Supporting Information

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Supporting Information

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I. General. All commercial materials (Sigma-Aldrich, Fluka, and Novabiochem) were used without further purification. All solvents were reagent or HPLC (Fisher) grade. All reactions were performed under air in glass vials. Conversions refer to chromatographically pure compounds; % conversions were obtained by comparison of HPLC peak areas of products and starting materials. HPLC was used to monitor reaction progress.

II. Materials. Fmoc-amino acids, Rink amide resin, 3-[Bis(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU), 1-Hydroxy-7-azabenzotriazole (HOAt), *N,N'*-iisopropylcarbodiimide (DIC), and N,N-Diisopropylethylamine (DIEA) were obtained from CreoSalus (Louisville, Kentucky). 4-Dimethylaminopyridine (DMAP), Piperidine, Trifluoroacetic acid (TFA), Di-tert-butyl dicarbonate (BOC_2O), 4-methyl morpholine (NMM) and Hydrazine monohydrate were obtained from Alfa Aesar (Ward Hill, Massachusetts). N,N-Dimethylformamide (DMF), Dichloromethane (DCM), Methanol (MeOH), Acetonitrile (ACN), Sodium cyanoborohydride, Tetrahydrofuran (THF), and Fmoc-Ala-aldehyde were obtained from VWR (100 Matsonford Road Radnor, Pennsylvania). Dithiocarbamate (DTC) was obtained from Sigma-Aldrich (St. Louis, Missouri). Water was purified using a Millipore Milli-Q water purification system.

III. Purification. Purification was performed with high performance liquid chromatography (HPLC) on an Agilent 1100/1200 series HPLC equipped with a 5 mm C-18 reversed-phase column. All separations involved a mobile phase of 0.1% formic acid (v/v) in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). The HPLC method employed a linear gradient of 0–80% solvent B over 30 minutes at ambient temperature with a flow rate of 1.0 mL min^{-1}. The separation was monitored by UV absorbance at both 220 and 254 nm unless otherwise noted.

IV. Analytical Methods.

HPLC: Peptide compositions were evaluated by high performance liquid chromatography (HPLC) on an Agilent 1100/1200 series HPLC equipped with a 5.0 um C-18 reversed-phase column (Agilent Eclipse Plus C18 4.6 x 100mm). All separations used mobile phases of 0.1% formic acid (v/v) in water (solvent A) and 0.1% formic acid (v/v) in acetonitrile (solvent B). A linear gradient of 0–80% solvent B in 30 minutes at room temperature with a flow rate of 1.0 mL min^{-1} was used. The eluent was monitored by UV absorbance at 220 nm unless otherwise noted.

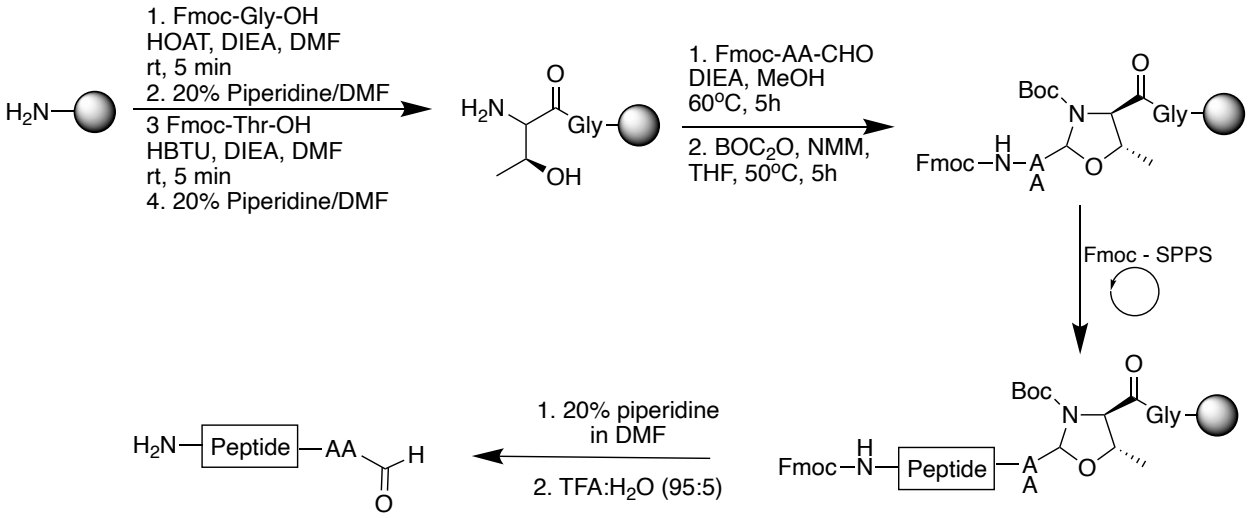
LC-MS: Mass spectrometry to check reaction mixtures was performed using an Agilent 1100 Series HPLC with MSD VL mass spectrometer using positive polarity electrospray ionization (+ESI).

HRMS: High resolution MS data were acquired on Thermo Exactive Plus using a heated electrospray source. The solution was infused at a rate of 10-25 ul /min/Electrospray using 3.3 KV. The typical settings were Capillary temp 320 ^{o}C. S-lens RF level was between 30-80 with an AGC setting of 1 E-6. The maximum injection time was set to 50 ms. Spectra were taken at 140,000 resolution at m/z 200 using Tune software and analyze with Thermo's Freestyle software.

V. Fmoc Solid-Phase Peptide Synthesis (Fmoc-SPPS).[1] Peptides were synthesized manually on a 0.25 mM scale using Rink amide resin. Resin was swollen with DCM for 1h at room temperature. Fmoc was deprotected using 20% piperidine–DMF for 5 min to obtain a deprotected peptide-resin. First Fmoc-protected amino acid (1.25 mM/5equiv.) was coupled using HOAt (1.25 mM/5equiv.) and DIC (1.25 mM/5equiv.) in DMF (final conc. 0.05M) for 15 min at room temperature. Fmoc-protected amino acids (0.75 mM/3equiv.) were sequentially coupled on the resin using HBTU (0.75 mM/3equiv.) and DIEA (1.5 mM/6equiv.) in DMF (final conc. 0.05M) for 5 min at room temperature. Peptides were synthesized using standard protocols.[1] Any Fmoc-protected amino acid added after Fmoc-proline was subjected to the conditions of the first amino acid coupling. Peptides were cleaved from the resin using a cocktail of 95:5, trifluoroacetic acid: water for 2 h. The resin was removed by filtration and the resulting solution was concentrated. The residue was diluted with ACN/water mixture. The resulting solution was purified by semi-preparative chromatography.

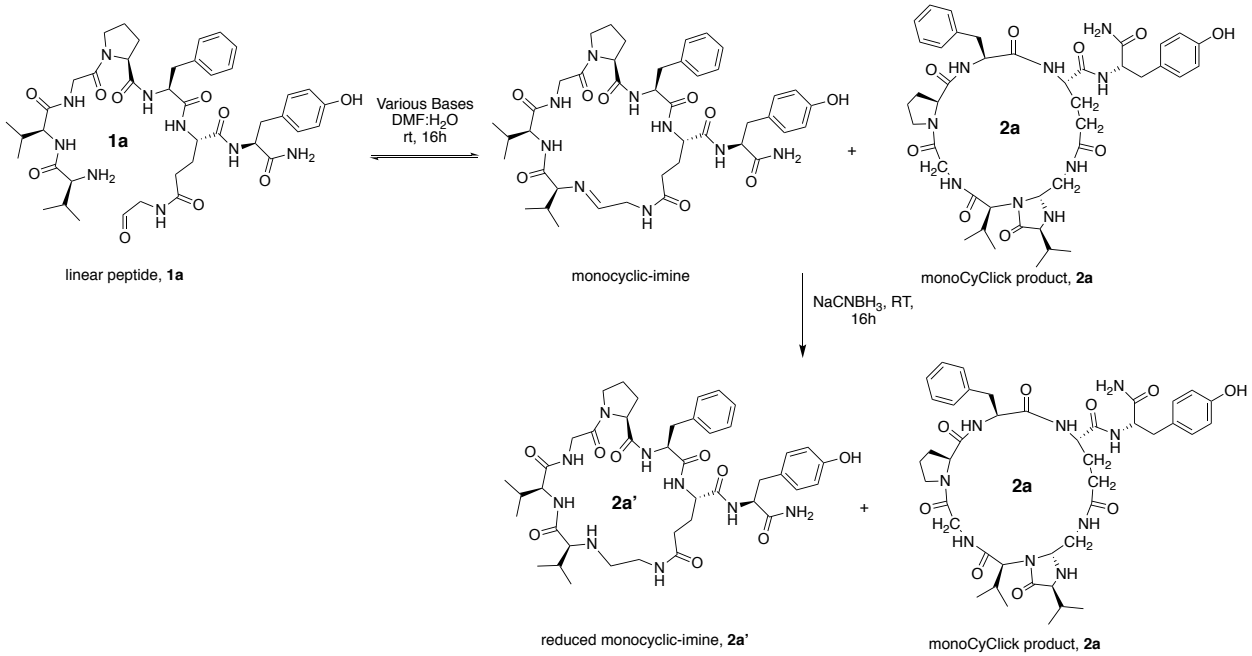
VI. Procedure for synthesis of C-terminal peptide aldehydes.[2] Fmoc-Gly-OH and Fmoc-Thr-OH were coupled with the general peptide synthesis procedure to swollen Rink resin (0.5mM/g). Following Fmoc-deprotection, the resin (500mg) was added to a solution of Fmoc-AA-CHO (alanine aldyhyde) (1 mM/4equiv.) in 1% DIEA v/v in MEOH (2.5mL/final conc. 0.1M) and rocked for 5h at 60°C. The resin was washed with MeOH (5 x 3mL), DMF (5 x 3mL), DCM (5 x 3mL), and THF (5 x 3mL). The resin was rocked for 5h at 50°C in a solution of Boc anhydride BOC_2O (1.25 mM/5equiv.), NMM (1.25 mM/5equiv.) in THF (2.5mL/final conc. 0.1M). The resin was washed with THF (5 x 3mL), DCM (5 x 3mL), and DMF (5 x 3mL) followed by coupling of rest of Fmoc-amino acid residues using normal Fmoc SPPS. After Fmoc-SPPS, the resulting resin was washed three times with DMF, DCM and MeOH. This was followed by the cleavage of the C-terminal peptide aldehyde from solid support using cleavage cocktail TFA:H_2O (95:5) for 2h at room temperature. The peptide aldehyde was analyzed by LCMS and purified by HPLC followed by lypholization to obtain pure peptide aldehyde.

VII. Supplementary Figure 1: Solid phase synthesis procedure for C-terminal peptide aldehydes.



VIII. General procedure for macrocyclization in solution: Lyophilized peptide aldehyde (2mg) was mixed with 21 equiv. DIEA in a 1:1 DMF/H_2O solution (final conc. 3mM). The reaction was shaken at room temperature for 16 h. The product was analyzed with HPLC, NMR, and MS. The presence of [M+2H]2+ of a cyclic dimer will give the same nominal mass as the $[M+H]^+$ for the monocyclic compound; however, the doubly-charged dimer will have a different isotopic pattern with 0.5 Da separation compared to the monocyclic compound making the two compounds discernable.

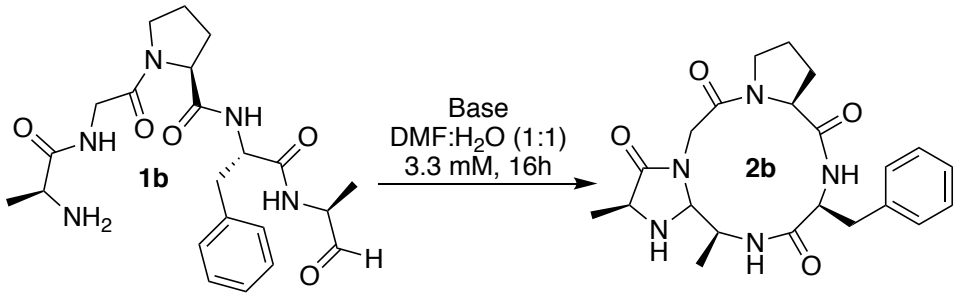
IX. Supplementary Figure 2: Optimization of Peptide aldehyde **1a** macrocyclization using different bases.



Procedure for base studies: To a lyophilized peptide aldehyde VVGPFEY (2mg) **1a** in 1:1 DMF/H_2O solution (final conc. 3mM), different bases such as DMAP, Imidazole, pyrrolidine, or DBU were added (7 equivalent) and the reactions were shaken at room temperature for 16 h. To differentiate the mono-cyclic imine from mono-CyClick product, sodium cyanoborohydride (10 equiv.) was added to the reaction mixtures and reactions were shaken at room temperature for another 16 h. The reactions were analyzed with HPLC and MS.

S/N	Base	Linear (%) 1a	Reduced monocyclic imine 2a' (%)	Mono-CyClick 2a (%)
1	Imidazole	17.34	54.32	28.34
2	Pyrrolidine	44.17	36.00	30.48
3	DBU	29.88	42.44	27.68
4	DMAP	17.95	48.25	33.80

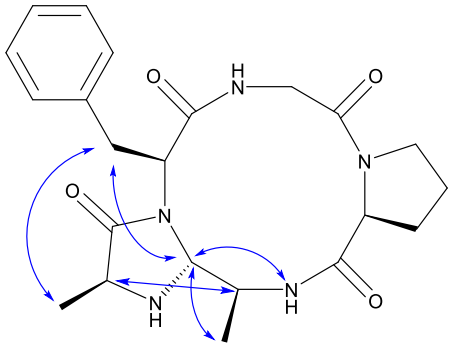
X. Supplementary Figure 3: Optimization of bases for the cyclization of head-to-tail cyclic tetrapeptide product.



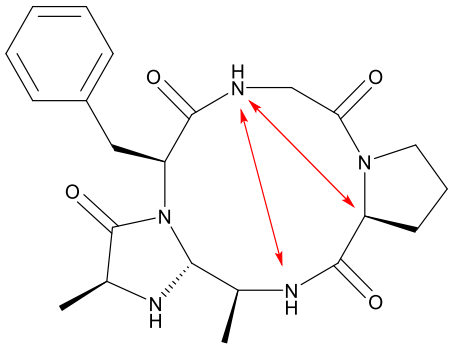
Procedure for base optimization: To a lyophilized peptide aldehyde **1b** (1mg) in 1:1 DMF/H_2O solution (final conc. 3mM), DIEA or DMAP at different equivalents (7-21 equivalent) were added and the reactions were shaken at different temperatures (room temperature or 60 ^{o}C) for 16 h. The reactions were analyzed with HPLC and MS.

S/N	Base	Equivalent	Temperature	Mono-CyClick 2b (%)
1	No Base	0	RT	13
2	DMAP	7	RT	58
3	DMAP	21	RT	82
4	DIEA	21	RT	73
5	DIEA	21	60 ^{o}C	89

XI. Supplementary Figure 4: **Structure Elucidation of cyclic tetrapeptide cyc-4-Imz-A(GPFA) 2b via NMR.** The sample (ca. 1-2 mg) was dissolved in 0.15 mL of DMSO-d6, and the solution was then transferred to a 3-mm NMR tube. 1H, 13C, TOCSY, HSQC, HMBC, and ROESY spectra were acquired at ambient temperature (298 K) using a 5-mm inverse Prodigy probe on a 500 MHz Bruker AVIII HD NMR spectrometer. Proton chemical shifts were referenced to residual DMSO-d5 at 2.50 ppm, and carbon chemical shifts were referenced to DMSO-d6 at 39.52 ppm. Spectra were processed using Mnova ver. 12.0.4. A TOCSY spectrum was acquired using a 80 ms mixing time, 50% non-uniform sampling, and 2048 x 400 increments. An adiabatic, gradient, multiplicity-edited HSQC spectrum was acquired using a J-optimization of 145 Hz, 25% non-uniform sampling, and 2048 x 512 increments; the spectrum was then linear predicted to 1024 points in F1. A gradient, echo/antiecho with 3-fold low-pass J-filter HMBC spectrum was acquired using a J-optimization of 8 Hz, 33% non-uniform sampling, and 4096 x 256 increments; the spectrum was then processed using magnitude mode along F2, a 90° sine square apodization in F1, and linear prediction to 512 points in F1. A ROESY spectrum was acquired using a 200 ms mixing time and 2048 x 256 increments, and 90° sine squared apodizations were applied in both dimensions.



ROEs to determine stereochemistry: blue double arrows

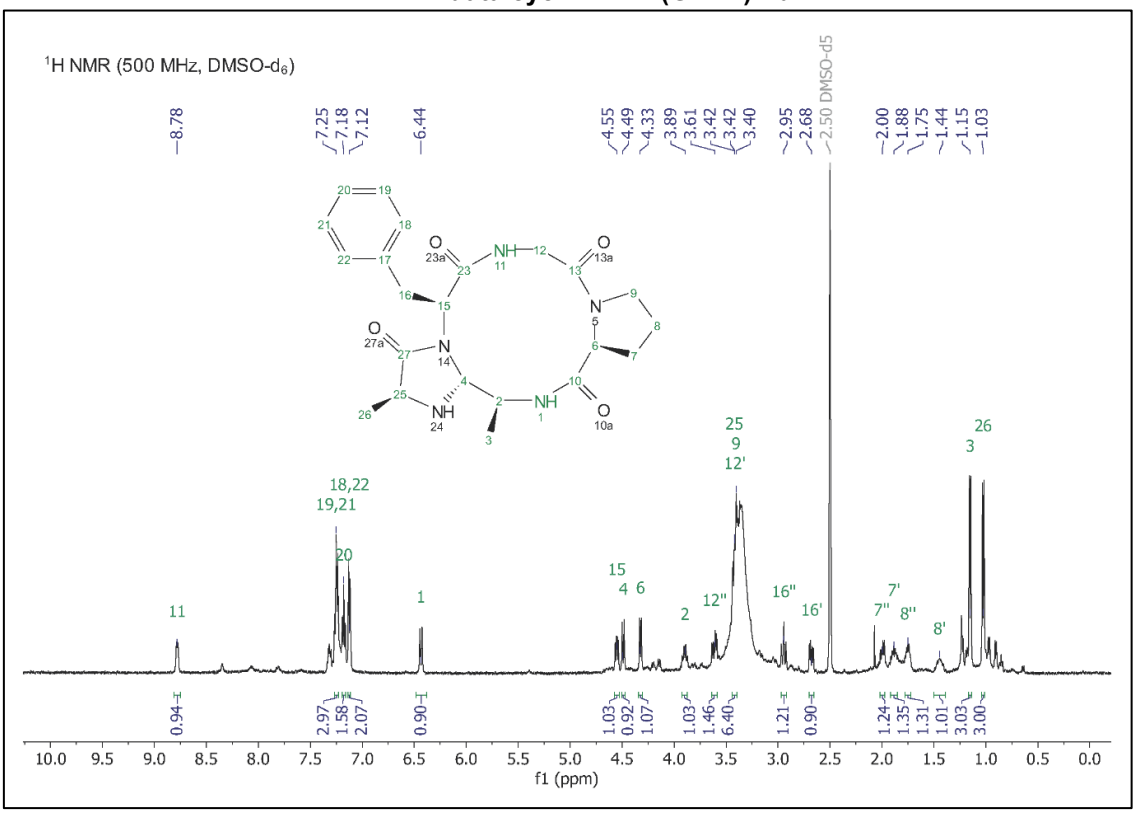


long range ROEs indicating solution structure: red double arrows

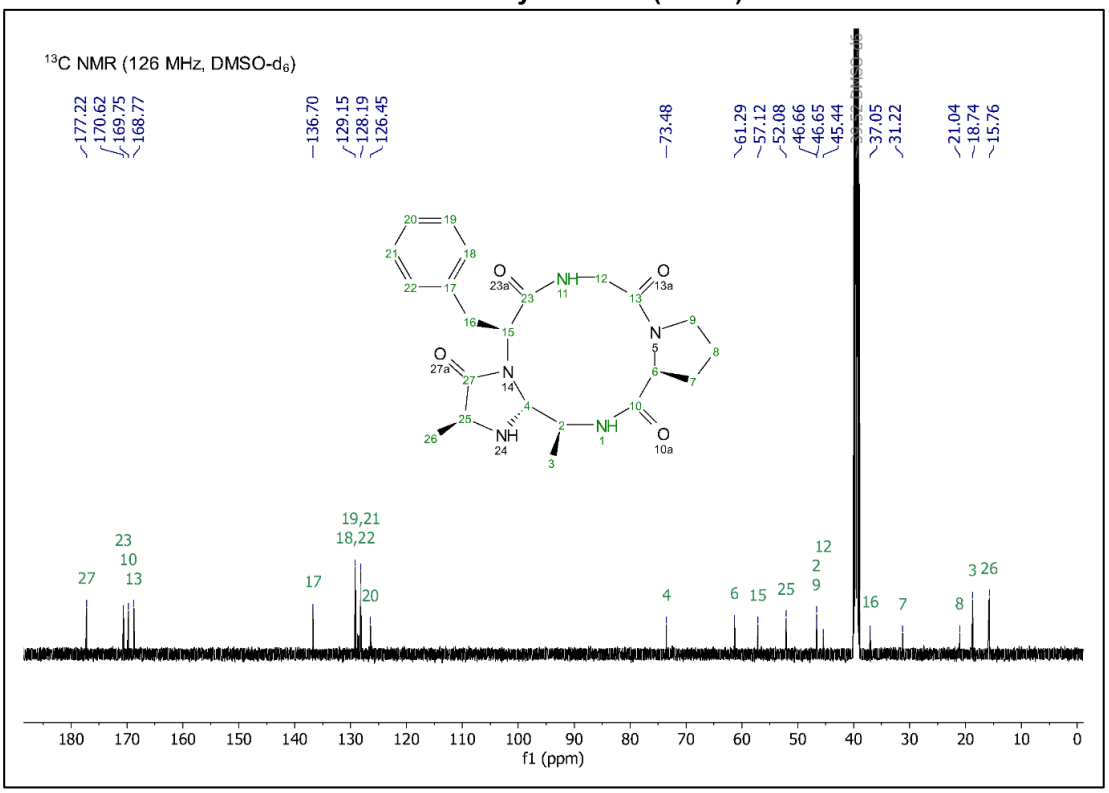
NMR data for cyc-4-Imz-A(GPFA) 2b

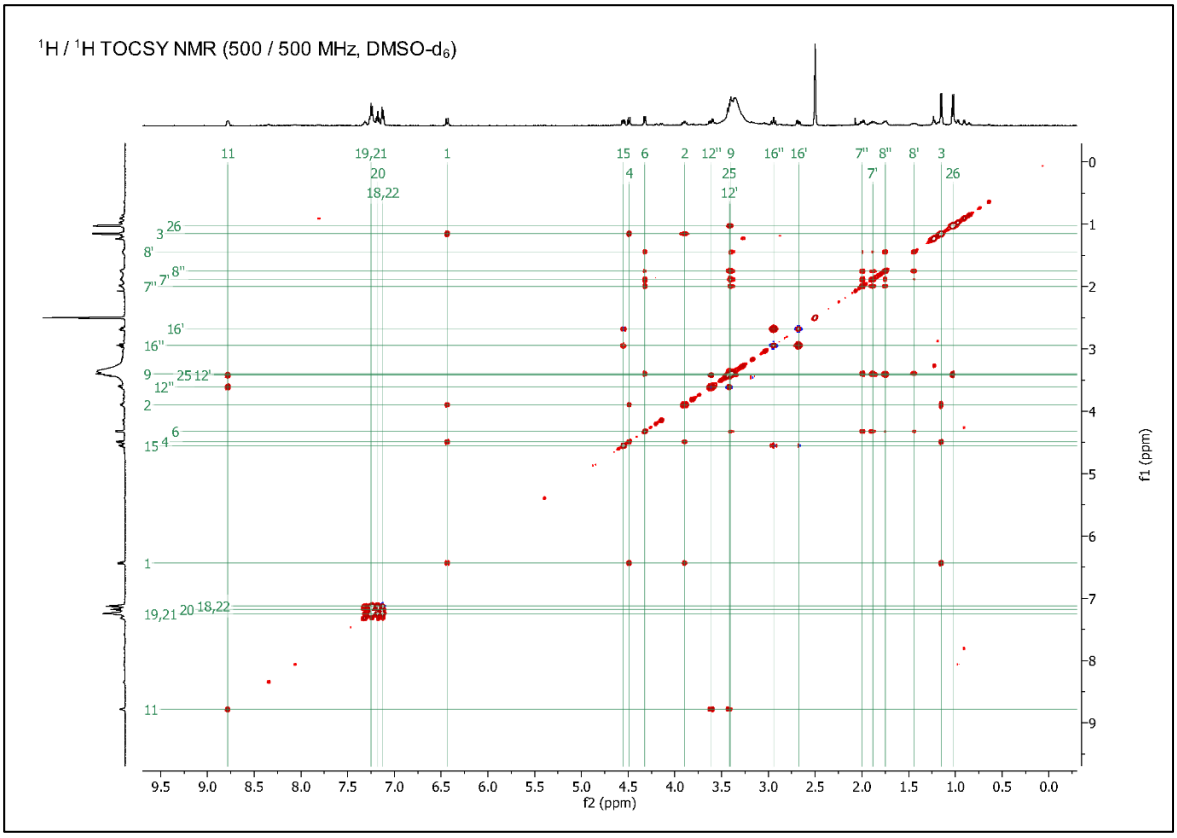
Residue	Atom Name	Numbering	δ_H (ppm), multiplicity	δ_C (ppm)
Ala	NH	1	6.44, d (*J* = 9.9 Hz)	--
	$C_\alpha H$	2	3.89, m	46.65
	$C_\beta H_3$	3	1.15, d (*J* = 6.7 Hz)	18.74
	CH	4	4.49, d (J = 9.1 Hz)	73.48
Pro	N	5	--	--
	$C_\alpha H$	6	4.33, d (*J* = 7.9 Hz)	61.29
	$C_\beta H_2$	7	1.88, m; 2.00, dd (*J* = 12.4, 6.4 Hz)	31.22
	$C_\gamma H_2$	8	1.44, m; 1.75, m	21.04
	$C_\delta H_2$	9	3.40, m	46.66
	CO	10	--	169.75
Gly	NH	11	8.78, dd (*J* = 6.9, 3.2 Hz)	--
	$C_\alpha H_2$	12	3.42, m; 3.61, dd (*J* = 15.8, 7.0 Hz)	45.44
	CO	13	--	168.77
Phe	N	14	--	--
	$C_\alpha H$	15	4.55, dd (*J* = 9.4, 5.5 Hz)	57.12
	$C_\beta H_2$	16	2.68, dd (*J* = 12.9, 5.8 Hz); 2.95, dd (*J* = 12.8, 9.5 Hz)	37.04
	C_ι	17	--	136.70
	C_2H, C_6H	18, 22	7.12, d (*J* = 7.6 Hz)	129.15
	C_3H, C_5H	19, 21	7.25, t (*J* = 7.3 Hz)	128.19
	C_4H	20	7.18, t (*J* = 7.1 Hz)	126.45
	CO	23	--	170.62
Ala	NH	24	--[a]	--
	$C_\alpha H$	25	3.42, m	52.08
	$C_\beta H_3$	26	1.03, d (*J* = 6.9 Hz)	15.76
	CO	27	--	177.22

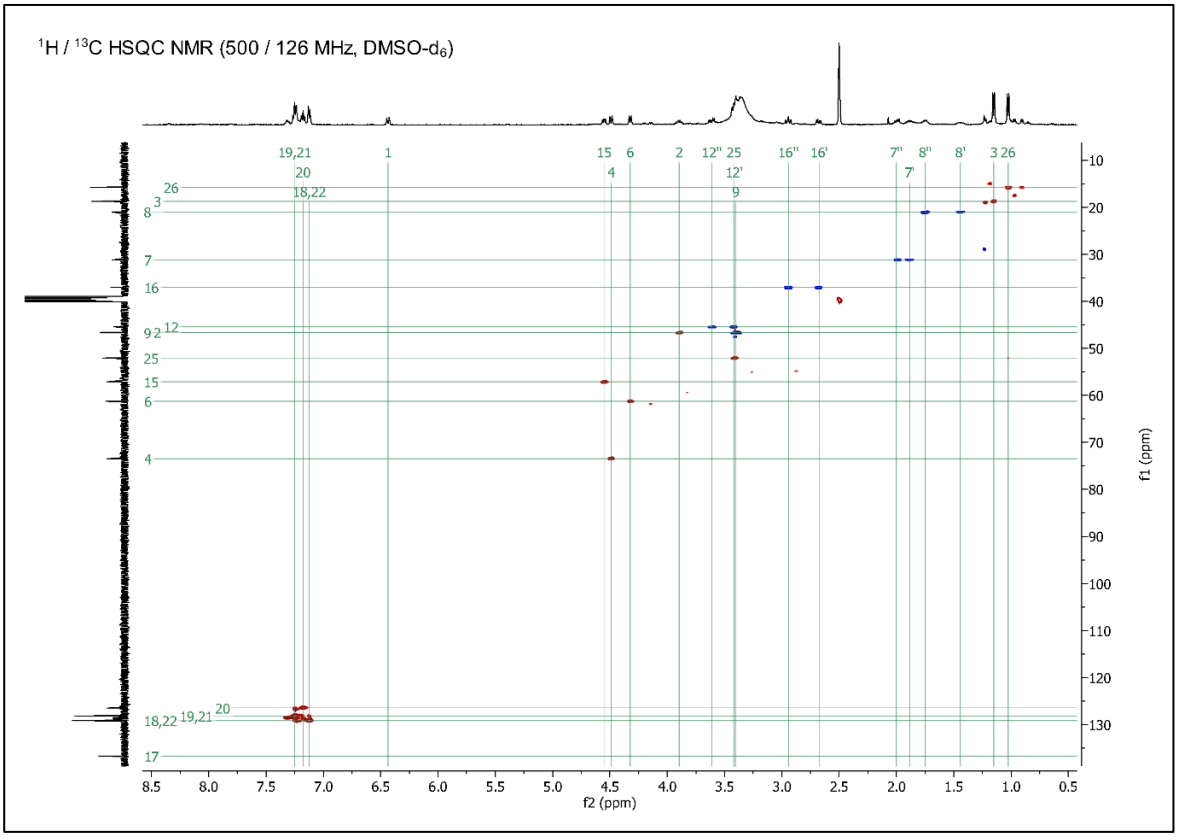
^{1}H NMR data cyc-4-Imz-A(GPFA) 2b

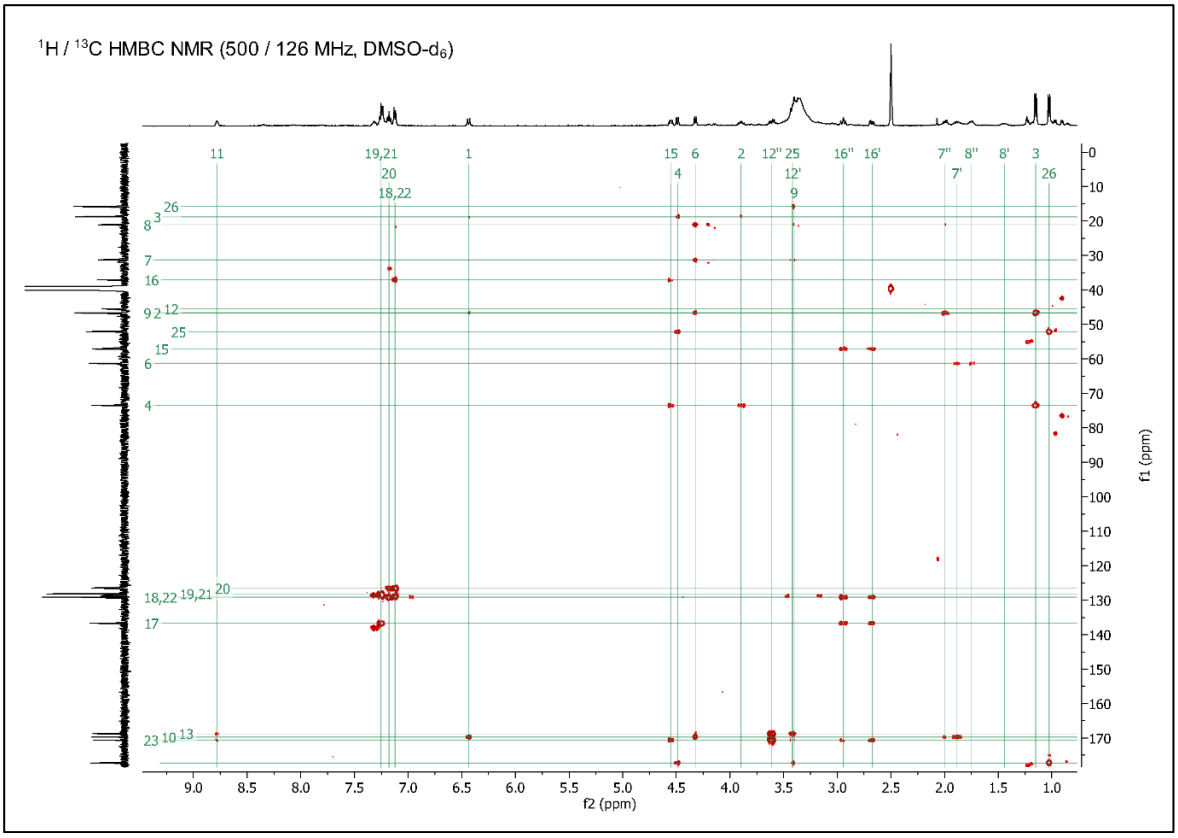


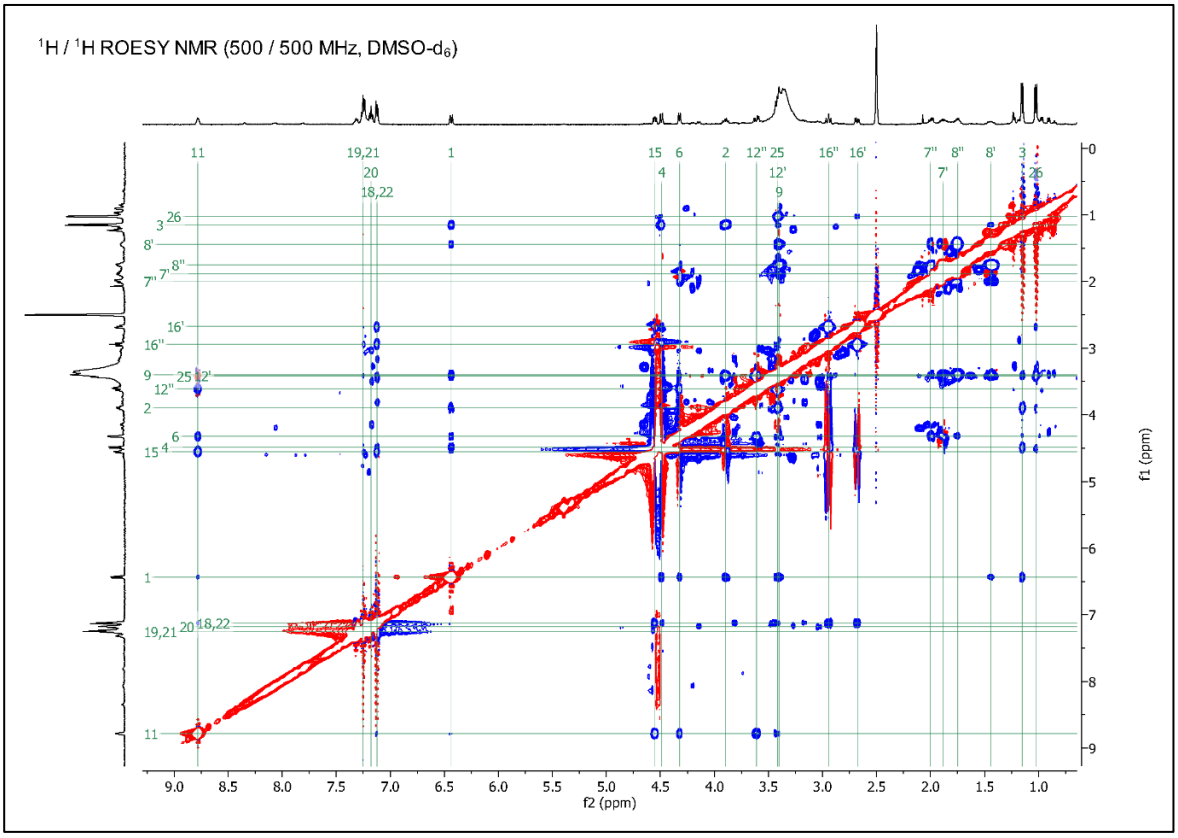
^{13}C NMR data cyc-4-Imz-A(GPFA) 2b



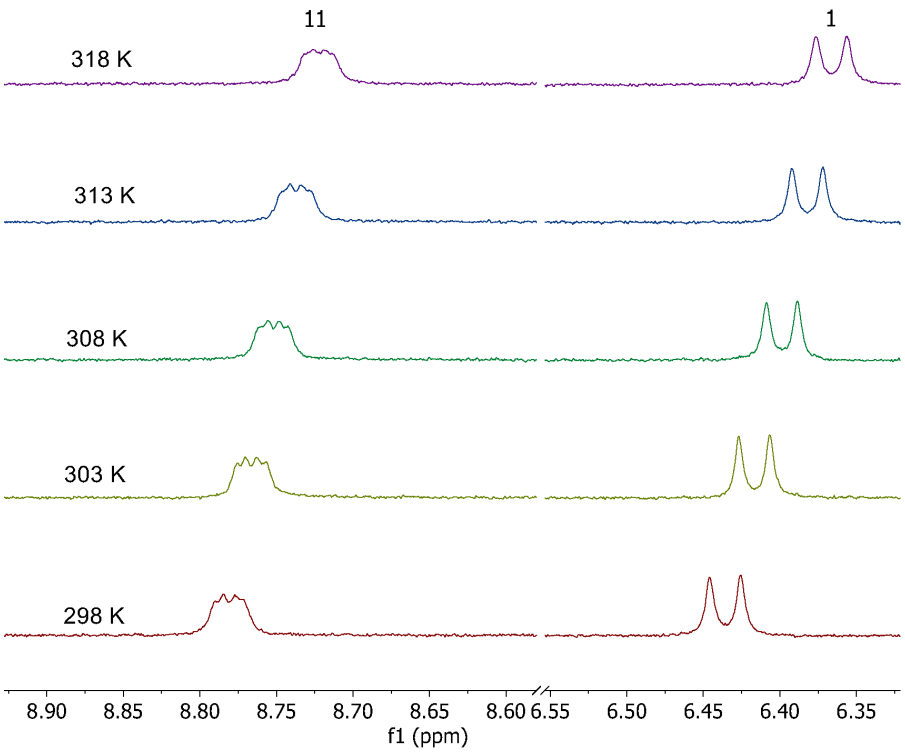

^{1}H / ^{1}H TOCSY NMR (500 / 500 MHz, DMSO-d$_6$)
f2 (ppm)
f1 (ppm)

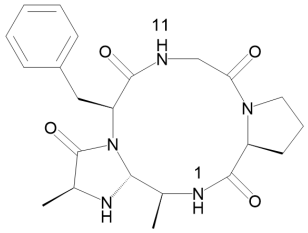

^{1}H / ^{13}C HSQC NMR (500 / 126 MHz, DMSO-d$_6$)
f2 (ppm)
f1 (ppm)


^{1}H / ^{13}C HMBC NMR (500 / 126 MHz, DMSO-d_6)
f2 (ppm)
f1 (ppm)


^{1}H / ^{1}H ROESY NMR (500 / 500 MHz, DMSO-d_6)
f2 (ppm)
f1 (ppm)

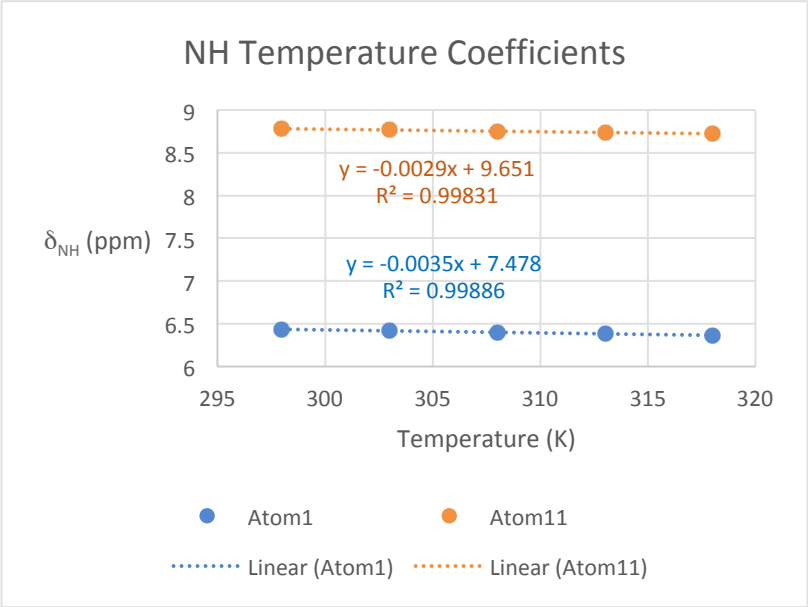
XII. Supplementary Figure 5: **VT NMR Study of cyclic tetrapeptide cyc-4-Imz-A(GPFA) 2b**
Solvent = DMSO-d_6
Temperatures (K): 298, 303, 308, 313, 318



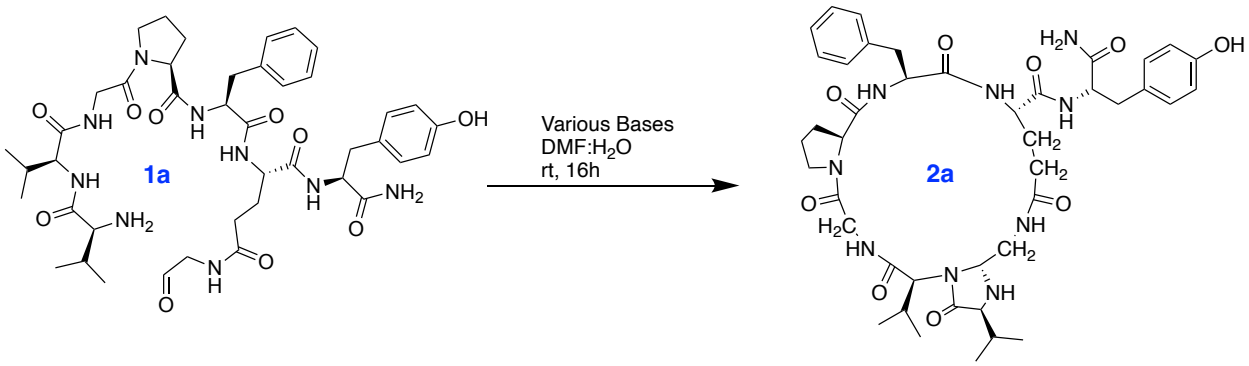


Atom:	1	11
Temp (K)	δ_{NH} (ppm)	
298	6.436	8.781
303	6.417	8.767
308	6.399	8.75
313	6.382	8.737
318	6.366	8.723
$\Delta\delta_{NH}/\Delta T$ (ppb/K):	-3.5	-2.9

H-bonding for $\Delta\delta_{NH} / \Delta T > -4.6$

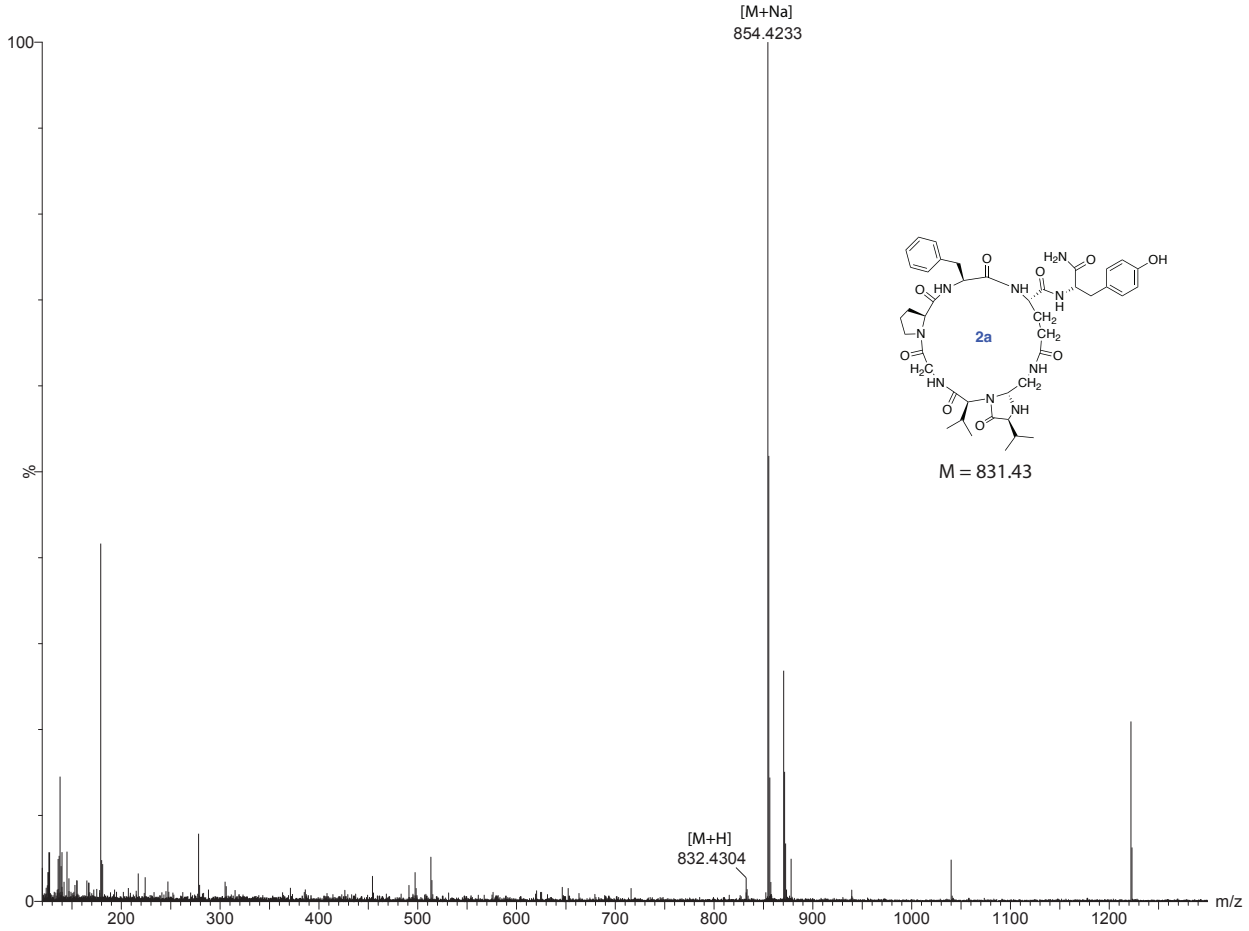


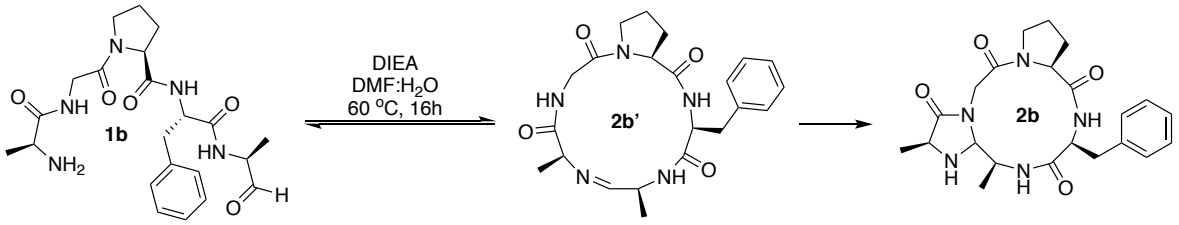
XIII. Supplementary Figure 6: **Substrate Scope of the formation of head-to-tail-cyclic tetrapeptides. Peptide Characterization and HPLC Traces**



Cyc-4-Imz-Val(Val-Gly-Pro-Phe-Glu-Tyr) (2a). LCMS: *m/z* 832.4304 (calcd $[M+H]^+$ = 832.44), *m/z* 854.4233 (calcd $[M+Na]^+$ = 854.44, Purity: >95% (HPLC analysis at 220 nm). Retention time: 14.7 min

HRMS spectra of the macrocyclized product **2a**

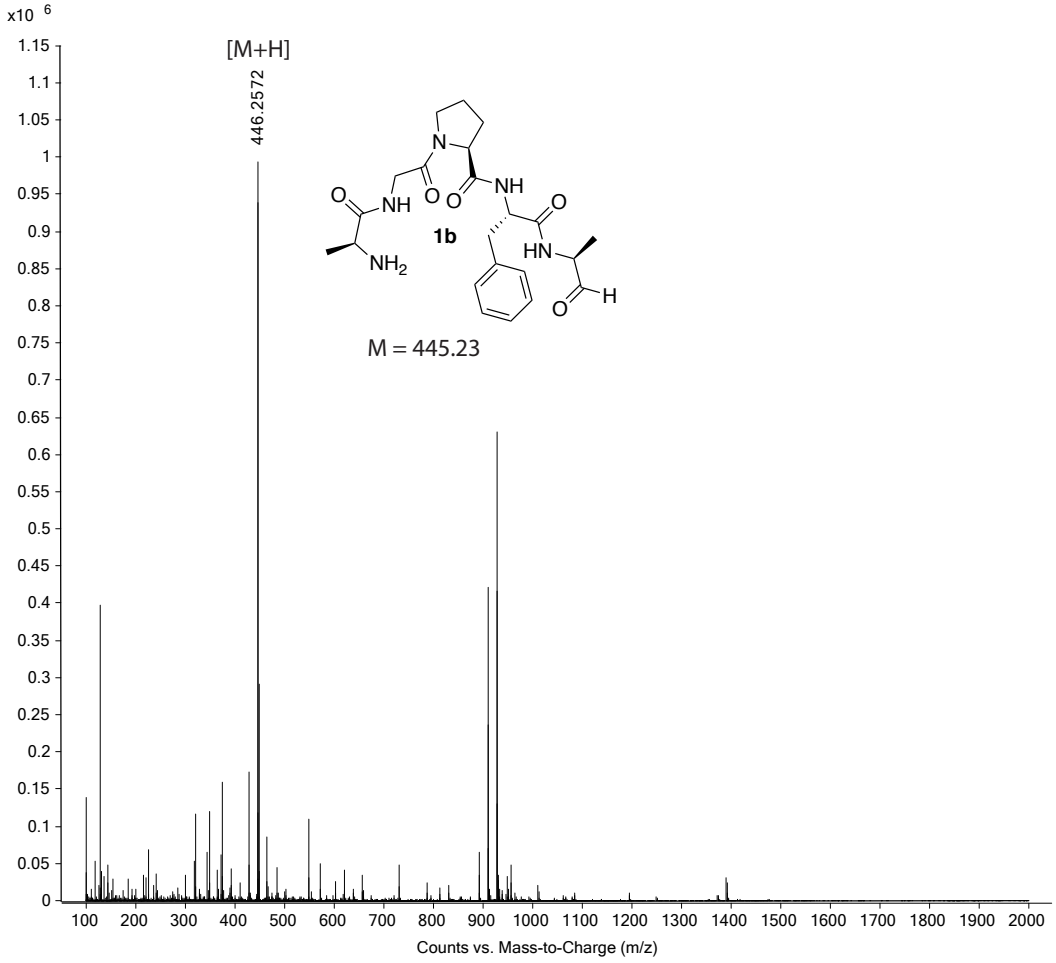




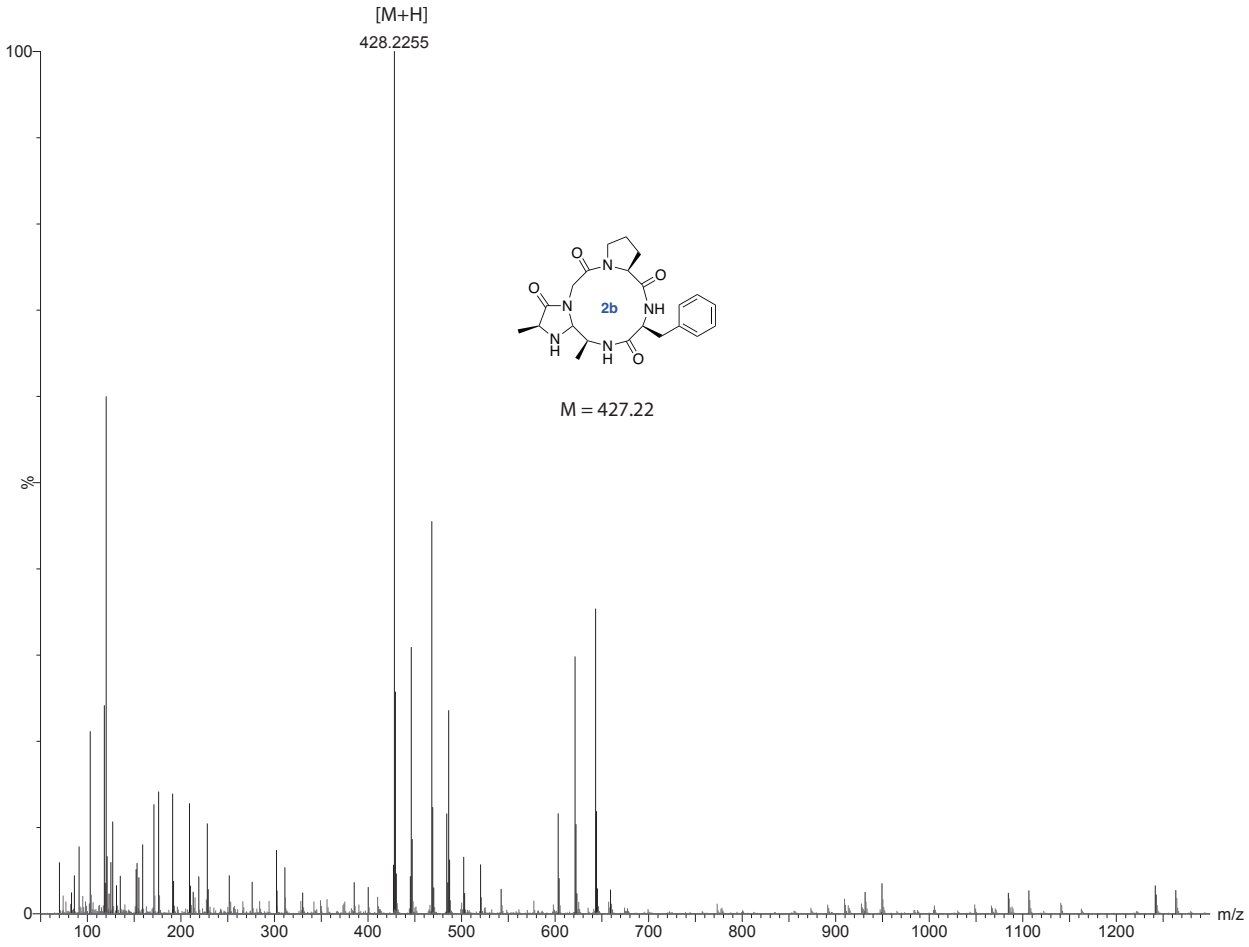
Ala-Gly-Pro-Phe-Ala (1b). LCMS: *m/z* 446.2572 (calcd $[M+H]^+$ = 446.24), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.2 min

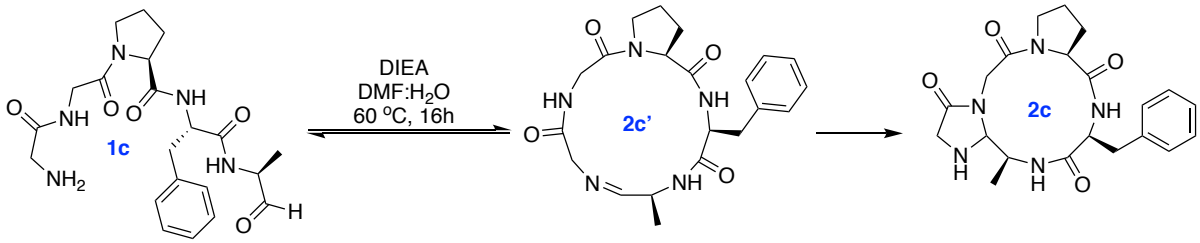
***cyc*-4-Imz-Ala(-Gly-Pro-Phe-Ala) (2b).** LCMS: *m/z* 428.2255 (calcd $[M+H]^+$ = 428.23), Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.78 min

HRMS spectra of the linear peptide **1b**



HRMS spectra of the macrocyclized product **2b**

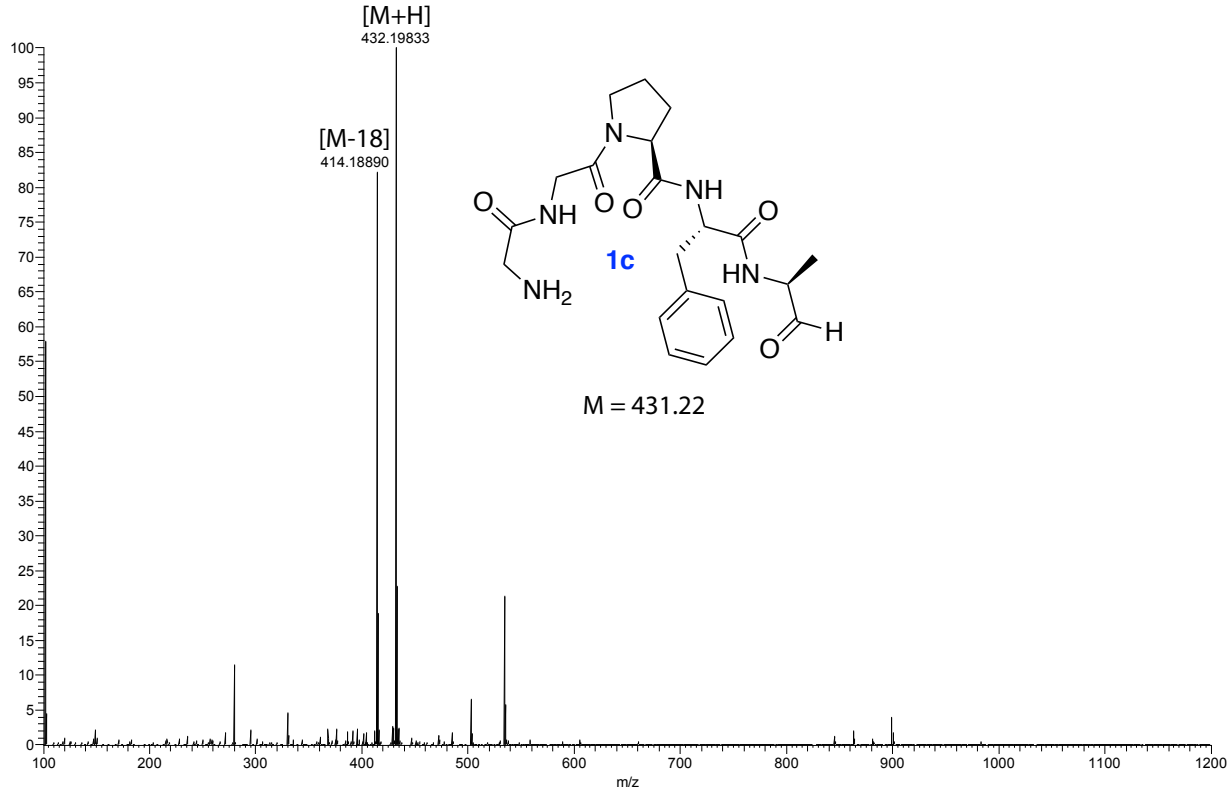




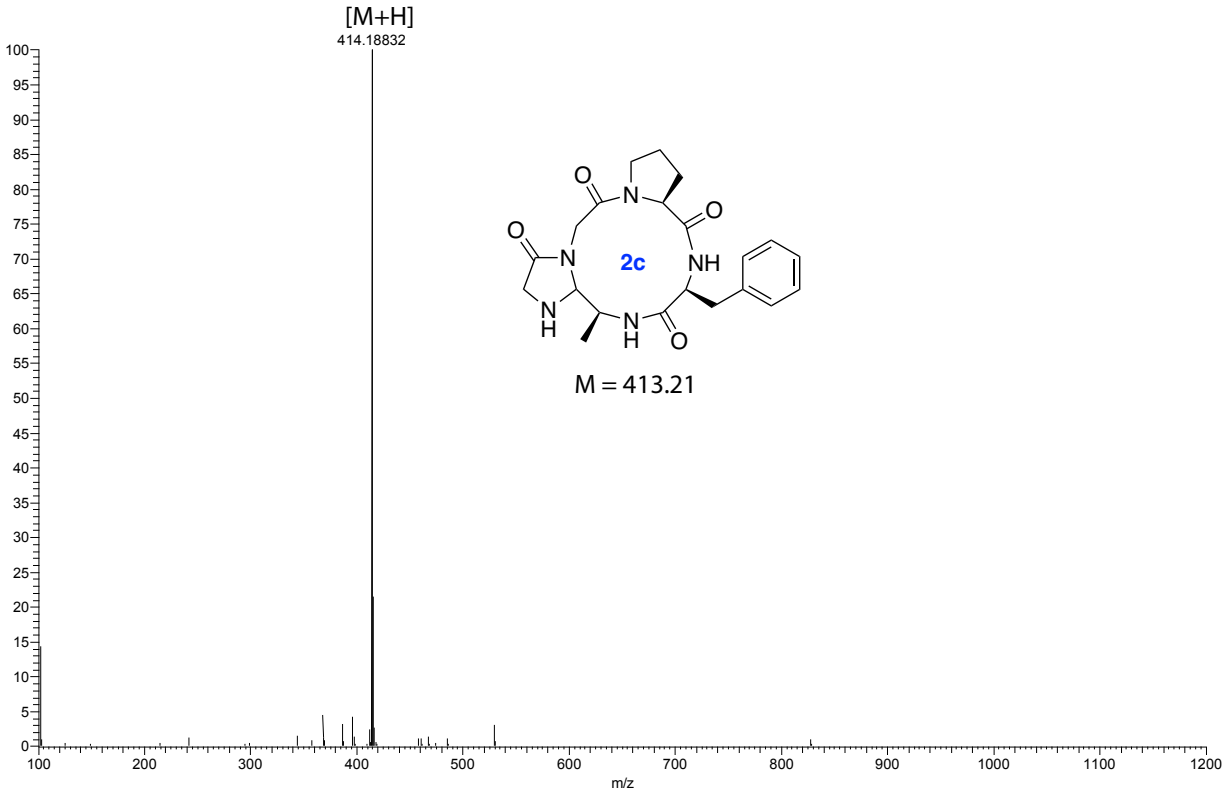
Gly-Gly-Pro-Phe-Ala (1c). LCMS: *m/z* 432.1983 (calcd $[M+H]^+$ = 432.22), *m/z* 414.1889 (calcd $[M-18]^+$ = 414.22), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.9 min

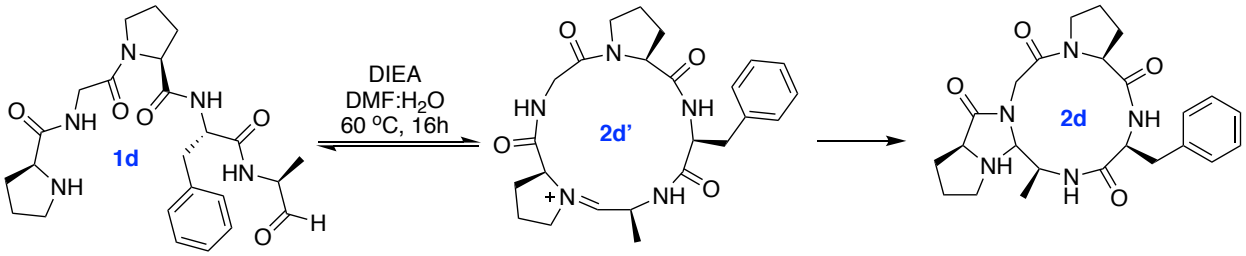
***cyc*-4-Imz-Gly(-Gly-Pro-Phe-Ala) (2c).** LCMS: *m/z* 414.1883 (calcd $[M+H]^+$ = 414.21), Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.81 min

HRMS spectra of the linear peptide **1c**



HRMS spectra of the macrocyclized product **2c**

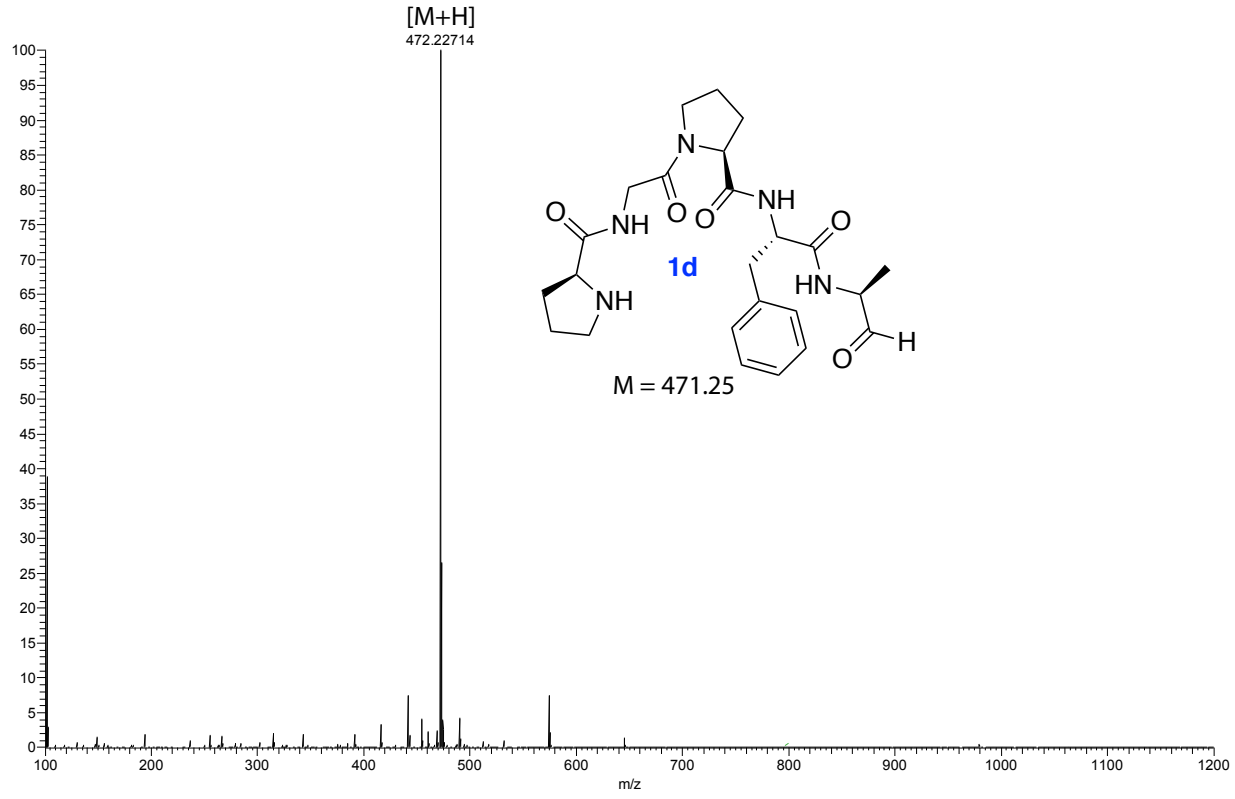




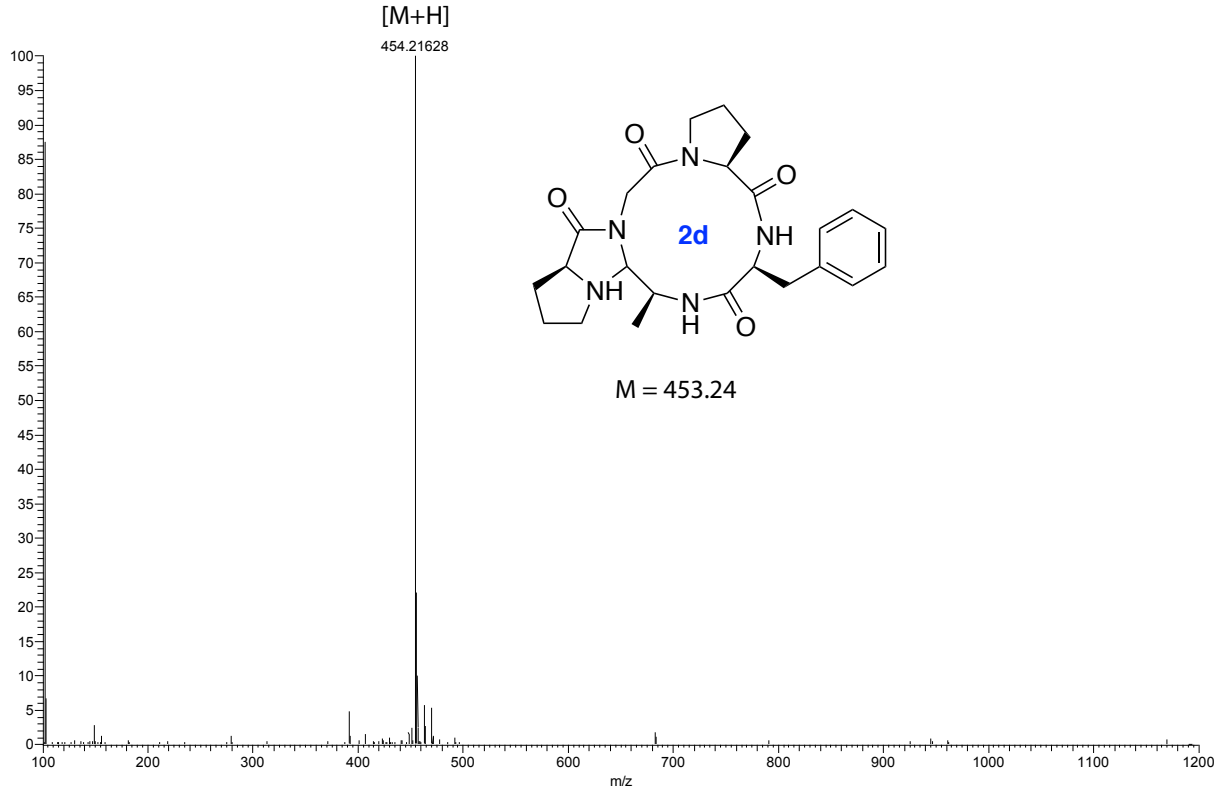
Pro-Gly-Pro-Phe-Ala (1d). LCMS: *m/z* 472.2271 (calcd $[M+H]^+$ = 472.26), Purity: >95% (HPLC analysis at 220 nm). Retention time: 8.5 min

***cyc*-4-Imz-Pro(-Gly-Pro-Phe-Ala) (2d).** LCMS: *m/z* 454.2162 (calcd $[M+H]^+$ = 454.25), Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.59 min

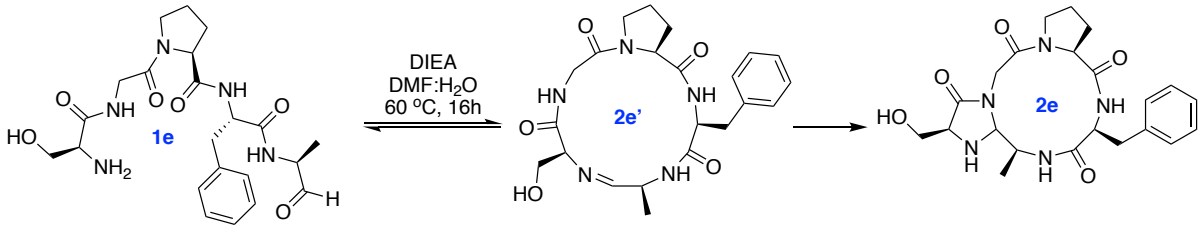
HRMS spectra of the linear peptide **1d**



HRMS spectra of the macrocyclized product **2d**



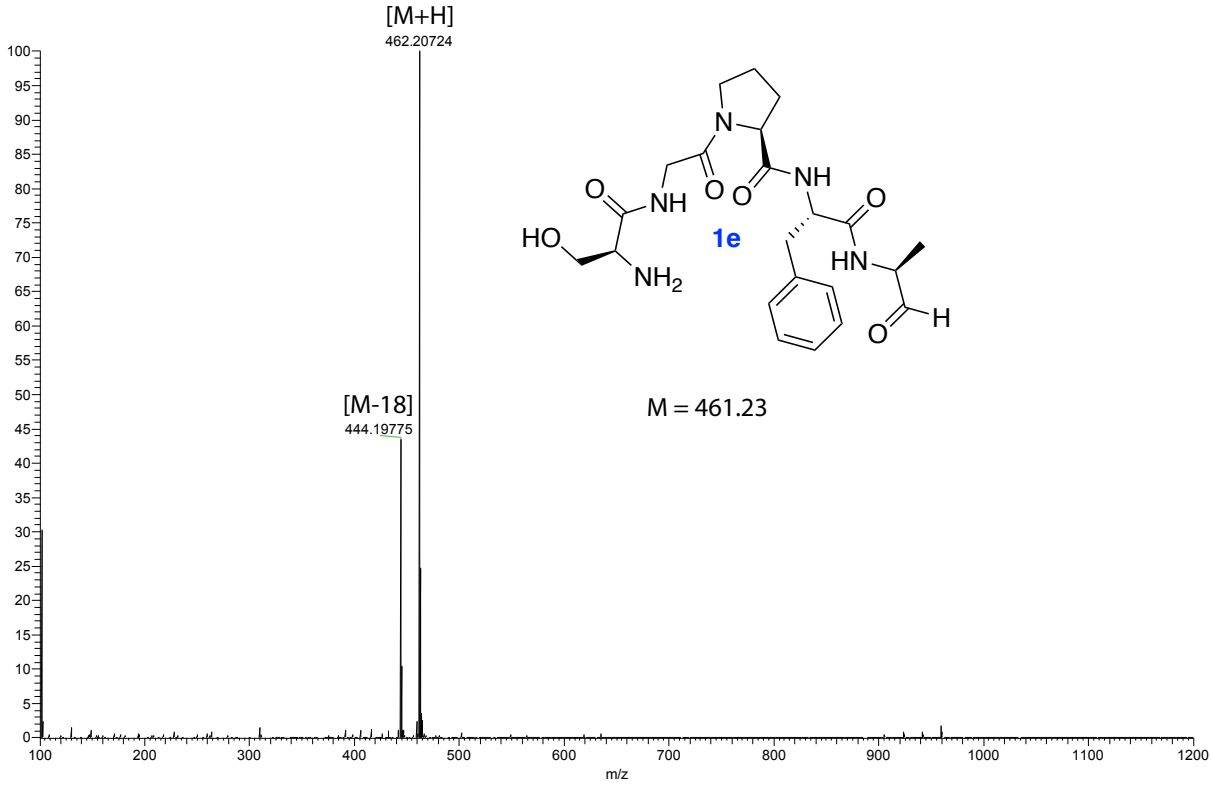
M = 453.24



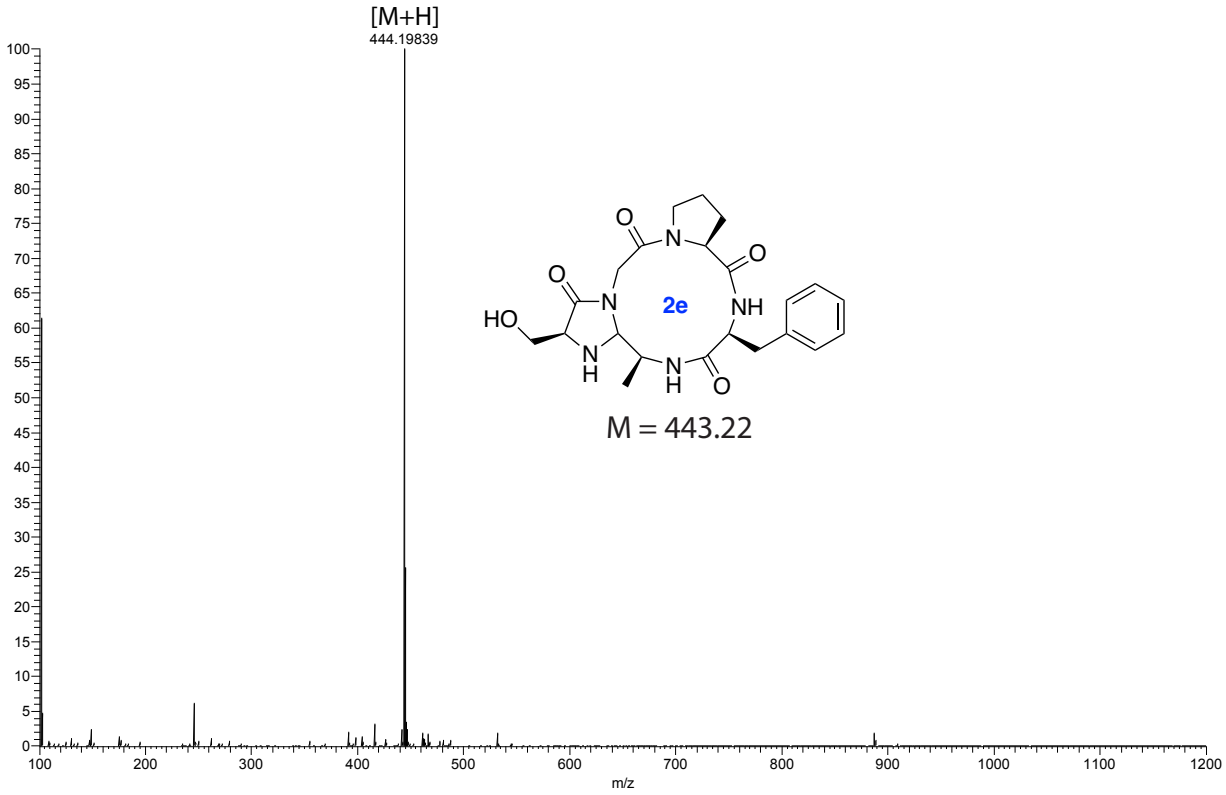
Ser-Gly-Pro-Phe-Ala (1e). LCMS: *m/z* 462.2072 (calcd $[M+H]^+$ = 462.24), *m/z* 444.1977 (calcd $[M-18]^+$ = 444.24), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.5 min

***Cyc*-4-Imz-Ser(-Gly-Pro-Phe-Ala) (2e).** LCMS: *m/z* 444.1983 (calcd $[M+H]^+$ = 444.22), Purity: >95% (HPLC analysis at 220 nm). Retention time: 11.26 min

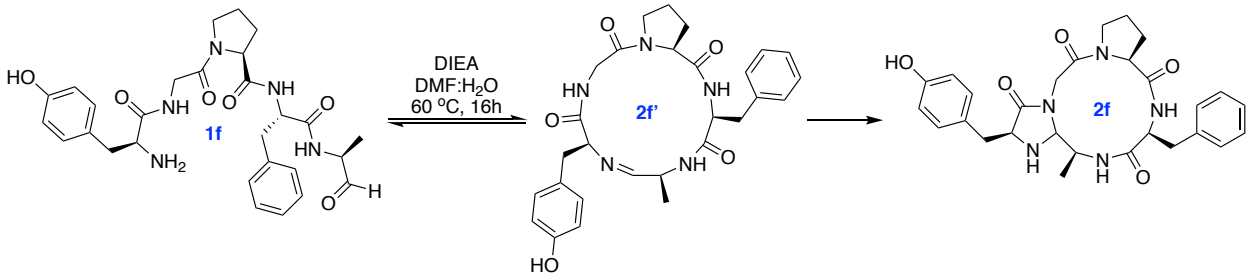
HRMS spectra of linear peptide **1e**



HRMS spectra of the macrocyclized product **2e**



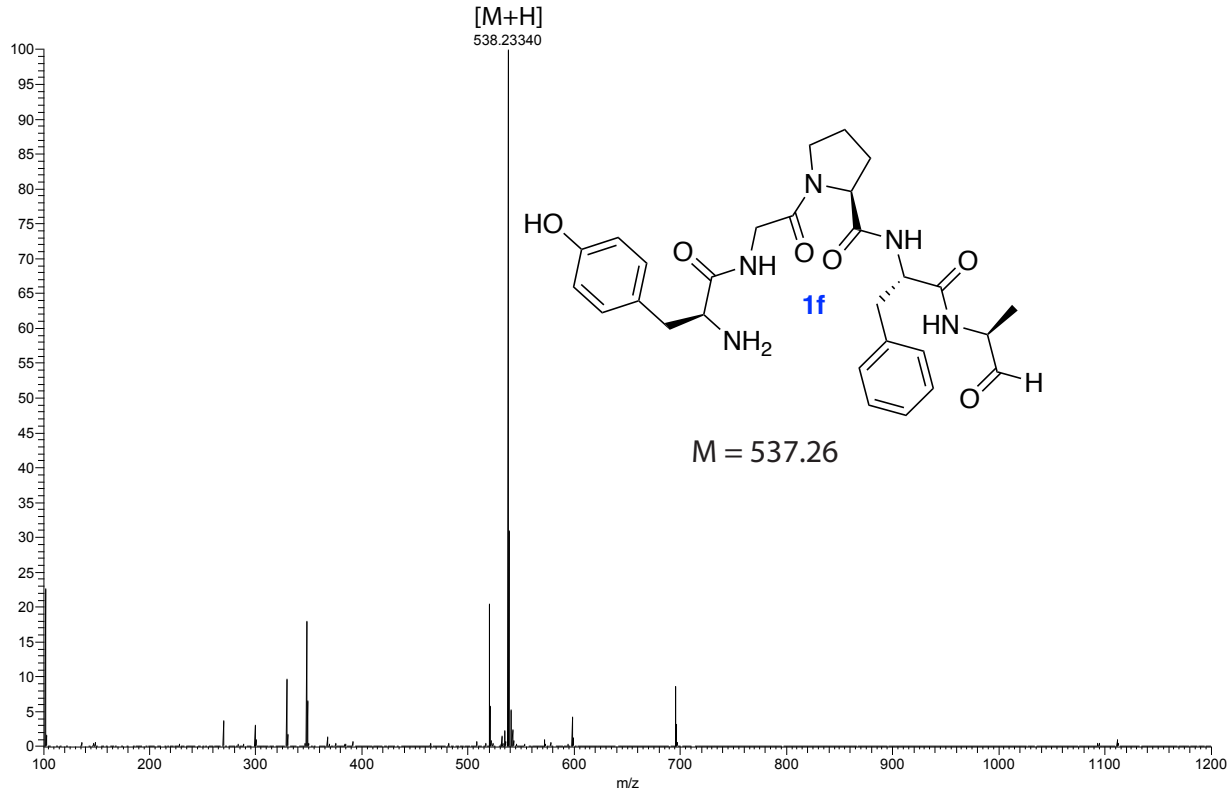
M = 443.22



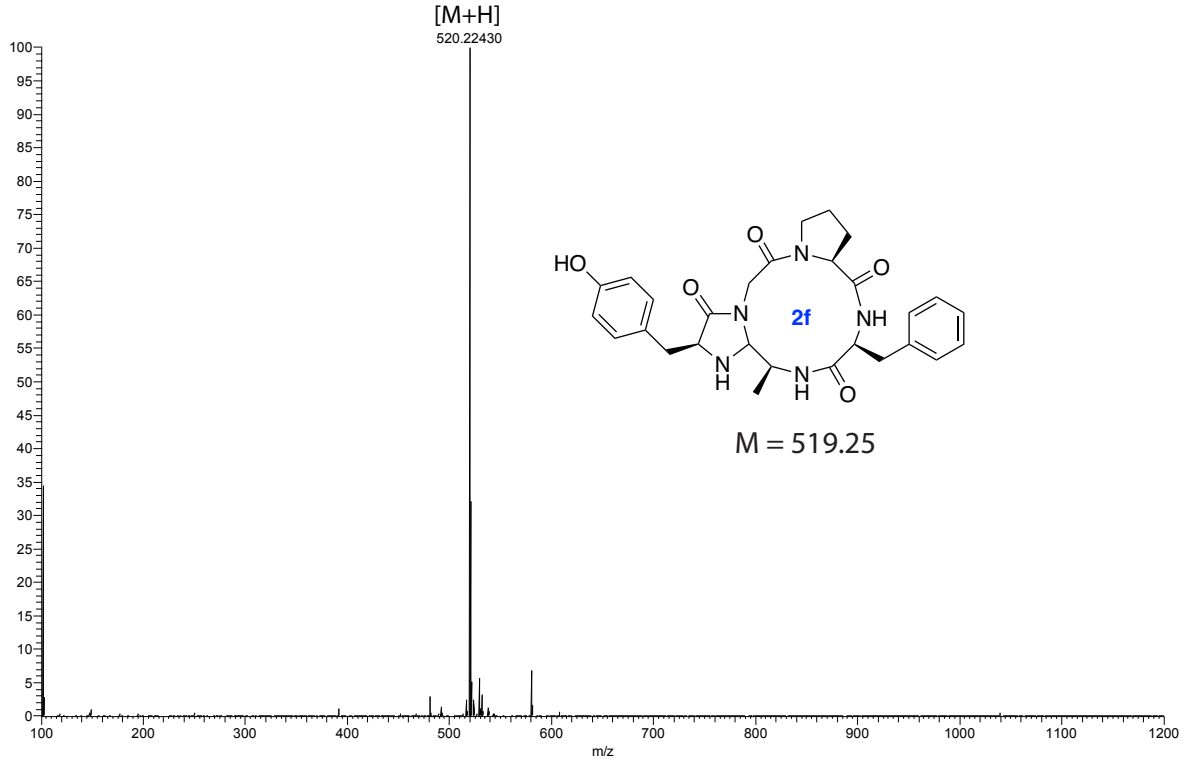
Tyr-Gly-Pro-Phe-Ala (1f). LCMS: *m/z* 538.2334 (calcd $[M+H]^+$ = 538.27), Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.4 min

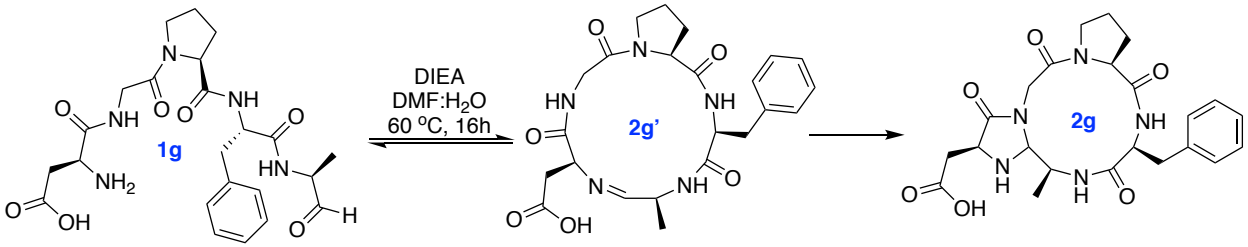
***cyc*-4-Imz-Tyr(-Gly-Pro-Phe-Ala) (2f).** LCMS: *m/z* 520.2243 (calcd $[M+H]^+$ = 520.26), Purity: >95% (HPLC analysis at 220 nm). Retention time: 13.98 min

HRMS spectra of linear peptide **1f**



HRMS spectra of the macrocyclized product **2f**

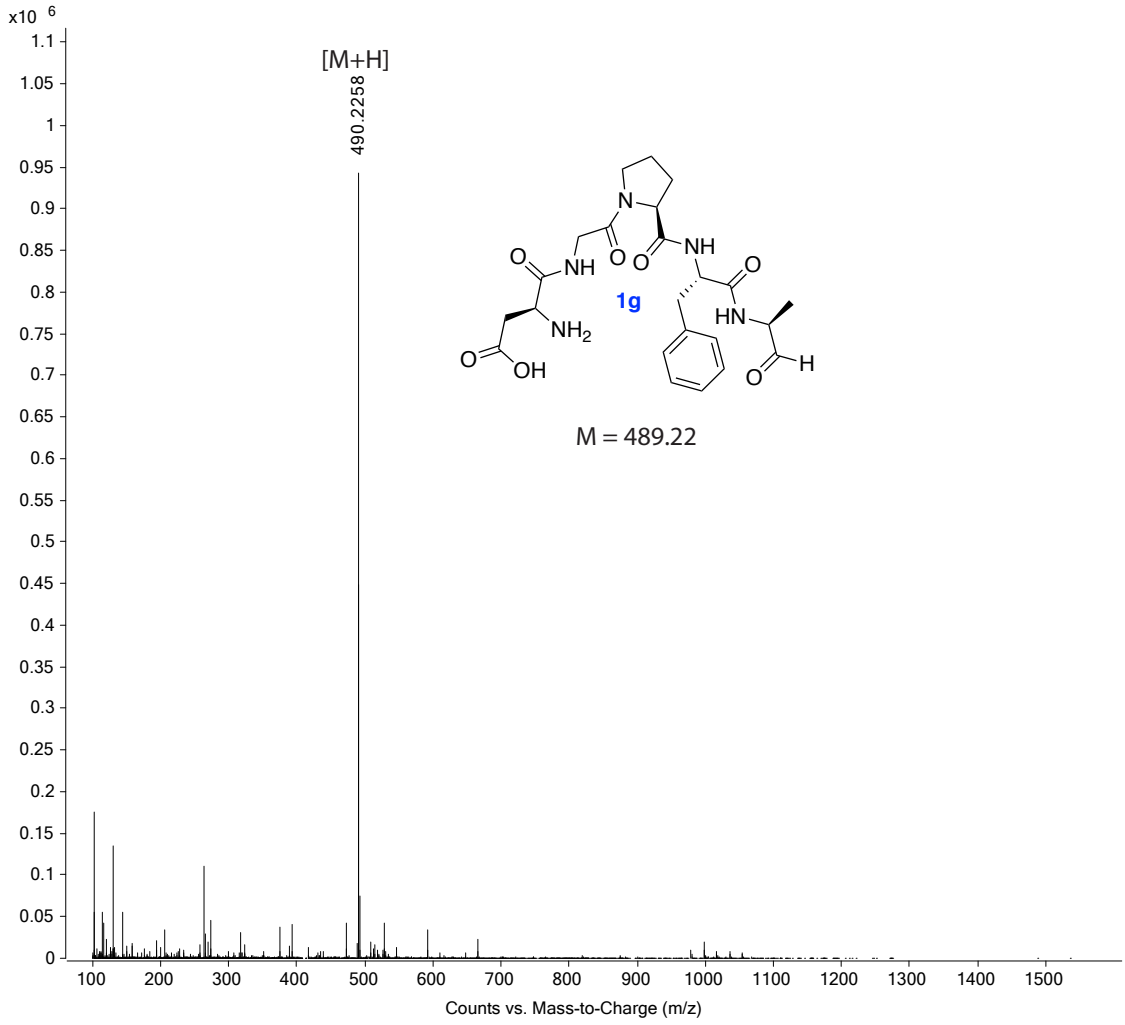




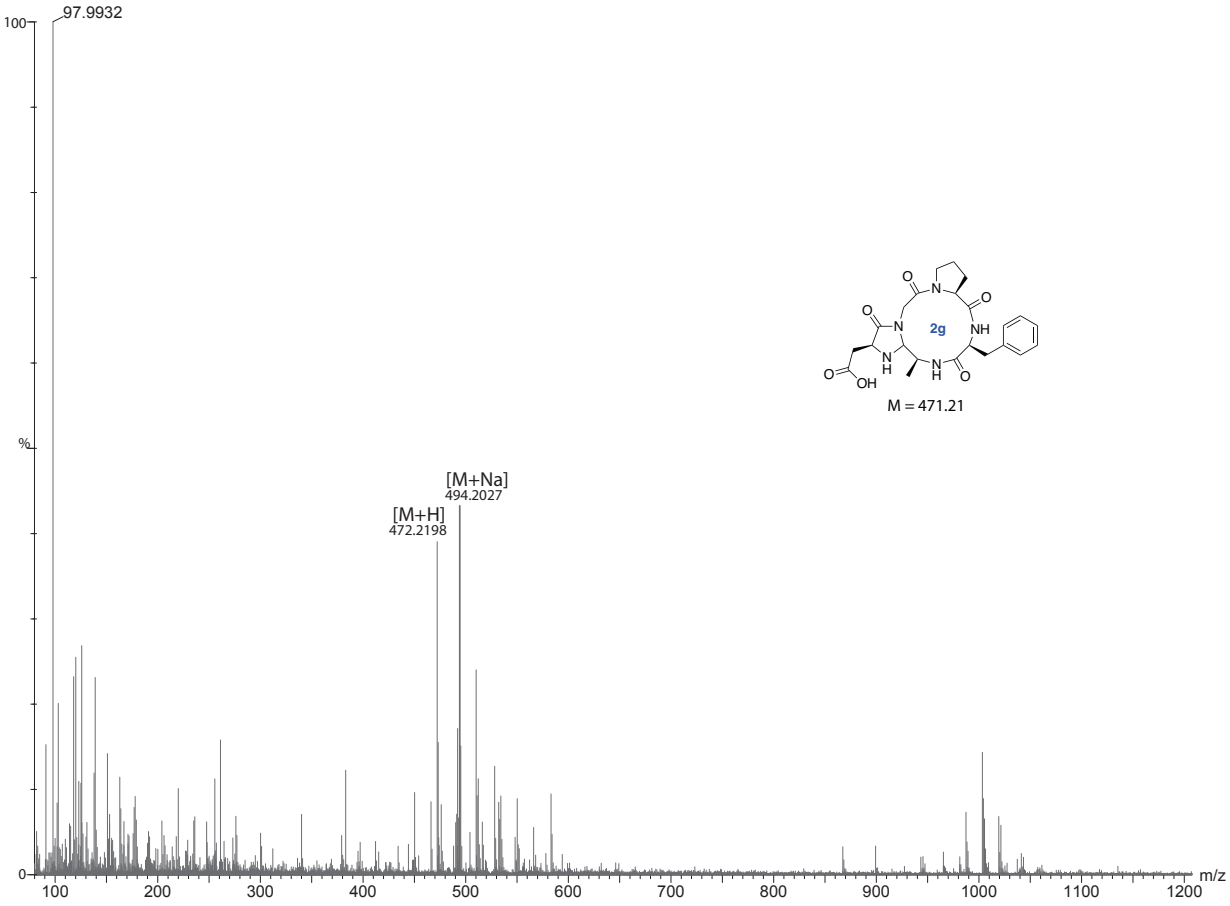
Asp-Gly-Pro-Phe-Ala (1g). LCMS: *m/z* 490.2258 (calcd $[M+H]^+$ = 490.23), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.62 min

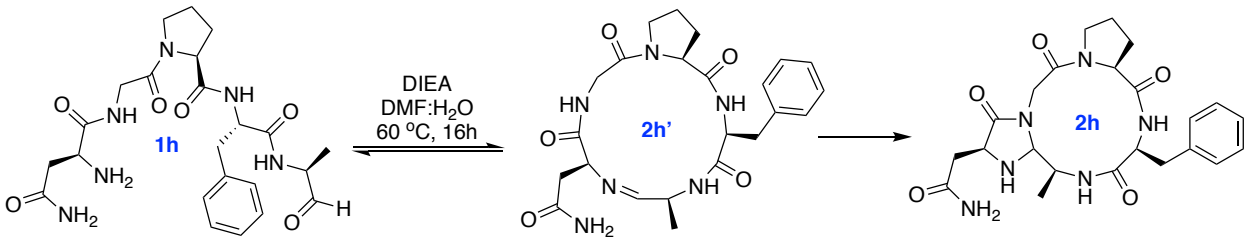
***Cyc*-4-Imz-Asp(-Gly-Pro-Phe-Ala (2g).** LCMS: *m/z* 472.2198 (calcd $[M+H]^+$ = 472.22), *m/z* 494.2027 (calcd $[M+Na]^+$ = 494.22), Purity: >95% (HPLC analysis at 220 nm). Retention time: 12.19 min

HRMS spectra of linear peptide **1g**



HRMS spectra of the macrocyclized product **2g**

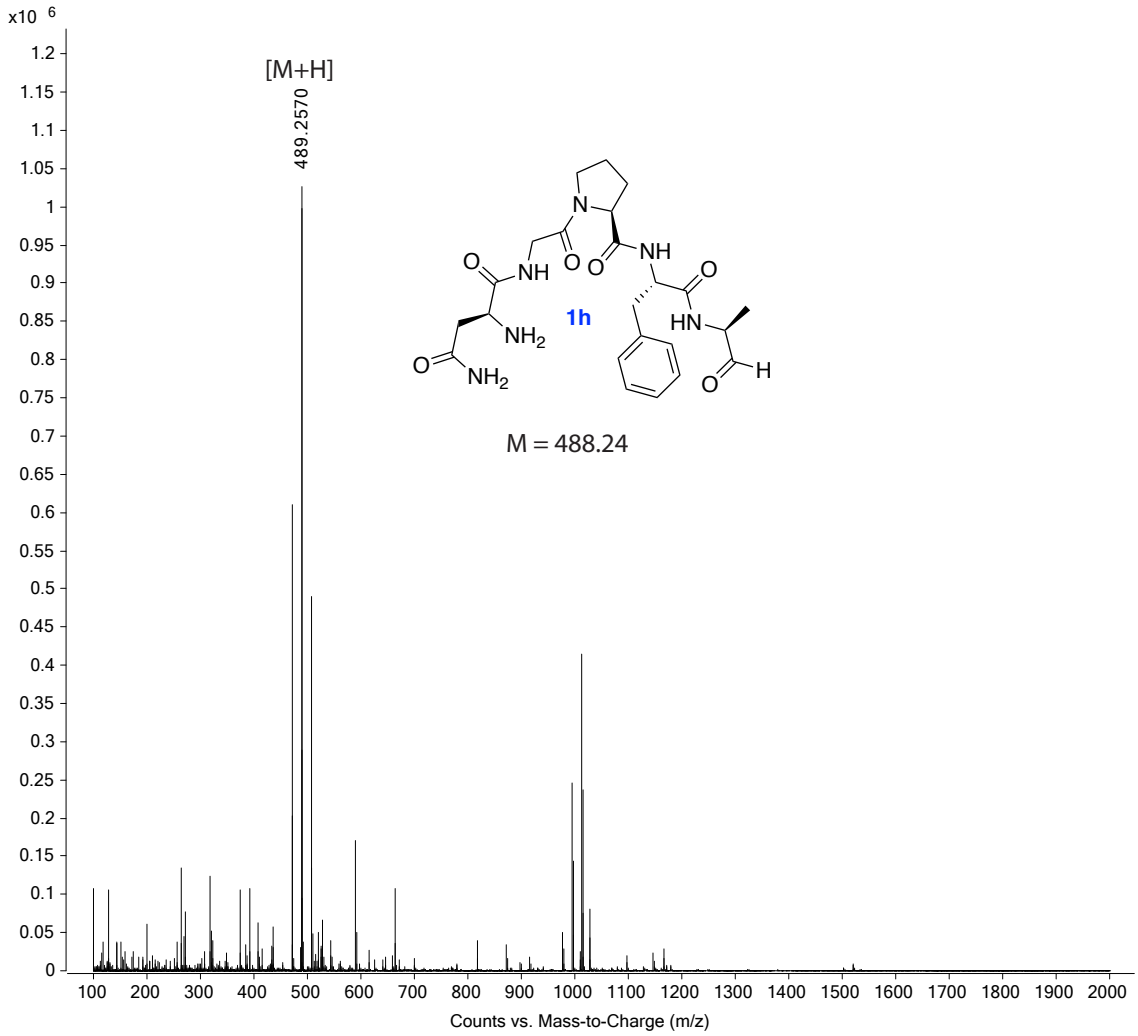




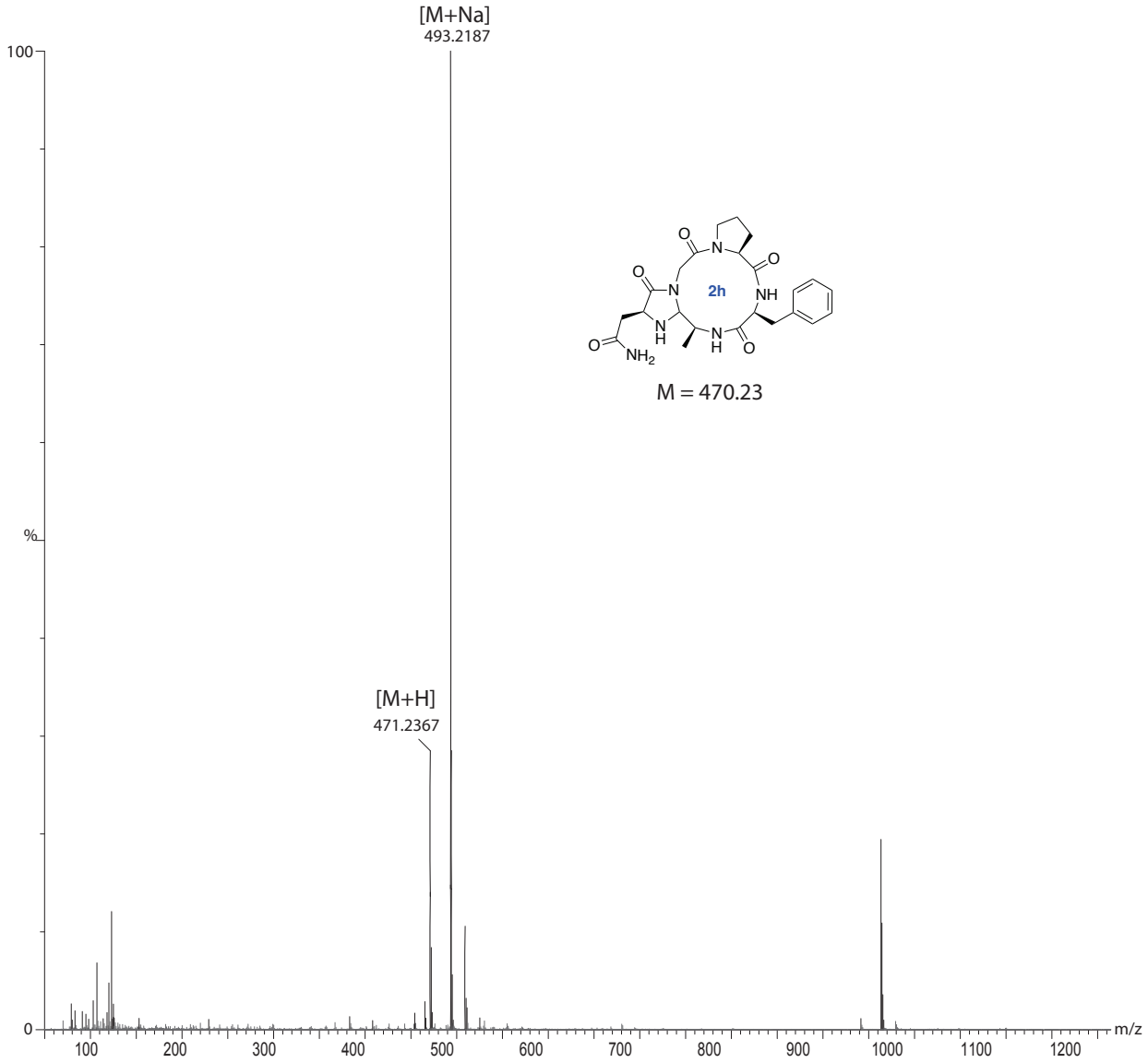
Asn-Gly-Pro-Phe-Ala (1h). LCMS: *m/z* 489.2570 (calcd $[M+H]^+$ = 489.25), Purity: >95% (HPLC analysis at 220 nm). Retention time: 6.13 min

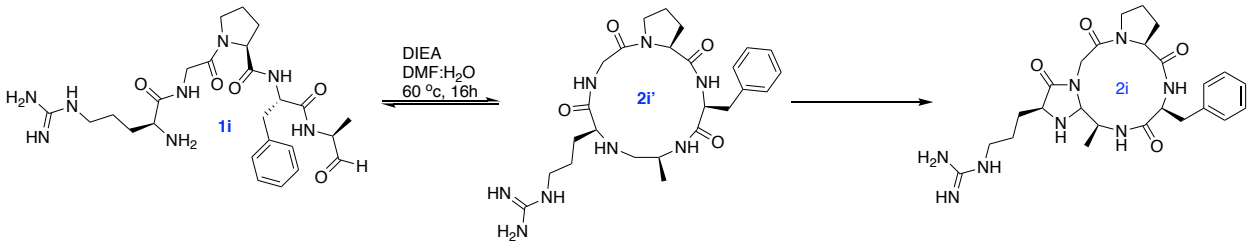
***Cyc*-4-Imz-Asn(-Gly-Pro-Phe-Ala) (2h).** LCMS: *m/z* 471.2367 (calcd $[M+H]^+$ = 471.24), *m/z* 493.2187 (calcd $[M+Na]^+$ = 493.24), Purity: >95% (HPLC analysis at 220 nm). Retention time: 10.18 min

HRMS spectra of linear peptide **1h**



HRMS spectra of the macrocyclized product **2h**

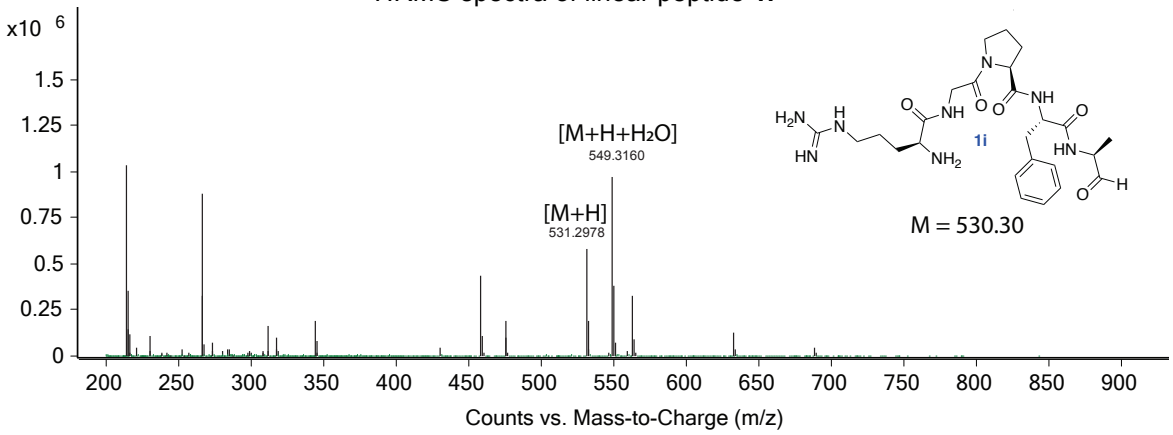




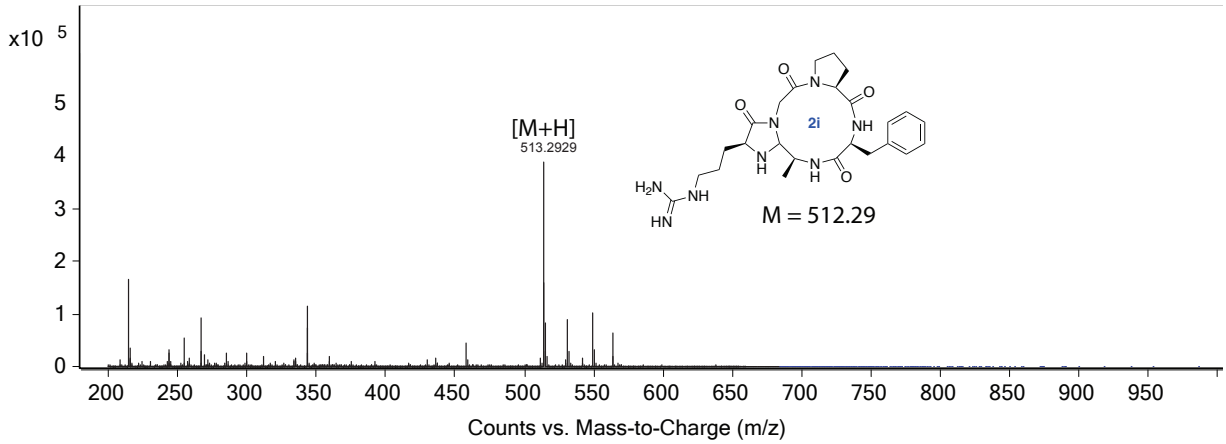
Arg-Gly-Pro-Phe-Ala (1i). LCMS: *m/z* 531.2978 (calcd $[M+H]^+$ = 531.30), m/z 549.3160 (calcd $[M+H+H_2O]^+$ = 549.31), Purity: >95% (HPLC analysis at 220 nm). Retention time: 5.5 min

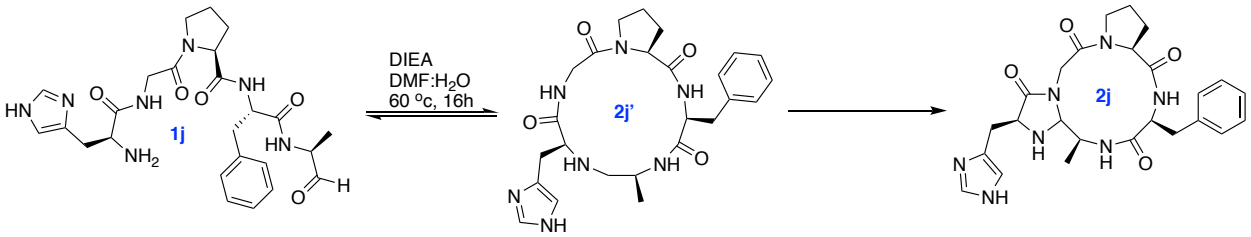
***Cyc*-4-Imz-Arg(-Gly-Pro-Phe-Ala) (2i).** LCMS: *m/z* 513.2929 (calcd $[M+H]^+$ = 513.29 Purity: >95% (HPLC analysis at 220 nm). Retention time: 8.90 min

HRMS spectra of linear peptide **1i**



HRMS spectra of the macrocyclized product **2i**

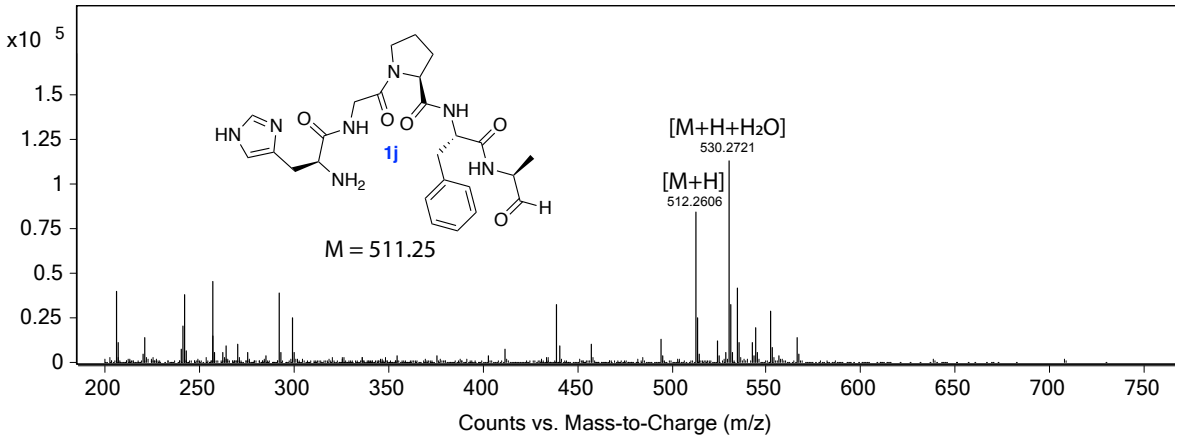




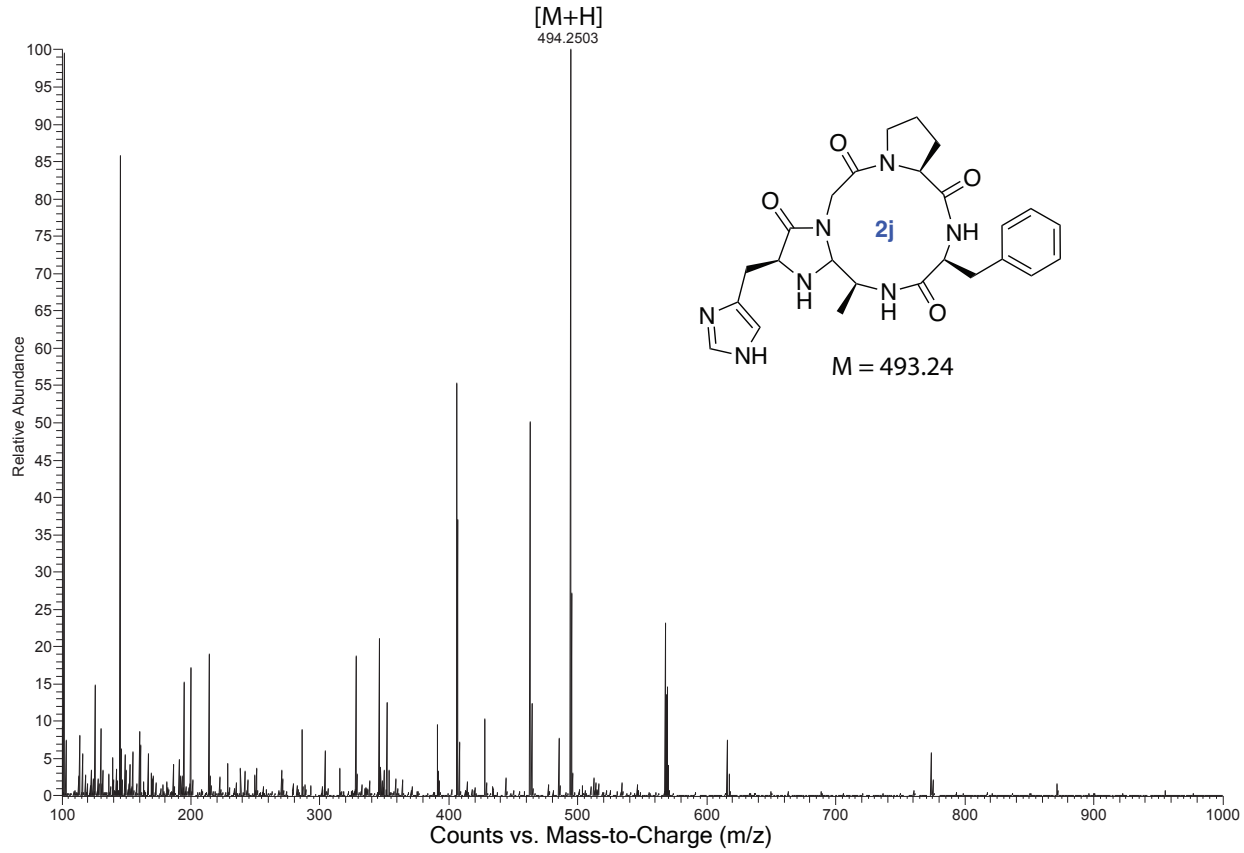
His-Gly-Pro-Phe-Ala (1j). LCMS: *m/z* 489.2570 (calcd $[M+H]^+$ = 489.25), m/z 530.2721 (calcd $[M+H+H_2O]^+$ = 530.27), Purity: >95% (HPLC analysis at 220 nm). Retention time: 6.13 min

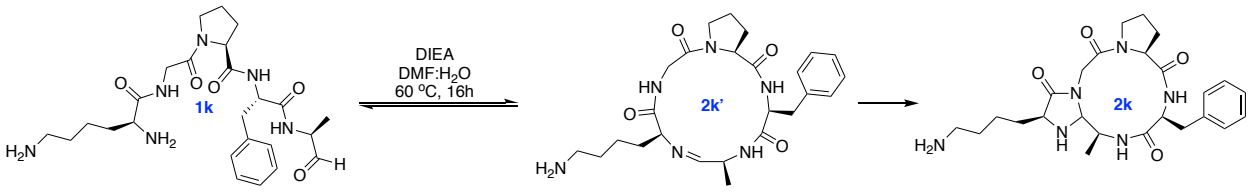
***Cyc*-4-Imz-His(-Gly-Pro-Phe-Ala) (2j).** LCMS: *m/z* 494.2503 (calcd $[M+H]^+$ = 494.24), *m/z* 515.2222 (calcd $[M+Na]^+$ = 516.24), Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.2 min

HRMS spectra of linear peptide **1j**



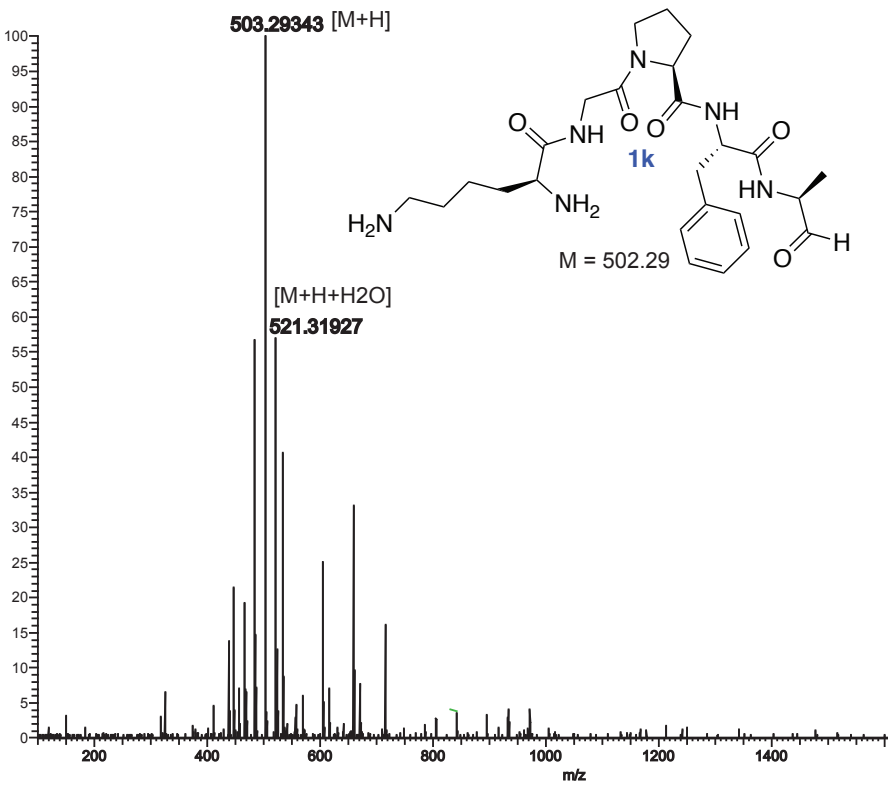
HRMS spectra of the macrocyclized product **2j**

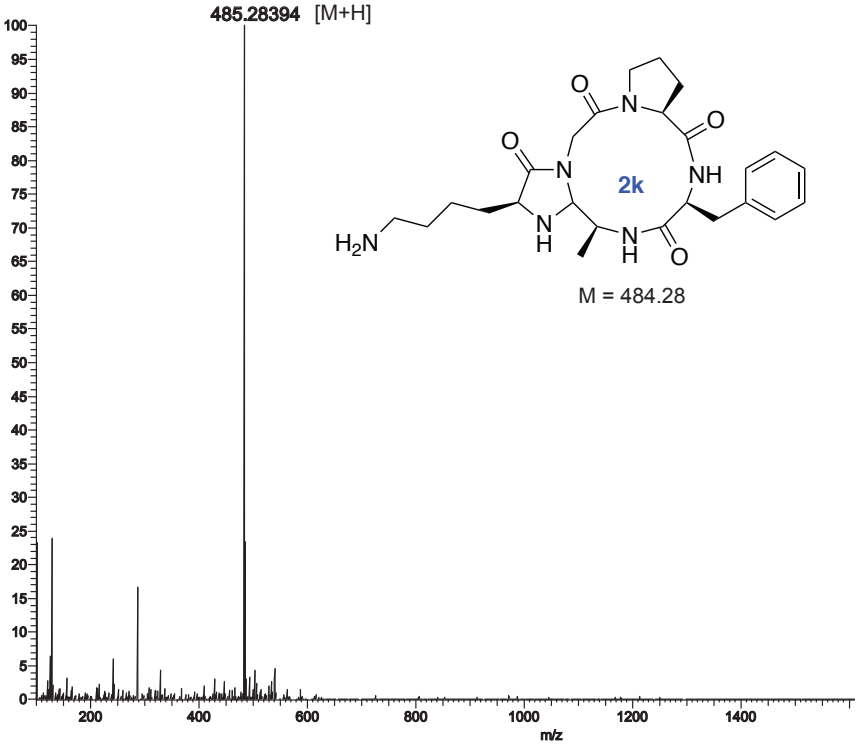


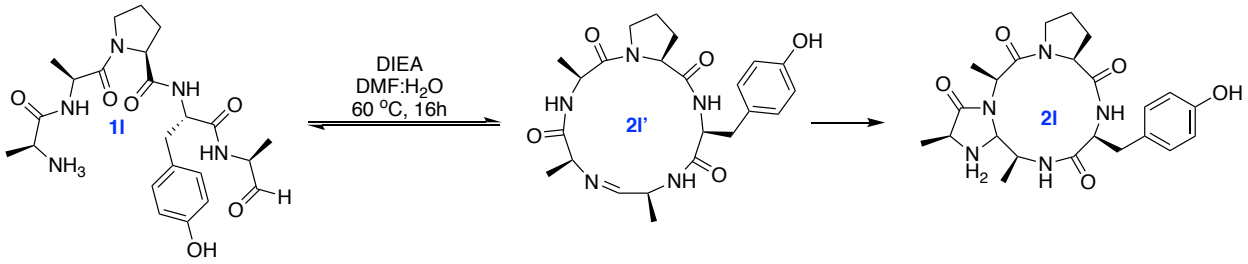


Lys-Gly-Pro-Phe-Ala (1k). LCMS: *m/z* 503.2934 (calcd $[M+H]^+$ = 503.30), m/z 521.31927 (calcd $[M+H+H_2O]^+$ = 521.30), Purity: >95% (HPLC analysis at 220 nm). Retention time: 5.5 min

***Cyc*-4-Imz-Lys(-Gly-Pro-Phe-Ala) (2k).** LCMS: *m/z* 485.2839 (calcd $[M+H]^+$ = 485.29),) Purity: >95% (HPLC analysis at 220 nm). Retention time: 8.5 min

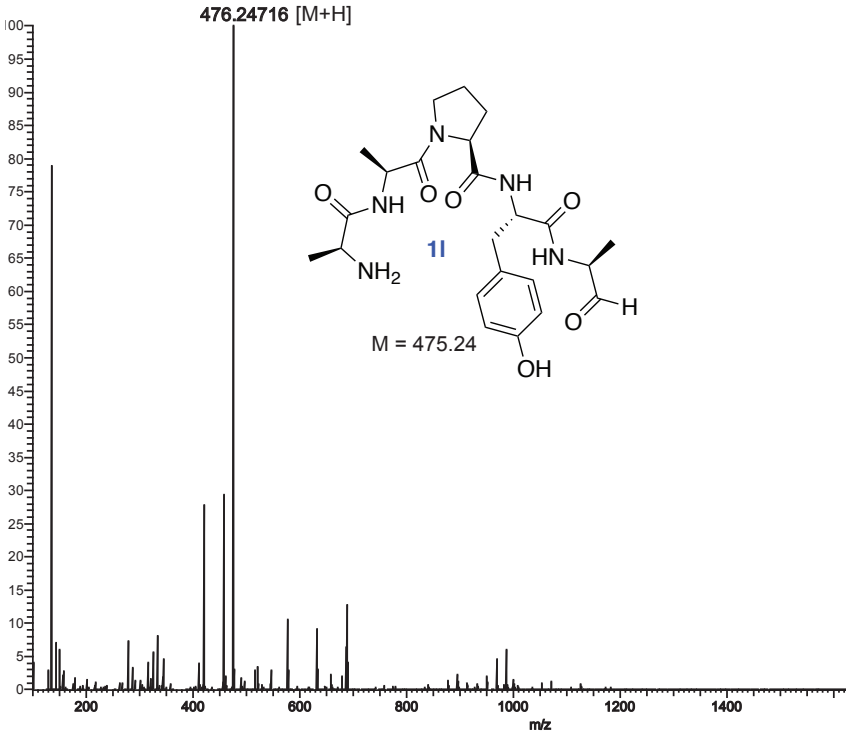

503.29343 [M+H]
[M+H+H2O]
521.31927
1k
M = 502.29
m/z

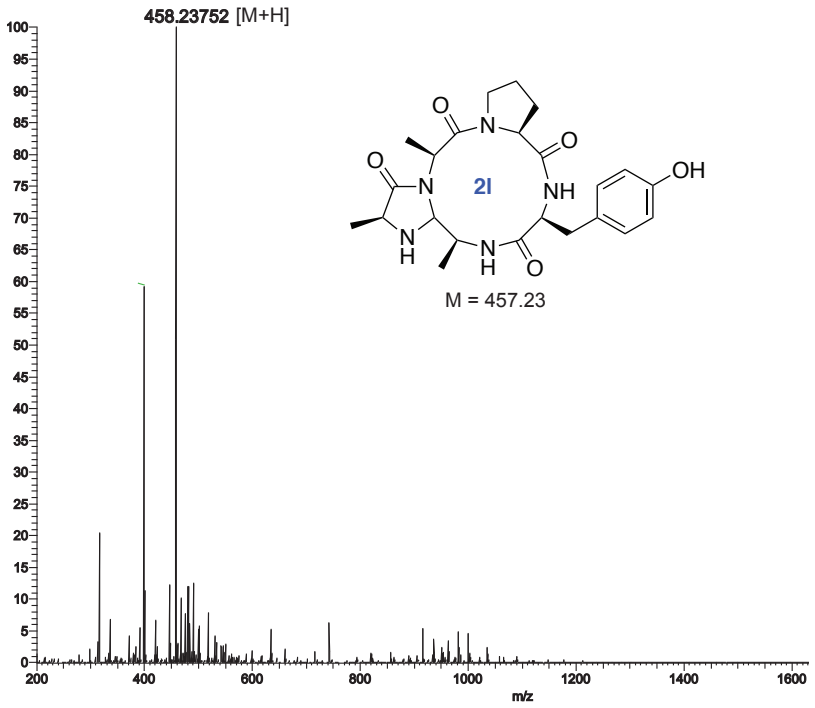

485.28394 [M+H]
2k
M = 484.28
m/z



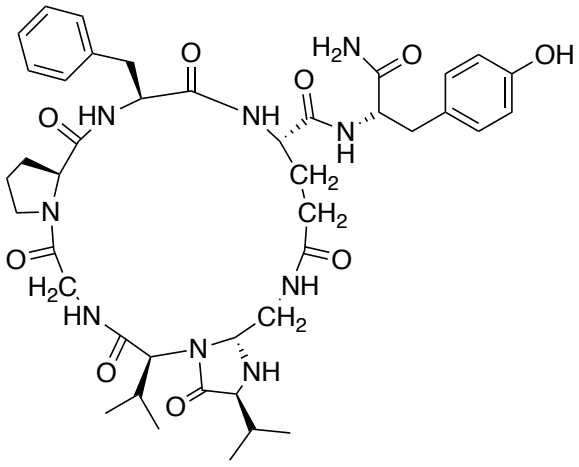
Ala-Ala-Pro-Tyr-Ala (1l). LCMS: *m/z* 476.2471 (calcd $[M+H]^+$ = 476.25), Purity: >95% (HPLC analysis at 220 nm). Retention time: 6.0 min

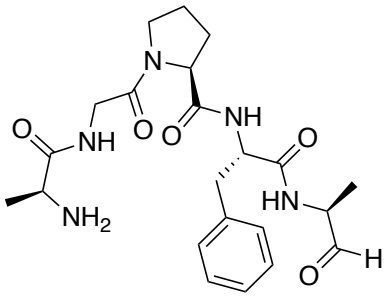
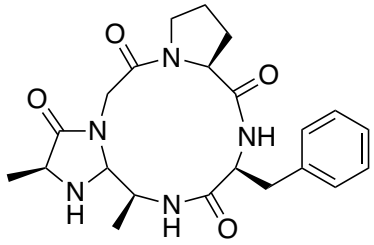
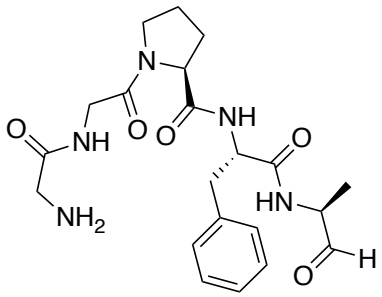
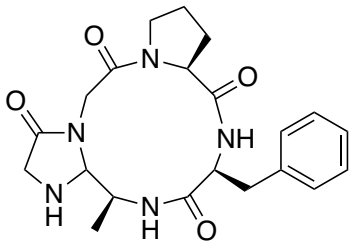
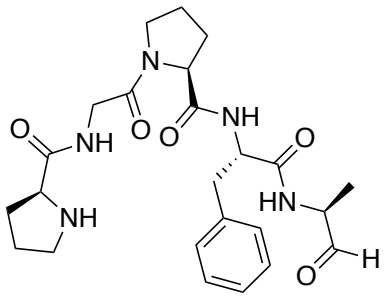
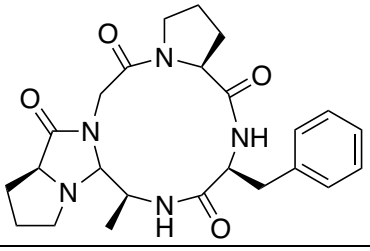
***Cyc*-4-Imz-Ala(-Ala-Pro-Tyr-Ala) (2l).** LCMS: *m/z* 458.2375 (calcd $[M+H]^+$ = 458.24) Purity: >95% (HPLC analysis at 220 nm). Retention time: 9.0 min

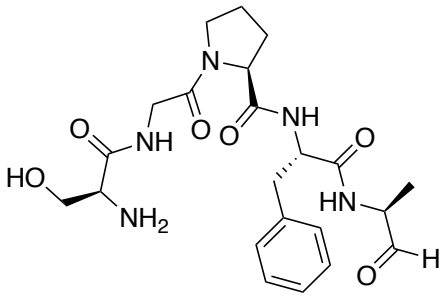
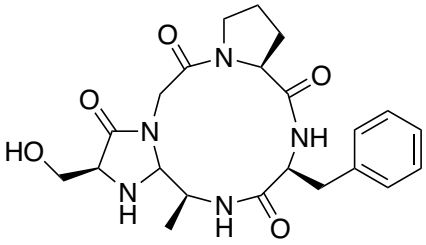
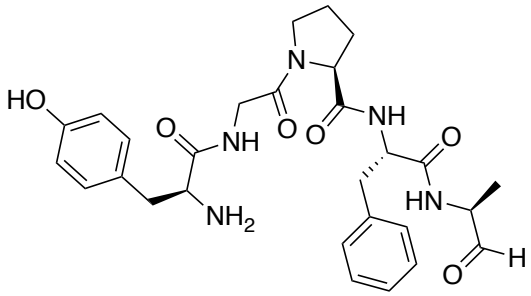
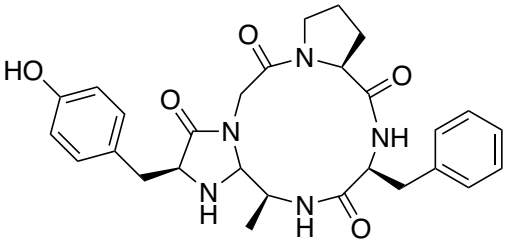
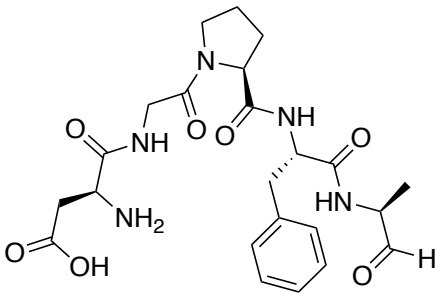
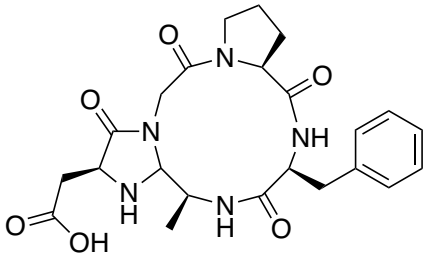


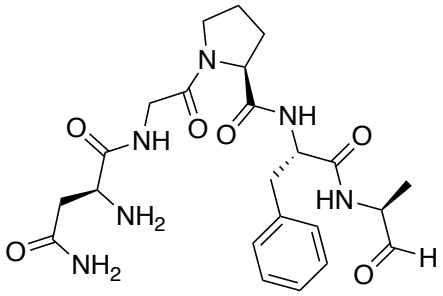
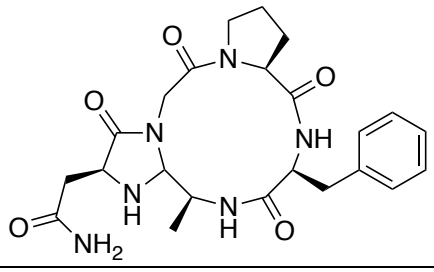
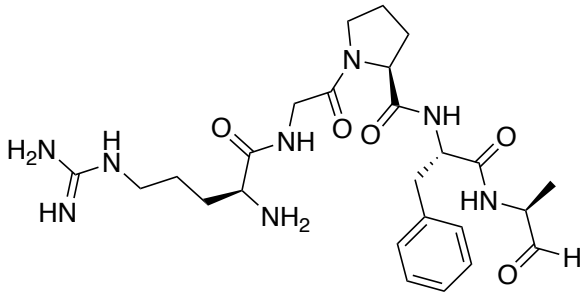
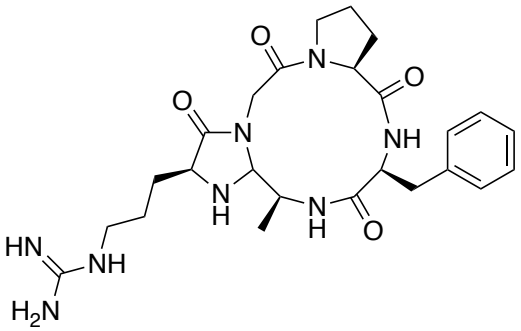
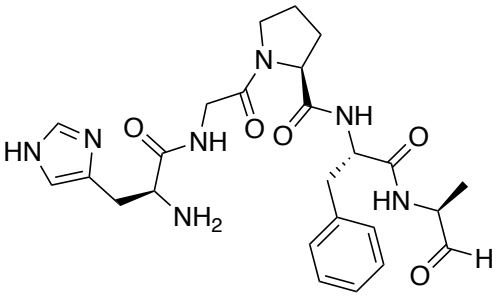


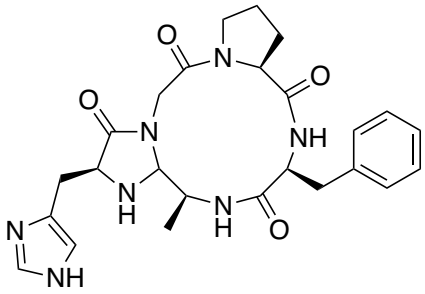
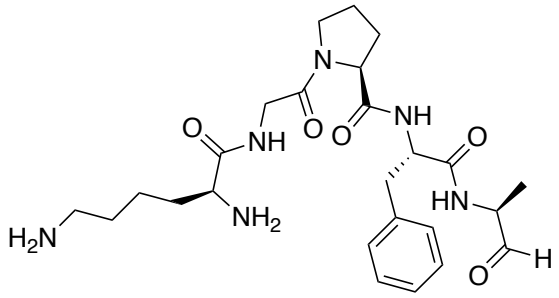
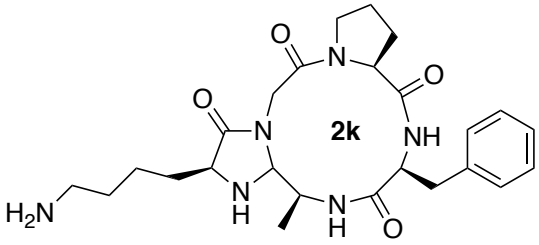
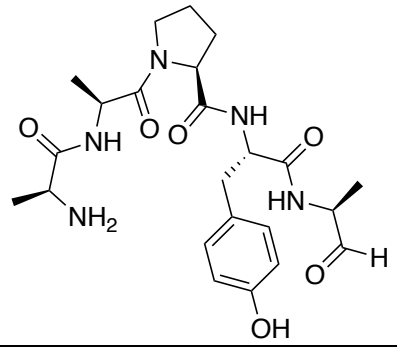
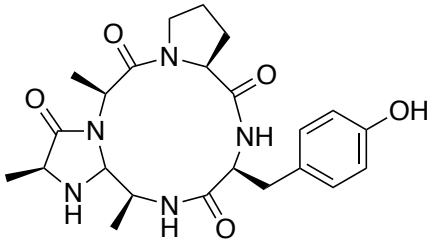
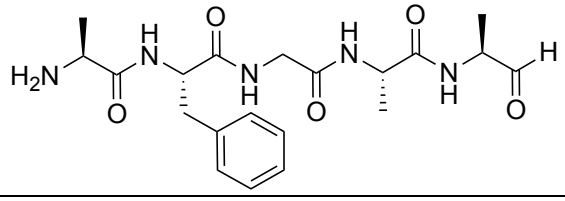
XIV: Supplementary Table 1: Molecular structures and high resolution mass spectrometry

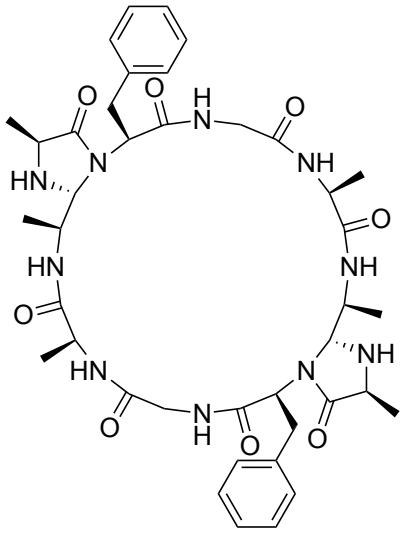
Entry	Structure	Calc. m/z	Obs. m/z
2a		832.44	832.4304

1b		446.24	446.2572
2b		428.23	428.2255
1c		432.22	432.1983
2c		414.21	414.1883
1d		472.26	472.2271
2d		454.25	454.2162

1e		462.24	462.2072
2e		444.22	444.1983
1f		538.27	538.2334
2f		520.26	520.2243
1g		490.23	490.2258
2g		472.22	472.2198

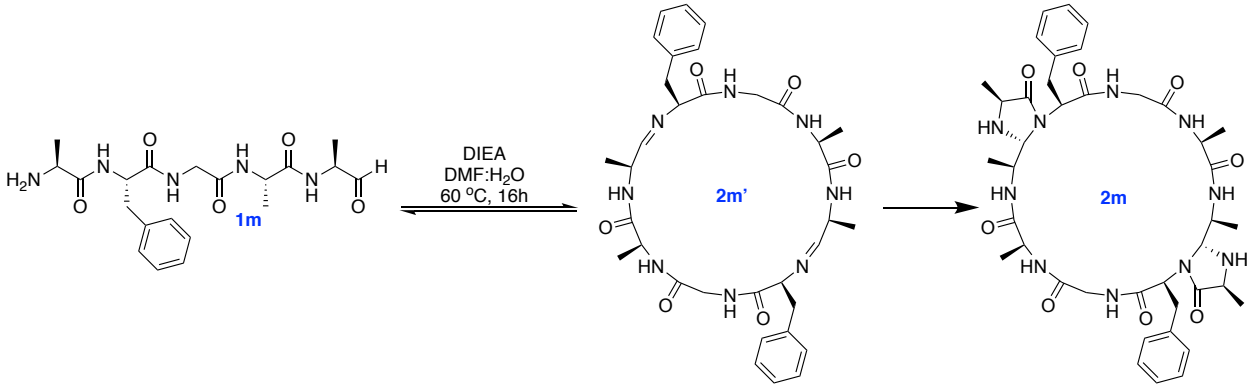
1h		489.25	489.2570
2h		471.24	471.2367
1i		531.30	531.2978 $[M+H_2O]^+$ 549.3160
2i		513.29	513.2929
1j		512.25	512.2626 $[M+H_2O]^+$ 530.2721

2j		494.25	494.2503
1k		503.30	503.2934
2k		485.29	485.2839
1l		476.25	476.2471
2l		458.24	458.2375
1m		420.22	420.2421

2m		803.42	803.4087 [(M+2)/2] 402.2083

XV. Supplementary Figure 7. Cyclization of peptide AFGAA (1m) without a Turn Inducing Element.

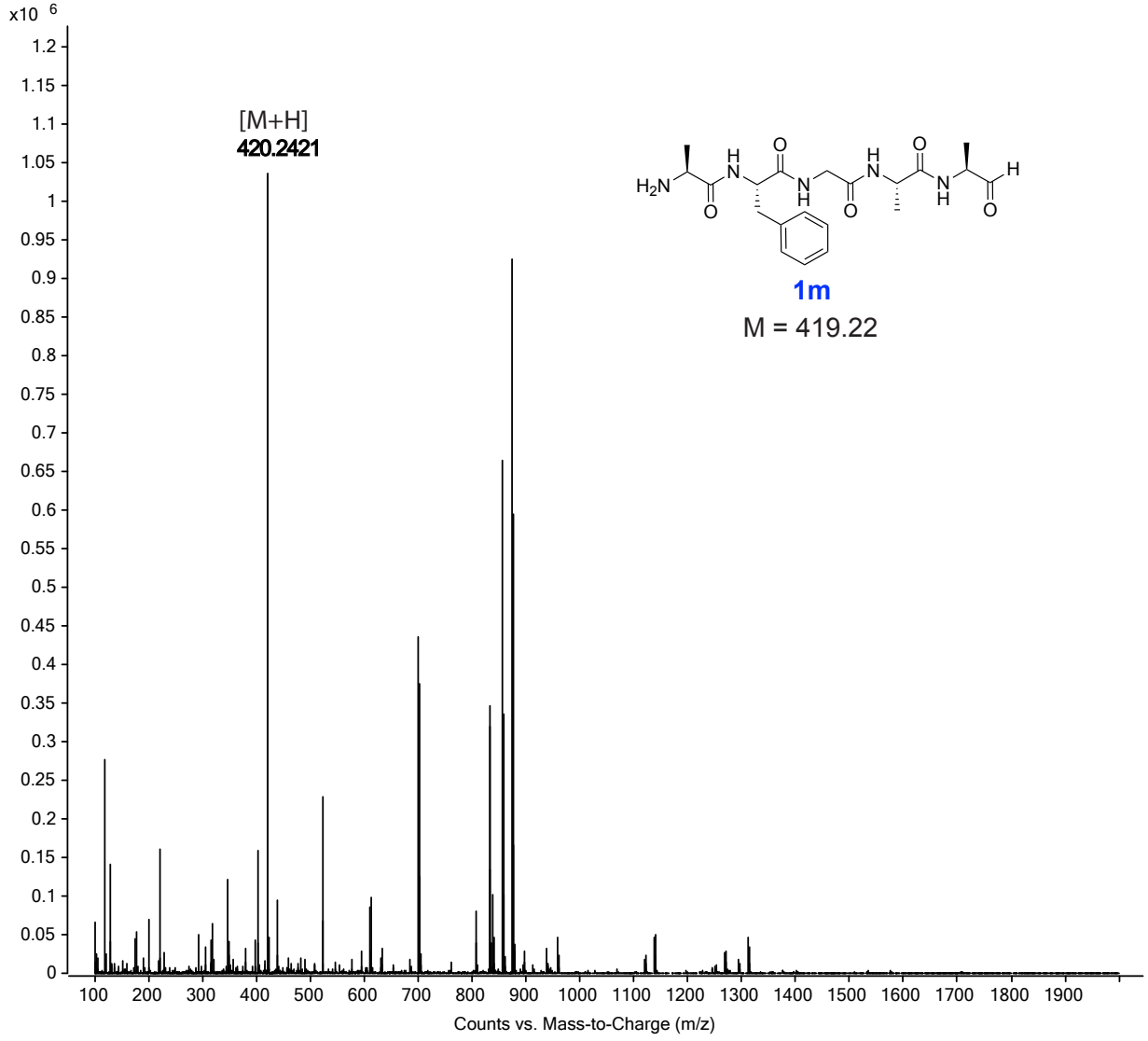
Lyophilized peptide aldehyde AFGAA **1m** (1mg) was solubilized in a 1:1 DMF/H_2O solution (3mM conc.), DIEA (21 equiv.) was added, and the reaction was shaken at room temperature or 60 ^{o}C for 16 h. The product was analyzed with HPLC and MS. We did not observe the formation of CyClick monomer but observed the formation of CyClick dimer (59 %).



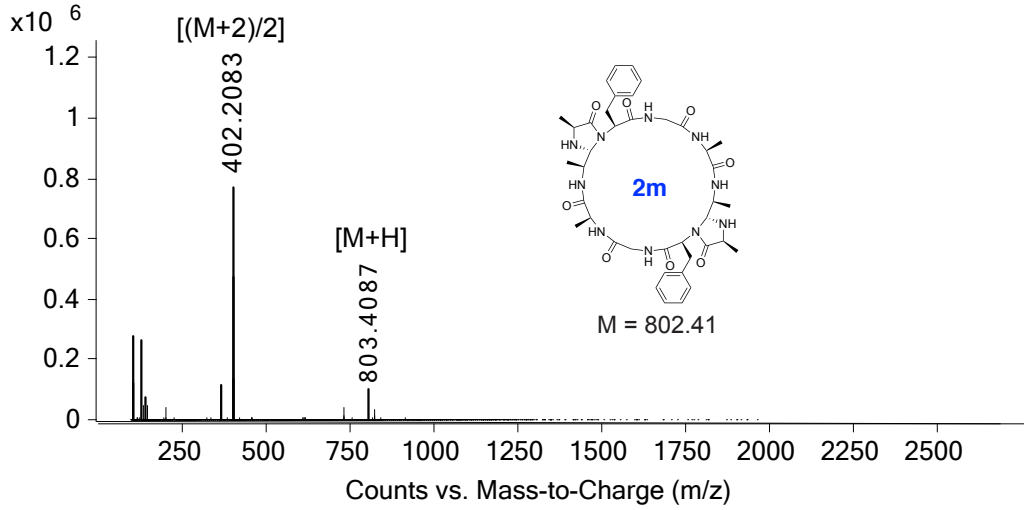
Ala-Phe-Gly-Ala-Ala (1m). LCMS: *m/z* 420.2421 (calcd $[M+H]^+$ = 420.22), *m/z* 494.2027 (calcd $[M+Na]^+$ = 494.21), Purity: >95% (HPLC analysis at 220 nm). Retention time: 7.1 min

***Cycdimer-(*4-Imz-Ala(-Phe-Gly-Ala-Ala)$_2$ (2m).** LCMS: *m/z* 803.4087 (calcd $[M+H]^+$ = 803.41), *m/z* 402.2083 (calcd $[(M+2)/2]^+$ =402.205), Purity: >95% (HPLC analysis at 220 nm). Retention time: 15.1 min

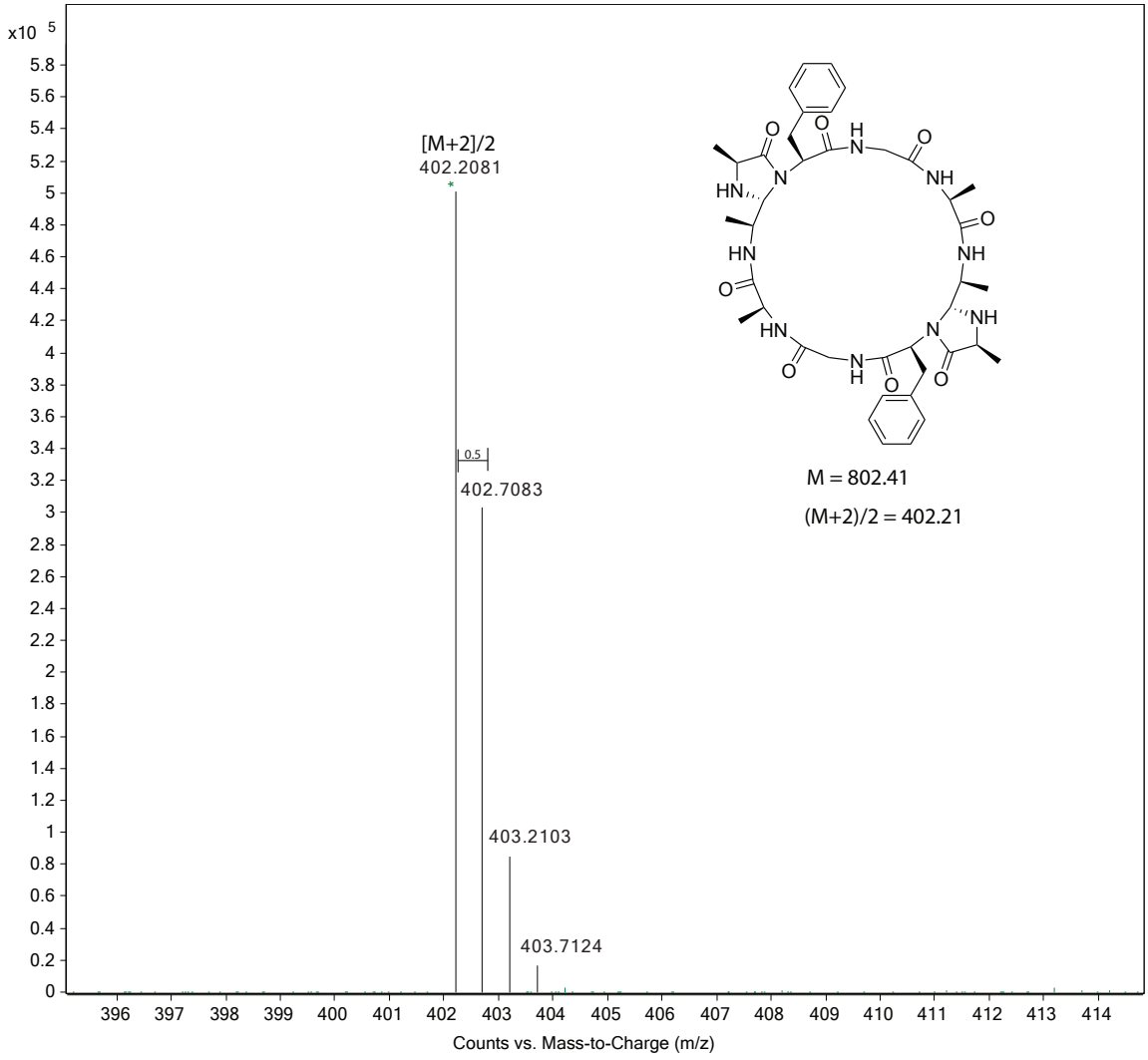
HRMS spectra of linear peptide **1m**



HRMS spectra of CyClick dimer product **2m**



HRMS Splitting Pattern **2m**



XVI. Supplementary Figure 8. Cyclization of peptide AGPFA 1b at High Concentration (25 mM)

Procedure for cyclization at high concentration. Lyophilized peptide aldehyde AGPFA **1b** (4mg) was solubilized in a 1:1 DMF/H_2O (362µL) solution to make a concentration of 25mM, DIEA (21 equiv.) was added, and the reaction was shaken at 60 ^{o}C for 16 h. The product was analyzed with HPLC and MS. The reaction proceeded smoothly with conversion of 89.5% to monoCyClick product with cyclodimer ratio of >17:1.

XVII. References

1. W. C. Chan, P. D. White, Fmoc Solid Phase Peptide Synthesis: A Practical Approach (Oxford Univ. Press, New York, 2000).
2. A. Moulin, J. Martinez, J.-A. Fehrentz, *J. Pept. Sci*. **2007**, *13*, 1-15.