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Chemical synthesis of DNA oligomers containing cytosine arabinoside

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ABSTRACT

The solid phase phospite triester synthesis of oligodeoxynucleotides containing cytosine arabinoside (araC) is described. A protected araC phosphoramadite was prepared for the introduction of araC residues at 5'termini and internucleotide positions in DNA oligomers. These oligomers were utilized to demonstrate the formation of correct 3'-5' linkages, to test for alkaline lability at the araC site, and to study the stability of duplexes containing araC-G base pairs. For the introduction of araC residues at 3' terminal positions, a protected derivative of araC was coupled to functionalized silica. This material was used to prepare a test oligomer which was characterized enzymatically.

INTRODUCTION

AraC (I, scheme 1) differs from deoxyC solely in the configuration at C_2' of the sugar moiety, where it carries an additional -OH group which is trans to the -OH group at C_3'. It is a potent antileukemic agent as well as a general inhibitor of DNA replication(1-4). Its mechanism of action is controversial (4); however, there is evidence to suggest that its misincorporation into DNA as a fraudulent nucleotide is directly related to its cytotoxic effect(5,6). To aid the investigation into the manner in which sites of araC misincorporation create sites of DNA disfunction, oligonucleotides containing araC at specific locations are required.

We report here the preparation of the necessary reagents for the introduction of araC residues into DNA oligomers by the solid phase phosphite triester synthesis. The utility of these reagents is demonstrated by synthesis and characterization of oligomers containing araC at either terminal or internucleotide linkages. In another study, we use these araC containing oligomers for in vitro study of the molecular consequences of araC misincorporation on DNA chain elongation, replication fidelity, and fragment ligation (7).

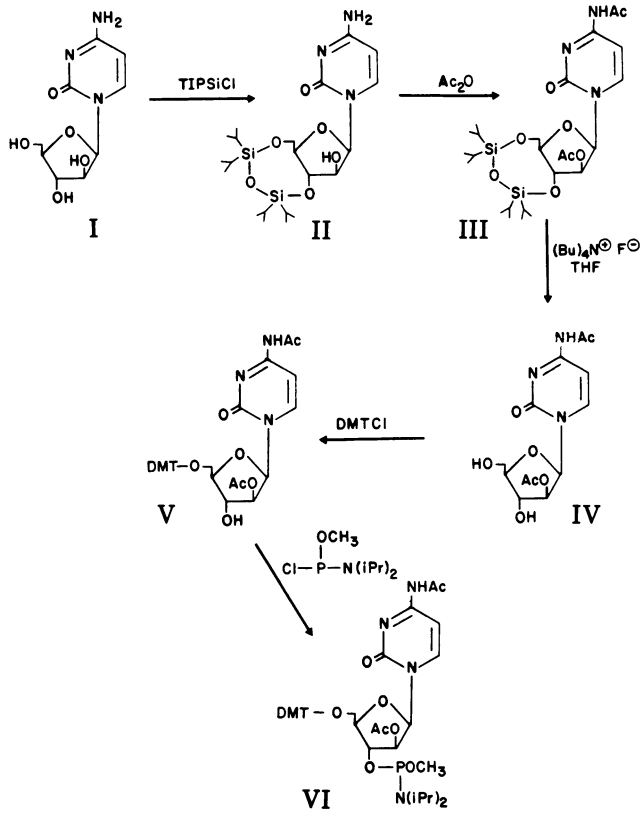
MATERIALS AND METHODS

Chemical Reagents and Enzymes Cytosine arabinoside(I), 1,3-dichloro-1,1,3,3-tetraisopropyl-disiloxane, and tetrabutylammonium fluoride were obtained from the Aldrich Chemical Company. Macroporous silica gel (Vydac TP20) was obtained from The Separations Group. Dimethoxytrityl chloride, phosphoramidites and silica coupled nucleosides for introduction of T,G,C, and A were obtained from ChemGenes Corp. Other chemicals and solvents were reagent grade and obtained from Fisher Scientific. 3' araCMP was a generous gift of Dr. E. Wechter of the Upjohn Laboratories. Snake venom phosphodiesterase, bacterial alkaline phosphatase and phosphodiesterase II were purchased from Sigma Chemical Corporation. T4 polynucleotide kinase was purchased from New England Biolabs.

3',5'-O-(tetraisopropyl disiloxane-1,3-diyl) cytosine arabinoside, II, was prepared as described by Markiewicz et al. (8), yielding highly crystalline material(from ethanol/H_2O), m.p. 223-225°. ^{1}H-NMR (DMSO d_6): 7.44 (H_6, d, 7.5), 7.13, 7.08(NH_2), 6.07($H_{1'}$, d, 6.2), 5.64($H_{5'}$, d, 7.5), 4.26($H_{2'}$), 4.08, 3.6 ($H_{5',5''}$), 3.96($H_{4'}$), 3.96 ($H_{3'}$), 1.07-.99 ($-CH(CH_3)_2$).

N^4, $O^{2'}$-diacetyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl) cytosine arabinoside, III, was prepared from II as previously described(8). Recrystallization from ethanol/water, yielded colorless needles, m.p. 235- 236°. ^{1}H-NMR ($DMSO_6$): 10.96(NH), 8.01(H_6, d, 7.5), 7.25(H_5, d, 7.5), 6.16 ($H_{1'}$, d, 6.4), 5.7($H_{2'}$), 4.3($H_{3'}$), 4.16($H_{4'}$), 4.0 ($H_{5',5''}$), 2.1, 1.83(acetyl groups), 1.07-.94($CH(CH_3)_2$).

N^4,$O^{2'}$-diacetyl cytosine arabinoside, IV, was obtained by dissolution of III (4 grams) in 10ml of 1 M tetrabutylammonium fluoride. The solution was stirred at room temperature for 1/2 hour and evaporated to a viscous oil under reduced pressure. This material was redissolved in chloroform and applied to a 3 x 38 cm silica gel column and eluted first with chloroform (200 ml), 5% ethanol in chloroform (300 ml), and finally 10% ethanol in chloroform (700 ml). Fractions were monitored by silica gel TLC developed with 10% ethanol and chloroform. Fractions containing product (Rf 0.18) were combined and evaporated to dryness under reduced pressure to yield 2 grams of crystalline material which contained only trace amounts of impurites as judged by TLC. This material could be further purified by preperative HPLC on a 9.4 mm by 25 cm C18 column eluted with 6% acetonitrile in water. Material obtained in this manner was homogeneous to TLC and analytical HPLC and readily crystallized from ethanol or methanol to yield colorless needles m.p. 186-188 degrees. ^{1}H-NMR (DMSO d_6): 10.88(NH), 8.2(H_6, d, 7.5),



Scheme 1.
Synthesis of araC phosphoramadite

7.2(H_5, d, 7.5), 6.16($H_{1'}$, d, 4.8), 5.23($H_{2'}$), 4.08($H_{3'}$), 3.87 ($H_{4'}$), 3.6($H_{5'5''}$), 2.08, 1.87(acetyl groups).

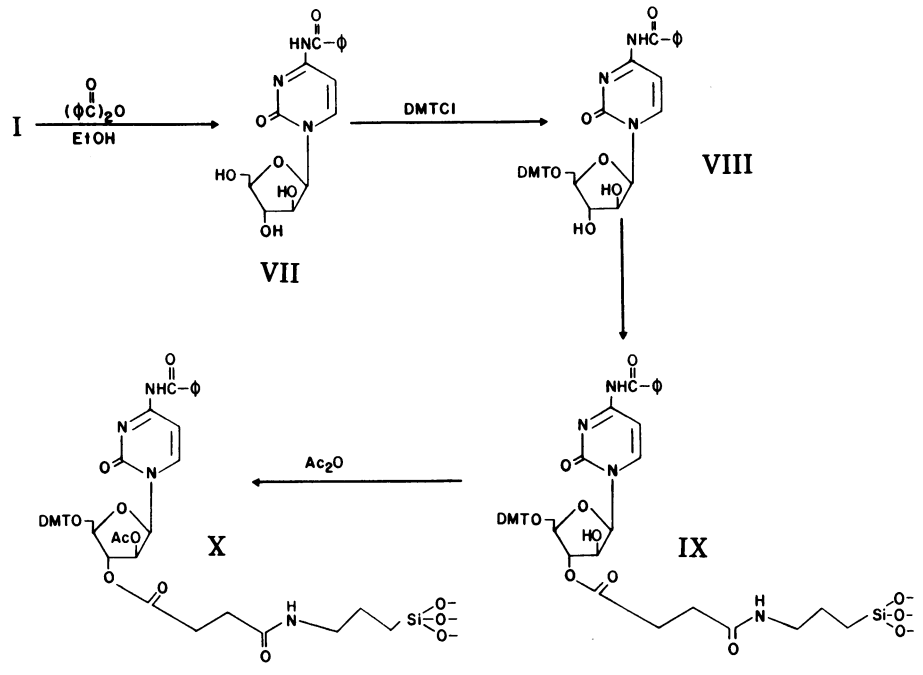
5'-Dimethoxytrityl-N^4,$O^{2'}$-diacetyl-cytosine arabinoside, V, was prepared from IV (1.5g) by a reaction with 1.1 equivalents of dimethoxy- trityl chloride in 25 ml pyridine at room temperature overnight. The reaction mixture was poured into ice cold water, extracted into chloroform, dried over Na_2SO_4, and evaporated under reduced pressure. The residual pyridine was removed by further co-evaporation with toluene. The yellow oily residue was applied to a 3 x 38 cm column of silica gel eluted with chloroform (300 ml) and 5% ethanol in chloroform(600 ml). Fractions were monitored by silica gel TLC developed with 5% ethanol and chloroform. Fractions containing product (Rf 0.35) were combined and evaporated under reduced pressure to yield 1.8 grams of a pale yellow solid which could not be induced to crystallize. This

material was homogenous to TLC and gave a strong positive trityl reaction with phophoric acid. ^{1}H-NMR (DMSO d_6): 10.88(NH), 7.85(H_6, d, 7.5), 7.1 (H_5, d, 7.5), 7.4-6.9(dimethoxytrityl & benzoyl groups), 6.18($H_{1'}$, d, 4.8), 5.22(H_2, H_3, $H_{4'}$), 3.7(-OCH_3) 3.4($H_{5'5''}$), 2.08, 1.7 (acetyl groups).

5'-Dimethoxytrityl-N^4,$O^{2'}$-diacetylcytosine arabinoside-3 -N,N-diisopropyl methyl phosphoramidite, VI, was obtained from the dimethoxytrityl diacetyl compound, V, by reaction with chloromethoxy-N,N-diisopropylaminophosphine in chloroform solution for 45 minutes at room temperature under an argon atmosphere following the method of Beaucage and Caruthers (9). The chloroform was evaporated under reduced pressure and the residue taken up in toluene and reprecipitated by the addition of hexane at -70 degrees. The solid was removed by filtration, dried in vacuo, and stored at -20 degrees under argon.

5'-Dimethoxytrityl-N^4-benzoyl araC, VIII, was prepared from araC in two steps. AraC was converted to its N^4-benzoyl derivative, VII, as described by Lin and Prussoff(10). N^4-benzoyl araC (1.5g) was then reacted with 1.1 equivalents of dimethoxytritylchloride in 25 ml of pyridine at room temperature over night. The reaction mixture was poured into water, extracted into chloroform, washed once with water, dried over Na_2SO_4 and evaporated under reduced pressure. Residual pyridine was removed by coevaporation with toluene. The product was applied to a 3 x 38 cm column of silica gel and eluted with chloroform(300 ml) and 5% ethanol in chloroform (600 ml). Fractions were monitored by silica gel TLC developed with 5% ethanol in chloroform. Those containing the product (Rf 0.41) were combined and evaporated to afford 2.0g of a pale yellow solid. This material was homogenous to TLC and gave a strong positive trityl reaction with phosphoric acid. ^{1}H-NMR (DMSO d_6): 11.2(NH), 8.0 (H_6, d, 7.5), 7.6(H_5, d, 7.5) 7.4-6.9(dimethoxytrityl & benzoyl groups), 6.1($H_{1'}$, d, 4.3), 4.08($H_{2'}$), 4.0($H_{3'}$), 3.9($H_{4'}$), 3.7 (-OCH_3), 3.4($H_{5'5''}$).

Silica coupled 5'-dimethoxytrityl-N^4-benzoyl araC, IX. Hemisuccinoyl aminopropyl silica was prepared from macroporous silica gel as described by Matteucci and Caruthers (11), yielding material containing 191 micromole of -CO_2H per gram. The 5'-dimethoxytrityl- N^4-benzoyl araC (728 mg) was coupled by stirring together 700 mg of the functionalized silica, 40 microliters 2-dimethylamino pyridine, and 1.3g of dicyclohexylcarbodimide in 10 ml of pyridine for 40 hours at room temperature. p-Nitrophenol (1.2g) was added and the mixture stirred an addition 16 hrs. Piperidine (0.50 ml) was then



Scheme 2.
Synthesis of araC functionalized silica

added and stirring continued for 1 hr. Solid material was separated by filtration and washed repeatedly with methanol followed by diethyl ether.

Acetylation of Silica Coupled 5'- dimethoxytrityl-N^4-benzoyl araC. The coupled product, IX, (500 mg) was stirred in 5 ml of 10% acetic anhydride in pyridine at room temperature overnight. Solid material was separated by centrifugation and washed repeatedly with pyridine, tetrahyrodrofuran, and finally with diethyl ether. The solid was further dried in vacuo and stored at -20 degrees under argon. This material, X, contained 31 micromole of dimethoxytrityl nucleoside/gram when tested with perchloric acid (12).

Synthesis of Oligonucleotides Containing araC

The above reagents were utilized in conjuction with T, G, C, and A phosphoramidites and silica-coupled nucleotides in standard solid phase phosphite triester syntheses (9). The oligomers were cleaved from the solid support by agitation overnight in 28% aqueous ammonia prior to removal of the 5'- terminal dimethoxytrityl group. Dimers and trimers were purified as the 5'-dimethoxytrityl derivatives by HLPC on C8 silica eluted with a gradient of

acetonitrile and 0.1 M tetraethylammonium bicarbonate, pH 7. Following HPLC purification, the oligomers were detritylated by exposure to 80% acetic acid for 20 minutes at room temperature, evaporated to dryness under reduced pressure, and repurified by HPLC on C_8 silica as above. Dimers and trimers were also analyzed for purity by reverse phase HPLC using C18 silica eluted with a mixture of acetonitrile and 10 mM potassium phosphate buffer, pH 5.1. The self complimentary 12mers, 5'CGCGAATTaraCGCG 3' and 5'CGCGAATTCGCG 3', used in the alkaline lability and duplex stability studies, were purified by eletrophoresis through 20% denaturing polyacrylamide gels. Oligomers were 5' end labeled using [γ^{32}P]ATP and polynucleotide kinase and analyzed by electrophoresis using 20% denaturing gels(7).

Enzymatic Hydrolysis

Enzymatic hydrolysis of the araC containg dimer and trimer to nucleosides was carried out using snake venom phosphodiesterase followed by bacterial alkaline phosphatase(13). The nucleosides were analyzed by reverse phase HPLC(Varian 5500 series) on C18 silica using a gradient of acetonitrile and 10mM KH_2PO_4 pH 5.1. Enzymatic hydrolysis of the araC containg dimer to 3'-nucleotide monophosphates was carried out using Calf spleen phosphodiesterase II(7). These were then analyzed by ion-exchange HPLC using an NH_2 column and an elution buffer of 10 mM KH_2PO_4, pH 2.85.

Melting temperature studies

The absorbance versus temperature profile for the self complimentary 12mers was measured at 260 nm on a Perkin Elmer Lambda 3B Spectrophotometer equipped with water jacketted cuvette holder. The temperature gradient was controlled using circulating water from a Neslab RTE-9DD circulating bath and Neslab Temperature Programmer.

Approximately 1 O.D. of the self complimentary 12mers were separately annealed in a buffer of 1 mM $NaPO_4$, 0.1 mM EDTA pH 7, 0.3 M NaCL, in order to produce the following duplexes:

5'CGCGAATTCGCG	and	5'CGCG AATTC*GCG
3'GCGCTTAAGCGC		3'GCGC*TTAAG CGC

where C* indicates araC. The annealing was done by heating the solutions to 80°C and slow cooling to 4°C. The UV absorbance at 260 nm was then measured for each sample over a range from 10°C to 77°C. The temperature was increased linearly at a rate of 1.4°/min.

RESULTS AND DISCUSSION

The synthesis of appropriate chemical precursors for the introduction of araC residues into DNA oligomers is complicated by the presence of the additional hydroxyl group in the 2' position. Use of the bifunctional silylating reagent to temporarily protect the 3' and 5' positions avoids this problem by directing an incomming additional protecting group to the 2' position. Moreover, this simplifies the overall synthesis of the precursor since both the 2' and N^4 protecting groups can be introduced in a single step.

Acyl or aroyl groups are unsuitable as protecting groups in ribo nuceotide synthesis because of facile ester interchange between the 2' and 3' positions. We reasoned that acyl protecting groups should be satisfactory in the ara series, since 2' to 3' acyl transfer is sterically impossible and 2' to 5' acyl transfer is sterically unlikely. Although benzoyl groups have generally been used for protection of the exocyclic amino group on the cytosine base, the simplicicity of introducing an acetyl protecting group at this site and at the 2'-OH position in one step was pursued and indeed found to be adequate.

The stringent requirements for regioselectivity are considerably relaxed in the case of the precursor required for introduction of araC at 3' termini. In this case it is of no consequence whether the protected araC nucleoside is coupled to silica via the 2' or 3' hydroxyl group, since both will be freed in one of the final steps in the overall oligomer synthesis. In either case, after coupling to the silica, the additional hydroxyl group is subsequently protected by acetylation.

Since the completion of this work we have become aware of a recently described method for the synthesis of 2'-O-(4-Methoxytetrahydropyranyl)-N-4-anisoylarabinocytidine which has been used to introduce araC at internucleotide positions in DNA oligomers via the hydroxybenzotriazole phosphotriester approach(14). This synthetic method was developed primarily for the synthesis of DNA-RNA hybrid oligomers, which are much more susceptible to alkali induced degradation, and require a gentler synthetic scheme(14).

To test the utility of our reagents for the introduction of araC into DNA oligomers, short test sequences were first prepared. The dimer, dAaraC was prepared from our protected araC coupled silica, and the standard dA phosphoramidite reagent. The product was homogeneous on HPLC. Hydrolysis to nucleosides with snake venom phopshodiesterase and bacterial alkaline phosphatase completely converted this material to a mixture consisting only of

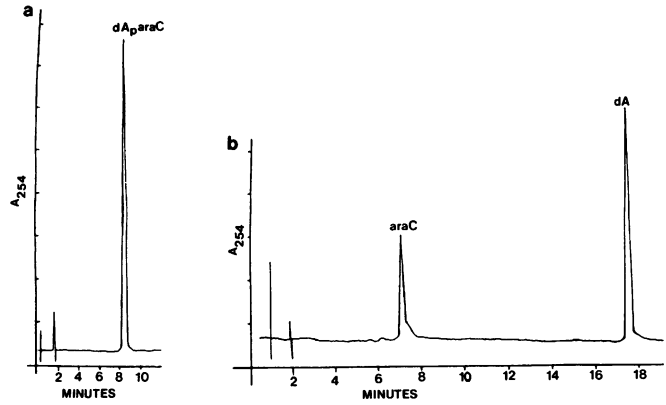


Figure 1.
Reverse phase HPLC elution profile of dAaraC before hydrolysis to nucleosides (a), and after(b).

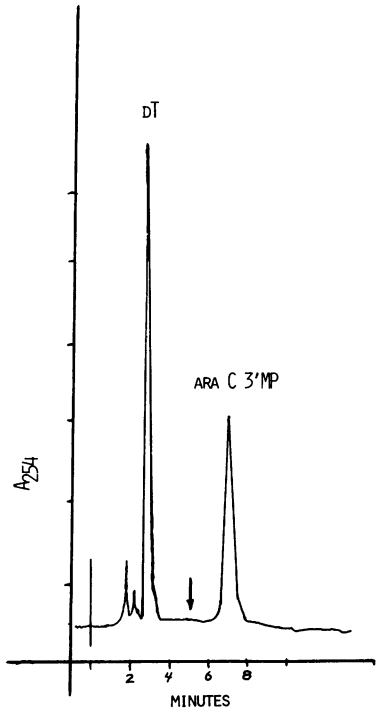


Figure 2.
Ion-exchange HPLC elution profile of araCdT previously digested with Calf spleen phospodiesterase 2. Arrow marks the position of where araC 2'MP would elute.

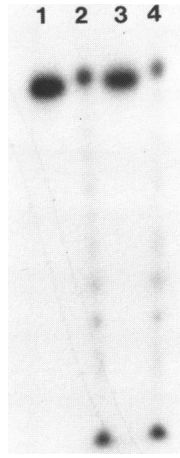


Figure 3.
Autoradiograph showing the extent of base induced cleavage for the control (lanes 1 and 2) and araC 12mer(lanes 3 and 4). Prior to incubation with 1 M NaOH at 45 C for 18 hours(1,3) and after(2,4).

araC and dA in equimolar amounts as determined by reverse phase HPLC (figure 1).

The araC phosphoramidite, VI, was tested by preparation of the dimer, araCdT. This yielded a product which was homogeneous by reverse phase and ion-exchange HPLC. This material was converted to an equimolar mixture of araC and dT upon hydrolysis to nucleosides as above (data not shown). The araCdT dimer was also subjected to hydrolysis with spleen phosphodiesterase. Wechter has shown that araC 2', 5' dinucucleotides are hydrolyzed slowly by this enzyme (13). The araCdT dimer was completely converted to dT and araC 3'-monophosphate as determined by ion-exchange HPLC (figure 2). No araC- 2'-monophosphate was formed(arrow), nor was there any detectable residual dimer. These results demonstrate that our reagent affords only the desired araC 3', 5' linkages.

A trimer containing araC in an internucleotide position, dAaraCdT was also prepared. This material, which was homogeneous on HPLC, afforded only dA, araC and dT in equimolar quantities upon hydrolysis to nucleosides followed by reverse phase HPLC(data not shown).

Assay for alkaline lability of araC containing oligomers

It has been proposed that araC at internucleotide linkages in DNA is akali labile and results in strand scission(15). Base induced chain cleavage via formation of a 2', 3' cyclic phosphodiester as occurs in polyribonucleotides,

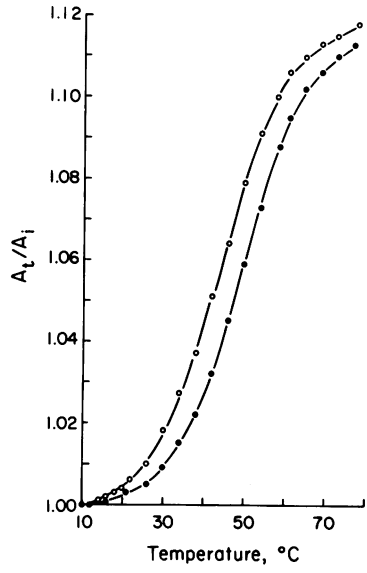


Figure 4.
Normalized melting curves for araC (-O-) and control (-●-) selfcomplementary duplexes.

is sterically precluded in the ara series, however an analogous 2',5' cyclic intermediate is possible. Another mechanism which has been proposed(15,16) involves the formation of a 6,2'-anhydro derivative which could lead to cleavage of the cytosine ring, followed by alkline induced strand breakage at the resultant apyrimidic site.

To test for an increase in alkaline lability at the araC site, we 5'end labeled the araC and control 12mers and then incubated them separately in 1 M NaOH at 45 C for 18 hours. Analysis of oligomers by denaturing polyacrylamide gel electrophoresis followed by autoradiographic visualization showed that both oligomers under went similar amounts of base induced chain cleavage (figure 3). Any increased lability at the araC site would have been easily detected by this method. That none was observed suggests that araC misincorporation into DNA does not result in chain scission under alkaline conditions. Deamination of araC to araU may occur, however, and would not have been detected in these experiments.

Melting temperature studies

Figure 4 shows the normalized melting profiles for the two duplexes. The T_m for the control duplex was 52 C, and the T_m for the araC containing duplex was 48 C. This 4 C difference in transition midpoint indicates only

a slight decrease in duplex stability resultant from the replacement of two deoxyC-G base pairs by araC-G base pairs. By comparison, it has been shown that two G-T mispairs introduced into the above 12mer results in a 20 C drop in transition midpoint(17).

That the araC-G base pairs do not significantly disturb DNA duplex stability is not suprising. The cytosine base is normal and crystallographic(18) and NMR(19) studies with the free nucleoside indicate that the arabinose sugar does not significantly alter the conformational possibilities of the ara nucleoside relative to its deoxyribose counterpart.

Our biochemical studies(7) demostrate that polymerases with associated exonuclease activity, carrying out DNA synthesis on a primer/template substrate which contains an araC residue in the template overhang, are partially arrested after inserting G opposite araC. However this does not appear to be on the basis of thermodynamic instability of the base pair. The same experiments with polymerases which lack an associated exonuclease showed no synthesis arrest opposite araC. These results suggest that synthesis arrest is due to an effect of the araC residue on the associated exonuclease, rather than the instability of the araC-G base pair, as the latter would result in synthesis arrest independent of which polymerase was employed.

The most dramatic effect on DNA synthesis was observed when araC was situated at the 3' terminus of a DNA primer. All polymerases tested utilized araC terminated primer/template substrates extremely inefficiently, with greater than 100 fold reductions in the rate of next nucleotide addition compared with controls. This same effect on primer extension was also found using terminal deoxynucleotide transferase incubated with the single strand primer alone, as this enzyme does not require a template or paired terminus to catalyze next nucleotide addition. This general effect of the araC nucleotide on primer extension, by polymerases requiring and not requiring a template(or paired terminus), suggests that it is very unlikely that this inhibition is due to the slight instability we observed for the araC-G base pair relative to its natural counterpart.

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