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Synthesis of allobetulin-based asialoglycoprotein receptor-targeted glycoconjugates

Egor I. Seleznev, Emil Yu. Yamansarov, Elena V. Lopatukhina, Anton V. Lopuhov, Dmitry A. Skvortsov, Sergei A. Evteev, Elvira T. Yamansarova, Amina Yu. Adelgareeva, Natalia L. Klyachko, Elena K. Beloglazkina, Yan A. Ivanenkov and Alexander G. Majouga

1. Chemistry

General: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 MHz spectrometer in $CDCl_3$. Chemical shifts (δ) in ppm are reported relative to the residual signals of chloroform-*d* (7.25 for ^{1}H NMR and 77.0 for ^{13}C NMR) or methanol-d_4 (3.31 for ^{1}H NMR and 49.0 for ^{13}C NMR) as internal references. The coupling constants (*J*) are given in Hertz. IR spectra were recorded on Thermo Nicolet IR 200. Melting points were determined with automated melting point system OptiMelt. Optical rotations were measured on High-precision automatic polarimeter A.KRUSS Optronic® R-800. Combustion analysis was performed with Vario-II Elemental Analyzer. ESI-HRMS spectra were measured with ThermoScientificOrbitrapElite. The silica gel used for column chromatography was MN Kieselgel 60 0.04-0.063 mm/230-400 mesh ASTM. TLC analysis was performed on Merck TLC Silica gel 60 F254 plates, and the solvent system was CH_2Cl_2 for compounds **5** or CH_2Cl_2-MeOH (5:1) for compounds **6-8**. Compounds were detected by H_2SO_4 solution (10%) with subsequent heating at 100-120°C for 2-3 minutes. Organic chemicals with purity no less than 95% were used as purchased. Solvents were purified routinely and freshly distilled prior to use. Hex-5-ynoyl chloride[S1], azido sugars **2-4**[S2] and allobetulin **1**[S3] were obtained according to published literature.

Synthesis of compound 5

Hex-5-ynoyl chloride (0.2 g, 1.5 mmol, 1.5 eq.) and DMAP (cat. amounts) were added dropwise to a solution of allobetulin **1** (0.44 g, 1 mmol) in anhydrous pyridine (10 ml). The mixture was refluxed for 12 h, poured into 2 M HCl solution (150 ml), and the precipitate was filtered off, washed with water until neutral pH, air dried, and purified by column chromatography on silica gel using dichloromethane as the eluent.

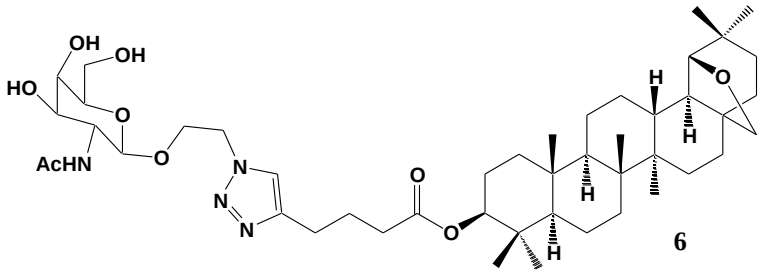
3β-O-(Hex-5-ynoyl)-19β,28-epoxy-18*α*-oleane 5

Yield: 0.47 g, 88%, as a white solid. m.p. 273 °C. R_f 0.46. $[\alpha]_D^{20}$ +35.0° (c 0.02, $CHCl_3$). ν_{max} (cm^{-1}, ZnSe) 3286, 2942, 2925, 2851, 1722, 1447, 1384, 1252, 1232, 1186, 1165, 1139, 1032, 1007, 970. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.49 (t, 1H, H-3), 3.77 (d, *J* = 7.7 Hz, 1H, H_a-28), 3.52 (s, 1H, H-19), 3.44 (d, *J* = 7.7 Hz, 1H, H_b -28), 2.44 (t, *J* = 7.3 Hz, 2H, CH_2), 2.26 (td, *J* = 6.9, 2.5 Hz, 2H, CH_2), 1.97 (t, *J* = 2.4 Hz, 1H, -C≡<u>CH</u>), 1.89 – 1.81 (m, 2H, CH_2), 1.76 – 1.03 (m, 24H, CH, CH_2), 0.97 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.84 (2s, 6H, $2CH_3$), 0.79 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.8 (-CH_2(O)<u>C</u>O-), 87.9 (C-19), 82.0 (-<u>C</u>≡CH), 80.9 (C-3), 71.3 (C-28), 69.1 (-C≡<u>CH</u>), 55.6, 51.0, 46.8, 41.5, 40.7, 40.6, 38.6, 37.9, 37.2, 36.7, 36.3, 34.1, 33.8, 33.4, 32.7, 28.8, 28.0,

26.4, 26.3, 24.5, 23.8, 23.7, 21.0, 20.9, 18.1, 17.9, 16.6, 16.5, 15.7, 13.5. Anal. Calcd. for $C_{36}H_{56}O_3$ (Mr 536.42): C, 80.54; H, 10.51. Found: C 80.58, H 10.37.

Synthesis of compound 6-8

Azido sugar **2-4** (1.2 mmol, 1.2 eq.), DIPEA (cat. amounts) and CuI (cat. amounts) were added to a solution of alkyne **5** (0.53 g, 1 mmol) in dry DMF (5 ml), and this was stirred in ambient conditions under argon atmosphere overnight. The mixture was evaporated under reduced pressure and co-evaporated with toluene twice. The crude residue was purified by column chromatography on silica gel using mixture of CH_2Cl_2 and MeOH (10:1) as the eluent.



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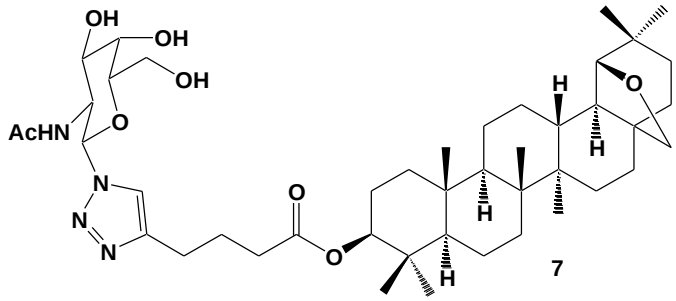
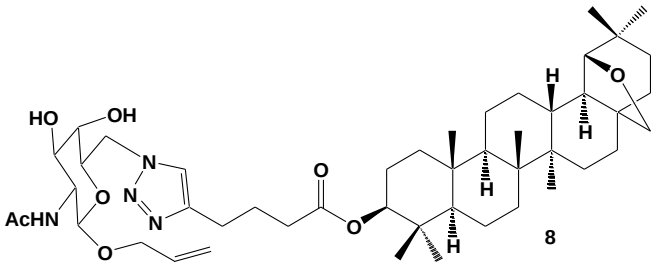
2-{4-[4-(19β,28-Epoxy-18α-olean-3α-yloxy)-4-oxobutyl]-1*H*-1,2,3-triazol-1-yl}ethyl 2-acetamido-2-deoxy-D-galactopyranoside 6. Yield 0.5 g (64%), white solid. m.p. 163°C. R_f 0.28. $[\alpha]_D^{20}$ -5.0° (*c* 0.02, $CHCl_3$ – CH_3OH 1:1). ν_{max} (cm^{-1}, ZnSe) 3330, 2927, 2856, 1726, 1654, 1452, 1376, 1270, 1120, 1072, 1033, 1008, 980. ^1H NMR (400 MHz, CD_3OD): δ 8.14 (s, 1H, -N-CH=C-), 7.65 (m, 1H, NHAc), 4.65 (d, *J* = 8.4 Hz, 1H, -O-CH(O)-), 4.48 (dd, *J* = 10.7, 5.6 Hz, 1H, H-3), 4.37 (s, 1H, CH), 4.25 (m, 1H, CH), 4.21 (m, 3H, CH, CH_2), 3.94 (m, 2H CH_2), 3.83 (d, *J* = 3.2 Hz, 1H, CH), 3.78 (m, 2H, CH_2), 3.73 (m, 1H, H_a-28), 3.55 (s, 1H, H-19), 3.47 (d, *J* = 7.7 Hz, 1H, H_b-28), 2.82 (m, 2H, CH_2), 2.43 (m, 2H, CH_2), 2.03 (m, 2H, CH_2), 1.94 (s, 3H, NHAc), 1.79–1.20 (m, 27H, CH, CH_2), 1.01 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.90 (s, 6H, $2CH_3$), 0.86 (s, 6H, $2CH_3$), 0.82 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 172.8 (-CH_2(O)CO-), 167.7 (NHAc), 130.6 (-N-CH=C-), 128.1 (-N-CH=C-), 101.1 (C-1'), 87.8 (C-19), 80.8 (C-3), 75.0, 71.2, 70.5, 67.8, 67.5, 66.1, 60.8, 55.1, 50.5, 49.5, 48.9, 46.3, 40.9, 40.1, 40.1, 38.3, 37.2, 36.6, 35.9, 35.5, 33.8, 33.2, 32.0, 31.7, 29.8, 28.4, 27.7, 27.0, 25.8, 23.3, 23.2, 22.3, 20.5, 17.5, 15.5, 14.7, 12.9, 12.5, 9.9. ESI-HRMS m/z: $[M-H]^-$ Calcd for $C_{46}H_{74}N_4O_9$ 825.5383; Found 825.5378.



7

19β,28-Epoxy-18α-olean-3α-yl 4-[1-(2-acetamido-2-deoxy-D-galactopyranosyl)-1*H*-1,2,3-triazol-4-yl]butanoate 7. Yield 0.52 g (73%), white solid. m.p. 132 °C. R_f 0.35. $[\alpha]_D^{20}$ +32° (*c* 0.02, $CHCl_3$ – CH_3OH 1:1). ν_{max} (cm^{-1}, ZnSe) 2942, 2924, 2865, 2852, 1721, 1660, 1644, 1548, 1463, 1442, 1366, 1304, 1246, 1123, 1095, 1033, 1009, 961, 890, 884. ^1H NMR (400 MHz, CD_3OD): δ 7.85 (s, 1H, -N-CH=C-), 7.54 (m, 1H, NHAc), 5.66 (d, *J* = 9.7 Hz, 1H, -O-CH(N)-), 4.45 – 4.38 (m, 1H, H-3), 4.38 (d, *J* = 8.3 Hz, 1H, CH), 4.11 (m, 2H, CH), 4.02 (d, *J* = 2.9 Hz, 1H, CH), 3.80 (m, 2H, CH_2), 3.68 (d, *J* = 7.7 Hz, 1H, H_a-

28), 3.45 (s, 1H, H-19), 3.36 (d, *J* = 7.8 Hz, 1H, H_b-28), 2.69 – 2.63 (m, 2H, CH_2), 2.28 (t, *J* = 7.4 Hz, 2H, CH_2), 1.89 (t, *J* = 6.5 Hz, 2H, CH_2), 1.73 (s, 3H, NHAc), 1.66–1.11 (m, 27H, CH, CH_2), 0.88 (s, 3H, CH_3), 0.82 (s, 6H, $2CH_3$), 0.78 (s, 3H, CH_3), 0.74 (2s, 6H, $2CH_3$), 0.71 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 172.6 (-CH_2(O)CO-), 167.5 (NHAc), 130.5 (-N-CH=C-), 128.3 (-N-CH=C-), 101.5 (C-1'), 87.7 (C-19), 81.1 (C-3), 76.9, 70.8, 67.8, 60.5, 55.1, 50.5, 46.3, 41.7, 41.0, 40.2, 40.1, 38.2, 38.1, 37.4, 36.7, 36.2, 35.8, 33.7, 33.3, 33.1, 32.2, 29.9, 28.4, 28.3, 27.5, 25.9, 25.7, 24.0, 23.2, 22.5, 20.5, 17.6, 16.0, 15.2, 13.5, 13.0, 10.4. ESI-HRMS m/z: $[M+Cl]^-$ Calcd for $C_{44}H_{70}N_4O_8$ 817.4882; Found 817.4887.



Allyl 2-acetamido-6-{4-[4-(19β,28-epoxy-18α-olean-3α-yloxy)-4-oxobutyl]-1*H*-1,2,3-triazol-1-yl}-2,6-dideoxy-D-galactopyranoside 8. Yield 0.51 g (62%), white solid. m.p. 149 °C. R_f 0.65. $[\alpha]_D^{20}$ +20° (*c* 0.02, $CHCl_3$ – CH_3OH 1:1). ν_{max} (cm^{-1}, ZnSe) 3253, 2893, 2822, 1705, 1631. 1439, 1361, 1258, 1125, 1109, 1060, 1022. ^{1}H NMR (400 MHz, CD_3OD): δ 7.74 (s, 1H, -N-CH=C-), 7.64 (m, 1H, NHAc), 6.00 – 5.85 (m, 1H, -CH=CH_2), 5.32 – 5.02 (m, 2H, -CH=CH_2), 4.65 (d, *J* = 8.9 Hz, 1H, -O-CH(O)-), 4.48 (m, 1H, H-3), 4.39 (d, *J* = 7.2 Hz, 1H, CH), 4.36 – 4.23 (m, 1H, CH), 4.19 (dt, *J* = 8.4, 4.1 Hz, 3H, CH, CH_2), 4.04 (d, *J* = 5.3 Hz, 1H, CH), 3.91 – 3.80 (m, 2H, CH_2), 3.77 (d, *J* = 7.7 Hz, 1H, H_a-28), 3.53 (s, 1H, H-19), 3.45 (d, *J* = 7.8 Hz, 1H, H_b-28), 1.96 (s, 3H, NHAc), 1.77 – 1.21 (m, 32H, CH, CH_2), 0.99 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.90 (2s, 6H, $2CH_3$), 0.89 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.80 (s, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 172.8 (-CH_2(O)CO-), 167.6 (NHAc), 131.7 (-CH=CH_2), 130.6 (-N-CH=C-), 128.2 (-N-CH
=C-), 116.0 (-CH=CH_2), 99.9 (C-1'), 87.8 (C-19), 80.7 (C-3), 72.6, 71.0, 70.5, 69.1, 68.1, 67.5, 55.1, 52.2, 50.5, 46.3, 40.9, 40.1, 40.0, 38.3, 38.0, 37.2, 36.6, 35.9, 35.5, 33.8, 33.2, 32.0, 29.8, 28.4, 27.7, 27.1, 25.8, 25.4, 23.4, 23.2, 22.3, 20.5, 17.5, 15.6, 14.7, 12.9, 12.5, 9.9. ESI-HRMS m/z: $[M+Na]^+$ Calcd for $C_{47}H_{74}N_4O_8$ 845.5399; Found 845.5405.

2. Cytotoxicity measurements.

The measurements were carried out using the standard MTT method.[S4] All the manipulations described below were performed using JANUS G3 Automated Workstation. 3 000 cells per well for PC3 and HEK 293T, 8 000 cells per well for Huh7 and 25 000 cells per well for HepG2 were plated out in 140 µl of media in 96-well plate. DMEM-F12 media (Gibco) supplemented with 10% FBS (Gibco), 2 mM L-Glutamine (Gibco) and 1% PenStrep (10000:10000, Gibco) was used for all cell lines. Cells were incubated at the 37 ºC in the 5% CO_2 incubator for first 16 h without treating. Then 11 µl of test substance media-DMSO solutions were added to the cells (the final DMSO concentrations (v/v) in the media were 0.5% or less) and cells were treated for 72 h (triplicate each test substance concentration). After that the MTT reagent (Dia-M) was added to cells up to the final concentration of 0.5 mg ml^{-1} (10x stock solution in PBS was used) and cells were incubated for 1.5 h at 37 ºC in the 5% CO_2 incubator. The MTT solution was discarded and 140 µl of DMSO was added. The plates were swayed on a shaker (90 rpm) for 15 min to solubilize the formazan. The absorbance was measured using a microplate reader (VICTOR X5 Plate Reader) at a wavelength of 555 nm (in order to measure the formazan concentration). The results were used to construct a four-parameter dose-response logistic curve and to estimate IC_{50} value by RStudio software using "drc" and "drexplorer" packages.

Table S1. Cytotoxicity of allobetulin conjugates **6-8** against HepG2, Huh7, PC3 and Hek293 cell lines

Compound	IC_{50} (μM)			
	HepG2	Huh7	PC3	Hek293
6	>100	>100	>100	>100
7	>100	>100	>100	>100
8	>100	>100	>100	>100

3. Surface plasmon resonance (SPR).

Experiment was carried out on a Biacore X100 machine (Biacore AB, Uppsala, Sweden) using CM5 chip. Protein ASGP-R was immobilized according standard amine-coupling protocol. Running buffer mixture containing 150 mM NaCl, 50 mM $CaCl_2$, 50 mM Tris, pH 7.4 was used. Concentration of ligands were presented in range from 10^{-2} M to $5*10^{-11}$ M. Compound solutions were injected at a flow rate 20 μl min^{-1} at 25 °C for 180 s followed by 60 s dissociation. The regeneration of the sensor chip was obtained by injection of 20 μl of 20 mM EDTA. Raw data obtained was analyzed using BIAevaluation 3.0 software

SPR results

The obtained K_d values are listed in Table S2.

Table S2. The binding potency of the representative compounds **6-8**.

Compound	K_d, nM
6	10.6
7	8.3
8	2.0

4. Molecular docking studies

Receptor model preparation

The ASGP-R model was prepared using Protein Preparation Wizard tool in the Maestro software (Schrodinger Inc.) based on crystal structure 5JPV.[S5] The developed docking model was validated by redocking of the crystallographic ligand using the UCSF Chimera program.[S6] Since the ligands are rather large, the energy grid was built within a cubic box of dimensions 36 × 36 × 36 Å.

Ligands preparation

Ligands were designed using ChemDraw software. 3D-optimization was carried out using Maestro software (Schrodinger Inc.).

Molecular docking

Molecular docking was performed by Glide module in Maestro software (Schrodinger Inc.). Visualization of docking results was performed using UCSF Chimera software.

Docking results

The list of E^{score} for ligands binding to the receptor surface is provided in Table S3.

Table S3. List of energy score values for conjugates **6-8**.

Compound	E^{score}, kkal/mol
6	-4.23
7	-5.11
8	-5.21

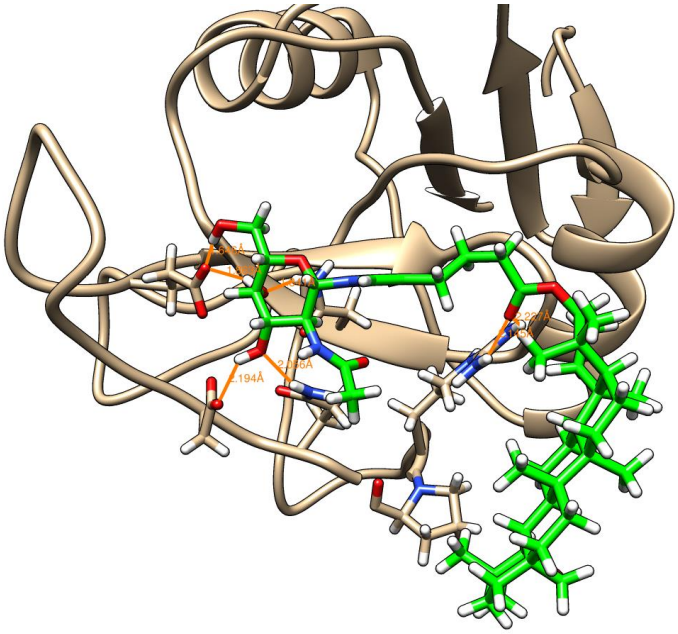


Figure S1. The mode of interaction between conjugate **7** and the receptor.

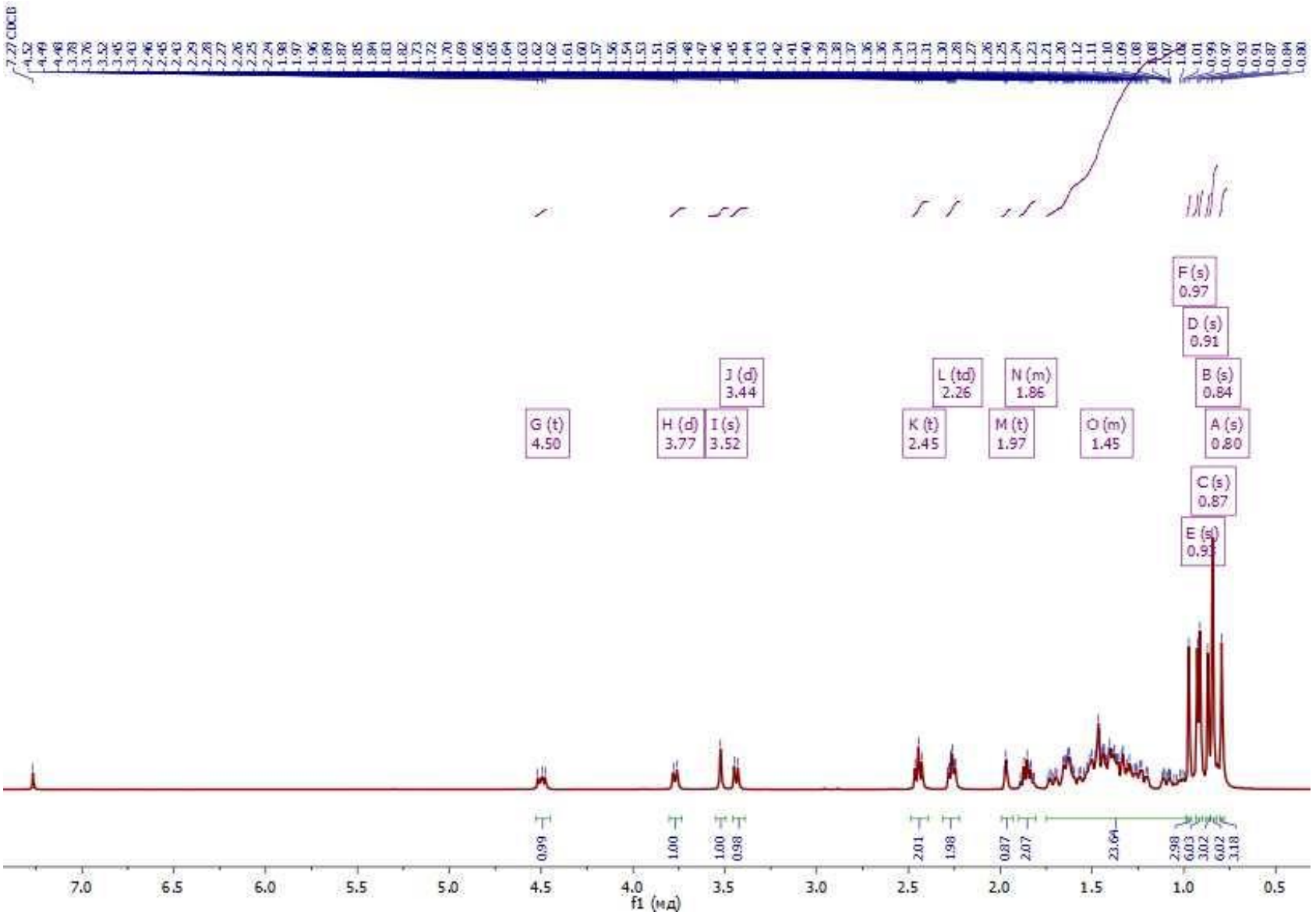


Figure S2. ^{1}H NMR of Compound **5**.

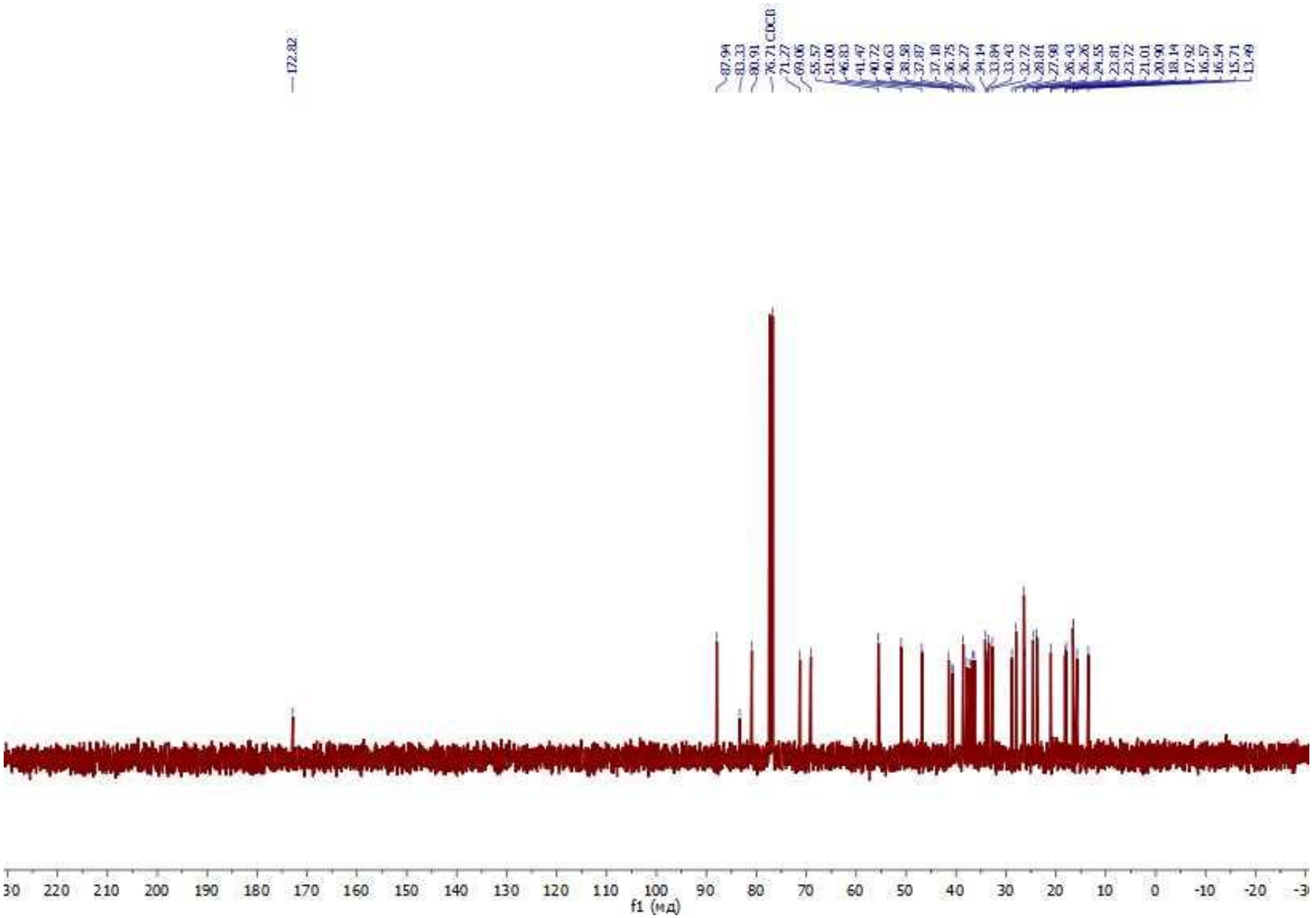


Figure S3. ^{13}C NMR of Compound **5**.

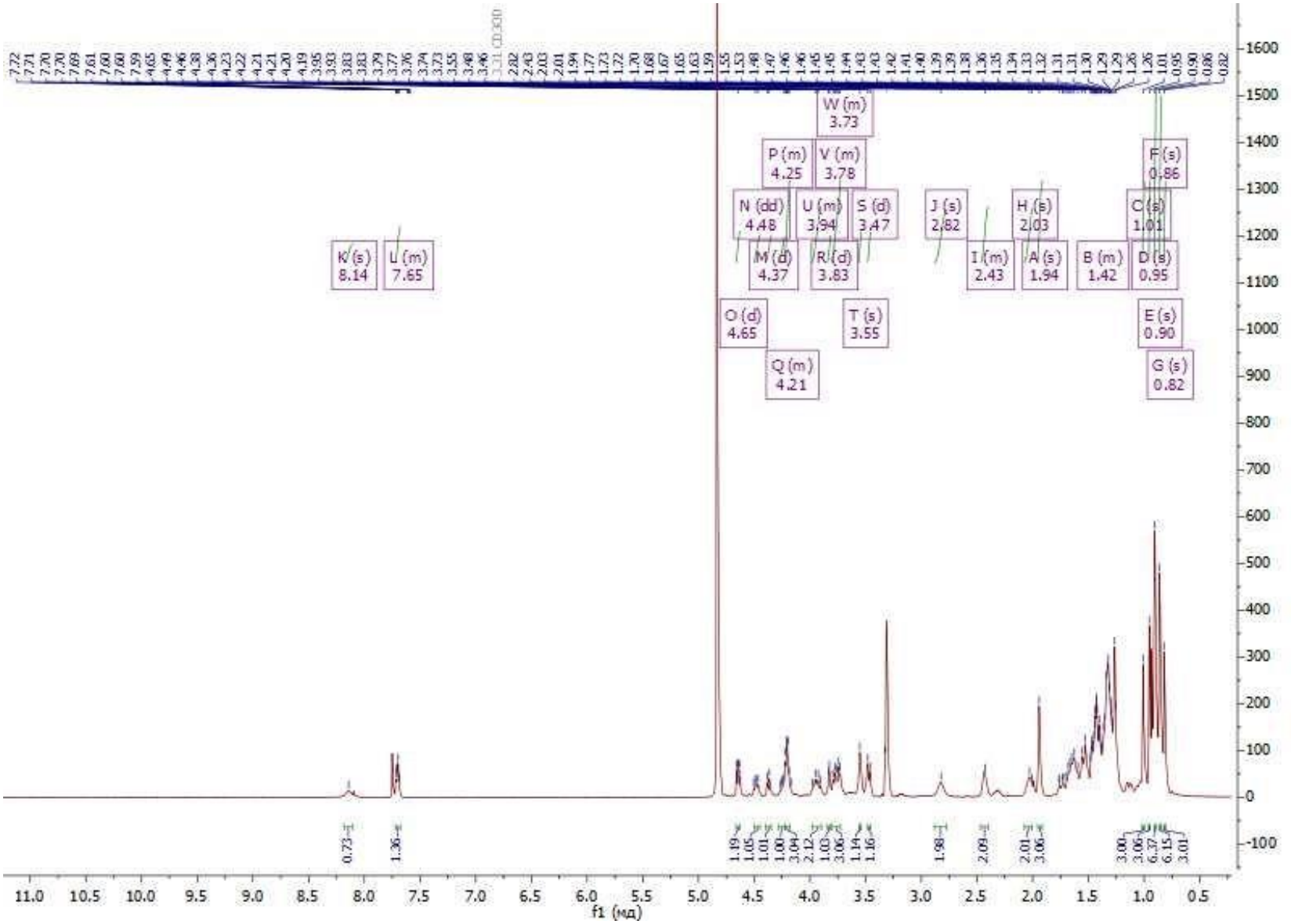


Figure S4. ^{1}H NMR of Compound **6**.

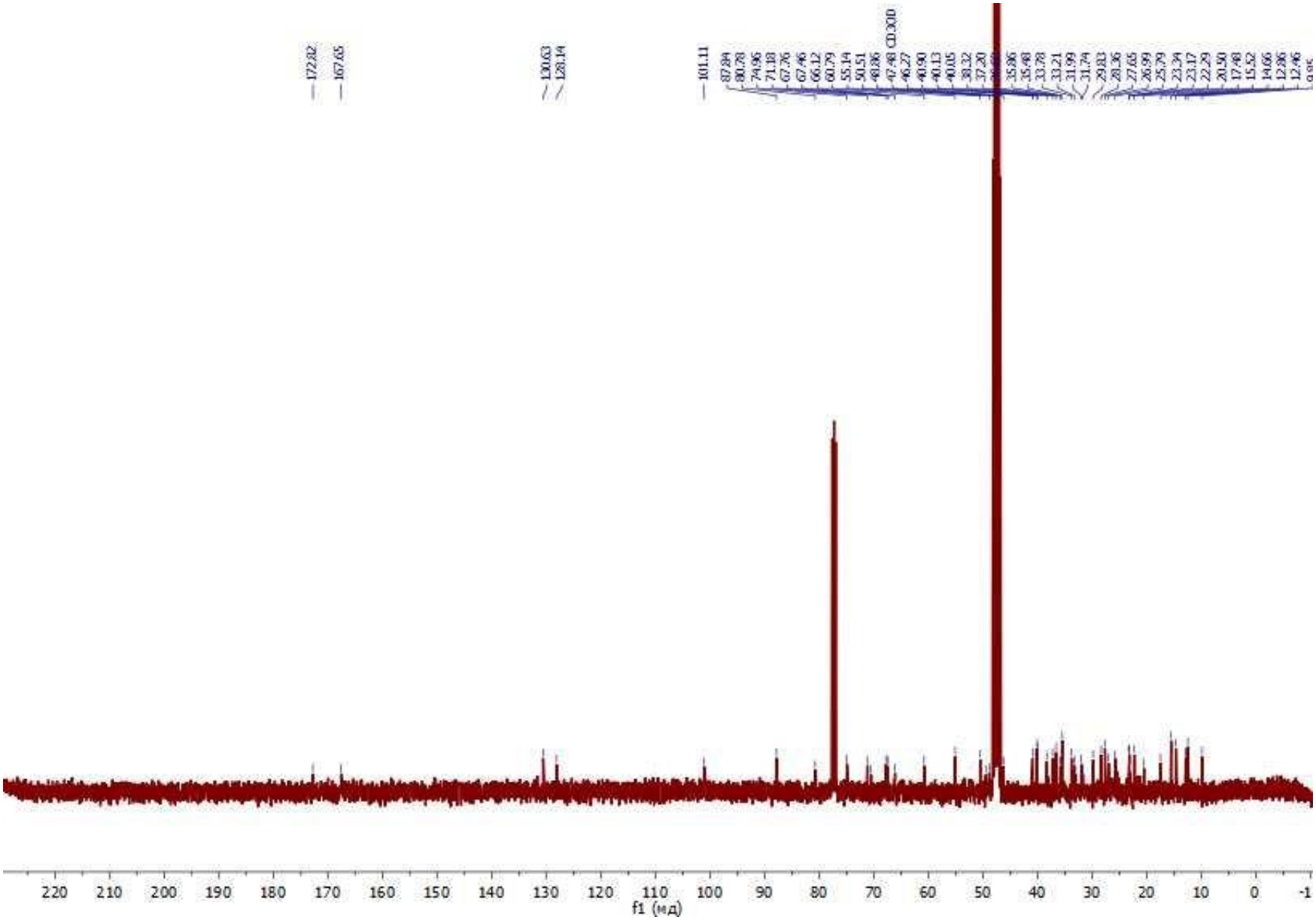


Figure S5. ^{13}C NMR of Compound **6**.

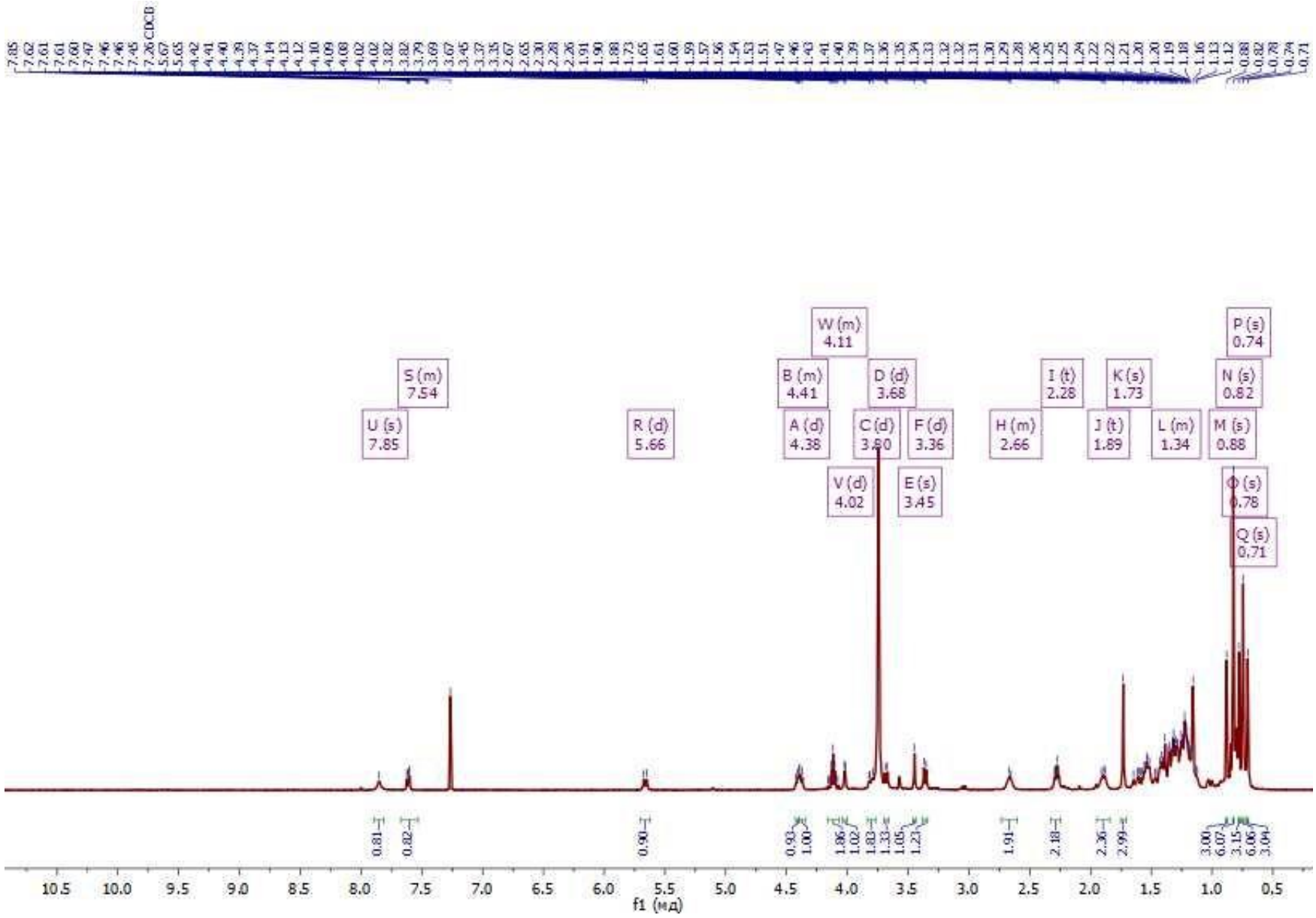


Figure S6. ^{1}H NMR of Compound **7**.

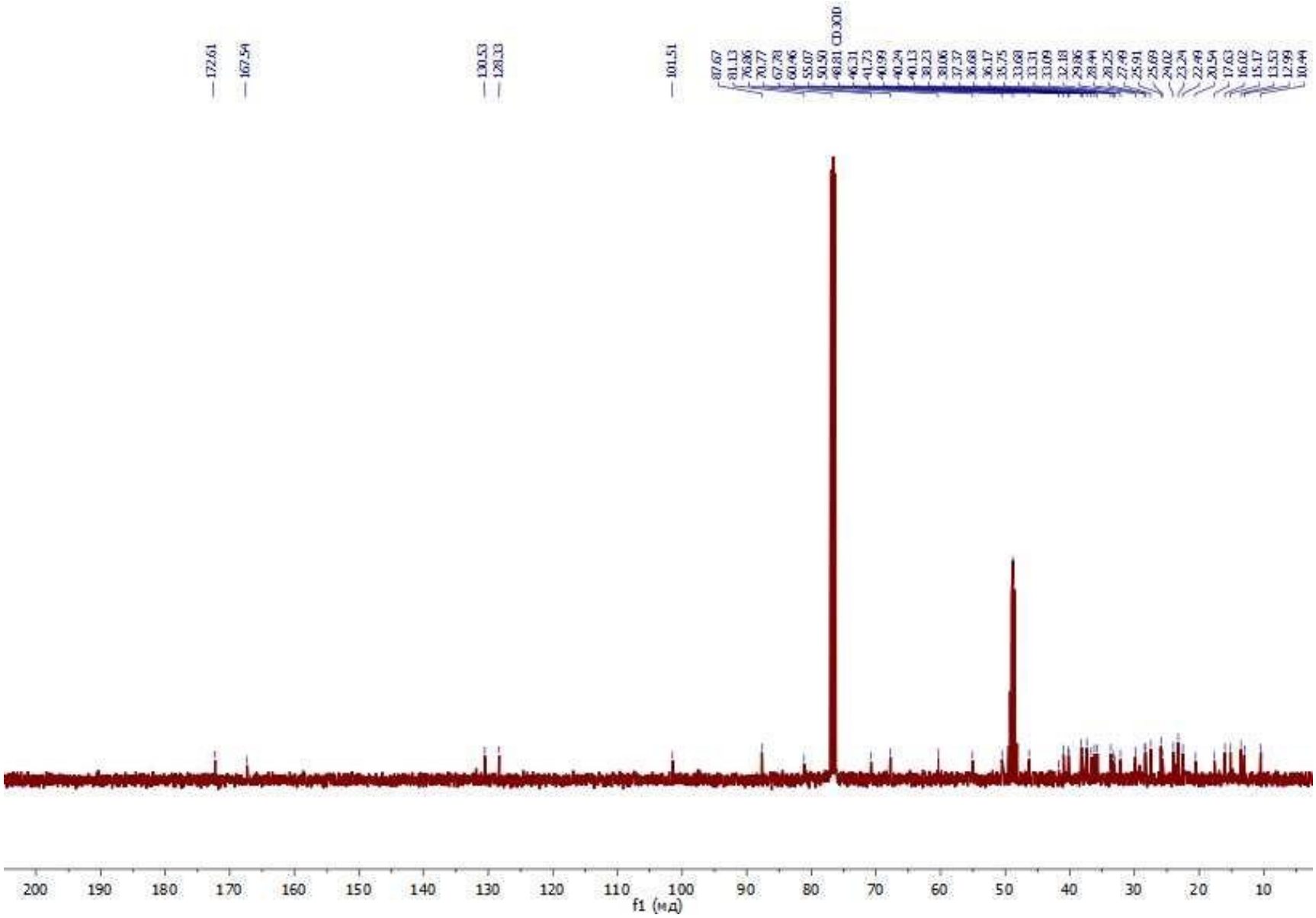


Figure S7. ^{13}C NMR of Compound **7**.

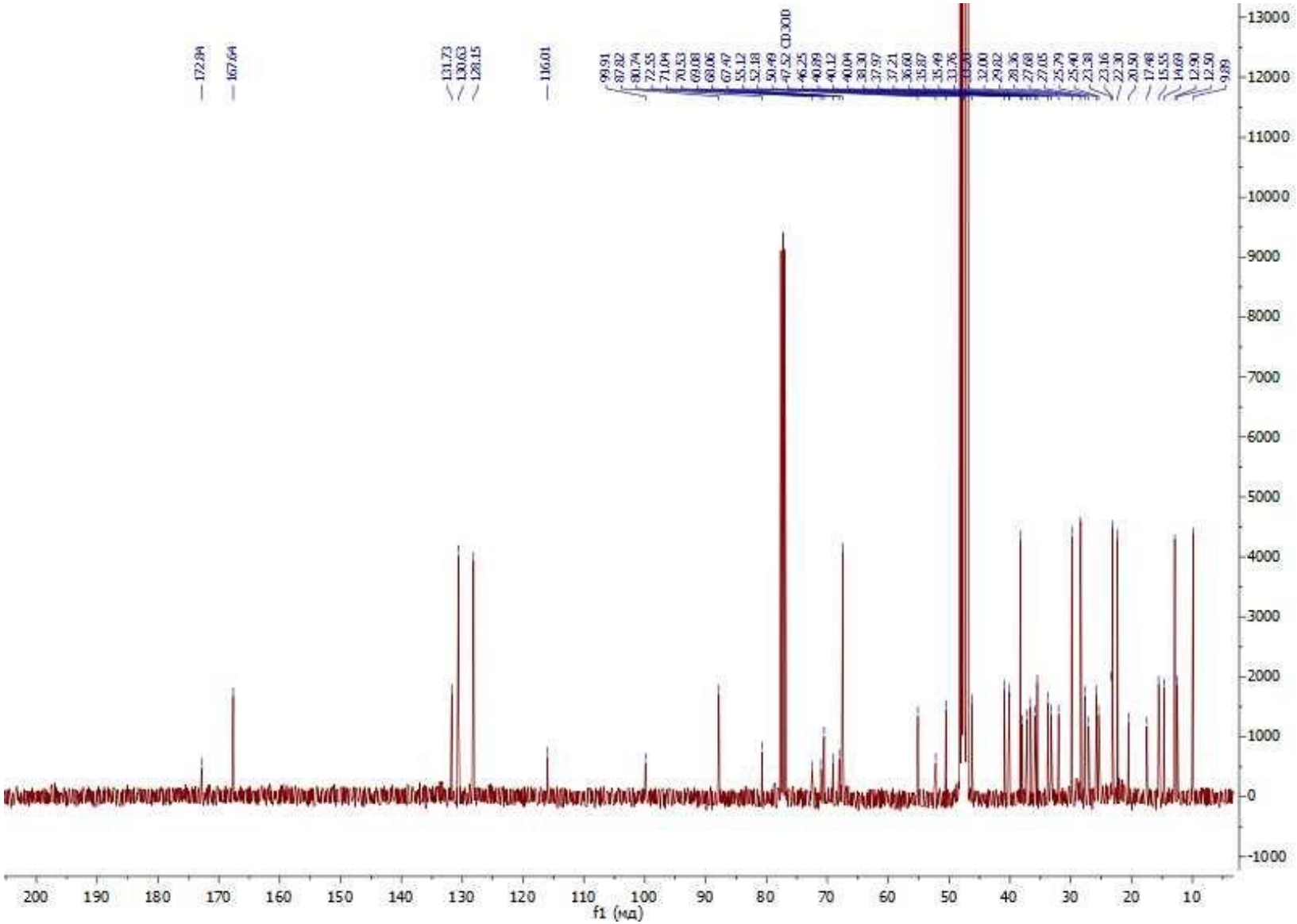


Figure S8. ^{13}C NMR of Compound **8**.

References

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