**Supplementary data**

# Thermally reversible and irreversible interstrand photocrosslinking of 5-chloro-2'-deoxy-4-thiouridine modified DNA oligonucleotides

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## 1) Synthesis of 5'-*O*-dimethoxytrityl-2'-deoxy-3'-*O*-[(2-cyanoethoxy),(N,N-diisopropylamino)phosphino]-4-(2-cyanoethylthio)-5-chlorouridine (4)

### 5-Chloro-3',5'-di-*O*-acetyl-2'-deoxyuridine (1)

5-Chloro-3',5'-di-*O*-acetyl-2'-deoxyuridine was synthesized according to described procedure,[1] starting from acetylated 2'-deoxyuridine (Sigma-Aldrich).

### 5-Chloro-2'-deoxy -5'-*O*-dimethoxytrityl-3'-*O*-*t*-butyldimethylsilyluridine (2)

5-Chloro-3',5'-di-*O*-acetyl-2'-deoxyuridine (**1**) (500 mg, 1.45 mmol) was treated with $NH_3$ (25%, 5 ml ) in MeOH (10 ml) for 3h. After evaporation to dryness the product was purified by flash chromatography using a gradient of MeOH in $CH_3Cl$ (eluting with 5% MeOH), TLC $R_f = 0.19$ ($CH_3Cl$: MeOH; 9:1).

5-Chloro-2'-deoxyuridine (368 mg, 1.4 mmol) was evaporated with pyridine and 20 ml of anhydrous pyridine was added followed by DMTCl (712 mg, 2.1 mmol). After 5h of stirring the mixture was evaporated to dryness and pure product (480 mg, 0.84 mmol) was received after purification by flash chromatography using a gradient of MeOH in $CH_3Cl$ (eluting with 2.5% MeOH), TLC $R_f = 0.3$ ($CH_3Cl$: MeOH; 95:5).

5-Chloro-2'-deoxy-5'-*O*-dimethoxytrityluridine (250 mg, 0.44 mmol) and imidazole (125 mg, 1.84 mmol) were dried in vacuo for 2h. Anhydrous DMF (1 ml) and t-butyldimethylsilyl chloride (120 mg, 0.8 mmol) were added and reaction mixture was left overnight at rt.

Saturated $NaHCO_3$ was added and the mixture was extracted with ethyl acetate, organic layer was washed with $H_2O$, dried with $MgSO_4$ and evaporated. Product **2** was purified by flash chromatography (eluting in $CH_3Cl$) (240 mg, 0.36 mmol), TLC $R_f$ = 0.57 ($CH_3Cl$: MeOH; 95:5).
$^1H$ NMR ($CDCl_3$): 8.04 (s, 1H); 7.26-7.41 (m, 9H); 6.85 (m, 4H); 6.27 (t, 1H); 4.48 (m, 1H); 4.01 (m, 1H); 3.79 (s, 6H); 3.38 (m, 2H); 2.38 (m, 1H); 2.28 (m, 1H); 0.84 (s, 9H); 0.04 (s, 3H); 0.03 (s, 3H); HRMS (ESI) *m/z* calcd for $C_{36}H_{43}ClN_2O_7SiNa$ ($[M+Na]^+$) 701.2426, found 701.2439.

**5-Chloro-2'-deoxy-5'-*O*-dimethoxytrityl-3'-*O*-*t*-butyldimethylsilyl-4-(1,2,4-triazol-1-yl)uridine (3)**

1,2,4-Triazole (5.83 mg, 8.44 mmol) was dried in vacuo for 3 h. Dry acetonitrile was added (1.75 ml) followed by triethylamine (1.95 ml) and flask was cooled in ice. Phosphoryl chloride (0.2 ml) was added and after stirring for 10 min at 0°C, compound **2** (350 mg, 0.52 mmol) in solution 1:2 of acetonitrile and pyridine (5 ml) was added and reaction mixture was left at rt overnight. Water (0.5 ml) was added and after 30 min mixture was evaporated, satd $NaHCO_3$ was added and residue was extracted with ethyl acetate. Organic layer was washed with $H_2O$, dried with $MgSO_4$ and evaporated to give product **3** (350 mg), TLC $R_f$ = 0.5 ($CH_3Cl$: MeOH; 9:1).
$^1H$ NMR ($CDCl_3$): 9.16 (s, 1H); 8.66 (s, 1H); 8.16 (s, 1H); 7.23-7.40 (m, 9H); 6.85 (m, 4H); 6.22 (t, 1H); 4.48 (m, 1H); 4.16 (m, 1H); 3.77 (s, 6H); 3.38 (m, 2H); 2.38 (m, 1H); 2.19 (m, 1H); 0.84 (s, 9H); 0.04 (s, 3H); 0.03 (s, 3H).

**5'-O-dimethoxytrityl-2'-deoxy-3'-*O*-[(2-cyanoethoxy),(N,N-diisopropylamino)phosphino]-4-(2-cyanoethylthio)-5-chlorouridine (4)**

Compound **3** (330 mg) was dissolved in dry acetonitrile (1.5 ml), 2-cyanoethanethiol (0.4 ml) and DIPEA (0.24 ml) were added and reaction mixture was left at rt for 1.5 h. After evaporation, $CH_2Cl_2$ was added and solution was washed with 0.5 M $KH_2PO_4$ and evaporated. Product **3_1** was purified by flash chromatography (eluting in $CH_3Cl$) (328 mg), TLC $R_f$ = 0.65 ($CH_3Cl$: MeOH; 95:5).
$^1H$ NMR ($CDCl_3$): 8.23 (s, 1H); 7.26-7.32 (m, 9H); 6.85 (m, 4H); 6.25 (t, 1H); 4.48 (m, 1H); 4.03 (m, 1H); 3.79 (s, 6H); 3.38 (m, 2H); 2.82 (m, 2H); 2.94 (m, 2H); 2.20 (m, 2H); 0.83 (s, 9H); 0.032 (s, 3H); 0.030 (s, 3H).
Product (328 mg, 0.44 mmol) was treated with $Et_3N{\times}3HF$ (0.275 ml) and was left for 3h. Pure product **3_2** (180 mg, 0.28 mmol) was received after purification by flash chromatography using a gradient of MeOH in $CH_3Cl$ (eluting with 1% MeOH), TLC $R_f$ = 0.32 ($CH_3Cl$: MeOH; 95:5).
$^1H$ NMR ($CDCl_3$): 8.14 (s, 1H); 7.26-7.40 (m, 9H); 6.84 (m, 4H); 6.19 (t, 1H); 4.52 (m, 1H); 4.14 (m, 1H); 3.79 (s, 6H); 3.40 (m, 4H); 2.89 (m, 2H); 2.85 (m, 1H); 2.45 (m, 1H).
Product (180 mg, 0.28 mmol) was dried in vacuo for 3 h. Anhydrous, freshly distilled THF (2 ml) was added in argon atmosphere followed by DIPEA (40µl) and chlorophosphine (2-cyanoethyl N,N-diisopropylchlorophosphoramidite) (25 µl). After 3h reaction was completed and methanol (0.5 ml), triethylamine (0.5 ml) and $CH_2Cl_2$ (30 ml) were added. Solution was washed with satd $NaHCO_3$, brine, dried over $MgSO_4$ and evaporated. Products (two diastereoizomers) were purified by flash chromatography using a gradient of $CH_2Cl_2$ (20-40%) in cyclohexane containing 2% of triethylamine (eluting with 25-30% $CH_2Cl_2$), TLC $R_f$ = 0.42 (**4a**), 0.48 (**4b**) ($CH_3Cl$: MeOH; 95:5). Yield 175 mg (0.21 mmole) of **4**.
$^{31}P$ NMR(CDCl3): 151.16, 150.60; HRMS (ESI) *m/z* calcd for $C_{42}H_{49}ClN_5O_7PSNa$ ($[M+Na]^+$) 856.2676, found 856.2675.

## 2) Methods

High-performance liquid chromatography (HPLC) was performed with an Agilent 1200 system with a binary gradient-forming module and diode-array UV–Vis and fluorescence detectors.

Steady-state photochemical irradiation experiments were carried out in 1 cm × 1 cm rectangular fluorescence cell with stirring bar on a standard optical bench system equipped with an Coherent Genesis CX-355-100 cw laser and with the temperature-controlled cell holder (Quantum Northwest, model TC125).

The melting curves and absorption spectra were measured on a JASCO V750 spectrophotometer equipped with a temperature controller. The melting temperatures were determined from the maximum of first derivative of the melting curves. Heating and cooling curves were registered and calculated $T_m$ values were in agreement within 2˚C. The range of temperature of the measurement was 10-90˚C, with step 0.5˚C /min.

## 3) Synthesis of oligonucleotides 5 and 6

Syntheses were performed on DNA/RNA Synthesizer H-6 (K&A Laborgeraete) applying 0.2 μmol protocol and commercial CPG supports.
Oligonucleotide **5** was synthesized using previously published method.[2] Oligonucleotide **6** was synthesized using standard phosphoramidite chemistry protocol.
High-performance liquid chromatography (HPLC) was performed with an Agilent 1200 system with a binary gradient-forming module and diode-array UV–Vis and fluorescence detectors.

Oligodeoxynucleotides were purified by reversed phase HPLC (Waters XBridge OST C18 Column, 2.5μm, 10 x 50 mm, 40°C with mobile phases: A = 0.01M $CH_3COONH_4$, B = 0.01M $CH_3COONH_4$/ acetonitrile, 50/50, v/v, with a diode array detector monitoring at 260 nm) using the following solvent gradient: 0% to 10%B in 10 minutes, flow rate: 1 ml/min. The residue was evaporated and dissolved in 0.7 ml $H_2O$ and passed through a NAP 25 column to remove $CH_3COONH_4$. The column was washed with 0.1M phosphate buffer (pH=7) and 7 fractions of 1.2 ml were collected and checked by UV. Fraction 3-5 containing oligomer were combined.

## 4) Irradiation experiments and isolation of photocrosslink 8

Oligonucleotide **5** was subjected to hybridization with small excess of **6** (1.2eq), ($A_{260}$ of **5**+**6** =2.3), by heating to 90°C and slow cooling to room temperature in an aqueous phosphate buffer (pH 7.0). Irradiations near UV light were carried out under aerobic conditions at 15°C with 80mW optical laser power. The progress of the reaction was monitored by absorption and fluorescence spectra (the reaction was carried out until no changes in spectra were noticed (120 min)) and by HPLC (all analyses were performed on a Agilent Poroshell 120 EC-C18 Column, 2.7μm, 4.6 × 150 mm, 40°C, eluted with 0.1M TEAA, using a linear gradient of 5%-15% of acetonitrile over 20 min, flow rate: 0.5 ml/min).

The reaction mixture was concentrated to small volume and photocrosslink **8** was separated by reversed phase HPLC (Agilent Poroshell 120 EC-C18 Column, 2.7μm, 4.6 x 150 mm, 40°C) with mobile phases: A = 0.1M $CH_3COONH_4$/ acetonitrile, 95/5, v/v, B = 0.1M $CH_3COONH_4$/ acetonitrile, 50/50, v/v with a diode array detector monitoring at 260 nm using the following solvent gradient: 0% to 15%B in 25 minutes, flow rate: 0.5 ml/min. The residue was evaporated and passed through Waters XBridge OST C18 Column, 2.5μm, 10 x 50 mm, 40°C, with mobile phases: A = 0.01M phosphate buffer (pH = 7), B = acetonitrile/water, 80/20, v/v with a diode array detector monitoring at 260 nm using the following solvent gradient: 0% to 20%B in 12 minutes, flow rate: 1.5 ml/min to remove $CH_3COONH_4$.

## 5) Thermal instability of photocrosslink 7

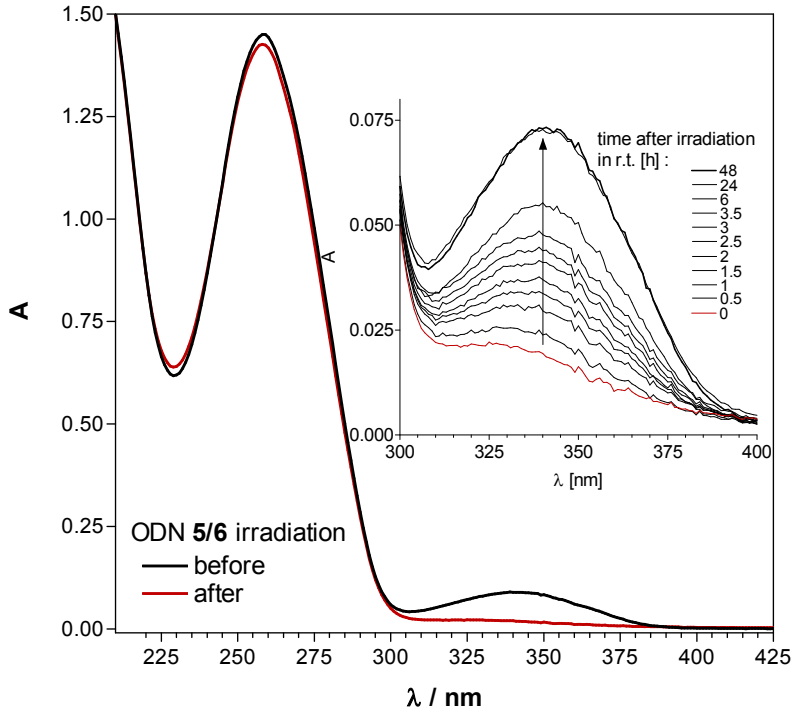


**Figure 1** Changes in absorption spectra of duplex ODN **5/6** after 4.5 min of irradiation and during next 48 hours in room temperature (inset). Conditions: 25°C, 0.1 M phosphate buffer, pH 7.0.

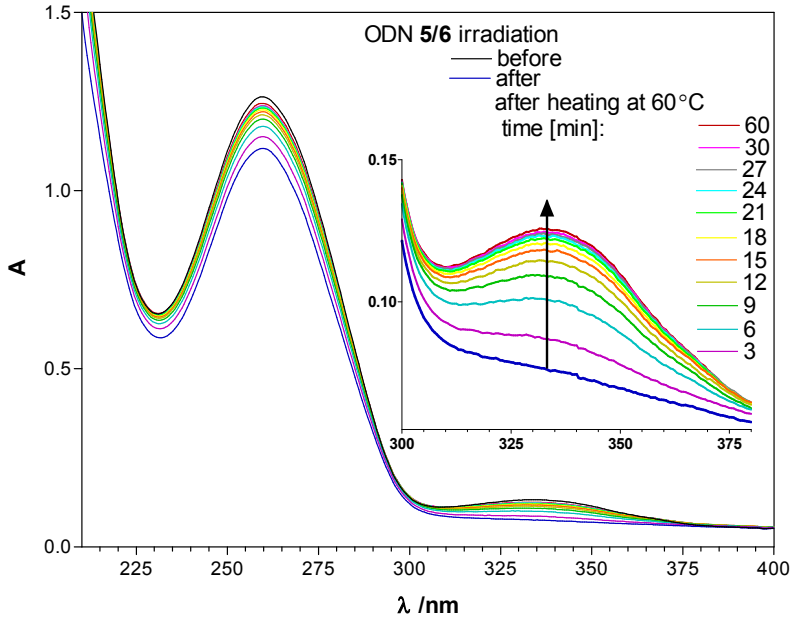


**Figure 2** Changes in the absorption spectra of duplex ODN **5/6** after 4.5 min of irradiation and during heating in 60˚C for following 60 min. Conditions: 60°C, 0.1 M phosphate buffer, pH 7.0.

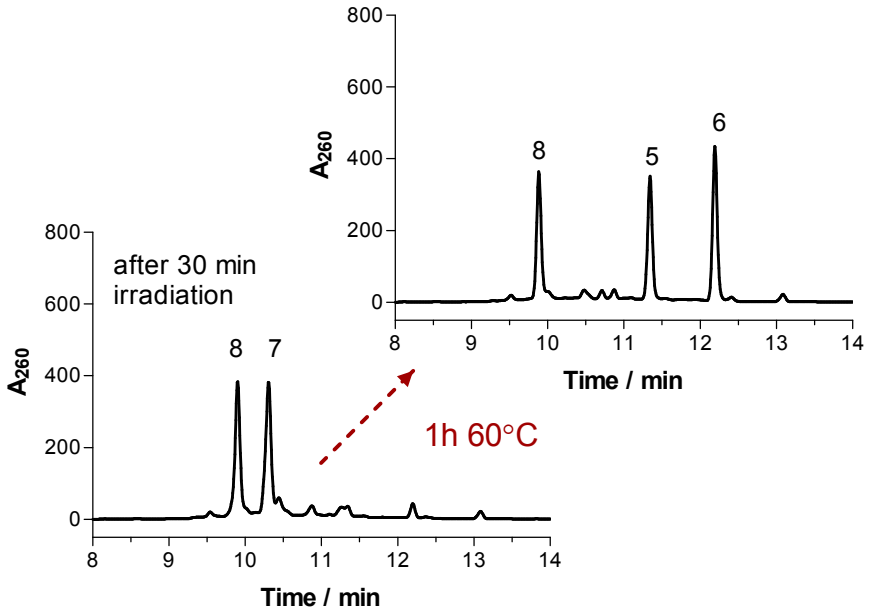


**Figure 3** HPLC analysis of the reaction mixture containing ODN **5** and ODN **6** after 30 min of irradiation (80 mW, 355 nm, 15 °C), the inset shows HPLC analysis after 30 min of irradiation and 1h of heating of irradiated solution at 60°C.

**Table 1** Area of peaks obtained in HPLC analyzes.

| | Area of peak at 260 nm | | |
|---|---|---|---|
| | **5** | **6** | **7** (without heating) |
| Before irradiation | 3226 | 4029 | 0 |
| After 4.5 min of irradiation and 1h in 60˚C | 2701 | 3209 | 3436 |
| After 30 min of irradiation and 1h in 60˚C | 1626 | 1888 | 2108 |

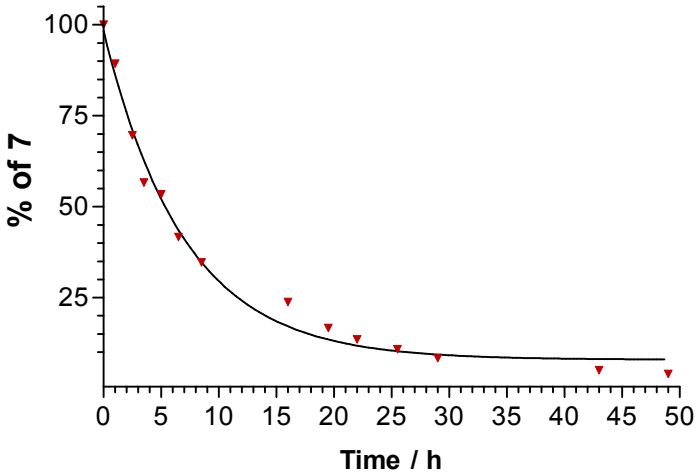


**Figure 4** Thermal reversion of interstrand photo-cross-linked DNA oligonucleotide **7** to starting oligonucleotides **5**+**6**. Conditions: 25°C, 0.1 M phosphate buffer, pH 7.0.

## 6) Melting temperature measurements

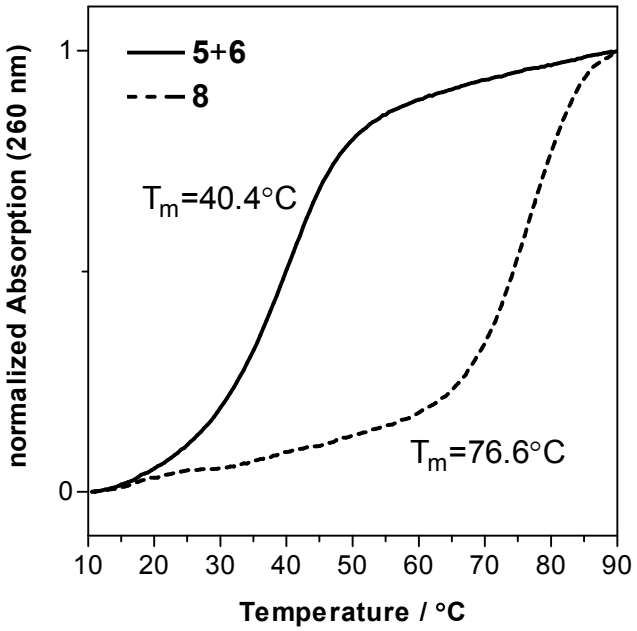


**Figure 5** Melting profiles of duplex ODN **5/6** before (solid line) and after (dotted line) irradiation (photocrosslink **8**). Conditions: 2 μM duplex, 0.1M phosphate buffer, pH=7.0.

## 7) Enzymatic digestion of photocrosslink 8

0.2 OD of photocrosslink **8** in 150 μl of buffer (10mM $KH_2PO_4$, 10mM $MgCl_2$, pH=7) was digested with alkaline phosphatase bovine intestinal mucosa (27 DEA units, Sigma-Aldrich, BioUltra) and phosphodiesterase I from Crotalus adamanteus venom (0.0055 units, Sigma-Aldrich, Purified) for 20h at 37˚C.

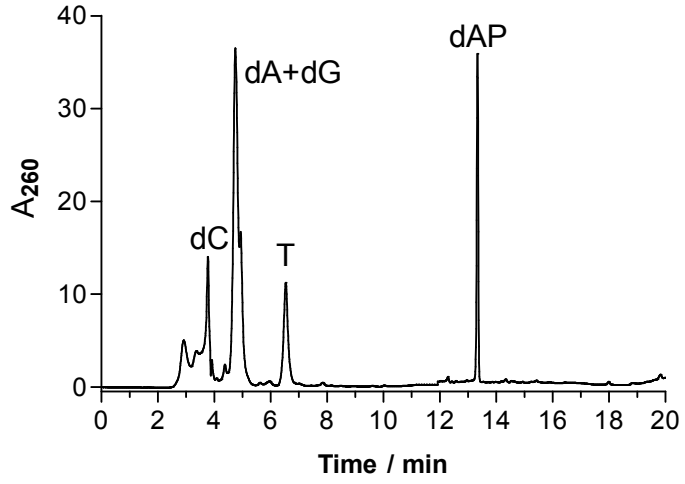


**Figure 6** HPLC analysis of photocrosslink **8** after digestion (Agilent Poroshell 120 EC-C18 Column, 2.7μm, 4.6 x 150 mm, 40˚C) with mobile phases: A = 0.1M $CH_3COONH_4$/ acetonitrile, 95/5, v/v, B = 0.1M $CH_3COONH_4$/ acetonitrile, 50/50, v/v with a diode array detector monitoring at 260 nm using the following solvent gradient : 0% to 15%B in 10 minutes, than to 100%B in 10 minutes, flow rate: 0.7 ml/min.

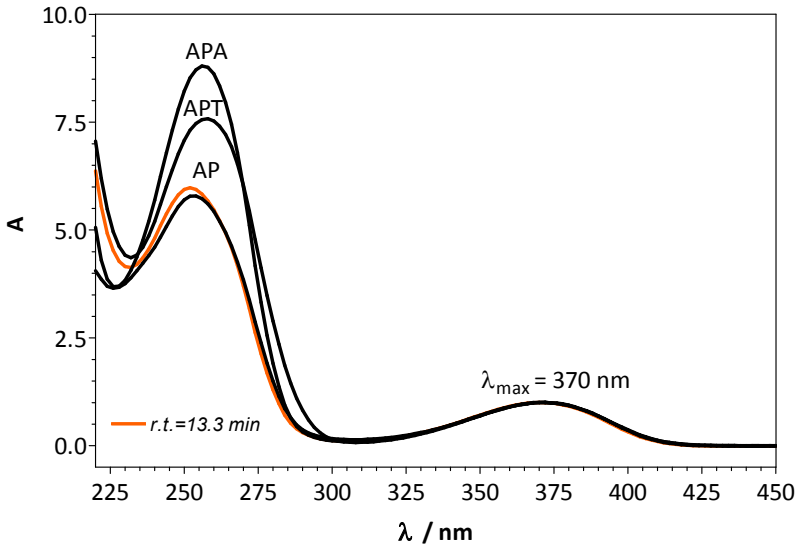


**Figure 7** Comparison of UV absorption spectra of fragments APA, APT, AP containing photoadduct **P** [3] with spectrum of undigested fragment of photocrosslink **8** (orange).

## 8) Spectra

$^{1}H$ and $^{31}P$ NMR spectra were recorded on Varian 300 MHz Mercury system in $CH_3Cl$. All chemical shifts are reported in parts per million relative to TMS ($^{1}H$) or 85% $H_3PO_4$ ($^{31}P$).

The MALDI-TOF MS analyses were performed using the MALDI-TOF MS instrument model Autoflex II equipped with a reflectron (resolution about 5000 at m/z 1000), on a MALDI metal target plate (Bruker, Bremen, Germany). The instrument was equipped with a SmartBeam laser and operated under FlexControl. Spectra were calibrated in FlexAnalysis using the Protein Calibration Standard I from Bruker and 3-hydroxypicolinic acid was used as matrix.

High resolution ESI mass spectra (HRMS) were obtained using Impact HD mass spectrometer (Q-TOF type instrument equipped with electrospray ion source; Bruker Daltonics, Germany). The sample solutions (DCM:MeOH) were infused into the ESI source by a syringe pump (direct inlet) at the flow rate of 3 µL/min. The instrument was operated under the following optimized settings: end plate voltage 500 V; capillary voltage 4.2 kV; nebulizer pressure 0.3 bar; dry gas (nitrogen) temperature 200 °C; dry gas flow rate 4 L/min. The spectrometer was previously calibrated with the standard tune mixture.

## 9) References

[1] J. Asakura, M. J. Robins, *J. Org. Chem*. **1990**, *55*, 4928-4933.
[2] J. Milecki, J. Nowak, B. Skalski, S. Franzen, *Bioorgan. Med. Chem.* **2011**, *19*, 6098–6106.
[3] J. Nowak-Karnowska, Z. Chebib, J. Milecki, S. Franzen, B. Skalski, *ChemBioChem* **2014**, *15*, 2045 – 2049.

Display Report

**Analysis Info**

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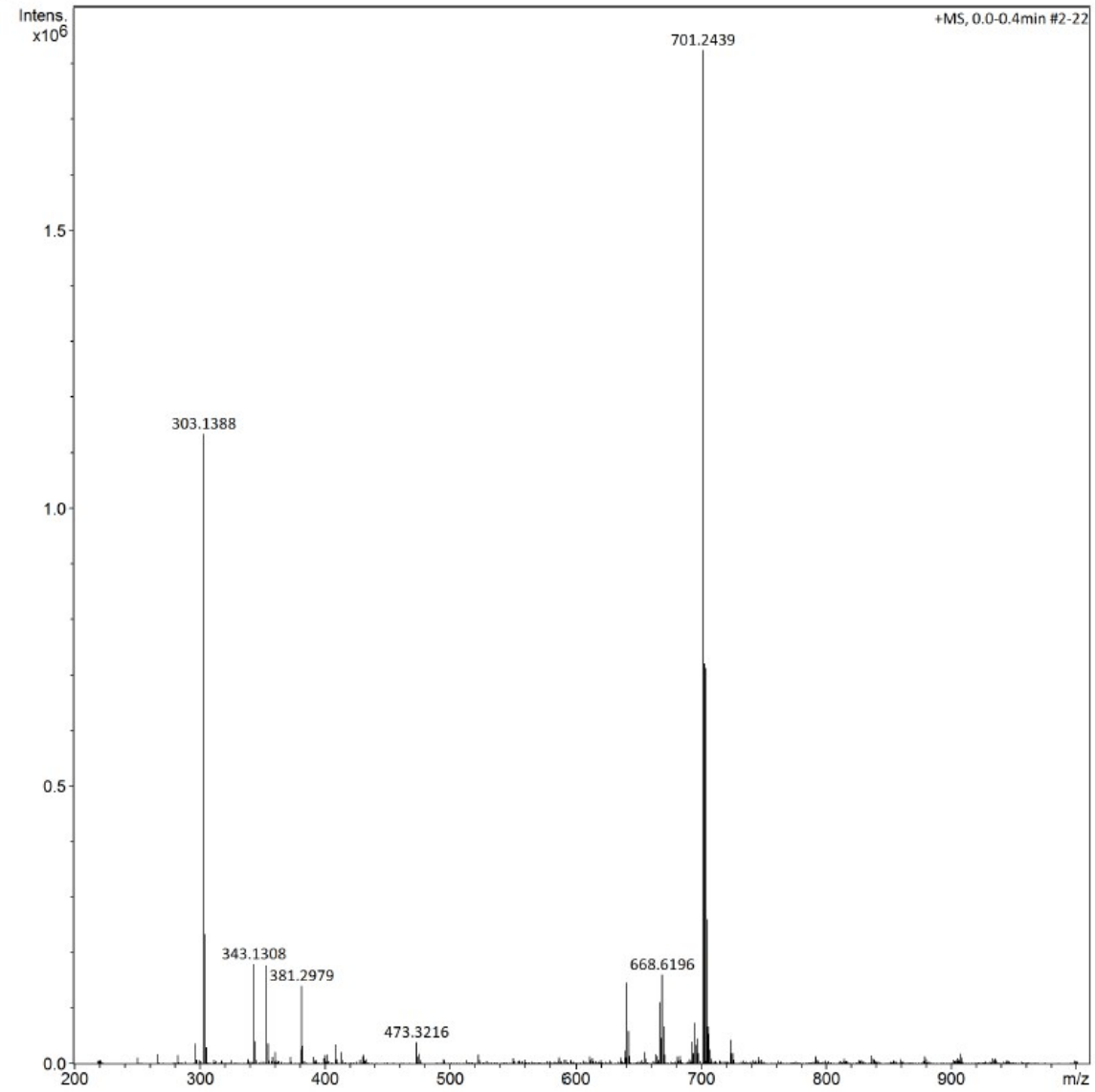


**Figure 8** ESI MS spectrum of **2**.

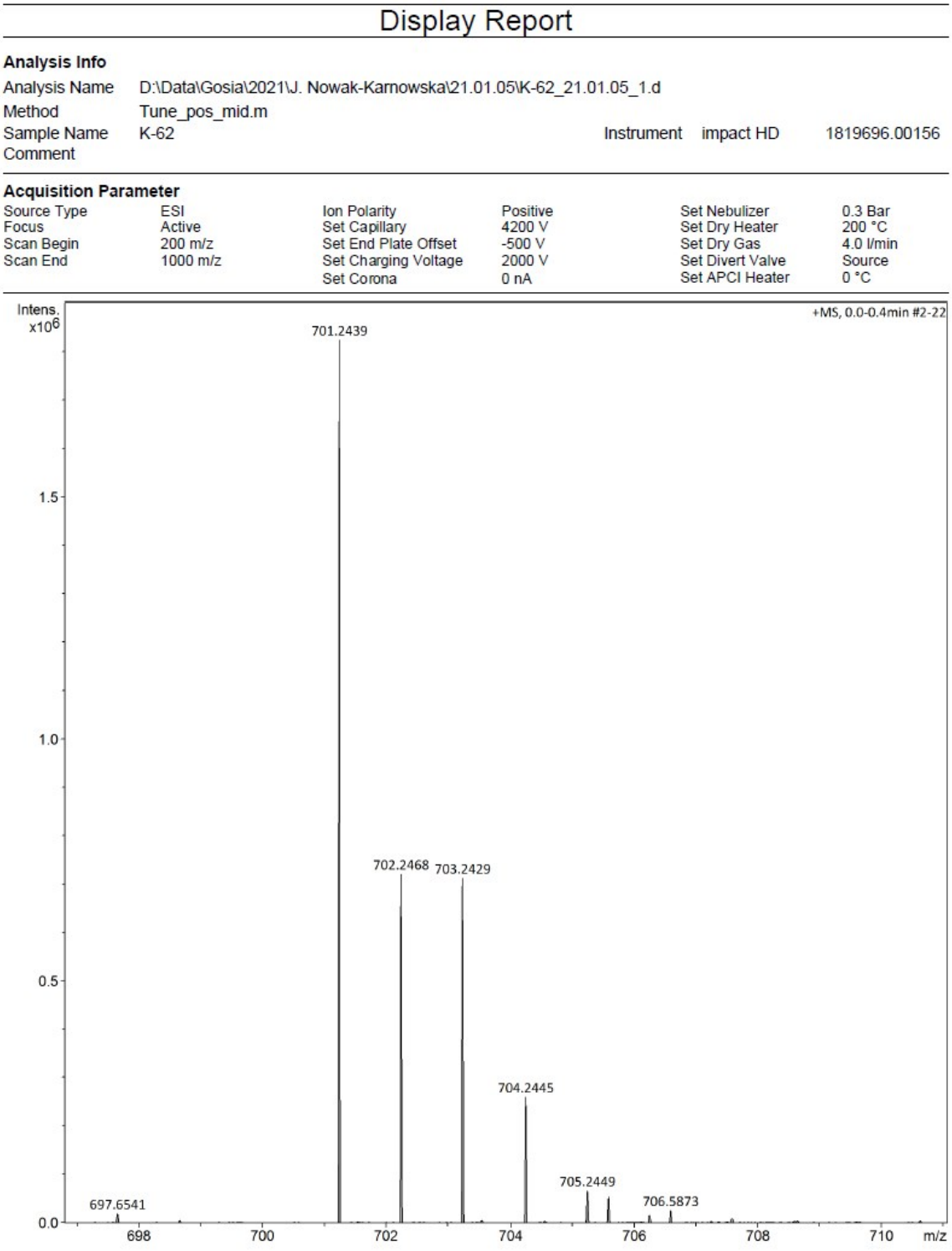


**Figure 9** ESI MS spectrum of **2 (**zoom around m/z 701).

Display Report

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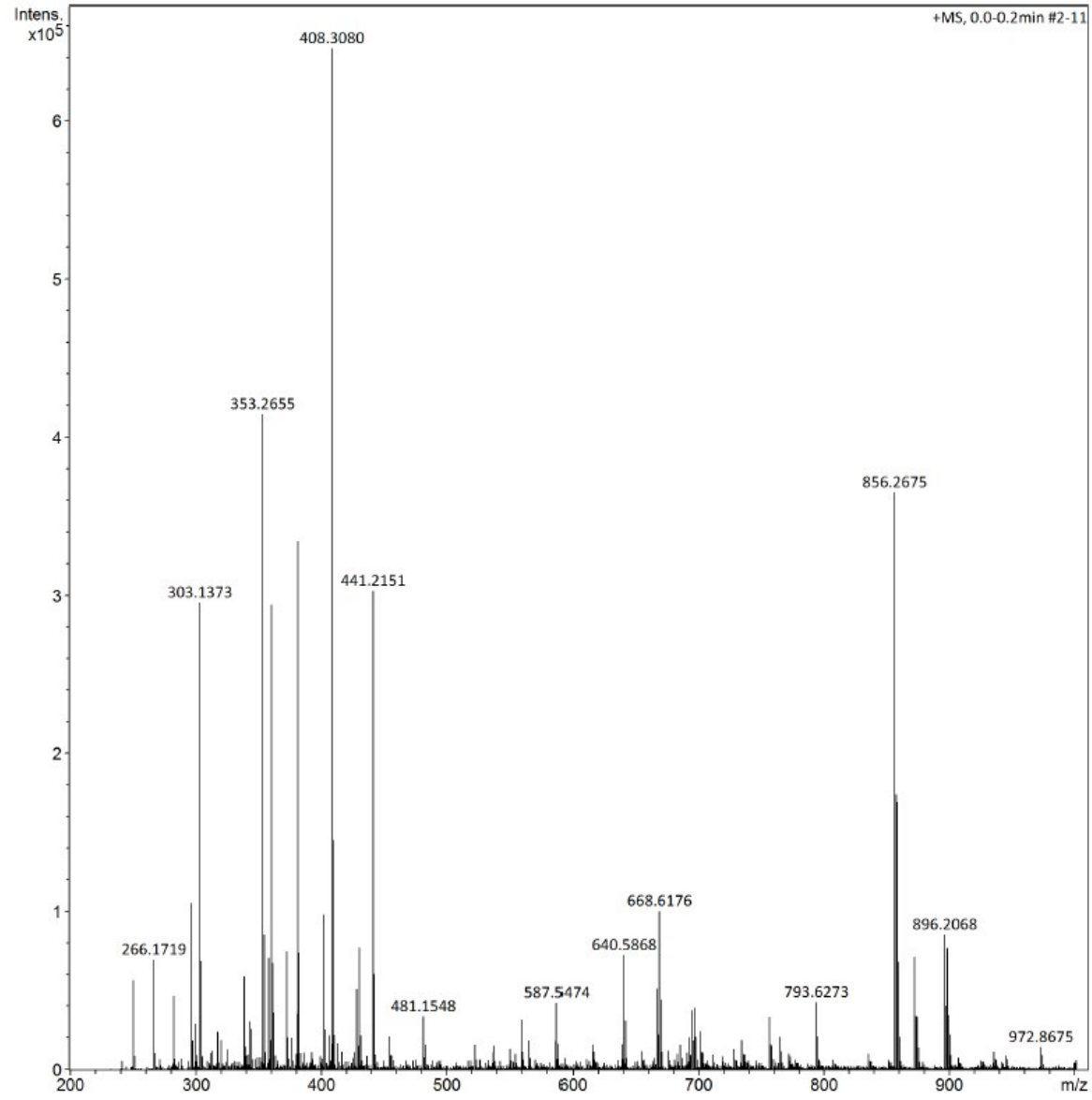


**Figure 10** ESI MS spectrum of **4**.

Display Report

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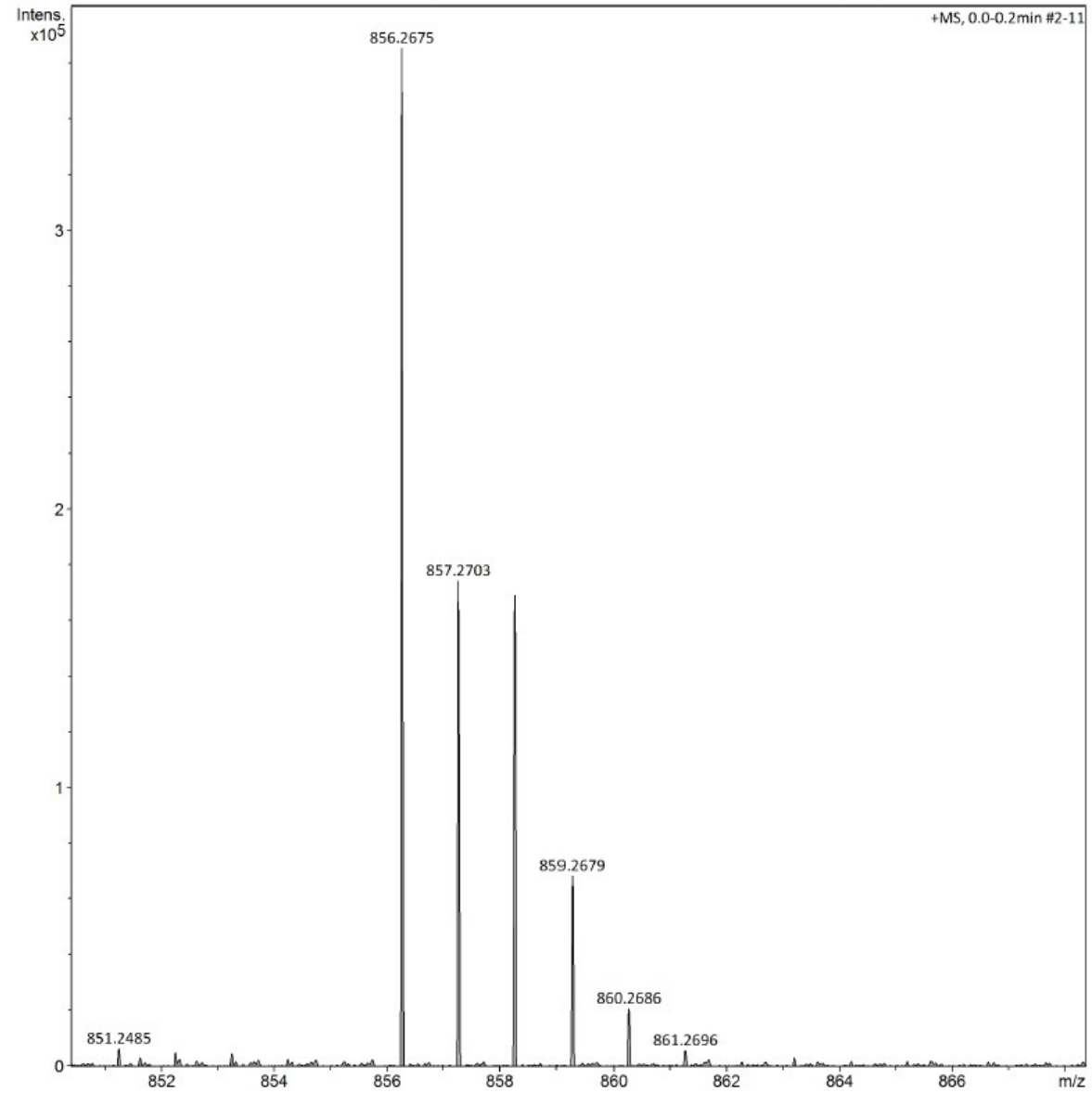


**Figure 11** ESI MS spectrum of **4 (**zoom around m/z 856).

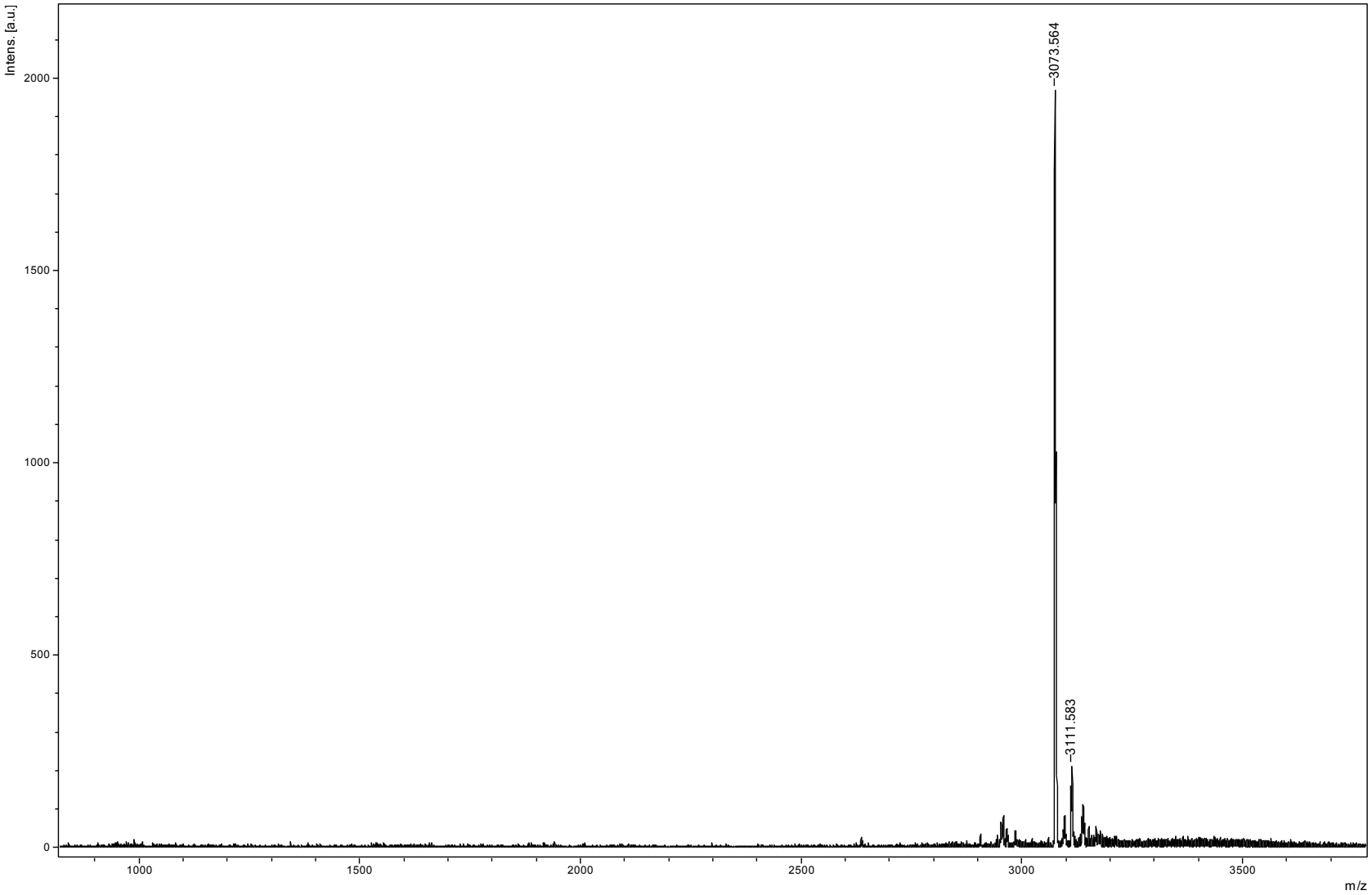


**Figure 12** Maldi TOF spectrum of oligonucleotide **5**.
MW=3072.5 (3073.5 calcd for $[M+H]^+$, 3111.5 calcd for $[M+K]^+$)

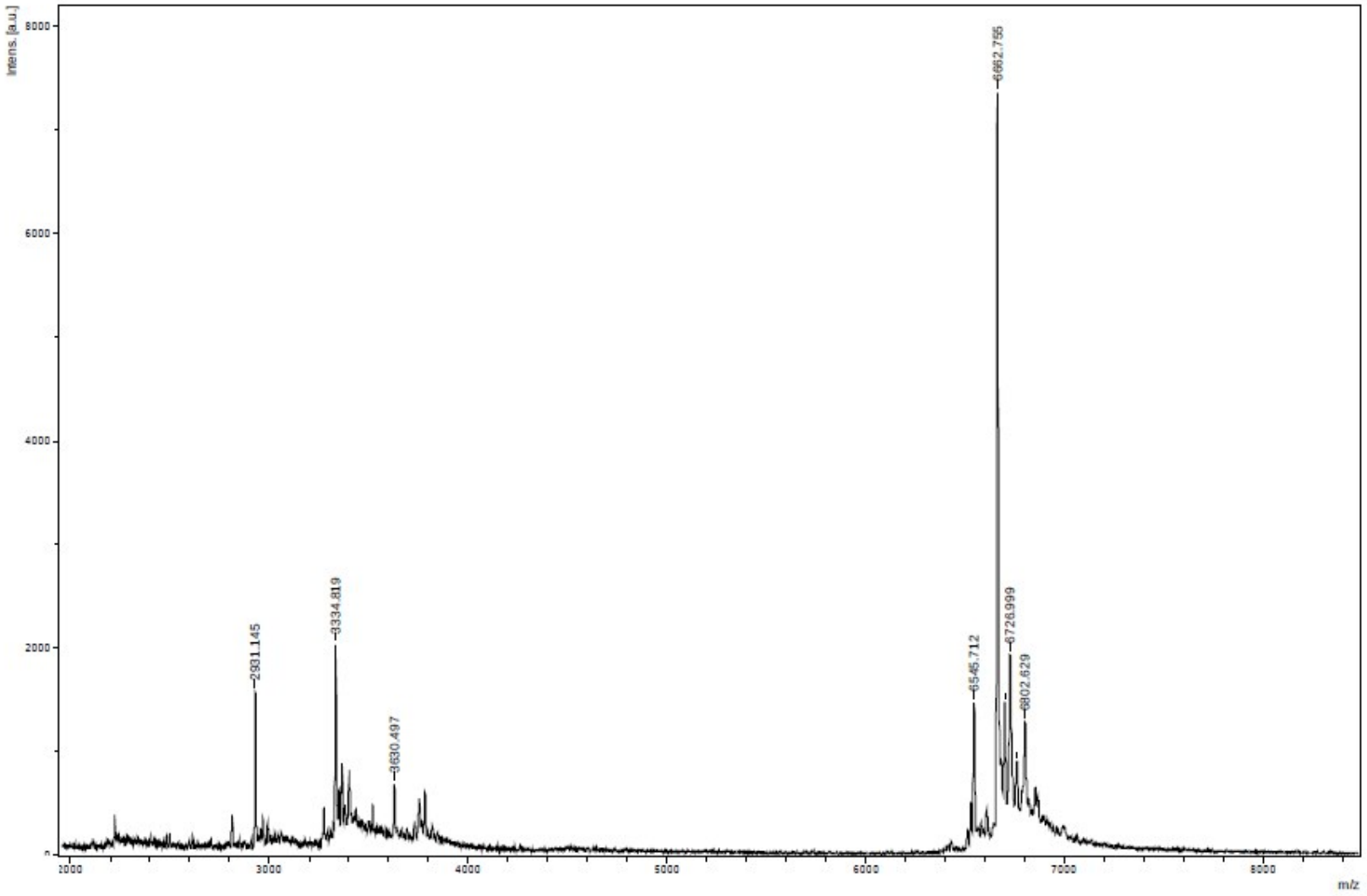


**Figure 13** Maldi TOF spectrum of photocrosslink **8**.
MW=6662.7 (6662.4 calcd for $[M+H]^+$)

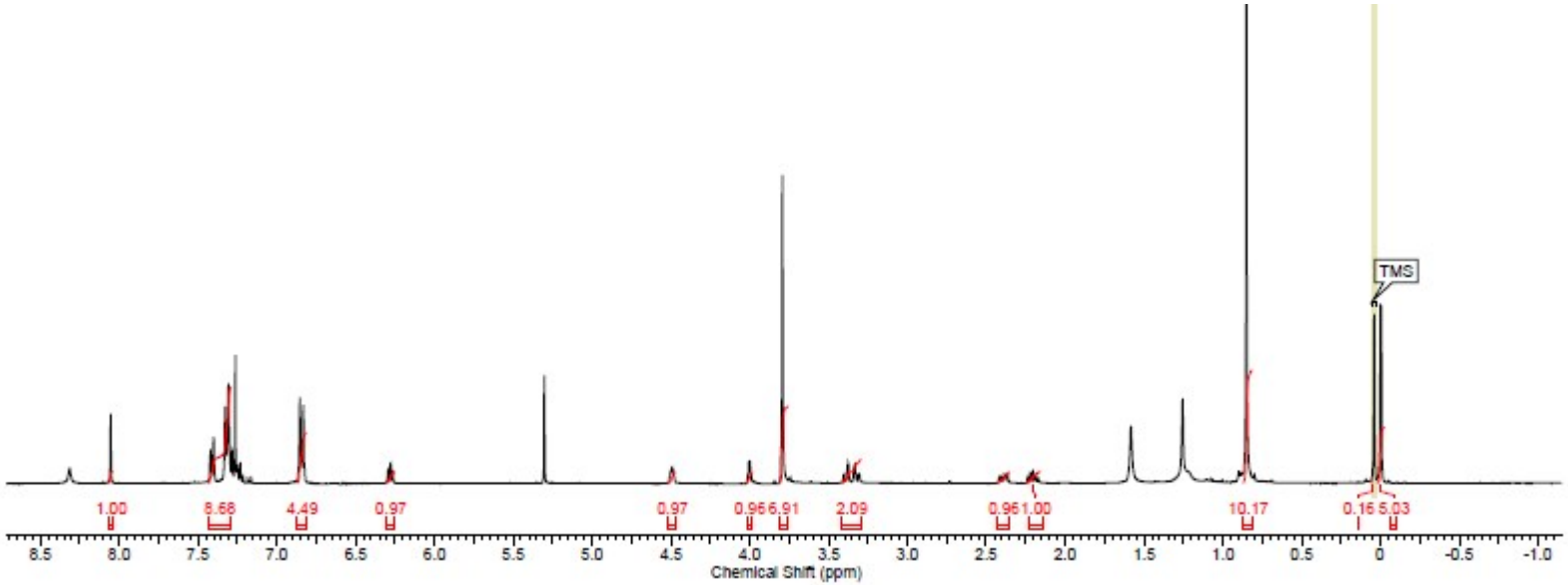


**Figure 14** $^{1}$H NMR spectrum of **2**.

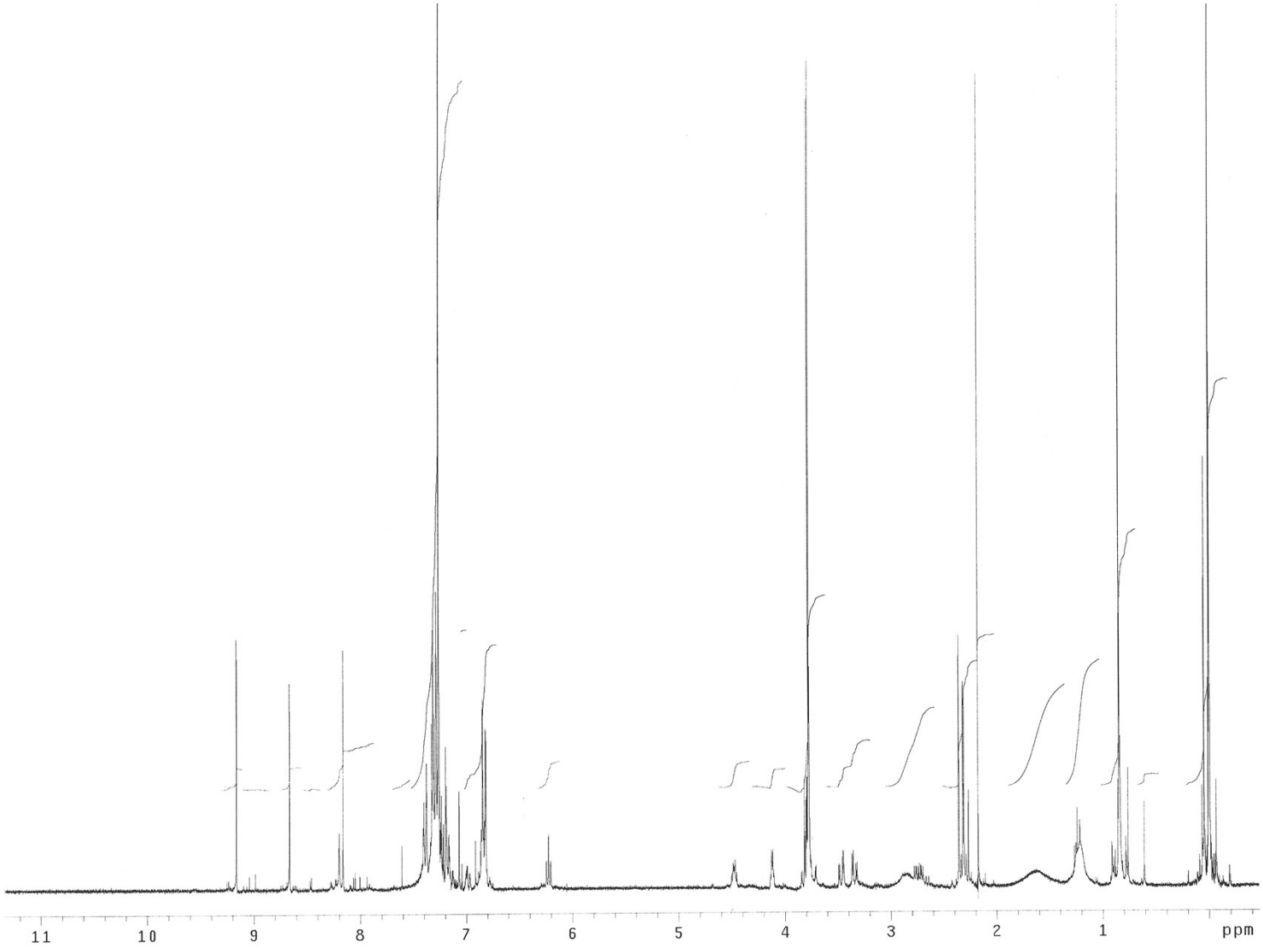


**Figure 15** $^{1}$H NMR spectrum of **3**.

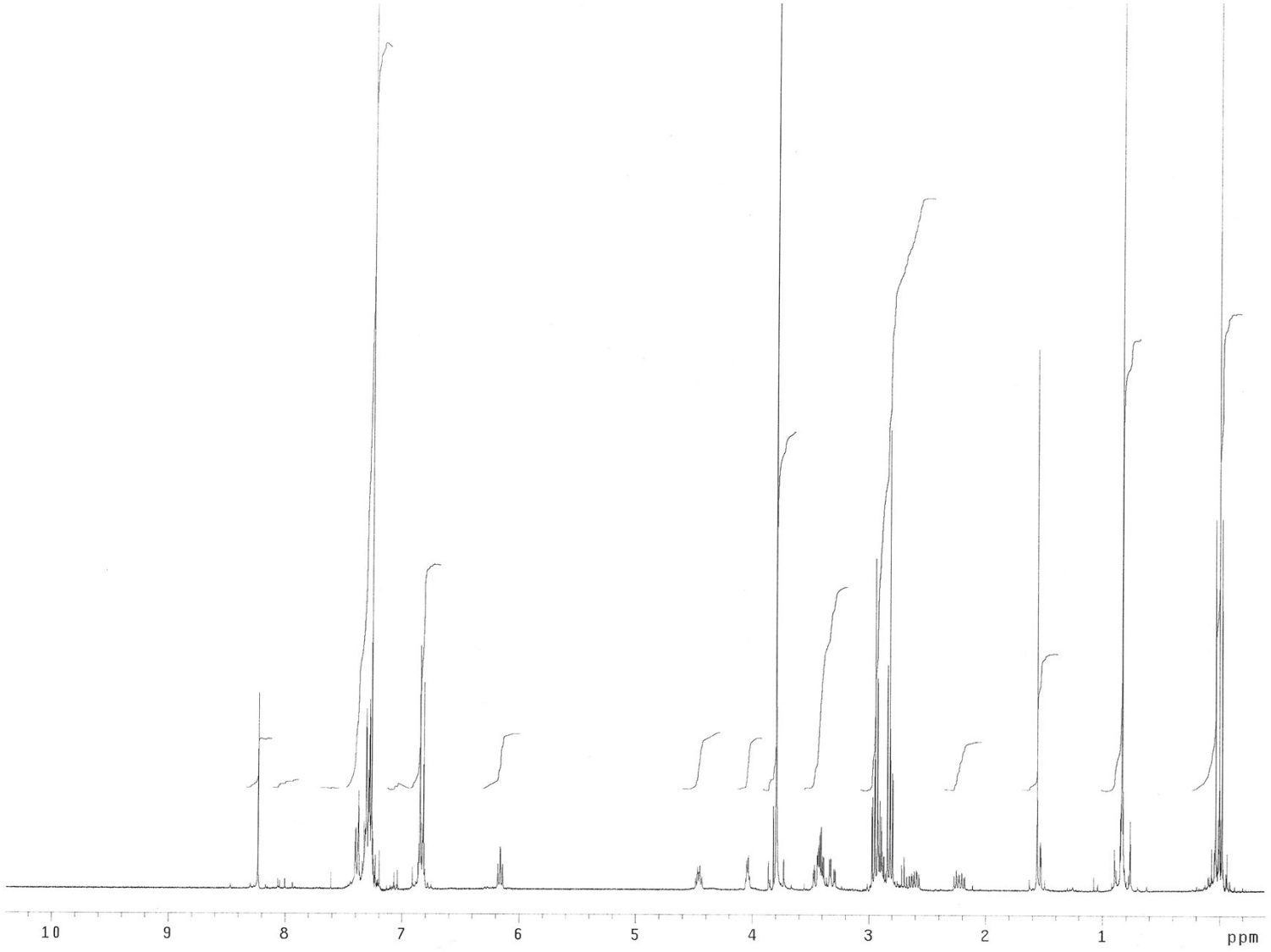


**Figure 16** $^{1}$H NMR spectrum of **3_1**.

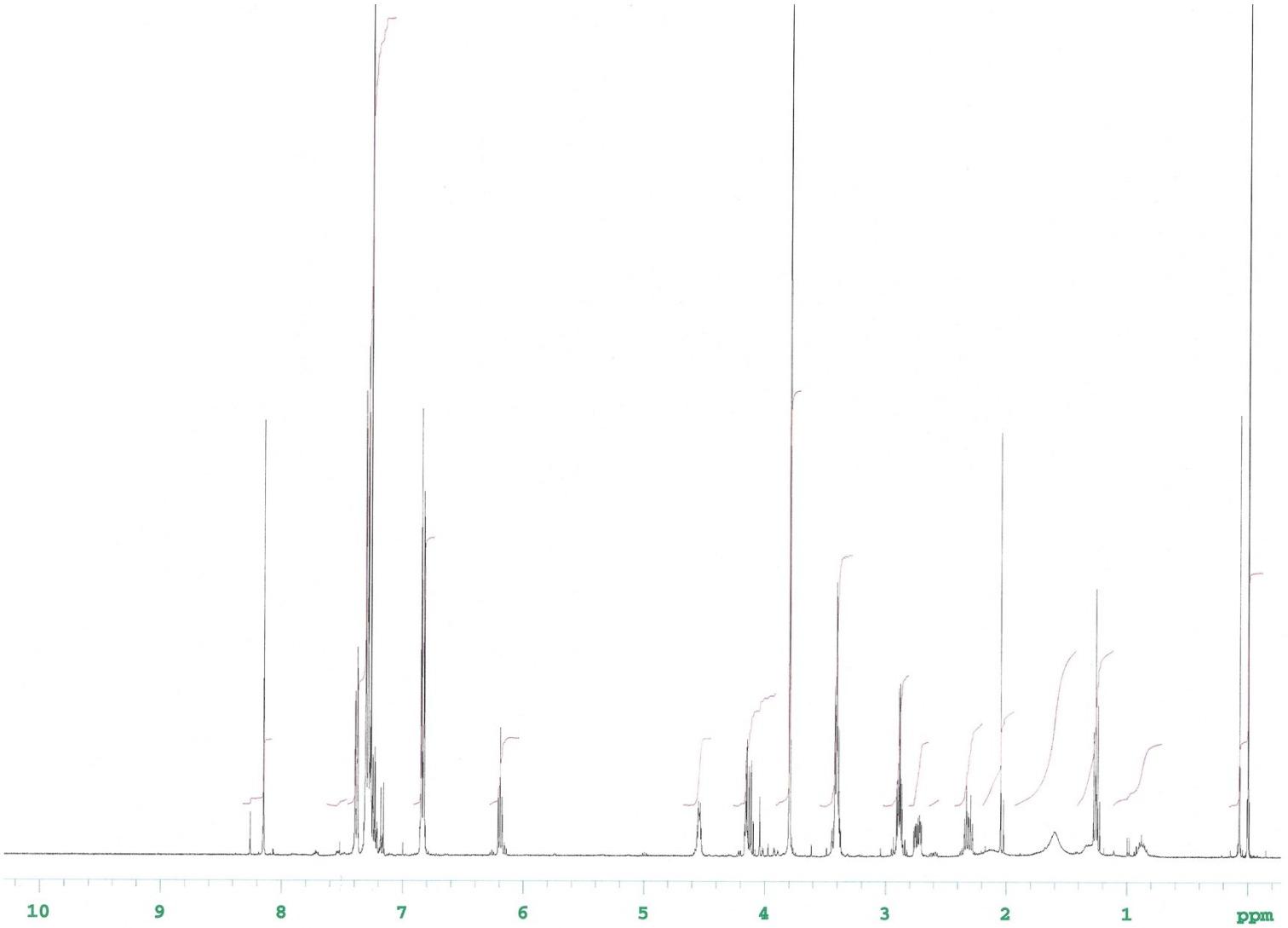


**Figure 17** $^{1}$H NMR spectrum of **3_2**.

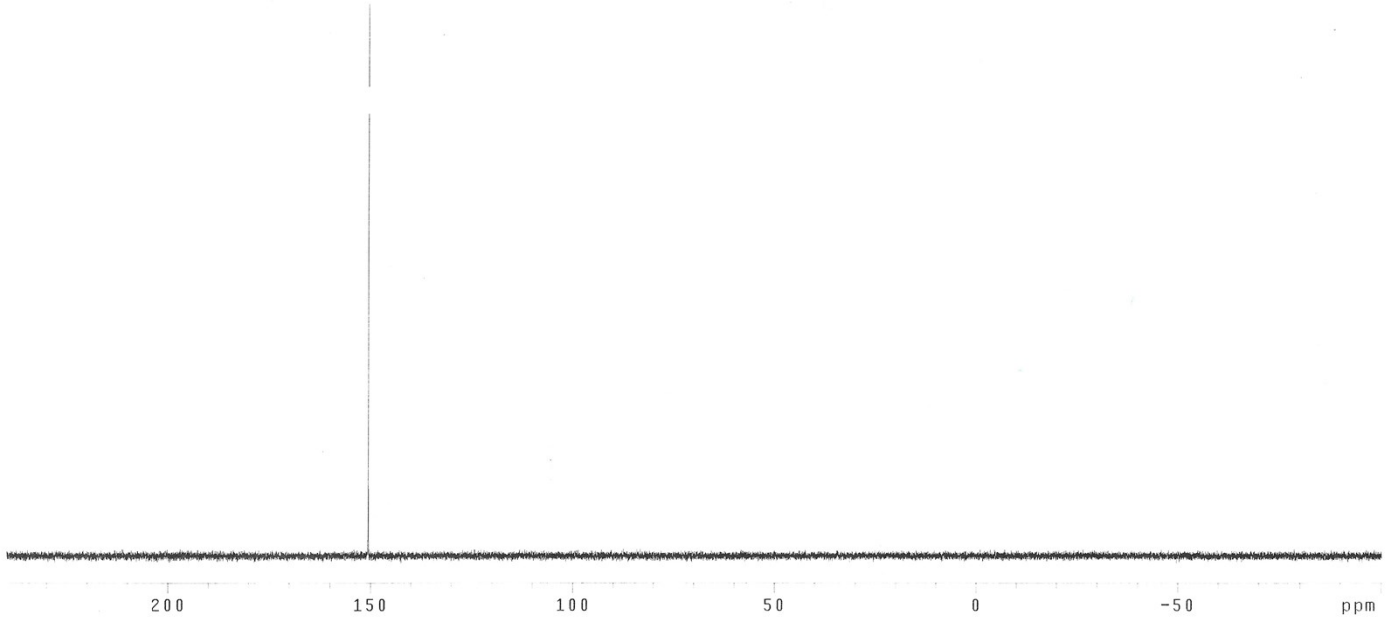


**Figure 18** $^{31}$P NMR spectrum of **4a**.

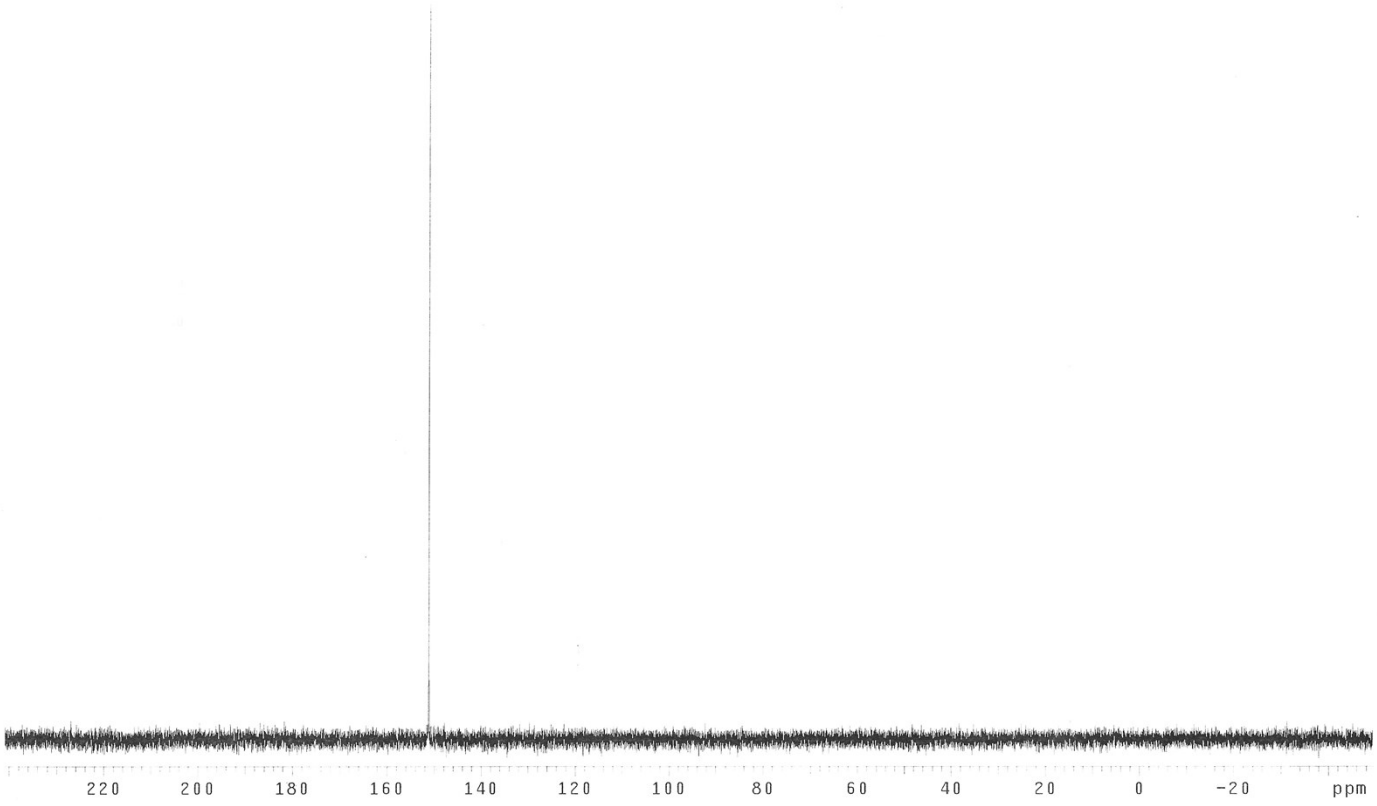


**Figure 19** $^{31}$P NMR spectrum of **4b**.