Supporting Information

Withaferin A analogs that target the AAA+ chaperone p97

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Supporting Methods

Supporting Figures

A Chemical synthesis. Withaferin A (**1**) and its analogues **2**–**8** and **10**–**16** were obtained from aeroponically grown *Withania somnifera*, microbial biotransformation of **1** followed by acetylation, and chemical transformation and/or derivatization of **1,** as described previously.[S1–S3] Unless otherwise stated, chemicals were of reagent grade and used as obtained from commercial sources without further purification. Solvents purchased from commercial sources were redistilled before use. Analytical and preparative thin layer chromatography (TLC) was performed on pre–coated 0.20 mm thick plates of Silica gel 60 F_{254} (EM Sciences) followed by staining with acidic anisaldehyde. All compounds were purified by preparative TLC (pTLC) using 250 µm thick preparative silica gel TLC plate (EM Sciences). NMR spectra were recorded in $CDCl_3$ using residual solvent as internal standard on a 400 MHz Avance III spectrometer (Bruker). The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants (J values) are in Hz. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High resolution MS analyses were recorded on HX110A (JEOL) and TOF spectrometer equipped with an ESI source in positive and negative modes (Shimadzu). Optical rotations were measured in MeOH or $CHCl_3$ with a DIP–370 digital polarimeter (Jasco). IR spectra for KBr discs were recorded on an FTIR–8300 spectrometer (Shimadzu).

A.1. Preparation 4,22(23),27–tridehydrowithaferin A (9) and 4–dehydrowithaferin A (10): Activated MnO_2 (50 mg) was added to solution of withaferin A (**1**; 10 mg, 21.3 µmol) in $CHCl_3$ (1.0 mL) and EtOAc (1.4 mL). After 14 h at rt, the reaction mixture was filtered, filtrate was evaporated under reduced pressure, and the crude material was purified by pTLC eluting with 8% MeOH in CH_2Cl_2 to afford 1.6 mg (24%) 4,22(23),27–tridehydrowithaferin A (**9**) and 4.6 mg (69%) of known 4–dehydrowithaferin A (**10**).[S3] Spectroscopic data for **9** has been provided.

4,22(23),27–Tridehydrowithaferin A (9): white solid; R_f = 0.87 (8% MeOH in CH_2Cl_2); $[\alpha]_D^{25}$ = +132 (*c*, 1.0, MeOH); IR (KBr): ν_{max} 2925, 1718, 1689, 1618, 1527, 1456, 1379, 1263, 1112, 1056 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 10.24 (s, 1H, H–27), 6.85 (d, J = 10.4 Hz, 1H, H–2), 6.84 (d, J = 10.4 Hz, 1H, H–3), 5.93 (s, 1H, H–23), 3.41 (brd, J = 2.2 Hz, 1H, H–6), 2.51 (s, 3H, H_3–28), 2.48 (dq, J = 10.6, 6.8 Hz, 1H, H–20), 2.13 (dt, J = 14.9, 3.3 Hz, 1H, H–7a), 2.04 (dt, J = 9.1, 3.0 Hz, 1H, H–11a), 1.95 (dd, J = 9.1, 3.0 Hz, 1H, H–12a), 1.74 (m, 1H, H–17), 1.62–1.50 (m, 3H, H–8, H–15a, H–16a), 1.50–1.39 (m, 4H, H–7b, H–9, H–11b, H–12b), 1.38 (s, 3H, H_3–19), 1.28 (d, J = 6.8 Hz, H_3–21), 1.22 (m, 1H, H–15b), 1.11–1.05 (m, 2H, H–14, H–16b), 0.17 (s, 3H, H_3–18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.9 (C, C–1), 193.8 (C, C–4), 190.5 (CH, C–27), 174.3 (C, C–22), 163.5 (C, C–24), 163.1 (C, C–26), 141.6 (CH, C–3), 139.2 (CH, C–2), 115.2 (C, C–25), 108.3 (CH, C–23), 64.0 (C, C–5), 63.5 (CH, C–6), 55.4 (CH, C–14), 52.7 (CH, C–17), 49.8 (C, C–10), 43.6 (CH, C–9), 42.6 (CH, C–20), 42.2 (C, C–13), 39.2 (CH_2, C–12), 30.4 (CH_2, C–7), 29.7 (CH, C–8), 27.5 (CH_2, C–15), 24.1 (CH_2, C–16), 23.4 (CH_2, C–11), 21.0 (CH_3, C–28), 19.1 (CH_3, C–19), 18.2 (CH_3, C–21), 12.1 (CH_3, C–18); HRMS (FAB) calcd for $C_{28}H_{33}O_6$ $[M+H]^+$: 465.2277; found 465.2292.

A.2. Preparation of 27–acetyl–3β–azido–2(3)–dihydrowithaferin A (17) and 3β,27–diazido–2(3)–dihydro–27–deoxywithaferin A (19): Triethylamine was added drop wise to a solution of $TMSN_3$ (30 μL, 226.4 µmol) in anhydrous MeOH (0.4 mL) at rt to maintain the pH of 8.5. A solution of 27–acetylwithaferin A (**2**)[S4] (10.0 mg, 19.5 μmol) in anhydrous MeOH (0.6 mL) was added to the methanolic solution of $TMSN_3$. After stirring for 4 h at rt (progress of the reaction was monitored by TLC), the reaction mixture was evaporated under reduced pressure, H_2O (2.5 mL) was added to the residue and extracted with $CHCl_3$ (3 × 5 mL). The $CHCl_3$ layers were combined, dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by pTLC eluting with 3% MeOH in Et_2O to afford 2.9 mg (27%) of 27–acetyl–3β–azido–2(3)–dihydrowithaferin A (**17**) and 1.7 (16%) of 3β,27–diazido–2(3)–dihydro–27–deoxywithaferin A (**19**).

27–Acetyl–3β –azido–2(3)–dihydrowithaferin A (17): white solid, R_f = 0.73 (3% MeOH in Et_2O); $[\alpha]_D^{25}$ = +9 (*c*, 3.0, $CHCl_3$); IR (KBr): v_{max} 3415, 2945, 2098, 1737, 1712, 1398, 1380, 1234, 1028 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.88 (d, *J* = 11.7 Hz, 1H, H–27a), 4.84 (d, *J* = 11.7 Hz, 1H, H–27b), 4.39 (dt, *J* = 13.2, 3.5 Hz, 1H, H–22), 4.07 (dt, *J* = 7.0, 4,3 Hz, 1H, H–3), 3.45 (t, *J* = 4.3 Hz, 1H, H–4), 3.24 (brs, 1H, H–6), 3.08 (dd, *J* = 15.8, 7.0 Hz, 1H, H–2a), 2.52 (dd, *J* = 15.8, 4.3 Hz, 1H, H–2b), 2.51 (m, 1H, H–23a), 2.18 (dt, *J* = 13.4, 2.2 Hz, 1H, H–7a), 2.06 (s, 3H, H_3–28), 2.04 (s, 3H, OAc), 2.03–1.95 (m, 2H, H–20, H–23b), 1.91 (dt, *J* = 12.8, 3.4 Hz, 1H, H–12a), 1.70–1.60 (m, 2H, H–15a, H–16a), 1.45–1.32 (m, 5H, H–7b, H–8, H_2–11, H–15b), 1.30 (s, 3H, H_3–19), 1.26 –1.05 (m, 4H, H–9, H–12b, H–16b, H–17), 0.97 (d, *J* = 6.6 Hz, 3H, H_3–20), 0.96 (m, 1H, H–14), 0.65 (s, 3H, H_3–18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.4 (C, C–1), 170.9 (C, OAc), 165.3 (C, C–26), 156.9 (C, C–24), 121.9 (C, C–25), 78.2 (CH, C–22), 75.2 (CH, C–4), 64.0 (C, C–5), 60.2 (CH, C–6), 58.9 (CH, C–3), 58.0 (CH_2, C–27), 56.0 (CH, C–14), 51.9 (CH, C–17), 50.4 (C, C–10), 42.7 (C, C–13), 42.6 (CH, C–9), 39.1 (CH_2, C–12), 38.8 (CH, C–20), 38.6 (CH_2, C–2), 30.9 (CH_2, C–23), 29.9 (CH_2, C–7), 29.3 (CH, C–8), 27.2 (CH_2, C–14), 24.2 (CH_2, C–15), 21.7 (CH_2, C–11), 20.9 (CH_3, OAc), 20.7 (CH_3, C–28), 16.0 (CH_3, C–19), 13.3 (CH_3, C–21), 11.6 (CH_3, C–18); HRMS (ESI) *m/z* calcd for $C_{30}H_{41}N_3NaO_7$ $[M+Na]^+$: 578.2838; found 578.2847.

3β,27–Diazido–2(3)–dihydro–27–deoxywithaferin A (19): white solid, R_f = 0.83 (3% MeOH in Et_2O); $[\alpha]_D^{25}$ = +13.5 (*c*, 2.0, $CHCl_3$); IR (KBr): v_{max} 3415, 2945, 2096, 1708, 1458, 1396, 1255, 1130 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.40 (dt, *J* = 13.2, 3.4 Hz, 1H, H–22), 4.10 (s, 2H, H_2–27), 4.08 (dt, *J* = 6.9, 4,5 Hz, 1H, H–3), 3.45 (t, *J* = 4.5 Hz, 1H, H–4), 3.24 (s, 1H, H–6), 3.08 (dd, *J* = 15.7, 7.0 Hz, 1H, H–2a), 2.52 (dd, *J* = 15.7, 4.6 Hz, 1H, H–2b), 2.51 (m, 1H, H–23a), 2.17 (dt, *J* = 13.5, 2.3 Hz, 1H, H–7a), 2.05 (s, 3H, H_3–28), 2.02–1.95 (m, 2H, H–20, H–23b), 1.90 (dt, *J* = 12.6, 3.4 Hz, 1H, H–12a), 1.70–1.58 (m, 2H, H–15a, H–16a), 1.44–1.32 (m, 5H, H–7b, H–8, H_2–11, H–15b), 1.30 (s, 3H, H_3–19), 1.28 –1.03 (m, 4H, H–9, H–12b, H–16b, H–17), 0.98 (d, *J* = 6.6 Hz, 3H, H_3–20), 0.96 (m, 1H, H–14), 0.65 (s, 3H, H_3–18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.4 (C, C–1), 165.8 (C, C–26), 156.1 (C, C–24), 121.8 (C, C–25), 78.5 (CH, C–22), 75.3 (CH, C–4), 64.0 (C, C–5), 60.1 (CH, C–6), 58.9 (CH, C–3), 56.0 (CH, C–14), 51.9 (CH, C–17), 50.4 (C, C–10), 45.6 (CH_2, C–27), 42.6 (C, C–13), 42.5 (CH, C–9), 39.0 (CH_2, C–12), 38.7 (CH, C–20), 38.6 (CH_2, C–2), 30.9 (CH_2, C–23), 29.9 (CH_2, C–7), 29.2 (CH, C–8), 27.2 (CH_2, C–14), 24.2 (CH_2, C–15), 21.6 (CH_2, C–11), 20.7 (CH_3, C–28), 15.9 (CH_3, C–19), 13.3 (CH_3, C–21), 11.6 (CH_3, C–18); HRMS (ESI) *m/z* calcd for $C_{28}H_{38}KN_3O_5$ $[M+K]^+$: 577.2535; found 577.2525.

A.3. Preparation of 27–acetyl–2(3)–dihydrowithaferin A (18). Et_3N (60 μL, 431 μmol) and 10% Pd on C (2.0 mg) were added to solution of withaferin A (**1**; 10 mg, 21.3 μmol) in EtOH (1.0 mL). The mixture was degassed and charged with an atmosphere of H_2. After 30 min, the reaction mixture was filtered and filtrate was evaporated under reduced pressure to afford 10.0 mg (99%) of 2,3–dihydrowithaferin A (**13**).[S4] Ac_2O (5 μL, 61.8 μmol) was then added to a stirred solution of **13** (10 mg, 21.2 μmol) in pyridine (100 µL) at 0°C. After 1 h at 0°C, the reaction mixture was evaporated under reduced pressure. Repetitive evaporation of EtOH further facilitated the removal of pyridine. The residue was purified by pTLC eluting with 8% MeOH in CH_2Cl_2 to afford 2.8 mg (76%) of 27–acetyl–2(3)–dihydrowithaferin A (**18**).

27–Acetyl–2(3)–dihydrowithaferin A (18): white solid, R_f = 0.69 (8% MeOH in CH_2Cl_2); $[\alpha]_D^{25}$ = 0 (*c*, 3.0, $CHCl_3$); IR (KBr): v_{max} 3465, 2947, 1740, 1709, 1458, 1398, 1380, 1234, 1190, 1028 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.88 (d, *J* = 11.9, 1H, H–27a), 4.84 (d, *J* = 11.9, 1H, H–27b), 4.39 (dt, *J* = 13.3, 3.3 Hz, 1H, H–22), 3.50 (t, *J* = 3.8 Hz, 1H, H–4), 3.13 (brs, 1H, H–6), 2.65 (ddd, *J* = 16.0, 8.8, 7.4 Hz, 1H, H–2a), 2.55–2.45 (m, 2H, H–2b, H–23a), 2.19 (ddd, *J* = 14.9, 4.2, 2.0 Hz, 1H, H–7a), 2.14– 2.08 (m, 2H, H–3a, H–20), 2.06 (s, 3H, H_3–28), 2.04 (s, 3H, OAc), 1.93–2.01 (m, 2H, H–3b, H–23b), 1.90 (dt, *J* = 9.6, 3.4 Hz, H–12a), 1.72–1.58 (m, 2H, H–15a,

H–16a), 1.42–1.31 (m, 4H, H–8, H_2–11, H–15b), 1.30 (s, 3H, H_3–19), 1.26 (m, 1H, H–7b), 1.17–1.02 (m, 4H, H–9, H–12b, H–16b, H–17), 0.97 (d, J = 6.6 Hz, H_3–21), 0.93 (m, 1H, H–14), 0.65 (s, 3H, H_3–18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 211.5 (C, C–1), 170.9 (C, OAc), 165.3 (C, C–26), 156.9 (C, C–24), 121.9 (C, C–25),78.2 (CH, C–22), 72.7 (CH, C–4), 66.5 (C, C–5), 59.1 (CH, C–6), 58.0 (CH_2, C–27), 56.2 (CH, C–14), 51.9 (C, C–10), 50.5 (CH, C–17), 43.0 (CH, C–9), 42.7 (C, C–13), 39.1 (CH_2, C–12), 38.8 (CH, C–20), 31.8 (CH_2, C–2), 31.4 (CH_2, C–7), 30.1 (CH_2, C–23), 29.3 (CH, C–8), 27.3 (CH_2, C–15), 26.5 (CH_2, C–3), 24.2 (CH_2, C–16), 21.5 (CH_2, C–11), 20.9 (CH_3, OAc), 20.6 (CH_3, C–28), 15.6 (CH_3, C–19), 13.6 (CH_3, C–21), 11.5 (CH_3, C–18); HRMS (ESI) *m/z* calcd for $C_{28}H_{43}O_6$ $[M+H]^+$: 515.2931; found 515.2962.

K. Additional References

[S1] Y. Xu, M. T. Marron, E. Seddon, S. P. McLaughlin, D. T. Ray, L. Whitesell, A. A. L. Gunatilaka, *Bioorg. Med. Chem*. **2009**, *17*, 2210–2214.

[S2] Y. Xu, S. Gao, D. P. Bunting, A. A. L. Gunatilaka, *Phytochem*. **2011**, *72*, 518–522.

[S3] E. M. K. Wijeratne, Y. Xu, R. Scherz–Shouval, M. T. Marron, D. D. Rocha, M. X. Liu, L. V. Costa–Lotufo, S. Santagata, S. Lindquist, L. Whitesell, A. A. L. Gunatilaka, *J. Med. Chem*. **2014**, *57*, 2851–2863.

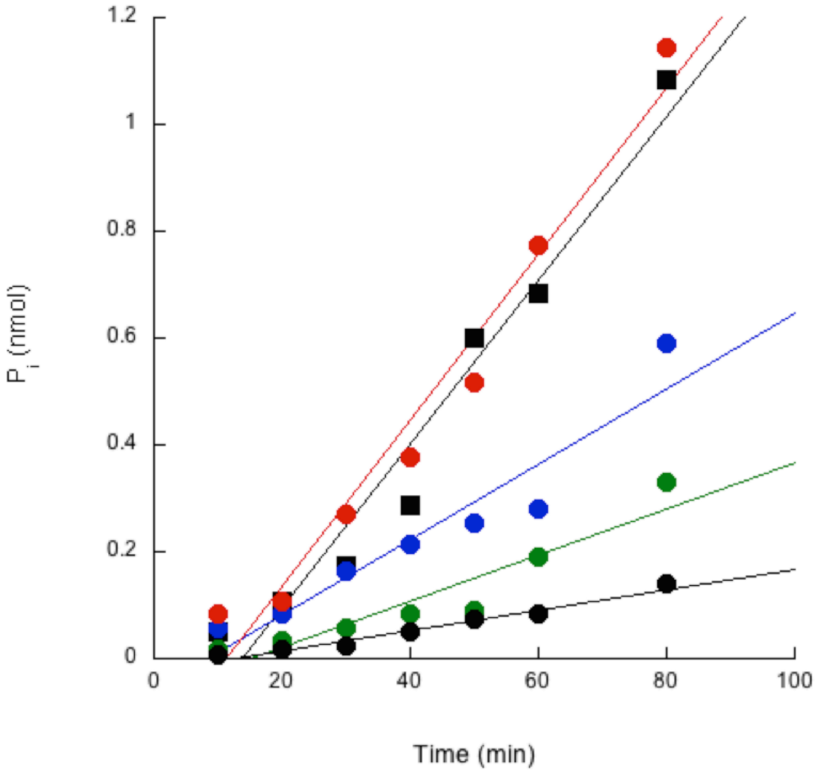

Figure S1. ATPase activities after prolonged p97–withanolide incubation and extensive dialysis. DMSO is given by black squares; **1** by blue circles; **4** by red circles; **6** by green circles; and **9** by black circles.

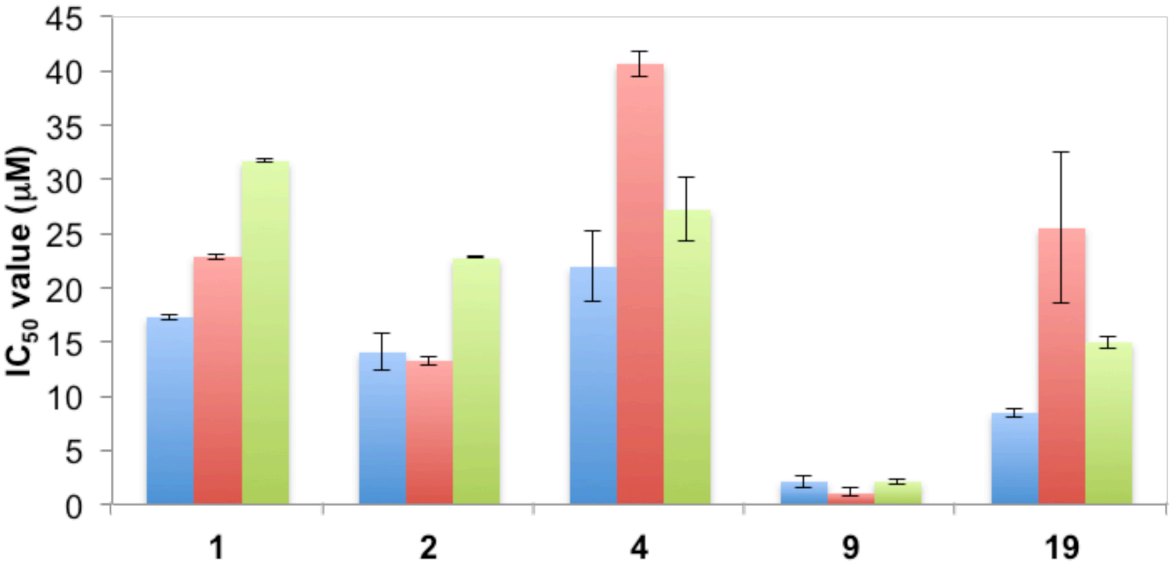

Figure S2. IC_{50} values of compounds at varying concentrations of ATP. Each bar is given by: 100 mM ATP (blue); 500 mM ATP (red); and 1 mM ATP (green).

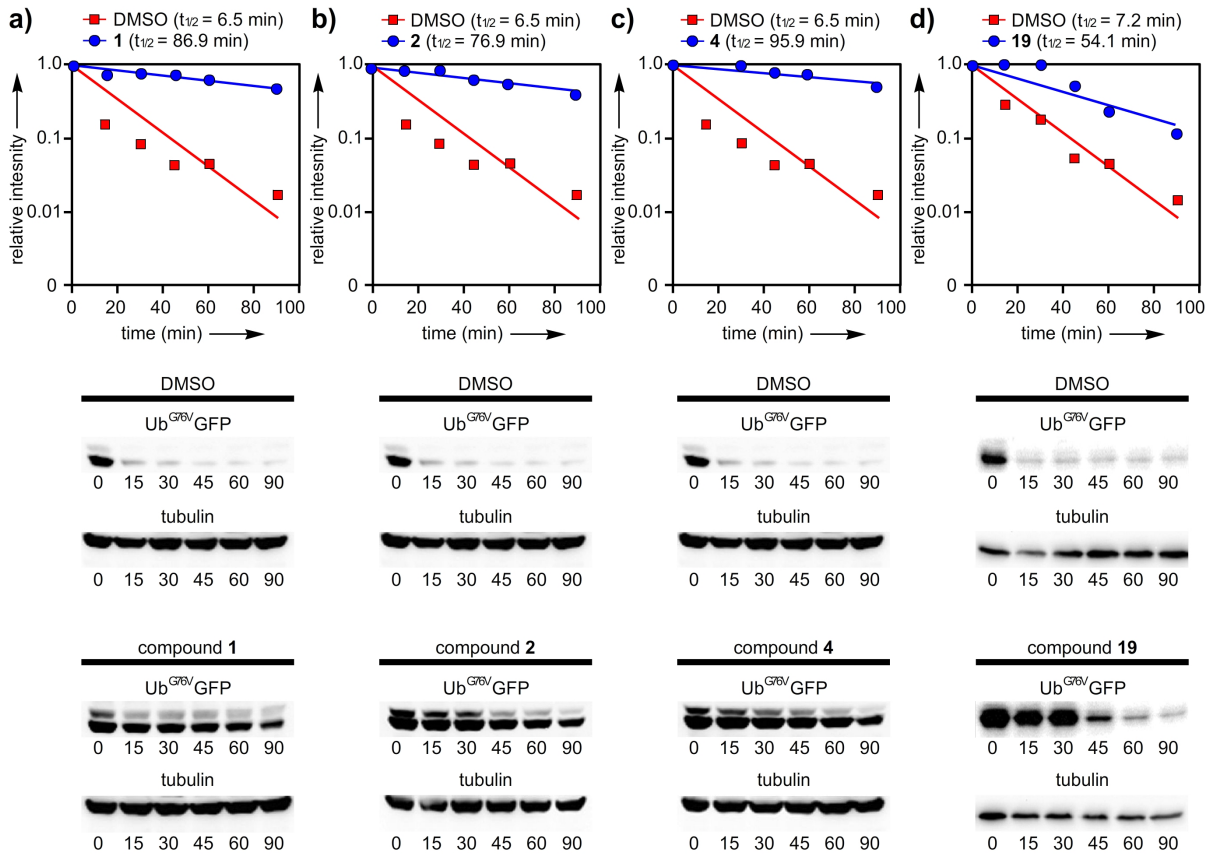

Figure S3. Half–life measurements for $Ub^{G76V}GFP$ degradation. The rate of degradation of $Ub^{G76V}GFP$ was measured by Western blotting with anti–GFP antibodies at the indicated times and quantitating by densitometry. Measurements were made in the presence of the indicated molecules and compared to DMSO. (bottom) plots depicting data from densitometric analyses comparing compounds **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4** or **d)** 1 μM **19** against DMSO (negative control). (top) Images of Western blots generated from $Ub^{G76V}GFP$ degradation after treatment with **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4** or **d)** 1 μM **19**.

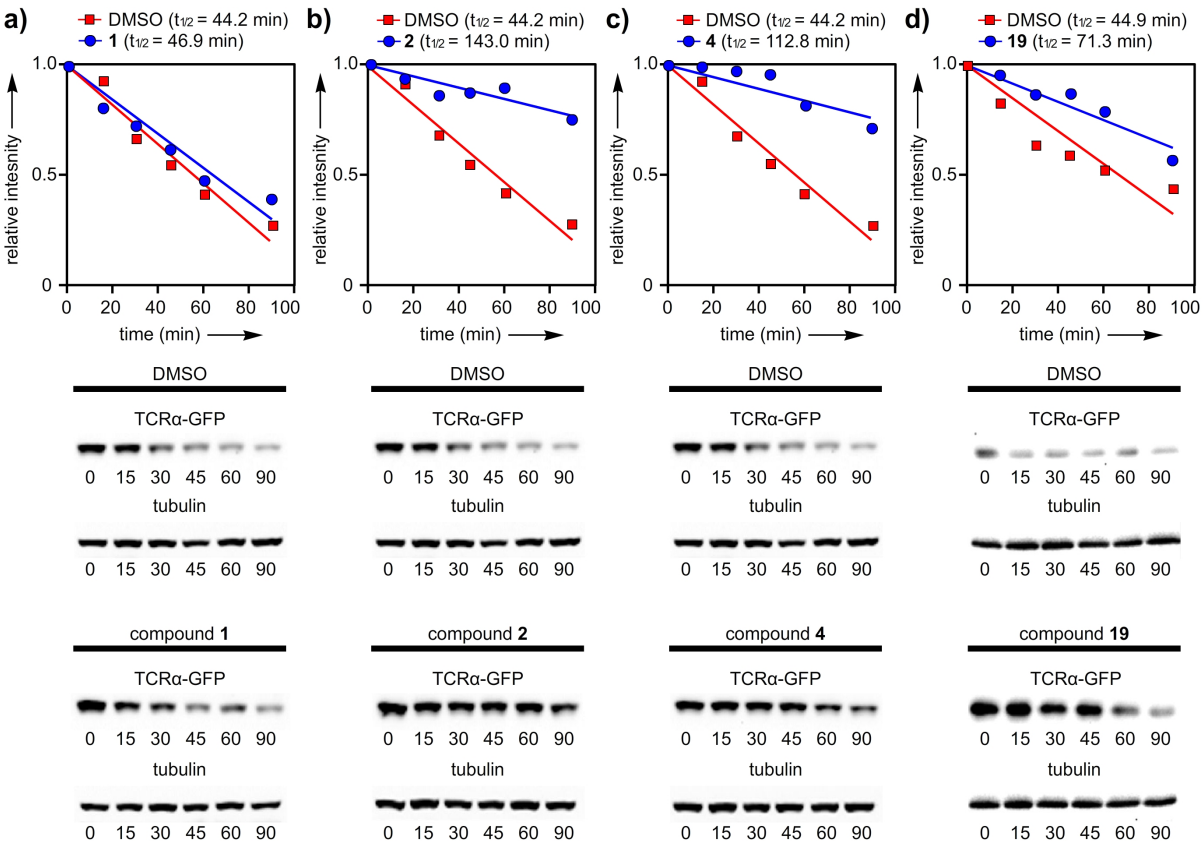

Figure S4. Half–life measurements for TCRα–GFP degradation. The rate of degradation of TCRα–GFP was measured by Western blotting with anti–GFP antibodies at the indicated times and quantitating by densitometry. (top) plots depicting data from densitometric analyses comparing compounds **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4** or **d)** 1 μM **19** against DMSO (negative control). (bottom) Images of Western blots generated from TCRα–GFP degradation after treatment with **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4** or **d)** 1 μM **19**.

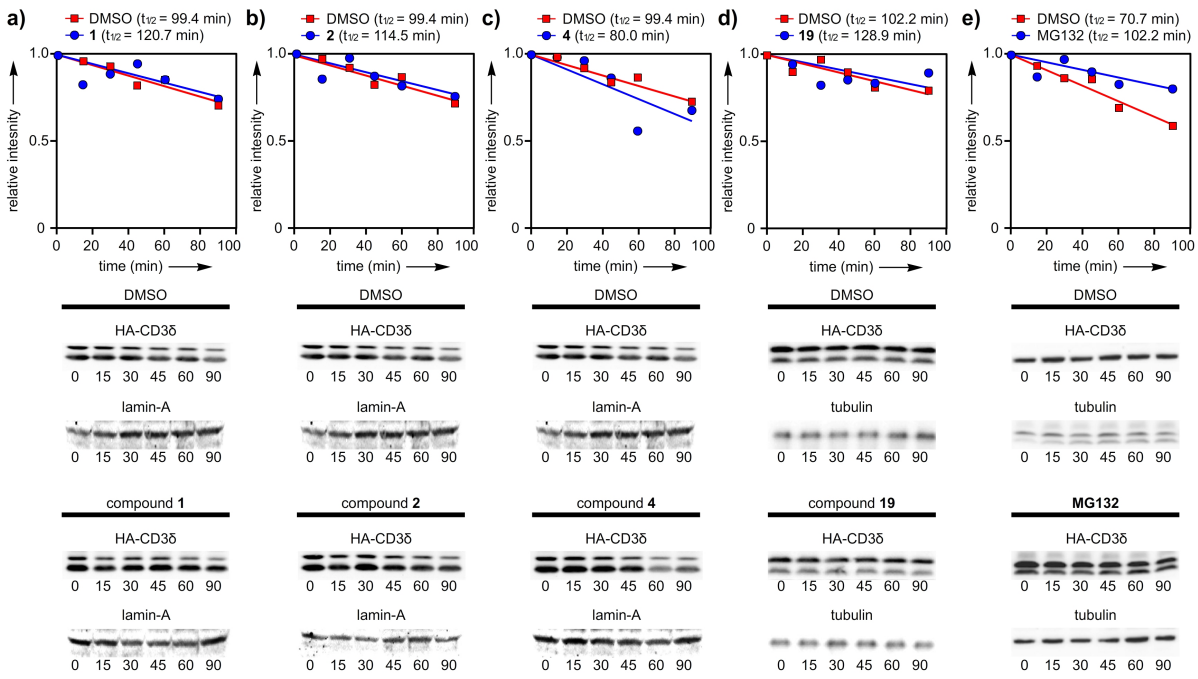

Figure S5. Half–life measurements for HA–CD3δ degradation. The rate of degradation of HA–CD3δ was measured by Western blotting with anti–HA antibodies at the indicated times and quantitating by densitometry. (top) Plots depicting data from densitometric analyses comparing compounds **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4** , **d)** 1 μM **19** or **e)** 5 μM MG132 (positive control) against DMSO (negative control). (bottom) Images of Western blots generated from HA–CD3δ degradation after treatment with **a)** 10 μM **1**, **b)** 1 μM **2**, **c)** 1 μM **4**, **d)** 1 μM **19** or **e)** 5 μM MG132 (positive control).

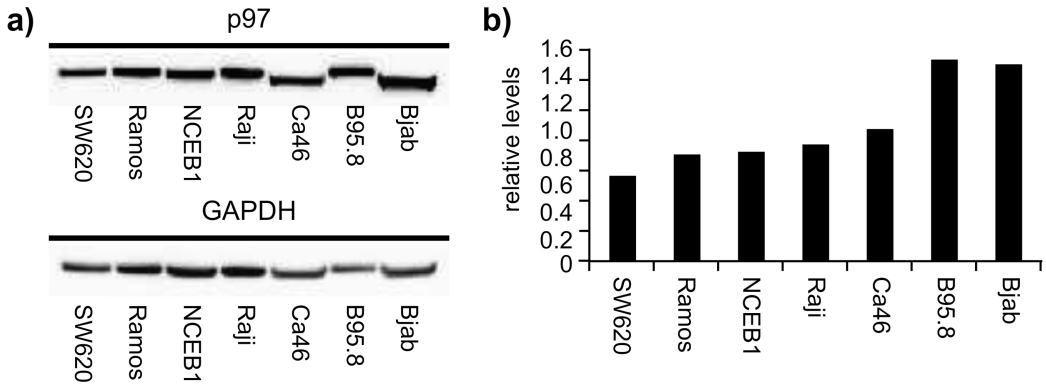

Figure S6. p97 levels in select cell lines. **a)** The level of p97 was measured by Western blotting with anti–p97 antibodies and **b)** quantified using densitometry, which is plotted relative to a GAPDH control.

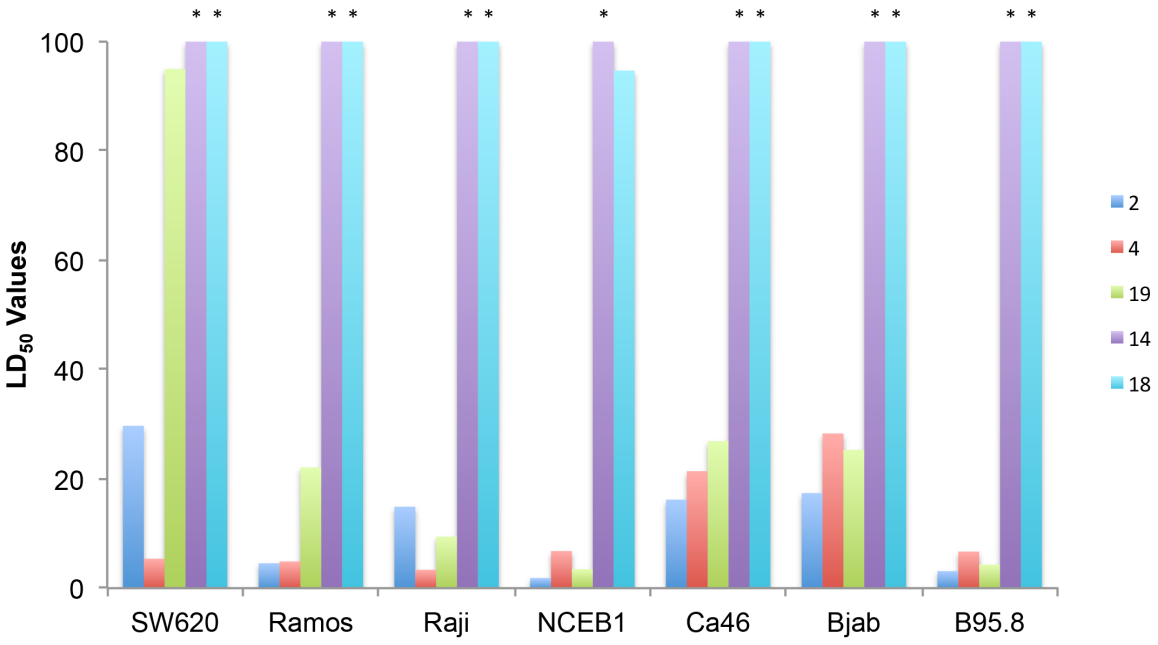

Figure S7. LD_{50} values versus a small panel of cancer cell lines.

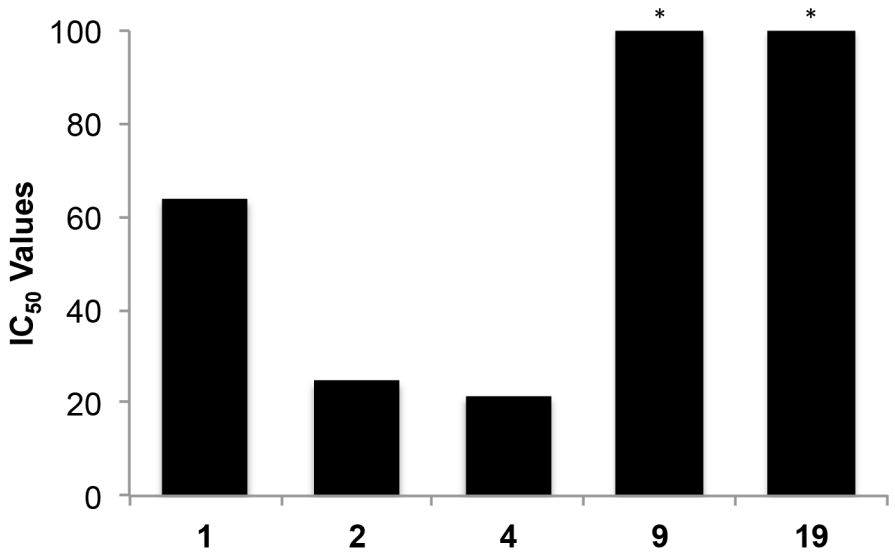

Figure S8. Inhibition of the 20S proteasome. IC_{50} values were calculated from 12–point dose–responses.

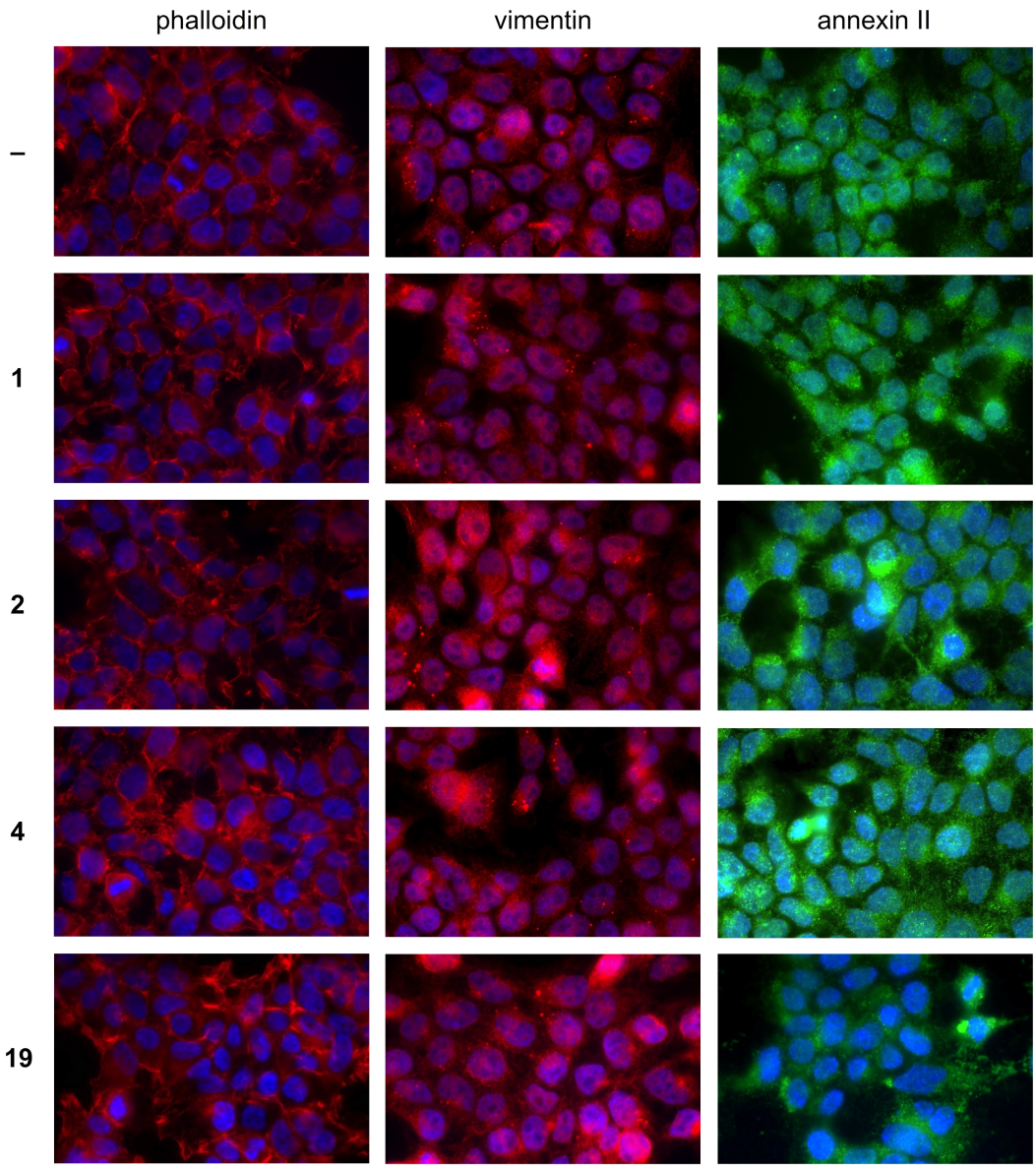

Figure S9. Confocal fluorescence microscopic imaging of HEK293 cells after staining with phalloidin (red), annexin II (green) or vimentin (red) with the nucleus stained using DAPI (blue). Cells were treated with either 10 μM **1**, 1 μM **2**, 1 μM **4**, or 1 μM **19** for 4 h prior to staining.

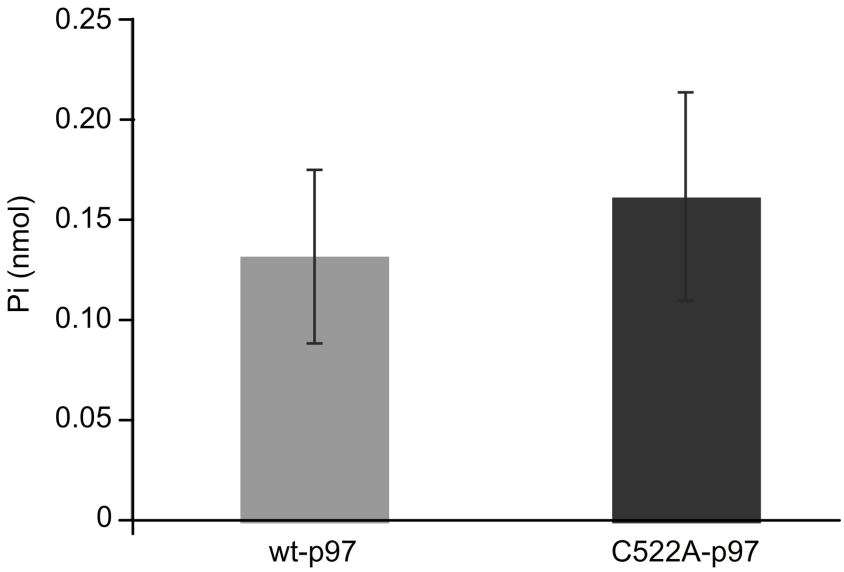

Figure S10. Comparative ATPase activity of wt-p97 and C552A-p97.

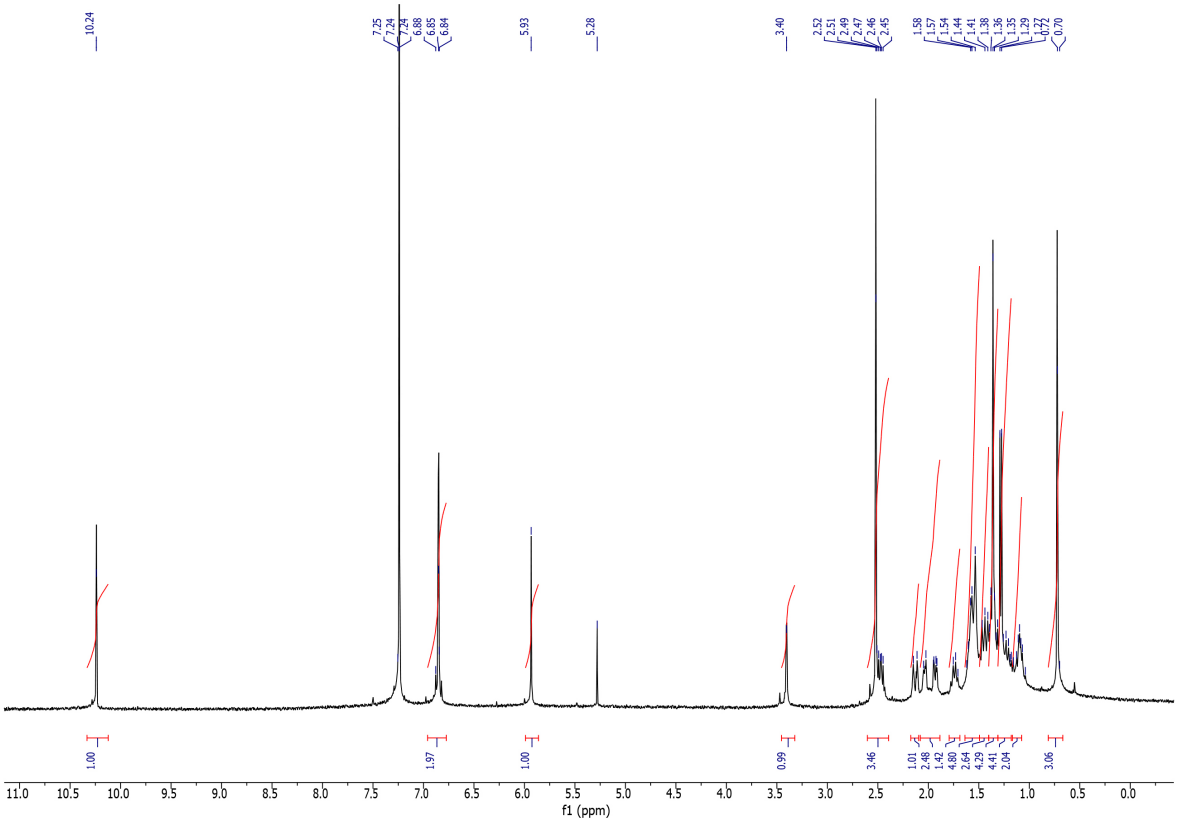

Figure S11. ^{1}H NMR spectrum of **9** in $CDCl_3$.

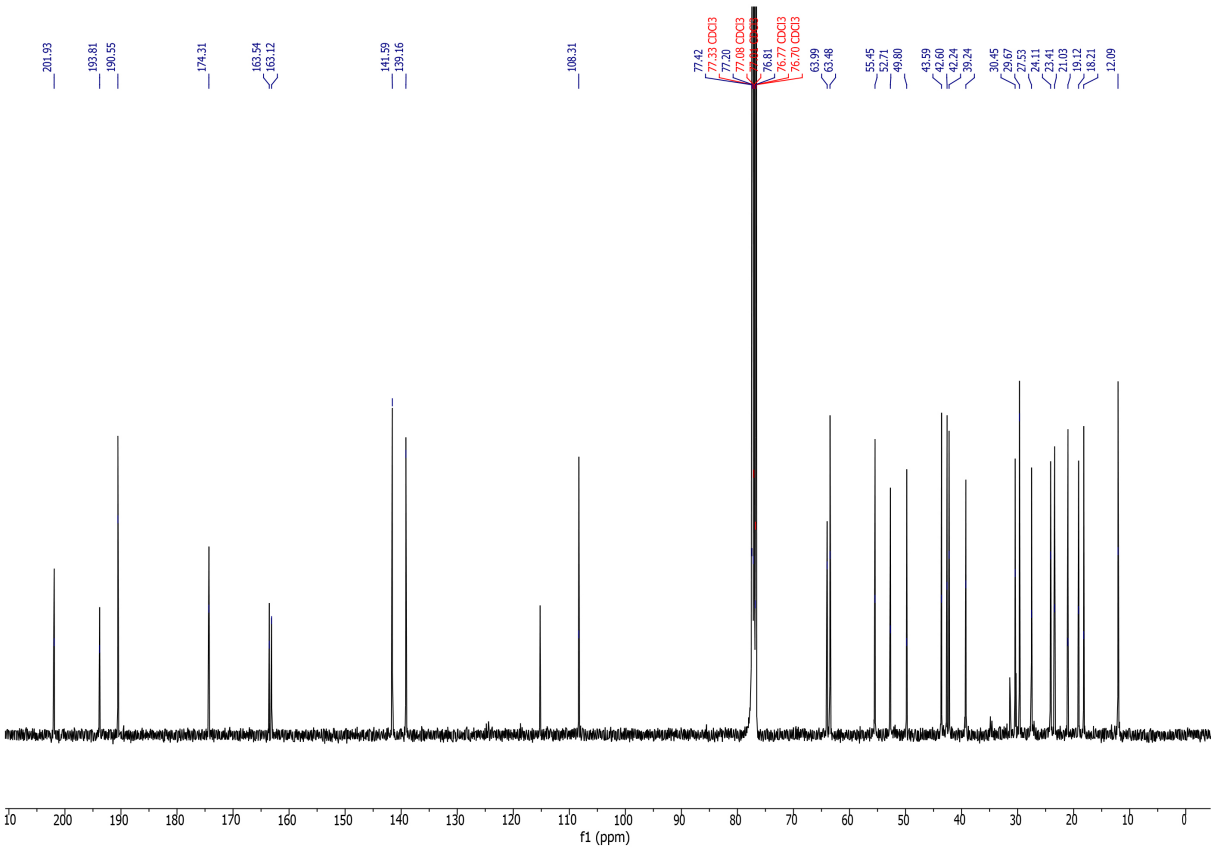

Figure S12. ^{13}C NMR spectrum of **9** in $CDCl_3$.

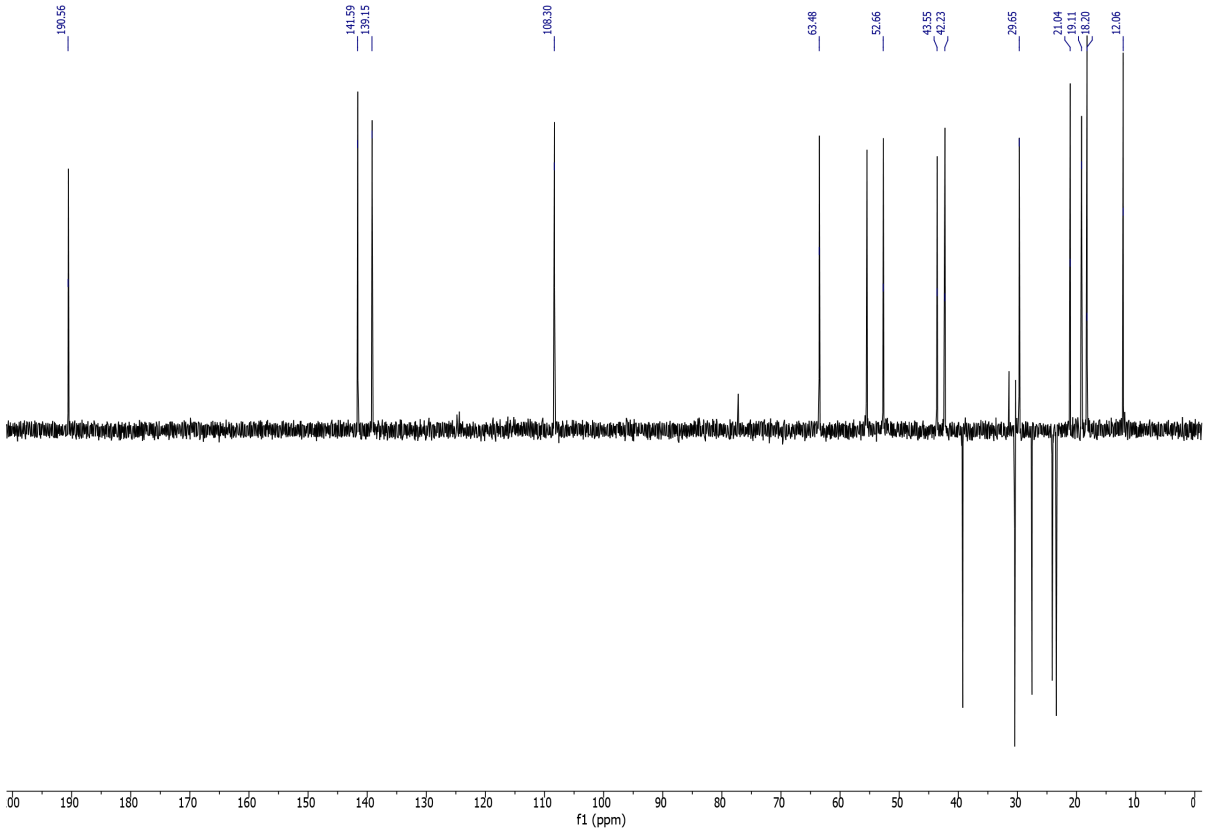

Figure S13. DEPT 135 spectrum of **9** in $CDCl_3$.

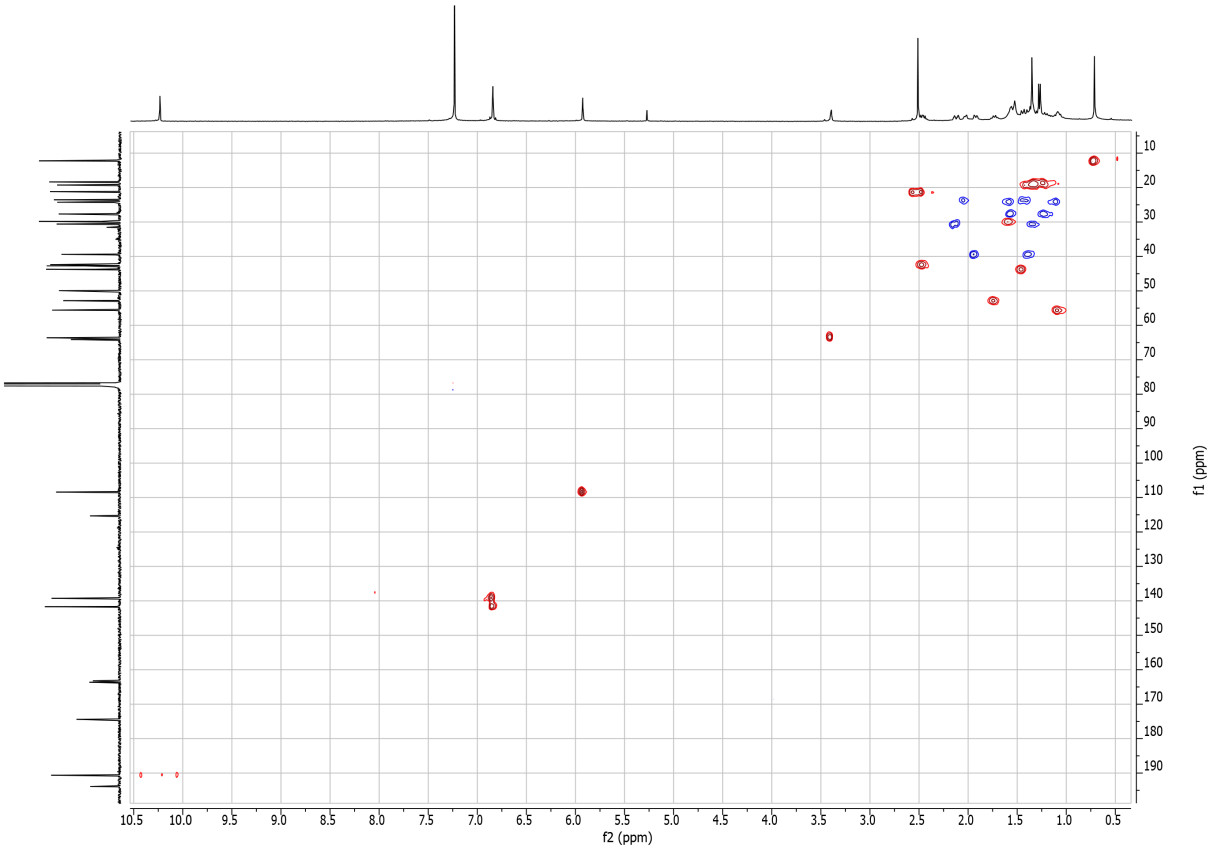

Figure S14. HSQC spectrum of **9** in $CDCl_3$.

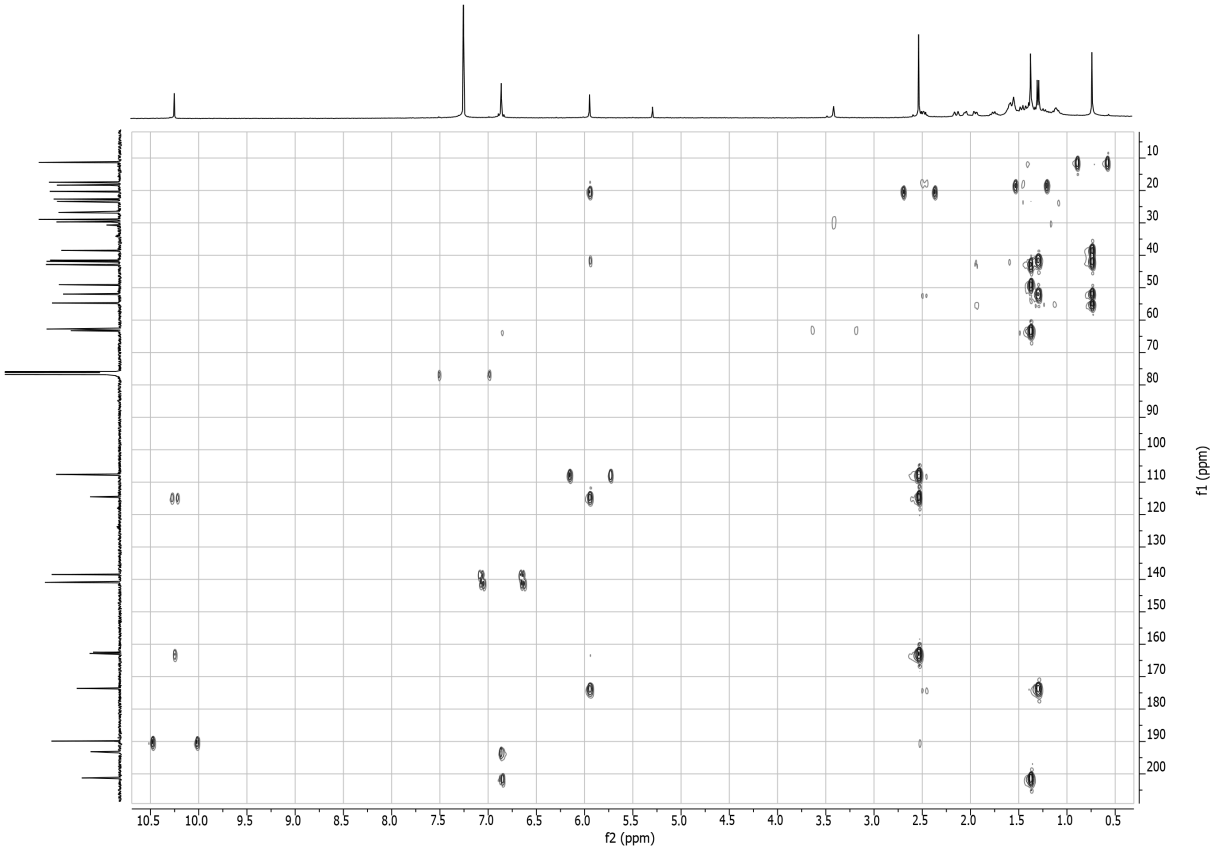

Figure S15. HMBC spectrum of **9** in $CDCl_3$.

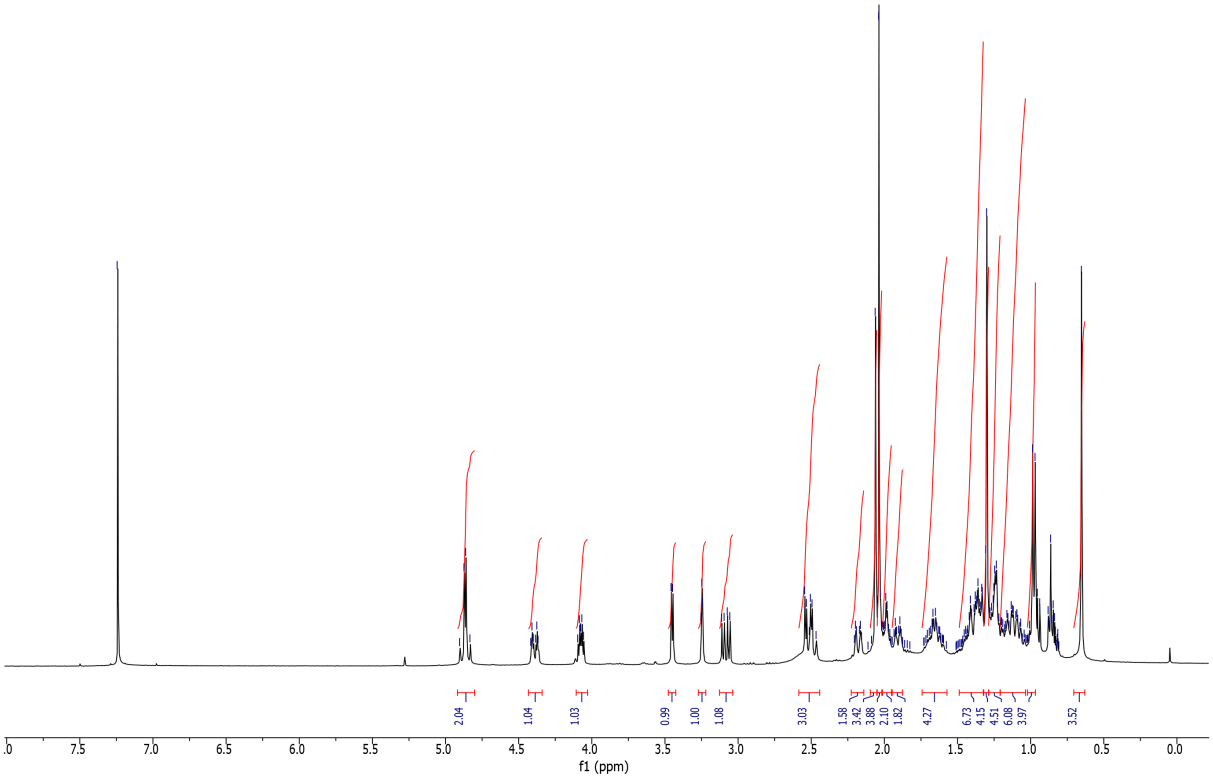

Figure S16. ^{1}H NMR spectrum of **17** in $CDCl_3$.

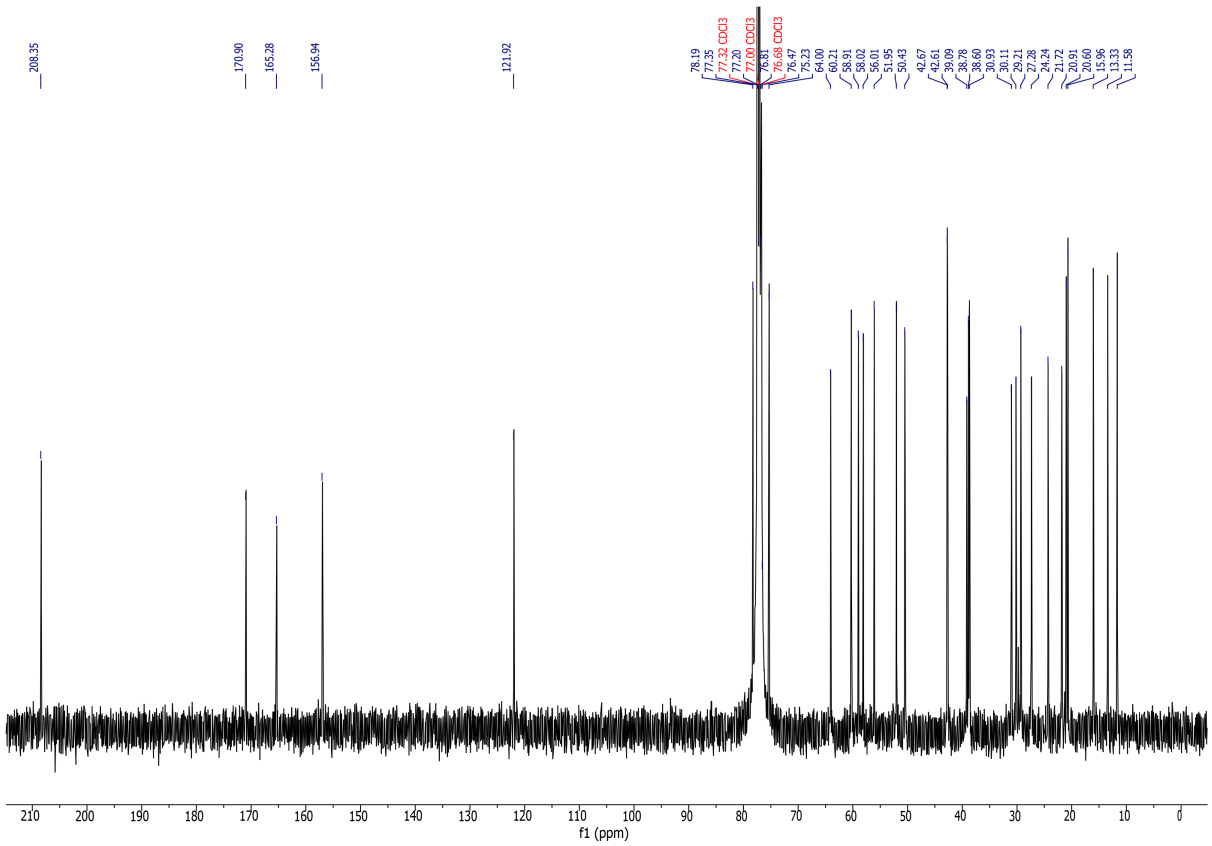

Figure S17. ^{13}C NMR spectrum of **17** in $CDCl_3$.

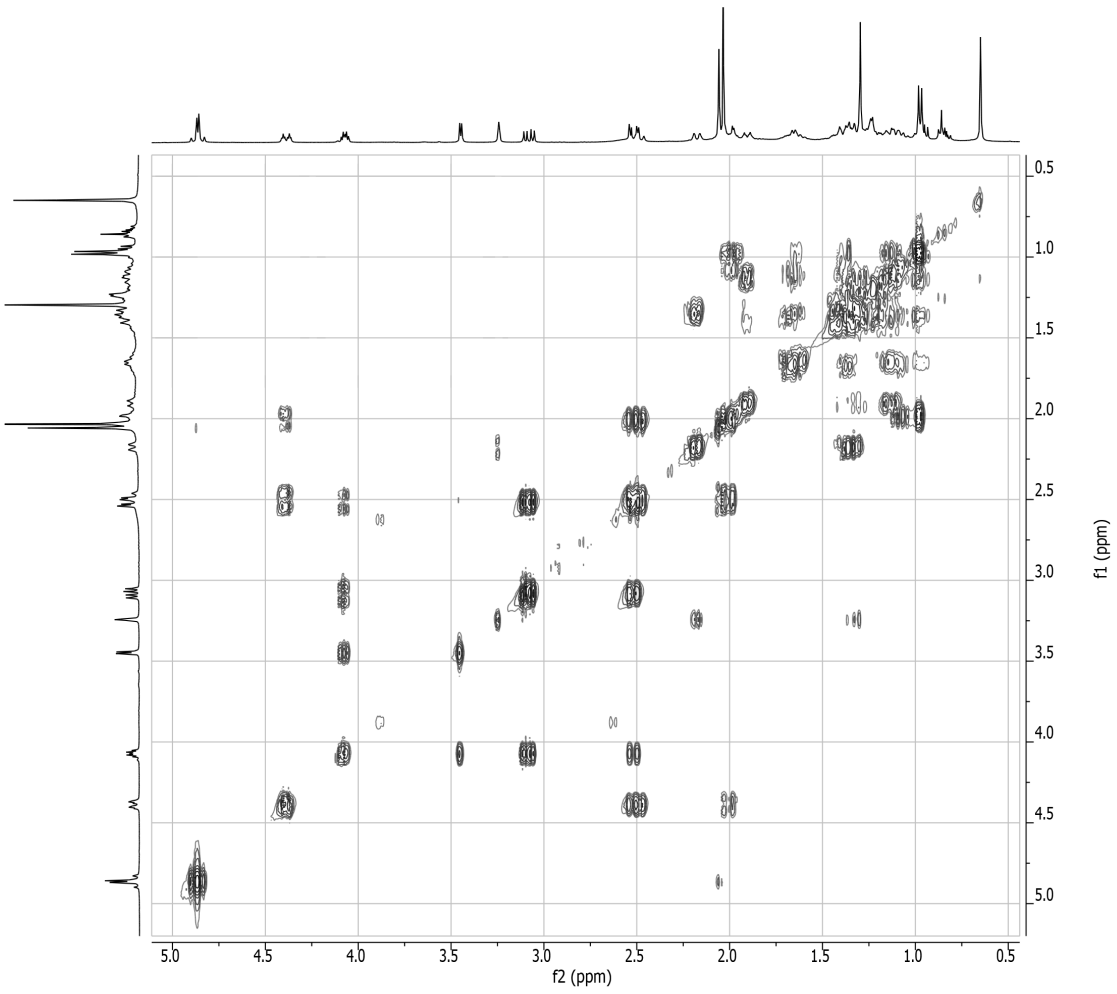

Figure S18. ^{1}H–^{1}H COSY spectrum of **17** in $CDCl_3$.

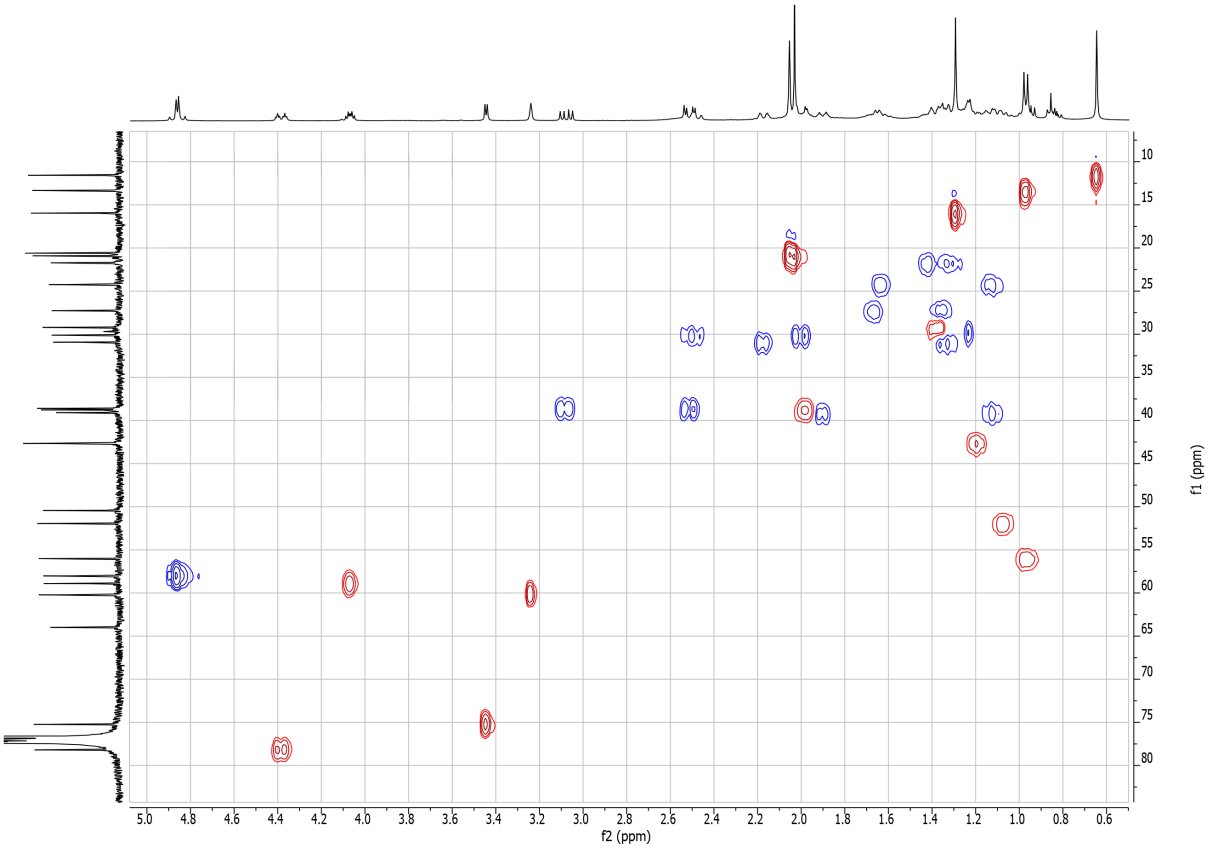

Figure S19. HSQC spectrum of **17** in $CDCl_3$.

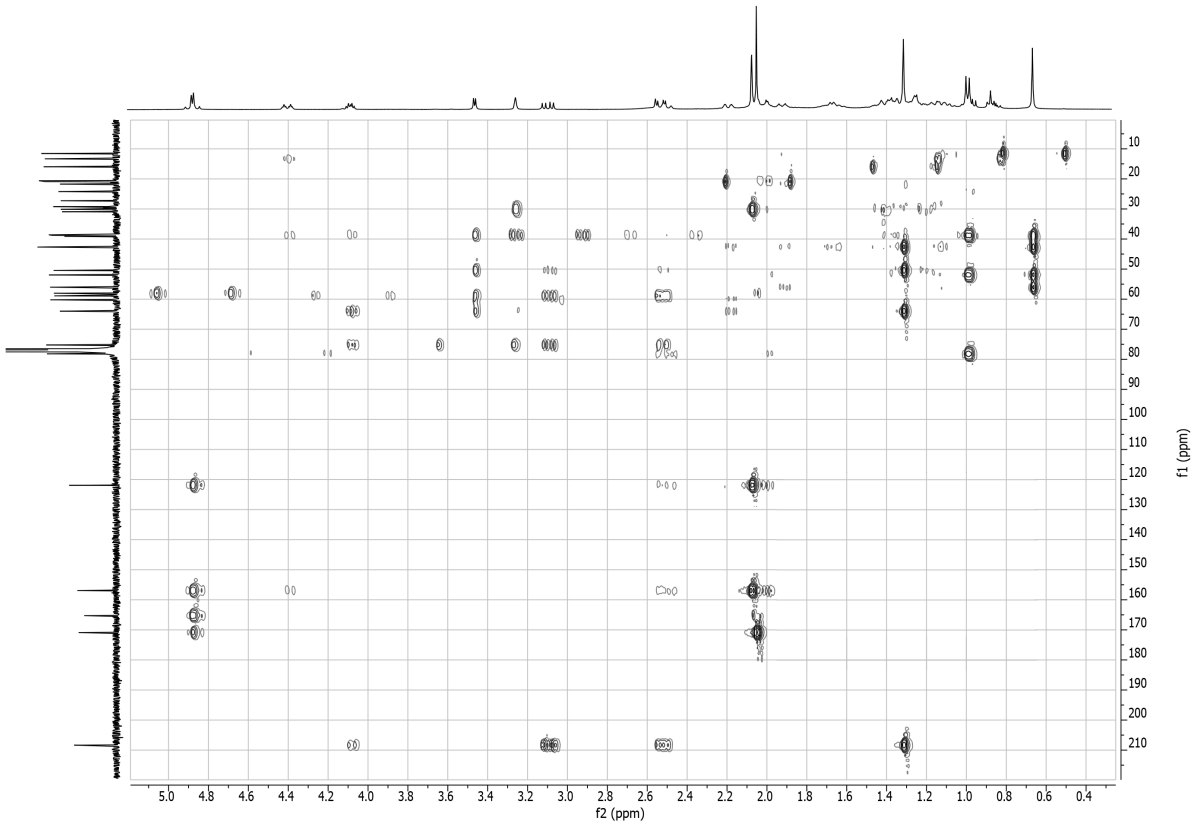

Figure S20. HMBC spectrum of **17** in $CDCl_3$.

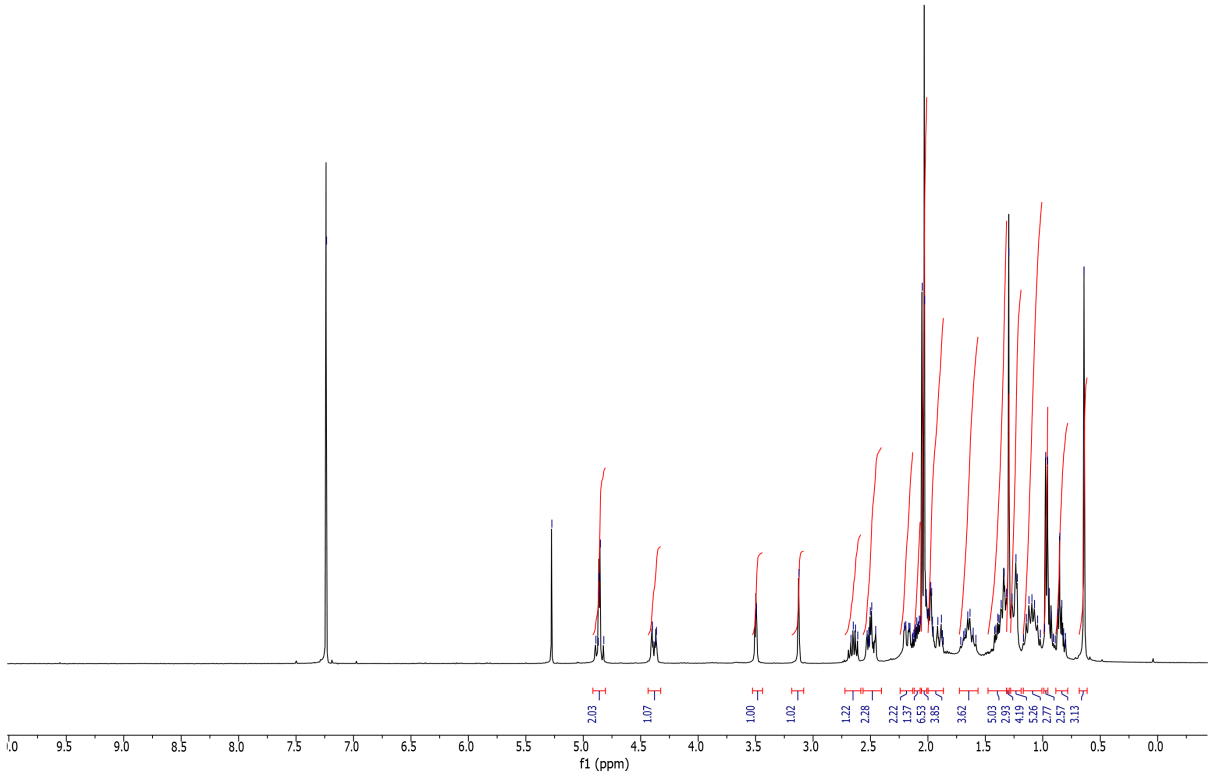

Figure S21. ^{1}H NMR spectrum of **18** in $CDCl_3$.

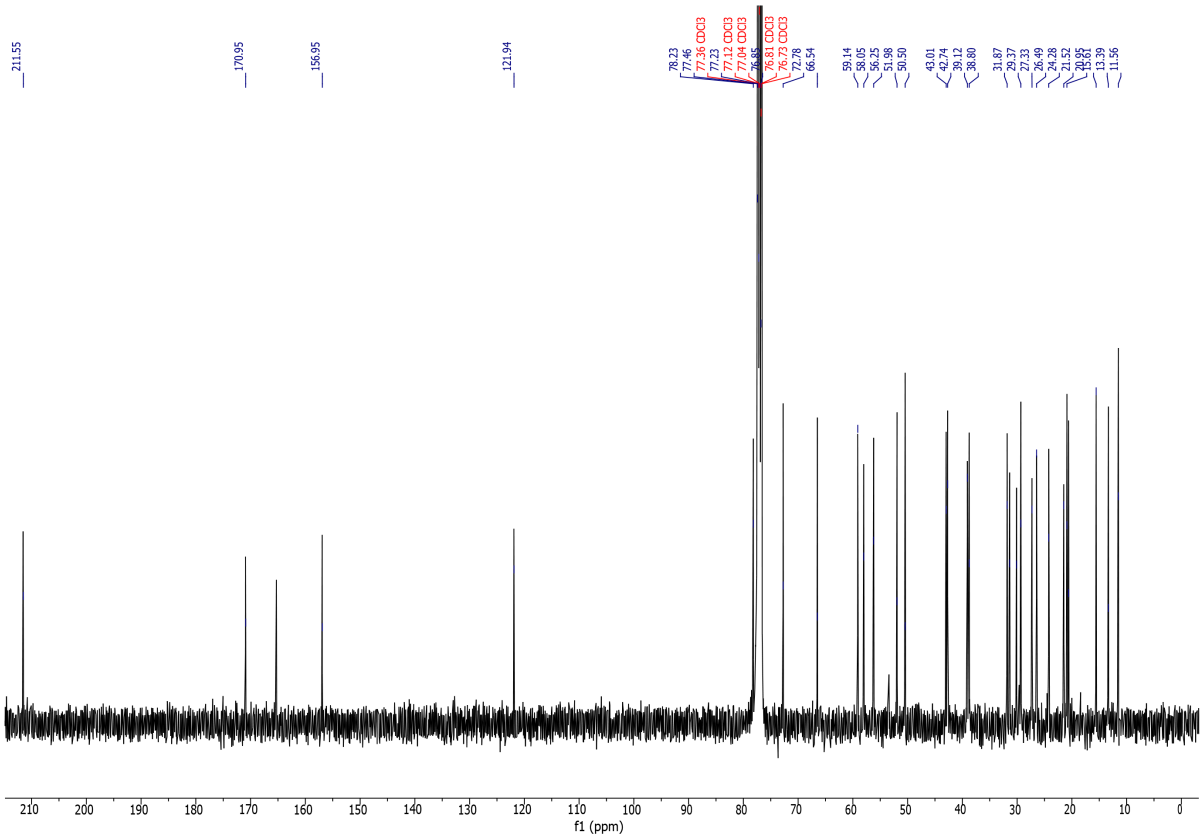

Figure S22. ^{13}C NMR spectrum of **18** in $CDCl_3$.

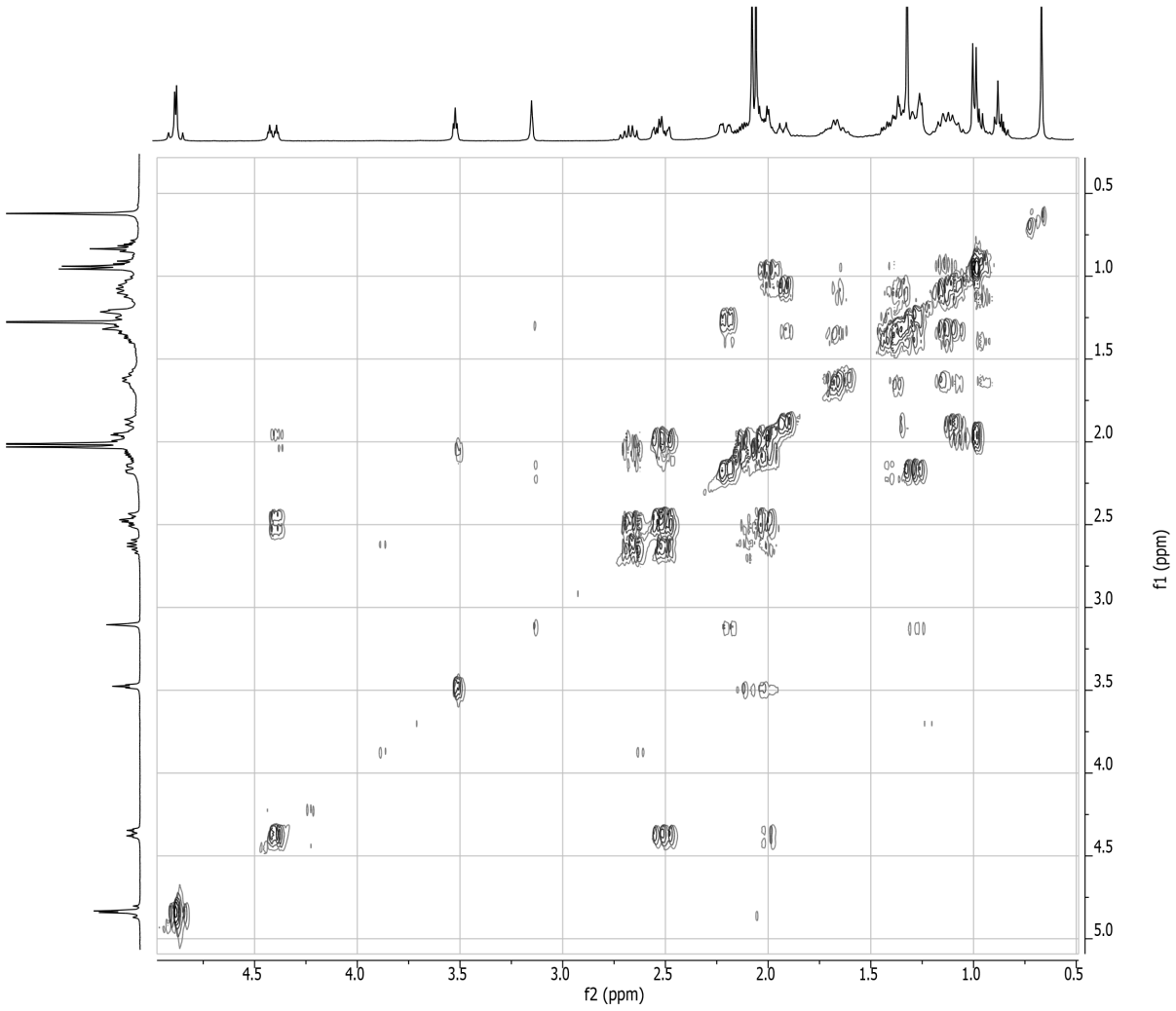

Figure S23. ^{1}H–^{1}H COSY spectrum of **18** in $CDCl_3$.

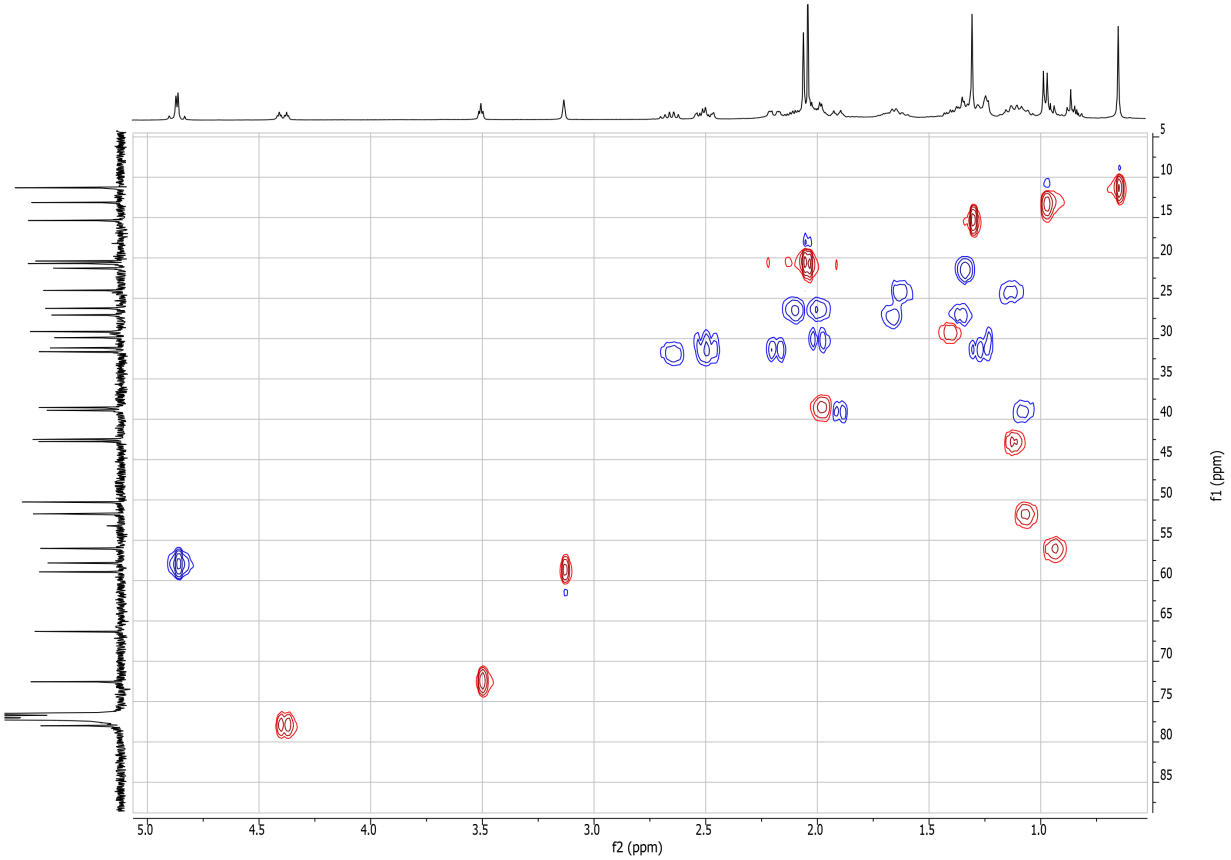

Figure S24. HSQC spectrum of **18** in $CDCl_3$.

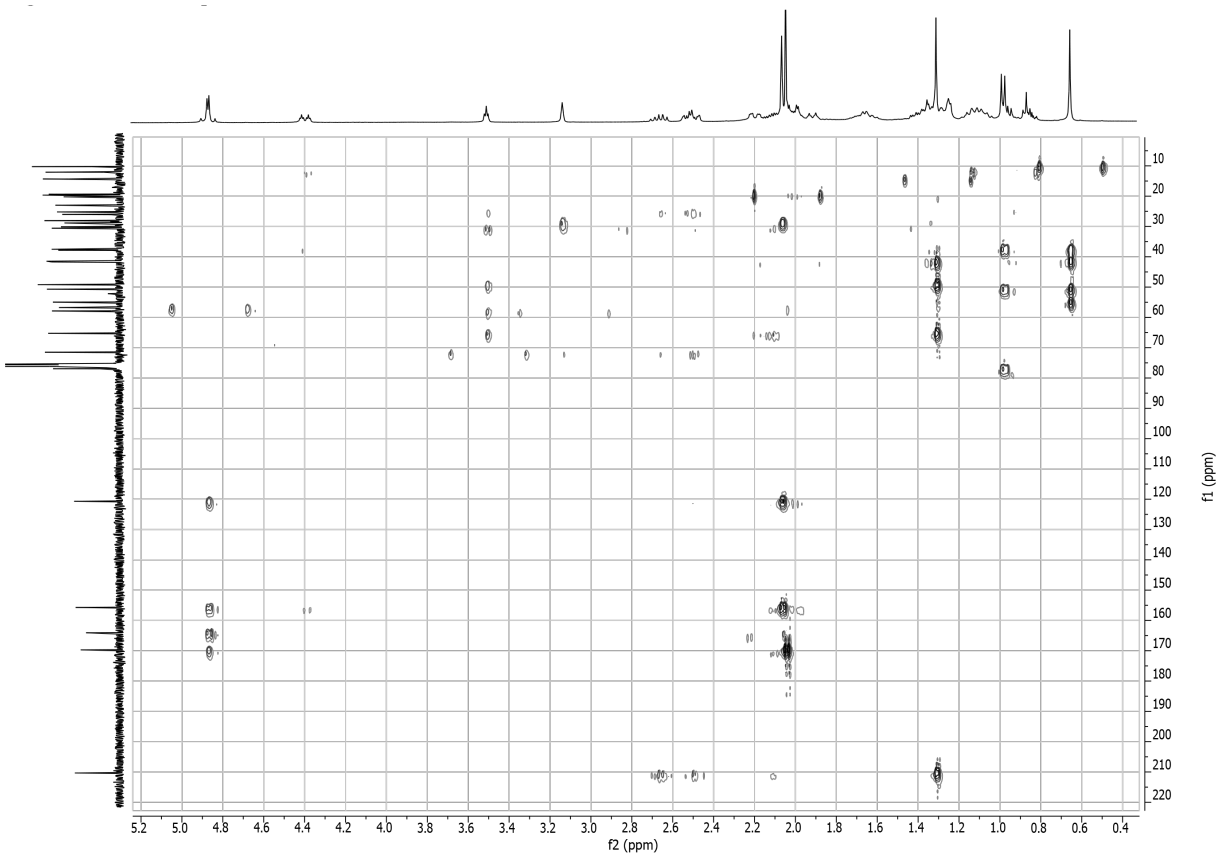

Figure S25. HMBC spectrum of **18** in $CDCl_3$.

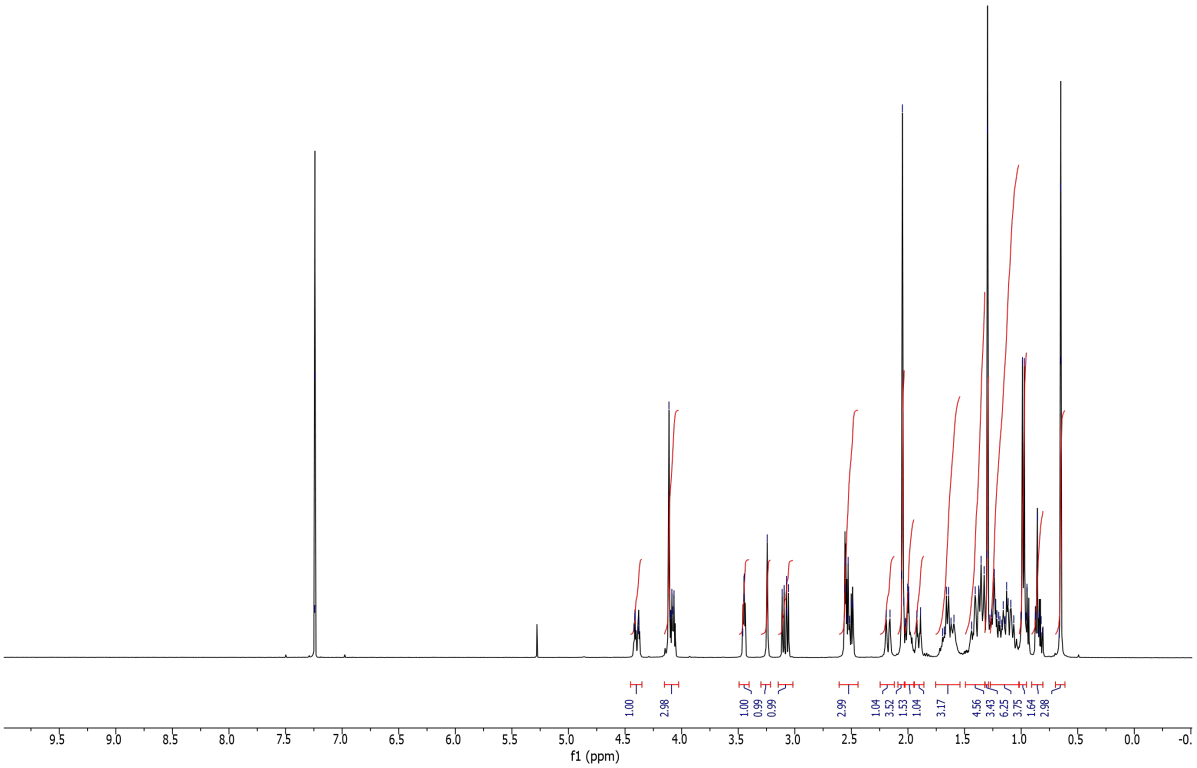

Figure S26. ^{1}H NMR spectrum of **19** in $CDCl_3$.

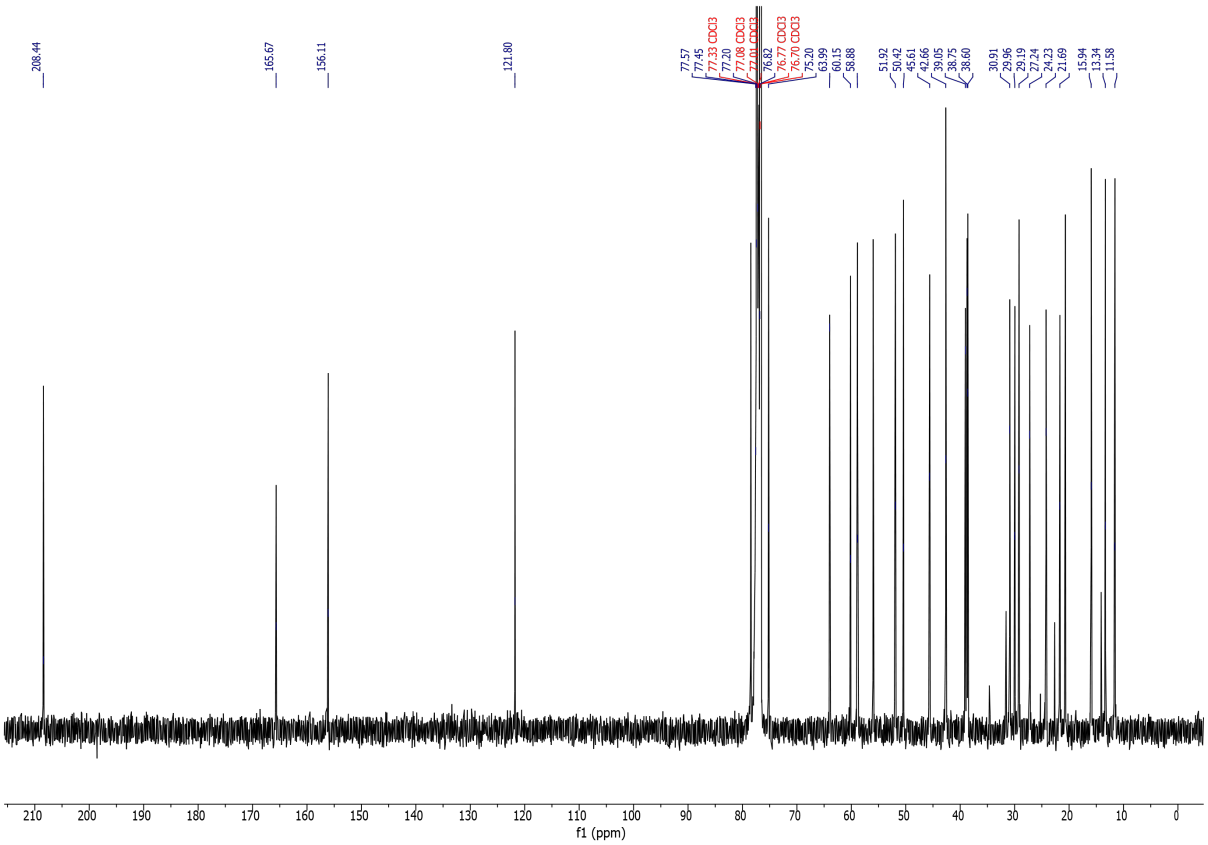

Figure S27. ^{13}C NMR spectrum of **19** in $CDCl_3$.

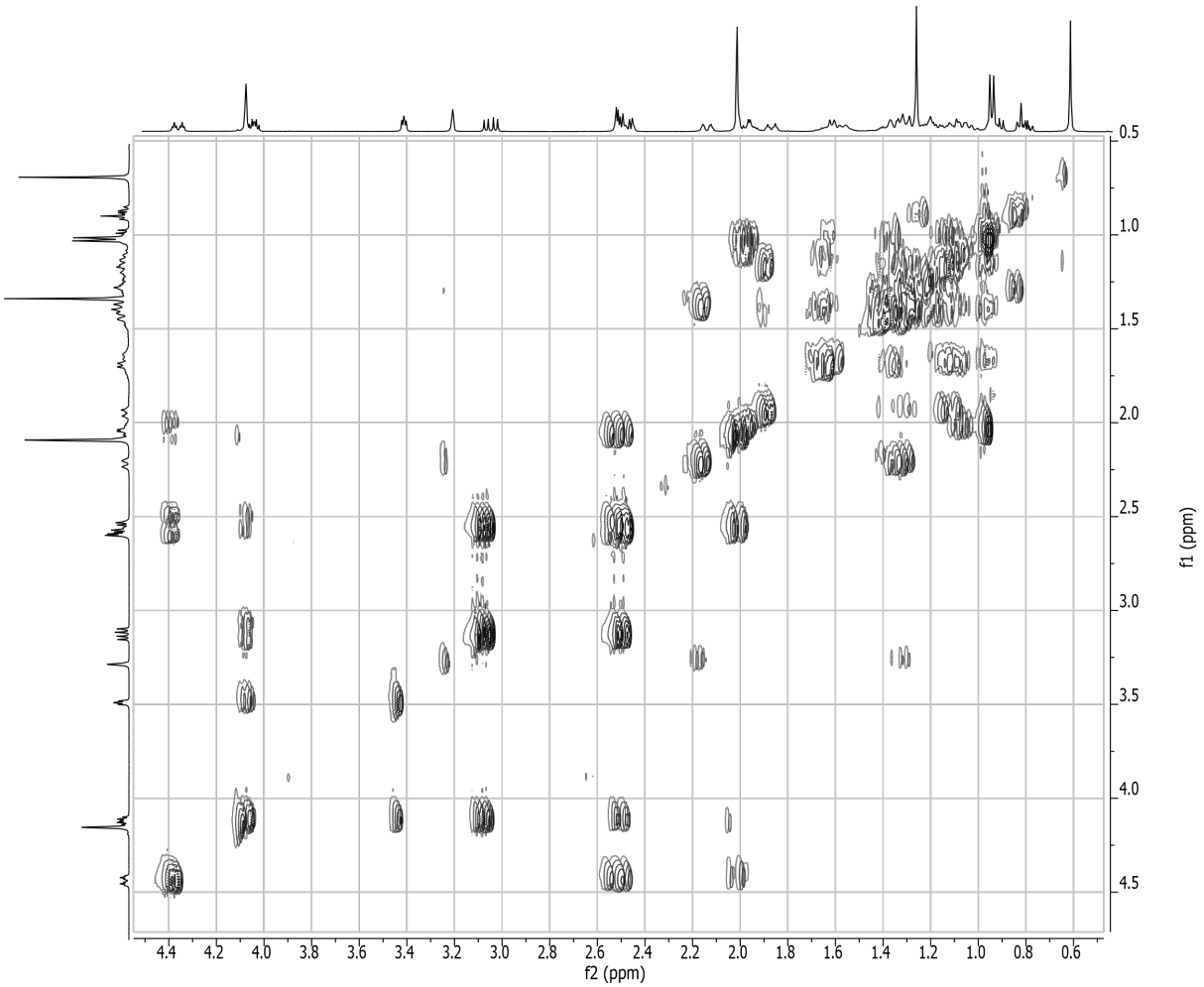

Figure S28. ^{1}H–^{1}H COSY spectrum of **19** in $CDCl_3$.

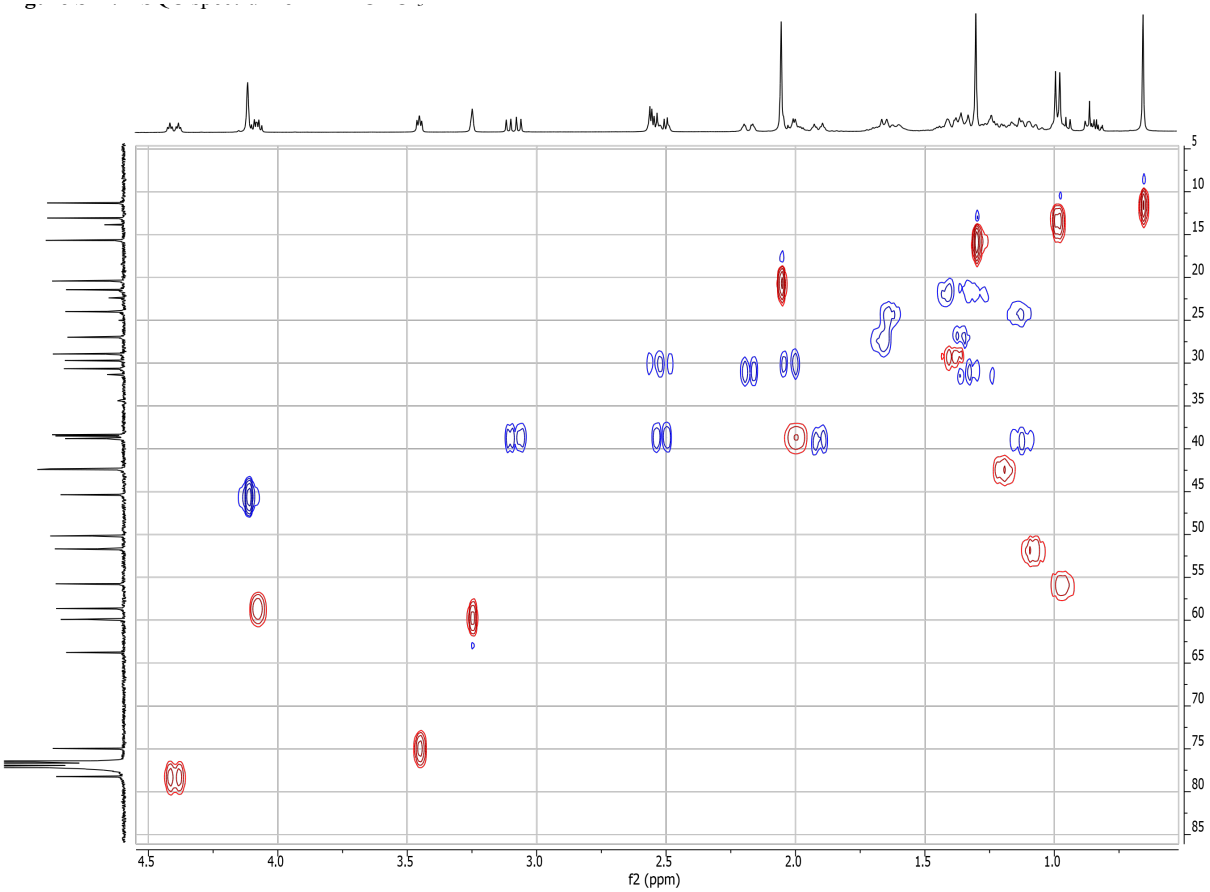

Figure S29. HSQC spectrum of **19** in $CDCl_3$.

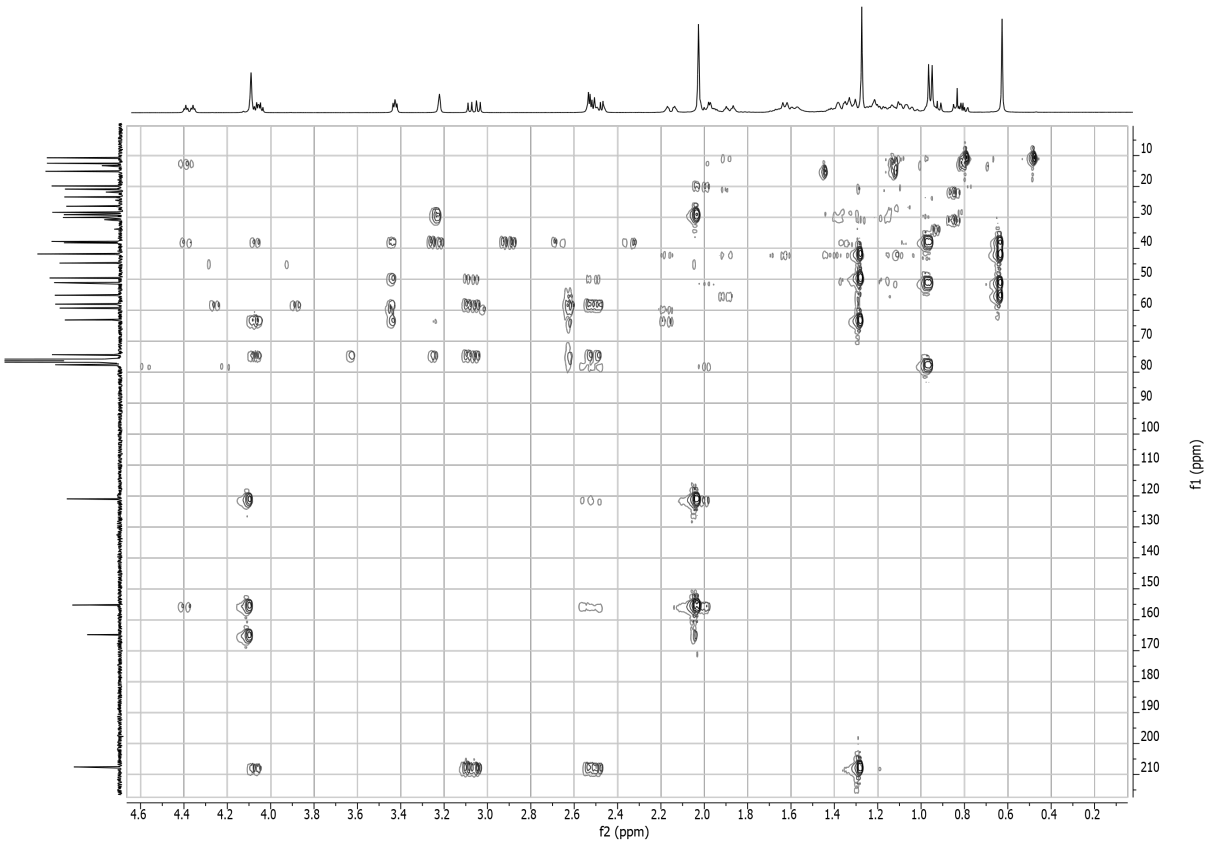

Figure S30. HMBC spectrum of **19** in $CDCl_3$.