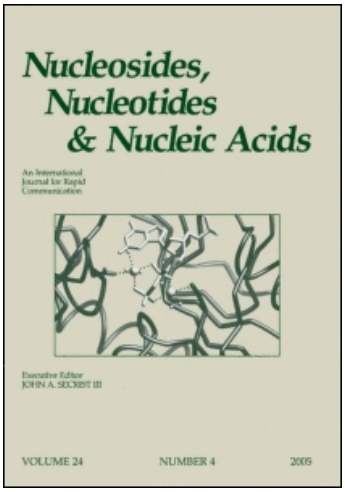

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INHIBITION OF (CYTOSINE C5)-METHYLTRANSFERASE BY OLIGONUCLEOTIDES CONTAINING FLEXIBLE (CYCLOPENTANE) AND CONFORMATIONALLY CONSTRAINED (BICYCLO[3.1.0]HEXANE) ABASIC SITES

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[a] ISIS Pharmaceuticals, Inc., California, U.S.A. [b] Department of Biochemistry and Molecular Biology and UNMC/Eppley Cancer Center, University of Nebraska Medical Center, Nebraska, U.S.A. [c] Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland, U.S.A.

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INHIBITION OF (CYTOSINE C5)-METHYLTRANSFERASE BY OLIGONUCLEOTIDES CONTAINING FLEXIBLE (CYCLOPENTANE) AND CONFORMATIONALLY CONSTRAINED (BICYCLO[3.1.0]HEXANE) ABASIC SITES

Victor E. Marquez,[1,*] Peiyuan Wang,[1] Marc C. Nicklaus,[1] Martin Maier,[2] Muthiah Manoharan,[2] Judith K. Christman,[3,*] Nilesh K. Banavali,[4] and Alexander D. Mackerell, Jr.[4,*]

[1]Laboratory of Medicinal Chemistry, Division of Basic Sciences, NCI-FCRDC, Boyles St., Bldg. 376, Frederick, Maryland 21702
[2]ISIS Pharmaceuticals, Inc., 2292 Faraday Ave., Carslbad, California 92008
[3]Department of Biochemistry and Molecular Biology and UNMC/Eppley Cancer Center, University of Nebraska Medical Center, 600 South 42nd St., Nebraska 68198
[4]Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201

ABSTRACT

Pseudorotationally locked sugar analogues based on bicyclo[3.1.0]-hexane templates were placed in DNA duplexes as abasic target sites in the M.*Hha*I recognition sequence. The binding affinity of the enzyme increases when the abasic site is constrained to the South conformation and decreases when it is constrained to the North conformation. A structural understanding of these differences is provided.

Several lines of evidence suggest that the control of gene expression in mammalian cells is related to the pattern of DNA methylation found in more than 70%

*Corresponding authors.

of the CpG residues present in the genome (1). The methylation of DNA, which involves the covalent addition of a methyl group to the 5-position of the cytosine ring, is a post-replicative modification since all mammalian DNA is synthesized with deoxycytidine triphosphate as a precursor.

The replication of a DNA that is methylated on both chains at CpG doublets produces two new hemimethylated helices. Subsequently, an enzyme with preferential affinity for hemimethylated sites (maintenance DNA methylase or DNMT1) preserves the methylation pattern with high fidelity from one cell generation to the next (2). Different protooncogenic pathways can up-regulate DNMT1 expression (3) and high levels of DNMT1 RNA have been observed in many cancer cells (4). The resulting hypermethylation at CpG islands in the promoter region of a growth-regulatory gene can silence its expression and provide a growth advantage to transformed cells. However, since methylation changes are reversible, inhibition of aberrant DNMT1 activity could reactivate the expression of the growth-regulatory gene, highlighting the importance of DNMT1 as a therapeutic target (1).

The mechanism of the methylation reaction proceeds via a 5,6-dihydropyrimi dine intermediate formed after nucleophilic attack of an enzyme's cysteinyl thiolate group on carbon 6 (C6) of the target cytosine (5,6). After methyl transfer from S-adenosyl-L-methionine (AdoMet) to C5, sp^3 to sp^2 rehybridization occurs by removal of the C5 hydrogen (5,6). This transient 5,6-dihydropyrimidine intermediate has been trapped with oligodeoxynucleotides (ODNs) containing 5-fluorocytosine as a suicide substrate, and crystal structures of the chemically trapped complex with prokaryotic methylases (M.*Hha*I and M.*Hae*III) reveal that the target cytosine exhibits a flipped-out conformation (7,8). ODNs containing 5-fluorocytosine can thus function as stoichiometric inhibitors of the enzyme (9). An ideal ODN inhibitor, however, should not be a substrate and should exhibit a high affinity for the enzyme with a slow off rate. Since the tightest binding of M.*Hha*I occurs when either the target base or the target nucleotide is missing (abasic site or gap) ODNs containing abasic tetrahydrofuran (THF) sites replacing the target cytosine (C) are considered ideal candidates (10). Furthermore, crystallographic analysis of the ternary complex containing M.*Hha*I, the cofactor S-adenosylhomocysteine (AdoHcy) and an ODN with a THF abasic target site reveal that the flexible sugar surrogate (THF) is also in a flipped-out conformation (11). In this crystal structure with a resolution of 2.3 Å(11), the conformation of the THF appears to adopt a North conformation as defined in the pseudorotational cycle (12). Since in the most common form of DNA (B-DNA), the sugar moieties favors the antipodal South conformation, we decided to investigate the effect of locking the conformation of the target abasic site (North or South) on the interaction with M.*Hha*I. Our results demonstrate that the binding affinity of M.*Hha*I for abasic sites increases when the abasic target sugar is constrained to the South and decreases when it is constrained to the North. The use of the bicyclo[3.1.0]hexane template for the construction of conformationally locked abasic sites, as well as the apparent discrepancy with the crystal structure of M.*Hha*I complexed with an ODN containing a North THF conformation site will be discussed.

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THE DESIGN OF TARGET ODN SEQUENCES

ODN 13-mers containing abasic pseudosugars (X) in place of the target C residue in the M.*Hha*I recognition sequence (5′-GXGC-3′) were synthesized (Fig. 1). Except for the nature of X, these 13-mer ODNs are identical to the ones used in the elucidation of the ternary structure of M.*Hha*I with the cofactor AdoHcy and DNA (ODNs, X = 5-fluorocytosine (7) or X = THF (11)). When the complementary strand contains a 5-methylcytosine (M in the sequence 5′-GMGC-3′) the residue X indicated by the arrow becomes the only target for flipping. ODNs incorporating pseudosugars locked into one the two antipodal North and South hemispheres of the pseudorotational cycle (Fig. 2) were compared with an ODN containing the flexible cyclopentane ring. The stable pseudoboat conformation of the bicyclo[3.1.0]hexane template ensures that the conformations remain locked North and South as depicted in Figure 2 (13).

CHEMISTRY

(*1S,2R*)-2-[(Benzyloxy)methyl] cyclopent-3-enol (**1**) was selected as the chiral starting material for the synthesis of the conformationally locked South- and North-type phosphoramidites **5** an **15** (Schemes 1 and 2). Compound **1** was prepared by a modification of the procedure originally reported by Biggadike et al. (14). Synthesis of the South-type phosphoramidite **5** started with homoallylic hydroxyl-directed cyclopropanation of **1** under Simmons-Smith conditions with diethylzinc and diiodomethane to give carbobicyclic intermediate **2** (Scheme 1). Deprotection of the benzyl group with palladium black in methanol provided diol **3**, which after dimethoxytritylation produced the protected alcohol **4**. The South-type phosphoramidite **5** was finally completed from **4** using standard reaction conditions with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite.

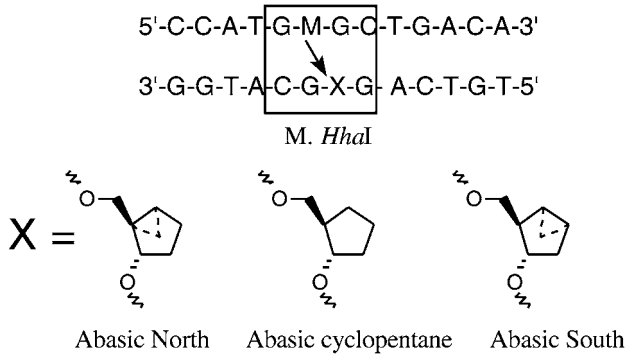

Figure 1. Modified ODN structures with abasic moieties at the M.*Hha*I site consisting of a flexible cyclopentane pseudosugar and two conformationally rigid bicyclo[3.1.0]hexane templates locked in the North and South conformations.

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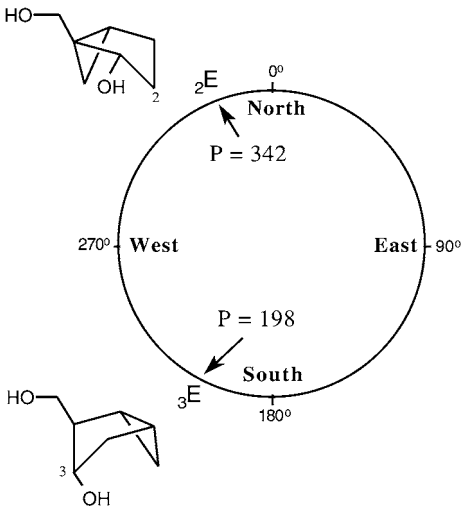
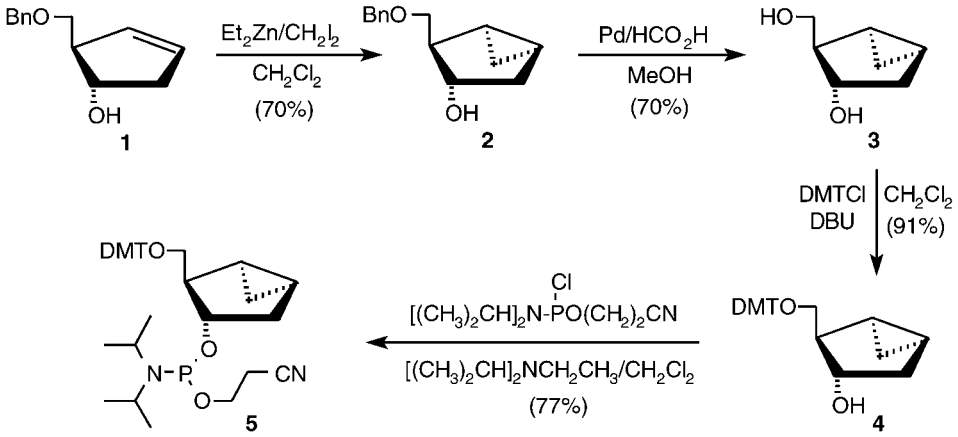


Figure 2. Fixed locations of bicyclo[3.1.0]hexane envelope conformations (E) in the pseudorotational cycle.

The approach to the North-type phosphoramidite **15** started from chiral intermediate **6** (15), which was easily obtained from compound **1**. Treatment of **6** with $PhSeCl/AgCF_3CO_2$ followed by hydrolysis of the trifluoroacetate ester gave the trans-2-(phenylselenyl)-cyclopentan-1-ol (**8**). The regio- and stereoselectivity of this addition reaction has been reported (13). Following oxidation of the phenylselenide group, unidirectional elimination of PhSeOH gave the corresponding allylic alcohol **9** exclusively. Deoxygenation of this allylic alcohol with sulfur trioxide-pyridine complex, followed by $LiAlH_4$ reduction afforded compound **10**. Allylic alcohol **11** was obtained after deprotection of **10** with triethylamine trihydrofluoride and subjected to hydroxyl-directed cyclopropanation of under Simmons-Smith



Scheme 1.

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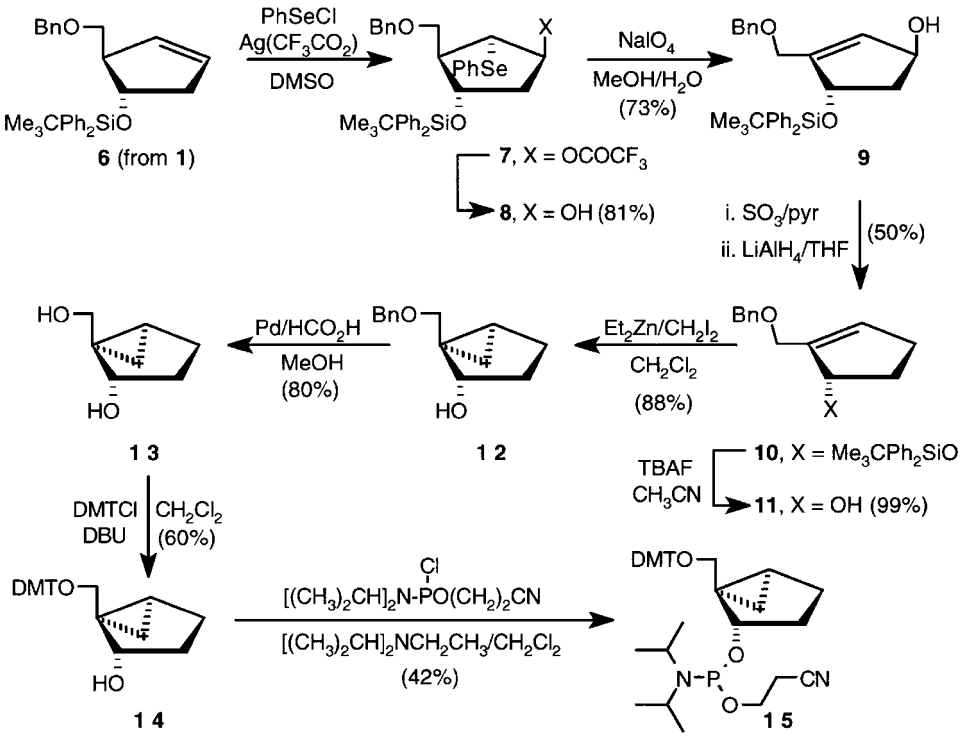
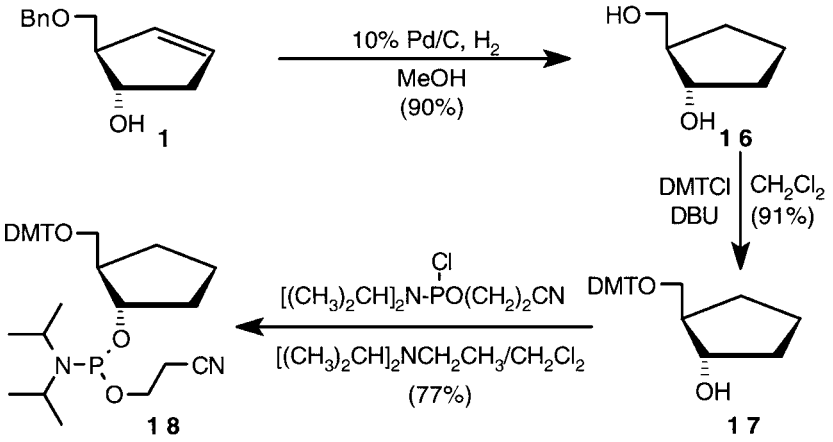


Scheme 2.

conditions to give the North-type intermediate **12**. Deprotection of the benzyl group, followed by dimethoxytritylation provided compound **14**, which was converted to the North-type analogue phosphoramidite **15** by the standard methodology.

A synthetic method for the preparation of cyclopentane phosphoramidite **18** has been reported (16). However, as outlined in Scheme 3, a simpler approach from **1** provided the same material.

Phosphoramidites **5**, **15** and **18** were used in a conventional DNA synthesizer to prepare the corresponding 13-mers containing the South- (ODN-South), the North- (ODN-North) and the cyclopentane (ODN-cyclopentane) abasic sites. The sequence of the target oligos synthesized was 5′-TGTCAGXGCATGG-3′ where X corresponds to the abasic sites shown in Figure 1. The coupling yields for the modified abasic pseudosugars were >95% which represents a significant improvement from the coupling yields obtained in our first approach (17). All 13-mer ODNs were annealed with equimolar amounts of the complementary strand containing the hemimethylated *Hha*I site (GMGC where M = 5-methylcytosine) after heating at 90°C for ten minutes and slowly cooling to room temperature. The three double-stranded ODNs gave similar melting curves (not shown) with almost identical Tm values: ODN-South (Tm = 38.2°C), ODN-North (Tm = 39.6°C) and ODN-cyclopentane (Tm = 37.1°C). Irrespective of their chemical nature, the presence of an abasic site strongly destabilizes the duplex relative to control ODN



Scheme 3.

(X = cytosine, Tm = 56.1°C) due to the loss of base stacking and Watson-Crick hydrogen bonding. The Tm for the ODN-cyclopentane was virtually indistinguishable from that of the THF-containing ODN (17).

INHIBITION OF M.*Hha*I METHYLASE

Inhibition assays were performed as described (17) with reaction mixtures containing 0.5 μg of each ODN, ~0.6 unit of DNA M.*Hha*I (1 unit transfers 1 pmol to the substrate per min) and 2.8 μCi (1Ci = 37 GBq) of [methyl-^{3}H] AdoMet (8 μM). Substrate = AMp:A'; A = 5′-ATT**GMGC**ATTCMGGATCMGMGATC-3′ and A′ = 3′-TAA**CGCG**TAAGGCCTAGGCGCTAG-5′ (target C underlined).

The inhibition curves for M.*Hha*I transferase activity exhibited by the three abasic ODNs (Fig. 3) shows that ODN-South has the highest inhibitory activity (IC_{50} of 14 nM) followed by ODN-cyclopentane (IC_{50} of 48 nM). ODN-North, on the other hand, had no detectable activity below 75 nM. The inhibitory activity of the flexible ODN-cyclopentane falls between that of ODN-South and ODN-North and is indistinguishable from that measured for ODN-THF (17).

Native gel-shift assays with M.*Hha*I (not shown) demonstrated that the ODN-South was able to form a "closed" complex with the enzyme even in the absence of cofactor (AdoMet or AdoHcy), whereas the ODN-North remained mainly in the "open" form (17). Although the binding of ODN-South and ODN-THF appeared essentially irreversible, ODN-South binds tighter. Indeed, it takes more than 110 h for 50% dissociation of ODN-South versus 55 h for ODN-THF to dissociate from M.*Hha*I when incubated in the presence of a 100-fold excess of cold competitor ODNs (17).

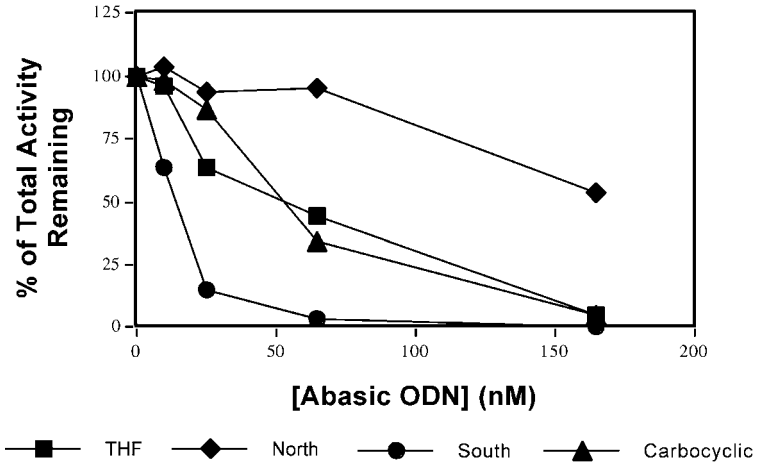


Figure 3. Inhibition of methyltransferase activity of M.*Hha*I by ODNs containing abasic sites.

THE FLIPPING MECHANISM

Investigation of the influence of a conformationally locked pseudosugar structure on the torsional properties of the γ dihedral was performed via *ab initio* calculations (Gaussian 94 or Gaussian 98 programs with the HF/6-31+G* basis set) of model compounds A, B, and C (Fig. 4). Since the behavior of the ODN-THF and ODN-cyclopentane was identical, only the THF moiety was studied as a flexible abasic site (17).

The γ dihedral energy surface (Fig. 5) for the three abasic sugar moieties was obtained by constraining the C3′-C4′-C5′-O5′ dihedral in 30° increments from 0° to 360°. In all cases, the following additional constraints were included: C4′-C5′-O5′-P = 168°, C5′-O5′-P-O = 298°, O5′-P-O-C = 262°, and C4′-C3′-O3′-H = 187°,

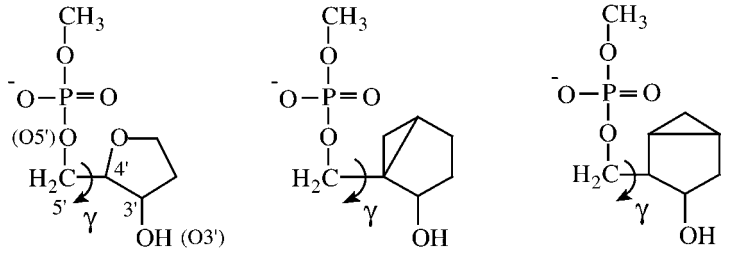


Figure 4. Model compounds to study the γ dihedral energy surface in the abasic moieties.

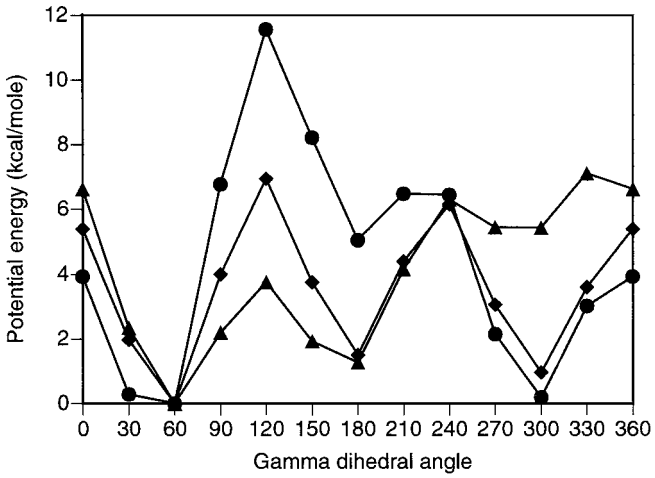


Figure 5. Potential energy as a function of the γ dihedral (◆ = model A (THF); ▲ = model B (North); ● = model C (South).

while the remainder of the molecule was allowed to relax. These constraints correspond to modal values of the β, α, ζ and ε crystallographic dihedral distributions in B-DNA (12), and are introduced to prevent variations in the other dihedral degrees of freedom from interfering with the γ energy surface, as previously performed (18). Due to the flexibility of the THF ring and to allow for a systematic analysis of the effect of sugar conformation on the behavior of γ, additional ring constrains were applied to C3′-C4′-O4′-C1′ to explicitly sample the C2′-endo (South) and C3′-endo (North) conformations in A.

Comparison of the three surfaces (Fig. 5) shows the structure of the sugar moiety to significantly influence the energetic properties. Common to all surfaces are the three minima that occur around 60°, 180° and 300°, corresponding to the $+sc$, *ap* and $-sc$ states, respectively. The $+sc$ minimum corresponds to the conformation observed in experimental DNA duplex structures (12). For the North model compound B, the $+sc$ to $-sc$ barrier (ca. 6 kcal/mole) is higher than the $+sc$ to *ap* barrier (ca. 4 kcal/mole) and, more importantly, the energy of the $-sc$ minimum is ca. 6 kcal/mol greater than that of both the THF and South model compounds, A and C, respectively. These energy differences are suggested to disallow the transition to the $-sc$ conformation for γ in the North DNA system and prevent flipping. What these results suggest is that flipping probably begins with the torsion angle γ rotating to the $-sc$ state and proceeding to *ap* state, which corresponds to the conformation observed in the crystal structure of the ternary complex (11). Both of these transitions are permissible for the flexible THF and the rigid South conformer, and are energetically more favorable than a direct $+sc$ to *ap* rotation. After the conclusion of the flipping event, the sugar is able to readjust its pucker to a North conformation only in the case of the flexible THF. This obviously cannot occur with the rigid South conformer and suggests that changes in pseu-

dorotation after the flipping event is concluded might be irrelevant for the stability of the final "closed" conformation of the complex.

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