

Reliable Diagnosis of Murine Type 1 Diabetes Using A Panel of Autoantigens and “Antigen Surrogates” Mounted Onto A Liquid Array

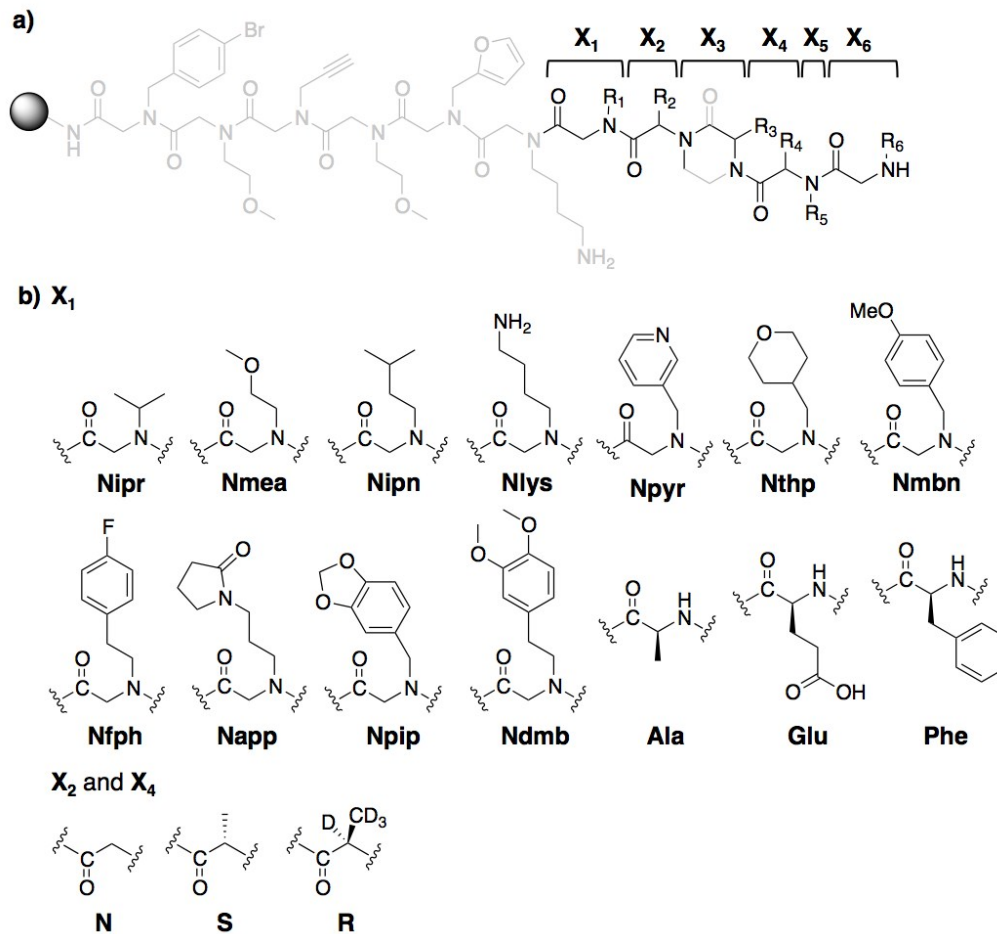
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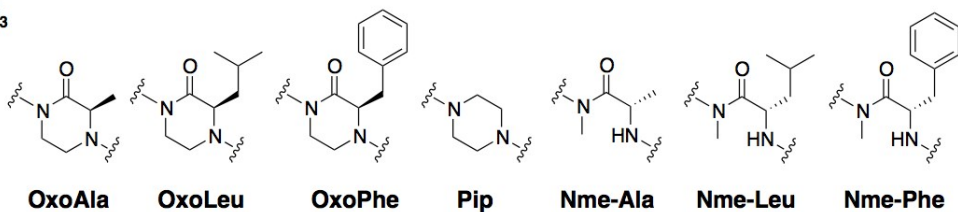
ELECTRONIC SUPPORTING INFORMATION

Figure S1. Detailed Library Design	S2
Figure S2. Sequence elucidation of three random library beads	S4
Figure S3. Characterization of compound 1	S7
Figure S4. Characterization of compound 2	S8
Figure S5. Characterization of compound 3	S9
Figure S6. Anti-MOG(35–55) titers in C57BL/6 mice after immunization	S10
Figure S7. Natural history of the IgG response to 1 over time in Swiss mice	S11
Figure S8. Natural history of the IgG response to 1 over time in NOD mice.....	S12
Figure S9. Cross-competition between compound 1 , compound 3 and GAD65	S13
Figure S10. NOD mouse hyperglycemia with respect to time.....	S14
Figure S11. Highest titers of the IgG that recognize 3 in 5–8 week old NOD and Swiss mice	S15
Figure S12. Highest titers of the IgG that recognize GST-GAD65 fusion protein in 5–8 week old NOD and Swiss mice	S16
Supplementary Materials and Methods	S17
Supplementary References	S27

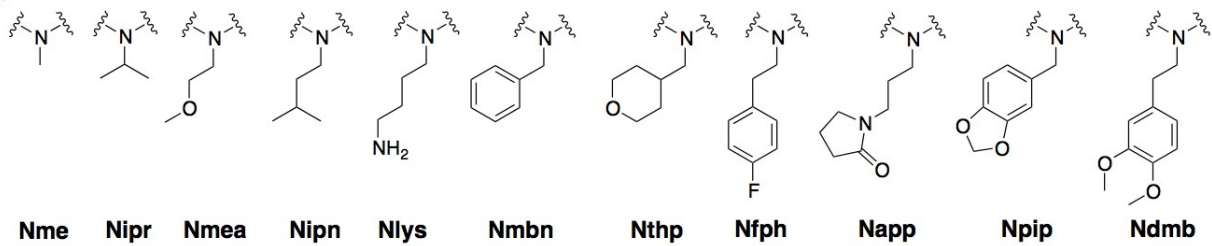
Figure S1. Detailed library design. a) General structure of PTA-OP-PTA library. The invariant linker structure is depicted in gray. b) Components of each position (X1–X6).



X₃



X₅



X₆

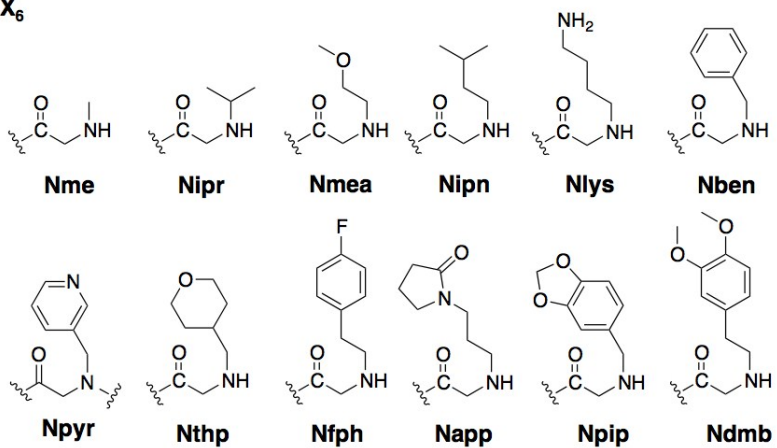


Figure S2. Structures of three representative compounds in the library.

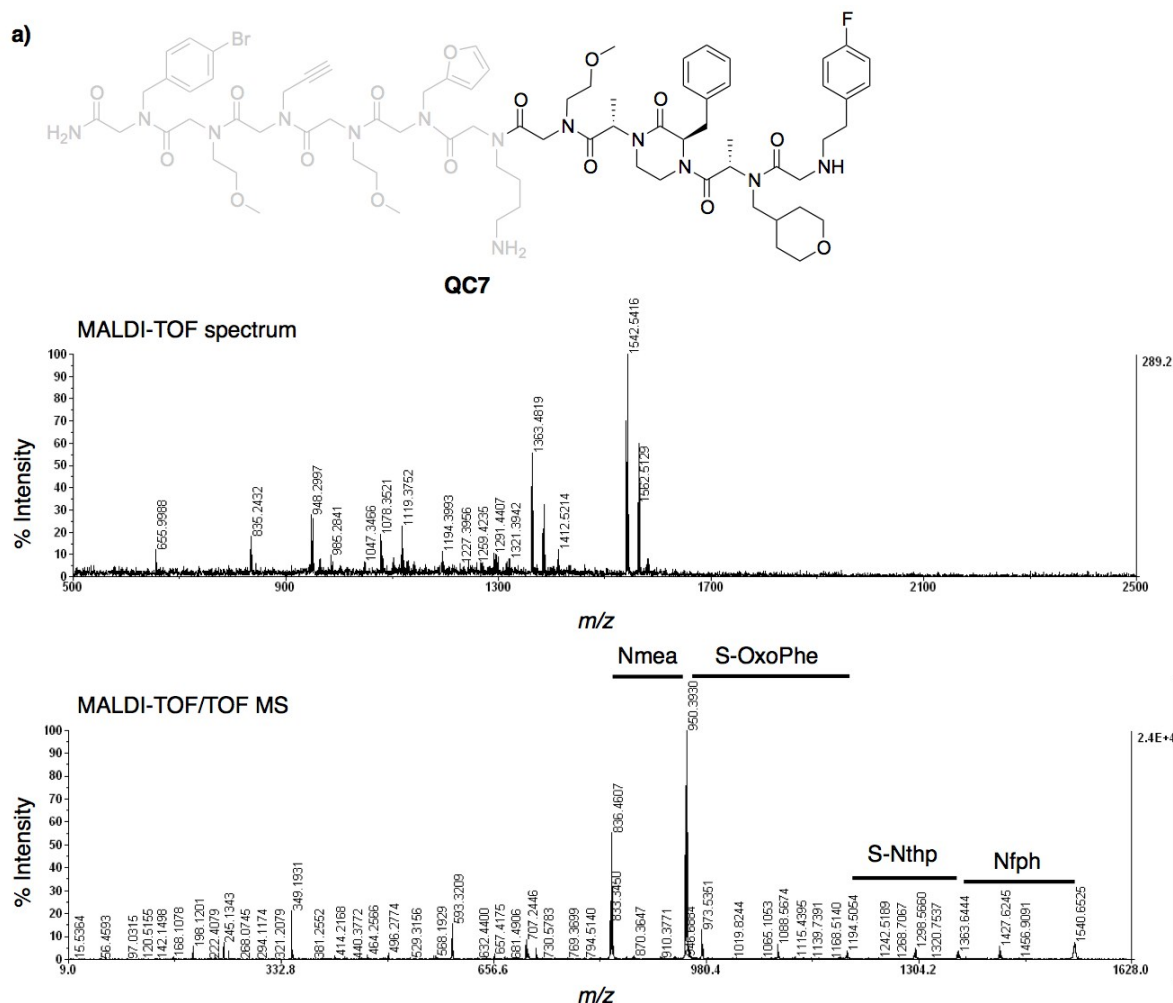
Quality Control Analysis:

Cleavage of compounds on beads. 35 of the deprotected beads were separated into a 96 microwell plate (one bead per well) and dried under vacuum. The plate was covered with aluminum foil and cooled at 4 °C for 15 min. ~50 μL of cooled 50% TFA/DCM was dropped to each well and the plate was covered with aluminum foil and shaken gently at 4 °C for 15 min. The plate was dried under vacuum and the cleaved compound in each well was dissolved in 20 μL of 50% acetonitrile/water.

MALDI-TOF mass analysis of cleaved compounds. 0.5 μL of each compound was spotted on MALDI plate and mixed with 1 μL of 50 % acetonitrile/water containing 4 mg mL^{-1} α -cyano-4-hydroxycinnamic acid (CHCA) on the plate. After the solution was dried, compounds were analyzed by MALDI-TOF and -TOF/TOF.

Linkers are indicated in gray in the structures. These compounds were identified during a quality check of the library. a) QC7 contains two PTAs and 2-oxopiperazine. $[\text{M}+\text{H}]^+$ Calcd. 1540.7, Obsd. 1540.5. b) QC16 contains one PTA and piperazine. $[\text{M}+\text{H}]^+$ Calcd. 1396.6, Obsd. 1396.4

c) QC19 contains 2 PTAs. $[\text{M}+\text{H}]^+$ Calcd. 1529.7, Obsd. 1529.5



c)

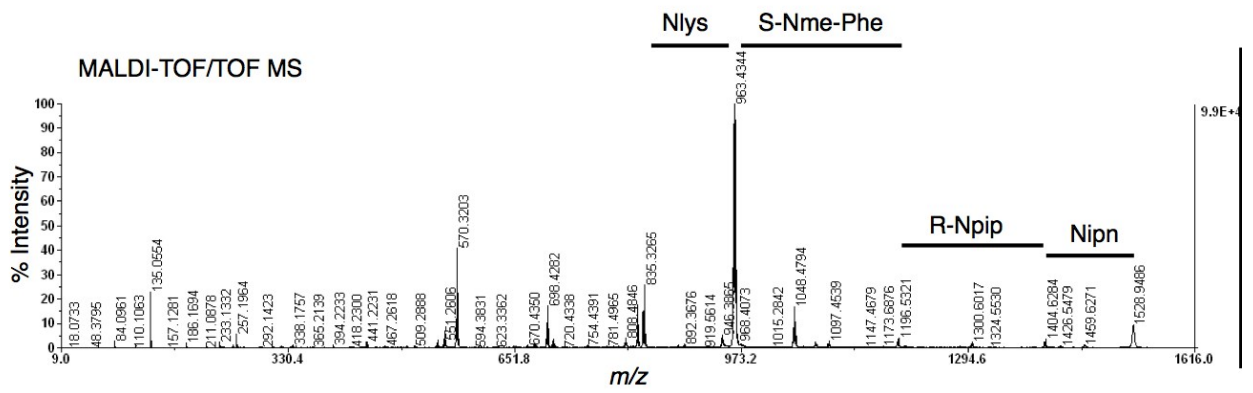
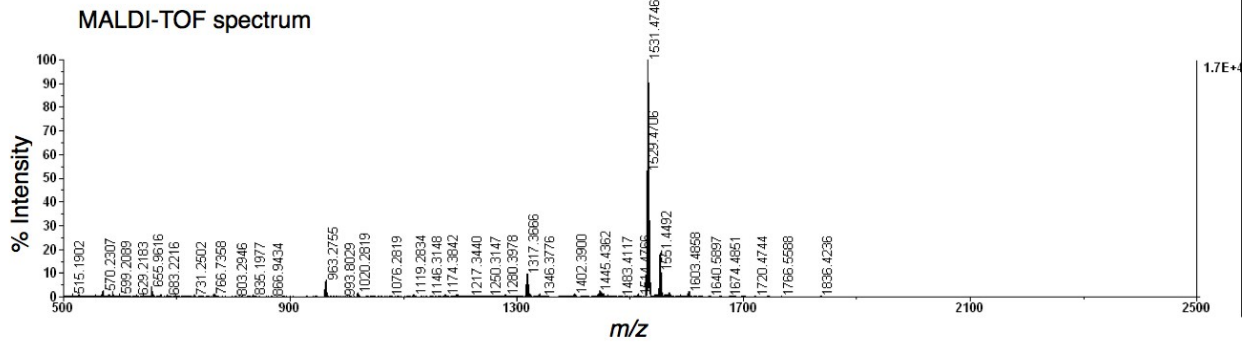
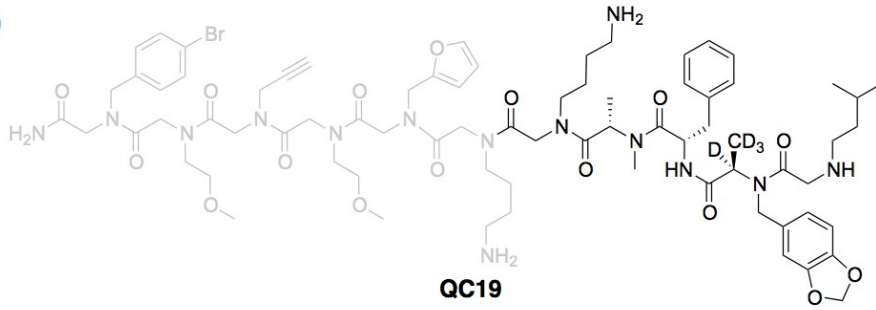


Figure S3. Characterization of Compound **1**. a) Chemical structure of **1**. b) HPLC chromatogram of purified **1**. c) MALDI-TOF MS spectrum of **1**. Calculated m/z : 1238.65. Observed m/z : 1239.68.

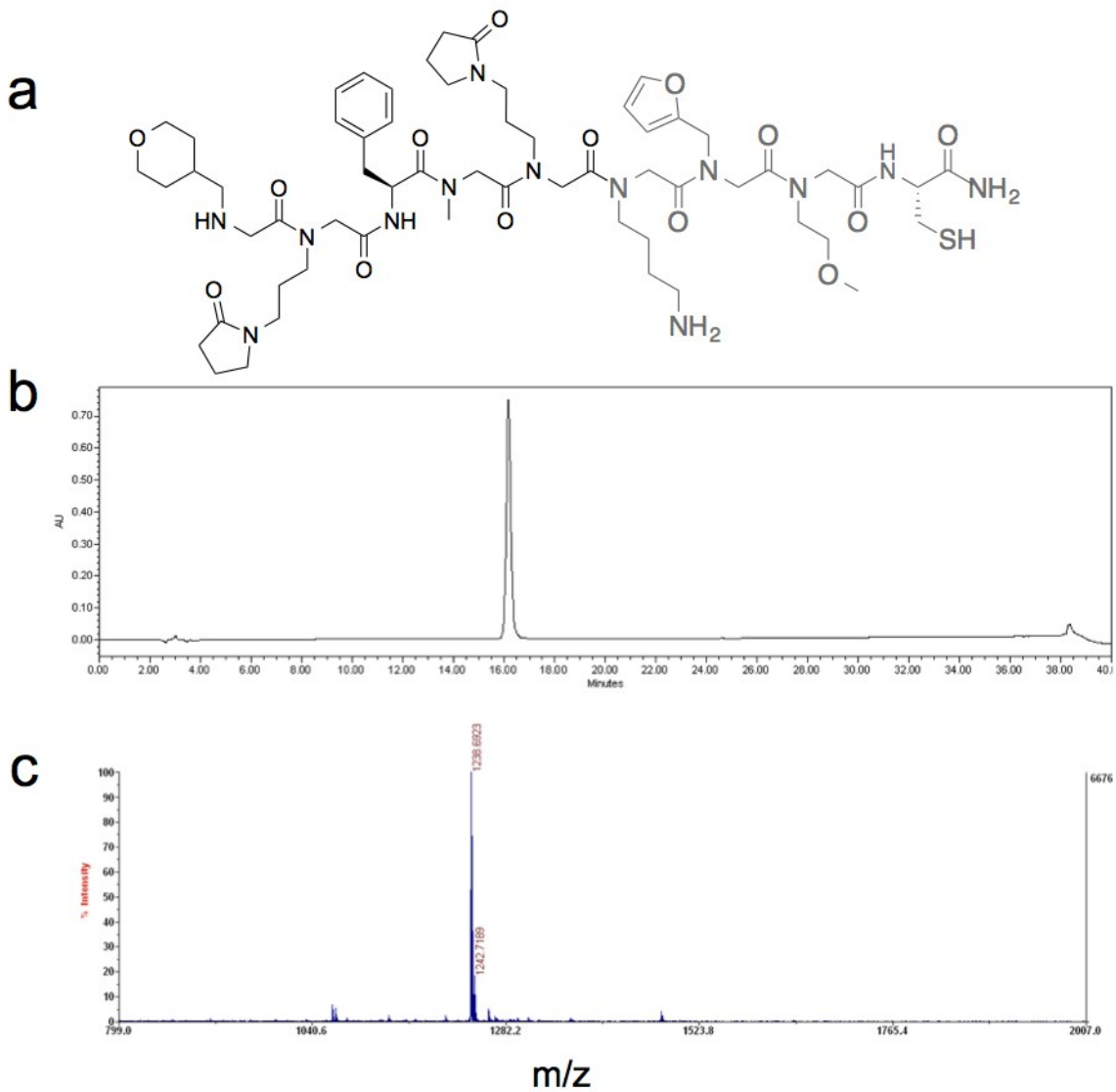


Figure S4. Characterization of Compound **2**. a) Chemical structure of **1**. b) HPLC chromatogram of purified **2**. c) MALDI-TOF MS spectrum of **2**. Calculated m/z: 1069.58. Observed m/z: 1069.64

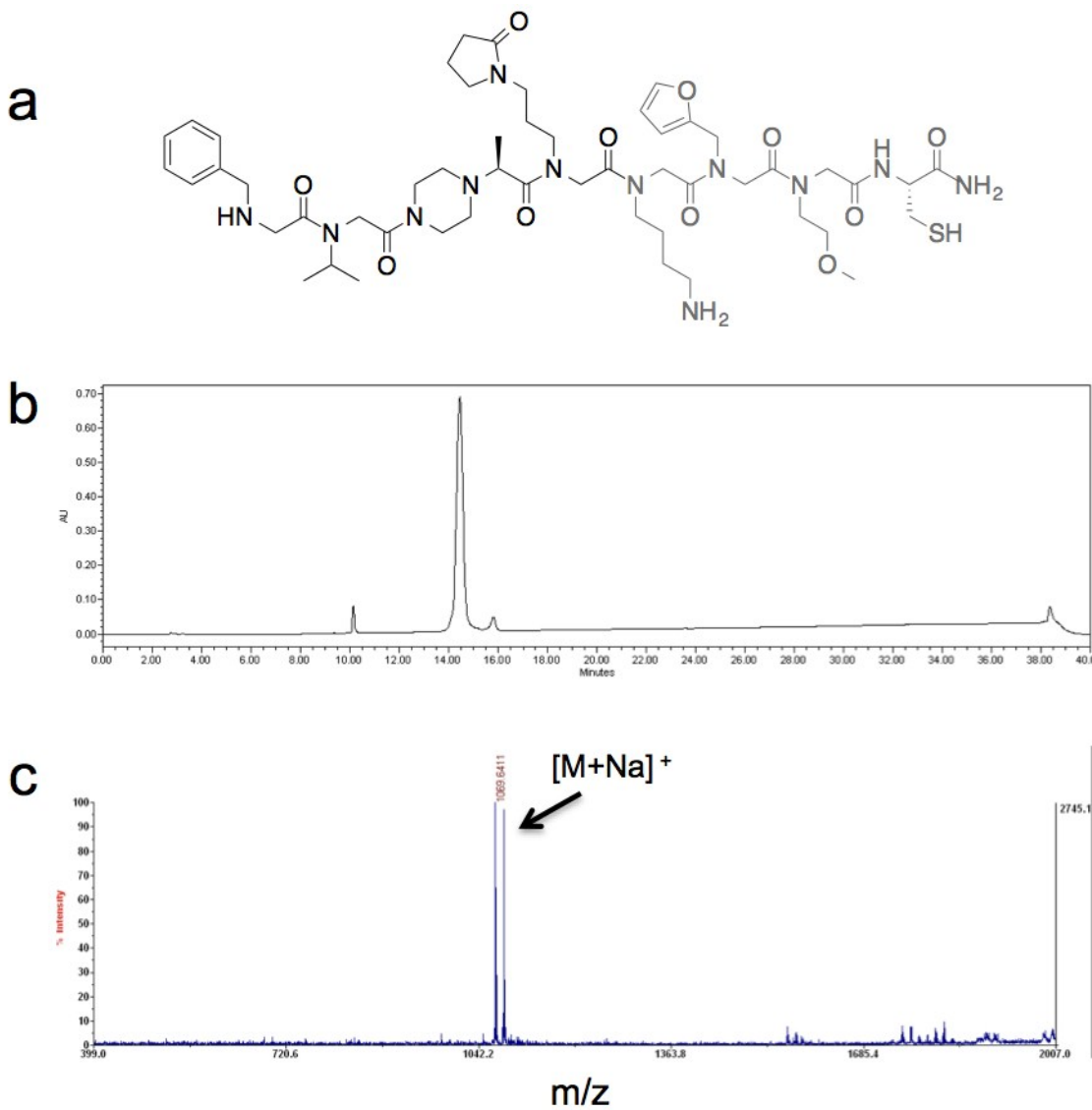


Figure S5. Characterization of Compound **3**. a) Chemical structure of **3**. b) HPLC chromatogram of purified **3**. c) MALDI-TOF MS spectrum of **3**. Calculated m/z : 1650.84. Observed m/z : 1650.90

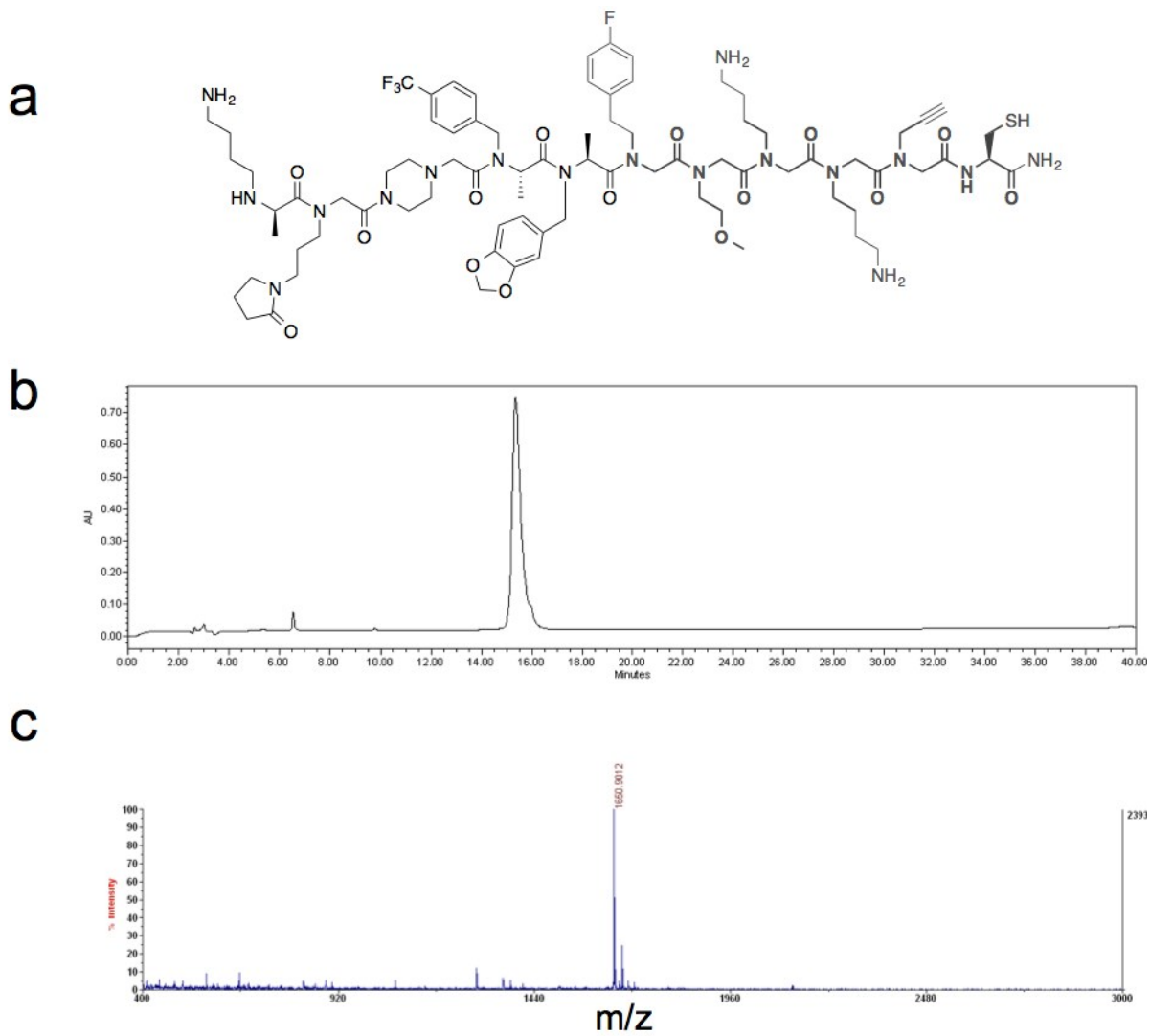


Figure S6. Anti-MOG(35–55) titers in C57BL/6 mice after immunization.

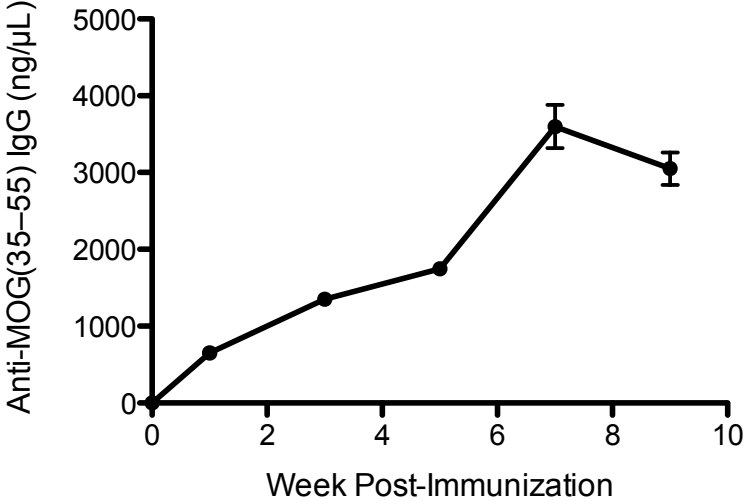


Figure S7. Natural history of Swiss mouse IgG response to antibodies that bind 1. Serum IgG antibody binding for each mouse at every blood draw available. The y -axes in figures S7–S8 are normalized so that relative titers can be compared between plots. A) IgG antibody binding to 1 for Swiss1–Swiss5; B) Swiss6–Swiss10; C) Swiss11–Swiss15; D) Swiss16–Swiss20. The 5σ threshold is indicated by a dashed line in each plot. Lines that terminate before the end of 24 weeks are from mice that expired or were sacrificed prior to the completion of the study.

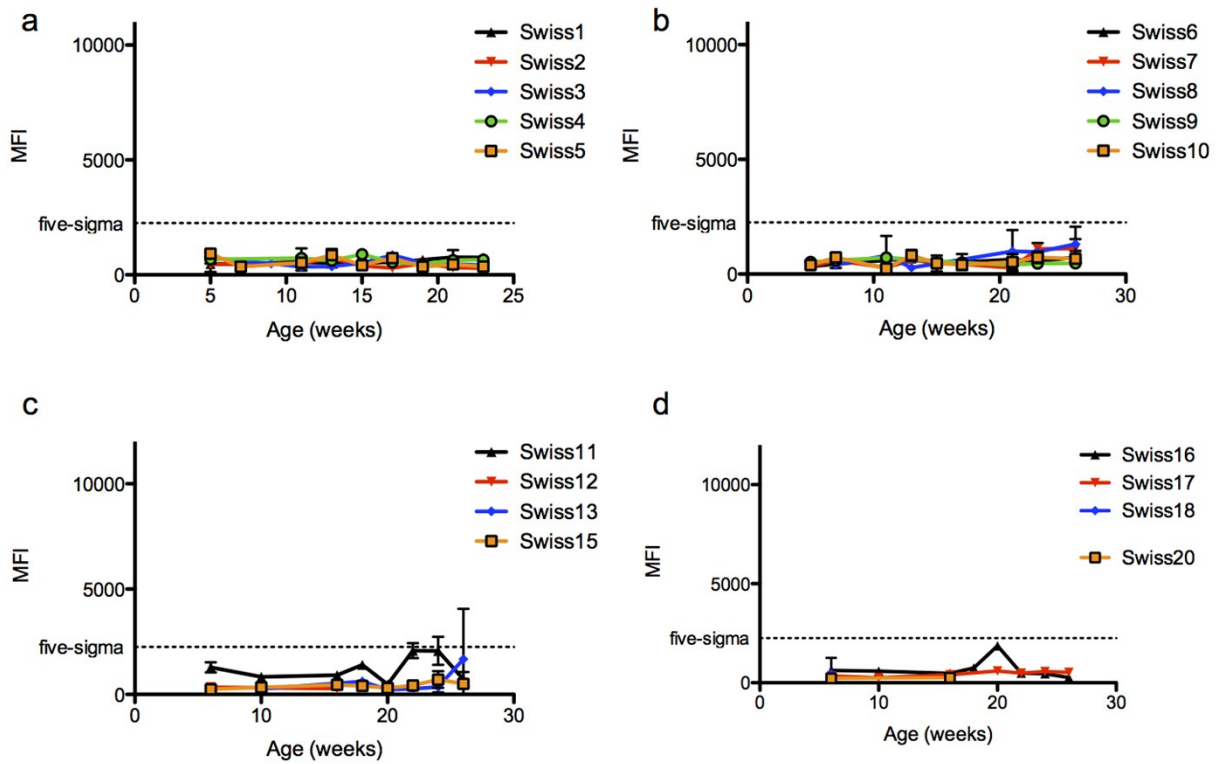


Figure S8. Natural history of NOD mouse IgG response to antibodies that bind 1. Serum IgG antibody binding for each mouse at every blood draw available. The y -axes in figure S7–S8 are normalized so that relative titers can be compared between plots. A) IgG antibody binding to 1 for NOD1–NOD5; B) NOD6–NOD10; C) NOD11–NOD15; D) NOD16–NOD20. The 5σ threshold is indicated by a dashed line in each plot. Lines that terminate before the end of 24 weeks are from mice that expired or were sacrificed prior to the completion of the study.

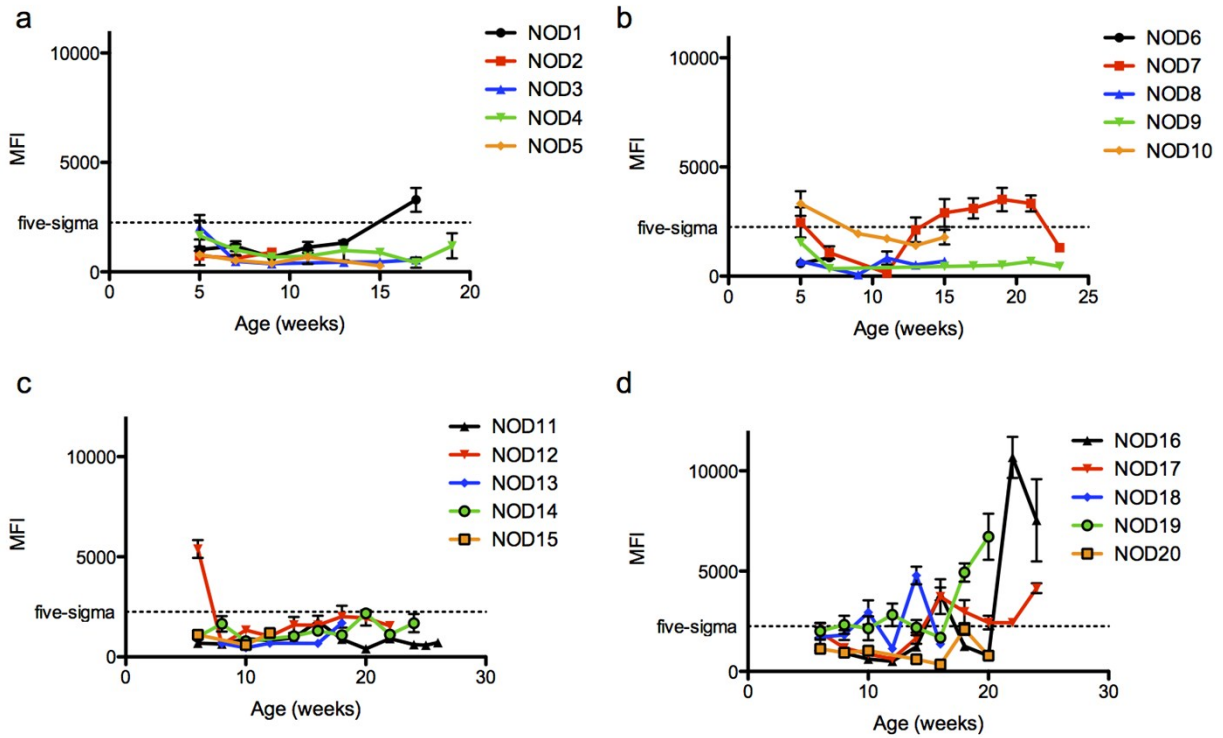


Figure S9. Cross-competition between compound 1, compound 3 and GAD65. A) Compound 1 and B) compound 3 were mounted onto color-coded TentaGel beads.

Serum from mice that showed high titers for compound 1 (A) or compound 3 (B) was pre-incubated with 250 nM of each competitor. The beads containing 1 and 3 were added to each serum sample containing the various competitors and binding of serum antibodies to each ligand was monitored using the cytometry assay. The titer of each antibody is depicted in the presence of each competitor where a gray bar indicates competition with the soluble form of the immobilized ligand (self-competition). Competition of antibody binding to each immobilized ligand is only observed when serum antibodies were pre-incubated with soluble self, but not the other antigen surrogate or GAD65, indicating that each ligand recognizes a unique paratope or paratopes. Data presented represent the average of three individual binding experiments. Statistical significance was determined using an unpaired t-test: ns, not significant; * $P < 0.01$; ** $P < 0.05$.

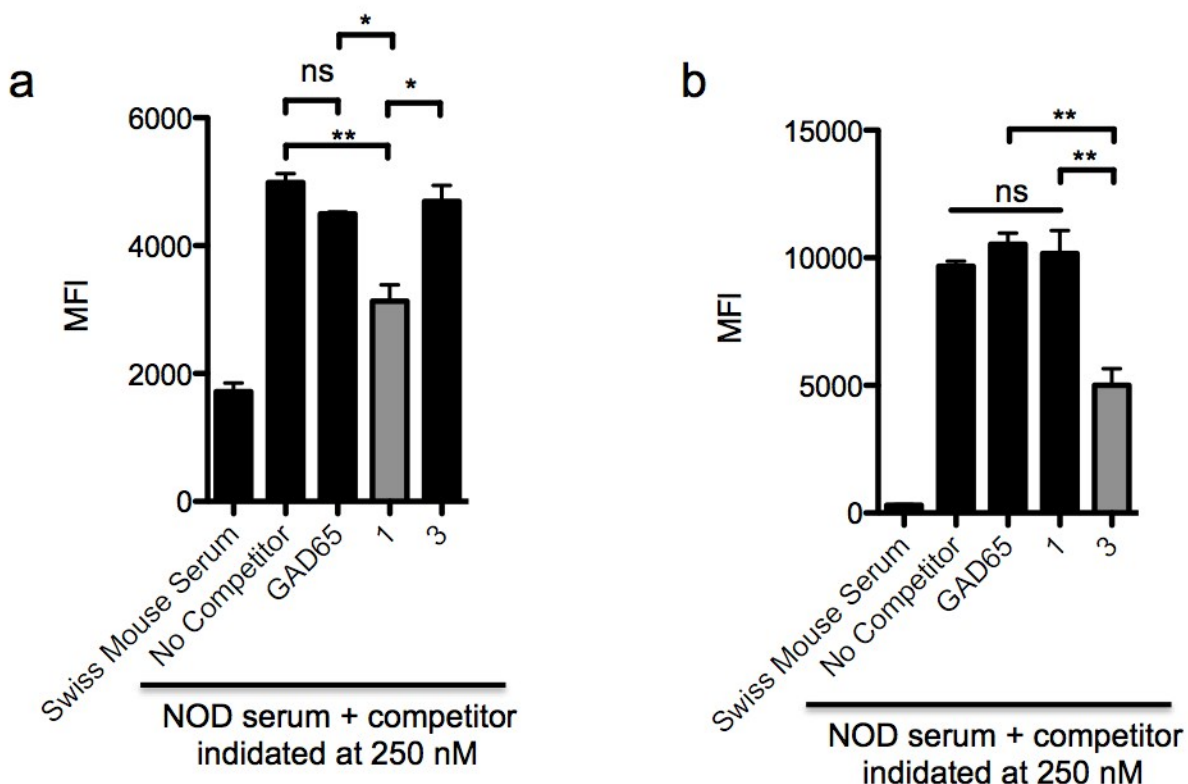


Figure S10. NOD and Swiss mouse hyperglycemia with respect to time.

A) NOD mouse blood sugar for: NOD1–NOD10; B) NOD11–NOD20; C) Swiss1–Swiss10; D) Swiss11–Swiss20. Blood sugar monitoring began at age 9.

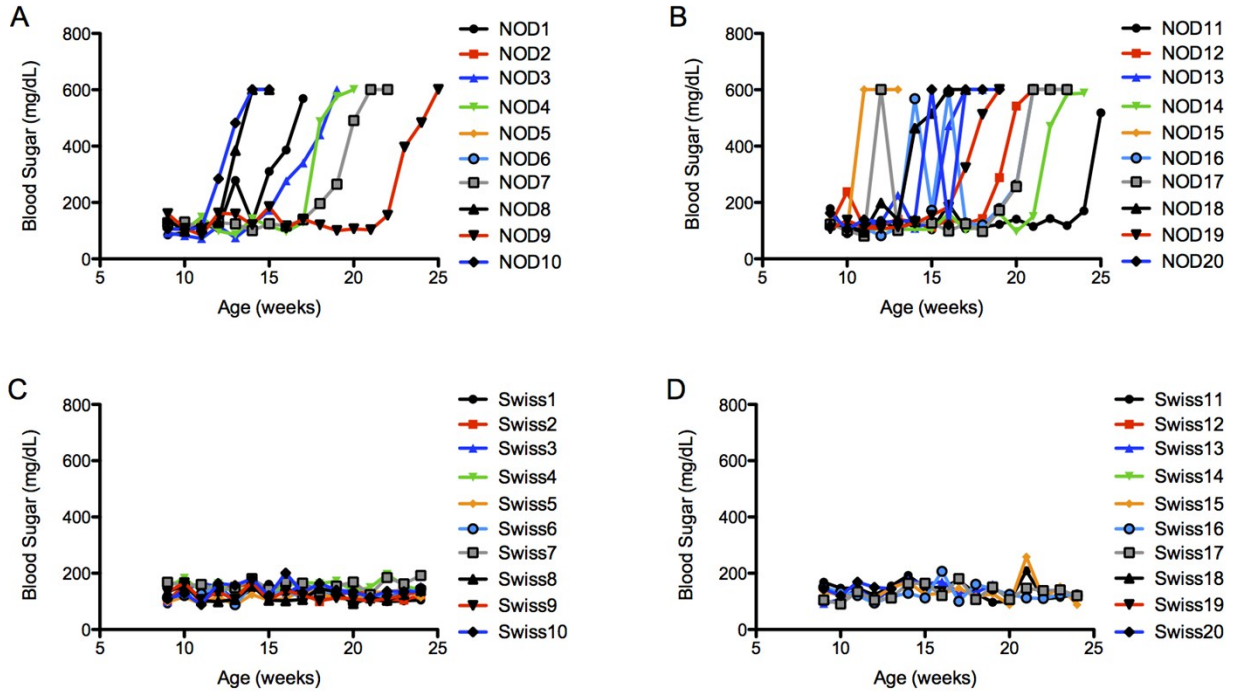


Figure S11. Highest titers of the IgG that recognize 3 in 5–8 week old NOD and Swiss mice. The three-sigma threshold is indicated by a dotted line.

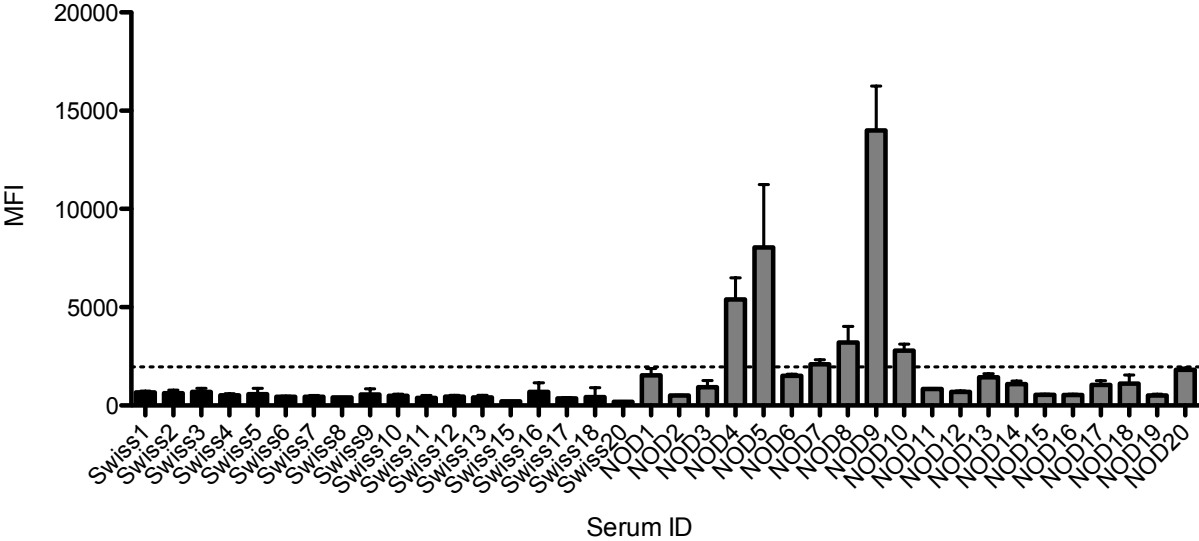
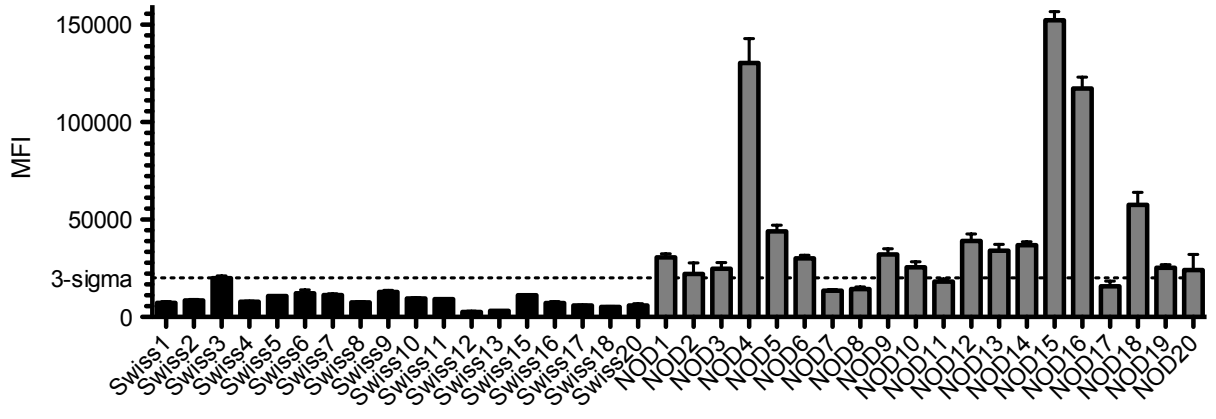
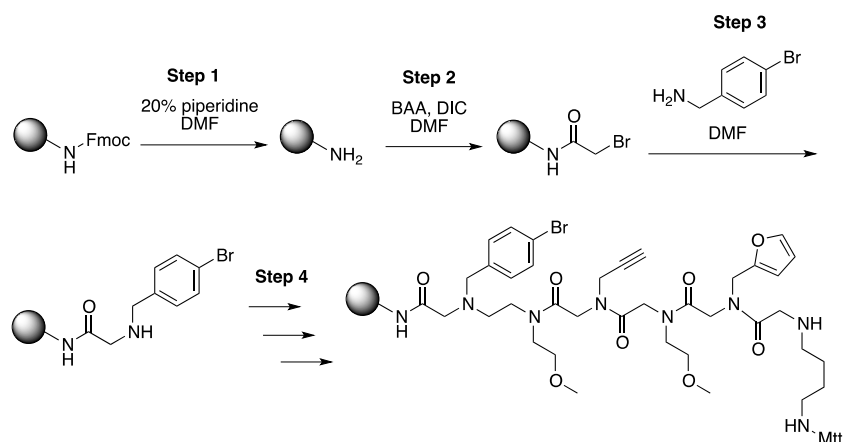


Figure S12. Highest titers of the IgG that recognize GST-GAD65 fusion protein in 5–8 week old NOD and Swiss mice. The three-sigma threshold is indicated by a dotted line.



Supplementary Methods: Detailed PTA-OP library construction.

Part 1. Linker synthesis



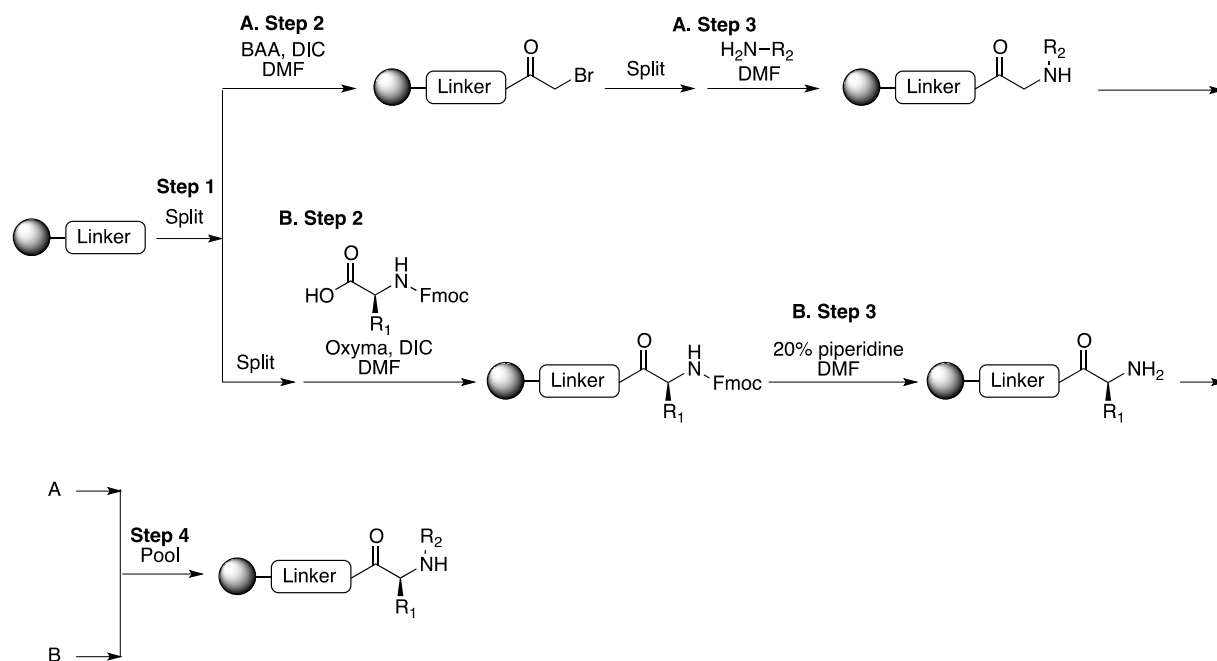
Step 1. Fmoc deprotection: TentaGel resin (2 g, 0.36 mmol) was swelled in anhydrous DMF for 30 min. The Fmoc group on the resin was deprotected by exposing the beads to 15 mL of 20% piperidine in dimethylformamide (DMF) for 3 min and, after washing with DMF once, to 15 mL of 20% piperidine in DMF for 12 min. The beads were washed with DMF three times.

Step 2. Bromoacetic acid coupling: 7.2 mL of 2 M bromoacetic acid in DMF and 7.2 mL of 1 M diisopropylcarbodiimide (DIC) in DMF were mixed in a 50 mL tube and pre-incubated for 5 min. The solution was applied to the beads and the reaction was carried out at 37 °C for 10 min with continuous shaking. The beads were washed with DMF three times.

Step 3. Amine displacement: The beads were incubated with 16 mL of 1 M 4-bromobenzylamine in DMF at 37 °C for 1 h with continuous shaking. The beads were washed with DMF three times.

Step 4. Further chain elongation: Step 2 and Step 3 were repeated using 2-methoxyethylamine, propargylamine, 2-methoxyethylamine, furfurylamine, and Mtt-1,4-diaminobutane for step 3.

Part II. X₁ synthesis (See Fig. S1)



Step 1. The beads were split 11:3 to distribute into two reaction vessels (1571 mg [A] and 429 mg [B]).

[A] Step 2. Bromoacetic acid coupling: 5.7 mL of 2 M bromoacetic acid in DMF and 5.7 mL of 1 M DIC in DMF were mixed in 50 mL tube and pre-incubated for 5 min. The solution was applied to the beads and the reaction was carried out at 37 °C for 10 min with continuous shaking. The beads were washed with DMF three times.

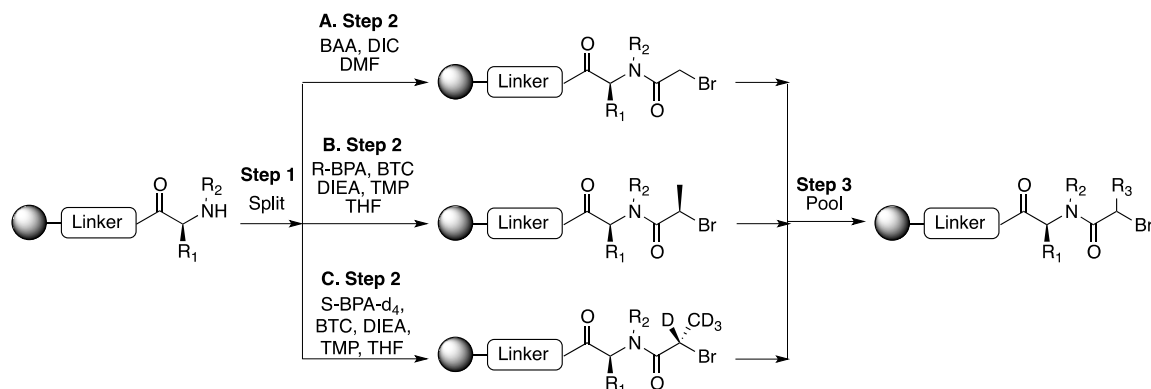
[A] Step 3. Amine displacement: The beads were split equally to distribute into eleven reaction vessels (143 mg each). The beads were incubated with 1 mL of 1 M amine (isopropylamine, 2-methoxyethylamine, isoamylamine, Mtt-1,4-diaminobutane, *N*-(3-aminopropyl)-2-pyrrolidinone, 4-fluorophenethylethylamine, 3-picolylamine, 4-methoxybenzylamine, piperonylamine, 3,4-methoxyphenethylamine, or 4-aminomethyltetrahydropyran) in DMF at 37 °C for 1 h with continuous shaking. The beads were washed with DMF three times.

[B] Step 2. Amino acid coupling: The beads were split equally to distribute into three reaction vessels (143 mg each). 8 equivalents of amino acid (Fmoc-Ala-OH, Fmoc-Phe-OH, or Fmoc-Glu(O-2-PhiPr)-OH) and 8 equivalents of oxyma (29 mg) were dissolved in 1 mL of DMF. 8 equivalents of DIC (32 μ L) was added and the solution was pre-incubated at RT for 5 min. The solution was applied to the beads and the reaction vessel was shaken at 37 $^{\circ}$ C for 2 hrs. The beads were washed with DMF three times.

[B] Step 3. Fmoc deprotection: The beads were pooled together. The Fmoc group on the resin was deprotected by exposing the beads to 3 mL of 20% piperidine in DMF for 3 min and, after washing with DMF once, to 3 mL of 20% piperidine in DMF for 12 min. The beads were washed with DMF three times.

Step 4. The beads from [A] step 3 and [B] step 3 were pooled together and mixed thoroughly.

Part III. X₂ synthesis



Step 1. The beads were split equally to distribute into three reaction vessels (667 mg each, [A], [B], and [C]).

[A]-Step 2. Bromoacetic acid coupling: 2.4 mL of 2 M Bromoacetic acid in DMF and 2.4 mL of 1 M DIC in DMF were mixed in 50 mL tube and preincubated for 5 min. The solution was

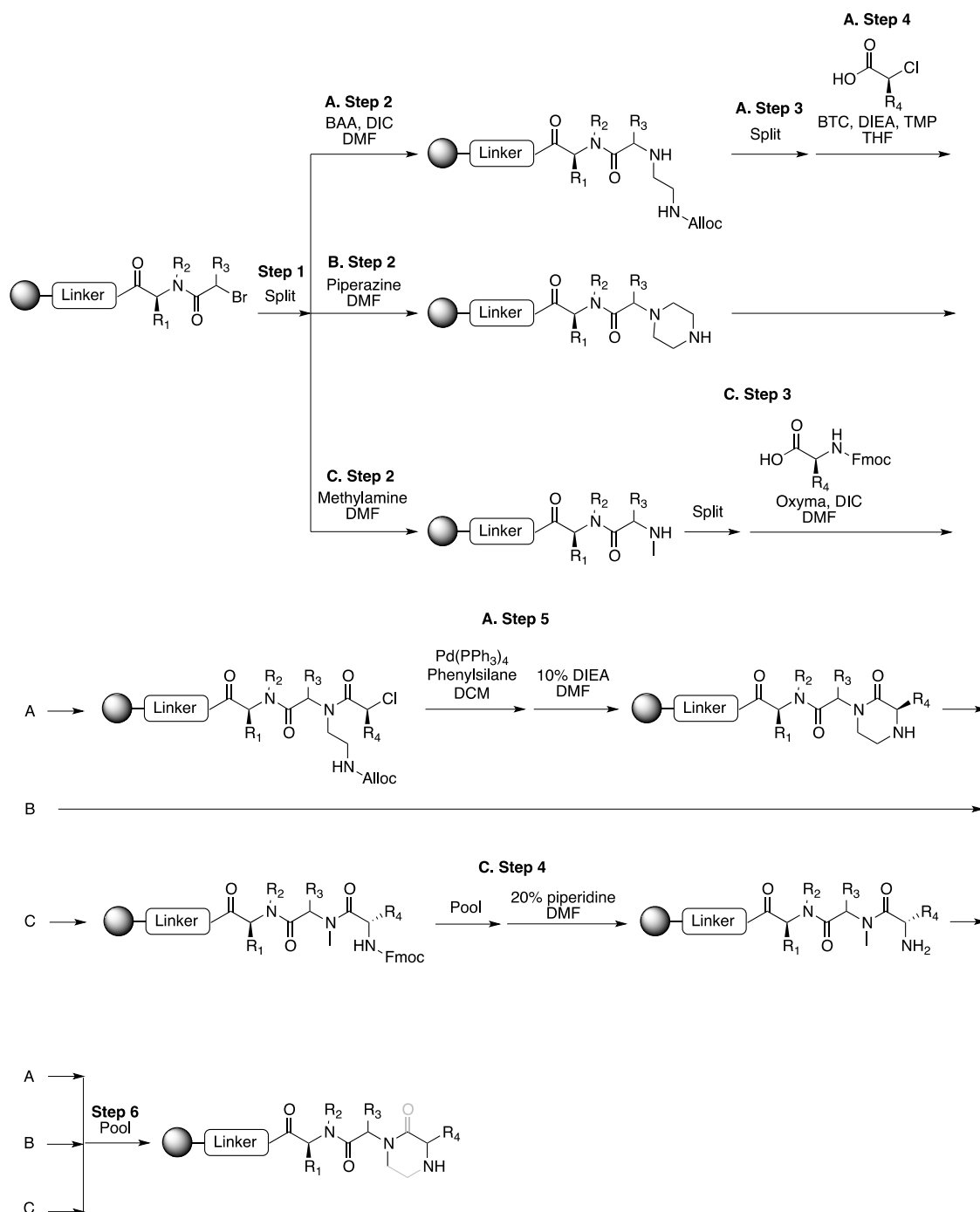
applied to the beads and the reaction was carried at 37 °C for 10 min with continuous shaking. The beads were washed with DMF three times.

[B]-step 2. (R)-(+)-2-bromopropionic acid (R-BPA) coupling: The beads were washed with DCM and anhydrous tetrahydrofuran (THF) three times each. The beads were swelled in 2 mL of anhydrous THF and 21 equivalents of diisopropylamine (DIEA) (439 μ L). 3 equivalents of bis(trichloromethyl) carbonate (BTC) (107 mg) was dissolved in 5.5 mL of anhydrous THF. 9 equivalents of R-BPA (97 μ L) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of 2,4,6-trimethylpyridine (TMP) (428 μ L) and applied to the swelled beads. The reaction was carried out at RT for 2 hrs with continuous shaking. The beads were washed with THF, DCM, and DMF three times each.

[C]-step 2. Deuterated (S)-(-)-2-bromopropionic acid (S-BPA-d₄) coupling: The beads were washed with DCM and anhydrous THF three times each. The beads were swelled in 2 mL of anhydrous THF and 21 equivalents of DIEA (439 μ L). 3 equivalents of BTC (107 mg) was dissolved in 5.5 mL of anhydrous THF. 9 equivalents of deuterated S-BPA-d₄ (97 μ L) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of TMP (428 μ L) and applied to the swelled beads. The reaction was carried out at RT for 2 h with continuous shaking. The beads were washed with THF, DCM, and DMF three times each.

Step 3. The beads were pooled together and mixed thoroughly.

Part IV. X₃ synthesis



Step 1. The beads were split 3:1:3 to distribute into three reaction vessels (857 mg [A], 286 mg [B], and 857 mg [C]).

[A] Step 2. *N*-Alloc-1,4-ethylenediamine displacement: 10 equivalents of *N*-Alloc-1,4-ethylenediamine hydrochloride (279 mg) was dissolved in 3.1 mL of DMF and 20 equivalents of DIEA (538 μ L) was added to the solution. The solution was applied to the beads and amine displacement reaction was carried out at 60 °C overnight with continuous shaking. The beads were washed with DMF and DCM three times each.

[A] Step 3. The beads were split equally to distribute into three reaction vessels (286 mg each, [A-a], [A-b], and [A-c]).

[A-a] Step 4. (*S*)-(-)-2-Chloropropionic acid (S-CPA) coupling: The beads were washed with anhydrous THF three times. The beads were swelled in 1 mL of anhydrous THF and 21 equivalents of DIEA (188 μ L). 3 equivalents of BTC (46 mg) was dissolved in 2.5 mL of anhydrous THF. 9 equivalents of S-CPA (42 μ L) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of TMP (183 μ L) and applied to the swelled beads. The reaction was carried out at RT for 3 h with continuous shaking. The beads were washed with THF and DCM three times each.

[A-b] Step 4. (*S*)-2-Chloro-3-phenylpropanoic acid (S-CPhA) coupling: The beads were washed with anhydrous THF three times. The beads were swelled in 1 mL of anhydrous THF and 21 equivalents of DIEA (188 μ L). 3 equivalents of BTC (46 mg) was dissolved in 2.5 mL of anhydrous THF. 9 equivalents of S-CPhA (67 μ L) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of TMP (183 μ L) and applied to the swelled beads. The reaction was carried out at RT for 3 h with continuous shaking. The beads were washed with THF and DCM three times each.

[A-b] Step 4. (*S*)-2-Chloro-4-methylvaleric acid (S-CVA) coupling: The beads were washed with anhydrous THF three times. The beads were swelled in 1 mL of anhydrous THF and 21 equivalents of DIEA (188 μ L). 3 equivalents of BTC (46 mg) was dissolved in 2.5 mL of anhydrous THF. 9 equivalents of S-CVA (64 μ L) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of

TMP (183 μ L) and applied to the swelled beads. The reaction was carried out at RT for 3 h with continuous shaking. The beads were washed with THF and DCM three times each.

[A] Step 5. Alloc deprotection and intramolecular amine displacement: The beads were pooled together and swelled in DCM for 15 min. Solution of 3 equivalents of tetrakis(triphenylphosphine)palladium(0) (535mg) and 12 equivalents of phenylsilane (228 μ L) in 15 mL of DCM was added to the beads and the reaction vessel was shaken at RT for 30 min. The beads were washed with DCM, DMF, and 10% DIEA in DMF three times each. The beads were shaken at 37 $^{\circ}$ C overnight and washed with DMF three times.

[B] Step 2. Piperazine displacement: 20 equivalents of piperazine (78 mg) was dissolved in 900 μ L of *N*-methyl-2-pyrrolidone and applied to the beads. The reaction was carried at 60 $^{\circ}$ C overnight with continuous shaking. The beads were washed with DMF three times.

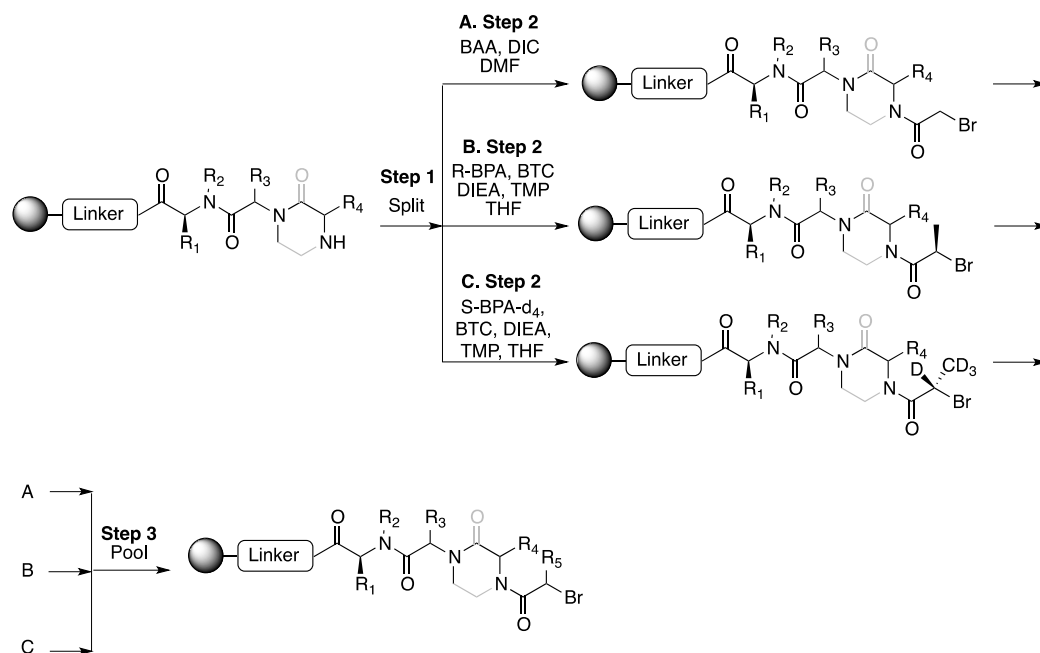
[C] Step 2. Methylamine displacement: 500 μ L of 2 M methylamine solution in water was diluted with 4.5 mL of DMF. The amine solution was added to the beads and reaction was carried at 37 $^{\circ}$ C overnight with continuous shaking.

[C] Step 3. Amino acid coupling: The beads were split equally to distribute into three reaction vessels (286 mg each). 8 equivalents of Fmoc protected amino acid (Fmoc-Ala-OH, Fmoc-Leu-OH, or Fmoc-Phe-OH) and 8 equivalents of oxyma (58 mg) were dissolved in 2 mL of DMF. 8 equivalents of DIC (64 μ L) was added and the solution was pre-incubated at RT for 5 min. The solution was applied to the beads and the reaction vessel was shaken at 37 $^{\circ}$ C for 2 h. The beads were washed with DMF three times. This coupling step was repeated again.

[C] Step 6. Fmoc deprotection: The beads were pooled together. The Fmoc group on the resin was deprotected by exposing the beads to 6 mL of 20% piperidine in DMF for 3 min and, after washing with DMF once, to 3 mL of 20% piperidine in DMF for 12 min. The beads were washed with DMF three times.

Step 6. The beads were pooled together and mixed thoroughly.

Part V. X₄ synthesis



Step 1. The beads were split equally to distribute into three reaction vessels (667 mg each, [A], [B], and [C]).

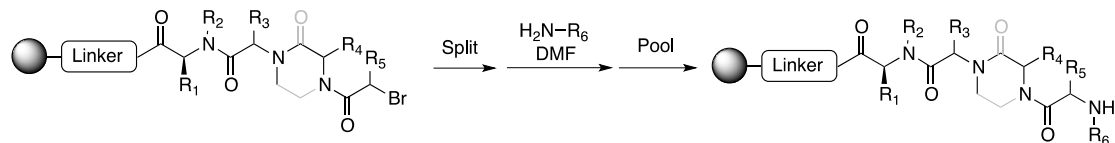
A-Step 2. Bromoacetic acid coupling: 2.4 mL of 2 M bromoacetic acid in DMF and 2.4 mL of 1 M DIC in DMF were mixed in 50 mL tube and pre-incubated for 5 min. The solution was applied to the beads and the reaction was carried at 37 °C for 10 min with continuous shaking. The beads were washed with DMF three times.

B-step 2. R-BPA coupling: The beads were washed with DCM and anhydrous THF three times each. The beads were swelled in 2 mL of anhydrous THF and 21 equivalents of DIEA (439 µL). 3 equivalents of BTC (107 mg) was dissolved in 5.5 mL of THF. 9 equivalents of R-BPA (97 µL) was added to the BTC solution and the solutions was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of TMP (428 µL) and applied to the swelled beads. The reaction was carried out at RT for 3 h with continuous shaking. The beads were washed with THF, DCM, and DMF three times each.

C-step 2. S-BPA-d₄ coupling: The beads were washed with DCM and anhydrous THF three times each. The beads were swelled in 2 mL of anhydrous THF and 21 equivalents of DIEA (439 μ L). 3 equivalents of BTC (107 mg) was dissolved in 5.5 mL of THF. 9 equivalents of deuterated S-BPA-d₄ (97 μ L) was added to the BTC solution and the solution was cooled at -20 °C for 15 min. The cooled solution was mixed with 27 equivalents of TMP (428 μ L) and applied to the swelled beads. The reaction was carried out at RT for 3 h with continuous shaking. The beads were washed with THF, DCM, and DMF three times each.

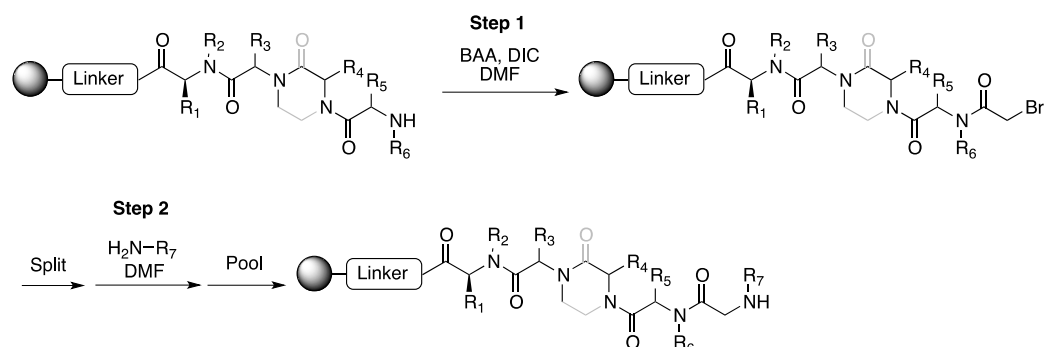
Step 3. The beads were pooled together and mixed thoroughly.

Part VI. X₅ synthesis



The beads were split equally to distribute them into 12 reaction vessels and subjected to the amine displacement reactions. The beads in each reaction vessel were treated with 1.2 mL of 1 M solution of amine (methylamine, isopropylamine, 2-methoxyethylamine, isoamylamine, Mtt-1,4-diaminobutane, *N*-(3-aminopropyl)-2-pyrrolidinone, 4-fluorophenylethylamine, 3-picolylamine, benzylamine, piperonylamine, 3,4-methoxyphenethylamine, or 4-aminomethyltetrahydropyran) in DMF. The reaction was carried out at 60 °C overnight with continuous shaking. The beads were washed with DMF three times, pooled together, and mixed thoroughly.

Part VII. X₆ synthesis



Step 1. Bromoacetic acid coupling: 7.2 mL of 2 M bromoacetic acid in DMF and 7.2 mL of 1 M DIC in DMF were mixed in 50 mL tube and preincubated for 5 min. The solution was applied to the beads and the reaction was carried at 37 °C for 1 h with continuous shaking. The beads were washed with DMF three times. This coupling step was repeated again.

Step 2. Amine displacement: The beads were split equally to distribute into 12 reaction vessels. The beads were incubated with 1.2 mL of 1 M amine (methylamine, isopropylamine, 2-methoxyethylamine, isoamylamine, Mtt-1,4-diaminobutane, *N*-(3-aminopropyl)-2-pyrrolidinone, 4-fluorophenylethylamine, 3-picolylamine, benzylamine, piperonylamine, 3,4-methoxyphenethylamine, or 4-aminomethyltetrahydropyran) in DMF at 37 °C for 2 h with continuous shaking. The beads were pooled together, washed with DMF and DCM three times each, and dried under vacuum.

Deprotection of side chain protecting groups: The library beads were preincubated with DCM at 4 °C for 30 min. DCM was removed and the side-chain protecting groups were deprotected by treating the beads with 1% trifluoroacetic acid (TFA) in DCM for 3 min six times. The library beads were washed with DCM and dried under vacuum. A few hundreds of beads were transferred to a 96 microwell plate to assess the quality of the synthesis (Fig S2).

Supplementary References

- 1) Gao, Y.; Kodadek, T. *Chem. Biol.* **2013**, *20*, 360.
- 2) Suwal, S.; Kodadek, T. *Org. Biomol. Chem.* **2013**, *11*, 2088.
- 3) Doran, T. M.; Simanski, S.; Kodadek, T. *ACS Chem. Biol.* **2015**, *10*, 401.