Supporting Information

Cross-Conjugation of DNA, Proteins and Peptides via the pH Switch

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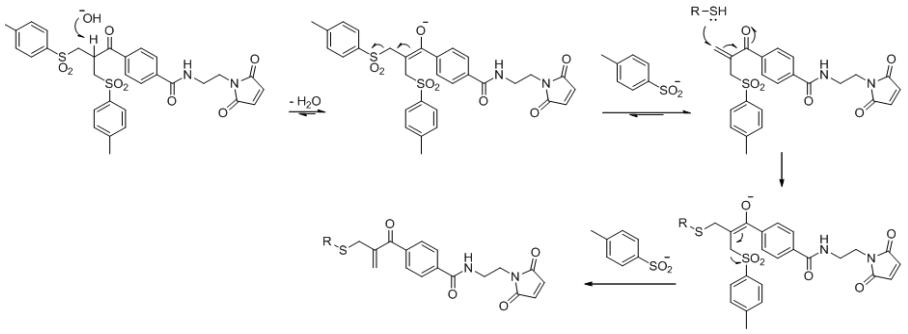
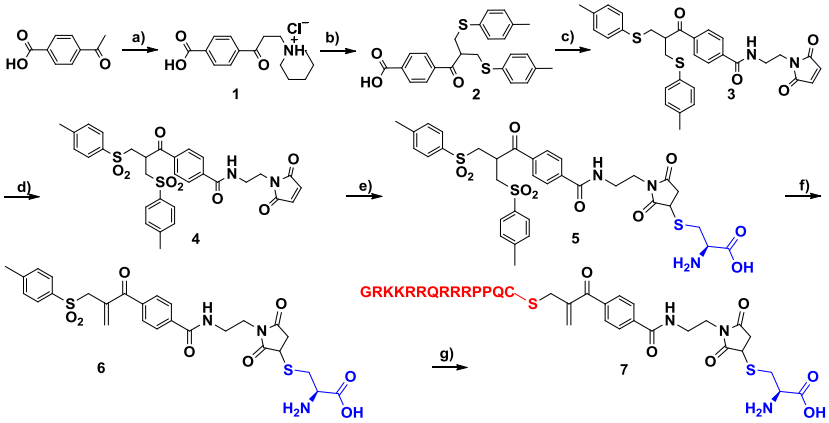
1. General experimental details

Unless otherwise noted, all operations were performed without taking precautions to exclude air and moisture. All solvents and reagents were purchased from commercial sources and were used without further purification. HIV TAT (GRKKRRQRRRPPQC-NH_2), and NLS (CGGGPKKKRKVED) were purchased from GL Biochem China. Oligonucleotide (C_6SH-5'-AATTGAATAAGCTGGTAT-3') was purchased from Biomers.net (Ulm, Germany). Reaction progress was monitored by thin layer chromatography (TLC) using Merck 60 F254 pre-coated silica gel plates illuminating under UV 254 nm or using appropriate stains. Flash column chromatography was carried out using Merck silica gel 70-230 mesh. NMR spectra were measured on Bruker 400 MHz or 500 MHz NMR spectrometer and the chemical shifts were referenced to residual solvent shifts in the respective deutero solvents. Chemical shifts are reported as parts per million referenced with respect the residual solvent peak. Mass spectra were acquired on a Finnigan Mat LCQ (ESI) spectrometer or a Bruker Reflex III (MALDI-TOF).

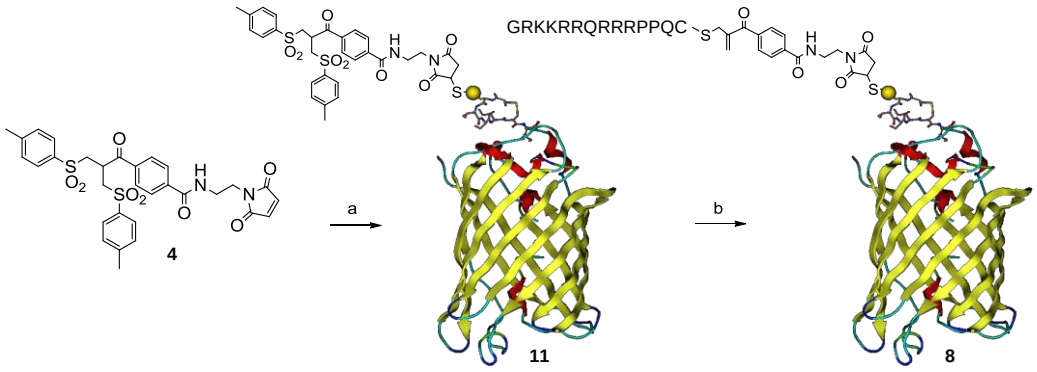
2. a) General Mechanism



2. b) General Reaction Scheme



Scheme 1. Synthesis of TCC (**4**) and cysteine-Tat conjugate (**7**). a) piperidine hydrochloride, paraformaldehyde, EtOH, 48%; b) 4-methylbenzenethiol, piperidine, 37 wt.% formaldehyde in water, EtOH-MeOH, 75%; c) N-(2-aminoethyl) maleimide trifluoroacetate salt, BOP, triethylamine, CH_3CN, 86%; d) OXONE, 2:1:1 MeOH-H_2O-$CHCl_3$, 88%; e) 1.5 eq. Cysteine, 40% CH_3CN in 50 mM PB, 10 mM EDTA, pH 6 buffer, 95%; f) 50 mM PB, 10 mM EDTA, pH 8 buffer; g) HIV TAT (GRKKRRQRRRPPQC-NH_2), 50 mM PB, 10 mM EDTA, pH 8 buffer, 57%.



Scheme 2. Conjugation of tagged EYFP (containing a free cysteine at N-terminal) and HIV TAT (GRKKRRQRRRPPQC-NH_2). a) 50 eq. TCC (**4**) 10% DMSO in 50 mM PB pH 6; b) 1.Bissulfone-EYFP (**11**), 50 mM PB pH 8; 2. 20 eq. HIV TAT (GRKKRRQRRRPPQC-NH_2),

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        10         20         30         40         50         60
CCTSICVSKG EELFTGVVPI LVELDGDVNG HKFSVSGEGE GDATYGKLTL KFICTTGKLP

        70         80         90        100        110        120
VPWPTLVTTF GYGLQCFARY PDHMKQHDFF KSAMPEGYVQ ERTIFFKDDG NYKTRAEVKF

       130        140        150        160        170        180
EGDTLVNRIE LKGIDFKEDG NILGHKLEYN YNSHNVYIMA DKQKNGIKVN FKIRHNIEDG

       190        200        210        220        230        240
SVQLADHYQQ NTPIGDGPVL LPDNHYLSYQ SALSKDPNEK RDHMVLLEFV TAAGITLGMD

       250
ELYKKLAAAL EHHHHHH
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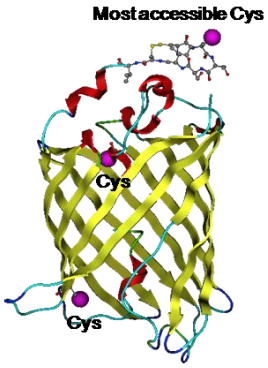
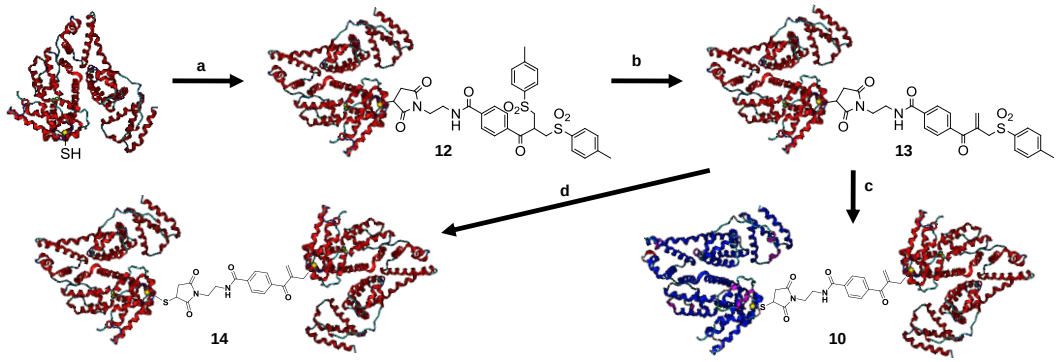


Figure 1. Amino acid sequence of the tagged EYFP. tag YFP His-tag



Scheme 3. Synthesis of HSA-BSA conjugate (**10**) and BSA dimer (**14**). a. 5 eq. **4**, 50 mM PB, 10 mM EDTA, pH 6; b. 50 mM PB, 10 mM EDTA, pH 8; c. 5 eq. HSA, 50 mM PB, 10 mM EDTA, pH 8. d. 3 eq. BSA, 50 mM EDTA, pH 8.

Scheme 4. Synthesis of oligonucleotide (SHC_6-5'aattgaataagctggtat-3')-NLS peptide (CGGGPKKKRKVED) conjugate (**10**). a. NLS (CGGGPKKKRKVED), 40% ACN in 50 mM PB pH 6， 46%; b. 1.Bissulfone-NLS (**15**), 50 mM PB pH 8; 2.Oligonucleotide SHC_6-5'aattgaataagctggtat-3', 10 eq. TCEP; 3.Reduced oligonucleotide, monosulfone-NLS

3. Experiment Protocol and Characterization

Synthesis of 1-[3-(4-carboxy-phenyl)-3-oxo-propyl]-piperidiniumHCl (1)[1]

4-Acetylbenzoic acid (500 mg, 3.045 mmol, 1 eq.), piperidine HCl (370 mg, 3.045 mmol, 1 eq.) and paraformaldehyde (274 mg, 9.135 mmol, 3 eq.) were added to a solution of 2 ml absolute ethanol. Then, concentrated HCl was added (30 µl) and the mixture was heated to 105℃ for reflux. After 4 h reaction time, paraformaldehyde (274 mg, 9.135 mmol, 3 eq.) was added and the reaction was refluxed for further 6 h. Thereafter, acetone was poured into the reaction mixture in order to allow precipitation of the product. A white precipitate was formed, filtered off and dried under the vacuum to afford 437 mg of **1** in 48% yields.

^{1}H NMR (300MHz, DMSO-d6): δ 1.5–1.8 (m, 6H), 3.23(s, 4H), 3.38 (t, 2H), 3.69 (t, 2H), 8.10 (m, 4H)；

ESI-MS (MeOH, 250°C): (-) m/z=260 [M-HCl].

Synthesis of 4-(3-(p-tolylthio)-2-((p-tolylthio)methyl)propanoyl)benzoic acid (2)[1]

To a solution of ethanol (1.2 ml) and methanol (0.8 ml), mannich salt (**1**, 350 mg, 1.17 mmol, 1 eq.) and 4-methylbenzenethiol (291mg, 2.348 mmol, 2 eq.) were added. Then, piperidine (0.05 ml) and 37% (wt/vol) aq. formaldehyde (0.35 ml) were introduced sequentially and the reaction mixture was heated to 105℃ for reflux. After 1 h, aq. formaldehyde (0.35 ml, 37 %, (wt/vol)) was added *via* a pipette through the top of the condenser. The reaction mixture was allowed to reflux for additional 3 h. The mixture was cooled to RT and then the solvent was evaporated at 40 °C. The resulting reaction mixture was stored in a refrigerator (4 °C) overnight. The formed solid was washed two times with methanol and the white solid was dried under vacuum to afford 384 mg of **2** as white solid in 75% yield.

^{1}H NMR (500 MHz, CDCl3): δ 2.38 (s, 6H), 3.16–3.31 (m, 4H), 3.85 (q, 1H), 7.15 (d, 4H), 7.18 (d, 4H), 7.64(d, 2H), 8.07 (d, 2H);

^{13}CNMR (125 MHz, CDCl3): δ 200.50, 137.24, 131.55, 131.15, 130.17, 129.86, 128.29, 45.88, 36.41, and 21.10

ESI-MS (MeOH, 250^{o}C): (-) m/z=435 [M-H].

Synthesis of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-(p-tolylthio)-2- ((p-tolylthio) methyl) propanoyl) benzamide (3)

2 (50 mg, 0.1145 mmol) and 2-aminoethylmaleimide (29 mg, 0.1145 mmol) were stirred in dry acetonitrile (5 ml) while BOP (Benzotriazol-1-yloxy-tris(dimethylamino)-phosphoniumhexafluorophosphate) reagent (51 mg, 0.1145 mmol) was added followed by triethylamine (32 µl). The reaction mixture was stirred at RT for 2 h. After removal of the solvent under vacuum, the residual mixture was purified by silica gel column using 2% MeOH in chloroform. 55 mg of **3** were isolated in 86% as light yellow foam.

^{1}H NMR (400 MHz, $CDCl_3$):δ 7.67 (d, 2H), 7.54(d, 2H), 7.10(d, 4H), 7.03(m, 5H, ArH and NH), 3.76(m, 3H), 3.61(m, 2H), 3.21(q, 2H), 3.11(q, 2H), 2.31(s, 6H).

^{13}CNMR (100 MHz, $CDCl_3$):δ 200.30, 171.02, 166.77, 138.69, 138.18, 137.19, 134.28, 131.45, 131.13, 129.88, 128.57, 127.22, 45.69, 39.83, 37.41, 36.33, 21.12.

FD-MS: (+) m/z=558.2

UV/VIS ($CHCl_3$): λmax(ε) 257.2(8239)

IR (cm^{-1}) 3364.28, 2921.68, 1710.58, 1682.62, 1537.02, 1493.63, 1436.74, 1406.84, 1294.03, 1220.74, 1168.67, 1098.28, 808.04, 695.23

HRMS (ESI) calc. 559.1725 exp. 559.1736

Synthesis of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamide (4)

3 (55 mg, 0.0985 mmol) was dissolved in 4 ml of MeOH/H_2O/$CHCl_3$ (2:1:1) and Oxone (363 mg, 0.5912 mmol) was added thereafter. The reaction mixture was stirred for 24h. Subsequently, the solvent was removed under vacuum and the mixture was poured into a separatory funnel and extracted with chloroform twice. Then, sufficient DI-water was added in order to dissolve the inorganic salts and extraction was performed twice. The organic extracts were combined and again extracted with Brine once. Thereafter, the organic layer was dried over sodium sulfate and the solvent was removed under vacuum to afford 54 mg of **4** as white solid in 88 % yield.

^{1}H NMR (400 MHz, $CDCl_3$):δ 7.74 (d, 2H), 7.70(d, 4H), 7.65(d, 2H), 7.36(m, 4H), 6.81(br, 1H), 6.76(s, 2H), 4.35(m, 1H), 3.86(m, 2H), 3.68(m, 2H), 3.62(q, 2H), 3.49(q, 2H), 2.49(s, 6H)

^{13}C NMR (75 MHz, $CDCl_3$) δ 209.78, 194.73, 170.71, 166.15, 145.28, 138.65, 135.87, 134.88, 133.98, 129.88, 128.35, 127.93, 127.21, 55.17, 39.44, 37.06, 35.26, 21.37, 8.82

UV/VIS (MeCN): λmax (ε) 223.6(10170), 255.6(4982)

IR (cm^{-1}) 3364.28, 2923.61, 1710.58, 1665.26, 1536.05, 1437.70, 1406.84, 1295.96, 1144.57, 932.43, 827.32, 694.26

HRMS (ESI) calc. 623.1522 exp. 623.1533

Synthesis of 2-amino-3-((2, 5-dioxo-1-(2-(4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamido) ethyl) pyrrolidin-3-yl) thio) propanoic acid (5)

Maleimide bissulfone (**4**, 10 mg, 0.016 mmol) was dissolved in 500 µl of 40% acetonitrile in phosphate buffer (50 mmol PB, 10 mM EDTA, pH 6) and cysteine (3 mg, 0.025 mmol) was added. The reaction mixture was incubated 1h at RT. After removal of the solvent *in vacuum*, the residual mixture was purified by silica gel column using Chloroform/Methanol/Acetonitril (5:3:1). 11.4 mg of compound **5** were isolated as white solid in 95% yield.

^{1}H NMR (250 MHz, THF-d8) δ 8.32-8.16 (m, 1H), 7.82 – 7.69 (m, 2H), 7.62 (d, 4H), 7.56 – 7.48 (m, 2H), 7.38 (d, *J* = 8.2 Hz, 4H), 4.50-4.33 (m, 1H), 4.31 – 4.08 (m, 2H), 3.76 – 3.46 (m, 11H), 3.31 -3.12 (m, 1H), 2.46 (s, 6H).

^{13}C NMR (100MHz, THF-d8:196.13, 179.91, 178.75, 175.58, 167.64, 146.28, 140.35, 137.66, 137.21, 131.05, 129.42, 129.31, 128.65, 56.18, 54.45, 53.73, 42.50, 40.53, 39.88, 38.54, 36.89, 21.81.

MALDI-TOF-MS: (+) m/z=744.27 (M+H)

HRMS (ESI) calc. 744.1718 exp. 744.1711

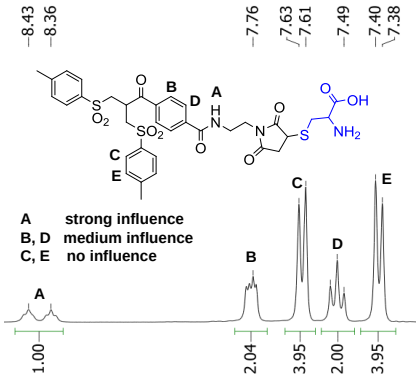


Figure 2. Influence of the difference in the magnetic environment according to the distance of the protons to the chiral centres of TCC (**4**).

Synthesis of cysteine TAT conjugate (7)

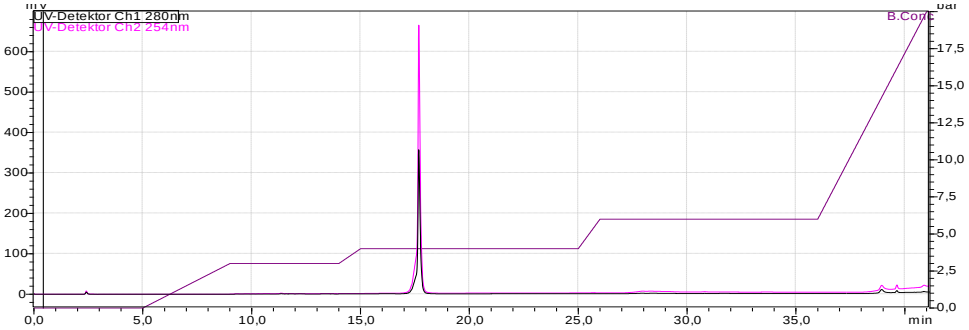


Figure 3. The HPLC profile of Cysteine TAT conjugate (**7**)

5 (0.82 mg, 1.098 µmol) were dissolved in 30% acetonitrile /PB (50 mM PB, 10 mM EDTA, pH 8) and HIV-TAT 48-60 Cys peptide (1 mg, 0.5488 µmol) was added. The resulting mixture was incubated overnight at RT. Thereafter, the product was purified by prep HPLC using an Atlantis Prep OBD T3 column (19×100 mm, 5 µm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-5 min) to 15% solvent B in 5mins, raising to 20% solvent B in 1min, 20% B for 10 mins, raising to 30% solvent B in 1 min, 30% B for 10 mins and finally reaching 100% solvent B in 5 mins with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for (**7**) was 17.68 min. 0.7 mg (0.3108 µmol) of (**7**) was isolated in 57 % yield after lyophilisation.

The reaction was also performed by using 2 eq. TAT peptide and the procedure is as follows:

5 (1 mg, 1.344 µmol) were dissolved in 1ml of 30% acetonitrile /PB (50 mM PB, 10 mM EDTA, pH 8) and HIV-TAT 48-60 Cys peptide (5 mg, 2.689 µmol) was added. The resulting mixture was incubated overnight at RT. Thereafter, the product was purified by prep HPLC using an Atlantis Prep OBD T3 column (19×100 mm, 5 µm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-5 min) to 15% solvent B in 5mins, raising to 20% solvent B in 1min, 20% B for 10 mins, raising to 30% solvent B in 1 min, 30% B for 10mins and finally reaching 100% solvent B in 5 mins with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for (**7**) was 17.68 min. 1.7 mg (0.755 µmol) of (**7**) was isolated in 56 % yield after lyophilisation.

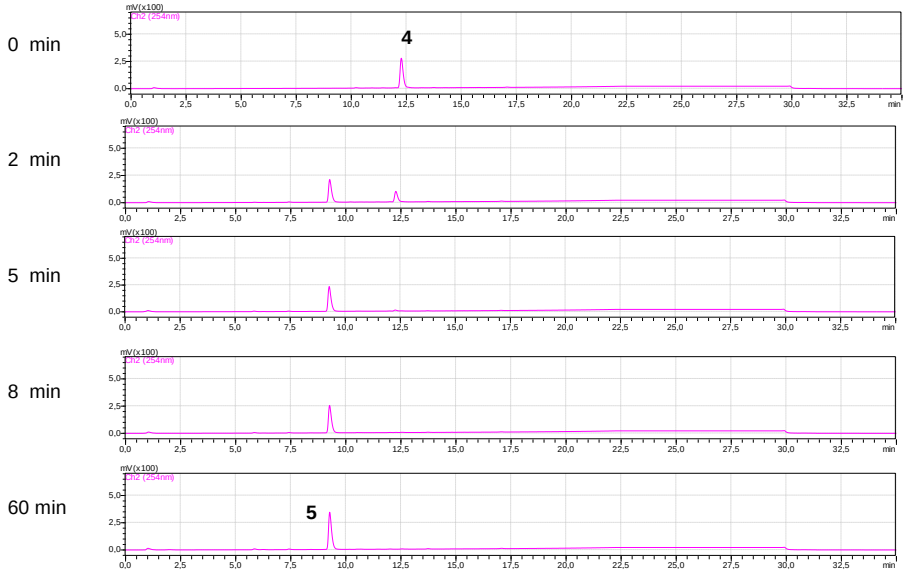
MALDI-TOF-MS: (+) m/z=2253(M+H)

LC-MS Studies of pH switch

*1. LC-MS study of the addition of thiol to maleimide in TCC (**4**)*

20 µL of 4 (10 mg/mL in ACN, 1 eq.) was added to 20 µl of PB (50 mM phosphate buffer, 10 mM EDTA, pH 6). Thereafter, 10 µL of cysteine (0.064 mmol/mL in PB, 2 eq.) was added and the mixture was stirred at RT. After 0, 2, 5, 8 or 60 min, 2 µL was taken from the mixture and diluted into 500 µL of MeOH. The reaction was quenched with 1µL of formic acid. LC-MS analysis was performed on a Shimadzu LC-MS 2020 equipped with an electrospray ionisation source and a SPD-20A UV-Vis detector (Shimadzu, Duisburg, Germany). Aliquots (10 µL) were injected onto Gemini C18 column (50 x 4.6 mm, 5 µm) (Phenomenex). The column temperature was set at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was linearly increased from 5% to 95% A over 20 min, held at 95% for an additional 8 min, and then immediately stepped back down to 5% for re-equilibration. The mobile phase flow rate was 0.4 mL/min. Identification of products was performed simultaneously by UV-VIS detection at 254 nm.

a. LC of the reaction of 4 and cysteine at different time point



b. ESI-MS of the two peaks in the LC

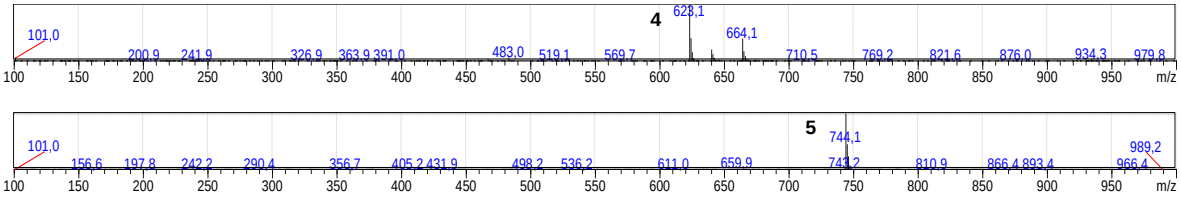
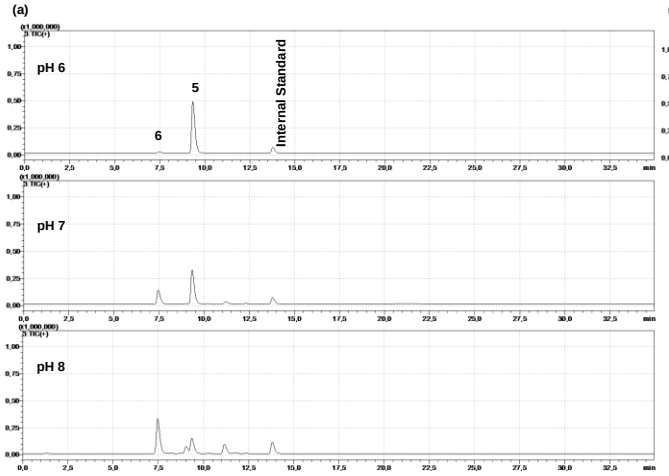


Figure 4. a. LC of the reaction mixture of TCC (4) and cysteine at 0, 6, 8 and 60 min separately; b. ESI-MS of the two peaks in the LC.

*2. LC-MS study for the elimination step (from **5** to **6**)*

4 µL of **4** (5 mg/mL in ACN, 1 eq.) was added to 5µl of buffer. Buffers over pH range 6-8 (50 mM phosphate buffer, 10 mM EDTA) were used for the investigation. Thereafter, 1 µL of cysteine (0.032 mmol/mL in MilliQ water, 1 eq.) was added and the mixture was stirred for 3 hr. The reaction was quenched with 1µL of formic acid and addition of 10 ppm of Fmoc-phenylalanine as internal standard. A solution of **5** at same concentration and quantity was also generated in situ using the above conditions as standard for subsequent quantification. LC-MS analysis was performed on a Shimadzu LC-MS 2020 equipped with an electrospray ionisation source and a SPD-20A UV-Vis detector (Shimadzu, Duisburg, Germany). Aliquots (20 µL) were injected onto Gemini C18 column (50 x 4.6 mm, 5 µm) (Phenomenex). The column temperature was set at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was linearly increased from 5% to 95% B over 20 min, held at 95% for an additional 8 min, and then immediately stepped back down to 5% for re-equilibration. The mobile phase flow rate was 0.4 mL/min. Identification of products was performed simultaneously by UV-VIS detection at 254 nm and selective ion monitoring (SIM) of the [M+H] + at m/z = 623 (**4**), 744 (**5**), 588 (**6**) and 388 (internal standard).The amount of **5** in each sample was determined as a ratio of **5** to the internal standard Fmoc-phenylalanine integrated according to SIM chromatogram. Calculations were made based on duplicate runs.



(c)

5 (for calibration)	**5**/IS 12.3	**5**/IS 14.7	Average 13.5		
pH	**5**/IS	**5**/IS	Average	%	Error (%)
6	11.4	14.0	12.7	94	14
7	5.2	5.9	5.6	41	3
8	1.04	0.6	0.8	6	3

Figure 5: (a) SIM profile of conversion of **5** to **6** at pH 6-8. (b) SIM profile of **5** as quantification standard and (c) Calculation of percentage of **5** from integration of SIM chromatogram.

*3. LC-MS Studies for addition of TAT to **5** at pH 6-8.*

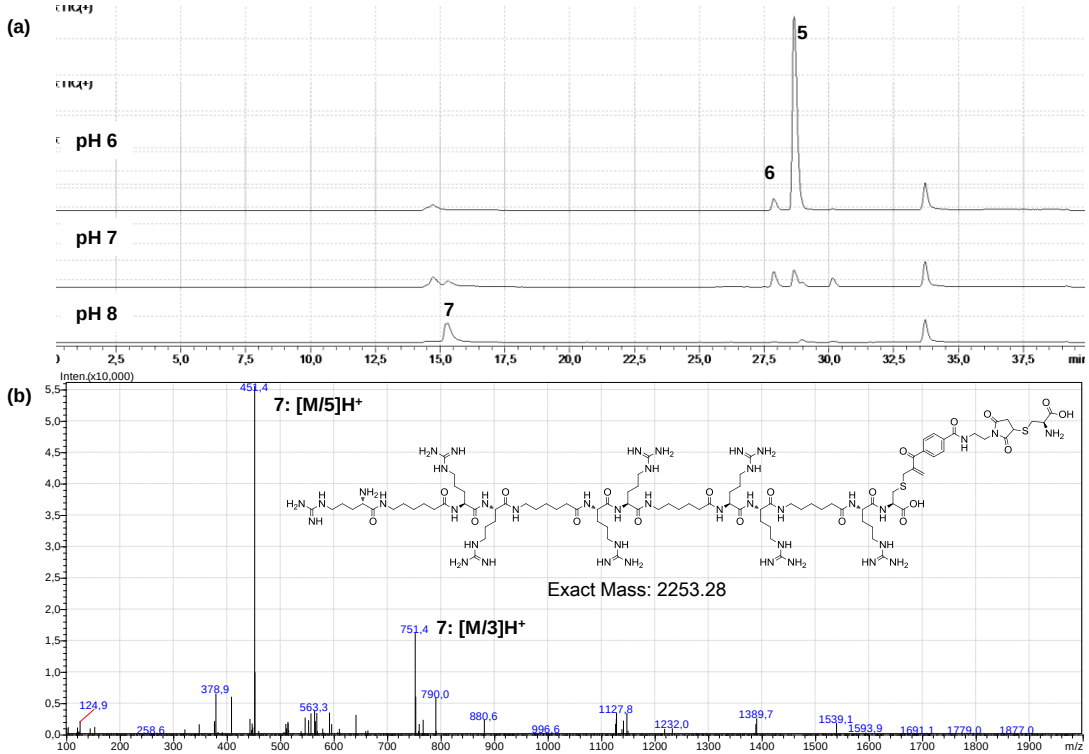


Figure 6: (a) SIM profile of effect of pH on reaction of **5** with TAT and (b) Mass spectrum of peak at tR = 15.2 mins corresponding to **7**.

4 µL of 4 (5 mg/mL in ACN, 1 eq.) was added to 5µl of buffer. Buffers over pH range 6-8 (50 mM phosphate buffer, 10 mM EDTA) were used for the investigation. 1 µL of cysteine (0.032 mmol/mL in MilliQ water, 1 eq.) was then added and the mixture was stirred for 1 h. Thereafter, 39 µL of TAT (3 mg/mL in MilliQ water, 2 eq.) was then added and the mixture was stirred overnight. The reaction was quenched with 1µL of formic acid. Duplicate sets were prepared and analysed by LC-MS. LC-MS analysis was performed on a Shimadzu LC-MS 2020 equipped with an electrospray ionisation source and a SPD-20A UV-Vis detector (Shimadzu, Duisburg, Germany). Aliquots (20 µL) were injected were injected onto a Atlantis T3 column (100 x 4.6 mm, 5 µm) (Waters). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was held at 0% B over 5 mins, linearly increased from 0% to 10% B over 7 min, held at 10% for an additional 8 min, stepped up to 100% over 10 mins and held over 15 mins and then immediately stepped back down to 0% for re-equilibration. The mobile phase flow rate was 0.4 mL/min. Identification of products was performed simultaneously by UV-VIS detection at 254 nm and selective ion monitoring (SIM) of the [M+H] + at m/z = 623 (4);744 (5); 588 (6); 607, 456 (TAT); 603, 482 (A) 751, 564, 452 (7); 815, 679 (B).

Synthesis of Bissulfone-NLS (15)

Maleimide bissulfone (**4**, 3 mg, 0.004283 mmol) was dissolved in 3ml of 40% acetonitrile in PB (50 mmol PB, 10 mM EDTA, pH 6) and NLS (3 mg, 0.00214 mmol) was added. The reaction mixture was incubated 2h at RT. After removal of the solvent in vacuum, the residual mixture was purified by Prep HPLC using an Atlantis Prep OBD T3 Column (19×100 mm, 5 µm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-5 min), raising to 20% solvent B in 1min, 20% solvent B for 10 mins, raising to 30% solvent B in 1min, 30% solvent B for 10 mins and finally reaching 100% solvent B in 5 mins with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for 11 was 27.07 min, and 27.91 min. 2 mg of the product **15** was obtained from lyophilisation with 46% yield. The Bissulfone-NLS (**15**) was characterized by MALDI-TOF-MS using α-Cyano-4-hydroxycinnamic acid as matrix. There is trace of monosulfone-NLS was observed in the MALDI-MS and the observed M.W. is 1890.88 g/mol (calcd. M.W. = 1866.88 g/mol) which corresponds to the sodium adduct.

MALDI-TOF-MS: (+) m/z=2024(M+H)

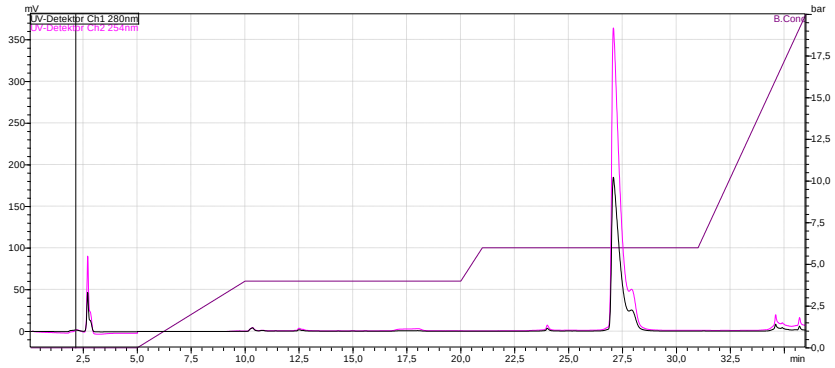


Figure 7. The HPLC profile of Bissulfone-NLS (**15**)

Synthesis of Oligonucleotide-NLS conjugate (10)

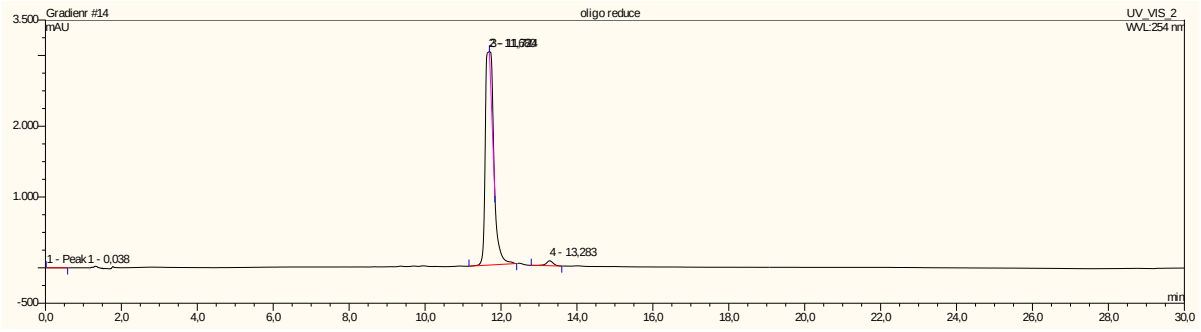


Figure 8. The HPLC profile of reduced oligonucleotide C_6SH-5'aattgaataagctggtat-3'

Bissulfone-NLS (**15**, 1.5 mg, 0.7411 µmol) was dissolved in 2 ml of 50 mM PB, 10 mM EDTA, pH 8 and incubated at RT for 24h. The oligonuclotide 5'-aattgaataagctggtat-3' (70 µl, 7 nmol) was dissolved in 10 mM PB, 2 mM EDTA, pH 8 and 20 µl of TCEP solution (3.5 mM) was added. The mixture was shaken at RT for 1h and purified by analytical HPLC using an MerckChroCART 125-4 Column with the mobile phase starting from 95% solvent A (0.1M triethylammonium acetate solution, pH 8) and 0% solvent B (acetonitrile) (0-2 min) to 30% solvent B in 20 mins, decreasing to 5% solvent B in 5 mins and finally balancing the column with 5% solvent B for 5 mins with a flow rate of 1ml/min. The absorbance was monitored at 254 nm. The retention time of reduced oligonucleotide was 11.68 min. The reduced oligonucleotide was immediately mixed with 200 µl of Bissulfone-NLS solution. The reaction mixture was shaken overnight and purified by analytical HPLC using the same condition of purification of reduced oligonucleotide. The retention time for oligonucleotide-NLS **10** was 11.71 min. The oligonucleotide-NLS **10** was characterized by MALDI-TOF MS using 3-hydroxypicolinic acid and ammo-

nium citrate dibasic as matrix. Matrix preparation: 3-hydroxypicolinic acid and ammonium citrate dibasic were separately dissolved in ACN/H_2O (3:7) and the concentration was 50mg/ml. 2µl 3-hydroxypicolinic acid and 0.5µl ammonium citrate dibasic were mixed and a little amount of cationic exchange resin was added. After 2 minutes, the ON was mixed with the supernatant of the matrix for recrystalization.

MALDI-TOF-MS: (+) m/z=7466.13

No.	Ret. Time (min)	Area (mAU*min)	Height (mAU)	Rel.Area (%)
1	3,028	3,6644	18,503	2,55
2	8,062	16,6407	24,397	11,56
3	11,722	83,8230	238,499	58,25
4	13,286	35,5692	160,930	24,72
5	13,760	2,3607	16,317	1,64
6	14,394	1,8449	10,563	1,28
Total		143,9030	469,208	100,00

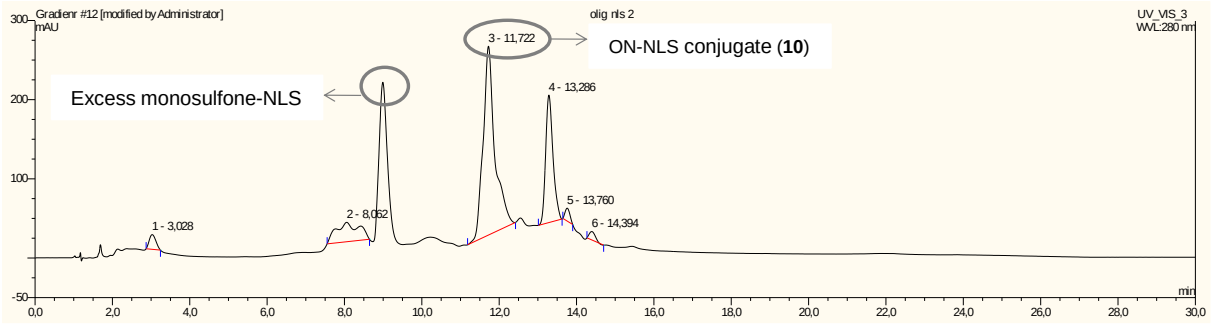


Figure 9. The HPLC purification for the reaction mixture of Bissulfone-NLS(**15**) and oligonucleotide

Synthesis of BSA dimer (14)

6 ml of PB (50 mM PB, 10 mM EDTA, pH 6) was degassed under Argon and bovine serum albumin (BSA, 10 mg, 0.15 µmol) was added, followed by adding 4ml of maleimide bissulfone (**4**, 1mg in 8 ml CH_3CN). The reaction mixture was stirred 3h at RT. The mixture was purified into MilliQ water by centrifugal filtration (MW 30000 cut off). The concentrated product was diluted in 20ml of PB (50 mM PB, 10 mM EDTA, pH 8) and the solution was stirred at RT overnight. BSA (10 mg) was added and the mixture was stirred overnight at RT. The mixture was purified and concentrated into MilliQ water by centrifugal filtration (MW 100000 cut off). The crude product was purified by FPLC (ÄKTA from GE) using Superose 6 10/300 GL column from GE Healthcare. The dimer BSA was characterized by SDS gel electrophoresis. Mobile phase: 20 mM PB, 150 mM NaCl, pH 7.4; isocratic flow rate: 0.5 ml/min. The effluent peaks were monitored at 280 nm.

Synthesis of HSA-BSA conjugate (9)

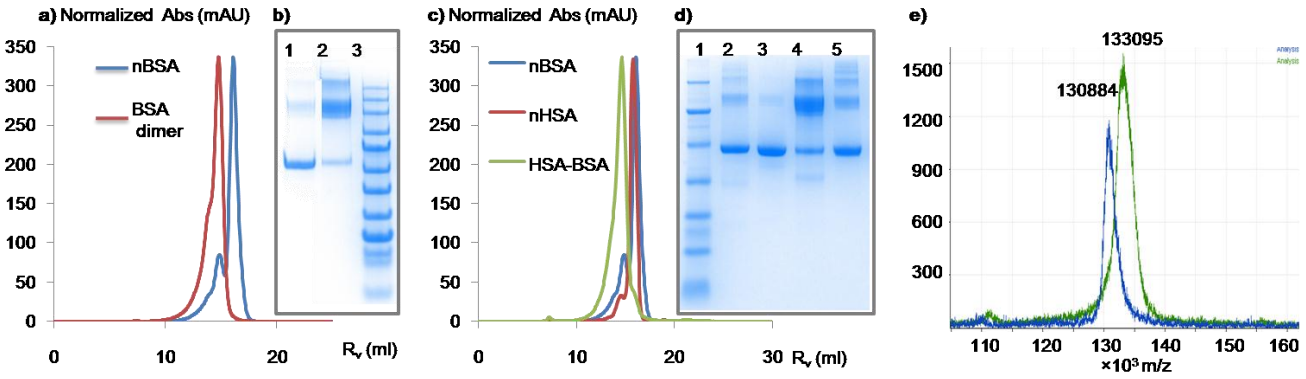


Figure 10. (a) The elution chromatogram of native BSA (nBSA, blue) and BSA dimer(**14**, red); (b) SDS-PAGE gel; lane 1: native BSA, lane 2: dimer BSA **14**, lane 3: protein markers (From top to the bottom: 240, 165, 125, 93, 72, 57, 42, 31, 24, 18, 15, 8 kD); (c) The elution chromatogram of HSA-BSA (**9**, green), native HAS (nHSA, red) and native BSA (nBSA, blue). (d) SDS-PAGE gel; lane 1: protein markers,(From top to the bottom: 200, 116, 68, 43, 30, 20, 14.4, 6.5 kD) lane 2: native BSA, lane 3: native HSA, lane 4: HSA-BSA conjugate **9**, lane 5: maleimide bissulfone-BSA; (e) MALDI-TOF mass spectrum of HSA-BSA conjugate (**9**, green) and BSA dimer (**14**, blue) with sinnapinic acid as matrix.

6ml of PB buffer (50 mM PB, 10 mM EDTA, pH 6) was degassed under Argon and BSA (5mg, 0.075µmol) was added, followed by adding 1ml of maleimide bissulfone (**4**, 1mg in 4ml CH_3CN). The reaction mixture was stirred 3h at RT. The mixture was purified

into MilliQ water by centrifugal filtration (MW 30000 cut off). The concentrated product was diluted in 10ml of PB (50 mM PB, 10 mM EDTA, pH 8) and HSA (25 mg) was added and the mixture was stirred overnight at RT. The mixture was purified and concentrated into MilliQ water by centrifugal filtration (MW 100000 cut off). The crude product was purified by FPLC (ÄKTA from GE) using Superose 6 10/300 GL column from GE Healthcare. The HSA-BSA **9** was characterized by SDS gel electrophoresis. Mobile phase: 20 mM PB, 150 mM NaCl, pH 7.4; isocratic flow rate: 0.5 ml/min. The effluent peaks were monitored at 280 nm. The product (2.2 mg, 0.016 µmol) was desalted, concentrated via Ultrafiltration (30k MWCO), and lyophilized, resulting in 10% yield.

Synthesis of TAT-EYFP conjugate (8)

100 µl of TCC (4, 0.34mg, 0.5931µmol, 50eq.) in DMSO were added to the solution of tagged EYFP (0.344 µg, 0.01186 µmol, 1 eq.) in 900 µl of 50 mM PB (pH 6). The resulting mixture was shaken at RT for 3h and concentrated by Ultra filatration (MW. 10000). The crude product was further purified by size exclusion chromatography using a Sepharose G-50 matrix and 50 mM PB (pH 8) as eluting solvent to afford bissulfone-EYFP (**11**). Bissulfone-EYFP (**11**) was incubated in 1 ml of 50 mM PB (pH 7.8) overnight to yield monosulfone-EYFP. TAT peptide (GRKKRRQRRRPPQC-NH2, 0.43 mg, 0.2361 µmol, 20 eq.) was added to a solution of monosulfone-EYFP in 1ml of 50 mM PB (pH 8). The mixture was incubated overnight at RT and purified by size exclusion chromatography using a Sepharose G-50 matrix and Mili Q water as eluting solvent to obtain TAT-EYFP conjugate (**8**). The TAT-EYFP conjugate (**8**) was characterized by MALDI-MS. The concentration of TAT-EYFP was determined by the absorbance at 514 nm. The absorbance of EYFP (1.146 mg/ml) and TAT-EYFP were 1.3797 and 0.8446 separately at 514 nm, so the concentration of TAT-EYFP was 0.7015 mg/ml. In addition, the volume of TAT-EYFP solution is 420µl. Therefore, 0.295mg TAT-EYFP was obtained and the yield is about 81%. Noteworthy, EYFP has an isotopic distribution in mass spectrum and in particular in the linear mode of the MALDI-MS, the peak is usually broad and the resolution is not as good as when applying ESI-MS. The width at the half height of the TAT-EYFP is 540Da. The observed M.W. of 30862 g/mol for TAT-EYFP is only about 50Da larger than the expected M.W. (30812 g/mol), which is within the error range.

Cells and media

The A-549 cell line originating from a human lung carcinoma was received from the DSMZ (German Collection of Microorganisms and Cell Culture). All media and supplements were obtained from Gibco (Invitrogen, Mannheim, Germany), Plastic material, well plates, culture flasks etc. were obtained from GreinerBioOne (Frickenhausen, Germany).

Cell culture

A-549 cells were cultured in DMEM-Medium, Dulbecco's Modified, Eagle Medium, supplemented with 10% FBS, 1% Penicillin/Streptomycin, and 1% MEM (non-essential amino acids with earl's salts). For experiments, the cells were seeded and incubated over night for attachment.

Microscopy experiments:

In an eight-well chambered cover glass (LabTek, Nunc,Langenselbold), 25 000 cells per well were seeded in 300 µl medium and after attachment, they were incubated with1.15 µM EYFP-TAT conjugate (**15**) for 13h. For investigation under the microscope, the cells were fixed with 80% methanol and 20% PBS for 10 min. An Olympus laser scanning setup, microscope IX70 with 40x water objective (Olympus) was used. The excitation wavelength was selected at 494 nm, the emission was detected using a 520 nm pass filter. For co-staining, fixed cells were incubated with a 1 µg/ml DAPI-solution and incubated for 15 min at 37°C. Then, the cells were washed once with methanol and kept in PBS during the microscopy experiments. For excitation experiments, the DAPI-chromophore, a mercury lamp and the filter cube WU (Olympus, ex: BP 330-385 nm, Em BA 420 LP) were applied.

4. Reference

[1] S. Brocchini, S. Balan, A. Godwin, J.-W. Choi, M. Zloh, S. Shaunak, *Nat. Protocols* **2006**, *1*, 2241.

5. NMR and Mass spectra

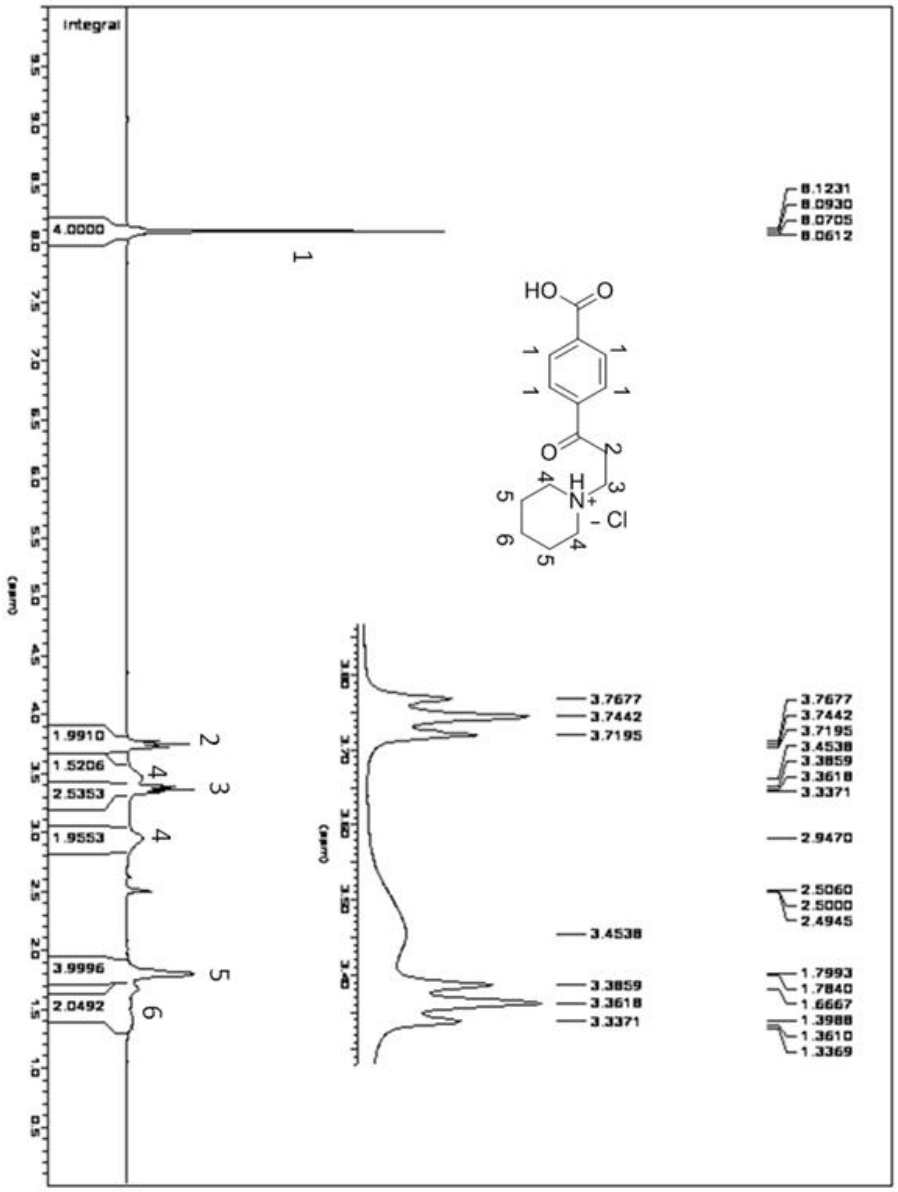


Figure 11. The 1H NMR spectrum of 1-[3-(4-carboxy-phenyl)-3-oxo-propyl]-piperidinium HCl (**1**)

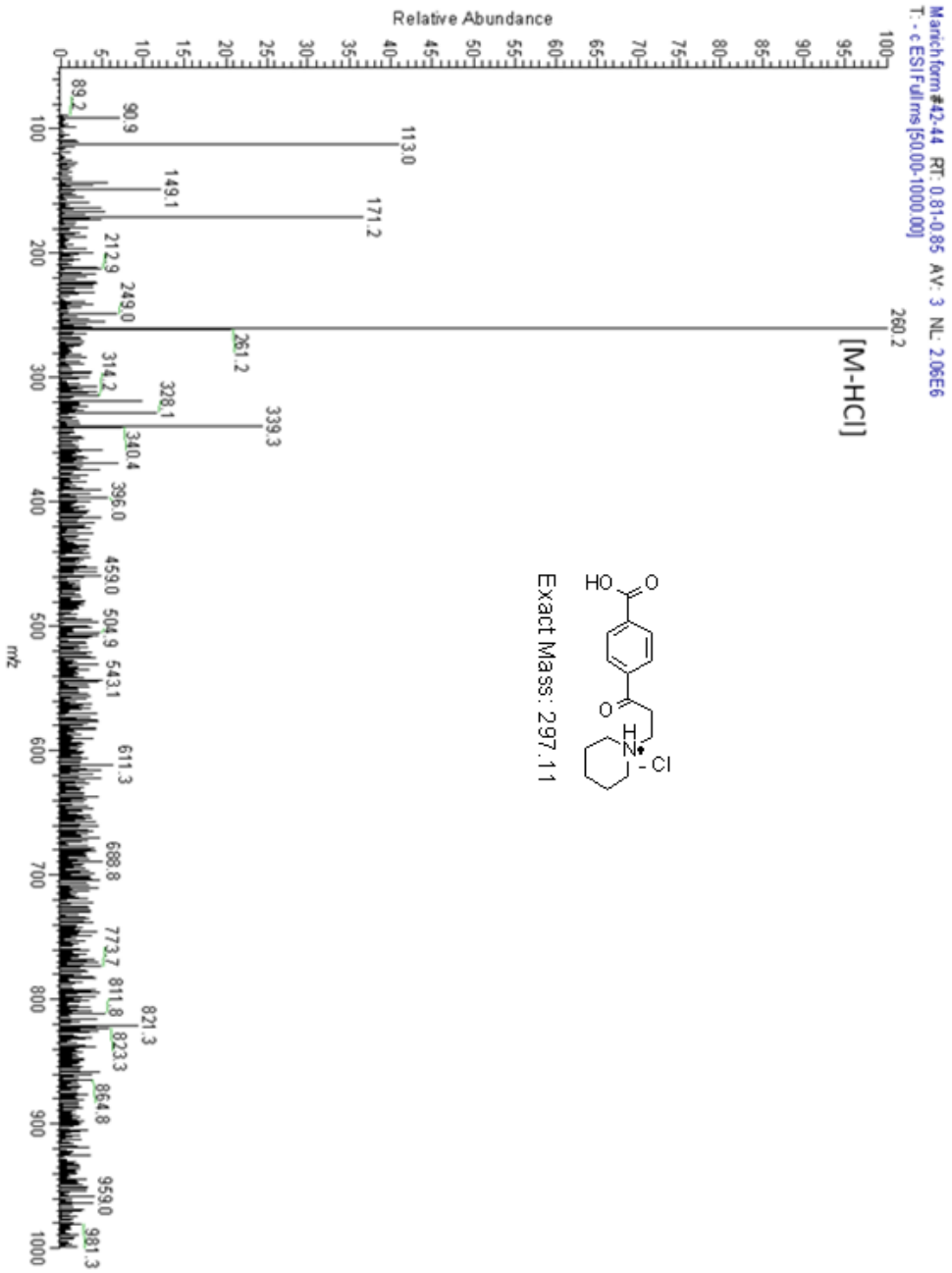


Figure 12. The ESI MS spectrum of 1-[3-(4-carboxy-phenyl)-3-oxo-propyl]-piperidinium HCl (**1**)

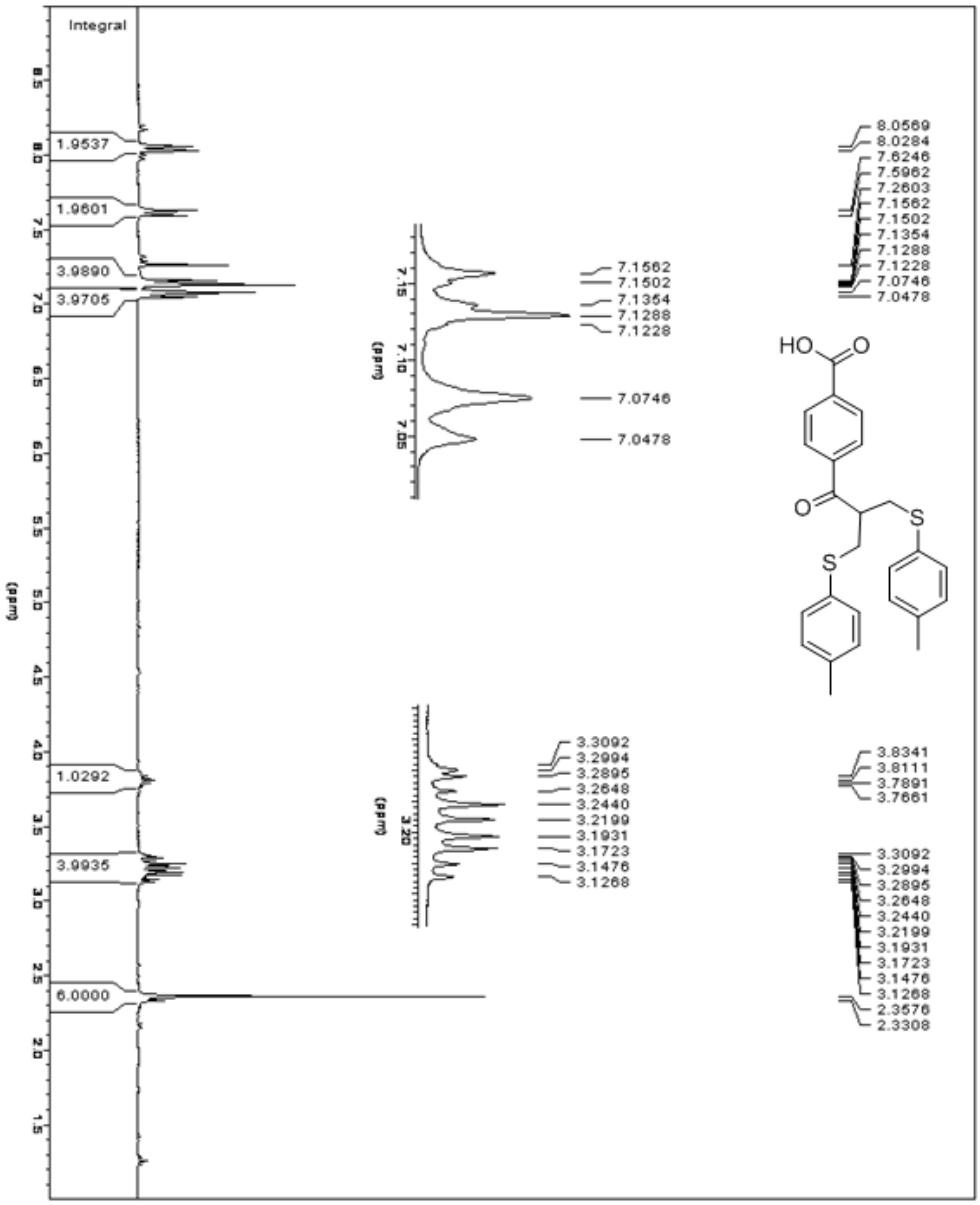


Figure 13. The ^{1}H NMR spectrum of 4-(3-(p-tolylthio)-2-((p-tolylthio)methyl)propanoyl)benzoic acid (**2**)

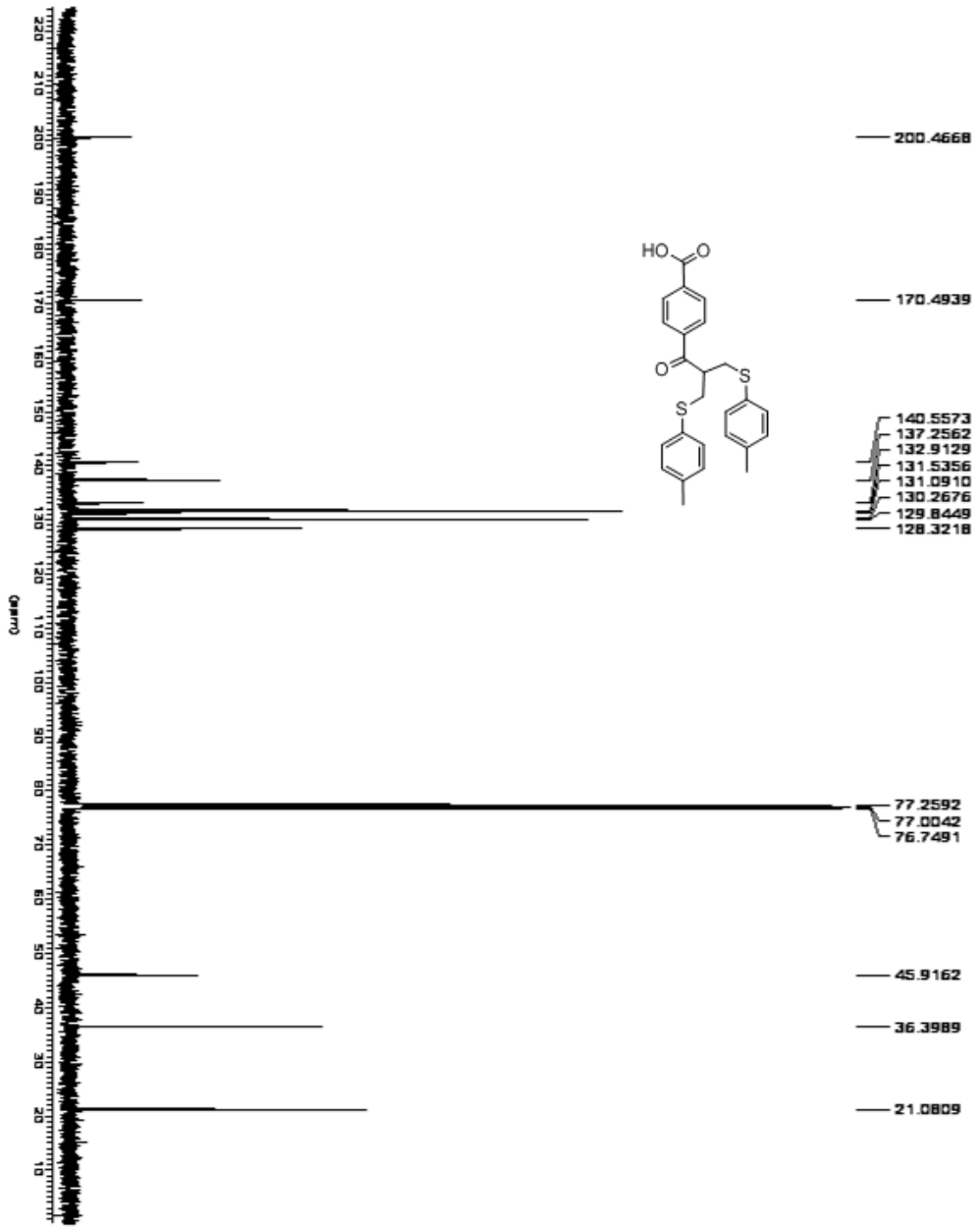


Figure 14. The ^{13}C NMR spectrum of 4-(3-(p-tolylthio)-2-((p-tolylthio)methyl)propanoyl)benzoic acid (**2**)

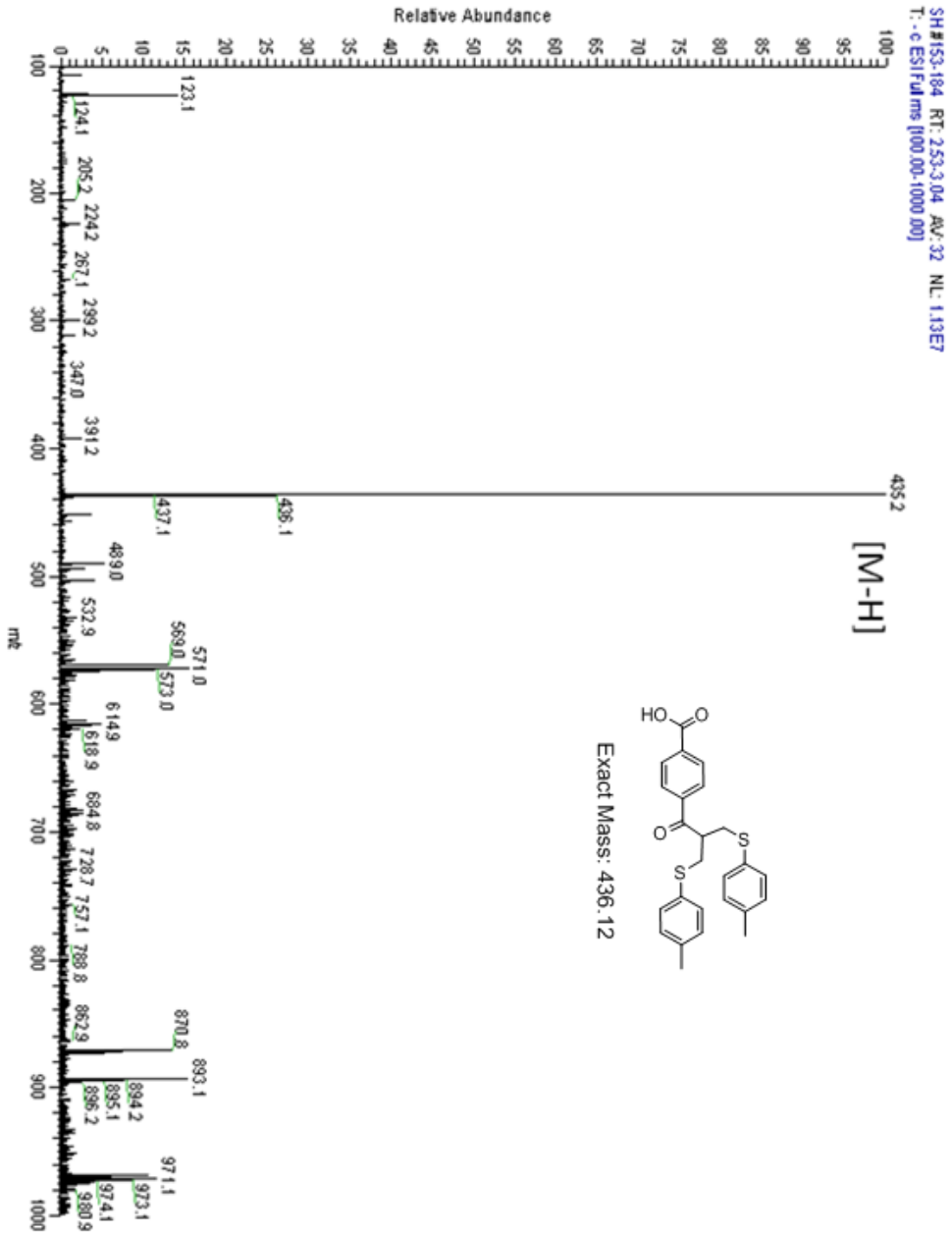


Figure 15. The ESI MS spectrum of 4-(3-(p-tolylthio)-2-((p-tolylthio)methyl)propanoyl)benzoic acid (**2**)

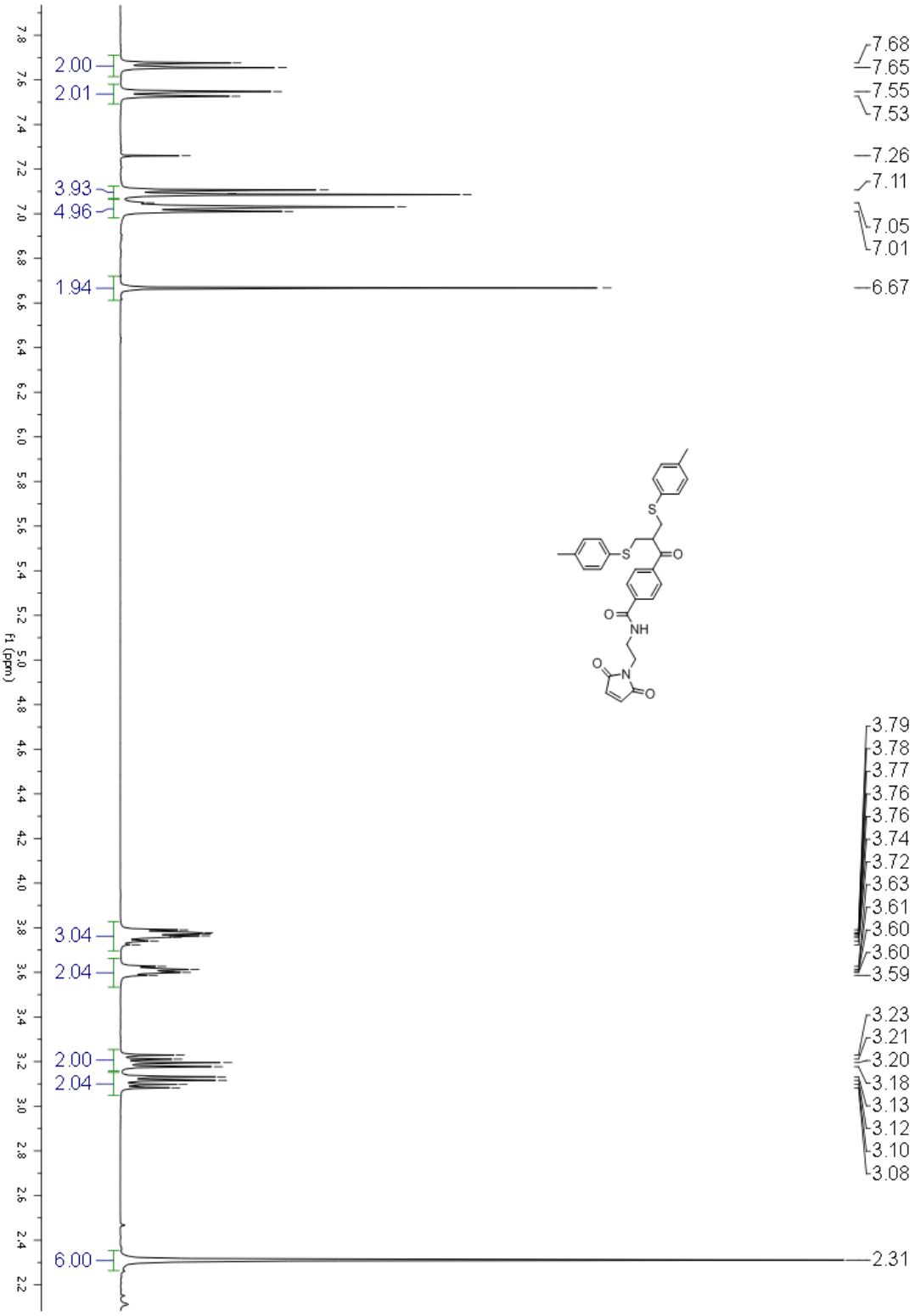


Figure 16. The ^{1}H NMR spectrum of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-(p-tolylthio)-2- ((p-tolylthio) methyl) propanoyl) benzamide (**3**)

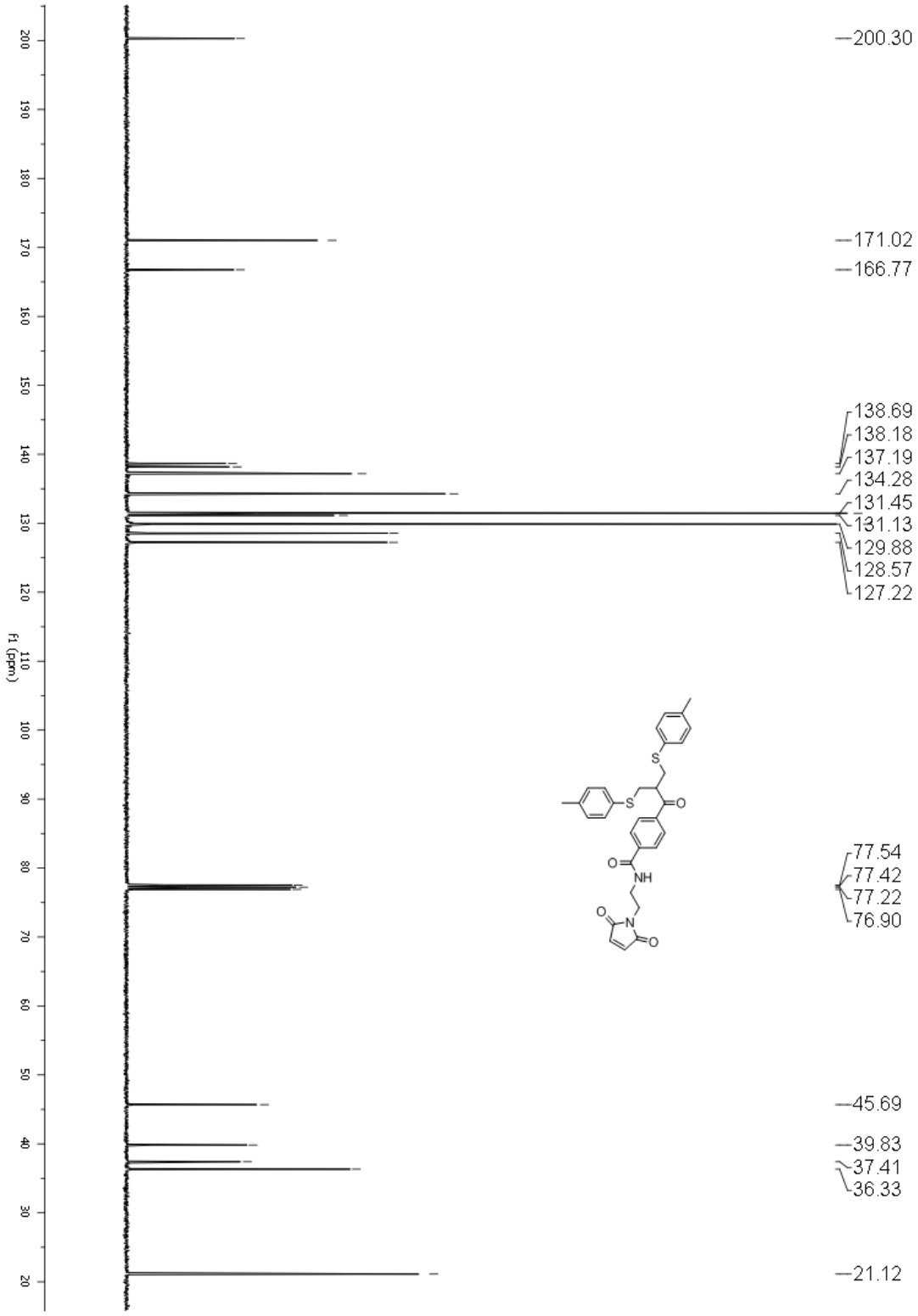


Figure 17. The ^{13}C NMR spectrum of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-(p-tolylthio)-2- ((p-tolylthio) methyl) propanoyl) benzamide (**3**)

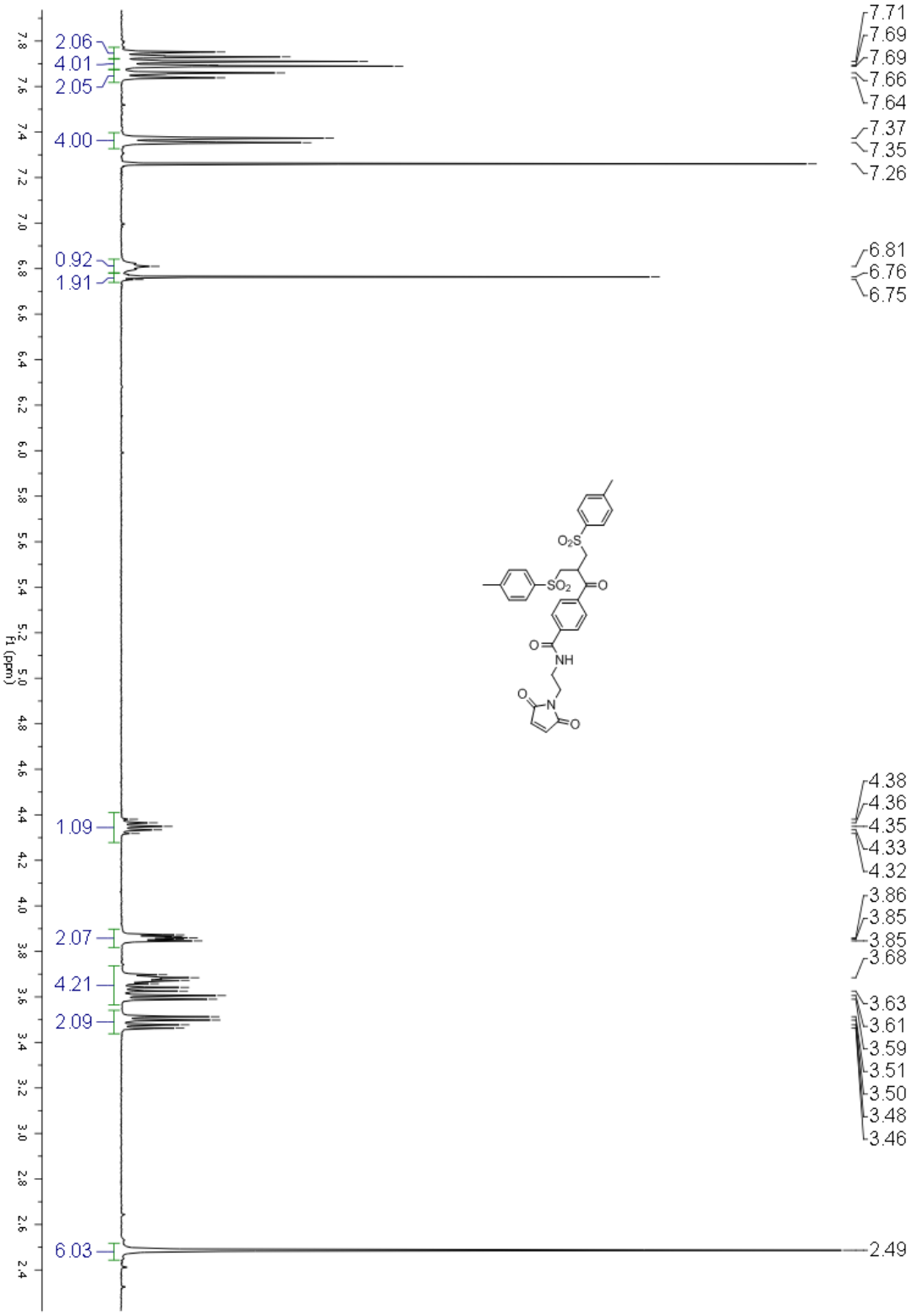


Figure 18. The ^{1}H NMR spectrum of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamide (**4**)

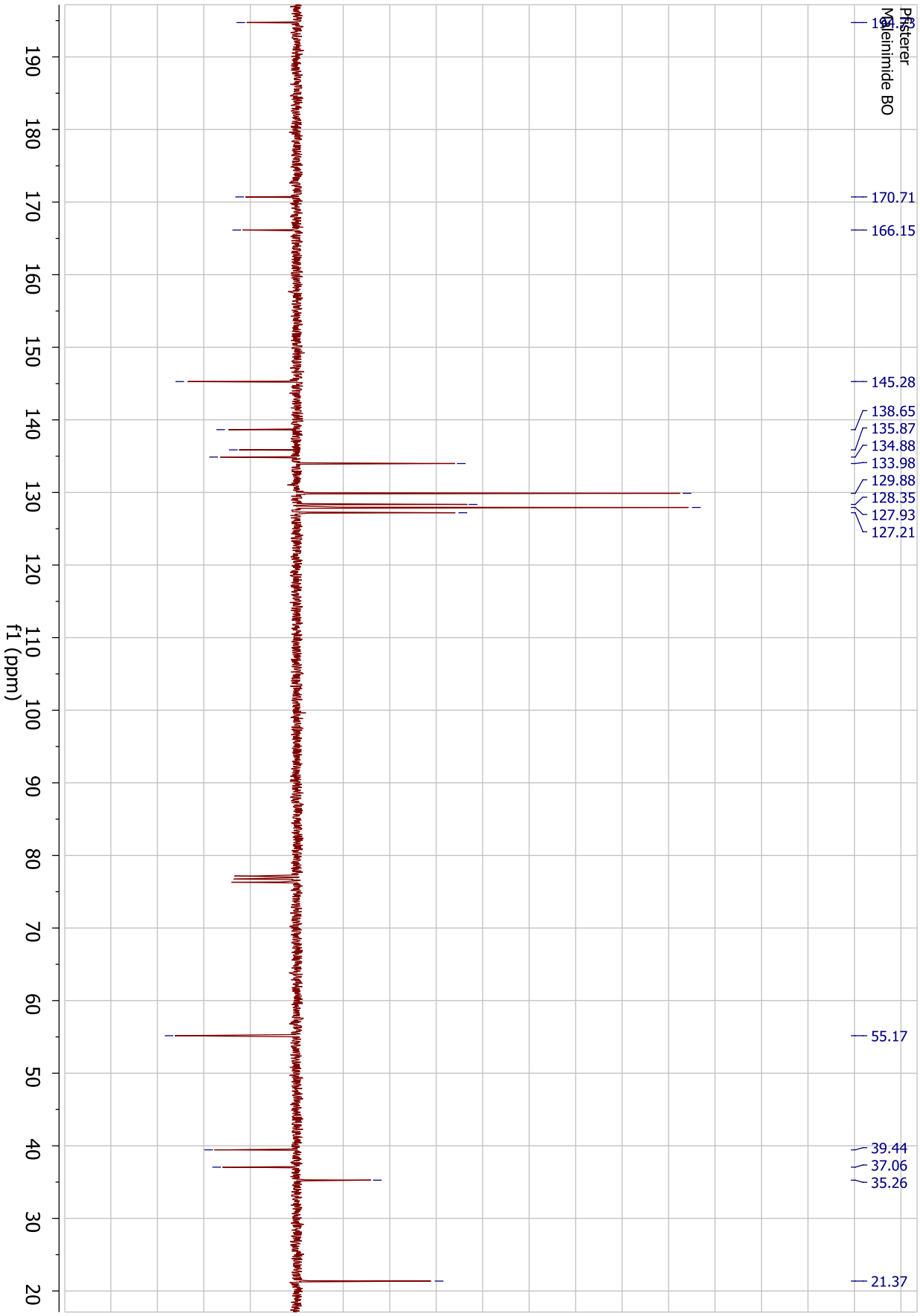


Figure 19. The ^{13}C NMR spectrum of N-(2-(2, 5-dioxo-2, 5-dihydro-1H-pyrrol-1-yl) ethyl)-4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamide (**4**)

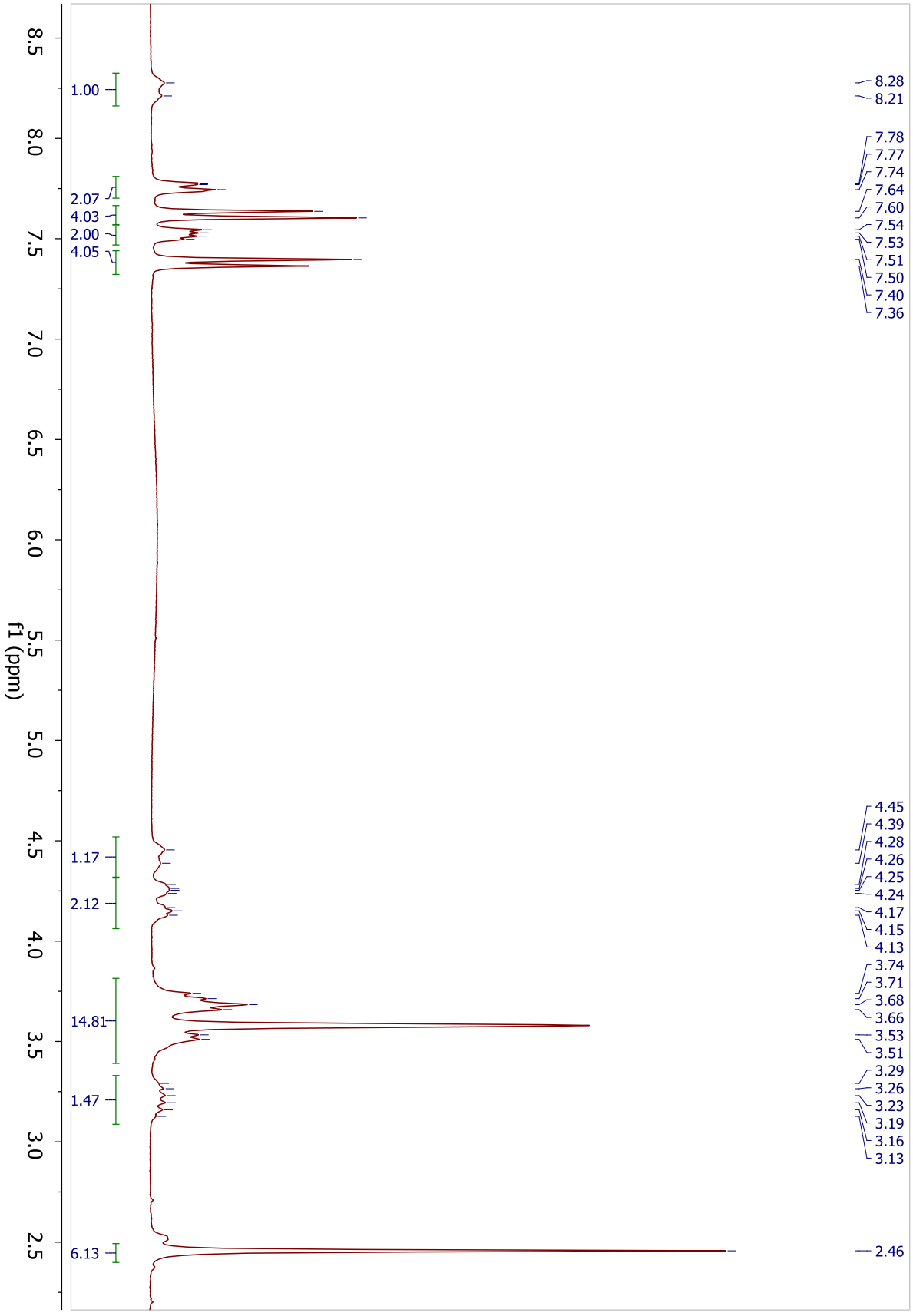


Figure 20. The ^{1}H NMR spectrum of 2-amino-3-((2, 5-dioxo-1-(2-(4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamido) ethyl) pyrrolidin-3-yl) thio) propanoic acid (**5**)

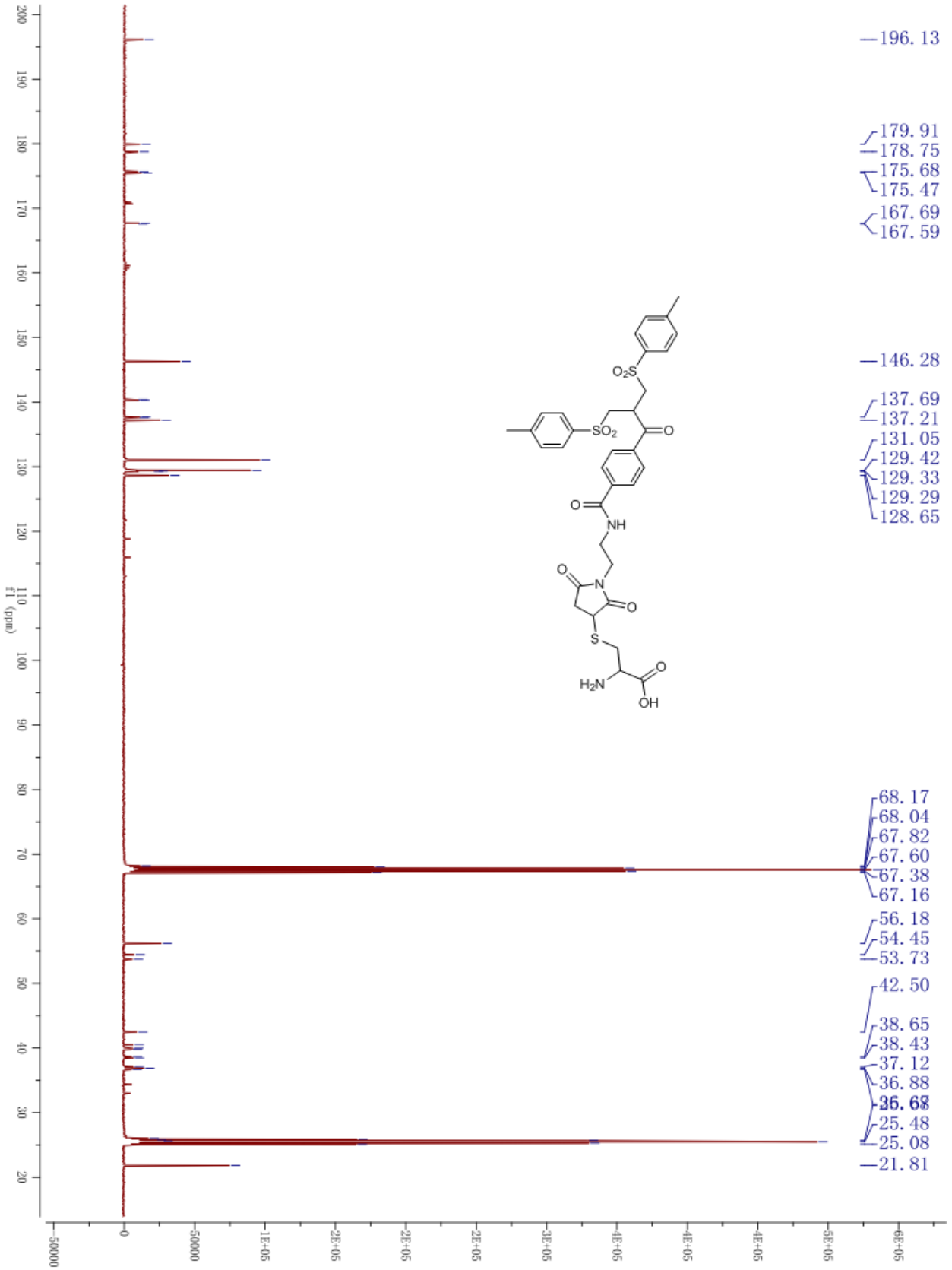


Figure 21. The ^{13}C NMR spectrum of 2-amino-3-((2, 5-dioxo-1-(2-(4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamido) ethyl) pyrrolidin-3-yl) thio) propanoic acid (**5**)

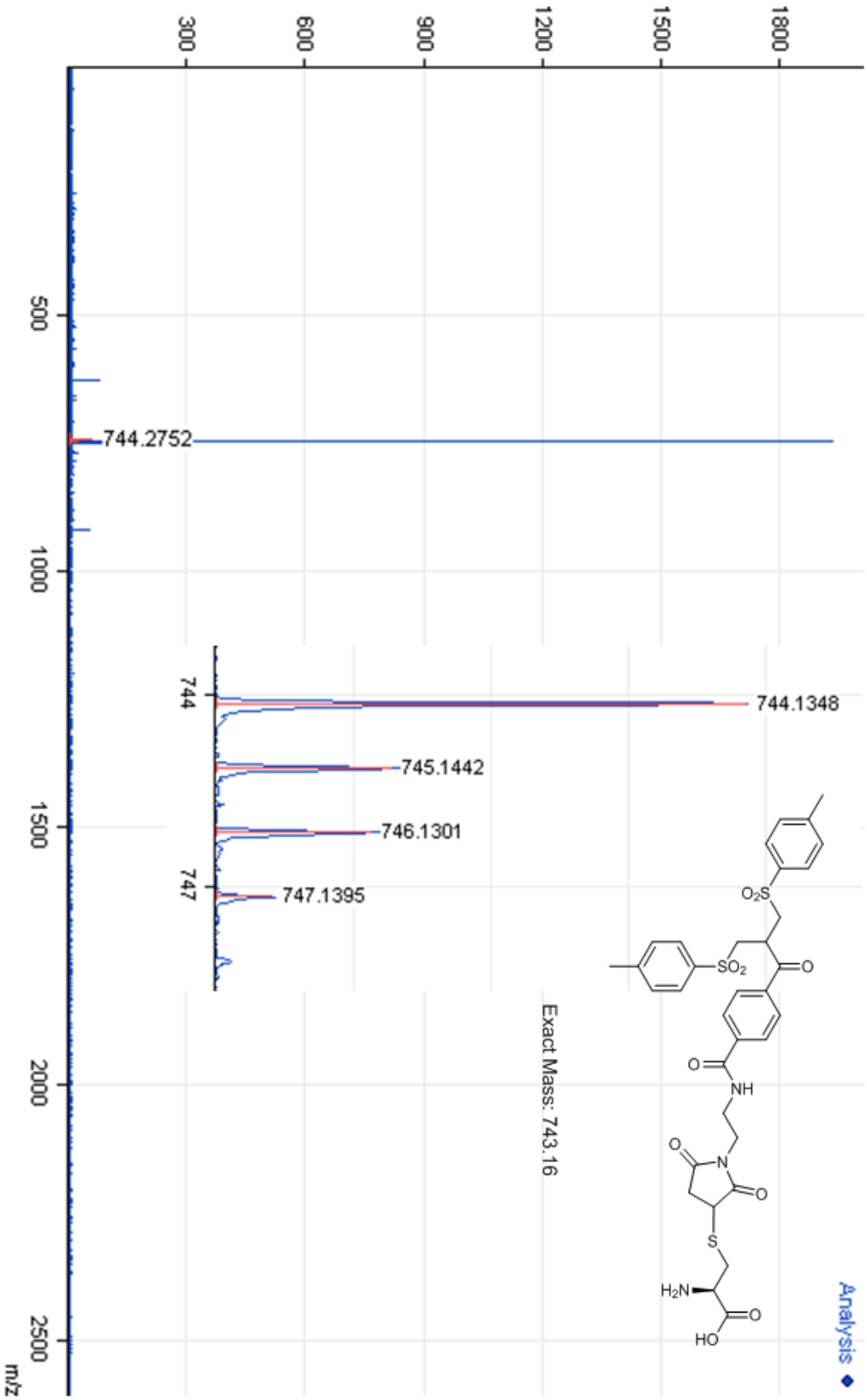


Figure 22. The MALDI-TOF mass spectrum of 2-amino-3-((2, 5-dioxo-1-(2-(4-(3-tosyl-2-(tosylmethyl) propanoyl) benzamido) ethyl) pyrrolidin-3-yl) thio) propanoic acid (**5**)

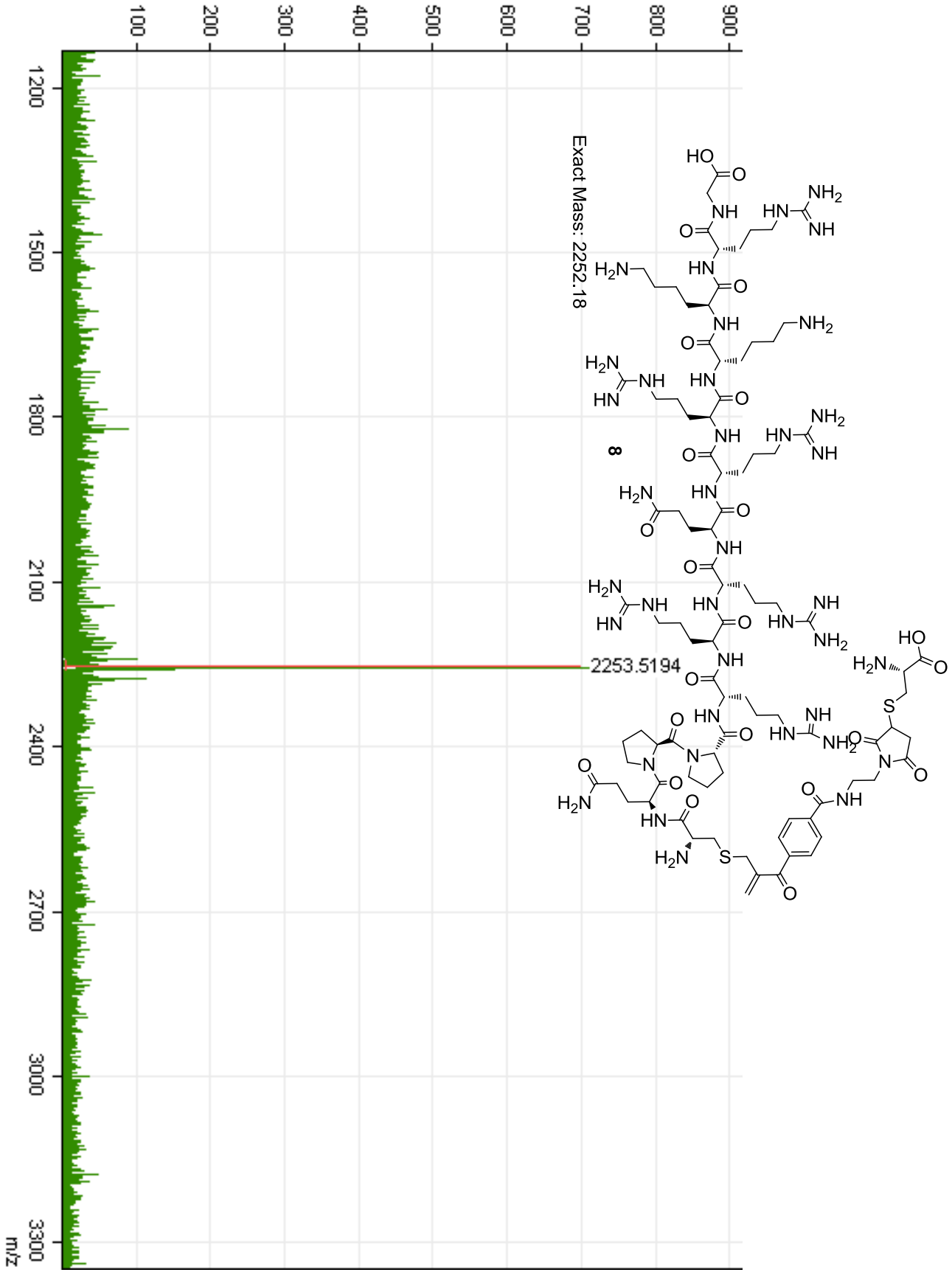


Figure 23. The MALDI-TOF mass spectrum of cysteine TAT conjugate (**7**)

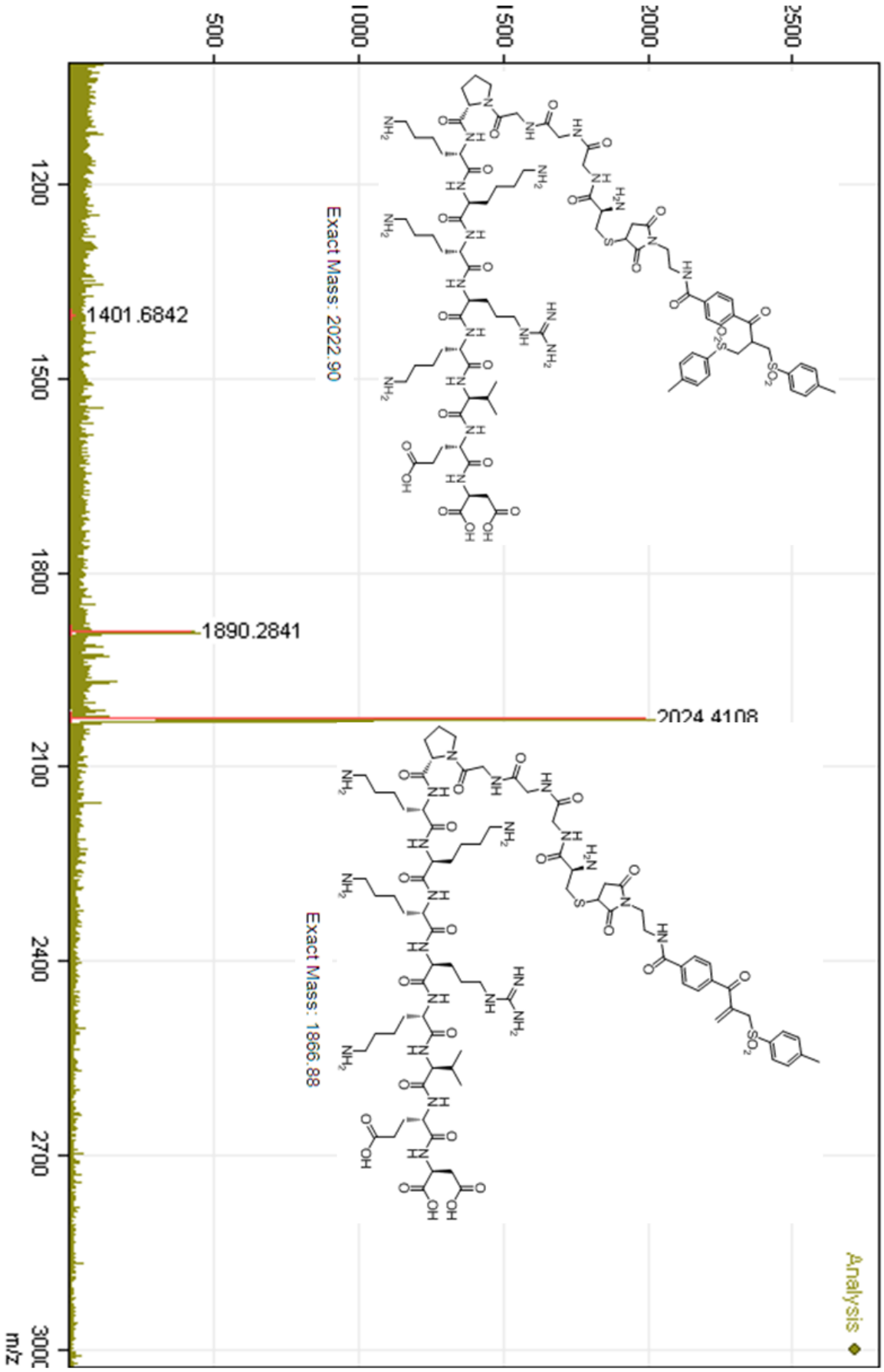


Figure 24. The MALDI-TOF mass spectrumof Bissulfone-NLS (**15**)

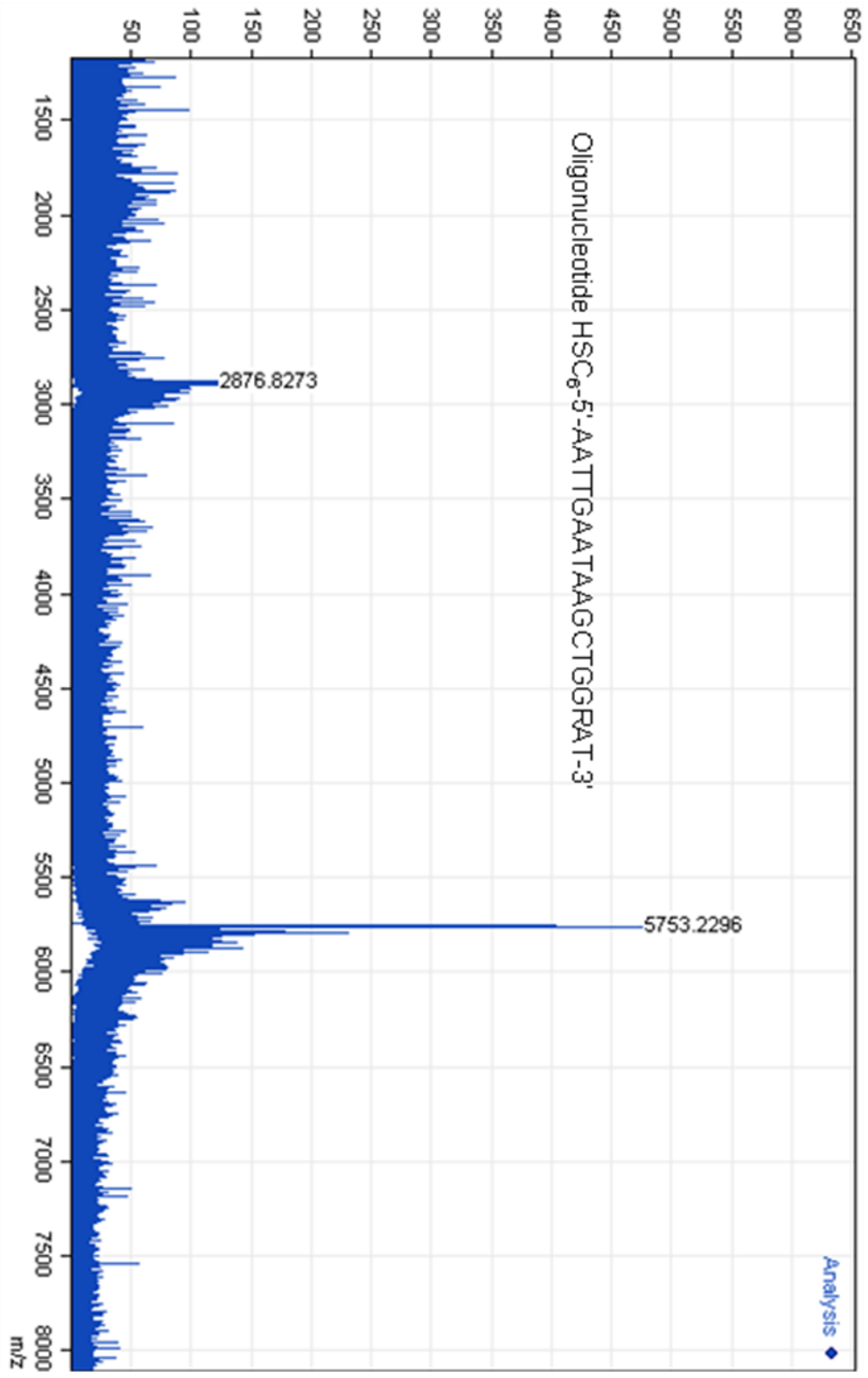


Figure 25. The MALDI-TOF mass spectrum of oligonucleotides (C_6SH-5'-AATTGAATAAGCTGGTAT-3')

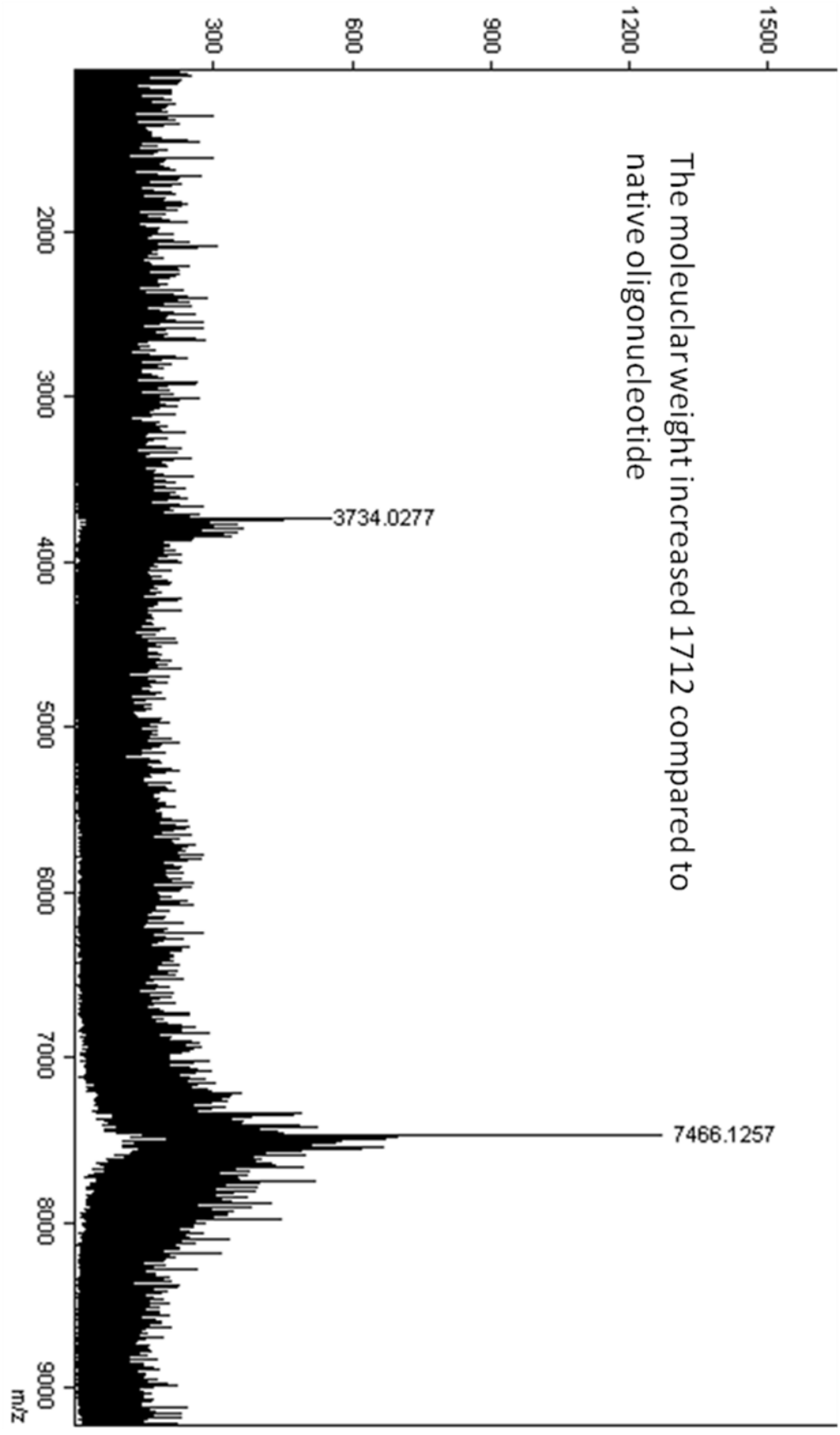


Figure 26. The MALDI-TOF mass spectrum of Oligonucleotide-NLS conjugate (**10**)

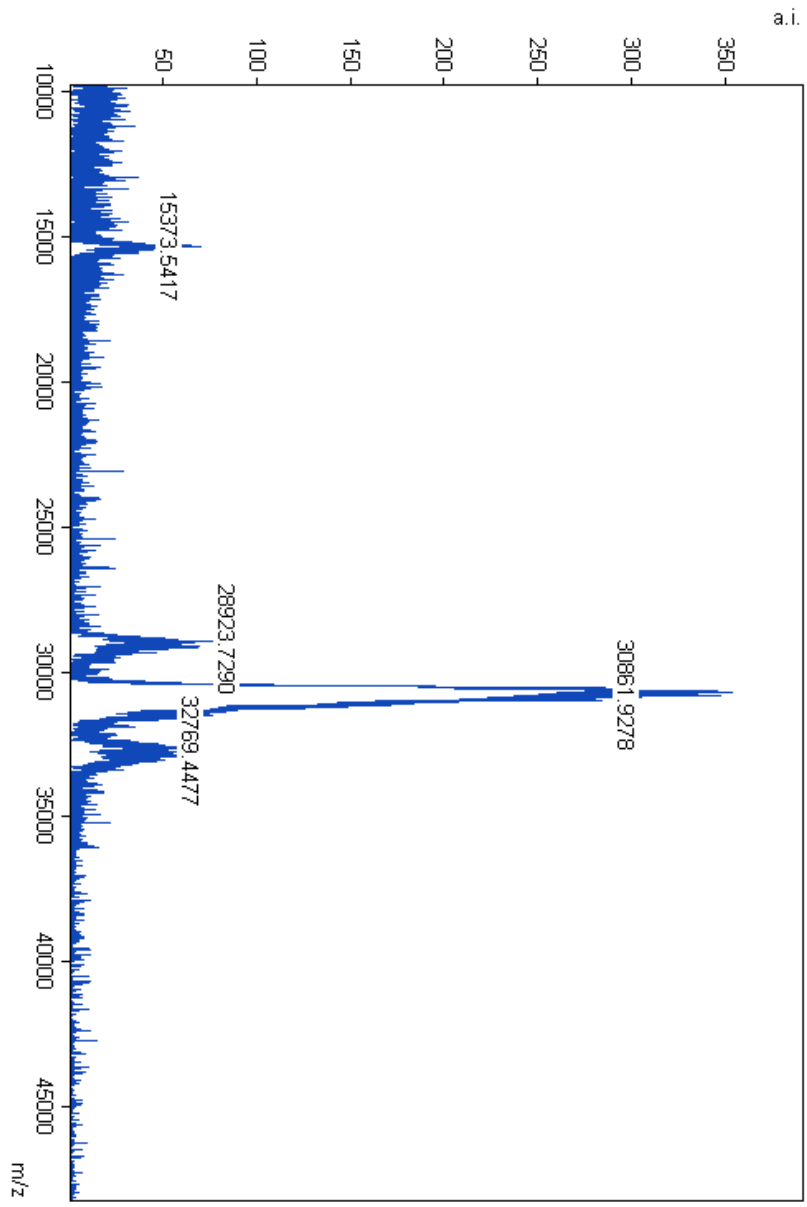


Figure 27. The MALDI-TOF mass spectrum of TAT-EYFP (**8**)