Supporting Information of

Direct Inhibition of Hypoxia-Inducible Transcription Factor Complex with Designed Dimeric Epidithiodiketopiperazine

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Contents

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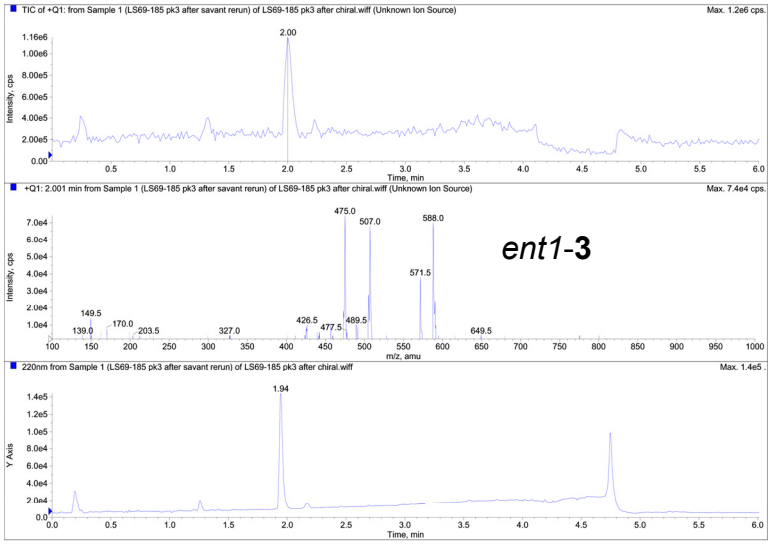

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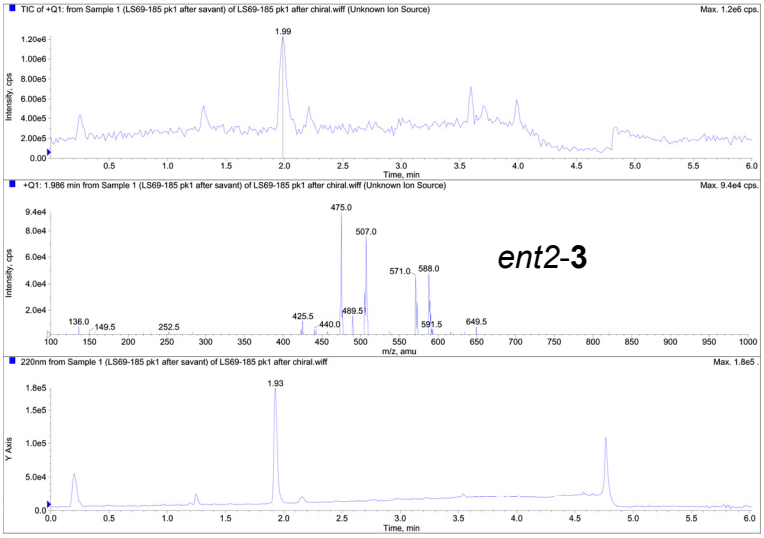

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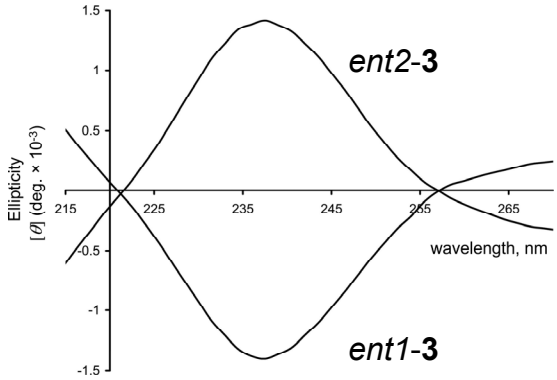


Figure S1. (a-b) Total ion chromatograms (top), mass spectra (middle) and reverse-phase HPLC traces at 220 nm (bottom) of the two separated enantiomers *ent1*-**3** and *ent2*-**3**. (c) CD spectra of the enantiomers.

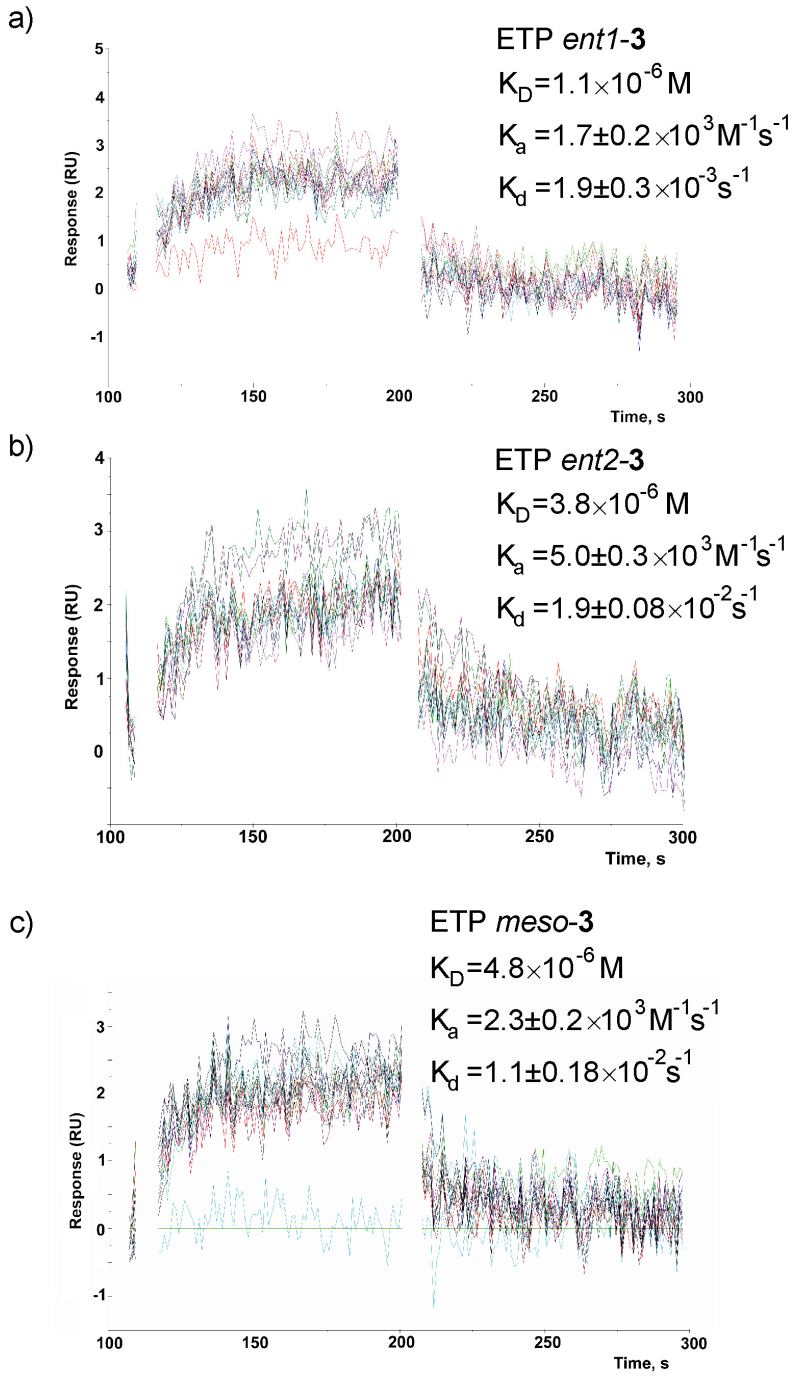
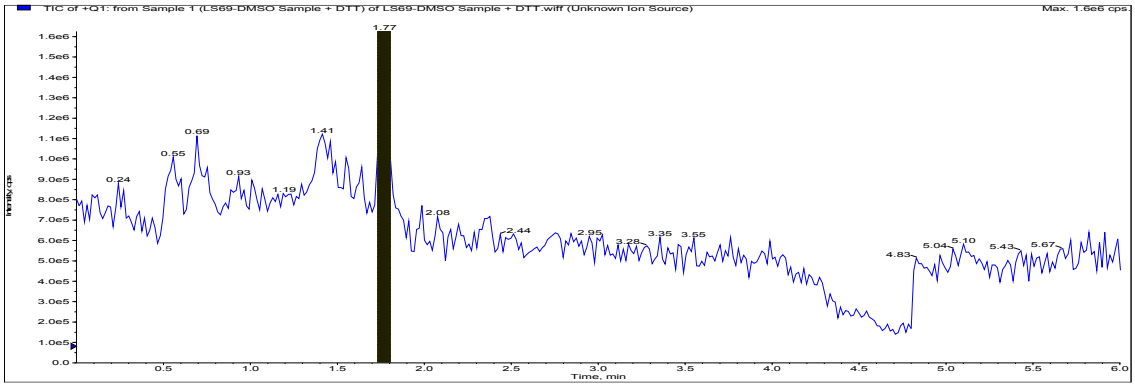


Figure S2. **Both enantiomers of ETP 3 and its meso-isomer bind target protein p300 CH1 domain with comparable affinities.** SPR sensorgrams for binding of (a) *ent1*-**3**, (b) *ent2*-**3**, and (c) *meso*-**3** to surfaces modified with p300-CH1-GST fusion protein in the presence of 100 μM concentration DTT. Curves represent the concentrations of compound tested ranging from 100 nM to 8 μM with all concentrations tested in duplicate.

a)



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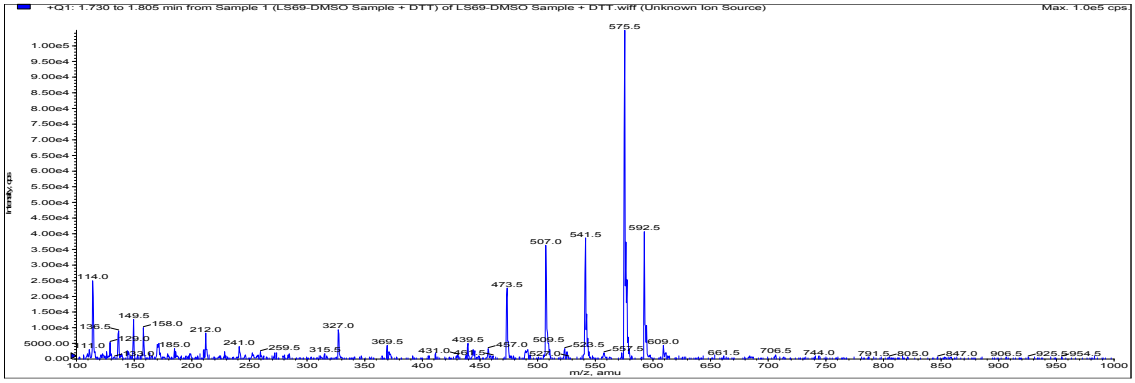


Figure S3. (a) LC–MS total ion chromatogram (TIC) showing the result from an MS detector (positive ion mode) from the sample of ETP **(±)-3** in the presence of 100 μM DTT. (b) The mass spectrum taken from the shaded region of the TIC indicating presence of the reduced tetrathiol form of **(±)-3**.

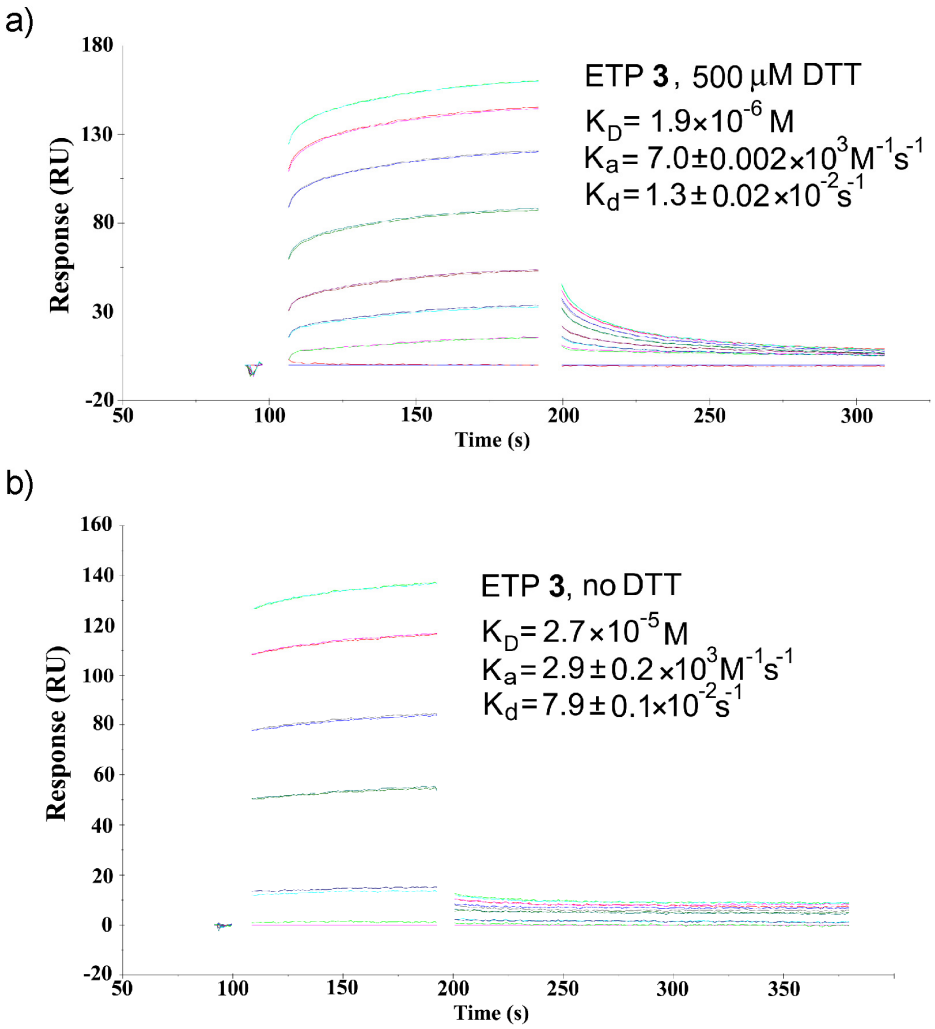


Figure S4. **Reducing conditions are necessary for ETP 3 to bind to its target with high affinity**. (a) SPR sensorgrams for binding of ETP **3** to surfaces modified with p300-CH1-GST fusion protein in the presence of 500 µM concentration DTT. (b) Sensorgrams for binding of ETP **3** to the chip surfaces in the absence of DTT under otherwise identical conditions. Curves represent the concentration of compound tested ranging from 50 nM, 200 nM, 500 nM, 1 µM, 10 µM, to 50 µM; all concentrations were tested in duplicate.

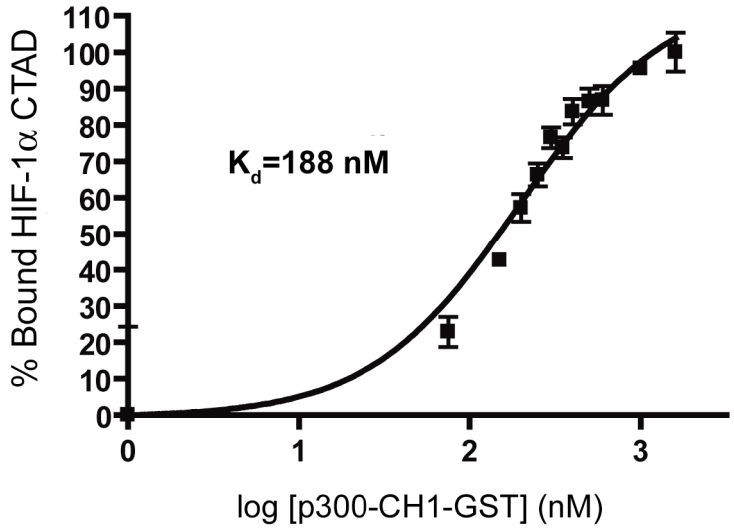


Figure S5. **Saturation binding curve for HIF-1α C-TAD fluorescent probe binding to p300-CH1-GST fusion protein.** Calculated K_d value was used to perform fluorescence polarization competition experiments to determine the binding affinity of synthetic ETP **3** and controls for p300. Each point represents the average of 3-5 experiments, error bars are ± s.e.m.

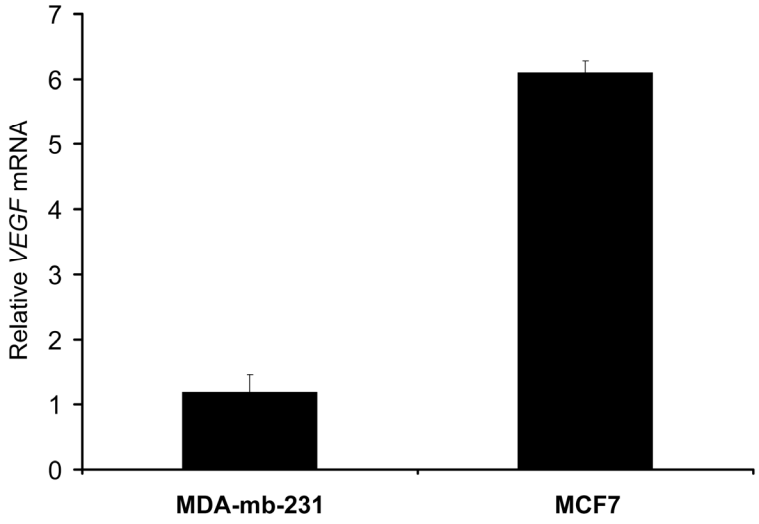


Figure S6. **Relative VEGF mRNA expression levels in MDA-MB-231 and MCF7 cell lines determined by qRT-PCR.** MCF7 cells exhibit a 4-fold greater induction as compared to MDA-MB-231 cells. Hypoxia was induced with 300 μM DFO. Error bars represent ± s.e.m. of experiments performed in triplicate.

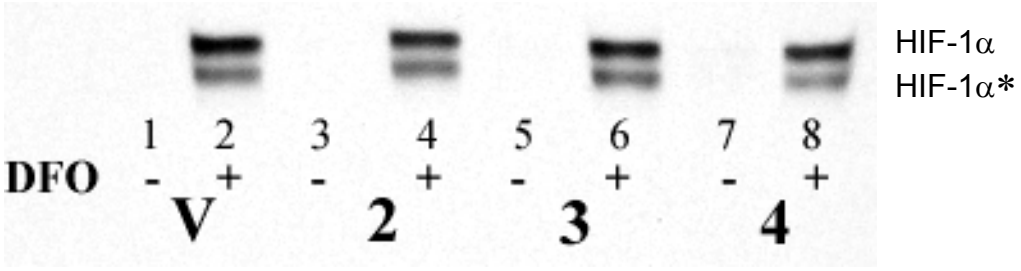


Figure S7. **ETP 3 and controls do not change the intracellular levels of HIF-1α.** Analysis of HIF-1α levels in nuclear extracts by Western blotting. Cells were incubated for a total of 24 hours with vehicle (0.1% DMSO), chetomin **2**, ETP **3** and control DKP **4**. All compounds were tested at 200 nM concentrations. After 6 hours, hypoxia was mimicked with DFO (300μM) for 18 additional hours. HIF-1α levels were determined 24 hours after treatment. The band that corresponds to phosphorylated HIF-1α is labeled as HIF-1α*.

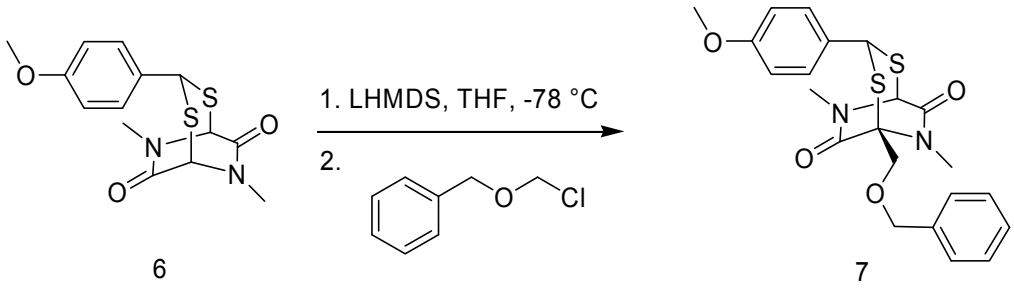
General Synthetic Methods

All reagents and solvents were obtained from commercial sources and were used as received unless otherwise stated. Chetomin was purchased from EMD Biosciences. Dry THF was obtained by distillation with sodium and benzophenone. Dry CH_2Cl_2 was freshly distilled with calcium hydride. All reactions involving moisture-sensitive reagents were conducted under argon atmosphere with anhydrous solvents and flame-dried glassware. Hygroscopic liquids were transferred via a syringe and were introduced into reaction vessels through rubber septa. Reaction product solutions were concentrated using a rotary evaporator at 30-150 mm Hg. Column chromatography was performed on silica gel (230-400 mesh) using reagent grade solvents. Analytical thin-layer chromatography (TLC) was performed on glass-backed, pre-coated plates (0.25 mm, silica gel 60, F-254, EM Science). Analytical HPLC were performed on Microsorb-MV C_8 reverse-phase column (250 × 4.6 mm, Varian) using Shimadzu LC-10A VP pump and Shimadzu SPD 10A VP UV-vis variable-wavelength detector. Preparative HPLC purifications were carried out with C_8 reverse phase preparative column (Alltech/Grace Davison). The flow rate for preparative reverse-phase HPLC was 5 mL/min. In all cases, gradients of acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) were used as eluents. Water (18 MΩ) was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered. Nuclear magnetic resonance (NMR) spectra were collected on Bruker 500 MHz or 600 MHz instruments in the indicated solvents. The peak positions are reported with chemical shifts (δ) in parts per million (ppm) downfield from the signal for tetramethylsilane (0 ppm) and referenced to the signal resulting from the incomplete deuteration of a solvent used in the experiment ($CDCl_3$: 7.26 ppm, or the center line of the multiplet of DMSO-D_6: 2.50 ppm). Carbon-13 chemical shifts are reported as δ values in ppm and referenced to the cabon-13 signal

of a solvent used in the experiment ($CDCl_3$: 77.0 ppm, or the center line of the multiplet DMSO-D_6: 39.51 ppm). The coupling constants (*J*) are reported in Hertz (Hz). The following abbreviations are used: singlet (s), doublet (d), triplet (t), doublet of doublets (dd), multiplet (m). Mass spectra were obtained from the Mass Spectrometry Laboratory in the Department of Chemistry, University of Arizona. CD spectra were recorded on Olis DSM-20 CD spectrophotometer at the Analytical Biophysics Core Facility, Arizona Research Labs.

Compound **6** (*vide infra*),[s1] α,α'-diiodo-*p*-xylene[s2] and benzyl chloromethyl ether[s3] were prepared as described in the literature.

1-(Benzyloxymethyl)-3-(4-methoxyphenyl)-6,8-dimethyl-2,4-dithia-6,8-diazabicyclo[3.2.2]nonane-7,9-dione (**7**)

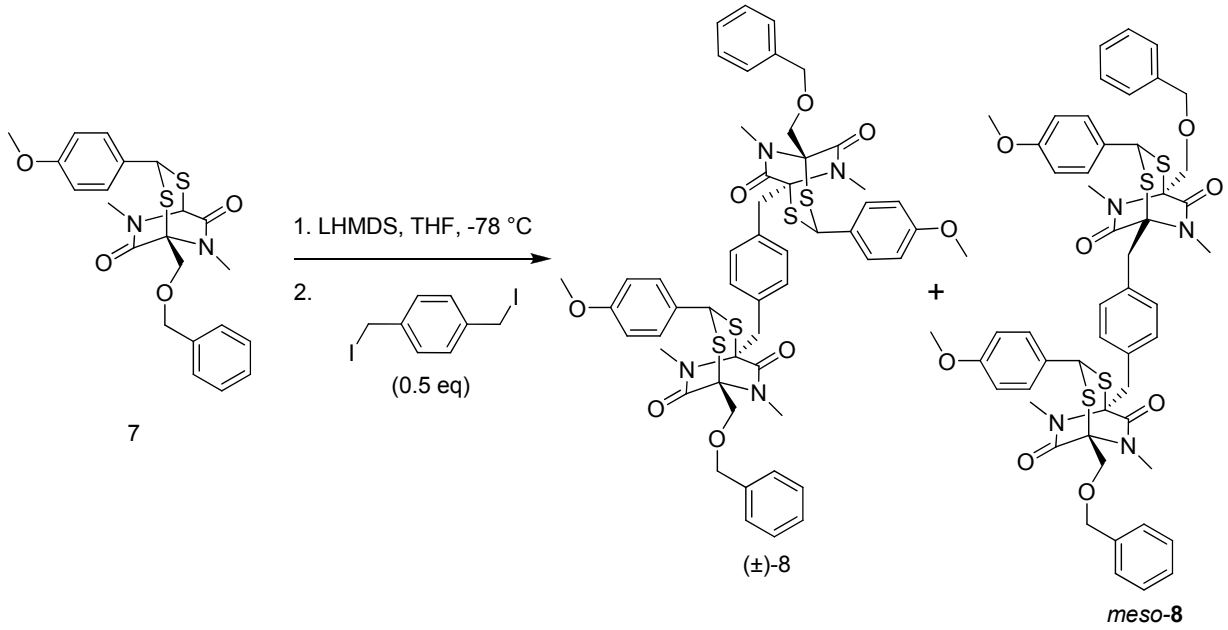


6 7

A solution of **6** (1.0 g, 3.2 mmol) in dry THF (80 mL) was cooled to -78 °C. LHMDS (1.0 M solution in THF) (3.8 mL, 3.8 mmol) was added dropwise over a period of 3 min with stirring. Next, benzyl chloromethyl ether (d = 1.527g/cm^3) (2.0 g , 1.3 mL, 13 mmol) was added into the resulting red, cloudy reaction mixture and the stirred mixture was allowed to slowly warm up to room temperature for 3 h. Water was added into the reaction and the mixture was extracted with dichloromethane (3 × 100 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The concentrated product was dissolved in a minimum amount of dichloromethane from which **7** (0.90 g, 69%) was obtained by precipitation with diethyl ether. ^{1}H NMR ($CDCl_3$, ppm) δ: 7.31 (m, 7H), 6.84 (d, *J* = 8.7 Hz,

2H), 5.11 (s, 1H), 5.04 (s, 1H), 4.74 (d, J = 11.2 Hz, 1H), 4.54 (d, J = 11.2 Hz, 1H), 4.22 (d, J = 10.5 Hz, 1H), 3.81 (d, J = 10.5 Hz, 1H), 3.78 (s, 3H), 3.22 (s, 3H), 3.10 (s, 3H). ^{13}C NMR ($CDCl_3$, ppm) δ: 164.89, 164.03, 160.57, 137.10, 130.44, 128.48, 128.03, 128.00, 126.55, 114.36, 74.10, 70.64, 68.43, 65.98, 55.33, 50.22, 33.09, 27.28. MS (FAB) *m/z*: calcd. for $C_{22}H_{25}N_2O_4S_2^+$ [M+H$^+$]: 445.1, Found: 445.1.

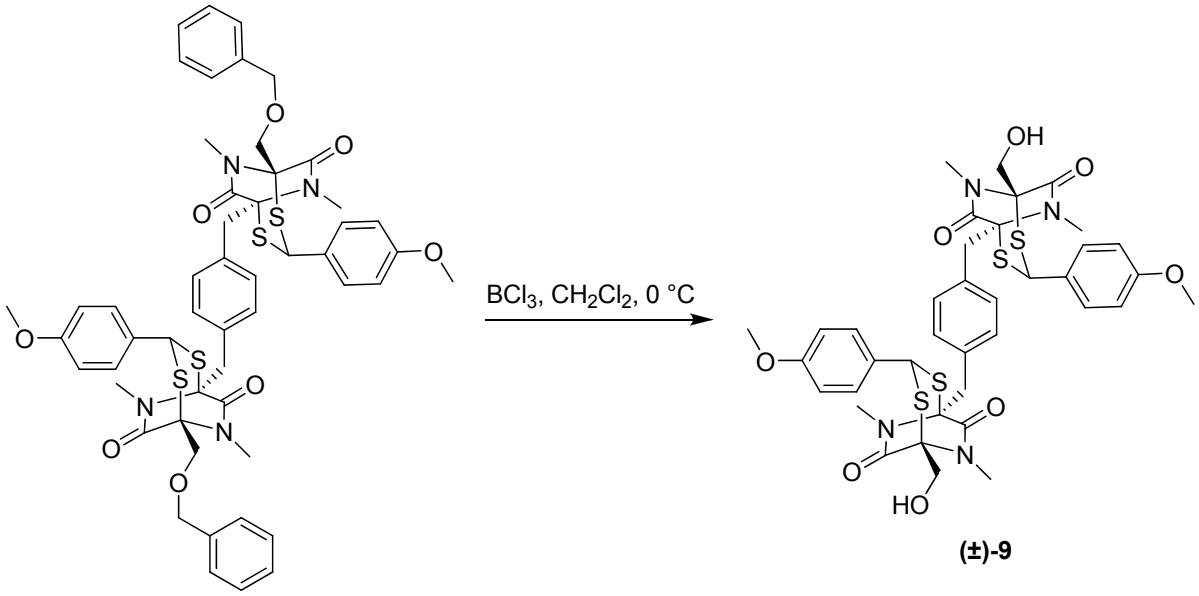
5,5'-(1,4-Phenylenebis(methylene))bis(1-(benzyloxymethyl)-3-(4-methoxyphenyl)-6,8-dimethyl-2,4-dithia-6,8-diazabicyclo[3.2.2]nonane-7,9-dione) (8)



A solution of **7** (0.33 g, 0.75 mmol) in dry THF (15 mL) was cooled to -78 °C. Next, 1 M solution of LHMDS in THF (1.0 mL, 1.0 mmol) was added dropwise over a period of 2 min with stirring. The α,α'-diiodo-*p*-xylene (88 mg, 0.25 mmol), dissolved in 2 mL of THF was then added dropwise into the reaction mixture and the solution was allowed to warm up to room temperature for 3 h. Water was added into the reaction and the mixture was extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. A mixture of diastereoisomers *meso*-**8**

and **(±)-8** (0.20 g, 80%) was separated by column chromatography on silica gel using CH_2Cl_2 : Hexane : EtOAc = 5 : 4 : 1 as an eluent. For **(±)-8** ^{1}H NMR ($CDCl_3$, ppm) δ: 7.33 (m, 14H), 7.07 (s, 4H), 6.84 (d, *J* = 8.8 Hz, 4H), 5.06 (s, 2H), 4.78 (d, *J* = 12.20 Hz, 2H), 4.56 (d, *J* = 12.20 Hz, 2H), 4.36 (d, *J* = 16.39 Hz, 2H), 4.28 (d, *J* = 10.67 Hz, 2H), 3.82 (d, *J* = 10.67 Hz, 2H), 3.80 (s, 6H), 3.34 (s, 6H), 3.08 (d, *J* = 16.39 Hz, 2H), 2.94 (s, 6H). ^{13}C NMR ($CDCl_3$, ppm) δ:165.76, 165.50, 160.45, 137.27, 133.72, 130.43, 128.78, 128.44, 127.92, 127.84, 126.52, 114.31, 73.97, 73.39, 71.00, 68.51, 55.35, 51.03, 40.23, 29.80, 28.08. HRMS (FAB) *m/z*: calcd. for $C_{52}H_{55}N_4O_8S_4^+$ [M+H$^+$]: 991.290, Found: 991.291.

(±)-5,5'-(1,4-Phenylenebis(methylene))bis(1-(hydroxymethyl)-3-(4-methoxyphenyl)-6,8-dimethyl-2,4-dithia-6,8-diazabicyclo[3.2.2]nonane-7,9-dione), (±)-9

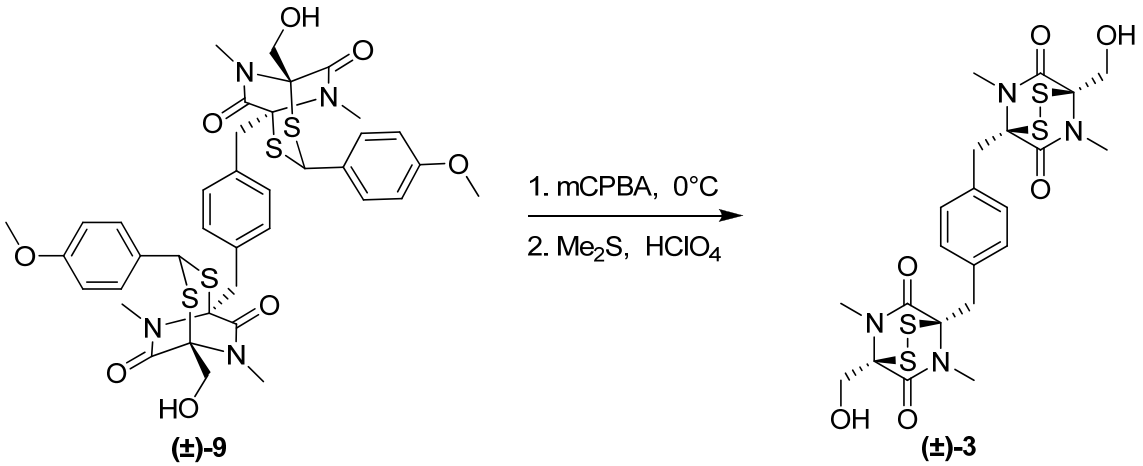


(±)-8 **(±)-9**

To a solution of **(±)-8** (0.13 g, 0.13 mmol) in dichloromethane (10 mL) cooled to 0 °C, boron trichloride (1M solution in CH_2Cl_2, 320 μL, 0.32 mmol) was added dropwise with stirring. The solution was stirred at 0 °C for additional 10 min and then poured into ice-cold water (10 mL)

and extracted with dichloromethane (25 mL). The organic layer was washed twice with water, dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to obtain crude product as a white solid. The crude product purified by column chromatography (silica gel, CH_2Cl_2/EtOAc = 7 : 3) to afford *rac*-**9** (93 mg, 91%). ^{1}H NMR ($CDCl_3$, ppm) δ: 7.32 (d, J=8.8 Hz, 4H), 7.03 (d, *J* = 8.8 Hz, 4H), 5.08 (s, 2H), 4.37 (d, *J* = 16.24 Hz, 1H), 4.33 (dd, *J* = 12.60 Hz and 5.54 Hz, 2H), 4.05 (dd, *J* = 12.6 Hz and 9.93 Hz, 2H), 3.81 (s, 6H), 3.42 (s, 6H), 3.06 (d, *J* = 16.24 Hz, 2H), 2.95 (dd, *J* = 9.93 Hz and 5.54 Hz, 2H), 2.91 (s, 6H). ^{13}C NMR ($CDCl_3$, ppm) δ:166.67, 165.83, 160.45, 133.70, 130.42, 128.80, 126.45, 114.39, 73.20, 71.12, 62.98, 55.39, 50.98, 40.42, 29.79, 27.98. HRMS (ESI) *m/z*: calcd. for $C_{38}H_{43}N_4O_8S_4^+$ [M+H$^+$]: 811.196, found: 811.195.

(±)-4,4'-(1,4-Phenylenebis(methylene))bis(1-(hydroxymethyl)-5,7-dimethyl-2,3-dithia-5,7-diazabicyclo[2.2.2]octane-6,8-dione), (±)-3



(±)-9 **(±)-3**

m-Chloroperbenzoic acid (22 mg, 77% max content, 0.10 mmol) was added to an ice-cold solution of **(±)-9** (33 mg, 0.040 mmol) in anhydrous dichloromethane (10 mL) with stirring. After 10 min of stirring at 0 °C, dimethyl sulfide (10 μL) was added, followed by treatment with 20 μL of a solution of 70% perchloric acid in methanol (1:5). The solution was allowed to stand at room temperature for 9 h. The reaction mixture was poured into a saturated sodium

bicarbonate. The solution was extracted with dichloromethane (3 × 30 mL). The combined organic extracts were combined, dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The glassy residue was purified by column chromatography (silica gel, CH_2Cl_2/EtOAc = 6 : 4) to afford **(±)-3** (11 mg, 33%). ^{1}H NMR (DMSO-D6, ppm) δ: 7.24 (s, 4H), 5.90 (t, *J* = 5.50 Hz, 2H), 4.33 (dd, *J* = 12.83 Hz and 5.50 Hz, 2H), 4.23 (dd, *J* = 12.83 Hz and 5.50 Hz, 2H), 3.89 (d, *J* = 16.04 Hz, 2H), 3.73 (d, *J* = 16.04 Hz, 2H), 3.13 (s, 6H), 2.82 (s, 6H). ^{13}C NMR ($CDCl_3$, ppm) δ:165.35, 164.92, 133.45, 128.89, 76.33, 75.91, 59.12, 35.50, 28.31, 27.89. HRMS (FAB) *m/z*: calcd. for $C_{22}H_{26}N_4O_6S_4Na^+$ [M+Na^+]: 593.063. Found: 593.063.

Chiral sepaparation of (±)-3.

A portion of purified racemate **(±)-3** was dissolved in methanol on heating. The solution was injected in several portions into HPLC system running isocratic method with 100% MeOH on a Chiralcel OD-H column (250 × 4.6 mm, 5μ particle size, Daicel). The flow rate of 1 ml/min was maintained by Shimadzu LC-10A VP pump with absorption measured by Shimadzu SPD 10A VP UV-Vis variable-wavelength detector. The first enantiomer, *ent1*-**3,** was eluted with retention time of 15.7 min, whereas the second, *ent2*-**3 -** with retention time of 22.2 min. Due to the small amount of the obtained enantiomers, we opted not to determine their signs of optical rotation by polarimetry, using *ent1*-**3** and *ent2*-**3** notations instead**.**

The separated enantiomers of *ent1*-**3** and *ent2*-**3** had virtually identical retention times within the error of the experiment - 1.99 and 2.00 min, respectively, on the total ion chromatogram in the reverse-phase HPLC. Compounds *ent1*-**3** and *ent2*-**3** also had identical *m/z* of their molecular ions and very similar fragmentation patterns in the mass spectra. Both *ent1*-**3** and *ent2*-**3** were dissolved in methanol to obtain solutions with concentrations of 450 μM and CD spectra of these

solutions were recorded. The opposite signs of the CD curves for *ent1*-**3** and *ent2*-**3** confirmed the enantiomeric relationship between the two compounds.

3. References:

(S1) Fukuyama, T.; Nakatsuka, S.; Kishi, Y. *Tetrahedron* **1981**, *37*, 2045-2078.

(S2) Kida, T.; Kikuzawa, A.; Higashimoto, H.; Nakatsuji, Y.; Akashi, M. S *Tetrahedron* **2005**, *61*, 5763–5768.

(S3) Shipov, A. G.; Savost'yanova, I. A.; Baukov, Yu. I. S. *Zhurn. Obshch. Khim.* **1989**, *59*, 1204-1205.

Complete Reference 22:

(22) Kung, A. L.; Zabludoff, S. D.; France, D. S.; Freedman, S. J.; Tanner, E. A.; Vieira, A.; Cornell-Kennon, S.; Lee, J.; Wang, B. Q.; Wang, J. M.; Memmert, K.; Naegeli, H. U.; Petersen, F.; Eck, M. J.; Bair, K. W.; Wood, A. W.; Livingston, D. M. *Cancer Cell* **2004**, *6*, 33-43.

4. NMR Spectra

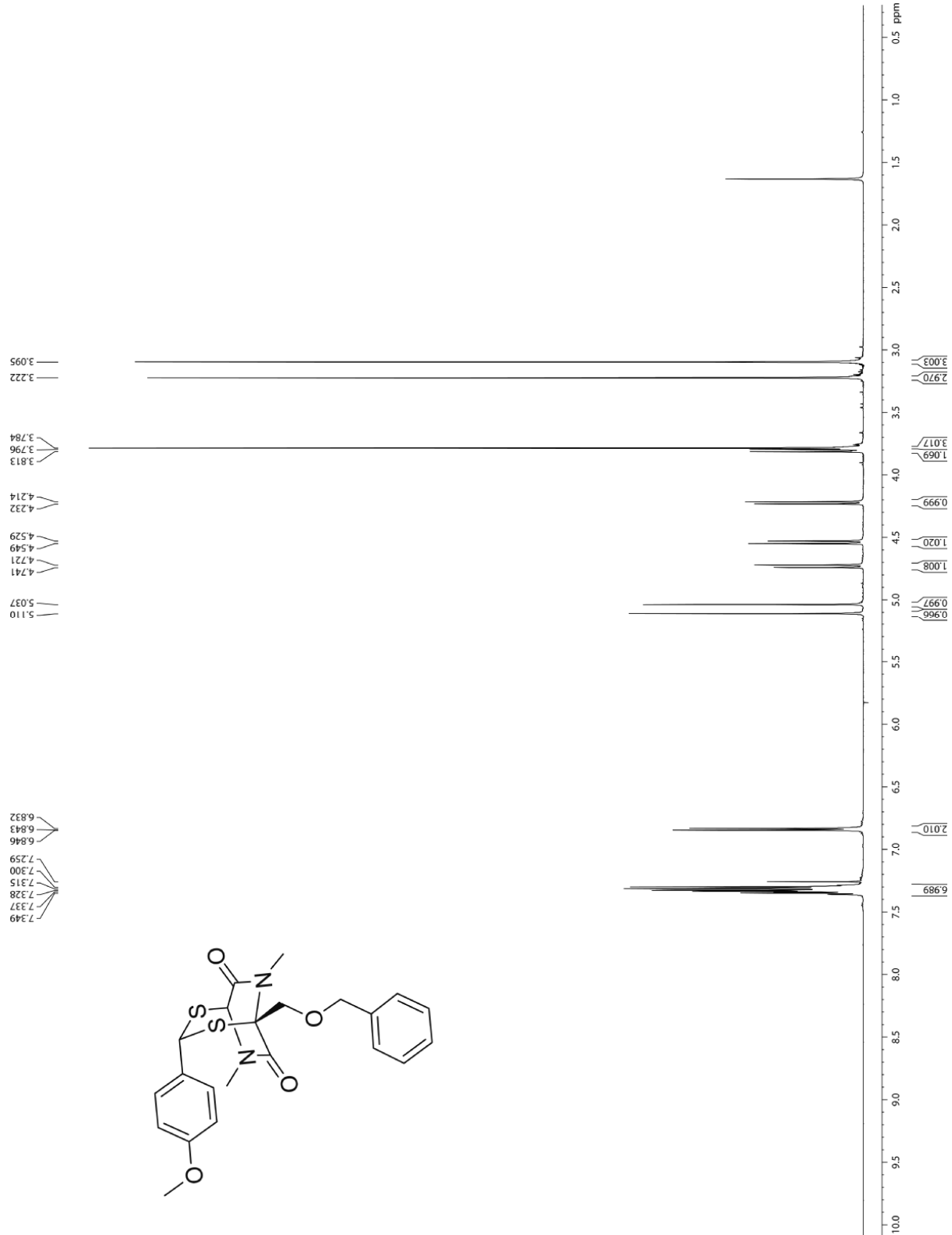


Figure N1. ^{1}H NMR spectrum of **7** (600 MHz, $CDCl_3$).

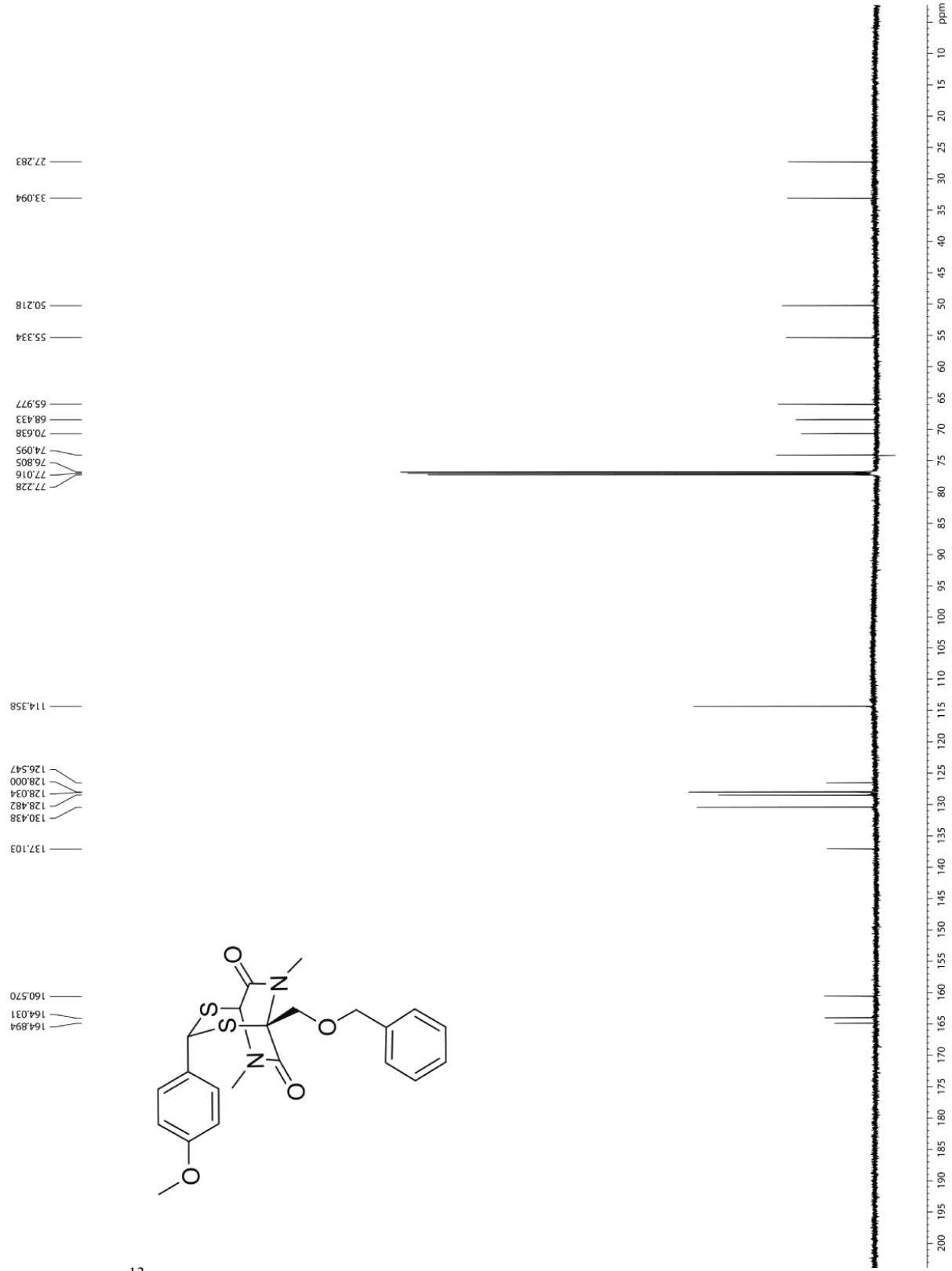


Figure N2. ^{13}C NMR spectrum of **7** (600 MHz, $CDCl_3$).

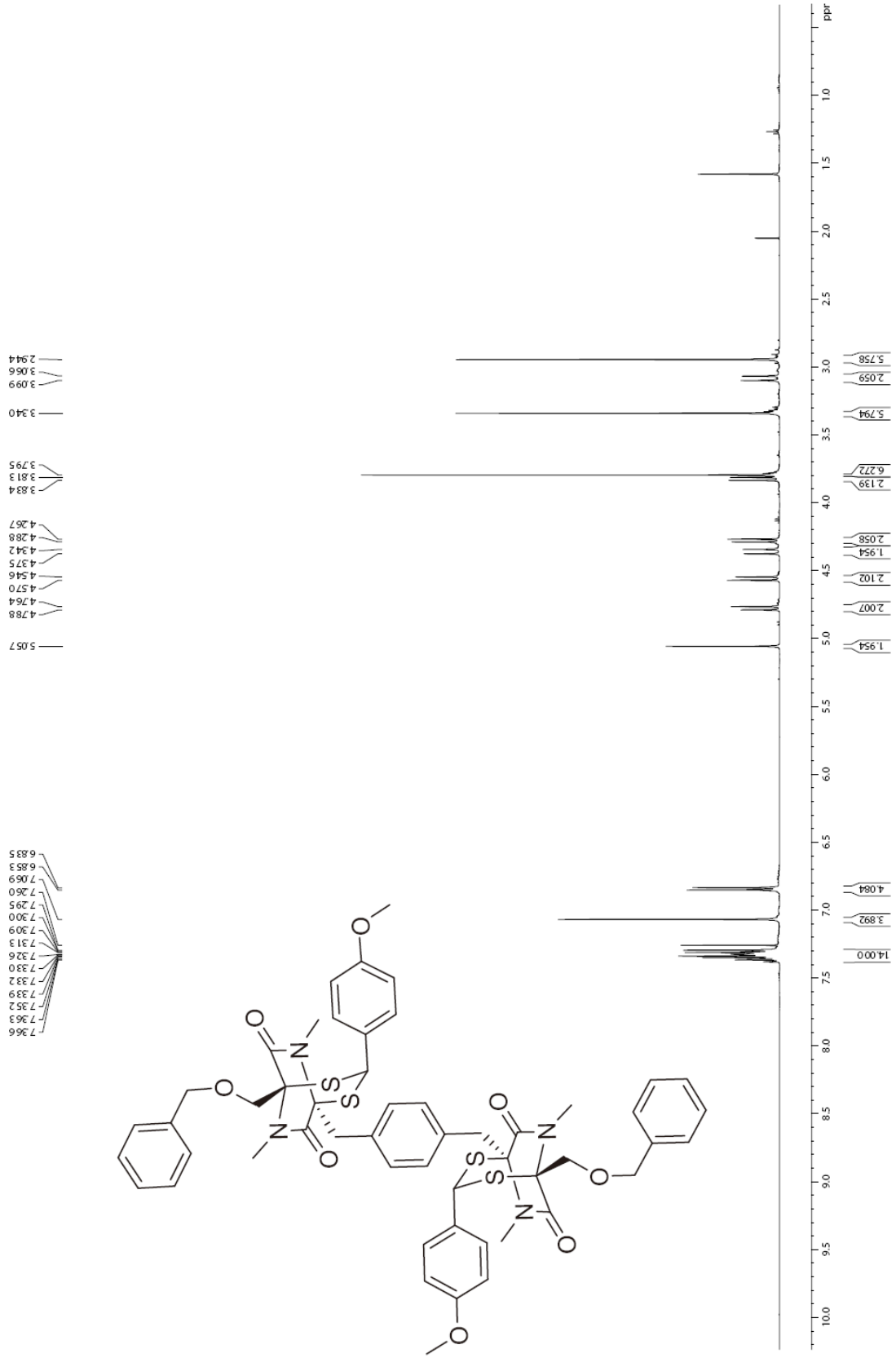


Figure N3. ^{1}H NMR spectrum of **(±)-8** (500 MHz, $CDCl_3$).

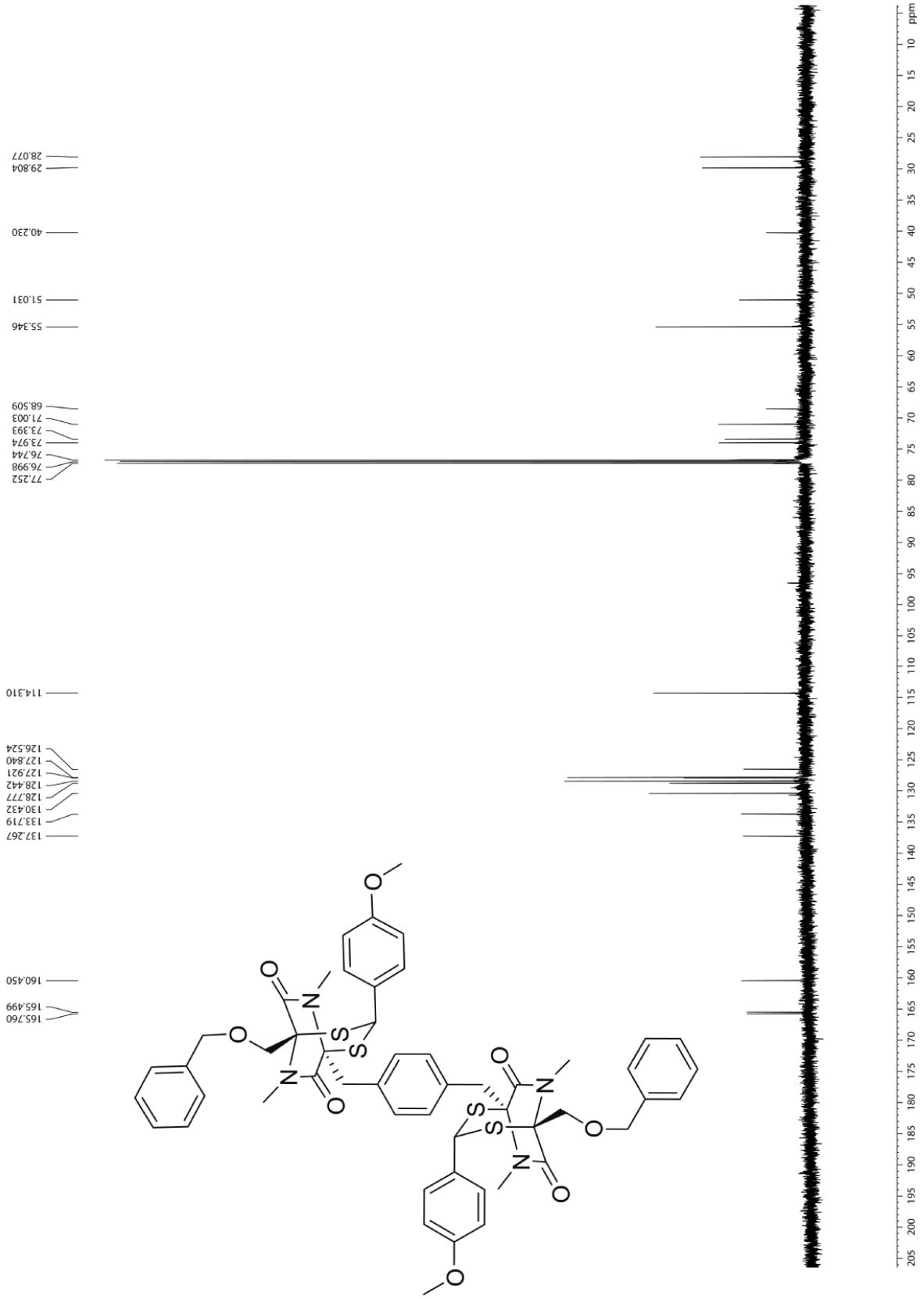


Figure N4. ^{13}C NMR spectrum of **(±)-8** (500 MHz, $CDCl_3$).

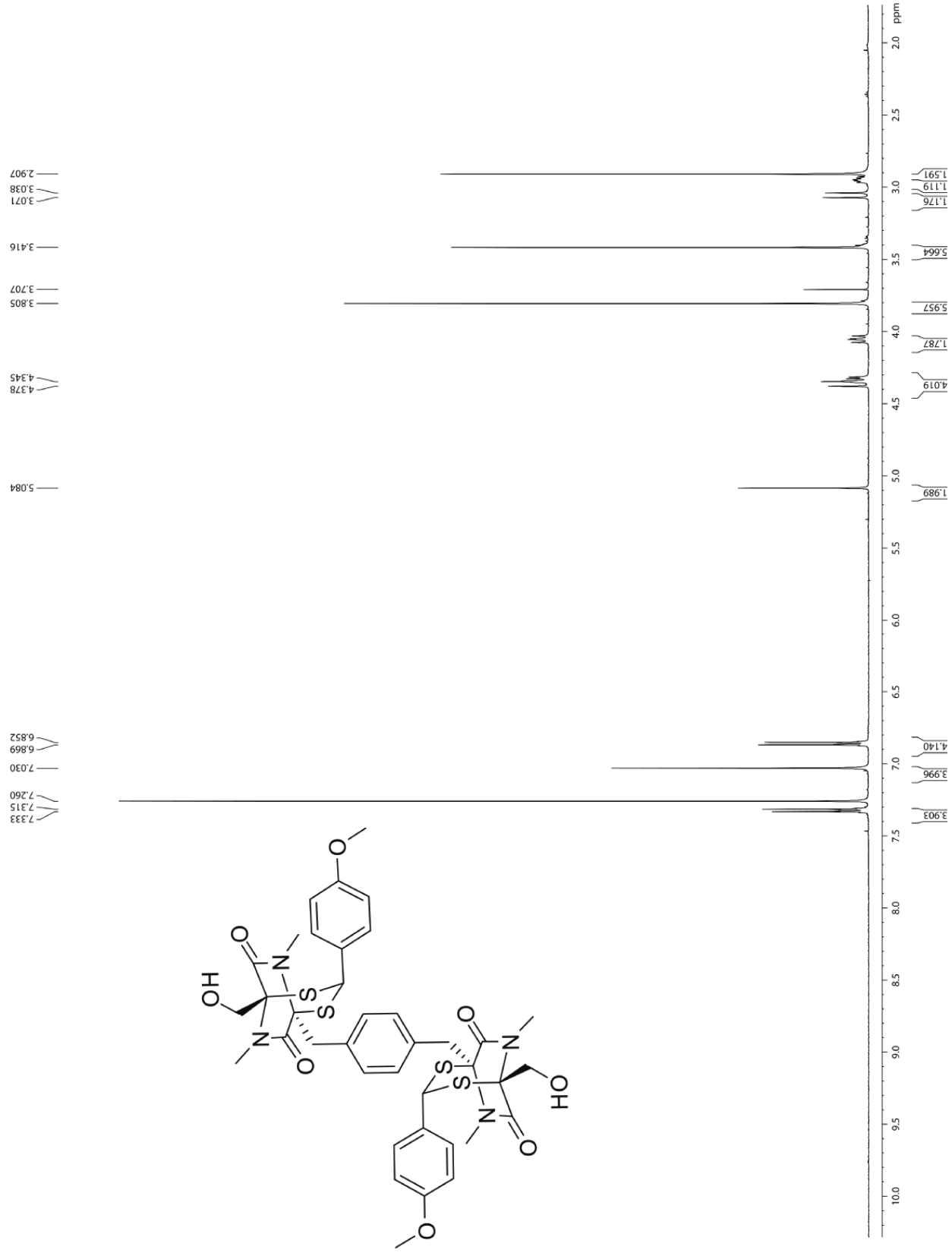


Figure N5. ^{1}H NMR spectrum of **(±)-9** (500 MHz, $CDCl_3$).

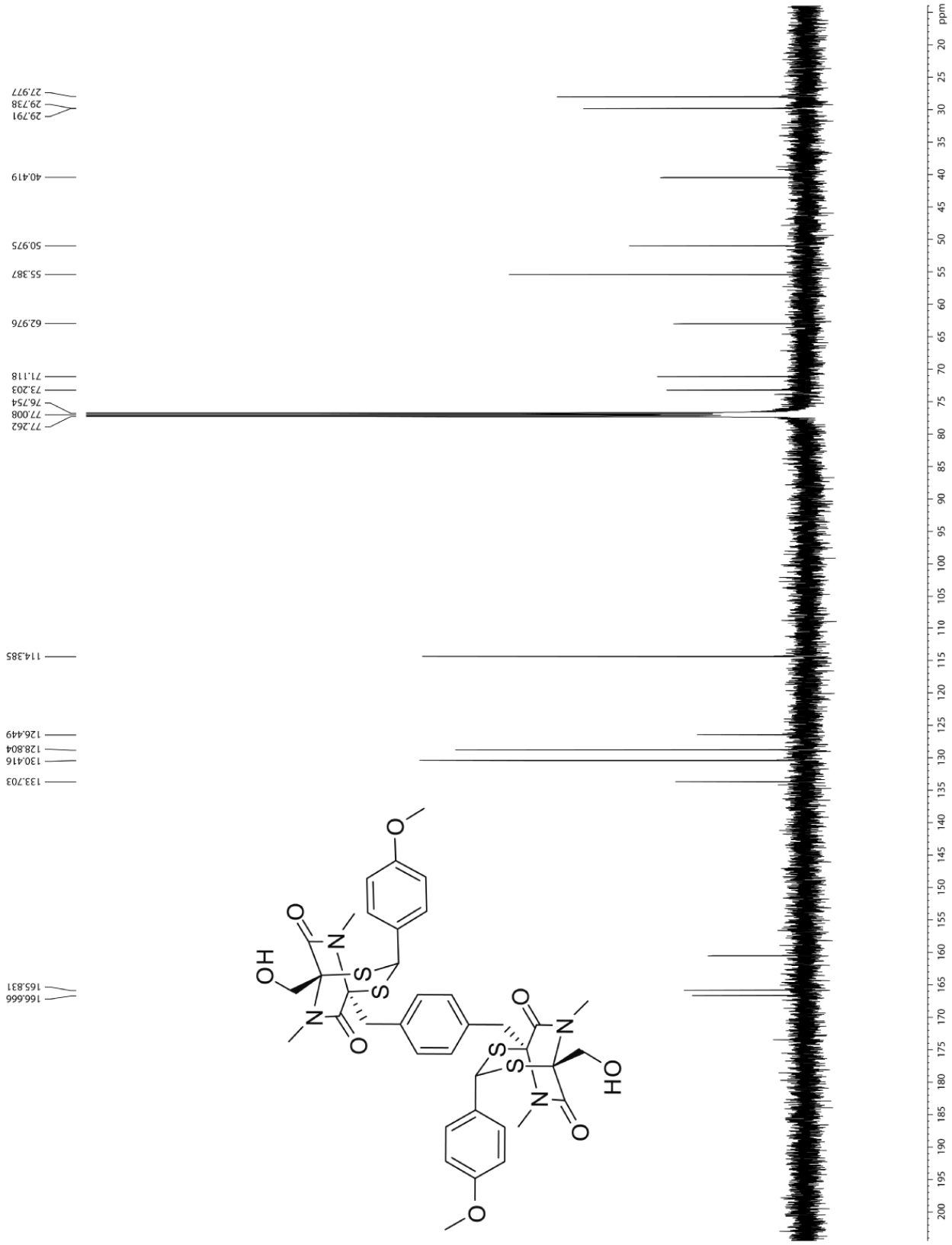


Figure N6. ^{13}C NMR spectrum of **(±)-9** (500 MHz, $CDCl_3$).

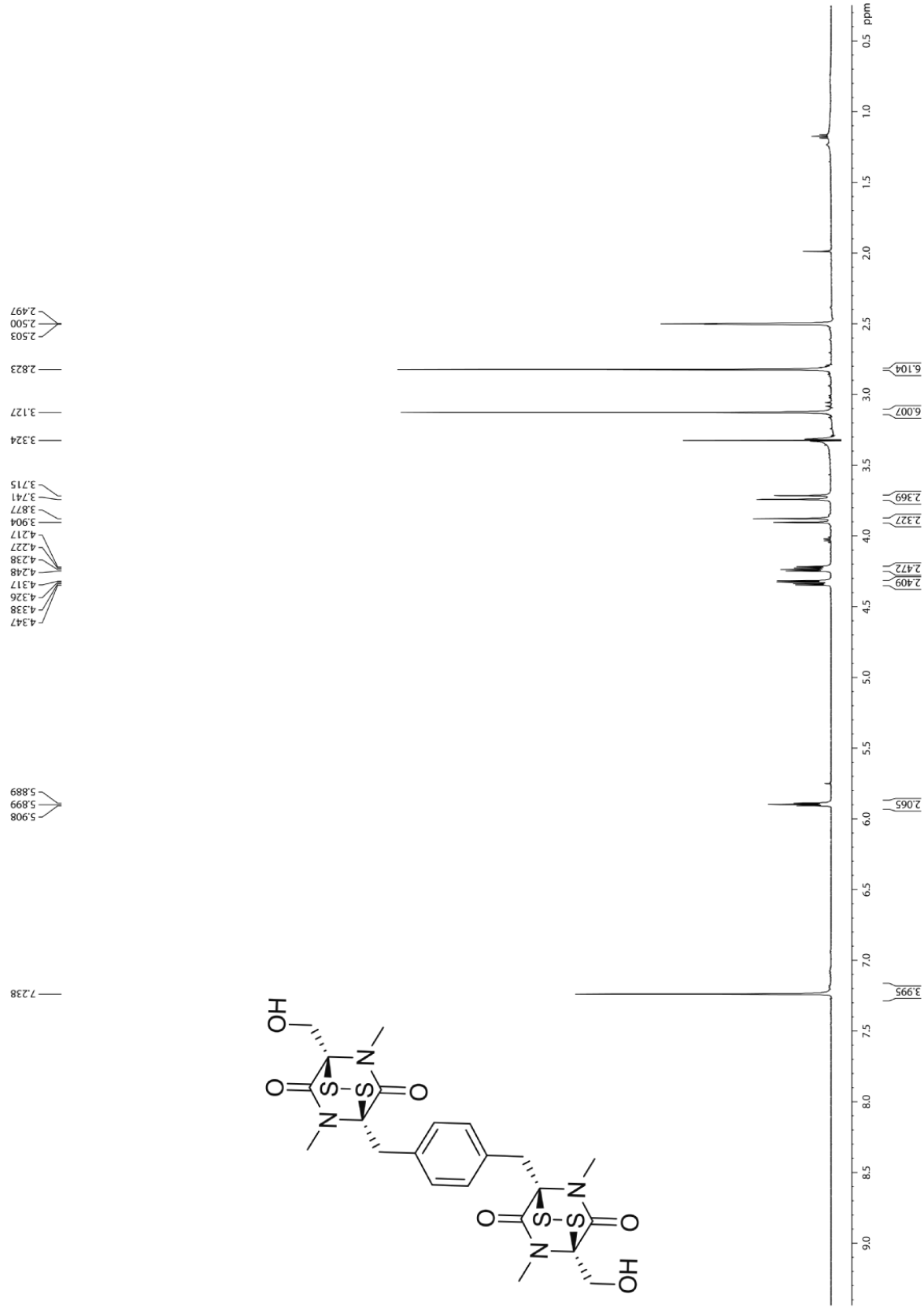


Figure N7. ^{1}H NMR spectrum of **(±)-3** (600 MHz, DMSO-D_6).

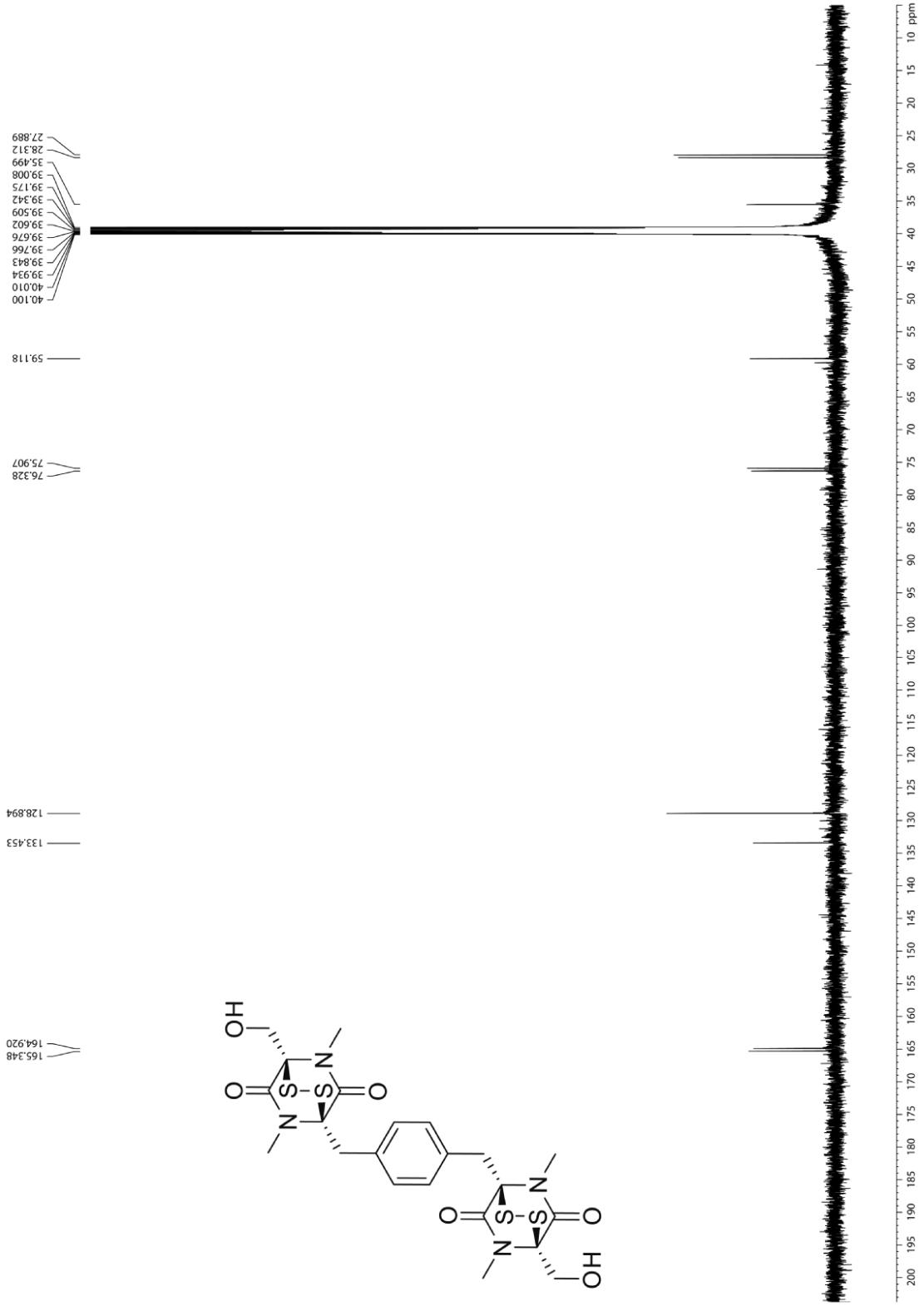


Figure N8. ^{13}C NMR spectrum of **(±)-3** (500 MHz, DMSO-D_6).

5. HPLC Analysis

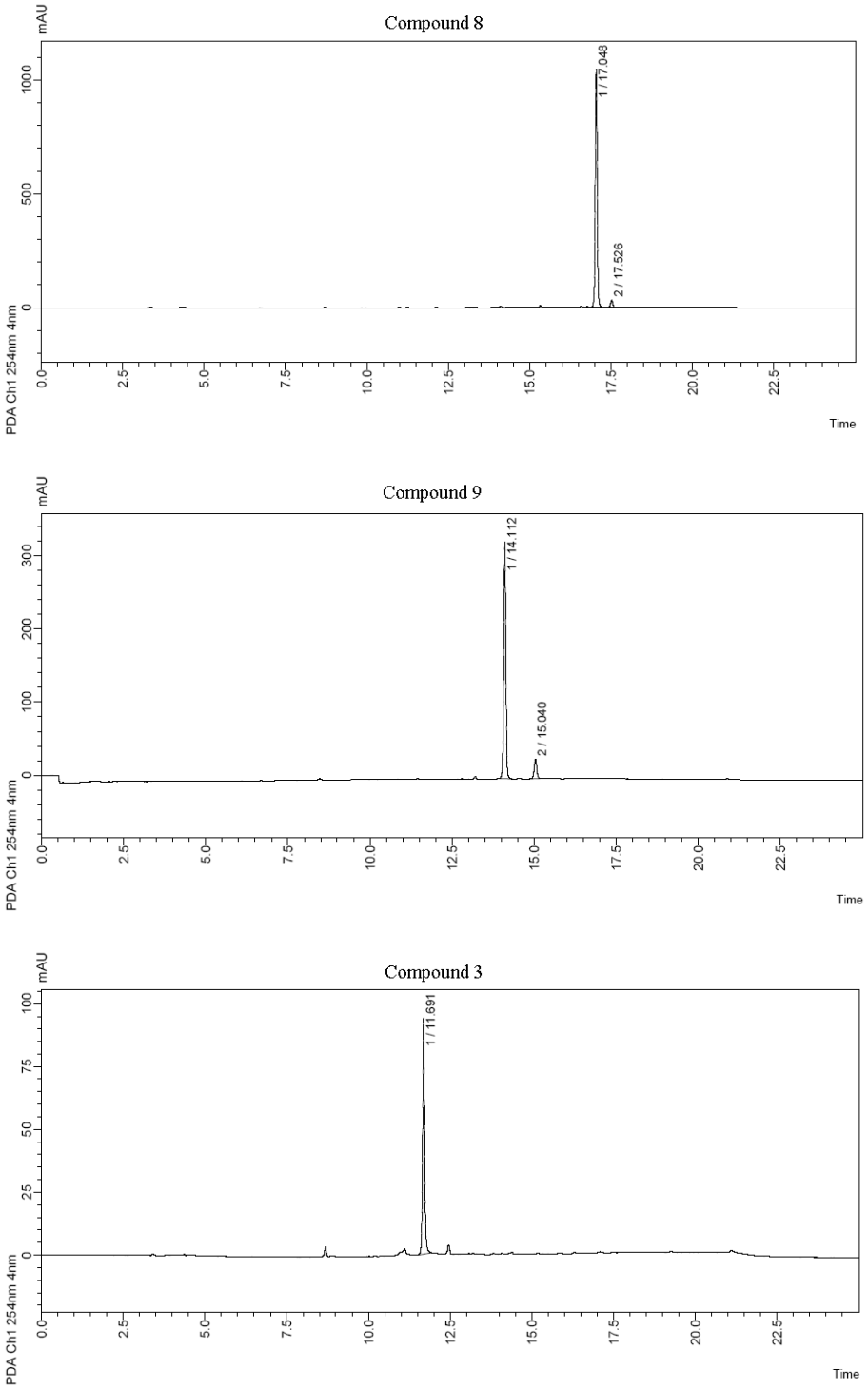


Figure H1. Reverse phase HPLC traces (254 nm) for compounds **(±)-8**, **(±)-9** and **(±)-3**. HPLC conditions: Apollo C_8 preparative reverse phase column, 5% B to 95% B in 15 min, 95% B to 95% B in 3 min, 95% B to 5% B in 5 min; A: 0.1% aqueous TFA, B: acetonitrile; flow rate: 5.0 mL/min; monitored at λ=254 nm.

Table S1. Change in the levels of expression of common transcription factors relative to vehicle in MCF7 cells treated with ETP **3** or DKP **4** under hypoxic conditions (300 μM DFO).

Gene Symbol	Fold Change by **3**	Fold Change by **4**	RefSeq	Name	Function
p53	-0.4	-0.2	NM_00546	Tumor Suppressor p53	DNA Damage, Cell Cycle/Growth Arrest
NFκβ	0.7	-0.3	NM_021975	Nuclear Factor κβ	Transcription Factor, regulates genes involved in inflammation
FOXA1	0.7	-0.4	NM_004496	Forkhead Box A1	Transcription Factor
CREB1	0.7	0.0	NM_004379	cAMP Responsive Element Binding Protein 1	Transcription Factor
E2F1	0.4	-0.5	NM_005225	E2F Transcription Factor	Cell Cycle Regulation/Tumor Suppressor
c-MYB	0.3	0.1	NM_005375	Myeloblastosis Viral Oncogene Homolog	Transcription Factor
ATF6	-0.4	-0.1	NM_007348	Activating Transcription Factor 6	Transcriptional Regulation of Endoplasmic Reticulum Proteins
ETS-2	1.2	0.0	NM_005239	Erythroblastosis Virus E26 Oncogene Homolog 2	Transcription Factor; regulates genes involved in senescence and death, tumorigenesis
ERBB2	-0.3	0.0	NM_001005862	Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2)	Growth Factor, Oncogene
GTF2F1	0.3	0.0	NM_002096	Transcription Factor II F (TFIIF)	General Transcription Factor
GTF35C	0.4	0.0	NM_001521	Transcription Factor II C (TFIIC)	General Transcription Factor
TAF13	0.9	0.0	NM_005645	TAF 13 RNA Polymerase II, TBP-associated Factor	General Transcription Factor
TAF10	0.1	-0.3	NM_006284	TAF 10 RNA Polymerase II, TBP-associated Factor	General Transcription Factor