Supporting Information

Cu-ligand clusters dictate size of cyclized peptide formed during alkyne-azide cycloaddition on solid support

R. Kandler, S. Das, A. Nag

Carlson School of Chemistry and Biochemistry, Clark University, Worcester, MA 01610, USA

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Abbreviations

AA	amino acid
ACN	acetonitrile
CuAAC	copper caltalyzed alkyne-azide cycloaddition
D	cyclic dimer
DCM	dichloromethane
DMF	dimethylformamide
DIEA	N,N-Diisopropylethylamine
ESI	Electrospray Ionization
eq.	equivalent
FT-IR	Fourier-Transform Infrared Spectroscopy
HATU	*N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
M	cyclic monomer
nm	nanometers
NMP	N-Methyl-2-pyrrolidone
SPE	Solid Phase Extraction
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoroacetic acid

Experimental details and calculations

S 1: Cyclization conditions Table 2B

The cyclization was done according to Turner (2007).[1] Solvents (DMF, ACN) were purged with Argon for 40 mins in round bottom flasks with septa. Every vial or tube was purged with Argon for several minutes before use. The swelling volume of DMF was calculated according to the reference and the following solutions were prepared to be added to that volume after swelling the resin. A CuBr solution was prepared with 4.81mg CuBr (33.5µmol, 1eq) per sample in Acetonitrile (481.1µL per sample). A 1% (w%/v) sodium ascorbate (19.9mg, 100.5µmol, 3eq) solution was prepared in DMF (1991µL). DIEA (10eq) and 2,6-lutidine (10eq), sodium ascorbate solution (20.5mM in total volume) and lastly the CuBr solution (6.84mM in total volume) were added were added through a septum into an SPE tube and the reaction solution was purged with Argon for several minutes. The tube was then rotated for 6 hours at room temperature followed by the workup protocol of Turner 2007. In the last step of the workup the sample is dried. The cyclized peptide is then swollen in N-Methyl-2-pyrrolidone (NMP) followed by 3 deprotection steps (10 min each) in 20% piperidine and 5 washes with NMP. The deprotected sample is then dried with Dichlromethane (DCM) and then cleaved.

S 2: Cyclization conditions Table 2C

The cyclization was done according to Jagasia (2009).[2] Solvents (DMSO, ACN) were purged with Argon for 40 mins in round bottom flasks with septa. Every vial or tube was purged with Argon for several minutes before use. On-bead linear sequence Fmoc-NH-Pra-YYTYT-Az4 (equivalent to 50mg starting resin) was swollen and stirred in 2.5mL of purged DMSO in a 20mL glass vial with septum under Argon. The DMSO was decanted and the resin washed with a mixture of DMSO: ACN of 1:4 (v:v). The solvent was removed under argon and washed with more of the solvent mixture before finally adding 3.35mL of a 5mM CuI solution (1:4 (v:v), DMSO:ACN). 7.8µL 2,6-lutidine (2 eq) are added and the vial is protected from light and the solution mixed for 16 hours under argon. The same procedure was also conducted by using 1 equivalent of sodium ascorbate. For the workup the resin and solution was transferred to an empty SPE tube.

S 3: Calculation of conversion% to cyclic products (Table 1, Table 2, Table 3)

The linear staring material, cyclic monomer and dimer fractions are identified in different samples by RP-HPLC (Agilent 1260 series), ESI LC-MS and FT-IR. The conversion is calculated by the detected ultraviolet signal (280nm) of the identified linear peptide sequence during the RP-HPLC related to the signals for cyclic monomer and cyclic dimer of the cyclized peptide sequence (chromatograms next page). The area% of the cyclic monomer and cyclic dimer are added up and are divided by the sum of the area% of the linear peaks in the starting material before the cyclization. The result is multiplied by 100 to get the conversion percentage. The average of multiple HPLC measurements of the same sample is used for the calculation. If the peptides where cyclized under the same conditions but from different batches of the same linear sequence, the individual conversion is calculated first and then the average conversion.

conversion% = [(average area% cyclic peak(s))/(average area% linear peak(s) starting material)] *100

calculation for peptide 1:
linear version of peptide 1: average % area of 74.24%
cyclic monomer: average % area of 52.23%
cyclic dimer: average % area of 12.62%

conversion% = [(52.23%+12.62%)/(74.24%)] *100 = 87.4%

calculation for peptide 3:
linear version of peptide 3: average % area of 75.24%
cyclic monomer : average % area of 27.69%
cyclic dimer : average % area of 12.56%

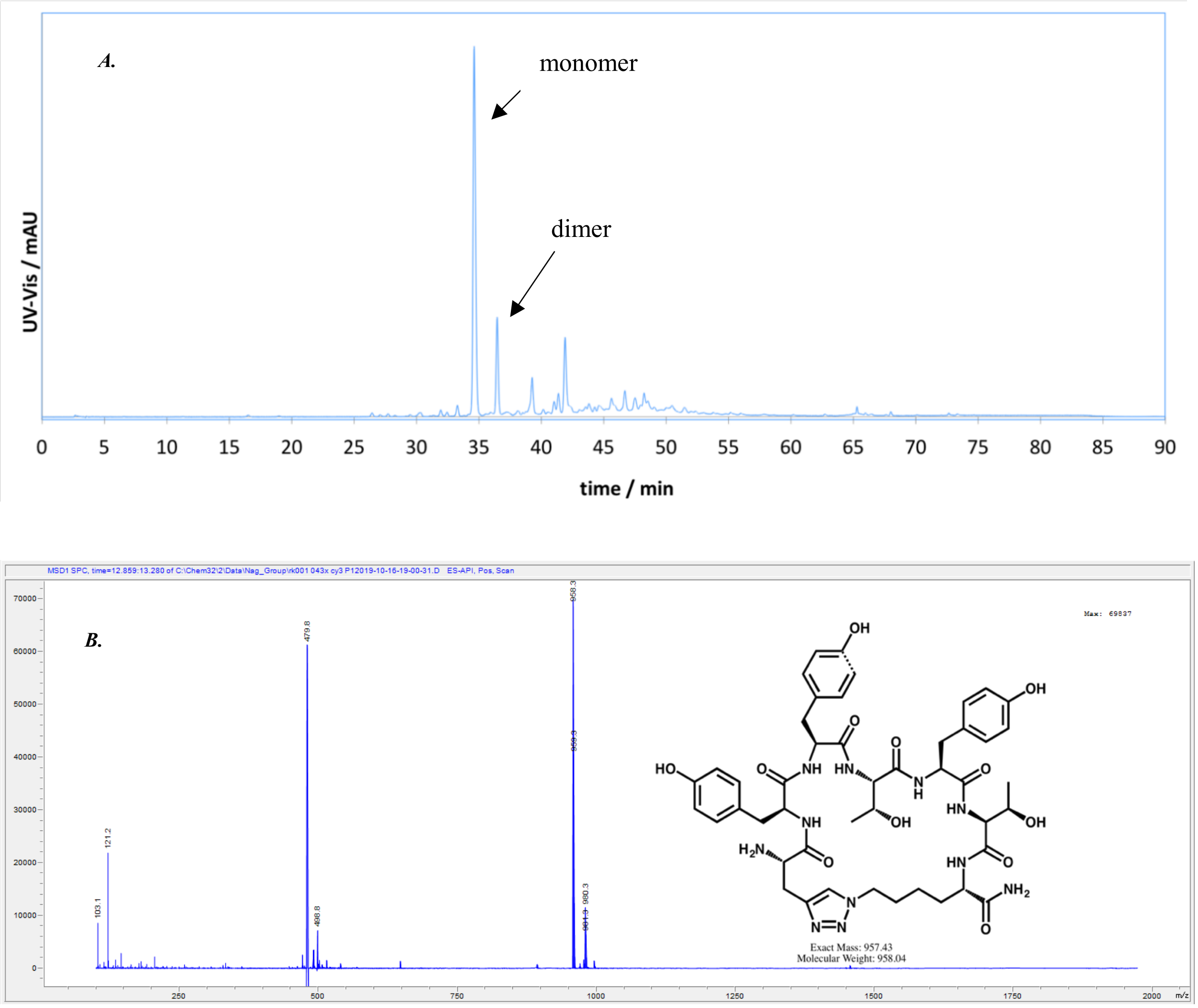
conversion% = [(27.69%+12.56%)/(75.24%)] *100 = 53.5%

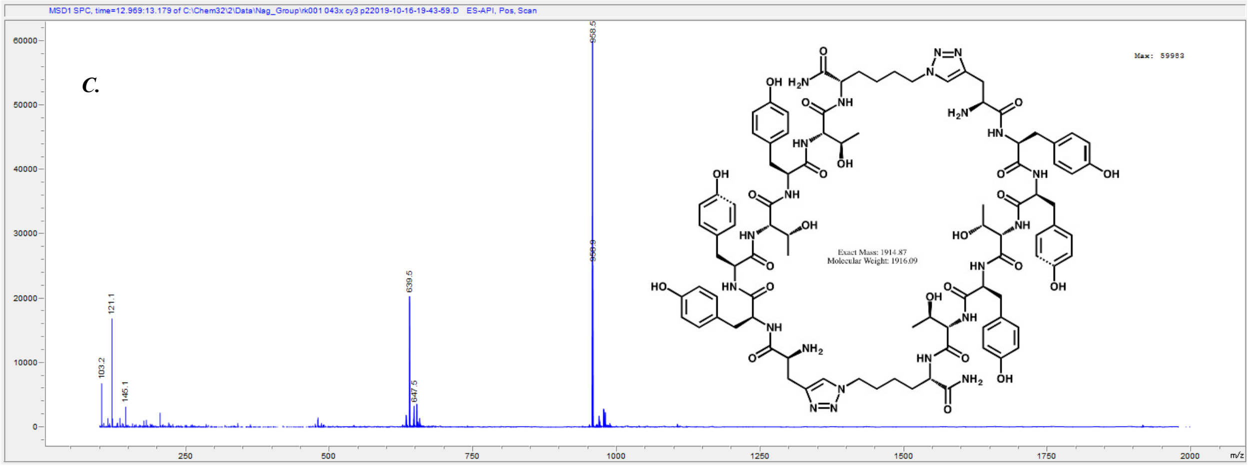
S 4: Calculation of cyclic monomer to cyclic dimer ratio

The cyclic monomer and dimer fractions are identified by RP-HPLC (Agilent 1260 series), ESI LC-MS and FT-IR. After identifying the cyclic monomer and cyclic dimer, the representative area% detected at 280 or 220 nm during the HPLC are related by adjusting them by the molar relation between a dimer to a monomer (1:2).

*Figure S. 1: HPLC and mass spectrometry analysis of cyclic peptide **1** cleaved from resin shows monomer (major product) and dimer.*

A. Analytical HPLC trace of cyclic peptide **1** cleaved from resin; B. LC/MS of fraction at 34.6 minutes corresponds to the monomer mass: m/z calculated for $[M+2]^{2+}$ expected: 479.7 found: 479.9, m/z calculated for $[M+H]^{+}$ expected: 958.4 found: 958.4; C. The LC/MS of fraction at 36.5 minutes cy[YYTYT-Tz4]2: m/z calculated for $[M+3H]^{3+}$expected: 639.4 found: 639.5, m/z calculated for $[M+2H]^{2+}$ expected: 958.4 found: 958.5 corresponds to the dimer mass.

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20000
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250
500
750
1000
1250
1500
1750
2000
m/z
Exact Mass: 1914.87
Molecular Weight: 1916.09
OH
HO
NH
HN
NH₂
H₂N
O
N
N=N
N—N

Figure S. 2: HPLC trace of linear peptide L-Pra-YYTYT-Az4 and mass spectrometry analysis of the main

A. Analytical HPLC trace of linear peptide **1** cleaved from resin; B. LC/MS the peak at 41.96 minutes corresponds to the mass of the linear peptide NH$_2$-Pra-YYTYT-Az$_4$: m/z calculated for $[M+2H]^{2+}$ expected: 479.7 found: 479.8, m/z calculated for $[M+H]^{+}$expected: 958.4 found: 958.4; the mass of the peak at 48.83 minutes has an increased mass of +106, 163 which comes from the cleavage byproducts of rink amide in 95%TFA.[3] The byproducts are sequence independent.

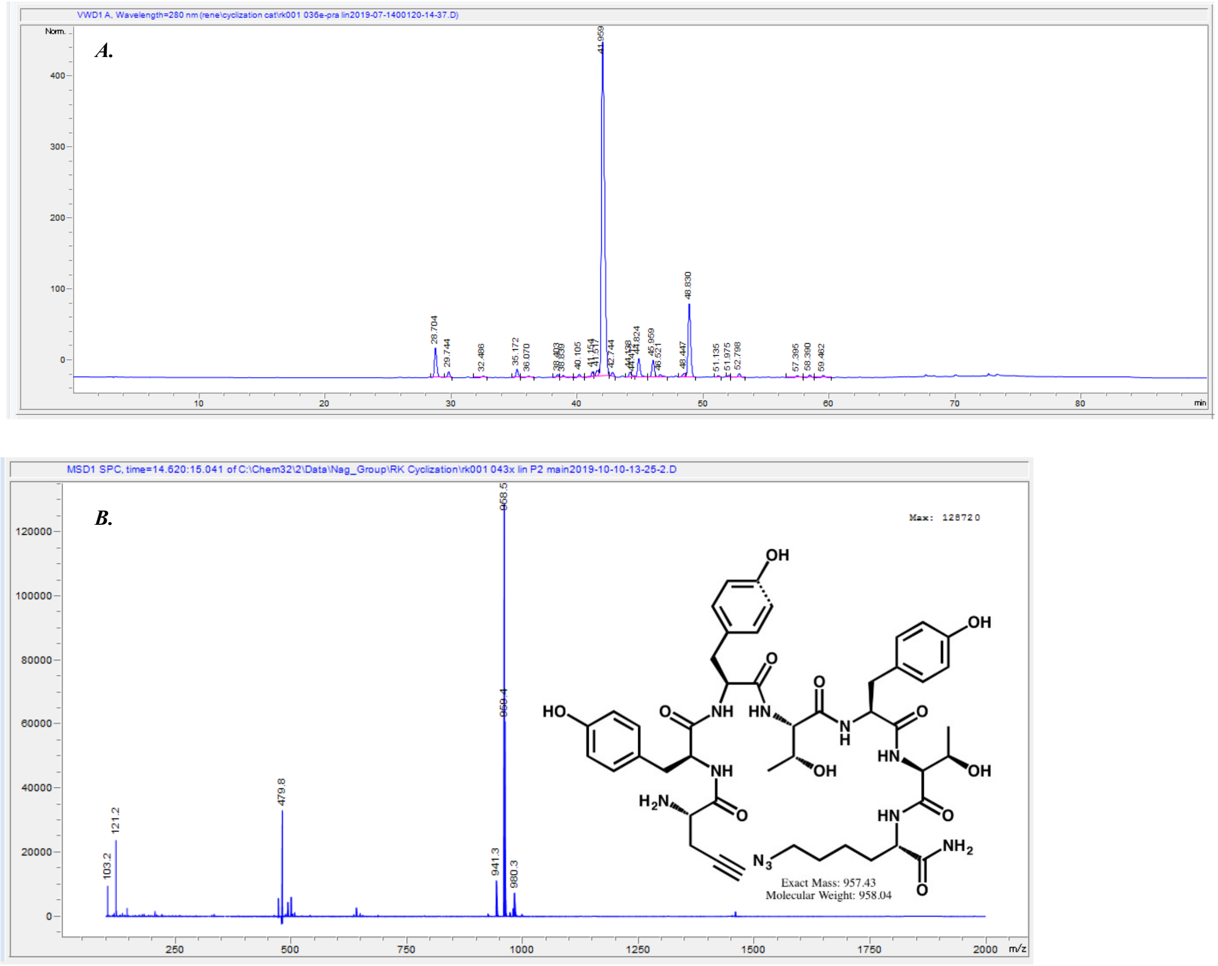


Figure S. 3: FT-IR spectra of linear and cyclic forms of peptide 1

A. FT-IR spectrum of linear NH_2-Pra-YYTYT-Az_4 . Azide triple bond rotation was detected at ~ 2100cm^{-1}.[4, 5] B. FT-IR spectrum of cyclic peptide 1 monomer (34.6 minutes HPLC fraction) C) FT-IR spectrum of cyclic peptide dimer (fraction at 36.5 Minutes)

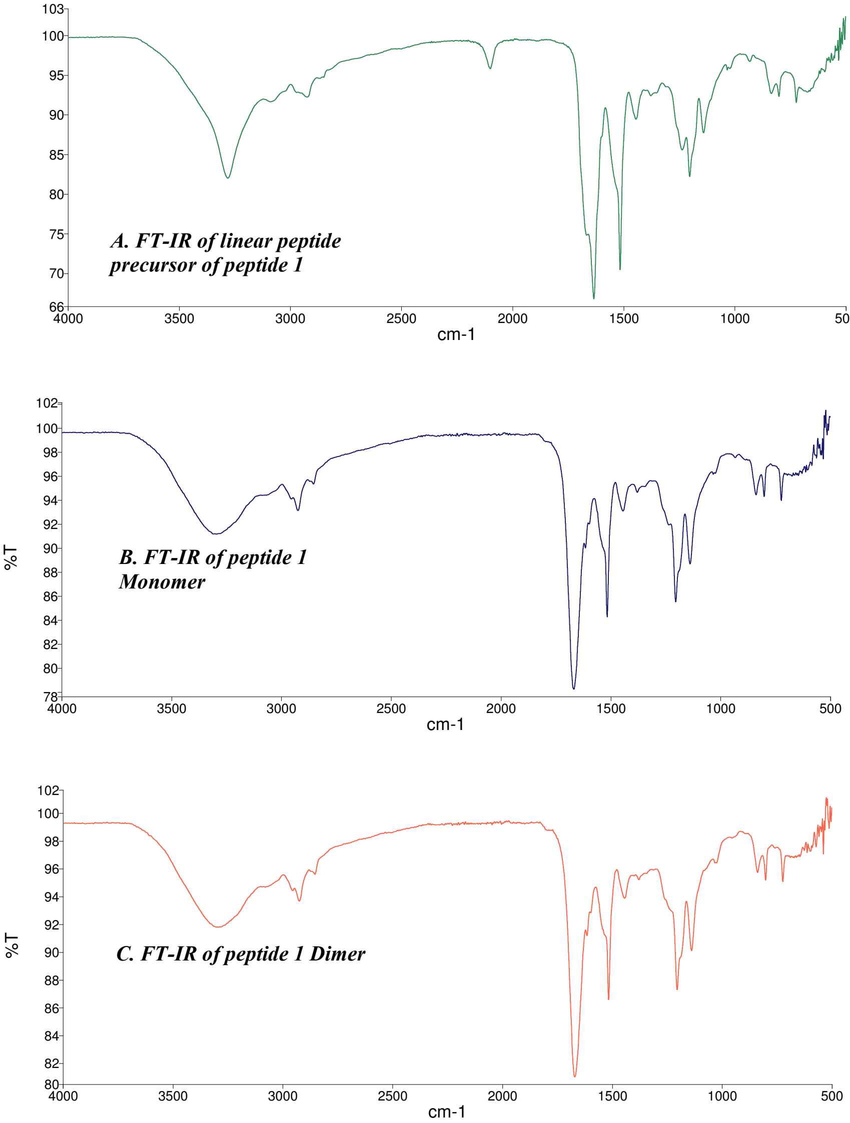


Figure S. 4: Cyclic voltammograms of CuI, on addition of 0-20 piperidine equivalents, in a reducing environment.

CV of Cu4I4Pip4 (blue diamonds) is also provided in this diagram.

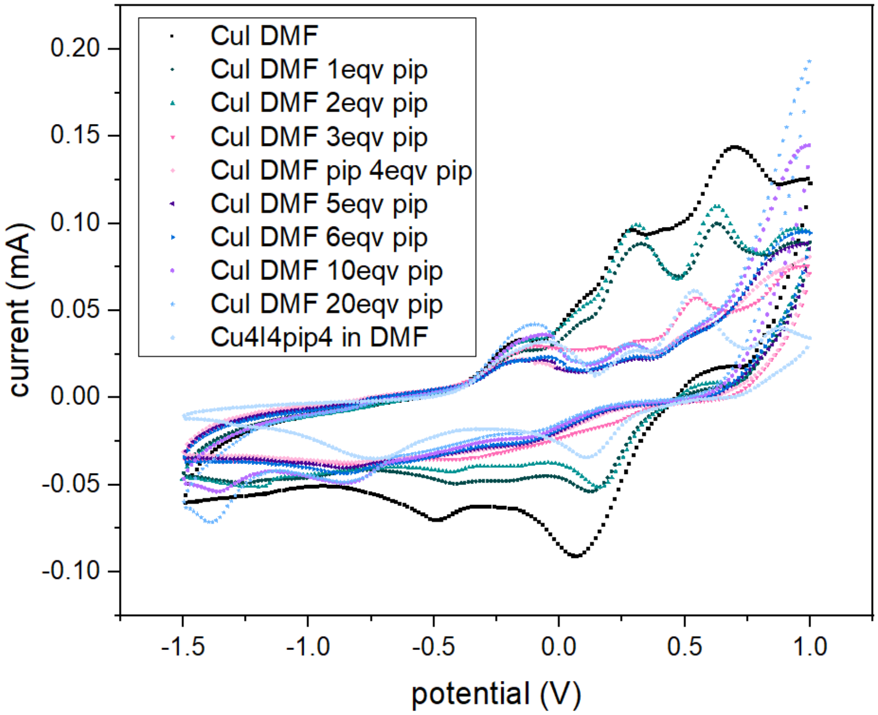


Figure S. 5: HPLC analysis of peptide 1 under different reaction conditions (Table 2B, 2C) Table 2B. HPLC trace 280nm: analytical column Phenomenex Luna C18

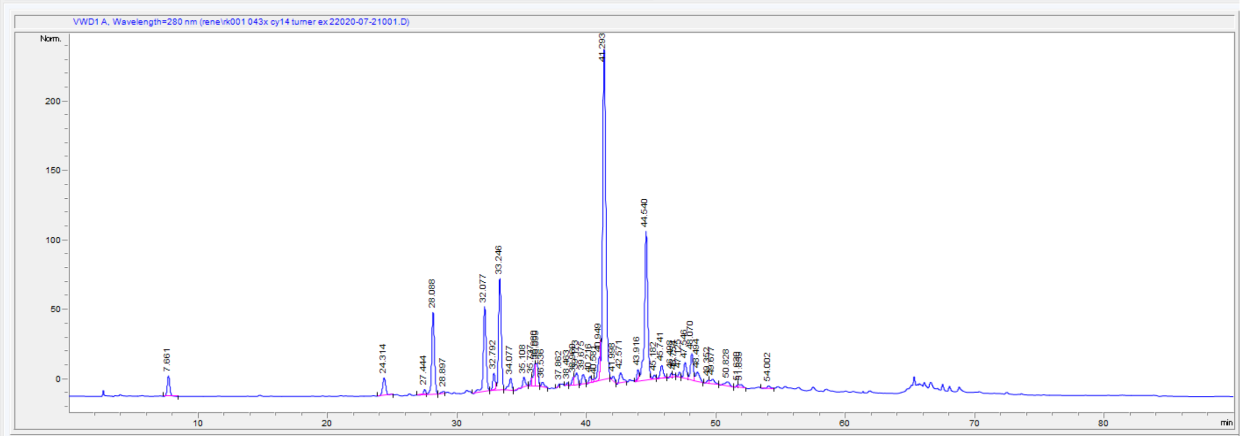


Table 2C. HPLC trace 280nm: analytical column Pursuit XRs C18

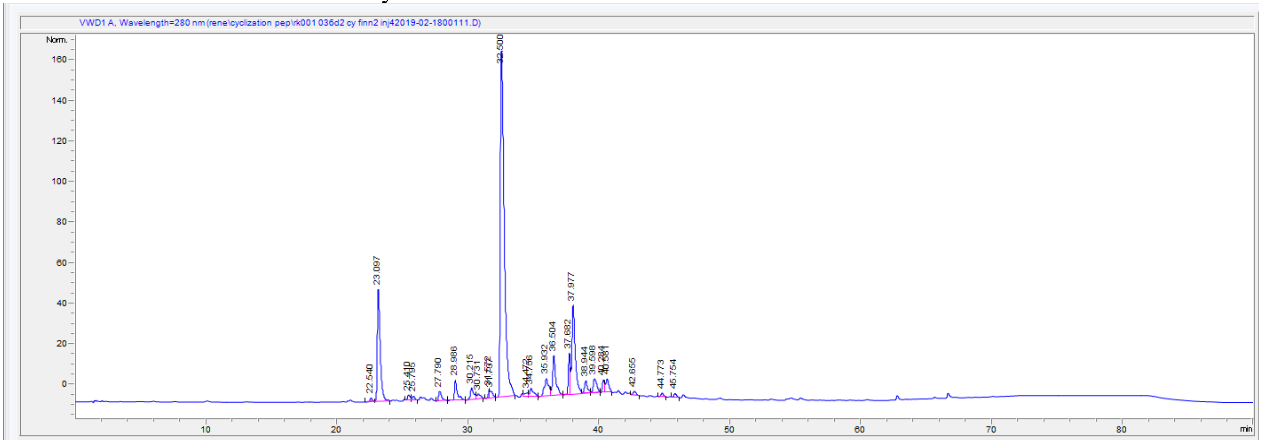


Figure S. 6: Time-dependence of cyclization reaction of peptide ***1*** *under reaction conditions given in Table 2A.*

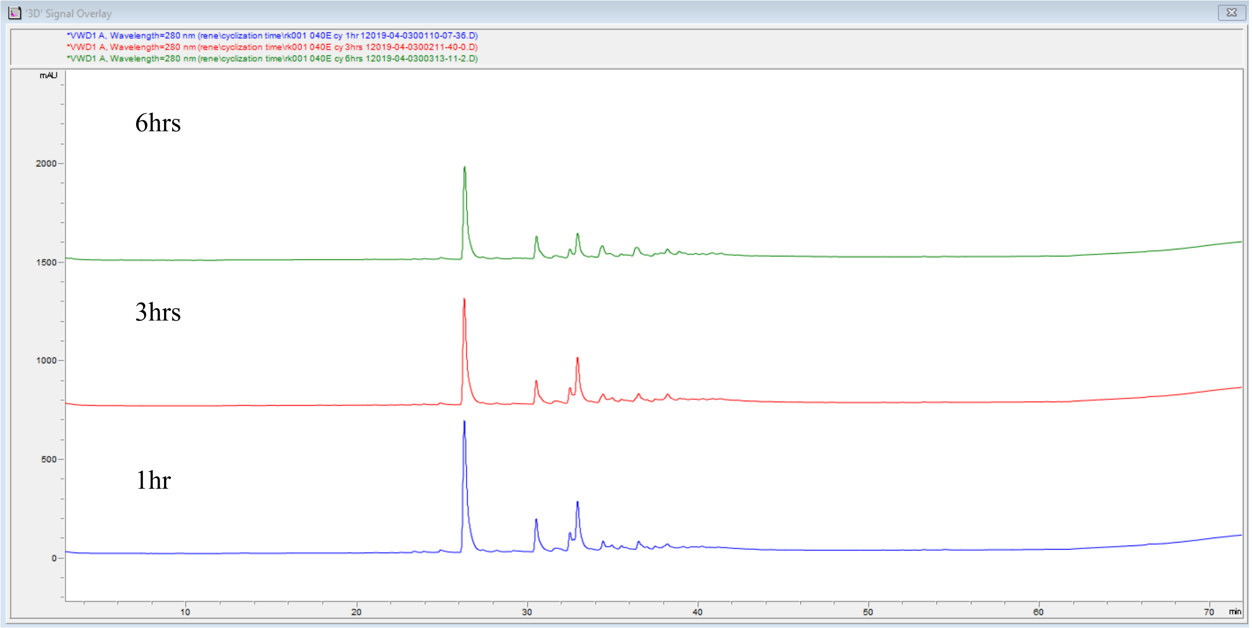
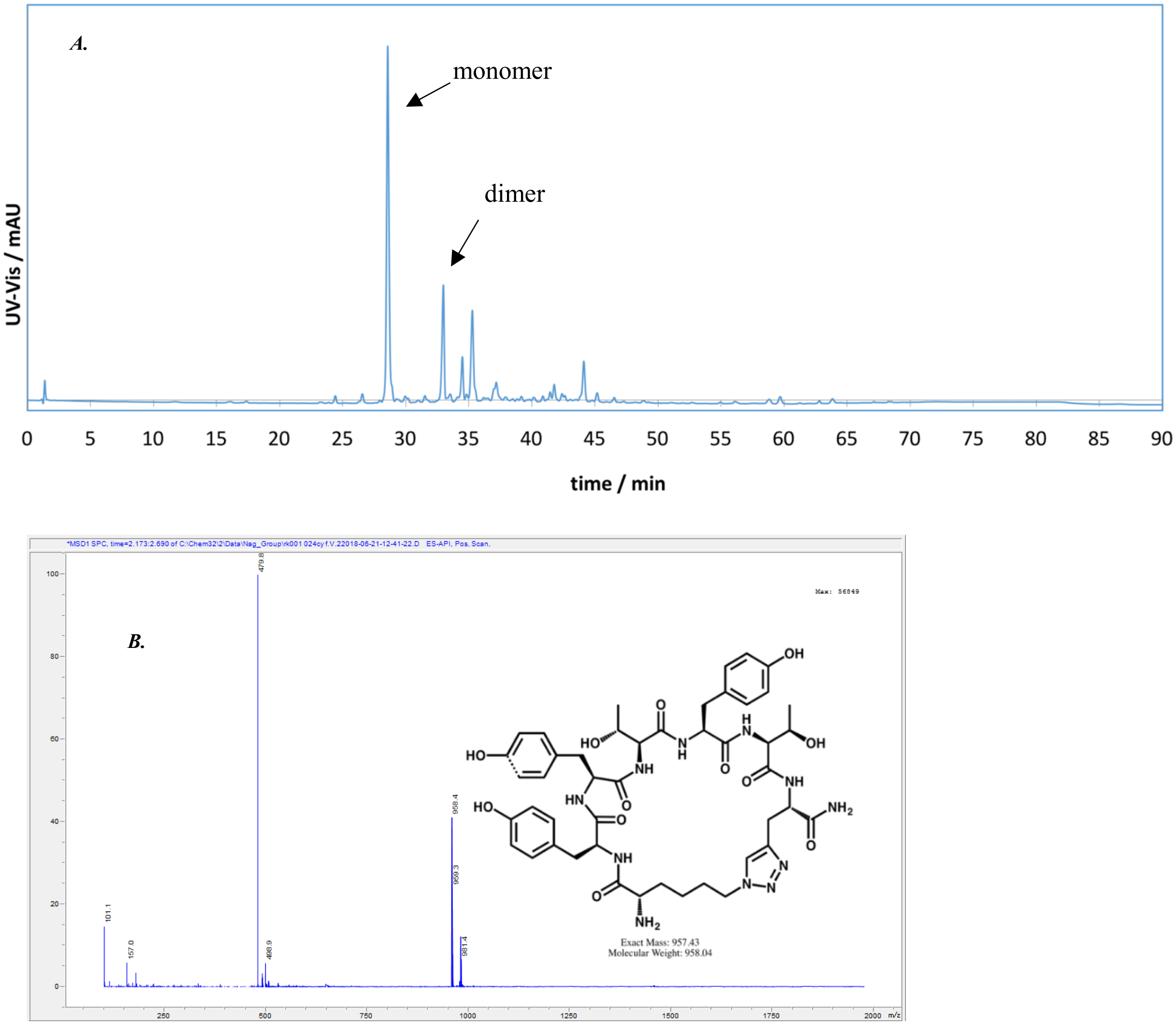
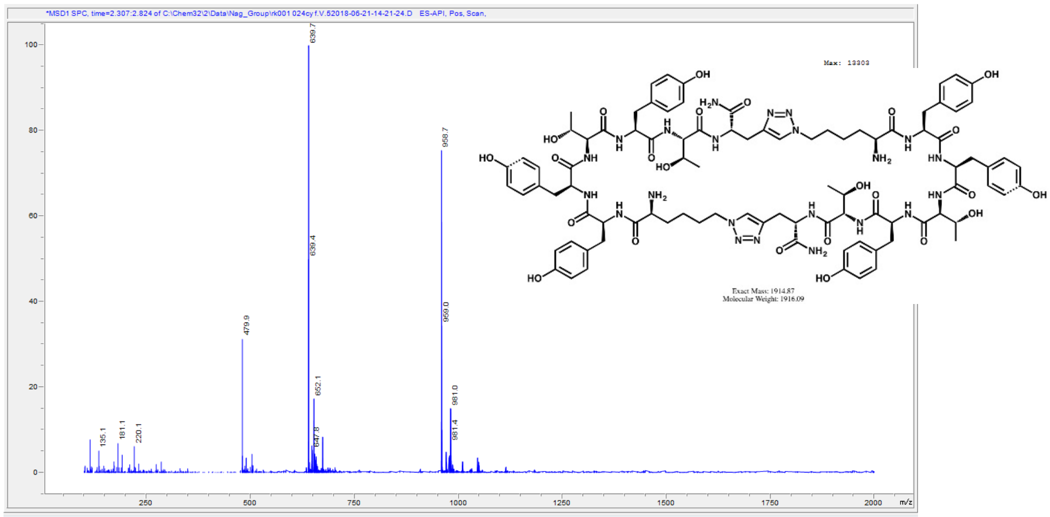


*Figure S. 7: HPLC and LC-MS analysis of cyclic peptide **2***

A. Analytical HPLC of peptide 2 with sequence $(\text{Tz}_4\text{-YYTYT})_{\text{cy}}$ B. LC-ESI-MS fraction from HPLC fraction at 28.5 minutes: m/z calculated for $[M+2H]^{2+}$ expected:479.7 found: 479.7, m/z calculated for $[M+H]^{+}$ expected: 958.4 found: 958.4; C. The LC/MS of fraction at 32.9 minutes corresponds to the dimer mass $\text{cy}[\text{YYTYT-Tz}_4]_2$: m/z calculated for $[M+4H]^{4+}$ expected: 479.7 found: 479.9, m/z calculated for $[M+3H]^{3+}$ expected: 639.4 found: 639.5 m/z calculated for $[M+2H]^{2+}$ expected: 958.4 found: 958.4 ,



C.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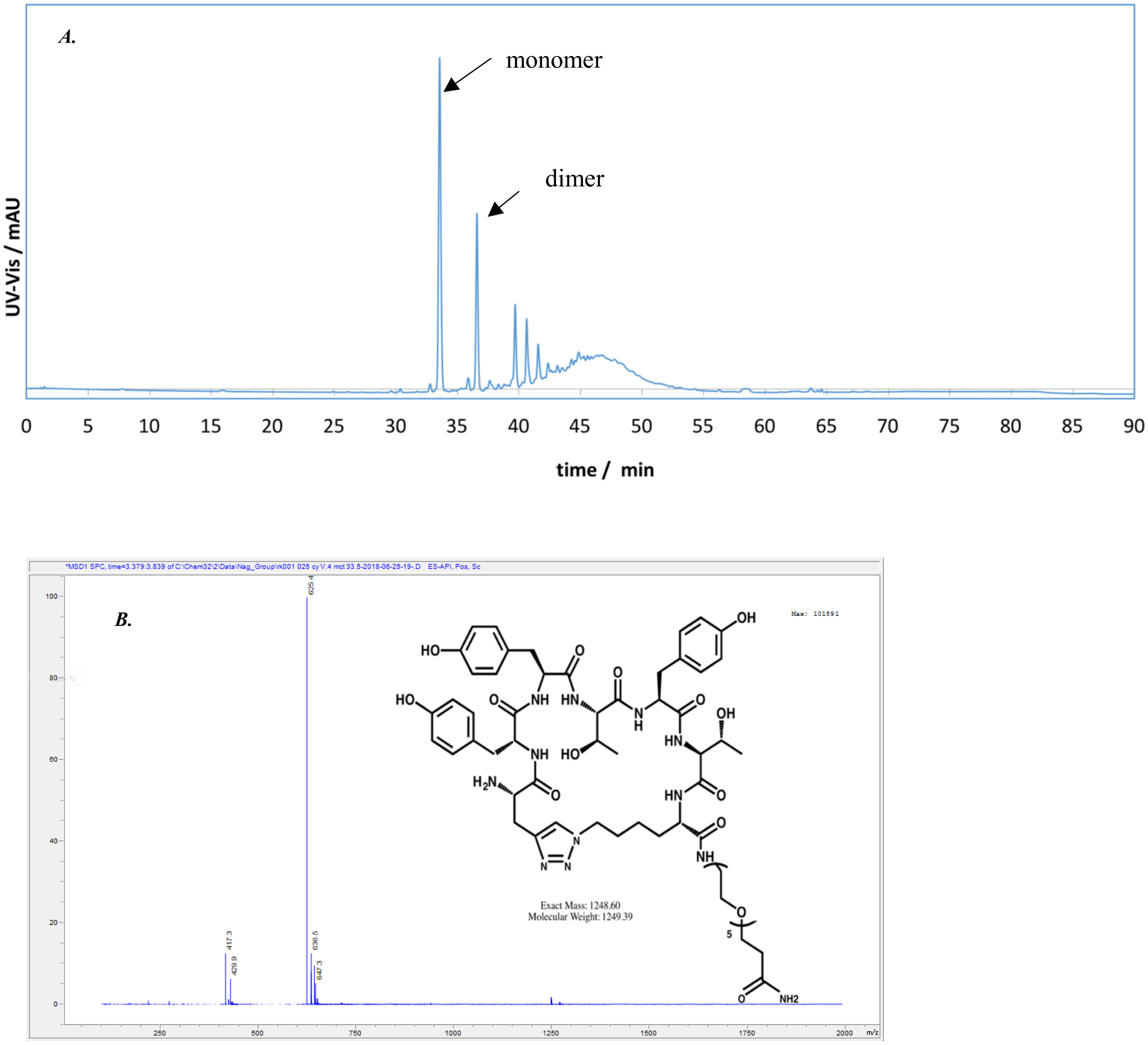
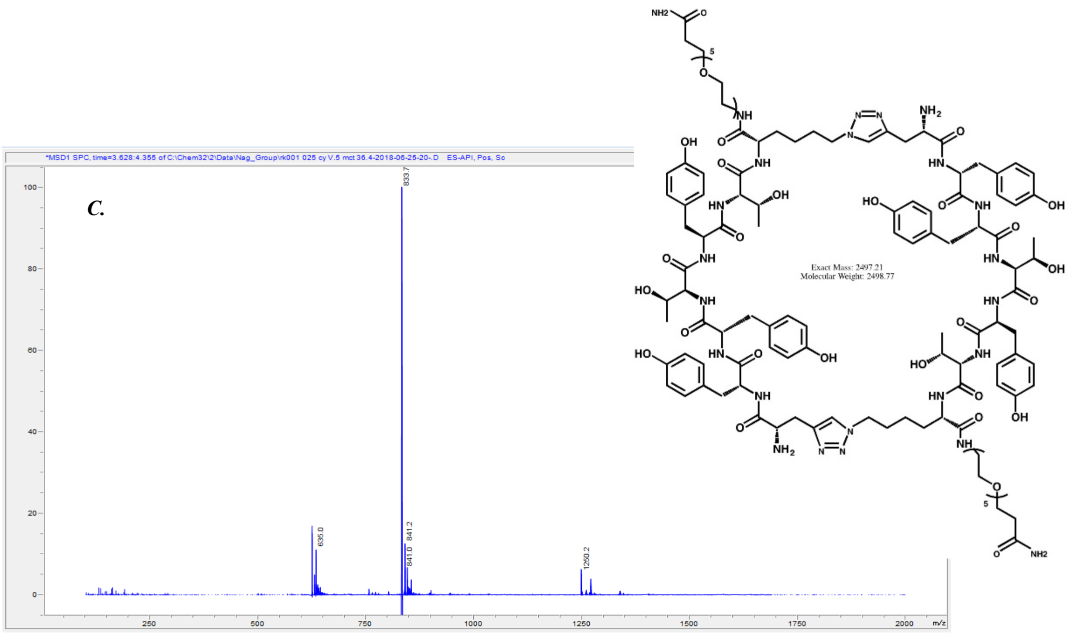
135.1
181.1
220.1
250
500
750
1000
1250
1500
1750
2000
m/z
HO
OH
H₂N
O
N=N
N
NH
NH₂
HN
Exact Mass: 1914.87
Molecular Weight: 1916.09

*Figure S. 8: HPLC and mass spectrometry analysis of cyclic peptide **3***

A. Analytical HPLC of cyclic peptide **3** with sequence $(Tz_4\text{-}YYTYT)_{cy}$ – $PEG_5\text{-}CONH_2$; B. LC-MS fraction from HPLC fraction at 33.5 minutes corresponding to monomer: m/z calculated for $[M+3H]^{3+}$expected: 417.2 found: 417.3, m/z calculated for $[M+2H]^{2+}$ expected: 625.3 found: 625.4; C. LC-MS fraction from HPLC fraction at 36.6 minutes corresponding to dimer: m/z calculated for $[M+3H]^{3+}$ expected: 833.5 found: 833.7, m/z calculated for $[M+2H]^{2+}$ expected: 1250.1 found: 1250.2; D. LC-MS fraction from HPLC fraction at 39.7 minutes corresponding to trimer: m/z calculated for $[M+5H]^{5+}$ expected: 750.4 found: 750.5 , m/z calculated for $[M+4H]^{4+}$ expected: 938.0 found: 938.0, m/z calculated for $[M+3H]^{3+}$ expected: 1250.1 found: 1250.2

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750
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1250
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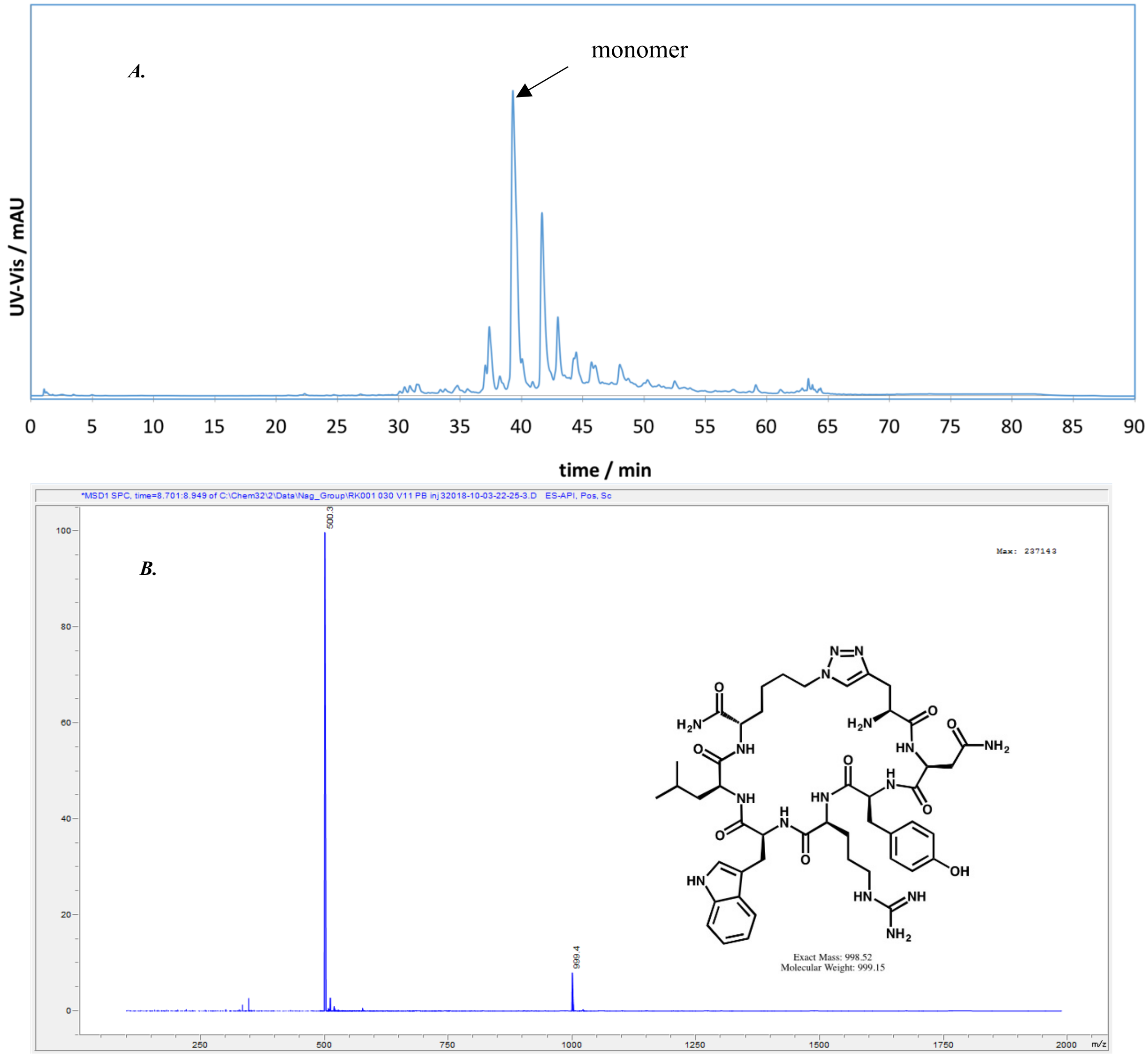
1750
2000
m/z
Exact Mass: 2497.21
Molecular Weight: 2498.77

*Figure S. 9: HPLC and mass spectrometry analysis of cyclic peptide **4***

A. Analytical HPLC of cyclic peptide **4** with sequence (NYRWL-Tz4)$_{cy}$ B. LC-MS fraction from HPLC fraction at 39.2 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 500.3 found: 500.3, m/z calculated for $[M+H]^{+}$ expected: 999.5 found: 999.4. LC-MS of other fractions did not yield mass corresponding to dimer.



B.

*Figure S. 10: HPLC and mass spectrometry analysis of cyclic peptide **5***

A. Analytical HPLC of cyclic peptide **5** with sequence (RYKHY -Tz$_{4}$)$_{cy}$ B. LC-MS fraction from HPLC fraction at 29.8 minutes corresponding to monomer: m/z calculated for $[M+3H]^{3+}$ expected: 338.9 found: 338.9, m/z calculated for $[M+2H]^{2+}$ expected: 507.8 found: 507.8, m/z calculated for $[M+H]^{+}$ expected: 1014.5 found: 1014.5. LC-MS of other fractions did not yield mass corresponding to dimer.

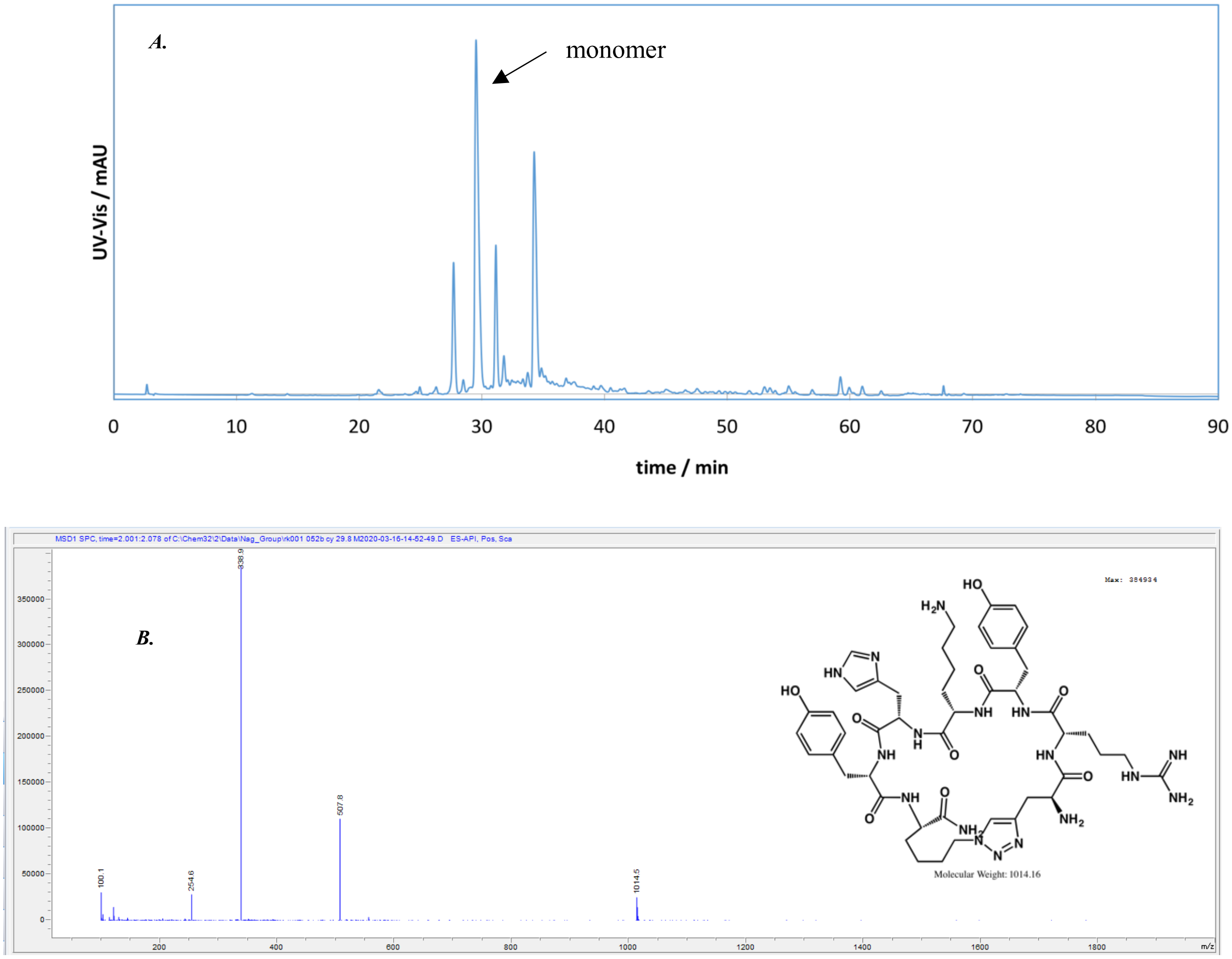
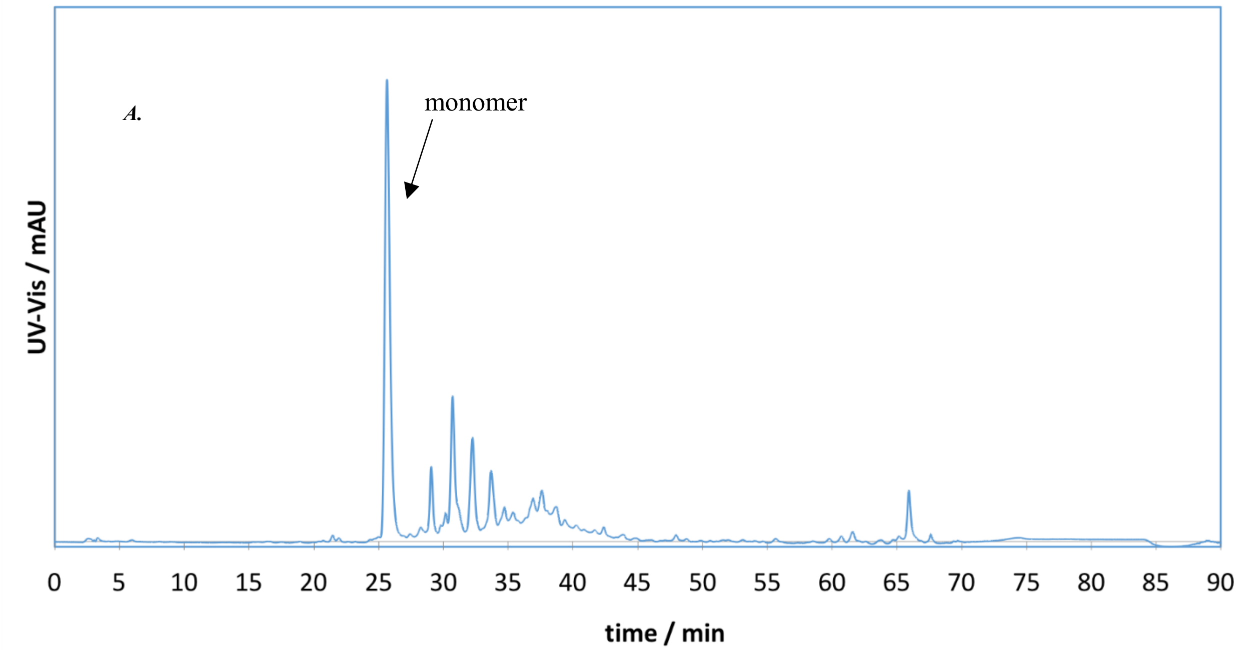


Figure S. 11: HPLC and mass spectrometry analysis of cyclic peptide ***6***

A. RP-HPLC of peptide **6** with sequence (YKYYR-Tz4)$_{cy}$; B LC-MS of HPLC fraction at 26.6 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{3+}$ expected: 347.5 found: 347.6, m/z calculated for $[M+2H]^{2+}$ expected: 520.8 found: 521.0, m/z calculated for $[M+H]^{+}$ expected:1040.5 found: 1040.4. LC-MS of other fractions did not yield mass corresponding to dimer.



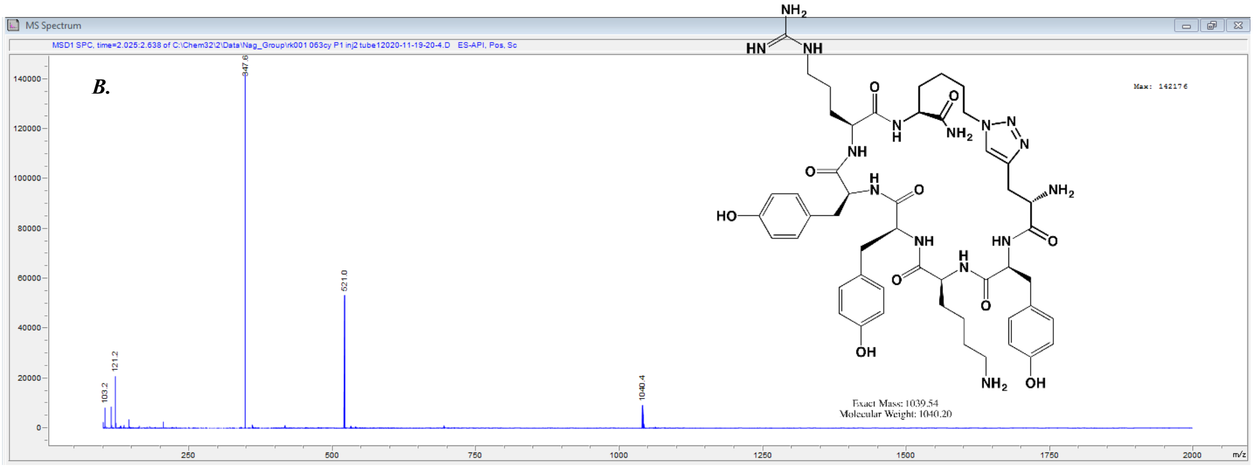
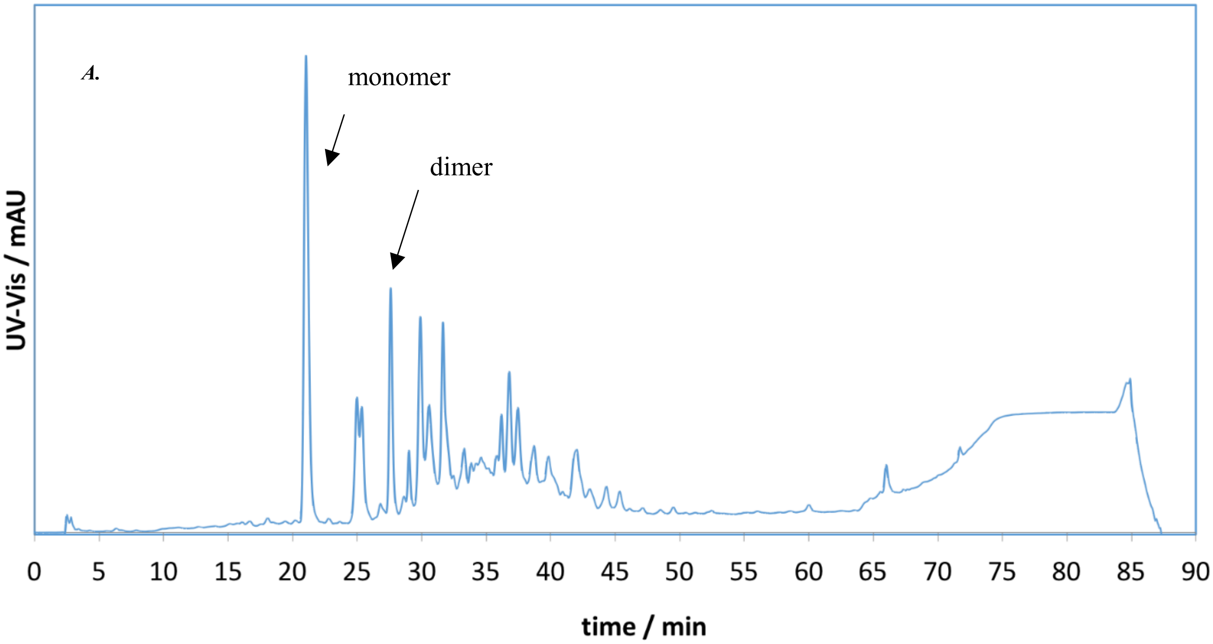
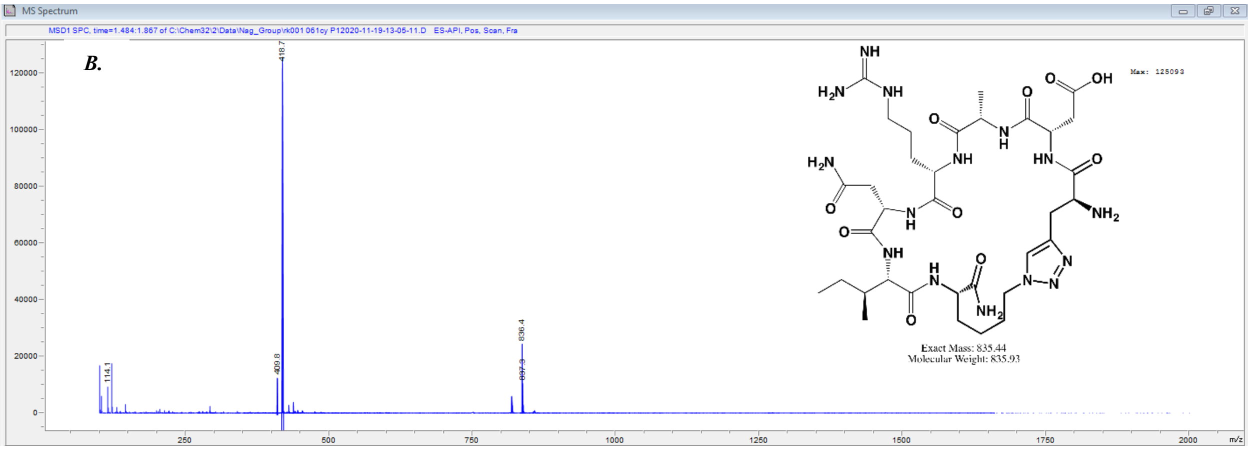


*Figure S. 12: HPLC and mass spectrometry analysis of cyclic peptide **7***

A. RP-HPLC of peptide **7** with sequence (DARNI-Tz4)cy ; B. LC-MS of HPLC fraction at 19.2 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 418.7 found: 418.8, m/z calculated for $[M+H]^{+}$ expected: 836.4 found: 836.4; C. LC- MS of HPLC fraction at 26.2 minutes corresponds to dimer mass: m/z calculated for $[M+4H]^{4+}$ expected: 418.7 found: 418.9, m/z calculated for $[M+3H]^{3+}$ expected: 558.0 found: 558.1, m/z calculated for $[M+2H]^{2+}$ expected: 836.5 found: 836.5





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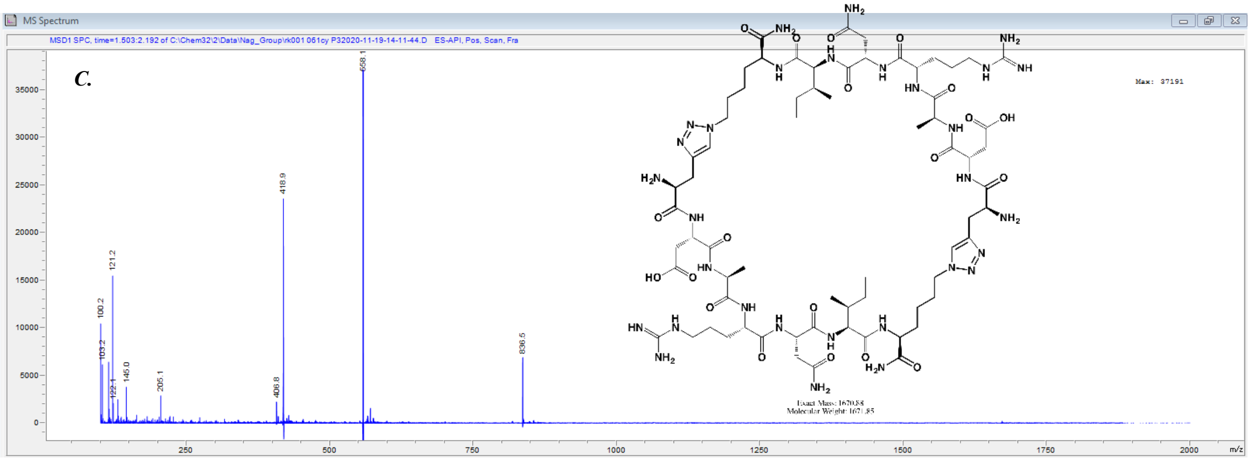

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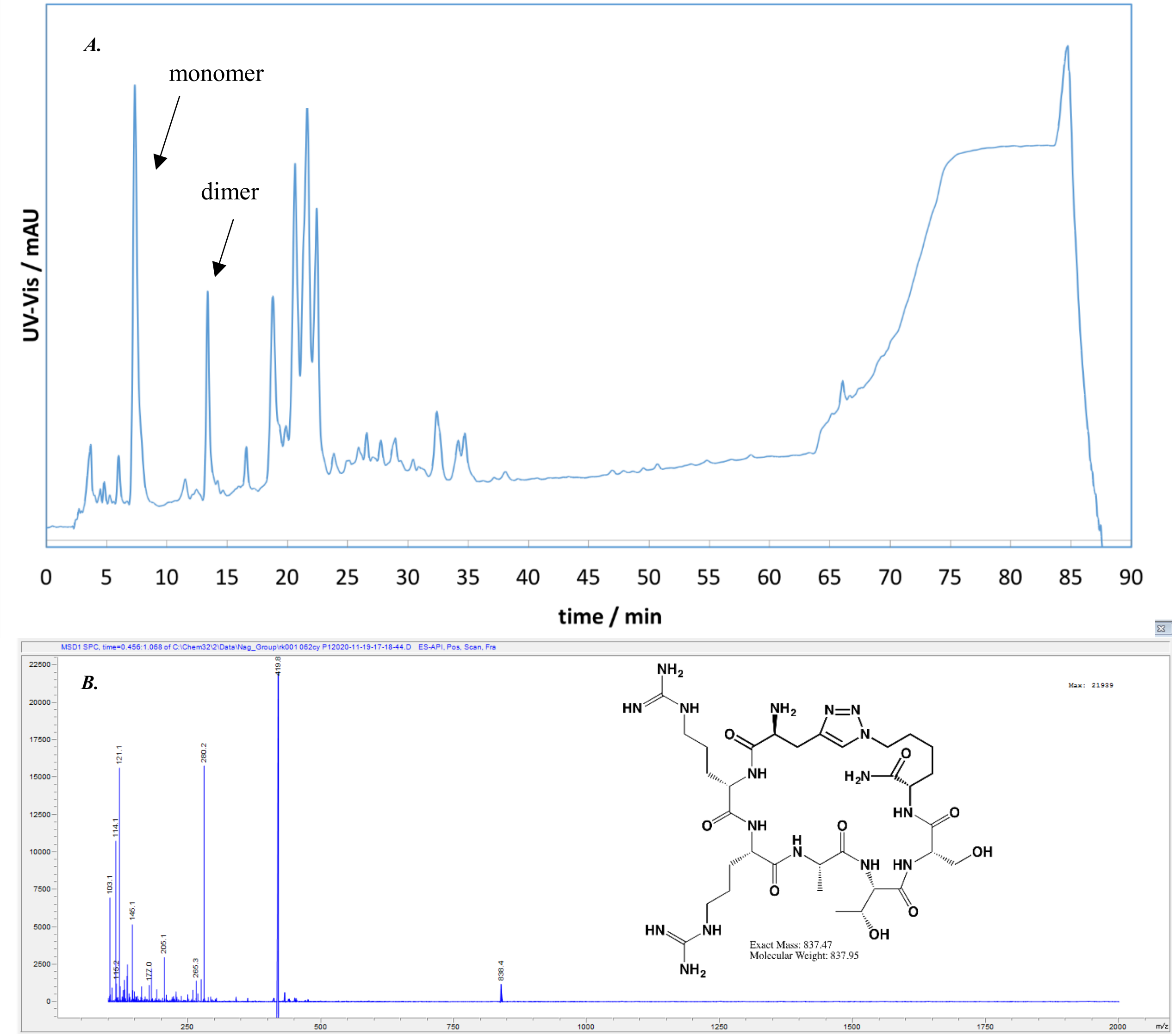
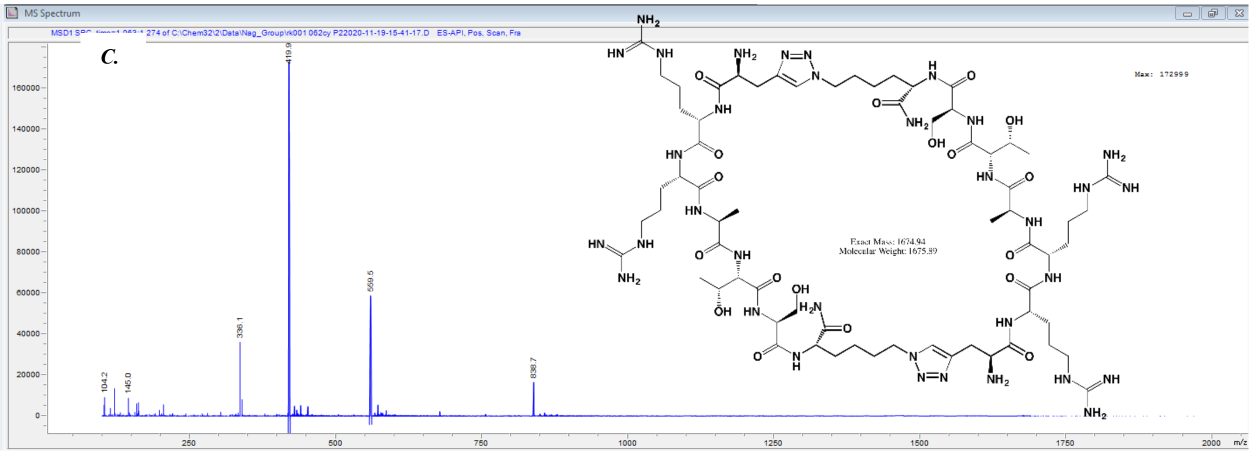
Exact Mass: 1670.88
Molecular Weight: 1671.85

Figure S. 13: HPLC and mass spectrometry analysis of cyclic peptide ***8***

A. RP-HPLC of peptide **8** with sequence (RRATS-Tz4)$_{cy}$; B. LC-MS of HPLC fraction at 7.3 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{3+}$ expected: 280.2 found: 280.2, m/z calculated for $[M+2H]^{2+}$ expected: 419.7 found: 419.8, m/z calculated for $[M+H]^{+}$ expected: 838.5 found: 838.4; C. LC- MS of HPLC fraction at 13.4 minutes corresponds to dimer mass: m/z calculated for $[M+5H]^{5+}$ expected: 336.0 found: 336.0, m/z calculated for $[M+4H]^{4+}$ expected: 419.7 found: 419.9 $[M+4H]^{4+}$, m/z calculated for $[M+3H]^{3+}$ expected: 559.3 found: 559.5, m/z calculated for $[M+2H]^{2+}$ expected: 838.5 found: 838.4

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1500
1750
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m/z
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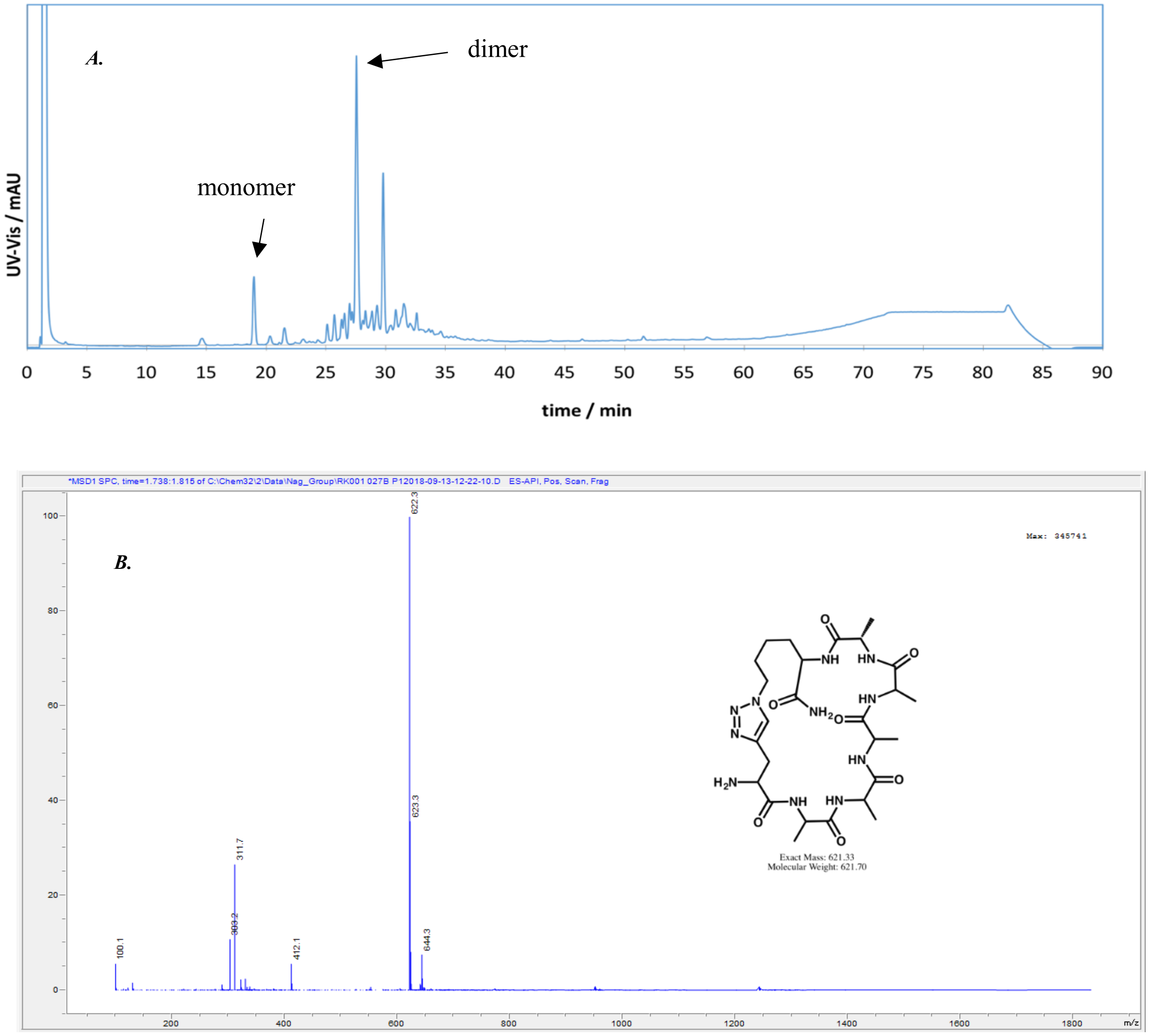
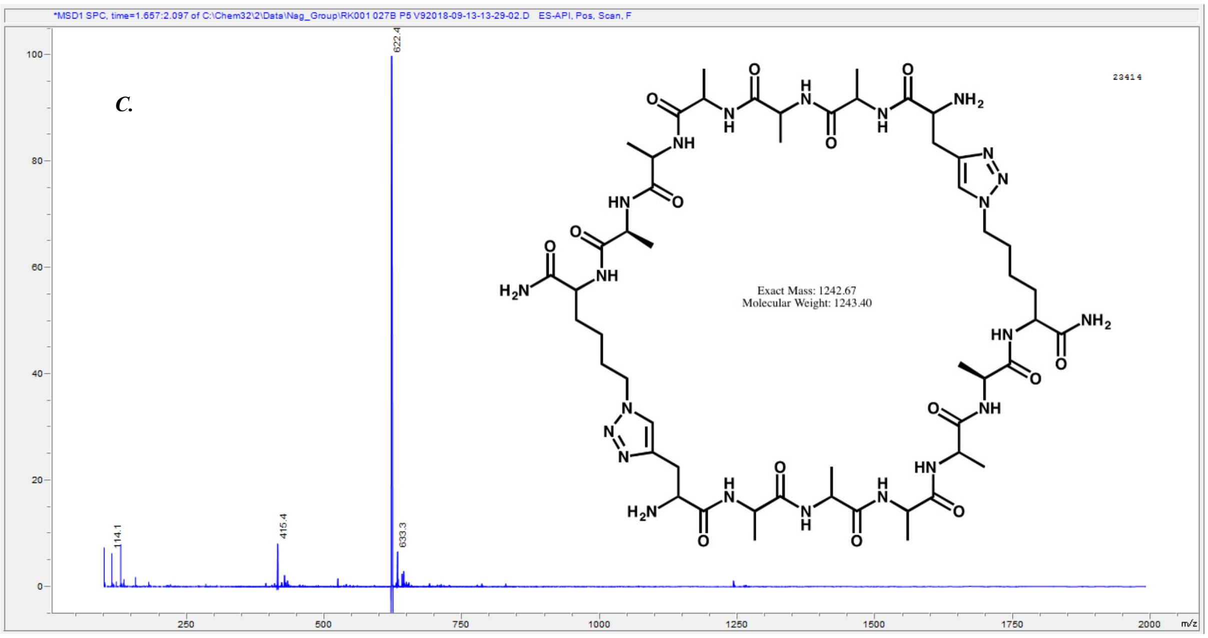
Molecular Weight: 1675.89

Figure S. 14: HPLC and mass spectrometry analysis of polyalanine containing peptide

A. HPLC of Polyalanine containing cyclic peptide; B. LC-MS fraction from HPLC fraction at 18.2 minutes corresponding to monomer (AAAAA-Tz$_4$)$_{cy}$: m/z calculated for $[M+2H]^{2+}$ expected: 311.7 found: 311.7, m/z calculated for $[M+H]^{+}$ expected: 622.3 found: 622.3; C. LC-MS fraction from HPLC fraction at 27.5 minutes corresponds to dimer mass cy[AAAAA-Tz$_4$]$_2$: m/z calculated for $[M+3H]^{3+}$ expected: 415.2 found: 415.4, m/z calculated for $[M+2H]^{2+}$ expected: 622.3 found: 622.4

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60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z

*Figure S. 15: HPLC and mass spectrometry analysis of cyclic peptide **9***

A. RP-HPLC chromatogram of peptide **9** with sequence (AYYTYT-Tz$_4$)$_{cy}$; B. LC-MS of fraction at 29.6 minutes corresponds to monomer mass m/z calculated for $[M+5H]^{5+}$ expected: 205.9 found: 204.9, m/z calculated for $[M+2H+K]^{3+}$ expected: 356.5 found: 356.5, m/z calculated for $[M+2H]^{2+}$ expected: 515.2 found: 515.3, m/z calculated for $[M+H]^{+}$ expected: 1029.5 found: 1029.4; C. LC-MS of fraction at 33.7 minutes corresponds to dimer mass cy[AYYTYT-Tz$_4$]$_2$: m/z calculated for $[M+4H]^{4+}$ expected: 515.2 found: 515.4, m/z calculated for $[M+3H]^{3+}$ expected: 686.6 found: 686.6, m/z calculated for $[M+2H]^{2+}$ expected: 1029.5 found: 1029.6

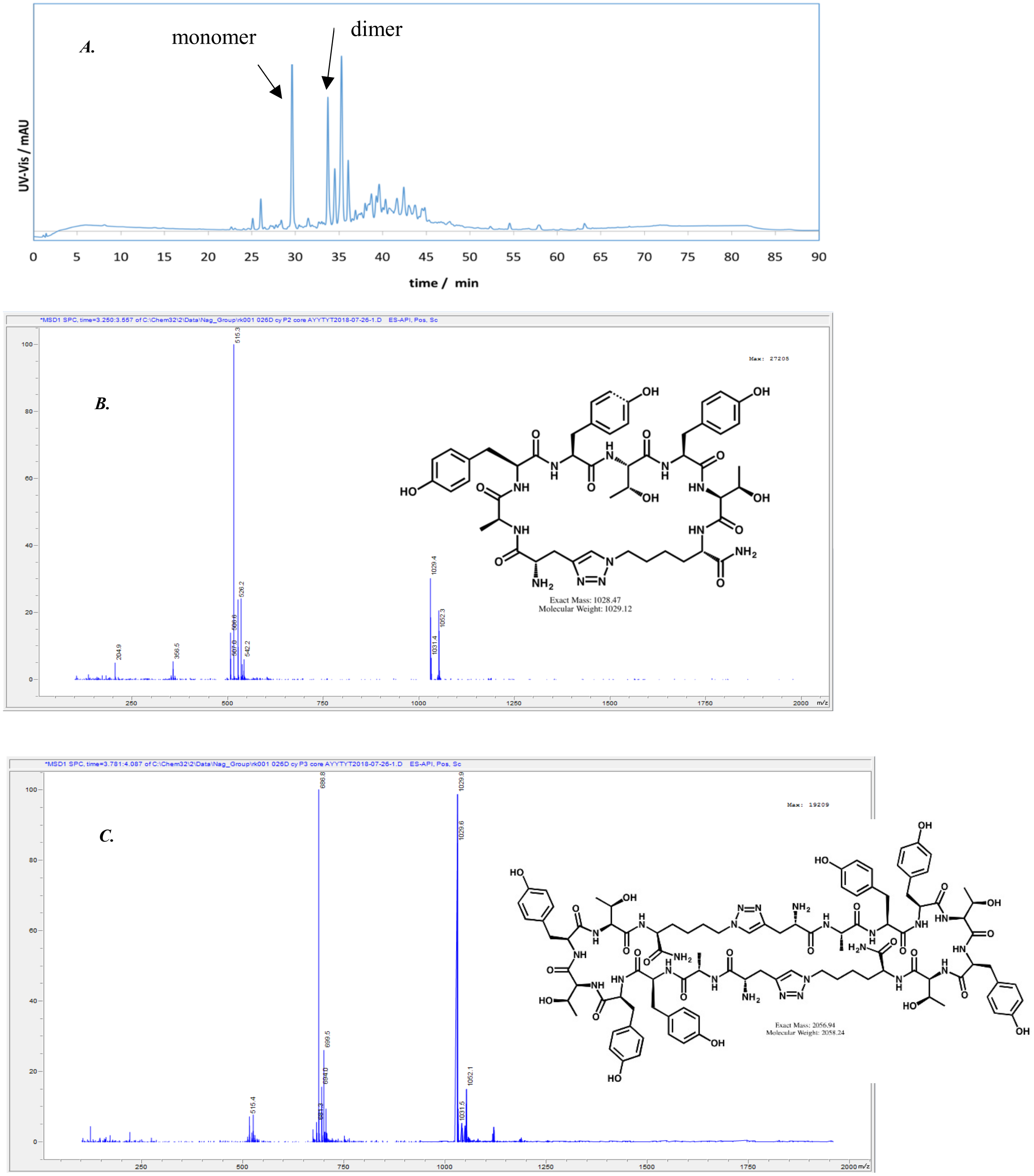
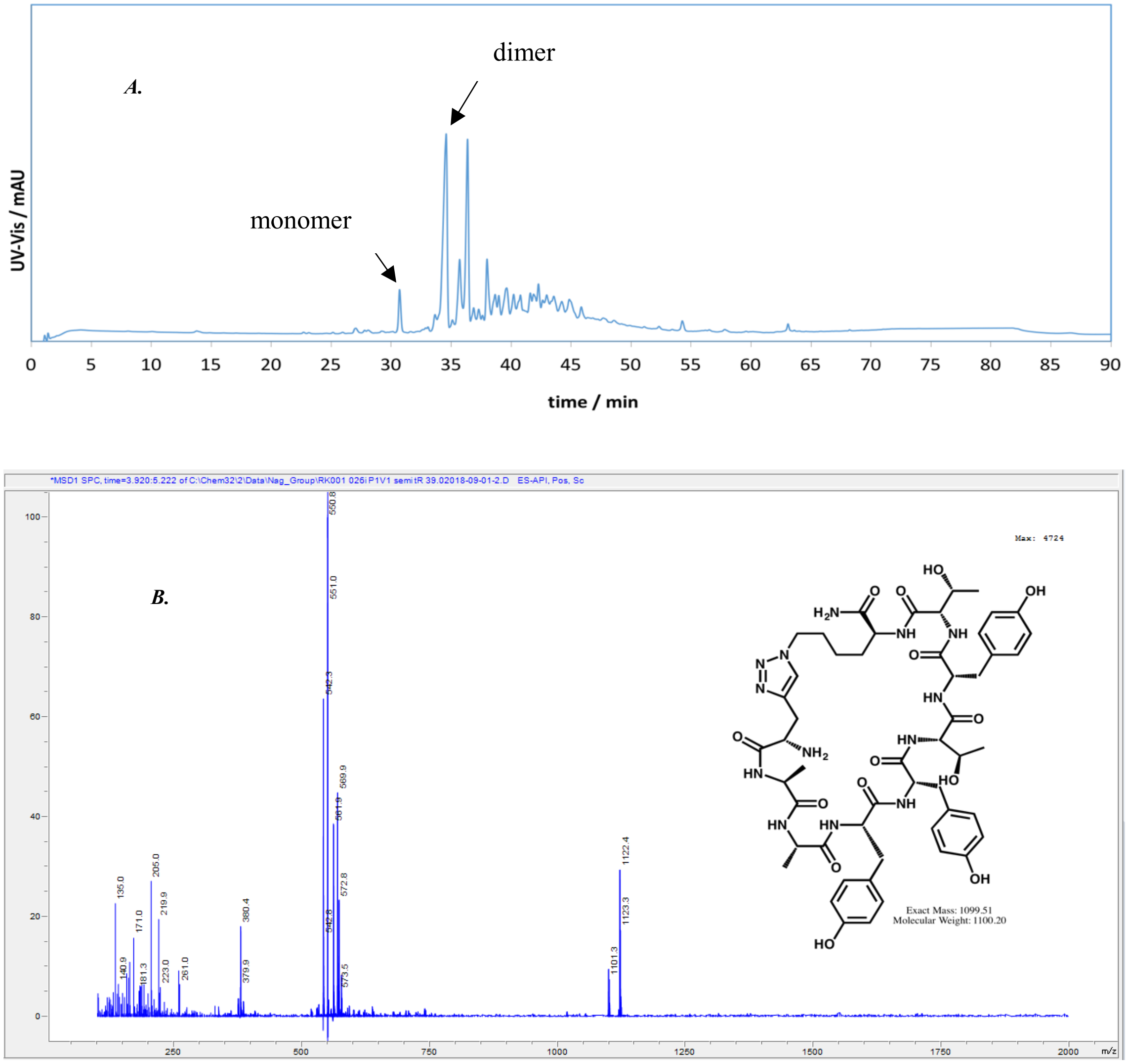
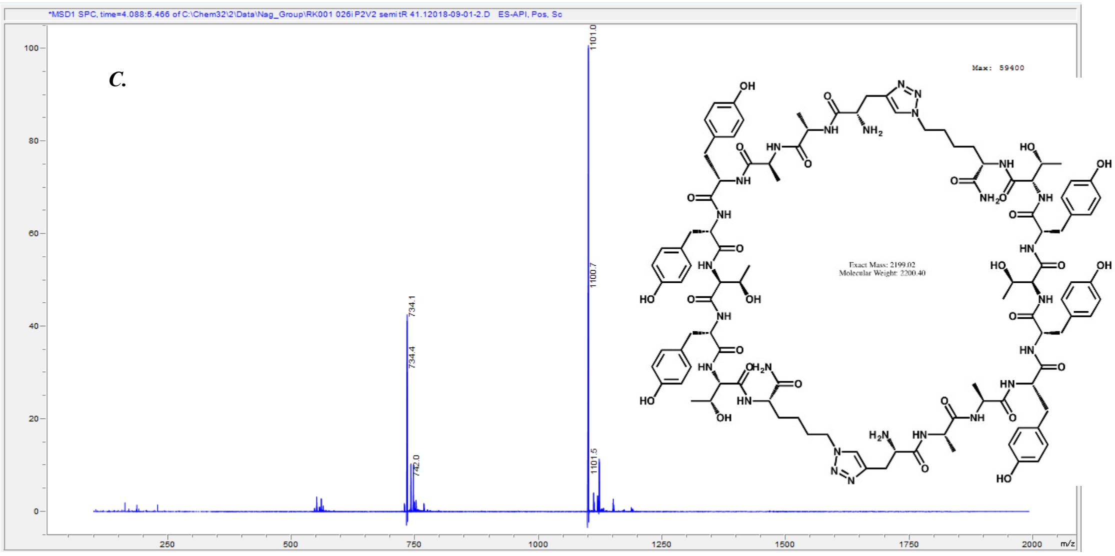


*Figure S. 16: HPLC and mass spectrometry analysis of cyclic peptide **10***

A. Analytical RP-HPLC chromatogram of cyclic peptide 10 with sequence (AAYYTYT-Tz4)cy; B. LC-MS of fraction at 30.7 minutes gives mass corresponding to cyclic monomer: m/z calculated for $[M+2H]^{2+}$ expected: 550.8 found: 550.8, m/z calculated for $[M+H]^{+}$ expected: 1001.5 found: 1101.3, m/z calculated for $[M+Na]^{+}$ expected: 1122.5 found: 1122.4; C. LC-ESI-MS of fraction at 34.5 minutes gives mass corresponding to cyclic dimer cy[AAYYTYT-Tz4]2: m/z calculated for $[M+3H]^{3+}$ expected: 734.0 found: 734.1, m/z calculated for $[M+2H]^{2+}$ expected: 1101.0 found: 1101.0

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734.4
742.0
Exact Mass: 2199.02
Molecular Weight: 2200.40
100
80
60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z

*Figure S. 17: HPLC and mass spectrometry analysis of cyclic peptide **11***

A. Analytical RP-HPLC chromatogram of peptide **11** with sequence (AAAYYTYT-Tz4)$_{cy}$; B. LC-MS of fraction at 38.7 minutes corresponds to mass of dimer cy[AAAYYTYT-Tz4]$_2$: m/z calculated for $[M+3H]^{3+}$ expected: 781.4 found: 781.5, m/z calculated for $[M+2H]^{2+}$ expected:1171.6 found: 1171.9; the small peak at 36.1 minutes was identified as the monomer (AAAYYTYT-Tz4)$_{cy}$. The fraction at 35 minutes is a compound with a lower molecular weight.

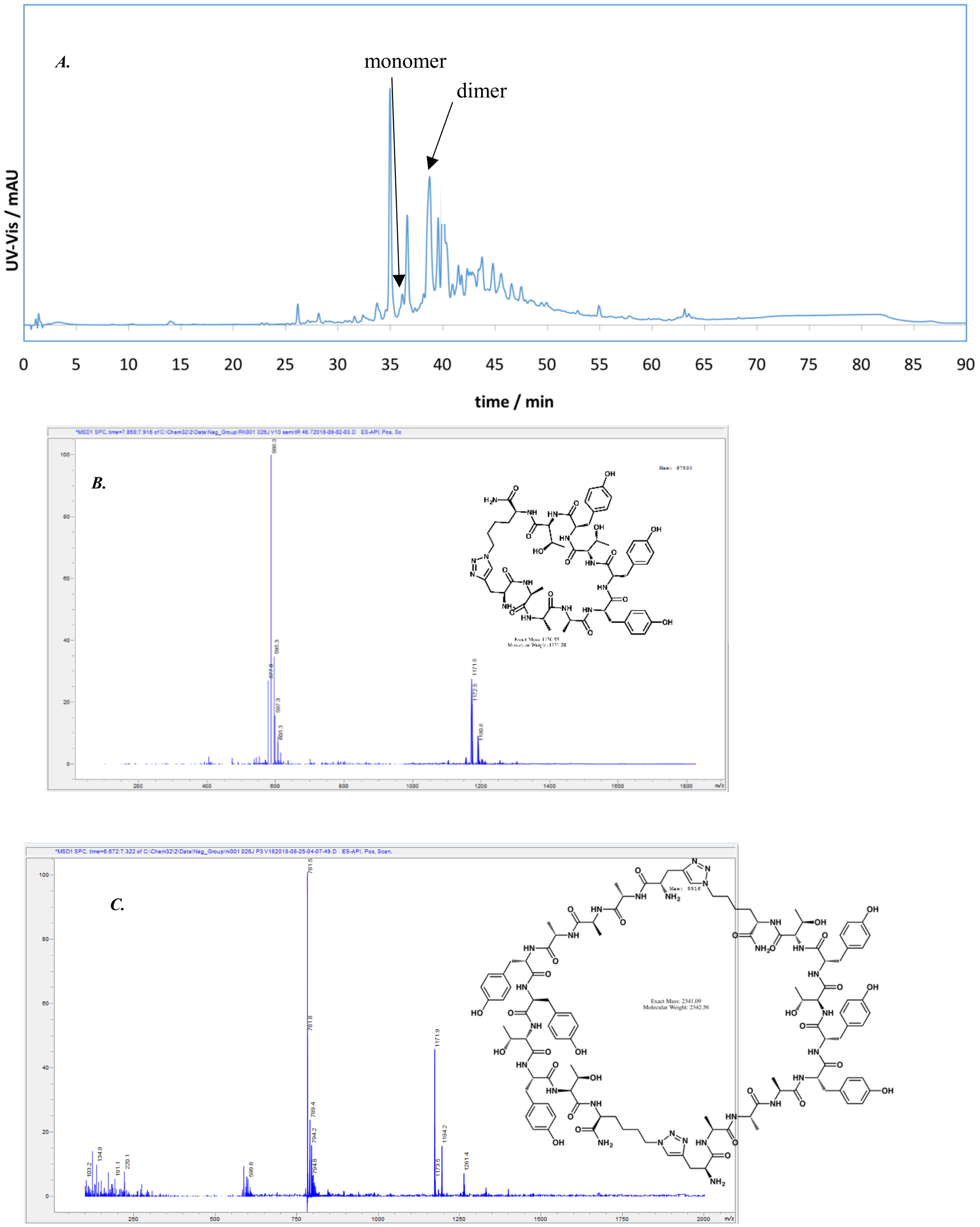
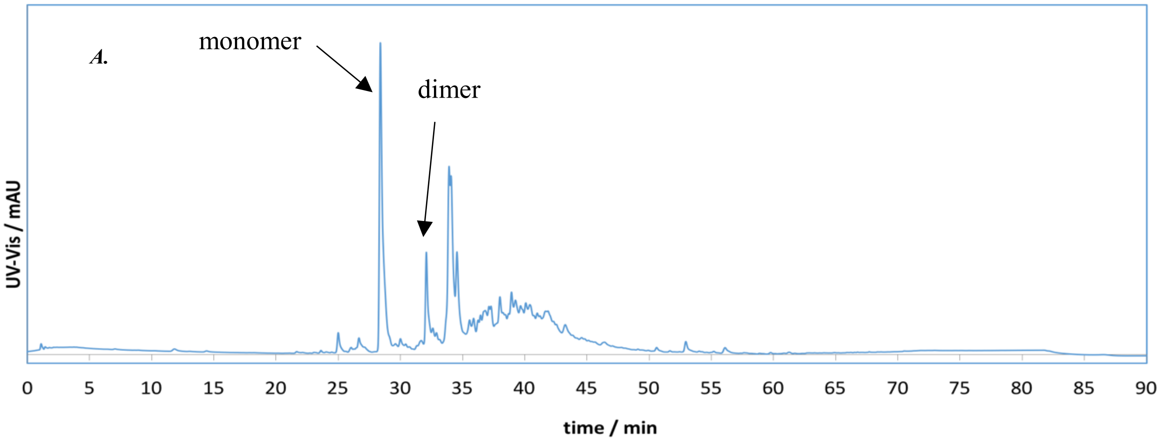
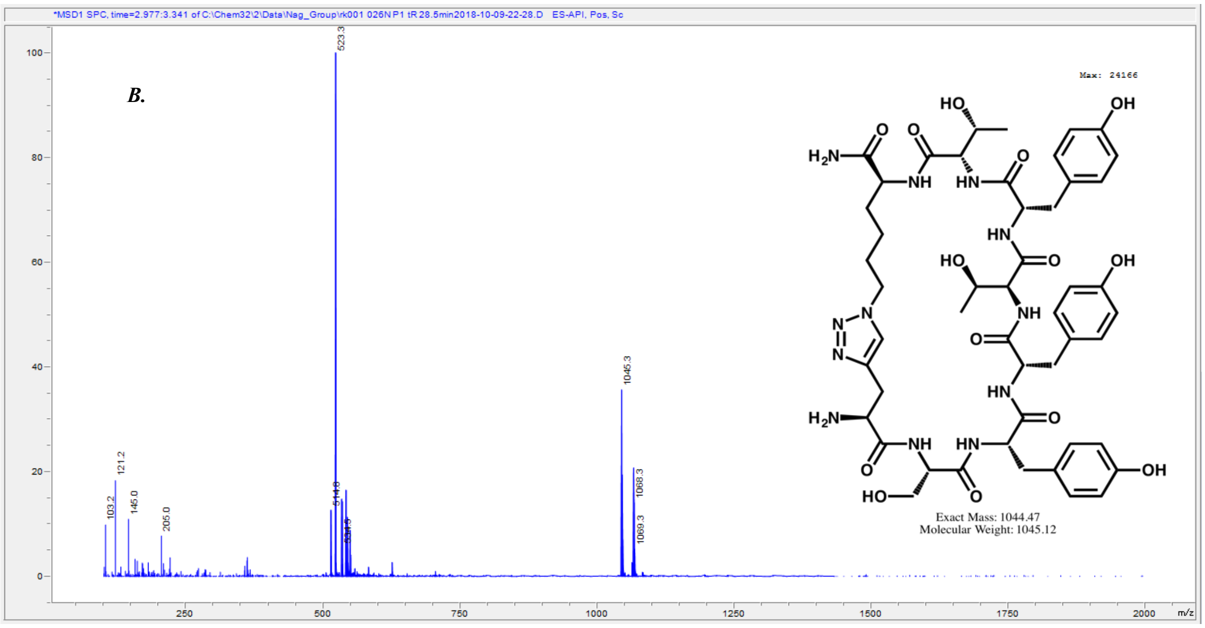


Figure S. 18: HPLC and mass spectrometry analysis of cyclic peptide ***12***

A. Analytical RP-HPLC chromatogram of cyclic peptide **12** with sequence (SYYTYT-Tz4)$_{cy;}$ B. LC-MS of fraction at 28.4 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 523.2 found: 523.3, m/z calculated for $[M+H)]^{+}$ expected: 1045.5 found: 1045.3; C. LC-MS of fraction at 32.3 minutes corresponds to dimer mass: m/z calculated for $[M+3H]^{3+}$ expected: 697.3 found: 697.6, m/z calculated for $[M+2H)]^{2+}$ expected: 1045.5 found: 1045.9





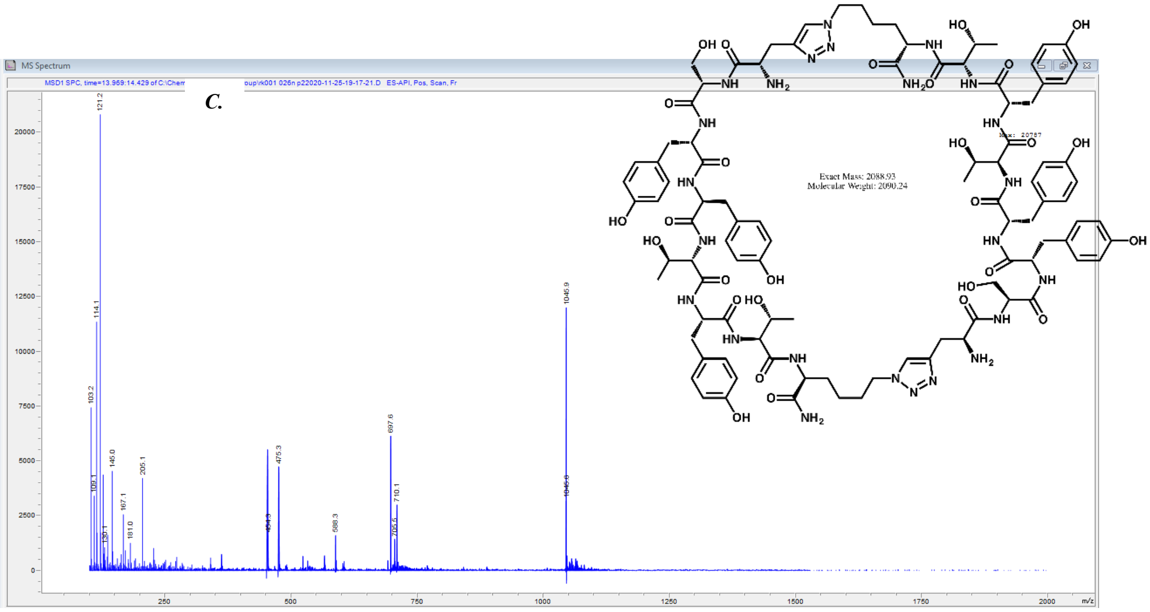
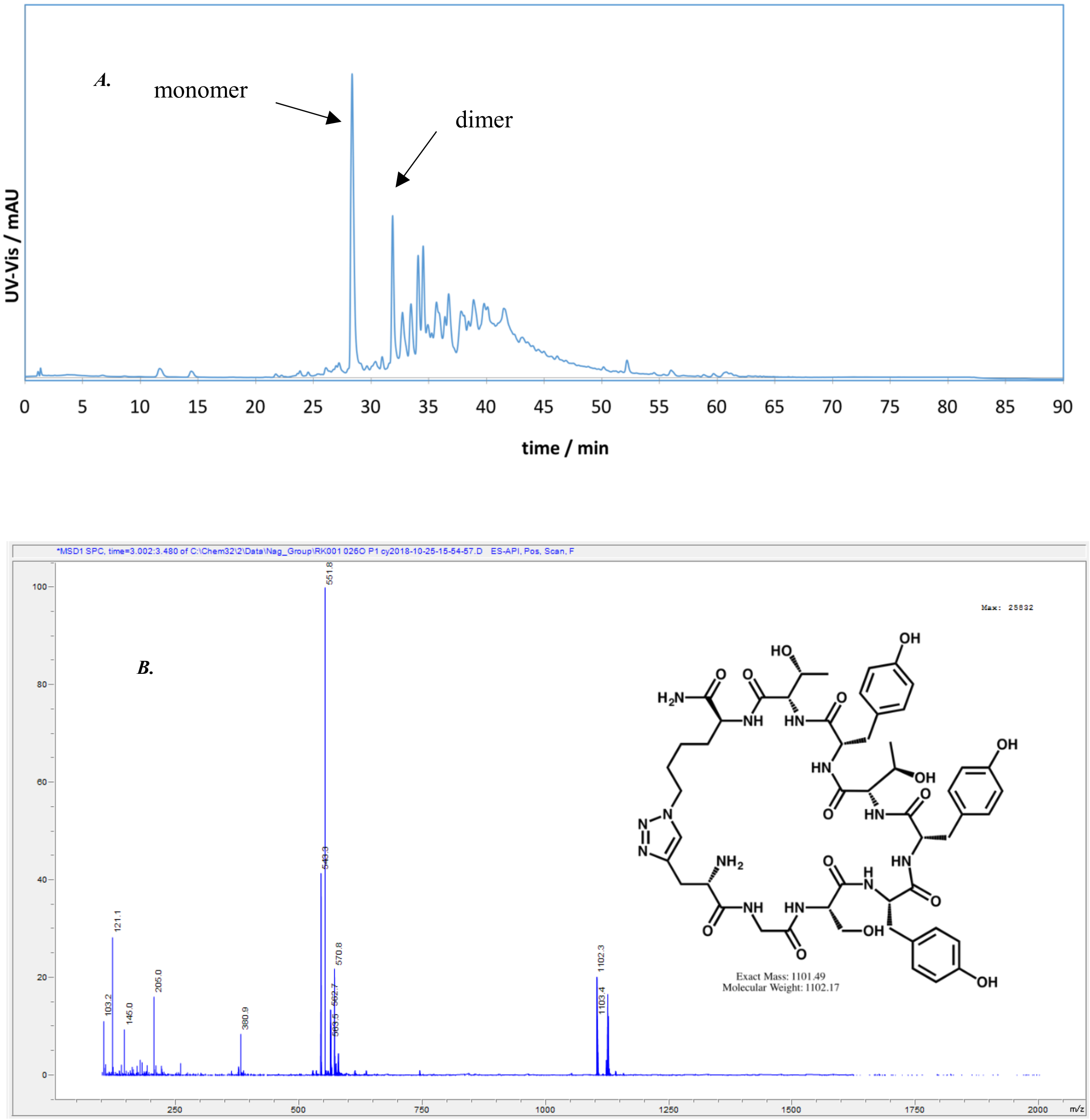
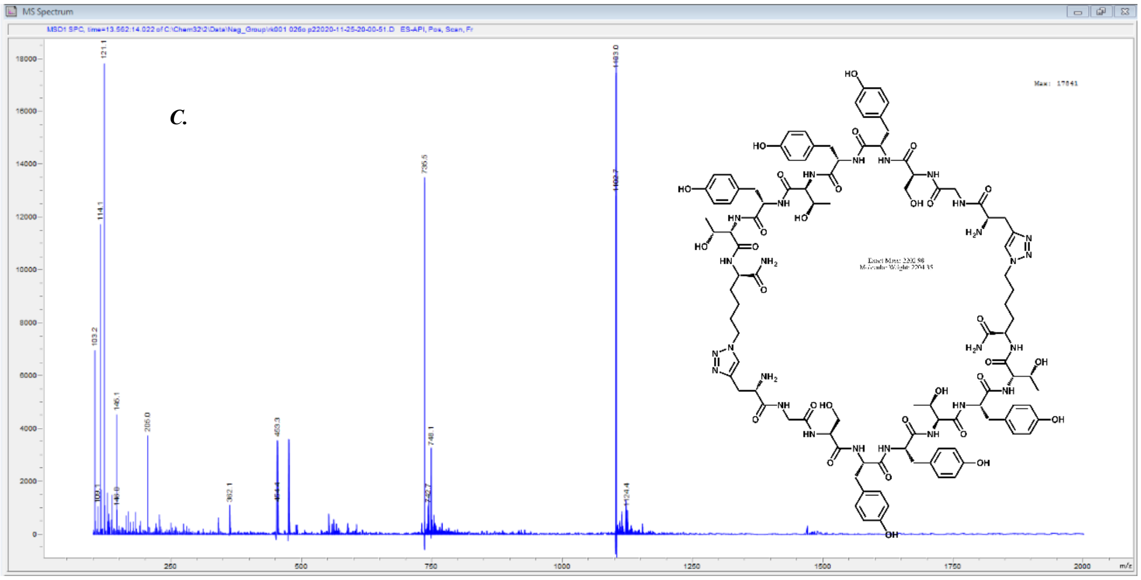

C.
MS Spectrum
MSD1 SPC, time=13.969:14.429 of C:\Chem oup\rk001 026n p22020-11-25-19-17-21.D ES-API, Pos, Scan, Fr
121.2
114.1
103.2
109.1
130.1
145.0
167.1
181.0
205.1
454.3
475.3
588.3
697.6
705.5
710.1
1045.9
1045.0
20000
17500
15000
12500
10000
7500
5000
2500
0
250
500
750
1000
1250
1500
1750
2000
m/z
Exact Mass: 2088.93
Molecular Weight: 2090.24
OH
NH
NH2
NH2O
HO
HN
O
N
HO
OH
NH2

*Figure S. 19: HPLC and mass spectrometry analysis of cyclic peptide **13***

A. Analytical RP-HPLC chromatogram of peptide **13** with sequence (GSYYTYT-Tz$_4$)$_{cy}$; B. LC-MS of HPLC fraction at 28.3 minutes corresponds to mass of monomer: m/z calculated for $[M+2H]^{2+}$ expected: 551.8 found: 551.8, m/z calculated for $[M+H]^{+}$ expected:1102.5 found: 1102.3; B. LC-MS of HPLC fraction at 31.8 minutes corresponds to mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 735.3 found: 735.5, m/z calculated for $[M+2H]^{2+}$ expected:1103.0 found: 1103.0

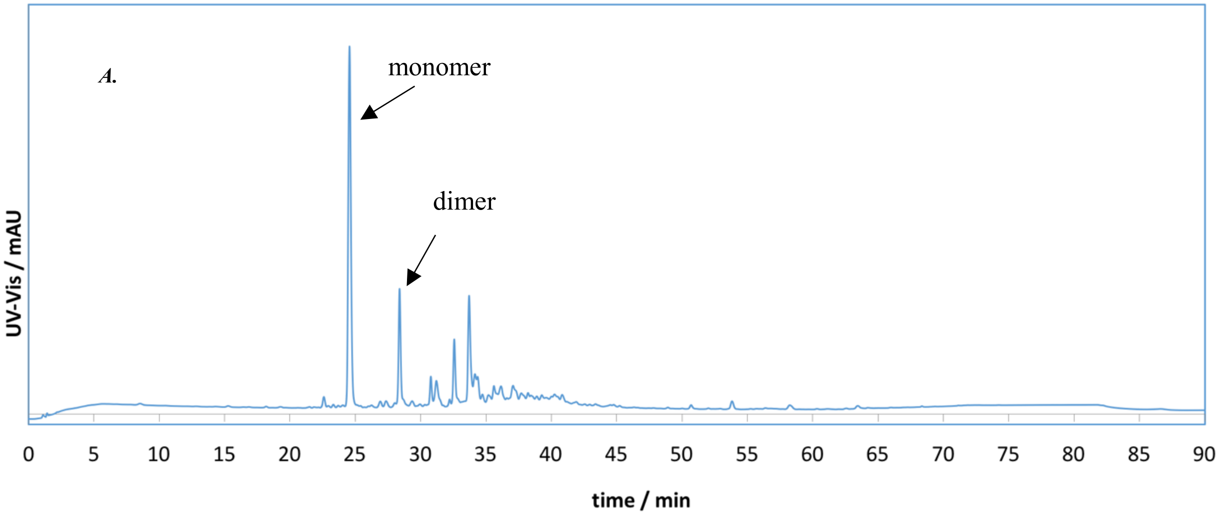


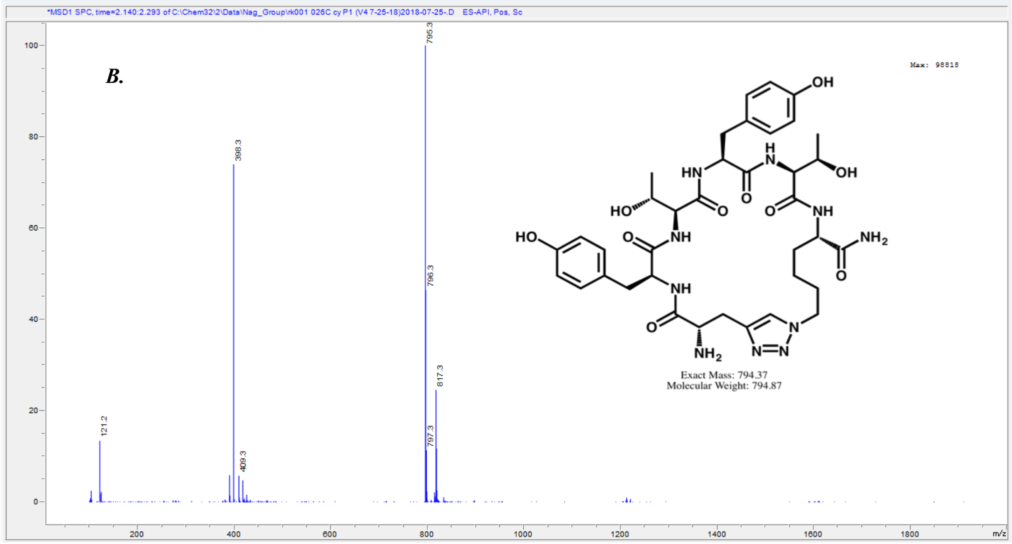


MSD1 SPC, time=13.562:14.022 of C:\Chem32\2\Data\Nag_Group\rk001 026o p22020-11-25-20-00-51.D   ES-API, Pos, Scan, Fr

*Figure S. 20: HPLC and mass spectrometry analysis of cyclic peptide **14***

A. Analytical RP-HPLC chromatogram of peptide **14** with sequence (YTYT-Tz4)$_{cy}$. B. LC-MS of HPLC fraction at 24.5 minutes corresponds to mass of monomer: m/z calculated for $[M+2H]^{2+}$ expected: 398.2 found: 398.3, m/z calculated for $[M+H]^{+}$ expected: 795.4 found: 795.3; C. LC-MS of HPLC fraction at 28.4 minutes corresponds to mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 530.6 found: 530.8, m/z calculated for $[M+2H]^{2+}$ expected: 795.4 found: 795.5





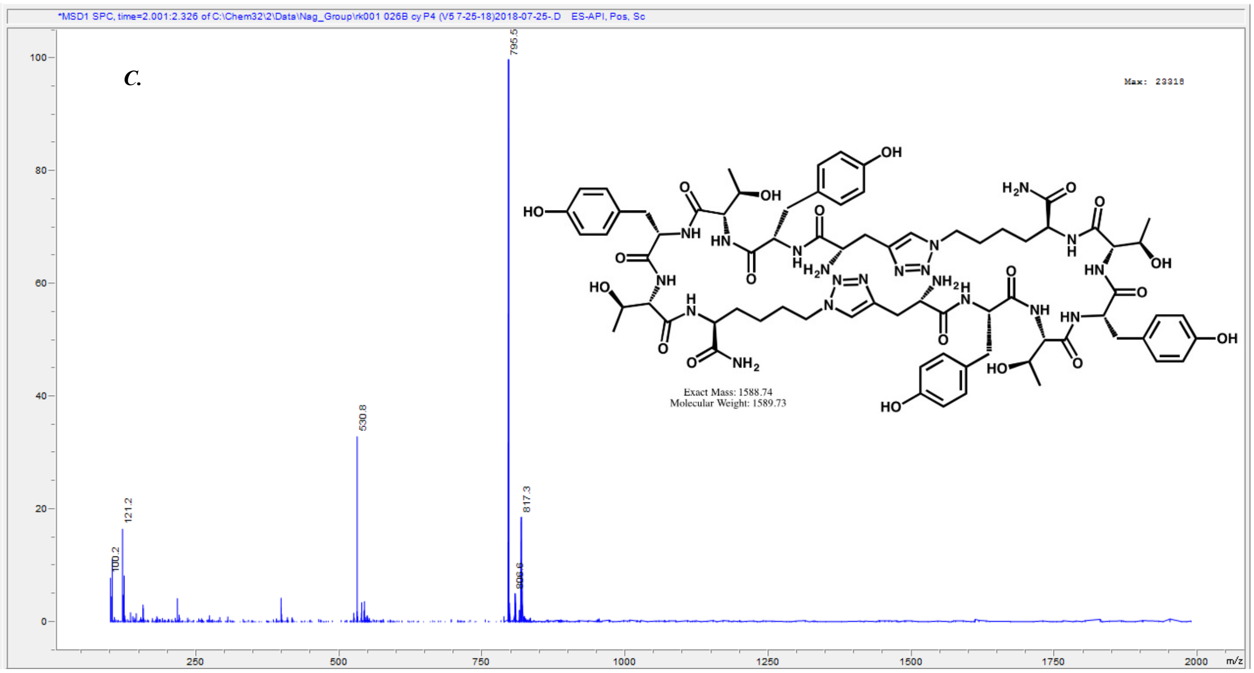
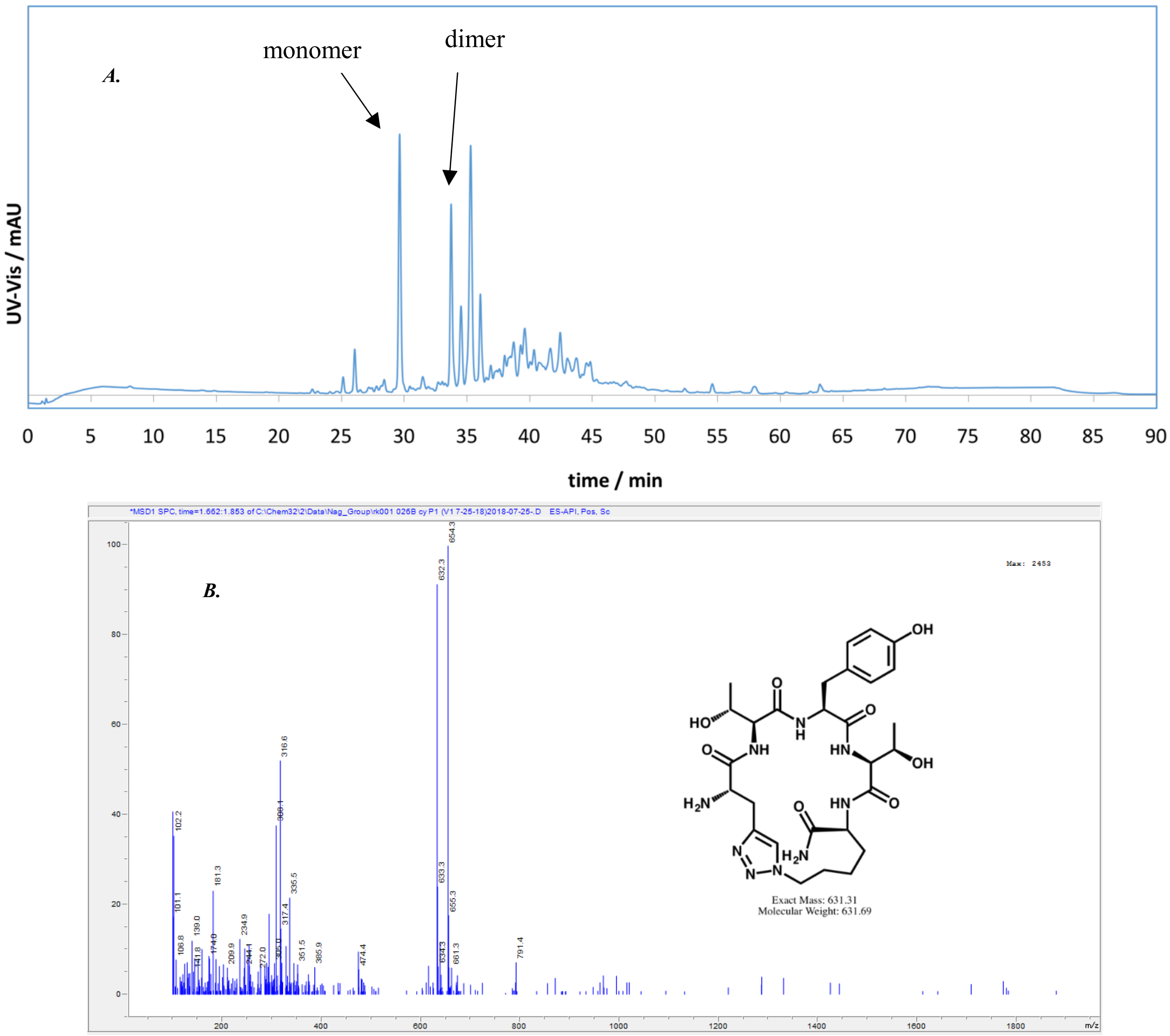

*MSD1 SPC, time=2.001:2.326 of C:\Chem32\2\Data\Nag_Group\rk001 026B cy P4 (V5 7-25-18)2018-07-25-.D ES-API, Pos, Sc
C.
Max: 23318
795.5
530.8
817.3
806.6
121.2
100.2
100
80
60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z
Exact Mass: 1588.74
Molecular Weight: 1589.73

Figure S. 21: HPLC and mass spectrometry analysis of cyclic peptide ***15***

A. RP-HPLC of cyclic peptide **15** with sequence (TYT-Tz$_4$)$_{cy}$; B. LC-MS of HPLC fraction at 29.6 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 316.7 found: 316.6, m/z calculated for $[M+H]^{+}$expected: 632.3 found: 632.3, m/z calculated for $[M+Na]^{+}$ expected: 654.3 found: 654.3; C. LC-MS of HPLC fraction at 33.7 minutes corresponds to dimer mass: m/z calculated for $[M+3H]^{3+}$ expected: 421.9 found: 421.9, m/z calculated for $[M+2H]^{2+}$ expected: 632.3 found: 632.3, m/z calculated for $[M+2Na]^{2+}$ expected: 654.3 found: 654.3



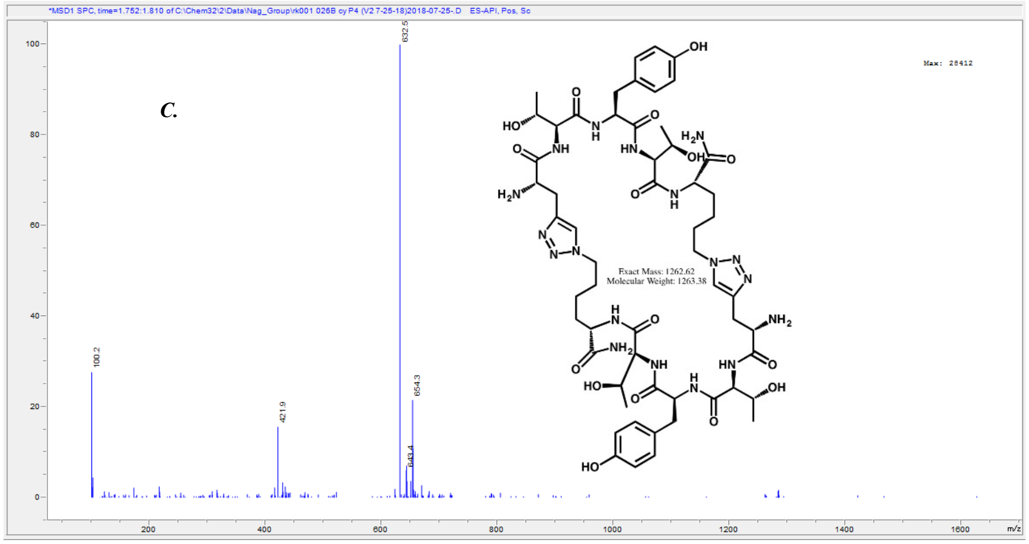
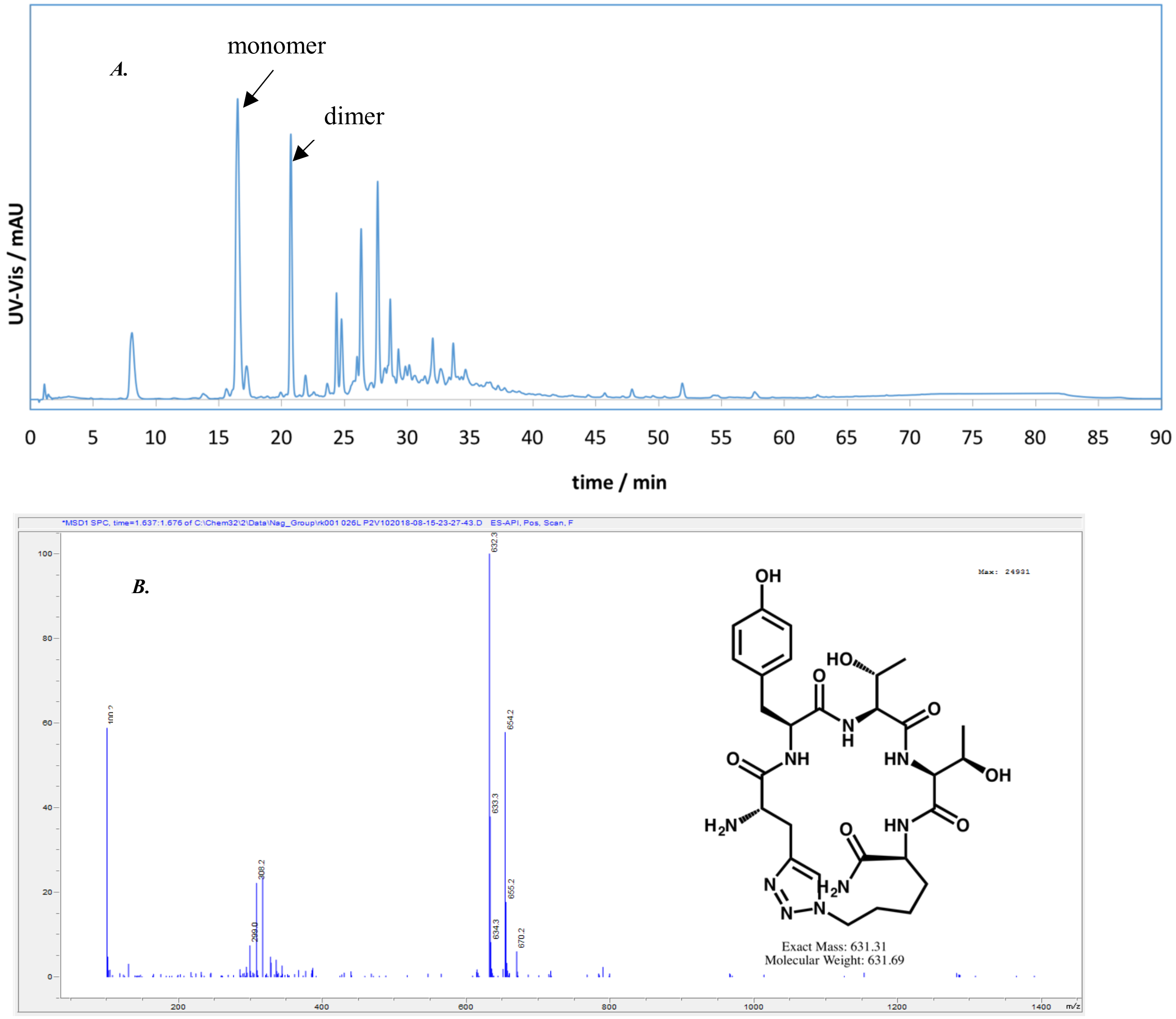
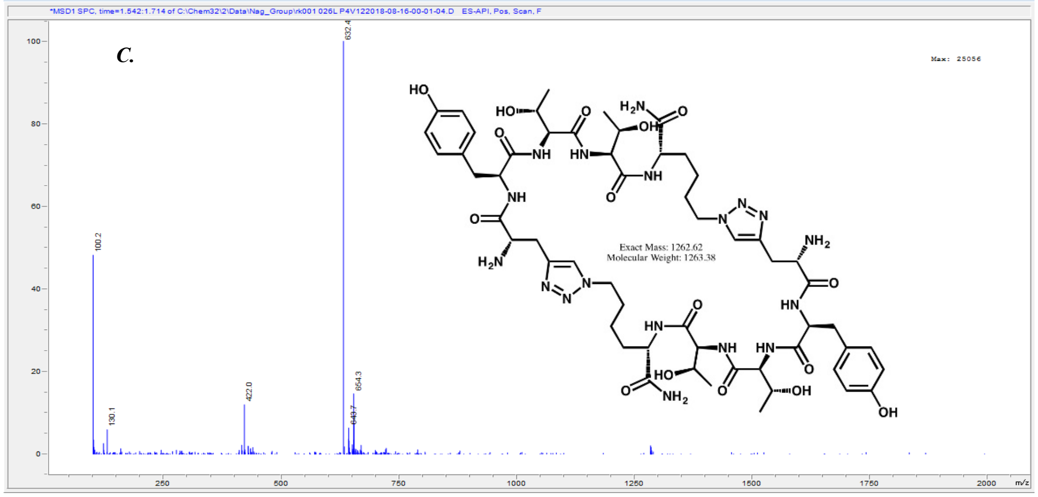

*MSD1 SPC, time=1.752:1.810 of C:\Chem32\2\Data\Nag_Group\rk001 026B cy P4 (V2 7-25-18)2018-07-25-.D ES-API, Pos, Sc
C.
Max: 28412
100
80
60
40
20
0
100.2
421.9
632.5
643.4
654.3
200
400
600
800
1000
1200
1400
1600
m/z
OH
HO
O
N
H
NH
H$_2$N
HN
OH
O
O
N
H
N
N
N
N
N
N
Exact Mass: 1262.62
Molecular Weight: 1263.38
NH$_2$
HN
O
NH$_2$
HO
O
NH
H
N
HN
OH
O
HO

*Figure S. 22: HPLC and mass spectrometry analysis of cyclic peptide **16***

A. RP-HPLC of cyclic peptide **16** with sequence $(YTT\text{-}Tz_4)_{cy}$; B. LC-MS of HPLC fraction at 16.5 minutes corresponds to monomer mass: m/z calculated for $[M+2H-H_2O]^{2+}$ expected: 308.2 found: 308.2, m/z calculated for $[M+H]^{+}$ expected: 632.3 found: 632.3; C. LC-MS of HPLC fraction at 20.7 minutes corresponds to dimer mass: m/z calculated for $[M+3H]^{3+}$ expected: 421.9 found: 422.0, m/z calculated for $[M+2H]^{2+}$ expected: 632.3 found: 632.4

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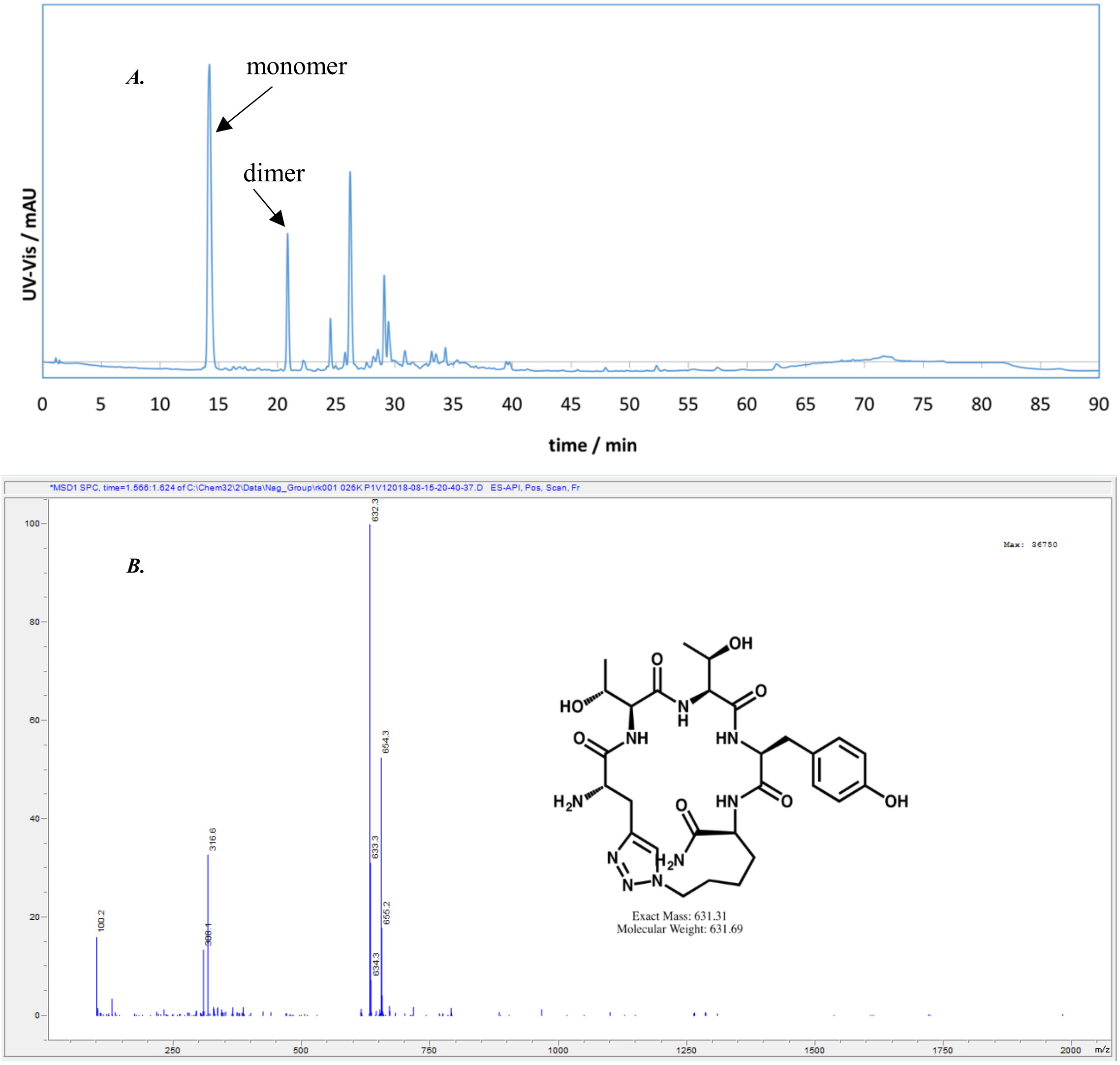
C.
Max: 25056
632.4
100.2
130.1
422.0
643.7
654.3
100
80
60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z
HO
HO
OH
H2N
O
NH
HN
N
NH2
Exact Mass: 1262.62
Molecular Weight: 1263.38
OH

*Figure S. 23: HPLC and mass spectrometry analysis of cyclic peptide **17***

A. RP-HPLC of cyclic peptide **17** with sequence (TTY-Tz$_4$)$_{cy}$; B. LC-MS of HPLC fraction at 14.1 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 316.6 found: 316.6, m/z calculated for $[M+H]^{+}$ expected: 632.3 found: 632.3, m/z calculated for $[M+Na]^{+}$ expected: 654.3 found: 654.3; C. LC-MS of HPLC fraction at 20.8 minutes corresponds to dimer mass: m/z calculated for $[M+3H]^{3+}$ expected: 421.9 found: 421.9, m/z calculated for $[M+2H]^{2+}$ expected: 632.3 found: 632.4, m/z calculated for $[M+2Na]^{2+}$ expected: 654.3 found: 654.3



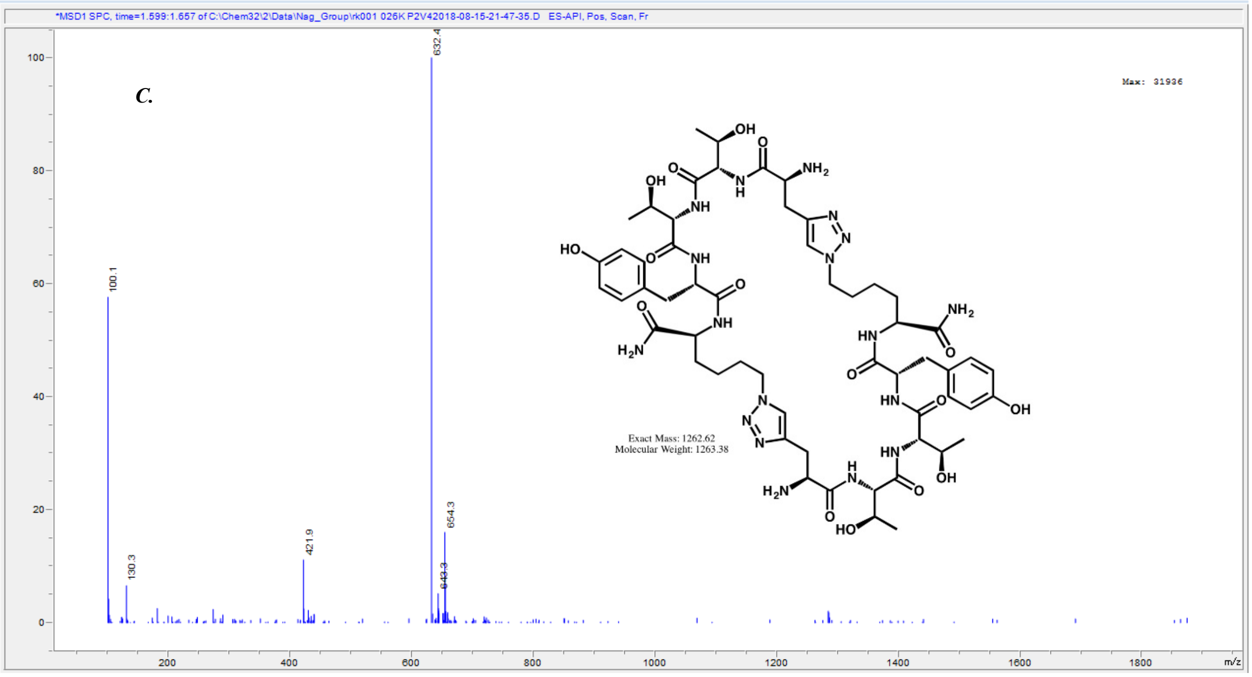
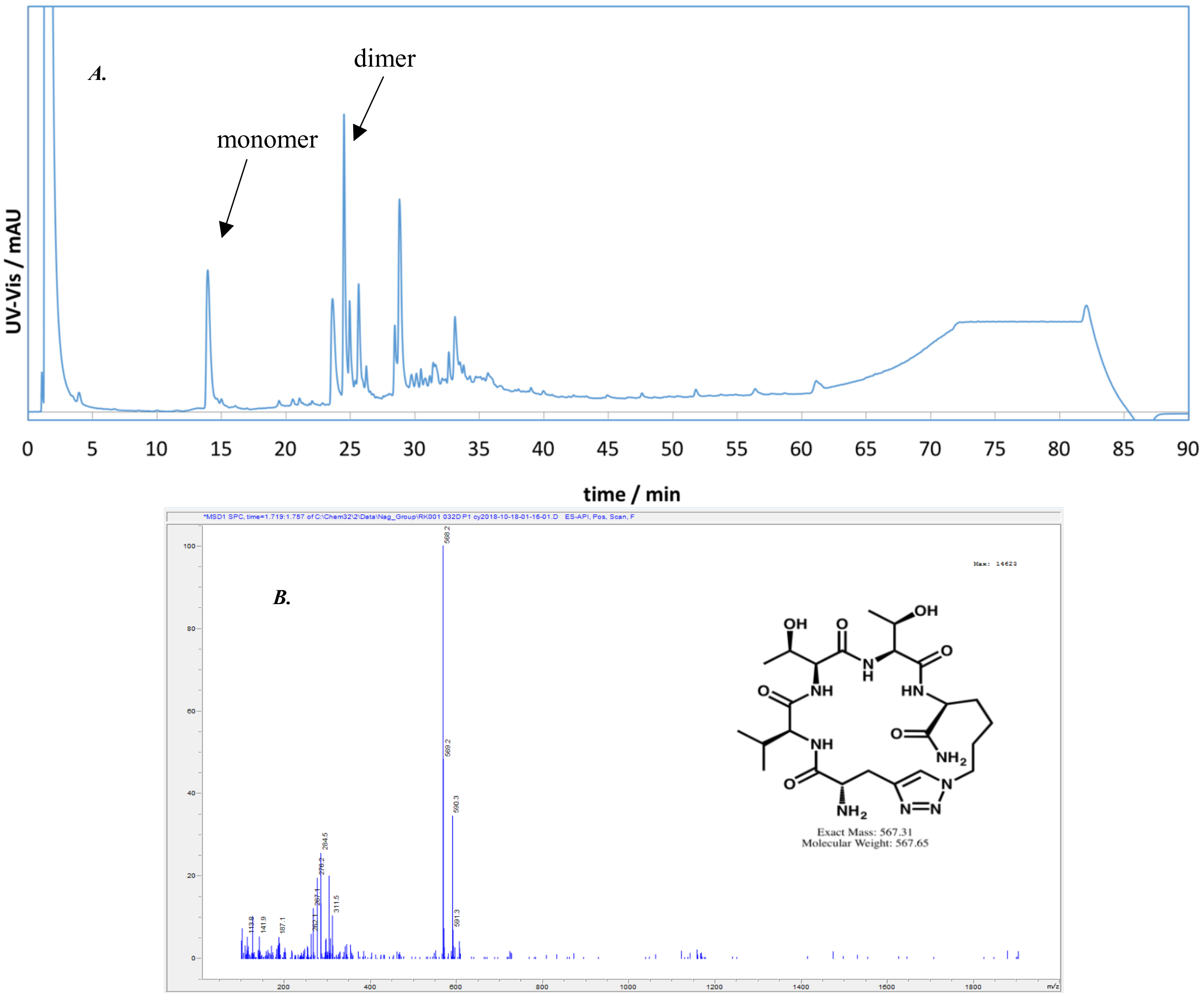

*MSD1 SPC, time=1.599:1.657 of C:\Chem32\2\Data\Nag_Group\rk001 026K P2V42018-08-15-21-47-35.D   ES-API, Pos, Scan, Fr
C.
Max: 31936
632.4
100.1
130.3
421.9
643.3
654.3
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
m/z
OH
O
NH2
NH
H
N
N
N
N
HO
NH
O
O
H2N
NH
NH2
HN
O
O
HN
O
OH
HN
OH
H2N
O
HO
Exact Mass: 1262.62
Molecular Weight: 1263.38

*Figure S. 24: HPLC and mass spectrometry analysis of cyclic peptide **18***

A. RP-HPLC of peptide **18** with sequence (VTT-Tz4)$_{cy}$; B. LC-MS of HPLC fraction at 13.9 minutes corresponds to monomer mass: m/z calculated for $[M+2H]^{2+}$ expected: 284.7 found: 284.5, m/z calculated for $[M+H]^{+}$ expected: 568.3 found: 568.2; C. LC-MS of HPLC fraction at 24.5 minutes corresponds to dimer mass: m/z calculated for $[M+3H]^{3+}$ expected: 379.3 found: 379.5, m/z calculated for $[M+2H]^{2+}$ expected: 568.3 found: 568.3



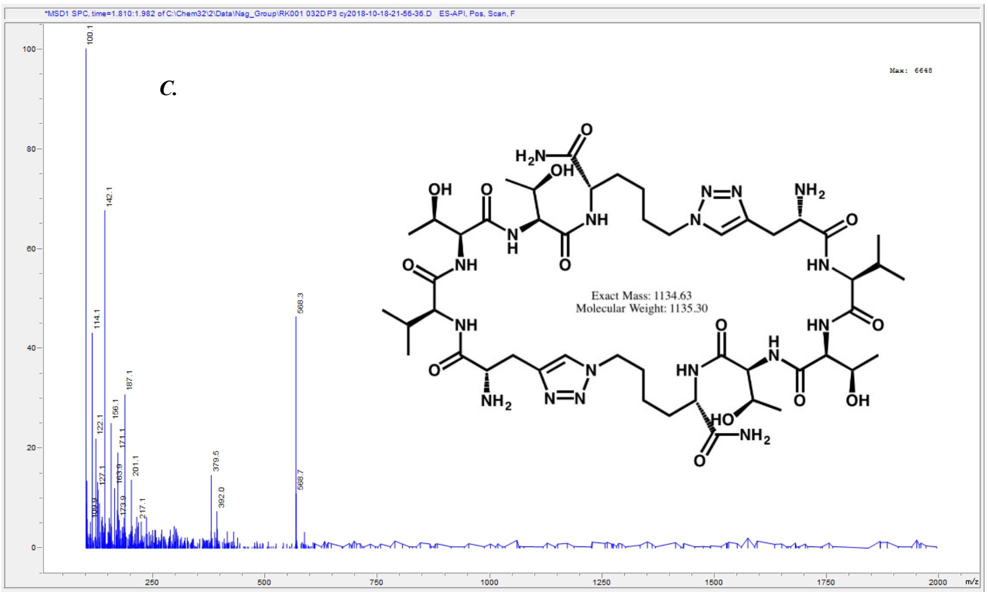
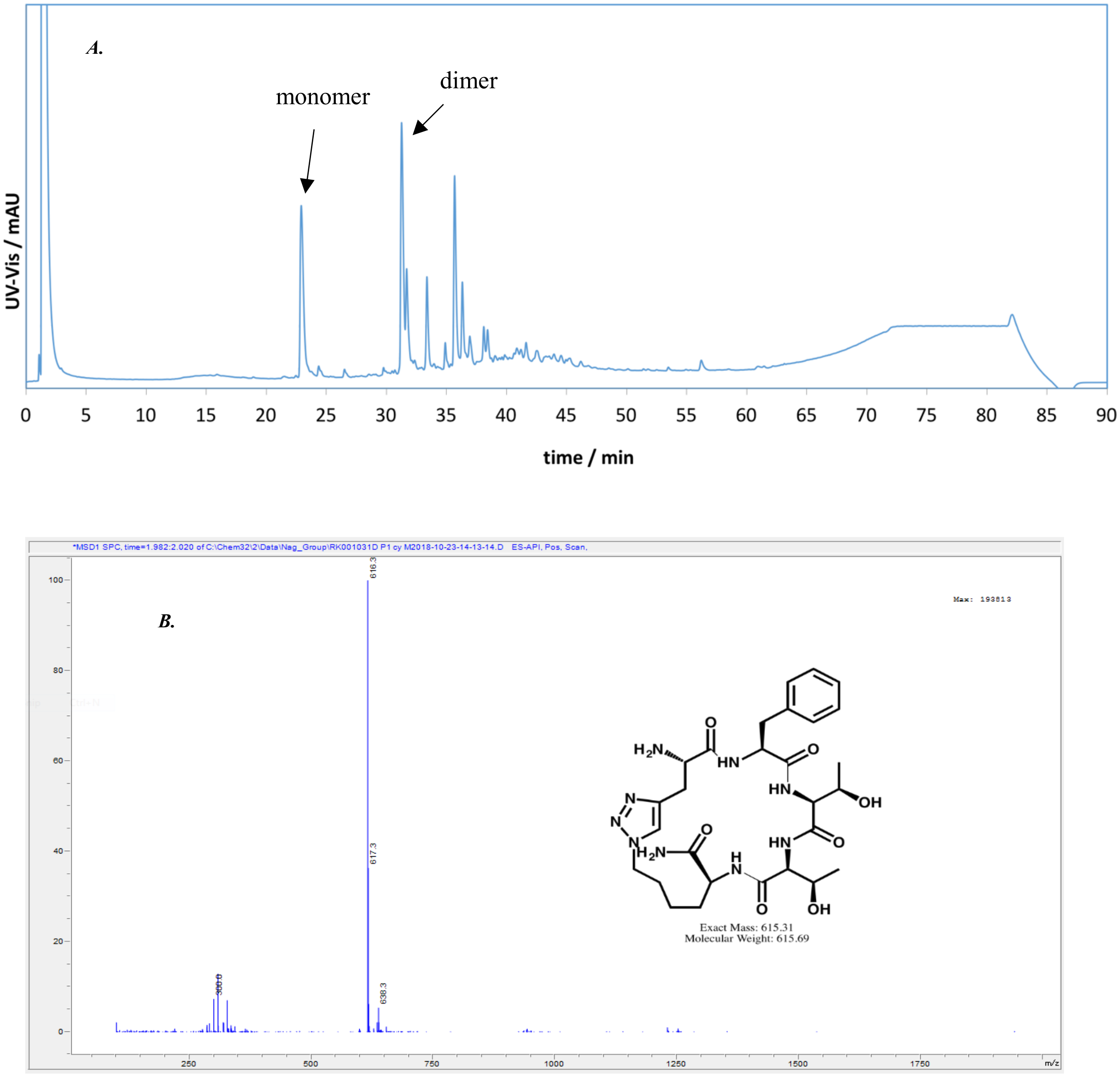
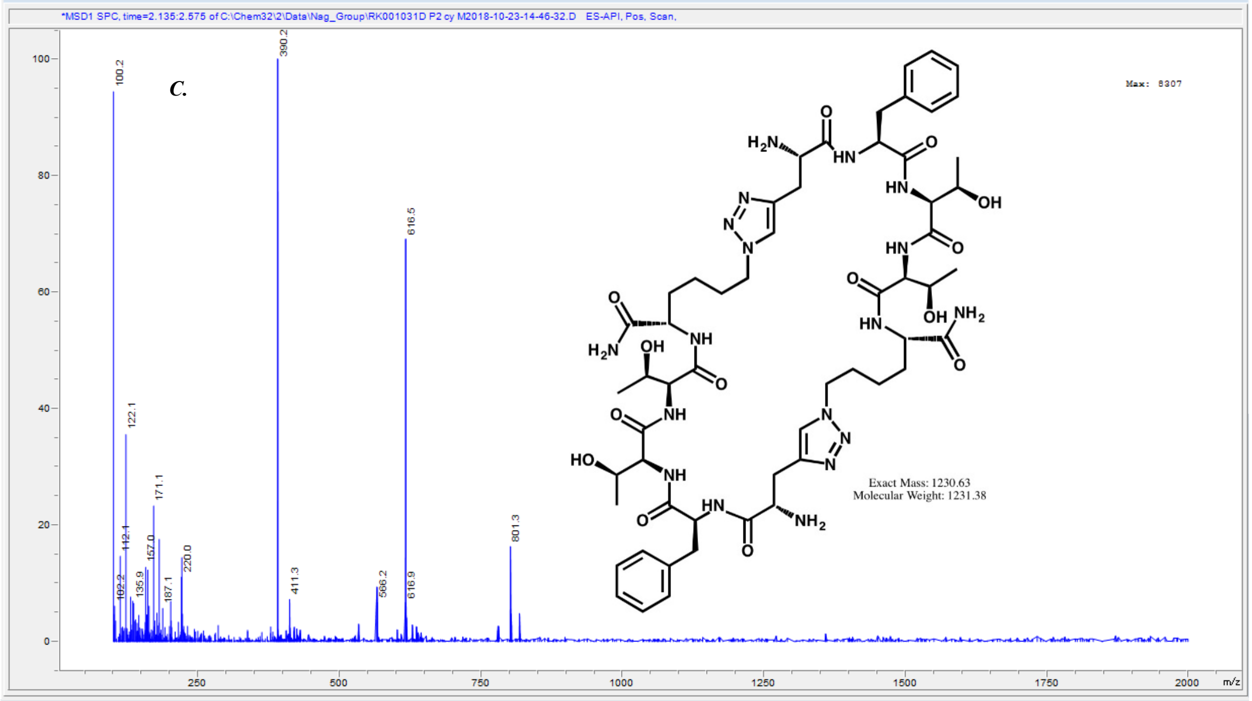

*MSD1 SPC, time=1.810:1.982 of C:\Chem32\2\Data\Nag_Group\RK001 032D P3 cy2018-10-18-21-56-36.D ES-API, Pos, Scan, F
C.
Max: 6648
100
80
60
40
20
0
100.1
142.1
114.1
122.1
127.1
109.9
156.1
163.9
171.1
173.9
187.1
201.1
217.1
379.5
392.0
568.3
568.7
250
500
750
1000
1250
1500
1750
2000
m/z
H2N
O
OH
OH
O
NH
N
N
N
NH2
O
H
N
O
NH
HN
O
NH
HN
O
Exact Mass: 1134.63
Molecular Weight: 1135.30
O
H
N
HN
O
NH
HN
O
N
O
NH2
N
N
HO
O
OH
O
NH2

Figure S. 25: HPLC and mass spectrometry analysis of cyclic peptide ***19***

A. RP-HPLC chromatogram of cyclic peptide **19** with sequence (FTT-Tz4)$_{cy}$; B. LC-MS of HPLC fraction at 22.9 minutes yields mass of monomer: m/z calculated for $[M+H]^+$ expected: 616.3 found: 616.3, m/z calculated for $[M+Na]^+$ expected: 638.3 found: 638.3; C. LC-MS of HPLC fraction at 31.2 minutes yields mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 411.2 found: 411.3, m/z calculated for $[M+H]^{2+}$ expected: 616.3 found: 616.5

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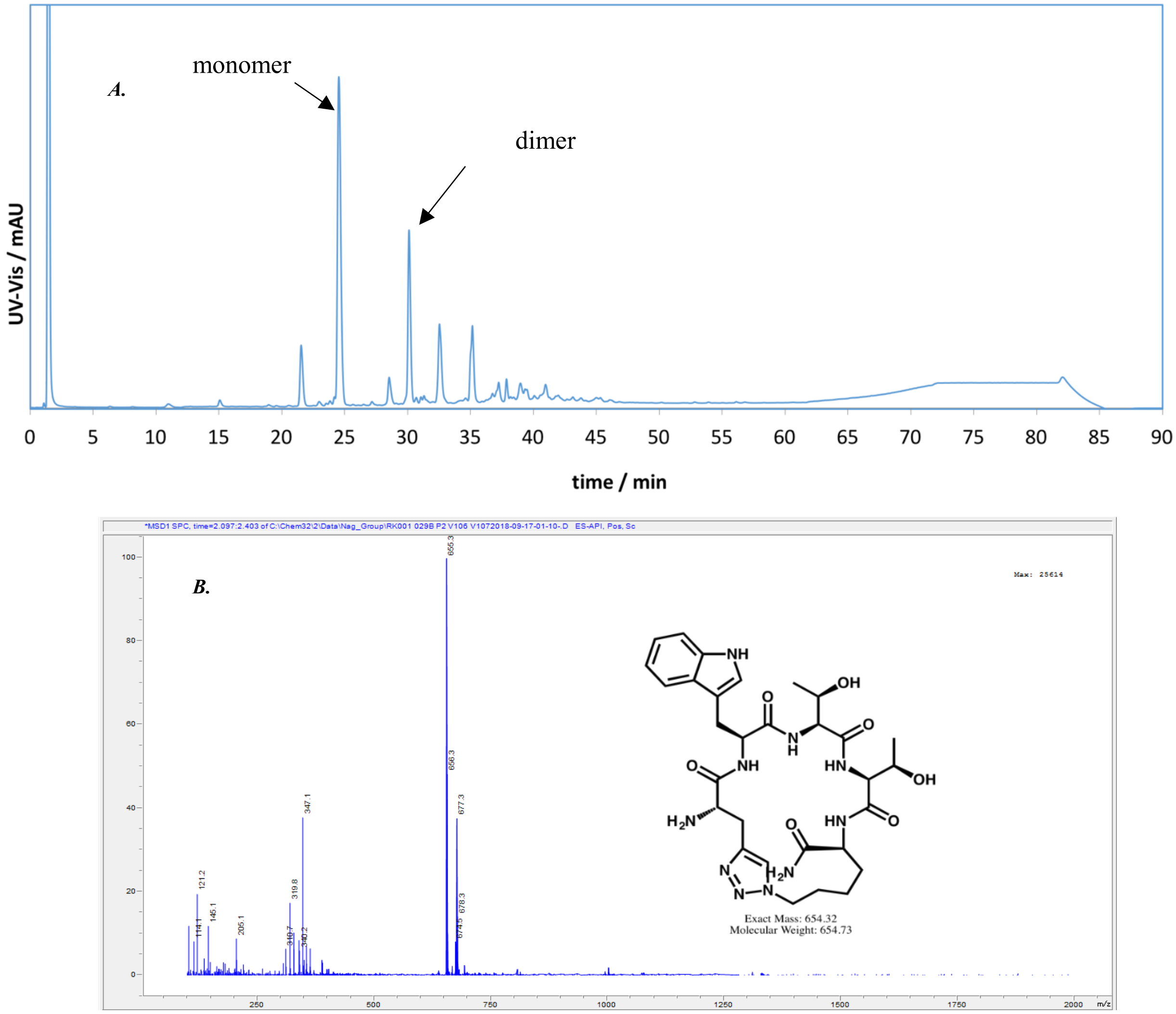
C.
Max: 8307
100
80
60
40
20
0
100.2
102.2
112.1
122.1
135.9
157.0
171.1
187.1
220.0
390.2
411.3
566.2
616.5
616.9
801.3
250
500
750
1000
1250
1500
1750
2000
m/z
H2N
HN
O
NH
OH
N
N
N
NH2
OH
Exact Mass: 1230.63
Molecular Weight: 1231.38
HO

*Figure S. 26: HPLC and mass spectrometry analysis of cyclic peptide **20***

A. RP-HPLC chromatogram of cyclic peptide **20** with sequence $(WTT\text{-}Tz_4)_{cy}$; B. LC-MS of HPLC fraction at 24.5 minutes yields mass of monomer: m/z calculated for $[M+H+K]^{2+}$expected: 347.2 found: 347.1, m/z calculated for $[M+H]^{+}$ expected: 655.3 found: 655.3; C. LC-MS of HPLC fraction at 30.1 minutes yields mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 437.3 found: 437.5, m/z calculated for $[M+2H]^{2+}$ expected: 655.3 found: 655.3, m/z calculated for $[M+2Na]^{2+}$ expected: 677.3 found: 677.3



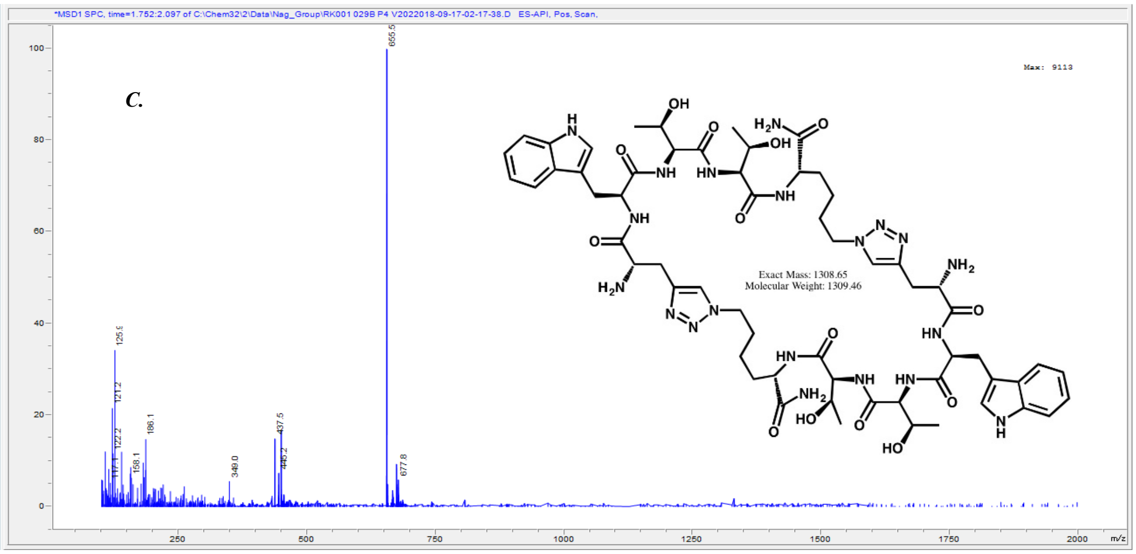
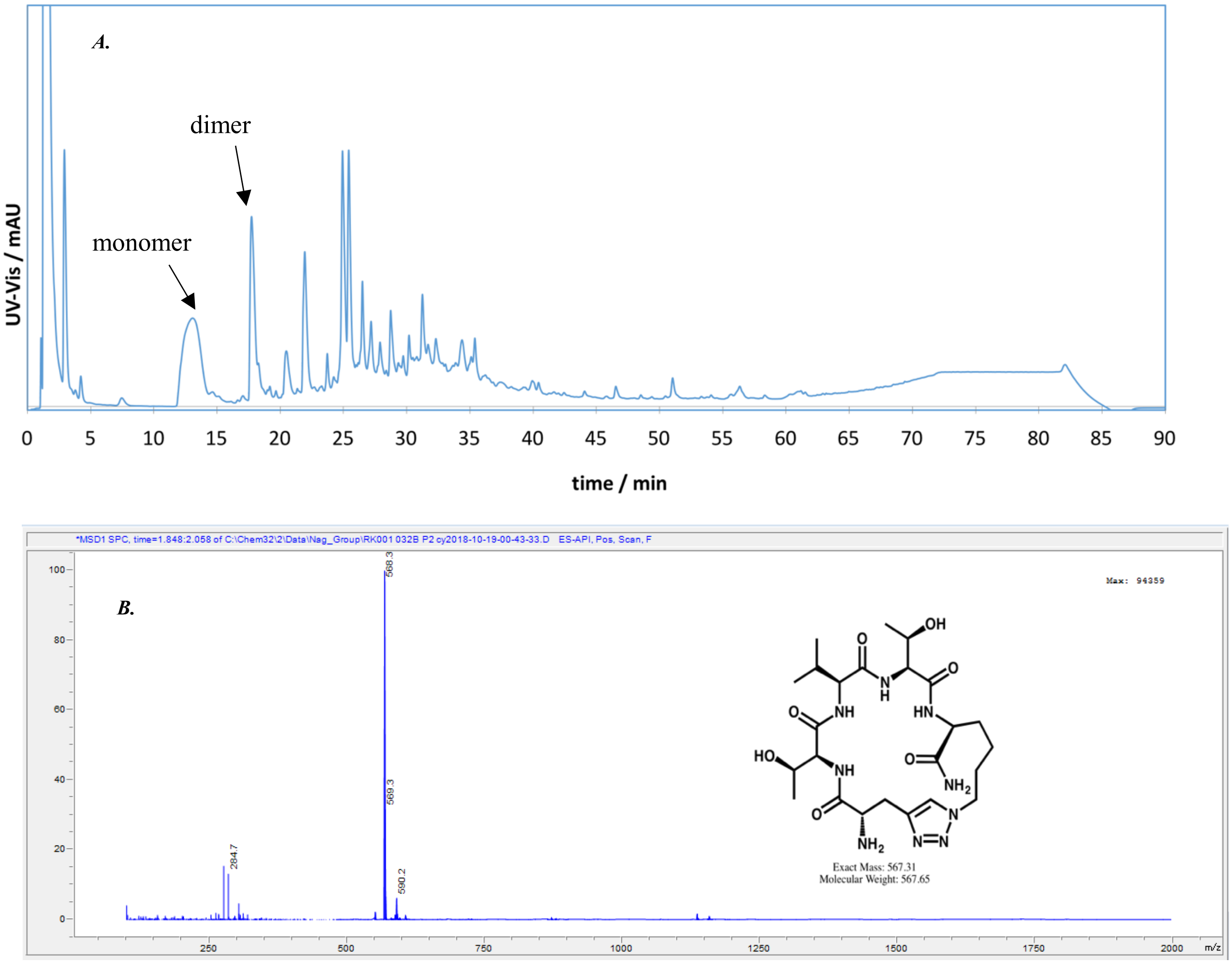

*MSD1 SPC, time=1.752:2.097 of C:\Chem32\2\Data\Nag_Group\RK001 029B P4 V2022018-09-17-02-17-38.D ES-API, Pos, Scan,
C.
Max: 9113
655.5
125.9
121.2
122.2
117.1
158.1
186.1
349.0
437.5
445.2
677.8
Exact Mass: 1308.65
Molecular Weight: 1309.46
0
20
40
60
80
100
250
500
750
1000
1250
1500
1750
2000
m/z

Figure S. 27: HPLC and mass spectrometry analysis of cyclic peptide ***21***

A. RP-HPLC of peptide **21** with sequence (TVT-Tz$_4$)$_{cy}$; B. LC-MS of HPLC fraction at 12.9 minutes yields mass corresponding to monomer: m/z calculated for [M+2H]$^{2+}$ expected: 284.7 found: 284.7, m/z calculated for [M+H]$^{+}$ expected: 568.3 found: 568.3, m/z calculated for [M+Na]$^{+}$ expected: 590.3 found: 590.2; C. LC-MS of HPLC fraction at 17.7 minutes yields mass corresponding to the mass of dimer $_{cy}$[YTT-Tz$_4$]$_2$: m/z calculated for [M+2H]$^{2+}$ expected: 568.3 found: 568.3, m/z calculated for [M+H+Na]$^{2+}$ expected: 579.3 found: 579.3, m/z calculated for [M+Na]$^{+}$ expected: 1158.6 found: 1158.5



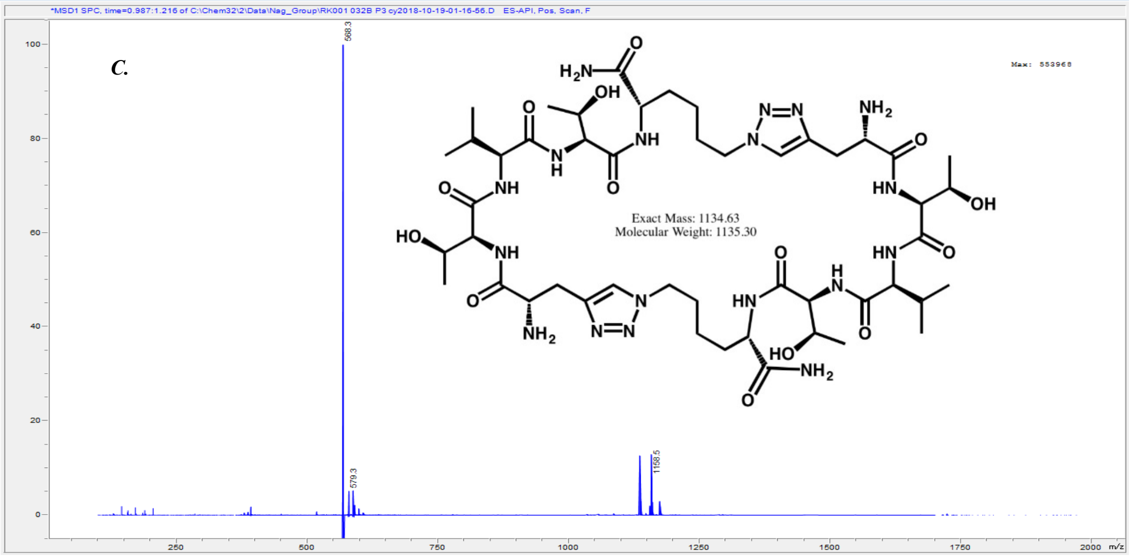
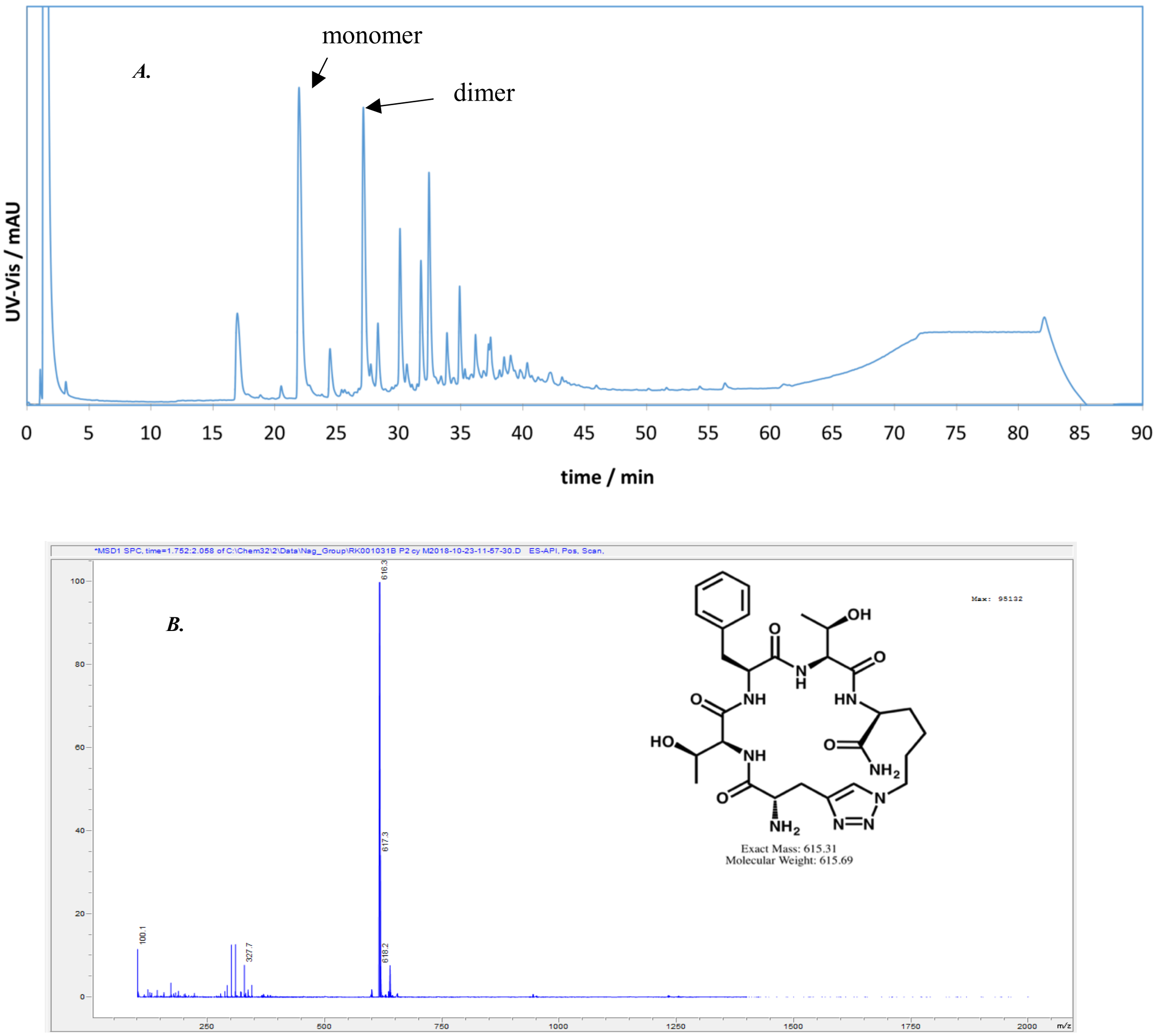

*MSD1 SPC, time=0.987:1.216 of C:\Chem32\2\Data\Nag_Group\RK001 032B P3 cy2018-10-19-01-16-56.D ES-API, Pos, Scan, F
C.
Max: 553968
568.3
579.3
1158.5
100
80
60
40
20
0
250
500
750
1000
1250
1500
1750
2000
m/z
H₂N
O
OH
NH
N=N
N
NH₂
O
HN
OH
HN
O
H
N
O
HN
O
HO
NH₂
O
NH
N
N=N
NH₂
O
NH
HO
O
NH
Exact Mass: 1134.63
Molecular Weight: 1135.30

Figure S. 28: HPLC and mass spectrometry analysis of cyclic peptide ***22***

A. RP-HPLC of peptide **22** with sequence (TFT-Tz4)$_{cy}$ B. LC-MS of fraction at 21.9 minutes yields mass corresponding to monomer: m/z calculated for $[M+2H]^{2+}$ expected: 308.7 found: 308.7, m/z calculated for $[M+H]^{+}$ expected: 616.3 found: 616.3; C. LC-MS of fraction at 27.1 minutes is corresponding to the mass of dimer: m/z calculated for $[M+H]^{2+}$ expected: 616.3 found: 616.4



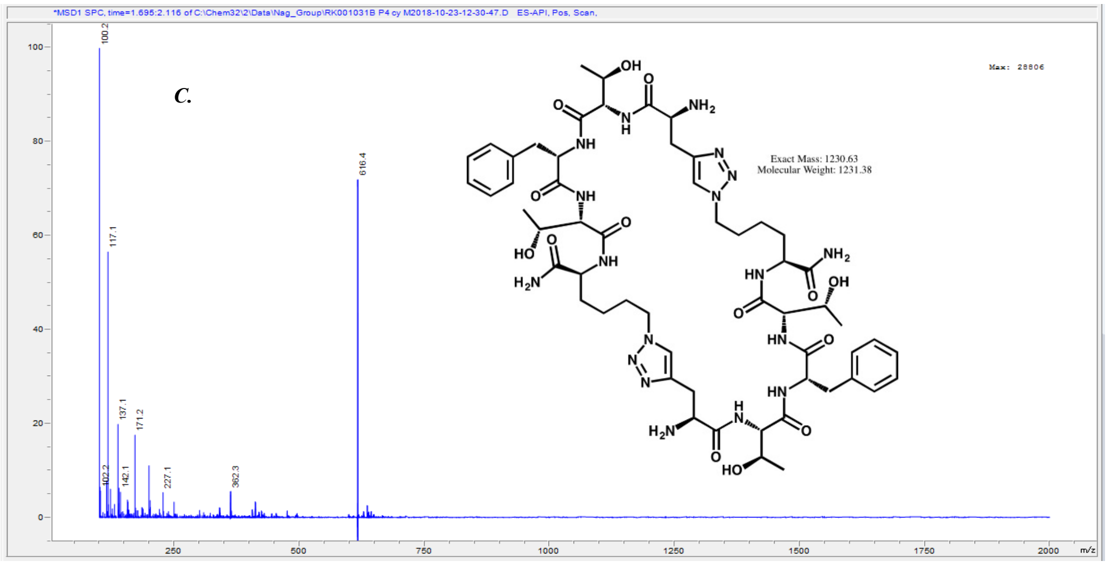
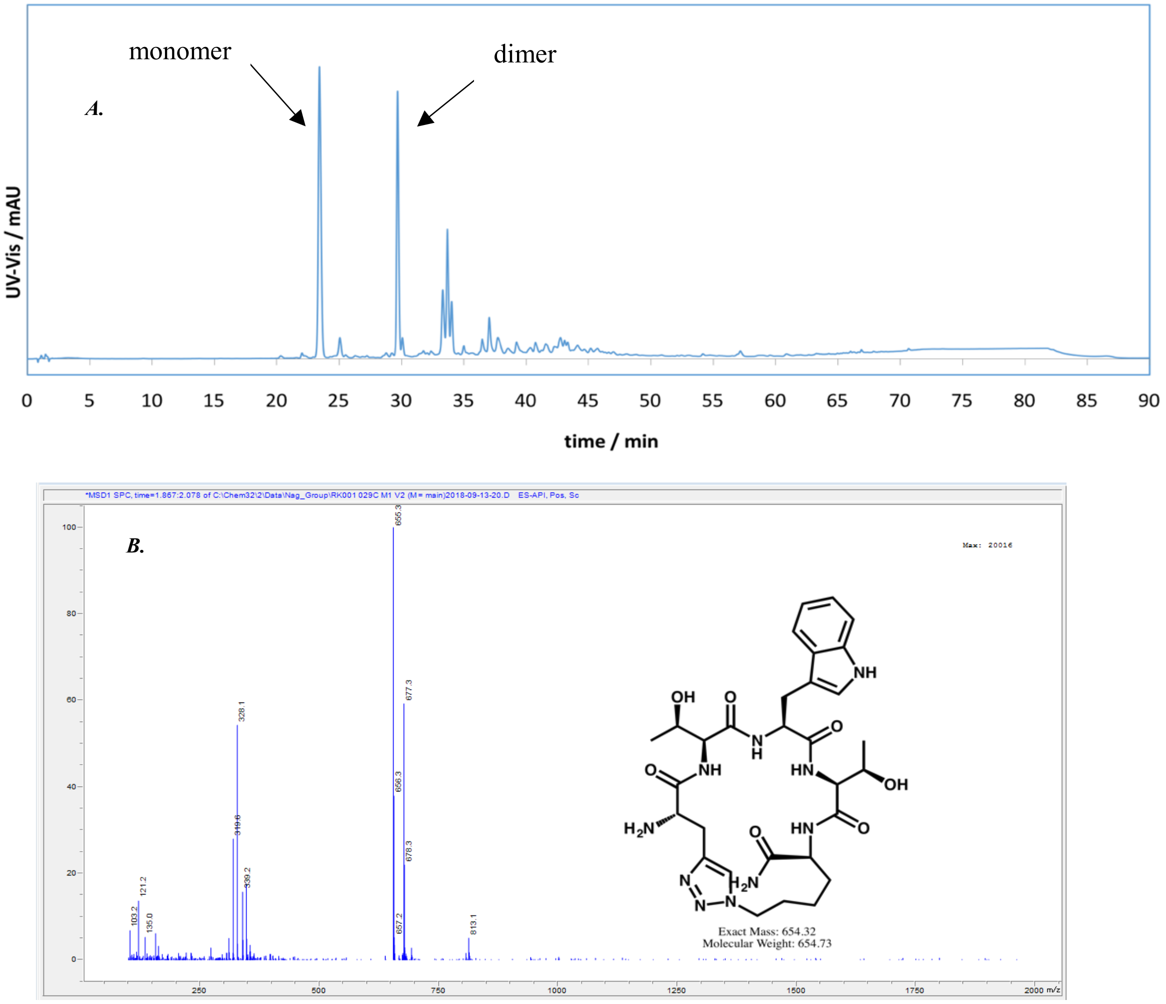
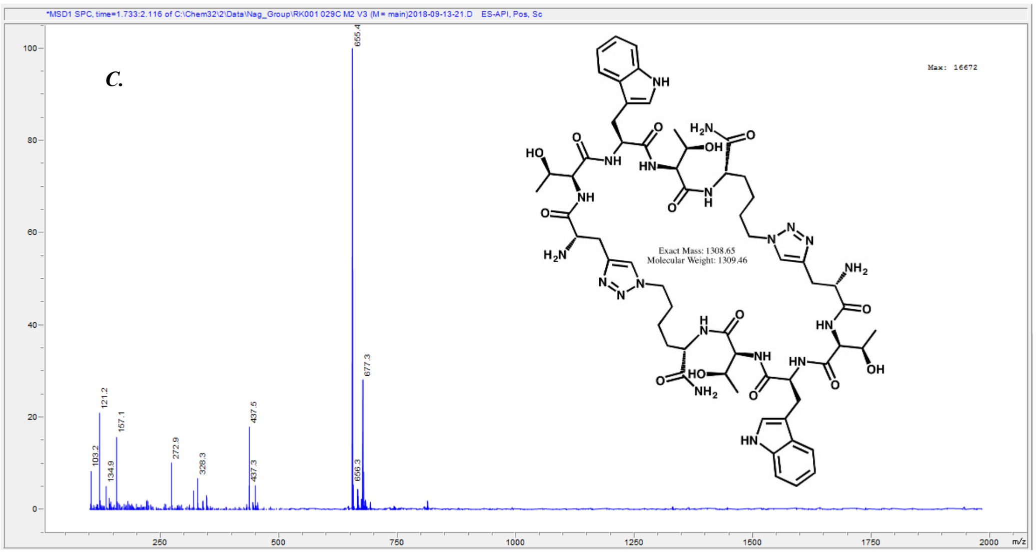

*MSD1 SPC, time=1.695:2.116 of C:\Chem32\2\Data\Nag_Group\RK001031B P4 cy M2018-10-23-12-30-47.D ES-API, Pos, Scan,
C.
Max: 28806
100
80
60
40
20
0
100.2
117.1
137.1
171.2
102.2
142.1
227.1
362.3
616.4
250
500
750
1000
1250
1500
1750
2000
m/z
OH
O
O
NH2
N
H
NH
N
N
N
O
NH
O
HO
O
NH
H2N
NH2
HN
OH
O
O
HN
O
N
N
N
HN
O
H
N
H2N
O
O
HO
Exact Mass: 1230.63
Molecular Weight: 1231.38

*Figure S. 29: HPLC and mass spectrometry analysis of cyclic peptide **23***

A. RP-HPLC of peptide **23** with sequence (TWT-Tz4)$_{cy}$ B. LC-MS of fraction at 23.4 minutes yields mass corresponding to monomer: m/z calculated for $[M+2H]^{2+}$ expected: 328.2 found: 328.1, m/z calculated for $[M+H]^{+}$ expected: 655.3 found: 655.3; C. LC-MS of fraction at 29.7 minutes yields mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 437.2 found: 437.5, m/z calculated for $[M+2H]^{2+}$ expected: 655.3 found: 655.3

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C.
Max: 16672
100
80
60
40
20
0
655.4
677.3
121.2
157.1
103.2
134.9
272.9
328.3
437.5
437.3
656.3
250
500
750
1000
1250
1500
1750
2000
m/z
Exact Mass: 1308.65
Molecular Weight: 1309.46
NH
HN
HO
OH
NH
HN
H2N
NH2
O
N
H

*Figure S. 30: HPLC and mass spectrometry analysis of cyclic peptide **24***

A. RP-HPLC of peptide **24** with sequence (TTW-Tz4)cy; B. LC-MS of HPLC fraction at 22.7 minutes yields mass corresponding to monomer: m/z calculated for $[M+2H]^{2+}$ expected: 328.2 found: 328.2, m/z calculated for $[M+H]^{+}$ expected: 655.3 found: 655.3, m/z calculated for $[M+Na]^{+}$ expected: 678.3 found: 678.2

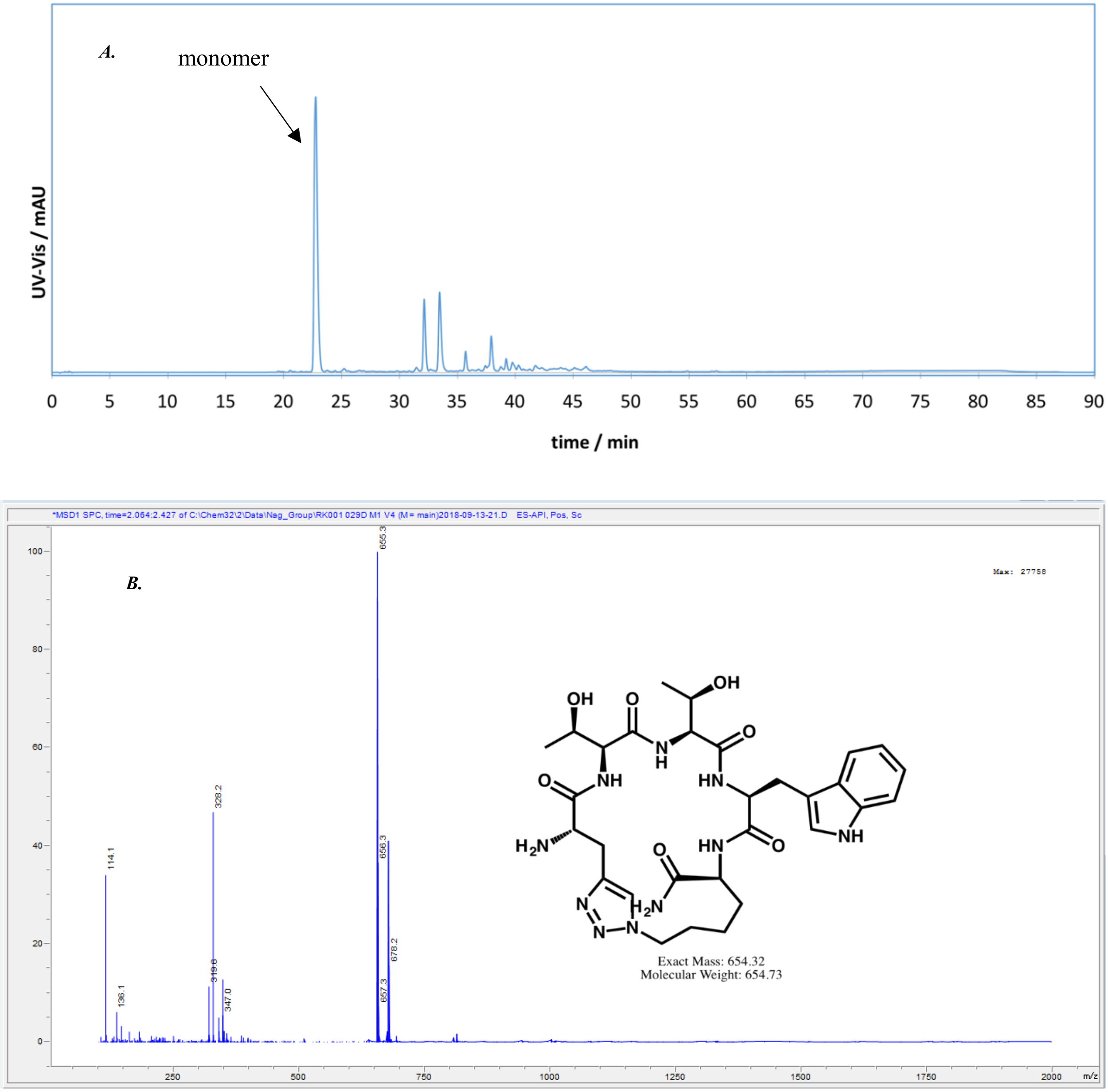
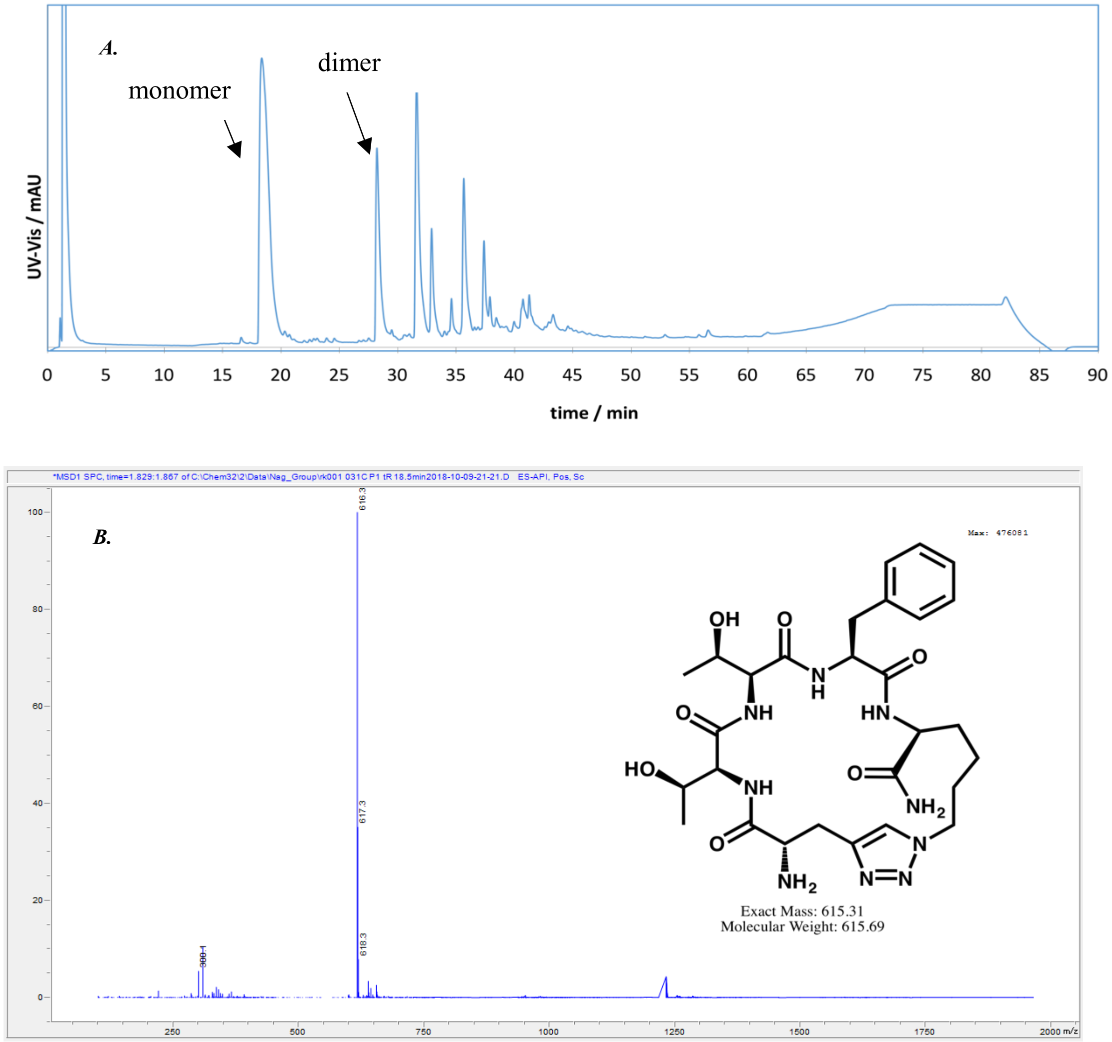
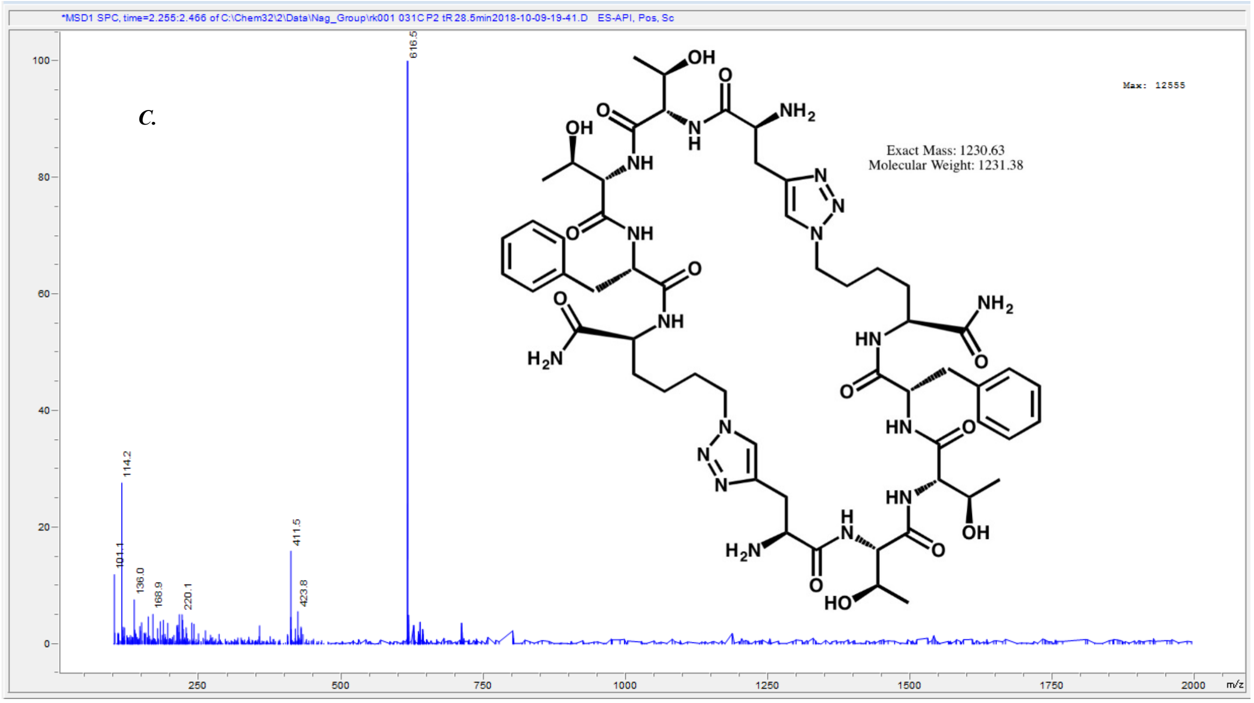


Figure S. 31: HPLC and mass spectrometry analysis of cyclic peptide ***25***

A. RP-HPLC of peptide **25** with sequence (TTF-Tz4)cy B. LC-MS of HPLC fraction at 18.3 minutes yields mass of monomer: m/z calculated for $[M+H]^+$ expected: 616.3 found: 616.3; C. LC-MS of fraction at 28.2 minutes yields mass of dimer: m/z calculated for $[M+3H]^{3+}$ expected: 411.3 found: 411.5, m/z calculated for $[M+2H]^{2+}$ expected: 616.3 found: 616.5





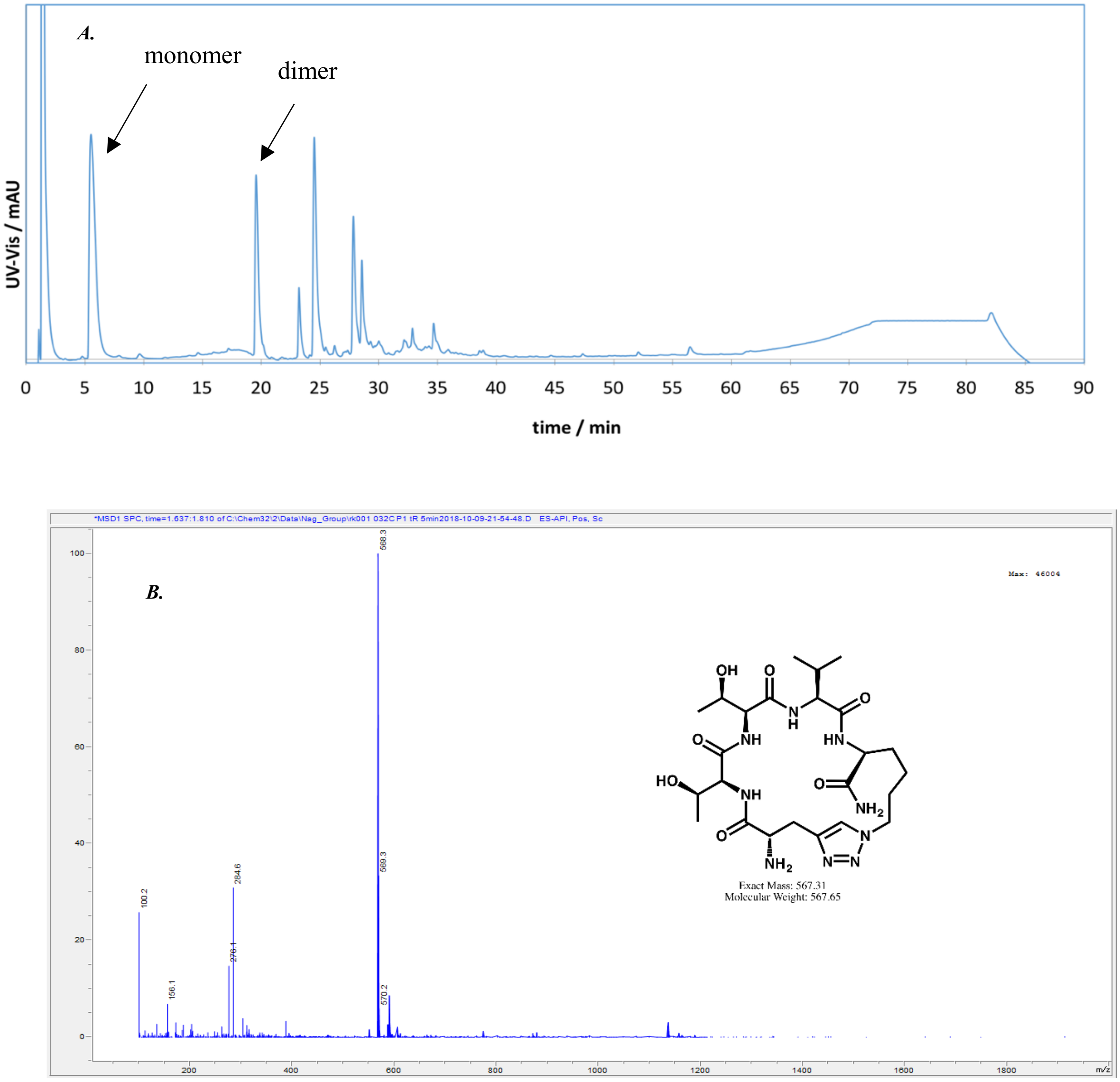
*MSD1 SPC, time=2.255:2.466 of C:\Chem32\2\Data\Nag_Group\rk001 031C P2 tR 28.5min2018-10-09-19-41.D ES-API, Pos, Sc

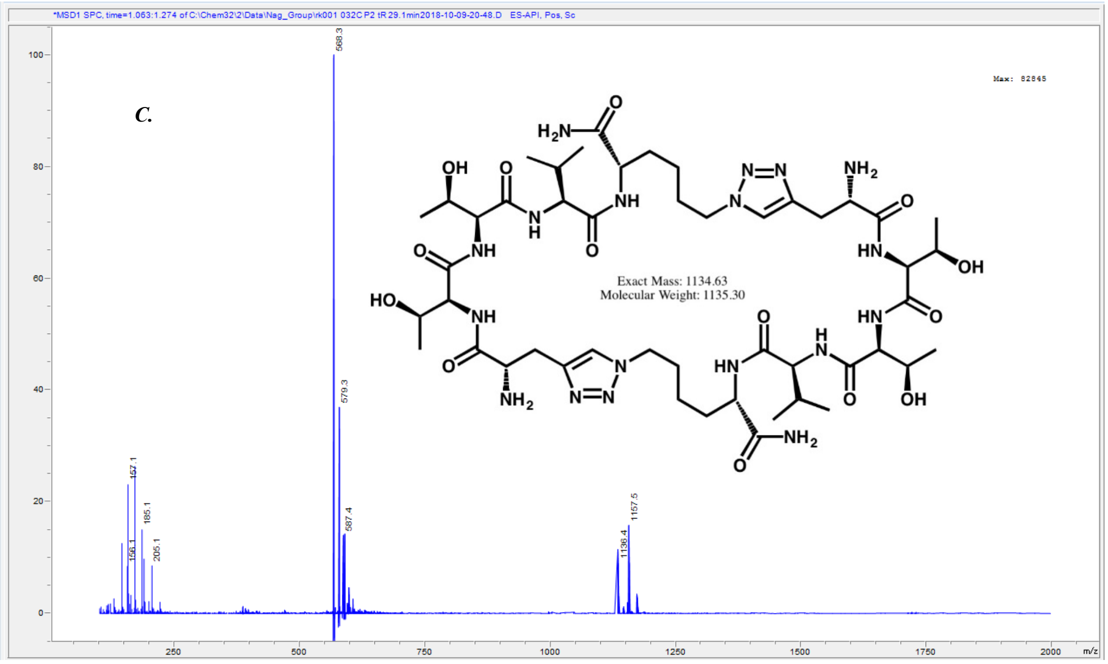
Max: 12555

Exact Mass: 1230.63
Molecular Weight: 1231.38

*Figure S. 32: HPLC and mass spectrometry analysis of cyclic peptide **26***

A. RP-HPLC of peptide **26** with sequence (TTV-Tz4)$_{cy}$ B. LC-MS of HPLC fraction at 5.5 minutes yields mass of monomer: m/z calculated for $[M+2H]^{2+}$ expected: 284.7 found: 284.6, m/z calculated for $[M+H]^{+}$ expected: 568.3 found: 568.3; C. LC-MS of fraction at 19.5 minutes yields mass of dimer: m/z calculated for $[M+2H]^{2+}$ expected: 568.3 found: 568.3, m/z calculated for $[M+1H+1Na]^{2+}$ expected: 597.3 found: 579.3, m/z calculated for $[M+H)]^{+}$ expected: 1136.6 found: 1136.4





*MSD1 SPC, time=1.063:1.274 of C:\Chem32\2\Data\Nag_Group\rk001 032C P2 tR 29.1min2018-10-09-20-48.D ES-API, Pos, Sc

References

1. Turner, R. A.; Oliver, A. G.; Lokey, R. S., Click chemistry as a macrocyclization tool in the solid-phase synthesis of small cyclic peptides. *Org Lett* **2007,** *9* (24), 5011-4.

2. Jagasia, R.; Holub, J. M.; Bollinger, M.; Kirshenbaum, K.; Finn, M. G., Peptide cyclization and cyclodimerization by Cu(I)-mediated azide-alkyne cycloaddition. *J Org Chem* **2009,** *74* (8), 2964-74.

3. Splith, K.; Neundorf, I.; Hu, W.; N'Dongo, H. W. P.; Vasylyeva, V.; Merz, K.; Schatzschneider, U., Influence of the metal complex-to-peptide linker on the synthesis and properties of bioactive CpMn(CO)3 peptide conjugates. *Dalton Transactions* **2010,** *39* (10), 2536-2545.

4. Patai, S., The Chemistry of Azido Group. *John Wiley & Sons Ltd* **1971**, 195-197.

5. Yuran, S.; Razvag, Y.; Das, P.; Reches, M., Self-assembly of azide containing dipeptides. *J Pept Sci* **2014,** *20* (7), 479-86.