Electronic Supplementary Information for

Carbon Dioxide Enhances Sulfur-Selective Conjugate Addition Reactions

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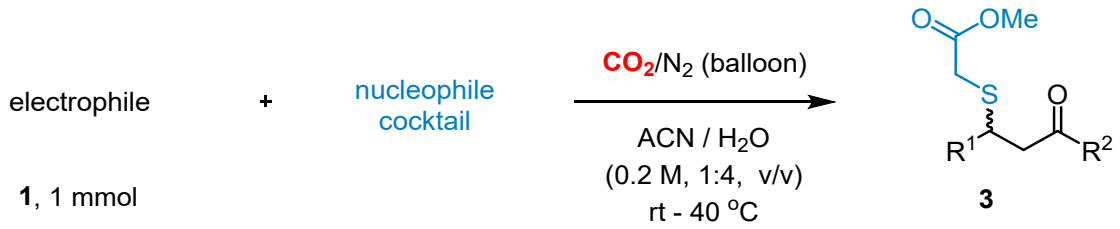
Contents

1. General methods S3
2. General procedure S4
3. Analysis of Crude Reaction Mixtures for Conjugate Addition Reactions S7
4. Mechanistic Studies S10
5. Human Serum Albumin (HSA) Cysteine Modification and LC Traces S14
6. Synthesis of FKVCF and Cys-conjugation with N-phenylmaleimide S48
7. ^{1}H and ^{13}C NMR Spectra S57
8. References S63

1. General methods

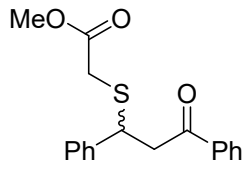
All chemicals, unless stated otherwise were purchased from commercial suppliers and used without further purification. CO_2 was directly used from a CO_2 cylinder of 99.7% purity without any treatment for reactions. Solvents used were HPLC grade and dried on molecular sieves (4Å) at least 2 days before use. The water concentrations of solvents were all measured on a Karl Fischer titrator (831 KF Coulometer) and used if the water content was below 15 ppm. Analytical thin layer chromatography was done on Merck DC-Alufolien SiO_2 60 F254 0.2 mm thick pre-coated TLC plates. Column chromatography was performed using SiO_2 from ROCC (SI 1721, 60 Å, 40−63 μm). ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) spectra were recorded with 500 MHz Ultrashield Plus 500 spectrometer and 125 MHz on a Bruker. ^{1}H, ^{13}C and ^{19}F NMR spectra were also recorded at 500 MHz, 126 MHz and 470 MHz, respectively, on a Bruker Avance 3 spectrometer with a BBFO probe. All chemical shifts (δ) are quoted in ppm and all coupling constants (*J*) are expressed in Hertz (Hz). The following abbreviations are used for multiplicity for NMR resonances: s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet and m = multiplet. LC-MS (Liquid Chromatography-Mass Spectroscopy). LC-MS analyses were carried out by connecting the above mentioned HPLC apparatus to a Bruker MicrOTOF-QII system equipped with an ESI source with nebulizer gas at 1.2 bars, dry gas at 10 L/min, dry temperature at 200 °C, capillary at 4500 V and end plate offset at –500 V. The ion transfer was conducted with funnel 1 and funnel RF's at 200.0 Vpp and hexapole RF at 100.0 Vpp while the quadrupole ion energy was set at 5.0 eV with a low mass cut-off at 100.00 m/z. In the collision cell, collision energy was set at 8.0 eV, collision RF at 100.0 Vpp, and a transfer time of 80.0 μs and pre-pulse storage of 1.0 μs were used. *N*-phenylmaleimide and *N*-ethylmaleimide used for HSA modification were ordered from Sigma Aldrich and TCI Europe respectively.

2. General procedure



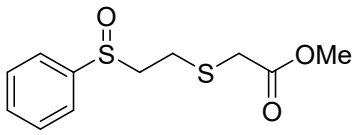
A mixture of nucleophiles was kept stirring for 10 min in water and MeCN (20% v/v, 0.2 M) as a solvent under CO_2 or N_2 atmosphere at rt. Then electrophile **1** (1.0 mmol) was added. The resulting mixture was kept stirring at rt for 14-24 h. The reaction mixture was extracted with dichloromethane (3 x 30 mL). The organic phases were combined, washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by silica column chromatography.

*Methyl 2-((3-oxo-1,3-diphenylpropyl)thio)acetate (**3ab**)*[1]



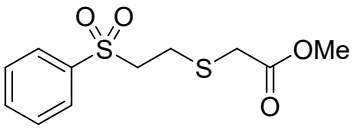
The title compound was purified by column as a brown oil (216.8 mg, 69%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.94 – 7.88 (m, 2H), 7.58 – 7.51 (m, 1H), 7.47 – 7.40 (m, 4H), 7.31 (t, *J* = 8.4 Hz, 2H), 7.25 – 7.21 (m, 1H), 4.74 (t, *J* = 7.1 Hz, 1H), 3.65 (s, 3H), 3.58 (dd, *J* = 7.0, 1.3 Hz, 2H), 3.11 (d, *J* = 15.0 Hz, 1 H), 3.01 (d, *J* = 15.0 Hz, 1 H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 196.4, 170.7, 140.9, 136.7, 133.4, 128.8, 128.7, 128.2, 128.1, 127.7, 52.5, 44.9, 44.8, 33.0. **HRMS**: $[M+H]^+$ Calculated for $C_{18}H_{19}O_3S$ 315.1055, found 315.1049.

*Methyl 2-((2-(phenylsulfinyl)ethyl)thio)acetate (**3bb**)*



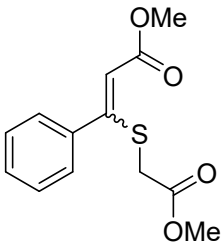
The title compound was purified by column as a brown oil (221.7 mg, 86%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.65 – 7.60 (m, 2H), 7.56 – 7.48 (m, 3H), 3.70 (s, 3H), 3.24 (d, 2H), 3.14 – 3.07 (m, 1H), 3.06 – 2.98 (m, 2H), 2.86 – 2.74 (m, 1H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.6, 143.2, 131.3, 129.5, 124.1, 56.2, 52.6, 33.8, 25.2. **HRMS**: $[M+H]^+$ Calculated for $C_{14}H_{15}O_3S_2$ 259.0463, found 259.0456. **IR** (neat): *ν* = 2951, 1723, 1477, 1442, 1275, 1139, 1084, 1008, 746, 689, 580, 521, 467, 412 cm^{-1}. **Elemental analysis:** calculated C: 51.14, H:5.46, S: 24.82, found C: 49.70, H:5.40, S: 24.79.

*Methyl 2-((2-(phenylsulfonyl)ethyl)thio)acetate (**3cb**)*



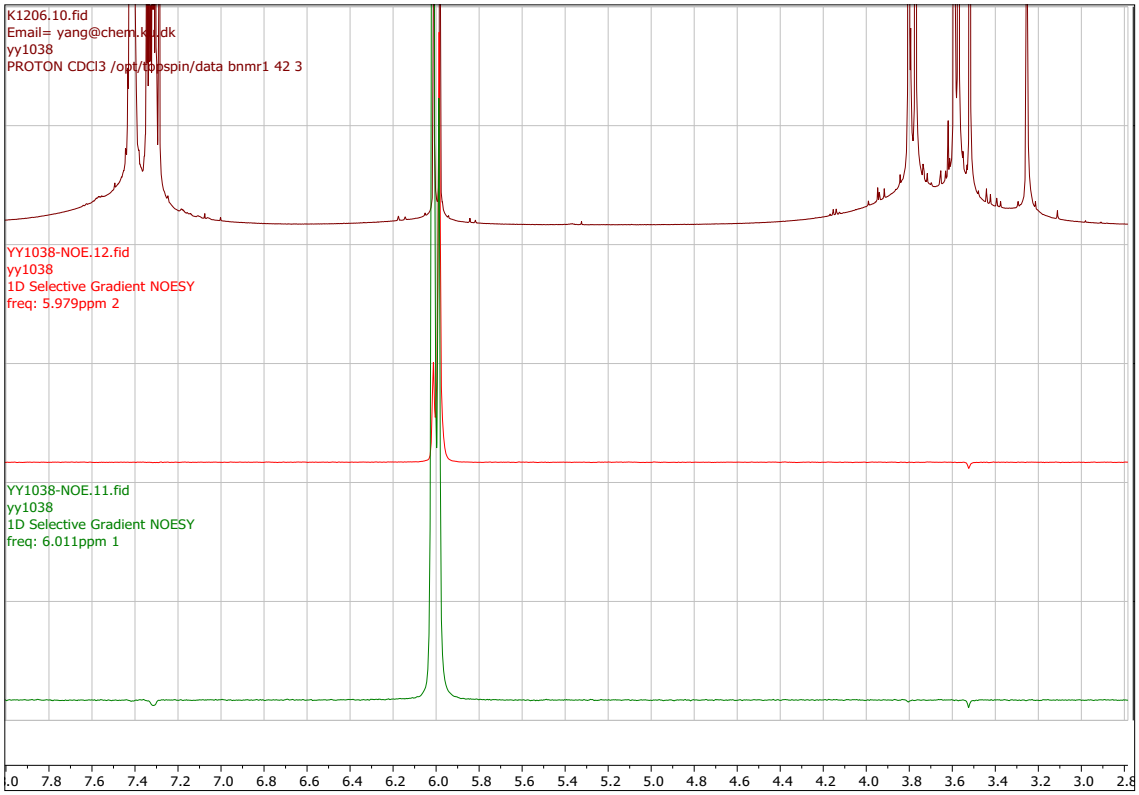
The title compound was purified by column as a brown oil (227.6 mg, 83%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.97 – 7.84 (m, 2H), 7.75 – 7.64 (m, 1H), 7.61 – 7.57 (m, 2H), 3.69 (s, 3H), 3.47 – 3.32 (m, 2H), 3.21 (s, 2H), 3.02 – 2.83 (m, 2H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 170.4, 138.8, 134.2, 129.6, 128.3, 56.0, 52.7, 33.7, 25.4. **HRMS**: $[M+H]^+$ Calculated for $C_{14}H_{15}O_4S_2$ 275.0412, found 275.0404. **IR** (neat): ν = 2953, 1731, 1446, 1436, 1308, 1285, 1215, 1144, 1084, 1000, 732, 688, 579, 551, 528, 445, 427 cm^{-1}. **Elemental analysis:** calculated C: 48.16, H:5.14, S: 23.37, found C: 47.86, H:6.67, S: 21.64.

*(Z, E)-Methyl 3-((2-methoxy-2-oxoethyl)thio)-3-phenylacrylate (**3db**)*[2]

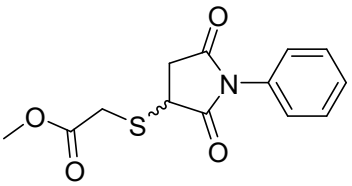


E/*Z* mixture

The title compound was purified via silica column chromatography as pink powder (239.7 mg, 90%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.46 – 7.35 (m, 6H) (major, minor), 7.33 – 7.27 (m, 4H) (major, minor), 5.98 (s, 1H) (major), 5.96 (s, 1H) (minor), 3.78 (s, 3H) (major), 3.74 (s, 3H) (minor), 3.56 (s, 3H) (major), 3.54 (s, 3H) (minor), 3.49 (s, 2H) (minor), 3.23 (s, 2H) (major). **^{13}C NMR** (126 MHz, $CDCl_3$) δ (major) 169.4, 166.2, 158.56, 137.7, 129.3, 128.8, 128.3, 116.8, 52.6, 51.6, 34.5; (minor) 168.9, 164.6, 157.9, 136.1, 129.4, 128.6, 128.2, 112.5, 52.9, 51.3, 34.6. **HRMS**: $[M+H]^+$ Calculated for $C_{13}H_{15}O_4S$ 267.0691, found 267.0688. The configuration of the isomers was determined by NOE experiments, Mix = 300 msec.

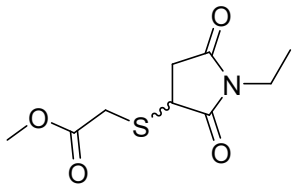


*Methyl 2-((2,5-dioxo-1-phenylpyrrolidin-3-yl)thio)acetate (**3eb**)*[3]



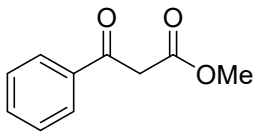
The title compound was purified via silica column chromatography as a brown oil (170.2 mg, 61%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 7.53 – 7.44 (m, 2H), 7.45 – 7.38 (m, 1H), 7.34 – 7.28 (m, 2H), 4.20 (dd, *J* = 9.3, 3.9 Hz, 1H), 3.96 (d, *J* = 15.9 Hz, 1H), 3.77 (s, 3H), 3.44 (d, *J* = 15.9 Hz, 1H), 3.33 (dd, *J* = 18.8, 9.4 Hz, 1H), 2.70 (dd, *J* = 18.9, 4.0 Hz, 1H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 175.3, 173.5, 170.2, 131.6, 129.3, 128.9, 126.5, 52.8, 38.6, 35.6, 32.9. **HRMS**: $[M+H]^+$ Calculated for $C_{13}H_{14}NO_4S$ 280.0643, found 280.06434.

*Methyl 2-((1-ethyl-2,5-dioxopyrrolidin-3-yl)thio)acetate (**3fb**)*



The title compound was purified via silica column chromatography as a colorless oil (202,1 mg, 87%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 4.01 (dd, *J* = 9.2, 3.9 Hz, 1H), 3.92 (d, *J* = 15.8 Hz, 1H), 3.77 (s, 3H), 3.57 (q, *J* = 7.2 Hz, 2H), 3.39 (d, *J* = 15.8 Hz, 1H), 3.13 (dd, *J* = 18.7, 9.2 Hz, 1H), 2.50 (dd, *J* = 18.7, 3.9 Hz, 1H), 1.18 (t, *J* = 7.2 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 176.3, 174.3, 170.2, 52.8, 38.5, 35.5, 34.3, 33.0, 12.9. **IR** (neat): *v* = 2976, 2953, 1774, 1734, 1697, 1440, 1402, 1225,1127, 1006, 791, 686 cm^{-1}. **Elemental analysis:** calculated C: 46.74, H:5.67, N, 6.06, S: 13.86, found C: 46.33, H:5.73, N, 6.00, S: 12.13. **HRMS**: $[M+H]^+$ Calculated for $C_9H_{14}NO_4S$ 232.0638, found 232.0639

Methyl (Z)-3-hydroxy-3-phenylacrylate[4]



The title compound was purified by column as white crystals (73.6 mg, 41%). **^{1}H NMR** (500 MHz, Chloroform-*d*) δ 9.62 (s, 1H), 7.69 – 7.55 (m, 2H), 7.48 – 7.33 (m, 3H), 6.98 (s, 1H), 3.91 (s, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 164.9, 149.6, 133.23, 129.5, 129.2, 126.1, 115.0, 52.0. **HRMS**: $[M+H]^+$ Calculated for $C_{10}H_{11}O_3$ 227.1072, found 227.1097.

3. Analysis of Crude Reaction Mixtures for Conjugate Addition Reactions

When the reactions were completed, the reaction mixtures were extracted with dichloromethane (10 mL). The organic phases were collected, and the solvent was removed. Analysis of the crude mixtures by ^{1}H NMR spectroscopy was performed in $CDCl_3$.

Note: Reactions under CO_2 were generally found to be more selective (see the following Figures) toward *S*-alkylation products. Quantitative yields were detected by NMR spectroscopy when sulfone **1b**, sulfoxide **1c**, and N-ethylmaleimide **1f** were used as electrophiles.

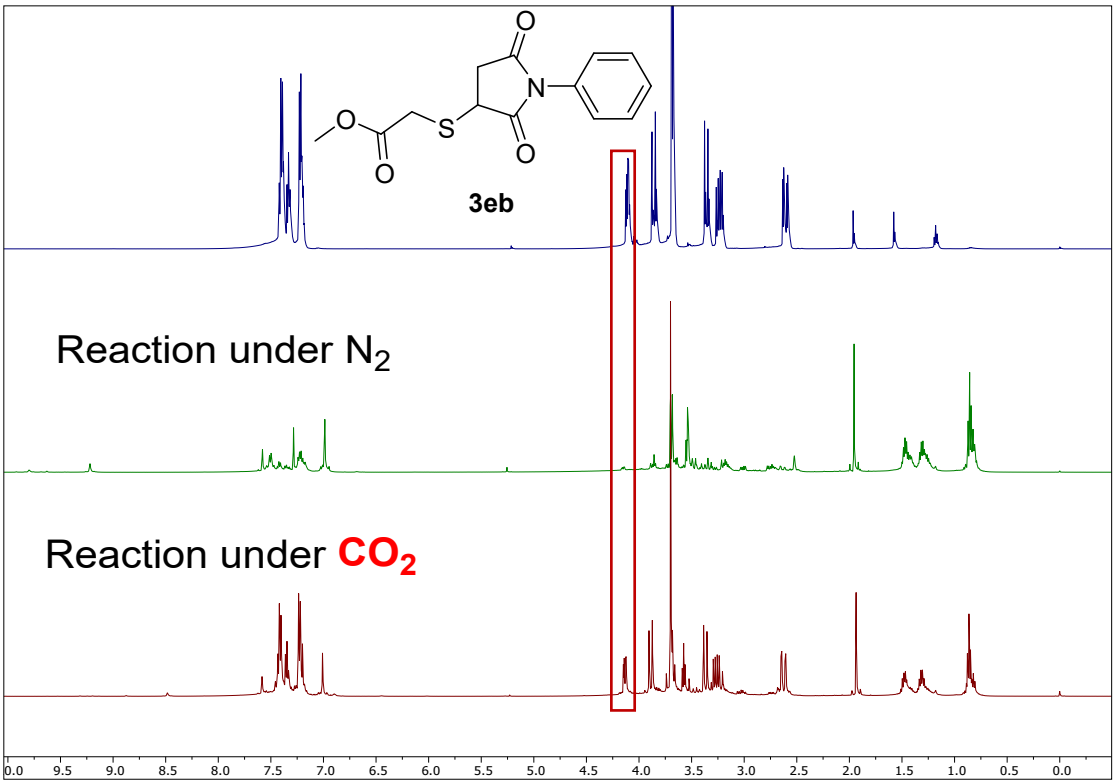


Fig. S1. Top: ^{1}H NMR spectrum of isolated product **3eb** from the reaction under CO_2, middle: crude ^{1}H NMR spectrum under N_2 (green), bottom: crude ^{1}H NMR spectrum under CO_2 (maroon).

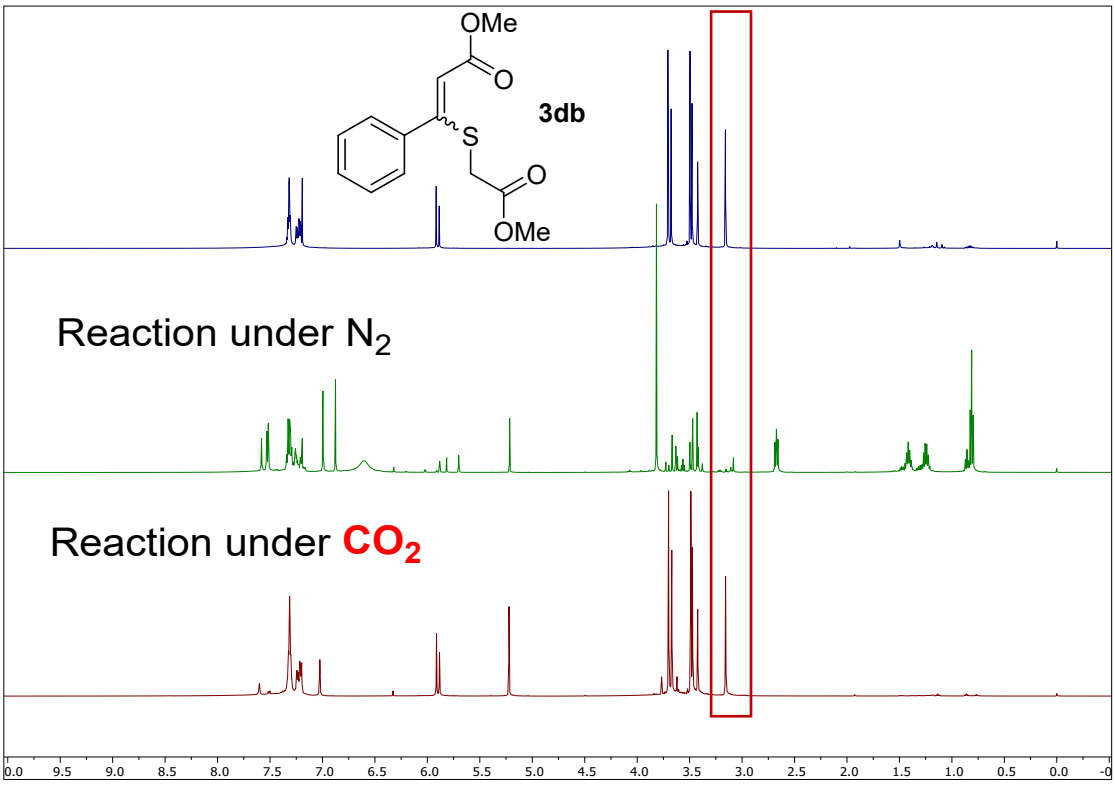


Fig. S2. Top: ^{1}H NMR spectrum of isolated product **3db** from the reaction under CO_2, middle: crude ^{1}H NMR spectrum under N_2 (green), bottom: crude ^{1}H NMR spectrum under CO_2 (maroon).

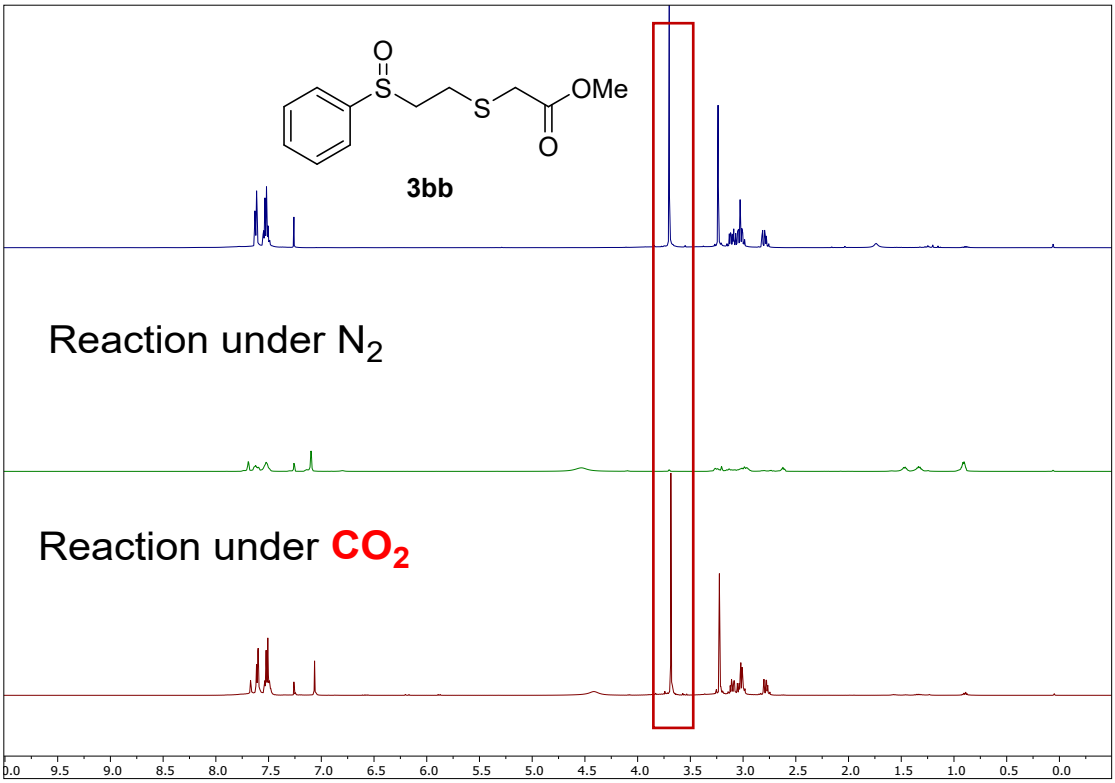


Fig. S3. Top: ^{1}H NMR spectrum of isolated product **3bb** from the reaction under CO_2, middle: crude ^{1}H NMR spectrum under N_2 (green), bottom: crude ^{1}H NMR spectrum under CO_2 (maroon).

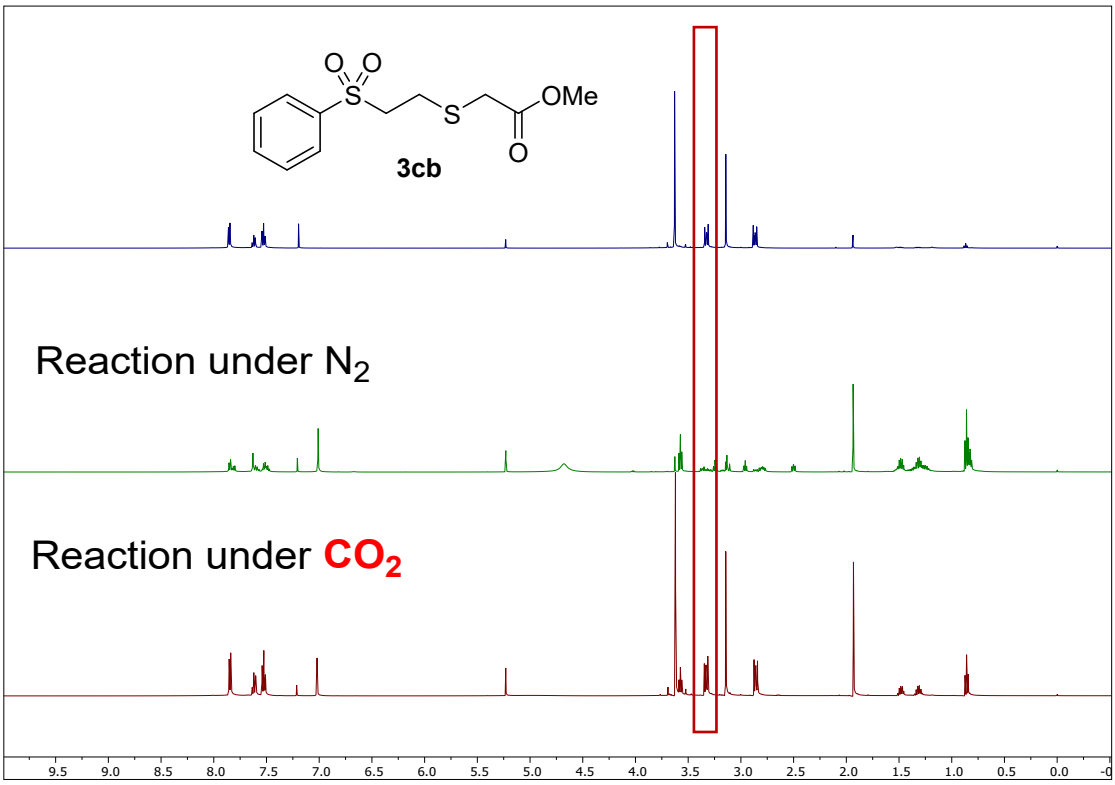


Fig. S4. Top: ^{1}H NMR spectrum of isolated product **3cb** from the reaction under CO_2, middle: crude ^{1}H NMR spectrum under N_2 (green), bottom: crude ^{1}H NMR spectrum under CO_2 (maroon).

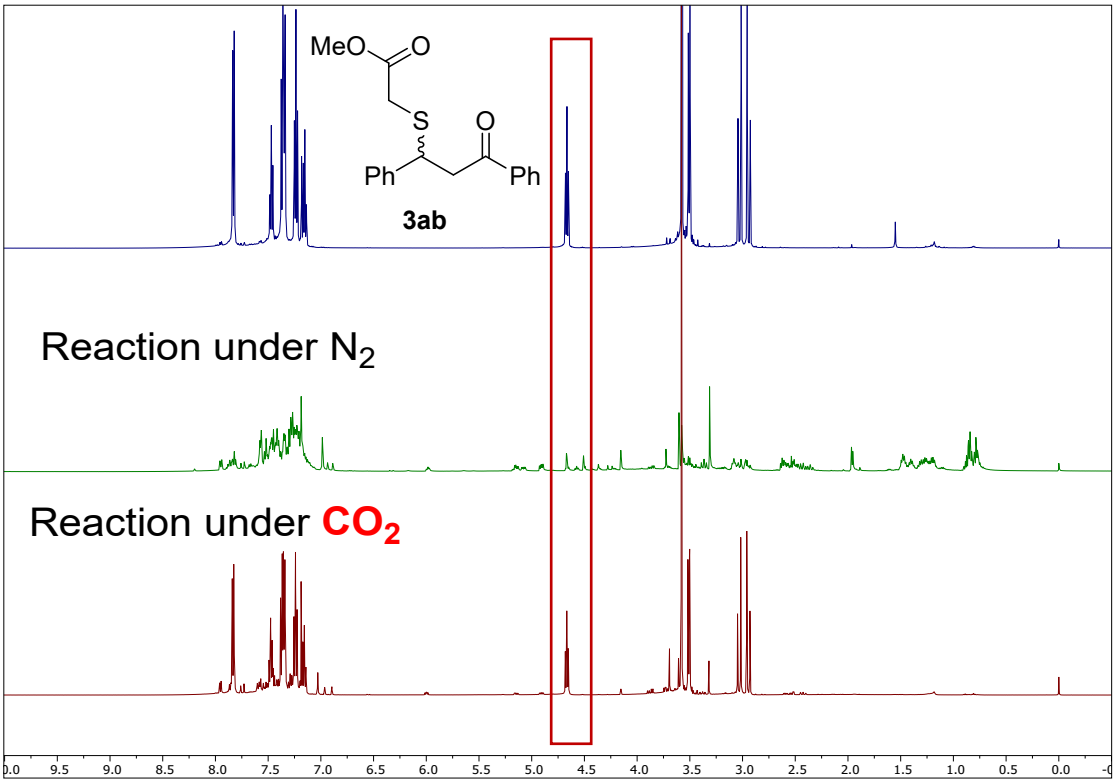


Fig. S5. Top: ^{1}H NMR spectrum of isolated product **3ab** from the reaction under CO_2, middle: crude ^{1}H NMR spectrum under N_2 (green), bottom: crude ^{1}H NMR spectrum under CO_2 (maroon).

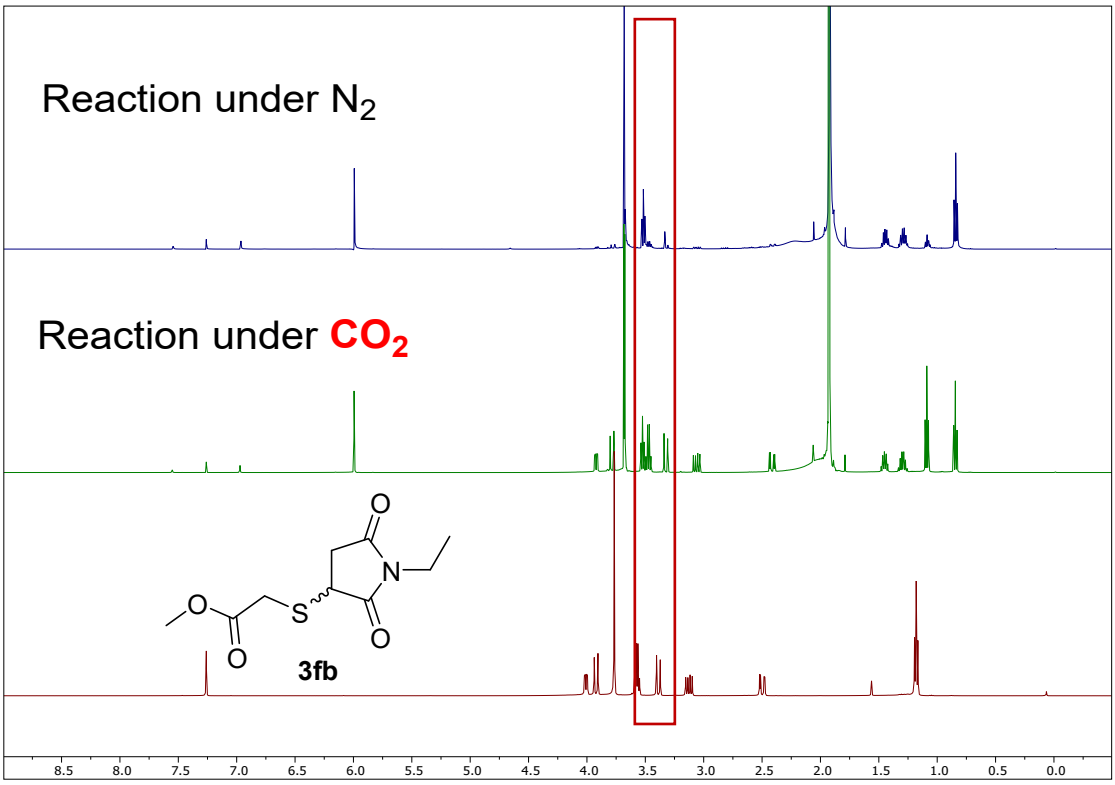


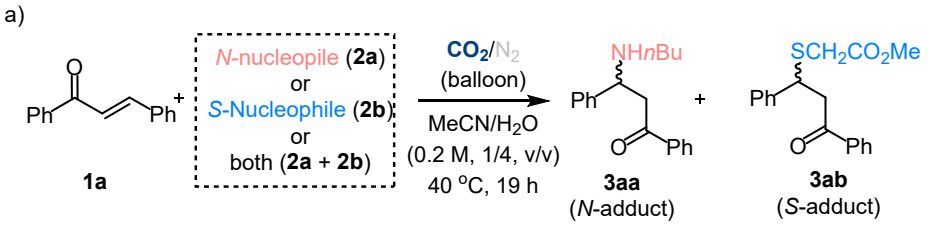
Fig. S6. Top: crude ^{1}H NMR spectrum under N_2 (blue), middle: crude ^{1}H NMR spectrum under CO_2 (green). Bottom: ^{1}H NMR spectrum of isolated product **3fb** from the reaction under CO_2, bottom:

4. Mechanistic Studies

4.1 Reactions of chalcone 1a and nucleophile 2a or 2b under various pHs

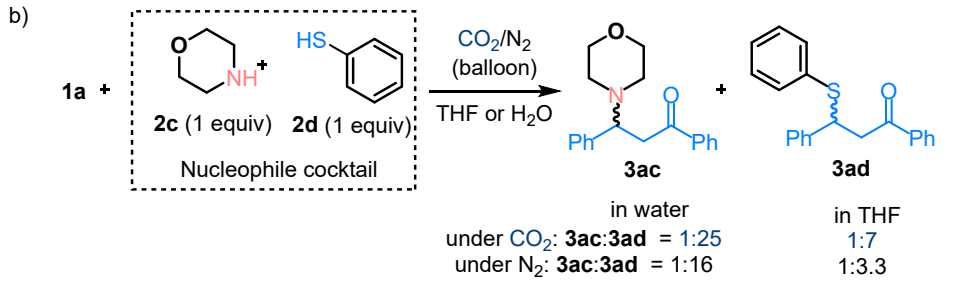
Gneral procedure: The pH of freshly prepared 20% ACN/PBS solution was detected to be 7.8. The solution of different pHs were prepared by simply bubbling CO_2 (10 min, to reach pH 6.0) or adding 1 M HCl (to afford pH 7.3, 6.5, 6.0, under a nitrogen atmosphere). The CO_2 saturated ACN/PBS solution was bubbled N_2 to remove CO_2 for 30 min (pH 6.9), 1 h (pH 7.5), 2 h (pH 7.6) respectively. To a 8 mL vail equipped with a stir bar, was added chalcone **1a** (0.4 mmol), 20% ACN/PBS solution of different pHs (2 mL), and *n*-butylamine **2a** (1 equiv.) or thiol **2b** (1 equiv.). The reaction mixture was blowed with N_2 and sealed thereafter. The reaction mixture was heated at 40 °C for 19 h. After the reaction was cooled down to room temperature, 1,3,5-trimethoxylbenzene (22.4 mg) in $CDCl_3$ (2 mL) was added to the reaction mixture and then the reaction mixture was extracted, and the organic phase was further diluted twice before ^{1}H NMR spectra were recorded.

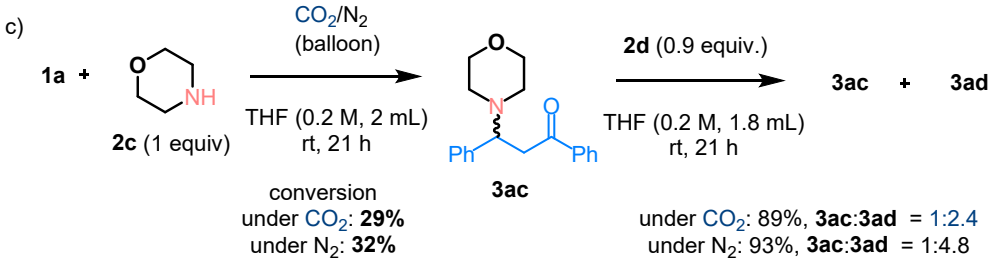
4.2 Reactions of chalcone 1a and S- and/or N- nucleophiles



Entry	2a (equiv.)	2b (equiv.)	Atm.	1a (%) [a]	3aa (%) [a]	3ab (%) [a]
1	1	0	**CO_2**	**>99**	**0**	X
2			N_2	76	24	
3	0	1	**CO_2**	**87**	X	**13**
4			N_2	90		10
5	1	1	**CO_2**	**9**	**0**	**91**
6			N_2	20	1	12
7 [b]	0	1	**CO_2**	**24**	X	**76**
8 [c]			N_2	71		29
9 [d]			N_2	14		86

a, ^{1}H NMR percent yields were determined by using 1,3,5-trimethoxylbenzene as an internal standard in $CDCl_3$. **2a**: *n*-butylamine, **2b**: $HSCH_2CO_2Me$. Phosphate buffer solution (PBS/ACN,1/4, v/v, pH 7.8) was used b) under CO_2 afford pH 6.0 reaction media, c) under N_2 (pH adjusted to 6.0 with HCl, d) under N_2.





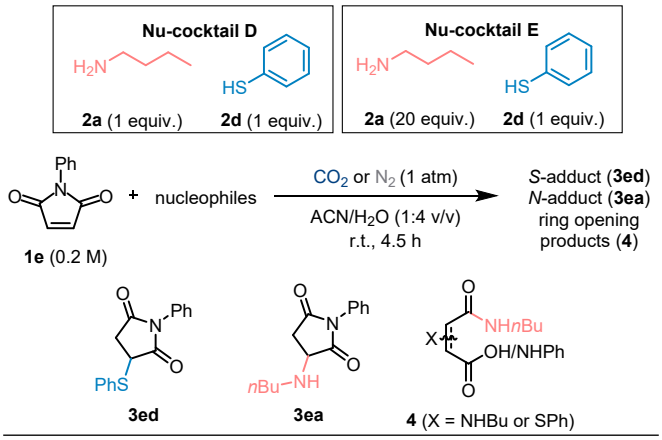
Scheme S1. Control experiments with electrophile **1a** and amines or thiols.

Note: To gain insights into the positive role of CO_2, we performed control experiments (Scheme S1, a). When only N-butylamine used (Entries 1 and 2), there is no reaction observed in the presence of CO_2, potentially due to the formation of amine-CO_2 adducts. Thiol afforded identical conversion, showing negligible role of CO_2 (Entries 3 and 4). Generally faster consuming of the starting material **1a** was detected (entries 1-8) compared to the control experiments under N_2, except entry 9 with pH 7.8 reaction media.

To generalize the concept, we performed the control experiments with morpholine and thiophenol (see ESI, experiments **b**), two-step, one-pot). Reversible *N*-addition was confirmed in the presence of thiol nucleophile under N_2 atmosphere to afford ratio of **3ac**:**3ad** around 1:5 (should be around 1:2 if it's completely irreversible, reaction **b**, second step). It was not, or less, reversible under CO_2 atmosphere to afford ratio of **3ac**:**3ad** around 1:2.4 (reaction **b**, second step), indicating the potential interaction between CO_2 and morpholine. In contrast to lower selectivity of *S*-alkylation product by running the reaction stepwise, higher selectivity toward thiol when a mixture of **2c** and **2d** was used under CO_2 atmosphere (reaction **a**, **3ac**:**3ad** around 1:7) was detected. Interestingly, the 30% conversion of 1a in the presence of CO_2 when secondary amine used, where potential iminium catalysis would dominate the reaction.

4.3 Reactions of N-phenylmaleimide 1e and nucleophile 2a or 2d or nucleophiles

Table S1. Control experiments of cyclic imide with N and S nucleophiles

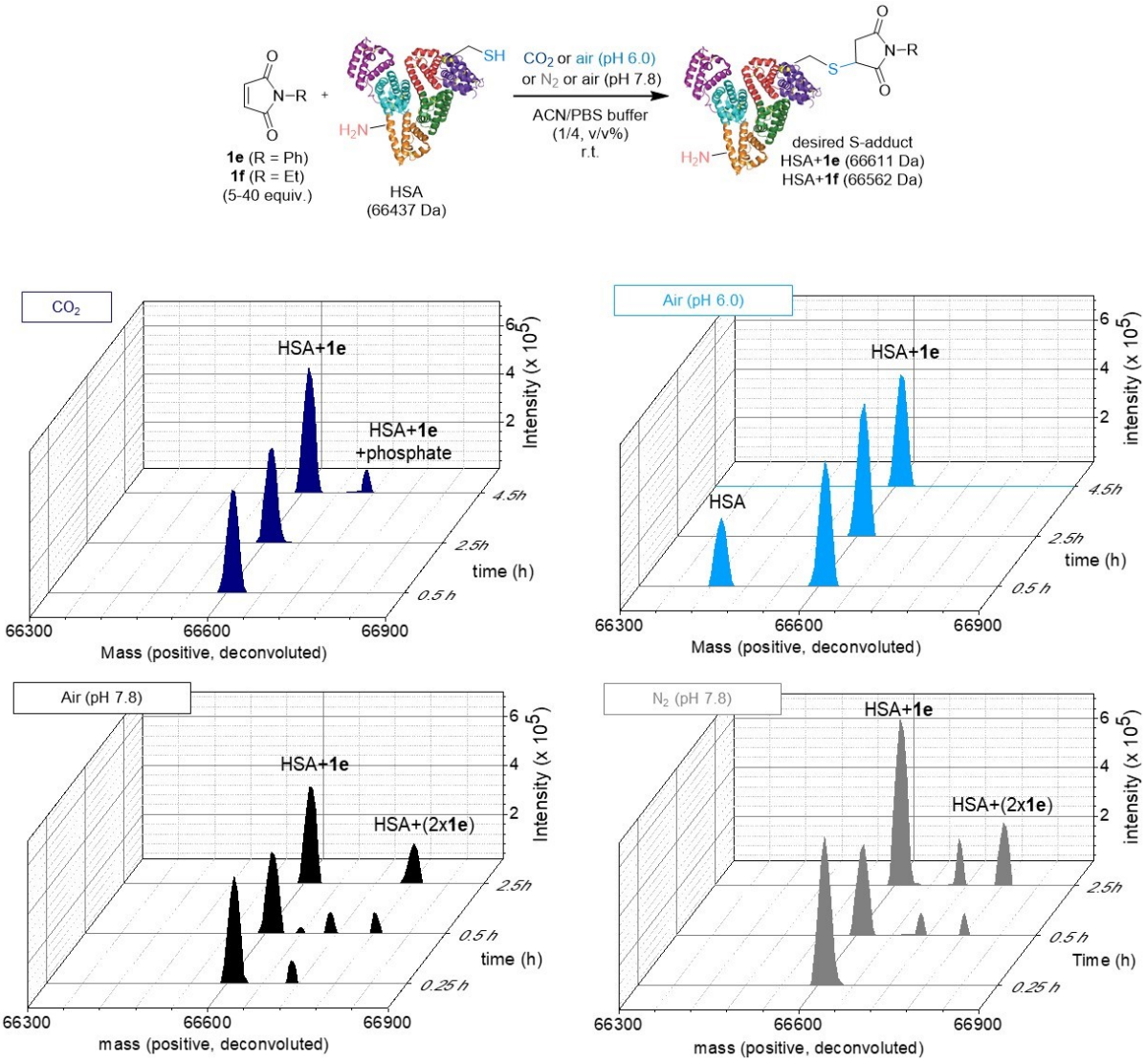


entry	Nucleophiles	atm	**1e** (%)	**3ed** (%)	**3ea** (%)	**4** (%)
1	**2a**	CO_2	23%	n/a	34%	<1%
2		N_2	0%	n/a	23%	11%
3	**2d**	CO_2	0%	74%	n/a	n.d.
4		N_2	0%	71%	n/a	n.d.
5	**NuD**	CO_2	35%	**47%**	17%	<1%
6		N_2	0%	38%	<1%	10%
7	**NuE**	CO_2	0%	**25%**	10%	27%
8		N_2	0%	<1%	<1%	10%

*^{1}H NMR percent yields were determined with 1,3,5-trimethoxylbenzene as an internal standard. n/a: not available, n.d.: not detected.

Note: We performed reactions with amine (**2a**) and sulfur (**2d**) nucleophiles separately and together (**NuD**). We also tested reactions with the same nucleophile cocktail but with a 20 to 1 ratio of amine to sulphur nucleophiles (**NuE**), to more closely mimic the reaction environment of HSA. With amine nucleophile **2a** reactions were slower under CO_2 conditions than a N_2 atmosphere. The same reaction under N_2 led to higher conversion, but lower selectivity to conjugate addition products due to the formation of ring-opening products (**4**). With *n*-butylamine as the sole nucleophile, no appreciable amounts of ring-opening products were observed under CO_2 while 11% of **4** was observed under a N_2 atmosphere (entries 1 and 2, Table S1). Thiophenol **2d** is highly nucleophilic explaining no effect of CO_2 (entries 3-4). With nucleophile cocktail **NuD**, reactions was slower under CO_2 (remaining **1e**: 35%) than under N_2 (full conversion), while preventing the formation of ring-opening product (**4**) (entries 5-6). Noticeably, the reaction with **NuE** (20 equiv. **2a** + 1 equiv. **2d**) showed 25% sulfur-conjugate addition product **3ed** under CO_2 conditions (entry 7). Under N_2 conditions (entry 8), a complicated reaction mixture was obtained, where aminolysis of the sulfur-conjugation products were converted to ring-opened products (**4**). The absence of both *S*- and *N*-adducts under a N_2 atmosphere highlights the advantage of a CO_2 atmosphere, which showed higher reaction rate and selectivity with HSA and various conjugate addition electrophiles.

5. Human Serum Albumin (HSA) Cysteine Modification and LC Traces



Scheme S2. Time-dependent LC-MS traces with 5 equiv. of **1e**.

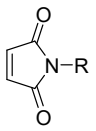
A solution of HAS ([HSA] = 100 µM, in PBS buffer, pH = 7.8) was prepared as a stock solution. The HSA solution (7 µL, 0.7 nmol, 1 equiv.) was added to a solution of PBS in 20% MeCN (pH 7.8, 81.8 µL) in an LC-MS vial equipped with a 200 µL insert. To the mixture was added a solution of maleimide (**1e** or **1f**) in MeCN (2.5 mM, 11.2 µL, 28 nmol, 40 equiv.) to obtain a final volume of 100 µL of reaction mixture and a concentration of HSA at 7 µM. The vial was shaken at 400 mot/min (mot: at 25 °C for 0.5 h (or 15 min) under CO_2 or N_2 atmosphere. The conjugation product was identified by LC-MS analysis (3 µL for each injection every half an hour, the temperature of the LC-MS room is 21 °C, fast wide positive mode) with deconvolution.

HSA modification with **NPM 1e**

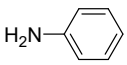
HSA	HSA + CO_2	HSA + H_3PO_4	**P** (mono-addition)	**P2** (double-addition)	**P3** (triple-addition)	**P4** (quad-addition)	**P5** (quint-addition)
66438 DA	66482 DA	66535 DA	66611 DA	66784 DA	669574 DA	67130 DA	67303 DA

HSA modification with **NEM 1f**

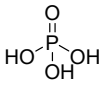
HSA	**P** (mono-addition)	**P2** (double-addition)	**P3** (triple-addition)	**P4** (quad-addition)	**P5** (quint-addition)
66438 DA	66562 DA	66687 DA	66812 DA	66938 DA	67063 DA



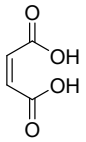
Molecular Weight:
R = Ph, NPM 173,1710
R = Et, NEM 125.0477



Molecular Weight:
93,1290



Molecular Weight:
97,9938

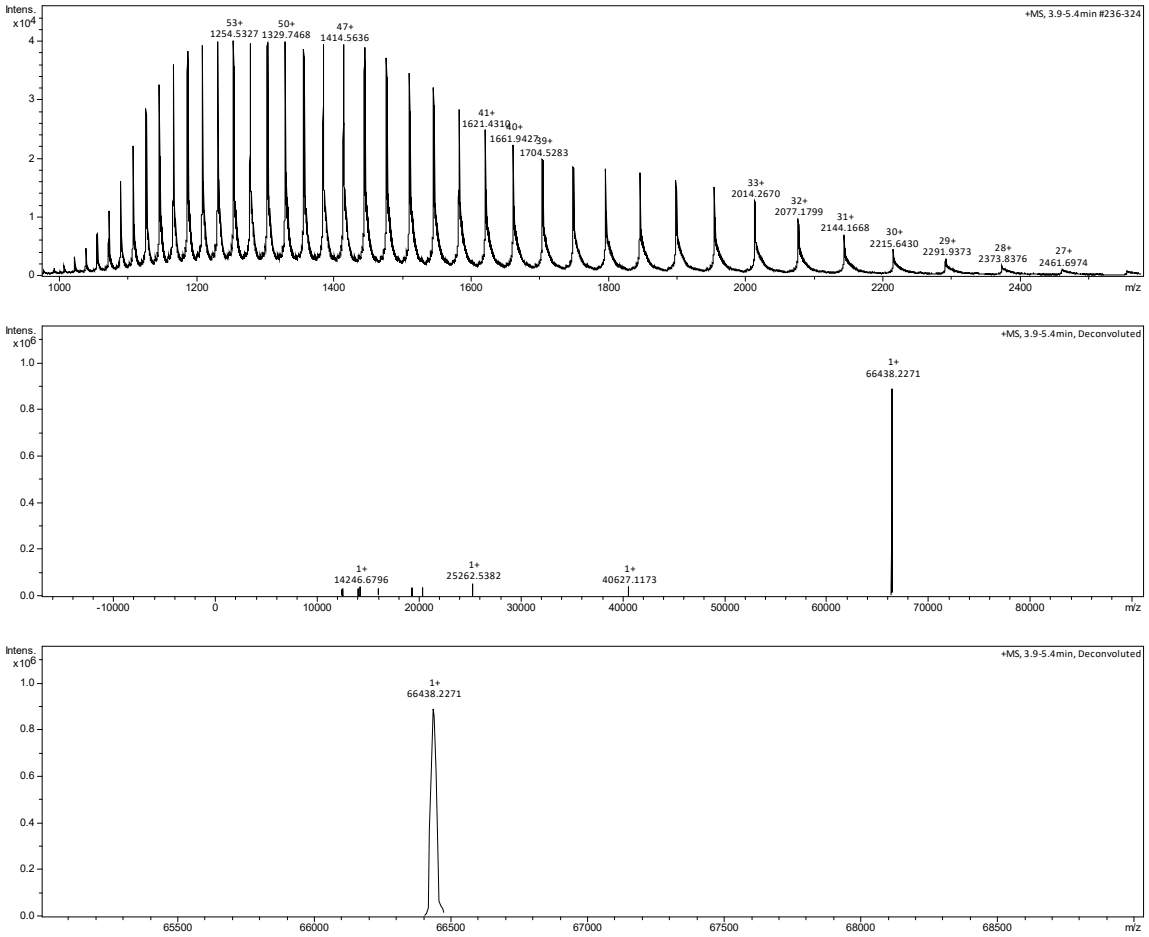


Molecular Weight:
116,0720

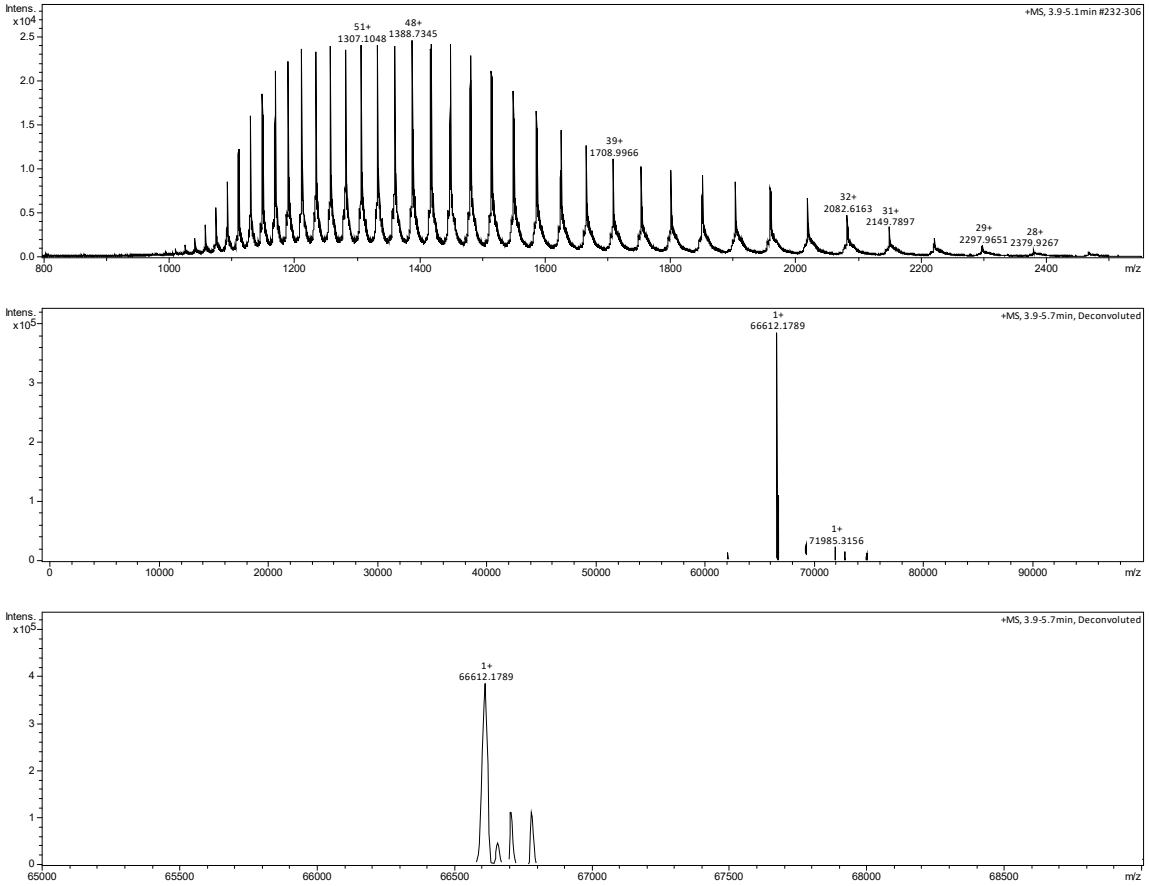
Fig. S7. Possible species detected by LCMS with the LCMS trace bellow.

LC-MS trace for Cys modification of HSA with imide 1e

Fresh HSA-pH 7.8-0.5 h

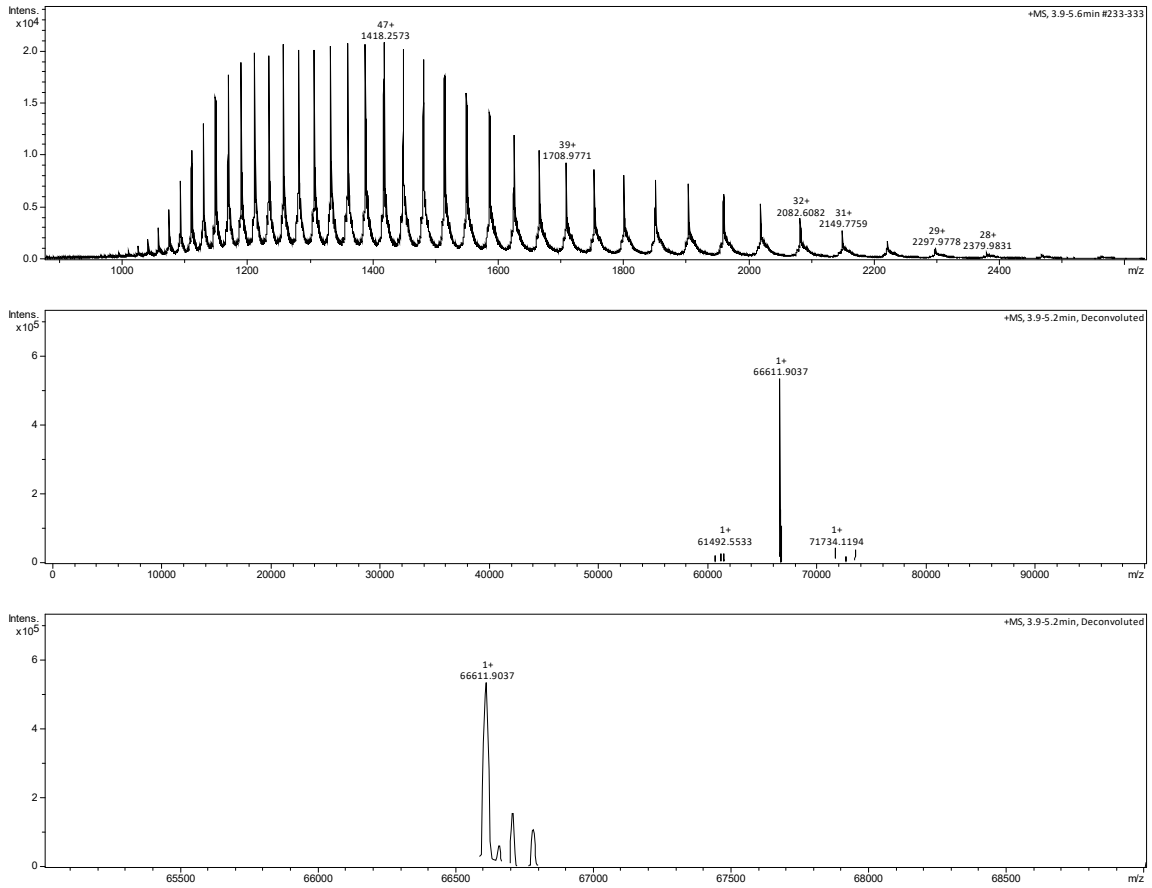


HSA-NPM-5eq-air-pH 7.8-0.5 h



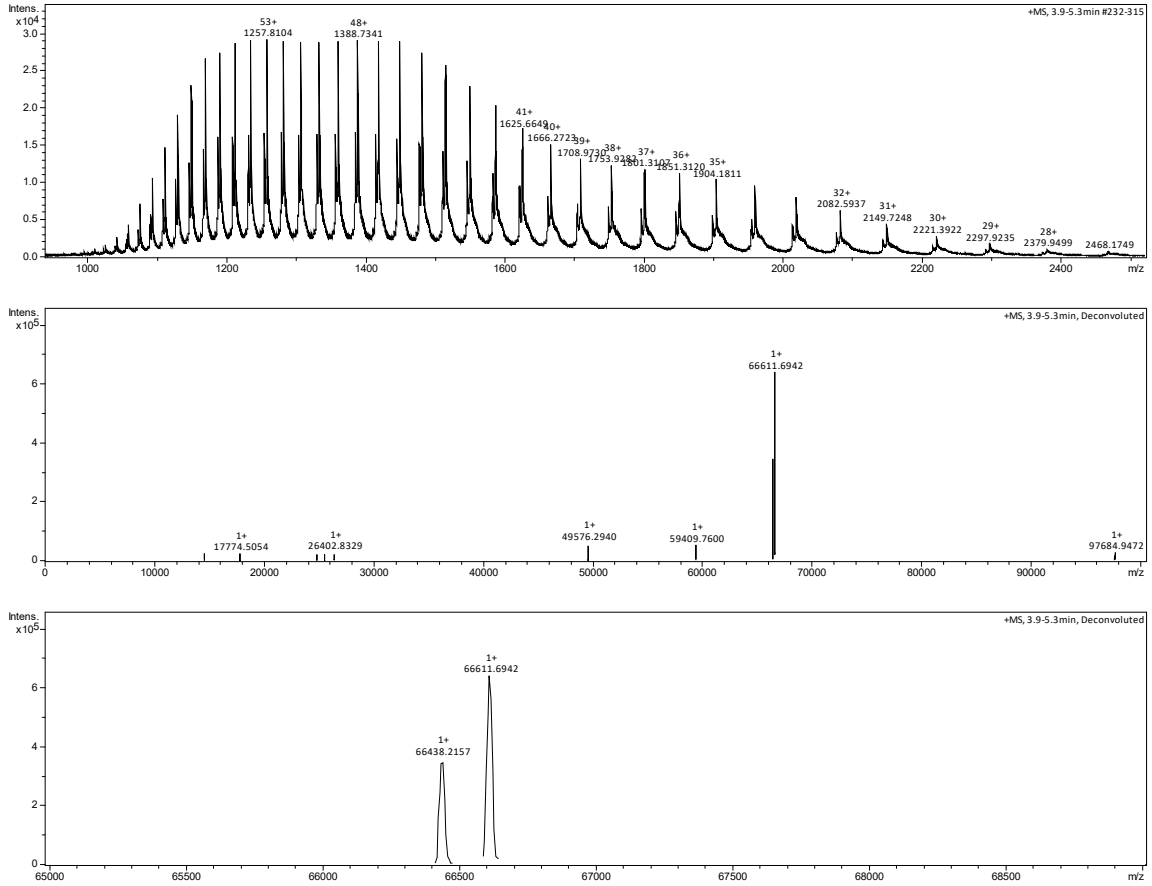
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.1789	**P**	58.5	83.0
2	66656.5904	**P** + CO_2	5.4	
3	66708.0995	**P** + H_3PO_4	19.2	
4	66782.4493	**P2**	17.0	

HSA-NPM-5eq-N_2-pH 7.8-0.5 h



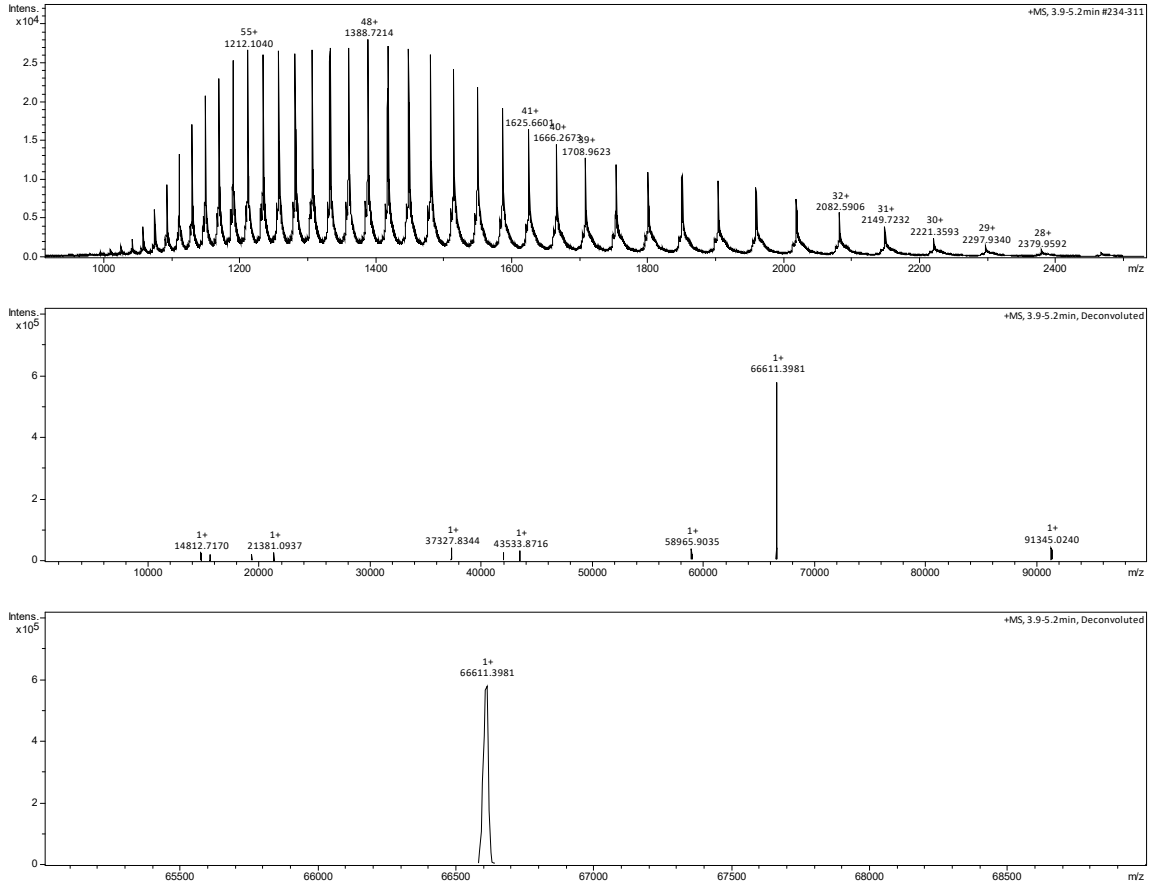
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66611.9037	**P**	61.0	87.7
2	66655.5432	**P** + CO_2	7.0	
3	66708.4525	**P** + H_3PO_4	19.7	
4	66782.5902	**P2**	12.3	

HSA-NPM-5eq-pH 6.0-0.5 h



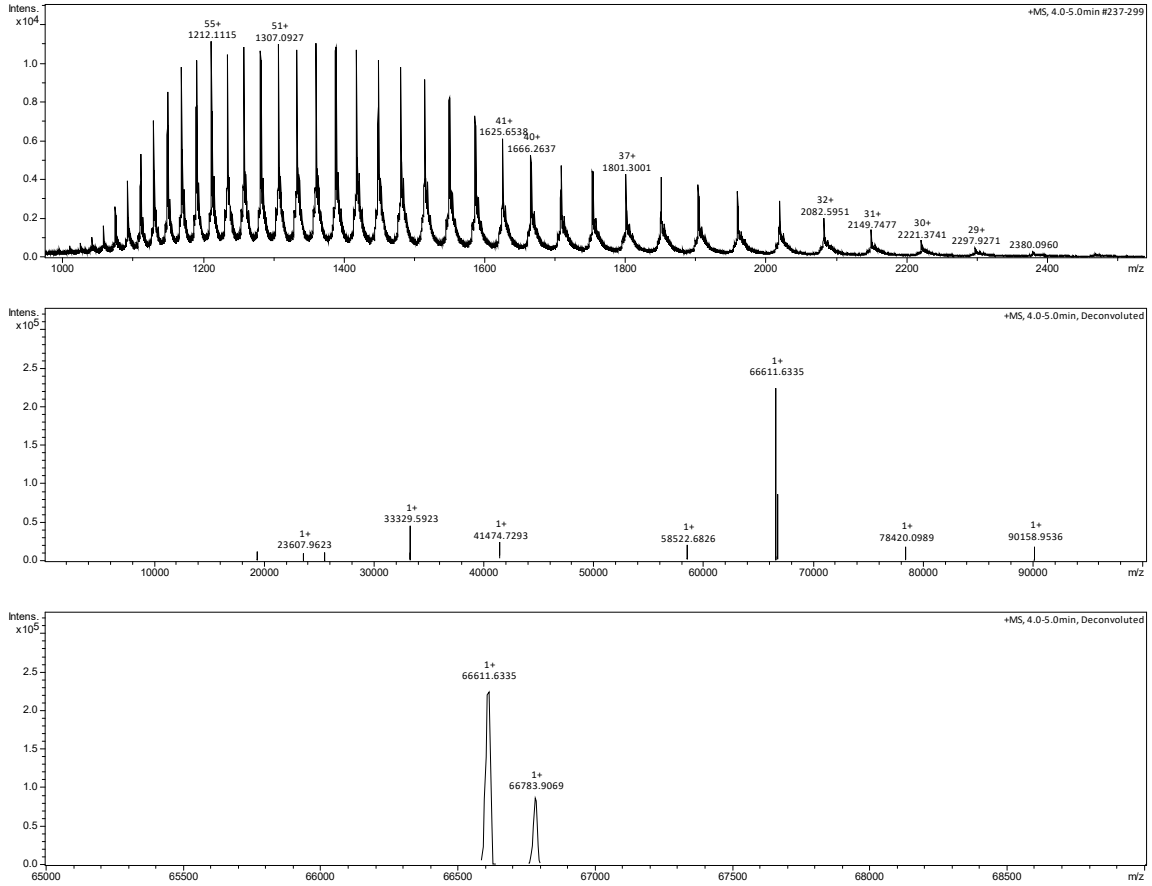
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66438.2157	**HSA**	36,1	63.9
2	66611.6942	**P**	63.9	

HSA-NPM-5eq-CO_2-0.5 h



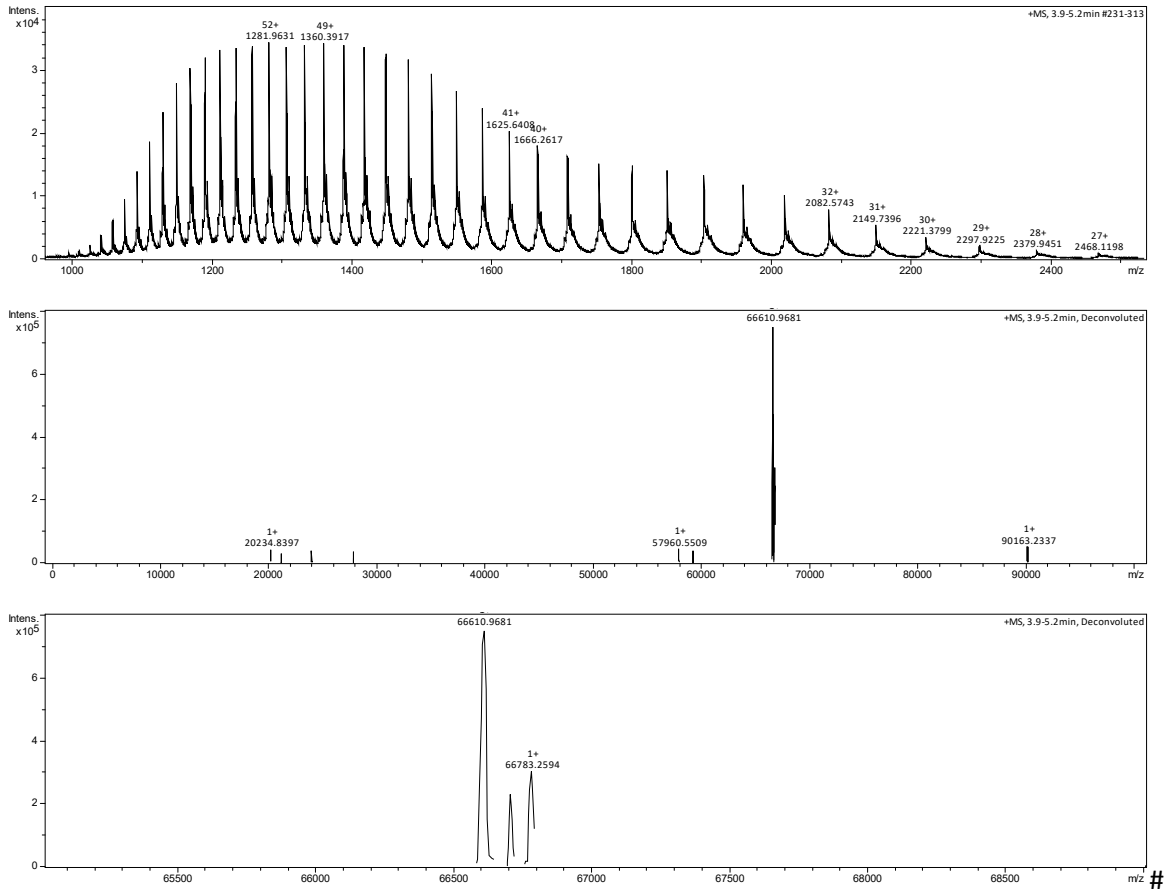
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66611.3891	**P**	100	100

HSA-NPM-5eq-air-pH 7.8-2.5 h



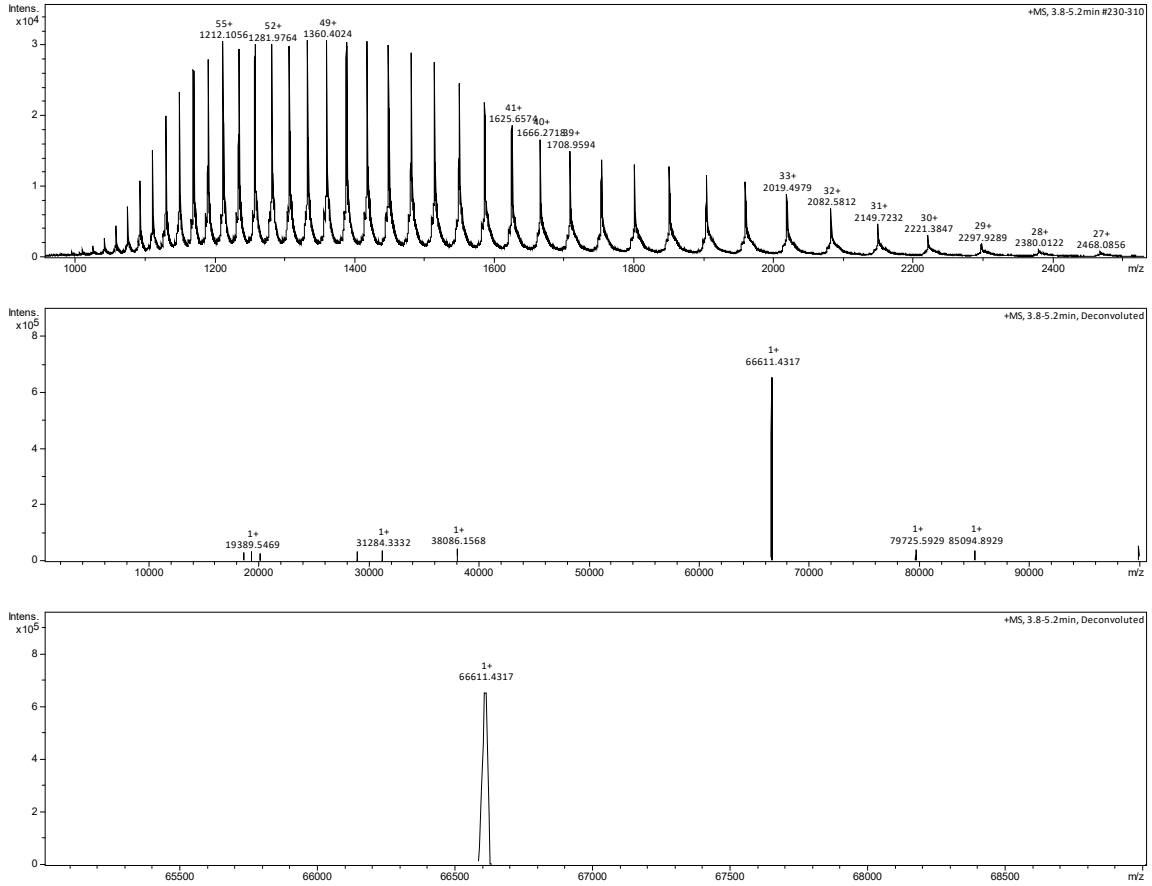
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66611.6335	**P**	71.7	71.7
2	66783.9069	**P2**	28.3	

HSA-NPM-5eq-N_2-pH 7.8-2.5 h



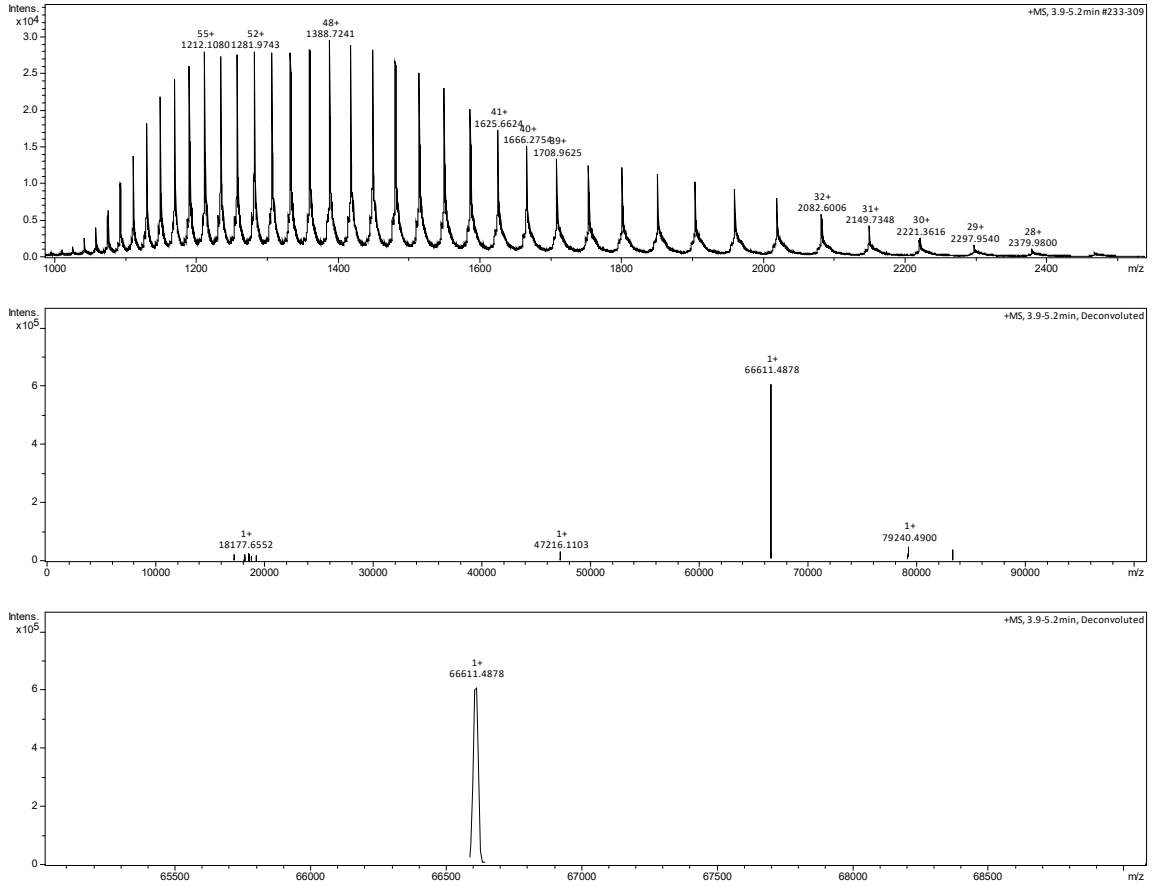
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.9681	**P**	59.3	76.9
2	66708.2809	**P** + H_3PO_4	17.6	
3	66783.2594	**P2**	23.1	

HSA-NPM-5eq-pH 6.0-2.5 h



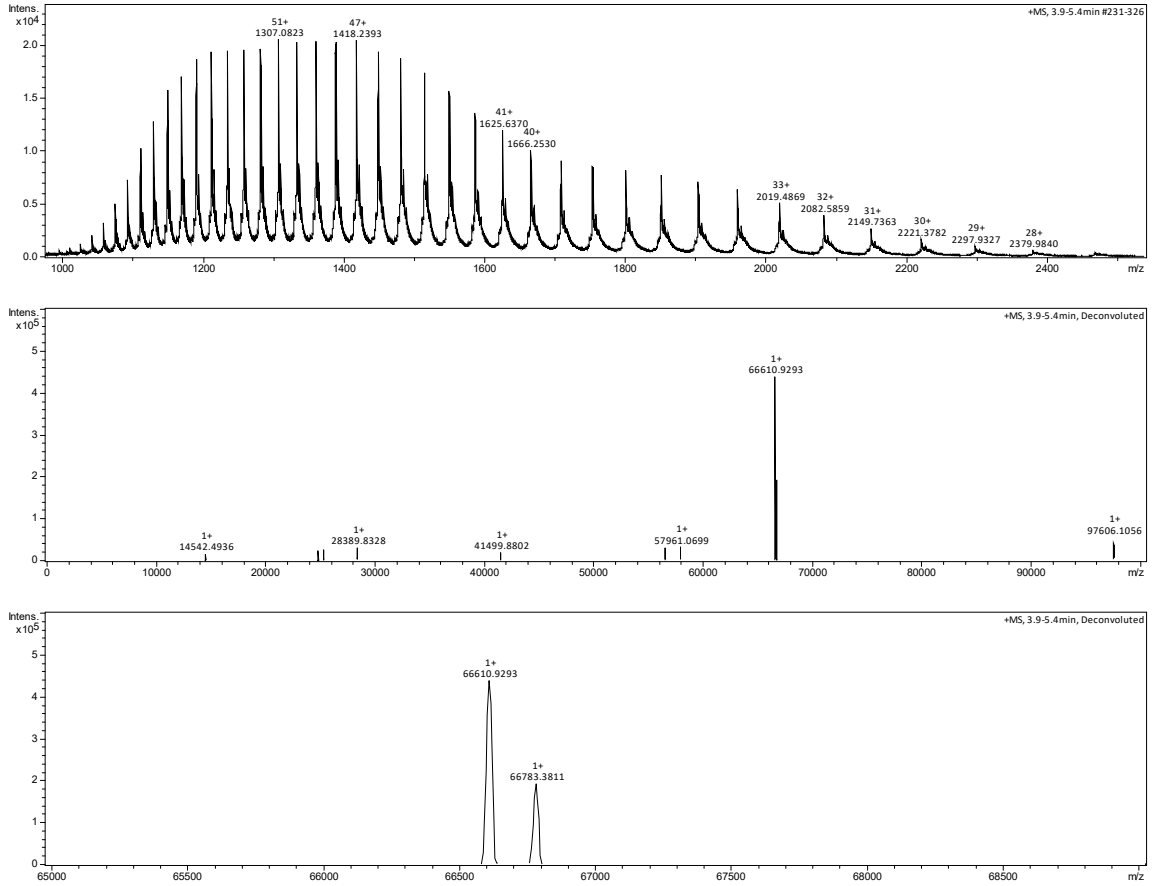
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66611.4317	**P**	100	100

HSA-NPM-5eq-CO_2-2.5 h



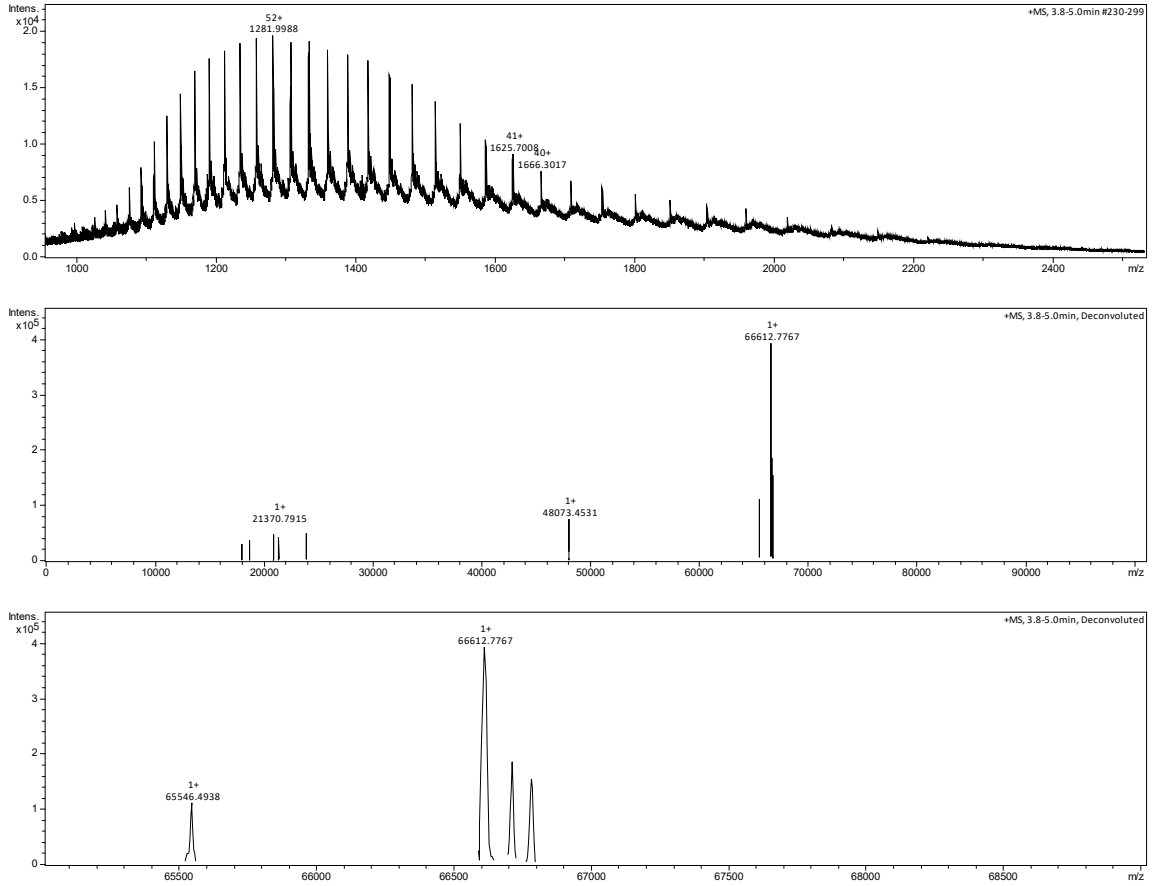
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66611.4878	**P**	100	100

HSA-NPM-40eq-air-pH 7.8-15 min



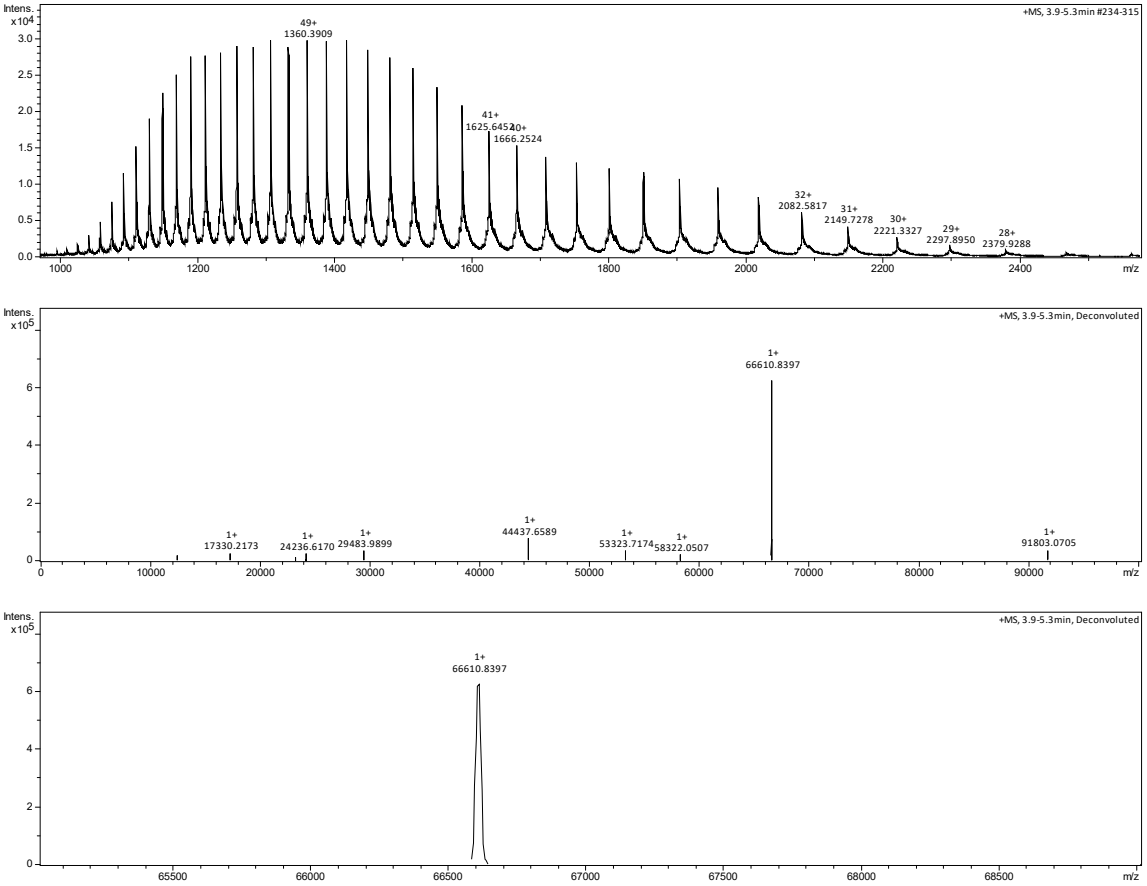
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.9293	**P**	69.5	69.5
2	66783.3811	**P2**	30.5	

HSA-NPM-40eq-N_2-pH 7.8-15 min



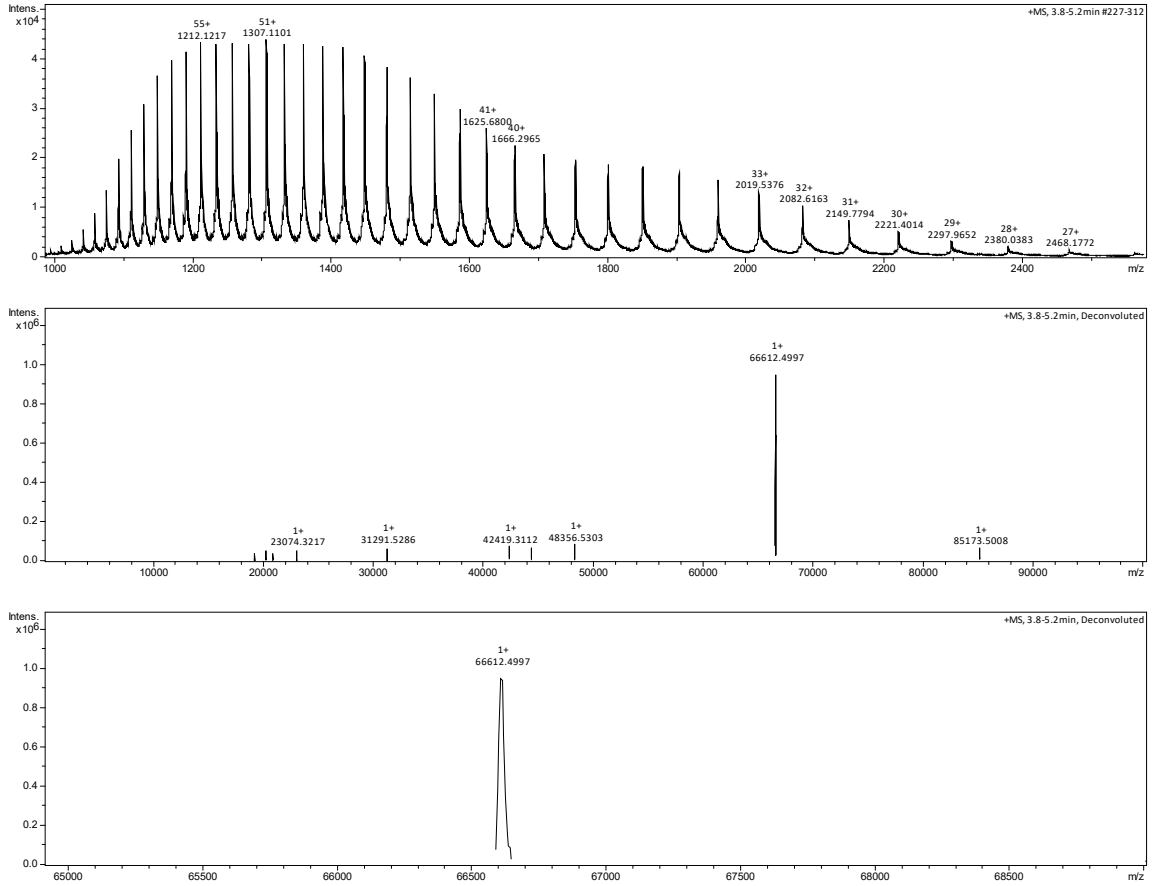
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.7767	**P**	53.5	78.8
2	66711.9662	**P** + H_3PO_4	25.3	
3	66784.9050	**P2**	21.2	

HSA-NPM-40eq-CO_2-15 min



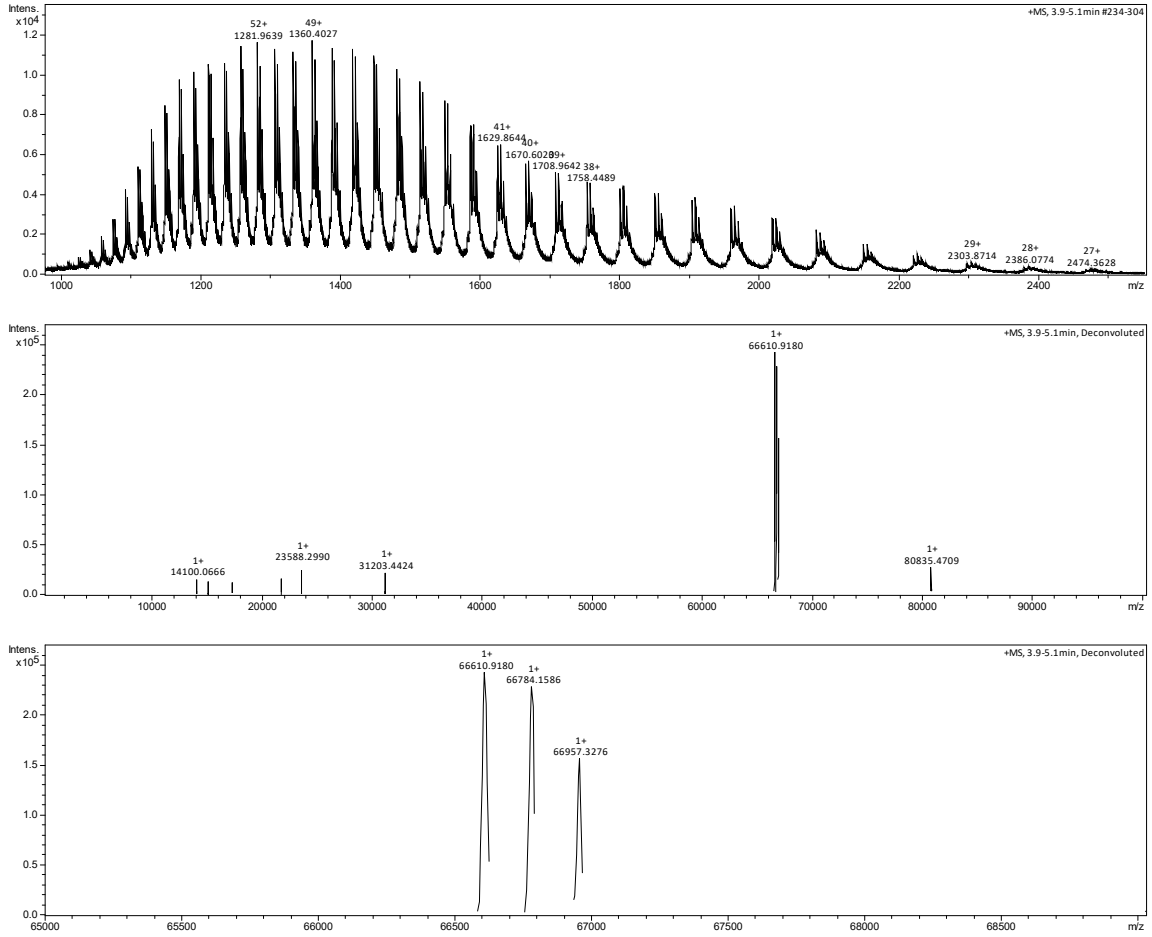
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.8397	**P**	100	100

HSA-NPM-40eq-pH6.0-15 min



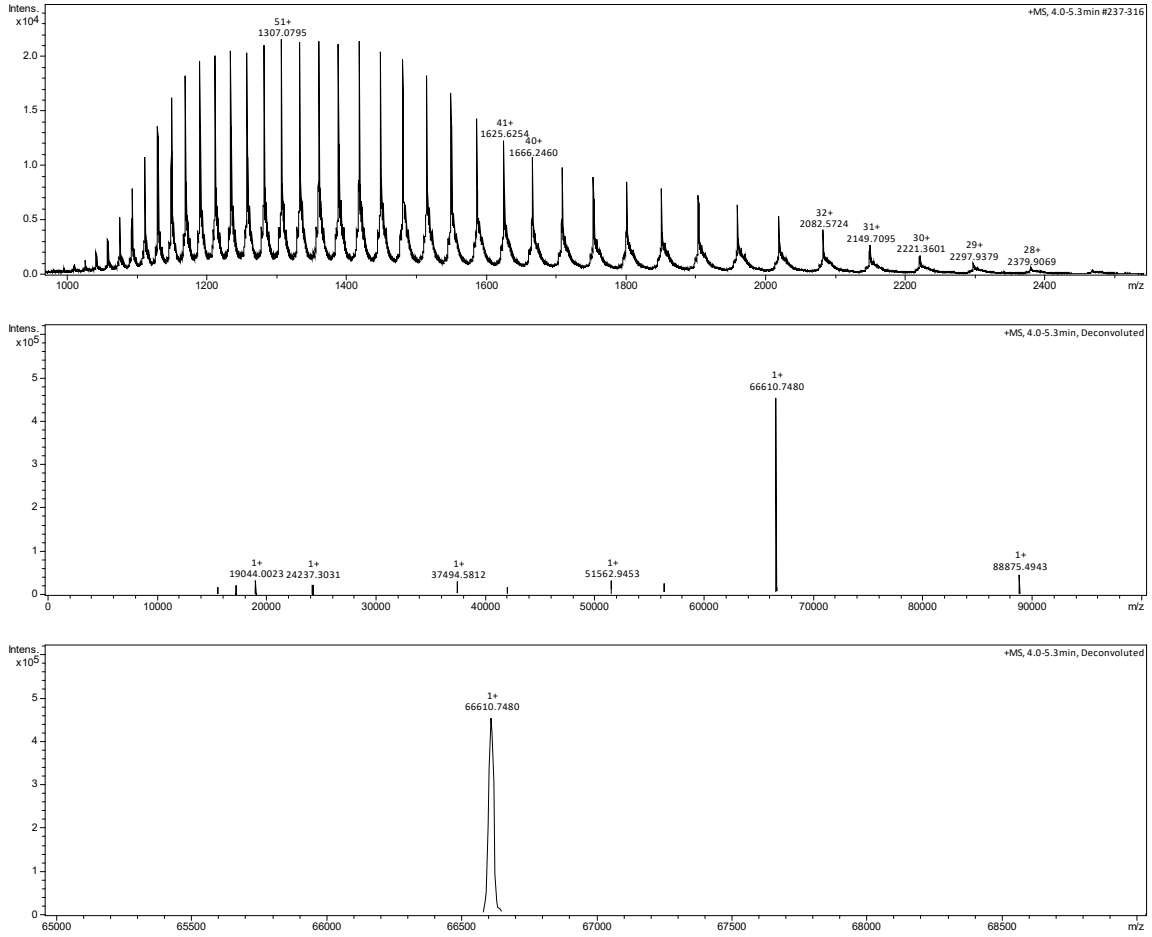
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.4997	**P**	100	100

HSA-NPM-40eq-air-pH 7.8-2 h



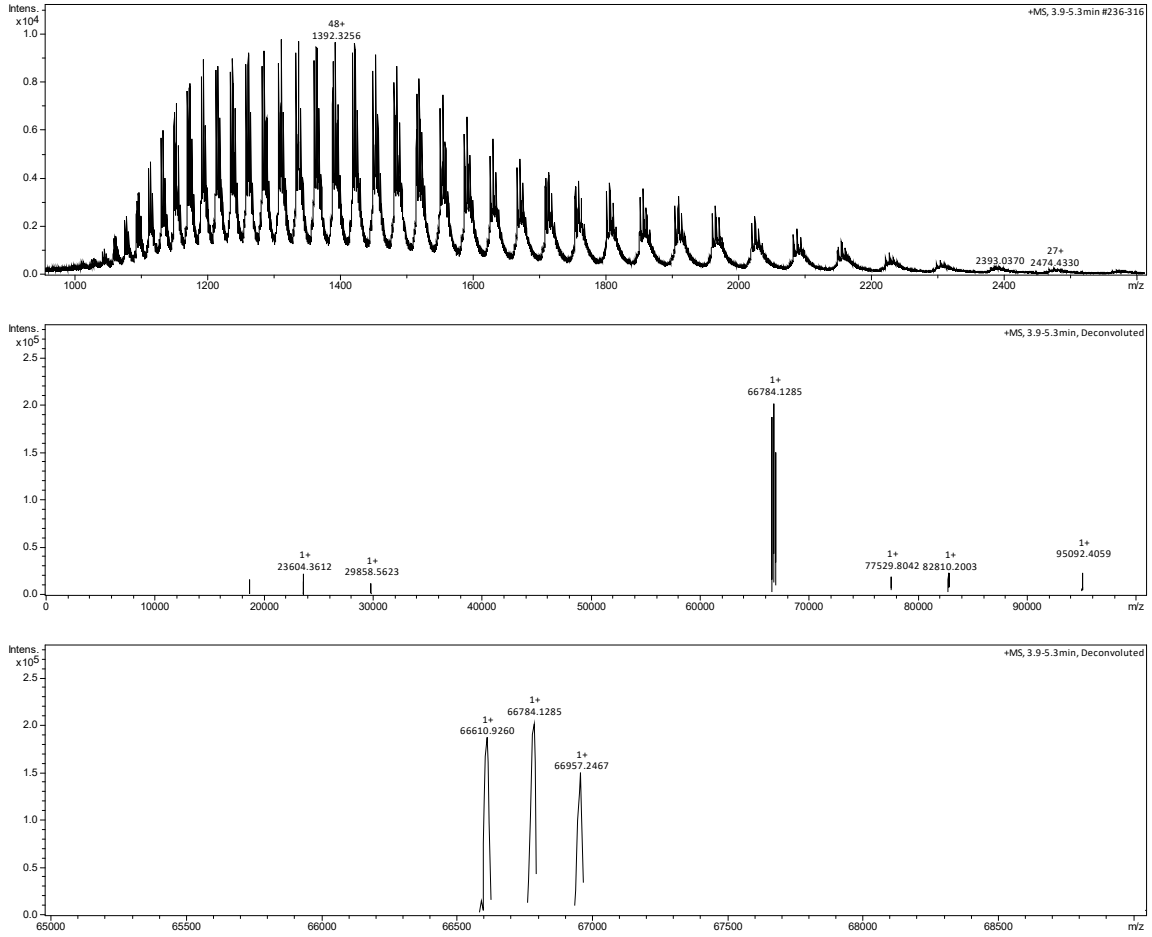
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.9180	**P**	38.7	38.7
2	66784.1586	**P2**	36.4	
3	66957.3276	**P3**	24.9	

HSA-NPM-40eq-CO_2-2 h



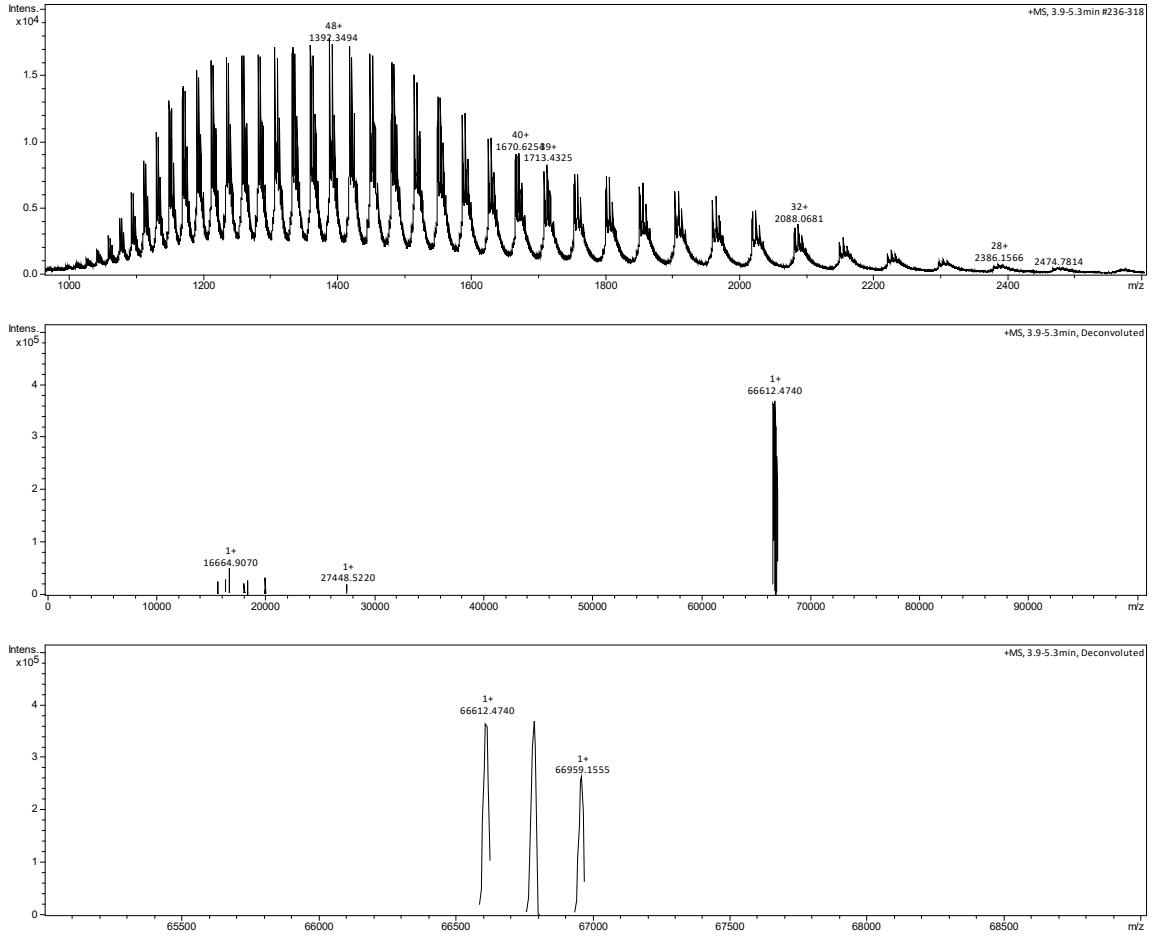
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.7480	**P**	100	100

HSA-NPM-40eq-air-pH 7.8-2.5 h



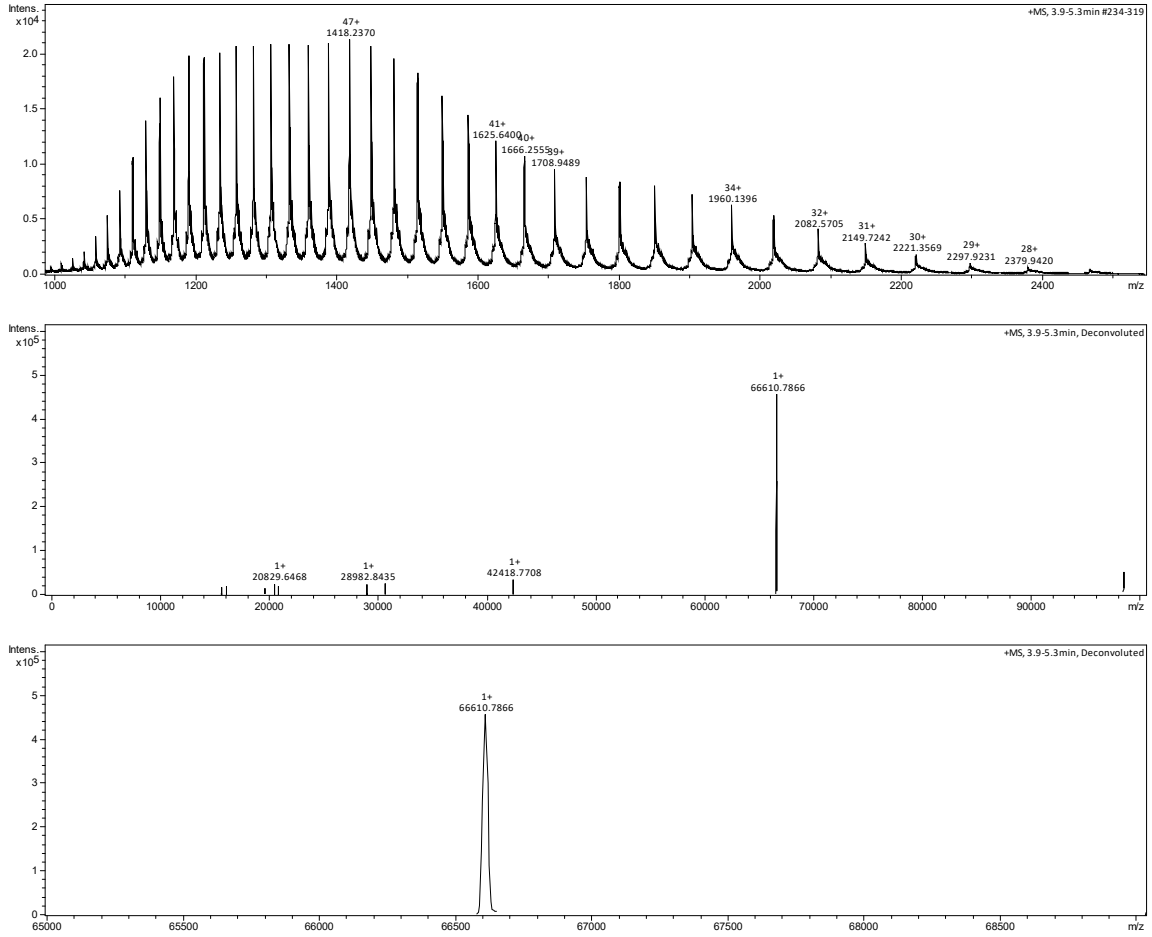
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.9260	**P**	34.5	34.5
2	66784.1285	**P2**	38.0	
3	66957.2467	**P3**	27.5	

HSA-NPM-40eq-N_2-pH 7.8-2.5 h



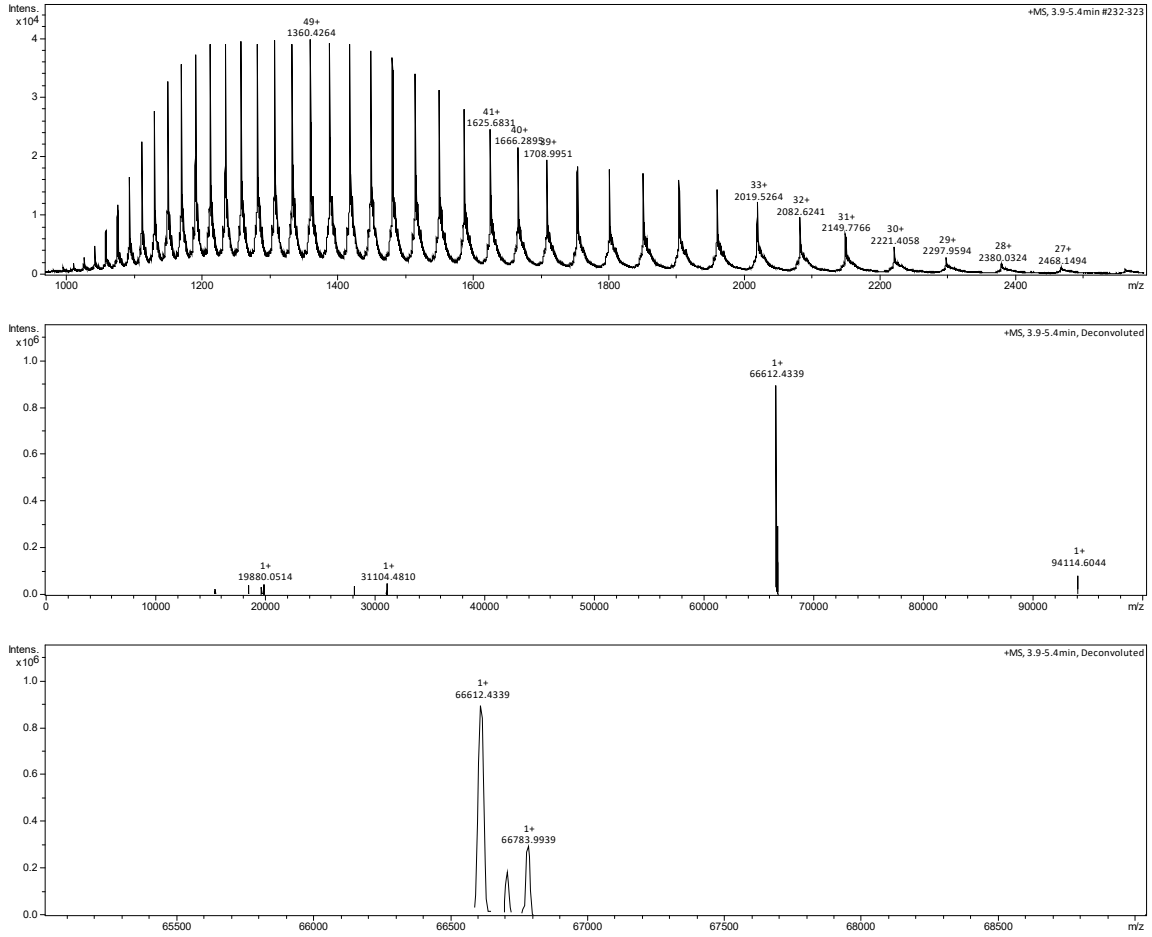
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.4740	**P**	37.3	37.3
2	66785.7307	**P2**	36.5	
3	66959.1555	**P3**	26.2	

HSA-NPM-40eq-CO_2-2.5 h



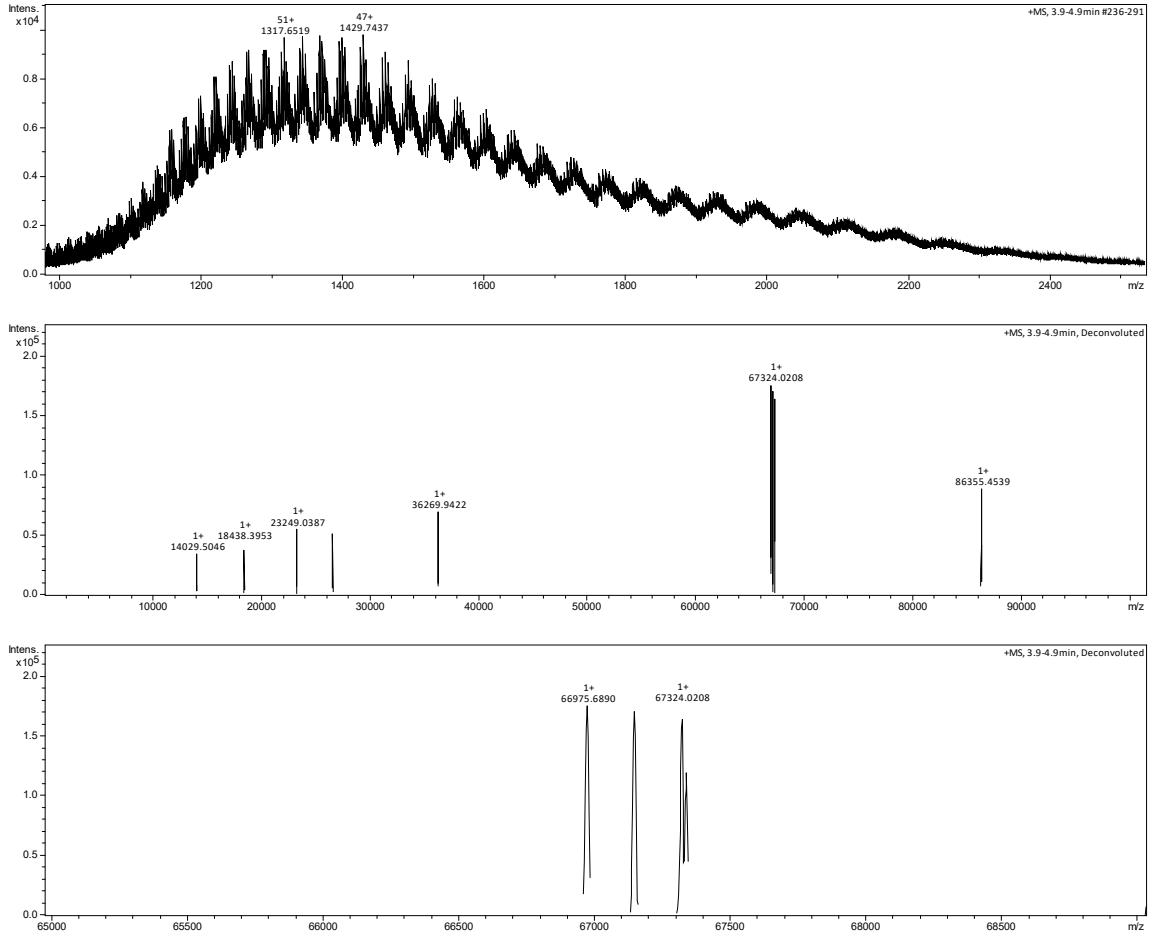
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66610.7866	**P**	100	100

HSA-NPM-40eq-pH 6.0-2.5 h



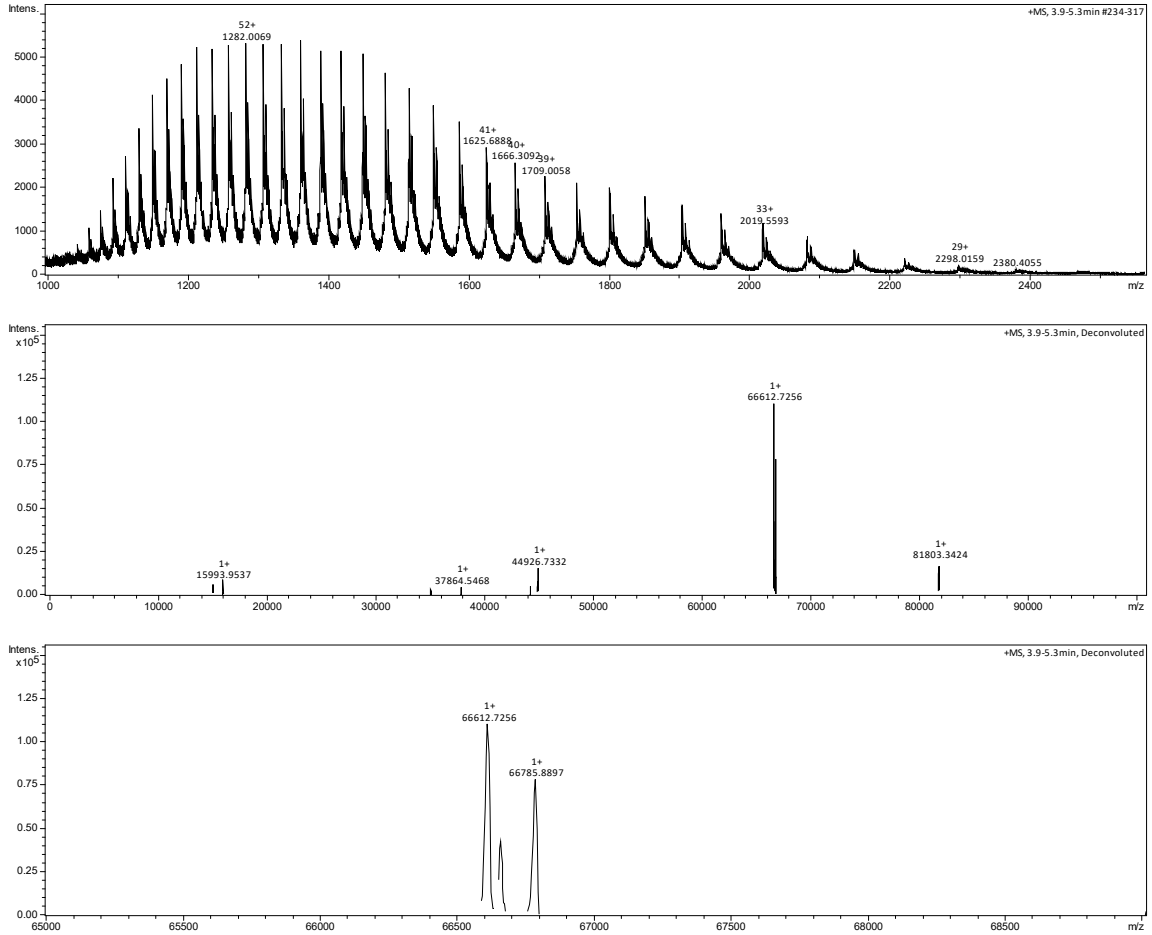
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.4339	**P**	65.6	78.8
2	66709.0095	**P** + H_3PO_4	13.2	
3	66783.9939	**P2**	21.2	

HSA-NPM-40eq-N_2-pH 7.8-14.5 h



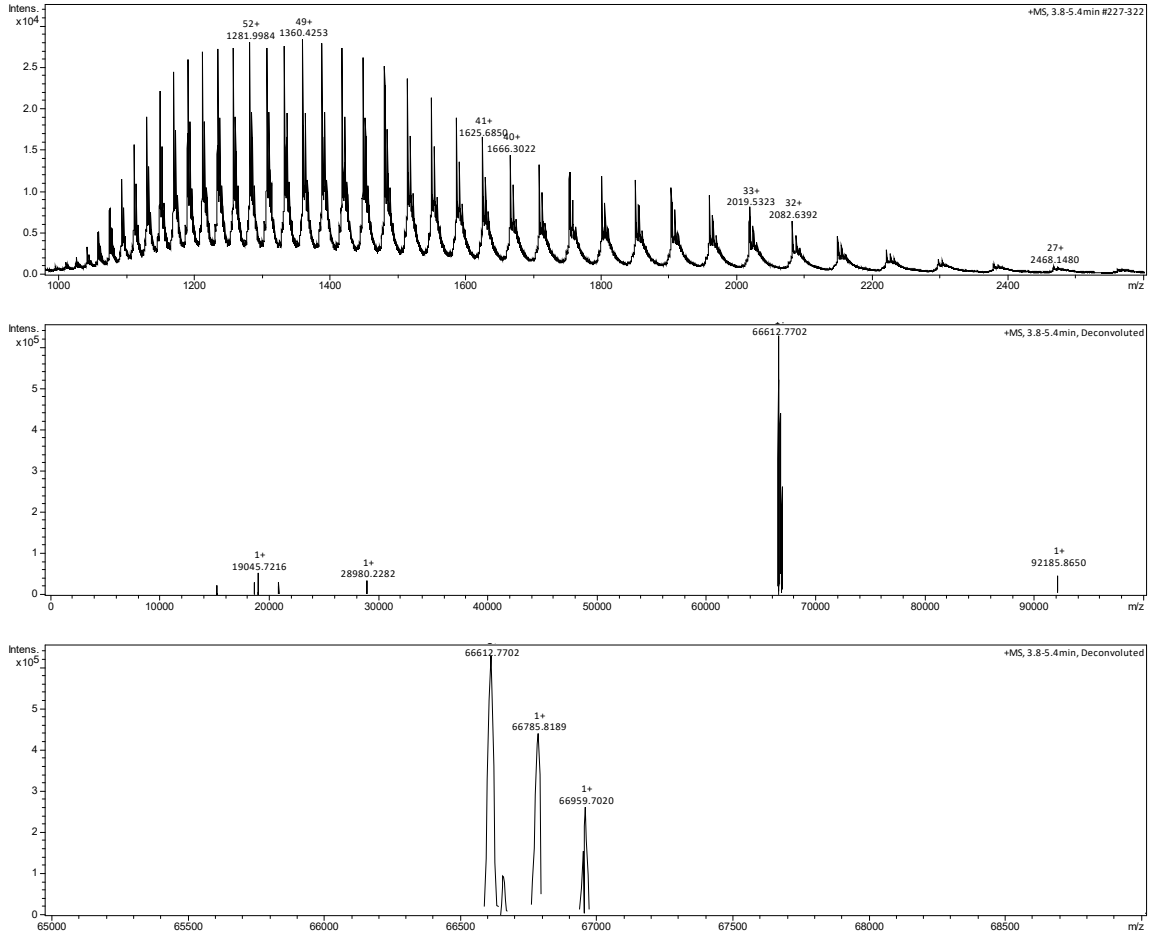
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66975.6890	**P3**	26.9	0
2	67149.8918	**P4**	26.2	
3	67324.0208	**P5**	27.0	
4	67339.4726	**P5** + H_2O	19.9	

HSA-NPM-40eq-CO_2-14.5 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.7256	**P**	47.7	66.1
2	66659.9112	**P** + CO_2	18.4	
3	66785.8897	**P2**	33.9	

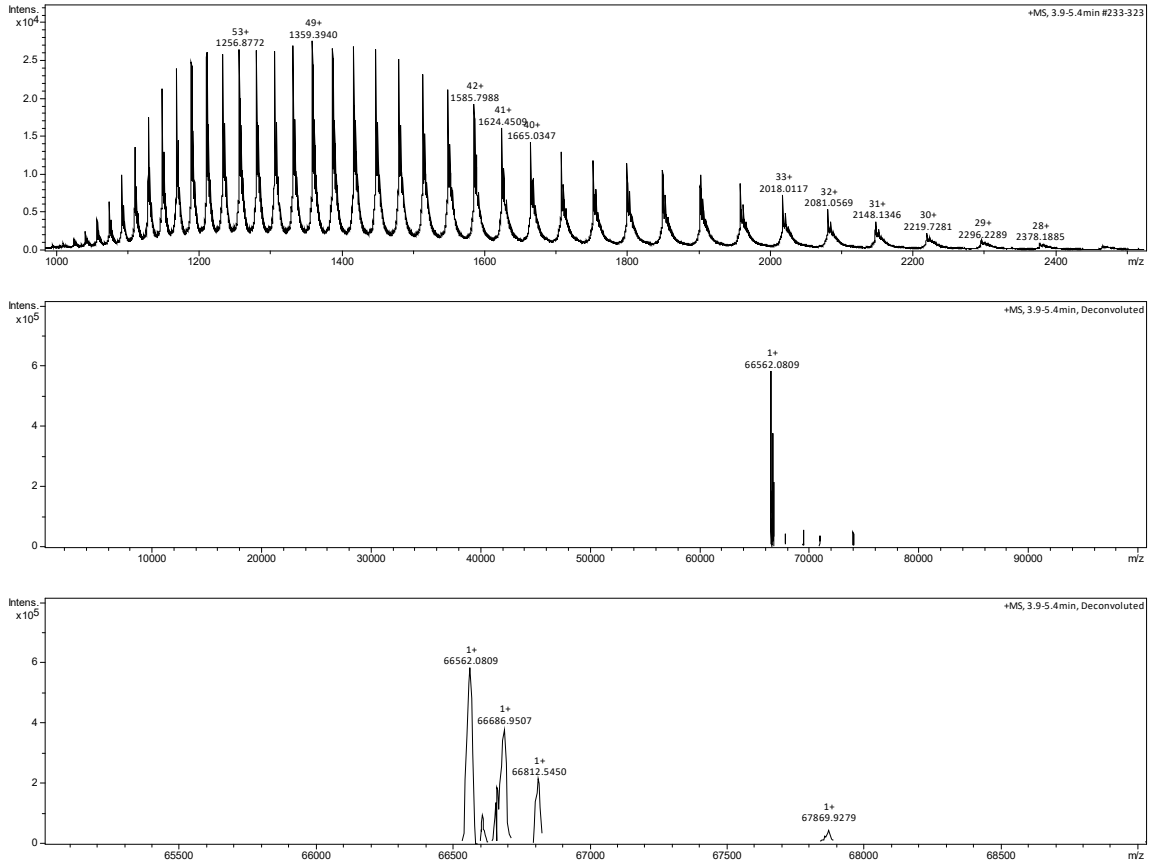
HSA-NPM-40eq-pH 6.0-14.5 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66612.7702	**P**	44.0	50.7
2	66657.7203	**P** + CO_2	6.7	
3	66785.8189	**P2**	30.9	
4	66959.7020	**P3**	18.3	

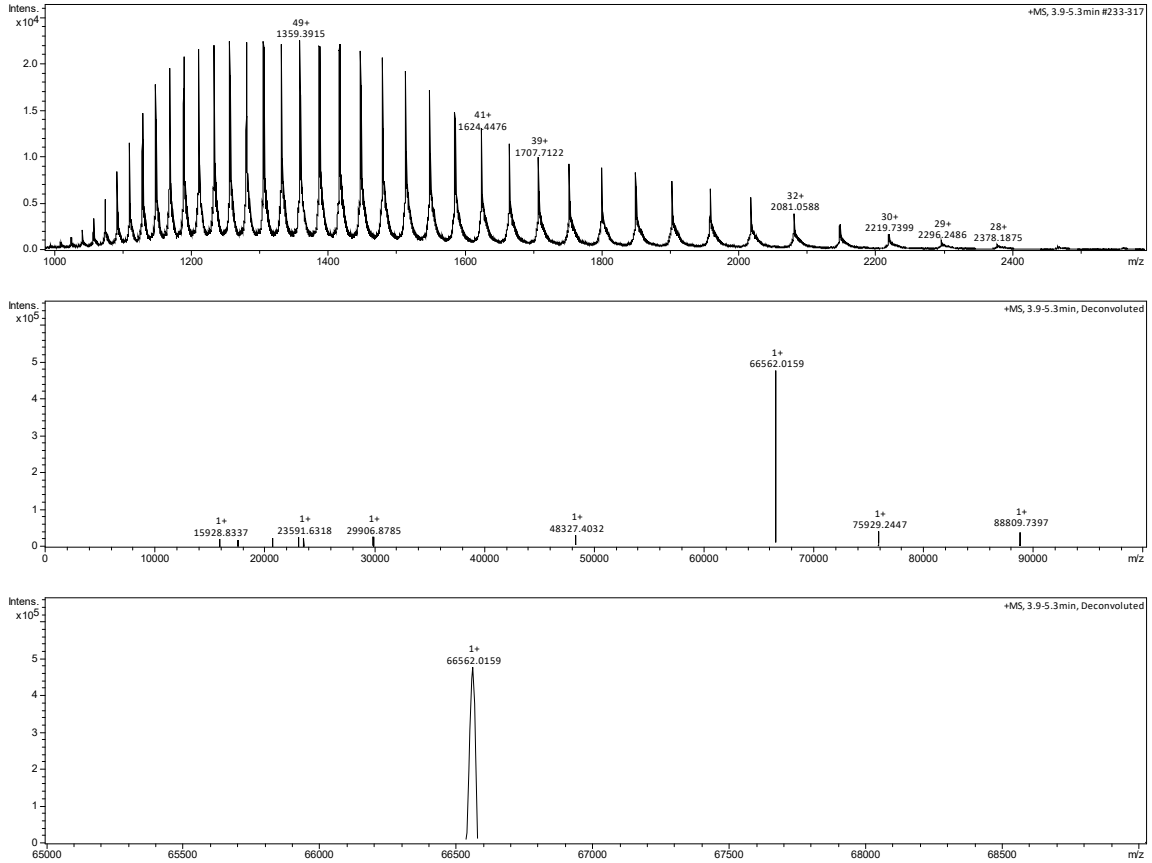
LC-MS trace for Cys modification of HSA with imide 1f

HSA-NEM-40 eq-N_2-Ph 7.8-4.5 h



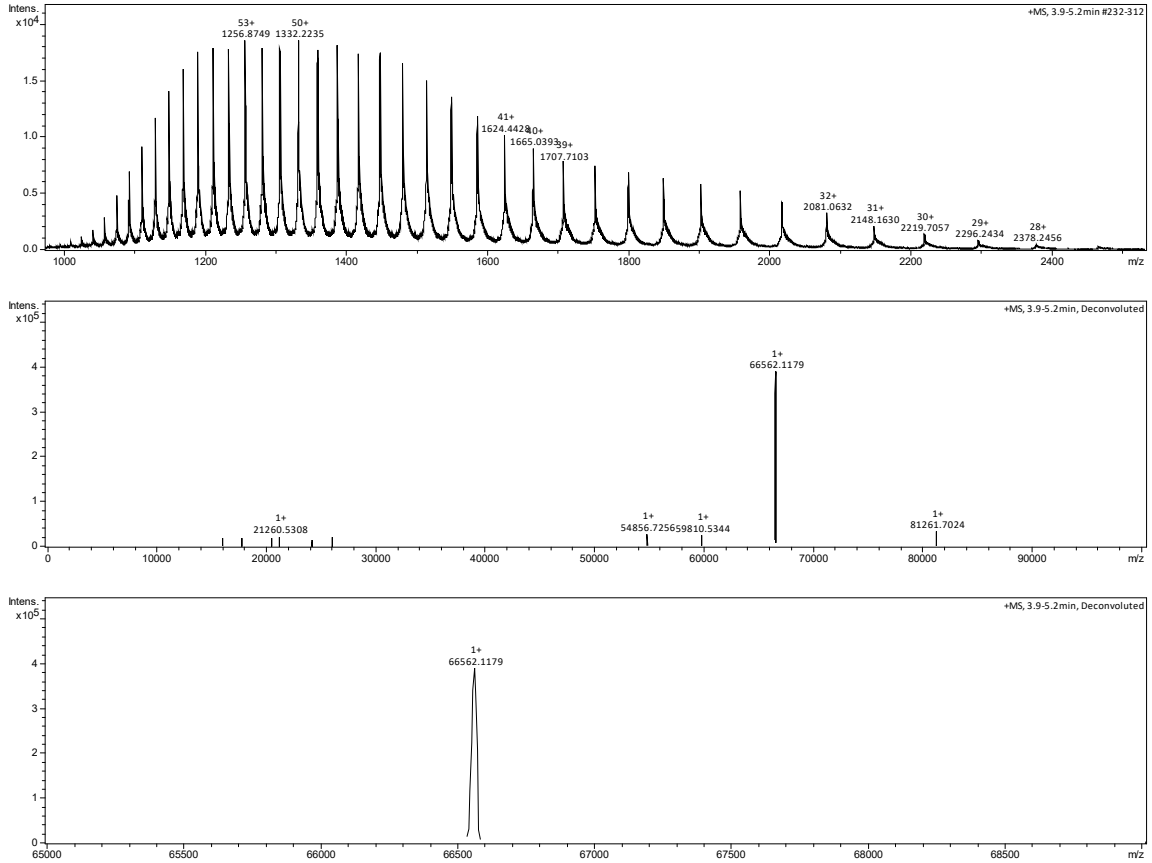
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.0809	**P**	37.5	61.4
2	66608.5156	**P** + CO_2	6.0	
3	66608.7893	**P** + CO_2	6.0	
4	66660.4480	**P** + H_3PO_4	11.9	
5	66686.9507	**P2**	24.7	
6	66812.5450	**P3**	13.8	

HSA-NEM-40 eq-CO_2- 4.5 h



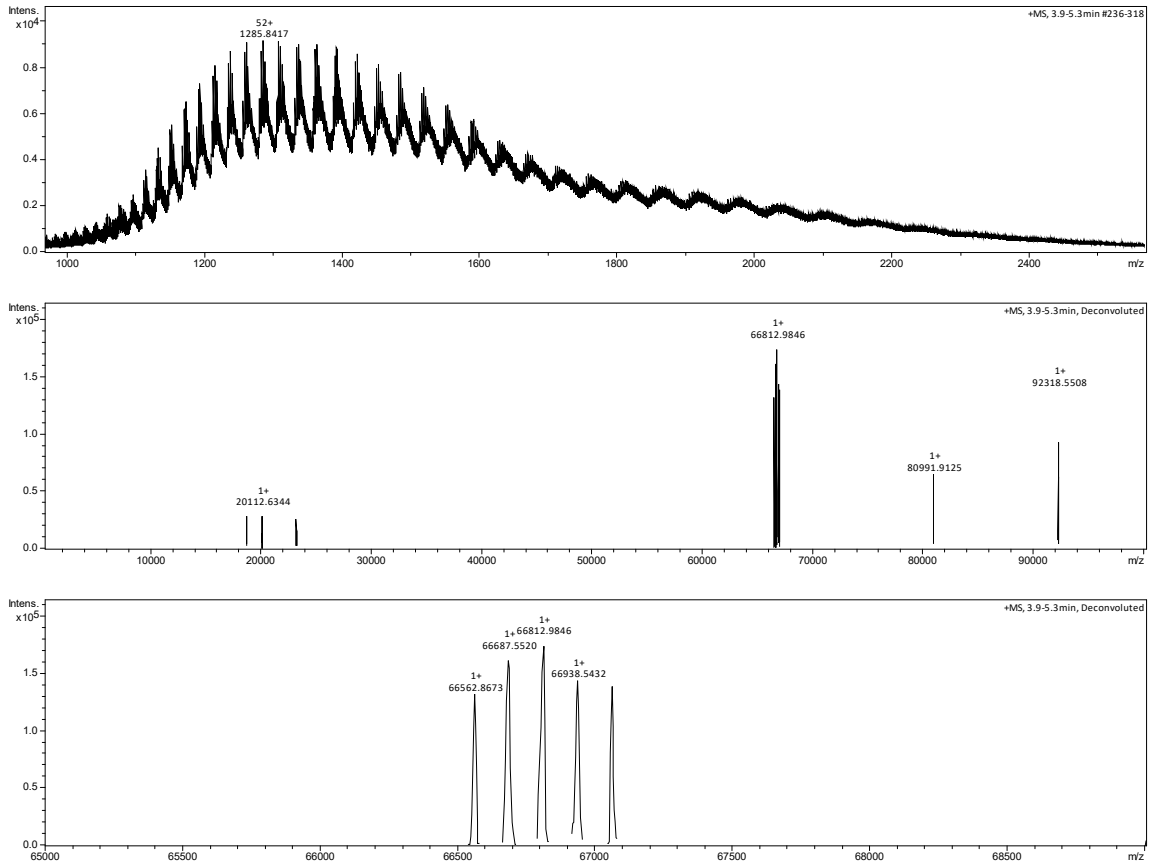
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.0159	**P**	100	100

HSA-NEM-40 eq-PH6.0- 4.5 h



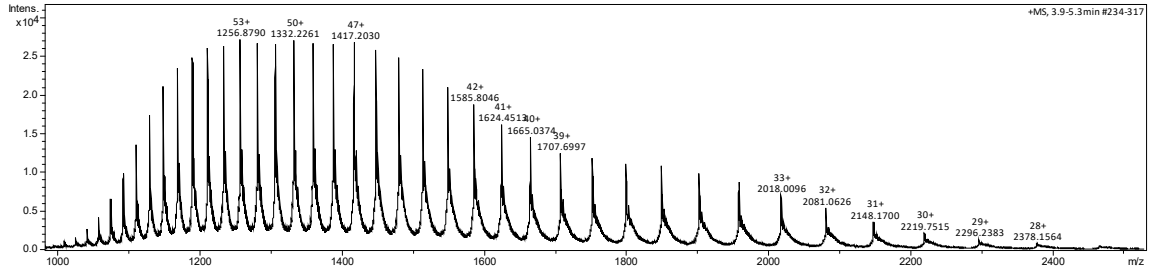
Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.1179	**P**	100	100

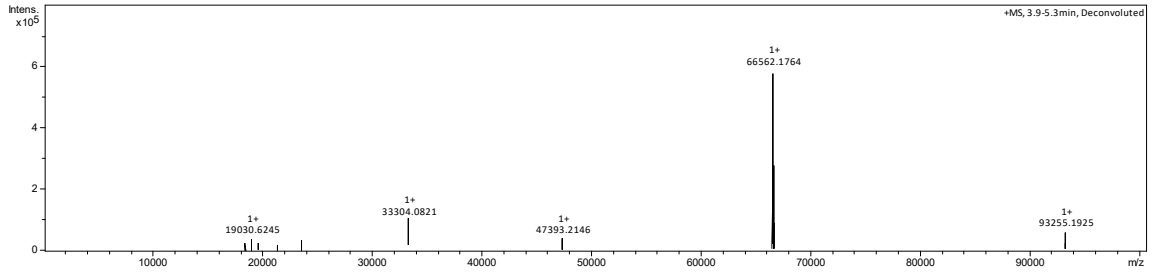
HSA-NEM-40 eq-N_2-pH7.8- 23 h

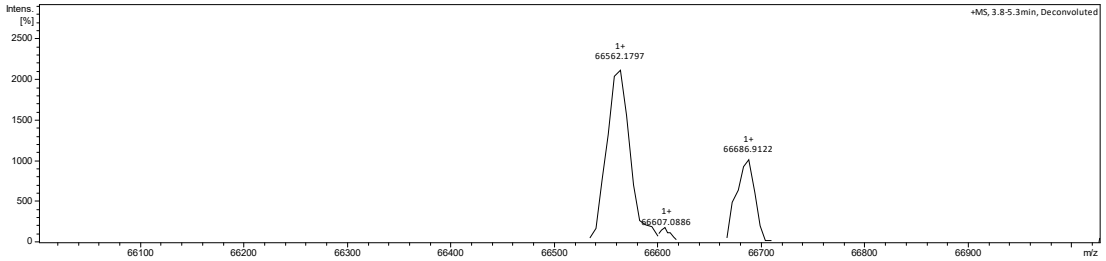


Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.8673	**P**	17.3	17.3
2	66687.5520	**P2**	22.0	
3	66812.9846	**P3**	23.7	
4	66938.5432	**P4**	18.8	
5	67063.3584	**P5**	18.1	

HSA-NEM-40 eq-CO_2- 23 h

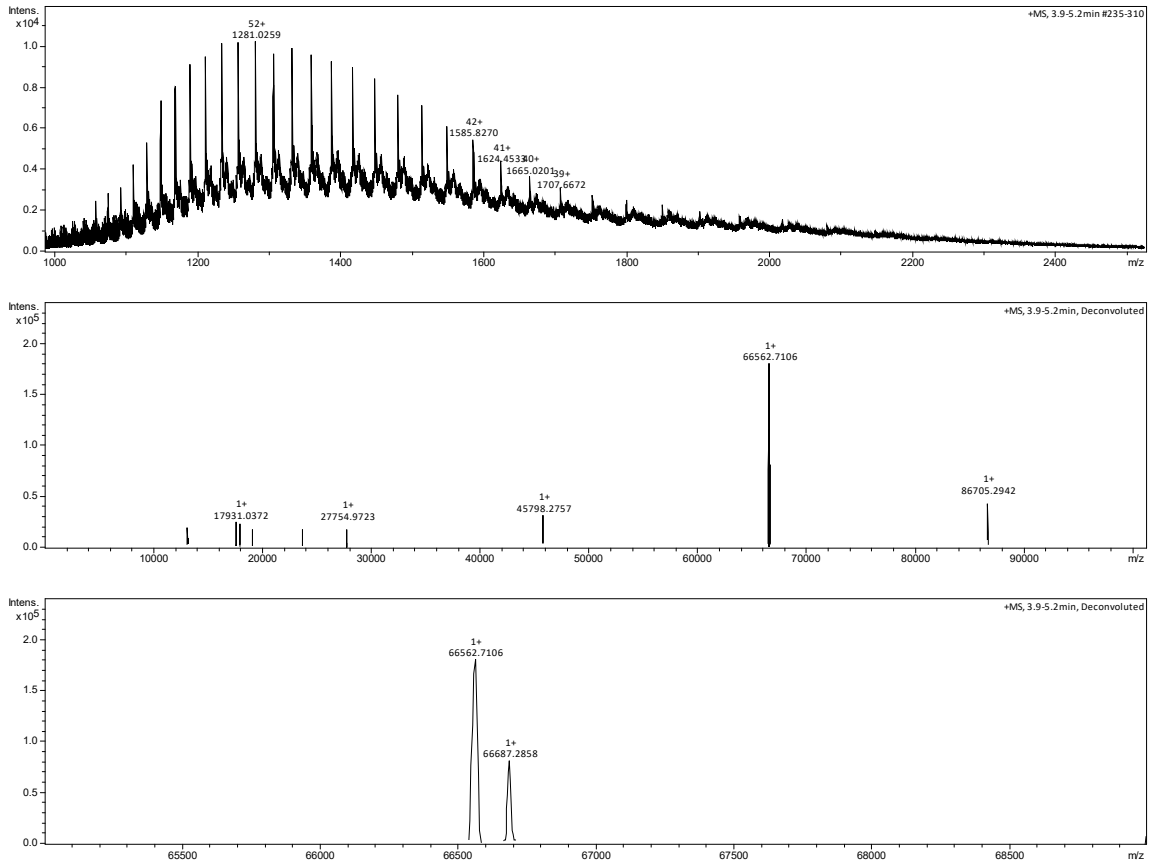






Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.1797	**P**	63.9	69.3
2	66607.0886	**P** + CO_2	5.4	
3	66686.9122	**P2**	30.7	

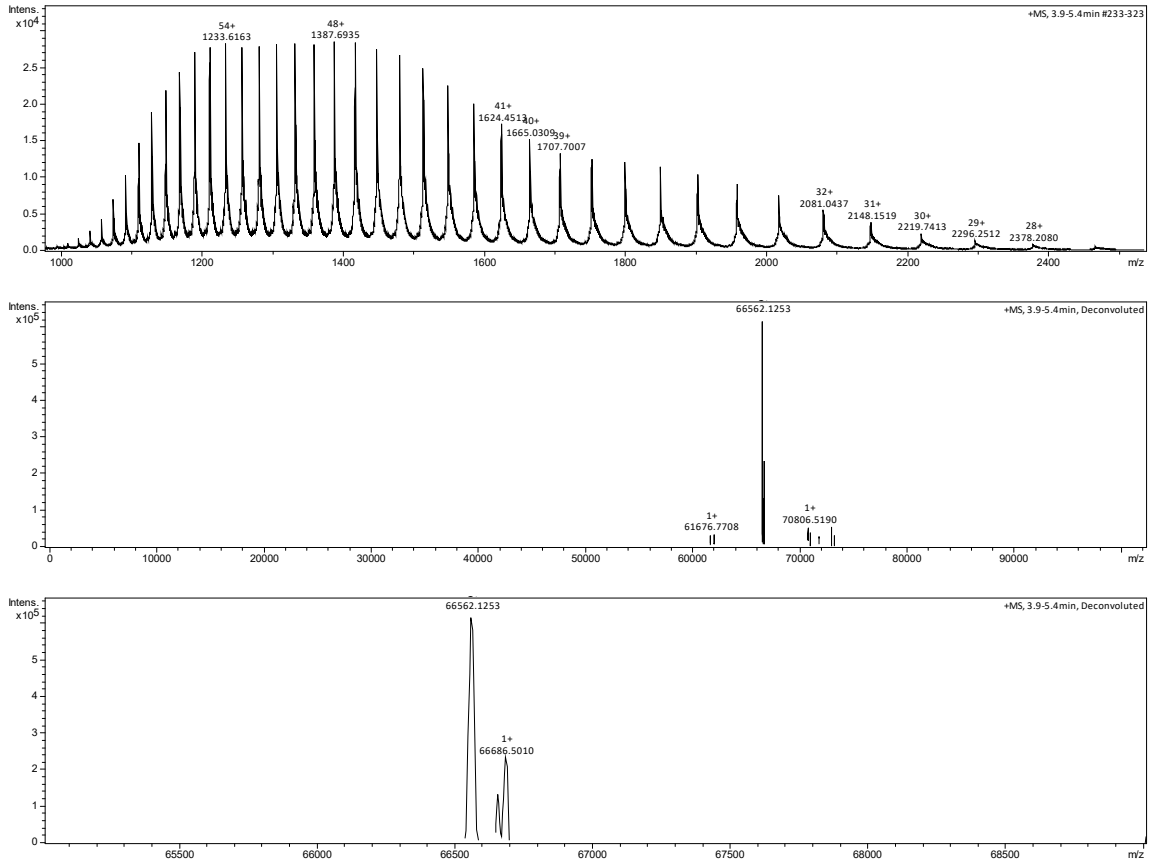
HAS-NEM-40 eq-PH 6.0- 23 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.7106	**P**	69.0	69.0
2	66687.2858	**P2**	31.0	

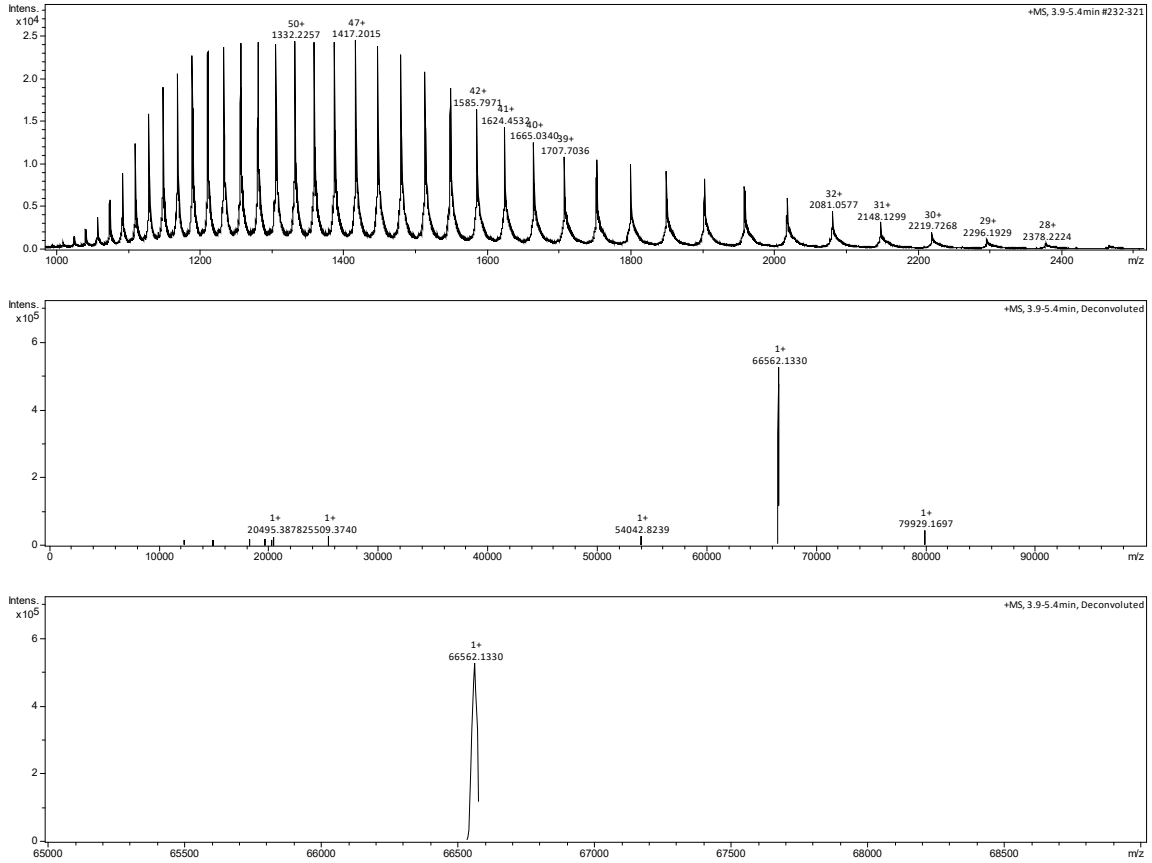
Note: Considering that *N*-ethylmaleimide (**1f**) is more reactive than *N*-phenylmaleimide (**1e**), we also performed the reactions with decreased amounts of *N*-ethylmaleimide (10 equiv.). CO_2 suppressed the double addition (4.5 h) and triple addition (23 h) from HSA to **1f**.

HSA-NEM 10 eq-N_2-Ph7.8-4.5 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.1253	**P**	63.4	77.0
2	66658.2835	**P** + H_3PO_4	13.3	
3	66686.5010	**P2**	23.0	

HSA-NEM 10 eq-CO_2- 4.5 h



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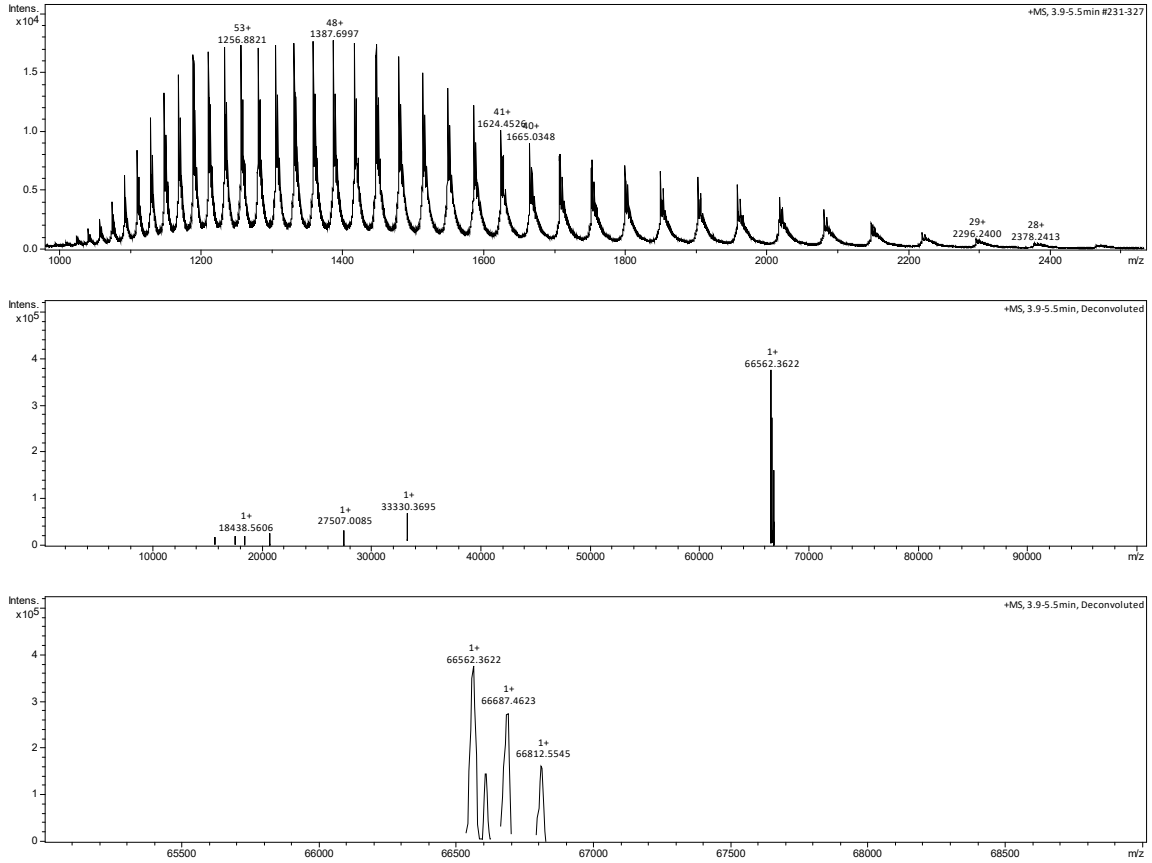
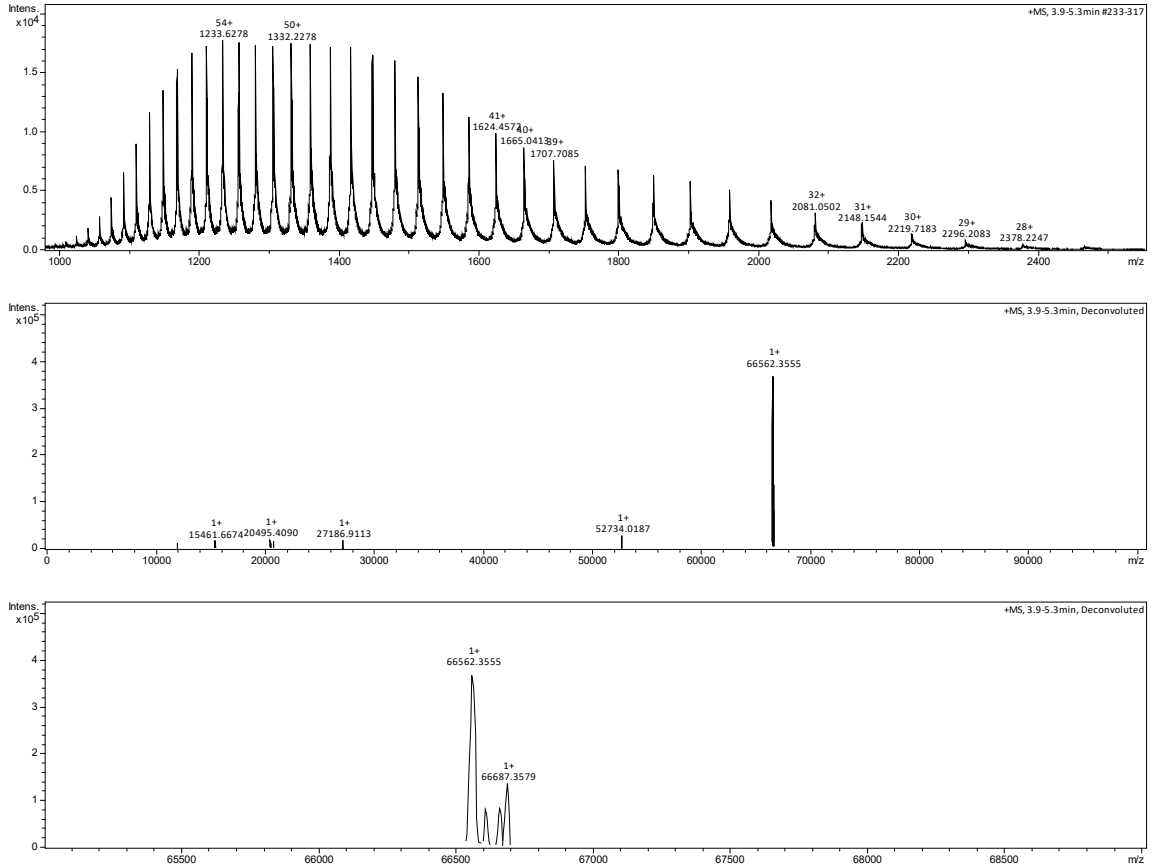
1	66562.1330	**P**	100	100

HSA-NEM 10 eq-N_2-Ph7.8-23 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.3622	**P**	37.5	53.9
2	66609.7506	**P** + CO_2	16.4	
3	66687.4623	**P2**	28.8	
4	66812.5545	**P3**	17.3	

HSA-NEM 10 eq-CO_2-23 h



Peak Number	m/z	Attribute	INTE %	Selectivity (**P**) %
1	66562.3555	**P**	55.9	80.0
2	66609.4149	**P** + CO_2	11.9	
3	66659.4546	**P** + CO_2+ H_3PO_4	12.3	
4	66687.3579	**P2**	20.0	

6. Synthesis of FKVCF and Cys-conjugation with N-phenylmaleimide

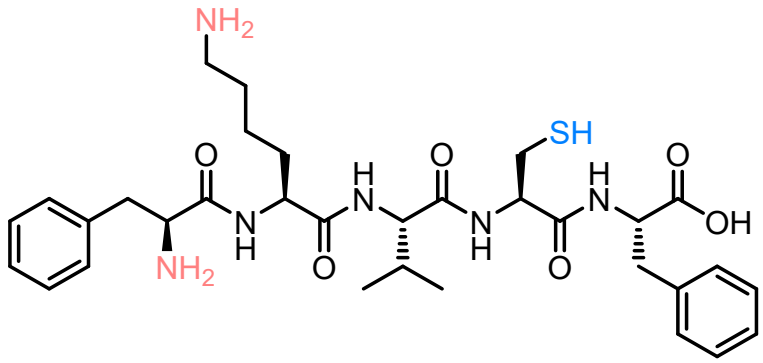
6.1 General methods

A Bruker instrument with an Acclaim™ RSLC 120 RSLC 120 C_{18} column (2.2 µm, 120 Å, 2.1 · 100 mm) was used for the recording of LC-MS spectra. A semipreparative Gilson RP-HPLC instrument with a UV detector and a stationary phase of C_{18}-modified silica (XTetra RP 18 column, 10 µm, 19 · 150 mm) was used for the purification of peptides with the mobile phase consisting of buffer A (0.1 % trifluoroacetic acid in Milli-Q water) and B (0.1 % trifluoroacetic acid in 9:1 MeCN/Milli-Q water).

HPLC analysis was performed on an Agilent 1100 HPLC using a 4.6 x 100 mm XBridge C18 column. A linear gradient of acetonitrile in water with 0.1% TFA was used, running from 0% to 90% acetonitrile, 1 mL/min over 10 min. The detection was performed by measurement of absorbance of UV-light light at 215, 230, and 254 nm.

Preparative HPLC purification was performed with a Gilson liquid handler employing a 10 µm 19 x 150 mm XTerra Prep RP18 column. A custom gradient of ACN/H_2O with 0.1% TFA and the flow rate 15 ml/min were applied. The gradients varied in the range of 0% to 90% ACN with runtime 30 min. Detection was performed by Gilson 170 Diode Array measuring absorbance of UV light at 220 and 280 nm.

6.2 Solid phase-peptide synthesis of Phe-Lys-Val-Cys-Phe or FKVCF



(S)-6-amino-N-((S)-1-(((R)-1-(((S)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-3-mercapto-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-2-((S)-2-amino-3-phenylpropanamido)hexanamide

The peptide was synthesized in polyethylene fritted filtration columns connected to Teflon tubes for solvent drainage through suction. **FKVCF** was synthesized on Rink Amide MBHA

resin with loading capacity 0.64 mmol/g. The resin was swelled in DMF (4 mL) for 15 min and stirred manually which was drained through suction after swelling. The first amino acid in the sequence Fmoc-Phe-OH was activated using TBTU (2.9 equiv, 83.43 mg) and DIPEA (4 equiv, 62.45 μL) in 2 mL DMF for 5 min with manual stirring. After swelling the Fmoc terminal amine of the resin is deprotected with 20% piperidine in DMF (2 mL) and stirred for 2 min, then the solvent was drained, and the resin was washed with DMF for 2 min (3x), after washing 20% piperidine (2 mL) was added again and left for 20 min followed by washing with DMF for 2 min (3x). The solvent was drained from the resin and the activated amino acid was added to the resin and left to react for 1 hour with manual stirring. After coupling the mixture was washed with DMF (3x) for 2 min.

The Fmoc protected amino acid was then deprotected with 20% piperidine for 2 min washed with DMF (3x) and then for another 20 min. After deprotection the resin was washed with DMF again for 2 min (3x) the next amino acid (Fmoc-Cys(Trt)-OH) was activated and added to the mixture. This procedure was repeated until the complete sequence of the peptide was obtained and then the resin was washed with DCM (5 mL) for 2 min 3x. At each step the solvent was removed by suction.

Amino acid	FW (g/mol)	Equiv.	Wt (mg)
Fmoc-Phe-OH	387,4	3	104,1
Fmoc-Cys(Trt)-OH	585,7	3	157,4
Fmoc-Val-OH	339,2	3	91,2
Fmoc-Lys(Boc)-OH	468,5	3	125,9
Fmoc-Phe-OH	387,4	3	104,1

FKVCF was cleaved from the resin using 5 mL reagent B (trifluoroacetic acid 88% v/v, phenol 5% v/v, milli-Q water 5% v/v, and triisopropylsilane 2% v/v) which also cleaved the protection groups of the side chains of the amino acids. The mixture was left to stir for 40 min and then filtered to remove the resin. The yellow liquid residue was concentrated *in vacuo* resulting in a brown oil which was dissolved in 4 mL acetonitrile/milli-Q water 80%/20% and purified on RP-HPLC and freeze-dried overnight affording pale yellow crystalline **FKVCF**-trifloroacetic acid salt, yield: 38.3 mg, 56.6%. **HRMS**: $[M+H]^+$ Calculated for $C_{32}H_{46}N_6O_6S$ 643.3278, found 643.3477.

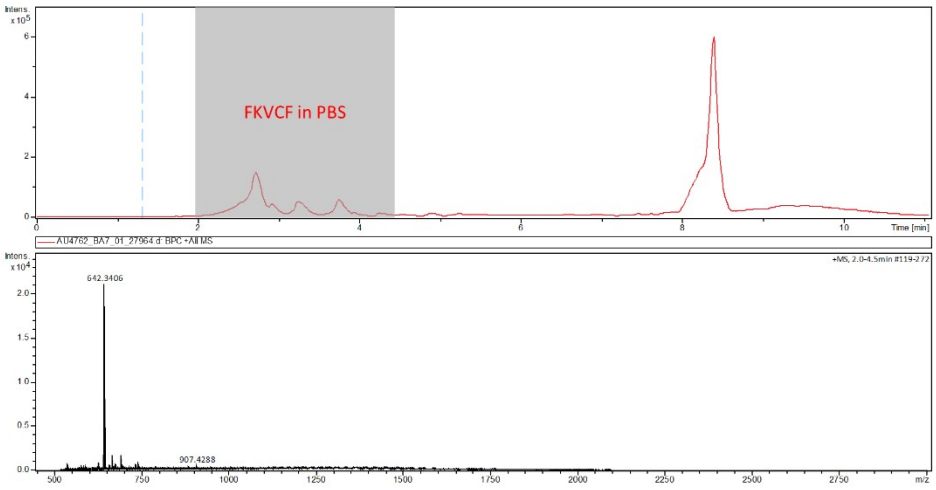


Fig. S8. LC-MS trace.

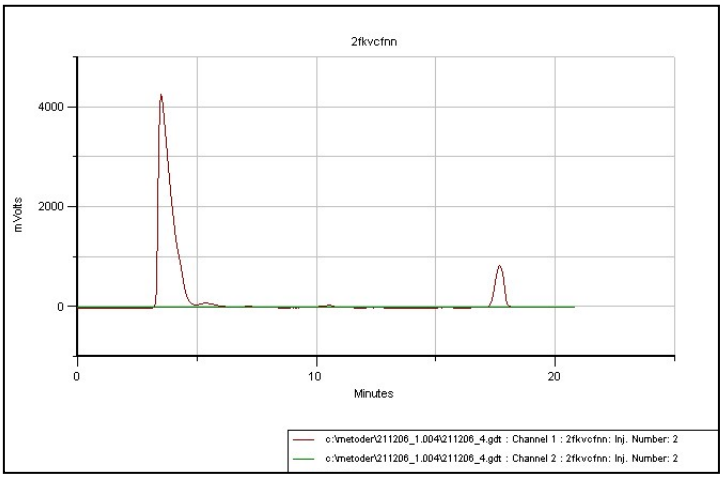


Fig. S9. Preparative HPLC trace.

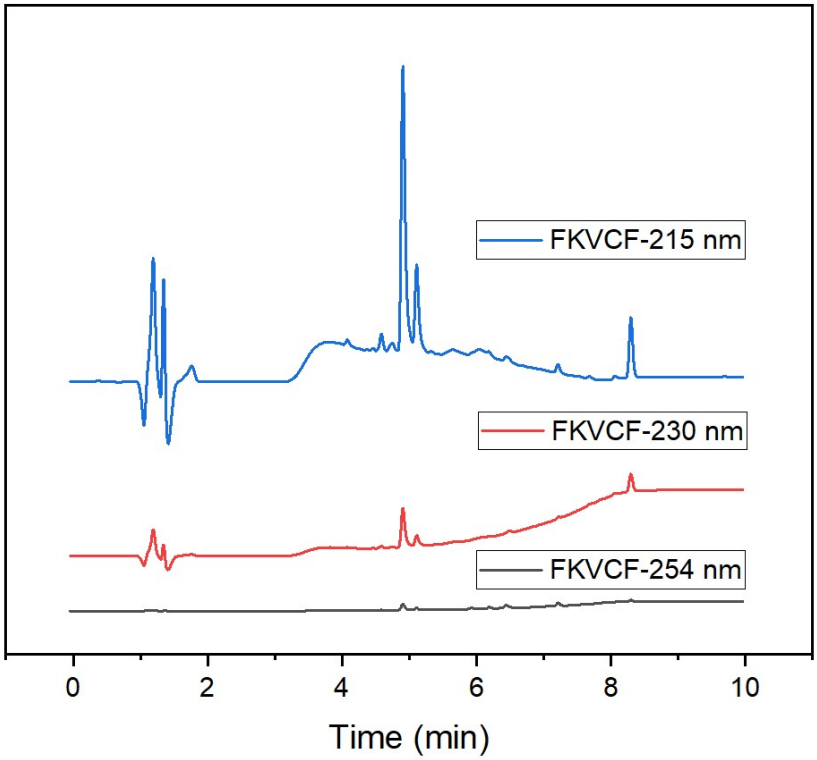


Fig. S10. HPLC analysis of **FKVCF** after purification.

6.4 Conjugation of FKVCF with N-phenylmaleimide

A 16 mM solution of **FKVCF** as trifloroacetate salt in PBS/MeCN (1/4, v/v%) was prepared after purification. The **FKVCF** solution (7 µL, 0.7 nmol, 1 equiv.) was added to a solution of PBS in 20% MeCN (pH 7.8, 79.0 µL) in an LC-MS vial equipped with a 200 µL insert. To the mixture was added a solution of maleimide (**1e**) in MeCN (80 mM, 14 µL, 10 equiv.) to obtain a final volume of 100 µL and a concentration of 1.12 mM **FKVCF**. The vial was shaken at 400 mot/min at 25 °C for 0.5 h under CO_2 or N_2 atmosphere. The reaction mixture was diluted (10 µL of the reaction mixture was dissolved in 90 µL of PBS in 20% MeCN) for LC-MS analysis (3 µL injection, the temperature of the LC-MS room is 21 °C, fast wide positive mode) (Fig. S11, S12). The rest of the reaction mixture was used for HPLC analysis (Fig. S13).

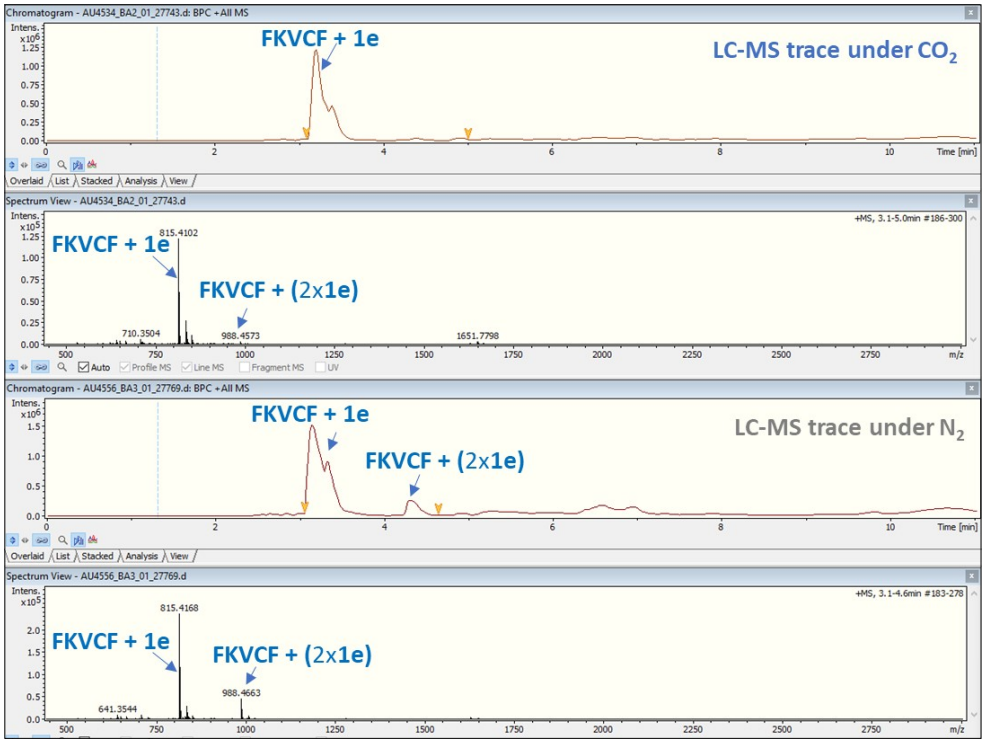


Fig. S11. LC-MS analysis of **FKVCF** conjugation after 30 min (significant amount of double addition product detected under N_2 atmosphere).

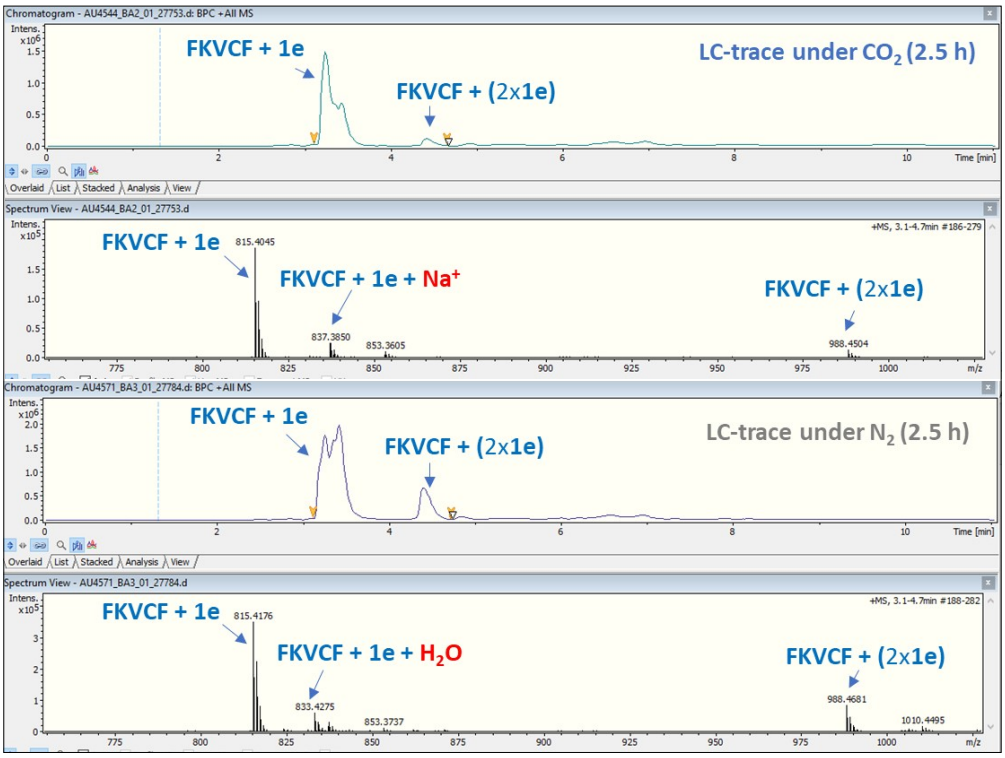


Fig. S12. LC-MS analysis of **FKVCF** conjugation after 2.5 h (hydrolyzed mono-addition product was detected under N_2 atmosphere, which was suppressed by CO_2, see also the HPLC analysis Fig. S13).

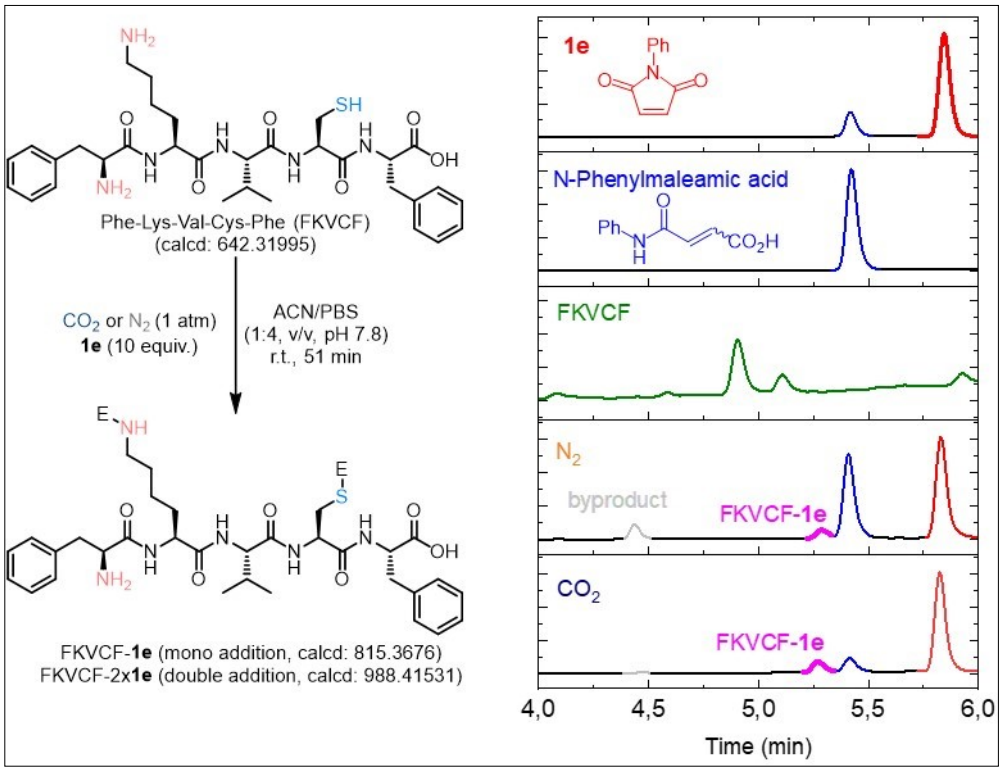


Fig. S13. HPLC traces of reaction mixture at 51 min.

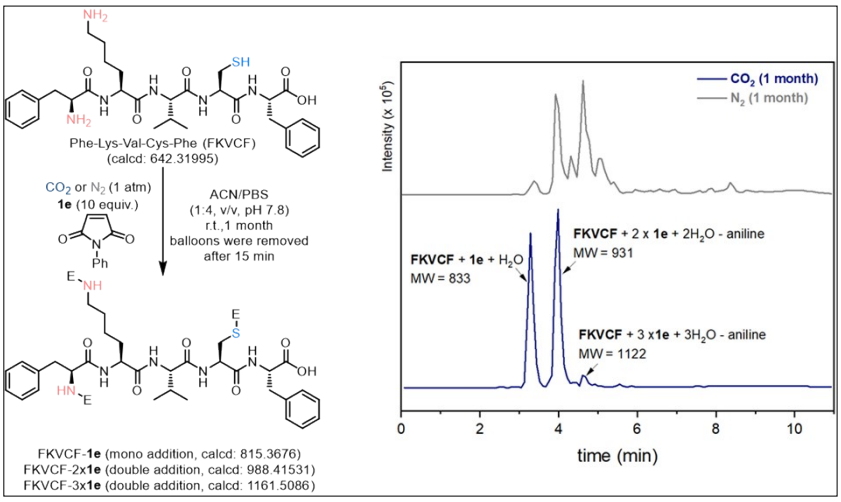


Fig. S14. **FKVCF** conjugate addition reaction using maleimide **1e** and LC traces of reaction mixture of after 1 month.

Note: We measured LC-MS for **FKVCF** modification reaction mixtures under CO_2 and N_2 atmospheres. After 1 month, the stored samples showed difference in terms of product stability: the reaction under CO_2 conditions showed two major peaks related to hydrolysis whereas the reaction under N_2 showed unidentifiable peaks as shown in the LC traces.

6.4 Hydrolysis of N-phenylmaleimide

We observed significant amount of impurity when checking HPLC by dissolving pure N-phenylmaleimide in PBS/MeCN (4/1, v/v), which we postulated to be the hydrolyzed product of *N*-phenylmaleimide. So, we performed the hydrolysis experiment with *N*-phenylmaleimide. *N*-phenylmaleimide (10 mg) was treated with PBS/MeCN (0.5 mL, 4/1, v/v) at 38 °C for overnight. The hydrolyzed product was isolated by preparational thin layer chromatography. Then the sample was extracted from silica gel with 0.7 mL of DMSO-d_6 as a salt. ^{1}H and ^{13}C NMR spectra were recorded after filtration of the reaction mixture with a syringe filter. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 15.37 (s, 1 H), 7.58 (d, J = 7.9 Hz, 2H), 7.27 (t, J = 7.4 Hz, 2H), 6.99 (t, J = 7.5 Hz, 1H), 6.13 (d, J = 13.4 Hz, 1H), 5.66 (d, J = 13.4 Hz, 1H), 5.32 (s, 1H). **^{13}C NMR** (126 MHz, DMSO-d_6) δ 168.1, 164.0, 140.3, 140.1, 129.6, 128.7, 122.6, 118.9. **HRMS**: $[M+Na]^+$ Calculated for $C_{10}H_9NO_3Na$ 214.0480, found 214.0475.

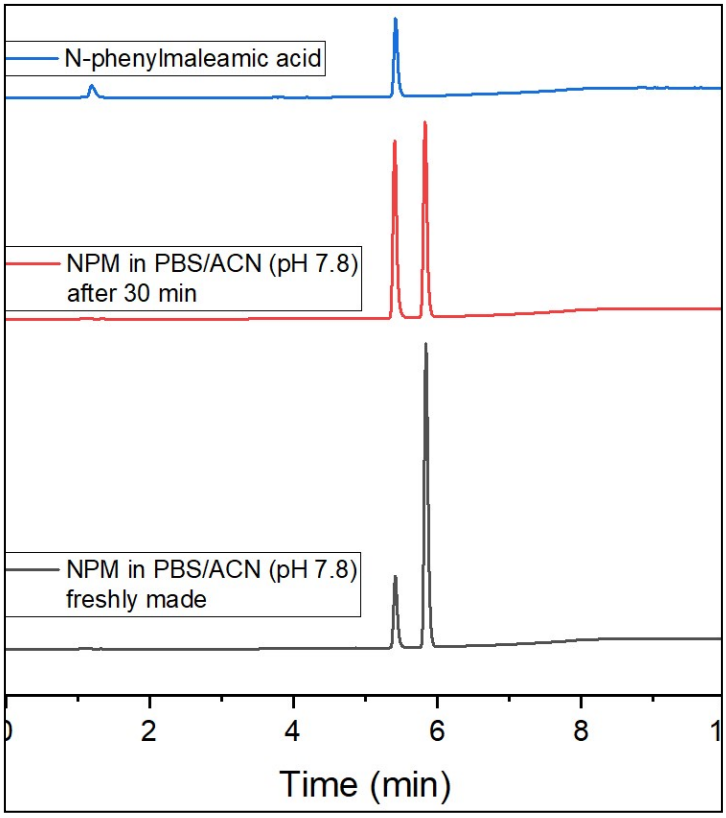


Fig. S15. HPLC traces of hydrolysis experiments with N-phenylmaleimide.

Note: NPM was hydrolyzed as a freshly prepared sample in PBS/ACN (4/1, v/v, pH 7.8).

6.5 LC-MS/MS analysis after conjugation reaction with N-phenylmaleimide (1e)

After the conjugation reaction of **FKVCF** with *N*-phenylmaleimide (10 equiv.) under a CO_2 atmosphere for 1 h, we separated the mono-addition product by preparative HPLC, then subjected purified mono-addition containing fraction for LC-MS/MS analysis. We analyzed the fragments detected from LC-MS/MS. We found fragments indicating Cys-conjugation product under CO_2 (Fig. S15), supporting the site-selective in modification of pentapeptide **FKVCF** on Cys residue.

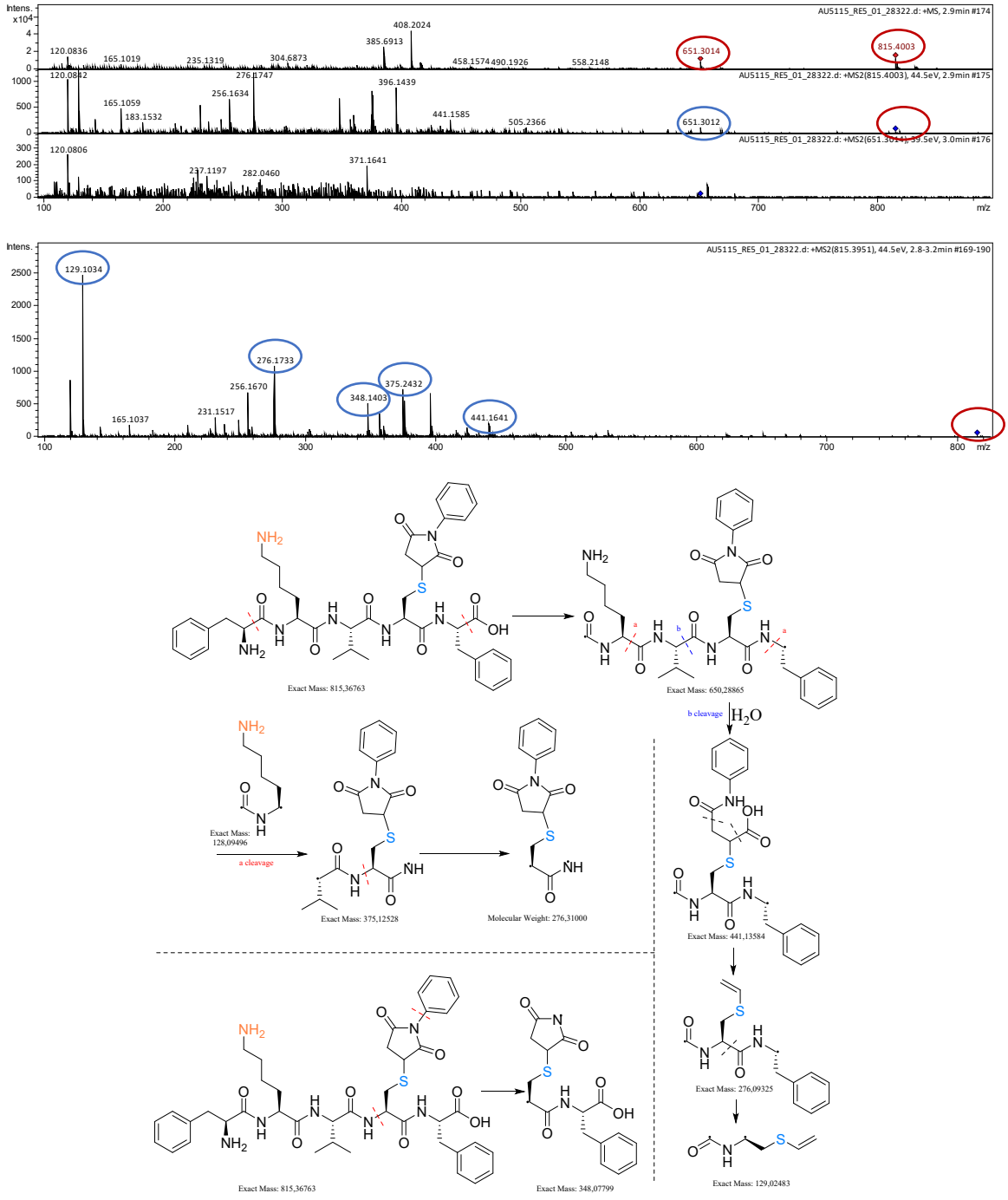
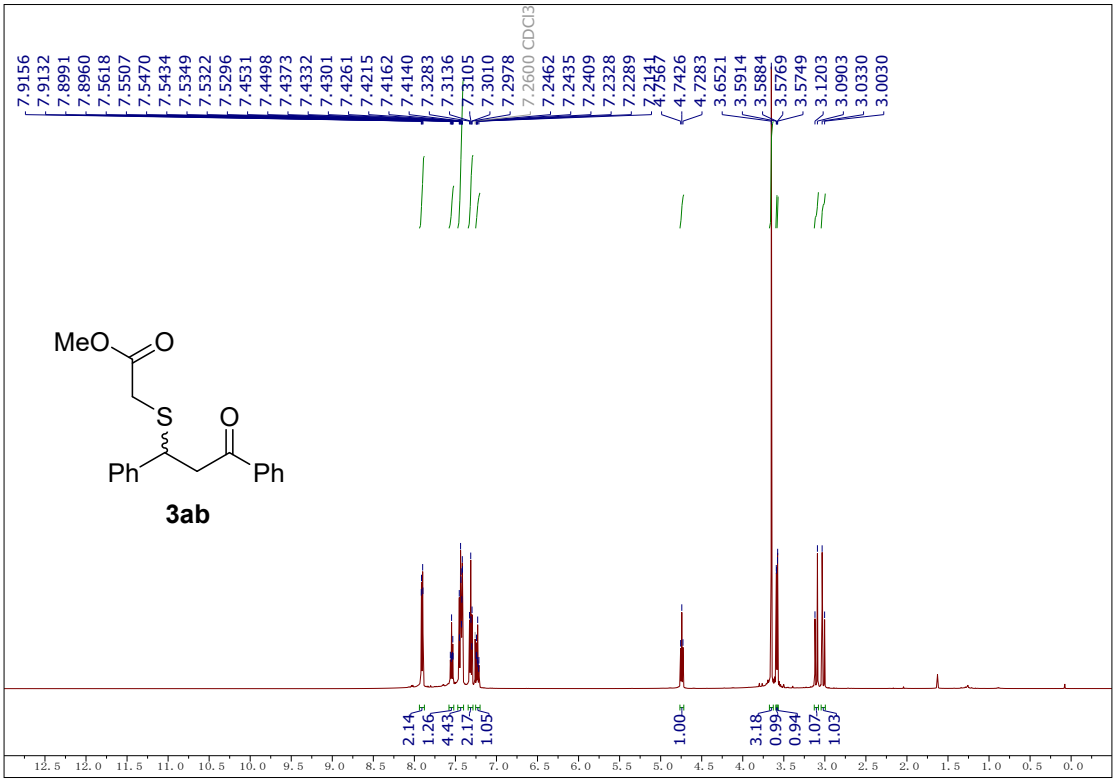
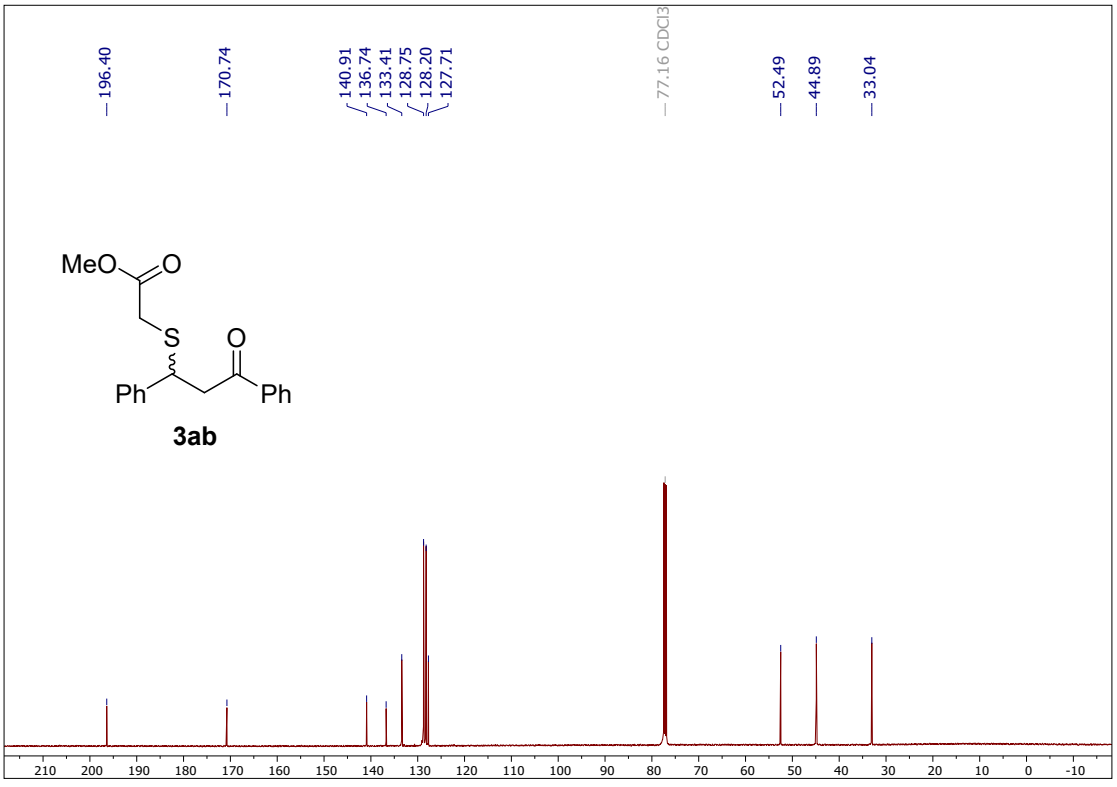
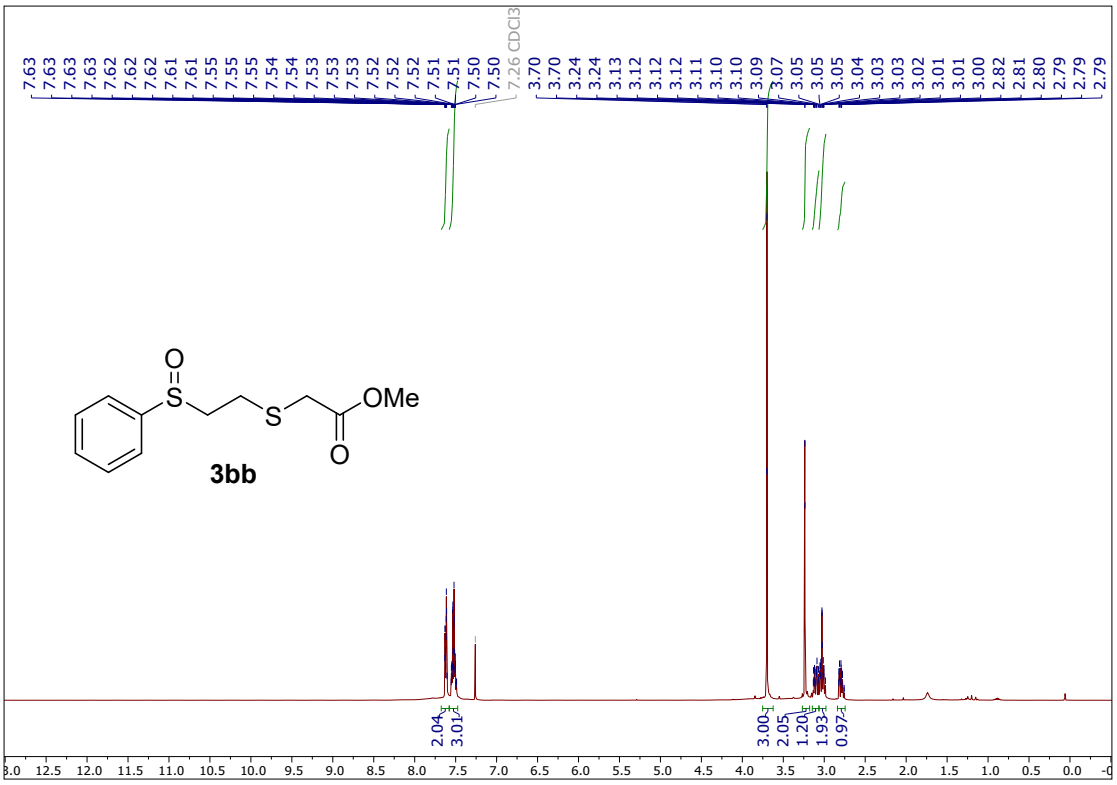


Fig. S16. Fragments detected by LC-MS/MS.

7. ^{1}H and ^{13}C NMR Spectra



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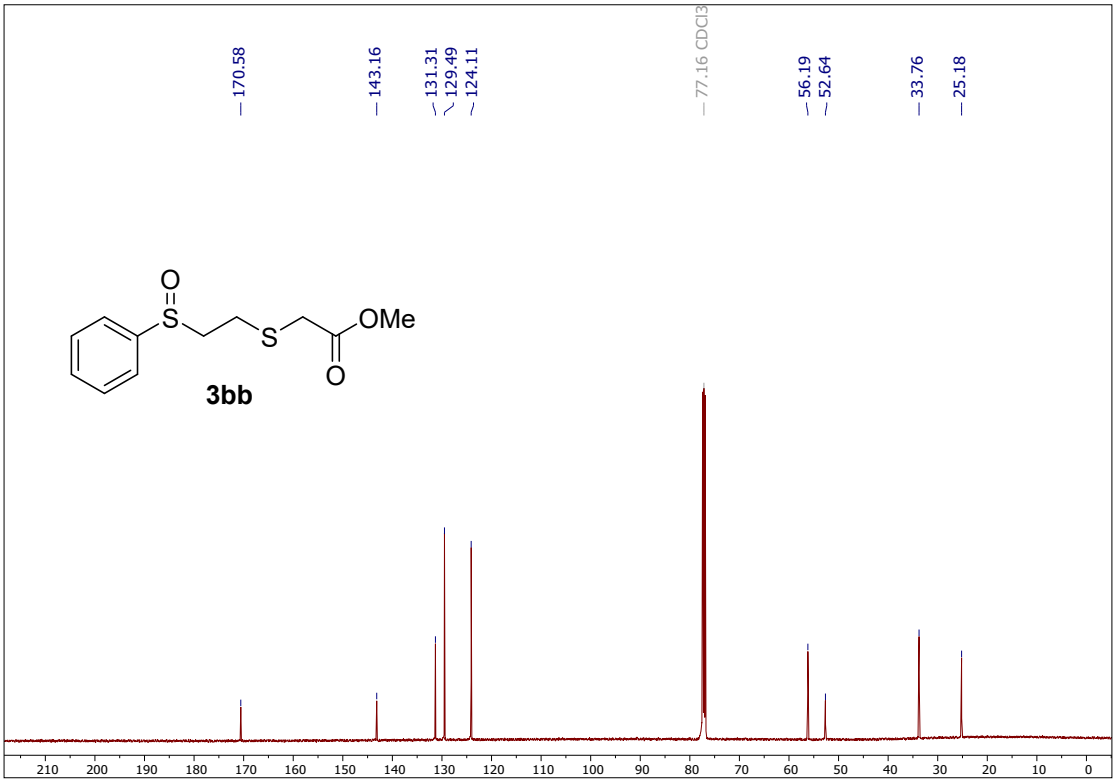
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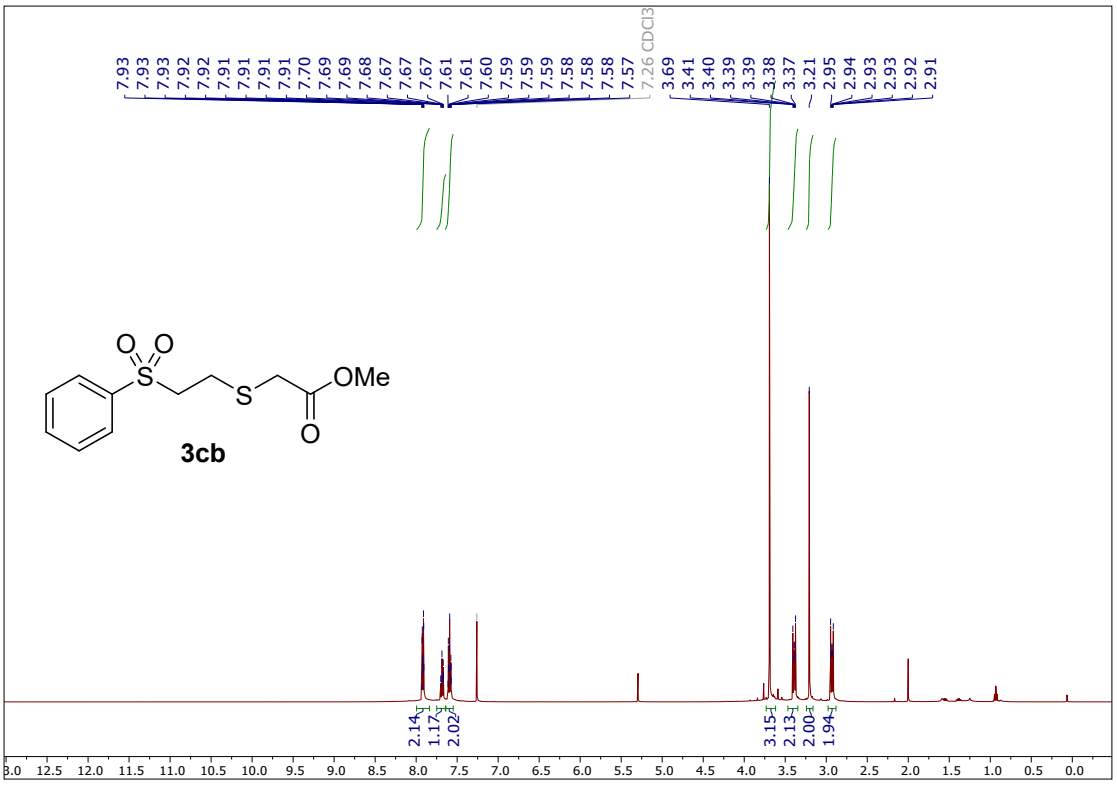
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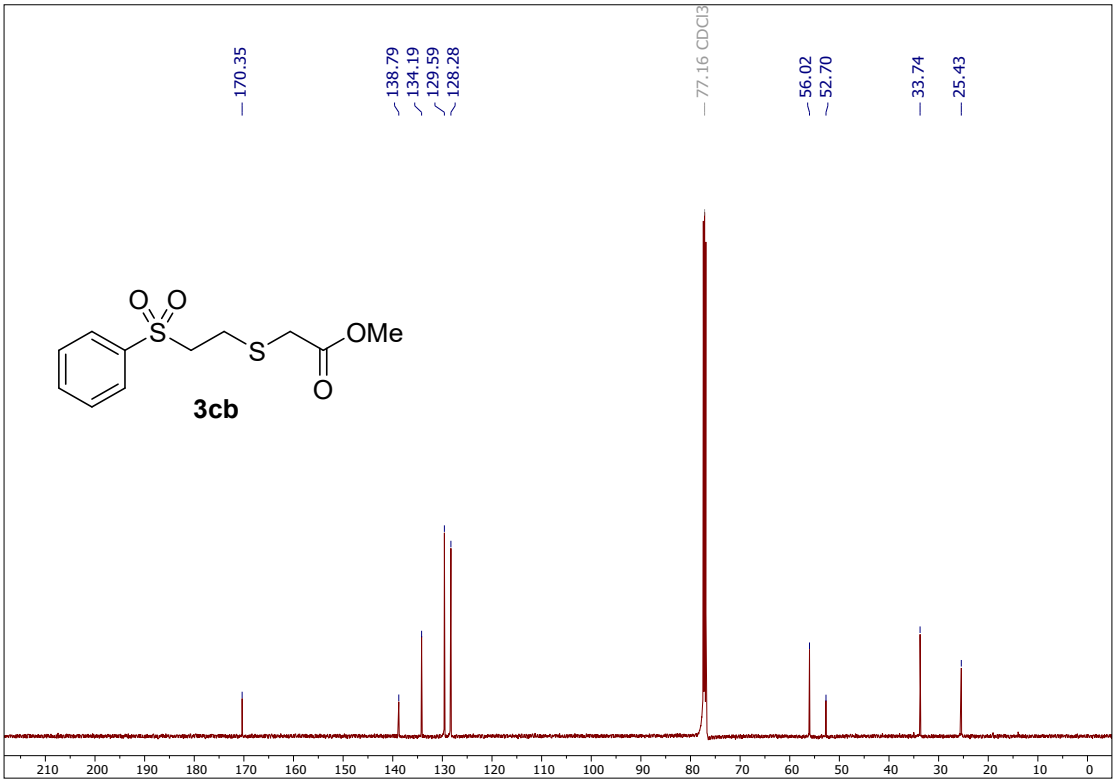
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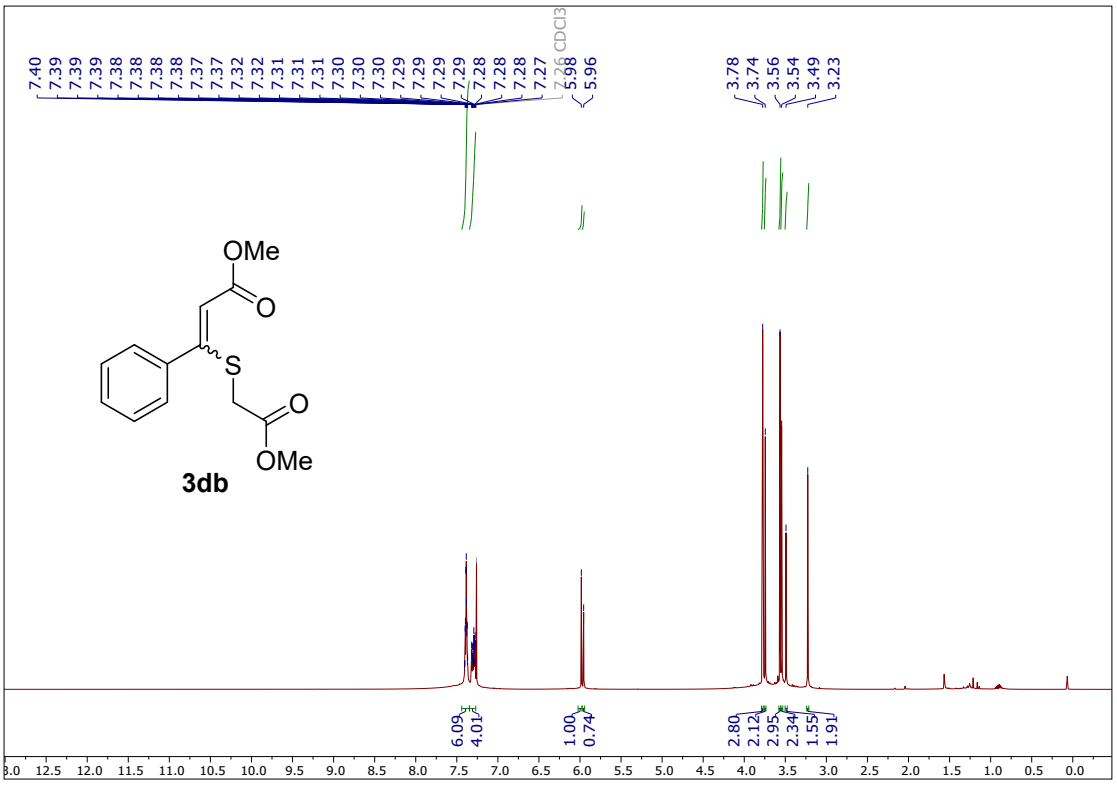
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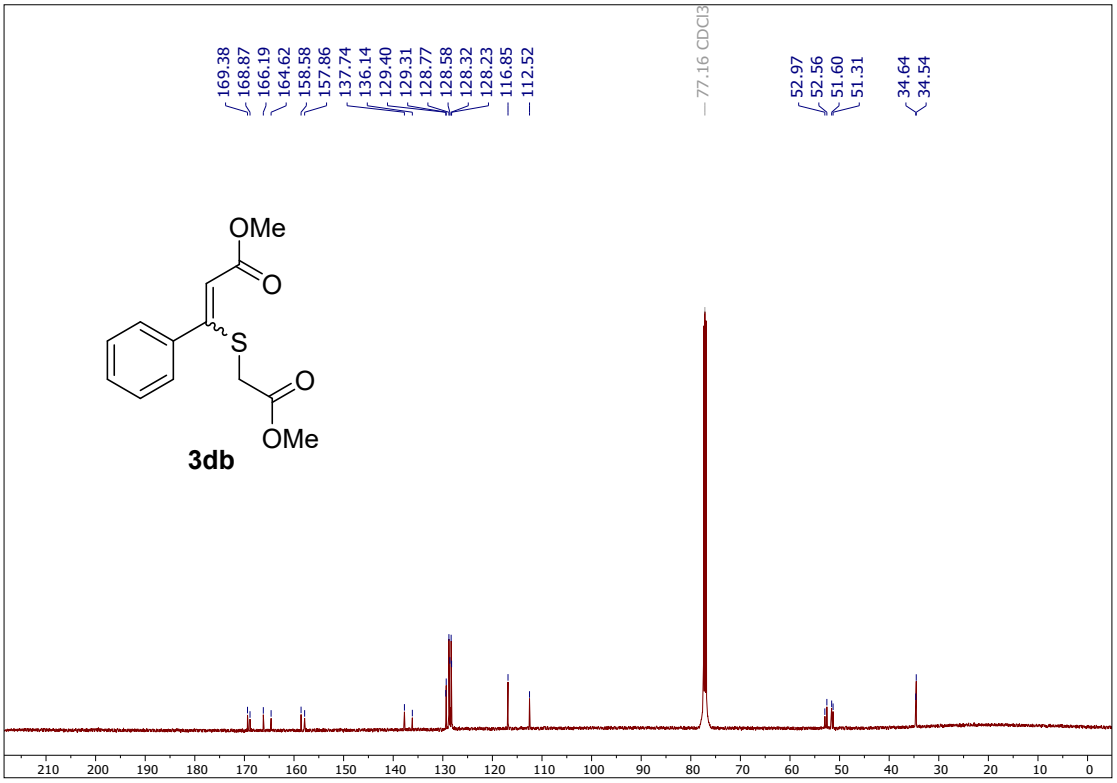
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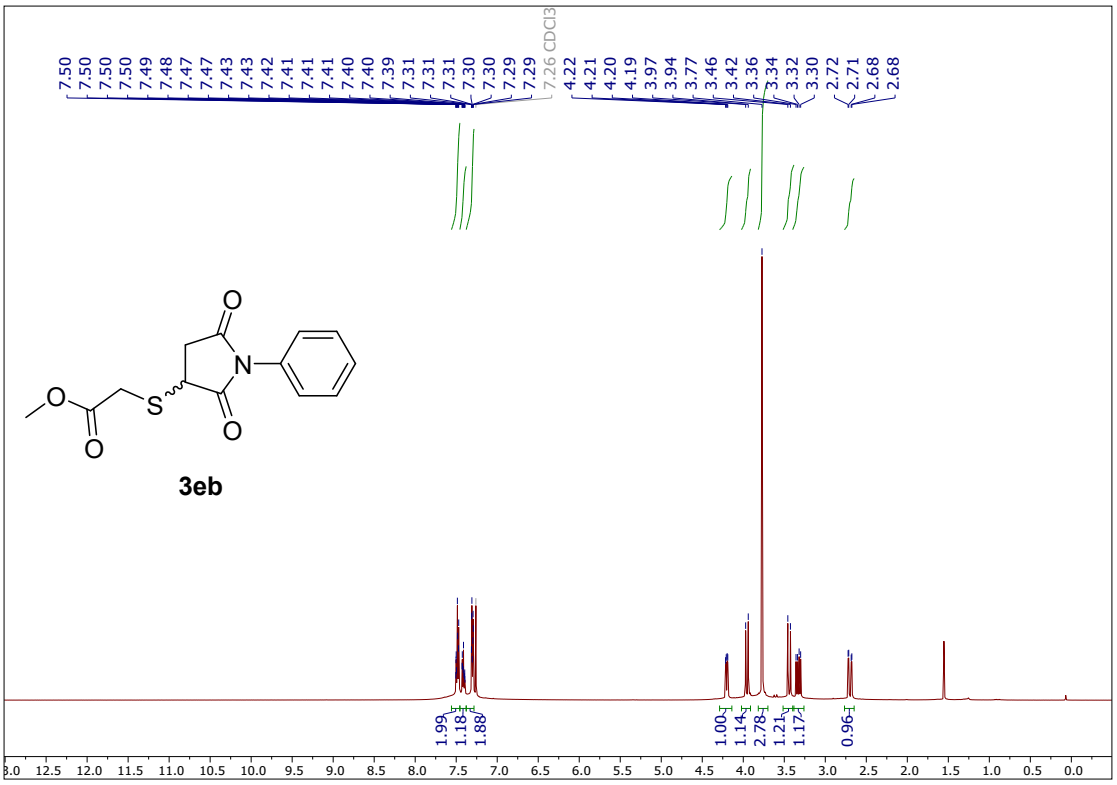
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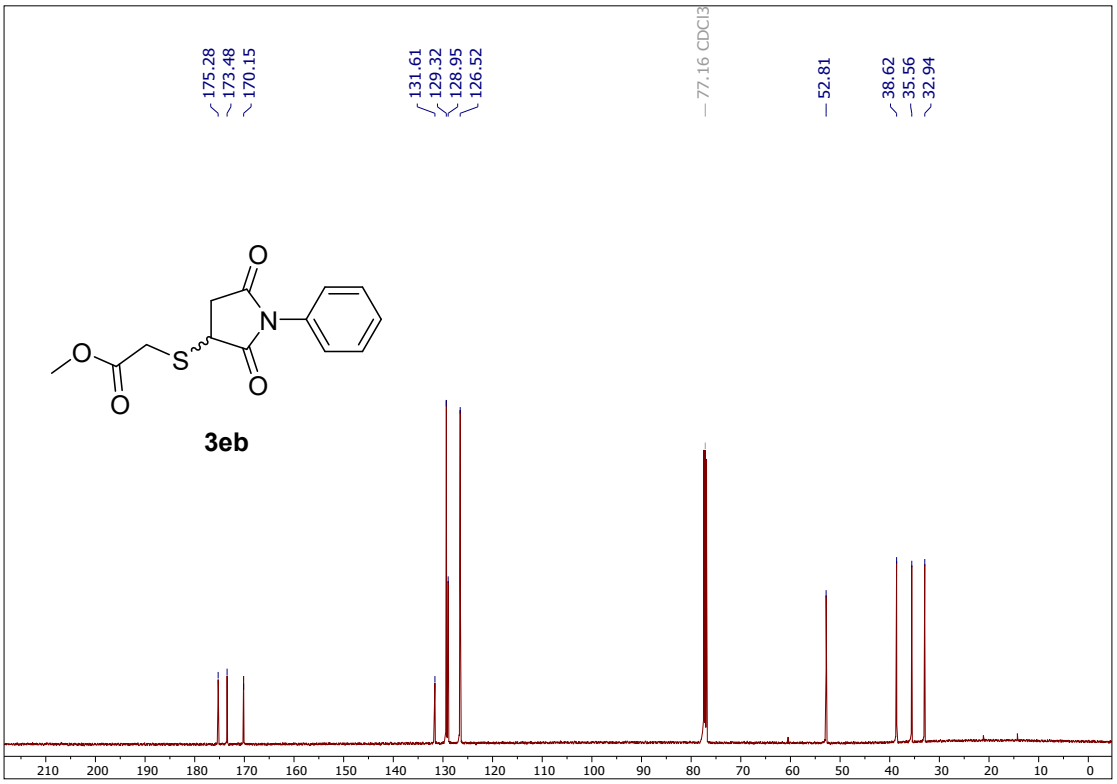
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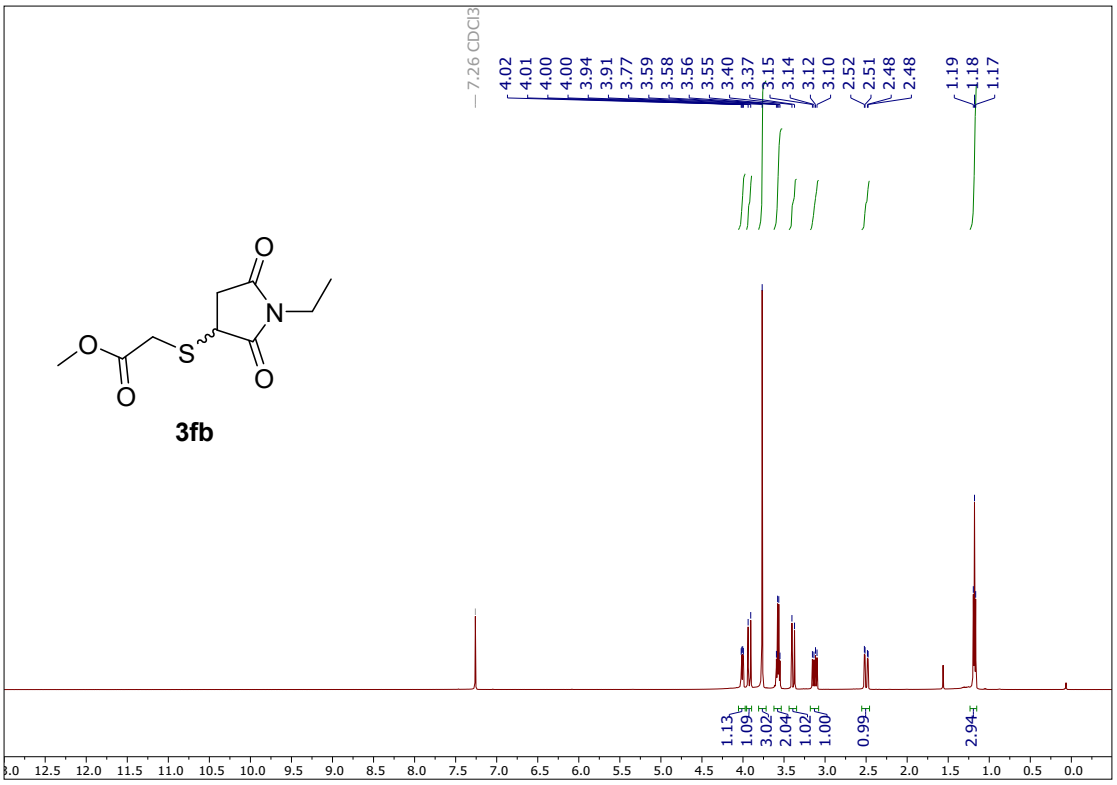
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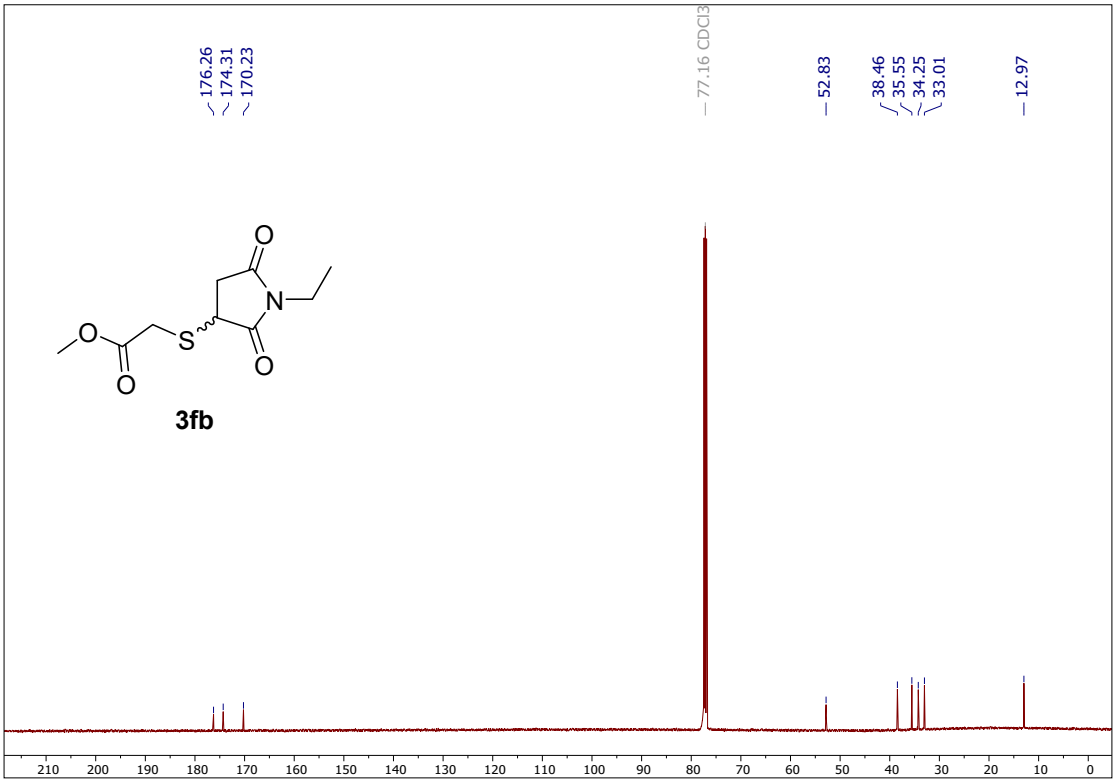
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