reacted at a concentration of 0.5 mM in the presence of nucleophile **11** in a concentration of 175 mM and CPY (10 U/ml) at a pH of 8.2 in a HEPES buffer, containing EDTA (Scheme 5). Since hydrolysis had to be assumed to be the thermodynamically preferred reaction-pathway, the reaction was monitored by capillary electrophoresis (CE) and MALDI-TOF. During the transpeptidation the C-terminus was being changed to an amide, leading the net-loss of one negative charge. It was possible to separate these hGH-derivatives with one charge difference well in a CE-electrophoresis, making this technology the tool of choice for the quick quantification of formed product. It was not expected that an unwanted di-transpeptidated



Scheme 4. Reagents: (a) CPY, **11**.

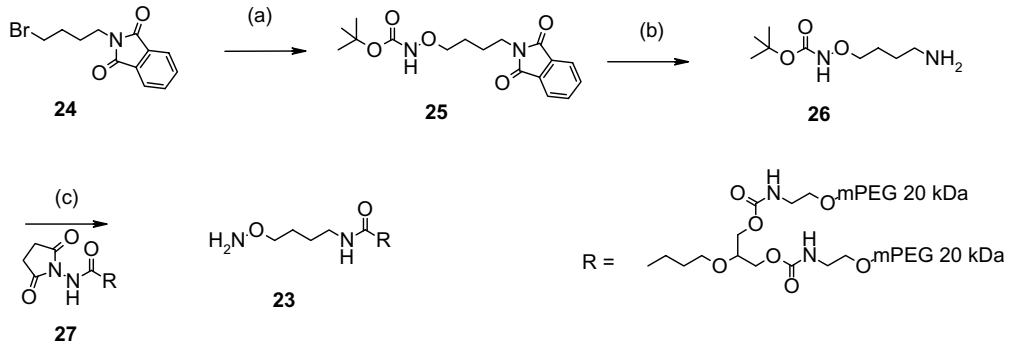


Scheme 5. Reagents and conditions: (a) CPY, **11**; (b) pH 6, 30 °C, 10 days.

compound could have been identified in a CE-electrophoresis because it would have the same net-charge as the desired product **22**. We were, however, never able to detect a mass corresponding to a di-transpeptidated product in the MALDI-TOF spectrum. When a yield of 80% of the desired product **22** was formed, as judged by CE-electrophoresis, the reaction was stopped by addition of the known irreversible serine-protease inhibitor phenylmethylsulfonyl fluoride (PMSF).[17,18] The excess nucleophile **11** and PMSF were removed from product **22** by gel-chromatography utilizing a Sephadex G-25 gel 2-(4-morpholino)ethanesulfonic acid (MES) buffer.

Unexpectedly, the amount of hydrolyzed product hGH-Leu-OH slowly increased, while the amount of desired product **22** decreased in batches, which had been subjected to this kind of gel-chromatography. Because the reaction had been stopped completely according to CE before the gel-chromatography it was evident that CPY did not show activity prior to but low activity after the chromatography. This rather peculiar observation was explained by a consideration, that nucleophile **11** was a competitive ligand in the active site for PMSF, which therefore was not as effective as expected and had not fully inhibited the enzyme. Thus the observed inhibition of the enzyme in the reaction mixture had been a combination of irreversible inhibition by PMSF and reversible inhibition by the nucleophile **11**. During the gel-chromatography the reversible inhibitor, nucleophile **11**, was removed, leaving a part of the CPY uninhibited, which caused the problems with degradation of the keto-protein **22**. Therefore, we added PMSF immediately to those fractions of the gel-chromatography containing the desired keto-protein **22**. This PMSF was subsequently destroyed by raising the pH to pH 8 for 3 h, after which the pH was adjusted back to pH 6.

Having introduced a keto-group into the C-terminus of hGH, we now wanted to use this group in a second step to attach a PEG-moiety an oxime-forming reaction. *N*,*N*-Dimethylformamide was added to the solution in order to ensure solubility of the protein during the reaction. The large branched mPEG 40 kDa moiety[19] was chosen as a suitable group because it was known in the literature that a high molecular weight correlates with low renal clearance.[3,20–22] The corresponding alkoxylamino PEG-reagent **23** was accessible as described in Scheme 6. Our synthetic strategy to PEG-reagent **23** had to take into account that phthaloyl-groups can migrate from alkoxylamine-groups to amino-groups.[23] Therefore, we protected the alkoxylamine with a BOC-group during synthesis. Commercially available bromide **24** was reacted with

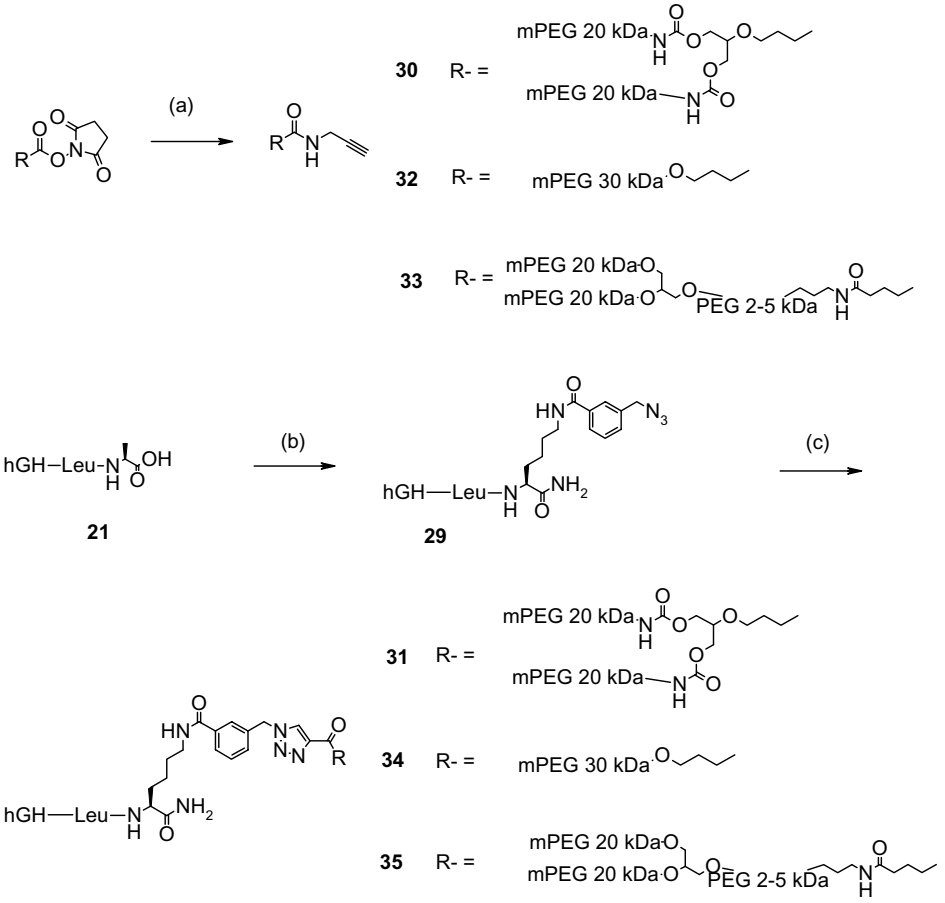


Scheme 6. Reagents: (a) *t*-BuOC(=O)NHOH, DBU; (b) N_2H_4; (c) i—DIPEA; ii—TFA/CH_2Cl_2.

BOC-protected hydroxylamine in the presence of DBU as base to yield alkoxylamine **25**. Liberation of the amine moiety by treatment with hydrazine resulted in amine **26**, which first was reacted with commercially available PEG-containing NHS ester **27** and subsequently treated with trifluoroacetic acid for removal of the BOC protective group. The resulting alkoxylamino PEG-reagent **23** was added to the hGH-analogue **22** at pH 6. The reaction proceeded very slowly; it had to be run for 10 days at 30 °C, to yield substantial amounts of the desired C-terminally PEGylated hGH-analogue **28**. This was isolated by gel-chromatography on a Superdex G-200 gel followed by an ion-exchange chromatography (Scheme 5).

Having shown the feasibility of this two-step approach, the second step had to optimized, because the low yield after 10 days reaction time was unacceptable. As an alternative for the oxime-forming reaction a copper(I) catalyzed [2+3]-cycloaddition reaction of an azide and an alkyne was considered.[24–28] Initial attempts to incorporate an alkyne to the C-terminus of a hGH-derivative led consistently to easily precipitating compounds. On the other hand, when the azide **12** was used as nucleophile in the CPY-catalyzed reaction of hGH-Leu-Ala (**21**), compound **29** could be prepared as precursor for a copper(I) catalyzed [2+3]-cycloaddition reaction without problems with precipitation under conditions very similar to those described for nucleophile **11**.

Another feature of amino amide **12** in the CPY catalyzed transpeptidation reaction turned out to be very attractive: it was observed that the reaction proceeded very slowly, compared to those reactions with other nucleophiles. Apparently, the enzyme was inhibited by nucleophile **12**, because the hydrolysis reaction leading to hGH-Leu-OH was equally slow. More interestingly, the product **29** was not a good substrate for the inhibited enzyme: no significant hydrolysis of protein **29** to hGH-Leu-OH was observed when running the reaction for 16 h. Therefore, close monitoring of the reaction was not needed anymore. Consequently, CPY-catalyzed transpeptidation reactions with amino amide **12** as nucleophile were routinely run overnight, followed by the addition of the irreversible enzyme inhibitor PMSF. However, after removal of amino amide **12** by gel-chromatography on a Sephadex G-25 gel, slow hydrolysis of product **29** was again observed. The inhibition of CPY by the nucleophile **12** reduces the activity of the enzyme enough to prevent hydrolysis of product **29**. Upon removal of amino amide **12** during the gel-chromatography, the activity of the CPY is enhanced, leading to the observed hydrolysis after gel chromatography. The hydrolysis of product **29** was again easily avoided by immediate addition of PMSF to the product-containing fractions of the gel-chromatography. This PMSF was removed by a second gel-chromatography on a Sephadex G-25 gel, giving rise to a stable solution of product **29**, in which



Scheme 7. Reagents: (a) Propargylamine, DIPEA; (b) CPY, **12**; (c) **30**, **32**, or **33**, $CuSO_4 \cdot 7\ H_2O$, ascorbic acid, 2% 2,6-lutidine.

hydrolysis did not occur any longer. The compound was characterized by MALDI-MS.

A copper(I)-catalyzed [2+3]-cycloaddition reaction between the protein-azide **29** and alkyne-containing branched PEG-reagent **30** with an average molecular weight of 40 kDa was used as ligation reaction. The PEG reagent **30** had been prepared straightforwardly from the corresponding commercially available *N*-hydroxysuccinimide ester (NHS ester) by reaction with propargylamine[29] (Scheme 7). Unlike the oxime-forming reaction, we were able to perform it in a aqueous buffer of 2,6-lutidine, far away from the isoelectric point of hGH, and thereby reducing the risk of unwanted precipitation of the protein. In fact, we never encountered problems with precipitation under these conditions. The PEGylated hGH-derivative **31** was isolated by gel-chromatography on a Superdex G-200 gel followed by ion exchange chromatography on a MonoQ column, which effectively separated the PEGylated and the unPEGylated hGH-derivatives.

In order to study the influence of the structure of the PEG-moiety on the activity of C-terminally PEGylated hGH-derivatives, other types of PEG-reagents were used in the copper(I) catalyzed [2+3]-cycloaddition reaction with azide-protein **29** (Scheme 7). The linear PEG-reagent **32** with an average molecular weight of 30 kDa and the PEG-reagent **33**, which has a molecular weight of approximately 40 kDa, but which is branched at position further away from the attachment point to the protein, were used analogously giving rise to hGH-derivatives **34** and **35**, respectively.

The hGH-analogues **28**, **31**, **34**, and **35** (Fig. 1) were tested in a functional BAF-hGH-receptor assay. Their in vitro potencies were reduced compared to wild-type hGH, which was run in the same experiment. The ratios of the EC_{50}, found for the test compounds, to that of hGH are depicted in Table 1.

The data show clearly a remarkable drop in activity, when hGH is C-terminally PEGylated with a branched PEG, which has a molecular weight of around 40 kDa and where the point of branching is close to the protein. Both proteins **28** and **31** are at least 100-fold less active

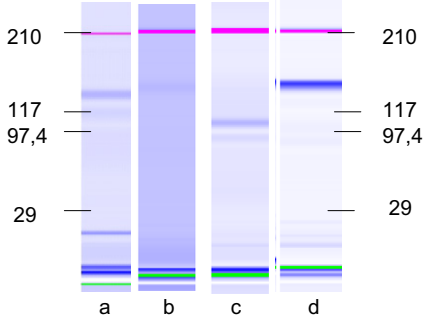


Figure 1. Results of Bioanalyzer, apparent MW by marker Mark 12™ (Invitrogen): (a) Reaction mixture of protein **28** after 7 days; (b) purified protein **31**; (c) purified protein **34**; (d) purified protein **35**.

Table 1. Screening results of PEGylated hGH-derivates

Compound	Ratio EC_{50} test compound/EC_{50} hGH	SD[a]	*N*[b]
28	200	20	4
31	100	10	4
34	30	4	3
35	50	7	3

[a] Standard deviation.
[b] Number of experiments.

than wild-type hGH. Interestingly, compound **34**, in which a linear PEG with an average molecular weight of 30 kDa was attached to the protein, was much more active. A hint whether this is merely due to the size of the PEG-moiety or predominantly due to its shape may be deduced from the results obtained with compound **35**. Its PEG-moiety is linear close to the protein but has a branching point further away from the protein. Even though the PEG-moiety in hGH-derivative **35** has the same size as those of compounds **28** and **31**, it is at least double as active as these analogues. Clearly, the shape of the PEG-reagent has a great influence on the activity of a C-terminally PEGylated hGH-derivative.

3. Summary

As we have shown in this paper, a CPY-catalyzed transpeptidation reaction can be performed on a hGH-analogue which is elongated with Leu-Ala at its C-terminus, changing the C-terminal amino acid to an amino acid amide. Using this strategy, reaction handles such as keto-groups or azides were introduced at the C-terminus of hGH. PEG-moieties were attached to these handles giving rise to PEGylated hGH-derivatives. Their in vitro biological activities seem to be governed by a combination of both the size and the shape of the PEG-group attached: All herein described hGH-derivatives have PEG-moieties attached with an average molecular weight of 30–40 kDa. Their in vitro potency was found to be between 30 and 180 times reduced compared to native human growth hormone. The influence of the size, shape, and the position of a PEG-moiety on the pharmacokinetic properties of hGH-derivatives has to be examined in due course.

4. Experimental

4.1. CE analysis method

The capillary electrophoresis was performed using a Hewlett Packard 3D CE system equipped with a diode array detector.

The fused silica capillary (Agilent) used had a total length of 64.5 cm, an effective length of 56 cm, an inner diameter of 50 μm. Samples were injected by pressure at 50 mbar for 4 s. Separations were carried out at 30 °C, under a tension of +25 kV, using phosphate buffer 50 mM, pH 7, as electrolyte. The analysis was moni-

tored at 200 nm. Between runs, an acidic and a basic washes were performed: the capillary was rinsed with water for 2 min, then with phosphoric acid 0.1 M for 2 min, then with water for 2 min; this was followed by rinsing with sodium hydroxide (0.1 M) for 3 min, and water for 2 min, before equilibrating the capillary with the electrolyte.

4.2. MALDI-TOF

MALDI-TOF spectra were obtained by using α-cyano-4-hydroxy-cinnamic acid (CHCA) as matrix on a Bruker autoflex MALDI-TOF spectrometer.

4.3. HPLC-methods

4.3.1. Method A. The reversed phase-analysis was performed using Waters 2690 systems fitted with a Waters 996 diode array detector. UV detections were collected at 214, 254, 276, and 301 nm on a 218TP54 4.6 × 250 mm 5μ C-18 silica column (The Separations Group, Hesperia), which was eluted at 1 ml/min at 42 °C. The column was equilibrated with 10% of a 0,5 M ammonium sulfate, which was adjusted to pH 2.5 with 4 M sulfuric acid. After injection, the sample was eluted by a gradient of 0–60% acetonitrile in the same aqueous buffer during 50 min.

4.3.2. Method B. The reversed phase-analysis was performed using Waters 2690 systems fitted with a Waters 996 diode array detector. UV detections were collected at 214, 254, 276, and 301 nm on a 218TP54 4.6 × 250 mm 5μ C-18 silica column (The Separations Group, Hesperia), which was eluted at 1 ml/min at 42 °C. The column was equilibrated with 5% acetonitrile, which was buffered with 0.1% trifluoroacetic acid, in a 0.1% aqueous solution of trifluoroacetic acid in water. After injection, the sample was eluted by a gradient of 0–90% acetonitrile, which was buffered with 0.1% trifluoroacetic acid, in a 0.1% aqueous solution of trifluoroacetic acid in water during 50 min.

4.3.3. BaF-hGH-receptor assay. The BaF-hGH-R assay is an in vitro proliferation assay. By stably transfecting BaF3 cells with the human growth hormone receptor (by electroporation), the cell line has been modified to be dependent on human growth hormone for growth and survival.

The cells were cultured at 37 °C and 5% CO_2 in growth medium containing RPMI1640+Glutamax (Gibco cat # 61870-010), 10% heat-inactivated FCS (Fetal calf serum, Gibco cat # 10084-077), 100 U/ml Penicillin/Streptomycin (Gibco Cat # 15140-114),1% mM NEAA (nonessential amino acids, Gibco cat # 11140-035), and 10 ng/ml recombinant hGH (from Novo Nordisk A/S). The assay was initiated by starving the cells in starvation medium (culture medium without hGH) for 24 h at 37 °C and 5% CO_2. Then, the cells were washed, resuspended in starvation medium, and seeded in 96-well microtiter plates (Nunclon cat # 167008) with 20.000 cells/well. After adding relevant test compounds, the plates were incubated for 68 h at 37 °C and 5% CO_2. Alamar Blue™ (BioSource cat # Dal 1025) was used to measure the proliferation. Cells were incubated with Alamar Blue™ for 4 h and the fluorescence was measured in a fluorescence plate reader using an excitation filter of 544 nM and an emission filter of 590 nM. The absorbance of the samples was plotted as a function of the concentration of hGH or compound. From these dose–response curves the EC_{50} were calculated.

4.3.4. 3-(Azidomethyl)benzoic acid (3). Sodium azide (5.68 g, 87 mmol) was added to a solution of methyl 3-(bromomethyl)benzoate (5.00 g, 22 mmol) in *N*, *N*-dimethylformamide (50 ml). Tetrabutylammonium iodide (81 mg, 0.22 mmol) was added. The reaction mixture was heated to 60 °C for 16 h. It was cooled to room temperature and poured onto water (200 ml). This mixture was extracted with ethyl acetate (400 ml). The organic layer was washed with water (3× 200 ml) and successively dried over sodium sulfate. The solvent was removed in vacuo to give 4.11 g of crude methyl 3-(azidomethyl)benzoate, which was used without further purification. MS: $m/z = 192$. ^{1}H NMR ($CDCl_3$): δ 3.92 (s, 3H); 4.40 (s, 2H); 7.50 (m, 2H); 8.00 (m, 2H).

A solution of lithium hydroxide (3.81 g, 21.5 mmol) in water (25 ml) was added to a solution of crude methyl 3-(azidomethyl)benzoate (4.11 g, 21.5 mmol) in 1,4-dioxane (25 ml). Water and 1,4-dioxane were added until a clear solution was obtained. The reaction mixture was stirred for 16 h at room temperature. An 1 N aqueous solution of sodium hydroxide (100 ml) was added. The reaction mixture was washed with *tert*-butyl methyl ether (2× 100 ml). The aqueous phase was acidified with a 10% aqueous solution of sodium hydrogensulfate. It was extracted with ethyl acetate (2× 200 ml). The combined ethyl acetate phases were dried over magnesium sulfate. The solvent was removed in vacuo to give 3.68 g of crude acid **3**, which was used without further purification. MS: $m/z = 150$. ^{1}H NMR ($CDCl_3$): δ 4.57 (s, 3H); 7.55 (m, 2H); 8.00 (m, 2H); 13.10 (br, 1H).

4.3.5. 4-Acetylbenzoic acid 2,5-dioxopyrrolidin-1-yl ester (4). 2-Succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU, 18.5 g, 60.9 mmol) was added to a solution of 4-acetylbenzoic acid (**2**, 10.0 g, 60.9 mmol) and triethylamine (8.49 ml, 60.9 mmol) in *N*,*N*-dimethylformamide (50 ml). The reaction mixture was stirred for 2 h at room temperature. It was diluted with ethyl acetate (400 ml) and washed with water (3× 200 ml). The organic layer was dried over sodium sulfate. The solvent was removed in vacuo to give 13.38 g of ester **4**. ^{1}H NMR ($CDCl_3$): δ 2.67 (s, 3H); 2.93 (s, 4H); 8.05 (d, 2H); 8.20 (d, 2H). MS: $m/z = 284$ $(M+Na)^+$.

4.3.6. Pyrrolidin-2,5-dione-1-yl 3-(azidomethyl)benzoic ester (5). TSTU (32.52 g, 107 mmol) was added to a solution of acid **3** (19.01 g, 107 mmol) and triethylamine (14.96 ml, 107 mmol) in *N*,*N*-dimethylformamide (50 ml). The reaction mixture was stirred for 16 h at room temperature. It was diluted with ethyl acetate (250 ml) and washed with water (3× 120 ml). The organic layer was washed with a saturated aqueous solution of

sodium hydrogencarbonate (150 ml) and dried over sodium sulfate. The solvent was removed in vacuo to give 25.22 g of ester **5**. ^{1}H NMR ($CDCl_3$) δ 2.92 (m, 4H); 4,45 (s, 2H); 7.55 (t, 1H); 7.65 (d, 2H); 8.10 (m, 2H).

4.3.7. ((*S*)-5-(*tert*-Butoxycarbonylamino)-5-(carbamoyl)pentyl)carbamic acid benzyl ester (7). Commercially available 2,5-dioxopyrrolidin-1-yl (*S*)-6-((benzyloxycarbonyl)amino)-2-((*tert*-butoxycarbonyl)amino)hexanoate (**6**, 15 g, 31 mmol) was dissolved in dichloromethane (50 ml). A 25% solution of ammonia in water (21 ml) was added. The reaction mixture was stirred vigorously for 16 h at room temperature. The solvent was removed in vacuo to yield 18.77 g of crude amide **7**, which was used in the next step without further purification. ^{1}H NMR (DMSO-d_6): δ 1.2–1.6 (m, 6H); 1.37 (s, 9H); 2.95 (q, 2H); 3.80 (td, 1H); 5.00 (s, 2H); 6.70 (d, 1H); 6.90 (s, 1H); 7.20–7.40 (m, 7H). MS: *m*/*z* = 280.

4.3.8. ((*S*)-5-Amino-1-(carbamoyl)pentyl)carbamic acid *tert*-butyl ester (8). Crude amide **7** (11.92 g, 31.41 mmol) was suspended in methanol (250 ml). Palladium on coal (50% wet, 1.67 g) was added. The reaction mixture was subjected to hydrogenation under pressure for 16 h. It was filtered through a plug of Celite. The solvent was removed in vacuo to give 13.13 g of crude amine **8**, which was used in the next step without further purification. ^{1}H NMR (DMSO-d_6): δ 1.30–1.60 (m, 6H); 1.37 (s, 9H); 2.65 (t, 2H); 3.80 (dt, 1H); 5.70 (br, 2H); 6.80 (d, 1 H); 6.95 (s, 1H); 7.30 (s, 1H).

4.3.9. (*S*)-6-(3-(Aminomethyl)benzoylamino)-2-(*tert*-butoxycarbonylamino)hexanoic amide (10). Crude amine **8** (10.26 g, 41.82 mmol) was dissolved in *N*,*N*-dimethylformamide (150 ml). Ester **5** (11.47 g, 41.822 mmol) and ethyldiisopropylamine (21.48 ml, 125.5 mmol) were added successively. The reaction mixture was stirred for 16 h at room temperature. It was diluted with ethyl acetate (500 ml) and washed first with a 10% aqueous solution of sodium hydrogensulfate (200 ml), water (3× 250 ml), and a saturated aqueous solution of sodium hydrogencarbonate (200 ml). It was dried over sodium sulfate. The solvent was removed in vacuo to give 6.05 g of amide **10**. ^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9H); 1.63 (m, 4H); 1.83 (m, 2H); 3.43 (q, 2H); 4.15 (m, 1H); 4.37 (s, 2H); 5.56 (d, 1H); 6.08 (s, 1H); 6.75 (s, 1H); 7.00 (s, 1H); 7.43 (m, 2H); 7.77 (m, 2H). MS: *m*/*z* = 427 $(M+Na)^+$, 305 $(M-Boc)^+$.

4.3.10. (*S*)-6-(4-(Acetyl)benzoylamino)-2-aminohexanoic amide (11). Ester **4** (8.21 g, 31.4 mmol) was added to a suspension of crude amine **8** (7.71 g, 31.4 mmol) in *N*,*N*-dimethylformamide (200 ml). Ethyldiisopropylamine (16.14 ml, 94.3 mmol) was added. The reaction mixture was stirred for 3 days at room temperature. The solvent was removed in vacuo at 70 °C. The residue was dissolved in dichloromethane (50 ml). Trifluoroacetic acid (50 ml) was added. The reaction mixture was stirred for 1 h at room temperature. The solvent was removed in vacuo. The residue was taken up in dichloromethane (200 ml). A 10% aqueous solution of sodium hydrogensulfate (50 ml) was added. The mixture was extracted with water (200 ml). The aqueous phase was concentrated in vacuo to approximately 60 ml. It was divided into three parts. Each part was purified by HPLC-chromatography on a C_{18} reversed phase column, using a gradient of 0–20% of acetonitrile in water, which was buffered with 0.1% trifluoroacetic acid, to give together 8.00 g of amino amide **11**. ^{1}H NMR (DMSO-d_6, TFA-salt): δ 1.35 (m, 2H), 1.55 (m, 2H); 1.75 (m, 2H); 2.62 (s, 3H); 3.30 (q, 2H); 3.70 (m, 1H); 7.55 (s, 1H); 7.80 (s, 1H) 7.95 (d, 2H); 8.00 (d, 2H); 8.00 (br, 3H); 8.65 (t, 1H). MS: *m*/*z* = 292.

4.3.11. (*S*)-2-Amino-6-(3-(azidomethyl)benzoylamino)-hexanoic amide (12). Gaseous hydrogen chloride was bubbled two times for 15 min each through a suspension of amide **10** (6.05 g, 14.96 mmol) in ethyl acetate (75 ml). The solvent was removed in vacuo. The crude product was purified by 9 runs of a HPLC-chromatography on a C18-reversed phase column, using a gradient of 8–28% acetonitrile in water, which was buffered with 0.1% trifluoroacetic acid, to give together 5.03 g of the trifluoroacetic acid salt of amino amide **12**. ^{1}H NMR (DMSO-d_6, TFA-salt): δ 1.36 (m, 2H); 1.55 (m, 2H); 1.75 (m, 2H); 3.26 (q, 2H); 3.70 (m, 1H); 4.53 (s, 2H); 7.52 (m, 3H); 7.84 (m, 3H); 8.06 (br, 3H); 8.54 (t, 1H). MS: *m*/*z* = 305.

4.3.12. (hGH(178-191)yl)Alanine (13). A peptide H-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe-Ala-OH was synthesized on an ACT-peptide-synthesizer, applying a preloaded Fmoc-Ala-Wang-polystyrene resin (0.25 mmol), suitable α-Fmoc-protected amino acids, and standard coupling protocols with the use of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBT) as coupling reagents, and piperidine as reagent for removal of the Fmoc-groups. It was cleaved from the resin in a mixture of trifluoroacetic acid (10 ml), water (0.25 ml), and triisopropylsilane (0.250 ml). The reaction mixture was stirred for 1 h at room temperature. The solvent was collected. It was concentrated under a stream of nitrogen to approx. 5 ml. Ether (40 ml) was added. The precipitation was isolated by centrifugation and washed twice with ether (40 ml). The crude peptide was dissolved in a mixture of water (44 ml), acetonitrile (5 ml), and an aqueous 6 M solution of guanidine hydrochloride (1 ml). The pH was adjusted to pH 8–9 by addition of 2.5% aqueous ammonia. Solid potassium hexacyanoferrate(III) was added, until the color persisted. The material was purified on a reversed phase C18-HPLC-column, using a gradient of 14–34% acetonitrile in water in a buffer of 0.1% trifluoroacetic acid to give 15 mg of peptide **13**. MS: *m*/*z* = 806, 536. HPLC: t_R = 18.76 min (method A) t_R = 20.94 min (method B).

4.3.13. Analytical experiment: reaction of peptide 13 with amino amide 11 under catalysis of CPY. A solution of CPY (64 U in 40 μl) in water was added to a solution of the trifluoroacetate salt of amino amide **11** (8.1 mg, 20,000 nmol) and peptide **13** (0.16 mg, 100 nmol) in a buffer (60 μl) of 250 mM HEPES and 5 mM EDTA, which had been adjusted to pH 7.8 by addition of a 1 N aqueous solution of sodium hydroxide. After 2 h,

peptides **14**, **15**, and **16** were identified by HPLC and LC–MS.

4.3.14. [($N^{181\alpha}$-AcetylhGH(181-191)yl)leucyl]alanine (17). A peptide Ac-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe-Leu-Ala-OH was synthesized on an ACT-peptide-synthesizer, applying a preloaded Fmoc -Ala-Wang-polystyrene resin (0.25 mmol), suitable α-Fmoc-protected amino acids, and standard coupling protocols with the use of HBTU and HOBT as coupling reagents, and piperidine as reagent for removal of the Fmoc-groups. It was cleaved from the resin in a mixture of trifluoroacetic acid (10 ml), water (0.25 ml), and triisopropylsilane (0.250 ml). The mixture was stirred for 1 h at room temperature. The solvent was collected. It was concentrated under a stream of nitrogen to approx. 5 ml. Ether (40 ml) was added. The precipitation was isolated by centrifugation and washed twice with ether (40 ml). The precipitation was air-dried and dissolved in a mixture of water (10 ml), 25% aqueous ammonia (approx. 30 μl), and acetonitrile (1.5 ml). Solid potassium hexacyanoferrate(III) was added until the yellow color persisted. The mixture was stirred for 1 h at room temperature and subsequently diluted with water to a total volume of 20 ml. The mixture was subjected to chromatography on a reversed phase C_{18}-HPLC-column, using a gradient of 15-35% acetonitrile in water, which both were buffered with 0.1% trifluoroacetic acid, for elution to give 13 mg of peptide **17**. MS: m/z = 1398, 700. HPLC: t_R = 21.8 min (method A), t_R = 23.2 min (method B).

4.3.15. Analytical experiment: reaction of peptide 17 with amino amide 11 under catalysis of CPY. A solution of CPY (4 U) in water (5 μl) was poured to a solution of the trifluoroacetate salt of amino amide **11** (4 mg, 0.01 mmol) and peptide **17** (0.140 mg, 100 nmol) in a buffer consisting of 250 mM HEPES and 5 mM EDTA (95 μl), which had been adjusted to pH 8.02 by addition of an 1 N aqueous solution of sodium hydroxide and ethyldiisopropylamine. After 65 min a mixture was obtained in which the transpeptidated product **19** (85%) and the hydrolyzed product **18** (10%) were the main components according to HPLC. The di-transpeptidated product **20** could not be detected by either HPLC or mass spectroscopy.

4.3.16. hGH-Leu-Ala (21). A cloning strategy based on pNNC13, a pET11a derived vector already containing Zbasic2mt-D4K-hGH[30], has been utilized. Using pNNC13 as template and a PCR primer set flanking the SacII and BamHI restriction sites, a 628 bp amplicon has been generated encoding two additional amino acids (Leucine and Alanine) in the C-terminal end of hGH. This PCR amplicon was then cloned back into pNNC13 using the existing SacII and BamHI sited to generate pNNC13.4 encoding Zbasic2mt-D4K-hGH-Leu-Ala. The integrity of the resulting clones was confirmed by DNA sequencing of the coding region.

Escherichia coli BL21(DE3) was transformed with pET11a-Zbasic2mt-D4K-hGH-Leu-Ala. A single colony was inoculated into 100 ml LB media with 100 μg/ml ampicillin and grown at 37 °C until OD_{600} reached 0.6. The cell culture temperature was reduced to 20 °C and the cells were induced with 1 mM IPTG for 6 h at 20 °C. The cells were harvested by centrifugation at 3000*g* for 15 min.

The cell pellet was re-suspended in cell lysis buffer (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7, 5 mM EDTA, 0.1% Triton X-100), and the cells were disrupted by cell disruption at 30 kpsi (Constant Cell Disruption Systems). The lysate was clarified by centrifugation at 10,000*g* for 35 min and the supernatant was used for purification.

Zbasic2mt-D4K-hGH-Leu-Ala was purified on SP Sepharose FF using a step gradient elution (buffer A: 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7; buffer B: 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7, 1 M NaCl). The protein was subsequently cleaved using Enteropeptidase for the release of hGH-Leu-Ala. After digestion, hGH-Leu-Ala was further purified on a Butyl Sepharose 4FF column to separate the product from the Zbasic2mt-D4K domain and Enteropeptidase (buffer A: 100 mM HEPES, pH 7.5, 2 M NaCl; buffer B: 100 mM HEPES, pH 7.5, a linear gradient was used).

As digestion was not complete, remaining Zbasic2mt-D4K-hGH-Leu-Ala had to be separated from protein **21** and this was done by loading the protein onto the SP Sepharose FF column again. Buffer exchange to 25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7, was performed on a Sephadex G-25 Medium column before purification on the SP Sepharose FF column. Zbasic2mt-D4K-hGH-Leu-Ala bound to SP Sepharose FF, whereas protein **21** was found in the flow through.

The buffer of protein **21** was exchanged to 50 mM NH_4HCO_3, pH 7.8. The solution was lyophilized.

4.3.17. (*S*)-N-((*S*)-5-((hGHleucinyl)amino)-5-carbamoylpentyl)-4-acetyl benzamide (22). A solution of amino amide **11** (127 mg, 314 μmol) in a buffer consisting of 250 mM HEPES and 5 mM EDTA (0.97 ml), which had been adjusted to pH 8.5 by addition of a 1 N aqueous solution of sodium hydroxide, was added to a solution of hGH-Leu-Ala (20 mg, 0.9 μmol) in mixture of water (0.6 ml) and diisopropylamine (6 μl). The pH was adjusted to 8.2 by addition of sodium hydroxide (10 M in water). The reaction volume was adjusted to 1.77 ml by addition of a buffer consisting of 250 mM HEPES and 5 mM EDTA, which had been adjusted to pH 8.5 by addition of a 1 N aqueous solution of sodium hydroxide. The reaction was started by addition of a solution of CPY (Fluka #21943, 18 U) in water (30 μl). The reaction mixture was incubated at 30 °C. The reaction was monitored by capillary electrophoresis. When the yield of transpeptidated product had reached >80%, the reaction was stopped by inactivating the enzyme by addition of a freshly prepared 200 mM solution of PMSF in dry isopropanol (36 μl). A gel filtration on Sephadex G-25, using a buffer consisting of Tris hydrochloride (2-amino-2-(hydroxymethyl)-1,3-propanediol, 50 mM, pH 8.5), was performed. A freshly

prepared 200 mM solution of PMSF in dry isopropanol was added to the fractions containing compound **22**, leading to a concentration of 1 mM of PMSF in the fractions. The solution obtained was used in the next step without further purification. MALDI-TOF: (CHCA) found: *m*/*z* 11156 [**21**, $(M+2H)^{2+}$], required: 11155; found: *m*/*z* 11257 [**22**, $(M+2H)^{2+}$], required: 11257; found: *m*/*z* 11120 [hGH-Leu $(M+2H)^{2+}$], required: 11120.

4.3.18. *N*-(4-Aminoxybutyl)-4-(2-(*N*-(20 kDa mPEGyl)-carbamoyloxy)-1-(*N*-(20 kDa mPEGyl)carbamoyloxymethyl)ethoxy)butanamide (23). Commercially available 2,5-dioxopyrrolidin-1-yl 4-(2-(*N*-(20 kDa mPEGyl)carbamoyloxy)-1-(*N*-(20 kDa mPEGyl)carbamoyloxymethyl)ethoxy)butanoate (**27**, 2.5 g, 0.062 mmol) and ethyldiisopropylamine (0.053 ml, 0.04 mmol) were dissolved in dichloromethane (25 ml). Amine **26** (51 mg, 0.248 mmol) was added. The reaction mixture was stirred for 16 h at room temperature. Ether (200 ml) was added. The mixture was cooled to 0 °C and left for 1 h. The formed precipitation was isolated by filtration and washed with ether (50 ml). It was dissolved in dichloromethane (10 ml). Ether (200 ml) was added. The mixture was cooled to 0 °C. The formed precipitation was isolated by filtration. It was dissolved in dichloromethane. Ion-exchange material Amberlyst 15, which had been washed with dichloromethane (20 ml) and ethanol (20 ml), was added to the solution. The mixture was gently stirred for 20 min. The ion-exchange material was removed by filtration and washed with dichloromethane (5 ml). The dichloromethane-phases were combined. Ether (250 ml) was added. The mixture was cooled to 0 °C. The formed precipitation was isolated by filtration and dried in vacuo. The obtained material was dissolved in dichloromethane (10 ml) and trifluoroacetic acid (10 ml) was added. The reaction mixture was stirred for 30 min at room temperature. Ether (300 ml) was added. The mixture was cooled to 0 °C. The formed precipitation was isolated by filtration, washed with ether (50 ml), and dried in vacuo to give 1.75 g of PEG reagent **23**.

4.3.19. 2-(4-(*tert*-Butoxycarbonylaminoxy)butyl)isoindole-1,3-dione (25). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 15.0 ml, 101 mmol) was added in portions to a stirred mixture of *N*-(4-bromobutyl)phthalimide (**24**, 18.9 g, 67.0 mmol), acetonitrile (14 ml), and *N*-Boc-hydroxylamine (12.7 g, 95.4 mmol). The resulting mixture was stirred at 50 °C for 24 h. Water (300 ml) and 12 M HCl (10 ml) were added, and the product was extracted three times with ethyl acetate. The combined extracts were washed with brine, dried over magnesium sulfate, and concentrated in vacuo. The resulting oil (28 g) was purified by chromatography (140 g SiO_2, gradient elution with heptane/AcOEt) to give 17.9 g of carbamate **25** as an oil. ^{1}H NMR (DMSO-d_6) δ 1.36 (s, 9H), 1.50 (m, 2H), 1.67 (m, 2H), 3.58 (t, J = 7 Hz, 2H), 3.68 (t, J = 7 Hz, 2H), 7.85 (m, 4H), 9.90 (s, 1H).

4.3.20. *N*-(4-Aminobutoxy)carbamic acid *tert*-butyl ester (26). Hydrazine hydrate (20 ml) was added to a solution of **25** (8.35 g, 25.0 mmol) in ethanol (10 ml). The mixture was stirred at 80 °C for 38 h. It was concentrated and the residue coevaporated with ethanol and toluene. Ethanol (50 ml) was added to the residue, and the precipitated phthalhydrazide was filtered off and washed with ethanol (50 ml). Concentration of the combined filtrates yielded 5.08 g of an oil. This oil was mixed with a solution of potassium carbonate (10 g) in water (20 ml), and the product was extracted with dichloromethane. Drying over magnesium sulfate and concentration in vacuo yielded 2.28 g of amine **26** as an oil, which was used without further purification. ^{1}H NMR (DMSO-d_6): δ 1.38 (m, 2H), 1.39 (s, 9H), 1.51 (m, 2H), 2.51 (t, J = 7 Hz, 2H), 3.66 (t, J = 7 Hz, 2H).

4.3.21. (*S*)-2-{(hGHylleucinyl)amino}-6-{4-(1-(4-(4-(2-(*N*-(20 kDa mPEGyl)carbamoyloxy)-1-((*N*-(20 kDa mPEGyl)carbamoyloxy)methyl)ethoxy)butyrylamino)-butoxyimino)ethyl)benzoyl amino}hexanoic amide (28). A solution of the crude protein **22** (18 mg, 800 nmol) in a 50 mM Tris–hydrochloride buffer (6 ml), which had been adjusted to pH 8.5, was cooled to 0 °C. Ice cold DMF (1.32 ml, 15% final concentration) was added. PEG reagent **23** (281 mg, 7025 nmol) was dissolved in a 0.14 M solution of 3-(methylsulfanyl)-1-propanol in water (1.0 ml). This solution was added to the solution of the protein. The volume was adjusted to 8.8 ml by addition of MES buffer 50 mM, pH 6. The final pH of the reaction mixture was 6. The reaction mixture was incubated at 30 °C under nitrogen for 10 days, while the reaction progress was followed by running analyses on Agilent 2100 Bioanalyzer. Subsequently, the product was isolated first by size exclusion chromatography (Superdex G-200 26/26, GE-Healthcare, eluent: Tris–HCl 50 mM pH 8.5, 2.5 ml/min), followed by an ion exchange chromatography (MonoQ 10/100 GL, GE Healthcare, A buffer: Tris 50 mM, pH 8.5, buffer B: Tris 50 mM, 0.2 M NaCl, pH 8.5l, 0–100% B over 100 column volumes with a flow of 0.5 ml/min). 2 mg of the PEGylated protein **28** was isolated and identified by SDS-gel.

4.3.22. (*S*)-2-((hGHylleucinyl)amino)-6-(3-(azidomethyl)-benzoylamino)hexanoic amide (29). hGH-Leu-Ala (60 mg, 2689 nmol) was dissolved in water (1.80 ml) and diisopropylethylamine (0.0020 ml). A solution of amino amide **12** (380 mg, 0.91 mmol) in 0.480 ml of a buffer of 0.25 M HEPES and 5 mM EDTA, which had been adjusted to pH 8 with a 1 N solution of sodium hydroxide, was added. The solution was filtered and diluted with a buffer of 0.25 M HEPES and 5 mM EDTA, which had been adjusted to pH 8 with a 1 N solution of sodium hydroxide, and a 1 N solution of sodium hydroxide in order to obtain 5.08 ml of a solution with a pH of 7.8. A solution of CPY (200 U/ml, 0.300 ml, 60 U) was added. The reaction mixture was gently shaken at 30 °C for 48 h. A freshly prepared 100 mM solution of phenylmethanesulfonyl fluoride (0.054 ml) in isopropanol was added. It was chromatographed on a HiPrep 26/10 desalting column, using a 50 mM ammonium bicarbonate buffer in water as eluent. A freshly prepared 100 mM solution of phenylmethanesulfonyl fluoride (0.0045 ml per 0.5 ml fraction-volume) in isopropanol was added to the frac-

tions immediately, containing the protein. These fractions were combined and were subjected to ultracentrifugation, using an Amicon Ultra-15 vial with a cut off of 10 kDa filter. They were diluted with a 50 mM solution of ammonium bicarbonate and lyophilized to give 52 mg of a mixture of protein **29** and starting material **21**. MALDI-TOF: (CHCA) found: *m/z* 11266 $(M+2H)^{2+}$, required: 11258; found: *m/z* 22523 $(M+H)^{+}$, required: 22515.

4.3.23. 4-(bis(20 kDa mPEGylcarbamoyloxymethyl)-methoxy)-*N*-prop-2-ynylbutyric amide (30). Commercially available 4-(bis(20 kDa mPEGylcarbamoyloxymethyl)methoxy)butyric acid (2 g, 0.05 mmol) was dissolved in dichloromethane (25 ml). Ethyldiisopropylamine (0.042 ml, 0.248 mmol) and propargylamine (0.014 ml, 0.198 mmol) were added successively. The reaction mixture was stirred for 3 days. Diethyl ether was added until a precipitation occurred. The mixture was cooled to 0 °C and was filtered through a P1-glass-filter. The precipitation was collected and dissolved in dichloromethane (18 ml) and ethanol (2 ml). Amberlyst 15 (1.0 g) was washed with ethanol and added. The reaction mixture was stirred gently for 30 min and was filtered. The solvent was removed in vacuo. Diethyl ether (50 ml) was added. The precipitation was isolated by filtration and dried 2 days in vacuo to give 1.36 g of PEG reagent **30**.

4.3.24. (*S*)-2-((hGHylleucinyl)amino)-6-(3-((4-((4-(bis(20 kDa mPEGylcarbamoyloxymethyl)methoxy)butyrylamino)methyl)triazol-1-yl)methyl)benzoylamino)hexanoic amide (31). A solution of copper(II) sulfate pentahydrate (41 mg, 0.16 mmol) in water (9.17 ml) was prepared. A solution of ascorbic acid (145 mg, 0.82 mmol) in water (8.94 ml) and 2,6-lutidine (0.229 ml) was prepared. A part of the solution of ascorbic acid (3.51 ml) was added to a part of the solution of copper(II) sulfate (3.51 ml). This mixture was left at room temperature for 5 min to form a copper(I)-salt solution. A solution of azido protein **29** (7.08 mg, 314 nmol) in a mixture of water (0.862 ml) and 2,6-lutidine (0.18 ml) was added to a solution of PEG-reagent **30** (127 mg, 0.003 mmol) in water (0.700 ml). A part of the copper(I)-salt solution (0.35 ml) was added. The reaction mixture was gently shaken for 20 h. The reaction mixture was filtered and was diluted to 2 ml with a 10 mM buffer of Tris, which had been adjusted with 1 N hydrochloric acid to pH 8.0. It was subjected to a gel chromatography, using a HiLoad 26/60 Superdex G-200 column, GE-Healthcare, in a 10 mM Tris buffer, which had been adjusted to pH 8 with 1 N hydrochloric acid. The fractions containing the desired protein were combined and concentrated by ultracentrifugation using an Amicon Ultra-15 vial with a cut off of 10 kDa. It was diluted with a 50 mM Tris buffer (20 ml), which had been adjusted to pH 8.5 with 1 N hydrochloric acid. It was purified by ion-exchange-chromatography on a MonoQ 10/100 GL column, using a 50 mM Tris buffer, which had been adjusted to pH 8.5 with 1 N hydrochloric acid, as buffer A and a 50 mM Tris/2 M sodium chloride buffer, which had been adjusted to pH 8.5 with 1 N hydrochloric acid, as buffer B. The fractions containing the desired protein were combined and concentrated by ultracentrifugation using an Amicon Ultra-15 vial with a cut off of 10 kDa. The buffer was changed to a 50 mM ammonium bicarbonate buffer by ultracentrifugation using an Amicon Ultra-15 vial with a cut off of 10 kDa and lyophilized to give 0.45 mg of PEGylated protein **31**. The SDS-gel showed a compound, which had the expected properties of the title compound and stained with both silver-staining and PEG-sensitive-staining.[31]

4.3.25. 4-(30 kDa mPEGyl)-*N*-(prop-2-ynyl)butanoic amide (32). Commercially available 2,5-dioxopyrrolidin-1-yl 4-(30 kDa mPEGyl)butanoic ester (2.5 g, 0.083 mmol) was dissolved in dichloromethane (25 ml). Ethyldiisopropylamine (0.071 ml, 0.413 mmol) and propargylamine (0.023 ml, 0.33 mmol) were added successively. The reaction mixture was stirred at room temperature overnight. Diethyl ether was added until a precipitation was formed. The mixture was cooled to 0 °C and the precipitation was isolated by filtration through a glass-filter P1. The isolated material was dissolved in a 10% solution of ethanol in dichloromethane (15 ml). Amberlyst 15 (2.0 g), which had been washed with a 10% solution of ethanol (20 ml) prior its use, was added. The mixture was stirred slowly for 30 min. The Amberlyst-material was removed by filtration and was washed with dichloromethane (20 ml). The solution was concentrated in vacuo. Ether was added, until a precipitation occurred. The mixture was cooled to 0 °C. The precipitation was isolated by filtration through a glass-filter P1 and dried in vacuo to give 2.04 g of PEG-reagent **32**.

4.3.26. 4-(*N*-(3-(*ω*-(2,3-bis(20 kDa mPEGyloxy)Porpoxy)-2-5 kDa PEGyloxy)propyl)carbamoyl)-*N*-(prop-2-ynyl)-butyric amide (33). Commercially available 2,3-bis (20 kDaPEGyloxy)-1-({3-[(1,5-dioxo-5-succinimidyl-oxypentyl) amino]propoxy} 2–5 kDa PEGyloxy)propane (1.00 g, 0.023 mmol) was dissolved in dichloromethane (10 ml). Ethyldiisopropylamine (0.019 ml, 0.113 mmol) and propargylamine (0.006 ml, 0.091 mmol) were added successively. The reaction mixture was stirred for 16 h at room temperature. Diethyl ether was added until a precipitation was obtained. The mixture was kept at 0 °C for 1 h. The precipitation was isolated by filtration through a filter paper P1. Amberlyst 15 ion-exchange material (1.0 g) was suspended in a mixture of dichloromethane (10 ml) and ethanol (1 ml). The mixture was stirred gently for 30 min. The Amberlyst was isolated by filtration. The precipitation of the PEG-reagent was dissolved in a mixture of dichloromethane (10 ml) and ethanol (1 ml). The Amberlyst material was added. The mixture was stirred gently for 30 min at room temperature. The Amberlyst was removed by filtration and washing with dichloromethane. The combined solutions were concentrated in vacuo to approx. 2 ml. Diethyl ether was added, until a precipitation was obtained. The mixture was kept at 0 °C for 1 h. The precipitation was isolated by filtration through a filter paper P1 and dried in vacuo to give 0.83 g of PEG-reagent **33**.

4.3.27. (*S*)-2-(hGHylleucinylamino)-6-(3-((4-((4-(30 kDa mPEGyloxy)butyrylamino)methyl)1,2,3-triazol-1-yl)-methyl)benzoylamino)hexanoic amide (34). A solution of azido protein **29** (11.0 mg, 488 nmol) was dissolved in a mixture of water (1.328 ml) and 2,6-lutidine (0.028 ml). This solution was filtered into a solution of PEG-reagent **32** (147 mg, 4860 nmol) in water (1.00 ml). A copper(I)-salt solution was prepared by addition of a solution of copper(II) sulfate pentahydrate (24.3 mg, 0.097 mmol) in water (5.44 ml) to a solution of ascorbic acid (85.0 mg, 0.488 mmol) in a mixture of water (5.30 ml) and 2,6-lutidine (0.135 ml). This copper(I)-salt solution was left for 5 min at room temperature. A part of this copper(I)-salt solution (0.544 ml) was added to the solution containing the protein. The reaction mixture was gently shaken for 22 h. The solution was filtered. The filter was washed with a buffer consisting of 25 mM Tris in water, which was adjusted to pH 8.5 with 1 N hydrochloric acid. The solution was run on a column, using a HiPrep26/10 desalting column with a flow of 20 ml/min and a buffer consisting of 25 mM Tris in water, which was adjusted to pH 8.5 with 1 N hydrochloric acid. The fractions, containing protein, were collected and combined. The protein was purified by ion-exchange chromatography using a MonoQ 10/100 GL column, a buffer consisting of 25 mM Tris in water, which was adjusted to pH 8.5 with 1 N hydrochloric acid, as buffer A, and a buffer consisting of 0.2 M sodium chloride and 25 mM Tris in water, which was adjusted to pH 8.5 with 1 N hydrochloric acid, as buffer B, applying a gradient of 0–100% buffer B over 100 column volumes with a flow of 0.50 ml/min. The fractions containing the desired protein were collected, combined, and concentrated via ultracentrifugation using Amicon Ultra centrifugation vials with a cut-off of 10 kDa. After concentration, the buffer was changed to a 50 mM ammonium hydrogencarbonate buffer in the same ultracentrifugation vials. The material was lyophilized to give 4.6 mg of PEGylated protein **34** compound. SDS-gels are in accordance with the expectation for the title compound. The characterization of the compounds was done by SDS-gel PAGE, using silver staining and a specific PEG staining.[31]

4.3.28. (*S*)-6-(3-((4-((4-(*N*-(3-(ω-(2,3-bis(20 kDa mPEGyloxy)Propoxy)2-5 kDa PEGyloxy)propyl)carbamoyl)-butyrylamino)methyl)triazol-1-yl)methyl)benzoylamino)-2-((hGHyl)leucinylamino)hexanoic amide (35). A solution of azido protein **29** (8.23 mg, 365 nmol) was dissolved in a mixture of water (0.996 ml) and 2,6-lutidine (0.021 ml). This solution was added to a solution of PEG-reagent **33** (161 mg, 3650 nmol) in water (0.75 ml). A copper(I)-salt solution was prepared by mixing of a solution of copper(II) sulfate pentahydrate (18.23 mg, 0.073 mmol) in water (4.08 ml) with a solution of ascorbic acid (64.52 mg, 0.366 mmol) in a mixture of water (3.98 ml) and 2,6-lutidine (0.10 ml). This solution was shaken for 5 min at room temperature. 0.41 ml of this copper(I) solution was taken and added to the solution containing the protein and the PEG-reagent. The reaction mixture was shaken gently for 16 h at room temperature. It was filtered through a 450 nm filter. A gel-chromatography was performed, using a HiPrep 26/10 desalting column (Amersham) and a buffer of 25 mM Tris, which had been adjusted to pH 8.5 with 1 N hydrochloric acid, at a flow of 10 ml/min. The fractions containing the desired compound were diluted with a buffer of 25 mM Tris, which had been adjusted to pH 8.5 with 1 N hydrochloric acid (45 ml). An ion-exchange chromatography was performed, using a MonoQ 10/100 GL column (Amersham) at a flow of 0.50 ml/min and a gradient of 0–100% of a buffer of 0.2 M sodium chloride and 25 mM Tris, which had been adjusted to pH 8.5 with 1 N hydrochloric acid, in a buffer of 25 mM Tris, which had been adjusted to pH 8.5 with 1 N hydrochloric acid over 25 column volumes. The fractions, containing the desired compound, were combined. This solution was divided into two parts. Each of these parts was subjected to a gel-chromatography, using a HiPrep 26/10 desalting column (Amersham) and a solution of 50 mM ammonium hydrogencarbonate at a flow of 10 ml/min. The fractions of both runs, which contained the desired product, were combined and lyophilized to give 2.6 mg of PEGylated protein **35**. The characterization of the compounds was done by SDS-gel PAGE, using silver staining and a specific PEG staining.[31]

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