Supporting Information (SI)

Conformation of the Macrocyclic Drug Lorlatinib in Polar and Nonpolar Environments: A MD Simulation and NMR Study

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1. REMD simulations
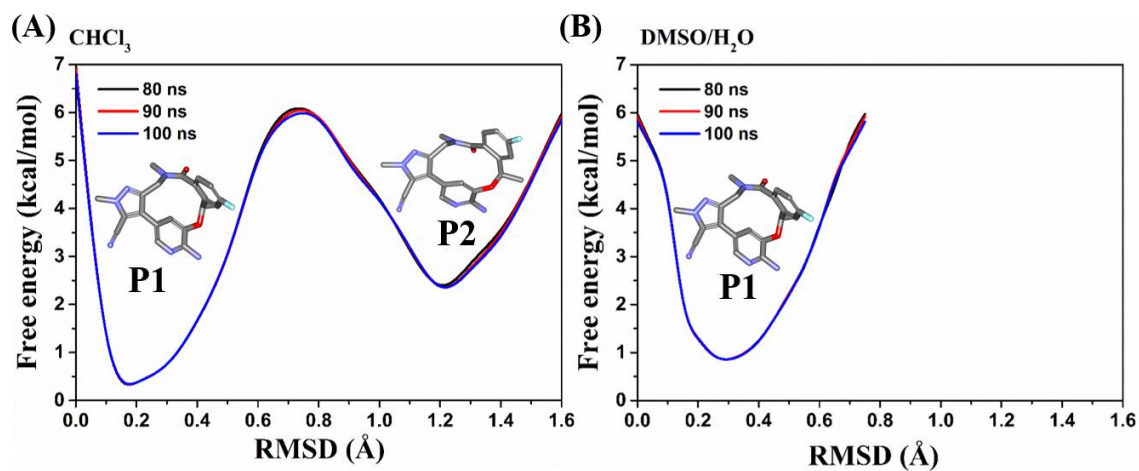

Figure S1. One-dimensional free energy profiles as the function of heavy-atom RMSD. The representative conformers in CHCl$_3$ (A), DMSO/H$_2$O (4:6) (B) are shown as sticks.
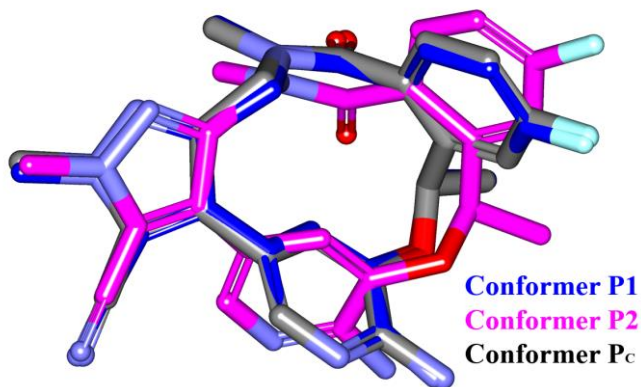

Figure S2. The representative conformers P1 (blue colored) and P2 (pink colored) in CHCl$_3$. The crystal conformer Pc is colored by gray.
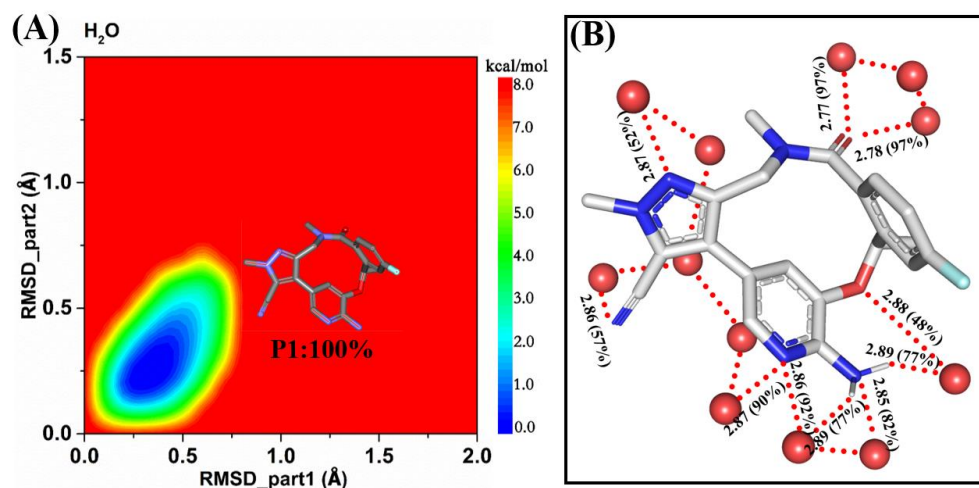
Figure S3. The simulation of lorlatinib in water. (A) Two-dimensional free-energy landscapes of the conformation distribution of lorlatinib in water. The lowest energy is assigned by the darkest color (red to blue). (B) Schematic hydrogen bond network formed by lorlatinib and water. The average distance between the HB donor and acceptor during the REMD simulations is provided along the dash line (Å). The percentage time of HB during the REMD simulations is shown in the brackets.
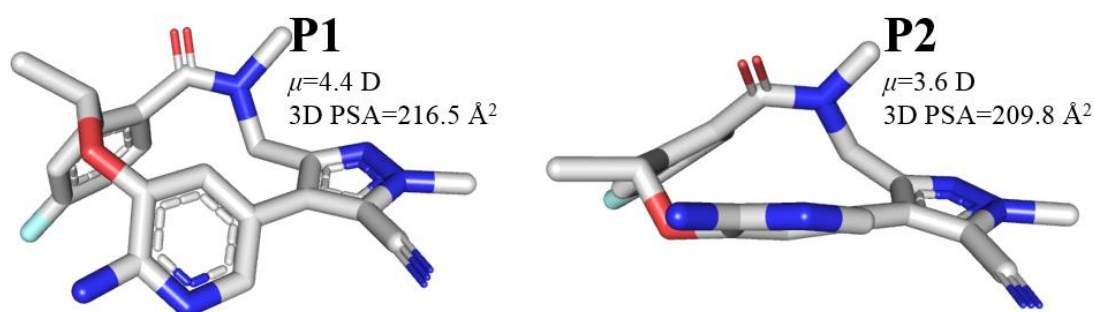

Figure S4. The conformers P1 and P2 after alignment. The solvent accessible surface area (SASA) of the carbonyl group calculated with Pymol and the overall dipole moment (μ) calculated with gaussian 09 at M062X/6-311G** level, are provided.

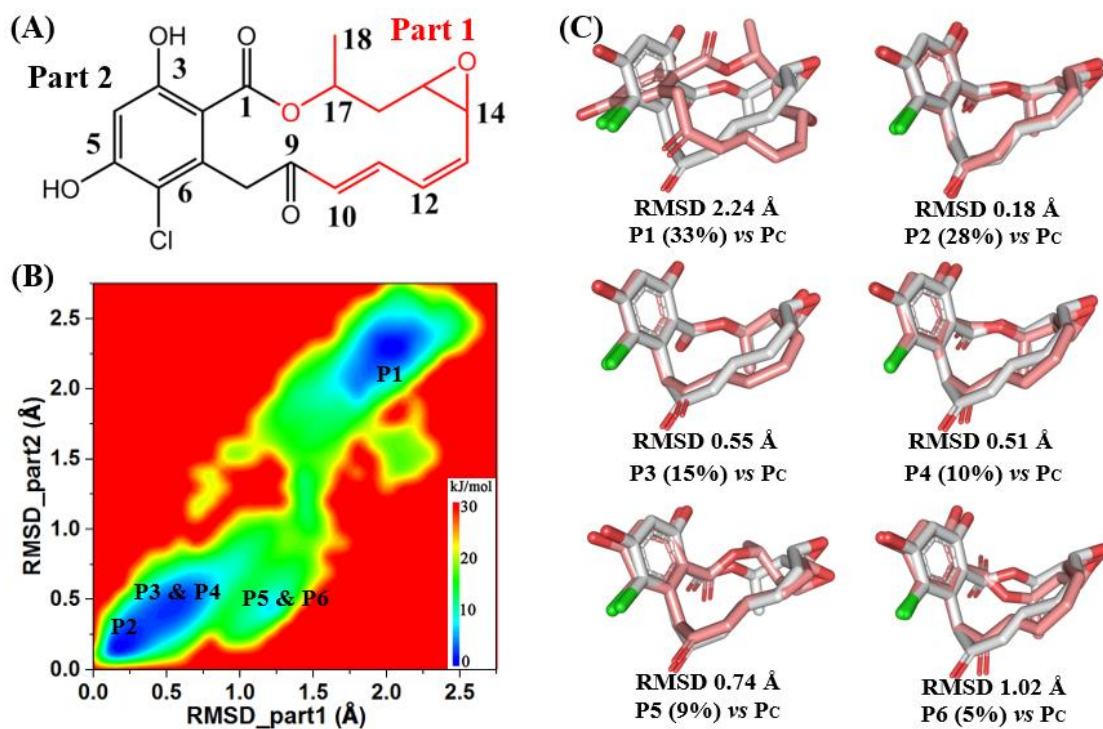Figure S5. The REMD simulation of radicicol in CHCl₃. (**A**) Radicicol and the two parts it was divided into for RMSD analysis; (**B**) Two-dimensional free energy landscape of the conformational distribution of radicicol in CHCl₃. (**C**) Representative conformers (P1-P6), with populations in %, from the REMD simulation compared to the crystal structure (Pc, colored in white) of radicicol (PDB ID: 1BGQ).
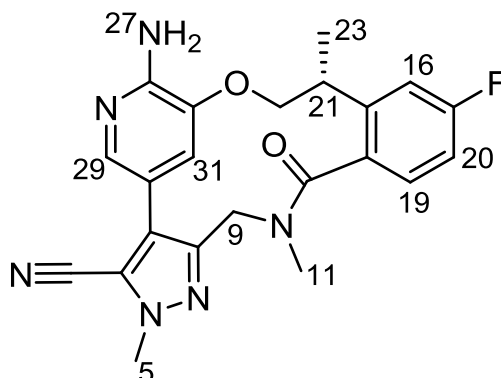
2. NMR analysis of Lorlatinib

Figure S6. Structure of Lorlatinib

2.1. ¹H-NMR assignments

Proton assignment (Table S1) for Lorlatinib (Figure S6) in D_2O/DMSO-d_6 (6:4) and $CDCl_3$ were deduced using 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, HSQC, HMBC and NOESY) NMR spectra recorded at 25 °C on a 800 MHz BRUKER Avance III HD NMR spectrometer equipped with a TCI cryogenic probe.

Table S1: ^1H-NMR assignment (ppm) of Lorlatinib in D_2O/DMSO-d_6 (6:4) and $CDCl_3$

[a]Proton	δ, D_2O/DMSO-d_6	δ, $CDCl_3$
5	4.17	4.07
9'	4.47	4.36
9"	4.59	4.45
11	3.19	3.13
16	7.66	7.29
19	7.31	7.00
20	7.55	7.21
21	5.75	5.73
23	1.87	1.79
27		4.94
29	7.74	7.82
31	6.97	6.86

[a]Numbering according to Figure S6.

2.2. Interproton distances

NOE build-ups were recorded without solvent suppression with alternated mixing times of 100, 200, 300, 400, 500, 600 and 700 ms. The relaxation delay was set to 2.5s, 16 scans were recorded with 8192 points in the direct dimension (F2) and 512 points in the indirect dimension (F1). Distances (Table S2 and S3) were calculated using ortho aromatic protons (ref=2.51 Å) and methine proton as reference distance (ref=2.43 Å).

$$NOE = \left(\frac{cross\ peak\ 1 * cross\ peak\ 2}{diagonal\ peak\ 1 * diagonal\ peak\ 2} \right)^{0.5}$$

...... Eq.1

NOE peak intensities were calculated using normalization of both cross peaks and diagonal peaks of the protons according to equation 1.

This resulted in seven normalized NOE intensities according to the recorded mixing times. At least four mixing times giving a linear initial NOE rate ($R^2 \geq 0.95$ for both solvents) were used to determine buildup-rates (σ) according to equation 2, where r_{ij} distances between protons i and j in Ångström and σ_{ref} is the buildup rate of the reference protons and σ_{ij} is the buildup rate of the protons. All buildups were based on normalized intensity obtained from the NOESY experiments.

$$r_{ij} = r_{ref} \left(\frac{\sigma_{ref}}{\sigma_{ij}} \right)^{1/6}$$

..... Eq.2

Table S2. Interproton distances (Å) for Lorlatinib derived from NOE build-up measurements in D$_2$O/DMSO-d_6 (6:4) (δ in ppm).

Dis. No.	Atom type	Proton i	Proton j	$\delta(^1H)\, i$	$\delta(^1H)\, j$	σ	R^2	Dis. rij [Å]
1	CHCH	16	21	7.66	5.75	7.26254E-06	0.97	4.18
2	CHCH	11	20	3.19	7.55	4.98398E-06	0.99	4.45
3	CH$_2$CH	9'	20	4.47	7.55	5.30124E-05	0.99	3.00
4	CH$_2$CH	9"	20	4.59	7.55	3.83095E-05	0.99	3.17
5	CH$_2$CH	9"	31	4.59	6.97	7.20326E-06	0.98	4.19
6	CH$_2$CH	9'	31	4.47	6.97	3.23764E-05	0.99	3.26
7	CHCH$_3$	31	23	6.97	1.87	3.82869E-06	0.97	4.65
8	CHCH$_3$	31	11	6.97	3.19	9.93347E-06	0.99	3.97
9	CHCH$_2$	31	21	6.97	5.75	0.000280931	0.99	2.27
10	CH CH$_3$	21	11	5.75	3.19	5.59412E-06	0.99	4.37
11	CHCH$_2$	21	9'	5.75	4.47	8.73313E-06	0.97	4.06
12	CH$_2$CH$_3$	9'	11	4.47	3.19	8.72449E-06	0.99	4.06
Ref.	CHCH	19	20	7.31	7.55	0.0001564	0.96	2.51

Table S3. Interproton distances (Å) for Lorlatinib derived from NOE build-up measurements in CDCl$_3$ (δ in ppm).

No.	Type	Proton i	Proton j	$\delta(^1\text{H})\,i$	$\delta(^1\text{H})\,j$	σ	R^2	Dis. rij [Å]
1	CHCH$_3$	16	23	7.29	1.79	7.17984E-05	0.99	2.47
2	CH CH$_2$	20	9'	7.21	4.36	3.22055E-05	0.96	2.83
3	CHCH$_2$	20	9"	7.21	4.45	4.90079E-05	0.98	2.64
4	CHCH$_3$	20	11	7.21	3.13	1.11725E-06	0.97	4.95
5	CHCH$_2$	31	9"	6.86	4.45	3.97831E-05	0.99	2.73
6	CHCH$_3$	31	11	6.86	3.13	4.74948E-06	0.97	3.89
7	CHCH	31	21	6.86	5.73	0.000145559	0.99	2.20
8	CHCH$_3$	21	23	5.73	1.79	7.97246E-05	0.98	2.43
9	CHCH$_3$	9'	11	4.36	3.13	1.69377E-05	0.98	3.15
Ref	CHCH$_3$	21	23	5.73	1.79	7.43545E-05	0.98	2.43

3. Monte Carlo molecular mechanics (MCMM) conformational search

The theoretical conformation ensembles of Lorlatinib was identified by performing careful Monte Carlo conformational analysis using five different (OPLS, OPLS-2005, OPLS3e, AMBER* and MMFF) force fields, each with the GB/SA solvation models chloroform and water[1]. These conformational search was done using the Monte Carlo algorithm with intermediate torsion sampling, 50 000 Monte Carlo steps and a RMSD cut-off set to 2.0 Å. A Molecular Mechanics energy minimization was performed as implemented in the Macromodel BatchMin V12.1 of the Schrödinger Package. Each conformation was energy minimized using Polak-Ribière type conjugate gradient (PRCG) with a maximum of 5000 iterative steps. All conformations within 42 kJ/mol from the global minimum were saved. Results of all the different conformational searches are given in Table S4. All ensembles generated by the conformational searches were combined and elimination of redundant conformations was performed by comparison of heavy atom coordinates applying an RMSD cutoff set to 1.0 Å, giving the final ensemble used for NAMFIS-analysis.

Table S4. Results of the MCMM conformational analysis.

Solvation Model	Sampling Method	Force field	Total[a]	Number of conformations	
				Within 12.6 kJ/mol[b]	Conformations added to final ensemble
	MCMM	OPLS-2005	5		5
CHCl₃	MCMM	Amber*	5		5
	MCMM	OPLS3	3		3
	MCMM	MMFF	6		6
	MCMM	OPLS	5	2	5
	MCMM	OPLS-2005	5		5
H₂O	MCMM	Amber*	5	2	5
	MCMM	OPLS3	3		3
	MCMM	MMFF	7	3	7
	MCMM	OPLS	5	2	5
	Total		49		49
	RCE		5		5[c]
	Final input ensemble for NAMFIS		5		

[a]Total unique conformations found.

[b]Number of conformations found that are within 12.55 kJ/mol (3 kcal/mol) of the global minimum.

[c]Conformations obtained after redundant conformation elimination with the root-mean-square deviation cutoff set to 1Å for heavy atoms.

4. Crystal Structures

In addition to the computational conformational search described above, 1 published crystal structures from the PDB were included (Table S5). All X-ray structures were derived from co-crystallization with the ribosomal subunit of the target protein of different bacteria.

Table S5: Crystal structures obtained from the PDB used in the ensemble

Structure PDB code	Resolution [Å]	R-value Free
4CLI	2.05	0.245

5. Molecular Dynamics

Three MD generated structures were also included in the final NAMFIS input ensemble with the MCMM generated conformers and crystal structures.

6. NAMFIS analysis

Molar fractions of conformations present in the two examined solvents were determined using an algorithm based analysis. NMR analysis of molecular flexibility in solution (NAMFIS) is a method that uses experimentally assigned distances and coupling constants and fits them to back-calculated values of computationally generated conformations.[1-2] For the NOE derived distances and ^3J-coupling derived dihedral angles, computational conformations were analyzed and the respective distances and dihedrals were measured.

CH$_2$-to-H distances were averaged according to the equation 3

$$d_{average} = \left(\frac{(d_1^{-6} + d_2^{-6})}{2}\right)^{-(1/6)}$$

...... Eq. 3

and CH$_3$-to-H distances according to equation 4.

$$d_{average} = \left(\frac{(d_1^{-6} + d_2^{-6} + d_3^{-6})}{3}\right)^{-(1/6)}$$

..... Eq. 4

In the NAMFIS-analysis the degree of matching between calculated and experimental values is expressed in the RMSD error. The validation of NAMFIS ensemble analyses were

performed using standard methods, that is, through evaluation of the reliability of conformational restraints by the additions of 10% random noise to the experimental distances, by the random removal of 10% of individual restrains, and by comparison of the experimentally observed and back-calculated distances. The result of the NAMFIS analysis is given in Table S7 and Figure S2 for D_2O/DMSO-d_6 (6:4) and $CDCl_3$.

Table S6. Experimentally determined and back-calculated distances (NAMFIS output) interproton distances (Å).

D_2O/DMSO-d_6 (6:4)			$CDCl_3$		
Interproton distances	Exp.	Calc.	Interproton distances	Exp.	Calc.
1	4.18	3.75	1	2.47	2.64
2	4.45	4.46	2	2.83	2.74
3	3.00	2.70	3	2.64	2.61
4	3.17	2.83	4	4.95	4.43
5	4.19	4.00	5	2.73	2.44
6	3.26	2.84	6	3.89	3.73
7	4.65	4.54	7	2.20	2.25
8	3.97	3.60	8	2.43	2.50
9	2.27	2.15	9	3.15	2.92
10	4.37	4.50			
11	4.06	4.19			
12	4.06	3.84			
RMSD		0.27	RMSD		0.23

Table S7. Conformational populations derived by NAMFIS-analysis of Lorlatinib in D_2O/DMSO-d_6 (6:4) and $CDCl_3$ solutions.
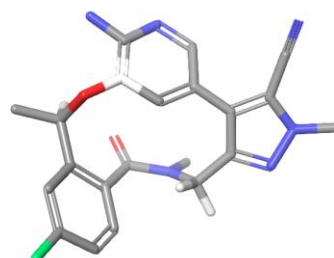
D_2O		$CDCl_3$	
Conformation Number	%[a]	Conformation Number	%[a]
1*	100	1*	76
		2	24

[a]Population of the indicated conformer in solution, as deduced by NAMFIS analysis.
*Conformers which are found in both solvents.
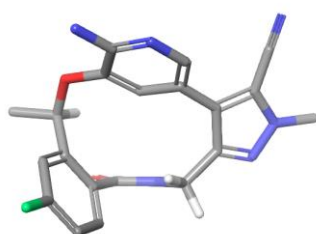
Conformer **1**   Conformer **2**

Figure S7. The solution conformations of Lorlatinib in D_2O/DMSO-d_6 (6:4) and $CDCl_3$ as selected by NAMFIS analysis. Population percentages are given in Table S7.

Table S8. RMSD comparison of the final conformations with the crystal structure based on the heavy atoms

	RMSD _Heavy atoms	
Conf. No	Crystal structure	1
Crystal structure	0	
1	0.349	0
2	1.022	1.235

Table S9. RMSD comparison of the final conformations with the crystal structure based on the macrocyclic atoms

	RMSD_MC atoms	
Conf.no	Crystal structure	1
Crystal structure	0	
1	0.1405	0
2	0.3619	0.4343

^{1}H NMR, COSY, TOCSY, NOESY, HSQC, HMBC Spectra of Lorlatinib in $CDCl_3$ and D_2O-DMSO are shown in Figure S8-19.

Original FIDs, along with the atomic coordinates of lorlatinib conformers identified by NAMFIS (mol2) are available, open access, at Zenodo with DOI: 10.5281/zenodo.3521650.
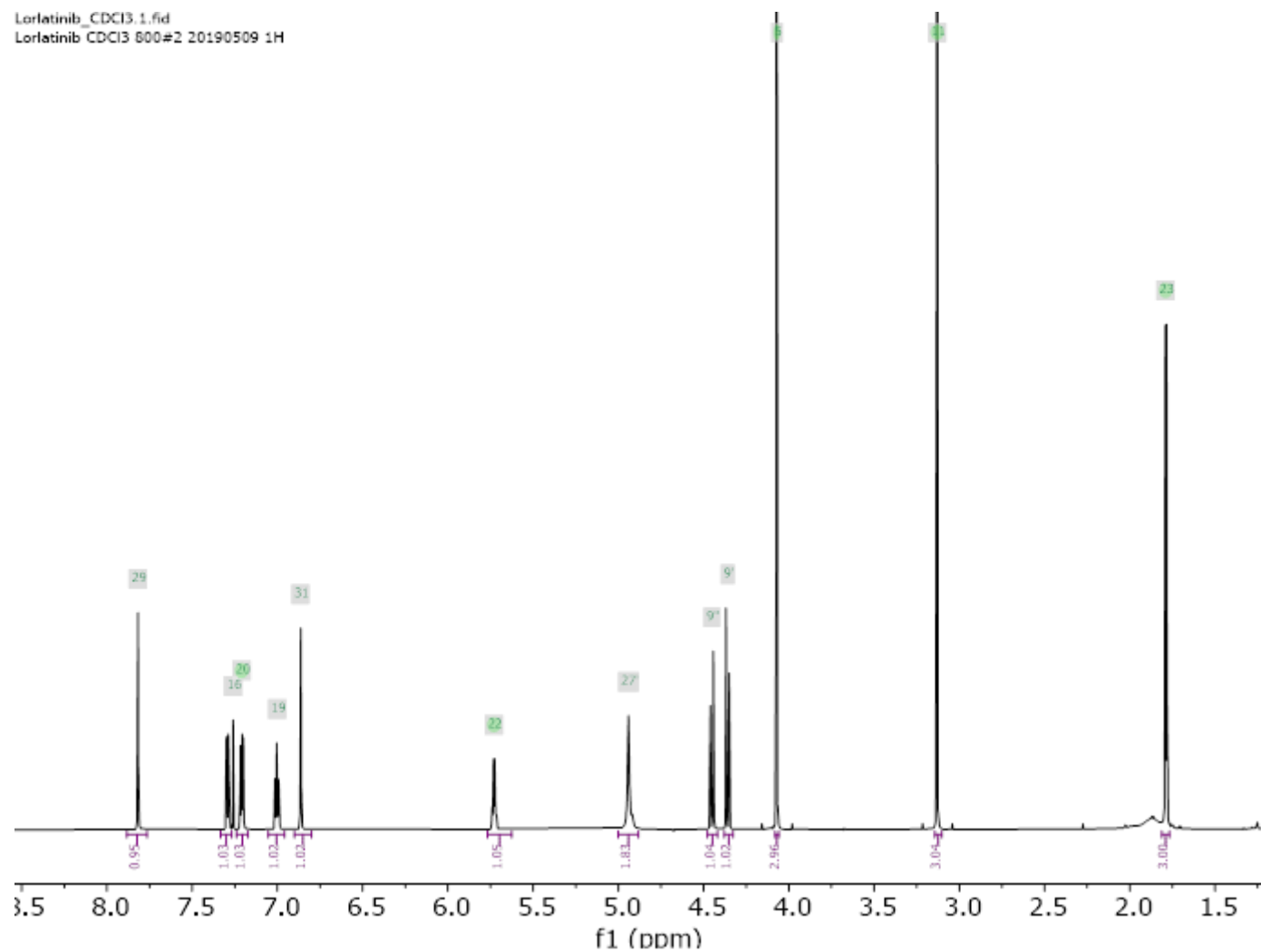
Figure S8: ^{1}H NMR Spectrum of Lorlatinib (CDCl$_3$)
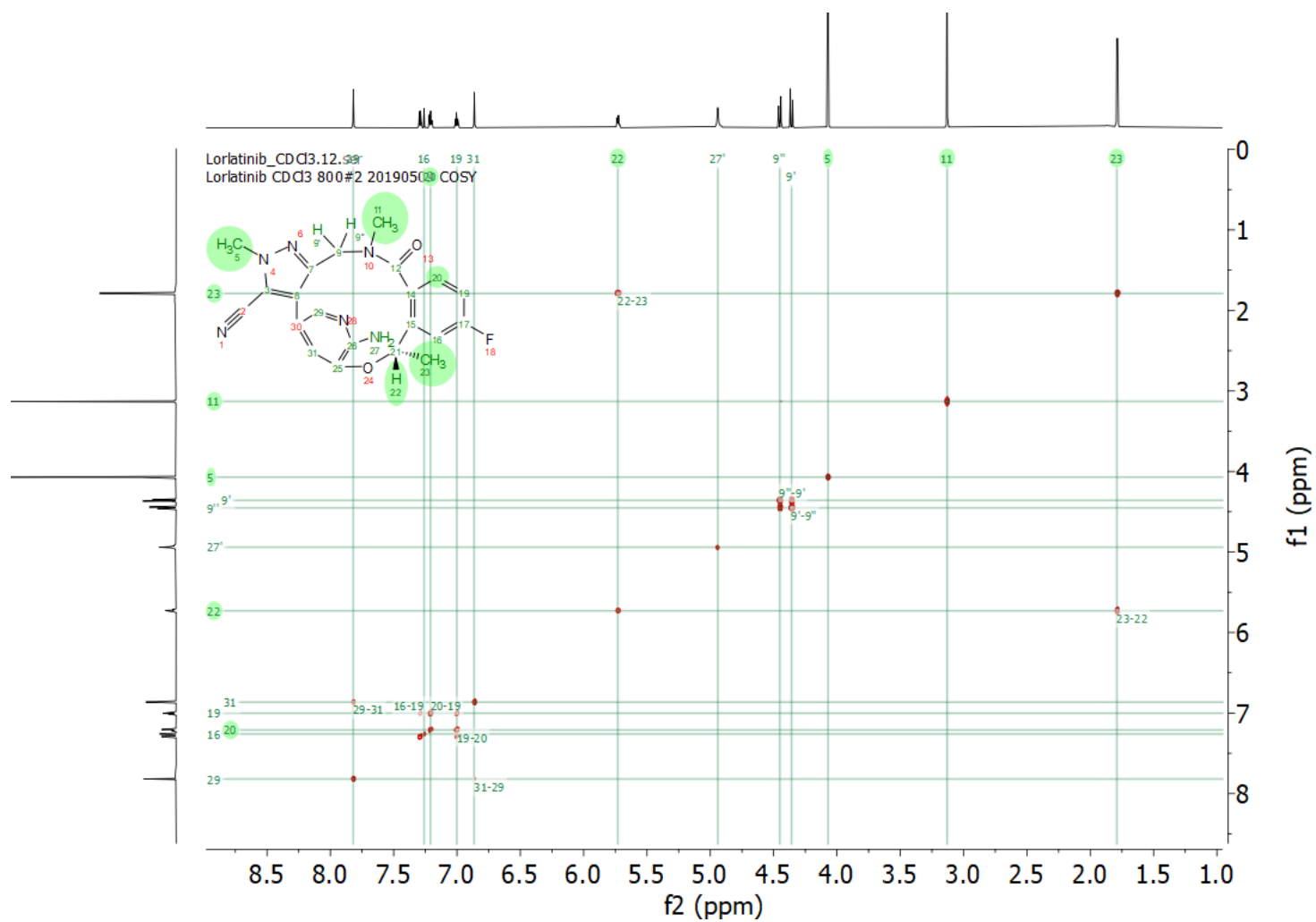
Figure S9: COSY Spectrum of Lorlatinib (CDCl₃)
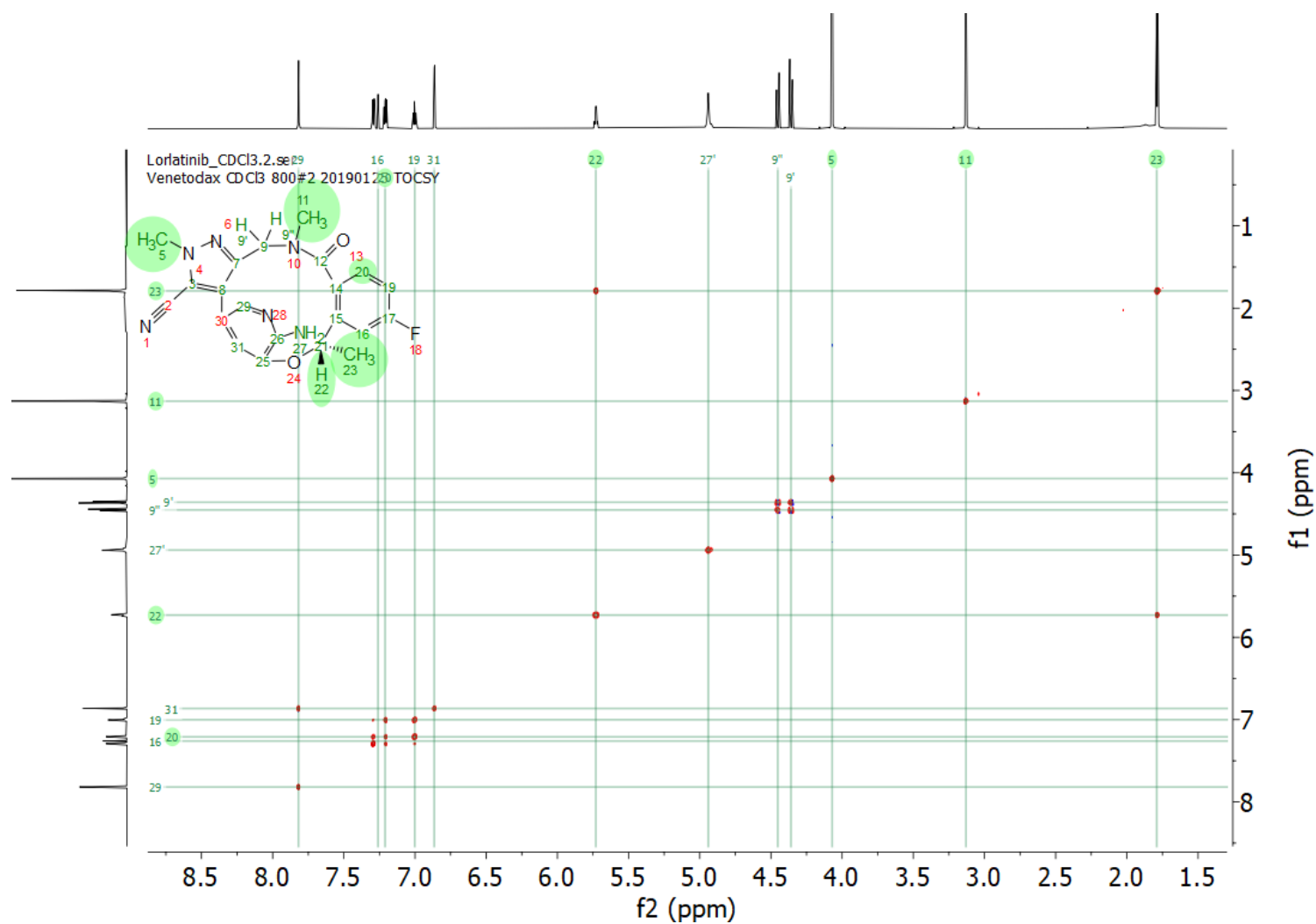
Figure S10: TOCSY Spectrum of Lorlatinib (CDCl₃)
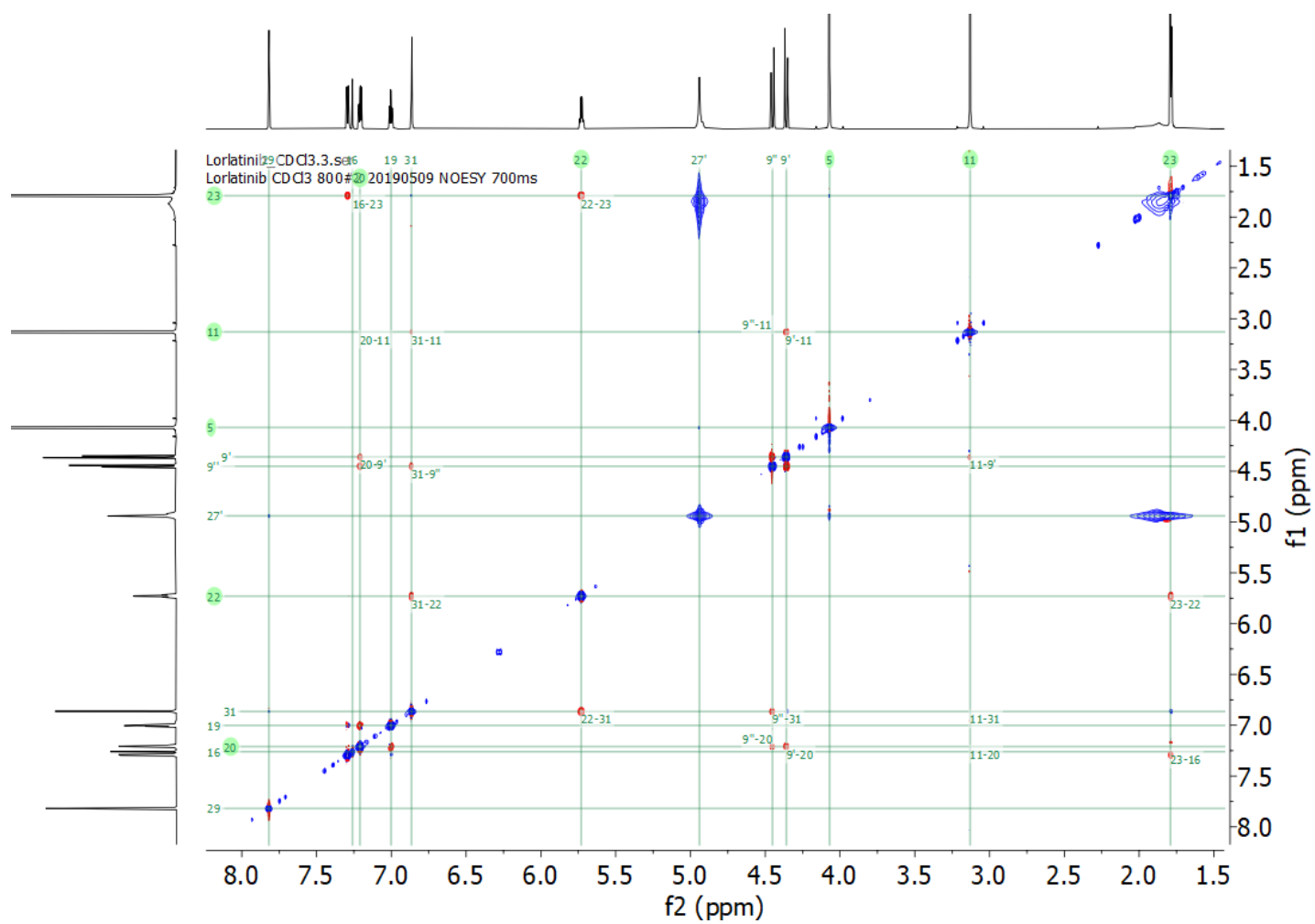
Figure S11: NOESY Spectrum of Lorlatinib (CDCl₃)

Figure S12: HSQC Spectrum of Lorlatinib (CDCl$_3$)
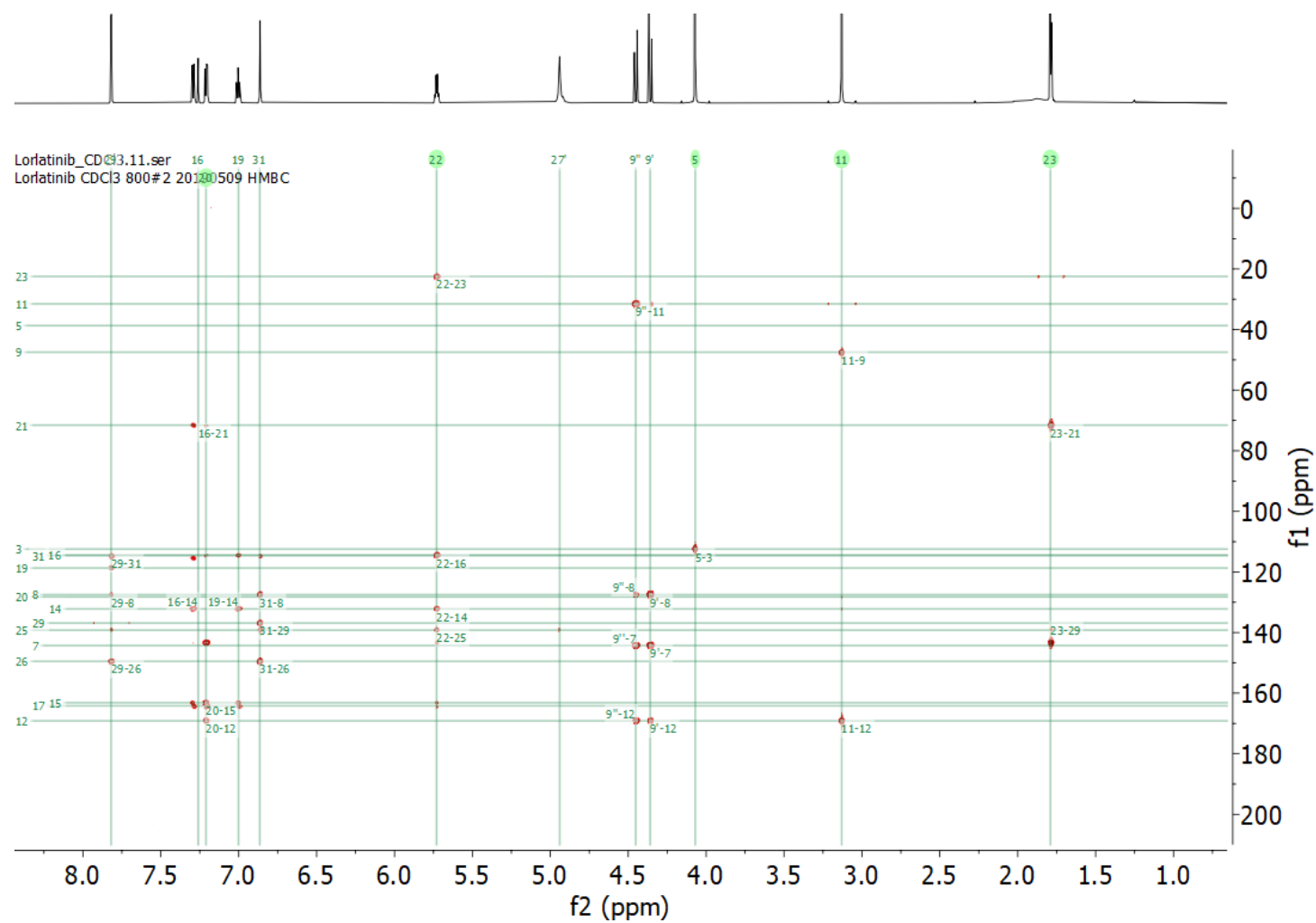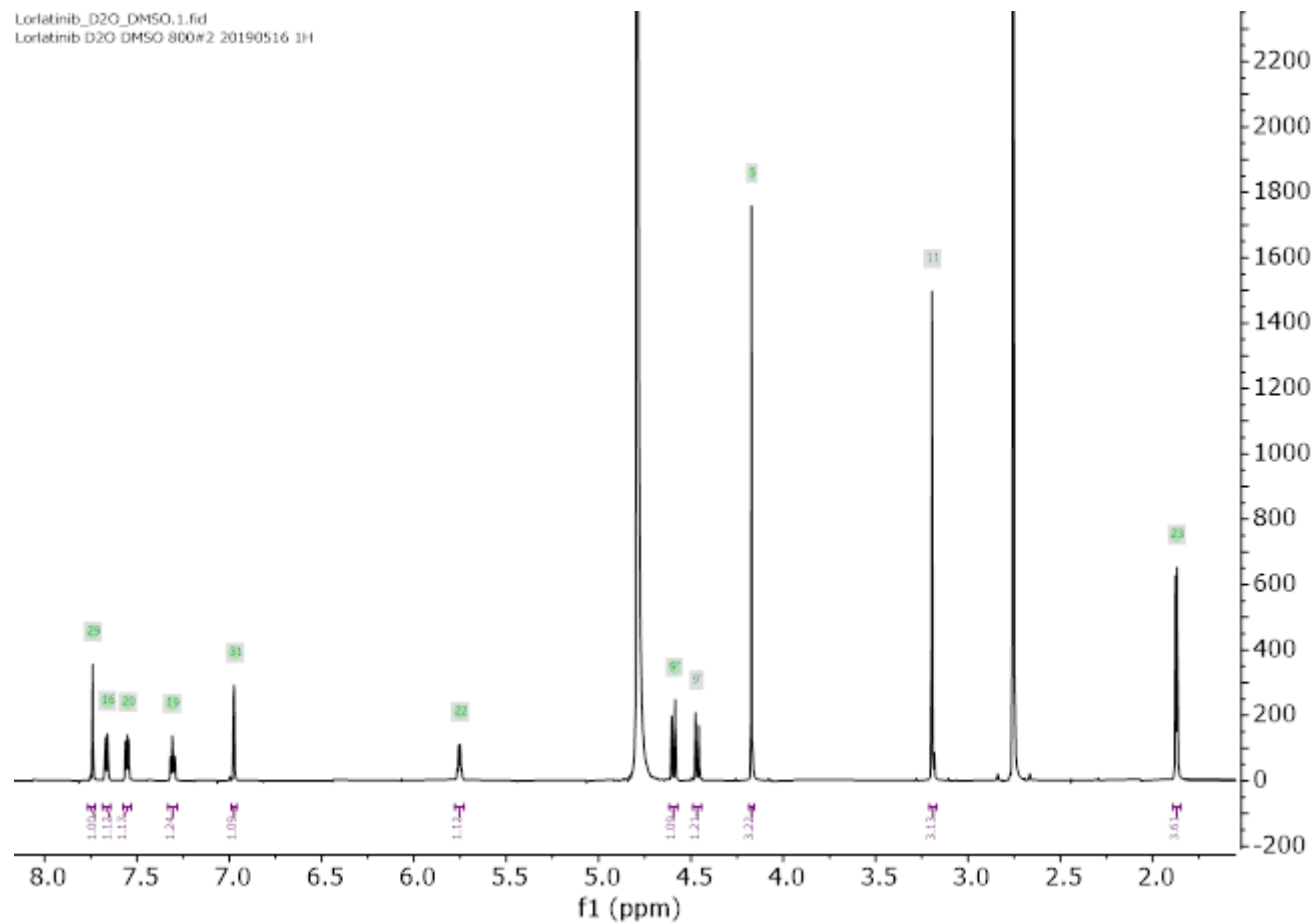
Figure S13: HMBC Spectrum of Lorlatinib (CDCl₃)

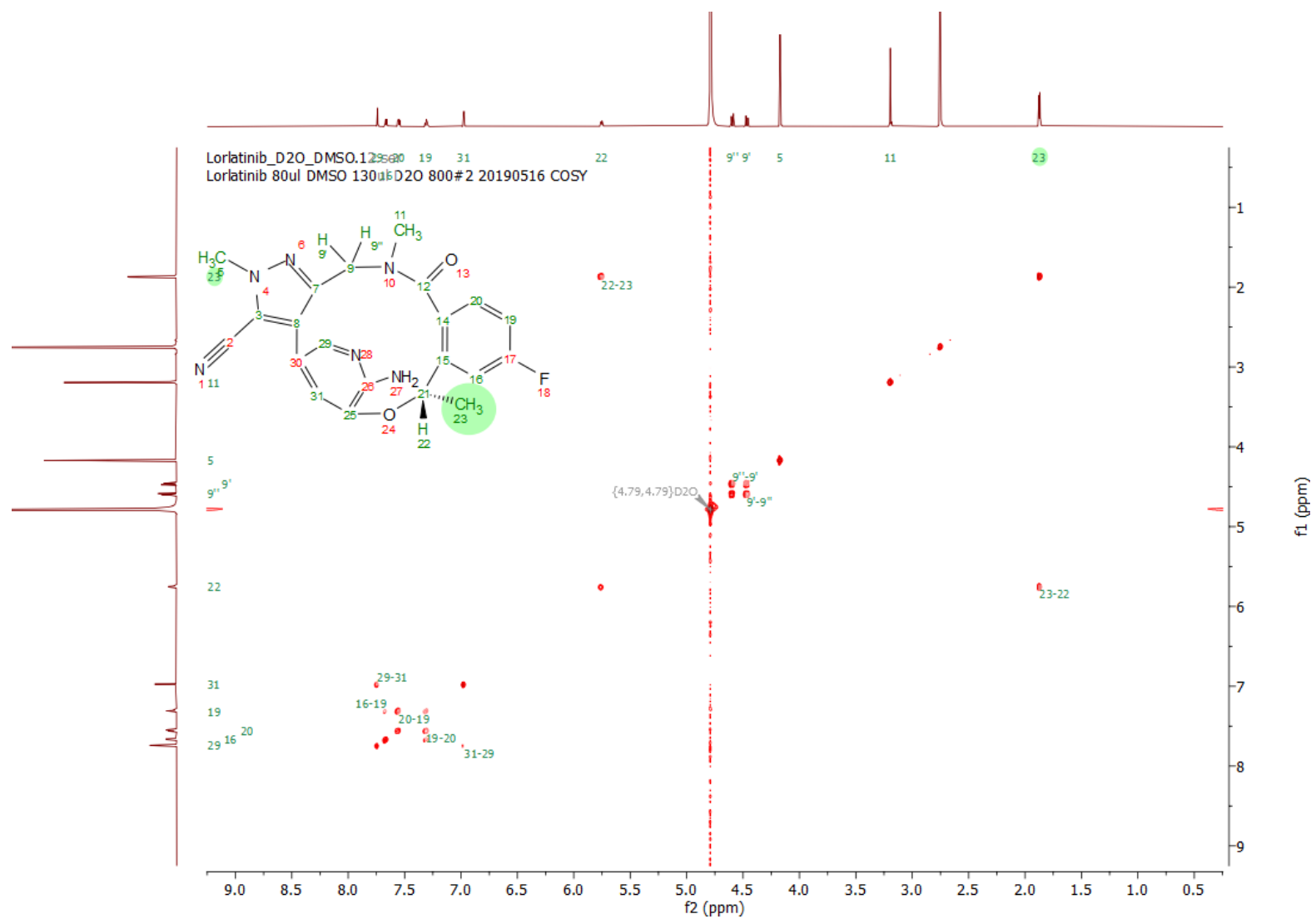Figure S14: ^{1}H NMR Spectrum of Lorlatinib (D$_2$O-DMSO)
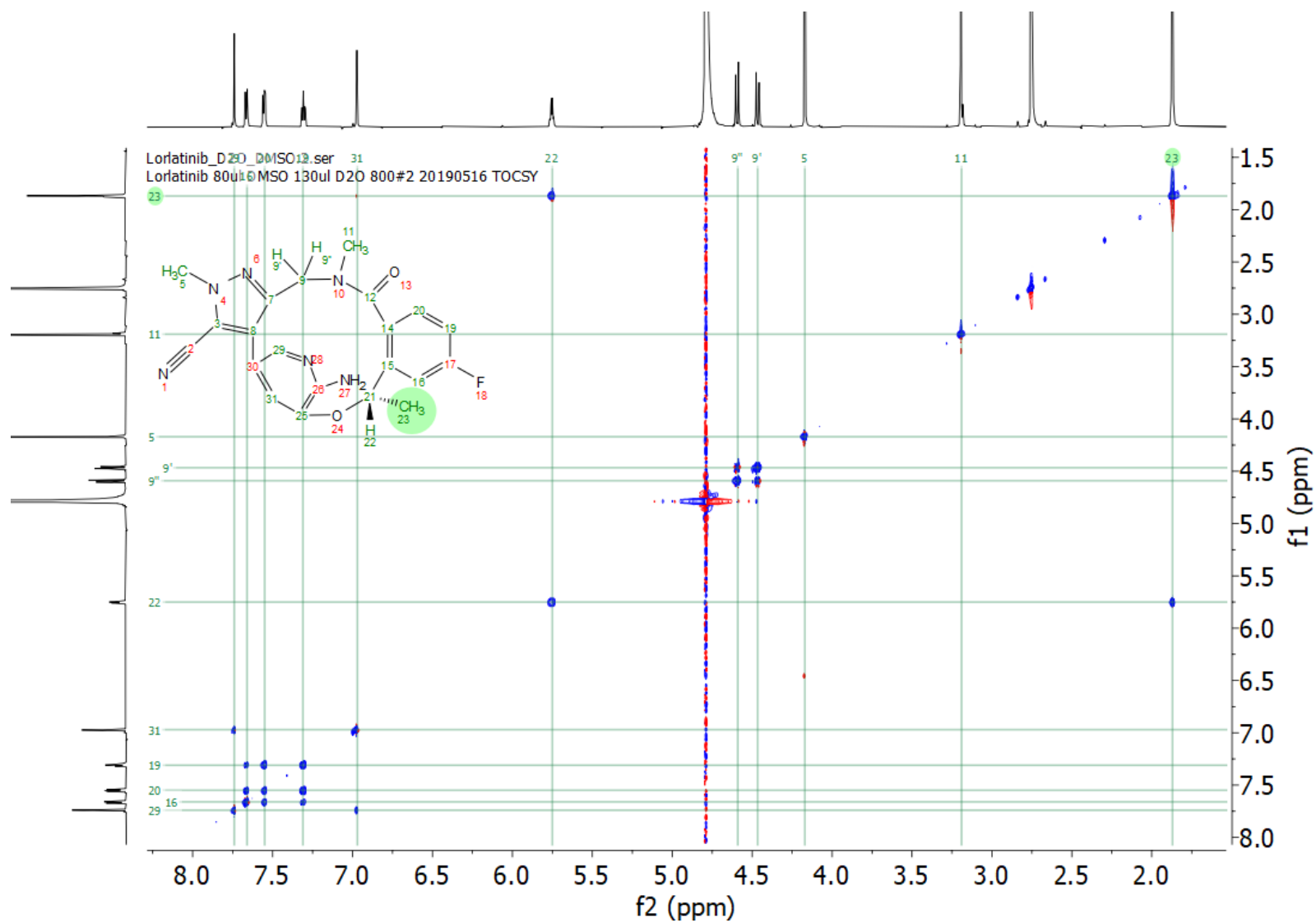
Figure S15: COSY Spectrum of Lorlatinib (D₂O-DMSO)
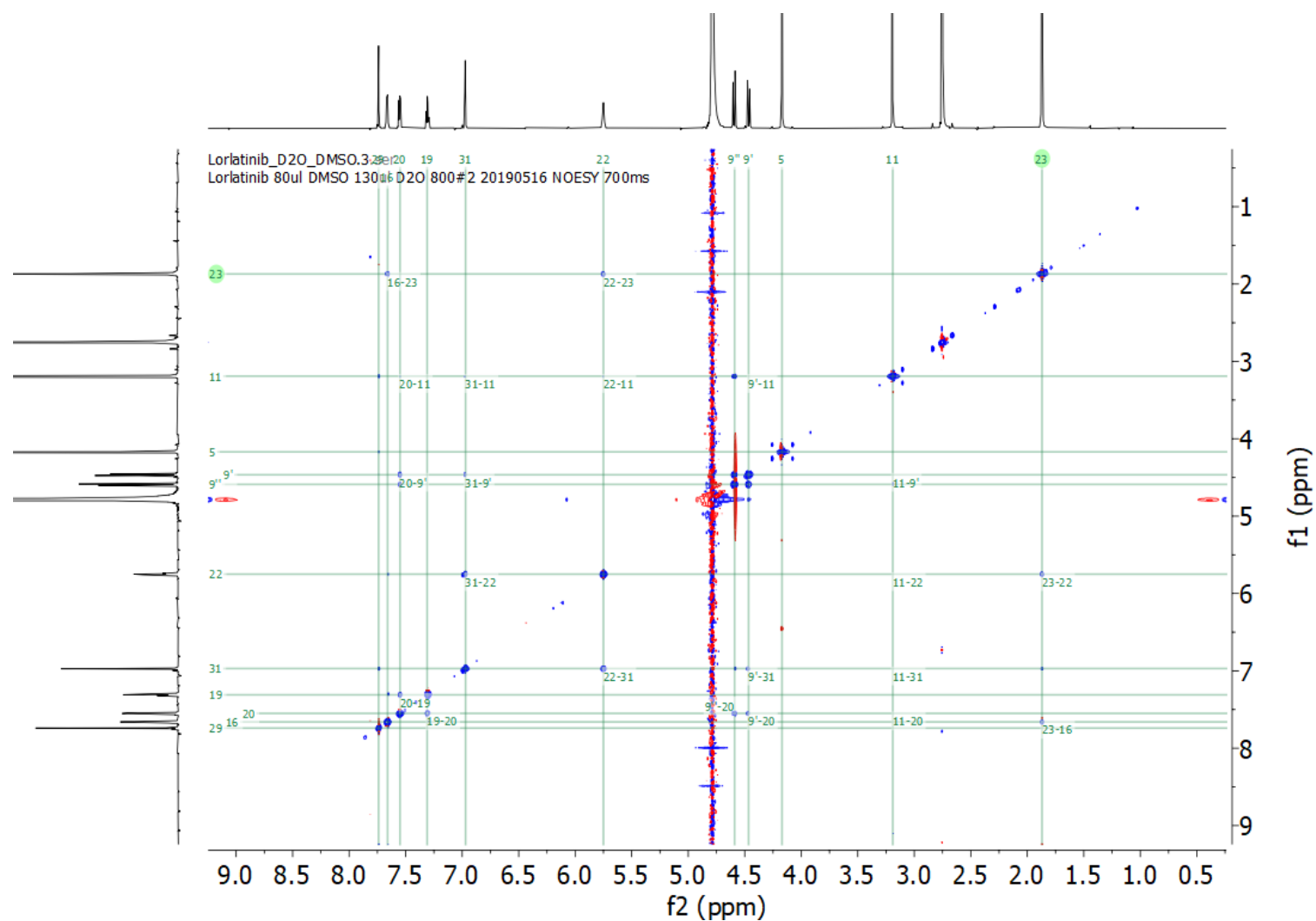
Figure S16: TOCSY Spectrum of Lorlatinib (D₂O-DMSO)

Figure S17: NOESY Spectrum of Lorlatinib (D$_2$O-DMSO)

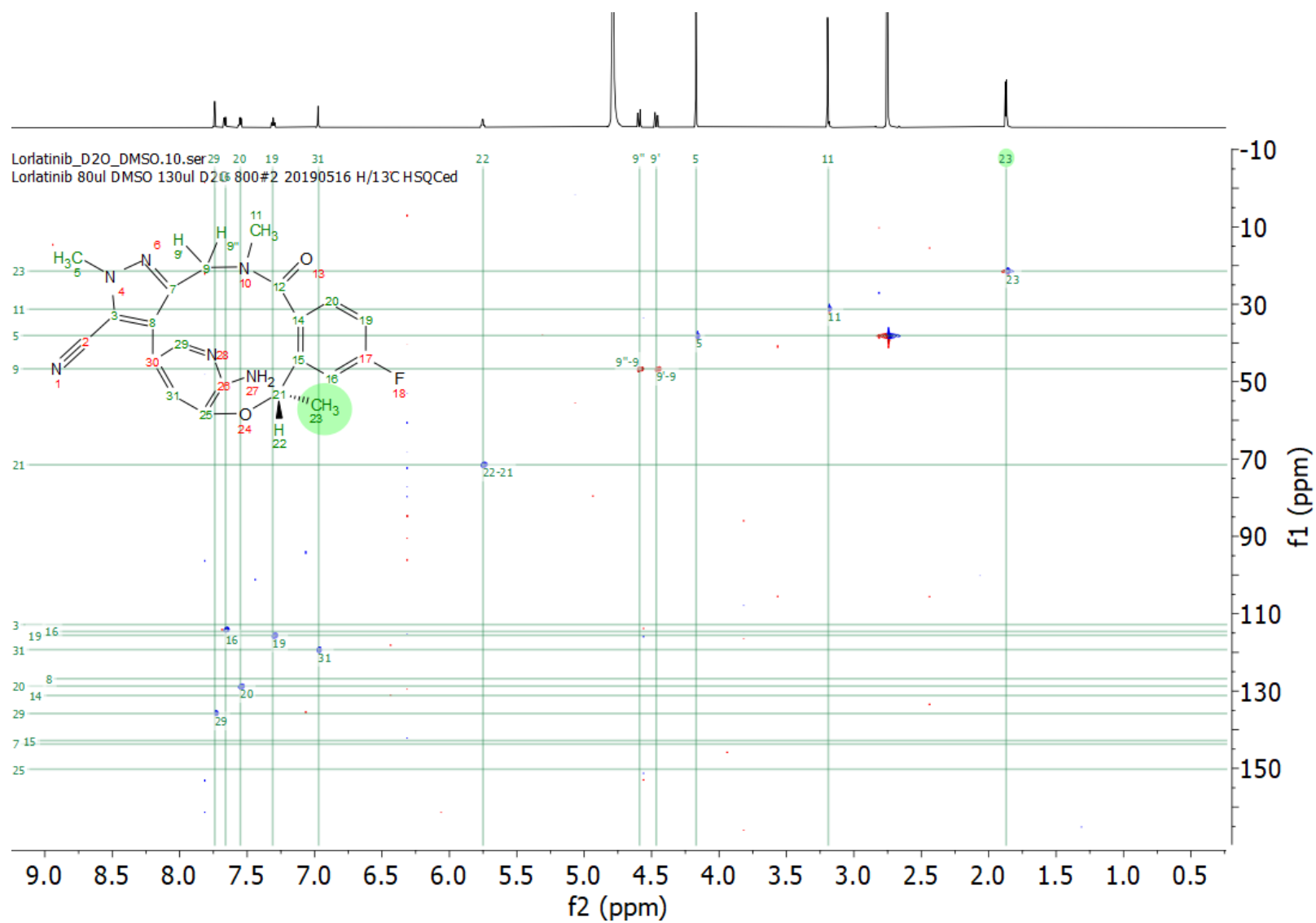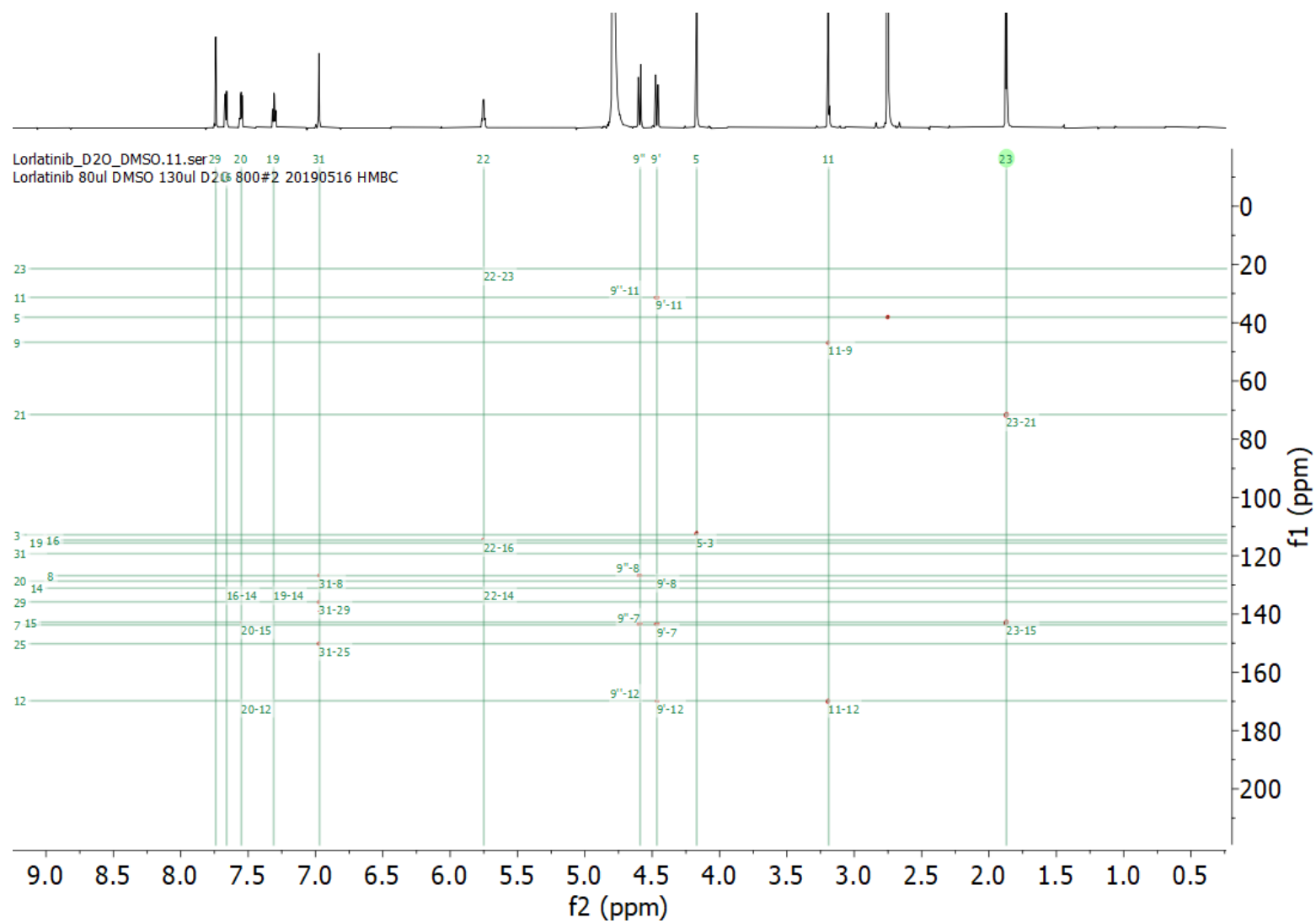Figure S18: HSQC Spectrum of Lorlatinib (D₂O-DMSO)

Figure S19: HMBC Spectrum of Lorlatinib (D₂O-DMSO)

References

1. Cicero, D. O.; Barbato, G.; Bazzo, R., NMR Analysis of Molecular Flexibility in Solution: A New Method for the Study of Complex Distributions of Rapidly Exchanging Conformations. Application to a 13-Residue Peptide with an 8-Residue Loop. *J. Am. Chem. Soc.* **1995**, *117* (3), 1027-1033.

2. Nevins, N.; Cicero, D.; Snyder, J. P., A Test of the Single-Conformation Hypothesis in the Analysis of NMR Data for Small Polar Molecules: A Force Field Comparison. *J. Org. Chem.* **1999**, *64* (11), 3979-3986.