Supplementary Figures

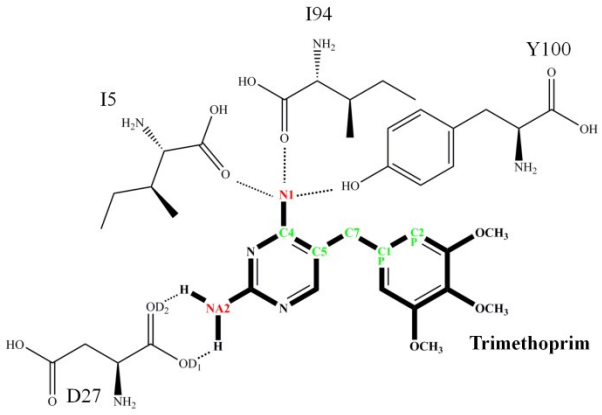

Figure S1. Binding site residues of TMP/TMP^{+} bound DHFR. Atom names involved in hydrogen bond formation are in red and atom names involved in torsional angles are in green.

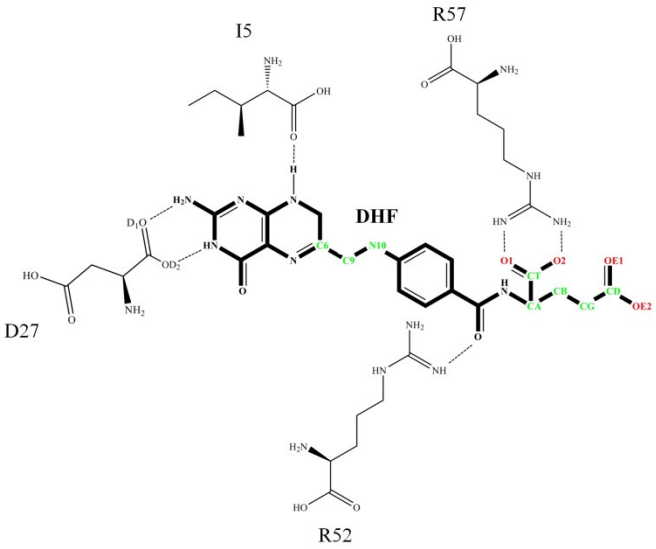

Figure S2. Binding site residues of DHF bound DHFR. Atom names involved in hydrogen bond formation in the original structure are in red and atom names involved in torsional angles are in green.

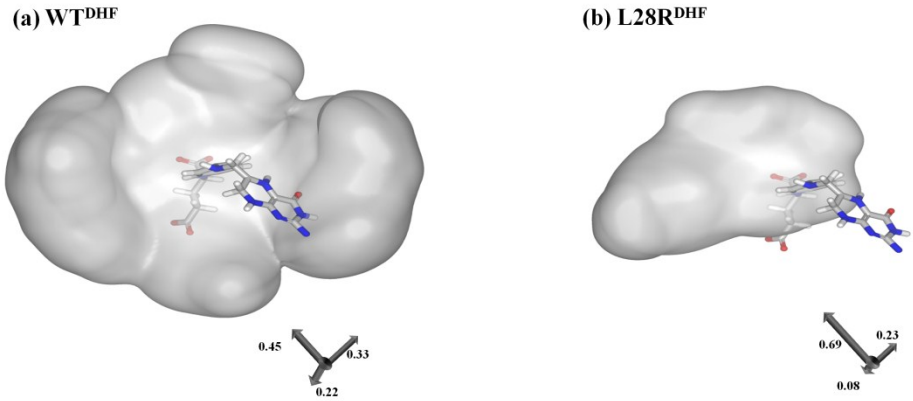

Figure S3. Rotational movements of DHF p-aminobenzoyl glutamate tail in (a) WT^{DHF} and (b) $L28R^{DHF}$ systems. The trajectory of the unit vector connecting N10 to CD atom position is shifted to (0,0,0) for better visualization of the tail movements which are projected on the x-y plane on the upper panels; color variations denote *z*-coordinate changes. The volume swept by the CD atom positions are displayed as a gray surface in the lower panels, where one of the poses of DHF is shown in stick representation. Principal axis contribution is 0.45, 0.33 and 0.22 for WT^{DHF}, which demonstrates a nearly free rotation of the tail in the binding site. Principal axis contribution is 0.69, 0.23 and 0.08 for $L28R^{DHF}$ revealing a stiffened tail with the motion predominantly confined to the major axis.

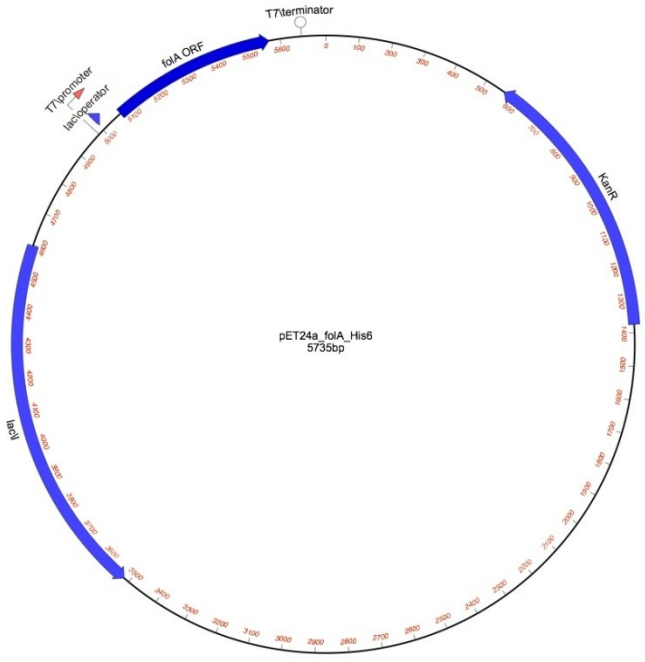

Figure S4. Full map of the pet24a-folA-kanR plasmid including T7 promoter and six His-tags on the C terminus used for cloning the *E. coli FolA* gene.

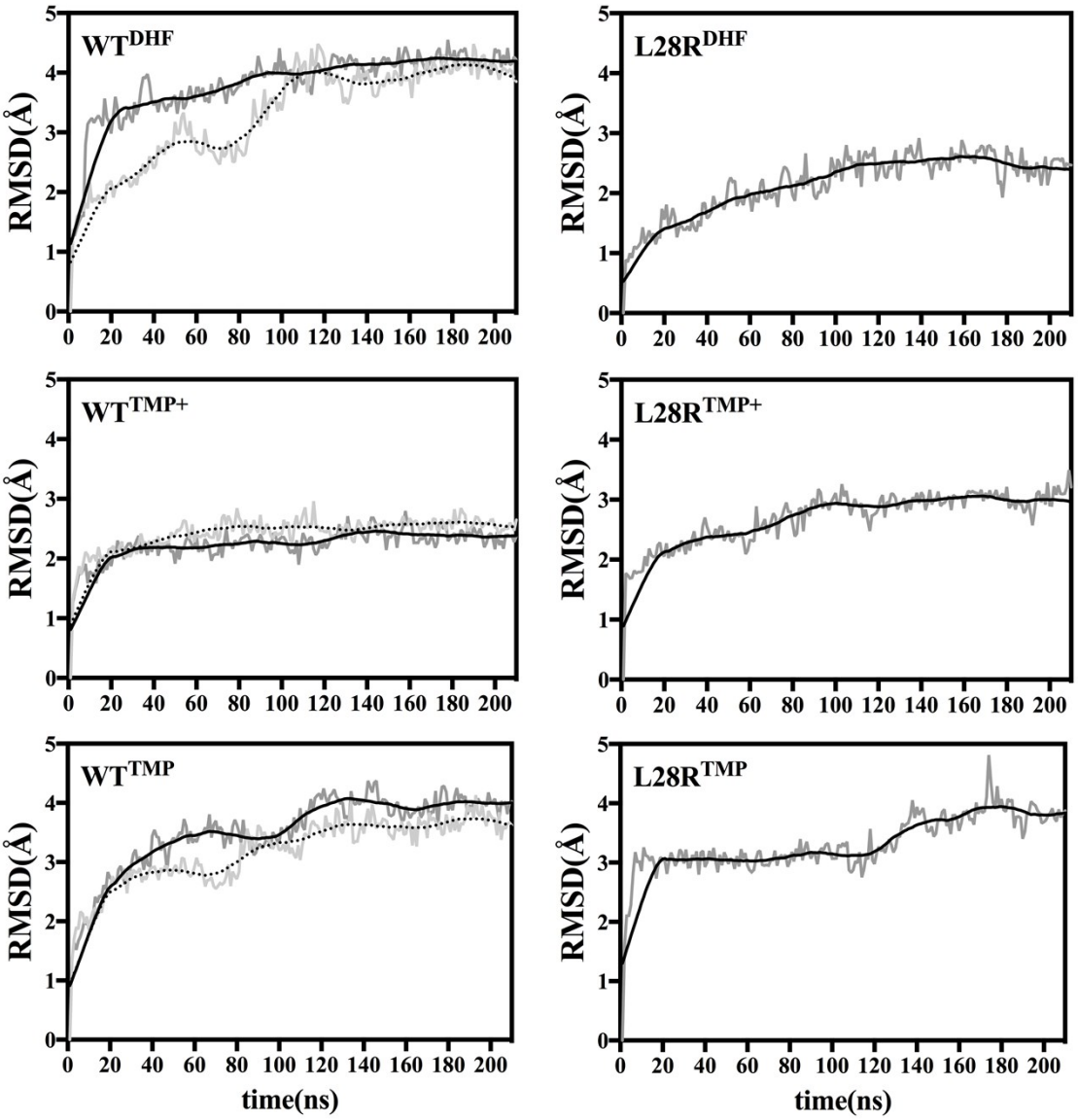

Figure S5. RMSD profiles for the 210 ns long MD simulations. On the left column, DHF, TMP^+ and TMP bound systems in WT are reported in dark and light gray, respectively, for the first and second runs. On the right column, DHF, TMP^+ and TMP bound systems in the L28R mutant are displayed in dark gray. Moving averages of all trajectories are shown in black.

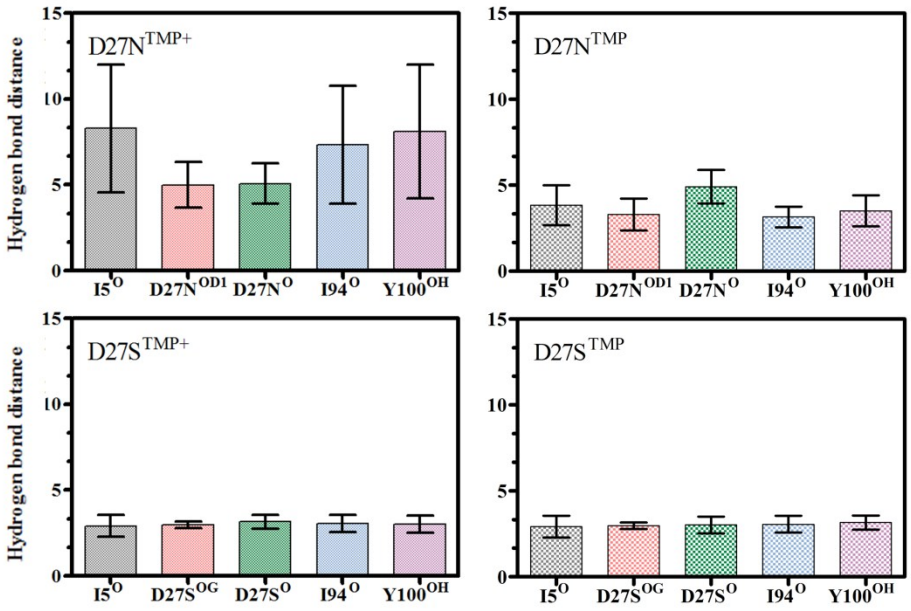

Figure S6. Average distances of characteristic binding pocket hydrogen bonds recorded in 50 ns trajectories of the D27N and D27S mutants for TMP^+ and TMP bound cases. Standard deviations are shown by vertical lines; color coding is the same as in figure 2. The more stable form of the drug is the one which maintains the characteristic hydrogen bonds. For D27N, this is the TMP bound form in contrast to the WT which accommodates TMP^+ (Figure 2); the D27S mutant binds both forms.

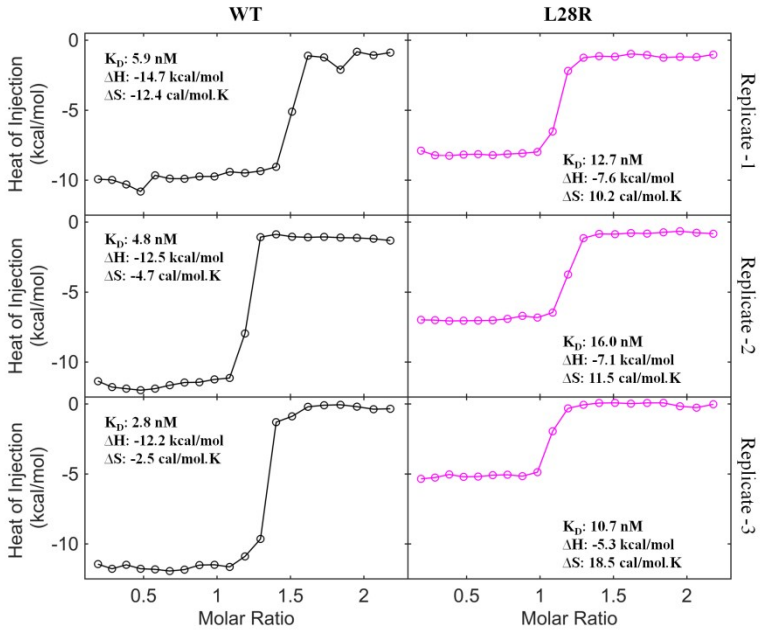

Figure S7. Isothermal Titration Calorimetry results for TMP binding are shown for WT and L28R DHFR. Each experiment is repeated in triplicate. All K_D, ΔH, ΔS and ΔG values are significantly different between the WT and the L28R mutant.

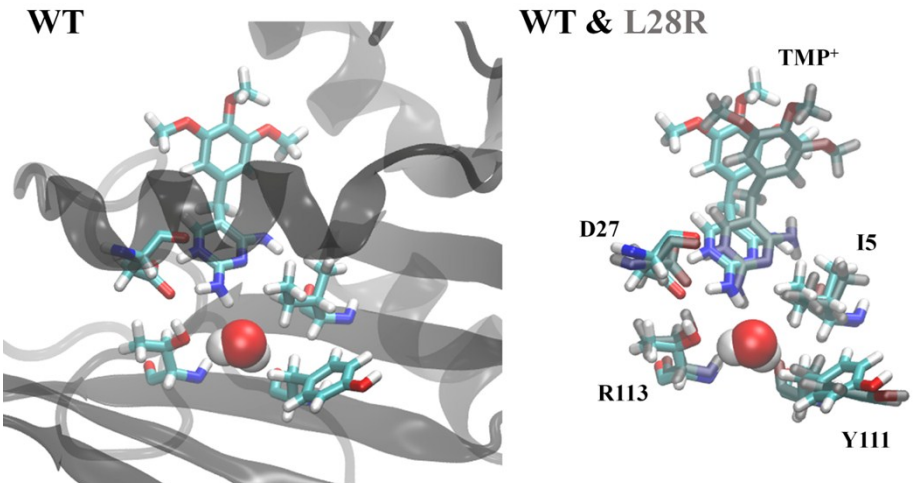

Figure S8. The environment of the coordinating water molecule in the WT is displayed on the left. Residues 5, 111 and 113 from the β sheet beneath the TMP binding site accommodate the water molecule that makes an additional hydrogen bond with trimethoprim. The interfacial water leads to negative entropy of binding and compensates by the additional enthalpic contribution. On the right, the shifts in the side chains of residues 5, 27, 111 and 113 and the coordinates of trimethoprim in the L28R mutant (in steel representation) are superimposed on those displayed on the left figure. In the L28R mutant the binding site is slightly tightened so that a water molecule may no longer squeeze between the β sheet, D27 and trimethoprim.

Supplementary text

For a proper representation of trimethoprim interactions with the protein, we have parameterized trimethoprim in two states (TMP and TMP^{+}). We described the parameterization protocol used for trimethoprim force filed under the Materials and Methods section.

S1. Topology

```
RESI TMP            0.0

ATOM    NA2  NN1T  -0.164000  !         HA41  HA42
ATOM    HA21 HN1T   0.148000  !           \   /
ATOM    HA22 HN1T   0.148000  !            NA4         H2P         H81
ATOM    C2   CAT   -0.096000  !             |           |           |
ATOM    N3   NC2T  -0.144000  !            C4   C7    C2P     O3P-C8-H81
ATOM    N1   NC2T  -0.039000  !           / \\ /  \  /  \\   /     |
ATOM    C6   CAT   -0.026000  !          N3  C5    C1P   C3P    H81
ATOM    H6   HPT    0.090000  !          ||   |    ||    |
ATOM    C4   CAT   -0.046000  !   HA21   C2   C6   C6P  C4P   H91
ATOM   NA4  NN1T   -0.185000  !    \   / \  //      \  // \   |
ATOM   HA41 HN1T    0.130000  !     NA2   N1         C5P  O4P-C9-H92
ATOM    HA42 HN1T   0.130000  !    /                 /        |
ATOM    C5   CAT   -0.010000  ! HA22              O5P        H93
ATOM    C7   CT2T  -0.075000  !                   |
ATOM    H71  HAT    0.090000  !              H101-C10-H102
ATOM    H72  HAT    0.090000  !                   |
ATOM    C1P  CAT    0.137000  !                 H103
ATOM    C2P  CAT   -0.095000  !
ATOM    H2P  HPT    0.090000  !
ATOM    C3P  CAT   -0.054000  !
ATOM    O3P  OST   -0.141000  !
ATOM    C8   CT3T  -0.137000  !
ATOM    H81  HAT    0.090000  !
ATOM    H82  HAT    0.090000  !
ATOM    H83  HAT    0.090000  !
ATOM    C4P  CAT   -0.066000  !
ATOM    O4P  OST   -0.055000  !
ATOM    C9   CT3T  -0.193000  !
ATOM    H91  HAT    0.090000  !
ATOM    H92  HAT    0.090000  !
ATOM    H93  HAT    0.090000  !
ATOM    C5P  CAT   -0.054000  !
ATOM    O5P  OST   -0.141000  !
ATOM    C10  CT3T  -0.137000  !
ATOM    H101 HAT    0.090000  !
ATOM    H102 HAT    0.090000  !
ATOM    H103 HAT    0.090000  !
ATOM    C6P  CAT   -0.095000  !
ATOM    H6P  HPT    0.090000  !

BOND   NA2 HA21  NA2 HA22 NA2 C2  C2 N1  N3 C4  C4 NA4  NA4 HA41  NA4 HA42
BOND   C5 C6  C6 H6  C5 C7  C7 H71  C7 H72  C7 C1P  C1P C2P  C2P H2P
BOND   C6P H6P C3P O3P  O3P C8  C8 H81 C8 H82  C8 H83  C3P C4P  C4P O4P  BOND
O4P C9  C5P C6P  C10 H103
```

```
BOND   C9 H91  C9 H92  C9 H93 C5P O5P  O5P C10  C10 H101  C10 H102
DOUBLE C2 N3  C4 C5  N1 C6  C2P C3P  C4P C5P  C1P C6P
DONOR  NA2 HA21  NA2 HA22

IC  CAT   CAT   CT2T  HAT     1.380 120.000 0.000 111.000 1.100
IC  HPT   CAT   CAT   CT2T    1.080 120.000 0.000 120.000 1.500
IC  CAT   OST   CT3T  HAT     1.340 112.000 0.000 108.500 1.500
IC  NN1T  CAT   CAT   CAT     1.400 122.000 180.0 120.000 1.380
IC  HPT   CAT   CAT   OST     1.080 120.000 180.0 120.000 1.340
IC  HPT   CAT   CAT   CAT     1.080 120.000 0.000 120.000 1.380
IC  OST   CAT   CAT   OST     1.340 120.000 0.000 120.000 1.340
IC  CAT   CAT   NN1T  HN1T    1.380 122.000 0.000 113.000 1.030
IC  CAT   CAT   CAT   CAT     1.380 120.000 180.0 120.000 1.380
IC  CAT   CAT   CT2T  CAT     1.380 120.000 180.0 111.000 1.500
IC  CT2T  CAT   CAT   CAT     1.500 120.000 180.0 120.000 1.380
IC  NC2T  CAT   NC2T  CAT     1.330 128.000 180.0 115.000 1.330
IC  NC2T  CAT   CAT   CAT     1.330 123.000 180.0 120.000 1.380
IC  NN1T  CAT   NC2T  CAT     1.400 116.000 180.0 115.000 1.330
IC  CAT   NC2T  CAT   HPT     1.330 128.000 180.0 115.500 1.080
IC  HN1T  NN1T  CAT   NC2T    1.030 113.000 0.000 116.000 1.330
IC  CAT   CAT   OST   CT3T    1.380 120.000 180.0 112.000 1.400
IC  NC2T  CAT   CAT   CT2T    1.330 123.000 180.0 120.000 1.500
IC  OST   CAT   CAT   CAT     1.340 120.000 180.0 120.000 1.380
IC  NN1T  CAT   CAT   CT2T    1.400 122.000 180.0 120.000 1.500
IC  CAT   NC2T  CAT   CAT     1.330 115.000 180.0 123.000 1.380

RESI TMP+           1.0

ATOM    NA2  NN1T   -0.213000 !
ATOM    HA21 HN1T   0.276000  !
ATOM    HA22 HN1T   0.276000  !
ATOM    C2   CAT   -0.085000  !
ATOM    N3   NC2T  -0.368000  !
ATOM    N1   NC2T  -0.004000  !
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ATOM    H6   HPT    0.090000  !
ATOM    C4   CAT    0.133000  !
ATOM    NA4  NN1T  -0.367000  !
ATOM    HA41 HN1T   0.291000  !
ATOM    HA42 HN1T   0.291000  !
ATOM   C5  CAT      0.096000  !
ATOM    C7   CT2T  -0.075000  !
ATOM    H71  HAT    0.090000  !
ATOM    H72  HAT    0.090000  !
ATOM    C1P  CAT   -0.276000  !
ATOM    C2P  CAT    0.119000  !
ATOM    H2P  HPT    0.090000  !
ATOM    C3P  CAT   -0.011000  !
ATOM    O3P  OST   -0.319000  !
ATOM    C8   CT3T  -0.146000  !
ATOM    H81  HAT    0.090000  !
ATOM    H82  HAT    0.090000  !
ATOM    H83  HAT    0.090000  !
ATOM    C4P  CAT    0.024000  !
ATOM    O4P  OST   -0.067000  !
ATOM    C9   CT3T  -0.088000  !
ATOM    H91  HAT    0.090000  !
ATOM    H92  HAT    0.090000  !
ATOM    H93  HAT    0.090000  !
ATOM    C5P  CAT   -0.011000  !
ATOM    O5P  OST   -0.319000  !
ATOM    C10  CT3T  -0.146000  !
ATOM    H101 HAT    0.090000  !
```

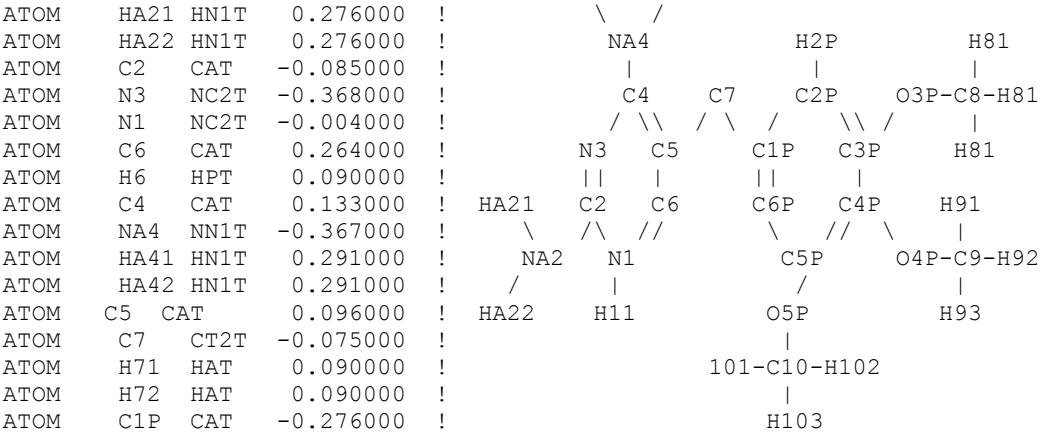

```
ATOM    H102 HAT    0.090000  !
ATOM    H103 HAT    0.090000  !
ATOM    C6P  CAT    0.119000  !
ATOM    H6P  HPT    0.090000  !
ATOM    H11  HN2    0.346000   !


BOND  NA2 HA21  NA2 HA22 NA2 C2  C2 N1  N3 C4  C4 NA4  NA4 HA41  NA4 HA42
BOND  C5 C6  C6 H6  C5 C7  C7 H71  C7 H72  C7 C1P  C1P C2P  C2P H2P
BOND  C6P H6P C3P O3P  O3P C8  C8 H81 C8 H82  C8 H83  C3P C4P  C4P O4P
BOND  C9 H91  C9 H92  C9 H93 C5P O5P  O5P C10  C10 H101  C10 H102
BOND  C10 H103  C5P C6P  H11 N1  O4P C9
DOUBLE C2 N3  C4 C5  N1 C6  C2P C3P  C4P C5P  C1P C6P
DONOR  NA2 HA21  NA2 HA22


IC  CAT   CAT   CT2T  HAT     1.380 120.000 0.000 111.000 1.100
IC  HPT   CAT   CAT   CT2T    1.080 120.000 0.000 120.000 1.500
IC  CAT   OST   CT3T  HAT     1.340 112.000 0.000 108.500 1.500
IC  NN1T  CAT   CAT   CAT     1.400 122.000 180.0 120.000 1.380
IC  HPT   CAT   CAT   OST     1.080 120.000 180.0 120.000 1.340
IC  HPT   CAT   CAT   CAT     1.080 120.000 0.000 120.000 1.380
IC  OST   CAT   CAT   OST     1.340 120.000 0.000 120.000 1.340
IC  CAT   CAT   NN1T  HN1T    1.380 122.000 0.000 113.000 1.030
IC  CAT   CAT   CAT   CAT     1.380 120.000 180.0 120.000 1.380
IC  CAT   CAT   CT2T  CAT     1.380 120.000 180.0 111.000 1.500
IC  CT2T  CAT   CAT   CAT     1.500 120.000 180.0 120.000 1.380
IC  NC2T  CAT   NC2T  CAT     1.330 128.000 180.0 115.000 1.330
IC  NC2T  CAT   CAT   CAT     1.330 123.000 180.0 120.000 1.380
IC  NN1T  CAT   NC2T  CAT     1.400 116.000 180.0 115.000 1.330
IC  CAT   NC2T  CAT   HPT     1.330 128.000 180.0 115.500 1.080
IC  HN1T  NN1T  CAT   NC2T    1.030 113.000 0.000 116.000 1.330
IC  CAT   CAT   OST   CT3T    1.380 120.000 180.0 112.000 1.400
IC  NC2T  CAT   CAT   CT2T    1.330 123.000 180.0 120.000 1.500
IC  OST   CAT   CAT   CAT     1.340 120.000 180.0 120.000 1.380
IC  NN1T  CAT   CAT   CT2T    1.400 122.000 180.0 120.000 1.500
IC  CAT   NC2T  CAT   CAT     1.330 115.000 180.0 123.000 1.380

PATCHING FIRST NONE LAST NONE
```

S2. Force field

```
BONDS


!V(bond) = Kb(b - b0)**2

!

!Kb: kcal/mole/A**2

!b0: A

!

!atom type Kb b0

!

NN1T  CAT   400.000    1.400
```

```
NN1T  HN1T  465.000    1.030
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CAT   CAT   340.000    1.380
CAT   HPT   380.000    1.080
CAT   CT2T  320.000    1.500
CT2T  HAT   365.000    1.100
CAT   OST   400.000    1.340

OST   CT3T  335.000    1.400
CT3T  HAT   355.000    1.090
NC2T  HN2   471.000    1.010


ANGLES


!

!V(angle) = Ktheta(Theta - Theta0)**2

!

!V(Urey-Bradley) = Kub(S - S0)**2

!

!Ktheta: kcal/mole/rad**2

!Theta0: degrees

!Kub: kcal/mole/A**2 (Urey-Bradley)

!S0: A

!

!atom types     Ktheta    Theta0   Kub     S0

!

!

NC2T  CAT   CAT   56.000    123.000
CAT   CAT   CAT   60.000    120.000
NC2T  CAT   NC2T  96.000    128.000
NN1T  CAT   NC2T  82.500    116.000
NN1T  CAT   CAT   101.000   122.000
OST   CAT   CAT   82.500    120.000
HN1T  NN1T  CAT   87.500    113.000
HN1T  NN1T  HN1T  90.000    115.000
CAT   NC2T  CAT   95.000    115.000
NC2T  CAT   HPT   89.000    115.500
HPT   CAT   CAT   48.000    120.000
CAT   CAT   CT2T  86.000    120.000
CAT   CT2T  CAT   145.000   111.000
CAT   CT2T  HAT   71.000    111.000
HAT   CT2T  HAT   64.000    105.000
CAT   OST   CT3T  129.000   112.000
OST   CT3T  HAT   77.000    108.500
HAT   CT3T  HAT   48.000    108.000
CAT   NC2T  HN2   45.000    115.600
```

```
DIHEDRALS

!

!V(dihedral) = Kchi(1 + cos(n(chi) - delta))

!

!Kchi: kcal/mole

!n: multiplicity

!delta: degrees

!

!atom types             Kchi    n   delta

!

CAT   CAT   CT2T  HAT   0.0000   3  0.0000
HPT   CAT   CAT   CT2T  0.0000   1  0.0000
CAT   OST   CT3T  HAT   0.0000   3  0.0000
NN1T  CAT   CAT   CAT   0.0000   2  180.00
HPT   CAT   CAT   OST   4.2000   2  180.00
HPT   CAT   CAT   CAT   0.0000   1  0.0000
OST   CAT   CAT   OST   0.0000   1  0.0000
CAT   CAT   NN1T  HN1T  0.0000   1  0.0000
CAT   CAT   CAT   CAT   1.9000   2  180.00
CAT   CAT   CT2T  CAT   1.6500   3  180.00
CT2T  CAT   CAT   CAT   2.9000   2  180.00
NC2T  CAT   NC2T  CAT   2.8000   2  180.00
NC2T  CAT   CAT   CAT   3.0000   2  180.00
NN1T  CAT   NC2T  CAT   2.9000   2  180.00
CAT   NC2T  CAT   HPT   2.9000   2  180.00
HN1T  NN1T  CAT   NC2T  0.5000   3  0.0000
CAT   CAT   OST   CT3T  1.8500   2  180.00
NC2T  CAT   CAT   CT2T  3.0000   2  180.00
OST   CAT   CAT   CAT   2.9000   2  180.00
NN1T  CAT   CAT   CT2T  3.0000   2  180.00
CAT   NC2T  CAT   CAT   2.9000   2  180.00
NN1T  CAT   NC2T  HN2   1.2000   2  180.00
NC2T  CAT   NC2T  HN2   0.0000   2  180.00
HN2   NC2T  CAT   HPT   2.5000   1  -64.16
HN2   NC2T  CAT   CAT   2.5000   2  180.00



NONBONDED nbxmod  5 atom cdiel shift vatom vdistance vswitch -

cutnb 14.0 ctofnb 12.0 ctonnb 10.0 eps 1.0 e14fac 1.0 wmin 1.5

!

!V(Lennard-Jones) = Eps,i,j[(Rmin,i,j/ri,j)**12 - 2(Rmin,i,j/ri,j)**6]

!

!epsilon: kcal/mole, Eps,i,j = sqrt(eps,i * eps,j)

!Rmin/2: A, Rmin,i,j = Rmin/2,i + Rmin/2,j
```

```
!
!atom  ignored    epsilon      Rmin/2   ignored   eps,1-4       Rmin/2,1-4
!
CAT    0.0  -0.070000  1.992400
CT2T   0.0  -0.055000  2.175000  0.0  -0.010000  1.900000
CT3T   0.0  -0.080000  2.060000  0.0  -0.010000  1.900000
HAT    0.0  -0.022000  1.320000
HN1T   0.0  -0.046000  0.224500
HPT    0.0  -0.030000  1.358200  0.0  -0.030000  1.358200
NC2T   0.0  -0.200000  1.850000
NN1T   0.0  -0.200000  1.850000
OST    0.0  -0.152100  1.770000
```