SUPPLEMENTARY MATERIAL (Supporting information)

An Investigation of the Predominant Structure of Antibiotic Azithromycin in Chloroform Solution through NMR and Thermodynamic Analysis

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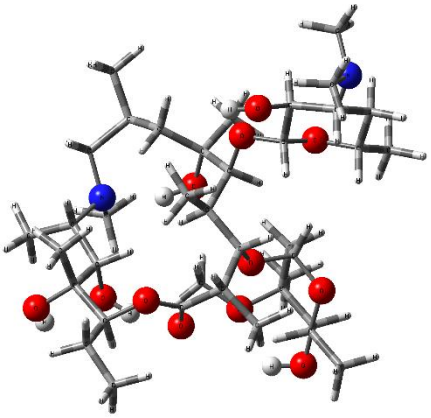
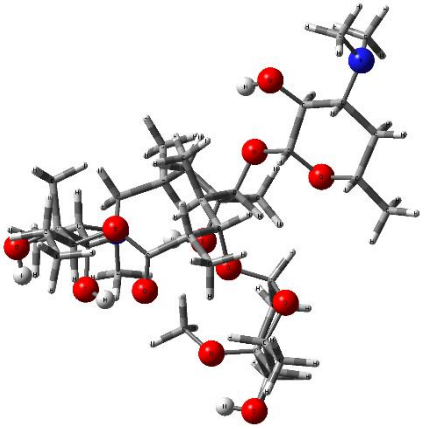


(a)**M1-A-C3SC5S**



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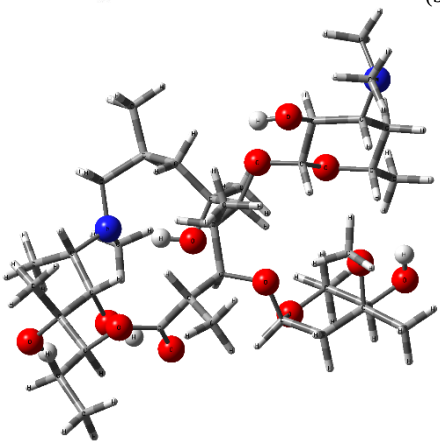

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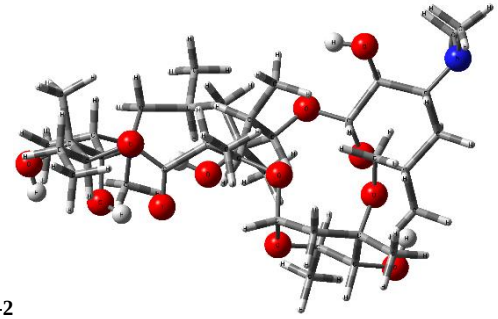

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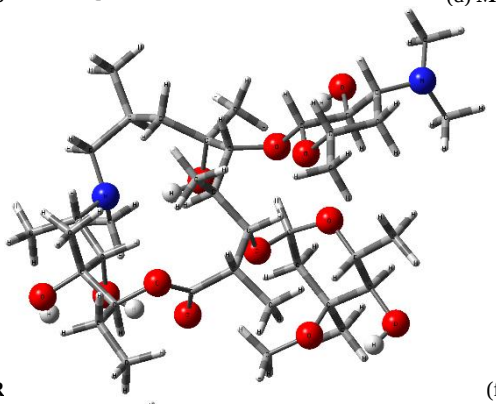

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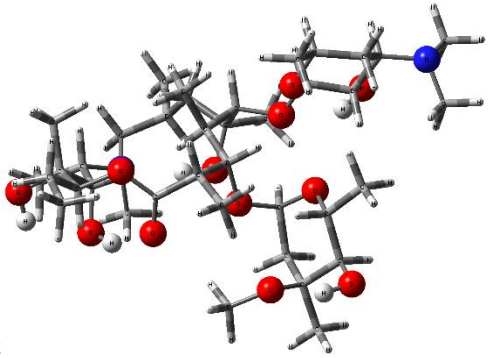

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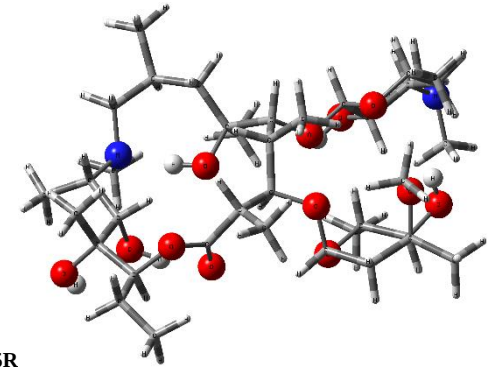

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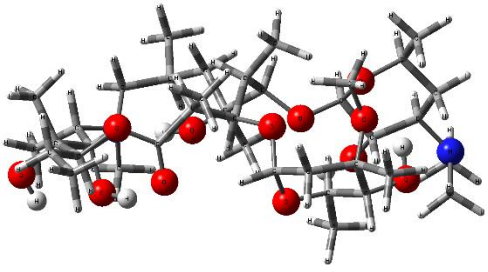

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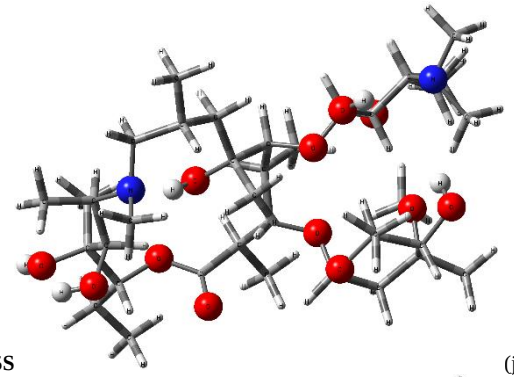

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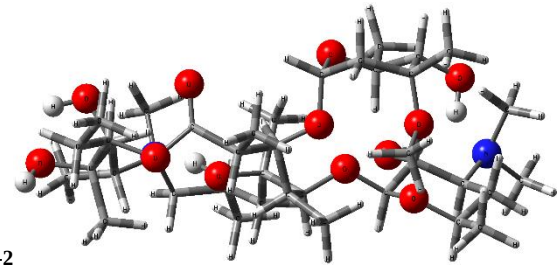

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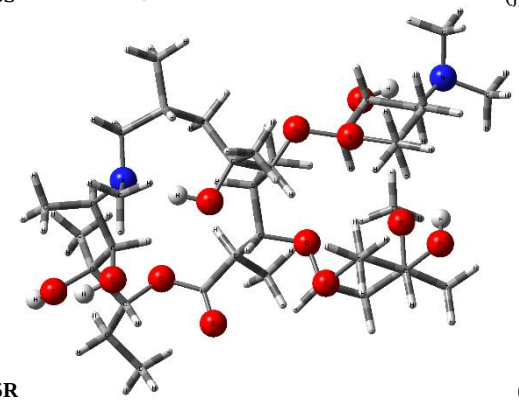

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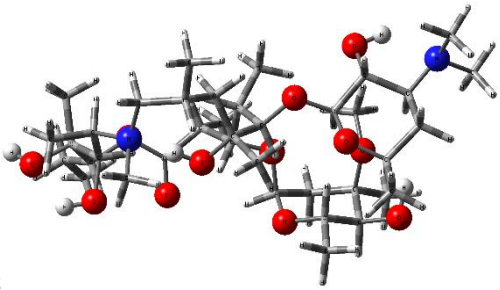

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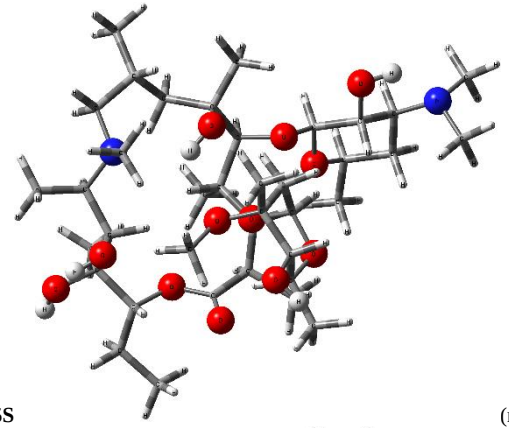

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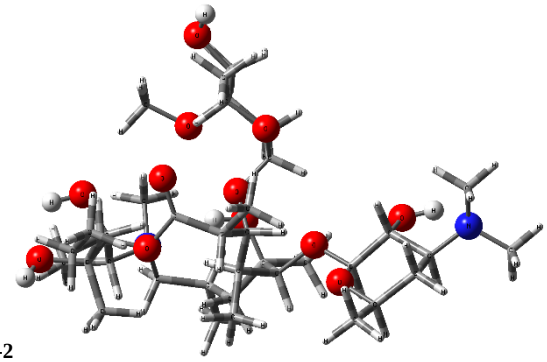
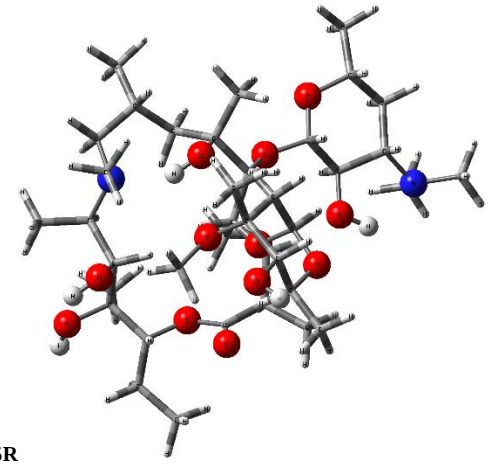
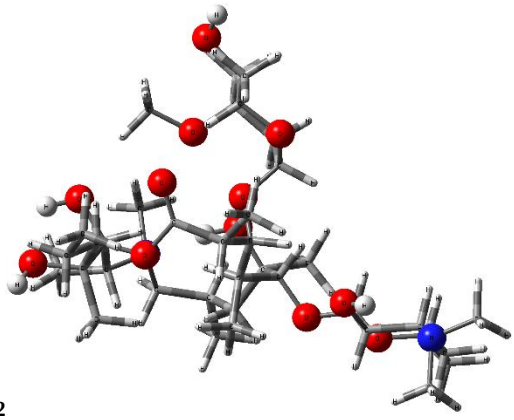


(n)**M3-B-C3RC5S-View-2**



(o)**M4-B-C3RC5R**



(p) **M4-B-C3RC5R-View-2**

Figure S1. ωB97x-D/6-31G(d, p) (PCM-Chloroform) fully optimized structures of azithromycin using as starting point X-Ray cartesian coordinates of enantiomers “A” (C3R-C5S) and “B” (C3S-C5R). Heteroatoms and O-H groups are highlighted to ease visualize conformational differences. All four possible configurations for C3 and C5 carbons atoms were explored.

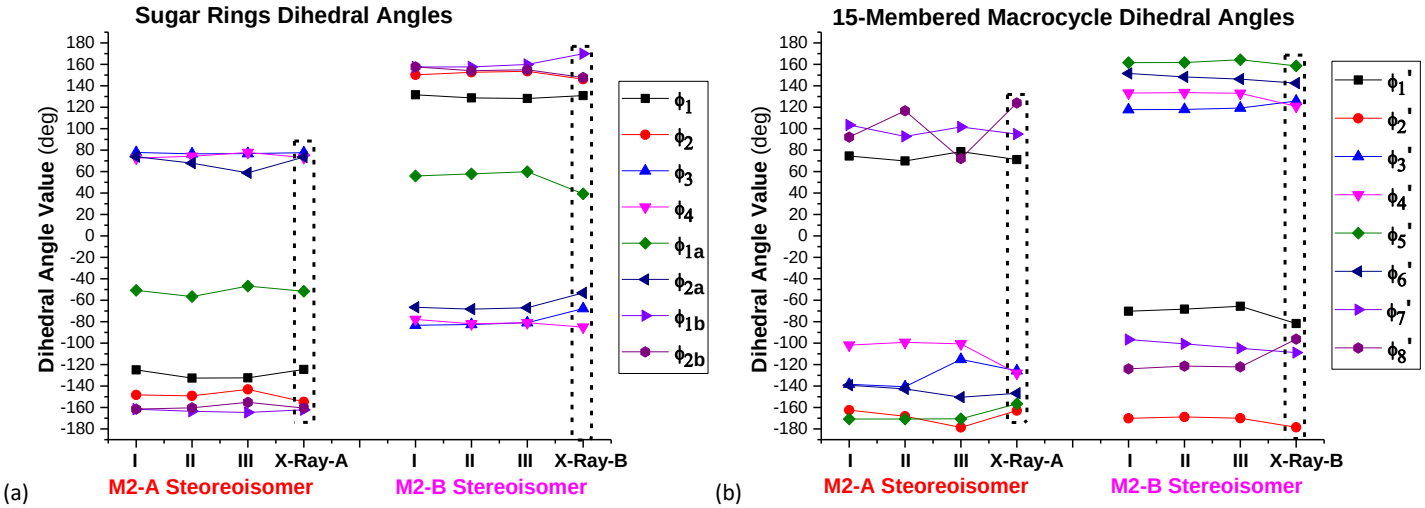


Figure S2. X-Ray and DFT (ωB97x-D/6-31G(d, p) optimized structures using as input X-Ray cartesian coordinates) torsion angles (º) for enantiomers "A" and "B" of azithromycin. X-Ray data for stereoisomers "A" and "B" are highlighted in the dashed rectangle.
2-I: geometry optimized in the vacuum.
2-II: geometry optimized using the PCM model (chloroform solvent) to include solvent effects.
2-III: PCM-Chloroform optimization including five explicit CHCl3 solvent molecules.

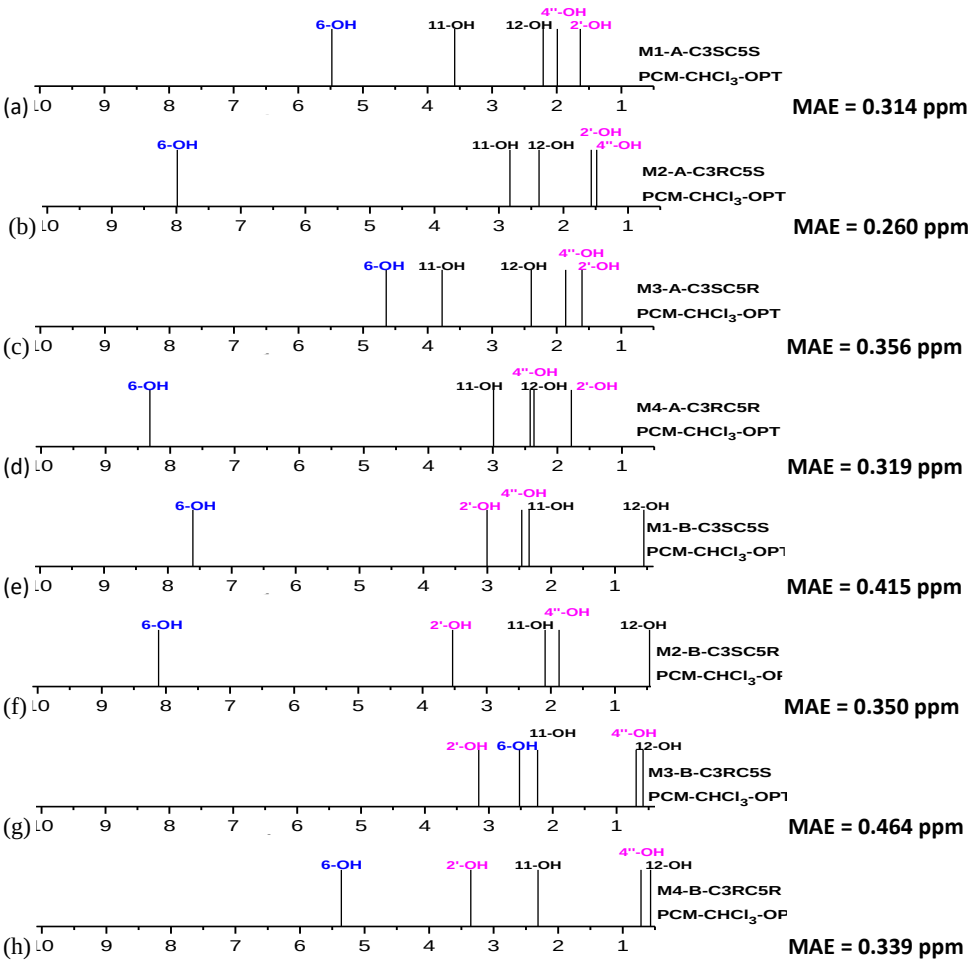


Figure S3. B3LYP/6-31G(d,p)-PCM-Chloroform ^{1}H NMR spectra for eight Azithromycin DFT fully optimized structures (stereoisomers "A" and "B").
(a) **M1-A-C3S-C5S** (b) **M2-A-C3R-C5S** (c) **M3-A-C3S-C5R** (d) **M4-A-C3R-C5R**
(e) **M1-B-C3S-C5S** (f) **M2-B-C3S-C5R** (g) **M3-B-C3R-C5S** (h) **M4-B-C3R-C5R**

Table S1. ωB97x-D/6-31G(d, p) (PCM-Chloroform) optimized O-H bond torsion angles (º) and O-H hydrogen bond distances (Å) for stereoisomers A and B of azithromycin optimized using as input X-Ray structures (cartesian coordinates). Results for geometries optimized in the vacuum, PCM-Chloroform, and including five explicit solvent molecules ($5CHCl_3$) are reported.

Structures	**M2-A Vac--OPT**	**M2-A PCM--OPT**	**M2-A PCM-OPT $5CHCl_3$**	**X-Ray Str.**	**M2-B Vac--OPT**	**M2-B PCM--OPT**	**M2-B PCM-OPT $5CHCl_3$**	**X-Ray Str.**
	Stereoisomer "A"				**Stereoisomer "B"**			
O-H Dihedral Angle (º)								
γ_1' [H-O-C2'-C1']	-51.9	-53.2	-51.3	179.9	-146.7	-149.3	-151.7	-61.1
γ_2' [H-O-C4''-C3'']	-38.6	-43.4	-46.0	-123.6	38.6	36.5	35.1	60.3
γ_3' [H-O-C6-C5]	137.0	124.2	111.8	-58.4	-132.6	-133.9	-126.6	-61.0
γ_4' [H-O-C11-C10]	141.6	146.2	144.8	179.8	87.0	86.0	88.1	65.0
γ_5' [H-O-C12-C11]	37.2	36.8	36.9	55.7	173.0	167.1	-169.7	180.0
H-bond Distance (Å)								
C2'-OH…O	2.37	2.33	2.33	NO H-Bond	2.38			NO H-Bond
C2'-OH…N	-	-	-	NO H-Bond		2.05	2.03	NO H-Bond
C4''-OH...O	2.09	2.18	2.16	NO H-Bond	2.10	2.07	2.06	NO H-Bond
C6-OH…N	1.77	1.73	1.82	NO H-Bond	1.90	1.72	1.69	2.62
C11-OH…O	2.00	2.03	1.88	NO H-Bond	1.93	2.01	1.99	2.27
C12-OH…O	2.11	2.10	2.10	2.21	2.11	NO H-Bond	NO H-Bond	NO H-Bond

Table S2. Selected X-Ray torsion angles (º) for stereoisomers A and B of azithromycin. Sugar rings and 15-membered macrolide units torsion angles are placed separately.

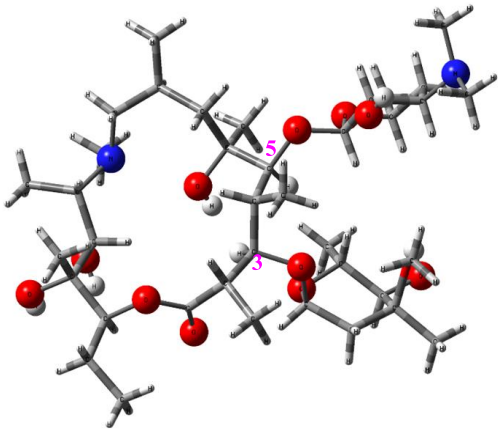
Structures	**Stereoisomer A C3R-C5S**			**Stereoisomer B C3S-C5R**					
	Ref. [7] Mon-Hyd	**Ref. [39] Di-Hyd**	**Ref. [41] 2007**	**Ref. [40] Di-Hyd**	**Ref. [8]**	**Ref. [9]**	**Ref. [36]**	**Ref. [37]**	**Ref. [38]**
	XR1-A	**XR2-A**	**XR3-A**	**XR4-B**	**XR5-B**	**XR6-B**	**XR7-B**	**XR8-B**	**XR9-B**
	Pure AZM	**Pure AZM**	**Pure AZM**	**Pure AZM**	**AZM interacting with other species**				
Dihedral Angle (º)	**X-Ray Strs. Sugar rings**								
φ_1 [C1'-O-C5-C6]	-124.5	-124.6	-124.3	133.3	130.9	119.4	123.2	100.1	123.4
φ_2 [C1''-O-C3-C4]	-154.7	-154.2	-153.0	145.2	146.1	144.9	147.3	146.1	142.0
φ_{1b} [C2'-C1'-O-C5]	-162.1	-161.7	-161.3	156.3	169.9	168.2	166.2	-152.6 [a]	164.9
φ_{2b} [C2''-C1''-O-C3]	-160.5	-161.3	-163.5	152.5	147.7	153.8	155.3	154.2	149.8
Dihedral Angle (º)	**15-Membered Macrocycle**								
φ_1' [C4-C5-C6-C7]	71.2	71.1	72.3	**-71.9**	-81.7	-73.0	-74.7	-69.6	-70.6
φ_3' [O-C1-C2-C3]	-125.7	-126.3	-126.4	121.9	125.8	124.5	120.4	121.4	129.7
φ_4' [C12-C13-O-C1]	-128.1	-126.2	-127.5	118.8	120.9	116.9	127.7	119.4	122.0
φ_6' [C9-N-C10-C11]	-146.8	-147.5	-145.3	158.5	142.5	142.0	144.9	156.7	132.7

[a] This large deviation from the other dihedral angles for enantiomer B causes a short contact between a pair of hydrogen atoms from C6' and C6'' methyl groups (H-H distance 0.72 Å) due to crystal packing effects for structure from Ref. [37]. This is not observed for the other X-Ray structures. Stereoisomer A (free AZM) exhibits larger H-H distances than B (interacting with biological targets).

Table S3. ωB97x-D/6-31G(d, p) (PCM-5$CHCl_3$) optimized torsion angles (º) for stereoisomers A and B of azithromycin. Sugar rings and 15-membered macrolide unit torsion angles are placed separately. O-H H-bond distances (Å) are given. X-Ray data are also given.

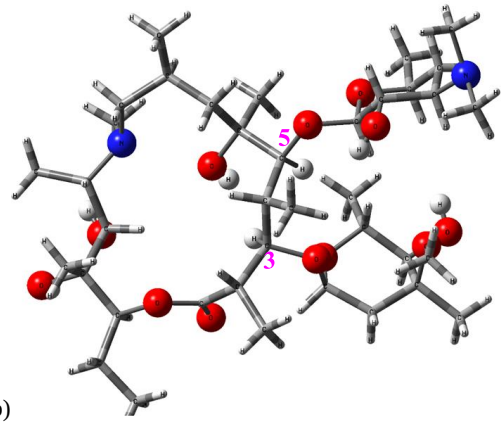
Structures	**M2-A PCM-OPT-5$CHCl_3$**	**X-Ray Str-A**[a] Mono-Hyd-Pure	**X-Ray Str-A**[b] Di-Hyd-Pure	**Deviation**[c]	**M2-B PCM-OPT-5$CHCl_3$**	**X-Ray Str-B**[d] **Di-Hyd-Pure**	**X-Ray Str-B**[e] **Complexed-Protein**	**Deviation**[f]
	Stereoisomer "A"				**Stereoisomer "B"**			
	Dihedral Angle (º): Sugar rings							
φ_1 [C1'-O-C5-C6]	-132.4	-124.5	-124.6	-7.9	124.8	133.3	130.9	8.5
φ_2 [C1''-O-C3-C4]	-143.1	-154.7	-154.2	11.6	144.3	145.2	146.1	0.9
φ_3 [O-C1'-O-C5]	76.8	77.6	77.7	-0.8	-81.2	-84.5	-67.7	-3.3
φ_4 [O-C1''-O-C3]	77.8	73.1	72.2	4.7	-76.2	-82.5	-85.1	-6.3
φ_{1a} [O-C5-C6-C7]	-46.8	-51.6	-51.7	4.8	44.3	52.4	39.2	8.1
φ_{2a} [O-C3-C4-C5]	58.8	73.6	74.9	-14.8	-67.1	-59.4	-53.1	7.7
φ_{1b} [C2'-C1'-O-C5]	-164.6	-162.1	-161.7	-2.5	158.5	156.3	169.9	-2.2
φ_{2b} [C2''-C1''-O-C3]	-155.2	-160.5	-161.3	5.3	157.5	152.5	147.7	-5
	Dihedral Angle (º): 15-membered macrocycle							
φ_1' [C4-C5-C6-C7]	78.6	71.2	71.1	7.4	-80.4	-71.9	-81.7	8.5
φ_2' [C2-C3-C4-C5]	-178.6	-162.9	-163.2	-15.7	169.8	177.2	-178.5	7.4
φ_3' [O-C1-C2-C3]	-115.2	-125.7	-126.3	10.5	124.1	121.9	125.8	-2.2
φ_4' [C12-C13-O-C1]	-100.6	-128.1	-126.2	27.5	99.5	118.8	120.9	19.3
φ_5' [C10-C11-C12-C13]	-170.5	-156.6	-157.9	-13.9	171.3	161.4	158.6	-9.9
φ_6' [C9-N-C10-C11]	-150.4	-146.8	-147.5	-3.6	145.1	158.5	142.5	13.4
	Dihedral Angle (º): O-H Groups							
γ_1' [H-O-C2'-C1']	-51.3	179.9	-0.1	-128.6	57.5	84.2	-61.1	26.7
γ_2' [H-O-C4''-C3'']	-46.0	-123.6	-124.4	77.6	37.6	20.8	60.3	-16.8
γ_3' [H-O-C6-C5]	111.8	-58.4	-59.9	53.4	-123.6	-118.4	-61.0	5.2
γ_4' [H-O-C11-C10]	144.8	179.8	0.7	-35	-144.6	-109.3	65.0	35.3
γ_5' [H-O-C12-C11]	36.9	55.7	55/0	-18.8	-38.1	-47.1	180.0	-9
	H-bond Distance (Å): O-H Groups							
	M2-A PCM-OPT-5$CHCl_3$	**X-Ray Str-A** Mono-Hyd-Pure	**X-Ray Str-A** Di-Hyd-Pure	-	**M2-B PCM-OPT-5$CHCl_3$**	**X-Ray Str-B Di-Hyd-Pure**	**X-Ray Str-B** Complexed-Protein	-
C2'-OH…O	2.33	3.56 (NO H-Bond)	2.50 (NO H-Bond)	-	2.43	2.75 (NO H-Bond)	NO H-Bond	-
C4''-OH...O	2.16	3.03 (NO H-Bond)	3.01 (NO H-Bond)	-	2.09	2.22	NO H-Bond	-
C6-OH…N	1.82	3.29 (NO H-Bond)	3.28 (NO H-Bond)	-	1.76	1.98	2.62 NO H-Bond	-
C11-OH…O	1.88	3.55 (NO H-Bond)	2.96 (NO H-Bond)	-	1.84	3.39 (NO H-Bond)	2.27	-
C12-OH…O	2.10	2.21	2.18	-	2.12	2.20	NO H-Bond	-

[a] See Ref. [7]
[b] See Ref. [41]
[c] Deviation with respect to X-Ray data from Ref. [7]
[d] See Ref. [40]
[e] See Ref. [8]
[f] Deviation with respect to X-Ray data from Ref. [35]



(a)

XR1-A: **Stereoisomer -A (C3R-C5S) Ref-[7]**



(b)

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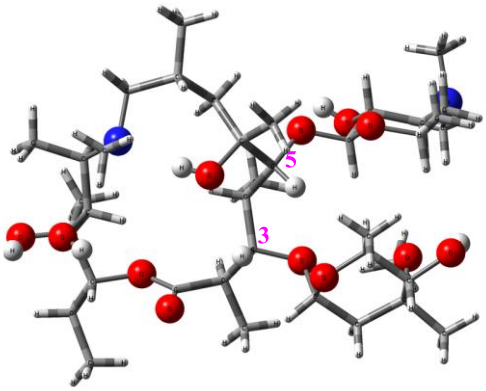
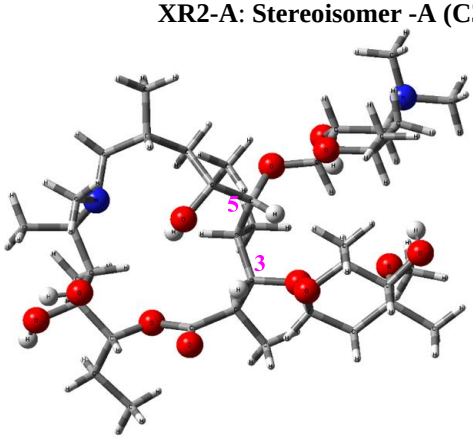


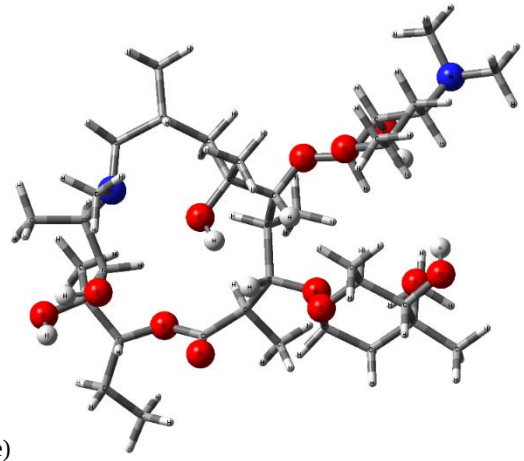
(c)

XR4-B: **Stereoisomer-B (C3S-C5R) Ref-[40]**



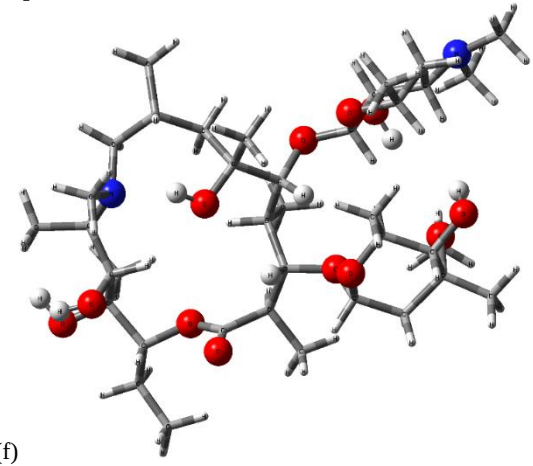
(d)

XR5-B: **Stereoisomer--B (C3S-C5S) Ref-[8]**



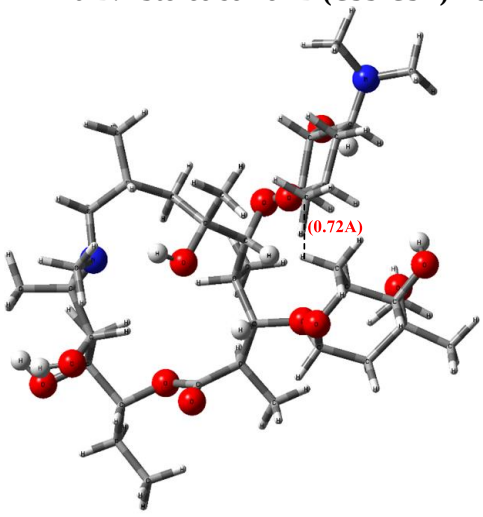
(e)

XR6-B: **Stereoisomer-B (C3S-C5R) Ref-[9]**



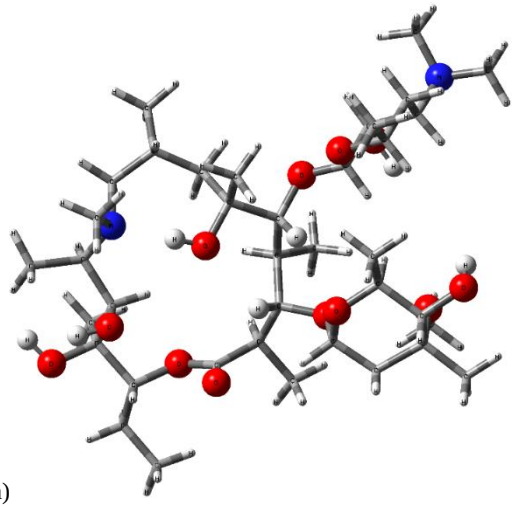
(f)

XR7-B: **Stereoisomer-B (C3S-C5S) Ref-[36]**



(g)

XR8-B: **Stereoisomer-B (C3S-C5R) Ref-[37]**



(h)

XR9-B: **Stereoisomer-B (C3S-C5S) Ref-[38]**

Figure S4. Azithromycin X-Ray structures for stereoisomer A (a,b) and B (c-h) B. Crystallographic data can be found in the references indicated in brackets. There is a short contact between a pair of hydrogen atoms from C6’ and C6’’ methyl groups (H-H distance 0.72 Å) for structure **XR7-B** due to crystal packing

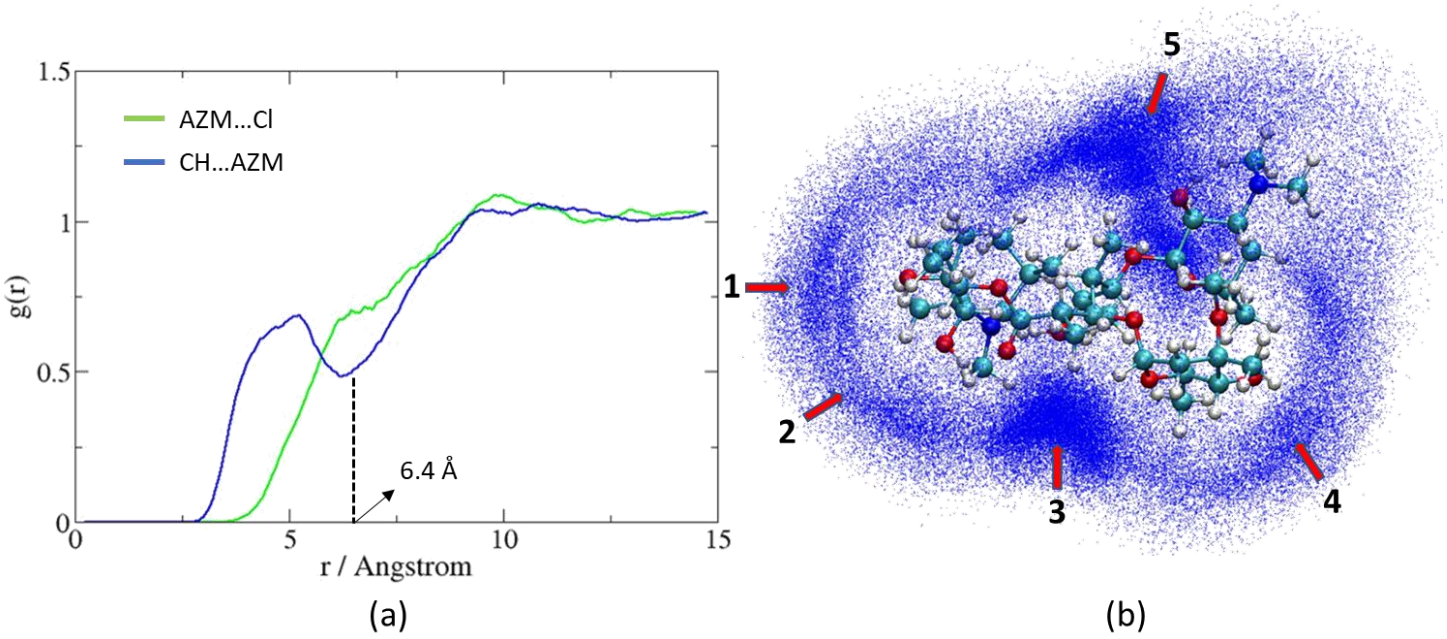


Figure S5. (a) Radial distribution function – g(r) – between AZM (solute) and $CHCl_3$ (solvent). The geometric center was used for the solute and H (blue line) or Cl (green line) for $CHCl_3$. (b) Spatial density map representing the overlap of 5000 frames with one solute and 5 $CHCl_3$ molecules each. The numbers indicate the most probable regions of each solvent molecule. Only the CH…AZM curve showed a peak centered at ~5 Å, which is due to the fact that $CHCl_3$ acts as H-donor. The integral of the first peak up to 6.4 Å gave 4 - 5 $CHCl_3$ molecules on average, which might be assigned as a 'micro solvation-shell'. The second solvation shell extends up to 10 Å and includes 24 solvent molecules. It is notable that the density of molecules is localized in some specific regions, close to the O atoms, which might be used as reference to include explicit solvent molecules.

Experimental Procedure

A sample of solid azithromycin was purchased and later extracted from compounding pharmacy pill. Thin-layer chromatography (TLC) was performed on TLC plates (silica gel 60 F254) and visualized by an ethanolic solution of sulfuric acid (20%), under heating, in order to confirm its purity. The ^{1}H NMR was measured on a Bruker Avance III HD magnetic resonance spectrometer and was recorded at 500 MHz and ^{13}C were recorded at 125 MHz. Chemical shifts were reported as δ (parts per million - ppm) relative to the signals of $CDCl_3$ at 7.26 ppm (singlet) and 77.0 (triplet) for ^{1}H and ^{13}C, respectively. Tetramethylsilane (TMS) was established as an internal reference. NMR chemical shifts are reported employing the following peak abbreviation pattern: s, singlet; d, doublet; dd, double doublet; t, triplet; tdd, triplet of doublet of doublets; dq, doublet of quartets; dt, doublet or triplet; dqd, a double quartet of doublet; ddd, double double doublet; and m, multiplet.

The ^{1}H NMR spectrum was deconvoluted with the aim of identifying the most complex signals, as well as identifying the hydroxyl 6-OH. The methyl groups were identified as (Me) and the hydrogen atoms were identified according to **Scheme 1**.

Characterization data for Azithromycin

^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.29 (s, 1H, 6-OH)*, 5.18 (d, *J* = 4.7 Hz, 1H, H-1”), 4.99 (s, 1H, 11-OH), 4.72 (dd, *J* = 9.8, 2.4 Hz, 1H, H-13), 4.46 (d, *J* = 7.3 Hz, 1H, H-1’), 4.28 (dd, *J* = 3.5, 1.9 Hz, 1H, H-3)*, 4.11 (dq, *J* = 11.9, 6.0 Hz, 1H, H-5”), 3.71 (s, 1H, H-5), 3.67 (d, *J* = 7.3 Hz, 1H, H-11), 3.54 (tdd, *J* = 13.0, 6.5, 2.1 Hz, 1H, H-5’), 3.36 (s, 3H Me-8”), 3.27 (dd, *J* = 10.0, 7.4 Hz, 1H, H-2’), 3.06 (d, *J* = 8.0 Hz, 1H, H-4”)*, 2.99 (s, 1H, 12-OH), 2.73 (m, 1H, H-2)*, 2.73 (m, 1H, H-10)*, 2.56 (d, *J* = 10.70 Hz, 1H, H-9a), 2.50 (m, 1H, H-3’)*, 2.38 (d, *J* = 15.3 Hz, 1H, H-2”a), 2.34 (s, 3H, Me-20), 2.33 (s, 6H, Me-7’, Me-8’), 2.20 (s, 1H, 2’-OH)*, 2.12 – 2.01 (m, 3H, H-8, H-9, 4”-OH), 1.98 (qd, 1H, *J* =7.5, 1.4 Hz, H-4) 1.92 (dqd, *J* = 14.8, 7.3, 2.3 Hz, 1H, H-14a)*, 1.81 (d, *J* = 14.6 Hz, 1H, H-7a), 1.70 (d, *J* = 11.9 Hz, 1H, H-4’a), 1.61 (dd, *J* = 15.2, 5.0 Hz, 1H, H-2”b), 1.48 (ddq, *J* = 14.2, 9.6, 7.2 Hz, 1H, H-14b), 1.37 – 1.30 (m, 7H, Me-18, Me-6”, H-7), 1.29 (d, *J* = 6.4 Hz, 1H, H-4’b), 1.27 (s, 3H, Me-7”), 1.25 (d, *J* = 6.1 Hz, 3H, Me-6’), 1.21 (d, *J* = 7.4 Hz, 3H, Me-16), 1.14 – 1.10 (m, 6H, Me-21, Me-22), 1.06 (d, *J* = 7.5 Hz, 3H, Me-17), 0.93 (d, *J* = 6 .2 Hz, 3H, Me-19), 0.91 (t, J = 7.3 Hz, 3H, Me-15). **^{13}C NMR (125 MHz, $CDCl_3$) δ**:178.78, 102.99, 94.54, 83.46, 78.14, 77.47, 77.44, 74.29, 73.8, 73.67, 72.99, 70.87, 70.08, 68.71, 65.63, 65.48, 62.37, 49.47, 45.29, 42.35, 42.11, 40.34, 35.8, 34.77, 28.82, 27.51, 26.79, 21.67, 21.34, 21.28, 21.20, 18.22, 16.18, 14.82, 11.27, 9.10, 7.33.

* Signals of deconvoluted spectrum

Table S4: Identification and comparison of experimental data with literature data[1]

	Ref B	A2	J (A2) / Hz	Multiplicity	Δδ
1'	4.47	4.46	7.30	d	-0.01
2'	3.23	3.27	10.0, 7.4	d	0.04
3'	2.48	2.50	-	m	0.02
4'a	1.69	1.70	11.9	d	0.01
4'b	1.27	1.29	6.4	d	0.02
5'	3.52	3.54	13.0, 6.5, 2.1	tdd	0.02
6'	1.21	1.25	6.10	d	0.04
7'/8'	2.30	2.33	-	s	0.03
1''	5.13	5.18	4.70	d	0.05
2”a	2.39	2.38	15.3	d	-0.01
2”b	1.59	1.61	15.2, 5.0	dd	0.02
4”	3.07	3.06	8.0	d	-0.01
5”	4.12	4.11	11.9, 6.0	dq	-0.01
6”	1.34	1.35	6.40	d	0.01
7”	1.27	1.27	-	s	0
8”	3.34	3.36	-	s	0.02
2	2.76	2.73	-	m	-0.03
3	4.30	4.28	3.5, 1.9	dd	-0.02
4	1.99	1.98	7.5, 1.4	qd	-0.01
5	3.65	3.71	-	s	0.06
7a	1.80	1.81	14.60	d	0.01
7b	1.33	1.35	-	m	0.02
8	2.01	2.05	-	m	0.04
9a	2.55	2.56	10.70	d	0.01
9b	2.09	2.05	-	m	-0.04
10	2.70	2.73	m	-	0.03
11	3.70	3.67	7.30	d	-0.03
13	4.68	4.72	9.8, 2.4	dd	0.04
14a	1.89	1.92	14.8, 7.3, 2.3	dqd	0.03
14b	1.52	1.48	14.6, 9.6, 7.3	ddq	-0.04
15	0.89	0.91	7.30	t	0.02
16	1.19	1.21	7.40	d	0.02
17	1.05	1.06	7.50	d	0.01
18	1.32	1.35	-	m	0.03
19	0.92	0.93	6.20	d	0.01
20	2.32	2.34	-	s	0.02
21	1.08	1.11	-	m	0.03
22	1.09	1.11	-	m	0.02
2'-OH	2.60	2.20	-	s	-0.4
4'' -OH	2.10	2.04	-	s	-0.06
6-OH	9.40	7.29	-	s	-2.11
11-OH	5.20	4.99	-	s	-0.21
12-OH	3.20	2.99	-	s	-0.21

^{1}H, ^{13}C NMR, COSY and NOESY

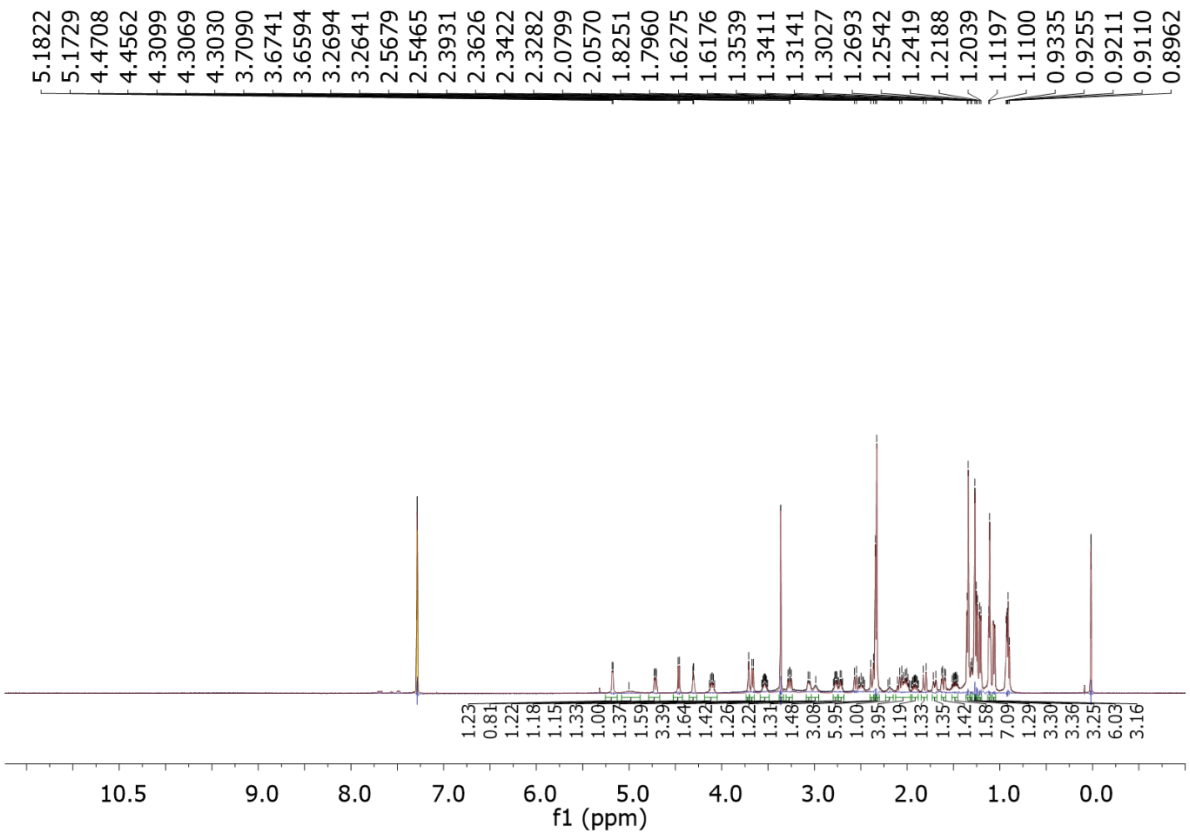


Figure S6. ^{1}H NMR spectrum of **Azithromycin** (500 MHz, $CDCl_3$).

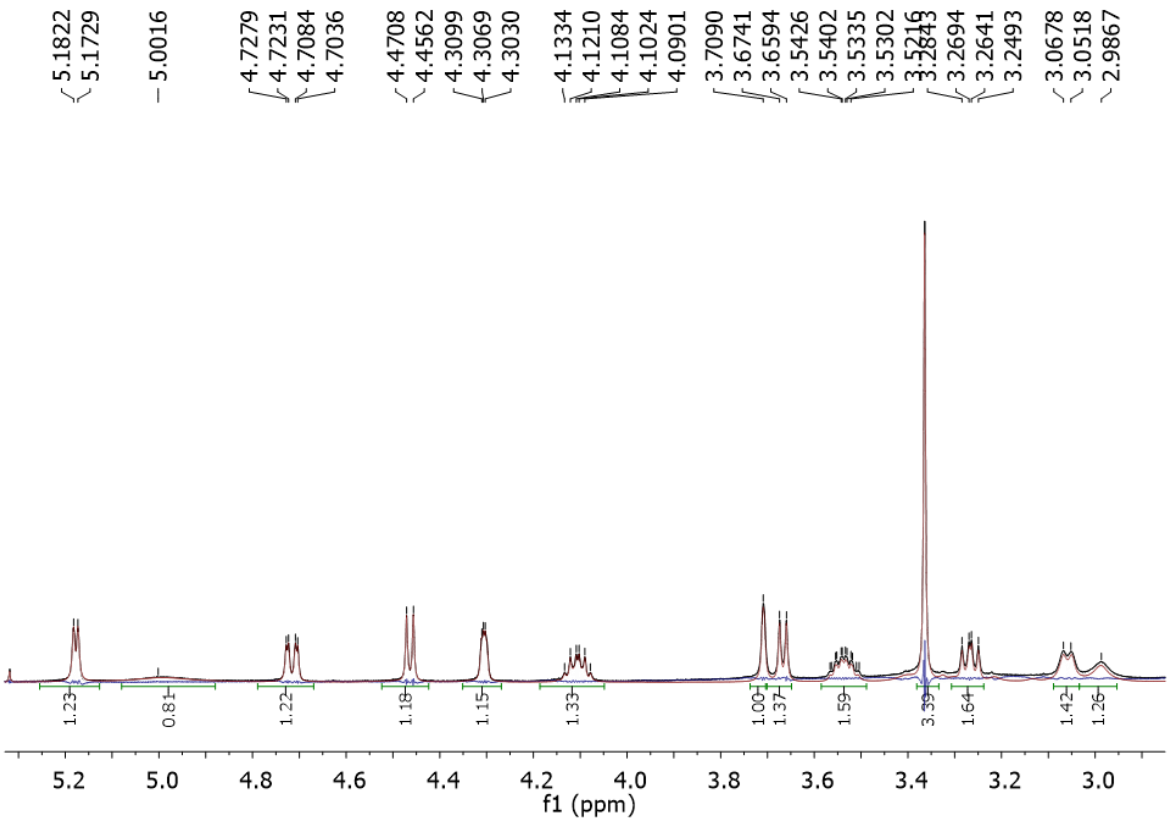


Figure S7. Expanded ^{1}H NMR spectrum of **Azithromycin** ranging from 5.2 to 3.0 ppm

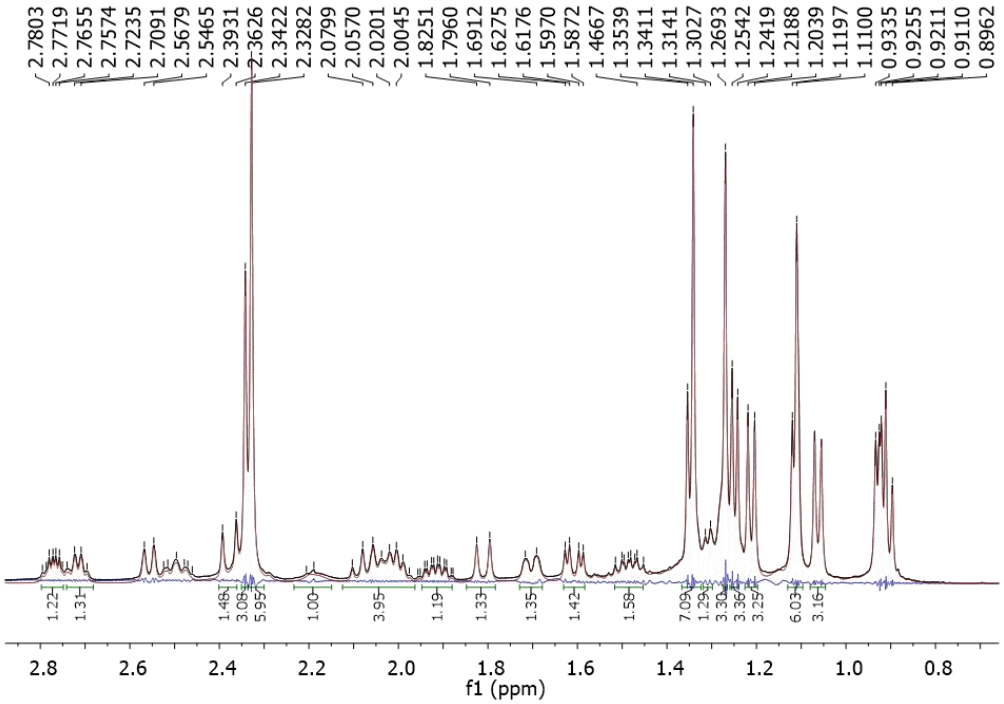


Figure S8. Expanded [1]H NMR spectrum of **Azithromycin** ranging from 3.0 to 0.8 ppm

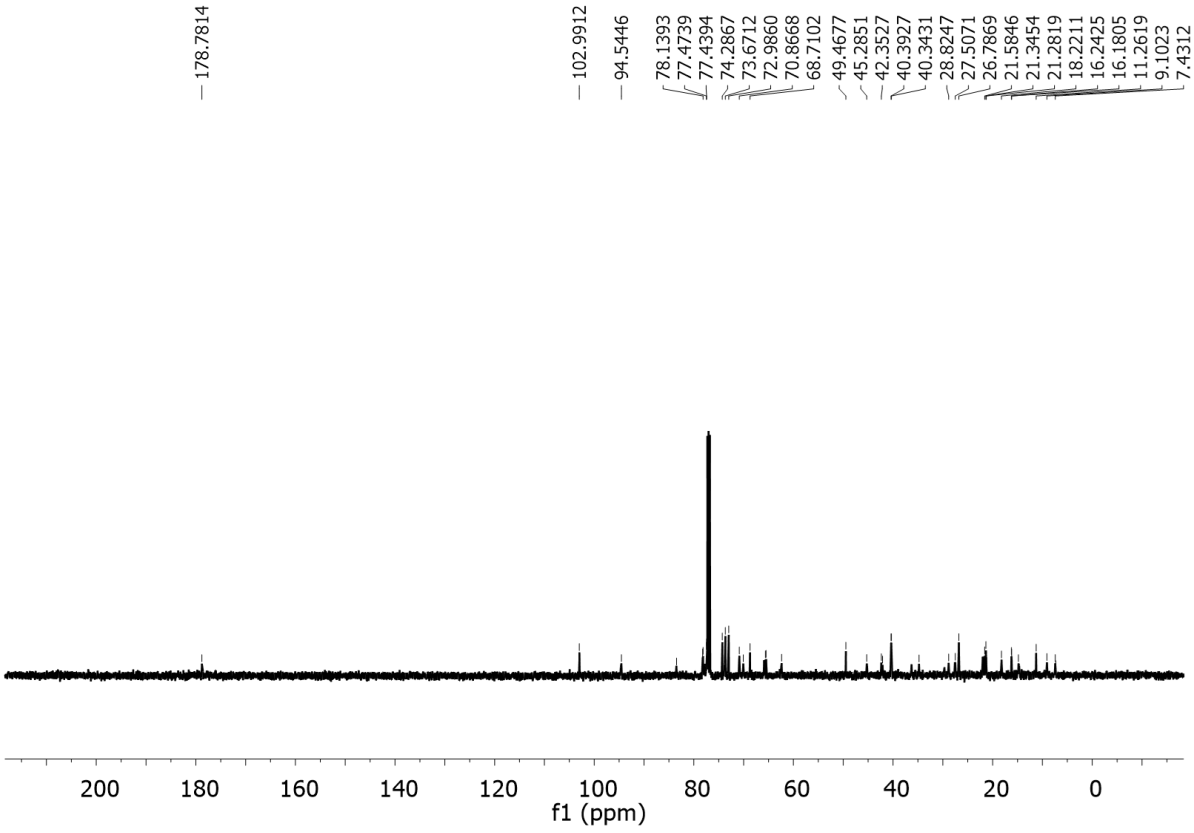


Figure S9. [13]C NMR spectrum of **Azithromycin** (125 MHz, $CDCl_3$)

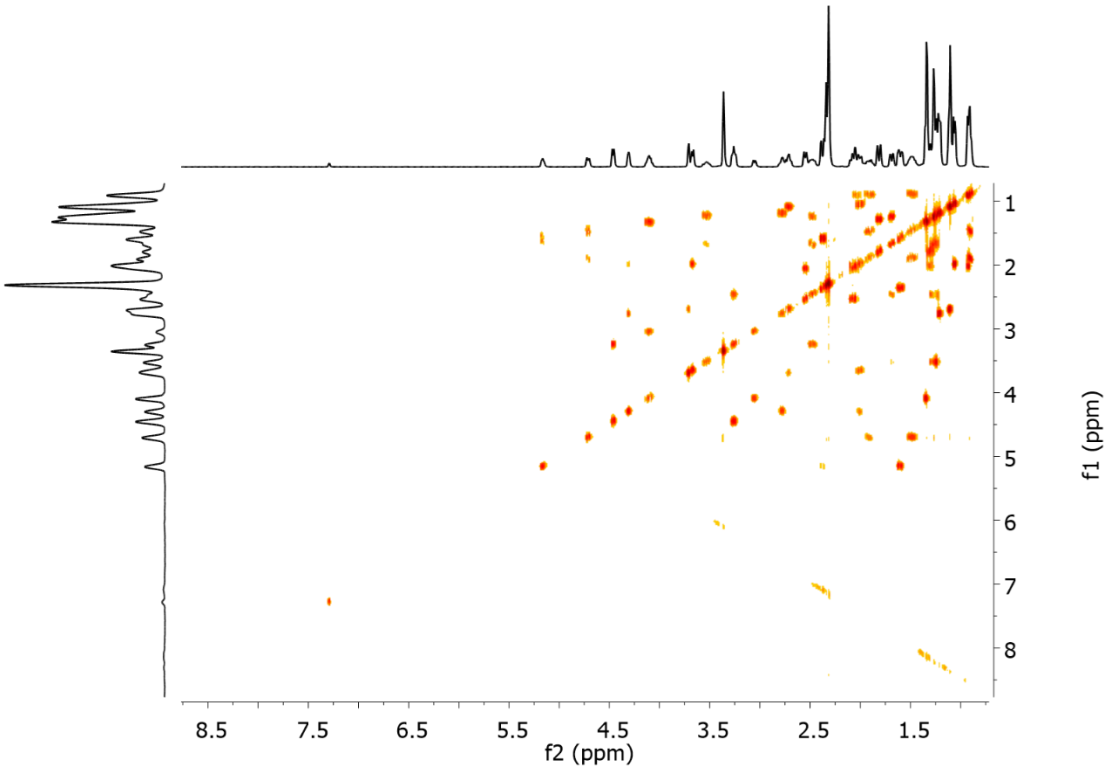


Figure S10. COSY contour map of **Azithromycin**.

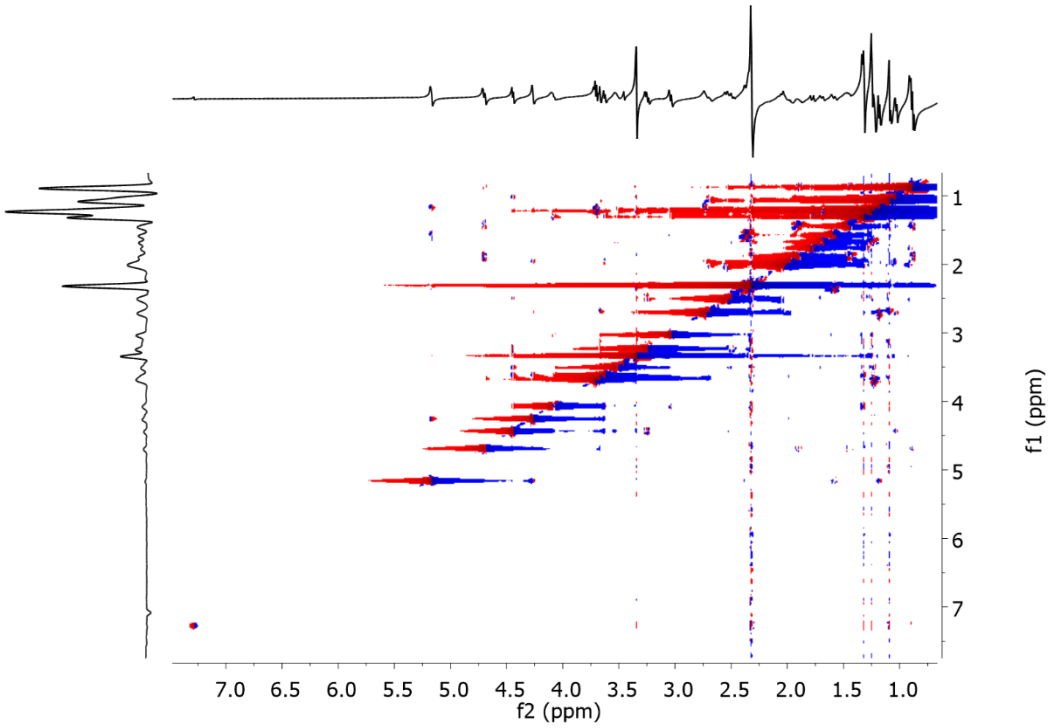


Figure S11. NOESY contour map of **Azithromycin**.

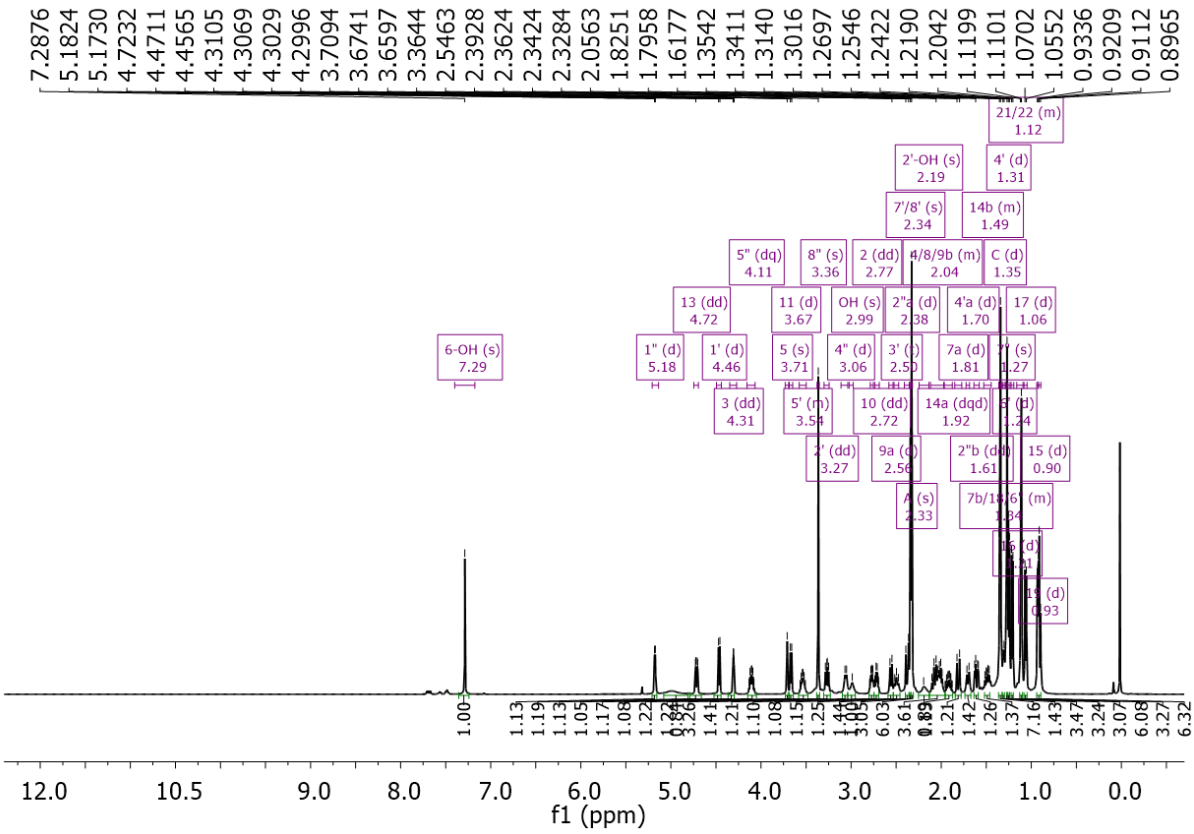


Figure S12. Deconvoluted ^{1}H NMR spectrum of **Azithromycin** (500 MHz, $CDCl_3$).