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Addition of Sialic Acid to Insulin Confers Superior Physical Properties and Bioequivalence

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Abstract

Native insulin is susceptible to biophysical aggregation and fibril formation, promoted by manual agitation and elevated temperatures. The safety of the drug and its application to alternative forms of administration could be enhanced through identification of chemical modifications that strengthen its physical stability without compromising its biological properties. Complex polysialic acids (PSAs) exist naturally and provide a means to enhance the physical properties of peptide therapeutics. A set of insulin analogues site-specifically derivatized with sialic acid were prepared in overall yield of 50-60%. Addition of a single, or multiple sialic acids conferred remarkable enhancement to biophysical stability of human insulin, while maintaining its potency. The time to the onset of fibrillation was extended by more than tenfold relative to native hormone. These results demonstrate that simplified sialic acid conjugates represent a viable alternative to complex natural PSAs in increasing the stability of therapeutic peptides.

Introduction

Diabetes is a metabolic disease associated with insufficient insulin action that has grown to global epidemic size.[1, 2] The discovery of insulin in 1921 by Banting and Best miraculously turned an untreatable disease to one that could be managed by daily injection of the hormone.[3-5] However, the therapeutic benefit of insulin has not yet been fully realized for multiple reasons, most notably its narrow therapeutic index and fragile physical properties. The pursuit of an insulin formulation or analog with enhanced stability under conditions of elevated temperature and agitation is a medicinal priority. Such an analog would extend the commercial life of the drug, simplify its transport and storage, and broaden its use most notably in pump administration.[6,7] These attributes would further strengthen the safety of insulin therapy and importantly extend its benefits in tropical climates or where refrigeration is limited.[8]

Several molecular approaches have been reported to enhance the stability of insulin, and these include single-chain insulin analogs,[7] four-disulfide bond insulin analogs,[9-10] and polyethylene glycol (PEG) conjugates.[11] The single-chain analogs employ a short peptide to link the A-chain *N*-terminus to the B-chain *C*-terminus. The resulting modification restricts conformational flexibility to minimize the formation of the fibrillation nucleus, an event that involves a large conformational change from native structure.[12] Four-disulfide bond insulin analogs achieve superior biophysical stability via a mechanism similar to the single chain analogs. PEGylation utilizes the shielding effect of the hydrophilic polymer, where the large hydrodynamic radius minimizes the self-association of insulin to inhibit aggregation.[13] Although all approaches can be effective, the potential of the former two is complicated by the risk of immune recognition of the non-native link and cross-reactivity with other endogenous single-chain insulin related peptides.[7, 9-10] The latter approach is compromised by the slow metabolism of the PEG-polymer leading to tissue accumulation and associated toxicity.[14]

Polysialic acid (PSA), facilitates bacterial escape immune surveillance, and has been proposed by Gregoriadis *et al*. in 1993 to serve a unique role in encapsulating therapeutic proteins.[15] It was proposed that the hydrophilic nature of PSA forms an aqueous layer that serves to prevent the interaction of the protein backbone with the biological milieu to enhance biological activity.[15-17] Given its natural character,

PSA has the additional virtue in being biodegradable minimizing the risk that otherwise plaques synthetic polymers with poor clearance from ligand directed target tissues. As such, PSA has been chemically conjugated to a number of proteins, including asparaginase, interferon alpha-2b, erythropoietin and insulin to increase stability, extend duration of action, and enhance therapeutic efficacy.[16, 18-21]

The use of naturally-sourced PSA in synthesis of medicinal agents has notable shortcomings since it is predominantly sourced from bacteria and of insufficient purity for drug development. The impurities which may contaminate protein-PSA conjugates present undefined risk for acute and chronic immune responses.[22] The microheterogeneity of naturally sourced PSA additionally poses a chemical challenge[22-24] since it complicates optimization of pharmacokinetics (PK) and pharmacodynamic (PD) performance. Finally, the poly-dispersity of PSA hinders its chemical characterization and reproducibility in synthesis of registered drugs.[25-28]

We hypothesized that a repeat polymer of mono-sialic acids site-specifically conjugated to a small peptide (termed here as "sialopeptide"), could mimic the favourable features of PSA yet be accessible by a highly defined synthetic approach to yield a single final product. Previous efforts to modify insulin by well-characterized carbohydrates includes enzymatic semi-synthesis with dendritic sialyloligosaccharides,[29] as well as the total insulin chemical synthesis incorporating GalNAc and mono, di, and tri-mannose glycans.[30] The semisynthetic glycol-insulin analogues displayed slightly extended duration in action while the synthetic glycosylated analogues possessed enhanced stability against oligomerization and proteolysis. Both methods provide encouragement for the identification of insulin analogues enhanced through carbohydrate modification and prepared by straightforward synthetic methods to yield well-defined chemical entities. We envisioned the chemical synthesis of a series of sialopeptide modified insulins in order to systematically assess the relationship between sialic acid density and the physical and biological properties of the resulting conjugates.

We report the synthesis of a set of sialopeptide-enhanced insulin analogues (termed as "Sialic-Ins") (**Figure 1**) with the characterization of their conformation, biophysical stability, and biological activity by *in vitro* and *in vivo* methods. The collective results indicate that Sialic-Ins represent a novel approach to

significantly improve peptide stability while retaining full biological potency. The subtle modification of peptides and proteins with a single, or several sialic acids offers a compelling route to achieving superior macromolecular drug candidates.

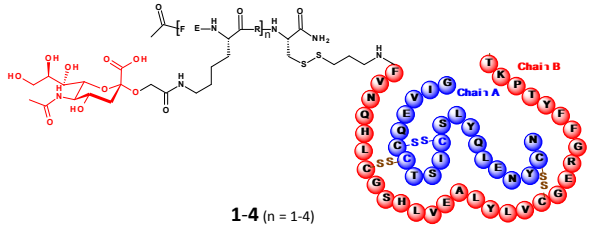
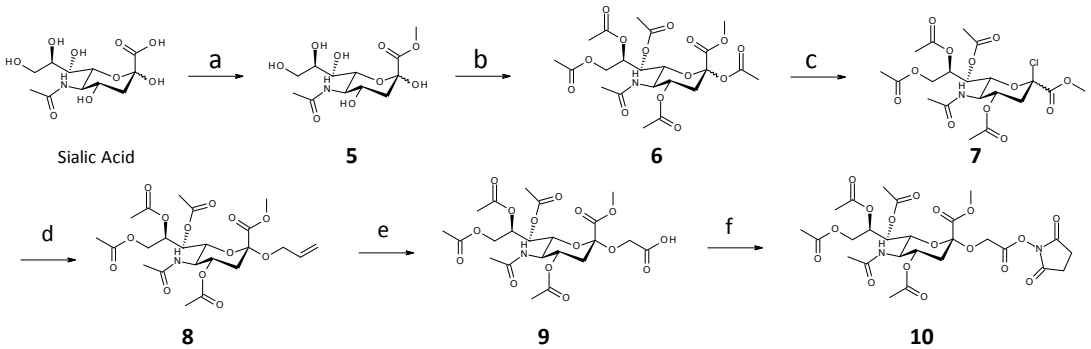


Figure 1. Sialopeptide-modified insulin, Sialic-Ins **1-4**

Results

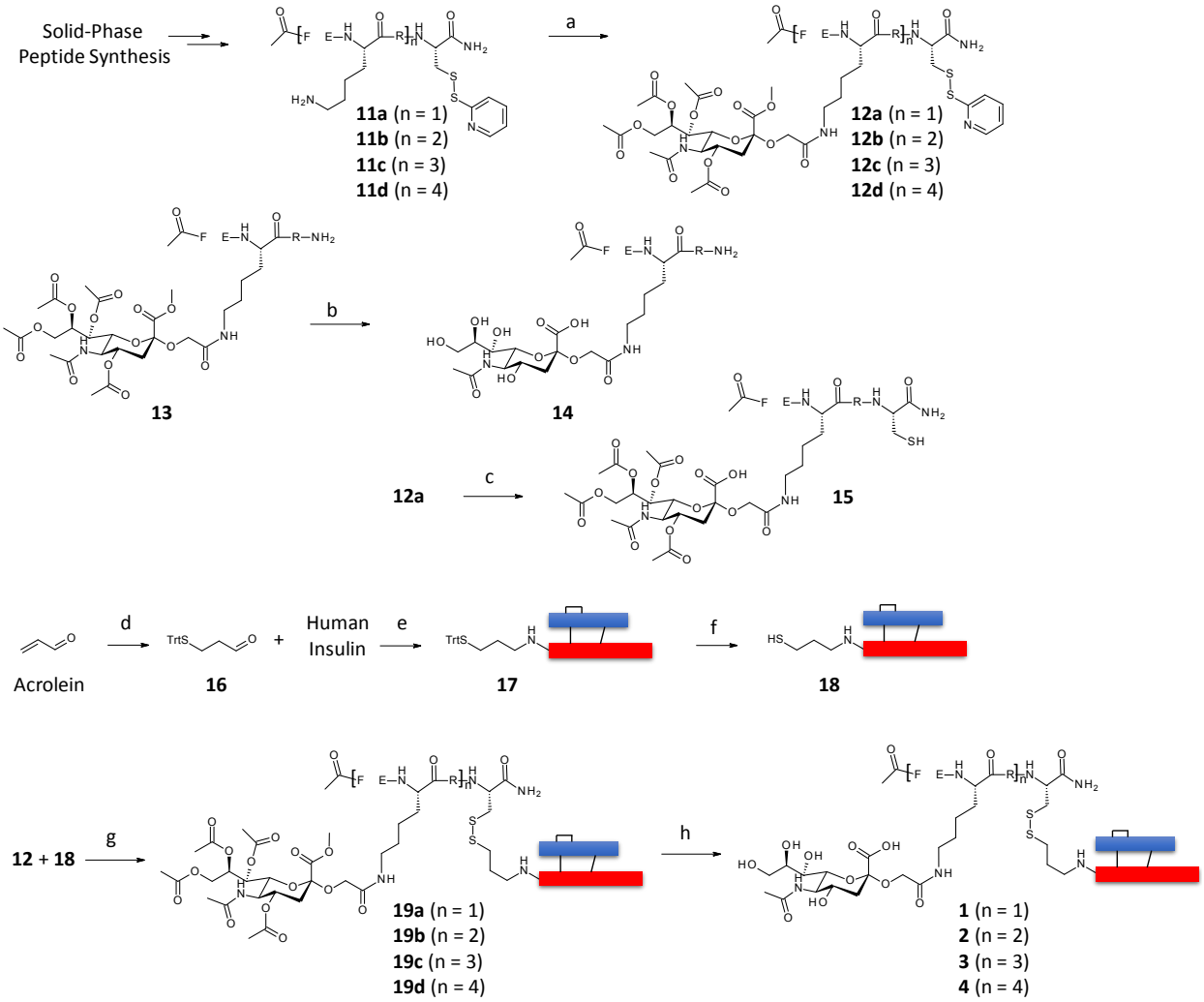
We select the tetrapeptide Phe-Glu-Lys-Arg representing a combination of charged, hydrophilic and hydrophobic amino acids as a core backbone, with the sialic acid covalently attached to the side chain amine of Lys (**Figure 1**). The resulting sialopeptide can be coupled repeatedly to provide a polysialic acid-peptide mimicking PSA. The sialopeptide is acetylated at the *N*-terminus and extended at the *C*-terminus with a Cys-amide providing a point for attachment to insulin through directed disulfide bond formation. Human insulin can be selectively modified at the B-chain *N*-terminal Phe to introduce a single thiol for conjugation with the sialopeptide C-terminal cysteine. The relatively labile disulfide bond was purposely chosen in order to minimize immunogenic risk as in metabolic degradation it represents a chemically less stable form of peptide attachment. Alternatively, a more stable form of attachment such as a thioether could prove superior in certain specific applications.



Scheme 1. Synthesis of sialic acid building block. Reagents and conditions: (a) MeOH, TFA, 25 °C, 48 h, 87% (b) Ac_2O, DMAP, pyridine, 25 °C, 85% (c) AcCl, MeOH 5 °C 48 h 96% (d) AllOH, silver salicylate, MS (4 Å), 23 °C, 4 h 82% (e) $NaIO_4$, $RuCl_3 \cdot XH_2O$, CCl_4:ACN:H_2O (2:2:3), 23 °C, 2 h, 80% (f) NHS, DCC, DMF, 23°C, 2 h, used without purification.

Acetylation is commonly employed to mask the numerous carbohydrate hydroxyl groups during the synthesis of glycopeptides.[31-34] Following the previously-established routes,[35-37] we started from sialic acid and prepared the fully-protected sialic acid building block **9** which was converted to the NHS activated ester form **10 (Scheme 1)**. Separately, peptides **11a-d** consisting of 1 to 4 repeats of Phe-Glu-Lys-Arg unit and derivatized by C-terminal Cys extension and N-terminal acetylation were prepared by Fmoc (**11a**) or Boc (**11b-d**) Chemistry-based solid-phase peptide synthesis (SPPS). The activation of Cys to Cys(thiolpyridine) was achieved during the resin cleavage by including 2,2‘-dithioldipyridine in the TFA cocktail (**11a**) or by treating the crude peptides with 2,2‘-dithioldipyridine in AcOH-containing aqueous acetonitrile (**11c-d**). Peptides **11a-d** were smoothly converted to the respective sialic acid-decorated forms **12a-d** by acylation with the activated ester **10** (1.5 equiv per Lys residue) in DMF with isolated yields of 80-90% (**Scheme 2**)

It has been reported that the deacetylation conditions are often problematic for glycopeptide synthesis, leading to β-elimination at residues such as Ser, Thr or Cys.[31, 32, 34] To identify a realiable deprotection protocol for the sialopeptides, a model peptide **13** was also prepared following a procedue similar to the synthesis of peptides **12**. Peptide **13** when treated with 40 mM sodium hydroxide (NaOH) at 4 °C for 3 h was found to completely liberate the acetyl and methyl esters to provide sialopeptide **14**. (**Scheme 2**). However, when the same deprotection condition was applied to the Cys(thiolpyridine)-extended peptide **12a**, the hydrolysis of the acetyl groups proceeded slowly, and prolonged treatment or higher concentrations of NaOH caused the degradation of pyridyl disulfide bond (**Scheme 2**). The mixed trial deprotection results here directed us to construct the disulfide linkage between the sialopeptide and insulin prior to deprotection of the sialic acids.



Scheme 2. **Synthesis of Sialic-Ins 1-4.** Reagents and conditions: (a) **10**, DIEA, DMF, 1 h, 83-94%; (b) 40 mM NaOH, ACN/H_2O (3:1), 3 h; (c) 40 mM NaOH, ACN/H_2O (3:1), 12 h; (d) Triphenylthiomethane, Et_3N, DCM, 1h, 90%; (e) $NaCNBH_3$ 25 mM acetate buffer (pH 4.0), 6 h, 75%; (f) TFA, TIPS, H_2O, 5 min, 93%; (g) 6.0 M Gn·HCl buffer, total peptide concentration 20 mg/mL, 1 h, 62-85%; (h) 15% NH_4OH, 0-4°C, 6-24 h, quant.

The single N-terminal insulin thiol was installed by reductive amination with Trt-S-CH_2CH_2CHO **16** which was prepared from triphenylthiomethane and acrolein (**Scheme 2**). The reaction was conducted in a mixed solvent of acetonitrile and water at pH 4.5 with sodium cyanoborohydride. The resulting insulin conjugate **17** was treated with trifluoroacetic acid (TFA), TIPS and H_2O to remove the Trt group and

provide thiopropyl-insulin **18** in 70% yield from human insulin. The attachment of the linker to the B1 position was unequivocally confirmed through endoprotease Glu-C mapping (**Figures S9-10**). The ligation of equal molar amounts of protected sialopeptides **12a-d** and thiopropyl insulin **18** was conducted in 6.0 M guanidine hydrochloride solution at pH 6.0 and 25 °C at a total peptide concentration of 20 mg/mL. Under such conditions, the reaction was typically completed within 1 h to provide Sialic-Ins **19a-d** as protected form in 70-85% isolated yields (**Figures S11-14**). Diluted NaOH treatment of Sialic-Ins **19a** resulted in a complex mixture of deprotected, and chemically degraded insulin derivatives. An alternative treatment with aqueous ammonium hydroxide (14-15% aq) at 4°C was found to completely remove the acetyl and methyl ester while preserving the insulin structure. All analogs **1-4** of Sialic-Ins were prepared by this method in nearly quantitative isolated step yields (**Figures S15-18**). The overall synthetic yield of each Sialic-Ins **1-4** was 50-60% from rDNA-derived human insulin.

Far-UV CD was employed to evaluate the impact of the sialopeptides upon insulin higher-order physical structure. The spectra of Sialic-Ins **1**, **2**, and **3** were similar to that of the human insulin implying the attachment of multiple sialic acids does not appreciably alter insulin structure. Interestingly, Sialic-Ins **4**, with four sialic acid moieties revealed a slightly altered spectrum, suggesting that there is a limit to the total sialic acid content that can be accommodated (**Figure 2A**)

Table 1. *In vitro* activity as determined by insulin receptor B autophosphorylation in three separate experiments.

	EC_{50} (nM)	**STDev (±)**
Insulin	0.315	0.079
Sialic-Ins **1**	0.200	0.070
Sialic-Ins **2**	0.354	0.096
Sialic-Ins **3**	0.378	0.165
Sialic-Ins **4**	1.560	0.128

The *in vitro* bioactivity of the Sialic-Ins analogs was assessed by their ability to stimulate the autophosphorylation of the insulin receptor isoform B in cells that over-express it. All Sialic-Ins analogues with the exception of **4** preserved potency comparable to native insulin. The single analogue **4** was approximately fivefold less potent suggesting that the subtle structural change determined by CD spectra is diminishing biochemical signaling at the insulin receptor (**Table 1**).

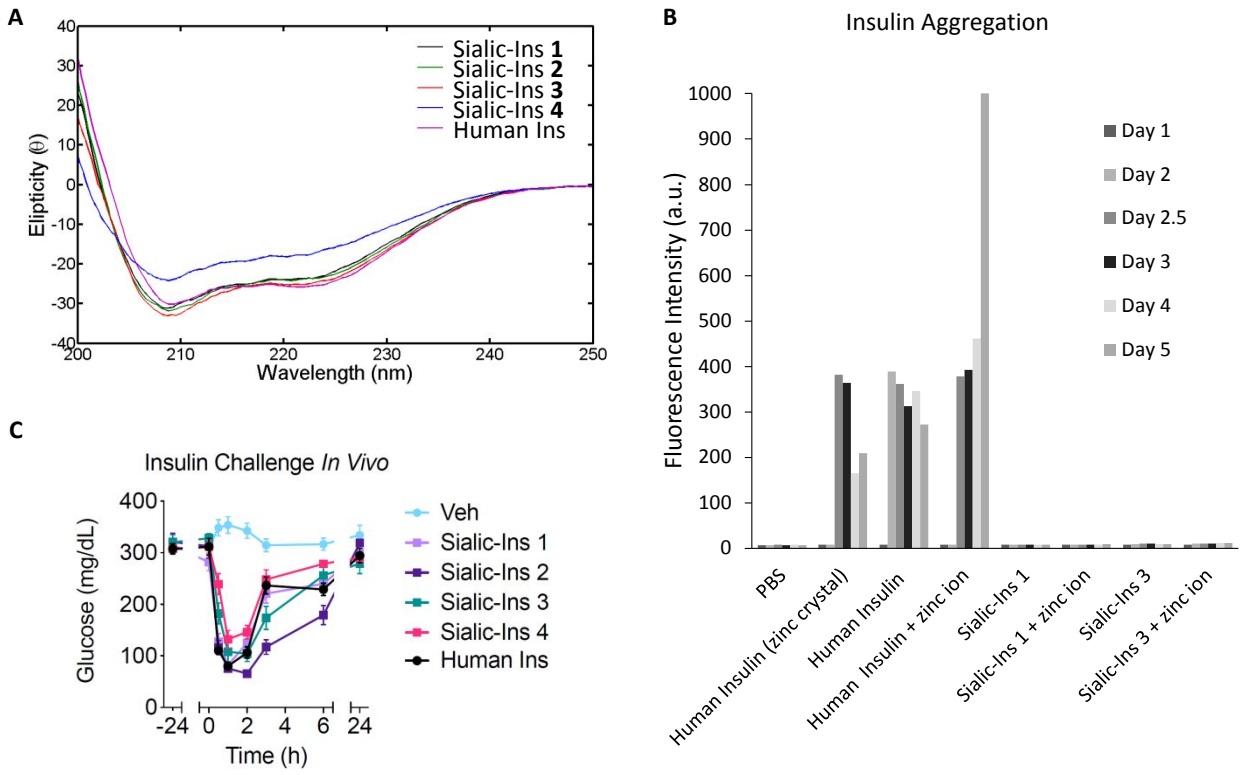


Figure 2. **A,** Circular dichroism profiles of Sialic-Ins **1**-**4**. **B**, Th-T anaylsis of aliquots at days 1-5. All samples were formulated in PBS at 1 mg/mL +/- 28 µg/mL zinc ion. The samples include PBS, Human Insulin-zinc crystal formulated directly; Human Insulin without zinc additive; Human Insulin with zinc additive; Sialic-Ins **1** without zinc additive; Sialic-Ins **1** with zinc additive; Sialic-Ins **3** without zinc additive; and Sialic-Ins **3** with zinc additive. **C**, Changes in blood glucose concentration after subcutaneous administration of insulin and Sialic-Ins in streptozotocin (STZ)-treated mice over 24 h. Data are expressed as mean·SEM; n=8 per group.

The influence of the sialic acid modification on the physical stability of the resulting insulin derivatives was determined for Sialic-Ins **1** and **3** as compared to native human insulin. Each peptide was formulated as a 1.0 mg/mL solution in PBS at pH 7.4 with and without $ZnCl_2$ (28 μg /mL, 3 zinc ions per insulin hexamer). Zinc at this concentration promotes insulin hexamer and thereby protects insulin from fibrillation.[38] These peptides were subjected to thermal and mechanical stress by incubation at 37 °C and 400 rpm in an orbital shaker. Aliquots were withdrawn every 24 h for analysis by Thioflavin T (ThT) assay, which quantifies aggregated peptide. The results indicate time to initial aggregation of human insulin without zinc was 48 h, followed by human insulin with zinc at around 60 h (**Figure 2B)**. Consistent with ThT assay results, turbidity and precipitation were also visually observed for these two treatments at 48 and 60 h respectively (**Figure 3**). In sharp contrast, Sialic-Ins **1** and **3** formulated even without zinc appeared physically stable through day 5 as only background signal was detected which remained unchanged as determined by ThT assay (**Figure 2B**). Continuous monitoring of these four samples up to four weeks did not identify any evidence of biophysical aggregation (**Figure 3**). The fact that no differences were observed in the absence of zinc suggests that Sialic-Ins are stable in molecular forms other than a hexamer. The possibility that they exist in monomeric form raises the potential that they are insulin analogs with a faster and more precise time action. Furthermore, Salic-Ins **1** performed equally to Salic-Ins **3** indicating that the enhanced stability could be achieved by incorporation of only a single sialic acid. Nevertheless, the exact mechanism of action remains to be established by systematic structural and biophysical characterization of Sialic-Ins **1**. Additional study specifically investigating the nature of the peptide linker relative to the carbohydrate remains a focus for continued investigation.

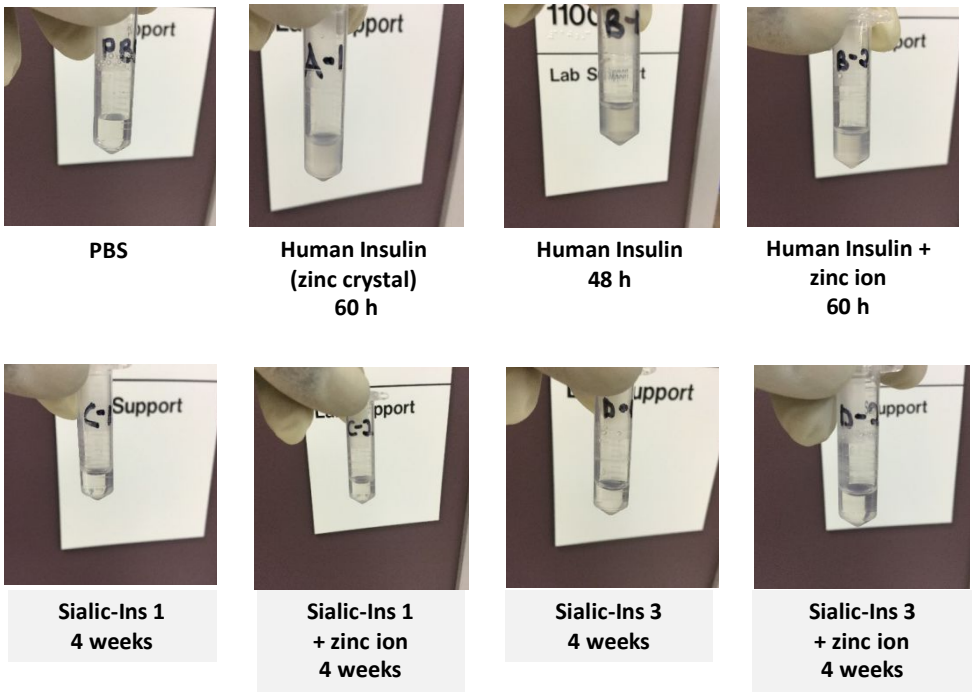


Figure 3. Photographs of insulin samples agitated at 37 °C and 400 rpm. The photo of human insulin samples were taken at the onset of cloudiness while the ones of Sialic-Ins samples were taken at 4 weeks. The photos include PBS; Human Insulin-zinc crystal formulated directly, 60 h; Human Insulin without zinc additive, 48 h; and Human Insulin with zinc additive, 60 h; Sialic-Ins **1** without zinc additive; Sialic-Ins **1** with zinc additive; Sialic-Ins **3** without zinc additive; and Sialic-Ins **3** with zinc additive.

The in vivo pharmacological effort of Sialic-Ins was assessed in a widely used mouse model of insulin deficiency. Insulin deficiency was achieved by treating mice with streptozotocin (STZ), a glucosamine-nitrosourea that induces selective cytotoxicity on the insulin-producing pancreatic beta-cells. STZ-treated mice exhibited hyperglycemia (>300mg/dl) and we monitored the reduction of blood glucose after subcutaneous injection of each of the Sialic-Ins **1**-**4** at 10 nmol/kg. All compounds demonstrated potent ability to lower glucose with a time action that was largely comparable to native hormone (**Figure 2C**). Specifically, the doubly substituted insulin conjugate, **2** exhibited a slight but statistically significant extended duration of action at 2 and 3 h. The most intensively modified analog **4,** which was of reduced *in vitro* potency, was of full *in vivo* potency (with the exception of a statistically significant delayed onset at

the initial 30 min), something that has been previously noted for other insulin analogs of comparably reduced potency.[39]

Discussion and Conclusions

Inspired by the potential of polysialic acid and seeking to mitigate their structural complexity, a set of sialopeptide-derivatized insulin analogs have been prepared in total yields of >50%. The core sialopeptide unit consists of a single sialic acid containing tetrapeptide. The attachment to human insulin of singular core sialic acid tetrapeptide and up to a total of four has little impact on structure as assessed by CD, or bioactivity as determined by *in vitro and in vivo* study, but confers remarkable improvement in biophysical stability. The presence of sugar extends the time to onset of fibrillation from 2 days for native insulin to longer than four weeks for Sialic-Ins. Of great significance is that the formulation with zinc was not required to achieve greater stability, and with only a single sialic acid adduct was sufficient to achieve full enhancement. The collective results support a simple sialopeptide introduction as a powerful technology to significantly strengthen insulin stability without compromising its structure or bioactivities. Hossain, Wade and colleagues have recently reported the synthesis of a sialylundecasaccharide-conjugated insulin using a large oligosaccharide complex isolated from egg yolk. The fully synthetic insulin was modified with a single additional cysteine orthogonally protected for subsequent sugar conjugation. This sialylundecasaccharide-conjugated insulin was equally potent to human insulin and more resistant to fibrillation for up to one week,[40] consistent with the findings reported here. Collectively, this work illustrates the power of sialic acid-based modifications to strengthen physical properties of insulin without altering biological function. The fact that the introduction of a single sialic acid can be employed to significantly optimize a drug as important as insulin suggests that the methodology is likely to be of similar value in enhancing other macromolecular drug candidates with chemistry much more defined than that historically employed.

Experimental Section

Reagents and solvents were bought and used without purification unless otherwise stated. Reagents including trifluoroacetic acid (TFA), piperidine, triisopropylsilane, (TIPS), diisopropylethyl amine (DIPEA), *N,N′*-Dicyclohexylcarbodiimide (DCC), sodium cyanoborohydride($NaBH_3CN$), allyl alcohol (AllOH), acetyl chloride (AcCl), sodium periodate ($NaIO_4$), ruthenium (III) chloride hydrate ($RuCl_3.XH_2O$), N-Hydroxysuccinimide (NHS), carbon tetrachloride (CCl_4), acetic anhydride (Ac_2O), guanidine hydrochloride (GnHCl), 2,2′-Dithiodipyridine (DTDP), and solvents including methanol (MeOH), acetonitrile (ACN), diethyl ether, tetrahydrofuran (THF) *N*-methyl-2-pyrrolidone (NMP), *N,N'*-dimethylforamide (DMF) and dichloromethane (DCM) were purchased from Sigma Aldrich. Fmoc-protected amino acids including Fmoc-L-Phe, Fmoc-L-Glu(O*t*Bu), Fmoc-L-Lys(Boc), Fmoc-L-Arg(Pbf), and Fmoc -L-Cys(Trt), and Boc-protected amino acids including Boc-Phe, Boc-Glu(OcHx), Boc-Lys(Cl-Z) , Boc-Arg(Tos) and Boc-Cys(MeBzl) were purchased from Midwest Bio-Tech Inc. LC-MS analysis was conducted on Agilent 1260 infinity LC system coupled to an Agilent 6120 quadrupole mass spectrometer with a analytical RP-HPLC column Phenomenex Kinetex C8 2.6 µm 100 Å (75 x 4.5 mm) using eluent gradient consisting of 10-80% B over 10 min (Buffer A: 0.05% TFA in H_2O; buffer B: 0.05% TFA in 90% aqueous ACN). The crude peptides were purified using preparative RP-HPLC system which employed a 2.0 x 25 cm Phenomenex C18 column in a 0.1% TFA buffer and an increasing ACN gradient. UV absorbance was measured at wavelengths 220 and 230 nm via Waters dual wavelength detector 2487. Purified fractions were collected at a flow rate of 8-10mL/min Prostar model 701 fractions collector.

The synthesis of sialic acid derivative was monitored using think layer chromatography (TLC) technique with Silica Gel HLTLC Plates (w/UV254, 250µm, 20 x 20 cm) from Sorbent Technologies. The chromatograms were developed, and TLC plates were visualized by dipping them in sulfuric acid dip followed by charring at 150 °C and their R_f values determined. Purification of all products was done using a medium pressure liquid chromatography (MPLC) system Teledyne ISCO (Teledyne Technologies Company, 4700 Superior St., CA, www.isco.com). The purity of all non-peptidic compounds were

determined by NMR to be > 95%. ^{1}H and ^{13}C NMR spectra were obtained using on a Varian VXR 400 (400 MHz/ 101 MHz), Varian INOVA 400 (400 MHz/ 101 MHz), Varian 500 (500 MHz/ 125 MHz) or Varian 600 (600MHz) instruments. Chemical shift values are measured and reported in parts per million (ppm) on the δ scale. Coupling constants and signal splitting patterns were recorded as J values in Hz. Abbreviations for multiplicities such as s = singlet, d = doublet, dd =double of doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet were used. ^{1}H NMR and ^{13}C NMR spectra taken for compounds in $CDCl_3$ were referenced to the solvent peak at 7.260 ppm (1H) and 77.16 ppm (^{13}C). On the other hands, those taken in CD_3OD were reported relative solvent peaks at 3.31 ppm (^{13}H) and 49.00 ppm (^{13}C).

Methyl 5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate (5). To a solution of N-acetalneuraminic acid (20.0 g, 64.7 mmol) in anhydrous methanol (400 mL) was added trifluoroacetic acid (0.2 mL) at 23 ^{o}C. The reaction mixture was allowed to stir at 23 ^{o}C for 48 h, after which TLC analysis TLC (EtOAc/*i*PrOH/H_2O: 2:2:1), confirmed the formation of the product. The reaction mixture was filtered over a pad of celite, concentrated and dried under vacuum. The dried material was dissolved in anhydrous pyridine and filtered via medium and the residue was washed several times with methanol. The filtrate was concentrated under reduced pressure to give the product, **5** as a white solid (18.2 g, 87%), whose data matched the reported ones.[35]

Methyl 5-Acetamido-2,4,7,8,9-penta-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate (6). To a solution of **5** (5.00 g, 15.5 mmol) in anhydrous pyridine (30 mL) was added acetic anhydride (11.8 g, 116 mmol) and catalytic amount of DMAP. The reaction mixture was at room temperature for 12 h after which TLC (acetone: toluene: 1:1) analysis confirmed the formation of the product. The reaction mixture was poured into a crashed ice and stirred until all the ice melted. The aqueous mixture was extracted with EtOAc (3x50 mL). The combined organic layers was washed with saturated solution of ammonium chloride, dried, filtered, concentrated under vacuum to give the crude, which was purified via MPLC using ISCO (using 0-50% acetone in toluene) to give the product, **6** (7.01 g, 85%), whose data agreed with the

literature report.[35] **^{1}H NMR (500 MHz, $CDCl_3$)** δ 5.78 – 5.64 (m, 1H), 5.33 (dd, *J* = 4.8, 1.5 Hz, 1H), 5.26 – 5.11 (m, 1H), 5.00 (ddd, *J* = 7.2, 4.8, 2.5 Hz, 1H), 4.45 (dd, *J* = 12.4, 2.6 Hz, 1H), 4.20 – 3.96 (m, 3H), 3.73 (s, 3H), 2.48 (dd, *J* = 13.4, 4.9 Hz, 1H), 2.09 (d, *J* = 2.4 Hz, 6H), 2.00 (s, 3H), 1.97 (d, *J* = 1.1 Hz, 6H), 1.83 (s, 3H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 170.89, 170.66, 170.53, 170.40, 170.32, 170.28, 170.19, 170.08, 170.04, 169.77, 169.65, 169.04, 168.60, 168.25, 168.21, 167.88, 166.31, 164.60, 97.45, 77.35, 77.30, 77.10, 76.85, 72.80, 71.49, 68.38, 68.36, 67.77, 62.11, 53.14, 49.09, 35.90, 23.06, 20.85, 20.84, 20.80, 20.77, 20.73, 20.72, 20.67, 10.56.

Methyl 5-Acetamido-2-chloro-4,7,8,9-penta-O-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosonate (7). A solution of **6** (2.00 g; 4.07 mmol) in a mixture of AcCl and HOAc in the ratio of 1:1 (40 mL) was saturated at 0 °C with anhydrous $HCl_{(g)}$ generated by addition of concentrated H_2SO_4 to anhydrous calcium chloride. The resulting mixture was stirred at 23 °C for 24 h, after which TLC analysis indicated complete formation of the chloride, which was concentrated under reduced pressure followed by co-evaporation with toluene three times to give a white foam as the product **7** (1.98 g; 3.88 mmol; 96%), which was taken to the next step without purification.[35]

Methyl (5-acetamido-2-allyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate (8). The chloride **7** (1.98 g; 3.88 mmol) was dissolved in allyl alcohol (21.6 mL; 318 mmol; 82 equiv) followed by the addition of silver salicylate (1.43 g; 5.82 mmol; 1.5 equiv) resulting in a suspension, that was stirred at 23 °C in the dark until TLC analysis confirmed product formation (2 h). The reaction mixture was filtered over a pad celite and the precipitate washed with chloroform. The filtrate was washed with cold sat. $NaHCO_3$ solution, 5% $Na_2S_2O_3$ and water. The organic layer was dried (Na_2SO_4), filtered, concentrated under reduced pressure to give the crude, which was purified via MPLC ISCO to afford the product **8** (1.69 g; 82%) whose data was in agreement with the published one.[36, 37] **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.26 (s, 1H), 5.95 – 5.76 (m, 1H), 5.44 – 5.22 (m, 4H), 5.22 – 5.11 (m, 2H), 4.86 (ddd, *J* = 12.1, 9.5, 4.6 Hz, 1H), 4.34 – 4.22 (m, 2H), 4.09 (td, *J* = 13.1, 12.6, 7.7 Hz, 3H), 3.87 (ddt, *J* = 12.9, 5.8,

1.5 Hz, 1H), 3.78 (s, 3H), 2.61 (dd, *J* = 12.8, 4.6 Hz, 1H), 2.14 (d, *J* = 6.4 Hz, 6H), 2.03 (d, *J* = 6.8 Hz, 6H), 1.87 (s, 3H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 170.95, 170.61, 170.22, 170.09, 168.33, 133.52, 117.22, 98.45, 77.33, 77.28, 77.08, 76.82, 72.52, 69.12, 68.73, 67.36, 65.85, 62.40, 52.62, 49.27, 38.01, 23.11, 21.08, 20.82, 20.80, 20.73, 20.68.

Methyl (2-Carboxylmethyl-5-acetamido-4,7,8,9-tetra-*O*- acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate (9). To a biphasic solution of the allyl product, **8** (500 mg; 0.941 mmol; 1.0 equiv) and $NaIO_4$ (800 mg; 3.76 mmol; 4 equiv) in CCl_4 (2 mL) acetonitrile (2 mL) and water (3 mL) was added $RuCl_3.XH_2O$ (9.76 mg; 0.0471 mmol; 0.05 equiv). The reaction solution was stirred vigorously at 23 ^oC for 2 h and then diluted with CH_2Cl_2 (30 mL) followed by the separation and extraction of the aqueous layer with CH_2Cl_2 (3 x 30 mL). The combined organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, filtered, concentrated and purified with MPLC (50-100% EtOAc/CH_2Cl_2) to give the product **9** (412 mg; 80%). The NMR agreed with the literally reported ones.[36] **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.26 (d, *J* = 1.0 Hz, 1H), 5.37 (ddt, *J* = 8.8, 5.8, 2.3 Hz, 2H), 5.29 (dd, *J* = 8.4, 1.5 Hz, 1H), 4.96 (ddd, *J* = 11.8, 9.4, 4.7 Hz, 1H), 4.35 (d, *J* = 16.7 Hz, 1H), 4.32 – 4.19 (m, 2H), 4.14 – 3.94 (m, 3H), 3.80 (s, 3H), 2.70 (dd, *J* = 13.0, 4.7 Hz, 1H), 2.19 – 1.94 (m, 14H), 1.89 (s, 3H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 171.74, 171.02, 170.80, 170.70, 170.28, 170.18, 167.59, 98.27, 77.27, 77.22, 77.02, 76.77, 72.55, 68.91, 68.45, 67.17, 62.41, 61.68, 53.12, 49.49, 37.38, 23.11, 21.09, 20.84, 20.76, 20.73.

Methyl 2-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethoxy-5-acetamido-4,7,8,9-tetra-*O*- acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosid)onate (10). To a solution of the acid **9** (1.15 g; 2.09 mmol; 1.0 equiv) in anhydrous DMF (15 mL) under argon were added NHS (0.240 g; 2.09 mmol, 1.0 equiv) and DCC (0.43 g; 2.09 mmol; 1.0 equiv). The resulting solution was stirred at 23 ^oC until TLC analysis showed a lower running spot corresponding to a complete transformation of the starting material in the desired product **10**. The reaction mixture was kept and used as stock solution for the subsequent steps.

Fmoc-based Synthesis of Peptide 11a

20% piperidine in DMF (10 mL) was added to the Polystyrene AM RAM resin (0.685 g, 0.50 mmol), the resulting mixture was shaken for 10 min on a rotator and then drained. This step was repeated one time. The Fmoc-deprotected resin was then washed with DMF (6 x 10 mL) followed by the addition Fmoc-Cys(Trt)-OH (1.464 g, 2.5 mmol, 5 equiv), DEPBT (0.748 g, 2.5 mmol, 5 equiv), DMF (10 mL) and DIPEA (0.646 g, 5 mmol, 10 equiv). The mixture was agitated on a rotator for 2 h. The resin was then drained and washed with DMF (3 X 10 mL), followed by the treatment of 20% piperidine in DMF (10 mL) for 10 min twice. The following couplings of Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Phe-OH were conducted following the same protocol. The resulting resin was then treated with Ac_2O (1.021 g, 10 mmol, 20 equiv.) in DMF (10 mL) for 2 h followed by washing with DMF and DCM. To cleave and activate the pentapeptide from the resin, DTDP (1.102 g, 5 mmol), TIPS (0.5 mL), H_2O (0.5 mL) and TFA (19 mL) were added to the resin, and the resulting mixture was agitated for 2 h followed by filtration and rotavapor to remove TFA. The resulting material was treated and washed with cold diethyl ether, dissolved in ACN/H_2O and purified by prep. RP-HPLC to give the pentapeptide **11a** 115 mg in a yield of 28% and purity by analytical HPLC of 99%. M.W. calculated: 832.0; observed: 831.4. **(Scheme S1 and Figure S1)**

Boc-based Synthesis of Peptides 11b-11d

0.2 mmol of 4-methyl benzhydrylamine resin (MBHA) was placed in a CSBio reaction vessel and 2 mmol tubes of Boc amino acids were aligned on the AA wheel to correspond to the desired sequence. 2.0 mmol acetic acid was placed in the last tube. 30 min couplings were run using 0.5M DEPBT and DIPEA in DMF on a CSBio synthesizer. At the completion of the chain assembly, the acetylated peptide resin was transferred to an HF reaction vessel after washing with DMF and DCM. The HF reaction vessel was attached to the HF apparatus, cooled in a dry ice/methanol bath, evacuated, and 5-7 ml of liquid hydrogen fluoride was condensed in. The reaction was stirred 60 min in an ice bath then the HF was removed under vacuum. The residue was suspended in ethyl ether (20-30 ml) and the product/resin suspension was filtered

and washed with ether. The peptide was extracted into 2% aqueous acetic acid/20% acetonitrile and S-pyridyl activation was achieved by the addition of DTDP (2 mmol) for 4 h. The resulting peptide was purified by prep. PR-HPLC followed by freeze-drying **(Scheme S2)**.

Peptide 11b. The above described general procedure was followed to synthesize peptide **11b** at 0.2 mmol scale which after prep. RP-HPLC purification offered 109 mg of product at 39% yield based on the resin substitution. Purity by analytical HPLC: 90%; M.W. calculated: 1392.7; observed: 1392.7 (**Figure S2**).

Peptide 11c. The above described general procedure was followed to synthesize peptide **11c** at 0.2 mmol scale which after prep. RP-HPLC purification offered 147 mg of product at 38% yield based on the resin substitution. Purity by analytical HPLC: 99%; M.W. calculated: 1952.3; observed: 1953.0 (**Figure S3**).

Peptide 11d. The above described general procedure was followed to synthesize peptide **11d** at 0.2 mmol scale which after prep. RP-HPLC purification offered 151 mg of product at 30% yield based on the resin substitution. Purity by analytical HPLC: 98%; M.W. calculated: 2513.9; observed: 2513.4 (**Figure S4**).

General procedure for preparing Sialopeptides 12a-d. The thiolpyridine peptides (**11a-d**) (1.0 equiv) was mixed with the activated sialic acid **10** (1.5 equiv per Lys) in anhydrous DMF under argon atmosphere. The reaction was stirred at rt until LC-MS analysis confirmed the complete conversion (1 h), and then diluted with 0.05%TFA/H_2O and purified by prep. RP-HPLC to give the products.

Peptide 12a. The above described general procedure was followed to synthesize sialopeptide **12a** from **11a** which after prep. RP-HPLC purification offered 68 mg of product at 89% step yield. Purity by analytical HPLC: 98%; M.W. calculated: 1363.5; observed: 1362.6 (**Figure S5**).

Peptide 12b. The above described general procedure was followed to synthesize sialopeptide **12b** from **11b** which after prep. RP-HPLC purification offered 61 mg of product at 83% step yield. Purity by analytical HPLC: 93%; M.W. calculated: 2455.6; observed: 2455.0 (**Figure S6**).

Peptide 12c. The above described general procedure was followed to synthesize sialopeptide **12c** from **11c** which after prep. RP-HPLC purification offered 85 mg of product at 94% step yield. Purity by analytical HPLC: 92%; M.W. calculated: 3547.7; observed: 3546.6. (**Figure S7**).

Peptide 12d. The above described general procedure was followed to synthesize sialopeptide **12d** from **11c** which after prep. RP-HPLC purification offered 78 mg of product at 85% step yield. Purity by analytical HPLC: 90%; M.W. calculated: 4639.8; observed: 4638.9. (**Figure S8**).

Synthesis of 3-(tritylthio)propanal 16

Triphenylthiomethane (10.0 g, 36.2 mmol, 8.3 equiv.) in anhydrous CH_2Cl_2 (80mL) was added to a solution of Et_3N (7.56 mL, 54.3 mmol, 12.5 equiv.) and acrolein (3.44 mL, 4.36 mmol, 1.0 equiv.). The reaction mixture was stirred at room temperature until TLC analysis confirmed the product, 3-tritylthiopropanal (1 h). The reaction mixture was concentrated under reduced pressure to give the crude 3-tritylthiopropanal which was recrystallized from ethyl acetate/hexane to obtain the pure product in 90% yield. **^{1}H NMR (500 MHz, $CDCl_3$)** δ 9.56 (t, *J* = 1.3 Hz, 1H), 7.54 – 7.37 (m, 7H), 7.39 – 7.12 (m, 11H), 2.48 (td, *J* = 7.0, 0.9 Hz, 3H), 2.37 (tt, *J* = 7.1, 1.1 Hz, 2H). **^{13}C NMR (126 MHz, $CDCl_3$)** δ 200.29, 144.54, 129.56, 128.00, 126.79, 77.32, 77.07, 76.81, 67.02, 42.69, 24.44.

Synthesis of 3-Thiopropyl Insulin 17

Human insulin (100 mg, 0.0172 mmol) was dissolved in 10 mM HCl (10 mL) and the solution neutralized with 10 mM NaOH (10 mL). To the resulting solution were added acetonitrile (25 mL), 25 mM NaOAc butffer (25 mL pH 4.0), 3-(tritylthio)propanal (5.73 mg, 0.0272 mmol, 1.0 equiv) and $NaCNBH_3$ (67.9 mg, 1.08 mmol, 20 equiv). The reaction was monitored with LC-MS at 30 min intervals. After 4 h, another 3-(tritylthio)propanal (5.73 mg, 0.0272 mmol, 1.0 equiv) was added and the reaction allowed to proceed until LC-MS analysis confirmed conversion of most of the insulin. The reaction mixture was diluted with buffer A containing 0.05% TFA in 10% acetonitrile/water. The mixture was purified via reverse phase chromatography using a 2.0x25cm Phenomenex C18 column in a 0.1%TFA buffer and an increasing

acetonitrile gradient to give the product **17** after freeze-drying (76 mg, 75%). Purity by analytical HPLC: 97%; M.W. calculated: 6124.5; observed: 6123.6 (**Figure S9**). **Endoprotease Glu-C digest.** 0.2 mg of **17** was dissolved in 0.5 mL buffer (25 mM NH_4HCO_3, pH 8.0) and 20 µL of GluC (Thermo Scientific, 20 µg dissolved in 500µL of 25 mM NH_4HCO_3 buffer, pH 8.0) was added to the solution. The reaction was incubated overnight at 37 °C. Reaction was analyzed by analytical LCMS (10-80% MeCN gradient over 10 min., 0.05% TFA, Kinetex C8/ 2.6µ/100A/ 75x4.60 mm column) (**Figure S9**).

Synthesis of 3-Thiopropyl Insulin 18

3-(Tritylthio)propyl insulin **17** (80.0 mg, 0.013 mmol) was dissolved in trifluoroacetic acid (TFA) containing triisopropylsilane (2%) and H_2O (2%) at 7.5 mg/mL concentration of the insulin derivative. After 5 mins, detritylated material was recovered by cold ether precipitation and centrifugation. The recovered material was washed three times with cold ether. LC-MS analysis confirmed that detritylation was quantitative. The material was dissolved in H_2O and freeze-dried directly without RP-HPLC purification to provide 71 mg **18** with 93% step yield. Purity by analytical HPLC: 99%; M.W. calculated: 5881.7; observed: 5881.8 (**Figure S10**).

General method of synthesizing protected Sialic-Ins 19a-d. The freeze-dried powder of 2-thiopyridyl activated sialopeptide (**12a-d**) (1.0 equiv) and thiopropyl insulin **18** (1.0 equiv) were combined in a 20 mL vial followed by the addition of 6.0 M guanidine hydrochloride buffer, pH 6.0 resulting in a reaction solution of total peptide concentration of 20.0 mg/mL. The reaction was stirred at rt until LC-MS analysis confirmed the complete conversion (60 min). The reaction mixture was then diluted with 0.05%TFA/H_2O and purified via prep. RP-HPLC to give the products **19a-d** after freeze-drying.

Sialic-Ins 19a. Sialopeptide **12a** (17.7 mg, 12.9 µmol) and thiopropyl insulin **18** (76.3 mg, 12.9 µmol) were ligated according to the above described general ligation procedure which after purification by prep. RP-

HPLC provided 65 mg protected Sialic-Ins **13** with 70% step yield Purity by analytical HPLC: 95%. M.W. calculated: 7134.5; observed: 7134.0 (**Figure S11**).

Sialic-Ins 19b. Sialopeptide **12b** (27.6 mg, 11.2 μmol) and thiopropyl insulin **18** (66.1 mg, 11.2 μmol) were ligated together according to the above described general ligation procedure which after purification by prep. RP-HPLC provided 56 mg protected Sialic-Ins **14** with 62% step yield. Purity by analytical HPLC: 95%. M.W. calculated: 8226.6; observed: 8226.5 (**Figure S12**).

Sialic-Ins 19c. Sialopeptide **12c** (42.6 mg, 11.4 μmol) and thiopropyl insulin **18** (67 mg, 11.4 μmol) were ligated together according to the above described general ligation procedure which after purification by prep. RP-HPLC provided 91 mg protected Sialic-Ins **15** with 82% step yield. Purity by analytical HPLC: 95%. M.W. calculated: 9318.7; observed: 9319.0 (**Figure S13**).

Sialic-Ins 19d. Sialopeptide **12d** (40.2 mg, 8.67 μmol) and thiopropyl insulin **18** (51 mg, 8.67 μmol) were ligated together according to the above described general ligation procedure which after purification by prep. RP-HPLC provided 76 mg protected Sialic-Ins **16** with 85% step yield. Purity by analytical HPLC: 94%. M.W. calculated: 10410.8; observed: 10410.0 (**Figure S14**).

General method of synthesizing Sialic-Ins 1-4. The sialic acid-protected Sialic-Ins (**19a-d**) was placed in a 20 mL vial at 0° C. To this was added pre-cooled solution 14-15% NH_4OH. The reaction was taken to and kept at 4 °C until LC-MS analysis revealed the complete hydrolysis of the acetate and methyl ester groups. The reaction mixture was neutralized with 0.1 M AcOH and diluted with 0.05% TFA/H_2O and purified via prep. RP-HPLC to give the products (**1-4**) after freeze-drying.

Sialic-Ins 1. The protected Sialic-Ins **19a** (20 mg, 2.80 μmol) was converted to Sialic-Ins **1** following the above described general hydrolysis condition providing 18 mg of **1** with step yield of 90%. Purity by analytical HPLC: 96%. M.W. calculated: 6952.3; observed: 6950.8 (**Figure S15**).

Sialic-Ins 2. The protected Sialic-Ins **19b** (21.3 mg, 2.59 µmol) was converted to Sialic-Ins **2** following the above described general hydrolysis condition providing 19.3 mg of **1** with step yield of 95%. Purity by analytical HPLC: 94%. M.W. calculated: 7862.2; observed: 7860.6 (**Figure S16**).

Sialic-Ins 3. The protected Sialic-Ins **19c** (24 mg, 2.58 µmol) was converted to Sialic-Ins **3** following the above described general hydrolysis condition providing 22 mg of **3** with step yield of 97%. Purity by analytical HPLC: 97%. M.W. calculated: 8772.2; observed: 8769.5(**Figure S17**).

Sialic-Ins 4. The protected Sialic-Ins **19d** (17.6 mg, 1.69 µmol) was converted to Sialic-Ins **4** following the above described general hydrolysis condition providing 16.1 mg of **4** with step yield of 98%. Purity by analytical HPLC: 97%. M.W. calculated: 9682.1; observed: 9680 (**Figure S18**).

Analysis of Sialic-Ins by CD Spectrometer

Circular dichroism. CD spectra were measured at 25 °C using a J-715 spectropolarimeter (Jasco, Tokyo, Japan) in a quartz cell of 10mm path length. Spectra were the average of 10 assessments over the λ = 190–370 nm range at 0.1 nm interval. Samples (~0.25 mg/mL) were prepared in PBS, pH 7.4. The CD results are presented as the mean residue molar ellipticity (Θ) in deg cm2/dmol.

Aggregation Study

Lyophilized insulin derivatives were formulated in PBS at concentration of 1 mg/mL, pH 7.4, with or without the additional $ZnCl_2$. Triplicate samples were prepared for each insulin or insulin derivatives. All samples were incubated at 37 °C and 400 rpm in a MaxQTM 4000 Benchtop Orbital Shaker. Aliquots (20 uL) were taken every 24 h and analyzed by Thioflavin T (ThT) assay, which measures changes in fluorescence intensity of ThT upon binding of the aggreated protein and following the modified Thioflavin-T fluorescence assay protocol (http://www.assay-protocol.com/biochemistry/protein-fibrils/thioflavin-t-spectroscopic-assay). 8 mg of Thioflavin-T (ThT) was dissolved in 10 mL of phosphate buffer (50 mM sodium phosphate, 150 mM of sodium chloride, pH 7.4). Solution was filtered through 0.22 µm syringe filter and stored at 4 °C in dark. Prior to experiment the 0.3 mL of ThT stock solution was further diluted

in 15 mL of the phosphate buffer. 5 μL of investigated peptide solution at 1 mg/mL was added to 400 μL of working solution of ThT in phosphate buffer. Solution was incubated for 20 to 30 min. Then fluorescence intensity was measured on Perkin-Elmer LS50B Luminescence Spectrometer (Perkin-Elmer, Waltham, MA) with following experimental parameters: 350 μL of peptide/ThT solution in sub-micro quartz cuvette [3 x 3 x 3 mm / Z = 9.85 (Hellna GmnG & Co. KG, Mullheim, DE)] excitation λ = 450 nm (slit width 5nm); emission λ = 482 nm (slit width 10 nm) integration 10 second. Signal was averaged from 4 consecutive points.

***In vitro* Characterization of Sialic-Ins 1-4 by Insulin Receptor Phosphorylation Assay**

To measure receptor phosphorylation, human insulin B receptor transfected HEK293 cells were plated in 96 well tissue culture plates (Costar #3596, Cambridge, MA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 IU/ml penicillin, 100 ug/ml streptomycin, 10 mM HEPES and 0.25% bovine growth serum (HyClone SH30541, Logan, UT) for 16-20 h at 37°C, 5% CO_2 and 90% humidity. Serial dilutions of recombinant human insulin or insulin analogs were prepared in duplicate in DMEM supplemented with 0.5% bovine serum albumin (Roche Applied Science #100350, Indianapolis, IN) and added to the wells with adherent cells. After 15 min incubation at 37 °C in humidified atmosphere with 5% CO_2 the cells were fixed with 5% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton-X100 for 25 min with 5 solution changes and blocked with 2% bovine serum albumin in PBS for 1 h. The plate was then washed three times with PBS pH 7.4 supplemented with 0.1% Tween-20 and filled with horseradish peroxidase-conjugated antibody against phosphotyrosine (Upstate biotechnology #16-105, Temecula, CA) at manufacturer's recommended dilution. After 3 h incubation at room temperature the plate was washed 4 times and 0.1 ml of TMB single solution substrate (Invitrogen, #00-2023, Carlbad, CA) was added to each well. Color development was stopped 5 min later by adding 0.05 ml 1N HCl. Absorbance at 450 nm was measured on Titertek Multiscan MCC340 (ThermoFisher, Pittsburgh, PA). Absorbance vs peptide concentration dose response curves were plotted and EC_{50} values

were determined by using logistic nonlinear 3 parameter regression in GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Each experiment was repeated at least three times.

Acute Insulin Tolerance Test in Mice by Subcutaneous Injection

All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male, 14 week-old, C57Bl6/J mice (Jackson Laboratories, ME) were housed on a 12 : 12 hours light-dark cycle (7 am - 7 pm lights on) at 22 °C and constant humidity with free access to standard chow (Teklad LM-485) and water. Mice were fasted overnight and received an injection of streptozotozin (STZ, 150mg/kg, ip, Sigma-Aldrich, MO) freshly dissolved in sodium citrate buffer (pH = 4.5), within the first 2 h following the onset of the light phase. After the injection, food was returned to the mice, and they additionally had access to a 10% sucrose solution for 72 h. Blood glucose was measured via tail laceration using a handheld glucometer (Freestyle, Abbot, IL) to confirm hyperglycemia. On the day of the study, the mice (BW=20.9±1.5 g) were deprived of food at the onset of the light phase. 3 h later, and they received a subcutaneous injection (100 µl) of vehicle (sterile, pyrogen-free phosphate buffer saline or different doses of the compounds indicated in the results section). The blood glucose was monitored 0.5, 1, 2, 3, 6 and 24 h post injection. The statistical analysis of the results obtained in the in vivo experiments was performed using Prism 8.3 h (GraphPad Software, CA) applying 2-way ANOVA for repeated measurements, followed by Dunnett's tests for multiple comparisons of the different treatments, using the human insulin group as control. P values lower than 0.05 were considered significant. The results are presented as means ± SEM of eight replicates per group.

Supporting Information Availability

Fmoc chemistry-based solid phase synthesis of peptide **11a** and Boc chemistry-based SPPS of peptides **11b-d**; UPLC and MS spectrums of peptides **11a-d**, **12a-d**, **17**, **18**, **19a-d** and **1-4**.

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Abbreviations Used:

SPPS, solid-phase peptide synthesis; Sialic-Ins, sialic acid-conjugated human insulin; STZ, streptozotocin; PSA, polysialic acids; rDNA, recombinant DNA; CD, circular dichroism; ThT, Thioflavin T.

Conflict of interest

V.M.G. and F.L. are currently full-time employees and shareholders of Novo Nordisk.

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