# Late-Stage Peptide Modification with Salicylaldehyde Tag Enhances Affinity for Nuclear Factor-kappa B Essential Modulator

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# Supplementary Information

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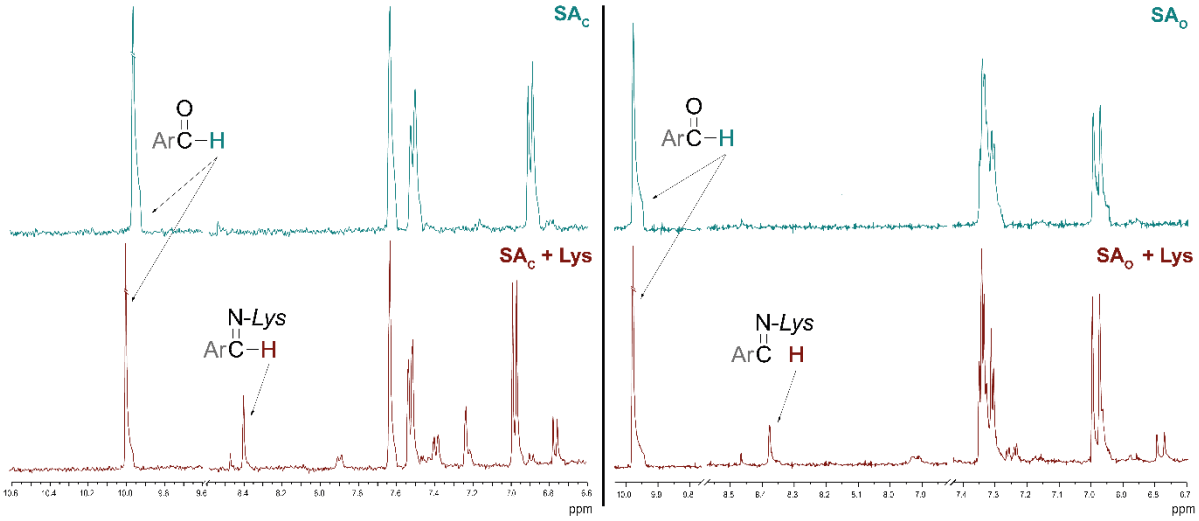
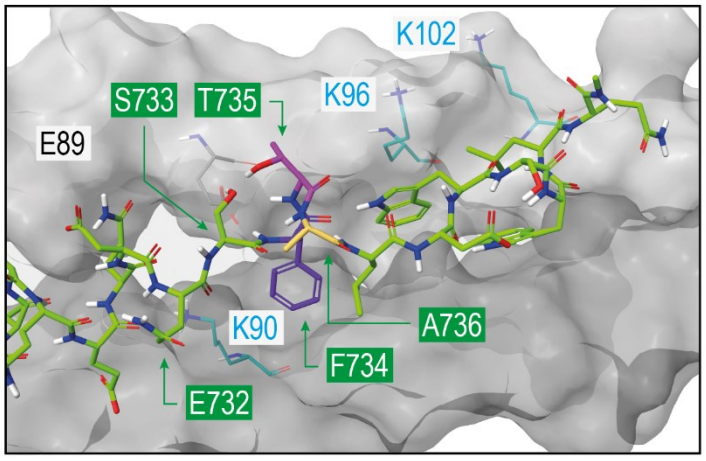



**Figure S1**. $^1$H NMR spectra of the reaction products of **SA$_C$** and **SA$_O$** (2 mM) with 20 mM of $N_\alpha$-acetyl-L-lysine (Ac-Lys-OH), recorded at room temperature in phosphate buffer (pH 7.4, $D_2O$). In both cases a stable imine product is observed, characterized by a distinctive signal at ≈8.4 ppm.




**IKKβ $C_\alpha$ - NEMO Lys(ε-$NH_3^+$) distances** (Å)

| | K90 | K96 | K102 |
|---|---|---|---|
| F734 | 07.09 | 09.85 | 18.98 |
| T735 | 10.07 | 07.99 | 18.32 |
| A736 | 10.19 | 11.04 | 21.45 |

**Relevant intermolecular H-bonds:**
E732(C=O backbone)-K90($NH_3^+$) = 2.10 Å
S733(OH)-E89($COO^-$) = 1.68 Å
F734(NH)-E89($COO^-$) = 1.80 Å

**Figure S2**. Crystal structure of the NEMO-IKKβ complex (PDB: 3BRV) originally-edited with Schrödinger Maestro graphical interface (Schrödinger Release 2025-3). The measured distances (Å) between the $C_\alpha$ atom of IKKβ residues F734, T735, A736 and the Lys(ε-$NH_3^+$) group are shown in the table. Relevant H-bonds detected in the PDB are also indicated, which results in the short distances listed.

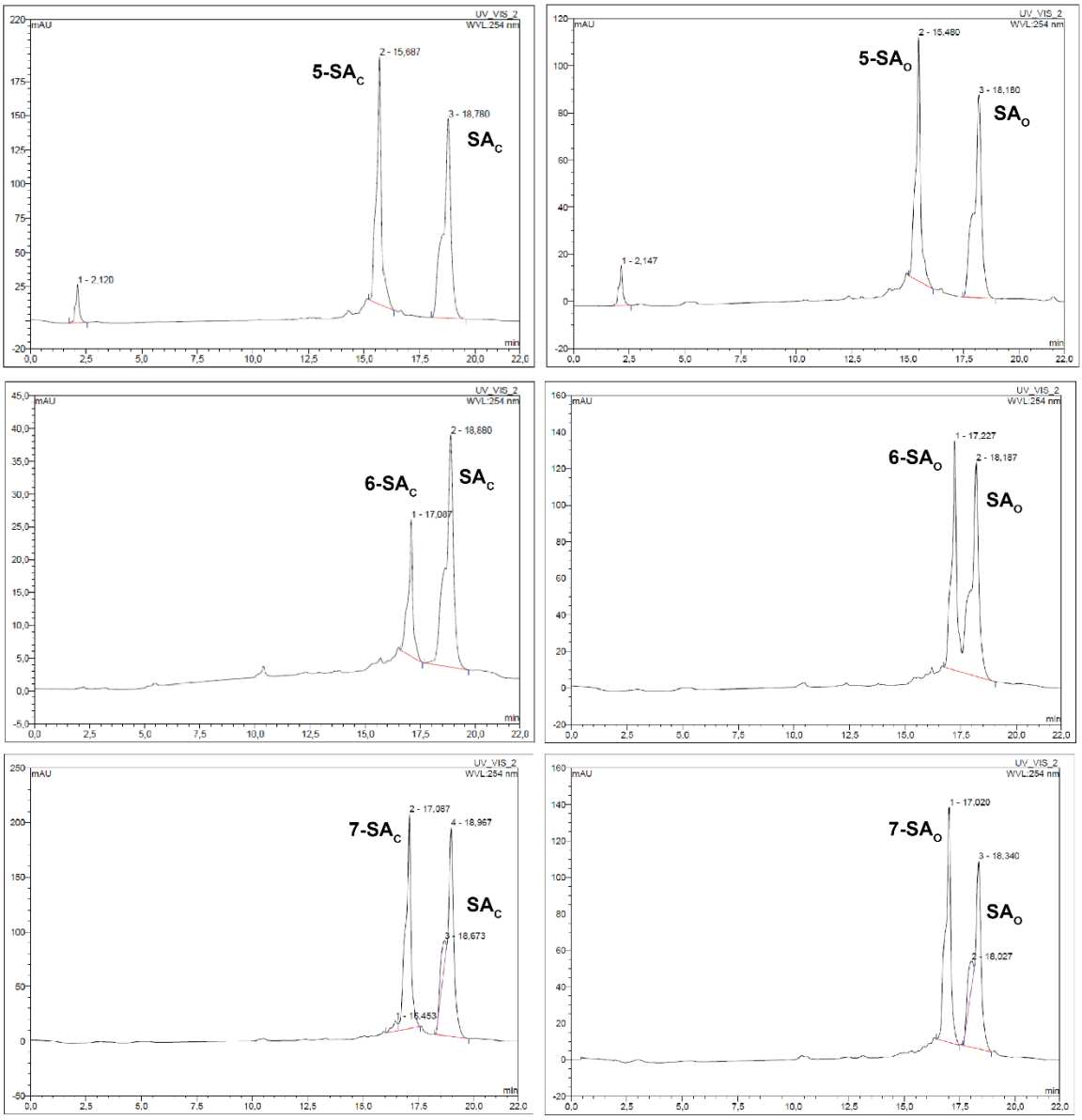



**Figure S3**. Semi-preparative HPLC traces of crude mixtures upon CuAAC reaction of aldehyde-azide units **SA$_C$** and **SA$_O$** with Pra-equipped peptides **5-7**. In all chromatograms, the UV peaks relative to the triazole products and the azide excess are visible, indicating full conversion of the starting alkyne peptide (see also the LC-MS analyses relative to the isolated product peaks, on page S21).

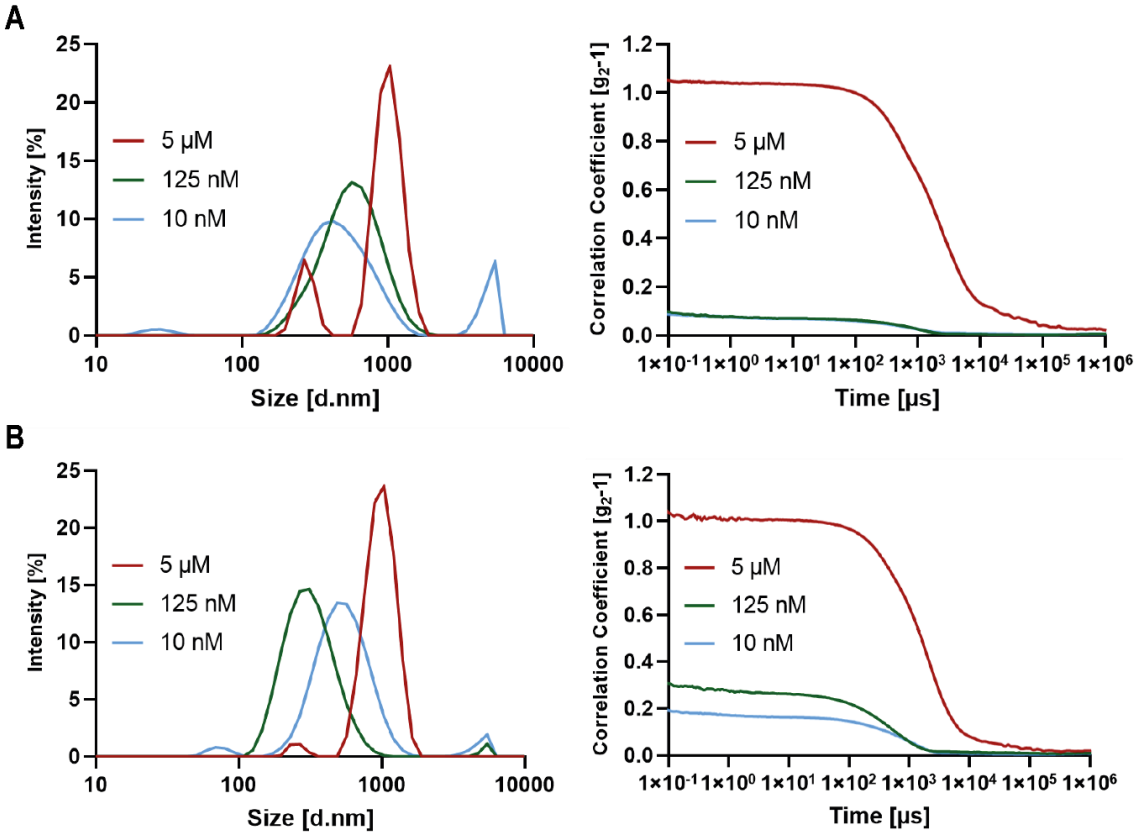



**Figure S4**. Dynamic Light Scattering (DLS) analysis of sample aggregation at different concentrations. A (**6**) and B (**6-SA$_C$**) correspond to two different samples, and each curve represents the average of triplicate measurements. The left panels show the intensity distribution (%) as a function of particle hydrodynamic size (d.nm), while the right panels report the correlogram (correlation coefficient *vs.* time). At 5 µM (red curves), the intensity distribution displays a sharp peak at large diameters and the correlogram shows a slow decay, both indicating the formation of large aggregates in solution. In contrast, at 125 nM (green curves) and 10 nM (light blue curves), the correlograms rapidly decay and the size distributions are shifted toward smaller species, consistent with the absence of significant aggregation.

## Materials and Methods

All manipulations requiring anhydrous conditions were carried out in flame-dried glassware, with magnetic stirring and under a nitrogen atmosphere. All commercially available reagents were used as received. Anhydrous solvents were purchased from commercial sources and withdrawn from the container by syringe, under a slight positive pressure of nitrogen. The reactions were monitored by analytical thin-layer chromatography (TLC) using silica gel 60 F254 pre-coated glass plates (0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molybdate solution, 2,4-dinitrophenylhydrazine, concentrated $H_2SO_4$ or ninhydrin. Flash column chromatography was performed using Chromagel 60 ACC (40-63 μm) silica gel. Proton chemical shifts are reported in ppm ($\delta$) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard ($CDCl_3$ $\delta$ = 7.26 ppm; $CD_2Cl_2$, $\delta$ = 5.32 ppm; $d_6$-DMSO, $\delta$ = 2.50 ppm; $CD_3OD$, $\delta$ = 3.33 ppm, $d_8$-THF $\delta$ = 3.58 ppm, 1.73 ppm). The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal, dd = doublet of doublet. Carbon NMR spectra were recorded on a spectrometer operating at 100.63 MHz, with complete proton decoupling. Carbon chemical shifts are reported in ppm ($\delta$) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, $\delta$ = 77.16 ppm; $CD_2Cl_2$, $\delta$ = 54.00 ppm; $d_6$-DMSO, $\delta$ = 39.51 ppm; $CD_3OD$, $\delta$ = 49.05 ppm; $d_8$-THF $\delta$ = 67.57 ppm, 25.37 ppm). HPLC purifications were performed on Dionex Ultimate 3000 equipped with Dionex RS Variable Wavelenght Detector (column: Atlantis Prep T3 OBDTM 5 μm 19 x 100 mm; flow 10 ml/min unless stated otherwise). LC-MS were recorded/acquired on Ultimate 3000 Thermo Scientific coupled with Fisons MD800 spectrometer and electrospray ion trap on a Finnigan LCQ advantage thermo-spectrometer and using a Phenomenex Luna - 5 μm - 4.6 x 150 mm column. High-resolution mass spectrometry analysis (HRMS, 4 decimal places) were performed on a Q-TOF Synapt G2-Si instrument available at the MS facility of the Unitech COSPECT at the University of Milan. Low resolution mass spectra (MS, 1 and 2 decimal places) were recorded on a Thermo Scientific LCQ Fleet Ion Trap Mass Spectrometer (ESI source). Confocal microscopy analysis was performed using Leica STELLARIS 8 Confocal Microscopy, equipped with 20x magnification objective and using LasX software (Leica). Dynamic light scattering analysis (DLS) was performed on a Malvern Zeta sizer Nano instrument (Malvern Panalytical, Ltd.) at 25 °C equipped with a 633 nm solid-state He−Ne laser at a scattering angle of 173°. 2-azidoethyl 4-methylbenzenesulfonate[1] was prepared following a published procedure.

[1] H. Choi, H. J. Shirley, P. A. Hume, M. A. Brimble, D. P. Furkert, *Angew. Chem. Int. Ed.* 2017, **56**, 7420-7424.

## List of Abbreviations and Symbols

| | | | |
|---|---|---|---|
| AcOEt | Ethyl Acetate | MeCN | Acetonitrile |
| aq. | Aqueous solution | MeOH | Methanol |
| $D_2O$ | Deuterium oxyde | min. | Minutes |
| DLS | Dynamic Light Scattering | MS | Mass Spectroscopy |
| DMEM | Dulbecco's Modified Eagle Medium | MW | Molecular weight |
| DMF | Dimethylformamide | NMR | Nuclear Magnetic Resonance |
| DMSO | Dimethylsulfoxide | o.n. | Overnight |
| EDTA | Ethylenediaminetetraacetic acid | PBS | Phosphate Buffer Saline |
| equiv. | Equivalents | PI | Polydispersity index |
| ESI | Electrospray ionization | ppm | Part per million |
| $Et_2O$ | Diethyl ether | r.t. | Room temperature |
| FA | Fluorescence Anisotropy | $R_f$ | Retention factor |
| FITC | Fluorescein isothiocyanate | SA | salicylaldehyde |
| Fmoc | Fluorenylmethyloxycarbonyl | *t*Bu | *tert*-Butyl |
| h | Hours | TEA | Triethylamine |
| Hex | *n*-Hexane | TFA | Trifluoroacetic acid |
| HPLC | High performance liquid chromatography | THF | Tetrahydrofuran |
| HRMS | High resolution mass spectroscopy | TIS | Triisopropylsilane |
| *J* | Scalar coupling constants | $t_R$ | Retention time |
| LC-MS | Liquid Chromatography-Mass Spectrometry | $\delta$ | Chemical shift |

## List of Amino acids

| AMINO ACID | ONE-LETTER CODE | THREE-LETTER CODE |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |
| Aspartic Acid | D | Asp |
| Cysteine | C | Cys |
| Glutamine | Q | Gln |
| Glutamic Acid | E | Glu |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

| | | |
|---|---|---|
| β-Alanine | β-A | β-Ala |
| Propargylglycine | | Pra |

# Synthetic procedures

## *General procedures*

**General procedure A for microwave-assisted SPPS:**
Microwave-assisted automated peptide synthesis was performed using the Fmoc/*t*Bu protection group strategy on a Rink amide resin (loading capacity = 0.55 mmol/g) with a Liberty Blue synthesizer using 0.1 mmol scale. The amino acids concentration was 0.2 M in DMF. DIC and Oxyma were used as coupling reagents (respectively 0.5 and 1 M in DMF) while a mixture of 20% piperidine in DMF and 0.1 M Oxyma was used for the deprotection. Couplings were performed at 75 °C using 170 W for 15 s and then at 90 °C using 40 W for 110 s. Deprotection was performed at 75 °C using 155 W for 15 s and then at 90 °C using 50 W for 50s.

A mini-cleavage of each peptide from the resin was performed: 10 mg of resin was suspended with 100 µL of cleavage cocktail (TFA, thioanisole and DODT in 90:7:3 ratio respectively). The supernatant was diluted with cold $Et_2O$, which favored the peptide precipitation (5 min, 15000 rpm). 2x1 mL washes with cold $Et_2O$ were performed; the pellet was dissolved in 1:1 $H_2O$:MeCN and analyzed by ESI-MS.

**General procedure B for FITC coupling on resin:**

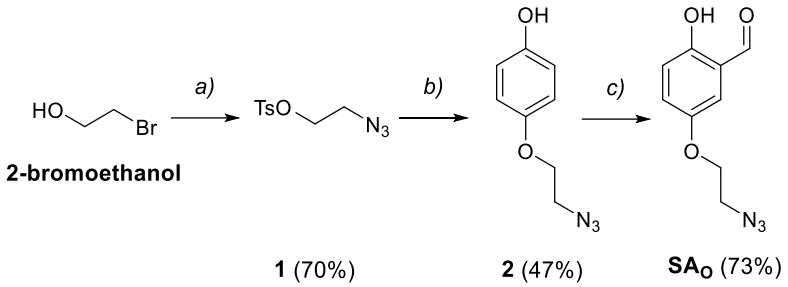
The resin was treated with a 5% *N*,*N*-diisopropylethylamine solution in 5 mL of DMF containing 2 equiv. of FITC and allowed to incubate overnight. The resin was then washed 3xDMF, 3X$CH_2Cl_2$ and 3x$Et_2O$.

**General procedure C for peptide cleavage from resin:**

The cleavage was performed using 4 mL of cleavage cocktail for each peptide (TFA/Phenol/TIS/Thioanisole/$H_2O$; 86:4:4:4:2) for 3 h at room temperature. After the cleavage, the peptides were precipitated from ice-cold $Et_2O$ and recovered by centrifugation at 4 °C. Three $Et_2O$ washes/centrifugation cycles were carried out to efficiently remove the scavengers.

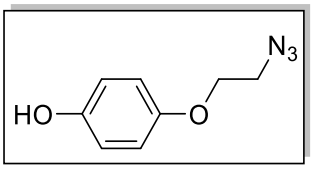
**General procedure D for CuAAC:**
Alkyne-containing peptide (1 equiv.) and azide (2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles. DMF (150 µL) and water (150 µL) were added, followed by $CuSO_4$ (0.5 equiv.) and Na ascorbate (0.6 equiv.) solutions (0.1 M in $H_2O$). The mixture was warmed to 40 °C and stirred overnight. The reaction was quenched by adding 1 equiv. of 500 mM EDTA solution (pH= 8.0). The solvent was removed, the crude product was purified through HPLC and then lyophilized.

***Synthesis of $SA_O$ azide***




**Scheme S1.** Reagents and conditions: a) [1] $NaN_3$, THF, $H_2O$, 0 °C to 50 °C, overnight; [2] TsCl, $Et_3N$, $CH_2Cl_2$, 0 °C to r.t., overnight; b) hydroquinone, $K_2CO_3$, $Bu_4NI$, DMF, 80 °C, overnight; c) paraformaldehyde, $MgCl_2$, $Et_3N$, THF, 70 °C, 3 h.

*Compound **2***

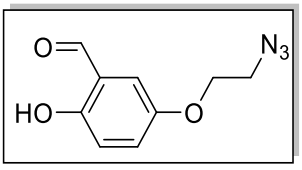



$C_8H_9N_3O_2$
MW: 179.18 g • $mol^{-1}$

To a solution of hydroquinone (236 mg, 2.14 mmol, 2 equiv.) in dry DMF (1.8 mL, 1.2 M), $K_2CO_3$ (163 mg, 1.18 mmol, 1.1 equiv.) and $Bu_4NI$ (41 mg, 0.11 mmol, 0.1 equiv.) were added under nitrogen atmosphere and the resulting suspension was stirred at r.t. for 10 min. A solution of **1** (257.8 mg, 1.07 mmol, 1 equiv.) in dry DMF (3.6 mL, 0.3 M) was added to the stirring suspension (final tosylate concentration: 0.2 M). The reaction mixture was stirred at 80 °C overnight. The solvent was removed in vacuo. 20 mL of AcOEt were added and washed with 1 M aq. solution of $KHSO_4$ (2x20 mL) and brine (1x20 mL), dried with anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (85:15 to 8:2 Hex/AcOEt). Compound **2** was obtained as a yellow oil (90 mg, 47%).

$R_f$ = 0.19 (8:2 Hex/AcOEt); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.85-6.80 (m, 2H), 6.79-6.74 (m, 2H), 4.55 (s, 1H), 4.10 (t, $J$ = 5.0 Hz, 2H), 3.56 (t, $J$ = 5.0 Hz, 2H) ppm; $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 152.30, 150.08, 116.26, 116.04, 67.81, 50.25 ppm. MS (ESI): *m/z* calcd. for $[C_8H_9N_3O_2]^-$: 178.06 [*M*-H]$^-$; found: 178.35.

***$SA_O$***




$C_9H_9N_3O_3$
MW: 207.19 g • $mol^{-1}$

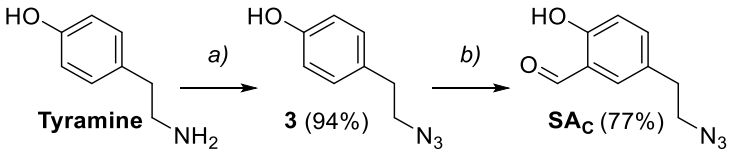
To a solution of paraformaldehyde (84 mg, 2.80 mmol, 5 equiv.) in dry THF (0.9 mL, 3 M) in a flame-dried Schlenk tube, anhydrous $MgCl_2$ (160 mg, 1.68 mmol, 3 equiv.) and dry $Et_3N$ (234 µL, 1.68 mmol, 3 equiv.) were added under nitrogen atmosphere. The resulting suspension was stirred at r.t. for 10 min. A solution of **2** (100 mg, 0.56 mmol, 1 equiv.) in dry THF (1.9 mL, 0.3 M) was added to the stirring suspension (final phenol concentration: 0.2 M). The reaction

mixture was stirred at 70 °C for 3 h. The solvent was removed in vacuo. 20 mL of HCl 1 M were added and the aqueous phase was extracted with AcOEt (2x20 mL). The combined organic phase was dried with anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The crude product was purified through flash chromatography (8:2 Hex/AcOEt). Compound **$SA_O$** was obtained as a pale-yellow oil (85 mg, 73%).

$R_f$ = 0.29 (7:3 Hex/AcOEt); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 10.67 (s, 1H), 9.86 (s, 1H), 7.19 (dd, *J* = 9.1, 3.1 Hz, 1H), 7.05 (d, *J* = 3.1 Hz, 1H), 6.96 (d, *J* = 9.1 Hz, 1H), 4.15 (t, *J* = 4.9 Hz, 2H), 3.61 (t, *J* = 4.9 Hz, 2H) ppm; $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 196.02, 156.36, 151.29, 125.67, 120.03, 118.77, 116.60, 67.94, 50.07 ppm. MS (ESI): *m/z* calcd. for $[C_9H_9N_3O_3]^-$: 206.06 [*M*-H]$^-$; found: 206.28.

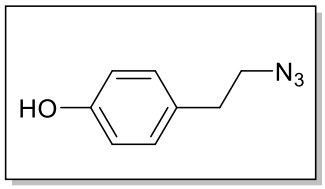
## ***Synthesis of $SA_C$ azide***

**$SA_C$** was prepared adapting a published procedure.[2]




**Scheme S2.** Reagents and conditions: a) Imidazole-1-sulfonylazide·$HSO^{4-}$, $NaHCO_3$, $CuSO_4·5H_2O$, MeOH, r.t., overnight.; b) Paraformaldehyde, $MgCl_2$, $Et_3N$, THF, 70 °C, 4 h.

*Compound **3***




$C_8H_9N_3O$
MW: 163.18 g • mol$^{-1}$

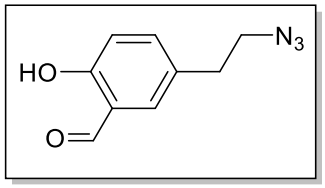
To a stirred solution of tyramine (200 mg, 1.46 mmol, 1 equiv.) and sodium bicarbonate (612 mg, 7.29 mmol, 5 equiv.) in anhydrous MeOH (4 mL) with nitrogen protection, imidazole-1-sulfonyl azide hydrogen sulfate (1.19 g, 4.37 mmol, 3 equiv.) was added into the mixture at room temperature followed by $CuSO_4·5H_2O$ (4 mg, 0.0146 mmol, 0.01 equiv.). The pH was controlled (acid, around 2-3) and adjusted with solid $NaHCO_3$ to pH 7-8. The solution was stirred at room temperature overnight. The mixture was concentrated, diluted with water (100 mL), acidified with 1 N HCl solution and extracted twice with AcOEt. The combined organic layers were washed with brine, dried with anhydrous sodium sulfate and evaporated. The crude product was purified through flash chromatography (25:75 Hex/AcOEt + 0.1% AcOH) affording **3** as a yellow/orange oil (223 mg, 94%).

$R_f$ = 0.27 (3:7 Hex/AcOEt); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.09 (d, *J* = 8.1 Hz, 2H), 6.79 (d, *J* = 8.1 Hz, 2H), 4.80 (s, 1H), 3.46 (t, *J* = 7.2 Hz, 2H), 2.83 (t, *J* = 7.2 Hz, 2H) ppm; $^{13}$C NMR (101

[2] Y. Zhang, Q. Zhang, C. T. T. Wong, X. Li, *J. Am. Chem. Soc.* 2019, **141**, 12274-12279.

MHz, $CDCl_3$) $\delta$ 154.27, 130.17, 129.97, 115.59, 52.64, 34.38 ppm. MS (ESI): *m/z* calcd. for $[C_8H_9N_3O]^-$: 162.07 [*M*-H]$^-$; found: 162.78.

*Compound* ***SA$_C$***




$C_9H_9N_3O_2$
MW: 191.19 g • mol$^{-1}$

To a 3 M solution of paraformaldehyde (205 mg, 6.83 mmol, 5 equiv.) in dry THF (2.3 mL) in a flame-dried Schlenk tube, anhydrous $MgCl_2$ (390 mg, 4.10 mmol, 3 equiv.) and dry $Et_3N$ (571 µL, 4.10 mmol, 3 equiv.) were added under nitrogen atmosphere. The resulting suspension was stirred at r.t. for 10 min. A 0.3 M solution of **3** (223 mg, 1.37 mmol, 1 equiv.) in dry THF (4.6 mL) was added to the stirring suspension (final phenol concentration: 0.2 M). The reaction mixture was stirred at 70 °C for 4 hours. The solvent was removed in vacuo. HCl 1 M was added and the aqueous phase was extracted with AcOEt (2x). The combined organic phase was dried over $Na_2SO_4$, filtered and the solvent was removed under reduced pressure. The crude product was purified through flash chromatography (9:1 Hex/AcOEt) affording **SA$_C$** as an orange oil (200 mg, 70%).

$R_f$ = 0.3 (9:1 Hex/AcOEt); $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ = 10.92 (s, 1H), 9.89 (s, 1H), 7.43-7.35 (m, 2H), 6.96 (d, *J*= 8.2 Hz, 1H), 3.52 (t, *J*= 6.9 Hz, 2H), 2.88 (t, *J*= 6.9 Hz, 2H) ppm; $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ = 196.44, 160.38, 137.47, 133.50, 129.61, 120.49, 117.85, 52.22, 34.09 ppm; MS (ESI): *m/z* calcd. for $[C_9H_9N_3O_2]^-$: 190.06 [*M*-H]$^-$; found: 190.71.

***NMR experiments***

**SA$_C$** and **SA$_O$** were dissolved in DMSO-$d_6$ at 0.1 M concentration. 10 µL of this solution were added to a 5 mm NMR tube containing 340 µL of $D_2O$ and 50 µL of 0.5 M phosphate buffer (pH 7.4) in $D_2O$. Additional 100 µL of $D_2O$ or a 100 µL of a 0.1 M solution of Ac-Lys-OH in $D_2O$ were added.

The mixture was left reacted for 1 h at room temperature and $^1$H spectra were recorded (200 scans; SW=20 ppm; D1=1.5 sec.; O1P=O2P=6 ppm).

Relative integration areas (%) of aldehyde and imine were calculated and plotted using Graphpad Prism software.

$$K_d = \frac{[\text{RCHO}][\text{Ac-Lys-OH}]}{[\text{Imine}]}$$

| | RCHO | | | Imine | | | |
|---|---|---|---|---|---|---|---|
| Sample | Rel. integral | % Area | Conc. [mM] | Rel. integral | % Area | Conc. [mM] | $K_d$ [mM] |
| $SA_O$ | 1.00 | 0.671 | 1.342 | 0.49 | 0.329 | 0.658 | 41 |
| $SA_C$ | 1.00 | 0.658 | 1.316 | 0.52 | 0.342 | 0.684 | 38 |

***Discovery of SA-Modified Anti-NEMO Peptides***

| Entry | Peptide | Sequence | Apparent $K_d$ (μM) |
|---|---|---|---|
| 1 | **4** | FITC-βAFTALDWSWLQTE-$NH_2$ | > 25 |
| 2 | **5** | FITC-βA-**Pra**-TALDWSWLQTE-$NH_2$ | N. D. |
| 3 | **6** | FITC-βAF-**Pra**-ALDWSWLQTE-$NH_2$ | > 25 |
| 4 | **7** | FITC-βAFT-**Pra**-LDWSWLQTE-$NH_2$ | > 25 |
| 5 | **5-SA$_C$** | FITC-βA-**SA$_C$**-TALDWSWLQTE-$NH_2$ | 2.07 ± 0.62 |
| 6 | **5-SA$_O$** | FITC-βA-**SA$_O$**-TALDWSWLQTE-$NH_2$ | 2.47 ± 0.47 |
| 7 | **6-SA$_C$** | FITC-βAF-**SA$_C$**-ALDWSWLQTE-$NH_2$ | 0.060 ± 0.009 |
| 8 | **6-SA$_O$** | FITC-βAF-**SA$_O$**-ALDWSWLQTE-$NH_2$ | 0.454 ± 0.210 |
| 9 | **7-SA$_C$** | FITC-βAFT-**SA$_C$**-LDWSWLQTE-$NH_2$ | 0.109 ± 0.024 |
| 10 | **7-SA$_O$** | FITC-βAFT-**SA$_O$**-LDWSWLQTE-$NH_2$ | 0.142 ± 0.045 |
| 11 | **6-3** | FITC-βAF-**3**-ALDWSWLQTE-$NH_2$ | > 25 |

*Compound **4***

General Procedure A and B were followed. After cleavage (General procedure D), the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 60% B (at min 12)]. The pure product was then lyophilized to give **4** as a yellow solid (26 mg).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 15.79 min; MS (ESI): *m/z* calcd. for $[C_{95}H_{114}N_{18}O_{26}S]^+$: 978.90 $[M+2H]^{2+}$; found: 979.00.

*Compound **5***

General Procedure A (double coupling of Fmoc-Pra-OH) and B were followed. After cleavage (General procedure C), 40 mg of the crude material were purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 16)]. The pure product was then lyophilized to give **5** as a yellow solid (7.63 mg).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 15.05 min; MS (ESI): *m/z* calcd. for $[C_{91}H_{110}N_{18}O_{26}S]^+$: 952.39 $[M+2H]^{2+}$; found: 953.00.

*Compound **6***

General Procedure A (double coupling of Fmoc-Pra-OH) and B were followed. After cleavage (General procedure C), 40 mg of the crude material were purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 16)]. The pure product was then lyophilized to give **6** as a yellow solid (6.00 mg).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.08 min; MS (ESI): *m/z* calcd. for $[C_{96}H_{112}N_{18}O_{25}S]^+$: 975.90 $[M+2H]^{2+}$; found: 975.99.

*Compound **7***

General Procedure A (double coupling of Fmoc-Pra-OH) and B were followed. After cleavage (General procedure C), 40 mg of the crude material were purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18)]. The pure product was then lyophilized to give **7** as a yellow solid (8.85 mg).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 14.15 min; MS (ESI): *m/z* calcd. for $[C_{97}H_{114}N_{18}O_{26}S]^+$: 990.90 $[M+2H]^{2+}$; found: 991.06.

*Compound **5-SA$_C$***

Peptide **5** (4.0 mg, 2.07 µmol, 1 equiv.) and **SA$_C$** (1.0 mg, 5.23 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 15.7 min]. The pure product was then lyophilized to give **5-SA$_C$** as a yellow solid (1.35 mg, 44%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 15.58 min; MS (ESI): *m/z* calcd. for $[C_{100}H_{119}N_{21}O_{28}S]^+$: 1048.42 $[M+2H]^{2+}$; found: 1048.50.

*Compound **5-SA$_O$***

Peptide **5** (3.2 mg, 1.68 µmol, 1 equiv.) and **SA$_O$** (1.0 mg, 4.83 µmol, 2.9 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 15.5 min]. The pure product was then lyophilized to give **5-SA$_O$** as a yellow solid (1.47 mg, 41%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 15.50 min; MS (ESI): *m/z* calcd. for $[C_{100}H_{119}N_{21}O_{29}S]^+$: 1056.42 $[M+2H]^{2+}$; found: 1056.50.

*Compound **6-SA$_C$***

Peptide **6** (4.0 mg, 2.07 µmol, 1 equiv.) and **SA$_C$** (1.0 mg, 5.23 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 17.1 min]. The pure product was then lyophilized to give **6-SA$_C$** as a yellow solid (1.18 mg, 27%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.69 min; MS (ESI): *m/z* calcd. for $[C_{105}H_{121}N_{21}O_{27}S]^+$: 1071.43 $[M+2H]^{2+}$; found: 1071.45.

*Compound **6-SA$_O$***

Peptide **6** (4.0 mg, 2.07 µmol, 1 equiv.) and **SA$_O$** (1.12 mg, 5.40 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 17.2 min]. The pure product was then lyophilized to give **6-SA$_O$** as a yellow solid (1.20 mg, 27%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.59 min; MS (ESI): *m/z* calcd. for $[C_{105}H_{121}N_{21}O_{28}S]^+$: 1079.43 $[M+2H]^{2+}$; found: 1079.58.

*Compound **7-SA$_C$***

Peptide **7** (4.0 mg, 2.02 µmol, 1 equiv.) and **SA$_C$** (1.0 mg, 5.23 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 17.1 min]. The pure product was then lyophilized to give **7-SA$_C$** as a yellow solid (1.34 mg, 31%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.57 min; MS (ESI): *m/z* calcd. for $[C_{106}H_{123}N_{21}O_{28}S]^+$: 1086.44 $[M+2H]^{2+}$; found: 1086.77.

*Compound **7-SA$_O$***

Peptide **7** (4.14 mg, 2.09 µmol, 1 equiv.) and **SA$_O$** (1.13 mg, 5.44 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 17.0 min]. The pure product was then lyophilized to give **7-SA$_O$** as a yellow solid (1.60 mg, 35%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.53 min; MS (ESI): *m/z* calcd. for $[C_{106}H_{123}N_{21}O_{29}S]^+$: 1094.43 $[M+2H]^{2+}$; found: 1094.56.

*Compound **6-3***

Peptide **6** (4.25 mg, 2.18 µmol, 1 equiv.) and **3** (1.0 mg, 5.66 µmol, 2.6 equiv.) were moved into a Schlenk and dried through $N_2$-vacuum cycles and treated following General Procedure D. After solvent removal, the crude material was purified by HPLC [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 15% B (at min 1) to 62% B (at min 18), $t_R$ (product): 16.60 min]. The pure product was then lyophilized to give **6-3** as a yellow solid (2.30 mg, 50%).

LC-MS analysis [eluent A: $H_2O$ + 0.1% TFA; eluent B: MeCN, ramp from 10% to 90% B in 25 min]: $t_R$= 16.60 min; MS (ESI): *m/z* calcd. for $[C_{104}H_{121}N_{21}O_{26}S]^+$: 1057.43 $[M+2H]^{2+}$; found: 1057.41.

## Prediction of lysine p$K_a$ values

The p$K_a$ values of the Lys(ε-$NH_2$) groups in the NEMO protein structure were predicted with the Rosetta p$K_a$ protocol[3] available on the Rosetta Online Server that Includes Everyone (ROSIE),[4] using the crystal structure of the NEMO-IKKβ complex (PDB: 3BRV) and giving flexibility to the side chain of all residues within 5 Å from the target residue. The structure shown in Figure 2 was prepared by the Protein Preparation Wizard of the Schrödinger Maestro graphical interface.[5]

## FA binding assays

Direct binding assays between the fluorescently labeled peptides and GST-NEMO (purchased from Biointron) were done in a 384-well black plate (Greiner) with a final volume of 10 µL. GST-NEMO protein stocks were allowed to thaw and a 2x serial dilution for 16 different concentration points was prepared. Each lane was filled with 5 µL of corresponding dilution point with the highest concentration in Row A being 53 µM. The peptides, previously dissolved in DMSO (500 µM), were diluted in PBS 1X (pH 7.4, Gibco) to a concentration of 250 nM. To the wells with previously loaded protein, 5 µL of the peptide was added for a final concentration of 125 nM for each peptide. Row P was treated as a control containing only buffer and tracer peptide. The plate was covered with a tin foil and centrifuged at 300 G for 5 min. After 30 minutes incubation at room temperature, FA was read on a Spark® Multimode Microplate Reader (TECAN) using polarized excitation at 485 nm with emission intensity measured through a parallel and perpendicularly polarized 535 nm filter (Gain= 67; Z-position= 23326; Settle time: 100 ms; T= 25 °C). Anisotropy values were plotted against NEMO concentration (µM) and fit into a non-linear regression curve utilizing GraphPad.

The data normalization used for the graphs in Figures 3B, 3C and 3E was performed by subtracting, from each recorded FA value, the mean FA of the three wells containing the lowest protein concentration. The resulting values were then divided by the highest subtracted anisotropy value, which corresponds to the well with the highest protein concentration, and expressed as a percentage.

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3 K. P. Kilambi, J. J. Gray, *Biophys. J.* 2012, **103**, 587-595.

4 S. Lyskov, F. C. Chou, S. Ó. Conchúir, B. S. Der, K. Drew, D. Kuroda, J. Xu, B. D. Weitzner, P. D. Renfrew, P. Sripakdeevong, B. Borgo, J. J. Havranek, B. Kuhlman, T. Kortemme, R. Bonneau, J. J. Gray, R. Das, *PLoS One* 2013, **8**, e63906.

5 Maestro, Schrödinger Release 2025-3, Schrödinger, LLC, New York, NY, 2025.

## Dynamic Light Scattering studies

Peptides **6** and **6-SA$_C$** were prepared in DMSO solutions at 500 μM, 12.5 μM, and 1 μM. Each solution was then dissolved in 1:100 in PBS to obtain final concentrations of 5 μM, 125 nM, and 10 nM, resulting in a final DMSO content of 1%.

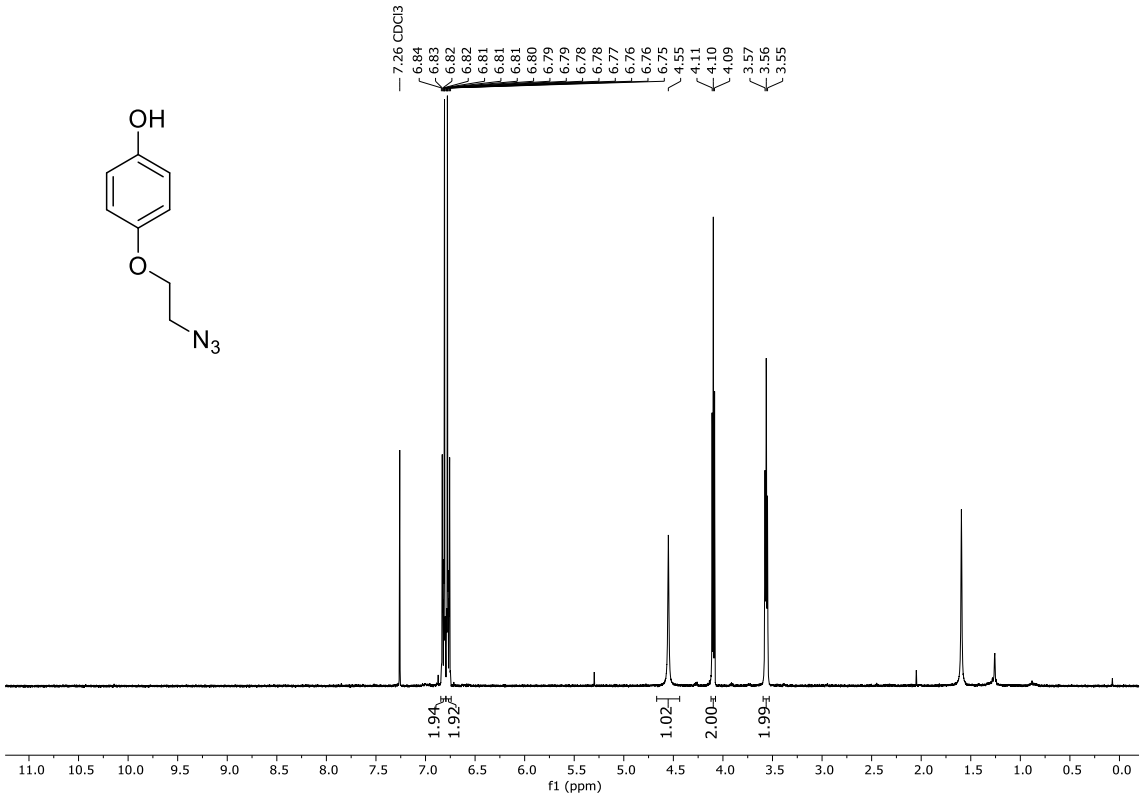
**Table S1**. Hydrodynamic diameter, and polydispersity index (PI) of compounds 3.51 and 3.55 at different concentrations in PBS/DMSO 99:1.

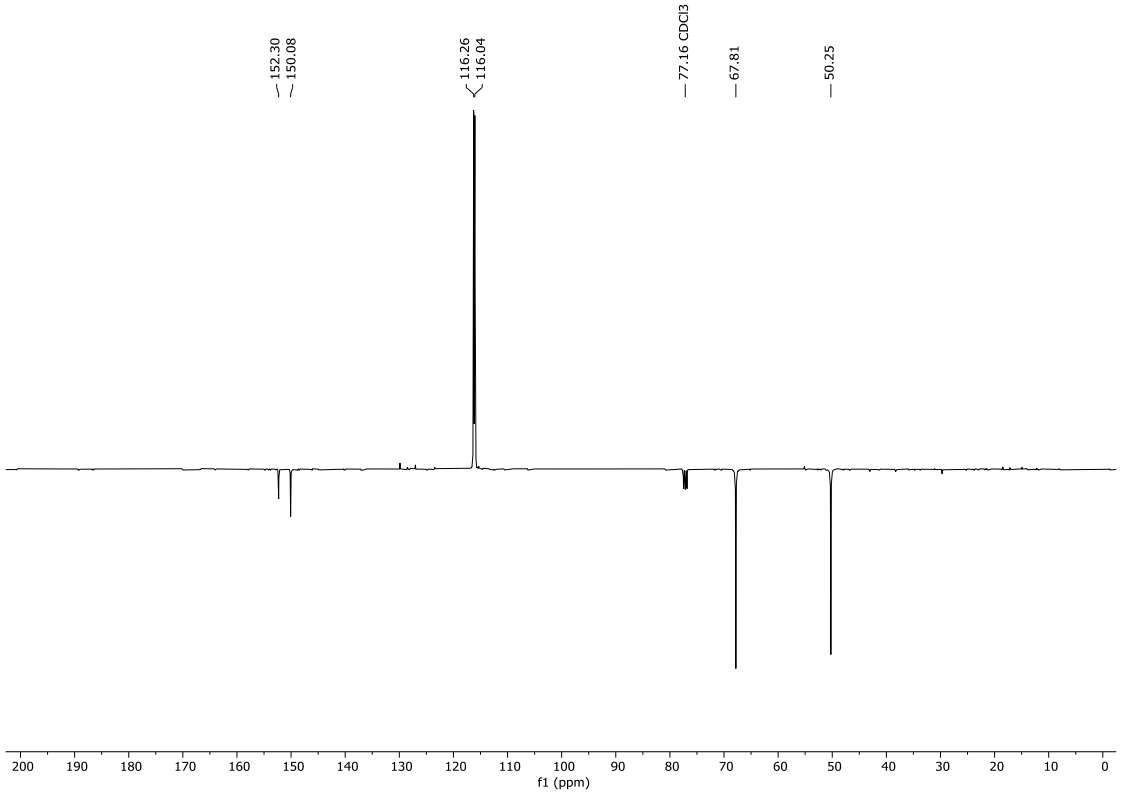
| Compound | Concentration (nM) | Diameter (nm) | PI |
|---|---|---|---|
| **6** | 5000 | 1246 ± 35 | 0.607 ± 0.093 |
| | 125 | 494.2 ± 144.3 | 0.207 ± 0.075 |
| | 10 | 359.3 ± 107.2 | 0.388 ± 0.188 |
| **6-SA$_C$** | 5000 | 1210 ± 187 | 0.352 ± 0.101 |
| | 125 | 315.9 ±18.4 | 0.282 ± 0.084 |
| | 10 | 485.7 ± 43.3 | 0.310 ± 0.067 |

# Appendix

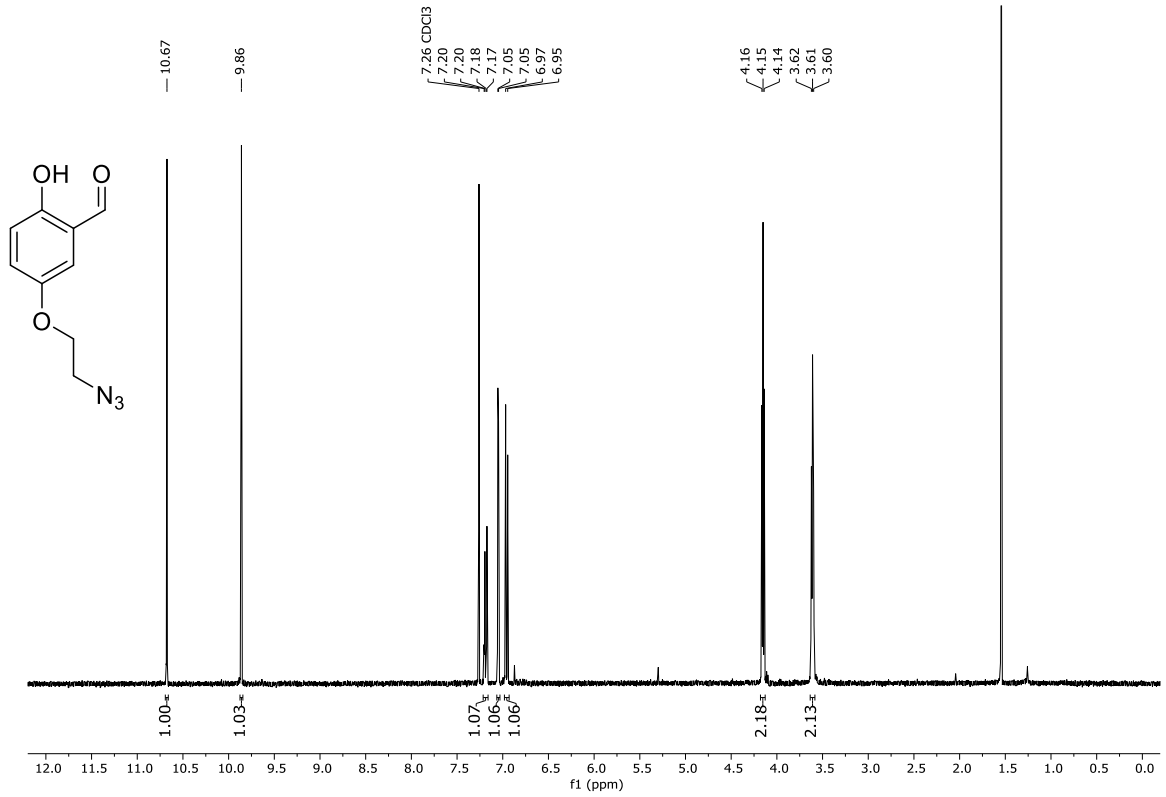
## ***NMR Spectra***

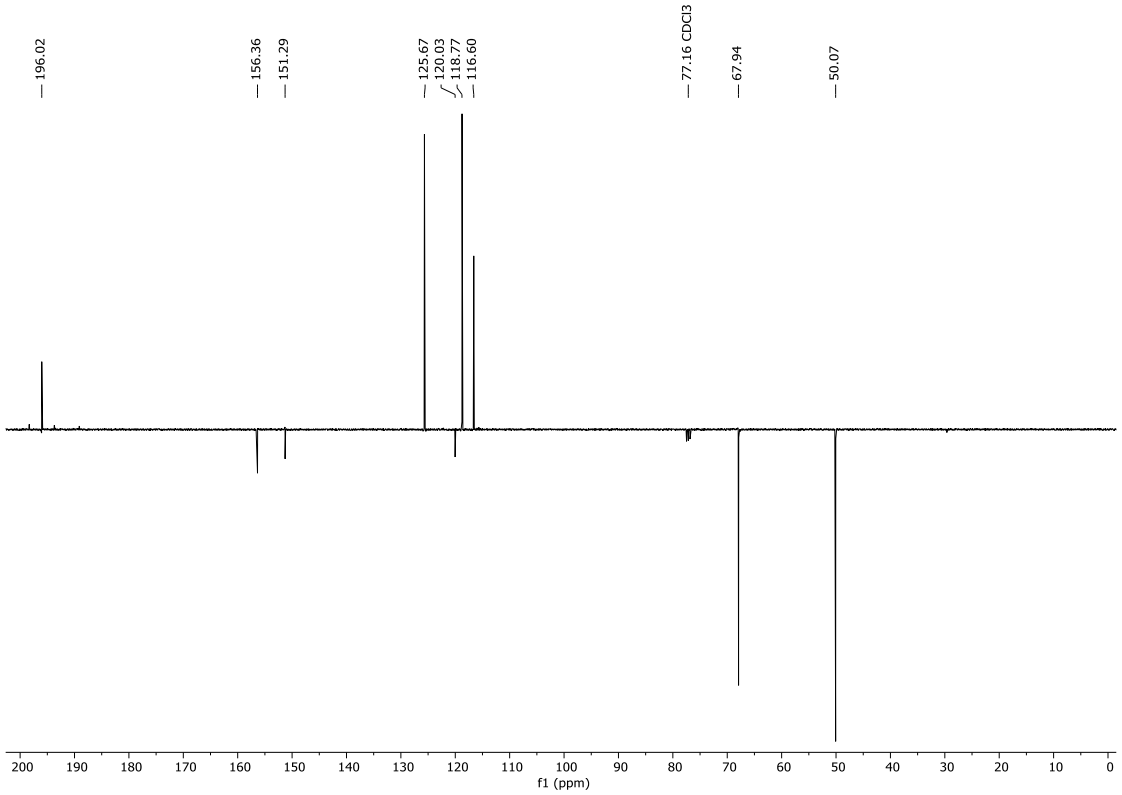
Compound **2**



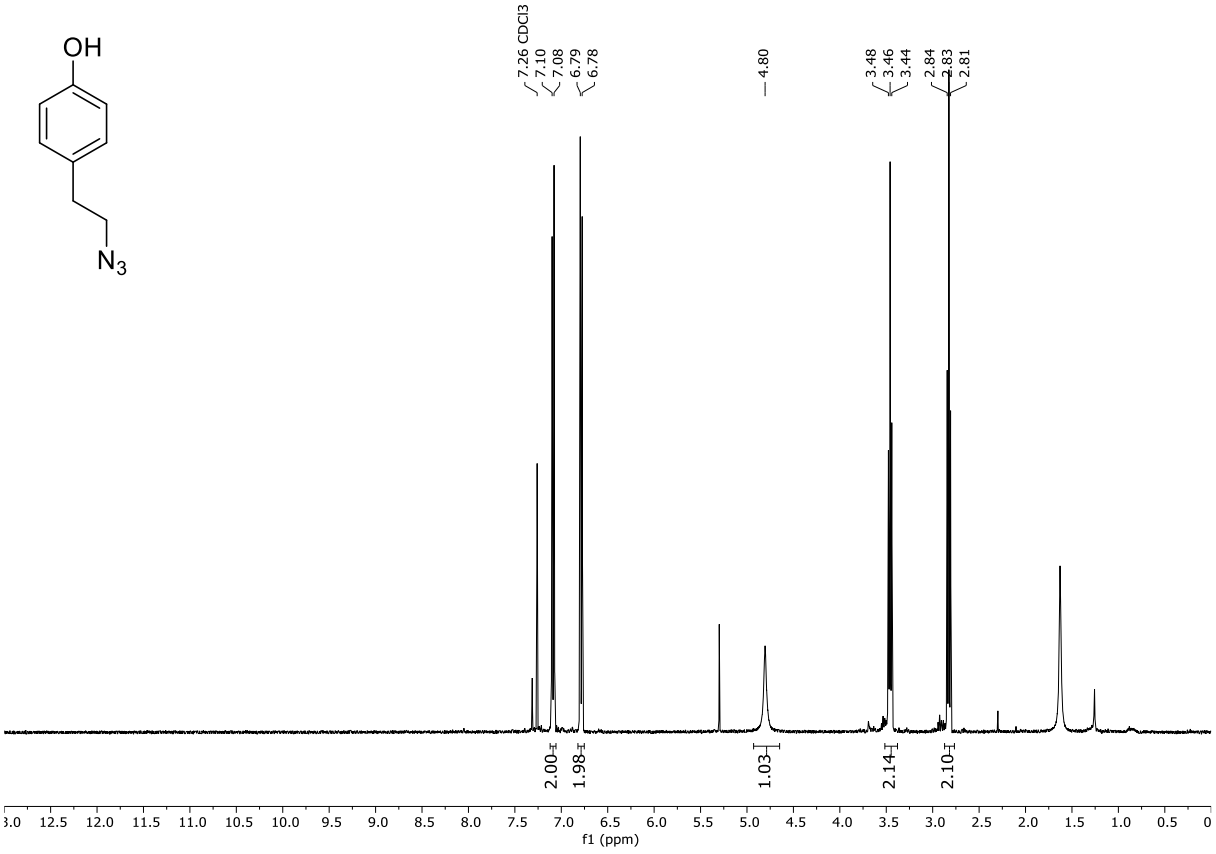



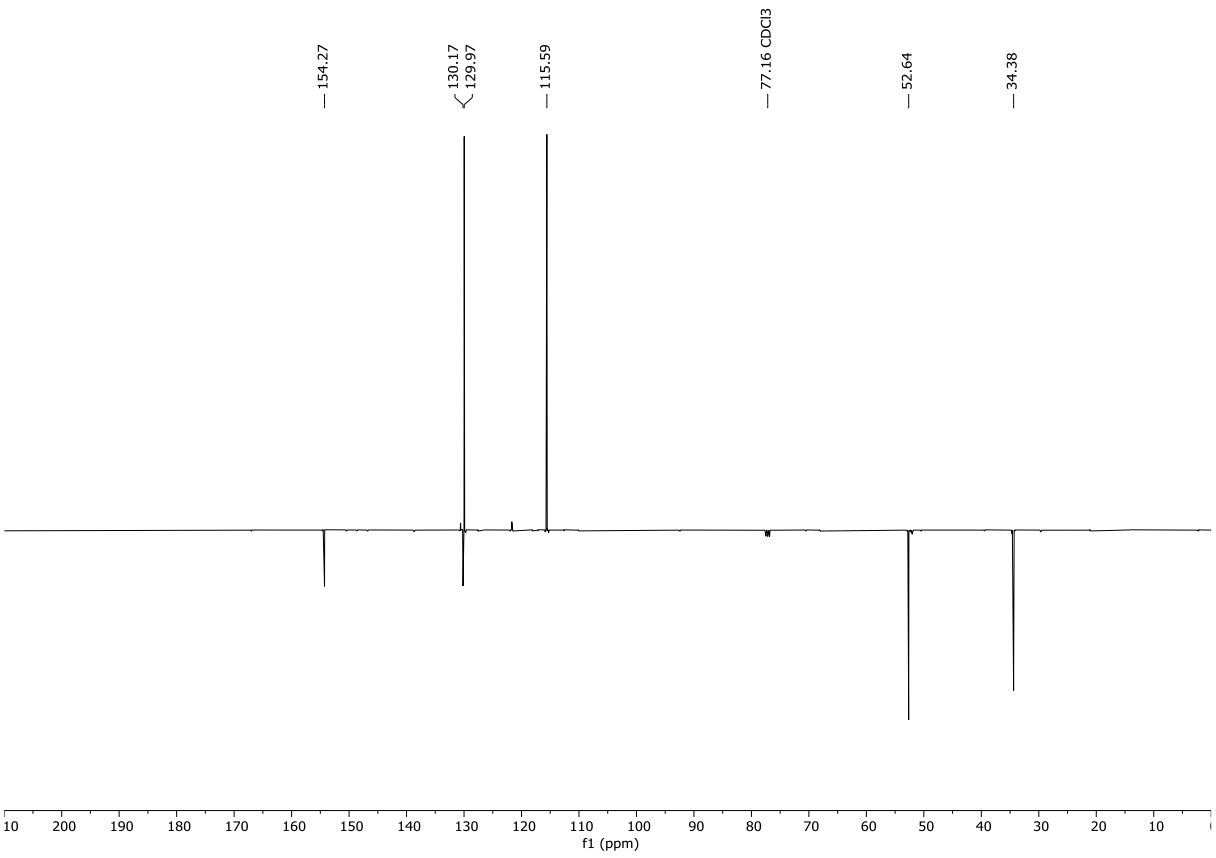
Compound **SA$_o$**



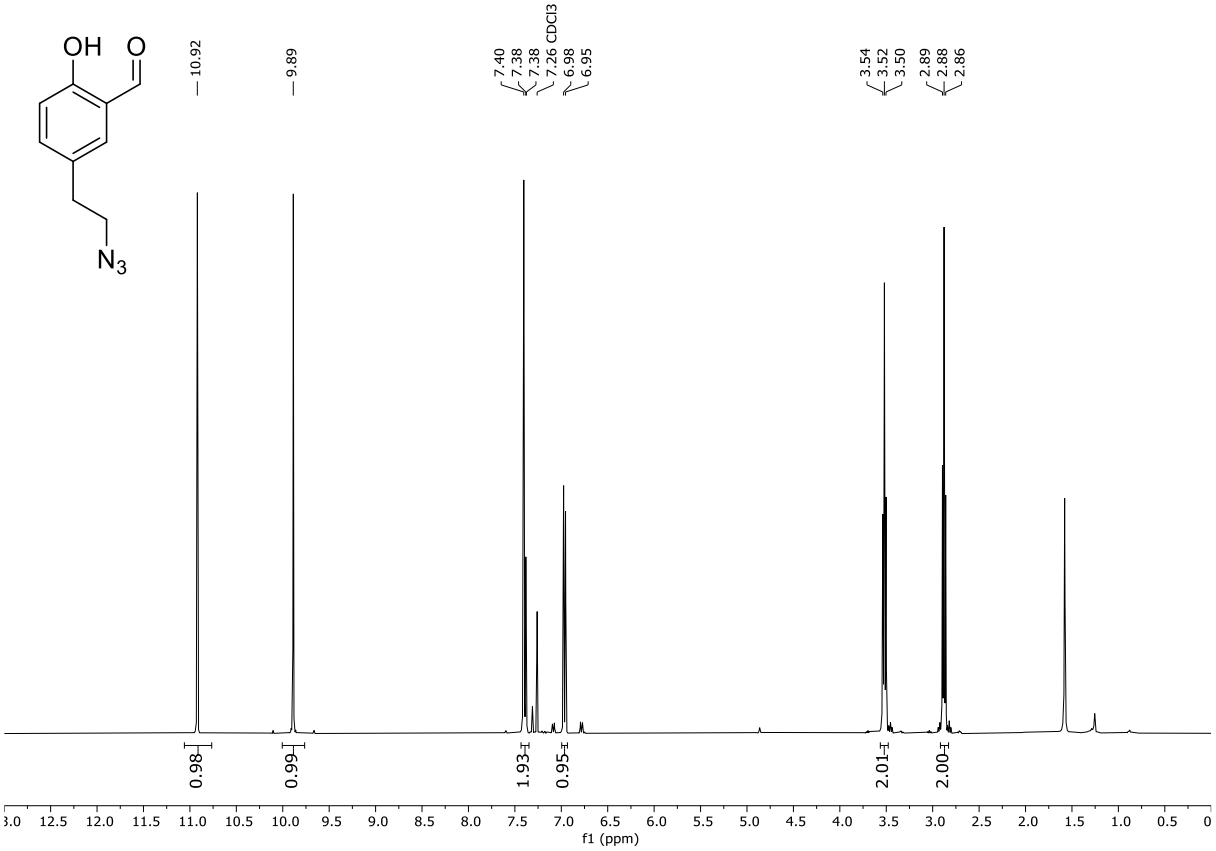



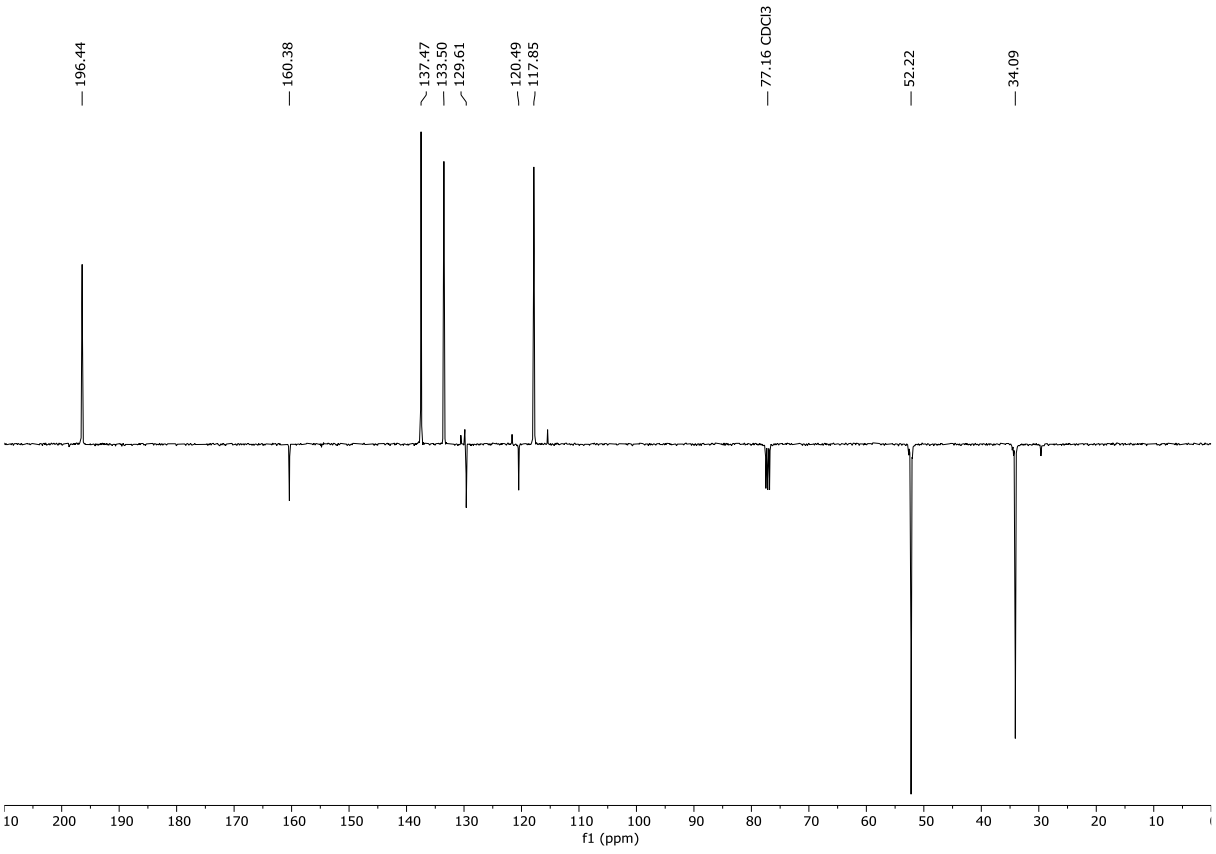
Compound **3**


OH
N3
7.26 CDCl3
7.10
7.08
6.79
6.78
4.80
3.48
3.46
3.44
2.84
2.83
2.81
2.00
1.98
1.03
2.14
2.10
f1 (ppm)


154.27
130.17
129.97
115.59
77.16 CDCl3
52.64
34.38
f1 (ppm)

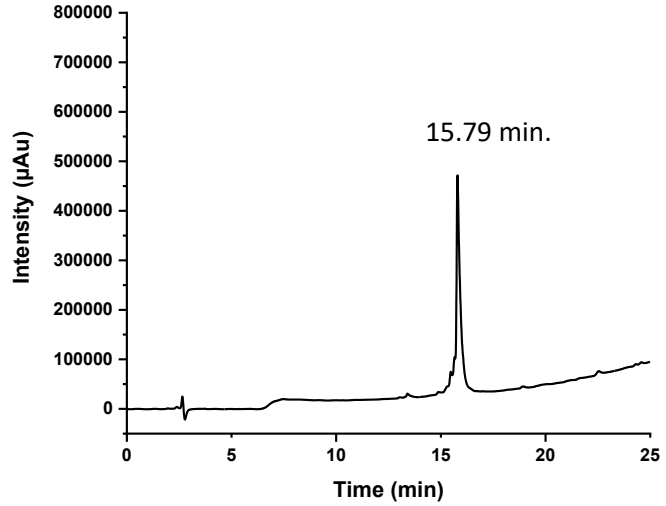
Compound **SA$_C$**

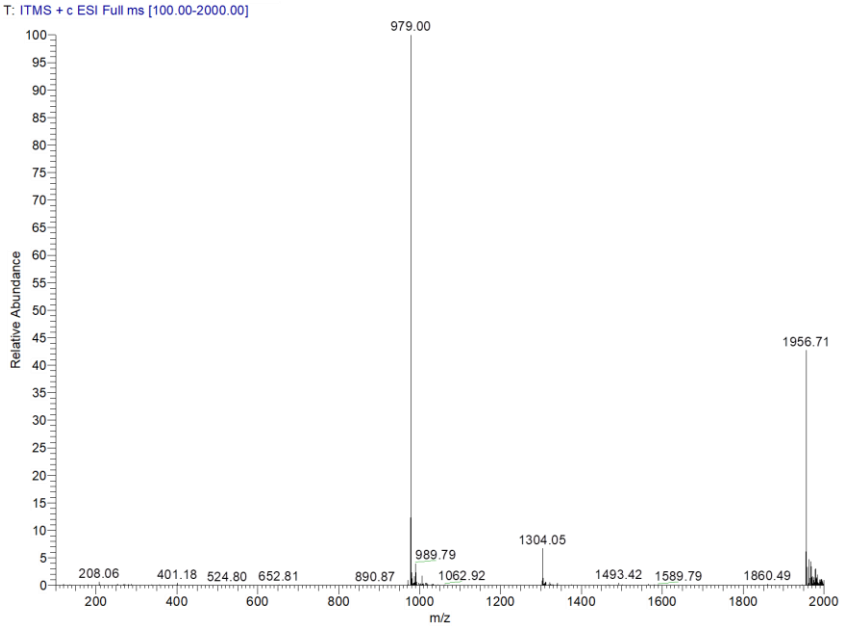


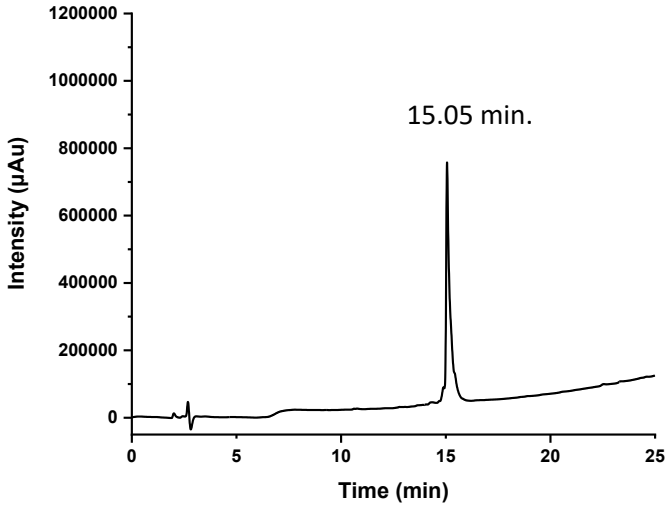
***LC-MS Spectra (Acquired at 220 nm)***

Compound **4**



Compound **5**


15.05 min.
Intensity (μAu)
Time (min)
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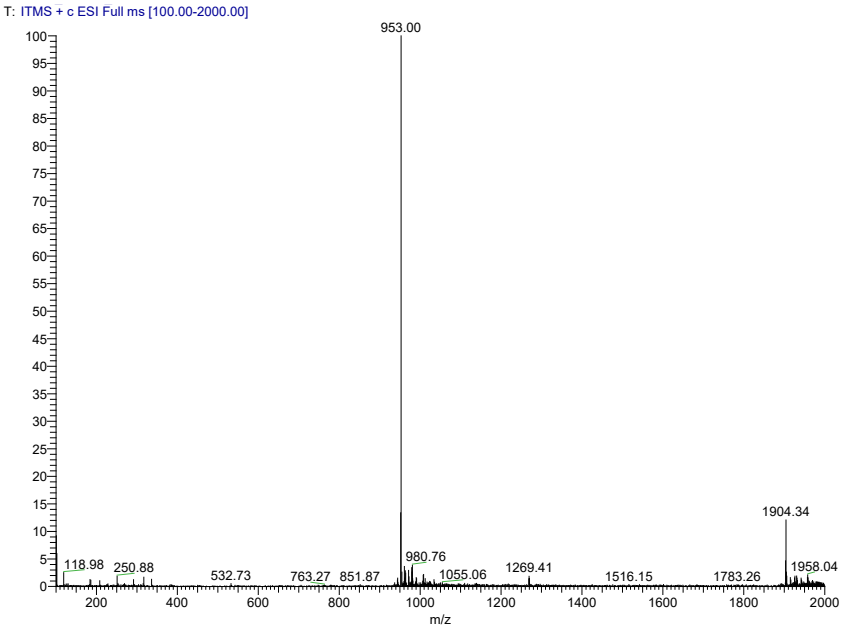
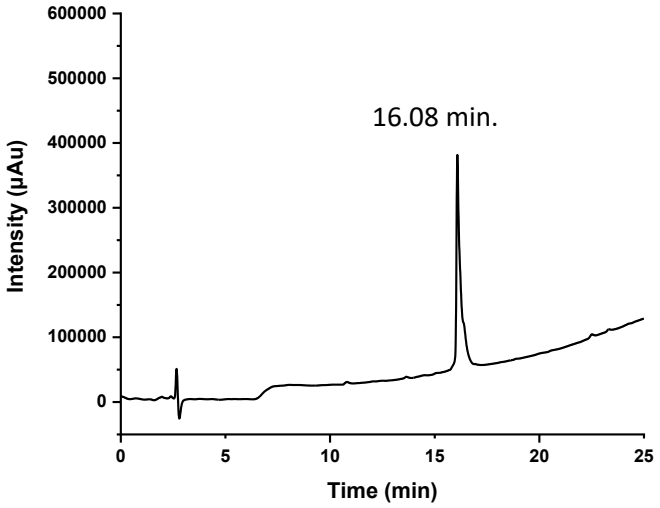
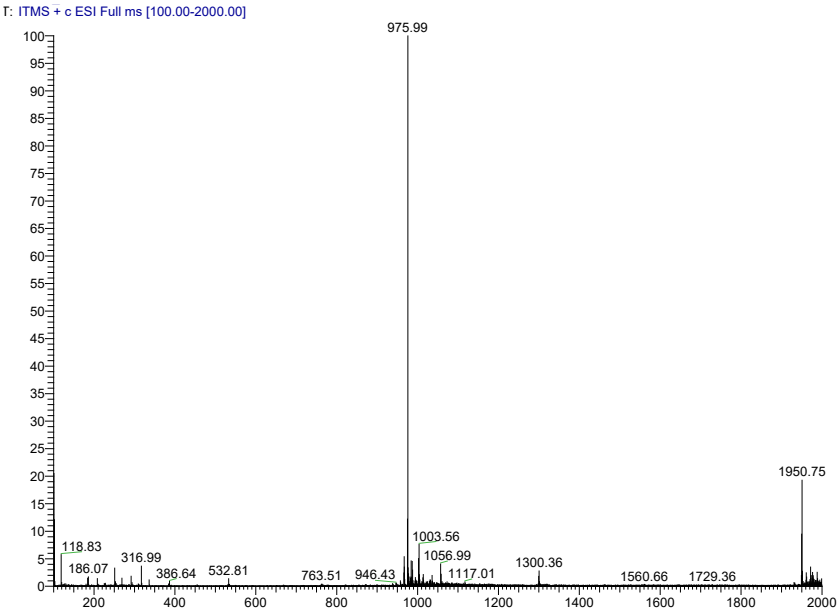
1000000
800000
600000
400000
200000
0
0
5
10
15
20
25

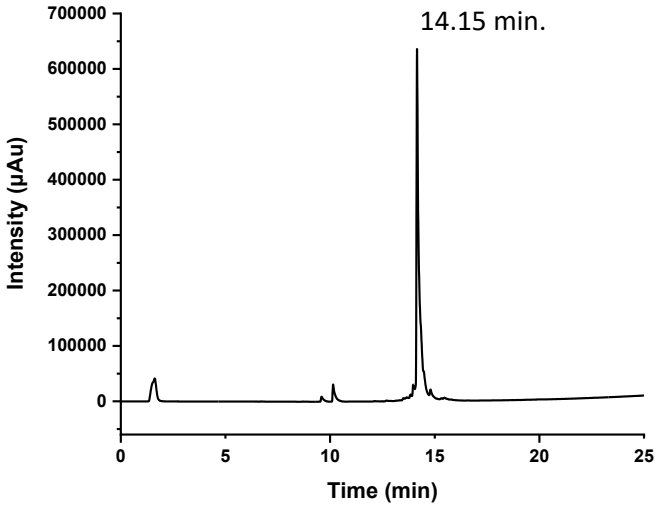

T: ITMS + c ESI Full ms [100.00-2000.00]
953.00
1904.34
980.76
1958.04
118.98
250.88
532.73
763.27
851.87
1055.06
1269.41
1516.15
1783.26
m/z

Compound **6**


16.08 min.
Intensity (µAu)
Time (min)


ITMS + c ESI Full ms [100.00-2000.00]
975.99
1950.75
1003.56
1056.99
1117.01
1300.36
1560.66
1729.36
118.83
186.07
316.99
386.64
532.81
763.51
946.43

Compound **7**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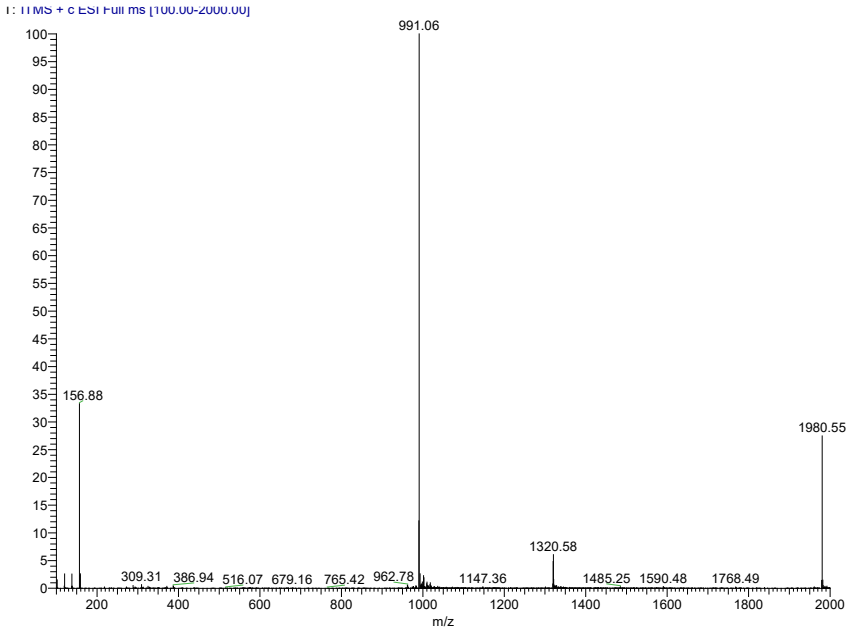
Intensity (µAu)
Time (min)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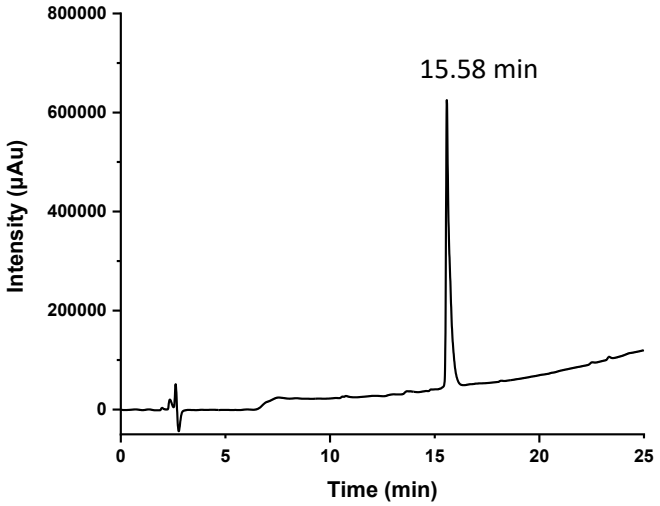
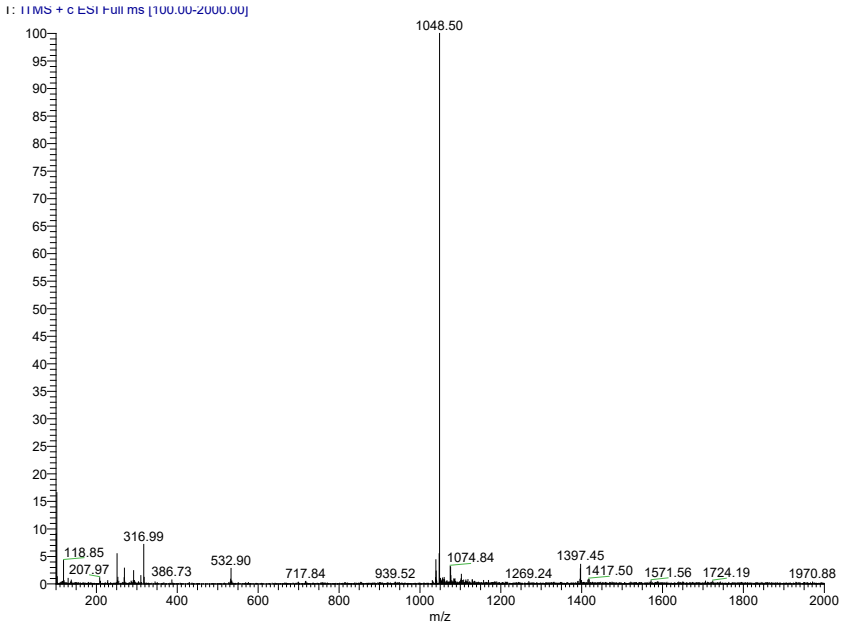
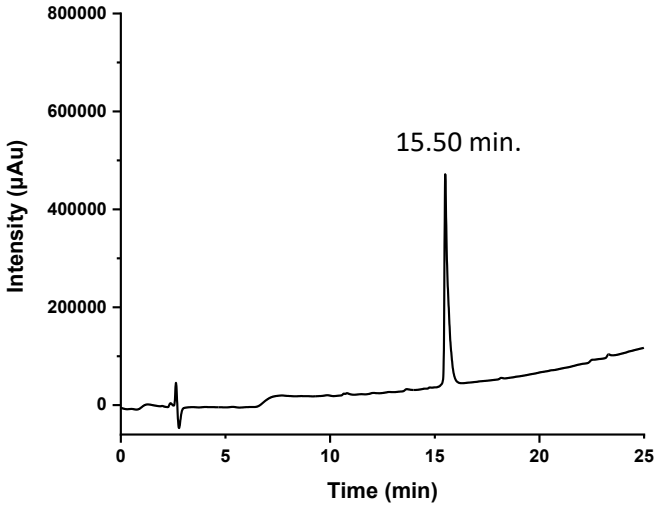
156.88
1980.55
1320.58
309.31
386.94
516.07
679.16
765.42
962.78
1147.36
1485.25
1590.48
1768.49
m/z

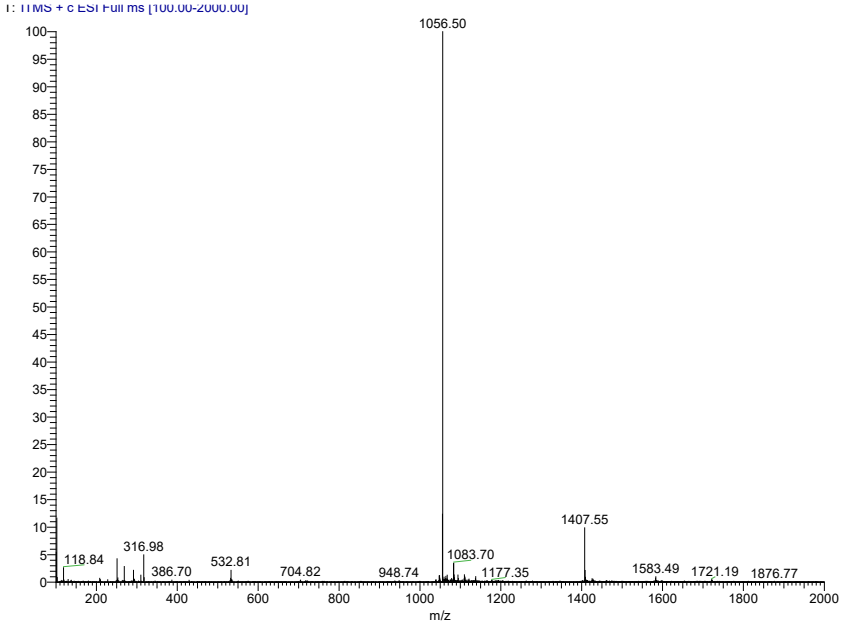
Compound **5-SA$_C$**



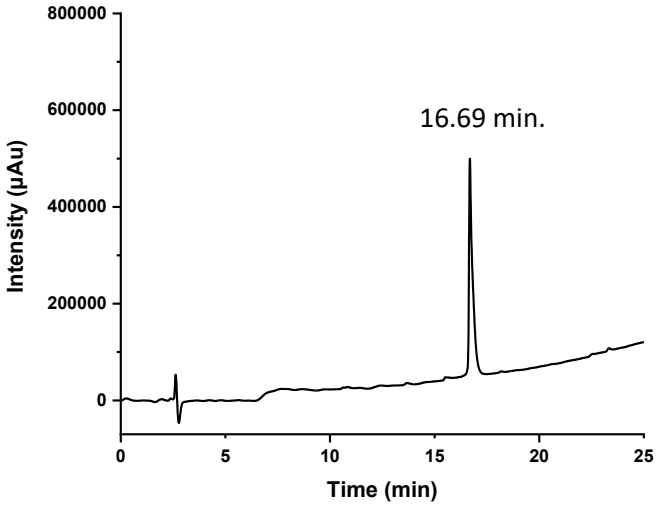



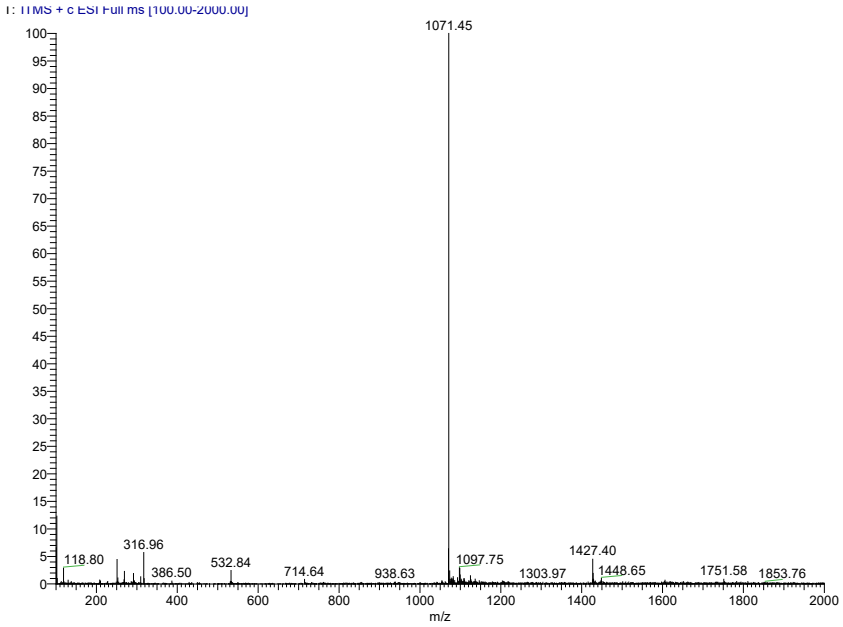
Compound **5-SA$_O$**



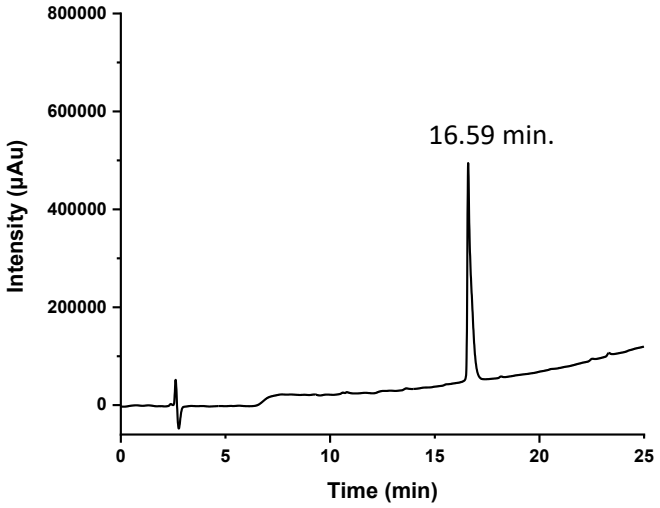



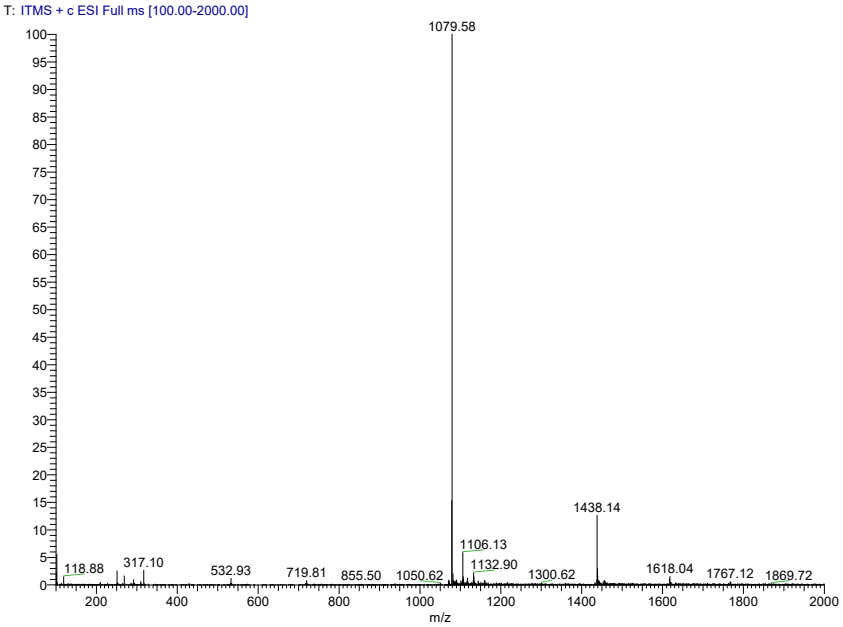
Compound **6-SA$_C$**



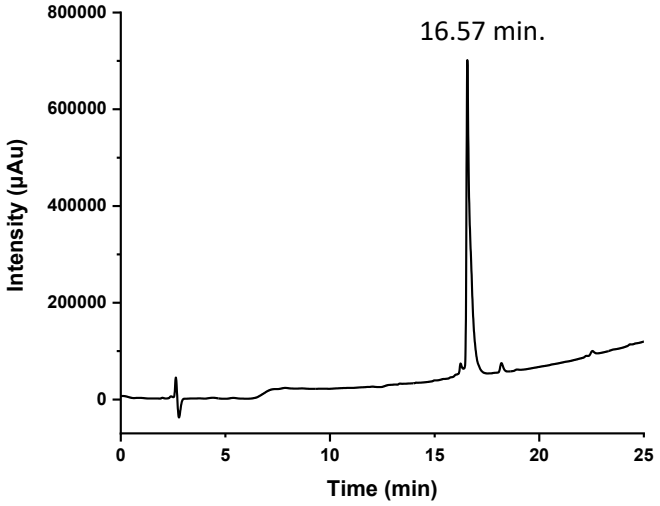



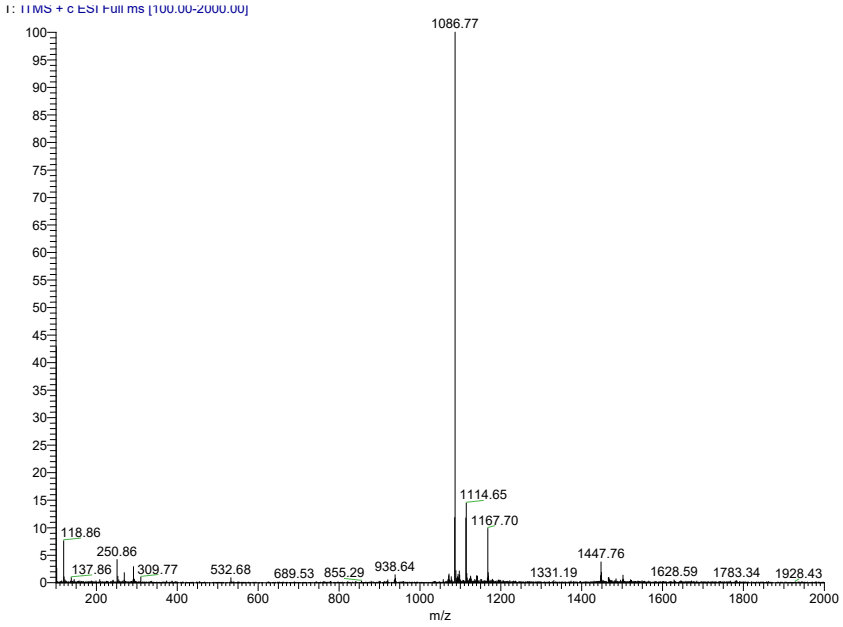
Compound **6-SA$_O$**



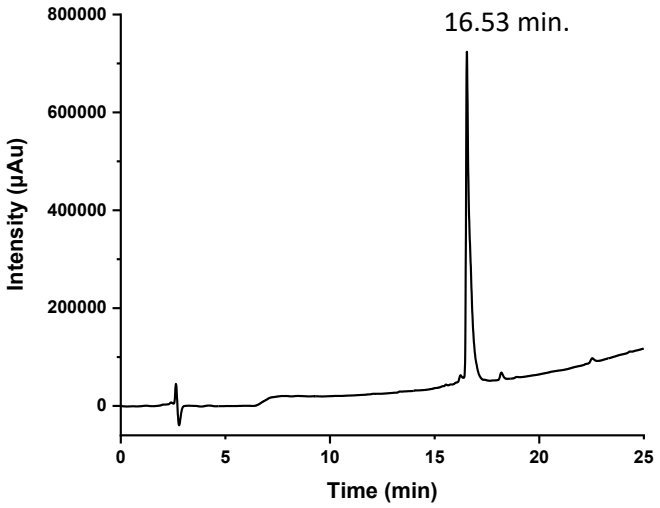



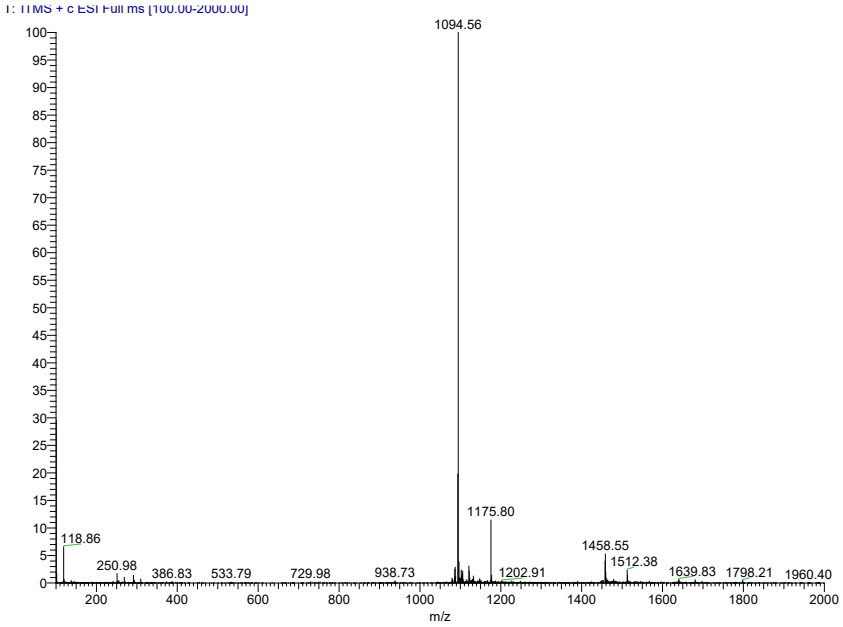
Compound **7-SA$_C$**



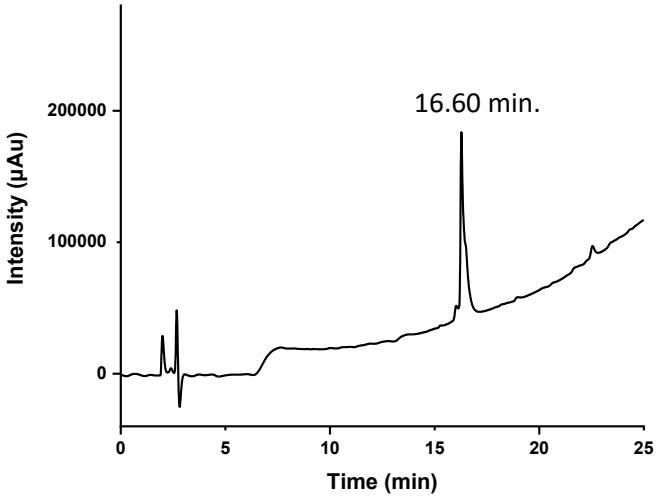



Compound **7-SA$_O$**



Compound **6-3**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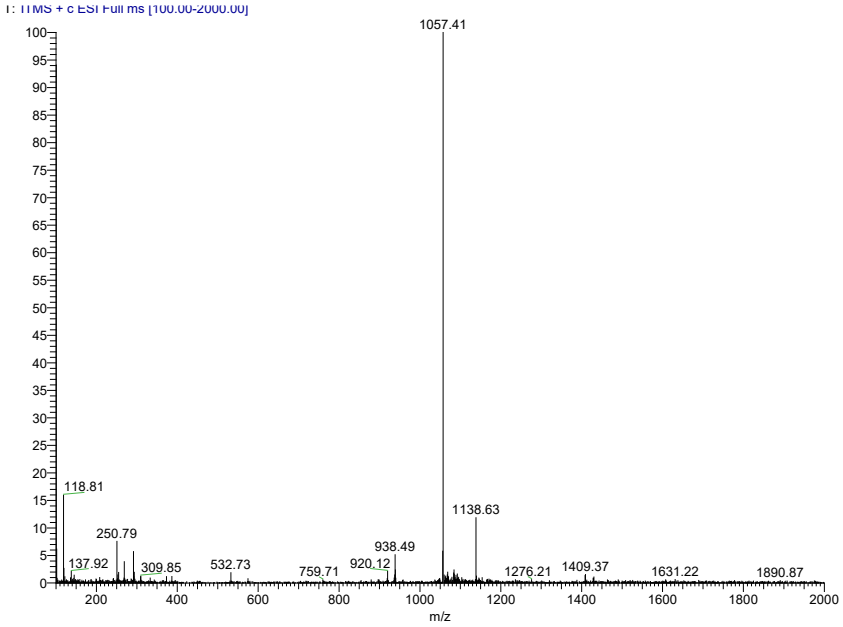
0
0
5
10
15
20
25
Time (min)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118.81
250.79
137.92
309.85
532.73
759.71
920.12
938.49
1138.63
1276.21
1409.37
1631.22
1890.87
m/z