

Supplementary Information

Stereochemical optimization of *N*,2-substituted cycloalkylamines as norepinephrine reuptake inhibitors

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Supplementary figures

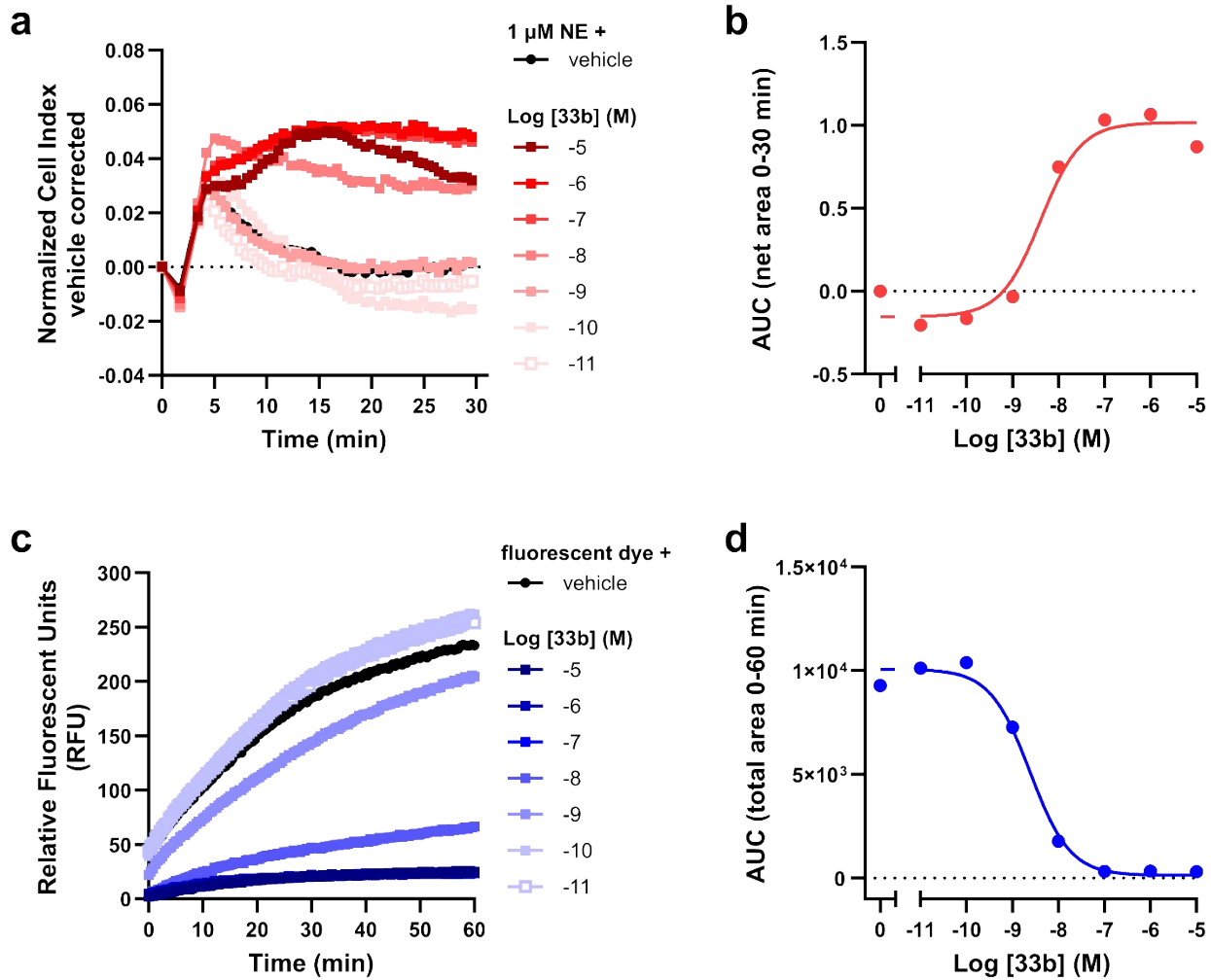


Figure S1. Potentiation of substrate-induced cellular response upon pretreatment with **33b** in dox-treated JumpIn-NET cells in the TRACT assay. (a) Representative vehicle-corrected normalized Cell Index traces are shown of dox-treated cells pretreated for 1 h with increasing concentrations of **33b** (red), stimulated with 1 μ M norepinephrine (NE) in TRACT assay. (b) Representative concentration-inhibition curve of **33b** upon stimulation with NE. Cellular response is expressed as the net AUC of the first 30 minutes after stimulation. Inhibition of fluorescent neurotransmitter-dye uptake in dox-treated JumpIn-NET cells in fluorescent uptake assay (c) Representative neurotransmitter-dye uptake traces corrected for non-NET mediated uptake are shown of dox-treated JumpIn-NET cells pretreated for 1 h with vehicle or increasing concentrations of **33b** (blue), followed by addition of fluorescent loading dye solution and uptake was monitored for 1 h. (d) Representative concentration-inhibition curve of **33b** in fluorescent uptake assay. Cellular response is expressed as the total AUC of the total 1 h after addition of the loading dye.

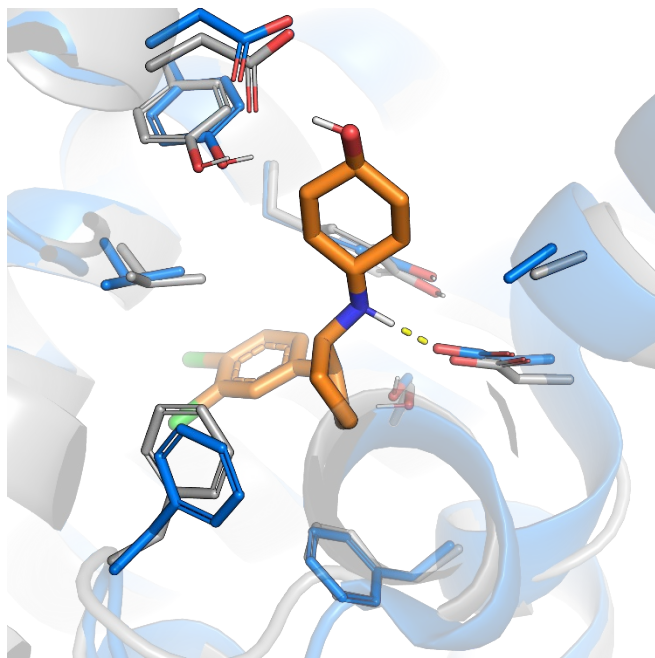


Figure S2. Alignment of the hNET AlphaFold model used in this study to dock compound **33b** (grey, compound in orange) aligned with a cryo-EM structure of the outward facing conformation of hNET with atomoxetine (PDB: 8Z1L)¹ in blue. The cryo-EM structure was aligned with PyMOL² with a total RMSD of 1.01 Å. Residues in the binding site are shown to closely overlap, validating the use of the AlphaFold model in this study.

- 1 H. Zhang, Y. L. Yin, A. Dai, T. Zhang, C. Zhang, C. Wu, W. Hu, X. He, B. Pan, S. Jin, Q. Yuan, M. W. Wang, D. Yang, H. E. Xu and Y. Jiang, Dimerization and antidepressant recognition at noradrenaline transporter, *Nature*, 2024, **630**, 247–254.
- 2 W. L. DeLano, The PyMOL Molecular Graphics System, Version 2.5.2, Schrödinger, LLC, New York, NY, 2021

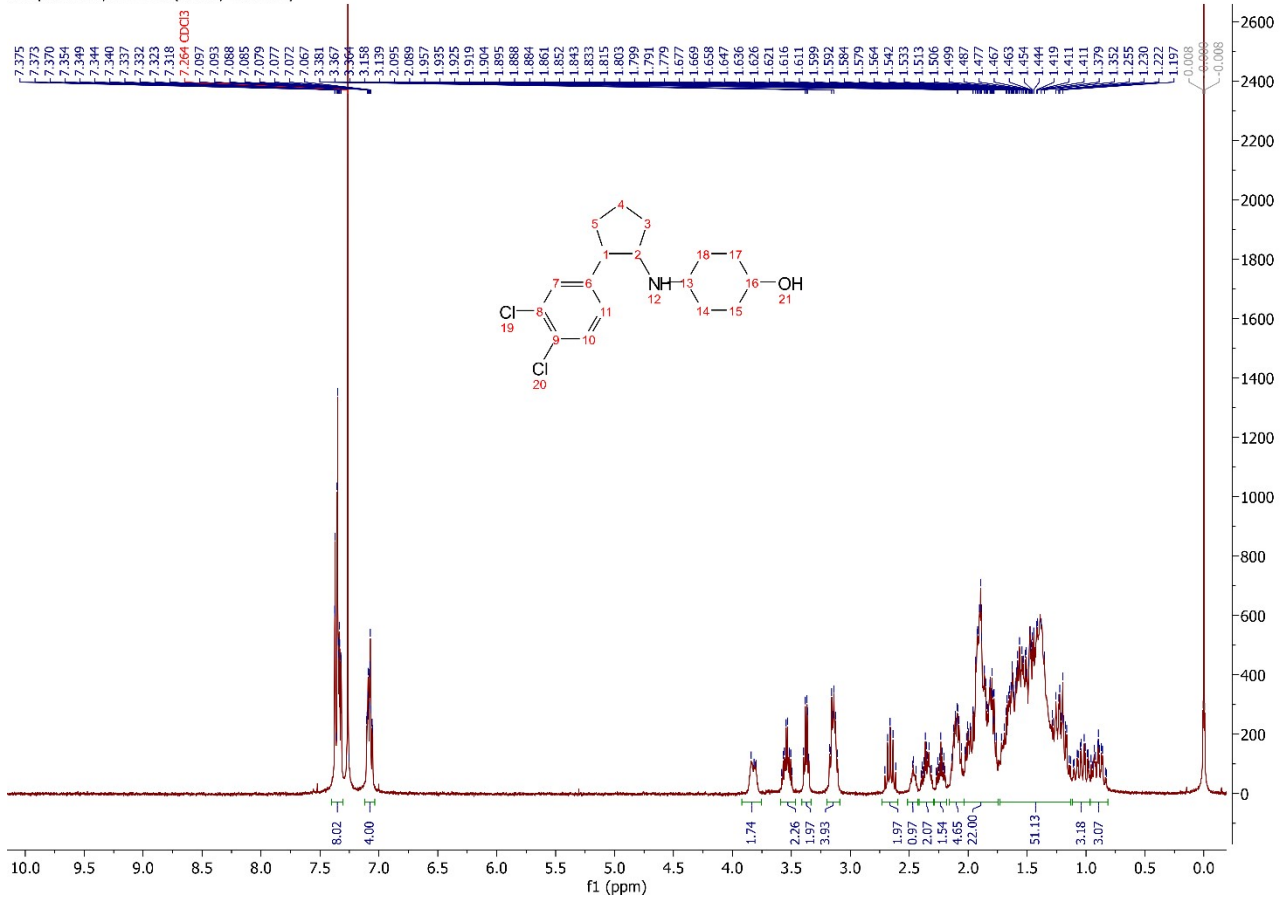
Supplementary experimental

1. Fluorescent uptake assay

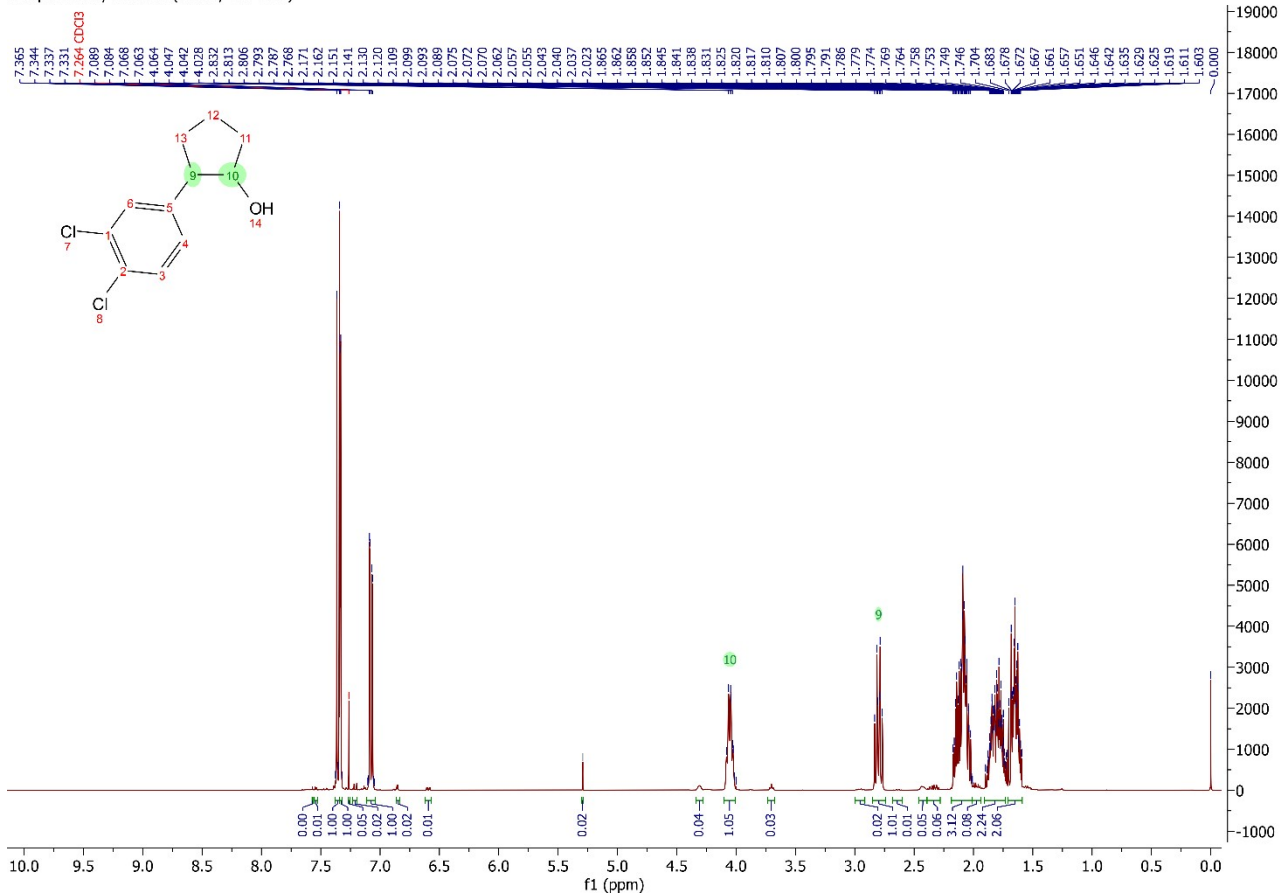
Fluorescent uptake assays were performed using the fluorescent neurotransmitter transporter uptake assay kit (Molecular devices San Jose, CA, USA) following the supplier's protocol. HEK293-JumpIn-NET cells were seeded (60.000 cells/well) in a poly-D-lysine coated black 96 wells plate and induced with doxycycline (1 $\mu\text{g}/\text{mL}$) to express the transporter of interest for 24 hours. After 24 h, medium was removed, and cells were preincubated by addition of 100 $\mu\text{L}/\text{well}$ inhibitor (at increasing concentrations ranging from 10^{-11} to 10^{-5}), inhibitor control (10 μM nisoxetine) or vehicle control (buffer only) in HBSS containing 20 mM HEPES for 1 h at 37 °C prior to initiation of uptake by addition of 100 μM fluorescent neurotransmitter dye. Uptake of dye was continuously monitored for 1 h at 37 °C on the FlexStation 3 Multi-Mode Microplate Reader (Molecular devices, San Jose, CA, USA). To obtain dose-response curves the total AUC over 60 minutes was calculated from the time traces and corrected for non-NET mediated uptake by subtracting uptake remaining in nisoxetine treated cells.

NMR spectra

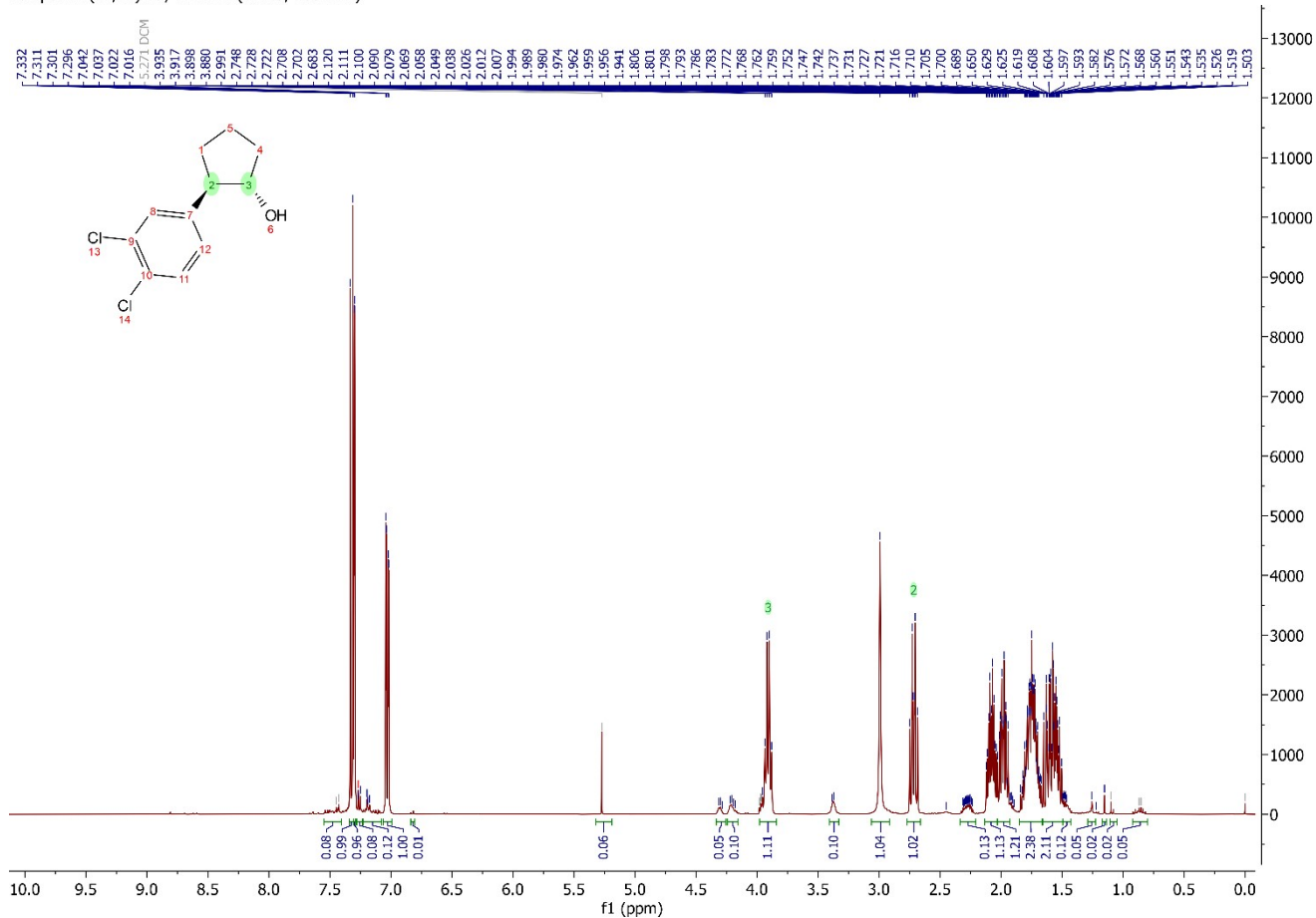
Compound 27; ¹H NMR (CDCl₃, 400 MHz)



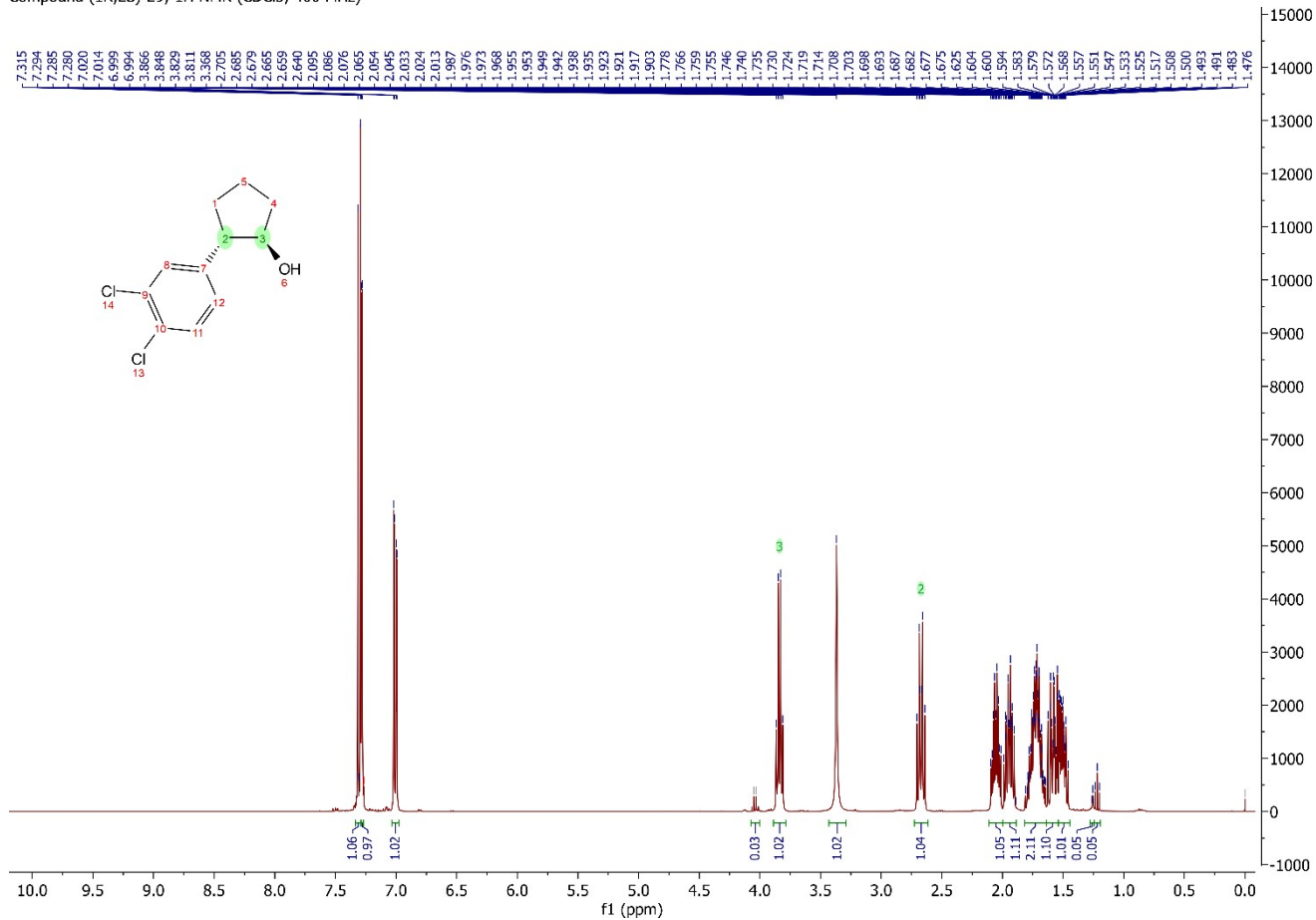
Compound 29; ¹H NMR (CDCl₃, 400 MHz)



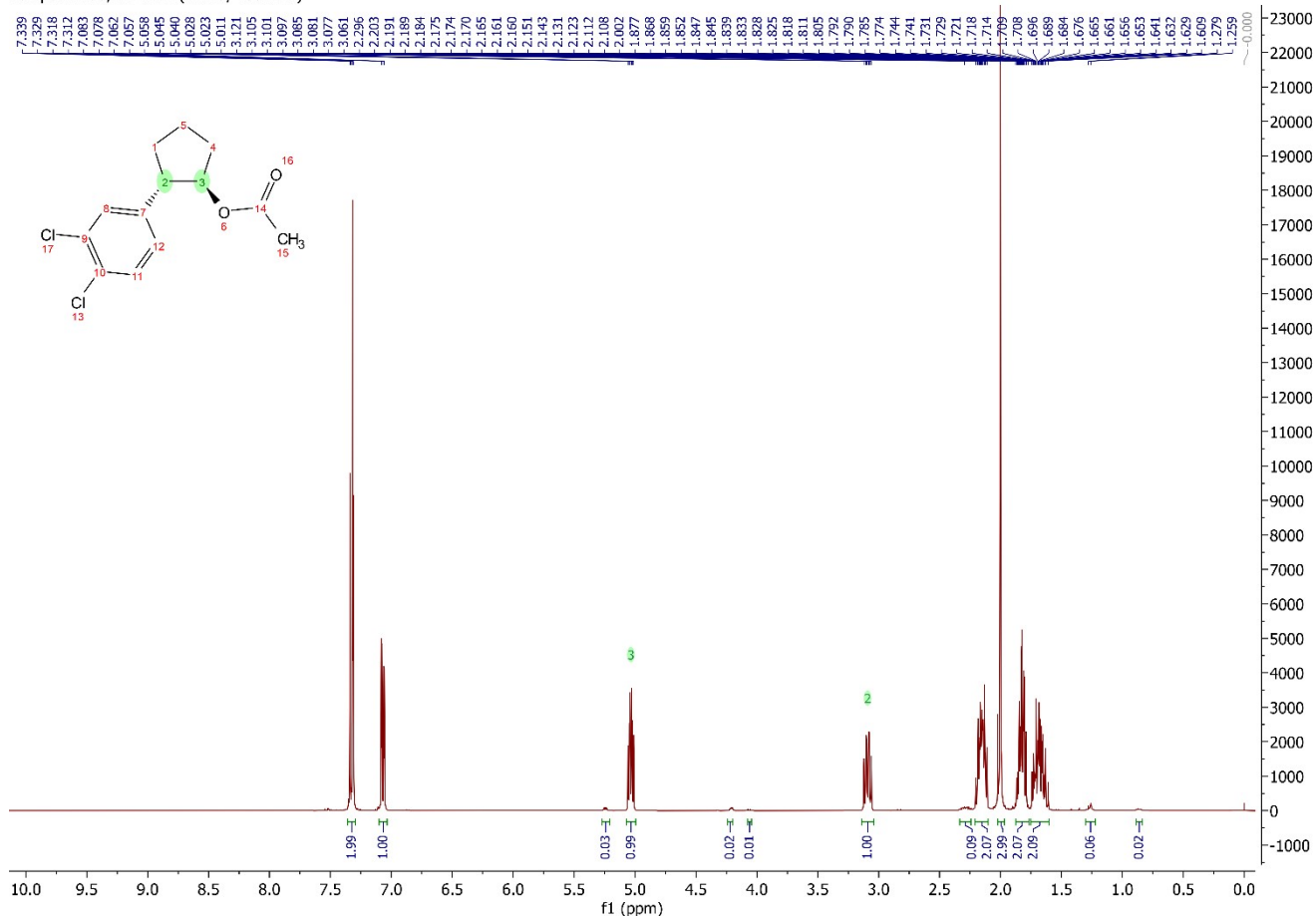
Compound (1S,2R)-29; 1H NMR (CDCl3, 400 MHz)



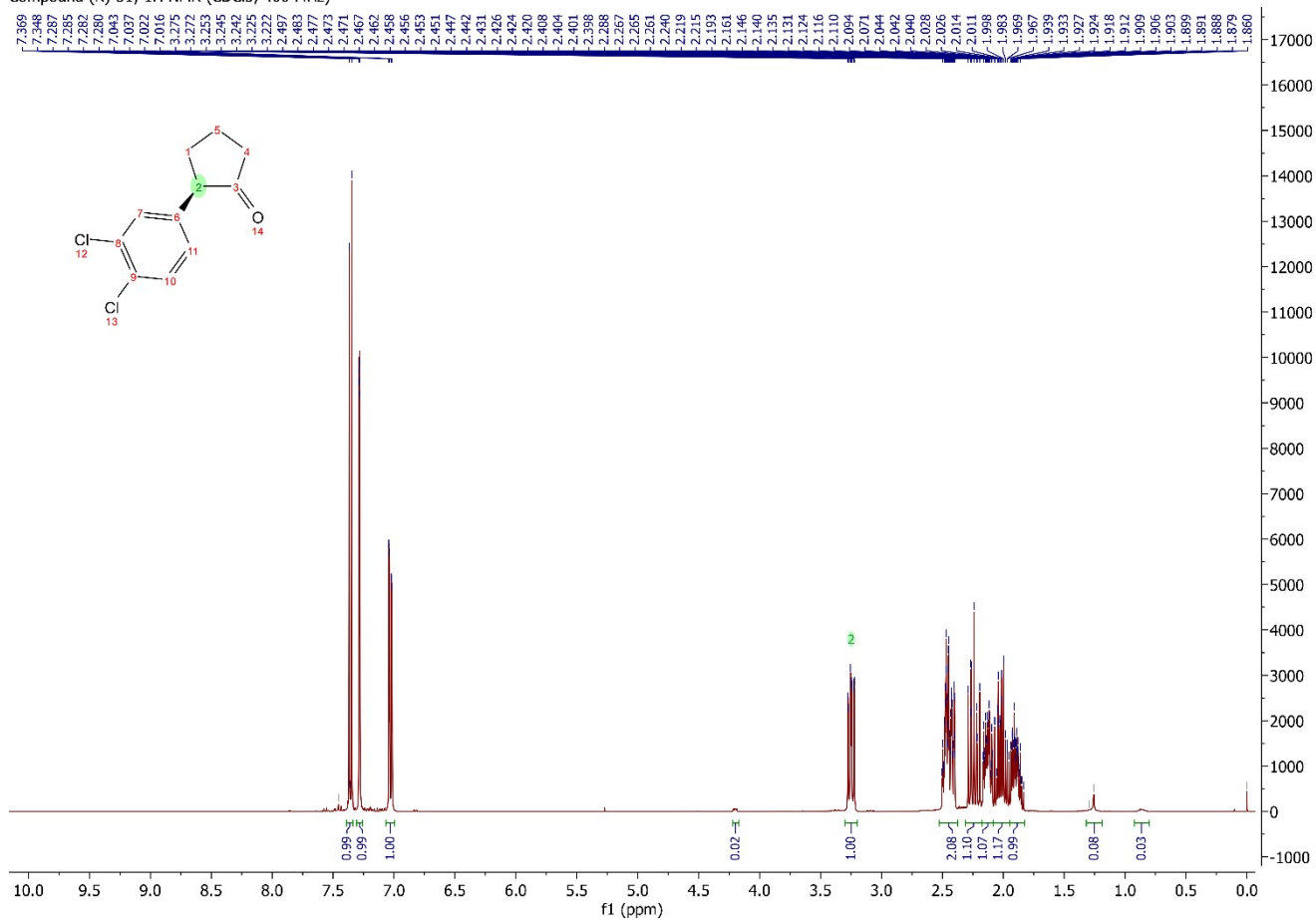
Compound (1R,2S)-29; 1H NMR (CDCl3, 400 MHz)



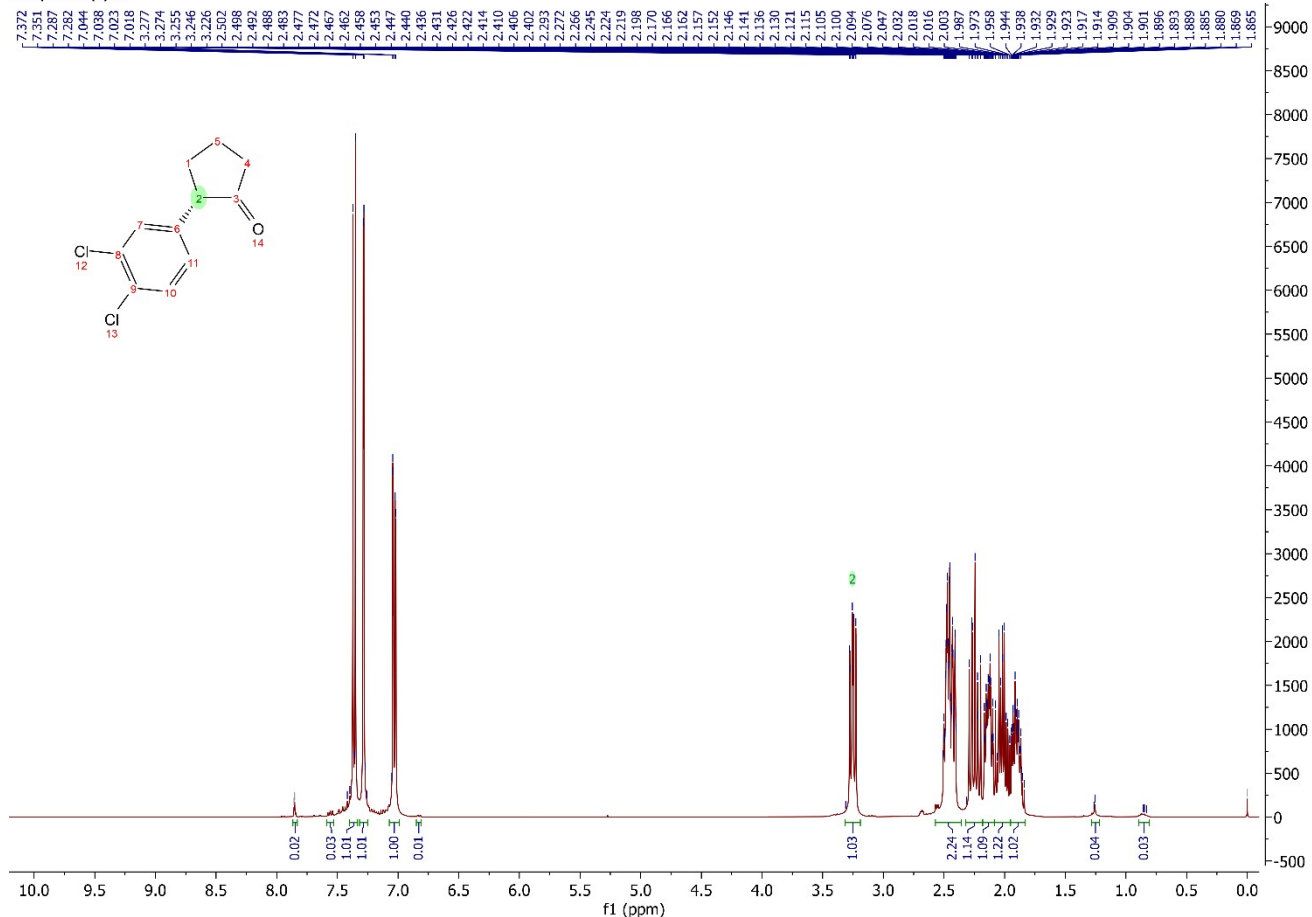
Compound 30; ¹H NMR (CDCl₃, 400 MHz)



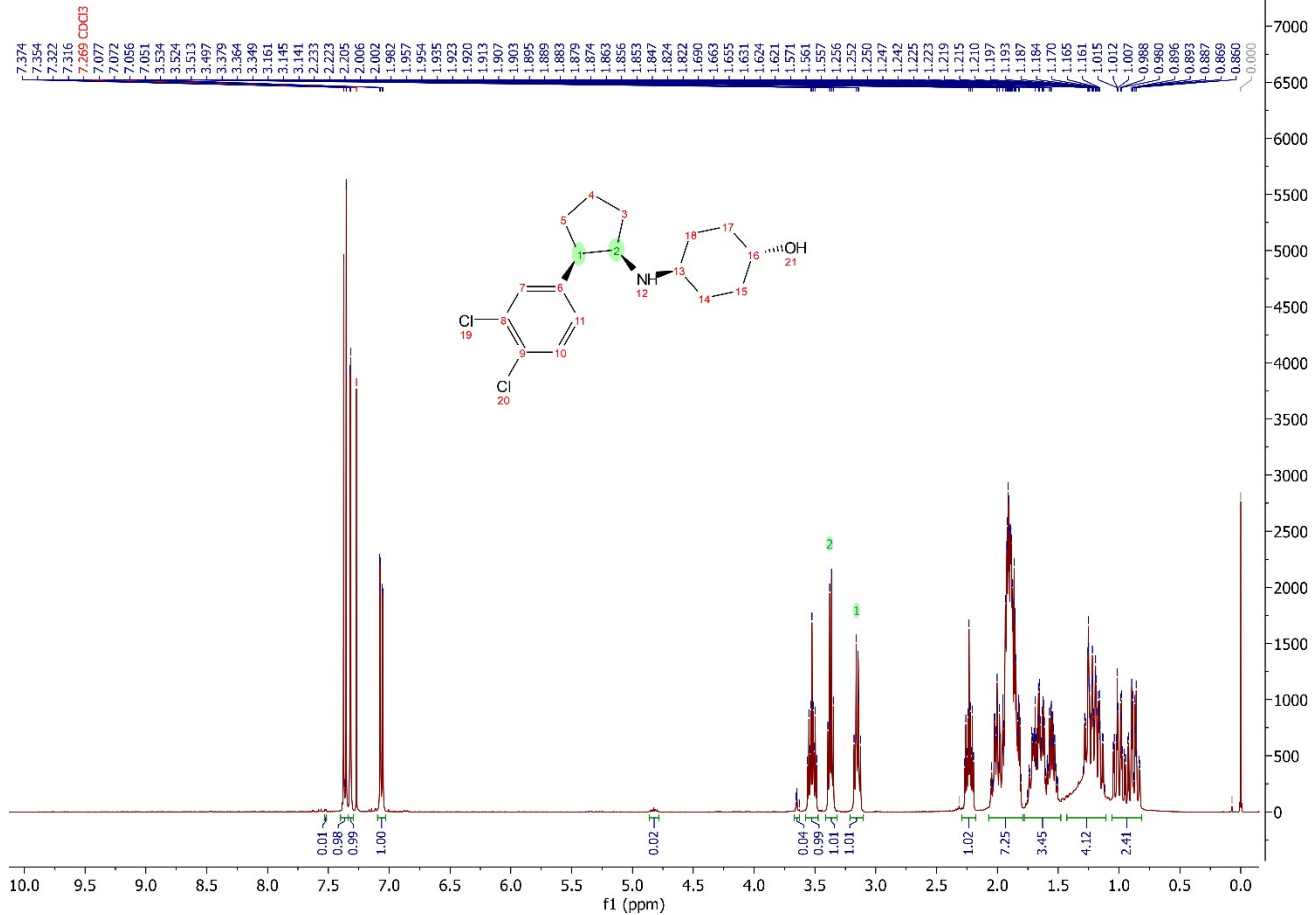
Compound (R)-31; ¹H NMR (CDCl₃, 400 MHz)



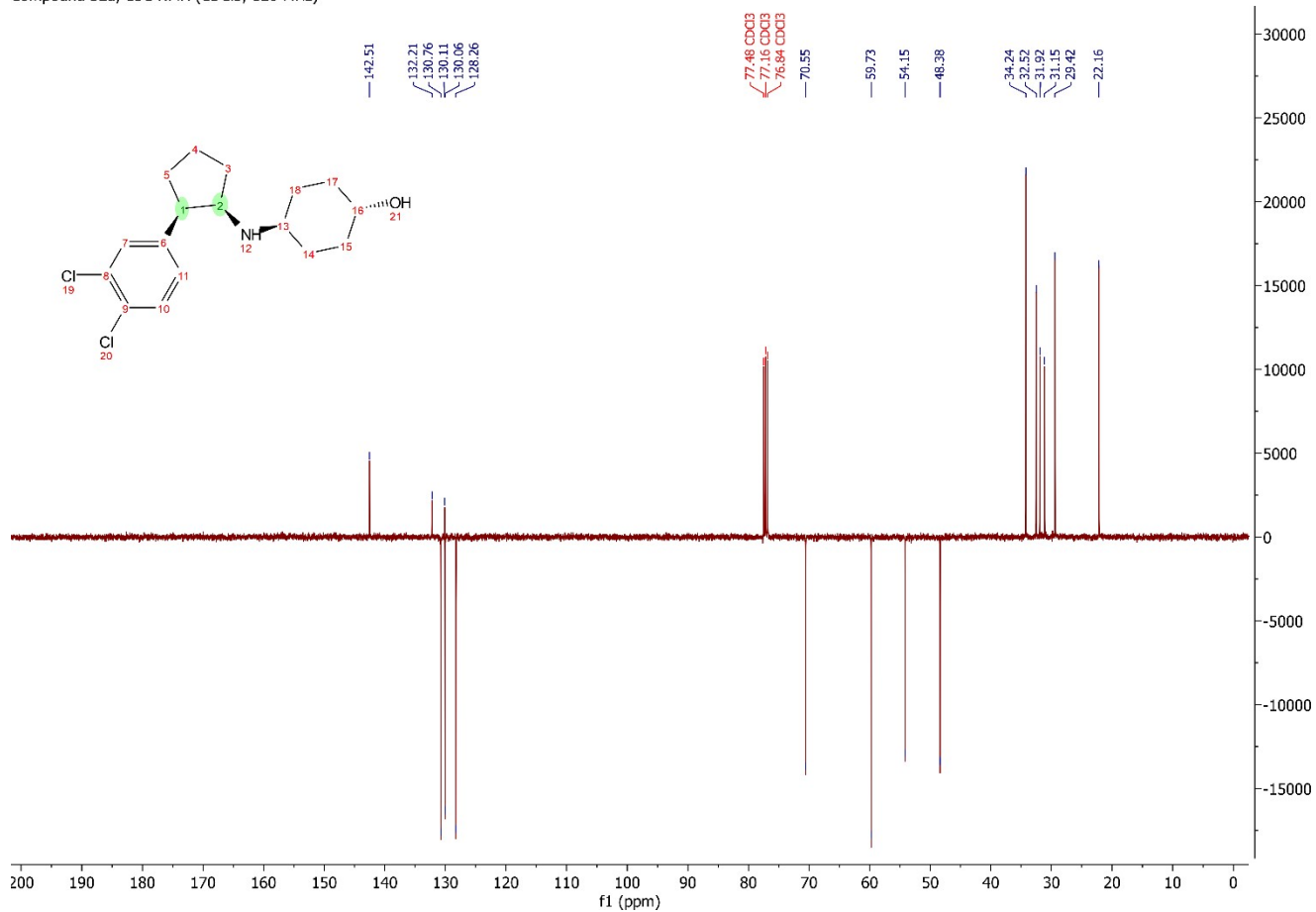
Compound (S)-31



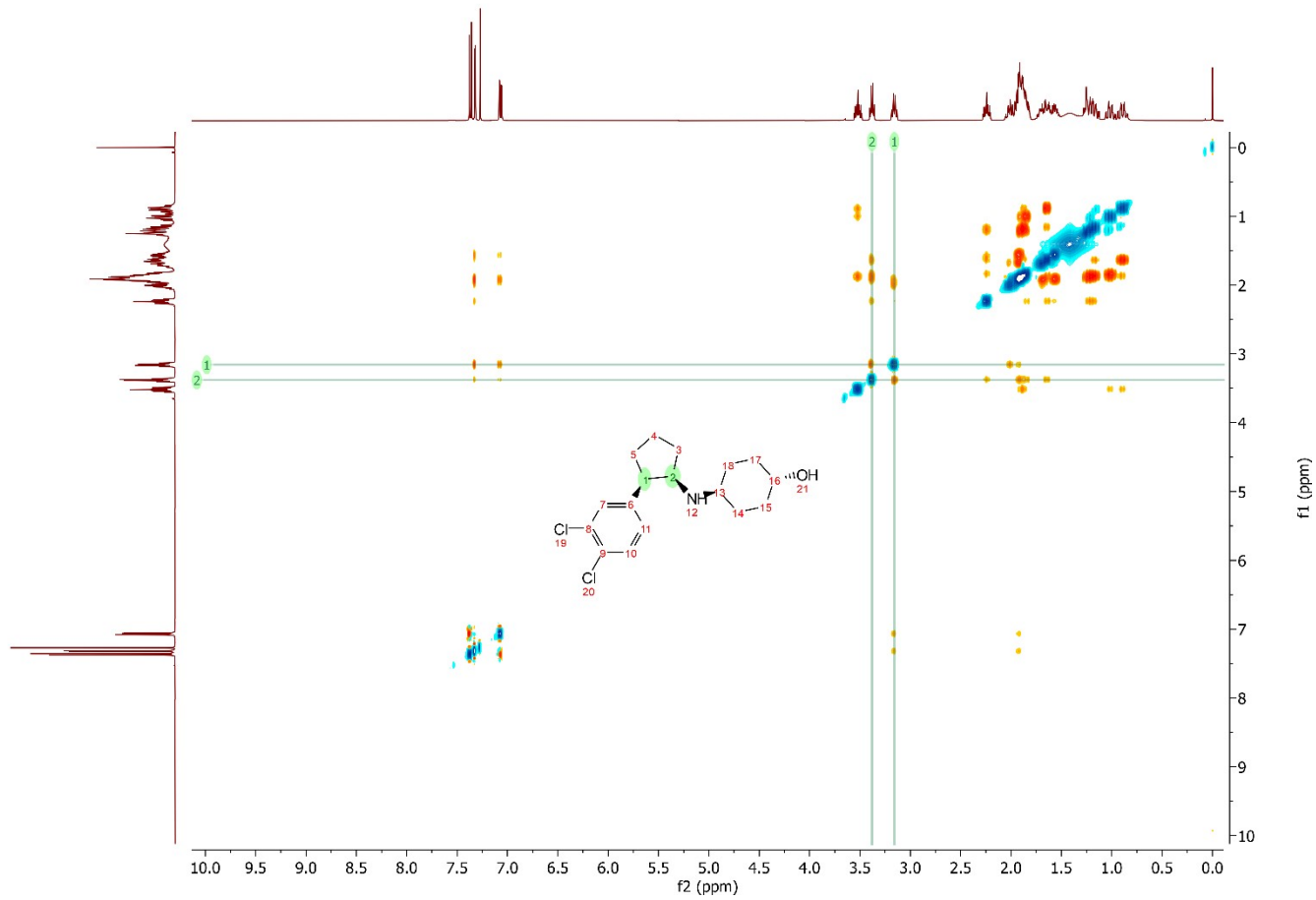
Compound 32a; ¹H NMR (CDCl₃, 400 MHz)



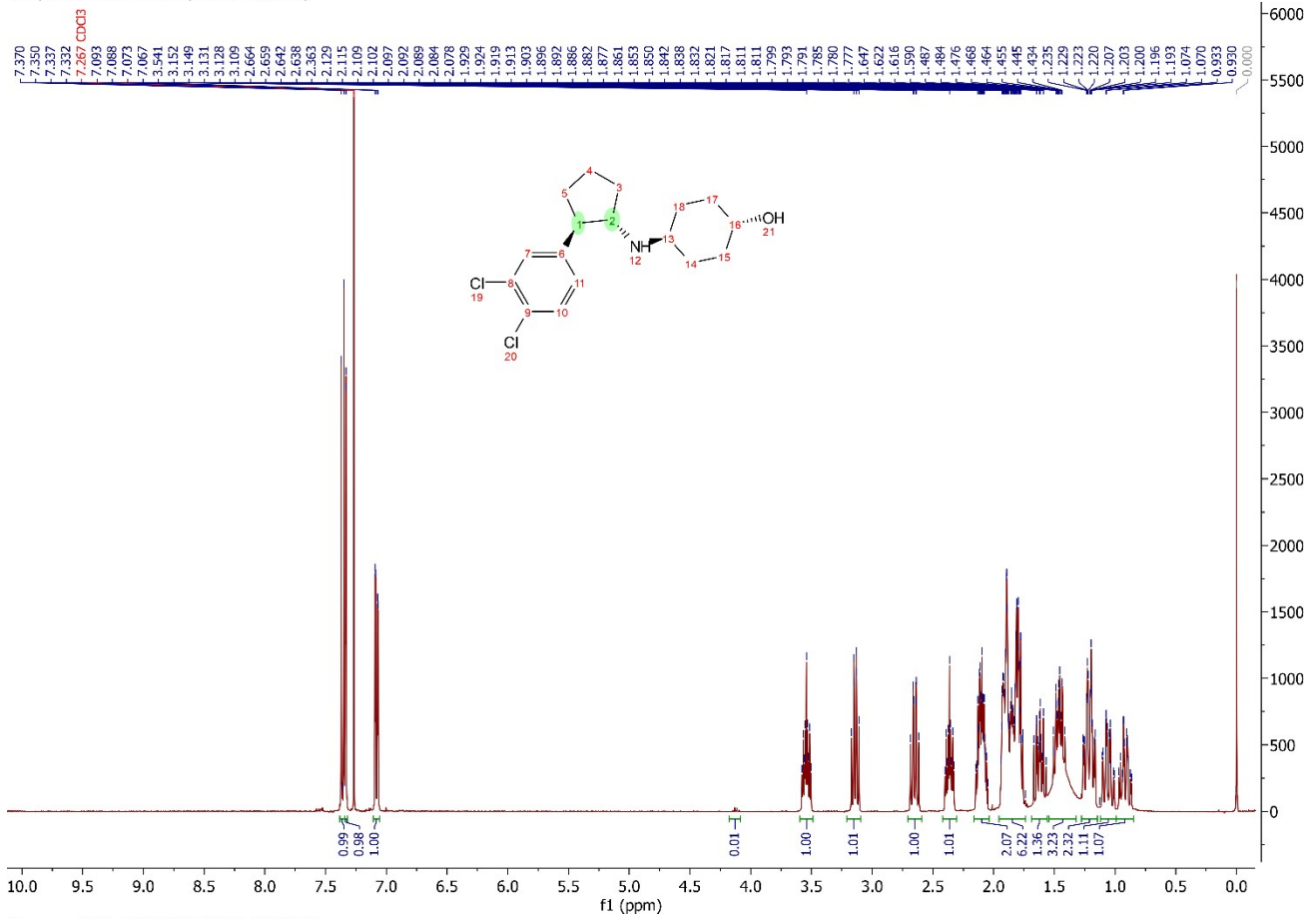
Compound 32a; ¹³C NMR (CDCl₃, 126 MHz)



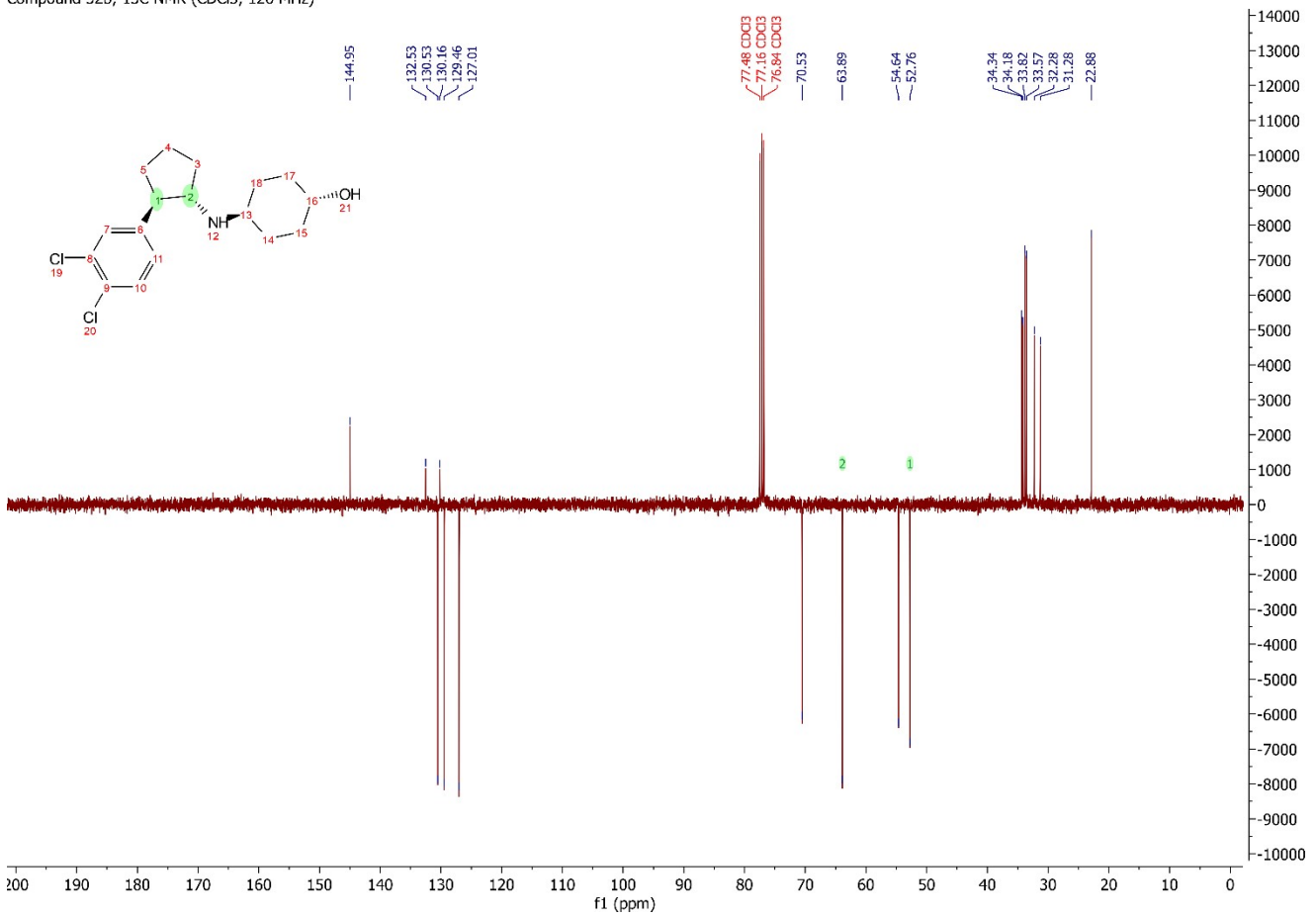
Compound 32a; NOESY (CDCl₃)

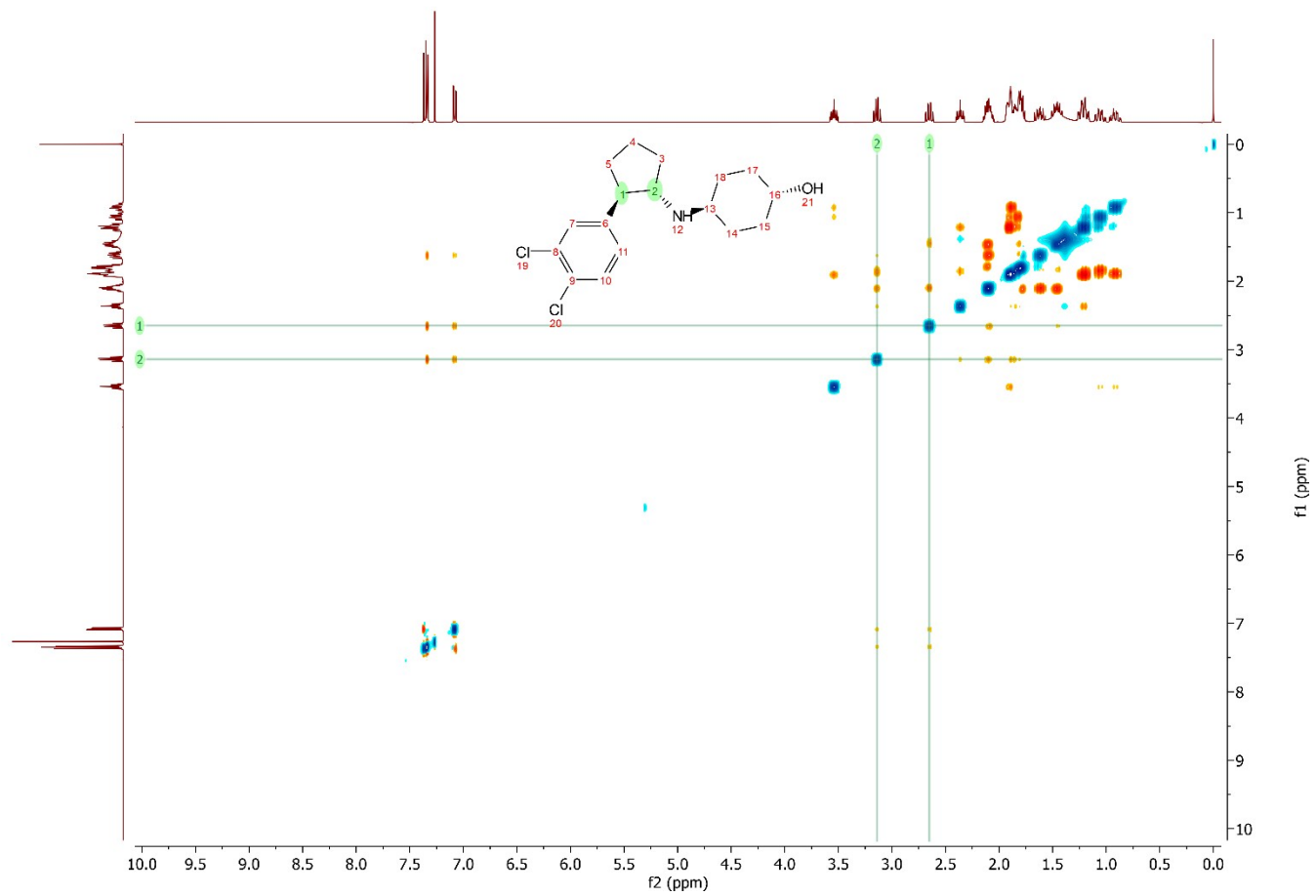


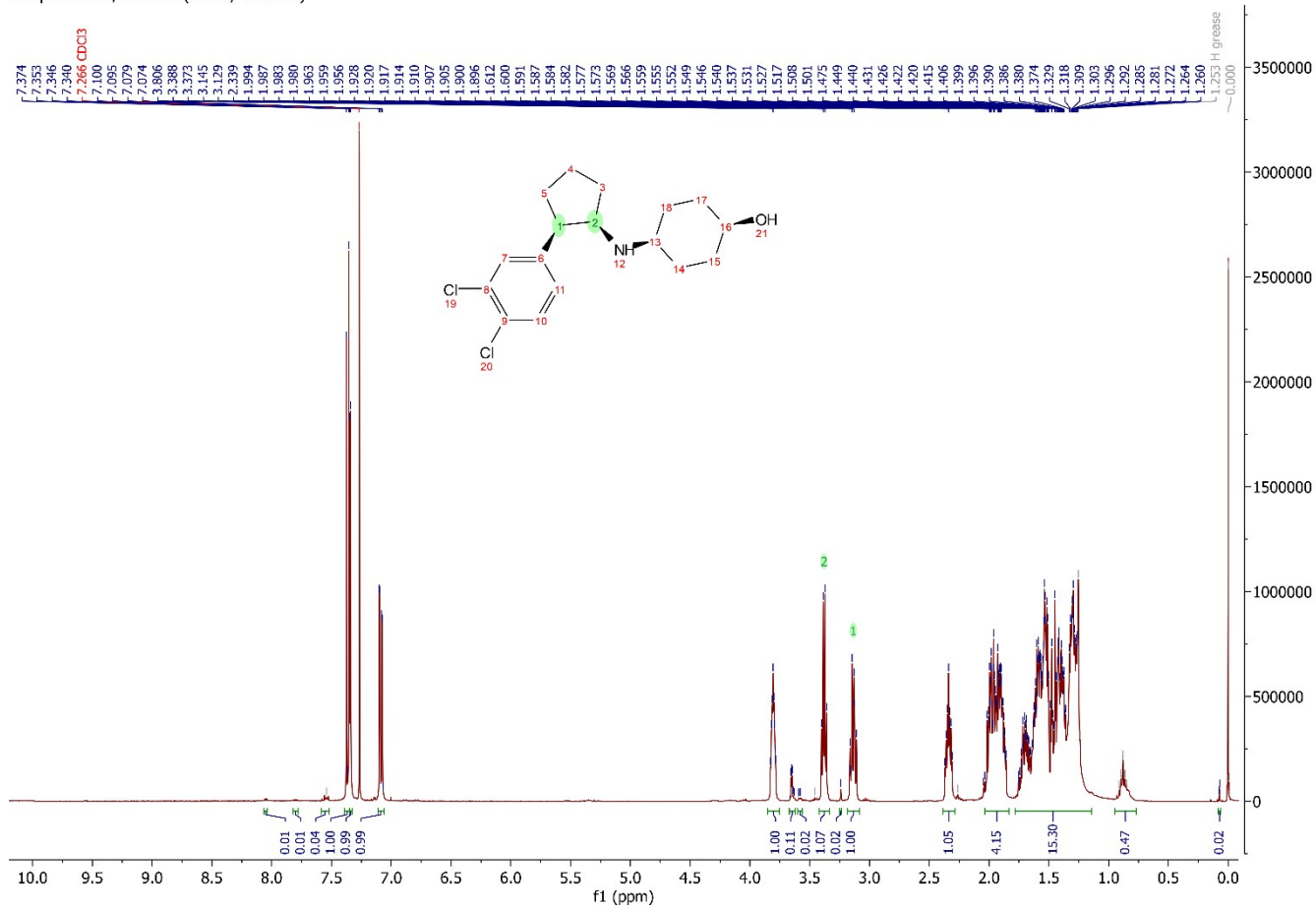
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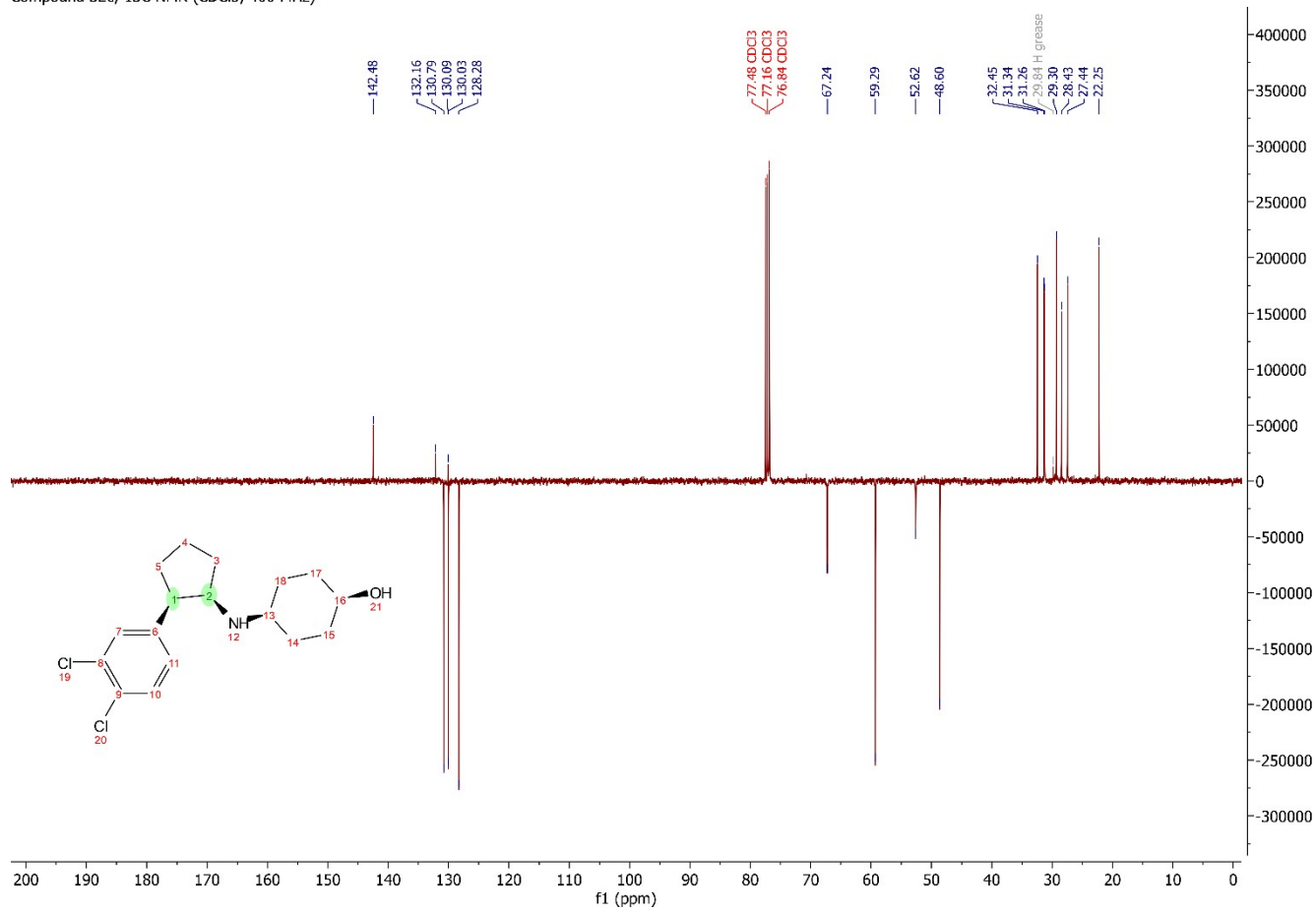
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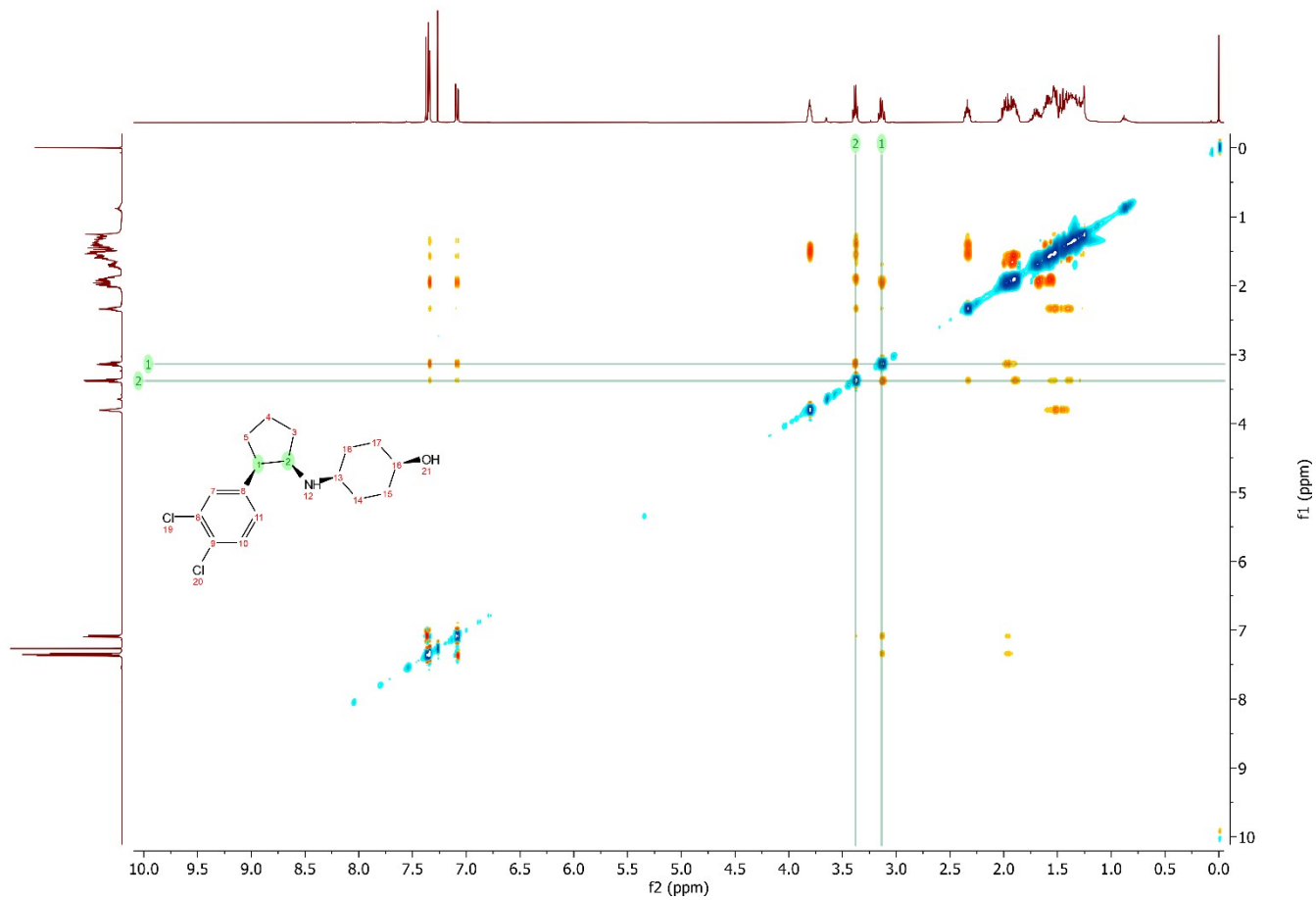




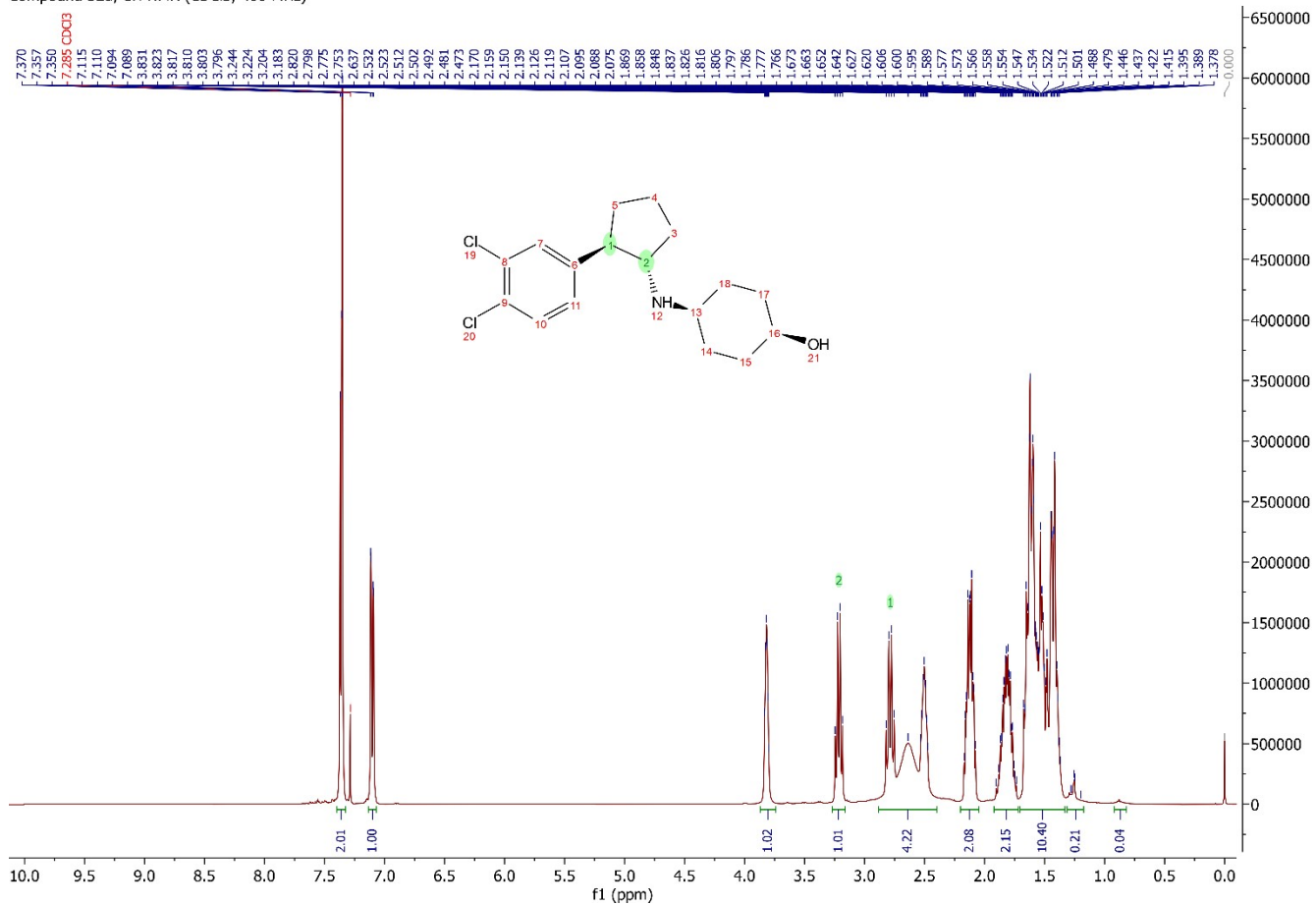


Compound 32c; ¹³C NMR (CDCl₃, 400 MHz)

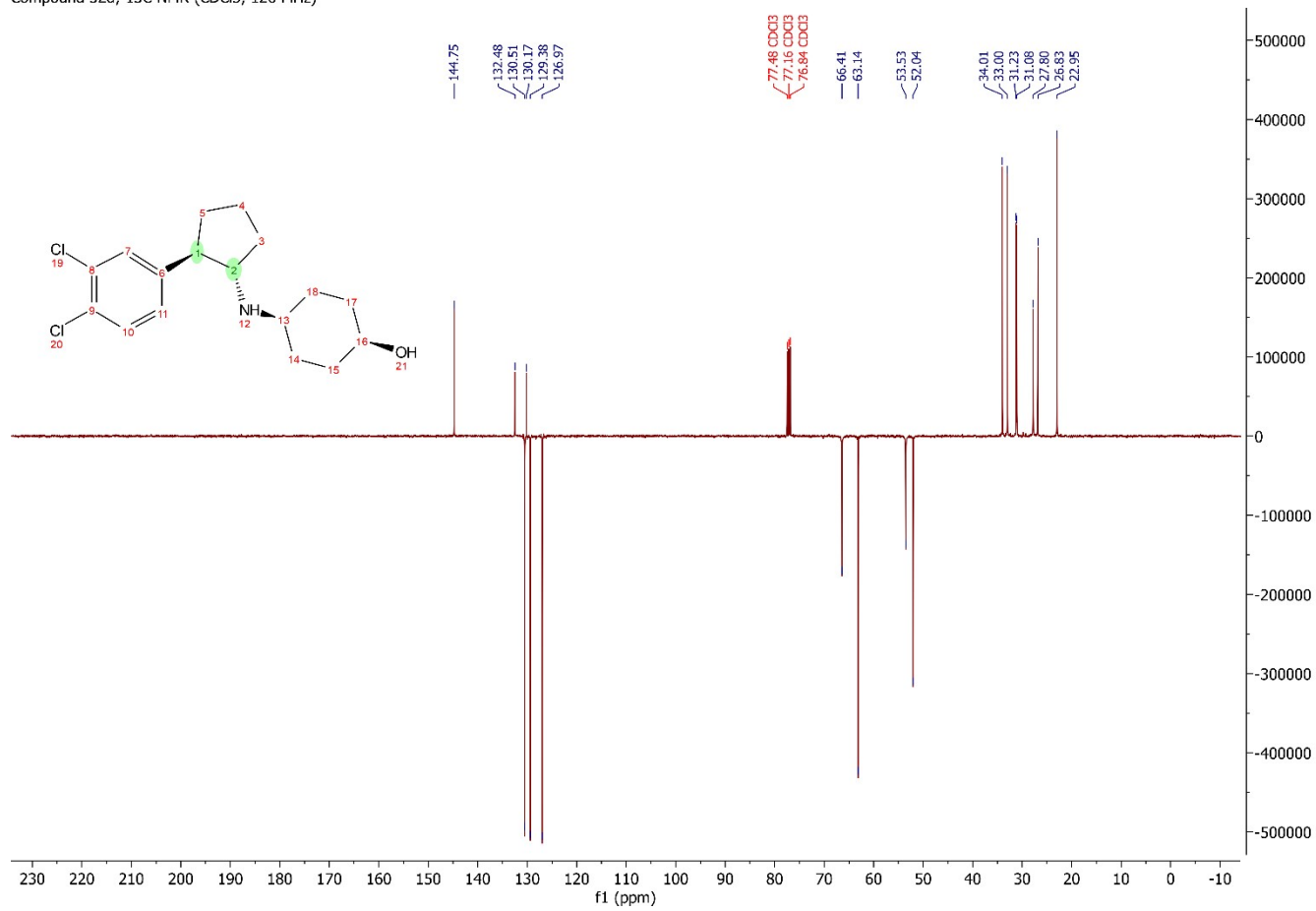


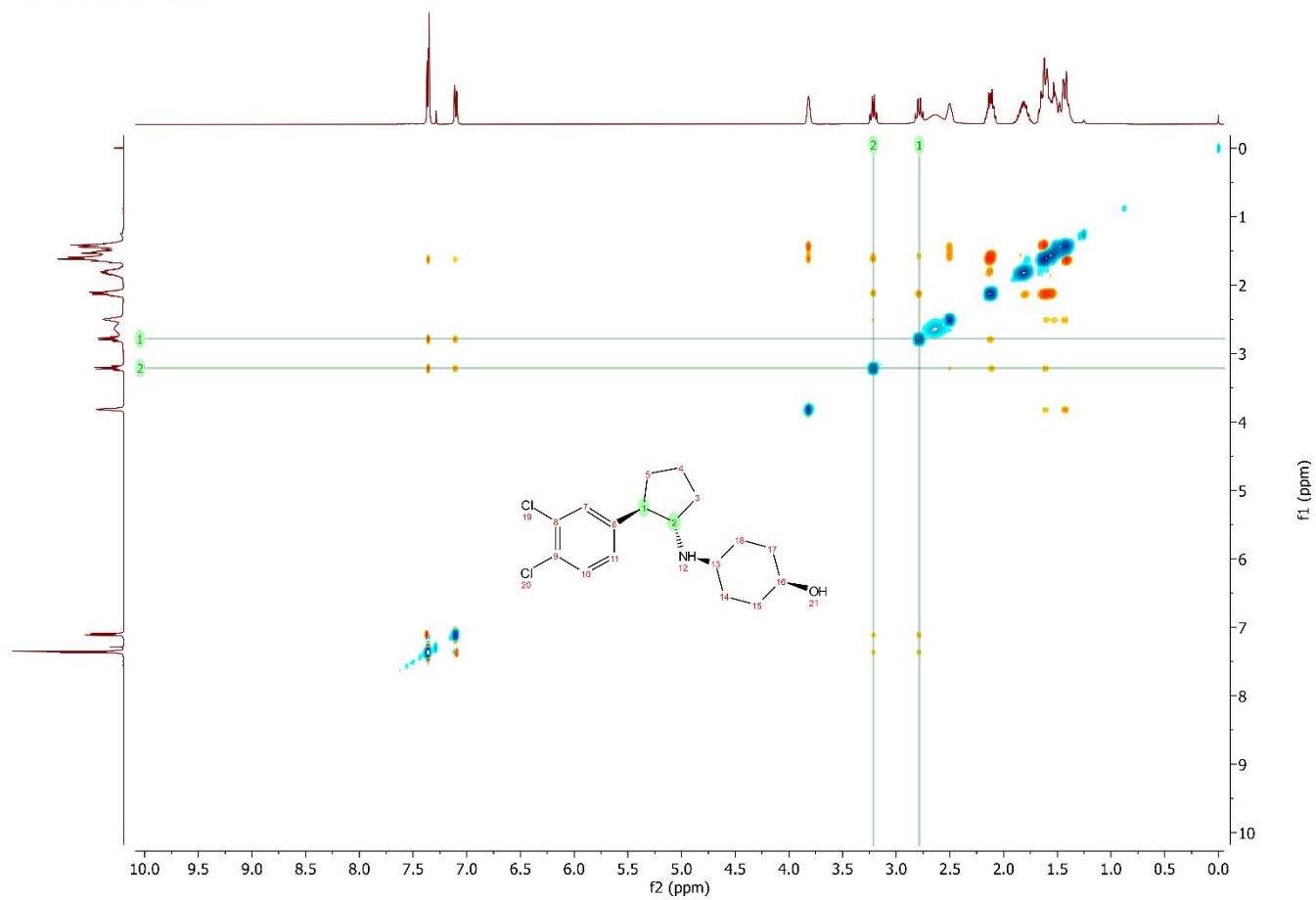


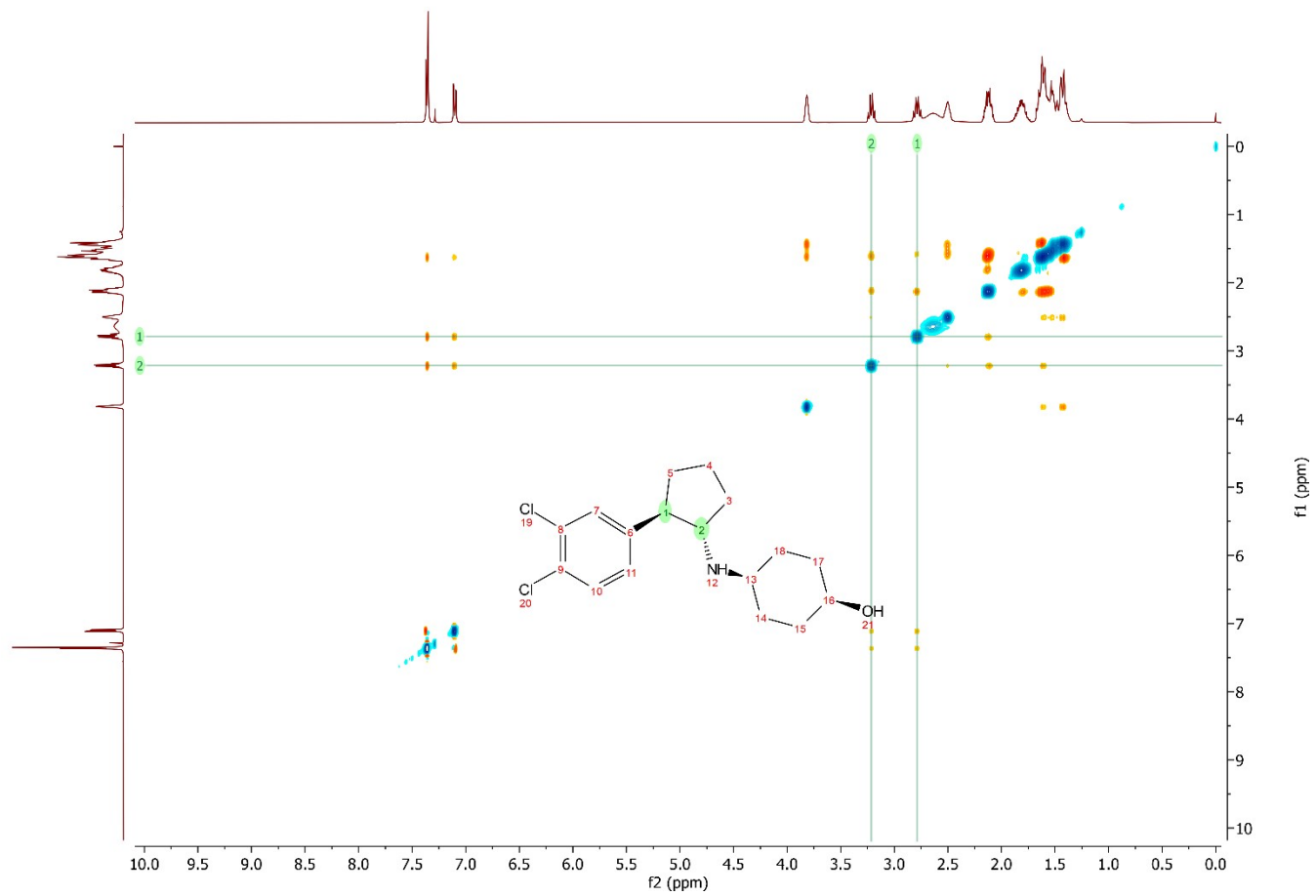
Compound 32d; 1H NMR (CDCl3, 400 MHz)



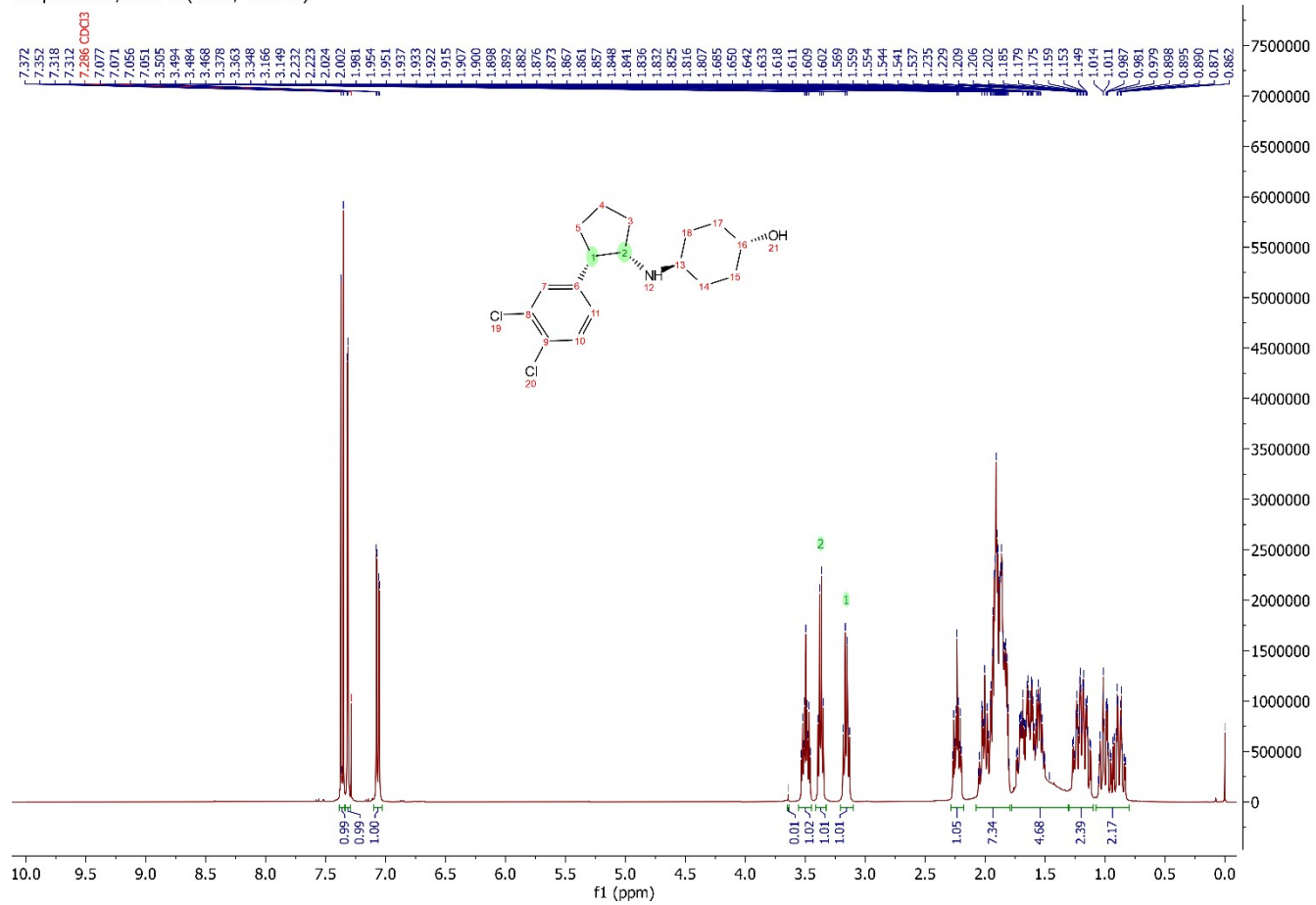
Compound 32d; ¹³C NMR (CDCl₃, 126 MHz)



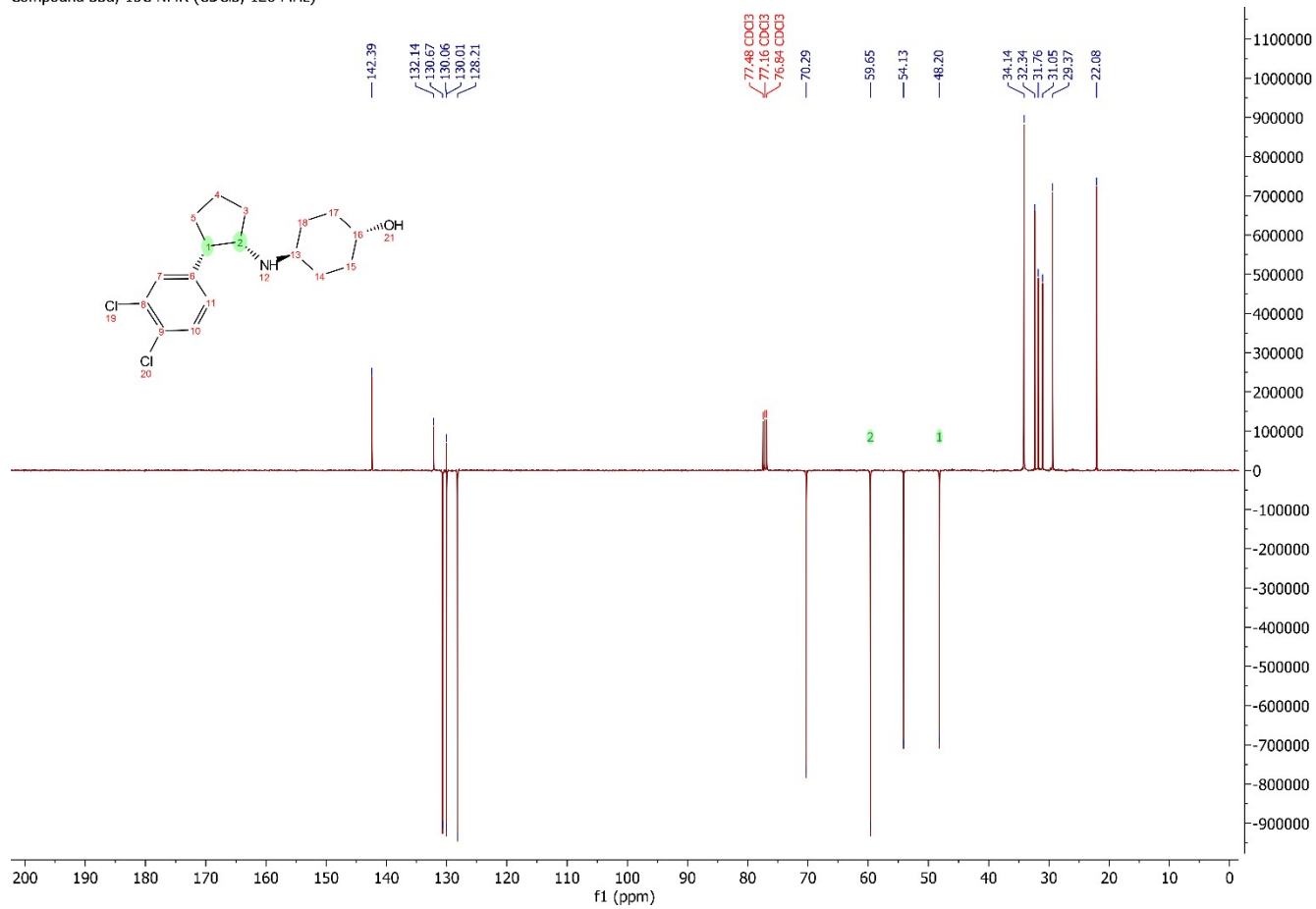


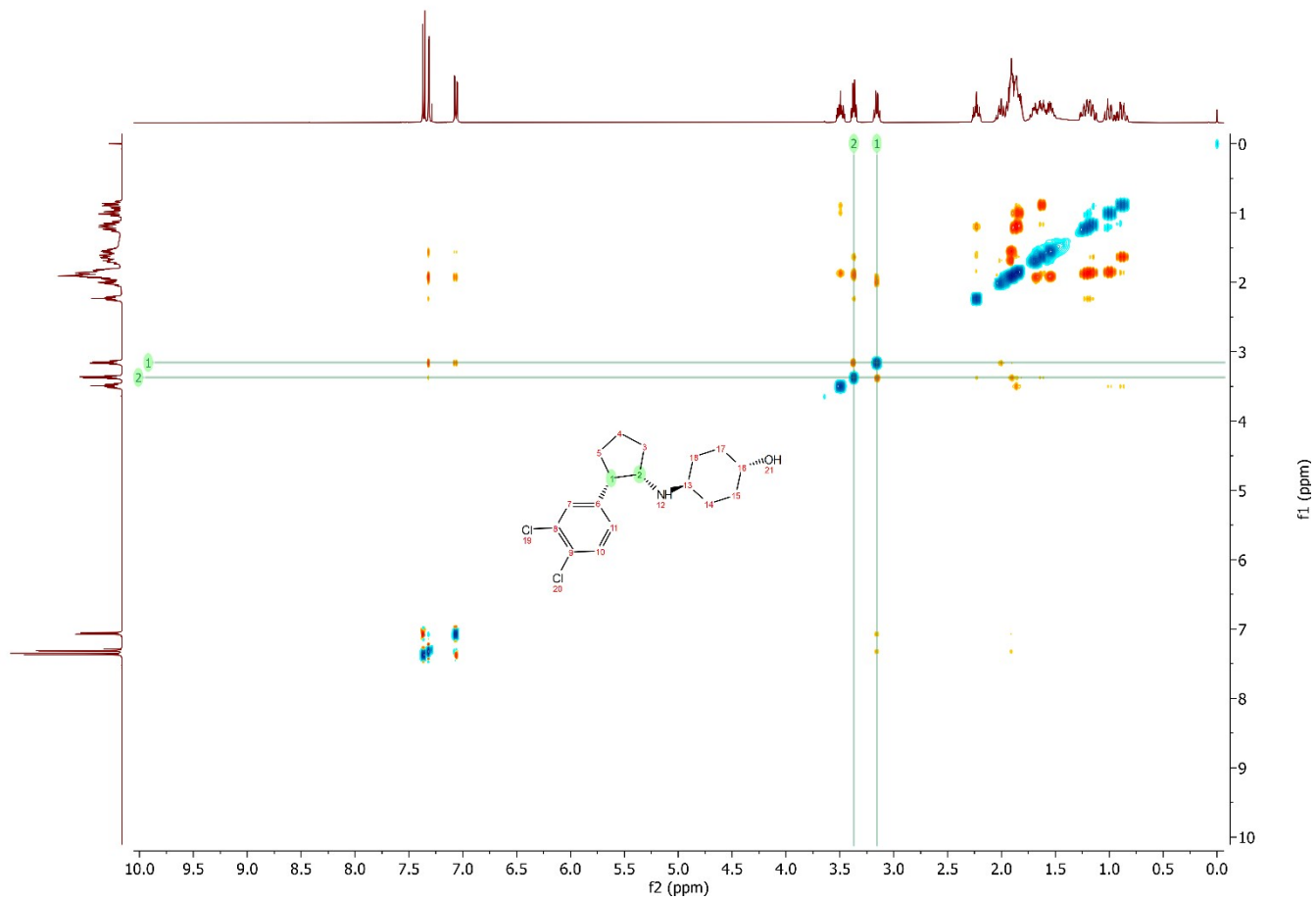


Compound 33a; ¹H NMR (CDCl₃, 400 MHz)

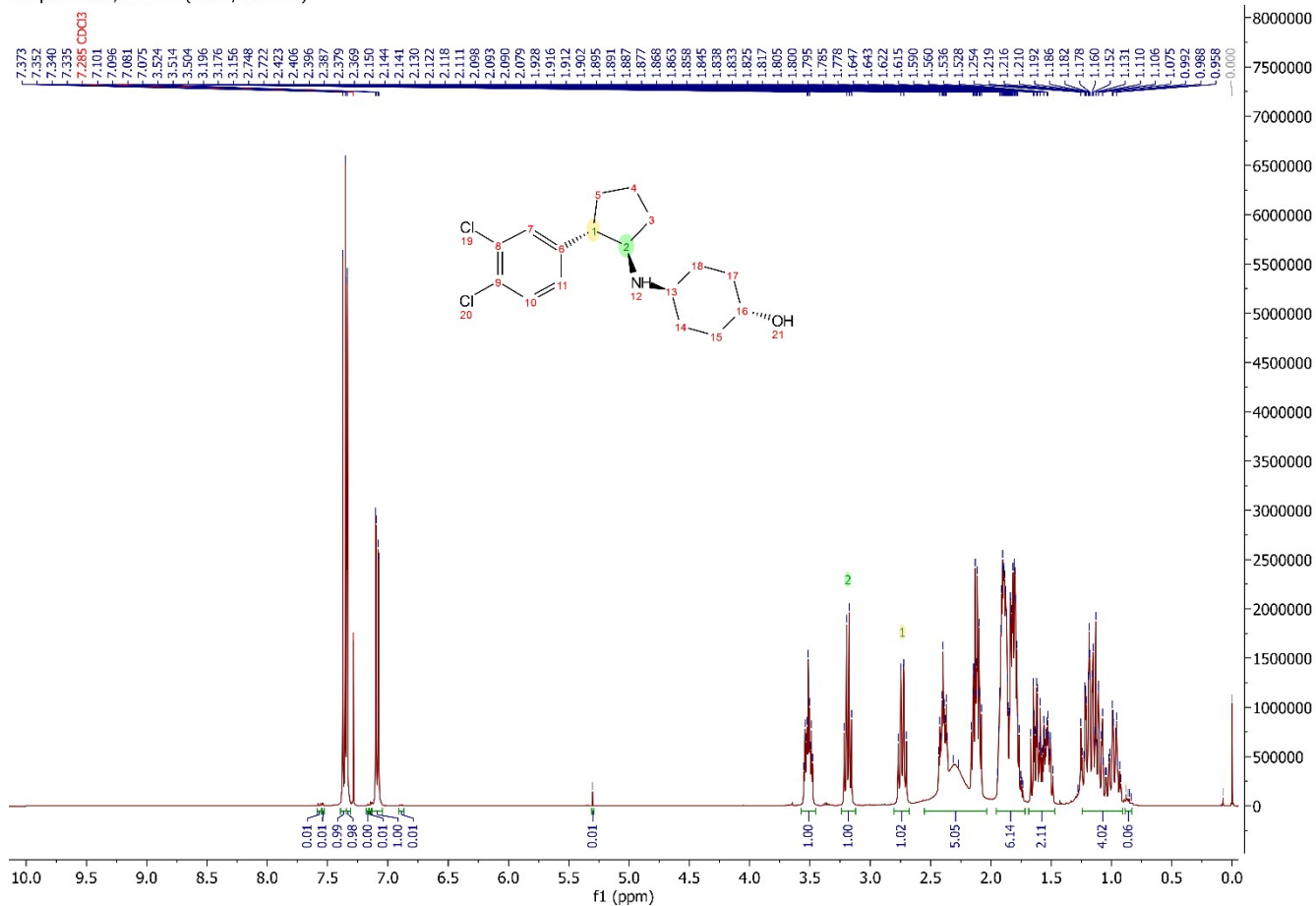


Compound 33a; ¹³C NMR (CDCl₃, 126 MHz)

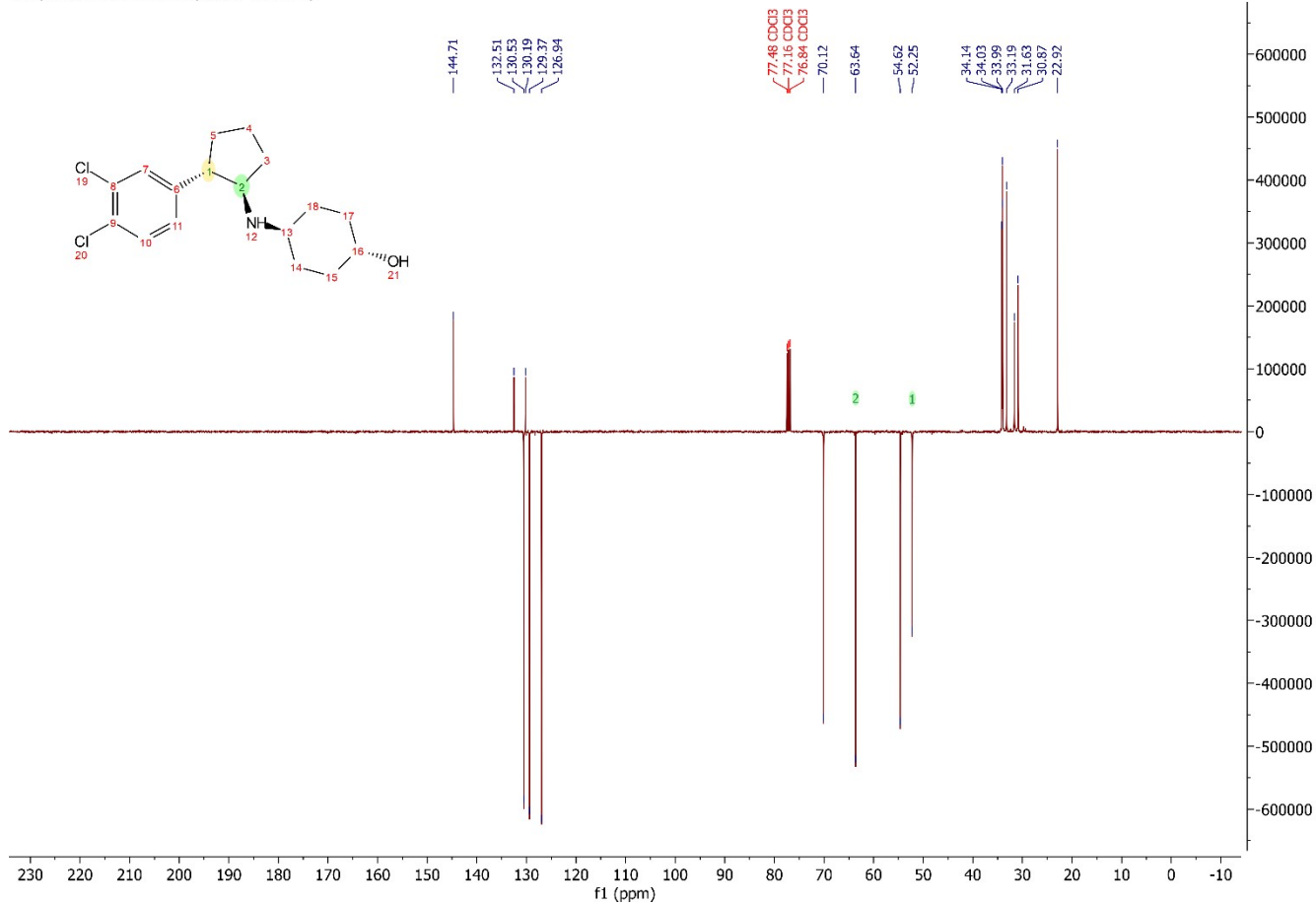




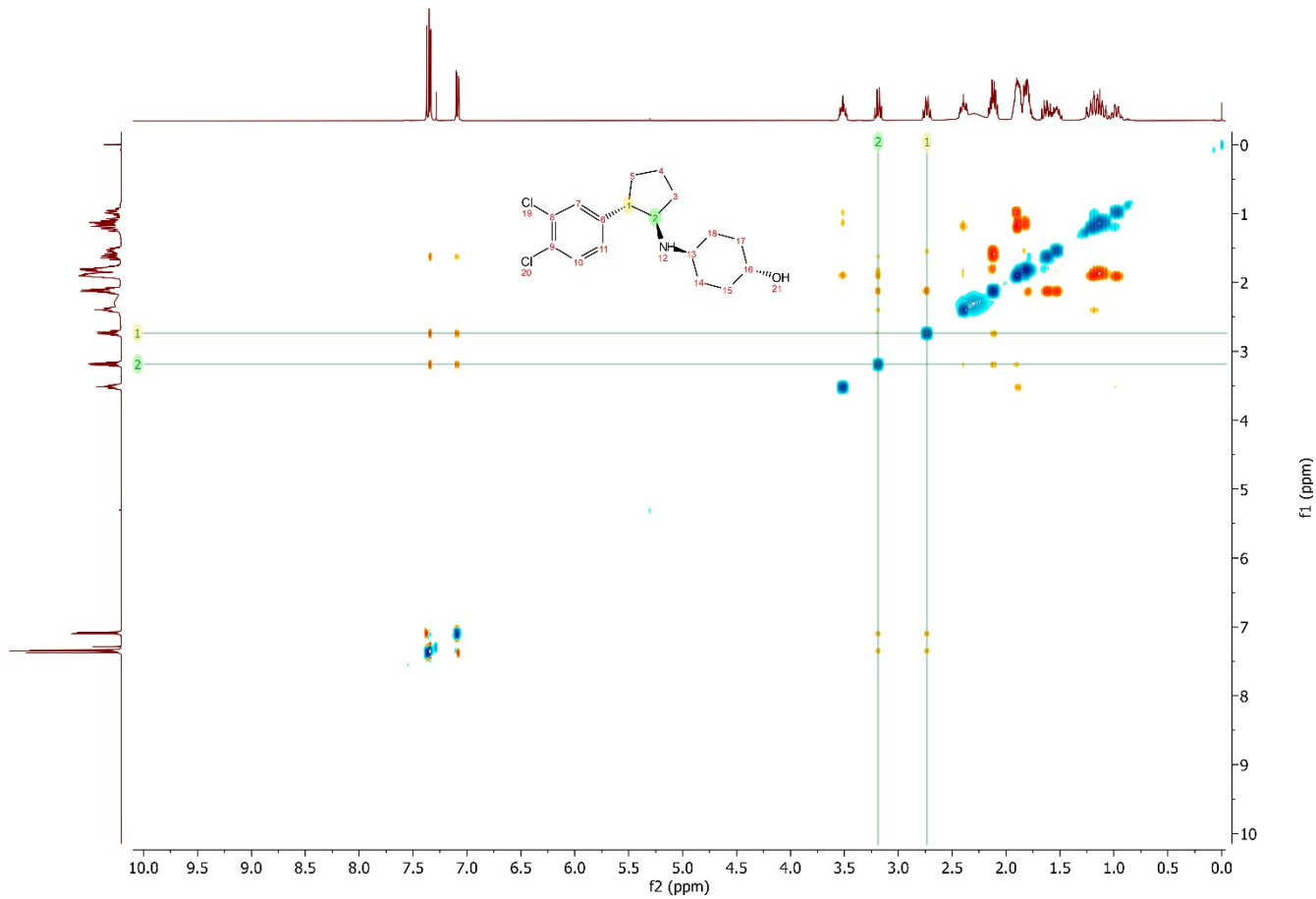
Compound 33b; 1H NMR (CDCl3, 400 MHz)



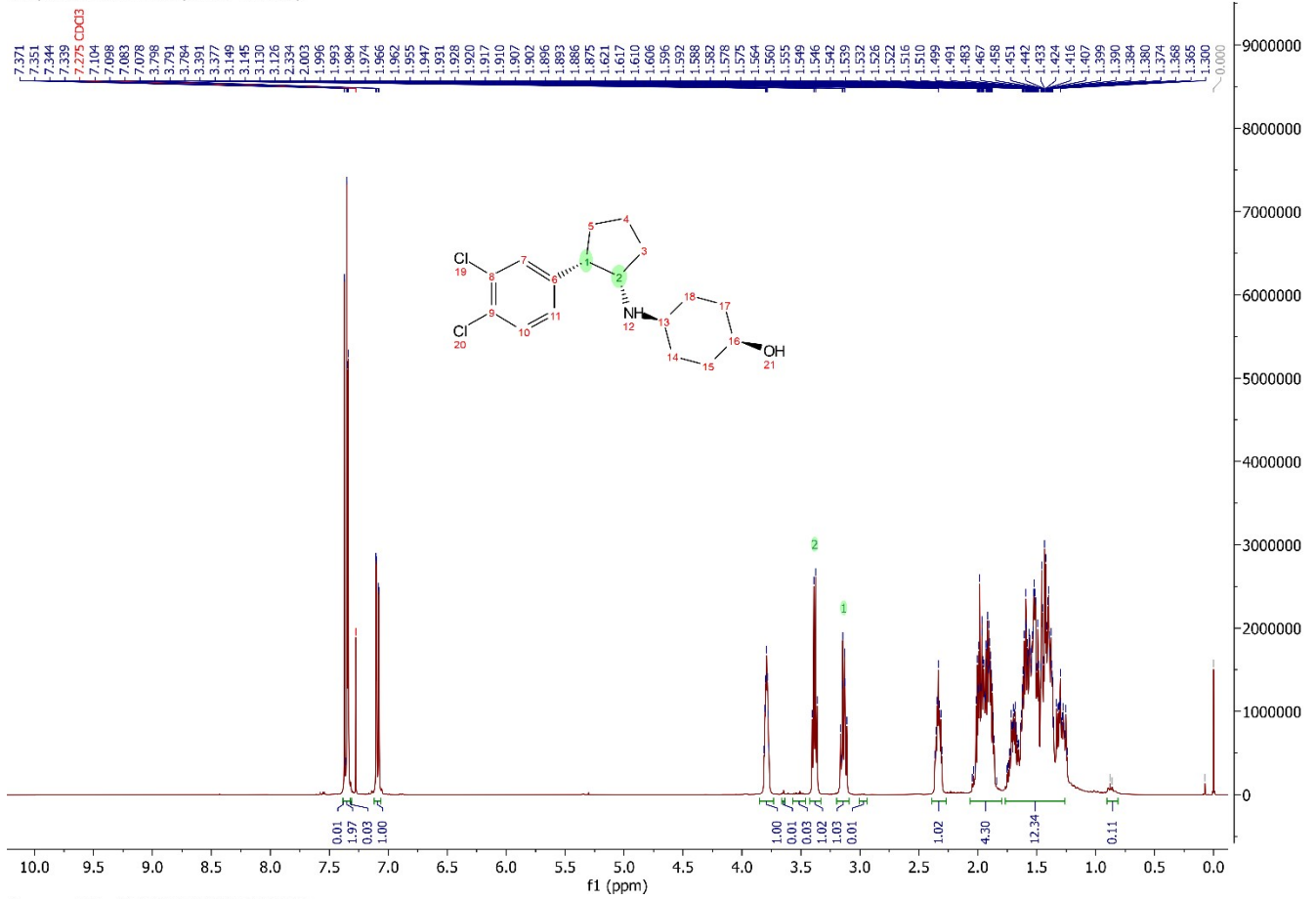
Compound 33b; ¹³C NMR (CDCl₃, 126 MHz)



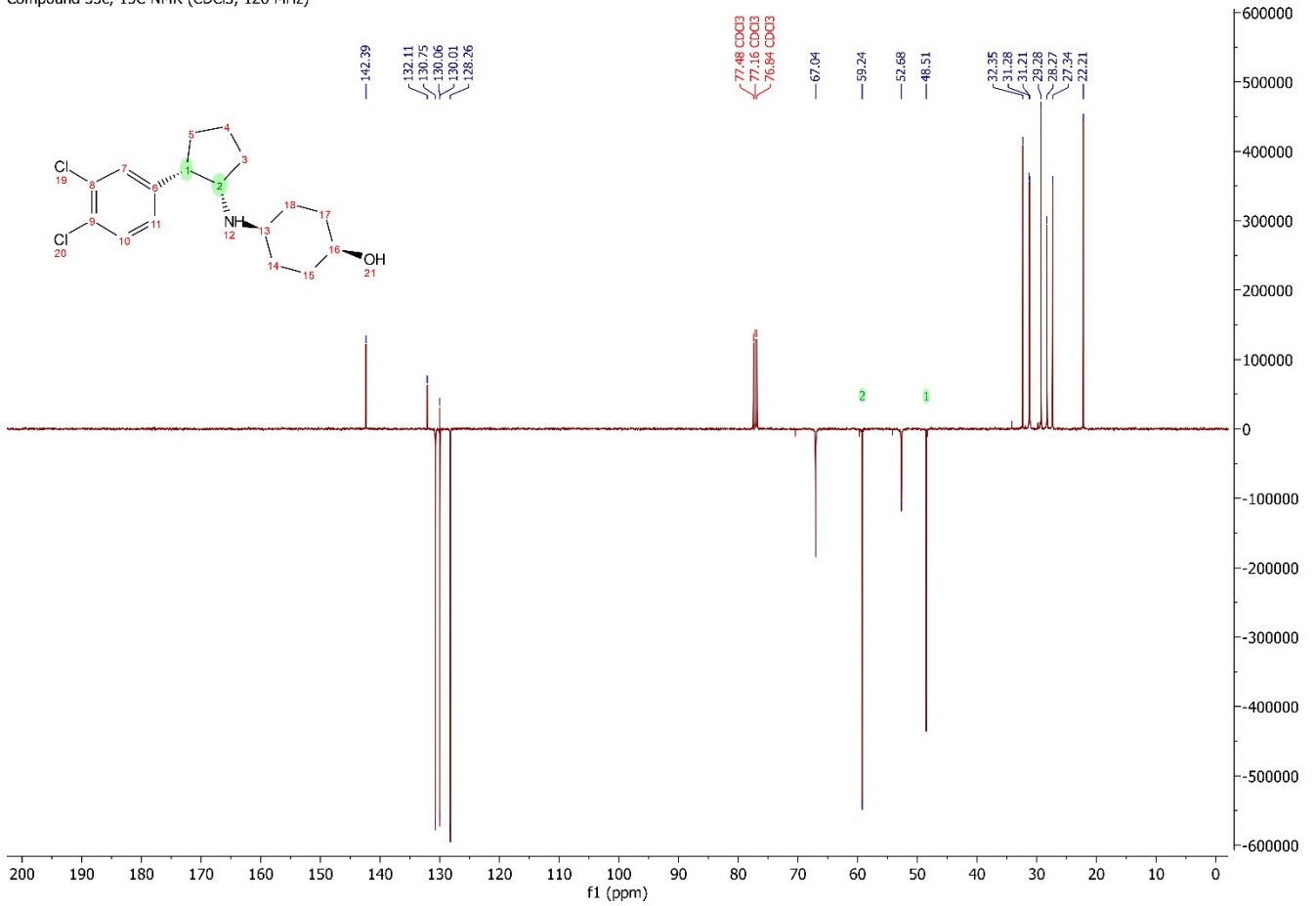
Compound 33b; NOESY (CDCl₃)



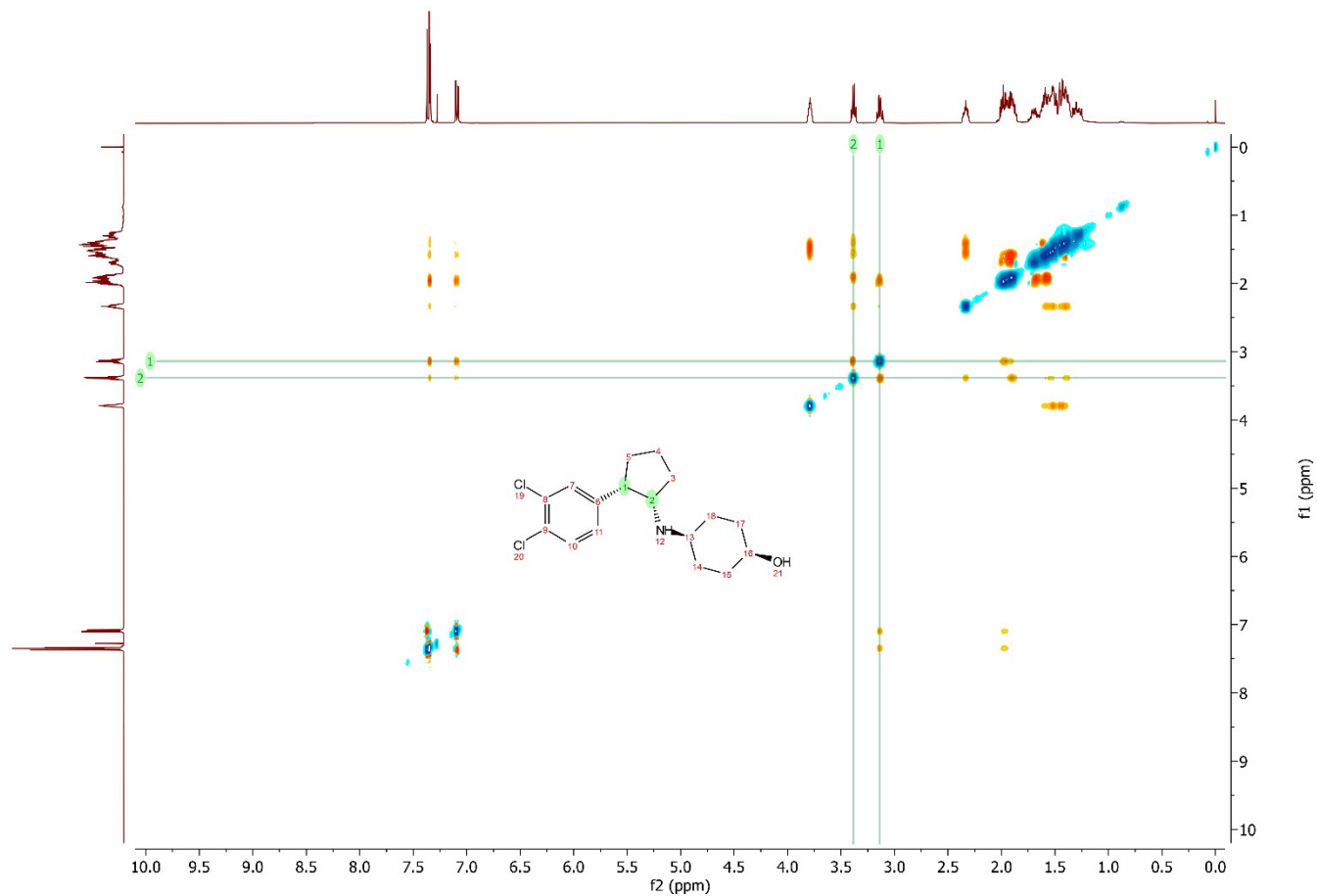
Compound 33c; ¹H NMR (CDCl₃, 400 MHz)



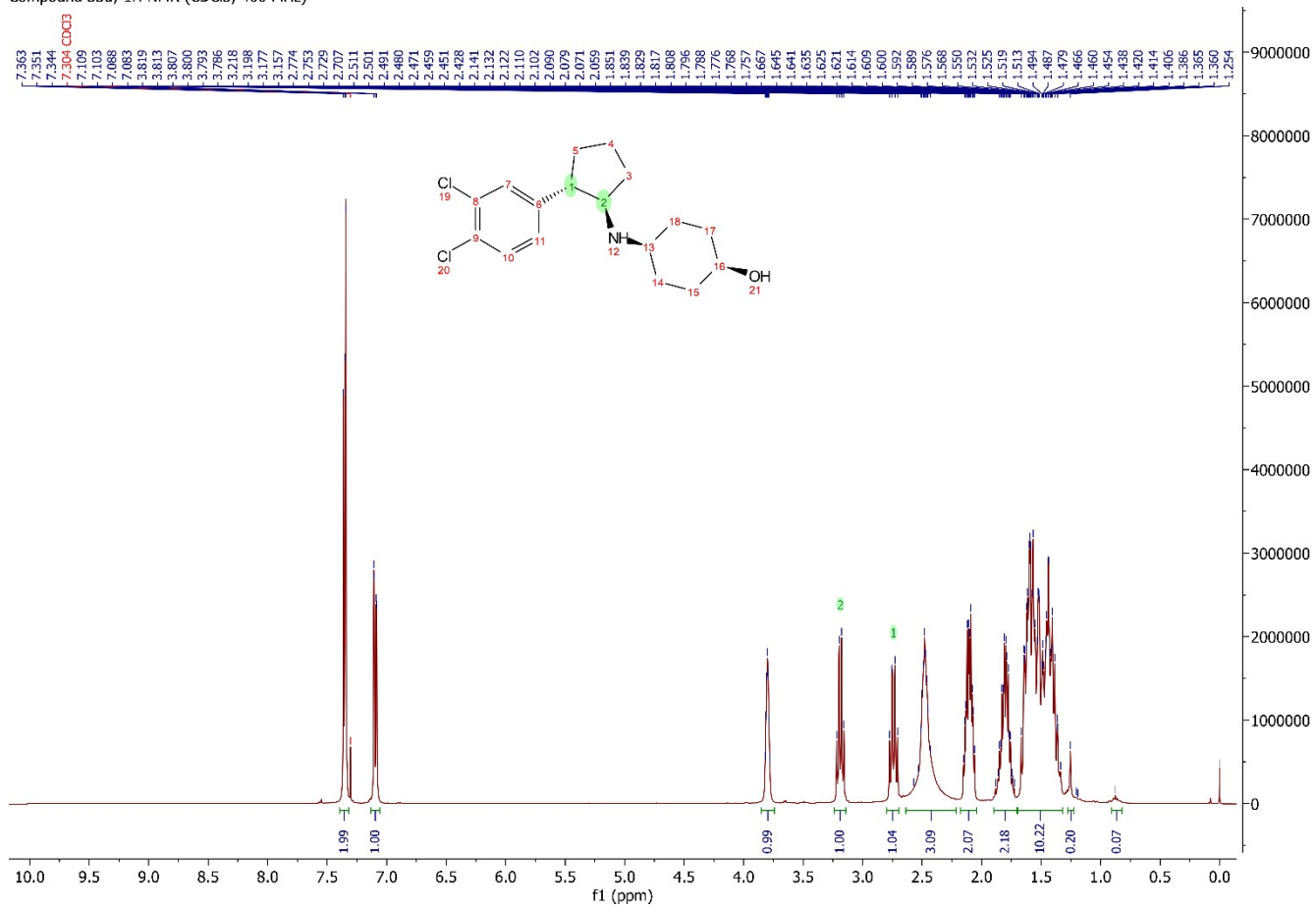
Compound 33c; ¹³C NMR (CDCl₃, 126 MHz)



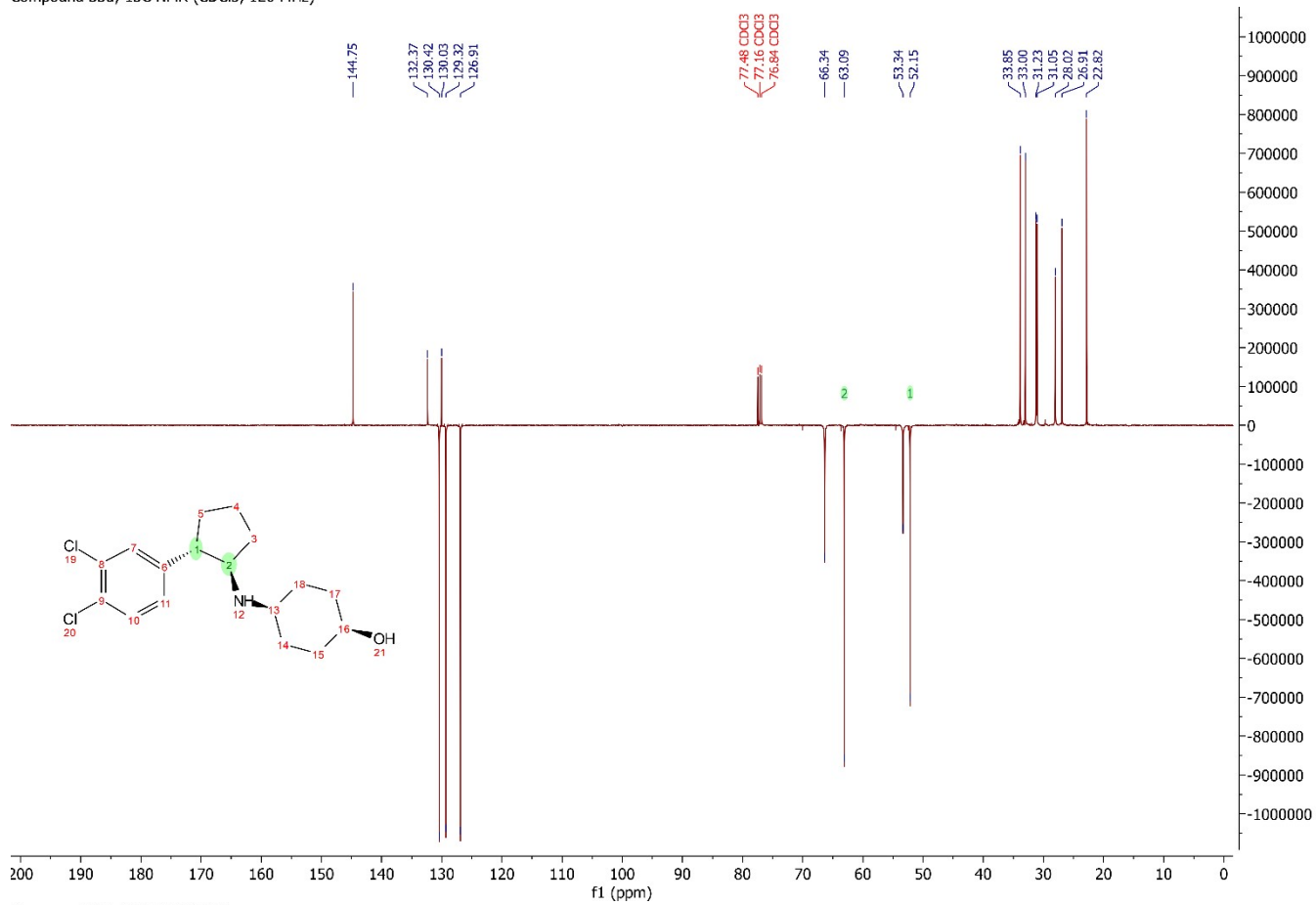
Compound 33c; NOESY (CDCl3)



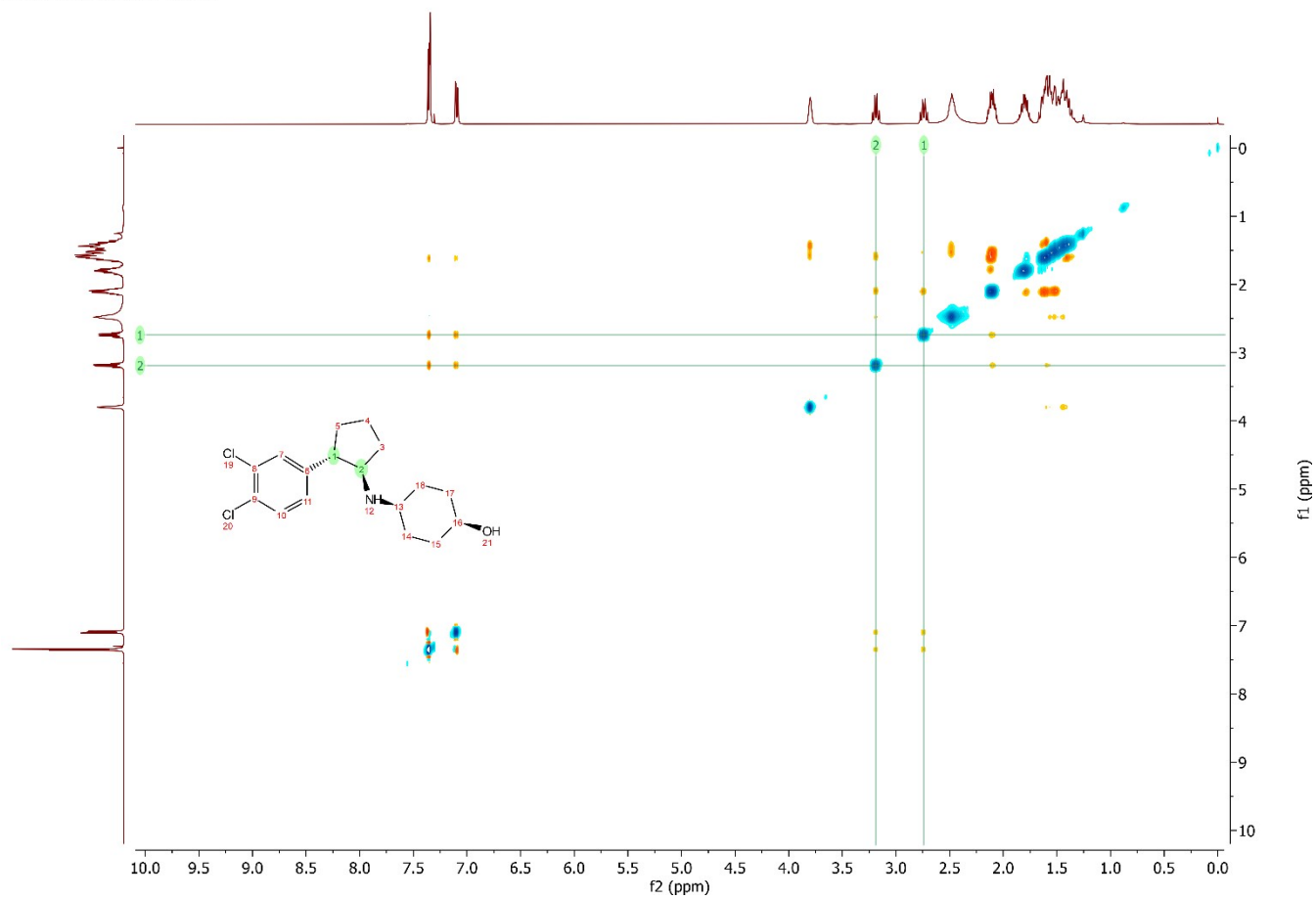
Compound 33d; 1H NMR (CDCl3, 400 MHz)



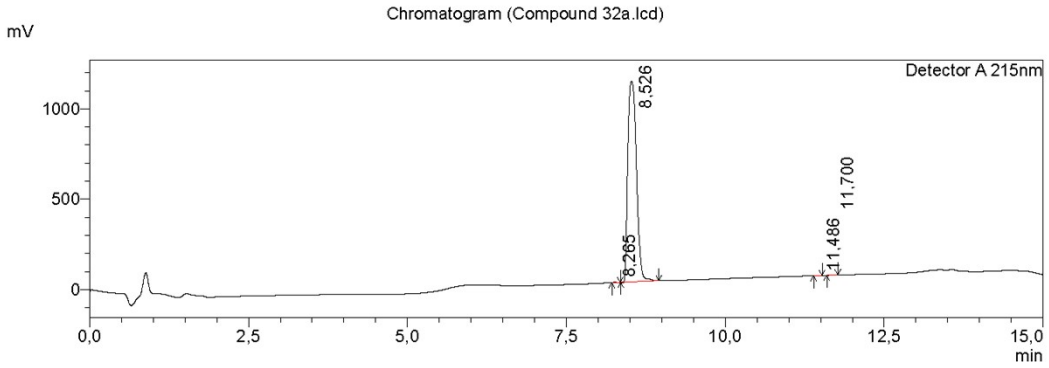
Compound 33d; ¹³C NMR (CDCl₃, 126 MHz)



Compound 33d; NOESY (CDCl₃)

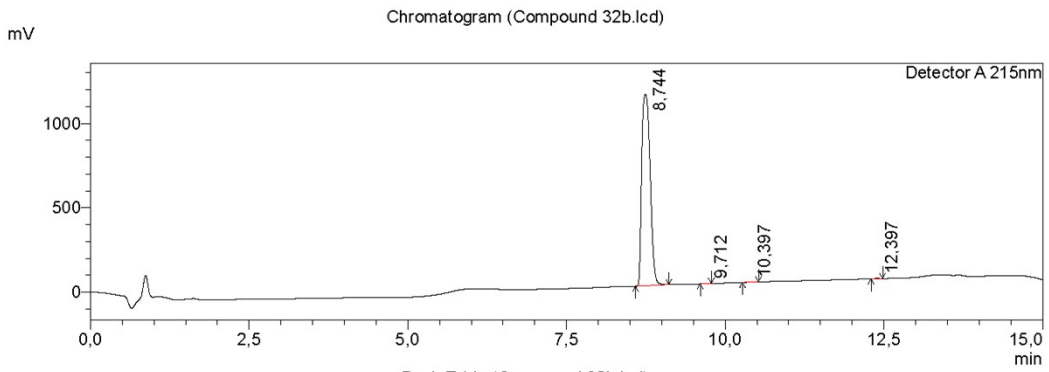


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Peak Table (Compound 32a.lcd)

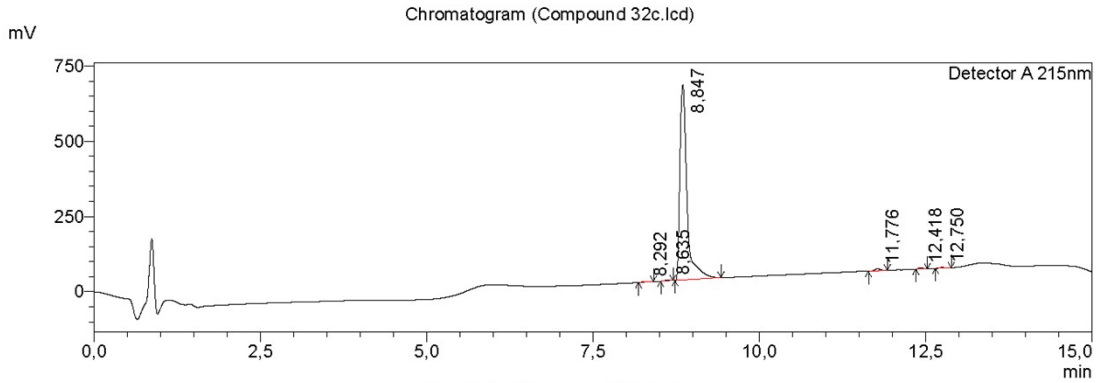
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|----------|---------|------|---------|---------|
| 1 | 8,265 | 8401 | 2089 | M | 0,080 | 0,080 |
| 2 | 8,526 | 10443769 | 1110104 | M | 99,737 | 99,737 |
| 3 | 11,486 | 4783 | 831 | M | 0,046 | 0,046 |
| 4 | 11,700 | 14387 | 2818 | M | 0,137 | 0,137 |
| Total | | 10471341 | 1115843 | | 100,000 | 100,000 |



Peak Table (Compound 32b.lcd)

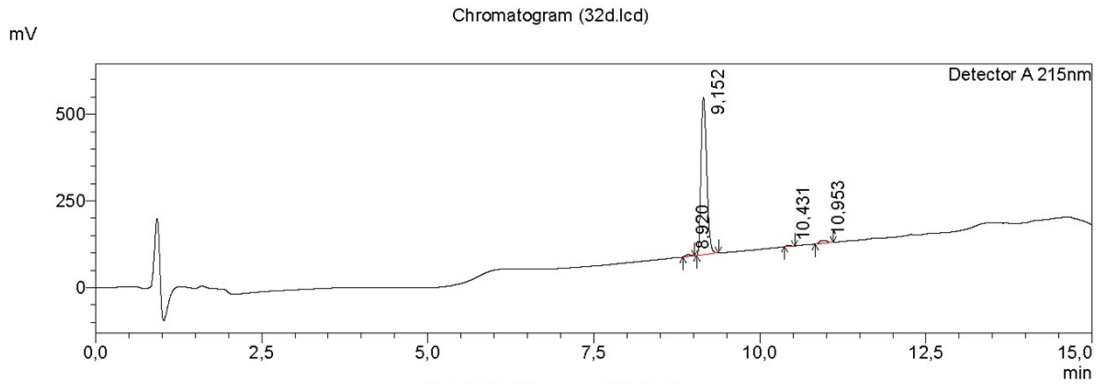
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|----------|---------|------|---------|---------|
| 1 | 8,744 | 10511737 | 1133982 | M | 99,632 | 99,632 |
| 2 | 9,712 | 10336 | 1902 | M | 0,098 | 0,098 |
| 3 | 10,397 | 16594 | 2688 | M | 0,157 | 0,157 |
| 4 | 12,397 | 11926 | 2117 | M | 0,113 | 0,113 |
| Total | | 10550593 | 1140689 | | 100,000 | 100,000 |

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Peak Table (Compound 32c.lcd)

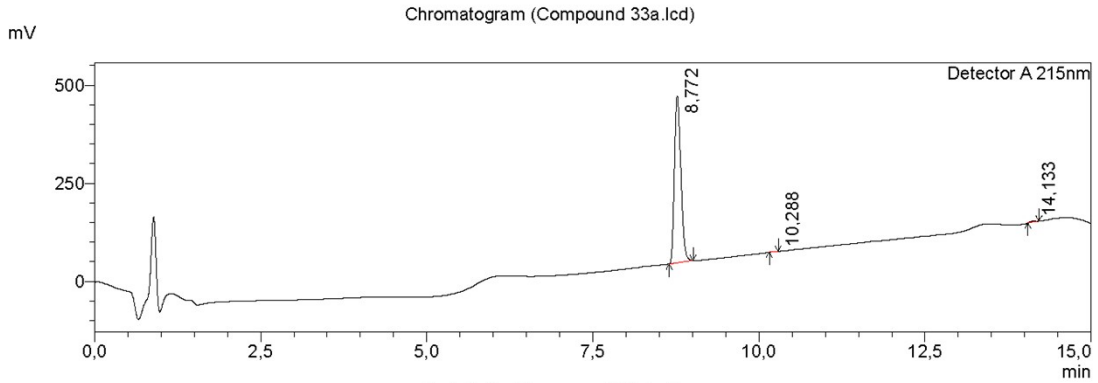
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|---------|--------|------|---------|---------|
| 1 | 8,292 | 10465 | 1556 | M | 0,217 | 0,217 |
| 2 | 8,635 | 15835 | 2741 | M | 0,328 | 0,328 |
| 3 | 8,847 | 4725232 | 648387 | M | 97,981 | 97,981 |
| 4 | 11,776 | 36523 | 6622 | M | 0,757 | 0,757 |
| 5 | 12,418 | 22949 | 4405 | M | 0,476 | 0,476 |
| 6 | 12,750 | 11599 | 2393 | M | 0,241 | 0,241 |
| Total | | 4822603 | 666104 | | 100,000 | 100,000 |



Peak Table (Compound 32d.lcd)

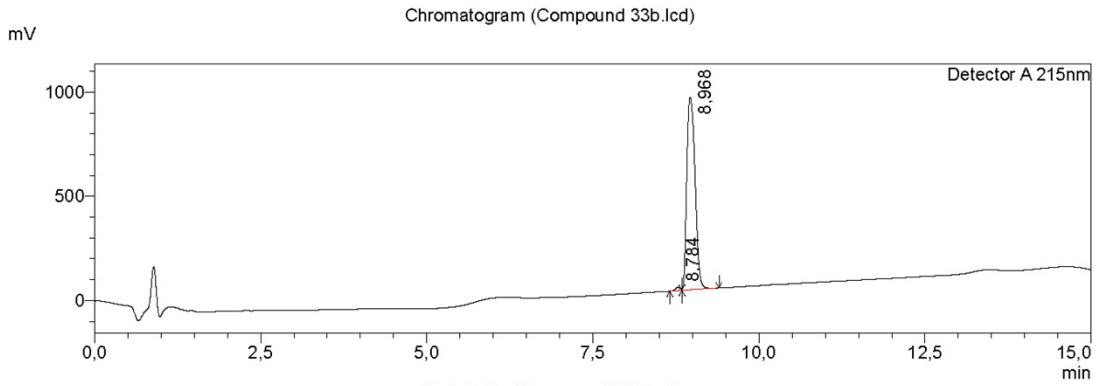
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|---------|--------|------|---------|---------|
| 1 | 8,920 | 29000 | 5216 | M | 1,046 | 1,046 |
| 2 | 9,152 | 2670648 | 455320 | M | 96,346 | 96,346 |
| 3 | 10,431 | 9245 | 1948 | M | 0,334 | 0,334 |
| 4 | 10,953 | 63031 | 8473 | M | 2,274 | 2,274 |
| Total | | 2771924 | 470957 | | 100,000 | 100,000 |

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Peak Table (Compound 33a.lcd)

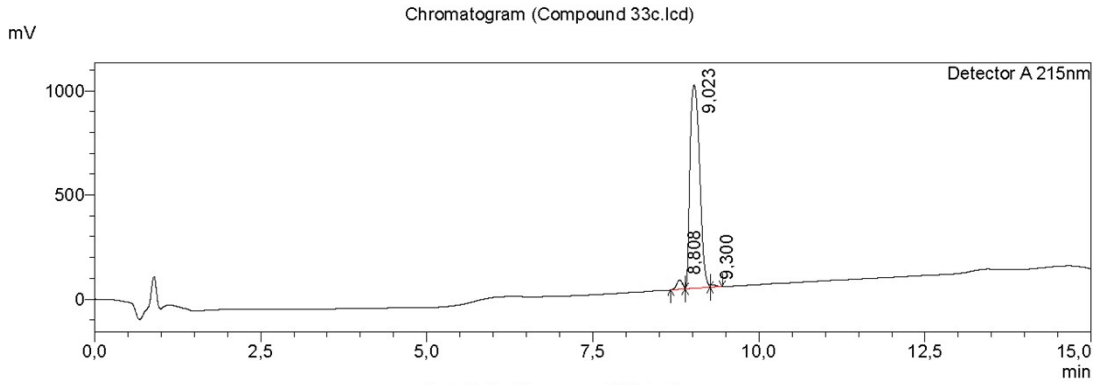
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|---------|--------|------|---------|---------|
| 1 | 8,772 | 2869474 | 425621 | M | 99,307 | 99,307 |
| 2 | 10,288 | 3954 | 31 | M | 0,137 | 0,137 |
| 3 | 14,133 | 16084 | 2554 | M | 0,557 | 0,557 |
| Total | | 2889512 | 428206 | | 100,000 | 100,000 |



Peak Table (Compound 33b.lcd)

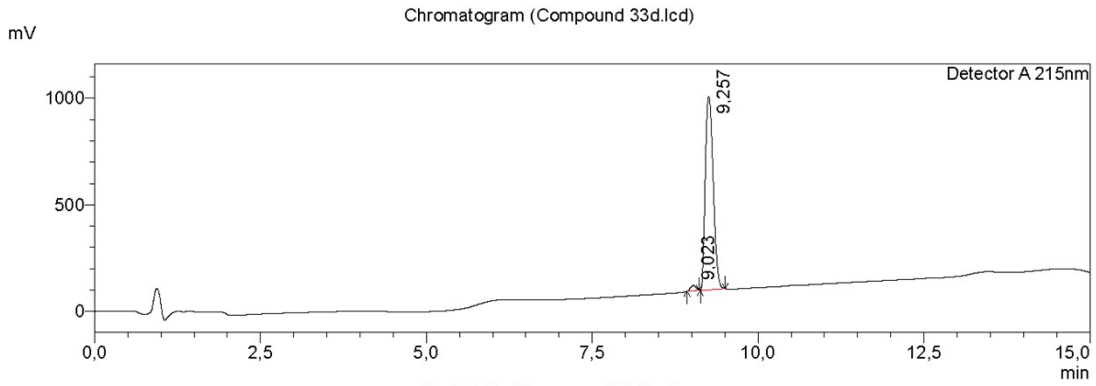
| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|---------|--------|------|---------|---------|
| 1 | 8,784 | 90877 | 16744 | M | 1,123 | 1,123 |
| 2 | 8,968 | 8001614 | 926334 | V M | 98,877 | 98,877 |
| Total | | 8092492 | 943077 | | 100,000 | 100,000 |

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Peak Table (Compound 33c.lcd)

| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|----------|---------|------|---------|---------|
| 1 | 8,808 | 266041 | 43158 | M | 2,632 | 2,632 |
| 2 | 9,023 | 9773699 | 977836 | V M | 96,710 | 96,710 |
| 3 | 9,300 | 66427 | 12362 | V M | 0,657 | 0,657 |
| Total | | 10106167 | 1033356 | | 100,000 | 100,000 |



Peak Table (Compound 33d.lcd)

| Peak# | Ret. Time | Area | Height | Mark | Conc. | Area% |
|-------|-----------|---------|--------|------|---------|---------|
| 1 | 9,023 | 139240 | 25461 | M | 1,808 | 1,808 |
| 2 | 9,257 | 7559980 | 906469 | M | 98,192 | 98,192 |
| Total | | 7699219 | 931929 | | 100,000 | 100,000 |