# *In vitro* characterization of inhibitors for lung A549 and leukemia K562 cell lines from fungal transformation of arecoline: Apoptosis mechanism, targeting M3-mAChR via *in silico* study and ADME prediction

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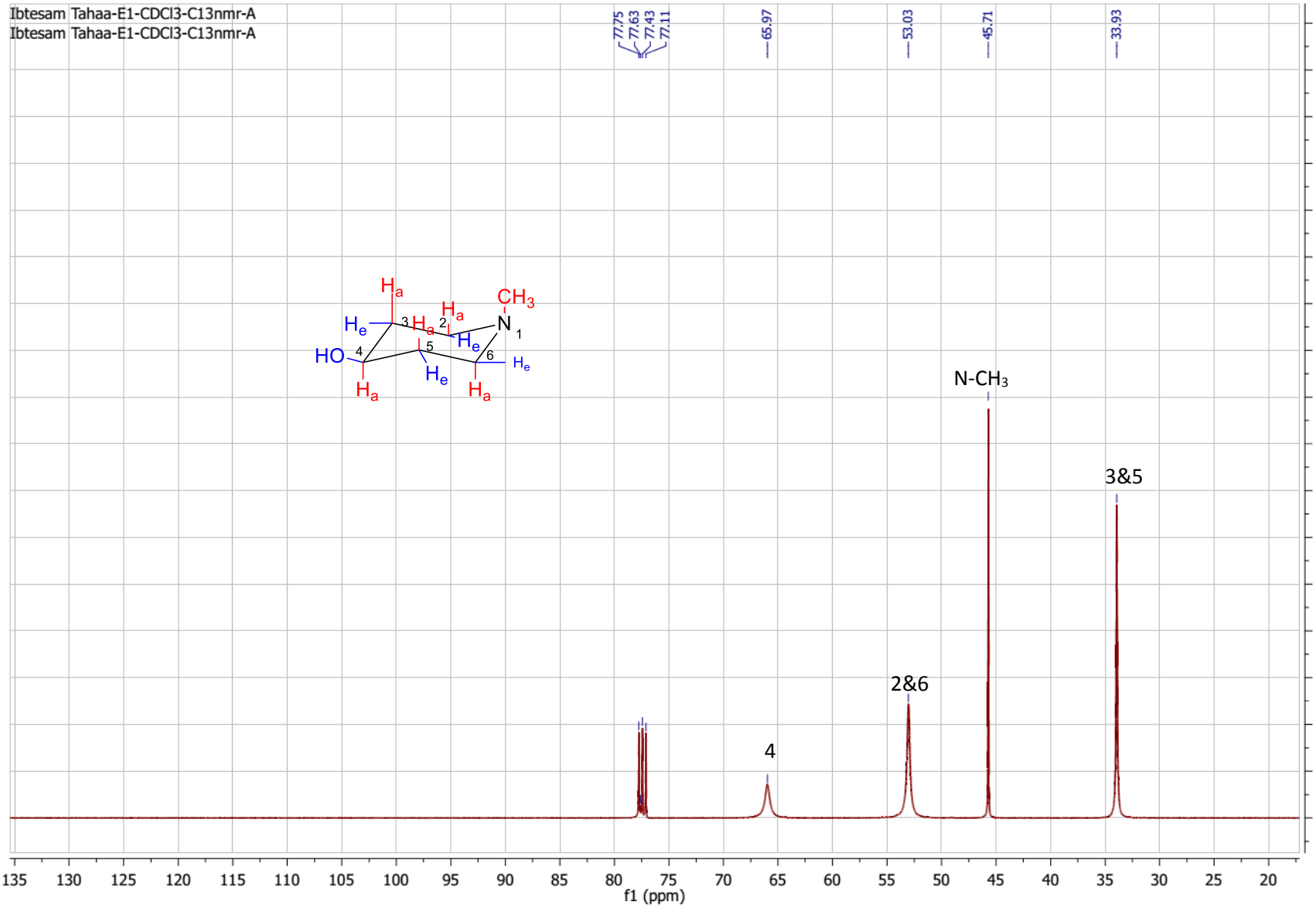



**Fig. S1:** $^{13}C$ NMR spectrum of **1** ($CDCl_3$, 100 MHz).

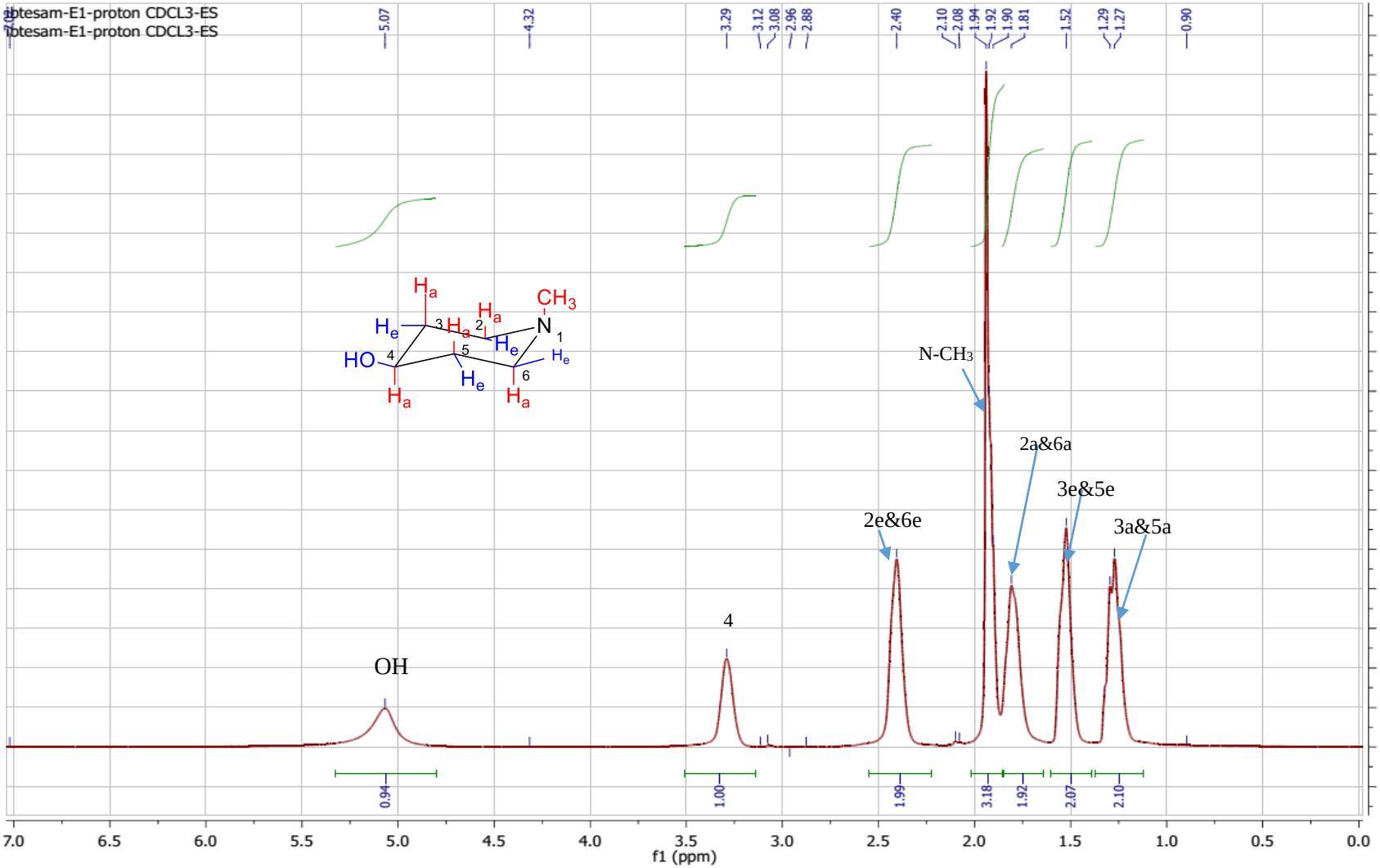



**Fig. S2:** $^{1}$H NMR spectrum of **1** ($CDCl_3$, 400 MHz).

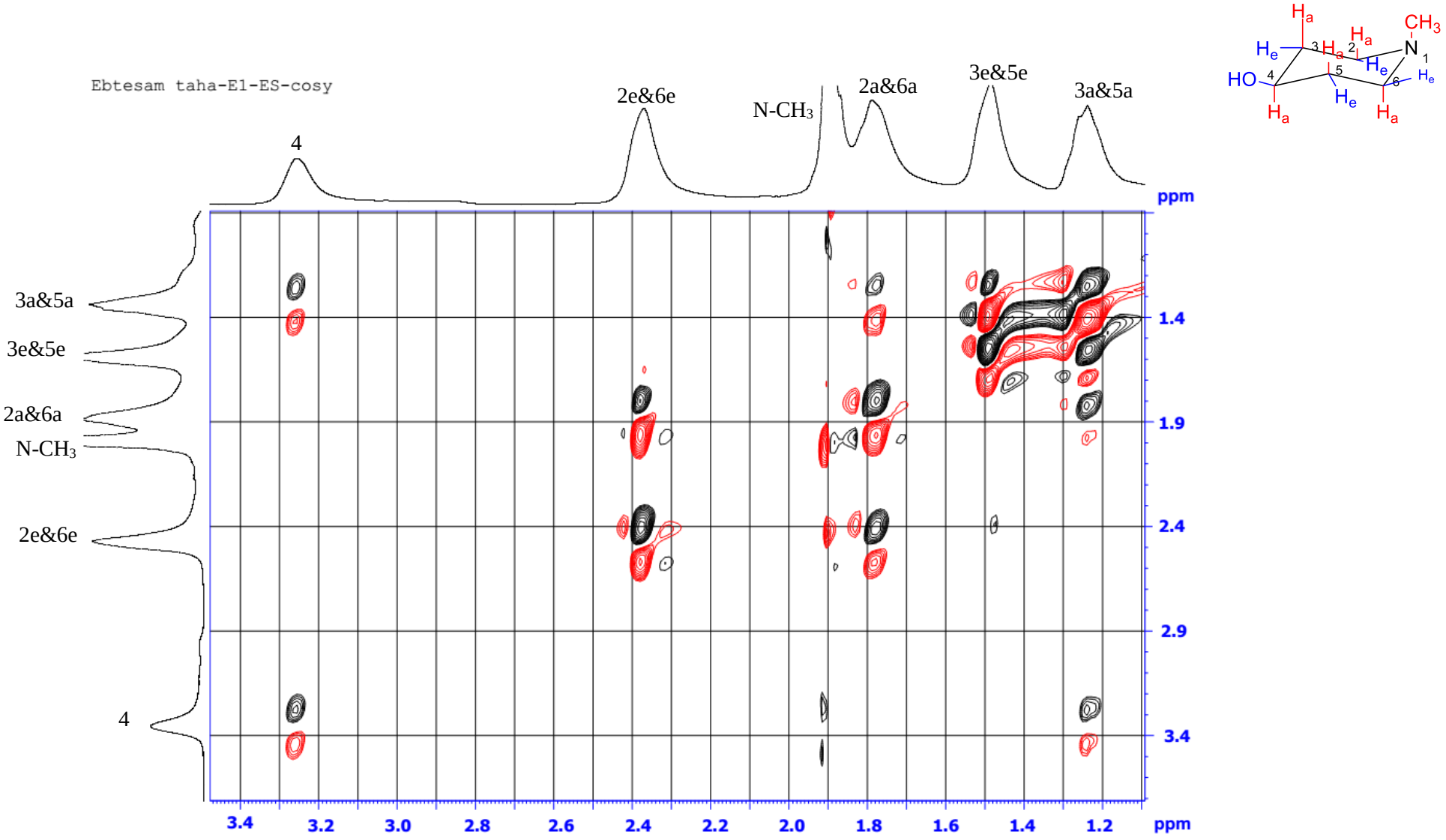



**Fig. S3:** COSY spectrum of **1** ($CDCl_3$).

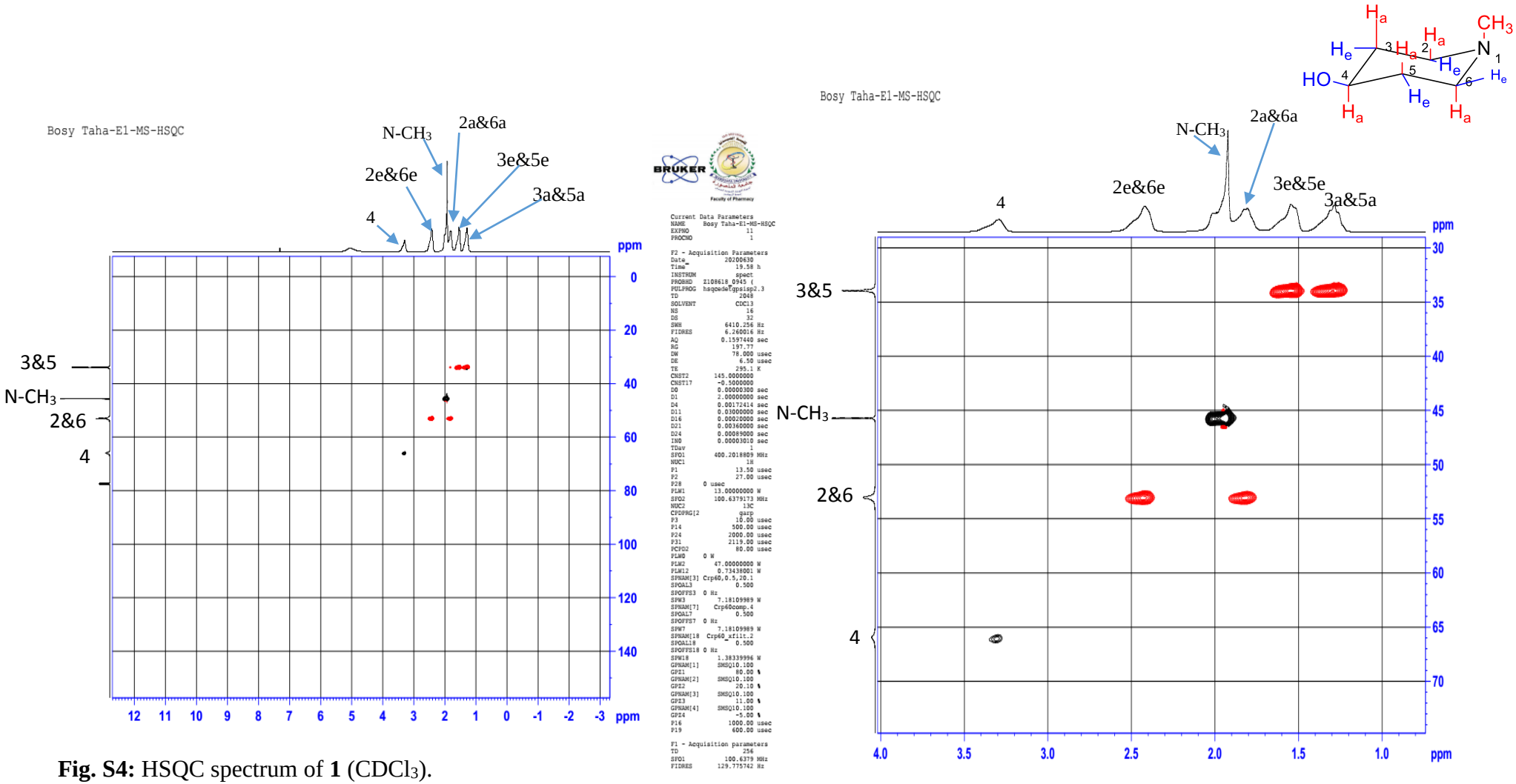



**Fig. S4:** HSQC spectrum of **1** ($CDCl_3$).

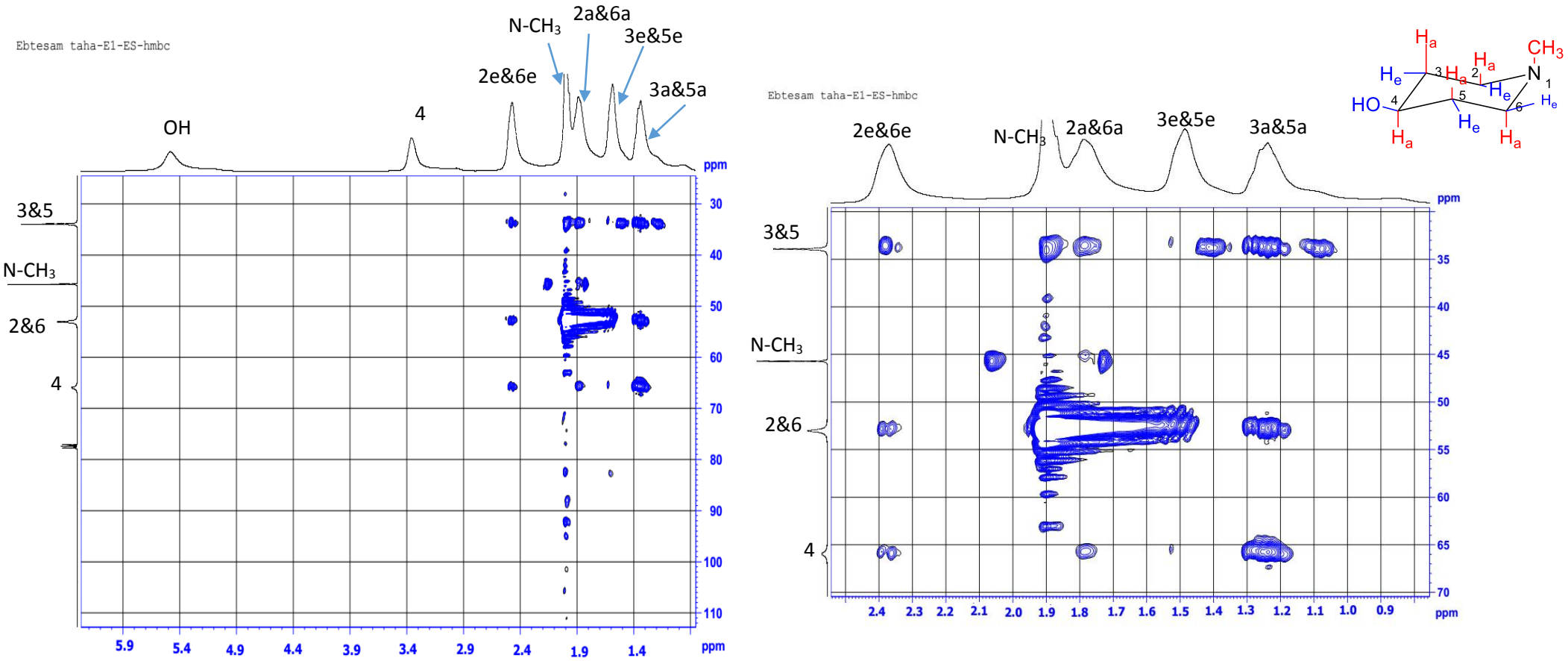



**Fig. S5:** HMBC spectrum of **1** ($CDCl_3$).

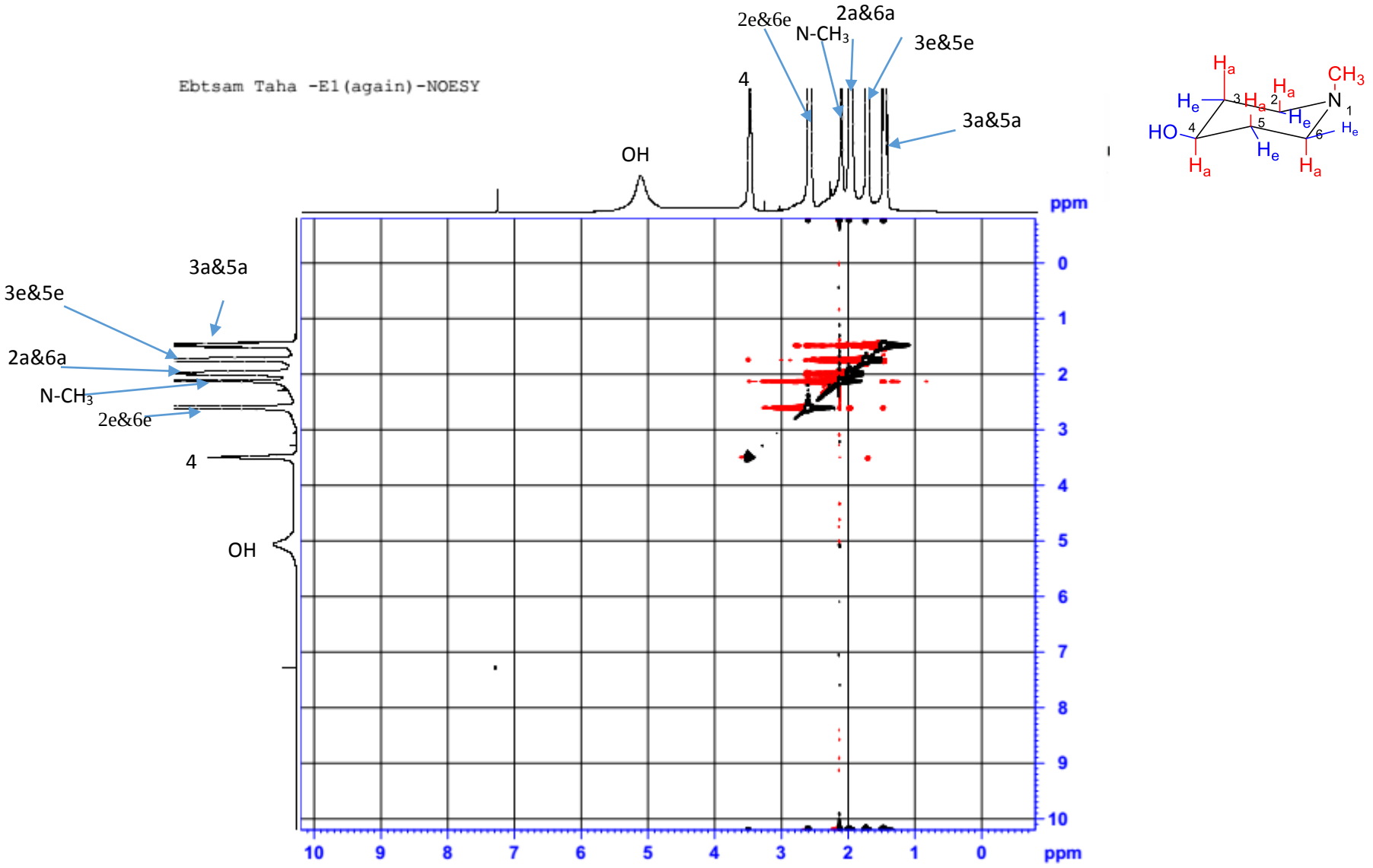



**Fig. S6:** NOE spectrum of **1** ($CDCl_3$).

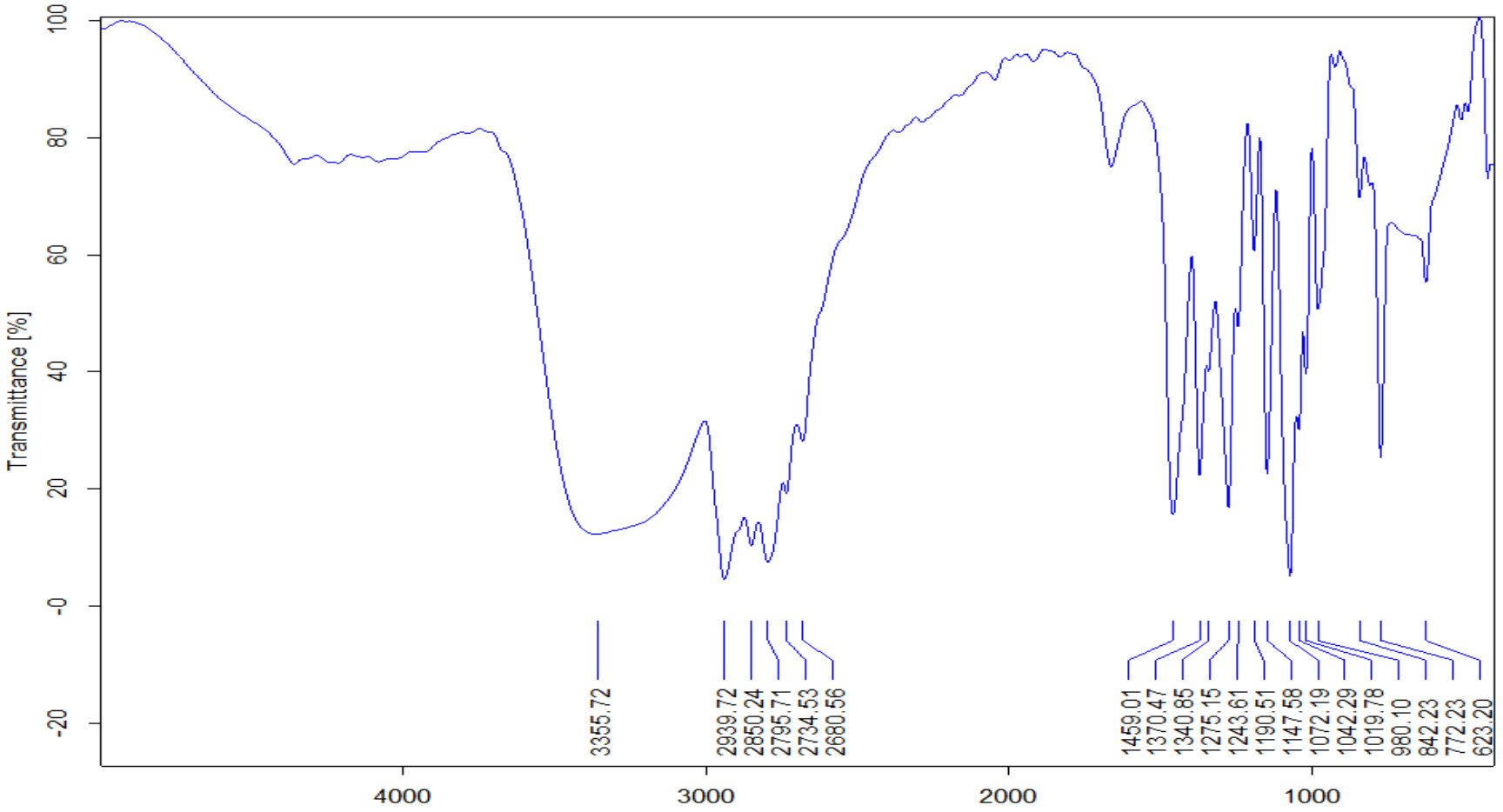



**Fig. S7:** IR spectrum of **1** (KBr disc).

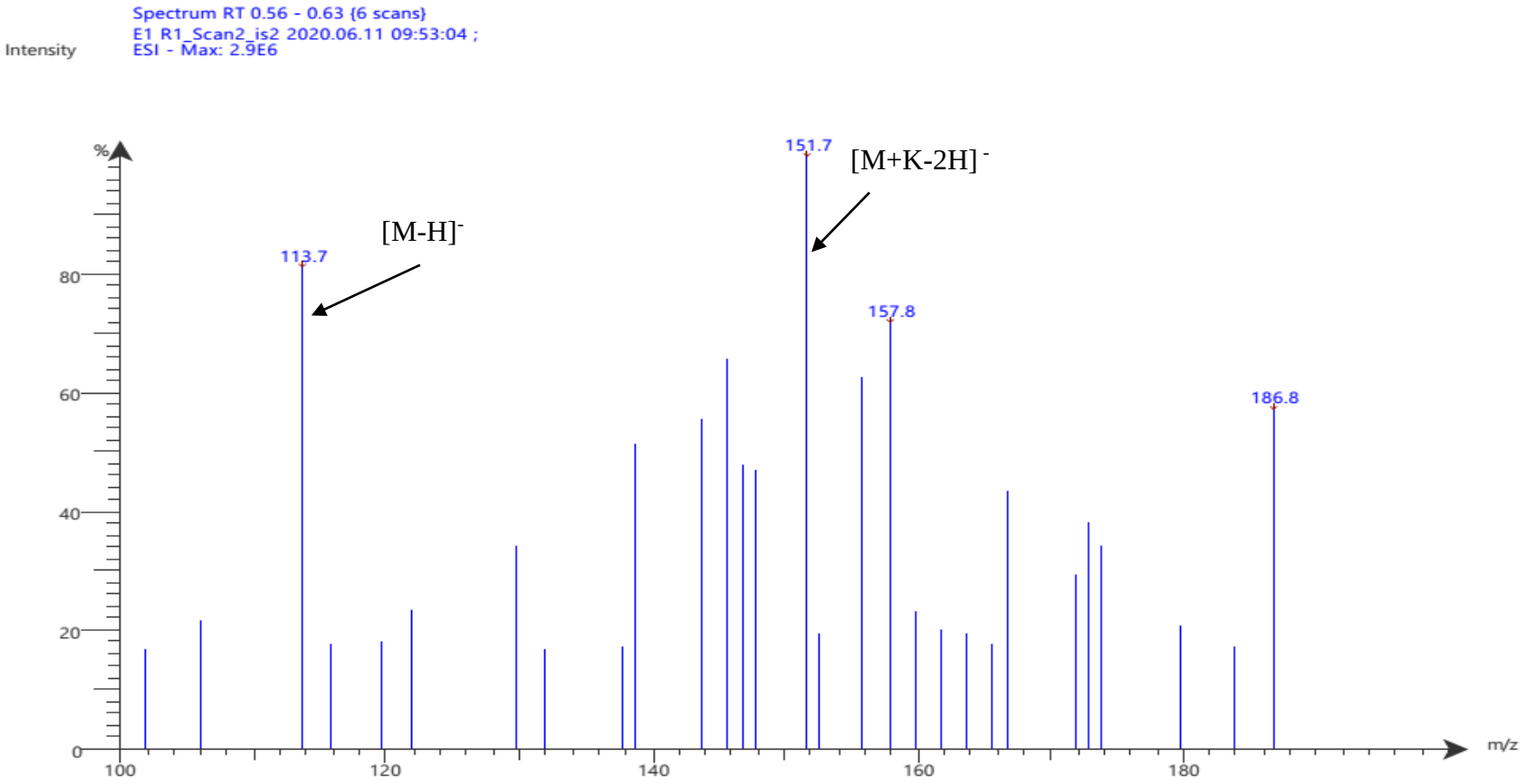



**Fig. S8:** ESIMS (-ve) spectrum of **1**.

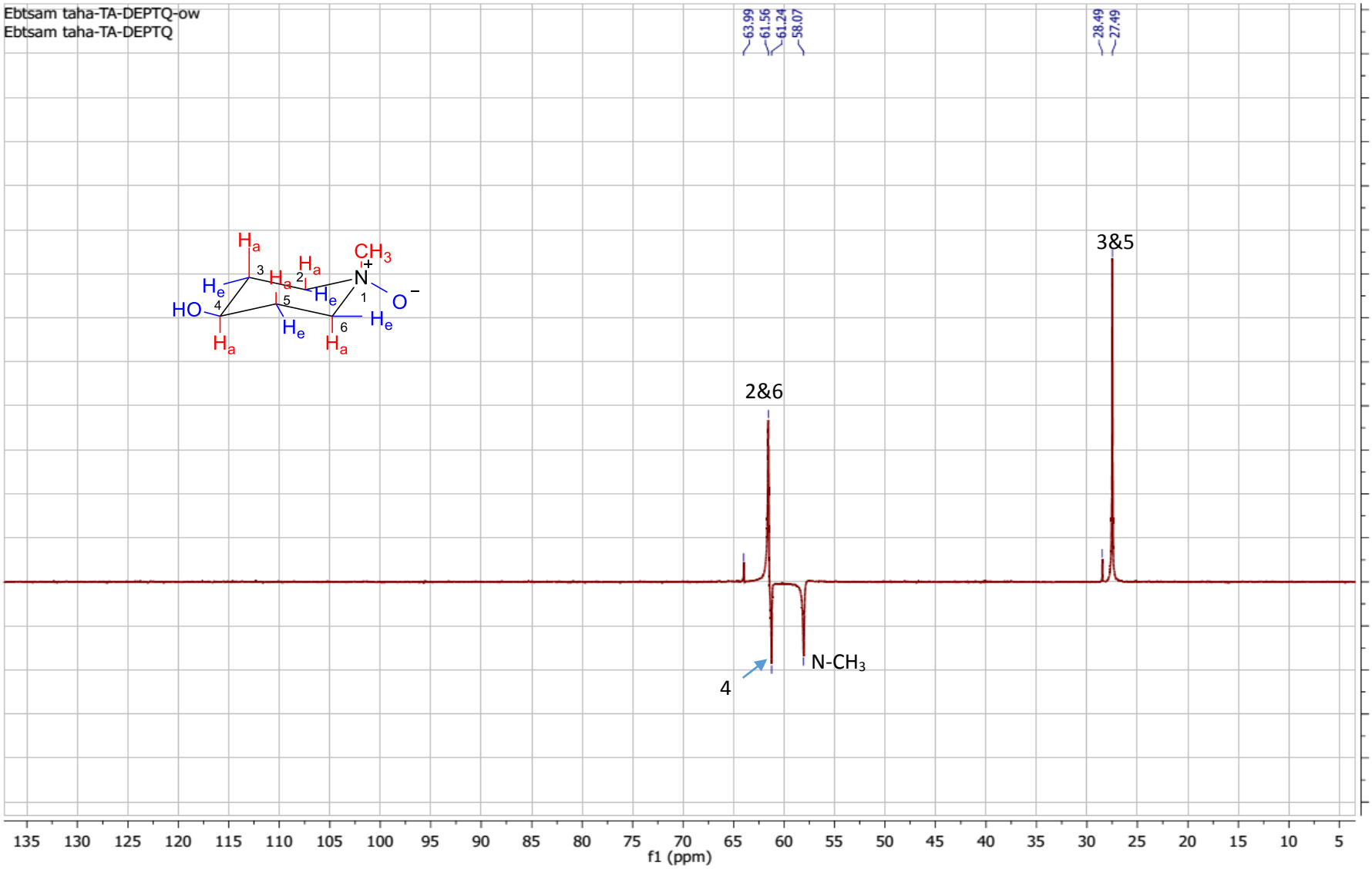



**Fig. S9:** DEPTQ-135 NMR spectrum of **2** ($D_2O$, 100 MHz).

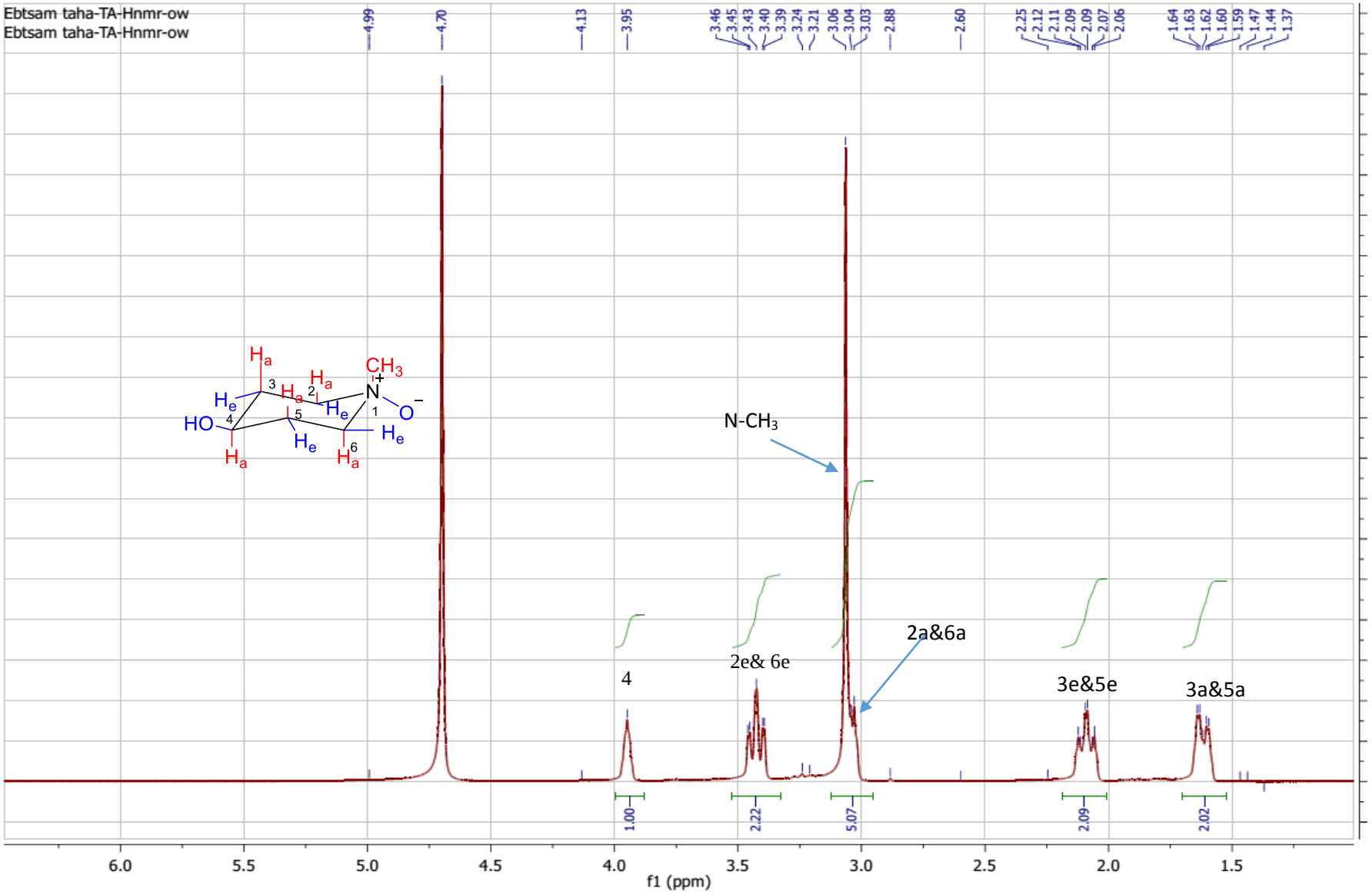



**Fig. S10:** $^{1}$H NMR spectrum of **2** ($D_2O$, 400 MHz).

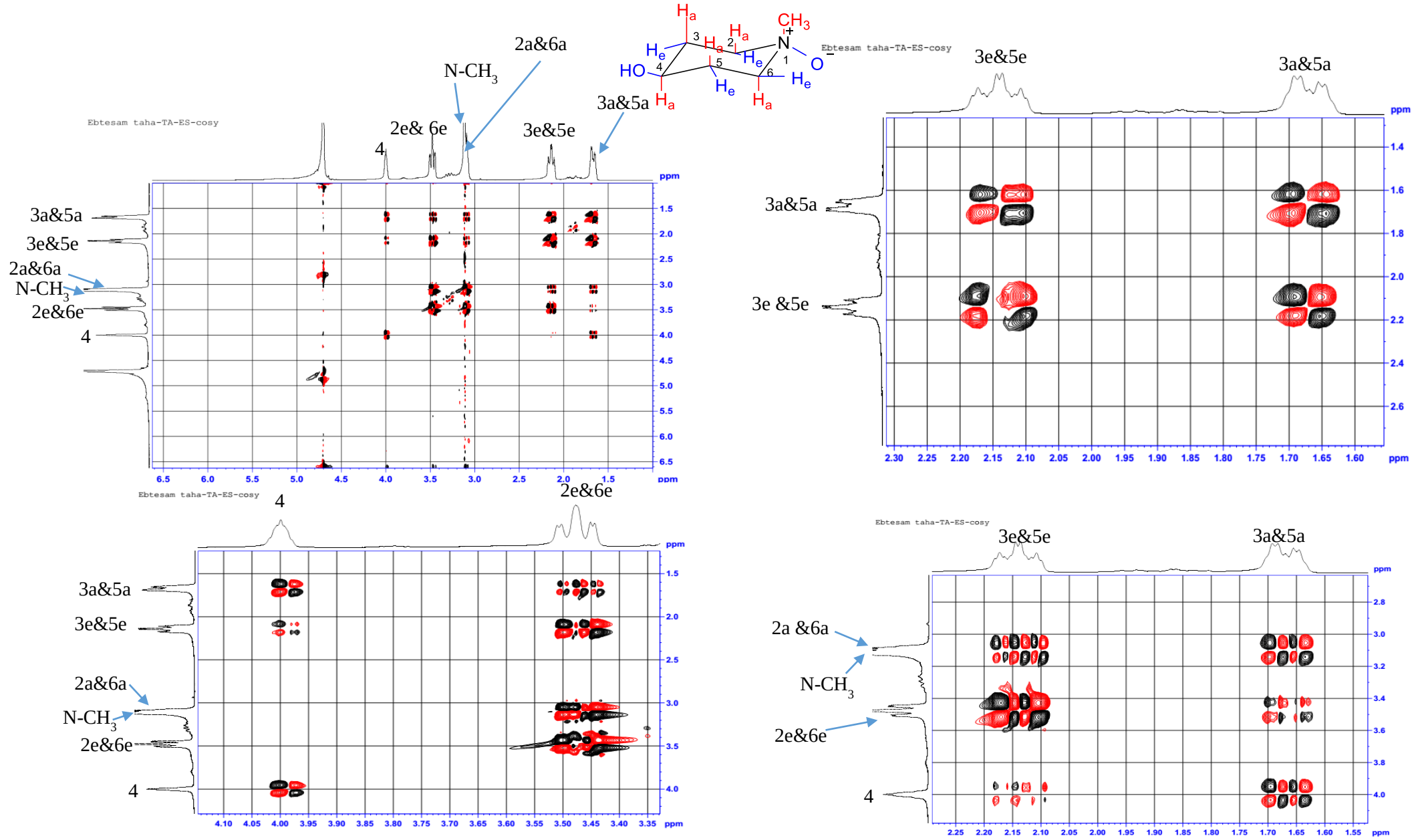



**Fig. S11:** COSY spectrum of **2** ($D_2O$).

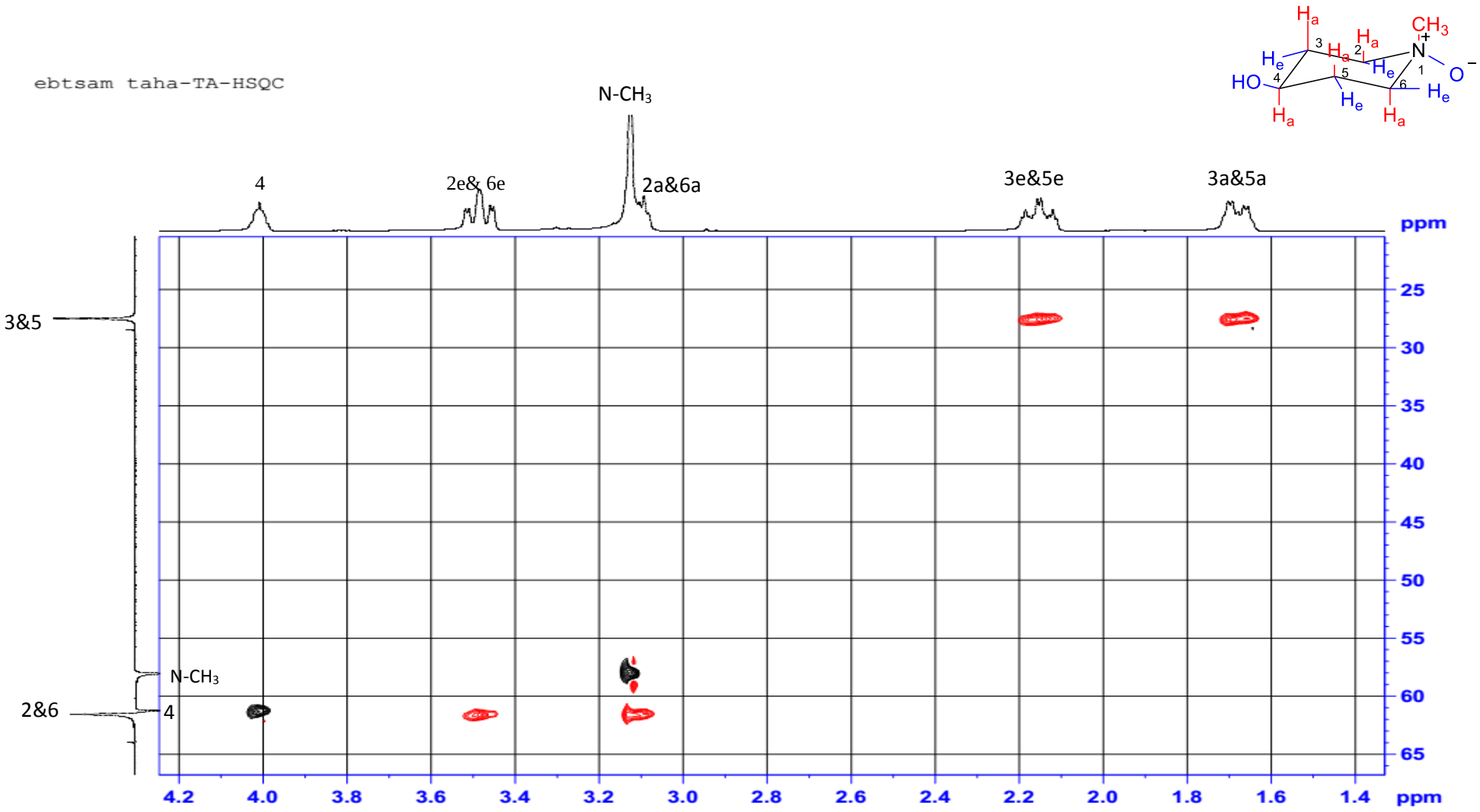



**Fig. S12:** DEPTQ-135 and $^{1}$H NMR correlation spectrum of **2** ($D_2O$).

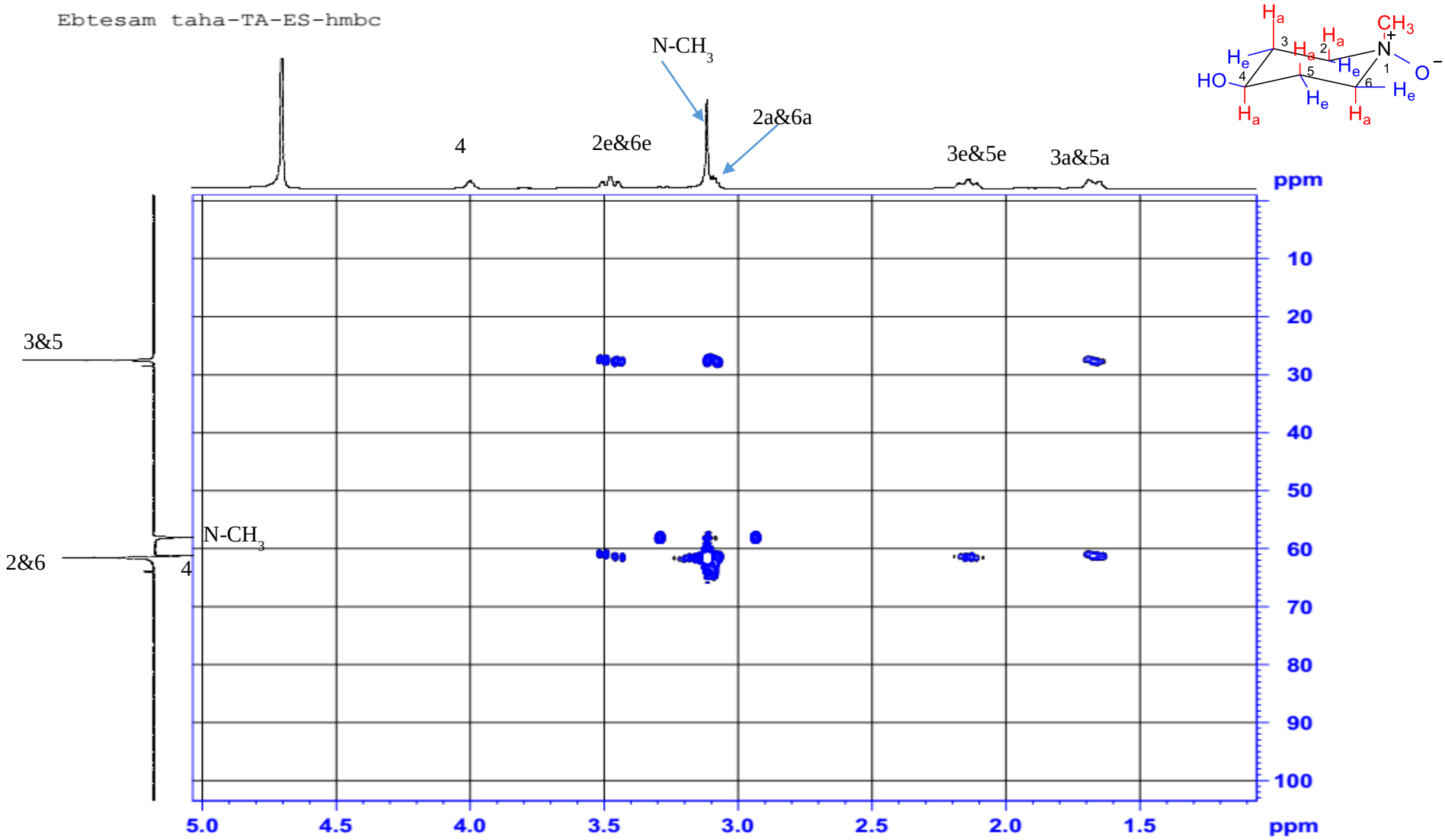



**Fig. S13:** HMBC spectrum of **2** ($D_2O$).

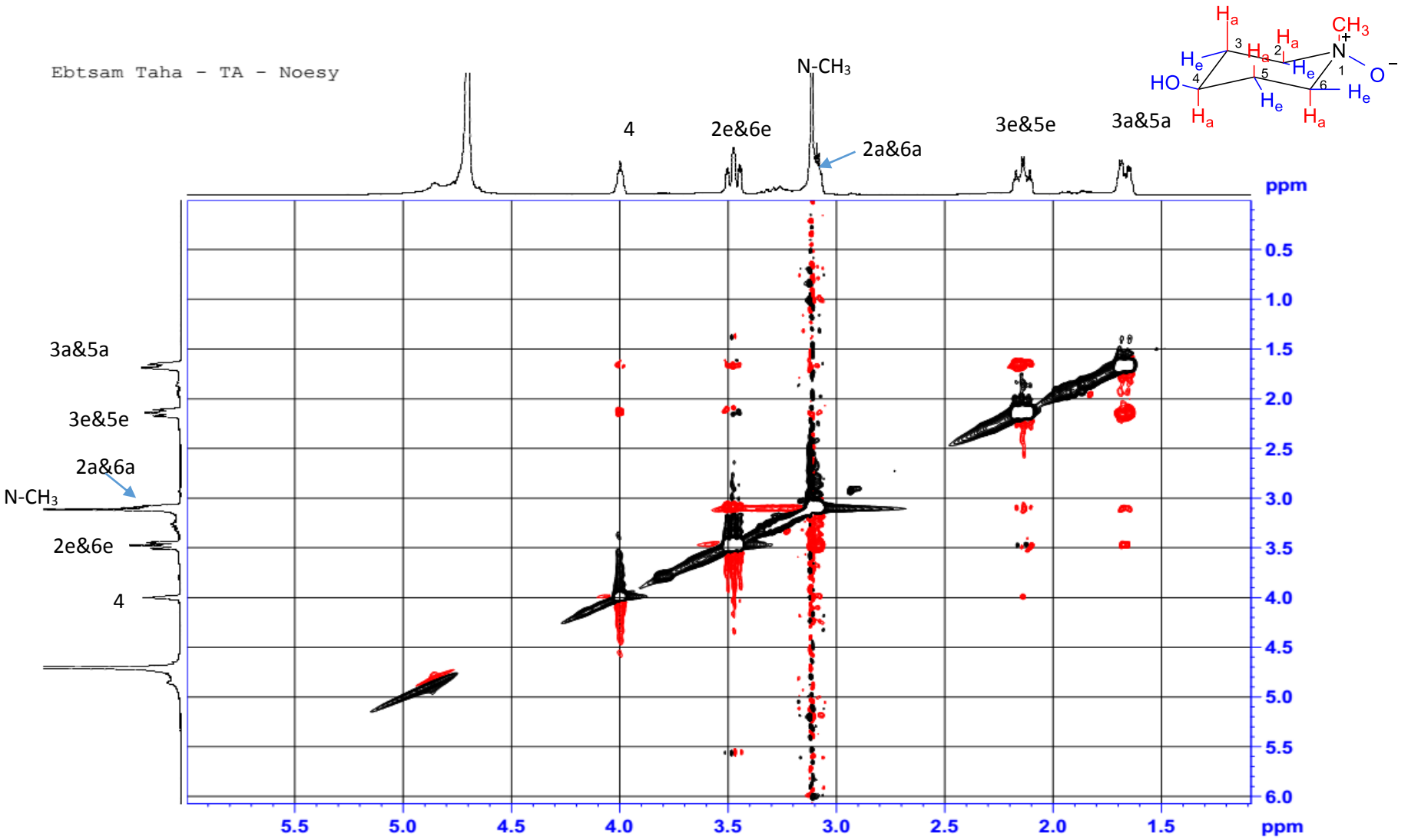



**Fig. S14:** NOE spectrum of **2** ($D_2O$).

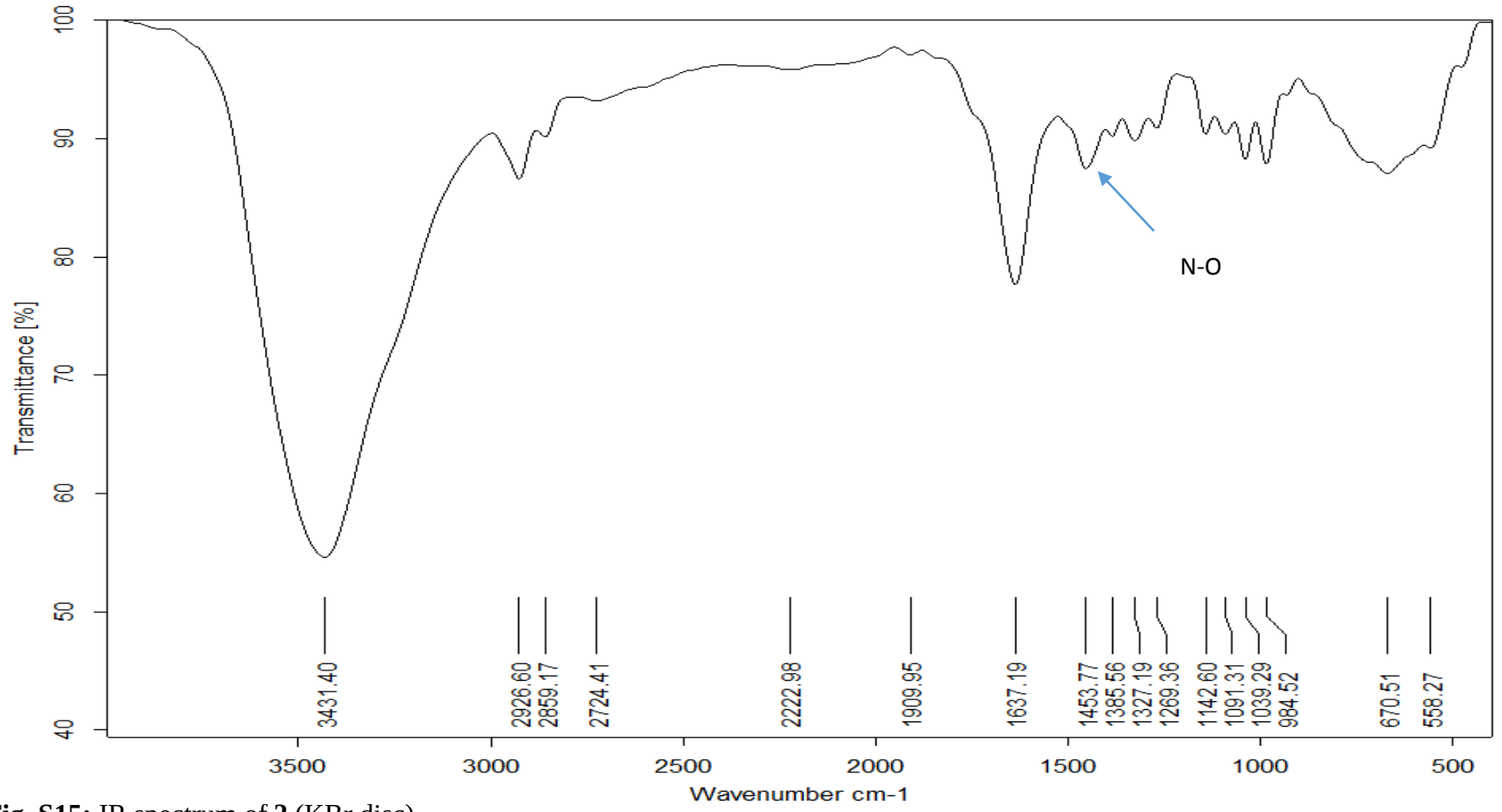



**Fig. S15:** IR spectrum of **2** (KBr disc).

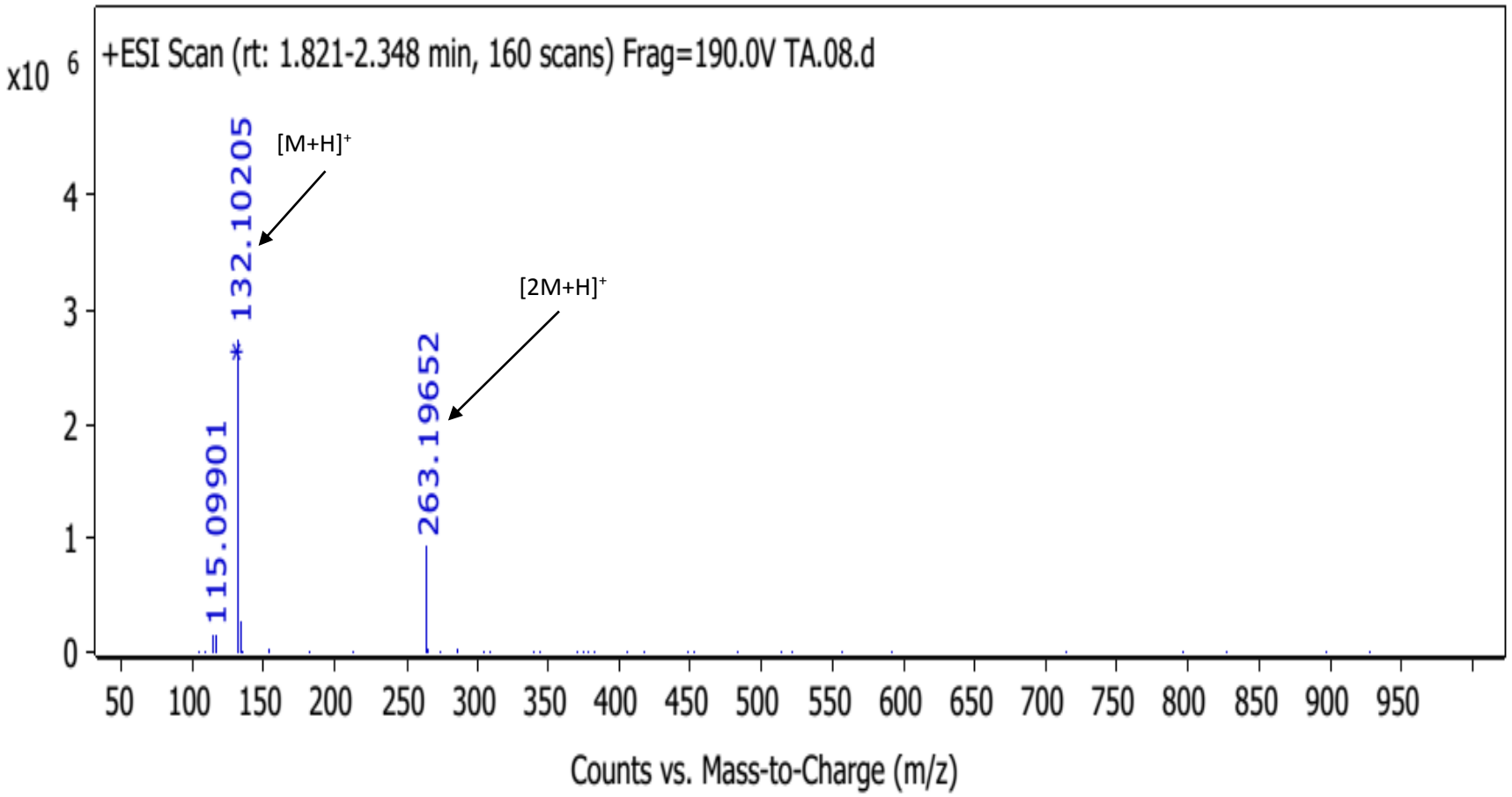



**Fig. S16:** HRESIMS (+ve) spectrum of **2**.

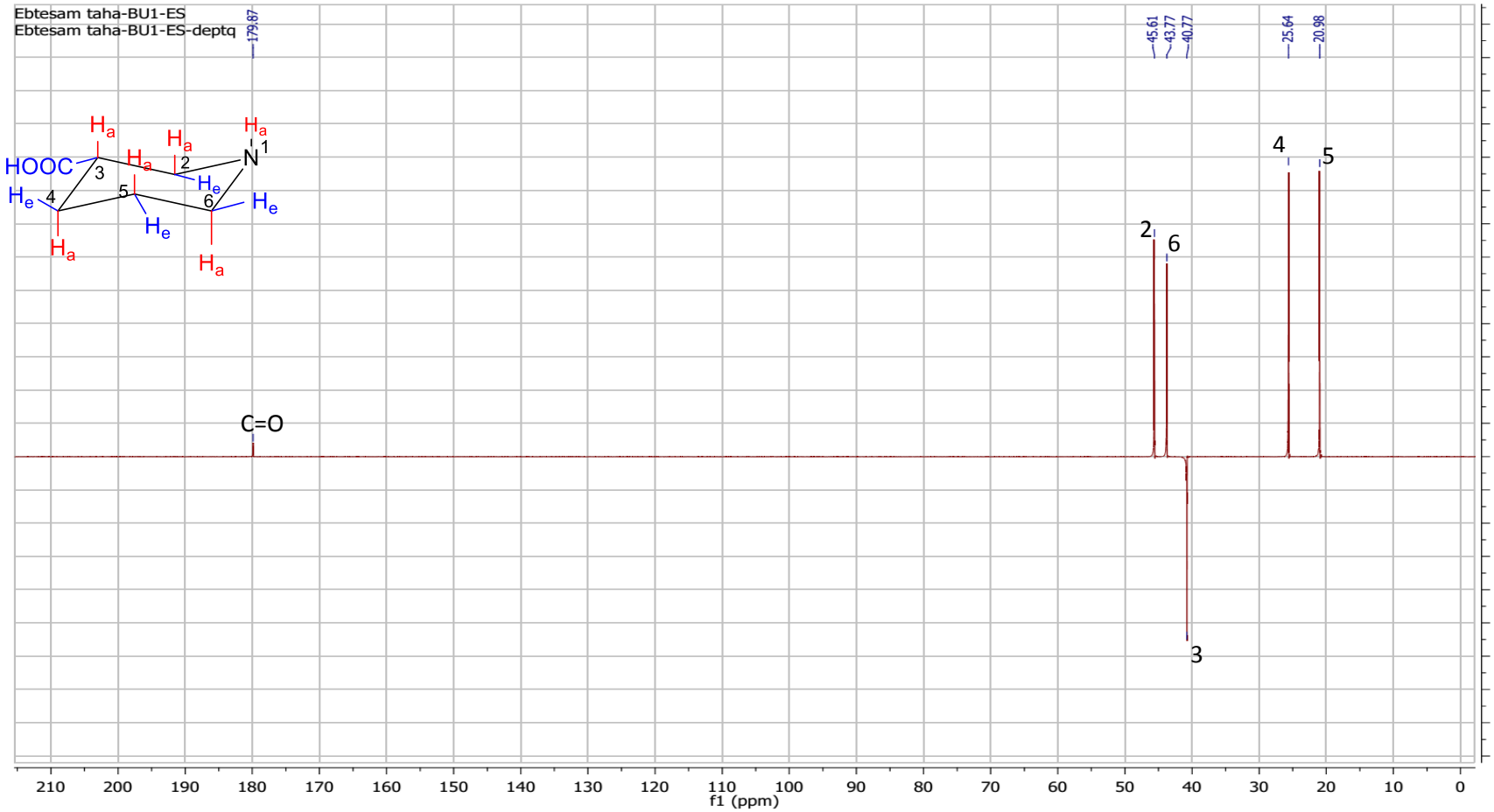



**Fig. S17:** DEPTQ-135 spectrum of **3** ($D_2O$, 100 MHz).

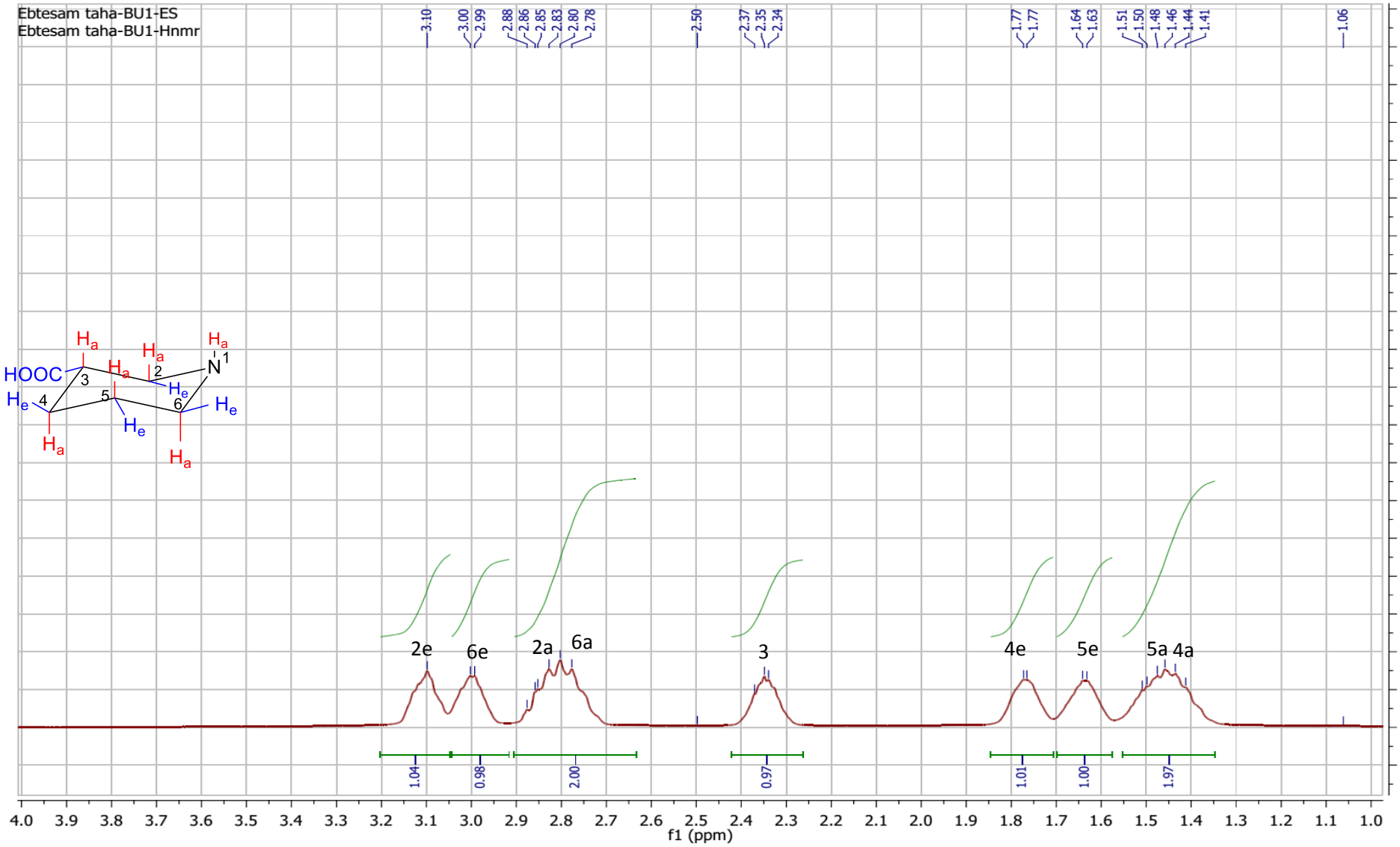



**Fig. S18:** $^{1}$H NMR spectrum of **3** ($D_2O$, 400 MHz).

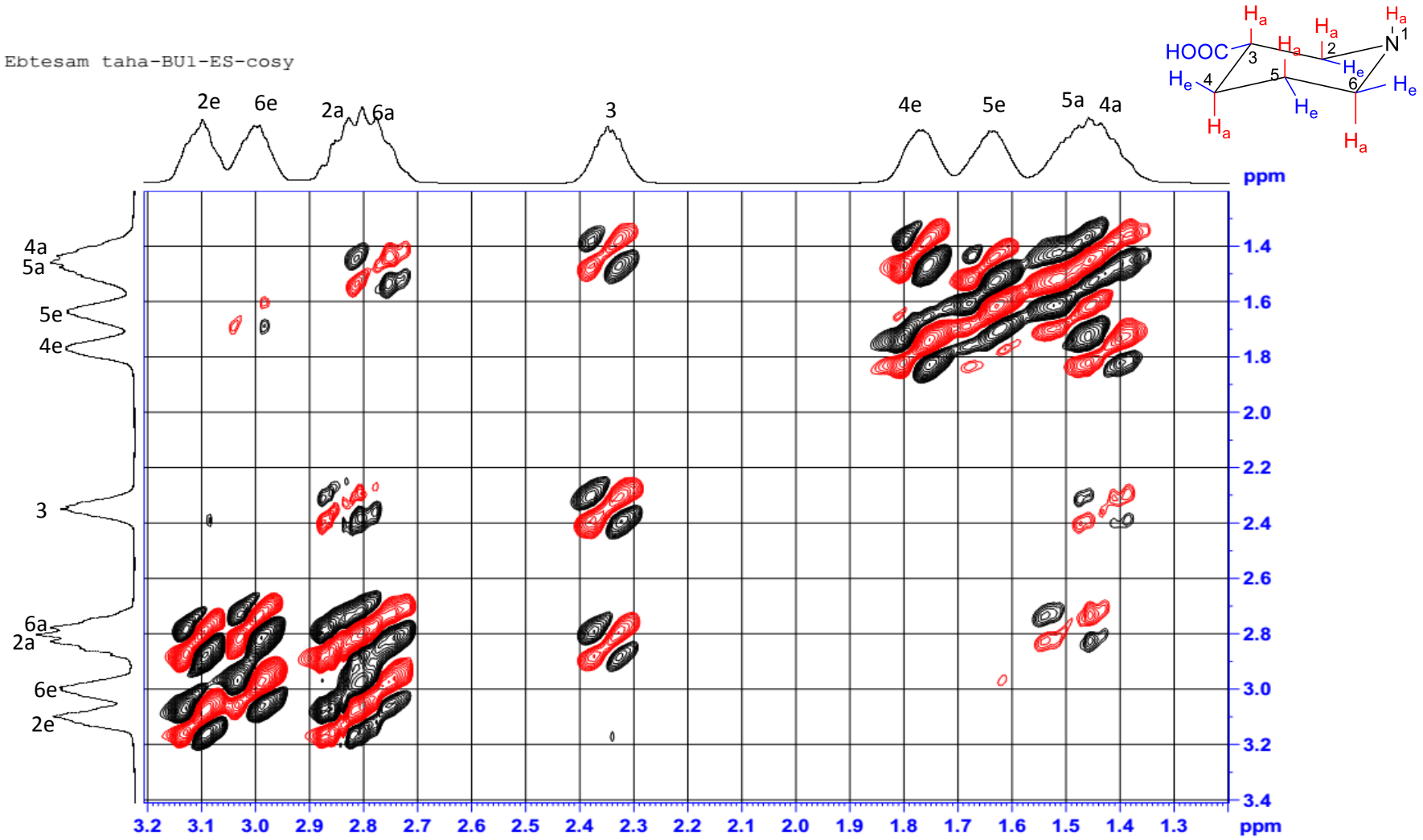



**Fig. S19:** COSY spectrum of **3** ($D_2O$).

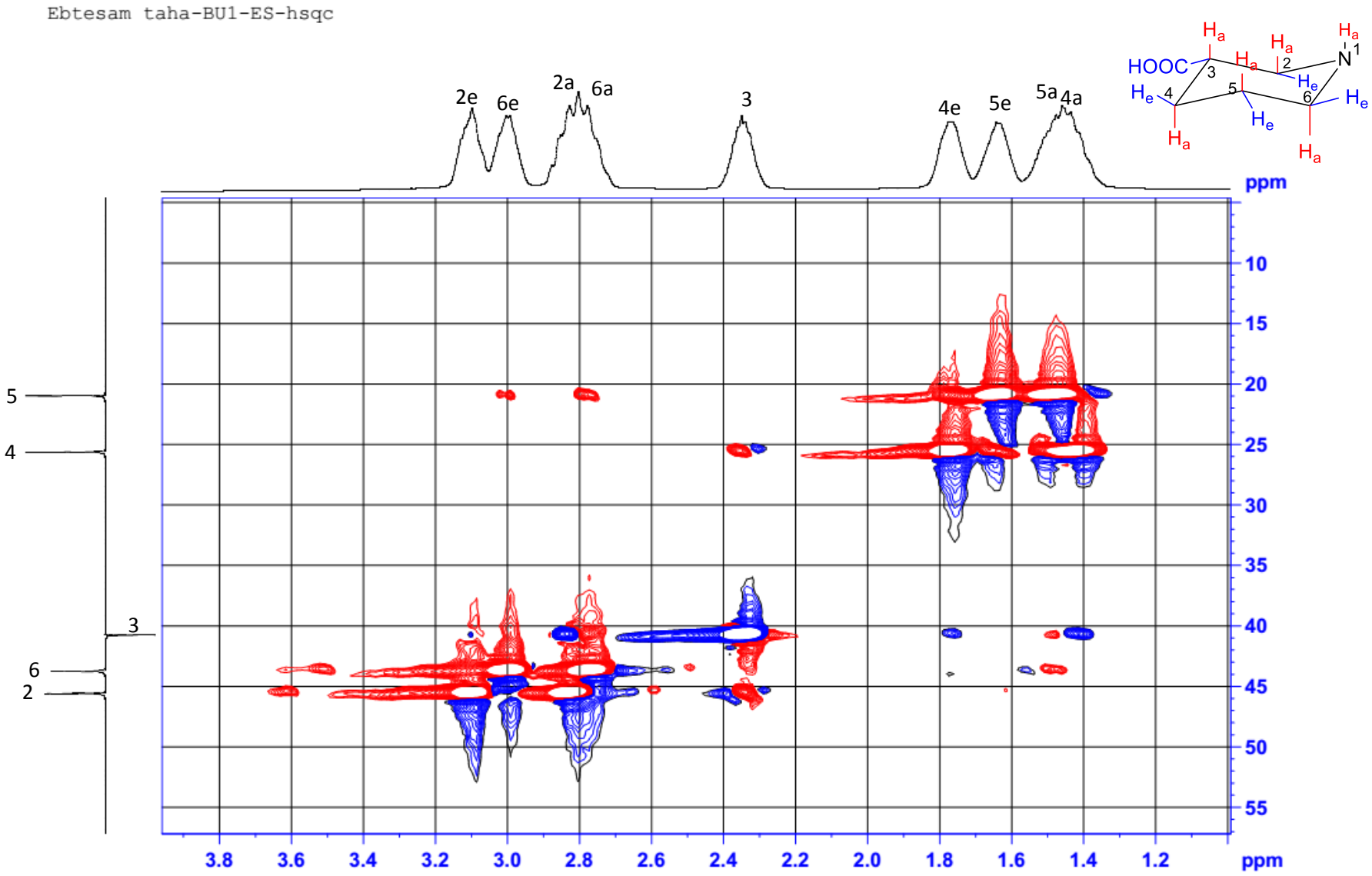



**Fig. S20:** DEPTQ-135 and $^{1}$H NMR correlation spectrum of **3** ($D_2O$).

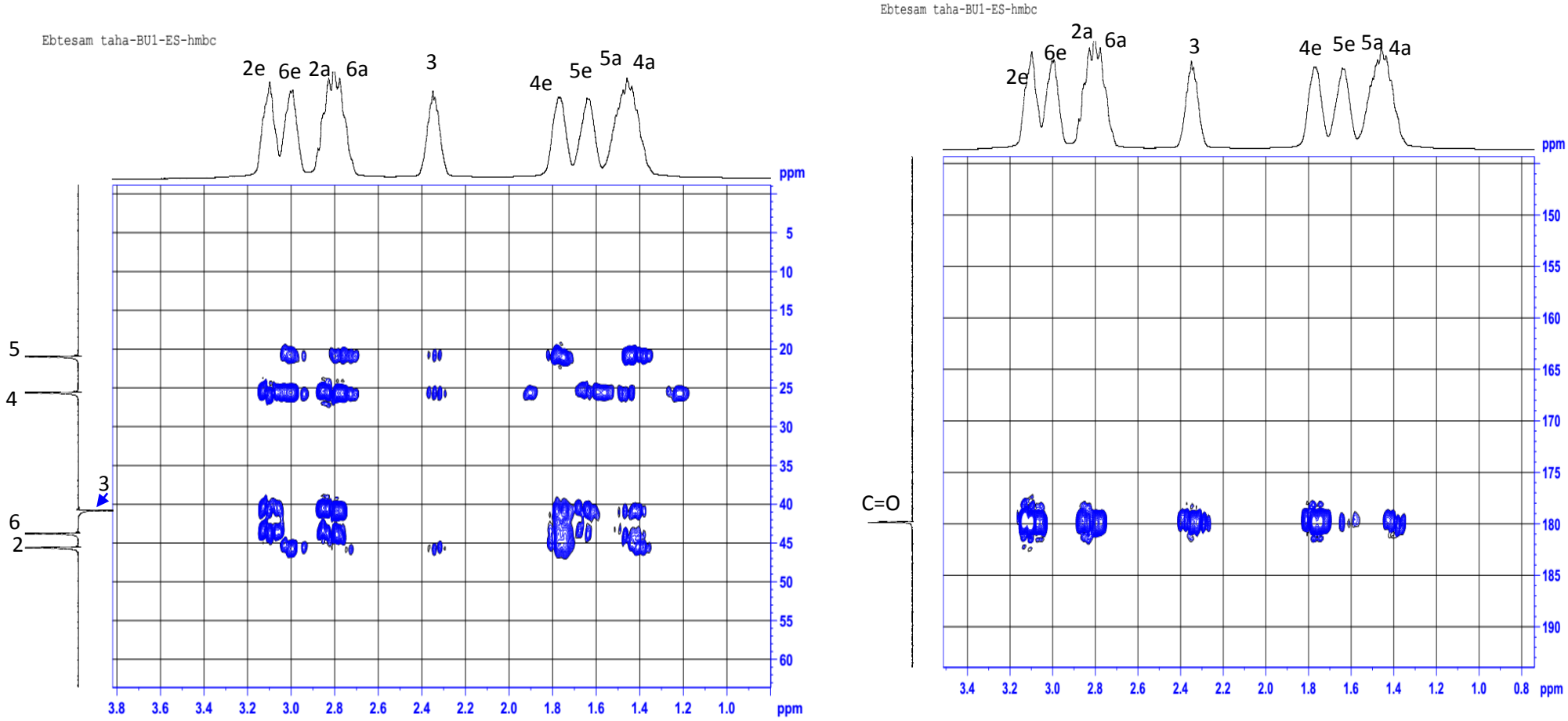



**Fig. S21:** HMBC spectrum of **3** ($D_2O$).

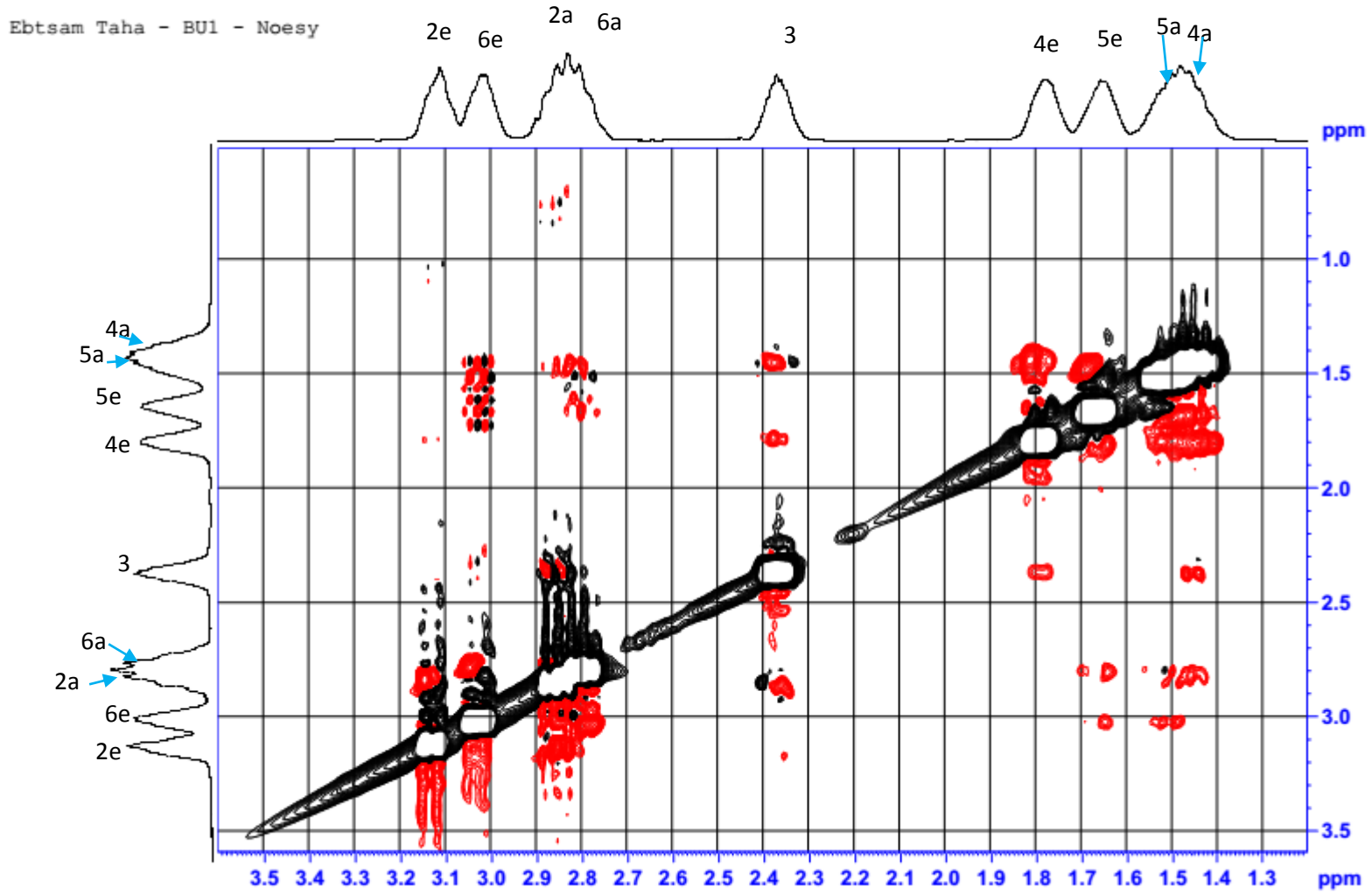



**Fig. S22:** NOE spectrum of **3** ($D_2O$).

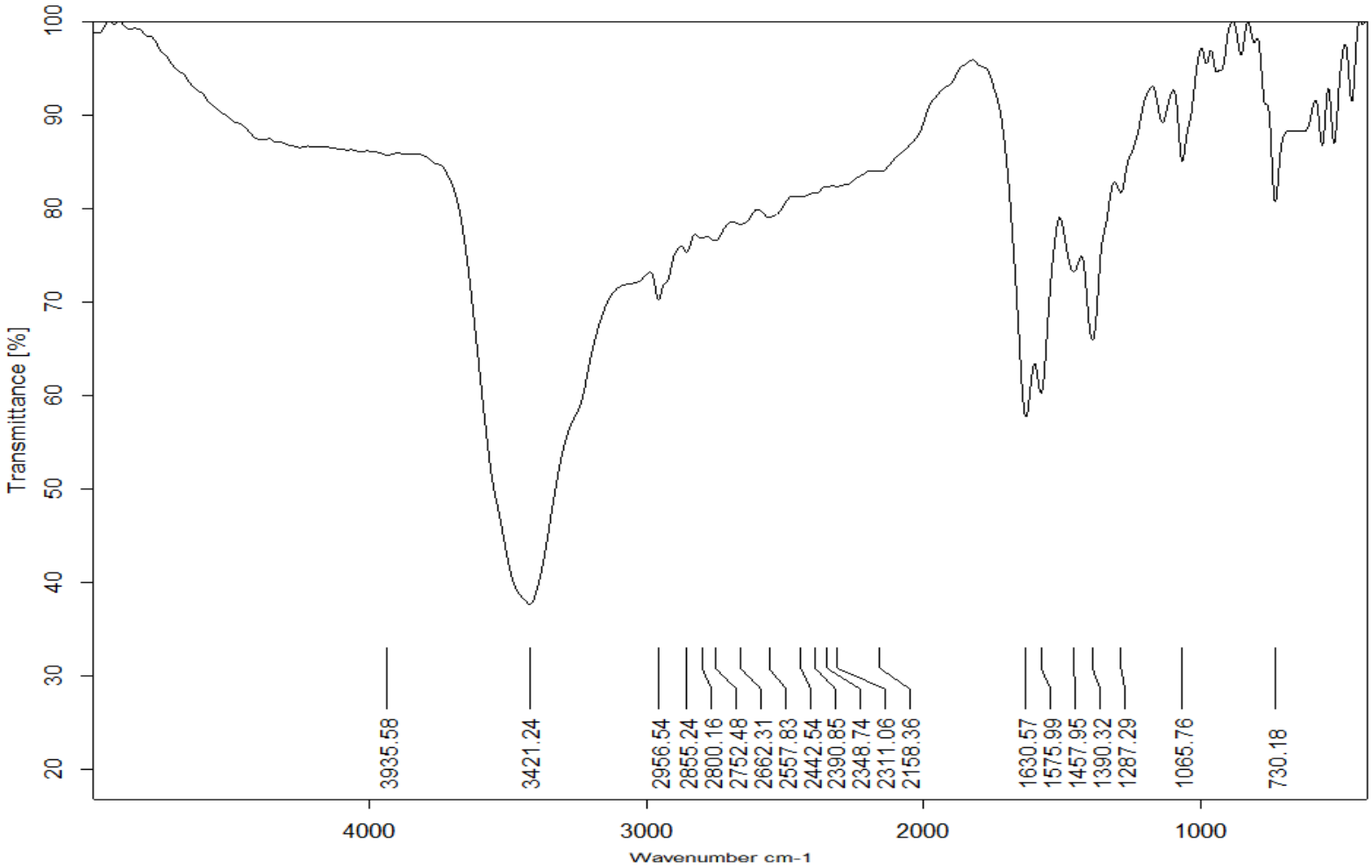



**Fig. S23:** IR spectrum of **3** (KBr disc).

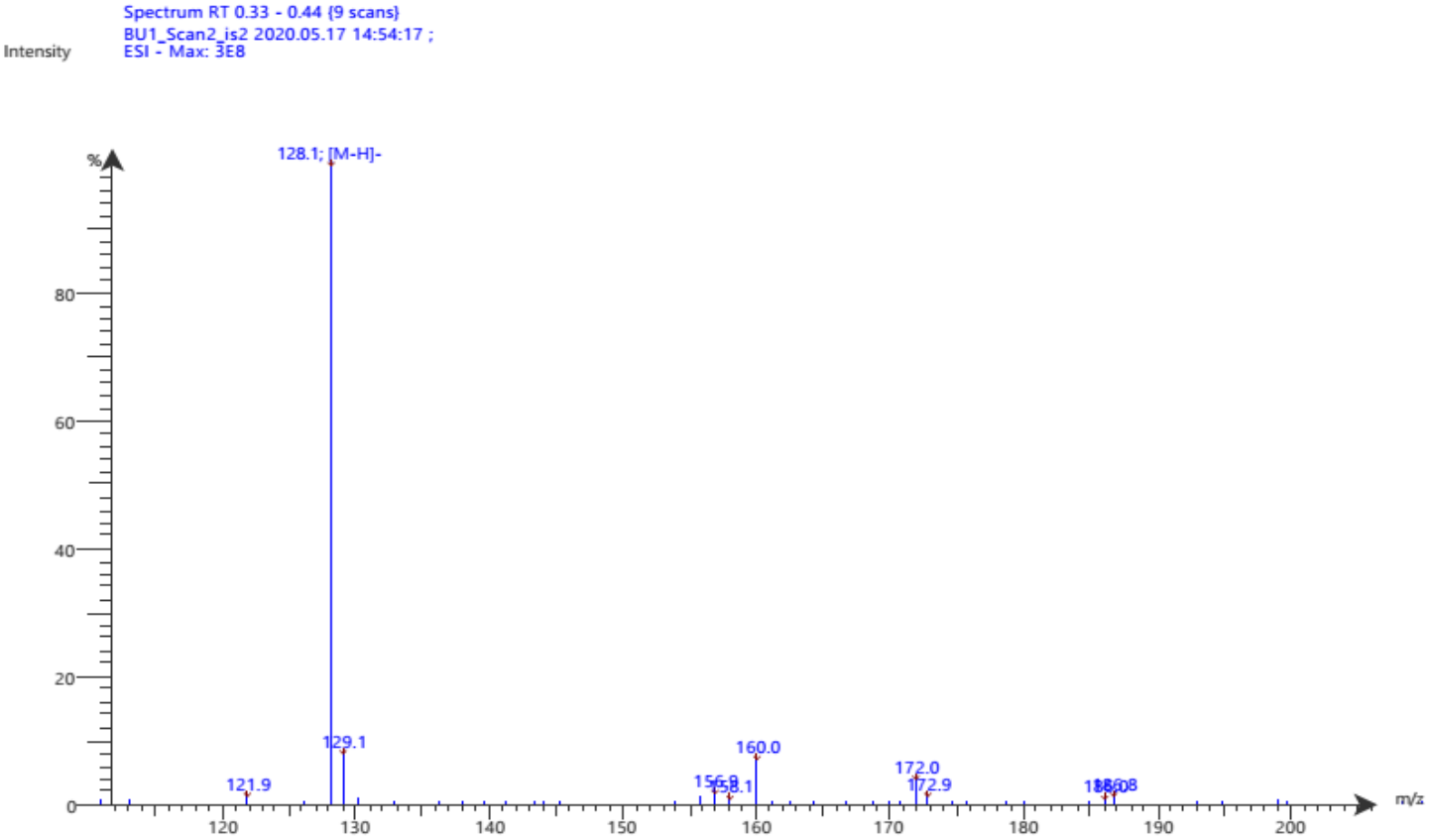



**Fig. S24:** ESIMS (-ve) spectrum of **3**.

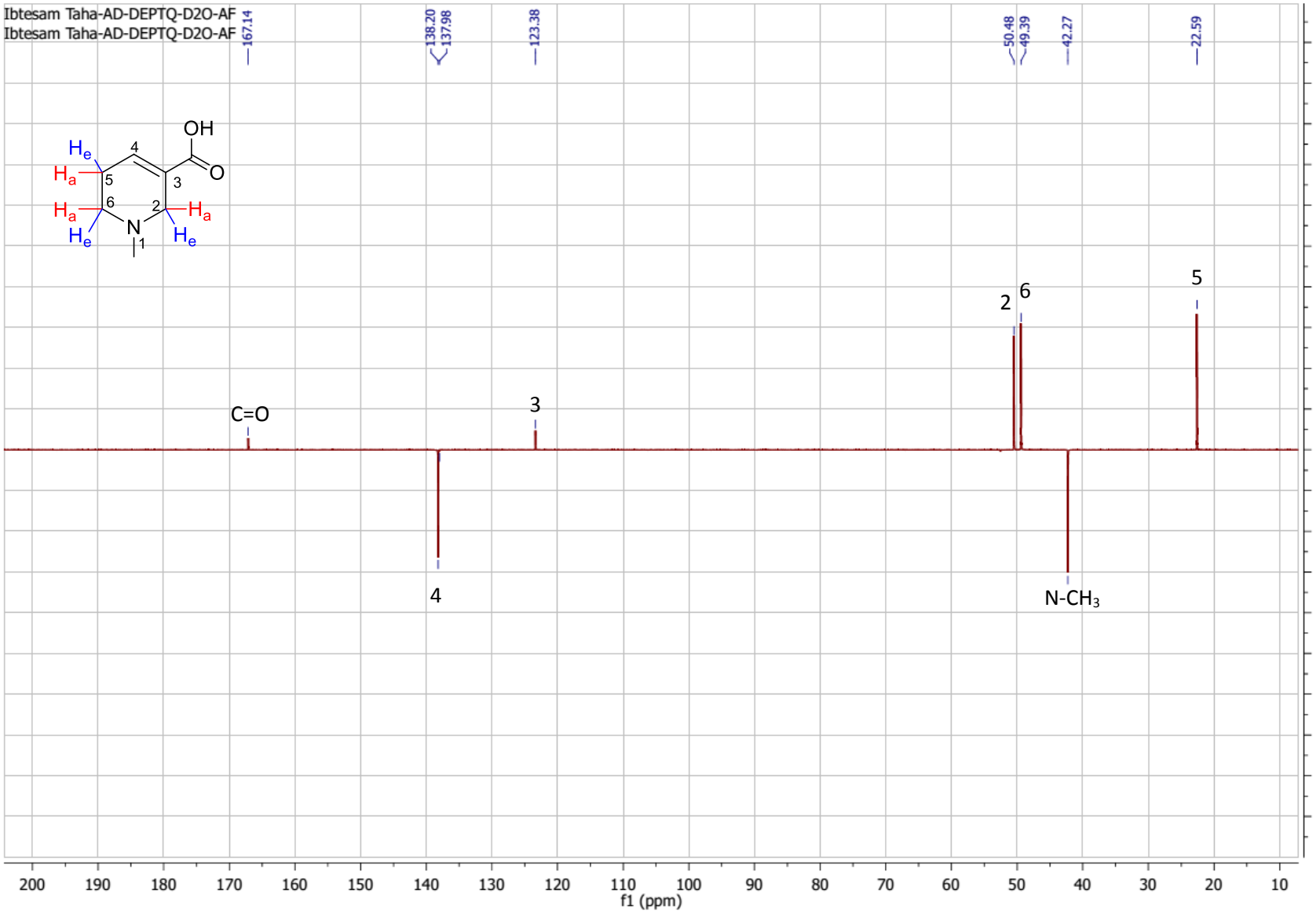



**Fig. S25:** DEPTQ-135 spectrum of **4** ($D_2O$, 100 MHz).

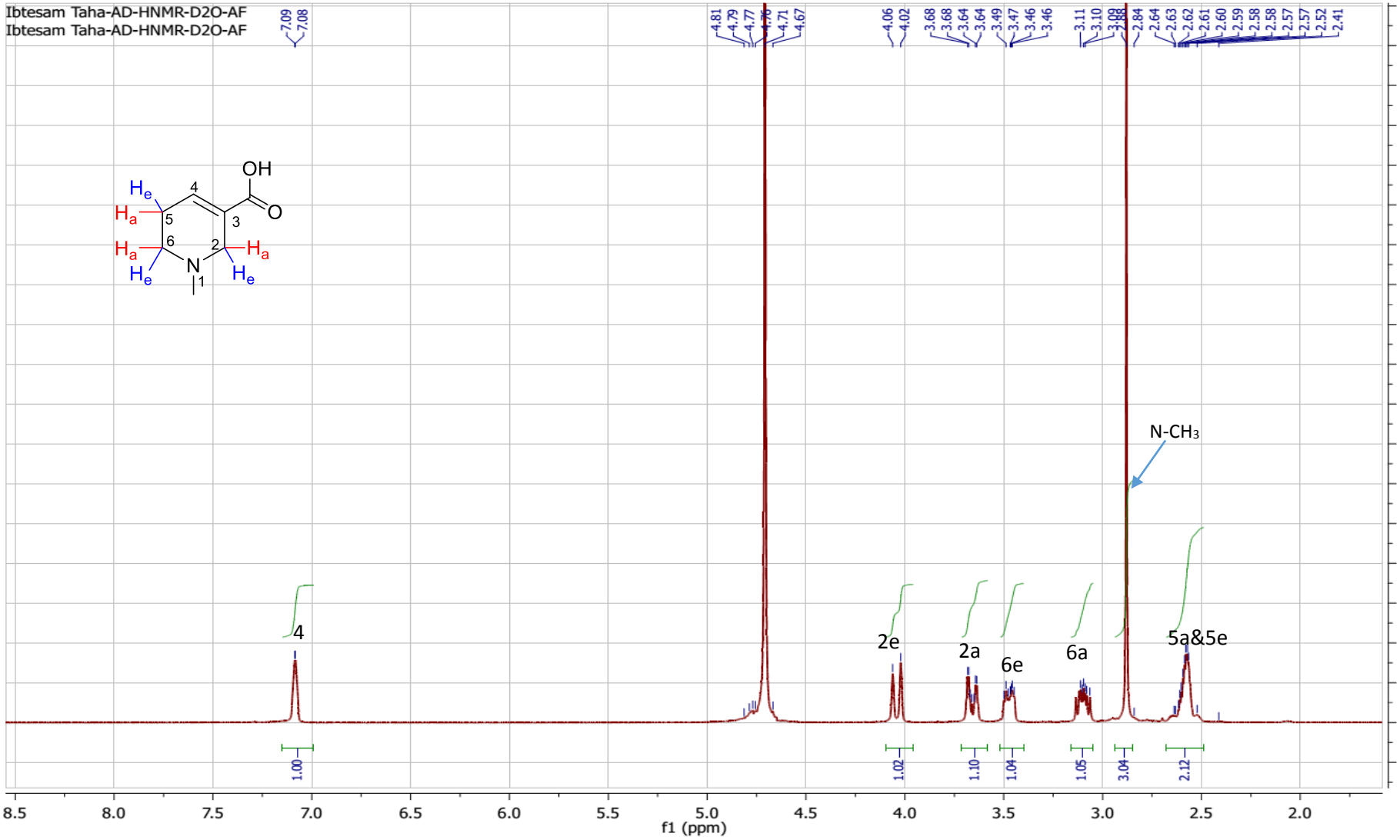



**Fig. S26:** $^{1}$H NMR spectrum of **4** ($D_2O$, 400 MHz).

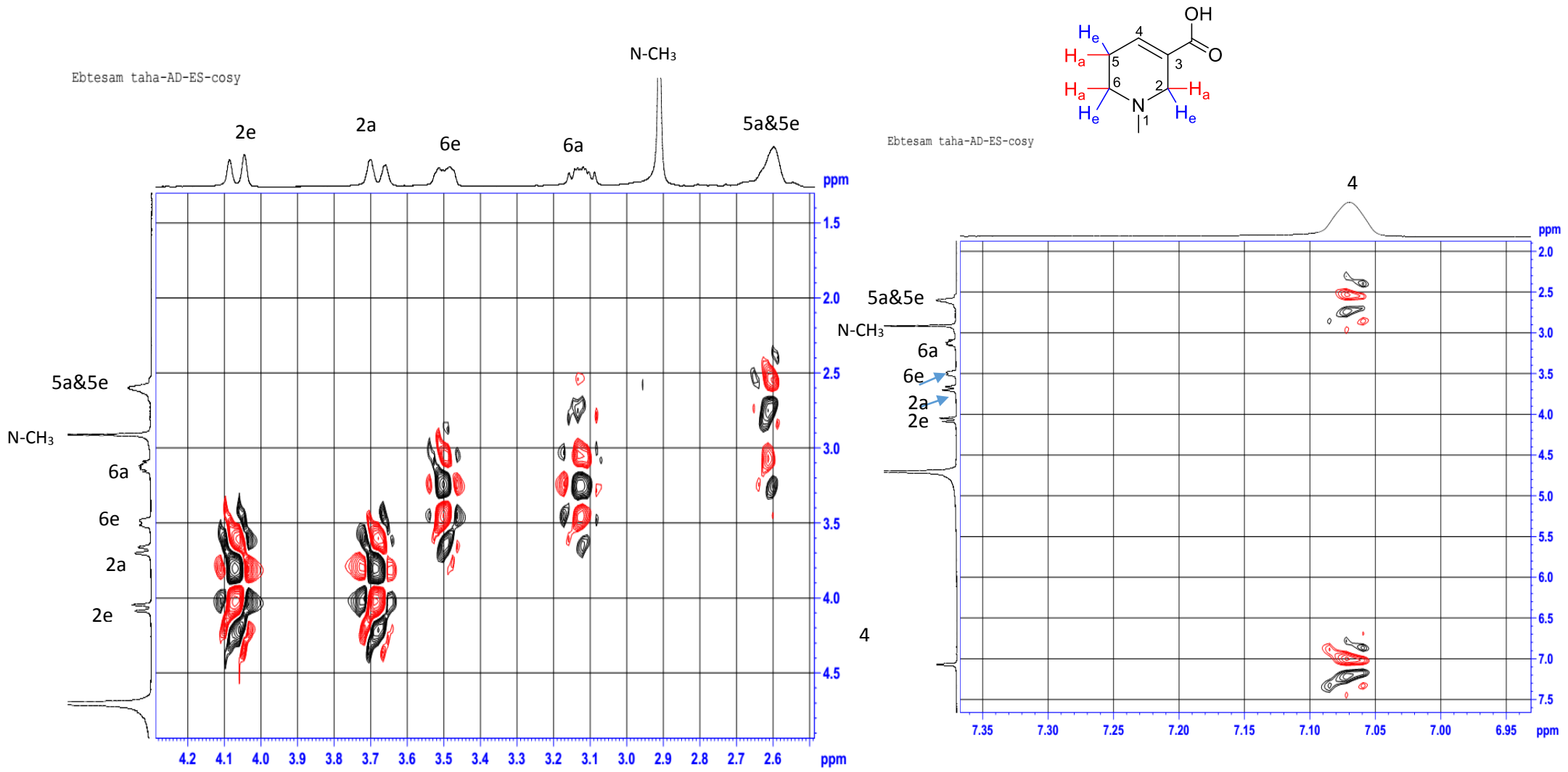



**Fig. S27:** COSY spectrum of **4** ($D_2O$).

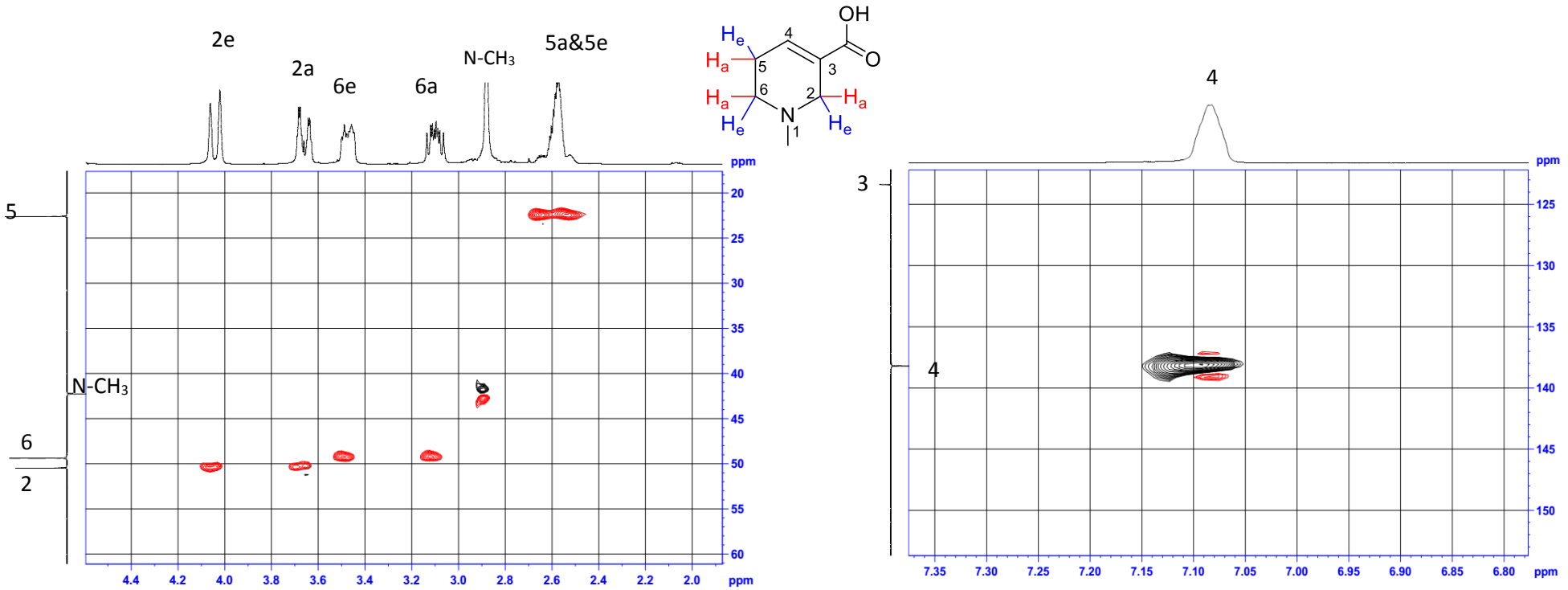



**Fig. S28:** DEPTQ-135 and $^{1}$H NMR correlation spectrum of **4** ($D_2O$).

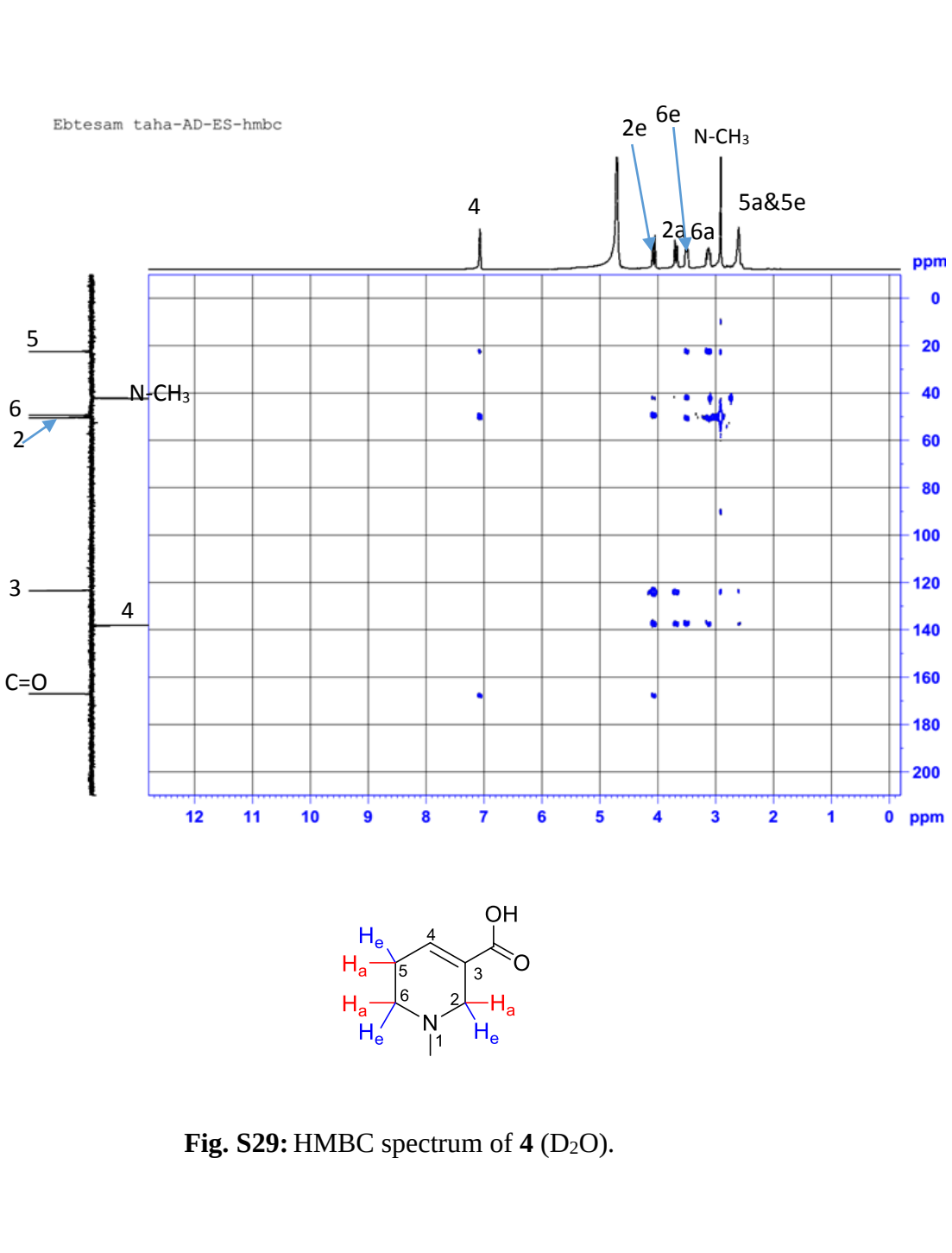
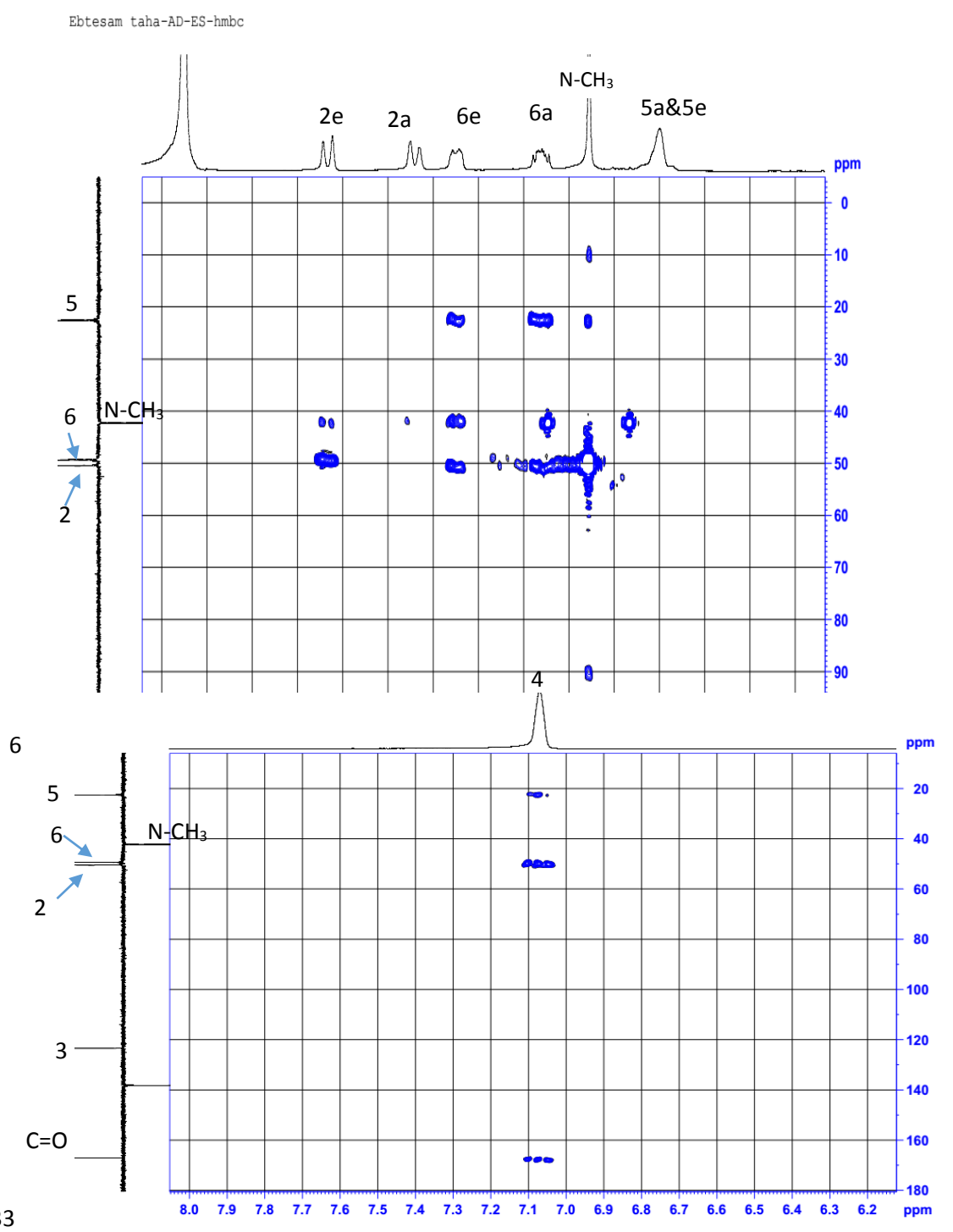






**Fig. S29:** HMBC spectrum of **4** ($D_2O$).



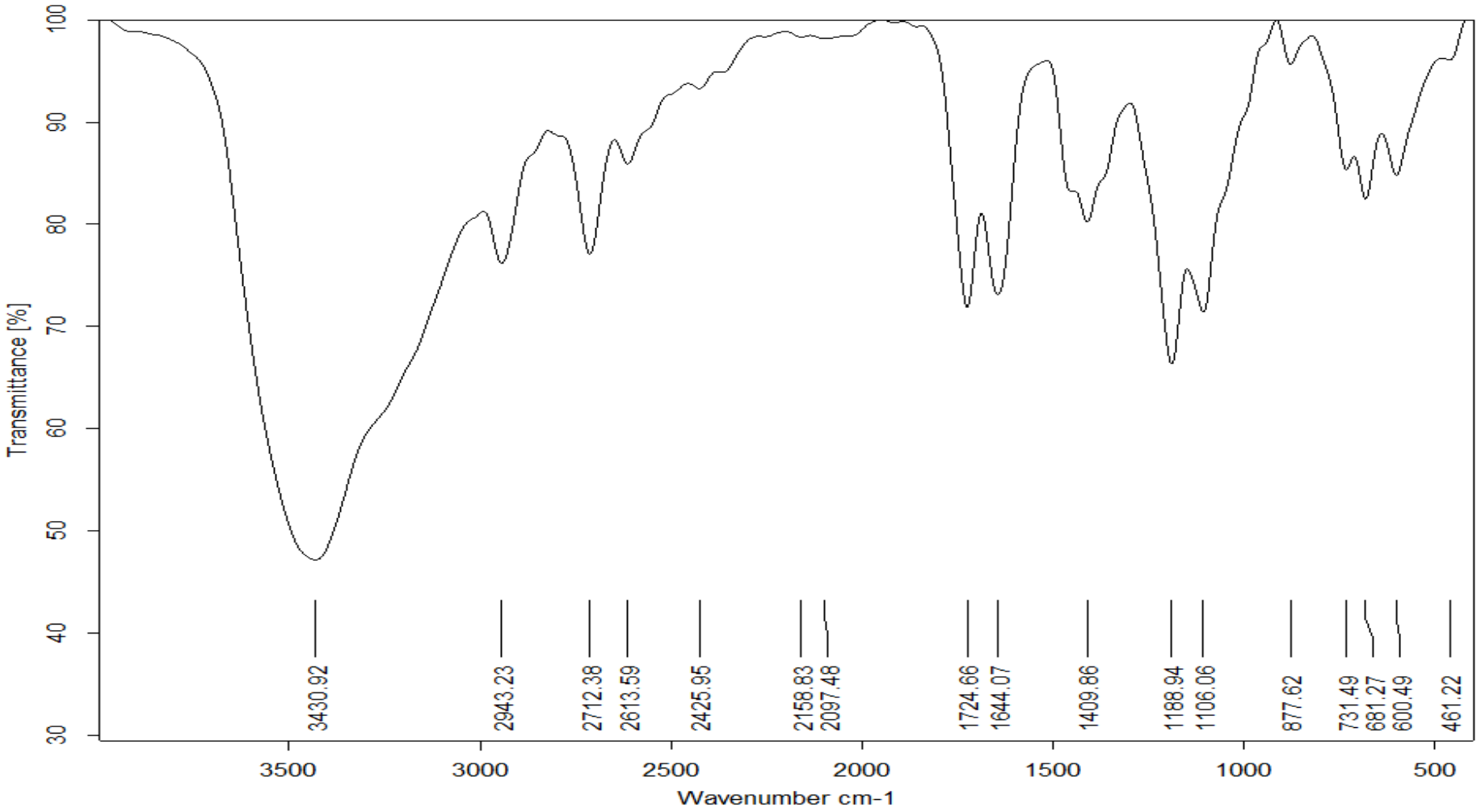



**Fig. S30:** IR spectrum of **4** (KBr disc).

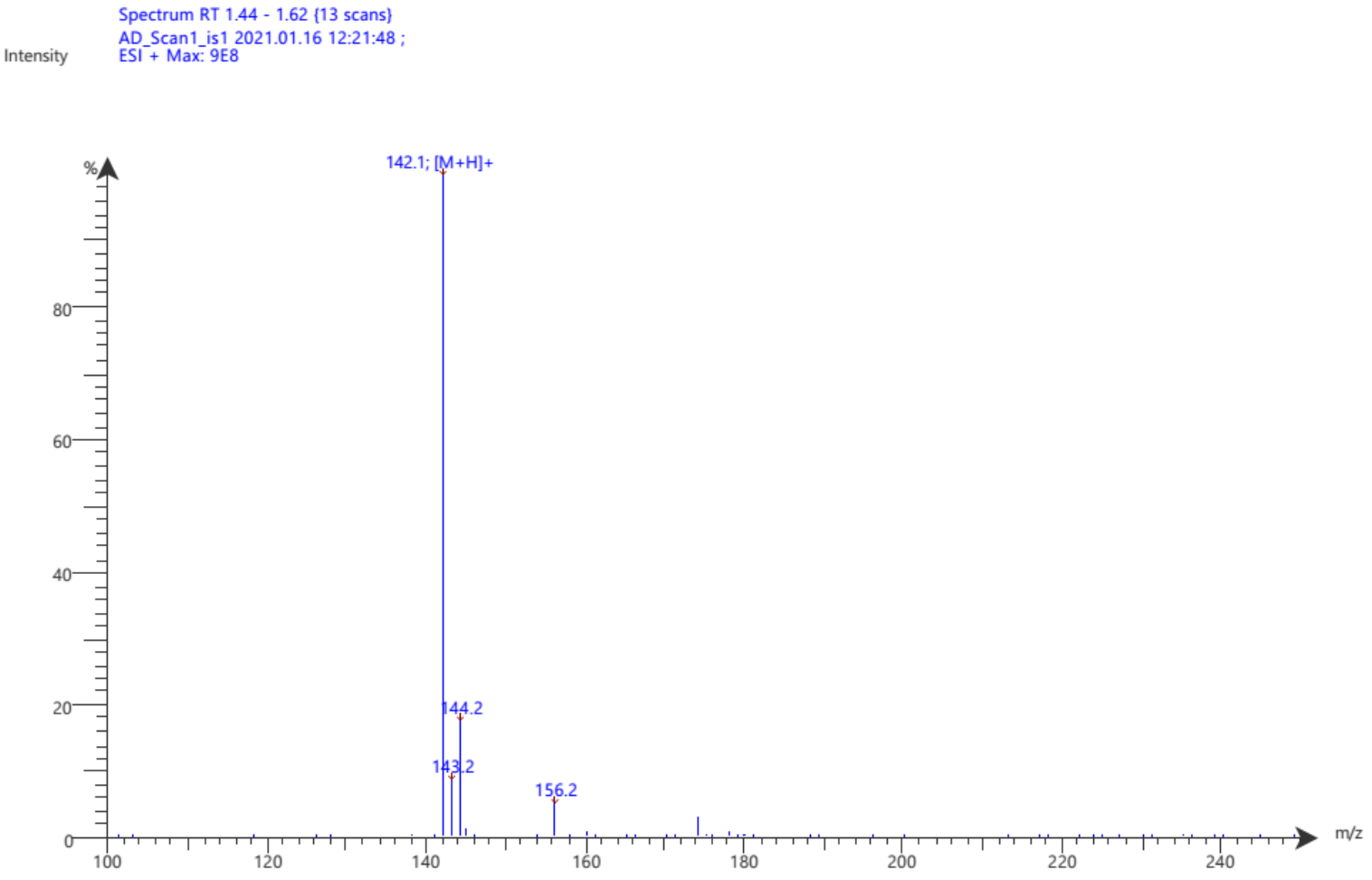



**Fig. S31:** ESIMS (+ve) spectrum of **4**.

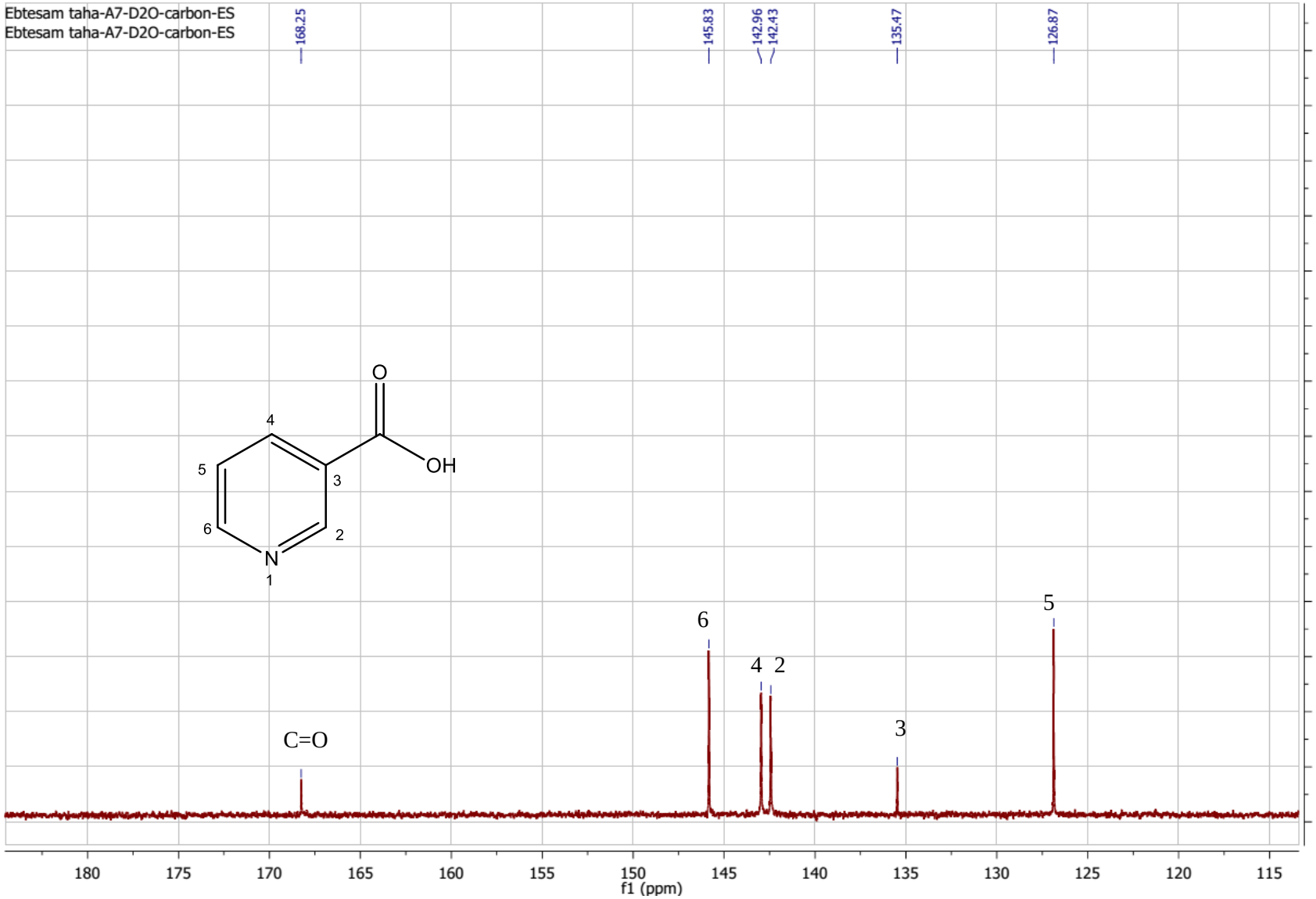



**Fig. S32:** $^{13}C$ NMR spectrum of **5** ($D_2O$, 100 MHz).

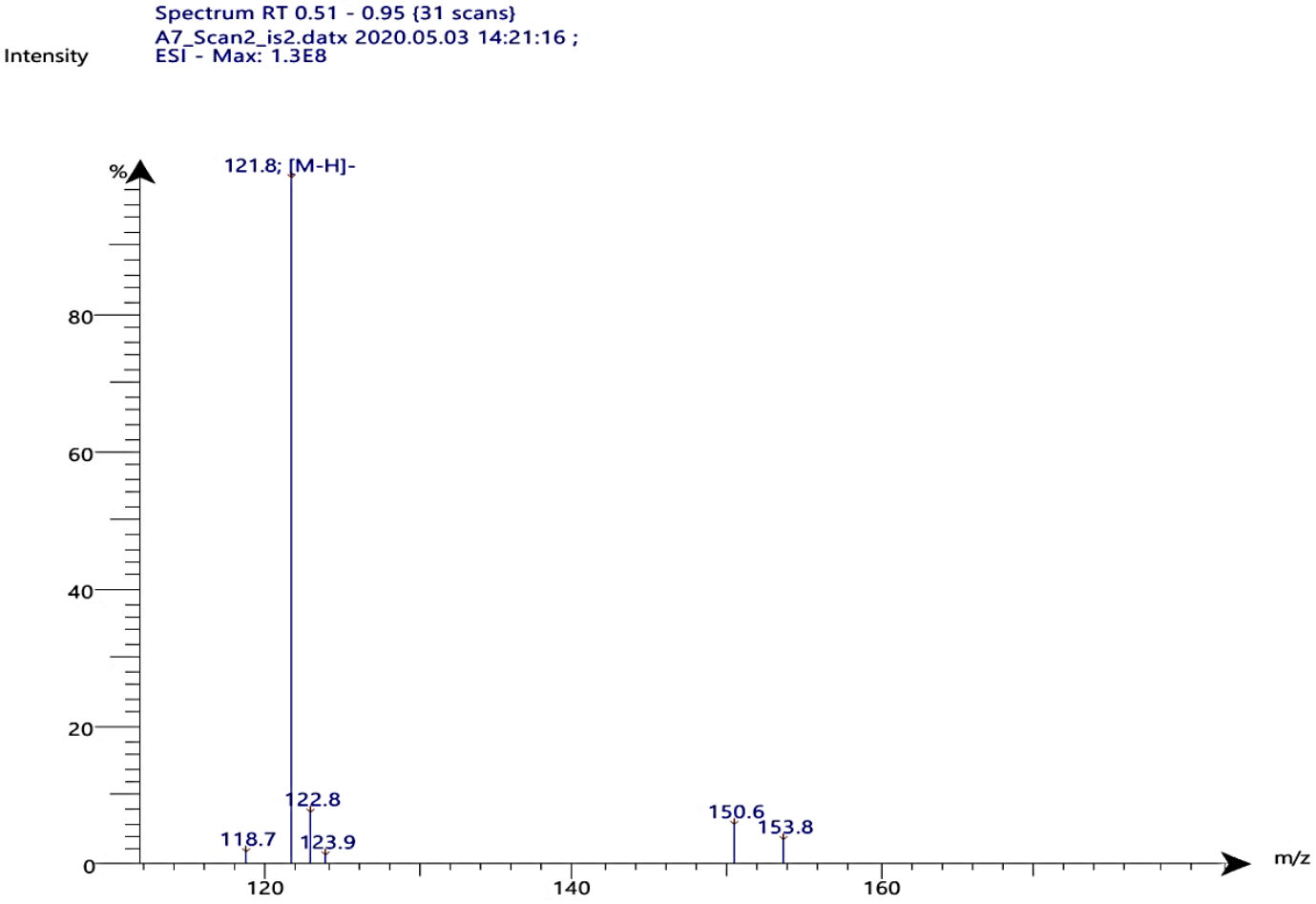



**Fig. S33:** ESIMS (-ve) spectrum of **5.**

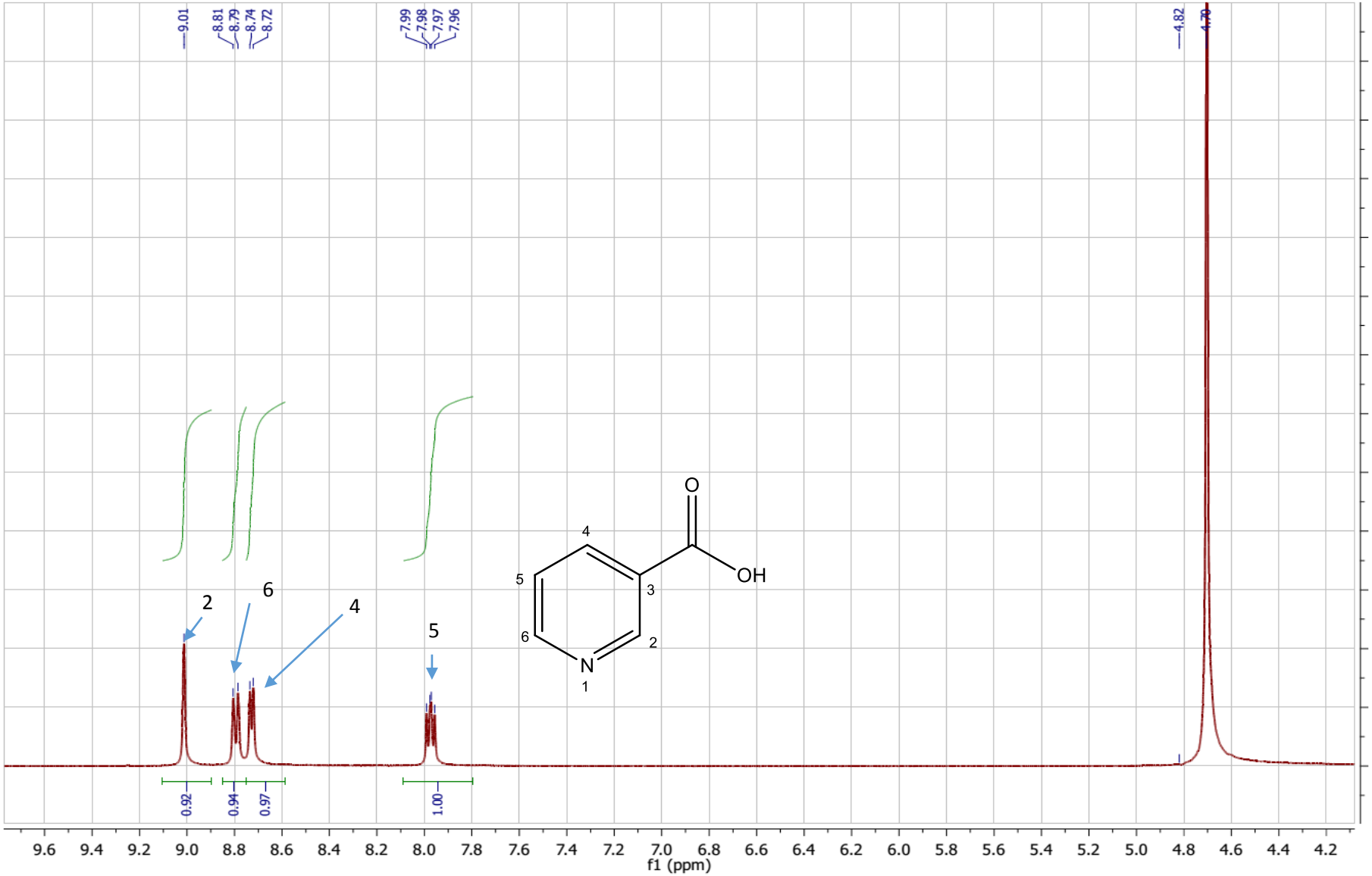



**Fig. S34:** $^{1}$H NMR spectrum of **5** ($D_2O$, 400 MHz).

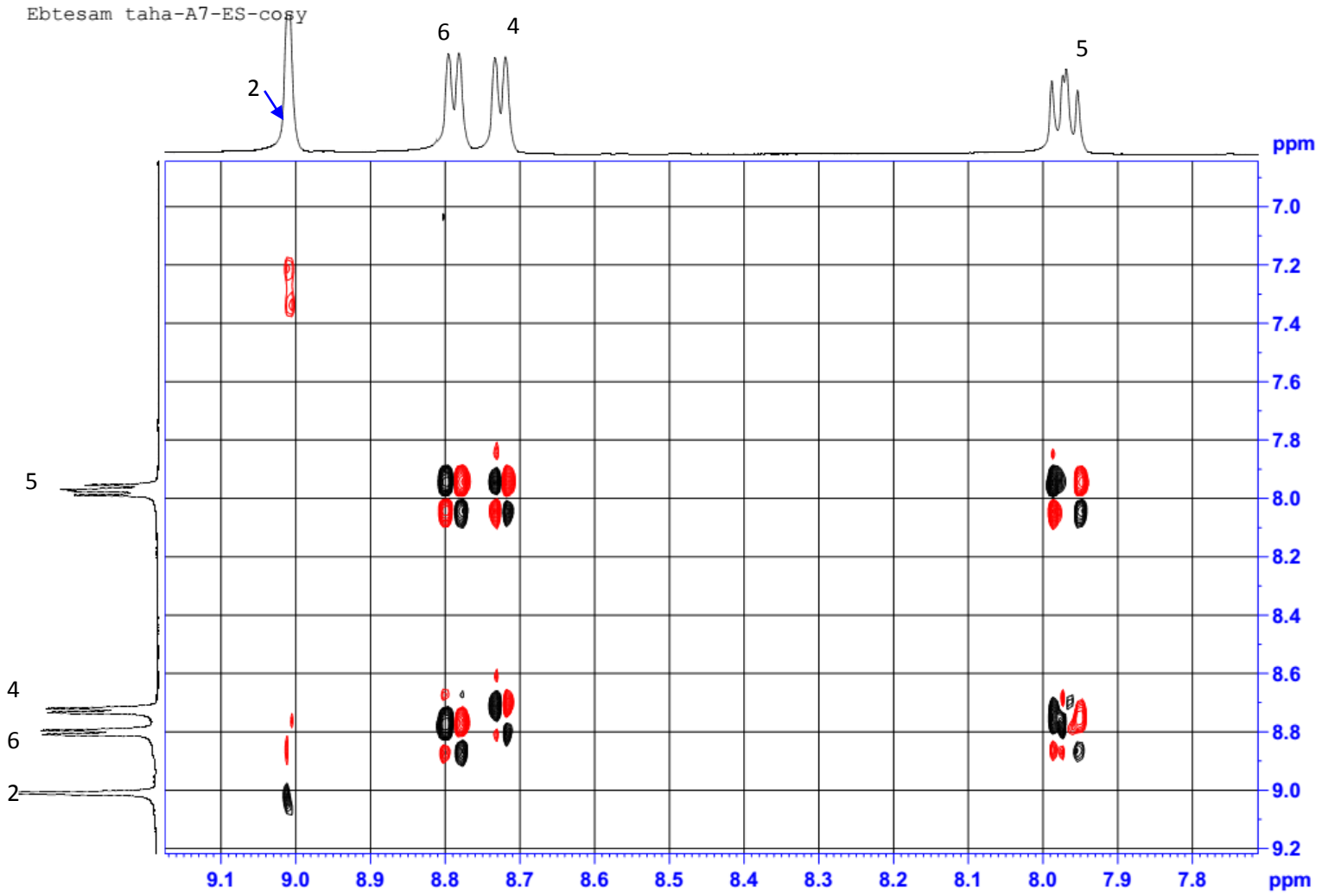



**Fig. S35:** COSY spectrum of **5** ($D_2O$).

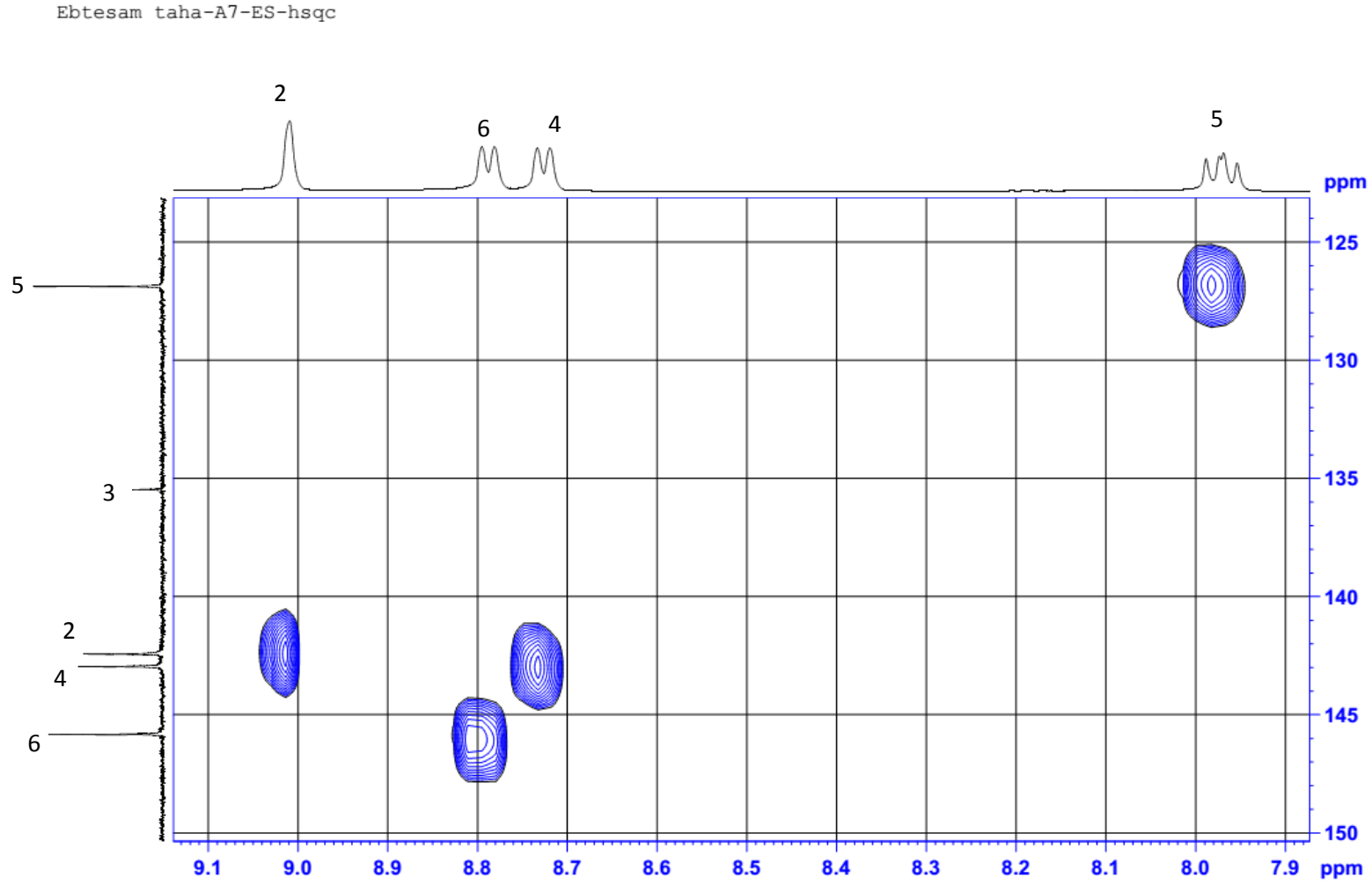



**Fig. S36:** HSQC spectrum of **5** ($D_2O$).

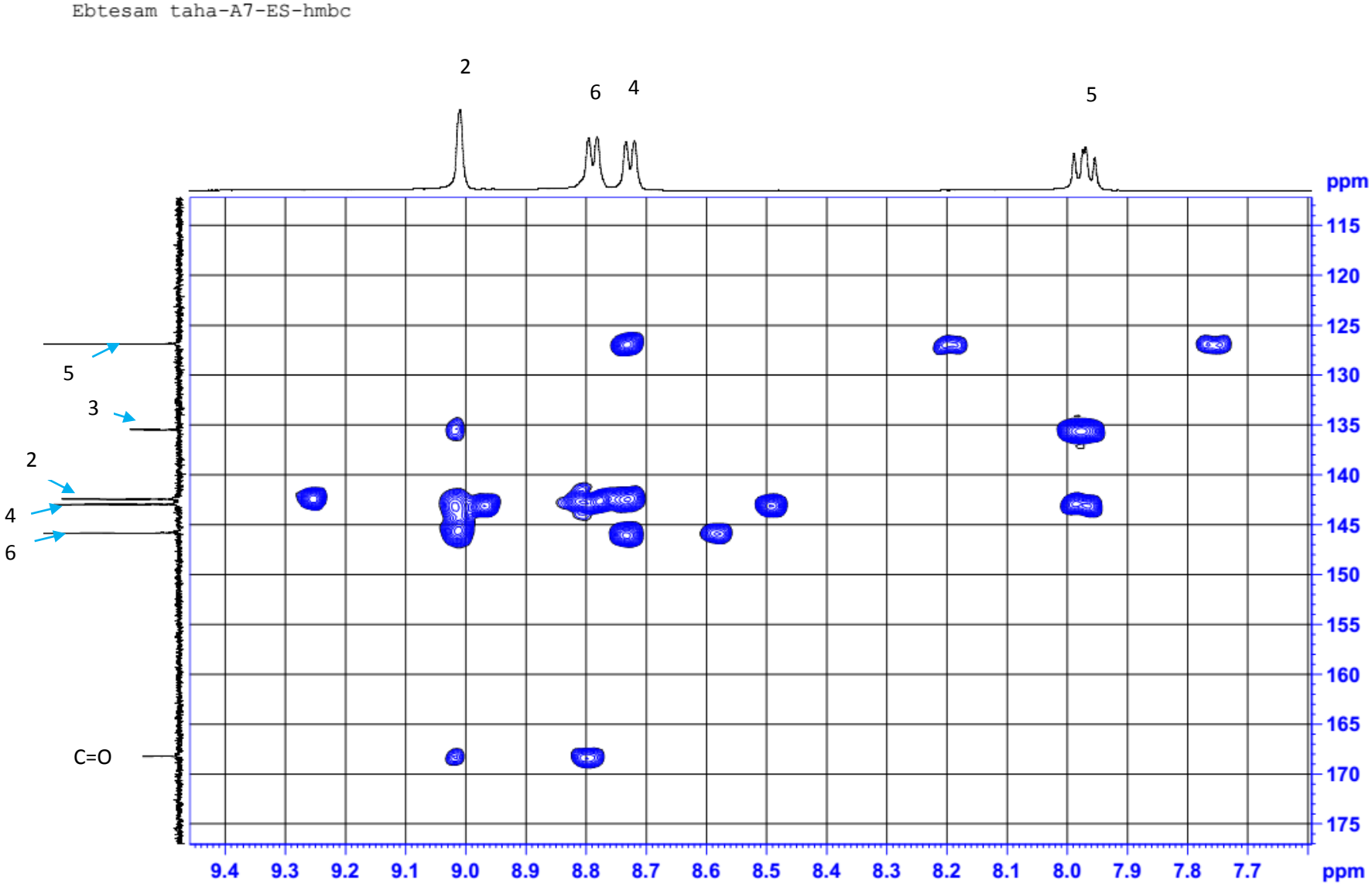



**Fig. S37:** HMBC spectrum of **5** ($D_2O$).

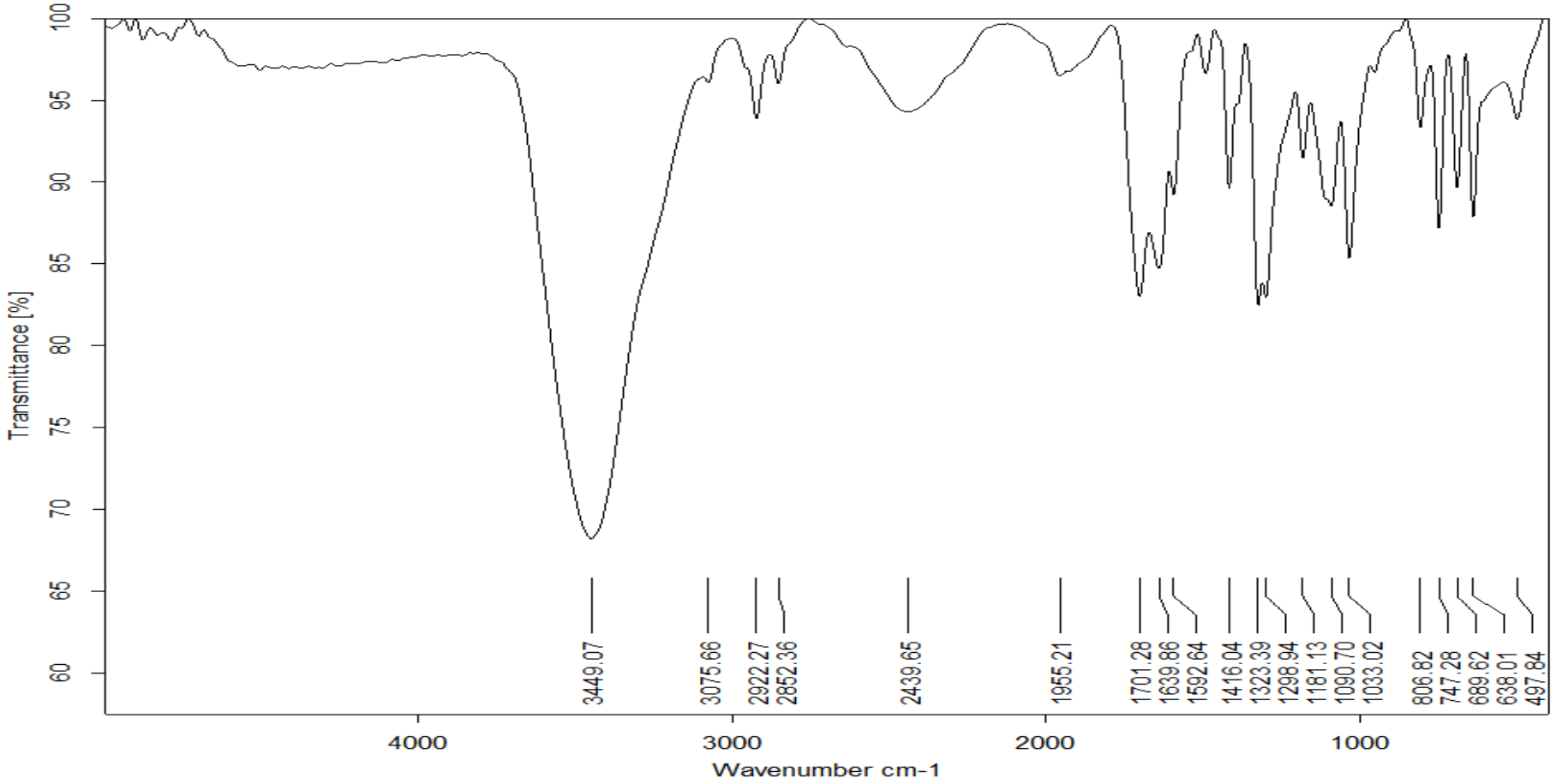



**Fig. S38:** IR spectrum of **5** (KBr disc).

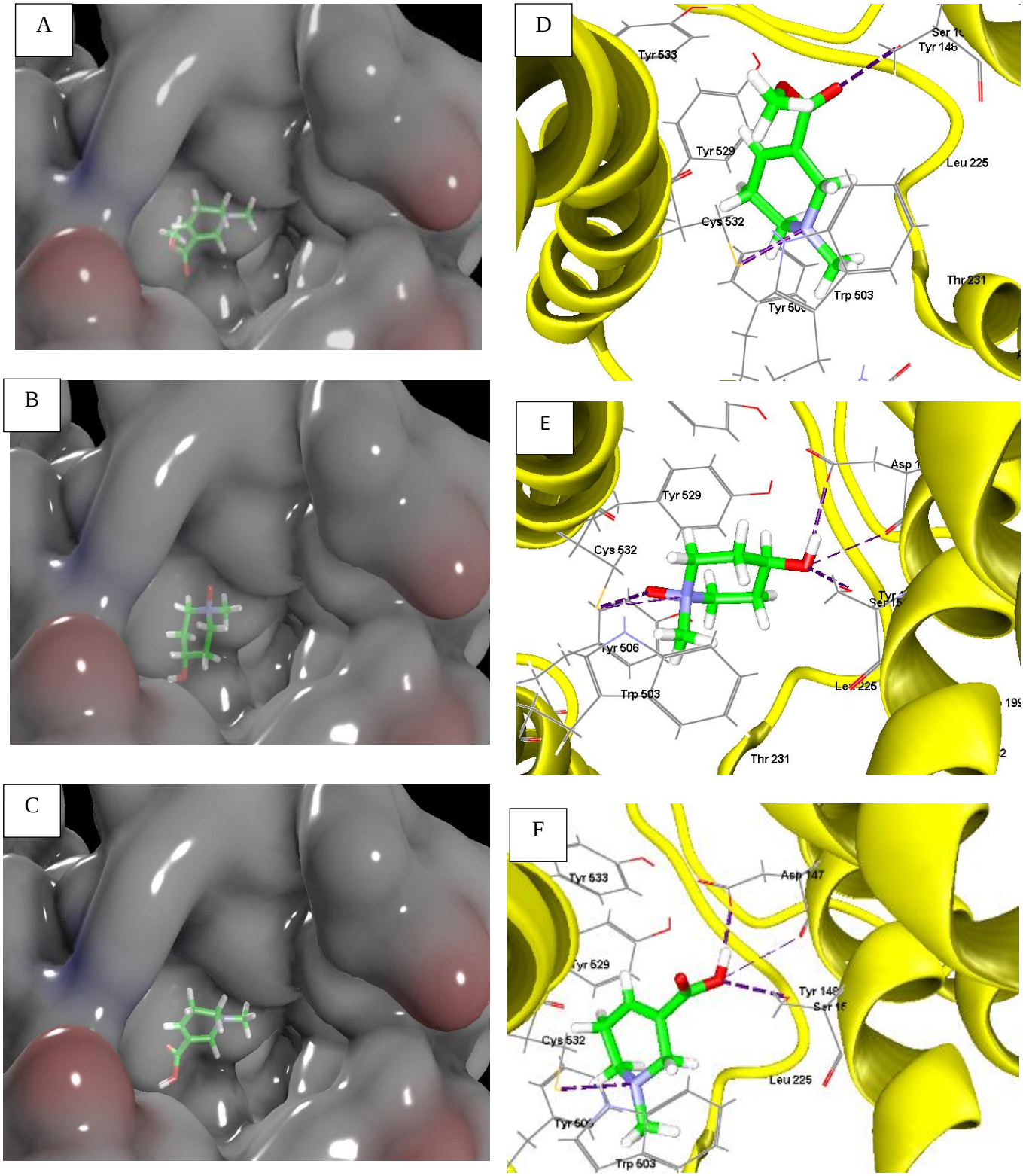



**Fig. S39:** The binding mode of **arecoline**, **2** and **4**. Electrostatic surface representation of ligand binding pocket of **arecoline** (A), **2** (B) and **4** (C). Amino acid residues involved in ligand interaction, hydrogen bonds are shown as blue dashed lines, **arecoline** (D), **2** (E) and **4** (F).

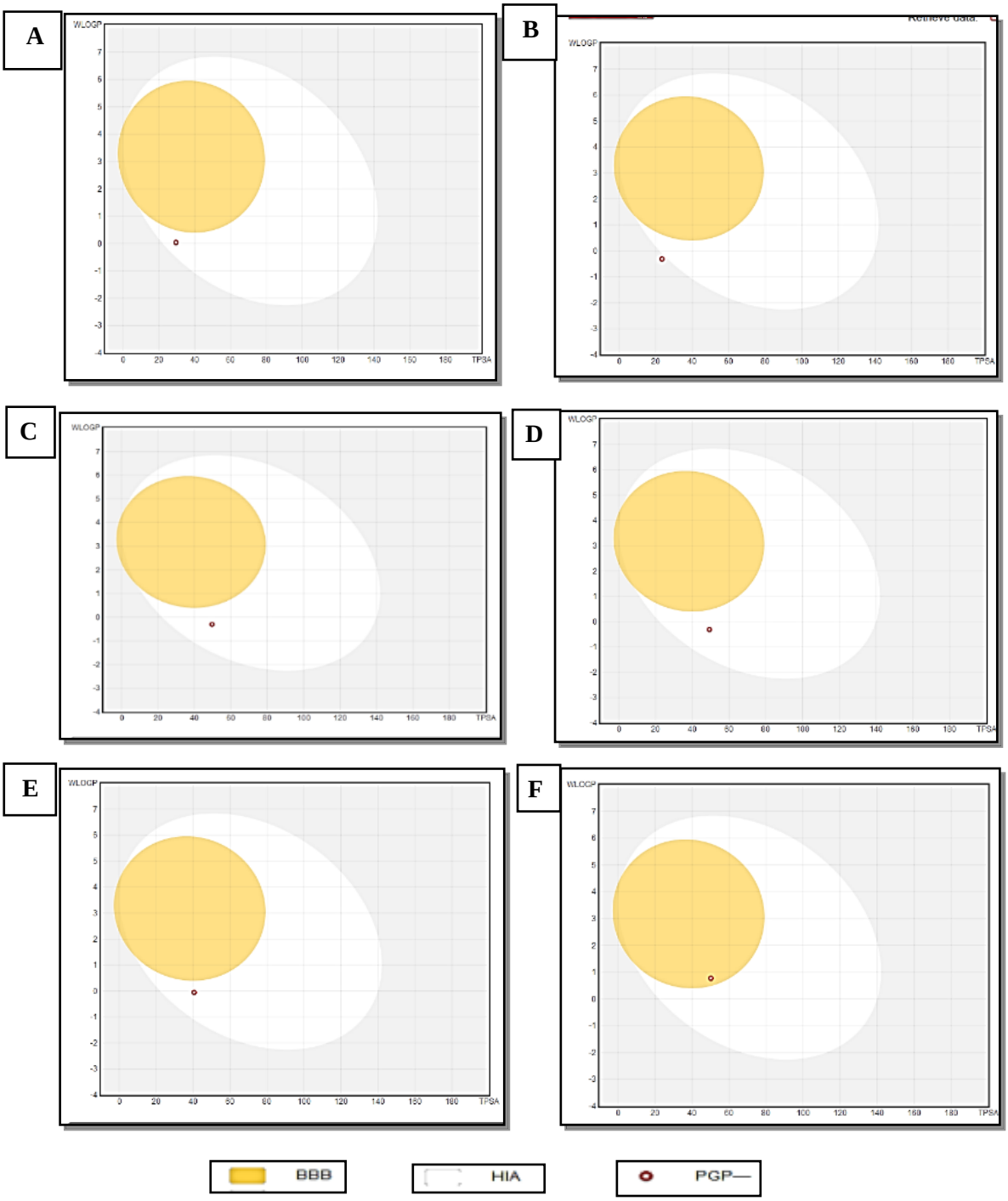



**Fig. S40:** SwissADME prediction, BOILED egg showing blood brain barrier permeability (yellow region) and human intestinal absorption (white region). Red dots represented molecules predicted not to be effluated from central nervous system by P-glycoprotein. **Arecoline** (A), **1** (B)**, 2** (C), **3** (D), **4** (E) and **5** (F).

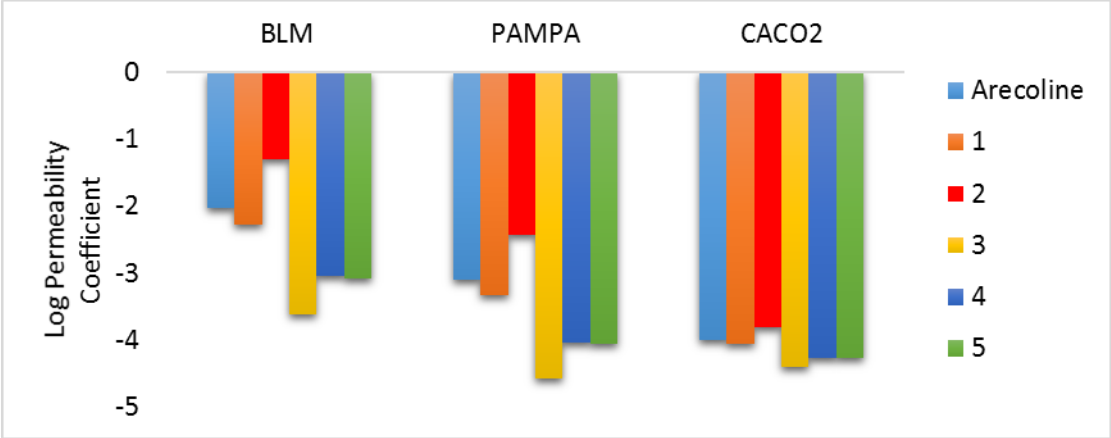



**Fig. S41:** Cell permeability prediction of arecoline and compounds **1-5** from PerMM web-based tool.

**Table S1: A:** Effect of **1** on the cell cycle of non-small cell lung cancer A549 using untreated lung cancer cells as a negative control. **B:** Effect of **3** and **5** on cell cycle of leukemia cancer K562 using untreated leukemia cancer cells as a negative control.

| Tested compounds | Cell cycle distribution (DNA content) | | | |
|---|---|---|---|---|
| | **%Pre-G1** | **%G0-G1** | **%S** | **%G2/M** |
| **A) Lung cancer A549 cell line** | | | | |
| **1** | 28.61 | 44.11 | 31.01 | 24.88 |
| Untreated lung A549 (**Control**) | 1.26 | 56.06 | 35.85 | 8.09 |
| **(B) Leukemia cancer K562 cell line** | | | | |
| **3** | 43.22 | 35.29 | 57.23 | 7.48 |
| **5** | 31.74 | 36.41 | 52.91 | 10.68 |
| Untreated leukemia K562 (**Control**) | 1.68 | 44.84 | 42.57 | 12.59 |

**Table S2**: **A**: Detection of apoptosis induced by **1** using non-small cell lung cancer A549 and untreated cells as a negative control. **B**: Detection of apoptosis induced by **3** and **5** using leukemia cancer K562 and untreated cells as a negative control. Fold increase than control was included.

| Test compound | % DNA content | | | |
|---|---|---|---|---|
| | Total | Apoptosis | | Necrosis |
| | | Early | Late | |
| **(A) Lung cancer A549 cell line** | | | | |
| **1** | 28.61 | 5.09 | 10.38 | 13.14 |
| Untreated lung A549 (Control) | 1.26 | 0.29 | 0.18 | 0.79 |
| Fold increase by **1** | 22.71 | 17.55 | 57.67 | 16.63 |
| **(B) Leukemia cancer K562 cell line** | | | | |
| **3** | 43.22 | 2.33 | 30.35 | 10.54 |
| **5** | 31.74 | 2.94 | 11.74 | 17.06 |
| Untreated leukemia K562 (Control) | 1.68 | 0.37 | 0.04 | 1.27 |
| Fold increase by **3** | 25.73 | 6.30 | 758.75 | 8.30 |
| Fold increase by **5** | 18.89 | 7.95 | 293.5 | 13.43 |

**Table S3:** Docking results of arecoline and compounds **1-5** to M3-mT4L (PDB ID: 4U15).

| Ligand | MolDock Score (kcal/mol) | Rerank Score (kcal/mol) | Interaction (kcal/mol) | H. Bond (kcal/mol) | LE1[a] | LE3 |
|---|---|---|---|---|---|---|
| Arecoline | -81.15 | -67.15 | -85.54 | -3.50 | -7.38 | -6.10 |
| **1** | -62.19 | -54.32 | -66.54 | -4.59 | -7.77 | -6.79 |
| **2** | -73.43 | -61.62 | -77.08 | -9.15 | -8.16 | -6.85 |
| **3** | -66.20 | -57.88 | -71.20 | -2.29 | -7.35 | -6.43 |
| **4** | -75.37 | -63.87 | -79.63 | -4.91 | -7.54 | -6.39 |
| **5** | -65.18 | -51.62 | -71.77 | -5.86 | -7.24 | -5.74 |

[a] LE: Ligand efficiency

**Table S4:** Amino acids involved in hydrogen bonding interaction of arecoline and compounds **1-5** with M3-mAChR.

| Ligand name | Amino acids involved in hydrogen bonding |
|---|---|
| Arecoline | Ser 151, Cys 532 |
| **1** | Ser 151, Asp 147 |
| **2** | Ser 151, Cys 532, Asp 147 |
| **3** | Ser 151, Tyr 529, Asp 147 |
| **4** | Ser 151, Cys 532, Asp 147 |
| **5** | Ser 151, Cys 532, Asp 147 |

**Table S5:** Physicochemical properties of arecoline and compounds **1-5** for detection of drug likeness and oral bioavailability.

| Tested compound | Size (M.W)[a] | Flexibility (number of rotatable bonds) | Hydrogen bond donor | Hydrogen bond acceptor | Polarity (TPSA)[b] | Partition coefficient WLogp |
|---|---|---|---|---|---|---|
| **Arecoline** | 155.19 | 2 | 0 | 2 | 29.54 | 0.04 |
| **1** | 115.17 | 0 | 1 | 2 | 23.47 | -0.31 |
| **2** | 131.13 | 1 | 1 | 2 | 49.66 | -0.30 |
| **3** | 129.16 | 1 | 2 | 3 | 49.33 | -0.31 |
| **4** | 141.17 | 1 | 1 | 3 | 40.54 | -0.05 |
| **5** | 123.11 | 1 | 1 | 3 | 50.19 | 0.78 |

[a]M.W: Molecular weight. [b]TPSA: Topological polar surface area.

**Table S6:** Results of ADME prediction of arecoline and compounds **1-5** from PreADMET web-based tool.

| Tested compound | ADME prediction | |
|---|---|---|
| | Absorption | Distribution |
| | Caco-2[a] cell permeability (nm/sec) | %PPB[b] |
| Arecoline | 26.33 | 8.14 |
| **1** | 37.30 | 0 |
| **2** | 6.71 | 14.13 |
| **3** | 20.74 | 0 |
| **4** | 21.02 | 91.95 |
| **5** | 20.75 | 4.51 |

[a]Caco-2: Colorectal carcinoma.

[b][o]%PPB: Plasma protein binding.

**Table S7:** Cell permeability prediction of arecoline and compounds **1-5** using PerMM web-based tool.

| Log of permeability Coefficient (kcal/mol) | **Arecoline** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|---|
| BLM [a] | -2.02 | -2.27 | -1.29 | -3.61 | -3.04 | -3.07 |
| PAMPA[b] | -3.09 | -3.33 | -2.42 | -4.56 | -4.03 | -4.06 |
| Caco-2[c] | -4.00 | -4.06 | -3.81 | -4.4 | -4.26 | -4.27 |

[a] BLM: Black liquid membrane.

[b] PAMPA: Parallel artificial membrane permeability assay.

[c] Caco-2: Colorectal carcinoma cell.

**Table S8:** List of microorganisms.

| | Microorganism | Code | Family |
|---|---|---|---|
| 1 | *Alternaria alternata* | AUMC 4685 | Pleosporaceae |
| 2 | *Aspergillus flavipes* | ATCC 11013 | Trichocomaceae |
| 3 | *A. ochraceous* | NRRL 405 | Trichocomaceae |
| 4 | *A. flavus* | AUMC 4787 | Trichocomaceae |
| 5 | *A. fumigatus* | ** | Trichocomaceae |
| 6 | *A. niger* | ATCC 10549 | Trichocomaceae |
| 7 | *A. niger* | AUMC 4156 | Trichocomaceae |
| 8 | *A. niger* | NRRL 328 | Trichocomaceae |
| 9 | *A. versicolor* | AUMC 4807 | Trichocomaceae |
| 10 | *Candida albicans* (lab isolate) | * | Saccharomycetaceae |
| 11 | *Chaetomium funicolum* | ** | Chaetomiaceae |
| 12 | *Cladosporium species* | ** | Davidiellaceae |
| 13 | *Cunninghamella blakesleeana* | MR 198 | Cunninghamellaceae |
| 14 | *C. blakesleeana* | NRRL 1369 | Cunninghamellaceae |
| 15 | *C. echinulata* | NRRL 1382 | Cunninghamellaceae |
| 16 | *C. elegans* | NRRL 1392 | Cunninghamellaceae |
| 17 | *C. elegans* | NRRL 2310 | Cunninghamellaceae |
| 18 | *Dreshella species* | ** | Pleosporaceae |
| 19 | *Gymnascella citrina* | NRRL 6050 | Gymnoascaceae |
| 20 | *Lindra pennispora* | NRRL 2237 | Lulworthiaceae |
| 21 | *Mucor species* | ** | Mucoraceae |
| 22 | *Penicillium chrysogenium* | ATCC 10002 | Trichocomaceae |
| 23 | *P. chrysogenium* | ATCC 9480 | Trichocomaceae |
| 24 | *P. glabrum* | ** | Trichocomaceae |
| 25 | *P. brevicompactum* | AUMC 2751 | Trichocomaceae |
| 26 | *P. vermiculatum* | NRRL 1009 | Trichocomaceae |
| 27 | *Rhizopus somniferum* | ATCC 36060 | Mucoraceae |
| 28 | *Rhodotorula rubra* | NRRL y 1592 | Sporidiobolaceae |
| 29 | *Saccharomyces cerivisae (Baker's yeast)* | Lyophilized yeast cell | Saccharomycetaceae |
| 30 | *Trichoderma virdie* | ** | Hypocreaceae |
| 31 | *Ulocladium botrytis* | ** | Pleosporaceae |

* Laboratory isolate supplied through the Department of Microbiology, College of Pharmacy, King Saud University.

**Isolate obtained from Assiut University Mycological Center (AUMC).