Electronic Supplementary Material (ESI) for Analyst.

Peptidylation for determination of low-molecule-weight compounds by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Preparation of isotope labeled internal standards

To obtain the internal standard in the MALDI-TOF-MS analysis, 100 μL isotope labeled P2 (^{7}P2, ^{15}N1 and ^{13}C6 labeled leucine, 100 μM) reacted with excessive 3-PA (^{7}P2:3-PA, 1:1000) at 25°C for 3 h, followed by removing the excessive 3-PA with vacuum centrifuge. The reaction product (^{7}P2-3-PA) was redissoved in 100 μL water and used as internal standard to optimize the reaction condition of peptidylation of 3-PA.

As shown in Figure S3, the dominant peak of the protonated ^{7}P2-3-PA appeared at *m/z* 1052, while the peak of protonated ^{7}P2 (*m/z* 963) disappeared, suggesting that ^{7}P2 fully reacted with 3-PA to form ^{7}P2-3-PA. We also examined the reactant (^{7}P2) and product (^{7}P2-3-PA) by electrospray ionization-high resolution mass spectrometer (ESI-HRMS). Electrospray ionization-high resolution mass spectrometry (ESI-HRMS) was carried out on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was controlled by the Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, San Jose, CA, USA). As shown in Figure S4, a major ion at *m/z* 536.2722 ($[^{7}$P2-3-PA + 2H$]^{2+}$) was observed in the ESI-HRMS spectrum, and no ion at *m/z* 481.7585 ($[^{7}$P2 + 2H$]^{2+}$) was observed. The results suggested that the internal standard of ^{7}P2-3-PA was successfully prepared.

Similarly, to obtain the internal standard to optimize the peptidylation conditions of the thiols, 100 μL isotope labeled P7 (^{7}P7, ^{15}N1 and ^{13}C6 labeled leucine, 100 μM) reacted with excessive ME (^{7}P7:ME, 1:1000) at 25°C for 1 h, followed by evaporating the reaction solution in a vacuum centrifuge to remove the excessive ME. The product (^{7}P7-ME) was

redissoved in 100 μL water and used as internal standard to optimize the condition of peptidylation of ME.

As shown in Figure S5, the dominant peak of the protonated ^{7}P7-ME appeared at *m/z* 1217, while the peak of protonated ^{7}P7 (*m/z* 1139) disappeared, suggesting that ^{7}P7 fully reacted with ME to form ^{7}P7-ME. We also examined the reactant (^{7}P7) and product (^{7}P7-ME) by ESI-HRMS. As shown in Figure S6, a major ion at *m/z* 608.8226 ($[^{7}\text{P7-ME} + 2\text{H}]^{2+}$) was observed in the ESI-HRMS spectrum, and no ion at *m/z* 569.8146 ($[^{7}\text{P7} + 2\text{H}]^{2+}$) was observed. The results suggested that the internal standard of ^{7}P7-ME was successfully prepared.

Optimization of the peptidylation conditions of thiols

For the peptidylation of the thiols, we first optimized the pH of the reaction. Our results demonstrated that the highest relative intensity of the P7 labeled ME can be achieved at pH 6 (Figure 3e). The pH higher than 7 may result in an oxidative disulfide side product.

Next, we optimized the reaction temperature. The results showed that the highest relative intensity of P7 labeled ME can be achieved at 4°C (Figure 3f). It is worth noting that with the increased temperature, the ratio of P7/7P7 decreased. We suspected that high temperature may result in the formation of oxidative disulfide. Therefore, the derivatization conditions for practical use should be performed at low temperature to avoid the destroy of thiols. In addition, the optimal concentration of P7 for derivatization of ME was also investigated. The results showed that the relative intensity of P7 labeled ME reached a plateau when the concentration of P7 was 50 μM (5 folds excess over ME) (Figure 3g). As shown in

Figure 3h, the relative intensity of P7 labeled ME increased with the increase of time from 5 to 180 min, and no obvious change was observed after 30 min. Taken together, the optimized conditions for the peptidylation of the thiols were under 4°C for 30 min with 50 μM P7 at pH 6. The derivatization reaction yield was calculated by comparing the peak intensity of P7 labeled ME with the internal standards of ^{7}P7-ME by MALDI-TOF-MS. The derivatization efficiency was calculated to be 100 ± 3 % for ME by P7.

Construction of calibration curve

To examine the dynamic range of the quantitative analysis by peptidylation with MALDI-TOF-MS, a series of solutions containing 0.1, 0.2, 0.5, 1, 5, 10, and 20 μM of Cys in 20 mM PBS at pH 6 were treated with 5 folds molar excess of P7 at 4°C for 30 min. 1 μM of Cys in 20 mM PBS at pH 6 was reacted with 5 folds molar excess of ^{7}P7 to form ^{7}P7 derivatized Cys (^{7}P7-Cys, 1 μM). Equal volumes of ^{7}P7-Cys standard solution and P7-Cys solution were mixed, and the resulting solution was analyzed by MALDI-TOF-MS. The calibration curves were constructed by plotting mean peak intensity ratios of light/heavy versus the mean concentration ratios of light/heavy based on data obtained from triplicate measurements.

As shown in Figure S2, the slopes of linear regressions of Cys was 0.992, which was approximate to 1.00, indicating the ratios of the peak intensity in MALDI-TOF-MS closely matched with the concentration ratios of the different isotope-labeled analytes. The coefficients of determination (R^2) were also found to be close to 1.00, demonstrating the good quantitative performance of peptidylation strategy coupled with MALDI-TOF-MS analysis.

Determination of creatinine

Creatinine was determined by a previously described method with slight modifications.[1] Briefly, 10 μL of urine was diluted by 80% ACN to 1 mL. Then 10 μL of the supernatant was injected into the HPLC–UV system for analysis (LC 20A, Shimadzu, Kyoto, Japan) after centrifugation (20,000 g, 4°C, 5 min). The separation was conducted using a Phenomenex Luna NH_2 column (150 × 4.6 mm, 5 μm) with ACN-phosphate buffer (1.0 mM) at pH 6.0 (80:20) as mobile phase at a flow rate of 1.0 mL/min. The column temperature was kept at 30°C, and the detection wavelength was set at 235 nm.

Table S1. The calculated energy of the peptide and protonated peptides, and the calculated proton affinity by PM3.

Peptide	Calculated energy (kcal/mol)	Calculated energy of protonated peptides (kcal/mol)								Proton Affinity (kcal/mol)
P1 (CFR_1GL)	-226.24	N		R_1						228.06
		-89.21		-99.27						
P2 (CFR_1GLR_2GF)	-303.56	N		R_1		R_2				251.55
		-175.33		-176.02		-200.08				
P3 ($CFR_1GLR_2GFR_3G$)	-364.05	N		R_1		R_2		R_3		249.07
		-228.34		-255.85		-253.85		-258.09		
P4 ($CFR_1GLR_2GFR_3GR_4G$)	-438.37	N		R_1		R_2		R_3	R_4	248.40
		-277.12		-295.67		-331.74		-316.57	-314.98	
P5 ($CR_1GR_2GR_3GR_4$)	-306.05	N		R_1		R_2		R_3	R_4	247.82
		-174.68		-190.42		-183.15		-198.84	-189.02	
P6 ($CR_1R_2R_3R_4R_5R_6R_7$)	-271.82	N	R_1	R_2	R_3	R_4	R_5	R_6	P_7	246.30
		-147.20	-148.28	-163.09	-157.50	-151.48	-161.89	-157.38	-162.33	

$PnH^+ \rightarrow Pn + H^+$, $\Delta E_{elec} = E(H^+) + E(Pn) - E(PnH^+)$, $E(H^+) = 353.58$ kcal/mol;

Proton affinity = $\Delta E_{elec} + \Delta(PV) + \Delta(ZPE) + \Delta E_{trans} + \Delta E_{rot} + \Delta E_{vib}$;

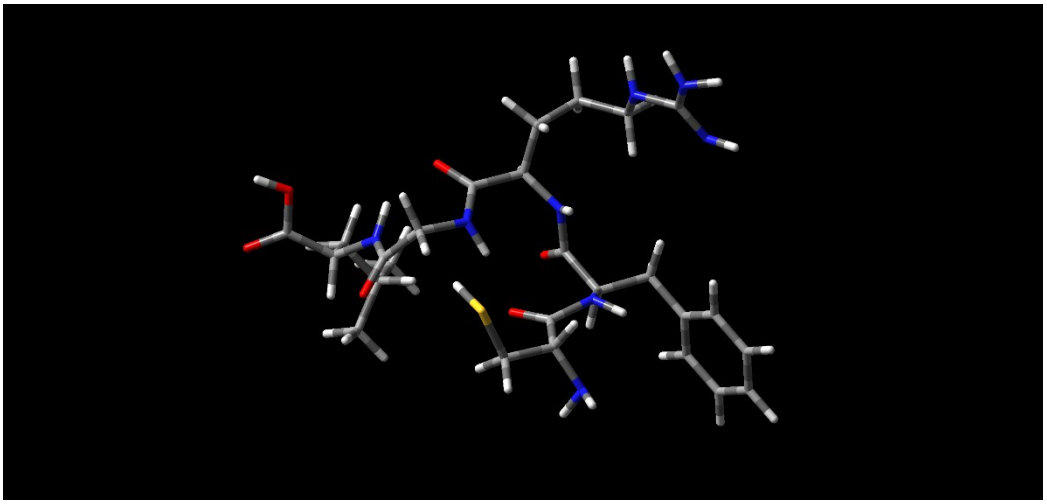
$\Delta(PV) = RT$, $\Delta E_{trans} = 1.5RT$, $RT = 0.58$ kcal/mol (298.15K). N represents the protonated site is amino nitrogen; R_n represents the protonated site is the sidechain of arginine n. Highlight in red are the lowest-energy structure in different protonation forms, which is used as the $E(PnH^+)$ to calculated proton affinity. The PM3 minimum-energy structures and calculated energies for different peptides were showed in Figure S1.

Table S2. Comparison of proton affinity of glycine calculated with PM3 method and previous reported results.

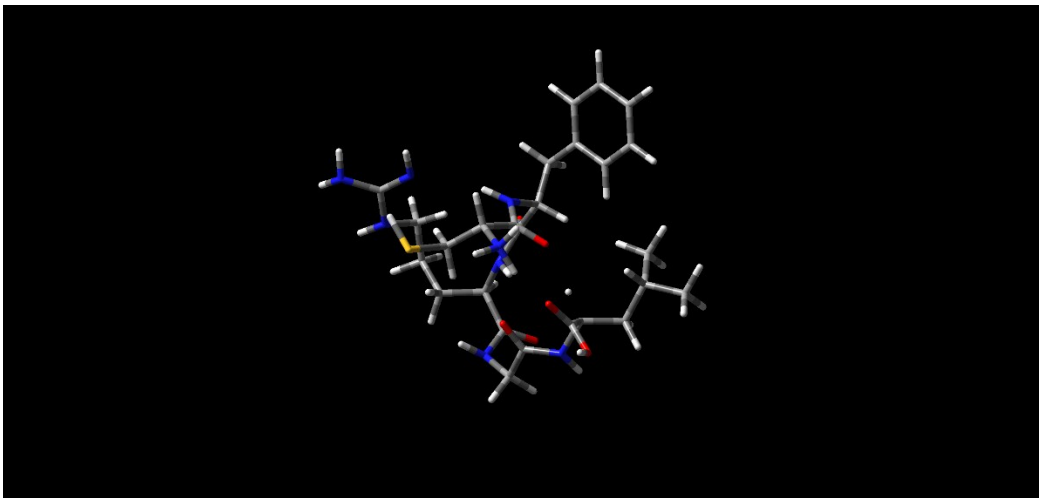
	PM3 method	References
Glycine	211.3 Kcal/mol	211.7 Kcal/mol [2]
Gly-Gly	221.7 Kcal/mol	223.5 Kcal/mol [3]

Table S3. The measured concentration of creatinine in each pooled urine samples.

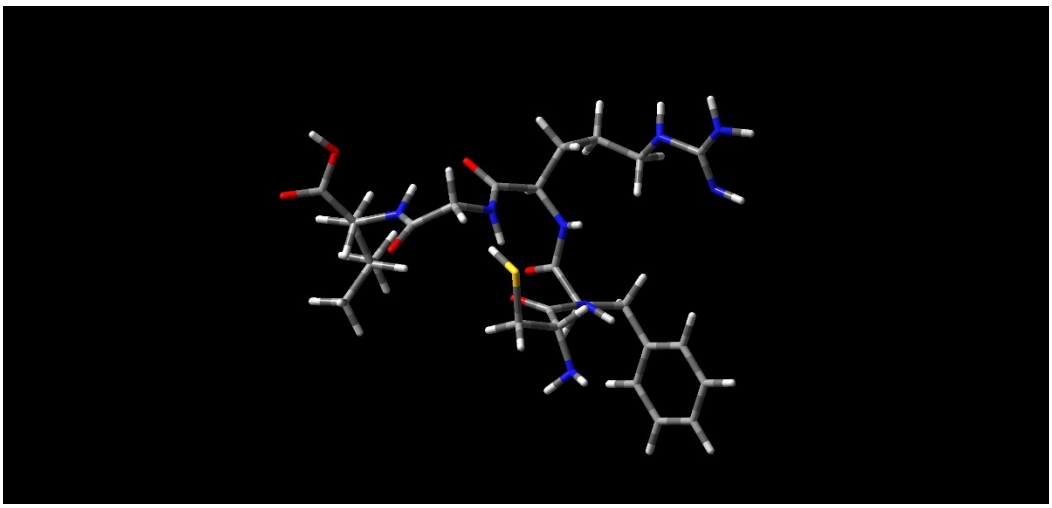
	Concentration (mM, n = 3)
Pooled urine sample of lung cancer patients	11.5 ± 0.1
Pooled urine sample of health controls	17.1 ± 0.1



1 (-226.24 Kcal/mol)

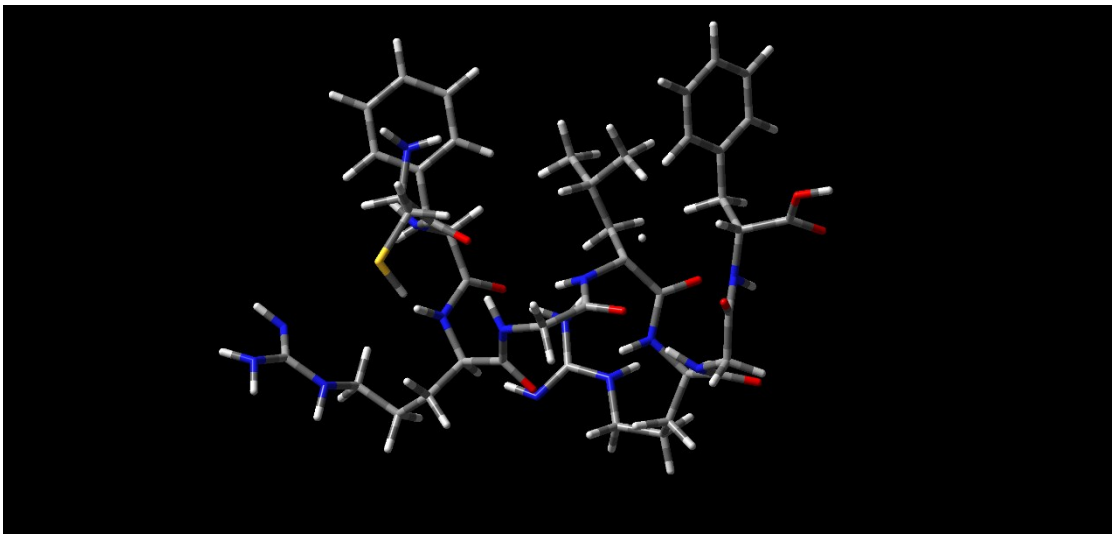


2 (-89.21 Kcal/mol)

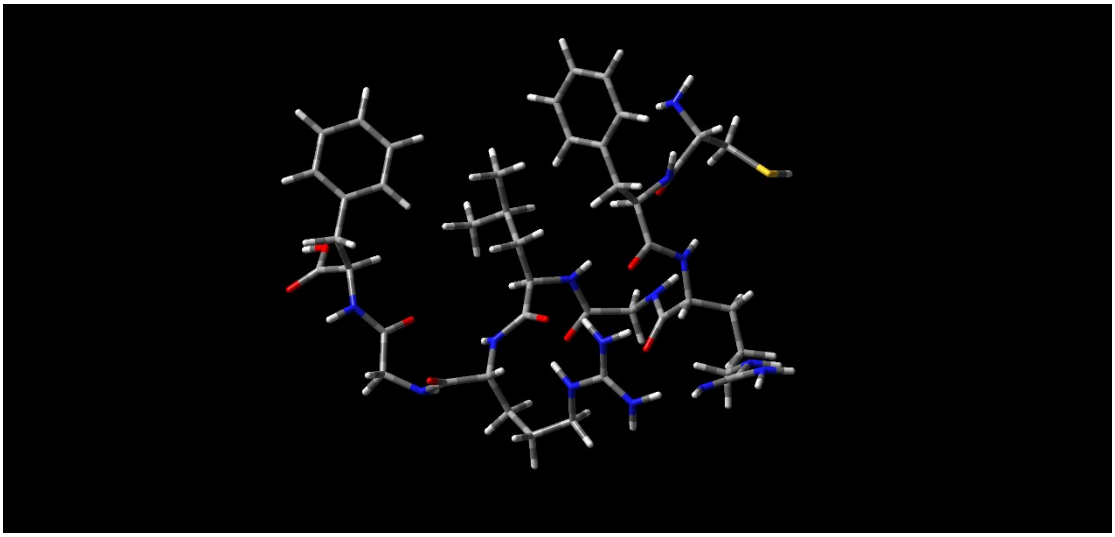


3 (-99.27 Kcal/mol)

Figure S1a. PM3 minimum-energy structures and calculated energies for P1 (*1*) and protonated P1 (*2* and *3*).

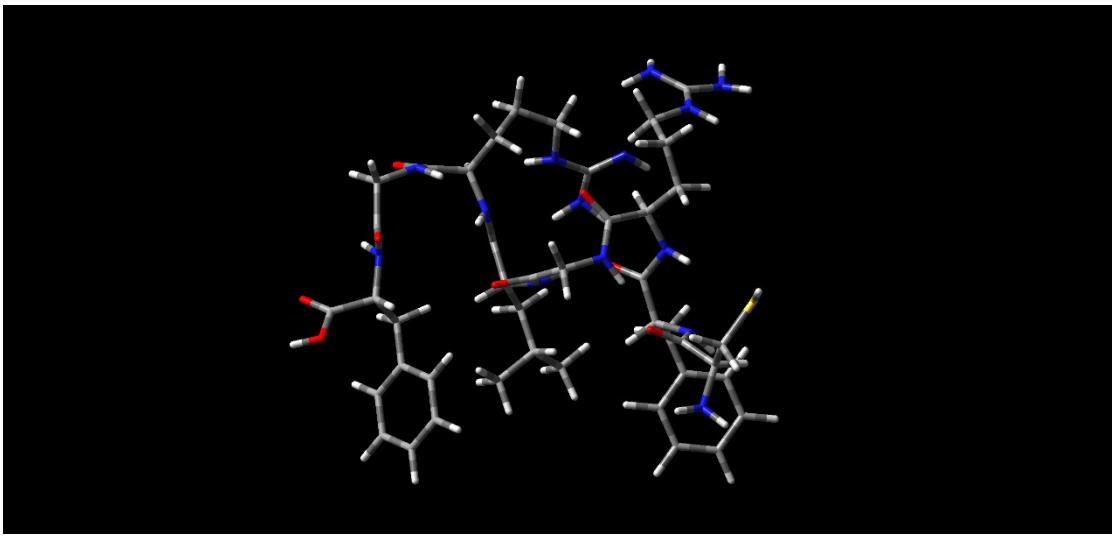


4 (-303.56 Kcal/mol)

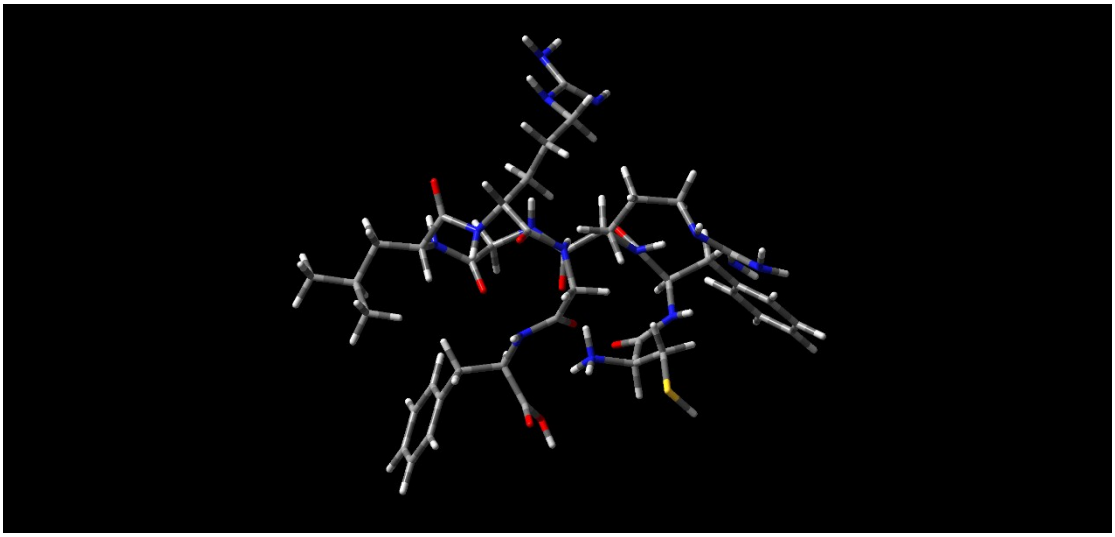


5 (-175.33 Kcal/mol)

Figure S1b. PM3 minimum-energy structures and calculated energies for P2 (*4*) and protonated P2 (*5*).

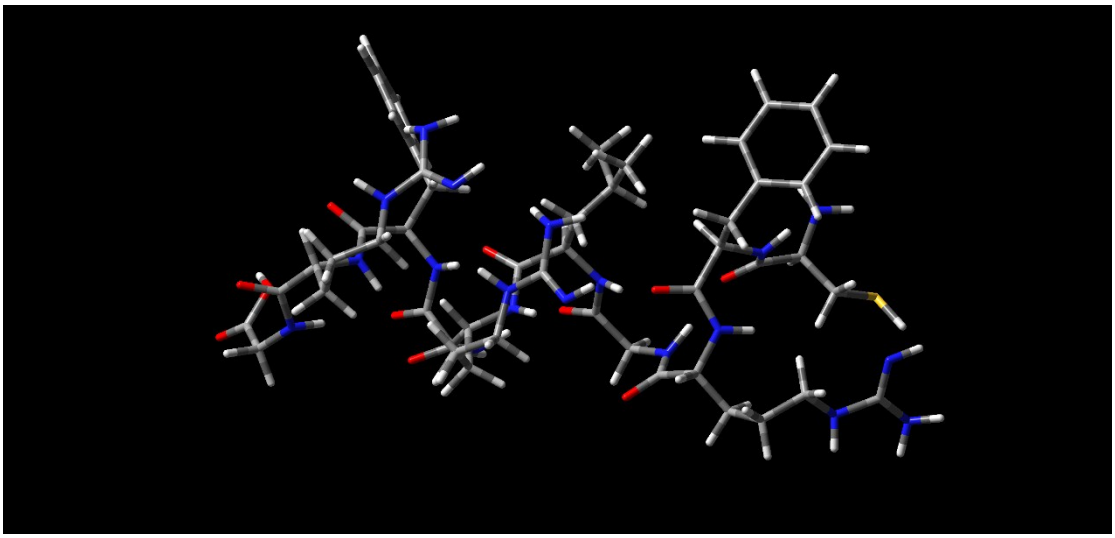


6 (-176.02 Kcal/mol)

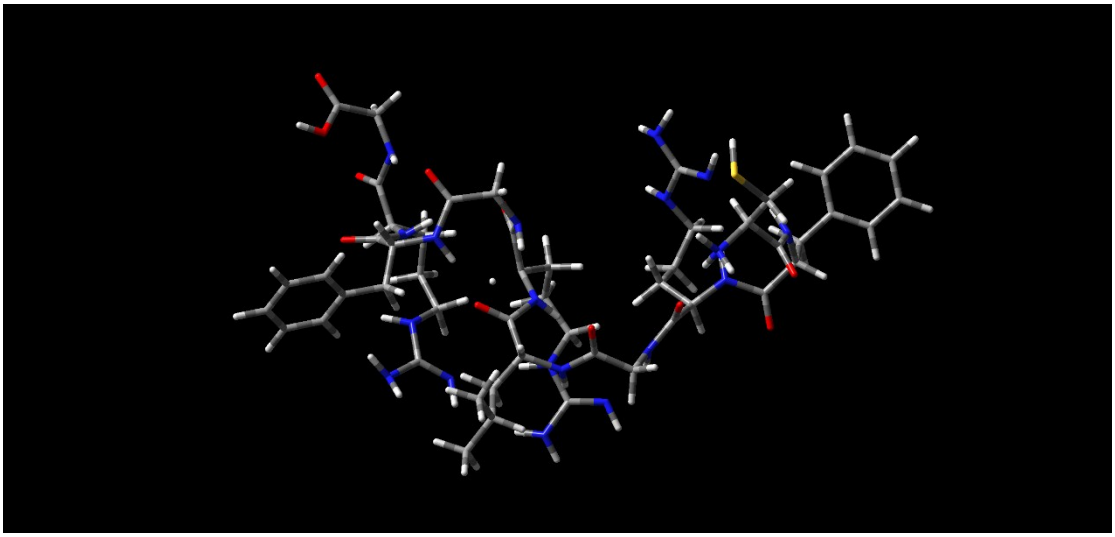


7 (-200.08 Kcal/mol)

Figure S1b, continued. PM3 minimum-energy structures and calculated energies for P2 and protonated P2 (*6* and *7*).

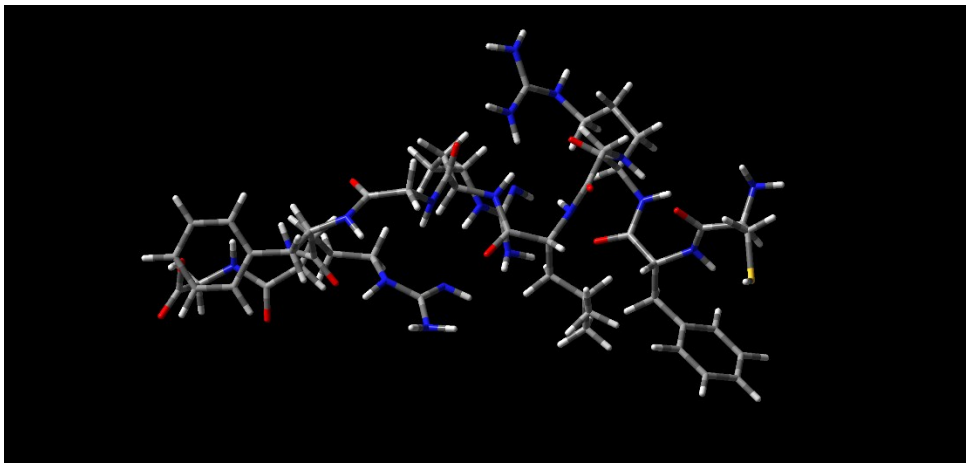


8 (-364.05 Kcal/mol)

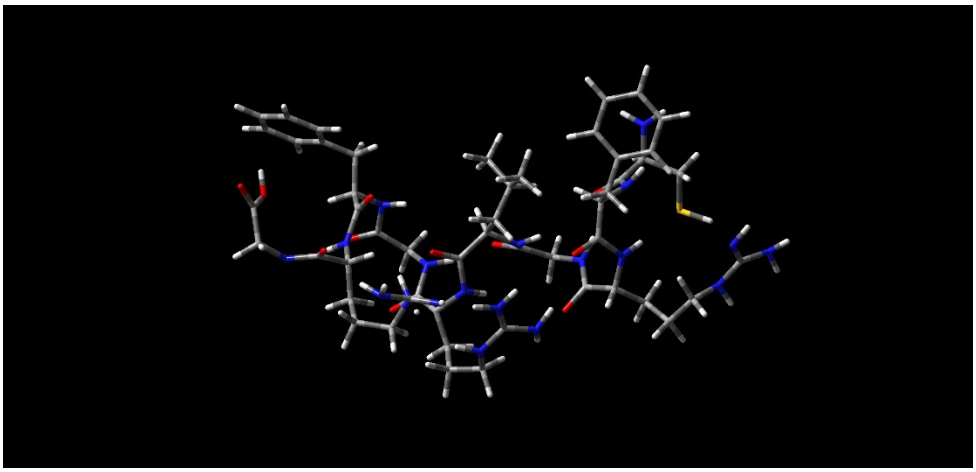


9 (-228.34 Kcal/mol)

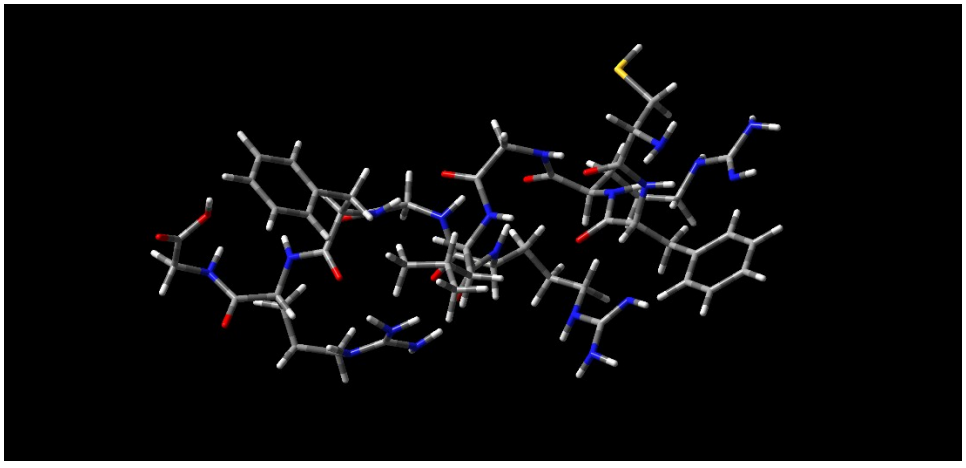
Figure S1c. PM3 minimum-energy structures and calculated energies for P3 (*8*) and protonated P3 (*9*).



10 (-255.85 Kcal/mol)

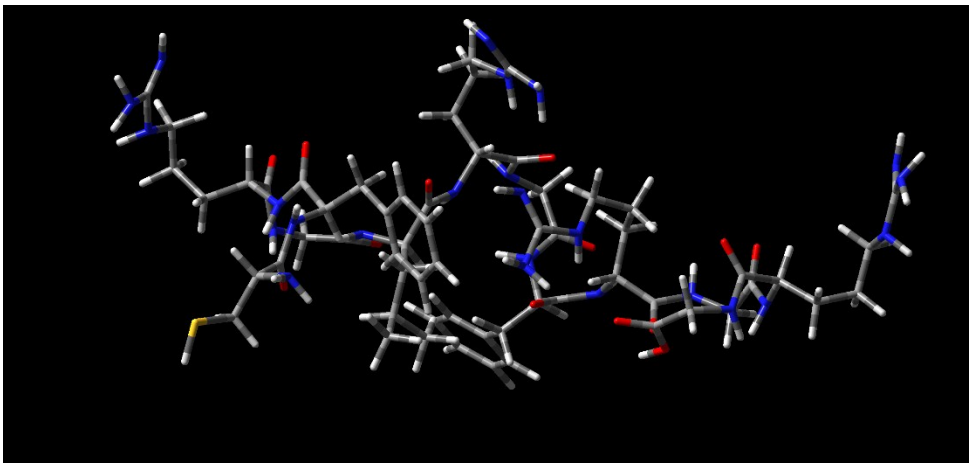


11 (-253.85 Kcal/mol)

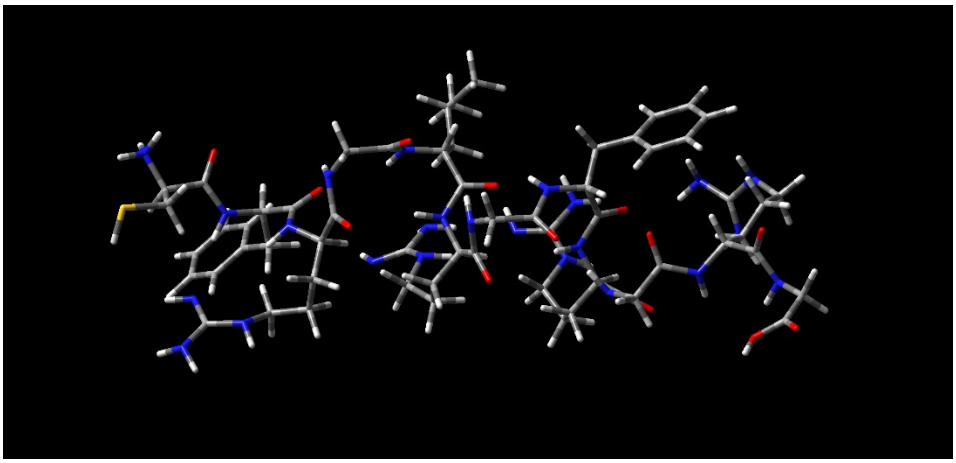


12 (-258.09 Kcal/mol)

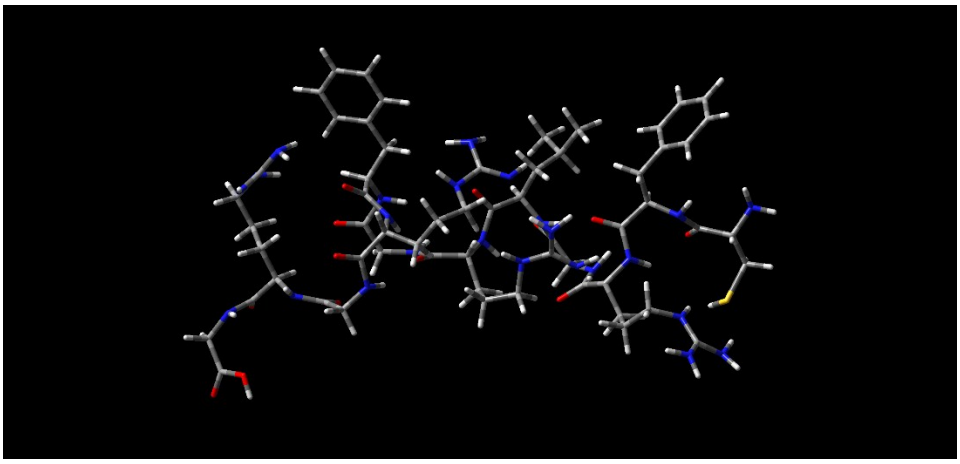
Figure S1c, continued. PM3 minimum-energy structures and calculated energies for P3 and protonated P3 (*10, 11* and *12*).



13 (-438.37 Kcal/mol)

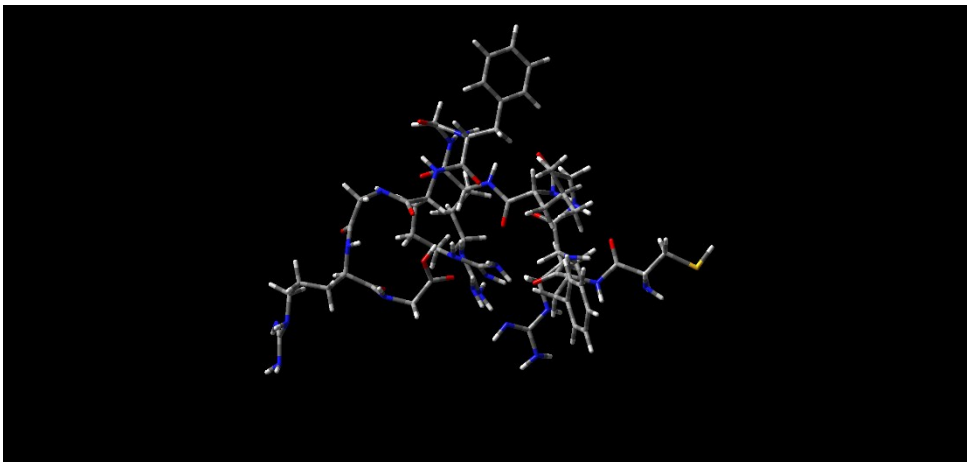


14 (-277.12 Kcal/mol)

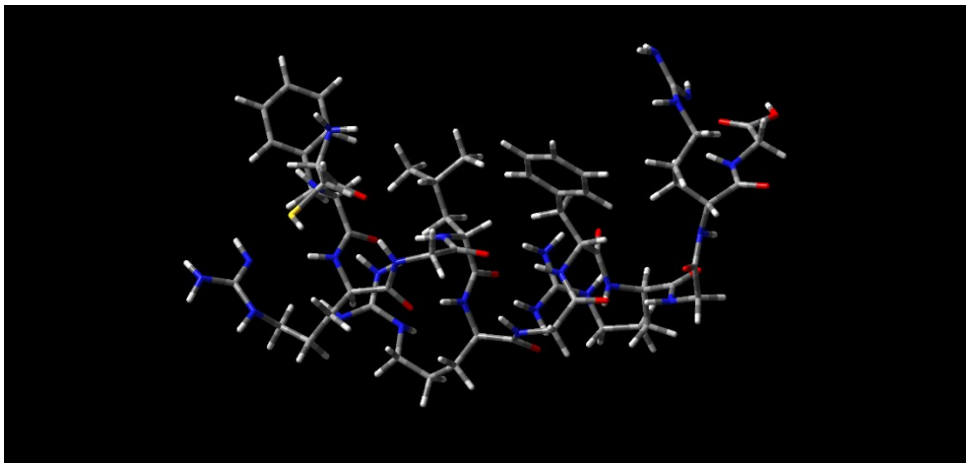


15 (-295.67 Kcal/mol)

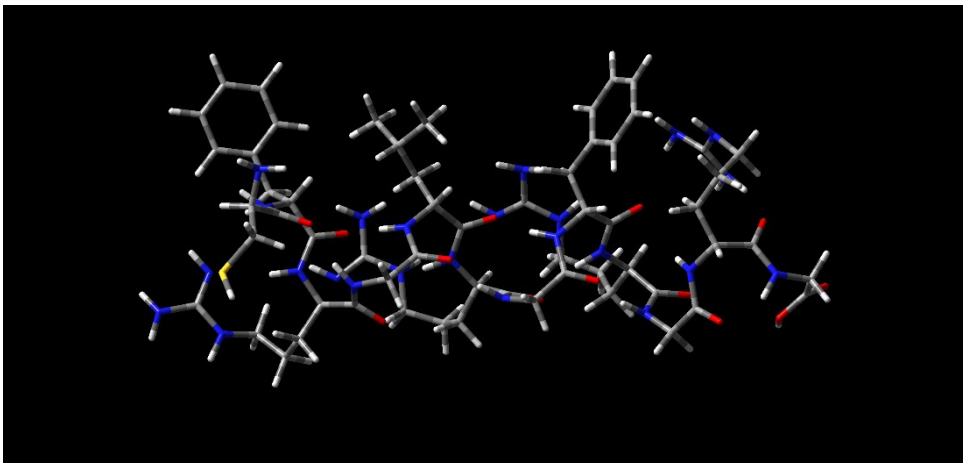
Figure S1d. PM3 minimum-energy structures and calculated energies for P4 (*13*) and protonated P4 (*14* and *15*).



16 (-331.74 Kcal/mol)

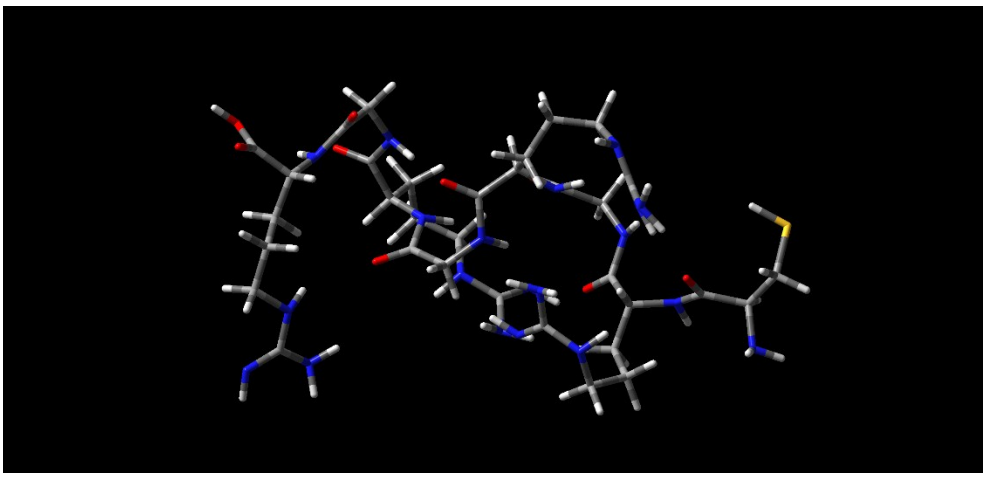


17 (-316.57 Kcal/mol)

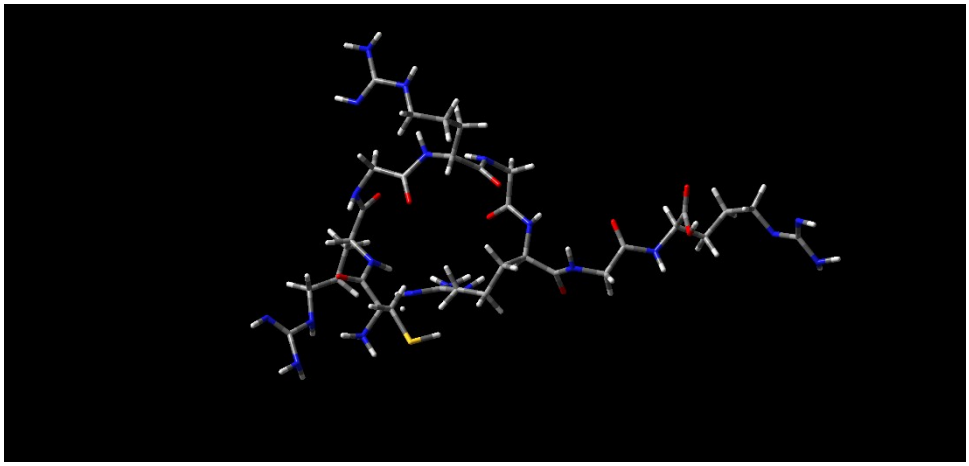


18 (-314.98 Kcal/mol)

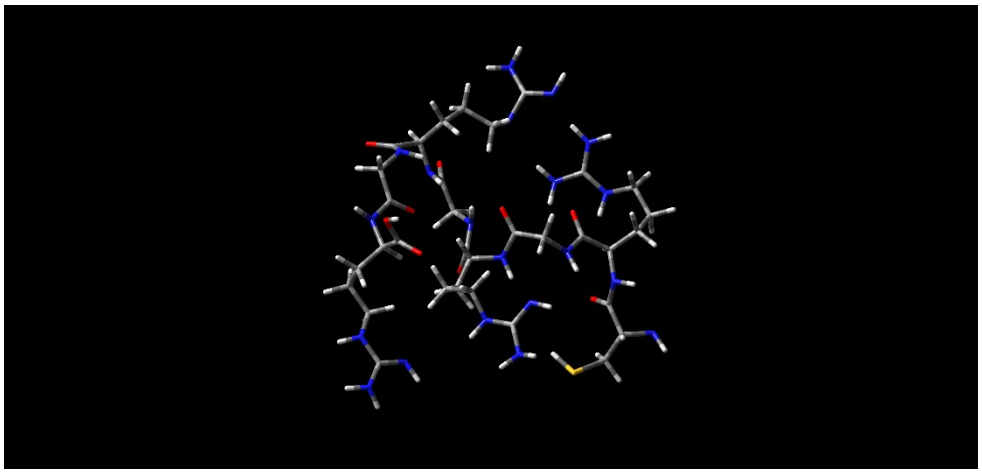
Figure S1d, continued. PM3 minimum-energy structures and calculated energies for P4 and protonated P4 (*16, 17* and *18*).



19 (-306.05 Kcal/mol)

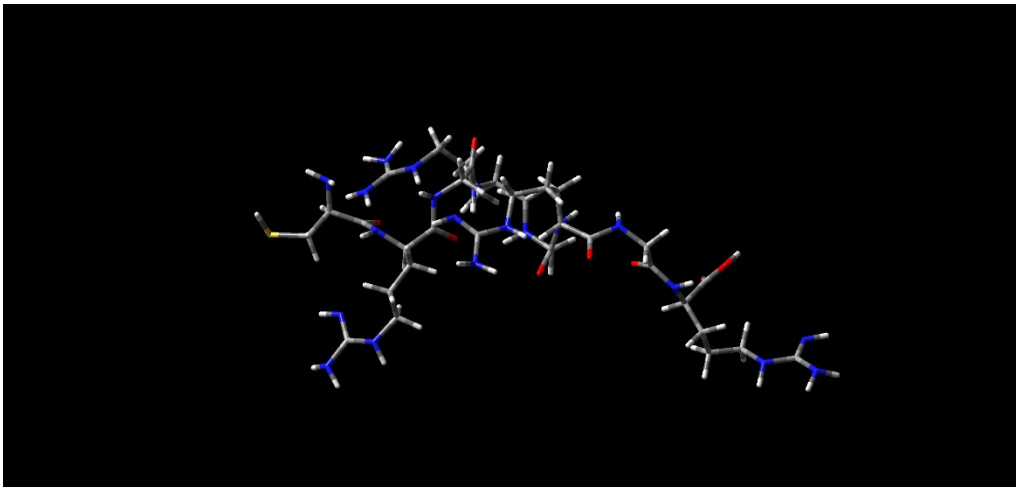


20 (-174.68 Kcal/mol)

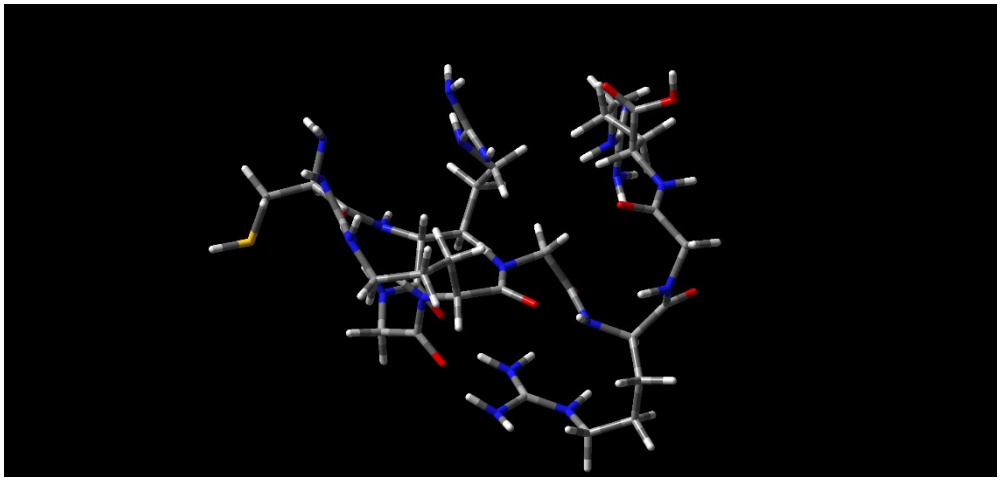


21 (-190.42 Kcal/mol)

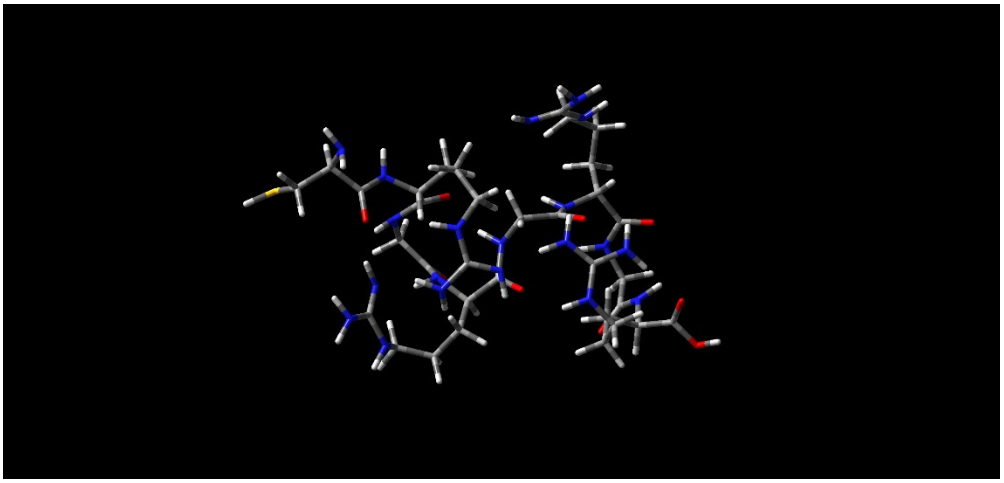
Figure S1e. PM3 minimum-energy structures and calculated energies for P5 (*19*) and protonated P5 (*20* and *21*).



22 (-183.15 Kcal/mol)

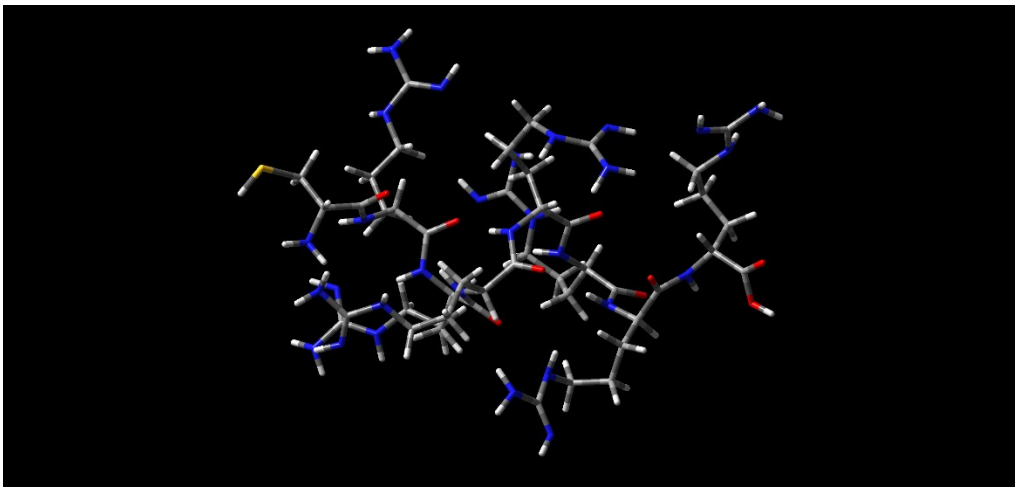


23 (-198.84 Kcal/mol)

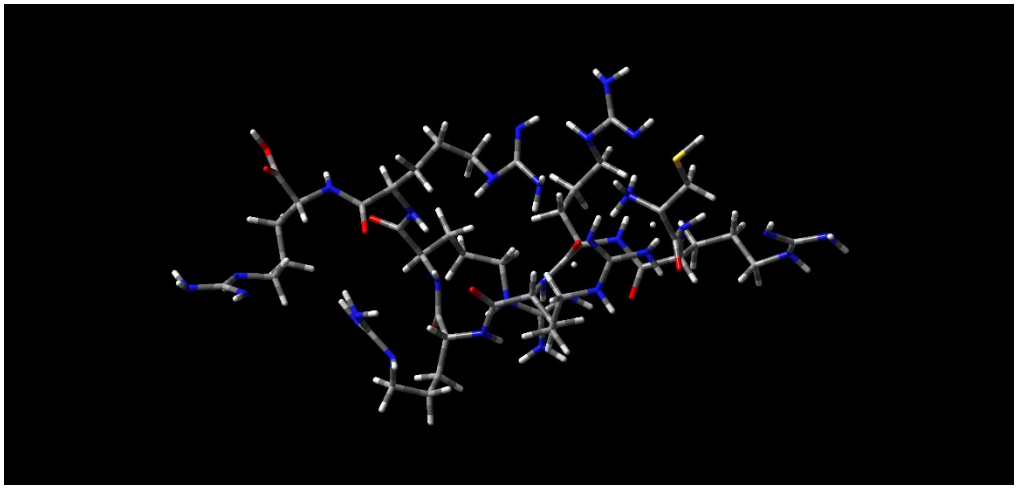


24 (-189.02 Kcal/mol)

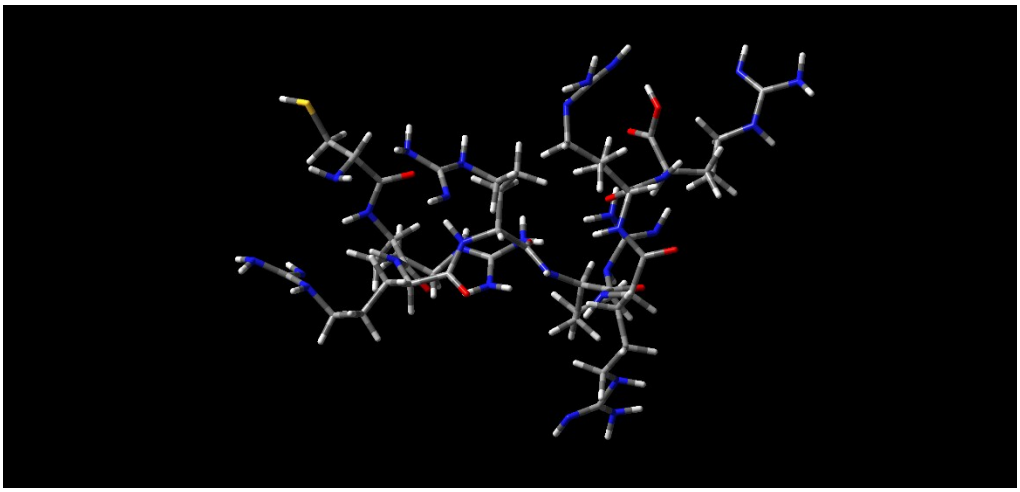
Figure S1e, continued. PM3 minimum-energy structures and calculated energies for P5 and protonated P5 (*22, 23* and *24*).



25 (-271.82 Kcal/mol)

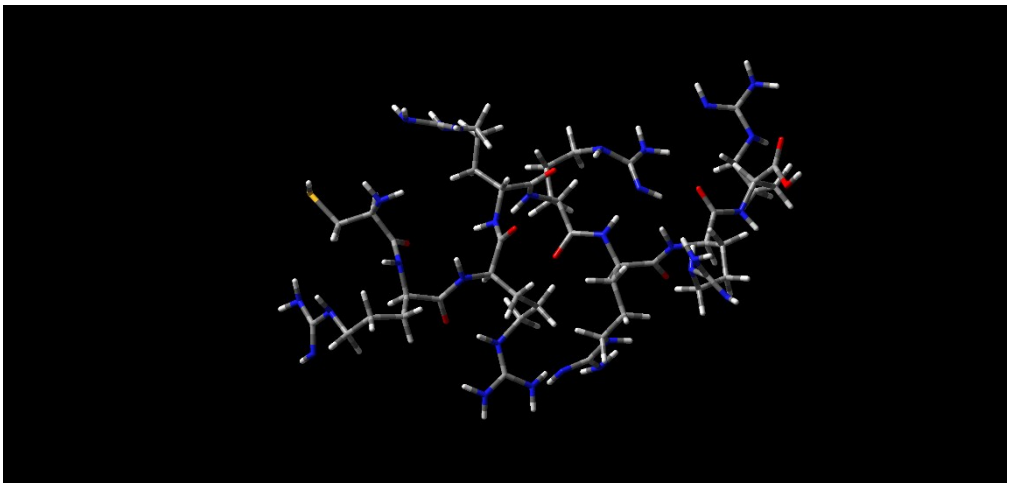


26 (-147.20 Kcal/mol)

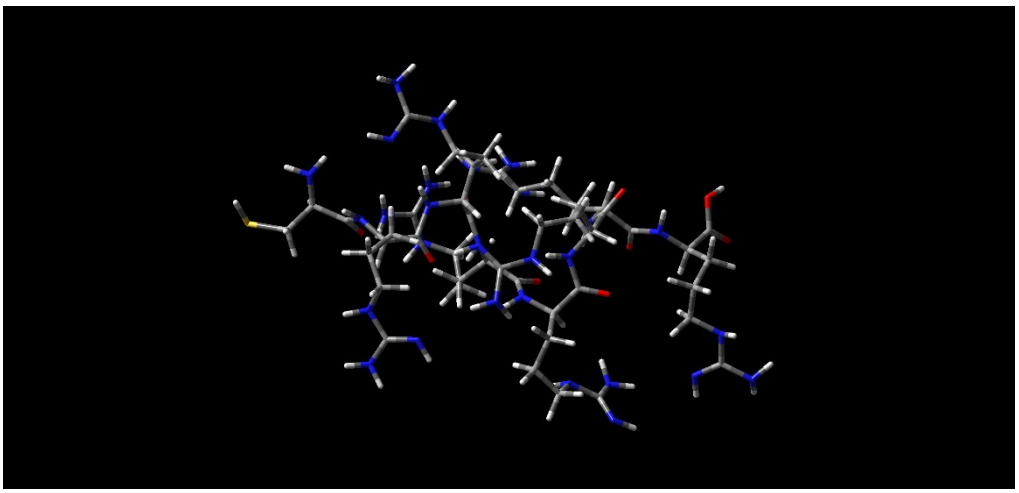


27 (-148.28 Kcal/mol)

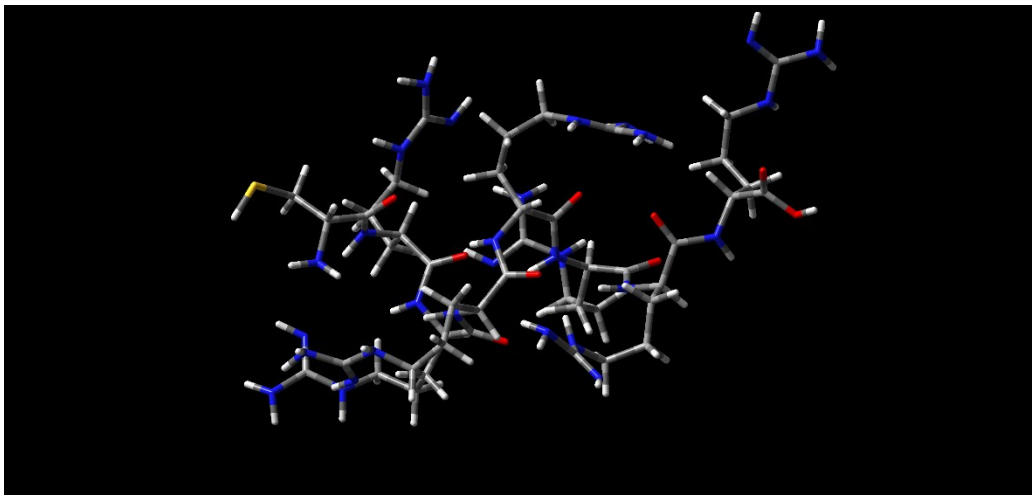
Figure S1f. PM3 minimum-energy structures and calculated energies for P6 (*25*) and protonated P6 (*26* and *27*).



28 (-163.09 Kcal/mol)

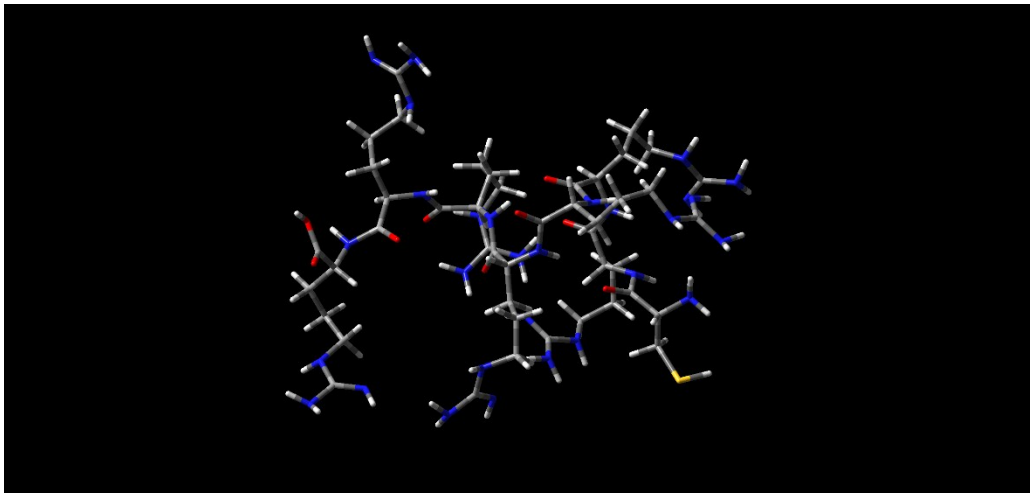


29 (-157.50 Kcal/mol)

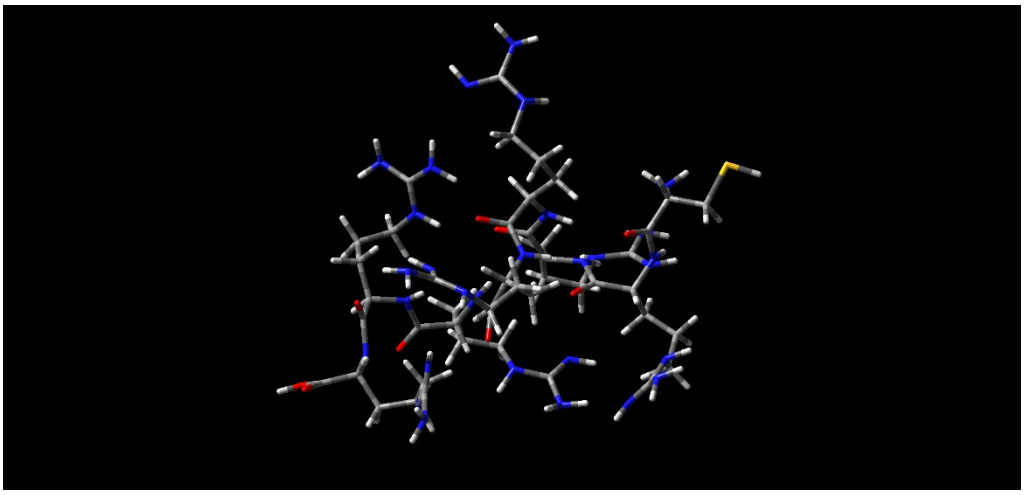


30 (-151.48 Kcal/mol)

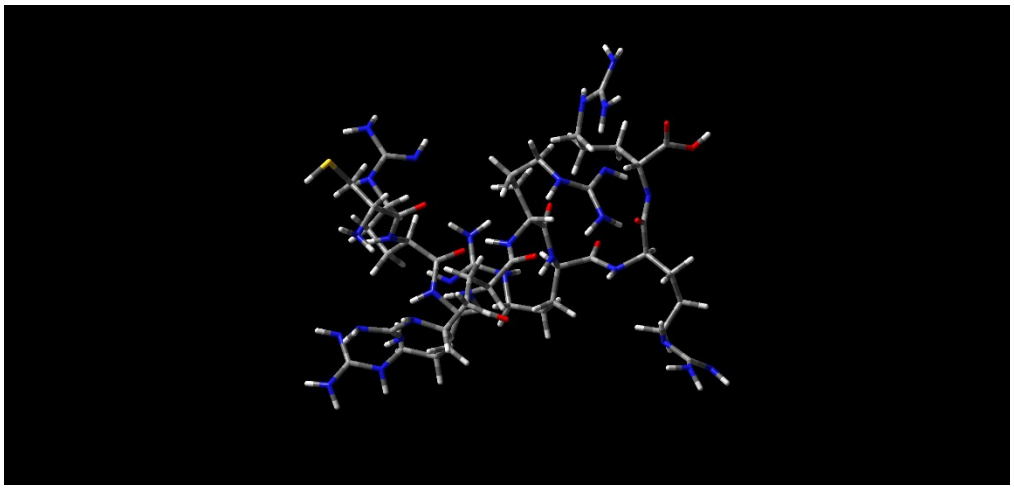
Figure S1f, continued. PM3 minimum-energy structures and calculated energies for P6 and protonated P6 (*28, 29* and *30*).



31 (-161.89 Kcal/mol)



32 (-157.38 Kcal/mol)



33 (-162.33 Kcal/mol)

Figure S1f, continued. PM3 minimum-energy structures and calculated energies for P6 and protonated P6 (*31, 32* and *33*).

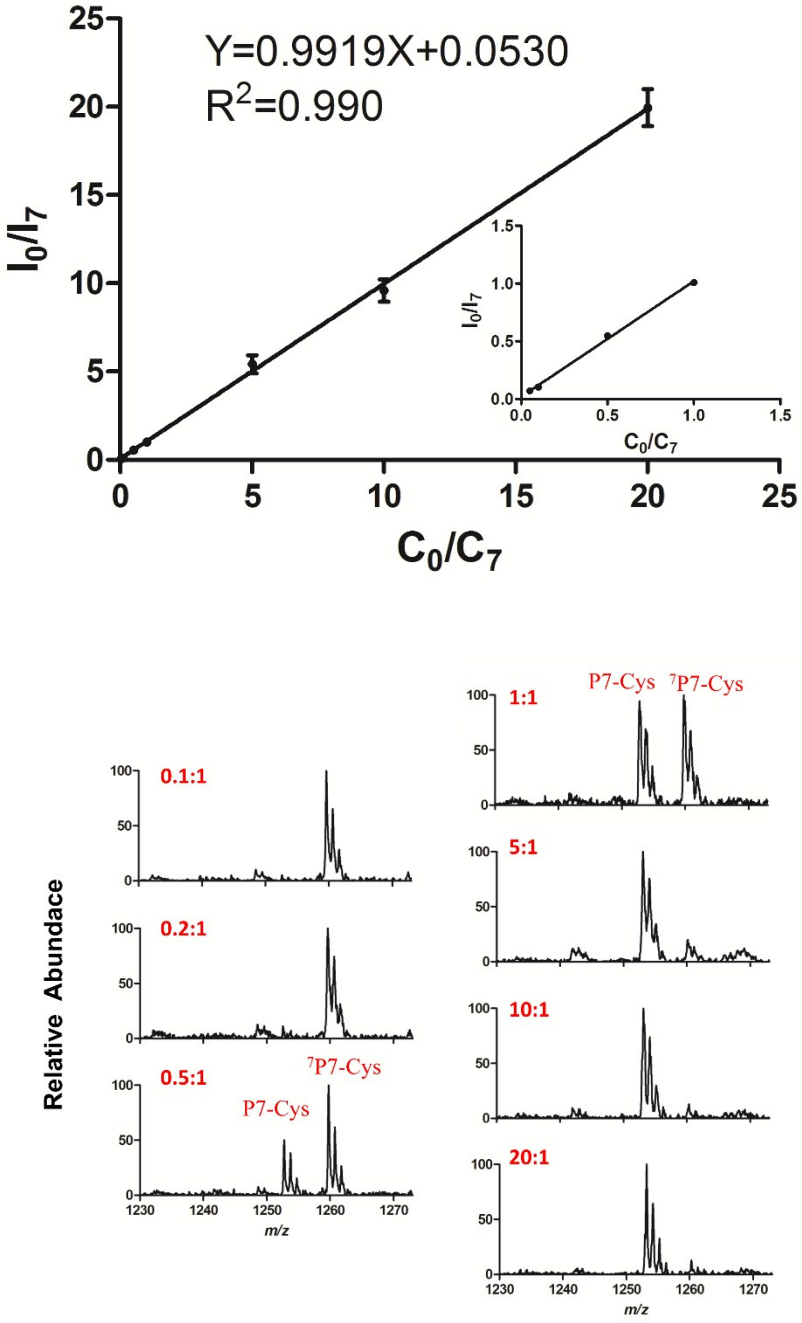


Figure S2. The regression line of the measured peak intensity ratios versus the concentration ratios (0.1:1, 0.2:1, 0.5:1, 1:1, 5:1, 10:1, 20:1) of P7/^{7}P7 labeled Cys. I_0/I_7, the peak intensity ratios of P7/^{7}P7 labeled Cys; C_0/C_7, the concentration ratios of P7/^{7}P7 labeled Cys. The concentrations of P7 labeled Cys are 0.1, 0.2, 0.5, 1, 5, 10, and 20 μM; the concentration of ^{7}P7 labeled Cys is 1 μM.

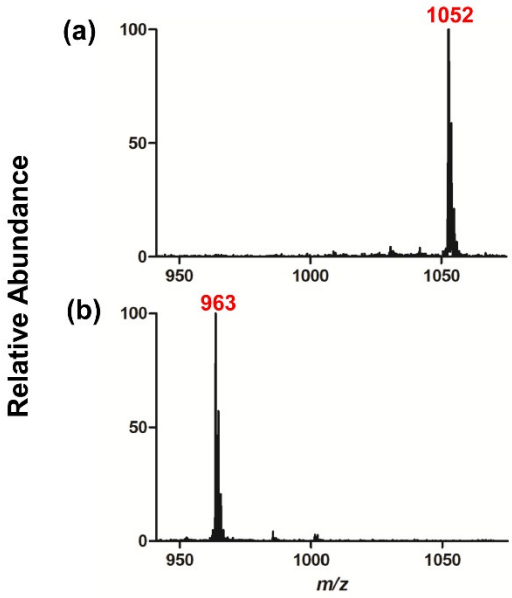


Figure S3. MALDI-TOF-MS spectra of (a) ^{7}P2-3-PA, (b) ^{7}P2. The theoretical *m/z* of ^{7}P2-3-PA is 1051.5 for $[M+H]^+$, and the theoretical *m/z* of ^{7}P2 is 962.5 for $[M+H]^+$.

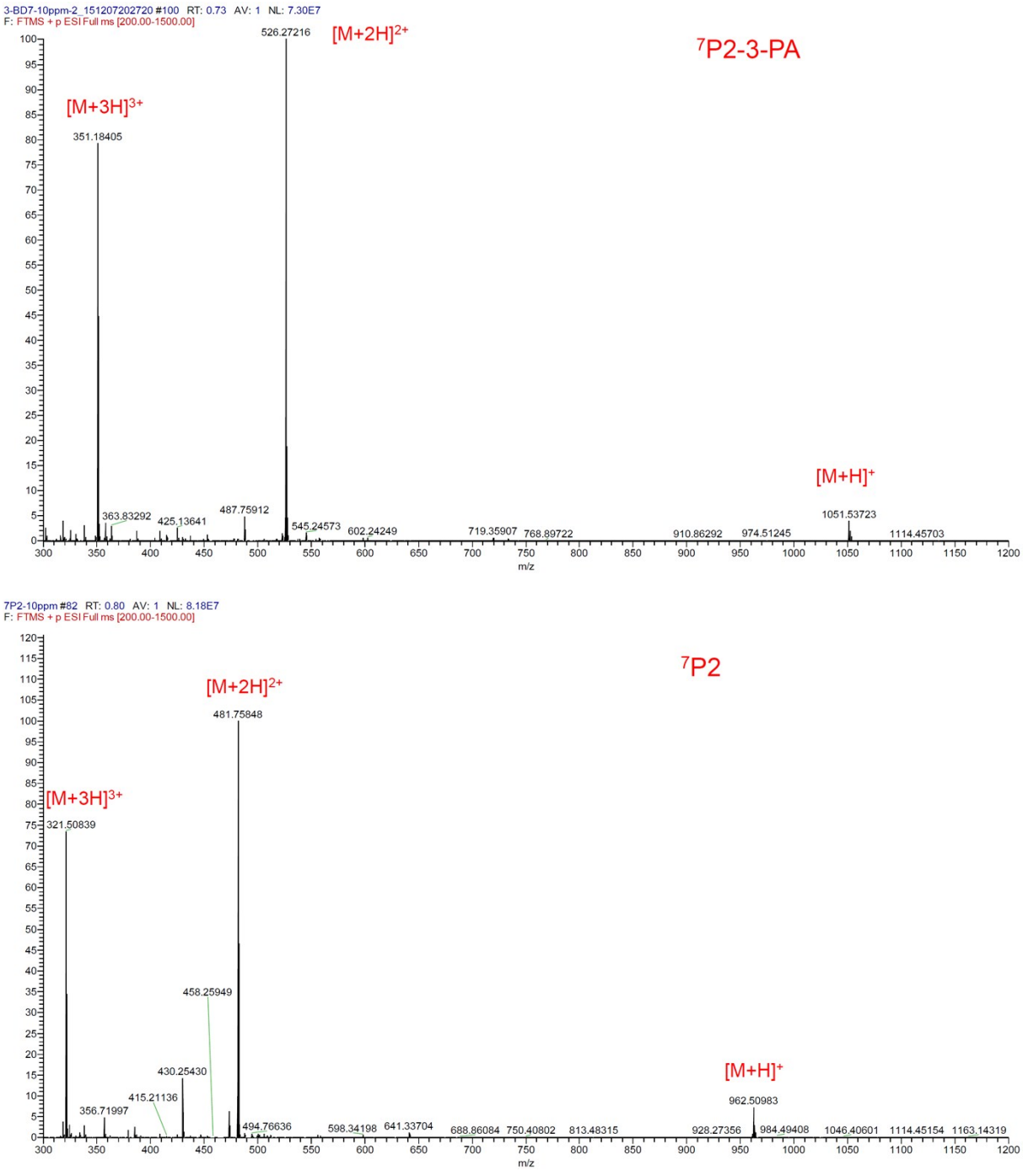


Figure S4. Electrospray ionization-high resolution MS spectra of ^{7}P2-3-PA and ^{7}P2. The theoretical *m/z* of ^{7}P2-3-PA is 1051.5373 for $[M+H]^{+}$, 526.2726 for $[M+2H]^{2+}$ and 351.1843 for $[M+3H]^{3+}$. The theoretical *m/z* of ^{7}P2 is 962.5108 for $[M+H]^{+}$, 481.7593 for $[M+2H]^{2+}$ and 321.5088 for $[M+3H]^{3+}$.

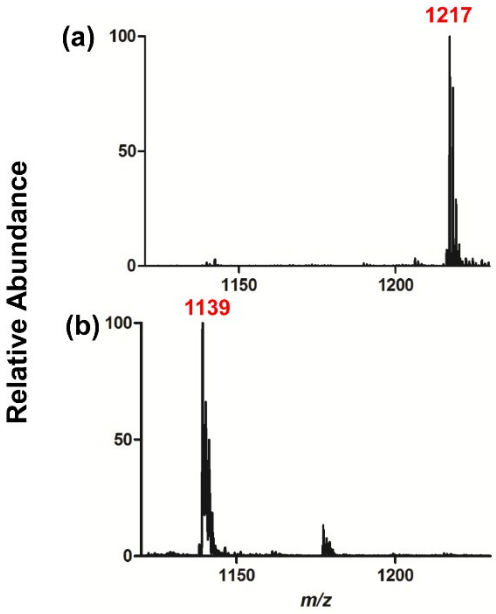


Figure S5. MALDI-TOF-MS spectra of (a) ^{7}P7-ME, (b) ^{7}P7. The theoretical *m/z* of ^{7}P7-ME is 1216.6 for $[M+H]^+$, and the theoretical *m/z* of ^{7}P7 is 1138.6 for $[M+H]^+$.

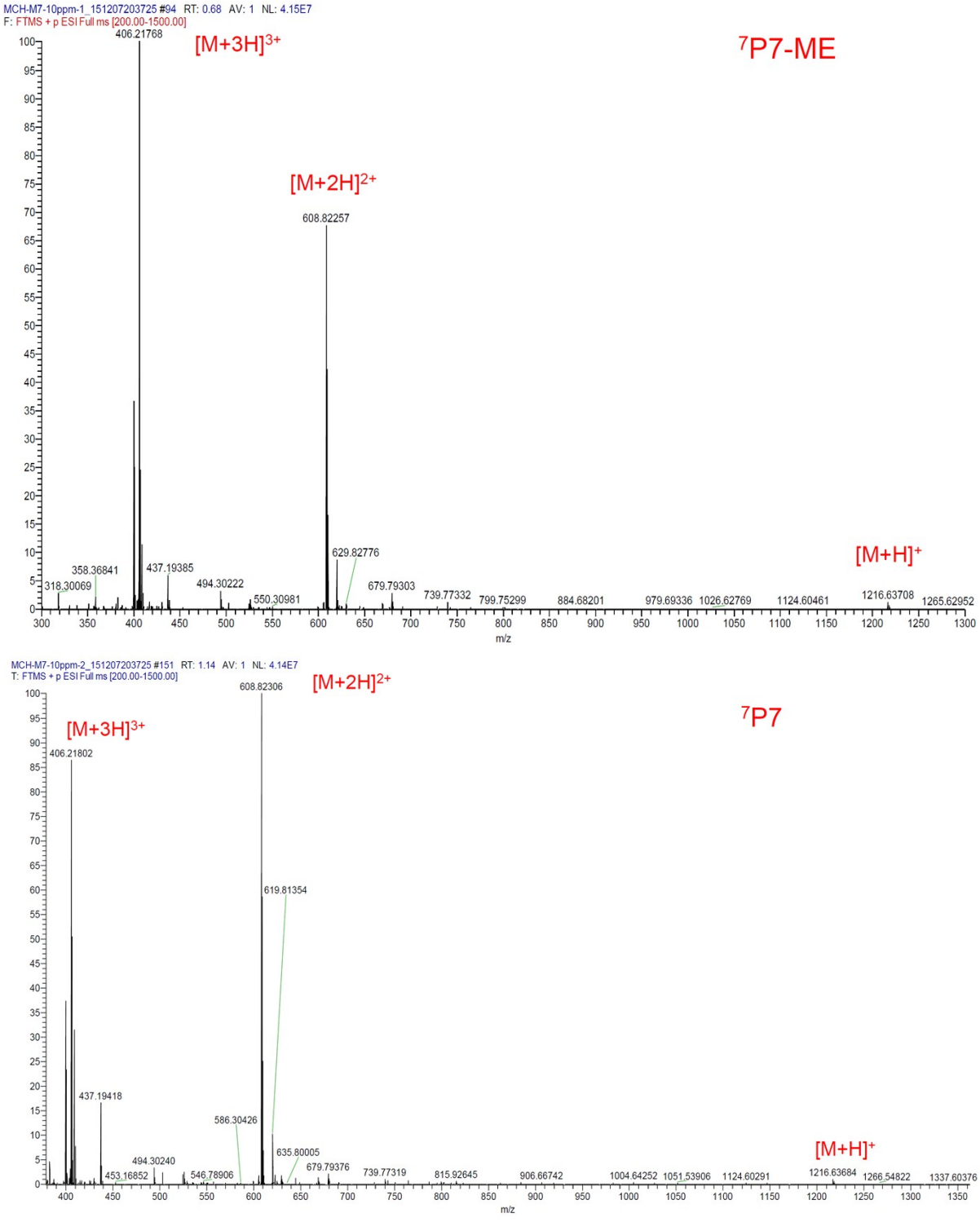


Figure S6. Electrospray ionization-high resolution MS spectra of ^{7}P7-ME and ^{7}P7. The theoretical *m/z* of ^{7}P7-ME is 1216.6374 for $[M+H]^{+}$, 608.8227 for $[M+2H]^{2+}$ and 406.2177 for $[M+3H]^{3+}$. The theoretical *m/z* of ^{7}P7 is 1138.6235 for $[M+H]^{+}$, 569.8157 for $[M+2H]^{2+}$ and 380.2131 for $[M+3H]^{3+}$.

References

1. Y. Q. Huang, G. D. Ruan, J. Q. Liu, Q. Gao and Y. Q. Feng, *Anal Biochem*, 2011, 416, 159-166.
2. G. S. Gorman, J. P. Speir, C. A. Turner and I. J. Amster, *J Am Chem Soc*, 1992, 114, 3986-3988.
3. K. Zhang, D. M. Zimmerman, A. Chungphillips and C. J. Cassady, *J Am Chem Soc*, 1993, 115, 10812-10822.