Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

An Unnatural Amino Acid Modified Human Insulin for Visual Monitoring of Insulin Aggregation

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1. General Information.

Sigma Aldrich (Cat. No. 91077c) was the source of Human insulin (HI). N-Hydroxy succinimide (NHS), Boc-anhydride, and Trifluoroacetic acid (TFA) were procured from Avra synthesis Pvt. Ltd. (Hyderabad, India), while S. D. Fine-Chem Pvt. Ltd and Finar chemicals were the respective suppliers of HPLC grade acetonitrile (ACN) and dry *N, N*-dimethylformamide (DMF). Thioflavin T (ThT) and *N, N*′-Dicyclohexylcarbodiimide (DCC) were sourced from Sisco Research Laboratories Pvt. Ltd. (SRL). Reactions were monitored by TLC (thin layer chromatography) and carried out on readymade TLC silica gel 60F254 plates from Merck, Dermstadt, Germany. UV light (254 nm) was used to visualize the presence of the compounds. Jasco V-670 spectrophotometer with a 10 mm quartz cell at 25 °C was used for recording UV-Vis absorption spectra. JEOL-JNM spectrometer operating at 400 MHz at 25 °C was used for recording ^{1}H and ^{13}C NMR spectra using 2-10 mM concentration in appropriate solvents using TMS as internal standard or the solvent signals as secondary standards, with the chemical shifts (δ) displayed in ppm scales. ESI-MS or MALDI-ToF-MS were utilized for recording mass spectra. The multiplicities of NMR signals were indicated by s (singlet), d (doublet), and m (multiplet, for unresolved lines). SpectraMax iD3 Multi-Mode Microplate Reader was used for recording ThT fluorescence. Complete proton decoupling was employed in recording ^{13}C NMR spectra at 100 MHz. CD spectra were recorded on JASCO J-815 CD spectrometer.

2. Synthesis:

Synthesis of BHBY-OSu

BHBY (100 mg, 0.241 mmol), synthesized using a reported procedure [***Chem. Commun.***, 2021,**57**, 5290-5293, Ref. 40 of manuscript], was transferred to a 25 mL round bottomed flask containing anhydrous dichloromethane (5 mL), followed by addition of *N*-Hydroxysuccinimide (33 mg, 0.2897 mmol). This solution was cooled to 0°C and to this solution was added *N, N*′-Dicyclohexylcarbodiimide (DCC, 206 mg, 0.386 mmol). The resultant reaction mixture was stirred at 0°C for 30 minutes and was transferred to room temperature for overnight stirring. The progress of reaction was followed with thin layer chromatography (TLC) towards complete transformation of reactants to product. After completion of the reaction, the reaction mixture was passed through celite bed to remove dicyclohexylurea. Filtrate was extracted using water and dichloromethane layer was evaporated using a rotary evaporator to get crude BHBY-OSu. Crude product was purified using column chromatography to get pure BHBY-OSu. Yield = 90 mg (73%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.50 (bs, 1H), 7.99 (d, 1H, *J* = 8.0 Hz), 7.92 (d, 1H, *J* = 8.0 Hz), 7.57 (s, 1H), 7.51 (t, 1H, *J* = 7.6 Hz), 7.42 (t, 1H, *J* = 8.0 Hz), 7.32 (dd, 1H, *J* = 8.4 Hz), 7.07 (d, 1H, *J* = 8.4 Hz), 4.98 (s, 1H), 3.35-3.20 (m, 2H), 2.87 (s, 4H), 1.43 (s, 9H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.25, 168.63,

157.42, 151.85, 134.24, 132.83, 129.53, 126.77, 125.64, 122.28, 121.64, 118.32, 116.85, 80.79, 52.63, 37.52, 29.76, 28.32, 25.68 ppm; HRMS-ESI $[M+H]^+$ calculated for $C_{25}H_{26}N_3O_7S^+$, *m/z* = 512.1486; experimental = 512.1485.

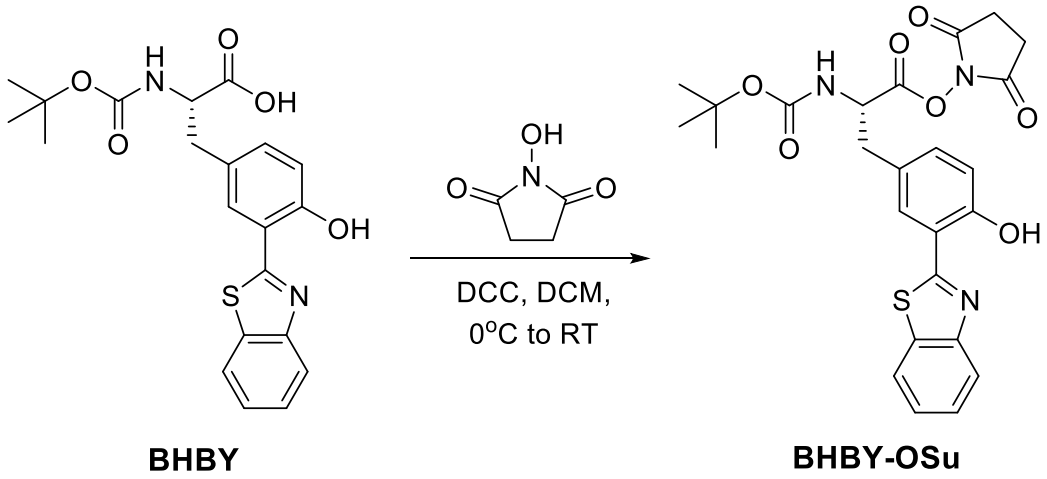


Figure S1. Synthesis of BHBY-OSu.

Synthesis of Lys B29 conjugated Boc-3-(2-Benzothiazolyl)-tyrosine-human insulin (Boc BT-HI)

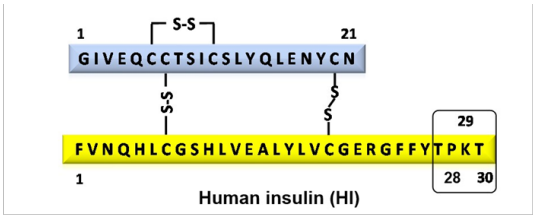
Human insulin (20 mg, 3.448 µmol) solubilized in 10 mM HCl solution (2 mL) and to this 14 mL of 100 mM carbonate-bicarbonate buffer (pH 10) was added and adjusted to the final pH of the reaction mixture to pH 10. BHBY-OSu (5.28 mg, 3 equiv., 10.344 µmol) was dissolved in 100 µL of dimethylformamide and added to the reaction mixture (figure S2). Addition of extra BHBY-OSu carried out at 2 hours intervals twice. Reaction was carried out at room temperature with moderate stirring. Desired product formed would be BHBY-HI (Boc BT-HI). Extent of reaction was screened through the analytical high-performance liquid chromatography using C18 reverse phase analytical column.

The formation of product can be analysed through the appearance of a new peak around in addition to the peak of human insulin in HPLC C18 analytical column elution. Reaction mixture was lyophilized, and the dried reaction mixture was dissolved in 1:1 mL of HPLC grade water: Acetonitrile. This solution was injected in the preparative C18 reverse phase column and corresponding product (Boc BT-HI) peak was collected. Acetonitrile present in the collected fraction was evaporated at 40 °C under reduced pressure and afterwards the sample was lyophilized, and we got Boc BT-HI with overall yield of 69.17 % (14.8 mg, 2.385 µmol).

Synthesis of Lys B29 conjugated 3-(2-Benzothiazolyl)-tyrosine-human insulin (BT-HI)

Boc deprotection was carried out by treating Boc BT-HI (14.8 mg, 2.385 µmol) with 2 mL of trifluoroacetic acid at room temperature for 20 min (figure S2). Trifluoroacetic acid was evaporated at 40 °C under reduced pressure and afterwards Boc deprotected insulin derivative was precipitated with anhydrous diethyl ether. Solvent was drained off to recover precipitated

insulin. Afterwards 2 mL of HPLC-grade water was added to solubilize insulin and afterwards lyophilized to get 3-(2-Benzothiazolyl)-tyrosine conjugated human insulin derivative (BT-HI) as powder with almost quantitative yield. BTHI was characterized through MALDI-MS analysis by observing the peak at 6097.037 Da (figure S4).



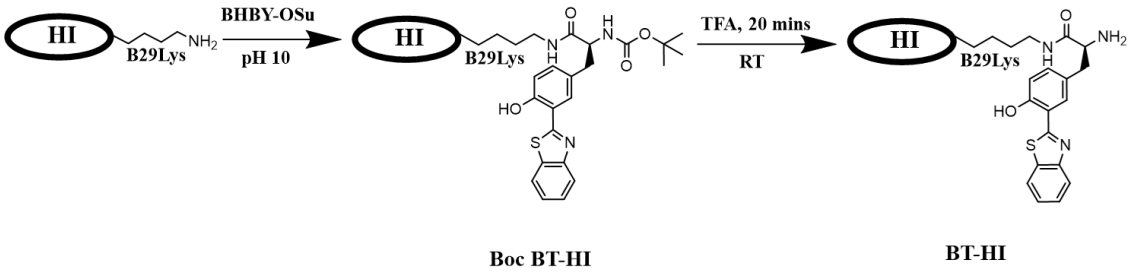


Figure S2. Synthesis of BT-HI.

3. Methods

High performance liquid chromatography (HPLC) analysis

HPLC analyses were performed with a HPLC system (Agilent technologies 1260 infinity) equipped with a quaternary pump (G1311B), auto liquid sampler (G1329B), Diode array detector (G1315D) and analytical scale-fraction collector (G1364C). Instrument control, data acquisition and data analysis was performed using a ChemStation software (Agilent Technologies, Workingham, UK). A ZORBAX Eclipse plus C18 (250 x 4.6 mm) column with 5 µm particle size at room temperature was used. Mobile phase consisted of acetonitrile/water with 0.1 % TFA and the flow rate was 1.0 mL/min. Injection volume was 10 µL and the column effluent was monitored at 220 nm.

Circular dichroism spectroscopy. Conservation of the structural integrity even after protein modification was confirmed by circular dichroism (CD) spectroscopy. Spectra were collected using JASCO J-815 CD spectrometer and a quartz cuvette of 1 mm path length. CD spectra of proteins (0.5 mg/mL or 85 µM) were collected between 195 to 300 nm and each spectrum represents the average of three scans. To avoid any instrumental baseline drift contributed by the working buffer, the background values were subtracted from each individual sample measurement.

UV-Vis spectroscopy. UV-Vis absorption spectra of HI as well as BT-HI (0.5 mg/mL or 85 µM) were analyzed on a UV-Vis spectrophotometer (VARIAN CARY 100 Bio) by using a 10 mm quartz cell at 25 ± 0.1 °C (figure 2a).

Fluorescence spectroscopy. Fluorescence spectra of incubated BT-HI (0.5 mg/mL or 85 µM) samples were measured at different time intervals (days) on a Varian Luminescence Cary Eclipse spectrophotometer using 10 mm quartz cells at 25 ± 0.1 °C.

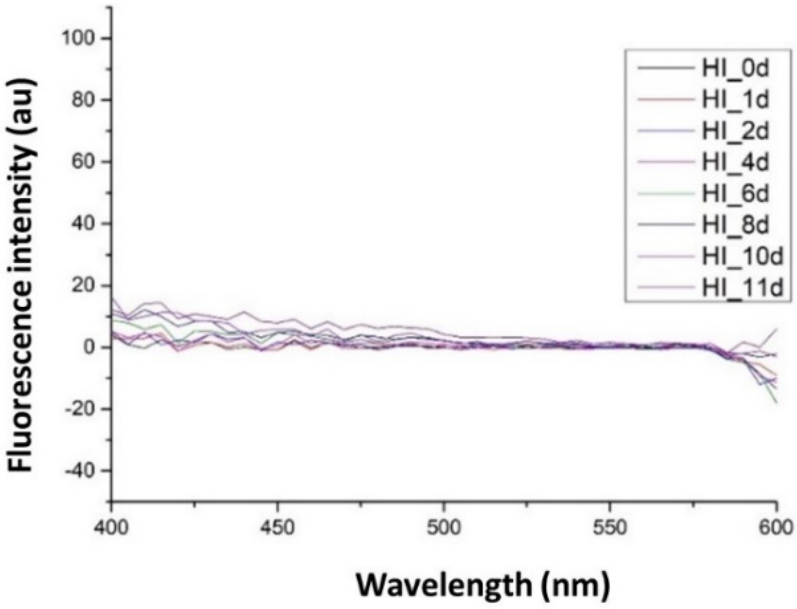


Figure S3. Time dependent fluorescence spectra (λ_{ex} 254 nm) of incubated HI sample.

Thioflavin T fluorescence assay. Thioflavin T (ThT) is an aggregation detection dye which demonstrates an enhancement in its fluorescence upon binding to insulin aggregates. 85 µM (0.5 mg/mL) solution of BT-HI has been prepared in water (pH 1.6). This solution was incubated at a higher temperature of 65 ^{o}C. From the incubated sample, 30 µL of different samples were taken out at different time intervals and mixed with 200 µL of 50 µM Thioflavin T (ThT) dye prepared in water (pH 1.6). Afterwards these ThT mixed insulin samples were kept for 10 min of incubation in dark. Using a Corning® 96 well clear flat bottom black polystyrene microplate corresponding emission spectra were collected from 450 to 560 nm with an excitation at 410 nm (λ_{ex}) in SpectraMax iD3 Multi-Mode Microplate Reader. The spectra were recorded at different time points (days) of incubation and the graph was plotted with emission value at 488 nm.

Plot of normalized autofluorescence intensity of incubated BT-HI at 65 °C were measured at different timepoints. Using a Corning® 96 well clear flat bottom black polystyrene microplate corresponding emission spectra were collected from 450 to 560 nm with an excitation at 254 nm (λ_{ex}) in SpectraMax iD3 Multi-Mode Microplate Reader.

The spectra were recorded at different time points (days) of incubation and the graph was plotted with emission value at 510 nm.

Atomic force microscopy (AFM). Comparative time dependent visualization of fibrillation were carried out by atomic force microscopy. 5 µL of incubated samples were drop casted on glass surface at room temperature. All samples were allowed to air dry at room temperature for overnight followed by subsequent drying under vacuum for 30 minutes prior to scanning. The samples were scanned with an atomic force microscope (Asylum Research, Oxford Instruments, MFP-3D Origin) at room temperature. Scanning was carried out under tapping mode with a force constant of 21 N/m. Silicon nitride cantilever from Nanosensors with following features was used; Resonance frequency: 170 kHz, Thickness :7.0 µm, length :225 µm, width: 38 µm.

Insulin Tolerance Test. The mice experiments were approved by the Institutional Animal Ethics Committee (protocol: IITK/IAEC/2018/1048). The animals were maintained at a 12:12-hr light-dark cycle at a constant temperature (22°C ± 1 °C), with ad libitum access to food and water. Three-to-four months-old wild-type males (C57BL6/J) were used for the intra-peritoneal administration of Streptozotocin (STZ, S0130, Sigma-Aldrich, 50 mg/kg for 7 days). Following this, the blood glucose levels of the STZ-treated and the vehicle-treated mice were monitored 7 days post-injection, every alternate day, for the onset of diabetes using a glucometer (LifeScan Inc.). For the insulin tolerance test, the STZ-treated mice were starved overnight and injected with either wild-type insulin (HI) or the modified BT-insulin analog (BT-HI) sub-cutaneously. The injection was administered at a dosage of 1 µg of HI or BT-HI per 100 µL of 0.9% saline water for every 30 grams of body weight. The blood glucose levels were monitored post-injection for the time points indicated until an increase in the dropped blood glucose level was observed (8 hr.) using the glucometer. The absolute blood glucose levels in mg/dl were plotted against time (hour) for both the experimental groups.

MTT assay. HEK 293T cells were grown at 37°C, 5% CO_2, in the incubator. A stock solution of BT-HI was prepared at a concentration of 1.6 mg per 80 µL using 10 mM HCl as the solvent. To obtain samples of the desired concentration, specific volumes were withdrawn from the stock solution and then diluted with PBS buffer at pH 7.4. Cells were treated with BT-HI for 24h at different concentrations. Post-treatment, the cells were incubated with 0.5 mg/ml MTT (thiazolyl blue tetrazolium bromide) for 3 h to assess the cell viability, which is a colorimetric assay. Once the media was removed, the cells were incubated with DMSO (100%) for 20 minutes to dissolve the purple-colour formazan crystals. The shift in optical density at 570 nm relative to a 650 nm reference was used to record using a spectrophotometer. Fold change in cell survival was calculated as a mean of three independent experiments and plotted with respect to the non-treated control.

Table S1: Estimation of Secondary structure of HI and BT-HI. % Secondary structure of HI and BT-HI were calculated for numerical comparison by using BestSel online server.[1,2]

% Secondary Structure	HI	BT-HI
Helix	27.4	23.8
Antiparallel	21.8	19.9
Parallel	5	3.6
Turn	13.4	13.3
Others	32.4	39.3

CD spectra of HI.

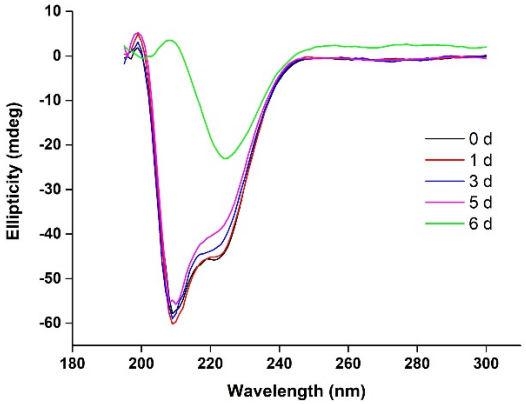


Figure S4. Circular Dichroism (CD) spectra of human insulin (HI) at different time points (in days). Solutions were prepared in 10 mmol HCl water (pH 1.6) to a protein concentration of 0.5 mg/mL (85 µM). Incubation condition: 65 °C and pH 1.6 at 85 µM concentration.

Blood glucose level of diabetic mice after treatment of BT-HI.

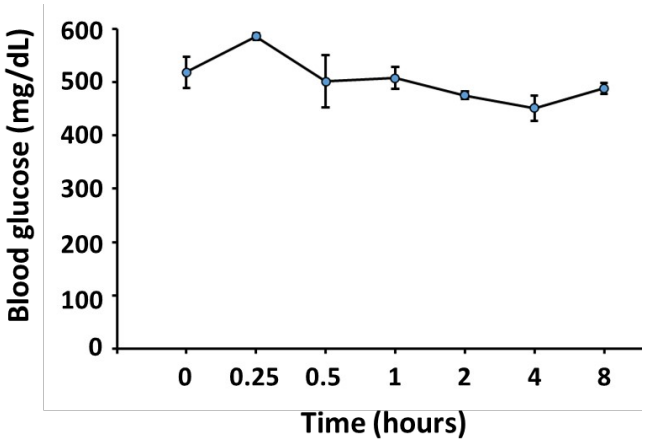


Figure S5. Blood glucose level after sub-cutaneous injection (1µg/100ul per 30 gm of mice) of BT-HI after incubating it for 11 days in diabetic mice. Data is represented as the mean of each group (n=4) for each time point. Error bars represent the S.E of mean.

CD and fluorescence spectra at pH 7.4. CD and fluorescence spectra of incubated BT-HI (Incubation condition: 65 °C and pH 7.4 at 85 µM concentration) samples were measured at different time intervals (days)

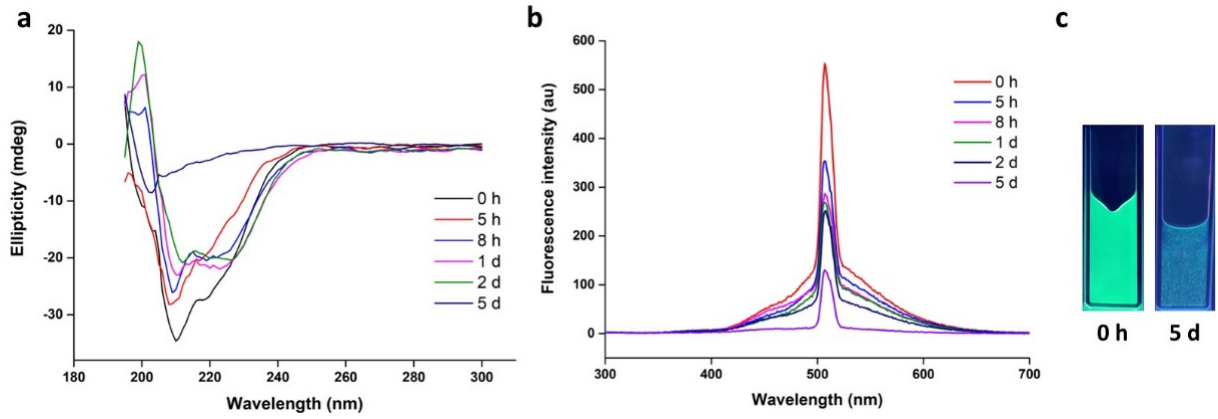


Figure S6. **a**. CD spectra of incubated BT-HI samples at different time-points (in days); **d.** Time dependent fluorescence spectra (λex 254 nm) of incubated BT-HI samples; **c.** UV-irradiated (254 nm) pictures of BT-HI samples after 0 and 5 days of incubation. Incubation condition: 65 °C and pH 1.6 at 85 µM concentration.

SDS-PAGE. Polyacrylamide gel electrophoresis was performed at a constant voltage at 60 mA with a Bio-Rad mini-PROTEIN II electrophoresis system using 15% Tris-HCl polyacrylamide gel. Aliquots of HI and BT-HI samples were loaded into separate wells of the gel. After completion the gel was stained with coomassie blue stain dye and further destained overnight. BT-HI shows equivalent molecular weight as well as purity as compared to the commercial HI sample.

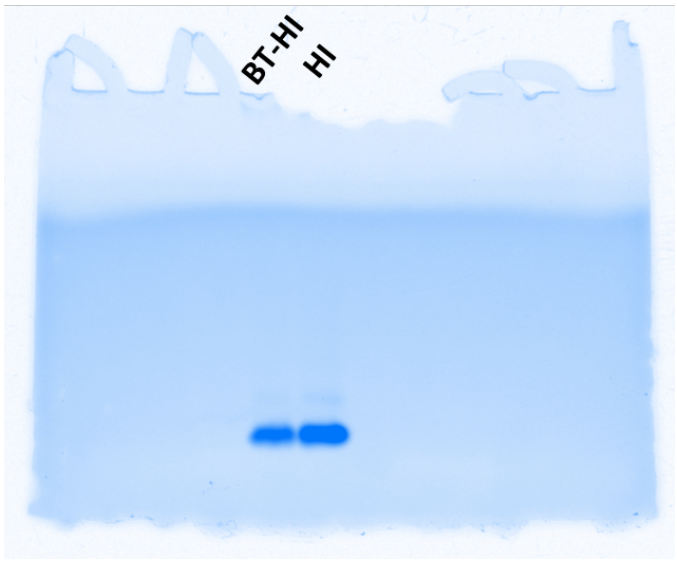


Figure S7. SDS PAGE of HI and BT-HI

Fluorescence excitation and emission spectrum of BT-HI.

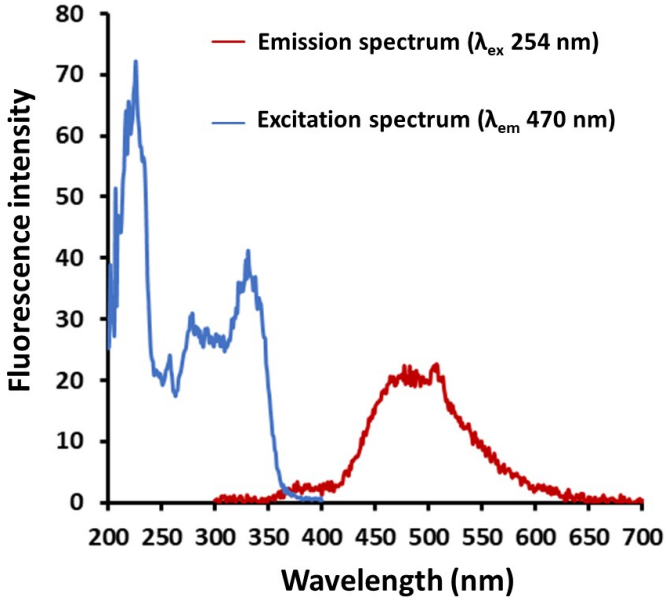


Figure S8. Fluorescence Excitation and emission spectrum of BT-HI.

Table S2: Summarized Photophysical properties

Probe	Excitation (λ_{ex})	Emission maxima (λ_{em})	Colour (Irradiated at 254 nm)
In soluble insulin	254 nm	460 nm	Cyan
In aggregated insulin	254 nm	510 nm	Green

4. Copy of mass spectrum:

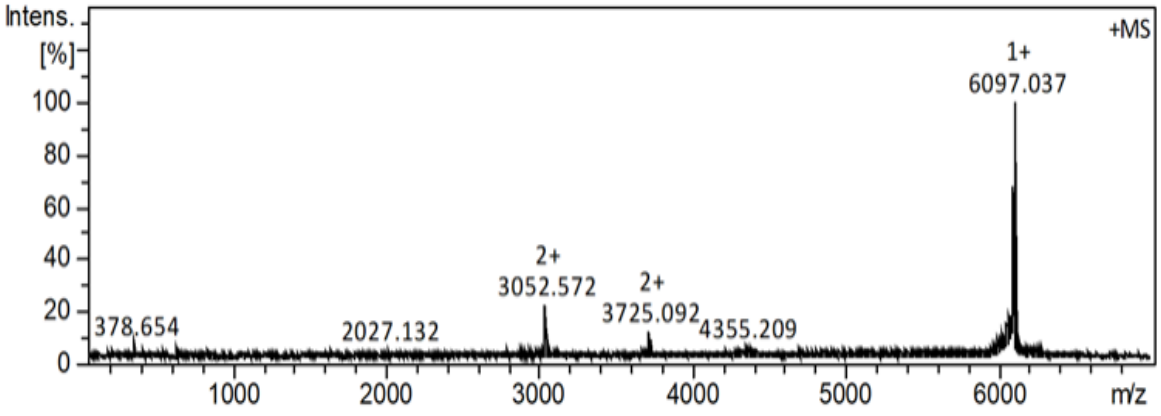


Figure S9. Mass spectrum of **BT-HI**

5. Copies of ^{1}H and ^{13}C NMR spectra

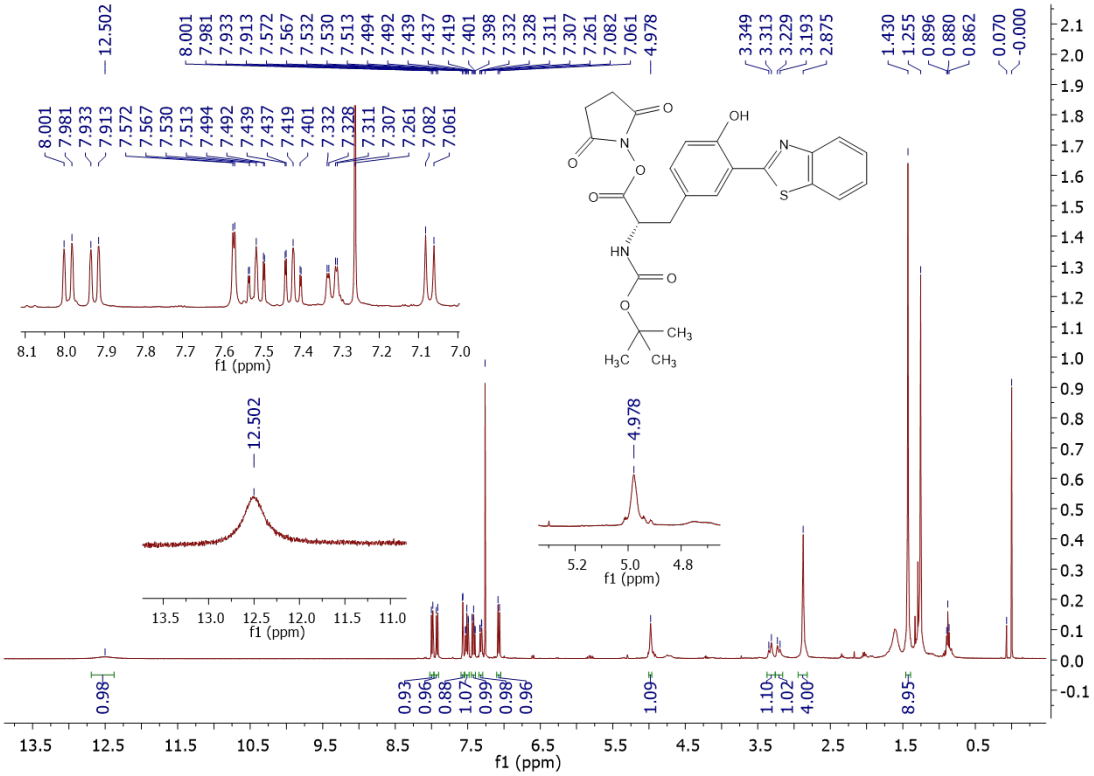


Figure S10. ^{1}H NMR spectrum of BHBY-OSu.

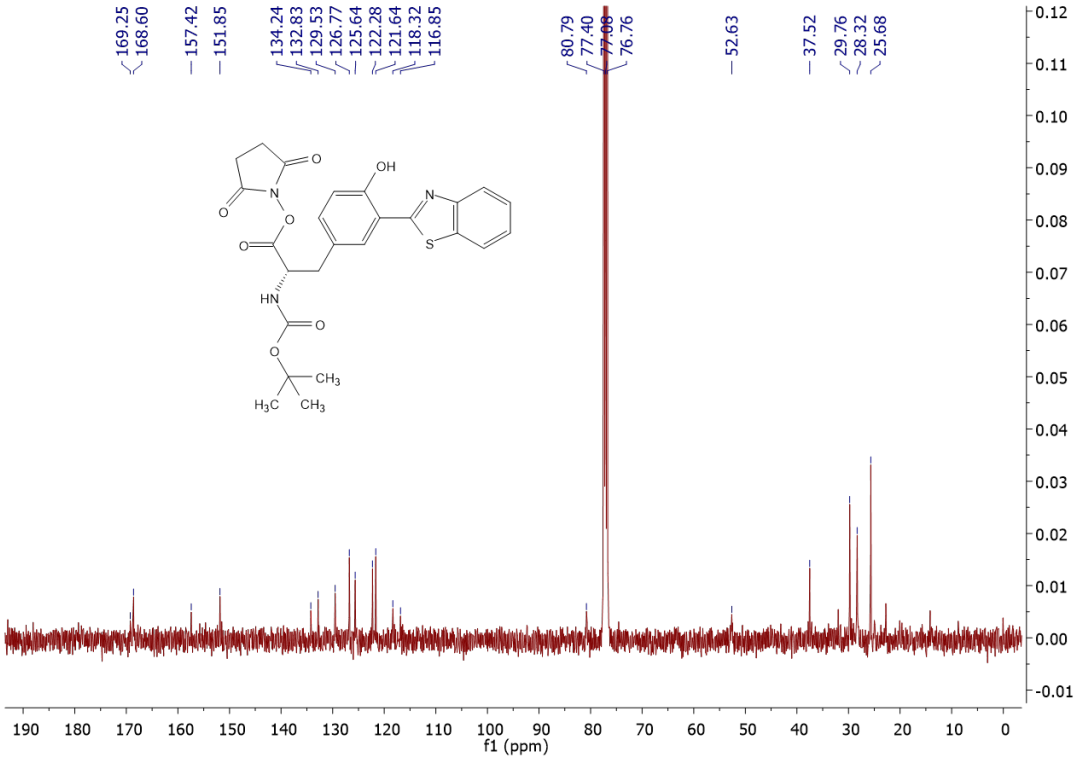


Figure S11. ^{13}C NMR spectrum of BHBY-OSu.

6. High performance liquid chromatography (HPLC) analysis

HPLC analyses were performed with a HPLC system (Agilent technologies 1260 infinity) equipped with a quaternary pump (G1311B), auto liquid sampler (G1329B), Diode array detector (G1315D) and analytical scale-fraction collector (G1364C). Instrument control, data acquisition and data analysis was performed using a ChemStation software (Agilent Technologies, Workingham, UK). A ZORBAX Eclipse plus C18 (250 x 4.6 mm) column with 5 µm particle size at room temperature was used. Mobile phase consisted of acetonitrile/water with 0.1 % TFA and the flow rate was 1.0 mL/min. Injection volume was 10 µL and the column effluent was monitored at 220 nm. Program set with the initial mobile phase composition of acetonitrile/water (25:75) with gradual increase in acetonitrile concentration from 25 % to 70 % within 20 min of run. Further acetonitrile concentration was increased from 70 % to 95 % within next 5 min of run.

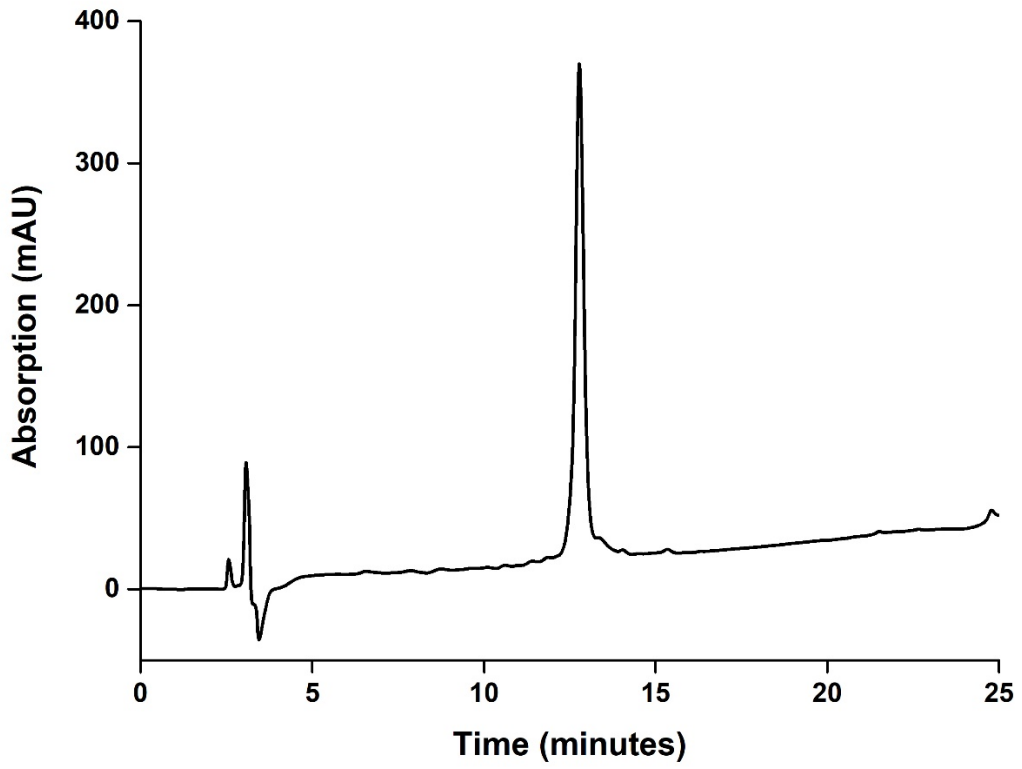


Figure S12. Analytical HPLC chromatogram of **BT-HI** at wavelength 220 nm.

7. References

1 A. Micsonai, F. Wien, É. Bulyáki, J. Kun, É. Moussong, Y.-H. Lee, Y. Goto, M. Réfrégiers and J. Kardos, *Nucleic Acids Res.*, 2018, **46**, W315–W322.

2 A. Micsonai, F. Wien, L. Kernya, Y.-H. Lee, Y. Goto, M. Réfrégiers and J. Kardos, *Proc. Natl. Acad. Sci.*, 2015, **112**, E3095–E3103.