N⁴-Methoxydeoxycytidine triphosphate is in the imino tautomeric form and substitutes for deoxythymidine triphosphate in primed poly d[A-T] synthesis with E. coli DNA polymerase I

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ABSTRACT

N^4-Methoxydeoxycytidine triphosphate (mo^4dCTP) substitutes for dTTP in poly d[A-T] synthesis with $\underline{E.\ coli}$ DNA polymerase I (Pol I). In parallel experiments using as template-primer, poly d[G-C], no incorporation of [^{14}C]mo^4dC was detected. This indicates that this deoxy derivative acts as the imino tautomer, as previously found for the riboderivative. Nearest neighbor analysis of transcripts of poly d[A-T] containing mo^4dC shows that the derivative substitutes for only one base. In replication, singlestranded mo^4dC-containing polymers gave little misincorporation, including that of dATP which can hydrogen-bond to mo^4dC in the imino form, if the methoxy group is $\underline{anti}$ to the N-3. It is therefore assumed that the methoxy group is constrained $\underline{anti}$ in a polymer such as d[A-T], but can be in the $\underline{syn}$ form in singlestranded polymers and not recognized by DNA polymerase. mo^4dC destabilizes the poly d[A-T] helix, as indicated by a lowered and less cooperative melting. Steric factors such as adjacent base displacement were invoked for similar findings with the doublestranded $r(U_{61}, mo^4C_{39}) \cdot r(A)$.

INTRODUCTION

Hydroxylamine and O-methylhydroxylamine (methoxyamine) have been useful reagents for studying the effect of tautomeric shift on mutation. Both form mono-substituted derivatives at the N^4 of C (or dC), concomitant with addition to the doublebond[1]. The N^6 of A (or dA) reacts to only form the mono substituted derivative, but its reaction is much slower than that of the N^4 of C[1]. The known tautomeric equilibrium between amino and imino of N^4-hydroxy C has led to the use of precursor N^4-hydroxy CDP or N^4-hydroxy dCTP in site-directed mutagenesis[2,3].

The methoxy analog, N^4-methoxy C (mo^4C), has been less studied but, in transcription, its presence has resulted in only ATP incorporation[4]. When poly (U_{61}, mo^4C_{39}) is transcribed by DNA-dependent RNA polymerase with only ATP present, the resulting doublestranded polymer has a hydrogen-bonded structure, further showing that mo^4C substitutes for U as a result of being the imino tautomer[5].

In this study we have prepared N^4-[^{14}C] methoxydeoxycytidine triphos-

phate by a new method and use it as a substrate for polymerases. The effects of mo^{4}dC on the template properties of resulting polymers are reported.

MATERIALS AND METHODS

0-[^{14}C]-methylhydroxylamine (methoxyamine) was obtained from New England Nuclear as a custom synthesis. Deoxynucleoside triphosphates were obtained unlabeled from Pharmacia P. L. and [^{3}H]-labeled from New England Nuclear at the highest specific activity available (10-30 Ci/mmol). [^{32}P] ribonucleo-side triphosphates were from Amersham (600-3000 Ci/mM). Polynucleotides, deoxyoligomers, the Klenow fragment of Pol I, terminal deoxynucleotidyl transferase (TdT), and E. coli DNA-dependent RNA polymerase were from Pharmacia P. L. Avian myeloblastosis virus (AMV) reverse transcriptase was from Seikhaku America, Inc. Highly purified Pol I was a generous gift from Dr. L. A. Loeb.

Preparation of N^4-[^{14}C]Methoxydeoxycytidine Triphosphate (mo^{4}dCTP)

25 mg dCTP (0.05 mmoles) were mixed with 0.15 ml 2.8 M pH 5.3 [^{14}C] methoxyamine HCl (0.42 mmoles, 0.12 mCi/mmol) and reacted for 18 h at 37°. To this was added 0.8 ml ethanol and 0.5 ml ether. The resulting oily pre-cipitate was dissolved in 1 ml H$_2$O. This crude product (yield 150 A$_{260}$ units, 6 x 10^4 cpm/λ_{max}) was largely disubstituted (bis), as judged from a comparison with the spectrum with that of the ribocytidylic acid derivative[6]. After evaporating to dryness with a N$_2$ stream, 0.5 ml 98% formic acid was added and the solution kept at 37° for 4 h, which almost quantitatively converted the bis to the N^4-monosubstituted derivative. The product was chromatographed, descending, on Whatman 3 MM paper with isoprop   alcohol: H$_2$O:saturated ammonium sulfate (30:15:0.2) as solvent. mo^{4}dCTP moved near the solvent front and was clearly separated from dCTP, dCDP and the bis derivative. The spectra of the two compounds, bis and mono, are very different (Figure 1) and contamination of the mono with the bis is expressed by the ratio of the λ_{max}220 to the λ_{max}283. The ratio obtained is 0.64, as contrasted to 0.75 for the previously reported analogous compound, N^4-methoxycytidine-5'-phosphate[6].

The final product, mo^{4}dCTP, in 0.1 N HCl had λ_{max} at 220 nm amd 283 nm, and λ_{min} at 219 nm and 245 nm. The integrity of the triphosphate was shown by chromatography on PEI-cellulose with 0.9 M LiBr which resolves mono-, di, and tri-phosphates. The specific activity was 2 x 10^4 cpm/A$_{283}$ (pH 1).

Synthesis of Alternating Poly d[A-T,mo^{4}C]

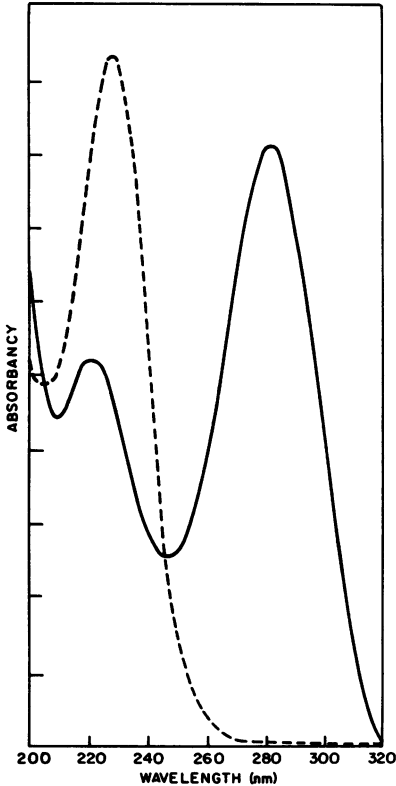Polymers were prepared using the same general method as previously

Fig. 1. UV absorption spectra in 0.1 N HCl of products of methoxyamine reaction with dCTP. N^4-methoxy dCTP (—); 6-methoxyamino-N-methoxy-5,6-dihydro-dCTP (– –).

described[7]. A typical reaction using Pol I contained (per ml): 50 mM Tris·HCl (pH 7.8), 2 mM MgCl$_2$, 0.15 A$_{260}$ of poly d[A-T], 0.1 μmol dATP, 0.05 μmol dTTP, 0.05 mol [^{14}C]mo^{4}dCTP (2 x 10^4 cpm/OD max) and 1.5 μg Pol I (30 units). After 75 min at 37°, the reaction was stopped and the polymer isolated[7]. The yield in O.D. units was 4-fold the starting material, without corrections for losses on handling. The newly synthesized polymer contained, by radioactivity measurement, 5.2% mo^{4}dC which represents about 10% of the pyrimidines. In another synthesis, using the Klenow fragment and stepwise proceedure[7] the net synthesis, after 8 h, was 7-fold without corrections, and 11.6% mo^{4}dC was incorporated. This represents 23% of the pyrimidines.

Attempted Synthesis of Poly d[G-C] Containing mo^{4}dC

Poly d[G-C], in the presence of dGTP, dCTP, and mo^{4}dCTP (2:1:1), was used under the same experimental conditions, but on a larger scale than used to prepare poly d[A-T,mo^{4}dC]. Little decrease in absorbance occurred over a 6-hour period and on on reisolation of the polymer, there was less than

two-fold synthesis. No [^{14}C]mo^{4}dC could be detected, although 1.5×10^5 cpm were in the reaction mixture.

Synthesis of Single-stranded Deoxypolymers Containing mo^{4}dC

Terminal deoxynucleotidyl transferase (TdT) was used to prepare polymers with oligo(dT)$_7$ as primer. Reaction mixtures contained, per ml: 100 mM cacodylate buffer (pH 7.2), 10 mM MgCl$_2$, 1 mM CoCl$_2$, 2 mM 2-mercaptoethanol, 0.4 - 1.0 μmol of a mixture of dATP and [^{14}C]mo^{4}dCTP (4:1 to 12:1 depending on the polymer desired), 0.1 A$_{260}$ primer and 1500 units TdT. The reaction was carried out in stoppered cuvettes at 37° and monitored by the decrease in absorbancy. Maximum decrease in absorbancy (30%) and maximum yield required 72 h. The polymer was isolated by first separating it from dNTPs on cellulose thin layer sheets using as solvent isopropanol:NH$_3$:H$_2$O (55:25:10)[8]. The polymer which remains at the origin was scraped off and eluted with water. The eluate was further purified by passing it through a Biogel P-150 column (7 x 25 cm) using 10 mM pH 7.8 Tris as eluant. The yield, without corrections for handling, was 50-75%, which represents a polymer containing 300-400 nucleotides. The content of mo^{4}dC was determined by counting an aliquot. Regardless of the input proportion of mo^{4}dC, the final polymer contained about 65% of this input. Control polymers lacking mo^{4}dC but containing unmodified triphosphates were prepared in a similar manner.

Attempts were made to use oligo (dC) or oligo (dA) as primers with mo^{4}dCTP and dCTP but the polymer yield was very low. Radioactivity representing [^{14}C]mo^{4}dC was incorporated but less than 15 nucleotides were added to the primer. These oligomers were too small to use as template.

Nearest Neighbor Analysis of Transcripts

Transcription and nearest neighbor analysis using [α ^{32}P]UTP or [α ^{32}P] ATP were according to Kuśmierek and Singer[9]. The incubation mixture contained 10 mM MgCl$_2$, 2 mM MnCl$_2$, 0.4 mM each of ATP, CTP, UTP, and GTP, with either UTP or ATP labeled, and _E coli_ DNA-dependent RNA polymerase.

Assay of Fidelity in Replication

Single-stranded deoxypolymers containing 4.5% or 8.5% mo^{4}dC, 2% dC, 9% dT, or 4% dG were used for replication as follows. A 50 μl reaction mixture contained 0.01 A$_{260}$ template, mixed with 0.001 A$_{260}$ oligo (dT)$_{12-18}$, 0.1 mM each dTTP and one other dNTP, 50 mM Tris·HCl, pH 7.8, 2 mM MgCl$_2$ and 0.4 units Pol I. Duplicate experiments were performed with each labeled dNTP. Incubation was 15 min at 37°. 40 μl reaction mixture was spotted on DEAE disks and washed repeatedly with 7% Na$_2$HPO$_4$, then with H$_2$O, followed by ethanol. The disks were dried and then radioactivity counted using a toluene scintillant.

Blanks values were obtained from reaction mixtures which did not contain
polymer. Misincorporation was considered significant only if the counts were
at least double the blank. Reverse transcriptase from AMV was used under the
same conditions except 20 units of enzyme were added and the reaction was for
30 min. One unit of reverse transcriptase is defined as incorporating
1 nanomole dTMP in 10 min, 35°, using an RNA template. One unit of Pol I
incorporates 10 nanomoles of dTMP in 30 min 37° using an activated DNA
template.

Thermal Denaturation Measurement

Measurements were made using Teflon-stoppered cuvettes in a dual-beam
Varian-Cary 219 spectrophotometer equipped with jacketed sample and reference
chambers. The temperature of the cell compartment was regulated by a Neslab
Eva-Endocal programmed at 1°/2 min. Up to four samples can be compared
simultaneously.

RESULTS

mo^4dCTP as a Substrate for Pol I

$[^{14}C]mo^4dCTP$ and its bis derivative (N^4-methoxyamino-5,6-dihydro dCTP)
were synthesized and characterized. The method of preparation of mo^4dCTP
utilized the fact that acid catalysis leads to reversal of the addition of
the second methoxy group to the C5-C6 double bond. By means of 98% formic
acid this result is achieved while avoiding any hydrolytic action on the
phosphotriester or glycosidic bonds. The spectral properties and the avail-
ability of the resultant triphosphate for enzymatic incorporation into
polynucleotides indicate a higher purity of this triphosphate than that of
the ribo triphosphate, mo^4CTP, previously reported[6,10]. The yield was almost
quantitative.

The monosubstituted triphosphate was a substrate for Pol I, was a
competitor of dTTP, and was incorporated into poly [A-T]. Synthesis, with
the two pyrimidines in a 1:1 ratio, was not decreased and mo^4dCTP was incor-
porated with about 10% efficiency compared to dTTP. Using the Klenow
fragment of Pol I, and increasing the amount of mo^4dCTP relative to dTTP, the
efficiency of incorporation of mo^4dC could be increased to about 25%. In
contrast, dCTP was not incorporated into poly d[A-T] at a level greater than
1/9000 bases synthesized. This is within the published values for the
fidelity of Pol I[11].

$[^{14}C]mo^4dCTP$ was not incorporated into poly d[G-C] with Pol I under the
same conditions and, unexpectedly, its presence inhibited the synthesis of
poly d[G-C].

Table 1. Nearest Neighbor Analysis of Transcripts of Poly (dA, mo^4dC) and of Poly d(A-T, mo^4dC) with $[\alpha^{32}P]$ UTP[a]

	Nearest Neighbor Sequence $(cpm \times 10^{-3})$[b]			
Polymer	$C^{32}pU$	$A^{32}pU$	$G^{32}pU$	$U^{32}pU$
Double-stranded[c]				
d[A-T]	0.21	904	2.7	4.4
d[A-T, mo^4dC]	0.5	626	3.8	3.6
Single-stranded[d]				
dA	0.24	0.48	0.55	433
dA, mo^4dC	0.25	_1.5_	0.65	350

a   Transcription conditions were according to Materials and Methods. DNA-dependent RNA polymerase, $[\alpha^{32}P]$ UTP, all four NTPs in equal molarity, 2 mM $MnCl_2$ and 10 mm Mg^{2+} were used.

b   The only significant misincorporation (> 2-fold that of the control polymer) resulting from mo^4dC is underlined. 0.32% of the total transcription is Ap which represents 1 Ap/30 mo^4dC.

c   Prepared by Pol I synthesis. Poly d[A-T , mo^4C] contained 9.4% mo^4dC.

d   Prepared using terminal deoxynucleotidyl transferase with oligo dT, as primer. Poly (dA, mo^4dC) contained 8.5% mo^4dC.

Effect of mo^4dC on Template Properties

DNA-dependent RNA polymerase was used to transcribe the mo^4dC-containing polymers. Nearest neighbor analysis of the transcripts of alternating poly d[A-T] containing mo^4dC, using $[\alpha^{32}P]$ UTP or ATP, showed that 99% of the ^{32}P was transferred to a single base, A_p or U_p respectively, (Tables 1, 2). These data indicate that the mo^4dC is not randomly inserted, but is in alternating sequence. The only indication of altered fidelity in transcription of poly d[A-T, mo^4C] is the occasional incorporation of GTP ($\backsim$ 1/100 mo^4dC) (Table 2). Single-stranded poly (dA, mo^4dC), synthesized with an oligo (dT) primer, directed some incorporation of ATP ($\backsim$ 1/38 mo^4dC) (Table 1).

The single-stranded polymers were also replicated using Pol I, but because of slippage commonly observed with stretches of dA hydrogen bonded to dT^{12}, the data are not easily interpreted. Although the presence of a few percent of dT in poly (dA) could easily be detected in this assay, mo^4dC residues did not direct a proportional amount of dATP (Table 3). The high

Table 2. Nearest Neighbor Analyses of Transcripts
of Poly d[A-T, mo^{4}C] with [^{32}P]ATP^a

Polymer	Nearest Neighbor Sequence (cpm x 10^{-4})							
	C^{32}pA	(%)^b	A^{32}pA	(%)	G^{32}pA	(%)	U^{32}pA	(%)
d[A-T]	0.025	(<0.01)	2.6	(0.35)	0.18	(0.038)	470	(>99.5)
d[A-T, mo^{4}dC]	0.03	(<0.01)	1.0	(0.25)	0.5	(0.12)	400	(>99.5)

a See Table 1 for description of methods and polymers
b Percent of total counts.

dATP incorporation with the control poly (dA) template made it impossible to ascertain whether any dATP incorporation could be attributed to mo^{4}dC. It was not possible to prepare polymers containing only dC and mo^{4}dC, which would have been advantageous in determining whether mo^{4}dC directed dA incorporation. No dCTP misincorporation was detected in poly (dA, mo^{4}dC) with either Pol I or AMV reverse transcriptase, which is more error-prone than Pol I[13] (Table 3). There was a small but definite misincorporation of dGTP (1/30 - 1/100 mo^{4}dC) that was detectible with both DNA polymerases, as well as by nearest neighbor analysis of transcripts using DNA-dependent RNA polymerase (Tables 2, 3).

Effect of mo^{4}dC on Thermal Denaturation of Poly [A-T]

Poly d[A-T, mo^{4}C] destabilized the helix, as measured by T_m, to a measurable extent, but reannealing was complete. With 19% of the pyrimidines as mo^{4}dC, the T_m was decreased and the cooperativity was diminished (Table 4). Figure 2 illustrates the melting behavior of poly d[A-T] and poly d[A-T, mo^{4}dC].

DISCUSSION

Although Pol I has exonucleolytic activity which can excise mismatched bases during synthesis[13], mo^{4}dCTP is an acceptable substrate in poly d[A-T] synthesis. This is in accordance with our earlier finding that mo^{4}C forms hydrogen-bonded basepairs with A[5]. This ability to basepair like T(U) can only occur if the derivative is the imino tautomer.

Both crystal[14] and nmr data[15,16] concur that mo^{4}C is in the imino form, but the exocyclic group prefers the rotational isomer which lies syn to the N-3 (Figure 3, top). Such a position would prevent basepairing. However, Engel and von Hippel[17] have shown that Pol I can rotate an exocyclic methyl group so that an adequate basepair can form (Figure 3, bottom). The ability

Table 3. Replication of Single-Stranded Deoxypolymers with
Pol I and Reverse Transcriptase[a]

Polymer[b] and Polymerase	dTTP + dATP dT (pmoles)	dA	dTTP + dCTP dT (pmoles)	dC	dTTP + dGTP dT (pmoles)	dG
Pol I						
dA	672	8.8	84	<0.026	148	<0.04
dA, mo^{4}dC (4.5%)	468	4.2	72	<0.027	84	0.17
dA, dC (2%)	336	6.6	48	<0.006	74	2.7 (1:0.5)[d]
dA, dT (9%)	240	12 (1:2.4)[d]				
dA, dG (4%)			54	0.2 (1:10)[d]		
Reverse Transcriptase						
dA			564	0.25	528	nd
dA, mo^{4}dC			372	0.25	324	<0.10
dA, dC					156	2.8 (1:1)[d]
dA, dG			312	1.6 (1:8)[d]		

a Replication was as described in Materials and Methods. 1200 pmoles dT
incorporation represents complete copying of the template. All values
are calculated after subtracting the blanks obtained in the absence of
polymer. Values less than double this number are indicated by a
"less than" symbol. Each set of data represents an independent experiment.

The extent of replication was variable.

b All polymers were synthesized using TdT and were initiated with
oligo(dT)$_7$ or oligo(d-T)$_{12-18}$ primers. The numbers in parentheses
refer to the percent of each minor base in the polymer.

c Pol I is highly purified E. coli DNA polymerase I. Reverse
transcriptase (lot 83A011) is the DNA polymerase from AMV.

d The numbers in parentheses refer to the incorporation of dA or dC
or dG relative to the amount of the complement in the template.
For example, with 9% T in a template and 3.7% A in the replicated
portion, the ratio (or efficiency) is 1 dA/2.4 dT after subtracting
the control, dA lacking dT.

Table 4. Thermal Denaturation of poly d[A-T] Containing mo^{4}dC

Polymer	15 mM NaCl/1.5 mM Na citrate, pH 7 T_m,°C	H,°C	H_{25-75}[b]	1 M NaCl[a] T_m,°C	H,°C	H_{25-75}
Poly d[A-T, mo^{4}dC][c]	42.4	39.4	4.2	71.4	40.7	6.2
Poly d[A-T]	46.1	41.9	1.1	74.8	40.5	2.8

a also contains 15 mM NaCl/1.5 mM Na citrate, pH 7.
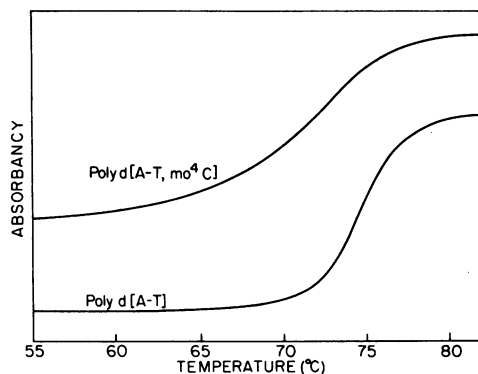b Width of transition (°C) from 25-75% of total hyperchromicity.
c Contains 9.4% mo^{4}C.

Fig. 2.   Thermal melting profiles in 1 M NaCl containing 0.1 x SSC.
Poly d[A-T, mo^{4}C] contains 11.5% mo^{4}dC, which is 23% of the
pyrimidines.

of a polymerase to rotate an exocyclic group may vary with the specific group
and may be energetically favorable in polymerization.

Once the modified nucleotide is incorporated into a doublestranded
structure it likely to continue to be held <u>anti</u> so that basepairing is main-
tained. This would, however, destabilize the polymer, which we find for the
alternating polymer, poly d[A-T, mo^{4}C], (Figure 2, Table 4).

The lack of significant A or dA incorporation in transcription and
replication of single-stranded dA polymers containing mo^{4}dC is not, we
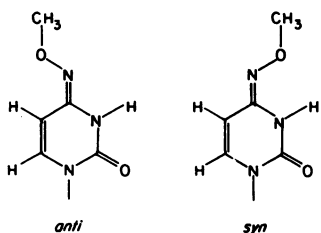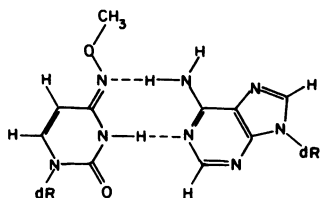

Fig. 3.   (Top) The <u>anti</u> and <u>syn</u> conformations of
the imino form of mo^{4}C.
(Bottom) Watson-Crick type basepairing
between dAdo and mo^{4}dC in the <u>anti</u> imino
form. The drawing is adapted from
Kierdaszuk <u>et al</u>[17].

believe, an accurate reflection of the nucleotide's ability to pair with A. This has been demonstrated with the same enzymes, Pol I and DNA-dependent RNA polymerase, in the experiments with the alternating polymer (Tables 1,2) and in earlier work from this laboratory[5]. Thus, it is likely that failure to observe dA incorporation using poly(dA, mo[4]dC)templates is a consequence of some aspect of the structure of the single-stranded polymer. There is significant stacking in poly (dA), even greater than observed in poly(rA)[18]. Therefore, mo[4]dC could be excluded by these interactions and not read by the polymerase. Indeed, inclusion of mo[4]dC might disrupt not only the specific stacking of two residues, but also delay the renucleation of stacking by distorting the alignment. It is well established that the pyrimidines do not stack as well as the purines[19], which could account for the observation that in copolymers with C appropriate mo[4]C•A pairing occurred[5]. Therefore, the expression of mo[4]C•A pairing may be sequence dependent.

A secondary factor which addresses the very low, but consistent, G incorporation directed by these polymers is the possibility that the small proportion of mo[4]C in the $\underline{anti}$ amino form can be read. Here again, the flexibility about the $N^4\text{-}\overline{O^4}$ bond would permit discrete insertion of the nucleotide in the helix, as if it were C. It appears unlikely that even the presumed minor proportion of mo[4]dCTP in the amino form can substitute for the unmodified triphosphate. In fact, the presence of mo[4]dCTP greatly inhibits Pol I synthesis of poly d[G-C]. A similar inhibition has been observed when m[6]dGTP is substituted for dGTP in poly d[G-C] synthesis[20].

It was suggested by Kierdaszuk and Shugar[16], from model studies in chloroform, that the presence of a methyl group on the C-5 would be even more energetically unfavorable for the methoxy group to rotate $\underline{anti}$. However, in unpublished experiments from this laboratory, we find that 5-methyl-N[4]-methoxy CTP, incorporated into ribopolymers, directs the incorporation of rA only slightly less frequently than does mo[4]C. Thus, we conclude that the methoxy group of mo[4]C which is predominantly imino can be rotated by polymerases in solution.

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