

Supporting Information

Fragment-based covalent targeting of lysines at the allosteric latch site of SHP2

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1. Supporting figures and tables

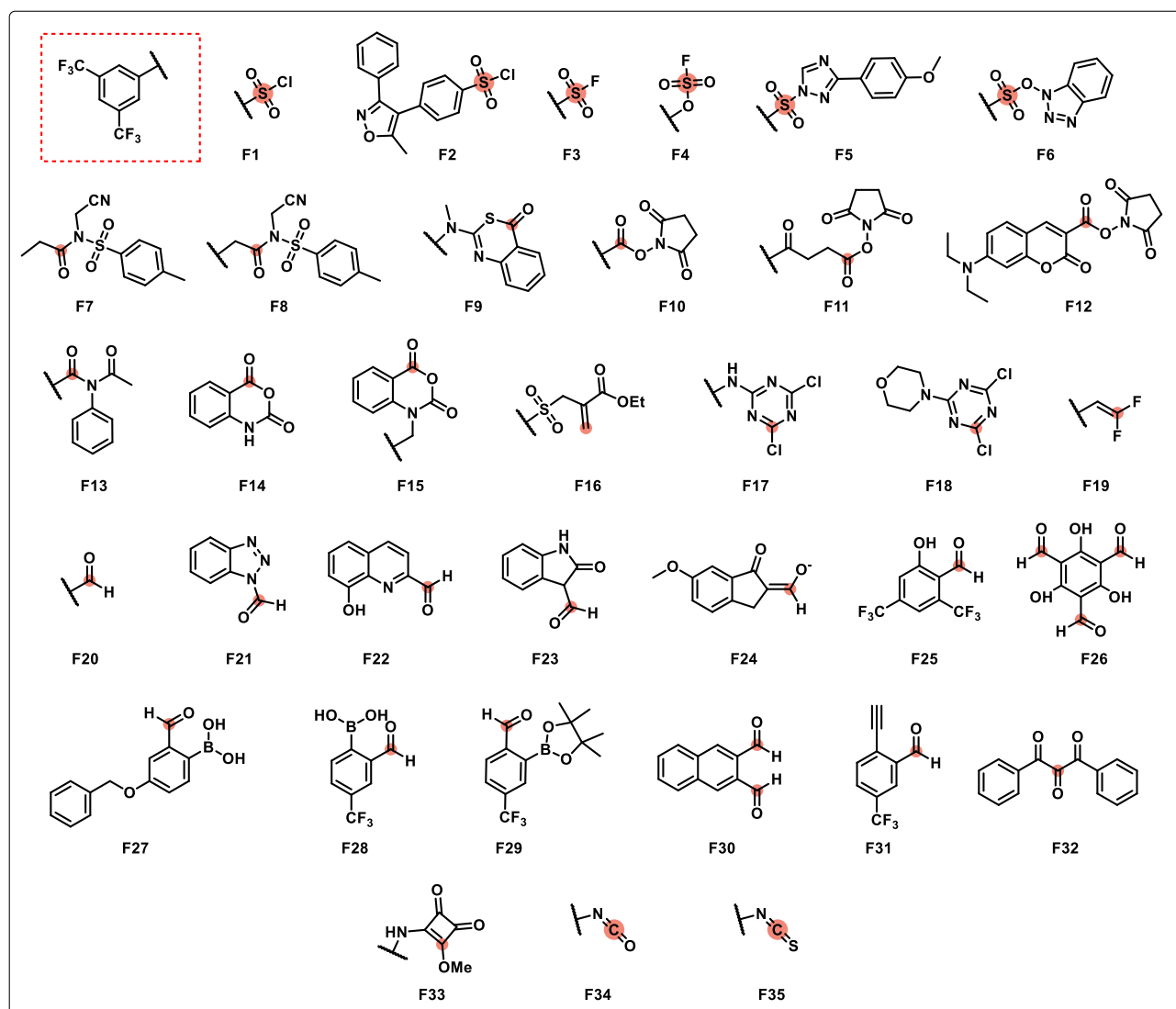


Figure S1 Lysine-targeting fragment library¹ Chemical structure of the lysine-targeting probes, the presumed electrophilic centers are highlighted by red.

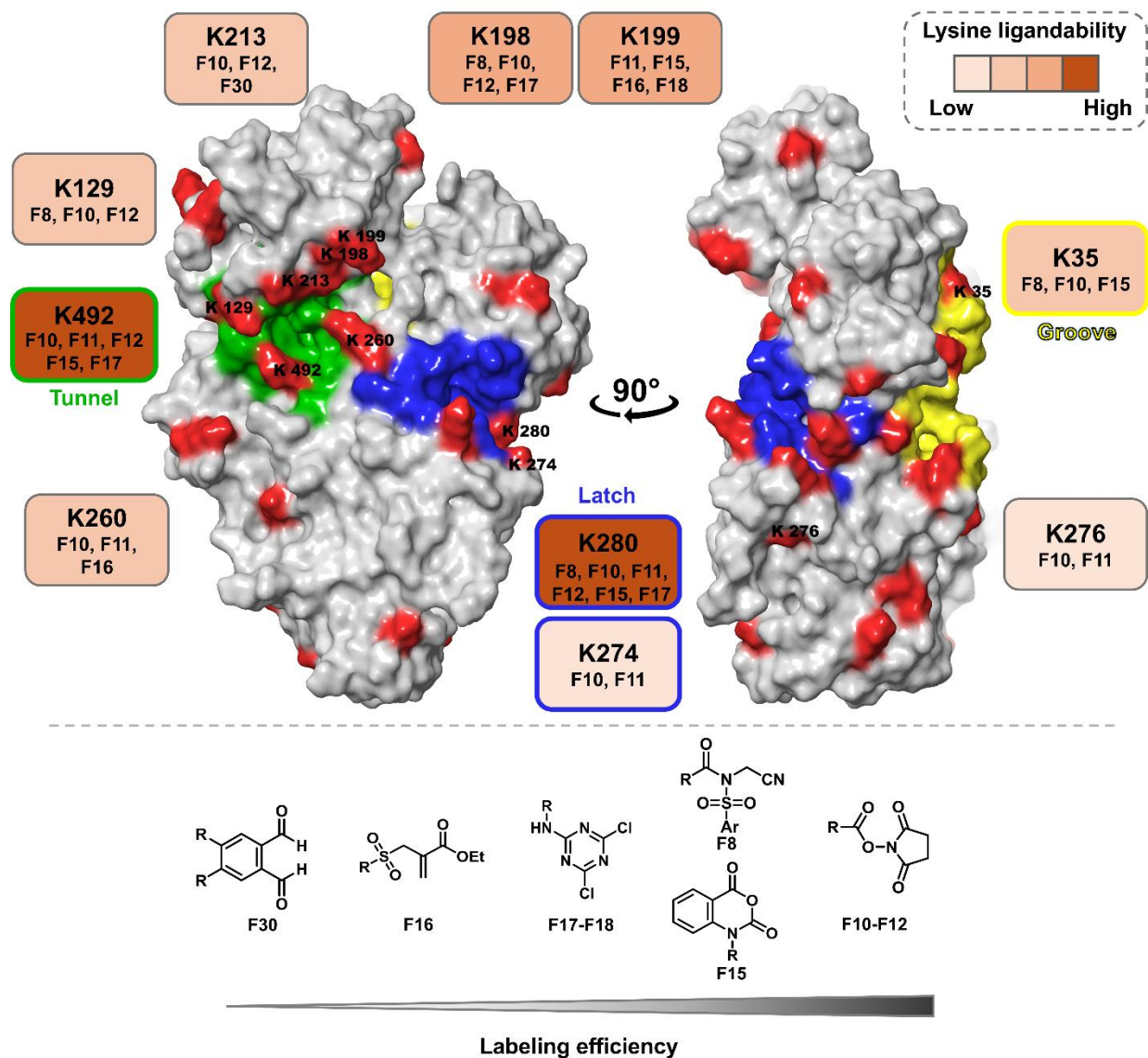


Figure S2 Lysine mapping results of SHP2. Full-length SHP2 (PDB ID: 6BMX) contains 34 lysine residues (shown with red highlight), of which 10 were labeled by fragments from our library following tryptic digestion (K35, K129, K198, K199, K213, K260, K274, K276, K280 and K492, respectively). Known allosteric sites of SHP2 are highlighted as follows: the latch (blue), the tunnel (green), and the groove (yellow). Lysine ligandability is indicated by increasing orange color intensity. Labeling efficiency by the warhead type of hit fragments is shown at the bottom.

Compound ID	k_{Lys} pH 10.2 [h ⁻¹]	k_{Blank} pH 10.2 [h ⁻¹]	$k_{Binding}$ pH 10.2 [h ⁻¹]	Lys- reactivity $t_{1/2}$, pH 10.2 [h]	Stability $t_{1/2}$, pH 10.2 [h]	Stability, pH 7.4 [%]
F1	-	-	-	<1min	<1min	8%
F2	3.5936	1.8704	1.7231	0.4023	0.3706	66%
F3	-	27.2410	-	<1min	0.0254	98%
F4	1.3672	0.1402	1.2271	0.5649	4.9447	75%
F5	1.2901	0.6490	0.6410	1.0813	1.0680	80%
F6	-	2.0090	-	<1min	0.3450	10%
F7	49.9687	14.6843	35.2845	0.0196	0.0472	80%
F8	6.6113	3.9798	2.6315	0.2634	0.1742	60%
F9	0.0382	0.0293	0.0089	77.9643	23.6209	91%
F10	-	-	-	<1min	<1min	52%
F11	-	-	-	<1min	<1min	35%
F12	-	8.3649	-	<1min	0.0829	1%
F13	3.0802	0.4796	2.6006	0.2665	1.4453	45%
F14	-	2.0545	-	<1min	0.3374	41%
F15	-	26.5429	-	<1min	0.0261	51%
F16	29.8739	0.4554	29.4185	0.0236	1.5219	87%
F17	8.8615	0.7139	8.1475	0.0851	0.9709	95%
F18	41.1428	0.1143	41.0284	0.0169	6.0627	86%
F19	0.7497	0.2086	0.5411	1.2811	3.3227	96%
F20	0.0012	0.1225	-	24h +	5.6599	83%
F21	0.0297	0.0046	0.0251	27.6669	150.3940	100%
F22	0.0045	0.0061	-	24h +	113.6511	94%
F23	0.0150	0.0045	0.0105	65.8466	154.2314	99%
F24	0.0064	0.0196	-	24h +	35.3681	100%
F25	0.0197	0.0028	0.0169	40.9295	246.7624	96%
F26	15.8164	0.0339	15.7825	0.0439	20.4624	86%
F27	0.0835	0.0640	0.0196	35.4082	10.8357	97%
F28	0.0078	0.0016	0.0063	110.5401	447.1690	100%
F29	-	-	-	-	<1min	0%
F30	-	0.0593	-	<1min	11.6925	82%
F31	3.0240	0.0451	2.9789	0.2327	15.3620	98%
F32	-	-	-	-	<1min	66%
F33	6.1278	0.0570	6.0708	0.1142	12.1672	34%
F34	-	-	-	<1min	<1min	73%
F35	6.1353	0.6697	5.4656	0.1268	1.0350	80%

Table S1. Overview of fragment reactivity profiling¹. Fragments were evaluated using an HPLC-based surrogate kinetic assay against *N*- α -acetyl-lysine, at pH 10.2 to determine pseudo first-order rate constants

(k) and the corresponding half-lives ($t_{1/2}$). In the case of competing reactions (reaction with *N*- α -acetyl-lysine and degradation), the apparent rate constant is defined as $k_{Lys} = k_{Blank} + k_{Binding}$. Experimentally determined half-lives were calculated as $t_{1/2}(Lys) = \ln 2 / (k_{Lys})$. Reactions were monitored for 24 h and performed in duplicates. The compounds' stability was also evaluated at pH 7.4 calculating the % decomposition of the compound within 1 h. Reactions were monitored at 0 and 1 h and performed in duplicates.

ID	Warhead	SHP2 inhibition at 100 μ M [%]	SHP2 IC ₅₀ [μ M]	Labelled AA
F1	Sulfonyl chloride	33% $\pm$ 4%	n.d.	-
F2	Sulfonyl chloride	85% $\pm$ 10%	>1000 μ M	-
F3	Sulfonyl fluoride	48% $\pm$ 8%	n.d.	-
F4	Fluorosulfate	28% $\pm$ 3%	n.d.	-
F5	Sulfonyl heterocycle (triazole)	7% $\pm$ 4%	n.d.	-
F6	<i>N,O</i> -sulfonate	53% $\pm$ 6%	>1000 μ M	-
F7	<i>N</i> -acyl- <i>N</i> -alkyl sulfonamide (NASA)	75% $\pm$ 0%	>1000 μ M	-
F8	<i>N</i> -acyl- <i>N</i> -alkyl sulfonamide (NASA)	100% $\pm$ 4%	66.9 μ M	K35, K129, K198, K280
F9	Benzothiazinone	4% $\pm$ 1%	n.d.	-
F10	<i>N</i> -succinimidyl ester	100% $\pm$ 2%	10.6 μ M	K35, K129, K198, K213, K260, K274 , K276, K280 , K492
F11	<i>N</i> -succinimidyl ester	100% $\pm$ 1%	29.2 μ M	K199, K260, K274 , K276, K492
F12	<i>N</i> -succinimidyl ester	100% $\pm$ 1%	18.5 μ M	K129, K198, K213, K280 , K492
F13	Bisacetanilide	54% $\pm$ 1%	>1000 μ M	-
F14	Isatoic anhydride	78% $\pm$ 1%	108.3 μ M	-
F15	Isatoic anhydride	78% $\pm$ 5%	43.7 μ M	K35, K199, K280 , K492
F16	Sulfonyl acrylate	100% $\pm$ 2%	2.8 μ M	K199, K260
F17	Dichlorotriazene	100% $\pm$ 6%	12.7 μ M	K198, K280 , K492
F18	Dichlorotriazene	95% $\pm$ 7%	24.8 μ M	K199
F19	Difluorostyrene	46% $\pm$ 7%	n.d.	-
F20	Aldehyde	11% $\pm$ 12%	n.d.	-
F21	<i>N</i> -formyl heterocycle	52% $\pm$ 3%	>1000 μ M	-
F22	Heterocyclic aldehyde	81% $\pm$ 11%	111.7 μ M	-
F23	Ketoaldehyde	69% $\pm$ 2%	>1000 μ M	-
F24	Ketoaldehyde (enolate)	78% $\pm$ 3%	319.7 μ M	-
F25	Hydroxy aldehyde (salicylaldehyde)	100% $\pm$ 2%	16.9 μ M	n.d.
F26	Acylphloroglucinol (salicylaldehyde)	100% $\pm$ 0%	14.4 μ M	n.d.
F27	Formylphenyl boronic acid	100% $\pm$ 2%	102.3 μ M	-
F28	Formylphenyl boronic acid	48% $\pm$ 9%	n.d.	-
F29	Formylphenyl boronic ester	51% $\pm$ 7%	347.4 μ M	-
F30	<i>ortho</i> -Phthalaldehyde (OPA)	100% $\pm$ 1%	0.22 μ M	K213
F31	Ethynyl aldehyde/formylacetylene	25% $\pm$ 15%	n.d.	-
F32	Polycarbonyl	66% $\pm$ 16%	504.0 μ M	-

F33	Squarate	52%±3%	>1000 μ M	-
F34	Isocyanate	28%±4%	n.d.	-
F35	Isothiocyanate	100%±1%	2.9 μ M	n.d.

Table S2. Summary of the warhead types of lysine-targeting covalent probes and corresponding fragment reactivity-profiling results against full-length SHP2 (SHP2^{FL}). Compounds were evaluated using a DiFMUP-based chromogenic assay,² with initial screening at fixed concentration (100 μ M), followed by IC₅₀ determination for the hits resulting >50% inhibition. The compounds were incubated with pre-activated SHP2^{FL} for 60 min at 37 °C, after which DiFMUP was added to assess inhibitory potency. All measurements were performed in triplicates. Compounds having IC₅₀ below 100 μ M were further evaluated by intact MS, followed by tryptic digestion to prove covalent labeling and identify the labelled sites after 60 min pre-incubation at 37 °C. (n.d. not determined)

ID	Stability at 1 h, pH 7.2 [%]
26	32
29	74
37	82

Table S3. Buffer stability of the hit latch compounds. The compounds' stability was also evaluated at pH 7.2 calculating the % decomposition of the compound corrected by the internal standard. Reactions were monitored at 0 and 60 min and performed in duplicates.

Compound	K_I [μM]	std (K_I)	k_{inact} [min⁻¹]	std (k_{inact})	R²	k_{inact}/K_I [μM⁻¹ min⁻¹]
26	0.037	10 ⁻⁶	0.037	10 ⁻⁷	0.95	1.000
37	2.000	10 ⁻⁴	0.033	10 ⁻⁶	0.84	0.017

Table S4. The k_{inact}/K_I of the hit compounds were calculated from time-dependent IC₅₀ measurements by fitting endpoint pre-incubation IC₅₀ data using the EPIC-Fit method.³ The std values are generated by calculating K_I and k_{inact} iteratively with different initial values, and the standard deviation of the resulting values is std. R² is the square of the correlation coefficient between the simulated effects with the fitted k_{inact} and K_I values and the measured effects.

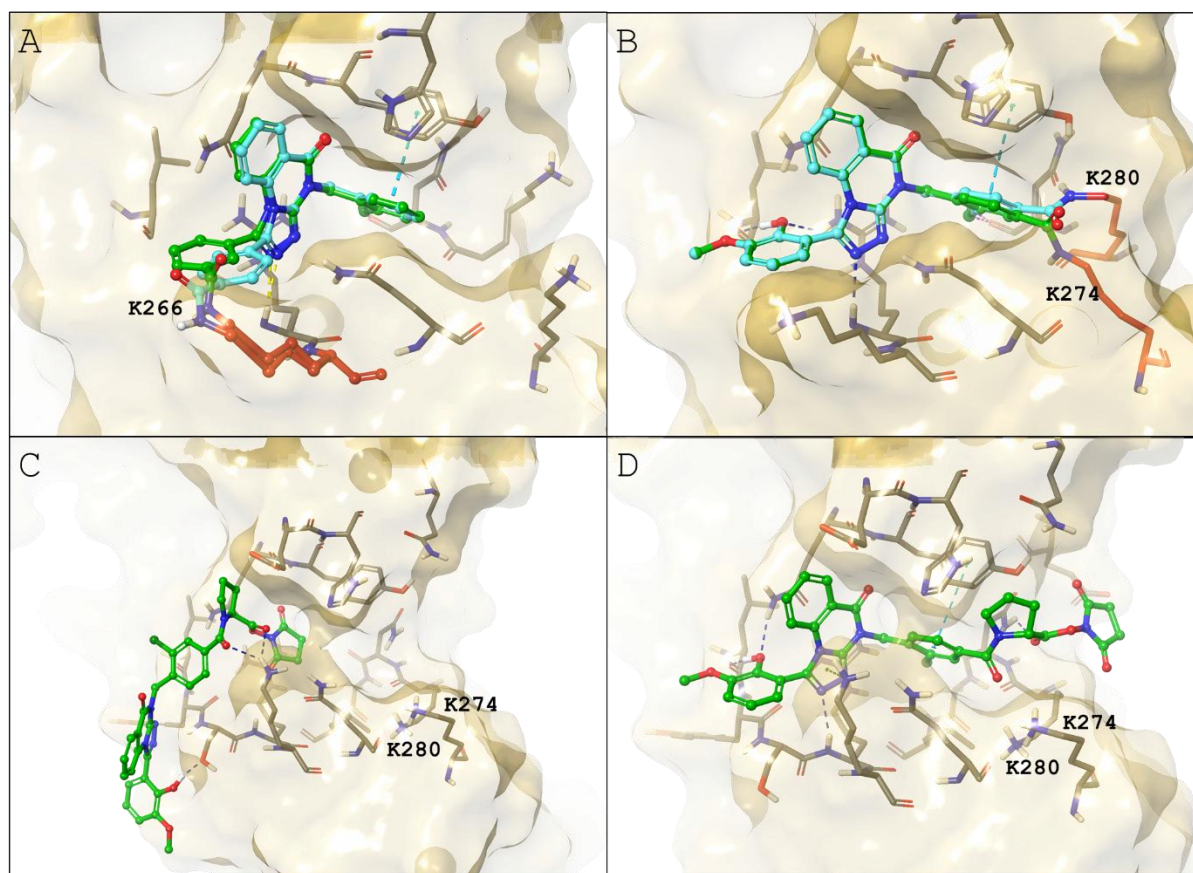


Figure S3. **A:** Covalent predicted binding mode of **SHP504** NHS-ester analogues modified at meta (**12**) or para (**13**) position (green and light blue, respectively), binding to K266 (PDB: 6BMV). **B:** Covalent predicted binding mode of **SHP244** NHS-ester analogue (**31**), binding to K274 or K280 (ligand colored green and light blue, respectively) (PDB: 6BMR). **C:** Best scoring non-covalent predicted binding mode of **30** (Glide Docking Score: -5.165) out of 10 predicted poses (PDB: 29CY). **D:** Best scoring non-covalent predicted binding mode of **26** (Glide Docking Score: -7.122) (PDB: 29CY).

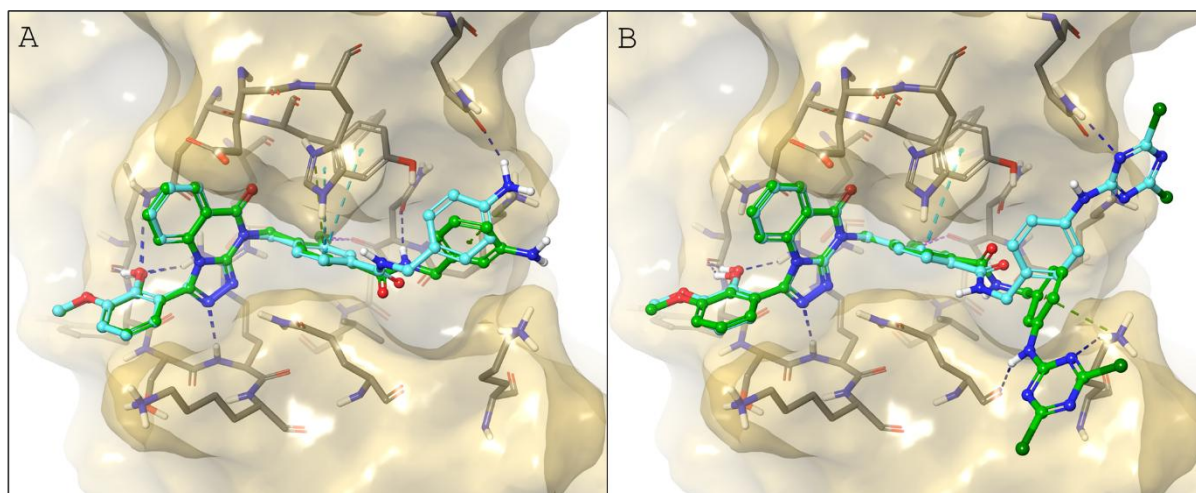


Figure S4. **A:** Predicted binding mode of **32**, and its constitutional isomer with the aniline in para position (green and light blue, respectively) (PDB: 6BMR). **32** fits better into the binding site, being less solvent exposed. **B:** Predicted binding mode of **34**, and its constitutional isomer with the aniline in para position (green and light blue, respectively) (PDB: 6BMR). On **34**, the dichlorotriazine motif establishes two hydrogen bonds with K274 and Q269, and the aniline ring also establishes a π -cation interaction with K274, while in the case of the para positioned isomer, the dichlorotriazine motif establishes only one hydrogen bond, with Q87. Interactions are shown as dashed lines, with light blue coloring corresponding to π - π stacking, dark blue coloring corresponding to hydrogen bond, green coloring corresponding to π -cation interactions and purple coloring corresponding to halogen bonds.

2. Materials and methods

Binding pose prediction using docking

Protein structures with PDB IDs 6BMR,² 6BMV,² 6BMX² and 29CY were prepared with Maestro's Protein Preparation Workflow,⁴ at a pH of 7.4, while ligands were prepared using LigPrep⁴ at a pH range of 7.4 $\pm$ 1.0. For all docking runs (non-covalent and covalent), the receptor grid was set by including the latch site, using the ligand found in the appropriate PDB structure for grid center. Non-covalent docking was performed using Glide SP, while covalent docking was performed using CovDock⁵ (Pose Prediction docking mode), targeting either residue K266, K274 or K280.

SHP2 overexpression, purification

Human SHP2 (aa 2-525) in a pQlinkH plasmid was recombinantly overexpressed in *E.coli* Rosetta DE3 with an N-terminal His7-tag containing a TEV protease cleavage site. Bacteria were cultured in TB-medium at 37 °C to an OD_{600nm} of 0.8, then shifted to 18 °C. Protein expression was induced overnight with 500 µM IPTG. Cells were harvested by centrifugation at 7,000 rpm for 15 min. Cells were lysed by two freeze-thaw cycles in lysis buffer (1x PBS, 0.5M NaCl, 5% Glycerol, 1 µg/ml lysozyme, 0.1% Triton X-100, 2 µg/ml DNaseI, 250 µM Pefabloc), followed by clarification of the lysate by centrifugation at 22,500 rpm for 45 min at 10 °C. The supernatant was supplemented with 15 mM imidazole and applied onto a HisTrapTM HP Ni-IMAC column (Cytiva) pre-equilibrated with buffer A (20 mM Tris pH 8, 0.5 M NaCl). His-tag bound SHP2 was eluted using an imidazole gradient from 15 mM up to 250 mM, and fractions containing the protein were combined.

His-tagged TEV-protease was added, and the sample was dialyzed over night at 4 °C against buffer A to remove imidazole and allow tag cleavage. The mixture was passed again over the Ni affinity column, and the flow-through containing tag-free SHP2 was collected and concentrated. This material was subjected to size-exclusion chromatography on a HiLoad Superdex 200 16/600 column in 20 mM Tris pH 8, 150 mM NaCl, 2 mM DTT. Fractions containing SHP2 were pooled, concentrated to 15 mg/mL, and purity was assessed by SDS-PAGE.

Crystallization and structure determination

Purified SHP2₍₂₋₅₂₅₎ was incubated with compound **26** at a final concentration of 1 mM in a solution containing 4% DMSO, which was subsequently used for crystallization. The complex was crystallized by the sitting-drop vapor-diffusion technique, in which 200 nL of the protein–ligand solution was mixed with 200 nL of reservoir solution composed of 20% (w/v) PEG 3350, 0.2 M sodium acetate, and 0.1 M Bis-Tris propane pH 8.5.

Drop setup was carried out using a Gryphon robotic dispenser (Matrix Technologies Co.), and plates were stored and monitored in a Rock Imager 1000 system (Formulatrix). Crystals grew within 7 days and were transferred into a cryoprotectant consisting of the crystallization mother liquor supplemented with 20% ethylene glycol and then rapidly frozen in liquid nitrogen.

Diffraction data were recorded to 2.6 Å at BL14.1 at BESSY II (Helmholtz-Zentrum Berlin, HZB), processed and scaled using XDSapp.⁶ The complex structure was solved by molecular replacement with Phaser⁷ using the SHP2 structure (PDB: 5EHR) as search model. The structure were manually

adjusted using COOT⁸ and iteratively refined using Phenix⁹ Rwork of 24.8% and Rfree of 29.6% (Table S5).

Data availability

The atomic coordinates of SHP2₍₁₋₅₂₅₎ with bound compound **26** have been deposited in the Protein Data Bank under accession code 29CY. All other data relevant to this work are available from authors on request.

Table S5 Data collection and refinement statistics

	SHP2 ₍₂₋₅₂₅₎ + compound 26
Data collection	
Space group	P2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	45.2, 213.0, 55.4
α , β , γ	90.0, 95.5, 90.0
Resolution (Å)*	44.9 - 2.62 (2.79-2.63)
<i>R</i> _{meas} *	14.8 (54.1)
$\langle I / \sigma(I) \rangle$ *	6.1 (1.2)
Completeness (%)*	84.8 (66.2)
Redundancy*	2.6 (2.0)
CC(1/2)	98.9 (72.0)
No. reflections	67388 (6520)
No. unique reflections	26089 (3264)
Refinement	
<i>R</i> _{work} / <i>R</i> _{free}	0.248/0.295
No. atoms	
Protein	8142
Ligand/ion	40
Water	145
Mean B factor (Å ²)	53.5
R.m.s deviations	

Bond lengths (Å)	0.005
Bond angles (°)	1.18
Ramachandran outlier (%)	0.2
Mol/AU	2

* Data in highest resolution shell are indicated in parenthesis.

HPLC-MS-based *N*- α -acetyl-lysine reactivity assay¹

HPLC-MS measurements were performed using a Shimadzu LCMS-2020 device equipped a positive–negative double ion source (DUIS $\pm$) and a quadrupole MS analyzer in the range of m/z 50–1000. The sample was eluted with gradient elution using eluent A (0.1% HCOOH in H₂O) and eluent B (0.1% HCOOH in MeCN). The column temperature was always kept at 30 °C; the injection volume was 10 μ L, and the flow rate was set to 1.5 mL/min. A Reprospher C18 (5 μ m, 100 mm $\times$ 3 mm) column was used along with the following gradient. The initial condition was 0% B eluent, followed by a linear gradient to 100% B eluent by 2 min; from 2 to 3.5 min, 100% B eluent was retained. From 3.5 to 4.5 min, the initial condition with 0% B eluent was restored and retained until 5 min.

For the reactivity and stability assay, in a glass LC-MS vial the internal standard (indoprofen or papaverine or 1,4-dicyanobenzene, 50 μ L, 2 mM in acetonitrile) and the 100 mM BBS buffer solution (900 μ L, pH 10.2) was mixed with or without 50 mM *N*- α -acetyl-lysine (providing results of reactivity or stability, respectively). Finally, to this mixture the corresponding electrophile was added (50 μ L, 20 mM in acetonitrile) right before the measurement. The vial was mixed, then analyzed by HPLC-MS (10 μ L injection volume) at intervals of approximately 60 min (0–4 h, 6 h, 8 h, 12 h, 24 h). Final volume was 1 mL. In the case of highly reactive compounds, the measurement was done within 1 h, monitored every 5 minutes. The AUC (area under the curve) values were determined via integration of HPLC or MS chromatograms and then corrected with the internal standard. The fragments' AUC values were subjected to ordinary least-squares (OLS) linear regression, and to compute the important parameters (kinetic rate constant and half-life time), an Excel sheet was applied. The data are expressed as means of duplicate determination. The kinetic rate constant for the degradation and corrected *N*- α -acetyl-lysine reactivity were calculated as follows. The reaction half-life for pseudo-first-order reactions ($t_{1/2}$) is $\ln 2/k$, where k is the reaction rate. In the case of competing reactions (reaction with *N*- α -acetyl-lysine and degradation), the apparent reaction rate is $k_{\text{Lys}} = k_{\text{Blank}} + k_{\text{Binding}}$. When half-lives are measured experimentally,

$t_{1/2}(\text{Lys}) = \ln 2 / (k_{\text{Lys}}) = \ln 2 / (k_{\text{Blank}} + k_{\text{Binding}})$. In our case, the corrected k_{Blank} and k_{Lys} (regarding blank and *N*- α -acetyl lysine-containing samples, respectively) can be calculated by linear regression of the measured kinetic data points. The corrected k_{Binding} is calculated as $k_{\text{Lys}} - k_{\text{Blank}}$, and finally, the half-life is determined using the equation $t_{1/2} = \ln 2 / k$.

Assessment of stability at pH 7.2

The intrinsic stability of compounds **26**, **29** and **37**, was characterized at pH 7.2 in HEPES buffer (60 mM HEPES, 75 mM NaCl, 75 mM KCl, 1 mM EDTA). A 10 mM solution of the compound (5 μL) in DMSO and 1 mM solution of indoprofen or papaverine in DMSO as the internal standard (5 μL) was added to HEPES buffer resulting in a final reaction volume of 500 μL with 2% DMSO. The reaction mixture was analyzed by HPLC-MS sampling after 0 and 1 h, respectively. The AUC (area under the curve) values were determined via integration of HPLC or MS chromatograms and then corrected with the internal standard.

Biochemical Assay²

SHP2 is allosterically activated upon binding of *bis*-tyrosyl-phosphorylated peptides to its Src Homology 2 (SH2) domains. This activation step results in the release of SHP2's auto-inhibitory interface, thereby activating the PTP domain and enabling substrate recognition and catalytic activity.

The inhibitory activity against SHP2^{FL} (amino acids 2-525) was evaluated *in vitro* by measuring its catalytic activity using DiFMUP (Sigma, SML2821) as the surrogate substrate in a modified fluorescence-based assay. The phosphatase reactions were performed in a 384-well, black polystyrene, flat bottom, non-binding surface plate (Greiner, Ref#781900) using a final reaction volume of 25 μL . SHP2^{FL} (5 nM) and *bis*-phosphorylated IRS-1 peptide (sequence: H₂N-LN(pY)IDL₂LDLV(dPEG8) LST(pY)ASINFQK-amide, GenScript, 2 μM) was incubated for 5 min at ambient temperature. The pre-activated protein master mix (24.5 μL) was added to the tested compounds (0.5 μL , in DMSO) – concentrations varying from 0.001 to 2000 μM – in the following assay buffer conditions: 60 mM HEPES, 75 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Tween-20 (pH=7.2). After 60 min incubation at 37 °C, DiFMUP (Sigma, Cat#SML2821, 200 μM) was added to the reaction wells and incubated for 30 min at 25 °C. The fluorescence signals were

measured (Ex:360/Em:450) using a microplate reader (Molecular Devices SpectraMax iD5 Multimode Microplate Reader, San Jose, CA, USA). The inhibitor dose-response curves were analyzed in GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) using a normalized nonlinear regression model to determine IC_{50} values (with 95% confidence intervals). Data was normalized to control conditions, where the negative control (containing DMSO and buffer only) was defined as 100% inhibition and the positive control (containing DMSO and protein without inhibitor) was defined as 0% inhibition. Normalized values were then fitted to a sigmoidal dose-response (variable slope) curve to calculate the half-maximal inhibitory concentration (IC_{50}) for each compound. All measurements were performed in triplicates.

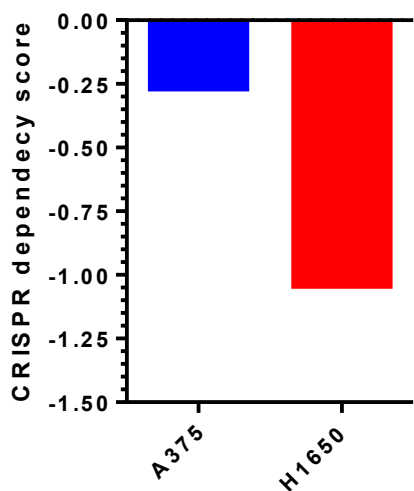
Time-dependent IC_{50} measurements and k_{inact}/K_I calculation

The phosphatase reactions were performed as described above, in a 384-well, black polystyrene, flat bottom, non-binding surface plate (Greiner, Ref#781900) using a final reaction volume of 25 μ L. SHP2^{FL} (5 nM) and *bis*-phosphorylated IRS-1 peptide (sequence: H₂N-LN(pY)IDL₂LDLV(dPEG8) LST(pY)ASINFQK-amide, GenScript, 2 μ M) was incubated for 5 min at ambient temperature. The pre-activated protein master mix (24.5 μ L) was added to the tested compounds (0.5 μ L, in DMSO) – concentrations varying from 0.001 to 2000 μ M – in the following assay buffer conditions: 60 mM HEPES, 75 mM NaCl, 75 mM KCl, 1 mM EDTA, 0.05% Tween-20 (pH=7.2). Following incubation periods of 15, 30, 45, and 60 minutes for compound **26**, and pre-incubation periods of 15, 30, 60, and 90 minutes for compound **37** at 37 °C, DiFMUP (Sigma, Cat#SML2821, 200 μ M) was added to the reaction wells and incubated for 30 min at 25 °C. The fluorescence signals were measured (Ex:360/Em:450) using a microplate reader (Molecular Devices SpectraMax iD5 Multimode Microplate Reader, San Jose, CA, USA). The inhibitor dose-response curves were analyzed in GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) using a normalized nonlinear regression model to determine IC_{50} values (with 95% confidence intervals). Data was normalized to control conditions, where the negative control (containing DMSO and buffer only) was defined as 100% inhibition and the positive control (containing DMSO and protein without inhibitor) was defined as 0% inhibition. Normalized values were then fitted to a sigmoidal dose-response (variable slope) curve to calculate the half-maximal inhibitory concentration (IC_{50}) for each compound. All measurements were performed in triplicates. The k_{inact}/K_I of the hit compounds were calculated from the time-dependent IC_{50} measurements by

fitting endpoint pre-incubation IC_{50} data using the EPIC-Fit method.³ The std values are generated by calculating K_I and k_{inact} iteratively with different initial values, and the standard deviation of the resulting values is std. R^2 is the square of the correlation coefficient between the simulated effects with the fitted k_{inact} and K_I values and the measured effects.

Cell Lines and Culture Conditions

For establishing suitable cell model system to test SHP2 inhibitors on the most appropriate way was used the Cancer Dependency Map (DepMap) available at <https://depmap.org/>. Based on CRISPR dependency score, where the lower score means more dependent (sensitive) on SHP2 inhibition, there were chosen a human lung cancer cell line sensitive on SHP2 inhibition (H1650) as well as melanoma cell line resistant on SHP2 inhibition (higher dependency score) such as A375.



Left: SHP2 CRISPR dependency of chosen cancer cell lines from DepMap. The lower value means that cell line is more dependent on SHP2 inhibition, meaning that it is more sensitive on SHP2 inhibition. Higher value means that cell line is less dependent on SHP2 inhibition, meaning that it is more resistant on SHP2 inhibition.

Human melanoma (A375), and human lung cancer (H1650) cell lines were cultured in RPMI 1640 Medium with glutamine (Roswell Park Memorial Institute Medium, Biosera, Nuaille, France), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Biosera) and 1% Penicillin/Streptomycin (Biosera). The cell lines were obtained from the American Type Culture Collection (ATCC). Cells were cultured in sterile T25 flasks with ventilation caps (Sarstedt, Nümbrecht, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂ within an ESCO Cell Culture Incubator (ESCO, Friedberg, Germany). All cell handling procedures were carried out in a biosafety cabinet (laminar flow), specifically the ESCO Sentinel Gold class II model AC2-4E8 (ESCO). Routine testing confirmed the absence of mycoplasma contamination in the cell cultures.

Real-time quantitative PCR (qPCR)

Cells were harvested using PBS, Trypsin, and EDTA (Biosera) and seeded in density of 0.3×10^6 cell per well, in 6-well plates (Sarstedt). Treatment was applied in concentration 1 μ M for 2 hours in FBS free medium. Control wells was treated with FBS free medium. After 2 hours, treatment was discarded, cells were washed in PBS (Serana, Pessin, Germany), and total RNA was isolated from cells using TRIzol reagent (Ambion, Kaufungen, Germany). Reverse transcription and synthesis of cDNA from mRNA was performed with Reverse Transcription System (Promega, Madison, WI, USA) kit, and Mastercycler gradient thermo cycler (Eppendorf, Hamburg, Germany). qPCR was conducted using SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) on CFX96 real-time PCR system (BioRad, Hercules, CA, USA). CFX Manager software (BioRad, Hercules, CA, USA) was used for determination of Cq values. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method where expressions were normalized to beta actin as a housekeeping gene and then expressions under treatments were normalized to the control for each cell line. A commonly used pharmacodynamic MAPK pathway marker downstream of SHP2 (DUSP6) mRNA expression levels were measured. Two different primer pairs of DUSP6 gene were applied. The primer sequences were as follows: Human DUSP6: forward1: 5'-AAC AGG GTT CCA GCA CAG CAG-3', reverse1: 5'-GGC CAG ACA CAT TCC AGC AA-3'; Human DUSP6: forward2 5'-CTC GGA TCA CTG GAG CCA AAA C-3', reverse2 5'-GTC ACA GTG ACT GAG CGG CTA A-3'; human beta actin: forward 5'- CAC CAT TGG CAA TGA GCG GTT C -3', reverse 5'- AGG TCT TTG CGG ATG TCC ACG T -3'.

The treatment concentration was chosen based on IC₅₀ values of 3 compounds. At 1 μ M concentration, compound **24** may show little or no effect on DUSP6 suppression, compound **37** should show moderate-to-strong suppression, while compound **26** should strongly suppress and DUSP6.

The 2 hours treatment duration was chosen based on the reference publication (Fodor et al, ref 2, PMID: 29304282), and another literature data where the effect the treatment on mRNA level of DUSP6 gene is measured after 2 hours of the treatment.

3. Biochemical assay results

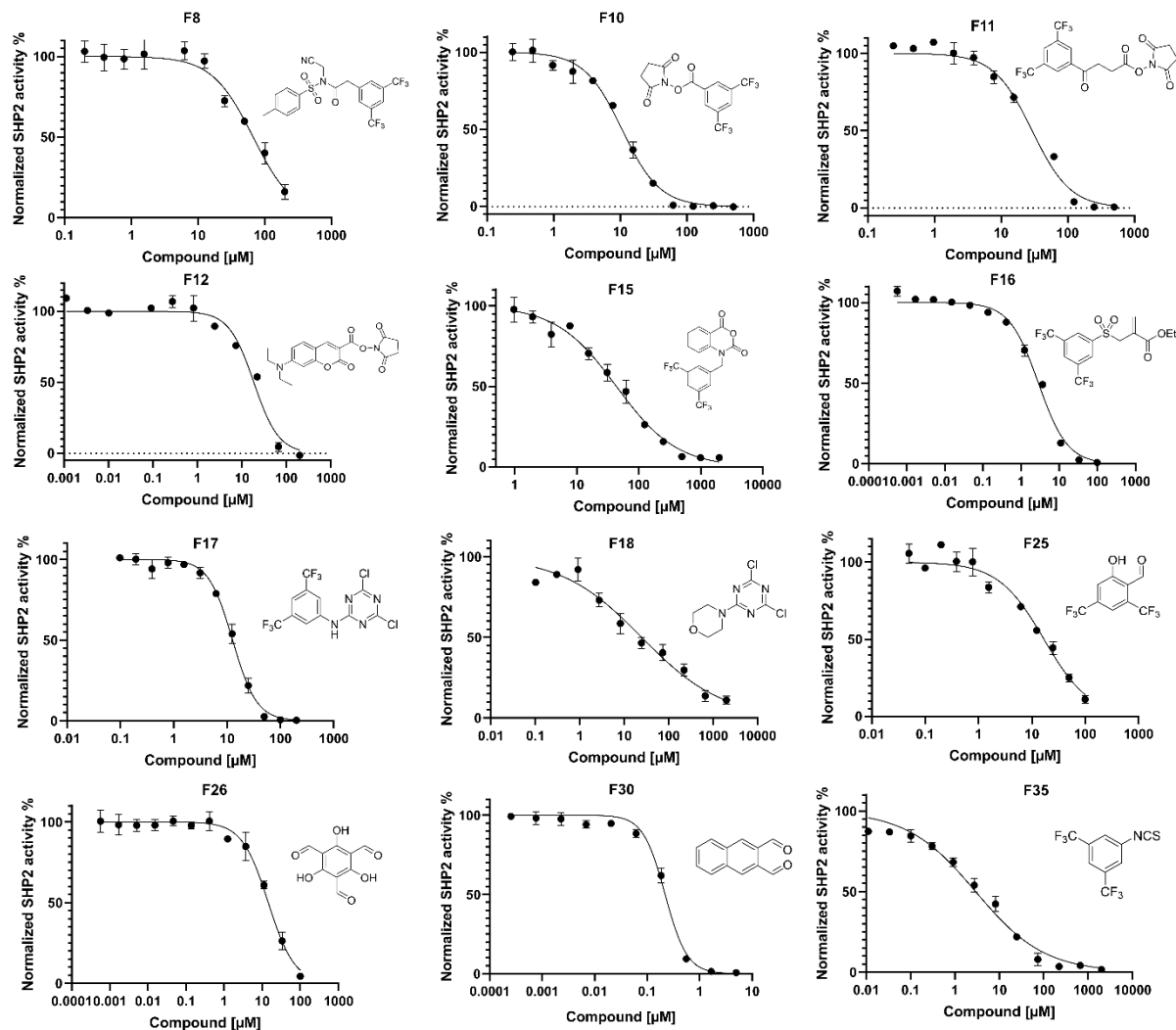


Figure S5. DiFMUP assay dose-response curves for fragments showing < 100 μM IC₅₀. Titration curves of the fragments [F8 (IC₅₀=66.9 ± 9.2 μM, R²=0.9543); F10 (IC₅₀=10.6 ± 0.98 μM, R²=0.9876); F11 (IC₅₀=29.2 ± 4.0 μM, R²=0.9876); F12 (IC₅₀=18.5 ± 3.6 μM, R²=0.9740); F15 (IC₅₀=43.7 ± 5.6 μM, R²=0.9806); F16 (IC₅₀=2.8 ± 0.3 μM, R²=0.9907); F17 (IC₅₀=12.7 ± 0.9 μM, R²=0.9930); F18 (IC₅₀= 24.8 ± 7.7 μM, R²=0.9633), F25 (IC₅₀=16.9 ± 3.3 μM, R²=0.9699), F26 (IC₅₀=14.4 ± 2.1 μM, R²=0.9778), F30 (IC₅₀=0.22 ± 0.02 μM, R²=0.9906), F35 (IC₅₀=2.9 ± 0.6 μM, R²=0.9813)] against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

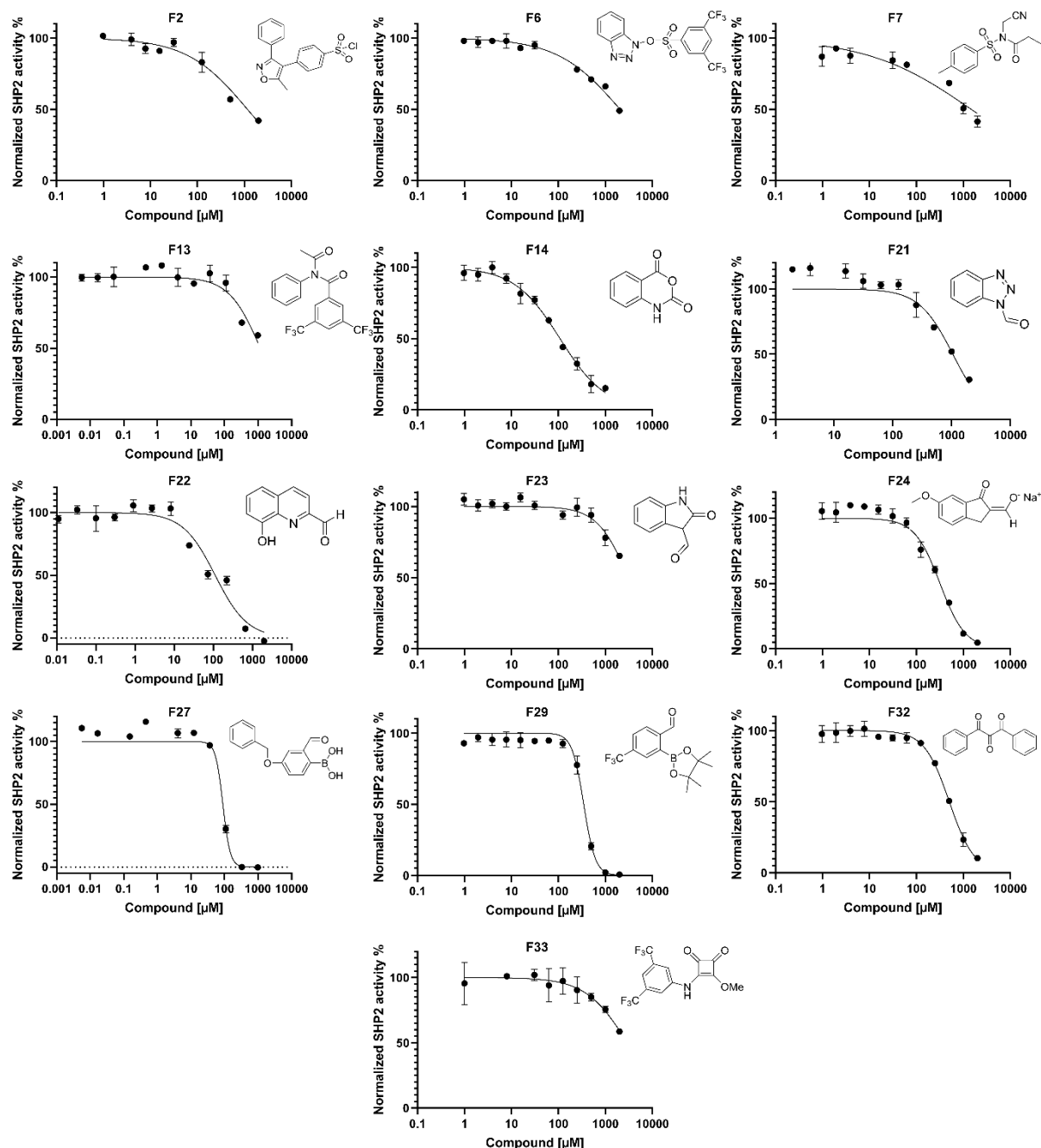


Figure S6. DiFMUP assay dose-response curves for fragments showing > 100 μM IC₅₀. Titration curves of the fragments [F2, F6, F7, F13, F21, F23, F33 (IC₅₀ >1000 μM); F14 (IC₅₀=108.3 ± 15.4 μM, R²=0.9805); F22 (IC₅₀=111.7 ± 31.5 μM, R²=0.9547); F24 (IC₅₀=319.7 ± 35.5 μM, R²=0.9787); F27 (IC₅₀=102.3 ± 15.7 μM, R²=0.9656); F29 (IC₅₀=347.4 ± 26.3 μM, R²=0.9822); F32 (IC₅₀=504.0 ± 40.2 μM, R²=0.9847)] against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

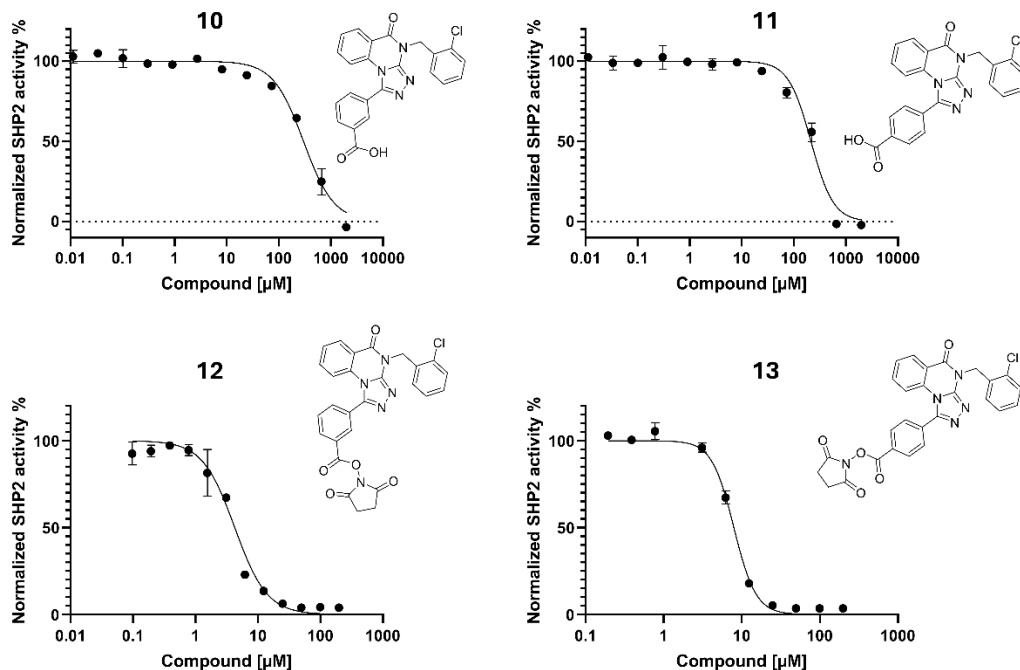


Figure S7. DiFMUP assay dose-response curves for compound 10-13. Titration curves of compound **10** (IC₅₀=299 ± 39 μM, R²=0.9769), **11** (IC₅₀=214 ± 27 μM, R²=0.9752), **12** (IC₅₀=4.3 ± 0.5 μM, R²=0.9842) and **13** (IC₅₀=7.9 ± 0.5 μM, R²=0.9940) against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

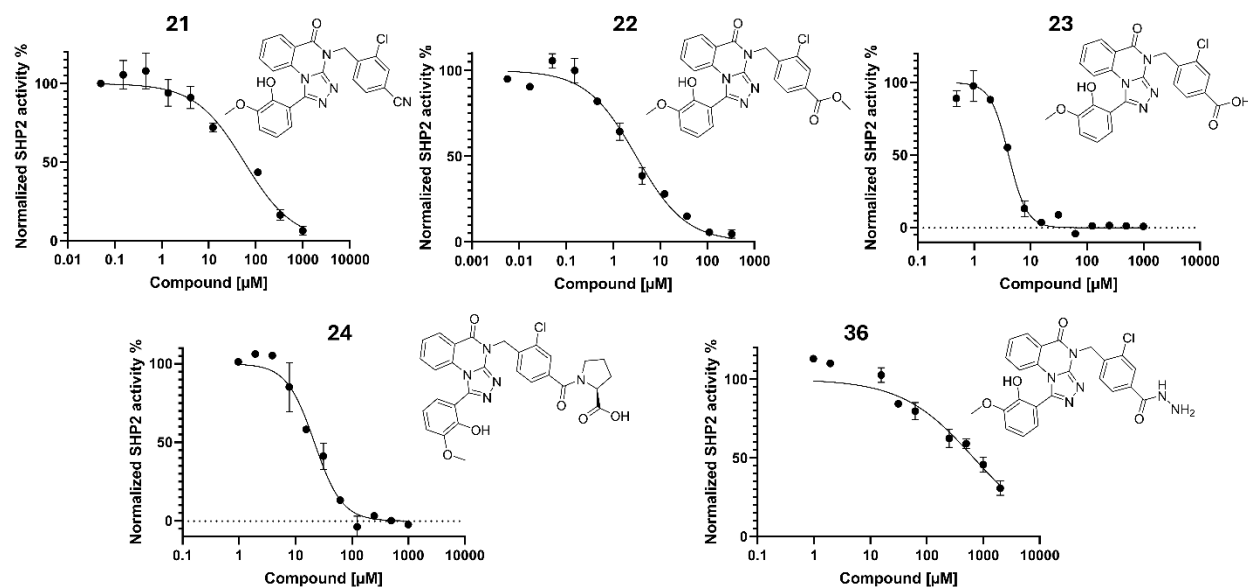


Figure S8. DiFMUP assay dose-response curves for compound 21-24 and 36. Titration curves of compound **21** (IC₅₀=57.3 ± 20.3 μM, R²=0.9710), **22** (IC₅₀=3.1 ± 0.7 μM, R²=0.9781), **23** (IC₅₀=4.1 ± 0.2 μM, R²=0.9783), **24** (IC₅₀=21.6 ± 3.5 μM, R²=0.9737) and **36** (IC₅₀=677 ± 180 μM, R²=0.9234) against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

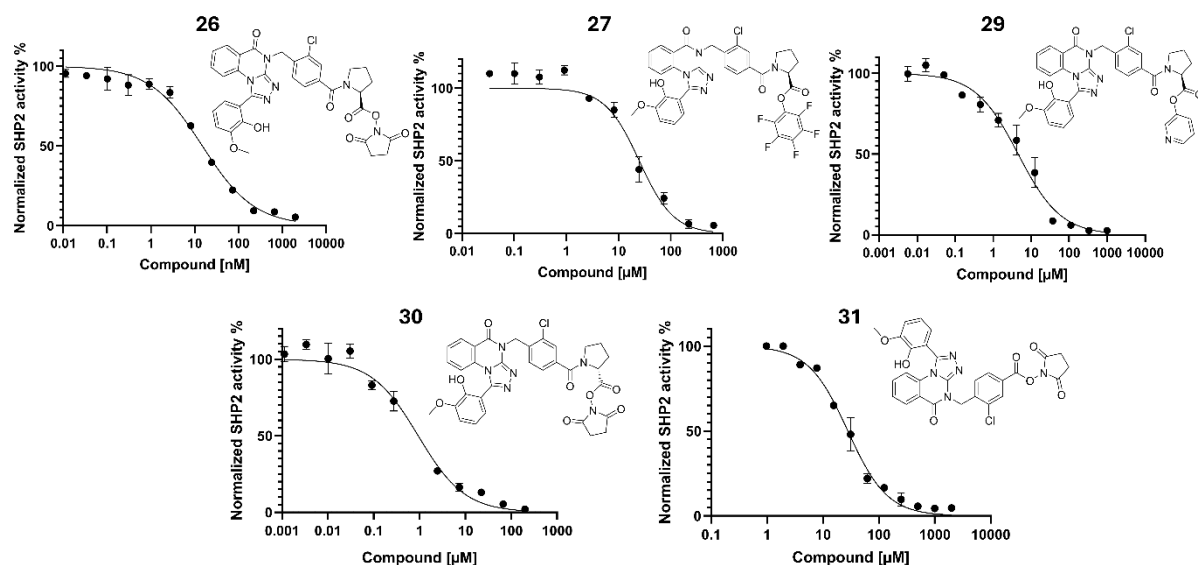


Figure S9. DiFMUP assay dose-response curves for compound 26, 27, 29-31. Titration curves of compound **26** ($IC_{50} = 15.0 \pm 2.3$ nM, $R^2 = 0.9852$), **27** ($IC_{50} = 25.1 \pm 5.3$ μ M, $R^2 = 0.9620$), **29** ($IC_{50} = 4.5 \pm 1.0$ μ M, $R^2 = 0.9817$), **30** ($IC_{50} = 0.94 \pm 0.24$ μ M, $R^2 = 0.9768$) and **31** ($IC_{50} = 28.0 \pm 3.3$ μ M, $R^2 = 0.9844$) against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

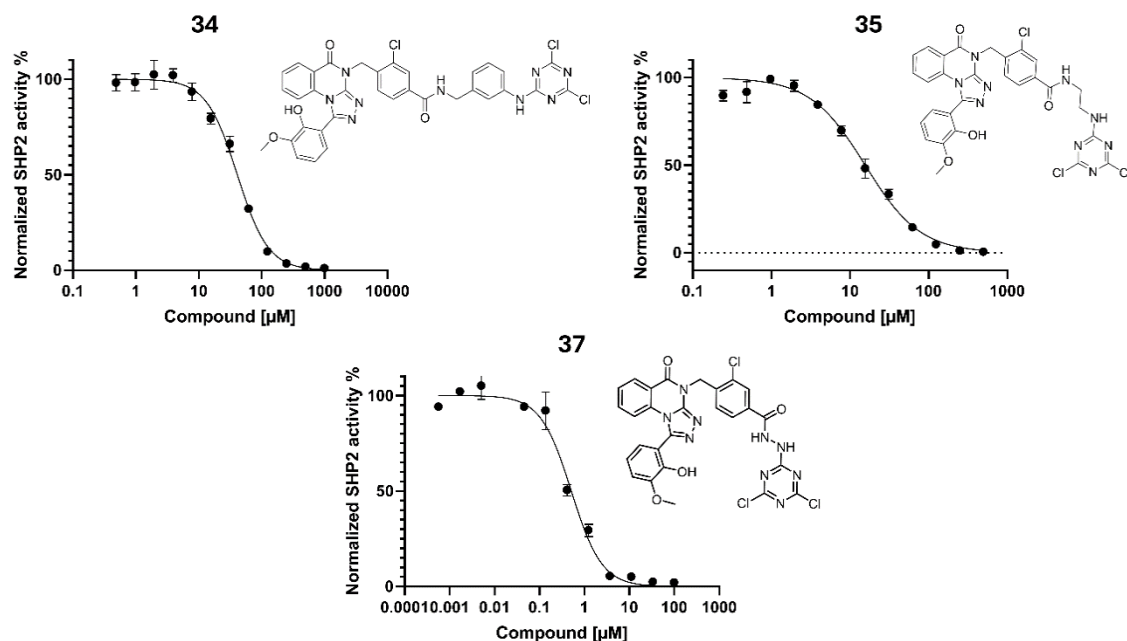


Figure S10. DiFMUP assay dose-response curves for compound 34, 35 and 37. Titration curves of compound **34** ($IC_{50} = 41.0 \pm 3.4$ μ M, $R^2 = 0.9911$), **35** ($IC_{50} = 15.6 \pm 1.6$ μ M, $R^2 = 0.9859$) and **37** ($IC_{50} = 0.51 \pm 0.08$ μ M, $R^2 = 0.9802$) against SHP2^{FL} in presence of DiFMUP and the activating bis-phosphorylated IRS-1 peptide. Error bars represent standard deviation (SD) from biological triplicates.

Figure S11. Results of the Reaction Biology phosphatase assays for compounds **SHP844** (**24**) and dichlorotriazine **37** against SHP1 (https://www.reactionbiology.com/datasheet/ptpn6_shp1_malvern_phos/) and PTP1B (https://www.reactionbiology.com/datasheet/ptpn1_ptp1b-fl_malvern_phos/).

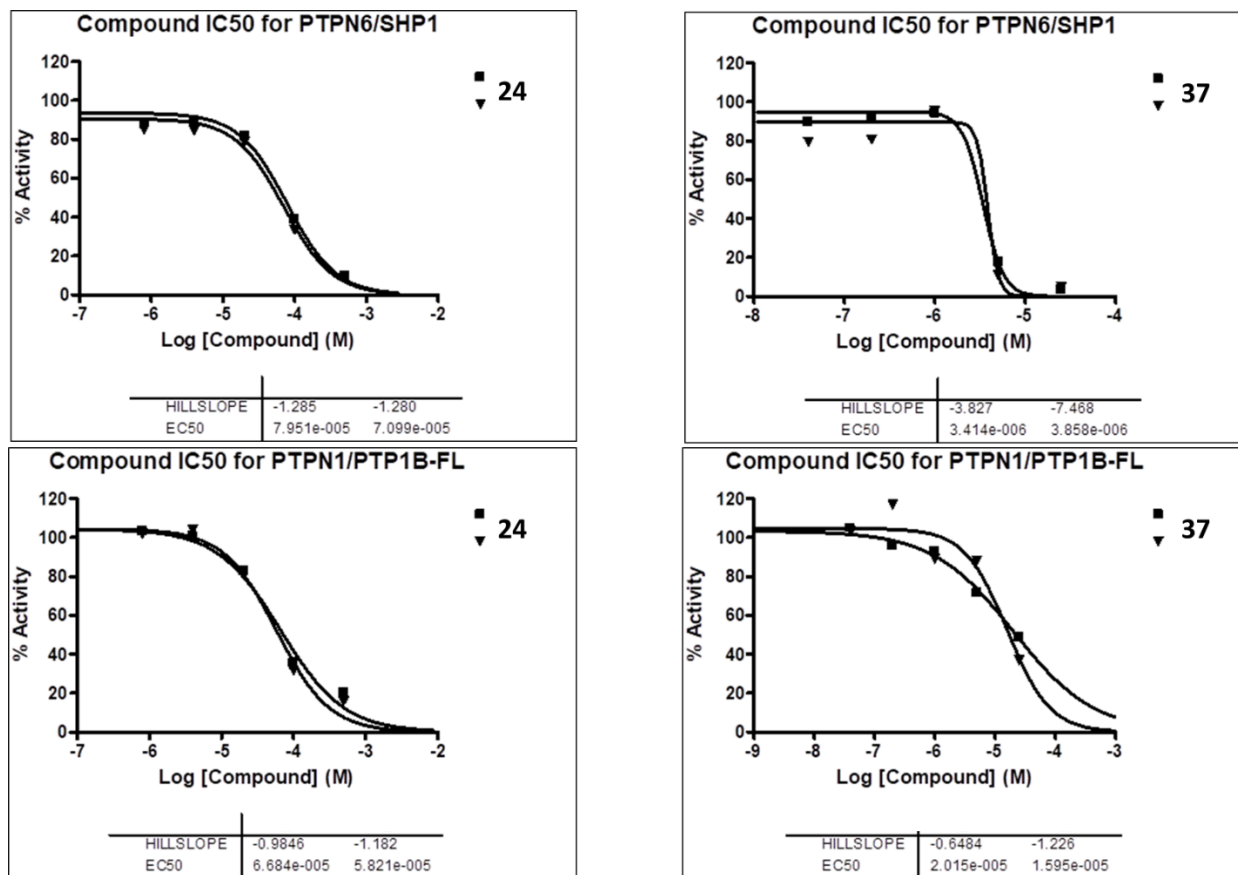
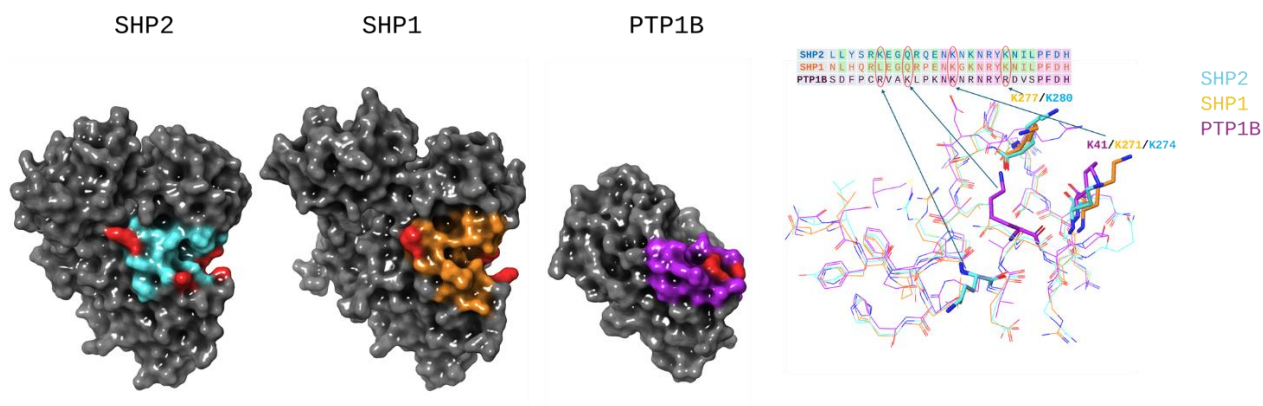


Figure S12. Comparison of the latch site in SHP1, SHP2 and PTP1B.



4. Mass spectrometry experiments and results

Covalent labeling of SHP2

The protein was diluted with HEPES buffer (60 mM HEPES, 75 mM NaCl, 75 mM KCl, 1 mM EDTA (pH=7.2) to 1 mg/mL. To a microcentrifuge tube (Eppendorf Protein LoBind tube, PCR clean, withstand, capacity 0.5 mL) 1 μ L of 40 mM fragment solution or 4 mM inhibitor solution in DMSO was added in each tube and diluted with the protein solution to 50 μ L (protein/compound ratio was 1:50 in case of the fragments and 1:5 in case of the latch-targeting compounds) and the final sample contained 2% DMSO). The samples were incubated at 37 °C for 60 minutes, then buffer exchange was performed three times with Sartorius Vivaspin 500 10000 MWCO at 12000 g for 6 minutes each time to remove the excess of the compounds. Afterwards, the concentrations were set to 1 mg/mL.

Intact mass spectrometry experiments

Next, 5 μ L of the samples were transferred into microvials, diluted with MilliQ water to 0.2 mg/mL, and the labeling was analyzed in intact mass spectrometry by using a Triple TOF 5600+ hybrid Quadrupole-TOF LC-MS/MS system (Sciex, MA, USA) equipped with a DuoSpray IonSource coupled with a Shimadzu Prominence LC20 UFLC (Shimadzu, Japan) system consisting of binary pump, an autosampler, and a thermostated column compartment, equipped with a Phenomenex SecurityGuard Widespore C4 4x3mm cartridge. The separation was achieved using mobile phase A (5% MeCN, 0.1% formic acid modifier) and B (95% MeCN, 5% water, 0.1% formic acid modifier) using a gradient elution. A 4 min gradient (both in solvent composition and flow rate) was used with an initial flow of 0.5 mL/min and 10% eluent B. A 2 min linear increase was applied to reach the final flow of 1 mL/min and maximum eluent composition of B at 65%. These parameters were held for 0.5 min, and a 0.5 min linear gradient was used to reach the initial flow rate and eluent composition. This was followed by a 1 min equilibrating part. Data acquisition and processing were performed using Analyst TF software version 1.7.1 (AB Sciex Instruments, CA, USA).

Digestion experiments

After the labeling was confirmed by intact MS, 25 μL of the sample and 5 μL 0.2% (w/v) RapiGest SF (Waters, Milford, USA) solution buffered with 50 mM ammonium bicarbonate were mixed (pH=7.8) and 3 μL of 45 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 were added and kept at 37.5 $^\circ\text{C}$ for 30 min. After cooling the sample to room temperature, 3.5 μL of 100 mM iodoacetamide in 100 mM NH_4HCO_3 were added and placed in the dark at room temperature for 30 min. The reduced and alkylated protein was then digested by 3.5 μL (1 mg/mL) trypsin (the enzyme-to-protein ratio was 1:10) (Sigma, St Louis, MO, USA). The sample was incubated at 37 $^\circ\text{C}$ for overnight. To degrade the surfactant, 3 μL of formic acid (500 mM) solution was added to the digested protein sample to obtain the final 40 mM concentration (pH $\approx$ 2) and was incubated at 37 $^\circ\text{C}$ for 45 min. For LC-MS analysis, the acid treated sample was centrifuged for 5 min at 13 000 rpm and the supernatant was pipetted into a microvial.

LC-MS/MS measurements

To get more precise information on the structure, samples were further analyzed by a Triple TOF 5600+ hybrid Quadrupole-TOF LC-MS/MS system (Sciex, MA, USA) equipped with a DuoSpray IonSource coupled with a Shimadzu Prominence LC20 UFLC (Shimadzu, Japan) system consisting of quaternary pump, an autosampler, and a thermostated column compartment.

Data acquisition and processing were performed using Analyst TF software version 1.7.1 (AB Sciex Instruments, CA, USA). Chromatographic separation was achieved on the Discovery® BIO Wide Pore C-18-5 (250 mm $\times$ 2.1mm, 5 μm , 300 $\text{\AA}$) HPLC column. Sample was eluted in gradient elution mode using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeCN). The initial condition was 5% B for 7 min, followed by a linear gradient to 90% B by 48 min, from 55 to 63 min 90% B was retained; and from 63 to 65 min back to initial condition with 5 % eluent B and retained for 10 min. Flow rate was set to 0.2 ml/min. The column temperature was 40 $^\circ\text{C}$ and the injection volume was 15 μL . Nitrogen was used as the nebulizer gas (GS1),

heater gas (GS2), and curtain gas with the optimum values set at 35, 35 and 35 (arbitrary units), respectively. The source temperature was 350 °C and the spray voltage was set to 5000 V.

Advanced Information Dependent Acquisition (IDA) mode was used on the TripleTOF 5600+ system to obtain MS/MS spectra on the 8 most abundant parent ions present in the TOF survey scan. In IDA LC-MS/MS experiments the mass spectra and tandem mass spectra were recorded in “high-sensitivity” mode with a resolution of ~35,000 full-width half-maximum.

In the first period (positive TOF MS mode) the data were acquired in the mass range of $m/z=300$ to 2500, with 0.1 s accumulation time. The declustering potential value was set to 60 V. The intensity threshold for precursor ion selection in TOF survey scan mode was 1000 cps. In MS2 experiments (Product Ion scan mode) the mass range was $m/z=50$ to 3000, with an accumulation time of 0.1 sec.

Initial data was handled with PeakView software (version 2.2, Sciex) and Sciex OS (version 3.4.5.828). Acquisition files were converted for open-source applications by MSConvert (version 3.0.25071, ProteoWizard). Bottom-up proteomics search was executed by SearchGUI (version 4.3.15, CompOmics) with four algorithms (Comet, Tide, MetaMorpheus, and Sage). Results were analyzed by PeptideShaker (version 3.0.11, CompOmics). Novel self-made software, developed with the Python programming language (version 3.11.0), pyOpenMS (version 3.3.0), and with the help of Spyder Integrated Development Environment (version 5.5.0), was used for the final evaluation. Graphs were prepared with Interactive Peptide Spectral Annotator.

Results of the mass spectrometry experiments

Intact MS spectra

- Lysine-targeting fragment hits**

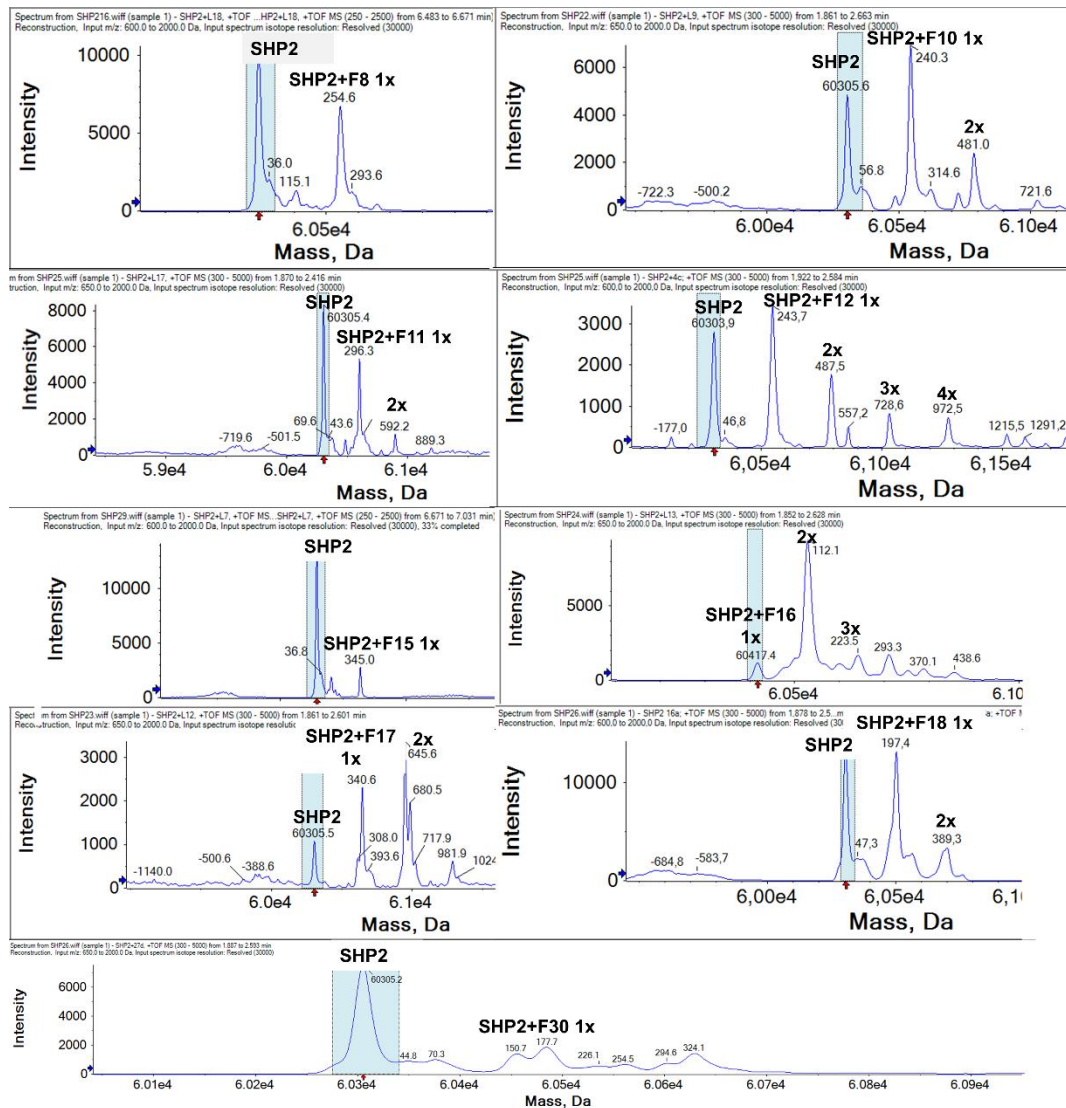


Figure S11. Intact MS hits of the lysine-targeting fragment library. Out of 12 assay hit 9 showed covalent labelling of SHP2 after 1h incubation with SHP2 FL on 37 °C. The intact MS hits were then digested via HPLC-MS/MS to identify the labelled site via tryptic digestion.

- Designed inhibitors**

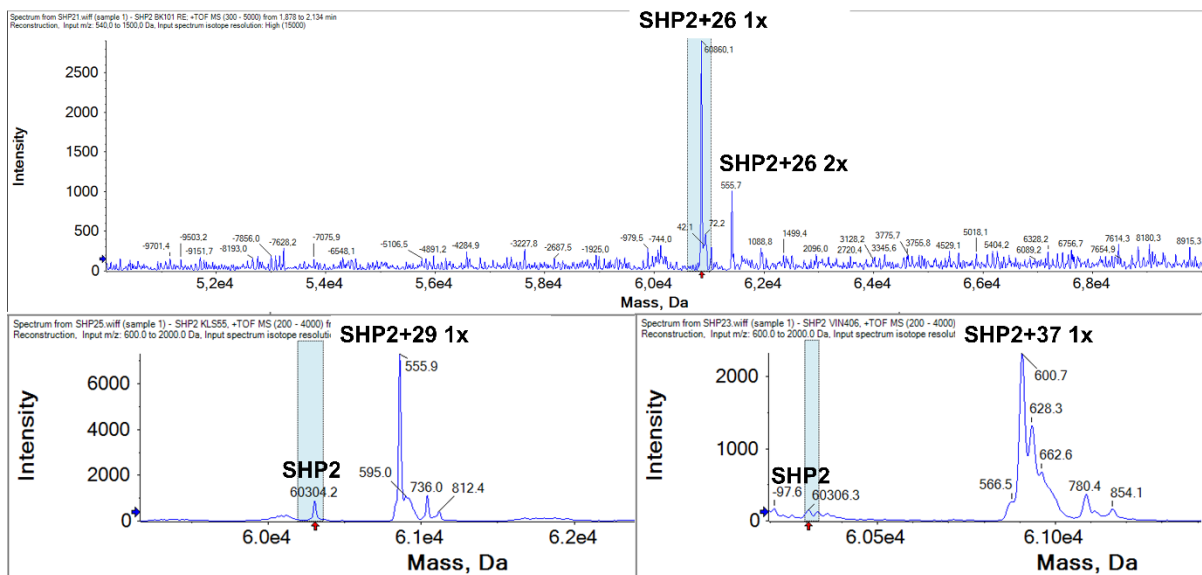
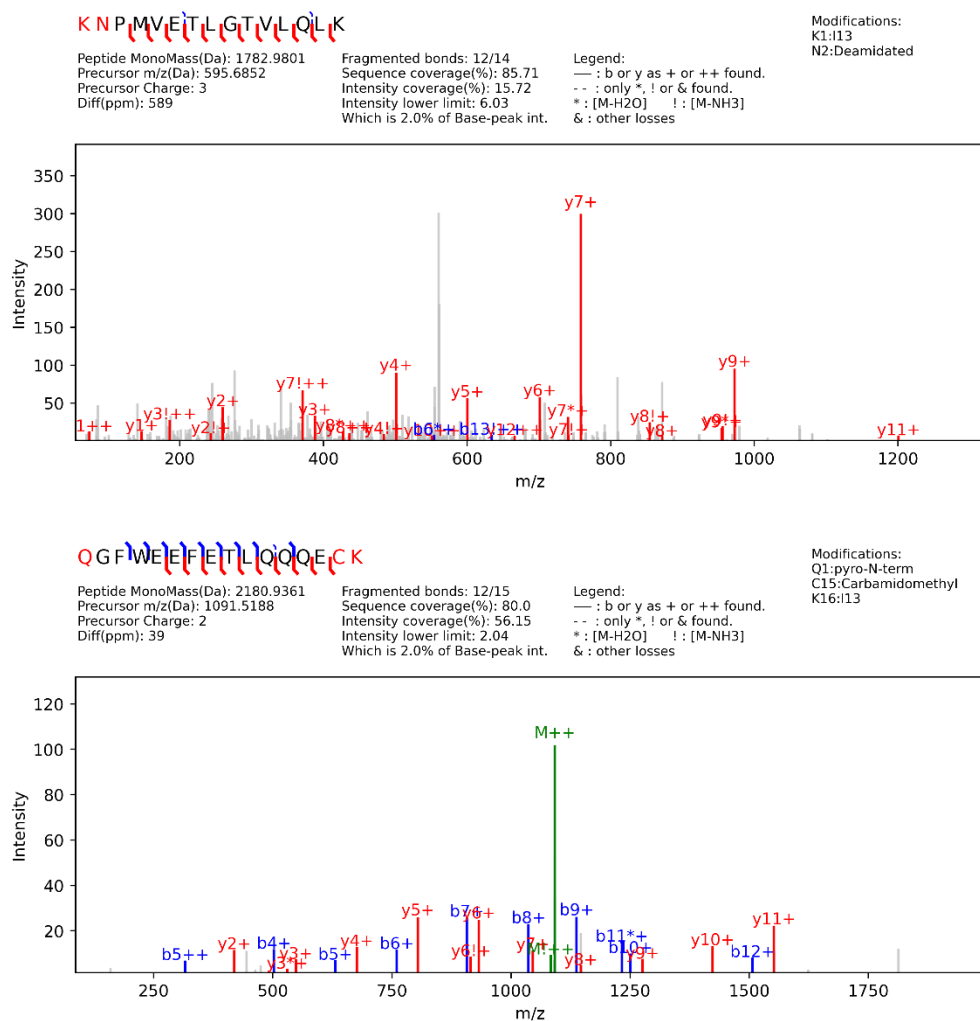


Figure S12. Intact MS spectra of the latch compounds having a low-micromolar or nanomolar activity.

Digestion results

- Lysine-targeting library



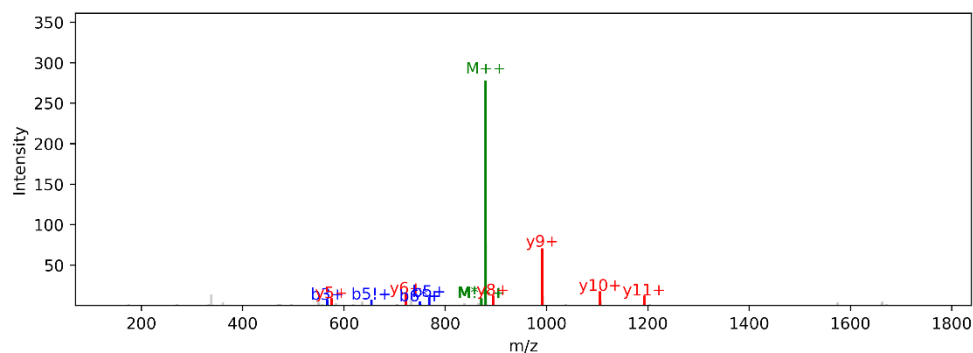
Sequence	Position start	Missed Cleavages	RT (min)
KNPMVETLGTVLQLK	199	1	24.78
QGFWEFETLQQECK	245	0	32.03

Figure S13. MS/MS spectra and analytical data of the peptides modified by **F16** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K199** and **K260**, respectively.

Peptide MonoMass(Da): 1757.7848
Precursor m/z(Da): 879.9102
Precursor Charge: 2
Diff(ppm): 11

Fragmented bonds: 6/13
Sequence coverage(%): 46.15
Intensity coverage(%): 66.02
Intensity lower limit: 5.0
Which is 1.8% of Base-peak int.

Legend:
 — : b or y as + or ++ found.
 - - : only *, ! or & found.
 * : [M-H₂O] ! : [M-NH₃]
 & : other losses

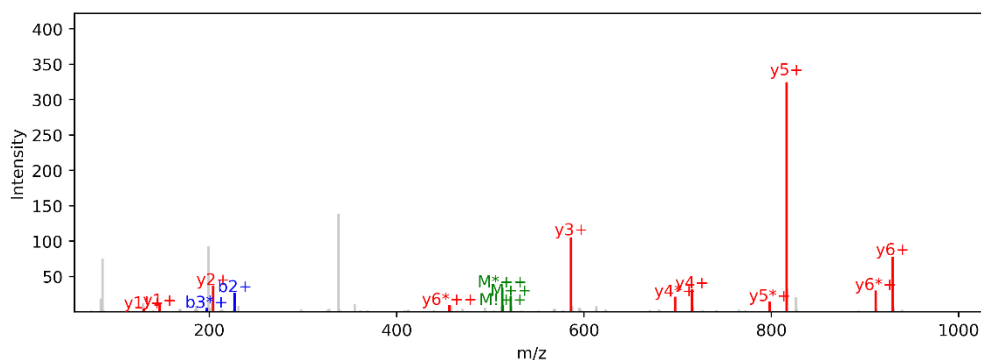


LLT¹EKGK

Peptide MonoMass(Da): 1041.497
Precursor m/z(Da): 521.7568
Precursor Charge: 2
Diff(ppm): 1

Fragmented bonds: 6/6
Sequence coverage(%): 100.0
Intensity coverage(%): 51.5
Intensity lower limit: 5.0
Which is 1.5% of Base-peak int.

Legend:
 — : b or y as + or ++ found.
 -- : only *, ! or & found.
 * : [M-H₂O] ! : [M-NH₃]
 & : other losses

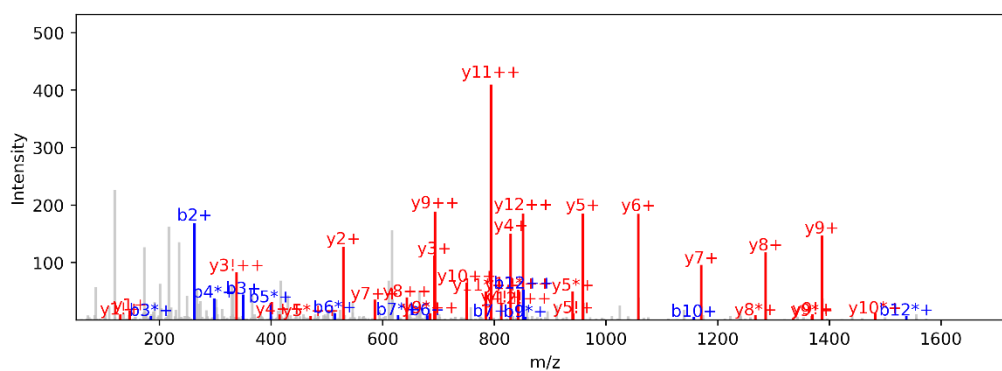


F D S L T D L V E H Y K K

Peptide MonoMass(Da): 1847.8205
Precursor m/z(Da): 616.9543
Precursor Charge: 3
Diff(ppm): 10

Fragmented bonds: 12/12
Sequence coverage(%): 100.0
Intensity coverage(%): 38.44
Intensity lower limit: 5.0
Which is 1.2% of Base-peak int

Legend:
 — : b or y as + or ++ found.
 - - : only *, ! or & found.
 * : [M-H₂O] ! : [M-NH₃]
 & : other losses



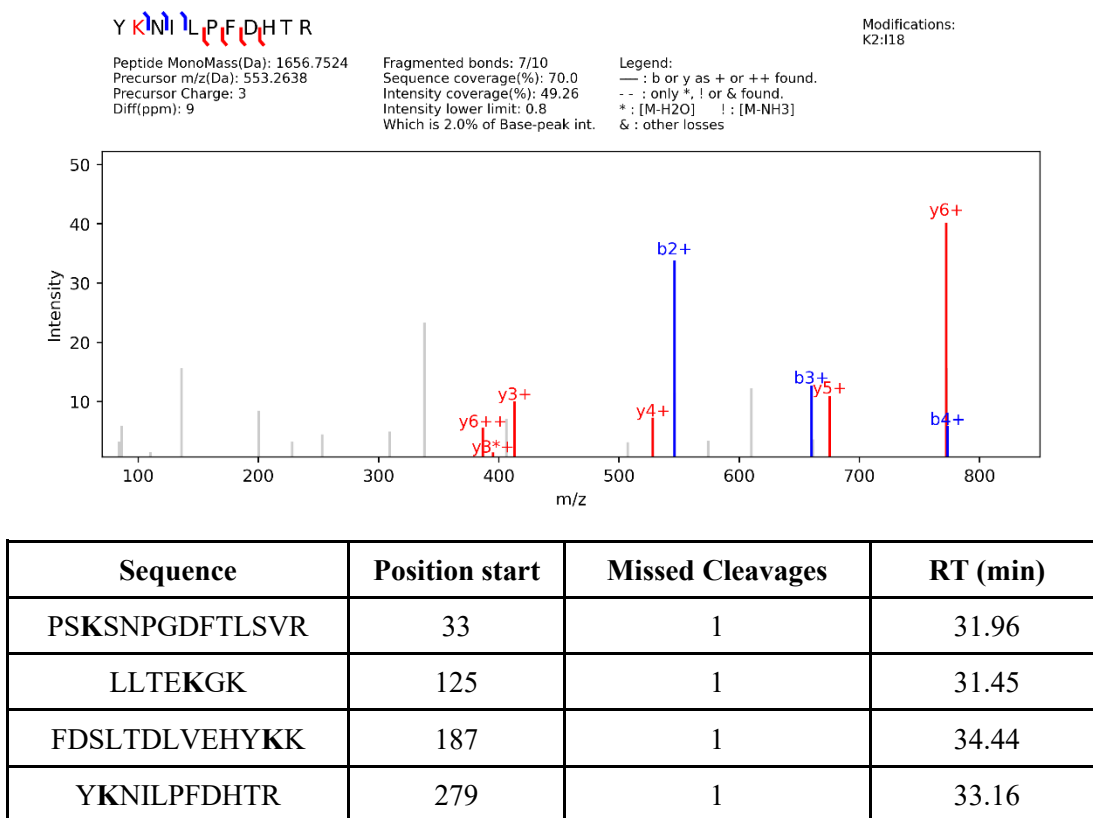
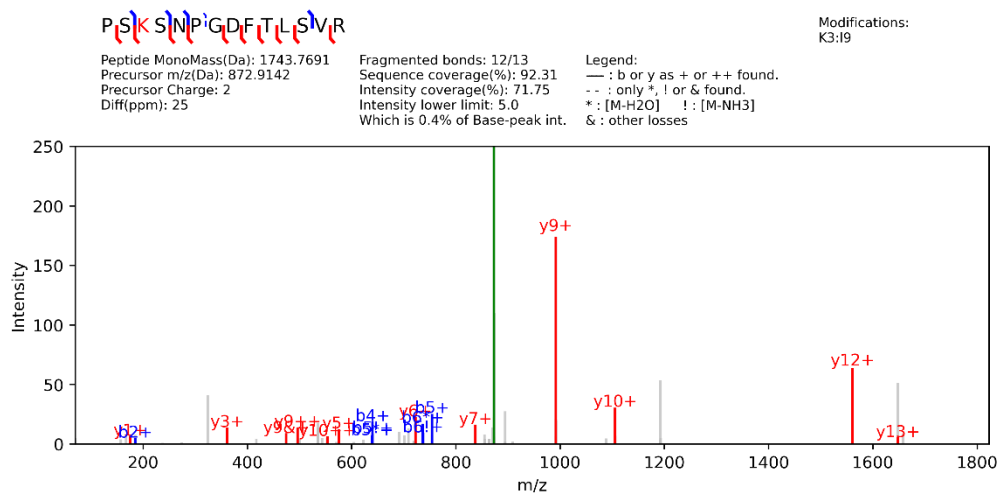
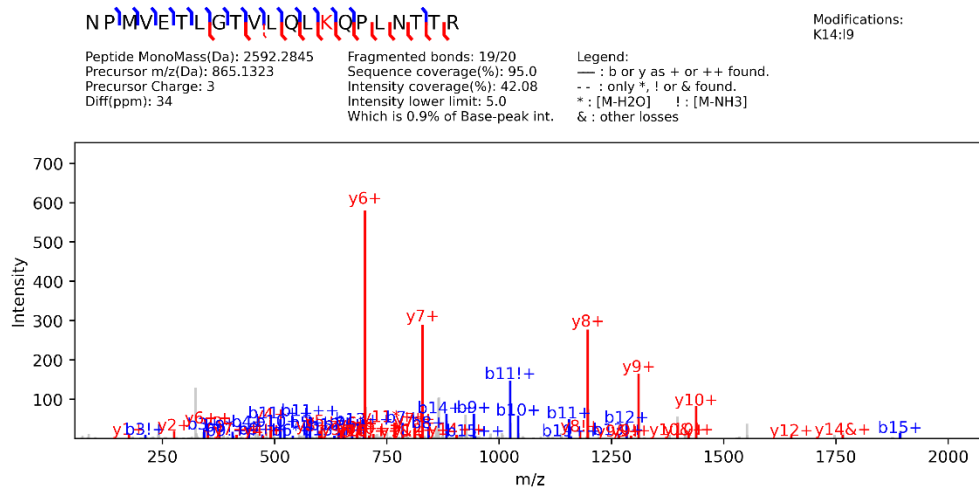
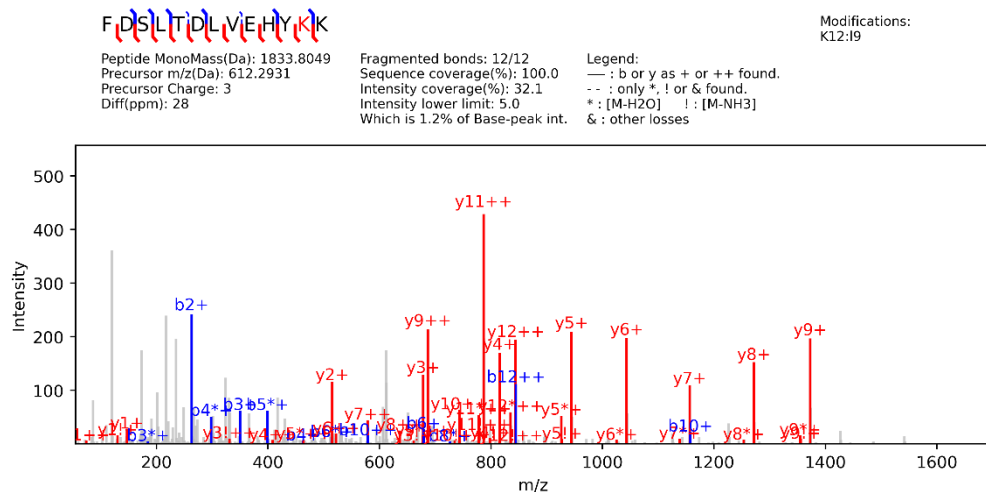
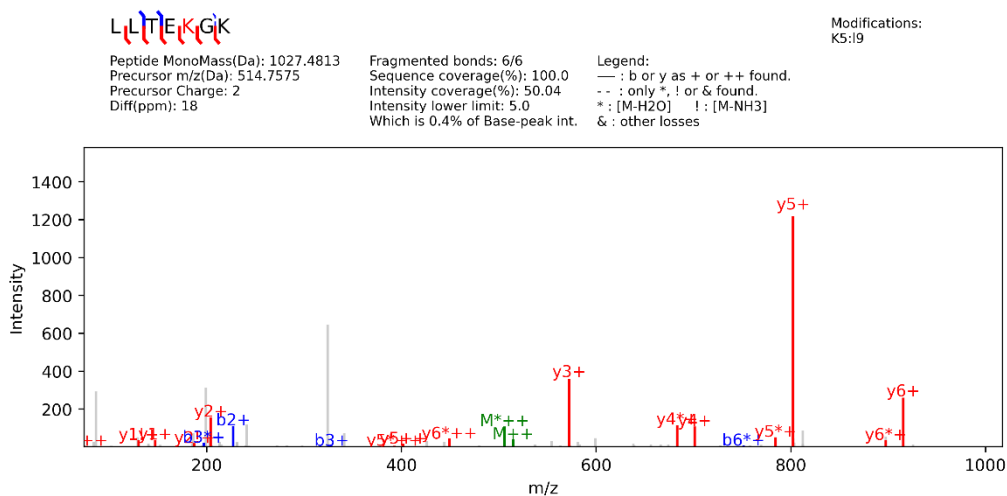
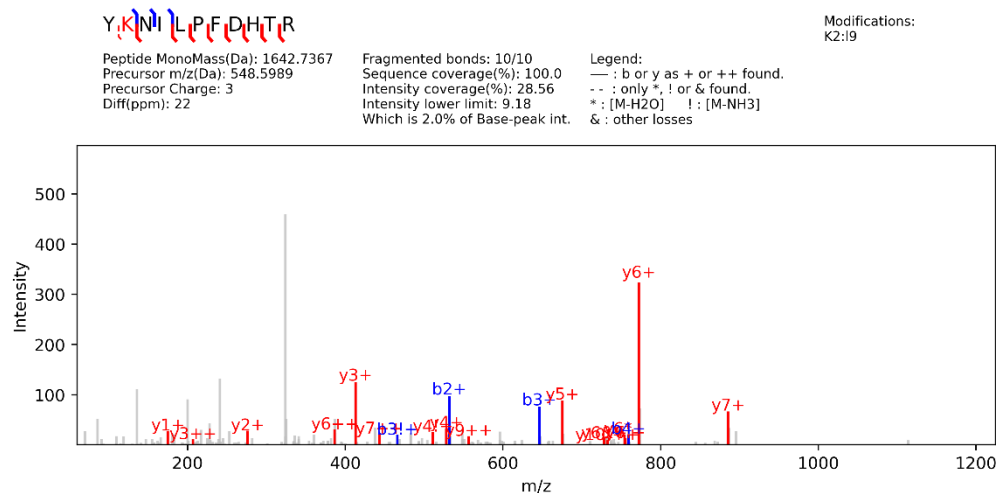
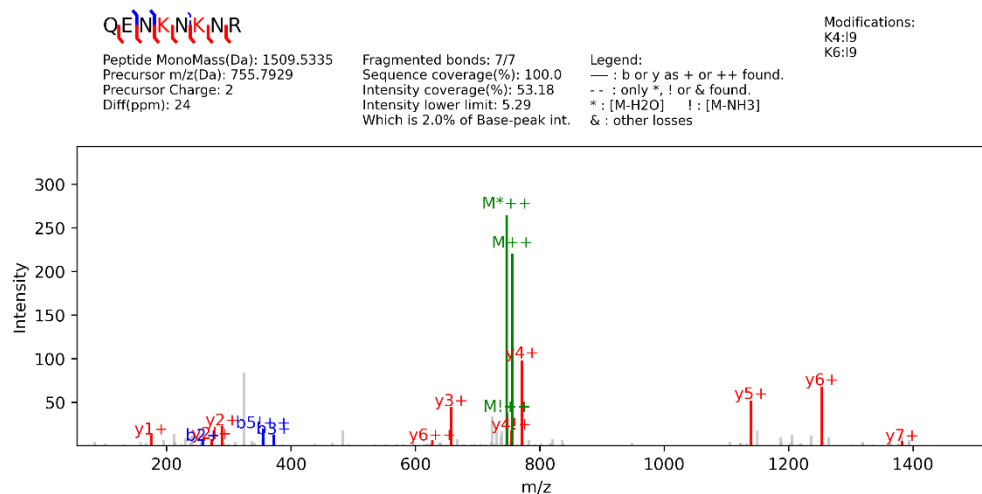
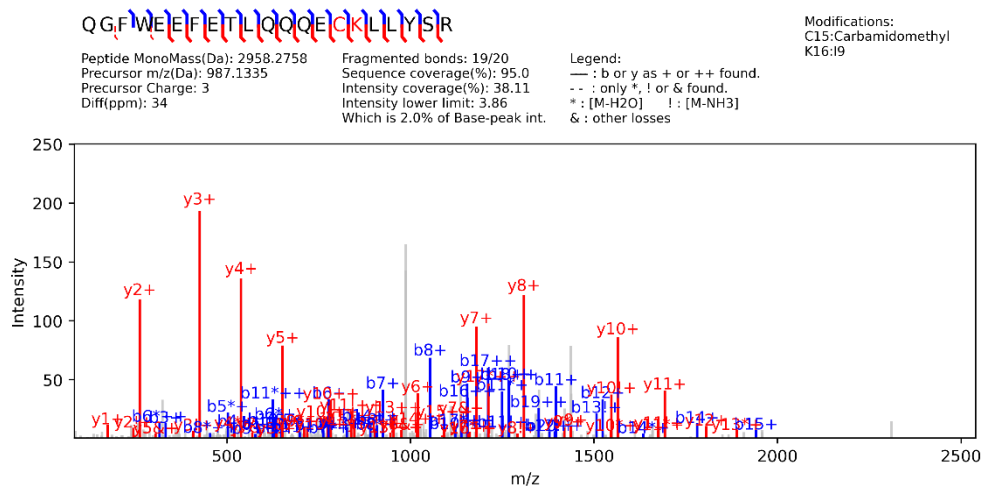
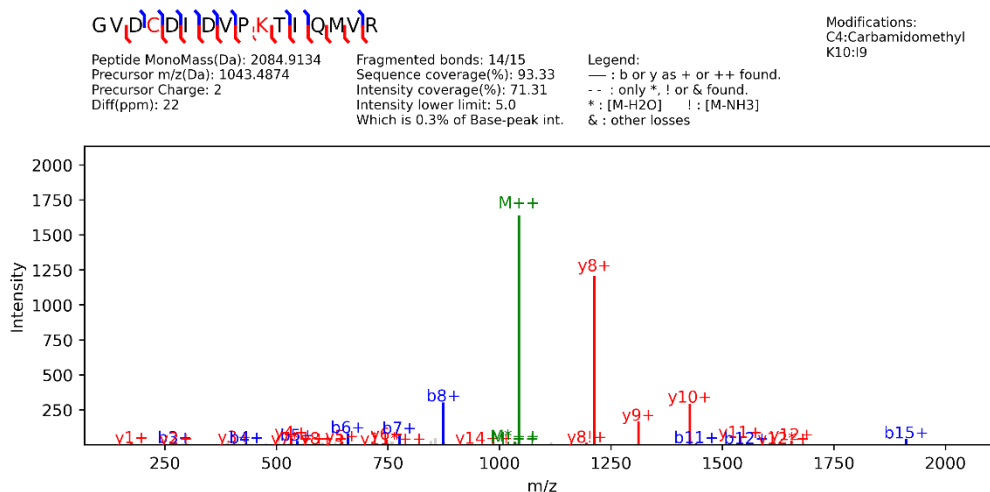


Figure S14. MS/MS spectra and analytical data of the peptides modified by **F8** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K35**, **K129**, **K198**, and **K280** (*Latch site*), respectively.



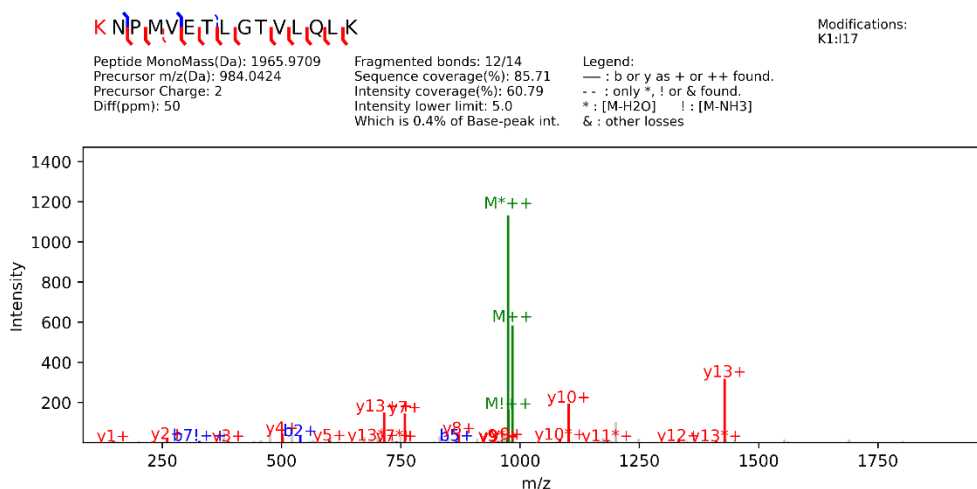


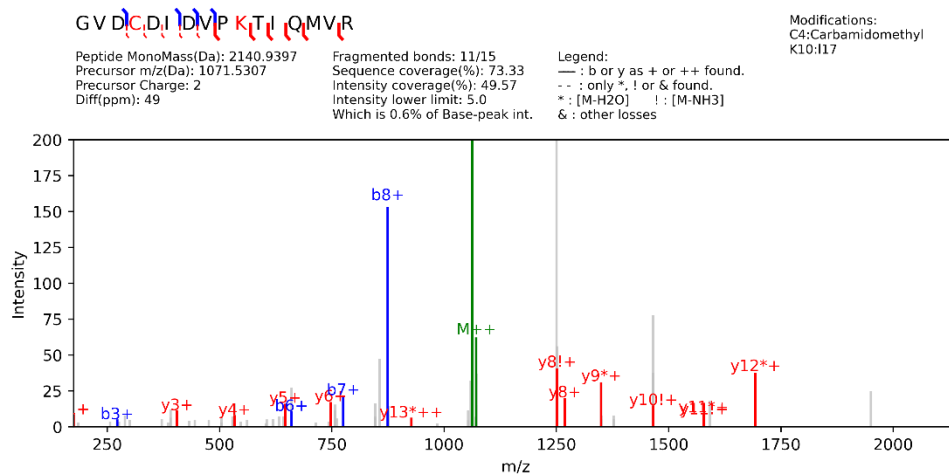
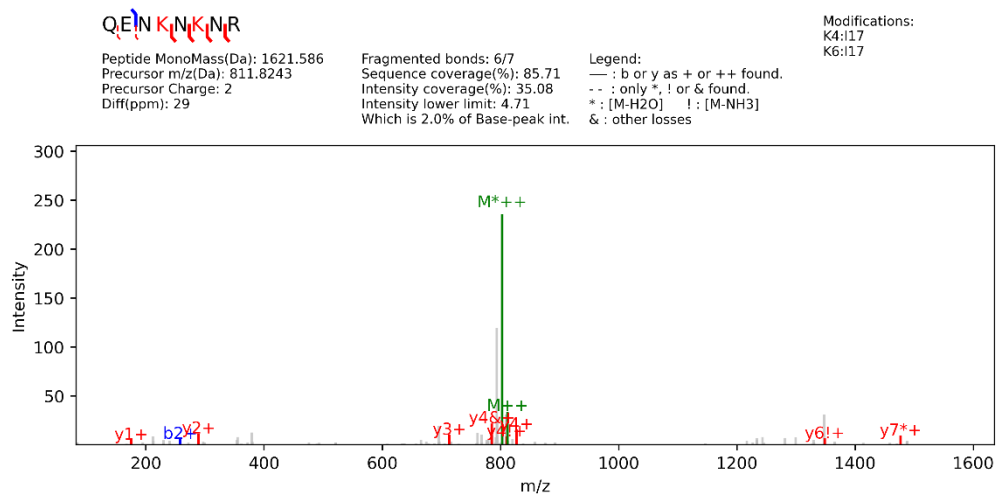
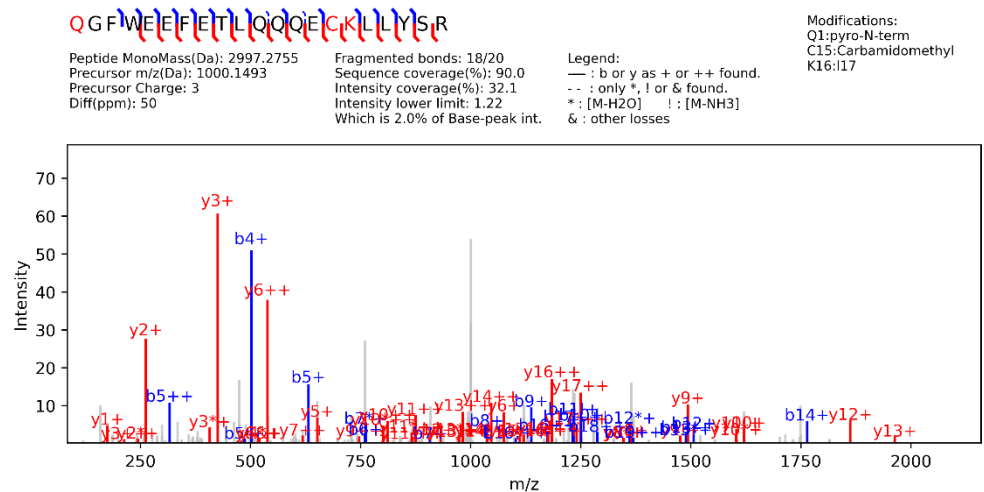




Sequence	Position start	Missed Cleavages	RT (min)
PSKSNPGDFTLSVR	33	1	31.66
LLTEKKGK	125	1	31.07
FDSLTDLVEHYKK	187	1	34.40
NPMVETLGTVLQLKQPLNTTR	200	1	38.02
QGFWEETFETLQQQECKLLYSR	245	1	35.03
QENKKNR	271	2	34.86
YKNILPFDHTR	279	1	32.81
GVDCIDIDVPKTIQMVR	483	1	32.92

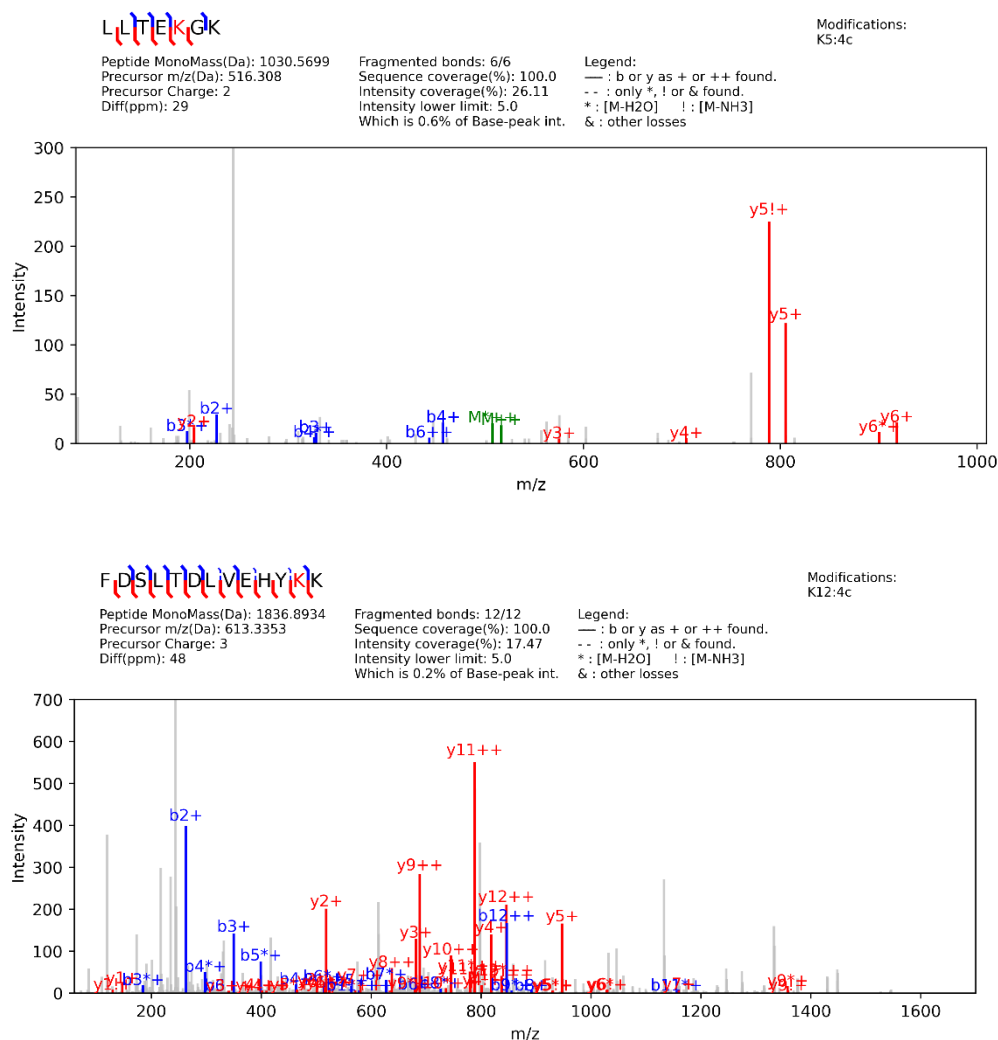
Figure S15. MS/MS spectra and analytical data of the peptides modified by **F10** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K35**, **K129**, **K198**, **K213**, **K260**, **K274** (*latch site*), **K276**, **K280** (*latch site*), and **K492** (*tunnel site*) respectively.





Sequence	Position start	Missed Cleavages	RT (min)
KNPMVETLGTVLQLK	199	1	36.73
QGFWEETFETLQQQECKLLYSR	245	1	37.25
QENKNKNR	271	2	35.74
GVDCDIDVPKTIQMVR	483	1	33.06

Figure S16. MS/MS spectra and analytical data of the peptides modified by F11 detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K199**, **K260**, **K274** (*latch site*), **K276**, and **K492** (*tunnel site*) respectively.



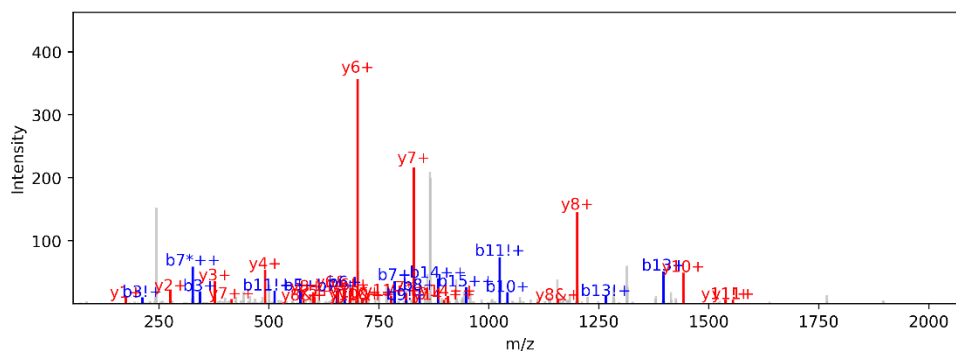
N P M V E T L G T V L I Q L K O P L N T T R

Peptide MonoMass(Da): 2595.3731
Precursor m/z(Da): 866.1806
Precursor Charge: 3
Diff(ppm): 56

Fragmented bonds: 17/20
Sequence coverage(%): 85.0
Intensity coverage(%): 31.78
Intensity lower limit: 5.0
Which is 1.4% of Base-peak int.

Legend:
— : b or y as + or ++ found.
- - : only *, ! or & found.
* : [M-H2O] ! : [M-NH3]
& : other losses

Modifications:
K14:4c



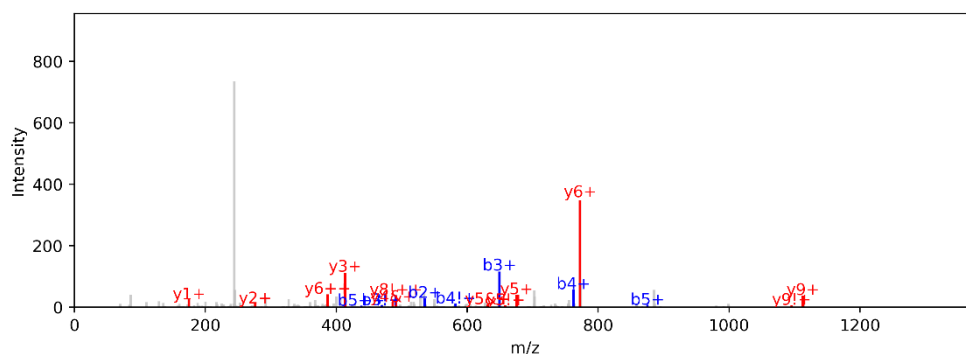
Y K N I L P F D H T R

Peptide MonoMass(Da): 1645.8253
Precursor m/z(Da): 549.6394
Precursor Charge: 3
Diff(ppm): 42

Fragmented bonds: 8/10
Sequence coverage(%): 80.0
Intensity coverage(%): 24.14
Intensity lower limit: 5.0
Which is 0.7% of Base-peak int.

Legend:
— : b or y as + or ++ found.
- - : only *, ! or & found.
* : [M-H2O] ! : [M-NH3]
& : other losses

Modifications:
K2:4c



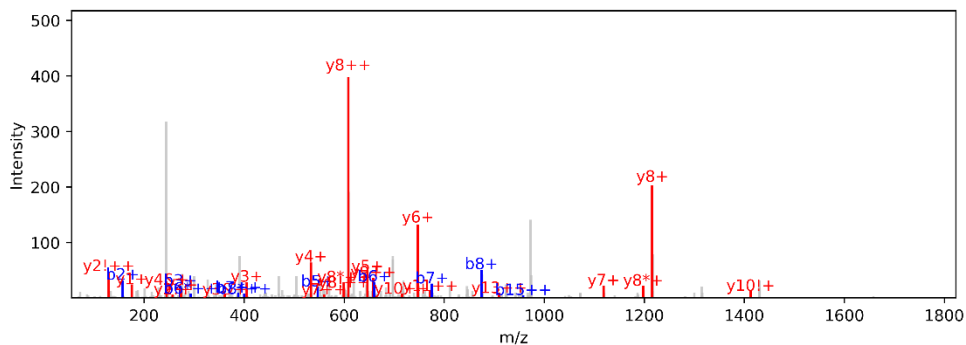
G V I D C D I D M P K T I Q M V R

Peptide MonoMass(Da): 2088.002
Precursor m/z(Da): 697.0422
Precursor Charge: 3
Diff(ppm): 48

Fragmented bonds: 13/15
Sequence coverage(%): 86.67
Intensity coverage(%): 28.05
Intensity lower limit: 5.0
Which is 1.3% of Base-peak int.

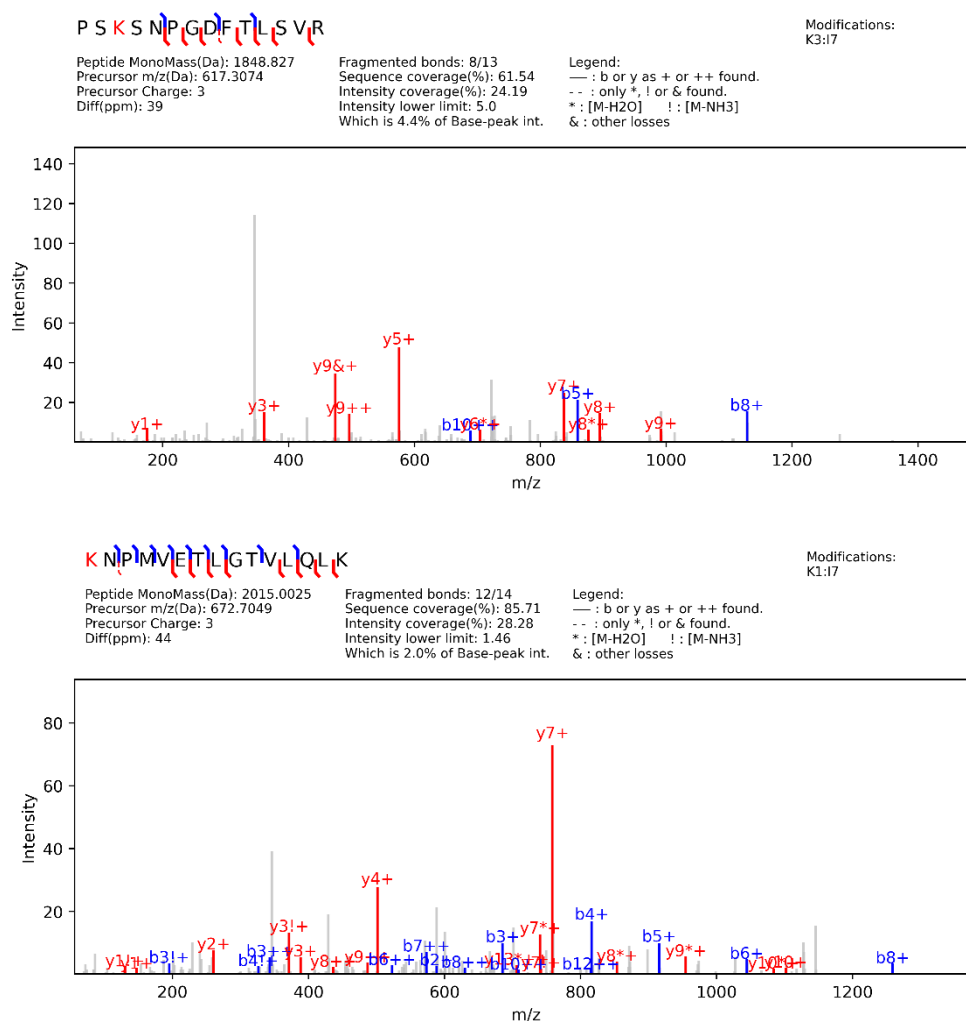
Legend:
— : b or y as + or ++ found.
- - : only *, ! or & found.
* : [M-H2O] ! : [M-NH3]
& : other losses

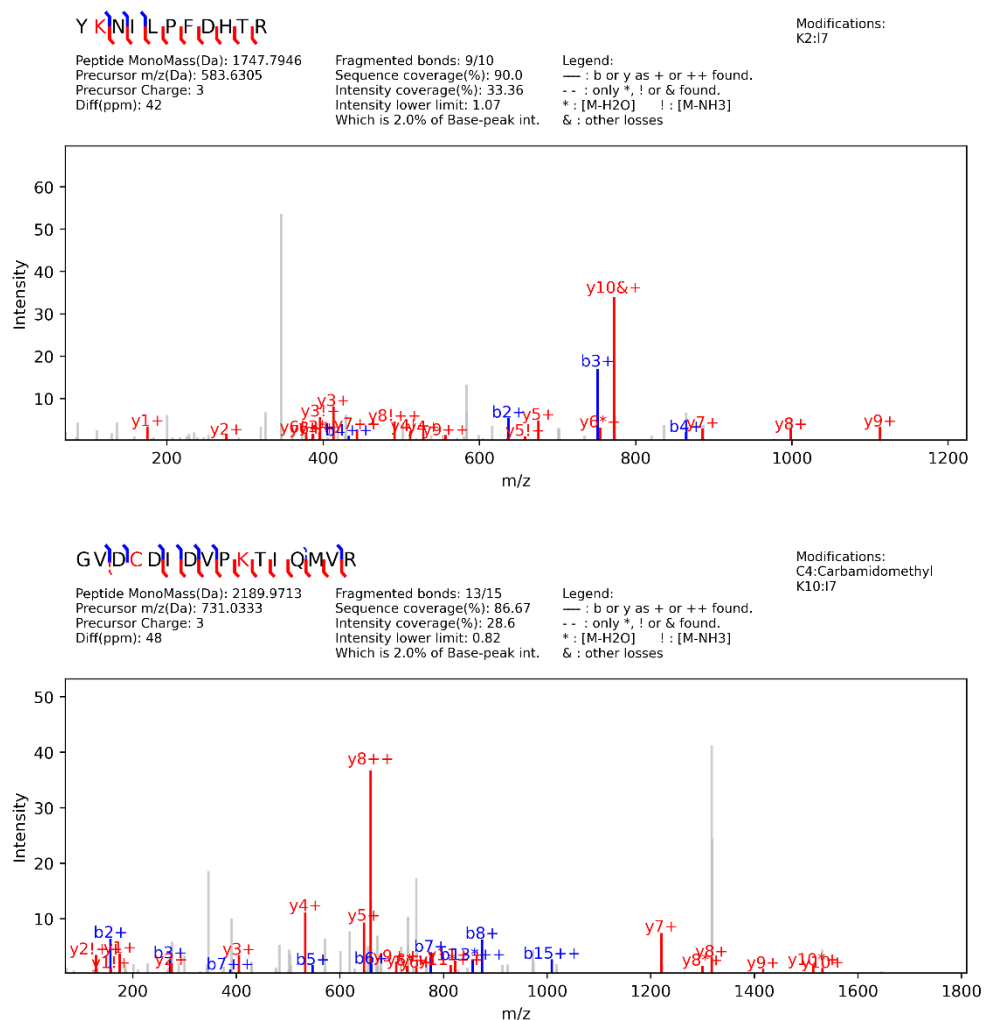
Modifications:
C4:Carbamidomethyl
K10:4c

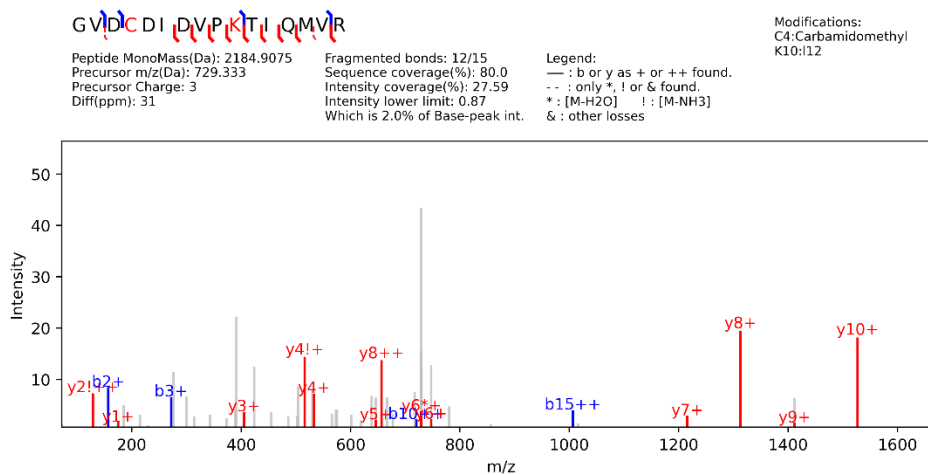
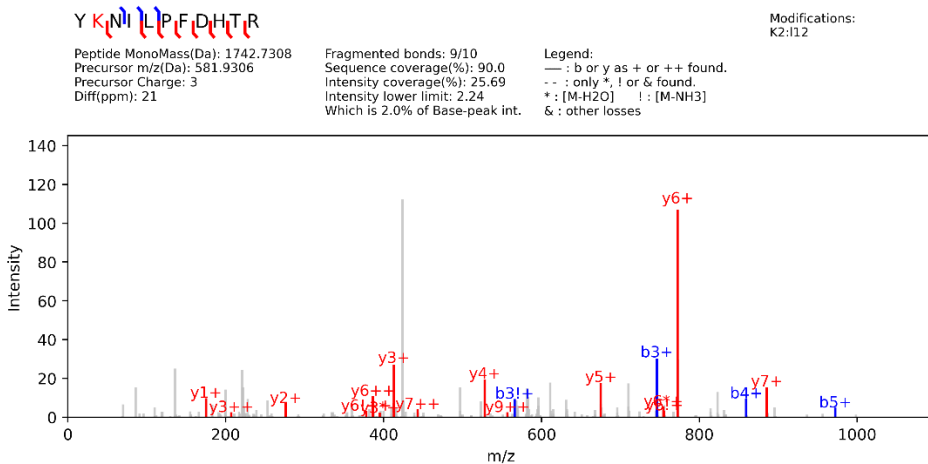
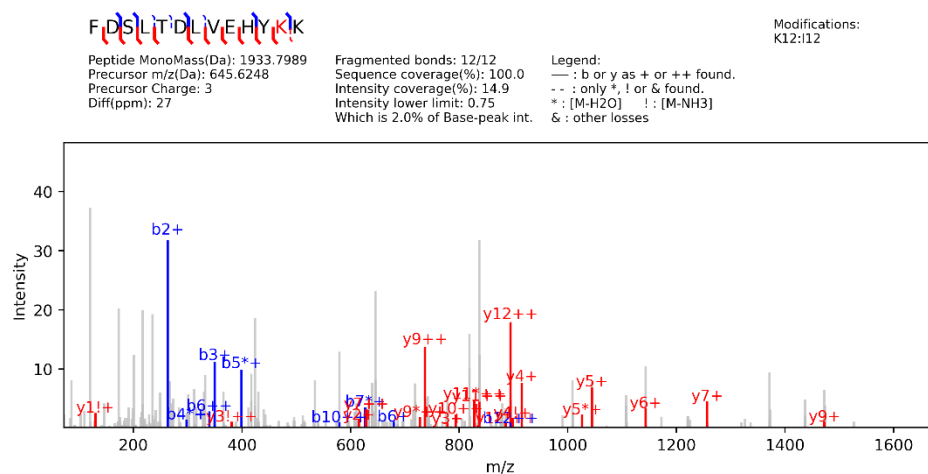


Sequence	Position start	Missed Cleavages	RT (min)
LLTEKKGK	125	1	31.13
FDSLTDLVEHYKK	187	1	33.95
NPMVETLGTVLQLKQPLNTTR	200	1	37.31
YKNILPFDHTR	279	1	32.73
GVDCDIDVPKTIQMVR	483	1	32.53

Figure S17. MS/MS spectra and analytical data of the peptides modified by **F12** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K129**, **K198**, **K213**, **K280** (*latch site*), and **K492** (*tunnel site*) respectively.

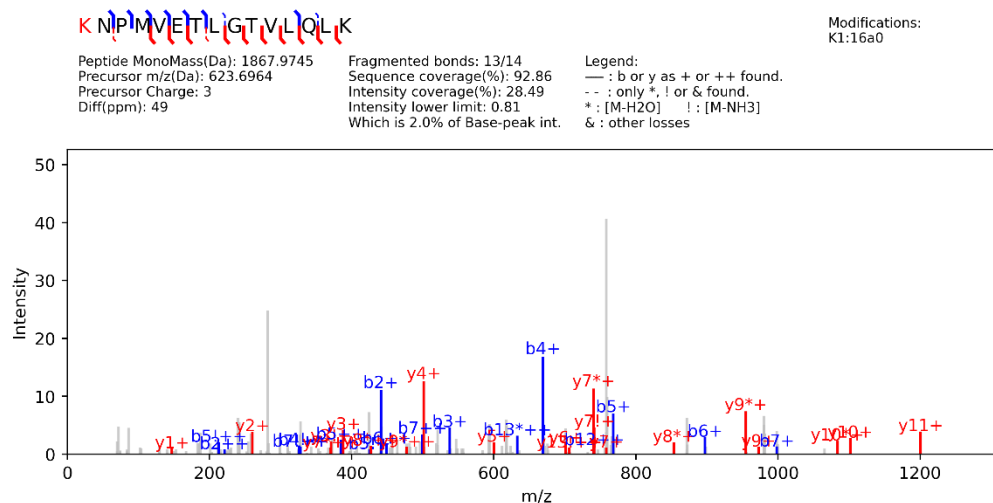






Sequence	Position start	Missed Cleavages	RT (min)
FDSLTLVEHY KK	187	1	36.91
Y KNILPFDHTR	279	1	36.28
GVDCDIDVP K TIQMVR	483	1	35.63

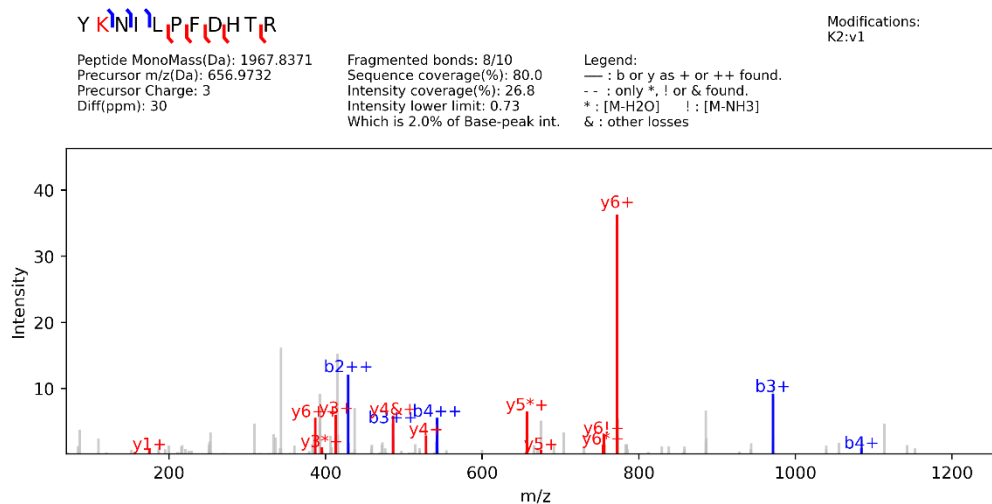
Figure S19. MS/MS spectra and analytical data of the peptides modified by **F17** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acids are **K198**, **K280** (*latch site*), and **K492** (*tunnel site*) respectively.



Sequence	Position start	Missed Cleavages	RT (min)
NPMVETLGTVLQLKQPLNTTR	200	1	35.68

Figure S21. MS/MS spectra and analytical data of the peptide modified by **F30** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acid is **K213**, respectively.

- Designed inhibitor

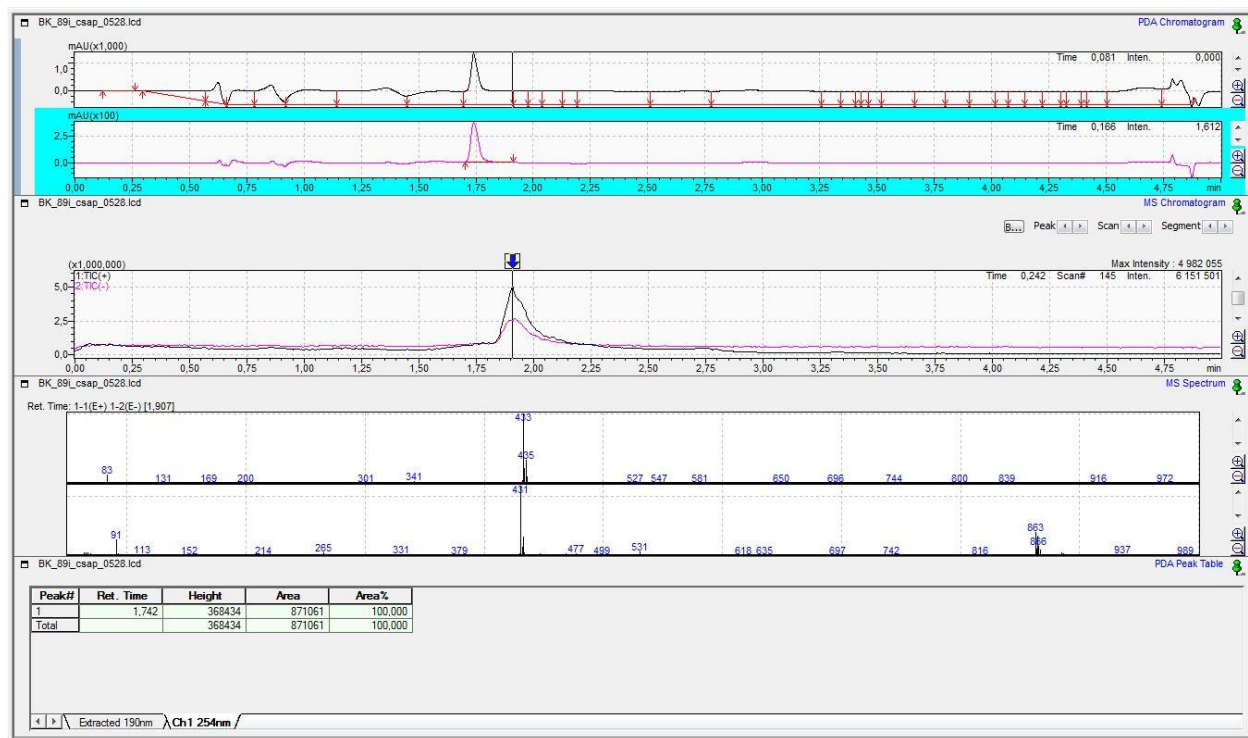
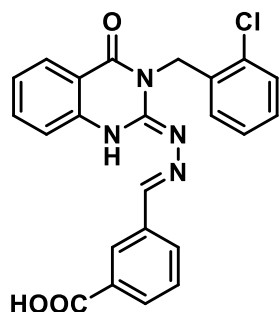


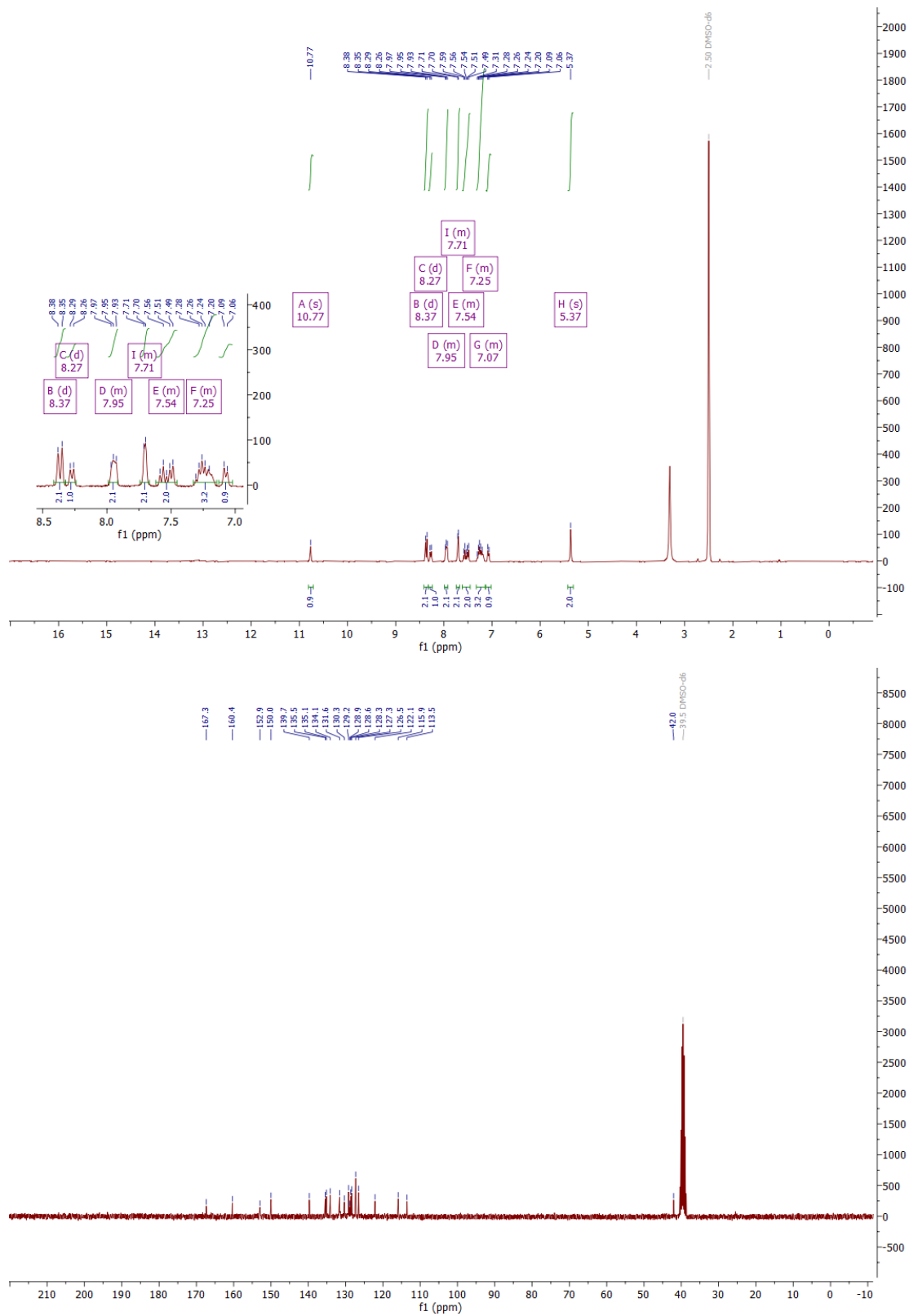
Sequence	Position start	Missed Cleavages	RT (min)
YKNILPFDHTR	279	1	32.49

Figure S22. MS/MS spectra and analytical data of the peptide modified by **37** detected in the peptide mapping analysis using tryptic digestion. Labelled amino acid is **K280** (*Latch site*), respectively.

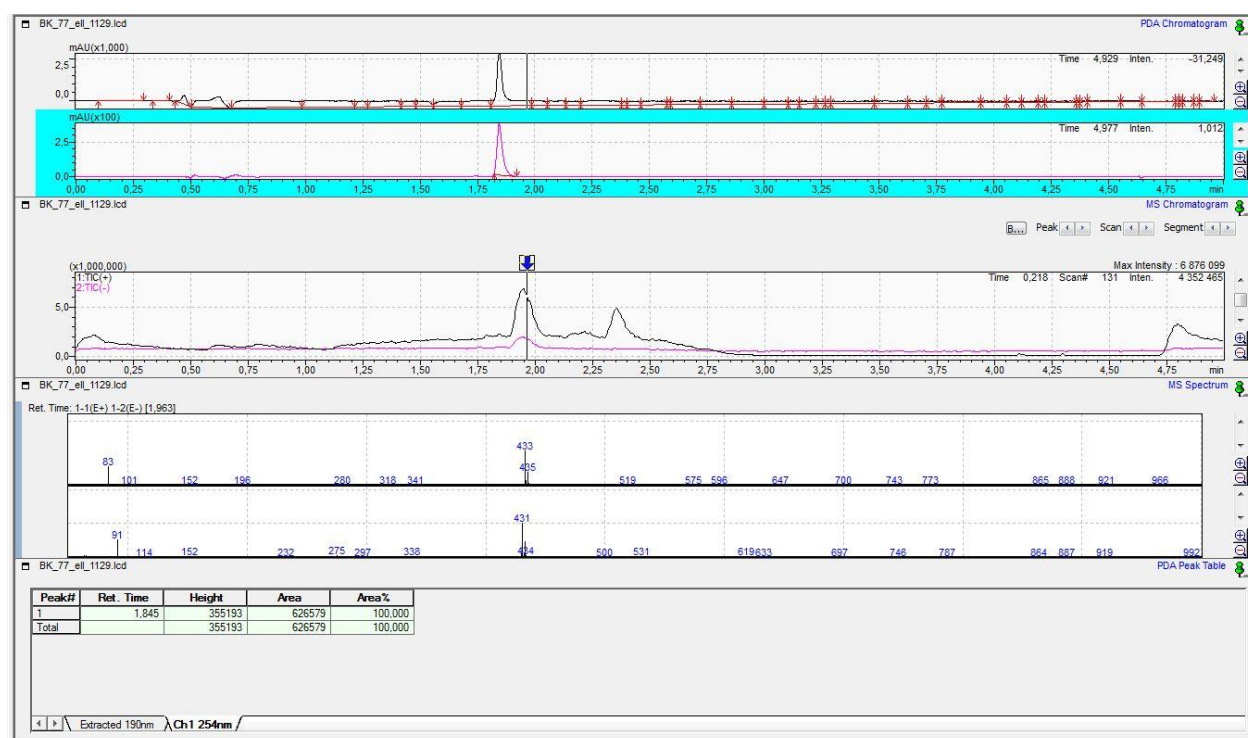
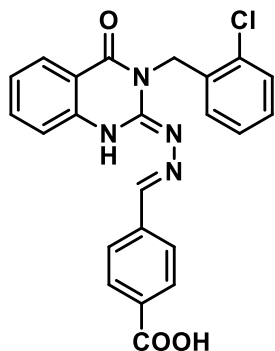
5. LC-MS chromatograms and spectra, ¹H NMR, ¹³C NMR, and HRMS spectra of the compounds

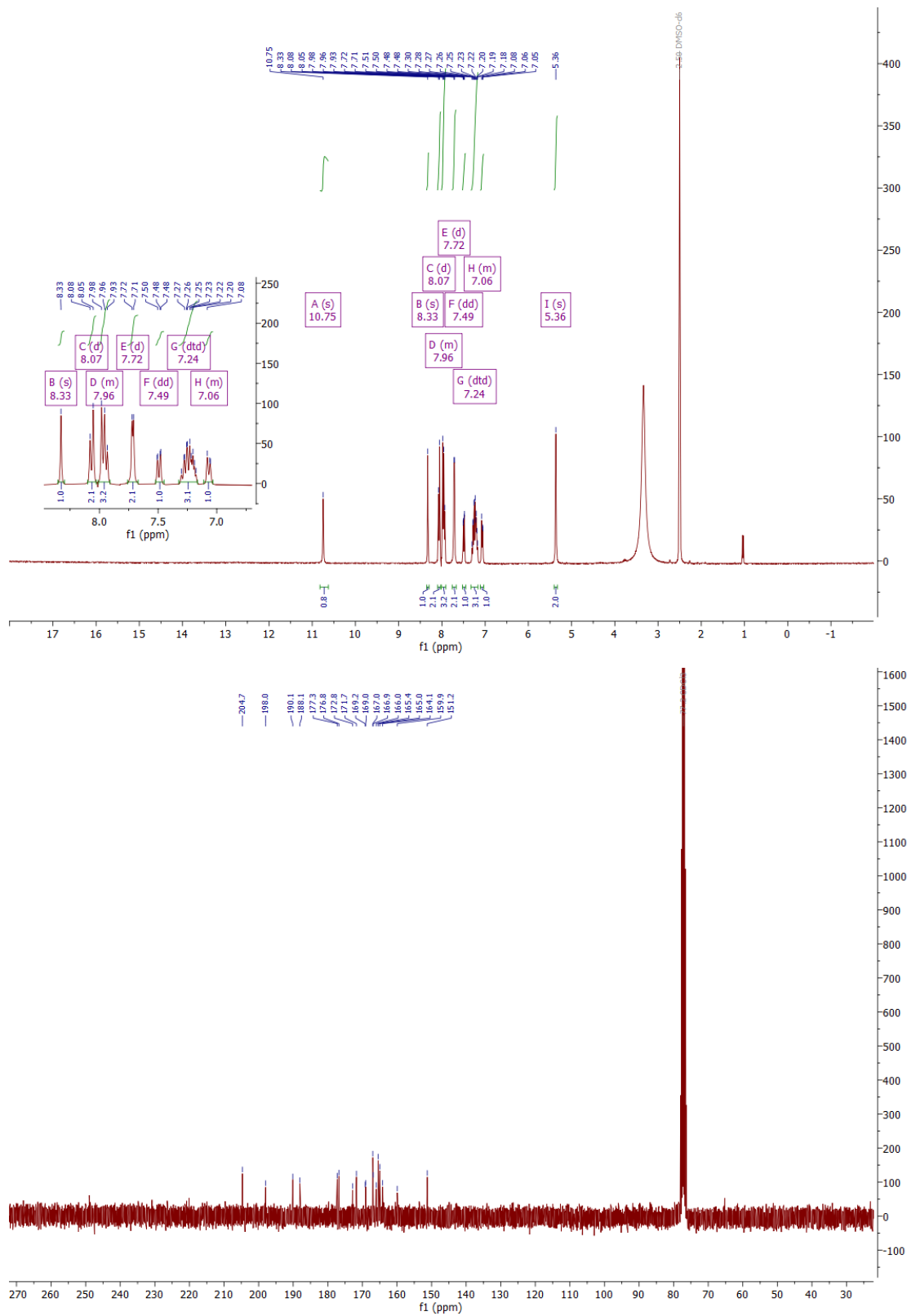
3-((*E*)-(((*E*)-3-(2-chlorobenzyl)-4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)hydrazineylidene)methyl)benzoic acid (**8**)



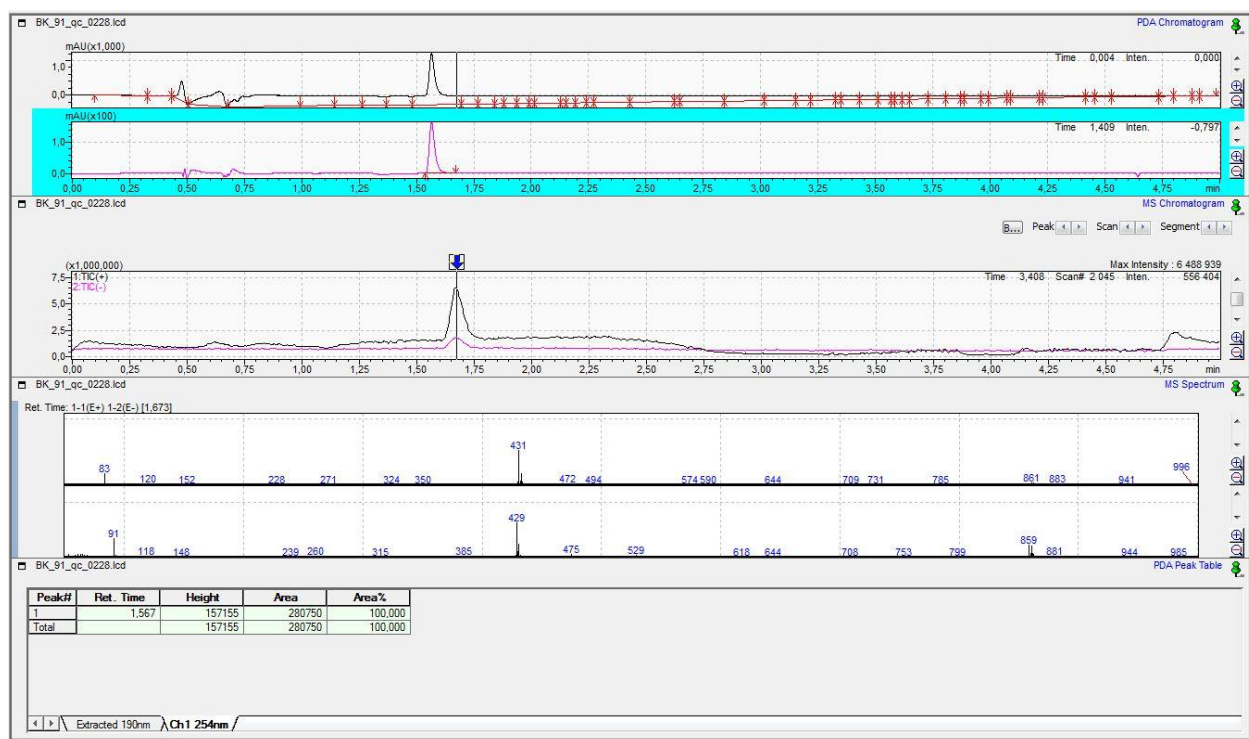
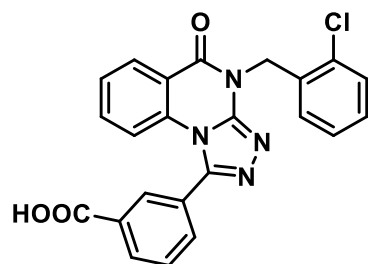


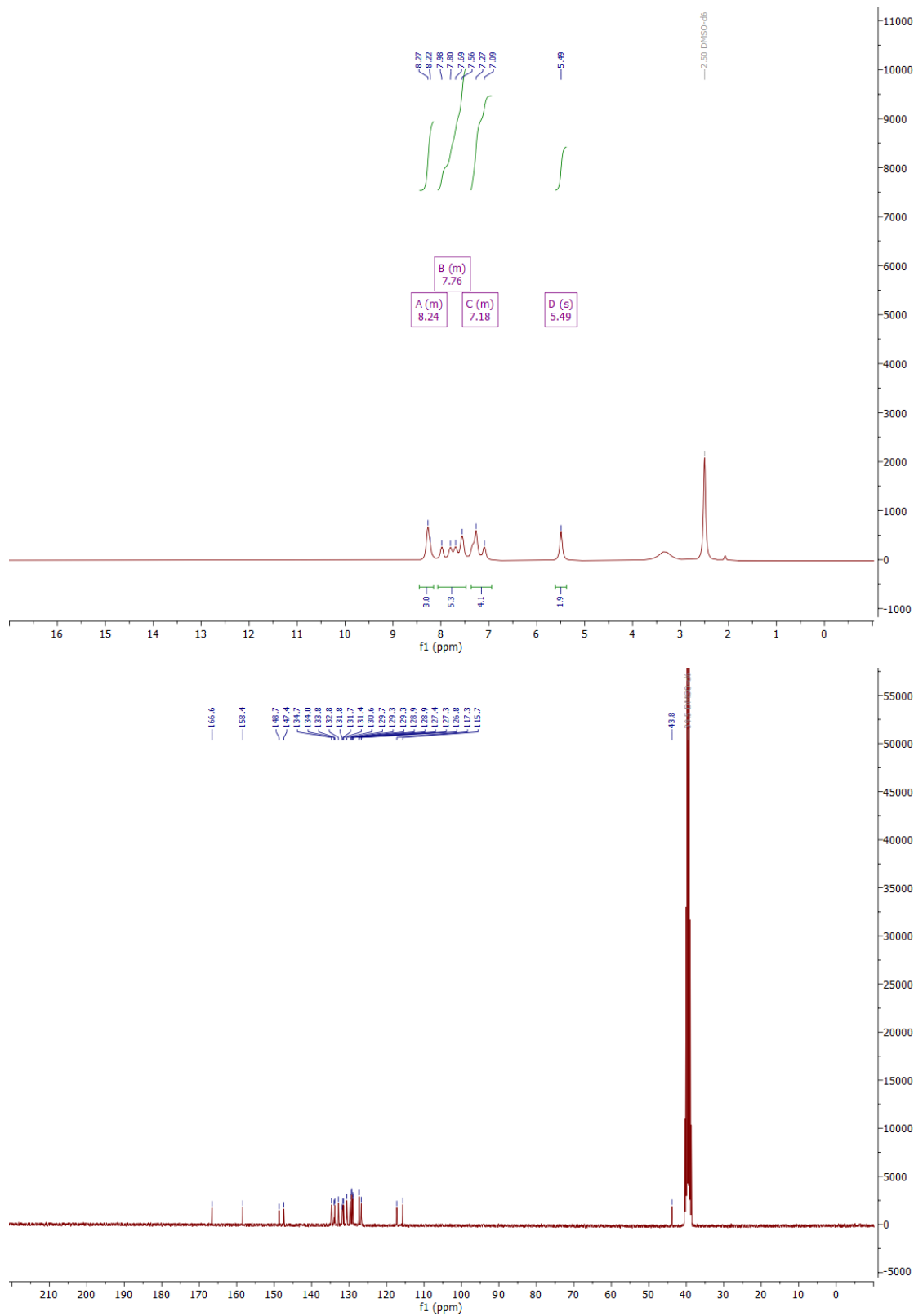
4-((E)-(((E)-3-(2-Chlorobenzyl)-4-oxo-3,4-dihydroquinazolin-2(1H)-ylidene)hydrazineylidene)methyl)benzoic acid (**9**)



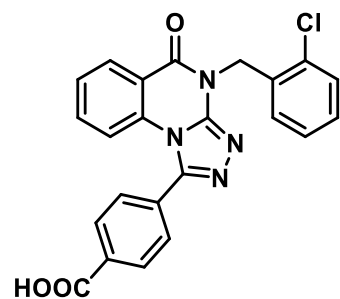


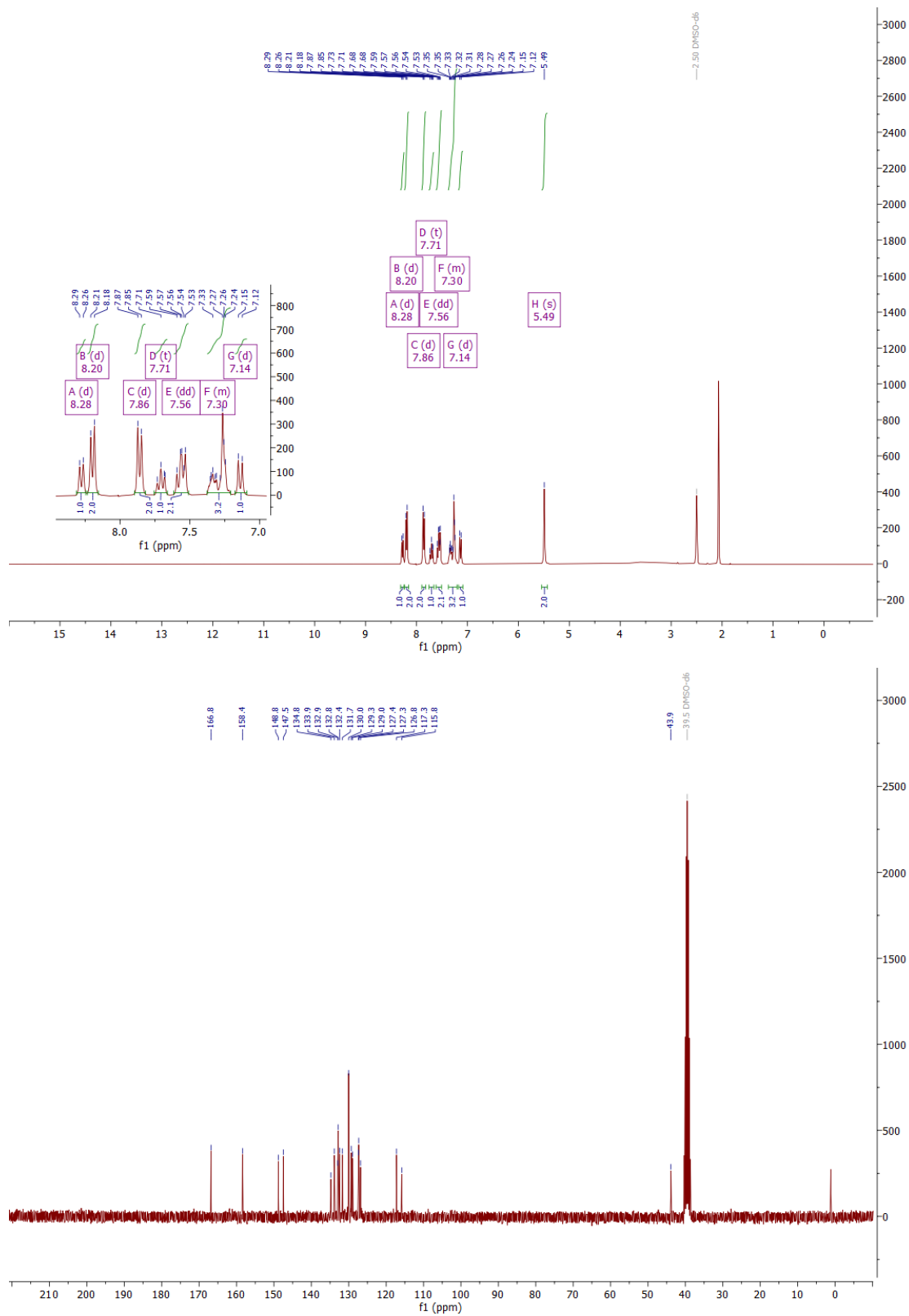
3-(4-(2-Chlorobenzyl)-5-oxo-4,5-dihydro-[1,2,4]triazolo[4,3-a]quinazolin-1-yl)benzoic acid (**10**)





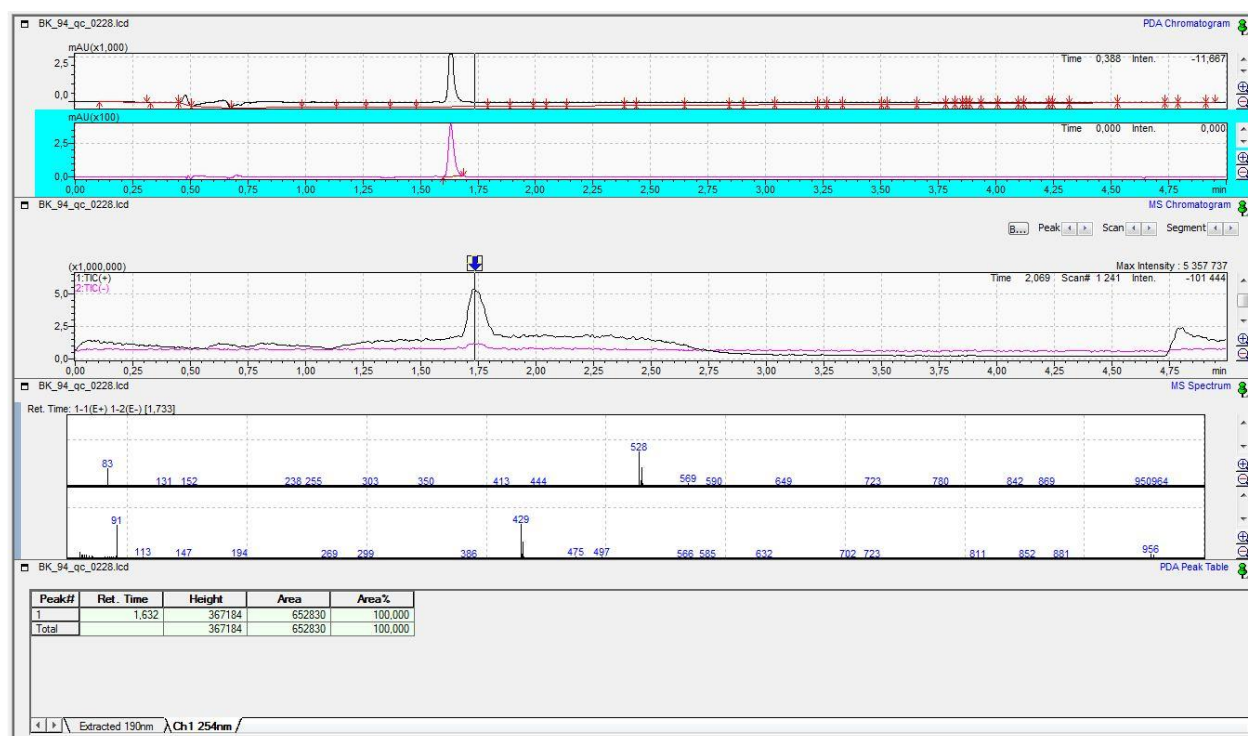
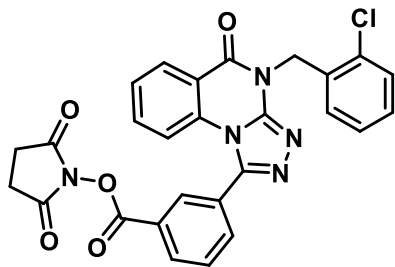
4-(4-(2-Chlorobenzyl)-5-oxo-4,5-dihydro-[1,2,4]triazolo[4,3-a]quinazolin-1-yl)benzoic acid (**11**)

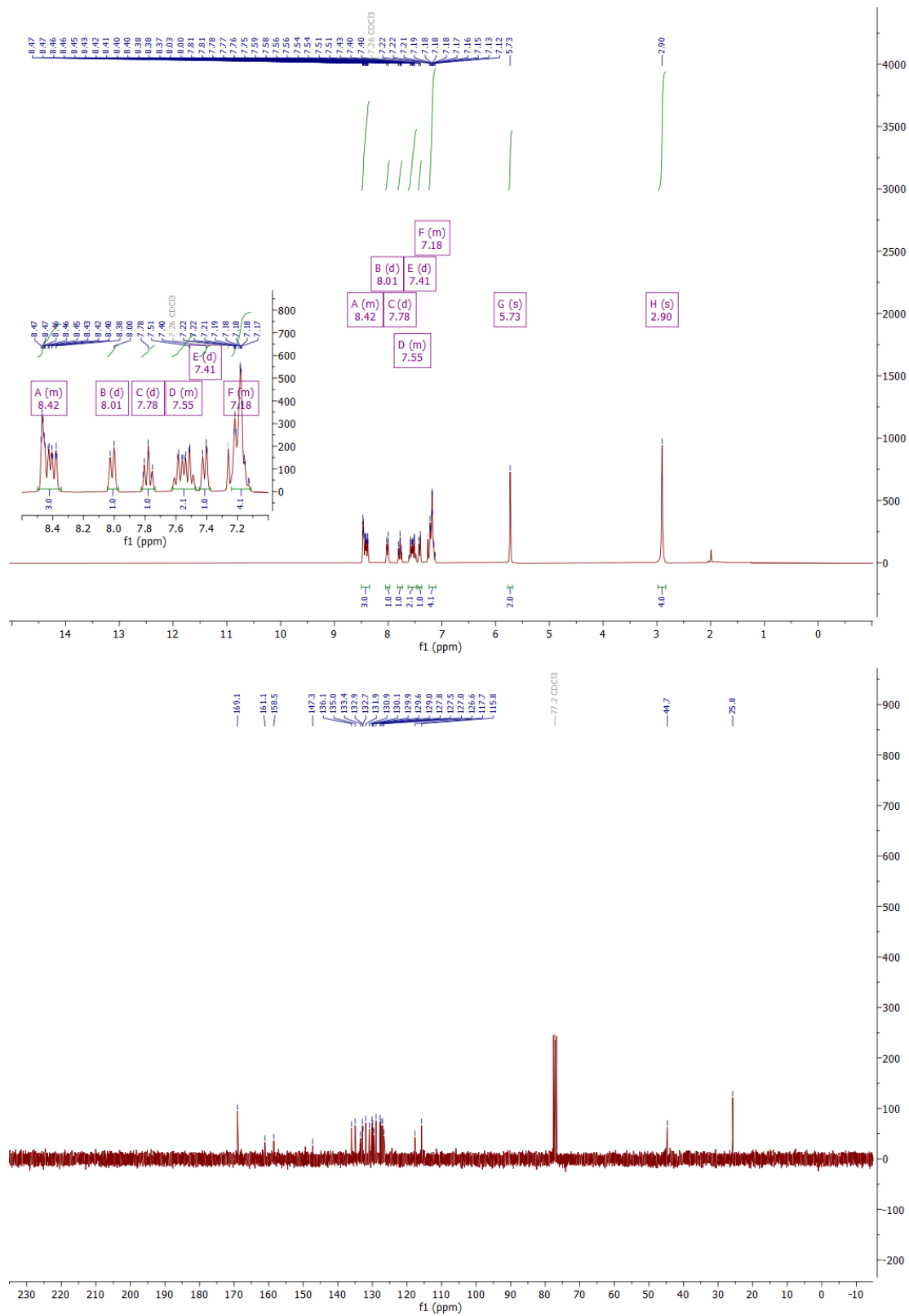




2,5-Dioxopyrrolidin-1-yl
a]quinazolin-1-yl)benzoate (**12**)

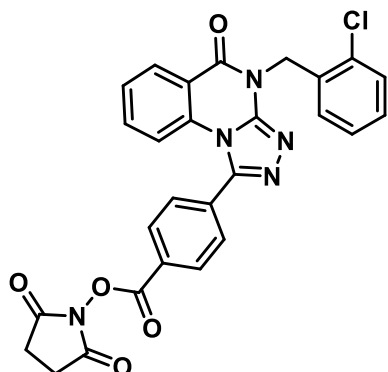
3-(4-(2-chlorobenzyl)-5-oxo-4,5-dihydro-[1,2,4]triazolo[4,3-

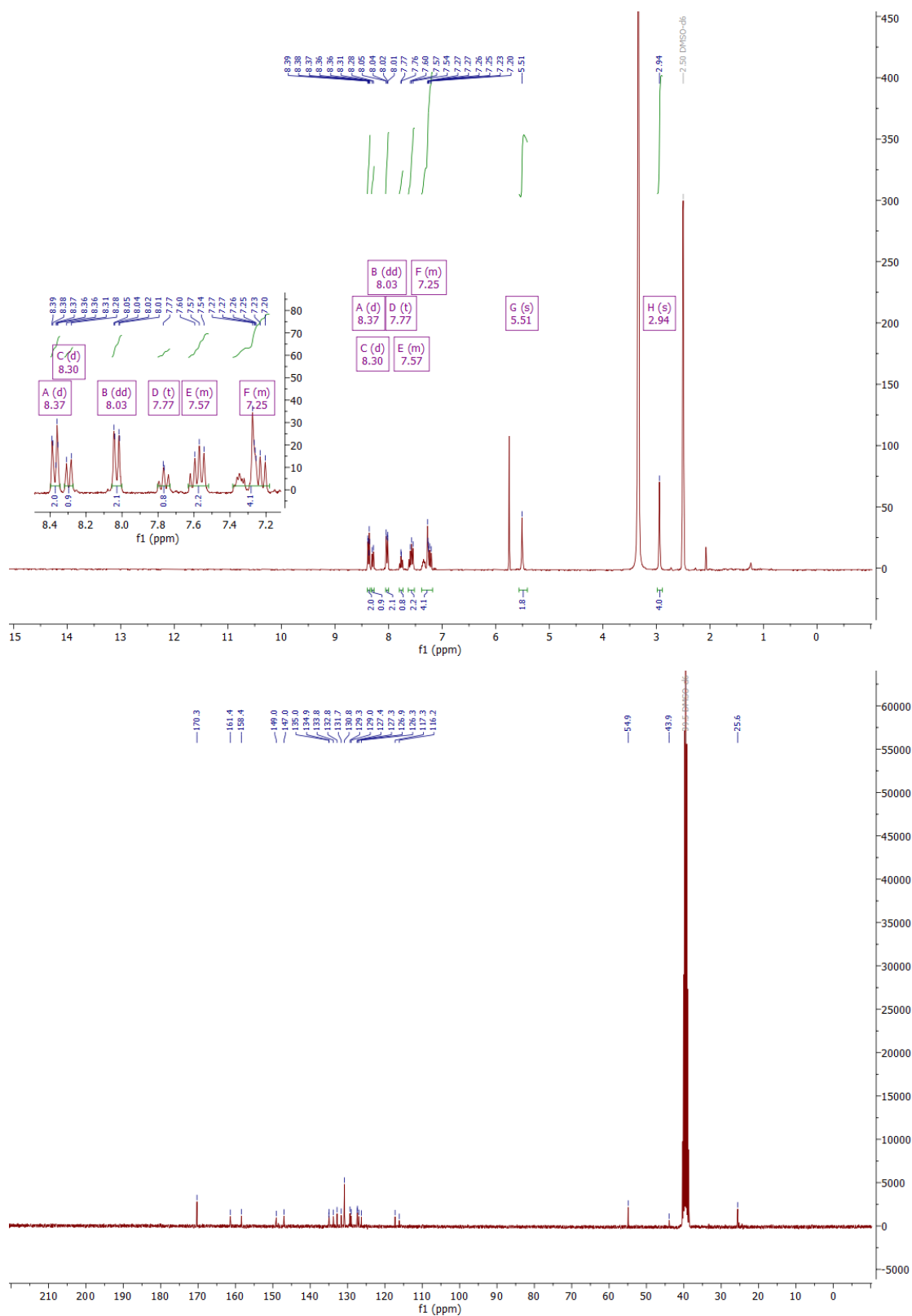




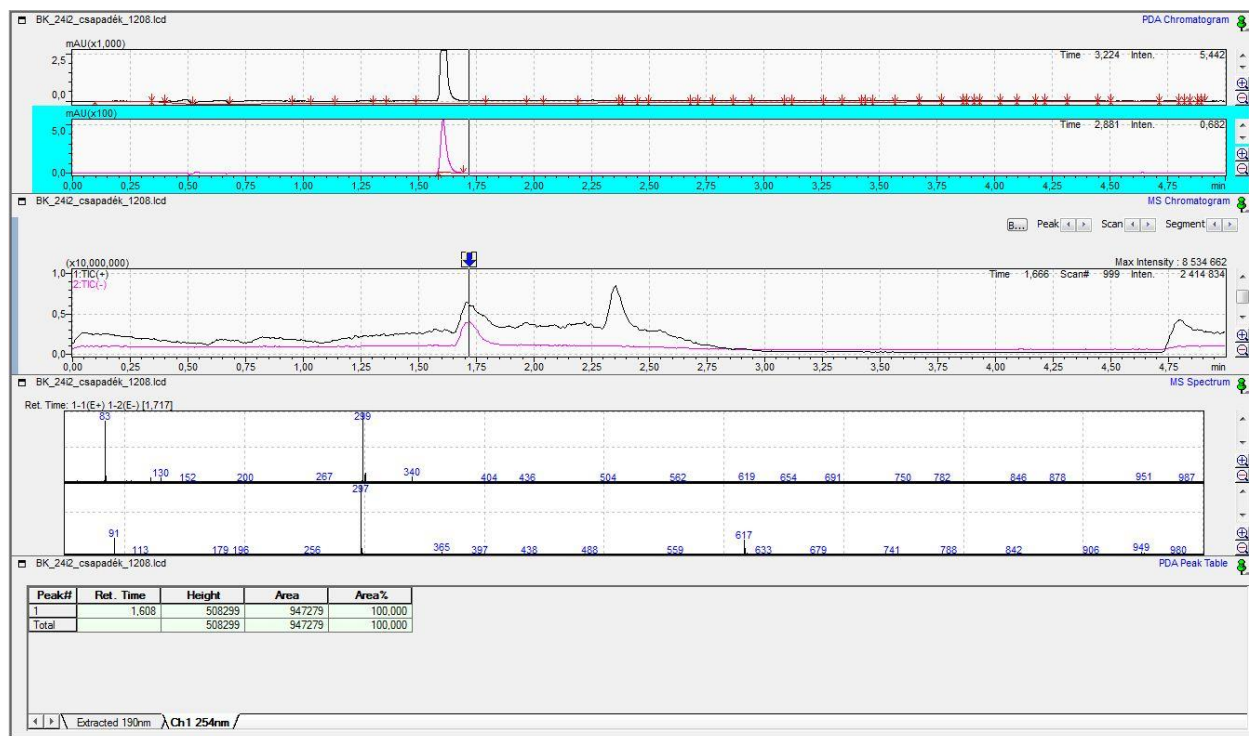
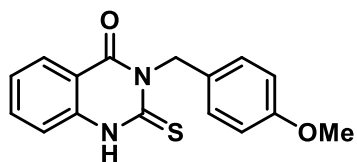
2,5-Dioxopyrrolidin-1-yl
a]quinazolin-1-yl)benzoate (**13**)

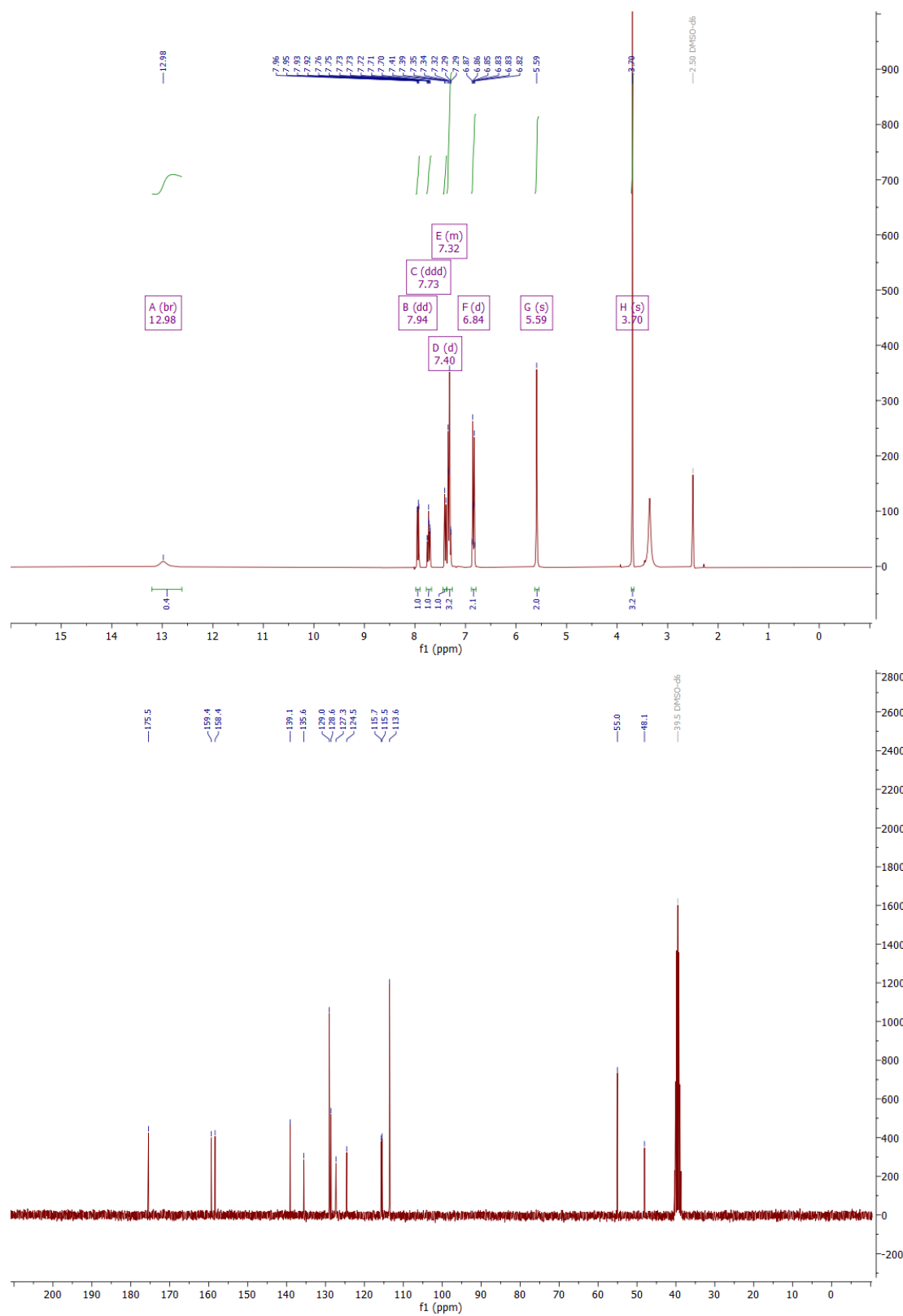
4-(4-(2-chlorobenzyl)-5-oxo-4,5-dihydro-[1,2,4]triazolo[4,3-



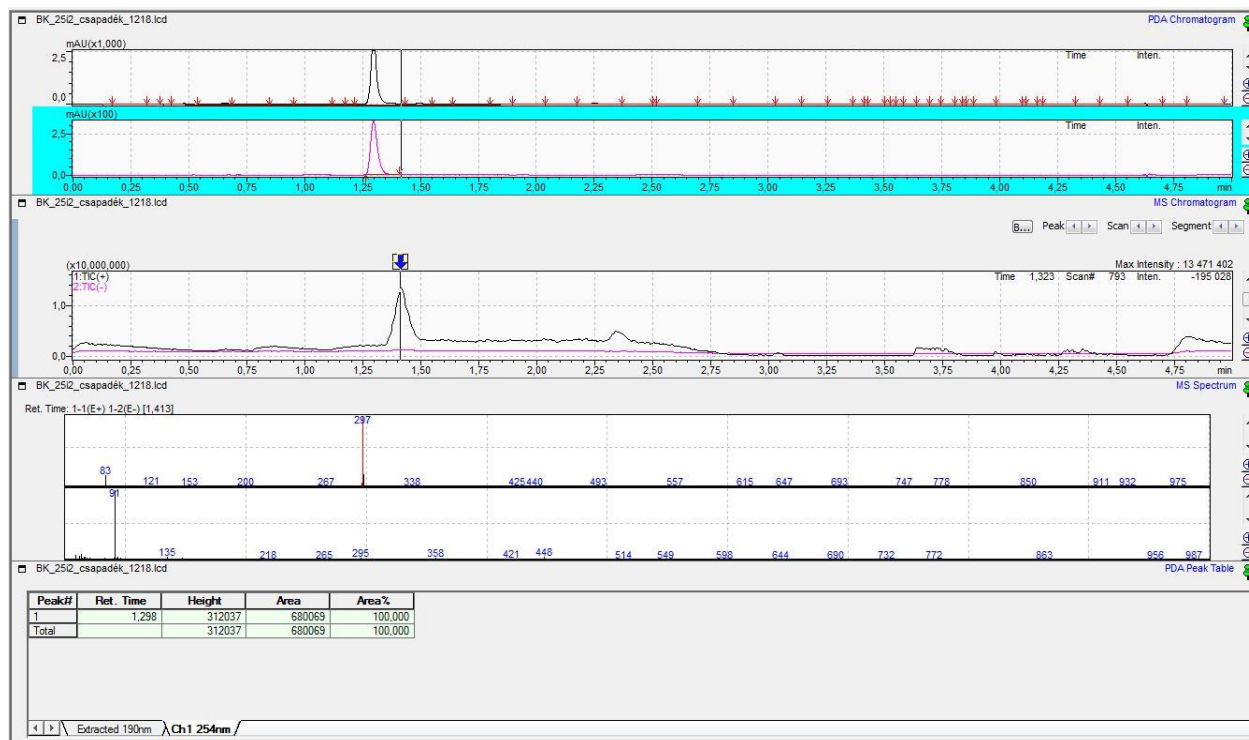
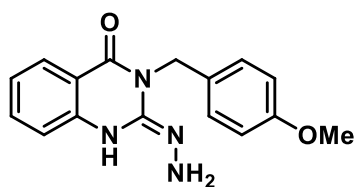


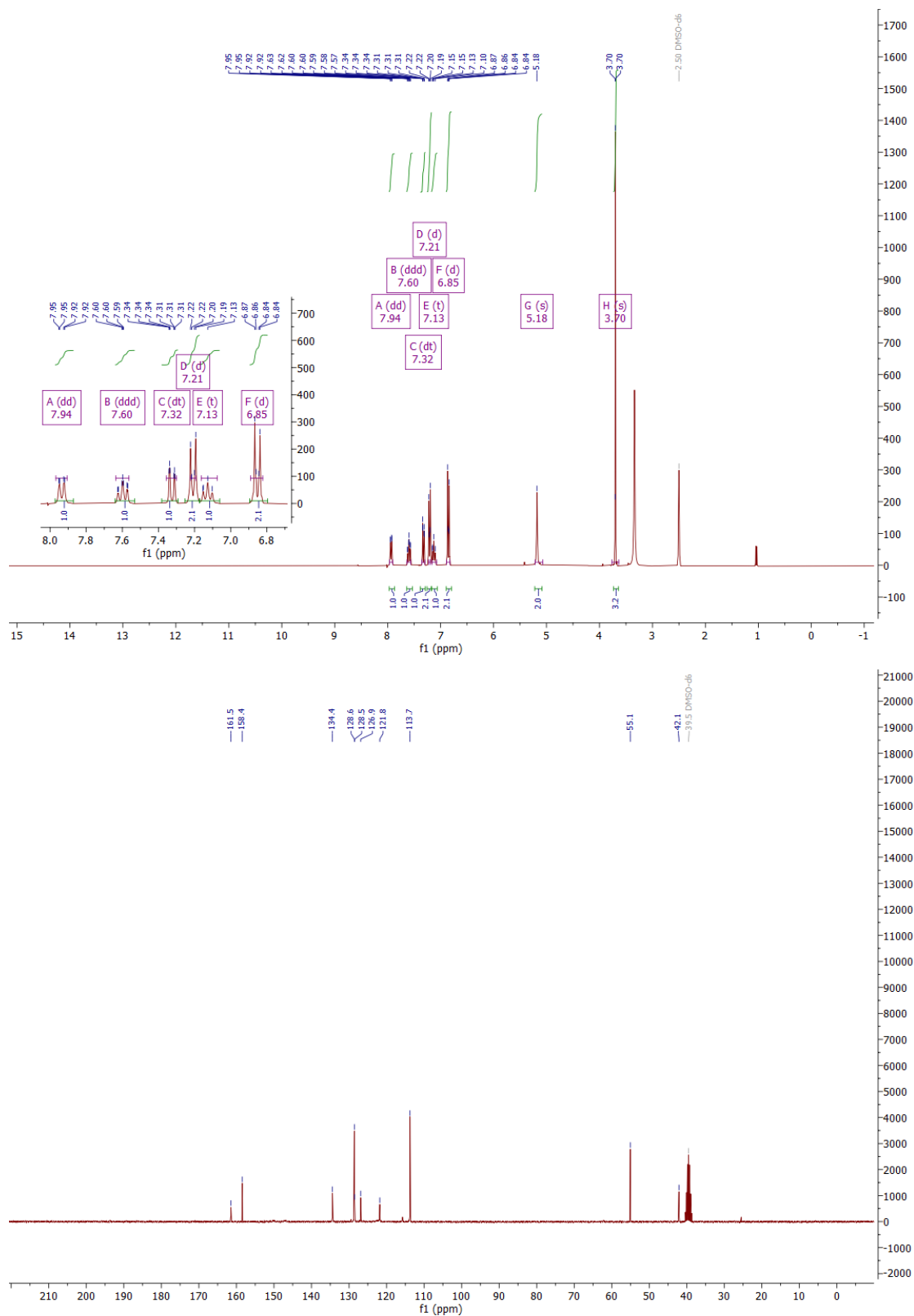
3-(4-Methoxybenzyl)-2-thioxo-2,3-dihydroquinazolin-4(1H)-one (**15**)



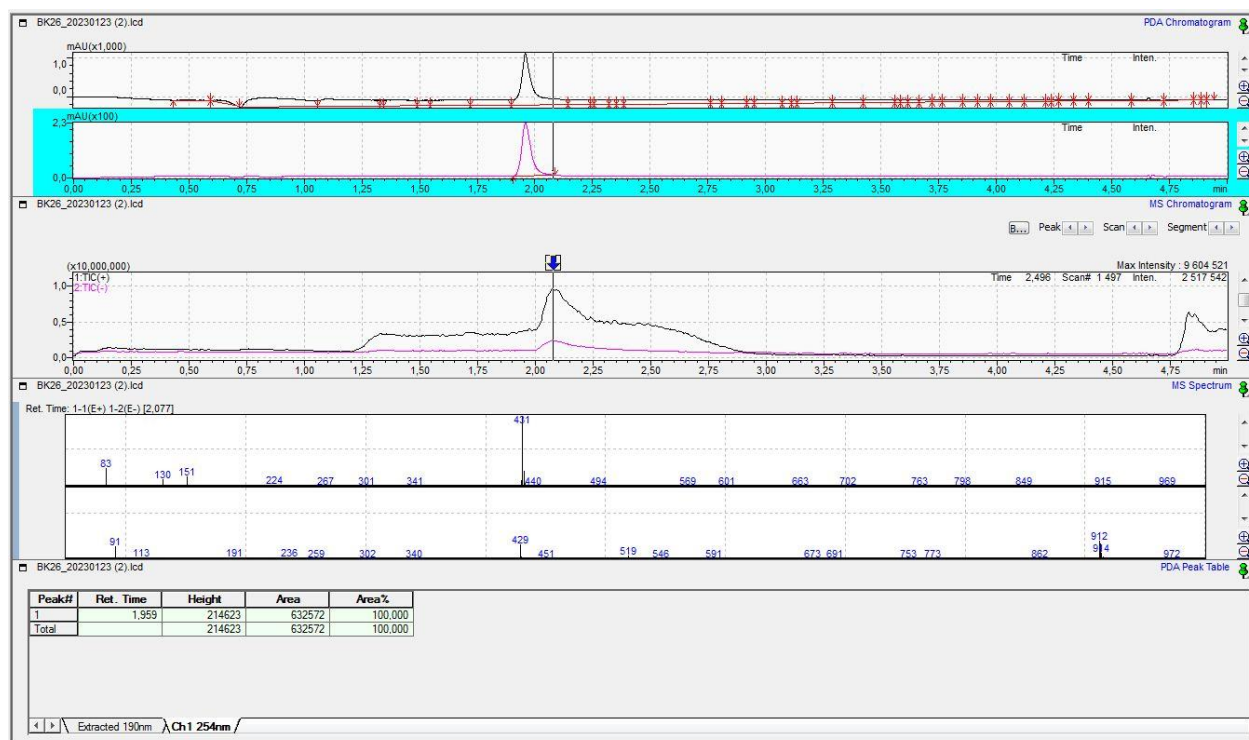
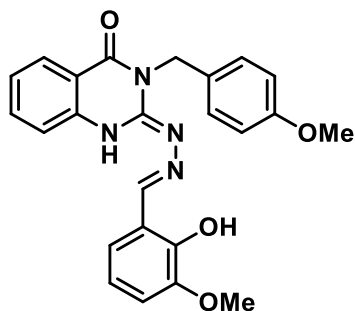


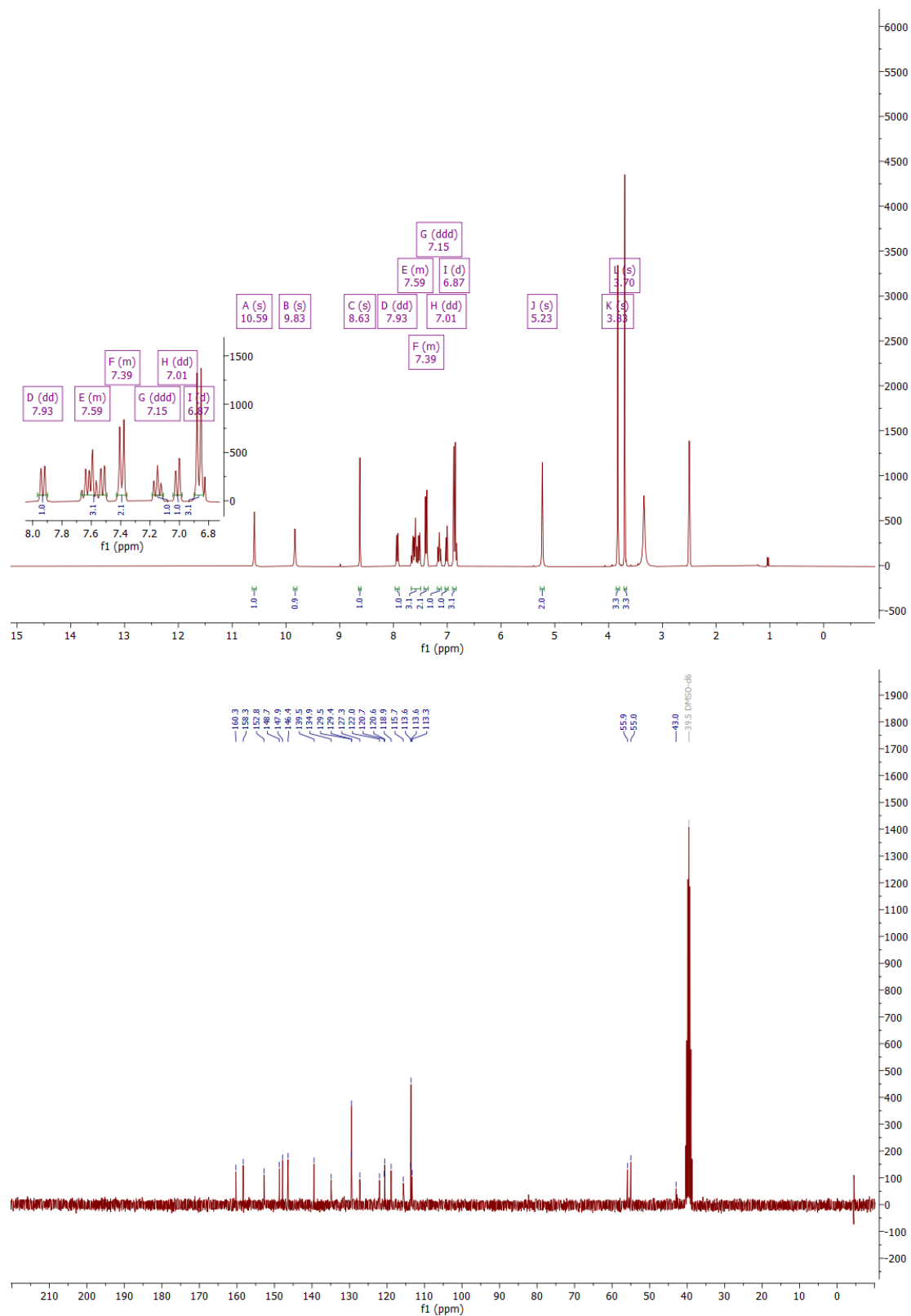
(E)-2-hydrazineylidene-3-(4-methoxybenzyl)-2,3-dihydroquinazolin-4(1H)-one (**16**)



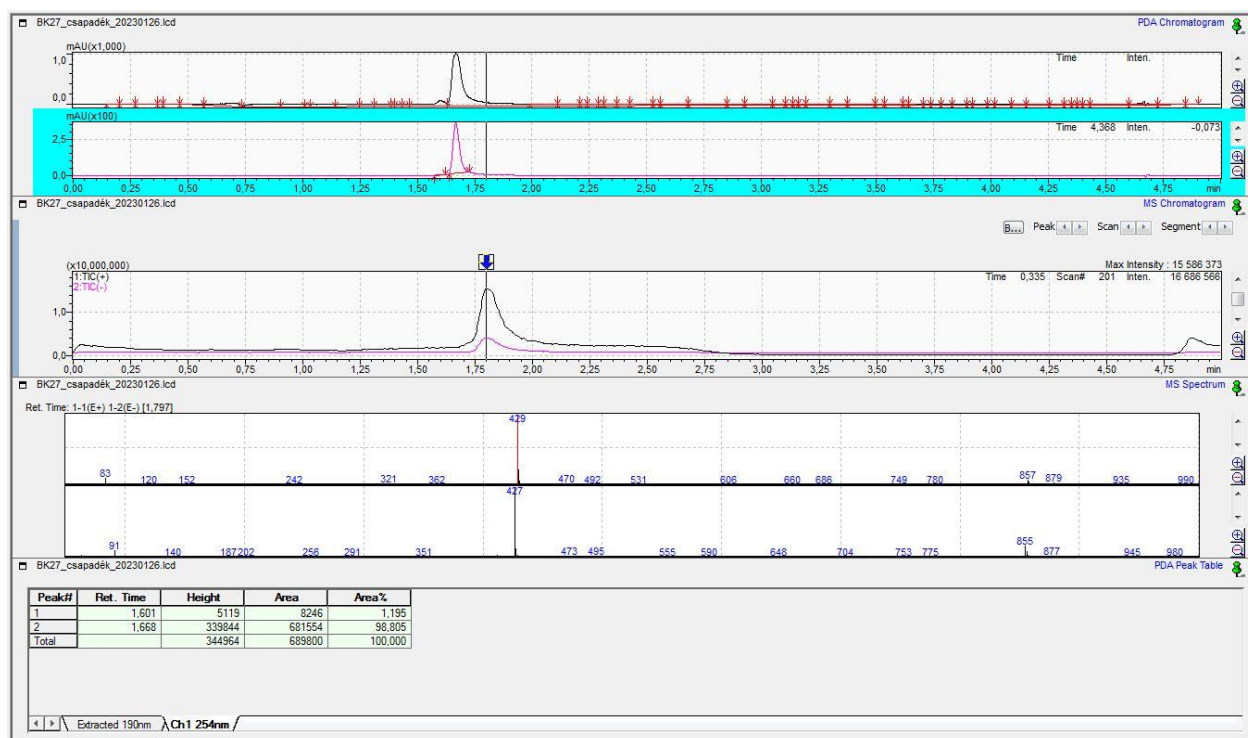
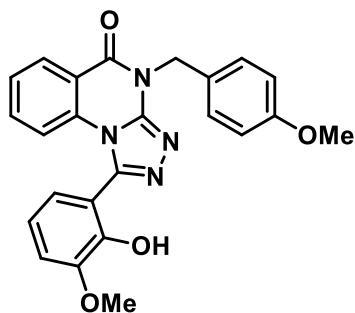


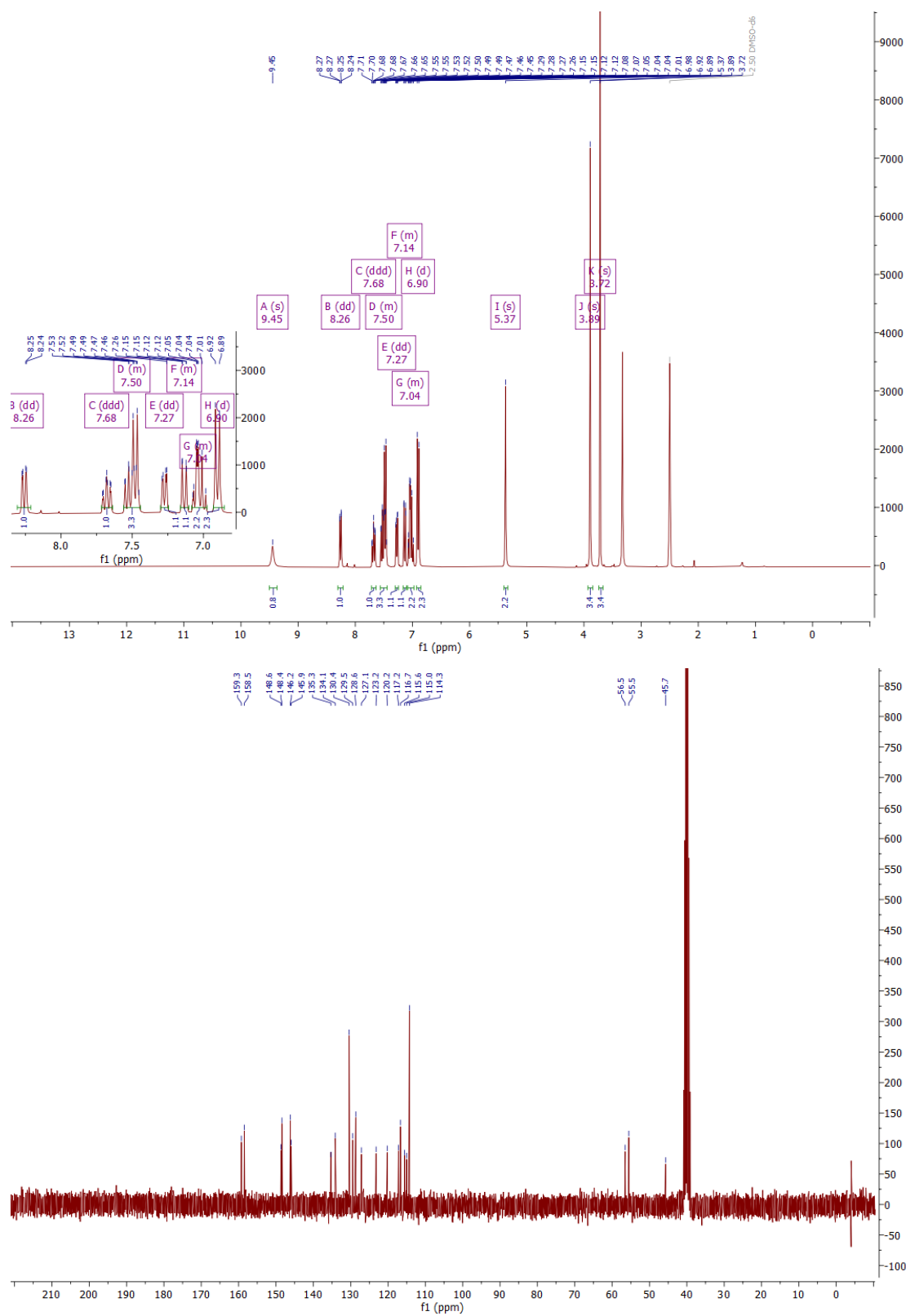
(E)-2-(((E)-2-hydroxy-3-methoxybenzylidene)hydrazineylidene)-3-(4-methoxybenzyl)-2,3-dihydro-quinazolin-4(1H)-one (**17**)



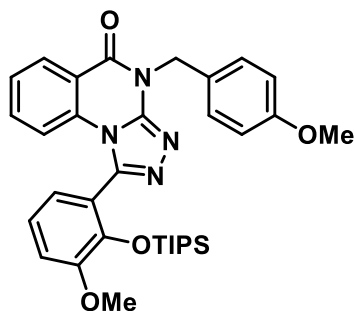


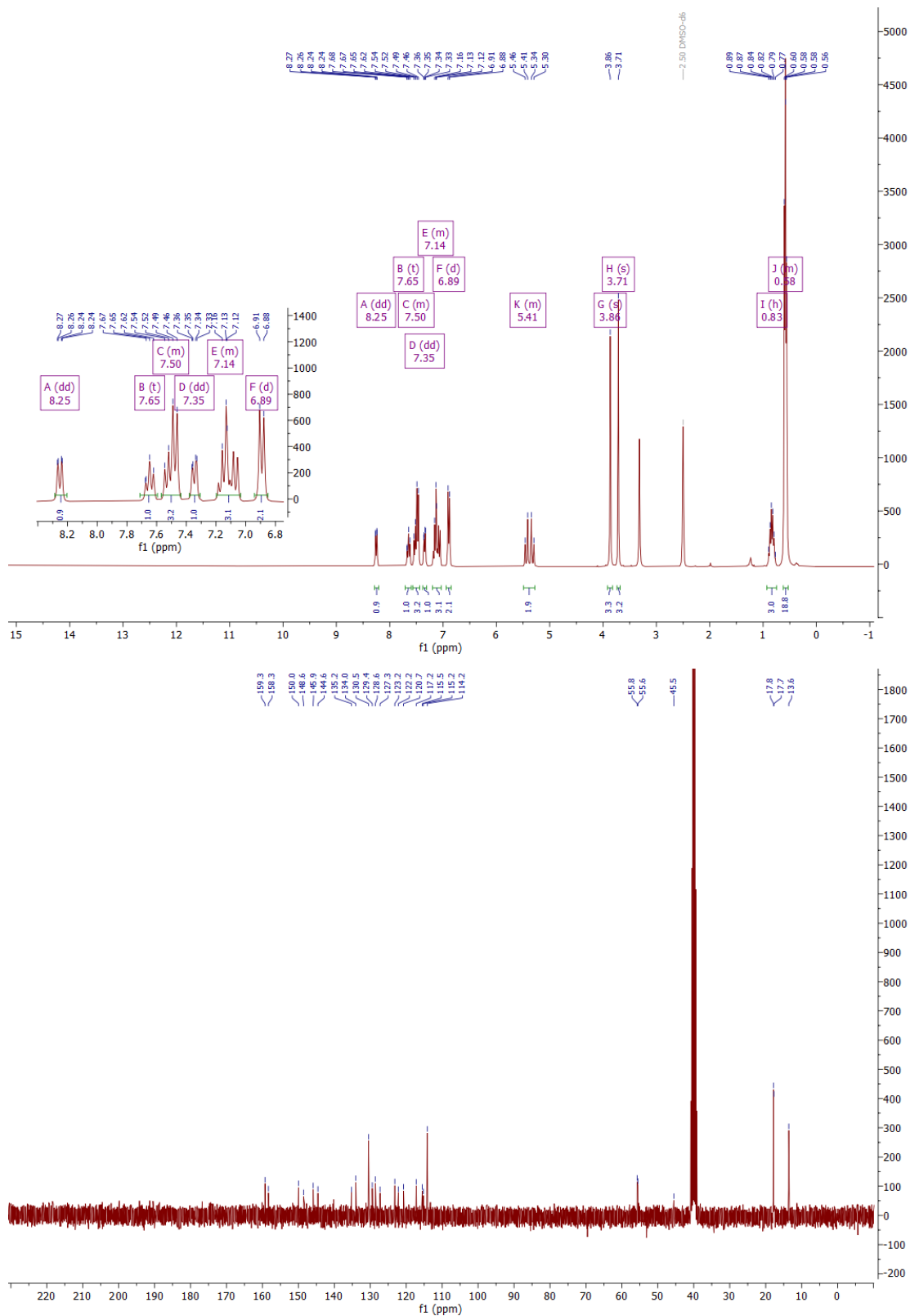
1-(2-hydroxy-3-methoxyphenyl)-4-(4-methoxybenzyl)-[1,2,4]triazolo[4,3-a]quinazolin-5(4H)-one (**18**)



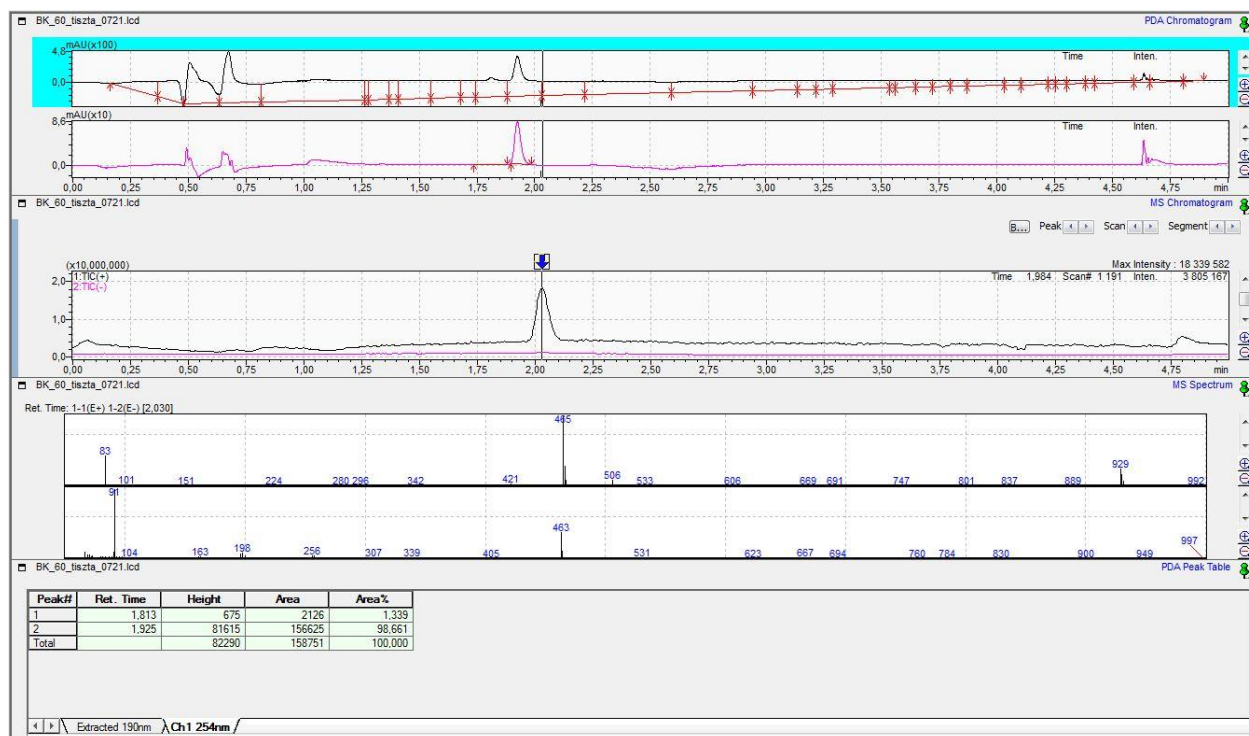
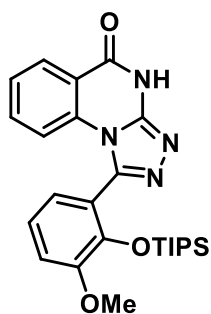


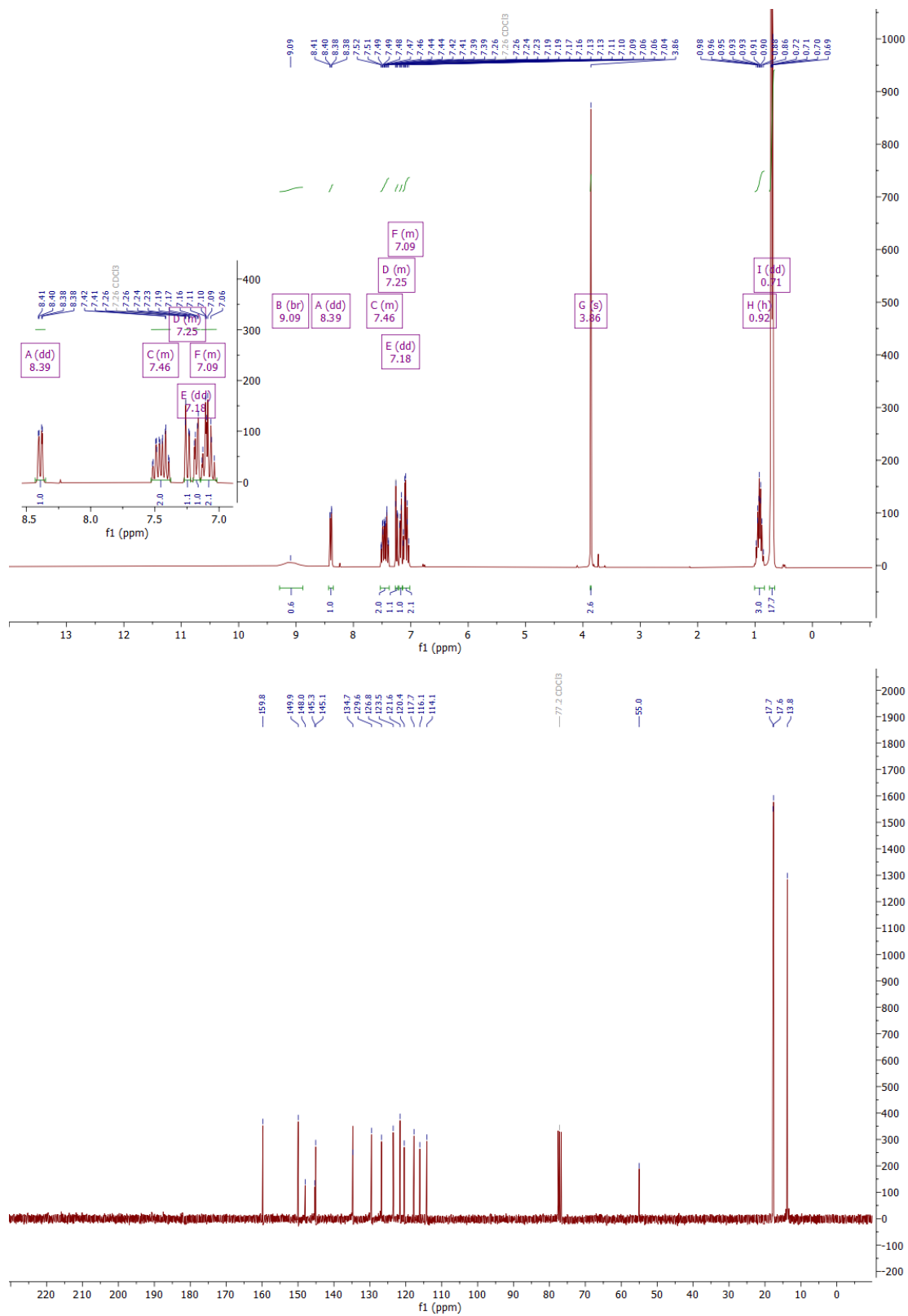
*1-(3-Methoxy-2-((triisopropylsilyl)oxy)phenyl)-4-(4-methoxybenzyl)-[1,2,4]triazolo[4,3-*a*]quinazolin-5(4*H*)-one (19)*



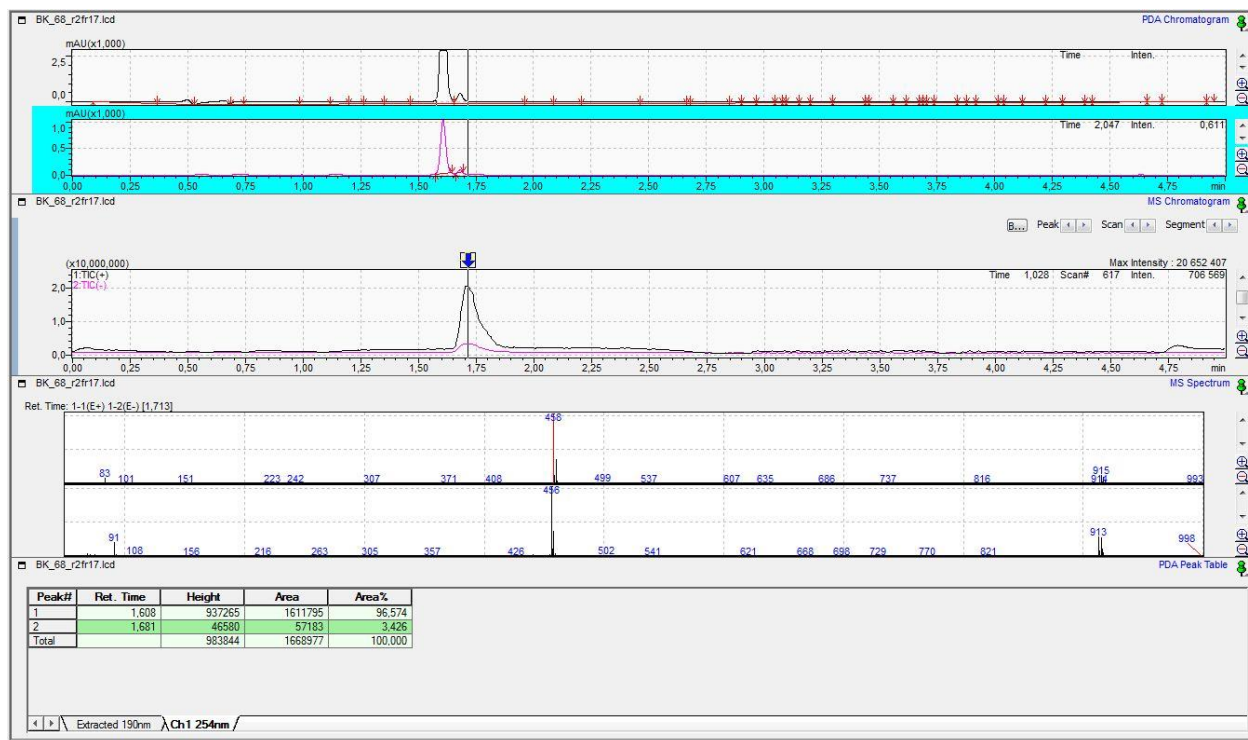
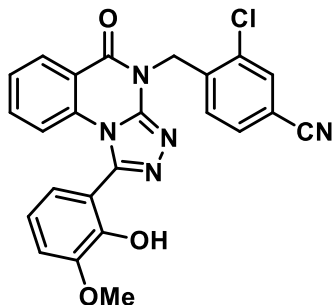


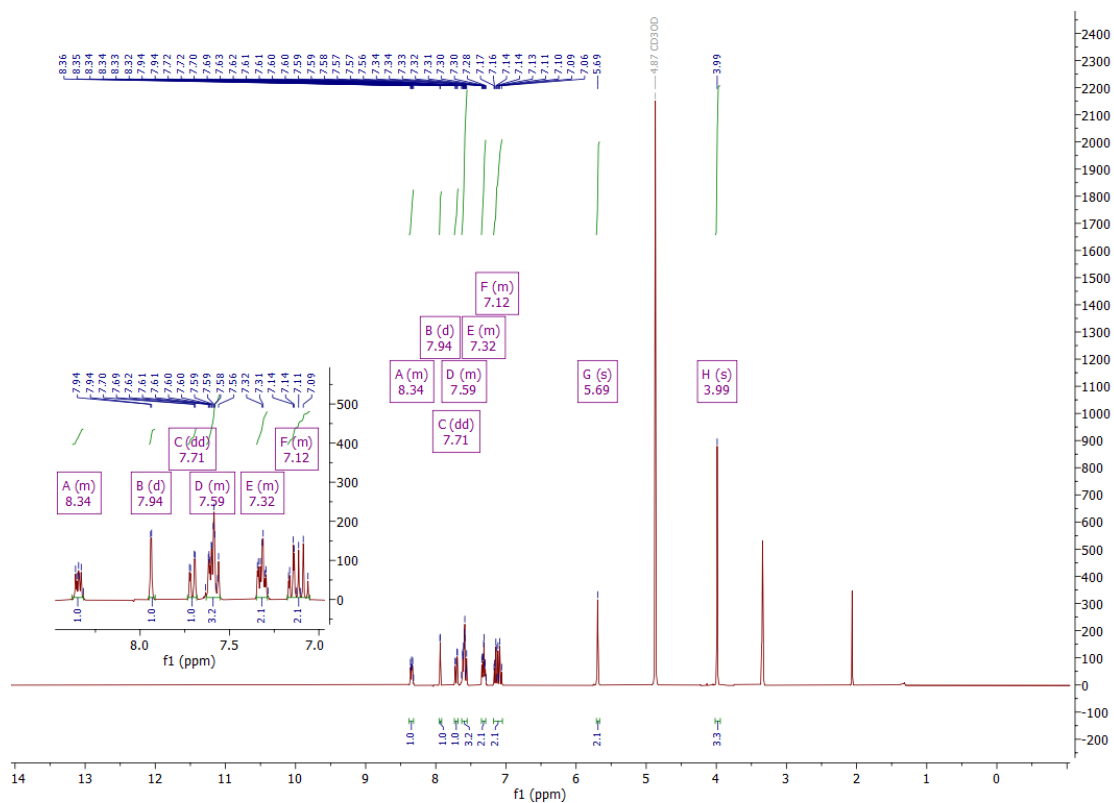
1-(3-Methoxy-2-((triisopropylsilyl)oxy)phenyl)-[1,2,4]triazolo[4,3-a]quinazolin-5(4H)-one (20)



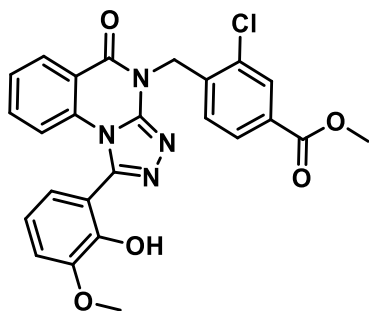


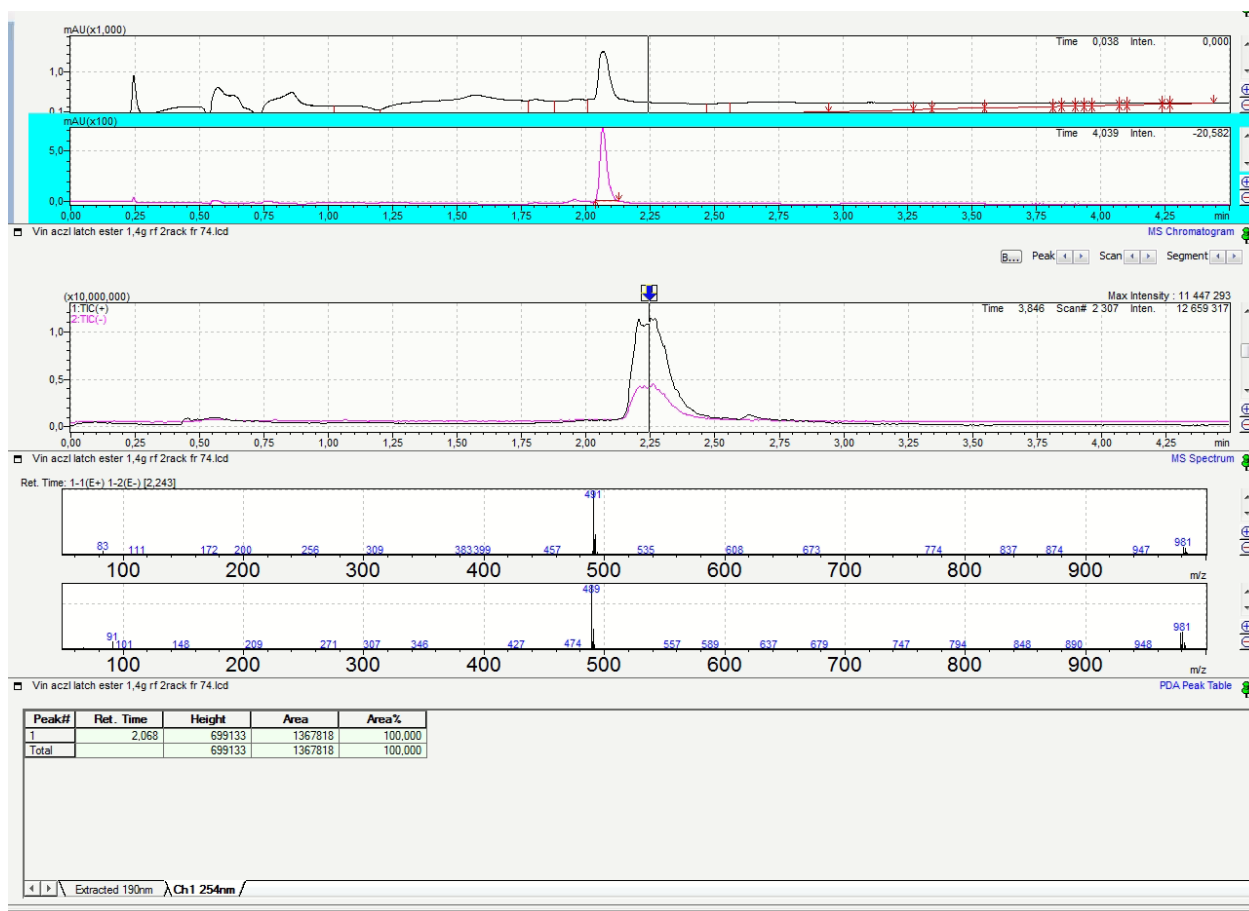
3-Chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzonitrile (**21**)

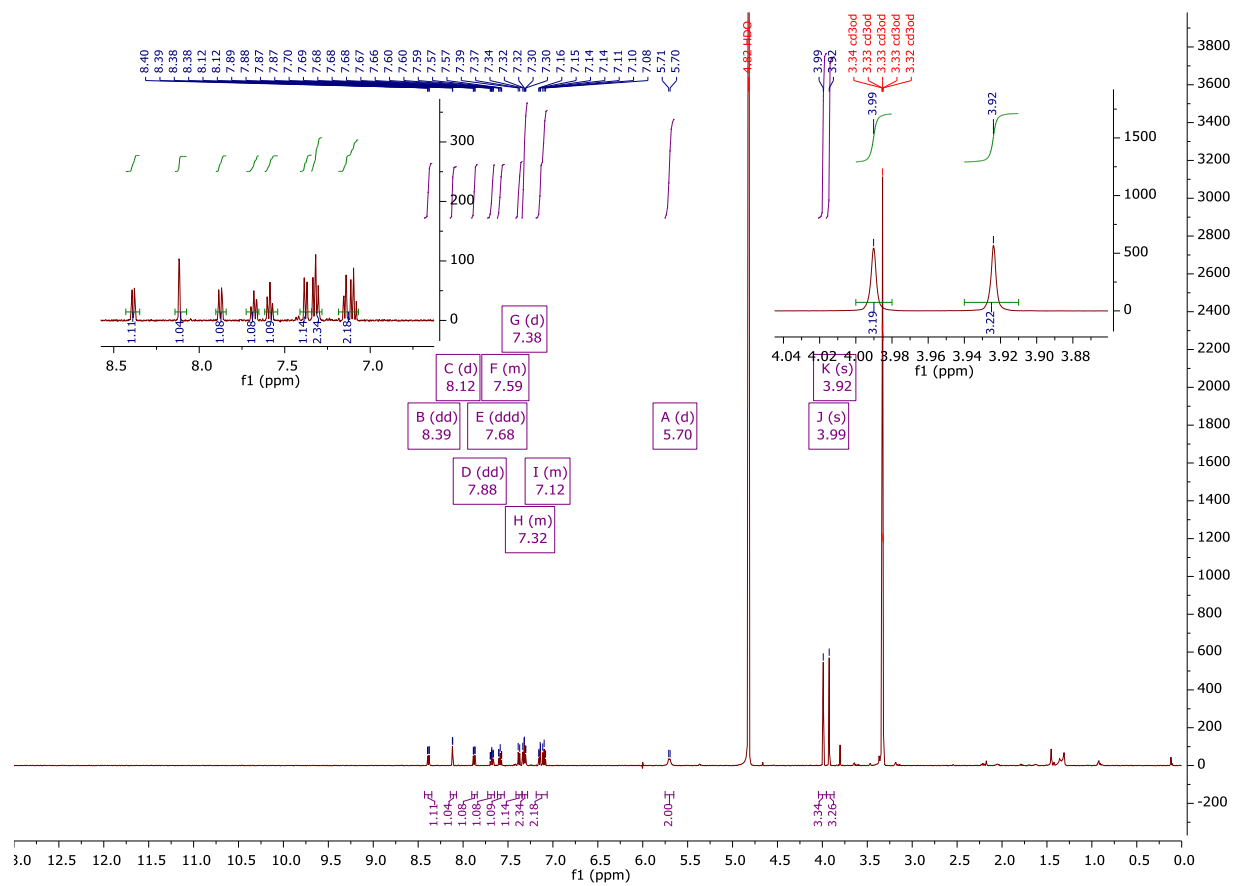


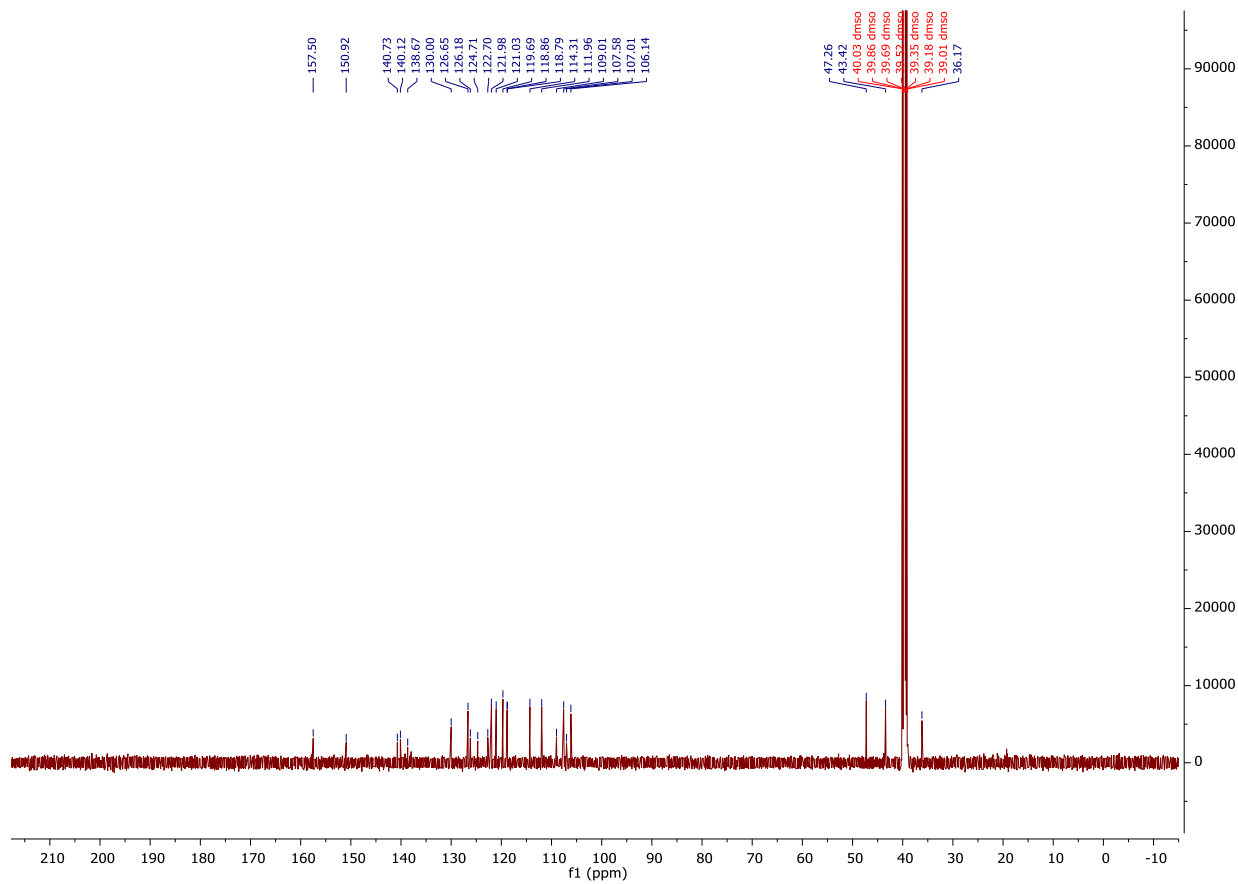


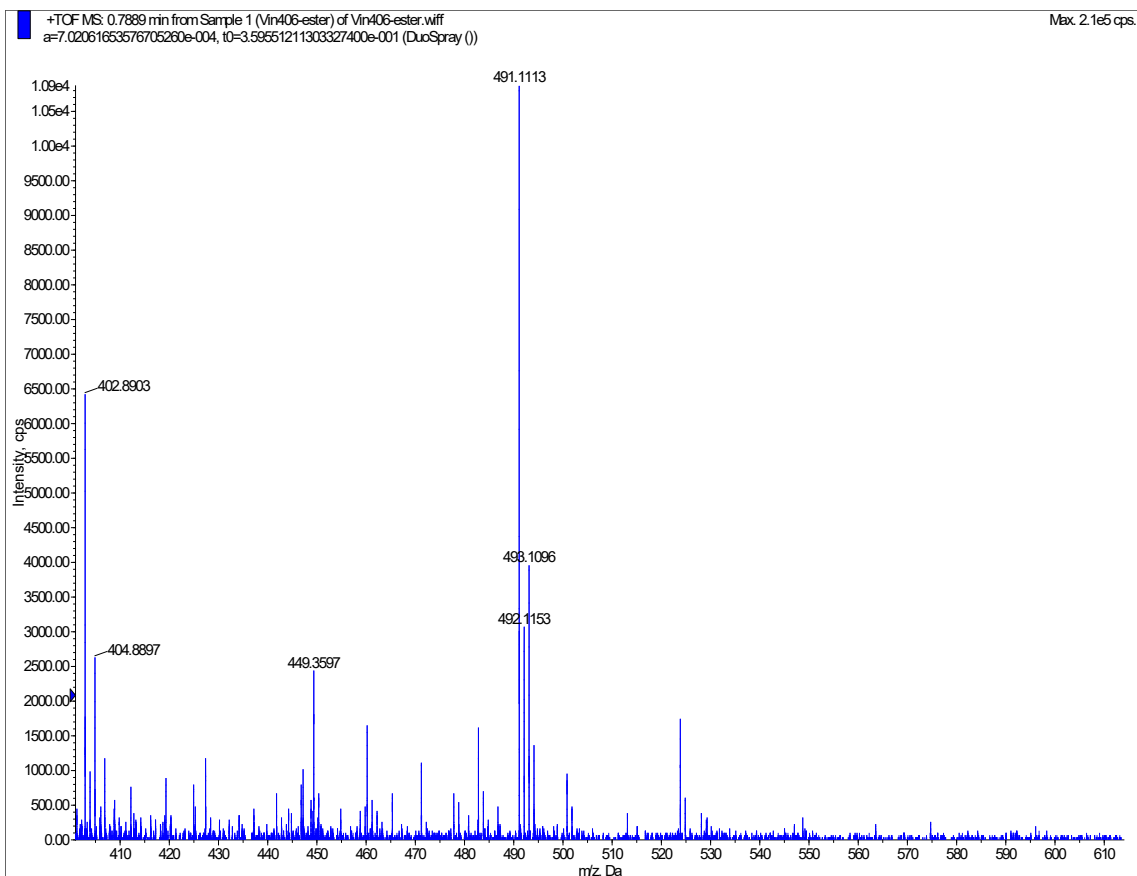
Methyl 3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoate (22)





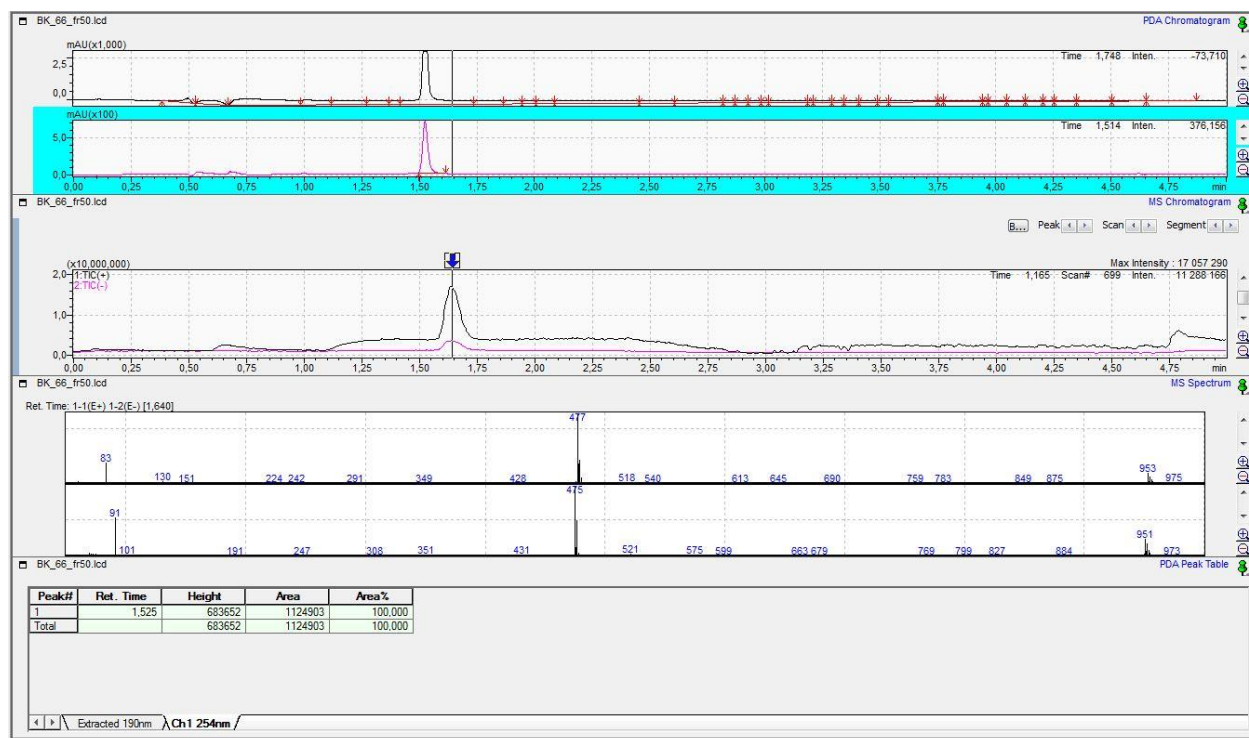
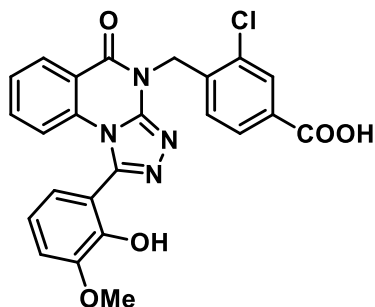


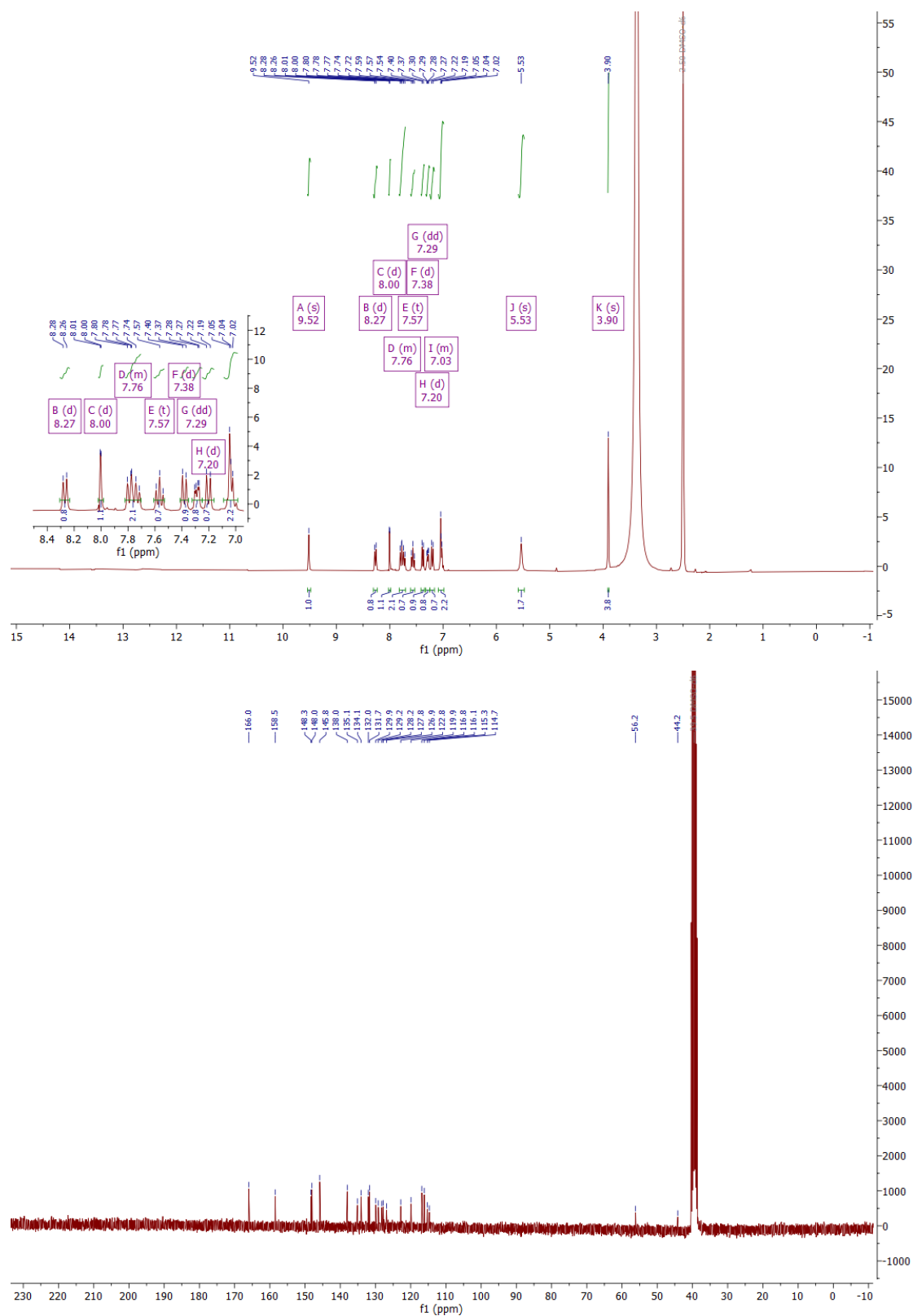




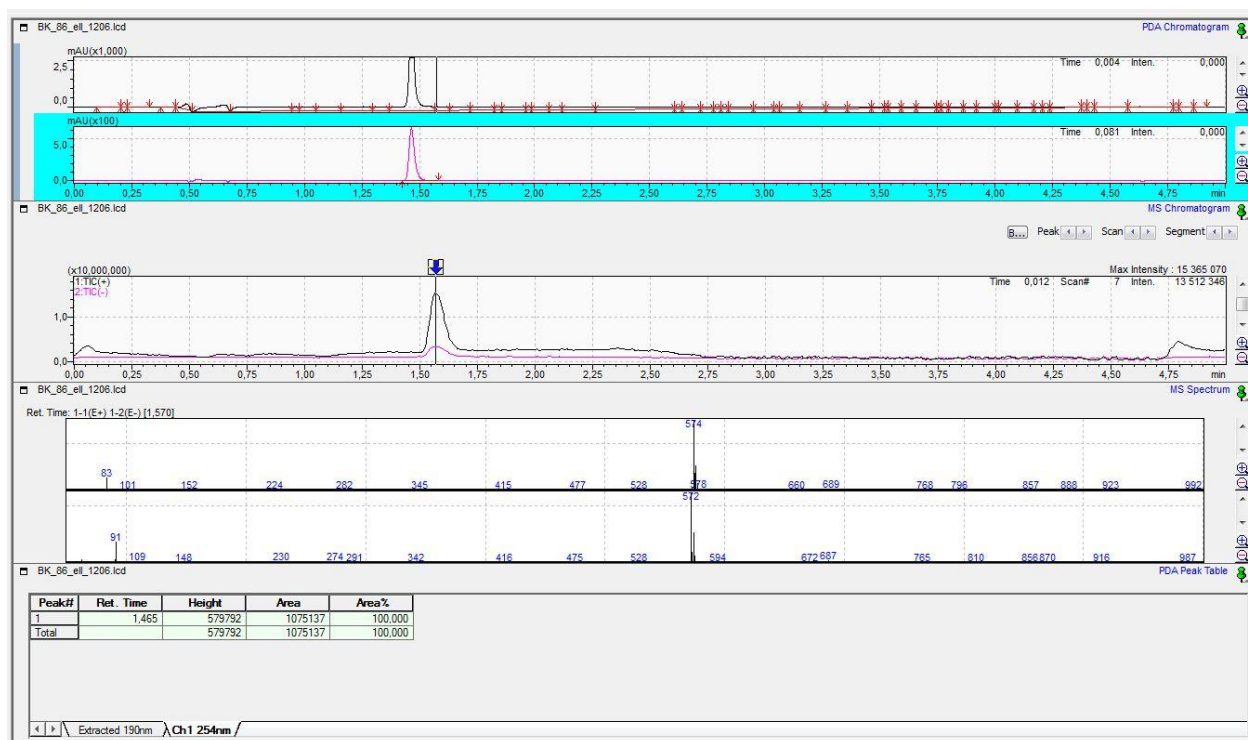
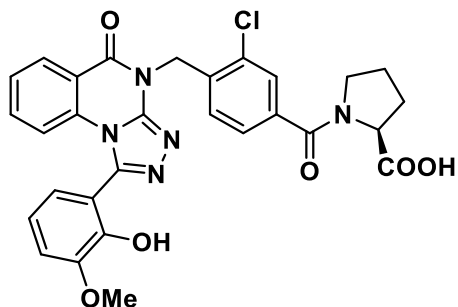
Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C25 H20 N4 O5 Cl	491.1116	-0.3741	-0.7619	17.5

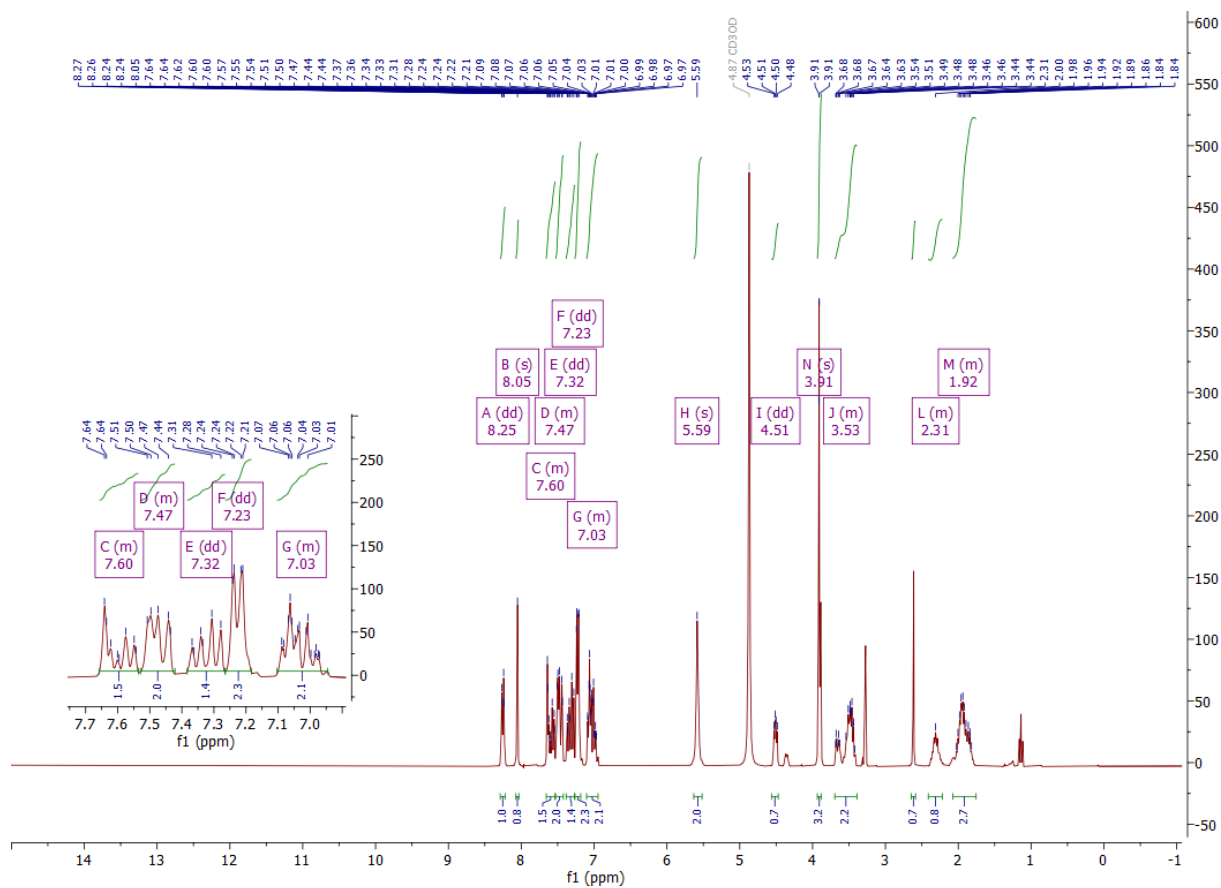
3-Chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoic acid (**23**)



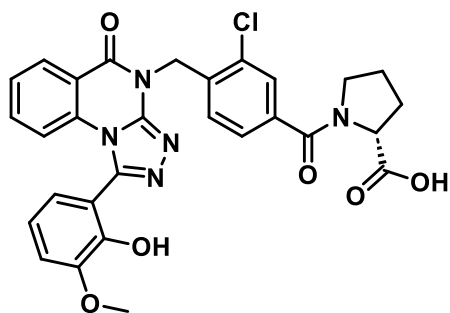


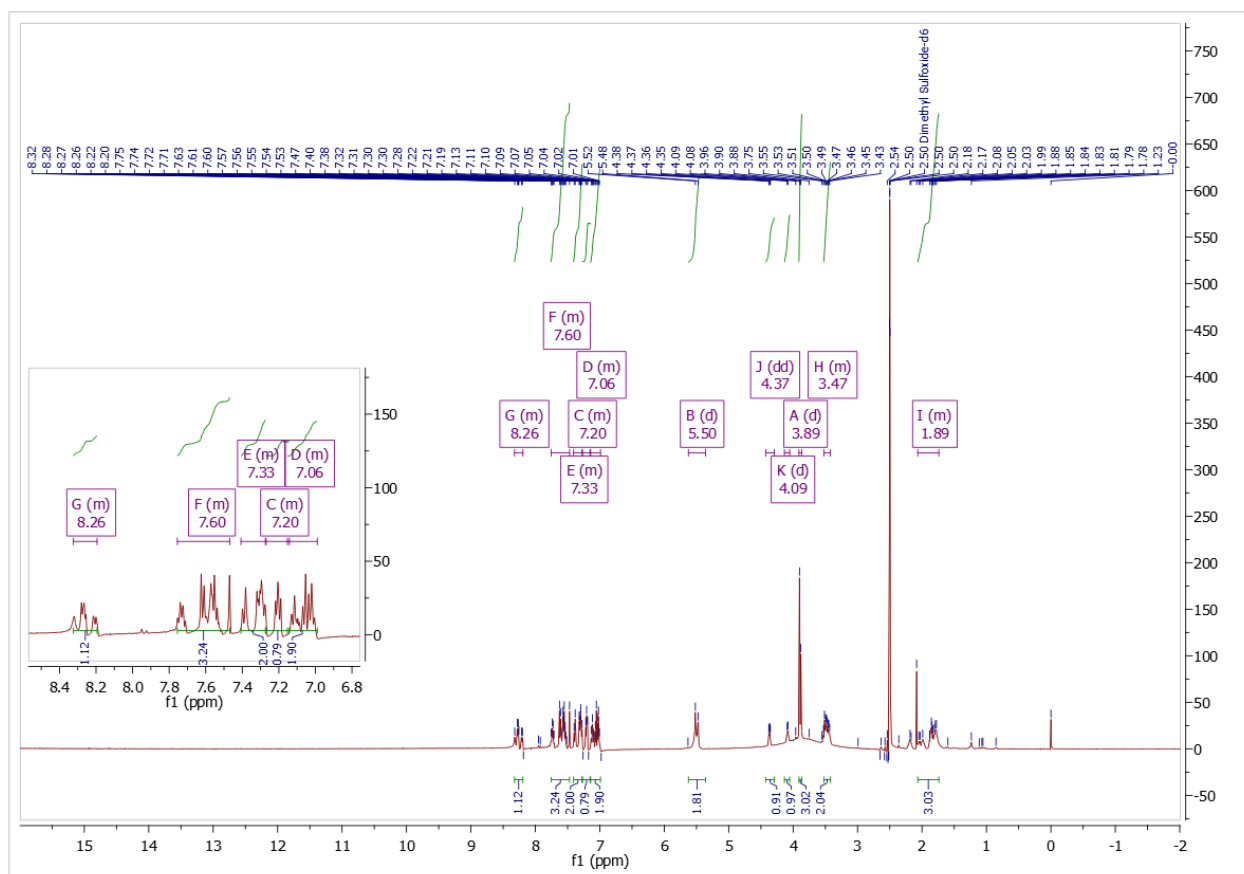
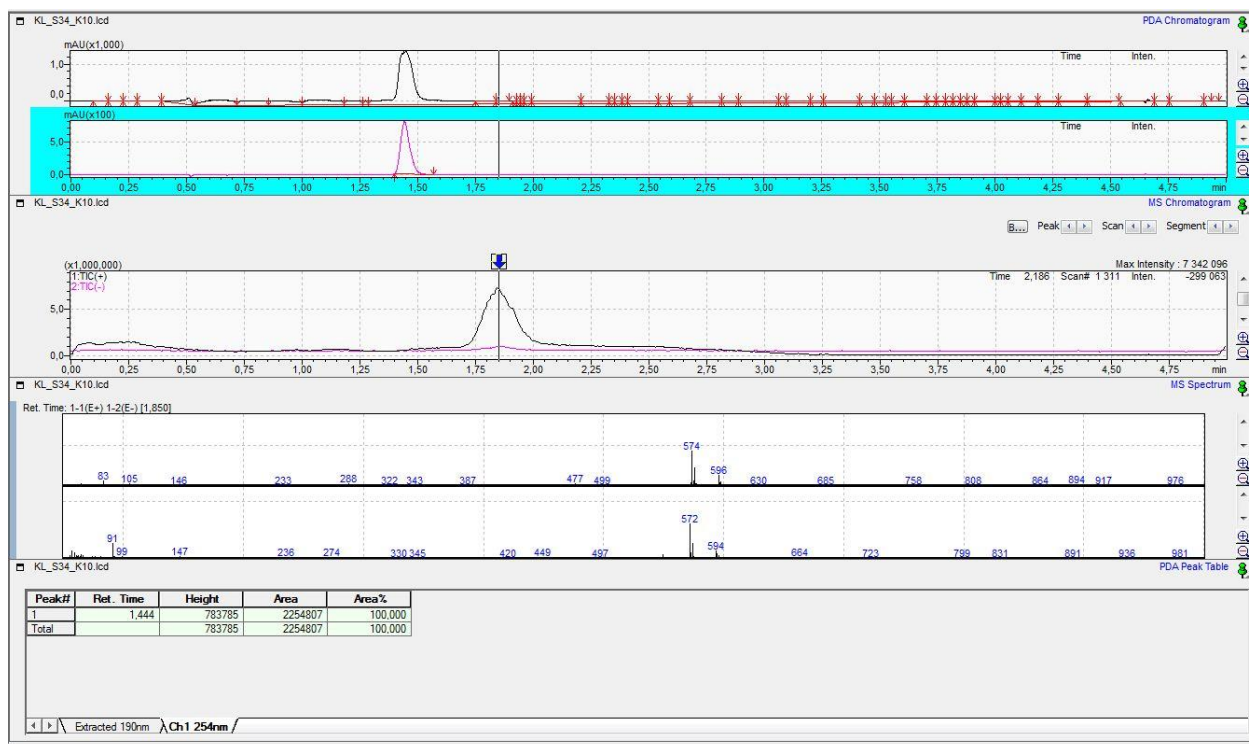
(3-Chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-L-proline (**24**)

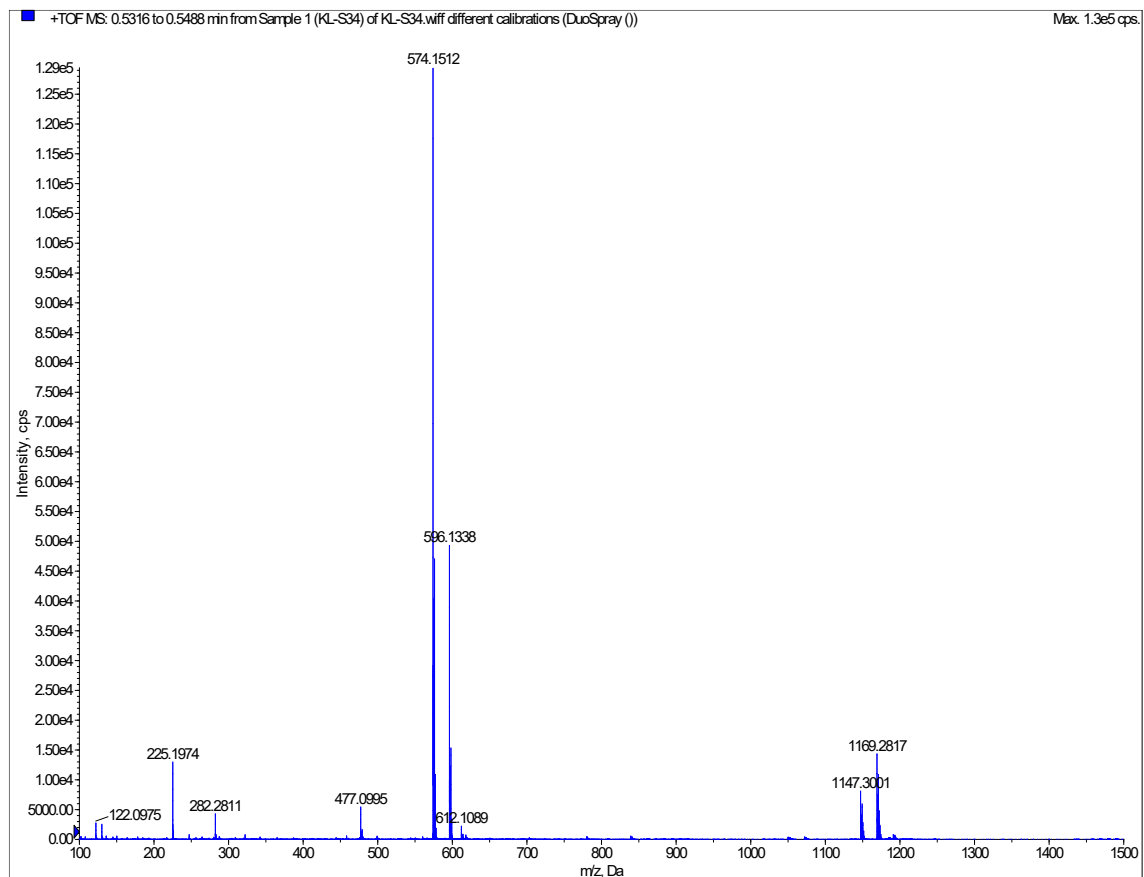




(3-Chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-D-proline (**25**)

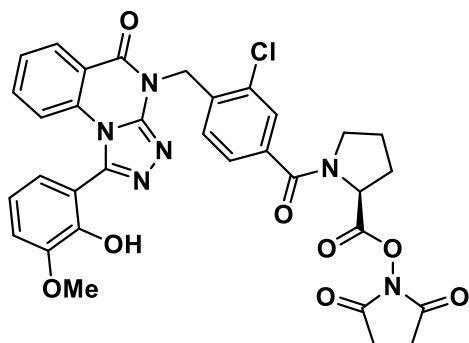


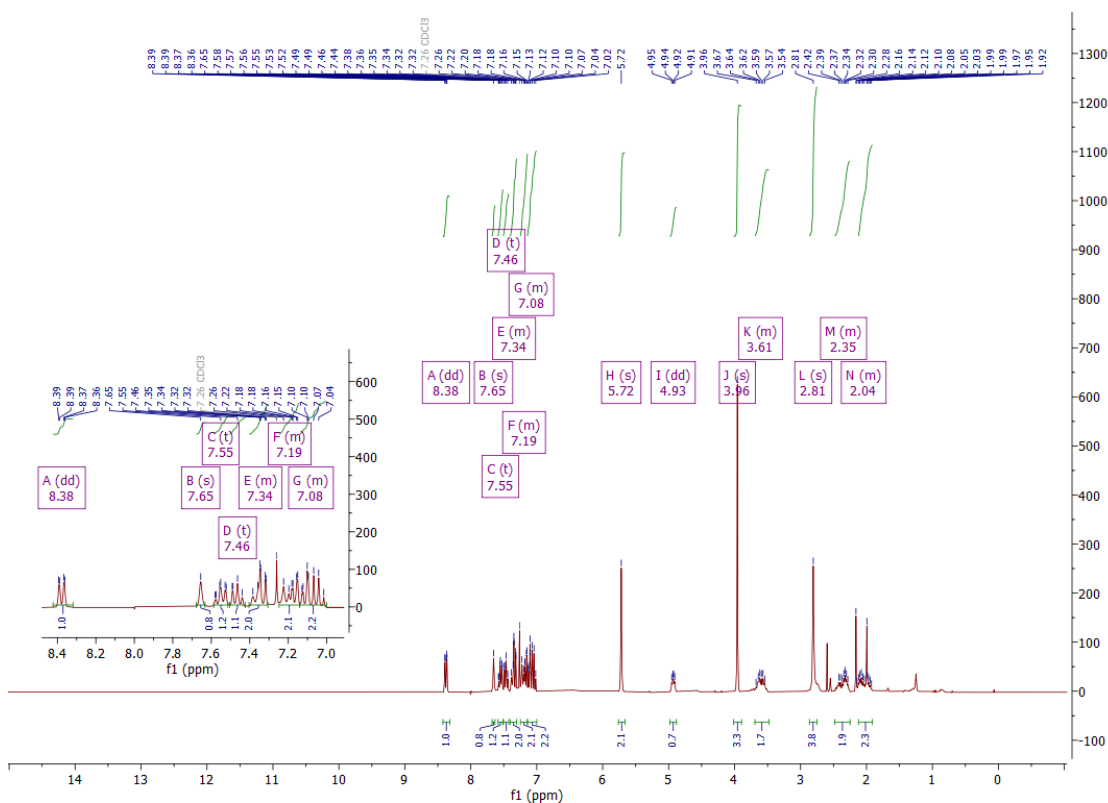
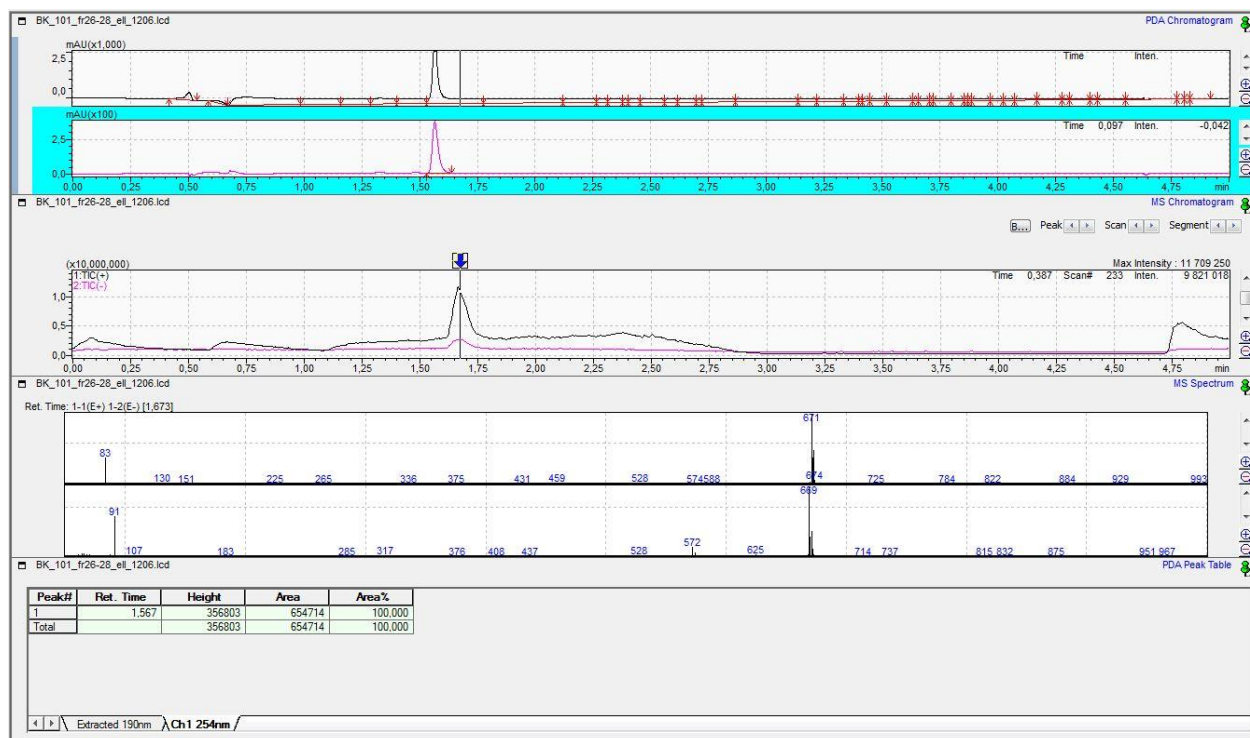




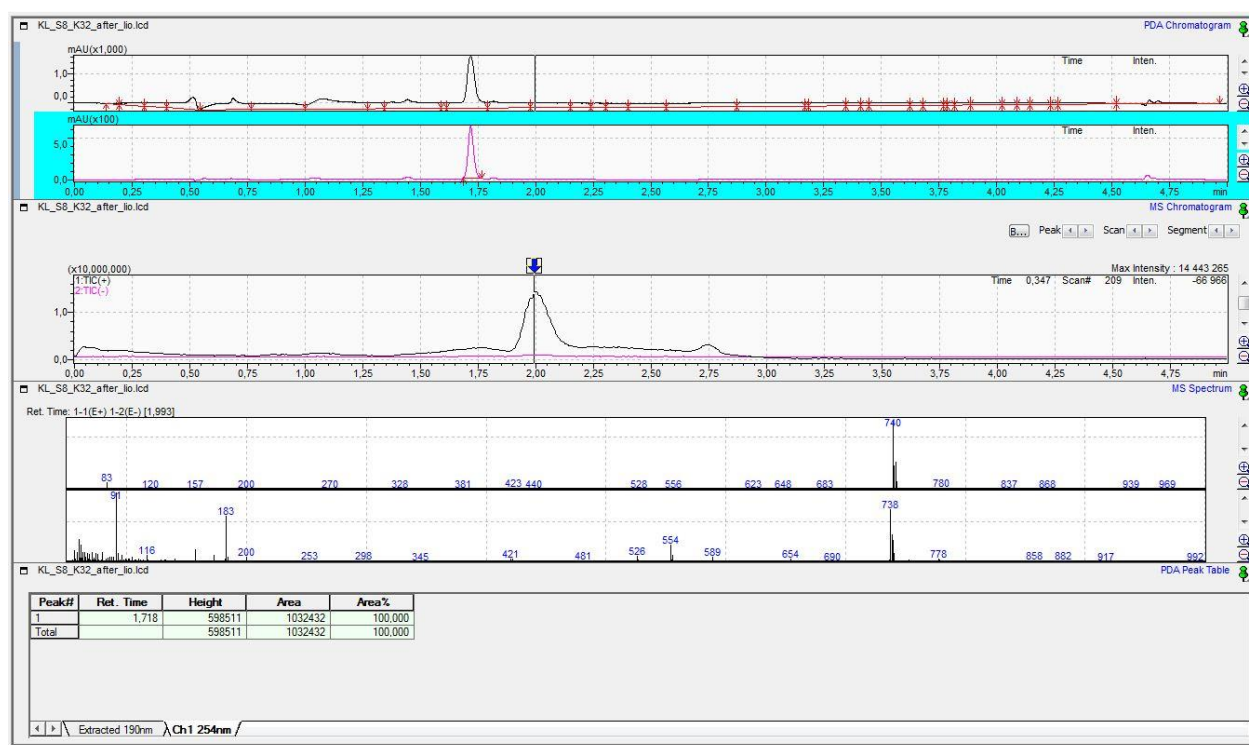
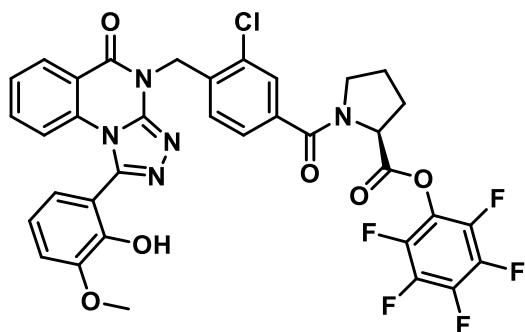
Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C ₂₉ H ₂₅ N ₅ O ₆ Cl	574.1487	2.4119	4.2009	19.5

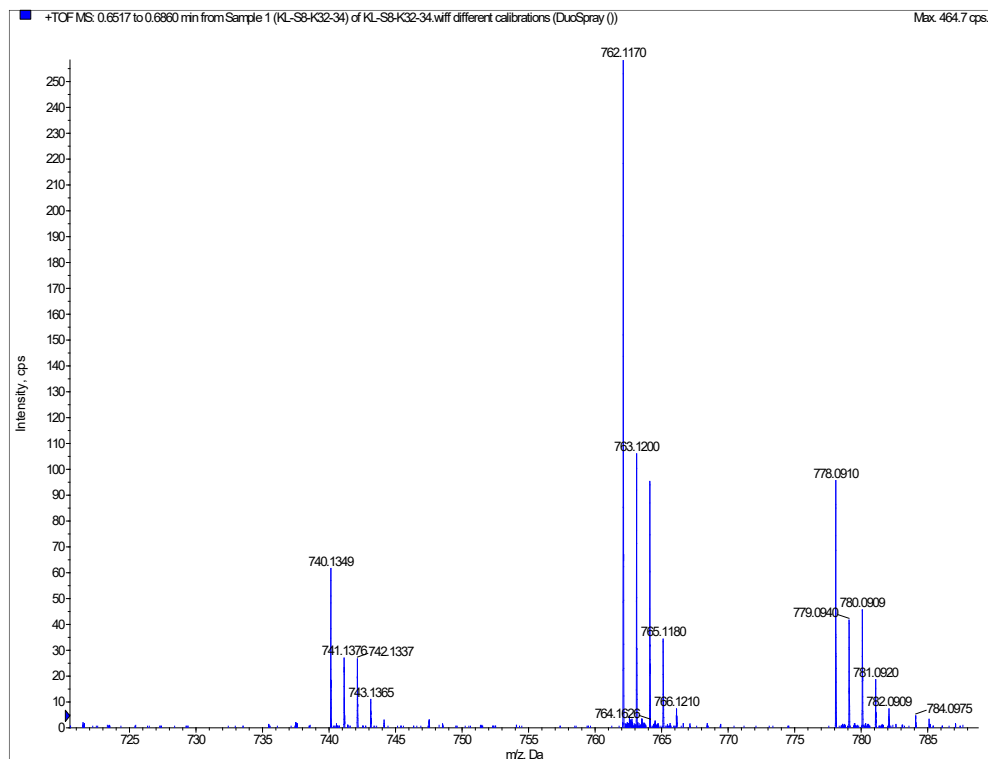
2,5-Dioxopyrrolidin-1-yl (3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-L-prolinate (**26**)





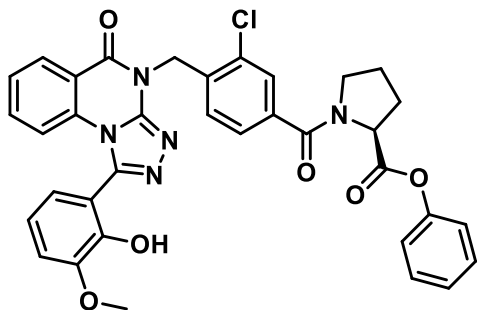
Perfluorophenyl (3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-L-prolinate (**27**)

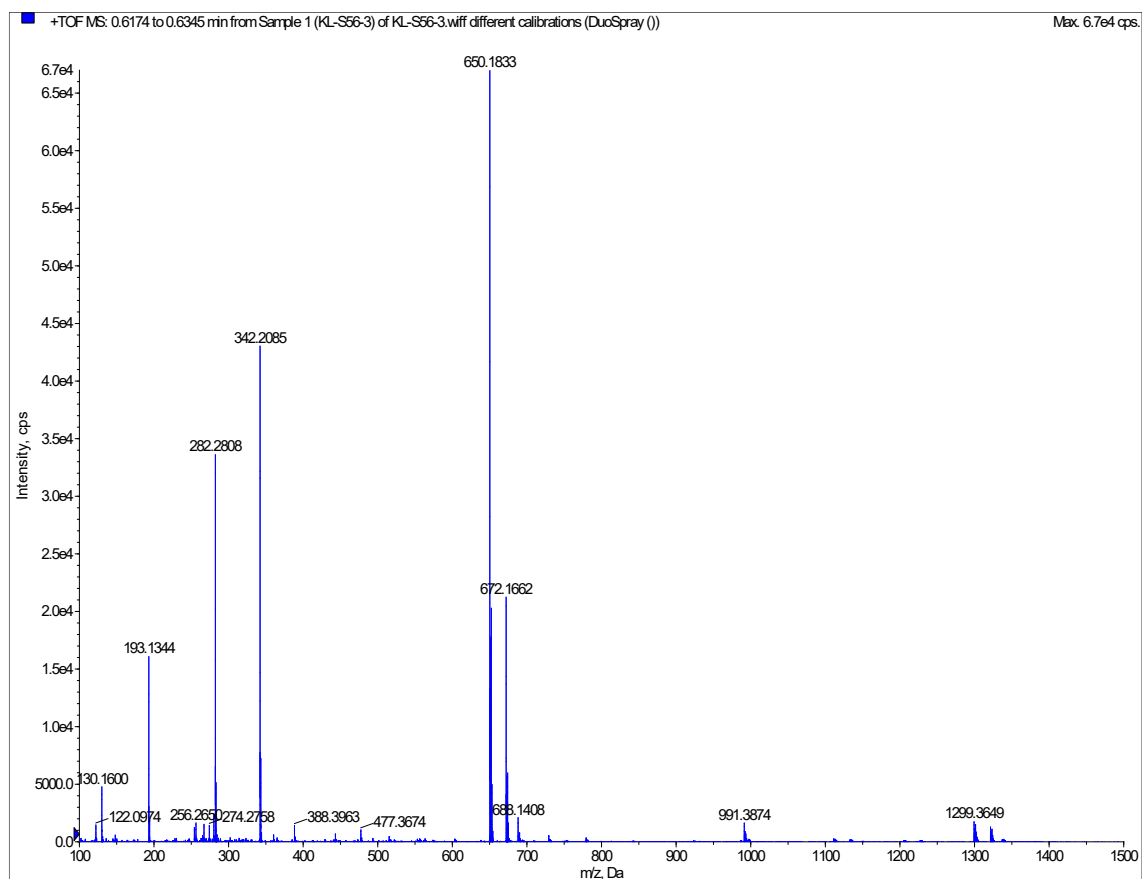
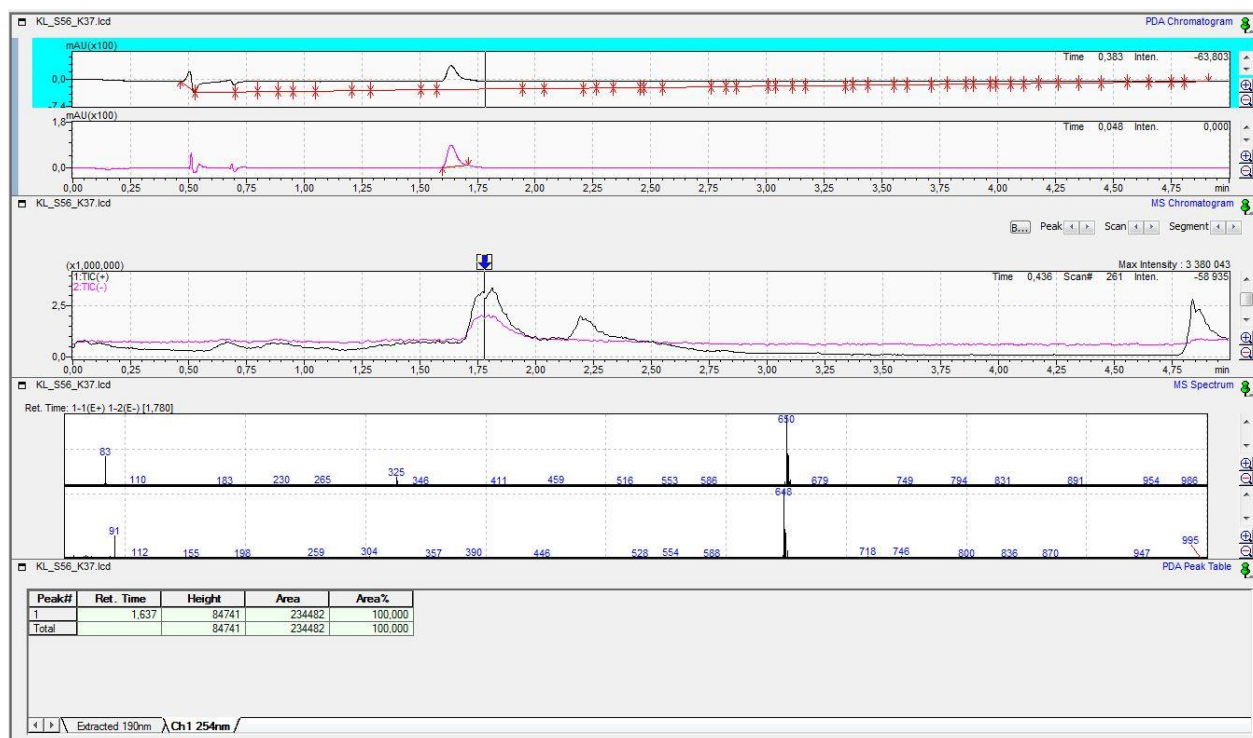




Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C35 H24 N5 O6 F5 Cl	740.1329	1.9207	2.5951	23.5

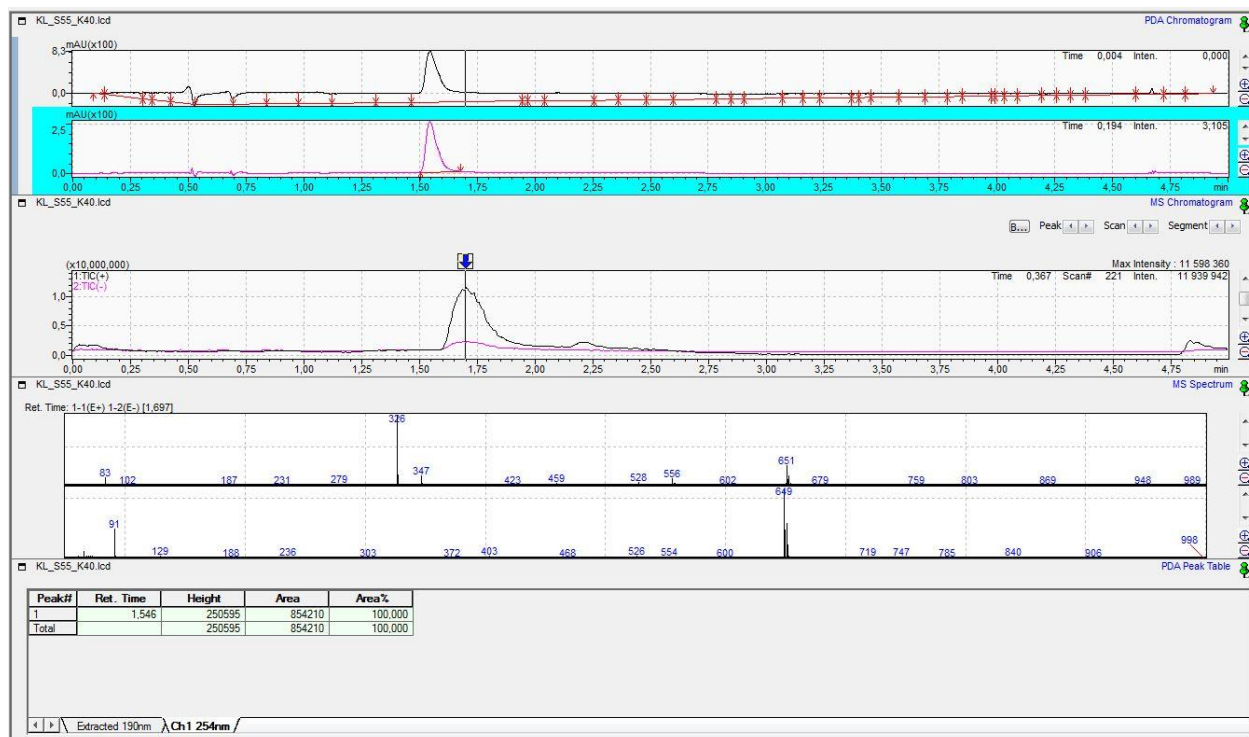
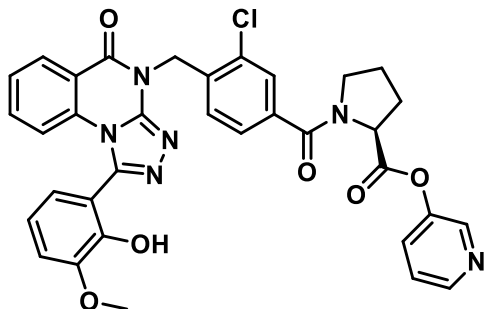
Phenyl (3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-L-prolinate (28)

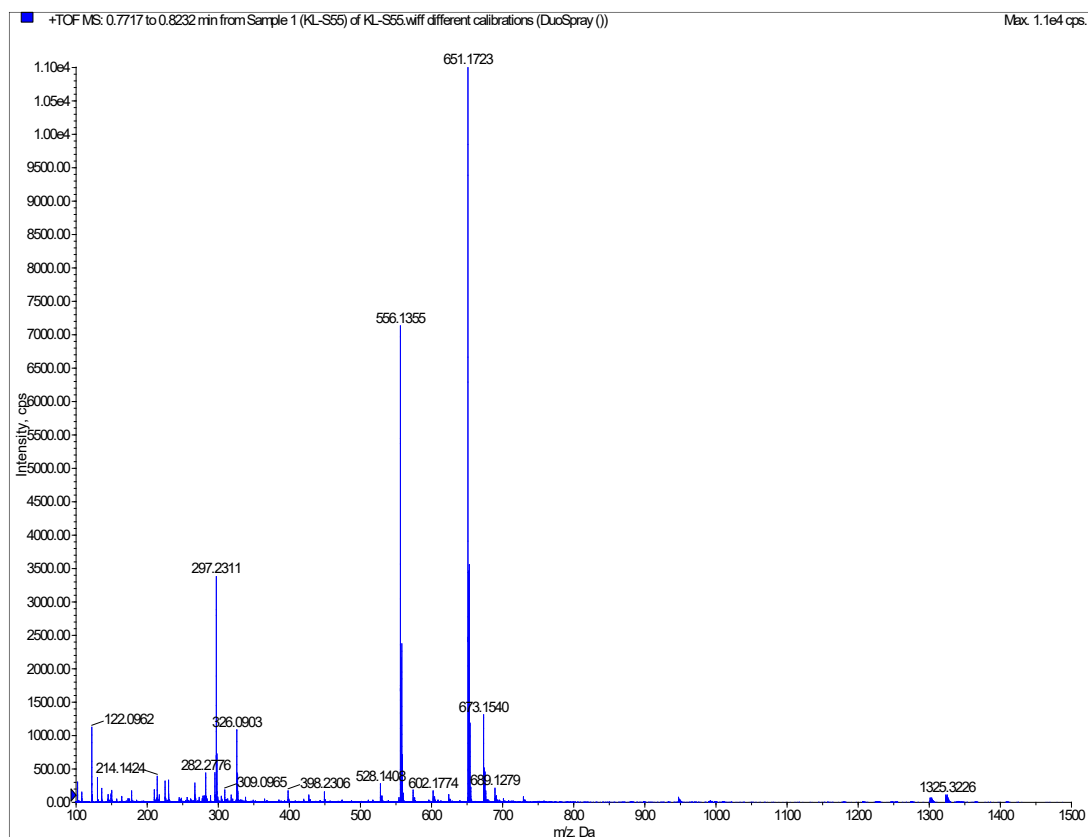




Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C35 H29 N5 O6 Cl	650.18	3.2118	4.9398	23.5

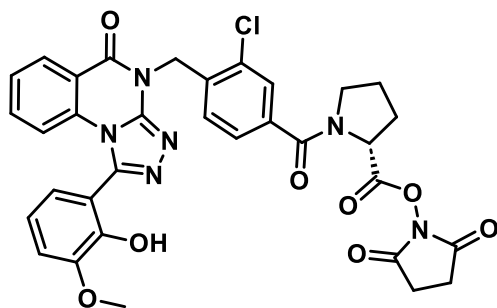
Pyridin-3-yl (3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzoyl)-L-prolinate (**29**)

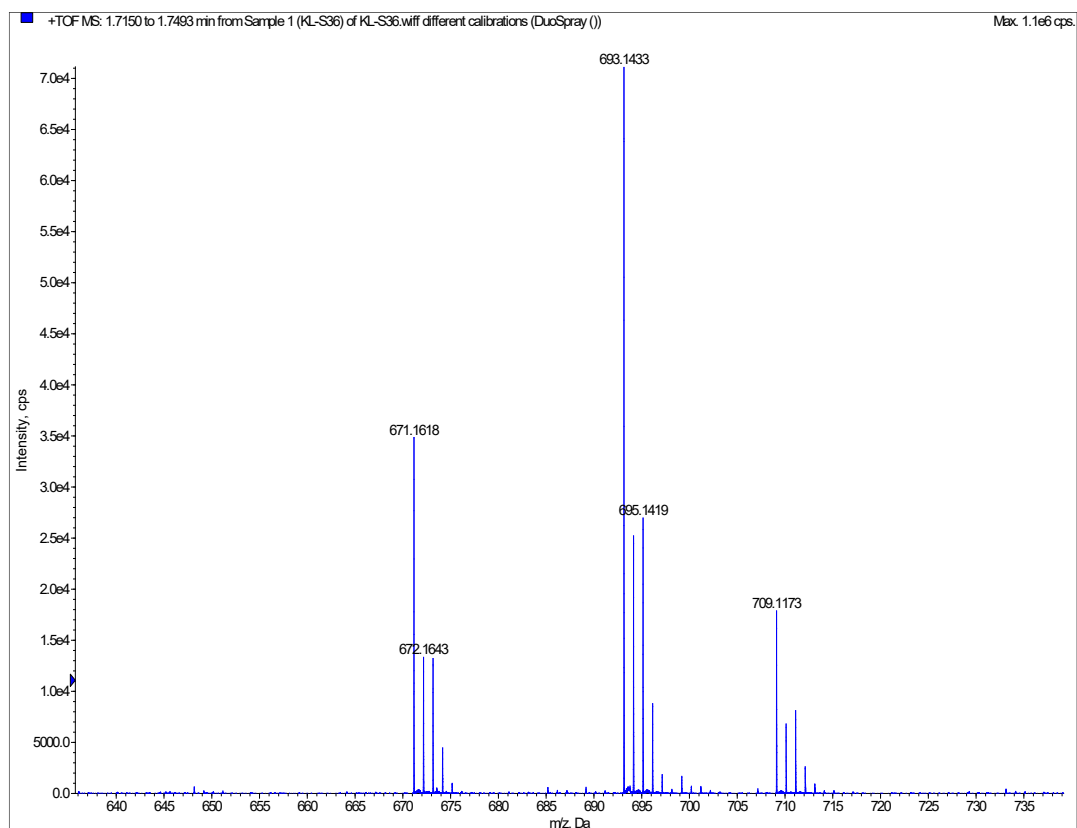
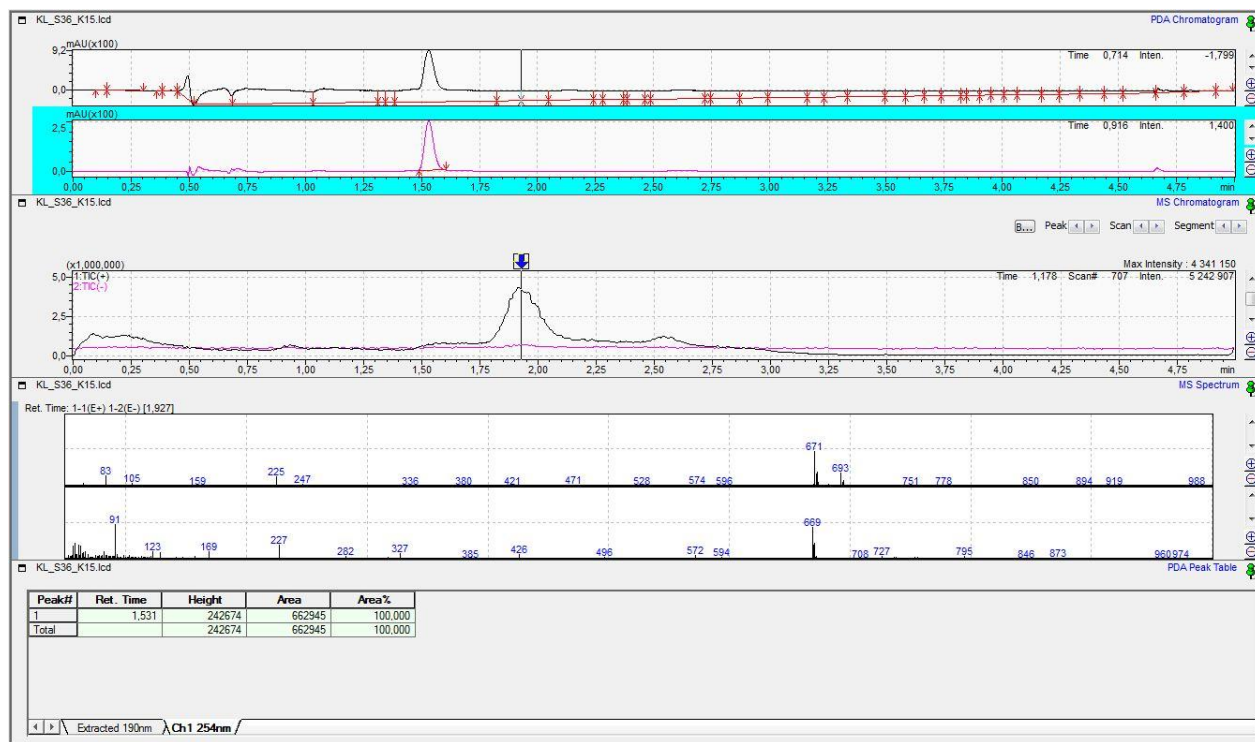




Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C ₃₄ H ₂₈ N ₆ O ₆ Cl	651.1753	-3.0371	-4.6641	23.5

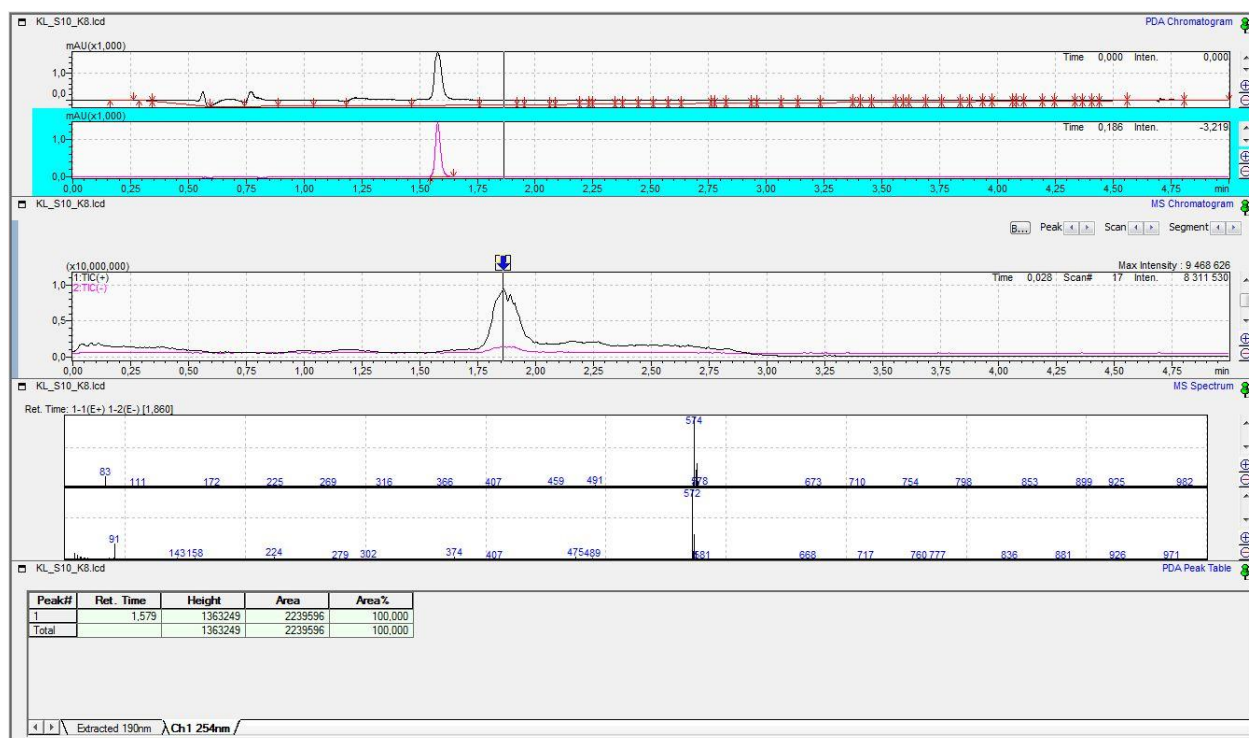
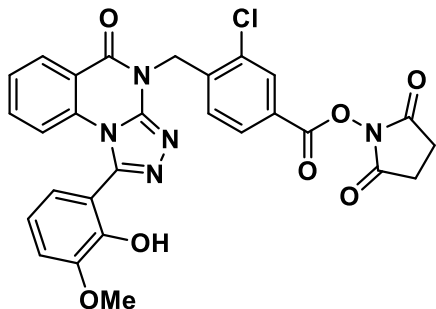
*2,5-dioxypyrrolidin-1-yl (3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-*a*]quinazolin-4(5*H*)-yl)methyl)benzoyl)-D-prolinate (30)*

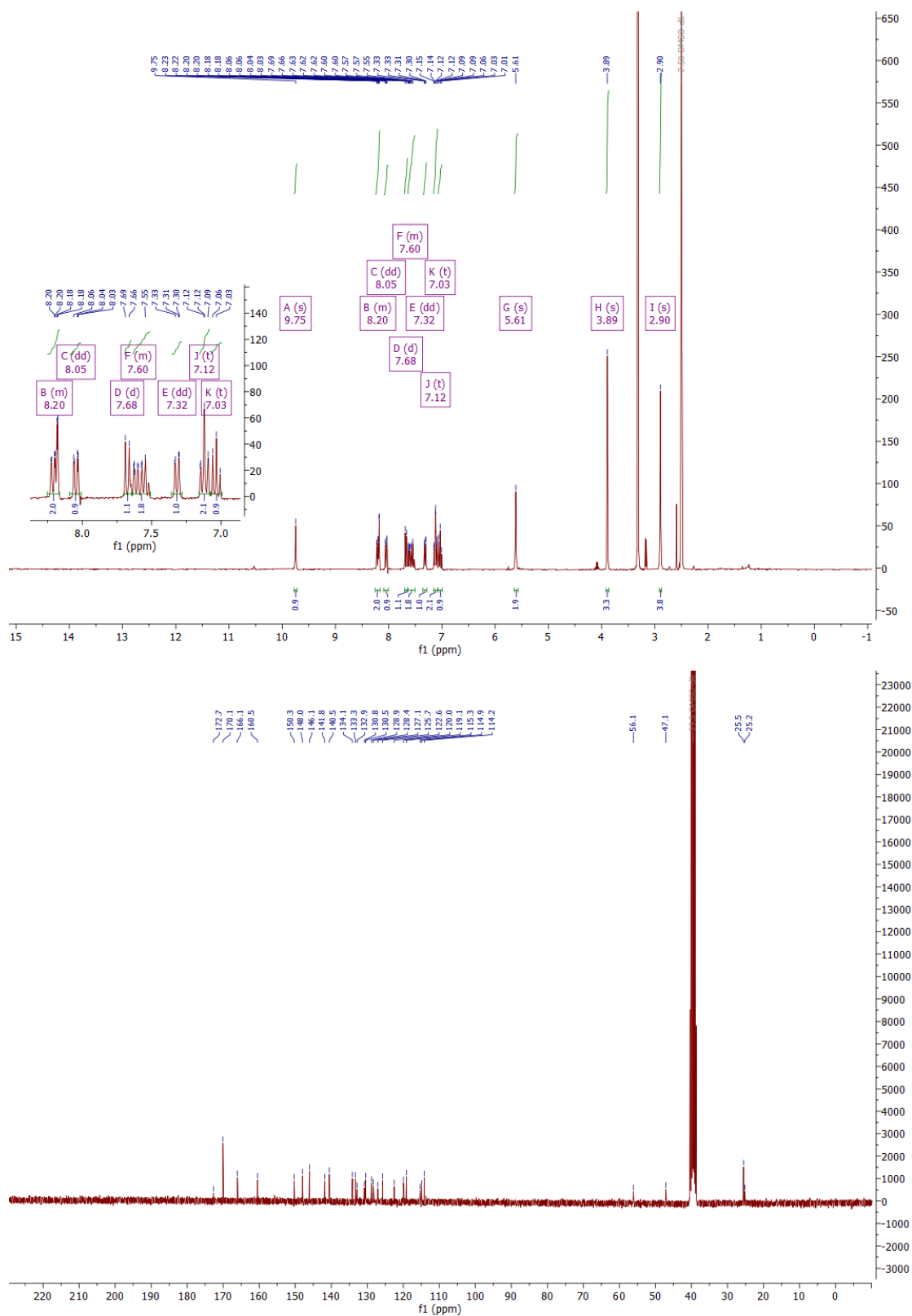




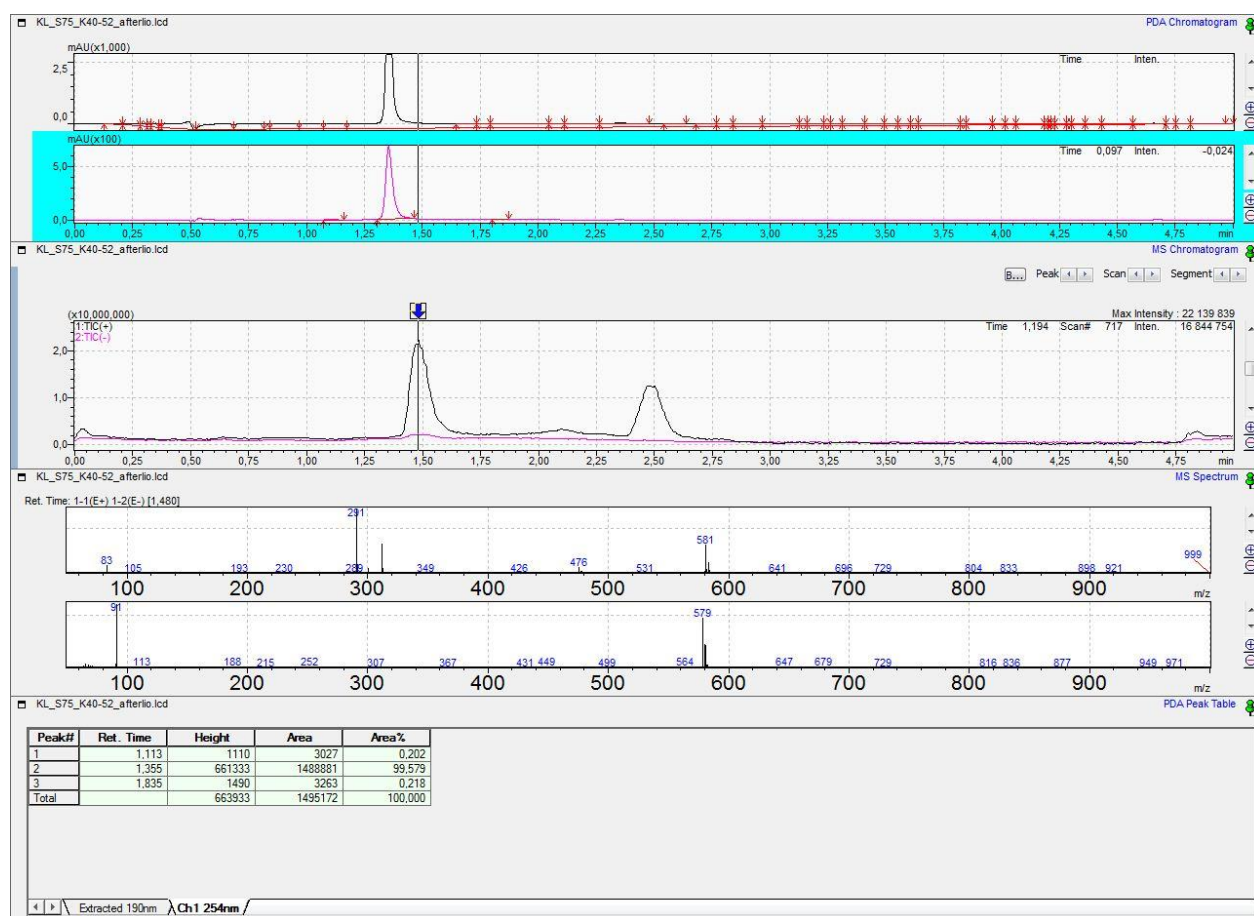
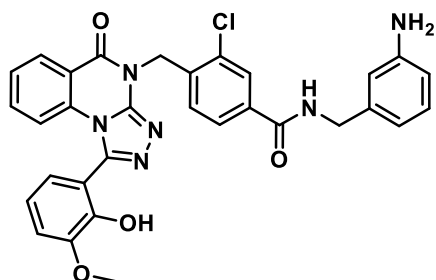
Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C33 H28 N6 O8 Cl	671.1651	-3.3664	-5.0158	22.5
C38 H21 N9 O2 Na Cl	693.1398	3.4049	4.9123	32

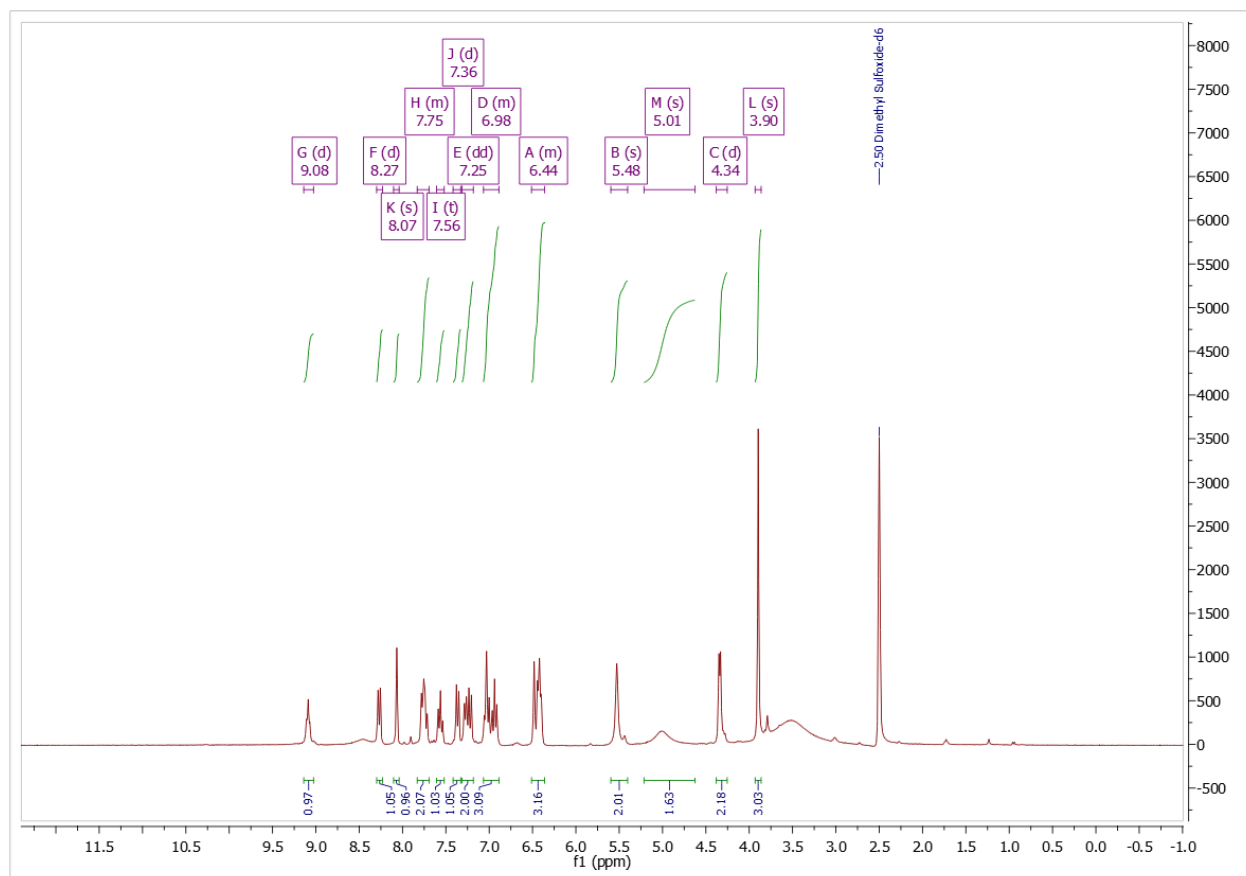
2,5-Dioxypyrrolidin-1-yl 3-chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-*a*]quinazolin-4(5*H*)-yl)methyl)benzoate (**31**)

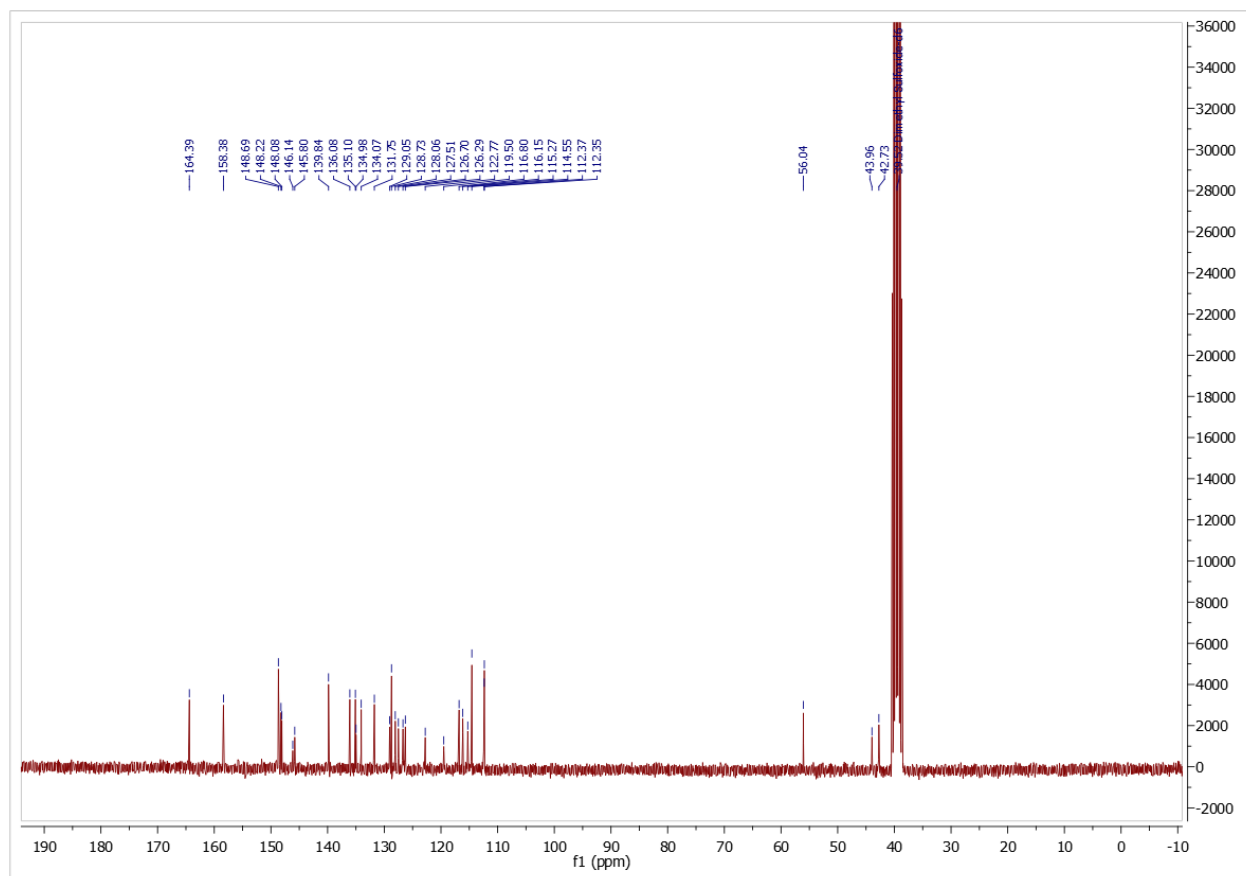




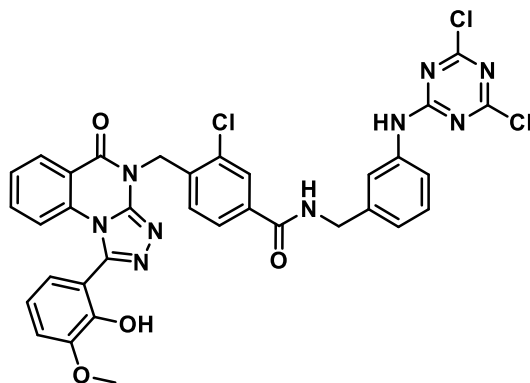
N-[(3-aminophenyl)methyl]-3-chloro-4-{{[1-(2-hydroxy-3-methoxyphenyl)-5-oxo-4*H*,5*H*-[1,2,4]triazolo[4,3-*a*]quinazolin-4-yl)methyl}benzamide (**32**)

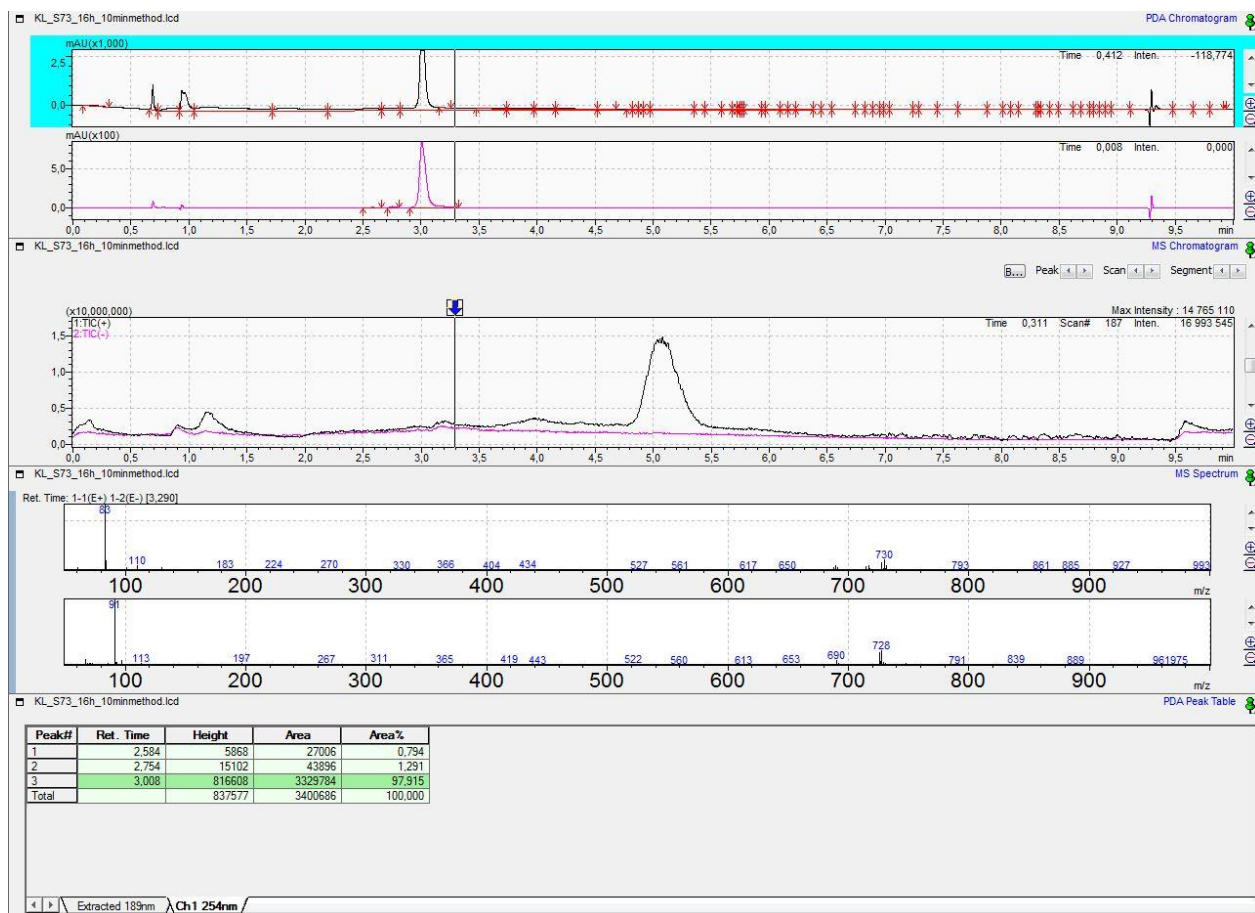


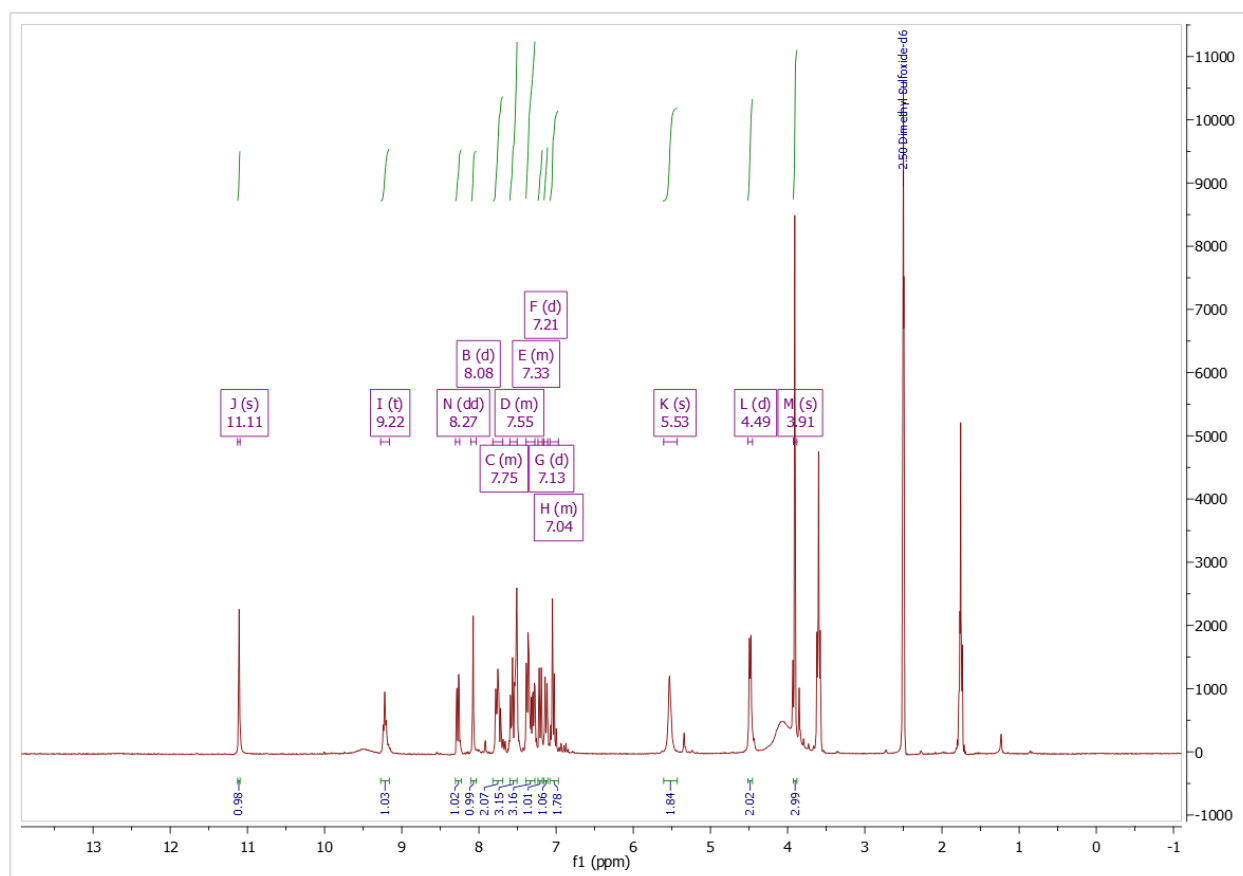




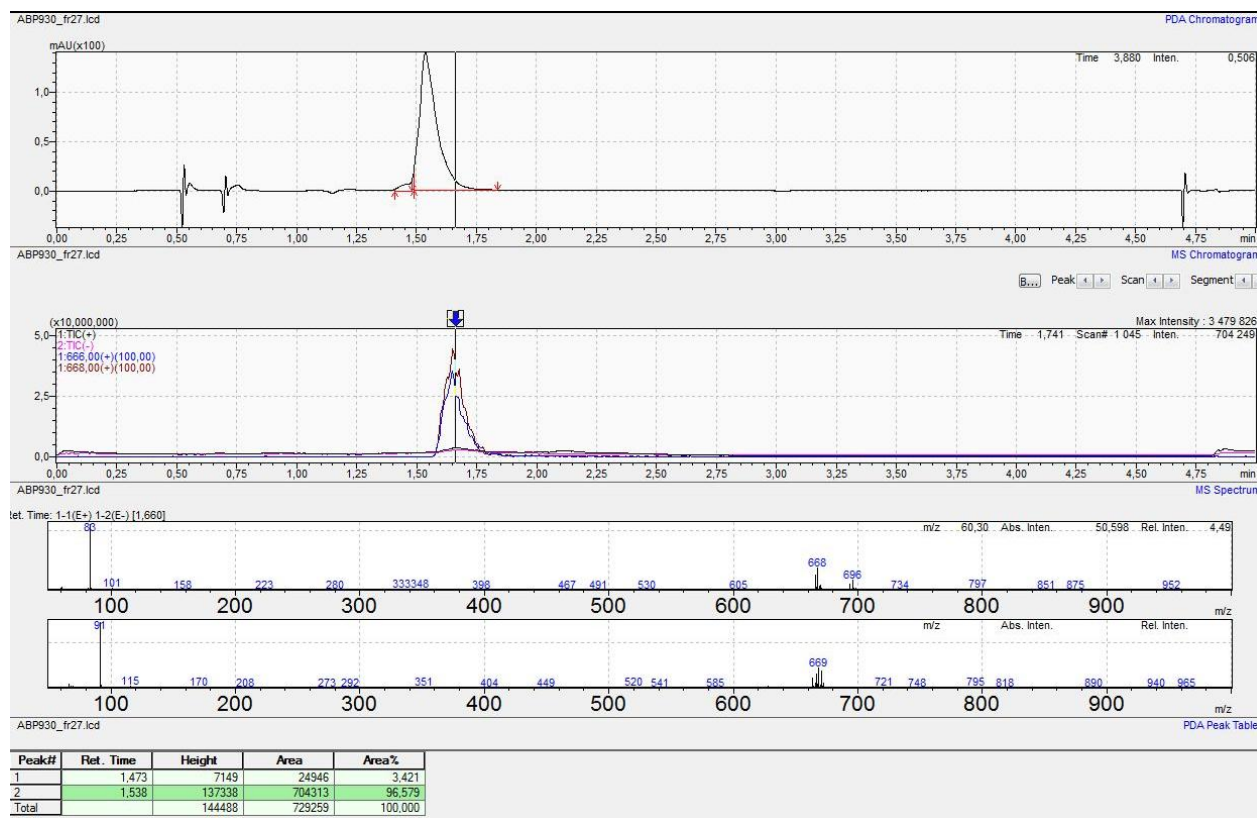
3-Chloro-*N*-($\{3-[(4,6\text{-dichloro-}1,3,5\text{-triazin-}2\text{-yl)amino}]phenyl\}$ methyl)-4- $\{[1-(2\text{-hydroxy-}3\text{-methoxyphenyl})\text{-}5\text{-oxo-}4H,5H\text{-}[1,2,4]\text{triazolo}[4,3-*a*]\text{quinazolin-}4\text{-yl}]methyl\}$ benzamide (**34**)

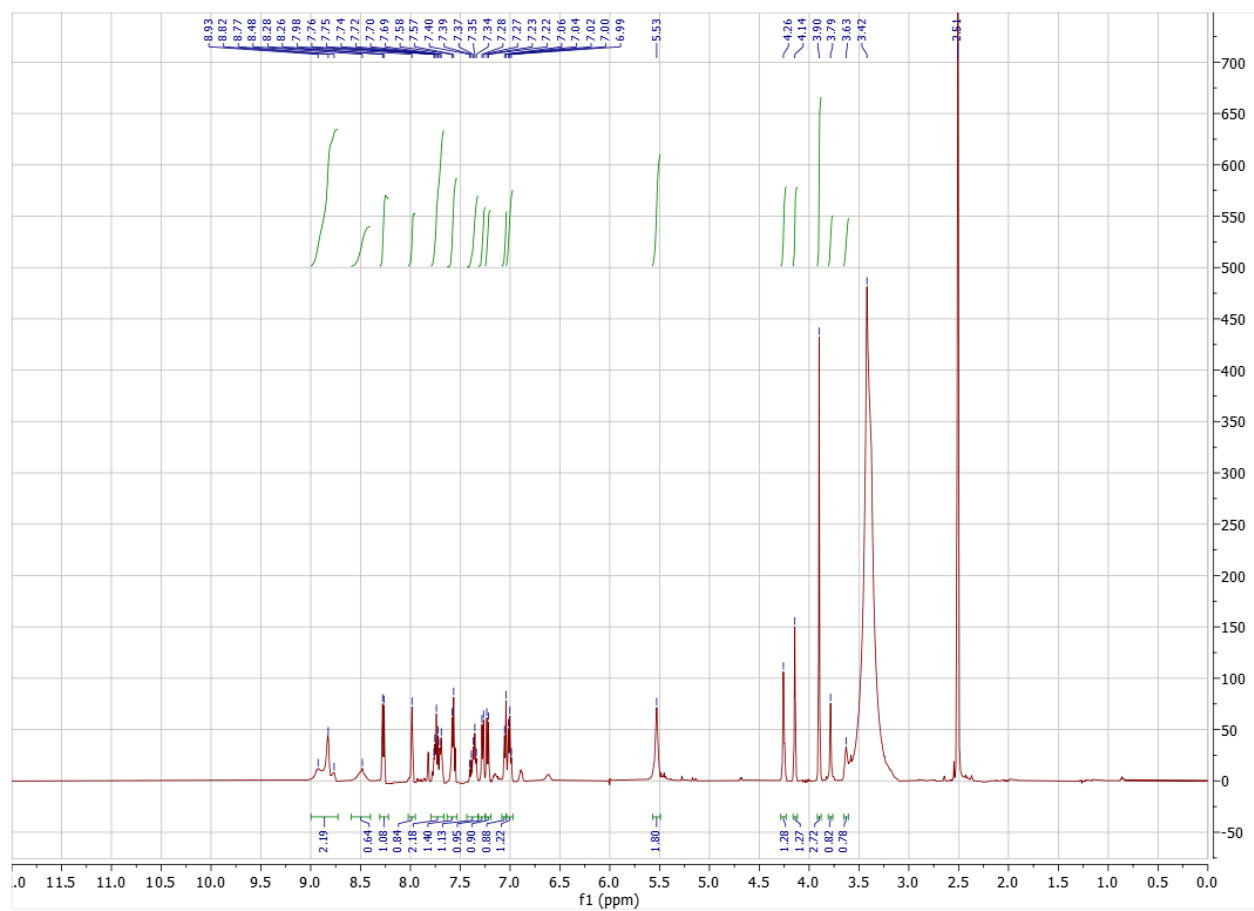


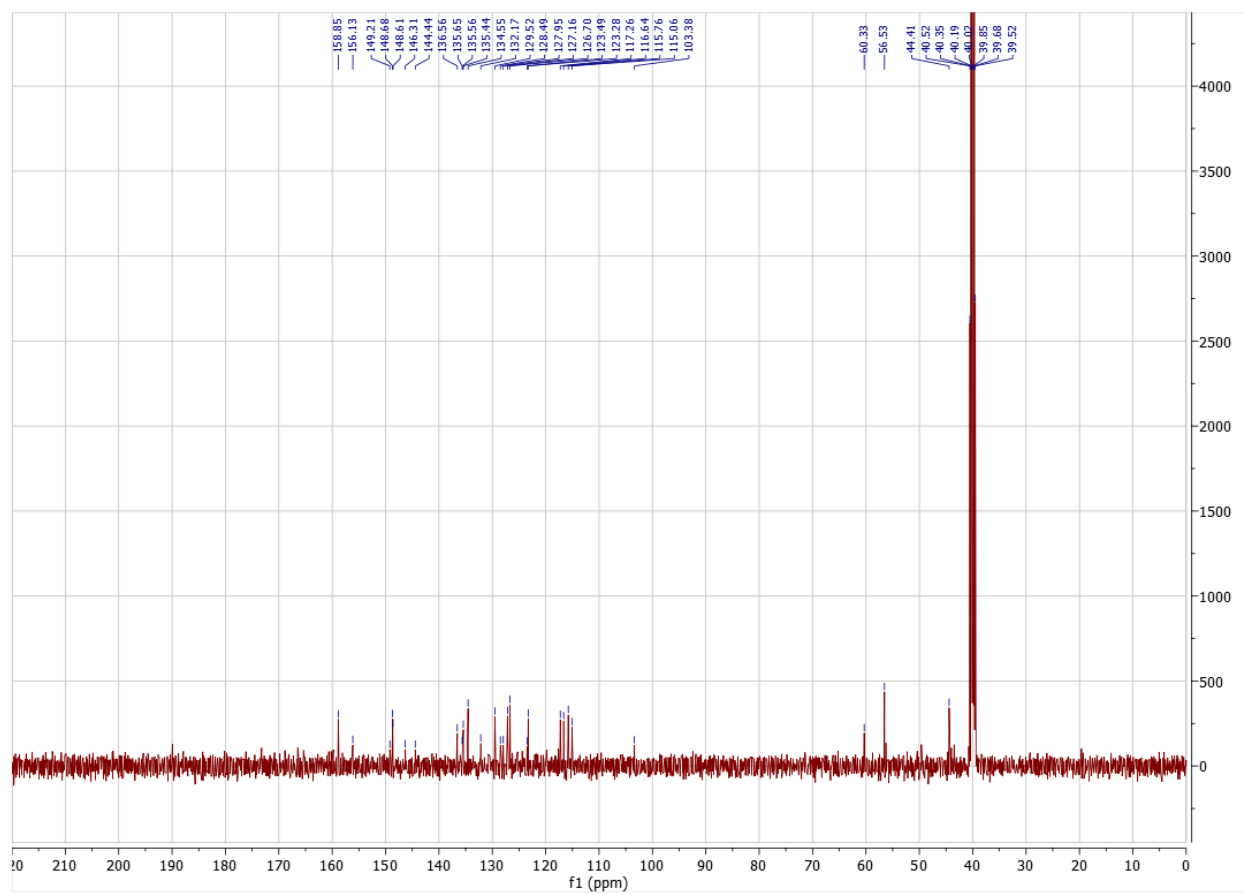




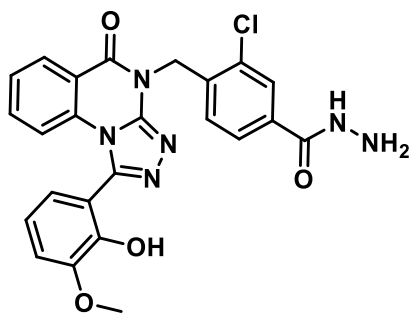
COc1ccc(O)c(c1)/N2C(=O)c3ccccc3N2C4=NC=NC=C4Cc5ccc(Cl)cc5C(=O)NCCNc6nc(Cl)nnc6Cl

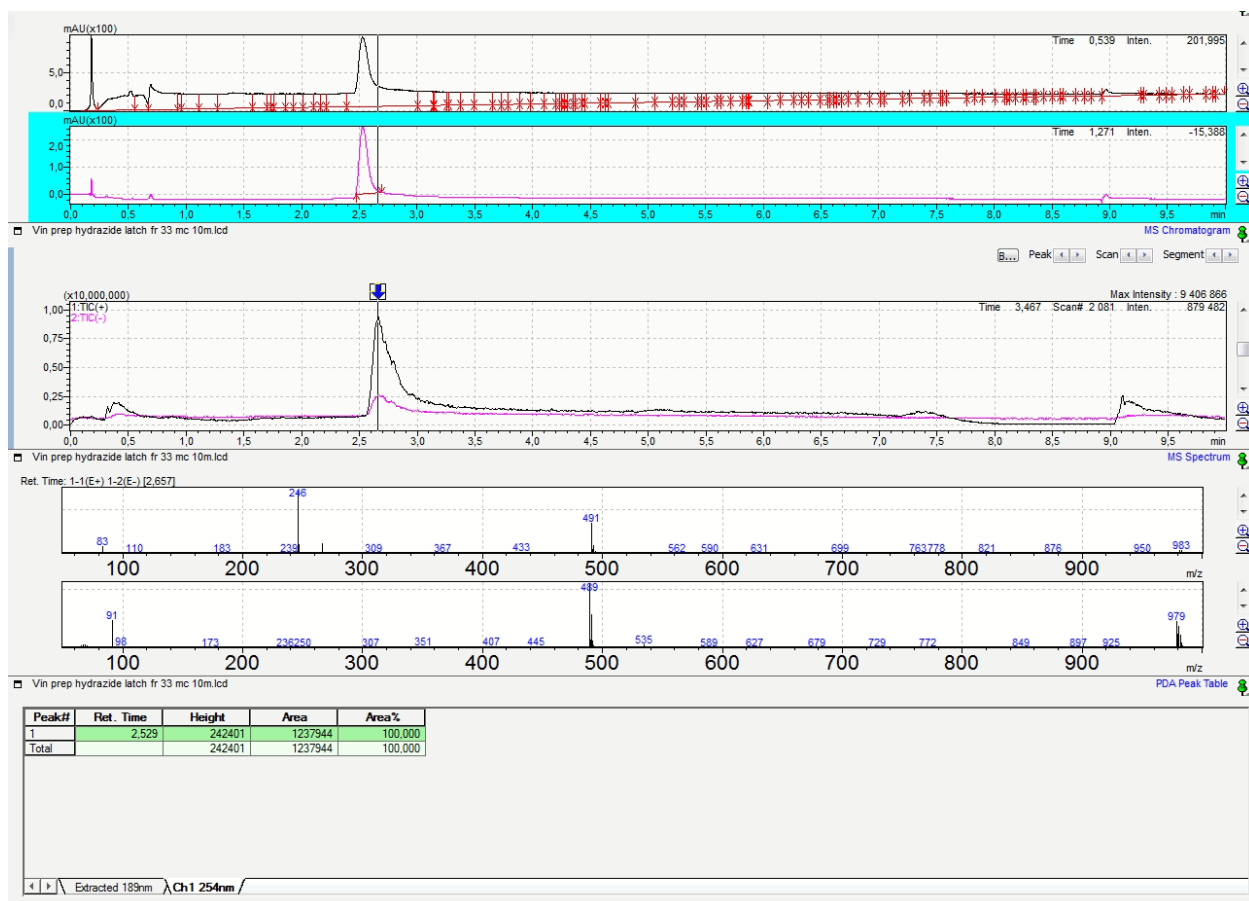


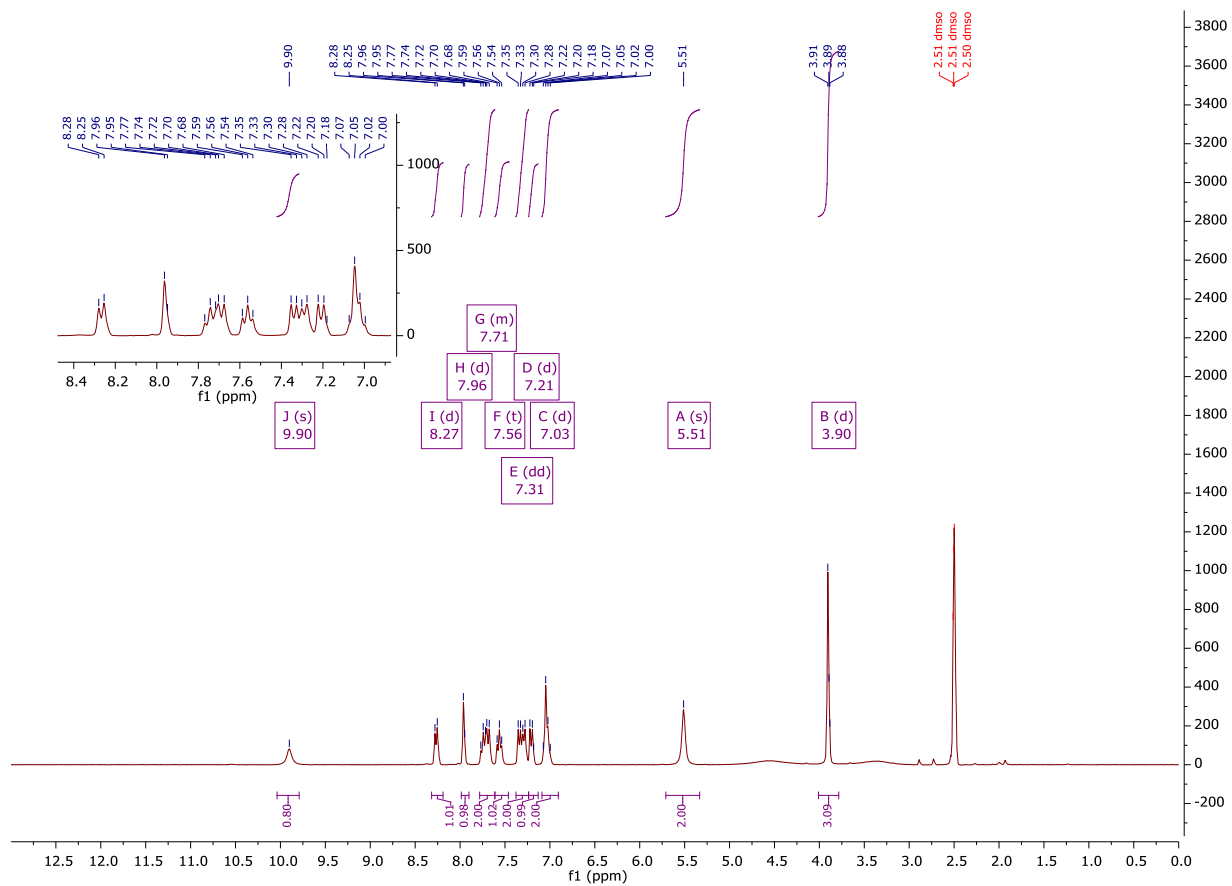


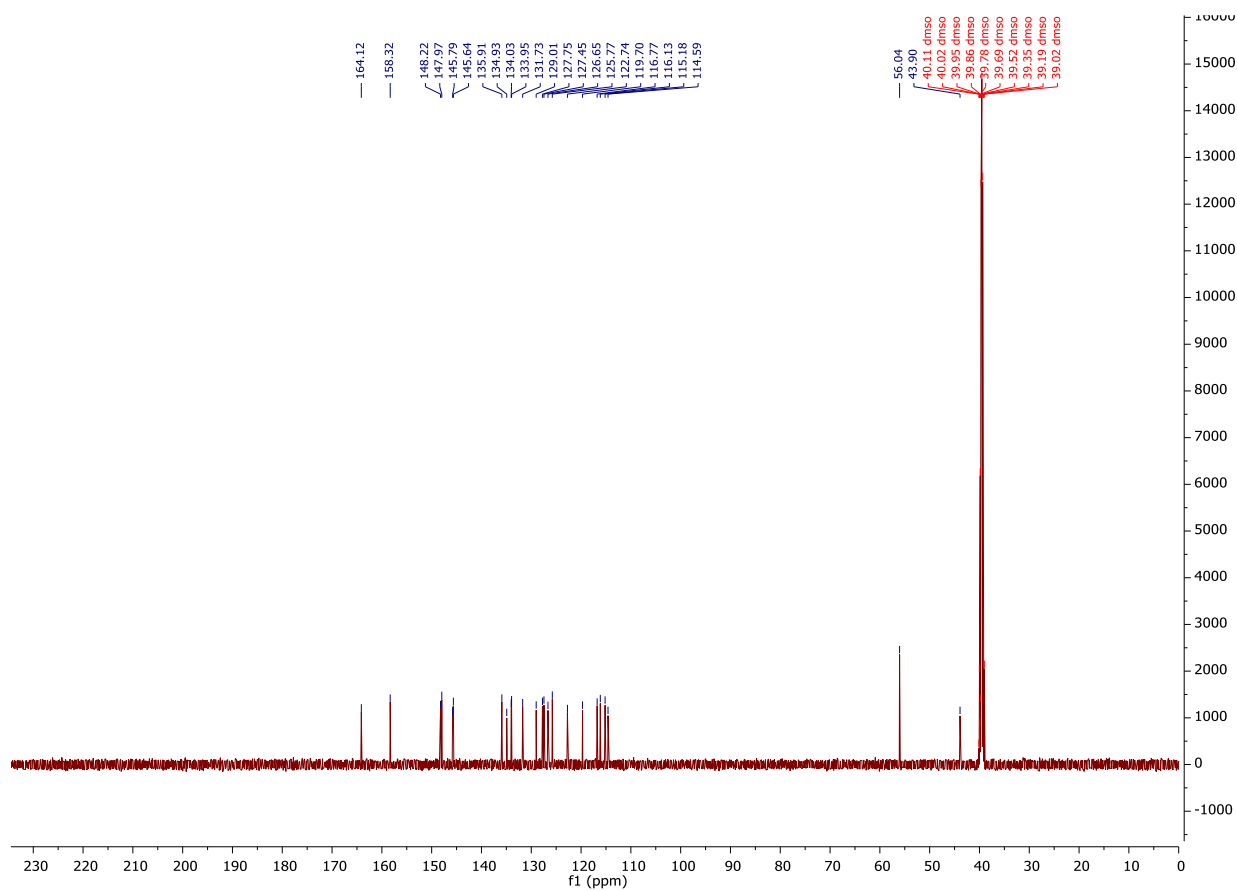


3-Chloro-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-a]quinazolin-4(5H)-yl)methyl)benzohydrazide (**36**)

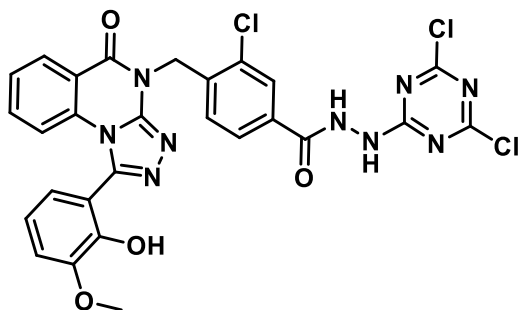


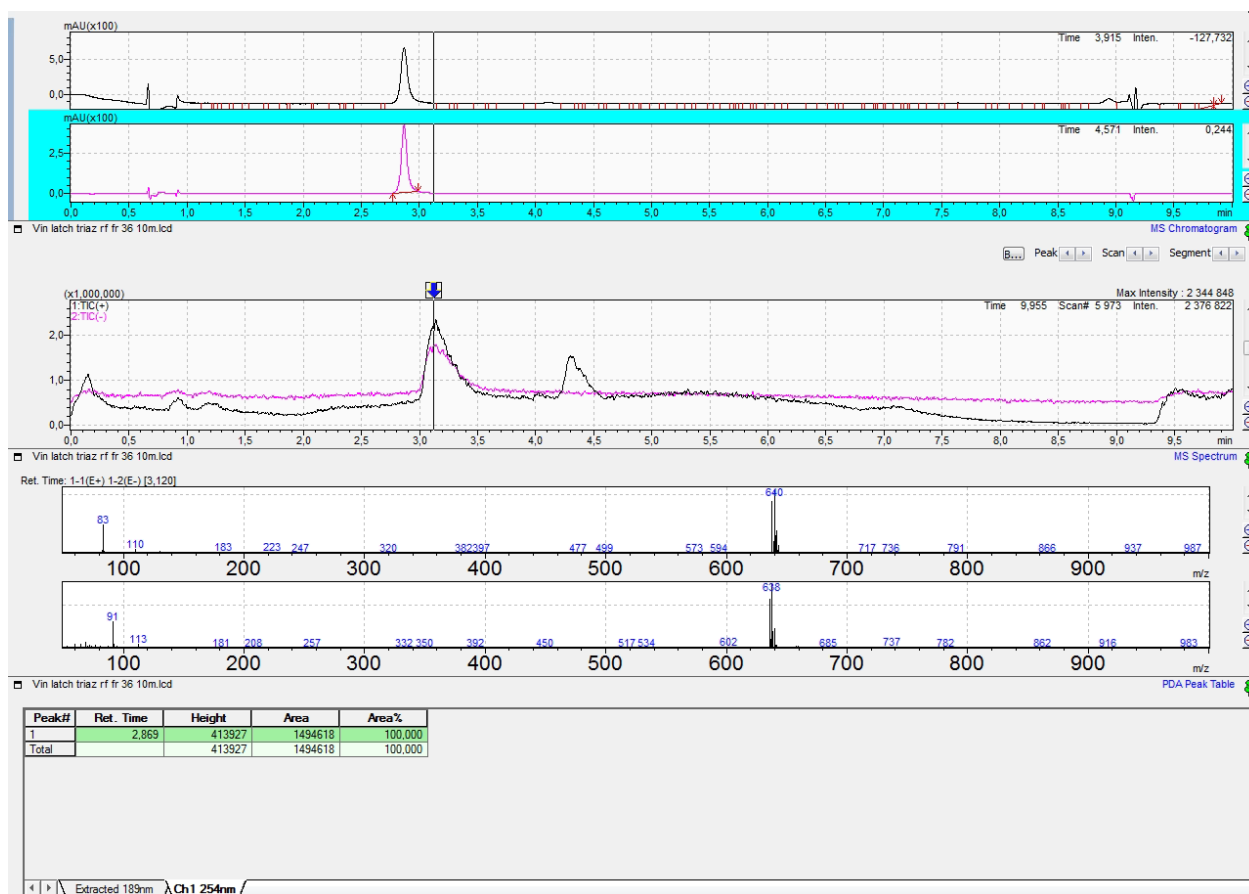


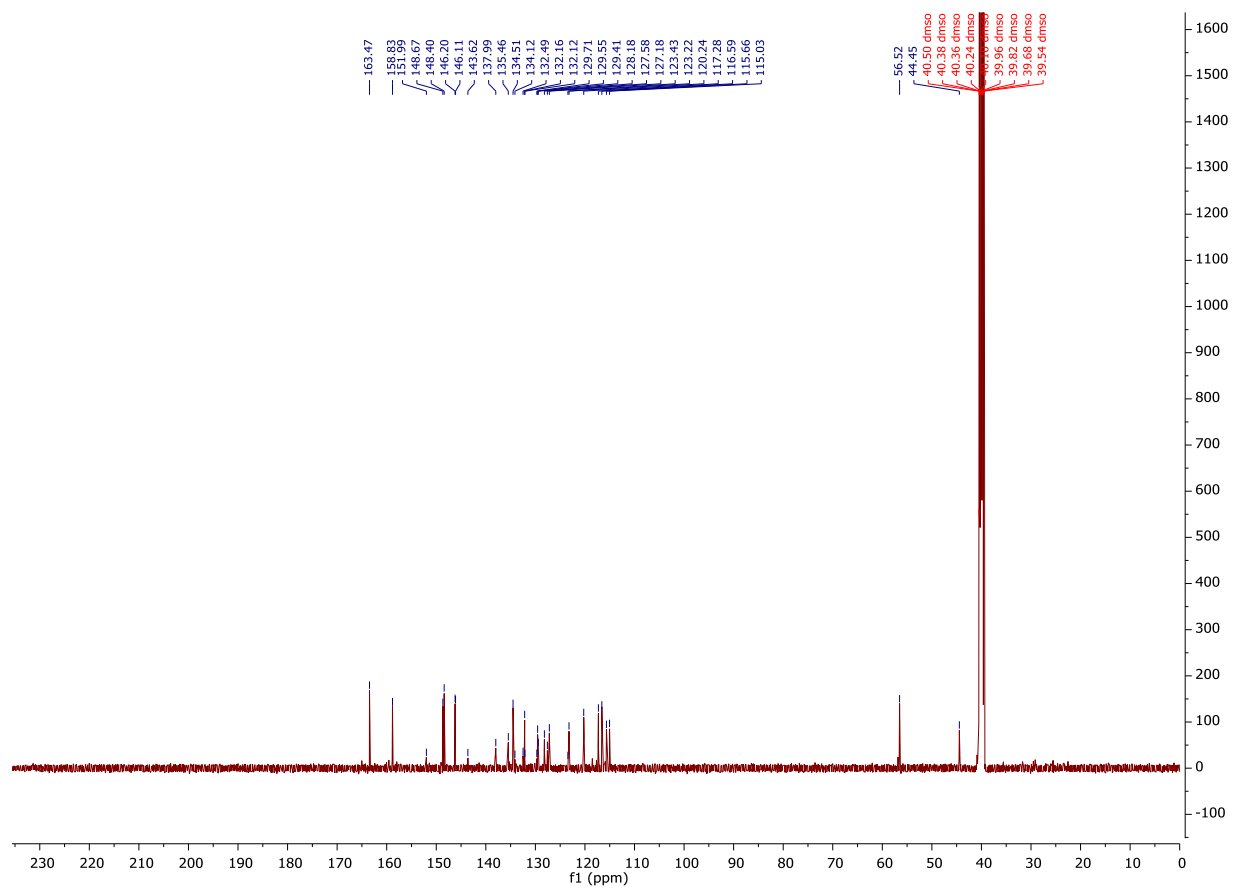




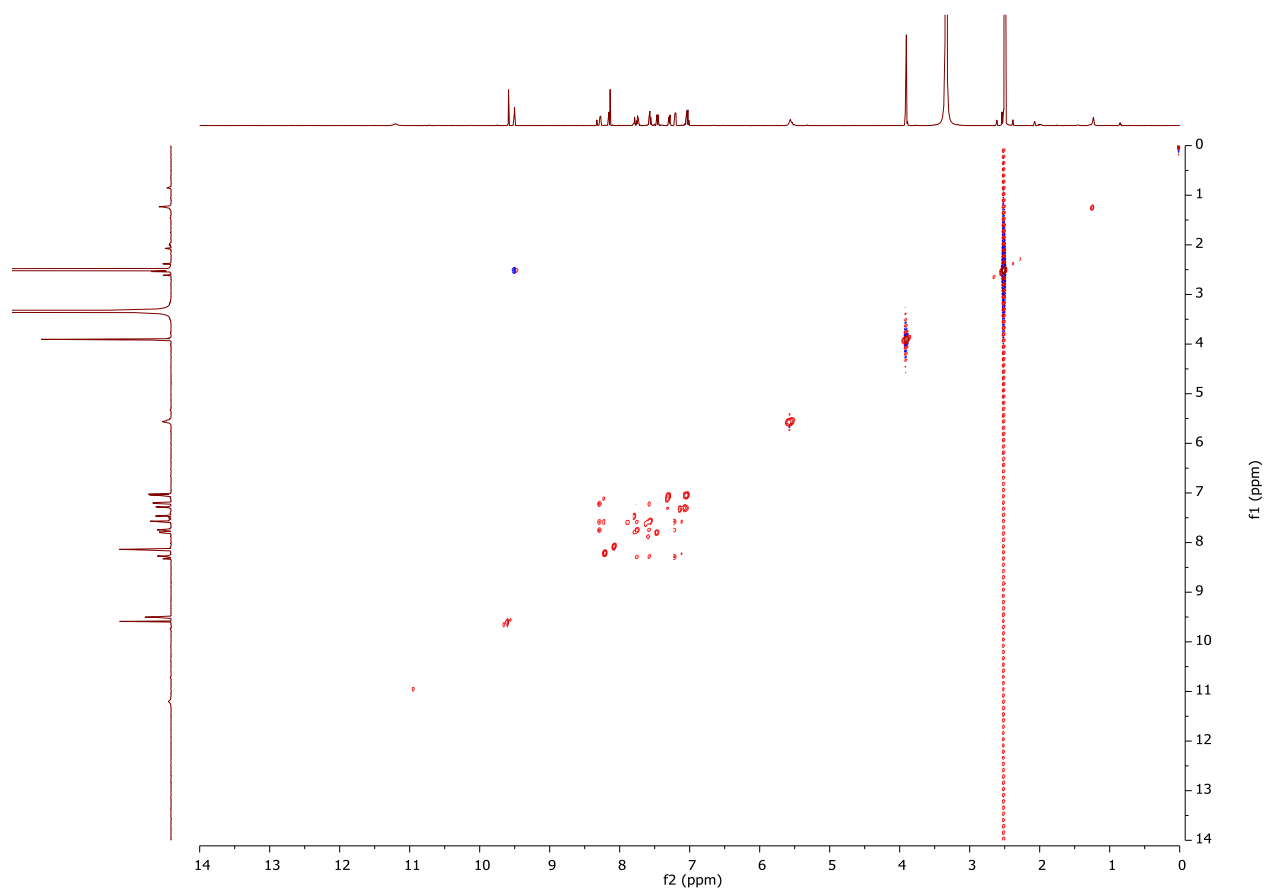
3-Chloro-*N'*-(4,6-dichloro-1,3,5-triazin-2-yl)-4-((1-(2-hydroxy-3-methoxyphenyl)-5-oxo-[1,2,4]triazolo[4,3-*a*]quinazolin-4(5*H*)-yl)methyl)benzohydrazide (**37**)



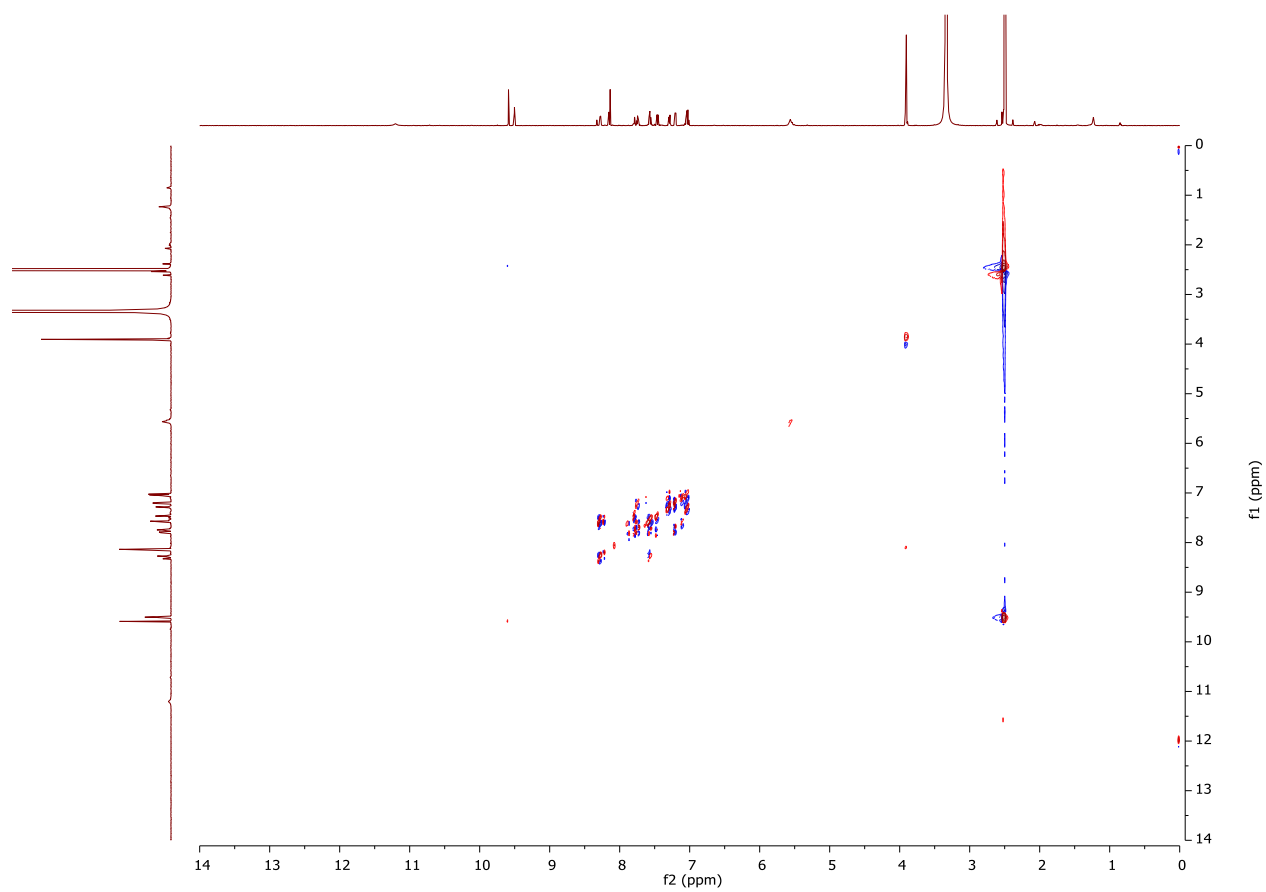




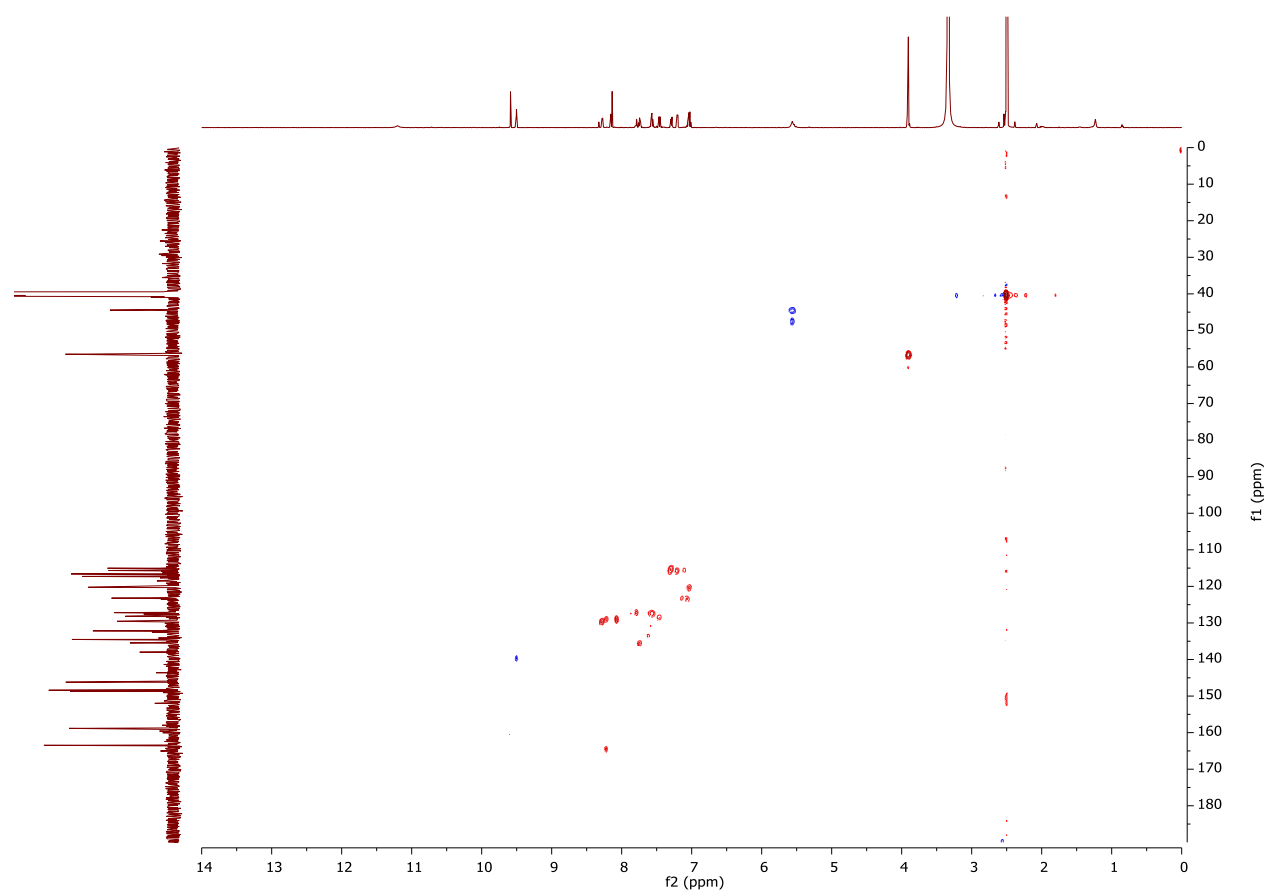
TOCSY

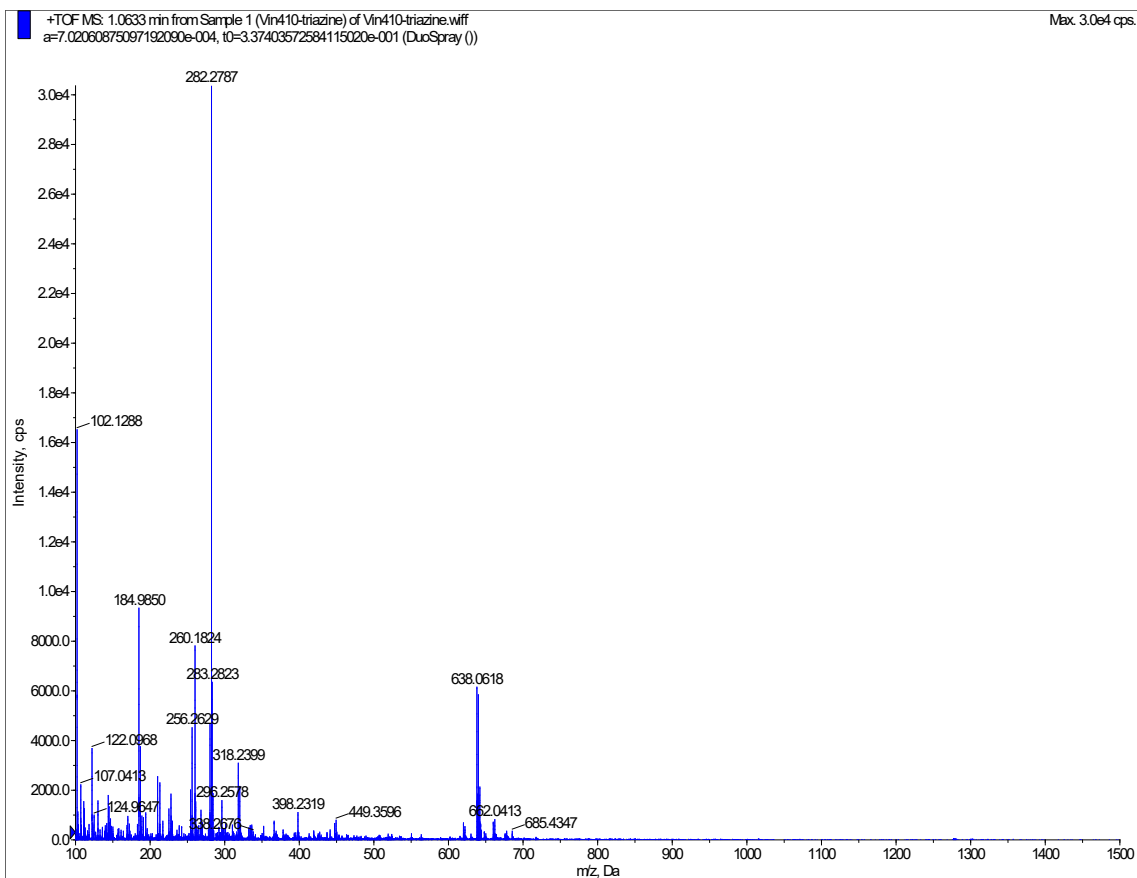


COSY



HSQC





Formula	Calculated mass	Error / mDa	Error / ppm	DBE
C27 H19 N9 O4 Cl3	638.062	-0.2099	-0.3291	21.5

6. References

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