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Supplementary Information for

Unnatural Enzyme Activation by a Metal-Responsive Regulatory Protein

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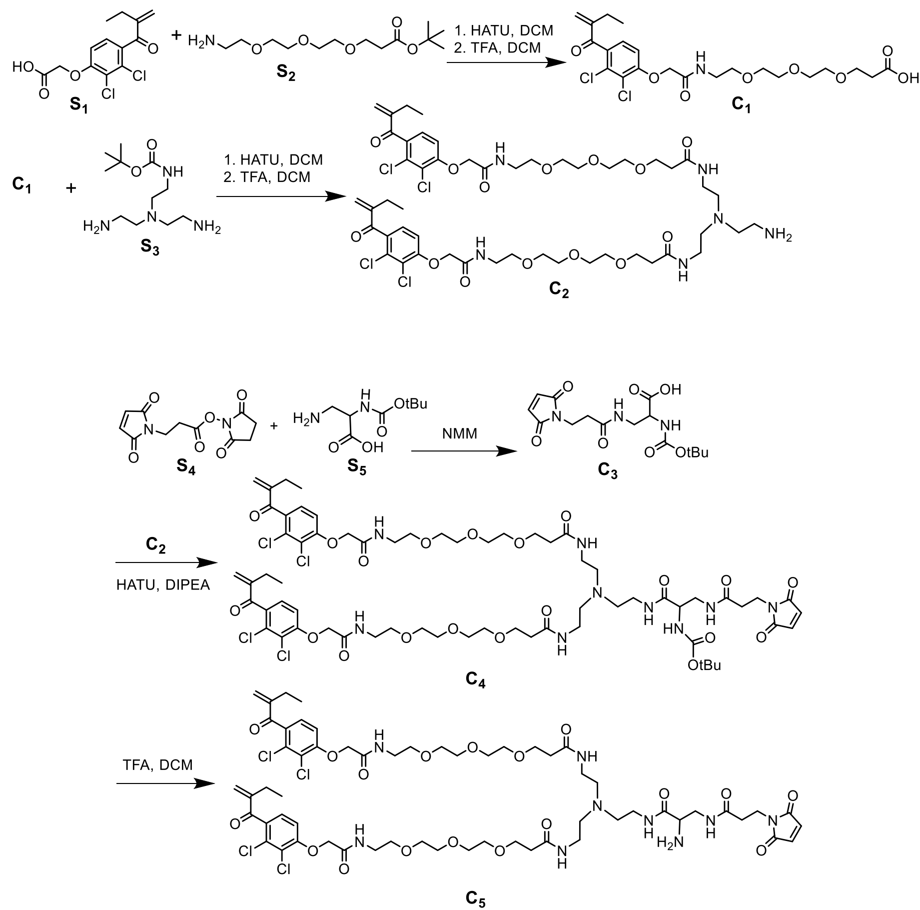
1. Materials and methods

All reagents and solvents were obtained from commercial suppliers. Recombinant human GST isoforms, GST-M1, GST-P1, and mouse recombinant His-Calmodulin were obtained from the Israel Structure Proteomics Center (Weizmann Institute of Science, Rehovot, Israel). Cys-modified mastoparan peptide (INLKALAALAKKILGC) was purchased from Shanghai Hanhong Scientific Co., Ltd. (Shanghai, China). Aluminum-backed silica plates (Merck silica gel 60 F254) were used for thin layer chromatography (TLC) to monitor the solution-phase reactions. TLC visualization was carried out using short wavelength ultraviolet (UV) light at 254 nm. The ^{1}H-NMR spectra were recorded using a 300 MHz, 400 MHz, or 500 MHz Bruker AvanceIII (300MHz, 400MHz) and Avance IIIHD (500MHz) NMR spectrometers; a 400 MHz Bruker Avance III NMR spectrometer was also used to record the ^{13}C{^{1}H}-NMR spectra. Chemical shifts are reported in ppm on the δ scale downfield from TMS as the internal standard. The following abbreviations were used to describe the peaks: s-singlet, d-doublet, dd-double doublet, t-triplet, q-quartet, quin-quintet, m-multiplet, and br-broad. Electrospray mass spectrometry was performed with a Micromass Platform LCZ-4000 instrument at the Weizmann Institute of Science mass spectrometry unit. Preparative HPLC was carried out using an Agilent 218 purification system equipped with an autosampler, a UV-Vis dual wavelength detector, and a 440-LC fraction collector. The analytical purity of all the final compounds was confirmed by using an Agilent 1260 infinity quaternary pump LC system and a maximum pressure 400 bar, equipped with a diode-array UV detector and autosampler. Analysis was performed using a 0.1% TFA water/acetonitrile gradient as the eluent and by detecting the absorbance at 220, 254, 360, 480, 550, and 630 nm. The enzymatic activity-based assays, fluorescence measurements, and fluorescence polarization assays were carried out using a BioTek synergy H4 hybrid multiwall plate reader in either clear flat-bottom polystyrene 384-well microplates (Greiner, for absorption assays) or black flat-bottom polystyrene NBS 384-well microplates (Greiner, for fluorescence assays). LC/MS runs were performed on a Waters ACUITY UPLC class H instrument. In-gel fluorescence detection was performed using a Typhoon™ FLA 9500 laser scanner.

2. Synthetic Procedures



S_6 + S_7 → (Acetic anhydride) → C_6

S_8 → (acetic anhydride, pyridine) → C_7 → (C_5; HATU, DIPEA) → bis-EA-a

bis-EA-a + HS–CGLIKKALAALAKLNI → (ACN, H_2O, DMSO, TEAA) → CT-3a

S_9 + S_{10} → DCM → C_8

→ C_2, HATU, DCM → C_9

→ TFA, DCM → C_{10}

→ S_{11}, HATU, DCM → **bis-EA-b**

→ HS–CGLIKKALAALAKLNI, ACN, H_2O, TEAA → **CT-3b**

Compounds **C_1** and **C_2** were synthesized according to previously reported procedures.[1]

Compound C_3. A solution of N-Succinimidyl 3-maleimidopropionate (**S_4**, 111.5 mg, 0.42 mmol) in DCM (5 mL) was added dropwise to a solution of N(α)-Boc-L-2,3-diaminopropionic acid (**S_5**, 134.5 mg, 0.66 mmol) and N-Methylmorpholine (46 µL, 0.42 mmol) in DMF (7 mL) at 0 °C. The reaction mixture was allowed to reach room temperature while stirring for 48 hours. After removing the solvent under high vacuum, the crude material was purified by RP-HPLC (162 mg, 87%). ^{1}H NMR (500 MHz, CD_3CN): δ 6.86 (s, 1H), 6.77 (s, 2H), 5.93 (d, J = 6.9 Hz, 1H), 4.22-4.15 (m, 1H), 3.71 (t, J = 7.0 Hz, 2H), 3.62-3.55 (m, 1H), 3.41-3.34 (m, 1H), 2.45 (t, J = 7.0 Hz, 2H), 1.44 (s, 9H). HRMS-ESI (*m/z*): Calcd. for $[M+Na]^+$ 378.1277; found 378.1283.

Compound C_4. **C_2** (494.1 mg, 0.44 mmol) and **C_3** (130.0 mg, 0.36 mmol) were dissolved in DMF (10 mL). HATU (173.3 mg, 0.45 mmol) and DIPEA (504 µL, 2.88 mmol) were added, and the reaction mixture was stirred under argon overnight. It was then dried under vacuum, redissolved in DCM (40 mL), and washed with 0.5 $M_{(aq)}$ HCl and brine. The organic phase was dried using Na_2SO_4, dried under reduced pressure, and purified by combi-flash column chromatography (silica gel, 9% MeOH in DCM) to afford **C_4** (115 mg, 22%). ^{1}H NMR (400 MHz, MeOD): δ 7.28 (d, J = 8.5 Hz, 2H), 7.13 (d, J = 8.5 Hz, 2H), 6.83 (s, 2H), 6.06 (s, 2H), 5.62 (s, 2H), 4.74 (s, 4H), 4.20 (t, J = 5.6 Hz, 1H), 3.80-3.73 (m, 6H), 3.67-3.41 (m, 38H), 2.58-2.41 (m, 10H), 1.46 (s, 9H), 1.16 (t, J = 7.4 Hz, 6H). ^{13}C{^{1}H}-NMR (100 MHz, MeOD): δ 195.81, 174.58, 173.09, 172.27, 170.84, 168.36, 156.24, 155.37, 150.28, 134.14, 133.62, 130.38, 128.65, 127.22, 122.40, 111.58, 79.69, 70.18, 70.08, 69.99, 69.89, 68.96, 67.99, 66.58, 54.64, 54.20, 40.65, 38.70, 35.89, 34.78, 34.62, 34.15, 33.89, 27.37, 23.10, 11.60. HRMS-ESI (*m/z*): Calcd. for $[M+Na]^+$ 1480.5032; found 1480.5028.

Compound C_7. **S_6** (201.8 mg, 0.80 mmol) and **S_7** (212.1 mg, 0.82 mmol) were dissolved in acetic anhydride (1 mL). The reaction was then heated to reflux and stirred under argon for 2 h. After the reaction mixture cooled to room temperature, EtOAc (20 mL) was added, and the precipitate (**C_6**) was collected on a Büchner funnel, washed again with EtOAc, and used without further purification in the next step. Next, **C_6** (267 mg, 0.63 mmol) was dissolved in acetic anhydride:pyridine (10 mL, 1:1), S_8 (222 mg, 0.628 mmol) was added, and the reaction was stirred under reflux for 4 h. After the reaction mixture was cooled to room temperature, EtOH (20 mL) was added, the precipitate was collected on a Büchner funnel, and dried under reduced pressure. The crude was first purified by combi-flash column chromatography (silica gel, 30% MeOH in DCM) and then by RP-HPLC to afford **C_7** (90.2 mg, 22%). ^{1}H NMR (400 MHz, MeOD): δ 8.33 (t, J = 12.3 Hz, 2H), 7.95-7.88 (m, 4H), 7.36 (dd, J_1 = 8.8, J_2 = 2.2 Hz, 2H), 6.70 (t, J = 12.4 Hz, 1H), 6.35 (d, J = 13.8 Hz, 2H), 4.14 (t, J = 7.1 Hz, 2H), 3.67 (s, 3H), 2.37 (t, J = 7.2 Hz, 2H), 1.90-1.66 (m, 16H), 1.56-1.43 (m, 2H). ^{13}C{^{1}H}-NMR (100 MHz, MeOD): δ 174.28, 173.87, 154.93, 154.81, 144.19, 143.54, 141.19, 141.11,

126.67, 126.25, 119.99, 119.88, 110.21, 110.06, 103.93, 103.72, 49.17, 43.56, 41.70, 33.06, 30.34, 29.37, 26.66, 26.42, 26.27, 25.85, 24.24. HRMS-ESI (*m/z*): Calcd. for $[M-H]^-$ 641.1991; found 641.1985.

Compound bis-EA-a. A solution of $\mathbf{C_4}$ (115 mg, 0.08 mmol) in TFA:DCM (6 mL, 1:1) was stirred at room temperature for 3 h. The solvent was then removed under high vacuum to afford $\mathbf{C_5}$. Next, $\mathbf{C_5}$ (25 mg, 18.4 µmol) and $\mathbf{C_7}$ (9.9 mg, 15.4 µmol) were mixed in DMF (1 mL). HATU (6.84 mg, 18.0 µmol) and DIPEA (7.8 µL, 4.50 µmol) were added, and the reaction was stirred overnight under argon at room temperature. After the reaction was completed, the crude material was purified by RP-HPLC, which afforded **bis-EA-a** (10.0 mg, 32%). ^{1}H NMR (400 MHz, $(CD_3)_2SO$): δ 8.35 (t, *J* = 13.0 Hz, 2H), 7.83 (s, 2H), 7.65 (d, *J* = 8.1 Hz, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.28 (d, *J* = 9.3 Hz, 2H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.97 (s, 2H), 6.55 (t, *J* = 12.3 Hz, 1H), 6.28 (t, *J* = 15.7 Hz, 2H), 6.07 (s, 2H), 5.56 (s, 2H), 4.73 (s, 4H), 4.26-4.17 (m, 3H), 3.63-3.55 (m, 7H), 3.52-3.34 (m, 24H), 3.33-3.20 (m, 12H), 2.42-2.31 (m, 10H), 2.12 (t, *J* = 6.8 Hz, 2H), 1.69 (s, 12H), 1.54 (q, *J* = 6.9 Hz, 2H), 1.40-1.29 (m, 2H), 1.29-1.21 (m, 2H), 1.07 (t, *J* = 7.5 Hz, 6H). ^{13}C{^{1}H}-NMR (100 MHz, $(CD_3)_2SO$): δ 195.61, 174.26, 173.24, 172.81, 171.99, 171.43, 171.25, 170.66, 167.15, 158.88, 158.53, 155.87, 154.75, 154.63, 149.83, 145.76, 145.33, 143.19, 142.64, 140.97, 140.92, 135.00, 132.99, 129.92, 129.83, 127.98, 126.57, 126.48, 126.12, 121.63, 120.40, 120.32, 127.85, 114.94, 112.45, 110.58, 104.15, 103.80, 70.19, 70.13, 70.04, 69.98, 69.26, 68.33, 66.96, 53.28, 52.60, 52.18, 49.37, 49.31, 43.84, 39.35, 38.84, 36.38, 35.37, 34.82, 34.33, 34.30, 34.16, 31.71, 27.57, 27.37, 27.10, 26.16, 25.09, 23.40, 12.82. HRMS-ESI (*m/z*): Calcd. for $[M+H]^+$ 1982.6652; found 1982.6624.

Compound CT-3a. bis-EA-a (16.4 mg, 8.25 µmol) was dissolved in ACN:DDW(400 µL, 1:1); then TEAA (50 µL) and DMSO (200 µL) were added. Next, cysteine-modified mastoparan peptide (5.1 mg, 3.10 µmol) was dissolved in DDW (300 µL) and added to the reaction mixture. The reaction was stirred at room temperature for 3 h and monitored by HPLC. After the reaction was completed, the crude material was immediately purified by RP-HPLC to afford **CT-3a** (3.6 mg, 32%). HRMS-ESI (*m/z*): Calcd. for $[M+H]^+$ 3622.71; found 3623.71.

Compound $\mathbf{C_8}$. A solution of dansyl chloride ($\mathbf{S_9}$, 708 mg, 2.63 mmol) in DCM (7.5 mL) was added dropwise to a solution of N(α)-Boc-L-2,3-diaminopropionic acid ($\mathbf{S_{10}}$, 590.0 mg, 2.89 mmol) and triethylamine (764 µL, 5.47 mmol) in DCM (7.5 mL) at 0 °C. The reaction mixture was allowed to reach room temperature while stirring under argon overnight. Next, the solvent was dried under reduced pressure and purified by combi-flash column chromatography (silica gel, 3% MeOH in DCM) to afford $\mathbf{C_8}$ (115 mg, 22%). ^{1}H NMR (400 MHz, MeOD): δ 8.58 (d, *J* = 8.5 Hz, 1H), 8.44 (d, *J* = 8.5 Hz, 1H), 8.25 (d, *J* = 7.3 Hz, 1H), 7.66 (t, *J* = 8.0 Hz, 2H), 7.43 (d, *J* = 7.6 Hz, 1H), 4.10 (t, *J* = 5.2 Hz, 1H), 3.27 (d, *J* = 4.5 Hz, 2H), 3.00 (s, 6H), 1.41 (s, 9H). ^{13}C{^{1}H}-NMR (100 MHz, MeOD): δ 172.00, 156.23, 151.81, 135.34, 129.97, 129.88, 129.49, 128.82, 127.83, 122.91, 119.04, 115.10, 79.35, 53.73, 44.40, 43.50, 27.27. HRMS-ESI (*m/z*): Calcd. for $[M-H]^-$ 436.1542; found 436.1537.

Compound C_9. C_2 (76.9 mg, 0.07 mmol) and C_8 (23.0 mg, 0.05 mmol) were first dissolved in DCM (3 mL), and then HATU (34.9 mg, 0.09 mmol) and DIPEA (65.80 µL, 0.38 mmol) were added. The reaction was stirred under argon overnight at room temperature. The mixture was then washed with water and sodium bicarbonate$_{(aq)}$. The organic phase was dried using Na_2SO_4, dried under reduced pressure, and purified by combi-flash column chromatography (silica gel, 5% MeOH in DCM) to afford C_9 (49 mg, 58.9%). ^{1}H NMR (400 MHz, MeOD): δ 8.58 (d, J = 8.5 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H), 8.18 (d, J = 7.5 Hz, 1H), 7.94 (t, J = 5.0 Hz, 1H), 7.61 (t, J = 8.3 Hz, 2H), 7.32 (d, J = 7.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 2H), 7.10 (d, J = 8.5 Hz, 2H), 6.05 (s, 2H), 5.60 (s, 2H), 4.72 (s, 4H), 4.20 (t, J = 5.0 Hz, 1H), 3.73 (t, J = 5.1 Hz, 6H), 3.65-3.41 (m, 36H), 3.21 (d, J = 4.7 Hz, 2H), 2.91 (s, 6H), 2.52 (t, J = 5.2 Hz, 4H), 2.46 (q, J = 7.5 Hz, 4H), 1.44 (s, 9H), 1.15 (t, J = 7.4 Hz, 6H). ^{13}C{^{1}H}-NMR (100 MHz, MeOD): δ 195.82, 174.78, 172.76, 168.38, 156.18, 155.33, 151.59, 150.27, 135.29, 133.61, 130.37, 129.93, 129.76, 129.43, 128.70, 128.66, 127.95, 127.20, 123.10, 122.40, 119.09, 115.28, 111.55, 79.83, 70.15, 70.05, 69.97, 69.87, 68.95, 67.97, 66.55, 54.77, 54.55, 44.47, 43.61, 38.67, 35.81, 34.96, 34.80, 27.35, 23.09, 11.59. HRMS-ESI (*m/z*): Calcd. for $[M+Na]^+$ 1562.5273; found 1562.5277.

Compound bis-EA-b. A solution of C_9 (74.8 mg, 0.05 mmol) in TFA:DCM (3 mL, 1:1) was stirred at room temperature for 3 h. The solvent was then removed under high vacuum. The resulting material (C_{10}) was redissolved in DCM (3 mL), and then HATU (26.6 mg, 0.07 mmol), DIPEA (36.0 µL, 0.21 mmol), and 3-maleimidopropionic acid (S_{11}, 11.3 mg, 0.07 mmol) were added, and the reaction mixture was stirred under argon overnight at room temperature. The mixture was then washed with water, and the organic phase was dried using Na_2SO_4, and dried under reduced pressure. The crude was then purified by combi-flash column chromatography (silica gel, 7% MeOH in DCM) to afford **bis-EA-b** (45 mg, 56.5%). ^{1}H NMR (400 MHz, MeOD): δ 8.57 (d, J = 8.5 Hz, 1H), 8.31 (d, J = 8.7 Hz, 1H), 8.19 (d, J = 6.5 Hz, 1H), 7.59 (t, J = 7.8 Hz, 2H), 7.28 (d, J = 7.6 Hz, 1H), 7.25 (d, J = 8.5 Hz, 2H), 7.10 (d, J = 8.5 Hz, 2H), 6.81 (s, 2H), 6.04 (s, 2H), 5.60 (s, 2H), 4.72 (s, 4H), 4.35 (t, J = 6.2 Hz, 1H), 3.81-3.66 (m, 6H), 3.66-3.46 (m, 26H), 3.30-3.15 (m, 8H), 2.91 (s, 6H), 2. 58 (t, J = 6.0 Hz, 4H), 2.51-2.41 (m, 10H), 1.15 (t, J = 7.4 Hz, 6H). ^{13}C{^{1}H}-NMR (100 MHz, MeOD): δ 195.81, 174.72, 171.89, 171.85, 170.94, 168.37, 155.33, 151.58, 150.27, 135.17, 134.17, 133.61, 130.37, 129.98, 129.75, 129.40, 128.86, 128.65, 127.97, 127.20, 123.14, 122.39, 119.10, 115.29, 111.56, 70.16, 70.05, 69.97, 69.86, 68.94, 67.97, 66.55, 54.92, 54.46, 53.61, 44.47, 43.04, 38.68, 35.86, 34.95, 34.71, 34.19, 33.72, 23.09, 11.58. HRMS-ESI (*m/z*): Calcd. for $[M+Na]^+$ 1613.5018; found 1613.5027.

Compound CT-3b. bis-EA-b (14.0 mg, 8.79 µmol) was dissolved in ACN (150 µL), and TEAA (12.5 µL) was added. Next, cysteine-modified mastoparan peptide (8.1 mg, 4.94 µmol) was dissolved in DDW (100 µL) and added to the reaction mixture. The reaction was stirred at room temperature for 8 h and monitored

by HPLC. After completion, the crude material was immediately purified by RP-HPLC to afford **CT-3b** (2.9 mg, 18.2%). HRMS-ESI (*m/z*): Calcd. for $[M+H]^+$ 3232.58; found 3232.55.

3. **GST inhibition assays.** The inhibitory effect of CT-3a and CT-3b on GST-M1-1 and GST-P1-1 activity was assessed according to the method established by Habig et al.[2] GST activity was determined spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) as substrates in phosphate-buffered saline (PBS) solution (4 mM, pH = 7.4). Briefly, GST was incubated with either CT-3a or CT-3b for 10 minutes at 25°C. Subsequently, CDNB and GSH were added, and the formation of the GS-CDNB conjugate was monitored using a microplate reader at λ=340 nm. To obtain the inhibition constants (K_i) of both CTs, GST-M1 at three different concentrations (5, 10, and 20 nM) was incubated with increasing concentrations of either CT-3a or CT-3b (0-1000 nM). The concentrations of CDNB and GSH were 1.5 mM and 3 mM, respectively. The data are expressed as the mean values ± std. deviation of triplicate measurements. The data were plotted and analyzed using GraphPad Prism 7.02 and fitted to the Morrison equation for tight binding substrates.[3-4]

$$Y = \frac{V_0\,(1 - (E + X + Q) - \{(E + X + Q)^2 - 4 \cdot E \cdot X\}^{1/2})}{2 \cdot E} \quad , \quad Q = K_i \cdot \left(1 + \frac{S}{K_m}\right)$$

where Y is the enzyme activity, X represents different concentrations of the inhibitor (CT-3a or CT-3b), E is the enzyme concentration, S is the concentration of substrate, K_m is the Michaelis-Menten constant determined in an experiment without an inhibitor, and V_0 is the initial velocity.

To obtain the half maximal inhibitory concentration (IC_{50}) of both CTs to GST-P1, GST-P1 (20 nM) was incubated with increasing concentrations of either CT-3a or CT-3b (0-1000 nM). The concentrations of CDNB and GSH were 1.5 mM and 3 mM, respectively. The data are expressed as the mean values ± std. deviation of triplicate measurements. The data were plotted and analyzed using GraphPad Prism 7.02 and fitted to a normalized dose-response inhibition curve.

$$Y = \frac{100}{1 + 10^{(\log X - \log IC_{50})}}$$

where Y is the relative calculated V_0 and X represents different concentrations of the inhibitor (CT-3a or CT-3b).

4. **Fluorescence polarization assays.** The dissociation constant between CT-3a and CaM was determined using a fluorescence polarization (FP) assay conducted at room temperature. The assay was performed with a BioTek Synergy H4 hybrid plate reader equipped with suitable excitation and emission polarizers. Initially, the fluorescence polarization of CT-3a (0-250 nM) in PBS buffer (4 mM, pH = 7.4) was measured

to identify the optimal concentration of CT-3a that produces a stable polarization signal. Subsequently, 50 nM of CT-3a was incubated for 10 minutes with increasing concentrations of CaM (0-1.28 μM) in PBS (4 mM, pH = 7.4), with or without $CaCl_2$ (0.3 mM). The degree of polarization was measured using excitation and emission filters of 635/20 and 671/20, respectively, along with a 660 nm cut-off mirror. The data corrections for increases in the fluorescence of CT-3a were applied following previously published procedures.[5-6] The results are presented as mean values ± standard deviation of hexa-replicate measurements. The data were analyzed using GraphPad Prism 7.02 and fitted to the following saturation equation:

$$X = \frac{[\text{Protein}]}{[\text{Protein}] + K_d}$$

where X represents the bound protein fraction.

5. Dissociation Constant of CT-3b and CaM (Ca^{2+}). To estimate the dissociation constant between CT-3b and CaM (Ca^{2+}), changes in the fluorescence signal of dansyl were monitored with increasing CaM (Ca^{2+}) concentrations. CT-3b (400 nM) was incubated for 10 min with increasing concentrations of CaM (0-1.28 μM) in PBS (4 mM, pH = 7.4) in the absence or presence of $CaCl_2$ (0.3 mM). Subsequently, the fluorescence intensity was recorded at 500 nm (λ_{ex}=340 nm). The data are presented as the mean values ± std. deviation of triplicate measurements. Analysis was performed using GraphPad Prism 7.02 and fitted to the following saturation equation:

$$IF = \frac{IF_{max} \cdot L}{k_d + L}$$

where IF represents the fluorescence intensity at a relative protein concentration (L), IF_{max} is the maximum fluorescence intensity at saturation, and L is the concentration of the protein.

6. GST-CaM(Ca^{2+}) Communication. The communication between GST and CaM was assessed by monitoring the activity of GST, following the previously described procedure. GST-M1 (10 nM) or GST-P1 (20 nM) was incubated for 10 minutes with either CT-3a or CT-3b (at concentrations of 200 nM for GST-M1 and 400 nM for GST-P1), along with CaM (1 μM) in PBS buffer (4 mM, pH = 7.4). Subsequently, $CaCl_2$ (0.3 mM) was added, and the samples were further incubated for 10 minutes. Then, CDNB (720 μM) and GSH (350 μM) were introduced, and the enzymatic reaction was monitored spectrophotometrically at 340 nm. Control experiments were conducted using compounds bis-EA-a and bis-EA-b, following a similar protocol.

7. LC/MS measurement. GST-M1 (1.25 µM) or GST-P1 (1.25 µM) was incubated for 10 min in the absence or presence of CT-3a (2.5 µM), and injected into LC/MS. The LC/MS analyses were performed on a Waters ACUITY UPLC class H instrument in a positive ion mode using electrospray ionization. Gradient separations were carried out a C_4 column (300 Å, 1.7 µm, 21 mm x 100 mm) using 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 0.4 mL/min. The column was held at 40 °C and the autosampler at 10 °C. Mass data were collected using a Waters SQD2 detector with an *m/z* range of 2-3071.98 at a range of 700-1300 *m/z*. The desolvation temperature was 500 °C with a flow rate of 1000 L/h. The capillary and cone voltages were maintained at 0.69 kV and 46 V, respectively. Raw data were processed using openLYNX, deconvoluted using MaxEnt, and plotted using GraphPad Prism 7.02.

8. Fluorescence in-gel detection. CT-3a (300 nM) was incubated with different combinations of GST-P1 (300 nM), CaM (300 nM), $CaCl_2$ (0.09 mM), and EDTA (9 mM). Next, the samples were subjected to electrophoresis on a 12% acrylamide gel that was prepared by mixing a 30% acrylamide solution in Tris-HCl (375 mM, pH = 8.8) supplemented with SDS (0.1%), APS (0.12%), and TEMED (0.1%). Electrophoresis was conducted at 120 V for 1 h. Subsequently, the gels were scanned using a TyphoonTM FLA 9500 laser scanner equipped with a 635/670 nm excitation/emission filter set.

9. Real-time changes in GST activity. To monitor real-time changes in GST activity, the enzymatic reaction was conducted simultaneously in two wells. Each well contained GST (10 nM), CT-3a or CT-3b (200 nM), and apoCaM (1000 nM). After a 10-minute incubation, CDNB (720 µM) and GSH (350 µM) were added, and the enzymatic activity of GSTs was measured. At 210 sec into the measurement, 0.3 mM $CaCl_2$ was added to one of the wells, and the enzymatic activity was continuously monitored in real time. In another assay, GST (10 nM) was incubated with CT-3a or CT-3b (200 nM), apoCaM (1000 nM), and $CaCl_2$ (0.3 mM). After a 10-minute incubation, CDNB (720 µM) and GSH (350 µM) were added, and the enzymatic activity of GSTs was measured. At 210 sec into the measurement, 3 mM EDTA was added to one of the wells, and the enzymatic activity was continuously monitored in real time.

10. Reversible switching of GST activity. GST-M1 (10 nM) was incubated with CT-3b (200 nM) and apoCaM (1 µM) for 10 min. Then $CaCl_2$ and EDTA were sequentially added to the mixture with a 3-minute interval between each addition. Finally, CDNB (720 µM) and GSH (350 µM) were added, and the initial velocities (V_0) were measured. The data are expressed as the mean values ± std. deviation of triplicate measurements.

Supplementary Figures

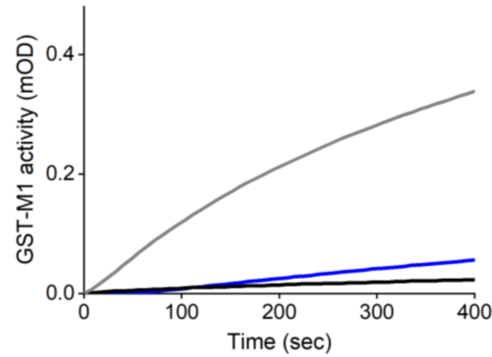


Fig. S1. Activity of GST-M1 before (grey line) and after the addition of 5 µM EA (blue line), or 200 nM CT-3a (black line).

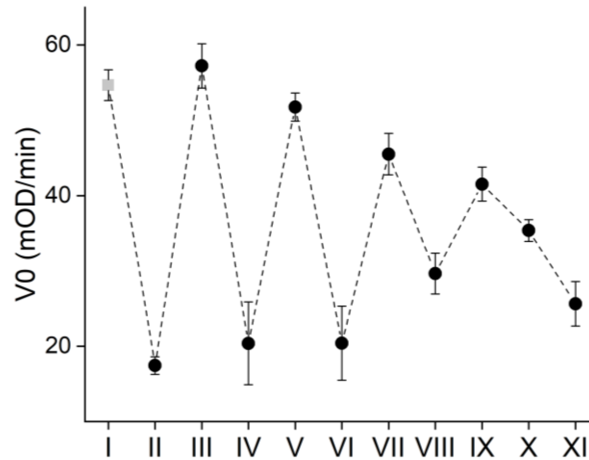


Fig. S2. Initial velocity (V0) measured before (I) and after (II) the addition of CT-3b to GST-M1 in the presence of CaM, followed by sequential additions of (III) Ca^{2+} (20 µM), (IV) EGTA (80 µM), (V) Ca^{2+} (100 µM), (VI) EGTA (400 µM), (VII) Ca^{2+} (500 µM), (VIII) EGTA (3 mM), (IX) Ca^{2+} (3.75 mM), (X) EGTA (22.5 mM), and (XI) Ca^{2+} (28 mM).

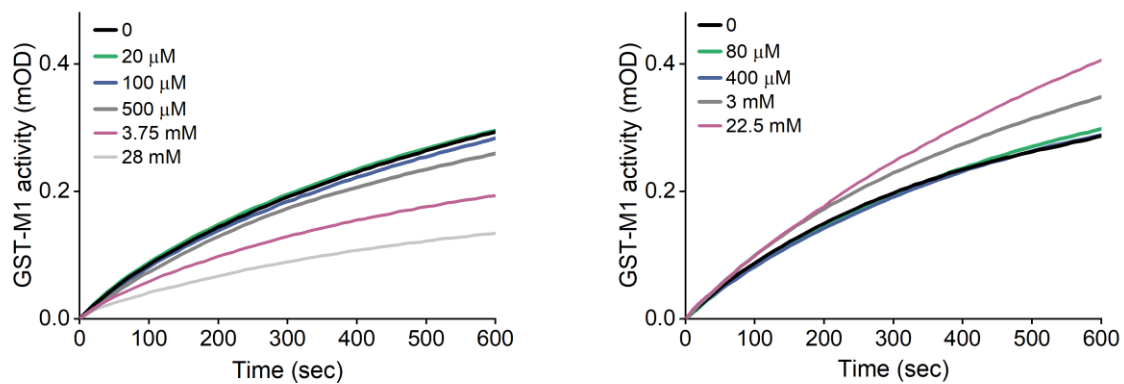
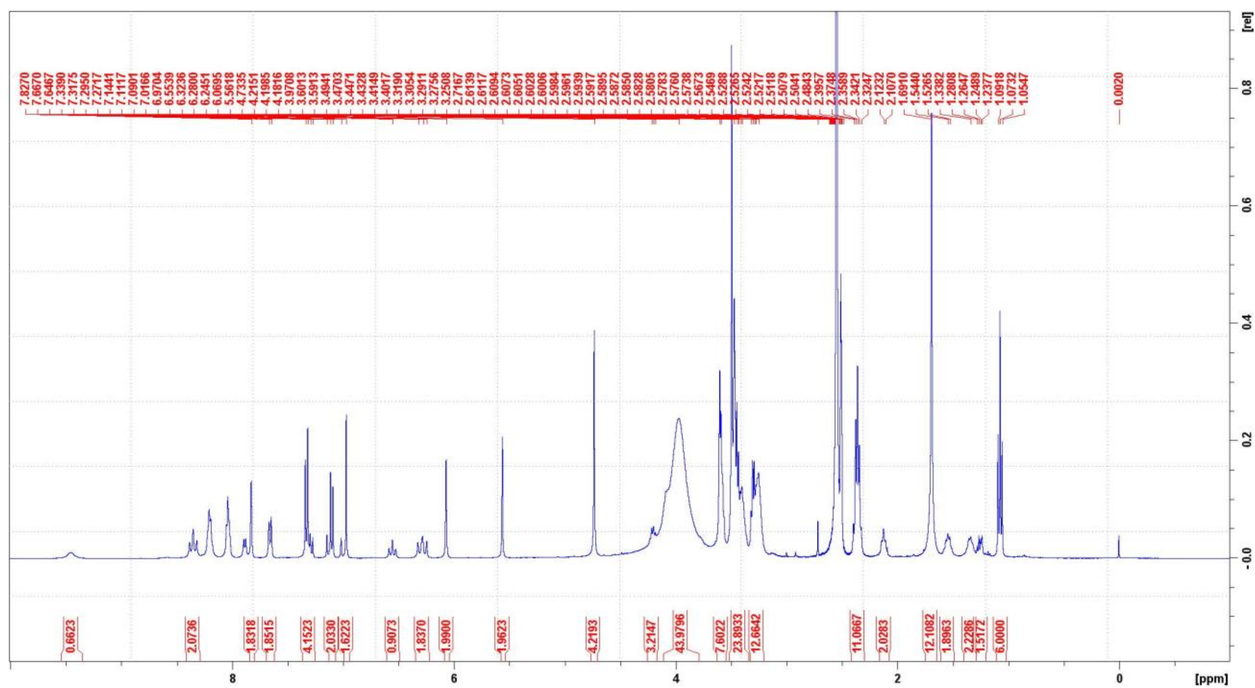
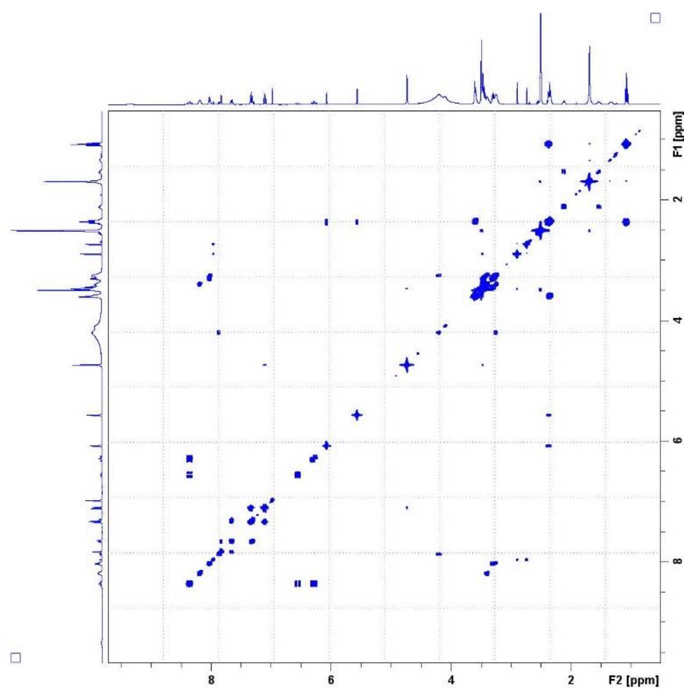


Fig. S3. Activity of GST-M1 upon addition of various concentration of Ca^{2+}(left) or EGTA (right).

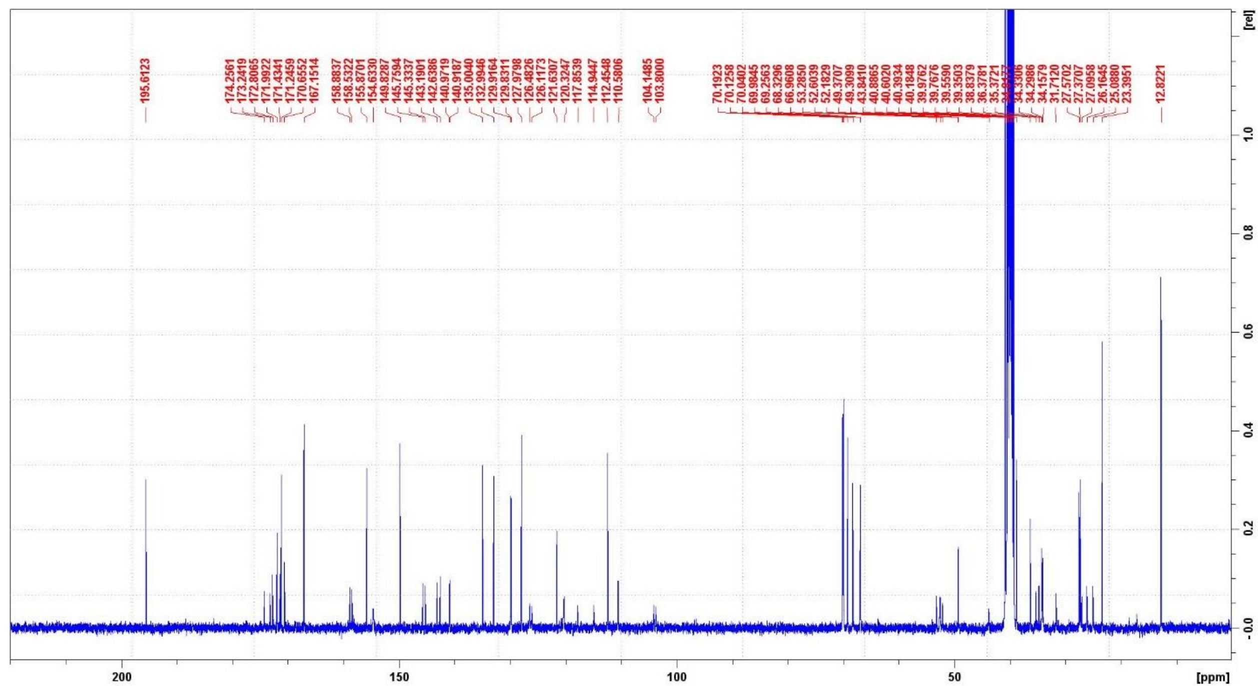
^{1}H NMR spectrum of compound bis-EA-a (400.13 MHz, DMSO-d_6)



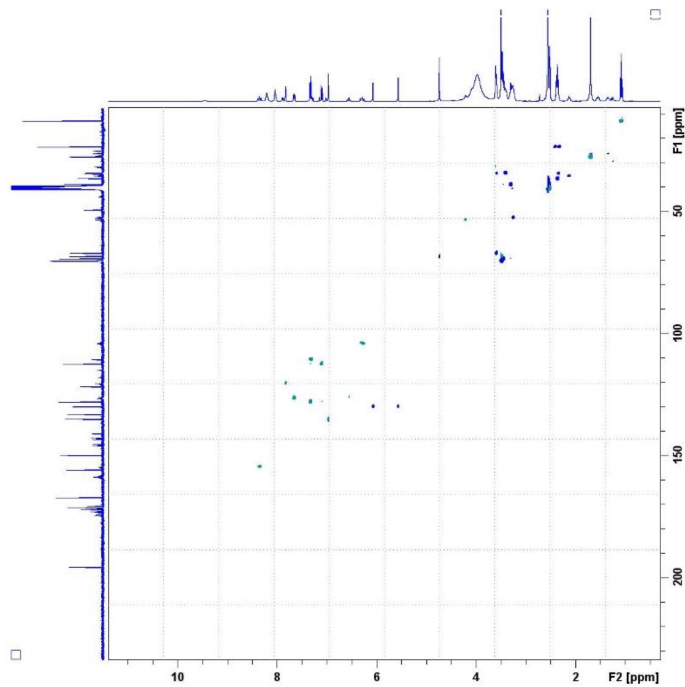
^{1}H-^{1}H COSY spectrum of compound bis-EA-a (400.13 MHz, DMSO-d_6)



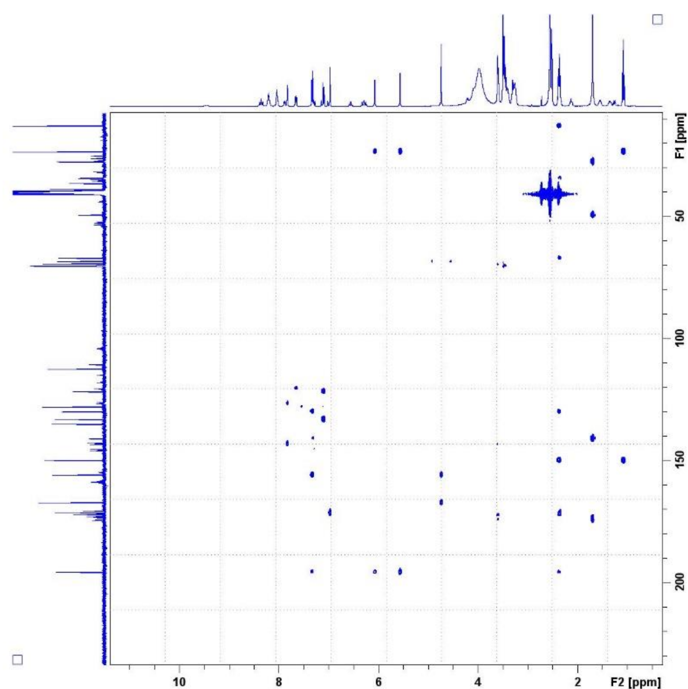
$^{13}C\{^{1}H\}$ NMR spectrum of compound bis-EA-a (400.13 MHz, DMSO-d_6)



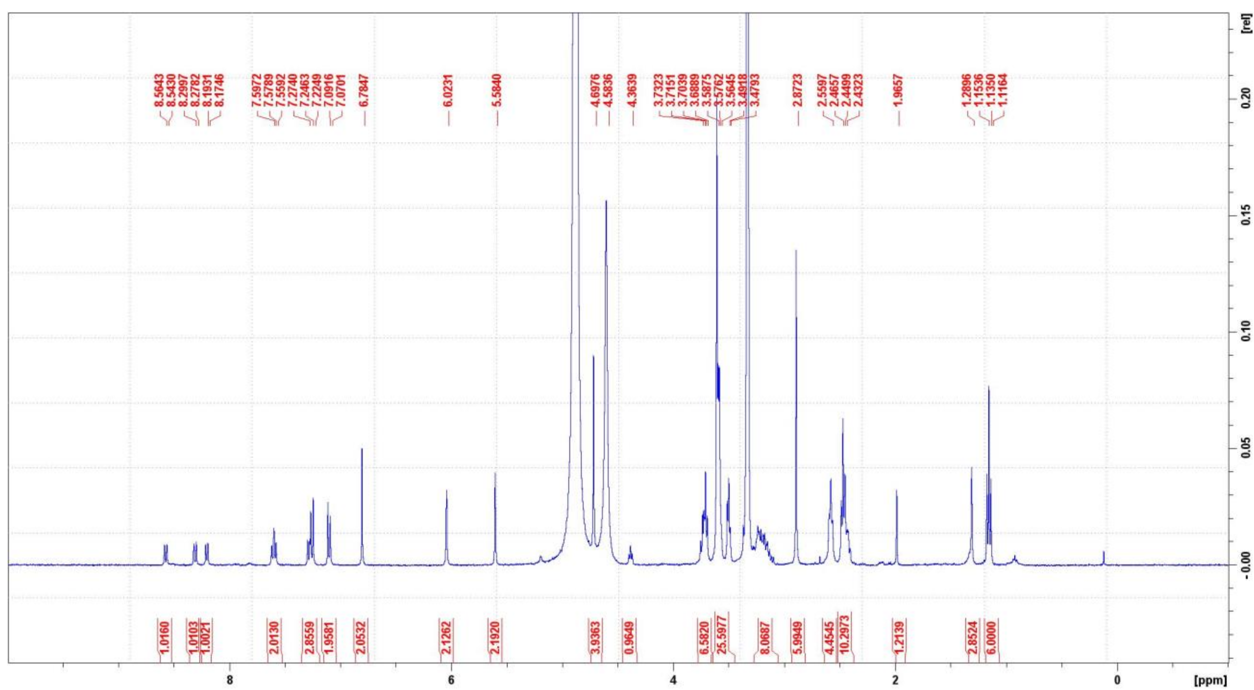
^{1}H-^{13}C phase sensitive HSQC of compound bis-EA-a (400.13 MHz, DMSO-d_6)



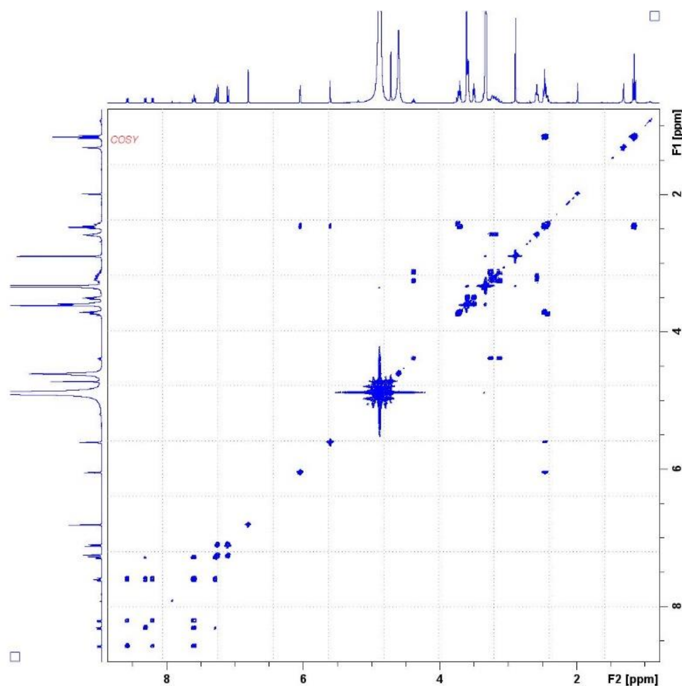
^{1}H-^{13}C HMBC of compound bis-EA-a (400.13 MHz, DMSO-d_6)



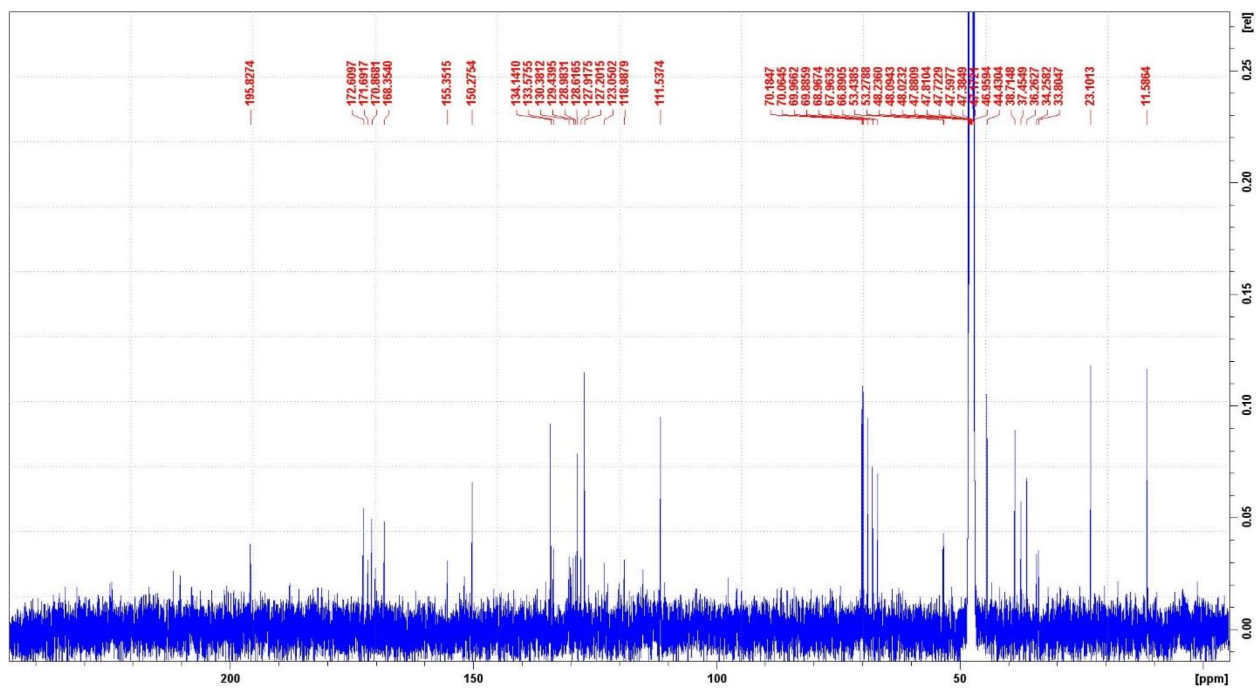
^{1}H NMR spectrum of compound bis-EA-b (400.13 MHz, CD_3OD)



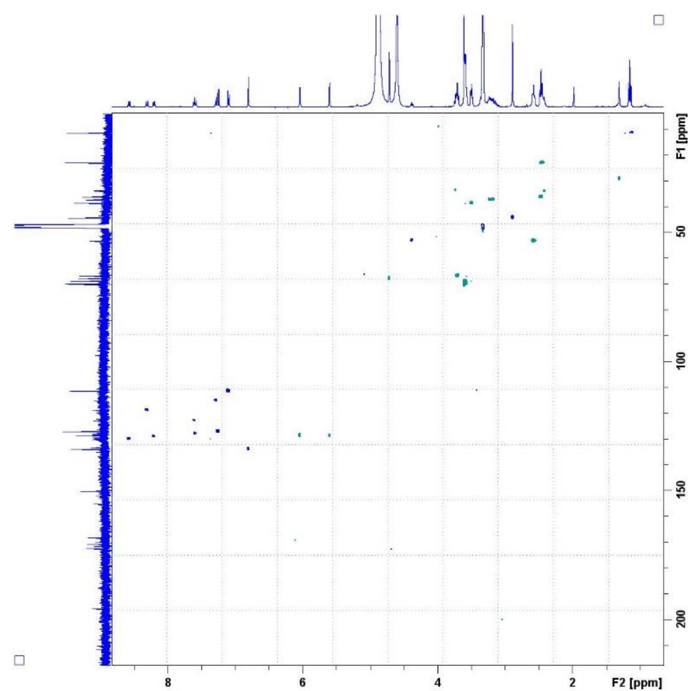
^{1}H-^{1}H COSY spectrum of compound bis-EA-b (400.13 MHz, CD_3OD)



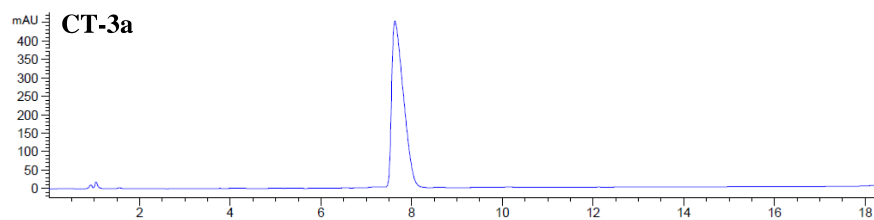
$^{13}C\{^{1}H\}$ NMR spectrum of compound bis-EA-b (100.67 MHz, CD_3OD)

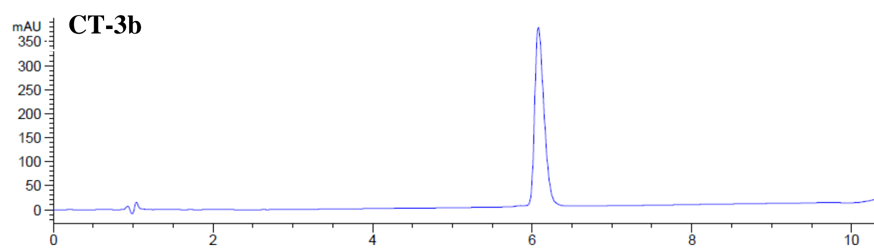


^{1}H-^{13}C **phase sensitive HSQC of compound bis-EA-b (400.13 MHz, CD_3OD)**

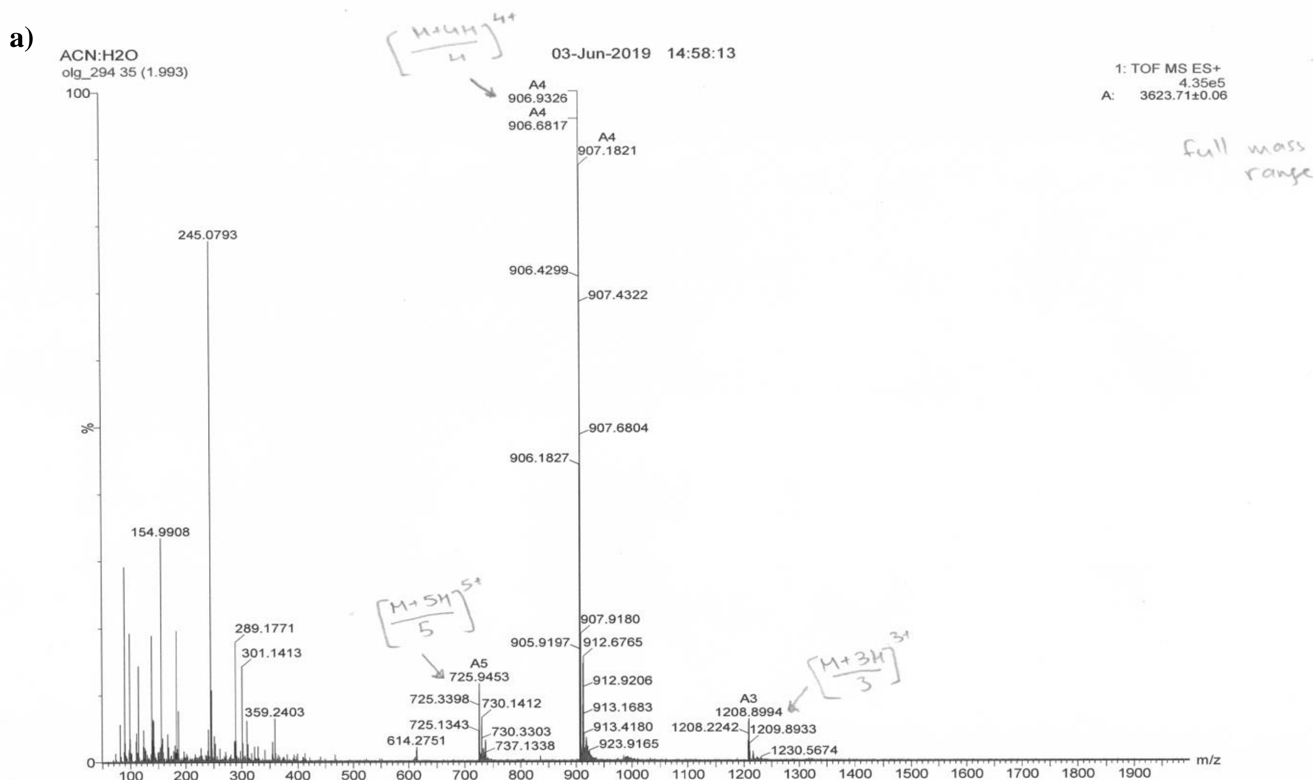


HPLC chromatograms of CT-3a and CT-3b.

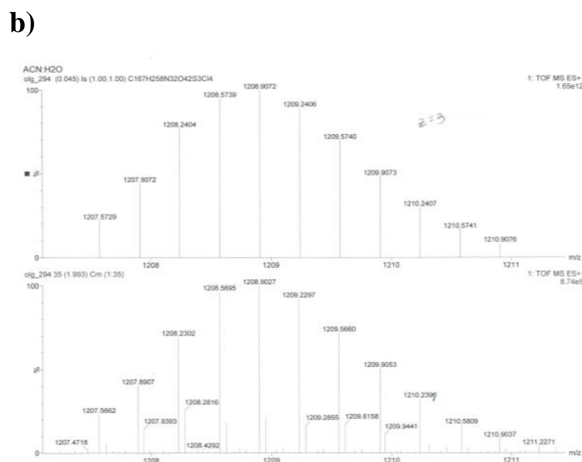




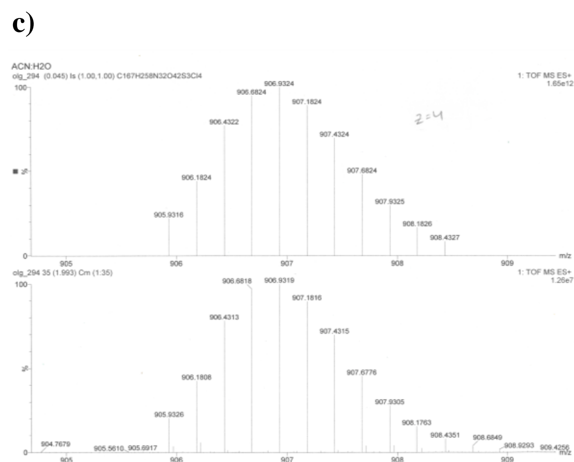
MS spectrum and isotopic patterns for CT-3a. (top) Full spectrum; (b) simulated mass (top), and experimental mass distribution (bottom) for $[M]^{3+}$, c) simulated mass (top), and experimental mass distribution (bottom) for $[M]^{4+}$.



b)

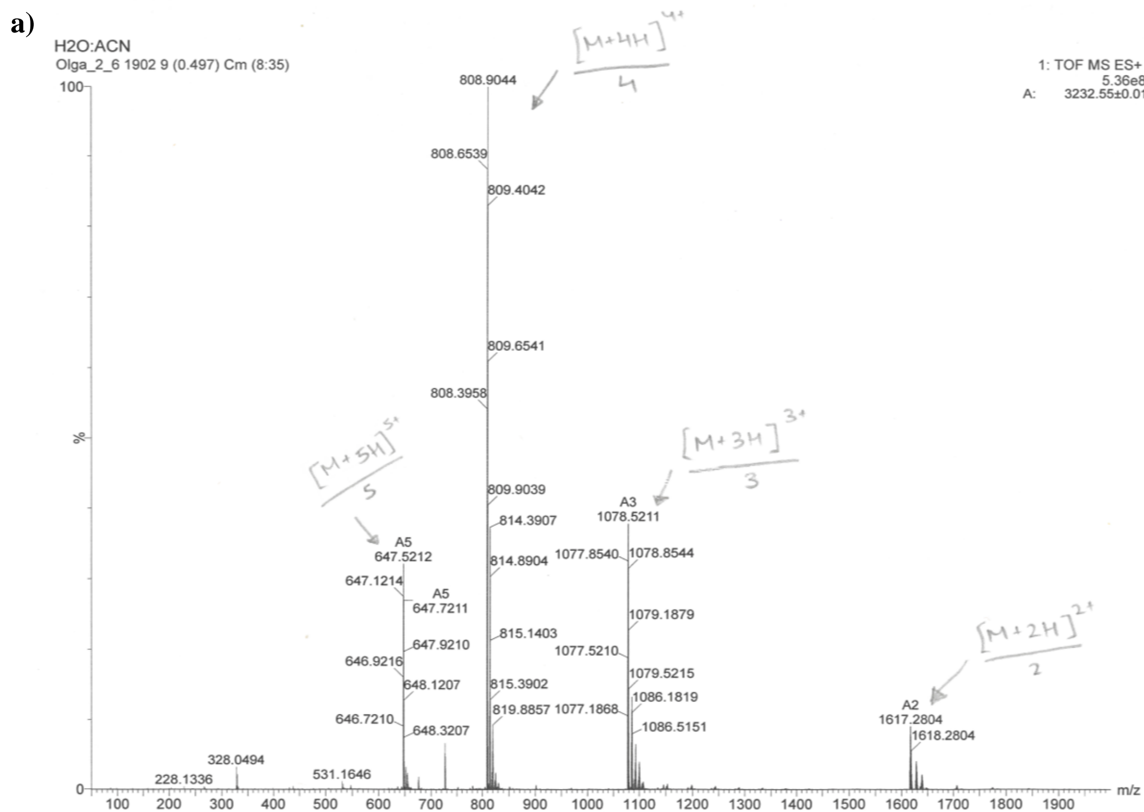


c)

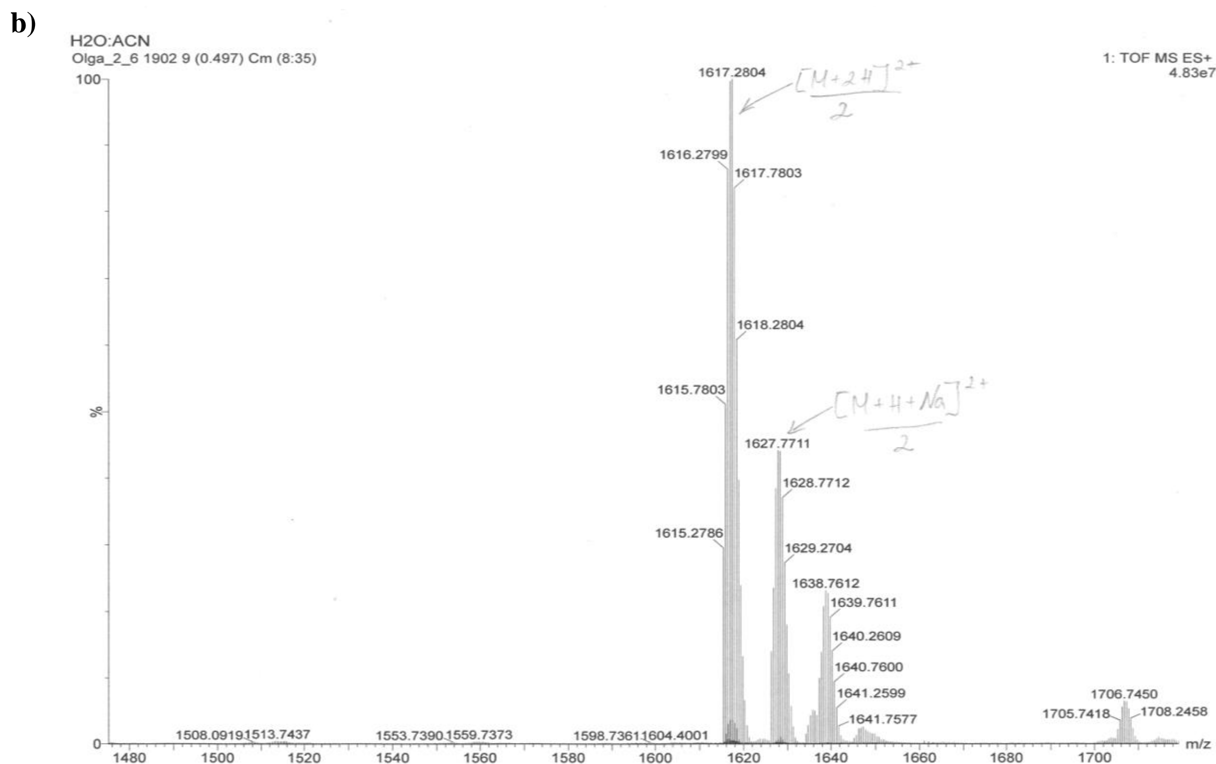


MS spectra for CT-3b. (a) Full spectrum; (b) magnification of the $[M]^{2+}$ peak range.

a)

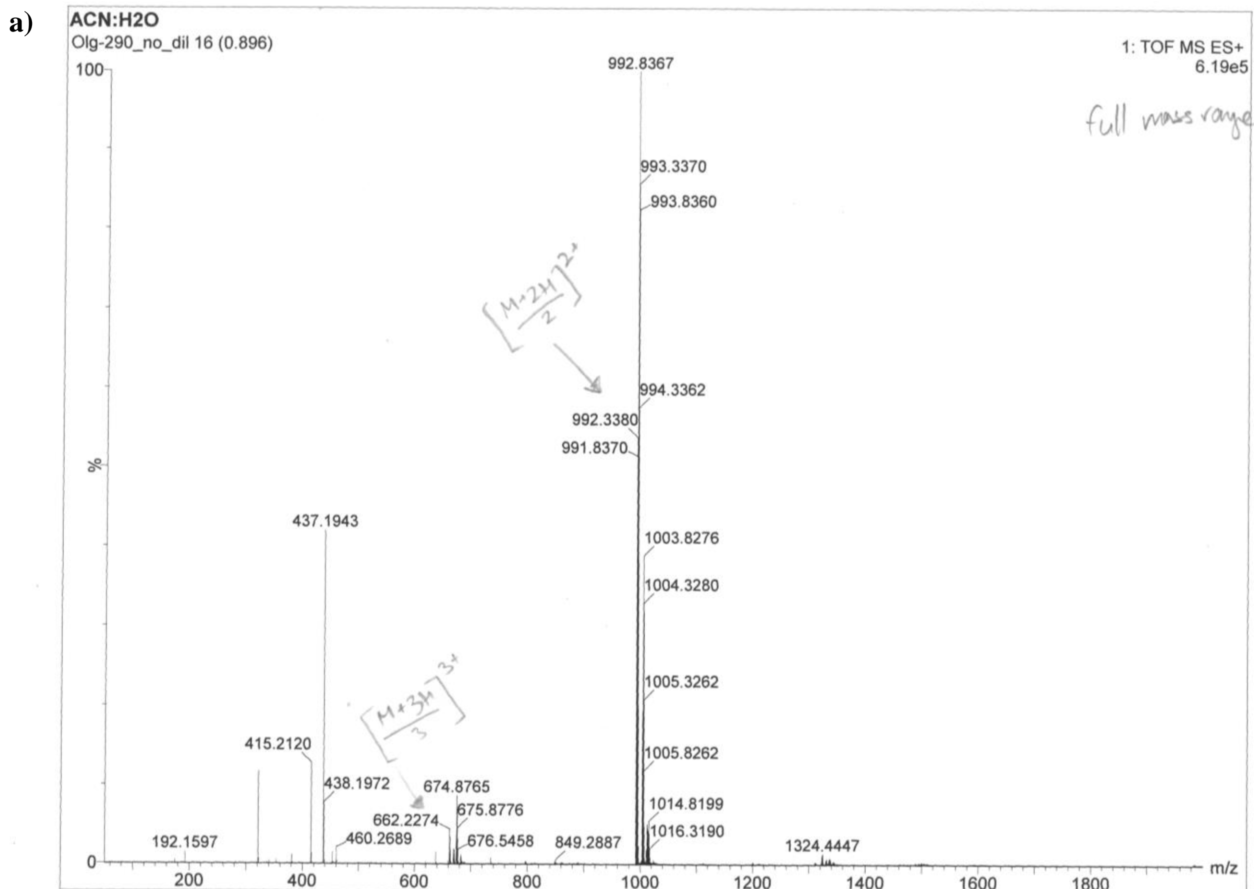


b)

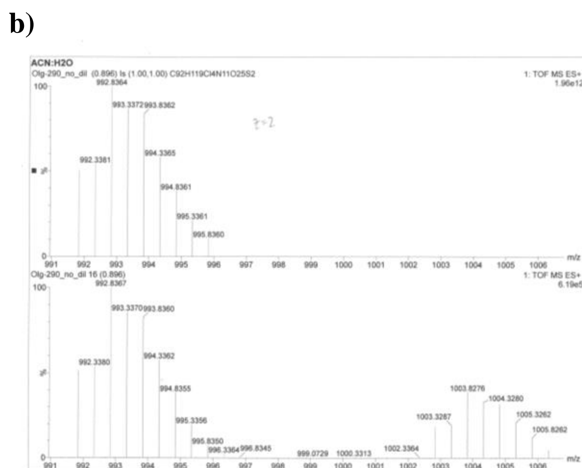


MS spectrum and isotopic patterns for bis-EA-a. (top) Full spectrum; (b) simulated mass (top), and experimental mass distribution (bottom) for $[M]^{2+}$, c) simulated mass (top), and experimental mass distribution (bottom) for $[M]^{3+}$.

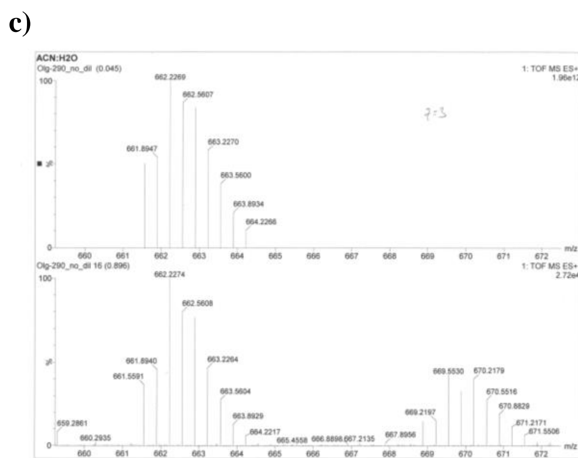
a)



b)

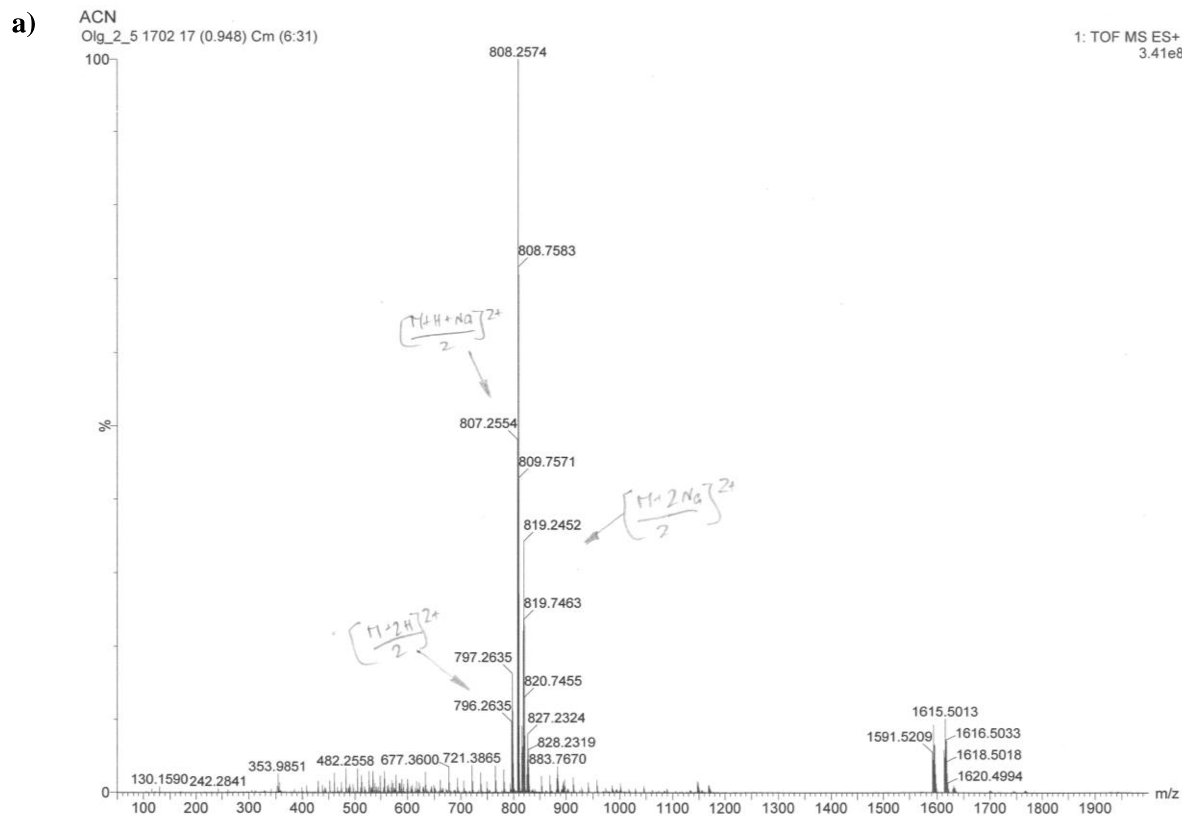


c)

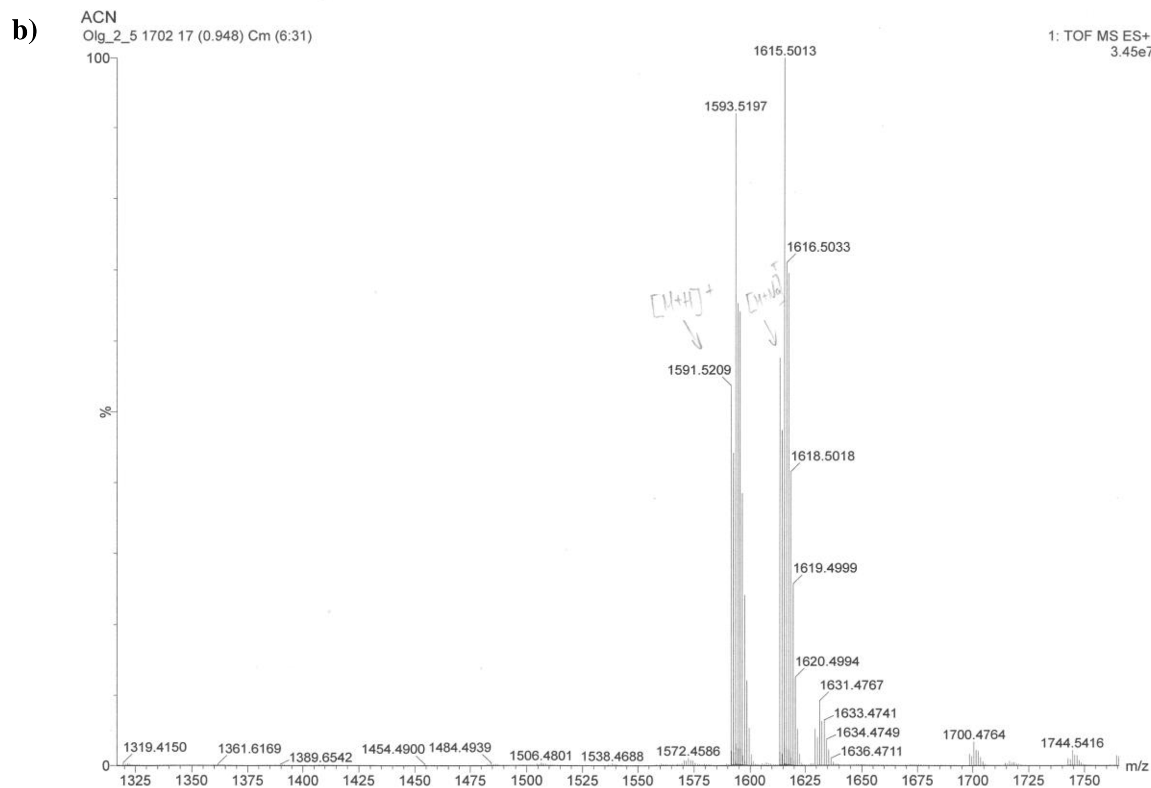


MS spectra for bis-EA-b. (a) Full spectrum; (b) magnification of the $[M]^+$ peak range.

a)



b)



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