Dually Reactive Long Recombinant Linkers for Bioconjugations as an Alternative to PEG

Thomas Kjeldsen, Wouter F. J. Hogendorf, Christian W. Tornøe, Jonathan Anderson, Frantisek Hubalek, Carsten E. Stidsen, Jan L. Sorensen, and Thomas Hoeg-Jensen*

ABSTRACT: Covalent cross-linking of biomolecules can be useful in pursuit of tissue targeting or dual targeting of two receptors on cell surfaces for avidity effects. Long linkers (>10 kDa) can be advantageous for such purposes, and poly(ethylene glycol) (PEG) linkers are most commonly used due to the high aqueous solubility of PEG and its relative inertness toward biological targets. However, PEG is non-biodegradable, and available PEG linkers longer than 5 kDa are heterogeneous (polydisperse), which means that conjugates based on such materials will be mixtures. We describe here recombinant linkers of distinct lengths, which can be expressed in yeast, which are polar, and which carry orthogonal reactivity at each end of the linker, thus allowing chemoselective cross-linking of proteins. A conjugate between insulin and either of the two trypsin inhibitor peptides/proteins exemplifies the technology, using a GQAP-based linker of molecular weight of 17 848, having one amine at the N-terminal, and one Cys, at the C-terminal. Notably, yeast-based expression systems typically give products with mixed disulfides when expressing proteins that are equipped with one unpaired Cys, namely, mixed disulfides with glutathione, free Cys amino acid, and/or a protein homodimer. To obtain a homogeneous linker, we worked out conditions for transforming the linker with mixed disulfides into a linker with a homogeneous disulfide, using excess 4-mercaptophenylacetic acid. Subsequently, the N-terminal amine of the linker was transformed into an azide, and the C-terminal Cys disulfide was reduced to a free thiol and reacted with halo-acetyl insulin. The N-terminal azide was finally conjugated to either of the two types of alkyne-containing trypsin inhibitor peptides/proteins. This reaction sequence allowed the cross-linked proteins to carry internal disulfides, as no reduction step was needed after protein conjugations. The insulin–trypsin inhibitor conjugates were shown to be stabilized toward enzymatic digestions and to have partially retained binding to the insulin receptor.

INTRODUCTION

Linkers based on poly(ethylene glycol) (PEG) are the most common types for chemical cross-linking of biomolecules.1 Long PEG chains (20–40 kDa) are also commonly used for modification of biomolecules in pursuit of prolonged half-life, due to slower absorption of large PEG derivatives from the relevant injection site, hindrance of kidney-mediated clearance, and/or partial protection from enzymatic breakdown.2,3 Advantages of PEG are its high solubility in water and most organic solvents, its large hydrodynamic volume, and its relative inertness to interactions with biomolecules. PEG is however nondegradable in vivo, so accumulation of PEG in various tissues upon chronic administration of PEGylated drugs has been reported in some cases,4 whereas excretion via bile has been described in other cases.5 PEG can be purchased with various functional groups at one or both ends, for making either PEGylated proteins or cross-linked constructs. Notably, long PEG chains are prepared by polymerization of ethylene oxide and fractioned for size, so long PEG chains (>10 kDa) are always heterogeneous, i.e., they consist of polydisperse mixtures of chain lengths with a polydispersity index of nearly 1.05 in best cases.

Polypeptide sequences with PEG-like properties, sometimes called “recombinant PEG”, have in recent years been described by Alvarez,6 Amumix (mixed sequences of...
GEDSTAP residues, termed “XTEN”),7 XL-protein (PAS repeats),8 Novo Nordisk (GQAP-like repeats),9,10 SOBI,11,12 and others. The advantages of these materials are their biodegradable nature and distinct size, i.e., one precise molecular weight. Furthermore, recombinant PEG can often be introduced by simple extension of the expressed protein (fusion protein), and the hydrodynamic volumes of the polar extensions are similar to what PEG provides. Immunogenicity of the non-native protein sequences can be a problem, but the risk seems to be low due to the polar nature of the sequences (no hydrophobic binding motifs) and disordered structures. XTEN fusions of other human growth hormones are in late-stage human clinical trials without serious immunogenicity problems.13

Both XTEN and PAS have high content of serine residues, and this can be a problem for expressions using yeast-based systems, since yeast typically gives mixtures of O-mannosylated products when there are many serine residues in the sequences.10,11,14 XTEN and PAS fusions are typically expressed in Escherichia coli-based systems where O-mannosylation is not a problem, but expression in E. coli typically gives protein products in inclusion bodies, which must be refolded and disulfide-paired, and this is often difficult. Expressions from yeast on the other hand often provide folded, disulfide-paired protein excreted into the medium.15 Another drawback of XTEN can be that the high content of charged amino acids, such as glutamate, can lead to an increase in viscosity of protein solutions/formulations when high protein concentrations are needed in pharmaceutical formulations, presumably due to the more extended character of the multicharged sequences vs uncharged sequences. For these reasons, we prefer sequences such as GQAP as recombinant PEG. The repeat sequences contain Gln/Q residues, which might be seen as a problem with regard to long-term stability, due to potential side-chain deamidations, but we have not encountered this problem during handling of the materials, and the half-life for Gln deamidations of peptides in aqueous solution at 37 °C is known to be in the range of 250 years.16 This is in sharp contrast to Asn/N, which often deamidates readily.16

XTEN, PAS, GQAP, etc. have mainly been used for making fusion proteins, but the sequences could also be used for making protein conjugations, i.e., used as “recombinant PEG linkers” for cross-linking biomolecules, via introduction of reactive residues along the sequence.17 We exemplify here the design and expression of GQAP-based linkers equipped with orthogonal reactivity at either end and use of such a linker for cross-linking prefolded cysteine-rich proteins, namely insulin and two trypsin inhibitor peptides/proteins. The insulin conjugates are not meant to be new drug candidates but were designed as model compounds for demonstrating the applicability of the homogeneous linkers in cross-linking complex biomolecules.

### RESULTS AND DISCUSSION

(GQAP)49-GQEP-Cys 1 (Scheme 1, MW 17 848) was expressed in yeast using a single-chain insulin precursor as an expression anchor, having GGGLG-(GQAP)49-GQEP-Cys as extension from A21, in 142 mg/L yield.15 The free linker 1 was isolated after cleavage at the lysine by Achromobactor lyticus protease (ALP) and purified by ion-exchange
chromatography. The reason for expressing the recombinant linker with insulin-GGGGK as the expression anchor is that this setup improves expression yields and gives the product that is secreted into the yeast medium. To be able to perform orthogonal conjugations with the linker, we did not require more than one amino group (the N-terminal on the linker). To allow the linker to be used for conjugations of prefolded, disulfide-paired proteins using robust conjugation reactions, we decided to transform the N-terminal amine into an azide, for performing triazole/click chemistry, CuAAC.21,22 But first we had to handle the Cys in the C-terminal of the linker. As expected for yeast-based systems, the expressed linker 1 was found to contain mixed disulfides at the unpaired Cys, namely, disulfides with both glutathions, free cysteine amino acid, and a Cys–Cys-linked homodimer.23 To simplify the planned protein conjugations and purification, we transformed the linker with mixed disulfides into a homogenous disulfide via treatment with excess 4-mercaptophenylacetic acid (MPAA) overnight at pH 9. MPAA has been promoted for use in native chemical ligation reactions and is an odor-free thiol.24 The homogeneous linker (GQAP)49-GQEP-Cys(MPAA) 1 was isolated in 64–69% yield by reversed-phase high-performance liquid chromatography (RP-HPLC) (see Scheme 1). Next, the N-terminal amine was transformed into an azide by treatment with excess imidazolyl-sulfonyl-azide hydrogen sulfate25 overnight in 10 mM phosphate buffer, pH 7.5, and the azido product 3 was isolated by RP-HPLC in 74–85% yield. The disulfide was next reduced to free thiol by treatment with a slight excess of dithiothreitol (DTT) for 2 h at pH 7.5. This reaction was performed gently and carefully followed by liquid chromatography mass spectrometry (LCMS), as DTT can reduce not just disulfides but also azides under rougher conditions. The azide-linker thiol 4 was isolated by RP-HPLC in 60–86% yield. B29Ne-iodoacetyl desB30 human insulin 5 was prepared by the reaction of desB30 human insulin with succinimidyl iodoacetate at pH 10.524 The amine in the lysine side chain is more basic than the two N-terminal amines in insulin (A1 and B1, pKₐ ∼ 7), and the lysine amine (pKₐ ∼ 10) is therefore the most reactive at pH above its pKₐ, so B29Ne products are always the main product from insulin acylation above pH 10.25 The thiol on the linker was reacted with B29Ne-iodoacetyl insulin 5 for 2 h at pH 7.5, and conjugate 6 was isolated by RP-HPLC in 44–52% yield. Finally, the azido group on the linker–insulin conjugate 6 was reacted under Cu catalysis (CuAAC, “click coupling”) with either the sunflower trypsin inhibitor (SFTI)26 or Bowman–Birk trypsin inhibitor (BBI)27 containing propargylglycine (Prg) in positions 14 and 27, respectively (7 and 8). The required alkyne-containing SFTI peptide 7 was prepared by solid-phase synthesis of CFP-Prg-GRCTKSIPPI-iodohydrazide, which was backbone-cyclized using the nitrite method,28 which transformed the acyl-hydrazide via acyl-azide into peptide thioester, which in turn spontaneously cyclized by internal native chemical ligation. The alkyne-containing BBI protein 8 was similarly prepared via hydrazides, by segmental native chemical ligations of DDESSKPCCDQCACTKSNPQPCR-hydrazide 8a with CSD-Prg-RNLSCHASCKSCIALSYPAQCF-hydrazide 8b and CVDITDFCYEPCKPSEDSDKEN 8c, as previously described for normal BBI.27

Upon CuAAC reaction between insulin-linker-azide 6 and either of the two alkyne enzyme inhibitors 7 and 8, using Cul and THPTA under N₂, the two final bivalent protein conjugates insulin-linker-SFTI 9 and insulin-linker-BBI 10 were isolated by RP-HPLC in 48–70 and 47–50% yield, respectively.

The human insulin receptor (HIR) affinities of the conjugates were measured as an assessment of preservation of insulin bioactivity: insulin-SFTI 9 and insulin-BBI 10 showed HIR affinities relative to human insulin of 8.5 and 7.4%, respectively. HIR affinities are classically reported relative to human insulin,29,30 and affinities in the given range will usually preserve full insulin bioactivity in vivo.31

Protection of the insulin conjugates against enzymatic breakdown was tested by treatments with chymotrypsin and trypsin, monitored using an HPLC method. First, it was examined whether conjugating SFTI and BBI to insulin had preserved the inhibitory activity against the enzymes. Indeed, insulin-SFTI 9 and insulin-BBI 10 were both found to be protected against tryptic degradation (see Figure 1 and Table 1).

![Figure 1. Chymotrypsin and trypsin degradation of insulin-SFTI 9 or insulin-BBI 10 and mixtures of human insulin with free Prg14-SFTI 7 or free Prg27-BBI 8, at enzyme/substrate ratios of 1:100 and 1:20, respectively.](https://dx.doi.org/10.1021/acsomega.0c02712)

**Table 1. Half-Lives of 9, 10, and HI +/− 7 or 8 Determined by Curve-Fitting Analysis**

| Protein Type | t₁/₂ chymo (min) | t₁/₂ trypsin (min) |
|--------------|-----------------|-------------------|
| insulin-SFTI 9 | 49.0            | >1000             |
| insulin-BBI 10 | 919.0           | >1000             |
| HI            | 8.4             | 25.8              |
| SFTI 7 + HI   | 7.1             | >1000             |
| BBI 8 + HI    | 398.6           | >1000             |

As expected, insulin-BBI 10 was also protected toward chymotryptic degradation, whereas insulin-SFTI 9 was only partially protected against chymotrypsin. Human insulin (HI) itself was rapidly degraded by both trypsin and chymotrypsin, whereas addition of equimolar concentrations of free BBI 8 to insulin considerably slowed down HI degradation by both enzymes, while addition of equimolar concentrations of free SFTI 7 only slowed down insulin degradation by chymotrypsin, not trypsin, as expected (Figure 1 and Table 1).
Next, it was examined whether there was an advantage of covalent attachment of SFTI to insulin over a simple mixture of the two compounds. In this experiment, the amount of trypsin was increased up to 10-fold. Compared to HI + free Prg-SFTI 7, the degradation half-life of insulin-SFTI 9 was twice as long as that using an enzyme/substrate ratio of 1:4 and 3 times as long as that using enzyme/substrate ratio of 1:2, thereby demonstrating an advantage of the covalent attachment of insulin and inhibitor under such harsh conditions (Figure 2 and Table 2). As a perspective, it could be imagined that the insulin-inhibitor conjugates would be better protected against enzymatic breakdown upon dramatic dilution, for example, upon oral dosing, since the conjugate would retain high local concentration of the inhibitor even after high dilution, whereas the free inhibitor would be diluted away from insulin when dosing the mixture of insulin and inhibitor to a large volume (the gut). We do not intend to pursue these compounds with oral dosing, but they illustrate the principle.

**CONCLUSIONS**

Long linkers of repeating GQAP units can be expressed in yeast, providing compounds with PEG-like properties. The linkers have a distinct molecular weight, contrary to PEG, and are expected to be biodegradable in vivo like XTEN and other recombinant PEG materials. Equipping the linkers with orthogonal functional groups at either terminal made them expected to be biodegradable, as exemplified using N-terminal amine and C-terminal thiol (Cys). To ensure robust conjugation chemistry, we transformed the N-terminal amine into an azide for the triazol/click reaction and used thiol alkylation chemistry at the C-terminus, via transformation of Cys mixed disulfides from yeast expression into a homogeneous MPAA disulfide. The azide/thiol-functionalized linker was used for making conjugates of insulin with peptide/protein-based enzyme inhibitors. It was shown that the conjugates were better protected toward enzymatic breakdown than simple mixtures of insulin and inhibitors, and that the conjugates bind to the insulin receptor.

It is possible to make functionalized linkers with amine on one end and a single carboxylate on the other end, so it should be possible to perform simple amide-based conjugations, although amide couplings are sometimes harder to achieve at high dilutions that are often necessary when handling big molecules, due to competing hydrolysis of active esters.

**EXPERIMENTAL PROCEDURES**

Solid-phase peptide synthesis (SPPS) was performed on a SymphonyX synthesizer from Gyros Protein Technologies (Tucson, AZ 85714) on a 450 μmol scale using a 5-fold excess of Fmoc-amino acids (300 mM in dimethylformamide (DMF) with 300 mM Oxyma Pure) relative to resin loading (typical loading of the hydrazine resin was 0.6 mmol/g). Fmoc-deprotection was performed using 20% piperidine in DMF with 0.1 M Oxyma Pure. Coupling was performed using amino acid/Oxyma Pure/DIC/collidine (3:3:3:4) in DMF. DMF and dichloromethane (DCM) washes (7 mL, 0.5 min, 2 × 2 each) were performed between deprotection and coupling steps. Coupling times were generally 120 min. After synthesis, the resin was washed with dichloromethane, and the peptide was cleaved from the resin by a 3 h treatment with trifluoroacetic acid/triisopropylsilane/dithiothreitol/water (92.5:2.5:2.5:2.5) followed by precipitation with diethyl ether. The peptide was dissolved in 0–20% acetonitrile in water and purified by standard RP-HPLC on a C18 column using acetonitrile/water/trifluoroacetic acid (TFA). LCMS was performed on a setup consisting of a Waters Acquity UPLC system and a LCT Premier XE mass spectrometer from Micromass. Eluents: A: 0.1% formic acid in water, B: 0.1% formic acid in MeCN. Column: Waters Acquity UPLC BEH, C-18, 1.7 μm, 2.1 mm × 50 mm. Gradient: Linear 5–95% acetonitrile during 40 min at 0.4 mL/min, detection at 214 nm.

**Expression of (GQAP)49-QGEP-Cys(mixed disulfide)**

1. An insulin precursor extended from A21 with GGGGGK-(GQAP)49-QGEP-Cys was expressed in yeast (Saccharomyces cerevisiae),15 142 mg/L, and cleaved with ALP to release the free linker (GQAP)49-QGEP-Cys (mixed disulfide) 1, which was isolated by ion-exchange chromatography as previously described.31 As expected from yeast-based expression, Cys in the linker was found to be mixed disulphide and glutathione, free Cys amino acid, and a homodimer.

**Transformation of 1 into (GQAP)49-QGEP-Cys(MPAA)**

2. (GQAP)49-QGEP-Cys(mixed disulfide) 1 (500 mg, ~28 μmol) was dissolved in 20 mM Tris, pH 8.5 (10 mL), with MeCN (2.5 mL). MPAA was added (47 mg, 280 μmol), pH was adjusted to 9, and the mixture was stirred at room temperature for 18 h. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving (GQAP)49-QGEP-Cys(MPAA) 2 (320 mg, 64% yield).

---

**Table 2. Half-Lives of 9 and HI + 7 at Higher Enzyme Concentrations Determined by Curve-Fitting Analysis**

|        | HI | Prg-SFTI 7 + HI | SFTI-insulin 9 |
|--------|----|----------------|----------------|
| t_{1/2} 1:2 (min) | 7.0 | 9.6 | 26.1 |
| t_{1/2} 1:4 (min) | n.d. | 16.3 | 29.9 |

**Figure 2. Degradation of SFTI-insulin 9 and HI + Prg14-SFTI 7 at higher trypsin concentrations.**

**Figure 2. Degradation of SFTI-insulin 9 and HI + Prg14-SFTI 7 at higher trypsin concentrations.**

ACS Omega 2020, 5, 19827

https://dx.doi.org/10.1021/acsomega.0c02712

ACS Omega 2020, 5, 19627–19833
Transformation of 2 into Azido-(GQAP)49-GQEP-Cys (MPAA) 3. (GQAP)49-GQEP-Cys (MPAA) 2 (320 mg, 18 μmol) was dissolved in 10 mM phosphate buffer, pH 7.5 (25 mL), and treated with 10 portions of imidazole-1-sulfonylazide hydrogen sulfate (10 x 15 mg, 888 μmol); pH was adjusted to 7.5 between each addition. The mixture was stirred at room temperature for 22 h. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving azido-(GQAP)49-GQEP-Cys (MPAA) 3 (236 mg, 74% yield). Deconvoluted LCMS showed 17 874.8, calculated 17 874.2 for C95H157N33O38S5, MW 2529.8.

Transformation of 3 into Azido-(GQAP)49-GQEP-Cys with Free Thiol 4. Azido-(GQAP)49-GQEP-Cys (MPAA) 3 (230 mg, 13 μmol) was dissolved in 20 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.5 (60 mL), and treated with DTT (2.5 mg, 16 μmol). The reaction was monitored by LCMS and stopped after 2 h. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving azido-(GQAP)49-GQEP-Cys 4 (196 mg, 74% yield). Deconvoluted LCMS showed 17 874.8, calculated 17 874.2 for AlaqCysGln4GluGly49Pro50C257H380N67O78S7.

Preparation of B29N-iodoacetyl desB30 Human Insulin 5. DesB30 human insulin (500 mg, 88 μmol) was dissolved in 0.2 M Na2CO3 (4.5 mL) and MeCN (1 mL), pH 10.5. Succinimidyl iodoacetate (37 mg, 131 μmol) was added, and the mixture was stirred for 30 min. The crude product was isoprecipitated by adjusting pH to 4.5 with cooling at 5 °C overnight. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving B29N-iodoacetyl desB30 human insulin 5 (350 mg, 68% yield). LCMS showed 1958.8 for [M + 3H]3+, 1469.6 [M + 4H]4+, calculated 1959.2 for [M + 3H]3+, 1469.6 [M + 4H]4+, for C355H522N84O82S6, MW 5874.4.

Transformation of 4 into Azido-(GQAP)49-GQEP-Cys(B29N-Acetyl desB30 Human Insulin) 6. Azido-(GQAP)49-GQEP-Cys 4 (75 mg, 42 μmol) was dissolved in 20 mM Tris, 10 mM EDTA buffer, pH 7.5 (5 mL) + MeCN (300 μL), treated with B29N-iodoacetyl desB30 human insulin 5 (100 mg, 17 μmol), and pH was readjusted to 7.5. The mixture was stirred for 18 h, and the product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving acido-linker-insulin 6 (51 mg, 52% yield). Deconvoluted LCMS showed 23 621.4, calculated 23 620.7 for AlaqCysGln4GluGly49Pro50C257H380N67O78S7.

Synthesis of 14-Proparglycine-SFTI Peptide 7. Crude CFP-Prp-GRCTKSIIPPi-hydrazone from SPPS (0.68 g, 0.45 mmol) was dissolved in a 9:1 mixture of 0.1 M NaH2PO4 in water and acetonitrile (1500 mL, pH 3.0) and cooled to 0 °C. Sodium nitrite (2.25 mL of a 1 M solution in water, 2.25 mmol) was added and the mixture was stirred for 20 min at 0 °C. Sodium 2-mercaptopethanesulfonate (3.69 g, 22.5 mmol) was added to the light pink solution and pH was adjusted to 6.7. The solution was stirred at 25 °C for 16 h, before the mixture was eluted from a C18 column using a gradient of 74:26 to 64:36 water/acetonitrile + 0.1% TFA over 20 min to isolate 14-proparglycine-SFTI peptide 7 (117 mg, 17% yield). LCMS showed 747.9 [M + 2H]2+, 1493.8 [M + 1H]+, calculated 747.7 for [M + 2H]2+, 1493.8 for [M + 1H]+ for C49H84Ne18O16S7.

Synthesis of 27-Proparglycine-BBI Protein 8. DDESSKPCDCQACHTKSNPPQRCSD-Prg-RLNSCHSACKSCICALSYPAQCF-hydrazide (8b) from SPPS was purified on a C18 column using a gradient of 76:24 to 66:34 water/acetonitrile + 0.1% TFA over 20 min to afford peptide 8a (195 mg, 17% yield). LCMS showed 1265.5 [M + 2H]2+, 844.1 [M + 3H]3+, 633.3 [M + 4H]4+, calculated 1265.9 [M + 2H]2+, 844.3 [M + 3H]3+, 633.5 [M + 4H]4+, 44.4, for C63H119N33O38S6, MW 2529.8.

CVDITDFCYEPC-PSEDKKEN (8c) from SPPS was purified on a C18 column using a gradient of 80:20 to 70:30 water/acetonitrile + 0.1% trifluoroacetic acid over 40 min to afford peptide 8c (311 mg, 28% yield). LCMS showed 1226.0 [M + 2H]2+, 817.7 [M + 3H]3+, calculated 1226.3 [M + 2H]2+, 817.9 [M + 3H]3+, for C49H187N37O37S6, MW 2920.4.

Deconvoluted LCMS showed 747.9 [M + 2H]2+, 1493.8 for [M + 1H]+ for C119H187N37O37S6, MW 2920.4.

Transformation of 8a into Azido-B29N-iodoacetyl desB30 Human Insulin 9. Azido-B29N-iodoacetyl desB30 human insulin 8a (236 mg, 74% yield). Deconvoluted LCMS showed 18 040.9, calculated 18 040.4 for C95H157N33O38S5, MW 2529.8.

Transformation of 8b into Azido-B29N-iodoacetyl desB30 Human Insulin 9. Azido-B29N-iodoacetyl desB30 human insulin 8b (137 mg, 47% yield). LCMS showed 1806.8 [M + 3H]3+, 1355.3 [M + 4H]4+, 1084.5 [M + 5H]5+, 903.9 [M + 6H]6+, calculated 1807.0 [M + 3H]3+, 1355.5 [M + 4H]4+, 1084.6 [M + 5H]5+, for C325H472N64O64S11, MW 5418.1.

Deconvoluted LCMS showed 1980.5 [M + 8H]8+, calculated 1960.0 [M + 4H]4+, 1568.2 [M + 5H]5+, 1307.0 [M + 6H]6+, 1120.5 [M + 7H]7+, 980.5 [M + 8H]8+, calculated 1960.2 [M + 4H]4+, 1568.3 [M + 5H]5+.
Conjugation of 6 and 7 to Give SFTI-Triazolyl-(GQAP)49-GQEP-Cys(B29Nε-Acetyl desB30 human insulin) 9. Azido-linker-insulin 6 (20 mg, 0.85 μmol) was dissolved in 2 M triethylamine buffer (4 mL) adjusted to pH 7.8 using acetic acid and purged with nitrogen. Propargyl-SFTI 7 (2 mg, 1.3 μmol) dissolved in 2 M triethylamine buffer (200 μL) adjusted to pH 7.8 using acetic acid was purged with nitrogen and added to 6. Triis(3-hydroxypropyltriazolylmethyl)amine (THPTA) was added (0.9 mg, 2 μmol), followed by Cu(II) (0.02 mg, 0.1 μmol), and the mixture was stirred under nitrogen for 2 h. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving SFTI-linker-insulin 9 (15 mg, 70%). LCMS showed 1196.3 [M + 4H]4+, 1565.3 [M + 5H]5+, 1304.5 [M + 5H]5+, 1304.8 [M + 6H]6+, for C316H474N90O115S14, MW 7822.6.

Conjugation of 6 and 8 to Give BBI-Triazolyl-(GQAP)49-GQEP-Cys(B29Nε-Acetyl desB30 human insulin) 10. Azido-linker-insulin 6 (15 mg, 0.64 μmol) was dissolved in 2 M triethylamine buffer (2 mL) adjusted to pH 7.8 using acetic acid and purged with nitrogen. Propargyl-BBI 8 (6.3 mg, 0.80 μmol) was dissolved in 2 M triethylamine buffer (2 mL) adjusted to pH 7.8 using acetic acid and was purged with nitrogen and added to 6. THPTA was added (0.6 mg, 1.3 μmol), followed by Cu(II) (0.1 mg, 0.5 μmol), and the mixture was stirred under nitrogen for 5 h. The product was purified by RP-HPLC on a C18 column using 0.1% TFA in water + 0.1% TFA in 80% MeCN/water as the eluent, followed by freeze-drying, giving BBI-SFTI linker-insulin 10 (10 mg, 50%). LCMS showed 1210.97 [M + 2H]2+, calculated 1196.96 [M + 2H]2+, deconvoluted MW 25116.0, calculated 25114.5 for Ala48CysGln38Glu66Pro88C37H854N157O193S21.

Human Insulin Receptor Binding Studies. Purification of solubilized human insulin receptor isoform A (HIR-A) expressed in baby hamster kidney cells by wheat germ agglutinin agarose chromatography and subsequent radio-ligand binding by scintillation proximity assays (SPA) were done as previously described.26 Briefly, a series of dilutions of human insulin and the insulin conjugates were used as competing agents against binding of 125I-TyrA14-HI to solubilized HIR-A. Binding was quantified following incubation at 25 °C for 22 h by counting scintillations emitted from SPA beads (PVT anti-mouse, GE Healthcare) in the presence of a mouse anti-IR antibody in binding buffer (100 mM HEPES, 100 mM NaCl, 10 mM MgSO4, and 0.025% (v/v) Tween-20, pH 7.8). All of the concentration–response relationships from binding assays were analyzed by application of a four-parameter logistic model assuming common slope and basal and maximal response level of the curves for human insulin and insulin analogues.26 Affinities of insulin conjugates are reported relative to human insulin [EC50 (HI)/EC50 (conjugate) × 100%].

Trypsin and Chymotrypsin Degradation Assays. Stabilities of SFTI-insulin 9, BBI-insulin 10, and mixtures of human insulin with free Prg14-SFTI 7 and Prg27-BBI 8 against degradation by chymotrypsin and trypsin were assessed using a liquid handling robot (Gilson) in a 96 deep well plate (2 mL) with 16 wells used in parallel. Human insulin, 9 or 10 (15 μM), was incubated with chymotrypsin (0.02 mg/mL) or trypsin (0.1 mg/mL) in 100 mM Heps, pH = 7.4 at 37 °C. Aliquots were taken after 1, 15, 30, 60, 120, and 240 min, and the reactions were quenched by addition of TFA. Concentrations of intact human insulin, 9 and 10, were determined by RP-HPLC using an Acquity UPLC system equipped with a BEH C4 1 × 50 mm² column (Waters) and a linear gradient of acetonitrile in 0.1% TFA. Degradation half-lives were calculated by exponential fitting of data using GraphPad Prism.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02712.

AUTHOR INFORMATION
Corresponding Author
Thomas Hoeg-Jensen — Novo Nordisk A/S, DK-2760 Måløv, Denmark; orcid.org/0000-0003-3882-6345; Email: tshj@novonordisk.com

Authors
Thomas Kjeldsen — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Wouter F. J. Hogendorf — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Christian W. Tornøe — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Jonathan Anderson — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Frantisek Hubalek — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Carsten E. Stidsen — Novo Nordisk A/S, DK-2760 Måløv, Denmark
Jan L. Sorensen — Novo Nordisk A/S, DK-2760 Måløv, Denmark

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c02712
The authors declare the following competing financial interest(s): All the authors are employees of the pharmaceutical company Novo Nordisk A/S, but Novo Nordisk has no direct economic interest in the described linkers.

REFERENCES

(1) Ramirez-Paz, J.; Saxena, M.; Delinois, L. J.; Joaquin-Ovalle, F. M.; Lin, S.; Chen, Z.; Rojas-Nieves, V. A.; Griebenow, K. Thiol-maleimide poly(ethylene glycol) crosslinking of L-asparaginase subunits at recombinant cysteine residues introduced by mutagenesis. PLoS One 2018, 27, No. e0197643.

(2) Turecek, P. L.; Bossard, M. J.; Schoetens, F.; Ivens, I. A. Pegylation of Biopharmaceuticals: A review of chemistry and nonclinical safety information of approved drugs. J. Pharm. Sci. 2016, 105, 460–475.

(3) Webster, R.; Didier, E.; Harris, P.; Seigel, N.; Stadler, J.; Tilbury, L.; Smith, D. Pegylated proteins: Evaluation of their safety in the absence of definitive metabolism studies. Drug Metab. Dispos. 2007, 35, 9–16.

(4) Knadler, M. P.; Ellis, B. B.; Brown-Augsburger, P. L.; Murphy, A. T.; Martin, J. A.; Wroblewski, V. J. Disposition of basal insulin peglispro compared with 27-kDa polyethylene glycol in rats following a single intravenous or subcutaneous dose. Drug Metab. Dispos. 2015, 43, 1477–1483.

(5) Alvarez, P.; Buscaglia, C. A.; Campetella, O. Improving protein pharmacokinetics by genetic fusion to simple amino acid sequences. J. Biol. Chem. 2004, 279, 3375–3381.

(6) Podust, V. N.; Sim, B. C.; Kothari, D.; Henthorn, L.; Gu, C.; Wang, C. W.; McLaughlin, B.; Schellenberger, V. Extension of in vivo half-life of biologically active peptides via chemical conjugation to XTEN protein polymer. Protein Eng. Des. Sel. 2013, 26, 743–753.

(7) Mortar, V.; Bolze, F.; Schlapschy, M.; Schneider, S.; Sédilény, F.; Seyfarth, K.; Klingenspor, M.; Skerra, A. PAsylation of murine leptin leads to extended plasma half-life and enhanced in vivo efficacy. Mol. Pharm. 2015, 12, 1431–1442.

(8) Hoeg-Jensen, T.; Kjeldsen, T.; Markussen, J. Peptide Extended Insulins. WIPO Patent WO200809471, priority date 2006-10-27, publication date 2008-05-02.

(9) Kjeldsen, T.; Hoeg-Jensen, T.; Vinther, T. N.; Hubalek, F.; Pettersson, I. Insulins with Polar Recombinant Extensions. WIPO Patent WO2016193380, priority date 2006-10-27, publication date 2008-05-02.

(10) James, S.; Kaiser, C.; Nilsson, J.; Nordling, E.; Strömberg, P.; Svensson Gelius, S.; Lethagen, S.; Cornvik, T.; Svensson, R. Fusion Proteins. WIPO Patent WO2019201866, priority date 2018-04-16, publication date 2019-10-24.

(11) Cornvik, T.; Nilsson, J.; Nordling, E.; Svensson Gelius, S. Coagulation Factor Based Fusion Protein with Half-Life Extending Polypeptides. WIPO Patent WO2019201868, priority date 2018-04-16, publication date 2019-10-24.

(12) Moore, W. V.; Nguyen, H. J.; Ketter, G. B.; Miller, B. S.; Rogers, D.; Ng, D.; Moore, J. A.; Humphris, E.; Cleland, J. L.; Bright, G. M. A randomized safety and efficacy study of somavaran (VRS-317), a long-acting rhGH, in pediatric growth hormone deficiency. J. Clin. Endocrinol. Metab. 2016, 101, 1091–1097.

(13) Neubert, P.; Halim, A.; Zauser, M.; Essig, A.; Joshi, H. J.; Zatorska, E.; Larsen, I. S.; Loibl, M.; Castells-Ballestre, J.; Aebi, M.; Clausen, H.; Strahl, S. Mapping the O-mannose glycoproteome in Saccharomyces cerevisiae. Mol. Cell. Proteomics 2016, 15, 1323–1337.

(14) Kjeldsen, T. Yeast secretory expression of insulin precursors. Appl. Microbiol. Biotechnol. 2000, 54, 277–286.

(15) Robinson, N. E.; Robinson, R. W.; Robinson, B. R.; Robinson, A. L.; Robinson, J. A.; Robinson, M. L.; Robinson, A. B. Structure-dependent nonenzymatic deamidation of glutaminyl and asparaginyl pentapeptides. J. Pept. Res. 2004, 63, 426–436.