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Supporting Information

for

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Binding Mode Determination of Benzimidazole Inhibitors of the Hepatitis C Virus RNA Polymerase Using a Structure and Dynamics Strategy

Steven LaPlante et al.

Three appendices are included which provide supporting information and experimental details.

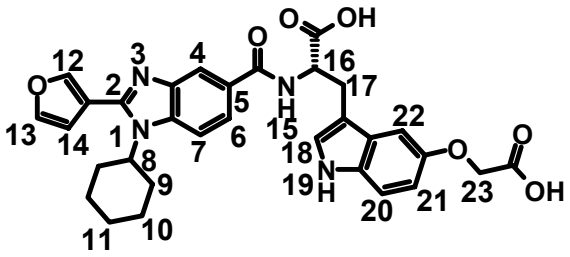
The syntheses and purification of the class of compounds described here have been reported elsewhere (ref. 7 and 8, US patents 6,448,281 and 6,479,508). NMR verification of the primary structures of the compounds is provided in Appendix 1 of the Supplementary Materials. The *in vitro* enzymatic assay used to determine the IC_{50} values has been published in ref. 4 and in US patents 6,448,281 and 6,479,508, and employed the His tagged HT-NS5B HCV polymerase construct. The IC_{50} values are an average of a minimum of three separate tests with a coefficient of variation below 25%.

A description of the biomolecular NMR experiments is provided in Appendix 2 of the Supplementary Materials. Also included are transferred NOESY spectra for compounds **1** and **2**, ROESY data for **2**, ^{13}C T_1 for free compounds **2** and **3** in DMSO solvent, and ^{13}C T_1 and transferred ^{13}C T_1 for compound **2** in aqueous buffer. All biomolecular NMR studies employed the NS5BΔ21C-HT construct[4] which is C-terminally truncated by 21 residues and capped with six His residues. A detailed comparison of the binding affinities of compound **2** versus various polymerase constructs has been described elsewhere.[4]

The molecular modeling procedures and data are given in Appendix 3 of the Supplementary Materials. This includes a description of the restrained simulated annealing calculations and the *ab initio* calculations. ROESY NMR data of an aza analogue is also included.

Appendix 1:

Included are the NMR spectra and resonance assignments of the compounds discussed in this paper, with the exception of compound **1** which has been characterized elsewhere.[7] The NMR spectra were acquired for the compounds dissolved in DMSO-d_6 solvent at 400 MHz and 300K. However, NMR spectra of compound **5** were acquired in aqueous solvent.



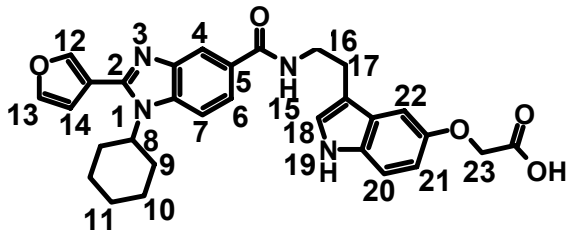
Compound 2

NMR resonance assignments and relaxation data.

^{1}H NMR Chemical Shifts, ^{13}C Chemical Shifts, and ^{13}C NT_1 times[a]

Atom ID	^{1}H δ	^{13}C δ	^{13}C NT_1
4	8.20	117.17	0.24
6	7.78	122.59	0.22
7	7.99	113.16	0.25
8	4.46	57.24	0.31
9	1.94, 2.27	30.31	0.40
10	1.43, 1.87	25.33	0.38
11	1.43, 1,67	24.27	0.24*
12	8.36	144.37	0.69
13	7.99	144.76	0.44
14	6.98	110.98	0.82
15	8.75		
16	4.61	54.07	0.28
17	3.17-3.28	26.57	0.28*
18	7.20	124.43	0.24
19	10.70		
20	7.21	111.99	0.24
21	6.72	111.30	0.24
22	7.15	101.43	0.24
23	4.61	65.36	0.59

[a] NMR data were collected in DMSO-d_6 solvent. Chemical shifts are given in ppm and are referenced to the DMSO-d_6 peak at 2.5 ppm. For the ^{13}C NT_1 data, N = number of attached hydrogens as applied in the NT_1 longitudinal relaxation times (in seconds).



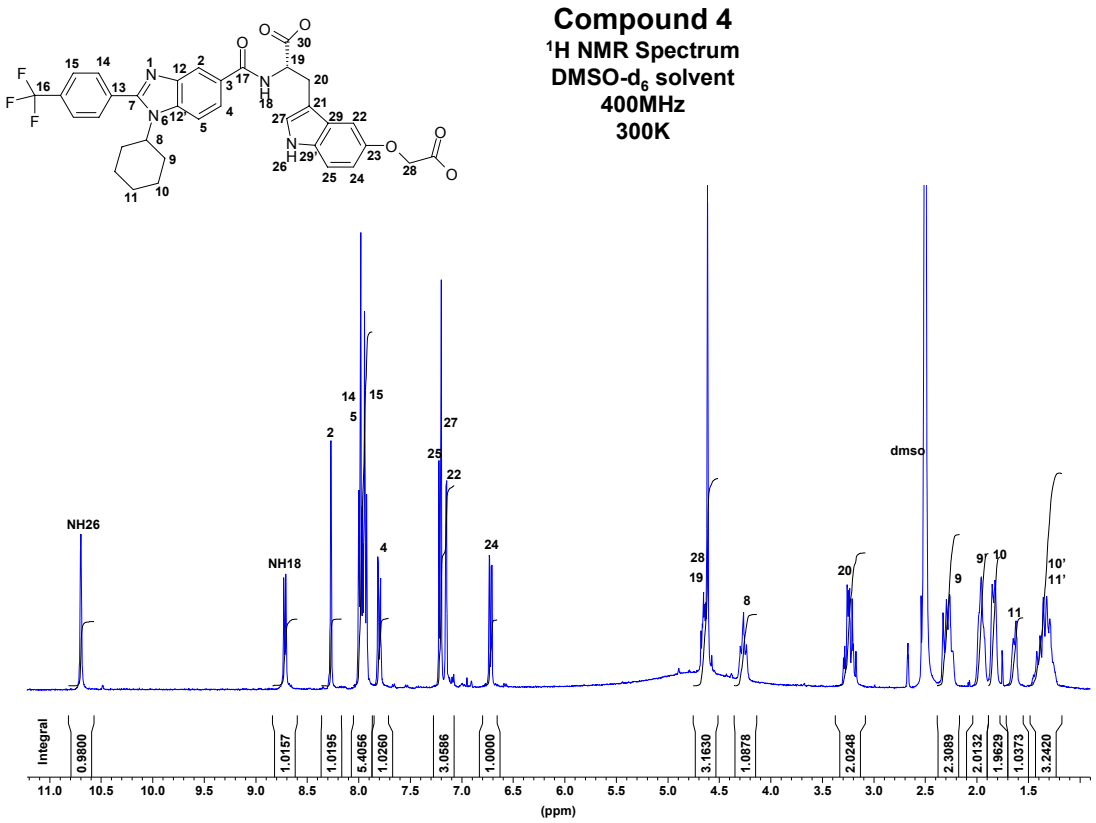
Compound 3

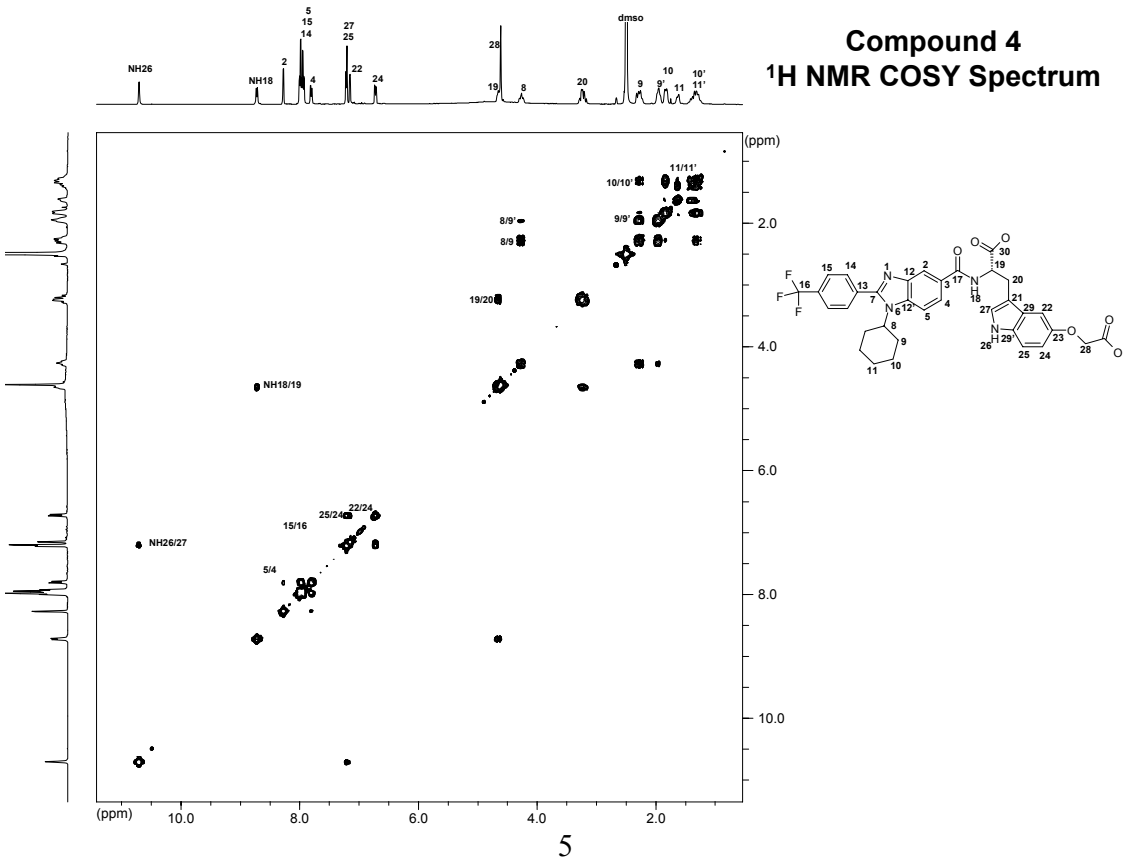
NMR resonance assignments and relaxation data.

^{1}H NMR Chemical Shifts, ^{13}C Chemical Shifts, and ^{13}C NT_1 times[a]

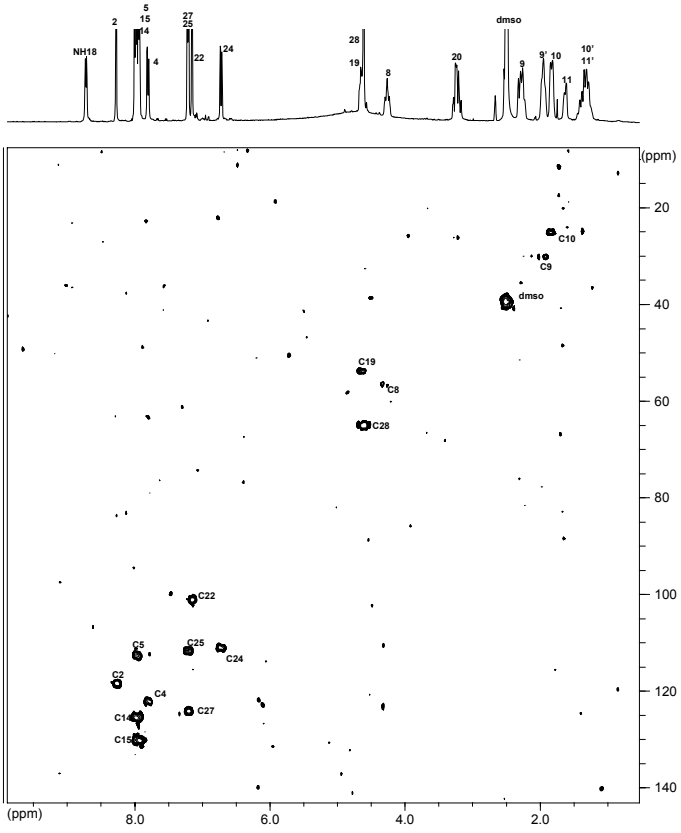
Atom ID	^{1}H δ	^{13}C δ	^{13}C NT_1
4	8.21	116.85	nd
6	7.85	122.32	0.23
7	7.99	113.13	0.27
8	4.47	57.19	0.28
9	1.96, 2.30	30.31	0.40
10	1.44, 1.88	25.30	0.30*
11	1.44, 1,69	24.25	0.26*
12	8.01	144.30	
13	7.99	144.41	0.46
14	6.99	111.00	0.89
15	8.67		
16	3.57	40.25	0.82
17	2.94	25.10	0.38*
18	7.17	123.52	0.27
19	10.69	111.94	0.24
20	7.25	111.93	0.26
21	6.75	111.18	0.26
22	7.06	101.62	0.26
23	4.59	65.38	0.68

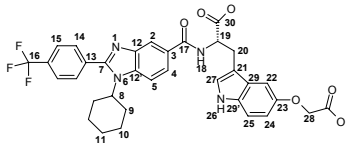
[a] NMR data were collected in DMSO-d_6 solvent. Chemical shifts are given in ppm and are referenced to the DMSO-d_6 peak at 2.5 ppm. For the ^{13}C NT_1 data, N = number of attached hydrogens as applied in the NT_1 longitudinal relaxation times (in seconds).


Compound 4
1H NMR Spectrum
DMSO-d6 solvent
400MHz
300K
NH26
NH18
dmso
Integral
0.9800
1.0157
1.0195
5.4056
1.0260
3.0586
1.0000
3.1630
1.0878
2.0248
2.3089
2.0132
1.9629
1.0373
3.2420
(ppm)

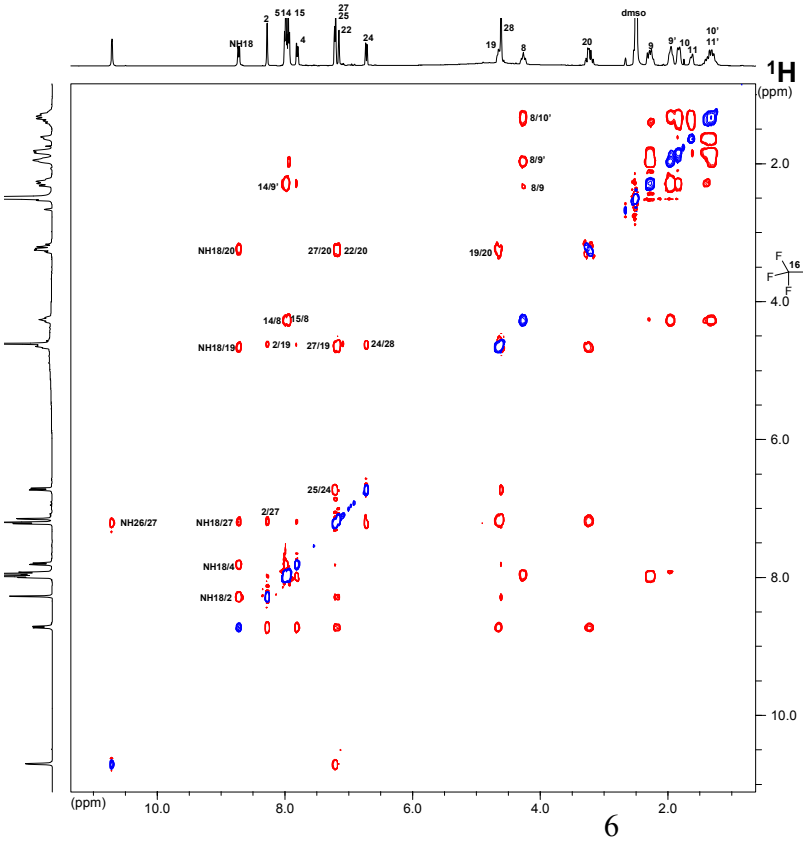

Compound 4
1H NMR COSY Spectrum
NH26
NH18
dmso
(ppm)
11/11'
10/10'
9/9'
8/9'
8/9
19/20
NH18/19
25/24
22/24
15/16
NH26/27
5/4

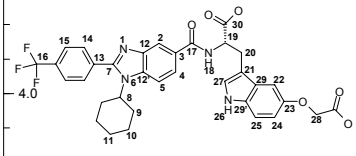
Compound 4
^{1}H-^{13}C NMR HMQC Spectrum

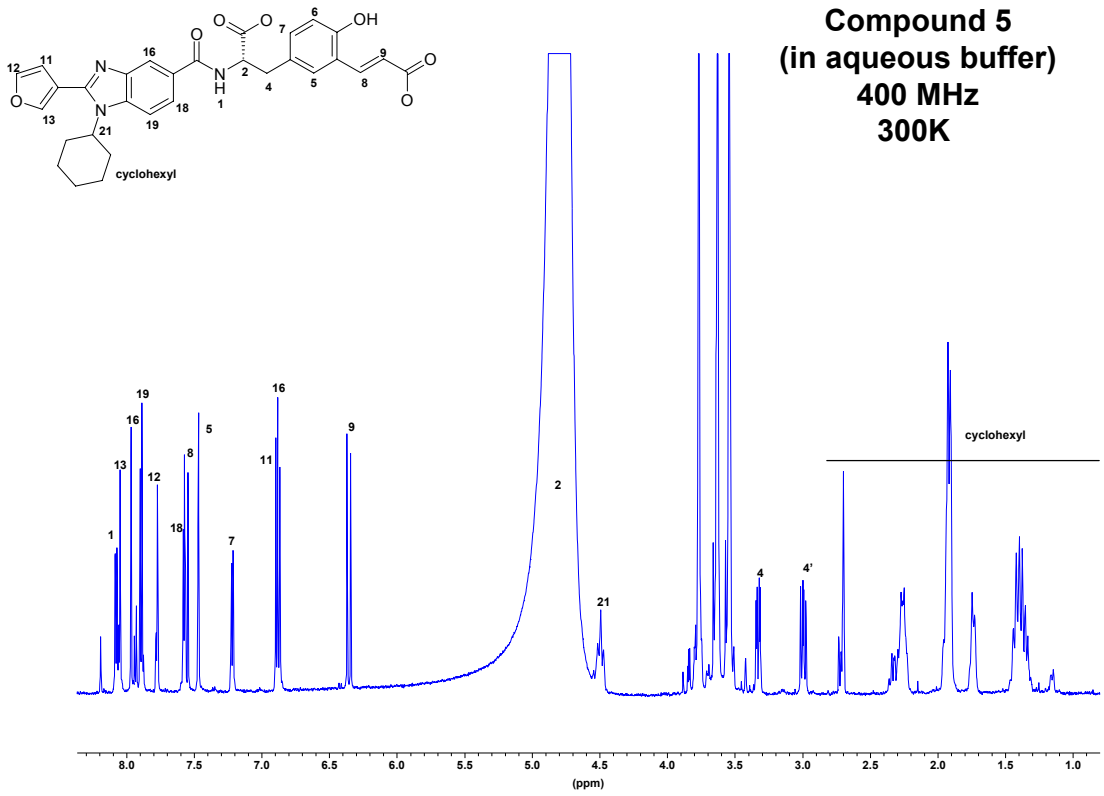


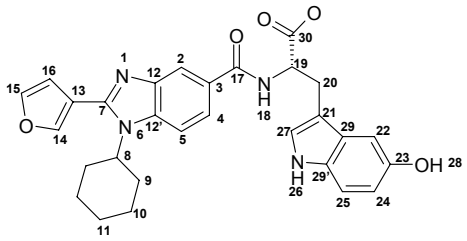


Compound 4
^{1}H NMR ROESY Spectrum

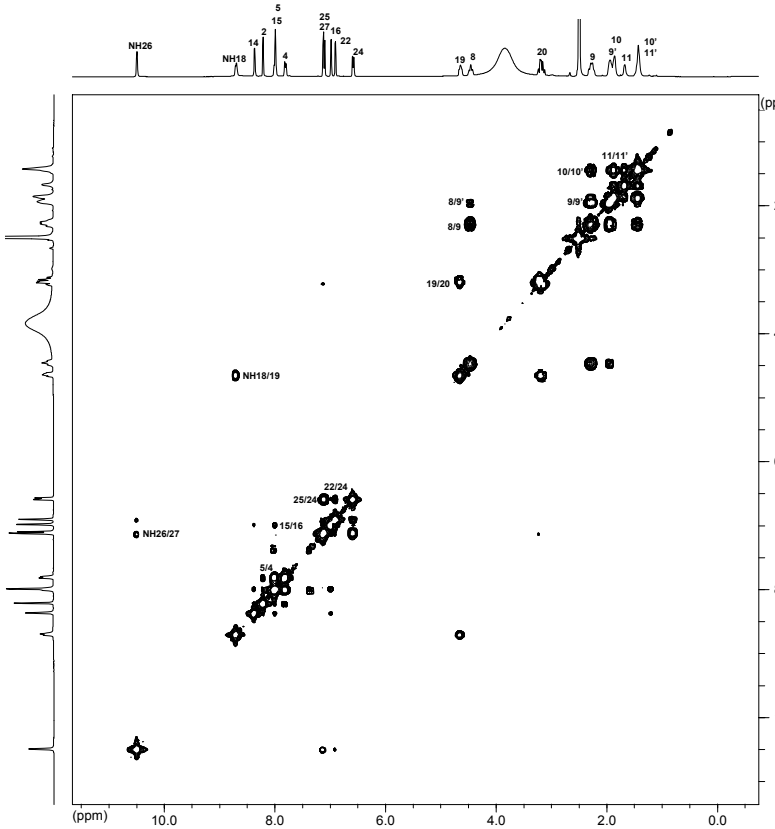




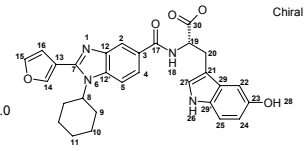

Compound 5
(in aqueous buffer)
400 MHz
300K
cyclohexyl
cyclohexyl
(ppm)



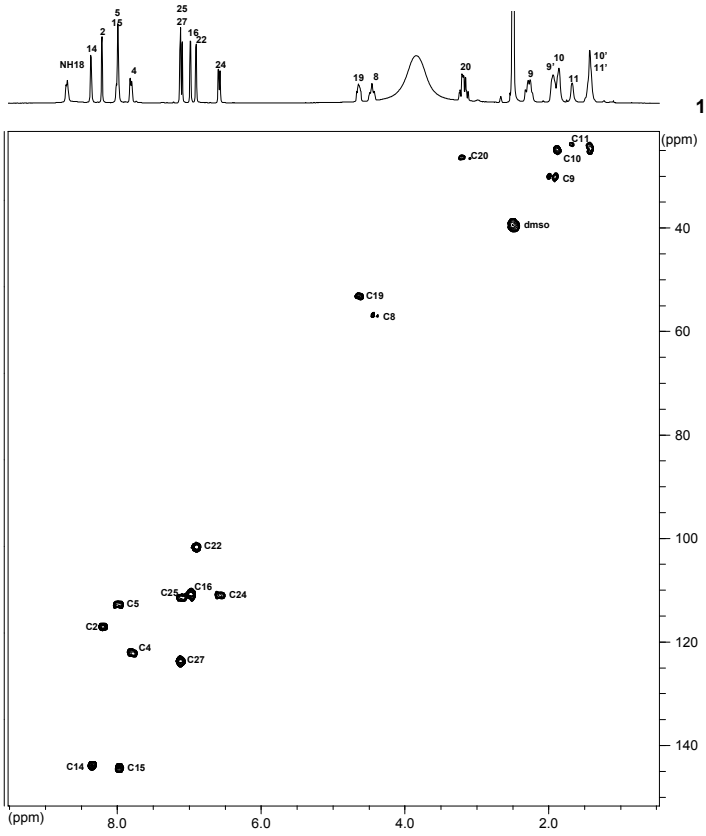
Compound 6
^{1}H NMR Spectrum
DMSO-d_6 solvent
400MHz
300K

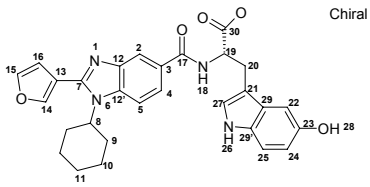


Compound 6
^{1}H NMR COSY Spectrum

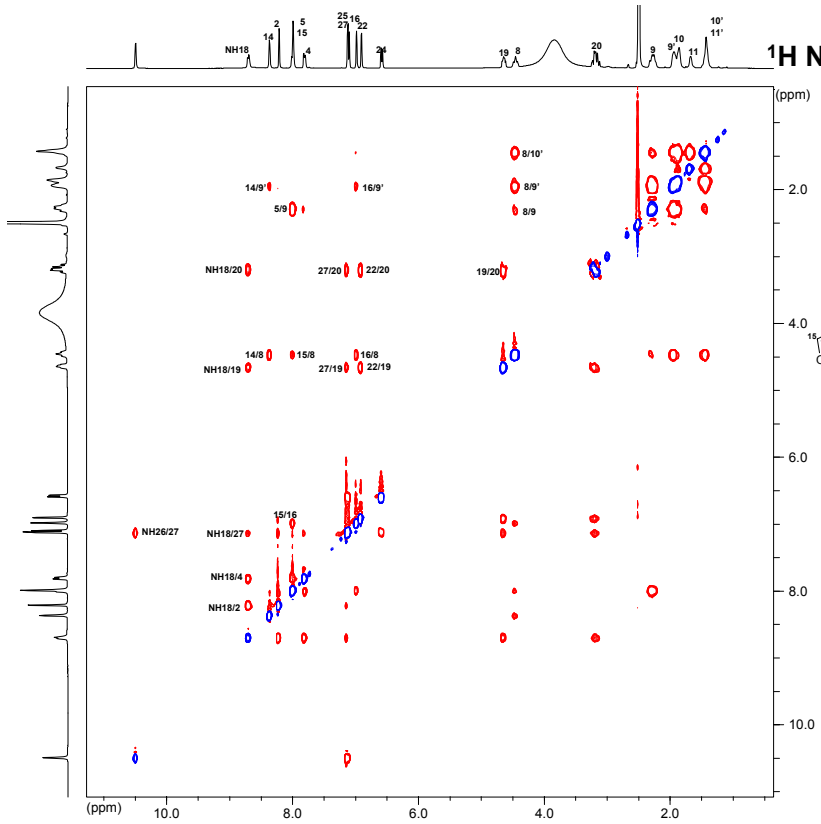


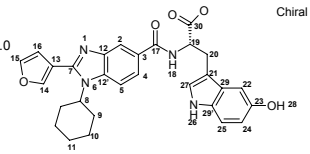
Compound 6
^{1}H-^{13}C HMQC NMR Spectrum

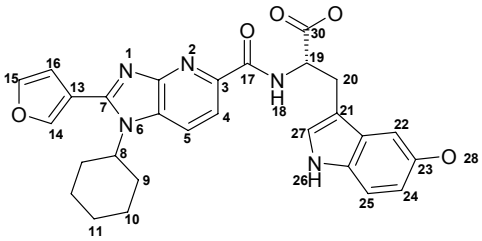




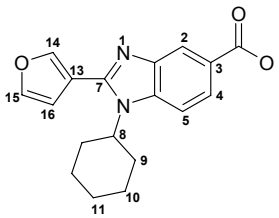
Compound 6
^{1}H NMR ROESY Spectrum



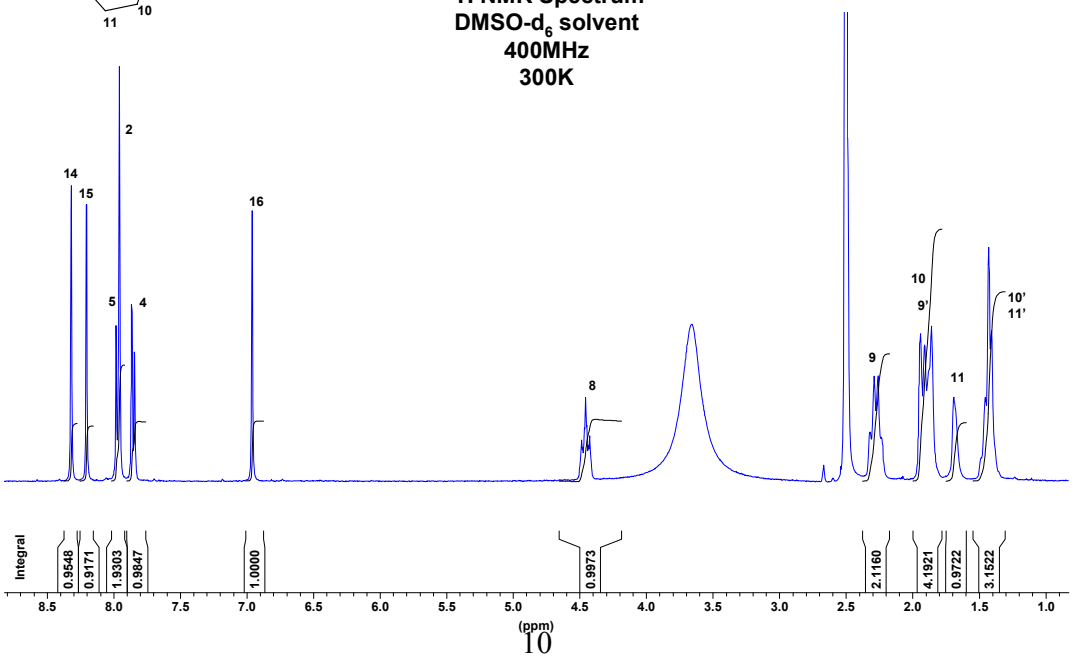


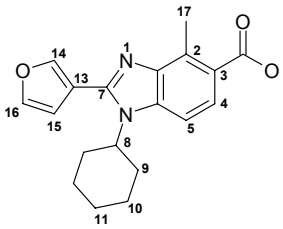


Compound 7
^{1}H NMR Spectrum
DMSO-d_6 solvent
400MHz
300K

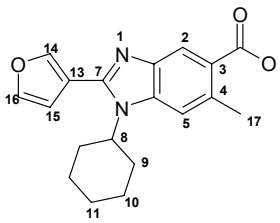


Compound 8
^{1}H NMR Spectrum
DMSO-d_6 solvent
400MHz
300K

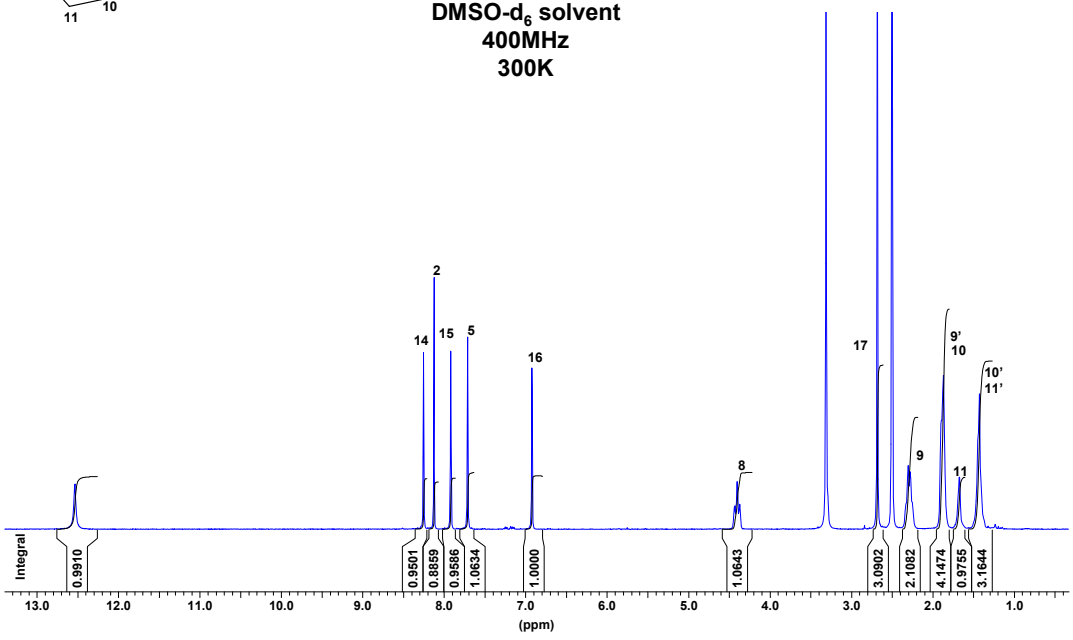




Compound 9
^{1}H NMR Spectrum
DMSO-d_6 solvent
400MHz
300K



Compound 10
^{1}H NMR Spectrum
DMSO-d_6 solvent
400MHz
300K



Appendix 2:

List of Contents

Appendix 2A:

A description of the experimental procedures used for NMR experiments is provided.

Appendix 2B:

Transferred NOESY spectrum of compound **1** in the presence of HCV polymerase.

Appendix 2C:

Transferred NOESY and ROESY spectra of compound **2**.

Appendix 2D:

^{13}C T_1 of free compounds **2** and **3**, and transferred ^{13}C T_1 of compound **2**.

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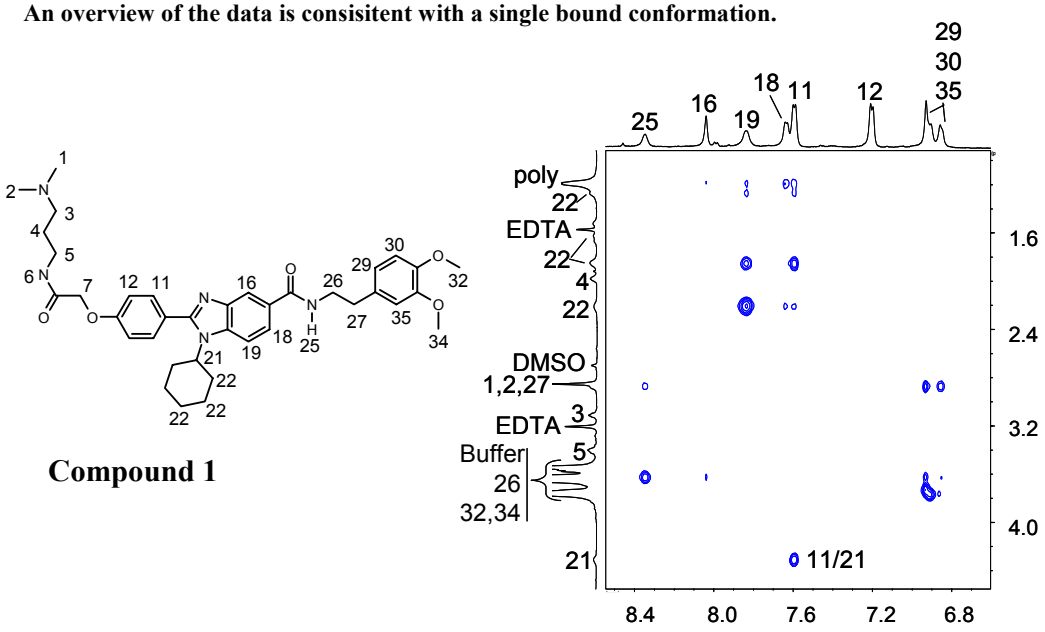
Appendix 2A: A description of the experimental procedures used for NMR experiments.

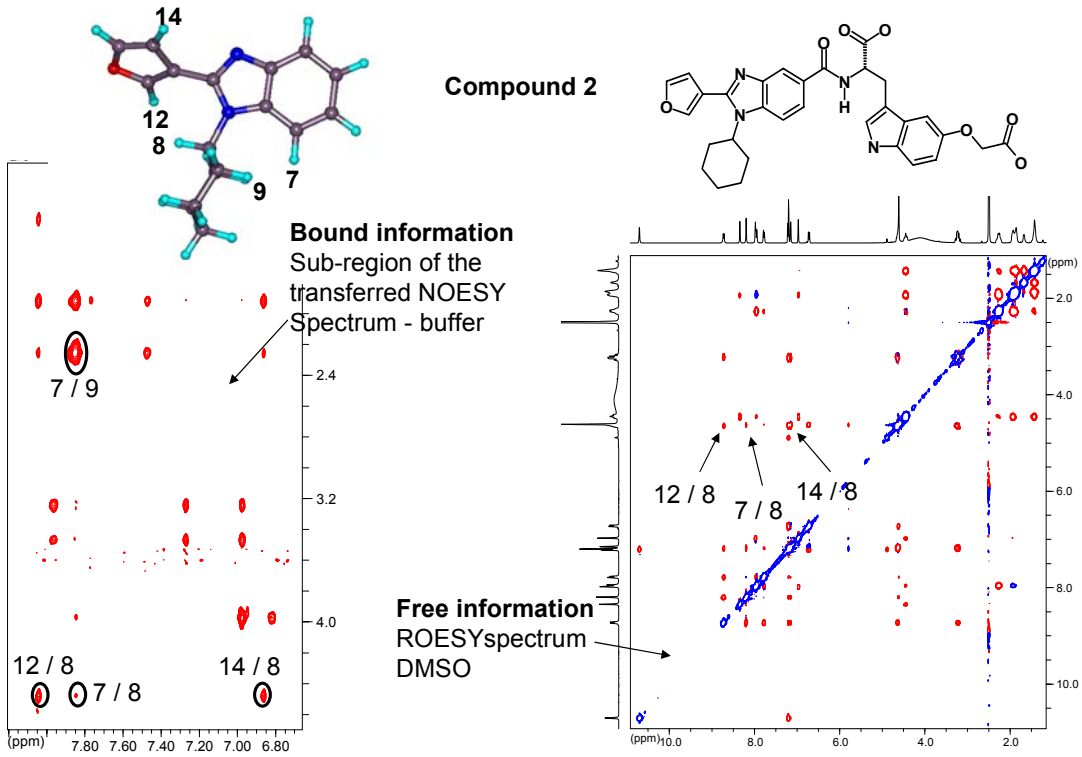
Samples containing compound **1** and **2** were prepared by adding 15 µl of concentrated solution in DMSO-d_6 (typically 0.3 mgs. of compound) to an aqueous buffer composed of 20 mM Tris-d_{11}, 2 mM DTT-d_{10}, 1 mM EDTA-d_{12}, 300 mM NaCl, and 10% (v/v) D_2O spiked with TSP-2,2,3,3-d_4. Buffer was added to a final volume of 600 µl**,** and the pH was adjusted to 6.0 with diluted HCl. Spectra of free compound were then acquired. A concentrated stock solution of HCV polymerase was then added to the NMR tube and spectra were acquired. Multiple additions of stock polymerase were added in some cases. The concentrated stock contained 21.7 mg/ml of HCV polymerase (NS5BΔ21C-His_6) in buffer consisting of 20 mM Tris-d_{11}, 2 mM DTT-d_{10}, 1 mM EDTA-d_{12}, 300 mM NaCl, and 10% (v/v) glycerol-d_8. For experiments involving tRNA (Sigma), stock solutions were prepared using the same concentration and buffer as that used for the polymerase stocks. For ^{19}F NMR experiments involving compound **4**, data were acquired directly on 0.3 ml of stock polymerase (in Shigemi tubes) after adding compound via 10 µl of DMSO-d_6 solvent such that a 1:1 compound:polymerase ratio was attained. A precipitant was subsequently observed after days at room temperature. NMR samples in DMSO-d_6 solvent were prepared by typically adding compound (approximately 0.5 mg) to 0.6 ml of solvent. Samples prepared for ^{13}C T_1 experiments were prepared as described elsewhere (LaPlante et al., *J. Biol. Chem* **1999**, *274*, 18618).

NMR spectra were acquired on Bruker AVANCE 400, 600 and 800 MHz NMR spectrometers at 22 and/or 27 ºC. Most NMR experiments for samples in DMSO-d_6 solvent were acquired using standard pulse sequences and conditions (i.e. 1D ^1H, COSY, ROESY, NOESY, and ^1H-^{13}C HMQC experiments). For samples in buffer, suppression of the solvent signal was achieved by the use of pre-saturation or by inserting a 3-9-19 WATERGATE module prior to data acquisition. NOESY experiments on sample tubes containing no polymerase resulted in the observation of no significant cross-peaks. On the other hand, NOESY spectra on sample tubes containing polymerase resulted in the observation of many cross-peaks which contained the valuable inter-hydrogen distance information of the compound when bound to HCV polymerase. Thus, a series of NOESY spectra were typically acquired on these latter samples which included the following mixing times 50, 75, 100, 150, 200 and 300 msec (at 600 and 800 MHz fields). A description of the conditions used to acquire data ^{13}C T_1 and transferred ^{13}C T_1 data has been provided elsewhere (LaPlante et al., *J. Biol. Chem* **1999**, *274*, 18618; LaPlante et al., *J. Am. Chem. Soc*. **2000**, *122*, 12530). Two-dimensional data sets were acquired with 2048 points in t_2 and 512 points in t_1. 128 scans were averaged for NOESY FIDs. Data were processed and analyzed (volumes, etc) using Bruker's XWinNMR and WinNMR software (Bruker Canada, 555 Steeles Ave. East, Milton, Ontario). Data sets were zero-filled to yield 2048 by 2048 real points after transformation using a phase-shifted sinebell window function.

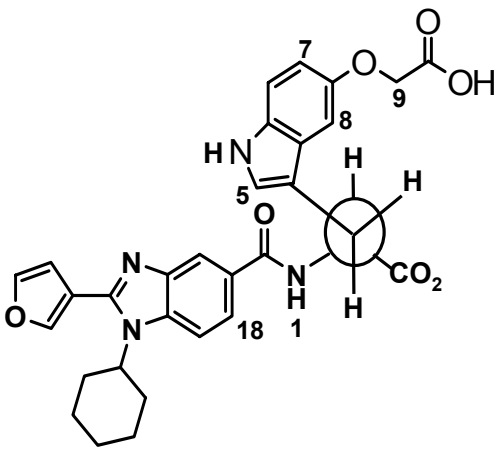
Appendix 2B: Transferred NOESY sub-spectrum showing specific inter-hydrogen distances of compound 1 when bound to HCV polymerase. An overview of the data is consisitent with a single bound conformation.



Appendix 2C: Left side conformation of compound 2 when bound to HCV polymerase and when free



Appendix 2C: Bound conformation of the right side differs from the free state

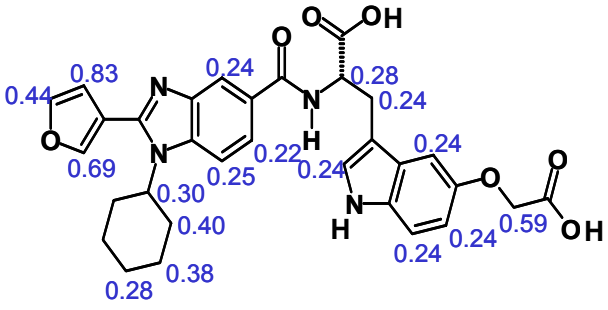


NMR-derived distances (Å)

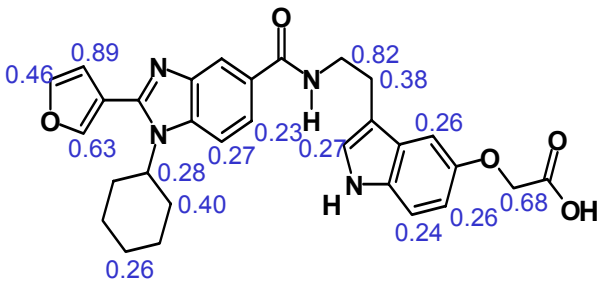
	Bound NOESY	Free ROESY
18 / 8	>5	3.4
18 / 9	>5	3.4
1 / 5	3.3	3.7
7 / 9	3.1	2.6
8 / 9	2.1	1.9

Appendix 2D: ^{13}C T_1 data for free compounds 2 and 3 to monitor flexibility

• *^{13}C T_1 time given in seconds. Data were measured in DMSO-d_6 solvent using similar sample and experimental conditions as described elsewhere (LaPlante et al., J. Biol. Chem.* **1999**, *274,* 18618*). Data were collected at 150 MHz.*



^{13}C T_1 (sec) of free compound **2**

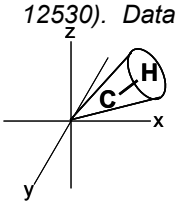


^{13}C T_1 (sec) of free compound **3**

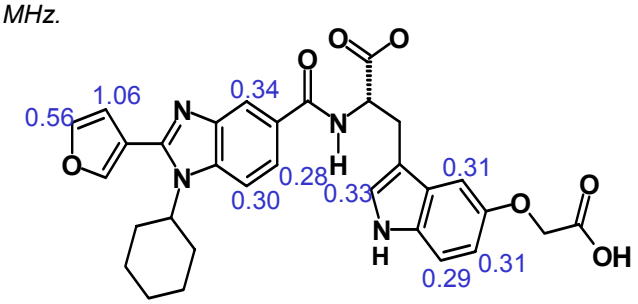
2640-079

Appendix 2D: Transferred ^{13}C T_1 data for compound 2 free and changes upon addition of HCV polymerase to probe dynamics changes upon binding

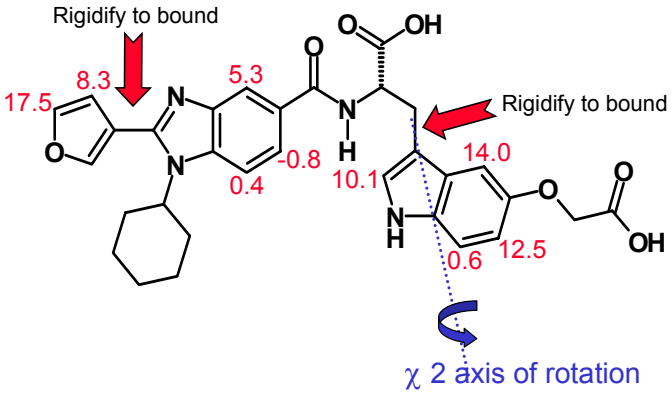
• *^{13}C T_1 time given in seconds. Data were measured in bufffer using the same NMR experimental conditions as described elsewhere (LaPlante et al, J. Am. Chem. Soc.* ***2000****, 122, 12530). Data were collected at 200 MHz.*



^{13}C T_1 (sec) of free compound 2



Percentage change in ^{13}C T_1 of protonated, aromatic carbons of compound 2 upon addition of HCV polymerase (70:1 ratio)



χ 2 axis of rotation

Appendix 3:

List of Contents

Appendix 3A:

A description is provided on the procedure used for determining the 3D structure of compound **2** when bound to HCV polymerase.

Appendix 3B:

Molecular modeling and NMR studies are shown which involve benzimidazole derivatives (methylation and aza compounds). Also included is an amide-benzimidazole torsion energy profile.

Appendix 3A:

The 3D, polymerase-bound structure of compound **2** was calculated by a simulated annealing protocol using the MOE molecular modeling program (CCG, Montreal, Qc, Canada). All calculations were performed using the mmff94 forcefield and a dielectric constant of 8. NMR-derived distance restraints were generated from the series of NMR NOESY data. The relative intensity of NOESY cross-peaks were classified into three categories which were then used as restraints, strong (1.8-2.7 Å), medium (1.8-3.5 Å) or weak (1.8-5.0 Å). Two principle calculations were run. One excluded a restraint involving H15-H4 and the other excluded H15-H6. A flat-bottomed potential was used with force constants that were increased during the cooling stages. A single, high temperature unrestrained dynamics run was performed at 1000 K, with 100 structures collected at 10 psec intervals to generate a starting set of conformations. Each structure was cooled and minimized using the following simulated annealing protocol. During the simulations the temperature, simulation time, and restraint weights were changed from 1000K to 50K, 50 to 10 psec, and from 1/1000000 to 20, respectively. The final structures were energy minimized, the total energies were calculated, the restraint energies were calculated, and the restraint violations were determined. NMR-consistent structures were isolated and a single family was identified for each set of calculations. Representative structures for each of the calculations is provided in Figures 2C (for the calculation in which an H15-H6 constraint was excluded), and Figure 2D (for the calculation in which an H15-H4 constraint was excluded).

Appendix 3B:

Modeling and NMR studies are shown which involve benzimidazole derivatives (methylation and pyridines). Also included is an amide-benzimidazole torsion energy profile.

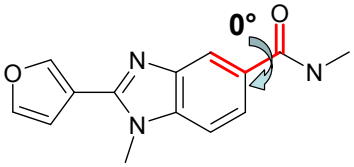
Ab initio calculations of the benzimidazole and azo-benzimidazole amide torsion profiles were performed at the RHF/6-31G** level of theory using Jaguar 5.5. Torsion profiles were calculated in both gas- and aqueous-phases (self-consistent reaction field method) with and without geometry optimization in 12 degree increments.

S1

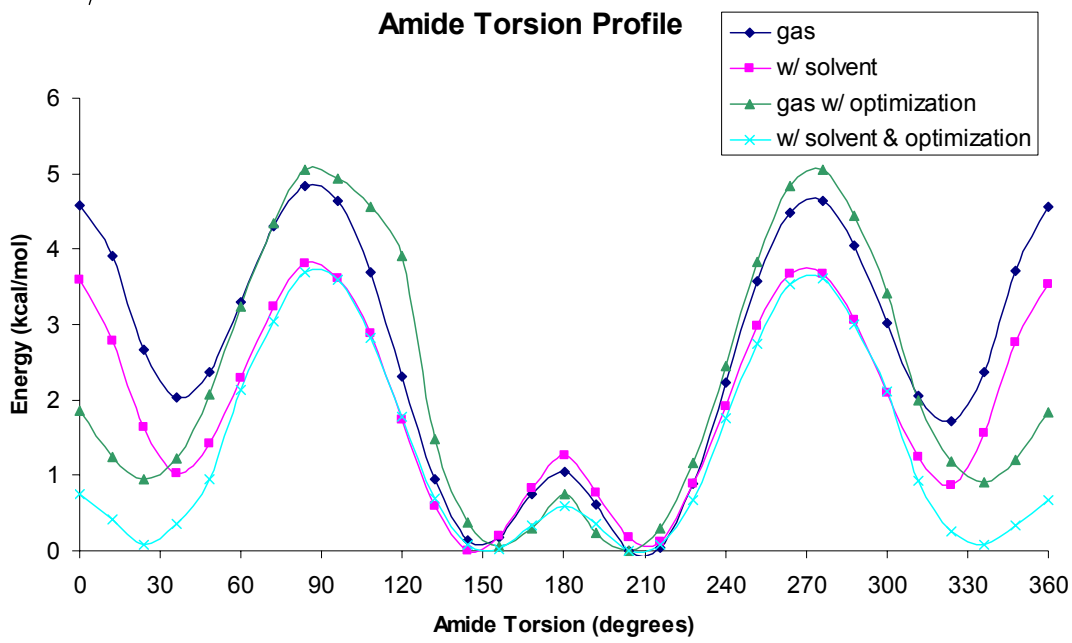
The S1 torsion profiles (defined as H-C8-N1-C2) for compounds **2** and the model compound were calculated in the gas-phase at the RHF/6-31G** level of theory using Jaguar 5.5. The torsion angles were sampled in 12 degree increments.

S2

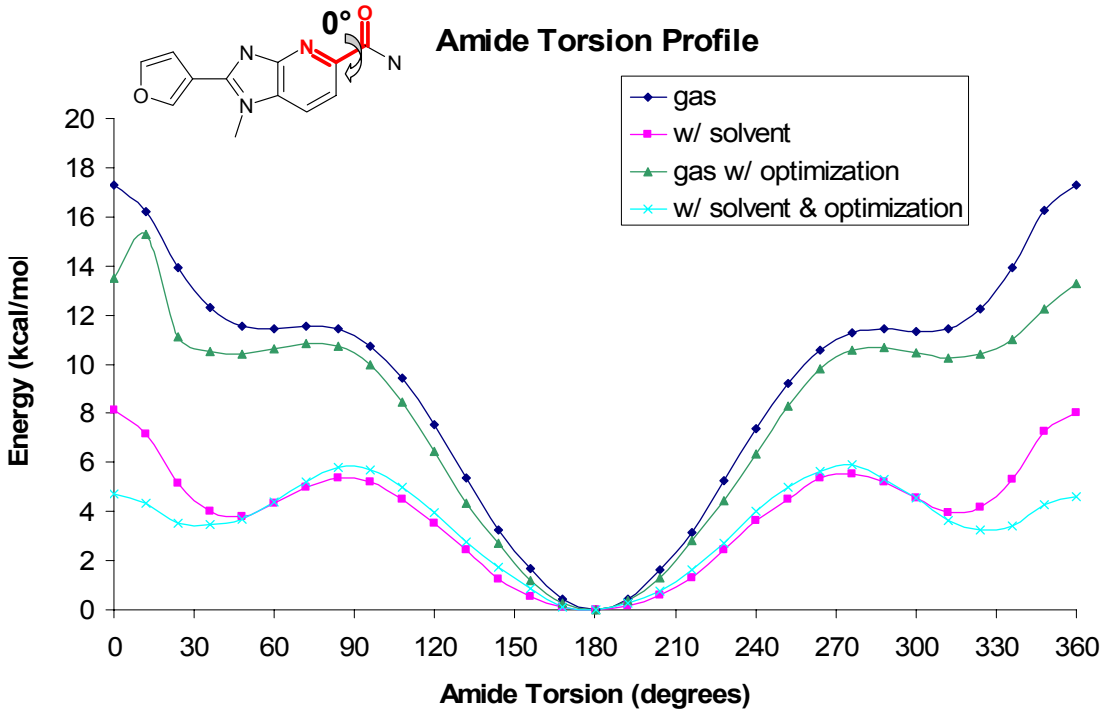
Molecular dynamic simulations of compounds **2** and **3** were performed using the MD module of MOE (version 2003.02) under constant volume and constant temperature conditions using MMFF94 (as implemented in MOE). The simulations consisted of a heating phase that gradually increased the system temperature from 0K to 298K in 10ps followed by a production phase at the target temperature of 298K for 10ns. The non-bonded cut-off radius was set to infinity, an implicit solvation model was used to mimic an aqueous environment, and a time step of 0.001ps was used to integrate the dynamics. During the simulation, snapshots were taken at 0.5ps. intervals and analyzed using the Conformation Geometries module.



Appendix 3B



Appendix 3B



Appendix 3B: Design of Aza-analogues to orient the amide