DNA-Encoded Library Hit Confirmation: Bridging the Gap Between On-DNA and Off-DNA Chemistry

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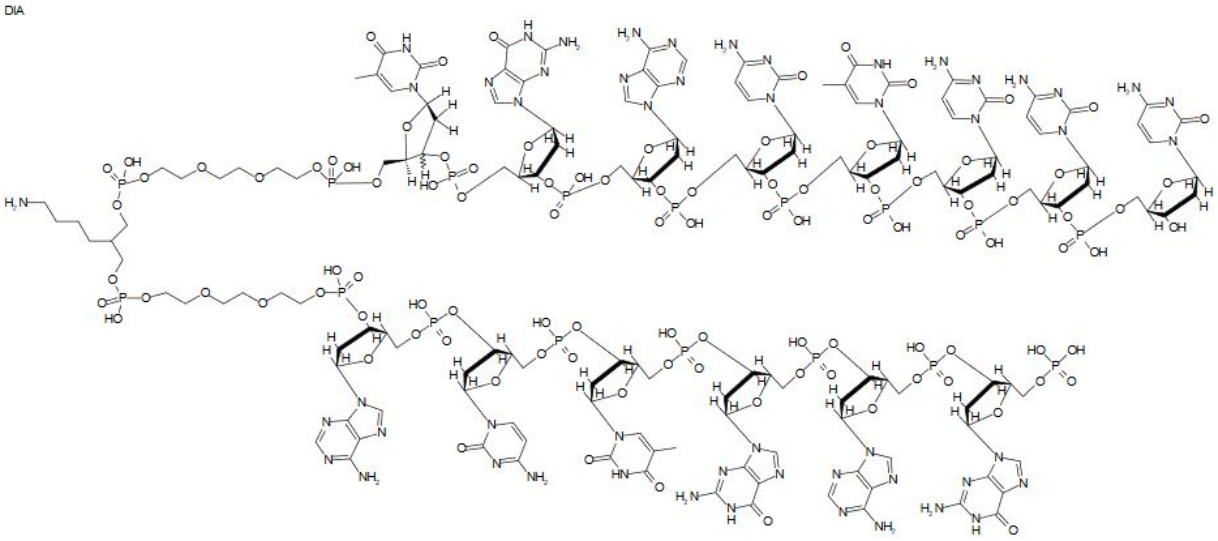
Part I: On-DNA chemistry

Analytical Methods

On-DNA reactions conducted were analyzed by LCMS. Samples (ca. 100 pmol) were injected onto a reverse-phase chromatography column (Targa C18, 5µ, 2.1 x 40 mm) and eluted (15 – 70% solvent B over 7 min, 0.36 mL/min flow rate; Solvent A: 0.75% hexafluoroisopropanol / 0.38% triethylammonium acetate /10 µM EDTA in deionized water; Solvent B: 0.75% HFIP/0.38% TEAA/10 µM EDTA in 90/10 methanol/water) with monitoring at 260 nm. Effluent was analyzed on a ThermoFinnigan Advantage electrospray mass spectrometer or microtof mass spectrometer in negative ion mode. When necessary, mass deconvolution was achieved using Bruker Compass DataAnalysis 4.4.

Materials

All solvents and reagents, unless otherwise described, were purchased through vendors and used as supplied. DNA headpiece (HP) was obtained from Biosearch Technologies, Novato, CA. **"Headpiece (HP)."** Sequence: 5'-/5Phos/GAGTCA/iSp9/iUniAmM/iSp9/TGACTCCC-3'



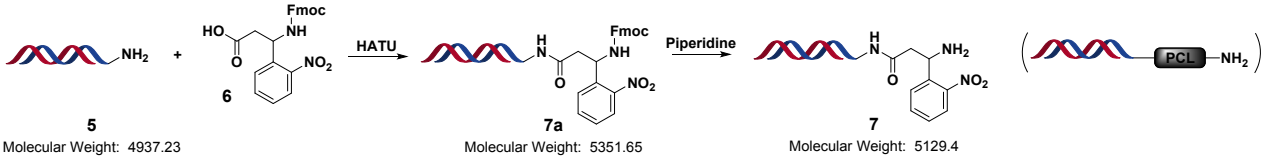
HP-NH$_2$ (5)

Figure S1. Sequence and structure of the "Headpiece (HP)". MW = 4937 D

General procedure for ethanol precipitation

To the reaction mixture containing DNA, one-tenth volume of NaCl (5M) was added, followed by the addition of 2.5 volumes of cold 100% ethanol. The mixture was left at -80 °C for 1 h and then centrifuged for 20 min at 4 °C in a microcentrifuge at 13000 rpm. The supernatant was then removed and the DNA pellet was afforded as a white solid after freeze-drying.

Synthesis of HP-PCL-NH$_2$



Scheme S1. Synthesis of **HP-PCL-NH$_2$**

In a plastic tube, 40 equiv. of 3-(9-Fluorenylmethyloxycarbonyl)amino-3-(2-nitrophenyl)propionic acid (**6**, FMOC-ANP-OH, Innovochem, CAS # 171778-06-6, 200 µmol in 1 mL of DMF) was mixed with 40 equiv. of N,N-diisopropylethylamine (200 µmol in 1mL of DMF), followed by 40 equiv. of HATU (1-[bis(dimethyl-amino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxidehexafluorophosphate, 200 µmol in 1 mL of DMF). The resulting mixture was quickly transferred into a solution of DNA headpiece (**HP**) (**5**, 5 µmol in 5 mL of 250 mM pH 9.4 sodium borate buffer) and vortexed. The reaction proceeded at room temperature for 30 min and was monitored by LCMS (estimated LCMS conversion >90%). Upon completion, the

reaction was subjected for ethanol precipitation and then reconstituted in water at 1 mM of **HP-PCL-NH-Fmoc** (**7a**). To this oligo solution was added 10% by volume of neat piperidine. The reaction was allowed to proceed at room temperature and monitored by LCMS (estimated LCMS conversion >95%). Once the reaction was complete, the "EtOH Precipitation Protocol" was followed, and then reconstituted in water at 2 mM of **HP-PCL-NH$_2$** (**7**) for further steps. Purity of the final product crude mixture was determined by LCMS total ion count.

General procedure for photocleavage

The oligo 0.25 mM solution in 4:1 (water:MeOH) was transferred to one well of a flat-bottomed, 24-well microtiter plate (Corning® Costar® clear polystyrene flat bottom, CLS3738). The reaction mixture was irradiated with 365 nm light at 4 °C for 1 h (light source should be positioned directly above transparent plate, ~2cm, UVP 95-0192-01 Model UVLS-225D Mineralight UV Display Lamp, 254/365nm Wavelength, 115V was used for photocleavage). Once the reaction was deemed complete, the "EtOH Precipitation Protocol" was followed.

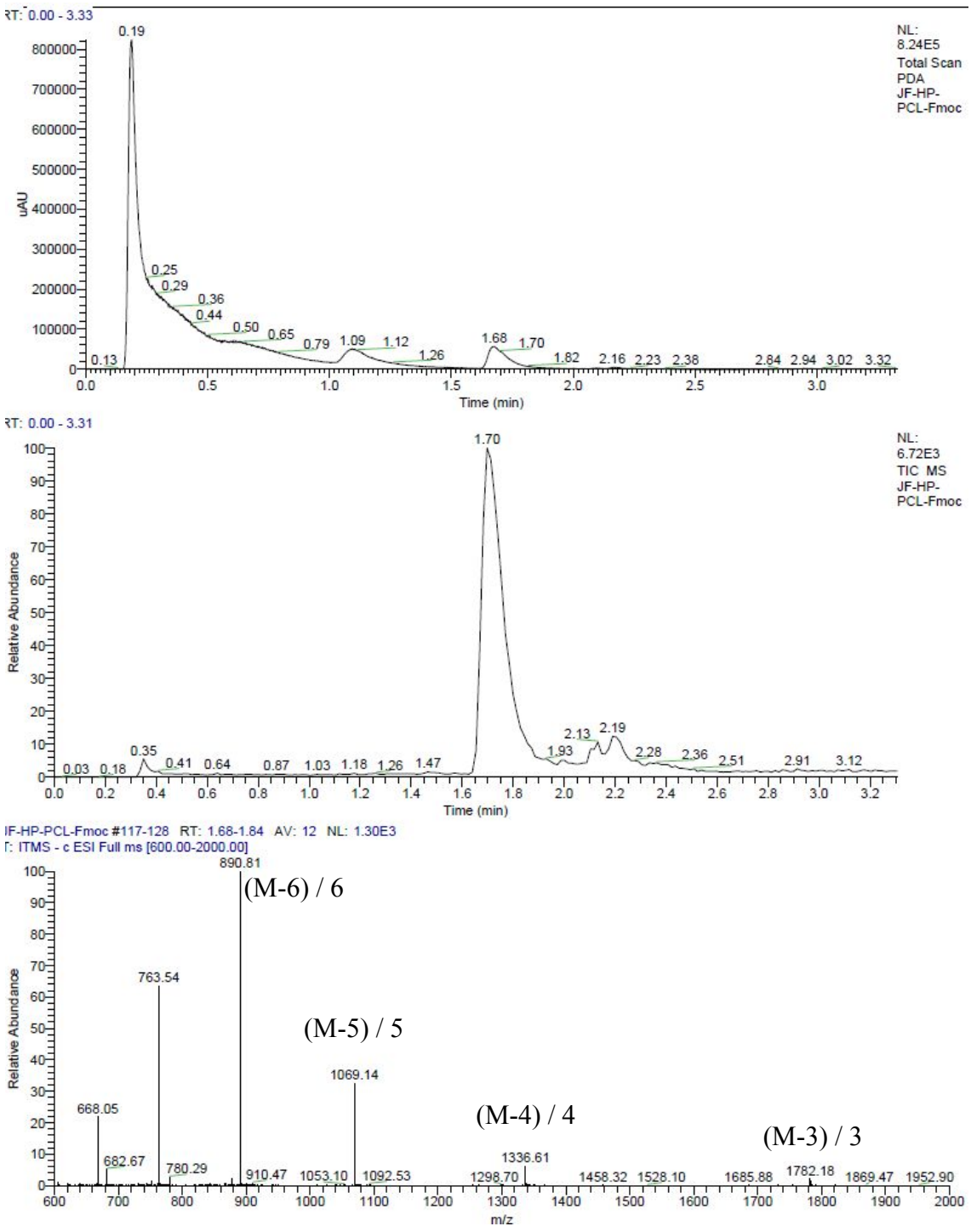


Figure S2. LCMS of **HP-PCL-NH-Fmoc (7a)**, MW 5351.65, $C_{178}H_{233}N_{54}O_{106}P_{17}$.

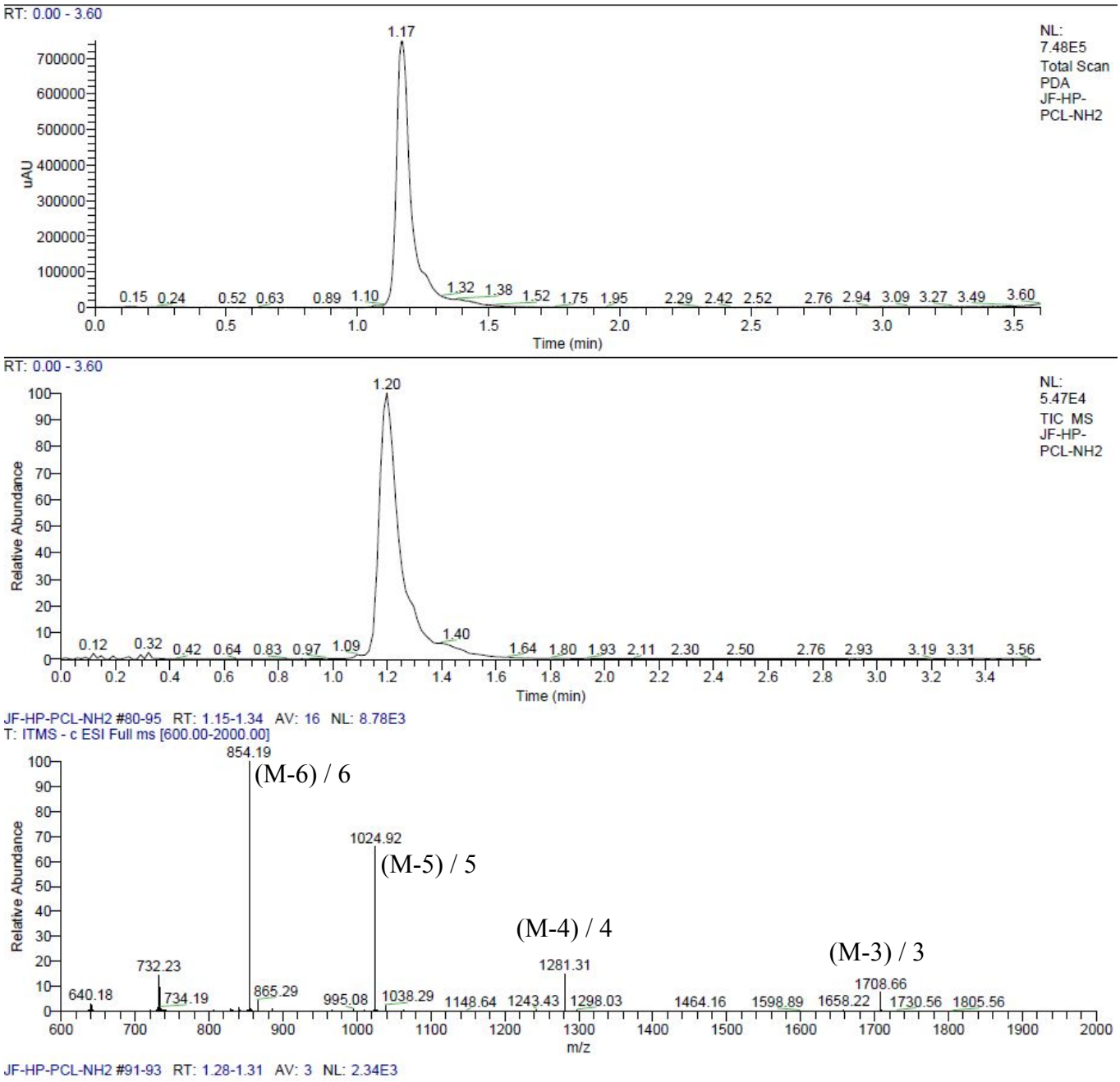
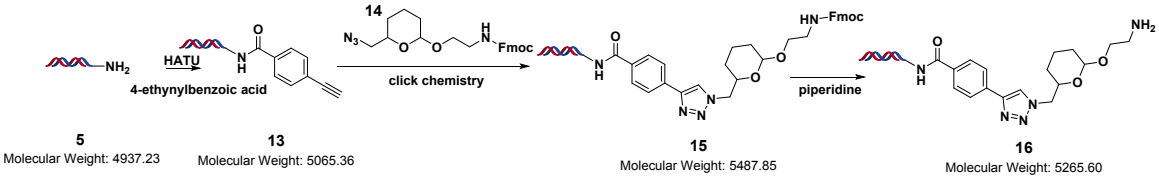


Figure S3. LCMS of **HP-PCL-NH$_2$ (7)**, MW 5129.40, $C_{163}H_{223}N_{54}O_{104}P_{17}$.

Synthesis of HP-THP-NH_2



Scheme S2. Synthesis of **HP-THP-NH_2**

In a plastic tube, 40 equiv. of 4-ethynylbenzoic acid (ASTA Tech CAS# 10602-00-3, 200 µmol in 1 mL of DMF) was mixed with 40 equiv. of N,N-diisopropylethylamine (200 µmol in 1mL of DMF), followed by 40 equiv. of HATU (1-[bis(dimethyl-amino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxidehexafluorophosphate, 200 µmol in 1 mL of DMF). The resulting mixture was quickly transferred into a solution of DNA headpiece (**5**, 5 µmol in 5 mL of 250 mM pH 9.4 sodium borate buffer) and vortexed. The reaction proceeded at room temperature for 30 min and was monitored by LCMS (estimated LCMS conversion >90%). Upon completion, the reaction was subjected for EtOH precipitation and then reconstituted in water at 2 mM.

To the 1 mM **HP-alkyne** (**13**) (5000 µL, 5 µmol) in 250 mM pH9.4 sodium borate buffer, was added 20 equiv. of 200 mM DMA solution of **14***, (9H-fluoren-9-yl)methyl (2-((6-(azidomethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)carbamate (custom synthesized by GVK Bio, 500 µL, 100 µmol), then added 10 equiv. of 200 mM water solution of copper(II) sulfate pentahydrate (250 µL, 50.0 µmol), followed by 15 equiv. 200 mM water solution of sodium ascorbate (sodium (R)-2-((S)-1,2-dihydroxyethyl)-4-hydroxy-5-oxo-2,5-dihydrofuran-3-olate) (375 µL, 75 µmol) and vortexed. The reaction proceeded at room temperature overnight. 1250 µL DMA was then added to facilitate solubility, and the reaction was monitored by LCMS (1 µL aliquot of reaction mixture was added to a small Eppendorf tube, followed by 1 µL of 100 mM sodium diethyldithiocarbamate, mixed well, then added 50 uL water, centrifuged using 30 µL clear

solution for LCMS) (estimated LCMS conversion >90%). Upon completion, the reaction was subjected to ethanol precipitation and then **HP-THP-NH-Fmoc** (**15**) was reconstituted in water at 2 mM. To this oligo solution was added 10% by volume of neat piperidine. The reaction was allowed to proceed at room temperature and monitored by LCMS (estimated LCMS conversion >95%). Once the reaction was complete, the "EtOH Precipitation Protocol" was followed, and then reconstituted in water at 2 mM of **HP-THP-NH_2** (**16**) for further steps. Purity of the final crude product mixture was determined by LCMS total ion count.

*See part II, off-DNA chemistry, for synthesis of **14**.

General procedure for THP cleavage

The oligo 0.25 mM in 0.1 % formic acid water solution was allowed to proceed at 60 °C for 1 h. Once the reaction was complete, the "EtOH Precipitation Protocol" was followed. For small scale reactions, the plate was directly evaporated under a gentle stream of nitrogen, and the dried pellet was submitted for testing by ASMS.

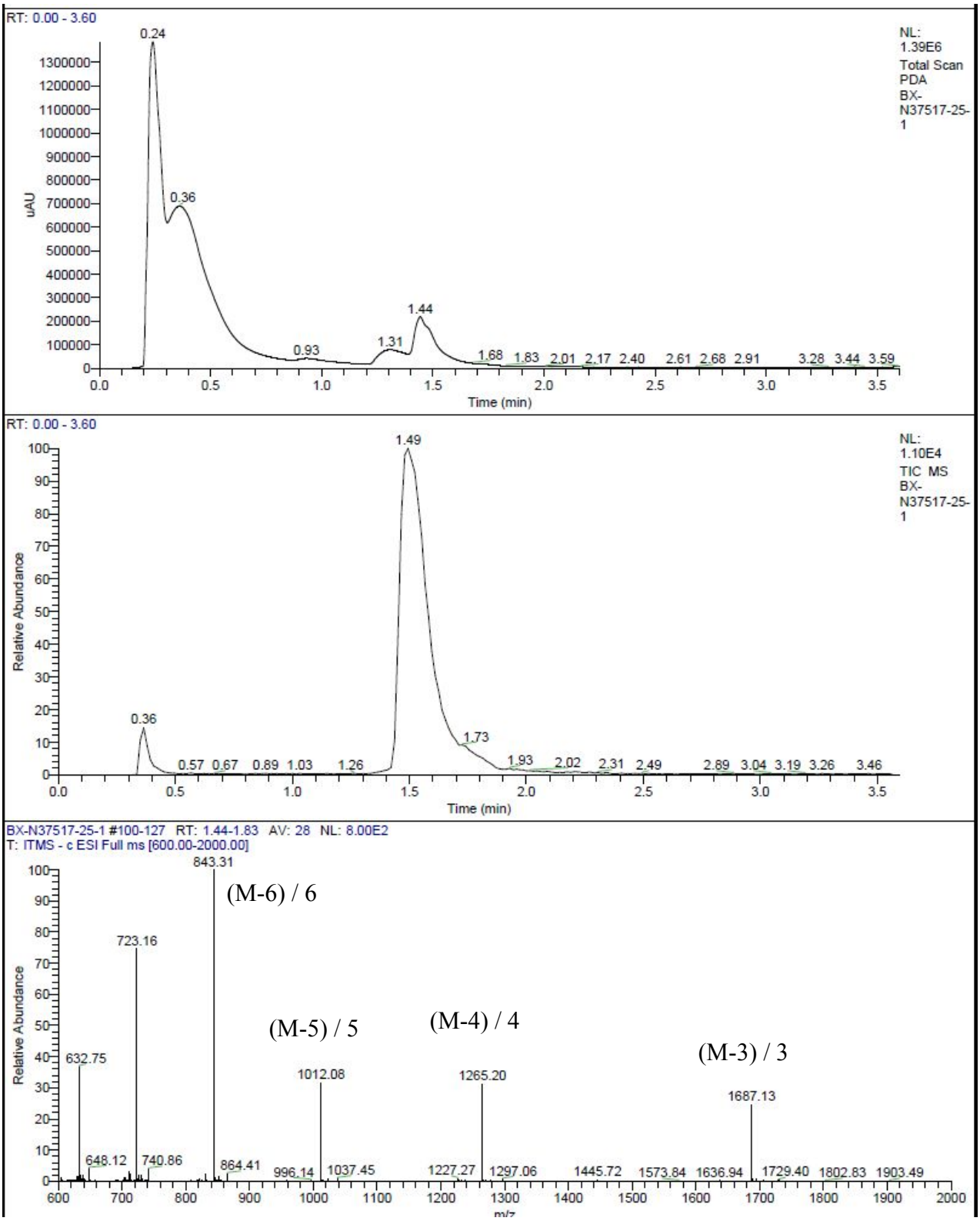


Figure S4. LCMS of **HP-alkyne** (**13**), MW 5065.36, $C_{163}H_{219}N_{52}O_{102}P_{17}$.

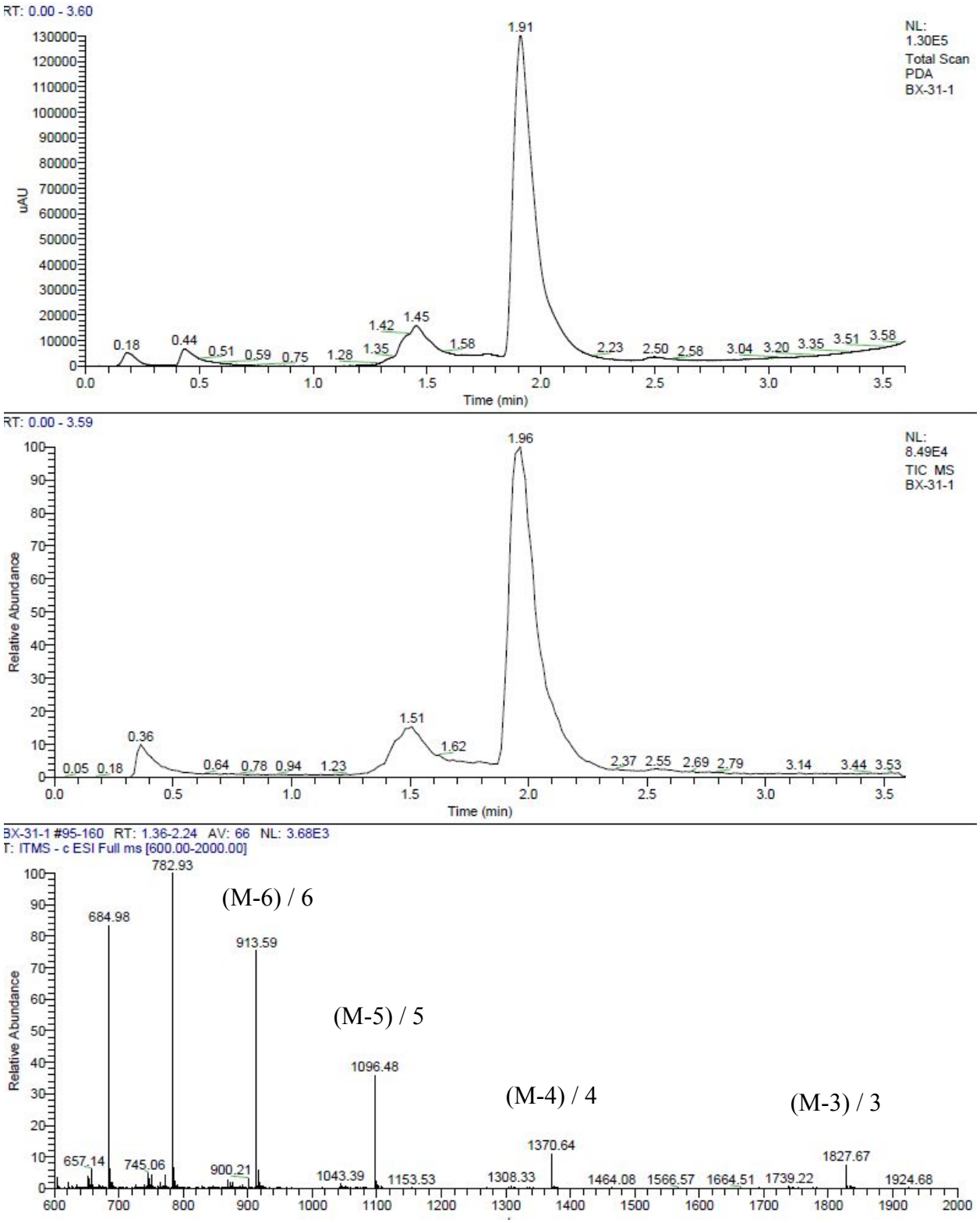


Figure S5. LCMS of **HP-THP-NH-Fmoc** (**15**), MW 5487.85, $C_{186}H_{245}N_{56}O_{106}P_{17}$.

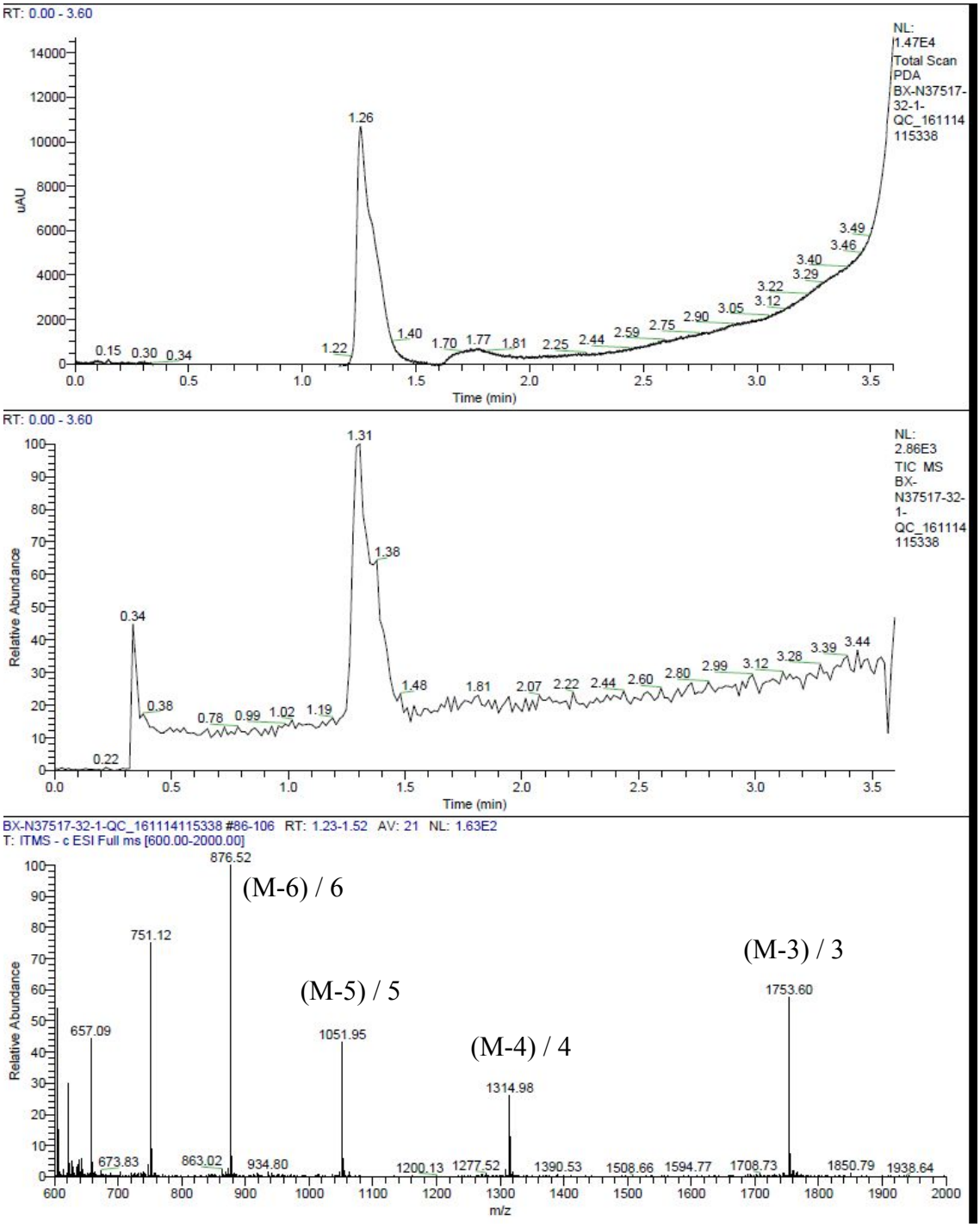
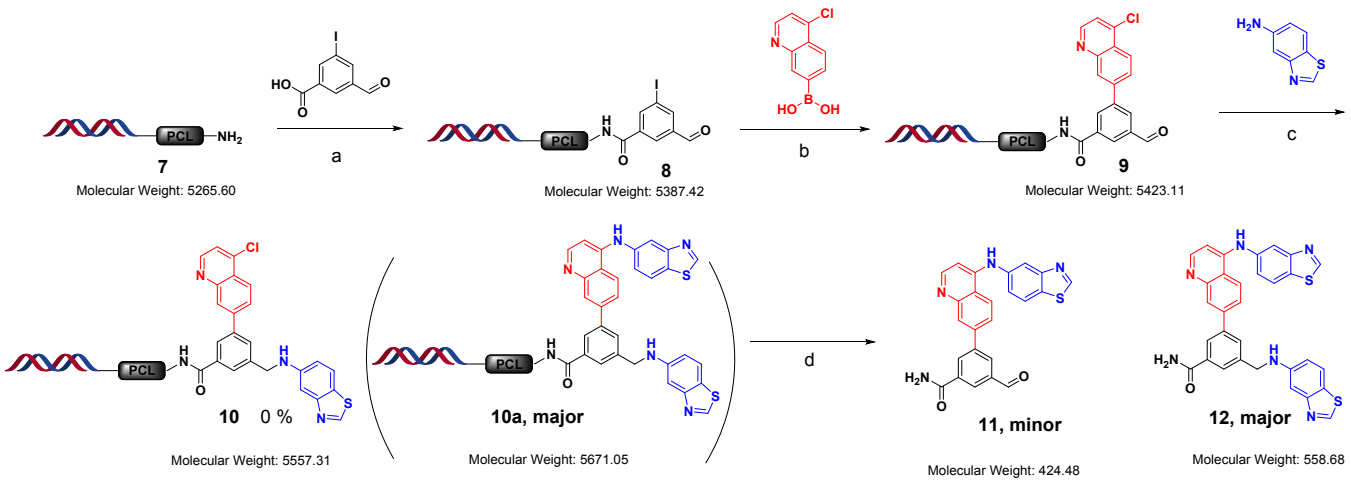


Figure S6. LCMS of **HP-THP-NH$_2$ (16)**, MW 5265.6, $C_{171}H_{235}N_{56}O_{104}P_{17}$.

Case study of Photocleavable Linker (PCL)



Scheme S3. Case study of Photocleavable Linker (**PCL**)

Synthesis of HP-PCL-Ph-I-CHO (8)

In a 2 mL plastic tube, 80 equiv. of 3-formyl-5-iodobenzoic acid (12 µmol in 60 µL of DMA) was added to a solution of **HP-PCL-NH$_2$** (**7**) (150 nmol in 150 µL of 250 mM pH 9.4 sodium borate buffer), followed by 80 equiv. of freshly prepared DMTMM water solution (12 µmol in 60 µL) and vortexed. The reaction proceeded at room temperature and was monitored by LCMS (estimated LCMS conversion >80%). Once the reaction was complete, the "EtOH Precipitation Protocol" was followed, and then reconstituted in water at 2 mM of **HP-PCL-Ph-I-CHO** (**8**) for the next step.

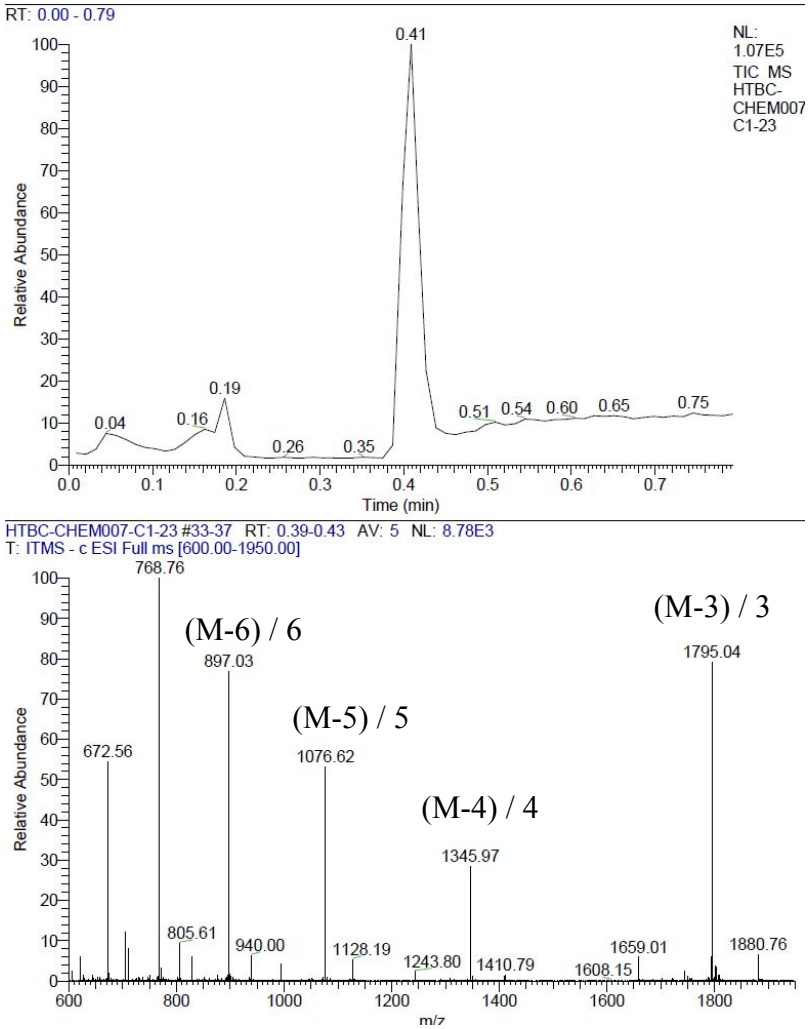


Figure S7. LCMS of **HP-PCL-Ph-I-CHO** (**8**), MW 5387.42, $C_{171}H_{226}IN_{54}O_{106}P_{17}$.

Synthesis of HP-PCL-Ph-Ar-CHO (9)

In a 2 mL plastic tube, 40 equiv. of (4-chloroquinolin-7-yl)boronic acid (6 μmol in 10 μL of DMA) and 80 equiv. of Na_2CO_3 (12 μmol in 20 μL of DMA) were added to a solution of **HP-PCL-Ph-I-CHO** (**8**) (150 nmol in 150 μL of water), followed by 0.5 equiv. of degassed $Pd(PPh_3)_4$ (75 nmol in 25 μL of acetonitrile) and vortexed. The reaction was allowed to proceed at 80 °C for 5 h, and was monitored by LCMS (estimated LCMS conversion >70%). Once the reaction was deemed complete, the reaction mixture was centrifuged and then the supernatant was carefully removed from the Pd-containing precipitate using a pipette. The "EtOH Precipitation Protocol"

was followed and the product reconstituted in water at 2 mM of **HP-PCL-Ph-Ar-CHO** (**9**) for the next step.

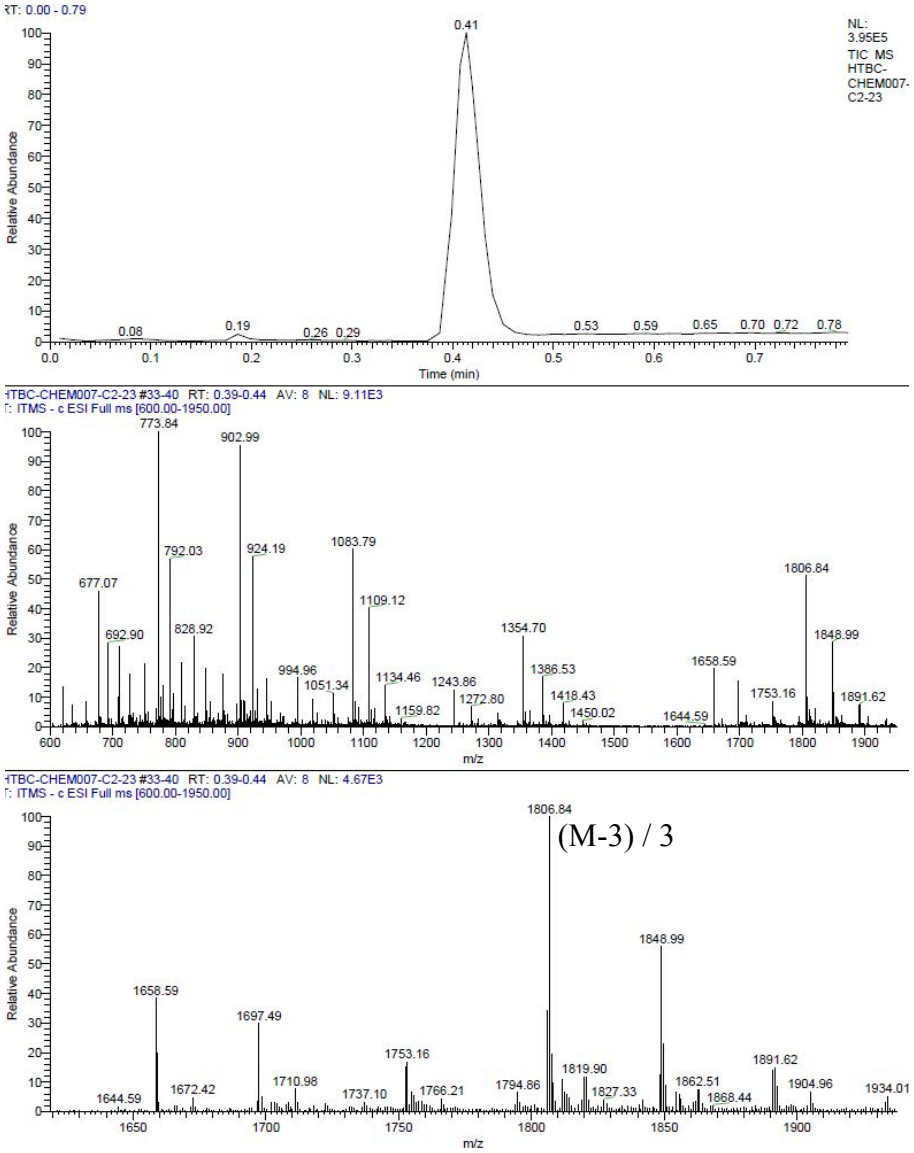


Figure S8. LCMS of **HP-PCL-Ph-Ar-CHO** (**9**), MW 5423.11, $C_{180}H_{231}ClN_{55}O_{106}P_{17}$.

Attempted synthesis of HP-PCL-Ph-Ar-NHAr (10)

In a 2 mL plastic tube, 80 equiv. of benzo[d]thiazol-5-amine (12 µmol in 60 µL of DMA) was added into a solution of **HP-PCL-Ph-Ar-CHO** (**9**) (150 nmol in 150 µL of 250 mM pH 5.5 sodium phosphate buffer), followed by 80 equiv. of freshly prepared sodium cyanoborohydride ($NaCNBH_3$) water solution (12 µmol in 30 µL) and vortexed. The reaction was allowed to proceed

at 60 °C for 16 h and was monitored by LCMS (estimated LCMS conversion >60%, **10a**). Once the reaction was complete, the "EtOH Precipitation Protocol" was followed and then reconstituted in water at 2 mM for the next step.

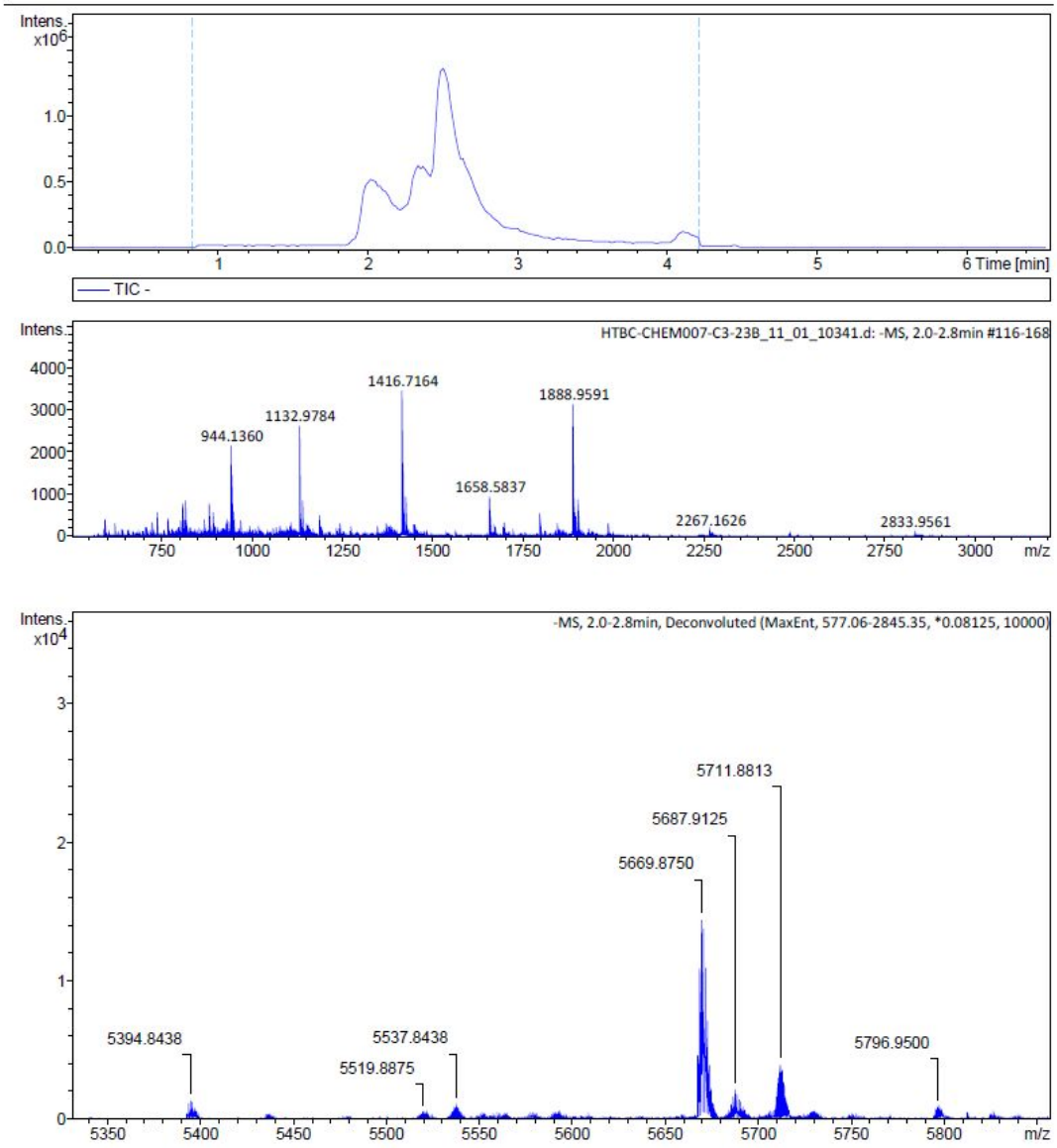


Figure S9. LCMS of attempted synthesis of **HP-PCL-Ph-Ar-NHAr** (**10**), MW 5557.31, $C_{187}H_{237}ClN_{57}O_{105}P_{17}S$; **10a**, MW 5671.05, $C_{194}H_{242}N_{59}O_{105}P_{17}S_2$.

Generation and detection of (11) and (12)

The "General procedure for PCL cleavage" described above was followed for the generation of **11** and **12**, also see the part III AS-MS. Compound **11** LCMS (*m/z*): $[M+H]^+$ calc'd for $C_{31}H_{22}N_6OS_2$, 559.13; found, 559.21. Compound **12** LCMS (*m/z*): $[M+H]^+$ calc'd for $C_{24}H_{16}N_4O_2S$, 425.10; found 425.23.

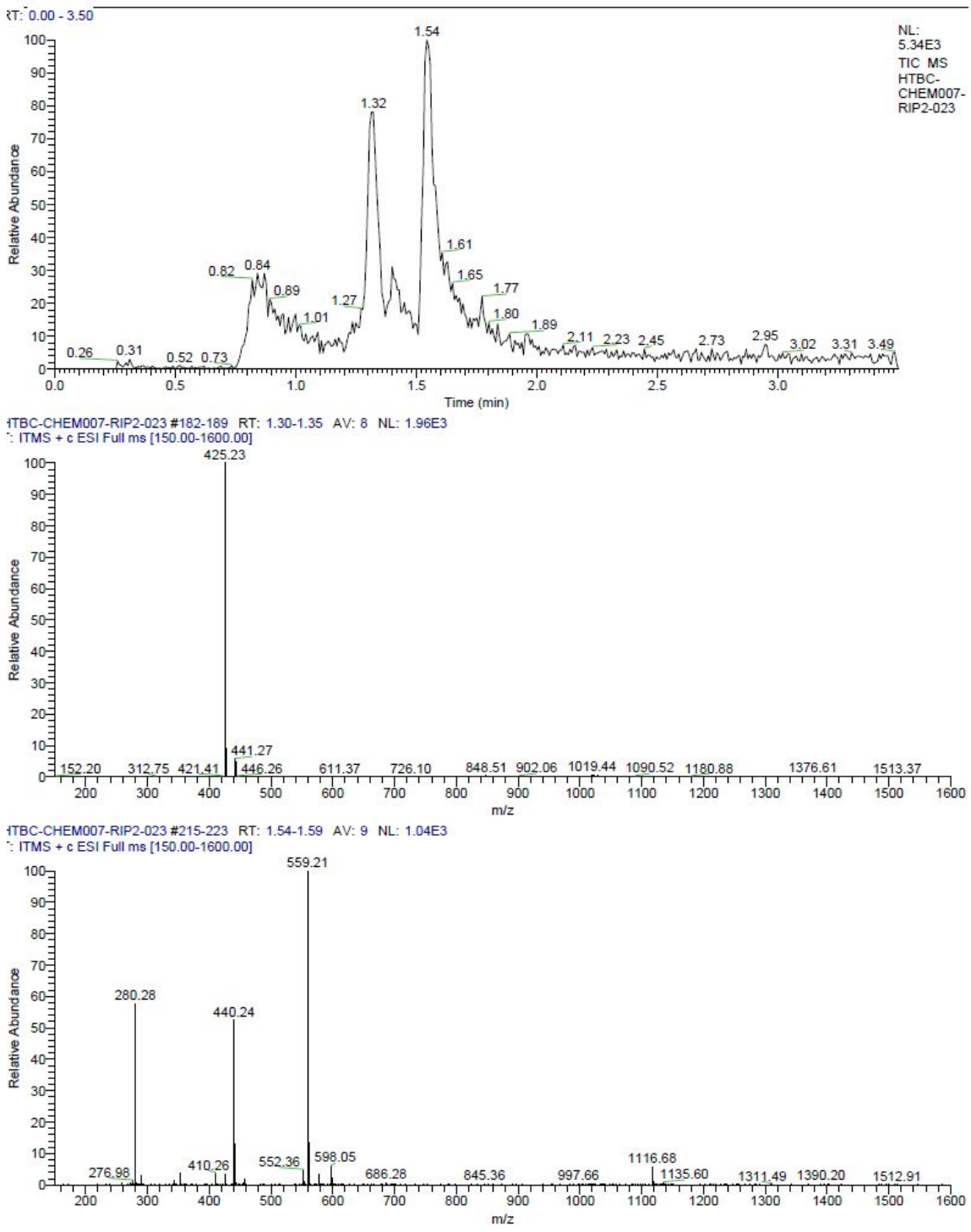
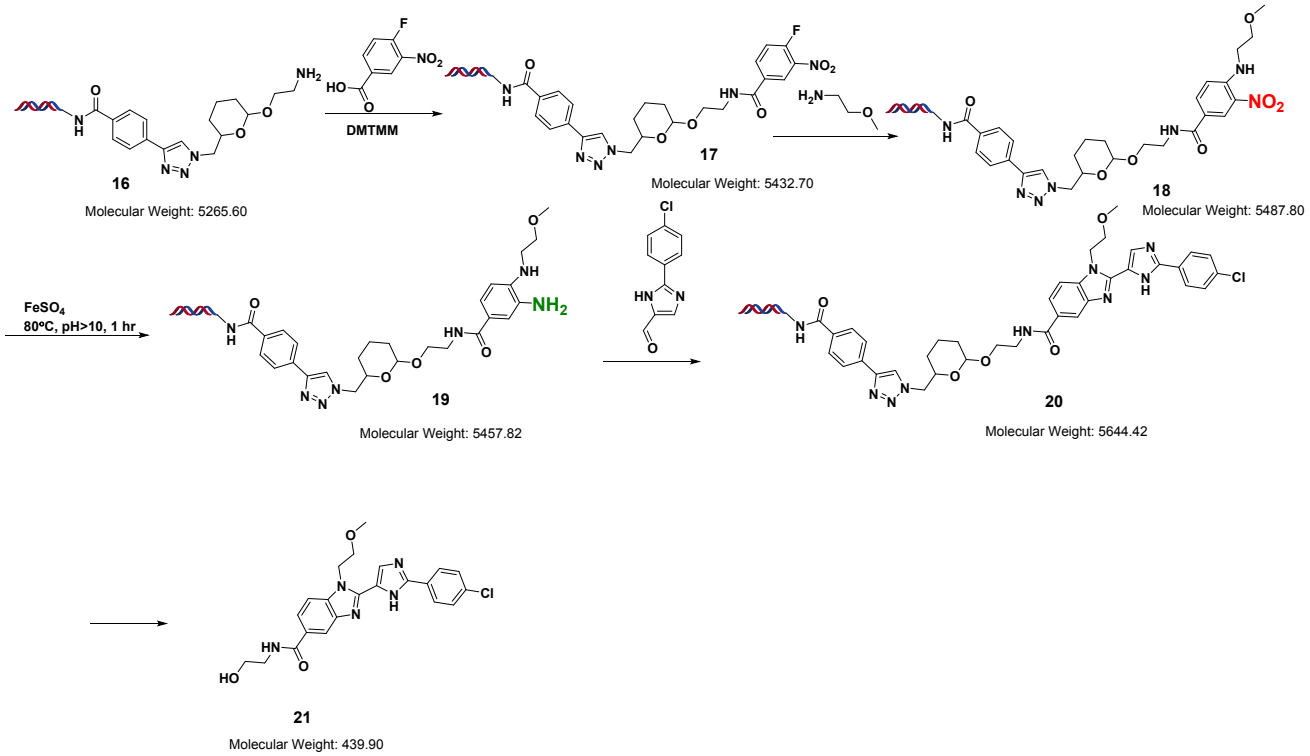


Figure S10. LCMS of **11** and **12**

Case study of THP



Scheme S4. Case study of **THP**

Synthesis of HP-THP-Ph-F-NO_2 (17)

In a 2 mL plastic tube, 40 equiv. of 4-fluoro-3-nitrobenzoic acid (100 µmol in 0.5 mL of DMA) was added into a solution of **HP-THP-NH_2 (16)** (1 µmol in 1 mL of 250 mM pH 9.4 sodium borate buffer), followed by 100 equiv. of freshly prepared DMTMM water solution (100 µmol in 0.5 mL) and vortexed. The reaction was allowed to proceed at room temperature and was monitored by LCMS(estimated LCMS conversion >80%). Once the reaction was deemed complete, the "EtOH Precipitation Protocol" was followed, and the product was reconstituted in water at 2 mM of **HP-THP-NH_2 (17)** for next step.

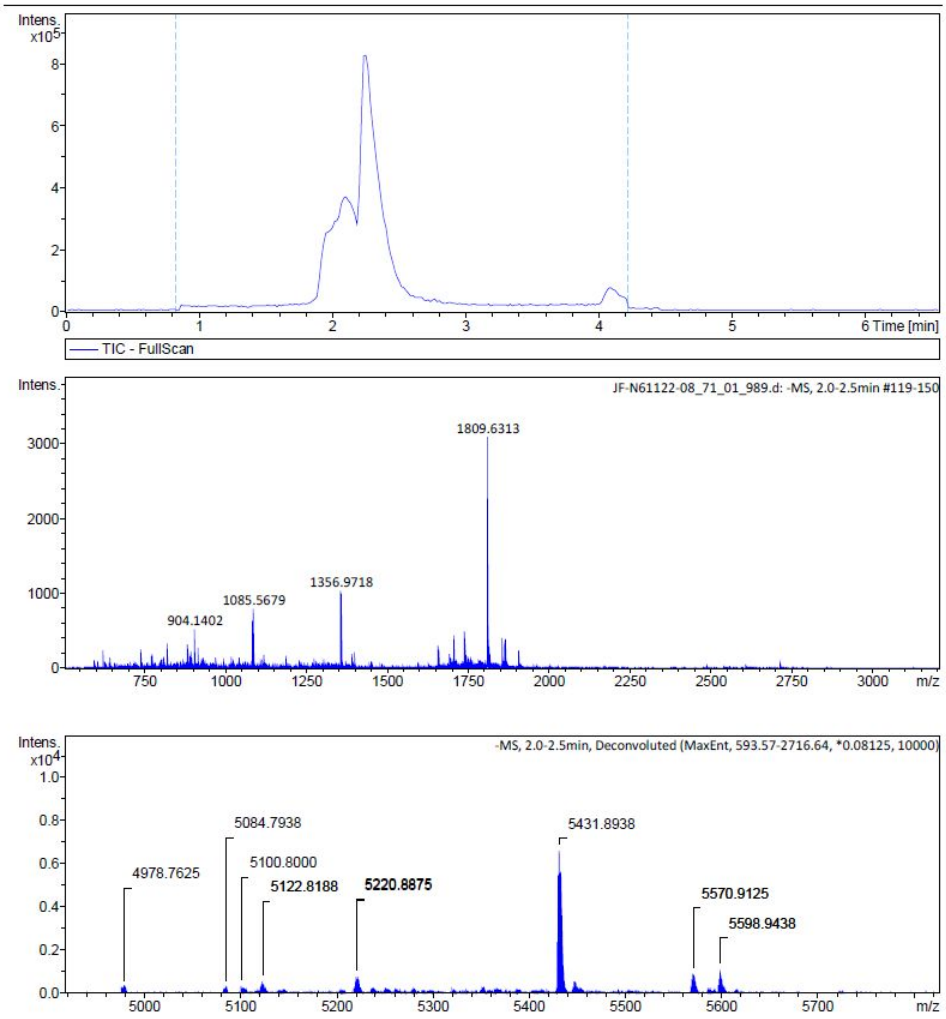


Figure S11. HP-THP-Ph-F-NO2 (**17**), MW 5432.70, $C_{178}H_{237}FN_{57}O_{107}P_{17}$.

Synthesis of HP-THP-Ph-NHR-NO_2 (18)

In a 2 mL plastic tube, 80 equiv. of 2-methoxyethan-1-amine (24 µmol in 120 µL of acetonitrile: water=50:50) was added to a solution of **HP-THP-Ph-F-NO_2 (17)** (300 nmol in 300 µL of 250 mM pH 9.4 sodium borate buffer) and vortexed. The reaction was allowed to proceed at 60 °C for 1 h and was monitored by LCMS (estimated LCMS conversion >85%). Once the reaction was deemed complete, the "EtOH Precipitation Protocol" was followed, and the product reconstituted in water at 2 mM of **HP-THP-Ph-NHR-NO_2 (18)** for the next step.

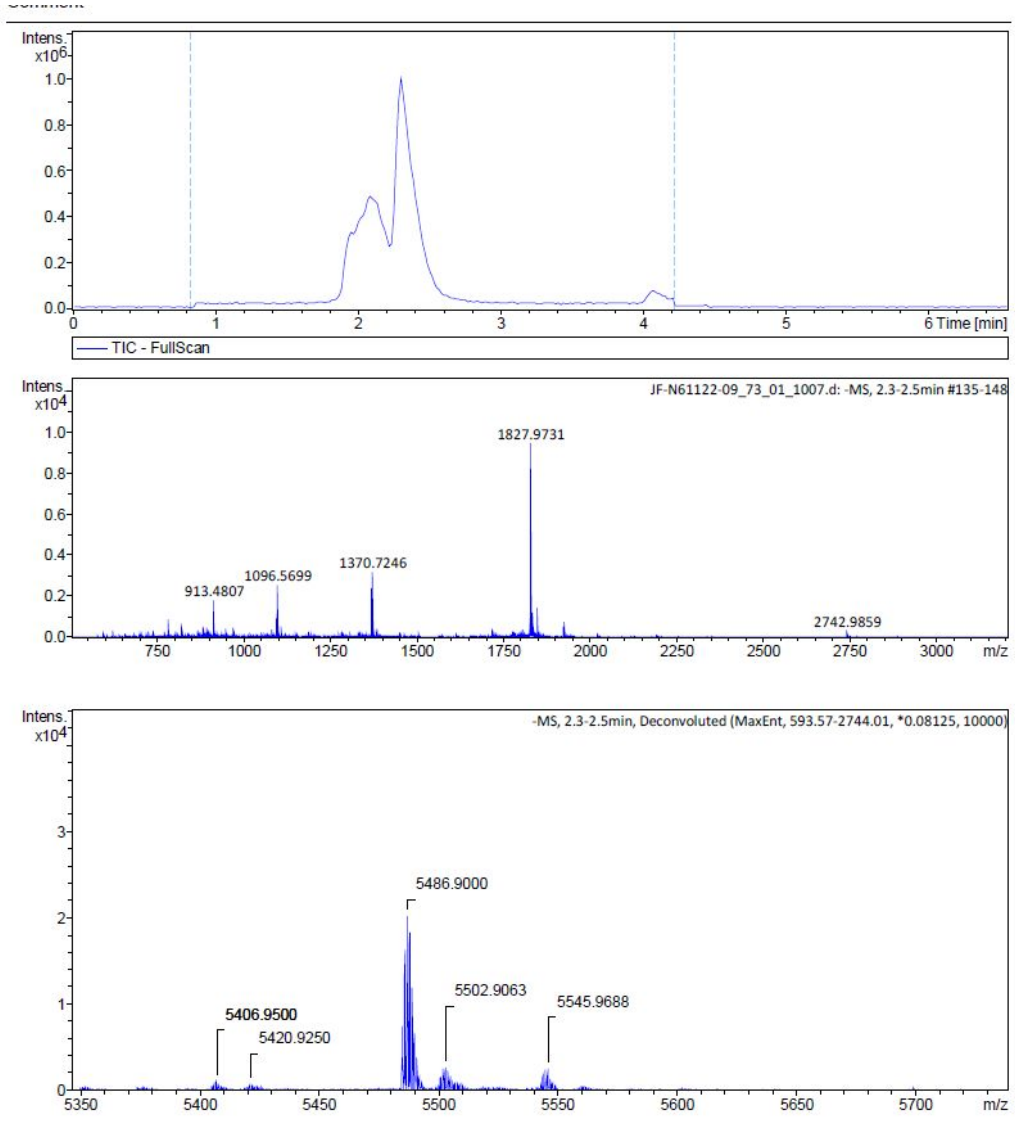


Figure S12. LCMS of **HP-THP-Ph-NHR-NO_2 (18)**, MW 5487.80, $C_{181}H_{245}N_{58}O_{108}P_{17}$.

Synthesis of HP-THP-Ph-NHR-NH_2 (19)

In a 2 mL plastic tube, 40 equiv. of $FeSO_4$ (12 µmol in 60 µL of water) was added to a solution of **HP-THP-Ph-NHR-NO_2 (18)** (300 nmol in 300 µL of 250 mM pH 9.4 sodium borate buffer, with 10% v/v 1M NaOH) and vortexed. The reaction proceeded at 80 °C for 2 h and was monitored by LCMS (estimated LCMS conversion >60%). Once the reaction was deemed complete, the reaction mixture was centrifuged and a pipette was used to carefully remove the supernatant from the precipitate to follow the "EtOH Precipitation Protocol". The product was reconstituted in water at 2 mM of **HP-THP-Ph-NHR-NH_2 (19)** for the next step.

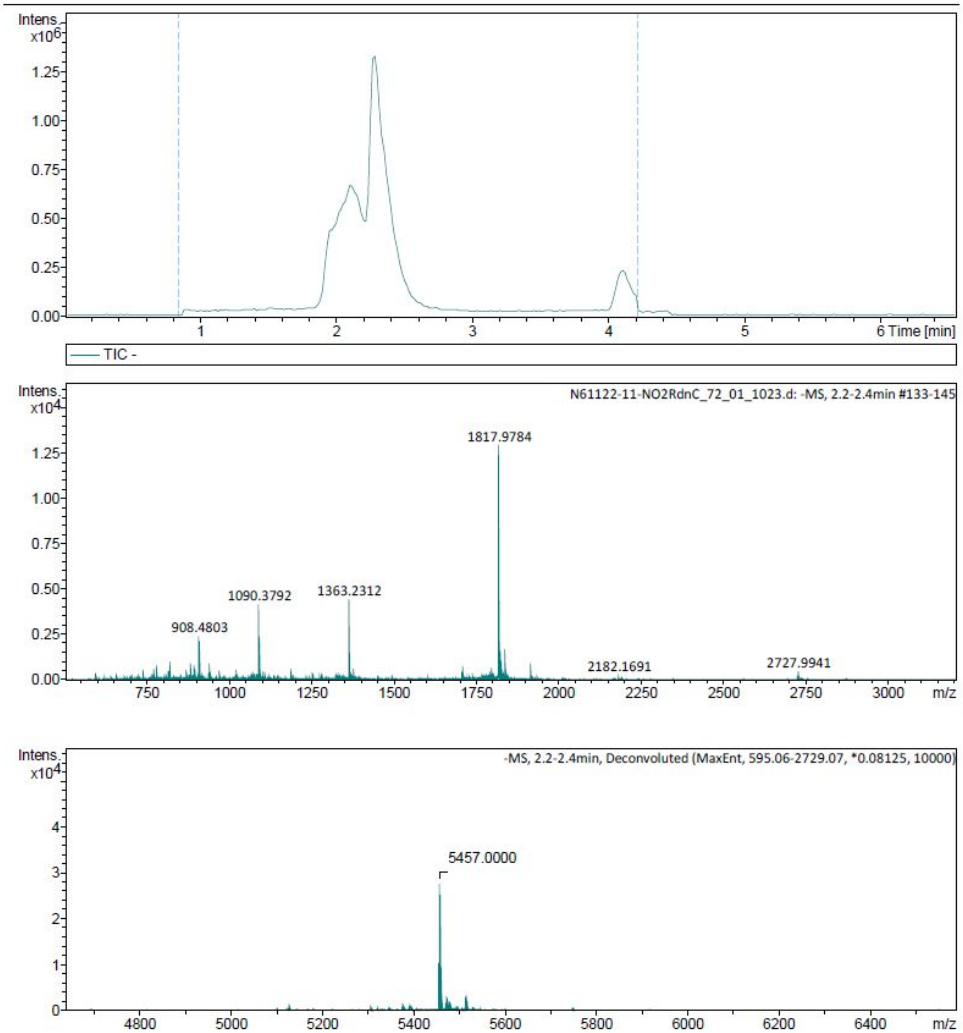


Figure S13. LCMS of **HP-THP-Ph-NHR-NH_2 (19)**, MW 5457.82, $C_{181}H_{247}N_{58}O_{106}P_{17}$.

Synthesis of HP-THP-Benzoimidazole (20)

In a 2 mL plastic tube, 60 equiv. of 2-(4-chlorophenyl)-1H-imidazole-5-carbaldehyde (18 μmol in 90 μL of DMA) was added to a solution of **HP-THP-Ph-NHR-NH_2** (**19**) (300 nmol in 300 μL of 250 mM pH 5.5 sodium phosphate buffer) and vortexed. The reaction was allowed to proceed at room temperature overnight and was monitored by LCMS (estimated LCMS conversion >60%). Once the reaction was complete, the "EtOH Precipitation Protocol" was followed and then the product was reconstituted in water at 2 mM **HP-THP-Benzimidazole** (**20**) for the next step.

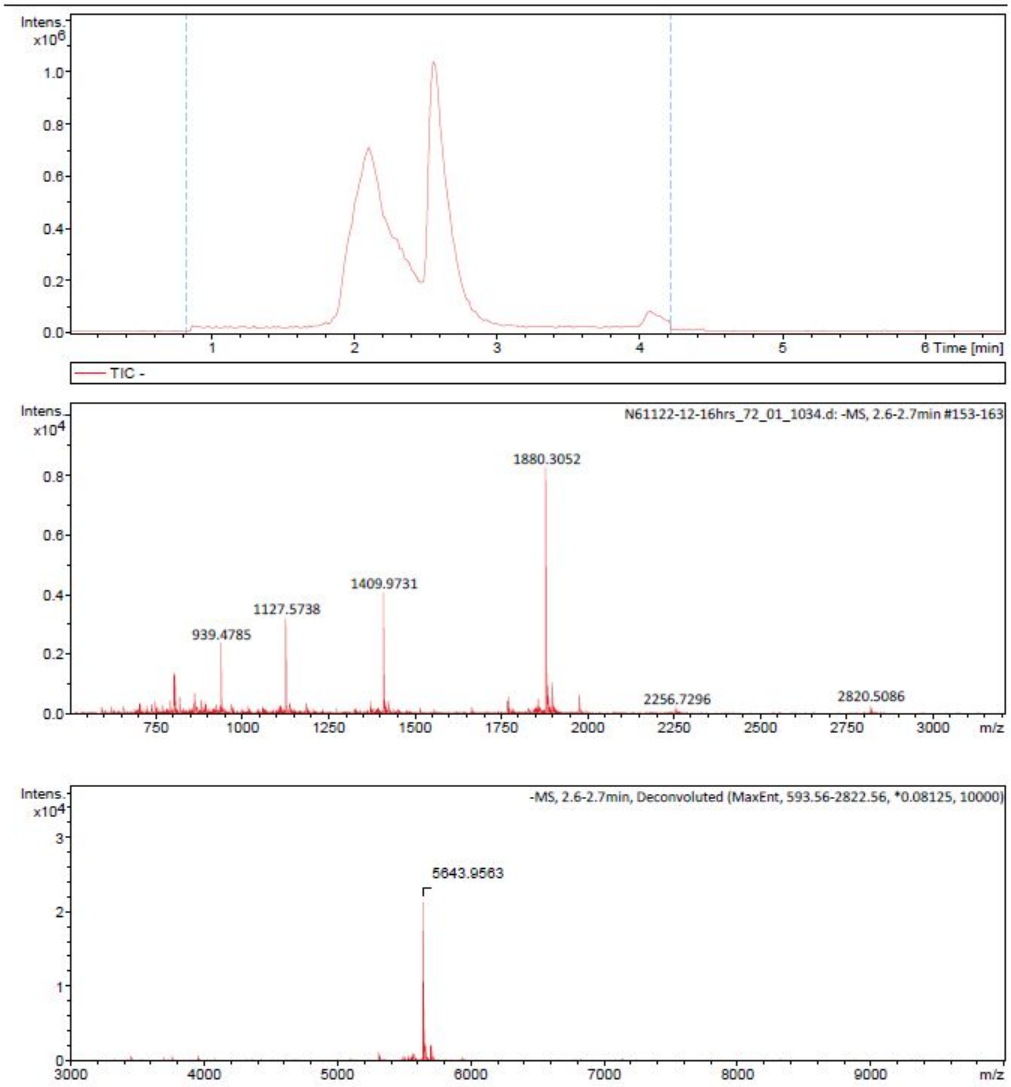


Figure S14. LCMS of **HP-THP-Benzimidazole** (**20**), MW 5644.42, $C_{191}H_{250}ClN_{60}O_{106}P_{17}$.

Detection of (21)

The "General procedure for THP cleavage" described above was followed for the generation and detection of **21**, also see part III AS-MS. LCMS (*m/z*): $[M+H]^+$ calc'd for $C_{22}H_{22}ClN_5O_3$, 440.14; found, 440.25.

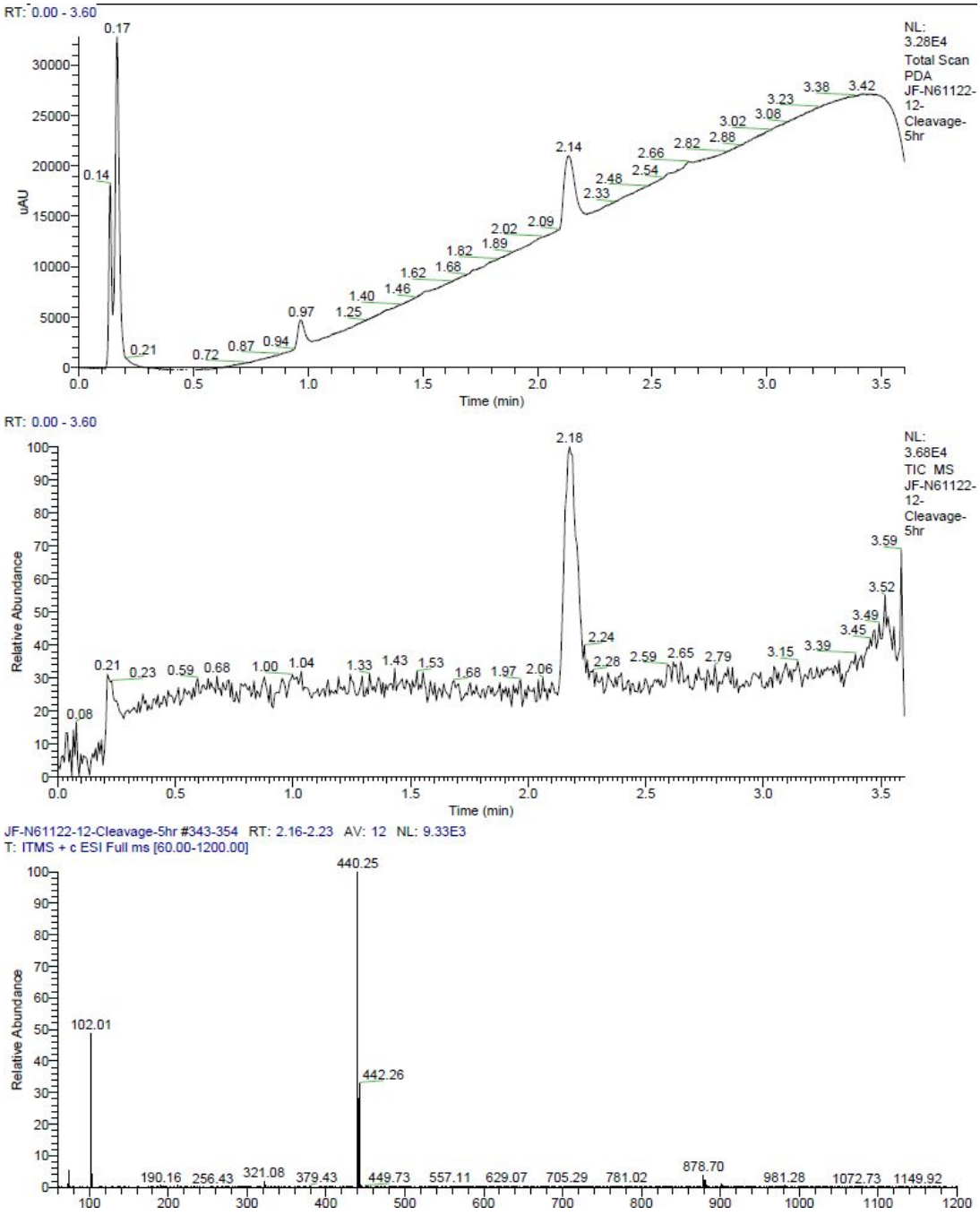


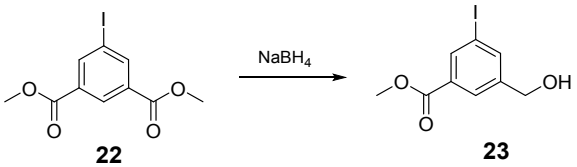
Figure S15. LCMS of **21**

Part II: Off-DNA chemistry

Proton (^{1}H) NMR spectra were recorded at 400 MHz at ambient temperature and chemical shifts are reported in parts per million. Data for ^{1}H NMR are reported as follows: chemical shift, , multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), integration and

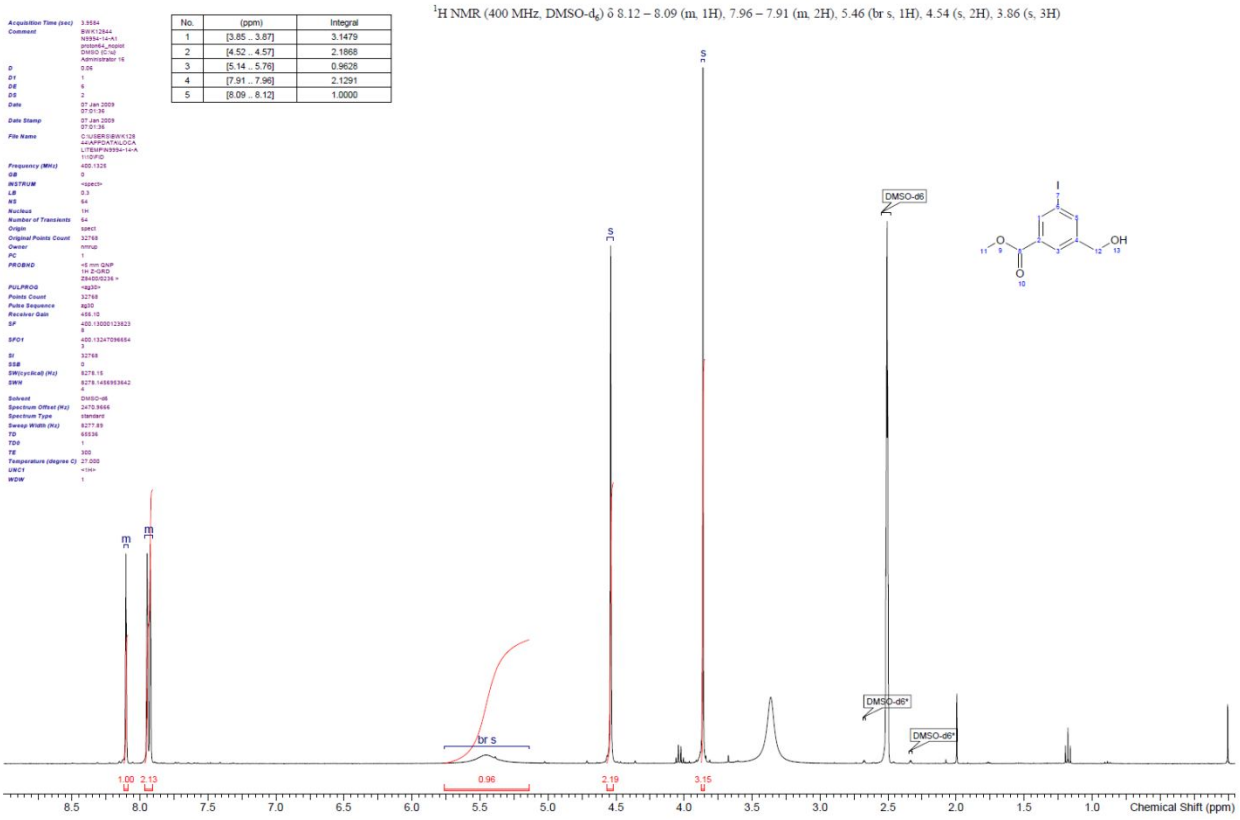
coupling constants. All reagents were used as supplied by Sigma-Aldrich, unless otherwise stated. All reactions were carried out in oven-dried glassware, under an argon atmosphere unless otherwise noted.

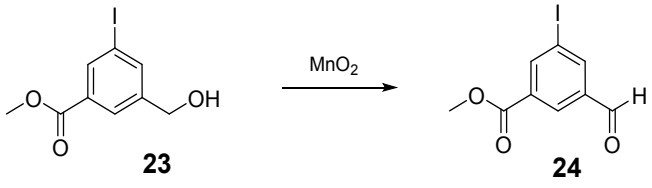
Synthesis of 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-(4-chloro-7-quinolinyl)-N-[2-(methyloxy)ethyl]benzamide (1).



Step 1: To a heterogeneous mixture of dimethyl 5-iodo-1,3-benzenedicarboxylate (**22**, 3.50 g, 10.93 mmol) and sodium borohydride (0.414 g, 10.93 mmol) in tetrahydrofuran (THF) (35 mL) was added methanol (5.0 mL) dropwise. Evolution of gas was observed. The mixture was heated at 50 °C for 1 h during which time the reaction became homogeneous. LCMS analysis indicated the reaction was ~60% desired product, 30% starting material, and 10% bis-reduced material. An additional 100 mg of sodium borohydride was added and the reaction allowed to stir at 50 °C for an additional h. LCMS analysis indicated that more of the starting material was consumed, but more undesired bis-reduced material was also present. A decision was made that the reaction had reached a point of diminishing returns. After cooling to room temperature, the solvent was removed under reduced pressure. The remaining residue was partitioned between ethyl acetate and water. The layers were separated, and the organic layer washed with brine and concentrated. Purification of the crude material by silica gel chromatography (CombiFlash; 10-30% ethyl acetate/hexanes) provided methyl 3-(hydroxymethyl)-5-iodobenzoate (**23**, 2.367 g, 74%) as a white solid. LCMS (*m/z*): $[M+H]^+$ calc'd for $C_9H_{10}IO_3$, 293.0; found, 292.8. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.12 – 8.09 (m, 1H), 7.96 – 7.91 (m, 2H), 5.46 (br s, 1H), 4.54 (s, 2H), 3.86 (s, 3H).

^{1}H NMR of **23**

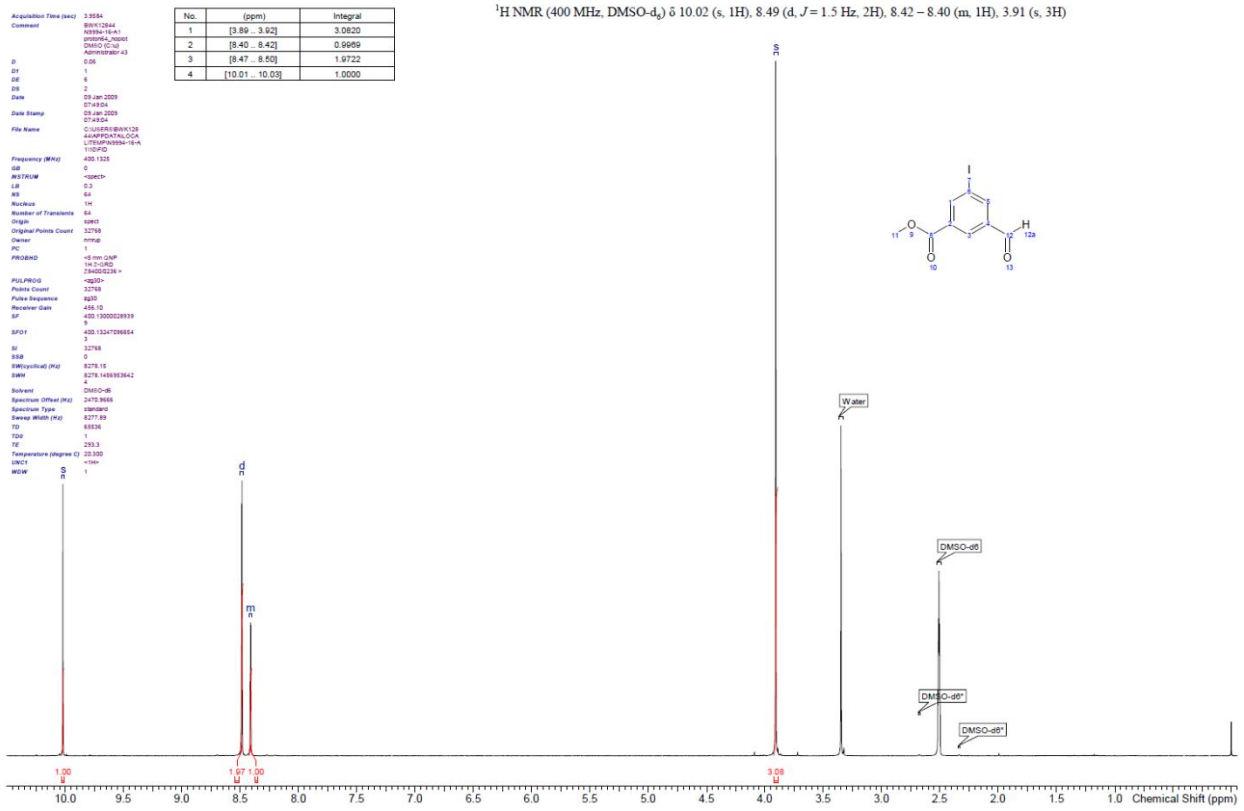


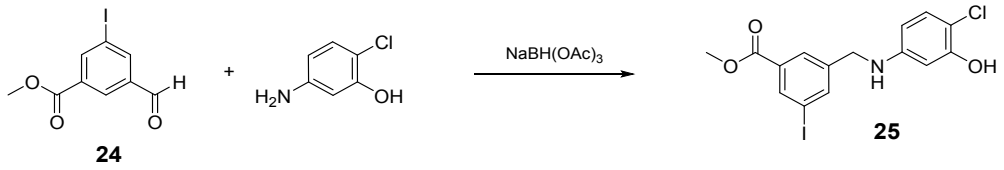


Step 2: A heterogeneous mixture of methyl 3-(hydroxymethyl)-5-iodobenzoate (**23**, 2.360 g, 8.08 mmol) and manganese dioxide (3.51 g, 40.4 mmol) in ethyl acetate (20 mL) was heated at reflux for 2.5 h. Analysis of the reaction by LCMS confirmed ~60% conversion to product. An

additional 2.0 g of manganese dioxide was added and the reaction allowed to heat at reflux overnight (~15 h). After cooling to room temperature, the reaction was filtered through a 0.45 micron PTFE filter and concentrated. Purification of the crude material by silica gel chromatography (CombiFlash; 0-35% ethyl acetate/hexanes) provided methyl 3-formyl-5-iodobenzoate (**24**, 1.360 g, 58%). LCMS (*m/z*): $[M+H]^+$ calc'd for $C_9H_8IO_3$, 290.9; found, 290.8. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.02 (s, 1H), 8.49 (d, J = 1.5 Hz, 2H), 8.42 – 8.40 (m, 1H), 3.91 (s, 3H).

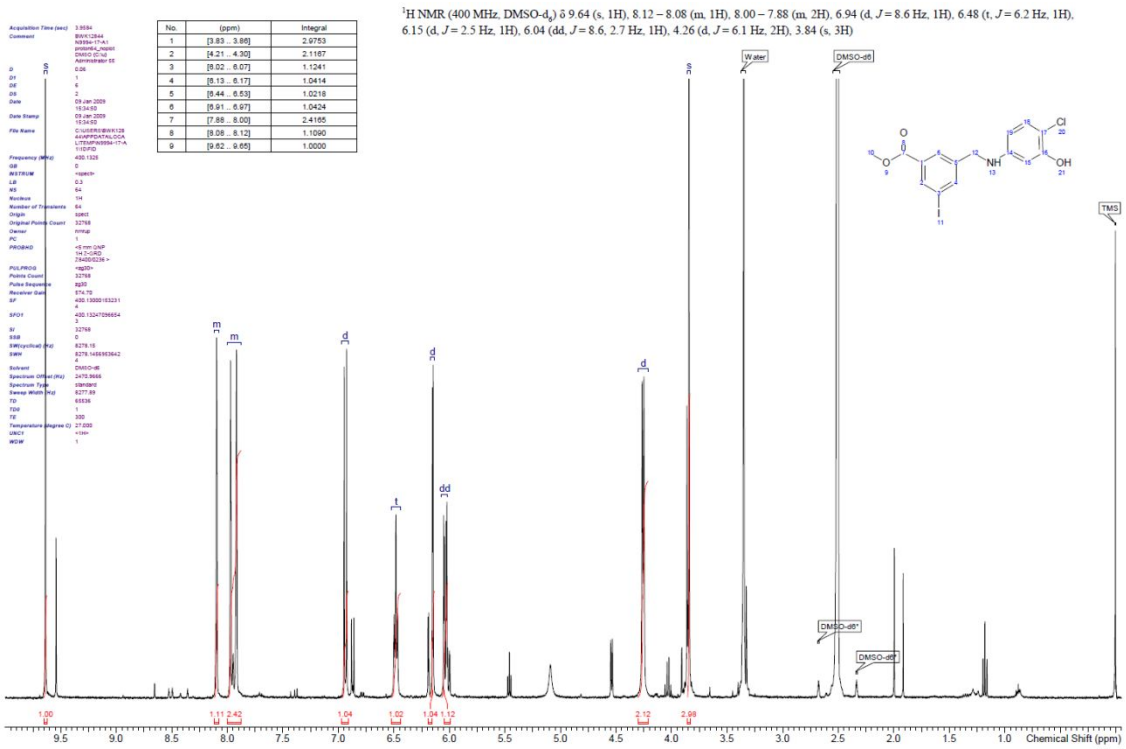
^{1}H NMR of **24**

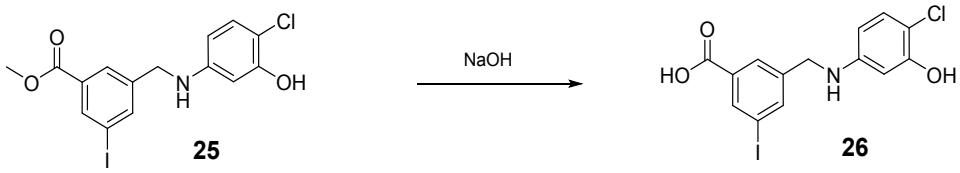




Step 3: To a heterogeneous mixture of methyl 3-formyl-5-iodobenzoate (**24**, 0.300 g, 1.034 mmol), 5-amino-2-chlorophenol (0.156 g, 1.086 mmol), and acetic acid (0.062 mL, 1.086 mmol) in 1,2-dichloroethane (DCE) (5 mL) was added sodium triacetoxyborohydride (0.263 g, 1.241 mmol) in one portion. Over the next 1.5-2 h, the reaction became homogeneous, then changed back to heterogeneous. The reaction was partitioned between ethyl acetate and 0.1 N NaOH solution, and the layers were separated. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated to give methyl 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodobenzoate (**25**, 430 mg, 100%). LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{15}H_{14}ClINO_3$, 418.0 and 420.0; found, 417.8 and 419.7. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.64 (s, 1H), 8.12 – 8.08 (m, 1H), 8.00 – 7.88 (m, 2H), 6.94 (d, J = 8.6 Hz, 1H), 6.48 (t, J = 6.2 Hz, 1H), 6.15 (d, J = 2.5 Hz, 1H), 6.04 (dd, J = 8.6, 2.7 Hz, 1H), 4.26 (d, J = 6.1 Hz, 2H), 3.84 (s, 3H).

^{1}H NMR of **25**

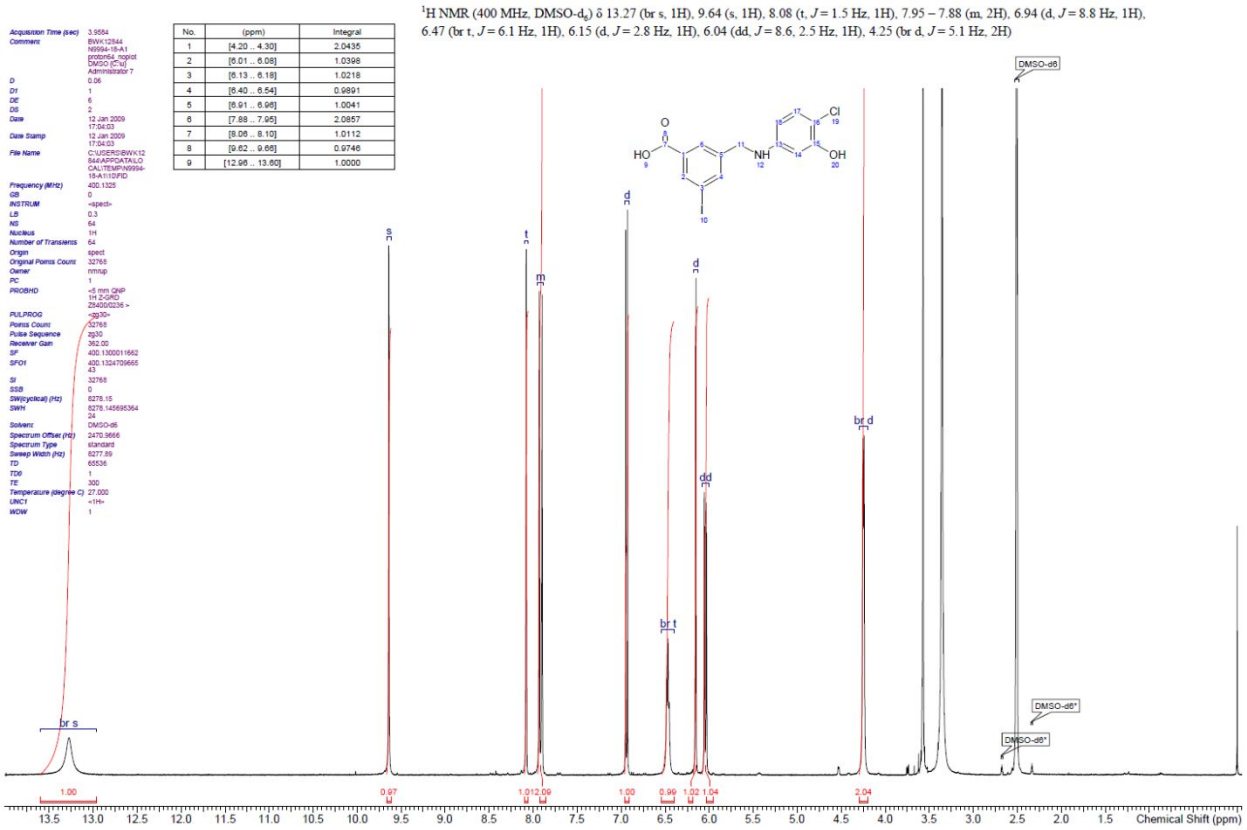


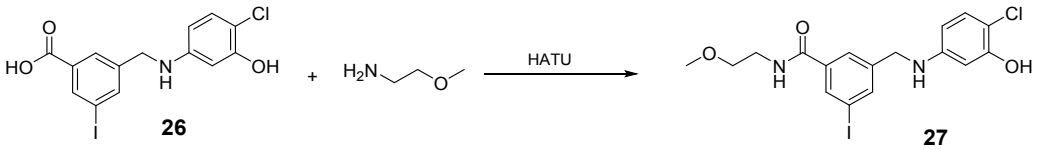


Step 4: A solution of methyl 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodobenzoate (**25**, 0.430 g, 1.030 mmol) in 1,4-dioxane (5 mL) and sodium hydroxide solution (2 M) (2.57 mL, 5.15 mmol) was allowed to stir for 2 h at room temperature. The reaction was acidified to pH ~4 by the addition of 2 M HCl solution (~2.6 mL). A precipitate began to form. The reaction vessel was cooled to 0 °C with an ice/water bath. The mixture was filtered through a fritted funnel and the collected solid washed with a minimal amount of water and thoroughly dried to give 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodobenzoic acid (**26**, 0.360 g, 87%). LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{14}H_{12}ClINO_3$, 404.0 and 406.0; found, 403.8 and 405.7. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.27 (br s, 1H), 9.64 (s, 1H), 8.08 (t, J = 1.5 Hz, 1H), 7.95 – 7.88

(m, 2H), 6.94 (d, J = 8.8 Hz, 1H), 6.47 (br t, J = 6.1 Hz, 1H), 6.15 (d, J = 2.8 Hz, 1H), 6.04 (dd, J = 8.6, 2.5 Hz, 1H), 4.25 (br d, J = 5.1 Hz, 2H).

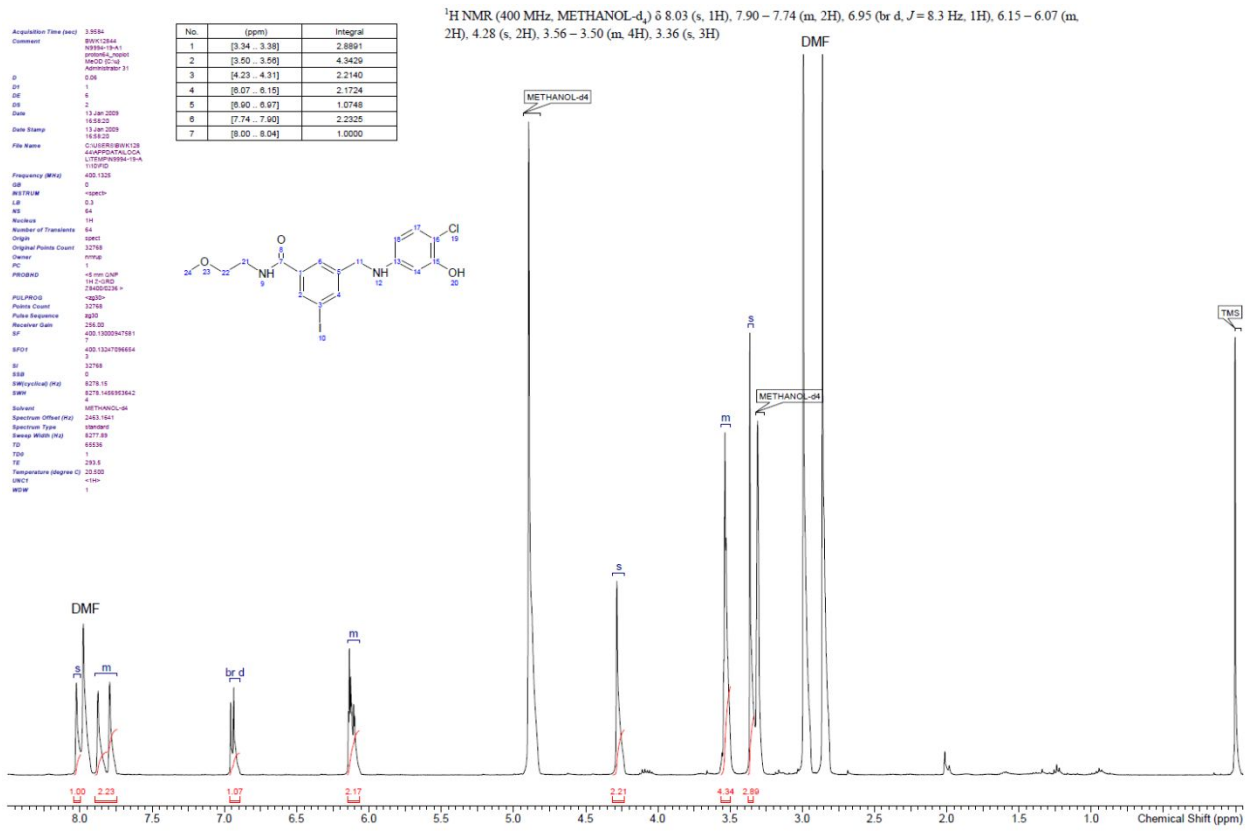
^{1}H NMR of **26**

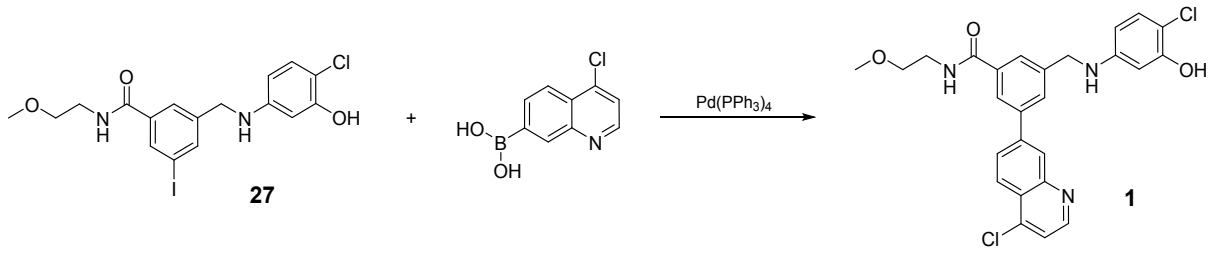




Step 5: To a solution of 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodobenzoic acid (**26**, 0.100 g, 0.248 mmol), 2-methoxyethylamine (0.023 mL, 0.260 mmol), and DIEA (0.043 mL, 0.248 mmol) in *N*,*N*-Dimethylformamide (DMF) (2 mL) was added HATU (0.099 g, 0.260 mmol) in one portion. The reaction was allowed to stir for 30 min. LCMS analysis indicated approximately 40% starting material remained. A second portion of 2-methoxyethylamine (0.023 mL) and HATU (99 mg) was sequentially added. The reaction was allowed to stir for 1 h. The reaction was partitioned between ethyl acetate and water. The layers were separated, and the organic layer washed with brine, and concentrated. Purification of the crude material by silica gel chromatography (CombiFlash; 20-60% ethyl acetate/hexanes) provided 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodo-N-[2-(methyloxy)ethyl]benzamide (**27**, 98 mg, 86%). LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{17}H_{19}ClIN_2O_3$, 461.0 and 463.0; found, 461.0 and 462.9. ^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.03 (s, 1H), 7.90 – 7.74 (m, 2H), 6.95 (br d, J = 8.3 Hz, 1H), 6.15 – 6.07 (m, 2H), 4.28 (s, 2H), 3.56 – 3.50 (m, 4H), 3.36 (s, 3H).

^{1}H NMR of **27**

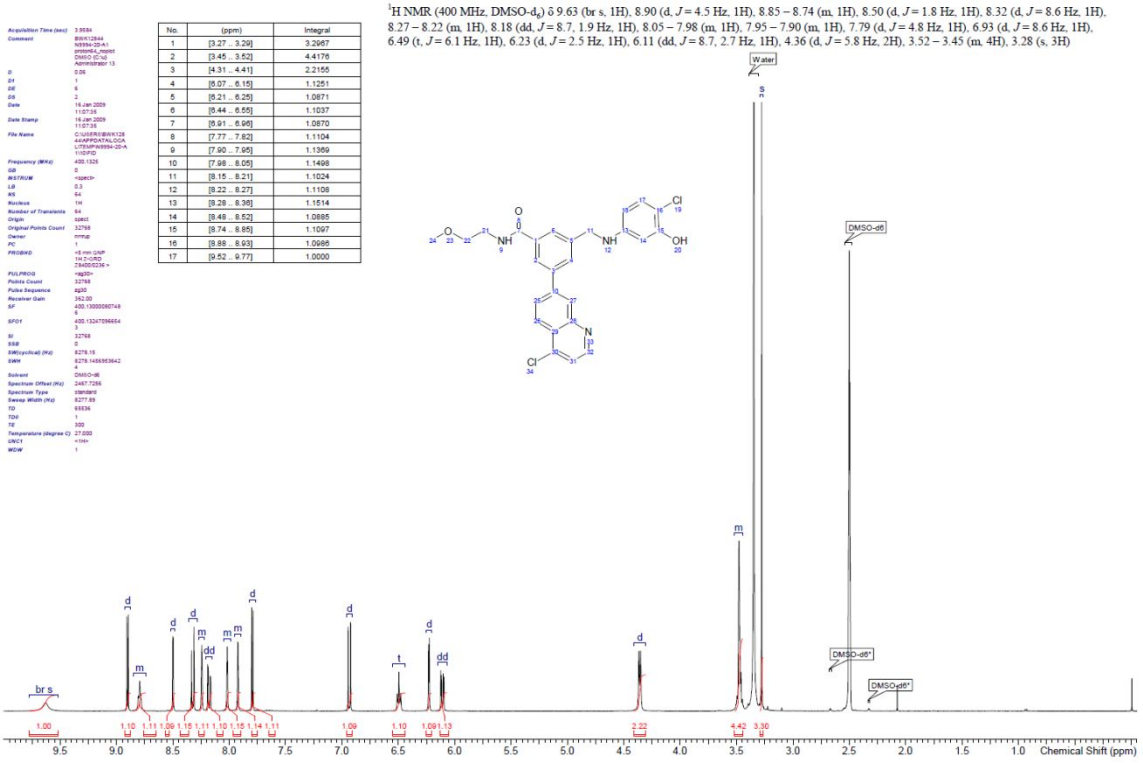




Step 6: To a solution of 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-iodo-N-[2-(methyloxy)ethyl]benzamide (**27**, 0.090 g, 0.195 mmol) in 1,2-dimethoxyethane (DME) (2 mL) was added palladium tetrakis (0.011 g, 9.77 µmol) in one portion. The reaction was purged with nitrogen gas and the vessel sealed. After stirring for ~10 min, (4-chloro-7-quinolinyl)boronic acid (0.041 g, 0.195 mmol) and sodium carbonate solution (0.195 mL, 0.391 mmol) were sequentially added. This heterogeneous mixture was irradiated in a microwave reactor at 100 °C for 30 min. The reaction was filtered through a 0.45 micron PTFE acrodisc and diluted with DMSO.

Purification of the crude material using reverse phase HPLC [40-65% acetonitrile:water (0.1% NH_4OH modifier), 30 mm Gemini column, 47 mL/min] gave 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-(4-chloro-7-quinolinyl)-N-[2-(methyloxy)ethyl]benzamide (**1**) (20 mg, 21%) (RT=6.9 min) as a white solid. LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{26}H_{24}Cl_2N_3O_3$, 496.1 and 498.1; found, 496.0 and 498.1. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.63 (br s, 1H), 8.90 (d, *J* = 4.5 Hz, 1H), 8.85 – 8.74 (m, 1H), 8.50 (d, *J* = 1.8 Hz, 1H), 8.32 (d, *J* = 8.6 Hz, 1H), 8.27 – 8.22 (m, 1H), 8.18 (dd, *J* = 8.7, 1.9 Hz, 1H), 8.05 – 7.98 (m, 1H), 7.95 – 7.90 (m, 1H), 7.79 (d, *J* = 4.8 Hz, 1H), 6.93 (d, *J* = 8.6 Hz, 1H), 6.49 (t, *J* = 6.1 Hz, 1H), 6.23 (d, *J* = 2.5 Hz, 1H), 6.11 (dd, *J* = 8.7, 2.7 Hz, 1H), 4.36 (d, *J* = 5.8 Hz, 2H), 3.52 – 3.45 (m, 4H), 3.28 (s, 3H). LCMS purity = 97% by UV at 214 nm.

^{1}H NMR of **1**



No.	(ppm)	Integral
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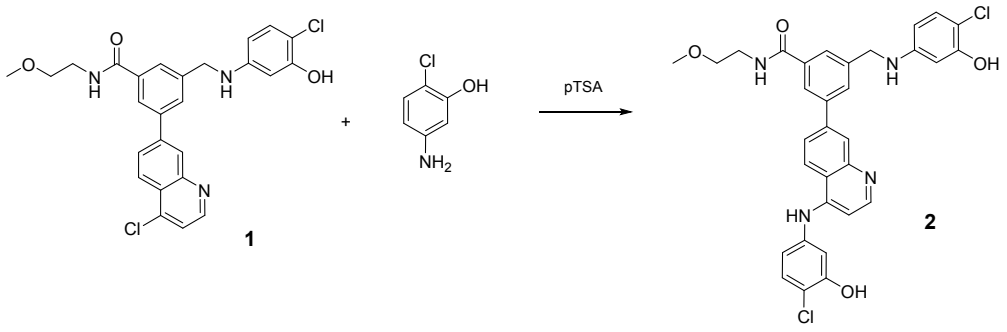
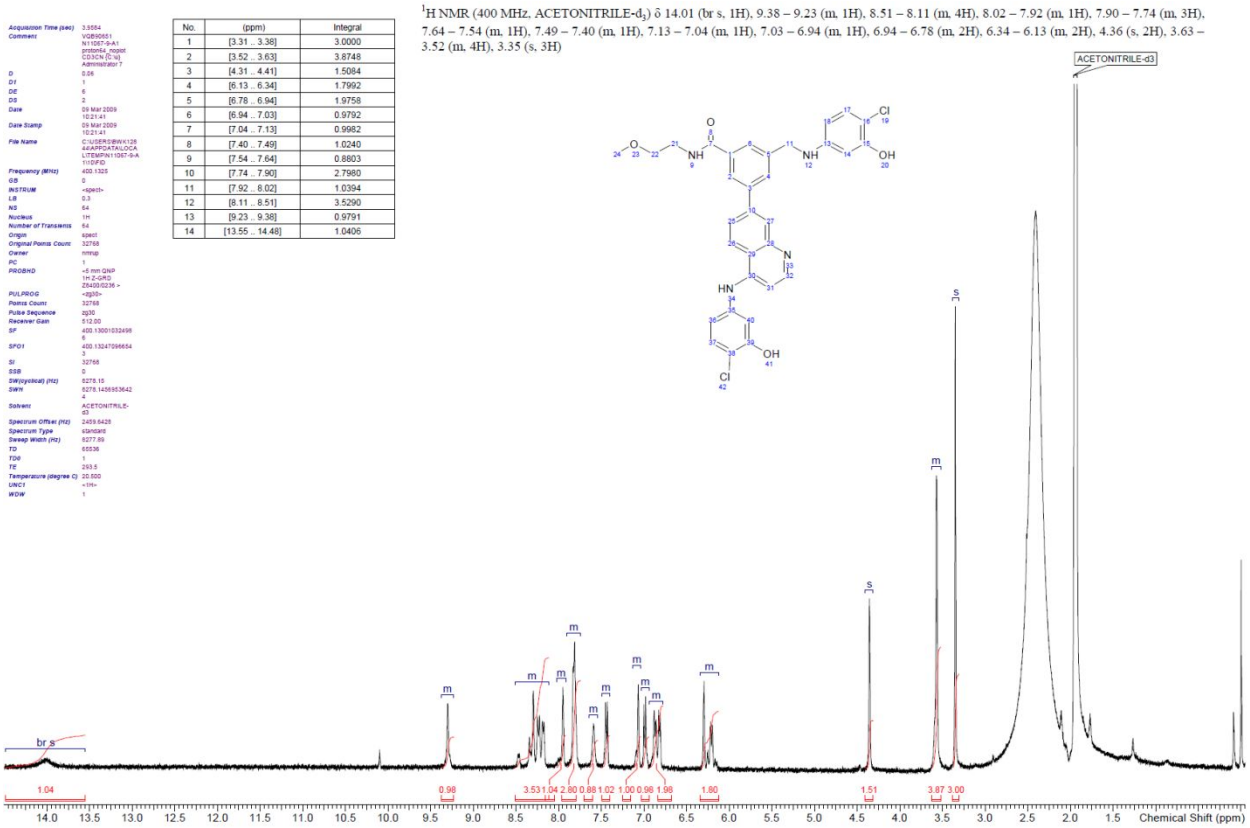
2	[3.45 .. 3.52]	4.4176
3	[4.31 .. 4.41]	2.2155
4	[6.07 .. 6.15]	1.1251
5	[6.21 .. 6.25]	1.0871
6	[6.44 .. 6.55]	1.1037
7	[6.91 .. 6.96]	1.0870
8	[7.77 .. 7.82]	1.1104
9	[7.90 .. 7.95]	1.1369
10	[7.98 .. 8.05]	1.1498
11	[8.15 .. 8.21]	1.1024
12	[8.22 .. 8.27]	1.1108
13	[8.28 .. 8.36]	1.1514
14	[8.48 .. 8.52]	1.0895
15	[8.74 .. 8.85]	1.1097
16	[8.88 .. 8.93]	1.0986
17	[9.52 .. 9.77]	1.0000

Synthesis of 3-(((4-chloro-3-hydroxyphenyl)amino)methyl)-5-(4-((4-chloro-3-hydroxyphenyl)amino)quinolin-7-yl)-N-(2-methoxyethyl)benzamide (2)



A solution of 3-{[(4-chloro-3-hydroxyphenyl)amino]methyl}-5-(4-chloro-7-quinolinyl)-N-[2-(methyloxy)ethyl]benzamide (**1**) (5 mg, 0.010 mmol), 5-amino-2-chlorophenol (1.45 mg, 0.010 mmol), and p-toluenesulfonic acid monohydrate (0.96 mg, 0.005 mmol) in dimethyl sulfoxide (0.5 mL) was irradiated in a microwave reactor at 80 °C for 30 min. Purification of the crude material using reverse phase HPLC [40-70% acetonitrile:water (0.1% TFA modifier), 30 mm Luna column, 47 mL/min] gave 3-(((4-chloro-3-hydroxyphenyl)amino)methyl)-5-(4-((4-chloro-3-hydroxyphenyl)amino)quinolin-7-yl)-N-(2-methoxyethyl)benzamide (**2**) (1.83 mg, 30%). LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{32}H_{28}Cl_2N_4O_4$, 603.1 and 605.1; found, 603.3 and 605.5. ^{1}H NMR (400 MHz, ACETONITRILE-d_3) δ ppm 14.01 (br s, 1H), 9.38 – 9.23 (m, 1H), 8.51 – 8.11 (m, 4H), 8.02 – 7.92 (m, 1H), 7.90 – 7.74 (m, 3H), 7.64 – 7.54 (m, 1H), 7.49 – 7.40 (m, 1H), 7.13 – 7.04 (m, 1H), 7.03 – 6.94 (m, 1H), 6.94 – 6.78 (m, 2H), 6.34 – 6.13 (m, 2H), 4.36 (s, 2H), 3.63 – 3.52 (m, 4H), 3.35 (s, 3H). LCMS purity = 97% by UV at 214 nm.

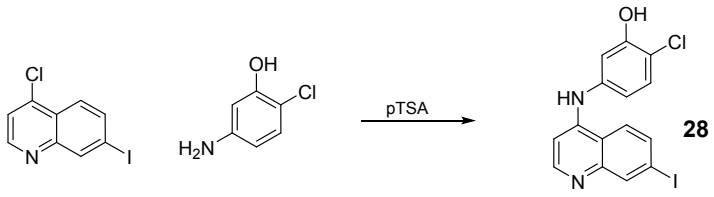
^{1}H NMR of **2**



No.	(ppm)	Integral
1	[3.31 .. 3.38]	3.0000
2	[3.52 .. 3.63]	3.8748
3	[4.31 .. 4.41]	1.5084
4	[6.13 .. 6.34]	1.7992
5	[6.78 .. 6.94]	1.9758
6	[6.94 .. 7.03]	0.9792
7	[7.04 .. 7.13]	0.9982
8	[7.40 .. 7.49]	1.0240
9	[7.54 .. 7.64]	0.8803
10	[7.74 .. 7.90]	2.7980
11	[7.92 .. 8.02]	1.0394
12	[8.11 .. 8.51]	3.5290
13	[9.23 .. 9.38]	0.9791
14	[13.55 .. 14.48]	1.0406

^{1}H NMR (400 MHz, ACETONITRILE-d_3) δ 14.01 (br s, 1H), 9.38 – 9.23 (m, 1H), 8.51 – 8.11 (m, 4H), 8.02 – 7.92 (m, 1H), 7.90 – 7.74 (m, 3H), 7.64 – 7.54 (m, 1H), 7.49 – 7.40 (m, 1H), 7.13 – 7.04 (m, 1H), 7.03 – 6.94 (m, 1H), 6.94 – 6.78 (m, 2H), 6.34 – 6.13 (m, 2H), 4.36 (s, 2H), 3.63 – 3.52 (m, 4H), 3.35 (s, 3H)

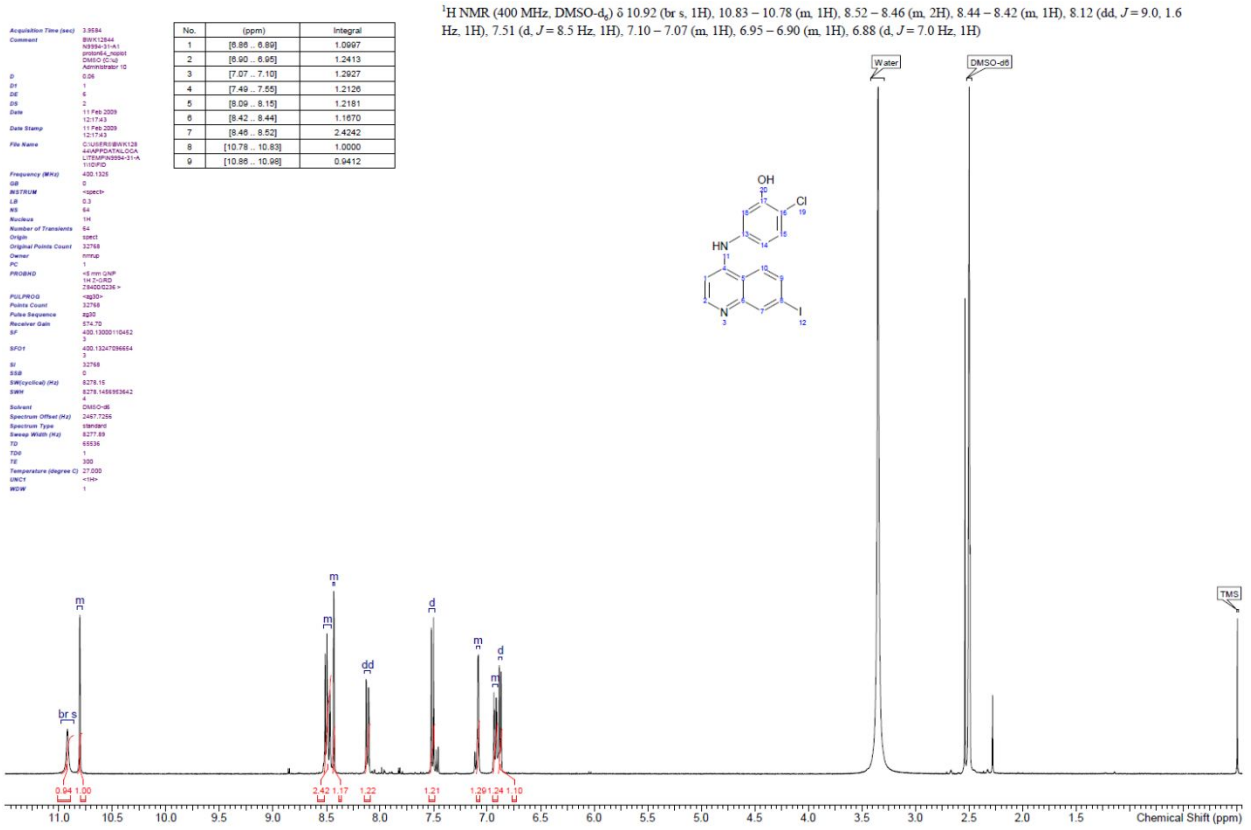
Synthesis 2-chloro-5-[(7-phenyl-4-quinolinyl)amino]phenol (3)

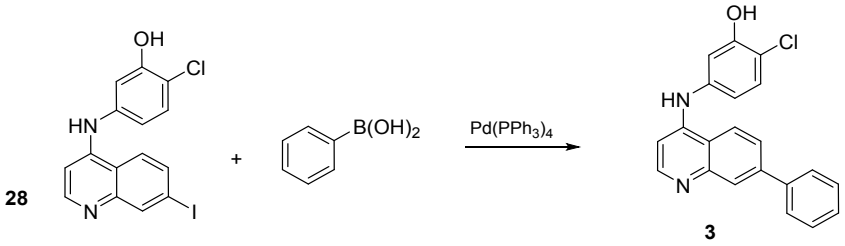


28

Step 1: A mixture of 4-chloro-7-iodoquinoline (0.135 g, 0.466 mmol), 5-amino-2-chlorophenol (0.067 g, 0.466 mmol), and p-toluenesulfonic acid monohydrate (4.44 mg, 0.023 mmol) in dimethyl sulfoxide (DMSO) (2 mL) was irradiated in a microwave reactor at 100 °C for 30 min. The addition of water resulted in a precipitation. The mixture was filtered and the collected solid washed with water and thoroughly dried to give 2-chloro-5-[(7-iodo-4-quinolinyl)amino]phenol (**28**, 165 mg, 89%). LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{15}H_{11}ClIN_2O$, 397.0 and 399.0; found, 396.5 and 398.7. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.92 (br s, 1H), 10.83 – 10.78 (m, 1H), 8.52 – 8.46 (m, 2H), 8.44 – 8.42 (m, 1H), 8.12 (dd, J = 9.0, 1.6 Hz, 1H), 7.51 (d, J = 8.5 Hz, 1H), 7.10 – 7.07 (m, 1H), 6.95 – 6.90 (m, 1H), 6.88 (d, J = 7.0 Hz, 1H).

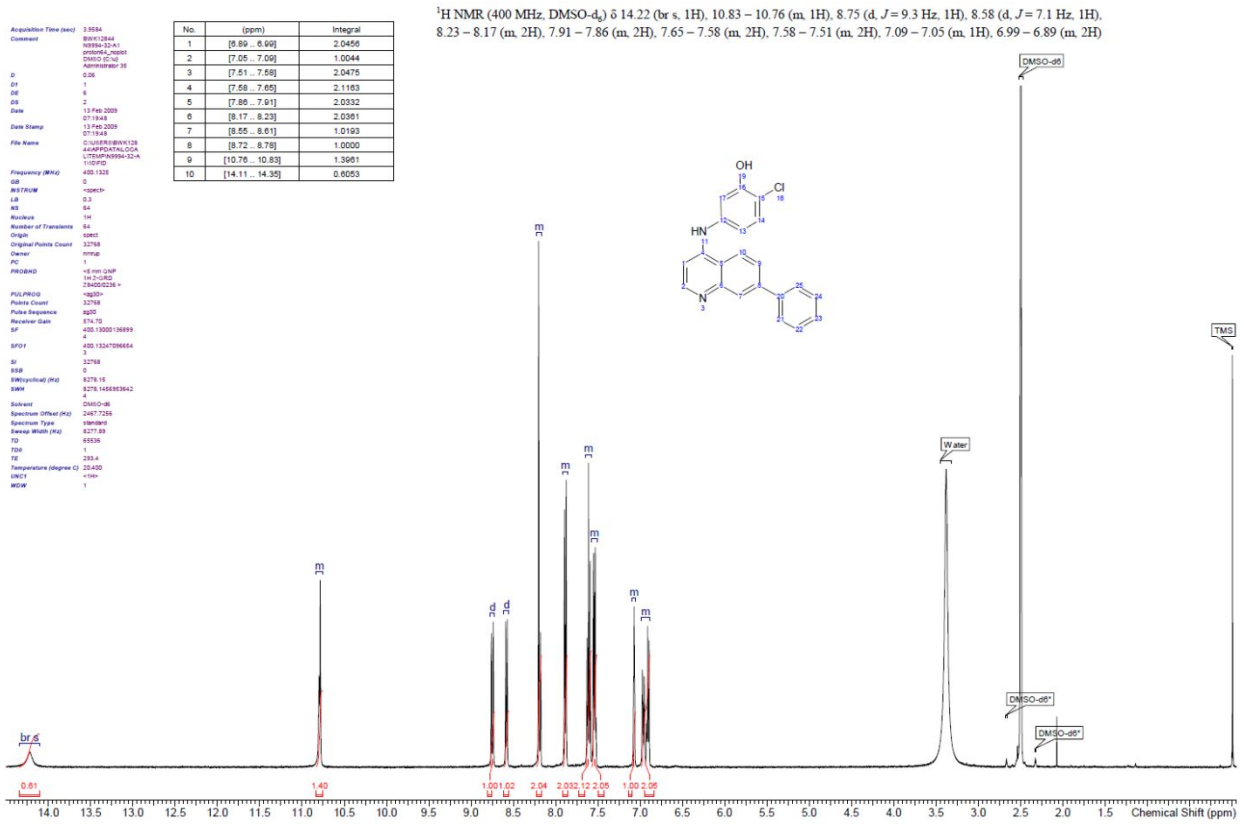
^{1}H NMR of **28**



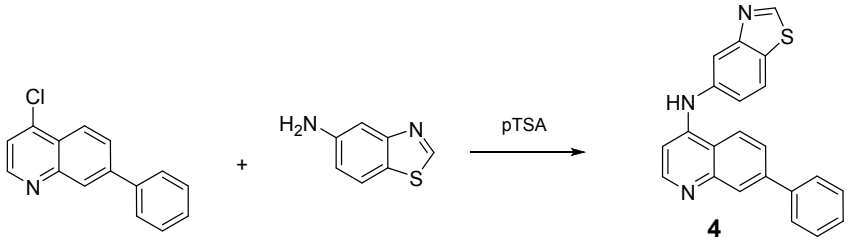


Step 2: A mixture of 2-chloro-5-[(7-iodo-4-quinolinyl)amino]phenol (**28**, 0.070 g, 0.176 mmol), phenylboronic acid (0.022 g, 0.176 mmol), and palladium tetrakis (10.20 mg, 8.82 μmol) in 1,2-dimethoxyethane (DME) (2 mL) and 2 M sodium carbonate solution (0.176 mL, 0.353 mmol) was irradiated in a microwave reactor at 100 °C for 30 min. Analysis of the reaction by LCMS indicated 2/3 starting material remained. Another 5 mg of palladium tetrakis was added and the mixture irradiated at 120 °C for 30 min. The reaction was filtered through a 0.45 micron PTFE acrodisc and diluted with DMSO. Purification of the crude material using reverse phase HPLC [30-60% acetonitrile:water (0.1% TFA modifier), 30 mm Luna column, 47 mL/min] gave 2-chloro-5-[(7-phenyl-4-quinolinyl)amino]phenol (**3**) (30 mg, 37%) (RT=5.8 min) as a light yellow solid. LCMS (*m/z*): $[M+H]^+$ and $[M+2+H]^+$ calc'd for $C_{21}H_{16}ClN_2O$, 347.1 and 349.1; found, 346.9 and 348.9. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 14.22 (br s, 1H), 10.83 – 10.76 (m, 1H), 8.75 (d, *J* = 9.3 Hz, 1H), 8.58 (d, *J* = 7.1 Hz, 1H), 8.23 – 8.17 (m, 2H), 7.91 – 7.86 (m, 2H), 7.65 – 7.58 (m, 2H), 7.58 – 7.51 (m, 2H), 7.09 – 7.05 (m, 1H), 6.99 – 6.89 (m, 2H). HRMS (*m/z*): $[M+H]^+$ calc'd for $C_{21}H_{16}ClN_2O$, 347.0951; found, 347.0951. LCMS purity = 100% by UV at 214 nm.

^{1}H NMR of **3**



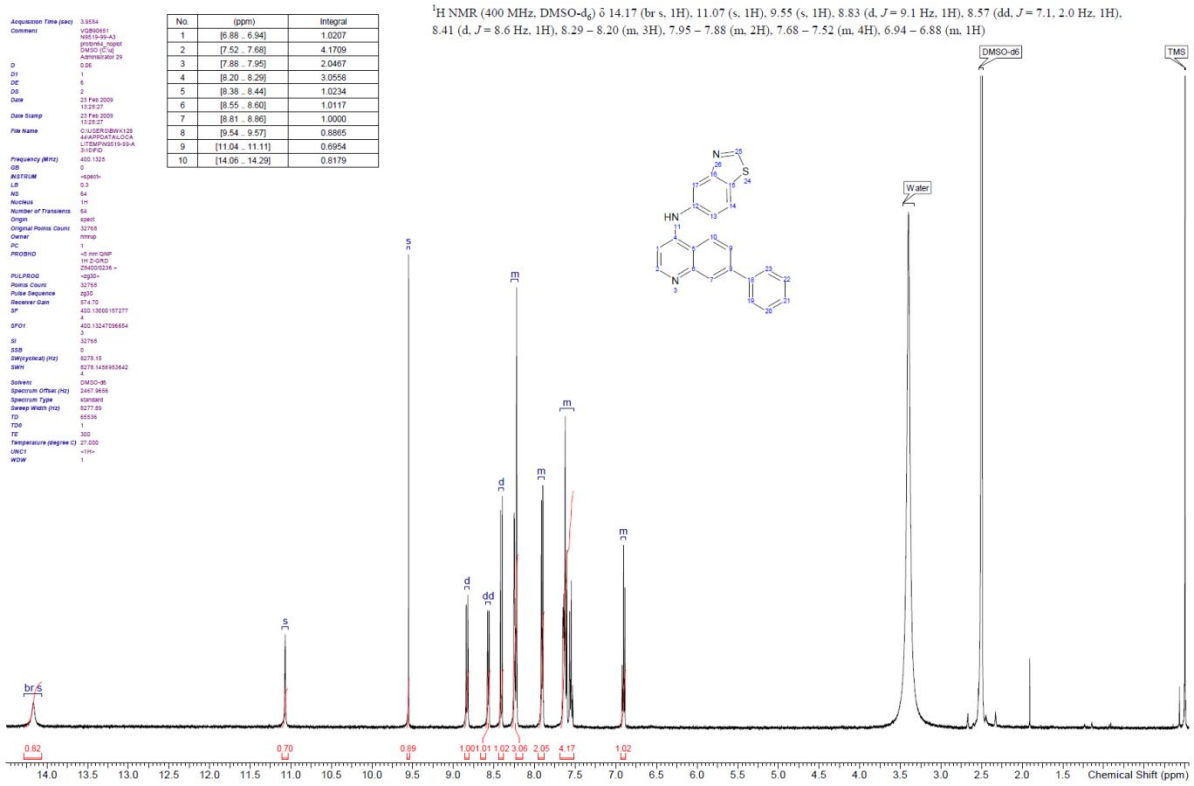
Synthesis of N-(7-phenylquinolin-4-yl)benzo[d]thiazol-5-amine (4)



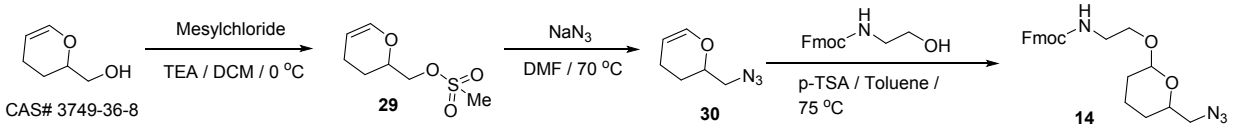
4

A solution of 4-chloro-7-phenylquinoline (12 mg, 0.050 mmol) (see *Chem. Commun.*, 2017, **53**, 13063-13066), benzo[d]thiazol-5-amine (7.5 mg, 0.050 mmol), and *p*-toluenesulfonic acid monohydrate (1.9 mg, 0.010 mmol) in dimethyl sulfoxide (DMSO) (1 mL) was irradiated in a microwave reactor at 80 °C for 30 min. Purification of the crude material using reverse phase HPLC [30-60% acetonitrile:water (0.1% TFA modifier), 30 mm Luna column, 47 mL/min] gave N-(7-phenylquinolin-4-yl)benzo[d]thiazol-5-amine (**4**) (18 mg, 78%) (mono TFA salt). LCMS (*m/z*): $[M+H]^+$ calc'd for $C_{22}H_{16}N_3S$, 354.1; found, 354.0. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 14.17 (br s, 1H), 11.07 (s, 1H), 9.55 (s, 1H), 8.83 (d, *J* = 9.1 Hz, 1H), 8.57 (dd, *J* = 7.1, 2.0 Hz, 1H), 8.41 (d, *J* = 8.6 Hz, 1H), 8.29 – 8.20 (m, 3H), 7.95 – 7.88 (m, 2H), 7.68 – 7.52 (m, 4H), 6.94 – 6.88 (m, 1H). HRMS (*m/z*): $[M+H]^+$ calc'd for $C_{22}H_{16}N_3S$, 354.1065; found, 354.1065. LCMS purity = 100% by UV at 214 nm.

^{1}H NMR of **4**



Synthesis of 9H-fluoren-9-yl)methyl (2-((6-(azidomethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)carbamate (14)



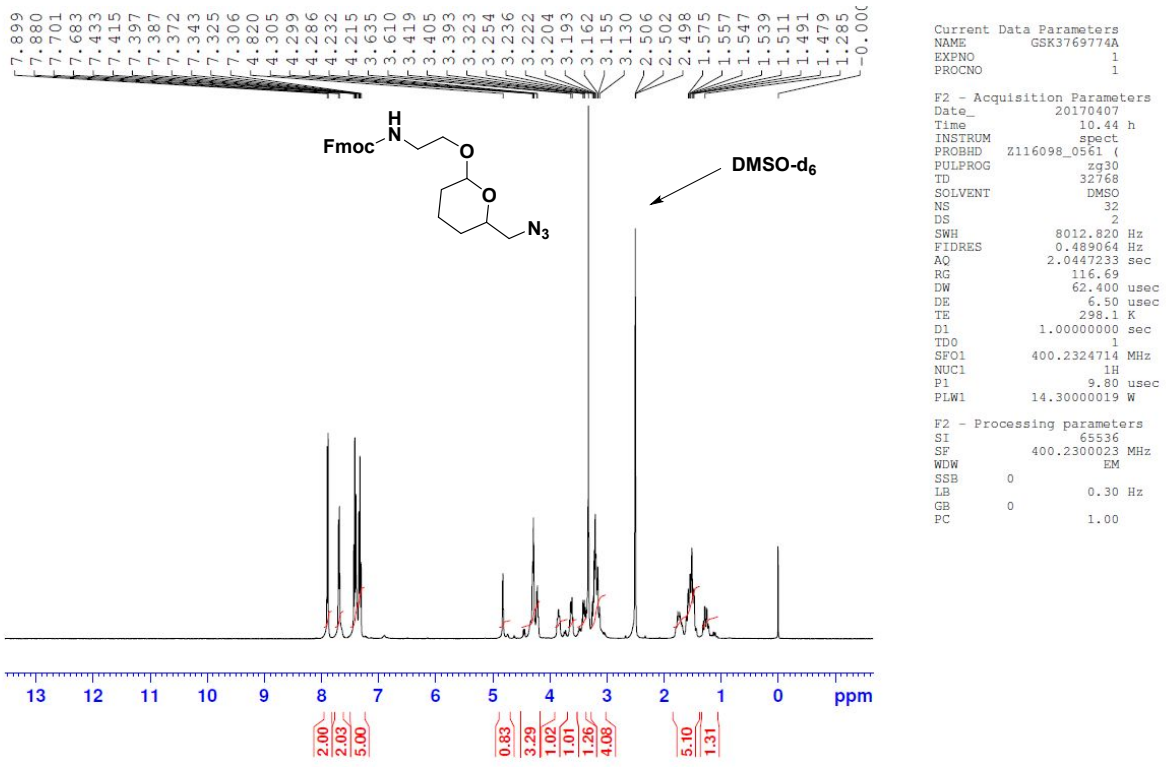
1g (3,4-dihydro-2H-pyran-2-yl)methanol and 4.8 mL TEA were added into 10 mL of DCM, and the solution was cooled to 0 °C under nitrogen atmosphere. 1 mL Mesylchloride was then added dropwise at 0 °C, and the mixture was stirred for 3 h at 0 °C. Reaction progress was monitored by TLC. After the reaction is completed, the reaction mixture was quenched with ice water and basified by adding sat. $NaHCO_3$ solution. The product was extracted by DCM (30 mL × 2). Organic layer was separated, washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to give crude product, and the crude product **29** was used in next step without further purification.

The crude **29** from previous reaction was dissolved in 10 mL DMF, then added 3.4 g sodium azide under nitrogen atmosphere. The reaction mixture was stirred at 60 to 70 °C for 36 h. Reaction progress was monitored by TLC. After the reaction is completed, the reaction mixture was cooled and poured into ice water. The product was extracted by diethylether (20 mL × 2). The organic layer was separated , dried over Na_2SO_4, and concentrated under reduced pressure to give crude product, and the crude product **30** was used in next step without further purification.

The crude **30** from previous reaction was dissolved in 10 mL toluene, then added 0.4 g p-TSA and 1g Fmoc glycinol at RT under nitrogen atmosphere. The reaction mixture was stirred at 70 to 80 °C for 5 h. Reaction progress was monitored by TLC. After the reaction is completed, the reaction mixture was cooled and poured into ice water. The product was extracted by ethylacetate (30 mL

× 2). The organic layer was separated, washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure to give crude product **14**. The crude product **14** was used in next step purified by 100-200 m silica gel column chromatography using 10-15% EtOAc in Hexanes as eluent. 0.1 g of (9H-fluoren-9-yl)methyl (2-((6-(azidomethyl)tetrahydro-2H-pyran-2-yl)oxy)ethyl)carbamate **14** was obtained with overall 2.7% yield, LCMS purity = 97.64 % at Maxplot, HPLC purity = 97.03% by UV at 215 nm, 97.66% by UV at 254 nm., as a mixture of isomers. LCMS m/z = 445.53/445.52 $[M+Na]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.89 (d, *J* = 7.6 Hz, 2H), 7.69 (d, *J* = 7.2 Hz, 2H), 7.42 (t, *J* = 7.2 Hz, 2H), 7.36 (t, *J* = 7.2 Hz, 2H), 4.82 (br, 1H), 4.22-4.31 (m, 3H), 3.85 (m, 1H), 3.63 (m, 1H), 3.25 (m, 1H), 3.38 (m, 4H), 1.74 (m, 1H), 1.58 (m, 5H), 1.28 (m, 1H)

^{1}H NMR of **14**

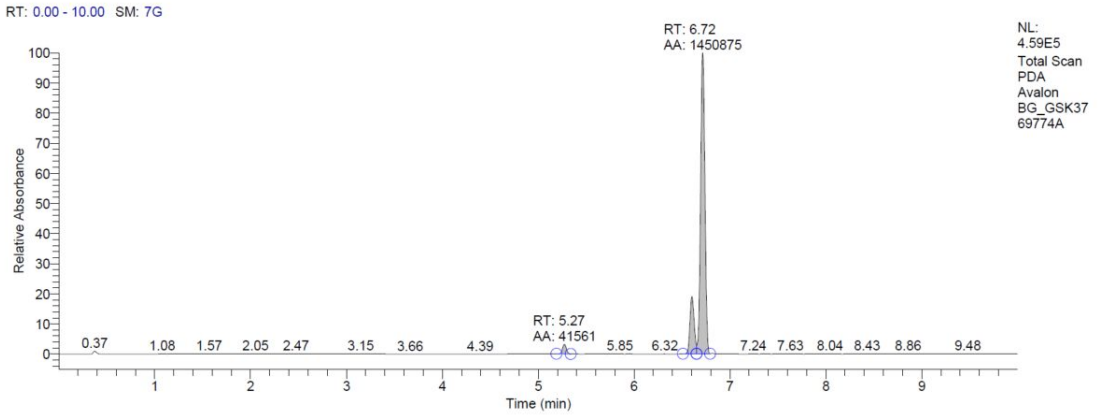


LCMS of **14**

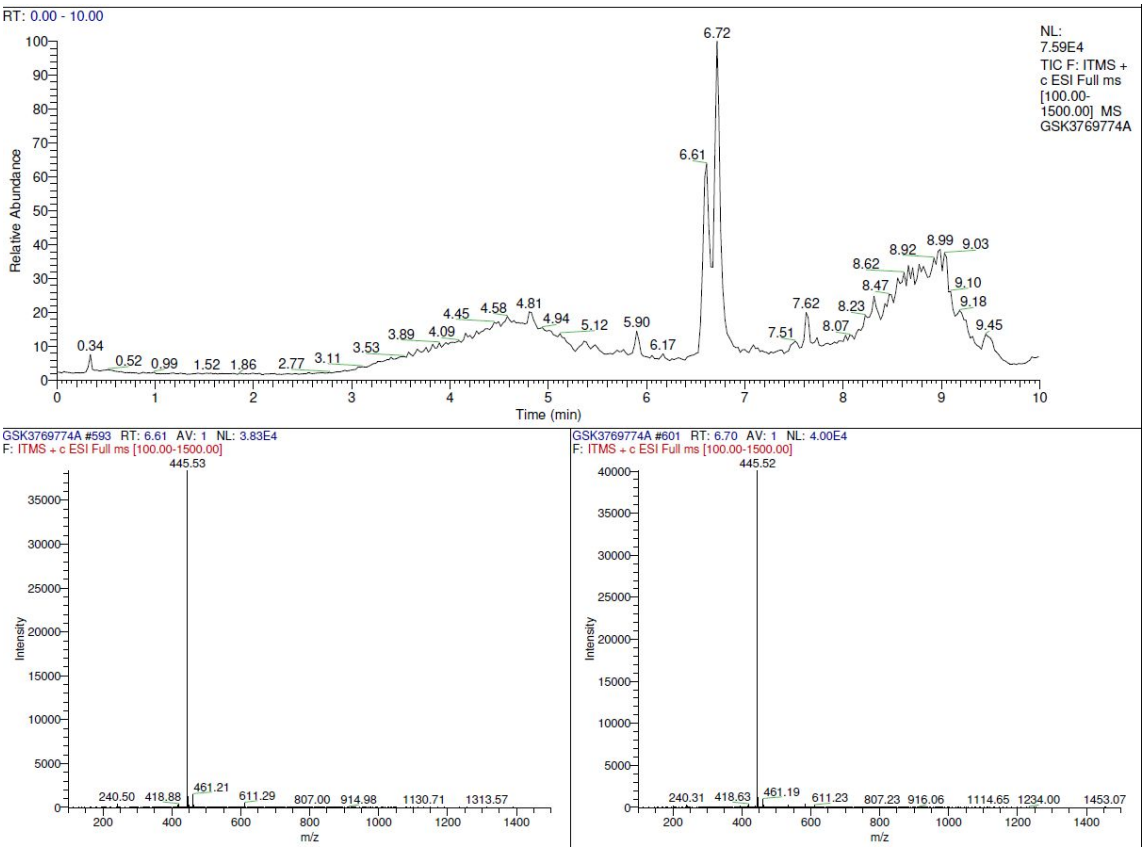
UV Chromatogram

Detector Type: PDA

Wavelength Range 1 (nm): N/A



RT	Peak Area	Area %
5.27	41560.70	2.36
6.60	270375.79	15.34
6.72	1450875.16	82.30



Part III: AS-MS

LC/MS: The HPLC system was a Thermo Scientific™ UltiMate™ 3000 Rapid Separation System. The reverse phase column used for the sample analysis was a Kinetex XB-C18 (1.7 μm, 100 Å, 2.1×50 mm) purchased from Phenomenex®. Solvent A was 0.1% formic in H_2O, and solvent B was 0.1% formic and 1% H_2O in CH_3CN. The elution program was 5% to 95% B in 2 min (hold final 0.4 min) at 0.6 mL/min. The mass analysis was carried out on a Thermo Orbitrap (Exactive), a benchtop LC/MS system that delivers high-resolution, accurate-mass (HR/AM) data with fast full-scan capabilities. The ionization source was heated electrospray ionization (HESI). The HESI source Exactive was tuned. The temperature of the probe heater and capillary was kept at 30 °C and 300 °C, respectively. Ions were collimated using a spray voltage of 3900 V and an S-lens RF voltage of 50 V. Sheath, Aux, and Spare gas were set at 20, and Xcalibur version 4.1.31.9 software (Thermo Fisher Scientific, Inc.) was used for data processing and peak integration. Affinity-MS was used for automatic data analysis. Affinity-MS is a bespoke Microsoft Windows-based software solution developed by GSK to register, manage, analyze, and report affinity selection mass spectrometry experiments and results.

AS-MS:

Sample preparation: The lyophilized samples (small molecule mixtures cleaved from DNA) were re-constituted in neat DMSO at a final concentration of 1 mM, and then further diluted in selection buffer at 100 μM for AS-MS experiments.

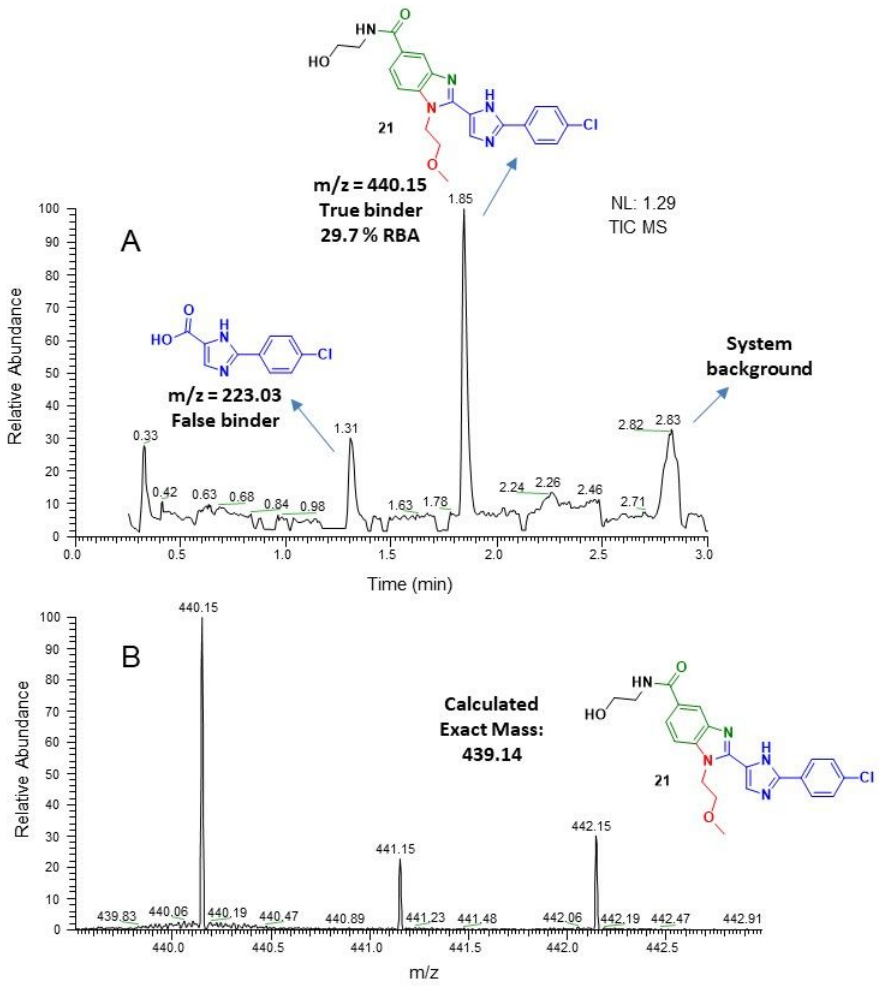
Incubation: A total of 2 μL of the compound solution in selection buffer was transferred to a 384-well microtiter plate (384 Well Polypropylene Microplates 781201, Greiner Bio-One, Winchester, MA), followed by the addition of 20 μL of AS-MS selection buffer with (selection sample) and without 10 μM of target protein (RIP2) for the no target control (NTC) sample. The plate was then sealed and the mixture incubated for 1 h at ambient temperature. These incubation

mixtures were also used as reference samples that were directly injected into the MS system, not loaded onto the SEC plates. The MS signals from the reference samples were used to compare MS signals from select samples loaded onto SEC columns. Relative Binding Affinity (%RBA) for cleaved products were then assessed for binding in AS-MS, which indicates that compound recovered in SEC.

Separation of bound and unbound compounds: After incubation of the target protein for ~1 h to achieve full binding of compounds in the mixtures in equilibrium, the incubation solutions were transferred to a 384-well SEC plate with pinholes in the well bottoms (Fisher Scientific, Multiscreen HTS 384-well filter plates, 0.45 μM Durapore, Cat# MZHVN0W50). The SEC plate was prepared with Bio-Gel P10 fine resin (Bio-Rad 150-4144) and spun at 1054 × g in a centrifuge (5810R, Eppendorf, Germany) for 3 min at 4 °C. The recovered NTC, selection samples, and reference (w/o SEC) were analyzed by LC/MS mentioned above.

AS-MS data analysis: Data processing was accomplished with Affinity-MS, a custom automated software solution developed by GSK. The extracted ion chromatogram (XIC) peak areas were used for data analysis. The test compounds were designated as True, False, or Indeterminate in the data analysis by comparing MS signals from the reference, NTC, and selection samples. A True binder was found in the protein-containing sample but absent in the control/NTC samples. On the other hand, the compound found in the protein fraction was also detected in the control/NTC samples. This compound passed through the SEC gel column in the absence of the protein and, therefore, is considered a false-positive hit. The relative binding affinity (RBA%) was calculated as: (MS signal from Selection samples – MS signal from NTC samples)/MS signal from Reference samples × 100%.

The AS-MS data for THP linker compound **21** is shown below as an example of how the compound is identified as a binder. LC/MS analysis of the reference sample indicated that compound **21** is the major cleaved product, which also includes some minor side products and originally produced intermediates (Figure S-3-1). The cleaved products were screened as small molecule mixtures by off-line AS-MS. Ligands that engage the target protein were identified by comparing their m/z values (mainly, molecular mass + H or Na) to the molecular masses of the enumerated compounds from that particular well using Affinity-MS software. The extracted ion chromatograms (XIC) of compound **21** in the reference, NTC, and selection samples are shown in Figure S16, which depicts it is a True binder as compound **21** (m/z 440.15) is found in the protein-containing sample but absent in the control/NTC samples. The RBA% of compound **21** is 29.7% (Figure S17).



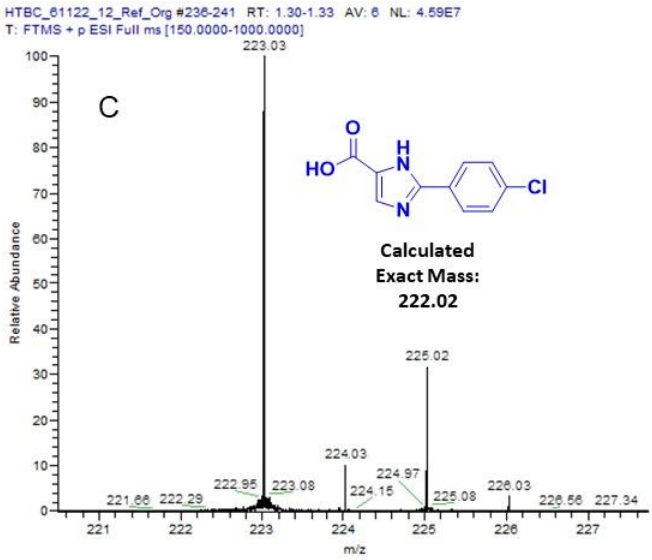
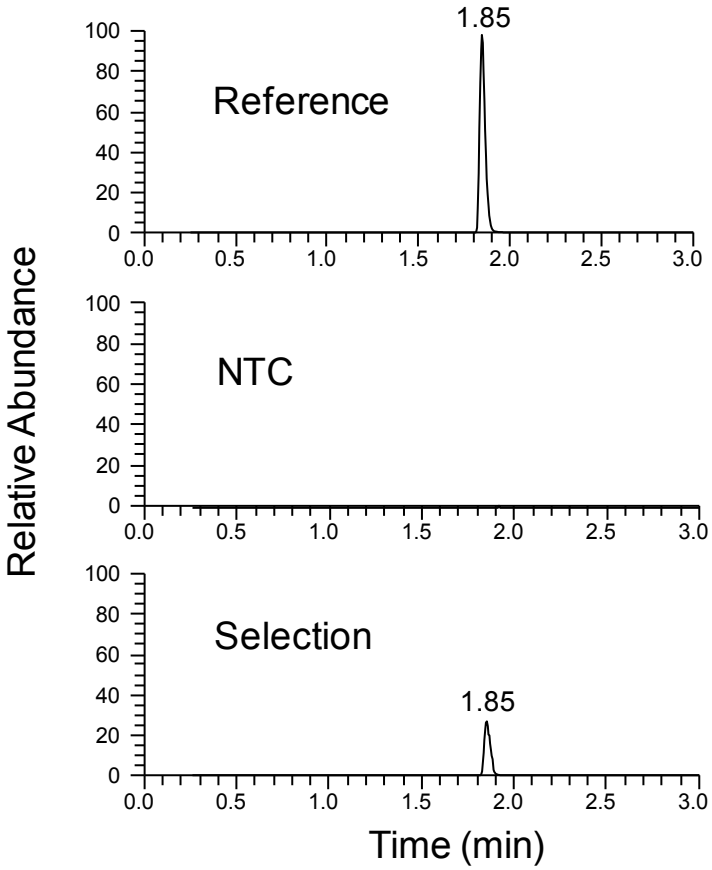


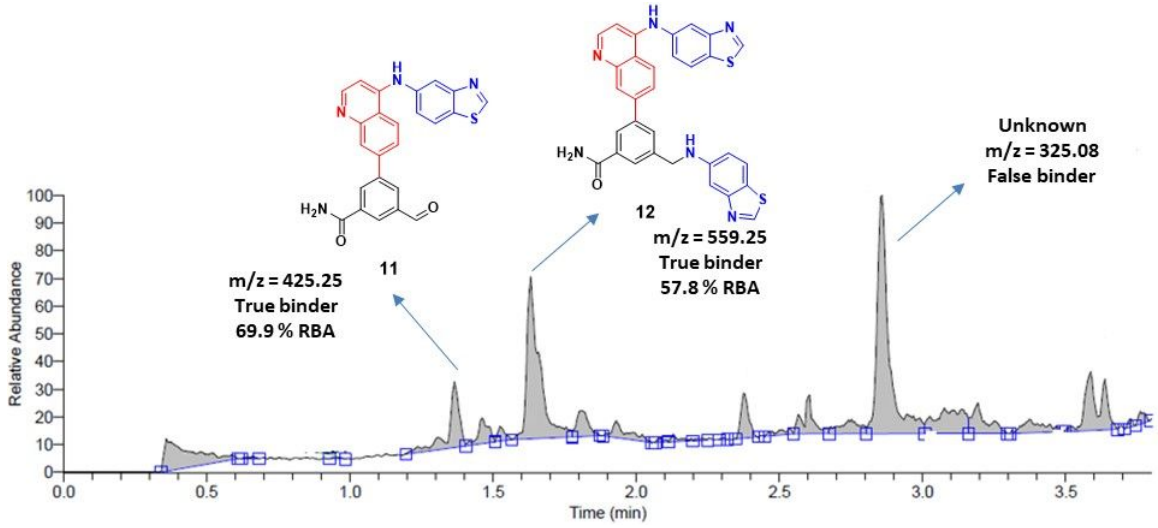
Figure S16. Total ion chromatogram (TIC) of the reference sample for the THP linker compound showing that compound **21** is the major cleaved product (A). The positive ion of the ESI mass spectrum of the peak at 1.85 min (B). Two peaks were separated by 2 m/z units with a peak height ratio of 3:1, indicating that the molecule contains 1 chlorine atom. The positive ion of the ESI mass spectrum of the peak at 1.31 min (C), similar to compound **21**, it must contain 1 chlorine atom since two peaks were separated by 2 m/z units with a peak height ratio of 3:1.



Compound 21	Retention time (min)	Peak Area	RBA (%)
Reference	1.85	5.15.E+08	29.7%
NTC	1.85	3.00.E+05	
Selection sample	1.85	1.54.E+08	

Figure S17. Identification of RIP2 binder **21** from the released small molecule mixture by AS-MS. Extracted ion chromatograms (XIC) shows compound **21** in the reference, NTC, and selection samples. The %RBA is calculated based on the peak area in the XIC of each sample.

Similarly, the RBAs of compounds **11** and **12** (after cleavage, shown in Figure S18) are calculated as following based on the peak area in the XIC. Other peaks including the peak at RT 2.86 min are not related to the desired compounds or side products and they were not observed in the LC/MS spectra including TIC and XIC after SEC.



Compound 11	Retention time (min)	Peak Area	RBA (%)
Reference	1.37	2.62E+06	69.9%
NTC	1.37	1.73E+05	
Selection sample	1.37	2.00E+06	

Compound 12	Retention time (min)	Peak Area	RBA (%)
Reference	1.64	1.67E+06	57.8%
NTC	1.64	1.12E+05	
Selection sample	1.64	1.07E+06	

Figure S18. Identification of RIP2 binders **11** and **12** from the released small molecule mixture by AS-MS. Extracted ion chromatograms (XIC) shows compound **11** and **12** in the reference, NTC, and selection samples. The RBAs are calculated based on the peak area in the XIC of each sample.