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Case study of Paliperidone

Verify the structure of your products by combining 1D and 2D NMR experiments

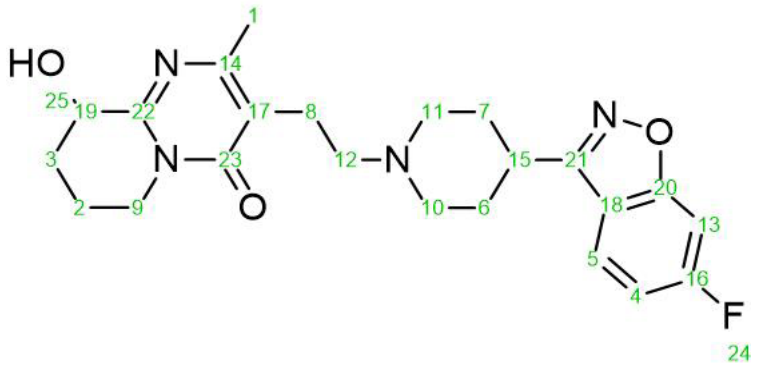

Figure 1: Chemical structure of Paliperidone ((3-(2-(4-(6-fluorobenzo[d]isoxazol-3-yl) piperidin-1-yl) ethyl)-9-hydroxy-2-methyl-6, 7, 8, 9-tetrahydro-4Hpyrido[1, 2- a]pyrimidin-4-one, $C_{23}H_{27}FN_4O_3$, MW 426.484 g mol^{-1}).

Paliperidone is the essential metabolite of risperidone, an atypical antipsychotic employed for the treatments of schizophrenia or bipolar disorders. Its chemical structure consists of a benzisoxazole moiety and it is less lipophilic than risperidone. Being an example for atypical antipsychotics, it acts more as a serotonin-receptor blocker than a dopamine-receptor one. The interested reader is referred to a review by Dolder et al. that describes the application of Paliperidone for the treatment of schizophrenia [1]. To perform pharmacokinetic and dynamic studies within clinical tests for the development of novel medical scaffolds, a complete structure verification of the target molecule is indispensable. Within this case study, a full peak assignment for structure verification of Paliperidone is performed utilizing a Spinsolve Carbon 80 MHz benchtop NMR system.

For a full and unambiguous assignment of the NMR signals to the according atoms of the molecule, a combination of 1D and 2D NMR techniques is crucial. The goal of this application note is to illustrate the steps required to perform the assignments and show how this can be used to verify the structure of a molecule.

NMR experiments

The Paliperidone samples were prepared in $CDCl_3$ with a concentration of 70 and 270 mM, respectively. ^1H and ^{13}C spectra were collected on a Spinsolve Carbon 80 MHz spectrometer with a ^1H frequency of 80.40 MHz and a ^{13}C frequency of 20.22 MHz. Table 1 lists all the experiments and parameters used to assign the NMR signals to the according atoms.

Table 1: NMR experiments and parameters used to assign the NMR signals of Paliperidone.

Protocol	Scans	NP in t_2	NP in t_1	Acquisition time (s)	Repetition time (s)	Total time (min.)
Proton+	1	32,768	...	6.4	10	0.18
Carbon+	2048	8,192	...	1.6	5	171
HSQC-ME	4	2,048	128	...	2	34.6
HMBC	8	1,024	128	...	2	34.5
Fluorine+	128	32,768	...	1.6	3	6.41
Fluorine HDEC	128	8,192	...	1.6	3	6.41

^1H Spectrum

Figure 2 shows the ^1H NMR spectrum of Paliperidone acquired in a single scan on the Spinsolve Carbon 80 MHz NMR system. To reference the chemical shift scale, TMS was added to the sample and its signal was set to 0.00 ppm. Thereafter, the chemical shifts of the proton peaks were assigned (blue shifts on top). After integrating the different peaks (violet areas at the bottom of the spectrum), the spectrum is ready for a first inspection.

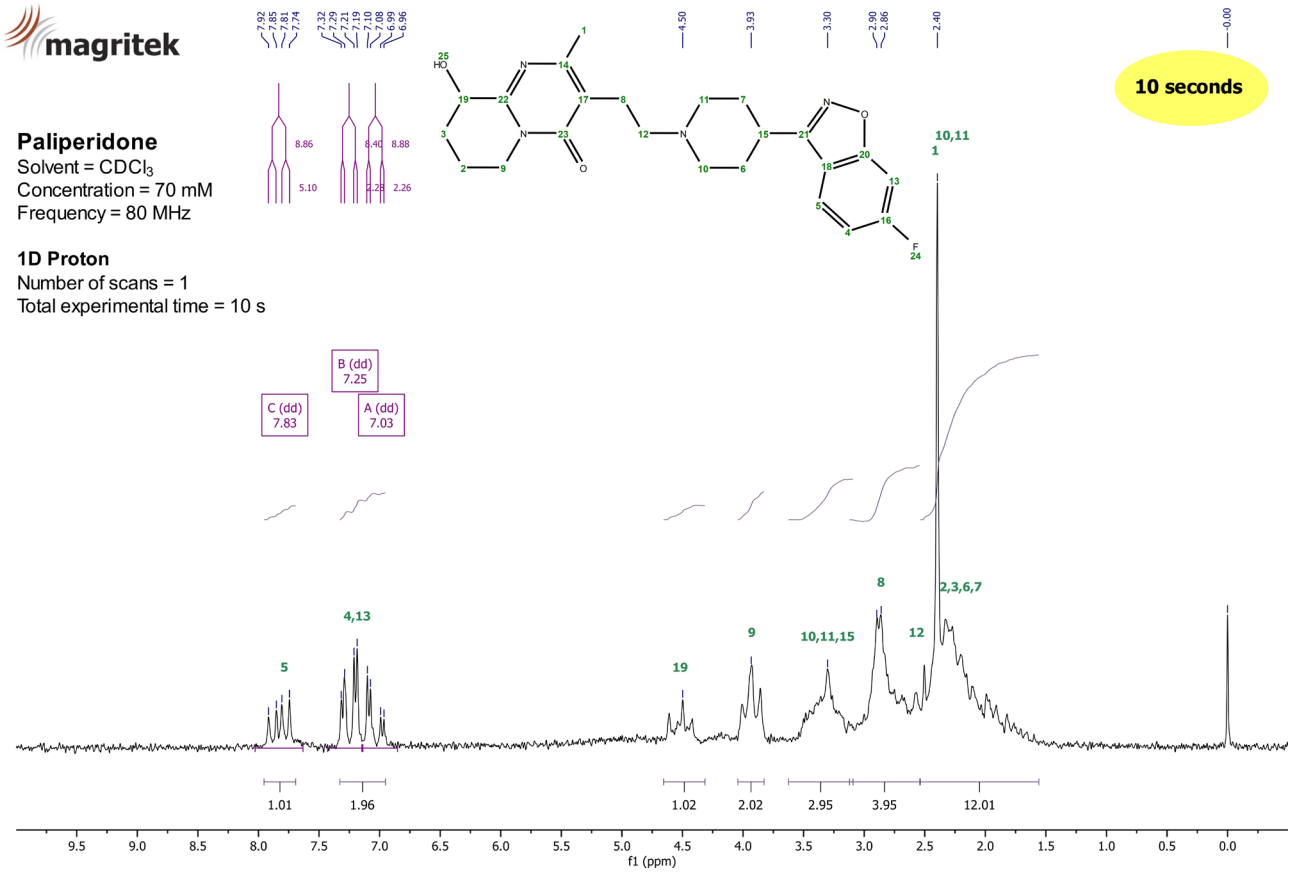

Figure 2: ^1H NMR spectrum of Paliperidone (70 mM) in $CDCl_3$.

In the aromatic region of the spectrum between 6 and 10 ppm, two sets of signals are visible. One appears at about 7.83 ppm and the other is situated around 7.19 ppm. Due to the integral values of 1.01 and 1.96, these two sets of signals belong to three protons in total. According to the structure, those three protons can only belong to the assigned protons at the positions **4**, **5**, and **13**. To assign these signals even more accurately, the splitting pattern as well as the coupling constants of the NMR peaks need to be taken into account. A characteristic splitting pattern should be detectable for the ^{1}H signals of the aromatic protons bearing a ^{19}F nucleus in its close proximity. In theory according to the n+1 rule, a doublet of doublets (dd) as splitting pattern is expected for protons **5** and **13** whereas a doublet of doublets of doublets (ddd) should appear for proton **4**. Since the ^{19}F nucleus has also a spin of ½, heteronuclear couplings between ^{1}H and ^{19}F are detectable.

^{13}C spectrum

Figure 3 shows the ^{13}C NMR spectrum of Paliperidone.

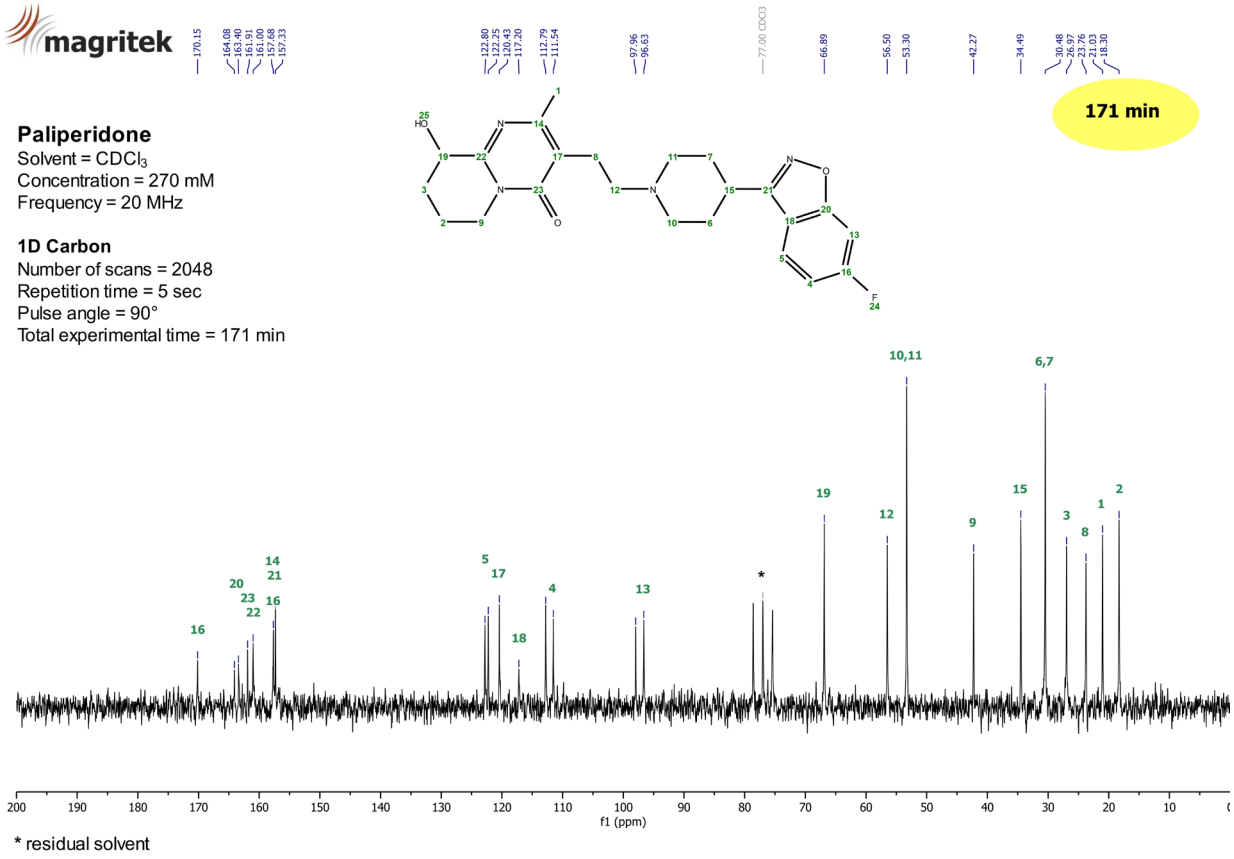

Figure 3: ^{13}C NMR spectrum of Paliperidone (270 mM) in $CDCl_3$.

According to the chemical structure of Paliperidone, 23 peaks within the ^{13}C spectrum are expected if every carbon species would be treated as chemically not equivalent. Due to a C2-symmetry at the piperidine moiety of the molecule, the carbons at the positions **6** and **7** and the ones at **10** and **11** will cause single peaks for each pair, respectively. Since each of these signals consists of two carbon moieties coupled to the protons attached to them, their signals in the ^{13}C spectrum are expected to have the highest intensities. Therefore, the peaks at 30.48 and 53.30 ppm can be assignment of those positions. Due to the close proximity to the nitrogen atom, the peak at 53.30 ppm must be assigned to the carbons **10** and **11** whereas the peak at 30.48 ppm then belongs to the carbons **6** and **7**.

Due to the afore mentioned symmetry, we would expect 21 carbon signals within the spectrum instead of 23, but the ^{13}C spectrum displayed 25 peaks. The reason for this is the fluorine atom in position 24 which causes a splitting of the carbon signals sitting in its close proximity. Consequently, we expect five more signals since the fluorine moiety is interacting with the carbons **4**, **5**, **13**, **16** and **20** creating a doublet peak for each carbon mentioned. From the inspection, 25 signals can be found, meaning that only one is potentially overlapping. Further assignments of the carbon signals at such an early stage of process are not possible. The 2D spectra of this case study will provide the missing information.

HSQC-ME

Figure 4 shows the HSQC-ME (multiplicity edited) spectrum of Paliperidone. The HSQC-ME protocol is a multiplicity edited version of the HSQC NMR. The HSQC-ME experiment provides multiplicity information, i.e. how many hydrogen atoms are attached to each carbon atom. In an HSQC-ME spectrum signals from CH and CH_3 groups are positive (coloured red), while signals from CH_2 groups are negative (coloured blue). HSQC-ME therefore provides the same multiplicity information as the DEPT-135 experiment, but an HSQC-ME spectrum can usually be obtained in significantly less time than a DEPT, making running a DEPT unnecessary in most cases.

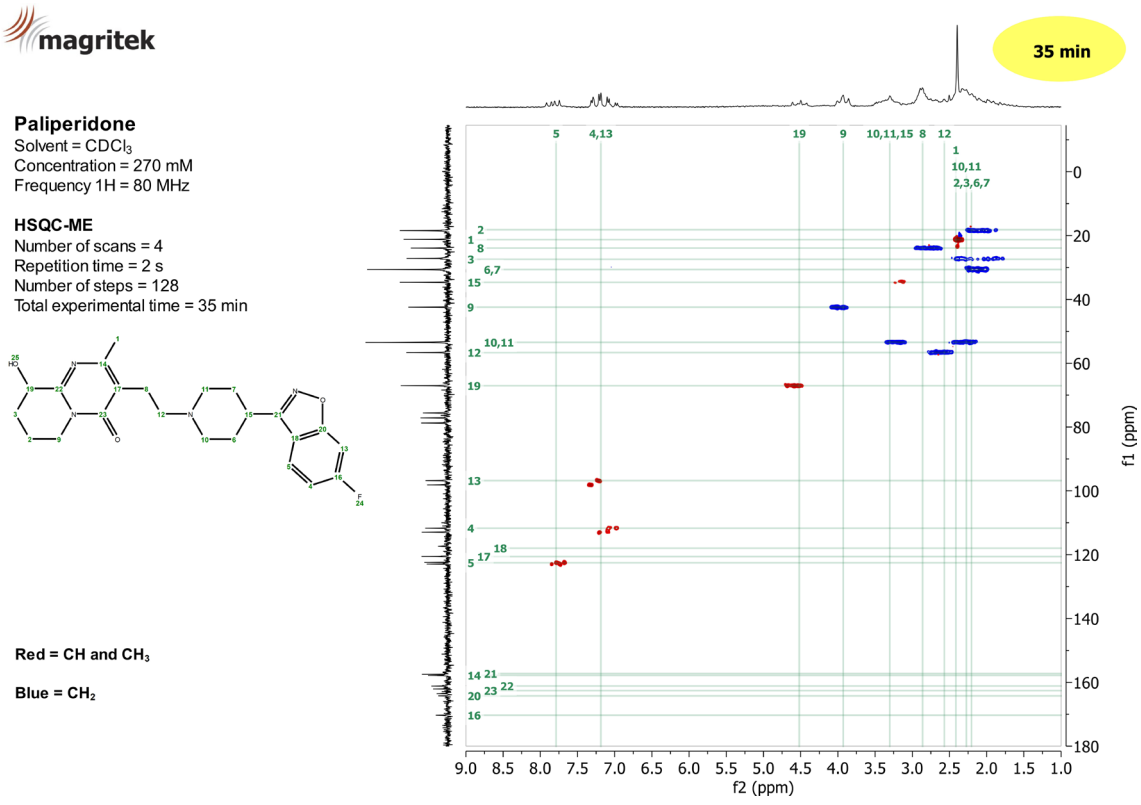

Figure 4: HSQC-ME NMR spectrum of Paliperidone (270 mM) in $CDCl_3$.

The two-dimensional spectrum displays the ^{1}H chemical shifts along the direct (f_2) dimension and the ^{13}C chemical shifts along the indirect (f_1) dimension. Cross-peaks in the spectrum confirm which ^{13}C and ^{1}H are coupled via a one-bond scalar coupling. Therefore, quaternary carbon peaks with no directly attached protons in the molecule do not give any cross-peaks within the HSQC-ME. So, the carbon peaks at about 160 ppm in the f_1 dimension display quaternary carbon atoms without detectable cross-peaks. These peaks will be highlighted in the HMBC section of this case study.

The first region of the HSQC-ME assignment is the aromatic region at about 7 ppm in the proton spectrum and 100 ppm within the carbon dimension. Figure 5 shows a zoom of the aromatic region of the HSQC-ME, where we can identify three group of signals that need to be assigned to protons 4, 5, and 13. All three aromatic protons correlate with a doublets along the carbon dimension. The splitting of the carbon signals is caused by the coupling of the different carbons with the fluorine atom. The signal at 7.83 ppm with the integral of 1.01 is displayed as a doublet of doublets (dd) with coupling constants of 8.86 Hz and 5.10 Hz. Therefore, this proton has two chemically not equivalent "neighbours" in its spin system with two different coupling constant values. This proton correlates with the carbon peaks at 122.25 and 122.80 ppm with a $J_{C,F}$ coupling of 11.0 Hz. The proton signal at 7.25 ppm has also a doublet of doublets (dd) structure with coupling constants of about 8.40 and 2.28 Hz and couples to the carbon peaks at 96.63 and 97.96 ppm with a $J_{C,F}$ coupling of 26.6 Hz. At about 7.03 ppm a multiplet with a splitting pattern of a doublet of doublets of doublets (ddd) that overlaps with the multiplet at 7.25 ppm along the proton dimension, but gets fully visible in the HSQC-ME. Its coupling constants are 2.26 and 8.88 Hz, respectively. This Proton correlates with the carbon peaks at 111.54 and 112.79 ppm with a $J_{C,F}$ coupling of 25.4 Hz.

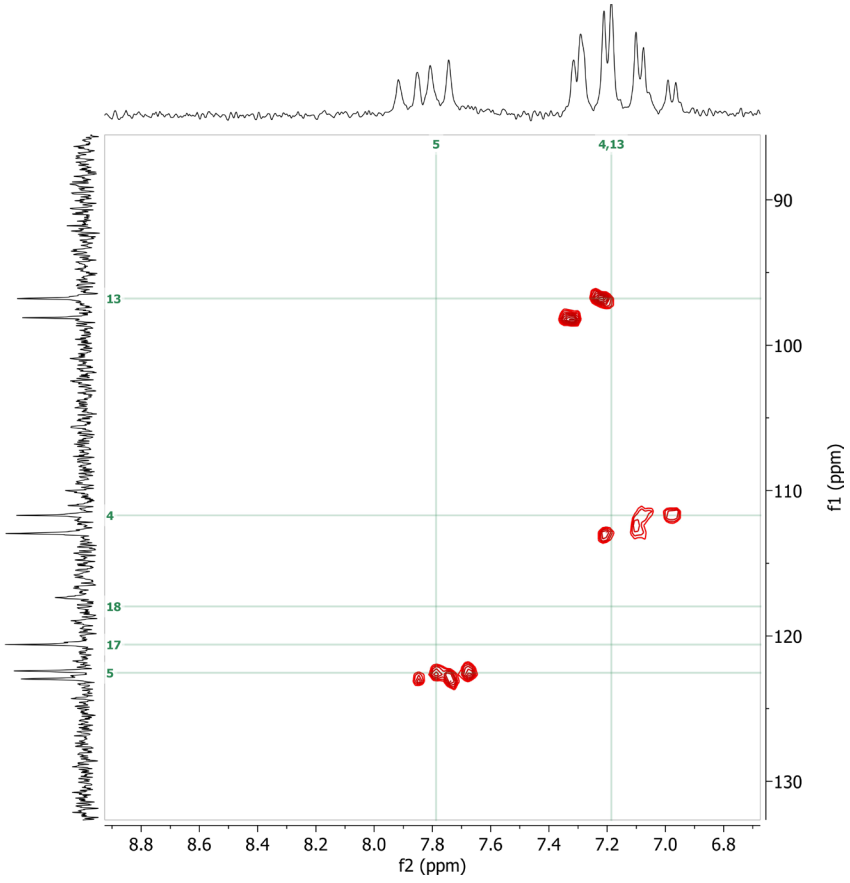

Figure 5: Zoom of the aromatic region of the HSQC-ME NMR spectrum of Paliperidone (270 mM) in $CDCl_3$.

From the size of the $J_{C,F}$ splitting of the carbon signals we can conclude that the proton at 7.85 must be proton 5 (smallest coupling corresponding to a $^3J_{C,F}$). The other two carbons show the same splitting constants (both $^2J_{C,F}$), but the signal at 7.03 can be assigned to proton 4, as this is the only one that is coupled to three different chemical groups to justify the ddd structure. In summary, proton 5 couples to proton 4 with a ^3J constant of 8.86 Hz and to the fluorine atom with a ^4J coupling constant of 5.10 Hz. Proton **13** couples with proton 4 with a ^4J constant of about 2.28 Hz and to the fluorine atom with a ^3J proton-fluorine coupling of 8.40 Hz. Proton 4 shows three coupling constants of 8.88 Hz, 2.28 Hz and 8.40 Hz, confirming all couplings observed on proton 4 and 13. The as the proton-fluorine constant of 8.40 Hz is similar to the proton-proton coupling with proton 5, the structure looks at first sight as a doublet of triplets, but is in reality a ddd multiplet.

For the assignments of the aliphatic signals, 1D and 2D NMR measurements were necessary since there is no clear coupling pattern visible due to a large number of protons in this area between 1 and 5 ppm in the proton spectrum (Fig. 2). Nevertheless, the signal at 4.50 ppm with an integral value of 1.12 could be assigned provisory to proton **19**. The direct electron withdrawing effect of the hydroxyl group **25** (inductive effect -I) could cause the large chemical shift and separate this signal significantly from the rest of the aliphatic protons. This assumption has to be validated with an additional 2D measurement.
The next cross-peak under investigation is the red signal at 66.89 ppm in the carbon dimension. This cross-peak is correlating with the proton previously assigned as number **19**. Since the cross-peak is displayed in red it must be a CH or a CH_3 group, the assignment for proton **19** at 4.50 ppm with an integral value of 1.12 in the proton dimension is verified and correct. Consequently, the carbon peak at 66.89 ppm is the CH carbon in position **19**, which is directly attached to the hydroxyl group causing the largest chemical shift in the aliphatic region.
Regarding the chemical structure of Paliperidone, the only CH and CH_3 groups left that were not assigned yet are the positions **1** and **15**. Position **1** which displays the methyl group can be easily assigned to the large singlet in the proton spectrum at 2.40 ppm (see Fig. 2). Again, the HSQC-ME verifies this assignment with a red cross-peak. The correlating carbon peak appears at 21.03 ppm. That leaves one red cross-peak corresponding to proton 15 with a chemical shift of 3.30 ppm in the proton dimension and 34.49 ppm in the carbon counterpart.
Another assignment that can be made at this stage of the structure verification is for the protons at 3.93 ppm with an integral of 1.82. Due to the large chemical shift in the proton dimension, this signal can be assigned to the protons **9**. The cross-peak within the HSQC-ME is displayed in blue which supports the assignment of this group to a CH_2 moiety. The carbon bearing these two protons is directly connected to the endocyclic nitrogen of the amide functionality which is the more electrophilic nitrogen species compared to the piperidine nitrogen. Therefore, the carbon peak at 42.27 ppm can be assigned to the position **9** of Paliperidone. The last position that can be assigned so far from the already measured spectra is the blue cross-peak with the largest chemical shift in the carbon dimension. This cross-peak appears at 56.50 ppm and displays a CH_2 moiety according to the HSQC-ME. The only CH_2 moiety that can be shifted so far "downfield" is the one that belongs to the exocyclic carbon atom adjacent to the piperidine nitrogen in position **12**. The unassigned CH_2 groups at positions **2**, **3**, and **8** are all surrounded by other carbon atoms which results in way lower chemical shifts, compared to position **12**. Its chemical shift in the f_2 dimension is 2.49 ppm. These assignments will finally be verified by the following HMBC study. Both assignments (proton 9 and 12) can be confirmed in the next section where the HMBC data is analysed.
The assignments made in the carbon spectrum (Fig. 3) for the positions **6**, **7**, **10**, and **11** can also be verified by the HSQC-ME. Both cross-peaks detected are displayed in blue which correlates perfectly with the assumption of having symmetrical CH_2 groups causing these peaks.

HMBC

The HMBC (Heteronuclear Multiple Bond Correlation) protocol provides a two-dimensional correlation where the ^{1}H spectrum is shown along the direct (f_2) dimension and the ^{13}C spectrum along the indirect (f_1) dimension. Cross-peaks within the spectrum reveal which ^{13}C peak is correlated to which ^{1}H peak via multiple bond coupling. Especially for quaternary carbons such a correlation reveals their surroundings and assignments. Figure 6 shows the HMBC spectrum of Paliperidone.

The carbon directly attached to the fluorine atom at position **16** is the first under investigation. Since it is directly coupled to a fluorine, its signal should split into a doublet with a large $^{1}J_{C,F}$ coupling constant. In theory, the value for this coupling constant should be approximately 250 Hz. Moreover, due to the close proximity to the fluorine moiety the chemical shift for the carbon peak in position **16** should be by far the most downfield shifted signal of the spectrum. Indeed, a coupling constant of 250.19 Hz was measured between the carbon peaks at 170.15 and 157.68 ppm which displays a typical value for a $^{1}J_{C,F}$ coupling. Moreover, there are three cross-peaks visible in the aromatic region of the HMBC all correlating to the proton signal at 7.83 ppm in position **5**. Besides carbon **16**, there is another quaternary carbon at position **20** within the same spin-system which has a $^{3}J_{C,H}$ coupling with proton **5**. Since this carbon atom is also at a reasonable coupling distance to the fluorine species with a $^{3}J_{C,F}$ coupling, a splitting of the carbon peak for position **20** was expected in a range between 10 – 15 Hz like the splitting of the carbon peak **5** (11 Hz). Again, two carbon peaks with a typical coupling constant of 13.31 Hz for a $^{3}J_{C,F}$ coupling were found at 164.08 and 163.40 ppm, respectively. Consequently, these carbon peaks can be assigned to position **20**.

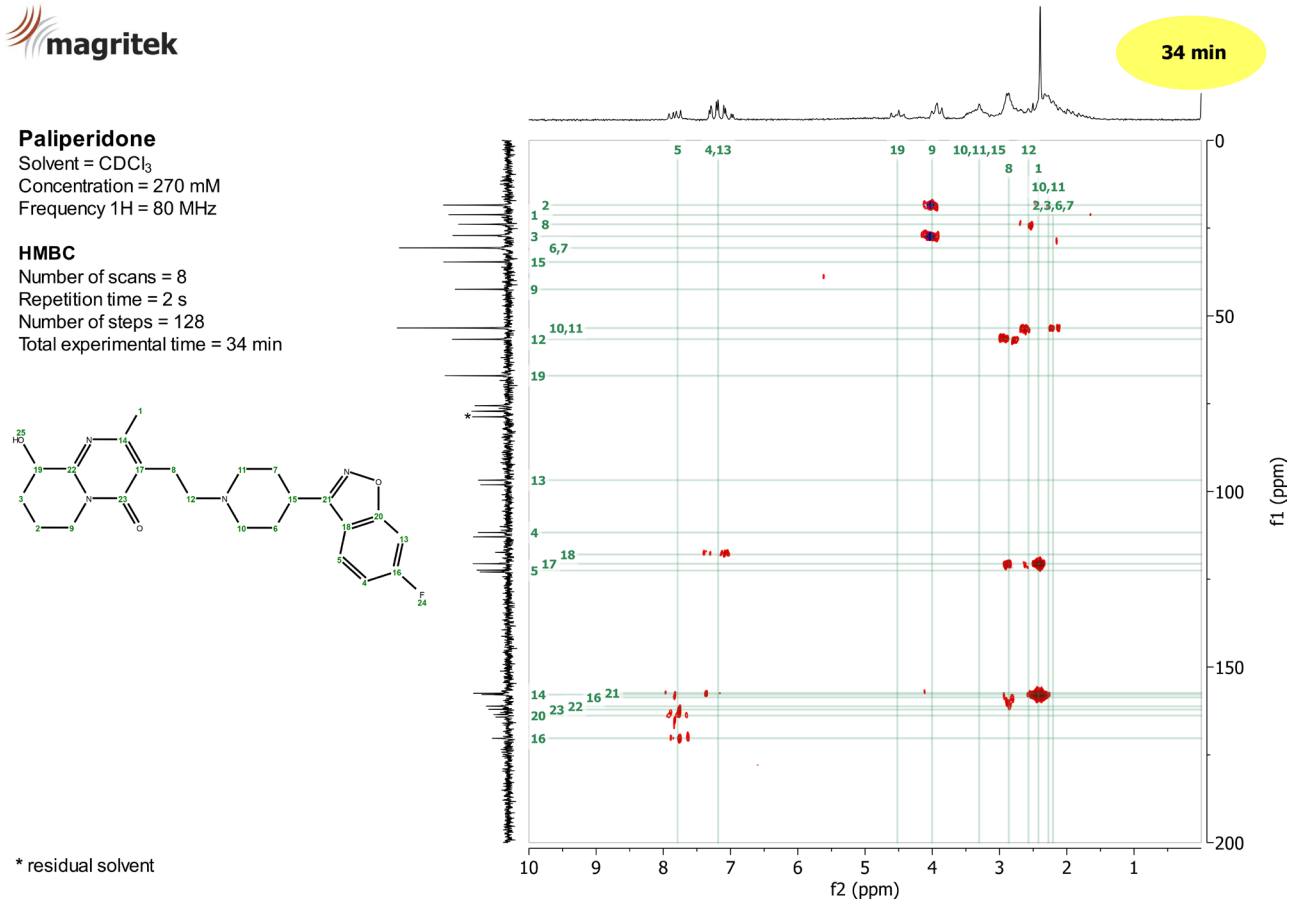

Figure 6: HMBC NMR spectrum of Paliperidone (270 mM) in $CDCl_3$.

Following this, additional cross-peaks for the correlation of the carbon at position **18** to the protons **4** and **13** are found as well. As for the carbon-proton correlations assigned before, two $^{3}J_{C,H}$ couplings between the protons in positions **4** and **13** and the carbon at position **18** should create cross-peaks within the HMBC. Only one cross-peak at 117.20 ppm with such a correlation is detectable. Consequently, the carbon atom creating this cross-peak can be assigned to position **18**.

Another strong coupling of the methyl group protons from position **1** with a quaternary carbon at 157.68 ppm is detectable within the HMBC spectrum. Moreover, a second cross-peak at this chemical shift is visible correlating to proton **8**. The only left carbon atom within a reasonable coupling range for such correlations is the quaternary carbon at position **14**. This carbon is assigned to the peak at 157.68 ppm.

The next carbon peak from the aromatic region with a chemical shift of 120.43 ppm that can be assigned undoubtedly, belongs to position **17**. The HMBC displayed three different cross-peaks for this carbon signal correlating to the already assigned proton signals at positions **1** and **12**, respectively. As before, both proton groups are in a $^{3}J_{C,H}$ coupling distance. The last cross-peak has a chemical shift of 2.90 ppm in f_2. The only signal with a chemical shift of 2.90 ppm in the proton dimension in the HSQC-ME spectrum in figure 4 is displayed as a blue signal. Therefore, this third cross-peak was created by the $^{2}J_{C,H}$ coupling in the HMBC of carbon **17** with the protons in position **8**. Since this moiety is a CH_2 group, a blue signal in the HSQC-ME spectrum was observed. Consequently, the carbon peak with the chemical shift of 23.76 ppm within the HSQC-ME correlating with these protons must be assigned to position **8**.

Two additional strong cross-peaks within the HMBC are visible for the correlation of protons **9** and the two carbon peaks at 18.30 and 26.97 ppm, respectively. The two carbon species left unassigned in close proximity to the protons **9** are the carbons **2** and **3**. Both carbon signals have strong cross-peaks to the two protons in position **9** due to $^{2}J_{C,H}$ and $^{3}J_{C,H}$ couplings. Due to the electron withdrawing hydroxy group in position **19**, the electron density in position **3** is lower than in position **2**. Therefore, the chemical shift for the carbon signal that belongs to position **3** must be shifted more downfield than the signal for position **2**. Consequently, the carbon peak at 26.97 ppm has to be assigned to position **3** whereas the peak at 18.30 ppm belongs to the position **2**.

The last quaternary carbons left that need to be assigned are the carbons at the positions **21**, **22**, and **23**. To facilitate the assignment, the HMBC spectrum was zoomed in the according region and is shown below.

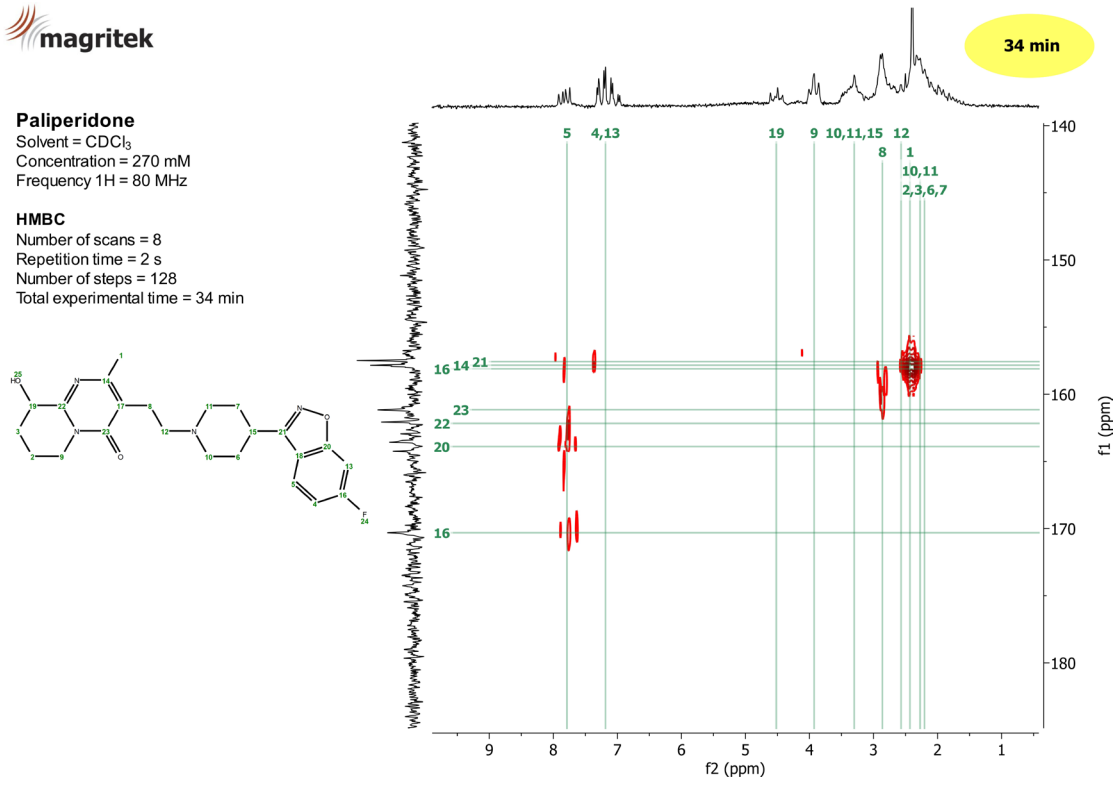

Figure 7: Zoomed HMBC NMR spectrum of Paliperidone (270 mM) in $CDCl_3$.

The first assignment was made for the position **23** which belongs to the carbon peak at the carbonyl moiety. Since only three quaternary carbons were left unassigned, the carbonyl peak usually belongs to the carbon signal which is shifted downfield the most in the carbon spectrum. In this example, carbon **22** is shifted slightly more downfield than **23** due to the two nitrogen atoms in its proximity instead of one (-I effect vs +M effect of the amide nitrogen at the carbonyl group). For the carbon signal at 161.00 ppm a cross-peak in the HMBC spectrum from figure 7 is detectable, which correlates with the proton signal at position 8. Only the carbonyl carbon is close enough for a detectable $^{3}J_{C,H}$ coupling within the HMBC. Therefore, the carbon peak at 161.00 ppm belongs to the carbonyl carbon in position **23**. Consequently, 161.91 ppm displays the chemical shift of carbon **22**. The carbon signal at 157.33 ppm displays two cross-peaks in the HMBC spectrum. One of the cross-peaks correlates to the proton in position **5** whereas the stronger one correlates with the aliphatic protons **6**, **7** and **10**, **11**. The only quaternary carbon atom correlating with both aromatic proton **5** and the aliphatic region of the piperidine scaffold is the carbon in position **21**. Both correlations are caused by $^{3}J_{C,H}$ and $^{4}J_{C,H}$ couplings. These assignments complete the structural verification of Paliperidone by 1D and 2D NMR spectroscopy.

^{19}F spectra

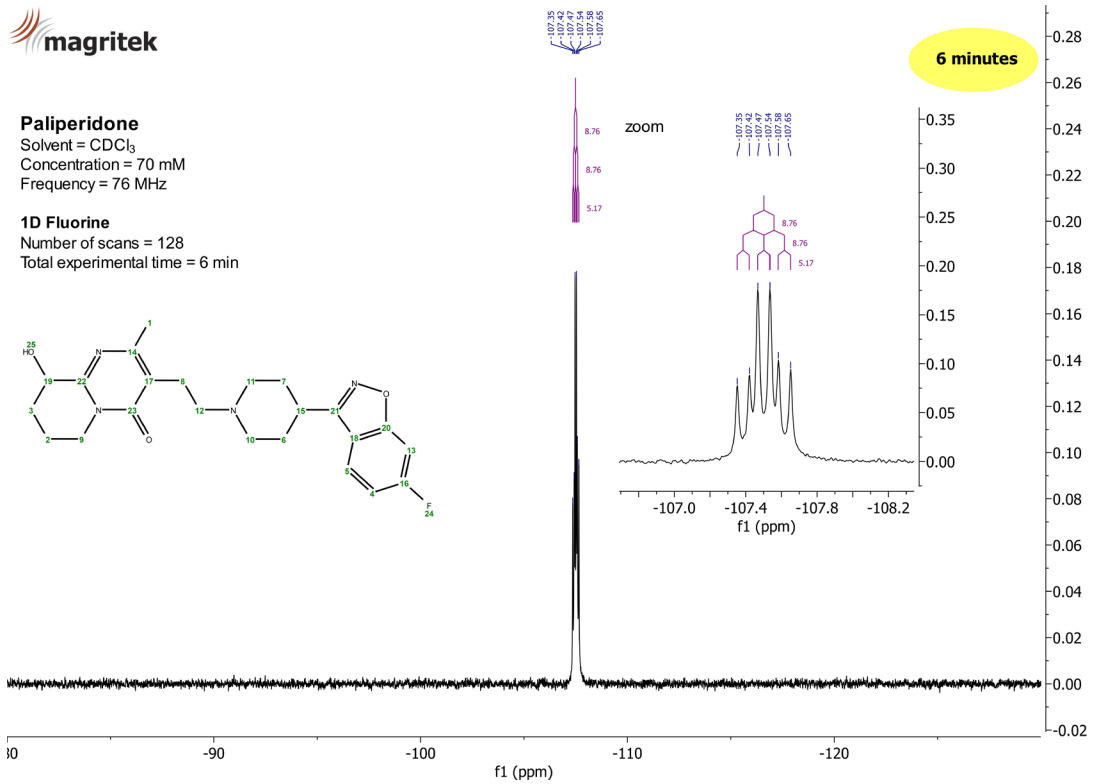

Figure 8: ^{19}F NMR spectrum of Paliperidone (70 mM) in $CDCl_3$.

Figure 8 shows the ^{19}F NMR spectrum of Paliperidone. It was recorded without ^{1}H decoupling to measure the ^{1}H and ^{19}F coupling constants. The multiplet structure of the fluorine peak at -107.54 ppm shows splitting values of 5.17 and 8.76 Hz, which are nearly identical to the values identified in the ^{1}H spectrum for the aromatic protons **4**, **5**, and **13** (fig. 2). The detected coupling pattern of the ^{19}F signal confirms the assignments for the aromatic protons **4, 5**, and **13** in the ^{1}H spectrum of Paliperidone. In contrast, figure 9 shows the ^{19}F spectrum of Paliperidone which was recorded with an activated ^{1}H decoupling protocol.

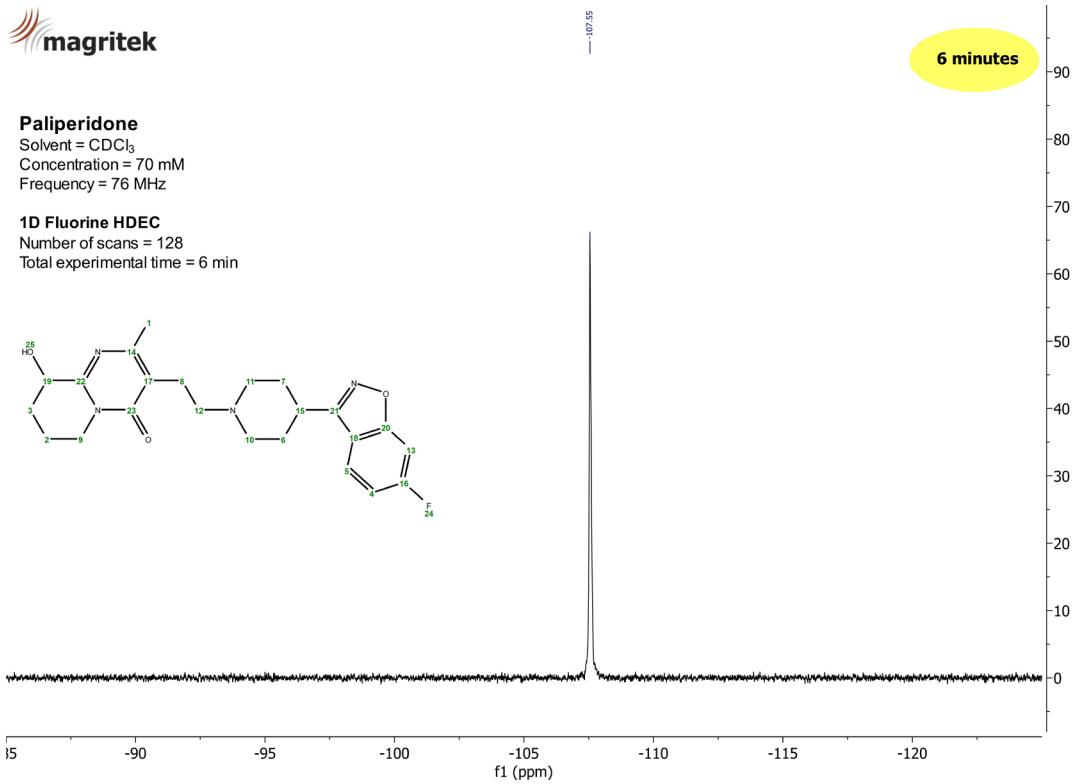

Figure 9: ^{19}F NMR spectrum of Paliperidone (70 mM) with activated ^{1}H decoupling in $CDCl_3$.

It has been shown how, through the application of 1D and 2D NMR methods, verification of the structure of paliperidone via the unambiguous assignment of its ^{1}H and ^{13}C peaks, can be performed using a Spinsolve 80 MHz spectrometer.

[1] C. Dolder, M. Nelson, Z. Deyo, Am. J. Health-Syst. Pharm., **2008**, 65, 403–413.