Supporting Information

Site-Selective Synthesis of Insulin Azides and Bioconjugates

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1. General procedures

All chemicals were purchased from commercial sources unless otherwise noted. Reactions were carried out at ambient temperature unless otherwise noted. Reactions sensitive to moisture or air were performed under nitrogen or argon using anhydrous solvents and reagents. Sequencing grade endoproteinase Glu-C was purchased from Promega. The progress of reactions was monitored by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). The ultra-performance liquid chromatography (UPLC) system was coupled to a Waters single quad detector operated under electrospray ionization (ESI) in positive ion detection mode.

The exact masses of intact insulin analogs were measured by a Waters Synapt G2-Si high-resolution mass spectrometer coupled to a Waters Acquity UPLC system. The mass spectrometer was operated in ESI positive mode. A Waters Acquity UPLC BEH C18 column (130 Å, 1.7 µm, 2.1 mm x 100 mm) was used for LC separation. Mobile phase A included water with 0.05% trifluoroacetic acid (TFA), and mobile phase B included acetonitrile with 0.05% TFA. The elution gradient was as follows: 5% B for 0.5 min, 5-95% B from 0.5 to 5 min, 95% B from 5 to 6 min, and 5% B from 6-8 min. The MS raw data was deconvoluted using the MaxEnt3 function on Waters UNIFI software to derive the mono-isotopic mass of the insulin analog molecules.

Preparative scale HPLC was performed on a Gilson 333-334 binary system using Waters DELTA PAK C4 15 µm, 300 Å, 50 x 250 mm column or KROMASIL® C8 10 µm, 100 Å, 50 x 250 mm column, flow rate = 85 mL/min, with gradient noted. Concentration of solutions was carried out on a rotary evaporator under reduced pressure or freeze-dried on a VirTis Freeze mobile Freeze Dryer (SP Scientific).

2. Preparation of buffer solutions

All buffers were prepared at 100 mM.

pH 5 (70 mL 0.1 M NaOAc and 30 mL 0.1 M HOAc)
pH 6 (6.15 mL 0.2 M Na2HPO4, 43.85 mL 0.2 M NaH2PO4; diluted to 100 mL with H2O)
pH 7 (30.5 mL 0.2 M Na2HPO4, 19.5 mL 0.2 M NaH2PO4; diluted to 100 mL with H2O)
pH 8 (47.35 mL 0.2 M Na2HPO4, 2.65 mL 0.2 M NaH2PO4; diluted to 100 mL with H2O)
pH 9 (10 mL 0.1 M Na2CO3 and 90 mL 0.1 M Na2HCO3)
pH 10 (60 mL 0.1 M Na2CO3 and 40 mL 0.1 M Na2HCO3)
3. Procedure for the synthesis of RHI mono-azides 4-6 and tri-azide 2

(a) imidazole-1-sulfonyl azide·HCl, CuSO₄·H₂O, aq. NaHCO₃, H₂O:MeOH (9:1 v/v), 6 h to overnight (b) phenyl acetylene, CuSO₄·H₂O (0.02 eq.), sodium ascorbate, DMSO, 2 h

1H-Imidazole-1-sulfonyl azide hydrochloride (102 mg, 0.485 mmol) was added to RHI (1, 3000 mg, 0.485 mmol), sodium bicarbonate (5.79 mg, 0.069 mmol) and copper(II) sulfate·5H₂O (0.619 mL, 0.097 mmol) dissolved in water (3.2 mL) and MeOH (0.8 mL). The reaction was maintained at pH ~9 by adding aqueous saturated NaHCO₃ solution (~0.5-1 mL) and stirred at room temperature for 6 hours. The reaction was monitored by LCMS for the formation of mono, di and tri azide products. After 6 h, a few drops of 1N aq. HCl were added in order to make the solution fully homogeneous and the solution was then filtered. Preparative reverse phase chromatography gave the purified azido RHI products.

A1-glycine azide RHI 4  Rₜ = 2.64 min.; Molecular formula: C₂₅₇H₃₈₁N₆₇O₇₇S₆; HRMS: Calculated protonated exact mass: 5830.6354; Observed protonated exact mass: 5830.6626

B29-lysine azide RHI 5  Rₜ = 2.70 min.; Molecular formula: C₂₅₇H₃₈₁N₆₇O₇₇S₆; HRMS: Calculated protonated exact mass: 5830.6354; Observed protonated exact mass: 5830.6558

B1-phenylalanine azide RHI 6 (Rₜ = 2.73 min.; Molecular formula: C₂₅₇H₃₈₁N₆₇O₇₇S₆; HRMS: Calculated protonated exact mass: 5830.6354; Observed protonated exact mass: 5830.6675

A1B1B29-tri-azide RHI 2  Rₜ = 2.95 min.; Molecular formula: C₂₅₇H₃₇₇N₇₁O₇₇S₆; HRMS: Calculated protonated exact mass: 5882.6169; Observed protonated exact mass: 5882.6567
Synthesis of B29-lysine azido RHI 5

RHI 1 (100 mg, 0.017 mmol) was dissolved in DMAc (0.4 mL), followed by the addition of pH 10 buffer (10 mL) and 1H-imidazole-1-sulfonyl azide·HCl salt (0.361 mL, 0.017 mmol, 10 mg/mL in MeOH). The reaction mixture was stirred at room temperature overnight (16 h). LCMS analysis showed formation of B29-lysine azide RHI 5 azide. After a few drops of 1 N aq. HCl were added to clarify the reaction mixture, the solution was filtered and purified by reverse phase chromatography. B29-lysine azide RHI 5 (24 mg, 32% yield based on recovered starting material) was isolated in addition to unreacted starting material (47 mg).

Synthesis of B1-phenylalanine azide RHI 6

RHI 1 (100 mg, 0.017 mmol) was dissolved in DMAc (0.4 mL) followed by the addition of pH 8 buffer (10 mL) and 1H-imidazole-1-sulfonyl azide·HCl salt (0.361 mL, 0.017 mmol, 10 mg/mL in MeOH). The reaction mixture was stirred at room temperature overnight (16 h). LCMS analysis showed formation of B1-phenylalanine azide RHI 6 azide. After a few drops of 1 N aq. HCl were added to clarify the reaction mixture, the solution was filtered and purified by reverse phase chromatography. B1-phenylalanine azide RHI 6 (16 mg, 30% yield based on recovered starting material) was isolated in addition to unreacted starting material (31 mg).

Synthesis of A1B1B29-tri-azide RHI 2

RHI 1 (100 mg, 0.017 mmol), sodium bicarbonate (5.79 mg, 0.069 mmol), and CuSO₄·5H₂O (0.550 mg, 3.44 µmol) were dissolved in water (0.800 mL) and MeOH (0.200 mL). To this mixture was added 1H-imidazole-1-sulfonyl azide·HCl salt (14.4 mg, 0.069 mmol) and the pH was maintained at pH 8-9 by the addition of aq. sat. NaHCO₃ solution. After stirring at room temperature for 6 h, LCMS analysis showed the formation of the A1B1B29-tri-azide RHI 2. After a few drops of 1 N aq. HCl were added to clarify the reaction mixture, the resulting solution was filtered and purified by reverse phase chromatography. A1B1B29-tri-azide RHI 2 was isolated in 72% (73 mg) yield.

4. Structural confirmation of 2, 4, 5, & 6 through Glu-C digestion

The structures of compounds 2, 4, 5 and 6 were confirmed by performing endoproteinase Glu-C digestion. Glu-C is a serine protease that specifically cleaves at the C-terminus of glutamic acid residues. The proteins of interest were dissolved in 50 mM ammonium bicarbonate buffer, and subjected to Glu-C digestion at 37 °C overnight (insulin to enzyme ratio is approximately 10 to 1 w/w). The peptide digests were separated by UPLC, and the total run time was 26 min (mobile phase A included water with 0.05% trifluoroacetic acid (TFA), and mobile phase B included acetonitrile with 0.05% TFA). The initial condition was 5-20% B for 3 min, 20-40% B from 3 to 15 min, 40-95% B from 15 to 20 min, and 95% B from 20 to 22 min, then B was dropped to 5% within 0.1 min and was kept at 5% until 26 min for equilibration. By comparing the LC/MS total ion chromatograms of the native human insulin and modified insulin analogs that were subject to Glu-C digestion separately, the peak(s) corresponding to peptide digests containing azide modification was identified. The exact masses of the characteristic insulin analog digests were used to pinpoint the exact location of the azide modifications.
5. General procedure for the synthesis of RHI triazole conjugates 7-9, 15-21

Mono-azide RHI 4/5/6 (1.7 µmol) was dissolved in DMSO (0.5 mL) under a constant flow of nitrogen. To this solution was added alkyne (R^6) (3.4 µmol) and freshly prepared CuSO₄·5H₂O in water (5.1 µmol). [Note: tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (5.14 µmol) can also be added] This was followed by dropwise addition of sodium 2-(1,2-dihydroxy-ethyl)-4-hydroxy-5-oxo-2,5-dihydro-furan-3-olate in water (6.9 µmol). After stirring the reaction mixture at room temperature for 2 h, LCMS analysis was used to track the formation of the desired triazole RHI product. After a few drops of 1 N aq. HCl were added to clarify the reaction mixture, the solution was filtered and purified by reverse phase chromatography.

For compound 19, no copper(II) catalyst was added (Strain-Promoted Azide–Alkyn Cycloaddition (SPAAC)).¹⁴
6. Procedures for site-selective bioconjugation: Synthesis of compounds 22, 23 and 24

Site-selective bioconjugation of A1B1B29 RHI

(a) imidazole-1-sulfonyl azide (1 eq.), CuSO₄·H₂O, pH 8 buffer, DMAc, RT, 2 d, 66%; (b) biotin propargyl alkyne (2 eq.), aq. CuSO₄·H₂O (0.02 eq.) Na-ascorbate, DMSO, RT, 2 h, 55%; (c) MeO-PEG5-NHS ester (2 eq.), Et₃N (6 eq.), RT, 2 h, 58%

Synthesis of B1-azido B29-phenyltriazole RHI 22

B1-phenyl-triazole RHI 8 (60 mg, 10.11 µmol) was dissolved in DMAc (0.4 mL), followed by addition of pH 8 buffer (10 mL) and 1H-imidazole-1-sulfonyl azide·HCl salt (10.11 µmol). After
stirring the reaction mixture at room temperature for 2 days and monitoring the formation of the desired product by LCMS, a few drops of 1 N aq. HCl was used to clarify the reaction mixture. This solution was filtered and purified by reverse phase chromatography to provide B1-azido B29-phenyltriazole RHI 22 (18.5 mg, 31% yield). Rt = 3.40 min.; Molecular formula: C_{265}H_{385}N_{69}O_{77}S_{6}; HRMS: Calculated protonated exact mass: 5958.6729; observed protonated exact mass: 5958.5557.

Synthesis of B1-biotin triazole-B29-phenyl triazole RHI 23

B1-azido B29-phenyltriazole RHI 22 (10 mg, 1.68 µmol) was dissolved in DMSO (0.5 mL) under a flow of nitrogen. To this solution was added 5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-N-(prop-2-yn-1-yl)pentanamide (biotin propargyl amide, 3.35 µmol) and freshly prepared CuSO₄ꞏ5H₂O in water (5.03 µmol). After adding a solution of sodium 2-(1,2-dihydroxy-ethyl)-4-hydroxy-5-oxo-2,5-dihydro-furan-3-olate dissolved in water (6.71 µmol), the reaction was stirred at room temperature for 2 h and the reaction progress was monitored by LCMS. The addition of a few drops of 1 N aq. HCl clarified the reaction mixture, which was then filtered and purified by reverse phase chromatography to give B1-biotin triazole-B29-phenyl triazole RHI 23 (5.5 mg, 53% yield). Rt = 3.09 min.; Molecular formula: C_{278}H_{404}N_{72}O_{79}S_{7}; HRMS: Calculated protonated exact mass: 6239.7927; observed protonated exact mass: 6239.6587.
Synthesis of A1-methoxy PEG5 amide-B1-biotin triazole-B29-phenyl triazole RHI 24

B1-Biotin triazole-B29-phenyl triazole RHI 23 (3 mg, 0.481 µmol) was dissolved in acetonitrile and water (9:1 v/v, 1 mL) under nitrogen flow. To this solution was added triethylamine (2.88 µmol) followed by dropwise addition of methoxy-PEG5-NHS ester (0.961, µmol) dissolved in acetonitrile. The reaction mixture was stirred at room temperature for 2 h, after which a few drops of 1 N aq. HCl was added to clarify the reaction mixture. The resulting solution was filtered and purified by reverse phase chromatography to give A1-methoxy PEG5 amide, B1-biotin triazole, B29-phenyl triazole RHI 24 (1.8 mg, 58 % yield). R<sub>t</sub> = 3.30 min.; Molecular formula: C<sub>290</sub>H<sub>426</sub>N<sub>72</sub>O<sub>85</sub>S<sub>7</sub>; HRMS: Calculated protonated exact mass: 6501.9343; Observed protonated exact mass: 6501.8330.
7. Human insulin receptor binding assay protocol

The IR binding assay was run in a scintillation proximity assay (SPA), 384-well format using cell membranes prepared from CHO cells overexpressing human IR(B) grown in F12 media containing 10% FBS and antibiotics (G418, penicillin/strepavidin). Cell membranes were prepared in 50 mM Tris buffer, pH 7.8, containing 5 mM MgCl₂. The assay buffer contained 50 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 5 mM MgCl₂, 0.1% BSA and protease inhibitors (Complete-Mini-Roche). Cell membranes were added to WGA PVT PEI SPA beads (5 mg/mL final concentration) followed by addition of insulin derivatives at appropriate concentrations. After 5-15 min incubation at room temperature,¹²⁵[I]-insulin was added at 0.015 nM final concentration for a final total volume of 50 μL. The mixture was incubated with shaking at room temperature for 1 to 12 hours followed by scintillation counting to determine ¹²⁵[I]-insulin binding to the IR.
8. Chromatograms of RHI 1 and mono-azido RHI products 4, 5, 6 and tri-azide RHI 2

| Compd. # | RHI Description |
|----------|-----------------|
| 1        | $R^1 = R^2 = R^3 = NH_2$ |
| 2        | $R^1 = R^2 = R^3 = N_3$ |
| 4        | $R^1 = N_3; R^2 = R^3 = NH_2$ |
| 5        | $R^3 = N_3; R^1 = R^2 = NH_2$ |
| 6        | $R^2 = N_3; R^1 = R^2 = NH_2$ |
Figure S1. Azidation of RHI (1) with one equivalent ISA
9. Structural confirmation of mono-azido RHI products 4, 5, 6 and tri-azide RHI 2 by Glu-C digestion

| Fragments | Exact Mass (unmodified) | Exact Mass (N3-modified) |
|-----------|------------------------|--------------------------|
| RHI       | 5803.63765             | 5829.62815               |
| F4        | 416.2271               | 442.2271 (on A1)         |
| F1-F5     | 2967.3024              | 2993.2929 (on B1)        |
| F2-F6     | 1376.5741              | N/A                      |
| F3        | 1115.5764              | 1141.5669 (on B29)       |

N/A Not Applicable
MS TIC for A1-glycine azide RHI 4 vs. RHI 1 after GluC Digestion

Expected m/z 443.2249
Observed m/z 443.2273

Compound 4

RHI 1
MS TIC for B29-lysine azide RHI 5 vs. RHI 1 after GluC Digestion

Expected m/z 1142.5742
Observed m/z 1142.5889

N$_3$ on F3 frag

Sample 2

Compound 5

RHI 1
MS TIC for B1-phenylalanine azide RHI 6 vs. RHI 1 after GluC Digestion

Conclusion: mono-N$_3$ modification on B chain N-terminus (B1) or Lys residue (B29)
MS TIC for A1B1B29-triazide RHI 2 and RHI 1 after GluC Digestion

**Compound 2**

- N3 on F1-F5 frag
  - Expected exact mass: 2993.2929
  - Observed m/z: 1497.9707 (z=2)

- N3 on F3 frag
  - Expected m/z: 1142.5742
  - Observed m/z: 1142.5837

**RHI 1**

- Tri-N₃ modification on A chain N-terminus, B chain N-terminus and Lys residue
10. Systematic Study of Azidation Conditions of RHI (1) with ISA·HCl at pH (5-10) and +/- Cu(II)

Conversion Area Percentage (CAP) on Y-axis was based on TIC determined by UPLC UV (210-400 nm) area%
Conversion Area Percentage (CAP) on Y-axis was based on UV area % determined by UPLC (DAD 210-400 nm)
11. HRMS Data

Recombinant Human Insulin (RHI) (1)
Compound 2

Chemical Formula: C_{20}H_{27}N_{5}O_{7}S_{2}
Expected Exact Mass: 5881.60914 Da

HRMS Deconvoluted Mass
Protonated Exact Mass
Expected: 5882.62 Da
Observed: 5882.66 Da

LC-MS TIC
Retention time [min]
TIC [Counts]

HRMS
Observed mass [m/z]
Intensity [Counts]

Deconvoluted mass
5882.65674 Da
Compound 3

Chromatogram: LC-MS TIC

Spectra:
- HRMS
- Deconvoluted mass

Protonated Exact Mass
Expected: 6188.76 Da
Observed: 6188.82 Da
Compound 4

Chemical Formula: C₄₀H₆₃N₂O₇S₈
Exact Mass: 5830.66 Da

Protonated Exact Mass:
Expected: 5830.64 Da
Observed: 5830.66 Da
Compound 5

Chemical Formula: \( \text{C}_{25}\text{H}_{38}\text{N}_{6}\text{O}_{5}\text{S}_{2} \)

Exact Mass: 5829.62815

Protonated Exact Mass
Expected: 5830.64 Da
Observed: 5830.66 Da
Compound 6

Chemical Formula C_{31}H_{30}N_{12}O_{14}S_{6}
Exact Mass: 5829.62815

Deconvoluted mass
Protonated Exact Mass
Expected: 5830.64 Da
Observed: 5830.67 Da
Compound 7

Chemical Formula: C_{28}H_{30}N_{2}O_{5}S_{2}

Exact Mass: 5931.67510

Deconvoluted mass

Protonated Exact Mass
Expected: 5932.68 Da
Observed: 5932.56 Da
Compound 8

Chemical Formula: C_{33}H_{55}N_{12}O_{11}S_{5}

Exact Mass: 5931.6750

Deconvoluted mass
Protonated Exact Mass
Expected: 5932.68 Da
Observed: 5932.70 Da
**Compound 9**

Chemical Formula: $\text{C}_{256}\text{H}_{375}\text{N}_{15}\text{O}_{57}\text{S}_{18}$

Exact Mass: 5931.67510

**LC-MS BPC**

**HRMS**

**Deconvoluted mass**

Protonated Exact Mass

Expected: 5932.68 Da

Observed: 5932.60 Da
Compound 15

Chemical Formula C_{31}H_{40}N_{10}O_{10}S_{3}
Exact Mass 6082.70 Da

Deconvoluted mass
Protonated Exact Mass
Expected: 6082.71 Da
Observed: 6082.73 Da
Compound 16

Chemical Formula: C_{94}H_{109}N_{33}O_{35}S_{5}
Exact Mass: 5982.79 Da

**LC-MS TIC**

**HRMS**

Deconvoluted mass

Protonated Exact Mass
Expected: 5982.79 Da
Observed: 5982.84 Da
Compound 17

**Chemical Formula:** C_{61}H_{47}N_{16}O_{24}S_{7}

**Exact Mass:** 6111.76 Da

**Deconvoluted mass**
- Protonated Exact Mass
  - Expected: 6111.76 Da
  - Observed: 6111.80 Da
Compound 18

**Chemical Formula:** C_{24}H_{39}N_{7}O_{6}S_{2}

**Exact Mass:** 6060.6954

**Deconvoluted mass**

Protonated Exact Mass
- Expected: 6061.70 Da
- Observed: 6061.75 Da
Compound 19

Chemical Formula: C_{36}H_{46}O_{20}N_{10}S_{9}

Exact Mass: 6970.77561

Protonated Exact Mass

Expected: 5980.74 Da

Observed: 5980.76 Da
**Compound 20**

### Chromatograms

**LC-MS TIC**

- Item name: 02Oct2017_008
- Channel name: 1: TOF MS TIC (100-3000) ESI+

### Spectra

#### HRMS

- Observed mass: 6048.74 Da

#### Deconvoluted mass

- Protonated Exact Mass
  - Expected: 6048.71 Da
  - Observed: 6048.74 Da
Compound 21

Chemical Formula: C_{24}H_{35}FeN_{5}O_{6}S_{5}

Exact Mass: 6040.64 Da

Deconvoluted mass

Protonated Exact Mass

- Expected: 6040.65 Da
- Observed: 6040.68 Da
Chemical Formula: C_{60}H_{148}N_{26}O_{77}S_{6}

Exact Mass: 5957.66559

Deconvoluted mass
Protonated Exact Mass
Expected: 5958.67 Da
Observed: 5958.56 Da
Chemical Formula: \( \text{C}_{278}\text{H}_{404}\text{N}_{72}\text{O}_{79}\text{S}_7 \)

Exact Mass: 6238.78539

Compound 23

Deconvoluted mass

Protonated Exact Mass

Expected: 6239.79 Da
Observed: 6239.66 Da
Disulfide Reduction of Compound 23

To compound 23 (5 µL of 0.1 mM stock solution, diluted in 44.9 µL of 50 mM ammonium acetate pH 7.4 to a final peptide concentration of 10 µM), was added 0.1 µL 500 mM TCEP (pH 7) (reducing agent final concentration 1 mM). Incubate on shaker at 50 °C for 20 minutes.

![Chemical structure of compound 23]

**compound 23**

Chemical Formula: C_{278}H_{404}N_{72}O_{79}S_{7}

Exact Mass: 6238.78539

![Chemical structure of compound 23a]

**compound 23a**

Fragment (A chain) @ Rt = 4.41 min.

Chemical Formula: C_{99}H_{155}N_{47}O_{35}S_{4}

Exact Mass: 2382.00002

![Chemical structure of compound 23b]

**compound 23b**

Fragment (B-chain) @ Rt = 4.70 min.

Chemical Formula: C_{179}H_{255}N_{47}O_{44}S_{3}

Exact Mass: 3862.83232
Compound 23a

Protonated Exact Mass
Expected: 3863.84 Da
Observed: 3863.86 Da

Compound 23b

Protonated Exact Mass
Expected: 2383.01 Da
Observed: 2383.00 Da

Deconvoluted mass
A chain

Deconvoluted mass
B chain with biotin

Protonated Exact Mass
Expected: 3863.84 Da
Observed: 3863.86 Da
Compound 24

Chemical Formula: C_{390}H_{424}N_{70}O_{29}S_{7}

Exact Mass: 5600.92703

Deconvoluted mass

Protonated Exact Mass
Expected: 6501.93 Da
Observed: 6501.83 Da
12. Human Insulin Receptor Binding

| Cmpd | IC50 (nM) |
|------|-----------|
| 1 (RHI) | 0.24 |
| 2 | 5.3 |
| 3 | 21 |
| 4 | 3.8 |
| 5 | 1.2 |
| 6 | 4.7 |
| 7 | 1.1 |
| 8 | 2.2 |
| 9 | 0.33 |
| Cmpd | IC50 (nM) |
|------|-----------|
| 1 (RHI) | 0.24 |
| 15 | 0.59 |
| 16 | 33 |
| 17 | 0.92 |
| 18 | 1.7 |
| 19 | 1.2 |
| 20 | 1.0 |
| 21 | 5.9 |
| 22 | 1.2 |
| 23 | 0.74 |
| 24 | 6.0 |