

# Supporting Information

## Identification and Validation of New Interleukin-2 Ligands using DNA-Encoded Libraries

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## 1. Abbreviations

A, Adenine; C, Cytosine; CAIX, Carbonic Anhydrase IX; Da, Dalton; DAD, Diode Array Detector; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; EDA, Extra-domain A of fibronectin; EDB, Extra-domain B of fibronectin; EF, Enrichment Factor; ELISA, Enzyme-Linked Immunosorbent Assay; F8, Anti-EDA antibody; Fmoc, Fluorenylmethyloxycarbonyl; G, Guanidine; HAS, Human Serum Albumin; IC<sub>50</sub>, Half maximal inhibitory concentration; IE-HPLC, Ion-Exchange-High Performance Liquid Chromatography; IL2, Interleukin-2; IL15, Interleukin-15; L19, Anti-EDB antibody; LC-ESI-MS, Liquid Chromatography-Electrospray Ionization Mass Spectrometry; min, minute; nm, nanometer; PBS, phosphate buffer saline; RP-HPLC, Reverse Phase-High Performance Liquid Chromatography; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; T, Thymine; TNF, Tumor Necrosis Factor; TIC, Total Ion Chromatogram; t<sub>R</sub>, Retention time; UV, Ultraviolet; μM, micromolar; UPLC, Ultra Performance Liquid Chromatography.

## 2. Oligonucleotides and library sequences.

Fmoc-amino acids were coupled to their corresponding amino-modified oligonucleotide **Code 1** (468). Phosphorylated oligonucleotides **Code 2** (1430) were ligated to the Pool 1 fractions using an oligonucleotide **Splint**.

### **Code 1:**

NH<sub>2</sub>-C6-5' -GGAGCTTCTGAATTCTGTGTGCTGXXXXXXCGAGTCCCATGGCGC-3' being XXXXXX the encoding region for each code

### **Code 2:**

Phospho-5' -CGGATCGACYYYYYYYYGCGTCAGGCAGC-3' being YYYYYYYY the encoding region for each code

### **Splint:**

5' -CGTCGATCCGGCGCCATGGACTCG-3'

### **Klenow Fill-in Code 3 for dsDNA screenings**

5' -GCTCTGCACGGTCGCCGTCAAGCTGCCTGACGC-3'

### **PCR1 Forward primers for ssDNA screenings:**

5' -TACACGACGCTTCCGATCTACACACGGAGCTTCTGAATTCTGTGTG-3' marked in red the encoding region of each primer

### **PCR1 Reverse primers for ssDNA screenings:**

5' -CAGACGTGTGCTTCCGATCCGATATGCTGCTGCCTGACGC-3' marked in red the encoding region of each primer

**PCR1 Forward primer for dsDNA screenings**

5' -TACACGACGCTCTCCGATCTACACACGGAGCTTCTGAATTCTGTGTG-3' marked in blue the encoding region of each primer

**PCR1 Reverse primer for dsDNA screenings:**

5' -CAGACGTGTGCTCTCCGATCCGATATGCTCTGCACGGTCGC-3' marked in blue the encoding region of each primer

**PCR2 Illumina Forward primer:**

5' -AATGATAACGGCGACCACCGAGATCTACACTTTCCCTACACGACGCTCTCCGATCT-3'

**PCR2 Illumina Reverse primer:**

5' -CAAGCAGAACGACGGCATACGAGAT333333GTGACTGGAGTTCAGACGTGTGCTCTCCGATC-3'

**PCR1 product example for ssDNA screenings:**

5' -TACACGACGCTCTCCGATCTACACACGGAGCTTCTGAATTCTGTGCTGTATCCACGAGTCCCATGGCGCCGGATCGACGGTCTCACGCGTCAGGCAGCAGCATATCGGATCGGAAGAGCACACGTCTG-3'  
3' -ATGTGCTGCGAGAAGGCTAGATGTGCTCGAACAGACTTAAGACACACGACATAGGTGCTCAGGGTACCGCGGCCTAGCTGCCAGAGTGCAGTCGTCGTATAGCCTAGCCTCTCGTGTGCAGAC-5'

**PCR2 product example for ssDNA screenings:**

5' -AATGATAACGGCGACCACCGAGATCTACACTTTCCCTACACGACGCTCTCGATCTACACACGGAGCTTCTGAATTCTGTGCTGTATCCACGAGTCCCATGGCGCCGGATCGACGGTCTCACGCGTCAGGCAGCAGCATATCGGATCGGAAGAGCACACGTCTG-3'  
3' -TTACTATGCCGCTGGCTCTAGATGTGAGAAAGGGATGTCTCGAACAGACTTAAGACACACGACATAGGTGCTCAGGGTACCGCGGCCTAGCTGCCAGAGTGCAGTCGTCGTATAGCCTAGCCTCTCGTGTGCAGACTTGAGGTAGAGCATACGGCAGAAC-5'

**PCR1 product example for dsDNA screenings:**

5' -TACACGACGCTCTCCGATCTACACACGGAGCTTCTGAATTCTGTGCTGTATCCACGAGTCCCATGGCGCCGGATCGACGGTCTCACGCGTCAGGCAGCAGTAGACGGCAGCGTGCAGAGCATATCGGATCGGAAGAGCACACGTCTG-3'  
3' -ATGTGCTGCGAGAAGGCTAGATGTGCTCGAACAGACTTAAGACACACGACATAGGTGCTCAGGGTACCGCGGCCTAGCTGCCAGAGTGCAGTCGTCGAATCTGCCGCTGGCACGTCGTATAGCCTAGCCTCTCGTGTGCAGAC-5'

**PCR2 product example for dsDNA screenings:**

5' -AATGATAACGGCGACCACCGAGATCTACACTTTCCCTACACGACGCTCTCGATCTACACACGGAGCTTCTGAATTCTGTGCTGTATCCACGAGTCCCATGGCGCCGGATCGACGGTCTCACGCGTCAGGCAGCAGCATATCGGATCGGAAGAGCACACGTCTG-3'  
3' -TTACTATGCCGCTGGCTCTAGATGTGAGAAAGGGATGTGCTCGAACAGACTTAAGACACACGACATAGGTGCTCAGGGTACCGCGGCCTAGCTGCCAGAGTGCAGTCGTCGAATCTGCCGCTGGCACGTCGTATAGCCTAGCCTCTCGTGTGCAGAC-5'

**5'-Amino-modified-12mer DNA:**

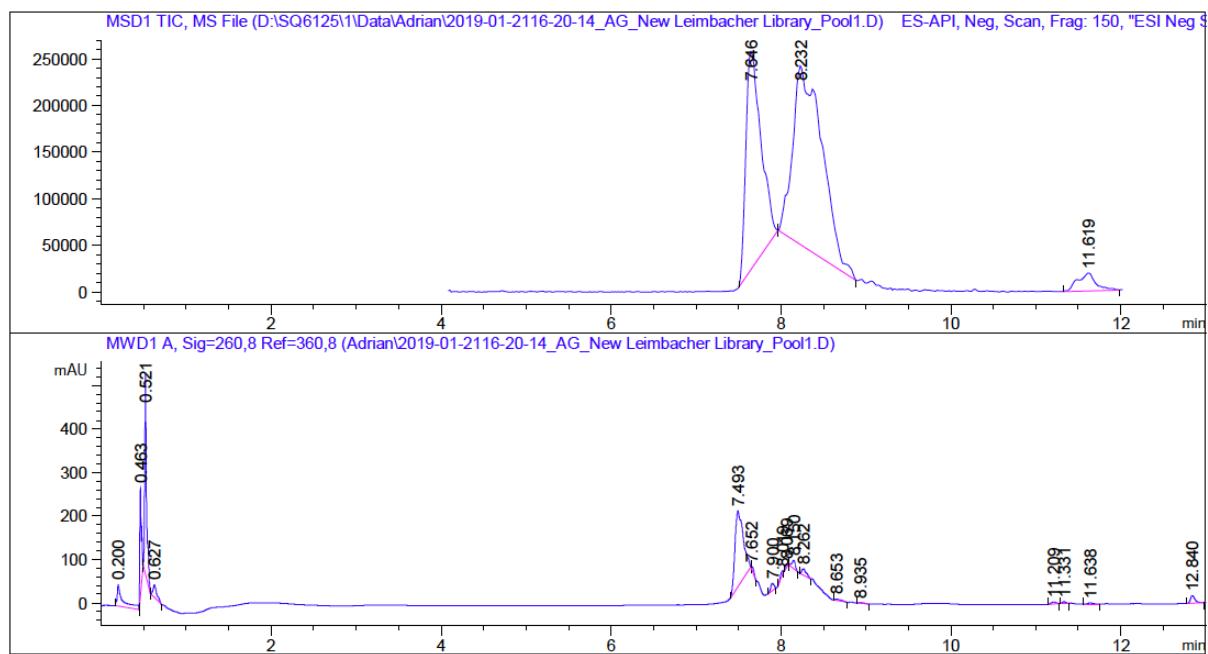
NH<sub>2</sub>-C6-5' -TAGTAGCCATCC-3'

**3'-Amino-modified-8mer LNA:**

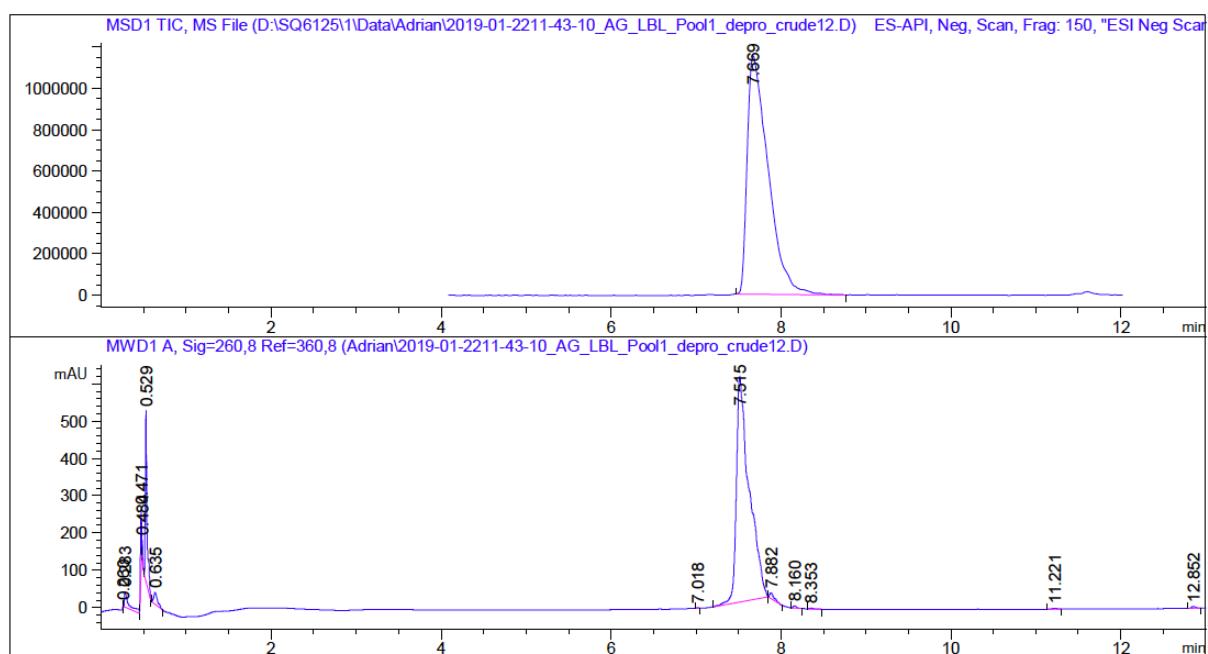
5' -GGCTACTA-3' -C6-NH<sub>2</sub>

### 3. Library synthesis, purification and characterization.

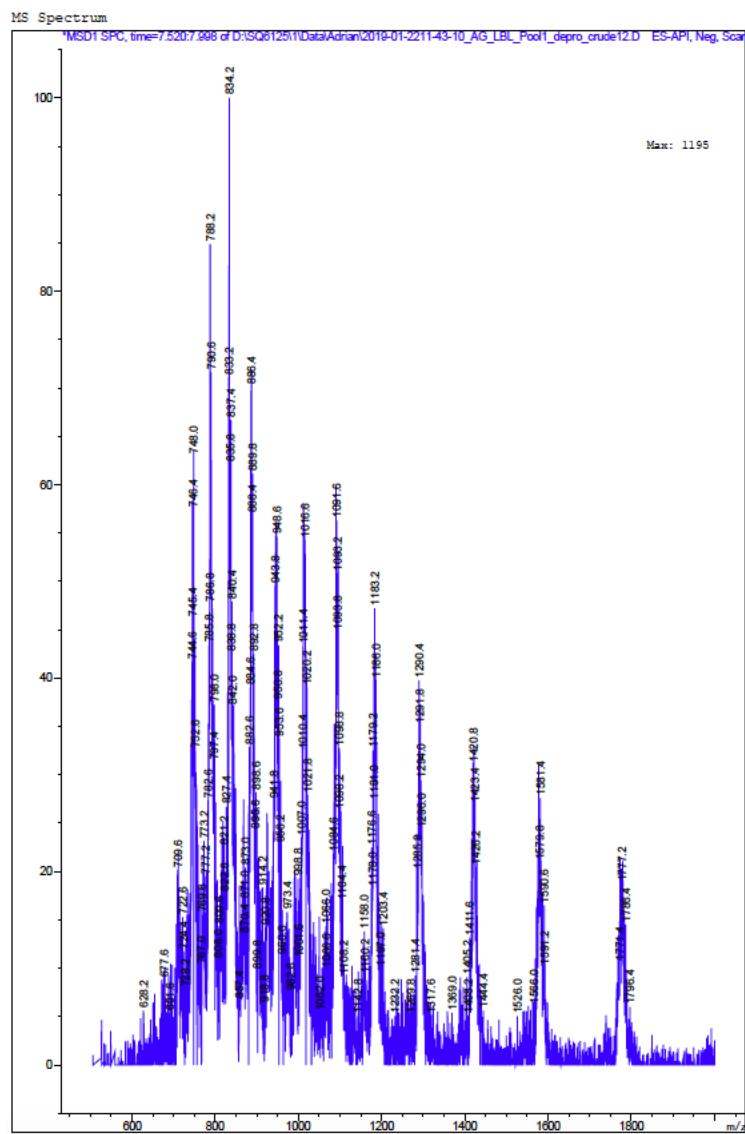
#### a. Characterization of Pool 1.



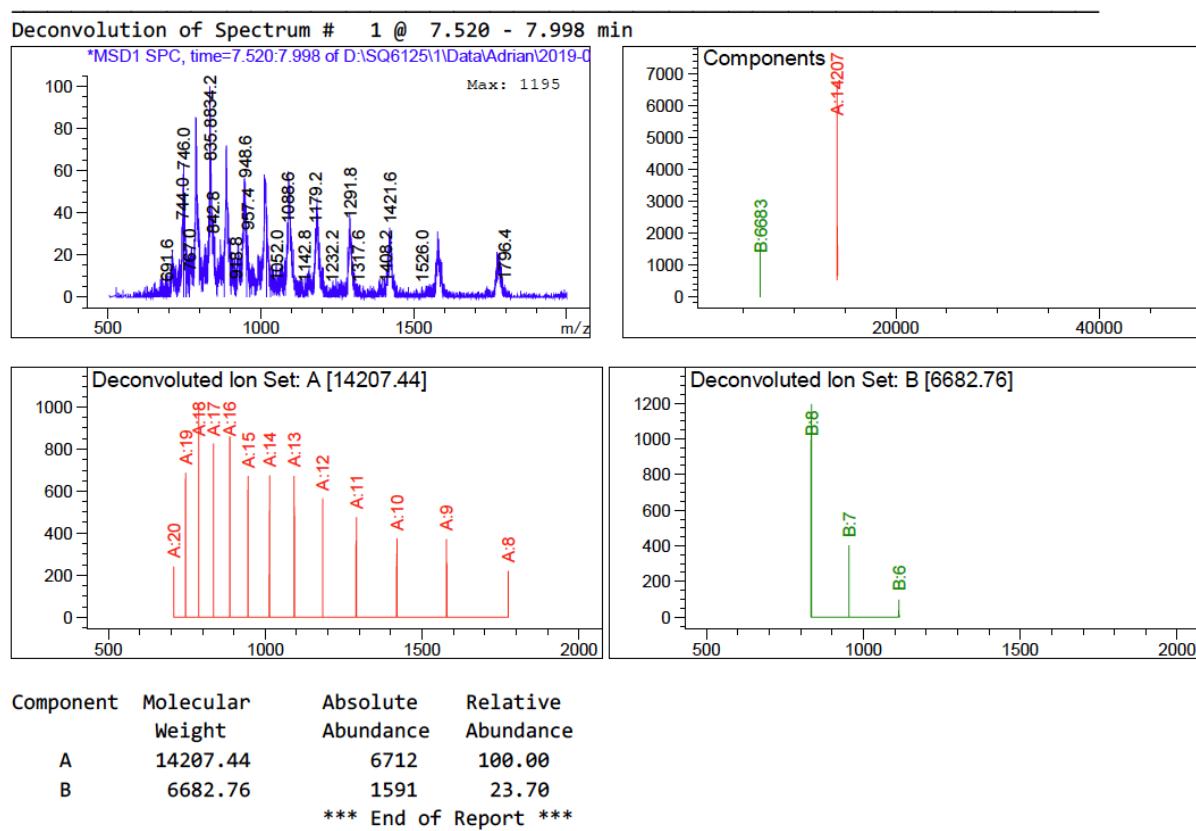
**Figure S1.** UV and MS traces of AG-DEL purified Pool 1 before fmoc deprotection. Fmoc-protected pool can be observed between 7.900 min to 8.935 min (DAD), 8.232 min (TIC). A fraction of Pool1 already deprotected after lyophilization of the single conjugates can be observed at 7.493 min (DAD), 7.646 min (TIC).



**Figure S2.** UV and MS traces of AG-DEL purified Pool 1 after fmoc deprotection

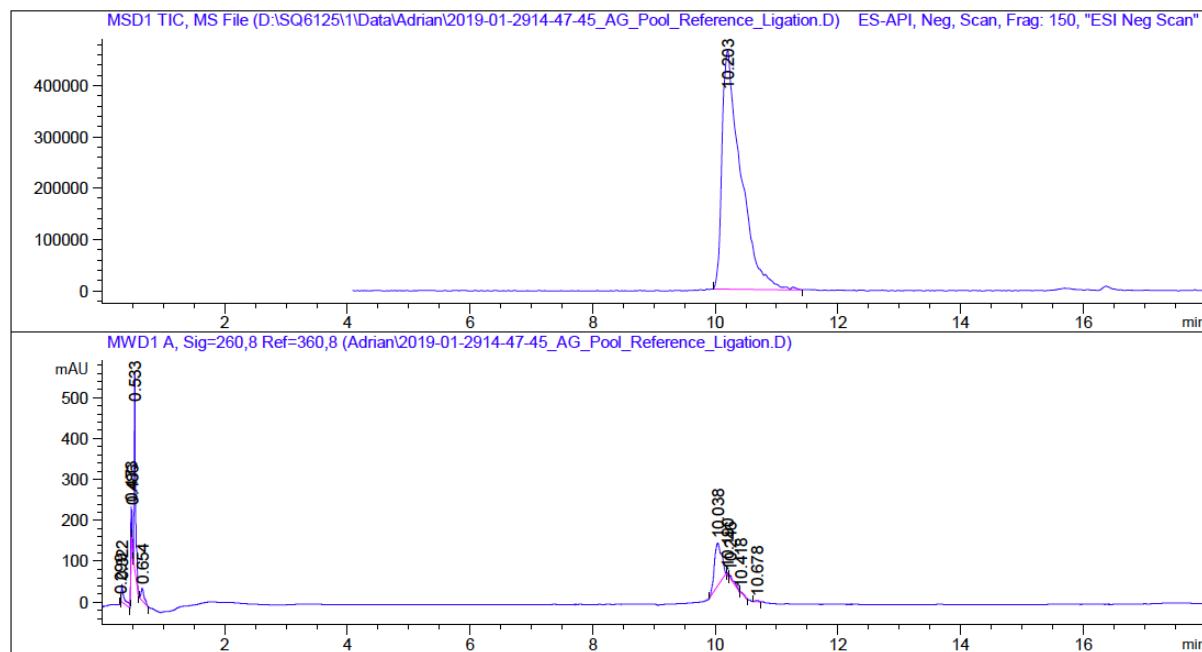


**Figure S3.** MS non-deconvoluted spectrum of AG-DEL purified Pool 1 after fmoc deprotection

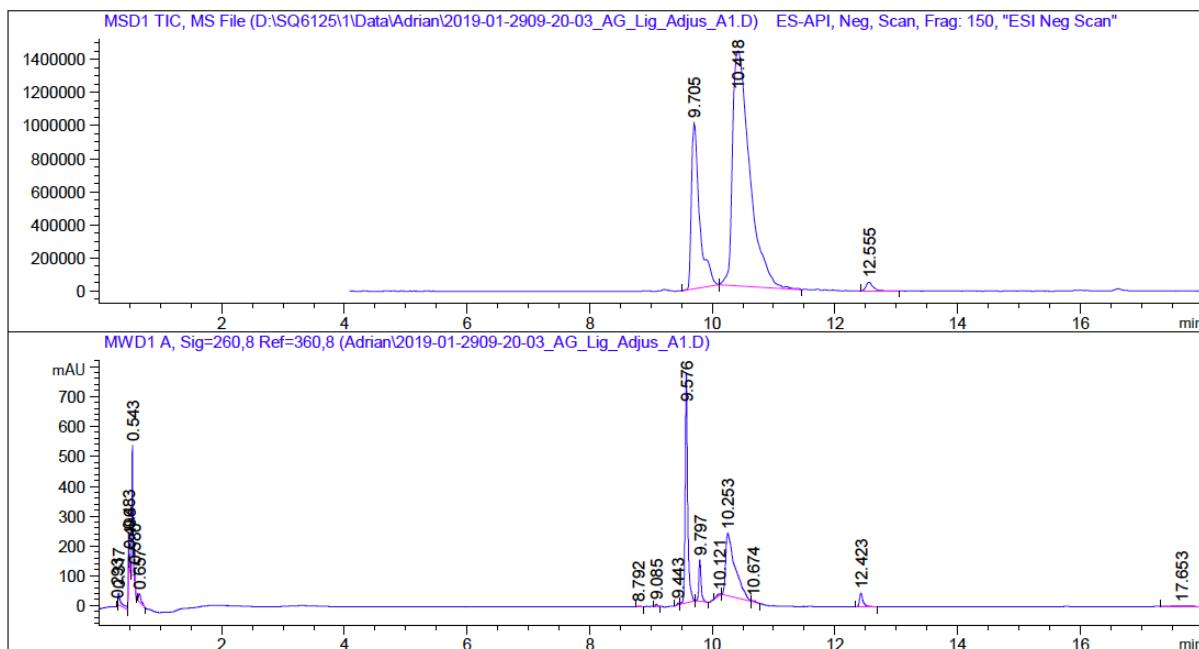


**Figure S4.** MS deconvoluted spectrum of AG-DEL purified Pool 1 after fmoc deprotection

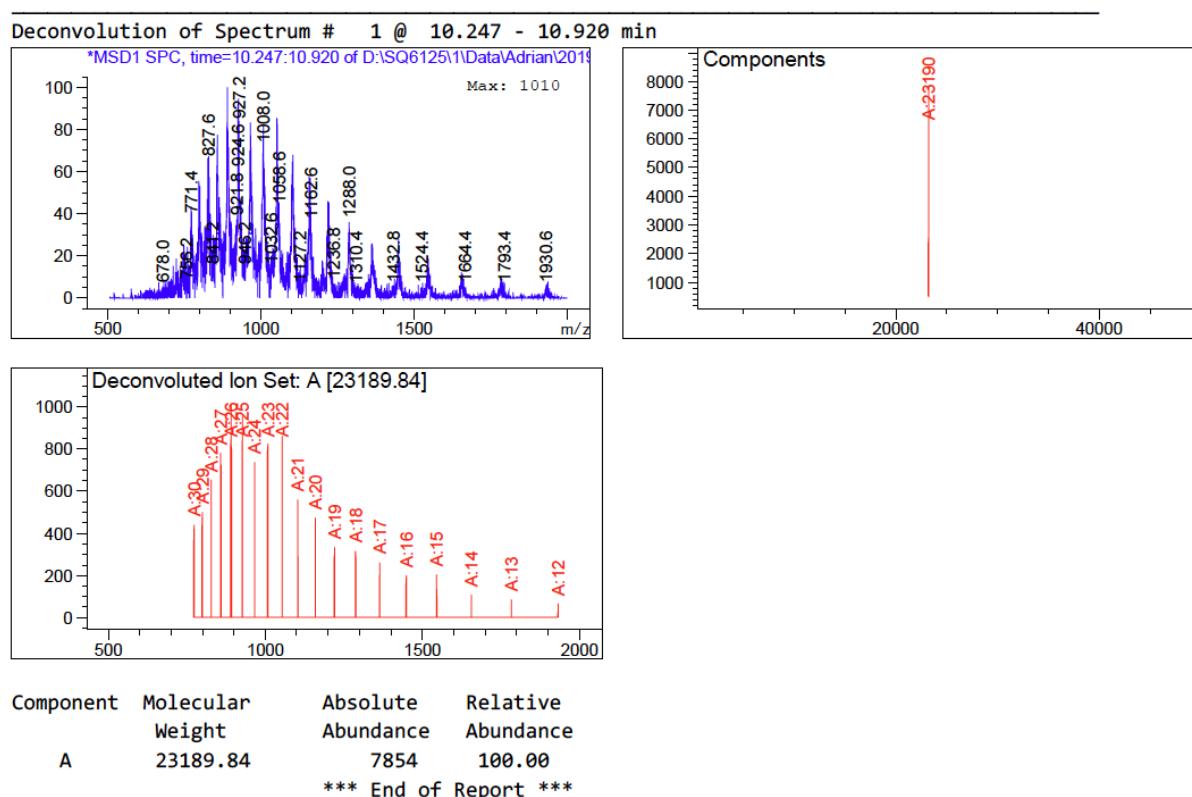
### b. Splint-mediated ligation of Code 2



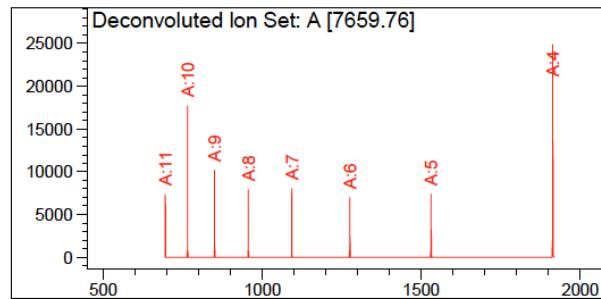
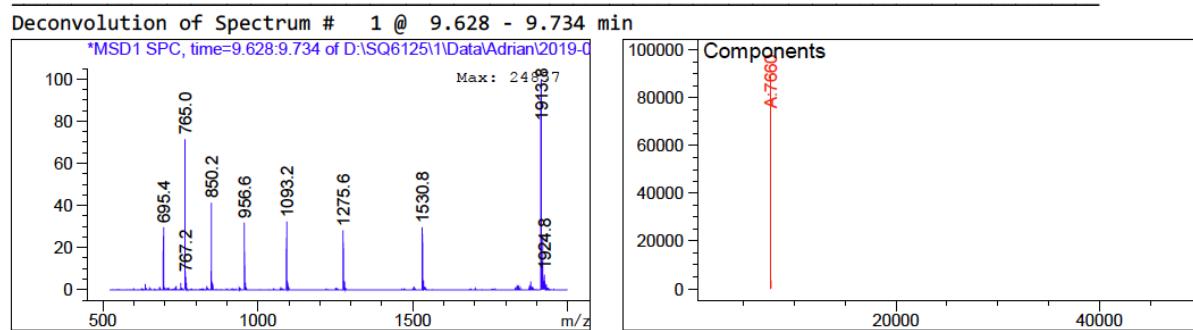
**Figure S5.** UV and MS traces of deprotected Pool 1 run on a different method serving as reference for ligation analysis



**Figure S6.** UV and MS traces of a representative example of splint-mediated ligation of Code 2 to Pool 1

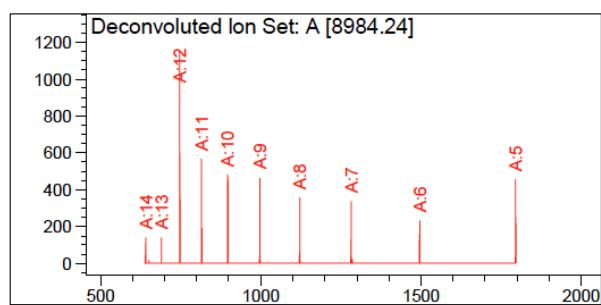
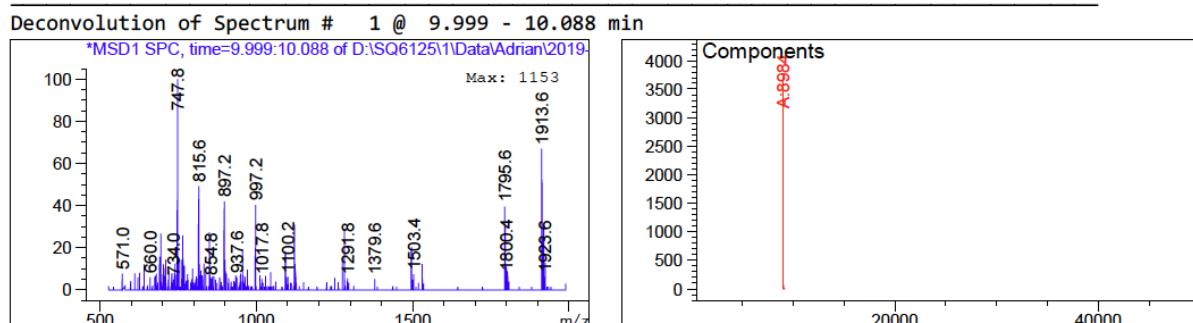


**Figure S7.** MS deconvoluted spectrum of a representative example of splint-mediated ligation of Code 2 to Pool 1



Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	7659.76	89025	100.00
*** End of Report ***			

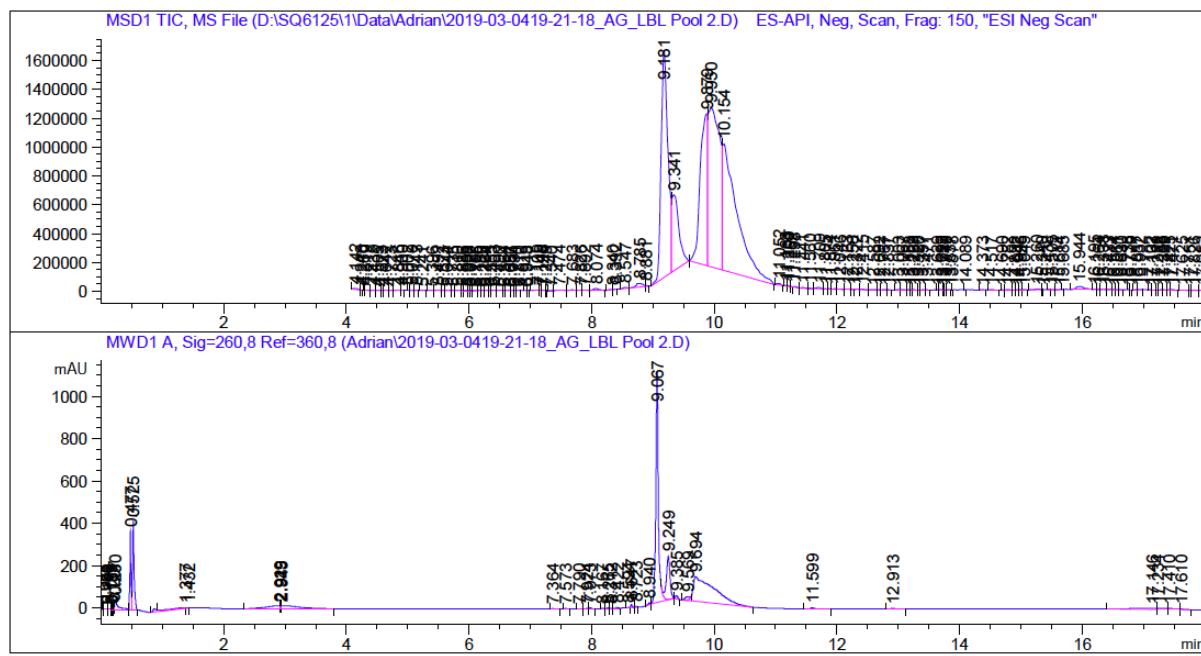
**Figure S8.** MS deconvoluted spectrum of the splint fraction

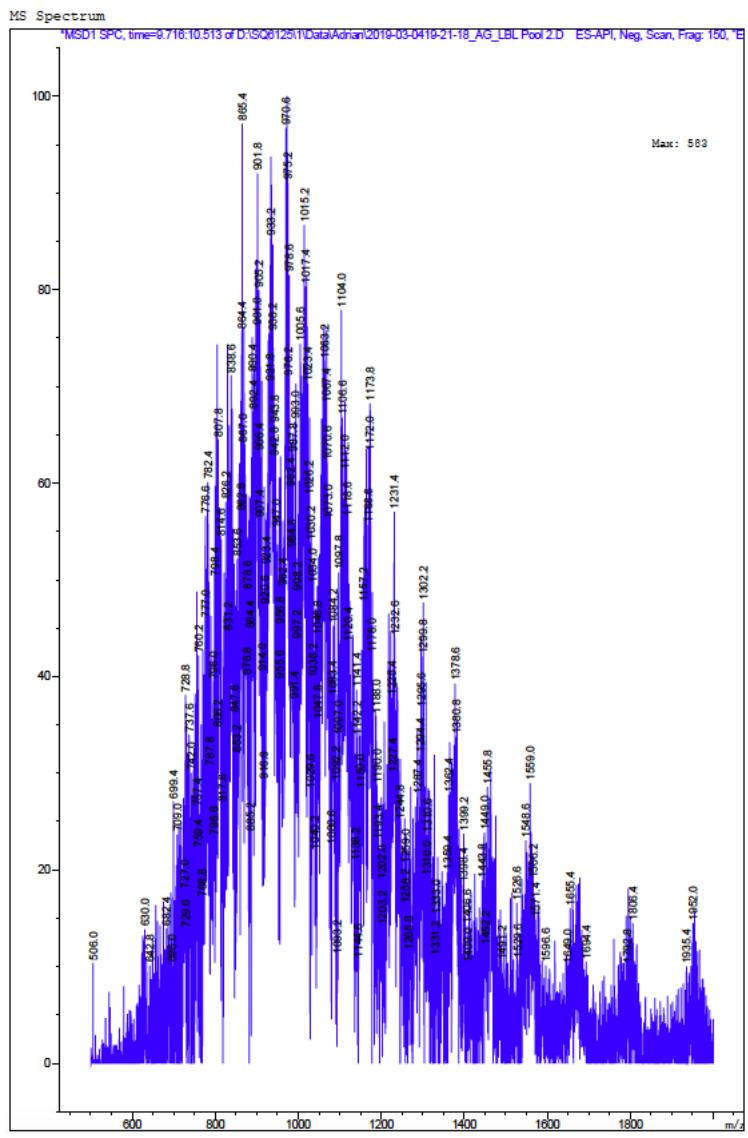


Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	8984.24	3729	100.00
*** End of Report ***			

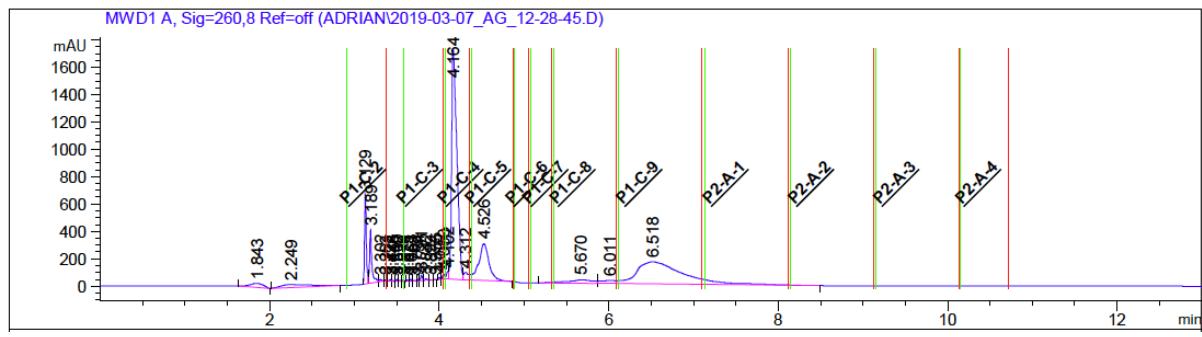
**Figure S9.** MS deconvoluted spectrum of the Code 2 fraction

**c. IE-HPLC Purification of Pool 2.**



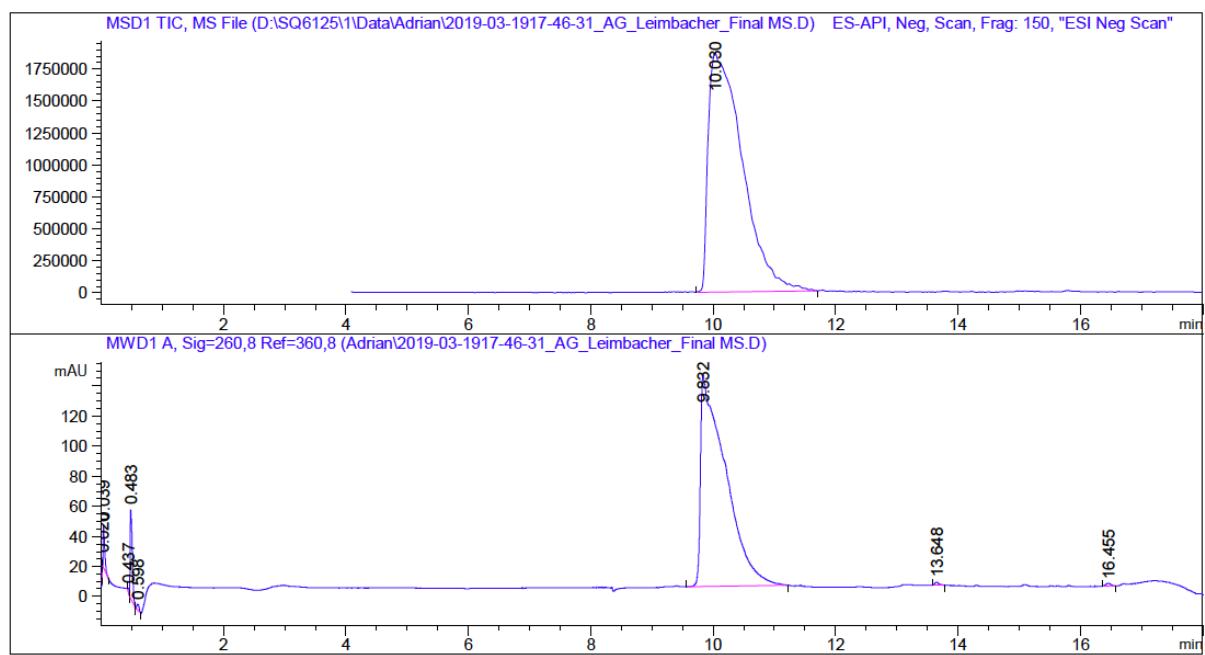


**Figure S11.** MS non-deconvoluted spectrum of AG-DEL Pool 2 before purification

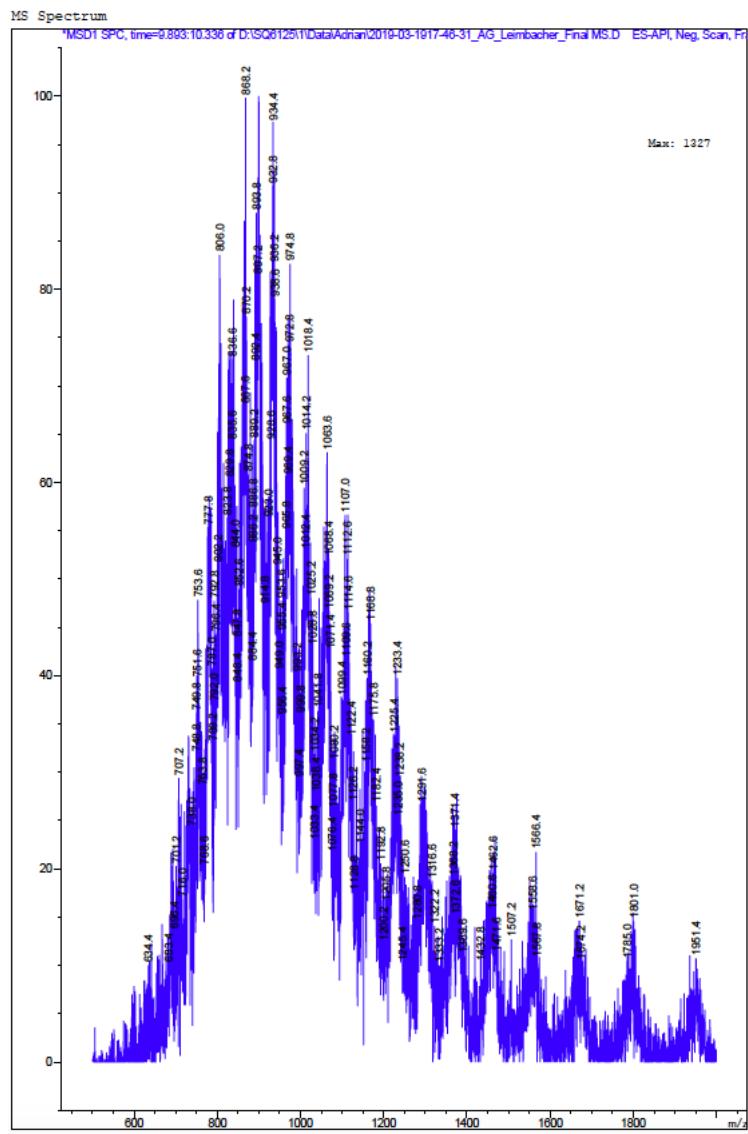


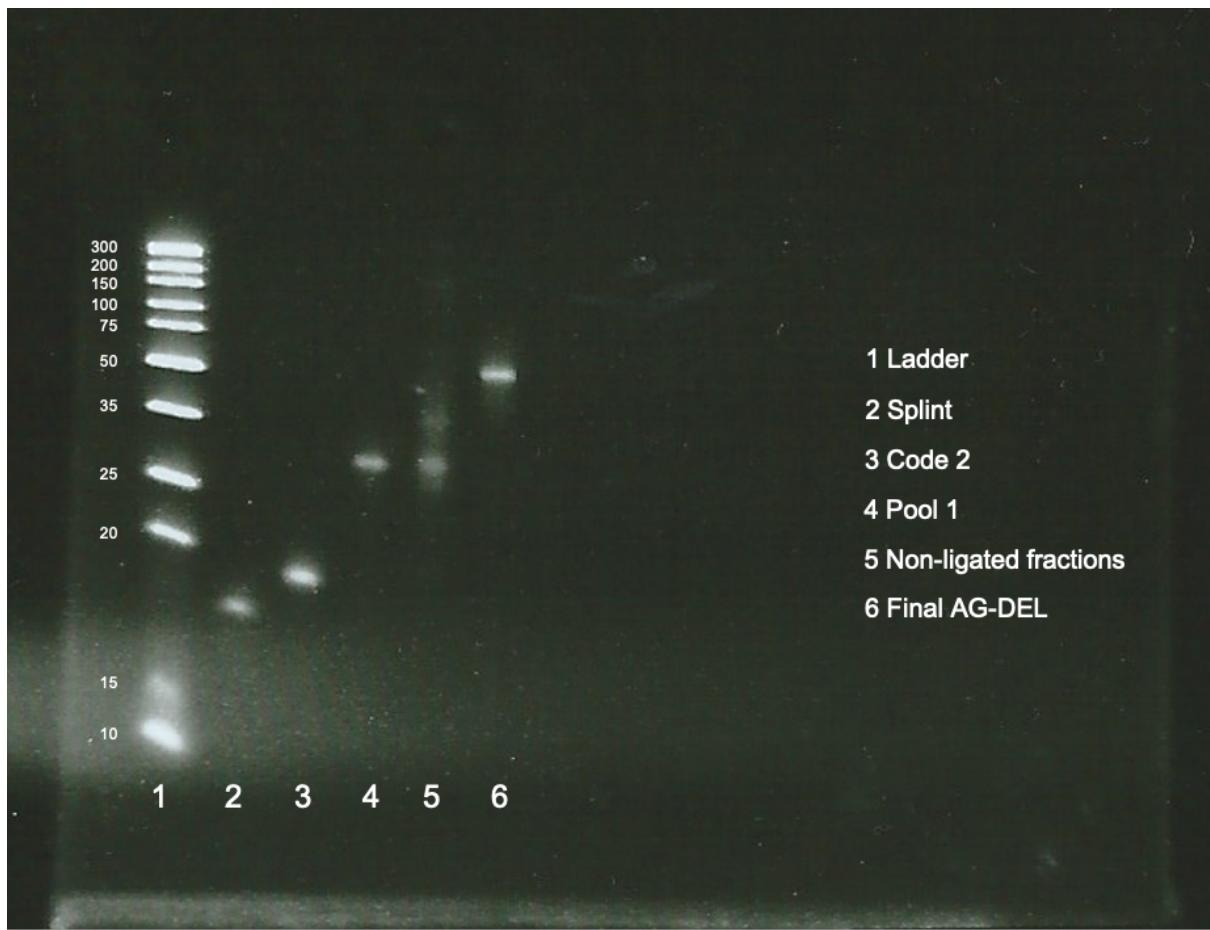
**Figure S12.** IE-HPLC purification traces of the AG-DEL Pool 2. Fractions P1C2 and P1C3 contain small molecules, fraction P1C4 contains the splint, fraction P1C5 contains the excess of different Codes 2, fractions P1C6 to P1C8 contain Code 2-non-ligated fractions, fractions P1C9 to P2A4 contain the final library pool.

#### d. Characterization of the final AG-DEL library



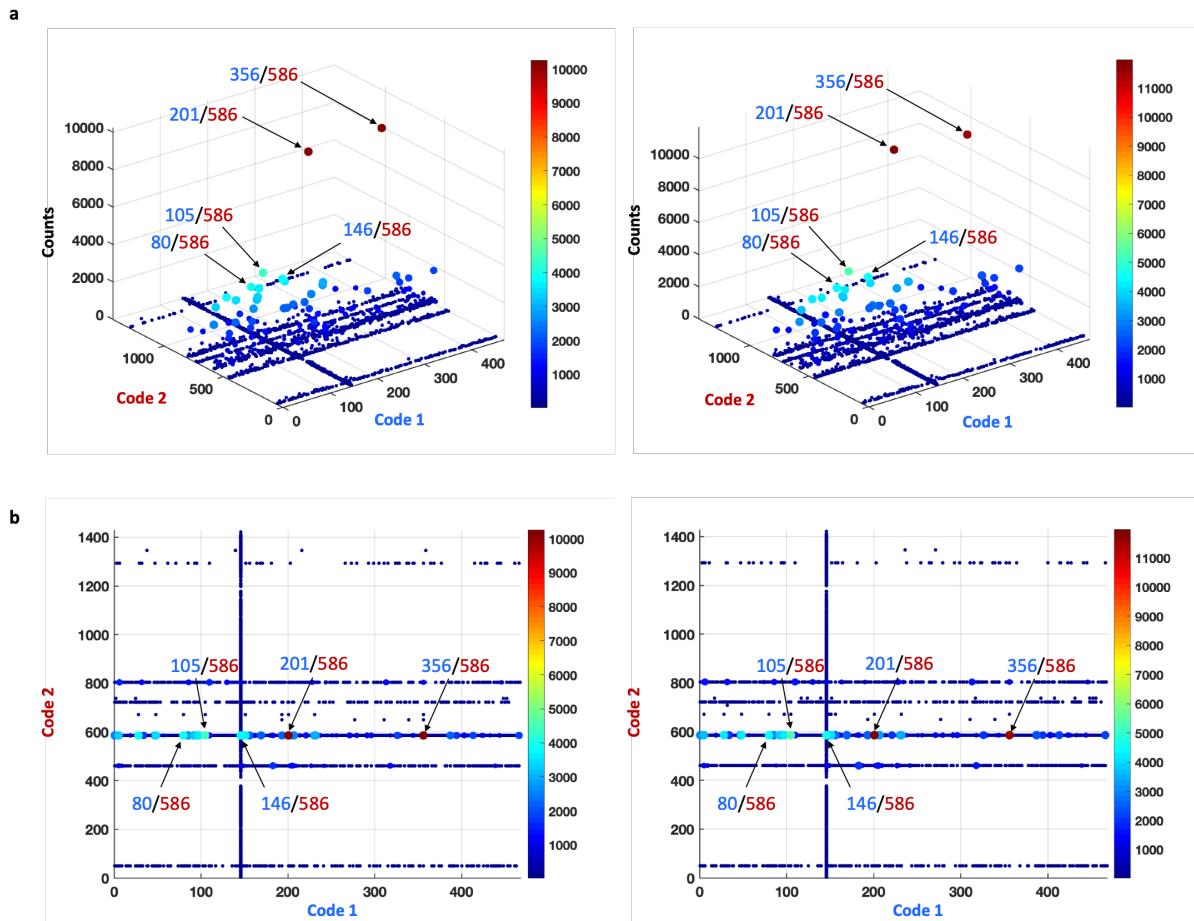
**Figure S13.** UV and MS traces of the final AG-DEL library





**Figure S15.** Gel electrophoresis of the final AG-DEL with the corresponding controls.

#### 4. Affinity Selection Fingerprints

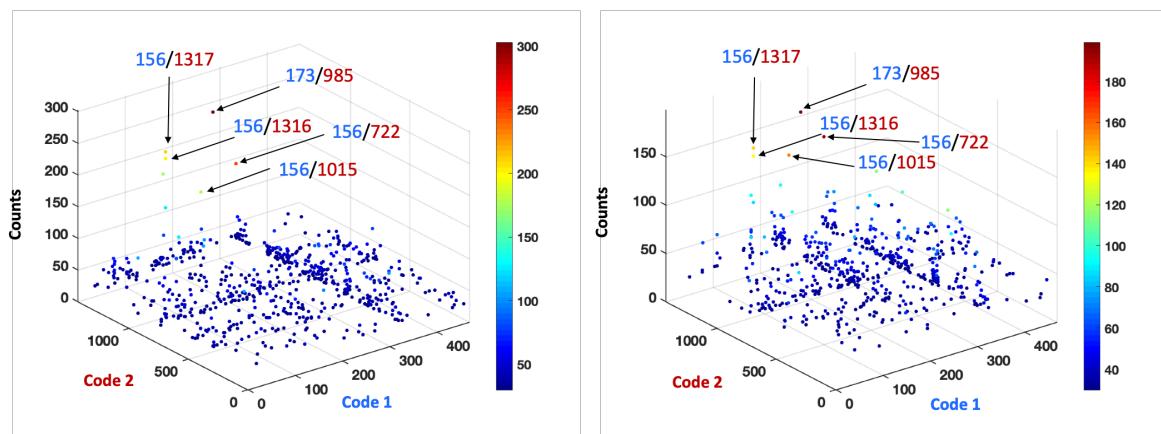
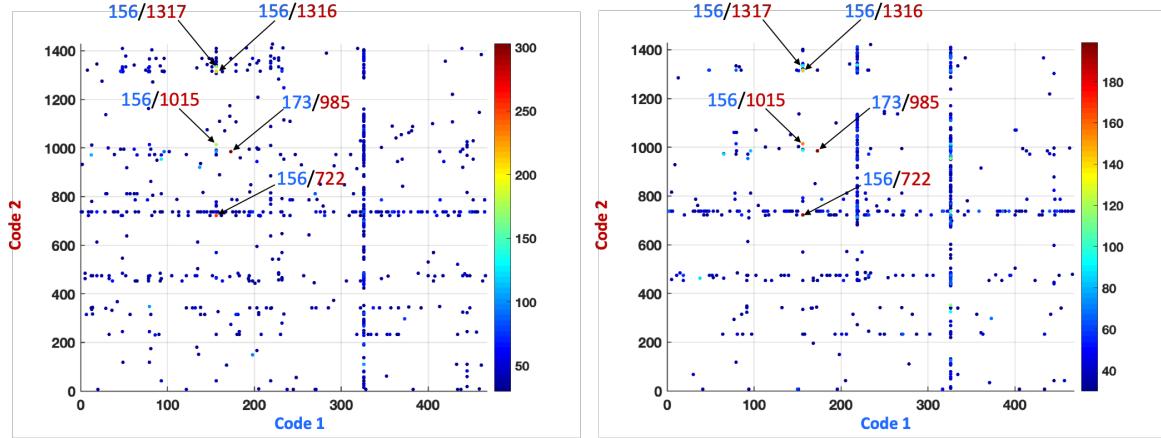


**c**

Counts (C)	Total Counts (T)	Library Size (S)	"Noise" (N)	Enrichment Factor (EF)	BB1	BB2
10258	581246	669240	0.86851653	11810.9439	356	586
10195	581246	669240	0.86851653	11738.4065	201	586
4456	581246	669240	0.86851653	5130.58746	105	586
3889	581246	669240	0.86851653	4477.75014	80	586
3805	581246	669240	0.86851653	4381.0335	146	586

$$EF = \frac{C}{N} = \frac{C}{(T/S)}$$

**Figure S16.** **a.** 3D plot of CAIX affinity selection fingerprint replicates. **b.** 2D plot of CAIX affinity selection fingerprint replicates, **c.** Table including the EF of each of the 5 most enriched combinations, calculated by the formula. Enrichment factors are consistent between both replicates

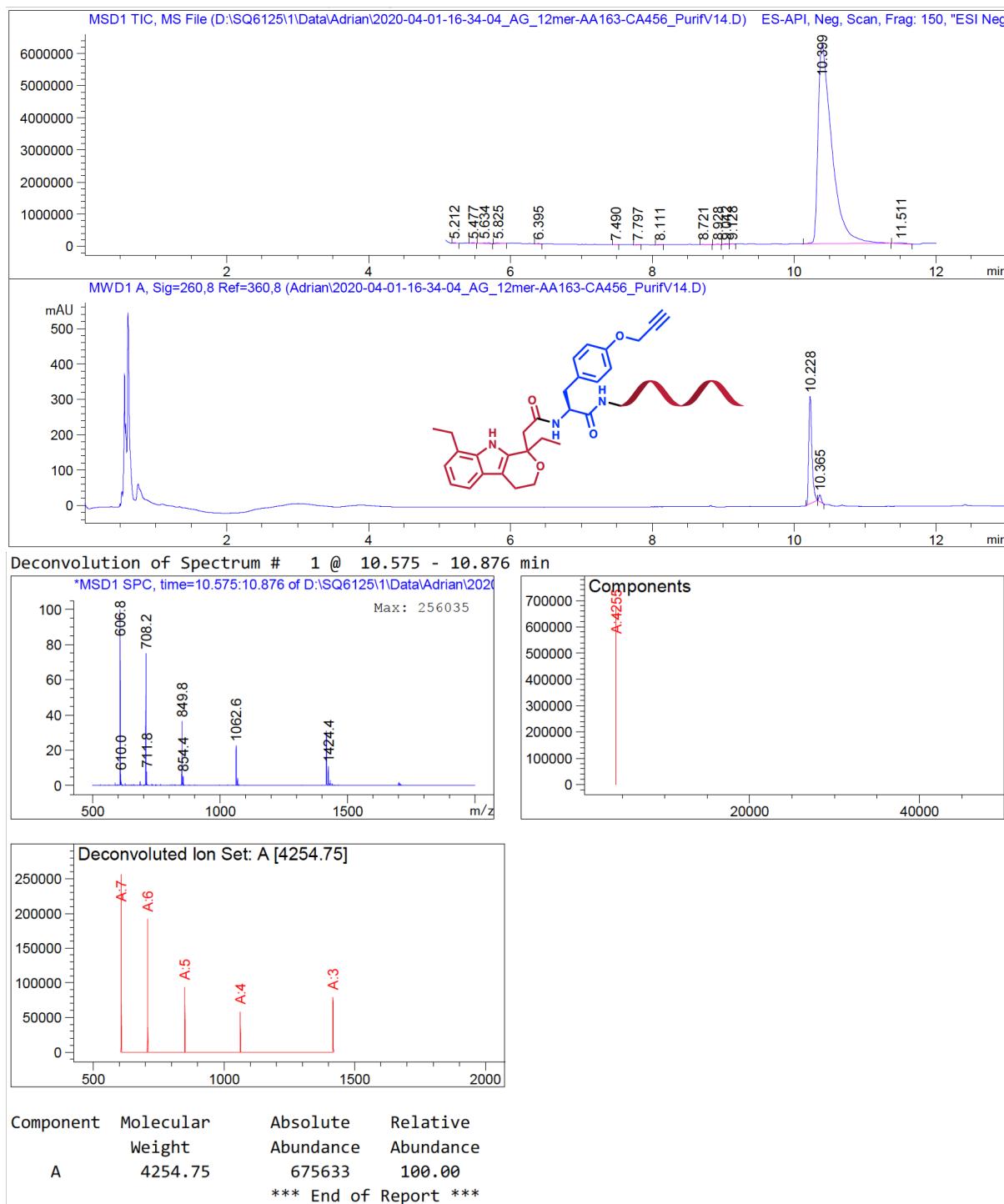
**a****b****c**

Counts (C)	Total Counts (T)	Library Size (S)	"Noise" (N)	Enrichment Factor (EF)	BB1	BB2
303	445983	669240	0.66640219	454.68038	173	985
251	445983	669240	0.66640219	376.649424	156	722
212	445983	669240	0.66640219	318.126207	156	1317
202	445983	669240	0.66640219	303.120253	156	1316
178	445983	669240	0.66640219	267.105966	156	1015

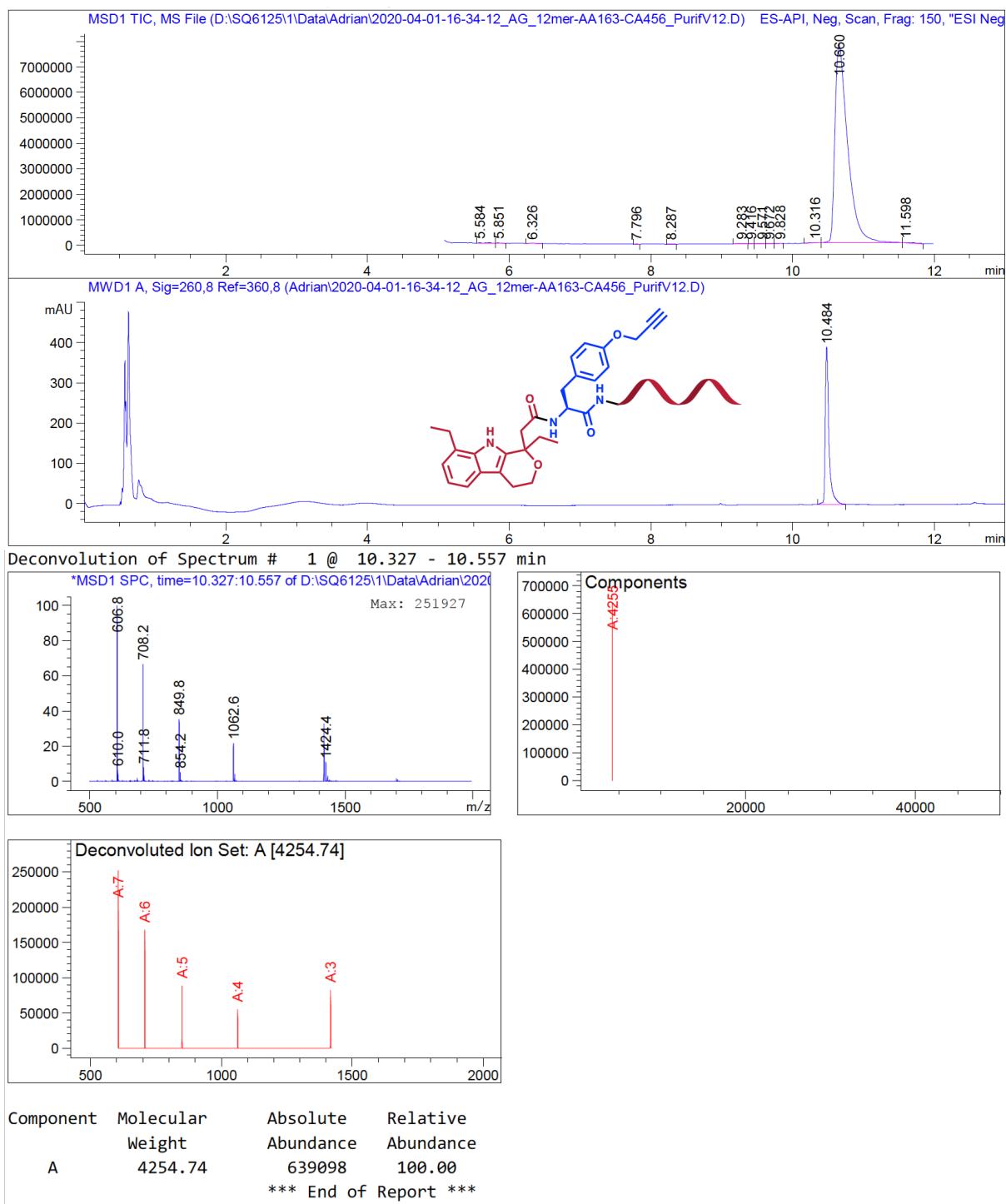
$$EF = \frac{C}{N} = \frac{C}{(T/S)}$$

**Figure S17.** **a.** 3D plot of L19-IL2 affinity selection fingerprint replicates. **b.** 2D plot of L19-IL2 affinity selection fingerprint replicates, **c.** Table including the EF of each of the 5 most enriched combinations, calculated by the formula. Enrichment factors are consistent between both replicates

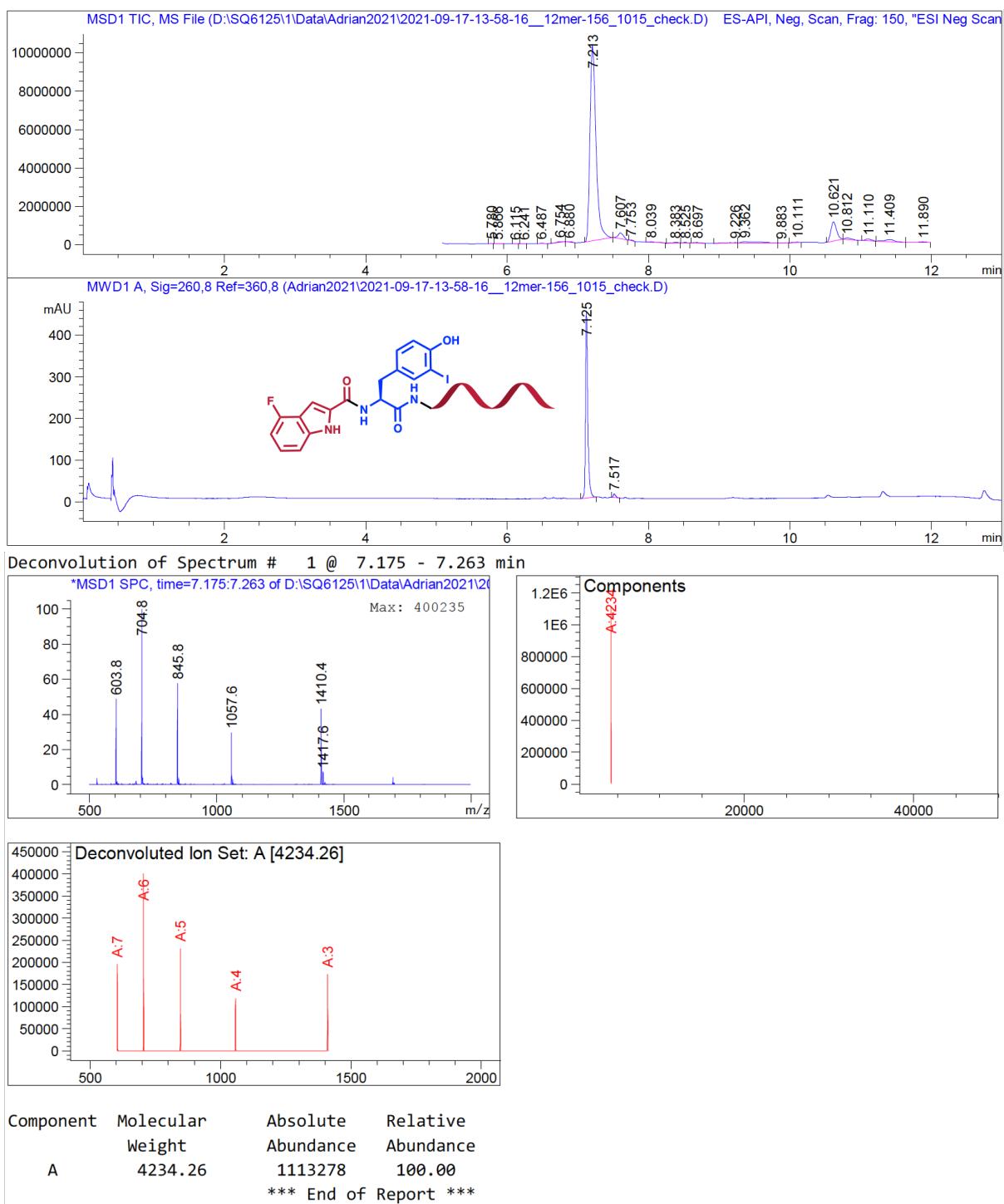
## 5. On-DNA resynthesis



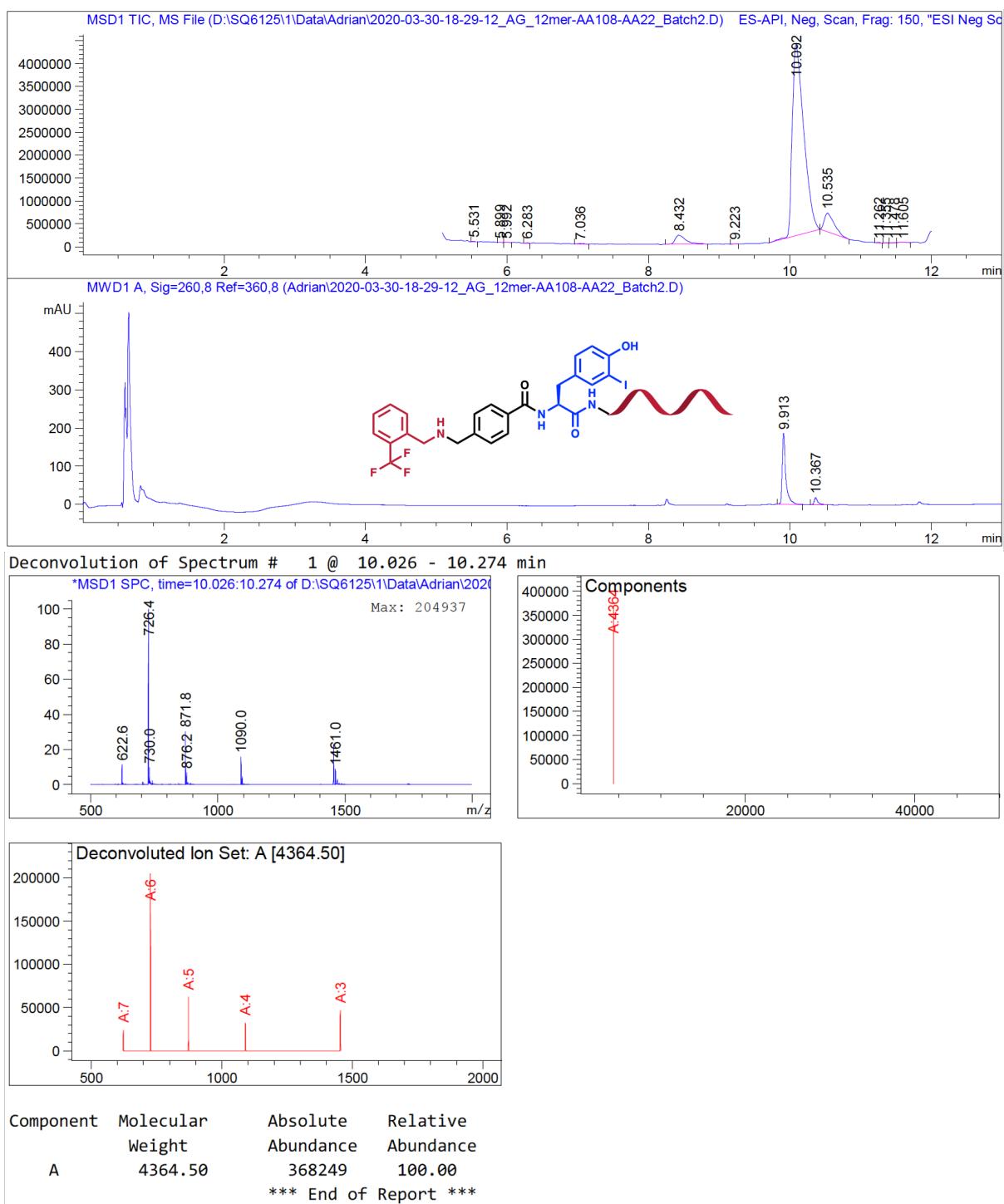
**Figure S18.** LC-ESI-MS chromatogram of compound **AG-173/985 Isomer 1**,  $t_R$ :10.228 min (DAD), 10.399 min (TIC). Expected mass 4255.18, observed 4254.75



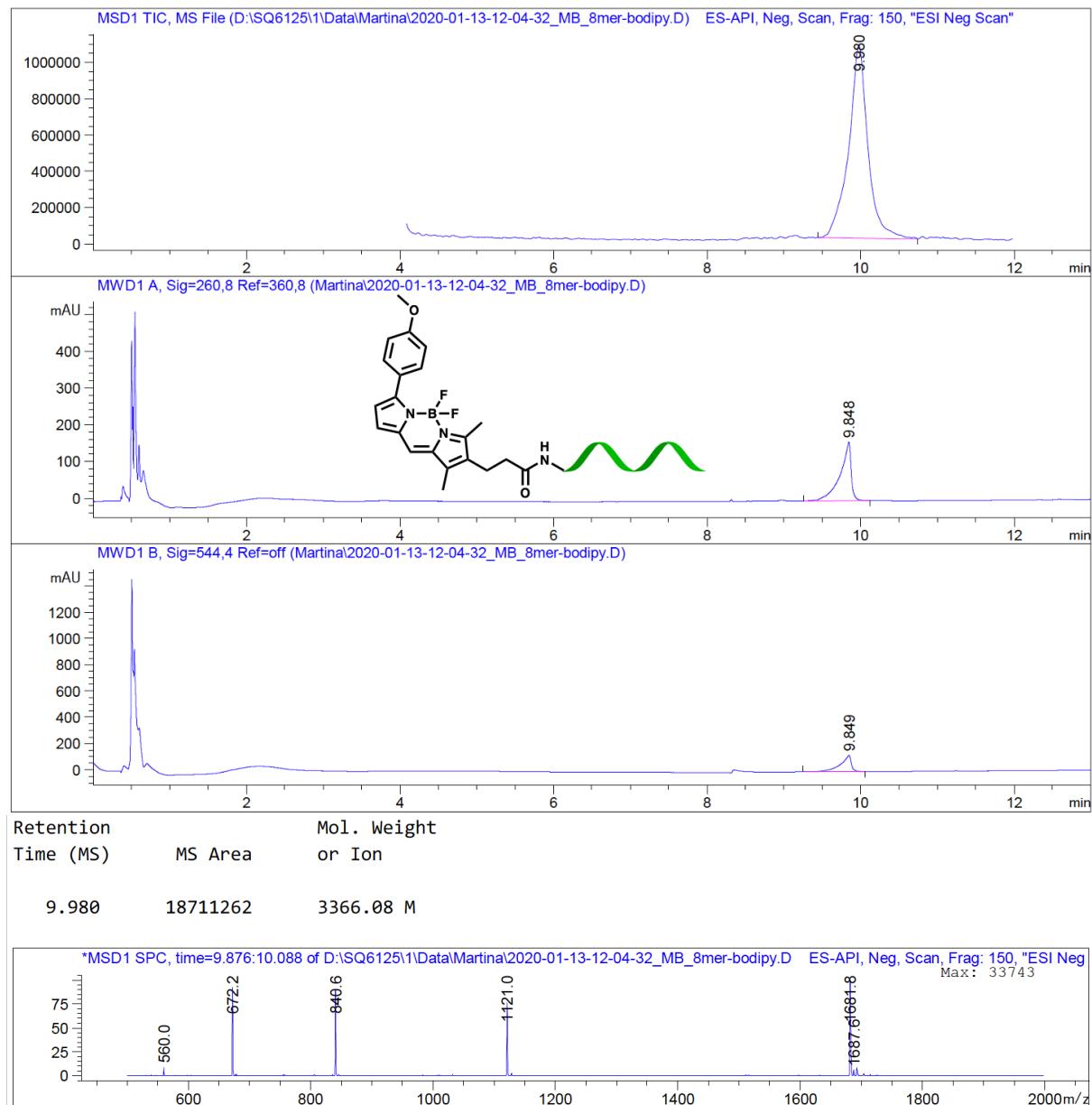
**Figure S19.** LC-ESI-MS chromatogram of compound **AG-173/985 Isomer 2**,  $t_R$ :10.484 min (DAD), 10.660 min (TIC). Expected mass 4255.18, observed 4254.74



**Figure S20.** LC-ESI-MS chromatogram of compound **AG-156/1015**,  $t_R$ :7.125 min (DAD), 7.213 min (TIC). Expected mass 4234.82, observed 4234.26

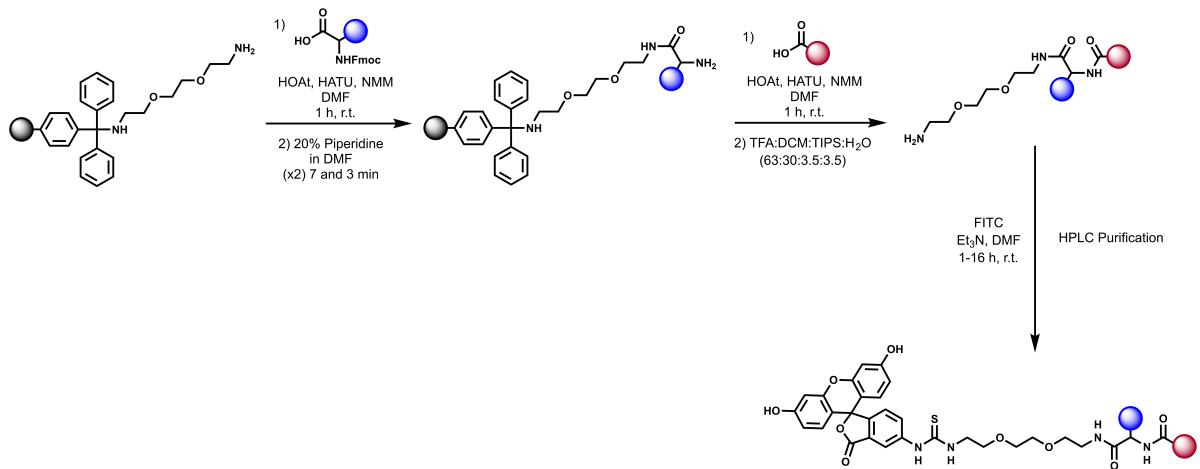


**Figure S21.** LC-ESI-MS chromatogram of compound AG-156/1317,  $t_R$ :9.913 min (DAD), 10.092 min (TIC). Expected mass 4364.96, observed 4364.50

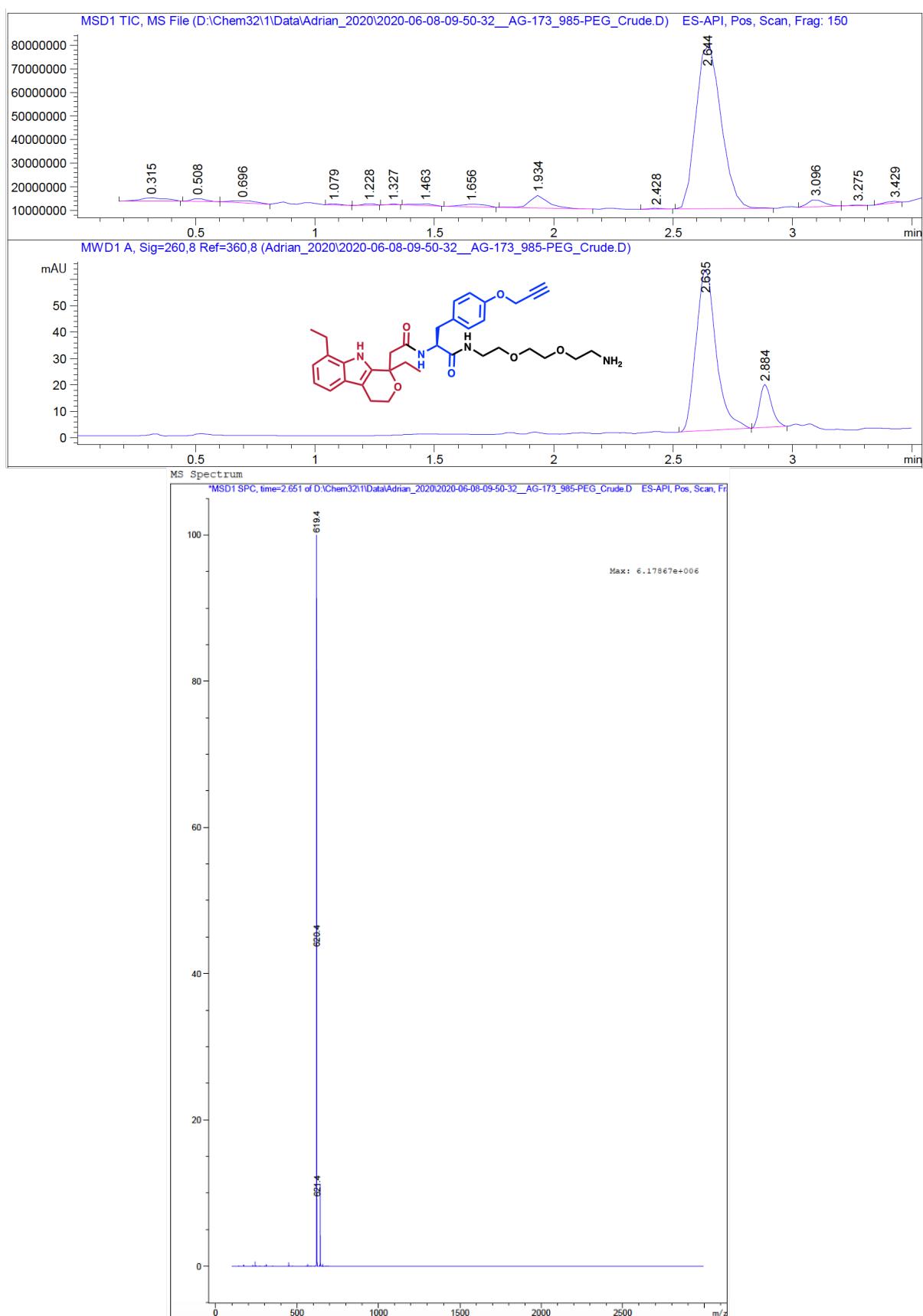


**Figure S22.** LC-ESI-MS chromatogram of compound **8mer LNA-BODIPY**,  $t_r$ :9.848 min (DAD), 9.980 min (TIC). Expected mass 3366.05, observed 3366.08

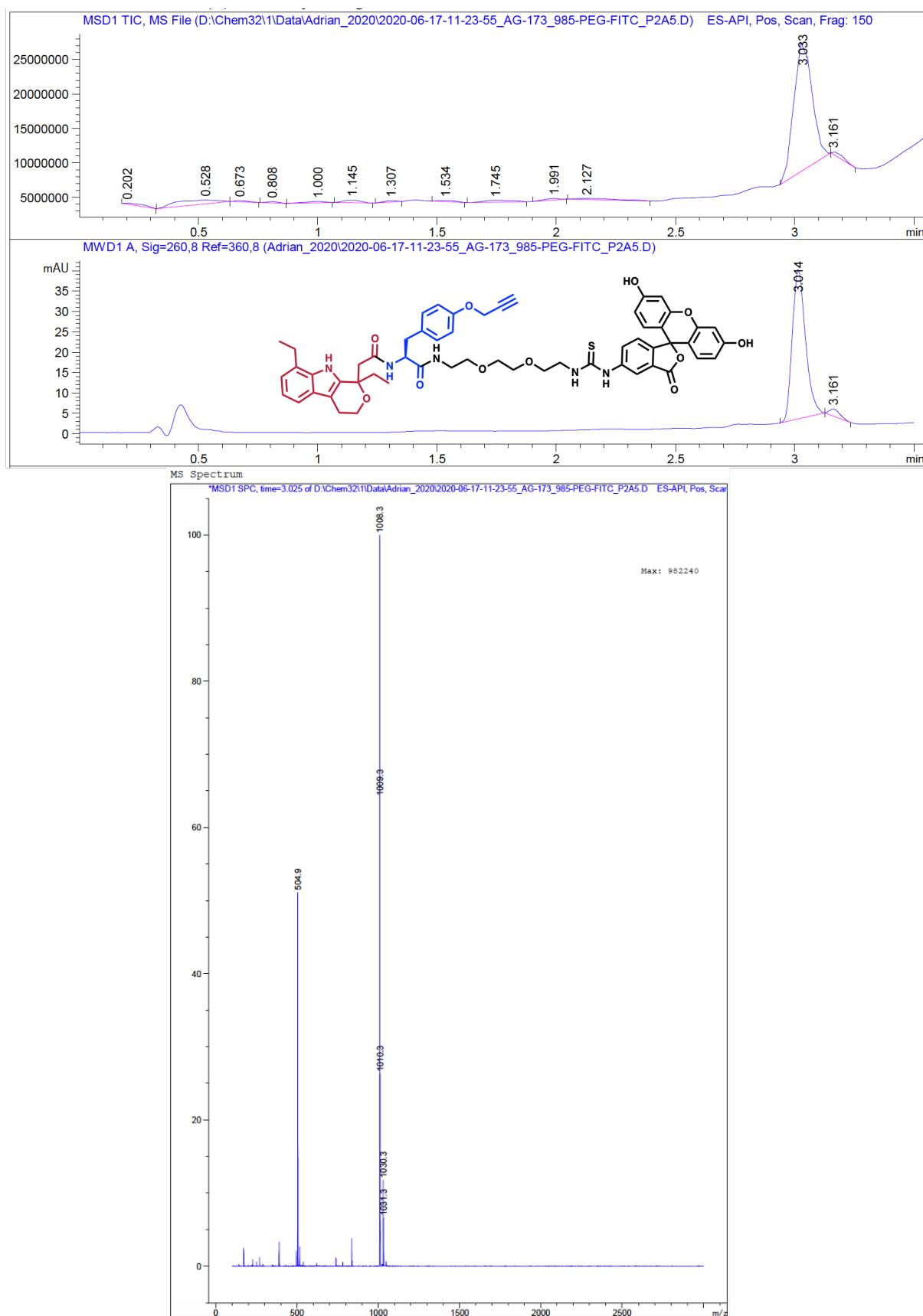
## 6. Off-DNA resynthesis



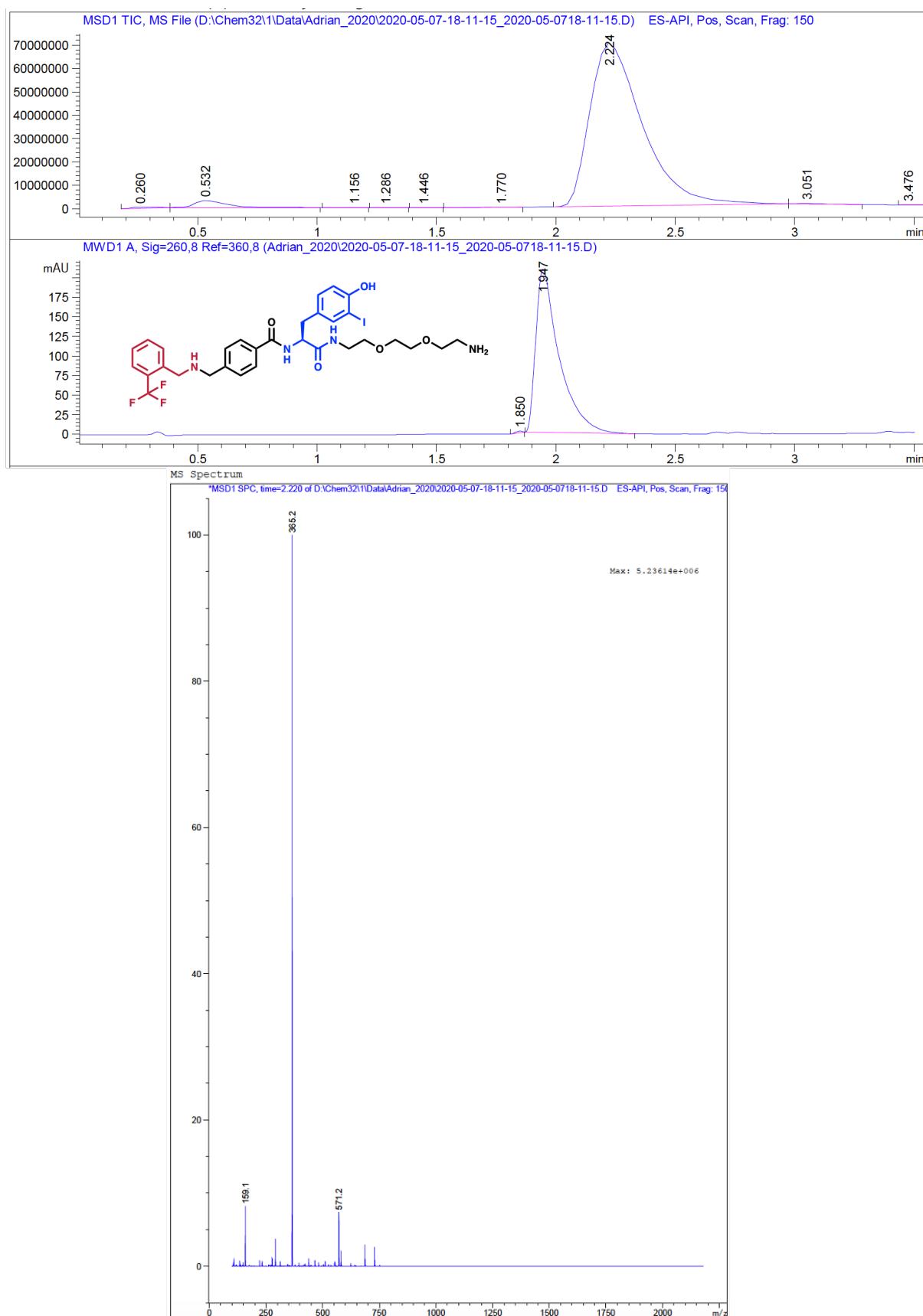
**Scheme S1.** Synthetic route for the synthesis of compounds **AG-173/985**, **AG-156/1317** and **AG-156/1015**. The grey ball represents the solid support, the blue ball represents the building block 1 and the red ball represents the building block 2



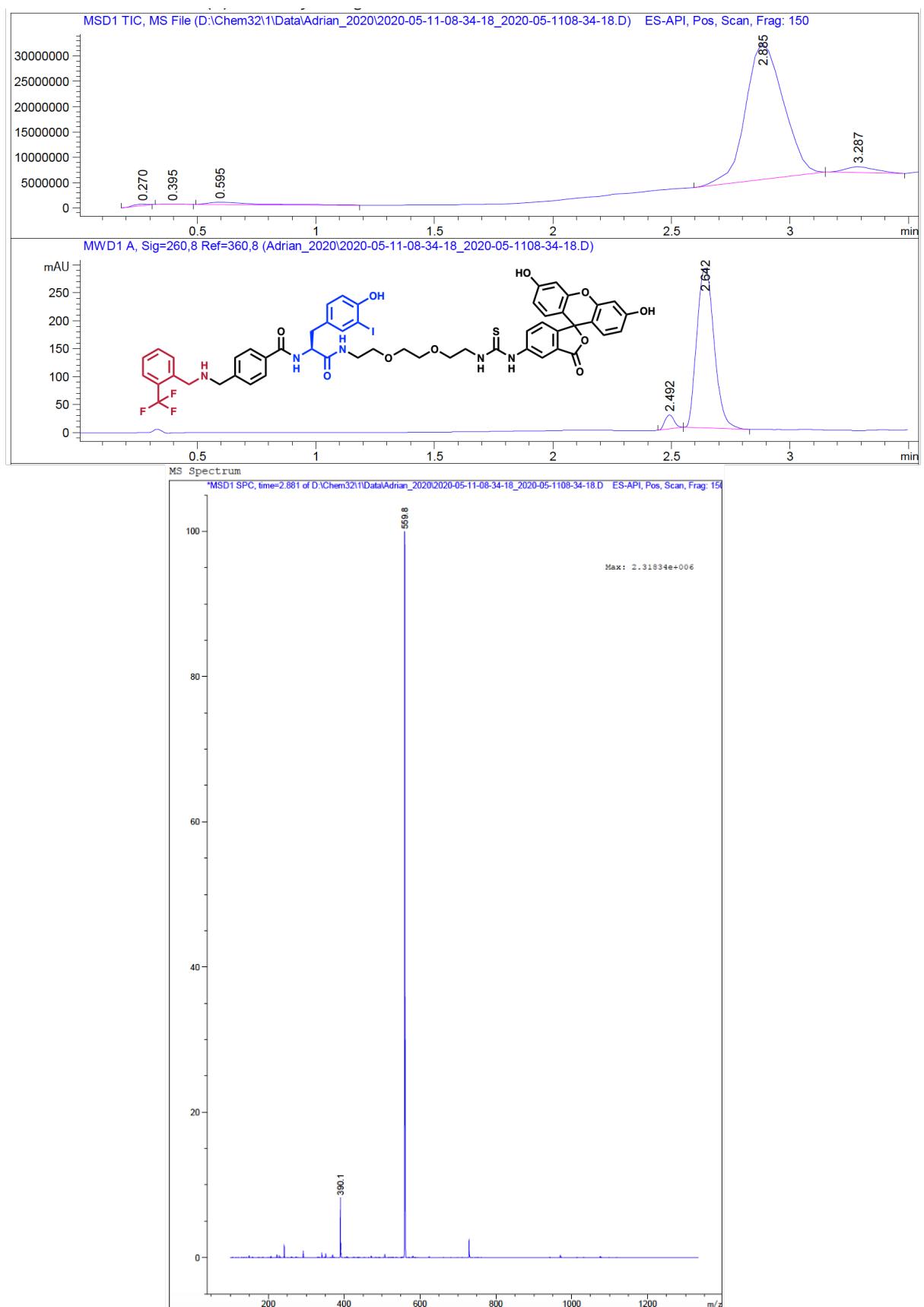
**Figure S23.** LC-ESI-MS chromatogram of compound AG-173/985-PEG<sub>2</sub>-NH<sub>2</sub>,  $t_R$ :2.635 min (DAD), 2.644 min (TIC). Expected mass 618.3, observed 619.4



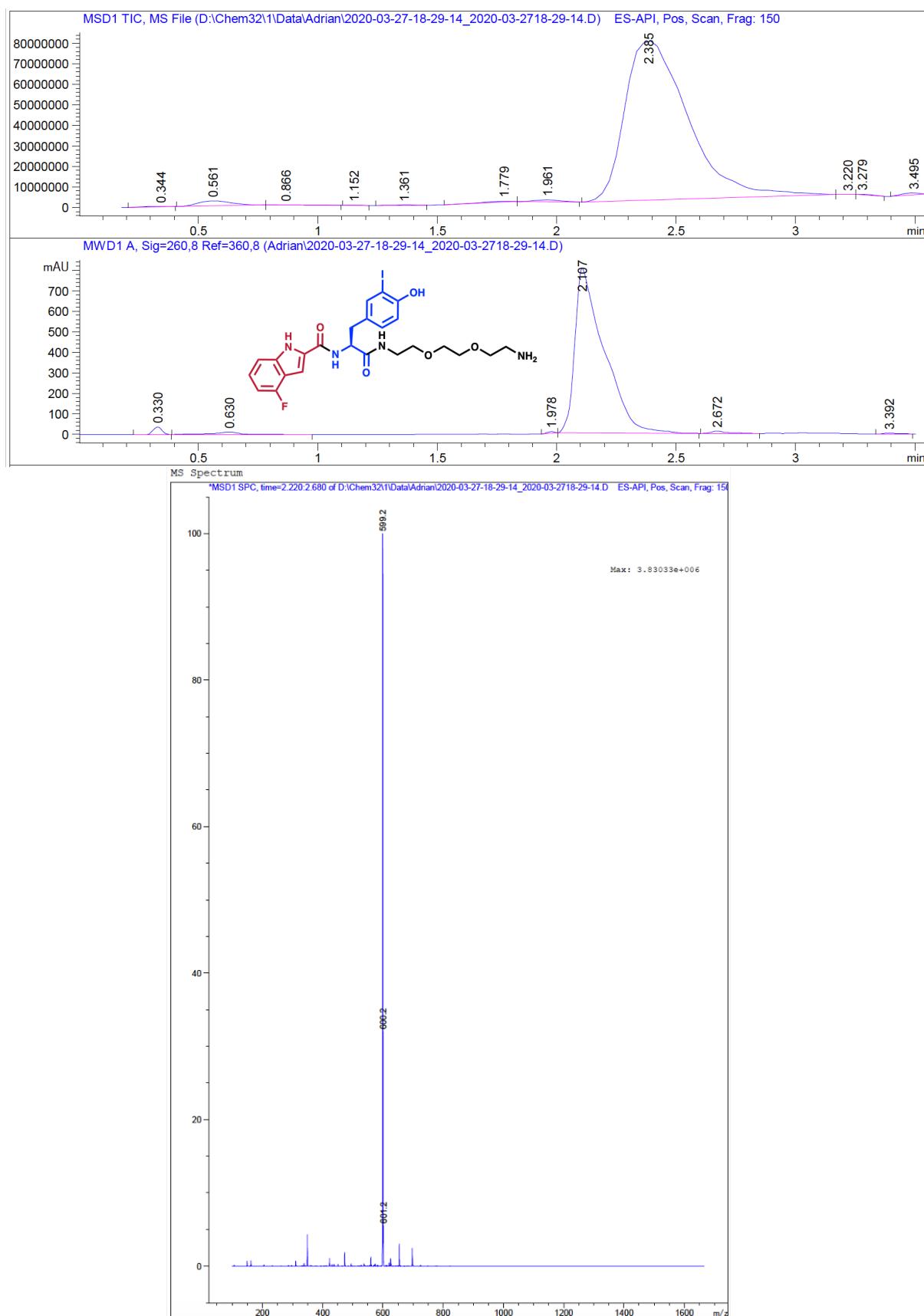
**Figure S24.** LC-ESI-MS chromatogram of compound **AG-173/985**,  $t_R$ :3.014 min (DAD), 3.033 min (TIC). Expected mass 1007.4, observed 1008.3



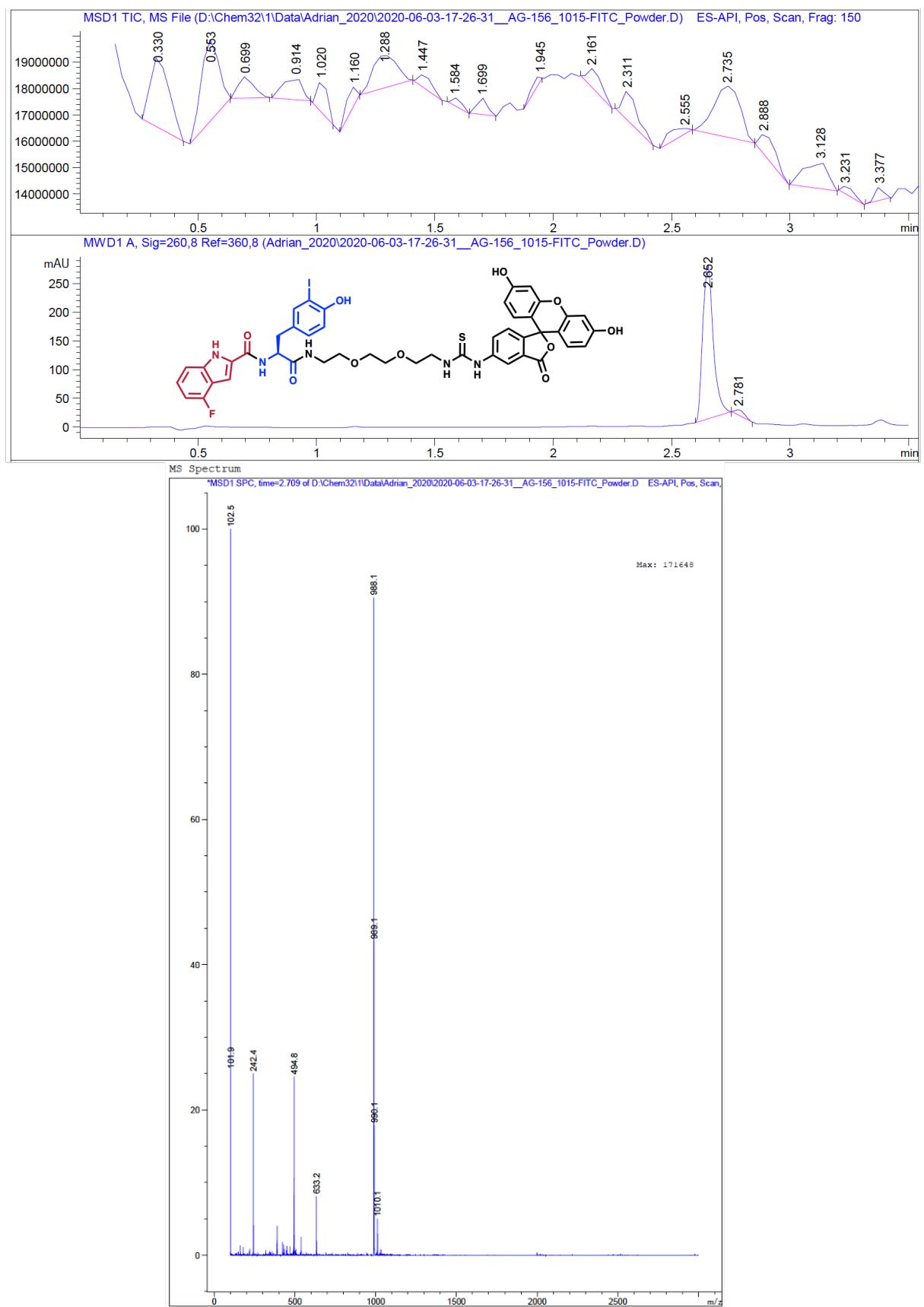
**Figure S25.** LC-ESI-MS chromatogram of compound **AG-156/1317-PEG<sub>2</sub>-NH<sub>2</sub>**, *t<sub>R</sub>*:1.947 min (DAD), 2.224 min (TIC). Expected mass 728.2, observed 365.2 (*z*=2) corresponding to 729.4



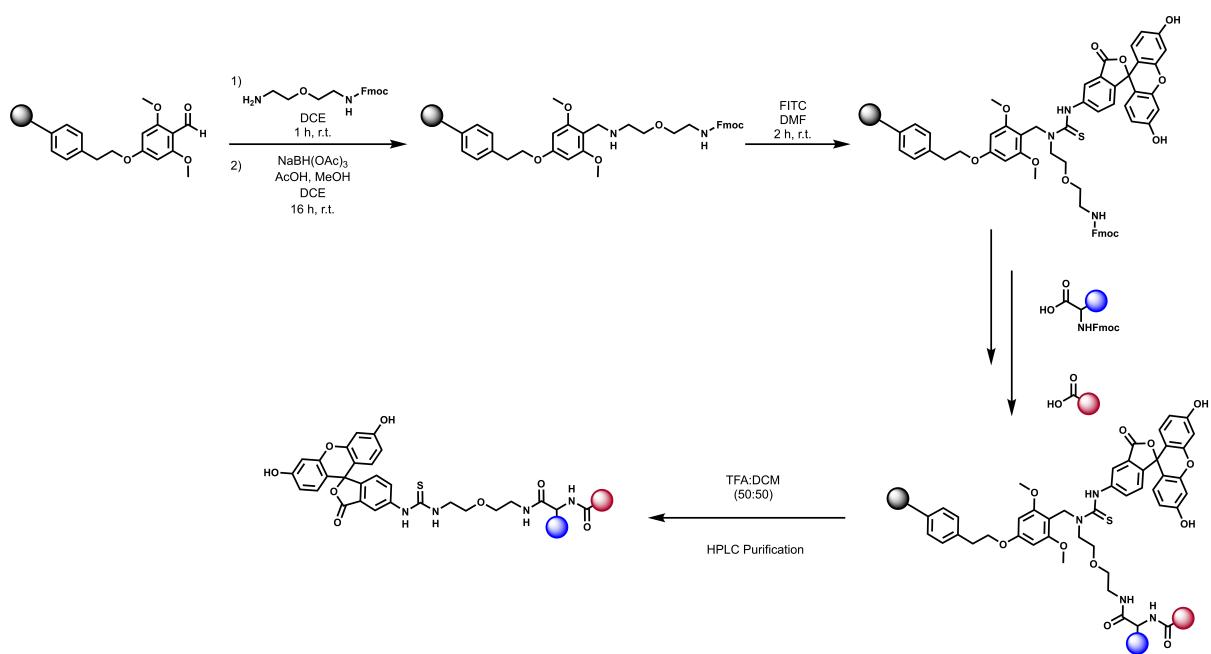
**Figure S26.** LC-ESI-MS chromatogram of compound **AG-156/1317**,  $t_R$ :2.642 min (DAD), 2.885 min (TIC). Expected mass 1117.2, observed 559.8 ( $z=2$ ) corresponding to 1118.6



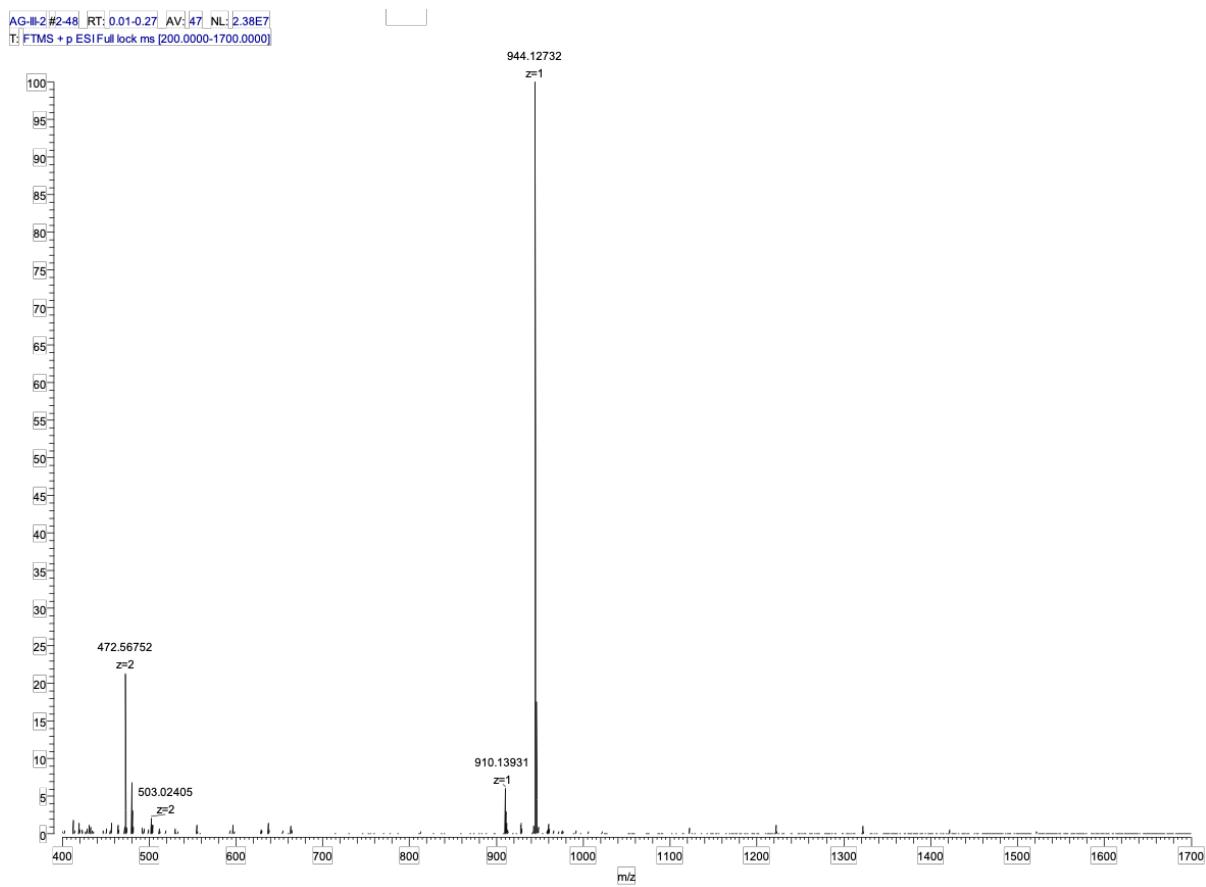
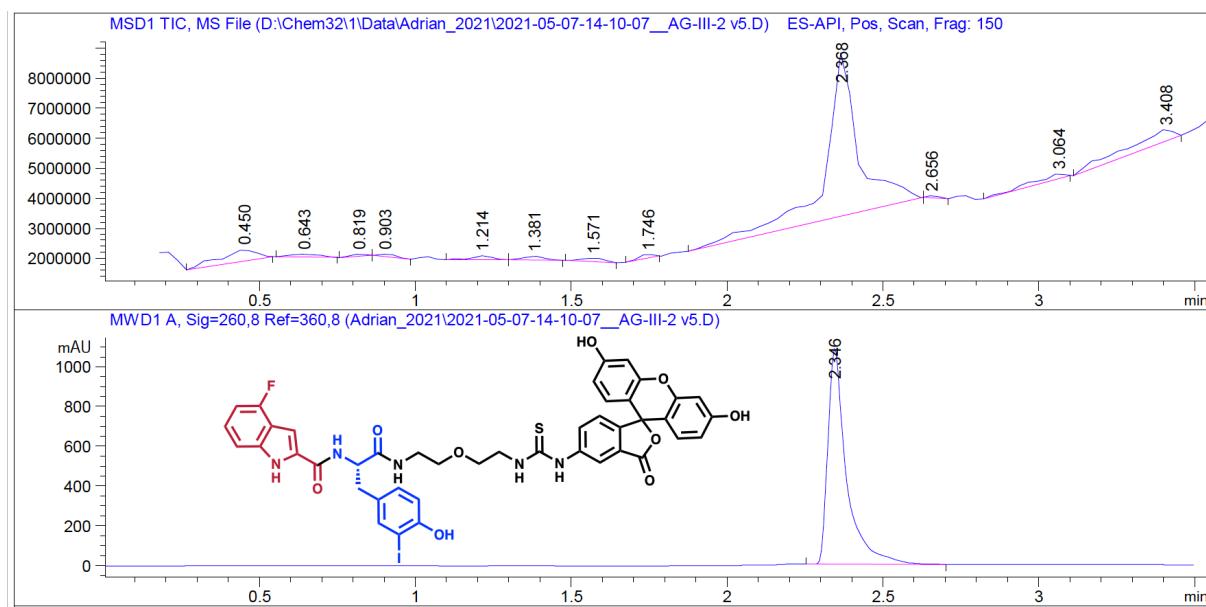
**Figure S27.** LC-ESI-MS chromatogram of compound **AG-156/1015-PEG<sub>2</sub>-NH<sub>2</sub>**,  $t_R$ :2.107 min (DAD), 2.385 min (TIC). Expected mass 598.1, observed 599.2



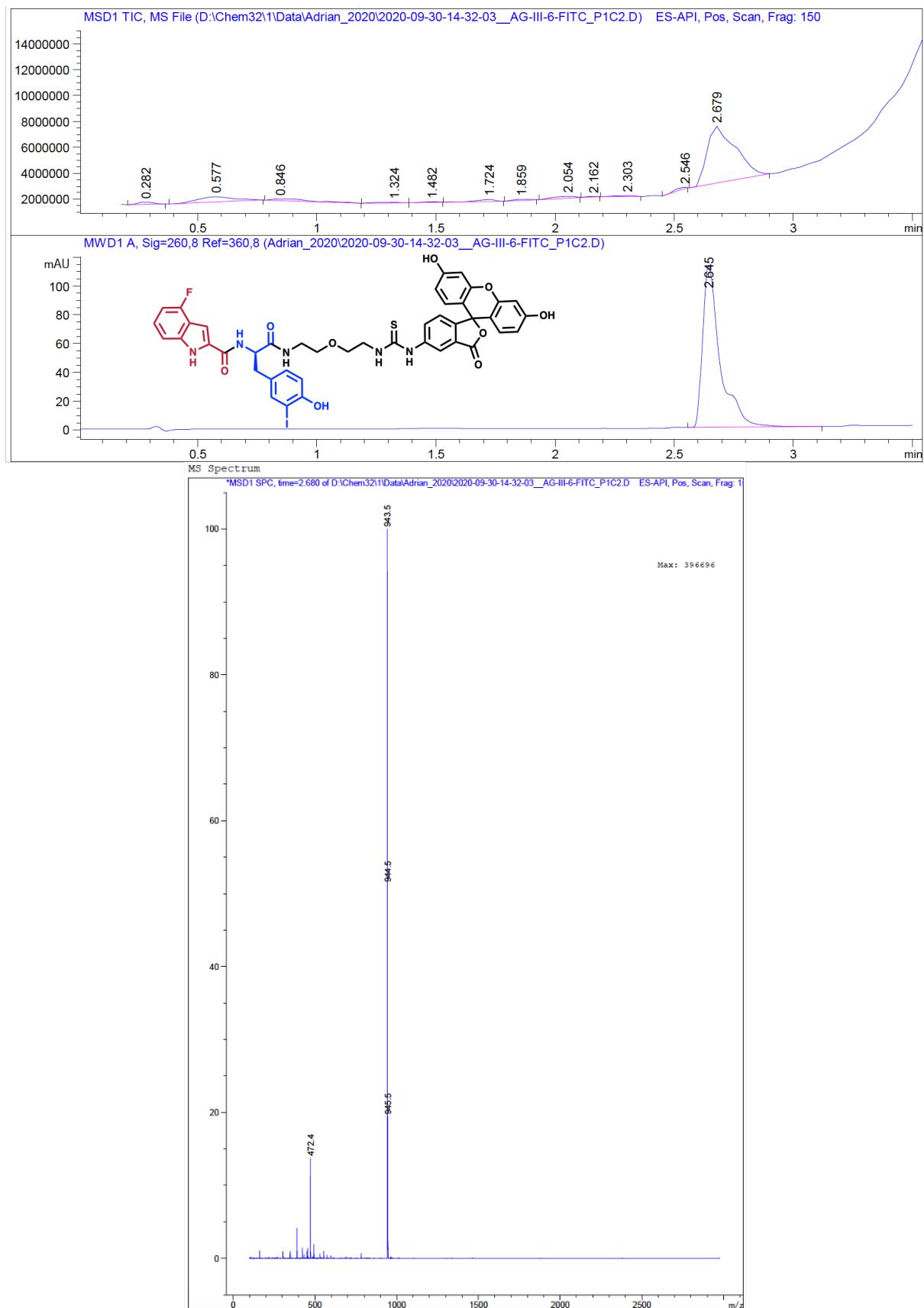
**Figure S28.** LC-ESI-MS chromatogram of compound AG-156/1015,  $t_R$ :2.652 min (DAD), 2.735 min (TIC). Expected mass 987.1, observed 988.1



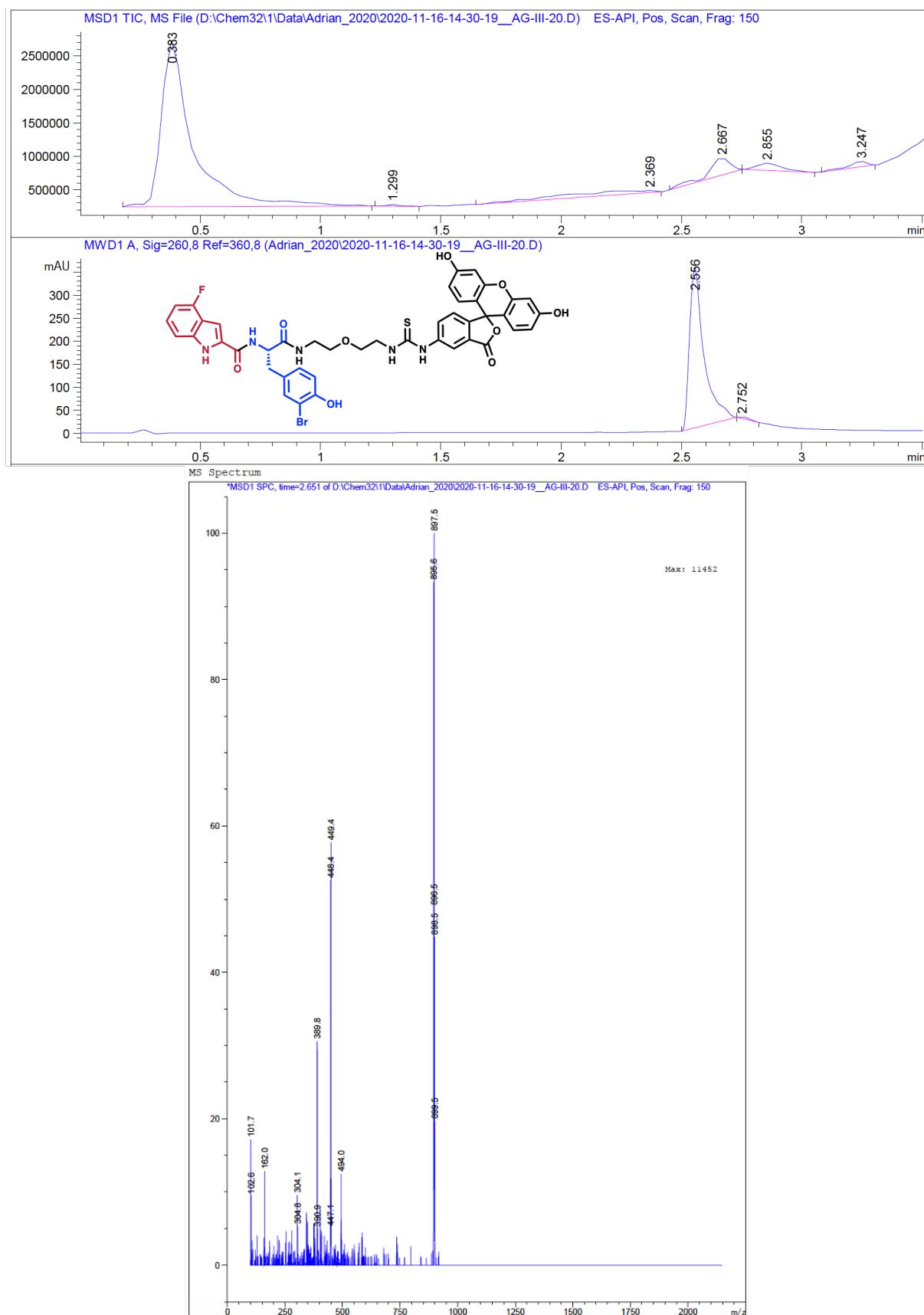
**Scheme S2.** Synthetic route for the synthesis of compounds **1-17** and **Ro26-4550**. The grey ball represents the solid support, the blue ball represents the building block 1 and the red ball represents the building block 2



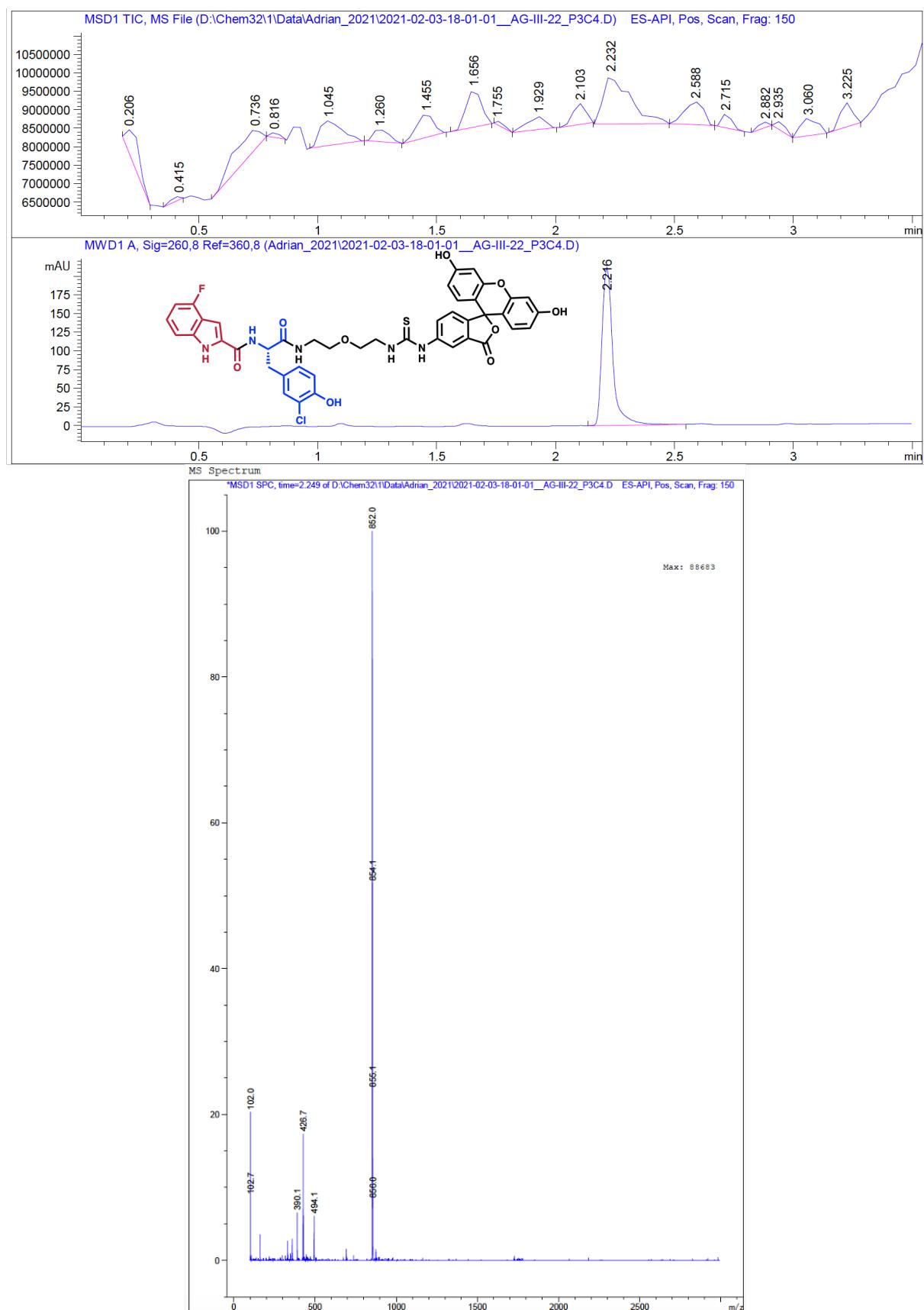
**Figure S29.** LC-HRMS chromatogram of compound **1**,  $t_R$ :2.346 min (DAD). Expected mass 943.11842, observed 944.12732



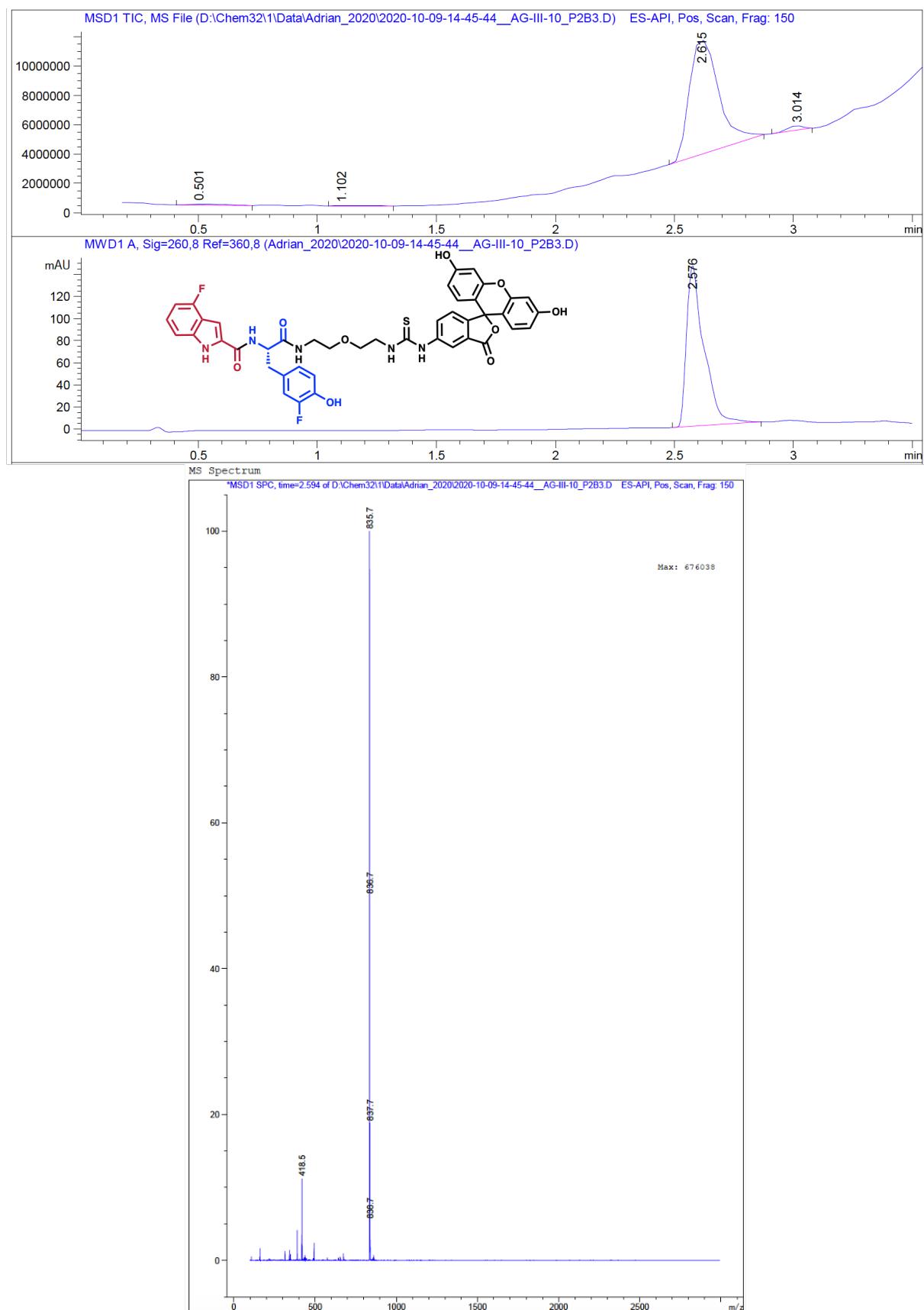
**Figure S30.** LC-ESI-MS chromatogram of compound **2**,  $t_R$ : 2.645 min (DAD), 2.679 min (TIC). Expected mass 943.1, observed 943.5



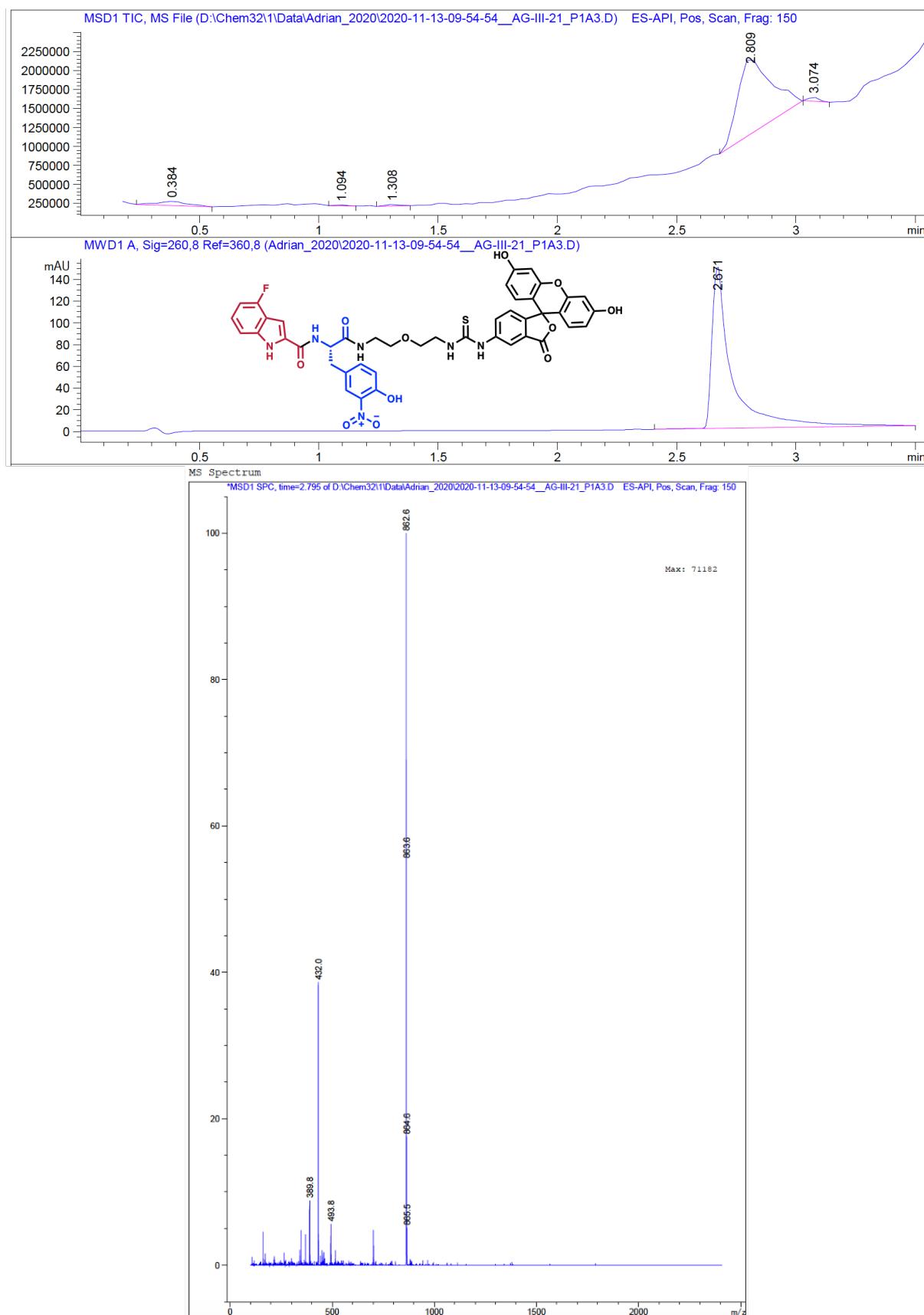
**Figure S31.** LC-ESI-MS chromatogram of compound **3**,  $t_R$ :2.556 min (DAD), 2.667 min (TIC). Expected mass 895.1, observed 897.5



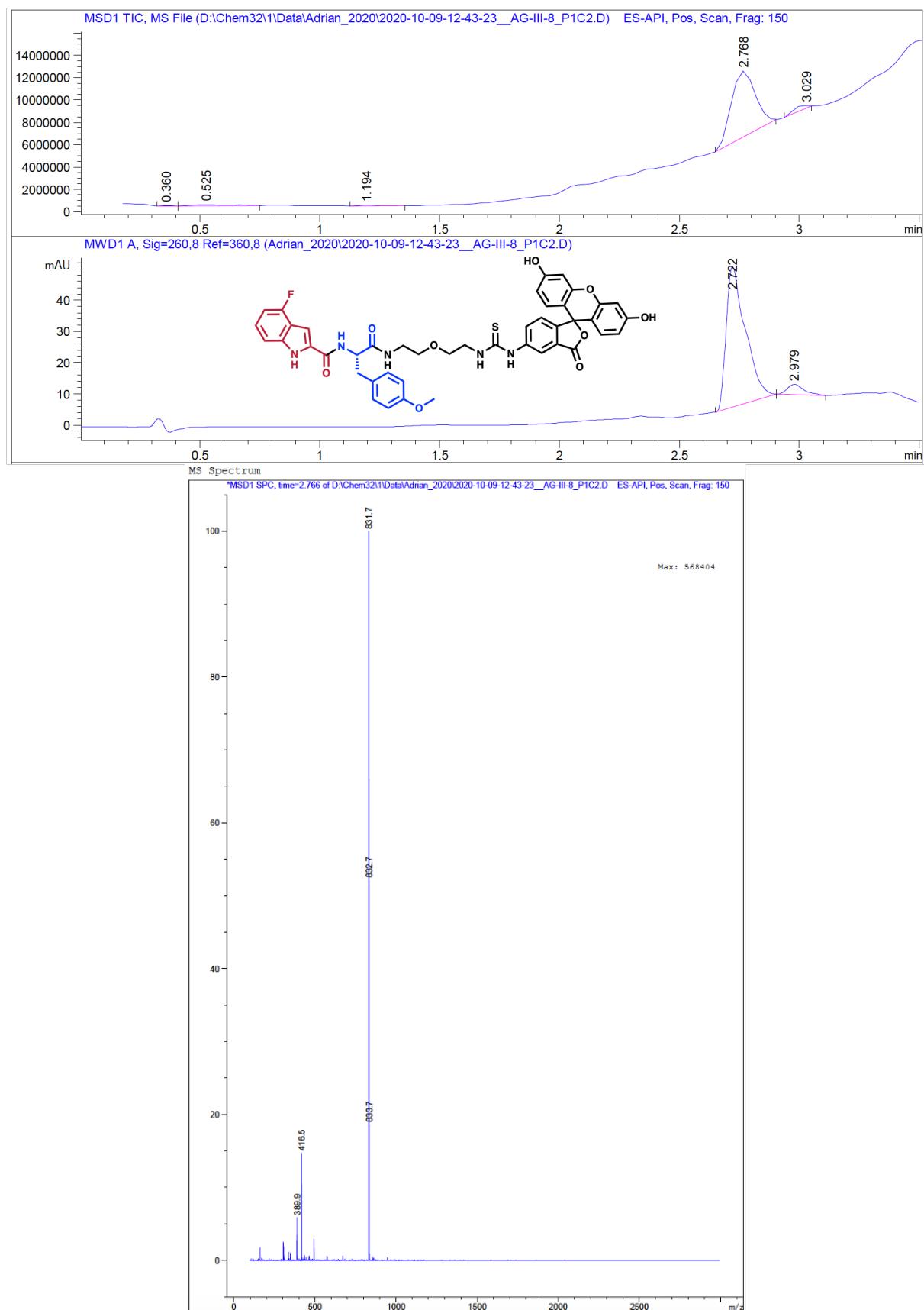
**Figure S32.** LC-ESI-MS chromatogram of compound **4**,  $t_R$ :2.216 min (DAD), 2.232 min (TIC). Expected mass 851.2, observed 852.0



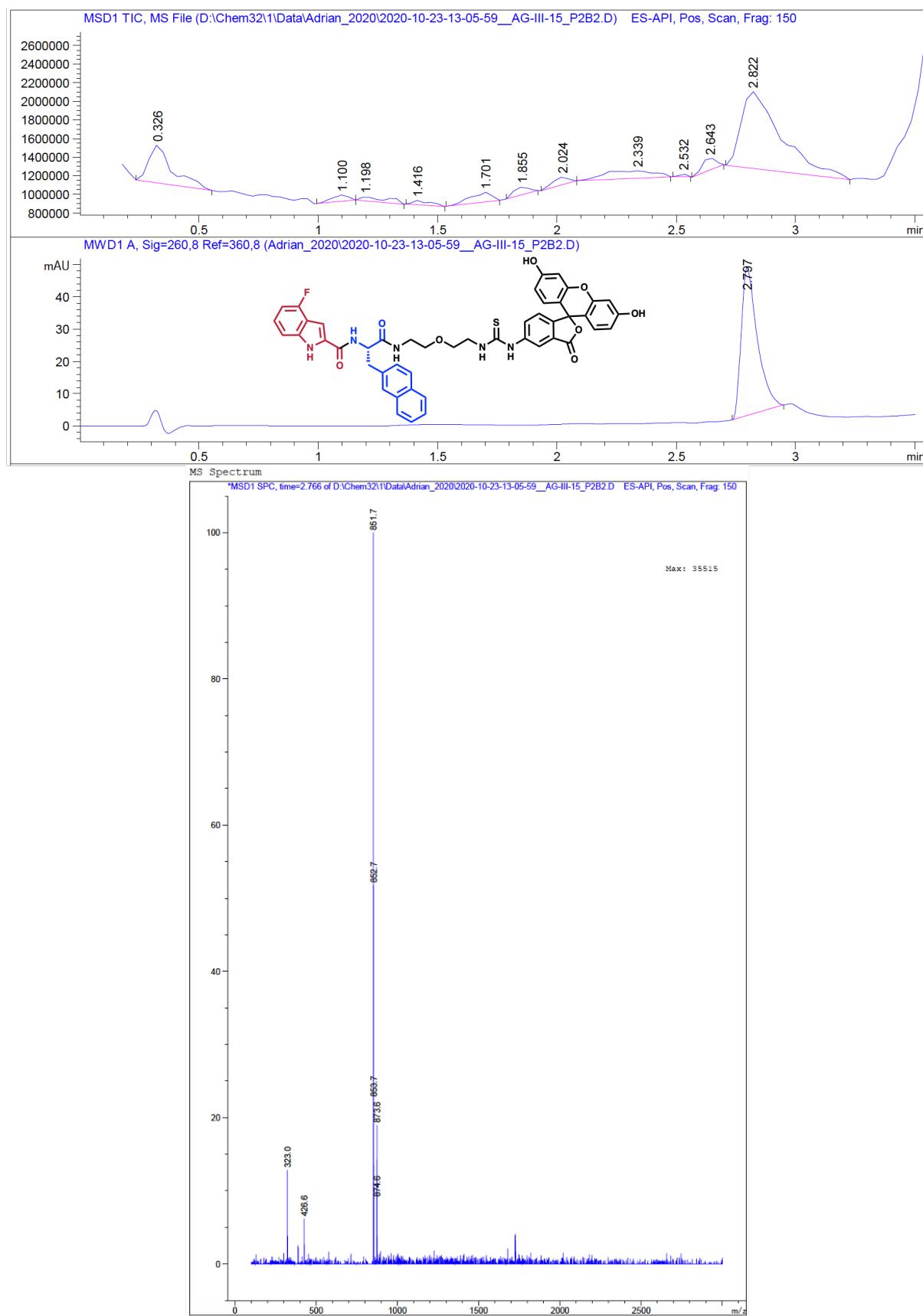
**Figure S33.** LC-ESI-MS chromatogram of compound **5**,  $t_R$ :2.576 min (DAD), 2.615 min (TIC). Expected mass 835.2, observed 835.7



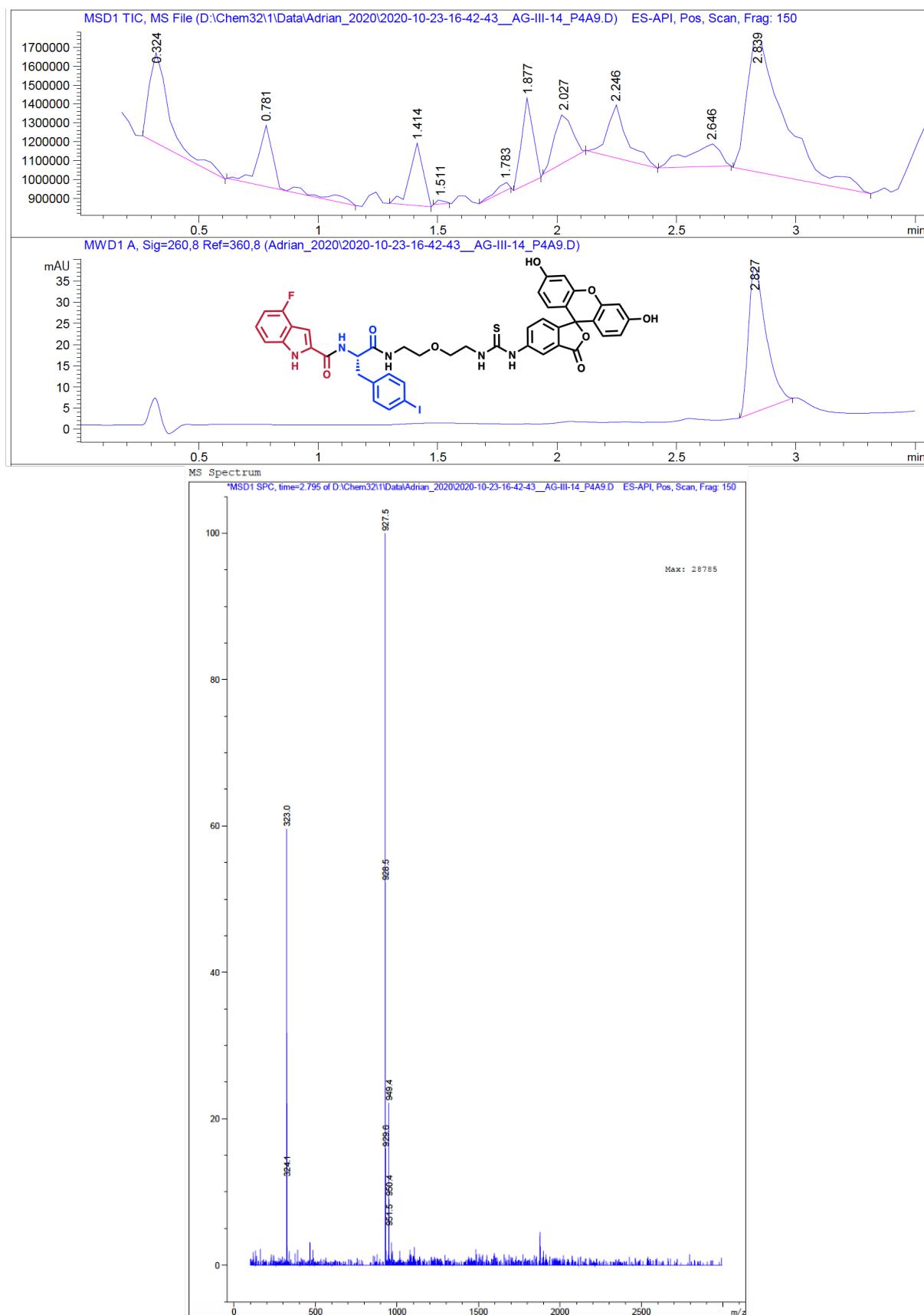
**Figure S34.** LC-ESI-MS chromatogram of compound **6**,  $t_R$ :2.671 min (DAD), 2.809 min (TIC). Expected mass 862.2, observed 862.6



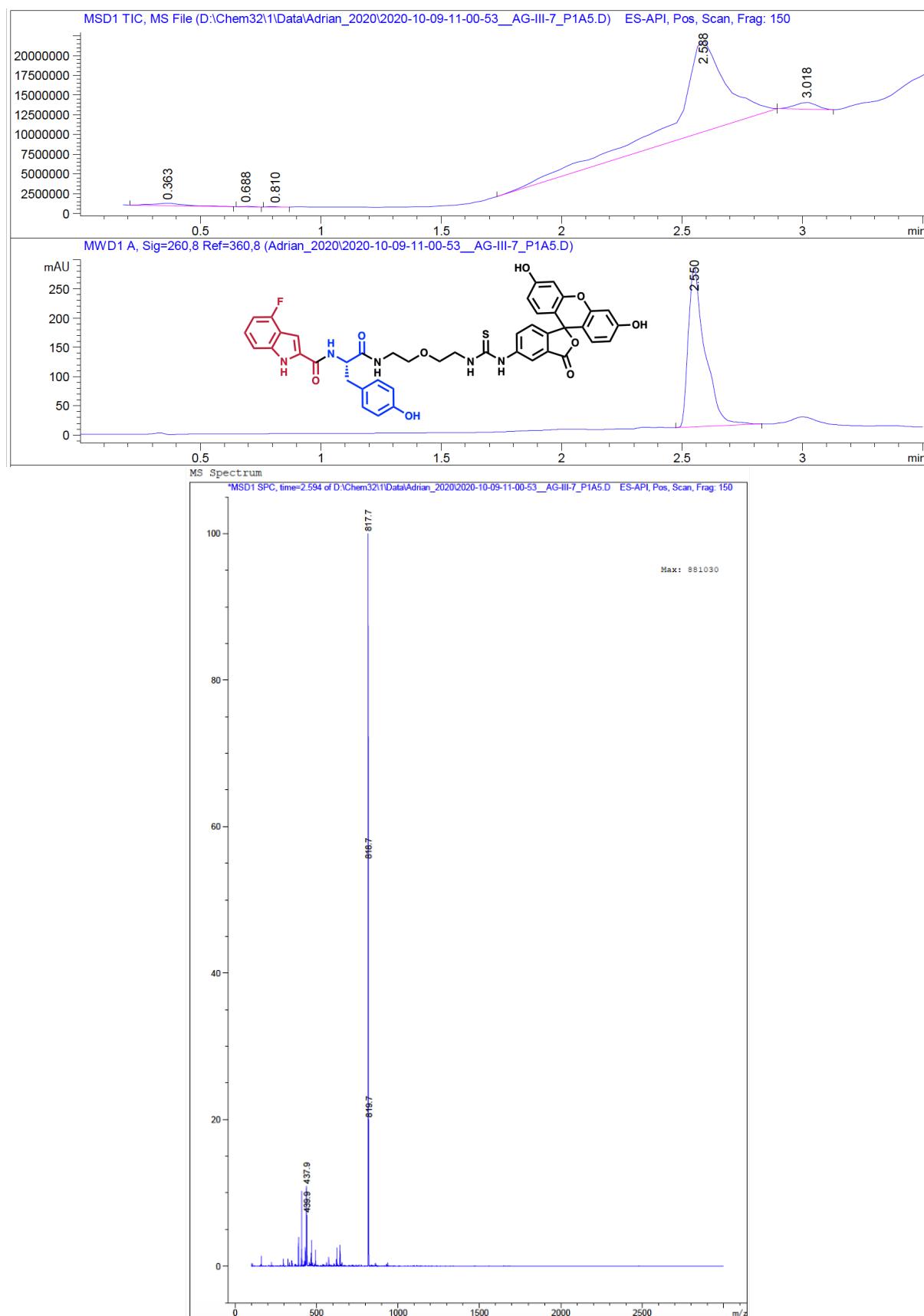
**Figure S35.** LC-ESI-MS chromatogram of compound 7,  $t_R$ :2.722 min (DAD), 2.768 min (TIC). Expected mass 831.2, observed 831.7



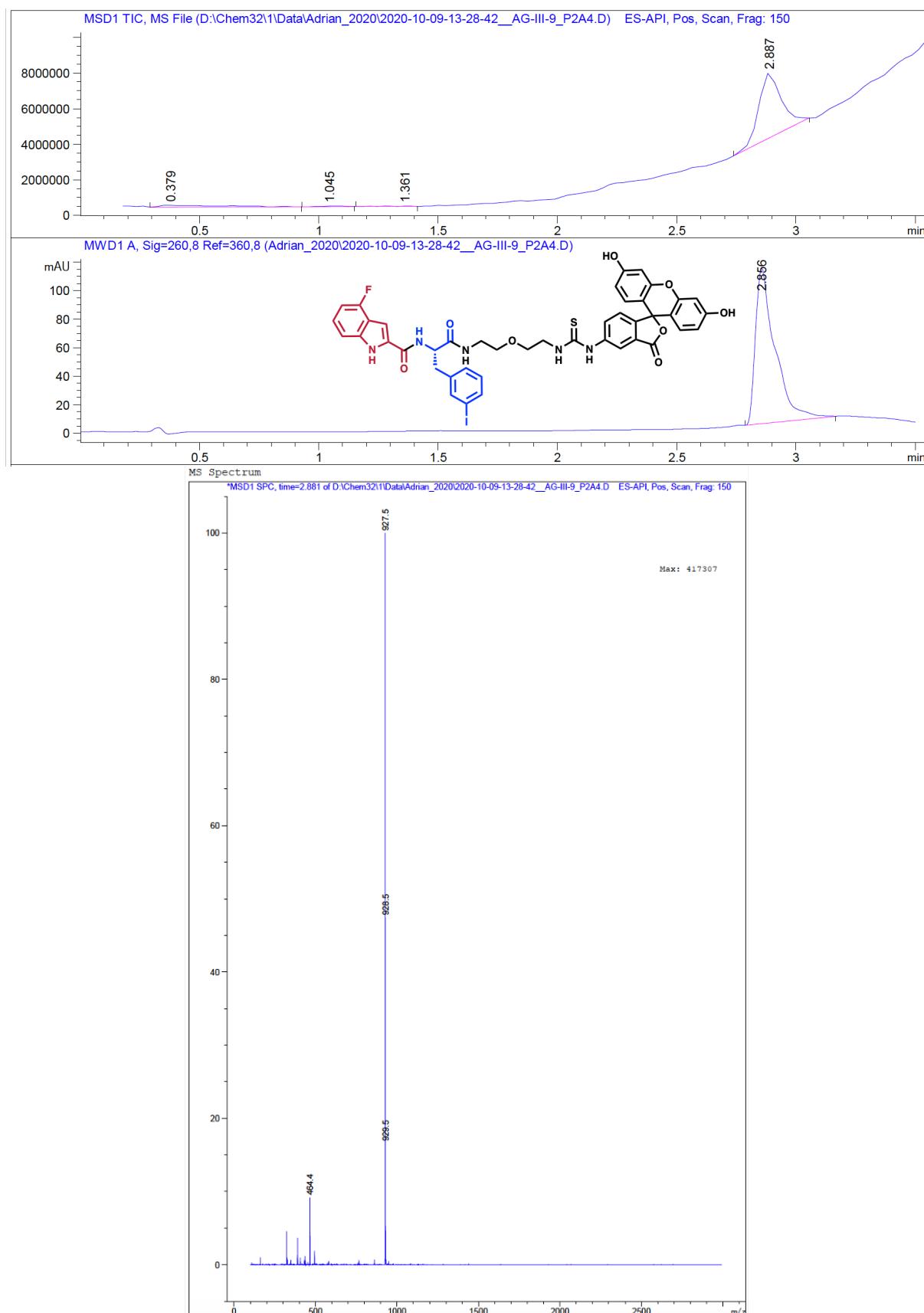
**Figure S36.** LC-ESI-MS chromatogram of compound **8**,  $t_R$ :2.797 min (DAD), 2.822 min (TIC). Expected mass 851.2, observed 851.7



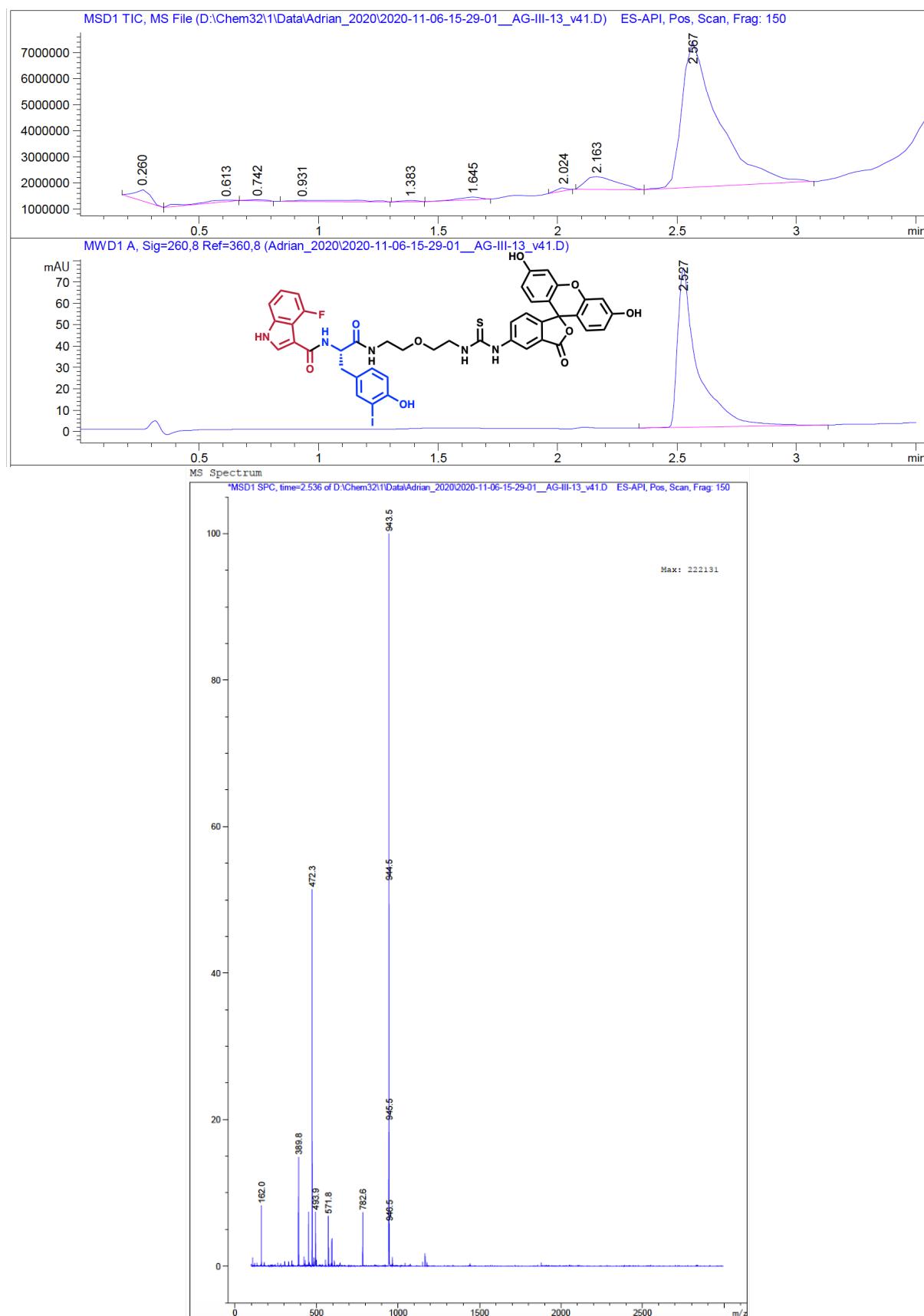
**Figure S37.** LC-ESI-MS chromatogram of compound **9**,  $t_R$ :2.827 min (DAD), 2.839 min (TIC). Expected mass 927.1, observed 927.5



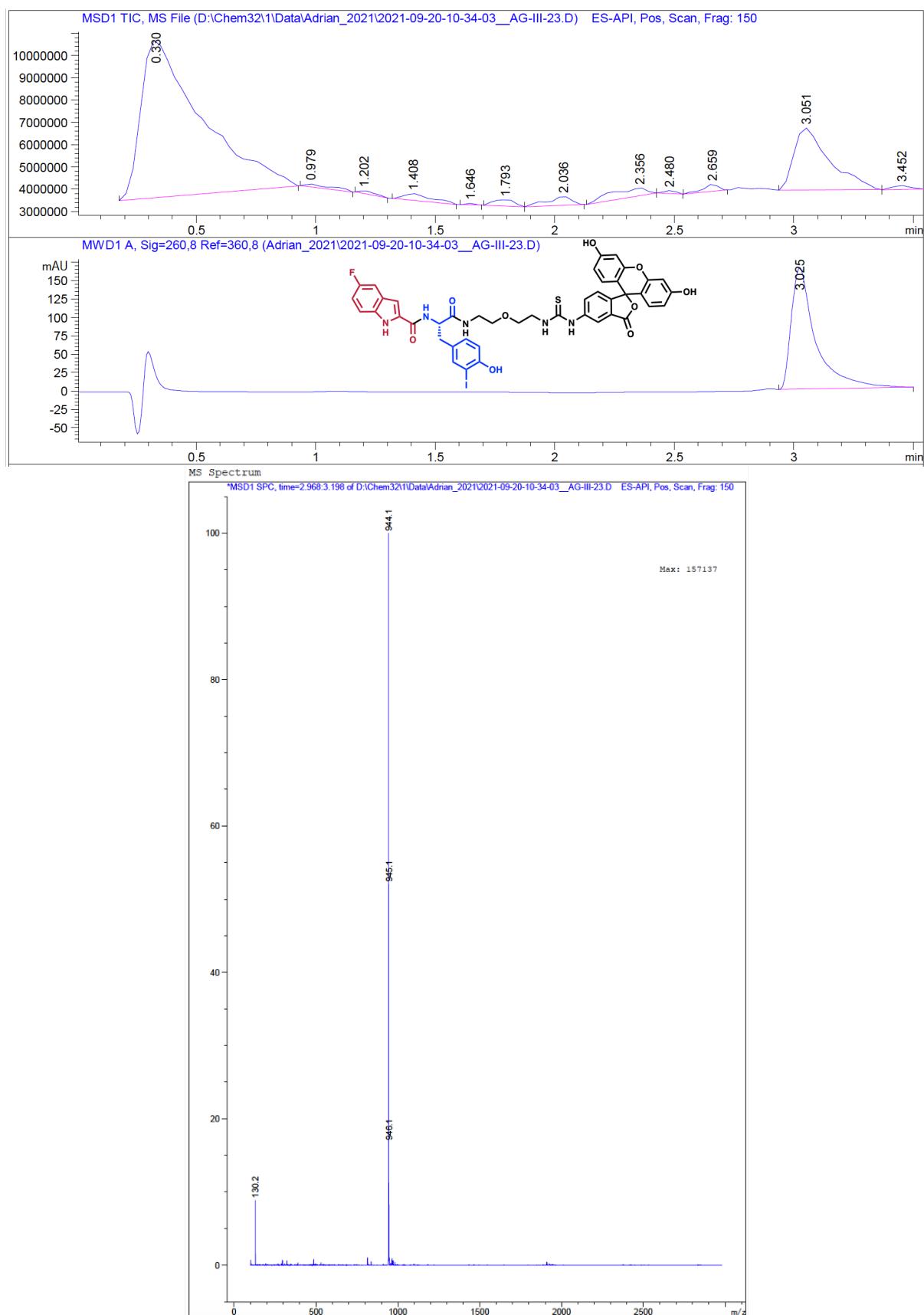
**Figure S38.** LC-ESI-MS chromatogram of compound **10**,  $t_{R}$ :2.550 min (DAD), 2.588 min (TIC). Expected mass 817.2, observed 817.7



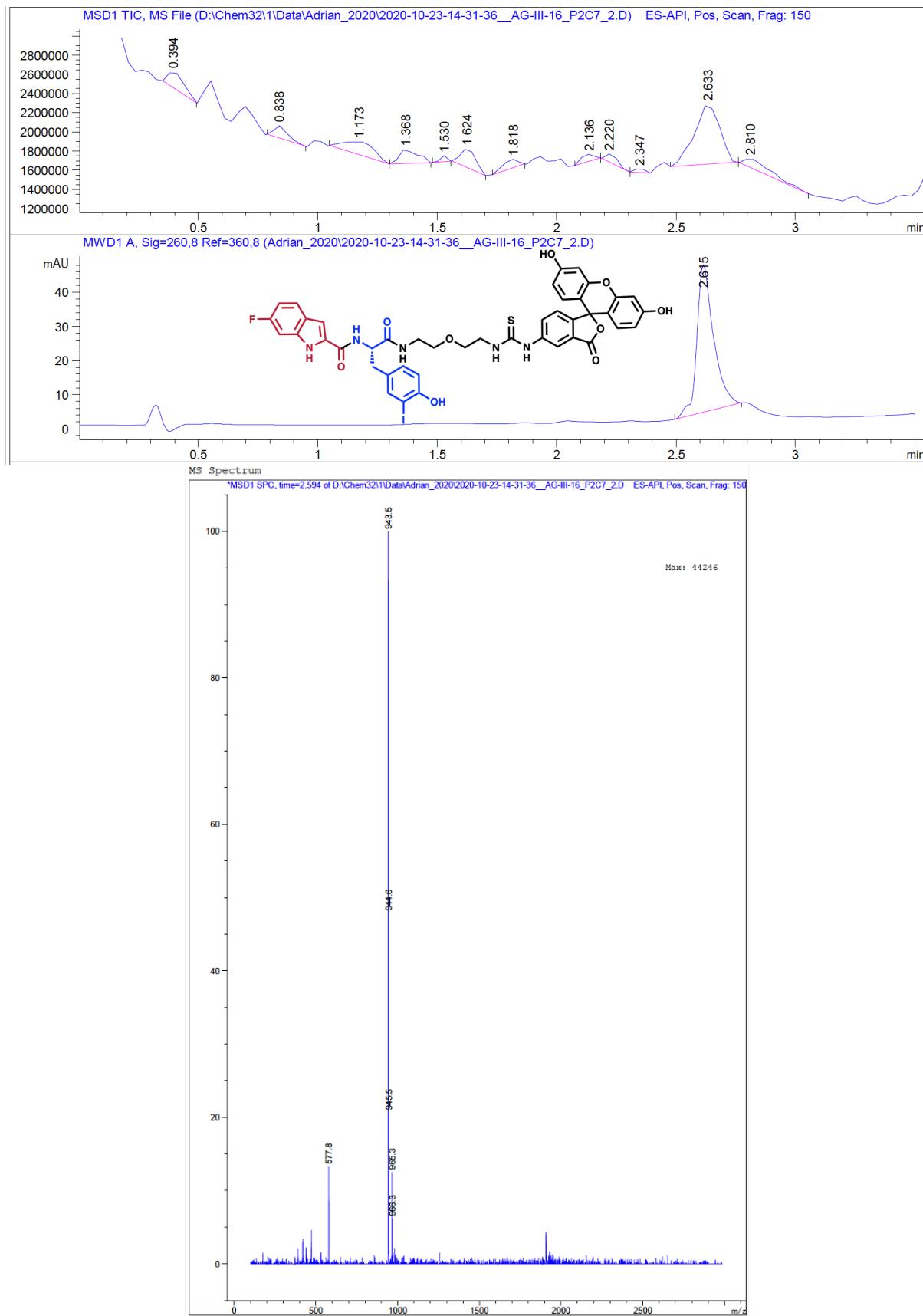
**Figure S39.** LC-ESI-MS chromatogram of compound **11**,  $t_R$ :2.856 min (DAD), 2.887 min (TIC). Expected mass 927.1, observed 927.5



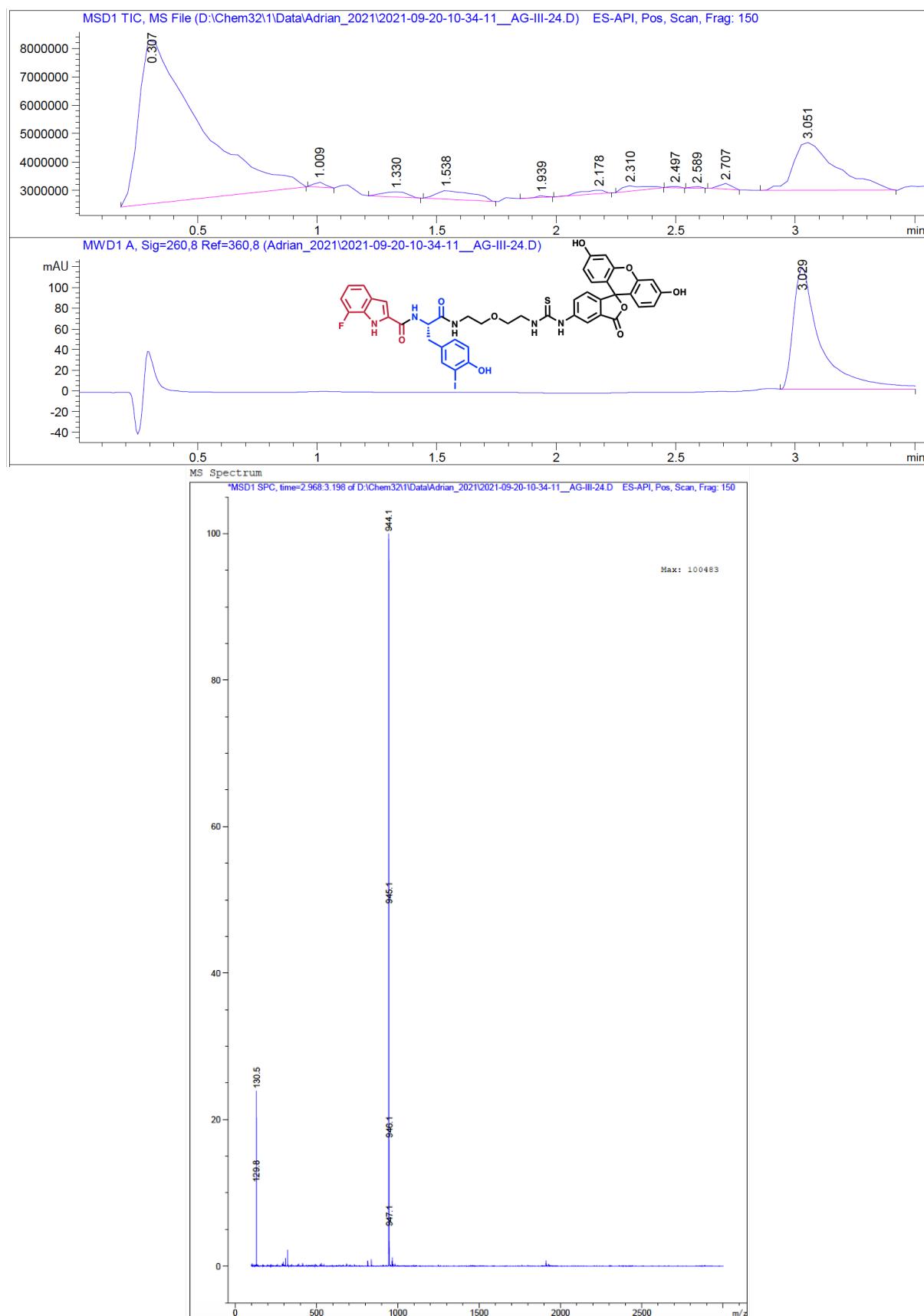
**Figure S40.** LC-ESI-MS chromatogram of compound **12**,  $t_R$ :2.527 min (DAD), 2.567 min (TIC). Expected mass 943.1, observed 943.5



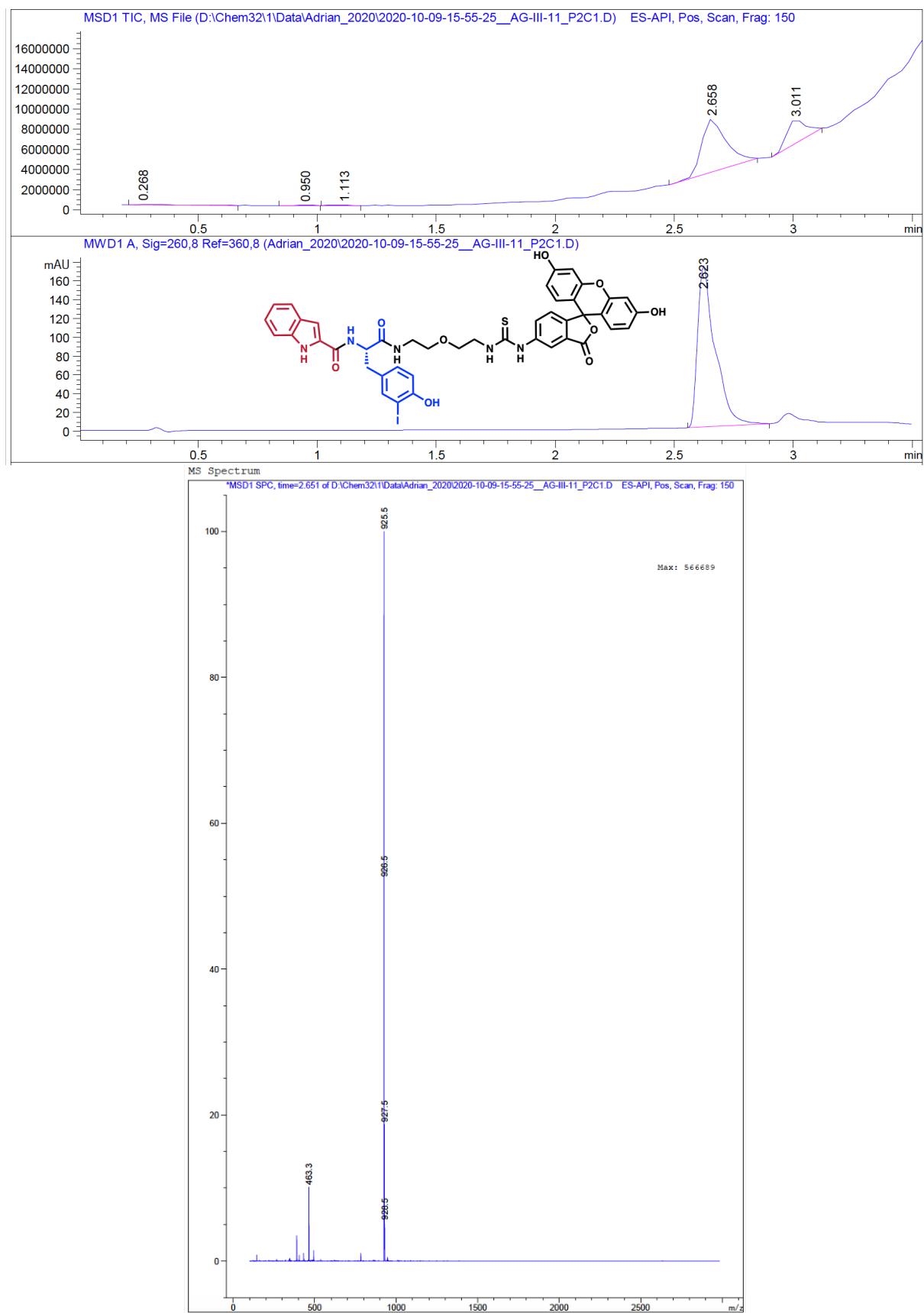
**Figure S41.** LC-ESI-MS chromatogram of compound **13**,  $t_R$ :3.025 min (DAD), 3.051 min (TIC). Expected mass 943.1, observed 944.1



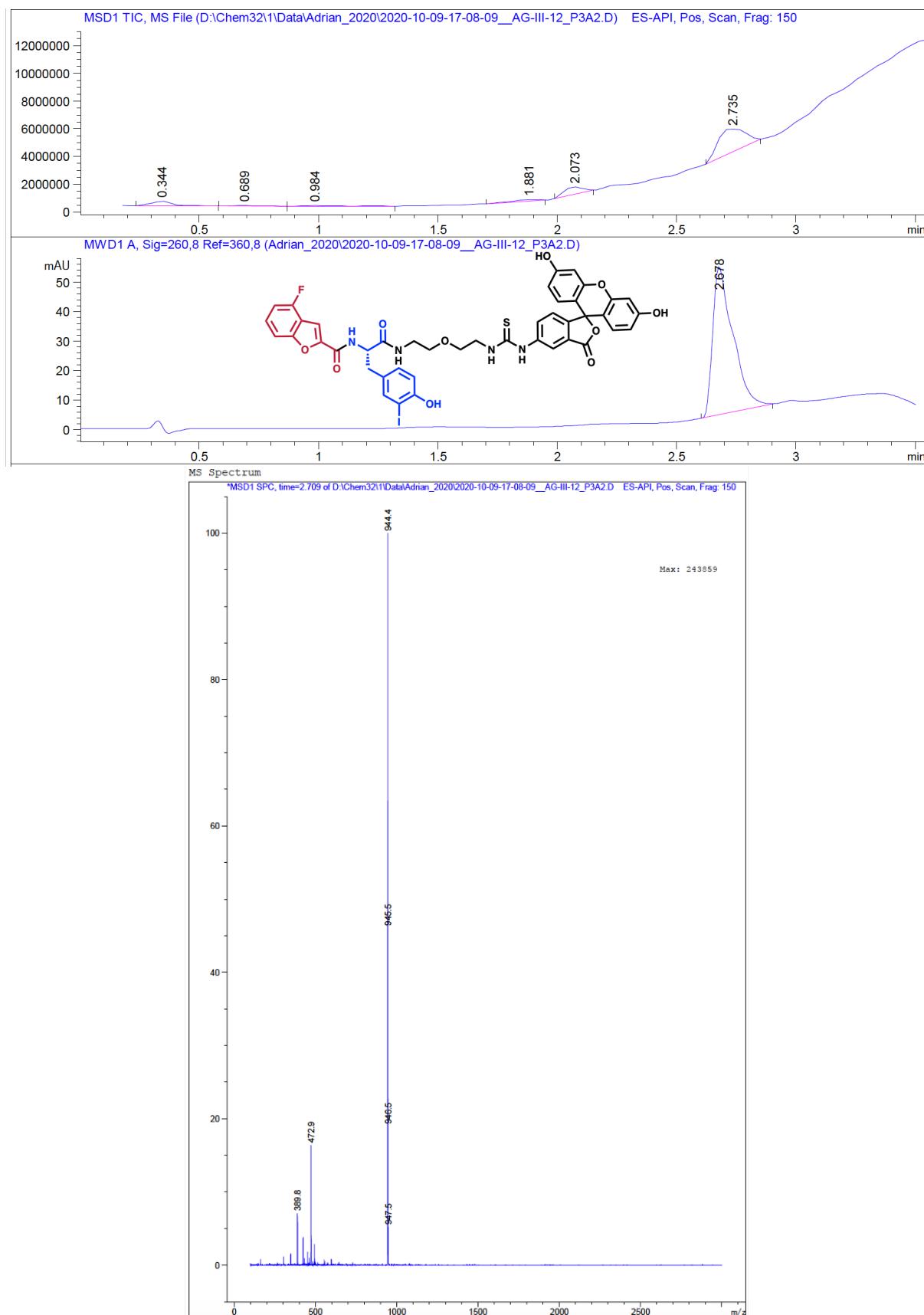
**Figure S42.** LC-ESI-MS chromatogram of compound **14**,  $t_{\text{R}}$ :2.615 min (DAD), 2.633 min (TIC). Expected mass 943.1, observed 943.5



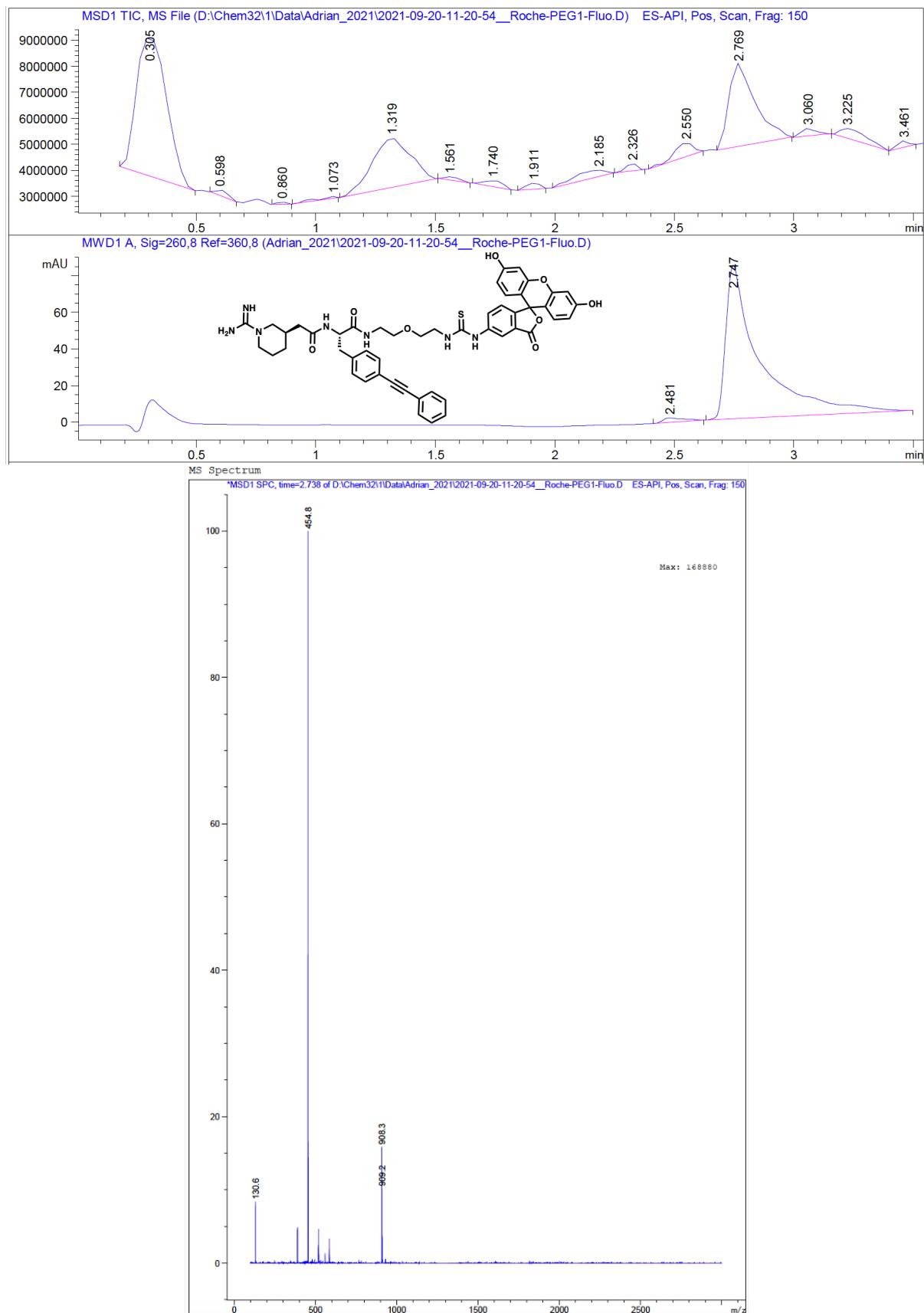
**Figure S43.** LC-ESI-MS chromatogram of compound **15**,  $t_R$ :3.029 min (DAD), 3.051 min (TIC). Expected mass 943.1, observed 944.1



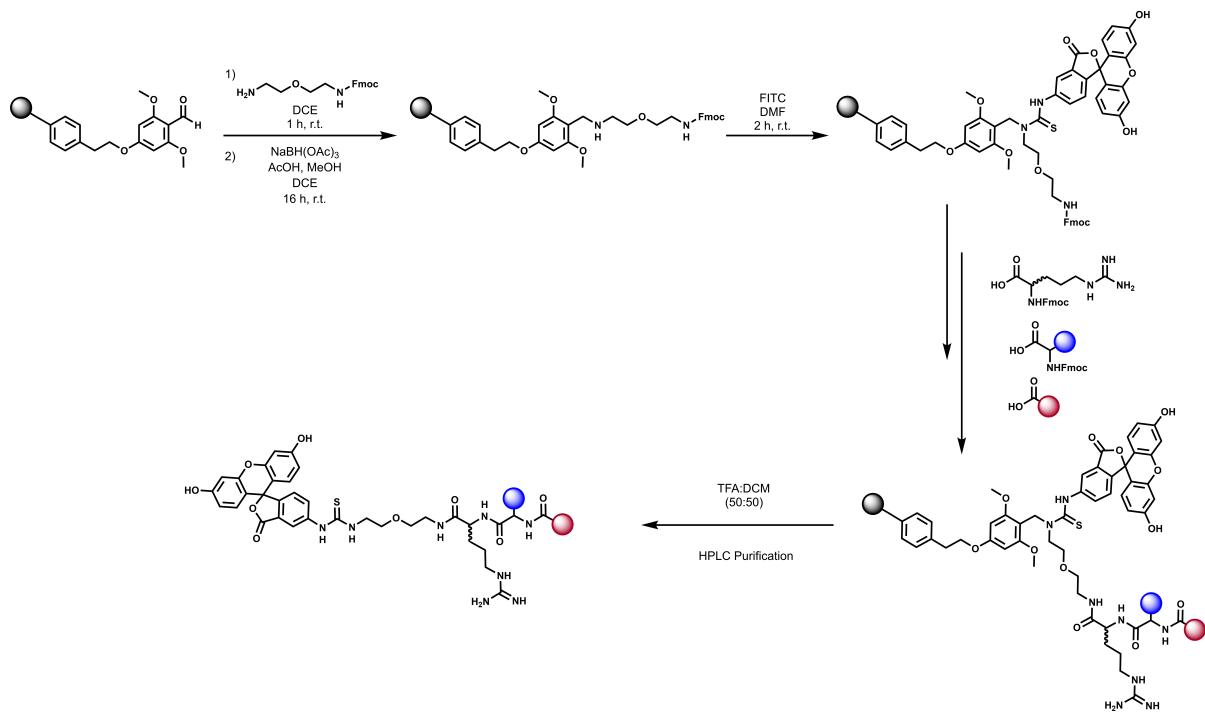
**Figure S44.** LC-ESI-MS chromatogram of compound **16**,  $t_R$ :2.623 min (DAD), 2.658 min (TIC). Expected mass 925.1, observed 925.5



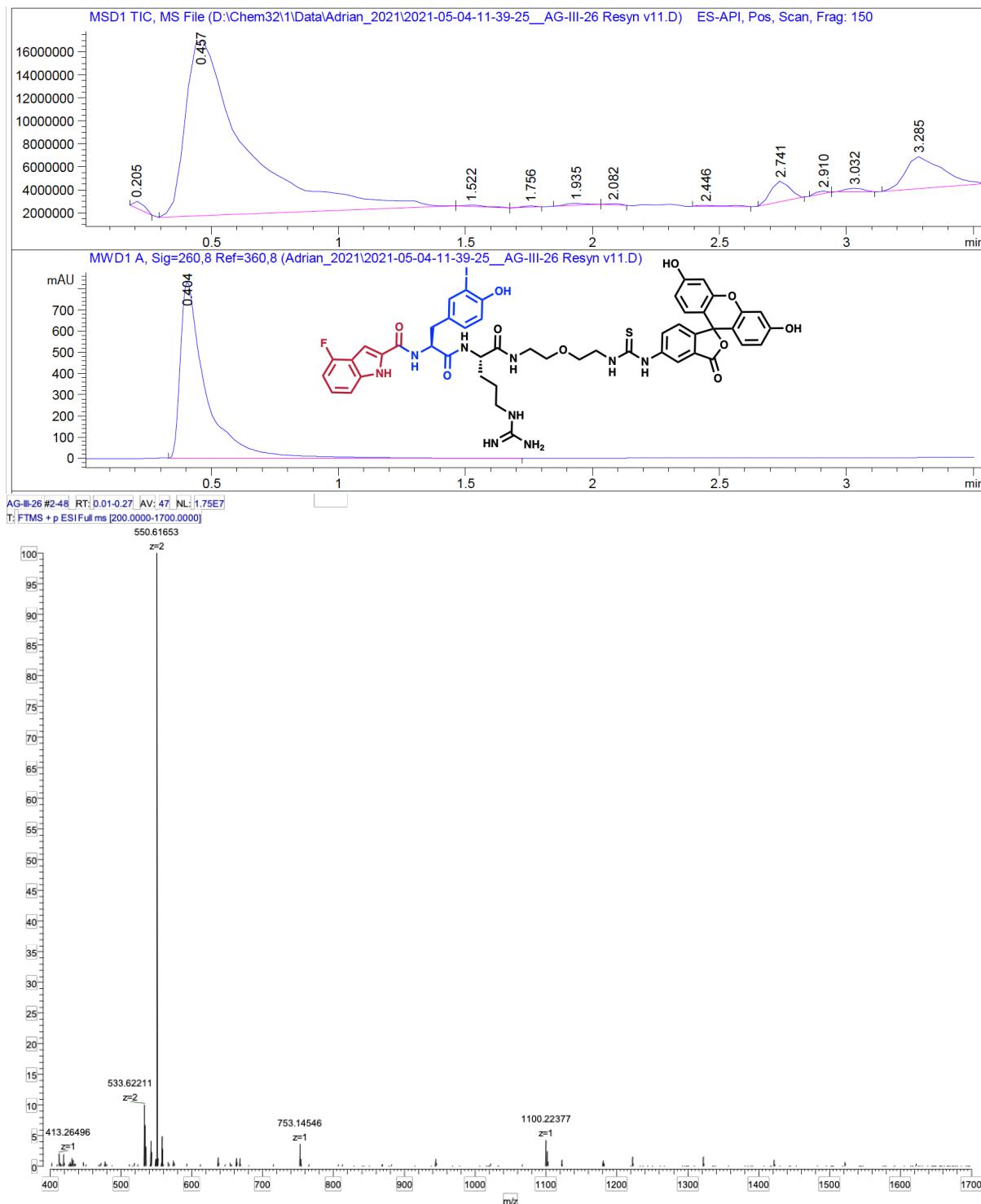
**Figure S45.** LC-ESI-MS chromatogram of compound **17**,  $t_R$ :2.678 min (DAD), 2.735 min (TIC). Expected mass 944.1, observed 944.4



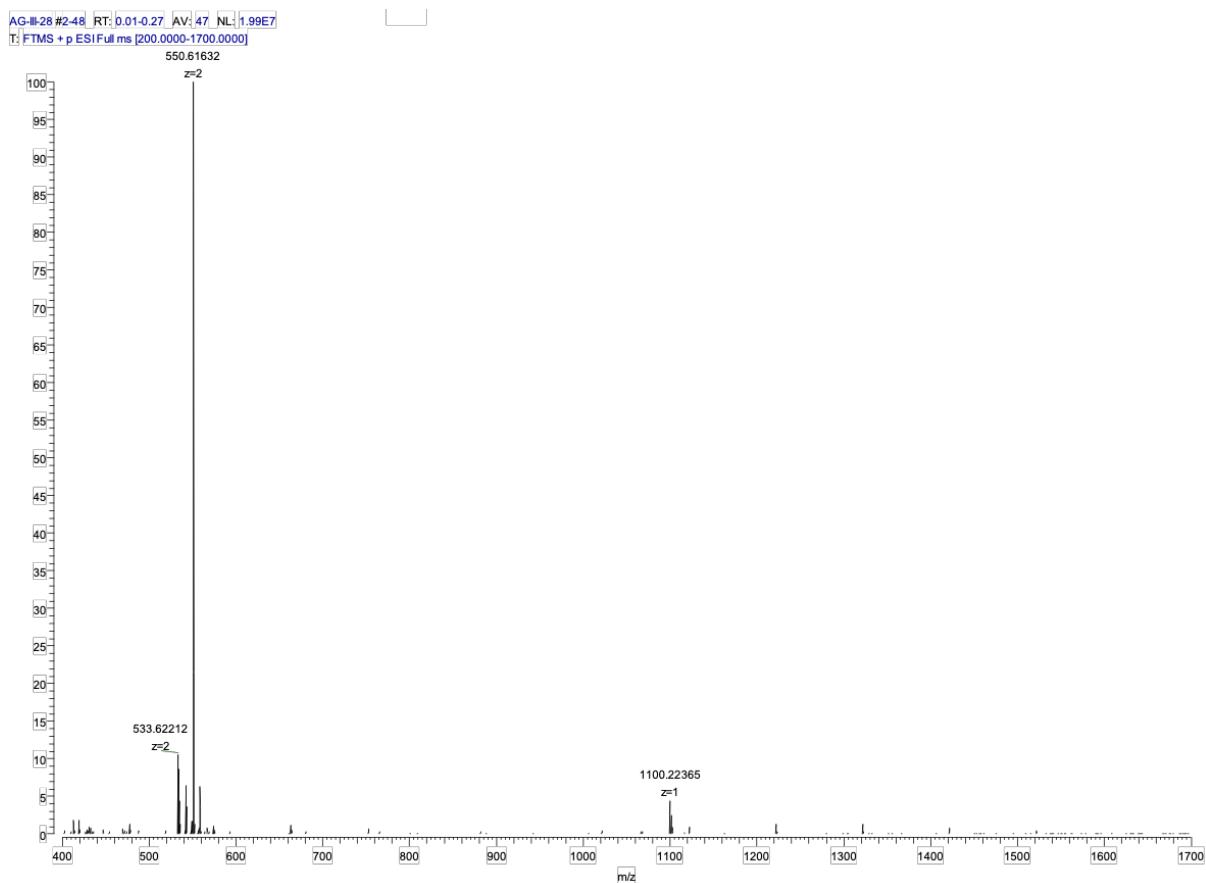
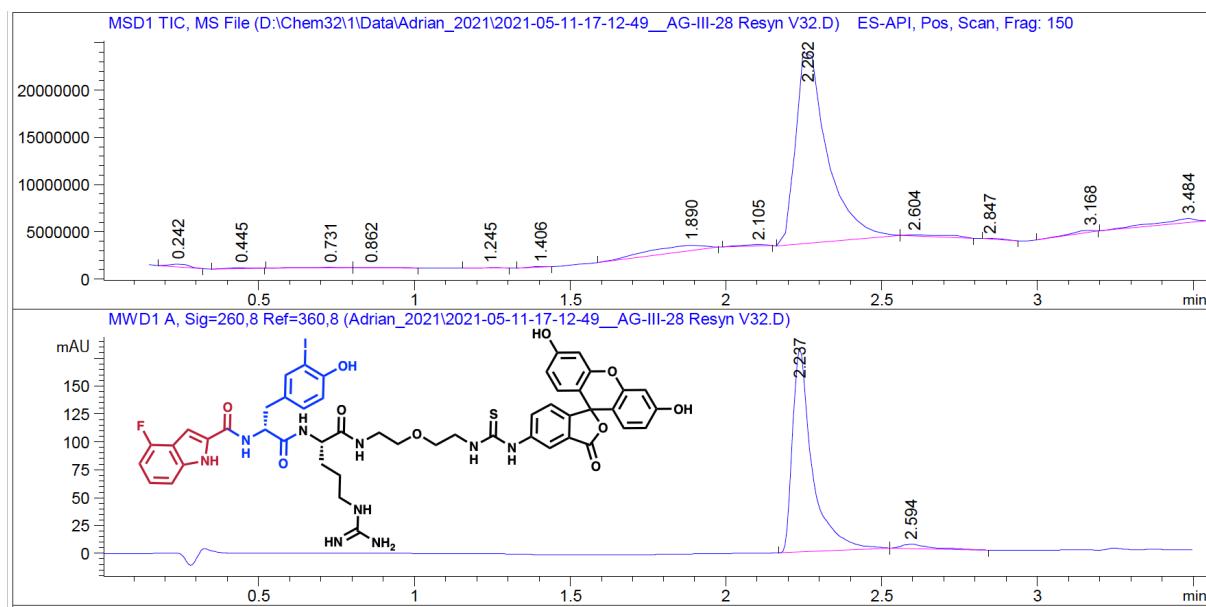
**Figure S46.** LC-ESI-MS chromatogram of compound **Ro26-4550**,  $t_R$ :2.747 min (DAD), 2.769 min (TIC). Expected mass 907.3, observed 908.3



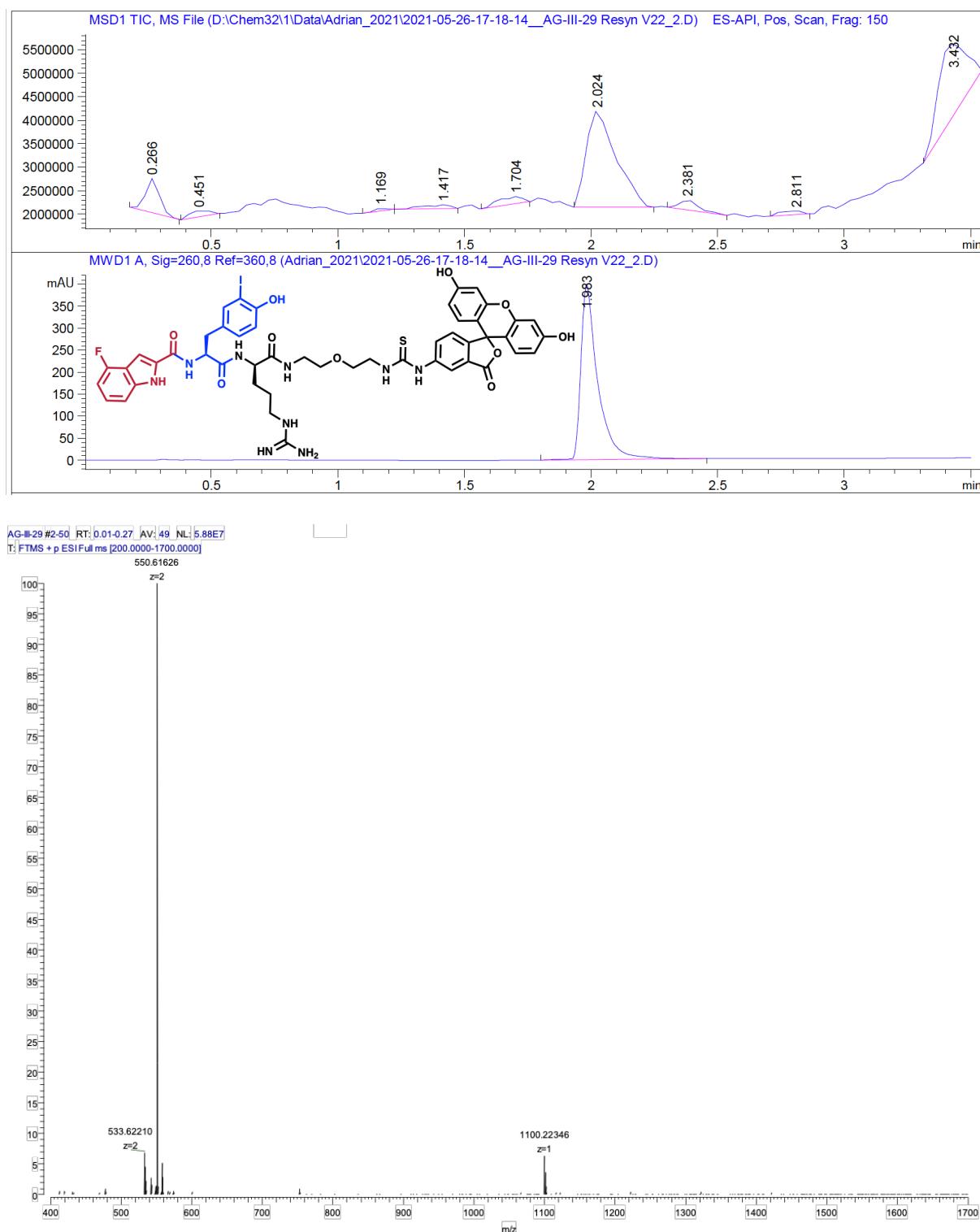
**Scheme S3.** Synthetic route for the synthesis of compounds **18-21**. The grey ball represents the solid support, the blue ball represents the building block 1 and the red ball represents the building block 2



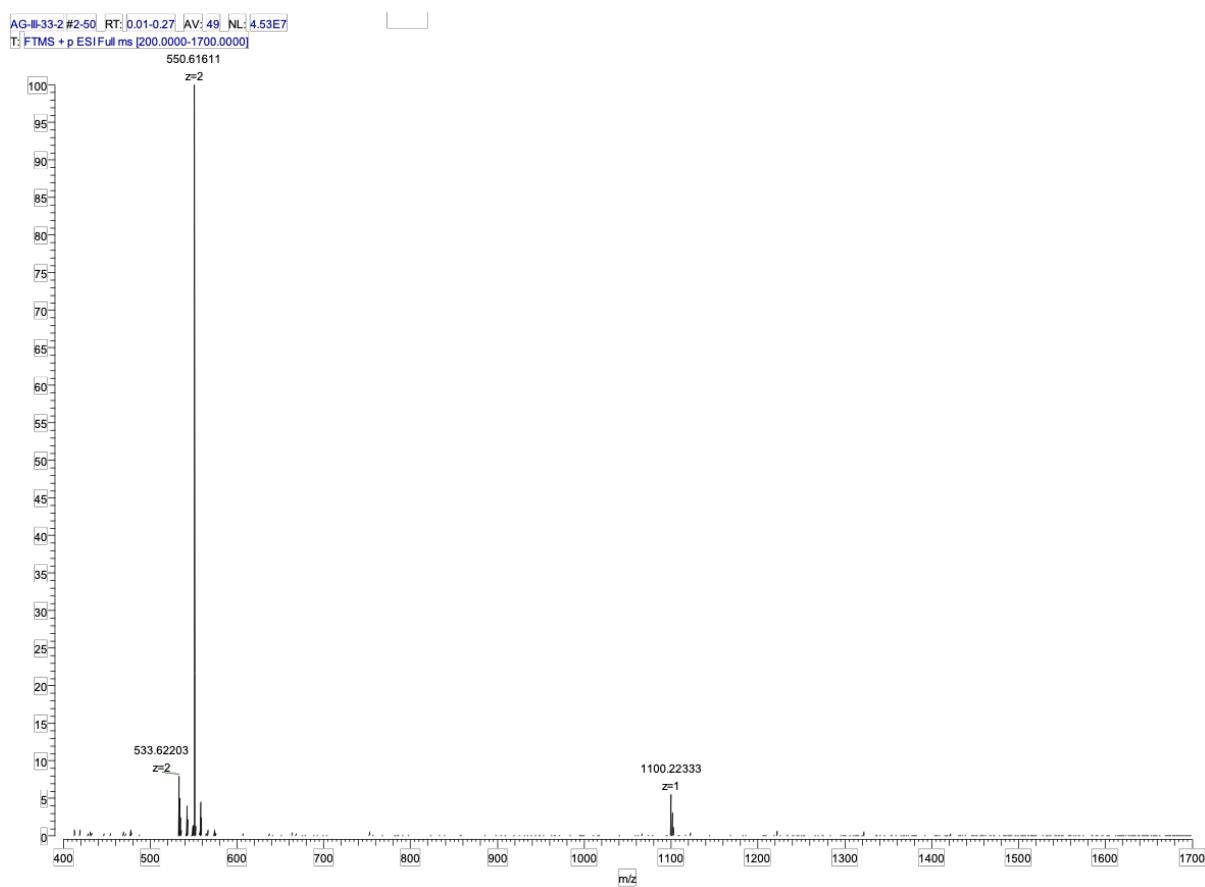
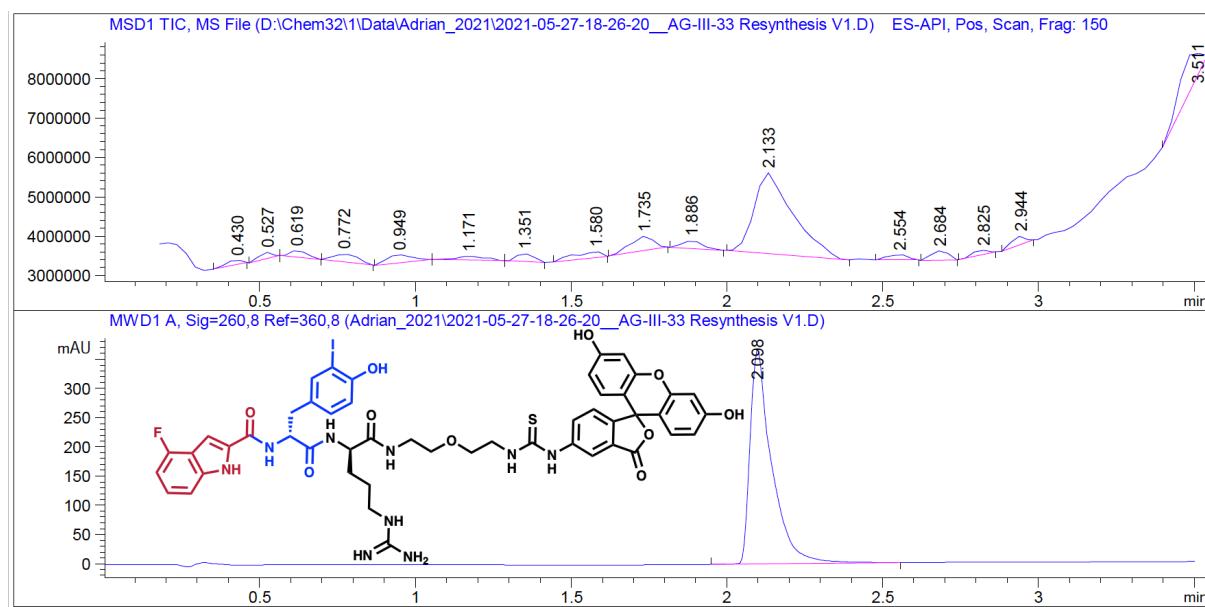
**Figure S47.** LC-HRMS chromatogram of compound **18**,  $t_R$ :0.457 min (DAD). Expected mass 1099.21953, observed 1100.22377



**Figure S48.** LC-HRMS chromatogram of compound **19**,  $t_R$ :2.237 min (DAD). Expected mass 1099.21953, observed 1100.22365



**Figure S49.** LC-HRMS chromatogram of compound **20**,  $t_R$ :1.983 min (DAD). Expected mass 1099.21953, observed 1100.22346



**Figure S50.** LC-HRMS chromatogram of compound **21**,  $t_R$ :2.098 min (DAD). Expected mass 1099.21953, observed 1100.22333

## 7. Recombinant proteins. Sequences and quality control

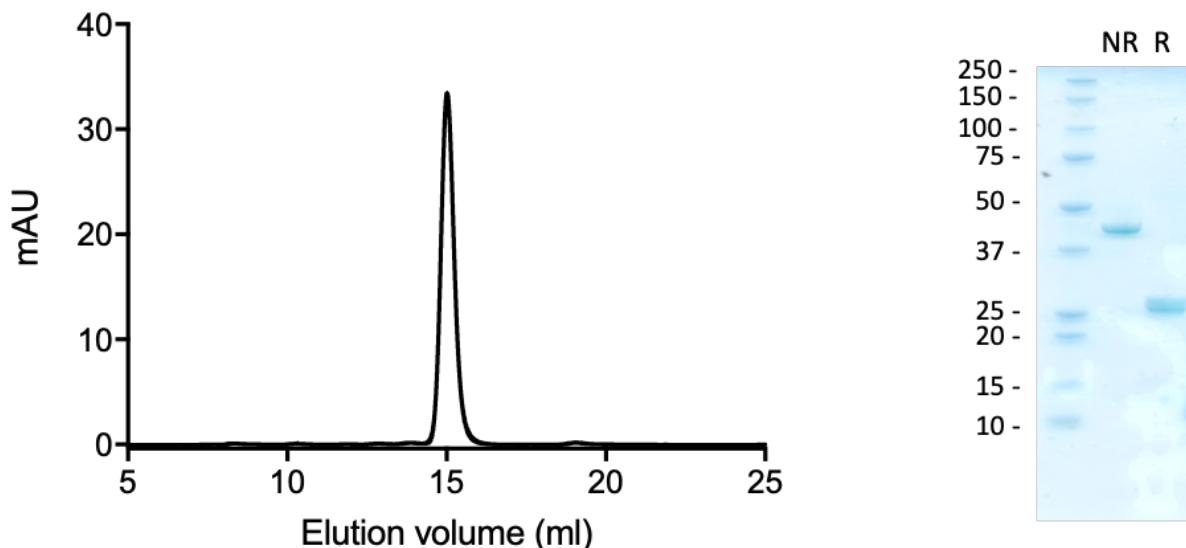
*NARA1 anti-IL2 antibody Fab format:*

Nara-1 (Fab): VL – mCL

DIVLTQSPASLAVSLGQRATISCKASQSVDYDGDSYMWNWYQQKPGQPPKLLIYAASNL  
 ESGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQSNEDPYTFGGGTKLEIKRADA  
 PTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVWKWIDGSERQNGVLNSWTQDSKD  
 STYSMSSTLTLKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

Nara-1 (Fab): VH – mCH1

QVQLQQSGAELVRPGTSVKVSCKASGYAFTNYLIEWVKQRPGQGLEWIGVINPGSGG  
 TNYNEKFKGKATLTADKSSSTAYMQLSSLTSDDSAVYFCARWRGDGYYAYFDVWGA  
 GTTVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLWNSGSLSSGV  
 HTFPAVLQSDLYTLSVVTVTSSTWPSQSITCNVAHPASSTKVDKIEPRGPTHHHHHH



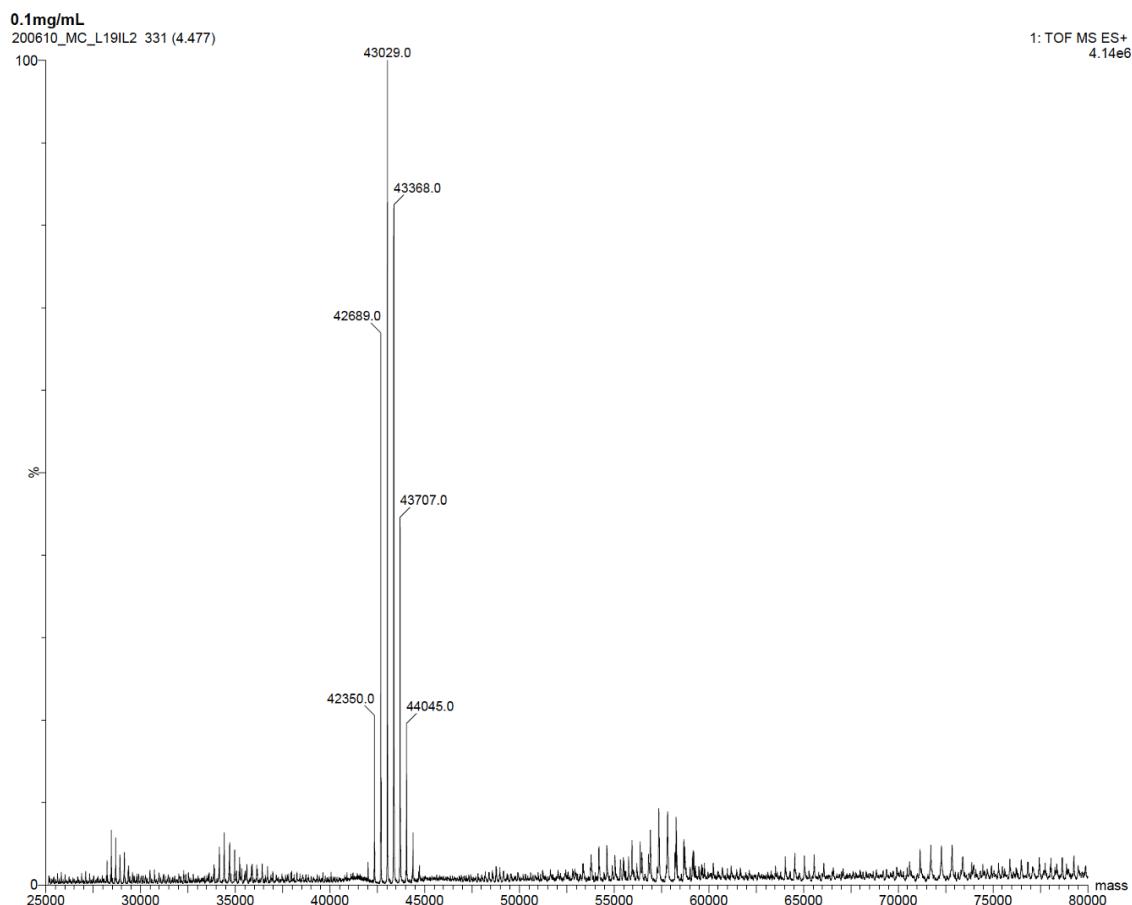
**Figure S51.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of NARA1. The size corresponds to the predicted molecular weight based on the sequence (48.47 kDa). As expected, under reducing conditions the Fab antibody format presents a single band on SDS-PAGE

### *L19-IL2 fusion protein:*

The protein was obtained from our GMP production plant in Siena. The sequence is indicated below and a MS spectrum serves as quality control for chemical biotinylation.

VH(L19) – linker – VL(L19) – linker – IL2  
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGT  
TYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWQGQLVT  
VSSGDGSSGGGASEIIVLTQSPGTLSSLSPGERATLSCRASQSVSSFLAWYQQKPGQ  
APRLLIYYASSRATGIPDRFSGSGSGTDFLTISRLEPEDFAVYYCQQTGRIPPTFGQGT  
KVEIKEFSSSGSSSGSSSGAPTSSSTKKTQLQLEHLLLQMLNGINNYKNPK  
LTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNI  
NVIVLELKGSETTFMCEYADETATIVEFLNRWITFCOSIISTLT

### *biot-L19-IL2 fusion protein:*

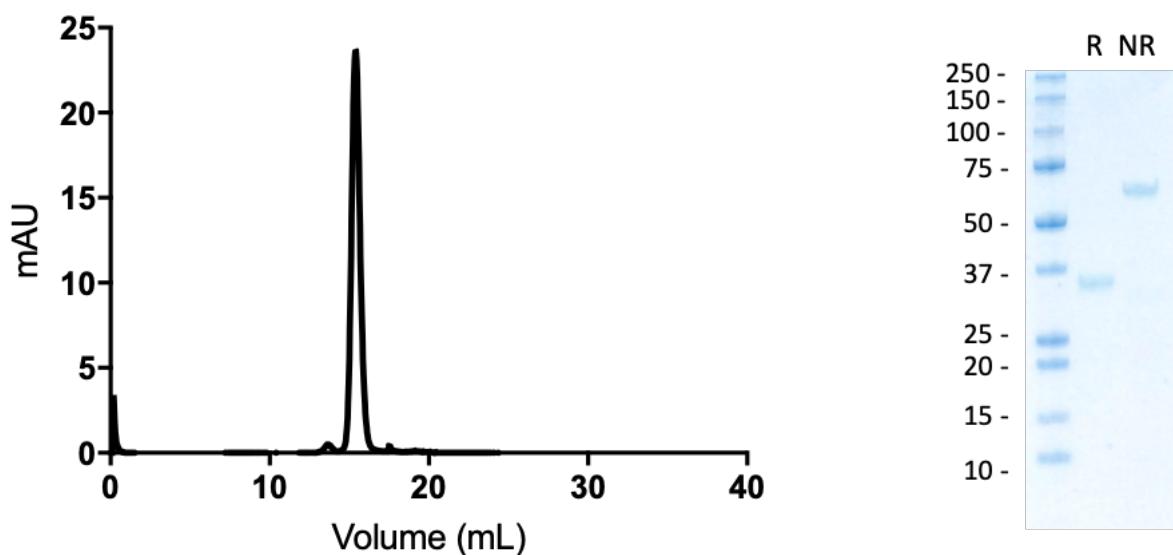


**Figure S52.** MS spectrum of biotinylated L19-IL2. Up to six biotin molecules can be observed

*Carbonic Anhydrase IX (CAIX):*

CAIX – HisTag

GDPQEPQNNAHRDKEGDDQSHWRYGGDPPWPRVSPACAGRQSPVDIRPQLAA  
FCPALRPLELLGFQLPPLPELRLRNNGHSVQLTLPPGLEMALGPGREYRALQLH  
LHWGAAGRPGSEHTVEGHRFPAEIHVVLSTAFARVDEALGRPGLAVLAFL  
EEGPREENSAYEQLLSRLEEIAEEGSETQVPGLDISALLPSDFSRYFQYEGSLTTPP  
CAQGVIWTVFNQTVM恐SAKQLHTLSDTLWGPGLSRLQLNFRATQPLNGRVIEA  
SFPAGVDSSHHHHHH

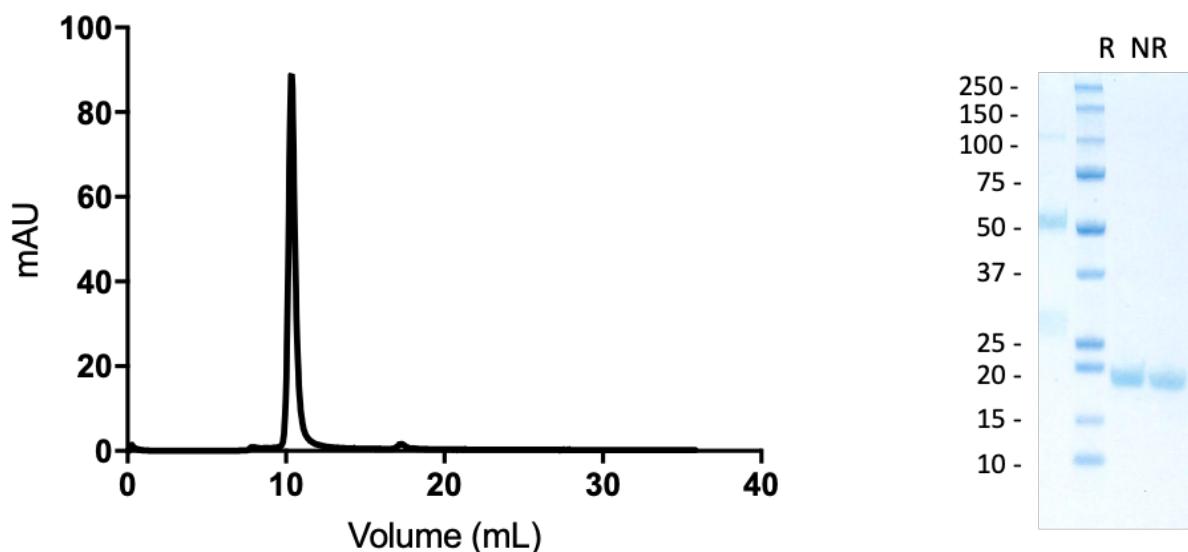


**Figure S53.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of CAIX. The size corresponds to the predicted molecular weight based on the sequence (Homodimer 62.47 kDa). As expected, under reducing conditions the disulfide-bridged homodimer presents a single band on SDS-PAGE.

**Tumor Necrosis Factor (TNF):**

**TNF – HisTag**

MGSHHHHHHRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQL  
VVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIA VSYQTKVNLLSAIKSPCQRE  
TPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGI AL

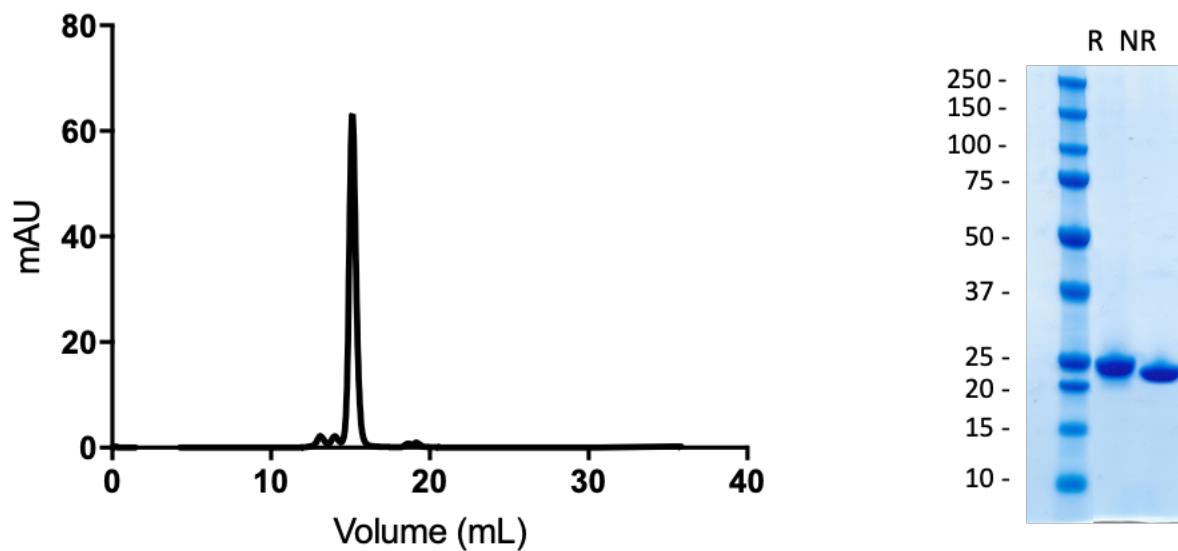


**Figure S54.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of TNF. The size corresponds to the predicted molecular weight based on the sequence (17.93 kDa). As expected, reducing and non-reducing conditions give the same bands on SDS-PAGE.

*F8 anti-EDA antibody (diabody format):*

VH(F8) – linker – VL(F8)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGS  
TYYADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGT  
LTVSSGGSGGEIVLTQSPGTLSSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRL  
LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKV  
EIK

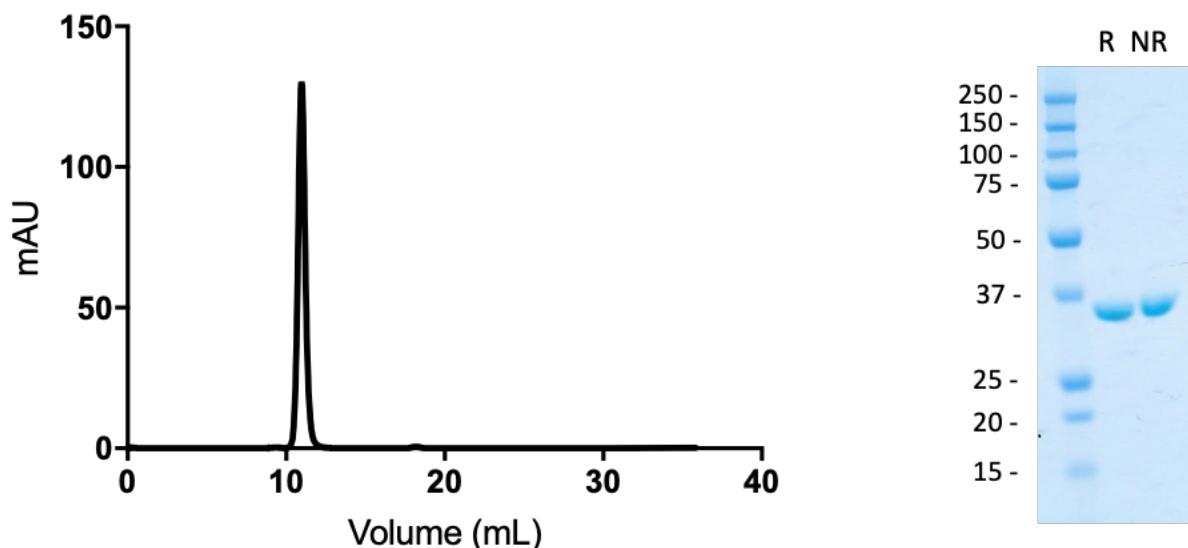


**Figure S55.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of F8. The size corresponds to the predicted molecular weight based on the sequence (24.79 kDa). As expected, reducing and non-reducing conditions give the same bands on SDS-PAGE.

*Extra domain A of Fibronectin (EDA):*

EDA – HisTag

MRGSYRTEIDKPSQMVTDVQDNSISVKWLPSSSPVTGYRVTTPKNGPGPTKT  
KTAGPDQTEMTIEGLQPTVEYVVSVYAQNPSGESQPLVQTAVTNIDRPKGGLAFT  
DVDVDSIKIAWESPQGQVSRVTVYSSPEDGIHELFPAPDGEEDTAELQGLRPGS  
EYTVSVVALHDDMESQPLIGTQSTAIPAPTDLKFTQVTPTSLSAQWTPPNVQLTG  
YRVRVTPKEKTGPMKEINLAPDSSSVVSGLMVATKYEVSVYALKDTLSRPAQ  
GVVTTLEENVRSHHHHHH

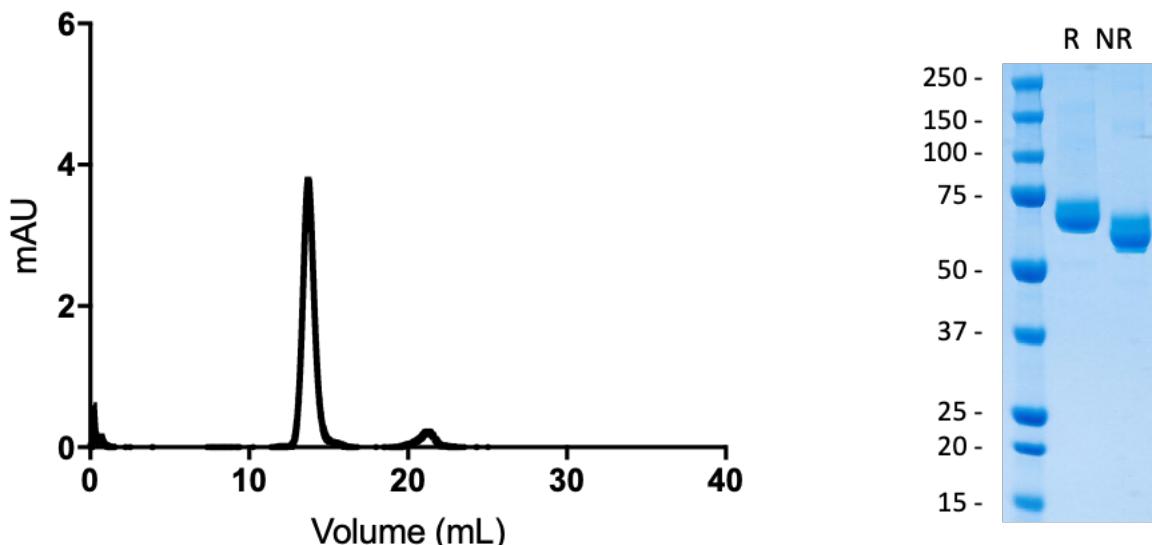


**Figure S56.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of EDA. The size corresponds to the predicted molecular weight based on the sequence (31.45 kDa). As expected, reducing and non-reducing conditions give the same bands on SDS-PAGE.

**F8F8-IL15 fusion protein:**

VH(F8) – linker – VL(F8) – linker – VH(F8) – linker – VL(F8) – *linker* – **IL15**

MGWSLILLFLVAVATGVHSEVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVR  
 QAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY  
 YCAKSTHLYLFDYWGQGTLTVSSGGSGGEIVLTQSPGTLSLSPGERATLSCRASQS  
 VSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV  
 YYCQQMQRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGEVQLLESGGGLVQPGGSLRLS  
 CAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNT  
 LYLMQNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLTVSSGGSGGEIVLTQSPGT  
 SLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSG  
 TDFTLTISRLEPEDFAVYYCQQMQRGRPPTFGQGTKVEIKGDGSSGGSGGASNWVNVIS  
 DLKKIEDLIQSMHIDATLYTESDVHPSKVTAMKCFLLELQVISLESGDASIHD  
 VENLILANNSSLSSNGNVTESGCCKECEELEEKNIKEFLQSFVHIVQMFINTS

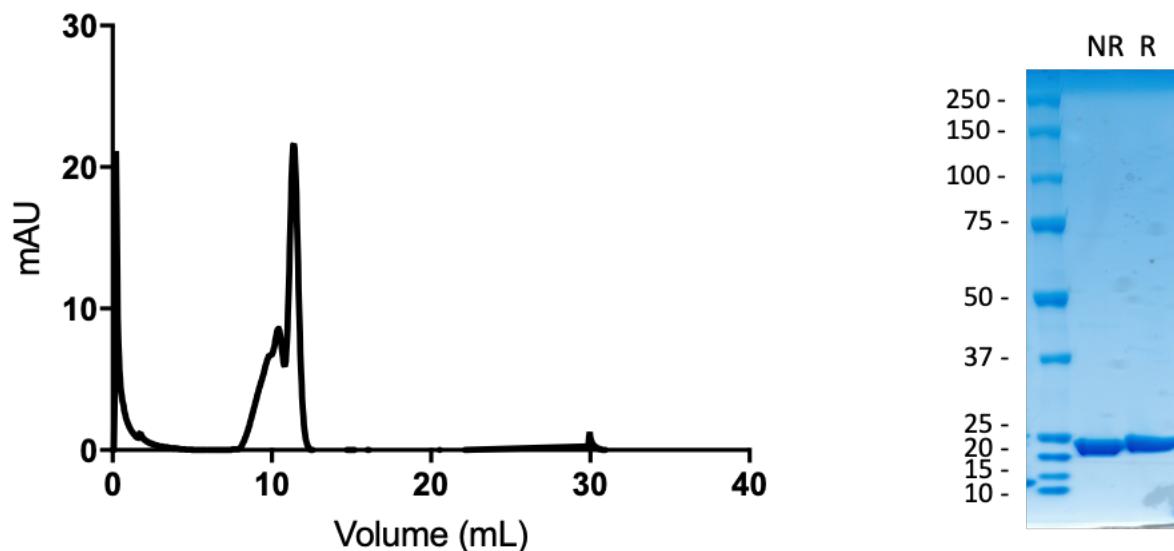


**Figure S57.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of F8F8-IL15. The size corresponds to the predicted molecular weight based on the sequence (66.40 kDa). As expected, reducing and non-reducing conditions give the same bands on SDS-PAGE.

**L19 anti-EDB antibody (diabody format):**

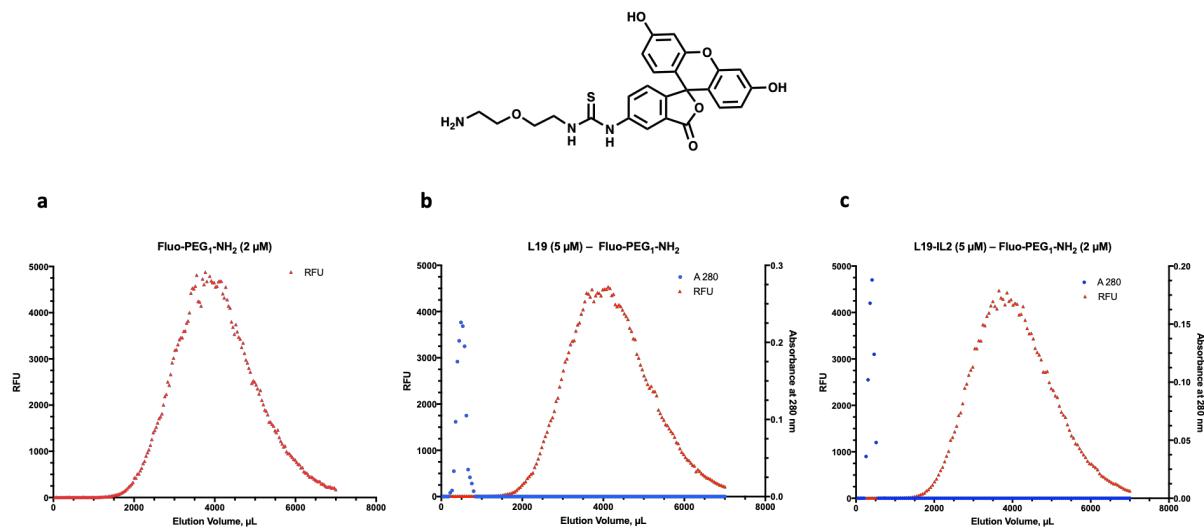
VH(L19) – linker – VL(L19)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGT  
TYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWQGQLVLT  
VSSGSSGGEIVLTQSPGTLSSLSPGERATLSCRASQSVSSFLAWYQQKPGQAPRLLIYY  
ASSRATGIPDRFSGSGSTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIK

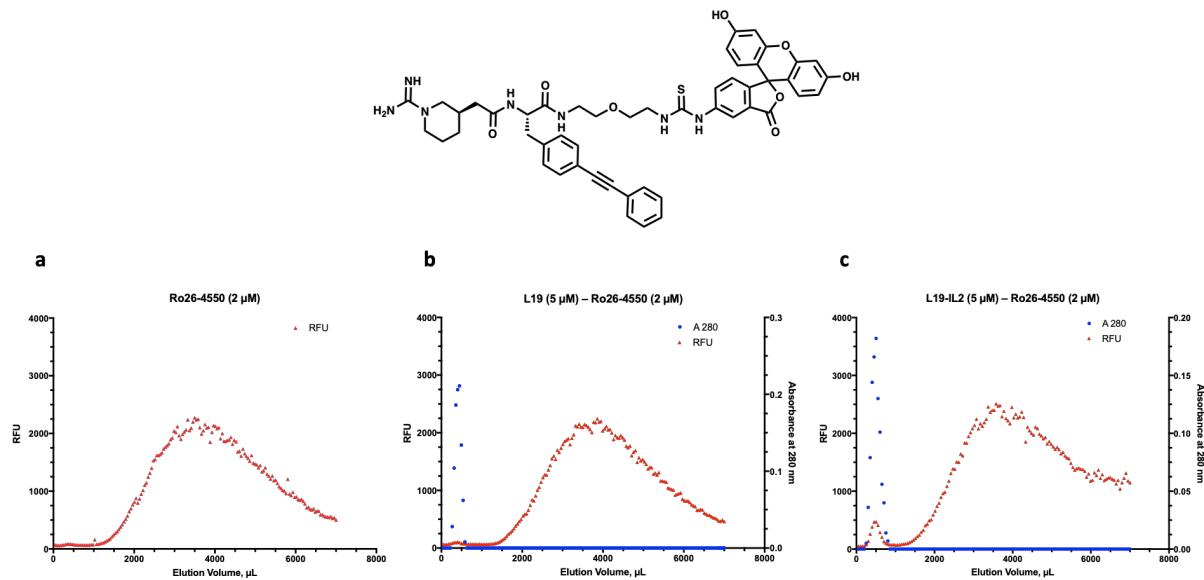


**Figure S58.** Size exclusion chromatography profile and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of L19. The size corresponds to the predicted molecular weight based on the sequence (24.61 kDa). As expected, reducing and non-reducing conditions give the same bands on SDS-PAGE.

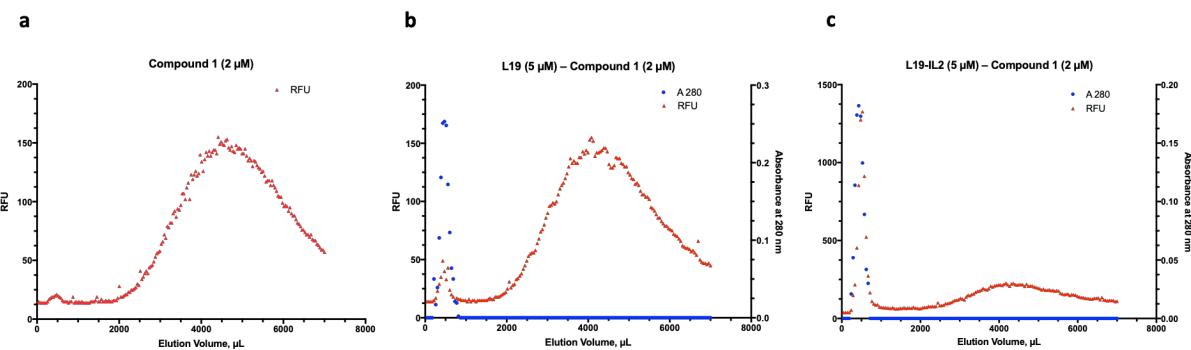
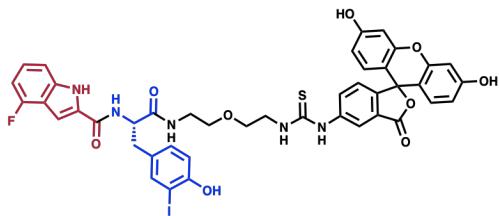
## 8. Coelution by gel filtration



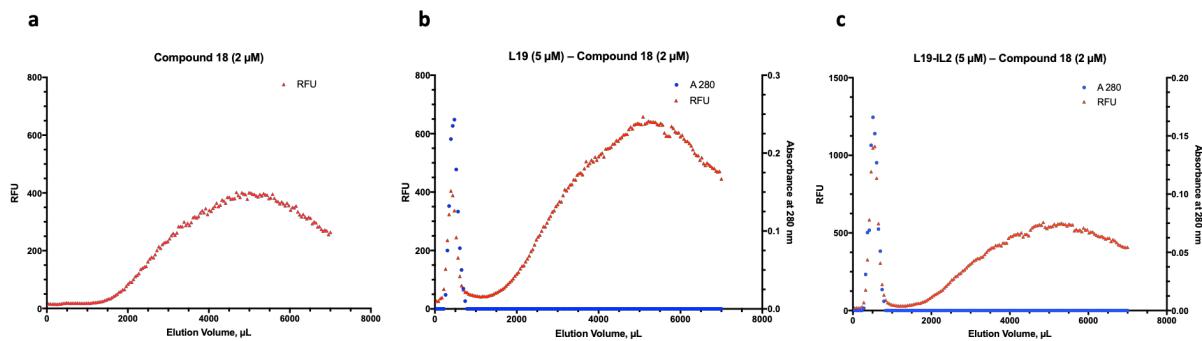
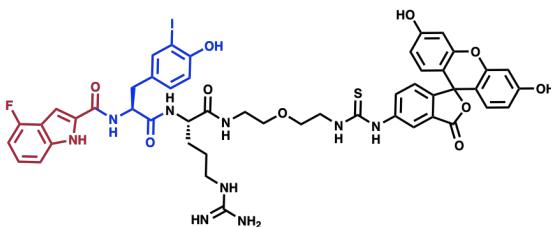
**Figure S59.** Gel filtration experiments using the negative control Fluo-PEG<sub>1</sub>-NH<sub>2</sub>. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Ligand alone, **b.** Preincubation with L19 antibody, **c.** Preincubation with L19-IL2.



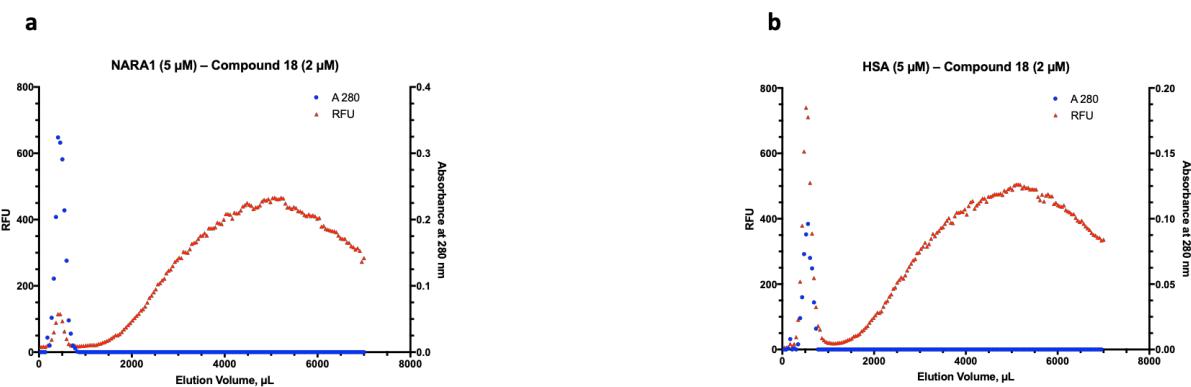
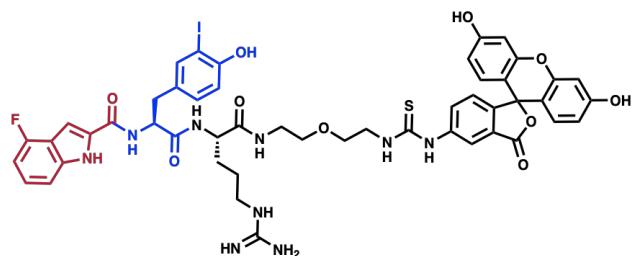
**Figure S60.** Gel filtration experiments using the positive control **Ro26-4550** as fluorescein conjugate. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Ligand alone, **b.** Preincubation with L19 antibody, **c.** Preincubation with L19-IL2



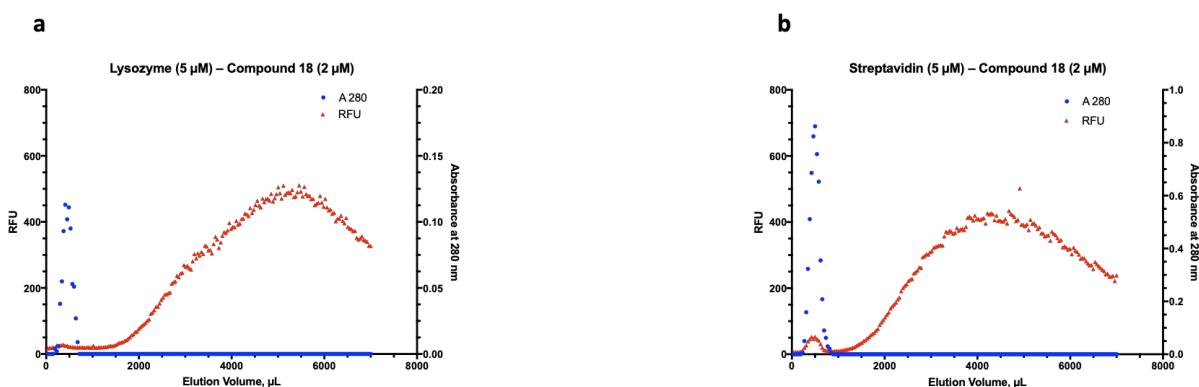
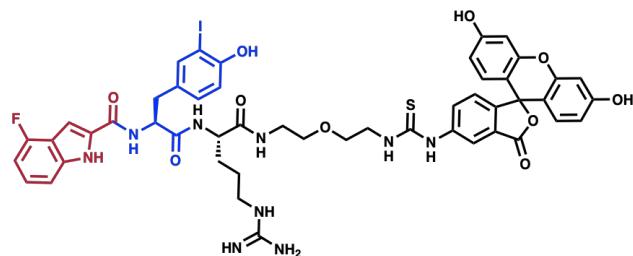
**Figure S61.** Gel filtration experiments using the positive control compound **1**. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Ligand alone, **b.** Preincubation with L19 antibody, **c.** Preincubation with L19-IL2



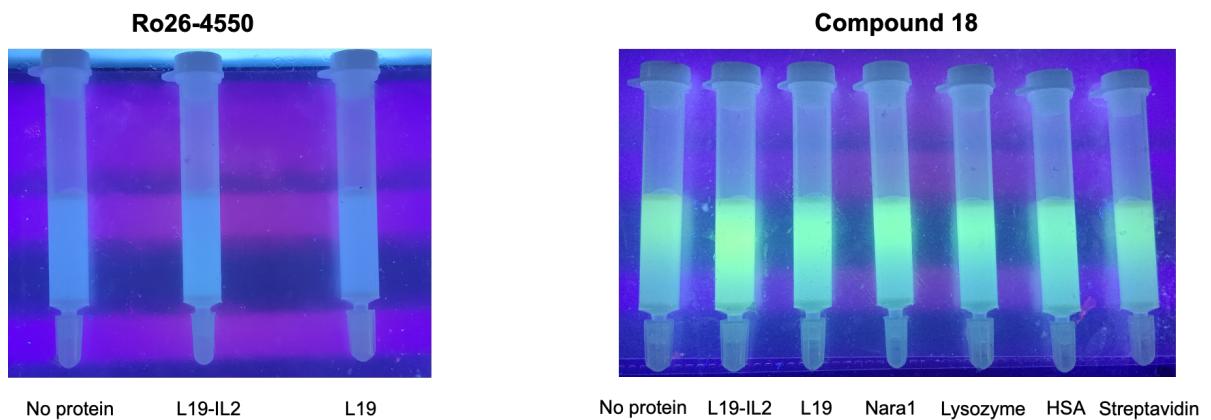
**Figure S62.** Gel filtration experiments using the positive control compound **18**. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Ligand alone, **b.** Preincubation with L19 antibody, **c.** Preincubation with L19-IL2



**Figure S63.** Gel filtration experiments using the positive control compound **18**. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Preincubation with NARA1 antibody, **b.** Preincubation with HSA

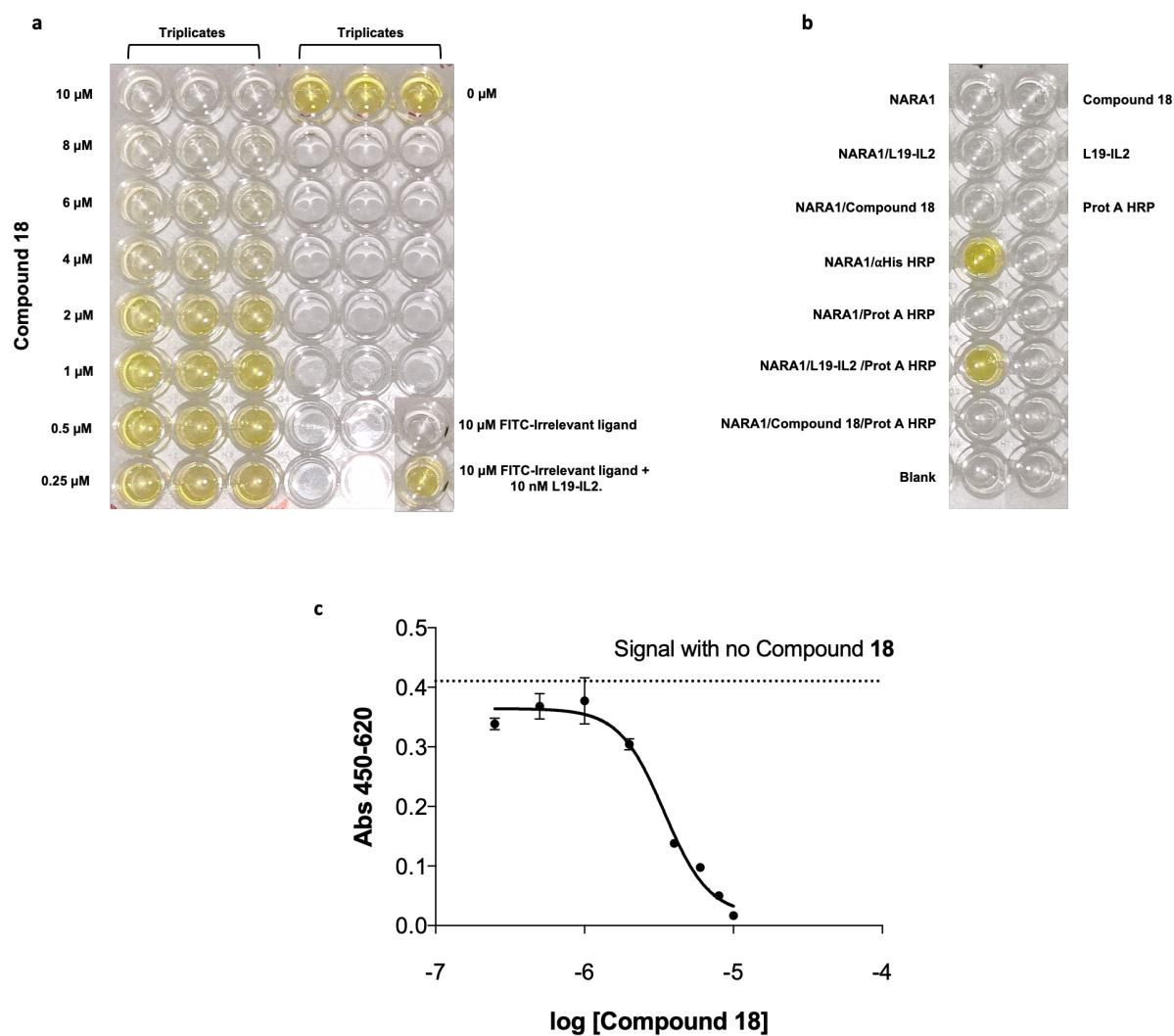


**Figure S64.** Gel filtration experiments using the positive control compound **18**. In blue absorbance at 280 nm (protein concentration); in red relative fluorescence units (ligand detection). **a.** Preincubation with Lysozyme, **b.** Preincubation with Streptavidin



**Figure S65.** UV light pictures of the NAP5 columns after gel filtration experiments.

## 9. Competition Experiment via ELISA



**Figure S66.** ELISA competition experiments. **a.** Experimental plate, **b.** Controls, **c.** Plotted data of the ELISA competition experiment. Data shows that compound **18** inhibits the interaction between IL2 and NARA1 with an inhibition constant ( $IC_{50}$ ) of approximately  $3.4 \mu M$