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Probing the dNTP Binding Region of *Bacillus subtilis*: DNA Polymerase III with Site-Directed Inhibitors: A Dissertation

Michelle Marie Butler
*University of Massachusetts Medical School*

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PROBING THE dNTP BINDING REGION OF BACILLUS SUBTILIS DNA POLYMERASE III WITH SITE-DIRECTED INHIBITORS

A DISSERTATION PRESENTED
BY
MICHELLE MARIE BUTLER

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in Partial Fulfillment of the Requirements for the Degree of:

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

March 13, 1992
Pharmacology
PROBING THE dNTP BINDING REGION OF BACILLUS SUBTILIS DNA POLYMERASE III WITH SITE-DIRECTED INHIBITORS
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MICHELLE MARIE BUTLER

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March, 1992
I would like to express my gratitude to those people who were instrumental to the completion of this thesis.

To Neal Brown, thanks for the inspiration you have given me as a scientist and a leader. Without your guidance and input, I would never have become the person I am today.

To George Wright, your vast chemistry and DNA polymerase knowledge has always kept me on my toes.

To Lech Dudycz, thank you for your tremendous assistance with my chemical synthetic work.

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To my parents, Adrienne and Armand Fontaine, I am forever grateful for your belief in the power of a good education. To all my family members, I thank you for your constant support and encouragement.

Finally, to my husband, Joe, without your support and partnership in my endeavors, I would never have achieved this goal. Thank you.
This thesis is dedicated to Joseph Jerome Butler
PROBING THE dNTP BINDING REGION OF *BACILLUS SUBTILIS*
DNA POLYMERASE III WITH SITE-DIRECTED INHIBITORS

March, 1992
Michelle Marie Butler
B.S., Rivier College, 1986
Ph.D., University of Massachusetts Medical School
Directed by Neal C. Brown, D.V.M., Ph.D.

![H2-HPUra](image)

**ABSTRACT**

6-(p-Hydroxyphenylhydrazino) uracil (H2-HPUra) is a selective and potent inhibitor of the replication-specific DNA polymerase III (pol III) of Gram+ bacteria such as *Bacillus subtilis*. Although a pyrimidine, H2-HPUra derives its inhibitory activity from its specific capacity to mimic the purine nucleotide, dGTP. The project described in this thesis dissertation involves the use of H2-HPUra-like inhibitors to probe the structure and function of the pol III active site. It consists of two separate problems which are summarized below.
Production of a potent *bona fide*, dGTP form of inhibitor. A method was devised to successfully convert the H2-HPUra inhibitor prototype to a *bona fide* purine, using N2-benzyl guanine as the basis. Structure-activity relationships of benzyl guanines carrying a variety of substituents on the aryl ring identified N2-(3,4-dichlorobenzyl) guanine (DCBG) as a compound equivalent to H2-HPUra with respect to potency and inhibitor mechanism. DCBdGTP, the 2'-deoxyribonucleoside 5'-triphosphate form of DCG, was synthesized and characterized with respect to its action on wild-type and mutant forms of pol III. DCBdGTP acted on pol III by the characteristic inhibitor mechanism and formally occupied the dNTP binding site with a fit which permitted its polymerization. The latter experiment identified the site for the binding of the inhibitor's aryl moiety as a distinct site located at a distance of approximately 6-7 Å from the base-paired 2-NH group of a bound dGTP.

Attempt to covalently label amino acid residue 1175, a putative participant in inhibitor binding. Azp-12, a point mutation of serine 1175, yields a form of pol III whose inhibitor sensitivity varies specifically as a function of the composition of the *para* substituent of the inhibitor’s aryl ring. On the basis of the latter behavior, residue 1175 was hypothesized to be a residue directly involved in the binding of the inhibitor's aryl moiety. To test this hypothesis, residue 1175 was specifically mutated to either cysteine or lysine, each of
which presents a side chain amenable to covalent bond formation with appropriately reactive inhibitor forms. Of the two mutant pol III forms, only the cysteine form (pol III-cys) was catalytically active. The kinetic properties and inhibitor sensitivity profile of pol III-cys identified it as a target suitable for potentially irreversible inhibitor forms containing the following groups in the meta position of the aryl ring: \(-\text{CH}_2\text{Br}\), \(-\text{CH}_2\text{Cl}\), and \(-\text{CH}_2\text{SH}\). None of the several inhibitors tested selectively or irreversibly inactivated pol III-cys. Possible bases for the failure of this group of inhibitors and for the redesign of more useful covalently reactive inhibitor forms are considered.
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<td>Amino Acid</td>
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<tr>
<td>AMP</td>
<td>Adenosine 5'-monophosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C.T. DNA</td>
<td>Calf Thymus DNA</td>
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<tr>
<td>dA</td>
<td>2'-Deoxyadenosine</td>
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<tr>
<td>dC</td>
<td>2'-Deoxyctydine</td>
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<td>dG</td>
<td>2'-Deoxyguanosine</td>
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<tr>
<td>dGTP</td>
<td>2'-Deoxyguanosine 5-triphosphate</td>
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<tr>
<td>dNMP</td>
<td>2'-Deoxyribonucleoside 5'-monophosphate</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-Deoxyribonucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>dT</td>
<td>2'-Deoxythymidine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>Exo</td>
<td>3'-5' Exonuclease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>FPLC</td>
<td>Fast Performance Liquid Chromatography</td>
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<td>HAc</td>
<td>Acetic Acid</td>
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<td>Nal</td>
<td>Nalidixic Acid</td>
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<td>OD</td>
<td>Optical Density</td>
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<td>PCR</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
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<td>Pol</td>
<td>DNA Polymerase; DNA polymerase activity</td>
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<td>PP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Pyrophosphate</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
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<td>TDC</td>
<td>Taurodeoxycholate</td>
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<td>TE</td>
<td>Tris-HCl, EDTA</td>
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### INHIBITOR ACRONYMS

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<td>BuPdGTP</td>
<td>N\textsuperscript{2}-(p-Butylphenyl)-2'-deoxyguanosine 5'-triphosphate</td>
</tr>
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<td>BuPG</td>
<td>N\textsuperscript{2}-(p-Butylphenyl)guanine</td>
</tr>
<tr>
<td>DCBdG</td>
<td>N\textsuperscript{2}-(3,4-Dichlorobenzyl)-2'-deoxyguanosine</td>
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<td>DCBdGTP</td>
<td>N\textsuperscript{2}-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DCBG</td>
<td>N\textsuperscript{2}-(3,4-Dichlorobenzyl)guanine</td>
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<td>HPUra</td>
<td>6-(p-hydroxyphenylazo)uracil</td>
</tr>
<tr>
<td>H\textsubscript{2}-HPUra</td>
<td>6-(p-hydroxyphenylhydrazino)uracil</td>
</tr>
<tr>
<td>H\textsubscript{2}-PUra</td>
<td>6-(phenylhydrazino)uracil</td>
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<tr>
<td>N\textsuperscript{2}-G</td>
<td>N\textsuperscript{2}-substituted Guanine</td>
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<tr>
<td>TMAU</td>
<td>6-(3,4-trimethyleneanilino)uracil</td>
</tr>
<tr>
<td>TMPG</td>
<td>N\textsuperscript{2}-(3,4-trimethylenephenyl)guanine</td>
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A. DNA-Dependent DNA Polymerases

All DNA polymerases are primer-driven, template-dependent enzymes which catalyze polymerization of the nucleoside 5'-monophosphate (dNMP) moiety of 2'-deoxyribonucleoside 5'-triphosphates (dNTPs) (Kornberg & Baker, 1992). They perform this function by catalyzing the extension of a 5' to 3' phosphodiester-linked polynucleotide along an antiparallel (3' to 5') template strand. DNA polymerases use Watson-Crick base pairing (Watson & Crick, 1953) furnished by the hydrogen bonding substituents of the bases to ensure correct dNTP selection (Figure I-A, page 2). With the exception of retroviral reverse transcriptases (Allaudeen, 1980) and the mammalian DNA polymerase γ (Fry & Loeb, 1986), both of which can use either a DNA or RNA template, DNA polymerases generally prefer to copy a polydeoxyribonucleotide template (Kornberg & Baker, 1992).

The salient features of the mechanism of DNA polymerase action are summarized in Figure I-A, page 2. Incorporation of a dNMP moiety occurs by nucleophilic attack of the 3'-OH group of the nucleotide at the primer terminus on the α phosphate of the incoming dNTP in the presence of Mg²⁺. The products of this reaction are a molecule of
Figure I-A: dNTP incorporation scheme. The template strand is that which furnishes the instructions for the sequence of nucleotides to be added to the primer strand. The primer strand is that which is growing at the 3'-OH terminus (Kornberg & Baker, 1992).

Reproduced with permission from Wright & Brown (1990).
pyrophosphate (PPi) and a template:primer whose primer moiety has been extended by one dNMP residue.

DNA polymerases cannot initiate a nucleotide chain \textit{de novo} from a single dNTP. They must be furnished with the 3'-OH of an NMP residue that has been linked, through its 5' position, to an oligo or polynucleotide (ribo or deoxyribo, Maki et al., 1985; Allaudeen, 1980) or a protein (Blanco & Salas, 1986; Nagata et al., 1983).

B. Bacterial DNA Polymerases

The Gram$^+$ bacterium, \textit{Bacillus subtilis}, like the Gram$^-$ organism, \textit{Escherichia coli} (Moses & Richardson, 1970; Kornberg & Gefter, 1971; Kornberg & Gefter, 1972; Wickner et al., 1972), has three distinct DNA-dependent DNA polymerases. The three enzymes are designated as polymerase (pol) I, pol II and pol III (Okazaki & Kornberg, 1964; Gass & Cozzarelli, 1973a; Ganesan et al., 1973; Laipis & Ganesan, 1972; Gass & Cozzarelli, 1973b) and are encoded by the respective genes, \textit{polA}, \textit{polB} and \textit{polC}. DNA polymerase I, like the corresponding \textit{E. coli} enzyme, is involved primarily in the repair synthesis of DNA (Okazaki & Kornberg, 1964; Bazill & Gross, 1973). Pol II has no known function in either \textit{E. coli} (Masker et al., 1973; Bonner et al., 1988) or \textit{B. subtilis}. Pol III, the subject of this work, is an essential enzyme, specifically required for the replicative synthesis of the host chromosomal DNA (Cozzarelli & Low, 1973; Neville & Brown, 1972; Brown, 1971).
C. B. subtilis Pol III: Target of this Study and Model for Gram⁺ Pol III

The structural gene for B. subtilis pol III, polC, has been cloned, sequenced, and engineered for overexpression as a functional recombinant protein in E. coli (Bares & Brown, 1983; Ott et al., 1986; Barnes et al., 1989; Hammond et al., 1991; Hammond & Brown, 1992; Barnes et al., 1992). The pol III protein is a 162.4 kDa polypeptide consisting of 1437 amino acids (Hammond et al., 1991). The protein possesses both polymerase (pol) and 3'-5' editing exonuclease (exo) activity (Clements et al., 1975; Low et al., 1976). The site of exo catalysis, a region displaying considerable primary structural homology with the model developed for the 3'-5' exo site of E. coli DNA pol I (Bernad et al., 1989; Beese & Steitz, 1991; Derbyshire et al., 1991), has recently been localized by the Brown group (Barnes et al., 1992) to a region within the enzyme encompassed by amino acids 420-510.

The integrated exo-pol structure of B. subtilis pol III is not mirrored in the structure of Gram⁻ pol IIIIs, exemplified by the enzymes of E. coli (Tomasiwicz & McHenry, 1987) and the nearly identical Salmonella typhimurium pol III (Lancy et al., 1989). Rather the two catalytic functions are carried on two physically and genetically distinct subunits-α, a 130 kDa protein subunit carrying the pol activity, and ε, a 27.5 kDa subunit (Echols et al., 1983; Scheuermann & Echols, 1984) carrying the exo activity.

Comparative amino acid sequence analysis has indicated significant homology (28% identity) between amino acids 613-1434 (Hammond et al., 1991) of B. subtilis pol III and the pol specific α subunits of the Gram⁻ pol IIIIs (Tomasiwicz & McHenry, 1987; Lancy et al., 1989). Figure I-B, pages 5-6, shows the sequence comparison made by Hammond et al. (1991). The results of this comparison and a wider comparison of the primary
Figure I-B: Comparison of the deduced amino acid sequences of *B. subtilis* pol III and the α (polymerase) subunit of *E. coli* pol III. *Upper panel:* Block diagram drawn to scale and showing arrangement and span of the regions of homology; solid lines, regions with no significant homology; shaded boxes, regions of homology detailed in lower panel; open box (exo), block of homology with the DNA ε exonuclease (detail not shown; Sanjanwala & Ganesan, 1989; Ito & Braithwaite, 1991; Barnes et al., 1992). *Lower panel:* Comparison of shaded blocks shown in upper panel. Last digits of numbers are aligned with corresponding amino acid (aa); colon, identical aa residues; single dot, either aa equivalence or codons differing in one base; dash, presence of gaps. Reprinted with permission by Hammond et al. (1991).
structures of bacterial pol IIIIs with those of >20 other DNA-dependent DNA polymerases strongly suggests that the pol IIIIs constitute a structurally unique family (Family C; Ito & Braithwaite, 1991) of enzymes, distinct from enzymes of eukaryotic and viral origin.

It is likely that many bacterial pol IIIIs will be added to this family as their sequences are elucidated (Ito & Braithwaite, 1991).

D. H2-HPUra-Type Inhibitors

Pol IIIIs of *B. subtilis* and other Gram+ bacteria display a property not observed for the corresponding replication-specific enzymes of Gram- organisms: they are uniquely sensitive, both *in vivo* (Brown, 1970) and *in vitro* (Bazill & Gross, 1972; Brown et al., 1972; Neville & Brown, 1972; Cozzarelli, 1977), to the inhibitory effects of 6-(arylhydrazino) pyrimidines, a novel class of antibacterial agents whose prototype is 6-(p-hydroxyphenylhydrazino)uracil (H2-HPUra).

The inhibitor family prototype, the "prodrug" azo form, 6-(p-hydroxyphenylazo)uracil (HPUra), was originally synthesized by Bernard Langley of Imperial Chemical Industries, Ltd. where it was observed to be a potent inhibitor of the growth of Gram+ bacteria and to be without significant effect on the growth of Gram- bacteria or animal cells. Its selective toxicity for Gram+ organisms prompted Brown and Handschumacher to explore its mechanism in the Gram+ bacterium, *Streptococcus faecalis*, using HPUra (Figure 1-C, page 8) as the model inhibitor. The results of their studies (Brown & Handschumacher, 1966) indicated that HPUra inhibited the replicative synthesis of DNA and, further, that it achieved this effect selectively, without inhibiting RNA, protein or cell wall synthesis, or the pathways involved in dNTP synthesis. Brown then extended this work to *Bacillus*
Prodrug (inactive form)

[H] (Reduction)

Active Form

Figure I-C: *Top:* Structure of HPUra, the oxidized inactive form of the drug. *Bottom:* Structure of H2-HPUra, the reduced form of the drug, which is active on the isolated enzyme. Reduction [H] *in vivo*, in the bacterial cell, occurs by an unknown mechanism. Reduction *in vitro* is readily accomplished with reducing agents such as sodium dithionite (Neville & Brown, 1972) or dithiothreitol (DTT) (Gass et al., 1973).
subtilis where he determined that DNA repair synthesis, unlike replicative synthesis, was completely resistant to HPUs (Brown, 1970; Brown, 1971).

Subsequent work with toluene-treated permeable B. subtilis revealed two important facts about drug action (Brown, 1972; Brown et al. 1972). First, it revealed that the azo form, HPUs, is inactive on its target and requires reduction to the hydrazino form (H2-HPUs, Figure I-C, page 8) to effect enzyme inhibition, and second, it indicated that this active hydrazino form acts by mimicking the purine dNTP, dGTP (Brown, 1972). The action of H2-HPUs is specifically and competitively reversed by dGTP. The specific target of H2-HPUs was elucidated by in vitro analysis of its action on the three isolated B. subtilis DNA polymerases (Neville & Brown, 1972; Gass et al., 1973; Mackenzie et al., 1973). H2-HPUs inhibited only pol III, and was without significant effect on either pol I or pol II.

Hydrogen bonding with template cytosine. The ability to mimic dGTP suggested that the inhibitory action of H2-HPUs resided, in part, in its ability to base pair, like guanine, with an unapposed template cytosine. The results of three sets of experiments confirmed this suggestion. The first experiment examined the effects of H2-HPUs on the activity of pol III in the presence of the synthetic template:primers, poly(dC):oligo(dG) and poly(dA):oligo(dT). H2-HPUs inhibited the poly(dC):oligo(dG)-mediated reaction and had no inhibitory effect on poly(dA):oligo(dT)-mediated synthesis (Clements et al., 1975). The second set of experiments analyzed interactions between H2-HPUs and cytosine via proton NMR. The results showed specific downfield changes in chemical shifts for the hydrazino N-6 and NH-1 proton signals of H2-HPUs in cytosine-containing solutions (Mackenzie et al., 1973). The third set consisted of studies of the crystal structure of H2-
HPUra itself. The results corroborated those established by NMR and confirmed the base pairing scheme shown in Figure I-D, page 11, (Coulter & Cozzarelli; 1974, Coulter & Cozzarelli, 1975), a scheme which clearly illustrated the purine-like behavior of H2-HPUra and its specific capacity to mimic dGTP.

**Sequestration.** During the course of investigation of drug mechanism, it became clear that H2-HPUra action did not depend solely on the capacity of this drug to compete with dGTP for the hydrogen bonding sites on template cytosine (Wright & Brown, 1990). The best evidence of a more complicated mechanism came from the observation that H2-HPUra, in the presence of natural cytosine-containing template:primer DNA, strongly inhibited the residual synthesis catalyzed by pol III under "truncated" (-dGTP) conditions (Wright & Brown, 1976). The ability of H2-HPUra to inhibit the dGTP-independent catalytic activity of pol III clearly suggested that the inhibitor, in the presence of a cytosine-containing template, induced the sequestration of pol III in an inactive DNA-enzyme-drug complex.

The first clear evidence for complex formation was obtained in an experiment which used poly(dA):oligo(dT) as the principal template:primer. As indicated above, this DNA cannot base pair with H2-HPUra and thus, dA:dT driven synthesis is resistant to inhibition. However, the addition of a small amount of activated (cytosine-containing) DNA, which alone produced an insignificant amount of dTMP incorporation, caused the main poly(dA):oligo(dT)-driven reaction to become drug sensitive (Gass et al., 1973). These results indicated that H2-HPUra sequestered enzyme on the cytosine-containing template:primer, leaving little enzyme available for incorporation of dTMP by the homopolymer template:primer.
Figure I-D: Proposed mechanism of H$_2$-HPUra:cytosine base pairing. The parts of the molecules which participate in base pairing are the 4, 3 and 2 moieties of cytosine and the 2, 1 and 6-NH positions of H$_2$-HPUra (left), which behave like the 6, 1 and 2 moieties of guanine (right). dNMP symbolizes the 2'-deoxyribosyl 5'-monophosphate moiety of each nucleotide.
The above results were corroborated by the results of a series of experiments that examined the effects of H2-HPUra on the elution behavior of pol III, DNA and pol III-DNA mixtures on an agarose gel column (Clements et al., 1975). In the absence of drug, both DNA and pol III eluted separately on this column, and H2-HPUra did not affect the elution profiles of DNA or enzyme alone. In the presence of drug, however, enzyme and DNA that had been loaded together were coeluted from the column (Clements et al., 1975) indicating that they had become complexed. Three ancillary observations supported these results. First, the inactive, azo form of drug, HPUra, did not promote complex formation. Second, the $K_i$ concentration of H2-HPUra and $K_{cx}$, the concentration of H2-HPUra required to induce half-maximal DNA-enzyme complex formation, were essentially the same (Clements et al., 1975). Third, dGTP, which specifically and competitively reversed drug-induced inhibition of pol III, also specifically antagonized complex formation (Clements et al., 1975).

E. H2-HPUra: Model for its Proposed Mechanism of Action

Working independently, Low et al. (1974) and Clements et al. (1975) proposed identical molecular models to explain the observed actions of H2-HPUra on pol III. The proposed model, taken from a schematic diagram of Brown and Wright (1977b), is shown in Figure I-E, page 13. The complex is ternary, composed of polymerase, template:primer DNA and a base-paired drug in a 1:1:1 stoichiometry. H2-HPUra behaves as a bridge, linking pol III and DNA by hydrogen bonding with a template cytosine immediately distal to the 3'-OH primer terminus and interacting, via its aryl moiety, with an enzyme site close
Figure I-E: Proposed model of H2-HPUra mechanism. Drug sequesters DNA and enzyme into a reversible ternary complex. This figure is copied with permission from Brown & Wright (1977b).
to, if not identical with, its dNTP binding site (Brown & Wright, 1977b). The interactions of DNA and enzyme have been assumed, in the model, to be the same as those which govern normal (drug-free) primer extension and which position the 3'-OH terminus of the DNA such that polymerization of the inhibitor could occur if it were in a bona fide dNTP form (see Figure I-E, page 13).

F. Structural Requirements for the Inhibitory Action of H2-HPUra and its Derivatives

H2-HPUra, as shown by NMR and crystal studies, must be able to hydrogen bond with template cytosine with its 6, 1 and 2 pyrimidine ring moieties (see Figure I-D, page 10). A variety of structural analogs of H2-HPUra affecting these substituents was synthesized in order to determine structure-activity relationships (Wright & Brown, 1974; Wright & Brown, 1976). Many substitutions can be made on the H2-HPUra structure without altering the guanine-like character of the inhibitor (Wright & Brown, 1977). Substitutions which change the ability of the inhibitor to base pair with cytosine include replacement of the oxo group at carbon 2 of the pyrimidine ring with an amino group. This rearrangement gives an equally potent inhibitor but changes its character to that of a dATP-specific analog (Wright & Brown, 1976; Mackenzie et al., 1973) which requires dATP to effect reversal of inhibition (Wright & Brown, 1976).

When the 6-NH moiety is alkylated or replaced with a methylene group, inhibitory activity is abolished. This is not the case for the NH moiety proximal to the aryl ring. This NH can be replaced with a methylene group or entirely removed. Appropriately substituted
benzylamino and anilino uracils are just as potent as their corresponding phenylhydrazino uracils (Brown et al., 1977).

Another structural requirement of the H2-HPUra inhibitor format is the presence of a planar, unsaturated aryl group. Removal of the aryl moiety and replacement with alkyl substituents or saturated cyclic substituents results in a loss of inhibitory activity (Wright & Brown, 1980; Brown et al., 1986). A variety of aryl ring substituents are compatible with maintenance of inhibitory potency. For example, most small meta or para alkyl or halo groups (which are hydrophobic in nature) maintain potency at the 0.5-2 μM Kᵢ range (Wright & Brown, 1977). Second, ortho substituents of any size or chemical nature are not tolerated and result in a major loss of potency (Wright & Brown, 1977), a loss likely due to steric hindrance of the mobility of the aryl moiety relative to the heterocyclic ring. Inhibitors with electron-withdrawing groups (i.e., NO₂ and CO₂H) also show remarkably decreased activity, likely resulting from electronic effects (Wright & Brown, 1977).

Wright and Brown sought to develop HPUrA-like 6-substituted uracils that would not require a chemical reduction to maintain inhibitory activity, and to pursue this goal, they synthesized a series of uracils which are summarized in Figure I-F, page 16. The azo/hydrazino form of uracil was altered to produce a series of 6-anilinouracils (1A) and 6-benzylaminouracils (1B) (Brown et al., 1977; Wright & Brown, 1980). Several 6-anilinouracils proved to be potent inhibitors, particularly those with larger hydrophobic groups such as 6-(3,4-trimethyleneanilino)uracil (TMAU). In general, benzylamino uracils with substituents equivalent to those of the phenylhydrazino uracils (Wright & Brown, 1977) had similar Kᵢs, indicating that the positioning of the aryl moieties of the two inhibitor types was similar.
Figure I-F: The evolution of H$_2$-HPUra-like inhibitors. Brackets indicate the base pairing domains of each prototype.
Following the development of the anilino and benzylamino derivatives, Wright and Brown sought to construct 2'-deoxyribonucleotidyl forms of the inhibitors. This construct was not possible with the uracil nucleus because pyrimidine glycosylation, which occurs at N1 of the heterocycle, would destroy the capacity of the pyrimidine to base pair with cytosine. Therefore, Wright and Brown used an alternative approach to nucleotide construction and developed a route based on the use of N2-substituted guanine (N2-G) as the heterocyclic nucleus. Because glycosylation of purines such as N2-G occurs at the 9 position, the N2-G nucleus provided a structural basis for nucleotide synthesis without compromising the integrity of the essential base pairing domain. Two specific classes of N2-substituted guanines, N2-arylguanines (2A) and N2-benzylguanines (2B), were synthesized. The most potent phenylguanine, N2-(3,4-trimethylenephenyl)guanine (TMPG), the guanine form corresponding to 6-(3,4-trimethyleneanilino)uracil (TMAU), was nearly as potent as TMAU on the isolated pol III in vitro, but was unable to penetrate intact cells (Wright et al., 1982).

G. Pol III Azp-12: a Drug-Resistant Mutant Enzyme

PolC azp-12 (Clements et al., 1975) and PolC22 (Cozzarelli & Low, 1973) are mutant pol III structural genes which were identified on the basis of the HPUr-resistant growth phenotype they confer upon their hosts. It has recently been determined, through sequencing, that these mutant polymerases result from the same amino acid change - serine
1175 to alanine (S1175A; Sanjanwala & Ganesan, 1989; Barnes et al., 1989; Barnes et al., 1992). S1175A has been useful to the study of polC and its enzyme product, pol III, in several ways. First, it established the central role of pol III in replication of B. subtilis chromosomal DNA (Clements et al., 1975; Cozzarelli & Low, 1973). Second, it became the means through which the Brown lab cloned polC and mapped its location on the B. subtilis chromosome (Love et al., 1976; Barnes & Brown, 1983; Ott et al., 1986). Third, the assessment of the mutant's catalytic properties has provided important information regarding the structure of the inhibitor binding site.

Pol III azp-12 is specifically resistant to H2-HPUra and other analogs carrying OH or other polar substituents in the para position (Brown & Wright, 1977a). Although resistant to this dGTP analog, the mutant enzyme maintains the same K_m for dGTP and other dNTP substrates, and it displays a near identical V_max for polymerization (Clements et al., 1975). The latter observations have suggested that the dNTP and inhibitor binding sites are not formally identical.

The structure-activity relationships indicated above in section F suggest several features of the aryl binding site. First, the binding site is hydrophobic and comprised of components which are likely arranged in a coplanar configuration, giving the site the ability to interact strongly with the phenyl ring of the inhibitors through van der Waals' interactions. Second, the S1175A mutation (pol III azp-12) reduces only the interaction of hydrophilic aryl substituents such as that on H2-HPUra, suggesting that a secondary, weaker hydrophilic site of interaction exists that is not in direct contact with the phenyl ring. It is postulated that the -CH2OH moiety of serine 1175 provides this interaction and
therefore, when it is changed to alanine (azp-12), the resultant methyl side chain may cause repulsion of the polar -OH substituent of H$_2$-HPUra, weakening its binding and potency as an inhibitor.
My thesis research project focuses on *B. subtilis* pol III. It is derived from the long range objective of the Brown laboratory to elucidate, in molecular detail, the structure and function of the dNTP binding domain of the polymerase active site and to determine the essential features of this domain that make Gram+ pol III's uniquely reactive with the H₂-HPUra inhibitor prototype.

My project has posed and addressed two major questions: (1) Physically, how are the aryl and dNTP binding sites of pol III related? Do they overlap or are they formally distinct? (2) Does there exist, among a collection of several untested inhibitor forms, any which is a strong candidate for use as a covalently reactive marker of the aryl binding site? How questions 1 and 2 were addressed experimentally and how each question was answered are presented, respectively, in Chapters III and IV.
CHAPTER III

ARE THE ARYL AND dNTP BINDING SITES OF POL III FORMALLY SEPARATE OR DO THEY OVERLAP?

A. Approach

To address this question experimentally, I proposed to synthesize and exploit an inhibitor form in which a pol III-specific aryl moiety was incorporated into the N² position of dGTP, the 2'-deoxyribonucleoside 5'-triphosphate with which the simple base forms of the H2-HPUra prototype are specifically competitive. This approach was analogous to that taken with the mammalian DNA polymerase α-specific inhibitor, N²-(p-n-butylphenyl)guanine (BuPG) (Khan et al., 1984). The conversion of BuPG to its corresponding 2'-deoxyribonucleoside 5'-triphosphate form increased its potency approximately 1000-fold, from a Kᵢ of 12 µM to less than 10 nM (Khan et al., 1984).

Specifically, I sought to determine for the pol III case: (a) if such a dNTP form would retain the characteristic, H2-HPUra-like mechanism of action, and (b) if the dNMP moiety of this dNTP form is polymerized to the 3'-OH group of the primer terminus while resident in the ternary enzyme: DNA: inhibitor complex. If the results of the determination of questions (a) and (b) were negative, no definitive information about site overlap would
likely be gained. However, if the determination of questions (a) and (b) yielded positive results, it could be firmly concluded that the dNTP and aryl moieties bind simultaneously to very closely neighboring, but formally separate, sites.

The first step was to screen existing compounds from the benzylguanine family to select one or more that were more potent or equally potent with the corresponding uracil derivative.

B. Results and Discussion

Screening for potent pol III inhibitors.

Table III-A, pages 23-24, displays the results from the search for a potent *bona fide* guanine inhibitor. In nearly all cases, the phenylhydrazinouracils such as H$_2$-HPUra and the benzylaminouracils such as $p$-OH BAU were more potent than their corresponding guanines. This held true for compounds substituted with both hydrophilic and hydrophobic groups. However, N$_2$-(3,4-dichlorobenzyl)guanine (DCBG) proved to be as potent as DCPUra and DCBAU on both wild type and azp-12 pol IIIIs. While azp-12 maintained resistance to those inhibitors with hydrophilic substituents such as H$_2$-HPUra and equal sensitivity to unsubstituted inhibitors such as H$_2$-PUra, it became more sensitive to inhibitors with hydrophobic substituents such as methyl groups. DCBG fell into the latter category in that it was more potent on the azp-12 enzyme.
<table>
<thead>
<tr>
<th>X Substituent</th>
<th>wt pol III Serine 1175</th>
<th>azp12 pol III Alanine 1175</th>
</tr>
</thead>
<tbody>
<tr>
<td>X= Hydrophilic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-OH (H₂-HPur) (a)</td>
<td>0.4</td>
<td>22</td>
</tr>
<tr>
<td>-OH (p-OHBAU) (b)</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>-OH (p-OHBG) (b)</td>
<td>80</td>
<td>700</td>
</tr>
<tr>
<td>-NH₂ (p-NH₂Ura) (a)</td>
<td>7</td>
<td>76</td>
</tr>
<tr>
<td>-NH₂ (p-NH₂BAU) (c)</td>
<td>17</td>
<td>156</td>
</tr>
<tr>
<td>X= H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-H (H₂-PUra) (a)</td>
<td>1.4</td>
<td>2.2</td>
</tr>
<tr>
<td>-H (BAU) (c)</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>-H (BG) (d)</td>
<td>300</td>
<td>500</td>
</tr>
<tr>
<td>X= Hydrophobic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CH₃ (H₂-TolUra) (a)</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>-CH₃ (p-MeBAU) (e)</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>-CH₃ (p-MeBG) (e)</td>
<td>20</td>
<td>6.0</td>
</tr>
<tr>
<td>X= 3,4-diCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-diCl (DCPUra) (a)</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>-diCl (DCBAU) (e)</td>
<td>0.25</td>
<td>0.075</td>
</tr>
<tr>
<td>-diCl (DCBG) (e)</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table III-A: Structure activity relationships of uracil and guanine analogs of H$_2$-HPUra. $K_i$s were determined by the truncated assay described in Chapter VI. The structural backbones of each inhibitor type are drawn above. (a) Synthesis was described by Wright and Brown, 1977. (b) Synthesized by Dr. L. Dudycz. (c) Synthesis was described by Brown et al., 1977. (d) Synthesis was described by Hildebrand et al., 1990. (e) Synthesis was described in Chapter VI of this thesis.
Properties of N²-(3,4-dichlorobenzyl)guanine, (DCBG), the model benzylguanine exploited for incorporation into a dGTP format.

(i) Action of DCBG on the isolated polymerase is characteristic of the H₂-HPUra prototype. My strategy for development of the nucleotide form of the inhibitor required that the simple N²-substituted guanine base on which it was to be constructed: (a) be potent (i.e., display a Kᵢ in the 0.5-2 μM range), (b) be specifically competitive with dGTP like the corresponding 6-(benzylamino) and 6-(arylhydrazino)uracils, and (c) be dependent on the presence of a cytosine-containing template. DCBG, one of many candidates available from Dr. George Wright, met all three requirements. First, DCBG was a characteristically potent inhibitor of wild-type pol III, displaying a Kᵢ of approximately 0.5 μM (see Table III-C, page 30). Second, its inhibition was specifically and competitively reversed by dGTP (Figure III-A, page 26); high concentrations of dATP, dCTP, or dTTP had no effect on its inhibitory capacity. Third, when the action of DCBG on pol III was assessed with either poly(dA):oligo(dT) or poly(dC):oligo(dG) as template:primer, it, like H₂-HPUra (Gass et al., 1973; Clements et al., 1975), inhibited only the latter reaction (Table III-B, page 27).

(ii) Action on intact cells: specificity for replicative DNA synthesis. DCBG, like HPUra, which is reductively activated in vivo, penetrated intact B. subtilis and inhibited its capacity to form colonies on media plates. Microscopic examination of B. subtilis grown in liquid medium for a period of time equal to four generations of growth, in the presence of 100 μM DCBG showed that the drug arrested cell division and effected a segmented morphology typical of HPUra-treated cells which are specifically unable to synthesize DNA
Figure III-A: Competitive inhibition of pol III by DCBG. Polymerase assays were performed as described in Chapter VI with varying concentrations of dGTP and the indicated concentrations of inhibitor, DCBG.
<table>
<thead>
<tr>
<th>System</th>
<th>dNMP incorporation (pmoles) (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>[α-³²P]dGTP → poly(dC):oligo(dG)(b)</td>
<td>12.5</td>
</tr>
<tr>
<td>[³H] dTTP → poly(dA):oligo(dT)(b)</td>
<td>12.6</td>
</tr>
</tbody>
</table>

Table III-B: Template dependence of inhibitor action. (a) based on an assay mix containing a specific activity of 825 cpm/pmole. (b) Polymer:oligomer assay conditions are described in Chapter VI, Section F.
(Brown, 1970). Figure III-B, page 29, summarizes the effect of DCBG on macromolecule synthesis in log-phase *B. subtilis*, based on analysis of incorporation of labeled adenine into RNA and DNA, and labeled leucine into protein. The results clearly indicated that DCBG, like HPUra (Brown & Handschumacher, 1966; Brown, 1970), inhibited only DNA synthesis and displayed no significant effect on the synthesis of RNA and protein.

**(iii) Utility of DCBG as an agent for selecting and identifying bacterial clones carrying mutations affecting the drug binding site of pol III.** Much of the value of the H2-HPUra inhibitor prototype as a genetic and structural probe of the replication-specific DNA pol III is derived from its "magic bullet" capacity to select, on drug-containing nutrient plates, bacterial clones which carry mutationally altered, drug-resistant forms of pol III. *B. subtilis* azp-12, a mutant displaying both an HPUra-resistant growth phenotype and an H2-HPUra-resistant pol III (Clements et al., 1975) exemplifies this strong correspondence of *in vitro* enzyme "phenotype" and the growth-response phenotype of its host. As shown in Table III-C, page 30, comparison of the action of DCBG and H2-HPUra on the wild-type and azp-12-specific systems indicated that DCBG also could consistently discriminate between wild-type and mutant forms of pol III, both *in vivo* and *in vitro*. The top row of data, displaying $K_i$ for *in vitro* enzyme inhibition, indicated that the azp-12 enzyme, relative to wild-type enzyme, showed approximately 55-fold resistance to H2-HPUra and, interestingly, 5-fold higher sensitivity to DCBG. The second row of data displayed the same trend *in vivo*; the growth of the azp-12 mutant host, relative to that of the wild-type host, was approximately 8 times more resistant to HPUra and 8 times more sensitive to DCBG.
Figure III-B: RNA, protein and DNA synthesis in the presence of DCBG or HPURa. Cultures of *B. subtilis* NB841 were grown and incorporation of [³H]-adenine into RNA and DNA and [³H]-leucine into protein were followed as described in Chapter VI. Inhibitors or control diluent were added at time zero.
Isolated pol III

\[ K_i (\mu M)^{(a)} \]

\[
\begin{array}{c|cc|cc}
 & \text{wild-type system} & & \text{azp-12 system} \\
 & H_2\text{-HPUra} & DCBG & H_2\text{-HPUra} & DCBG \\
\hline
\text{Isolated pol III} & & & & \\
[K_i (\mu M)^{(a)}] & 0.4 \pm 0.08 & 0.5 \pm 0.1 & 22 \pm 4 & 0.1 \pm 0.02 \\
\text{Bacterial division} & & & & \\
\text{CFU}_{50} (\mu M)^{(b,c)} & 2.5 & 40 & 20 & 5 \\
\end{array}
\]

Table III-C: In vitro vs in vivo effects of DCBG and H$_2$-HPUra. (a) $K_i$ was determined as described in Chapter VI. (b) CFU$_{50}$ is the concentration of inhibitor in minimal salts agar plates required to reduce the number of colony-forming units of $B. subtilis$ (strain BD54; Vrooman et al., 1978) to 50% of control values. (c) HPUra, in its oxidized native form, was used in the determination of bacterial growth.
Synthesis of N²-(3,4-dichlorobenzyl)-2'-deoxyguanosine 5'-triphosphate (DCBdGTP).

Given the favorable assessment of DCBG as an H₂-HPUra-like inhibitor, I synthesized and characterized its dNTP form, DCBdGTP. The synthetic pathway used to obtain DCBdGTP is illustrated in Figure III-C, page 32, and described in detail in Chapter VI, Section C. Step A was performed as described by Wright and Dudycz (1984). Step B involved chlorination of the benzylguanine with POCl₃ to produce the 6-chloro base. Glycosylation (Step C) involved reaction of the 6-chloro purine with a protected 2'-deoxyribofuranosyl chloride in the presence of sodium hydride (Wright et al., 1987). The resulting 9-β 6-chloro nucleoside was hydrolyzed to the deoxyguanosine derivative by reaction with 2-mercaptoethanol and sodium methoxide (Step D). Phosphorylation of the nucleoside with POCl₃ (Step E) gave the nucleoside 5'-monophosphate (Yoshikawa et al., 1967). In Step F, the monophosphate was pyrophosphorylated to generate the triphosphate, DCBdGTP (Ott et al., 1967; Blackburn et al., 1981). The ¹H and ³¹P NMR spectra served to identify each of the above compounds (see Chapter VI, Section C).

Properties of DCBdGTP action on its pol III target. The properties we sought in the dNTP form were: (a) a potency at least equivalent to that of the simple base form, DCBG; and (b) a mechanism equivalent to that of the base. The results of experiments described in subsections (i)-(v) below summarize the assessment of these properties.
Figure III-C: Summary of the synthetic pathway from 2-bromohypoxanthine to the final product, DCBdGTP. Details are presented in Section C of Chapter VI.
(i) Potency and discrimination of wild-type and mutant pol IIIIs. As Table III-D, page 34, indicates, the dNTP inhibitor form, DCBdGTP, was 4-5 fold more potent than DCBG for both wild-type and azp-12-specific enzymes. The presence of the dNTP moiety did not change the ability of the DCBG nucleus to discriminate the wild-type and azp-12 enzymes in vitro. The ratio of the \( K_i \) for azp-12 to the \( K_i \) for wild-type was the same for both DCBG and DCBdGTP (ie. 4-5 fold, Table III-D, page 34).

(ii) Specific reversal by dGTP. Like the parent compound, DCBG, the triphosphate form, DCBdGTP was specifically reversed by dGTP. High concentrations of dATP, dCTP, and dTTP under identical assay conditions had no effect on its potency (Table III-E, page 35).

(iii) The template cytosine requirement for DCBdGTP-induced inhibition is preferred but not absolute. Competition experiments, identical to those performed with DCBG, indicated that the inhibitory action of DCBdGTP, in the presence of activated natural (calf thymus) DNA, was competitively and specifically antagonized by dGTP as shown in Figure III-D, page 36. Given this observation, I exploited the homopolymeric template:primers, poly(dA):oligo(dT) and poly(dC):oligo(dG) to determine whether the action of the dNTP form, like that of the parent base, DCBG, absolutely required template cytosine to effect inhibition. The results of the homopolymer experiments are summarized in Table III-F, page 37. DCBG, like H2-HPUra (Gass et al., 1973; Clements et al., 1975), displayed no significant inhibition in the absence of template cytosine. In contrast, DCBdGTP, although considerably more potent in the poly(dC)-driven reaction (IC\(_{50} = 7 \mu M\)), displayed significant activity (IC\(_{50} = 200 \mu M\)) in the poly(dA)-directed reaction. The
Table III-D: Potencies of the base and nucleotide forms of DCBG. $K_i$s were determined as described in Chapter VI. The standard deviation for all $K_i$ values was no greater than +/- 20%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (μM)</th>
<th>wt pol III</th>
<th>azp12 pol III</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCBG</td>
<td>0.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>DCBdGTP</td>
<td>0.1</td>
<td>0.025</td>
<td></td>
</tr>
</tbody>
</table>
Table III-E: Effects of high concentrations of dNTPs on inhibition of pol III by DCBdGTP. (a) Incorporation of the appropriately labeled dNTP was monitored as described in Chapter VI, Section F, using activated calf thymus (C.T.) DNA. The labeled dNTP was [α-32P]dATP for the experiments assessing the effect of high dCTP, dTTP and dGTP concentrations and was [3H]dTTP for the experiment assessing the effect of high dATP concentration. The specific activity of each labeled dNTP was 625 cpm/µmole. (b) Control conditions consisted of the standard truncated (-dGTP) assay.
Figure III-D: Competitive inhibition of pol III by DCBdGTP. Polymerase assays were performed as described in Chapter VI, Section F.
<table>
<thead>
<tr>
<th>DNA System</th>
<th>dNMP incorporation (pmoles)(^{(a)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>[α-(^{32})P] dGTP → poly(dC):oligo(dG)</td>
<td>12.5</td>
</tr>
<tr>
<td>[(^{3})H] dTTP → poly(dA):oligo(dT)</td>
<td>13.7</td>
</tr>
</tbody>
</table>

Table III-F: Template dependence of inhibitor action. Polymer:oligomer assays were performed as described in Chapter VI in the presence of 20 μM [α-\(^{32}\)P] dGTP or [\(^{3}\)H] dTTP at a specific activity of 825 cpm/pmole.
basis for the template "independent" activity of DCBdGTP, although not clearly established, is likely related to its explicit and complete dNTP structure, which enhances its capacity to recognize and directly bind the enzyme's dNTP binding site. I tested this possibility by examining the effect of increasing the concentration of dTTP in the poly(dA):oligo(dT)-driven reaction on the sensitivity of the reaction to DCBdGTP. If DCBdGTP were competing with dTTP for the dNTP binding/polymerization site, its inhibitory capacity would be expected to be inversely related to dTTP concentration. The results of single point experiments summarized in Table III-G, page 39, indicated such an inverse relationship; only dTTP, the substrate for this reaction, reversed DCBdGTP action in this system.

(iv) DCBdGTP-induced inhibition in the presence of template cytosine apparently involves a sequestration mechanism. To assess sequestration, I exploited the "scavenging" method employed to ascertain the induction of DNA:enzyme complex formation by H2-HPUra (Gass et al., 1973; Clements et al., 1975) and TMAU (unpublished data). The specific experimental approach exploited poly(dA):oligo(dT)-driven incorporation of dTMP and simply asked whether enzyme, in the presence of inhibitor at a concentration causing minimal inhibition, can be sequestered by the addition of a small amount of a dC-containing template:primer, an amount too small per se to interfere with dTTP incorporation into the oligo(dT). The results of relevant experiments comparing DCBG, DCBdGTP and a TMAU control are summarized in Table III-H, page 40. All three inhibitors were used at 100 µM; at this concentration DCBG, DCBdGTP and TMAU, as expected from the results shown in Table III-F, page 37, inhibited dTMP incorporation by, respectively, 2, 40 and 5% (see top row Table III-H, page 40). The addition of cytosine-containing activated calf
<table>
<thead>
<tr>
<th>Conditions</th>
<th>dNMP incorporation (a) (pmoles)</th>
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</thead>
<tbody>
<tr>
<td>Control -DCBdGTP</td>
<td>11.9</td>
</tr>
<tr>
<td>Control + 100 µM DCBdGTP</td>
<td>6.0</td>
</tr>
<tr>
<td>1 mM dATP + 100 µM DCBdGTP</td>
<td>6.0</td>
</tr>
<tr>
<td>1 mM dCTP + 100 µM DCBdGTP</td>
<td>5.5</td>
</tr>
<tr>
<td>1 mM dTTP(b) + 100 µM DCBdGTP</td>
<td>11.7</td>
</tr>
<tr>
<td>1 mM dGTP + 100 µM DCBdGTP</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Table III-G: Effects of dNTPs on DCBdGTP induced inhibition of pol III (a)
Incorporation of [3H]dTTP was monitored as described in Chapter VI, Section F using poly(dA):oligo(dT) as template:primer and [3H]dTTP at a concentration of 20 µM (825 cpm/pmole). (b) Under conditions of increased dTTP, the specific activity was lowered to 100 cpm/pmole.
<table>
<thead>
<tr>
<th>Template:Primer</th>
<th>[³H]dTMP Incorporation (pmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>poly(dA):oligo(dT)</td>
<td>5.7</td>
</tr>
<tr>
<td>No homopolymer (Activated calf thymus DNA alone)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>poly(dA):oligo(dT) + Activated calf thymus DNA</td>
<td>4.6</td>
</tr>
<tr>
<td>poly(dA):oligo(dT) + Activated calf thymus DNA + dGTP (0.5 mM)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table III-H: Demonstration of sequestration. Poly(dA):oligo(dT) experiments were performed with wild-type enzyme as described in Chapter VI. Activated calf thymus DNA was used at a concentration of 9μg/ml (1:45 dilution of concentrations used in normal assays). (a) (%) = % inhibition; i.e. % of control.
thymus DNA at low concentration neither supported significant dTMP incorporation (see second row of data) nor drastically reduced incorporation of dTMP into poly(dA):oligo(dT) (see third row of data) in the absence of inhibitor; however, in its presence the effect was dramatic. With DCBG or the control inhibitor, TMAU, present, dTMP incorporation was inhibited by approximately 90%, and in the presence of DCBdGTP, inhibition of dTMP incorporation was increased from 40% to 87%. To determine if this drug-induced, DNA-dependent inhibition was, as expected, sensitive to the presence of a high concentration of dGTP (0.5 mM), we examined its effect and, as a control, that of dATP and dCTP (not shown). dGTP, when unaccompanied by inhibitor, exhibited a competitive action on incorporation of dTMP into poly(dA):oligo(dT) and decreased control values by 40%. However, only dGTP was effective as a competitor; it reduced inhibition induced by DCBG from 89 to 24%, that induced by DCBdGTP from 87 to 32% and that induced by TMAU from 85 to 21% (see bottom row Table III-H, page 40). In sum, these results strongly suggested that both DCBG and DCBdGTP, when provided with template cytosine in an appropriate structural context, act by a sequestration-specific mechanism typically displayed by the pyrimidine-based inhibitors such as TMAU.

**(v) DCBdGTP can serve as a substrate for its target enzyme.** To investigate the potential of DCBdGTP for polymerization, with the assistance of Dr. Naseema Khan, we exploited sequencing gel analysis of primer extension, using the template:primer depicted in the inset of Figure III-E, pages 42-43. Experimentally, I simply sought to determine if DCBdGTP, like dGTP, could be utilized to extend the primer by one nucleotidyl residue. The results are summarized in the gel autoradiogram in the lower part of Figure III-E, pages 42-43.
Figure III-E: Sequencing gel analysis of the substrate potential of DCBdGTP. Inset: structure of the template:primer. Lower panel: autoradiogram of the sequencing gel electropherogram of the products resulting from pol III action on the template:primer. Details of the methods are described in Chapter VI. Lanes: A, template:primer (T/P) control; B, T/P + pol III; C, D, E, T/P + pol III + DCBdGTP at 0.01, 1 and 10 μM, respectively; F, T/P + pol III + 25 μM dGTP; G, T/P + pol III + all 4 dNTPs at 25 μM each.
There was, as expected, no primer extension in the absence of dNTP (lane B); only partial degradation resulting from the activity of the enzyme's resident 3'-5' exonuclease (Low et al., 1976). The 17mer was extended to its full, 29mer length when all 4 dNTPs were present (lane G), and, as expected, was extended by one residue when dGTP was present as the sole dNTP substrate (lane F). The presence of DCBdGTP as the sole dNTP, at concentrations ranging from 0.1 to 100 times its $K_i$ (lanes C, D and E) resulted in the formation of a product which migrated at the position expected for that of the primer extended by one DCBdGMP residue. The $R_f$ of primer extended by one DCBdGMP residue is, under the conditions of electrophoresis I used, slightly, but consistently, lower ($\sim 0.96$) than that of the primer extended by one dGMP residue. The same is true when we polymerize multiple molecules of DCBdGTP with terminal deoxynucleotidyl transferase to obtain a ladder of bands on the electropherogram (data not shown). Each band runs slightly slower than that for the corresponding dGMP-terminated isomer, indicating that molecules containing DCBdGMP molecules are slightly larger and/or bulkier.

C. Conclusions

DCBdGTP behaved similarly to H$_2$-HPUra in the in vitro experiments, described in the previous section of this chapter, where it displayed a high degree of inhibitory potency on both wild type and azp-12 target pol IIIIs. DCBdGTP-induced inhibition of activated DNA-mediated synthesis was specifically reversed by dGTP, indicating that it, like H$_2$-HPUra,
has a requirement for template cytosine. Unlike H2-HPUra, however, DCBdGTP displayed nonspecific inhibition of poly(dA):oligo(dT)-mediated synthesis, indicating a strong affinity for the dNTP binding region of pol III. DCBdGTP appeared to act via a sequestration mechanism where it caused the formation of a reversible ternary complex with pol III and the DNA template:primer. The above information suggested that the model shown in Figure I-E, page 13, for H2-HPUra-induced inhibition applies equally well for DCBdGTP. Figure III-F, page 46, illustrates the earlier sequestration model adapted with the addition of a deoxyribosyl triphosphate group to the simpler base inhibitor.

The evidence for polymerization of DCBdGTP by pol III suggests that DCBdGTP formally occupies the dNTP binding site of pol III with a "fit" approximating that of the dNTP it mimics, dGTP. This fit, when considered in the context of the characteristic, H2-HPUra-like mechanism of DCBdGTP, which includes the ability to promote ternary complex formation, clearly permits the conclusion that the aryl and dNTP binding sites are formally distinct and not overlapping. The alternative, that, at the time of polymerization, the aryl group is displaced from its binding site to make room for the incoming deoxyribosyl triphosphate group, is less likely to occur. The reasoning behind this statement originates from the observed increase in $K_i$ of the dNTP inhibitor form over the $K_i$ for the base form, DCBG. This 5-fold increase (see Table III-D, page 34) is likely due to the addition of interactions of DCBdGTP with the amino acid side chains involved in binding the deoxyribosyl ring and its 5' triphosphates to the interactions with the guanine base and the N2-aryl substituents. If the inhibitor aryl domain were displaced during binding and polymerization, I would expect the $K_i$ for DCBdGTP to be in the range of the $K_m$ for dGTP, at approximately 1-5 μM. Instead, the observed $K_i$ for DCBdGTP is 0.1 μM. The additional observation that DCBdGTP had a five-fold greater potency on the
Figure III-F: Proposed model of the DCBdGTP mechanism of action. Inhibitor sequesters DNA and enzyme into a reversible ternary complex. Upon formation of the complex, the α-phosphate is positioned for nucleophilic attack by the 3'-OH group of the primer terminus. * indicates the base paired NH substituent which is a calculated distance of 6-7 Å from the aryl binding domain of pol III.
mutant azp-12 pol III, provides additional support to the hypothesis that the aryl domain binds simultaneously with the binding of the deoxyribosyl triphosphate moiety of DCBdGTP.

The fit described above and demonstrated in Figure III-F, page 46, suggests several other important conclusions regarding the structure of the enzyme:inhibitor:DNA complex and specific features of the enzyme's dNTP binding domain on which complex formation depends. First, because DCBdGTP behaves similarly to H2-HPUra, which has been shown, through NMR experiments, to base pair with cytosine in a manner similar to guanine, i.e. via Watson-Crick pairing, one may infer that the geometry of the base pair formed between the template cytosine and the N2-benzylguanine substituent closely approximates that of the G:C pair formed in the process of dGTP polymerization. Second, one may conclude that the N2-benzyl substituent does not formally occupy sites or space critical to the catalysis of dNTP polymerization; these include: (a) the amino acid side chain substituents involved in the binding of the deoxyribosyl ring and its 5'-phosphates; (b) the space about the 3'-OH of the primer terminus, and (c) the path between this OH group and the α phosphate of the incoming dNTP. Finally, as a corollary to the above conclusions, one also may envision more precisely the location of the major site of inhibitor:enzyme interaction, the so-called aryl binding site (Brown et al., 1986). Specifically, the model suggests that this site lies on the "edge" of the dNTP binding domain in space defined by a radius of 6-7 Ångstoms from the unpaired proton on the exocyclic 2-amino group of a base-paired dGTP molecule, or the base paired 2-NH of DCBdGTP (the * in Figure III-F indicates the position), as they await polymerization.
CHAPTER IV

SEARCH FOR A POL III INHIBITOR FORM POTENTIALLY USEFUL
AS A COVALENT MARKER OF THE ARYL BINDING SITE

A. Approach

Dr. George Wright, in collaboration with Dr. Brown, has synthesized a considerable number of inhibitors of Gram+ pol III substituted in the aryl ring with groups capable of forming a covalent bond with appropriately reactive amino acid side chains. Whereas the potential of these forms as covalent markers had not been systematically analyzed, I proposed such an analysis, and to facilitate the experiments, I proposed the design of an enzyme specifically mutated at serine 1175 (S1175).

The rationale for targeting amino acid 1175. The choice of S1175 was based on its specific effect on the drug sensitivity profile of pol III azp12, a mutant enzyme derived from a B. subtilis mutant selected for its ability to grow on medium containing HPUra (Clements et al., 1975). The point mutation of polC azp-12 results in a change of serine residue 1175 in the wild-type enzyme to alanine (S1175A) (Barnes et al., 1989; Sanjanwala & Ganesan, 1989). Table IV-A, page 49, compares the sensitivity of the serine and alanine enzymes toward H2-HPUra and 6-(phenylhydrazino)uracil (H2-PUra),
Table IV-A: Effects of H₂-HPUra and H₂-PUra on inhibition of wild-type vs azp-12 pol III's. Kᵢs were determined as described in Chapter VI.
6-(arylhydrazino)uracils differing only with respect to the presence/absence of the hydroxy substituent in the para position of the aryl ring.

As the results in Table IV-A, page 49, indicate, the mutation has little effect on the enzyme's ability to bind the unsubstituted ring of H2-PUra, while it reduces nearly fifty-fold the affinity of the p-hydroxylated H2-HPUra against which it was selected. Although there are many possible explanations for the differential reactivity to the hydroxyl form of the inhibitor, I proposed the following hypothesis as the basis. I suggested that the -CH2OH side chain of serine 1175 lies on the periphery of the aryl binding site, positioned such that it does not significantly influence the binding of the unsubstituted aryl ring but close enough to react with polar substituents (i.e. OH) which extend from the para and/or the meta positions of the ring (I included meta-substituted 6-anilinouracil analogs in this hypothesis because many such compounds are effective pol III inhibitors (Wright & Gambino, 1984). In the wild-type enzyme, the polar -CH2OH serine side chain is envisioned to form a hydrogen bond with the p-OH group, facilitating the binding of the aryl moiety. Conversely, in the mutant enzyme, the hydrophobic -CH3 side chain of the alanine is envisioned to repel the polar p-OH group of H2-HPUra, thus reducing the strength of binding of the aryl moiety.

If the above hypothesis regarding the positioning of residue 1175 were correct, an 1175 side chain would, in theory, be available for direct covalent attack by a suitably reactive group placed in the para and/or meta position on the inhibitor's aryl ring.

The collection of potentially reactive phenyl-substituted inhibitor forms which I had available to test my hypothesis fell into two classes: those with halomethyl substituents (i.e. -CH2Cl and -CH2Br) and those with a mercaptomethyl group (-CH2SH) (Table IV-B, pages 51-52). Because both classes of inhibitors were likely to be more reactive for the
<table>
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<th>Reactivity</th>
<th>Target Residues</th>
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<tr>
<td>1</td>
<td><img src="image1" alt="Structure 1" /></td>
<td>Direct</td>
<td>-CH$_2$SH (cys)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure 2" /></td>
<td>Direct</td>
<td>-CH$_2$SH (cys)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Structure 3" /></td>
<td>Requires photoactivation to nitrene</td>
<td>Cys, Ser, Tyr, Thr, Phe, Lys</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Structure 4" /></td>
<td>Direct</td>
<td>Forms a reducible Schiff base with lysine NH$_2$</td>
</tr>
</tbody>
</table>
Table IV-B: Potential covalent inhibitors of pol III-cys. Inhibitors from categories 1 and 2 have been synthesized. The last two forms of inhibitor can readily be synthesized. Synthesis of inhibitor 3 would involve treatment of $N^2$-(p-acetamidobenzyl)guanine (synthesized by reacting 2-bromohypoxanthine with the appropriate benzylamine) under basic conditions to yield the p-amino compound and subsequent treatment with sodium azide and nitrous acid to yield the azido compound. Synthesis of inhibitor 4 would involve the oxidation of $N^2$-(p-hydroxybenzyl)guanine (previously synthesized by reacting 2-bromohypoxanthine with the appropriate benzylamine) to yield the formyl compound.
cysteine side chain than for the serine side chain, I sought to construct the corresponding mutant enzyme S1175C. As an alternate, I sought to construct an additional mutant, S1175K, in order to have an enzyme form capable of reacting with a formyl-substituted inhibitor (Table IV-B, pages 51-52, inhibitor 4). Inhibitor 3, the p-azido-substituted compound, would be useful in labeling both cysteine and lysine mutant forms as well as the wild type enzyme form.

B. Results

Design of pol III-cys and pol III-lys.

(i) Site-directed mutagenesis. The mutagenesis system, obtained from Promega, exploited oligonucleotide-directed mutagenesis on a phagemid vector, p-SELECT, containing a single-stranded F1 origin of replication. This system, shown in Figure IV-A, page 54, relies on the use of a second mutagenic oligonucleotide to confer antibiotic resistance on the mutated DNA strand and thus provides a means to select and increase the yield of mutant clones. p-SELECT contains a functional tetracycline resistance gene for selection purposes and an inactivated ampicillin resistance gene for reactivation during mutagenesis (shown in Figure IV-B, page 55). A small Pol C fragment, EcoRV (nucleotide 2941) to BclI (nucleotide 3965) was inserted into the BamHI site of the multiple cloning site of p-SELECT. Single-stranded DNA was prepared from the resulting vector and isolated for the subsequent mutagenesis reactions. Site directed mutagenesis was performed, as shown in Figure IV-A, page 54, by annealing both mutagenic and ampicillin
Figure VI-A: Mutagenesis system obtained from Promega. Reproduced from the Promega manual.
Figure VI-B: p-SELECT plasmid map. Reproduced from the Promega manual.
repair oligonucleotides to the single-stranded DNA, synthesizing the mutant strand with T4 DNA polymerase, and sealing the mutant strand with T4 ligase. The design of the pol III-cys/pol III-lys mutagenic oligonucleotides is illustrated in Figure IV-C, page 57. The resulting DNA was transformed into a mismatch repair-deficient *E. coli* strain and selected on the basis of ampicillin resistance. The isolated mutant DNAs were identified on the basis of restriction site polymorphisms engineered into the mutagenic oligonucleotides (Figure IV-C, page 57). An existing SacI site was changed to a unique MaeI site in each of the two mutations. Sequencing of a 200 base pair region of the mutagenized *polC* gene served to screen for any other mutations. The sequenced region was then subcloned, through several steps (described in Chapter VI), into an *E. coli*-based overexpression plasmid.

(ii) Overexpression and purification. The cysteine-1175 and lysine-1175 forms of *B. subtilis* *polC* were generated by overexpression from the respective mutant expression plasmids, which contained the λ phage leftward promoter, pL, in an *E. coli* strain which contains the λ repressor, cI. Repression of the pL promoter by cI in this system is controlled simply by adding nalidixic acid (Nal) to an exponentially growing culture. Induction of this system inhibits replicative DNA synthesis, which in turn signals an SOS response in the host (Little & Mount, 1982). This response induces the activity of a protease which cleaves the cL repressor, relieving pL repression and thus, permits transcription of any genes under pL control (Shatzman & Rosenberg, 1987). Assay of a crude extract of the lysine 1175 form of pol III unfortunately displayed a very low level polymerase activity (< 1% of wild-type) and therefore, this enzyme was of no further use. However, the cys form, pol III-cys-1175, displayed excellent activity and therefore, was
Converted serine 1175 to cysteine: 2 bases changed; 3569 and 3570.
Deleted SacI site @ 3553-3558 by changing 3558.
Added MaeI site @ 3556-3559 by changing 355

\[
5'-\text{TCTGAGCTAGTTCAAGATTGTTGGCTTGTCCTACG}-3' \\
\text{(C)} \quad \text{MaeI} \quad \text{(CA)}
\]

3550 3560 3570 3580

Figure VI-C: Mutagenic oligonucleotide utilized to generate the change of serine 1175 to cysteine. Bases in parentheses are wild-type sequences that have been replaced by the bases in bold type. The oligonucleotide used to generate a serine to lysine change was identical to the one shown above except that the sequence at bases 3568-3570 was AAA (to code for lysine).
subsequently purified through several steps, including Cibacron Blue, Phenyl sepharose and Mono-Q columns (see Chapter VI), to yield a peptide of the expected 162.4 kDa size at a level of purity of approximately 98% (Figure IV-D, pages 59-60).

**Characteristics of pol III-cys.** I characterized the mutant protein in two contexts: *in vitro* and *in vivo*, in the natural *B. subtilis* host. The *in vivo* characterization included transformation of the mutant form of *polC* into wild-type *B. subtilis* and assessment of the impact of its pol III-cys-1175 product on the growth and inhibitor (HPUra type) response phenotype of the transformed host. The *in vitro* characterization included assessment of the basic catalytic properties of the purified protein. The results of the *in vivo* and *in vitro* assessments are summarized below and tabulated in Table IV-C, pages 61-62.

**In vivo assessment.** The *polC-cys* was transformed into competent wild-type *B. subtilis* by exploiting the HPUrA resistance it conferred upon the recipient host. In addition to the HPUrA-resistant phenotype (Table IV-C, pages 61-62) the mutant protein conferred a marginal, although significant, temperature sensitivity to its host; 51°C was required to demonstrate this phenotype. Growth at 45°C did not effect differentiation between wild-type and pol III-cys.

**In vitro assessment.** Relative to wild-type pol III, the mutant enzyme displayed a 60% reduction of the specific activity of the pol activity and only a 37% reduction in the exo activity. This resulted in a lower pol:exo ratio for pol III-cys. In effect, the pol activity was more significantly impaired than the exo activity. There were no significant differences with respect to the mutant enzyme’s $K_m$ for dNTPs and native and
Figure IV-D: SDS polyacrylamide gel electropherogram of Mono-Q FPLC-purified pol III-cys stained with Coomassie Blue. *Lanes*: A: molecular weight markers; B: Mono-Q column load volume; C-F: Mono-Q fractions 41-44. Only fractions 42 and 43 (Lanes D and E) were considered of sufficient purity for enzyme assays.
Table IV-C

<table>
<thead>
<tr>
<th>System</th>
<th>Characteristics</th>
<th>pol III-cys-1175</th>
<th>pol III wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>Growth at 45°C in <em>B. subtilis</em> (a)</td>
<td>Strong growth</td>
<td>Strong growth</td>
</tr>
<tr>
<td></td>
<td>Growth at 51°C in <em>B. subtilis</em> (a)</td>
<td>No growth</td>
<td>Strong growth</td>
</tr>
<tr>
<td></td>
<td>Growth on HPUra in <em>B. subtilis</em> (a)</td>
<td>Strong growth</td>
<td>No growth</td>
</tr>
<tr>
<td>In vitro</td>
<td>Pol Specific Activity (b) (units/mg)</td>
<td>2300</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>Exo Specific Activity (c) (units/mg)</td>
<td>33.4</td>
<td>53.4</td>
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<tr>
<td></td>
<td>Polymerase:Exonuclease ratio (d)</td>
<td>68.8</td>
<td>112.4</td>
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<td>Substrate $K_m$:</td>
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<tr>
<td></td>
<td>dATP (μM)</td>
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<td>dCTP (μM)</td>
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<tr>
<td></td>
<td>dGTP (μM)</td>
<td>2.8</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Activated C.T. DNA (μg/mL)</td>
<td>77</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Poly(dA):oligo(dT) (units/mL) (e)</td>
<td>0.2</td>
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</tr>
<tr>
<td></td>
<td>Poly(dC):oligo(dG) (units/mL) (e)</td>
<td>0.15</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Table IV-C: Characteristics of pol III-cys\textsuperscript{1175} vs pol III wild-type.

(a) \textit{B. subtilis} strain BD54 (Cozzarelli et al., 1975) (HPUra-sensitive) was transformed by wild-type (pWT73) or cys mutant (pWT7C3) DNA and selected at 30\degree C on C\textsuperscript{+}50 media containing 40 \mu M HPUra. HPUra-resistant colonies were picked onto C\textsuperscript{+}50 plates at 51\degree C, 45\degree C or 30\degree C or picked onto C\textsuperscript{+}50 plates containing 40 \mu M HPUra and grown at 30\degree C. All colonies picked onto C\textsuperscript{+}50 plates grew at 30\degree C.

(b) One unit of polymerase activity is the amount of enzyme that incorporates into activated calf thymus DNA 1 nmole of labeled dNTP into a cold TCA-insoluble form in 10 minutes, under the conditions described in Chapter VI. Protein quantities were assessed as described (Bradford, 1972).

(c) One unit of exonuclease activity is the amount of enzyme that releases 1.4 \mu g (3.5 \times 10^4 \text{cpm}) of \textsuperscript{3}H-labeled dTMP in 10 minutes, under the conditions described in Chapter VI.

(d) The pol:exo ratio is based on the ratio of polymerase activity (units/mg) by the method defined above in (b) to exonuclease activity (units/mg) defined above in (c).

(e) Units are OD\textsubscript{260} absorbing units defined in Chapter VI, section G.
homopolymeric template:primers (Table IV-C, pages 61-62). The most remarkable difference from wild-type enzyme involved inhibitor sensitivity. The pol III-cys-1175 displayed >200-fold resistance to H2-HPUra and 20-fold resistance to TMAU (Table IV-D, page 64). Interestingly, pol III-cys-1175 was indistinguishable from wild-type with respect to its susceptibility to DCBG. A screening of several other inhibitors, illustrated in Table IV-D, page 64, yielded results that were consistent with those described above. Those inhibitors with polar substituents were much less potent on pol III-cys (30-45-fold) and those with hydrophobic substituents were less potent on pol III-cys (about 10-fold). Again, those inhibitors which resembled DCBG, with chloro group substituents, were equally potent on both enzymes.

The properties outlined in Table IV-C and Table IV-D indicated that S1175C was a tolerable mutation which did not significantly impair the ability of the enzyme to bind template:primer and dNTPs. Although H2-HPUra-resistant, the enzyme could be completely inhibited by high concentrations of inhibitors of the H2-HPUra form. In sum, the latter properties and the relative stability of pol III-cys-1175 made the enzyme a suitable candidate as a target for irreversible inhibitor analysis.

Assessment of the potential irreversible inhibitors. The approach was based on the assumptions that covalent bond formation between an inhibitor substituent and a potentially reactive residue at or near the aryl binding site (i.e. side chain 1175): (1) would irreversibly inactivate the polymerase activity of the molecule of pol III in which bond formation occurred; (2) would show time-dependent inactivation; (3) would be enhanced
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K_i (μM)</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pol III-cys</td>
<td>pol III wild type</td>
</tr>
<tr>
<td>H2-HPUra (a)</td>
<td>90</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>TMAU (b)</td>
<td>8.5</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>DCBG (c)</td>
<td>0.8</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>p-OH benzylaminouracil (d)</td>
<td>270</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>p-OH benzylguanine (d)</td>
<td>840</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>m-Et p-Me anilino uracil (e)</td>
<td>6.7</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>p-Me anilino uracil (b)</td>
<td>160</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>p-Me phenylhydrazino uracil (a)</td>
<td>6.0</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>3,4-diCl anilinouracil (b)</td>
<td>0.9</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>3,4-diCl benzylaminouracil (c)</td>
<td>0.25</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

Table IV-D: Potencies of reversible inhibitors of pol III wild type and pol III-cys. K_i determinations were made as described in Chapter VI.  
(a) Synthesis was described by Wright and Brown, 1977.  
(b) Synthesis was described by Wright and Brown, 1980.  
(c) Synthesis was described in Chapter VI of this thesis.  
(d) Synthesized by Dr. L. Dudycz.  
(e) Synthesis was described by Wright and Gambino, 1984.
by, if not require, the presence of an excess of template:primer to promote ternary complex formation; and (4) inactivation would be specifically preventable by dGTP at high concentrations, but not reversible by dGTP once effected. If the bond, indeed, involved the side chain of residue 1175, one might also expect the -CH$_2$SH of the cysteine mutant enzyme to be considerably more susceptible to attack by electrophiles or sulfhydryl groups than the -CH$_2$OH of the wild-type form or the -CH$_3$ of the azp-12 (S1175A) form.

All potentially irreversible inhibitors were tested, under standard polymerase assay conditions, for their ability to inhibit both wild-type and pol III-cys enzymes. The resulting $K_i$s, shown in Table IV-E, page 66, ranged from 0.18 µM to 125 µM. Each of these inhibitors was considered to be sufficiently potent for use in the irreversibility assays.

**Assays for assessment of irreversible inhibition.** The standard polymerase assay (Section G, Chapter VI) was modified to obtain conditions suitable for covalent bond formation between inhibitor and mutant enzyme. Figure IV-E, pages 67-68, illustrates the experimental conditions. First, enzyme was incubated at 25°C in the presence of activated C.T. DNA, the required buffer and Mg$^{2+}$, and the inhibitor (Tube A). At various time points, samples were removed from the A tubes and added to B tubes which contained all four dNTPs (which specifically included a large excess of dGTP). Upon mixing the contents of tubes A and B, the standard polymerase assay was initiated (see Figure IV-E and Section G, Chapter VI). The DNA template:primer concentration was kept in large excess over enzyme concentration during the initial period of incubation with inhibitor. The rationale for the excess was to saturate the enzyme with DNA and inhibitor and provide maximal ternary complex formation and, thus, maximal opportunity for covalent inactivation by the inhibitor.
Table IV-E: Assessment of inhibitor potencies with wild-type and pol III-cys using the truncated (-dGTP) assay described in Chapter VI, Section G. (a) Synthesis was described in Chapter VI of this thesis. (b) Synthesis was described by Wright et al., 1980. (c) Synthesis was described by Gambino et al., 1992.
At 0, 1, 2 and 4 hours remove a sample from A and add to B

Assess polymerase activity as percent of control (sample from A containing no inhibitor)

Incubate:
Enzyme, DNA,
Buffer, Mg
+- Inhibitor
@ 25°C

Add to:

\[ ^{3}H\text{-dTTP (10 \mu M)} \]
\[ \text{dATP (25 \mu M)} \]
\[ \text{dCTP (25 \mu M)} \]
\[ \text{dGTP (750 \mu M)} \]

Incubate at 30°C for 10 min.
Standard polymerase assay
Figure IV-E: Conditions for assay for irreversible inhibition. Incubation mixtures (300 μL) of mix A contained 30 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)$_2$, 25% glycerol, 0.5 mg/mL activated calf thymus DNA and 1.6 μg/mL pol III-cys (approximately 0.02 units/mL). Incubations were performed at 25°C, and at 0, 1, 2, and 4 hours, 20 μL samples were removed and added to 5 μL of B mix containing 5x dNTP stock [30 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)$_2$, 125 μM dATP and dCTP, 50 μM [³H]-dTTP, (625 cpm/pmole), and 3750 μM dGTP] and incubated at 30°C for 10 minutes. Reactions were quenched and prepared for scintillation counting as described in Chapter VI.
The determination of activity, after incubation with inhibitor, as a percent of control activity, in the presence of a large excess of dGTP was used as an indication of irreversible inactivation of the polymerase. Table IV-F, pages 70-71, summarizes the results of these assays described above and in Figure IV-E, pages 67-68. The first line of the table indicates that control values (minus inhibitor) did not decrease significantly during the four hour incubation. TMAU (second line) was employed as a negative control because the nature of its aryl substituent makes it an unlikely candidate for covalent bond formation. Surprisingly, activity in the presence of TMAU increased during the four hour incubation. This effect was not examined carefully, but was likely due to an ability of the inhibitor to stabilize DNA:enzyme complex formation or to cause renaturation of a partially denatured mutant enzyme. Examination of the results obtained with inhibitors 1-5 indicated a pattern of increasing activity, during the four hour incubation, similar to that seen with TMAU, the negative control. The above results indicate a failure to demonstrate irreversible inactivation of pol III-cys with inhibitors 1-5.

C. Conclusions and Discussion

**Mutagenesis of pol III amino acid 1175.** Given the evidence presented in Section A, pages 49-54, I chose the side chain of residue 1175 as a likely candidate for an "anchor". For covalent tagging, I engineered a change of the moderately reactive serine residue at position 1175 to the more chemically reactive lysine and cysteine residues.

Although the lys form was expressed in high yield as a 162 kDa soluble protein, it was barely active in polymerization. The loss of activity could be due to any number of factors.
Table IV-F

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
</tr>
<tr>
<td>Control (no drug)</td>
<td>100</td>
</tr>
<tr>
<td>+ TMAU (25 μM)</td>
<td>102</td>
</tr>
<tr>
<td>+ 1, m-CH₂SH AU (30 μM)</td>
<td>96</td>
</tr>
<tr>
<td>+ 2, m-CH₂Cl AU (40 μM)</td>
<td>70</td>
</tr>
<tr>
<td>+ 3, m-CH₂Cl, p-Me AU (20 μM)</td>
<td>81</td>
</tr>
<tr>
<td>+ 4, m-CH₂Br AU (30 μM)</td>
<td>67</td>
</tr>
<tr>
<td>+ 5, m-CH₂Cl PG (20 μM)</td>
<td>97</td>
</tr>
</tbody>
</table>
Table IV-F: Results of the assessment of irreversible inhibition. Conditions for the assays are described in Figure IV-E, pages 67-68, and were performed in the presence of a large excess of dGTP (750 μM, 250x its K_m value) to effect reversal of inhibition by competitive, non-irreversible inhibitors. Percent activity was calculated by dividing activity (cpm) obtained in the presence of inhibitor by the control (-inhibitor) values and multiplying by 100.
For example, it is possible that the size or charge of lysine at position 1175 interfered with the catalytic function of the pol active site and its capacity to bind dNTP substrates. Alternately, the presence of lysine at position 1175 may have disturbed the folding of the polymerase as it was being synthesized. Or, the positively charged side chain may have disturbed both protein folding and catalysis by changing the ionic nature of its environment.

The replacement of serine 1175 with cysteine proved to be more successful in that it yielded an acceptably active (40% the activity of wild-type) polymerase. The cysteine, a more conservative substitution, was likely better tolerated, because the -CH$_2$SH side chain is much smaller than the lysine -(CH$_2$)$_4$-NH$_2$ side chain and much closer in size to the -CH$_2$OH of the native serine residue. The -SH of cysteine must not be in a position to form a disulfide bridge with another cysteine thiol; otherwise, it would likely have more profoundly perturbed the catalytic activity of the polymerase. The loss of 60% of the wild type activity upon mutation of serine 1175 to cysteine may have been a result of an overall impairment of the mutant polymerase to 40% of the wild type activity. Another possibility is that 60% of the enzyme molecules were denatured and the remaining 40% maintained the same activity as that of the wild type pol III. Evidence to support this possibility was displayed during assays for irreversible inhibition and will be discussed later. The pol:exo ratio, a measurement used to assess pol and exo domain mutants, of pol III-cys was less than that of the wild-type enzyme. This observation is consistent with other evidence (Hammond et al., 1991) that suggests that the two domains are closely associated in the *B. subtilis* pol III, but not so close that the pol and exo activities were equally affected by the mutation at position 1175. An overall denaturation of the mutant enzyme would, in theory, adversely affect the exo domain as well.
The negative results described in Section B of this chapter did not invalidate our theory that the side chain of residue 1175 is exposed in the aryl binding domain. While pol III-cys-1175 was highly resistant to polar aryl inhibitor substituents such as the hydroxyl of H2-HPUra, it also showed intermediate resistance to nonpolar aryl substituents such as the trimethylene group of TMAU. This latter form of inhibitor resistance is likely attributable to steric effects. Even though cysteine is a conservative substitution for serine, the atomic radius of sulfur is 0.4 Å larger than that of oxygen (He & Quiocho, 1991) and the cysteine side chain has bond lengths and angles significantly different from those of serine (Frey et al., 1973). The serine C\(_\beta\) to O bond length is 1.42 Å vs 1.82 Å for the cysteine C\(_\beta\) to S bond length. The serine O-H bond length is 1.00 Å vs 1.34 Å for the S-H bond length of cysteine. The greater resistance of the mutant enzyme for the inhibitors with polar substituents could also be due to the diminished hydrogen bonding ability of the thiol group of cysteine (Solomons, 1980) compared to that of the hydroxyl group of serine. The chloro-substituted inhibitors, such as DCBG, 6-(3,4-dichlorobenzylamino)uracil, and 6-(3,4-dichloroanilino)uracil, were equally potent on both wild-type pol III and pol III-cys (see Table IV-D, page 64) suggesting that the drug resistances discussed above were not due to some overall structural change occurring away from the aryl binding site but actually involved a change in a residue that directly contacts the aryl moieties of inhibitors. The ability of the chloro-substituted guanine and uracils to equally inhibit wild-type and pol III-cys was likely due either to favorable spacing of these inhibitors in the aryl binding domain or to an optimal chemical interaction between the -CH\(_2\)SH side chain of cysteine and the \textit{para} and/or \textit{meta} substituents of the inhibitors. The above conclusions are only speculation, since a complex quantitative structure activity relationship analysis has not been performed.
The tested inhibitors did not irreversibly inhibit pol III-cys. Each of the five potential irreversible inhibitors that I had available was substituted in the *meta* position with the alkylation or disulfide bond-forming moieties. It is possible that the geometry of these inhibitors was not appropriate; for example, the reactive species of inhibitor and side chain 1175 may either be not close enough or were too close for successful covalent bond formation. Had I had more time, I would have attempted to synthesize the corresponding *para*-substituted inhibitors to test the latter possibility; a change in positioning on the aryl ring may have made a significant difference in reactivity.

Another possible factor for failure of the alkylation inhibitors with -CH2Cl or -CH2Br substituents was pH. The thiolate (S⁻) form of cysteine is far more reactive than the thiol (SH) form (Lundblad & Noyes, 1984). Whereas the pKa of the cysteine functional group is 8.5, I attempted to perform the relevant incubations at pH 8.4 in order to obtain a higher proportion of ionized cysteine. However, pol III-cys was only marginally active at pH 8.4 and not active at higher pH (data not shown). The presence of a thiolate form of cysteine may, indeed, be a prerequisite for successful nucleophilic attack of a halomethyl-substituted inhibitor. An interesting experiment would have been to incubate the enzyme at pH 8.4 and then adjust assay conditions to pH 7.5. An appreciable amount of activity may then have been recovered.

The incubation conditions for irreversible inhibition assays were minus the reducing agent, DTT. I omitted DTT because I thought that it might prevent or reverse the formation of a disulfide bond between cysteine 1175 and inhibitor 1 (mercaptomethyl substituted) if it were present during the incubation stage. It is certainly possible that the inhibitor could dimerize by forming a disulfide bond between two identical inhibitor molecules. This possibility was supported by the fact that inhibition at zero time was 60-70 % and virtually
nonexistent after the preincubation for two hours, suggesting that the inhibitors were somehow being inactivated. I later attempted to improve conditions by varying the concentration of DTT (20 μM, 40 μM and 100 μM) during incubation to perhaps disrupt disulfide bonds between inhibitor molecules and allow the desired disulfide bonds between inhibitor and cysteine. This attempt proved unsuccessful (data not shown), yielding identical results as those obtained without DTT. The failure of the above attempts to optimize the conditions for covalent reaction may effectively eliminate a -CH₂SH side chain in \textit{B. subtilis} pol III as a potentially useful target for covalently reactive inhibitor forms.

An alternate explanation is that, if the alkylating/sulfhydryl substituents did not come close enough to cysteine 1175, they, as well as TMAU, may have been able to renature a partially denatured enzyme without being able to covalently inactivate pol III-cys. This phenomenon would likely increase enzyme activity by approximately 60%, the amount of activity lost by pol III-cys relative to wild type pol III. Results in Table IV-F, pages 70-71, indicate that several inhibitors actually succeeded in increasing enzyme activity to 160% of the control activity.

**Did the failure of the compounds to irreversibly inactivate pol III-cys activity represent a failure to form a covalent adduct at position 1175?** An alternate explanation for my failure to effect irreversible inactivation of pol III-cys is that a covalent adduct may have formed at residue 1175 but may \textit{not} have inactivated the polymerase, as previously hypothesized. It is possible that a covalently bound inhibitor adduct could swing out of the dNTP-binding region and not obstruct polymerization of dNMP molecules. The only way to determine if the above phenomenon were actually occurring would be to synthesize the inhibitor in a
radiolabeled form, subject enzyme and inhibitor to the incubation conditions described earlier, and determine whether radiolabel is actually bound to pol III-cys. Ideas for future experiments toward the goals of this project will be discussed in Chapter V.
CHAPTER V

FUTURE DIRECTIONS

A. Contributions to the Long Range Goals of the Brown Lab

The research presented in this thesis has contributed towards one of the long range objectives of the Brown lab, which is to locate and to characterize the molecular structure of the dNTP and aryl inhibitor binding sites of the B. subtilis DNA polymerase III pol domain. The major aim of my research was to determine, within the latter context, whether these two sites were separate and distinct, overlapping, or completely congruous. I successfully determined that the dNTP and aryl binding sites are formally distinct, but close, positioned such that the aryl moiety of a bound inhibitor molecule is but a few Å distant from space occupied by the hydrogen-bonded 2-NH group of a dNTP (i.e. dGTP) molecule as it sits in its prepolymerization position in the DNA:dNTP complex.

Using the latter information and structure activity relationships obtained with p-substituted phenylhydrazinouracils and the mutant, azp-12 enzyme, I hypothesized that the para region of the aryl group of bound inhibitor and the side chain of serine 1175 interacted directly in the ternary enzyme:inhibitor:DNA complex. On the basis of this working hypothesis, I attempted to redesign enzyme residue 1175 to optimize its covalent reactivity for a suitably tailored inhibitor. I generated both a lysyl and a cysteinyl mutant; of the two,
only the cysteine form, pol III-cys, was active and useful for study. Attempts to irreversibly inhibit pol III-cys with potential covalently reactive forms of the H2-HPUra-like inhibitors did not succeed. However, only a small number of inhibitors, mostly in the meta-substituted anilinouracils, were tested. Further promising candidates for covalent reaction could readily be synthesized.

B. Further Attempts to Define the Aryl Binding Site

Other potentially irreversible inhibitors. In the second half of Table IV-B, pages 51-52, I proposed the synthesis and testing of two potential irreversible inhibitor forms which had not been synthesized: 6-(p-azidoanilino)uracil and the 6-(p-formylanilino)uracil. The former compound has excellent potential to label a broad range of amino acid side chains and thus, could be used to label both wild-type pol III and pol III-cys-1175. The azido group requires photoactivation in order to generate the reactive moiety, a nitrene group (Rush & Konigsberg, 1990). The reversible ternary complex of enzyme:DNA:inhibitor would have to be formed in the dark to assure the specificity of the reaction upon exposure to light.

The second compound, 6-(p-formylanilino)uracil, is an aldehyde derivative like pyridoxal phosphate and thus, should be able to form a reversible Schiff base with proximal lysine residues (Mitra & Metzler, 1988). A reduction reaction of this intermediate with sodium borohydride (NaBH4) would form a covalent bond that cannot readily be reversed (Tagaya & Fukui, 1986; Basu et al., 1988).
Methods exist to synthesize 6-(p-azidoanilino)uracil, or the equivalent guanine analog, with a tritium label, to permit the detection of covalently linked inhibitor (see Section C of this chapter). The p-formyl-substituted inhibitor would not require synthesis of a tritium-labeled form. Reduction of the Schiff base intermediate with [3H] NaBH₄ (Basu et al., 1988) would introduce a tritium label into the lysyl-formyl double bond (-N=CH-) to yield a single bond with two tritium labels (-N³H-CH³H-).

Random mutagenesis of polC. Another approach to the tentative identification of aryl binding domain residues would be to perform random mutagenesis on small segments of the polC gene. The generation of random mutations could be accomplished through the use of both in vivo and in vitro techniques. For example, the target DNA can be propagated in E. coli mutD [(Echols et al., 1983), a dnaQ gene mutant which has a deficient editing exonuclease], for several generations to yield mismatch mutations (Schaaper, 1988). Alternatively, the target DNA can be propagated in a plasmid within an alkylation repair-deficient strain such as E. coli ada⁻ (Demple, 1986) and during propagation be subjected to treatment with an alkylating agent such as methylnitrosoguanidine. The resulting chemically modified DNA gives rise to frequent mismatches in the DNA and thus, the generation of mutations.

Mutated DNA obtained with either of the above methods can be inserted by transformation back into the homologous segment of polC of a suitable B. subtilis host (N.C. Brown, unpublished results) and transformants selected on the basis of resistance to a wide variety of structurally modified inhibitors in the benzylguanine, HPUrA or anilinouracil family (see Figure I-F, page 16). The latter approach should yield a variety of point mutations specifying several inhibitor response phenotypes. PCR-mediated
sequencing of these mutations would provide a simple means to identify the mutated amino acids specifying the phenotype and thus, yield, indirectly, a more detailed concept of the aryl binding site and identify promising residues for site specific attack.

C. Parallel Attempt to Label the dNTP Binding Site

dNTP analogs. A well established method to identify active site residues of dNTP binding proteins involves their covalent cross-linking by various dNTPs and dNTP analogs. One analog, 8-azido-dATP, has been used to covalently label the dNTP binding sites of E. coli pol I Klenow fragment (Rush & Konigsberg, 1990) and mammalian terminal deoxynucleotidyl transferase (TdT) (Evans & Coleman, 1989b) upon photoactivation of the azido group to a reactive nitrene. A related compound, 5-azido dUTP, also has been used as a photoaffinity label for E. coli pol I (Evans & Haley, 1987). The above compounds can be readily synthesized in a [γ-32P]-labeled form (Glynn & Chappell, 1964) to permit analysis of labeled amino acid residues (see page 81).

Alternatively, the nucleoside analog, 5’-(p-fluorosulfonyl)benzoyl-8-azido-adenosine, a bifunctional affinity label (Dombrowski & Colman, 1989), is potentially useful as an agent for covalent labeling experiments, perhaps as the 2’-deoxy derivative. The latter compound, which can be labeled at the 2-position with tritium, has been used to label bovine liver glutamate dehydrogenase. It contains a photoactivatable azido group, capable of reacting with many amino acid side chains, and an electrophilic fluorosulfonyl group that can react with a wide variety of side chains, including those of lysine, tyrosine, serine, cysteine and histidine.
Direct cross-linking of dNTPs. If the above approach with dNTP analog labeling does not work with wild-type or mutant *B. subtilis* pol III s, direct U.V.-induced cross-linking of dNTPs could be attempted. This is a well established technique that has been used to cross-link mammalian TdT and ribonucleotide reductase and *E. coli* pol I Klenow fragment with \( [\alpha{}^{32}\text{P}]\)-dTTP (Pandey & Modak, 1988; Pandey et al., 1987; Eriksson et al., 1982) and *E. coli* pol III holoenzyme with \( [\alpha{}^{32}\text{P}]\)-dATP (Biswas & Kornberg, 1984). The conditions required for the cross-linking experiments have been described previously (Eriksson et al., 1982).

Identification of labeled amino acid residues. If any of the above compounds successfully label the aryl and/or the dNTP binding sites, we would plan to identify the labeled amino acid(s). The next steps in processing appropriately labeled protein would involve its enzymatic cleavage into fragments, HPLC-identification and purification of the labeled fragment(s) (Kierdaszuk & Eriksson, 1988; Evans et al., 1989a), and sequencing of the purified peptide to identify its position in the protein (Rush & Konigsberg, 1990).

The experiments described in the previous two sections will likely directly identify key anchor residues in the dNTP and/or inhibitor binding sites and thus, provide a more accurate account of the location of the pol III active site and provide a concept that should facilitate the ultimate three-dimensional analysis of the enzyme structure by X-ray diffraction.
CHAPTER VI

MATERIALS AND METHODS

A. Chemicals and Reagents

Radioactive materials were purchased from New England Nuclear. Nucleotides and synthetic polynucleotides were obtained from P-L Laboratories. Calf thymus DNA was from Worthington. All chemicals, solvents and reagents used in syntheses were of analytical grade or better and were obtained from Sigma unless otherwise noted.

B. Chemical Analysis

Melting points were determined on a Mel-temp apparatus. Elemental analyses were performed by the Microanalysis Laboratory, University of Massachusetts, Amherst. Phosphorus analyses were performed as described by Peterson (1978). UV spectra were obtained with a Gilford Response UV-Vis Spectrophotometer. NMR spectra were obtained at 200 MHz with a Bruker ACP-200 instrument; chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (1H) or external 1% phosphoric acid (31P). Only characteristic chemical shifts (ppm) and coupling constants (Hz) are given. The
identity of the nucleosides as 9-β-(2-deoxyribofuranosyl) derivatives was based on the similarity of \(^1\)H NMR spectra of sugar ring protons to those of 2'-deoxyguanosine (Wright & Dudycz, 1984).

C. Chemical Syntheses

\(N^2-(3,4\text{-Dichlorobenzyl})\text{guanine (DCBG).}\) A solution of 2-bromohypoxanthine (10 g, 46.5 mmol) and 3,4-dichlorobenzylamine (16.4 g, 93 mmol) in 2-methoxyethanol (240 mL) and water (60 mL) was heated at reflux for 18 h. The reaction was stopped by addition of ice water (300 mL). The product was isolated by filtration and crystallized from methanol to yield 12.0 g (83%) of DCBG, melting point (m.p.) 282-285°C. \(^1\)H NMR (Me\(_2\)SO-d\(_6\)) \(\delta\) 12.42 (1H, s, 9-H), 10.69 (1H, s, 1-H), 7.65 (1H, s, 8-H), 7.32-7.56 (3H, m, Ph), 6.82 (1H, t, 2-NH), 4.49 (2H, d, CH\(_2\)). Found: C, 46.31; H, 3.09; N, 22.36. C\(_{12}\)H\(_9\)N\(_5\)OCl\(_2\) requires C, 46.47; H, 2.93; N, 22.58.

Synthesis of DCBdG, the nucleoside form of DCBG, was a four-step modification of the procedure described by Wright et al. (1987); the approach to the synthesis of the nucleoside mono- and triphosphates was based on that described by Wright and Dudycz (1984).

**Step 1: 2-(3,4-Dichlorobenzylamino)-6-chloropurine.** A solution of DCBG (7.53 g, 24.3 mmol) in phosphoryl chloride (74 mL) containing N,N-dimethylaniline (9 mL) was heated at reflux for 10 min. The reaction mixture was poured slowly into ice water (1400 mL). After 1 h the solution was brought to pH 4 with sodium acetate, and the solid was
collected by filtration. The solid was dissolved in 0.5 M NaOH (100 mL), and the solution was decolorized with carbon and acidified with acetic acid. The product was reisolated by filtration and crystallized from methanol to yield 6.65 g (84%) of product, m.p. 196-198°C. \(\lambda_{\text{max}}\) (EtOH) 249 nm (\(\varepsilon, 13700\)), \(\lambda_{\text{max}}\) (pH2) 247 nm (\(\varepsilon, 16900\)), \(\lambda_{\text{max}}\) (pH12) 230 nm (\(\varepsilon, 37100\)). \(^1\)H NMR (Me2SO-d6) \(\delta\) 8.12 (1H, s, 8-H) Found: C, 43.95; H, 2.62; N, 21.09. \(\text{C}_{12}\text{H}_{8}\text{N}_{5}\text{Cl}_{3}\) requires C, 43.86; H, 2.45; N, 21.31.

**Step 2:** 2-(3,4-Dichlorobenzylamino)-6-chloro-9-(2-deoxy-3,5-di-p-toluyl-\(\beta\)-D-ribofuranosyl)purine. A mixture of the step 1 product (5.65 g, 17.2 mmol) and sodium hydride (60% suspension in mineral oil, 0.72 g, 18.1 mmol) in dry acetonitrile (125 mL) was stirred at 25°C for 30 min. 2-Deoxy-3,5-di-p-toluyl-\(\alpha\)-D-ribofuranosyl chloride (6.7 g, 17.2 mmol) was added in small portions over a period of 50 min. The mixture was diluted with chloroform (100 mL) and stirred at 25°C overnight. The mixture was filtered through Celite. The filtrate was evaporated to dryness, and the residue was chromatographed on a silica gel column (15 x 3 cm). Elution with a gradient of toluene-20% acetone in toluene (4 L) gave two major products; the first, the desired product, was crystallized from methanol to give 7.11 g (61%) of the 9β isomer as colorless crystals with a m.p. of 83-85°C. \(\lambda_{\text{max}}\) (EtOH) 239 nm (\(\varepsilon, 41100\)), \(\lambda_{\text{max}}\) (pH2) 239 nm (\(\varepsilon, 41900\)), \(\lambda_{\text{max}}\) (pH12) 241 nm (\(\varepsilon, 41900\)). Found: C, 57.90; H, 4.13; N, 10.10. \(\text{C}_{33}\text{H}_{28}\text{N}_{5}\text{O}_{5}\text{Cl}_{3}\) requires C, 58.21; H, 4.14; N, 10.28. A second form, presumably the 7-\(\beta\) isomer, was isolated but was uncharacterized.

**Step 3:** 2-(3,4-Dichlorobenzylamino)-6-chloro-9-(2-deoxy-\(\beta\)-D-ribofuranosyl)purine. A stirred solution of 2-(3,4-dichlorobenzylamino)-6-chloro-9-(2-deoxy-3,5-di-p-toluyl-\(\beta\)-
D-ribofuranosyl)purine (6.8 g, 10 mmol) in methanol (400 mL) was treated at 25°C with a solution of 1 N sodium methoxide in methanol (20 mL). After 1 h the solution was neutralized with acetic acid, evaporated to dryness, and the residue was chromatographed on a silica gel column (10 x 2 cm). Elution with 10% methanol in chloroform (1.2 L) gave the product, which was crystallized from methanol yielding 4.11 g (92%) with a m.p. of 148-150°C. λ_max (EtOH) 254nm (ε, 15400), λ_max (pH2) 254nm (ε, 15700), λ_max (pH12) 254nm (ε, 15500). 1H NMR (Me2SO-d6) δ 8.28 (1H, s, 8-H), 6.23 (1H, t, Jav = 6.9 Hz, 1'-H), all other resonances as expected. Found: C, 45.61; H, 3.62; N, 15.47. C17H16N5O3Cl3 requires C, 45.91; H, 3.63; N, 15.75.

Step 4: N2-(3,4-Dichlorobenzyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine (DCBdG). A solution of the step 3 product (0.85 g, 1.9 mmol) in ethanol (50 mL) was treated with 2-mercaptoethanol (0.6 mL, 8.5 mmol) and a solution of 1 N sodium methoxide in methanol (6 mL) and heated at reflux for 26 h. The solution was diluted with water (80 mL), adjusted to pH 6.5 with acetic acid, and evaporated to dryness to yield a crude product. Crystallization from ethanol and then ethyl acetate gave 0.6 g (73%) of DCBdG with a m.p. of 212-214°C. λ_max (H2O) 255nm (ε, 11200), λ_max (pH2) 258nm (ε, 11500), λ_max (pH12) 261nm (ε, 10300). 1H NMR (Me2SO-d6) δ 11.19 (1H, s, 1-H), 7.83 (1H, s, 8-H), 7.32-7.61 (3H, m, Phe), 7.46 (1H, t, 2-NH), 6.13 (1H, t, Jav = 6.9 Hz, 1'-H), 5.27 (1H, d, 3'-OH), 4.87 (1H, t, 5'-OH), 4.50 (2H, d, CH2), 4.33 (1H, m, 3'-H), 3.81 (1H, q, 4'-H), 3.57 (1H, m, 5'-H), 3.47 (1H, m, 5''-H), 2.55 (1H, m, 2'-H), 2.18 (1H, m, 2''-H). Found: C, 46.02; H, 3.93; N, 15.54. C17H17N5O4Cl2.H2O requires C, 45.96; H, 4.31; N, 15.76.
**N^2-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-phosphate (DCBdGMP)**. Phosphoryl chloride (0.061 mL, 0.66 mmol) was added to a cold solution of DCBdG (256 mg, 0.6 mmol) in trimethyl phosphate (3.72 mL). The mixture was kept at 0-3°C for 4 h during which more phosphoryl chloride (0.061 mL, 0.66 mmol) was added. The mixture was kept at -20°C overnight, and poured onto ice (25 g) and neutralized with 1 N NaOH. The product was applied to a 24 x 3.5 cm DEAE-Sephadex column, and the column was eluted with a linear gradient of 0.2-1.0 M triethylammonium bicarbonate, pH 7.7 (total volume = 5.8 L), at a flow rate of 4 mL/min with collection of 15 mL fractions. Fractions containing the desired product were combined, evaporated to dryness, and the residue was dissolved in methanol (1.2 mL) and mixed with 0.5 M sodium perchlorate solution (5 mL). The resulting suspension was centrifuged in a conical glass tube at 5000 rpm for 10 min. Supernatant was removed and acetone (6 mL) was added to the pellet, and the suspension recentrifuged. The latter procedure was repeated twice and the resultant pellet was dissolved in H_2O (4 mL) and lyophilized. Recovery of DCBdGMP after conversion to the sodium salt was 181 mg (55%). \(\lambda_{max} (H_2O) 256\text{nm} (\epsilon, 12100), \lambda_{max} (pH2) 259\text{nm} (\epsilon, 12000), \lambda_{max} (pH12) 261\text{nm} (\epsilon, 10100)\). \(^{31}P\) NMR (D_2O) \(\delta 2.62\) (t). Found: P, 5.86. C_{17}H_{16}N_5O_7Cl_2PNa requires P, 5.87.

**N^2-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-triphosphate (DCBdGTP)**. A solution of DCBdGMP, as the triethylammonium salt (102 mg, 0.14 mmol), in hexamethylphosphoramide (0.78 mL), was treated with 1, 1'-carbonyldiimidazole (CDI, 112 mg, 0.7 mmol) at 25°C. A clear solution was obtained after 15 min, and after 1 h more CDI (41 mg, 0.25 mmol) was added. The reaction was quenched after 1.5 h by the addition of methanol (0.125 mL). TLC showed complete conversion of DCBdGMP to its
imidazolyl derivative. A solution of tributylammonium pyrophosphate, prepared from tetrasmium pyrophosphate (320 mg, 0.68 mmol) by applying the material to a Dowex column previously equilibrated with tributylamine, in hexamethylphosphoramide (6 mL) was added dropwise with stirring. After 8 h at 25°C, the reaction mixture was poured onto ice (25 g) and brought to 100 mL with water. The solution was applied to a 24 x 3.5 cm DEAE-Sephadex column and eluted with a linear gradient of 0.2-1.0 M triethylammonium bicarbonate, pH 7.7, (total volume = 3.9 L), at a flow rate of 2.7 mL/min with collection of 13 mL fractions. Fractions containing DCBdTTP, at 0.8-0.9 M buffer, were combined and evaporated. The product was converted to its sodium salt by the same procedure used for DCBdTMP. The product was lyophilized to give 64 mg (59%) of DCBdTTP. $\lambda_{max}$ (H2O) 256 nm ($\epsilon$, 11100), $\lambda_{max}$ (pH2) 259 nm ($\epsilon$, 11000), $\lambda_{max}$ (pH12) 261 nm ($\epsilon$, 10300). $^{31}$P NMR (D2O) δ -6.56 (1P, d, γ-P), -10.85 (1P, d, α-P), -21.66 (1P, t, β-P, $\delta_2$ 17.6 Hz). Found: P, 11.75. C17H16NSO13Cl2P3N4 requires P, 11.38.

$N^2$-(p-Methylbenzyl)guanine. A solution of 2-bromohypoxanthine (1.5 g, 6.98 mmol) and 4-methylbenzylamine (1.7 g, 13.96 mmol) in 2-methoxyethanol (36 mL) and water (9 mL) was heated at reflux for 18 h. The product was precipitated by the addition of an equal volume of cold water. The precipitate was isolated by filtration and crystallized from methanol to yield 0.95 g (54%) of product with a m.p. of 170-172°C. Found: C, 59.02; H, 5.48; N, 26.14. C13H13N5O1½H2O requires C, 59.08; H, 5.34; N, 26.50.

6-(p-Methylbenzylamino)uracil. A solution of 6-chlorouracil (1 g, 13.6 mmol) and 4-methylbenzylamine (3.3 g, 27.2 mmol) in 2-methoxyethanol (40 mL) was heated at reflux for 7 h. The solution was evaporated to one-half volume, and the product was precipitated
by the addition of an equal volume of cold water. The precipitate was isolated by filtration and crystallized from 50% acetic acid to yield 1 g (60%) of product which had a m.p. of 327-330°C. $^1$H NMR (Me$_2$SO-d$_6$) δ 7.12-7.15 (4H, m, Ph), 6.55 (1H, m, 6-NH), 4.38 (1H, d, 5-H), 4.20 (2H, d, CH$_2$), 2.28 (3H, m, CH$_3$). Found: C, 62.01; H, 5.52; N, 17.95. C$_{12}$H$_{13}$N$_3$O$_2$ requires C, 62.33; H, 5.67; N, 18.17.

6-(3,4-Dichlorobenzylamino)uracil (DCBAU). A solution of 6-chlorouracil (0.5 g, 3.4 mmol) and 3,4-dichlorobenzylamine (1.2 g, 6.8 mmol) in 2-methoxyethanol (15 mL) was heated at reflux for 18 h. The solution was cooled and an equal volume of cold water was added. The precipitate was isolated by filtration and crystallized from ethanol to yield 0.45 g (45%) of product with a m.p. of 316-317°C. $^1$H NMR (Me$_2$SO-d$_6$) δ 10.12 (1H, s, 1-H), 10.12 (1H, s, 3-H), 7.30-7.64 (3H, m, Ph), 6.56 (1H, t, 6-NH), 4.35 (1H, d, 5-H), 4.30 (2H, d, CH$_2$). Found: C, 45.80; H, 2.90; N, 14.54. C$_{11}$H$_9$N$_3$O$_2$Cl$_2$ requires C, 46.18; H, 3.17; N, 14.69.

6-(m-Bromomethylanilino)uracil (6-(m-CH$_2$Br) AU). A solution of 6-(m-hydroxymethylanilino) uracil (Wright & Brown, 1980), (100 mg, 0.43 mmol) in 3 mL HBr (48% in H$_2$O) was stirred at room temperature for 48 hours. The product began to precipitate during the reaction. The suspension was poured into 30 mL of cold water, and the precipitate was isolated by filtration and crystallized from 50% acetic acid (HOAc) to yield 103 mg (81%) of product with a m.p. > 350°C. $^1$H NMR (Me$_2$SO-d$_6$) δ 10.49 (1H, s, 3-H), 10.18 (1H, s, 1-H), 8.33 (1H, s, 6-NH), 7.12-7.39 (4H, m, Ph), 4.74 (1H, s, 5-H), 4.72 (2H, s, CH$_2$). Found: C, 44.99; H, 3.74; N, 13.90. C$_{11}$H$_{10}$N$_3$O$_2$Br requires C, 44.62; H, 3.40; N, 14.19.
6-(m-Mercaptomethylanilino) uracil. This synthesis involved two reactions: (1) A solution of 6-(m-CH2Br) AU (100 mg, 0.34 mmol) and potassium thiocyanate (44 mg, 0.45 mmol) in DMSO (2.5 mL) was stirred at room temperature for one hour. The solution was poured into 25 mL of cold water, chilled and filtered. Identification of the intermediate product, 6-(m-thiocyanatomethylanilino)uracil, was made by comparing mobility on TLC with an authentic product (prepared in the same manner by D. Trantolo, doctoral dissertation, Clark University, 1982). (2) A solution of the crude product of reaction (1) (55 mg, 0.20 mmol) in 2 N NaOH (6.25 mL) was heated at reflux for two hours. Glacial HOAc was added to the solution until the pH became neutral. The solution was chilled, and the resulting precipitate was filtered and crystallized from 50% HOAc to yield 23 mg (49%) of product with a m.p. of 288-290°C. 1H NMR (Me2SO-d6) δ 10.46 (1H, s, 3-H), 10.12 (1H, s, 1-H), 8.26 (1H, s, 6-NH), 7.04-7.34 (4H, m, Ph), 4.74 (1H, s, 5-H), 3.75 (2H, s, CH2).

D. Bacterial Growth and Transformation

E. coli strains BMH71-mutS (Kramer et al., 1984), JM109, (Hanahan, 1985), and XL1 Blue (Stratagene) were grown on LB broth (Sambrook et al., 1989) plates containing appropriate antibiotics and were made competent by the rubidium chloride method (Hanahan, 1985). E.coli AR120 was induced in maximal induction medium (Mott et al., 1985) and was made competent by the above method.
B. subtilis wild-type (Neville & Brown, 1972) and F2 (Gass et al., 1971) were grown in C+50 minimal salts medium (Anagnostopoulos & Spizizen, 1961) and were made competent by a method described previously (Stewart, 1969).

E. Assay of Nucleic Acid and Protein Synthesis in Intact B. subtilis

Wild type B. subtilis was grown to log phase at 37°C in C+50 medium. At a time designated as zero, either [³H] adenine (7 x 10⁵ cpm/pmole, 100 μM; for DNA and RNA) or [³H] L-leucine (1.4 x 10⁵ cpm/pmole, 10⁵ cpm/mL; for protein) was added to the cells along with either DCBG (75 μM), HPUra (75 μM) or DMSO, the inhibitor diluent (Neville & Brown, 1972). Periodically, duplicate samples (1 mL each) were removed to determine incorporation of radioactivity into RNA, DNA or protein as described previously (Brown, 1970). The radioactivity that was both cold TCA-precipitable and alkali-stable was counted as DNA incorporation. The alkali-soluble, cold TCA-precipitable material was counted as RNA incorporation and was calculated by subtracting counts that were TCA-precipitated but not alkali-treated (DNA + RNA) from counts that remained after alkali treatment (DNA). The hot TCA-insoluble radioactive material was counted as protein.

F. Analysis of Primer Extension

The method, performed by Dr. Naseema Khan in the Brown lab, involved the autoradiographic analysis of denaturing DNA sequencing gel electropherograms of products resulting from the extension of a 5' [³²P]-labeled 17 residue oligonucleotide
primer annealed to a 29 residue template. The structure of the primer (M13 sequencing primer #1211; Boehringer Mannheim) and template (Operon) is shown in the inset of Figure III-E, pages 42-43. The preparation of the sequencing gel (12% polyacrylamide, 8 M urea), 5'-labeling of the primer with polynucleotide kinase, primer:template annealing, sample preparation, and the conditions for gel development and autoradiography, based on work by Townsend & Cheng (1987), are described elsewhere (Khan et al., 1991). To assay for extension, 0.25 units of FPLC/MonoQ purified pol III (see Section L, Chapter VI, Hammond & Brown, 1992) were incubated in 10μL of standard assay buffer (Neville & Brown, 1972), [plus 2 mM adenosine 5'-monophosphate (AMP) to partially inhibit the associated exonuclease], in the presence of [32P]-primer:template, in slight excess over enzyme, and in the presence or absence of selected dNTPs including DCBdGTP. After 5 min. at 30°C, incubation mixtures were quenched and analyzed as described (Townsend & Cheng, 1987).

Calf thymus terminal deoxyribonucleotidyl transferase (TdT; New England Nuclear) was used to obtain a sequencing ladder for identifying the specific products of primer extension with DCBdGMP residues (Khan et al., 1991); 0.25 units of TdT were incubated at 37°C with 1μg of [32P]-labeled primer in the presence of 10 μM DCBdGTP in a volume of 10 μL in the assay buffer conditions described (Roychoudry & Wu, 1980). After 30 min. the incubation mixtures were quenched and subjected to analysis as described above for the products of pol III-catalyzed primer extension.
G. DNA Polymerase Assay

All assays measured the incorporation of the labeled dNMP moiety of a radioactive dNTP into a cold trichloroacetic acid (TCA)-insoluble form using various DNA template-primers. Insoluble DNA was filtered onto GF/A disks (Whatman) and dried. Filter-bound radioactivity was determined by scintillation counting of each disk in 2 mL of Optifluor (Packard). Standard assays were done as described by Barnes and Brown (1979).

Mixtures (25 µL each) contained 30 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, 4 mM DTT (Curtin Matheson Scientific), 20% glycerol, 0.4 mg/ml activated calf thymus DNA, 25 µM each of dATP, dCTP and dGTP, and 10 µM [3H]-dTT (625 cpm/pmol). Reactions were initiated by the addition of 0.05 units of enzyme and incubated at 30°C for 10 min. One unit of polymerase activity is the amount of enzyme that incorporates 1 nmol of labeled dNTP into a cold TCA-insoluble form in 10 minutes. Reactions were quenched and DNA rendered insoluble by the addition of an excess of cold 10% TCA containing 10 mM PPi, and a further incubation on ice for approximately 10 minutes.

Apparent inhibitor constants (K_i) were determined in the appropriate polymerase assay conditions by either conventional kinetic competition assay or by truncated, dGTP-deficient assay; both methods have been described (Wright and Brown, 1976).

Poly(dA):oligo(dT) and poly(dC):oligo(dG) were prepared by incubating at 50°C a mixture of 4 Optical Density (OD) units (260nm) of polymer and 0.625 OD units of oligomer in 10 mM Tris-Cl, pH 7.6, for 10 min. and allowing the mixture to cool to 25°C over an additional 10 min. period; 0.1µL of this mixture was used in a final assay volume of 25 µL. The labeled triphosphate, [3H]-dTTP or [3H]-dGTP, (20 µM, 875 cpm/pmole) was the only nucleotide used in experiments involving these homopolymeric templates.
H. Exonuclease Assay

The $^3$H-DNA template was prepared by scaling up a standard DNA polymerase assay (see Section G) to 5 mL, increasing the specific activity of dTTP to 1250 cpm/pmole and incubation time to 20 minutes. The labeled DNA was precipitated by adding 0.5 mL of 7.5 M ammonium acetate and 11 mL of ice cold ethanol and freezing at -20°C for 2 hours. After centrifugation, the pelleted DNA was resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), desalted on a Sephadex G-25 column and filtered. The DNA (specific activity, $2.6 \times 10^4$ cpm/µg) was dissolved in TE to generate a stock solution containing $1.5 \times 10^4$ cpm/µL. To generate single stranded DNA, the above solution was heated to 100°C for 5 min. and quenched on ice.

The exonuclease assay, which was a modified version of the procedure described by Low et al. (1976), was performed by adding 5 µL enzyme (0.05 units) to a 45 µL reaction mixture containing 30 mM Tris-HCl, pH 7.5, 6.5 mM MgCl$_2$, 3 mM DTT, 10% glycerol and 2.7 µg of single stranded $^3$H-DNA (approximately $7 \times 10^4$ TCA-precipitable cpm) and incubating at 30°C for 10 minutes. The reaction was terminated by adding 50 µL of 10 mg/mL bovine serum albumin (BSA), 10 mM PPi and 500 µL cold 10% TCA/10mM PPi. The mixture was centrifuged for 15 minutes and 400 µL of the resulting supernatant were counted in 2 mL of Optifluor (Packard).
J. Site-Directed Mutagenesis

The mutagenesis system, obtained from Promega, exploited oligonucleotide-directed mutagenesis on a phagemid vector, p-SELECT, containing a single-stranded F1 origin of replication. This system, shown in Figure IV-A, page 54, and Figure IV-B, page 55, is described in Chapter IV. Procedures involving manipulation and cloning of recombinant DNA were generally those described by Sambrook et al. (1989). Pol C fragment EcoRV (2941) to BclI (3965) was inserted into the BamHI site of the multiple cloning site of p-SELECT. Single-stranded DNA was prepared from the resulting vector by infecting E. coli JM109 with vector and the helper phage, R408, at a multiplicity of infection of 10 (10 phage molecules per bacterial cell). Single-stranded DNA was isolated according to the Promega manual, precipitated, and resuspended in TE.

Annealing was performed by mixing prepared single-stranded recombinant DNA, an ampicillin repair oligonucleotide and the appropriate mutagenic oligonucleotide (Operon), shown in Figure IV-C, page 57, in the presence of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 50 mM NaCl. This mixture was heated to 70°C for 5 min. and slowly cooled to room temperature. Subsequent synthesis and ligation of double-stranded mutant DNA was carried out by incubating the above mixture with 0.5 mM dNTPs, 1 mM ATP, 2 mM DTT, T4 DNA polymerase (10 units) and T4 DNA ligase at 37°C for 90 min.

Mutagenized DNA was transformed into E. coli BMH71-18 Mut S (a mismatch repair deficient strain). The cells were grown overnight and the double-stranded DNA was prepared in a mini-prep procedure based on the alkaline lysis method (Birboim & Doly, 1979). The DNA was then transformed into E. coli JM109 (a recA deficient strain) and 6 colonies were again miniprepped. The mutagenic oligonucleotide was designed to include
silent mutations which specified the loss of a SacI restriction site (GAGCTC) and the introduction of a unique MaeI site (CTAG). This restriction polymorphism provided a diagnostic tool with which to tentatively identify successful mutations for confirmatory sequencing. The dideoxy sequencing method (Sanger et al., 1977) was used according to the Sequenase Version 2.0 protocol (USB) to confirm the presence of the cysteine codon in the polC-specific fragment, BglII (3416) to SacI (3613). Sequencing primers, obtained from Operon, were employed by annealing one to the 3'-5' strand upstream of the BglII and the other to the 5'-3' DNA strand downstream of the SacI site by heating the DNA to 70°C for 5 min. and slowly cooling to room temperature (see Figure VI-A, page 96).

K. The Engineering of the Mutant Fragment into the polC Expression Plasmid, pKC30

The goal was to substitute the mutant BglII-SacI fragment for the equivalent fragment of an engineered version of wild-type polC which had previously been designed in the laboratory for construction of the pol III expression plasmid pRHpolCwt (Hammond & Brown, 1992). The essential features of the engineered polC fragment (nt 1-4311) are shown schematically in the structure of the recombinant plasmid pWT7C3 in Figure VI-B, page 97.

The specific procedure, which was dictated by consideration of the restriction site morphology, consisted of the following four steps. The E. coli host for cloning steps 1-3 was XL1 Blue. The host for step 4 was E.coli AR120, a phage λ lysogen which provides the pL repressor protein, cl, in trans. The product of step 4, pKBC6, must be propagated
Figure VI-A: p-SELECT vector containing the polC EcoRV to BclI fragment and the mutated codon 1175. Sequencing primers flanked the 197 b.p. BglII to SacI fragment. * indicates the site of mutation.
Figure VI-B: Structure of the pol III recombinant plasmid, pWT7C3, which contains the entire polC gene and the E. coli ribosomal binding site (RBS), for optimal expression of the gene inserted directly following it. * indicates the site of mutation. The met-glu codons ATG GAA represent the first six bases of native polC. DNA from bases 4312 to the BamHI site at nucleotide (nt) 4900 include a tandem array of 553 nt of contiguous, non-coding DNA from the B. subtilis genome and 36 nt of p-Bluescript DNA (this DNA does not affect the transcription of polC in the expression plasmid (Hammond et al., 1991)
in the presence of cl to prevent synthesis of *B. subtilis* pol III, which is toxic to *E. coli* (Barnes et al., 1989).

(1) Agarose gel separation and NACS column (Gibco BRL) purification of the mutant BglII-SacI fragment and insertion into a subcloned ClaI-BamHI (nt 2944-4864) fragment of *polC* to produce pCBC1.

(2) Digestion of pCBC1 with ClaI and SacII to generate a 907 bp fragment carrying the mutation. This was separated by agarose gel electrophoresis and purified by NACS column.

(3) Insertion of the above fragment into pWT73, shown in Figure IV-B, page 97, that was digested with ClaI and SacII to generate pWT7C3, the cysteine mutant * version of pWT73.

(4) Digestion of pWT7C3 with BamHI, purification of the liberated 5 kb *polC*-specific fragment, and insertion into the BamHI site of pKC30 (Rosenberg et al., 1983) downstream from its phage λ leftward promoter, pL, to generate the expression plasmid, pKBC6.
L. Induction, Overexpression, Purification and Characterization of Recombinant Pol III-Cys

The control of the λ promoter/repressor-controlled system employed in this overexpression is described in Chapter IV. Two 1 L flasks of maximal induction medium (Mott et al., 1985) containing ampicillin (150 µg/mL) were inoculated with 14 ml of overnight growth of AR120/pKBC6 and shaken at 32°C until an optical density at 600 nm of 1.0 was attained. At this time, nalidixic acid was added to a final concentration of 60 µg/mL and the incubation was continued for five hours. The cells were harvested by centrifugation, washed with cold phosphate buffered saline (PBS) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and stored as frozen pellets at -70°C.

Purification of pol III-cys was performed as described for wild-type pol III by Hammond et al. (1992). Essentially, cells were ruptured with a French pressure cell under 1000 psi of pressure and then centrifuged to remove cellular debris. The supernatant was then subjected to a series of column purification steps.

The first step involved tandem treatment with Cibacron Blue Sepharose (3G-A-agarose type 3000-CL, Sigma) and Phenyl Sepharose (CL-4B, Sigma). Cibacron Blue, a column with affinity for dNTP-binding proteins, allowed loading of the crude material at low salt and eluting pol III at 3 M NaCl. The eluent, which was loaded directly onto a phenyl sepharose column, with a hydrophobic column matrix, was not assayed for enzymatic activity due to the inhibitory effect of the 3 M NaCl. Upon elution of the phenyl sepharose column with low salt plus taurodeoxycholate (TDC), the eluate yielded pol III in
a small volume. Polymerase activity was determined to be that of *B. subtilis* pol III by the presence of >90% inhibition of activity in the presence of the pol III-specific inhibitor, TMAU.

The second step of purification involved removal of the TDC through use of a Sephadex G-25 (Pharmacia) gel filtration column. The polymerase activity eluted immediately following the void volume and was contained in a volume that was 1.5x the load volume. Smaller molecules, such as TDC, were bound on the G-25 column. The third step involved a final purification of pol III by a NaCl gradient elution on a MonoQ (Pharmacia) column attached to an FPLC (Pharmacia). The resultant protein, in pooled peak fractions, 42 and 43 (see Figure IV-D, pages 59-60), had a purity of greater than 98% and the correct, 162 KDa molecular weight compared to standard molecular weight markers.
CHAPTER VII

REFERENCES


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Development of novel inhibitor probes of DNA polymerase III based on dGTP analogs of the HPUra type: base, nucleoside and nucleotide derivatives of N²-(3,4-dichlorobenzyl)guanine

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INTRODUCTION

6-(p-Hydroxyphenylhydrazino)uracil (H₂-HPUra) is a selective and potent inhibitor of the replication-specific class III DNA polymerase (pol III) of G+ bacteria. Although formally a pyrimidine, H₂-HPUra derives its inhibitory activity from its specific capacity to mimic the purine nucleotide, dGTP. We describe the successful conversion of the H₂-HPUra inhibitor prototype to a bona fide purine, using N²-(benzyl)guanine (BG) as the basis. Structure-activity relationships of BGs carrying a variety of substituents on the aryl ring identified N²-(3,4-dichlorobenzyl)guanine (DCBG) as a nucleus equivalent to H₂-HPUra with respect to potency and inhibitor mechanism. DCDbGTP, the 2'-deoxyribonucleoside 5'-triphosphate form of DCG, was synthesized and characterized with respect to its action on wild-type and mutant forms of B. subtilis DNA pol III. DCDbGTP acted on pol III by the characteristic inhibitor mechanism and formally occupied the dNTP binding site with a fit which permitted its polymerization.

ABSTRACT

6-(p-Hydroxyphenylhydrazino)uracil (H₂-HPUra; ref 1), 6-(phenylhydrazino)uracil (PUra; ref 2), 6-(benzylamino)uracil (BAU) and 6-(3,4-trimethyleneamino)uracil (TMAU; ref 3) are members of a family of 6-substituted pyrimidines (cf., Fig. 1A) which share a unique biologic property: they are novel dGTP analogs which selectively inhibit the replication-specific DNA polymerase III (pol III) of B. subtilis and other G+ bacteria (4–6). The mechanism of inhibitory action common to this family of bases (5,6) is summarized schematically in panels B–D of Fig. 1. As shown in panel B, the inhibitor molecule consists of two essential domains: a base pairing domain equivalent to that of guanine, and an aryl domain from which it derives reactivity and selectivity for its enzyme target. Panel C displays the structure of the H-bonded, H₂-HPUra:cytosine base pair. and panel D summarizes how the base pairing and aryl domains of the molecule cooperate to effect inhibition. The inhibitor acts as a bridge between the template:primer and the polymerase, sequestering the enzyme into a reversible ternary complex. The base pairing domain forms one bridgehead with an unapposed template cytosine immediately distal to the 3'-OH terminus, while the aryl substituent forms the other through its reaction with the polymerase in the vicinity of its dNTP binding site.

We seek to characterize in B. subtilis DNA pol III the location and molecular structure of its inhibitor binding site and the precise relationship of the latter to the site of dNTP binding and polymerization. Our strategy has required the reconfiguration of the inhibitor prototype into a bona fide guanine (G) format to provide a structure useful for mutant enzyme selection and suitable for development of 2'-deoxyribonucleosidyl forms. Our first attempt to adopt the G format involved the anilino prototype and resulted in the conversion of TMAU to the N²-(aryl)G derivative, TMPG (cf., Fig. 1,E). TMPG displayed the appropriate mechanism and requisite enzyme selectivity. However, TMPG was considerably less potent than TMAU and penetrated cells very poorly, and therefore, it was not suitable for further development. As an alternative to the N²-(aryl)-guanine structure, we exploited the N²-(arylalkyl)guanine format to produce a series of N²-(benzyl)guanine (BG) derivatives. As indicated in the results presented below, the N²-benzyl format proved to be a fruitful basis for inhibitor development, yielding the novel base, N²-(3,4-dichlorobenzyl)guanine (DCBG), and a derivative, 2'-deoxyribonucleoside 5'-triphosphate form, DCDbGTP, which can be polymerized by its pol III target.

MATERIALS AND METHODS

Chemicals, reagents and bacterial strains

Radioactive materials were purchased from New England Nuclear. Nucleotides and synthetic polynucleotides were obtained from P-L Laboratories. Calf thymus DNA was from Worthington. All chemicals, solvents and reagents used in
syntheses were of analytical grade or better. B. subtilis strain NB841 (7), which was used as the source of pol III, was grown on minimal salts agar supplemented as described (8).

Chemical analyses
Melting points were determined on a Mel-temp apparatus. Elemental analyses were performed by the Microanalysis Laboratory, University of Massachusetts, Amherst. Phosphorus analyses were performed as described by Peterson (9). UV spectra were obtained with a Gilford Response UV-Vis Spectrophotometer. NMR spectra were obtained at 200 MHz with a Bruker AC-P200 instrument; chemical shifts are reported in ppm (6) relative to internal tetramethylsilane (TMS) or external phosphoric acid (H3PO4). Only characteristic chemical shifts (ppm) and coupling constants (Hz) are given. The identity of the nucleosides as 9-β-(2-deoxyribofuranosyl) derivatives was based on the similarity of 1H NMR spectra of sugar ring protons to those of deoxyguanosine and others (10).

Syntheses
N2-(3,4-Dichlorobenzyl)guanine (DCBG). Reaction of bromohypoxanthine and 3,4-chlorobenzaldehyde as described (10) gave, after crystallisation from methanol, DBCG, in 83% yield; m.p. 282–285°C. 1H NMR (Me2SO-d6) δ 12.42 (1H, s, 9-H), 10.69 (1H, s, 1-H), 7.65 (1H, s, 8-H), 7.32–7.56 (3H, m, Ph), 6.82 (1H, t, 2-NH), 4.49 (2H, d, CH2). Anal. Found: C, 46.31; H, 3.09; N, 22.36. C12H14N3OCl5 requires C, 46.47; H, 2.93; N, 22.58.

Synthesis of DBCG, the nucleoside form of DBCG, was based upon the procedure described in reference 11; the approach to the synthesis of the nucleoside mono and triphosphates was based on that described by Wright and Dudycz (10).

Step 1: 2-(3,4-Dichlorobenzylation)-5-chloropurine. Treatment of DBCG with phosphoryl chloride and crystallisation from methanol yielded 84% of product, m.p. 196–198°C. 1H NMR (Me2SO-d6) δ 8.12 (1H, s, 8-H). Anal. Found: C, 43.95; H, 2.62; N, 21.09. C12H14N3Cl5 requires C, 43.86; H, 2.45; N, 21.31.

Step 2: 2-(3,4-Dichlorobenzylation)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine. Treatment of the sodium salt of the step 1 product with 2-deoxy-3,5-di-t-butyl-β-D-ribofuranosyl chloride in acetonitrile gave two major products which were separated by silica gel chromatography. The major desired product was deblocked by treatment with sodium methoxide in

![Figure 1](image-url)
the mixture to cool to 25°C of oligomer in 10mM Tris-Cl (pH 7.4) and 0.1M NaCl, all other reagents omitted. Ana. Found: C, 45.61; H, 3.62; N, 15.47. C$_{21}$H$_{23}$N$_3$O$_7$Cl$_3$ requires C, 45.91; H, 3.63; N, 15.75.

Step 3: N$^6$-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-phosphate (DCBGMP). The step 2 product was converted to DCBG by treatment with 2-mercaptoethanol in a solution of sodium methoxide in methanol at reflux for 26h. Crystallization from ethanol and then ethyl acetate gave 76% of DCBGMP with m.p. 212-214°C. $^{1}H$ NMR (MeSO-d$_6$) δ 11.19 (1H, s, 1-H), 7.83 (1H, d, 8-H), 7.32-7.61 (3H, m, Ph); 7.46 (1H, t, 2-NH), 6.13 (1H, t, J$_{3,5}$ = 6.9 Hz, 1'-H), 5.27 (1H, d, 3'-OH), 4.87 (1H, t, 5'-OH), 4.50 (2H, d, CH$_2$), 4.33 (1H, m, 3'-H), 3.81 (1H, q, 4'-H), 3.57 (1H, m, 5'-H), 3.47 (1H, m, 5'-H), 2.55 (1H, m, 2'-H), 2.18 (1H, m, 2'-H). Ana. Found: C, 54.02; H, 3.93; N, 15.54. C$_{21}$H$_{23}$N$_3$O$_7$Cl$_3$ requires C, 45.96; H, 4.31; N, 15.76.

N$^6$-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-triphosphate (DCBGTP). A solution of DCBGMP and phosphoryl chloride in trimethyl phosphate was kept at 0-3°C for 4h during which more phosphoryl chloride was added. After chromatography on DEAE-Sephadex and elution with a linear gradient of 0.2-1.0M triethylammonium bicarbonate, pH 7.7, the product was converted to the sodium salt by dissolving in methanol and treatment with 0.5M sodium perchlorate solution. After centrifugation and extensive washing with acetone, the resulting pellet was dissolved in water and lyophilized. Recovery of DCBGMP after conversion to the sodium salt was 55%. $^{31}$P NMR (D$_2$O) δ 2.62 (s). Ana. Found: P, 5.86. C$_{21}$H$_{23}$N$_3$O$_7$P$_3$Na requires P, 5.87.

N$^6$-(3,4-Dichlorobenzyl)-2'-deoxyguanosine 5'-triphosphate (DCBGTP). A solution of DCBGMP as the triethylammonium salt in hexamethylphosphoramide, after activation by I$_2$ and 1'-carboxylidimide, was treated with tributylammonium pyrophosphate. Chromatography of the diluted solution on DEAE-Sephadex and elution with a linear gradient of 0.2-1.0M triethylammonium bicarbonate, pH 7.7, the product was converted to its sodium salt by the same procedure used for DCBGMP. The product was lyophilized to give 59% of DCBGTP. $^{31}$P NMR (D$_2$O) δ -6.56 (IP, d, γ-P), -10.85 (IP, d, α-P), -21.66 (IP, t, β-P). Ana. Found: P, 11.75. C$_{21}$H$_{23}$N$_3$O$_7$P$_3$Na requires P, 11.38.

Purification and assay of B. subtilis pol III

Purification of wild-type and mutant (azpI2) pol III was performed as described previously (12); unless specified otherwise, the DNA cellulose fraction V was used in all experiments. The standard assay for DNA polymerase III was performed in a final volume of 250mL as described (7). Apparent inhibitor constants (K$_i$) for each inhibitor were determined in the appropriate polymerase assay conditions either conventionally or by the truncated, dGTP-deficient assay method described by Wright and Brown (13). Poly(dA):oligo(dT) and poly(dC):oligo(dG) were prepared by incubating at 50°C a mixture of 4 OD units (260nm) of polymer and 0.625 OD units of oligomer in 10mM Tris-Cl (pH 7.6) for 10min and allowing the mixture to cool to 25°C over an additional 10 min period. 0.1mL of this mixture was used in a final assay volume of

0.025mL. The labeled triphosphate (20mM, 875 cpm/pmmole) was the only nucleotide used in experiments involving these homopolymeric templates.

Assay of nucleic acid and protein synthesis in intact B. subtilis

At time zero, [3H] adenine (7×10$^8$ cpm/mole, 0.1mM; for DNA and RNA) or [3H] L-leucine (1.4×10$^6$ cpm/mole, 10$^3$ cpm/ml; for protein) and DCBG (75μM), HP3ta (75μM) or DMSO (inhibitor diluent) were added to log phase cultures of B. subtilis growing at 37°C in minimal salts medium (14), and periodically, duplicate samples (1ml each) were removed to determine incorporation of radioactivity into alkali-soluble (RNA) and alkali-stable (DNA) fractions of cold TCA-insoluble material or into hot TCA-insoluble material (protein) as described elsewhere (14).

Analysis of primer extension

The method involved the autoradiographic analysis of denaturing DNA sequencing gel electropherograms of products resulting from the extension of a 5' $^{32}$P-labeled 17 residue oligonucleotide primer annealed to a 29 residue template. The structure of the primer (M13 sequencing primer #1211; Boehringer Mannheim) and template (Operon) is shown in the inset of Figure 3. The preparation of the sequencing gel (12% polyacrylamide), 5'-labeling of the primer with polynucleotide kinase, primer-template annealing, sample preparation, and the conditions for gel development and autoradiography, were performed as described in reference 15. To assay for extension 0.25 units of homogeneous pol III (Fr. VIIa, ref. 12) were incubated in 10μL of standard assay buffer (7) in the presence of $^{32}$P-primer/template in slight excess over enzyme, and in the presence or absence of selected dNTPs. After 5 min at 30°C, incubation mixtures were quenched and analyzed (15).

RESULTS

Properties of DCBG, the model benzylguanine

Following leads developed with 6-benzaminouracil derivatives as pol III inhibitors (3), we synthesized several analogous N$^6$-benzylguanines substituted in the 3, 4 and 5 positions of the benzyl moiety. Of these only DCBG displayed inhibitory potency similar to that of H$_2$HPUra and related compounds. The properties of DCBG are summarized in the following three subsections.

(i) Action of DCBG on the isolated polymerase is characteristic of the H$_2$HPUra prototype. Our strategy for pol III inhibitor development required that in its simplest base form, the product:

(1) at least as potent as the corresponding uracil, which typically display K$_i$ in the 0.5-2μM range; (2) be specifically competitive with dGTP like the corresponding 6-(benzylamino) and 6-(aryhydrizinhydrizin) uracils; and (3) be dependent on the presence of a cytosine-containing template. The results of these sets of experiments, which are not explicitly displayed in tabled form, indicated that DCBG fulfilled all these requirements. First, DCBG was a characteristically potent inhibitor of wild-type (wt) pol III, displaying a K$_i$ of approximately 0.5μM. Second, its inhibition was specifically and competitively reversed by dGTP; high concentrations of dATP, dCTP, or dTTP had no effect on its inhibitory capacity. Third, when the action of DCBG on pol III was assayed, using either poly(dA):oligo(dT) or poly(dC):oligo(dG) as template-primer, it, like H$_2$HPUra (16, 17), inhibited only the latter reaction.
(ii) Action on intact cells: specificity for replicative DNA synthesis. DCBG, like HPURA, the oxidized azo form of H₂-HPURA which is reductively activated in vivo, penetrated intact B. subtilis and inhibited its growth on plates. In liquid medium, microscopic exam showed that 75µM DCBG arrested cell division and effected a segmented morphology typical of HPURA-treated cells which are specifically unable to synthesize DNA (14). Figure 2 summarizes the effect of DCBG on macromolecule synthesis in log-phase B. subtilis, exploiting analysis based on incorporation of labeled adenine into RNA and DNA, and labeled leucine into protein. The clear indication that DCBG, like HPURA (1,14), inhibits only DNA synthesis and displays no significant effect on the synthesis of RNA and protein.

(iii) Utility of DCBG as an agent for selecting and identifying bacterial clones carrying mutations affecting the drug binding site of pol III. Much of the value of the H₂-HPURA inhibitor prototype as a genetic and structural probe of the replication-specific DNA pol III is derived from its 'magic bullet' capacity to clearly and specifically select, on drug-containing nutrient plates, bacterial clones which carry mutationally altered, drug-resistant forms of pol III. B. subtilis azp-12, a mutant displaying both an HPURA-resistant growth phenotype and an H₂-HPURA-resistant pol III (17) exemplifies this strong correspondence of in vitro enzyme 'phenotype' and the growth-response phenotype of its host. As shown in Table I, comparison of the action of DCBG and H₂-HPURA on the wt and azp-12-specific systems indicated that DCBG also can consistently discriminate between wt and mutant forms of pol III, both in vivo and in vitro. Table I compares the potency of H₂-HPURA and DCBG as inhibitors of the wt and azp-12-specific enzymes and their potency as inhibitors of growth of the respective wt and mutant host bacteria. The top row of data, displaying K₅₅, indicated that the azp-12 enzyme, relative to wt enzyme, shows approximately 55-fold resistance to H₂-HPURA and, interestingly, 5-fold higher sensitivity to DCBG. The second row of data display the same trend in vivo; the growth of the azp-12 mutant host, relative to that of the wt host, is 8 times more resistant to HPURA and 8 times more sensitive to DCBG.

Properties of DCBGdGTP action on its pol III target

Given the promising behavior of DCBG described above, we asked whether the base would retain its H₂-HPURA-like action once converted to the dNTP form. The properties we sought in the dNTP form were: (a) a potency at least equivalent to that of the simple base form, DCBG; (b) a mechanism consistent with that of the base, and (c) a structurally 'complete' inhibitor form that will formally occupy the pol III dNTP binding site in the manner of the dNTP it mimics. The results of experiments described in subsections (i)-(iv) below summarize the properties we found.

(i) Potency and discrimination of wt and mutant pol III's. Table II summarizes the effects of the 2'-deoxyribonucleoside of DCBG and its 5'-mono and 5'-triphosphates on the wt and azp-12-specific enzymes. The addition of the deoxyribosyl moiety to DCBG and, in turn, the monophosphorylation of the nucleoside, decreased the potency of the inhibitors for the wt polymerase and, interestingly, had little effect on their affinity for the mutant enzyme. Addition of the β,γ-phosphates to produce the ultimate dNTP analog, DCBGdGTP, reversed the trend to reduction of potency for the wt polymerase, yielding a K₅₅ five times lower than that of the simple base. Similarly, DCBGdGTP was four times more potent as an inhibitor of the azp-12-specific enzyme than of the wt enzyme.

(ii) The template cytosine requirement for DCBGdGTP-induced inhibition is not absolute. Competition experiments, identical to those performed with DCBG, indicated that the inhibitory action of DCBGdGTP, in the presence of activated natural (calf thymus) DNA, was competitively and specifically antagonized by GTP, dATP, dCTP, and dTTP at high concentrations in identical assay conditions had no effect on its potency. Given the latter observation, we exploited the homopolymeric template:primers, poly(dA):oligo(dT) and poly(dC):oligo(dG) to determine whether the action of the dNTP form, like that of the parent base, DCBG, absolutely required template cytosine to effect inhibition. The results of the homopolymer experiments are summarized in Table III. DCBG, like H₂-HPURA (16,17), displayed no significant inhibition in the absence of template cytosine. In contrast,

Figure 2. RNA, protein and DNA synthesis in the presence of DCBG or HPURA. Cultures of B. subtilis NB841 were grown and incorporation of [³H] adenine into RNA and DNA and [³H] leucine into protein was followed as described in the methods section. Inhibitors or control diluent were added at time zero. ○, control; ●, 75µM DCBG; ■, 75µM HPURA.
DCBdGTP, although considerably more potent in the poly(dC)-
driven reaction ($IC_{50} = 7 \mu M$), displayed significant activity
($IC_{50} = 200 \mu M$) in the poly(dA)-directed reaction. Given
the superior structural potential of DCBdGTP relative to that of
DCBG for template independent binding to the dNTP binding
site of pol III, we hypothesize that DCBdGTP-induced inhibition
of the poly(dA)-driven reaction occurred simply by direct
competition for the dNTP binding site with dTTP, the substrate
being polymerized. The results of competition experiments
employing the (dA):dT reaction and the four dNTPs were
consistent with this hypothesis: only dTTP was competitive with
DCBGdT action in this system (results are not shown).

(iii) DCBdGTP-induced inhibition in the presence of template
cytosine apparently involves a sequestration mechanism. To
assess sequestration we exploited the 'scavenging' method
employed to ascertain the induction of DNA-enzyme complex
formation by HJ-HPUra (16,17). The specific experimental
approach exploited poly(dA)-oligo(dT)-driven incorporation of
dTTP and simply asked whether enzyme, in the presence of
inhibitor at a concentration causing minimal inhibition, can be
sequestrated by the addition of a small amount of a dC-containing
template-primer-an amount too small per se to interfere with
dTTP incorporation into the oligo(dT). The results of relevant
experiments comparing DCBG and DCBdGTP are summarized
in Table IV. Both inhibitors were used at 100 \mu M; at this
conzentration DCBG and DCBdGTP, as expected, inhibited
dTMP incorporation by, respectively, 0 and 40% (cf., top row
Table IV). The addition of cytosine-containing activated calf
thymus DNA at low concentration neither supported significant
dTMP incorporation (cf., second row of data) nor drastically
reduced incorporation of dTMP into poly(dA):oligo(dT) (cf.,
third row) in the absence of inhibitor; however, in its presence
the effect was dramatic. With DCBG present, dTMP
incorporation was inhibited by approximately 90%, and in the
presence of DCBdGTP, inhibition of dTMP incorporation was
increased from 43 to approximately 87%. To determine if this
drug-induced, DNA-dependent inhibition was, as expected,
sensitive to the presence of a high concentration of dGTP
(0.5 mM), we examined its effect and, as a control, that of dATP
and dCTP. Only dGTP was effective as a competitor; it reduced
inhibition induced by DCBG from 90 to 25% and that induced
by DCBdGTP from 87 to 30% (cf. bottom row Table IV). In
sum, these results strongly suggested that both DCBG and
DCBdGTP, when provided with template cytosine in an
appropriate structural context, act by a sequestration-specific
mechanism typified by the pyrimidine-based inhibitors.

(iv) DCBdGTP can serve as a substrate for its target enzyme.
Unlike the parent base, DCBdGTP had the potential for
polymerization by pol III. To investigate this potential, we
exploited sequencing gel analysis of primer extension, using the
template-primer depicted in the inset of Figure 3. Experimentally,
we sought to determine if DCBdGTP, like dGTP, could be
utilized to extend the primer by one nucleotidyl residue.
The results are summarized in the gel autoradiogram in the lower part
of Figure 3.

There was, as expected, no primer extension in the absence of
dNTP (lane B)—only partial degradation resulting from the
activity of the enzyme's resident 3'-5' exonuclease (18) which
could be partially inhibited by the addition of 2 mM AMP (lane
C). The 17mer was extended to its full, 29mer length when all
4 dNTPs were present (lane H), and, as expected, was extended
by one residue when dGTP was present as the sole dNTP
substrate (lane D). The presence of DCBdGTP as the sole dNTP,
at concentrations ranging from 1 to 100 times its $K_i$ (lanes E,
F and G) resulted in the formation of a product which migrated
at the position expected for that of the primer extended by one
DCBdGMP residue (note: The $R_i$ of primer extended by one
DCBdGMP residue is, in the conditions of electrophoresis,

Table I. | In vitro vs in vivo effects of DCBG and HJ-HPUra

<table>
<thead>
<tr>
<th>Compound</th>
<th>Form</th>
<th>wt pol III</th>
<th>apz12 pol III</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCBG</td>
<td>Base</td>
<td>0.5 ± .1</td>
<td>0.1 ± .02</td>
</tr>
<tr>
<td>DCBdG</td>
<td>dNMP</td>
<td>0.1 ± .02</td>
<td>0.02 ± .005</td>
</tr>
<tr>
<td>DCBdGMP</td>
<td>dNTP</td>
<td>0.1 ± .02</td>
<td>0.02 ± .005</td>
</tr>
</tbody>
</table>

$K_i$ was determined as described in Materials and Methods. 13h CUU was the
concentration of inhibitor in minimal salts agar plates required to reduce the plating
efficiency of B. subtilis to 50% of control values. 18HPUra, the oxidized drug
form, was used in the plating experiments.

Table II. Potency of the base and higher forms of DCBG

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCBG</td>
<td>0.5 ± .1</td>
</tr>
<tr>
<td>DCBdG</td>
<td>0.1 ± .02</td>
</tr>
<tr>
<td>DCBdGMP</td>
<td>0.02 ± .005</td>
</tr>
</tbody>
</table>

$K_i$ was determined as described in Materials and Methods. Using activated calf
thymus DNA as template-primer.

Table III. Template dependence of inhibitor action

<table>
<thead>
<tr>
<th>IC_{50} (\mu M)</th>
<th>DCBG</th>
<th>DCBdGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\alpha = 32P] dGTP + poly(dC):oligo(dG)</td>
<td>250</td>
<td>7</td>
</tr>
<tr>
<td>[\beta] dTTP + poly(dA):oligo(dT)</td>
<td>&gt;1000</td>
<td>300</td>
</tr>
</tbody>
</table>

$IC_{50}$ is the concentration of inhibitor required to reduce product formation
by 50% of control values. 18 Wild type pol III. 22 Polymer:oligomer assay
conditions are described in the methods section.

Table IV. Demonstration of sequestration

<table>
<thead>
<tr>
<th>Template:Primer</th>
<th>[^{13}H]dTMP Incorporation (pmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>DCBG (100 \mu M)</td>
</tr>
<tr>
<td>poly(dA):oligodT</td>
<td>5.7</td>
</tr>
<tr>
<td>poly(dC):oligodT</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>poly(dA):oligodT + DNA</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>poly(dC):oligodT + DNA</td>
<td>4.6</td>
</tr>
<tr>
<td>poly(dA):oligodT + Activated calf thymus DNA</td>
<td>3.4</td>
</tr>
<tr>
<td>poly(dC):oligodT + Activated calf thymus DNA + dGTP (0.5 mM)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

[^13] Poly(dA):oligodT experiments were performed with wild type enzyme
as described in Materials and Methods. [^22]Activated calf thymus DNA was used
at a concentration of 9 ng/ml (1:45 dilution of concentrations used in normal assay).
effectively (cf., Table I and chemically it is clearly identical to those of H$_2$HPUra. DCBG, provides an excellent basis for the inhibitor series we seek. The properties of the simplest, base form are essentially those of the propenies of the simplest base form. which can specifically access and sense wild-type and mutant forms of increasing complexity. ranging from the simple base. which can be manipulated appropriately to probe the structure and function of the dNTP and inhibitor:enzyme:DNA complex and specific features of the polymerization:inhibitor:DNA complex and specific features of the enzyme's dNTP binding domain on which complex formation depends.

First, one may firmly conclude that the geometry of the base pair formed between the template cytosine and the N$_2$-benzylguanine substituent closely approximates that of the G-C pair formed in the process of dGTP polymerization. Second, one may conclude that the N$_2$-benzyl substituent does not formally occupy sites or space critical to the catalysis of dNTP polymerization; these include: (a) the substituents involved in the binding of the deoxyribosyl ring and its 5'-phosphates; (b) the space about the 3'-OH of the primer terminus, and (c) the path between this OH group and the a phosphate of the incoming dNTP. Finally, as a corollary to the above conclusions, one also may envision more precisely the location of the major site of inhibitor:enzyme interaction-the so-called aryl binding site (6).

Specifically, one may conclude that this site lies on the 'edge' of the dNTP binding domain in space immediately surrounding the unpaired H constituent of the exocyclic 2-amino group of an appropriately base-paired dGTP molecule.

DISCUSSION

The benzylguanine format provides structural versatility without disruption of the characteristic inhibitor mechanism.

The goal of this work was to expand the utility of the H$_2$-HPUra inhibitor class as pol III-specific genetic and structural probes. Specifically, we sought to develop a rationally structured series of inhibitor forms of increasing complexity. ranging from the simple base. which can be manipulated appropriately to probe the structure and function of the dNTP and inhibitor binding site of isolated enzyme.

The N$_2$-benzylguanine format, represented specifically by DCBG, provides an excellent basis for the inhibitor series we seek. The properties of the simplest, base form are essentially identical to those of H$_2$-HPUra. DCBG penetrates cells effectively (cf., Table I), and chemically it is clearly adaptable to 9-β-D-nucleosidation and nucleotidation without loss of its essential H$_2$HPUra 'character', including the ability to distinguish wild-type and mutant forms of pol III (cf., Tables I and II).

Significance of the polymerization of DCBdGTP

Although DCBdGTP is less effective as a pol III substrate than dGTP, it, nevertheless, participates successfully in the catalysis of polymerization (cf., Fig. 3). The polymerization of DCBdGTP indicates that it formally occupies the enzyme's dNTP binding site with a 'fit' approximating that of the dNTP it mimics. This fit, when considered in the context of the characteristic, base (HPUra/DCBG)-like behavior of DCBdGTP, permits several important conclusions regarding the structure of the enzyme:inhibitor:DNA complex and specific features of the polymerization:inhibitor:DNA complex and specific features of the enzyme's dNTP binding domain on which complex formation depends.

Potential of DCBG derivatives as probes for in vitro analysis of the structure of pol III and its dNTP binding site

The four major forms, DCBG, DCBdG, DCDdGMP, and DCBdGTP, provide a rich variety of options for fitting the inhibitor with useful 'reporter' groups, in particular groups capable of forming covalent bonds with the inhibitor:dNTP binding site. Among the positions most amenable to substitution are the carbons of the N$_2$-aryl ring, the 7, 8, and 9 positions of the base and, in the nucleoside and nucleotide forms, the 2', 3', and 5' carbons and the 5'-phosphoryl groups. Application of such derivatives in combination with the manipulation and in vitro expression of the pol III structural gene (19-21) should provide a facile means to map and directly identify the side chains of amino acids comprising the dNTP/inhibitor binding sites. Even in the absence of covalently reactive substituents, DCBG derivatives can be useful as probes of the dNTP binding site of pol III. For example, DCBdGMP or non-polymerizable dNTP forms thereof (i.e. α-thio or β,γ-methylene) have considerable potential as reagents that can be exploited to promote the crystallization, and in turn, the 3-D structural analysis of a pre-polymerization DNA:enzyme:deoxyribonucleotide complex.

The availability of DCBG and structural variants increases the utility of the HPUra inhibitor class as genetic in vivo probes of pol III structure

A rationally designed series of structural variants of a mechanically 'pure' inhibitor class can serve as a powerful set of tools with which to probe the structure of inhibitor-specific...
binding sites within a relevant target protein. The utility of multiple inhibitor forms becomes even greater when their use can be combined with manipulation of the cloned gene encoding the target protein and the selection of gene mutants displaying appropriate resistance/sensitivity phenotypes for the relevant inhibitor variants. *B. subtilis* wt and *B. subtilis* azp-12 and their respective responses to HPUra and DCBG exemplify the potential of such a combined approach. *B. subtilis* azp-12 (serine to alanine in codon 1175 of pol C; cf., refs. 19–21) was obtained by selection on HPUra: azp-12 confers upon pol II approximately 50-fold resistance to HPUra and, as shown in Table I, ≈ 8-fold hypersensitivity to DCBG. The HPUrar/DCBG’ phenotype of bacteria carrying azp-12 permits counterselection of an appropriately mutagenized population of *B. subtilis* azp-12 on DCBG (Butler and Brown, unpublished results), and, thus, provides a very convenient means to isolate and map both intracodon- and extracodon-specific pol C mutations accompanying the development of DCBG resistance. Replication of similar approaches with appropriate combinations of pol mutants and the severa score of inhibitor variants now available should provide a wealth of detail about the location and molecular structure of both the inhibitor and dNTP binding domains of the enzyme’s active site.

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