DNA-Compatible Copper-Catalyzed Oxidative Amidation of Aldehydes with Non-Nucleophilic Arylamines

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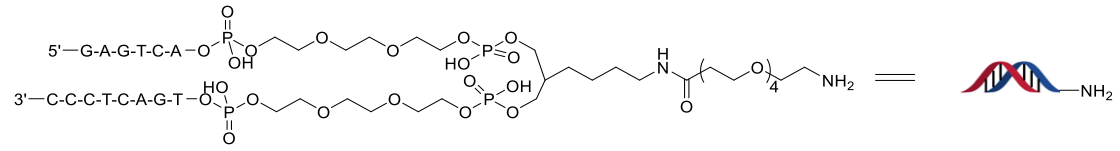
Supporting Information

Contents

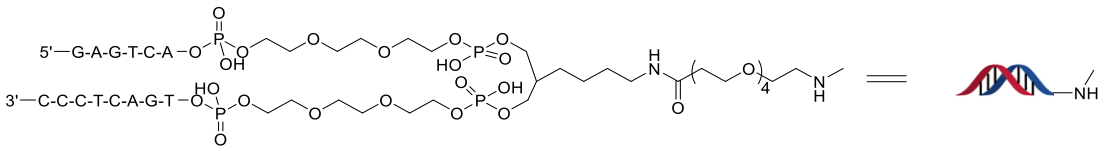
SI-1 General Experimental

Dimethylsulfoxide (DMSO), 1-methyl-2-pyrrolidinone (NMP), *N*,*N*-dimethylacetamide (DMAc), acetonitrile (CH_3CN), and EtOH were purchased from Sigma-Aldrich. EDCI (CAS: 25952-53-8), *N*-hydroxysulfosuccinimide sodium salt (*s*-NHS, CAS: 106627-54-7), *tert*-butyl-hydroperoxide (TBHP, 70% in water, 75-91-2), and NaCl were purchased from TCI. The H_2O used was obtained by passing through the Milli-Q Direct. The reaction buffers were purchased from Vazyme and the ligase and ligation buffer were purchased from Thermo. The other reagents were purchased from domestic vendors unless mentioned otherwise. On-DNA reaction conversions were determined by UV traces of LC/MS analysis. The centrifuge instruments were Allegra X-15R, eppendorf-5424R. The peak at 0.27 min sometimes seen in the provided LC/MS traces is attributed to inorganic salts and small molecules contaminants, according to our experience.

SI-2 S-HP (Spacer-headpiece) Material



S-HP (Exact Mass: 5184.48)



S-HP-1 (Exact Mass: 5198.48)

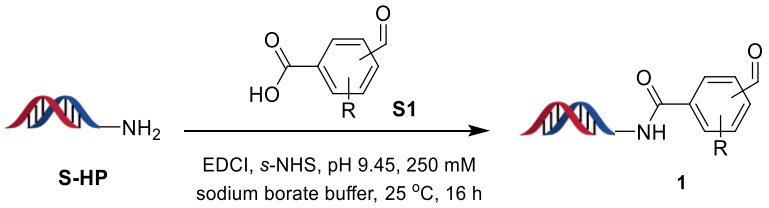
S-HP is composed of headpiece and PEG4 linker.

SI-3 General Procedure

SI-3-1 Ethanol Precipitation for DNA Compatible Reactions

To the reaction mixture was added 10% (v/v) 5 M NaCl aqueous solutions and 2.5-3 fold the volume of cold ethanol (stored at -20 ℃ before use). The colloidal solution was then allowed to sit at -80 ℃ for more than 2 hours. The solutions were centrifuged at 4 ℃ for 30 min at 4000 g. The supernatants were discarded, and the DNA pellet was dried at 30 ℃ for 1 hour in vacuo. Generally, ethanol precipitation was performed after each chemical reaction.

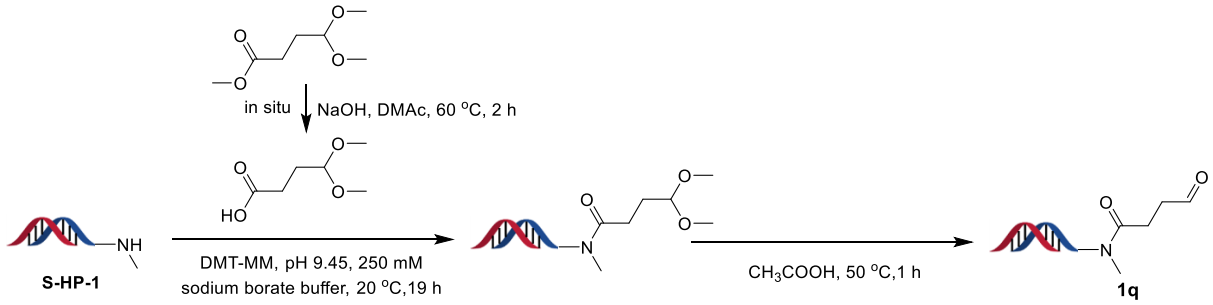
SI-3-2 General Procedure 1 for DNA conjugated aryl aldehydes



To a 15 mL tube was added a solution of EDCI (200 mM in DMSO, 125 μL, 50 equiv), *s*-NHS (200 mM in DMSO/H_2O=1/1, 75 μL, 30 equiv) and **S1** (200 mM in DMAc, 150 μL, 60 equiv). This solution was mixed and left to stand at 20 ℃ for 15 min to make the active ester.

Next, S-HP solution (1 mM in pH 9.5 sodium borate buffer (250 mM), 500 μL, 1 equiv) was added to the freshly prepared active ester solution. The solution was mixed and left to stand at 25 ℃ for 16 h (pH 9), with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

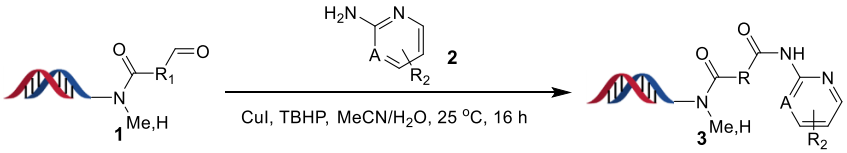
SI-3-3 General Procedure 2 for DNA conjugated aliphatic aldehyde 1q



The DNA-linked material **S-HP-1** (500 nmol) was dissolved in sodium borate buffer (pH 9.45, 250 mM, 500 μL) to make a 1 mM solution. To 4,4-dimethoxybutanoic acid methyl ester (200 mM in DMAc, 106.25 μL, 42.5 equiv) was added NaOH (200 mM in H_2O, 106.25 μL, 42.5 equiv); the solution was mixed and then stood at 60 ℃ for 2 h. To the DNA material **S-HP-1** was added the above solution and DMT-MM (200 mM in H_2O, 100 μL, 40 equiv). The solution was mixed and left to stand at 20 ℃ for another 3 h (pH 9) to make the precursor for **1q** in situ. The reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

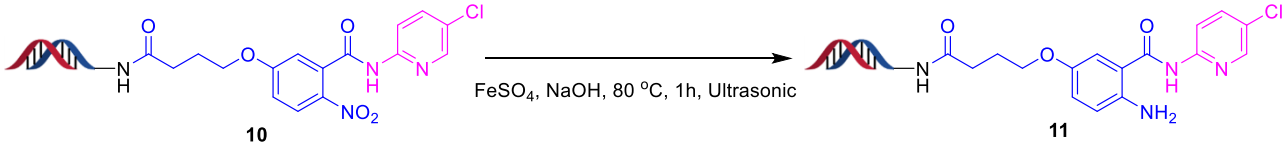
Next, the material was redissoved in H_2O (500 μL) and freshly prepared acetic acid solution (300 Mm in H_2O, 166 μL) was added. The solution was mixed and left to stand at 50 ℃ for 1 h (pH 9), with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

SI-3-4 General Procedure 3 for Oxidative Amidation.



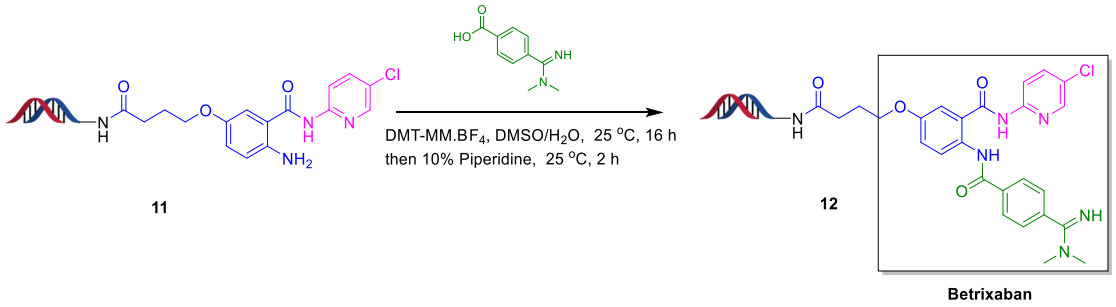
To the DNA-linked material **1** (10 nmol, 1 mM in H_2O, 10 μL, 1 equiv) was added aniline **2** (400.0 mM in CH_3CN, 5 μL, 200 equiv), CuI (50.0 mM in CH_3CN, 16 μL, 80 equiv) and TBHP (200 mM in H_2O, 5 μL, 100 equiv). The solution was mixed at 25 ℃ for 16 h in a shaker (pH 6~7). Next, to the solution was added ethyldithiocarbamate trihydrate (200.0 mM in H_2O, 12 μL, 240.0 equiv). The solution was mixed and left to stand at 80 ℃ for 30 min, with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

SI-3-5 Procedure for Compound 11.



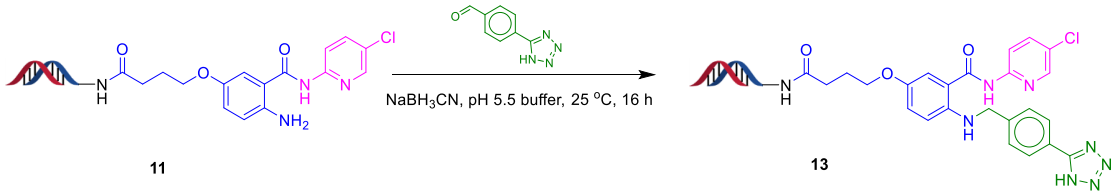
To the DNA starting material **10** (946 nmol, 1 mM in H_2O, 946 μL, 1 equiv) was added $FeSO_4.7H_2O$ (200.0 mM in H_2O, 378.4 μL, 80.0 equiv), and NaOH (2500.0 mM in H_2O, 189.2 μL, 500.0 equiv). The solutions was mixed at 80 ℃ for 1 h in an ultrasonic unit (pH 12). Next, to the solution was added ethyl-dithiocarbamate trihydrate (100.0 mM in H_2O, 567.6 μL, 60 equiv). The solution was mixed and left to stand at 80 ℃ for 30 min, with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**). The crude product was purified by reverse-phase HPLC to give 167.8 nmol of desired product **11**. Yield (17.7%).

SI-3-6 Procedure for Compound 12.



The DNA starting material **11** (5 nmol) was dissolved in MOPS buffer (pH 8.2, 50 mM, 5 μL). 4-(*N*,*N*-Dimethylcarbamimidoyl)benzoic acid (400.0 mM in H_2O, 2.5 μL, 200.0 equiv) and DMT-MM.BF_4 (400.0 mM in DMSO, 2.5 μL, 200 equiv) were then added. After the addition, the tube was centrifuged, eddied and re-centrifuged. The reaction mixture was kept at 25 ℃ for 16 h (pH 8). After EtOH precipitation, H_2O (5 μL) and 20% piperidine (5 μL) were added. The tube was centrifuged, eddied and re-centrifuged. The solution was mixed and left to stand at 25 ℃ for 2 h, with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

SI-3-7 Procedure for Compound 13.



The DNA starting material **11** (5 nmol) was dissolved in phosphoric buffer (pH 5.5, 250 mM, 5 μL). 4-(1*H*-Tetrazol-5-yl) benzaldehyde (200.0 mM in DMAc, 3 μL, 20.0 equiv) and $NaBH_3CN$ (400.0 mM in DMAc, 1.5 μL, 120 equiv) were then added. After the addition, the tube was centrifuged, eddied and re-centrifuged. The solution was mixed and left to stand at 20 ℃ for 16 h (pH 6), with reaction conversion monitored by LCMS. The reaction mixture was then precipitated with ethanol (see General Procedure **SI-3-1**).

SI-3-8 General Procedure 7 for Enzymatic Tagging

To DNA-linked starting materials (100 nmol, 1 mM in H_2O, 100 μL, 1 equiv) were added DNA tags (160 nmol, 1 mM in H_2O, 160 μL, 1.6 equiv), 10x T4 DNA ligation buffer (40 μL), T4 DNA ligase (4 μL, 30U/μL) and H_2O (96 μL). The solutions were mixed and stirred at 16 ℃ for 16 h. The reaction mixtures were precipitated with ethanol, according to General Procedure **SI-3-1**, and the samples were analyzed by 4% gel picture.

SI-4 DNA Damage Evaluation

Copper-mediated oxidative amidation of aldehyde reactions (abbreviated as "the oxidative amidation reaction" below) were performed with DNA conjugated compound with a double stranded DNA coding region to mimic the library component. The DNA conjugated compound was subjected to the oxidative amidation reaction under different conditions (Table S1) to determine DNA damage.

Table S1, sampling logic of the comparison.

ID	Condition No.	Condition Info.	Theoretical Input
A1	1	Normal reaction	5 nmol
A2	2	No CuI/TBHP	5 nmol
A3	3	No CuI	5 nmol
A4	4	No TBHP	5 nmol
A5	5[a]	formaldehyde(PC1)	5 nmol
A6	6[b]	No reaction(NC)	5 nmol
A7	7[c]	No reaction (Ab NC)	5 nmol

Note: [a] Compounds in this group act as positive control.[1] *[b] Compounds in this group act as negative control and go through the basic liquid transfer processes in the oxidative amidation reaction, but no reagent or material was added. [c] Compounds in this group don't go through the oxidative amidation reaction and are kept as an absolute negative control.*

[1] Huang, H., Hopkins, P. B. (1993) DNA interstrand cross-linking by formaldehyde: nucleotide sequence preference and covalent struc-ture of the predominant cross-link formed in synthetic oligonucleo-tides. *J. Am. Chem. Soc. 115*, 9402-9408.

The products of each reaction were visualized by an Agilent 2100 Bioanalyzer (Fig S1), then ligated to an oligonucleotide to generate a full-length DNA fragment, similar to DEL molecules. If there is DNA damage in the oxidative amidation reaction, the band of the experimental group may become vague or turn into a smear compared with the negative control. The shape of the band from the experimental group A1 is similar to the negative group A7, while the band of positive control of the formaldehyde treated group A5 is disappearing. These results indicate that normal reaction conditions will not affect the DNA as observed in condition A5. As the band of A2 to A4 are quite similar, it is clear that the different reagents in the oxidative amidation reaction will not affect the DNA. For subsequent qPCR analysis, only the groups of A1 (oxidative amidation reaction group), A5 (formaldehyde treated, positive control) and A7 (negative control) were used.

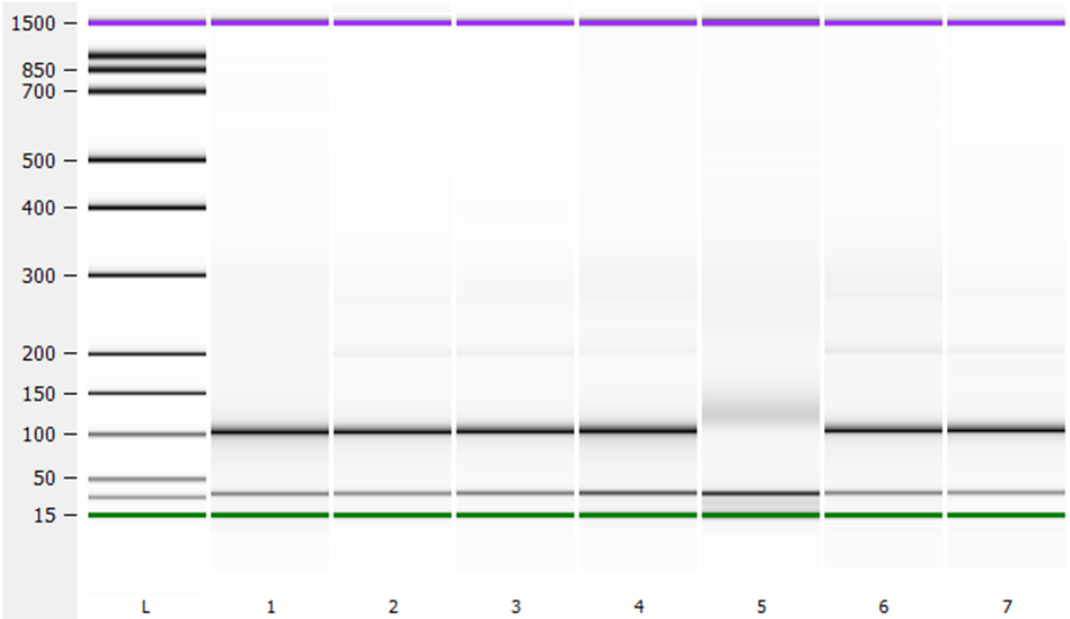


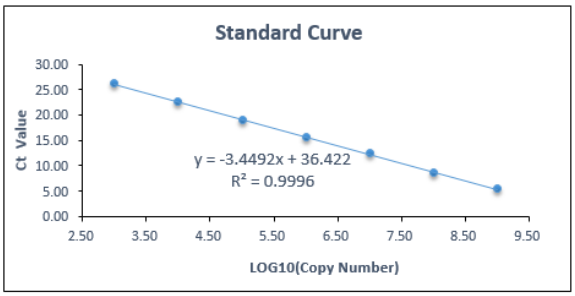
Figure S1. Gel image of different comparison groups. Lane 1 to 7 is the condition A1 to A7.

SI-4-1 QPCR Test

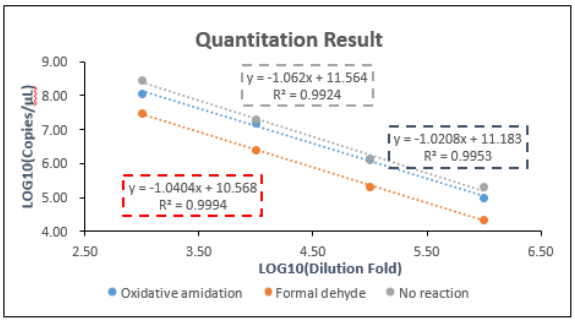
After EtOH Precipitation, according to General Procedure **SI-3-1**, the ligation products of A1 (oxidative amidation), A5 (formaldehyde treated) and A7 (negative control) were step-wise diluted 10 fold to a final 1e4 fold dilution. The serial dilutions were used as templates for qPCR testing using a SYBR Green Master Mix kit (Thermo) and a Real-Time PCR System (QuantStudio 7 Flex) (Table S2). All samples were run in triplicate and subjected to PCR cycles as follows: 95 ℃ heat activation for 5 min followed by 40 cycles of 95 ℃ denaturation for 10 seconds, 55 ℃ annealing for 15 seconds and extension at 72 ℃ for 30 seconds.

Table S2. Summary of qPCR Data Analysis.

qPCR Standard (Copy Number)	LOG10(Copy Number)	Average(Ct)
1.00E+09	9.00	5.46
1.00E+08	8.00	8.67
1.00E+07	7.00	12.49
1.00E+06	6.00	15.68
1.00E+05	5.00	18.96
1.00E+04	4.00	22.64
1.00E+03	3.00	26.18



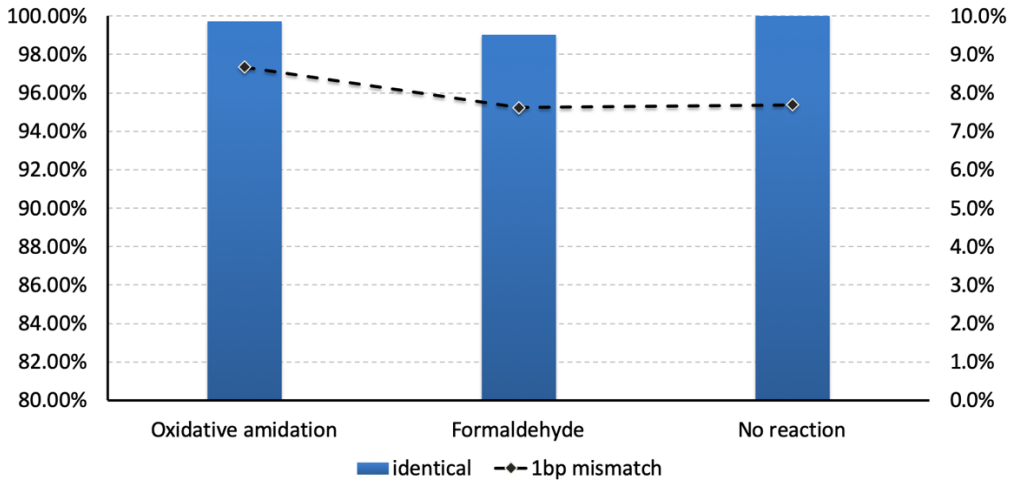
LOG10(Dilution Fold)	Group A1		Group A5		Group A7	
	Average(Ct)	Copies/μL	Average(Ct)	Copies/μL	Average(Ct)	Copies/μL
3.00	7.64	1.10E+08	9.61	2.97E+07	6.23	2.83E+08
4.00	10.63	1.50E+07	13.36	2.43E+06	10.26	1.92E+07
5.00	14.14	1.44E+06	17.00	2.14E+05	14.34	1.26E+06
6.00	18.21	9.55E+04	20.36	2.27E+04	17.08	2.02E+05



The results showed that no obvious concentration difference was observed between oxidative amidation and no reaction, but the concentration of formaldehyde at the same dilution ratio is significantly lower. The R^2 value are all above 0.99 in these three groups. This indicates that the oxidative amidation reaction did not affect the detectability of DNA tags.

SI-4-2 Next-generation sequencing.

1e7 copies of the three groups mentioned above were used as template for PCR amplification. To a PCR tube was added diluted sample (2 μL), 10x high fidelity PCR buffer (5 μL), 50 mM $MgSO_4$ (2 μL), 10 mM dNTP mix (1 μL), Platinum^{TM} Taq DNA Polymerase (0.2 μL), 10 μM forward primer (2 μL), 10 μM reverse primer (2 μL), and nuclease-free water (35.8 μL). After 20 cycles, the PCR products (204 bp, forward primer brought in additional 20 bp at 5' terminal, reverse primer brought in additional 66 bp at 3' terminal) were purified by the Agencourt AMPure XP Beads method. The purified samples were quantified by Qubit 4.0 to appear at 1.2 ng/μL. PCR products were diluted to 3nM and pooled together according to the ratio of PCR input copies, then provided for next-generation sequencing (Illumina HiSeq XTen). Bowtie2 were used to mapping the sequenced reads to reference by local alignment. The detailed mapping identity were extracted from CIGAR string and XM flag in the SAM format. The results of NGS showed that all samples retained the right sequence as expected (Fig. S2), indicating that the chemical reactions did not affect the encodability of DNA tags.



Condition	total Reads	perfect match	1bp mismatch
Oxidative amidation	43,502,085	32,818,659	3,771,280
Formaldehyde	47,738,282	35,756,941	3,637,258
No reaction	58,501,783	44,254,860	4,499,768

Figure S2. Statistics of next-generation sequencing results. The left Y-axis is the ratio of perfect match read count normalized by the negative control, while the right Y-axis is the fraction of 1bp mismatch.

In conclusion, our data revealed that the oxidative amidation reaction used in this paper caused no damage to DNA, and thus could be used for the encoded library construction.

SI-5 Selected examples of the active molecule

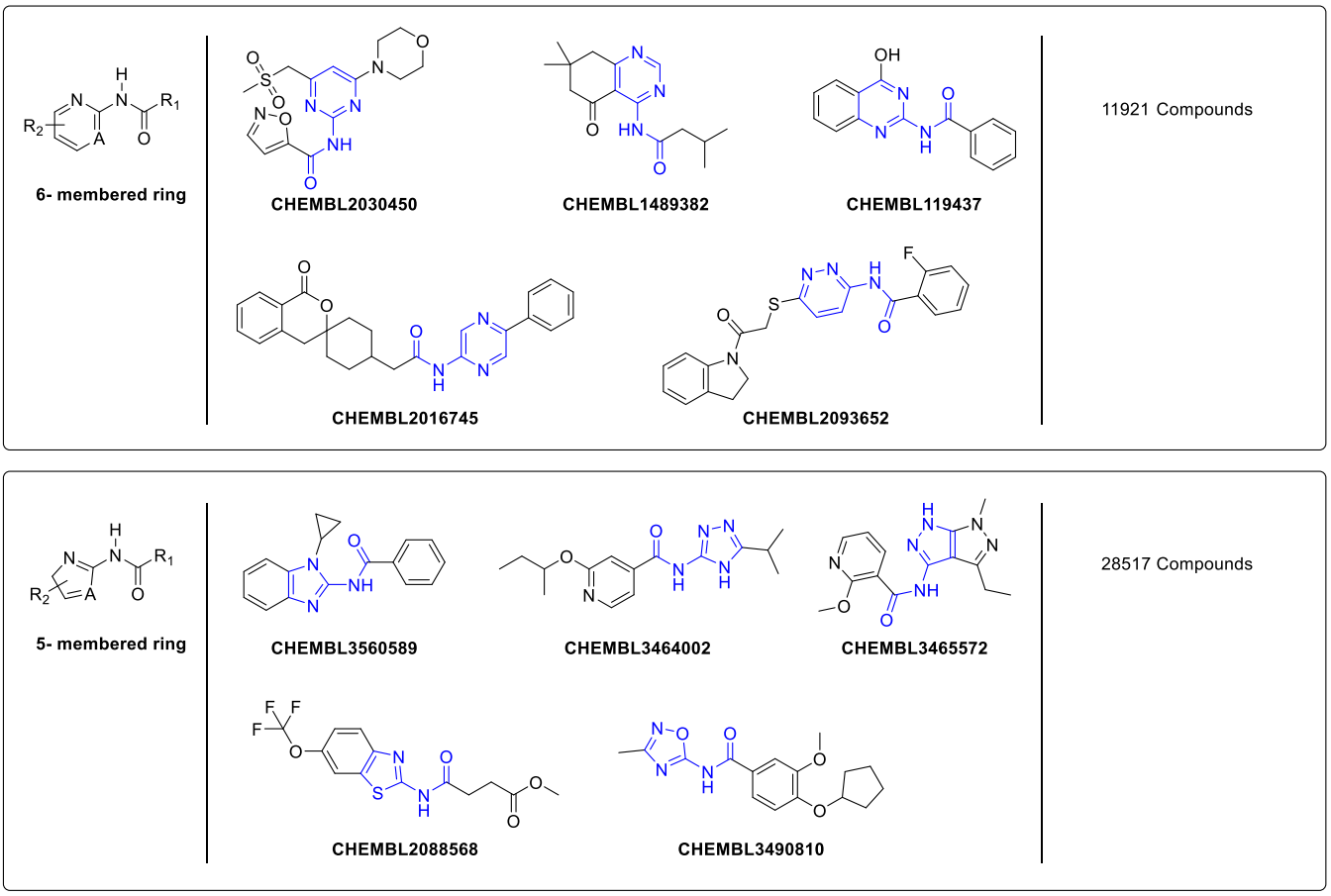


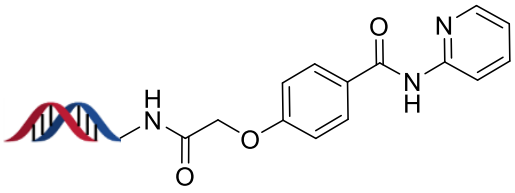
Figure S3. Selected examples of building blocks used in the Cu-catalyzed oxidative amidation reactions.

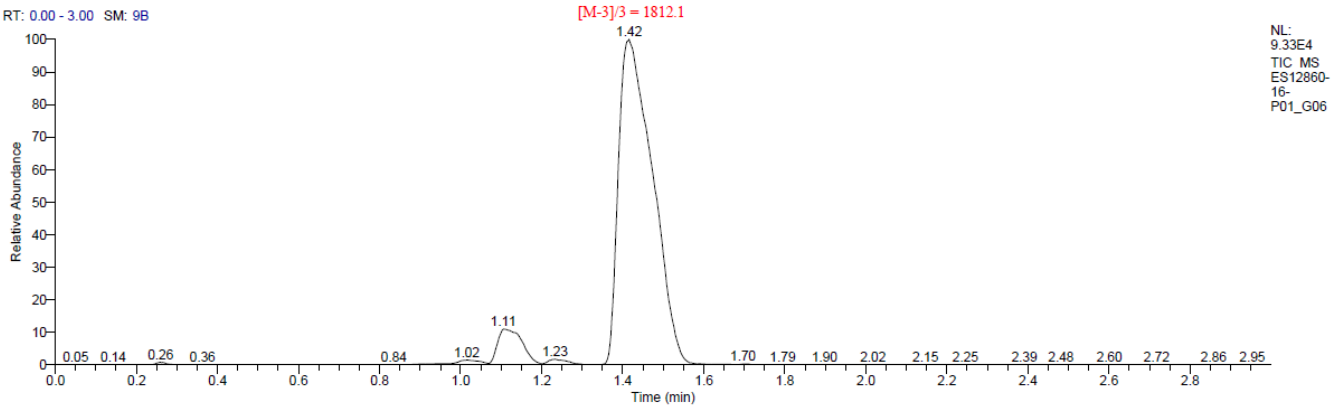
To investigate the molecules that could potentially be synthesized with the oxidative amidation reaction, the library of CHEMBL_25.sdf was searched through. 11921 (0.64% of the total) pyridin-2-yl analogous amides and 28517 (1.52% of the total) pyrrol-2-yl analogous amides were found, among which 283 molecules were counted in both of the categories. The total coverage of molecules that could be synthesized with the newly developed method is 2.15%. (CHEMBL database (CHEMBL 25) accessed on 2020. Feb. 26th under https://www.ebi.ac.uk/chembl/)

SI-6 LC Trace and Mass

SI-6-1 LC Trace and Mass of 3a

Following **General Procedure 3**
Yield: 92.31%
Exact mass: 5438.56
Triply charged mass [M-3]/3, calculated: 1811.9; observed: 1812.1.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	10532.76	0.05	22617.59	3.85
1.13	9454.39	0.08	19430.23	3.30
1.22	1549.48	0.03	1600.16	0.27
1.24	1387.02	0.02	1543.74	0.26
1.42	95603.18	0.25	542717.33	92.31

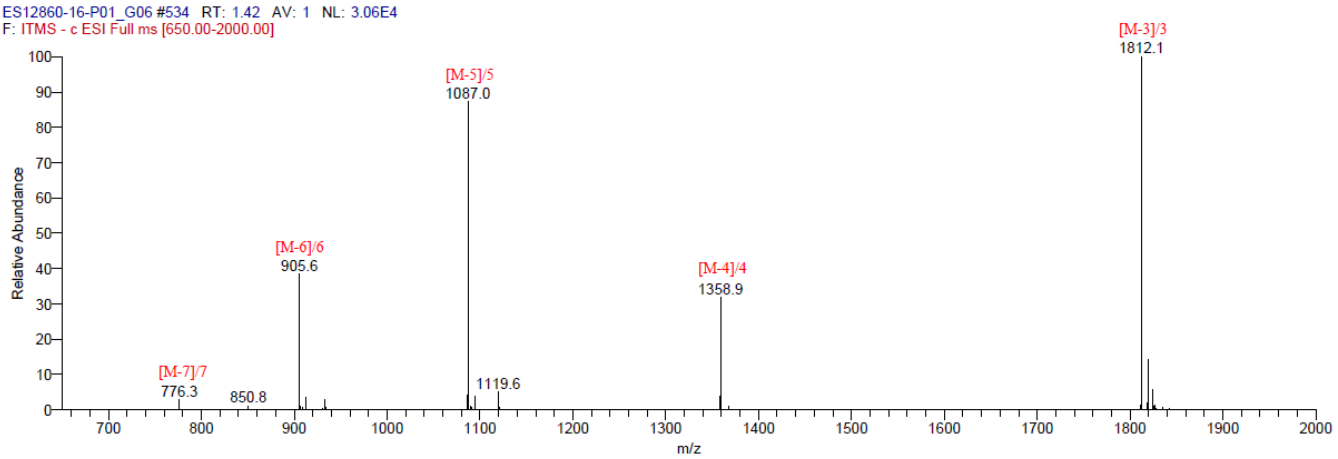
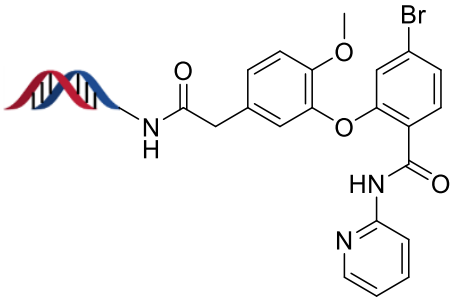
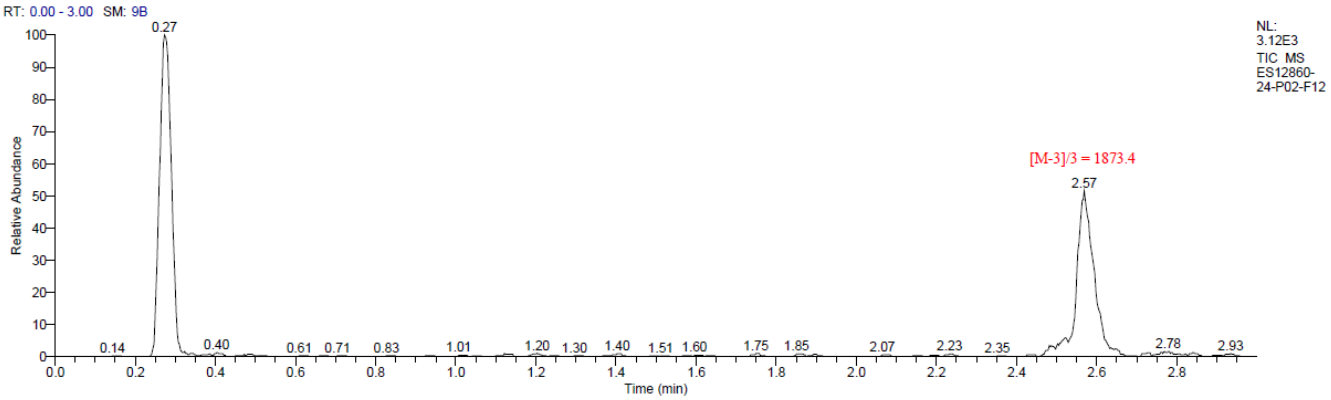


Figure S4. LC trace and mass of **3a**

SI-6-2 LC Trace and Mass of 3b

Yield: >99%
Exact mass: 5623.77
Triply charged mass [M-3]/3, calculated: 1873.6; observed: 1873.4.





RT	Peak Height	Peak Width	Peak Area	Area %
2.56	1704.14	0.06	3089.89	100.00

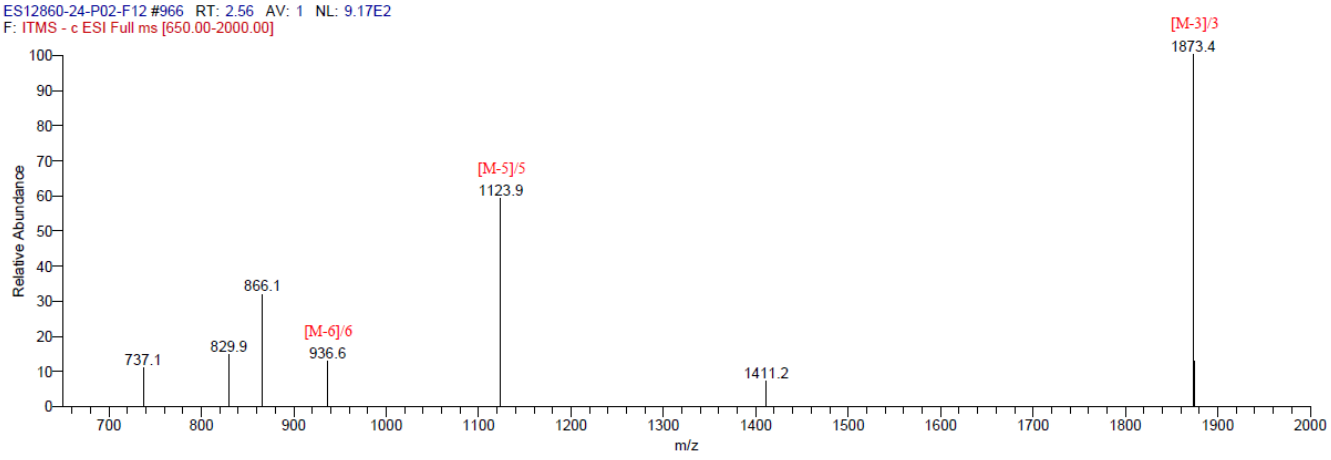
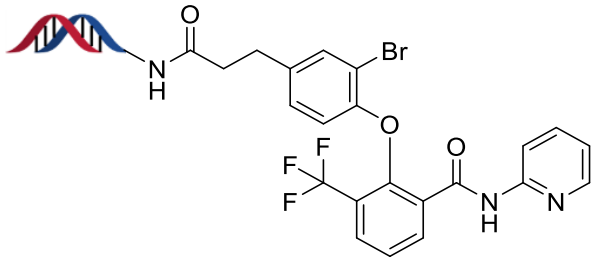
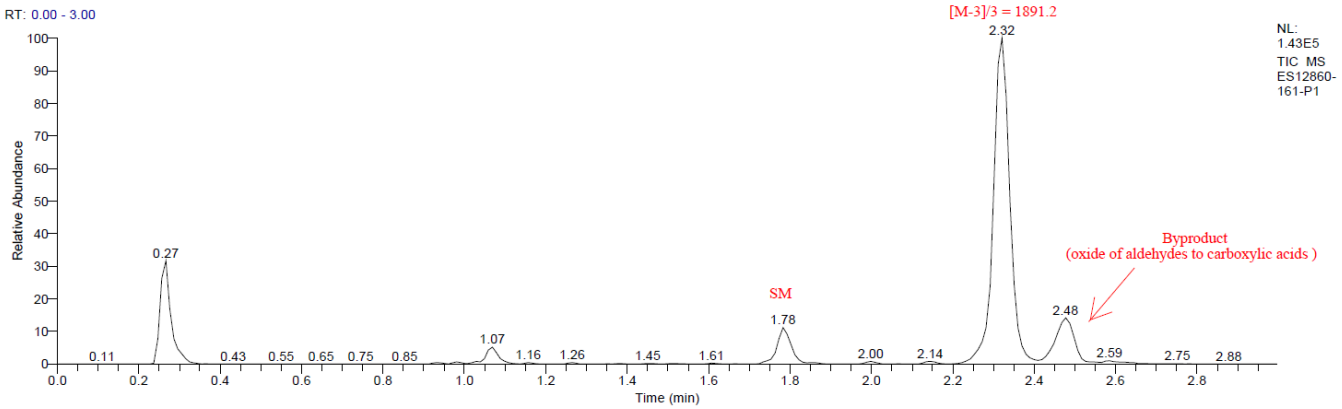


Figure S5. LC trace and mass of **3b**

SI-6-3 LC Trace and Mass of 3c

Yield: 77.13%
Exact mass: 5675.77
Triply charged mass [M-3]/3, calculated: 1890.9; observed: 1891.2.





RT	Peak Height	Peak Width	Peak Area	Area %
1.07	5449.05	0.04	9615.99	2.21
1.78	12123.61	0.07	33169.37	7.63
2.32	118829.77	0.07	335101.54	77.13
2.48	17404.78	0.08	56577.90	13.02

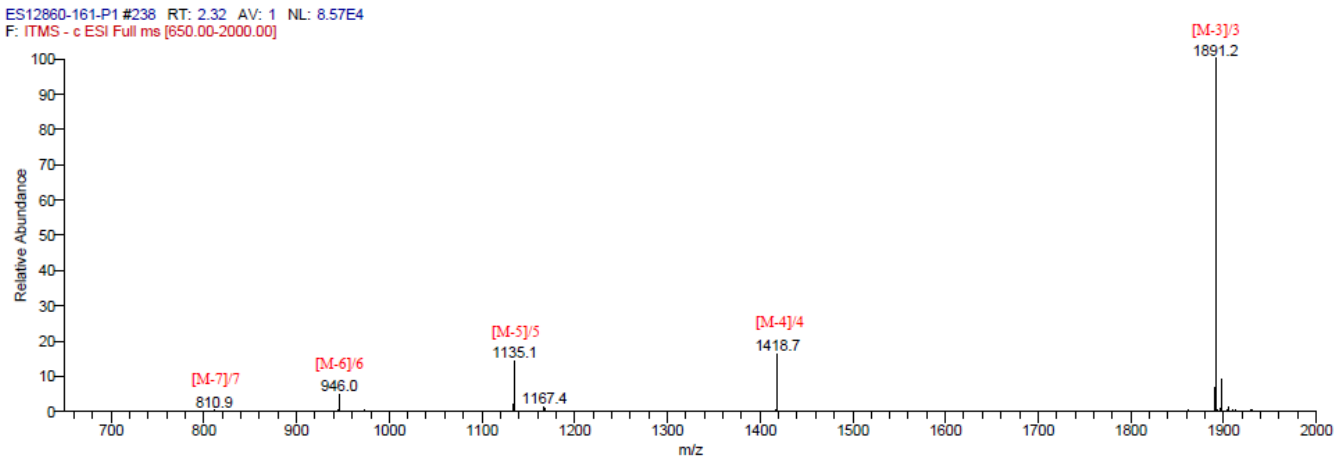
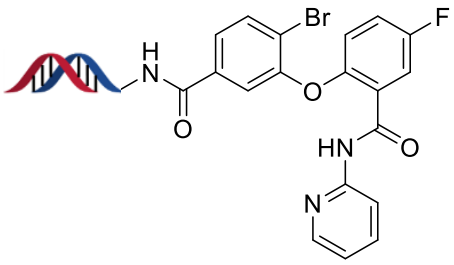
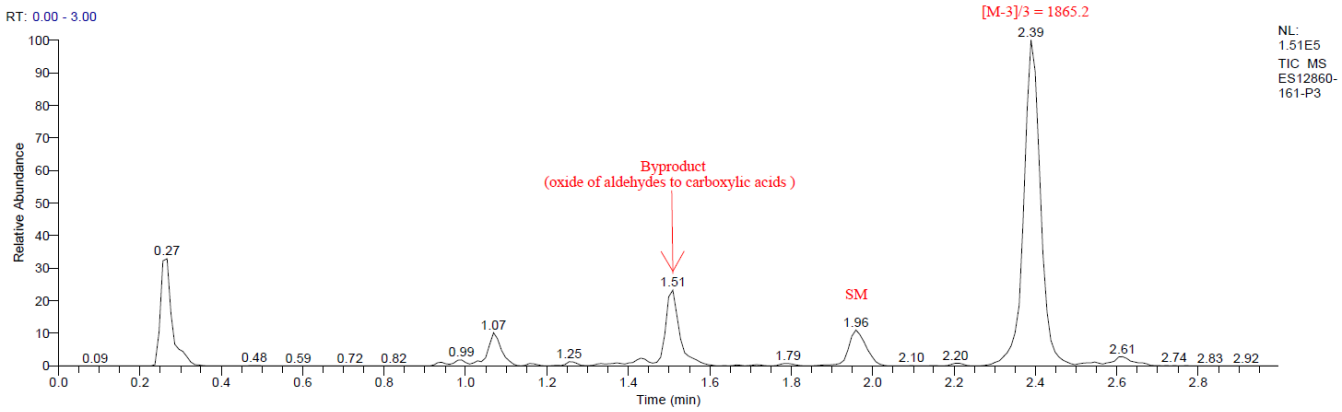


Figure S6. LC trace and mass of **3c**

SI-6-4 LC Trace and Mass of 3d

Following **General Procedure 3**
Yield: 69.87%
Exact mass: 5597.71
Triply charged mass [M-3]/3, calculated: 1864.9; observed: 1865.2.





RT	Peak Height	Peak Width	Peak Area	Area %
1.07	11266.70	0.07	30809.80	5.99
1.43	2867.77	0.02	3209.72	0.62
1.51	27035.24	0.06	67443.01	13.11
1.96	13789.12	0.07	41047.37	7.98
2.39	124240.47	0.07	359501.08	69.87
2.61	3490.03	0.09	12486.26	2.43

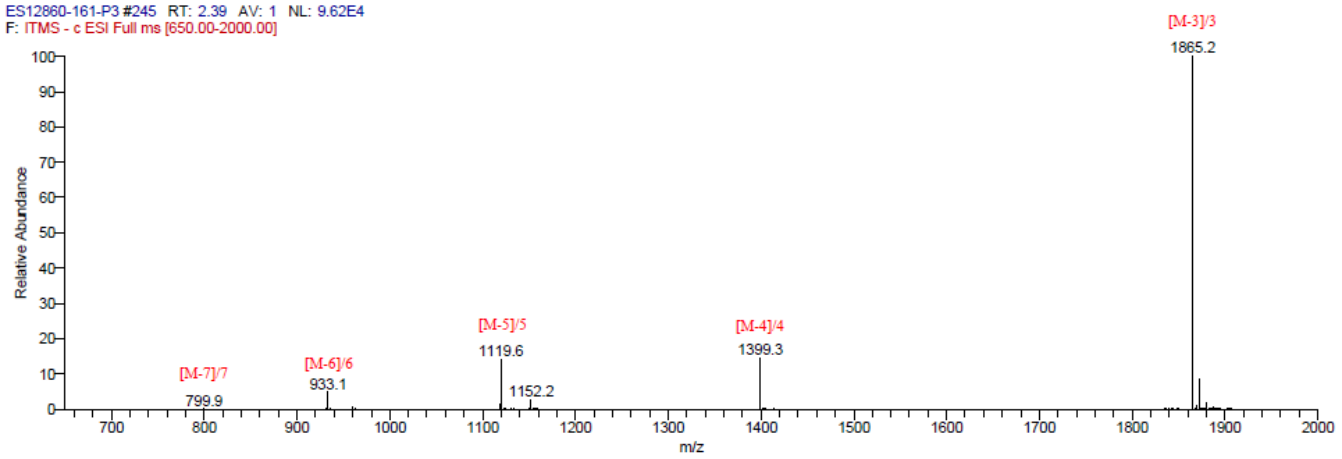
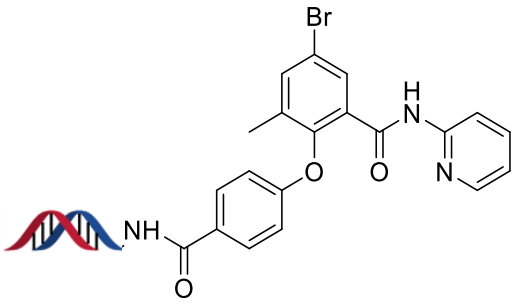
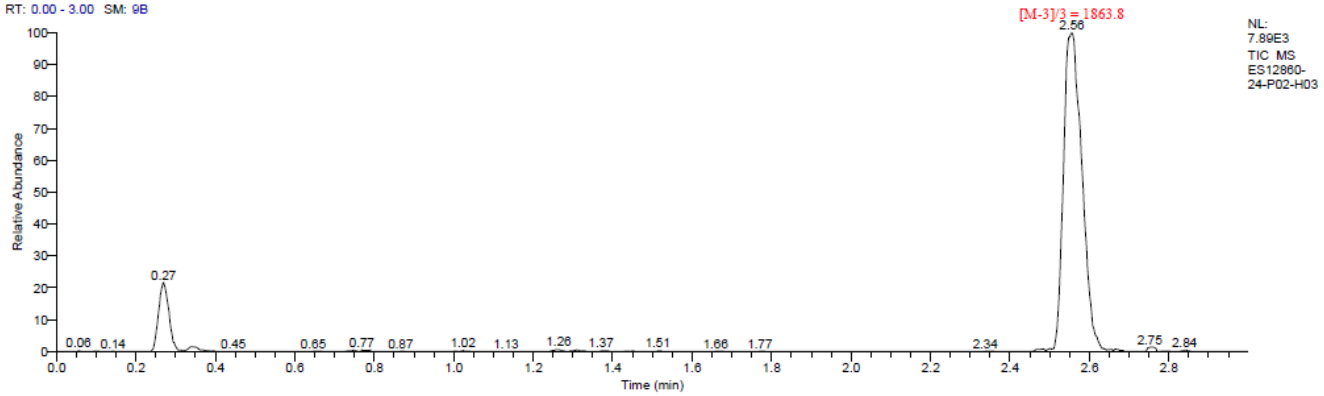


Figure S7. LC trace and mass of **3d**

SI-6-5 LC Trace and Mass of 3e

Following **General Procedure 3**
Yield: 99.12%
Exact mass: 5593.8
Triply charged mass [M-3]/3, calculated: 1863.6; observed: 1863.8.





RT	Peak Height	Peak Width	Peak Area	Area %
2.55	8130.75	0.06	17599.57	99.12
2.76	261.90	0.04	157.11	0.88

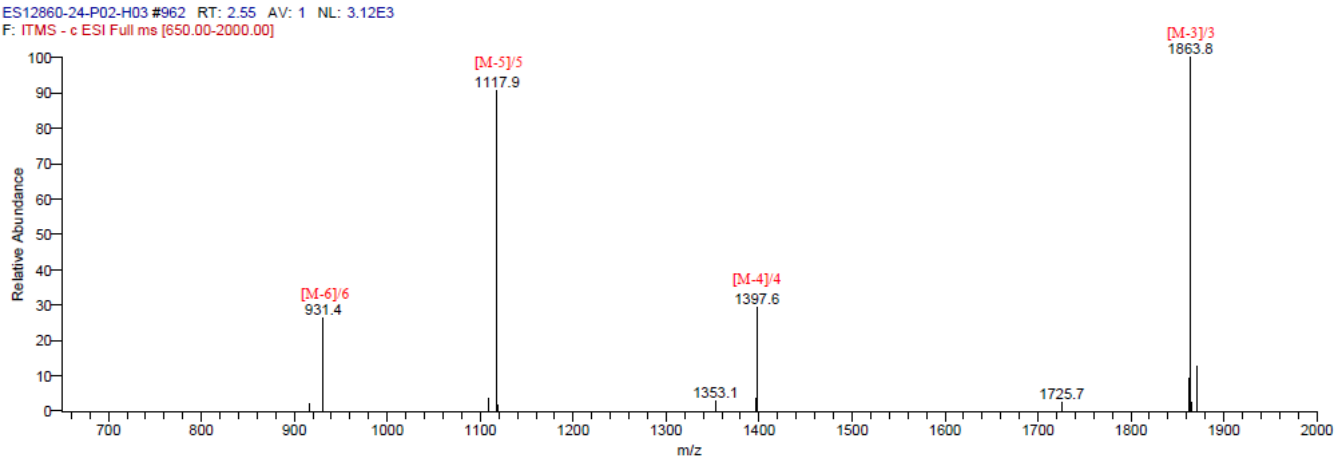
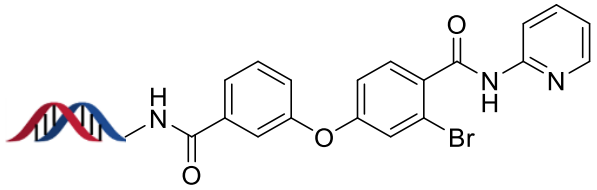
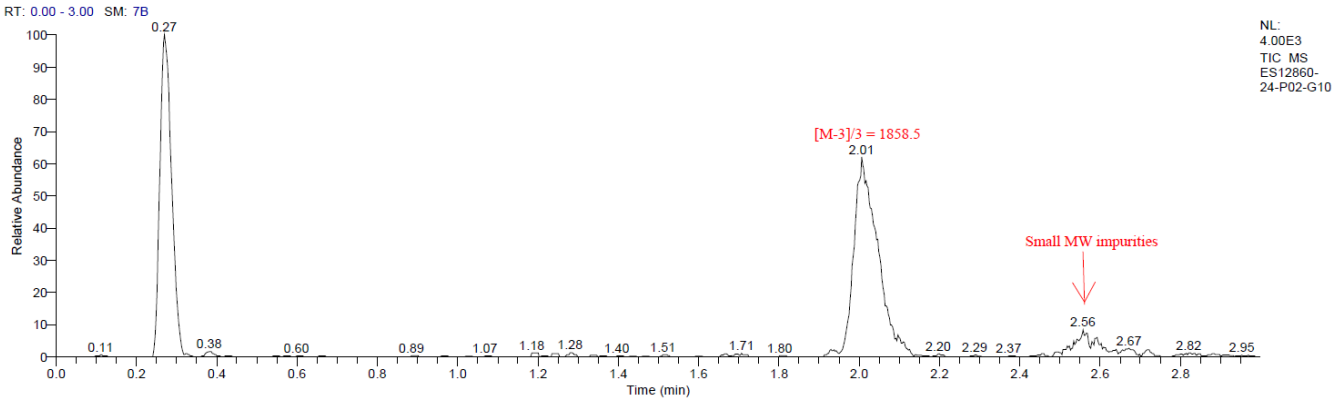


Figure S8. LC trace and mass of **3e**

SI-6-6 LC Trace and Mass of 3f

Following **General Procedure 3**
Yield: 88.75%
Exact mass: 5579.72
Triply charged mass [M-3]/3, calculated: 1858.9; observed: 1858.5.





RT	Peak Height	Peak Width	Peak Area	Area %
2.00	2431.68	0.23	10241.32	88.75
2.54	225.76	0.03	298.98	2.59
2.56	365.75	0.02	356.11	3.09
2.59	247.01	0.05	412.33	3.57
2.68	110.12	0.05	231.04	2.00

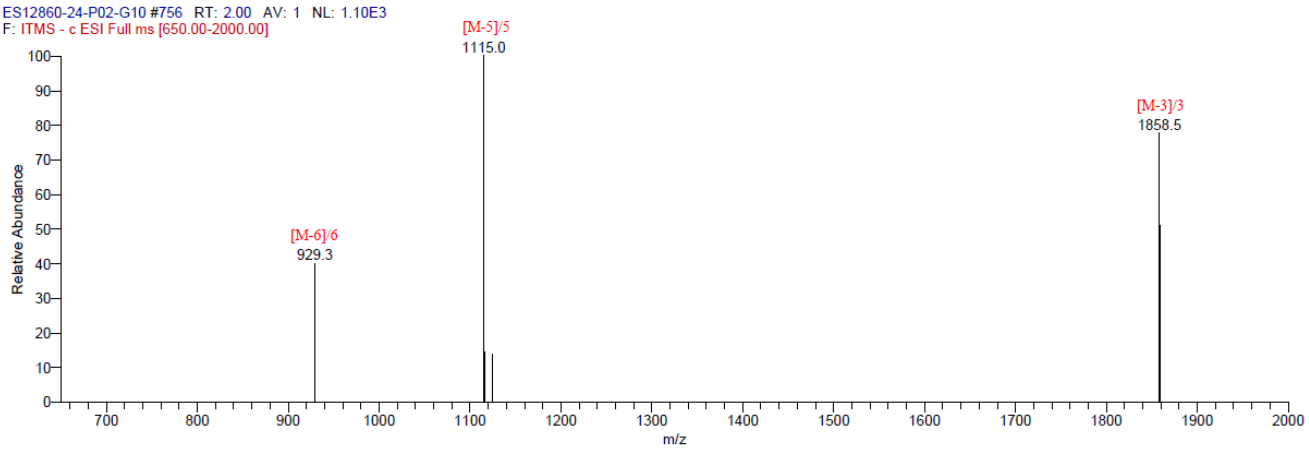
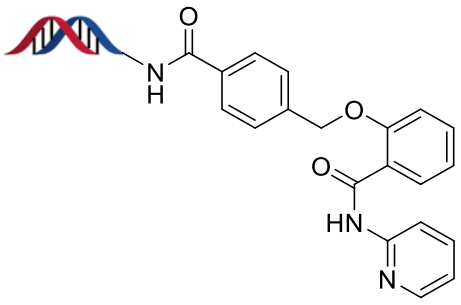
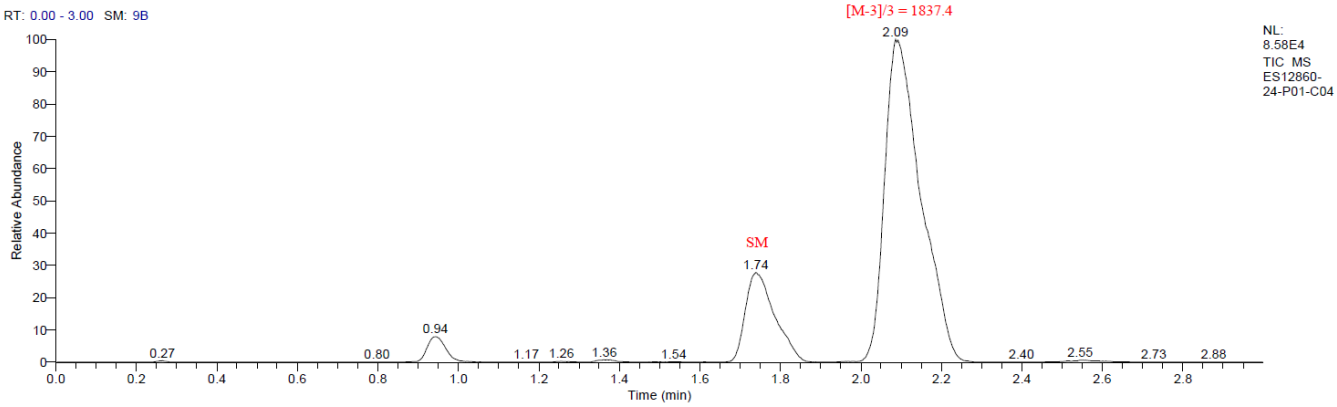


Figure S9. LC trace and mass of **3f**

SI-6-7 LC Trace and Mass of 3g

Following **General Procedure 3**
Yield: 80.94%
Exact mass: 5514.85
Triply charged mass [M-3]/3, calculated: 1837.3; observed: 1837.4.





RT	Peak Height	Peak Width	Peak Area	Area %
0.94	7032.50	0.12	21793.51	3.30
1.35	681.06	0.03	852.30	0.13
1.37	746.53	0.04	1128.36	0.17
1.74	23162.54	0.16	102069.69	15.46
2.09	87597.83	0.26	534512.89	80.94

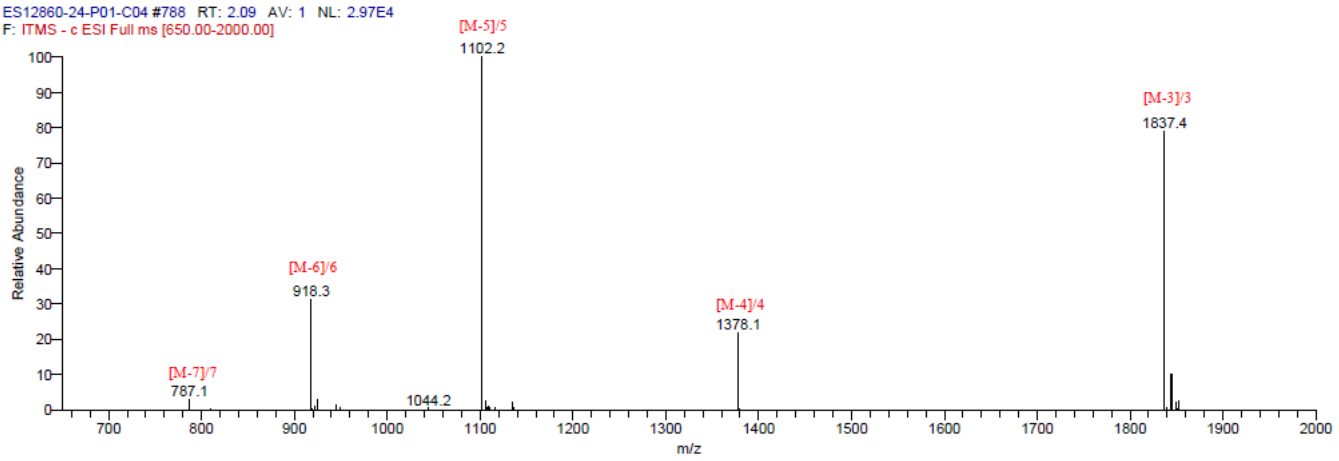
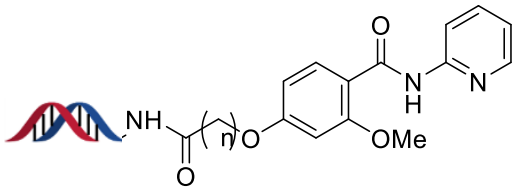
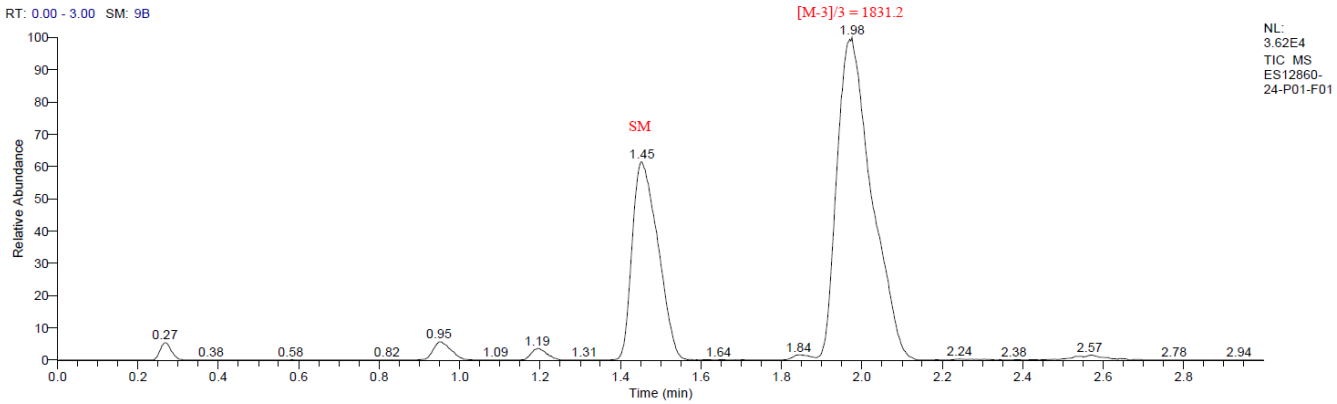


Figure S10. LC trace and mass of **3g**

SI-6-8 LC Trace and Mass of 3h

Following **General Procedure 3**
Yield: 66.01%
Exact mass: 5496.83
Triply charged mass [M-3]/3, calculated: 1831.3; observed: 1831.2.





RT	Peak Height	Peak Width	Peak Area	Area %
0.95	2148.58	0.14	6379.83	1.96
1.19	1344.31	0.10	3504.73	1.07
1.45	22825.66	0.20	99150.50	30.40
1.84	674.32	0.07	1818.23	0.56
1.97	36770.72	0.24	215286.07	66.01

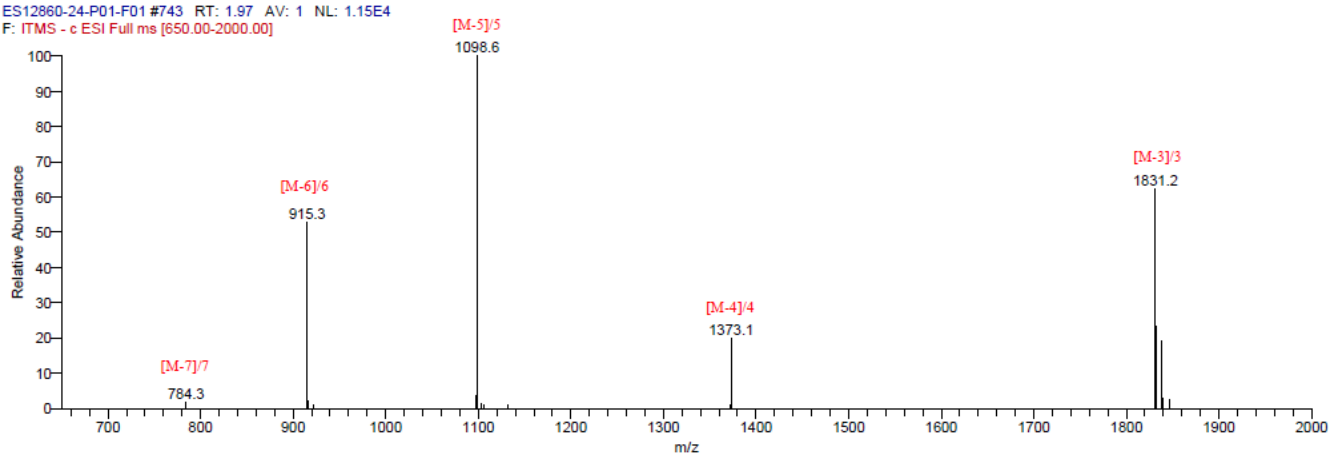
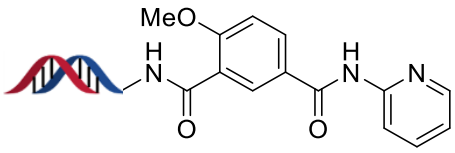
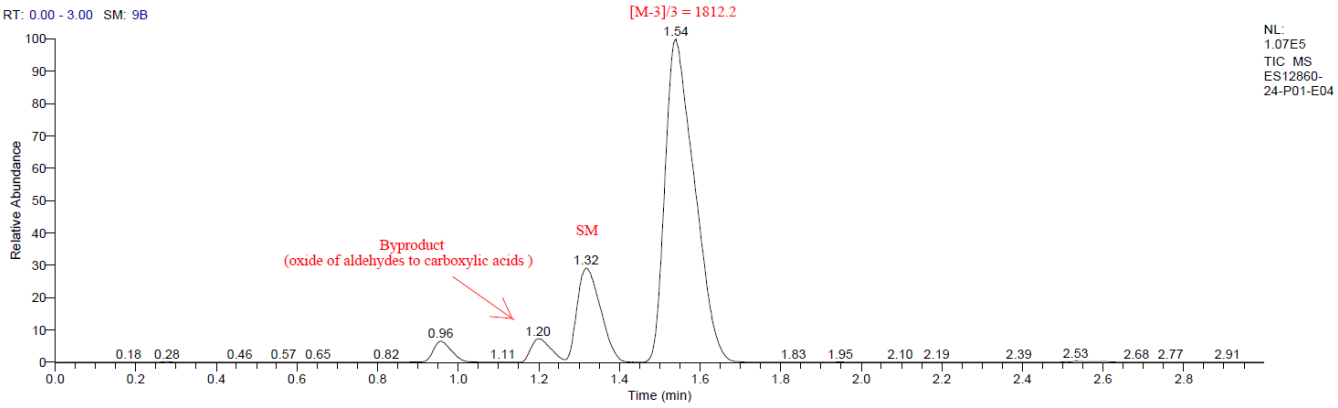


Figure S11. LC trace and mass of **3h**

SI-6-9 LC Trace and Mass of 3i

Following **General Procedure 3**
Yield: 74.54%
Exact mass: 5438.75
Triply charged mass [M-3]/3, calculated: 1811.9; observed: 1812.2.





RT	Peak Height	Peak Width	Peak Area	Area %
0.96	7289.20	0.12	21623.79	3.02
1.20	8430.40	0.11	26859.77	3.75
1.31	31803.26	0.18	125983.95	17.61
1.54	109754.25	0.24	540485.13	75.54
2.54	605.83	0.03	574.92	0.08

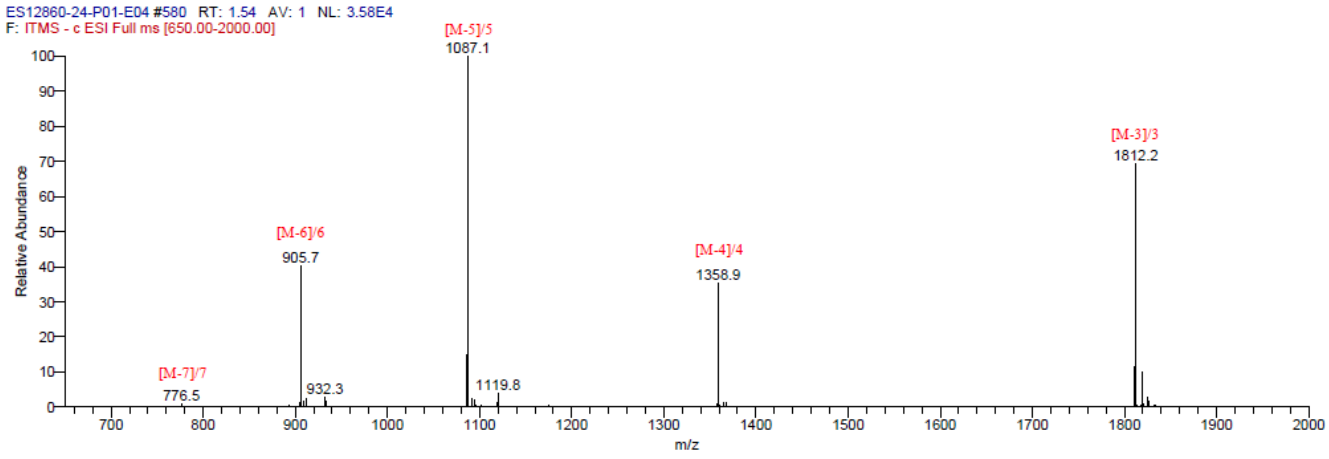
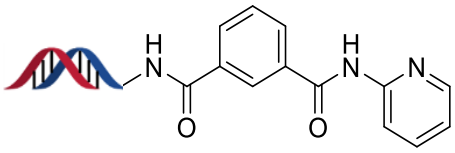
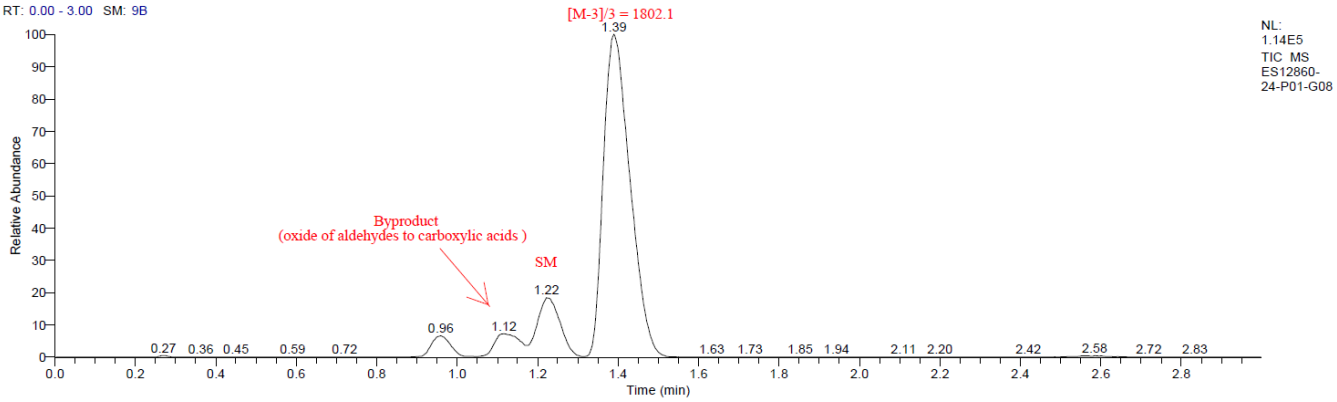


Figure S12. LC trace and mass of **3i**

SI-6-10 LC Trace and Mass of 3j

Following **General Procedure 3**
Yield: 79.33%
Exact mass: 5408.72
Triply charged mass [M-3]/3, calculated: 1801.9; observed: 1802.1.





RT	Peak Height	Peak Width	Peak Area	Area %
0.96	7887.26	0.09	15781.26	2.38
1.11	8647.07	0.09	21916.69	3.30
1.14	7914.56	0.05	16100.88	2.43
1.23	21720.58	0.14	83325.71	12.56
1.39	117877.47	0.23	526263.18	79.33

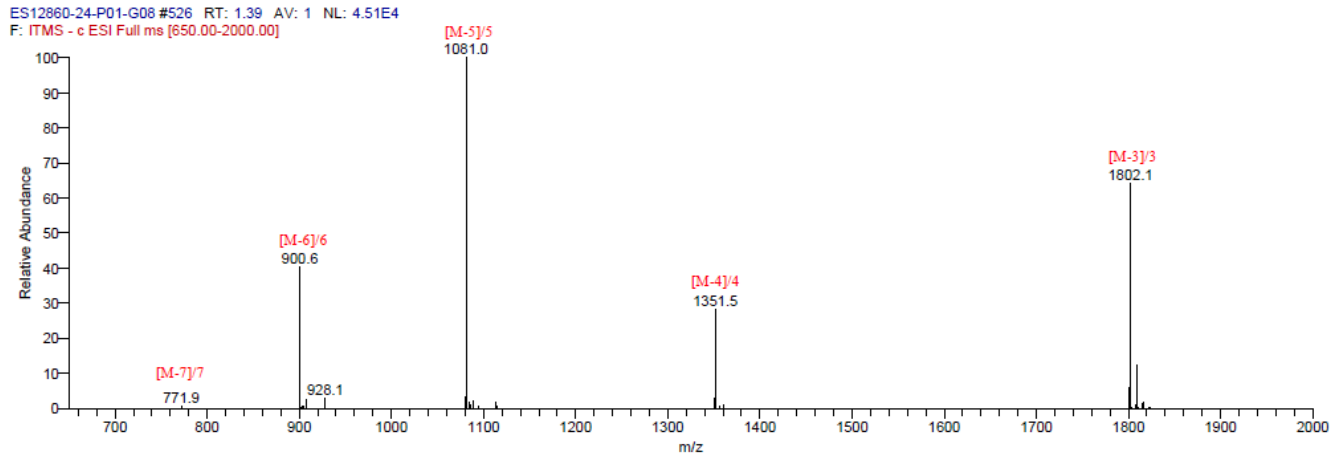
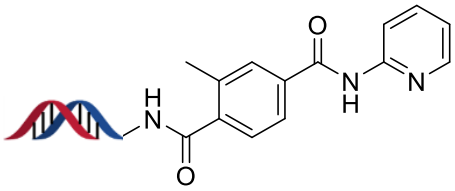
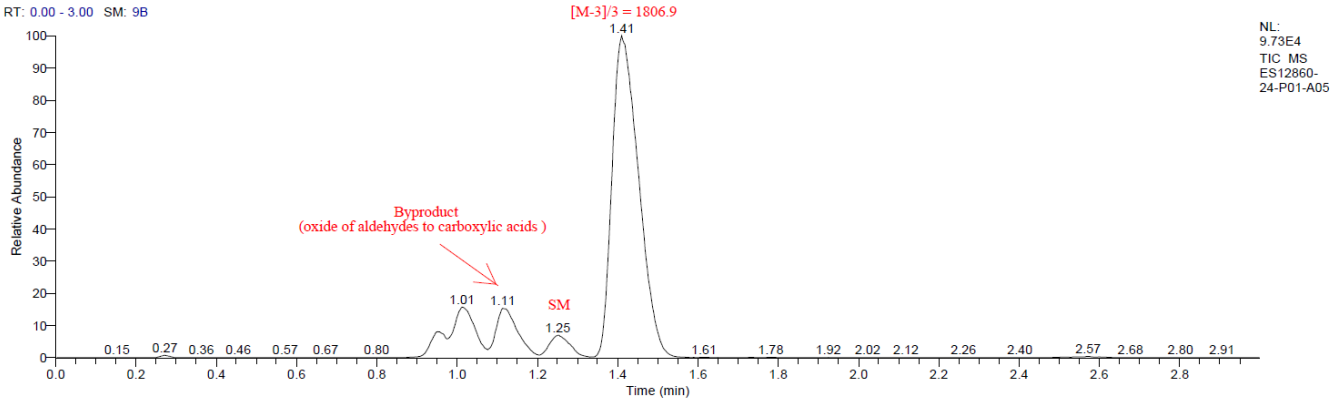


Figure S13. LC trace and mass of **3j**

SI-6-11 LC Trace and Mass of 3k

Following **General Procedure 3**
Yield: 74.7%
Exact mass: 5422.75
Triply charged mass [M-3]/3, calculated: 1806.6; observed: 1806.9.





RT	Peak Height	Peak Width	Peak Area	Area %
0.96	8508.54	0.09	22352.75	3.72
1.01	16147.25	0.10	55164.62	9.19
1.11	15442.75	0.12	56342.17	9.39
1.26	7186.23	0.06	17965.96	2.99
1.41	99061.92	0.23	448276.14	74.70

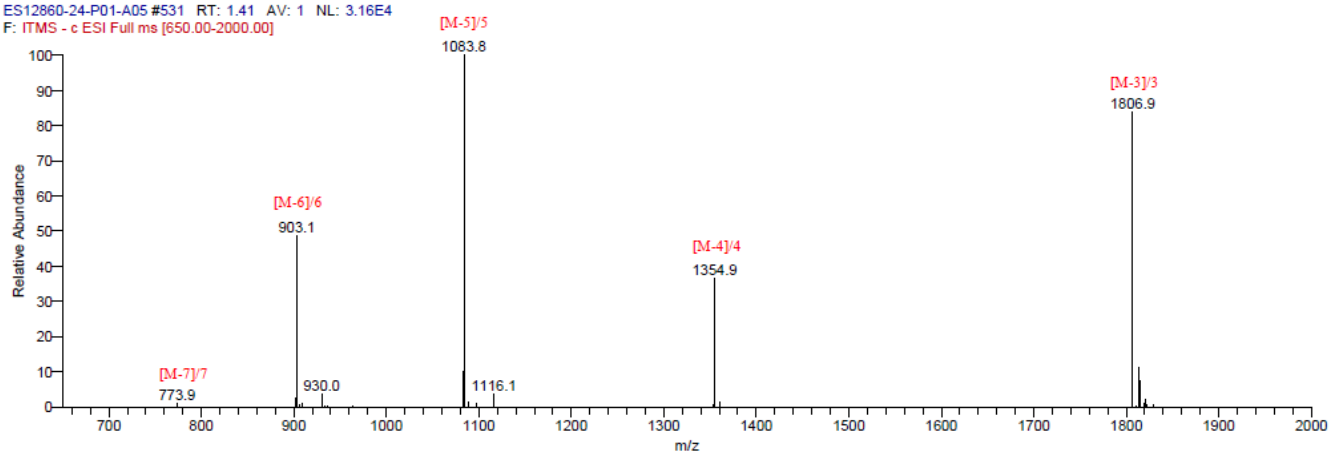
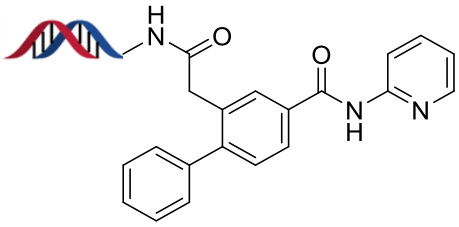
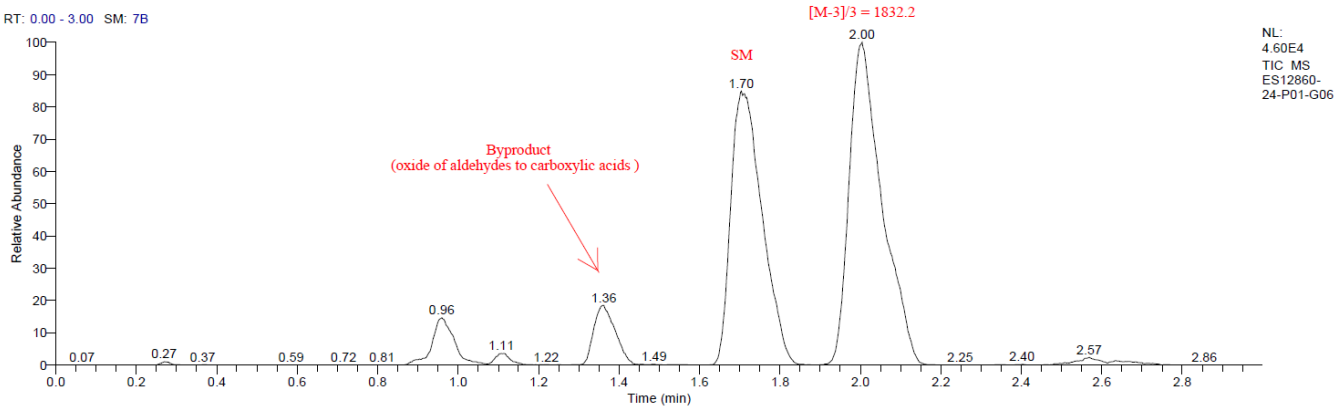


Figure S14. LC trace and mass of **3k**

SI-6-12 LC Trace and Mass of 3l

Following **General Procedure 3**
Yield: 48.70%
Exact mass: 5498.85
Triply charged mass [M-3]/3, calculated: 1832.0; observed: 1832.2.





RT	Peak Height	Peak Width	Peak Area	Area %
0.96	6883.74	0.17	24554.94	4.58
1.11	1727.52	0.11	4479.69	0.84
1.36	8561.72	0.17	31765.83	5.92
1.71	39313.66	0.27	211599.85	39.46
2.00	46443.14	0.35	261136.35	48.70
2.57	1056.73	0.07	2659.45	0.50

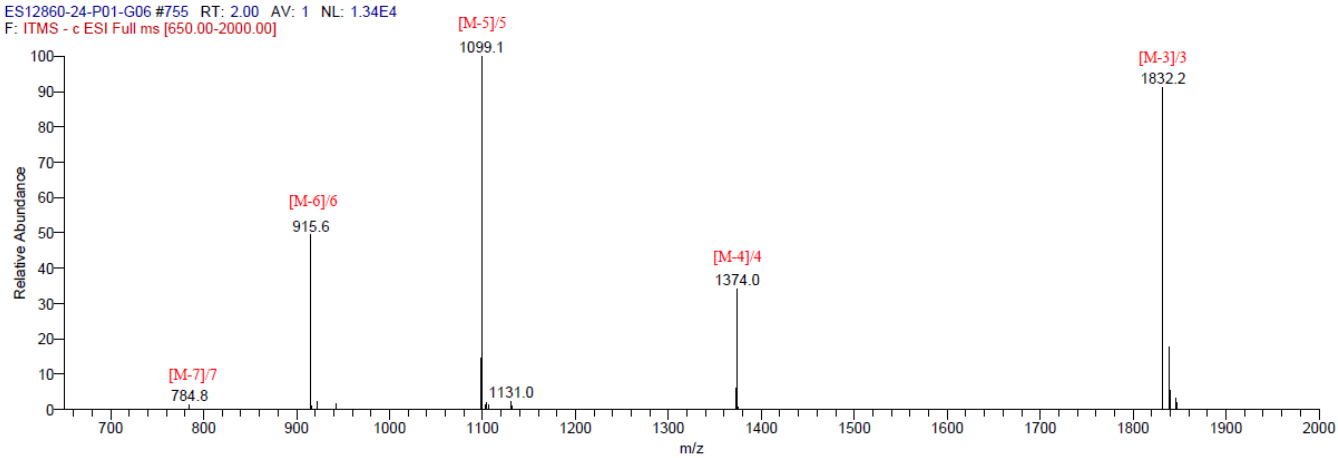
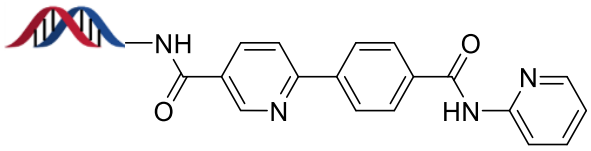
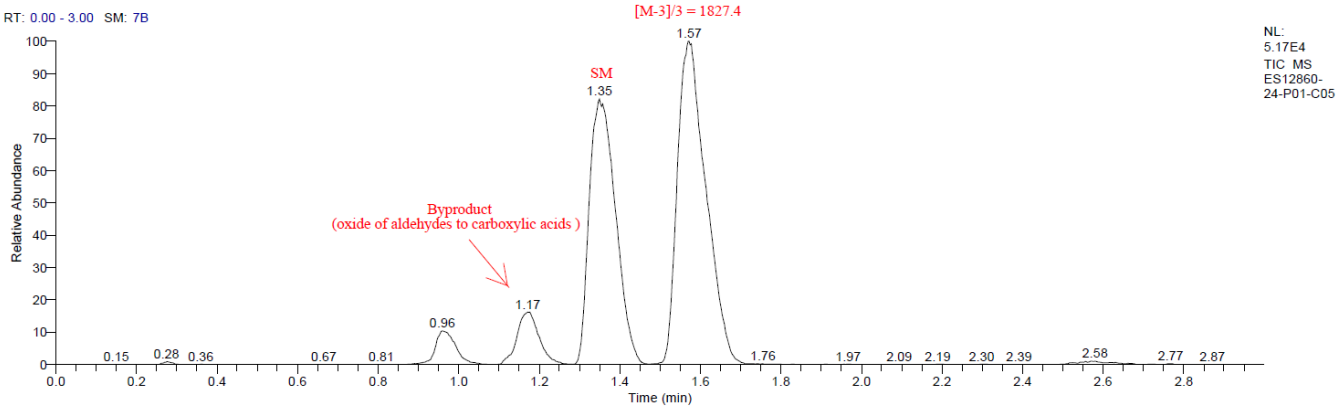


Figure S15. LC trace and mass of **3l**

SI-6-13 LC Trace and Mass of 3m

Following **General Procedure 3**
Yield: 51.06%
Exact mass: 5485.81
Triply charged mass [M-3]/3, calculated: 1827.6; observed: 1827.4.





RT	Peak Height	Peak Width	Peak Area	Area %
0.96	5342.52	0.16	18825.87	3.77
1.17	8535.55	0.18	32126.23	6.44
1.35	42700.50	0.17	193164.33	38.73
1.57	52206.65	0.23	254674.69	51.06

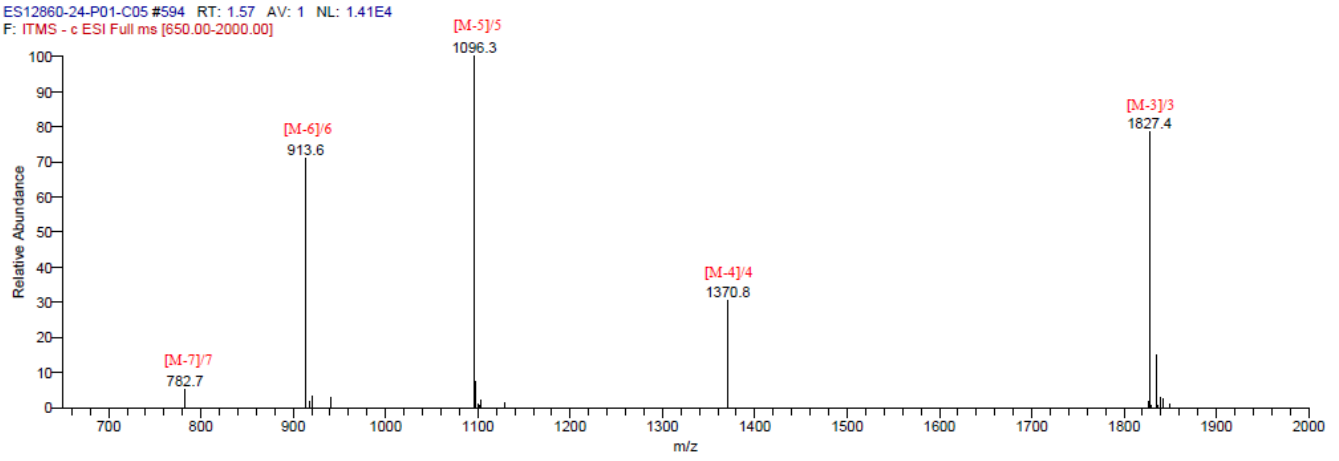
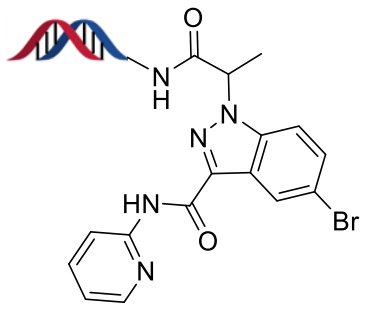
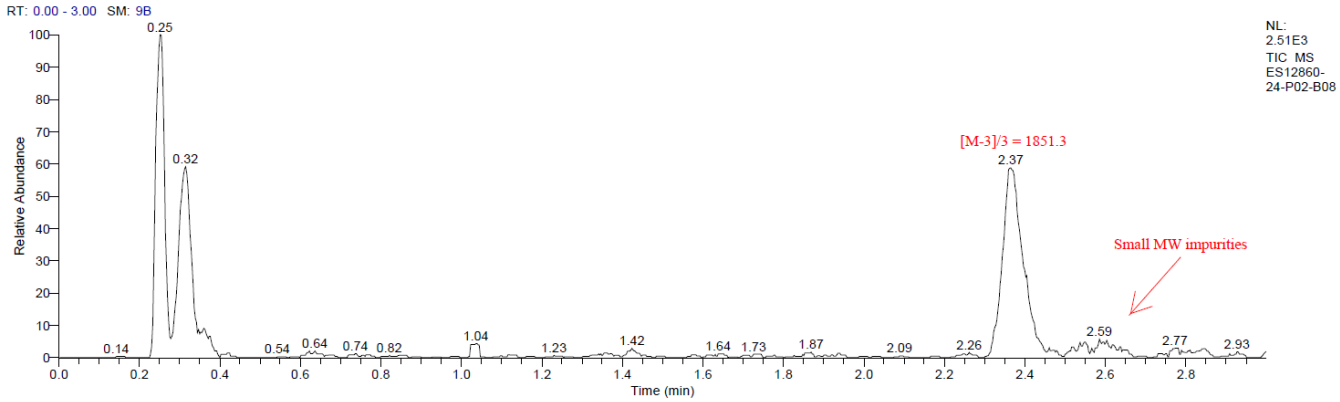


Figure S16. LC trace and mass of **3m**

SI-6-14 LC Trace and Mass of 3n

Following **General Procedure 3**
Yield: 85.95%
Exact mass: 5555.7
Triply charged mass [M-3]/3, calculated: 1850.9; observed: 1851.3.





RT	Peak Height	Peak Width	Peak Area	Area %
2.33	424.52	0.03	357.44	6.26
2.37	1648.07	0.11	4906.42	85.95
2.55	208.60	0.03	174.72	3.06
2.60	183.75	0.04	270.08	4.73

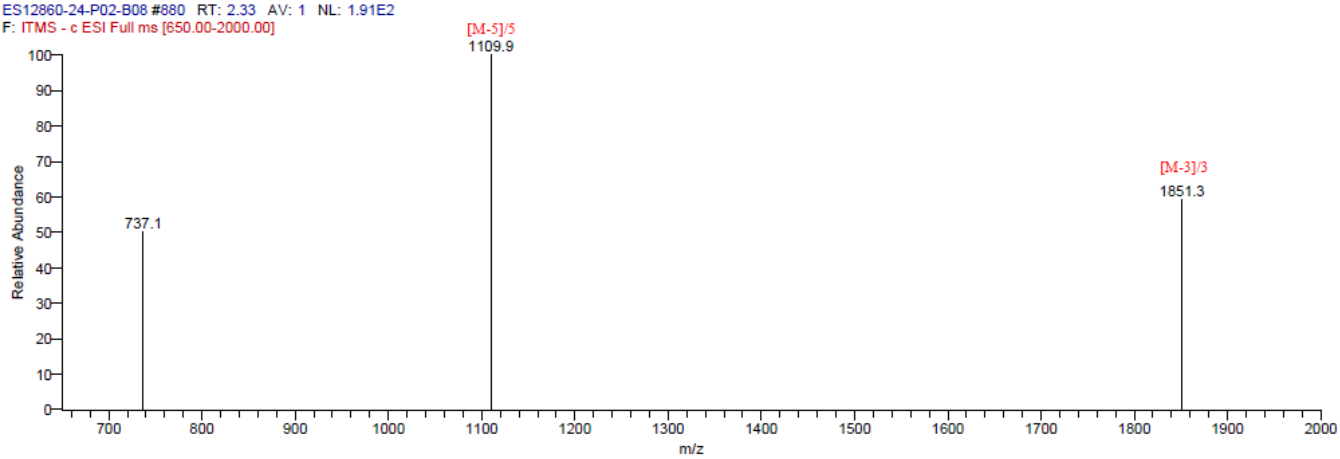
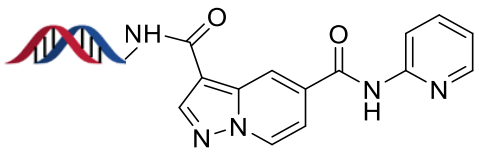
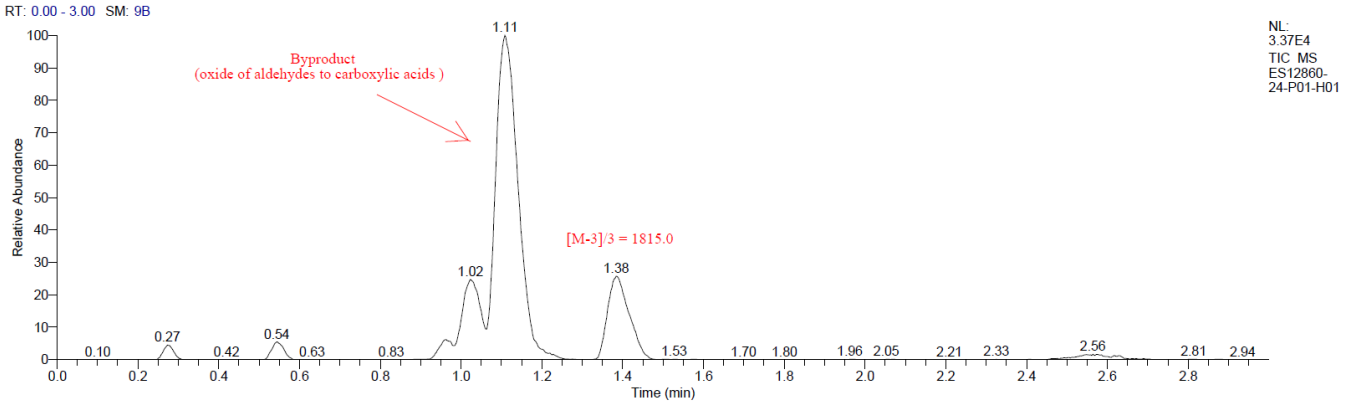


Figure S17. LC trace and mass of **3n**

SI-6-15 LC Trace and Mass of 3o

Following **General Procedure 3**
Yield: 17.14%
Exact mass: 5448.75
Triply charged mass [M-3]/3, calculated: 1815.3; observed: 1815.0.





RT	Peak Height	Peak Width	Peak Area	Area %
0.97	2407.84	0.08	4628.29	2.47
1.02	8502.55	0.08	25459.67	13.59
1.11	33789.24	0.12	124103.62	66.25
1.20	1128.32	0.03	1020.74	0.54
1.39	8927.77	0.16	32116.53	17.14

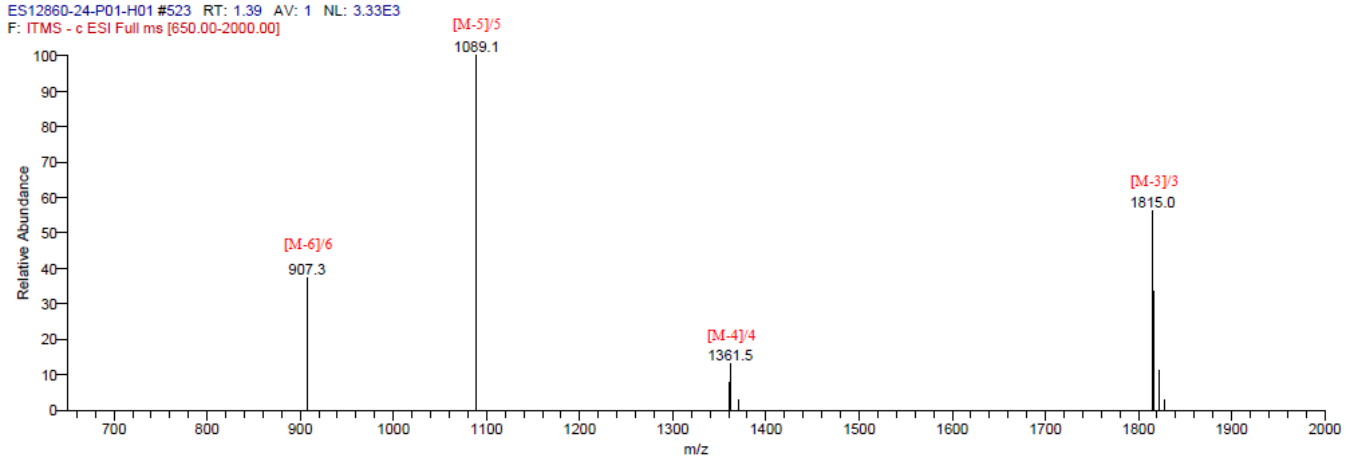
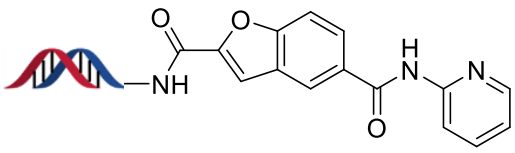
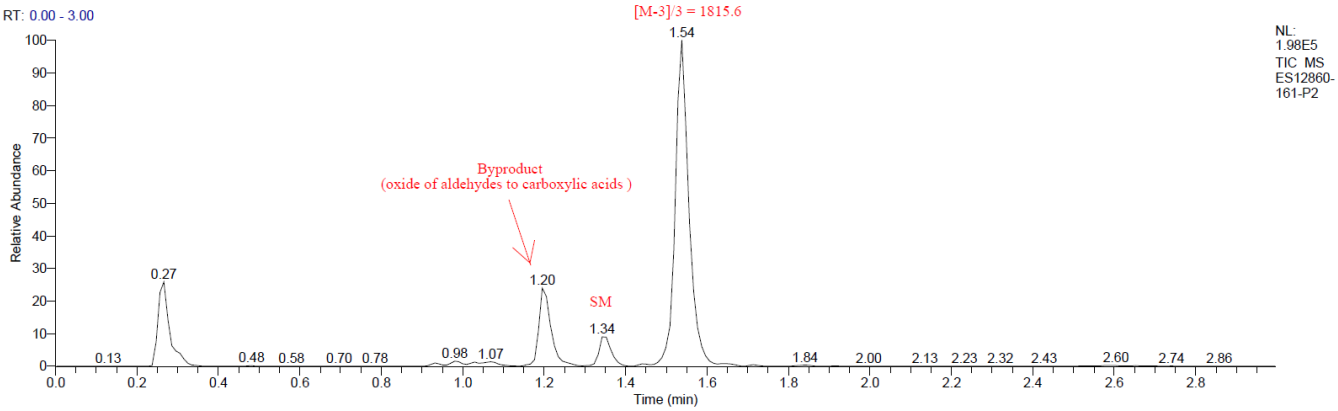


Figure S18. LC trace and mass of **3o**

SI-6-16 LC Trace and Mass of 3p

Following **General Procedure 3**
Yield: 76.37%
Exact mass: 5448.74
Triply charged mass [M-3]/3, calculated: 1815.3; observed: 1815.6.





RT	Peak Height	Peak Width	Peak Area	Area %
1.21	33952.81	0.06	83807.04	16.91
1.35	13692.19	0.06	33300.39	6.72
1.54	151616.92	0.06	378394.39	76.37

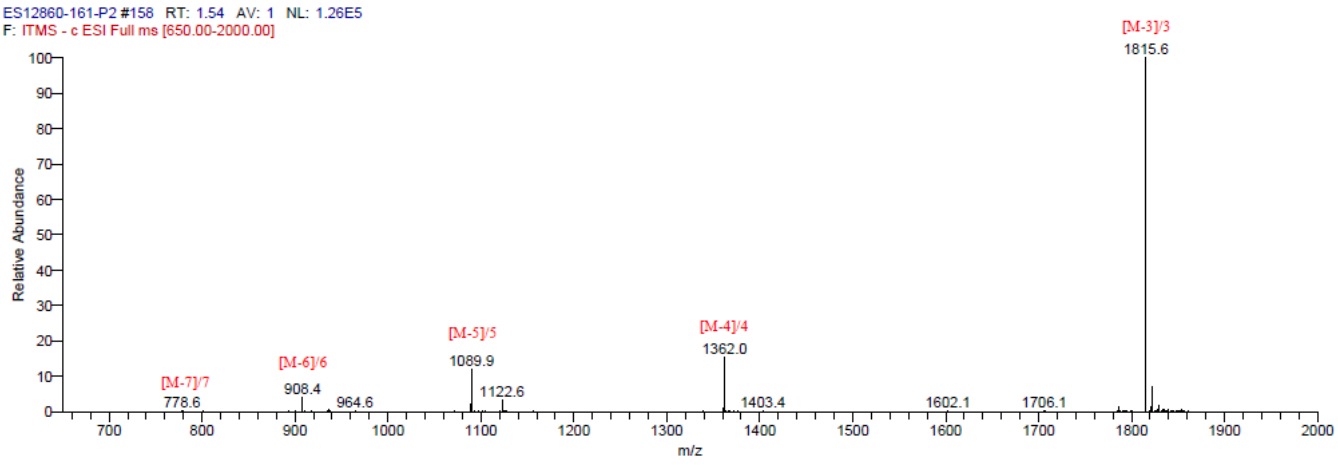
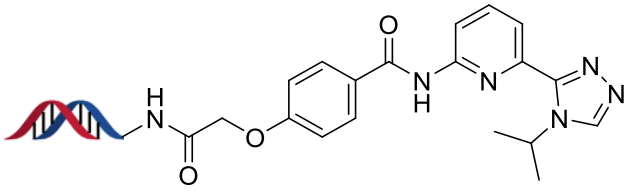
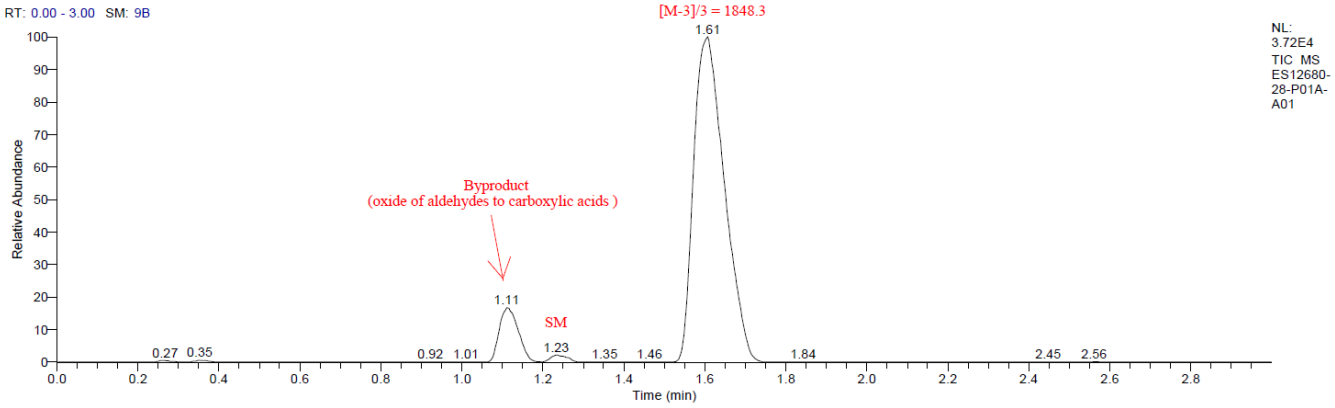


Figure S19. LC trace and mass of **3p**

SI-6-17 LC Trace and Mass of 4b

Following **General Procedure 3**
Yield: 92.75%
Exact mass: 5547.89
Triply charged mass [M-3]/3, calculated: 1848.3; observed: 1848.3.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	5654.43	0.06	12940.67	6.14
1.23	893.52	0.05	1555.02	0.74
1.26	715.89	0.03	793.59	0.38
1.60	37398.53	0.24	195595.26	92.75

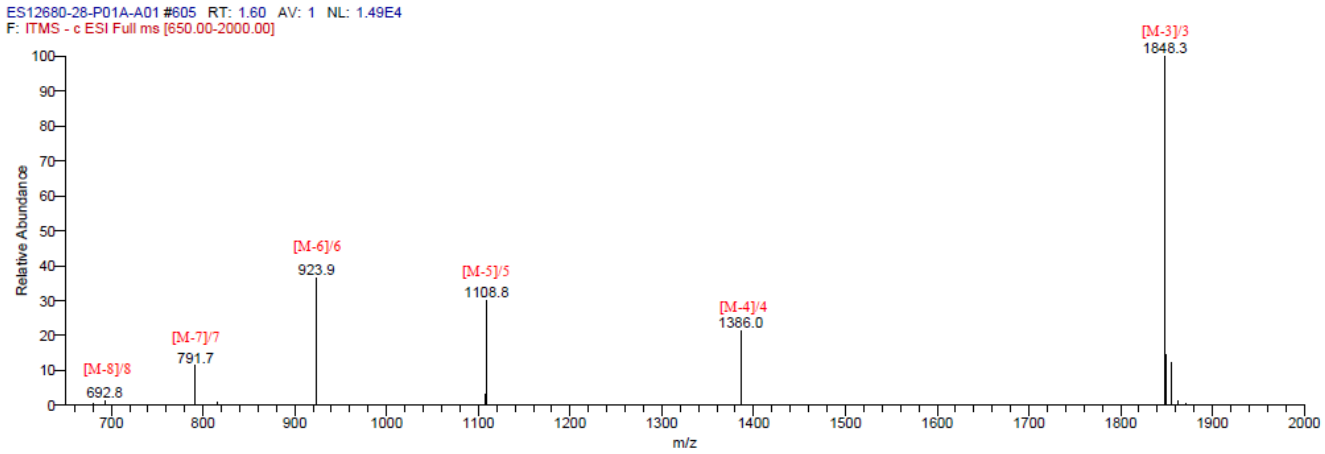
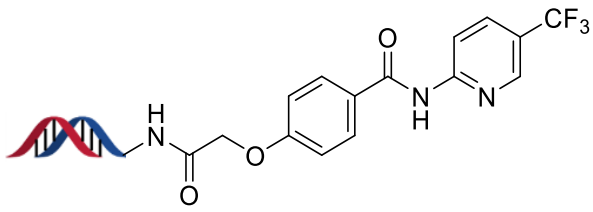
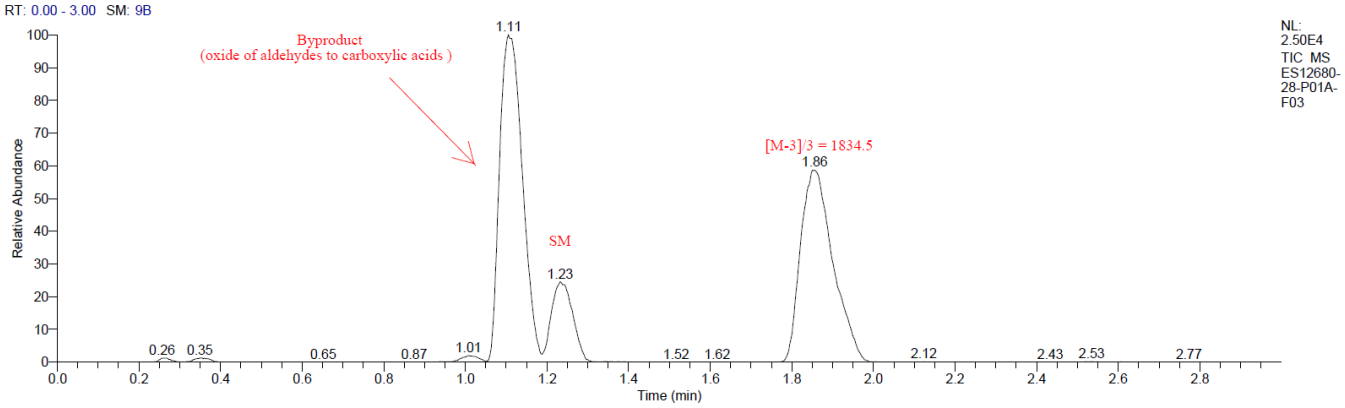


Figure S20. LC trace and mass of **4b**

SI-6-18 LC Trace and Mass of 4c

Following **General Procedure 3**
Yield: 42.26%
Exact mass: 5506.75
Triply charged mass [M-3]/3, calculated: 1834.6; observed: 1834.5.





RT	Peak Height	Peak Width	Peak Area	Area %
1.01	187.29	0.04	295.54	0.15
1.11	25181.37	0.14	96169.81	49.96
1.24	5112.23	0.07	14686.05	7.63
1.86	15531.02	0.24	81337.51	42.26

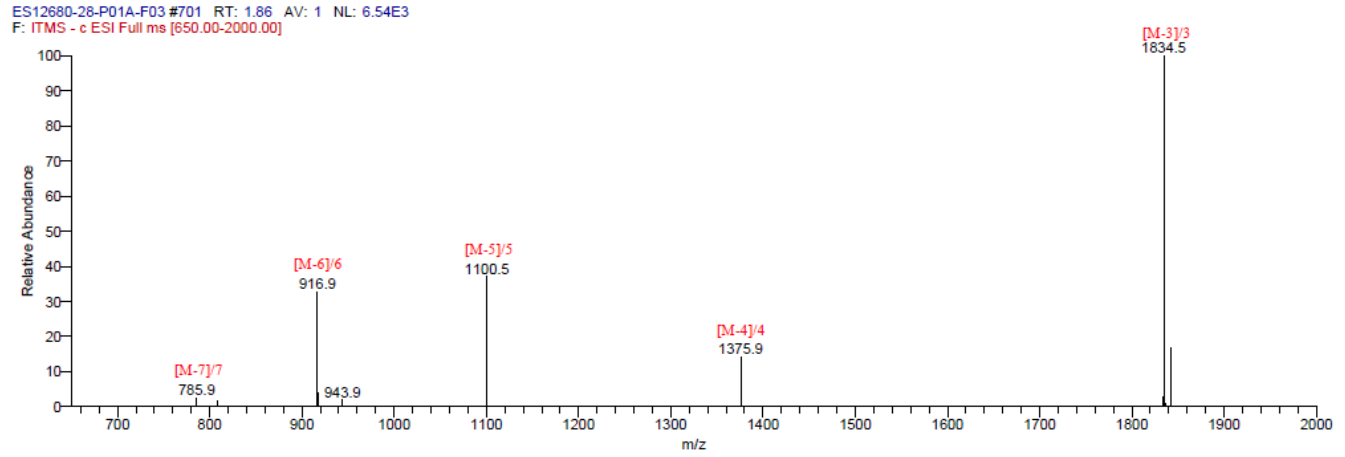
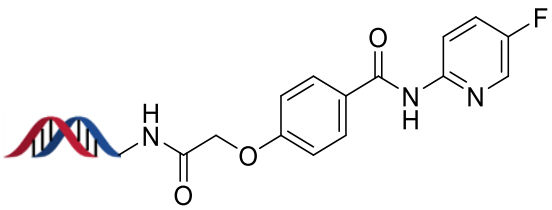
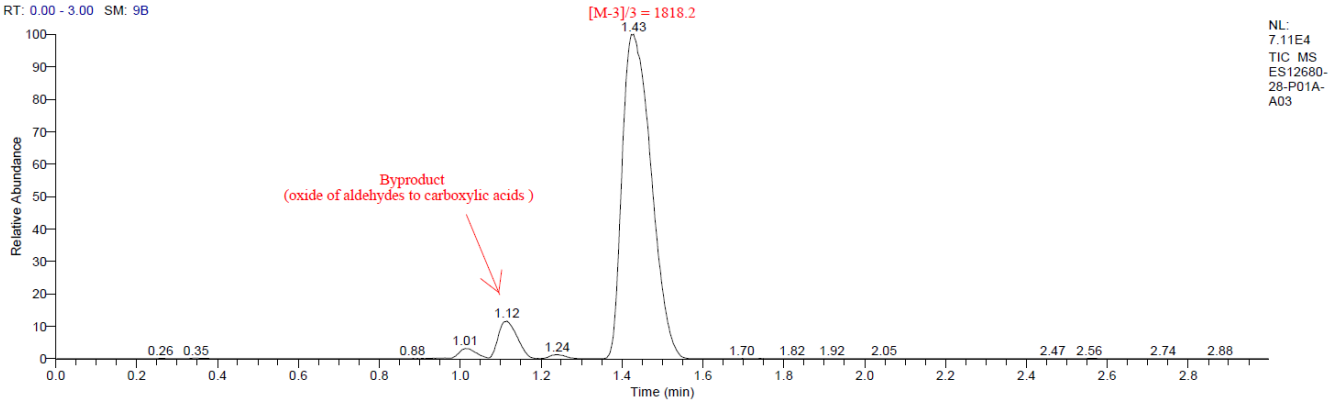


Figure S21. LC trace and mass of **4c**

SI-6-19 LC Trace and Mass of 4d

Yield: 90.67%
Exact mass: 5456.75
Triply charged mass [M-3]/3, calculated: 1817.9; observed: 1818.2.





RT	Peak Height	Peak Width	Peak Area	Area %
1.01	2544.67	0.10	6866.92	1.76
1.11	8726.11	0.12	26922.17	6.89
1.23	1104.40	0.05	1255.36	0.32
1.25	933.00	0.07	1381.28	0.35
1.42	71949.44	0.24	354047.05	90.67

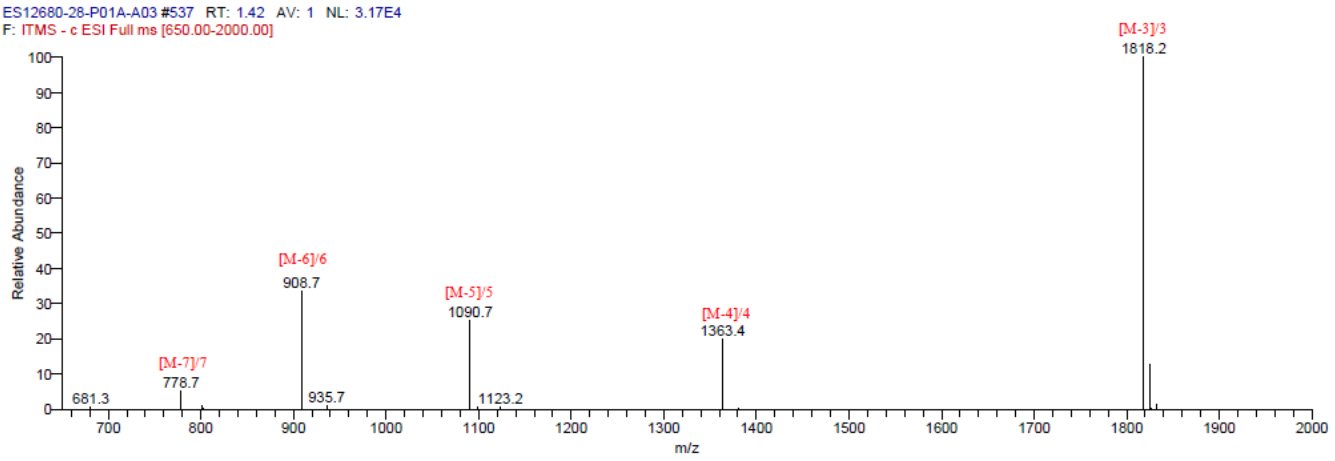
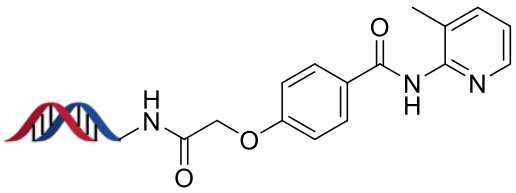
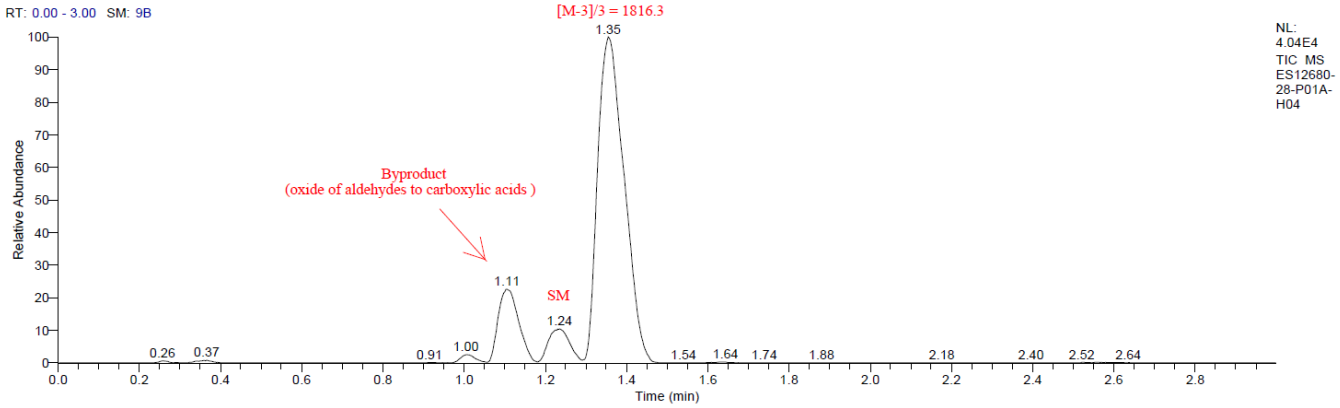


Figure S22. LC trace and mass of the mixture of **4d**

SI-6-20 LC Trace and Mass of 4e

Following **General Procedure 3**
Yield: 80.83%
Exact mass: 5452.78
Triply charged mass [M-3]/3, calculated: 1816.6; observed: 1816.3.





RT	Peak Height	Peak Width	Peak Area	Area %
1.01	1134.92	0.10	2610.65	1.16
1.11	9313.19	0.12	31896.02	14.16
1.23	3017.73	0.07	8661.97	3.85
1.36	41077.77	0.20	182032.78	80.83

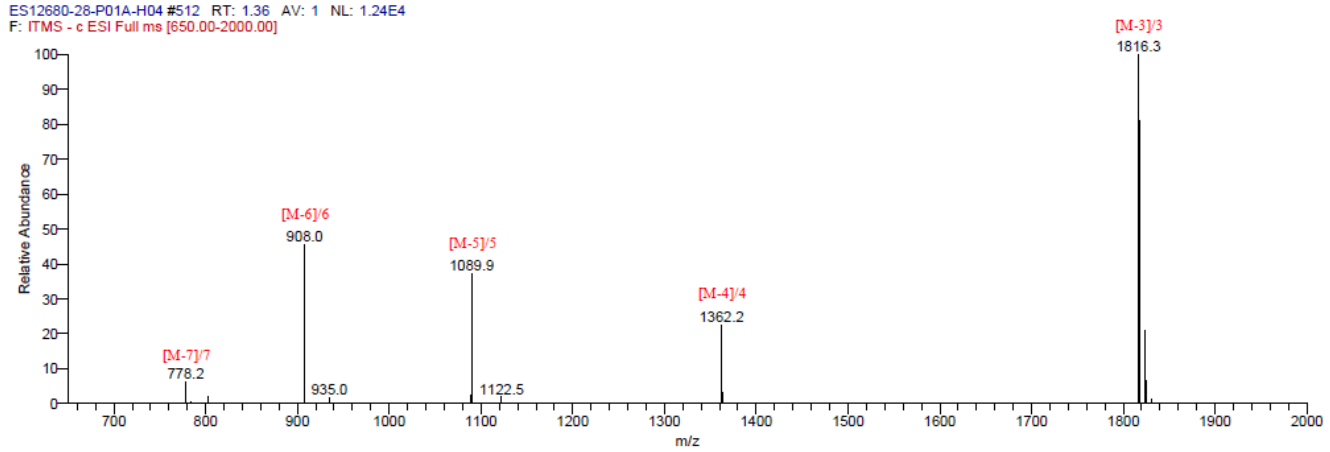
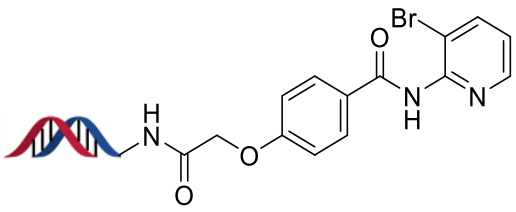
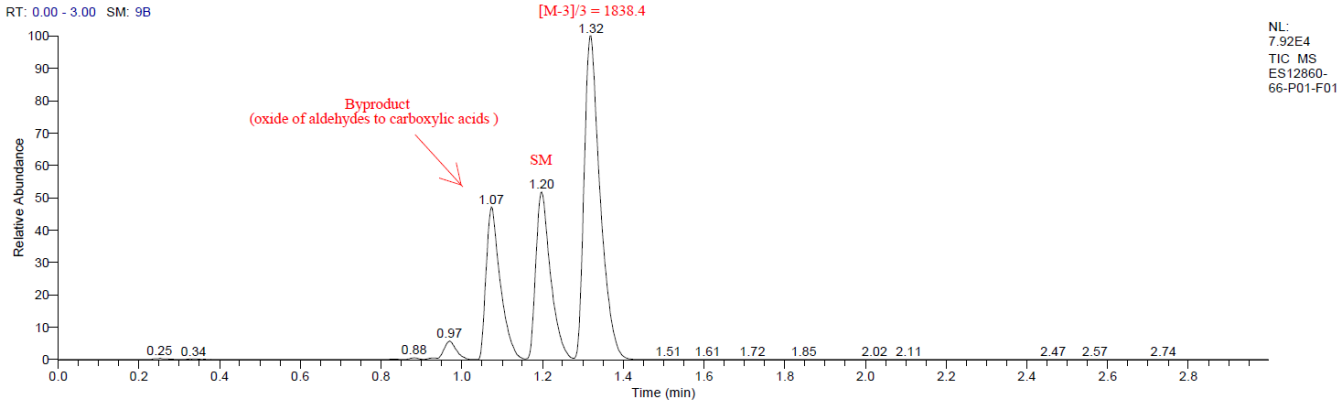


Figure S23. LC trace and mass of **4e**

SI-6-21 LC Trace and Mass of 4f

Following **General Procedure 3**
Yield: 51.46%
Exact mass: 5517.65
Triply charged mass [M-3]/3, calculated: 1838.2; observed: 1838.4.





RT	Peak Height	Peak Width	Peak Area	Area %
0.88	577.48	0.05	671.58	0.16
0.97	5145.44	0.08	9563.64	2.26
1.07	42676.07	0.11	91521.25	21.67
1.20	45873.01	0.12	103248.85	24.45
1.32	86032.43	0.16	217366.65	51.46

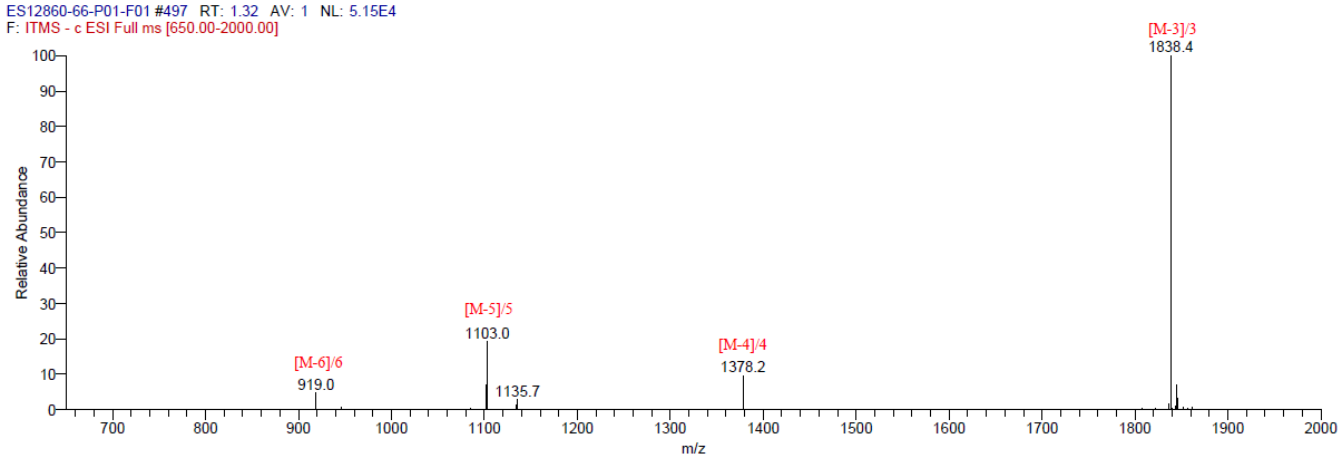
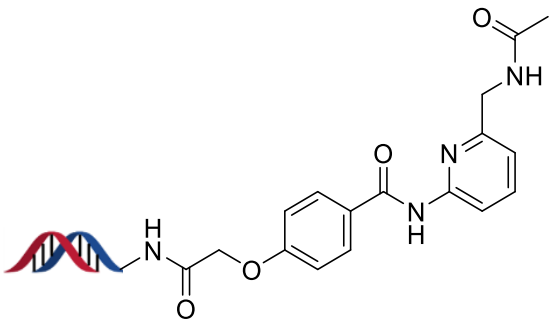
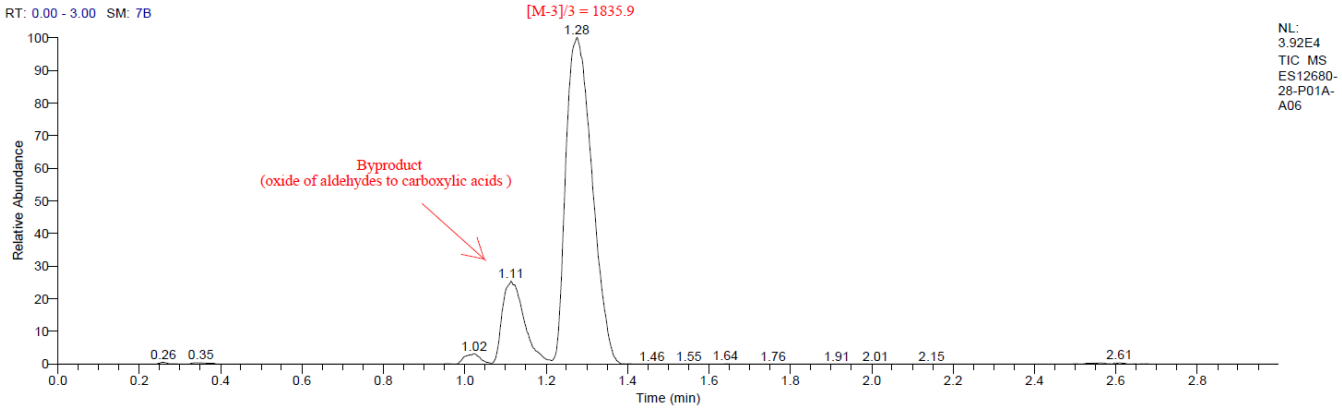


Figure S24. LC trace and mass of **4f**

SI-6-22 LC Trace and Mass of 4g

Following **General Procedure 3**
Yield: 88.49%
Exact mass: 5509.83
Triply charged mass [M-3]/3, calculated: 1835.6; observed: 1835.9.





RT	Peak Height	Peak Width	Peak Area	Area %
1.00	715.35	0.03	681.15	0.35
1.02	679.35	0.03	795.76	0.41
1.12	8233.04	0.06	20430.53	10.60
1.27	38737.88	0.16	170590.80	88.49
2.53	82.06	0.06	281.38	0.15

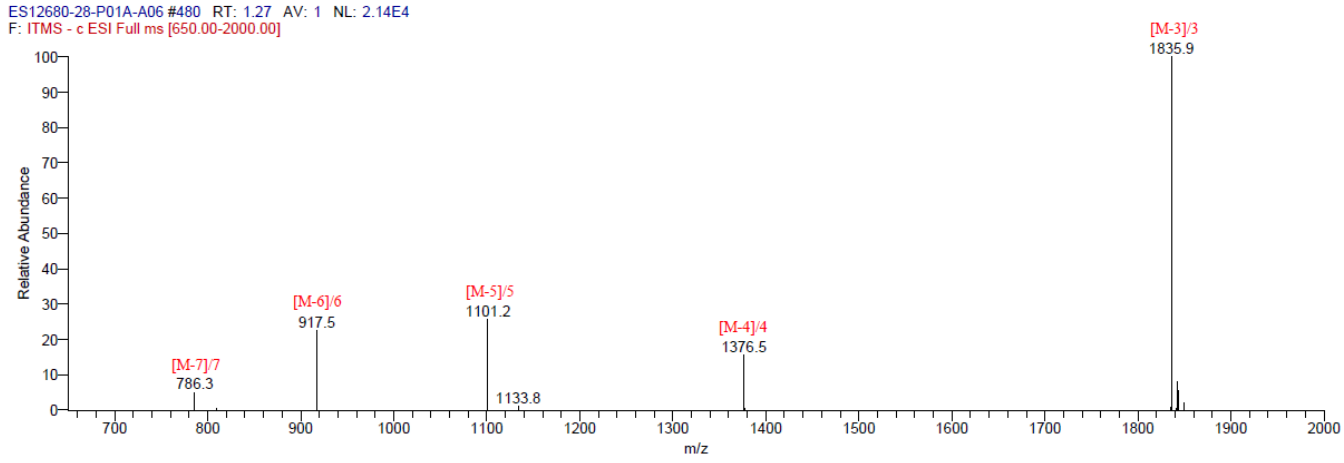
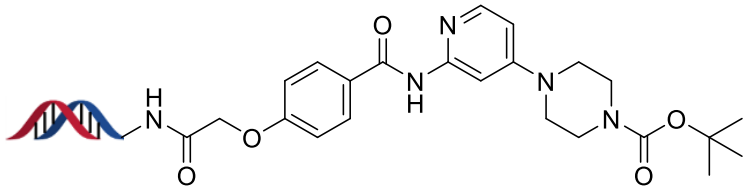
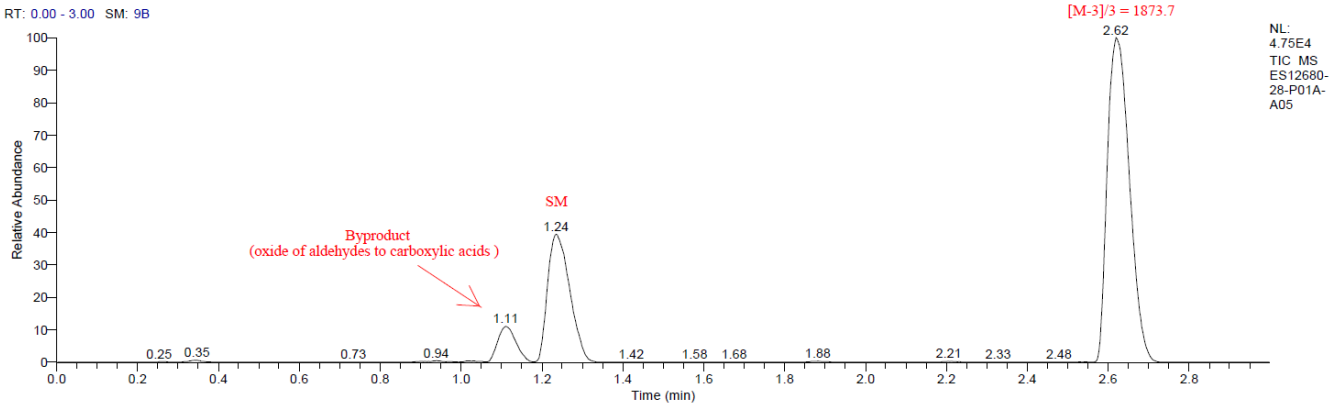


Figure S25. LC trace and mass of **4g**

SI-6-23 LC Trace and Mass of 4h

Following **General Procedure 3**
Yield: 77.95%
Exact mass: 5622.98
Triply charged mass [M-3]/3, calculated: 1873.3; observed: 1873.7.





RT	Peak Height	Peak Width	Peak Area	Area %
0.94	312.87	0.04	415.69	0.18
1.11	5496.11	0.11	16328.33	7.06
1.23	15639.75	0.06	33940.25	14.68
2.20	230.97	0.04	299.76	0.13
2.62	48222.98	0.17	180282.66	77.95

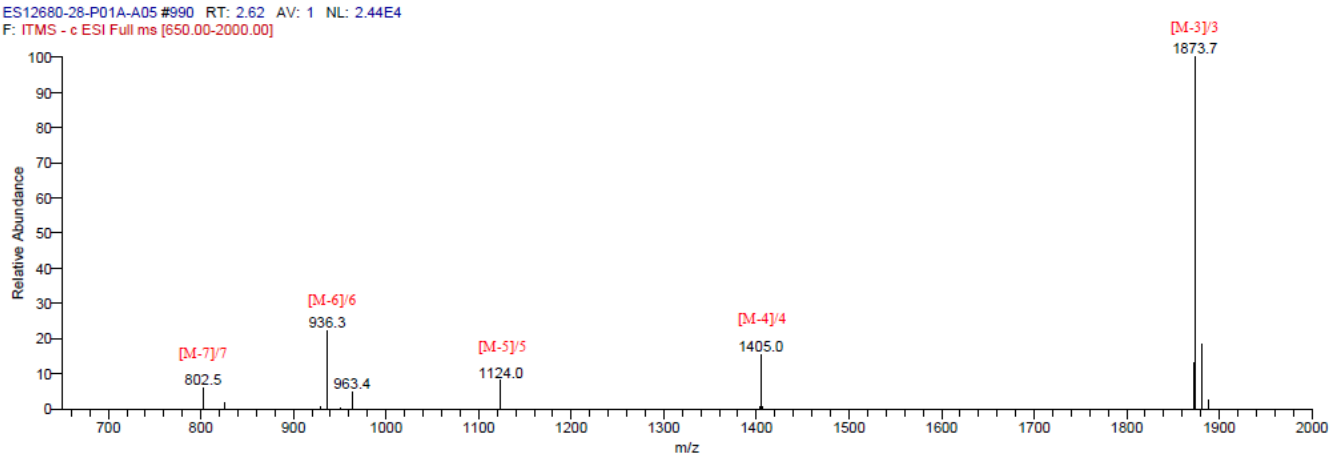
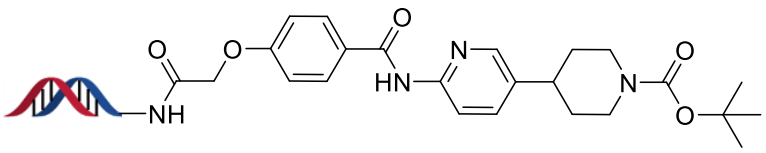
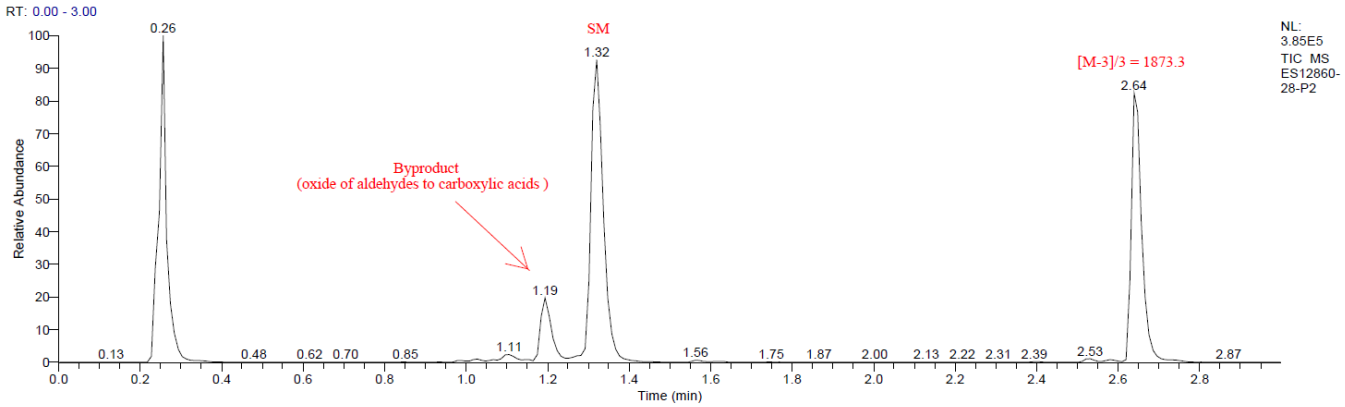


Figure S26. LC trace and mass of **4h**

SI-6-24 LC Trace and Mass of 4i

Following **General Procedure 3**
Yield: 39.03%
Exact mass: 5622.01
Triply charged mass [M-3]/3, calculated: 1873; observed: 1873.3.





RT	Peak Height	Peak Width	Peak Area	Area %
1.19	60908.26	0.10	135611.52	9.35
1.32	312247.00	0.20	748383.21	51.61
2.65	258629.40	0.20	565981.15	39.03

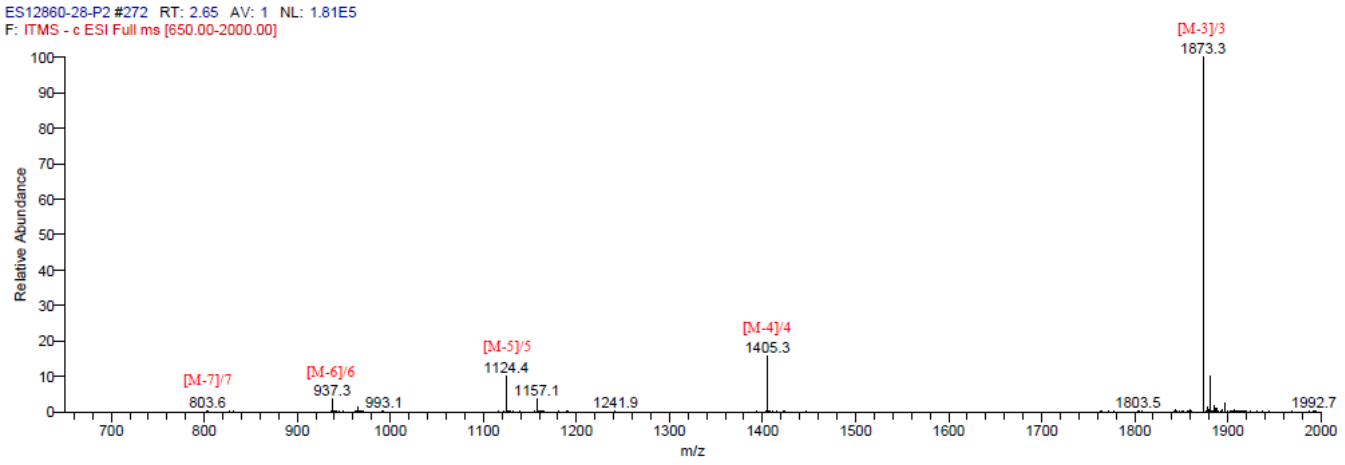
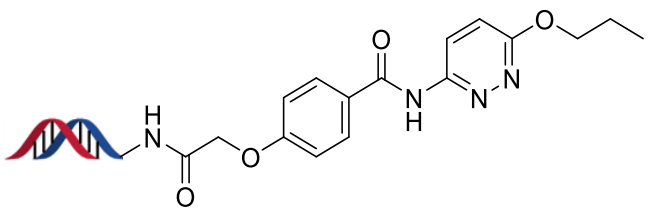
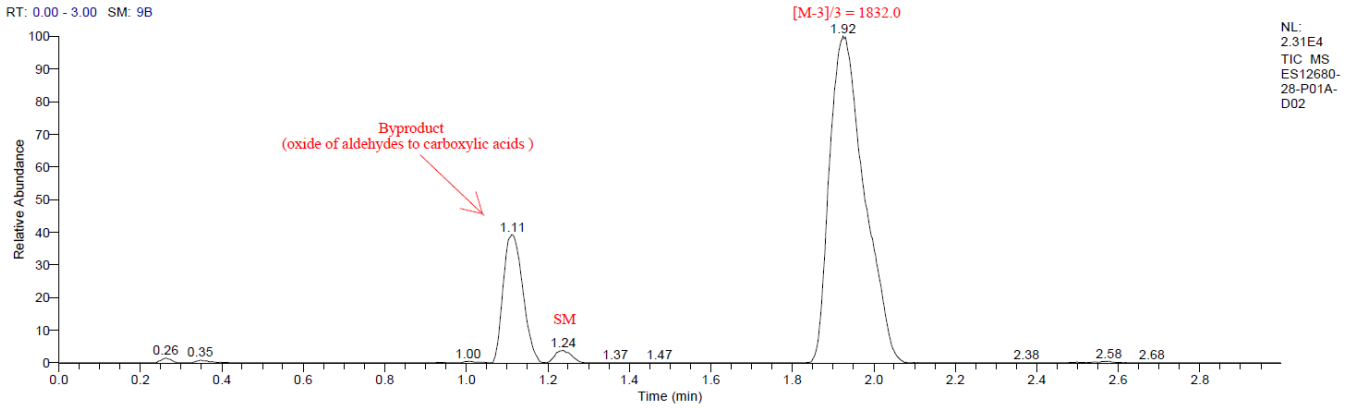


Figure S27. LC trace and mass of **4i**

SI-6-25 LC Trace and Mass of 4j

Following **General Procedure 3**
Yield: 80.82%
Exact mass: 5497.82
Triply charged mass [M-3]/3, calculated: 1831.6; observed: 1832.0.





RT	Peak Height	Peak Width	Peak Area	Area %
1.00	150.48	0.05	205.11	0.12
1.11	9482.25	0.13	30517.81	18.02
1.23	898.74	0.06	1596.42	0.94
1.92	23985.69	0.23	136886.17	80.82
2.57	183.63	0.03	162.79	0.10

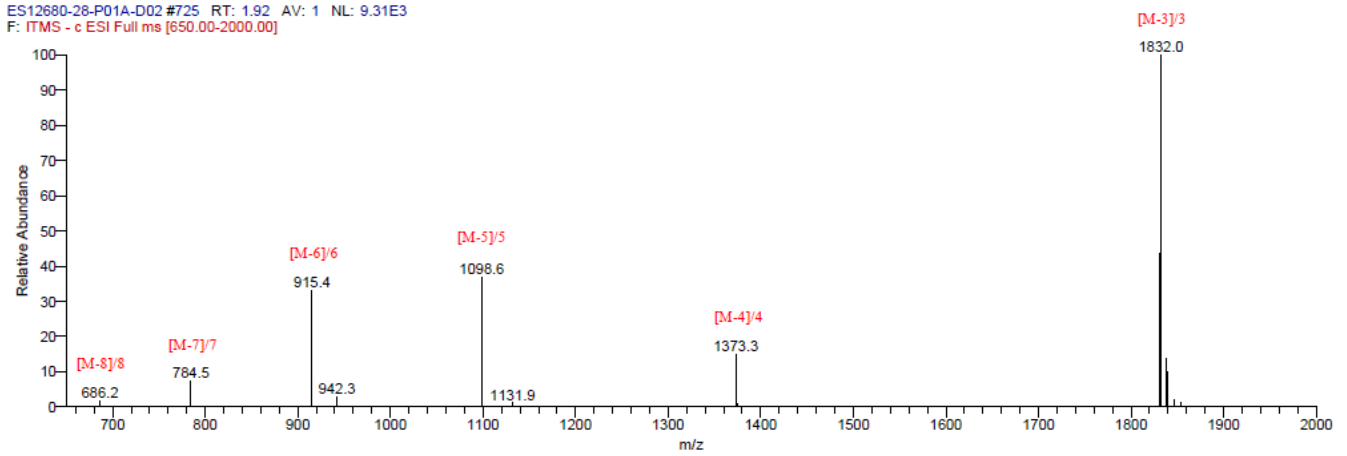
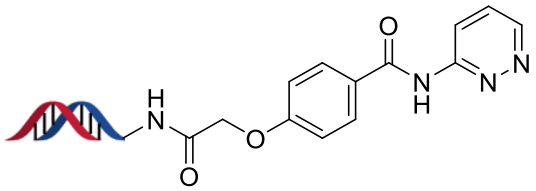
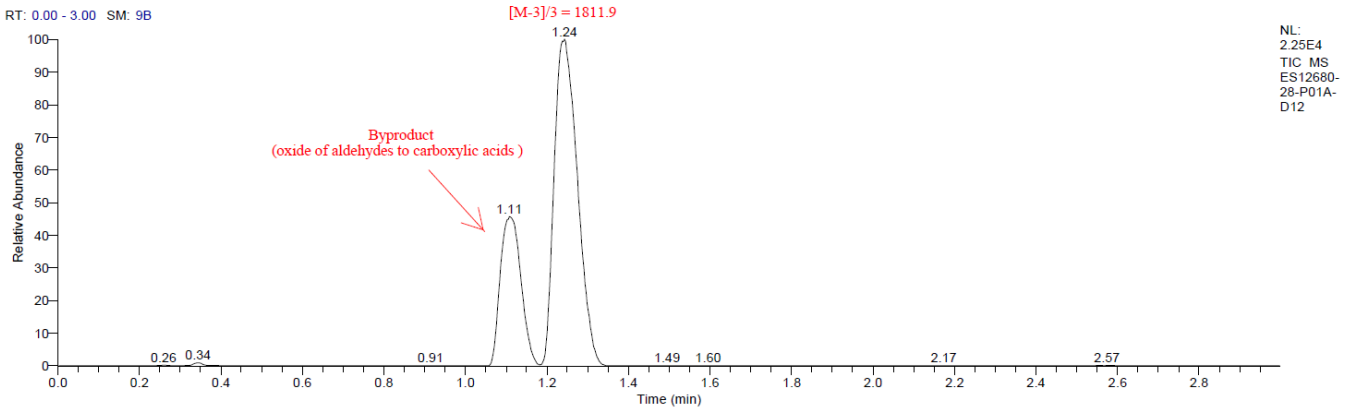


Figure S28. LC trace and mass of **4j**

SI-6-26 LC Trace and Mass of 4k

Following **General Procedure 3**
Yield: 71.01%
Exact mass: 5439.74
Triply charged mass [M-3]/3, calculated: 1812.3; observed: 1811.9.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	10464.48	0.12	36460.83	28.99
1.24	23698.82	0.18	89322.94	71.01

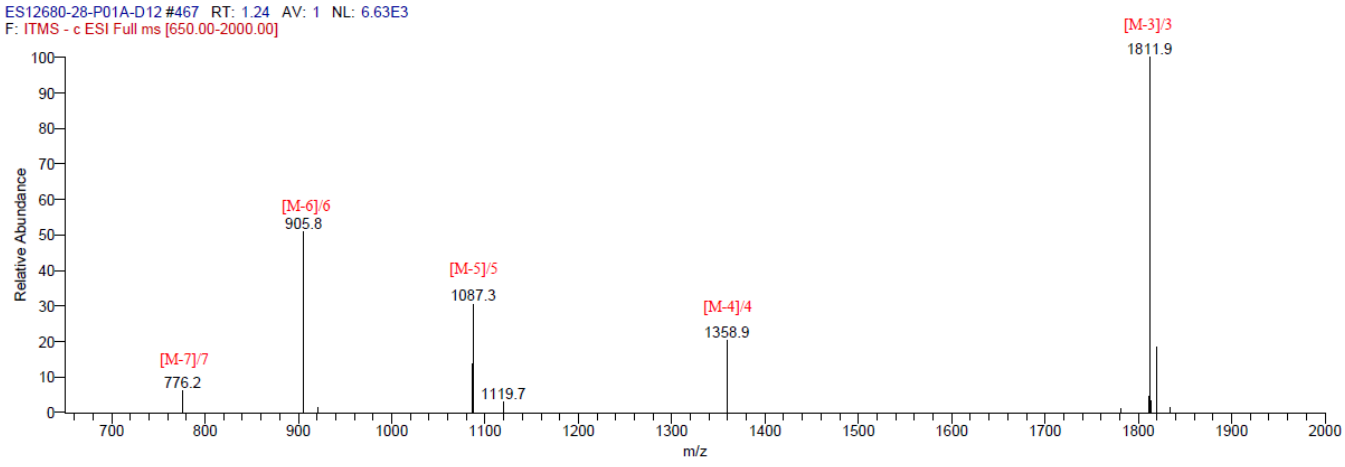
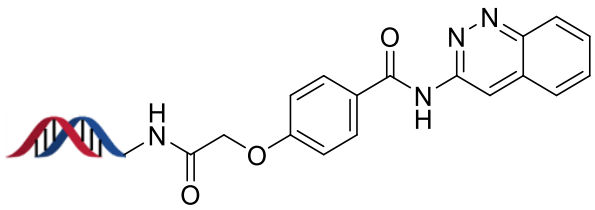
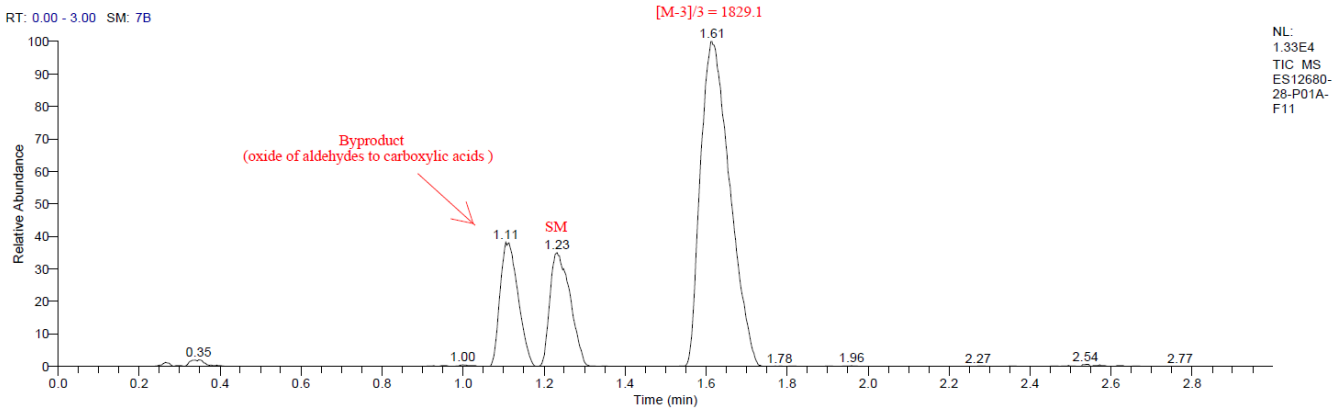


Figure S29. LC trace and mass of **4k**

SI-6-27 LC Trace and Mass of 4l

Following **General Procedure 3**
Yield: 67.64%
Exact mass: 5489.8
Triply charged mass [M-3]/3, calculated: 1828.9; observed: 1829.1.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	5362.52	0.14	15816.86	15.95
1.23	4845.87	0.14	16266.60	16.41
1.62	13410.25	0.27	67061.29	67.64

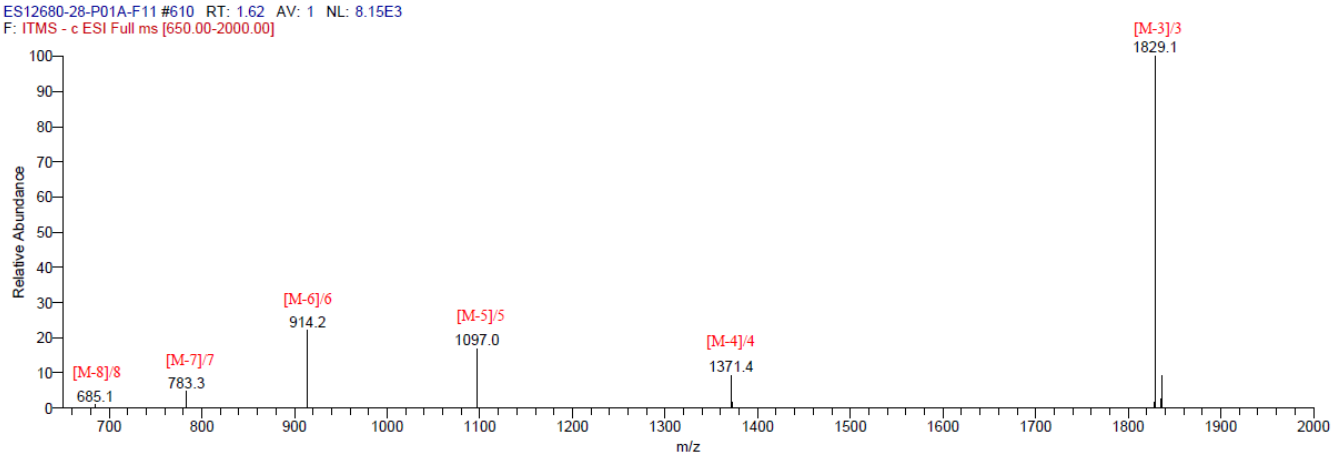
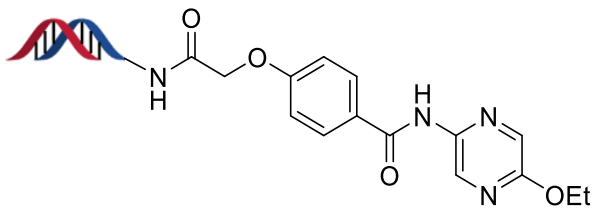
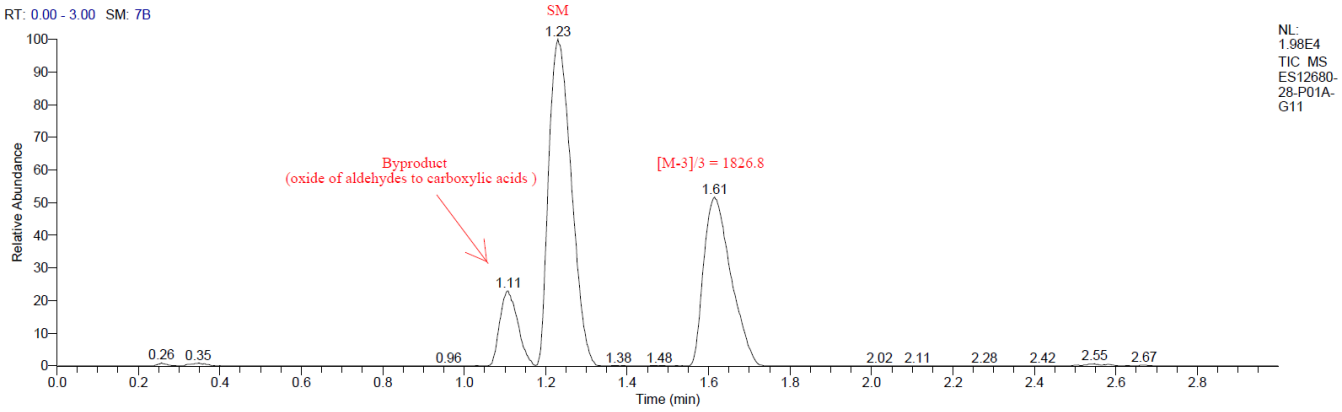


Figure S30. LC trace and mass of **4l**

SI-6-28 LC Trace and Mass of 4m

Following **General Procedure 3**
Yield: 34.39%
Exact mass: 5483.8
Triply charged mass [M-3]/3, calculated: 1826.9; observed: 1826.8.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	4667.48	0.12	13929.77	10.09
1.23	19788.08	0.18	76208.24	55.21
1.61	10177.09	0.20	47468.06	34.39
2.54	137.17	0.05	225.46	0.16
2.58	94.83	0.09	202.85	0.15

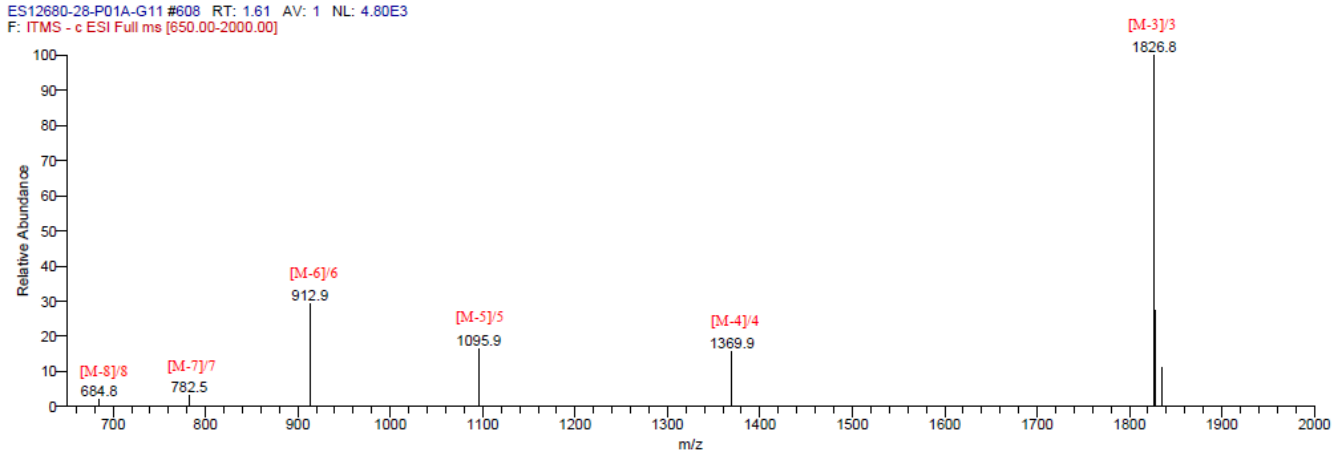
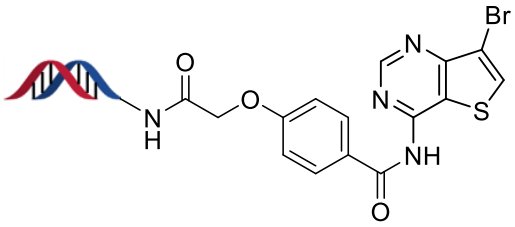
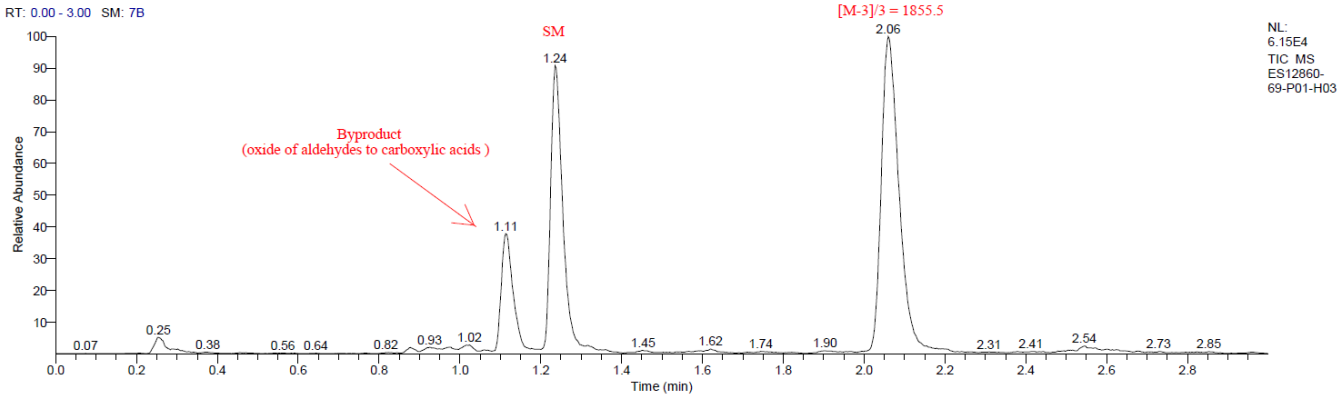


Figure S31. LC trace and mass of **4m**

SI-6-29 LC Trace and Mass of 4n

Following **General Procedure 3**
Yield: 51.95%
Exact mass: 5570.72
Triply charged mass [M-3]/3, calculated: 1855.9; observed: 1855.5.





RT	Peak Height	Peak Width	Peak Area	Area %
1.02	1637.85	0.05	3386.89	0.93
1.11	25100.11	0.11	48539.40	13.32
1.24	59123.12	0.16	118883.54	32.62
2.06	62736.48	0.29	189296.03	51.95
2.54	1439.44	0.12	4292.92	1.18

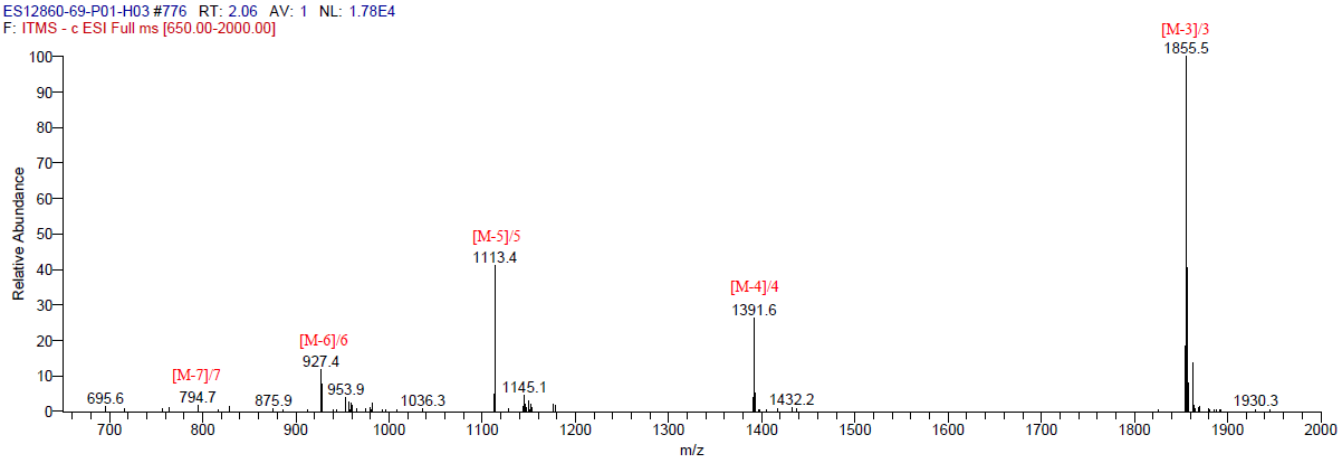
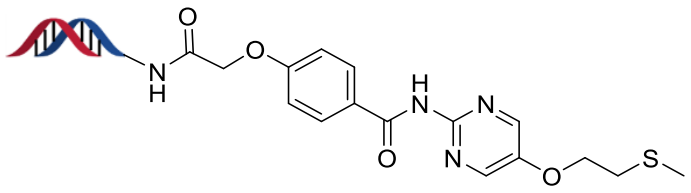
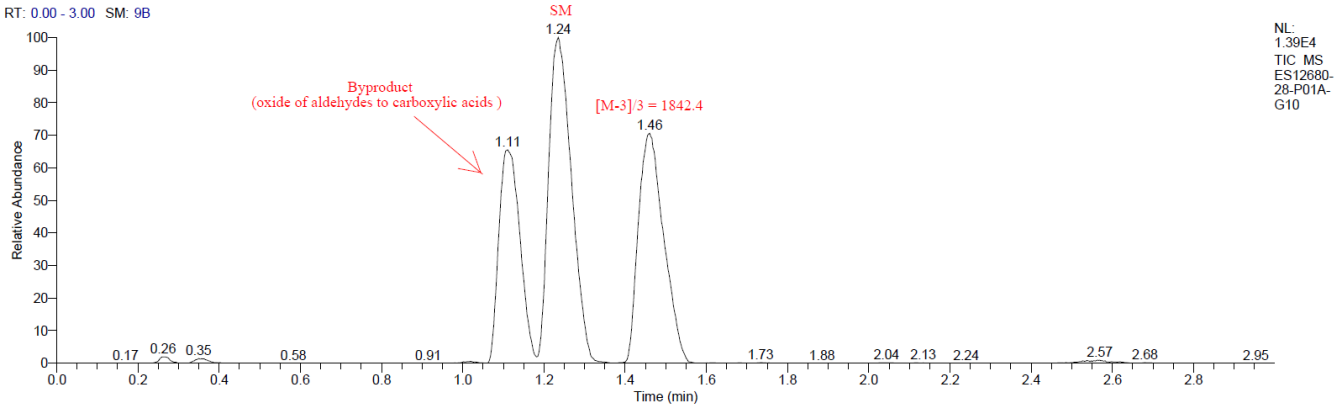


Figure S32. LC trace and mass of **4n**

SI-6-30 LC Trace and Mass of 4o

Following **General Procedure 3**
Yield: 35.27%
Exact mass: 5529.89
Triply charged mass [M-3]/3, calculated: 1842.3; observed: 1842.4.





RT	Peak Height	Peak Width	Peak Area	Area %
1.11	8659.37	0.07	24438.67	20.32
1.24	14163.64	0.17	53403.87	44.41
1.46	10078.75	0.17	42408.20	35.27

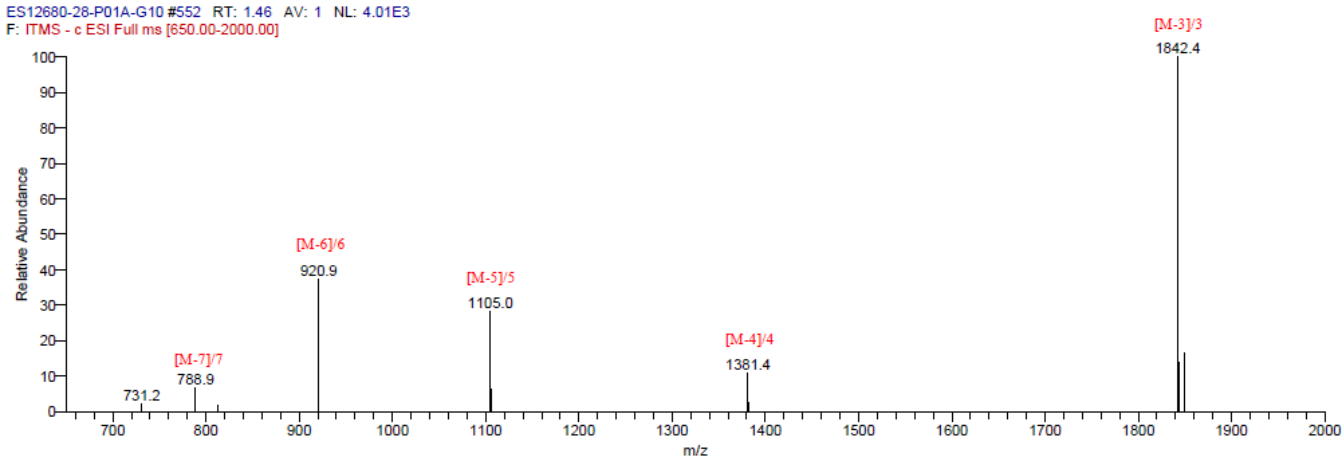
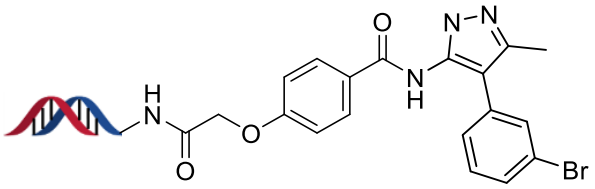
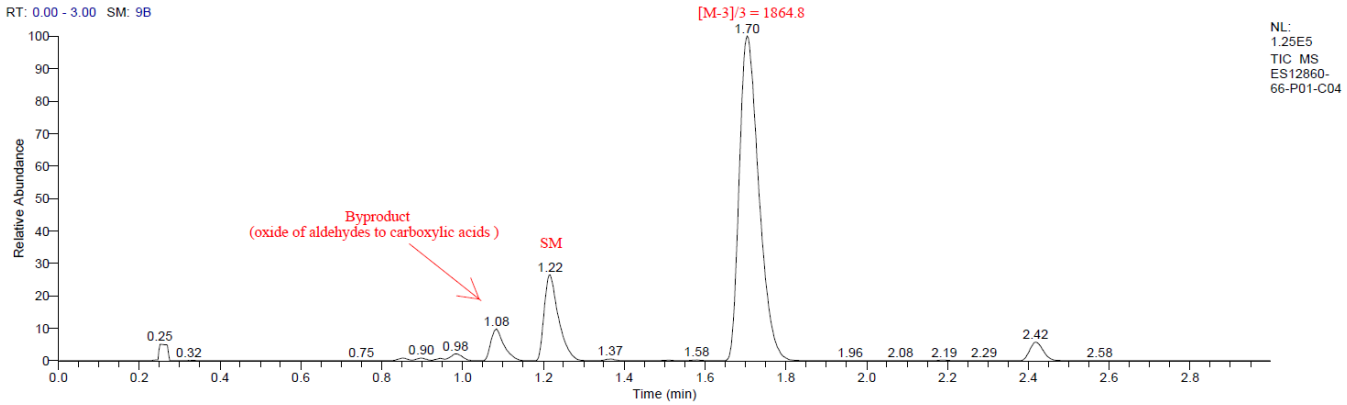


Figure S33. LC trace and mass of **4o**

SI-6-31 LC Trace and Mass of 4p

Following **General Procedure 3**
Yield: 76.37%
Exact mass: 5596.76
Triply charged mass [M-3]/3, calculated: 1864.6; observed: 1864.8.





RT	Peak Height	Peak Width	Peak Area	Area %
0.99	2895.28	0.06	3633.33	0.66
1.08	14257.14	0.11	27808.15	5.04
1.21	37646.65	0.14	82074.51	14.88
1.71	130066.36	0.19	421342.10	76.37
2.42	7885.36	0.10	16852.88	3.05

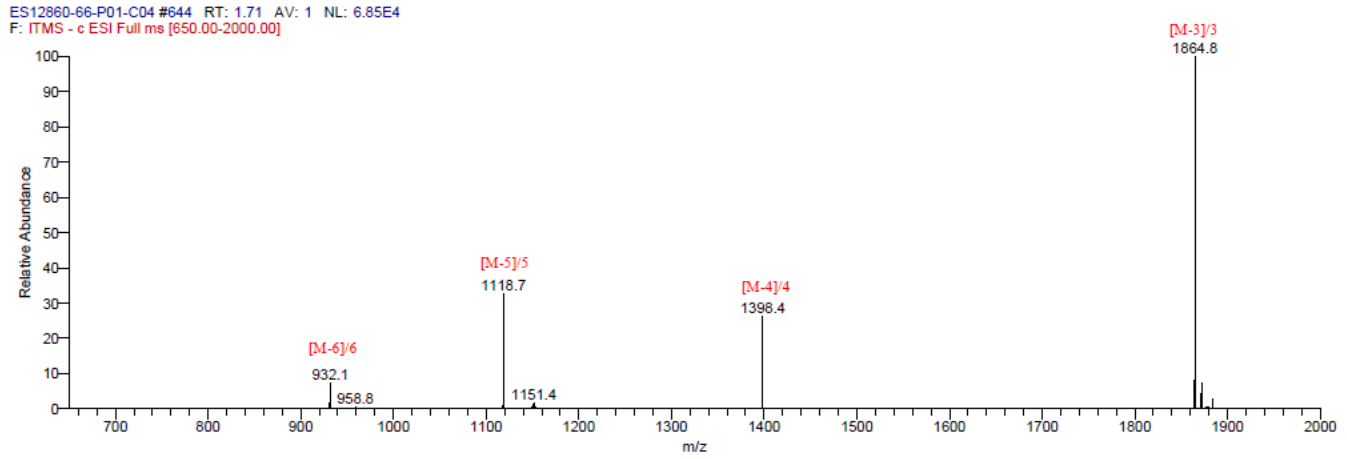
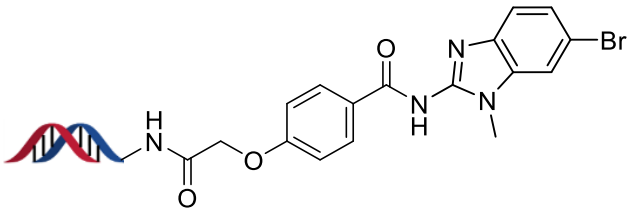
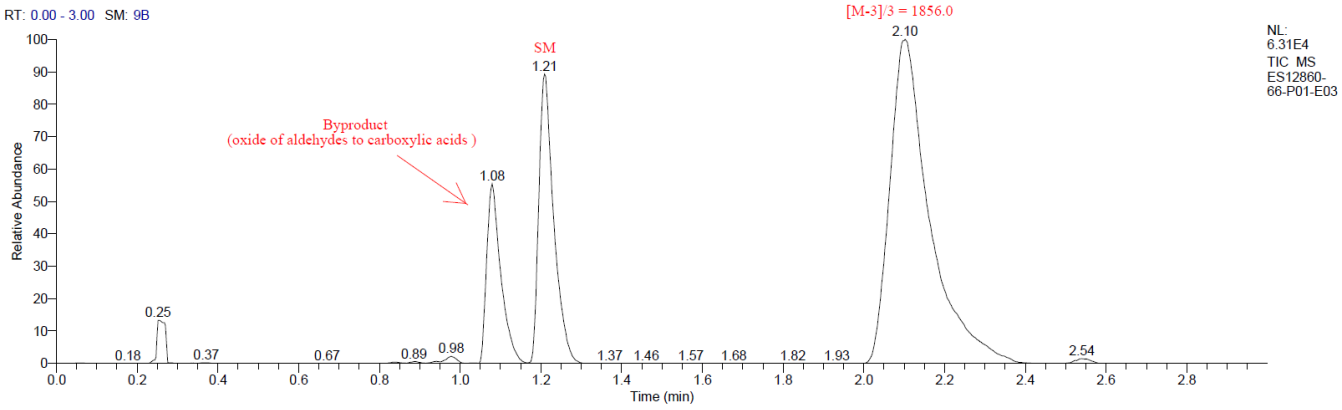


Figure S34. LC trace and mass of **4p**

SI-6-32 LC Trace and Mass of 4q

Following **General Procedure 3**
Yield: 64.7%
Exact mass: 5570.72
Triply charged mass [M-3]/3, calculated: 1855.9; observed: 1856.0.





RT	Peak Height	Peak Width	Peak Area	Area %
0.98	1478.83	0.06	2459.04	0.37
1.08	39674.60	0.12	85288.23	12.87
1.21	63026.84	0.15	144367.62	21.79
2.10	65277.70	0.35	428615.90	64.70
2.54	1076.53	0.07	1722.56	0.26

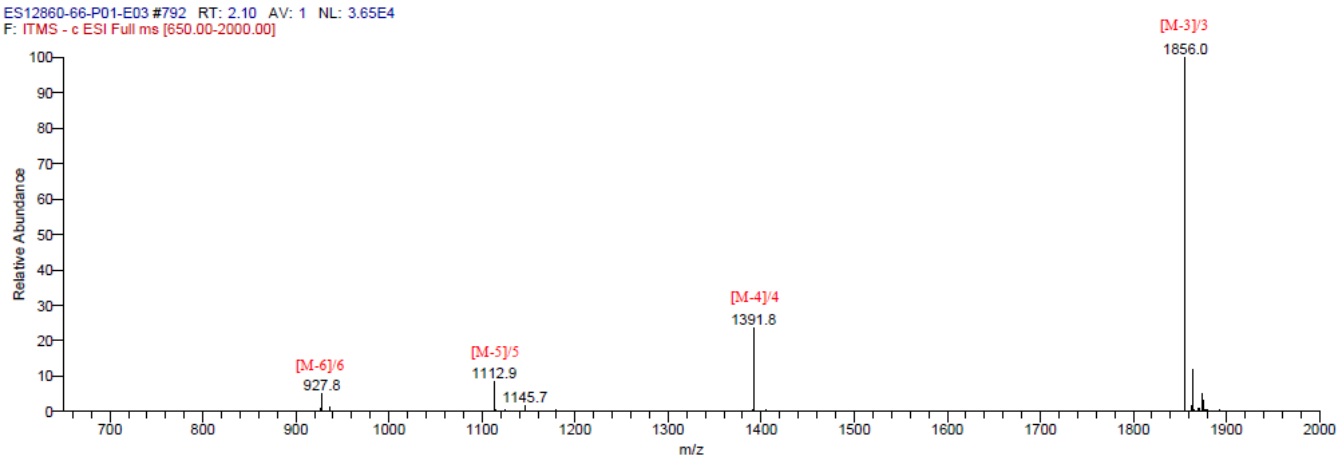
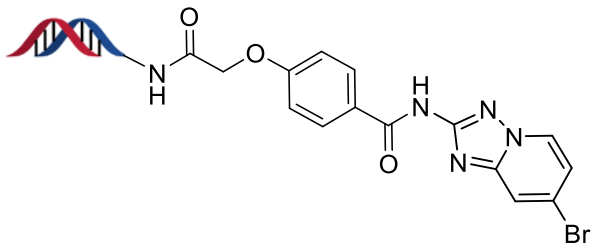
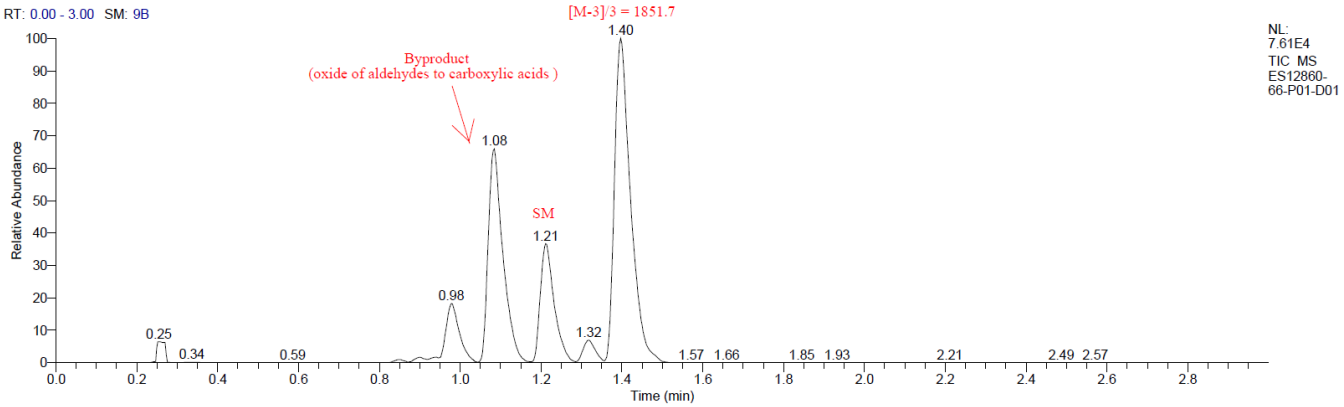


Figure S35. LC trace and mass of **4q**

SI-6-33 LC Trace and Mass of 4r

Following **General Procedure 3**
Yield: 46.72%
Exact mass: 5557.68
Triply charged mass [M-3]/3, calculated: 1851.6; observed: 1851.7.





RT	Peak Height	Peak Width	Peak Area	Area %
0.98	15565.34	0.09	32079.59	7.22
1.08	56989.87	0.13	127378.58	28.68
1.21	31286.65	0.11	66276.85	14.92
1.31	5913.83	0.07	10912.07	2.46
1.39	83570.02	0.11	207492.52	46.72

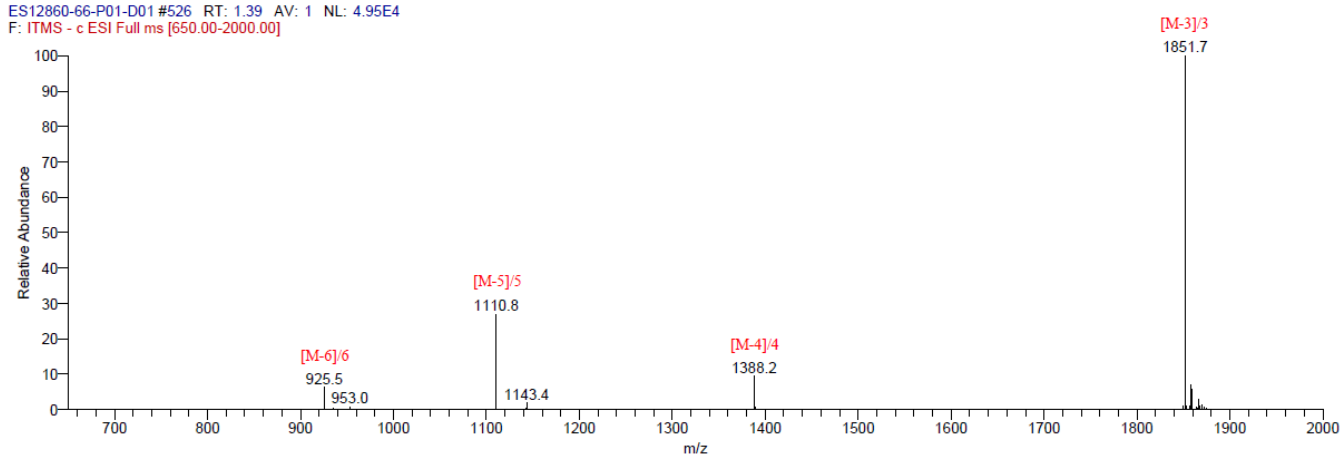
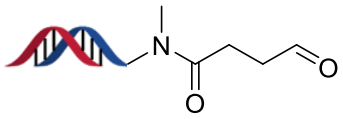
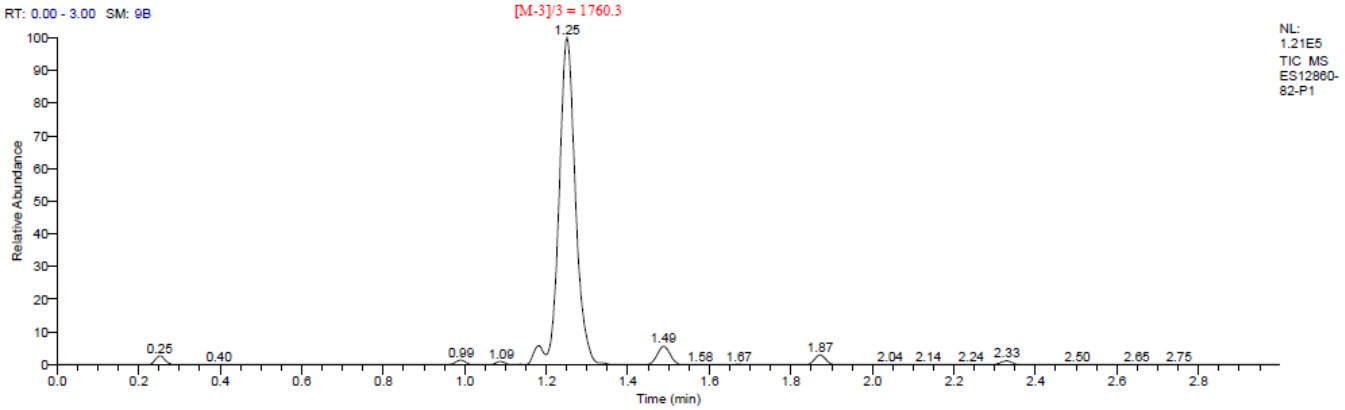


Figure S36. LC trace and mass of **4r**

SI-6-34 LC Trace and Mass of 1q

Following **General Procedure 2**
Yield: 89.23%
Exact mass: 5282.6
Triply charged mass [M-3]/3, calculated: 1759.9; observed: 1760.3.





RT	Peak Height	Peak Width	Peak Area	Area %
0.99	2160.43	0.06	2926.72	0.79
1.18	8457.62	0.05	12363.54	3.34
1.25	130655.03	0.12	330500.90	89.23
1.49	7485.38	0.09	15005.53	4.05
1.87	4083.14	0.07	6772.30	1.83
2.33	1655.51	0.08	2841.94	0.77

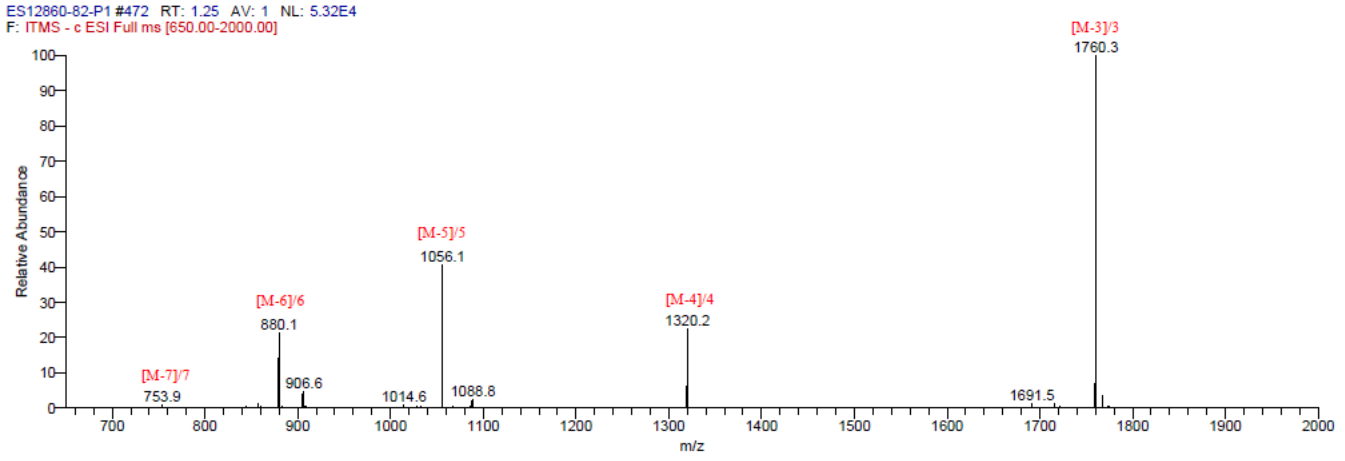
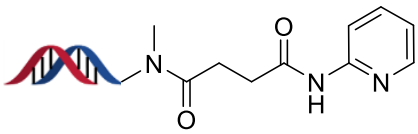
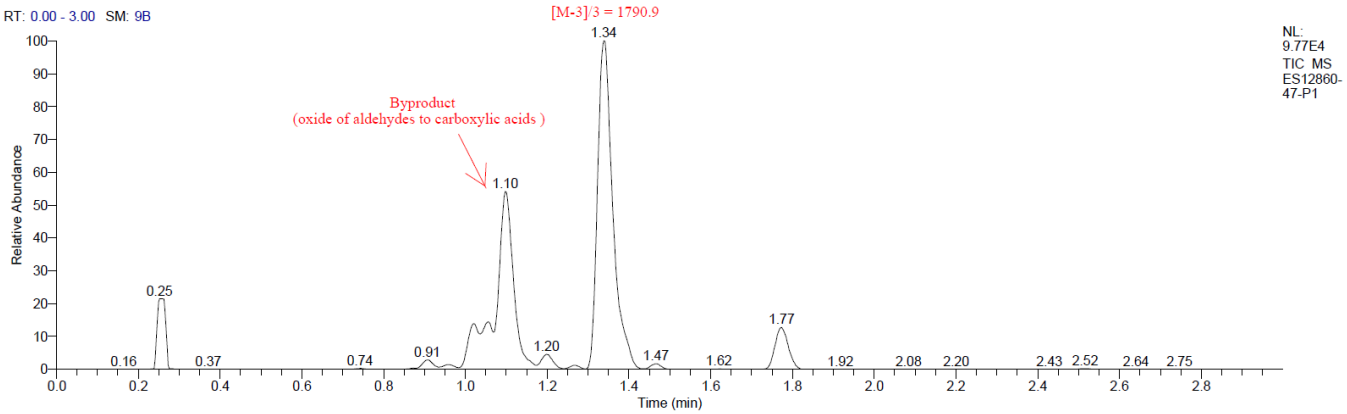


Figure S37. LC trace and mass of **1q**

SI-6-35 LC Trace and Mass of 5a

Following **General Procedure 3**
Yield: 55.91 %
Exact mass: 5374.67
Triply charged mass [M-3]/3, calculated: 1790.6; observed: 1790.9.





RT	Peak Height	Peak Width	Peak Area	Area %
1.02	14919.50	0.05	26832.58	5.89
1.06	16468.79	0.03	25657.35	5.63
1.10	59022.27	0.07	120156.47	26.38
1.34	109101.65	0.14	254680.86	55.91
1.77	13800.03	0.11	28230.09	6.20

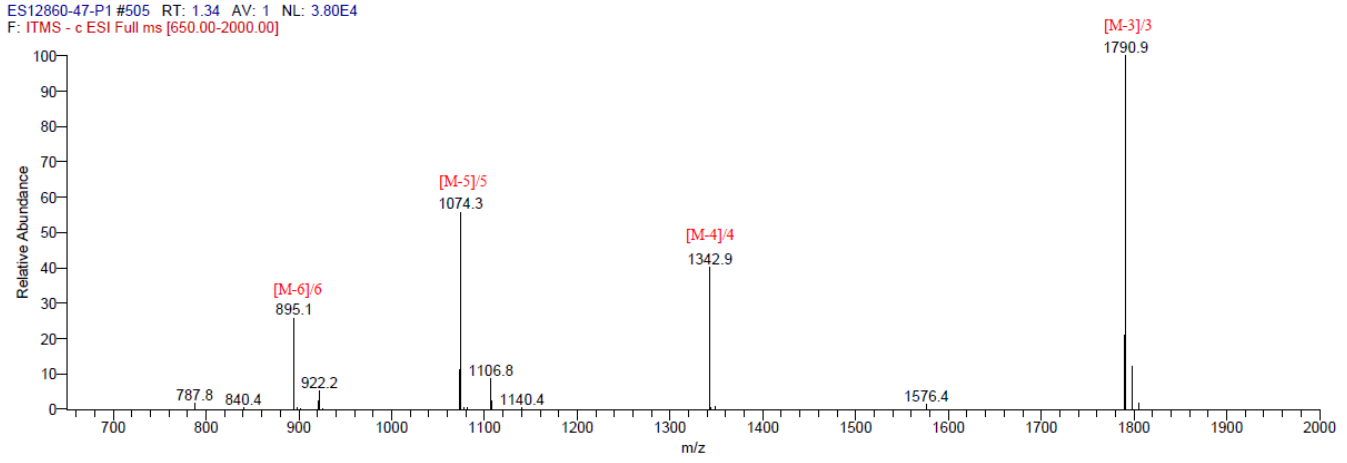
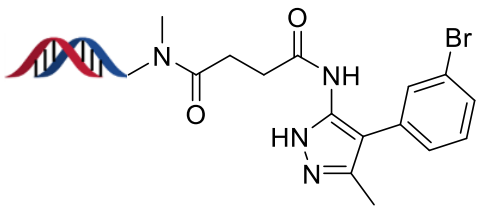
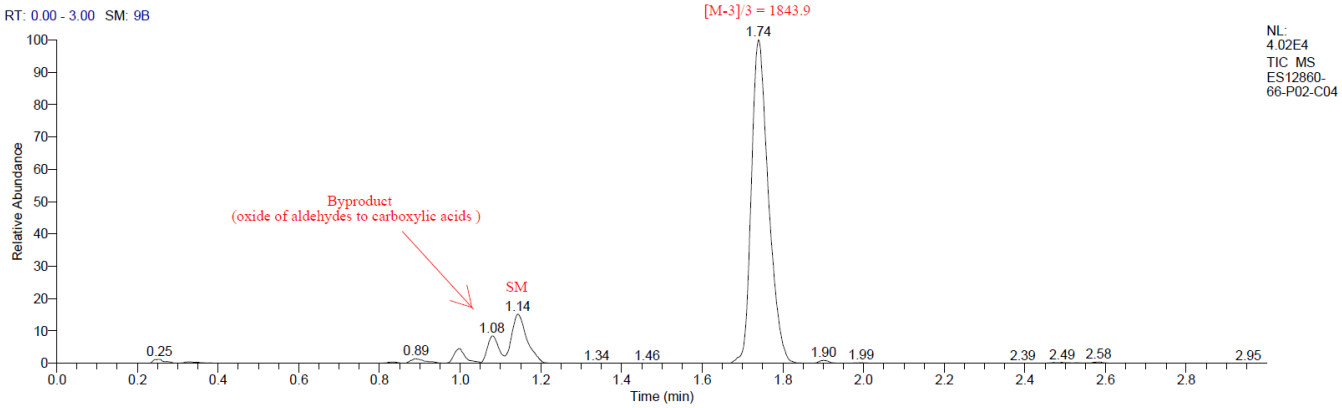


Figure S38. LC trace and mass of **5a**

SI-6-36 LC Trace and Mass of 5p

Following **General Procedure 3**
Yield: 80.97%
Exact mass: 5532.69
Triply charged mass [M-3]/3, calculated: 1843.2; observed: 1843.9.





RT	Peak Height	Peak Width	Peak Area	Area %
0.89	647.69	0.07	1310.58	0.90
1.00	2284.64	0.06	3337.05	2.30
1.08	4008.81	0.06	6828.89	4.71
1.14	6652.53	0.12	16104.94	11.11
1.74	42754.12	0.17	117384.13	80.97

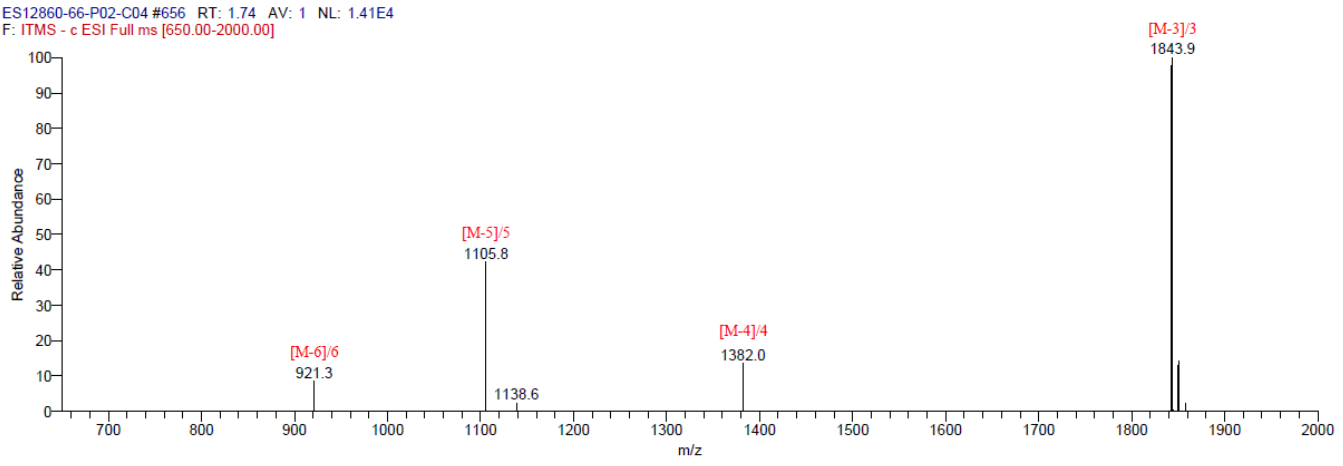
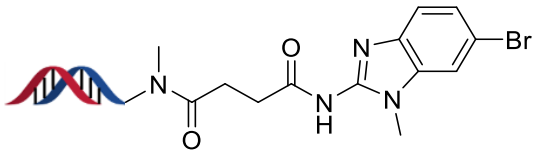
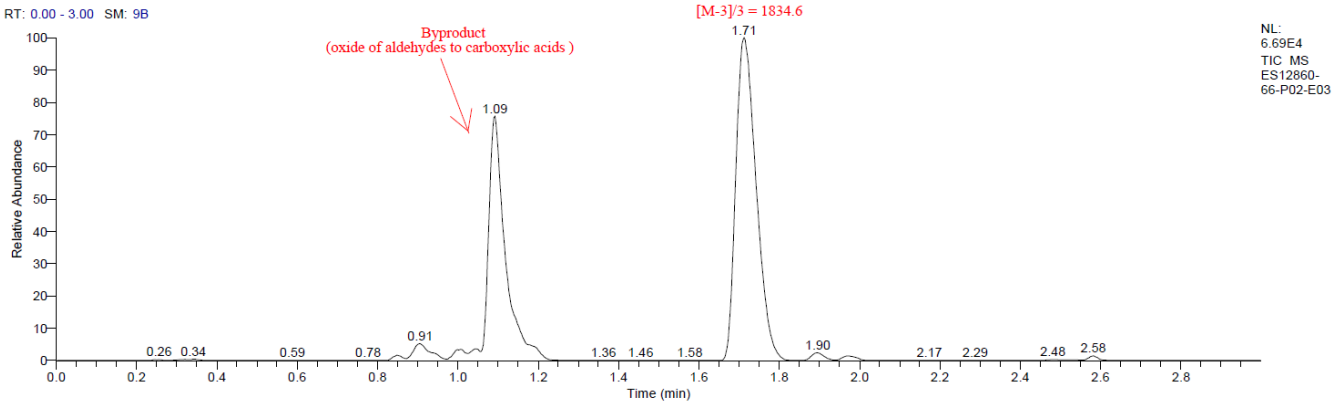


Figure S39. LC trace and mass of **5p**

SI-6-37 LC Trace and Mass of 5q

Following **General Procedure 3**
Yield: 59.97%
Exact mass: 5506.65
Triply charged mass [M-3]/3, calculated: 1834.6; observed: 1834.6.





RT	Peak Height	Peak Width	Peak Area	Area %
0.91	3828.52	0.06	7605.07	1.93
1.00	2788.29	0.05	5067.16	1.29
1.09	57178.77	0.12	139596.27	35.51
1.19	3439.52	0.05	5118.51	1.30
1.72	69507.01	0.17	235763.76	59.97

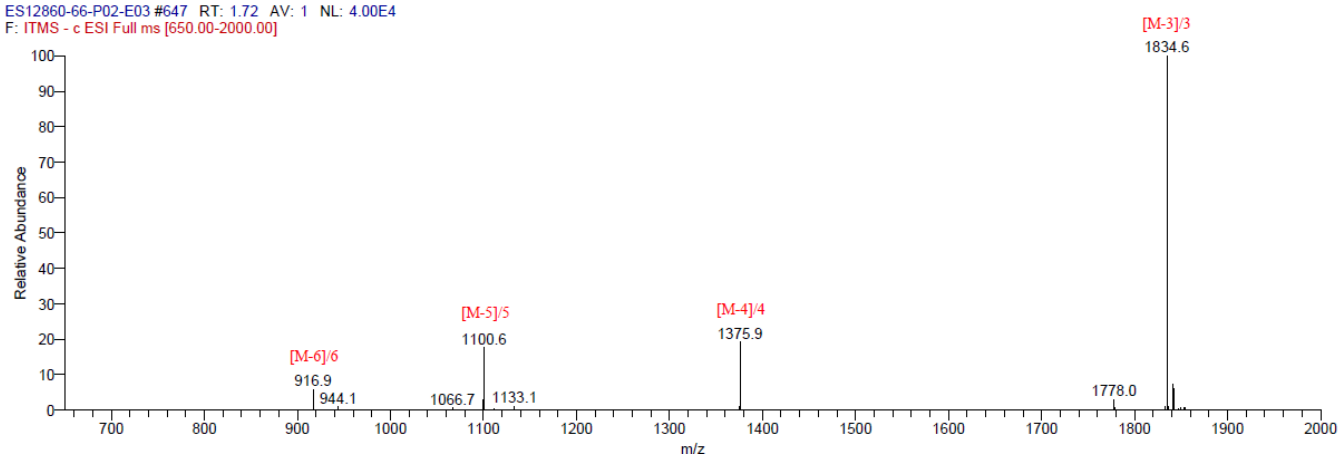
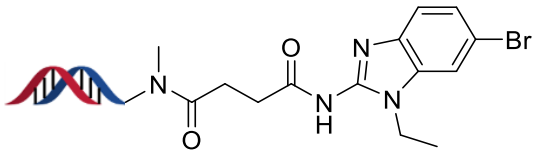
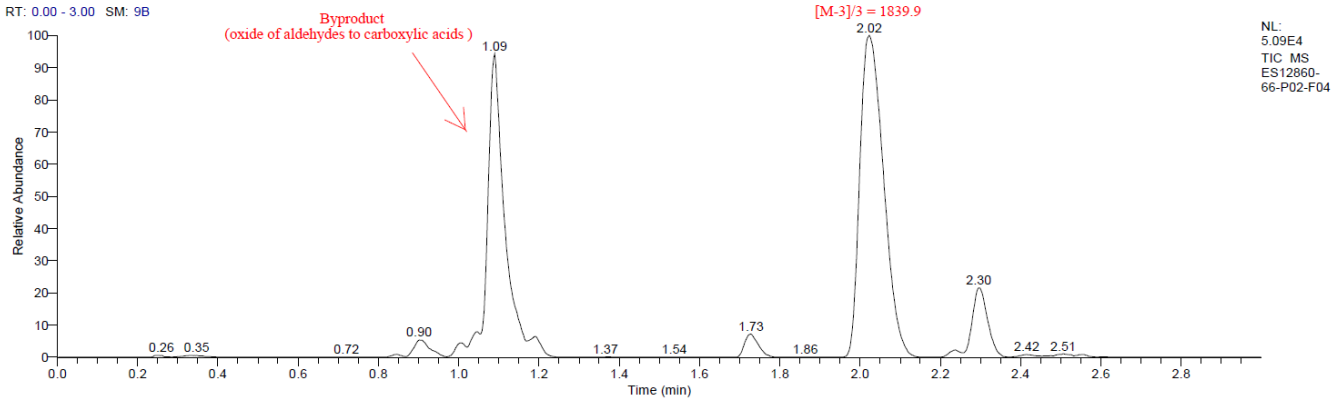


Figure S40. LC trace and mass of **5q**

SI-6-38 LC Trace and Mass of 5s

Following **General Procedure 3**
Yield: 54.07%
Exact mass: 5520.67
Triply charged mass [M-3]/3, calculated: 1839.2; observed: 1839.9.





RT	Peak Height	Peak Width	Peak Area	Area %
0.91	3125.64	0.11	7762.67	2.01
1.08	52788.20	0.12	131822.66	34.14
1.72	4167.45	0.10	8821.31	2.28
2.02	52101.25	0.20	208760.01	54.07
2.30	12053.66	0.12	28960.72	7.50

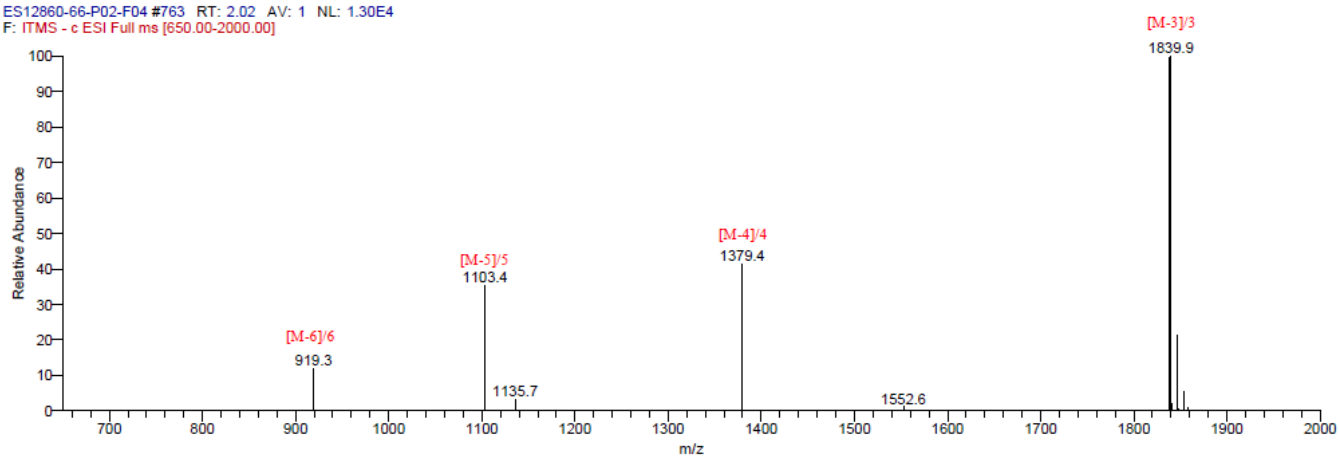
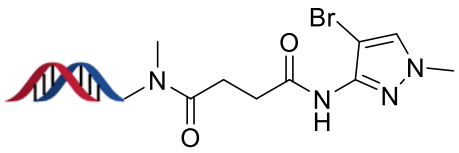
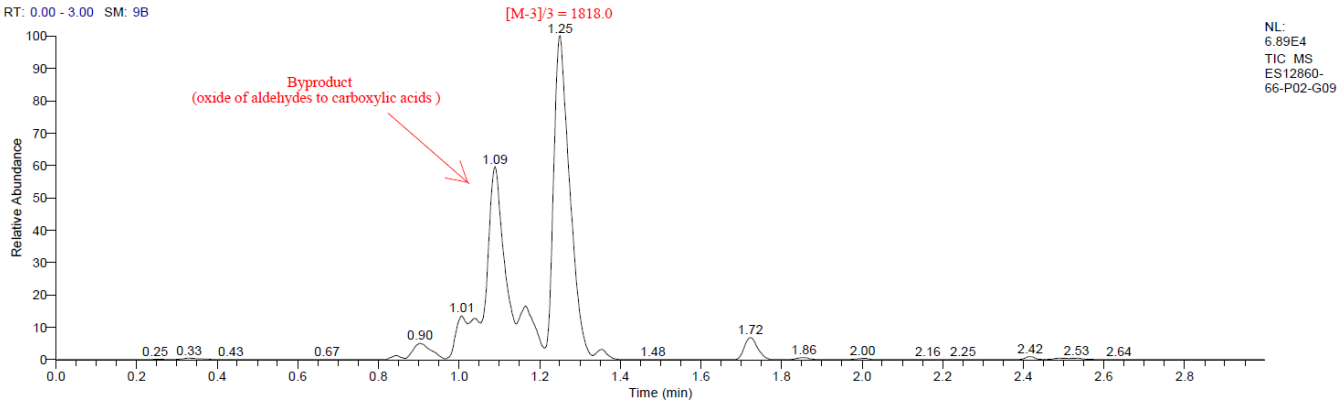


Figure S41. LC trace and mass of **5s**

SI-6-39 LC Trace and Mass of 5t

Following **General Procedure 3**
Yield: 53.04%
Exact mass: 5456.59
Triply charged mass [M-3]/3, calculated: 1817.9; observed: 1818.0.





RT	Peak Height	Peak Width	Peak Area	Area %
1.00	10860.67	0.05	16515.45	4.56
1.04	9166.91	0.04	18100.74	5.00
1.09	46744.61	0.09	115749.15	31.98
1.16	13273.99	0.03	19606.03	5.42
1.25	75004.20	0.12	192007.49	53.04

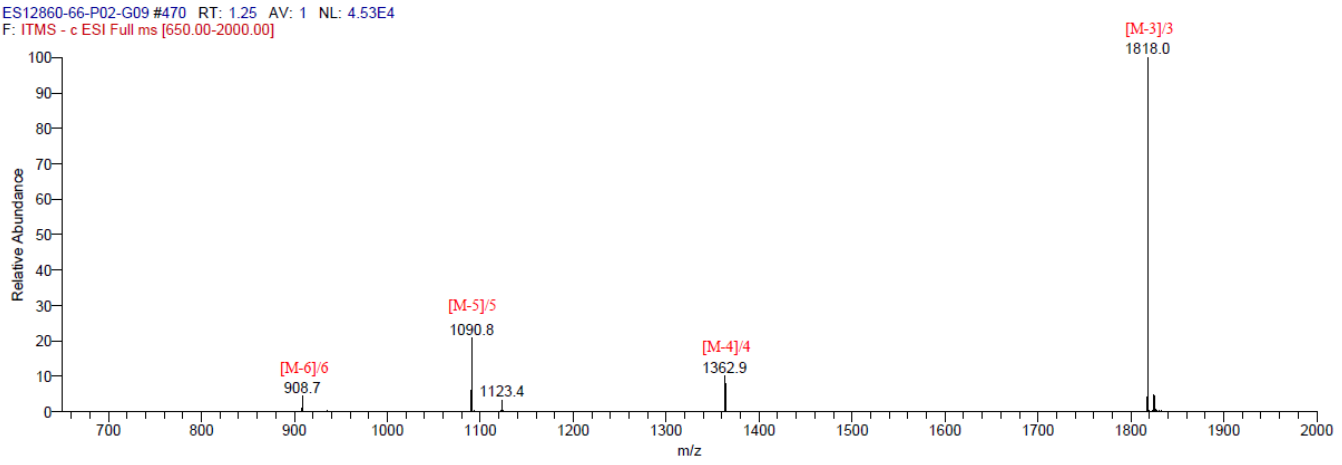
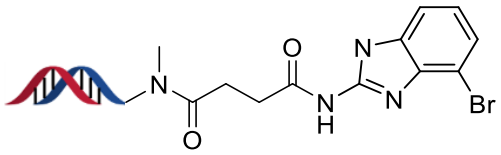
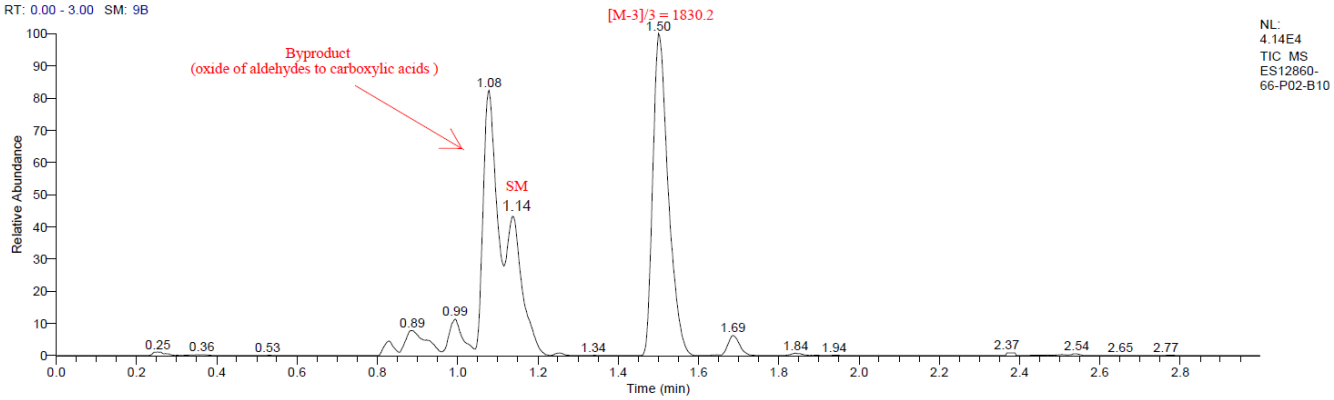


Figure S42. LC trace and mass of **5t**

SI-6-40 LC Trace and Mass of 5u

Following **General Procedure 3**
Yield: 42.77%
Exact mass: 5492.62
Triply charged mass [M-3]/3, calculated: 1829.87; observed: 1830.2.





RT	Peak Height	Peak Width	Peak Area	Area %
0.88	3329.07	0.06	7999.49	3.08
0.99	5344.80	0.06	9427.05	3.63
1.08	37834.73	0.07	81253.26	31.31
1.14	20044.93	0.12	49858.94	19.21
1.50	44815.01	0.16	110988.40	42.77

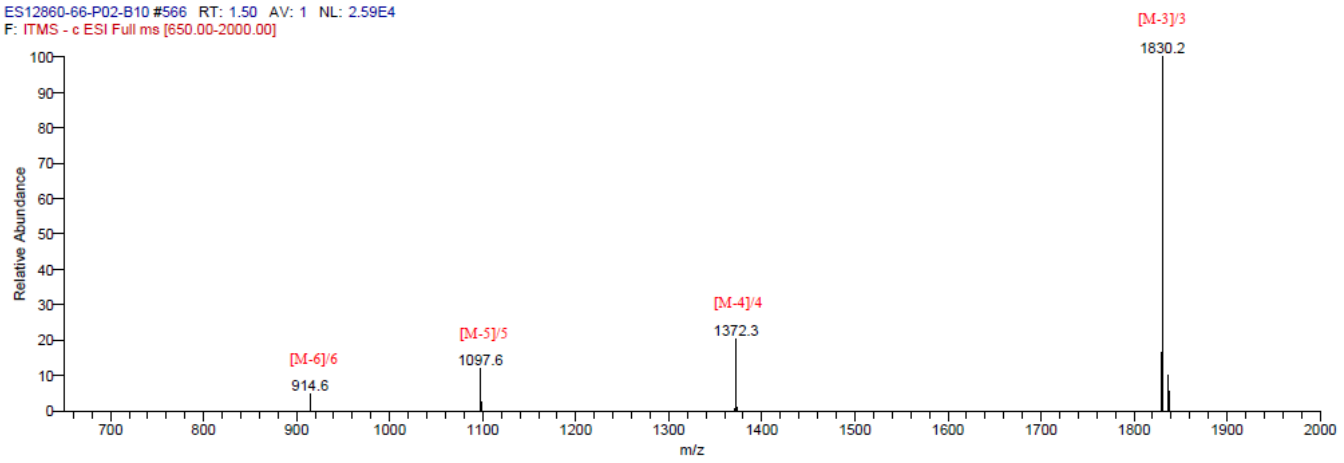
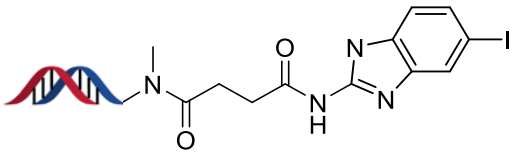
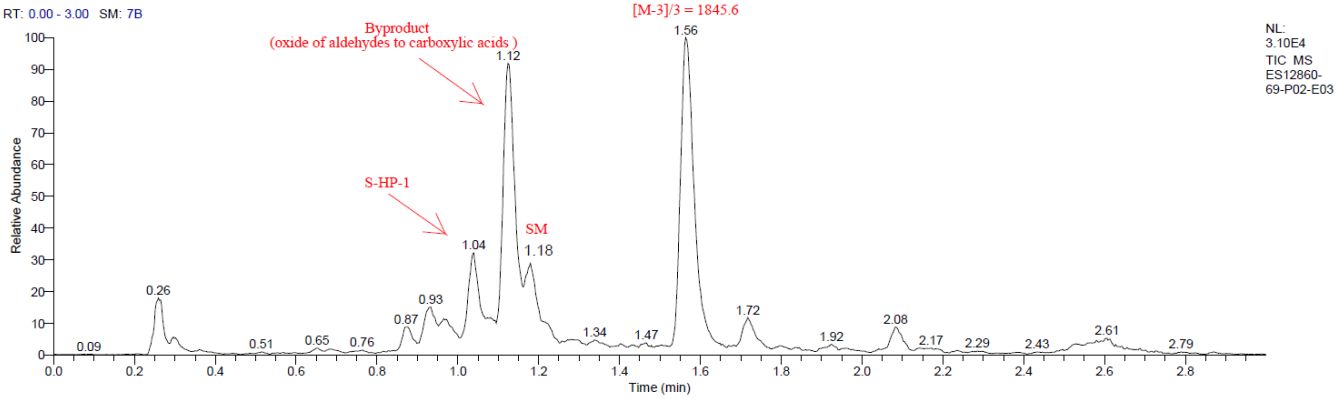


Figure S43. LC trace and mass of **5u**

SI-6-41 LC Trace and Mass of 5v

Following **General Procedure 3**
Yield: 38.71%
Exact mass: 5539.62
Triply charged mass [M-3]/3, calculated: 1845.5; observed: 1845.6.





RT	Peak Height	Peak Width	Peak Area	Area %
0.93	4277.40	0.11	15650.07	8.09
1.04	9776.77	0.09	23171.79	11.97
1.12	29721.31	0.07	60198.20	31.11
1.18	8672.55	0.11	19586.39	10.12
1.56	32017.25	0.25	74922.09	38.71

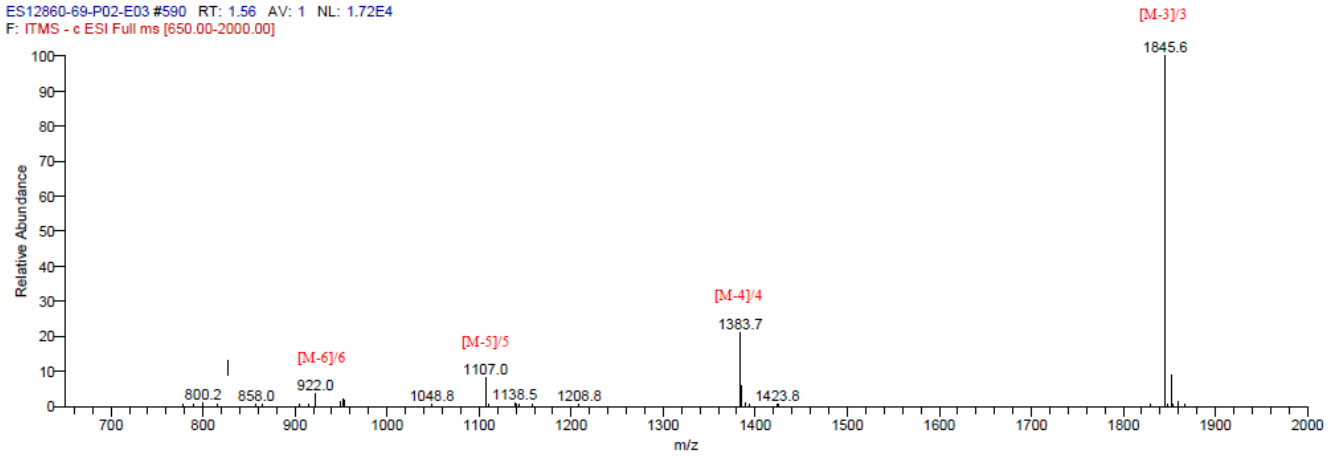
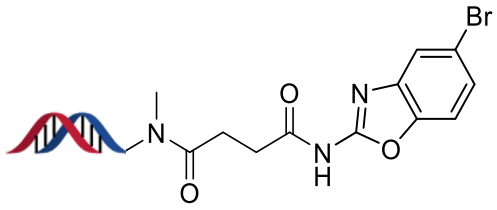
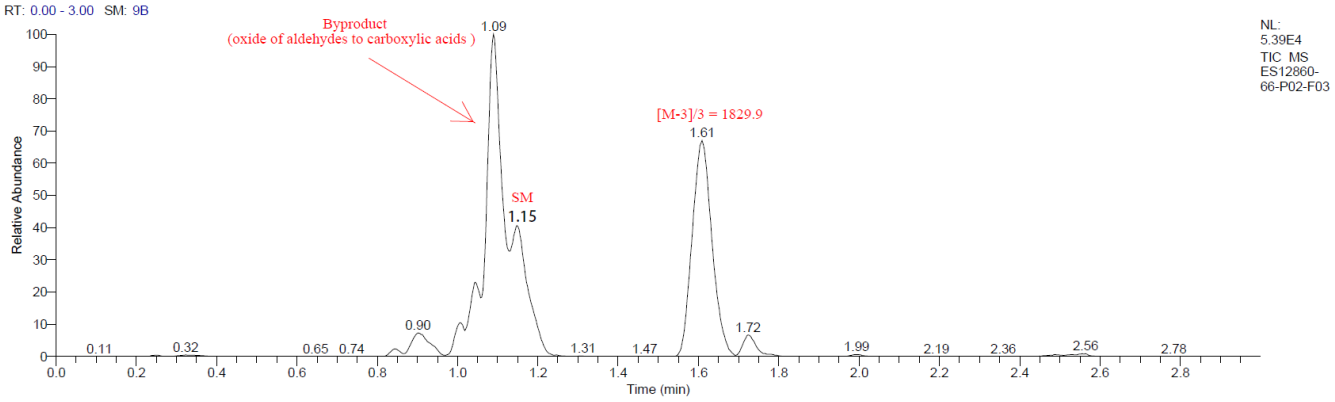


Figure S44. LC trace and mass of **5v**

SI-6-42 LC Trace and Mass of 5w

Following **General Procedure 3**
Yield: 34.93%
Exact mass: 5493.6
Triply charged mass [M-3]/3, calculated: 1830.2; observed: 1829.9.





RT	Peak Height	Peak Width	Peak Area	Area %
1.00	6713.67	0.05	10073.12	2.85
1.04	13595.09	0.04	22115.58	6.25
1.09	60252.92	0.07	132844.42	37.56
1.15	23591.89	0.12	65113.73	18.41
1.61	38158.99	0.15	123520.62	34.93

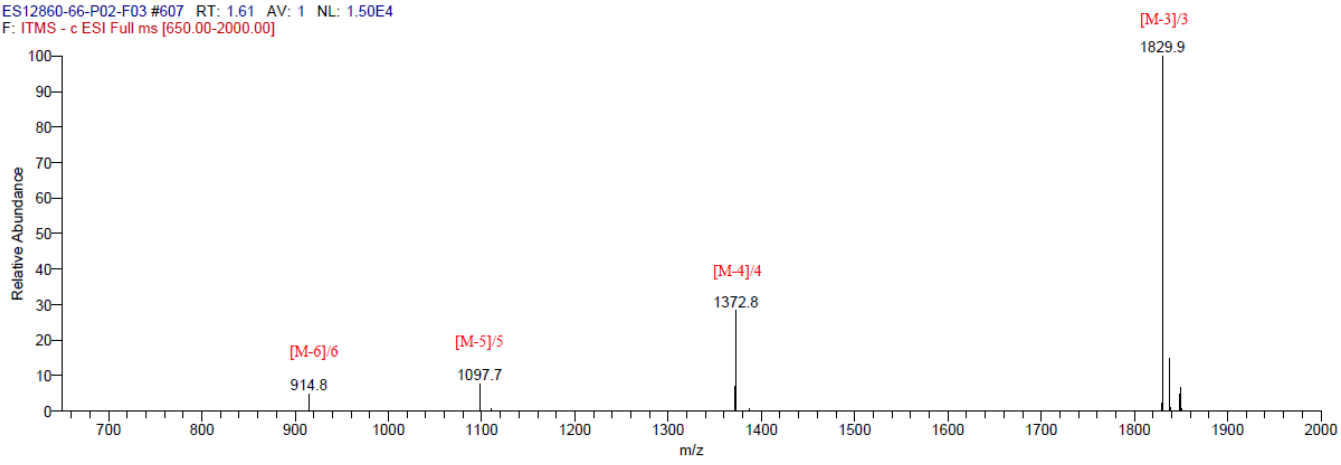
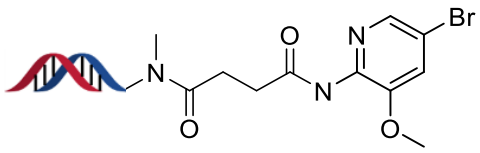
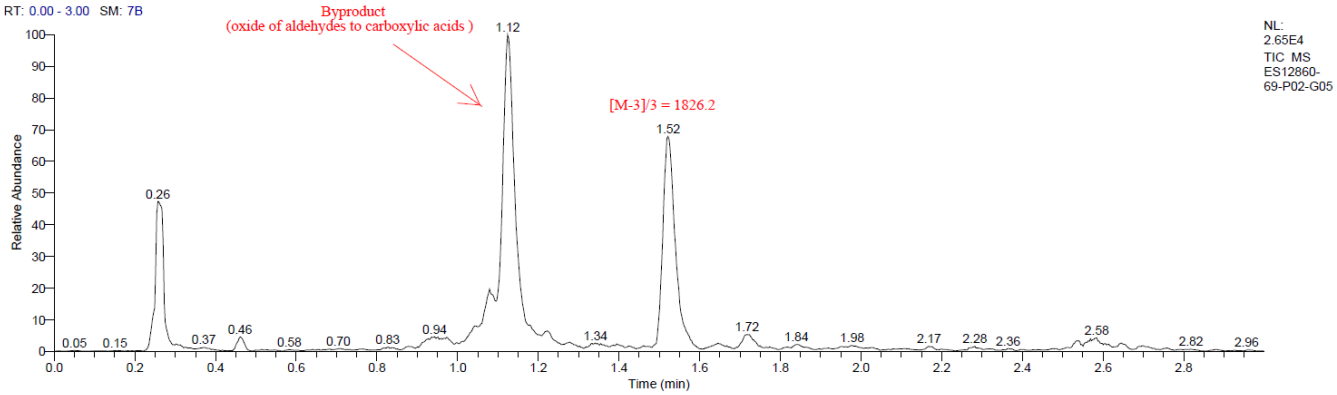


Figure S45. LC trace and mass of **5w**

SI-6-43 LC Trace and Mass of 5x

Following **General Procedure 3**
Yield: 32.22%
Exact mass: 5483.61
Triply charged mass [M-3]/3, calculated: 1826.8; observed: 1826.2.





RT	Peak Height	Peak Width	Peak Area	Area %
0.94	1054.67	0.13	4661.33	3.84
1.12	28426.36	0.20	71614.87	59.06
1.52	19746.94	0.15	39069.16	32.22
1.72	1454.67	0.11	2979.86	2.46
2.58	1172.44	0.08	2940.09	2.42

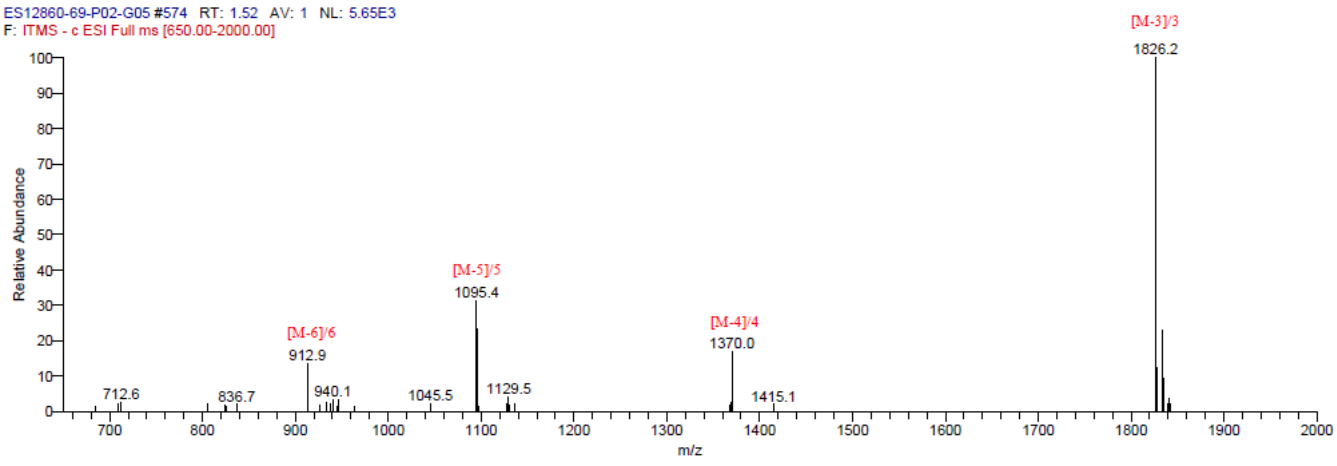
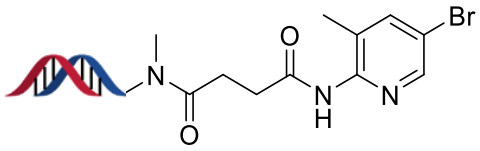
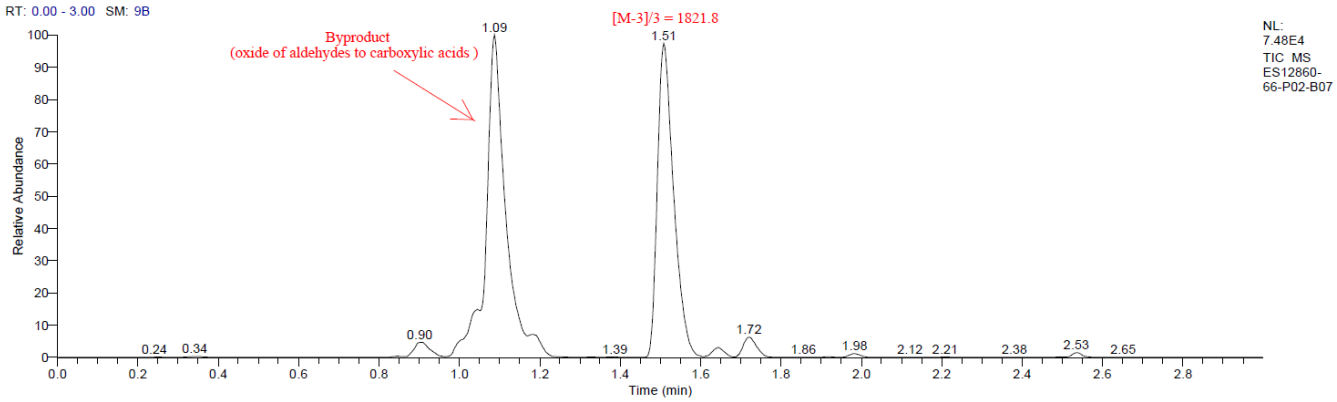


Figure S46. LC trace and mass of **5x**

SI-6-44 LC Trace and Mass of 5y

Following **General Procedure 3**
Yield: 43.69%
Exact mass: 5467.61
Triply charged mass [M-3]/3, calculated: 1821.5; observed: 1821.8.





RT	Peak Height	Peak Width	Peak Area	Area %
1.04	12289.97	0.04	21559.32	4.59
1.08	82251.20	0.12	221489.18	47.13
1.19	5848.31	0.07	10520.81	2.24
1.51	78816.03	0.15	205312.49	43.69
1.72	5391.25	0.11	11086.31	2.36

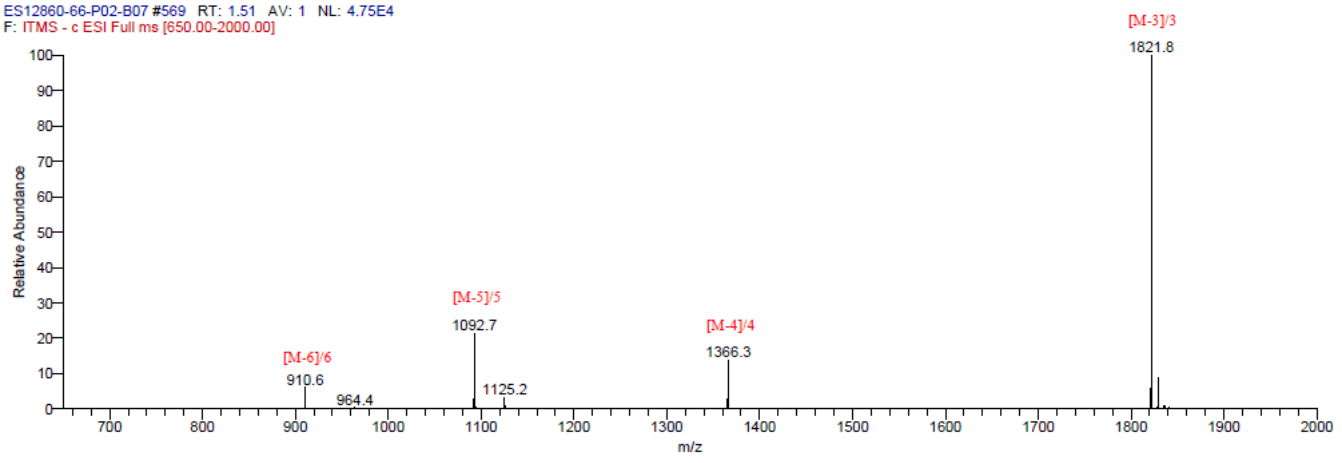
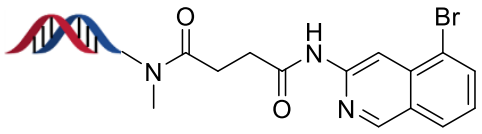
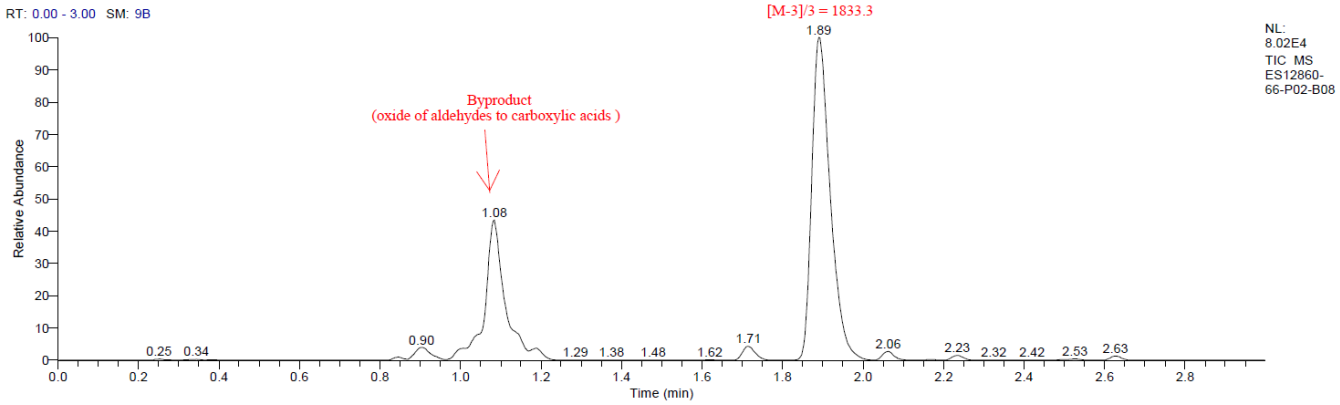


Figure S47. LC trace and mass of **5y**

SI-6-45 LC Trace and Mass of 5z

Following **General Procedure 3**
Yield: 67.24%
Exact mass: 5503.64
Triply charged mass [M-3]/3, calculated: 1833.6; observed: 1833.3.





RT	Peak Height	Peak Width	Peak Area	Area %
0.91	3626.73	0.10	8893.49	2.33
1.04	7091.04	0.04	12517.63	3.28
1.08	38892.69	0.08	90935.33	23.81
1.14	7854.61	0.04	12800.79	3.35
1.89	84721.24	0.21	256828.92	67.24

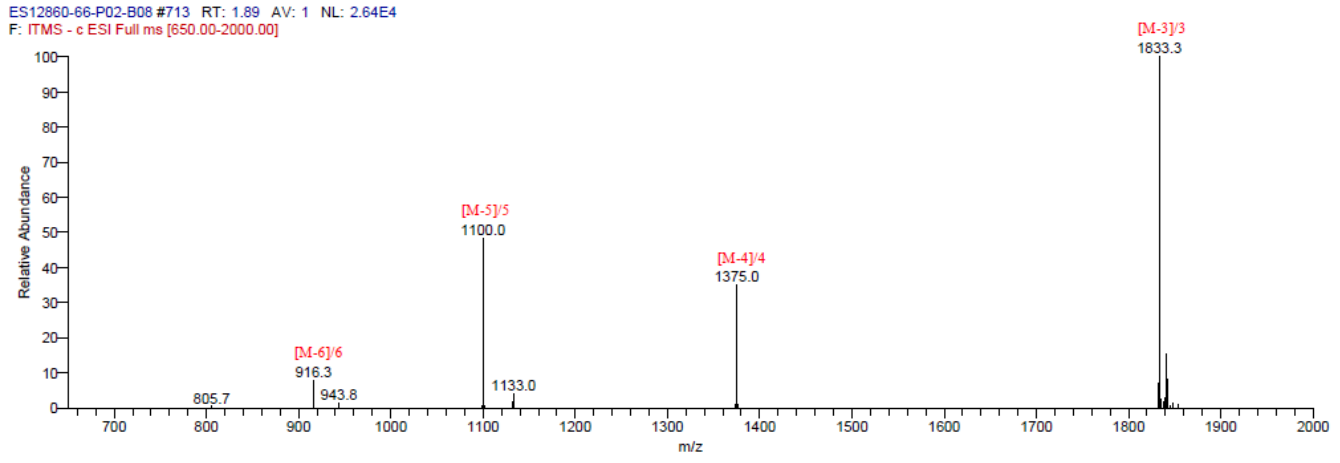
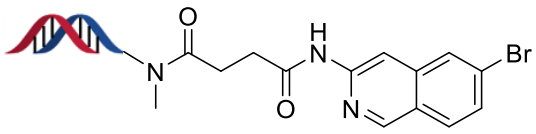
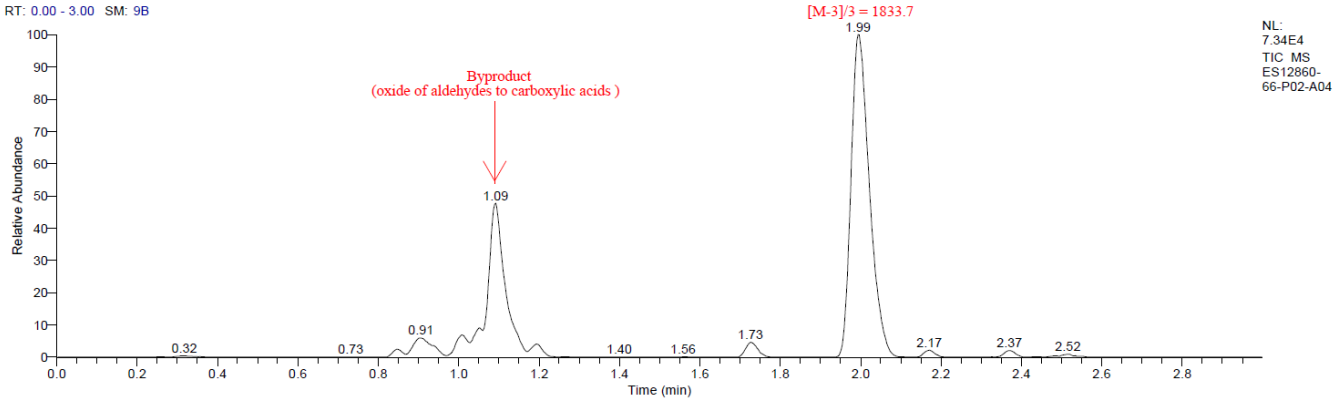


Figure S48. LC trace and mass of **5z**

SI-6-46 LC Trace and Mass of 5aa

Following **General Procedure 3**
Yield: 65.03%
Exact mass: 5503.64
Triply charged mass [M-3]/3, calculated: 1833.6; observed: 1833.7.





RT	Peak Height	Peak Width	Peak Area	Area %
1.00	5818.32	0.06	11149.92	3.10
1.05	7358.17	0.03	11813.32	3.29
1.09	39935.42	0.11	95483.57	26.57
1.73	3758.53	0.10	7223.36	2.01
1.99	78044.77	0.20	233687.57	65.03

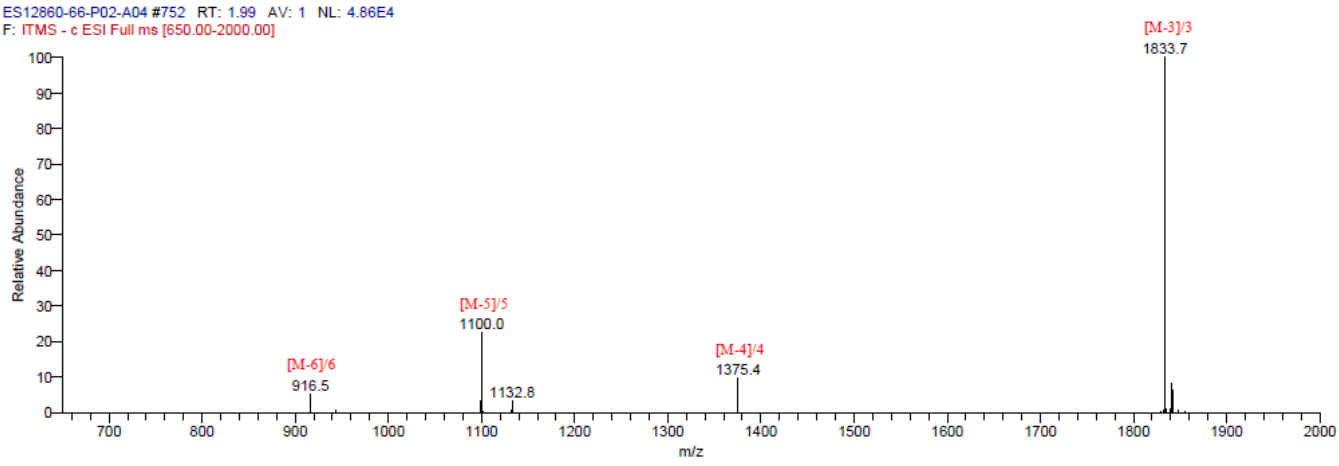
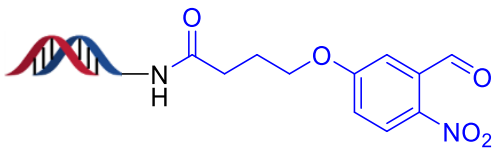
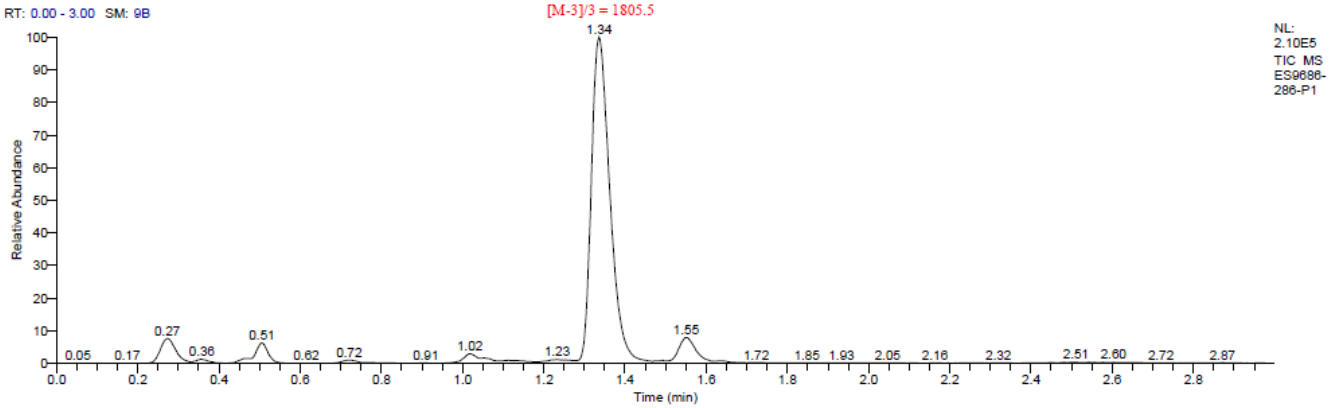


Figure S49. LC trace and mass of **5aa**

SI-6-47 LC Trace and Mass of 9

Following **General Procedure 1**
Yield: 89.75%
Exact mass: 5419.69
Triply charged mass [M-3]/3, calculated: 1805.6; observed: 1805.5.





RT	Peak Height	Peak Width	Peak Area	Area %
1.02	6581.54	0.09	14068.50	1.90
1.06	3757.80	0.05	6866.24	0.93
1.23	2549.44	0.07	6952.40	0.94
1.33	220719.88	0.17	665273.27	89.75
1.55	17195.12	0.11	48067.39	6.48

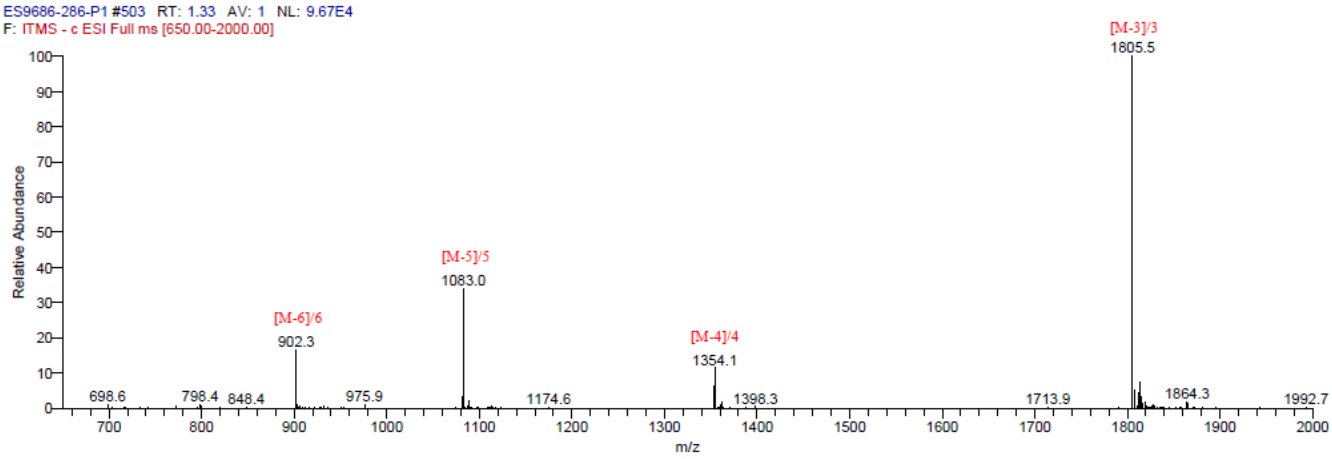
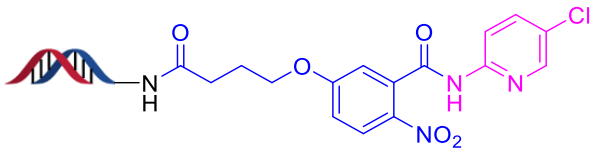
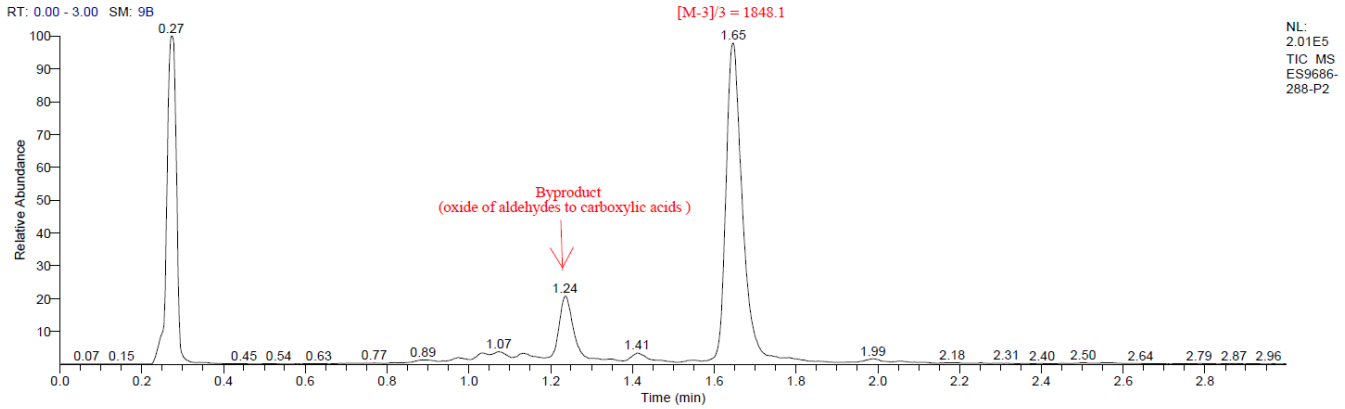


Figure S50. LC trace and mass of **9**

SI-6-48 LC Trace and Mass of 10

Following **General Procedure 3**
Yield: 79.02%
Exact mass: 5546.25
Triply charged mass [M-3]/3, calculated: 1847.8; observed: 1848.1.





RT	Peak Height	Peak Width	Peak Area	Area %
1.03	5519.99	0.05	10255.07	1.51
1.08	5947.64	0.06	14321.46	2.11
1.13	5610.63	0.08	15249.48	2.24
1.24	43893.53	0.18	102918.98	15.13
1.64	215852.78	0.23	537531.76	79.02

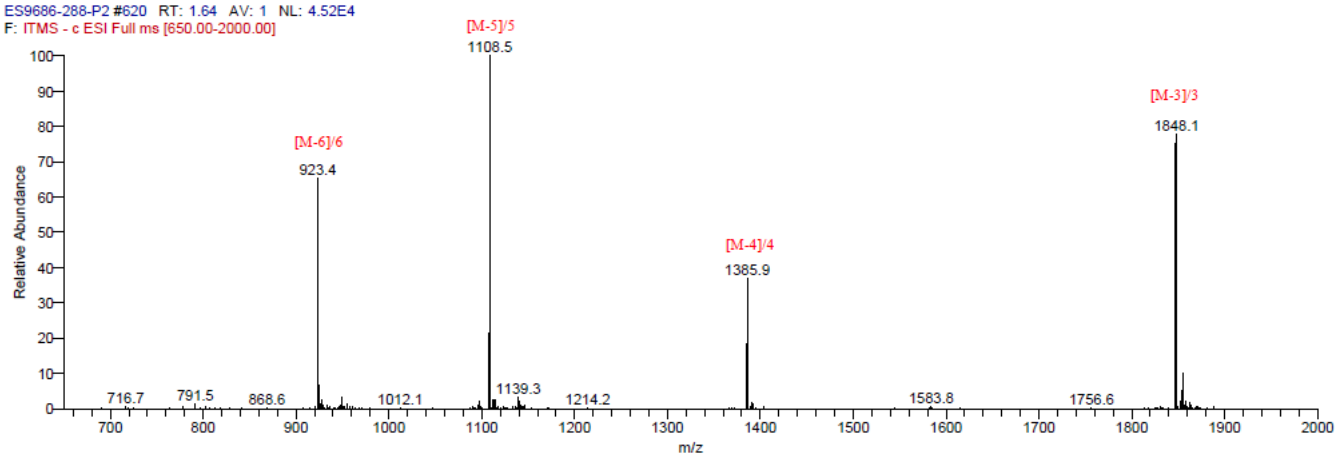
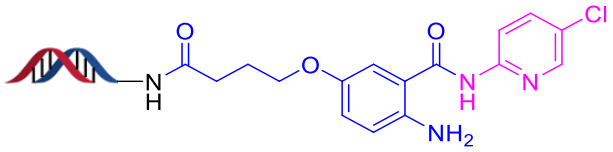
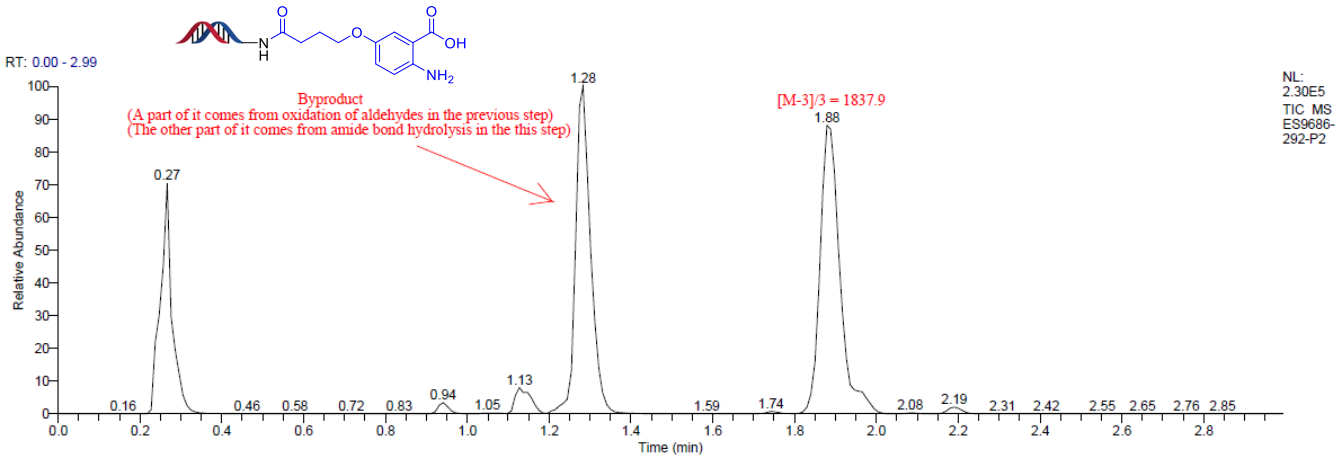


Figure S51. LC trace and mass of **10**

SI-6-49 LC Trace and Mass of 11

Yield: 51.36%
Exact mass: 5516.25
Triply charged mass [M-3]/3, calculated: 1837.8; observed: 1837.5.





RT	Peak Height	Peak Width	Peak Area	Area %
1.07	400.09	0.08	1169.15	0.09
1.13	15656.06	0.11	47898.96	3.58
1.28	208240.25	0.19	586089.48	43.80
1.74	1460.19	0.11	3396.34	0.25
1.89	191153.42	0.25	687256.43	51.36
2.08	594.23	0.09	1196.12	0.09
2.19	4261.78	0.13	11154.58	0.83

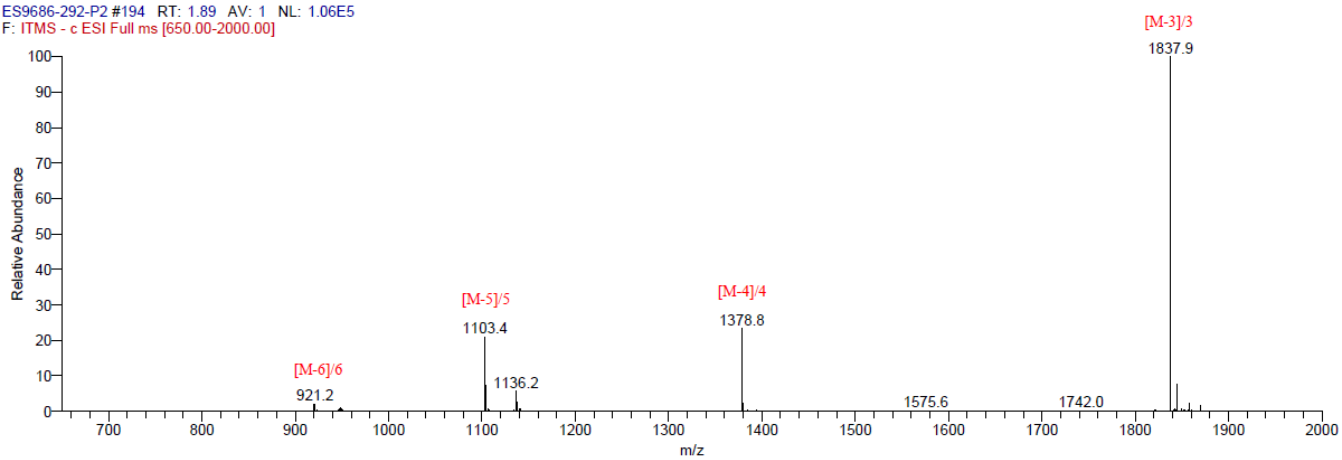
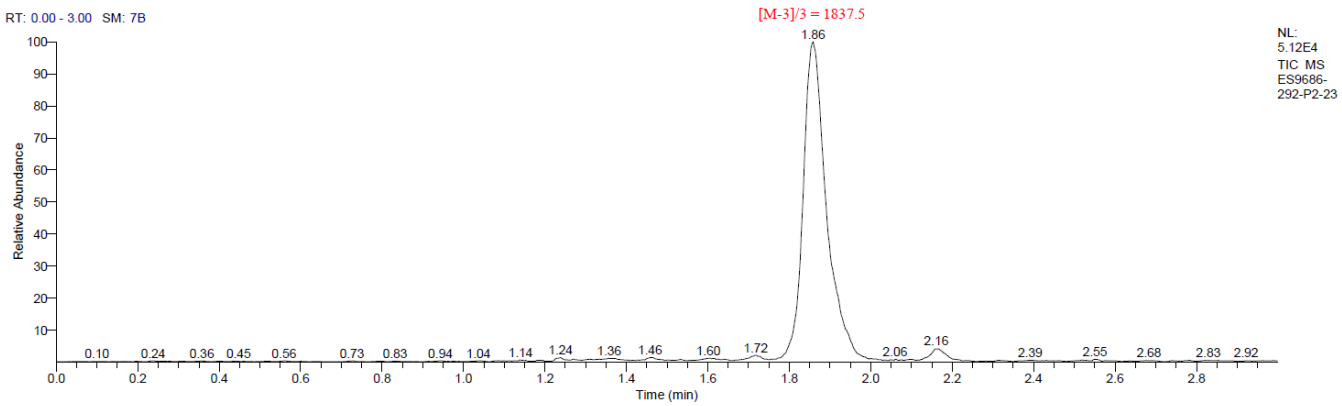


Figure S52. LC trace and mass of crude **11**



RT	Peak Height	Peak Width	Peak Area	Area %
1.86	51254.29	0.19	201126.67	98.43
2.16	1935.68	0.06	3205.87	1.57

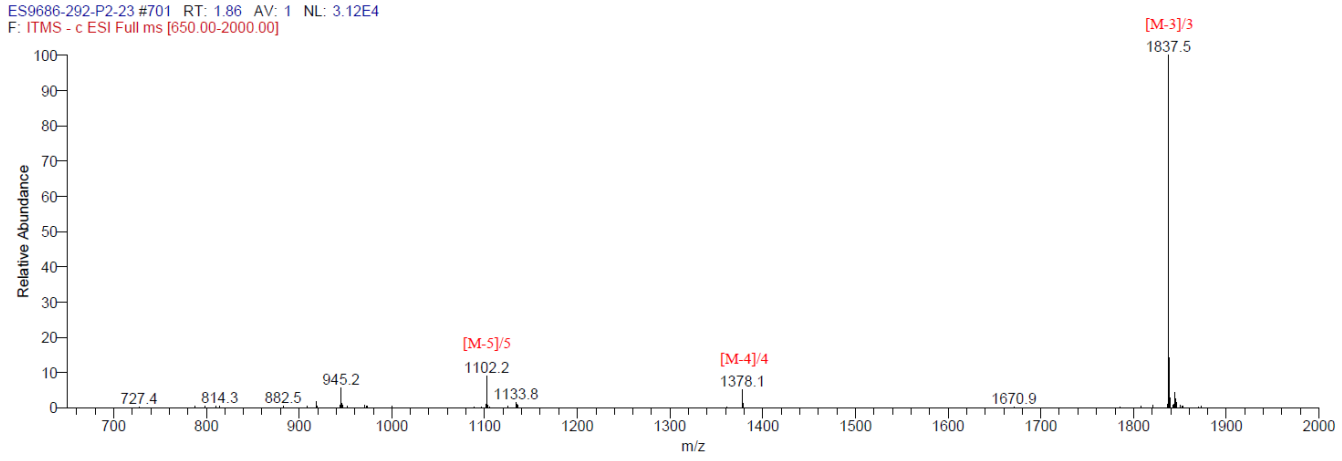
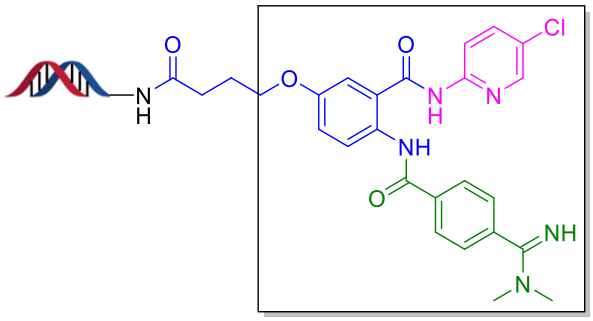


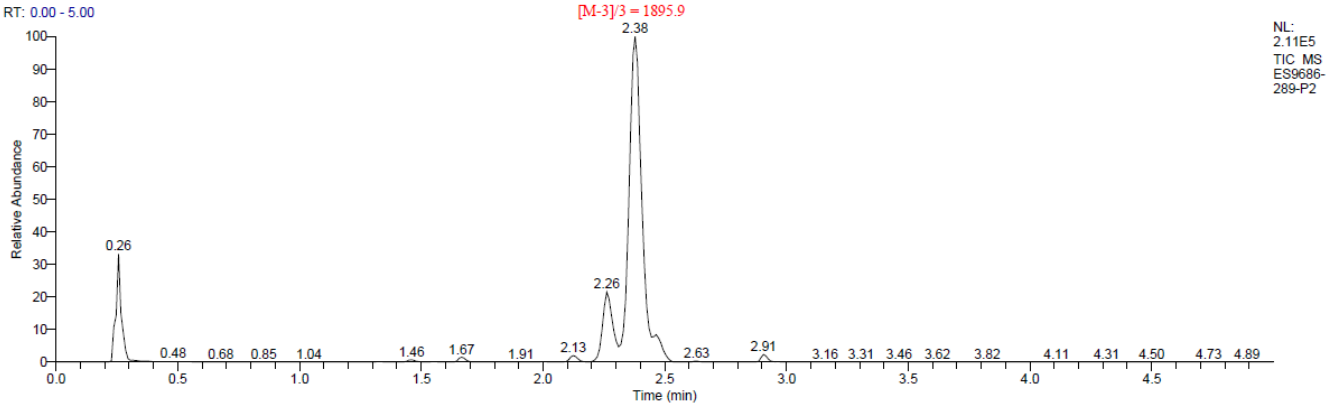
Figure S53. LC trace and mass of **11** after prep-HPLC

SI-6-50 LC Trace and Mass of 12

Yield: 83.16%
Exact mass: 5690.46
Triply charged mass [M-3]/3, calculated: 1895.8; observed: 1895.9.



Betrixaban



RT	Peak Height	Peak Width	Peak Area	Area %
1.46	1155.16	0.07	2482.92	0.26
1.67	2733.16	0.11	6192.08	0.65
2.13	3711.50	0.12	9391.39	0.99
2.26	41049.94	0.13	132121.96	13.93
2.38	200688.86	0.26	788979.70	83.16
2.63	428.25	0.07	762.72	0.08
2.91	3980.68	0.12	8866.90	0.93

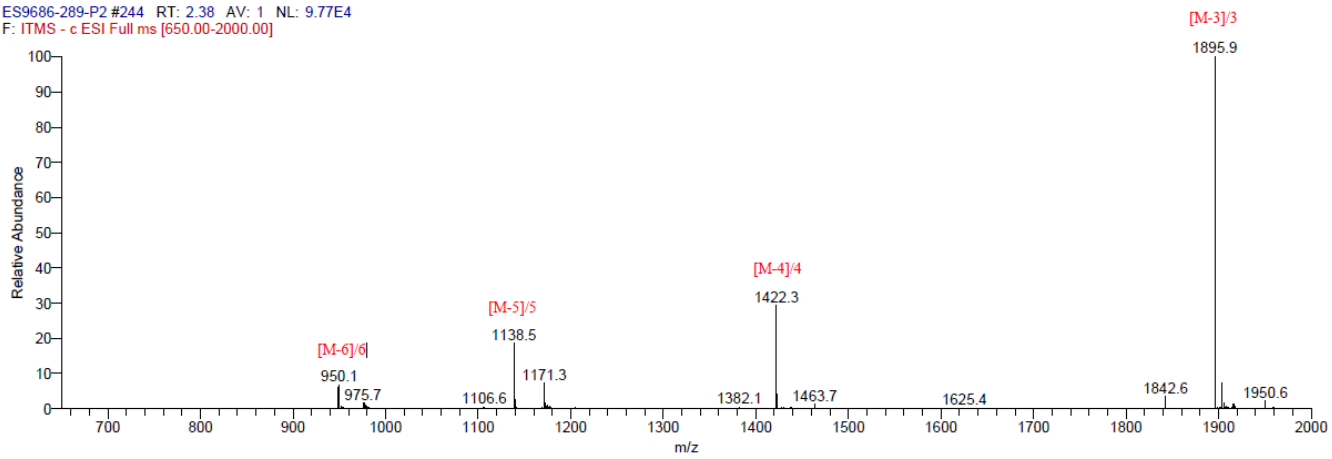
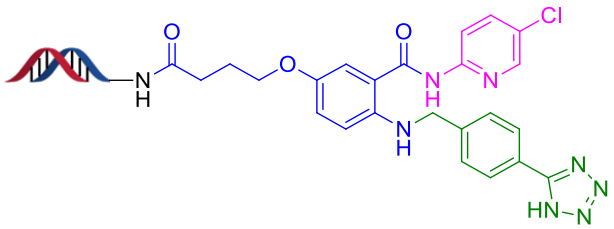
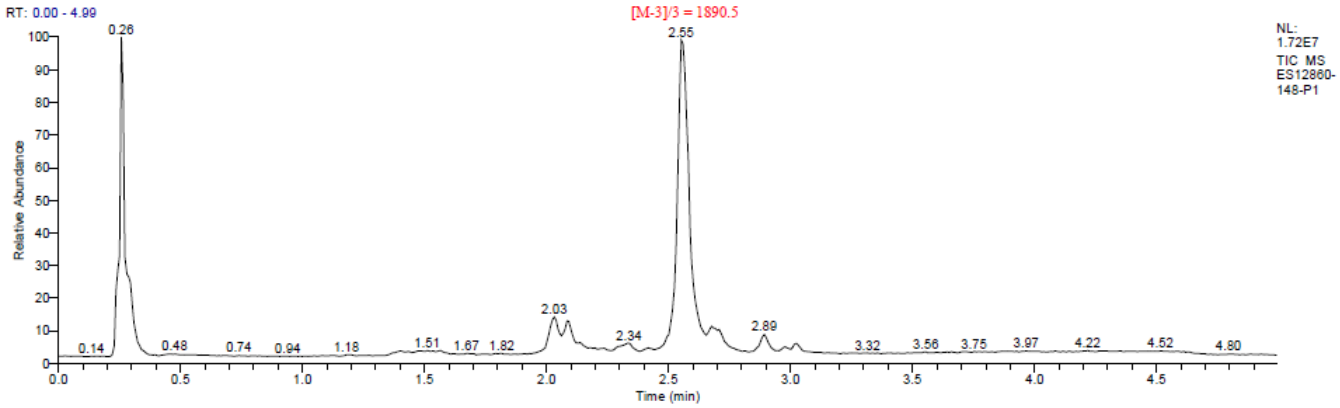


Figure S54. LC trace and mass of **12**

SI-6-51 LC Trace and Mass of 13

Yield: 70.44%
Exact mass: 5674.41
Triply charged mass [M-3]/3, calculated: 1890.5; observed: 1890.5.





RT	Peak Height	Peak Width	Peak Area	Area %
2.03	1835868.44	0.20	6704770.54	7.77
2.08	1618404.83	0.15	7367629.36	8.54
2.56	15416947.92	0.21	60795383.79	70.44
2.68	1450148.93	0.18	7890614.14	9.14
2.89	949073.17	0.12	3551273.78	4.11

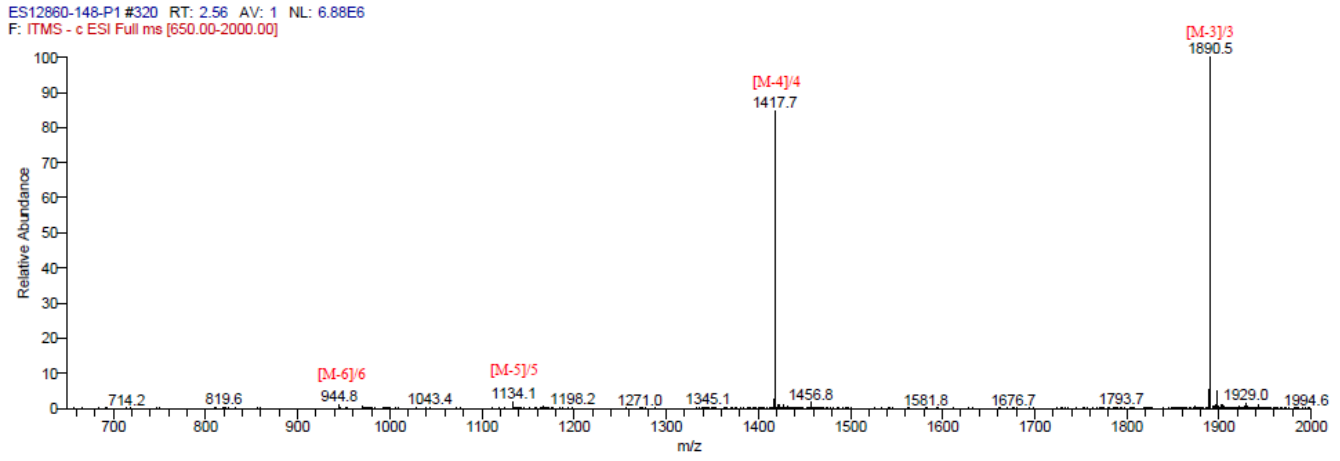


Figure S55. LC trace and mass of **13**