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New synthetic corticosteroids inhibit Epstein–Barr virus release

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Experimental Section

^{1}H and ^{13}C NMR spectra were recorded on a «Bruker AV-600» (600 and 150 MHz, respectively) and «Bruker AV-400» (400.1 and 100.6 MHz, respectively) spectrometers in $CDCl_3$ containing 0.05% Me_4Si as the internal standard. Assignments of ^{1}H and ^{13}C signals were made with the aid of 1D DEPT-135 and 2D COSY, ROESY, NOESY, HSQC and HMBC spectra.

All commercial reagents were used without further purification.

1. Corticosteroids synthesis

The synthesis of 5′-methyl-[2′-(*N*-arylcarbamoyl)]-3β,16β-dihydroxyspiroandrost-5-ene-17,6′-[1,3,4]thiadiazines **2a,b** was carried out according to a modified method [S1]. *p*-Toluenesulfonic acid (20 μL, 30 mol%) was added to a solution of 16β,17β-epoxy-17-isopregn-5-en-3β-ol-20-one **1** (330 mg, 1.0 mmol) and *N*-aryl-2-hydrazino-2-thioxoacetamide (1.2 mmol) in dry dioxane (15 mL) at room temperature. The mixture was heated at 40 °C for 10 h. The organic layer was treated with a 5% aqueous Na_2CO_3 solution (20 mL) and extracted with chloroform (3×40 mL). The combined organic layers were dried over Na_2SO_4. The crude product was purified by column chromatography using petroleum ether/ethyl acetate (3:1).

(3*S*,8*R*,9*S*,10*R*,13*S*,14*S*,16*R*,17*R*)-5′-Methyl-2′-(*N*-phenylcarbamoyl)-3,16-dihydroxy-spiroandrost-5-ene-17,6′-[1,3,4]thiadiazine (**2a**), mp 188–190 °C; yield 60% (305 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.78 (ddd, J = 4.8, 12.3, 15.5 Hz, 1H, 9-CH), 0.85 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.86–0.98 (m, 2H, 1-CH_2, 12-CH_2), 1.03–1.10 (m, 1H, 12-CH_2), 1.17–1.40 (m, 4H, 2-CH_2, 8-CH, 11-CH_2), 1.47–1.70 (m, 4H, 1-CH_2, 2-CH_2, 7-CH_2, 15-CH_2), 1.78 (ddd, J = 2.2, 10.4, 11.1 Hz, 1H, 14-CH), 1.83–1.96 (m, 2H, 7-CH_2, 15-CH_2), 2.04 (dd, J=8.7, 13.1 Hz, 1H, 4-CH_2), 2.12 (dd, J=5.0, 13.1 Hz, 1H, 4-CH_2), 2.47 (s, 3H, CH_3), 3.18–3.22 (m, 1H, 3-CH), 4.55 (d, J=4.2 Hz, 1H, 3-OH), 5.22–5.26 (m, 2H, 6-CH, 16-CH), 5.66 (d, J=4.8 Hz, 1H, 16-OH), 7.12 (t, J=7.8Hz, 1H, Ph), 7.34 (t, J=7.8 Hz, 2H, Ph), 7.85 (d, J=7.8 Hz, 2H, Ph), 10.5 (br.s, 1H, NH). ^{13}C NMR (150.9 MHz, DMSO-d_6): δ 14.8 (18-CH_3), 19.0 (19-CH_3),

19.9 (11-CH_2), 23.5 (CH_3), 31.2 (8-CH), 31.2 (7-CH_2), 31.3 (2-CH_2), 33.7 (15-CH_2), 34.6 (12-CH_2), 35.9 (10-C), 36.6 (1-CH_2), 42.0 (4-CH_2), 46.0 (14-CH), 48.8 (9-CH), 51.2 (13-C), 62.2 (17-C), 69.9 (3-CH), 72.5 (16-CH), 119.9 (6-CH), 120.3 (2CH, Ph), 124.1 (CH, Ph), 128.6 (2CH, Ph), 137.9 (C, Ph), 141.2 (5-C), 152.9 (2′-C), 153.9 (5′-C), 160.0 (CO). HRMS (ESI) for $C_{29}H_{38}N_3O_3S$ ($[M+H]^+$): calcd 508.2628, found 508.2626.

(3*S*,8*R*,9*S*,10*R*,13*S*,14*S*,16*R*,17*R*)-5'-Methyl-2'-[*N*-(4-fluorophenyl)carbamoyl)]-3,16-dihydroxyspiroandrost-5-ene-17,6'-[1,3,4]thiadiazine (**2b**), mp 211–214 °C; yield 65% (355 mg). ^{1}H NMR (600 MHz, DMSO-d_6): δ 0.78 (ddd, J = 4.9, 12.4, 15.4 Hz, 1H, 9-CH), 0.85 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.86–0.98 (m, 2H, 1-CH_2, 12-CH_2), 1.03–1.10 (m, 1H, 12-CH_2), 1.17–1.40 (m, 4H, 2-CH_2, 8-CH, 11-CH_2), 1.47–1.70 (m, 4H, 1-CH_2, 2-CH_2, 7-CH_2, 15-CH_2), 1.79 (ddd, J = 2.3, 10.4, 11.2 Hz, 1H, 14-CH), 1.84–1.97 (m, 2H, 7-CH_2, 15-CH_2), 2.04 (dd, J=8.7, 13.3 Hz, 1H, 4-CH_2), 2.13 (dd, J=4.9, 13.3 Hz, 1H, 4-CH_2), 2.47 (s, 3H, 21-CH_3), 3.19–3.22 (m, 1H, 3-CH), 4.50 (br.s, 1H, 3-OH), 5.22–5.28 (m, 2H, 6-CH, 16-CH), 5.67 (br.s, 1H, 16-OH), 7.18 (dd, J=8.4, 9.0 Hz, 2H, Ar), 7.89 (dd, J=4.8, 8.4 Hz, 2H, Ar), 10.6 (br.s, 1H, NH). ^{13}C NMR (150.9 MHz, DMSO-d_6): δ 14.8 (18-CH_3), 19.0 (19-CH_3), 20.0 (11-CH_2), 23.5 (CH_3), 31.1 (8-CH), 31.2 (7-CH_2), 31.3 (2-CH_2), 33.7 (15-CH_2), 34.6 (12- CH_2), 35.9 (10-C), 36.6 (1-CH_2), 42.1 (4-CH_2), 46.0 (14-CH), 48.8 (9-CH), 51.2 (13-C), 62.2 (17-C), 69.9 (3-CH), 72.5 (16-CH), 115.1 (d, $J_{C\text{-}F}$=23.0 Hz, 2CH, Ar), 120.0 (6-CH), 122.3 (d, $J_{C\text{-}F}$=6.9 Hz, 2CH, Ar), 134.4 (C, Ar), 141.2 (5-C), 152.8 (2′-C), 154.0 (5′-C), 158.5 (d, $J_{C\text{-}F}$=240.9 Hz, C, Ar), 160.0 (CO). HRMS (ESI) for $C_{29}H_{36}FN_3O_3SNa$ ($[M+Na]^+$): calcd 548.2354, found 548.2345.

2. Cell Culture and Reagents

B95-8 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml PenStrep (ThermoFisher). Spironolactone and its analogues were dissolved in DMSO at concentration 10 mM. When spironolactones were added to the cell culture medium at final concentration of 25 μM, concentration of DMSO was 0.025%, therefore one of the controls included cells incubated in the presence of DMSO in culture medium.

B95-8 cells were seeded in 48-well plate at concentration 200,000 cells/ml in 0.5 ml medium containing corresponding spironolactones for 14 days. Every 3-4 days half of the cell's suspension (0.25 ml) was removed for cell viability assay and collection of cell culture supernatant for EBV release measurement. 0.25 ml fresh medium supplemented with drugs was added back to the corresponding wells. Each compound was tested in triplicates. Cell viability and quantification was done using Cell Proliferation / Cytotoxicity Assay Kit CCK-8 (Dojindo Molecular Technologies, Cat.No. CK04-05).

3. DNA isolation

Cellular debris removal from the B95-8 cells culture medium was done by centrifugation at 10000 g for 5 min. Viral DNA was isolated using SubX™ cfDNA Isolation Kit (Capital Biosciences, Cat. CMDNA-0050).

4. qPCR

Aliquots from cell culture supernatant collected at days 4,7,11 and 14 were added directly into SYBR green qPCR with primers for EBNA 1 gene (Forward: 5'-ATGCCACCGAGGTTCTTG-3' and Reverse: 5'-TGACACCACCACCCTTCTA-3'; [S2]). The reaction conditions included initial heating (95 ºC; 60 sec) followed by 40 cycles of denaturation (95 ºC; 15 sec) and annealing with extension (58 ºC; 90 sec). To amplify target DNA from cell culture supernatant without purification, we used HighResist DNA polymerase mixture (Capital Biosciences, Cat.HRDP-0100) containing hybrid DNA polymerases, in which Taq catalytic domain is linked with non-specific DNA binding domains providing highly processive synthesis of DNA in the presence of common inhibitors of DNA polymerase reaction and biological fluids [S3,S4].

5. SPR inhibits EBV release from B95-8 cells

Verma *at al*. tested SPR and three structurally similar antimineralocorticoids for antiviral activity using GFP-EBV-infected gastric carcinoma cell line [S5]. Virion production was measured by expression of GFP in transduced 293 cells. Only SPR appeared to inhibit EBV release in this system. In contrast, we decided to test SPR directly on EBV producing B98-5 cells and estimated virus release by measurement of extracellular DNA in cell culture medium 4 days after treatment (Figure S1). DNA was isolated from cell culture supernatant and quantitated using Qubit 2.0 Fluorometer (Figure S1, *a*). Inhibitory effect of SPR was noticeable at concentration 1.5 μM and reached plateau at >3 μM. Measurement of EBV copies by SYBR green qPCR directly in supernatant also showed inhibition, and at 25 μM SPR virus DNA release in supernatant decreased by 69% (Figure S1, *b*). Thus, we have confirmed the antiviral activity of Spironolactone (SPR) against EBV (IC_{50} = 1.638 μM).

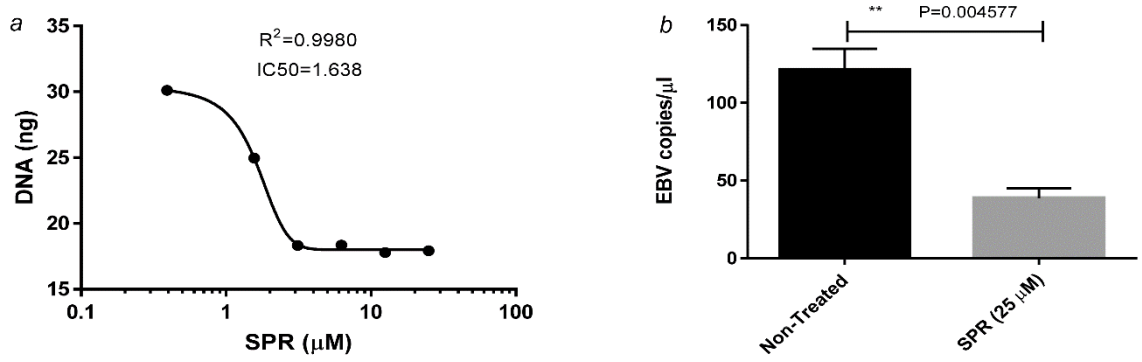

Figure S1. SPR anti-EBV activity in B95-8 cells. Cells were cultured in 48-well plate and DNA was isolated from 0.2 ml supernatant on day 5 after treatment with SPR (*a*). Inhibitory effect was confirmed by qPCR with EBNA1 primers directly from cell culture supernatant (*b*). DNA concentration was determined by Qubit HS DNA Assay. The 50% inhibitory concentration (IC50) was calculated using GraphPad PRISM version 6.05 software. Statistical significance calculated by student's T-test is indicated as: ** = $p<0.05$.

6. Cell toxicity

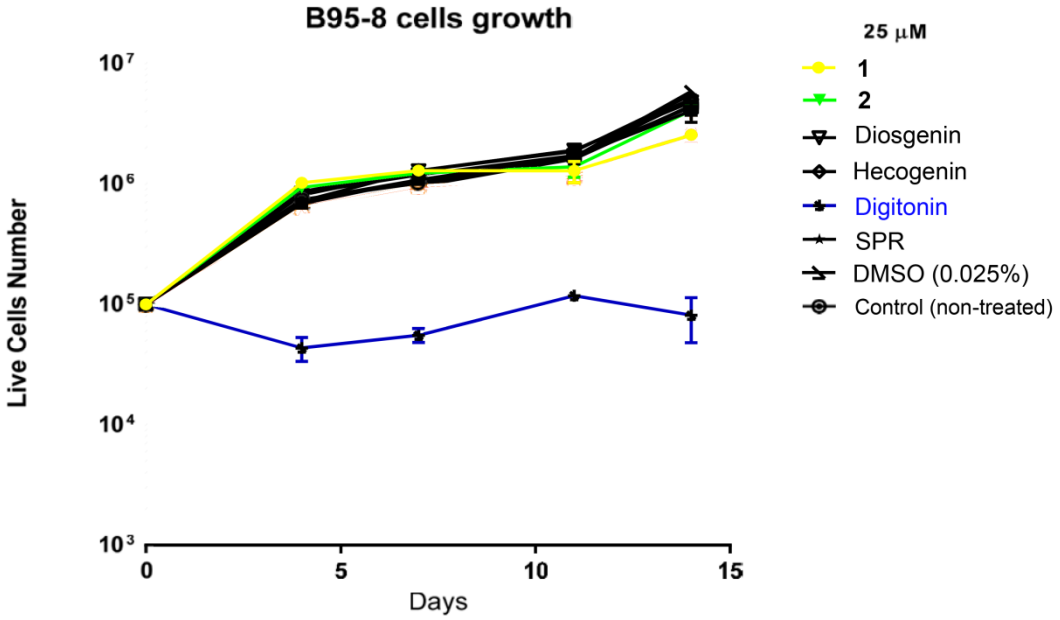

Figure S2. Extrapolated growth curve for cells treated with spironolactone and its analogues. The mean ± SD of three wells is shown. All steroids were tested at concentration of 25 μM.

References

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[S2] C. Ni, Y. Chen, M. Zeng, R. Pei, Y. Du, L. Tang, M. Wang, Y. Hu, H. Zhu, M. He, X. Wei, S. Wang, X. Ning, M. Wang, J. Wang, L. Ma, X. Chen, Q. Sun, H. Tang, Y. Wang, and X. Wang, *Cell Research*, 2015, **25**, 785.

[S3] A. R. Pavlov, G. I. Belova, S. A. Kozyavkin, and A. I. Slesarev, *Proceedings of the National Academy of Sciences*, 2002, **99**, 13510.

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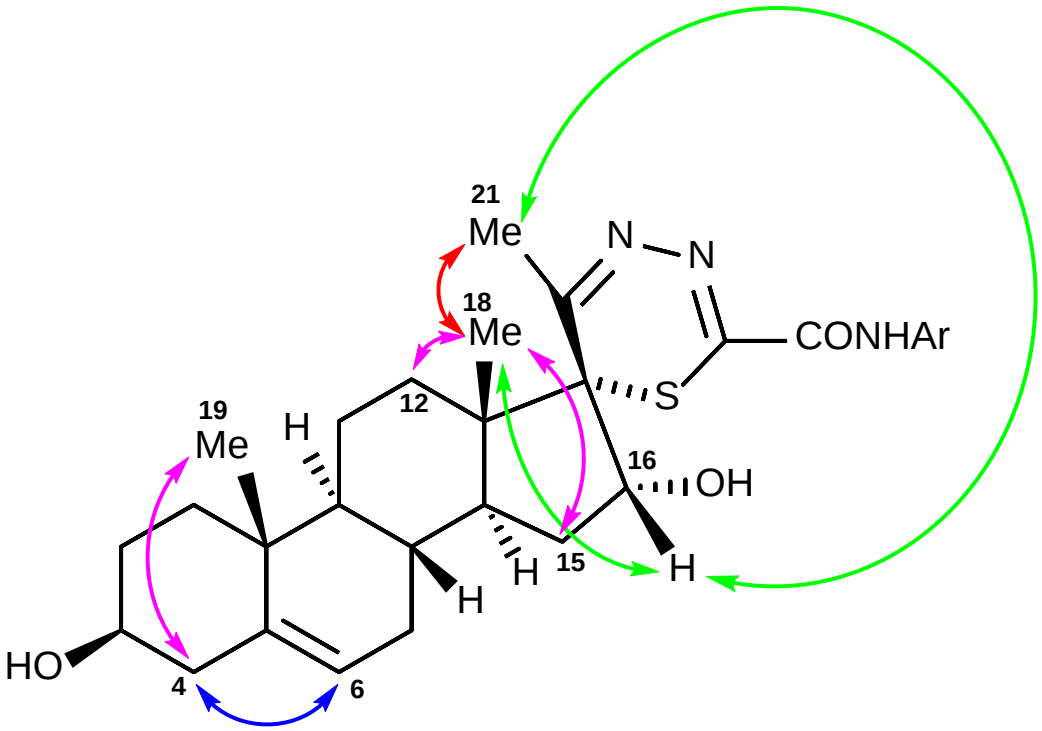
[S5] D. Verma, J. Thompson, and S. Swaminathan, *Proc. Natl. Acad. Sci. USA*, 2016, **113**, 3609.

Graphical interpretation of 2D NMR spectra for 2a,b.



NMR spectra for compound 2a (600 MHz).

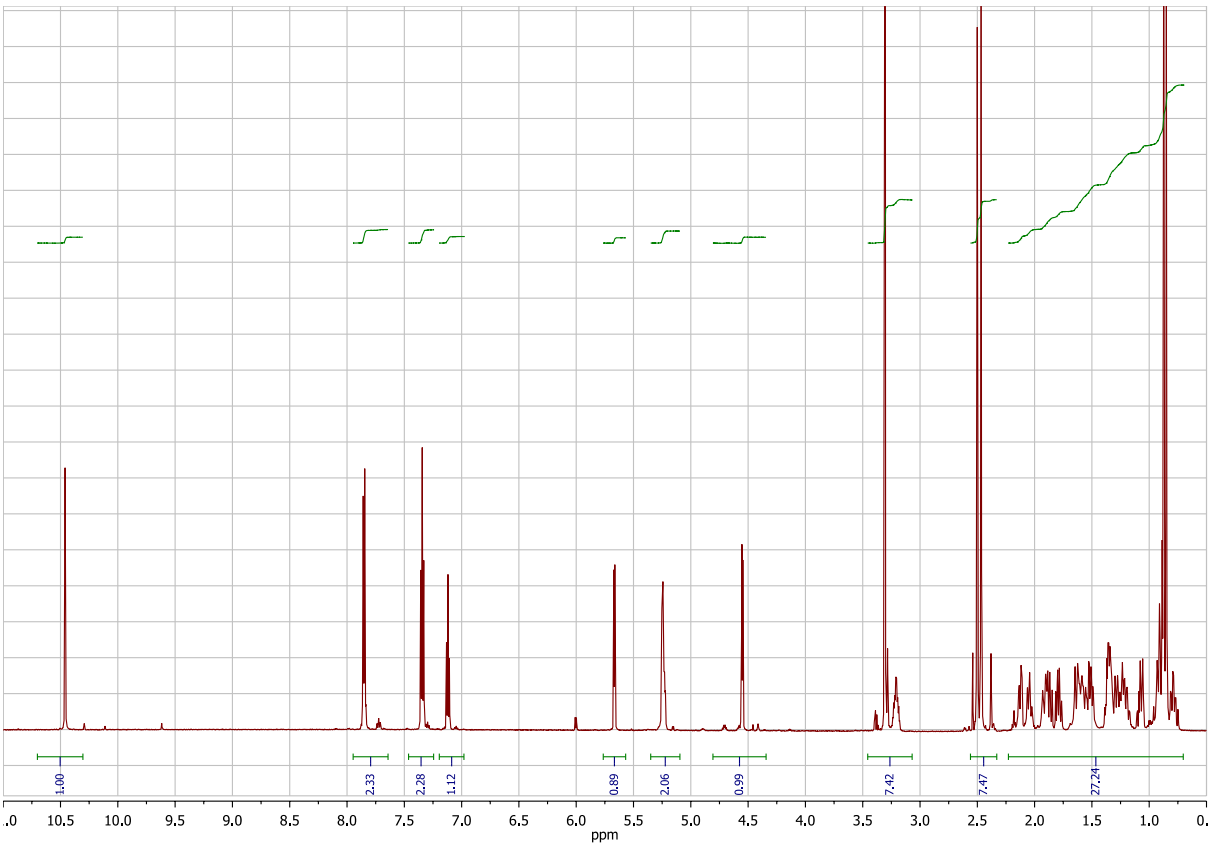


Figure S3: ^{1}H NMR spectrum of **2a**.

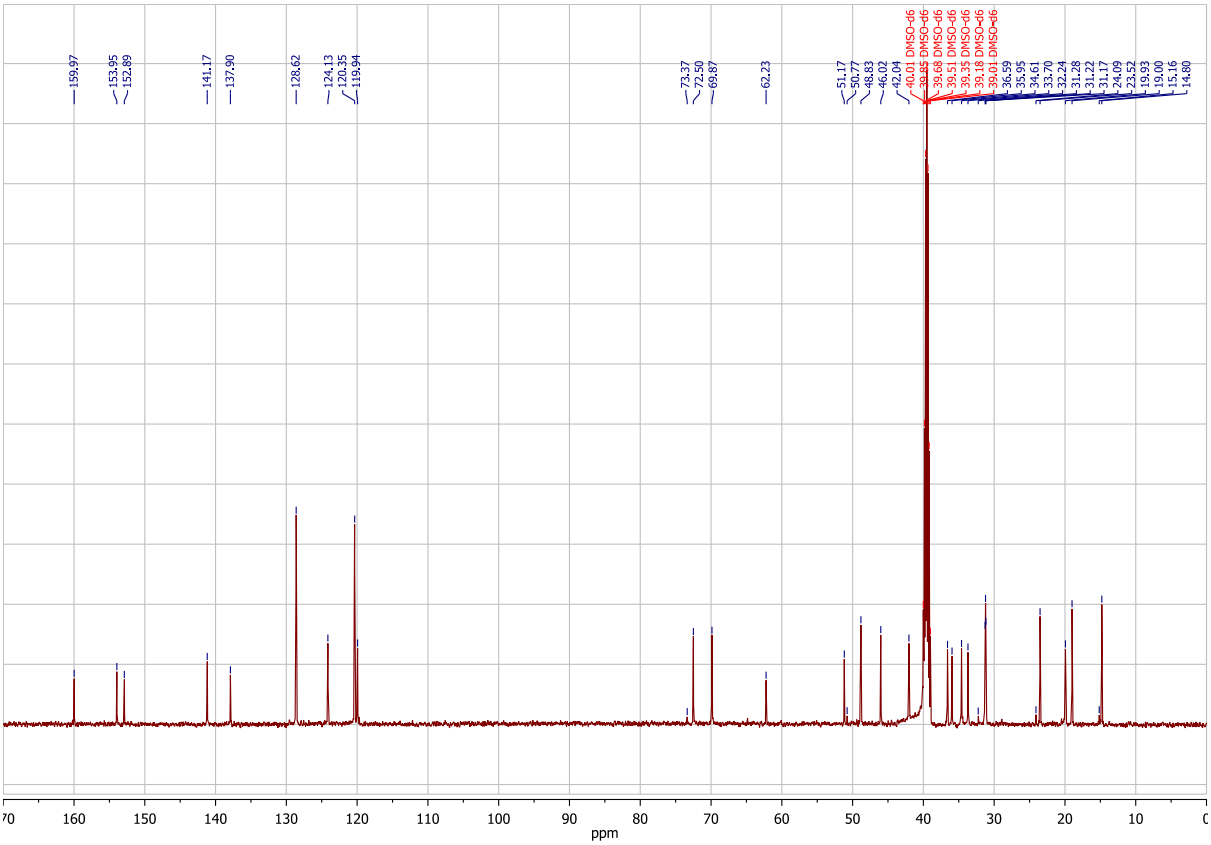


Figure S4: ^{13}C NMR spectrum of **2a**.

NMR spectra for compound 2a (400 MHz).

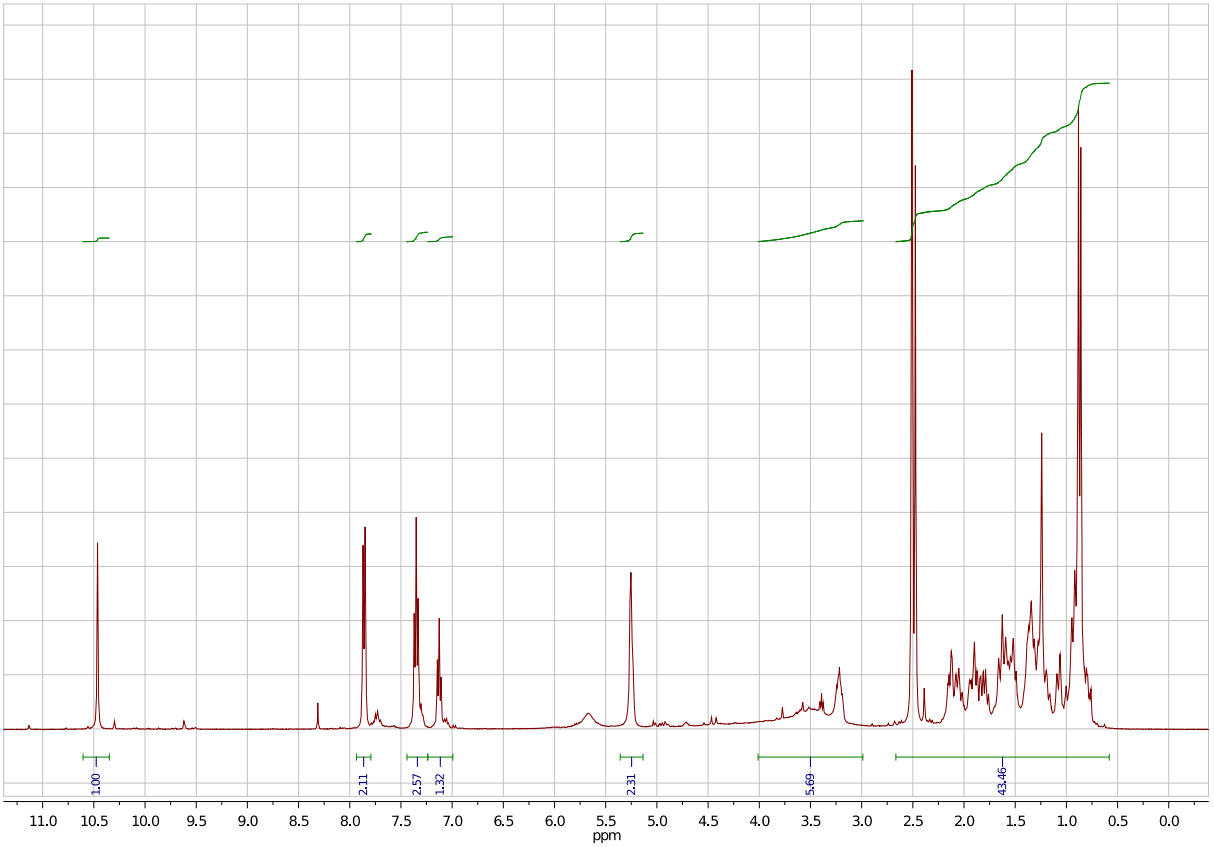


Figure S5: ^{1}H NMR spectrum of **2a**.

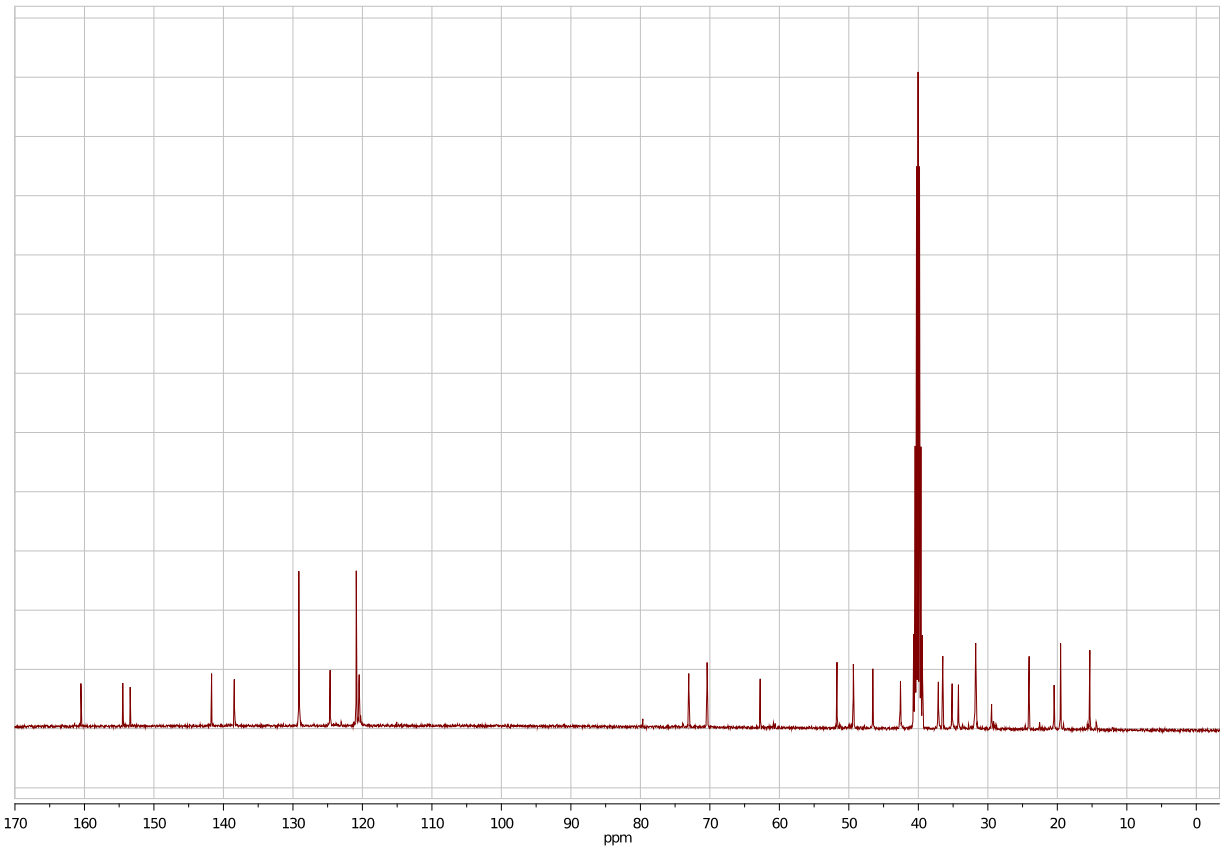


Figure S6: ^{13}C NMR spectrum of **2a**.

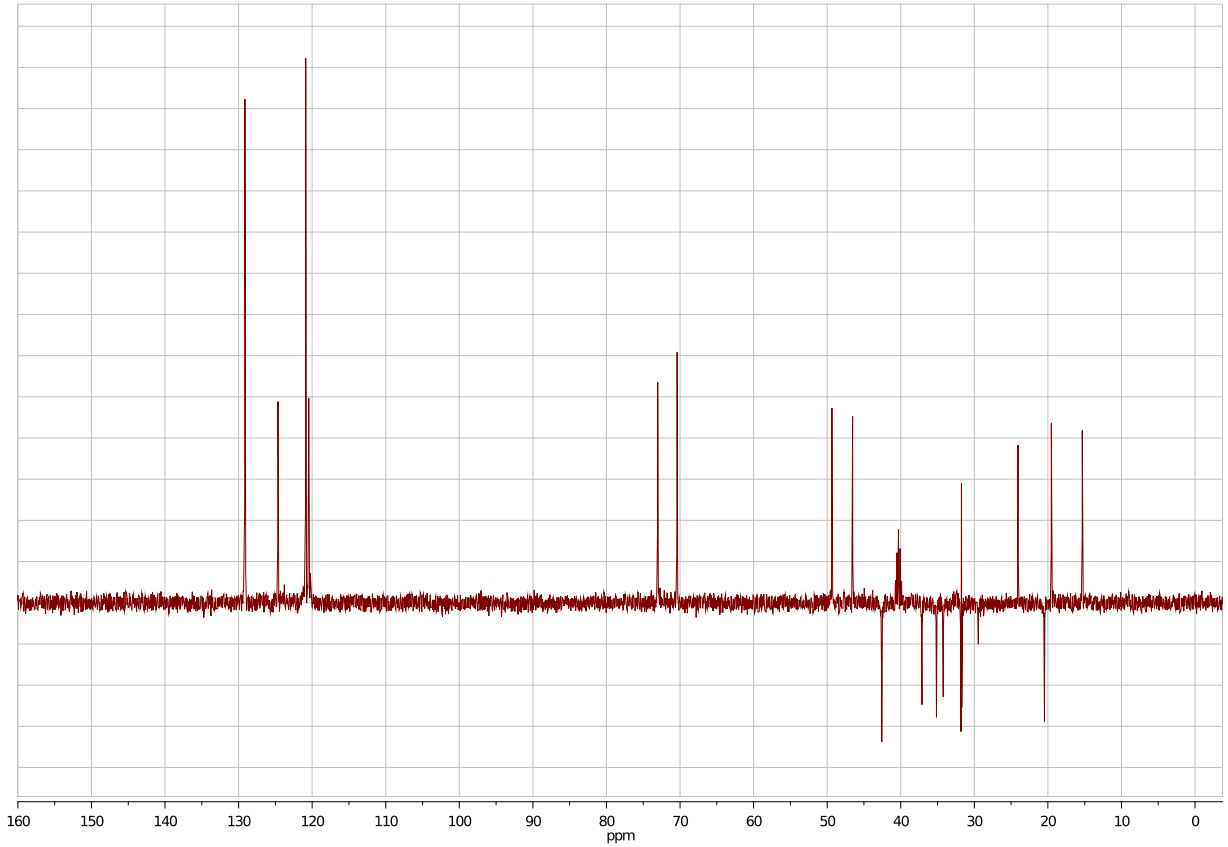


Figure S7: DEPT-135 NMR spectrum of **2a**.

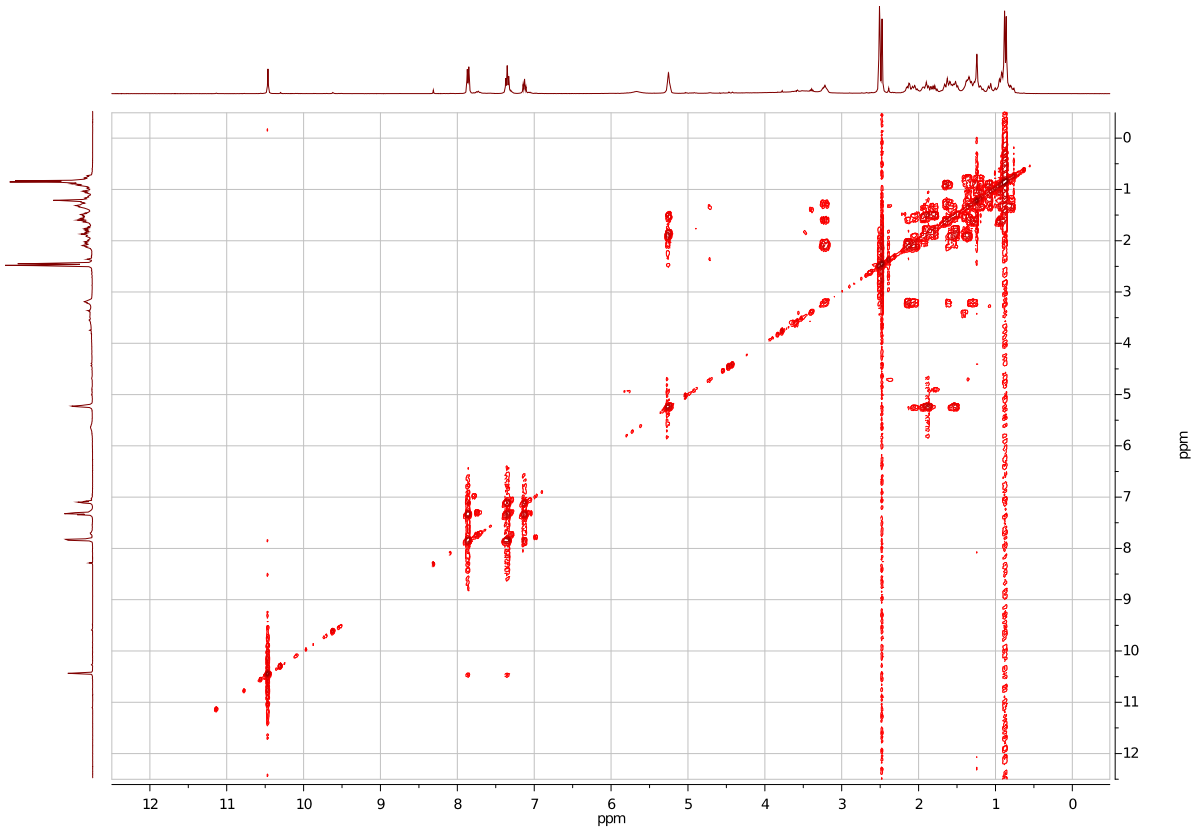


Figure S8: 2D ^{1}H-^{1}H COSY NMR spectrum of **2a**.

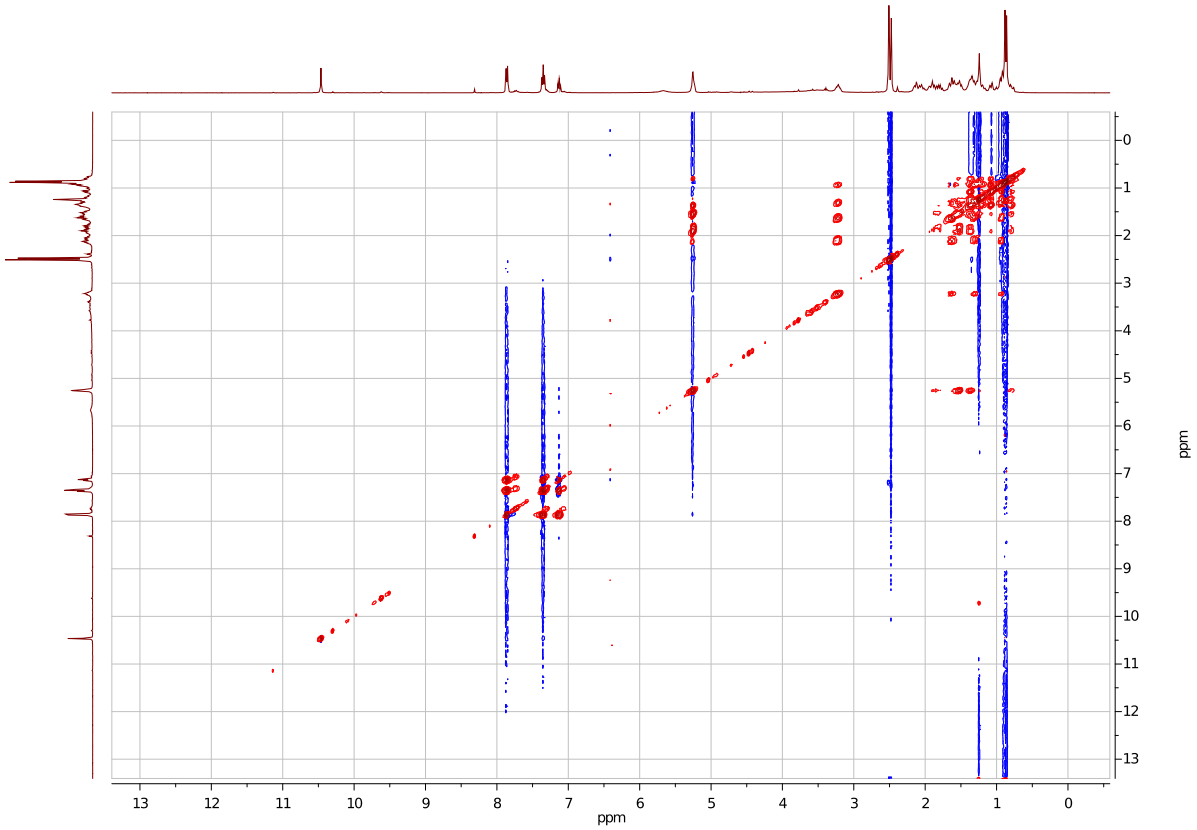


Figure S9: 2D ^{1}H-^{1}H TOCSY NMR spectrum of **2a**.

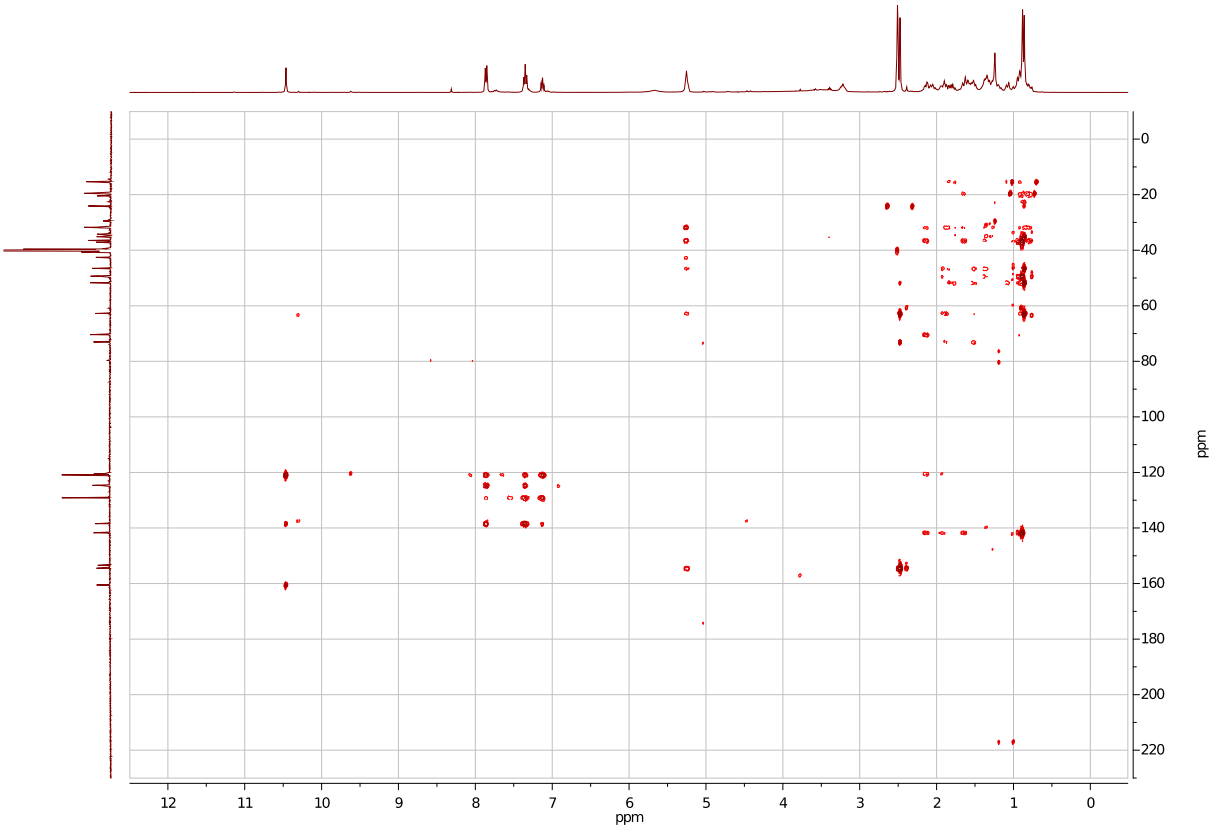


Figure S10: 2D ^{1}H-^{13}C HMBC NMR spectrum of **2a**.

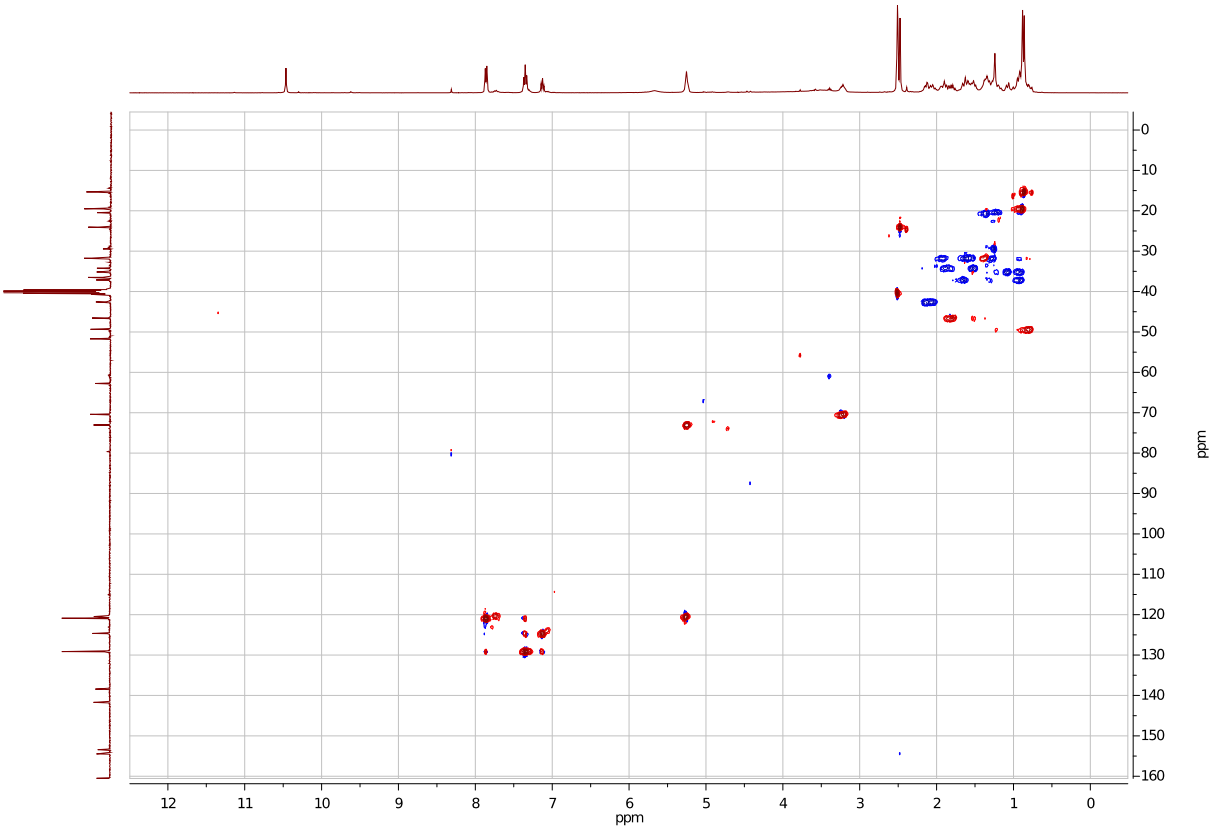


Figure S11: 2D ^{1}H-^{13}C HSQC NMR spectrum of **2a**.

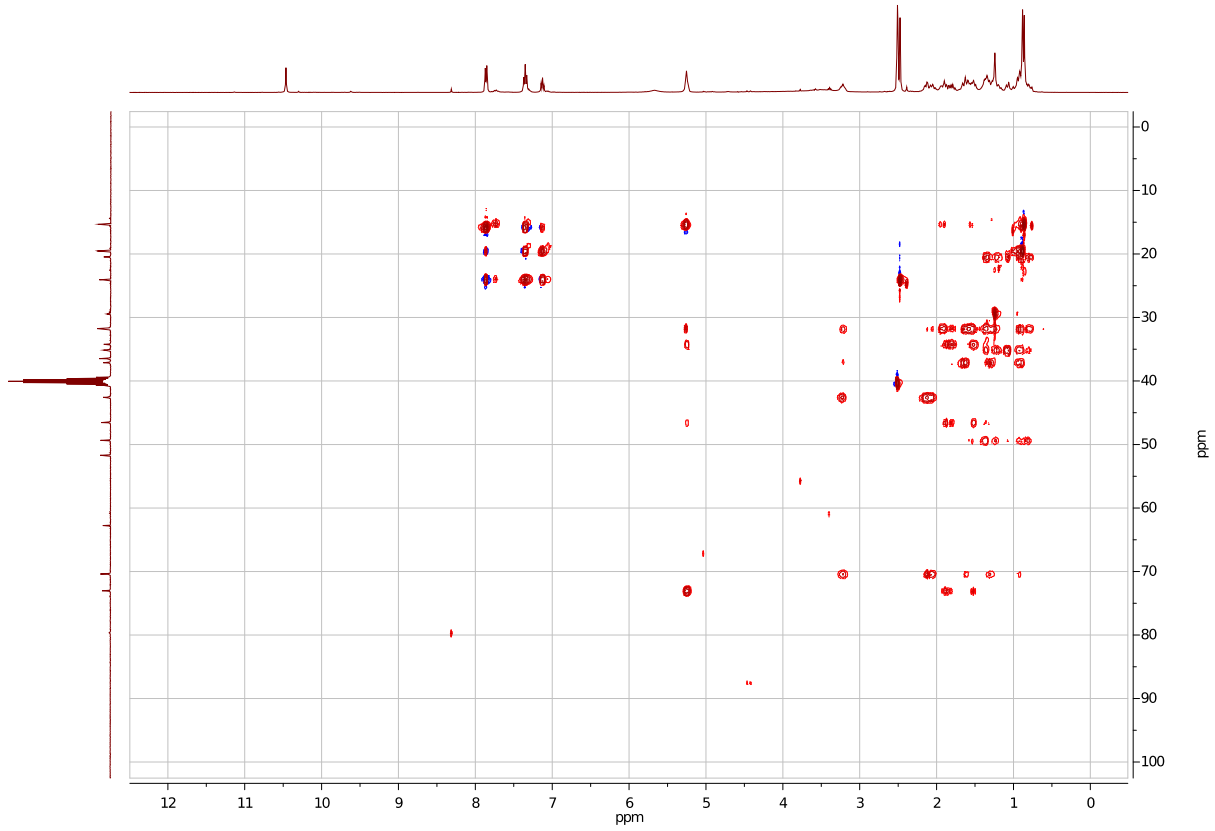


Figure S12: 2D ^{13}C–^{1}H HSQC-TOCSY NMR spectrum of **2a**.

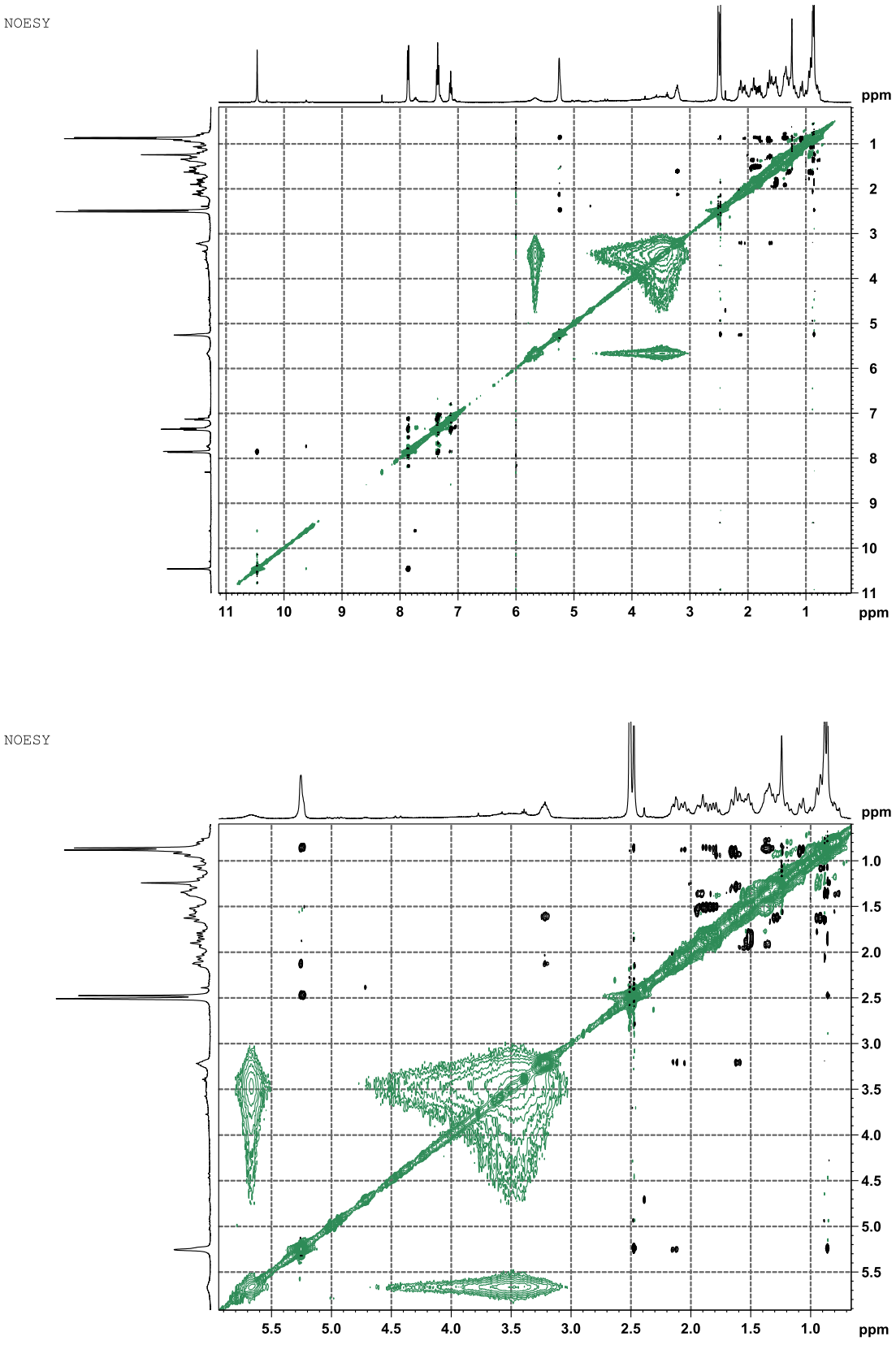


Figure S13: 2D 1H-1H NOESY NMR spectrum of the compound **2a**.

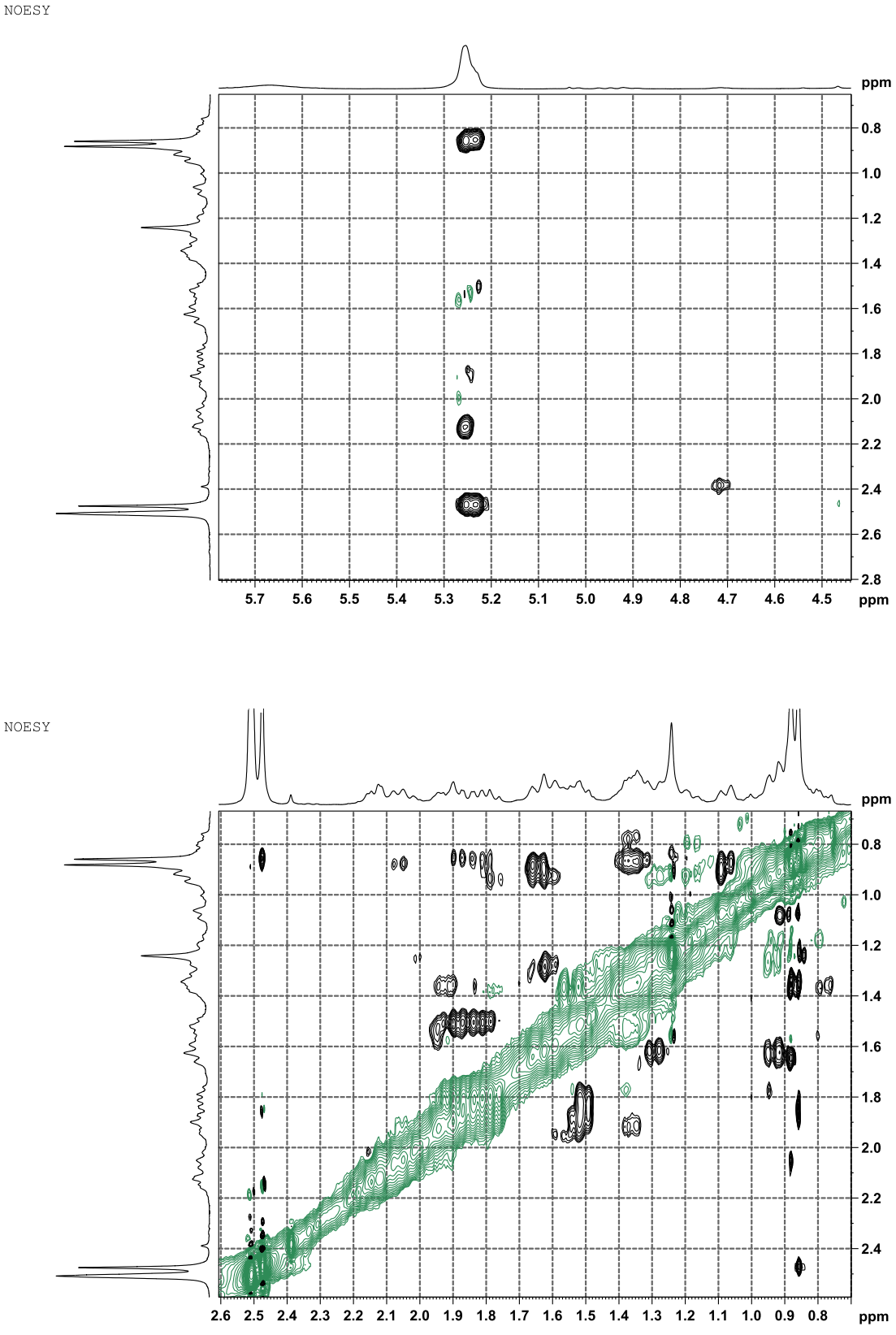


Figure S13 *(continued)*: 2D 1H-1H NOESY NMR spectrum of the compound **2a**.

NMR spectra for compound 2b (600 MHz).

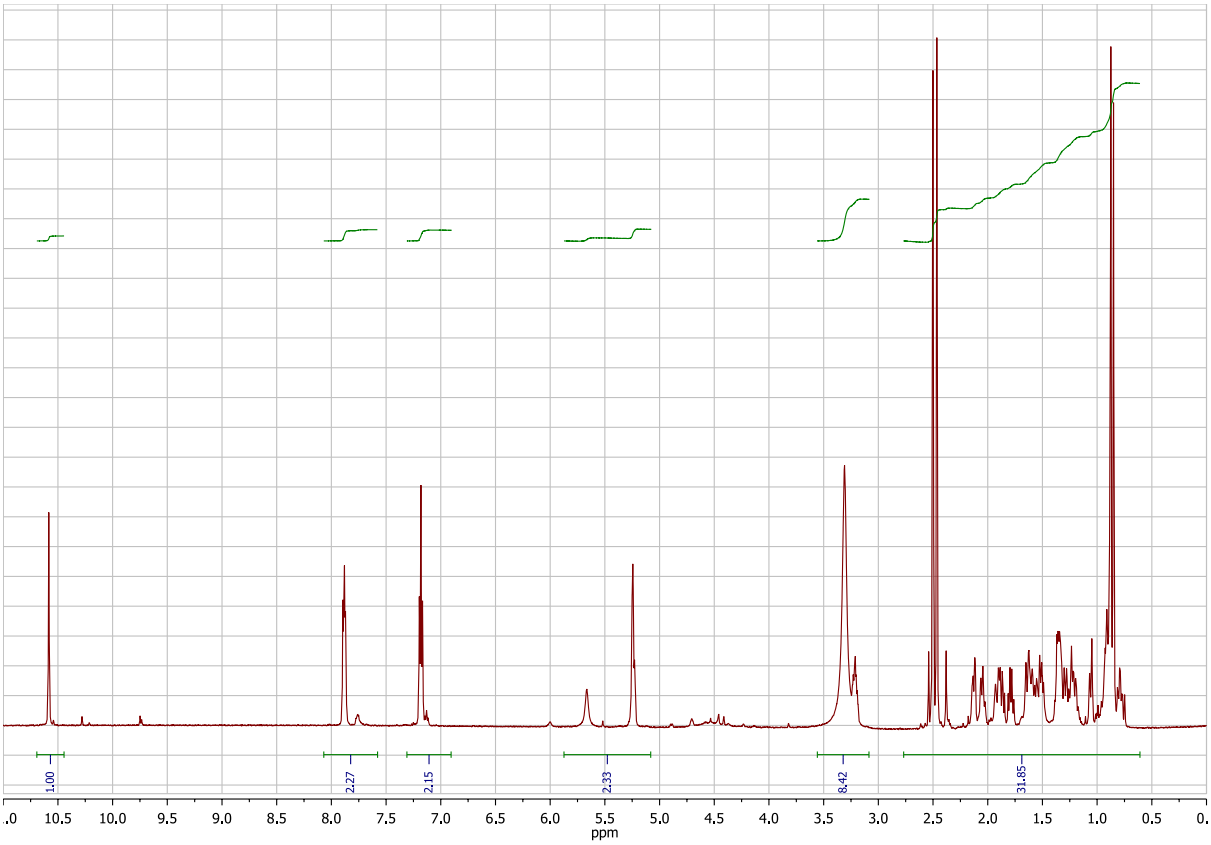


Figure S14: ^{1}H NMR spectrum of **2b**.

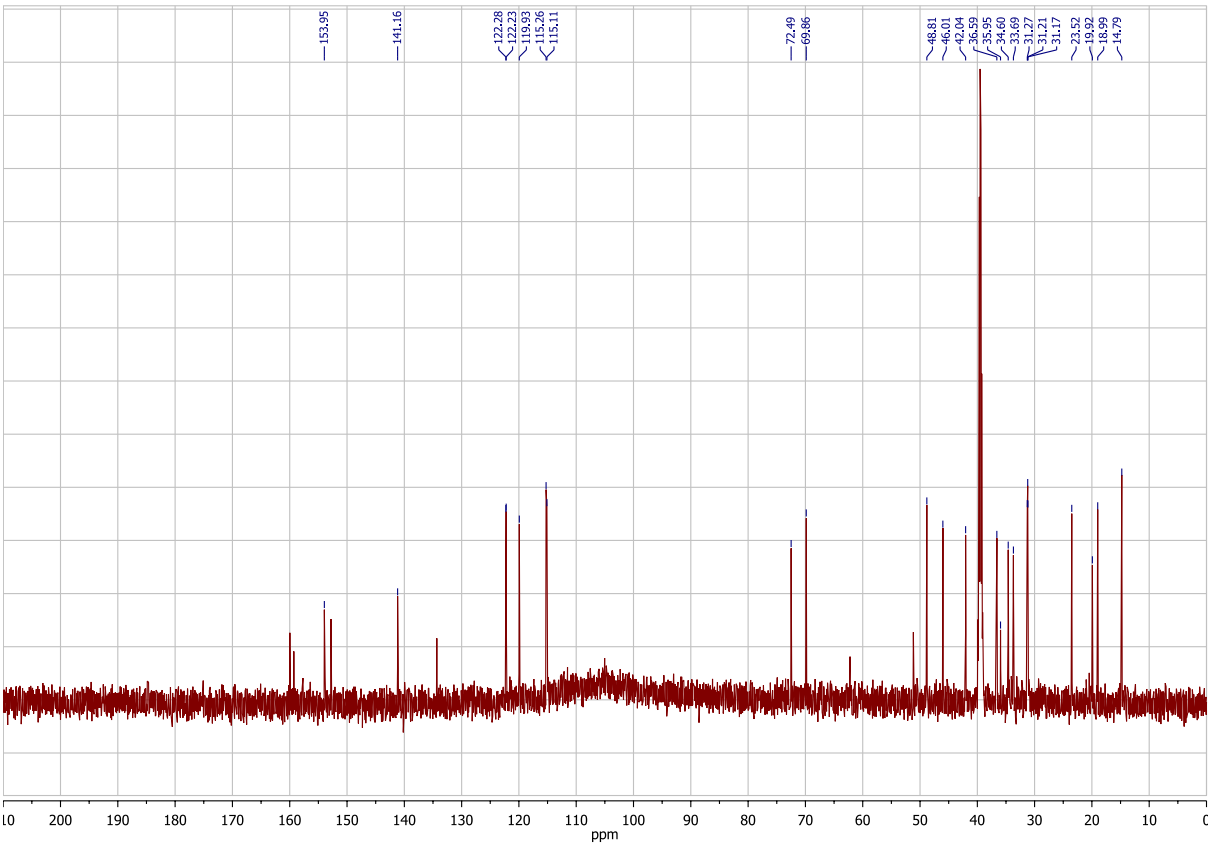


Figure S15: ^{13}C NMR spectrum of **2b**.

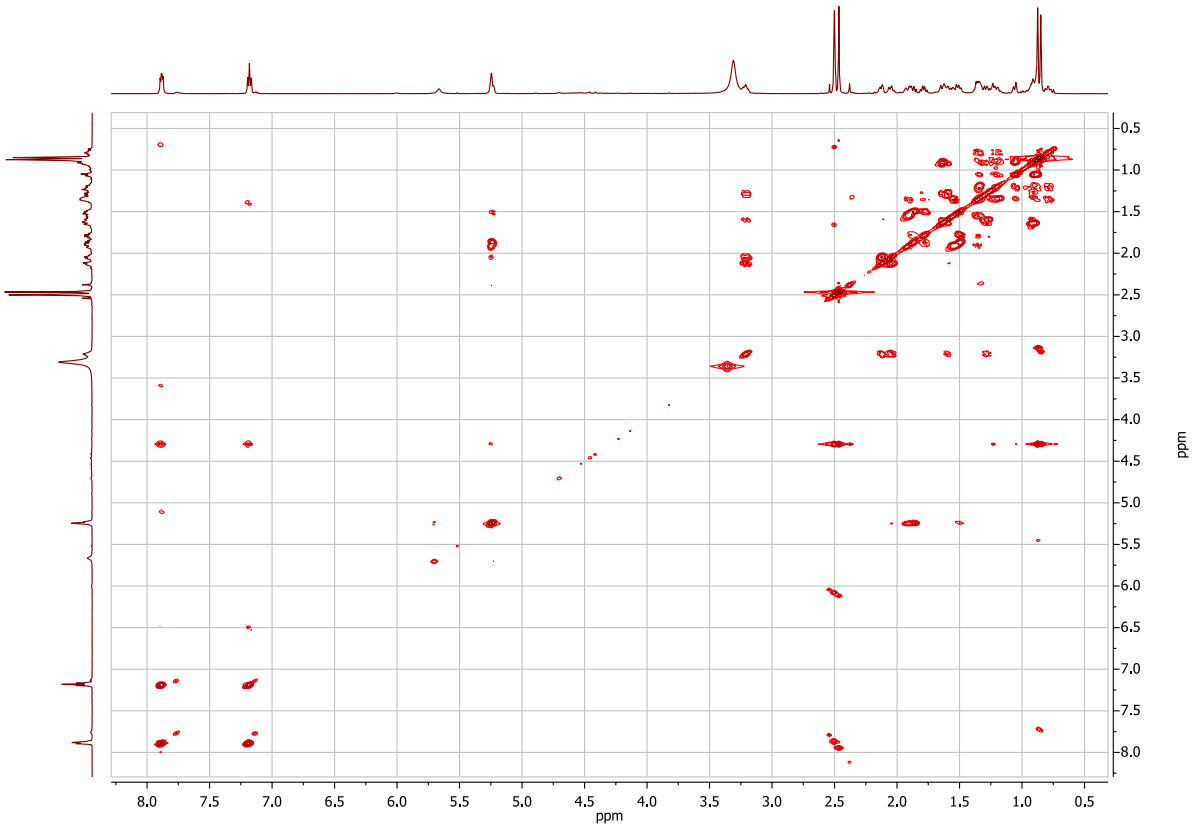


Figure S16: 2D ^{1}H-^{1}H COSY NMR spectrum of **2b**.

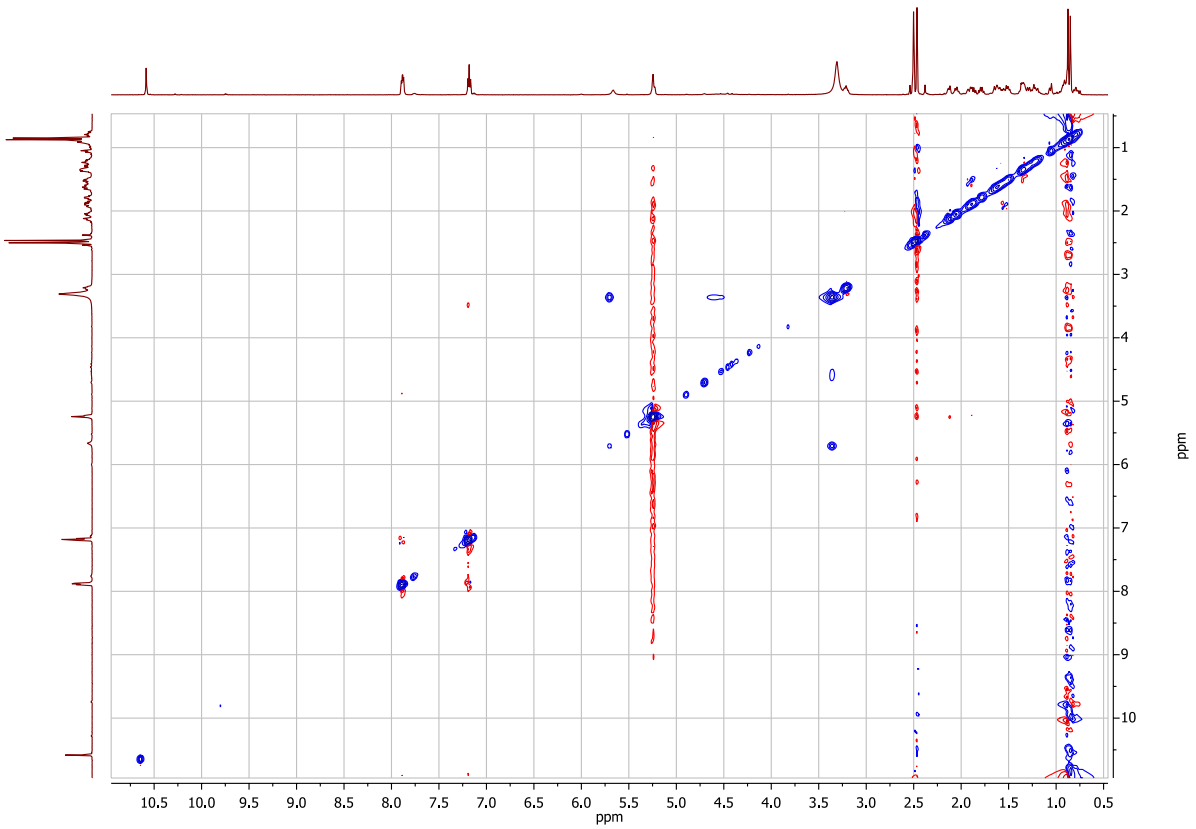


Figure S17: 2D ^{1}H-^{1}H ROESY NMR spectrum of **2b**.

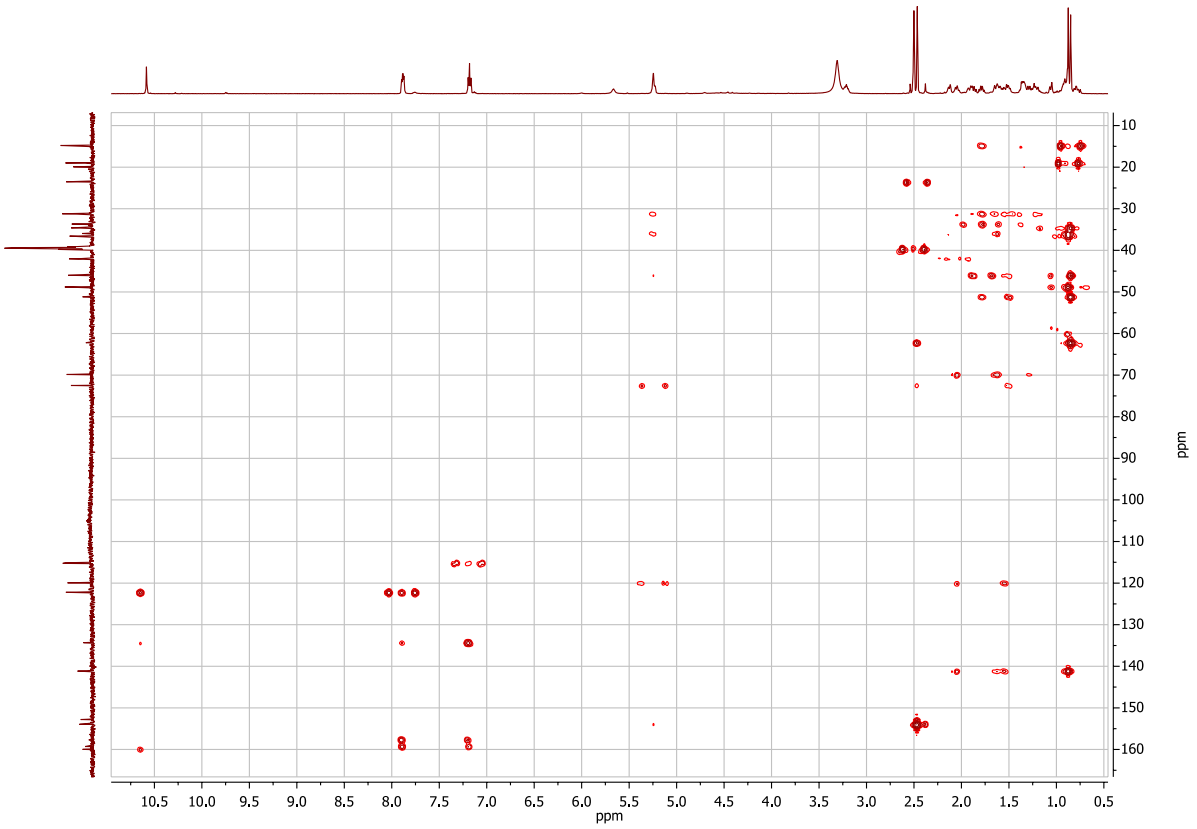


Figure S18: 2D ^{1}H-^{13}C HMBC NMR spectrum of **2b**.

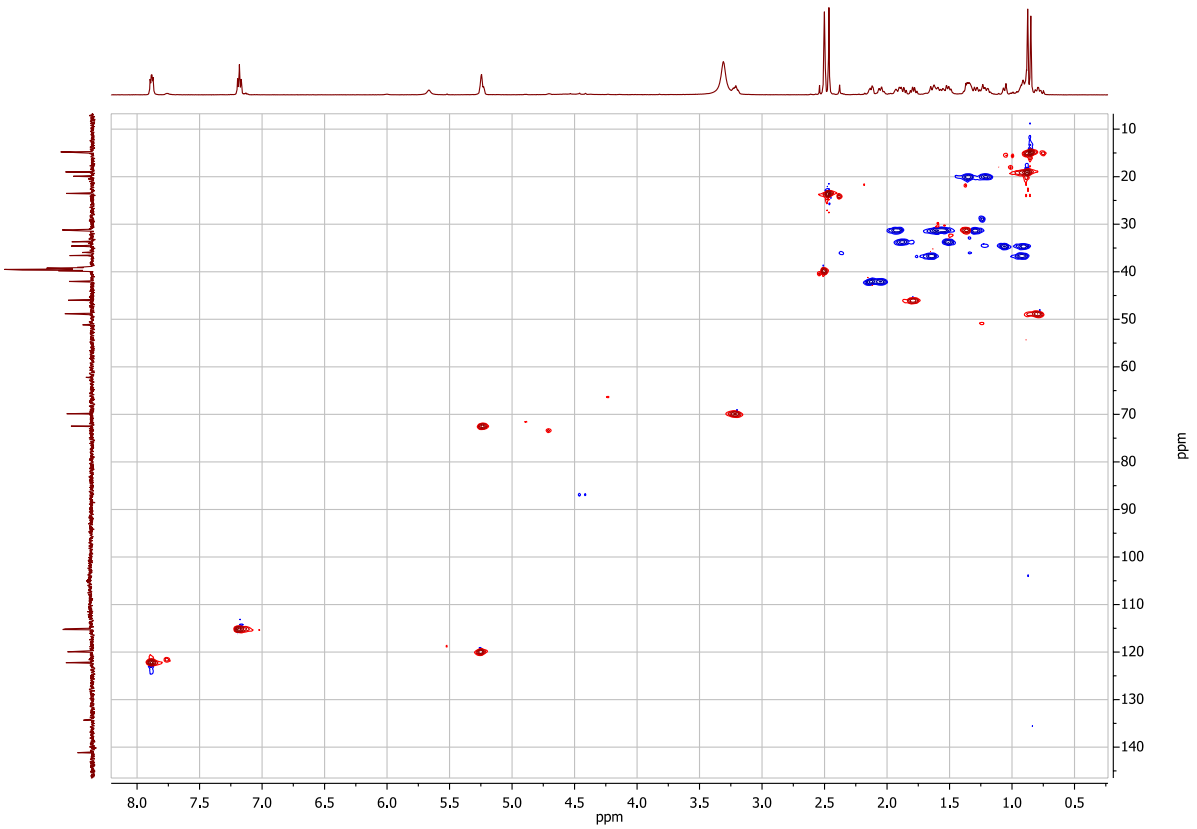


Figure S19: 2D ^{1}H-^{13}C HSQC NMR spectrum of **2b**.