Supporting Information

Discovery and optimization of a synthetic class of Nectin-4-targeted CD137 agonists for immuno-oncology

*Punit Upadhyaya†, Julia Kristensson‡, Johanna Lahdenranta†, Elizabeth Repash†, Jun Ma†, Jessica Kublin†, Gemma E. Mudd‡, Lia Luus†, Phil Jeffrey‡, Kristen Hurov†, Kevin McDonnell†, Nicholas Keen†**

† Bicycle Therapeutics, 4 Hartwell Place, Lexington, MA 02421 USA.

‡Bicycle Therapeutics, B900 Building, Babraham Research Campus, Cambridge, CB22 3AT, UK.

*Corresponding author: Nicholas.keen@bicycletx.com

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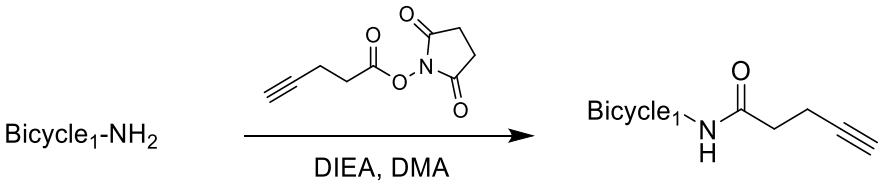
Supplementary Text

Synthesis of Bicyclic peptides

Bicycle peptides were synthesized as previously described (*36*). Briefly, linear peptide was synthesized on Rink amide resin or cysteamine loaded 2-chlorotrityl resin using standard Fmoc (9-fluorenylmethyloxycarbonyl) solid phase peptide synthesis, either by manual coupling or using a Gyros Protein Technologies Symphony X automated peptide synthesizer. The peptides were cleaved from resin using TFA cleavage cocktail containing appropriate protecting group scavengers and peptides were precipitated with diethyl ether and dissolved in 50:50 acetonitrile/water. The crude peptides were then cyclized with 1,3,5-Triacryloylhexahydro-1,3,5-triazine (TATA) at ~1 mM concentration peptide with 1.3 equivalents scaffold, using ammonium bicarbonate (100 mM) as base. Once complete, the cyclisation reaction was quenched using *N*-acetyl cysteine (10 equivalents). The solutions were lyophilized and purified by RP-HPLC. Peptide fractions of sufficient purity and correct molecular weight (verified by either MALDI-TOF or HPLC and LC-MS) were pooled and lyophilized. Bicyclic peptides synthesized for this work in listed in **table S7**.

General conjugation of 4-pentynoic acid to Bicyclic peptides

4-pentynoic acid (PYA) was incorporated into bicyclic peptide sequences to enable conjugation to a second bicyclic peptide containing a linker containing an azide group. PYA was incorporated either on solid phase by using orthogonal protecting groups on Dap, Lys or D-Lys or in solution after synthesis and purification of the bicyclic peptide. The general method for incorporation of PYA in solution is described below:



$Bicycle_1$ (1.0 eq) containing only one nucleophilic amine was dissolved in DMA (N-dimethylacetamide) and 4.0 eq of DIEA (N,N-diisopropylethylamine) was added and stirred at room temperature. 1.3 equiv. of the NHS (N-hydroxysuccinimide) ester of 4-pentynoic acid was and stirred overnight. The reaction mixture was purified by prep-HPLC.

Preparative HPLC conditions for purification

The *Bicycle* TICAs were purified using preparative HPLC setup was as follows:
Column: Phenomenex Gemini-NX C18 5um 110A 150 X 4.6 mm
Instrument: Agilent 1260 HPLC

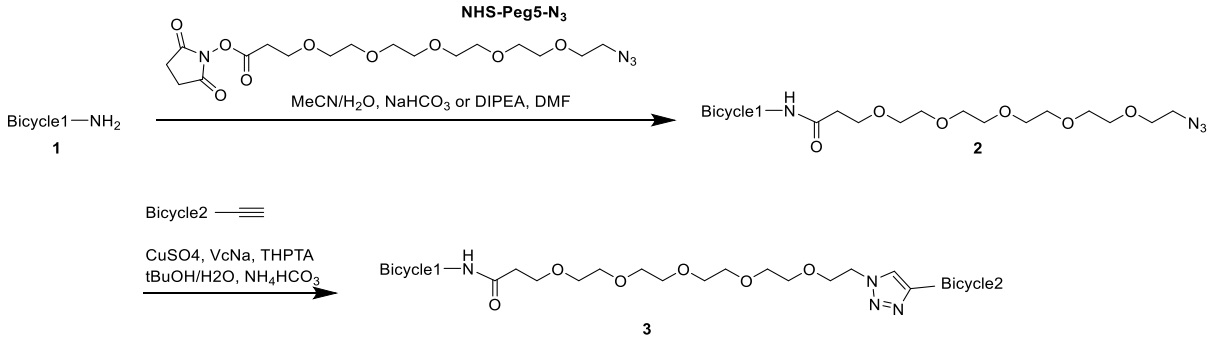
The mobile phase used are described in the table below:

Method	Mobile Phase
TFA	Mobile phase A: 0.1% TFA in H2O Mobile Phase B: Acetonitrile
Basic	Mobile phase A: 10 mM NH4HCO3 in H2O Mobile Phase B: Acetonitrile
Neutral	Mobile Phase A: H2O Mobile Phase B: Acetonitrile

General Synthesis of *Bicycle* TICAs:

The constituents Nectin-4 and CD137 *Bicycles* used in the synthesis of *Bicycle* TICAs are listed in **table S6**. **Table S7** reveals the sequence and cyclization scaffold of these constituent Nectin-4 and CD137 Bicycles along with the attachment point used for conjugation to the linker. **Table S8** has the % purity measured by analytical HPLC and observed m/z for the final molecule. The synthesis of the Bicycle TICAs are summarized using 4 general methods. For Bicycle TICAs whose synthesis is not covered by the four general methods, the specific method of synthesis is described.

Method A: Peg5 linker

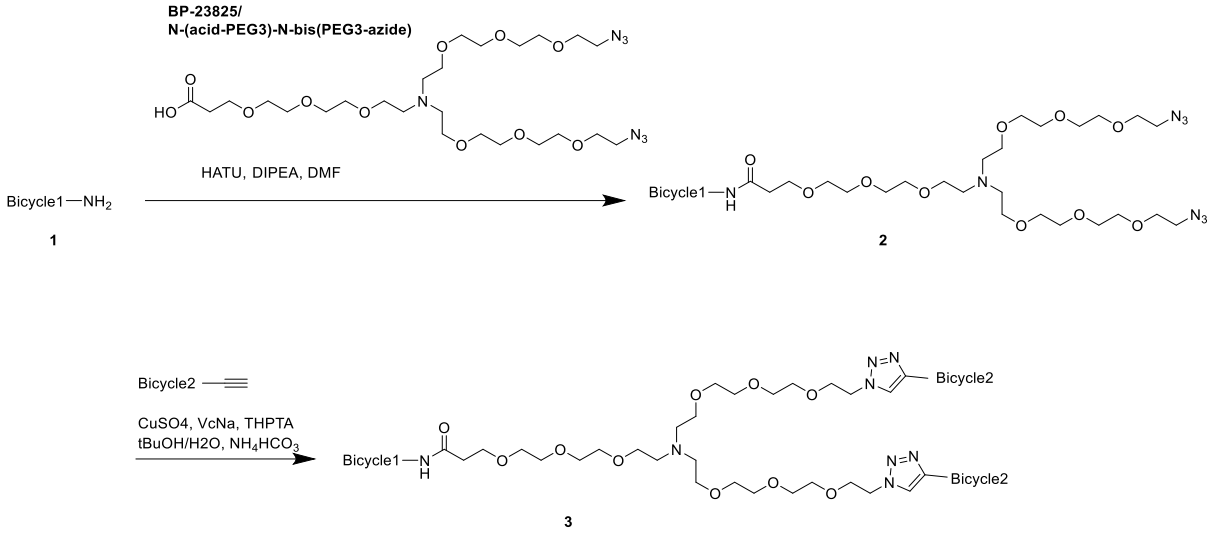


A mixture of Bicycle 1 (1.0 eq., Nectin-4 *Bicycle*) and NHS-PEG5-N3 (1.6 eq.) is dissolved in MeCN/H2O (1:1) or DMF, and the pH of the solution adjusted to 8 by dropwise addition of NaHCO3 (0.1 M) or DIPEA (5 eq). The reaction mixture is stirred at 30 °C for 2 hr then concentrated under reduced pressure to remove solvent and purified by prep-HPLC to give intermediate 2.

A mixture of intermediate 2 (1.0 eq) and Bicycle2 (1.0 eq., CD137 *Bicycle*) are dissolved in t-BuOH/H2O (1:1), and then CuSO4 (1.0 eq), VcNa (2.3 eq) (sodium ascorbate), and THPTA (1.0 eq) (tris-hydroxypropyltriazolylmethylamine) are added. Finally, 0.2 M NH4HCO3 is added to adjust pH to 8. The reaction mixture is stirred at 40°C for 16 hr under N2 atmosphere and purified by prep-HPLC.

The following *Bicycle* TICAs were synthesized using the above general method (Table S6): BCY10571, BCY10572, BCY10573, BCY11373, BCY11616, BCY11858, BCY12238, BCY12377, BCY12378, BCY12379, BCY12481, BCY12572, BCY12573, BCY12574, BCY12576, BCY12579, BCY12580, BCY12581, BCY12582, BCY12583, BCY12709 and BCY12710. The Bicyclic peptides used in the synthesis of the Bicycle TICAs are listed in Table S7.

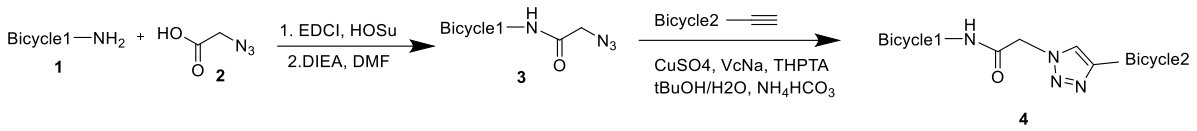
Method B (N-(acid-Peg3)-N-bis(Peg3-azide) hinge)



A solution of N-(acid-Peg3)-N-bis(Peg3-azide) (Broadpharm # BP23825, 1.0 eq), HATU (1.2 eq) and DIEA (2.0 eq) in DMF is mixed for 5 minutes, then Bicycle1 (Nectin-4 *Bicycle* or CD137 *Bicycle* for BCY11384*)*, 1.2 eq.) is added. The reaction mixture is stirred at 40°C for 16 hr. The reaction mixture is then concentrated under reduced pressure to remove solvent and purified by prep-HPLC to give intermediate 2.
A mixture of intermediate 2 (1.0 eq) and Bicycle2 (CD137 *Bicycle* or Nectin-4 *Bicycle* for BCY11384*)*, 2.0 eq) are dissolved in t-BuOH/H2O (1:1), and then CuSO4 (1.0 eq), VcNa (4.0 eq), and THPTA (2.0 eq) are added. Finally, 0.2 M NH4HCO3 is added to adjust pH to 8. The reaction mixture is stirred at 40°C for 16 hr under N2 atmosphere and purified by prep-HPLC.

The following *Bicycle* TICAs were synthesized using the above general method (Table S6): BCY11384, BCY11385, BCY11863, BCY11864, BCY12484, BCY12485, BCY12486, BCY12487, BCY12586, BCY12587, BCY12588, BCY12760, BCY12761, BCY12797. The Bicyclic peptides used in the synthesis of the Bicycle TICAs are listed in Table S7.

Method C (Azidoacetic acid linker)

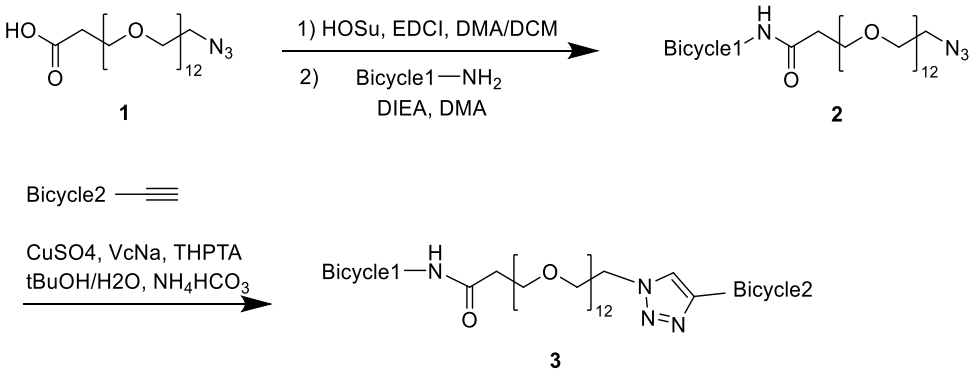


A mixture of azidoacetic acid (1.0 eq), EDCI (1.1 eq) and HOSu (1.0 eq) in DMF was stirred at 25-30 °C for 30 min. TLC indicated azidoacetic acid was consumed completely. A mixture of Bicycle1 (0.28 eq., Nectin-4 *Bicycle*) and DIEA (0.28 eq.) was added and stirred at 25-30 °C for 2 hr. The reaction mixture was concentrated and purified by prep-HPLC (TFA condition) to give intermediate **3**.

Intermediate **3** (1.0 eq.), Bicycle2 (1.0 eq., CD137 *Bicycle*), and THPTA (1.0 eq.) was dissolved in t-BuOH/H2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then CuSO4 (1.0 eq.) and VcNa (1.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH4HCO3 (in 1:1 t-BuOH/H2O), and the reaction mixture was stirred at 40 °C for 2 hr under N2 atmosphere, concentrated and purified by prep-HPLC (TFA condition).

The following *Bicycle* TICAs were synthesized using the above general method (Table S6): BCY11373, BCY11374 and BCY11375. The Bicyclic peptides used in the synthesis of the Bicycle TICAs are listed in Table S7.

Method D (Sar10-Peg12 linker)

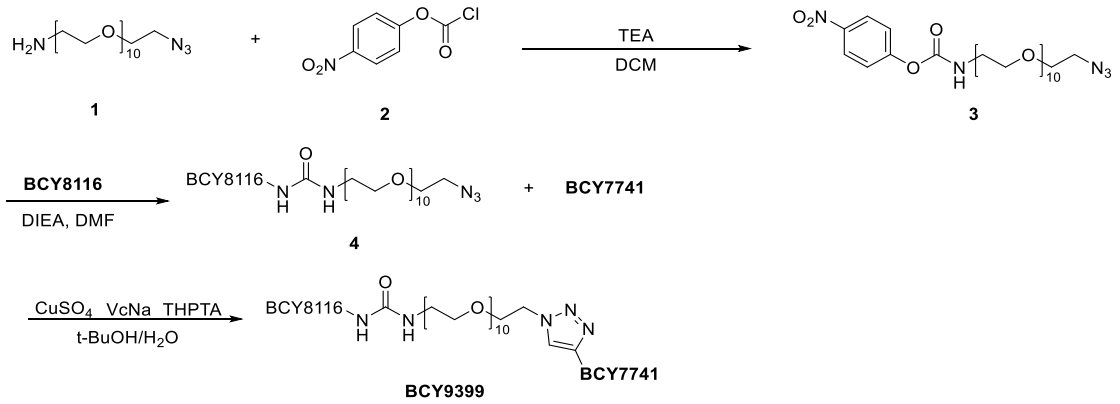


Compound **1** (1 eq, CD137 *Bicycle*), HOSu (1.5 eq) and EDCI (1.4 eq) in DMA/DCM (3:1 %v/v) was stirring at 20 °C for 2h. A mixture of Bicycle1 (1 eq, CD137 Bicycle) and DIEA (5 eq) in DMA was added to the reaction mixture, stirred for 5 h, concentrated and purified by prep-HPLC (TFA condition) to give intermediate **2**.

Intermediate **2** (1 eq) and Bicycle2 (1 eq, Nectin-4 *Bicycle* with Sar10-B-Ala-PYA on N-terminus) were dissolved in DMF, and aqueous ascorbic acid solution (1.0 eq) and CuSO4 (1.0 eq) were added and stirred for 2 h at 20 °C and purified by prep-HPLC (TFA condition)

The following *Bicycle* TICAs were synthesized using the above general method (Table S6 and S7): BCY8854, BCY10000 and BCY10569.

Synthesis of BCY9399

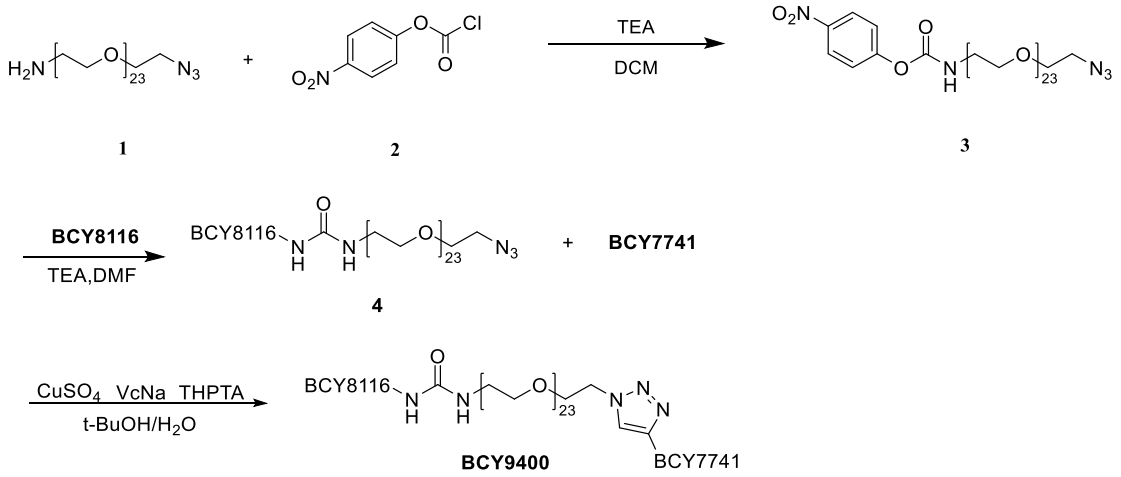


To a solution of **1** (30 mg, 56.7 µmol), **2** (17.22 mg, 85.45 µmol) in DCM (0.5 mL) was added TEA (8.65 mg, 85.45 µmol, 11.9 µL). The mixture was stirred at 25 °C for 1 hr. LC-MS showed **1** was consumed completely and one main peak with desired m/z (calculated MW: 691.72, observed m/z: 692.3([M+H]+) and 709.3 ([M+NH4]+)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (neutral condition). Compound **3** (30.5 mg) was obtained as a colorless oil.

To a solution of Compound **3** (15 mg, 21.68 µmol) and BCY8116 (47 mg, 21.68 µmol) in DMF (1 mL) was added DIEA (8.41 mg, 65.05 µmol, 11.33 µL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound 2 was consumed completely and one main peak with desired m/z (MW: 2725.1 observed m/z: 1362.7([M/2+H]+), 909.0([M/3+H]+)) was detected. The reaction mixture was filtered and concentrated under reduced pressure to give a residue. The crude product was purified by reversed-phase HPLC (TFA condition). Compound **4** (20 mg, 33.4% yield, 98.7% purity) was obtained as a white solid.

A mixture of Compound **4** (20.0 mg, 5.35 µmol, 1.0 eq), BCY7741 (13.0 mg, 5.70 µmol, 1.01 eq), and THPTA (0.4 M, 13.4 µL, 1.0 eq) was dissolved in t-BuOH/H2O (1:1, 2 mL, pre-degassed and purged with N2 for 3 times), and then CuSO4 (0.4 M, 13.4 µL, 1.0 eq) and VcNa (0.4 M, 26.8 µL, 2.0 eq) were added under N2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH4HCO3 (in 1:1 t-BuOH/H2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N2 atmosphere. The reaction mixture was purified by prep-HPLC (TFA condition). **BCY9399** (9.1 mg, 27.2% yield) was obtained as a white solid.

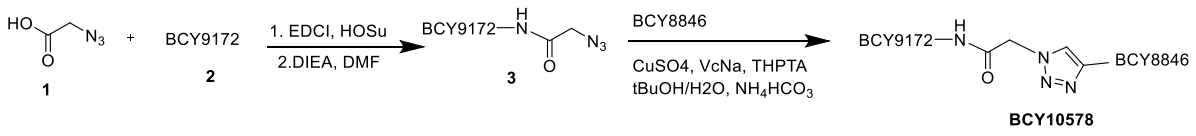
Synthesis of BCY9400



To a solution of Compound 1 (30.0 mg, 27.29 μmol), Compound 2 (8.3 mg, 40.94 μmol) in DCM (2 mL) was added TEA (4.14 mg, 40.94 μmol, 5.7 μL). Then the reaction mixture was stirred at 25-30 °C for 1 hr. LC-MS showed Compound 1 was consumed completely and one main peak with was detected. The reaction mixture was concentrated under reduced pressure and purified by prep-HPLC (neutral condition) to give compound 3 (18 mg) as a white solid.

To a solution of Compound **3** (15.5 mg, 7.12 μmol) and **BCY8116** (9 mg, 7.12 μmol) in DMF (2 mL) was added DIEA (1.4 mg, 10.68 μmol, 1.9 μL). The mixture was stirred at 30 °C for 2 hrs. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z (MW: 3297.78, observed *m/z*: 1099.7($[M/3+H]^+$)) was detected. The crude product was purified by reversed phase HPLC (TFA condition). Compound **4** (19.5 mg, 83.1% purity) was obtained as a white solid.
A mixture of Compound **4** (19.5 mg, 5.91 μmol), **BCY7741** (14 mg, 6.14 μmol, 1.01eq), and THPTA (0.4 M, 15 μL, 1 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 15 μL, 1 eq) and VcNa (0.4 M, 30 μL, 2 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY9400** (13.9 mg, 27.2% yield) was obtained as a white solid.

Synthesis of BCY10578

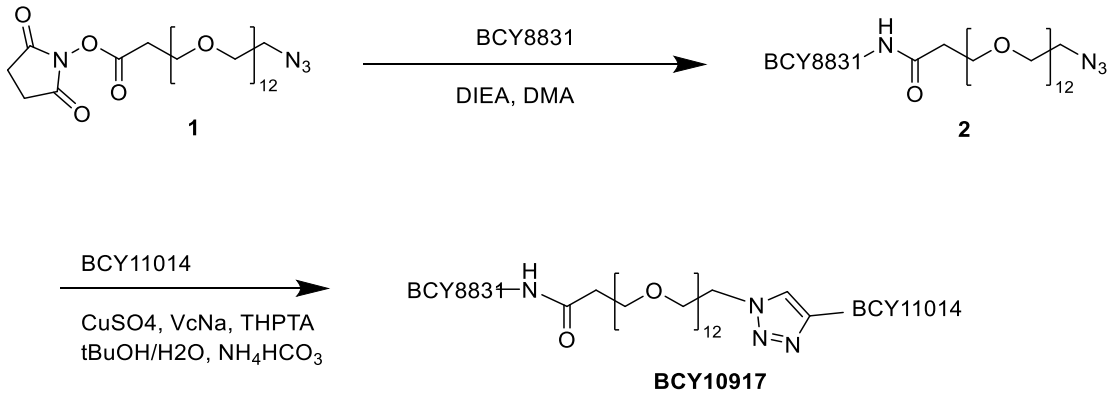


Compound **1** (5.0 mg, 49.5 umol, 1.0 eq) was first activated by mixing with EDCI (8.5 mg, 54.8 umol, 1.1 eq) and HOSu (5.7 mg, 49.5 umol, 1.0 eq)**.** The mixture was stirred at 25-30 °C for 30 min. TLC indicated compound **1** was consumed completely and one new spot formed. Then

compound **BCY9172** (80.0 mg, 38.18 μmol, 0.8 eq.) and DIEA (6.3 mg, 8.5 μL, 49.5 μmol, 1.0 eq.) were added to this mixture, and stirred at 40 °C for 1 hr, till LC-MS showed one main peak with desired m/z (calculated MW:2178.46, observed *m/z*: 1089.44 ($[M/2+H]^+$) was detected. The reaction mixture was then concentrated under reduced pressure to remove solvent and produced a residue, followed by purification by prep-HPLC (TFA condition). Compound **2** (15 mg, 6.88 μmol, 18.66% yield, 73.3% purity) was obtained as a white solid.

A mixture of compound **3** (9.8 mg, 4.5 μmol, 1.0 eq.), **BCY8846** (14.0 mg, 4.6 μmol, 1.0 eq.), and THPTA (2.0 mg, 4.6 μmol 1.0 eq.) was dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 12 μL, 1.0 eq.) and VcNa (0.4 M, 24 μL, 2.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 40 °C for 2 hr under N_2 atmosphere. The reaction mixture was filtered and concentrated under reduced pressure and purified by prep-HPLC (TFA condition), and **BCY10578** (13.78 mg, 58.66% yield) was obtained as a white solid.

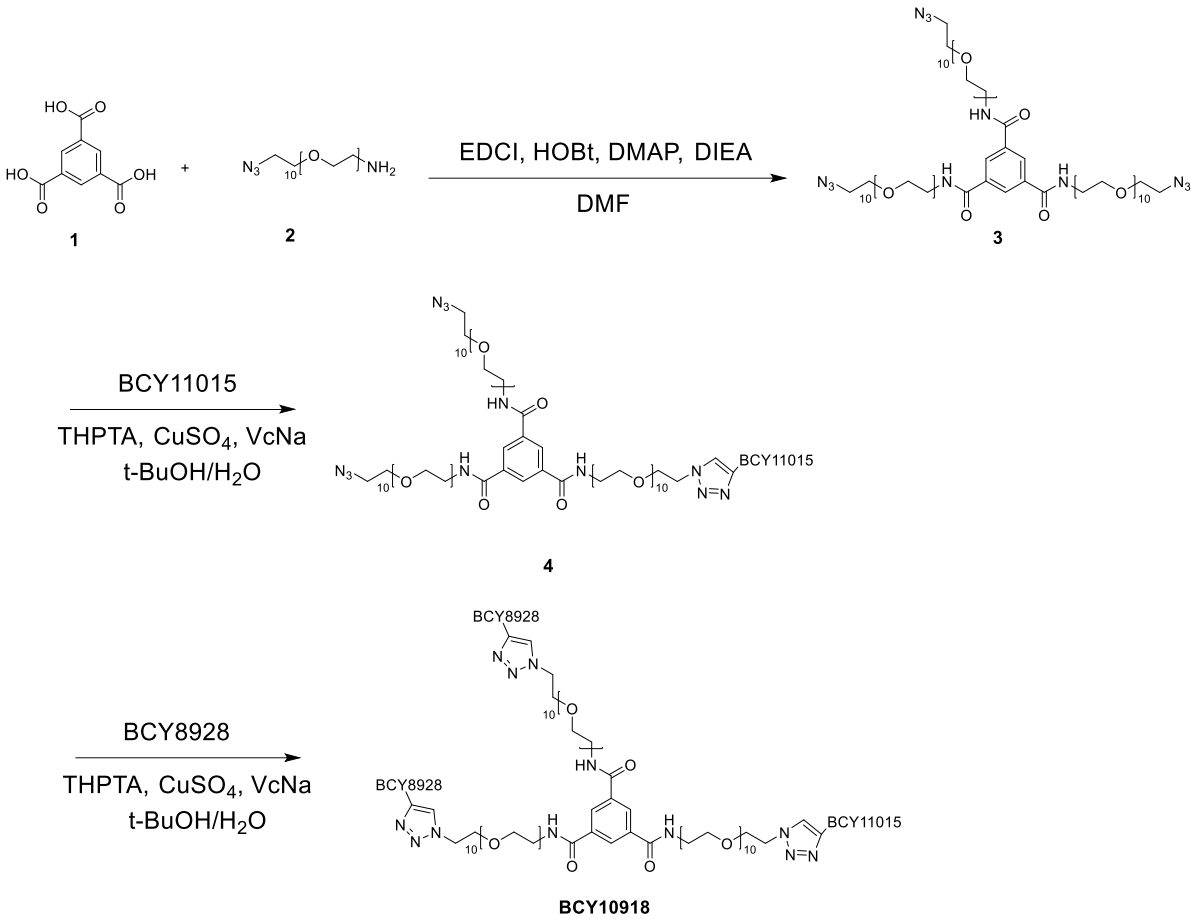
Synthesis of BCY10917



BCY8831 (40.0 mg, 13.3 μmol, 1.0 eq) and Compound **1** (10.5 mg, 14.2 μmol, 1.07 eq) were dissolved in DMF (1 mL). The solution was added with DIPEA (2.6 mg, 20.09 μmol, 3.5 μl, 1.5 eq), and then the mixture was stirred at 30°C for 16 hr. LC-MS showed **BCY8831** was consumed completely and one main peak with desired m/z (calculated MW: 3635.16 observed *m/z*: 1212.0($[M/3+H]^+$)) was detected. The reaction mixture was purified by prep-HPLC (TFA condition) and Compound **2** (22.0 mg, 5.83 μmol, 43.85% yield, 96.39% purity) was obtained as a white solid.

Compound **2** (10.0 mg, 2.75 μmol, 1.0 eq) and **BCY11014** (5.98 mg, 2.75 μmol, 1.0 eq), were dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 13.7 μL, 2.0 eq), VcNa（1.1 mg, 5.55 μmol, 2.0 eq） and THPTA (1.2 mg, 2.76 μmol, 1.0 eq) were added. Finally 1 M NH_4HCO_3 was added to adjust pH to 8. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY10917** (6.4 mg, 39.03% yield) was obtained as a white solid.

Synthesis of BCY10918



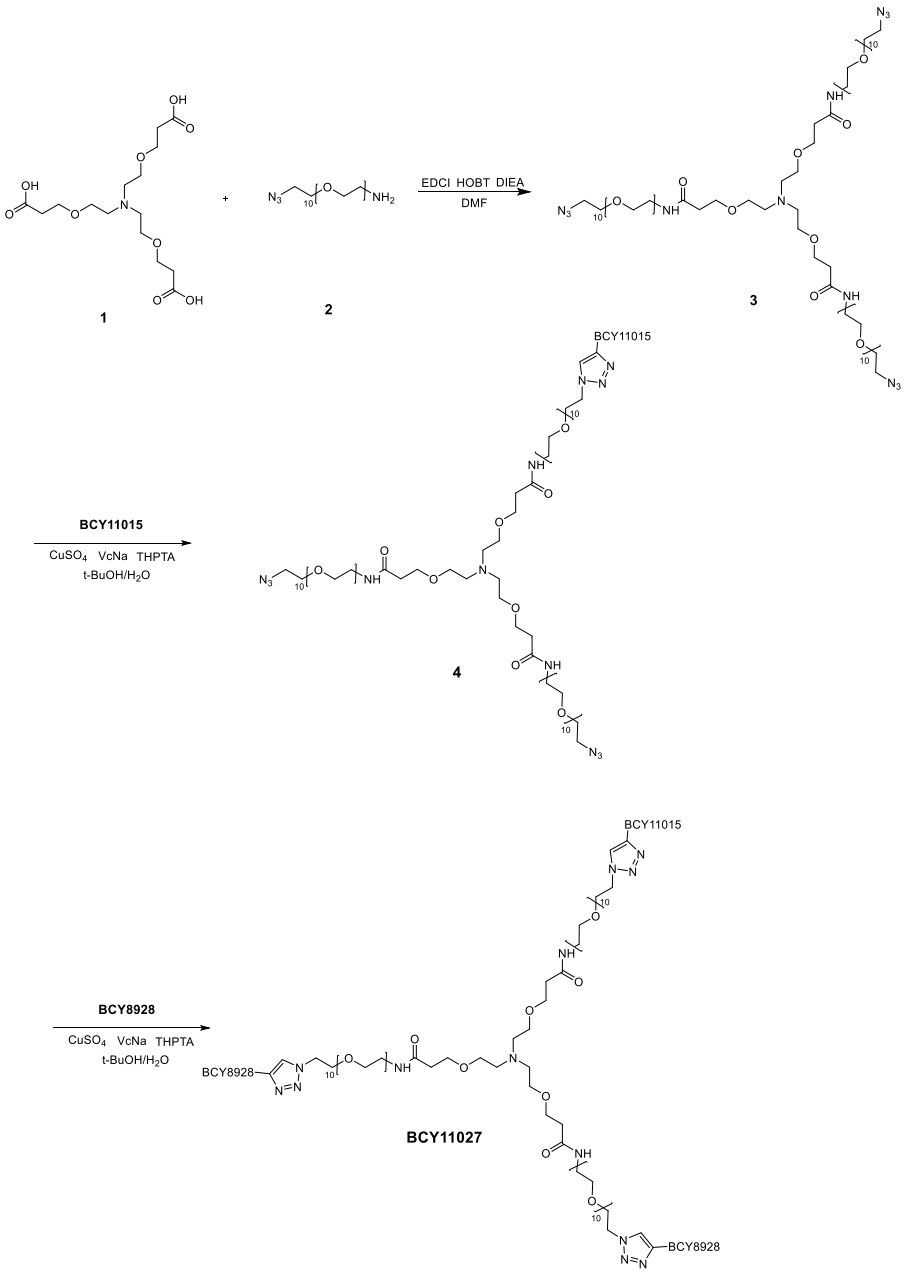
1 **2** **3** **4** **BCY10918**

To a solution of Compound **1** (20.0 mg, 95.2 μmol, 1.0 eq), Compound **2** (320.0 mg, 291.1 μmol, 3.06 eq) in DMF (5 mL) was added EDCI (60.0 mg, 313.0 μmol, 3.29 eq), HOBt (40.0 mg, 296.0 μmol, 3.11 eq), DMAP (10.0 mg, 81.8 μmol, 0.86 eq) and DIEA (44.5 mg, 344.5 μmol, 60 μL, 3.62 eq). The mixture was stirred at 30 °C for 12 hr. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z (calculated 30 MW: 3454.01, observed m/z: 1168.40([M/3+H2O]+)) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent to give a residue. The residue was purified by prep-HPLC (TFA condition). Compound **3** (200.0 mg, 57.9 μmol, 60.84% yield, 100% purity) was obtained as a white solid.

A mixture of Compound **3** (102 mg, 58.76 umol, 1.0 eq), **BCY11015** (92.6 mg, 41.13 umol, 0.7 eq) and THPTA (0.4 M, 146.9 uL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 146. 9 uL, 1.0 eq) and VcNa (0.4 M, 293.8 uL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. LC-MS showed Compound **3** was consumed completely and one main peak with desired m/z [MW: 3988.52 observed *m/z*: 1329.97([M/3+H^+]) and 990.56 ([M/4+H^+])] was detected. The reaction mixture was directly purified by prep-HPLC (TFA condition**)**. Compound **4** (60 mg, 13.61 umol, 23.16% yield, 90.45% purity) was obtained as a white solid.

A mixture of Compound **4** (60 mg, 15.04 umol, 1.0 eq), **BCY8928** (72.0 mg, 32.47 umol, 2.2 eq) and THPTA (0.4 M, 37.6 uL, 1.0 eq) was dissolved in t-BuOH/H_2O (1:1, 2 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 37.6 uL, 1.0 eq) and VcNa (0.4 M, 75.2 uL, 2.0 eq) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned to light yellow. The reaction mixture was stirred at 25-30 °C for 12 hr under N_2 atmosphere. The reaction mixture was directly purified by prep-HPLC (TFA condition). **BCY10918** (48 mg, 36.38% yield) was obtained as a white solid.

Synthesis of BCY11027

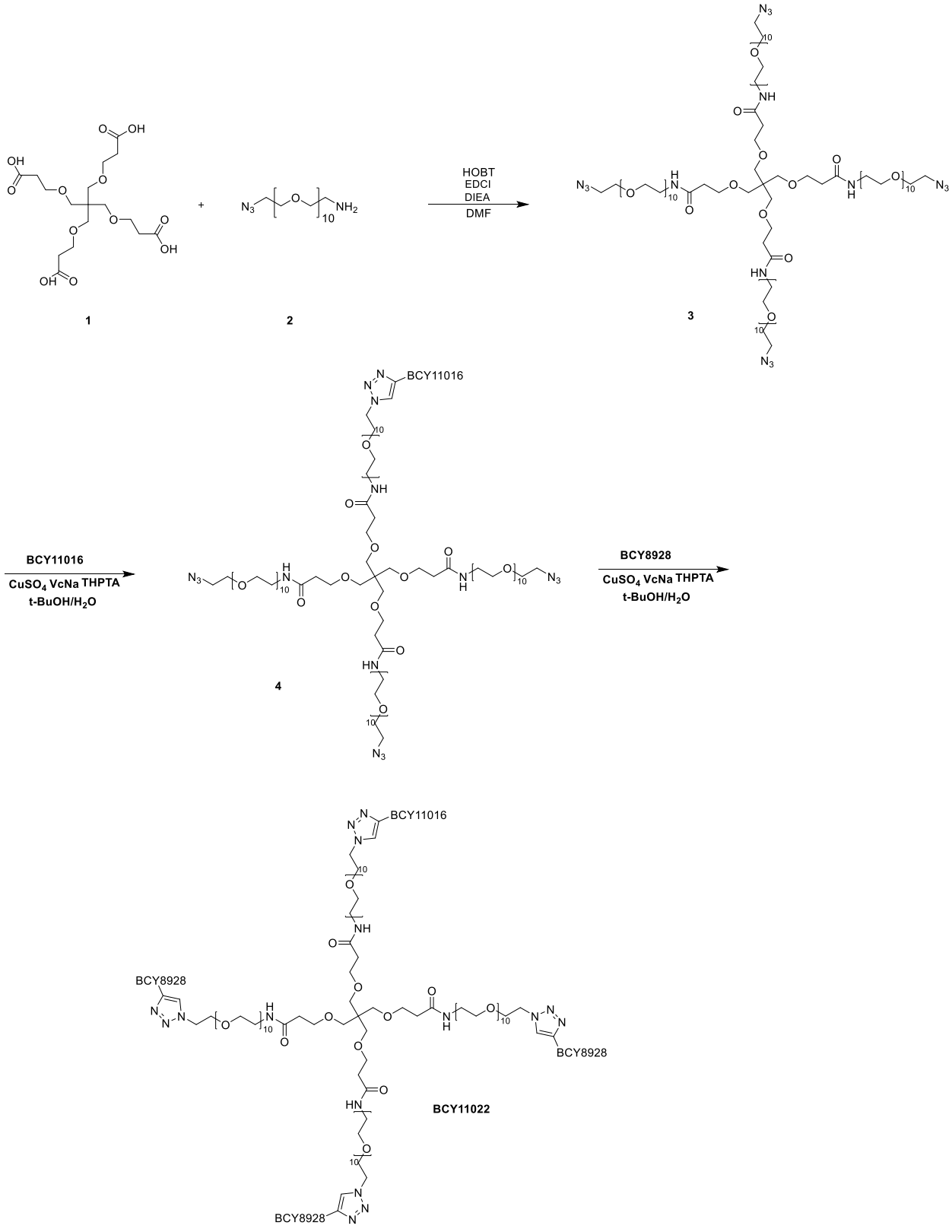


To a solution of Compound **1** (100 mg, 248.9 μmol, 1 eq, HCl) in DMF (1 mL) was added EDCI (160 mg, 834.6 μmol, 3.4 eq) and HOBt (110 mg, 814.1 μmol, 3.3 eq) and DIPEA (193 mg, 1.49 mmol, 260.08 μL, 6.0 eq), then Compound **2** (400 mg, 759.6 μmol, 3.1 eq) in DMF (1 mL) was added dropwise. The mixture was stirred at 25-30 °C for 12 hrs. LC-MS showed Compound **1** was consumed completely and one main peak with desired m/z was detected. The reaction mixture was purified by prep-HPLC (TFA condition) to give Compound **3** (128 mg, 64.3 μmol, 25.8% yield, 95% purity) as a colorless oil.

All solvents were degassed and purged with N_2 for 3 times. Compound **3** (22.0 mg, 10.58 μmol, 1.0 eq) and **BCY11015** (26.0 mg, 34.72 μmol, 1.1 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 26.4 μL, 1.0 eq), VcNa (4.2 mg, 21.2 μmol, 2.0 eq) and THPTA (4.6 mg, 10.58 μmol, 1.0 eq) were added. Finally, 1 M NH_4HCO_3 was added to adjust pH to 8. The reaction mixture was stirred at 30°C for 16 hr under N_2 atmosphere. LC-MS showed one main peak with desired m/z (calculated MW: 4143.75, observed *m/z*: 1040.50 ($[(M+18]/4+H]^+$), and 1381.27($[M/3+H]^+$)). The reaction mixture was purified by prep-HPLC (TFA condition) and Compound **4** (11.0 mg, 2.50 μmol, 23.66% yield, 94.26% purity) was obtained as a white solid.

Compound **4** (5.5 mg, 1.33 μmol, 1.0 eq) and **BCY8928** (5.9 mg, 2.66 μmol, 2.0 eq) were first dissolved in 2 mL of t-BuOH/H_2O (1:1), and then $CuSO_4$ (0.4 M, 10.0 μL, 3.0 eq), VcNa (1.0 mg, 5.05 μmol, 3.8 eq) and THPTA (1.0 mg, 2.30 μmol, 1.7 eq) were added. Finally, 1 M NH_4HCO_3 was added to adjust pH to 8. All solvents here were degassed and purged with N_2 for 3 times. The reaction mixture was stirred at 35°C for 16 hr under N_2 atmosphere. The reaction mixture was purified by prep-HPLC (TFA condition) and **BCY11027** (2.8 mg, 24.5% yield, 91.71% purity) was obtained as a white solid.

Synthesis of BCY11022

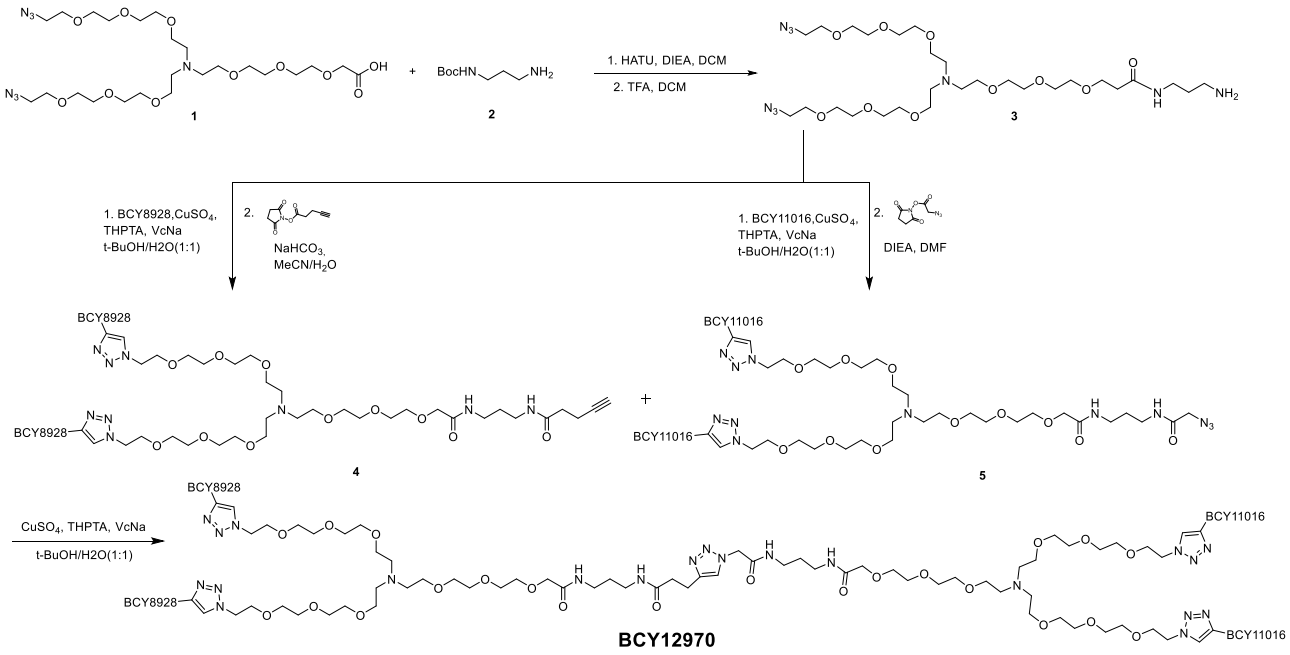


To a solution of compound **1** (100 mg, 235.63 μmol, 1 eq) in DMF (1 mL) was added EDCI (200 mg, 1.04 mmol, 4.43 eq) and HOBt (140 mg, 1.04 mmol, 4.4 eq) and DIPEA (185.50 mg, 1.44 mmol, 0.25 mL, 6.09 eq), then compound **2** (500 mg, 949.45 μmol, 4.03 eq) in DMF (1 mL) was added dropwise. The mixture was stirred at 25-30 °C for 12 hrs. The reaction mixture was purified by prep-HPLC (TFA condition) to give compound **3** (385 mg, 148.75 μmol, 63.13% yield, 95% purity) as a light yellow oil m/z (MW: 2458.85 observed *m/z*: 1229.40 ([M/2+H$^+$])).

Compound **3** (4.8 mg, 1.95 μmol, 1.0 eq.), **BCY11016** (4.1 mg, 1.76 μmol, 0.9 eq.), and THPTA (1.0 mg, 2.0 μmol, 1.0 eq.) were dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (10.0 μL, 0.4M, 2.0 eq.) and VcNa (0.4 M, 5.1 μL, 2.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned light yellow. The reaction mixture was stirred at 40 °C for 4 hr, till LC-MS showed one main peak with desired m/z (calculated MW: 4782.46, observed *m/z*: 1196.1 ([M/4+H]$^+$). The reaction mixture was then concentrated under reduced pressure and purified by prep-HPLC (TFA condition). Compound **4** (2.7 mg, 0.56 μmol, 27.3% yield, 94.5% purity) was obtained as a white solid after lyophilization.

Compound **4** (2.7 mg, 0.6 μmol, 1.0 eq.), **BCY8928** (5.3 mg, 2.38 μmol, 4.0 eq.), and THPTA (0.9 mg, 2.1 μmol, 3.5 eq.) were dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 6.0 μL, 4.0 eq.) and VcNa (0.4 M, 6.0 μL, 4.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH_4HCO_3 (in 1:1 t-BuOH/H_2O), and the solution turned light yellow. The reaction mixture was stirred at 40 °C for 4 hr under N_2 atmosphere. The reaction mixture was concentrated under reduced pressure and purified by prep-HPLC (TFA condition). **BCY11022** (1.9 mg, 23.2% yield) was obtained as a white solid after lyophilization.

Synthesis of BCY12970



Compound **1** (20.0 mg, 32.07 µmol, 1.0 eq.) was dissolved in DCM and HATU (13.4 mg, 35.27 µmol, 1.1 eq.) and DIEA (11.2 µL, 2.0 eq.) was added and stirred for 30 min. Compound **2** (6.7 mg, 38.48 µmol, 1.2 eq.) was added and stirred for 0.5 h. LC-MS showed compound 1 was consumed completely and one main peak with desired m/z (cal. MW: 779.92, observed m/z: [M+H]+=780.4) was detected. The reaction mixture was concentrated under reduced pressure to remove solvent and produced a residue. The crude product was used into next step without further purification by dissolving in 20% TFA/DCM (0.5 mL). The reaction mixture was stirred at 20-25 °C for 0.5 hr. LC-MS showed one main peak with desired m/z (MW:679.92, observed m/z: [M+H]+ 680.4) was detected. Compound **3** (21.8 mg, 32.07 µmol) was obtained as a yellow oil without further purification. A second batch of Compound **3** was also prepared.

(a) To first batch of compound **3 (**21.8 mg, 32.07 µmol, 1.0 eq.)**, BCY8928** (142.2 mg, 64.14 µmol, 2.0 eq.), and THPTA (28.0 mg, 64.14 µmol, 2.0 eq.) were dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 160.0 µL, 2.0 eq.) and VcNa (26.8 mg, 128.28 µmol, 4.0 eq.) were added under N_2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH4HCO3 (in 1:1 t-BuOH/H2O), and the solution turned to light yellow. The reaction mixture was stirred at 20-25 °C for 0.5 hr under N2 atmosphere. LC-MS showed compound **3** was consumed completely and desired m/z (cal. MW: 5114.96, observed m/z: 1705.7([M/3+H]+), 1279.3([M/4+H]+)) was detected. The reaction mixture was filtered to remove the undissolved residue. The crude product was purified by prep-HPLC (TFA condition), and an intermediate (68.4 mg, 12.85 µmol, 40.07% yield, 96.1% purity) was obtained as a white solid. Activated NHS ester of 4-penynoic acid (1.5mg, 7.31 µmol, 1.1 eq) was added to the purified intermediate (34.0 mg, 6.65 µmol, 1.0 eq.) in MeCN/H_2O and pH was adjusted to 8 using 0.4 M $NaHCO_3$ and stirred for 1 h and purified by RP-HPLC to obtain Compound **4** (31.3 mg, 5.42 µmol, 16.91% yield, 92.3% purity) (calculated MW: 5327.09, observed *m/z*: 1332.5($[M/4+H]^+$))

(b) To second batch of compound **3, BCY11016** (149.0 mg, 64.14 µmol, 2.0 eq.), and THPTA (13.9 mg, 32.07 µmol, 1.0 eq.) were dissolved in t-BuOH/H_2O (1:1, 1 mL, pre-degassed and purged with N_2 for 3 times), and then $CuSO_4$ (0.4 M, 160.0 µL, 2.0 eq.) and VcNa (25.4 mg, 128.28 µmol, 4.0 eq. were added under N_2. The reaction mixture was stirred at 20-25 °C for 0.5 hr under N2 atmosphere. LC-MS showed compound **3** was consumed completely and desired m/z (calculated MW: 5327.09, observed m/z: 1332.5([M/4+H]+)) was detected. The reaction mixture was filtered to remove the undissolved residue. The crude product was purified by prep-HPLC (TFA condition), and an intermediate (31.3 mg, 5.42 µmol, 16.91% yield, 92.3% purity) was obtained as a white solid. Activated NHS ester of azidoacetic acid (0.4 mg, 2.06 µmol, 1.1 eq.) was added to the purified intermediate (10.0 mg, 1.88 µmol, 1.0 eq.) in DMF and DIEA was added and stirred for 0.5 h and purified by RP-HPLC to obtain compound **5** (3.9 mg, 0.66 µmol, 35.29% yield, 91.9% purity) (cal. MW: 5410.14, observed *m/z*: 1803.5($[M/3+H]^+$), 1353.6($[M/4+H]^+$), 1083.0($[M/5+H]^+$)).

A mixture of compound **4** (3.74 mg, 0.72 µmol, 1.0 eq.), compound **5** (3.9 mg, 0.72 µmol, 1.0 eq.), and THPTA (0.3 mg, 0.72 µmol, 1.0 eq.) was dissolved in t-BuOH/H2O (1:1, 1 mL, pre-degassed and purged with N2 for 3 times), and then CuSO4 (0.4 M, 2.0 µL, 1.0 eq.) and VcNa (0.3 mg, 1.44 µmol, 2.0 eq.) were added under N2. The pH of this solution was adjusted to 8 by dropwise addition of 0.2 M NH4HCO3 (in 1:1 t-BuOH/H2O), and the solution turned to light

yellow. The reaction mixture was stirred at 20-25 °C for 1 hr under N2 atmosphere. LC-MS showed compound **4** was consumed completely and one main peak with desired m/z (cal. MW: 10605.18, observed m/z: 1768.5 ([M/6+H]+), 1515.7 ([M/7+H]+), 1526.4 ([M/8+H]+), 1589.3([M/9+H]+)) was detected. The reaction mixture was filtered to remove the undissolved residue. The crude product was purified by prep-HPLC (TFA condition), and **BCY12970** (4.5 mg, 55.04% yield) was obtained as a white solid.

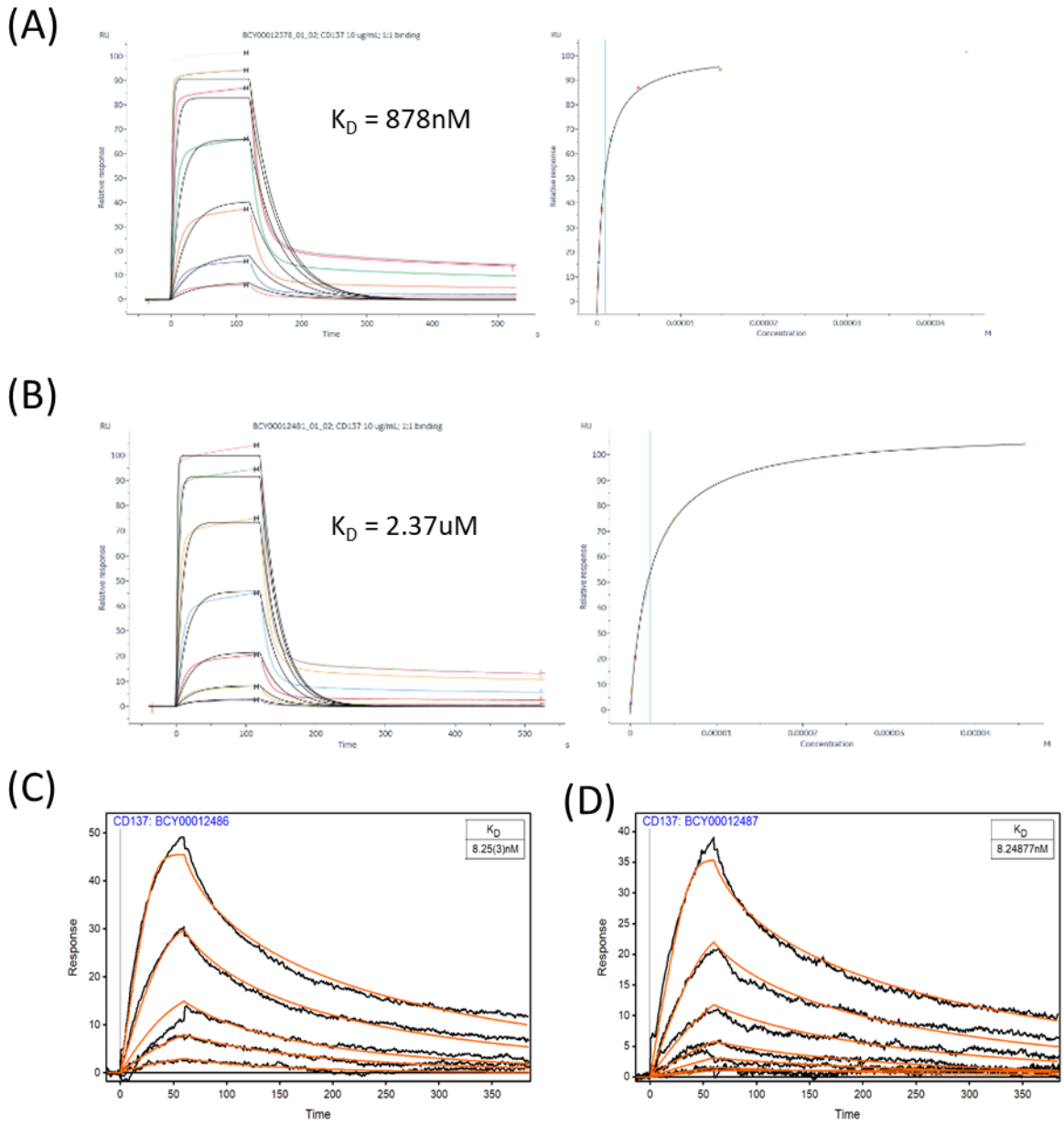


Fig. S1. SPR sensorgrams measuring CD137 binding of *Bicycle* TICAs incorporating weak CD137 binders in 1:1 and 1:2 format. (A and B) Sensorgram and K_D (nM) of 1:1 format *Bicycle* TICA (A) BCY12378 and (B) BCY12481 to immobilized CD137. (C and D) Sensorgrams of 1:2 Nectin-4/CD137 format *Bicycle* TICAs (C) BCY12486 and (D) BCY12487 incorporating the same CD137 binders as BCY12378 and BCY12481 and their enhanced affinity to immobilized CD137 due to avidity. The 'apparent' K_D of the dimeric CD137 binding motifs are shown.

Table S1. SPR binding affinity of 1:1 Nectin-4/CD137 *Bicycle* TICAs for Nectin-4 and CD137

BCY	CD137 K_D (nM)	Nectin-4 K_D (nM)
BCY8854	108	2.8
BCY9399	73	1.7
BCY9400	53	1.8
BCY10000	6.2	2.3
BCY10571	12	0.9
BCY10572	3.4	1.8
BCY10573	3.4	1.1
BCY11373	12	0.7
BCY11374	9.0	1.0
BCY11375	3.5	1.0
BCY11616	43	2.2
BCY11858	3.7	14
BCY12238	4.3	1.3
BCY12377	9.6	1.7
BCY12379	4.5	2.0
BCY12572	30	1.6
BCY12573	12	1.4
BCY12574	49	1.0
BCY12576	3.2	2.2
BCY12579	3.2	0.7
BCY12580	7.2	1.8
BCY12581	7.6	1.7
BCY12582	6.4	1.8
BCY12583	8.4	4.2
BCY12378	878	1.4
BCY12481	2370	2.5

Table S2. Geometric Mean of EC50 (nM) and Emax (fold induction over background) for HT1376/CD137 reporter coculture assay

BCY	n	EC50 (SD factor)	Emax (SD factor)
BCY8854	4	4.0 (1.3)	25 (1.2)
BCY9399	3	3.1 (1.4)	23 (1.5)
BCY9400	3	5.2 (1.5)	24 (1.4)
BCY10000	50	1.1 (1.7)	36 (1.5)
BCY10569	3	2.1 (1.3)	44 (1.4)
BCY10571	3	3.2 (1.3)	37 (1.4)
BCY10572	10	1.3 (1.7)	31 (1.3)
BCY10573	3	1.5 (1.3)	31 (1.5)
BCY10578	3	2.0 (1.4)	42 (1.3)
BCY10917	3	1.1 (1.5)	39 (1.3)
BCY10918	3	0.15 (1.5)	64 (1.2)
BCY11022	7	0.17 (1.6)	51 (1.8)
BCY11027	5	0.14 (1.7)	40 (1.4)
BCY11373	3	0.83 (1.6)	42 (1.4)
BCY11374	3	1.1(1.8)	44 (1.3)
BCY11375	3	0.85 (2.0)	37 (1.4)
BCY11384	3	1.6 (3.7)	21 (1.5)
BCY11385	3	0.20 (2.2)	59 (1.4)
BCY11616	7	2.4 (2.1)	28 (1.4)
BCY11858	3	7.0 (1.5)	33(1.3)
BCY11863	13	0.22 (2.4)	54 (1.5)
BCY11864	10	0.90 (2)	46 (1.6)
BCY12238	3	1.6 (1.6)	44 (1.4)
BCY12377	3	0.86 (1.7)	44 (1.3)
BCY12378	3	26.9 (1.9)	5.1 (1.6)
BCY12379	3	0.64 (1.6)	45 (1.3)
BCY12481	3	34 (3.2)	2.2 (1.2)
BCY12484	3	0.34 (1.5)	63 (1.3)
BCY12485	3	0.27 (1.2)	70 (1.3)
BCY12486	3	14 (1.7)	25 (1.5)
BCY12487	3	12 (1.6)	11 (1.6)
BCY12572	3	3.4 (1.4)	37 (1.4)
BCY12573	3	2.4 (1.4)	39 (1.3)
BCY12574	3	5.6 (1.5)	34 (1.3)
BCY12576	3	2.1 (1.4)	43 (1.3)
BCY12579	3	0.57 (1.5)	39 (1.2)
BCY12580	3	1.2 (1.3)	42 (1.2)
BCY12581	3	1.5 (1.4)	42 (1.2)
BCY12582	3	1.9 (1.2)	40 (1.2)
BCY12583	3	2.2 (1.3)	41 (1.2)
BCY12586	3	0.63 (1.4)	75 (1.2)
BCY12587	3	0.26 (1.4)	67 (1.2)
BCY12588	3	0.82 (1.4)	71 (1.2)
BCY12709	3	6.5 (1.3)	31 (1.5)
BCY12710	3	3.2 (1.4)	42 (1.2)
BCY12760	3	1.7 (1.3)	76 (1.2)
BCY12761	3	0.35 (1.3)	75 (1.1)
BCY12797	32	Not active (NA)	1.9 (1.3)
BCY12970	5	0.052 (1.6)	48 (1.1)

Table S3. EC50 (Mean ± SD) of *Bicycle* TICAs in the PBMC/HT1376 coculture assay (each BCY run in duplicate or triplicate in each experiment)

BCY	No. of independent experiments	INFy EC50 (nM)	IL-2 EC50 (nM)
BCY11385	4	0.43 ± 0.13	0.47 ± 0.30
BCY11863(24)	13	0.22 ± 0.12	0.36 ± 0.23
BCY11864	4	0.90 ± 0.21	0.83 ± 0.19
BCY10572	2	1.3	1.6
BCY10000	2	2.3	0.69
BCY12587	6	6.3 ± 5.7	4.4 ± 1.9
BCY8854	2	14	8.1
BCY13144	2	No Induction	No Induction

Table S4. Pharmacokinetic parameters of *Bicycle* TICAs in SD Rats

BCY	fu,p	n	CL (mL/min/kg)	Vdss (L/kg)	t½ (h)	CL, u (mL/min/kg)	Vdss, u (L/kg)
BCY10000	0.34	3	19 ± 2	0.46 ± 0.05	0.36 ± 0.04	55	1.4
BCY10572	0.37	3	18 ± 2	1.0 ± 0.3	0.93 ± 0.30	50	2.8
BCY10573	ND	3	16 ± 3	0.84 ± 0.08	0.96 ± 0.15	ND	ND
BCY10918	0.18	3	9.2 ± 1.4	1.0 ± 0.2	1.8 ± 0.2	51	5.3
BCY11027	0.24	3	12 ± 1	0.44 ± 0.02	0.59 ± 0.05	51	1.8
BCY11385	ND	3	8.7 ± 0.4	1.4 ± 0.13	3.6 ± 0.5	ND	ND
BCY11863	0.21	6	8.5 ± 1.5	1.5 ± 0.2	3.6 ± 0.6	41	7.0
BCY11864	0.2	3	9.8 ± 0.5	0.59 ± 0.08	0.95 ± 0.07	49	3.0
BCY12377	ND	3	7.8 ± 0.8	0.86 ± 0.02	1.7 ± 0.1	ND	ND
BCY12484	ND	3	11 ± 1	1.2 ± 0.2	2.1 ± 0.5	ND	ND
BCY12485	ND	3	13 ± 2	2.2 ± 0.6	3.1 ± 1.0	ND	ND
BCY12587	0.088	3	4.5 ± 0.5	4.8 ± 0.5	17 ± 1	51	54
BCY12590	ND	3	39 ± 1	1.0 ± 0.3	0.37 ± 0.06	ND	ND

ND: Not determined, fu,p: fraction unbound (Plasma protein binding), n: no. of animals, CL: Clearance, Vdss: Volume of distribution at steady state, t½: terminal half-life, CL,u :unbound clearance, Vdss,u: unbound volume of distribution

Table S5. Mean Pharmacokinetic parameters (mean ± SD) of BCY11863 in preclinical species

PK parameters	Mouse	Rat	NHP
CL (mL/min/kg)	16 ± 5	8.5 ± 1.5	4.0 ± 0.8
Vdss (L/kg)	2.3 ± 0.6	1.5 ± 0.2	0.88 ± 0.23
t½ (h)	2.3 ± 0.4	3.6 ± 0.6	6.2 ± 2.5

CL: Clearance, Vdss: Volume of distribution at steady state, t½: terminal half-life

Table S6. Valency, linker/hinge and identity of the Nectin-4 and CD137 binding bicycle incorporated in the *Bicycle* TICAs

BCY	Nectin-4: CD137 *Bicycle* Valency	Linker or Hinge	CD137 BCY	Nectin-4 BCY	General Synthesis Method
BCY8854	1:1	Sar10-Peg12	BCY7732	BCY8846	Method D
BCY9399	1:1	Peg10	BCY7741	BCY8116	Synthesis described
BCY9400	1:1	Peg23	BCY7741	BCY8116	Synthesis described
BCY10000	1:1	Sar10-Peg12	BCY9172	BCY8846	Method D
BCY10569	1:1	Sar10-Peg12	BCY8920	BCY8846	Method D
BCY10571	1:1	Peg5	BCY8927	BCY8116	Method A
BCY10572	1:1	Peg5	BCY8928	BCY8116	Method A
BCY10573	1:1	Peg5	BCY11014	BCY8116	Method A
BCY10578	1:1	Sar10	BCY9172	BCY8846	Synthesis described
BCY10917	1:1	Sar10-Peg12	BCY11014	BCY8831	Synthesis described
BCY10918	1:2	Trimesic-Peg10	BCY8928	BCY11015	Synthesis described
BCY11022	1:3	Tet-Peg10	BCY8928	BCY11016	Synthesis described
BCY11027	1:2	TCA-Peg10	BCY8928	BCY11015	Synthesis described
BCY11373	1:1	Azido acetic acid	BCY8927	BCY8116	Method C
BCY11374	1:1	Azido acetic acid	BCY8928	BCY8116	Method C
BCY11375	1:1	Azido acetic acid	BCY11014	BCY8116	Method C
BCY11384	2:1	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY9172	BCY11016	Method B
BCY11385	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY11014	BCY8116	Method B
BCY11616	1:1	Peg5	BCY7744	BCY8116	Method A
BCY11858	1:1	Peg5	BCY8928	BCY11414	Method A
BCY11863	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY8928	BCY8116	Method B
BCY11864	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY7744	BCY8116	Method B
BCY12238	1:1	Peg5	BCY8928	BCY12024	Method A
BCY12377	1:1	Peg5	BCY12143	BCY8116	Method A
BCY12378	1:1	Peg5	BCY12147	BCY8116	Method A
BCY12379	1:1	Peg5	BCY12149	BCY8116	Method A
BCY12481	1:1	Peg5	BCY12145	BCY8116	Method A
BCY12484	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12143	BCY8116	Method B
BCY12485	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12149	BCY8116	Method B
BCY12486	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12147	BCY8116	Method B
BCY12487	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12145	BCY8116	Method B
BCY12572	1:1	Peg5	BCY12352	BCY8116	Method A
BCY12573	1:1	Peg5	BCY12353	BCY8116	Method A
BCY12574	1:1	Peg5	BCY12354	BCY8116	Method A
BCY12576	1:1	Peg5	BCY8928	BCY12363	Method A
BCY12579	1:1	Peg5	BCY8928	BCY12366	Method A
BCY12580	1:1	Peg5	BCY8928	BCY12367	Method A
BCY12581	1:1	Peg5	BCY8928	BCY12368	Method A
BCY12582	1:1	Peg5	BCY8928	BCY12369	Method A
BCY12583	1:1	Peg5	BCY8928	BCY12370	Method A
BCY12586	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12352	BCY8116	Method B
BCY12587	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12353	BCY8116	Method B
BCY12588	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12354	BCY8116	Method B
BCY12709	1:1	Peg5	BCY12381	BCY8116	Method A
BCY12710	1:1	Peg5	BCY12382	BCY8116	Method A
BCY12760	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12381	BCY8116	Method B
BCY12761	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY12382	BCY8116	Method B
BCY12797	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY11506	BCY8116	Method B
BCY12970	2:2	Hinge 4 (Fig. 4)	BCY8928	BCY11016	Synthesis described
BCY13144	1:2	N-(acid-Peg3)-N-bis(Peg3-azide)	BCY8928	BCY11415	Method B

Table S7. Peptide sequence and resin used for solid phase synthesis of Nectin-4 and CD137 *Bicycles* incorporated into *Bicycle* TICAs. Attachment points to linkers are highlighted in bold.

BCY	Peptide Sequence, 1,3,5-Triacryloylhexahydro-1,3,5-triazine (TATA) cyclized	Target	Resin type
BCY7732	[Ac]ACIEEGQYCFADPY[Nle]C**[Dap][**CONH2]	CD137	Rink amide
BCY7741	[Ac]ACIEEGQYCFADPY[Nle]C**[Dap(PYA)]**[CONH2]	CD137	Rink amide
BCY7744	[Ac]ACIEE**[dK(PYA)]**QYCFADPY[Nle]CA[CONH2]	CD137	Rink amide
BCY8116	**NH2**-CP[1Nal][dD]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY8831	[Ac]CP[1Nal]**[dK(Sar10-B-Ala)]**CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY8846	**[PYA]**[B-Ala][Sar10]CP[1Nal][dD]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY8920	[Ac]C[tBuAla]PE**[dK]**PYCFADPY[Nle]CA[CONH2]	CD137	Rink amide
BCY8927	[Ac]C[tBuAla]P**K(PYA)**[dA]PYCFADPY[Nle]CA[CONH2]	CD137	Rink amide
BCY8928	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFADPY[Nle]CA[CONH2]	CD137	Rink amide
BCY9172	[Ac]C[tBuAla]PE[dA]PYCFADPY[Nle]C**[Dap]**[CONH2]	CD137	Rink amide
BCY11014	[Ac]C[tBuAla]PE[dA]PYCFADPY[Nle]C**[Dap(PYA)]**[CONH2]	CD137	Rink amide
BCY11015	**[PYA]**CP[1Nal][dD]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY11016	**[PYA]**[B-Ala]CP[1Nal][dD]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY11414	**NH2**-CPFGCM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY11415	**NH2**-CP[1Nal][dD]CM[HArg]D[dW]STP[HyP][dW]C[CONH2]	Nectin-4	Rink amide
BCY11506	[Ac][dA][dC][dI][dE][dE]**K(PYA)**[dQ][dY][dC][dF][dA][dD][dP][dY][dNle][dC][dA][CONH2]	CD137	Rink amide
BCY12024	[Ac]CP[1Nal]**[dK]**CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12143	[Ac]C[tBuAla]EE**[dK(PYA)]**PYCFADPY[Nle]C[CONH2]	CD137	Rink amide
BCY12145	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFAEPY[Nle]C[CONH2]	CD137	Rink amide
BCY12147	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFANPY[Nle]C[CONH2]	CD137	Rink amide
BCY12149	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFADPY[Nle]C[CONH2]	CD137	Rink amide
BCY12352	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFADPY[Nle][Cysam]	CD137	Cysteamine 2-chlorotrityl
BCY12353	[MerPro][tBuAla]PE**[dK(PYA)]**PYCFADPY[Nle]C[CONH2]	CD137	Rink amide
BCY12354	[MerPro][tBuAla]PE**[dK(PYA)]**PYCFADPY[Nle][Cysam]	CD137	Cysteamine 2-chlorotrityl
BCY12363	[MerPro]P[1Nal]**[dK]**CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12366	[Ac]CP[1Nal]**[dK]**CM[HArg]HWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12367	[Ac]CP[1Nal]**[dK]**CM[HArg]EWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12368	**NH2**-CP[1Nal][dE]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12369	**NH2**-CP[1Nal][dA]CM[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12370	**NH2**-CP[1Nal][dE]CL[HArg]DWSTP[HyP]WC[CONH2]	Nectin-4	Rink amide
BCY12381	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFAD[NMeAla]Y[Nle]C[CONH2]	CD137	Rink amide
BCY12382	[Ac]C[tBuAla]PE**[dK(PYA)]**PYCFAD[NMeDAla]Y[Nle]C[CONH2]	CD137	Rink amide

Ac = Acetyl, Nle = L-Norleucine, Dap = L-2,3-diaminopropionic acid, PYA = 4-pentynoic acid, CONH2 = C-terminal amide, 1-Nal = 3-(1-naphthyl)-L-alanine, HArg = L-homoarginine, [Hyp] = trans-4-Hydroxy-L-proline, Sar10-B-Ala = 10 X sarconine followed by beta alanine, B-Ala = Beta Alanine, tBuAla = t-butyl-L-alanine, Cysam = Cysteamine, MerPro = 3-mercaptopropanoic acid, d*X* = D-version of X amino acid, NMeAla = N-methyl-L-Alanine, NMeDAla = N-methyl-D-Alanine;NH2: free-N-terminal, (X) are prosthetic groups attached to side chain of the preceding amino acid. Amino acids highlighted in **Bold** indicate attachment points to the linkers.

Table S8. %Purity and m/z of *Bicycle* TICAs determined by HPLC (by batch) and MS

BCY	Calc. MW	Observed m/z	MS Detector	% HPLC Purity(batch)
BCY8854	5861.59	1173.3086,1466.3883,1954.8512	Q-TOF	95.3% (1), 95.9%(2)
BCY9399	5006.64	1002.3	Triple Quad	96.3% (1)
BCY9400	5579.33	1395.6175,1860.8205,2790.7182	Q-TOF	93.6% (1)
BCY10000	5755.55	1152.1068, 1439.8863, 1919.5150	Q-TOF	>98%(1), 95.0%(2)
BCY10569	5797.62	1160.7	Triple Quad	96.2% (1)
BCY10571	4649.36	1163.5	Triple Quad	96.5% (1)
BCY10572	4707.4	1570.0200, 2354.5282	Q-TOF	>95%(1,2,3,4,6), 93.2%(5)
BCY10573	4665.32	1555.9896,2333.4830	Q-TOF	>98% (1), 96.5(2)
BCY10578	5212.88	1304.0578, 1738.4109, 2607.6107	Q-TOF	96.2% (1)
BCY10917	5810.66	1163.1247, 1453.6590, 1937.8783	Q-TOF	97.5% (1), 97.6(2)
BCY10918	8423.67	1404.9618,1685.5564,2106.9487	Q-TOF	94.4% (1), 97.7%(2), 96.7%(3), 96.0%(4)
BCY11022	11435.22	1634.5985, 1906.8628, 2288.0332	Q-TOF	94.7% (1),91.3% (2)
BCY11027	8578.93	1430.8253, 1716.7918, 2145.7439	Q-TOF	95.9% (1), 93.5% (2)
BCY11373	4415.08	1472.2	Triple Quad	92.4% (1)
BCY11374	4473.12	1491.5	Triple Quad	92.0% (1)
BCY11375	4431.04	1477.9	Triple Quad	96.8% (1)
BCY11384	7348.4	1470.6174, 1838.0274, 2450.3695	Q-TOF	**87%* (1)** >90% purity not achieved
BCY11385	7129.18	1426.6269, 1783.2849, 2377.3764	Q-TOF	94% (1), 93.4% (2)
BCY11616	4827.47	1207.7584, 1610.0085, 2414.5036	Q-TOF	93.98% (1), 95.9% (2), 95.1%(3)
BCY11858	4599.31	1534.0000,2300.4983	Q-TOF	96.8% (1), >98% (2)
BCY11863	7213.34	1443.6375, 1804.2959, 2405.3879	Q-TOF	44 batches, all in-vivo batches >95%
BCY11864	7453.46	1491.6404, 1864.3041, 2485.4050	Q-TOF	89.2% (1), 92.3% (2), 95.6%(3)
BCY12238	4763.51	1588.3683, 2382.0505	Q-TOF	96.7% (1)
BCY12377	4668.33	1168	Triple Quad	95.3% (1)
BCY12378	4635.35	1546.6	Triple Quad	92.9% (1)
BCY12379	4636.33	1159.8	Triple Quad	91.5% (1), 95.5% (2)
BCY12481	4650.36	1550.9960, 2325.9929	Q-TOF	96.0% (1)
BCY12484	7135.17	1427.8198,1784.7698	Q-TOF	92.8% (1), 97.5% (2)
BCY12485	7071.18	1768.7	Triple Quad	92.0% (1), 95.6% (2)
BCY12486	7069.21	1768.2	Triple Quad	91.8% (1), 96.2% (2)
BCY12487	7099.23	1420.7882, 1775.7363, 2367.3136	Q-TOF	93.4% (1), 97.0% (2), 96.4%(3)
BCY12587	6957.08	1739.9936, 2319.6563	Q-TOF	90.1% (1), 90.9% (2)
BCY12588	6871.03	1718.7780, 2291.3712	Q-TOF	95.7% (1)
BCY12709	4624.32	1156.9935, 1542.3257, 2312.9870	Q-TOF	95.6% (1)
BCY12710	4624.32	1156.9956, 1542.3293, 2312.9922	Q-TOF	95.1% (1), >98% (2)
BCY12760	7047.16	1410.4137, 1762.7710, 2350.0283	Q-TOF	>98% (1)
BCY12761	7047.16	1410.2147, 1762.7707, 2350.0280	Q-TOF	94.8% (1)
BCY12970	10605.21	1515.9639, 1768.4578, 2121.9416	Q-TOF	92.5% (1)
BCY12797	7453.46	1491.6407, 1864.3017	Q-TOF	93.7% (1)
BCY12572	4593.31	1531.9920, 2297.4872	Q-TOF	97.3% (1)
BCY12573	4579.28	1527.3339, 2290.4998	Q-TOF	93.1% (1)
BCY12574	4536.25	1512.9840, 2268.9751	Q-TOF	94.0% (1)
BCY12576	4705.48	1569.3574, 2353.5350	Q-TOF	96.1% (1)
BCY12579	4784.58	1197.0372, 1595.7101	Q-TOF	91.5% (1),95.4% (2)
BCY12580	4776.56	1593.0535, 2389.0783	Q-TOF	96.5% (1)
BCY12581	4721.43	1181.2591, 1574.6800, 2361.5188	Q-TOF	93.6% (1)
BCY12582	4663.40	1166.7583, 1555.3457, 2332.5169	Q-TOF	92.0% (1)
BCY12583	4703.40	1176.7663, 1568.6894, 2352.5324	Q-TOF	92.4% (1)
BCY12586	6985.13	1397.8123, 1747.2672, 2329.3580	Q-TOF	95.1% (1)
BCY13144	7213.34	1443.6338, 1804.2928, 2405.3817	Q-TOF	92.8% (1)

HPLC traces of Bicycle TICAs used in in-vivo studies

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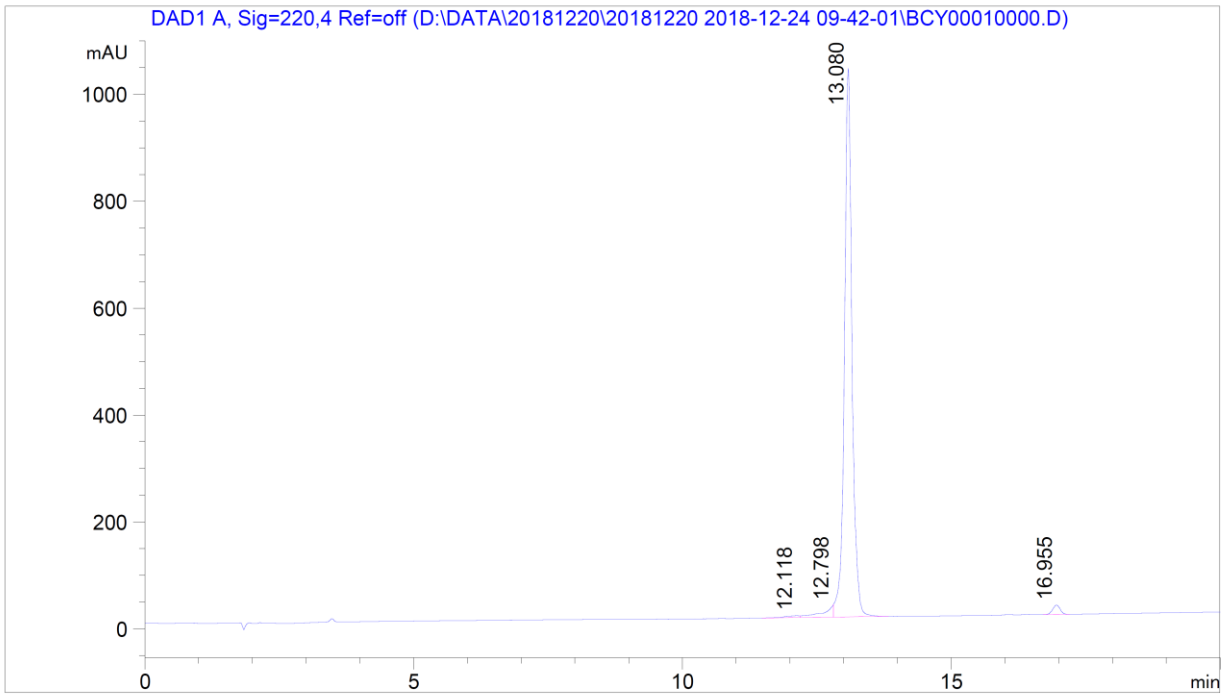

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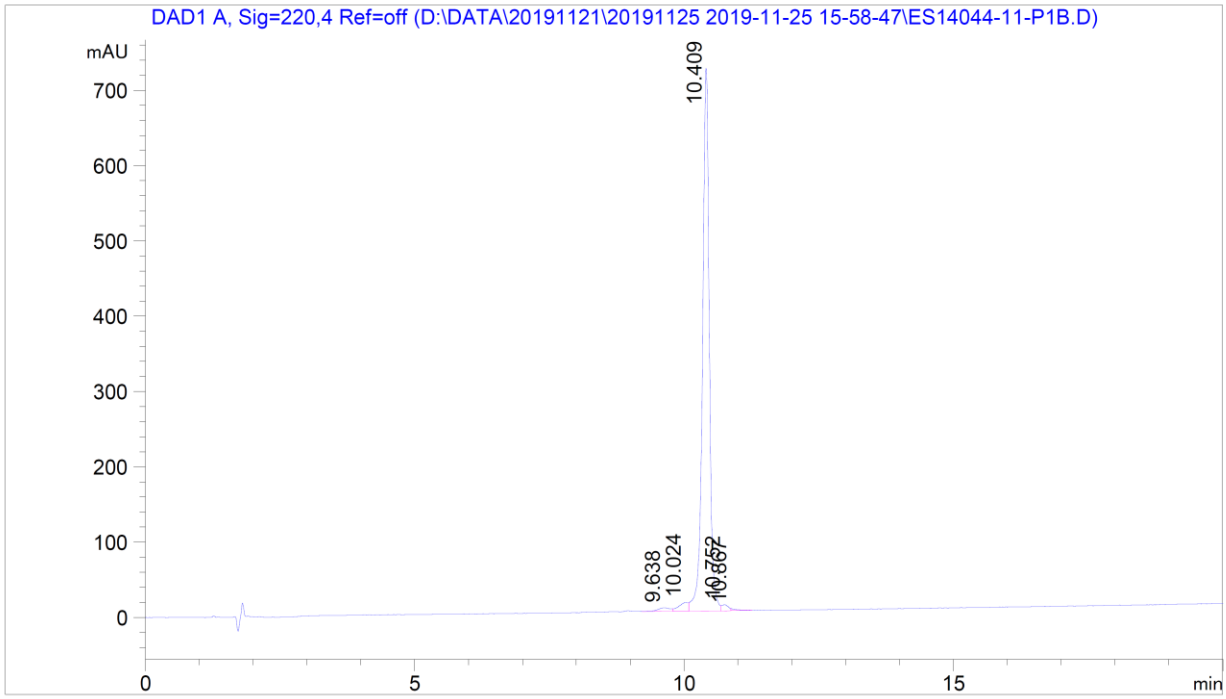

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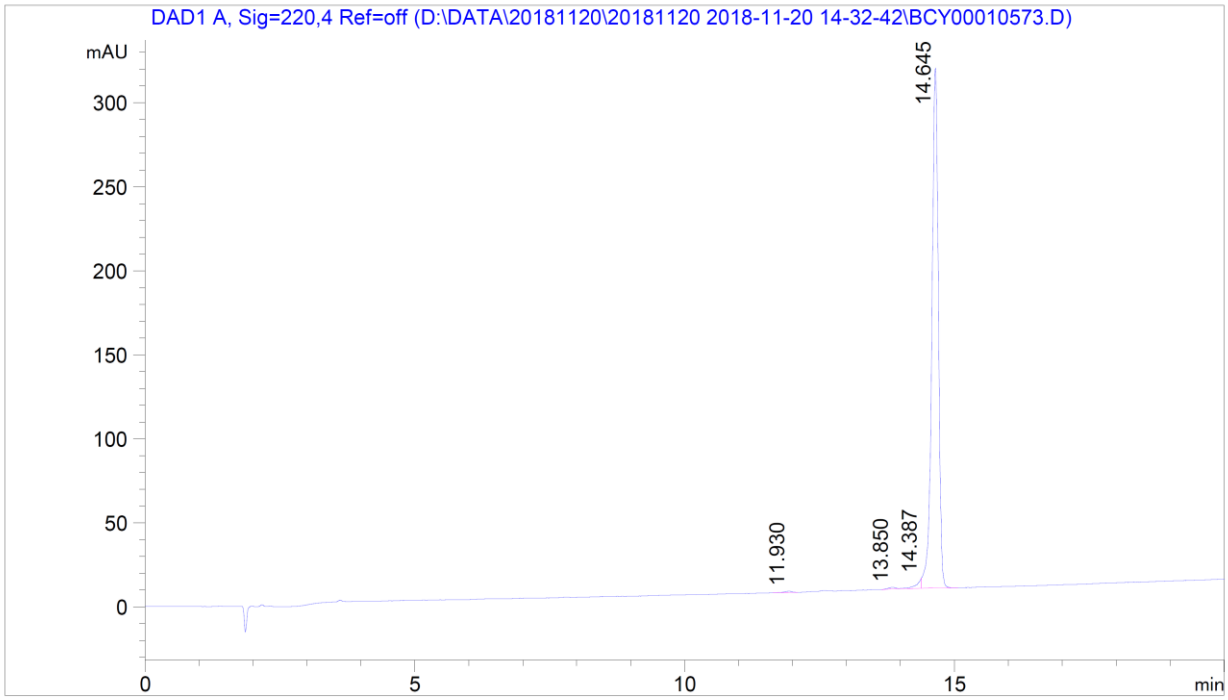

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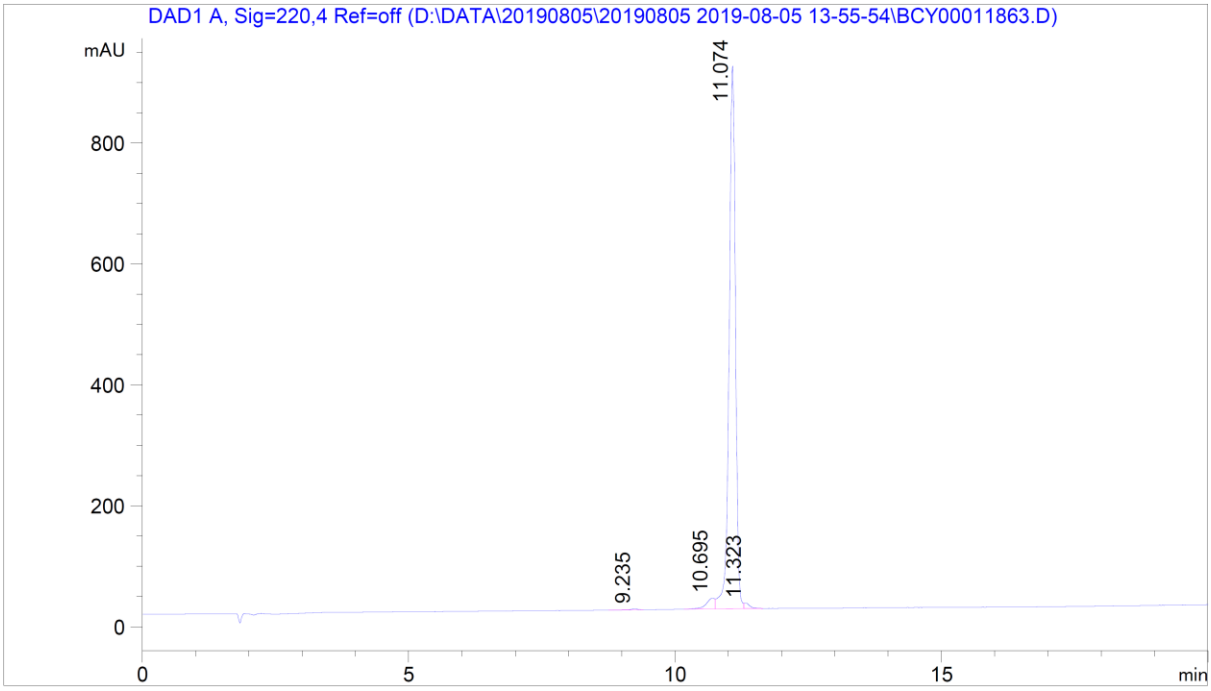

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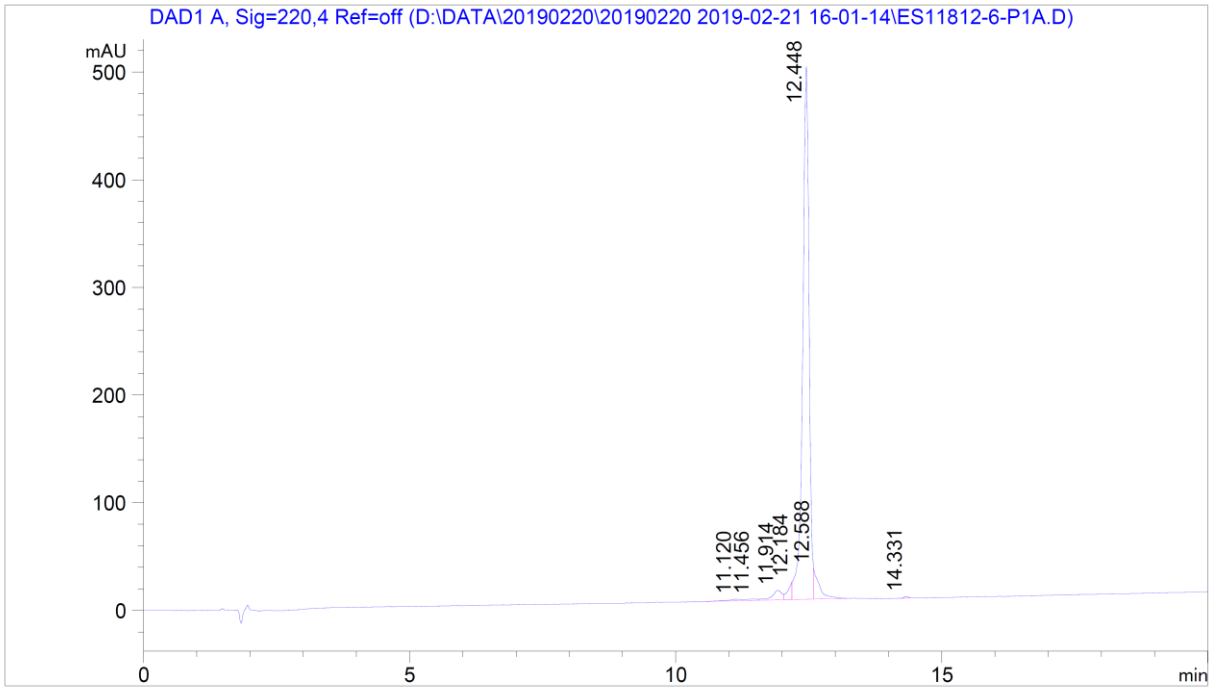

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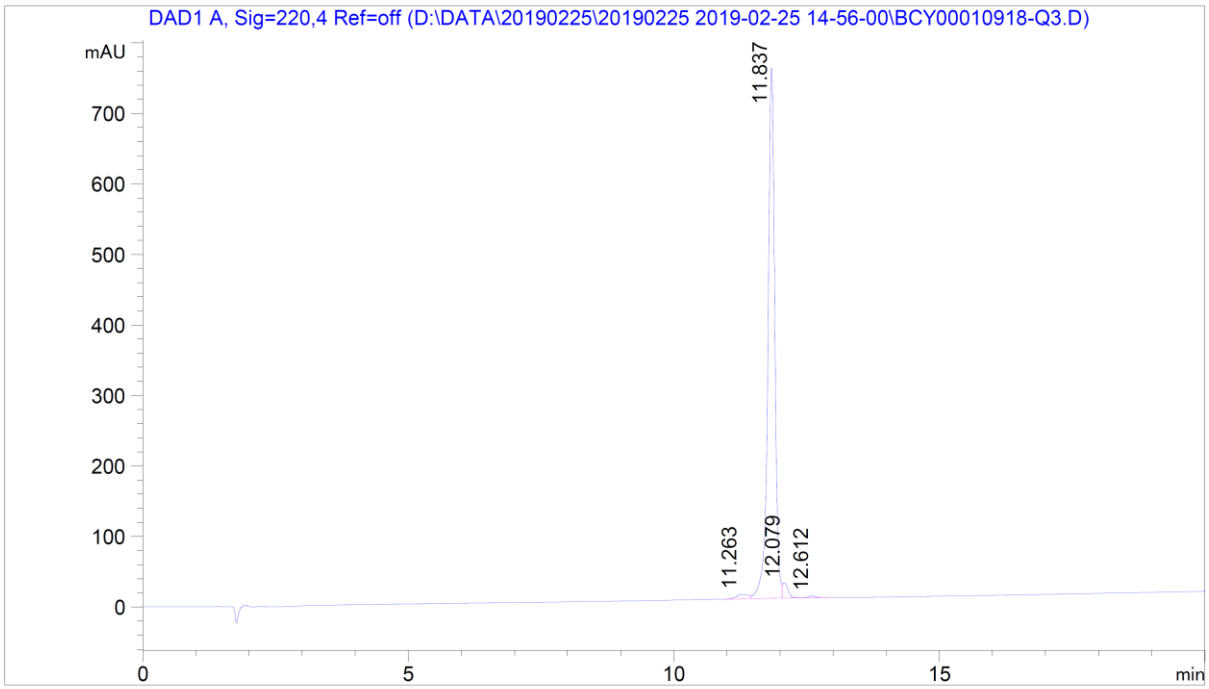

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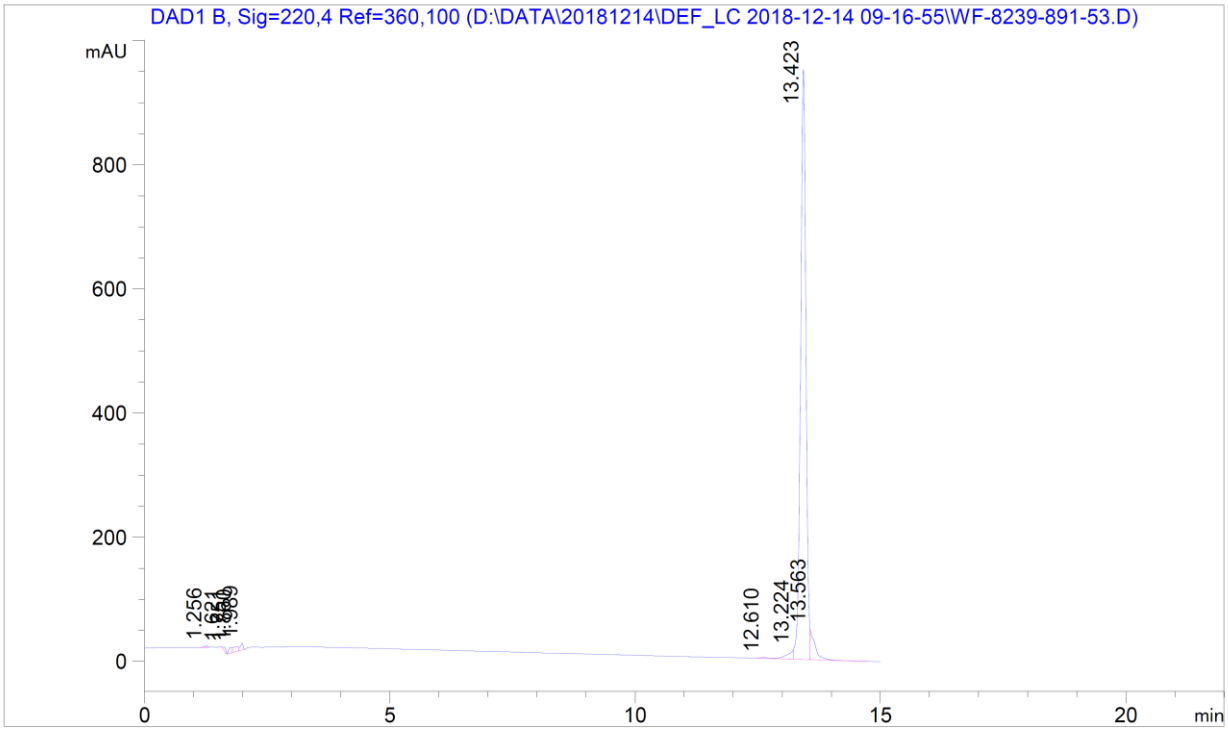

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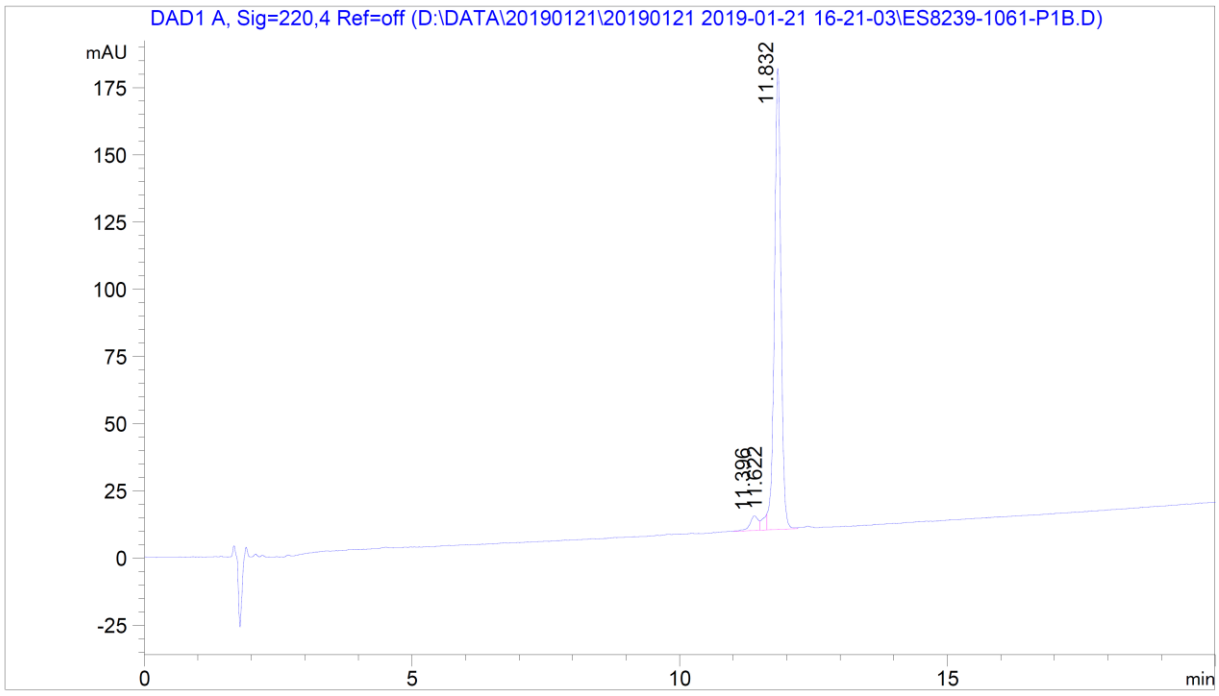

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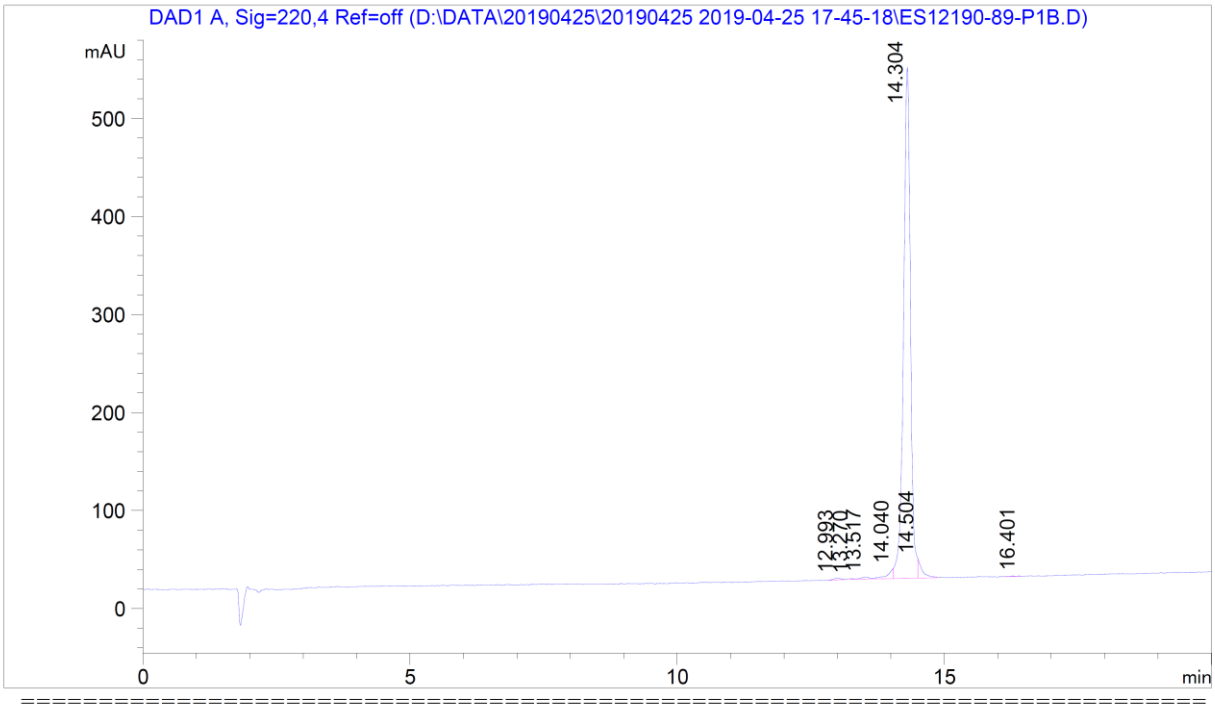

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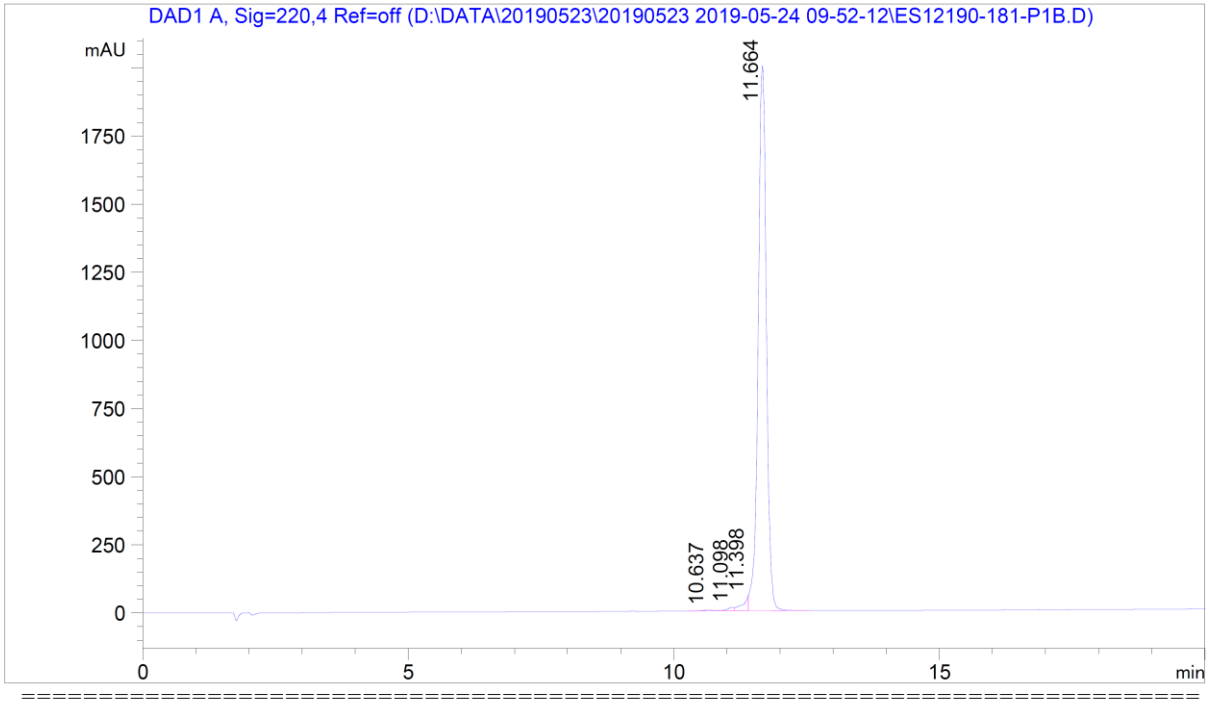

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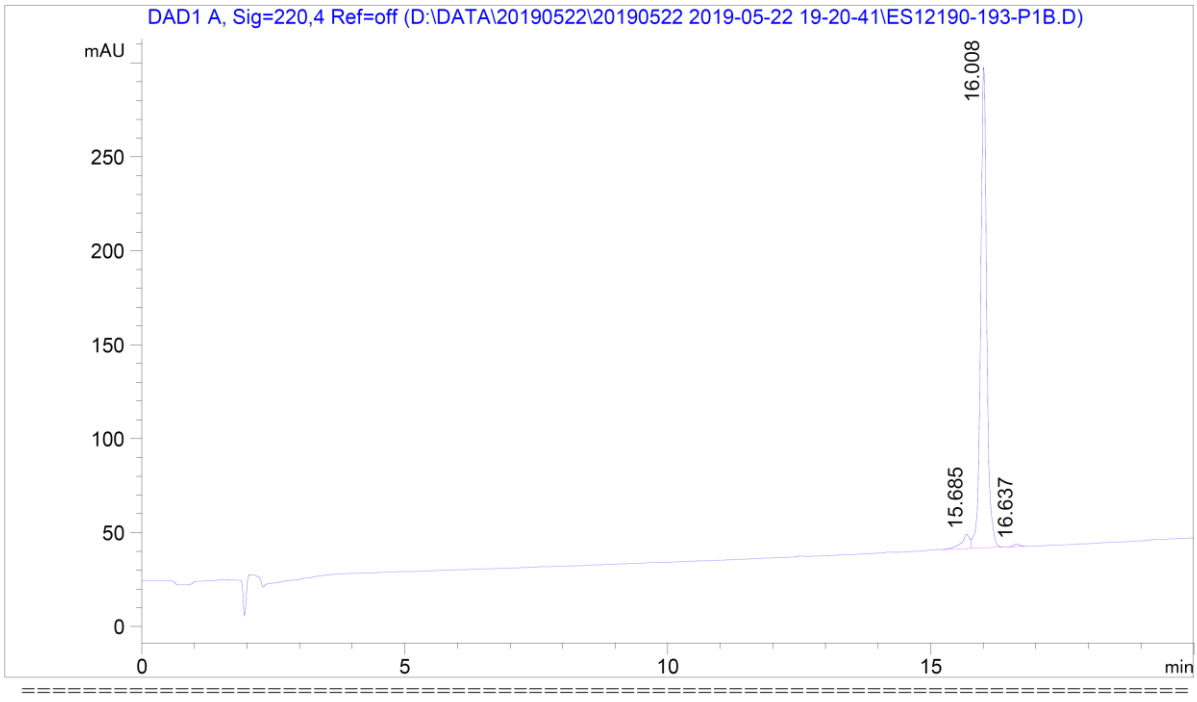

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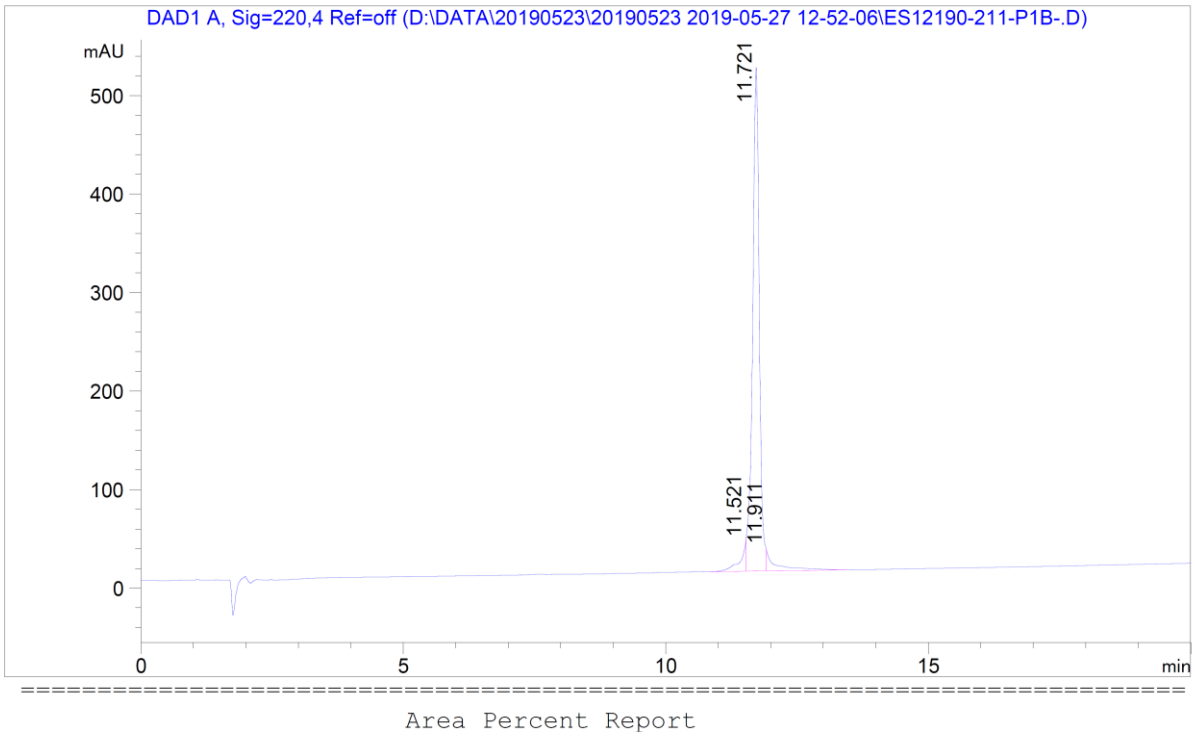

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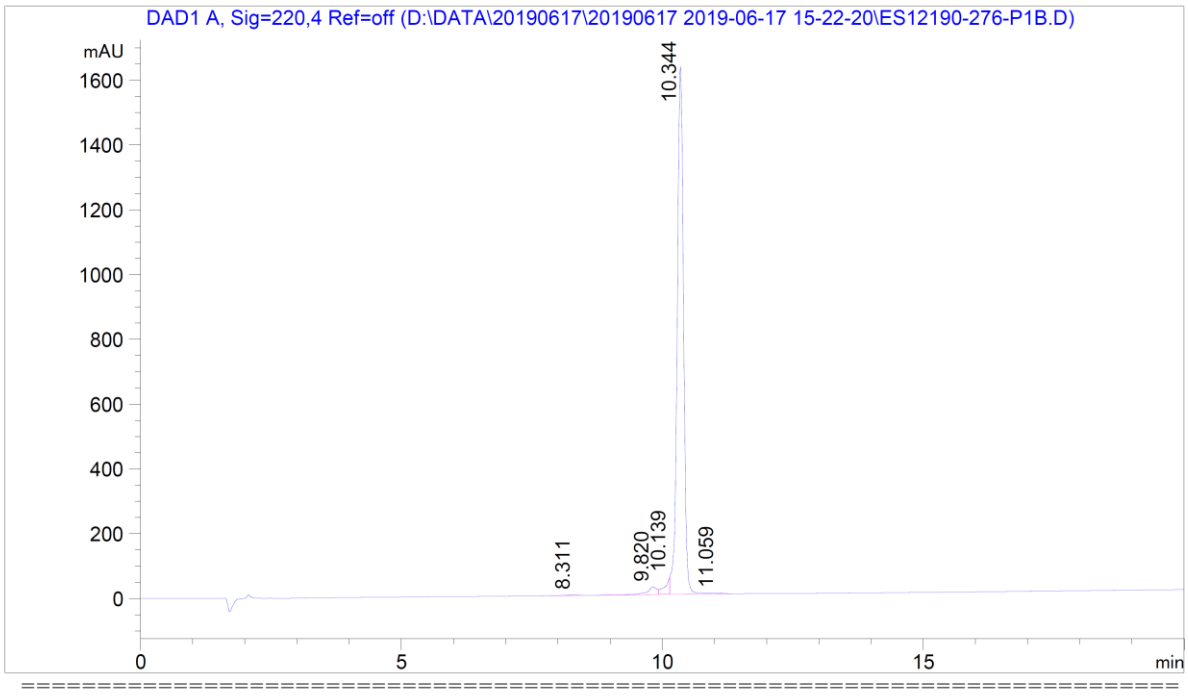
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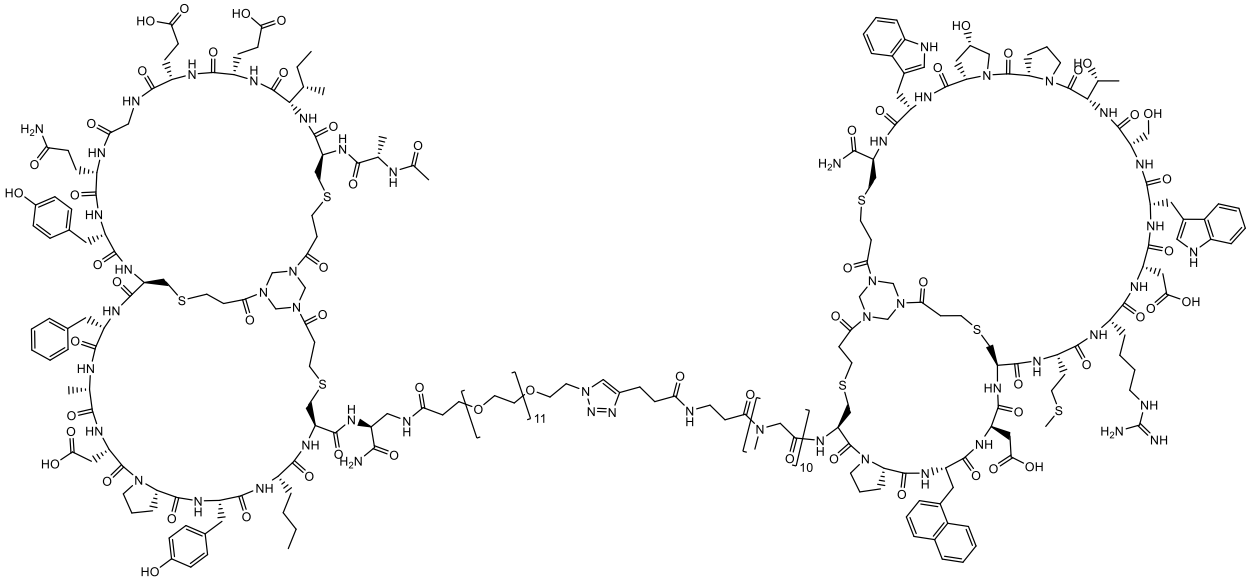
min

Structure of *Bicycle* TICAs

BCY8854



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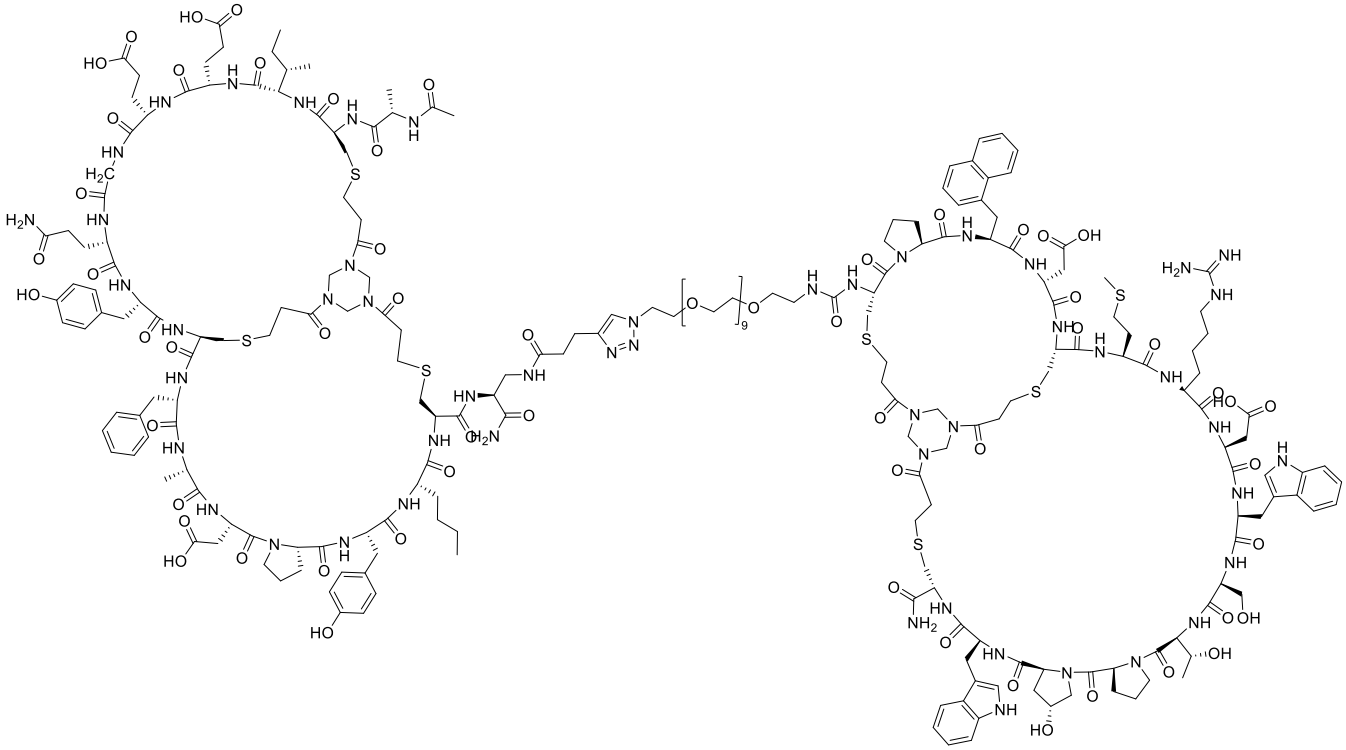

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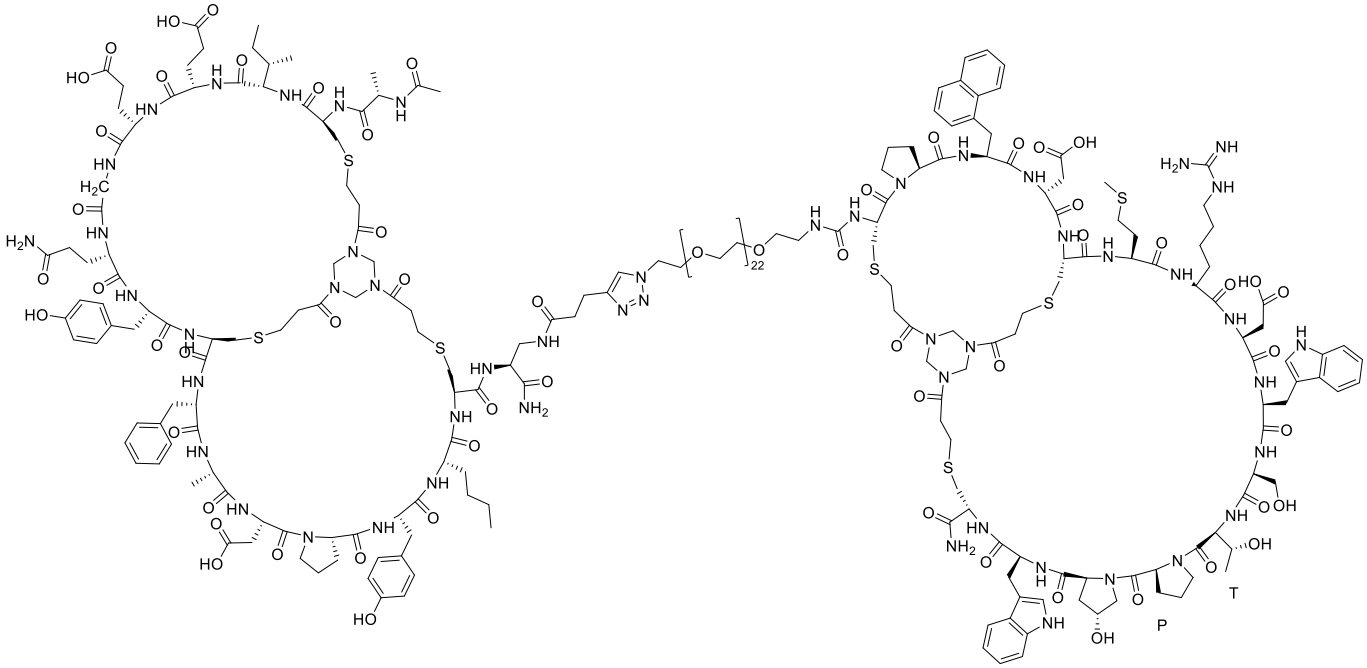

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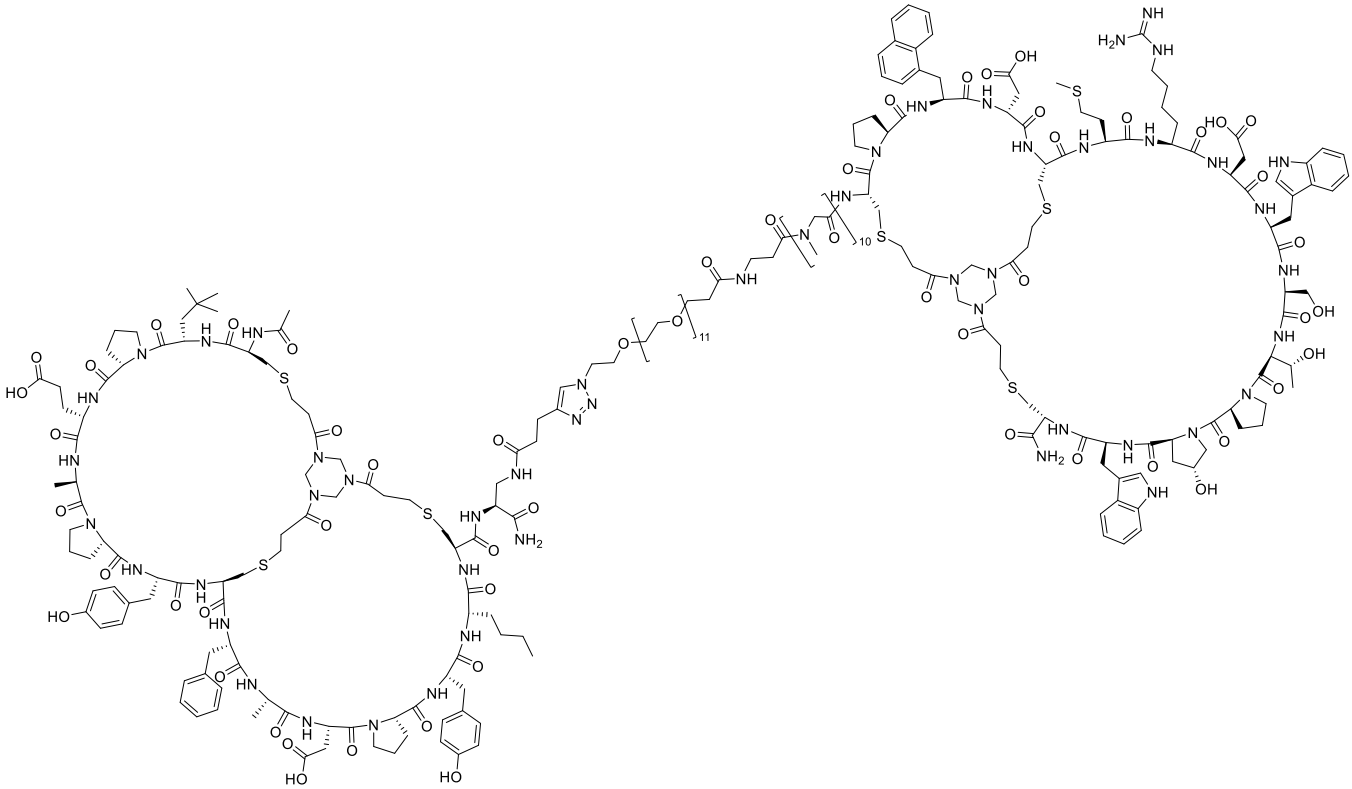

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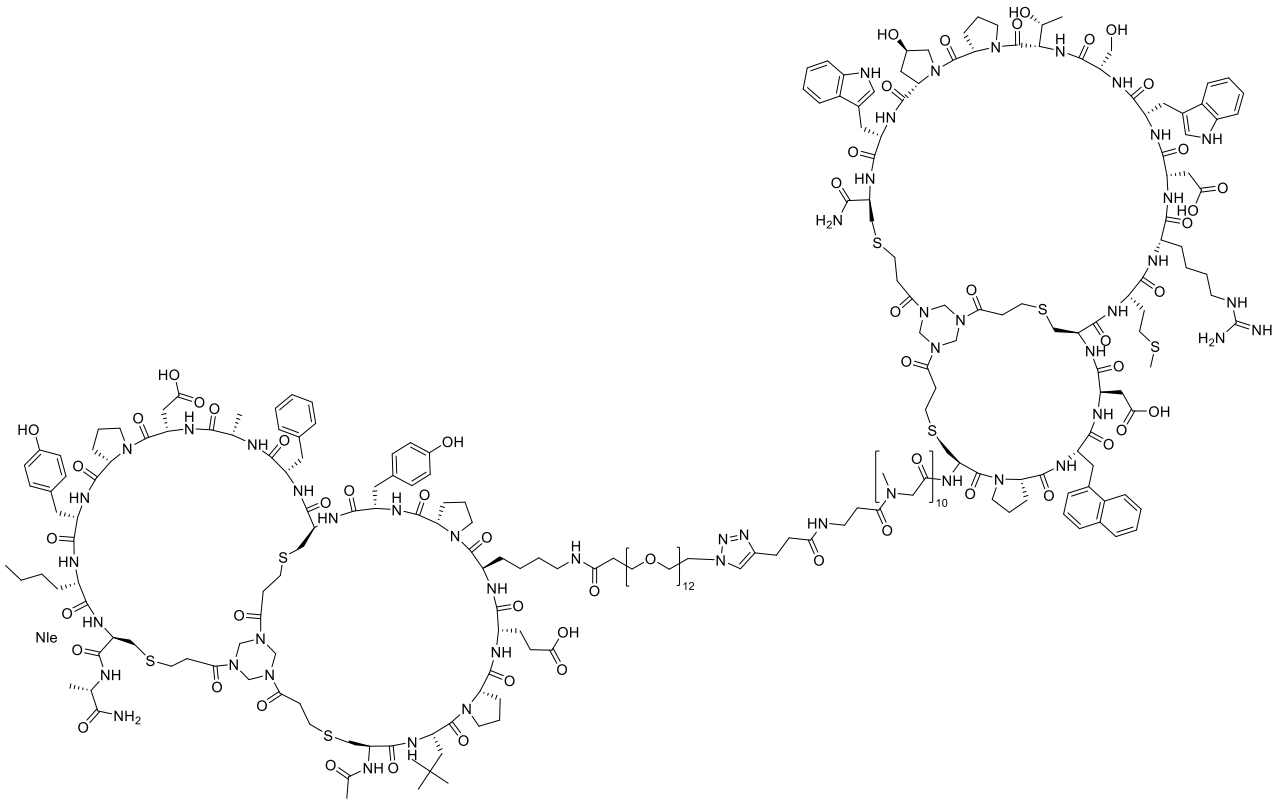

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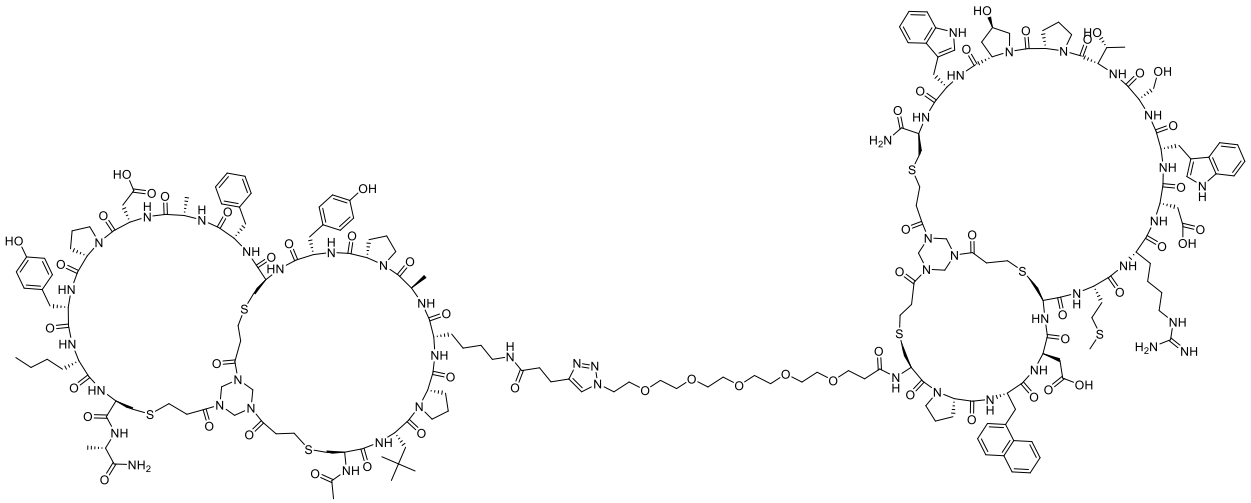

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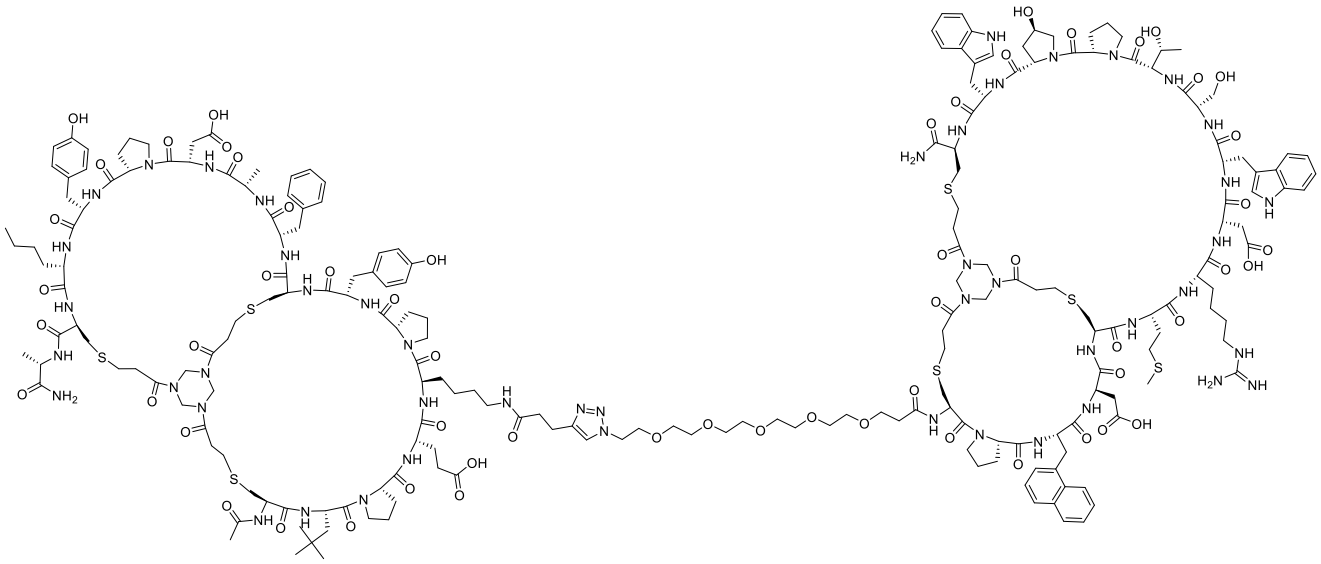

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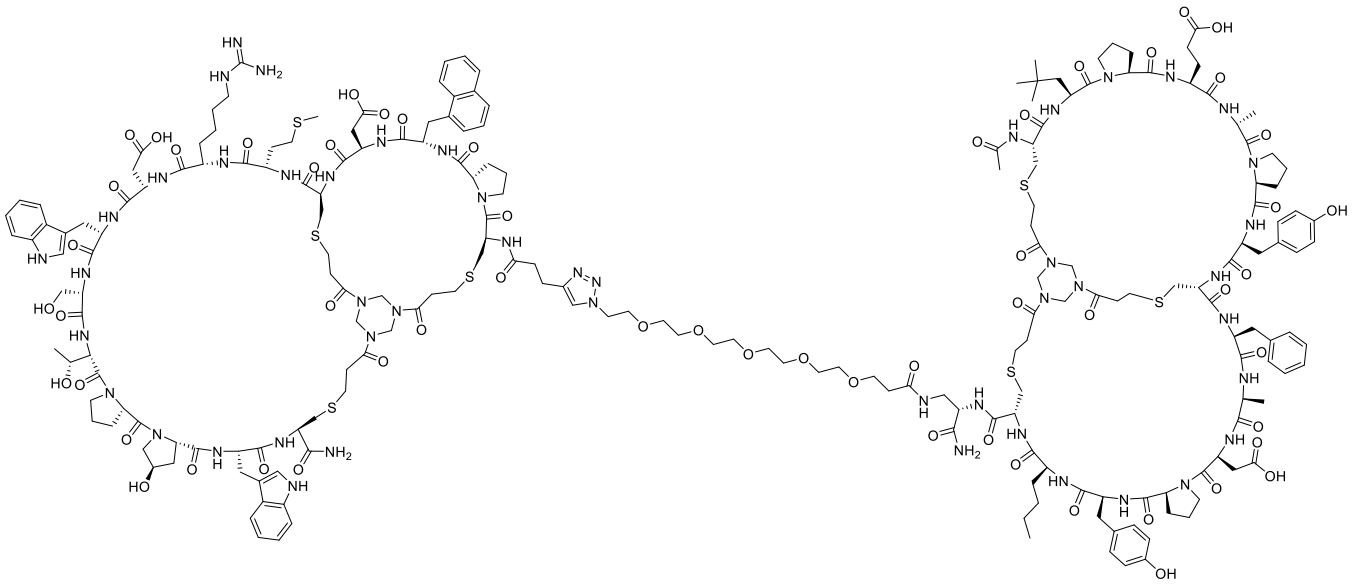

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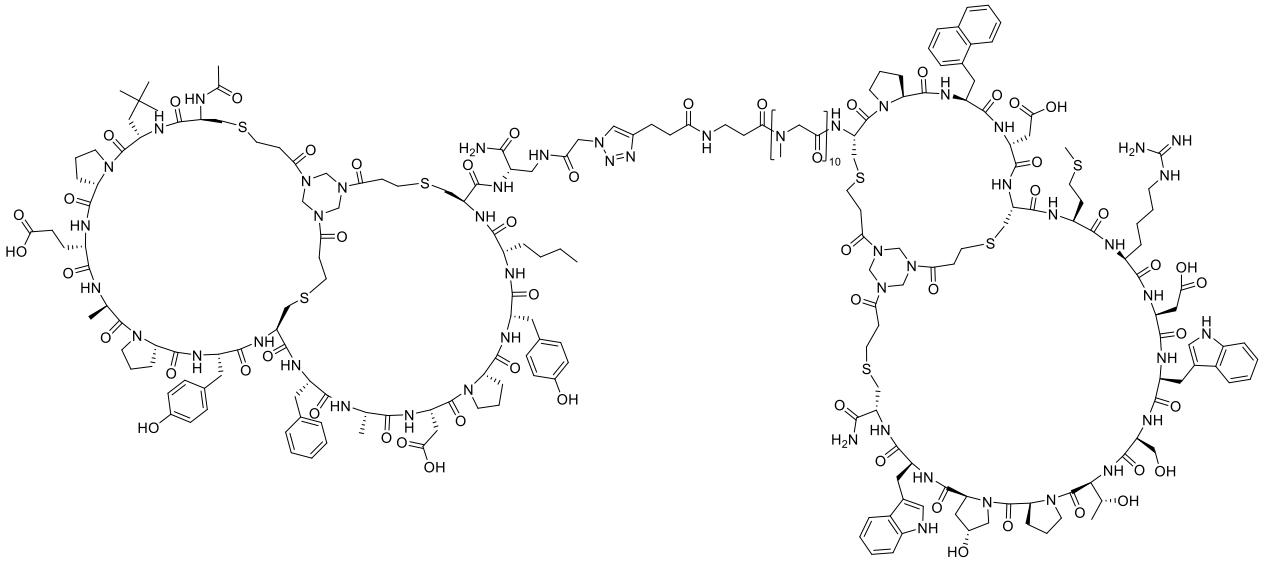

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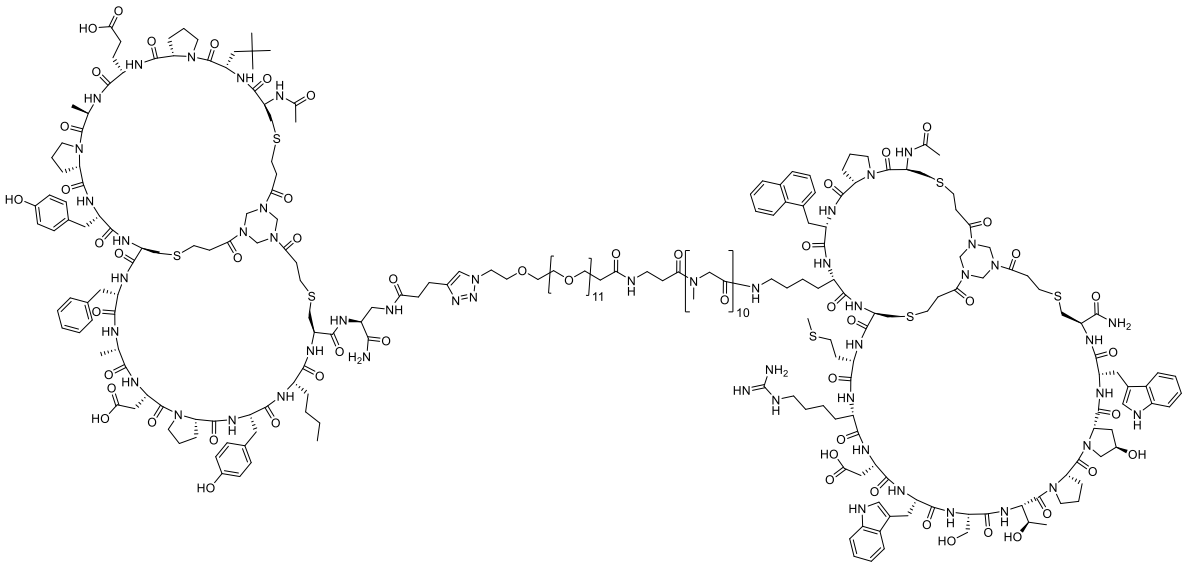

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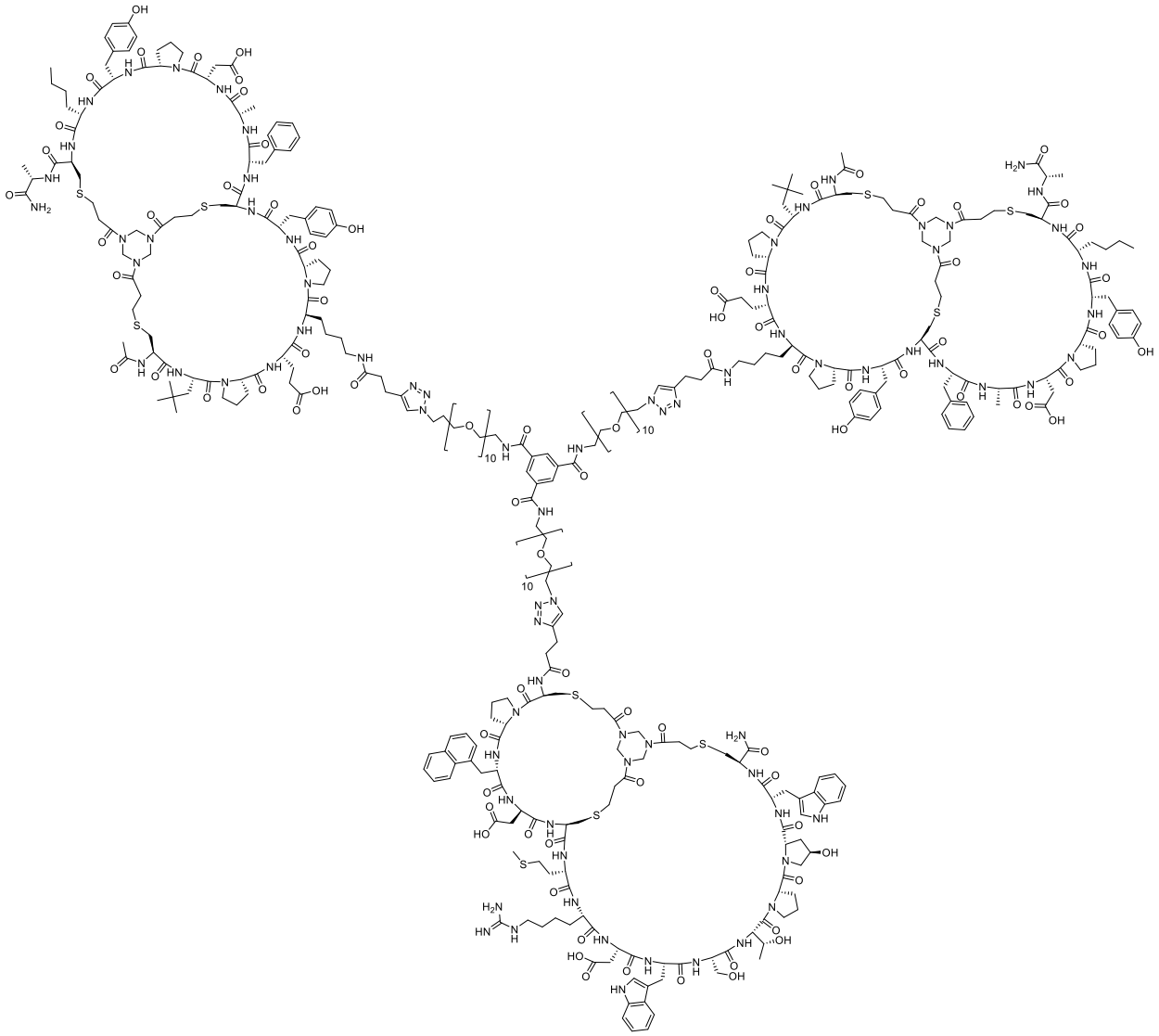

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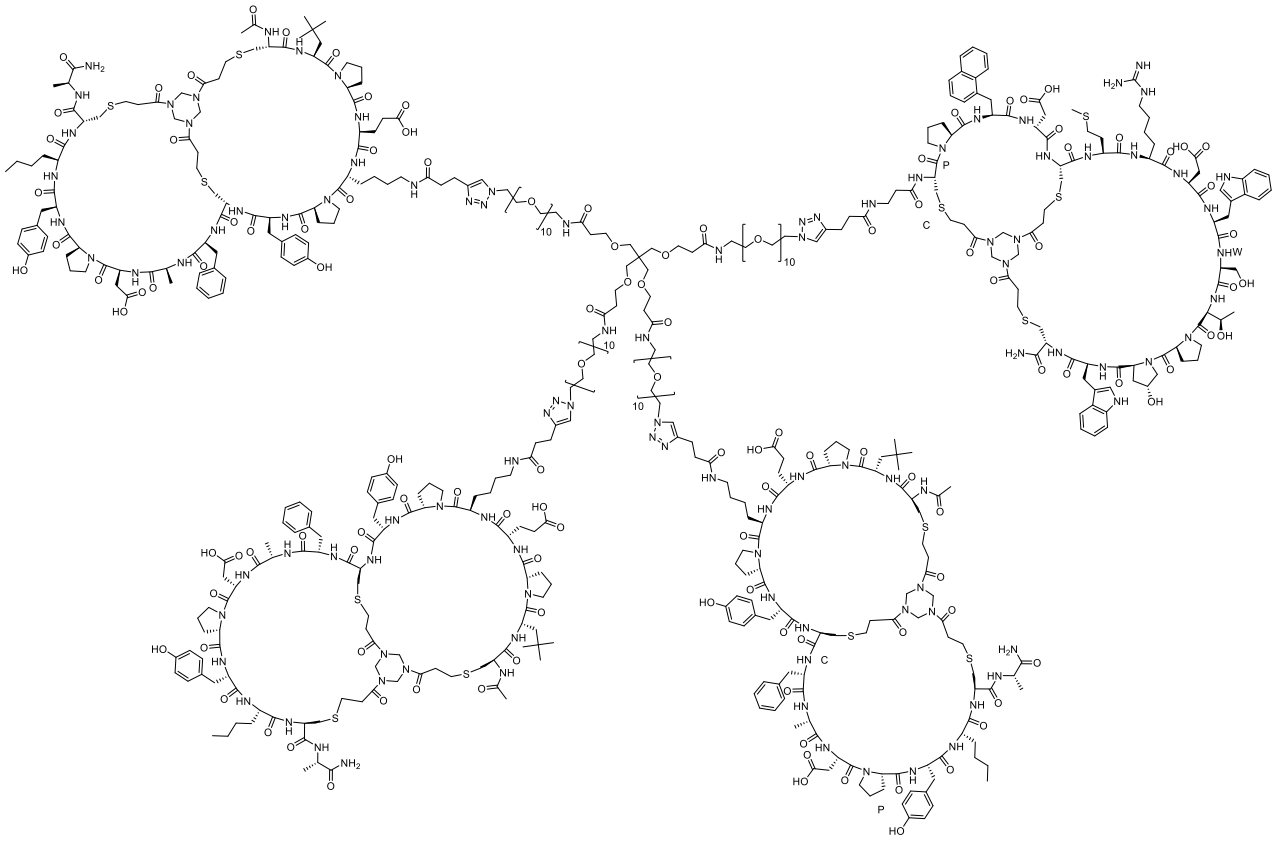

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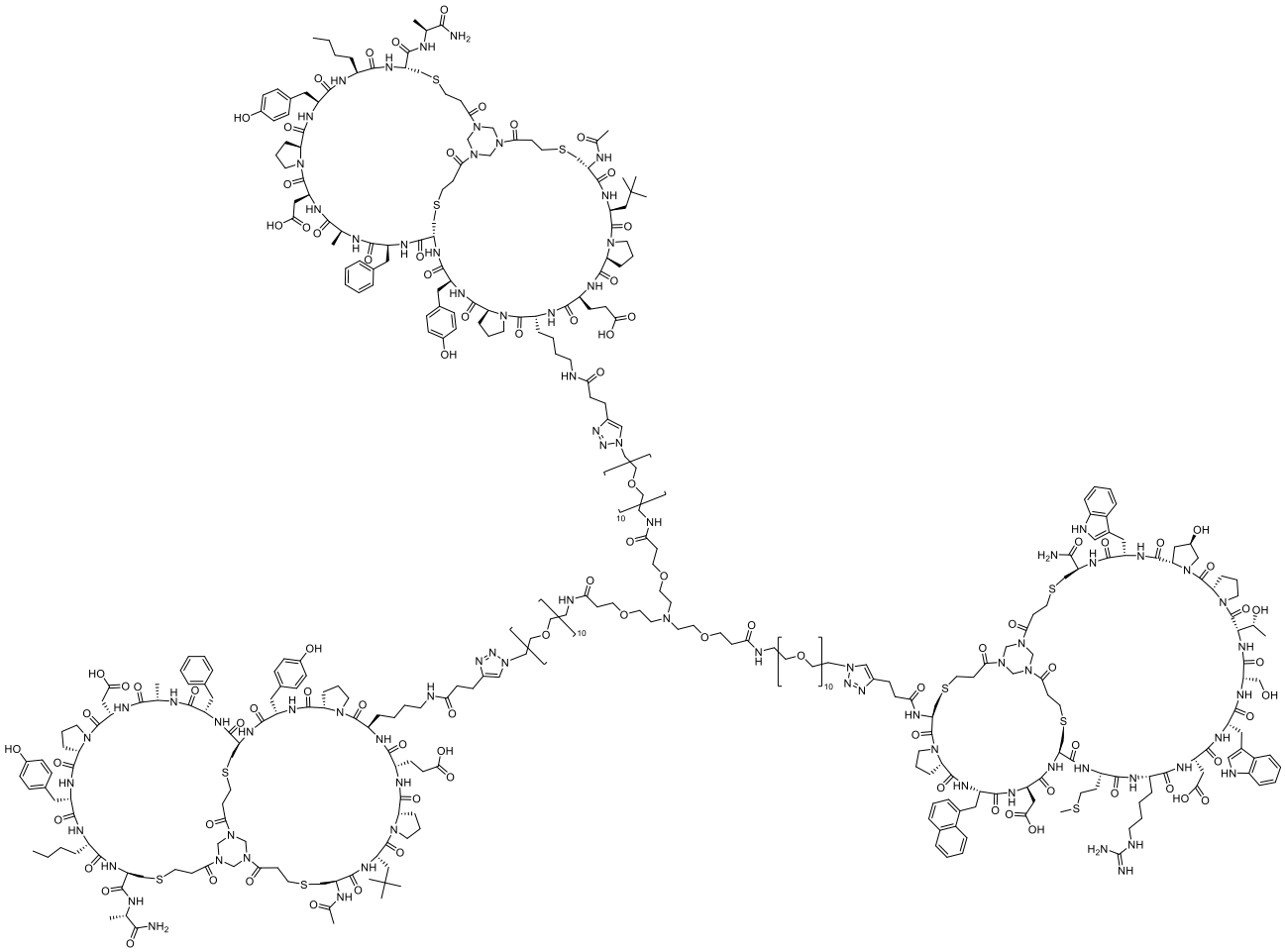

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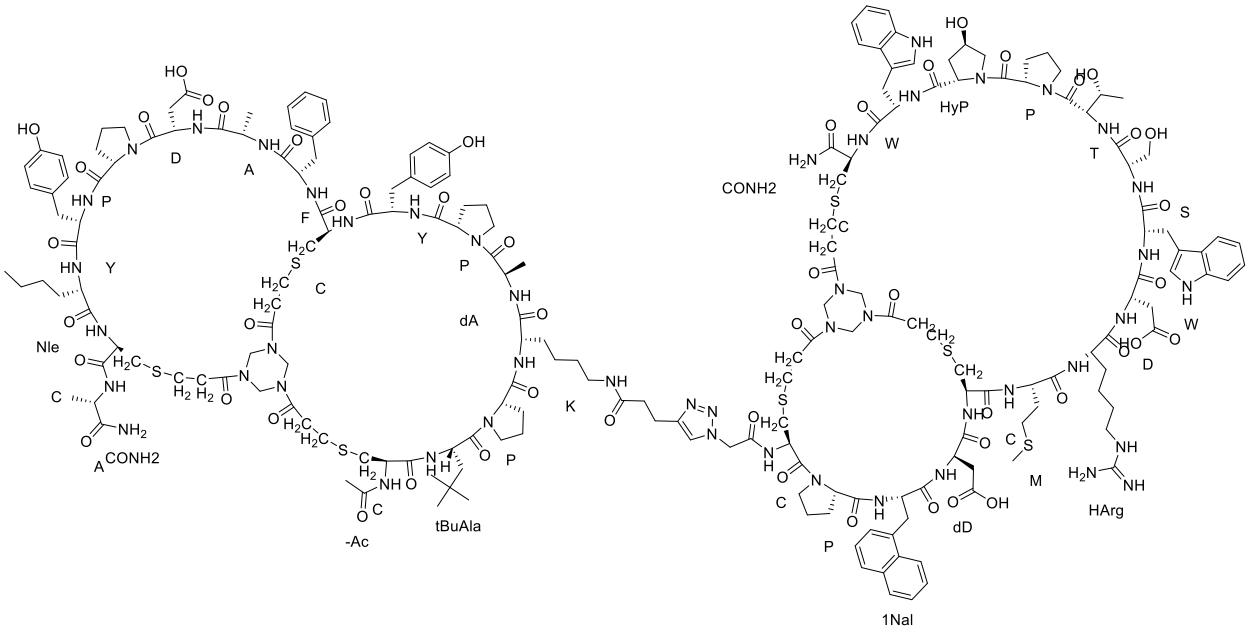

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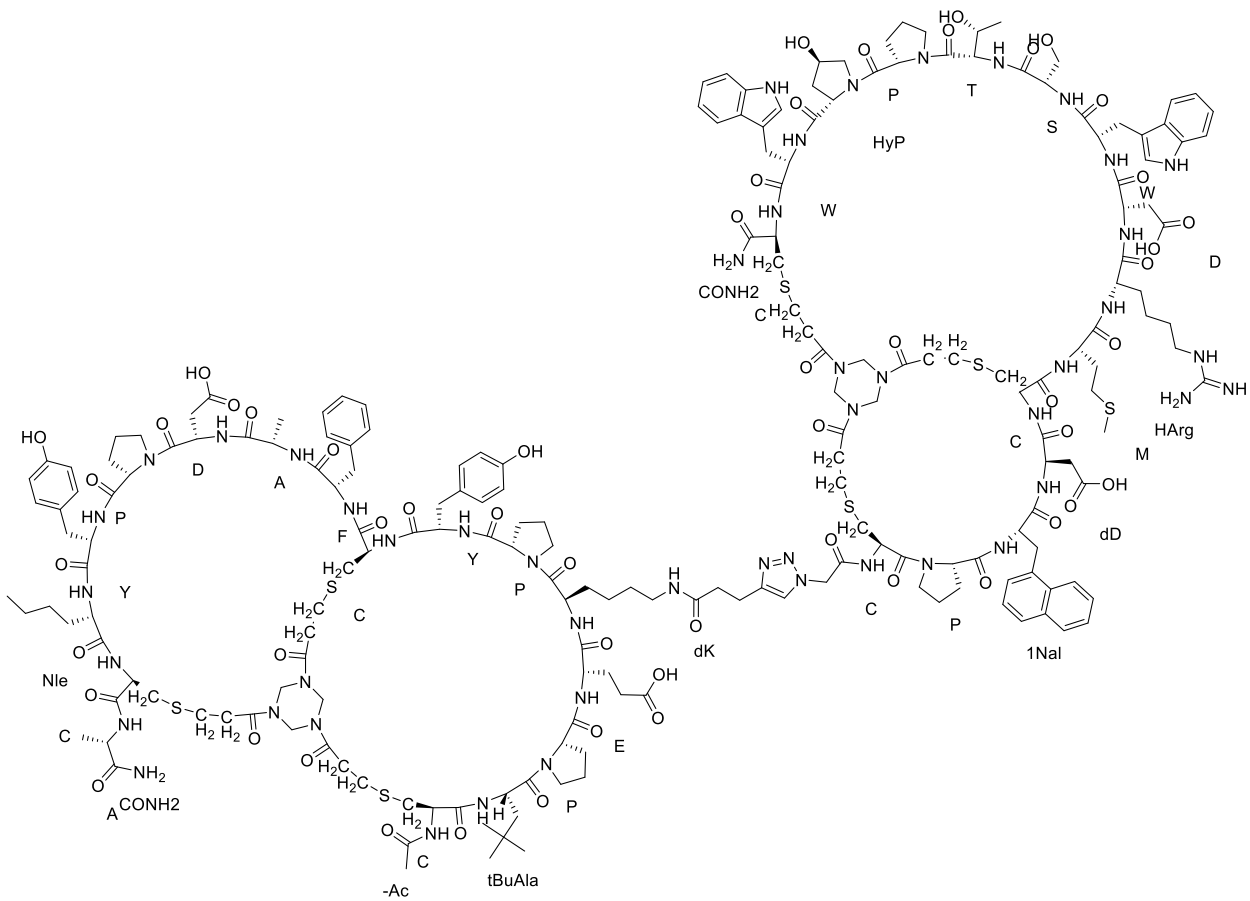

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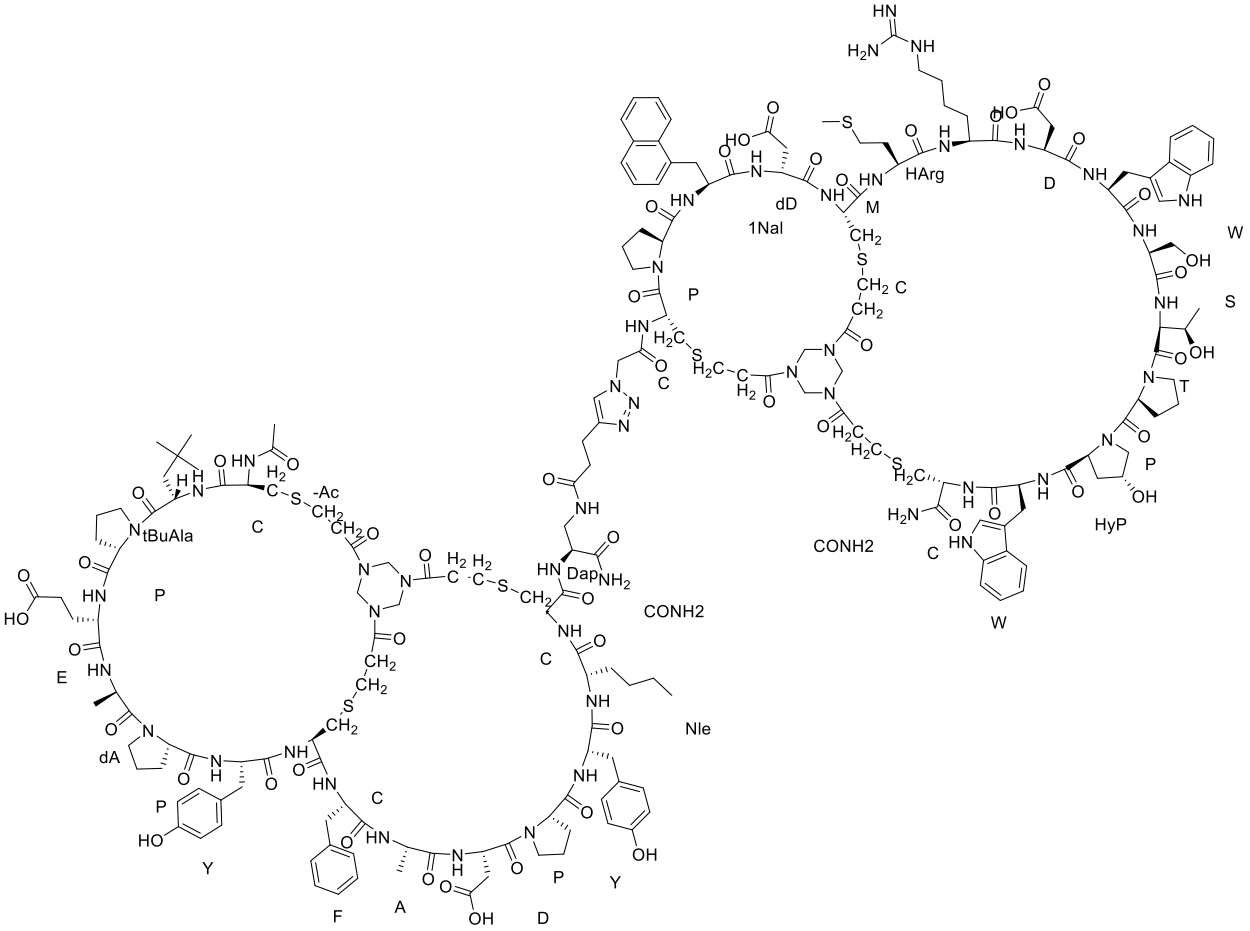
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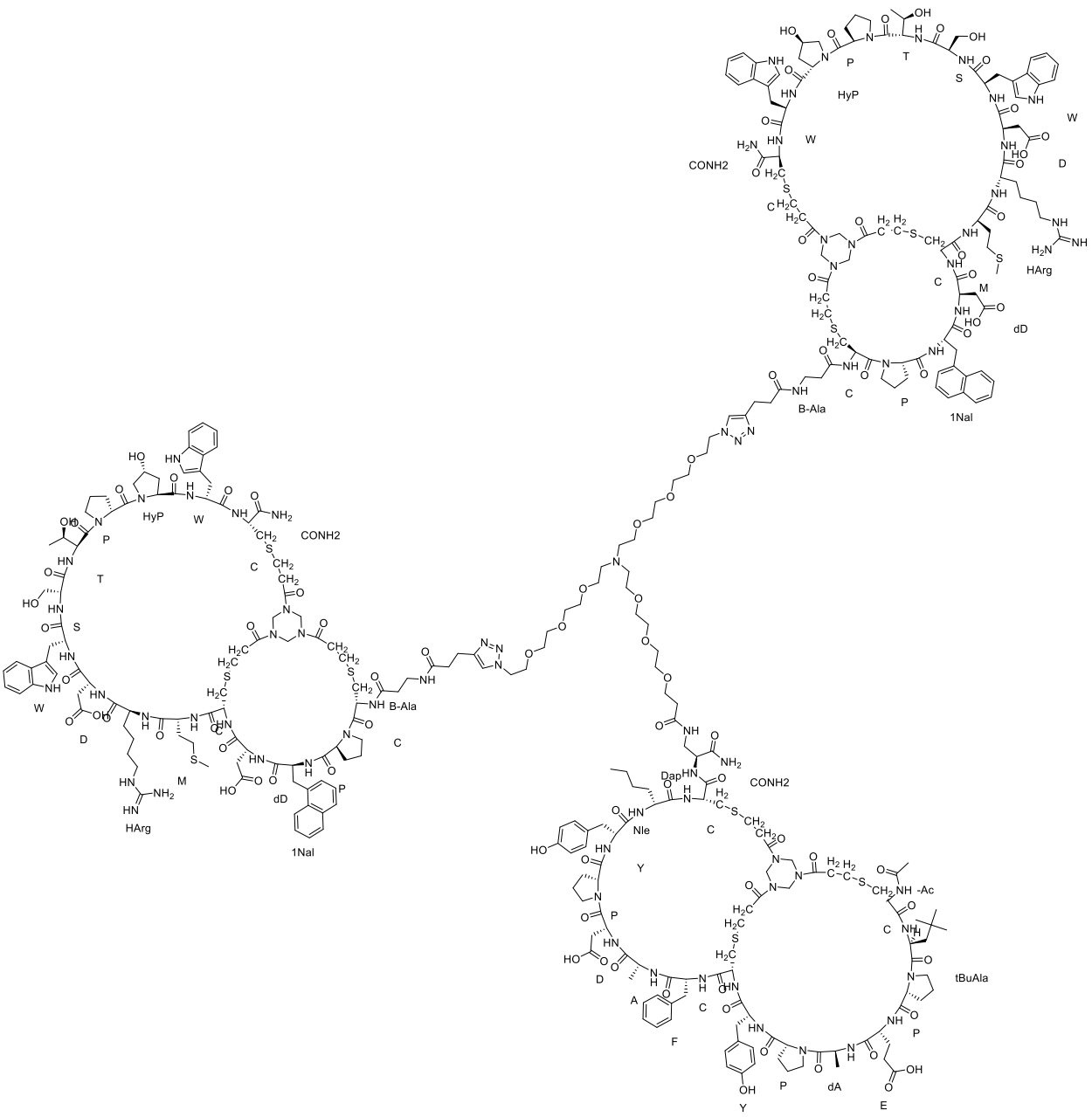
BCY11384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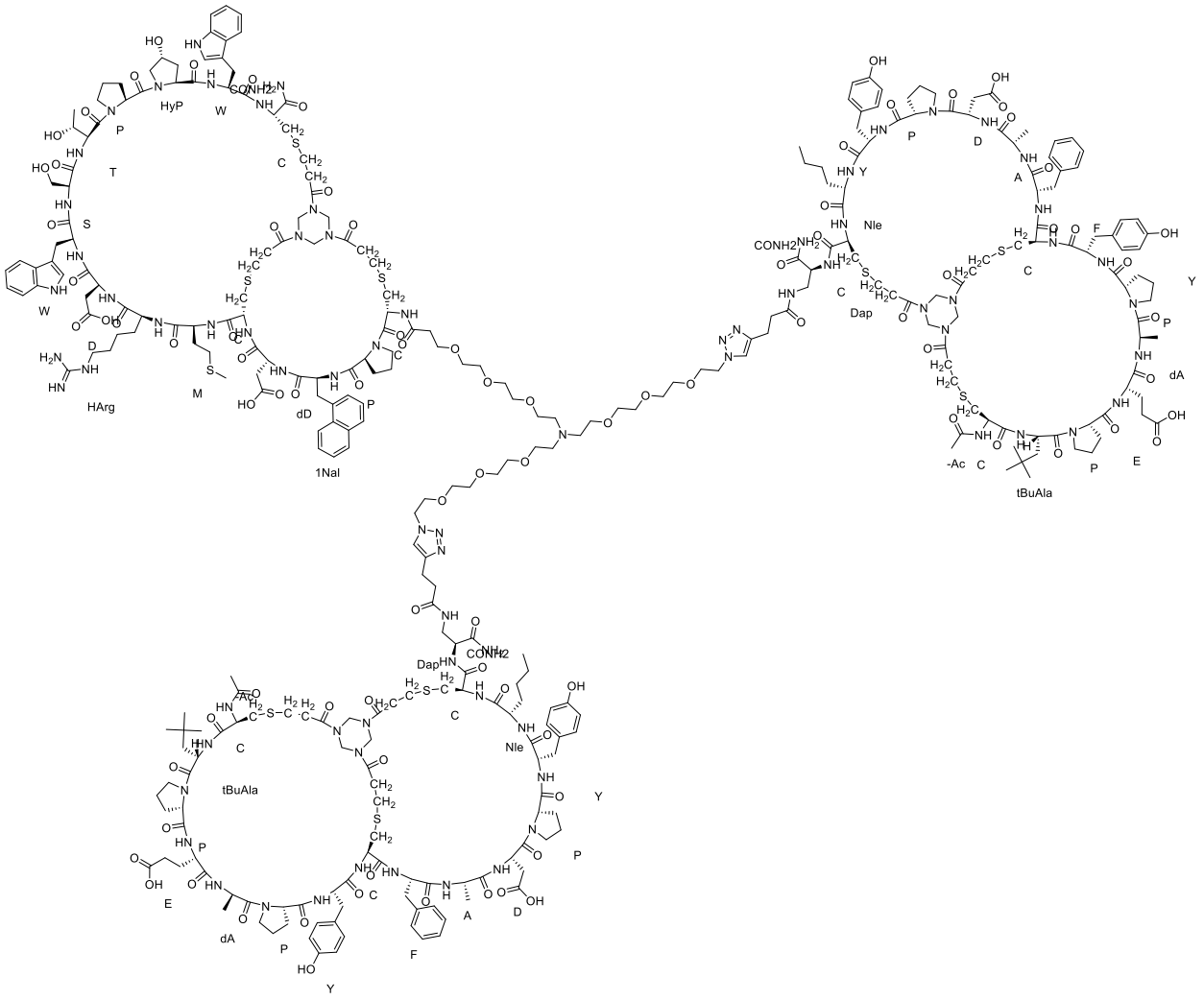
BCY11385



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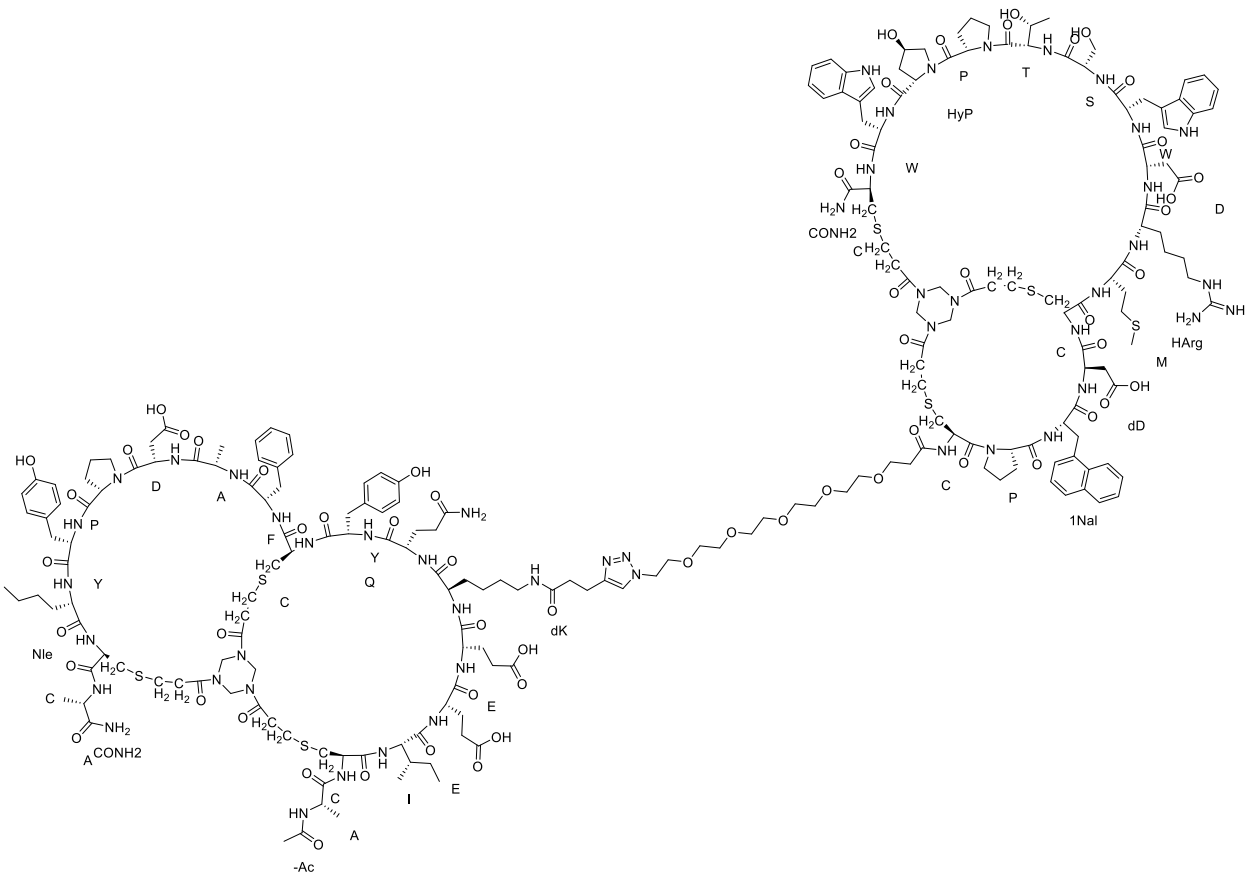

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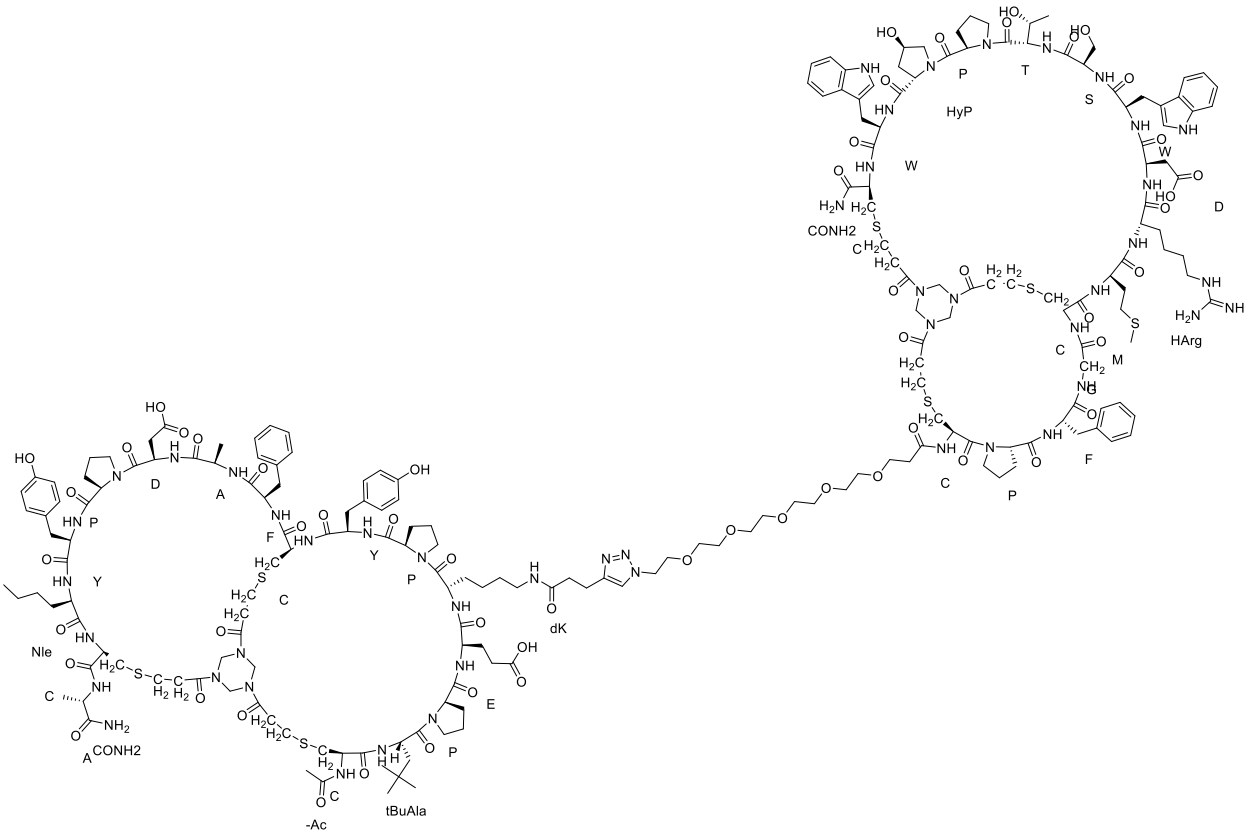

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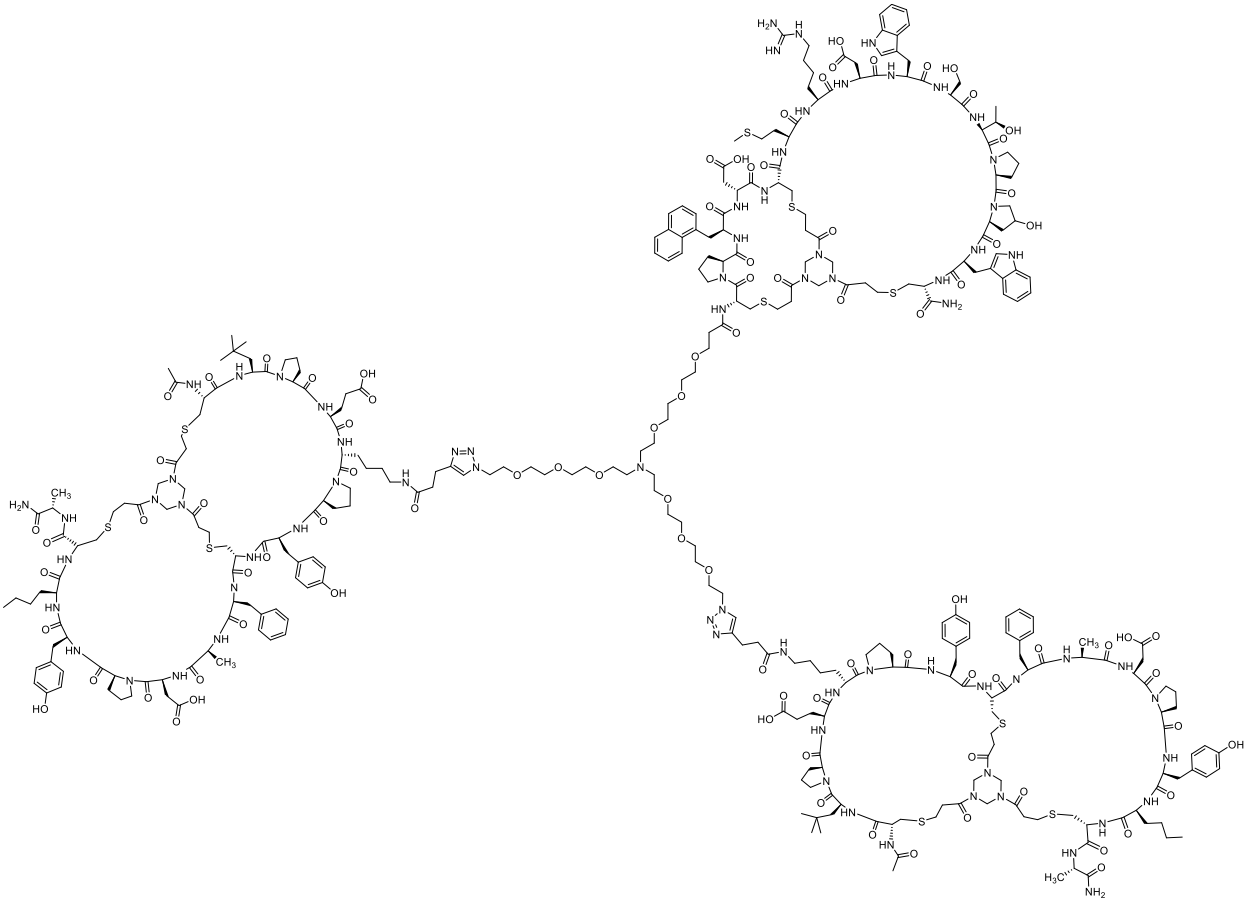

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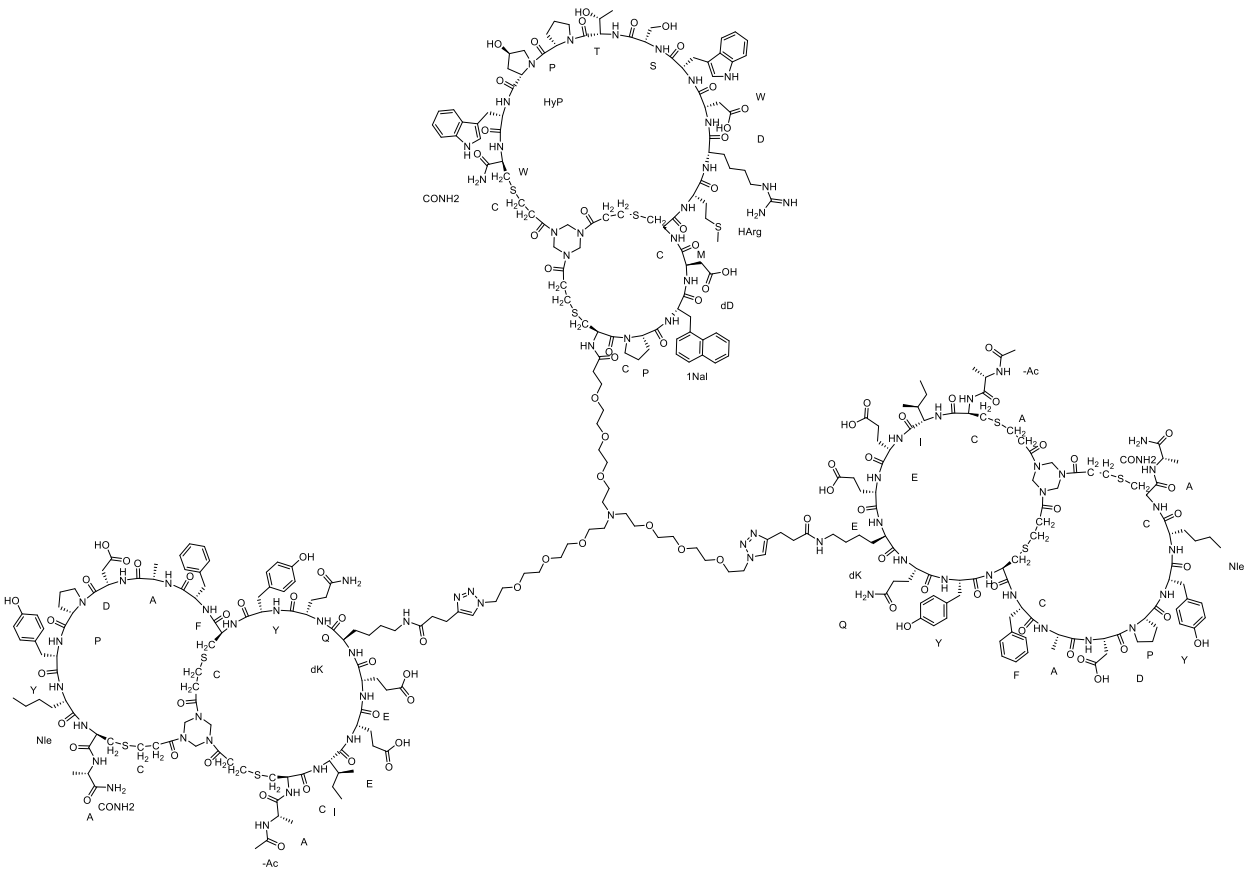

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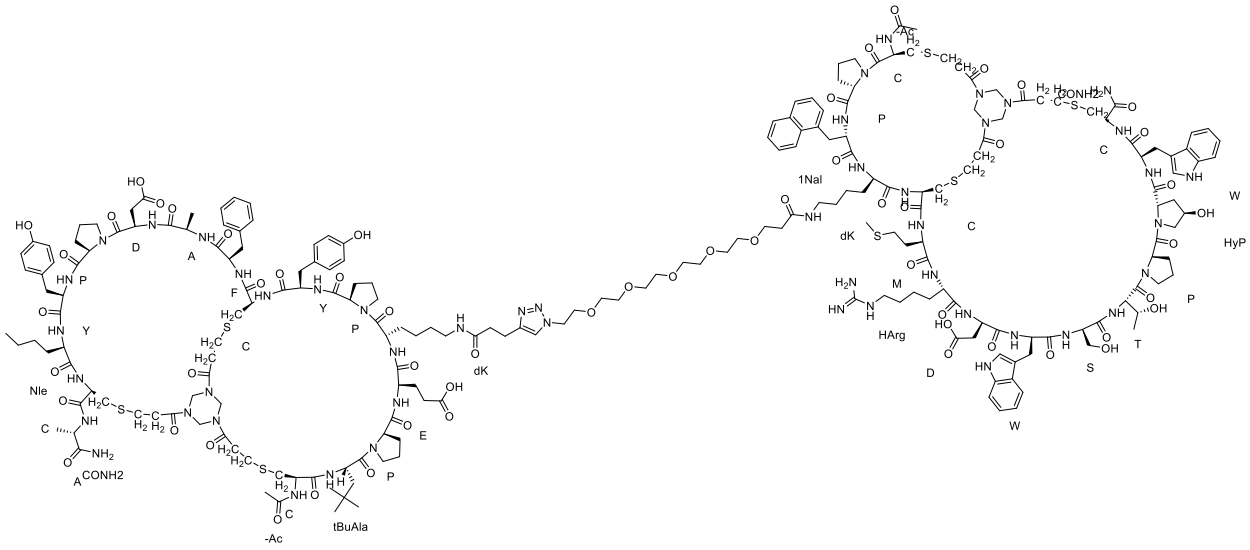

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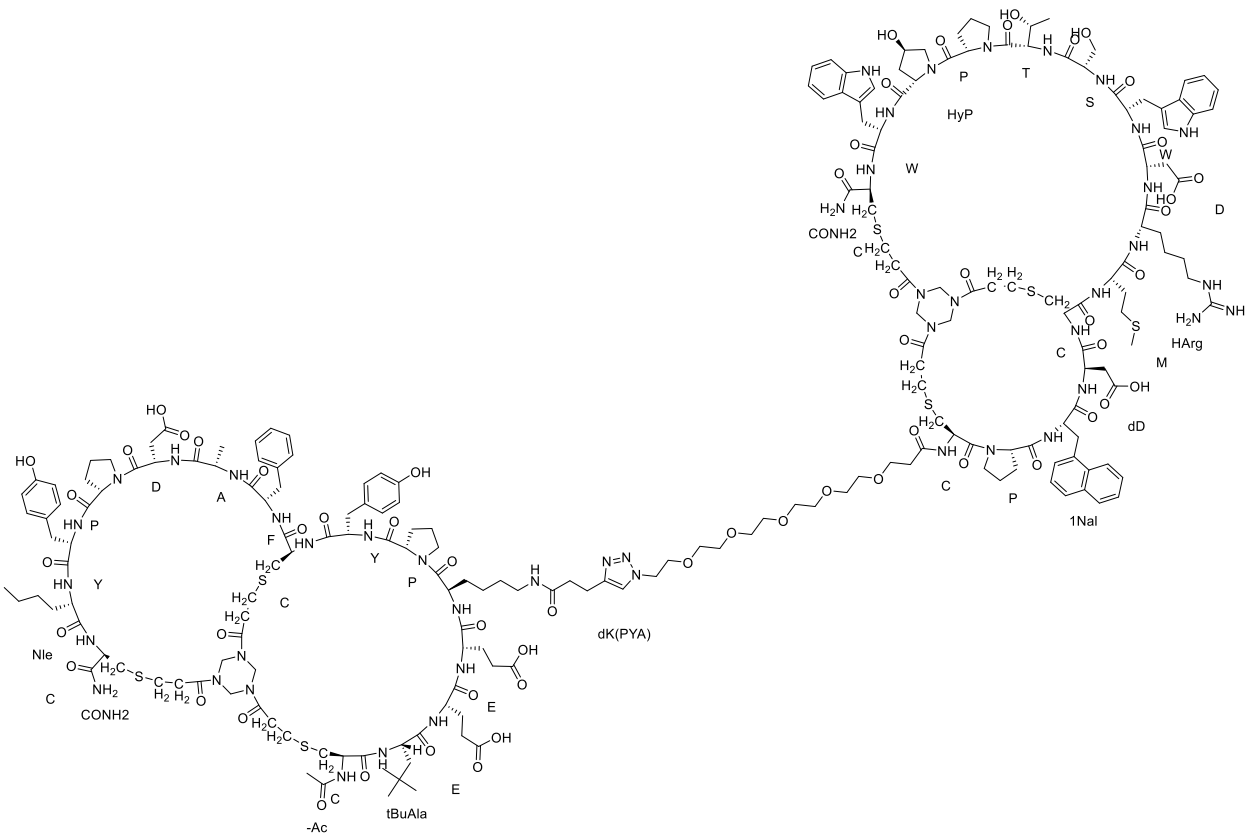
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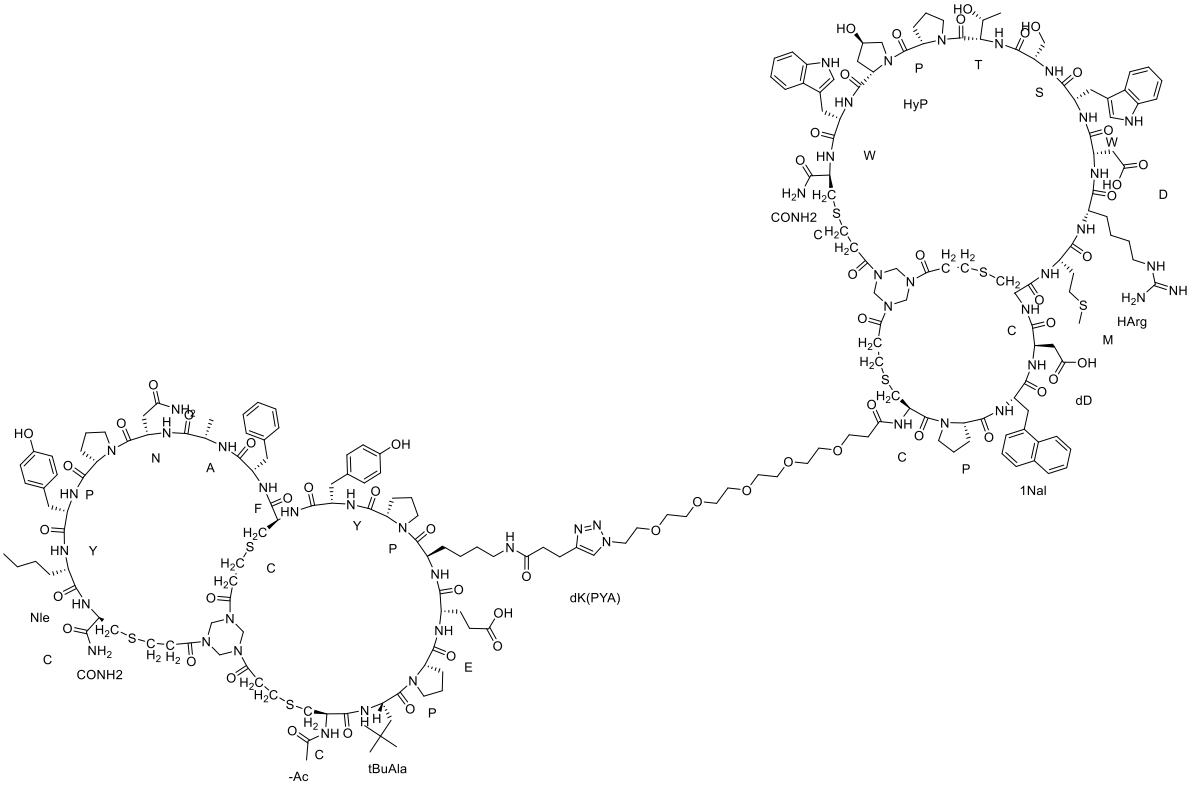

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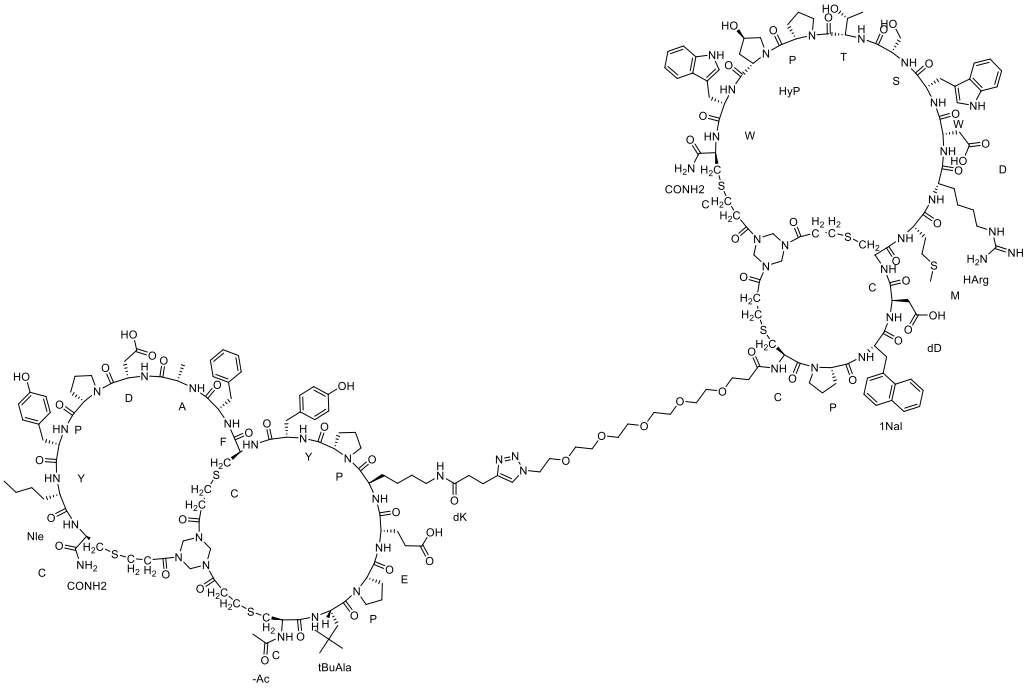

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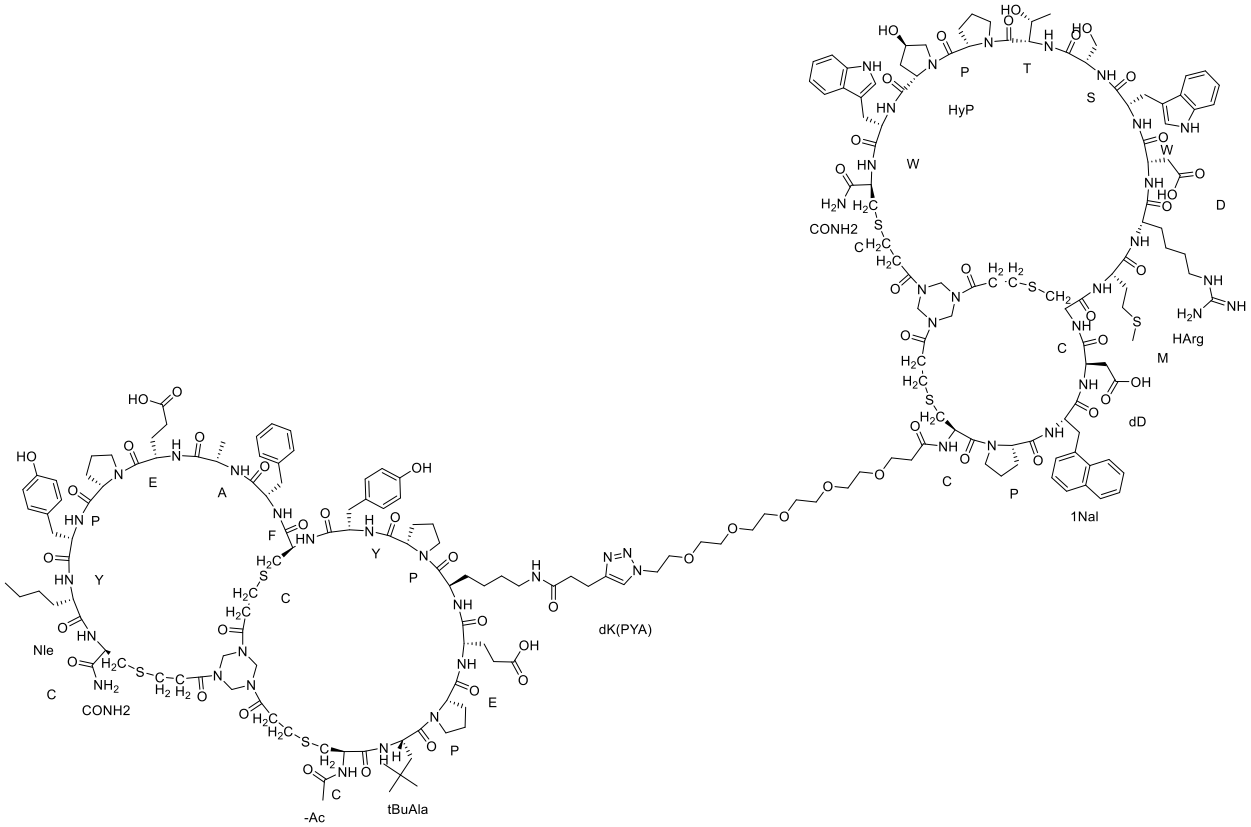
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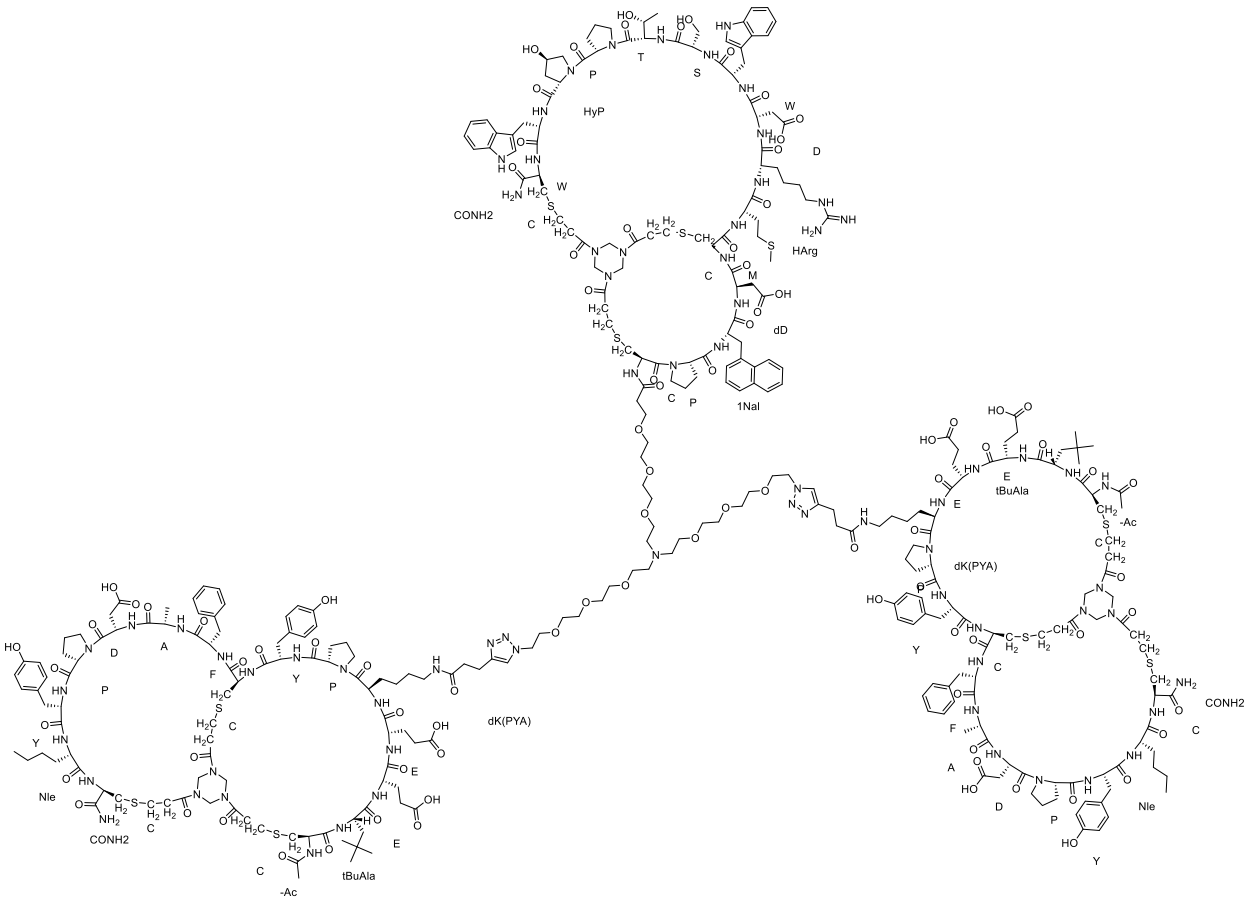

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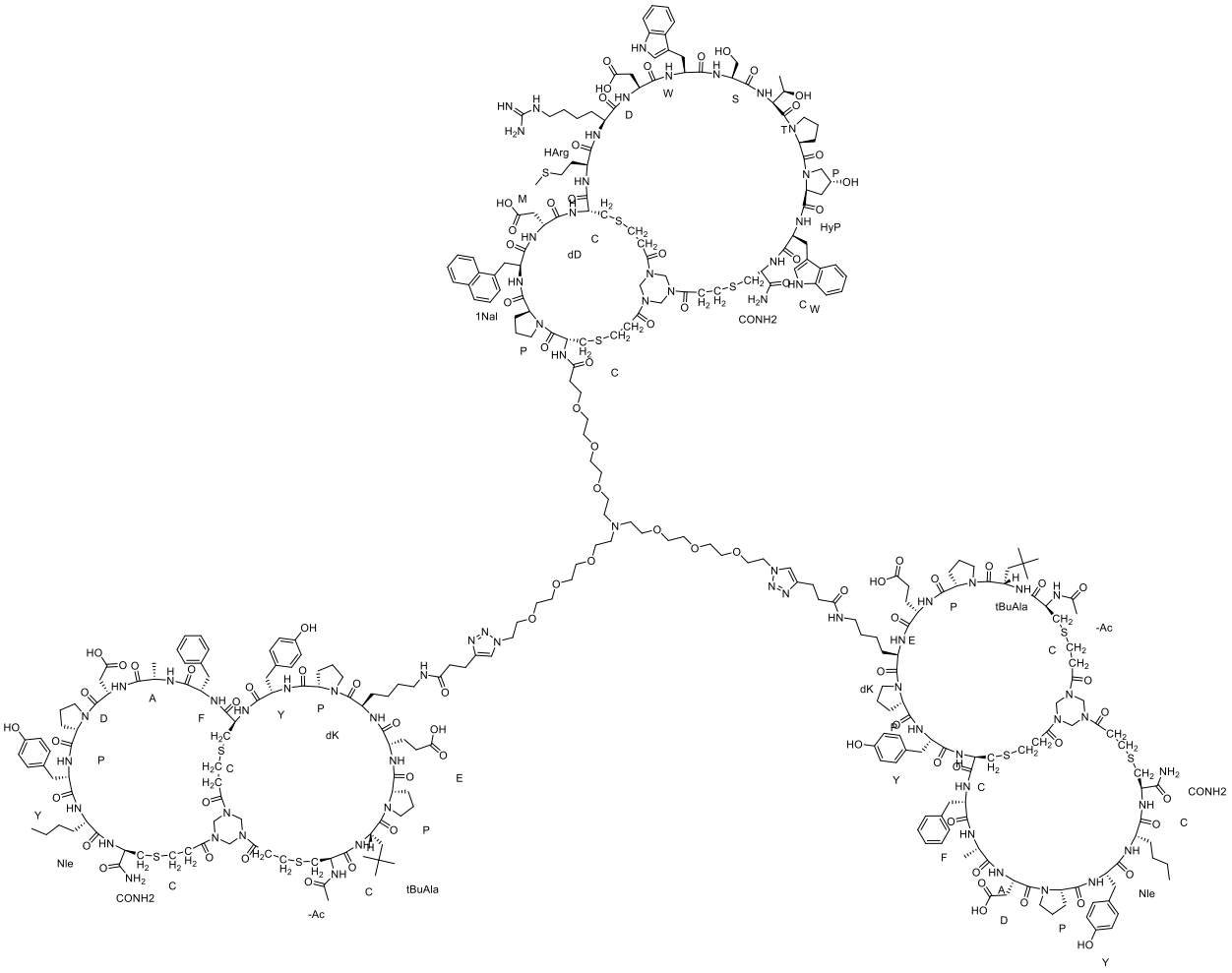

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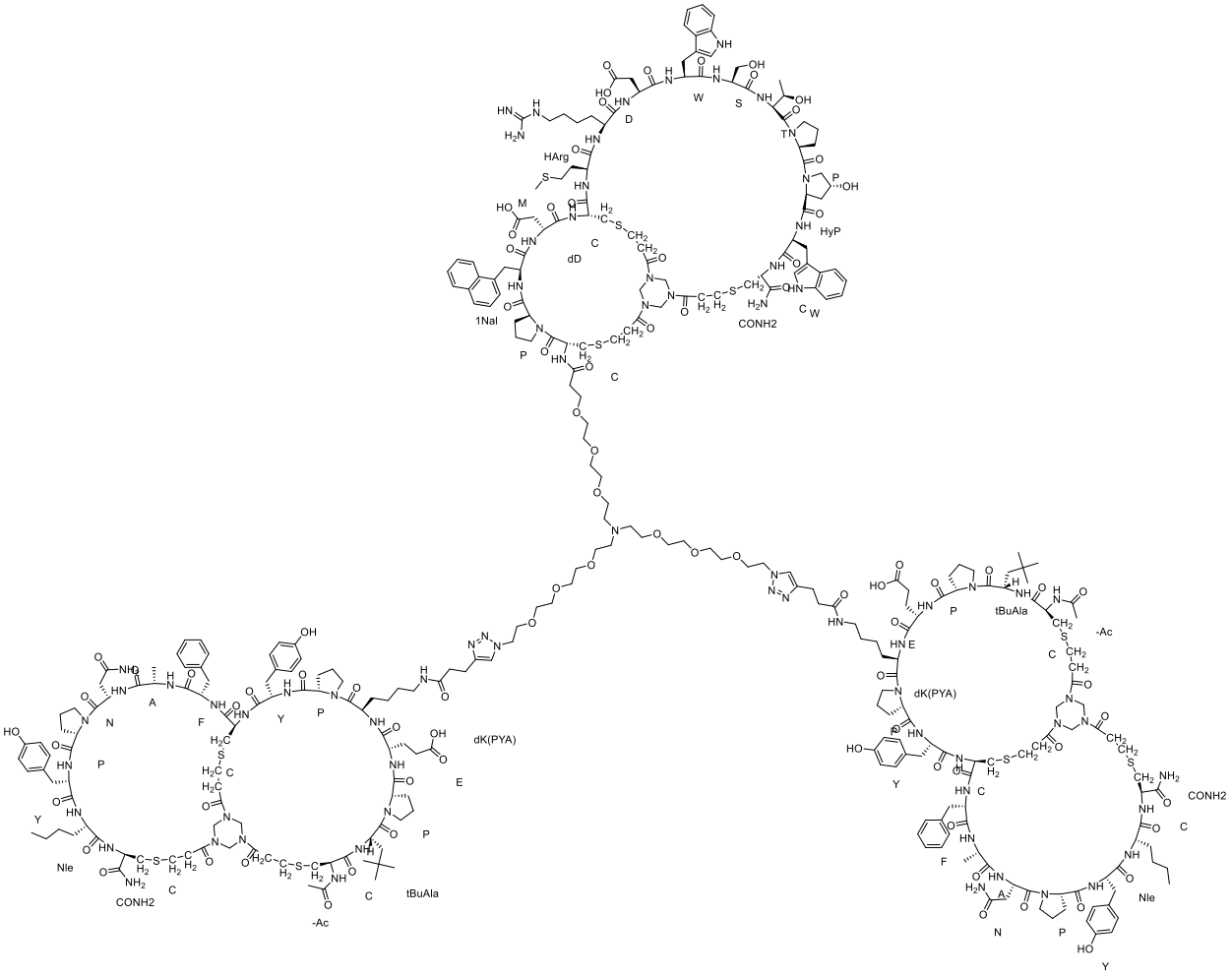

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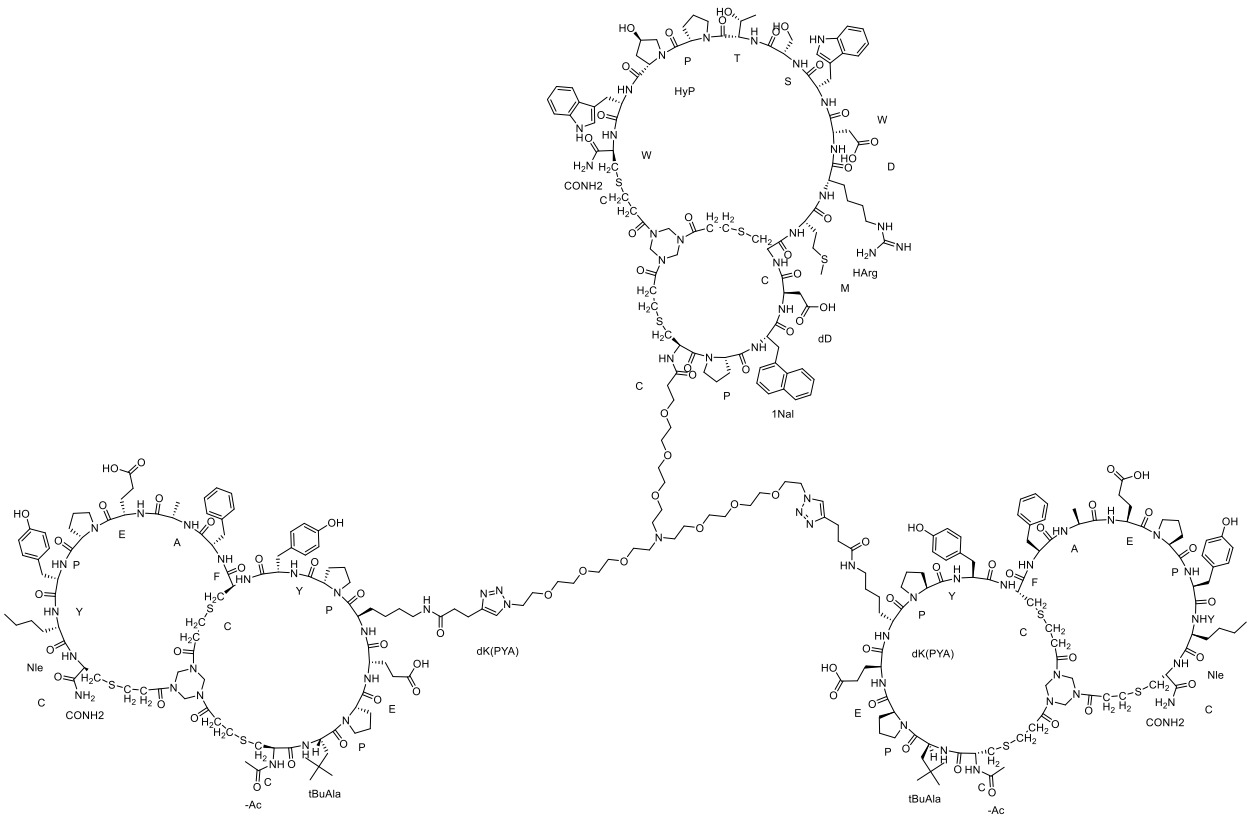
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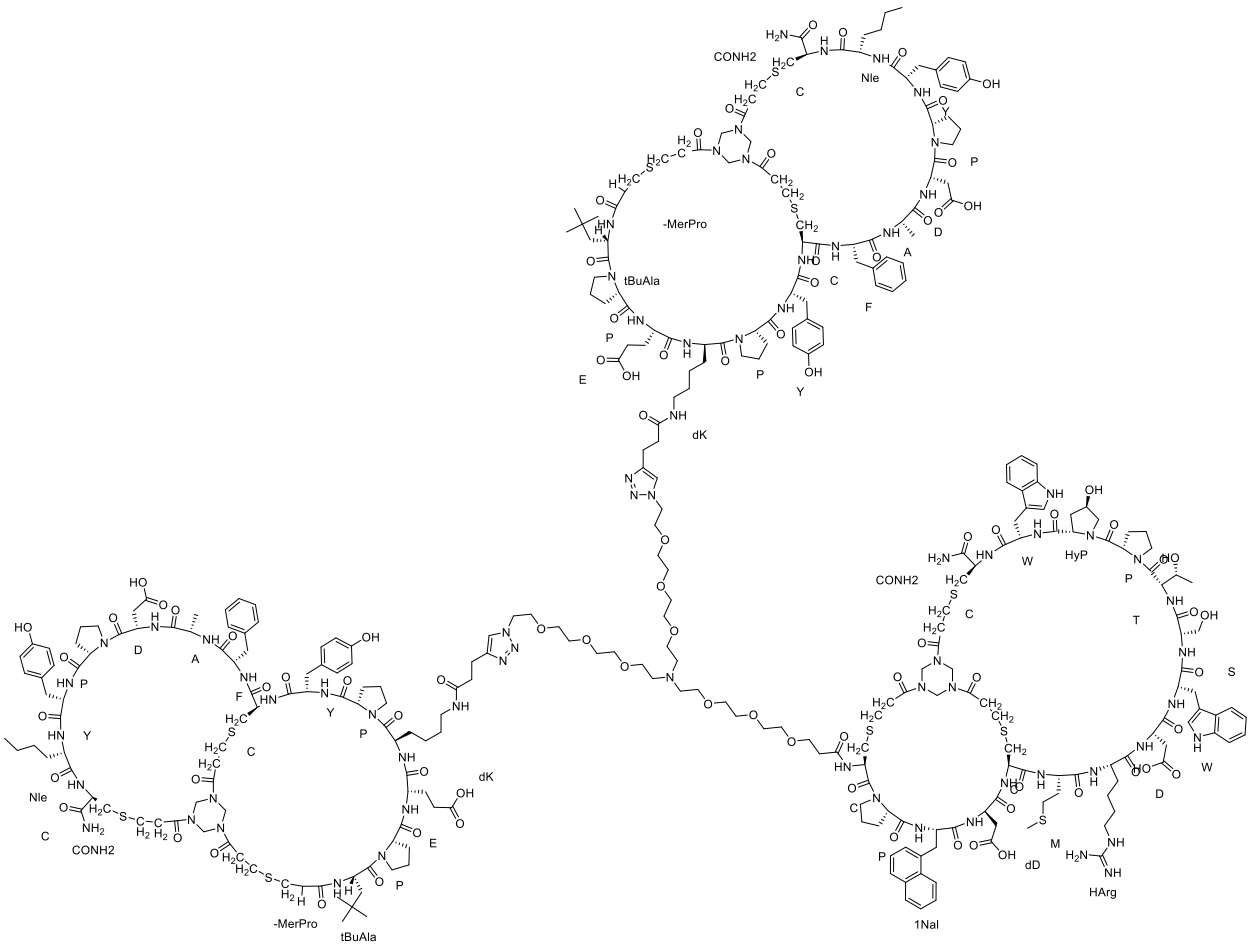

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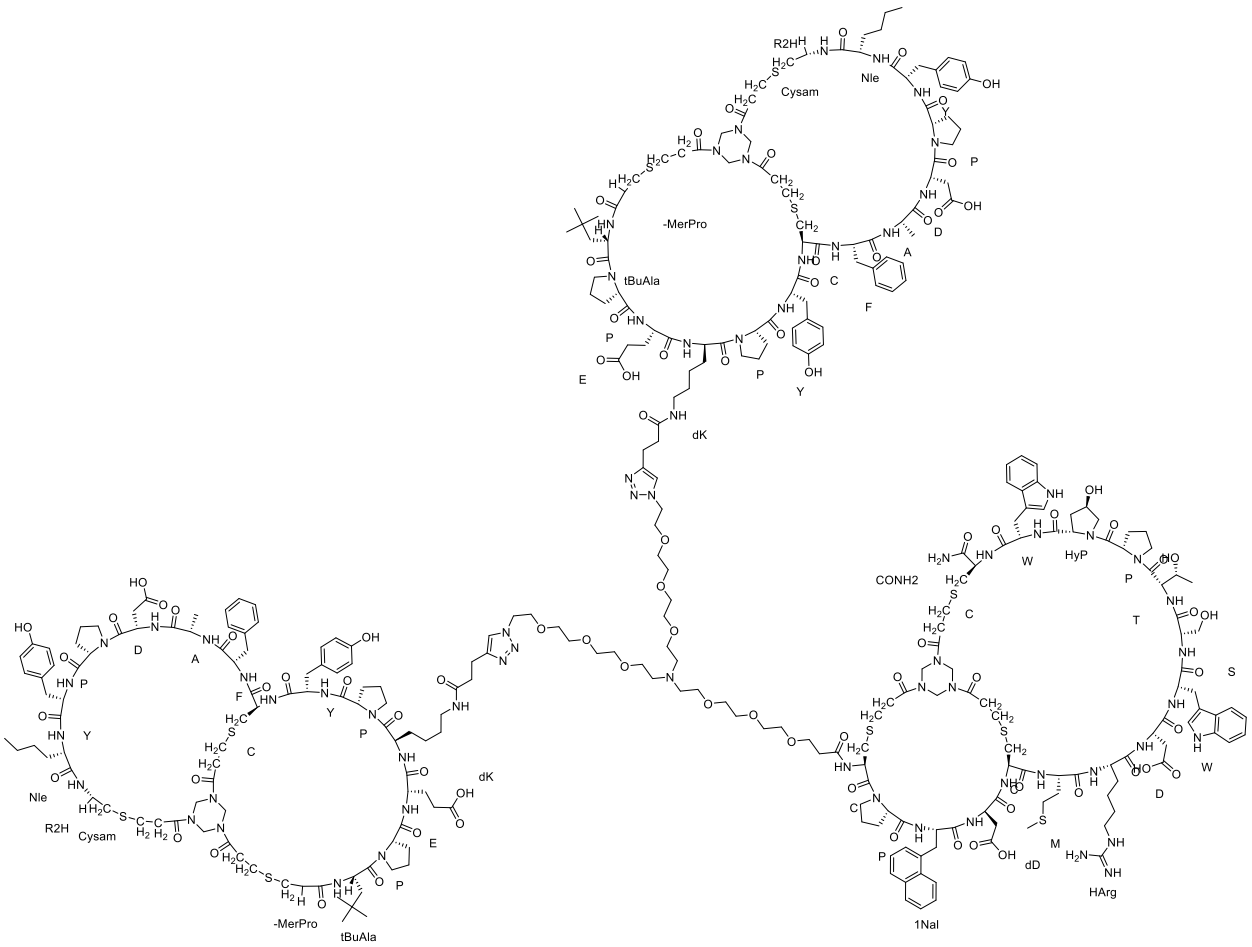

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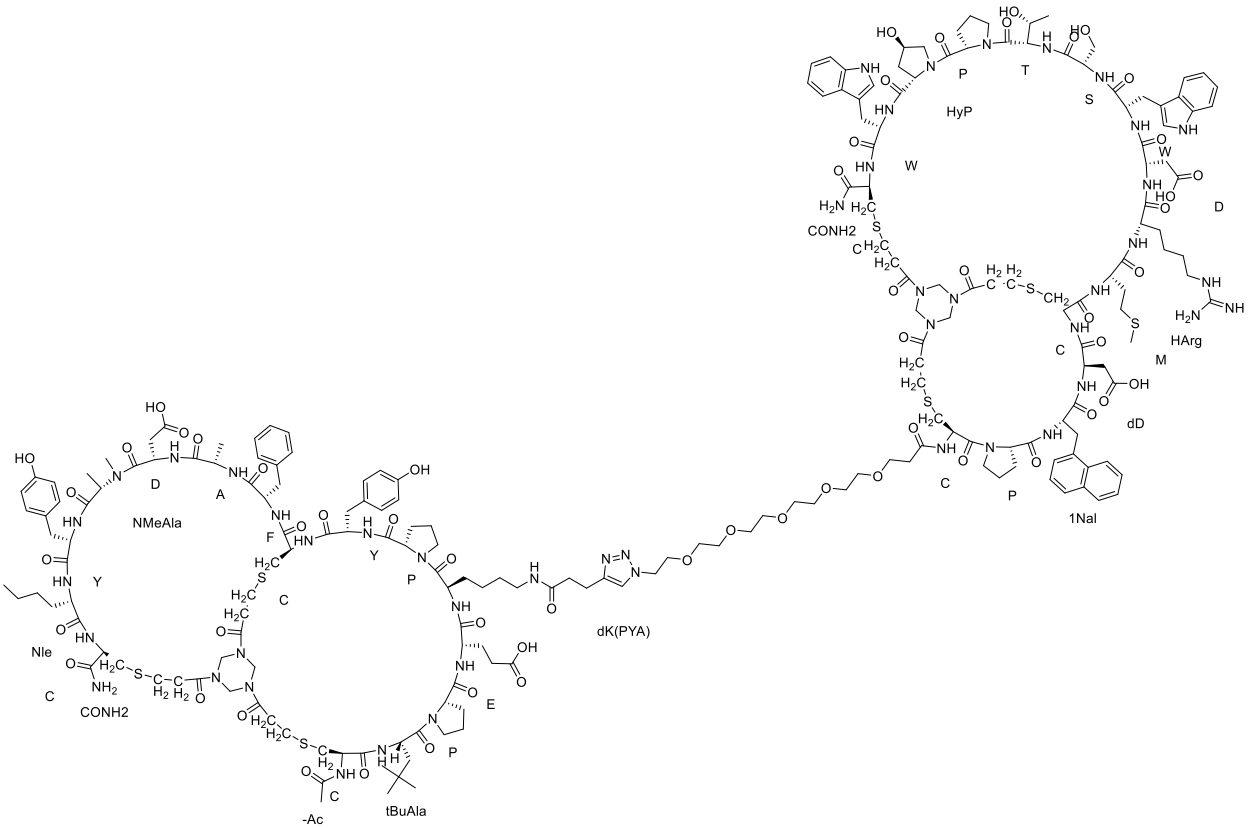
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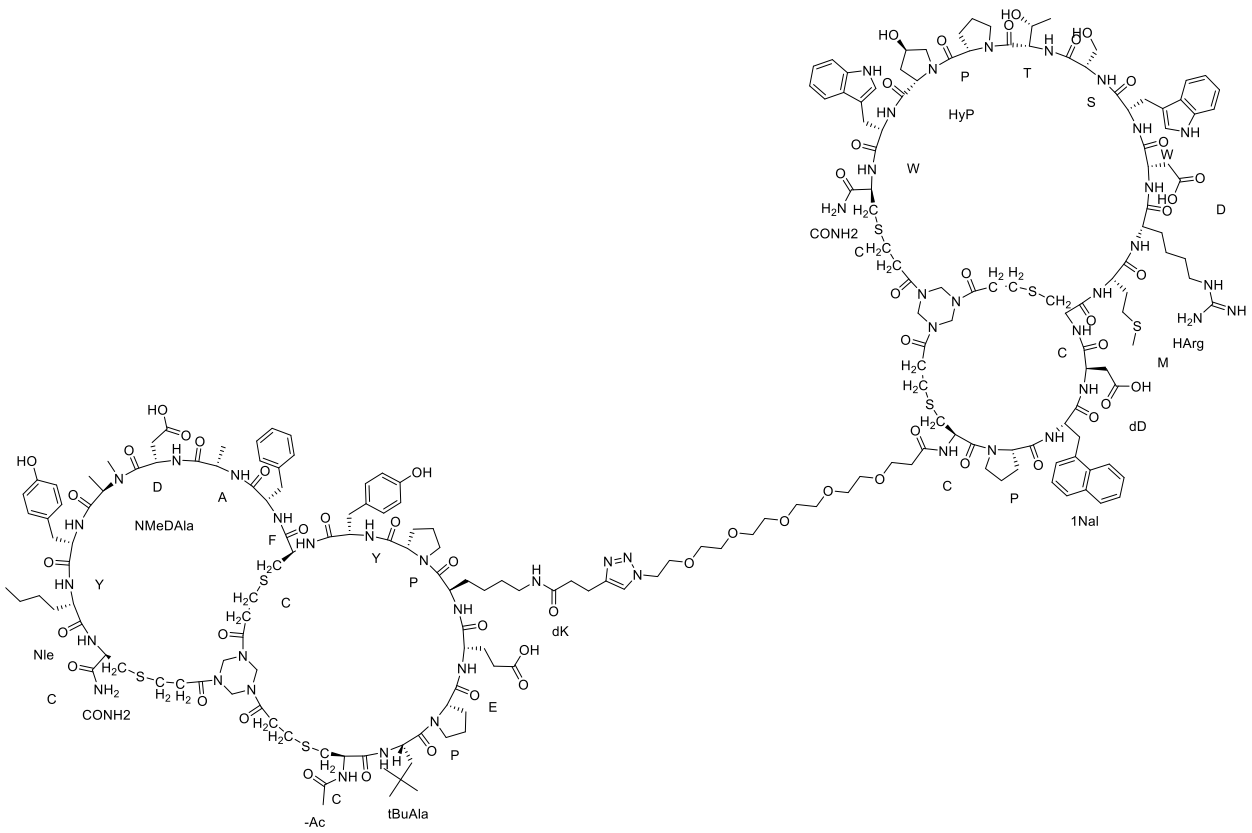

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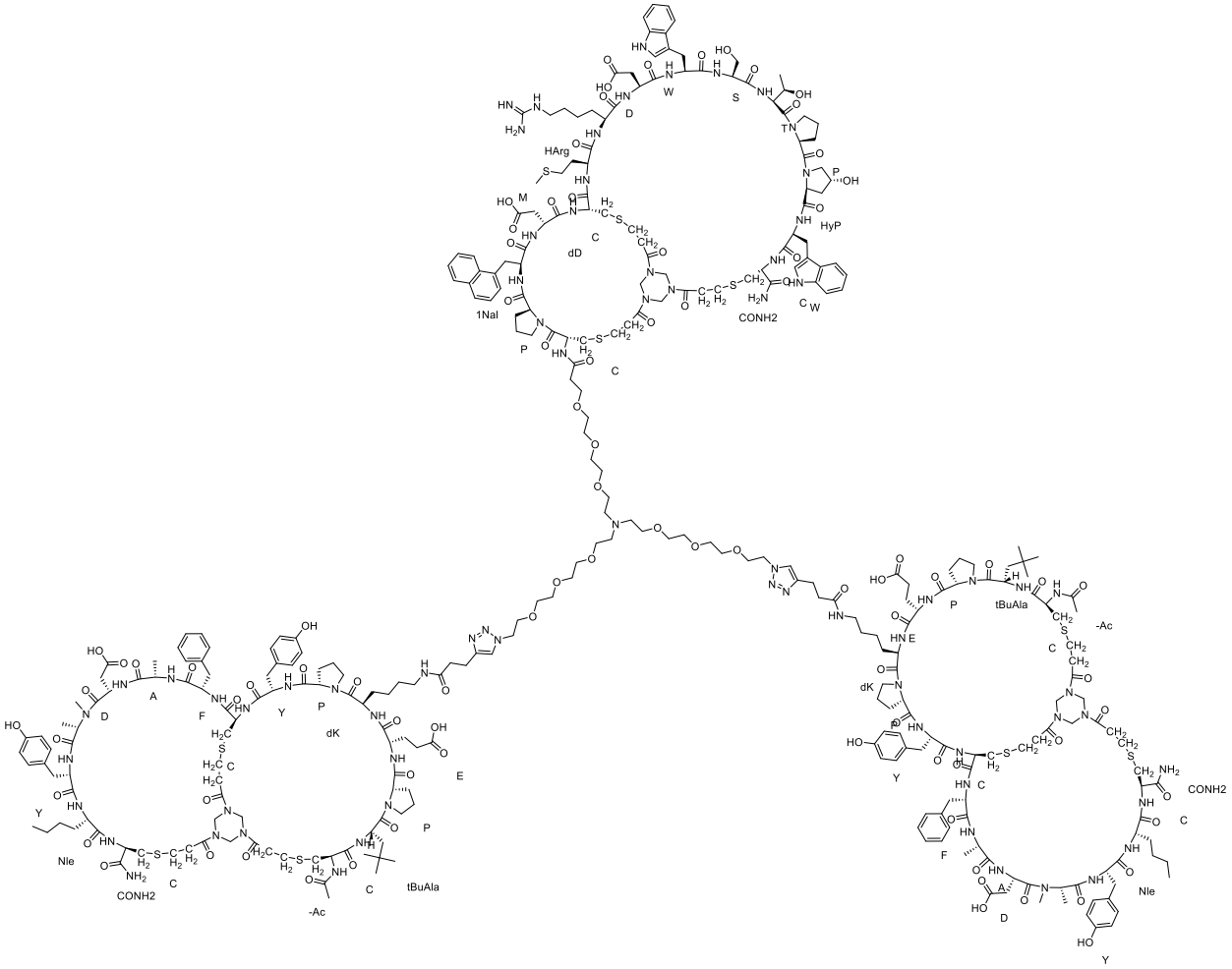

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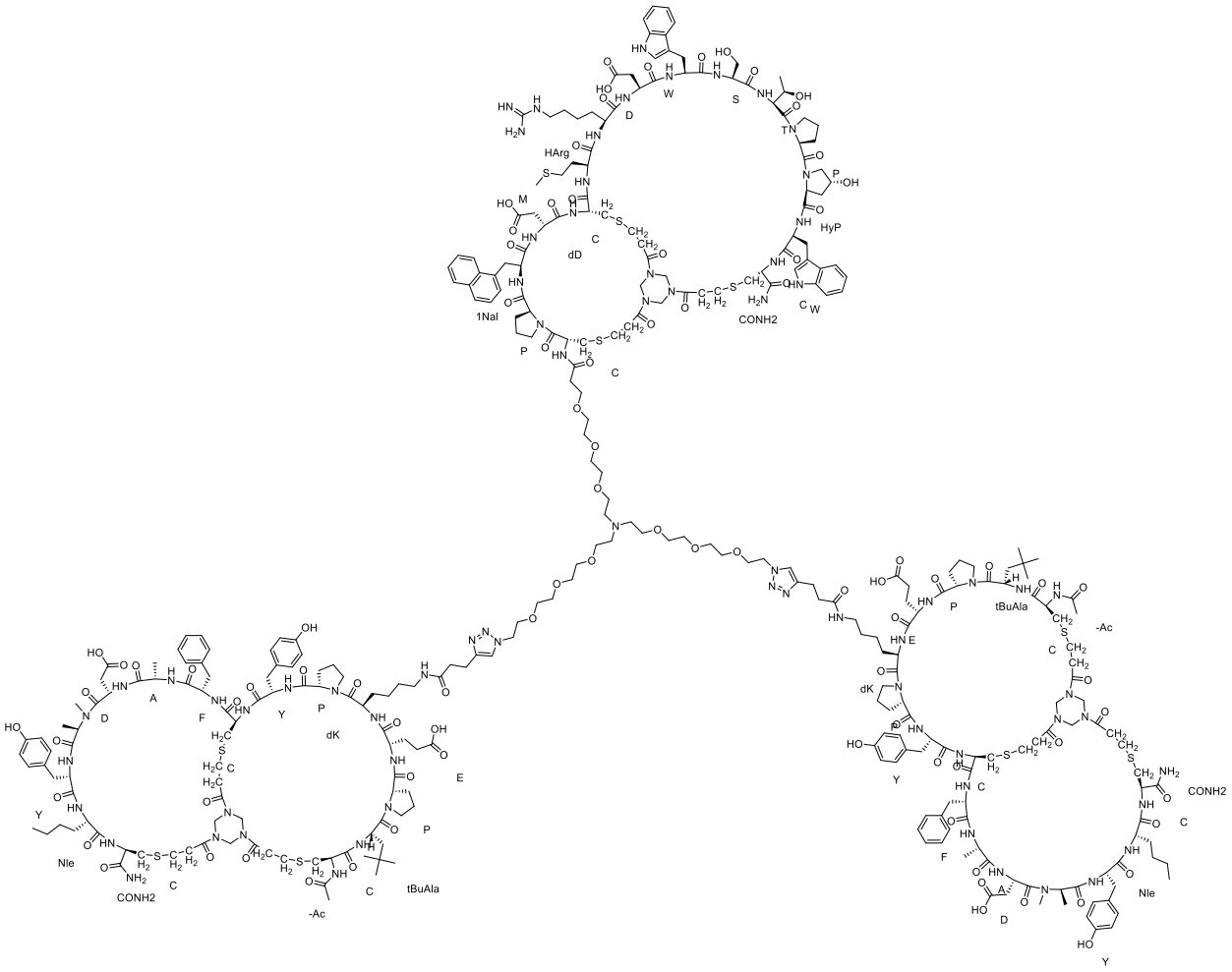

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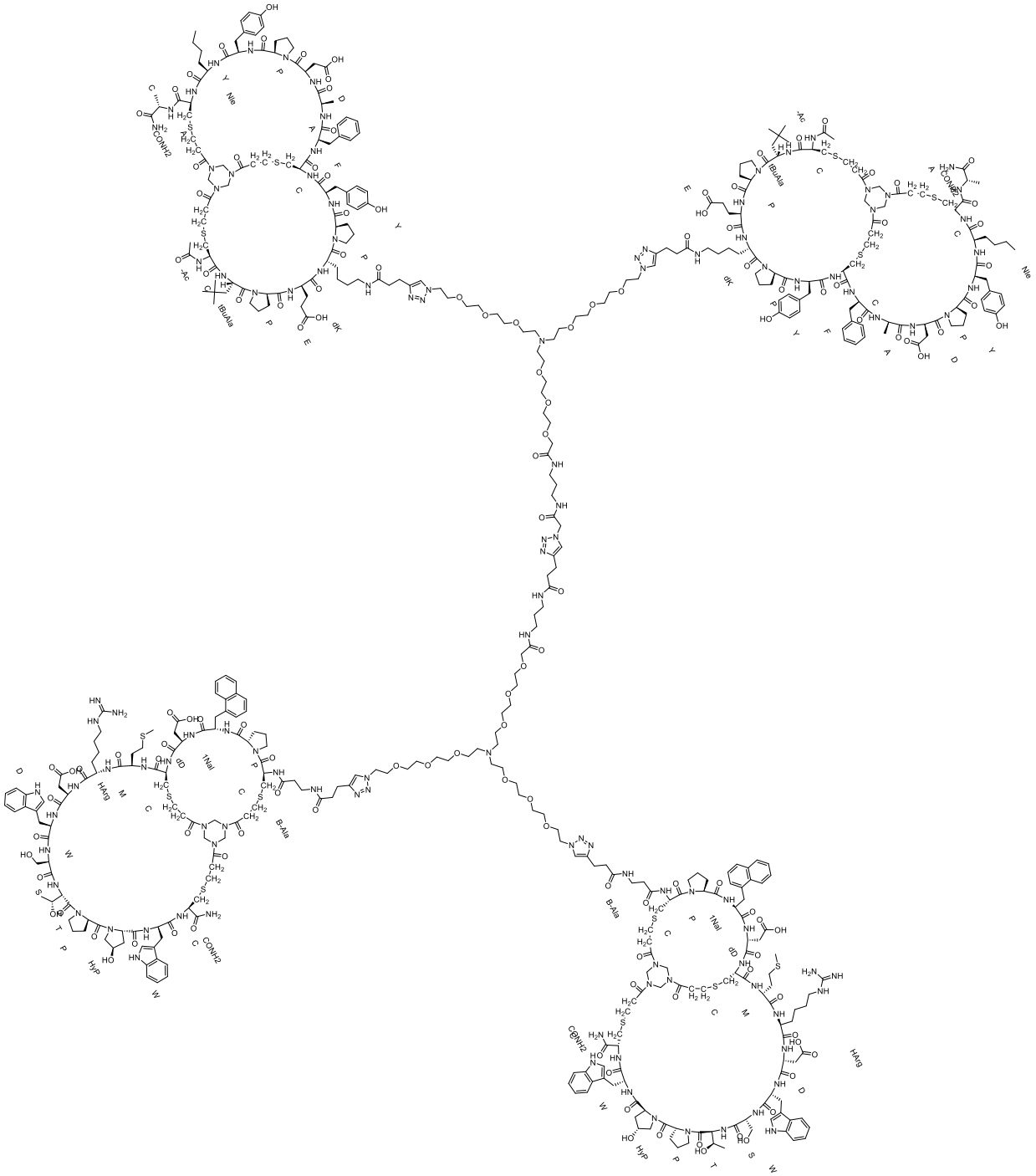

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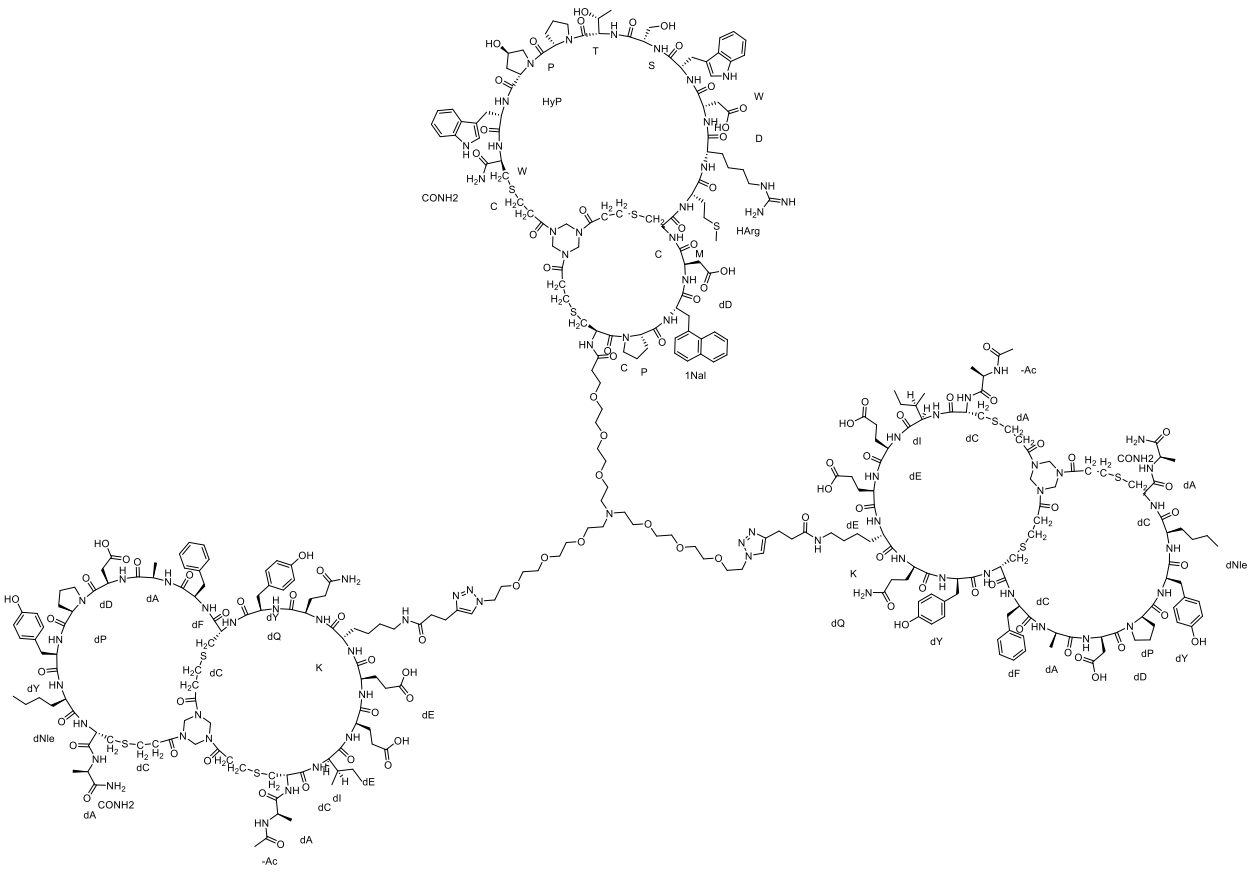

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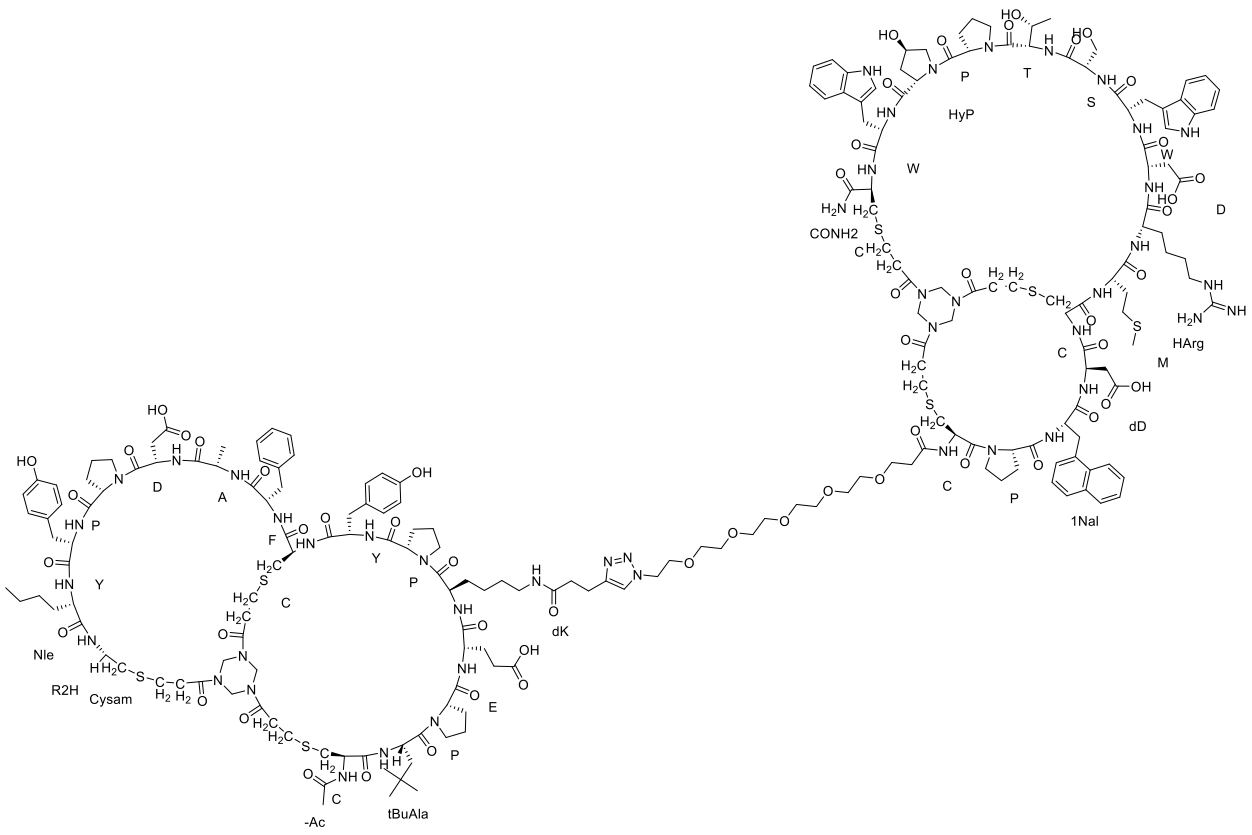
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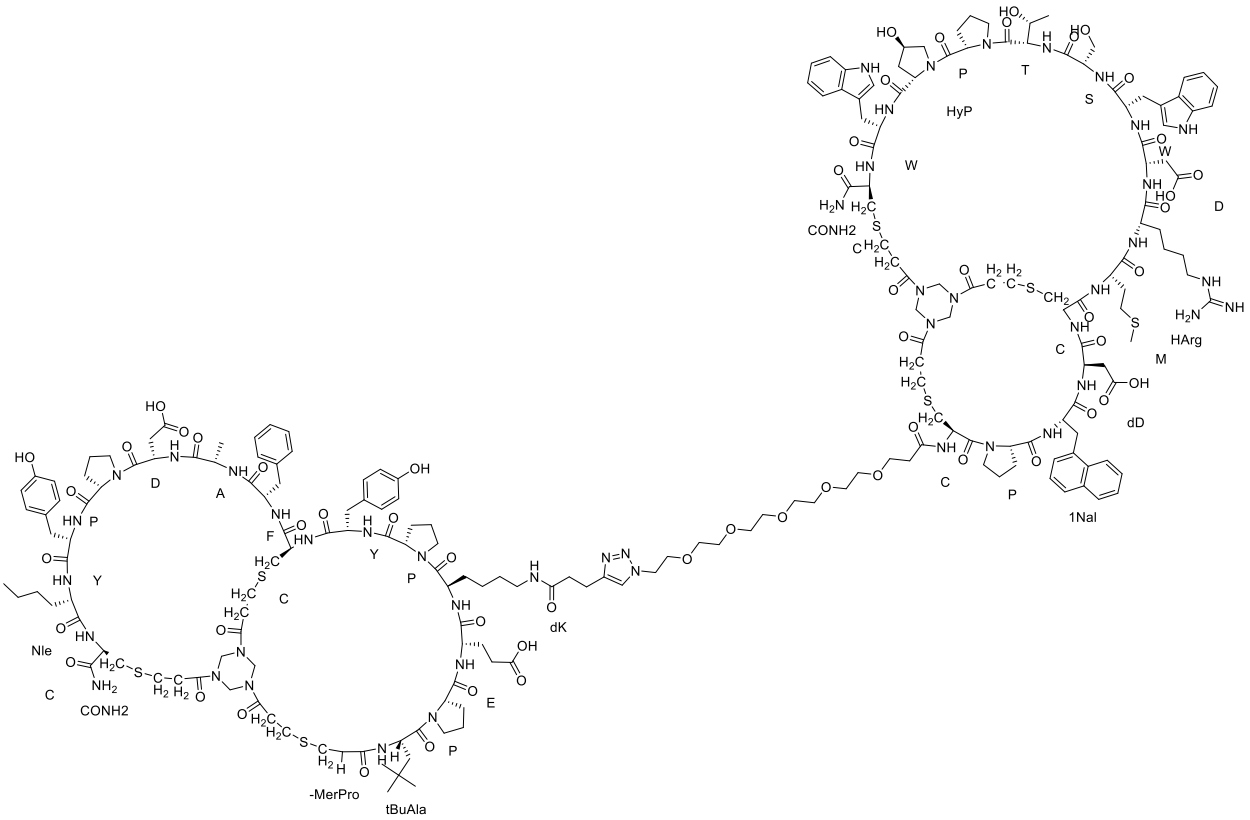

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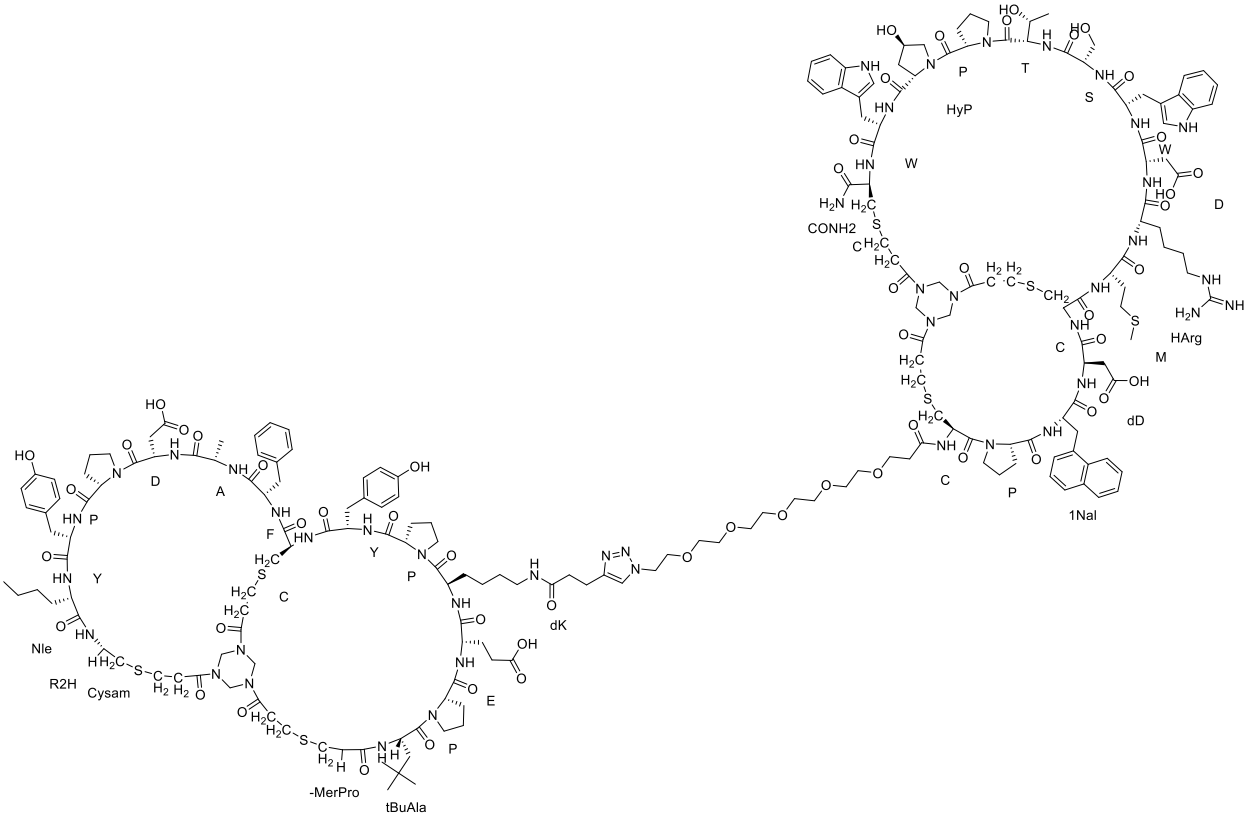

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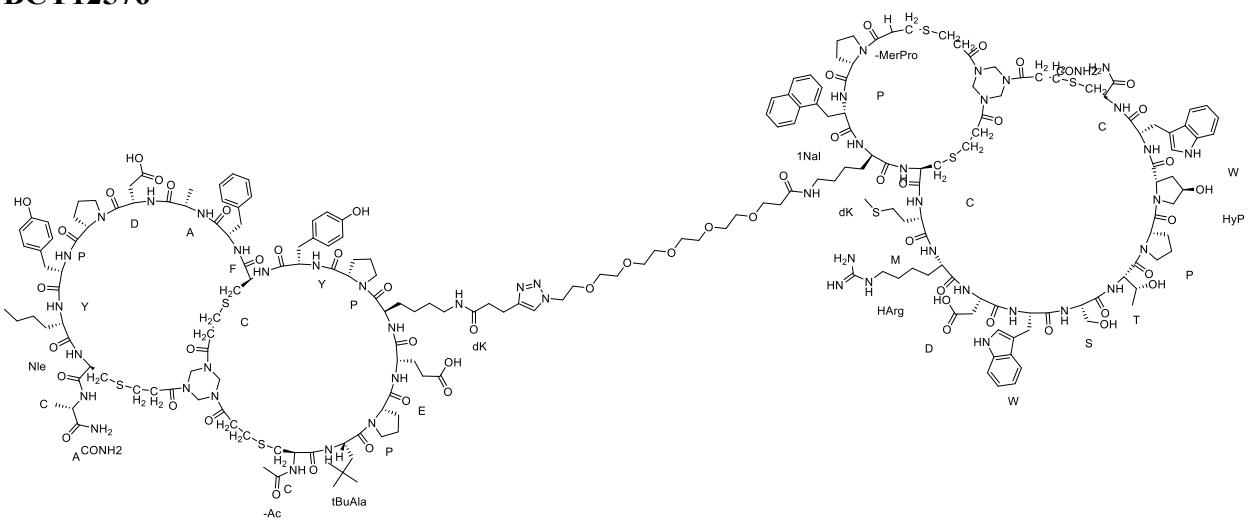

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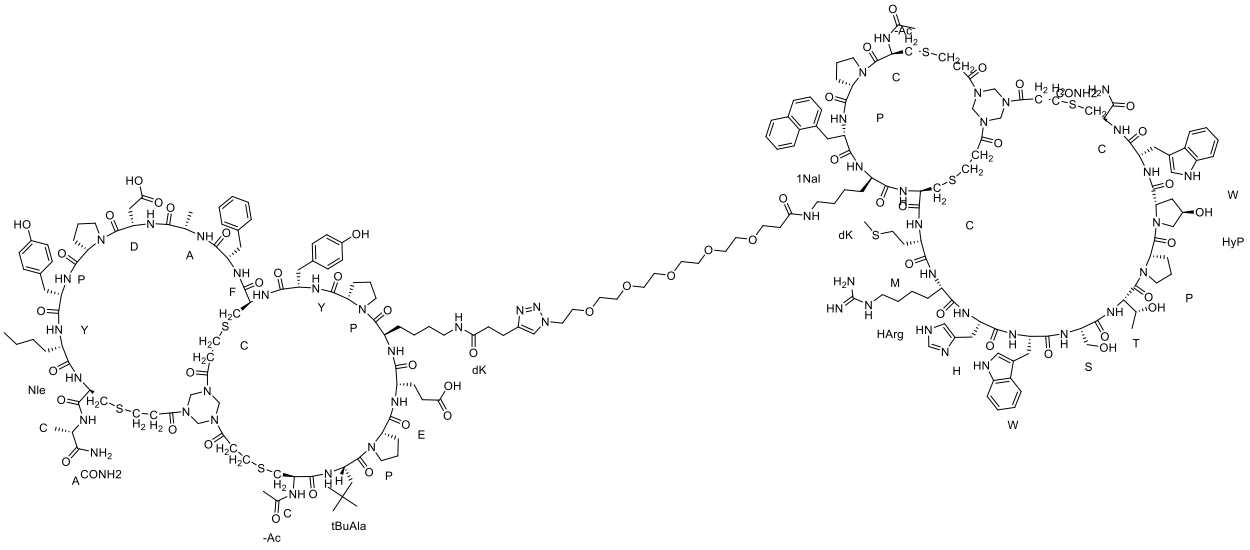

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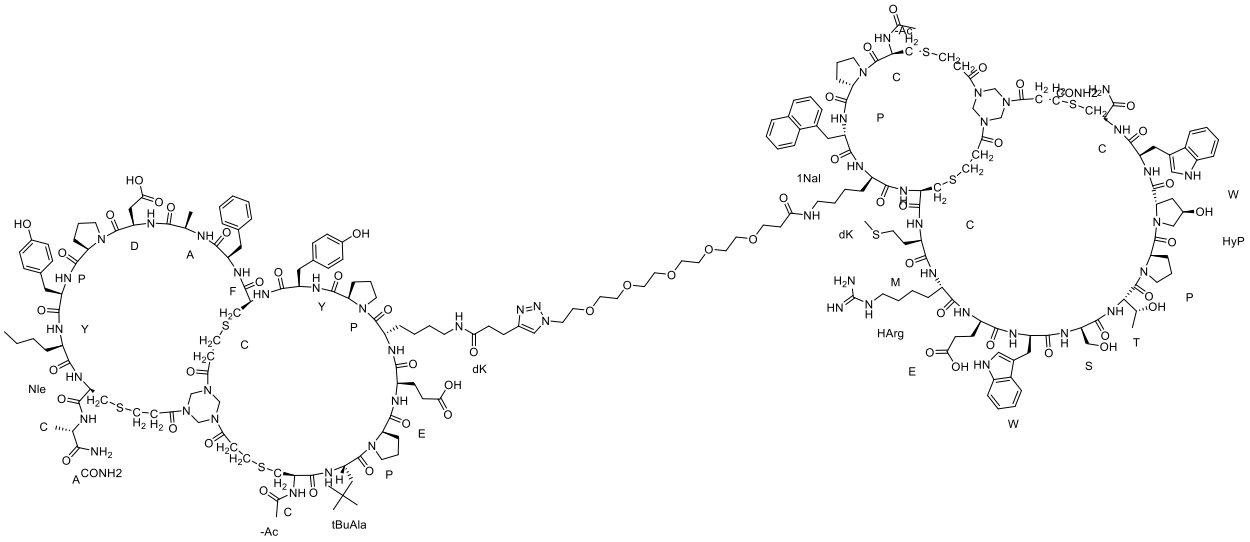

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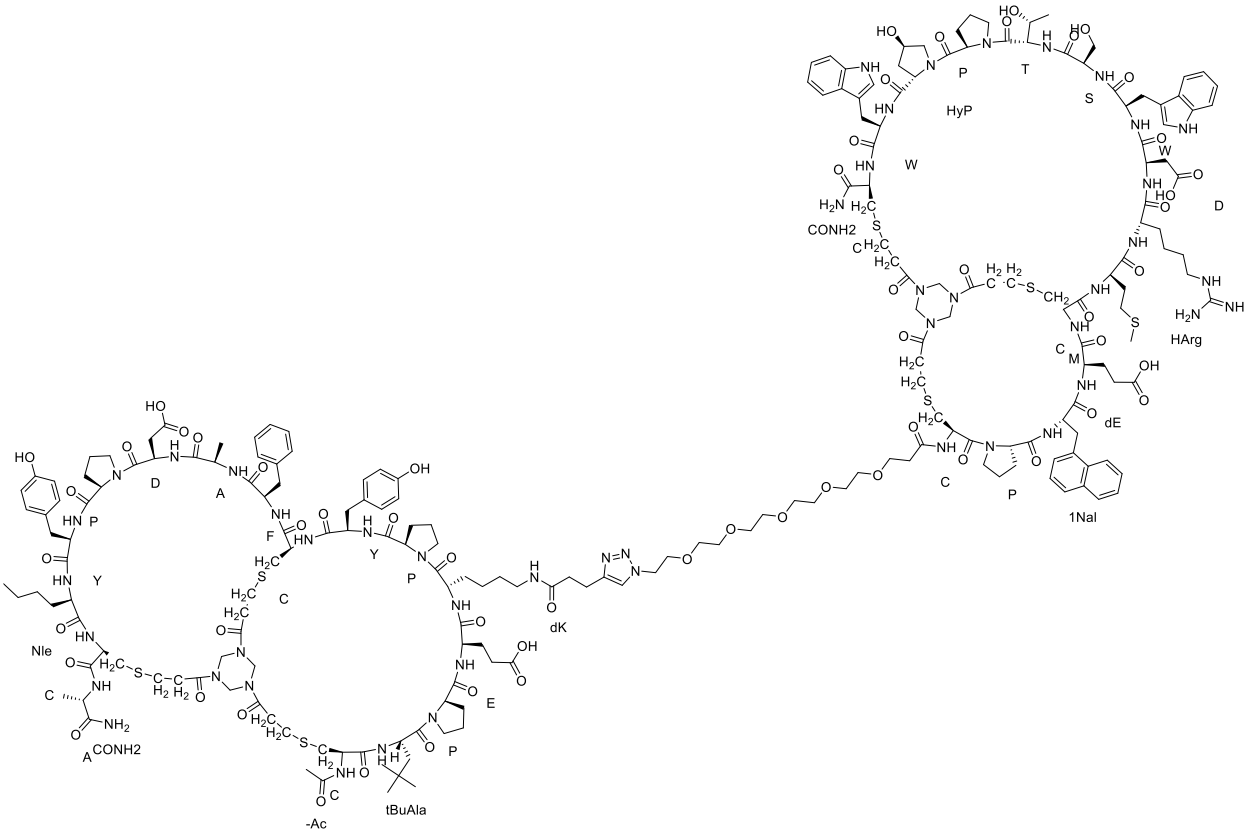

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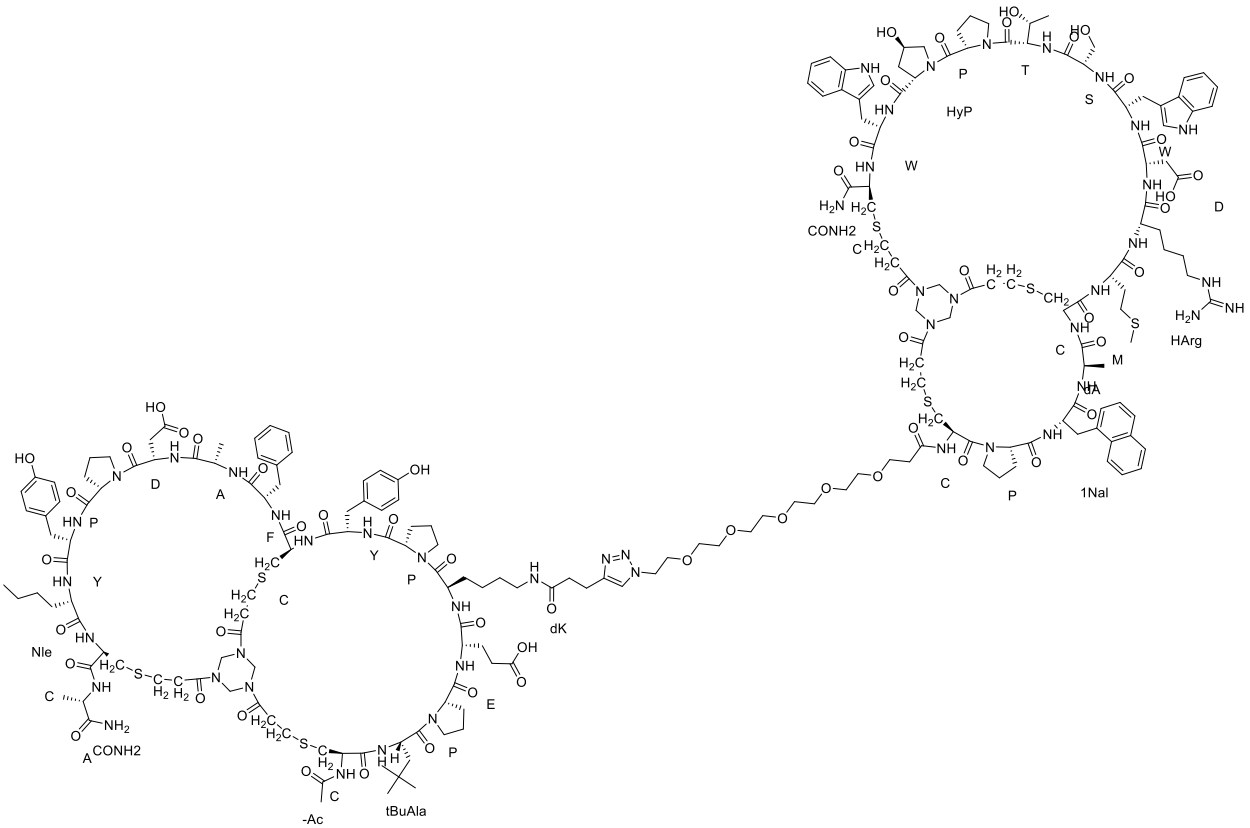

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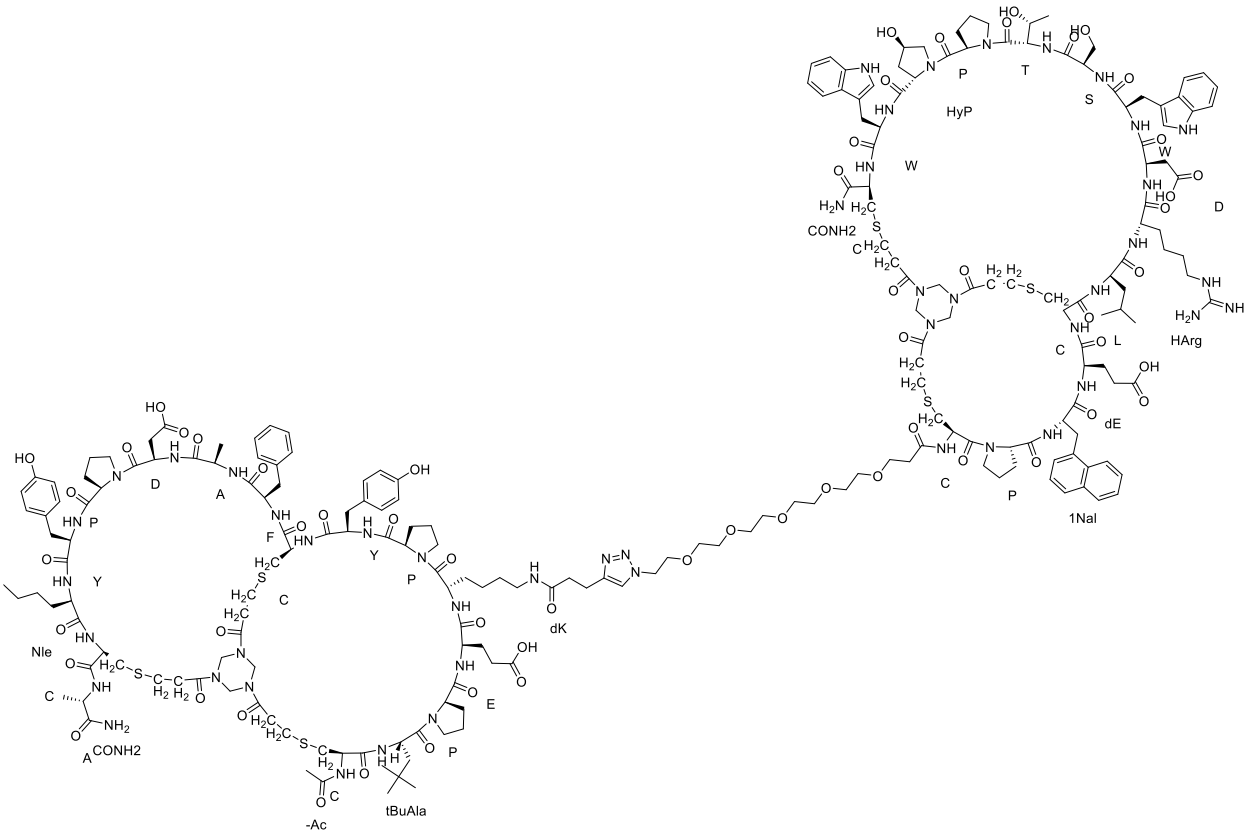

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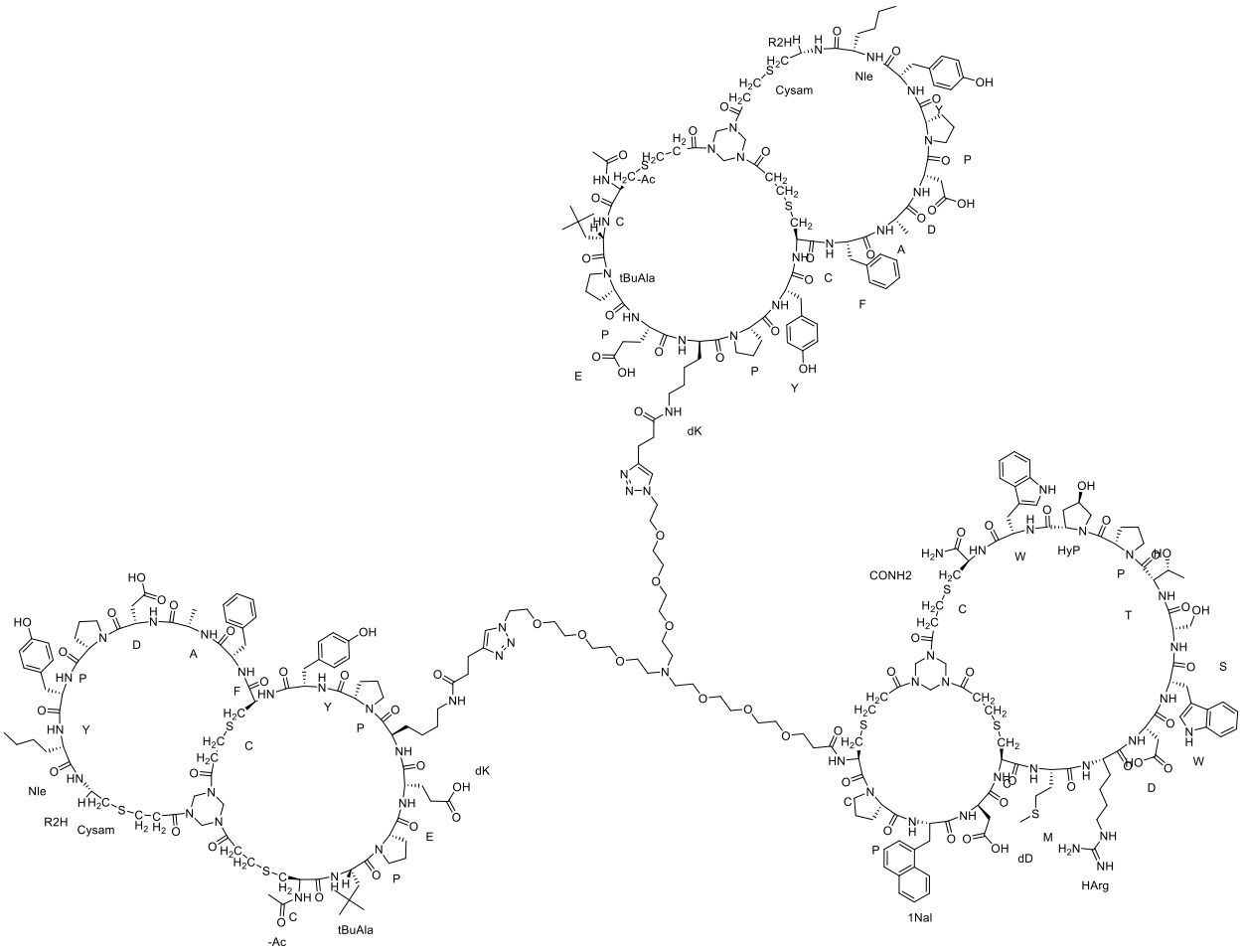

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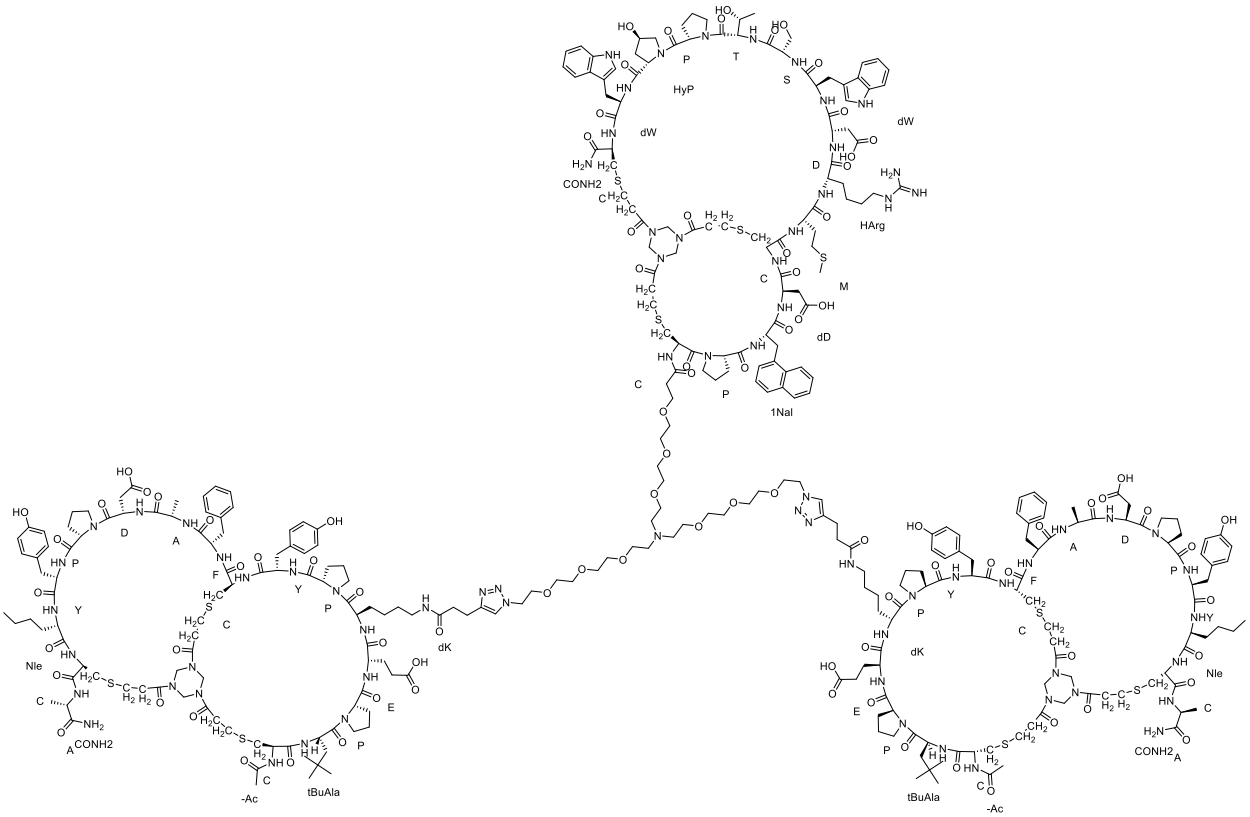

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1. V. Baeriswyl, H. Rapley, L. Pollaro, C. Stace, D. Teufel, E. Walker, S. Chen, G. Winter, J. Tite, C. Heinis, Bicyclic peptides with optimized ring size inhibit human plasma kallikrein and its orthologues while sparing paralogous proteases. *ChemMedChem* **7**, 1173-1176 (2012).