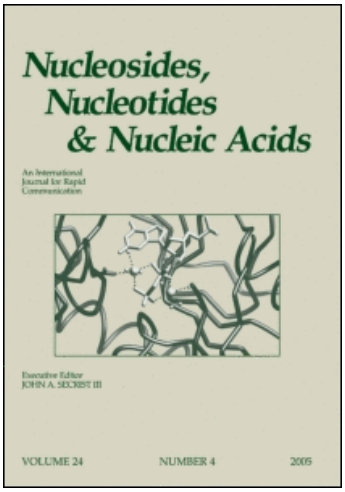


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Synthesis of 4'-Thio-β-D-arabinofuranosylcytosine (4'-Thio-ara-C) and Comparison of Its Anticancer Activity with That of Ara-C

Kamal N. Tiwari[a]; Anita T. Shortnacy-fowler[a]; Loredana Cappellacci[a]; William B. Parker[a]; William R. Waud[a]; John A. Montgomery[a]; John A. Secrist III[a]
[a] Southern Research Institute, Birmingham, Alabama

To cite this Article Tiwari, Kamal N. , Shortnacy-fowler, Anita T. , Cappellacci, Loredana , Parker, William B. , Waud, William R. , Montgomery, John A. and Secrist III, John A.(2000) 'Synthesis of 4'-Thio-β-D-arabinofuranosylcytosine (4'-Thio-ara-C) and Comparison of Its Anticancer Activity with That of Ara-C', Nucleosides, Nucleotides and Nucleic Acids, 19: 1, 329 — 340
To link to this Article: DOI: 10.1080/15257770008033012
URL: http://dx.doi.org/10.1080/15257770008033012

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(1&2), 329–340 (2000)

SYNTHESIS OF 4'-THIO-β-D-ARABINOFURANOSYLCYTOSINE (4'-THIO-ARA-C) AND COMPARISON OF ITS ANTICANCER ACTIVITY WITH THAT OF ARA-C

Kamal N. Tiwari, Anita T. Shortnacy-Fowler, Loredana Cappellacci, William B. Parker, William R. Waud, John A. Montgomery, and John A. Secrist III*
Southern Research Institute, P. O. Box 55305, Birmingham, Alabama 35255-5305

Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT: 4'-thio-β-D-arabinofuranosylcytosine was synthesized by a facile route in high yields. It was evaluated for antitumor activity against a panel of human tumors, both *in vitro* and *in vivo*.

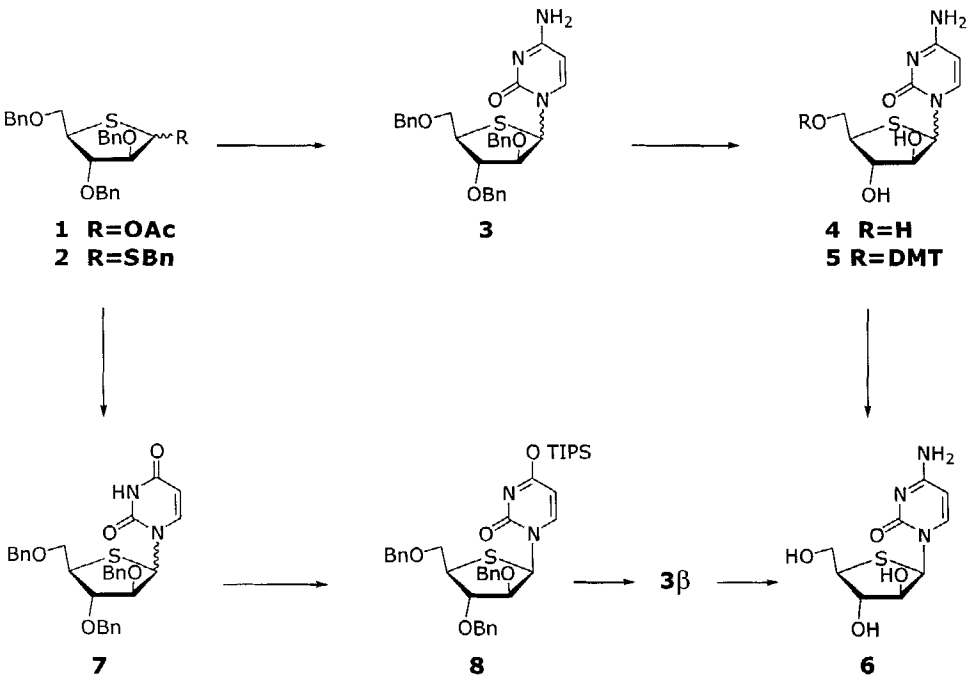
INTRODUCTION

A number of 2'-deoxyribonucleoside analogs have shown promise as anticancer agents.[1] The activity of 1-β-D-arabinofuranosylcytosine (ara-C, cytarabine), which is particularly useful against acute myelogenous leukemia, was the first to be observed in animals and in man. Ara-C is metabolized to its triphosphate, which inhibits DNA polymerase and, perhaps more importantly, is incorporated into DNA, resulting in the slowing of chain elongation as well as alterations in DNA repair.[1]

As a part of our ongoing program on the synthesis and biologic evaluation of 4'-thionucleosides,[2-7] we undertook the synthesis of 4'-thio-ara-C (**6**), first reported by Whistler, *et al.*[8,9] utilizing a lengthy route. Because of this long and arduous synthesis, only a very small amount of this nucleoside was prepared, enough to obtain cytoxicity in a single cell line (KB, EC_{50} = 0.42 μM) and no further biological results have been reported. We have developed a facile synthetic procedure that can be used to make gram amounts of 4'-thio-ara-C. With adequate quantities of the nucleoside, it was evaluated *in vitro* and *in vivo* against a panel of human cancers, and its biochemical pharmacology

was compared with that of ara-C in an effort to explain the differences observed in the antitumor activity of the two compounds. The details of the synthesis and initial antitumor activity are reported in this manuscript.

Scheme 1



CHEMISTRY

We have already reported the details of a convenient synthesis of a 4-thioarabinofuranose intermediate and its conversion to a series of purine nucleoside analogs.[7] Coupling of carbohydrate derivative **1** with silylated cytosine *in situ* using TMSOTf gave a 2:1 α,β mixture of the tri-*O*-benzyl cytosine nucleoside **3** in 77.5% yield. Separation of the anomers was unsatisfactory in several different solvent systems, so the anomeric mixture was deblocked using BCl_3 to afford compound **4** as an α,β mixture in 85% yield. Selective crystallization of the α,β mixture of **4** in water gave a poor recovery of pure β anomer **6** (13% overall, 40% based on 2:1 α/β mixture of **4**). In order to improve the separation of the anomeric mixture of **4**, it was converted to 5'-*O*-dimethoxytrityl derivative [**5**], which was separated on a silica gel column to obtain β-**5**

(90% yield based on the 2:1 α,β mixture of **4**). β-**5** was deblocked using trifluoroacetic acid in $CHCl_3$ in 85% yield after crystallization from water. In order to improve the β,α ratio, sugar **2** was coupled with silylated uracil using NBS and molecular sieves in acetonitrile. This coupling gave a much better β,α ratio (of **7**) (1.15:1 by HPLC), however coupling of the sugar **2** with cytosine as base using the same procedure gave a very poor yield of the cytosine nucleosides. Another advantage of the uracil coupling was the ease of separation of anomers (**7**) compared to the cytosine nucleosides (**3**), the separation of which was very difficult. The uracil nucleoside (β-**7**) was converted to cytosine nucleoside (β-**3**) via the 2,4,6-triisopropylbenzenesulfonate derivative (β-**8**) using the standard procedure. The cytosine nucleoside (β-**3**) was deblocked as described above using BCl_3 to give 4'-thio-ara-C (**6**), which upon crystallization from water had the same analytical properties as those obtained by the direct coupling of cytosine as reported above and in the literature.[9]

BIOLOGICAL DATA

The cytotoxicity of 4'-thio-ara-C was determined against eight different human cell lines,[10-12] identified in Table 1. For *in vivo* evaluation of the sensitivity of human tumors (Tables 2 and 3)[13] to 4'-thio-ara-C, NCr-nu athymic mice were implanted subcutaneously with tumor fragments. The day of implantation was designated Day 0. In each experiment, 4'-thio-ara-C was tested at several dosage levels. Treatment began when the median tumor size was approximately 100 mg. Antitumor activity was assessed on the basis of delay in tumor growth (T-C), which is the difference in the median of times poststaging for tumors of the treated (T) and control (C) groups to double in mass two or three times (depending on the growth rate of the tumor). Drug deaths and any animal that died whose tumor failed to grow to the evaluation size were excluded.

4'-Thio-ara-C was cytotoxic to most human tumor cell lines at micromolar concentrations, and ara-C was in general more cytotoxic. On the other hand, when 4'-thio-ara-C was administered intraperitoneally for nine successive days either as a single daily injection or three injections separated by 4h intervals, it exhibited excellent activity against HCT-116 colon and CAKI-1 renal tumors. 4'-Thio-ara-C was curative against HCT-116 colon tumor and effected complete regression of CAKI-1 renal tumors. In

Table 1
Cytotoxicity of 4'-Thio-ara-C and Ara-C in Eight Human Tumor Cell Lines

Cell Line	IC_{50}[a] (μM)	
	4'-Thio-Ara-C	Ara-C
CAKI-1 renal carcinoma	2.2	2.5
SNB-7 CNS tumor	1.8	1.1
NCI-H23 nonsmall cell lung adenocarcinoma	3.6	0.59
DLD-1 colon adenocarcinoma	39	>5
SK-MEL-28 melanoma	17	1.7
LOX IMVI amelanotic melanoma	13	2.0
ZR-75-1 breast carcinoma	2.5	0.80
PC-3 prostate adenocarcinoma	5.8	>5

[a] IC_{50} is defined as the concentration of drug inhibiting the growth of cells after 72 h to one-half that observed in the absence of drug. The values listed are the mean of three or more determinations.

contrast, araC was minimally active at best against these tumors (Table 2 & 3) at the maximum tolerated dose.

The biochemical pharmacology of 4'-thio-ara-C was compared to that of ara-C in CCRF-CEM cells in culture in an attempt to explain the differences in the utilization of these two agents by pyrimidine biosynthetic enzymes.[14] This study showed distinct differences in the metabolism of ara-C and 4'-thio-ara-C, although the mechanism of cell killing appears to be the same: phosphorylation to their respective triphosphates, which inhibit DNA replication, presumably after incorporation. A striking difference in the two is the retention of their cytotoxic metabolites, the triphosphates of the nucleosides, over a period of 72 hours in cells (Figure 1).[14] This difference could be one reason for the contrasting activities of the two drugs against solid tumors *in vivo*.

EXPERIMENTAL SECTION

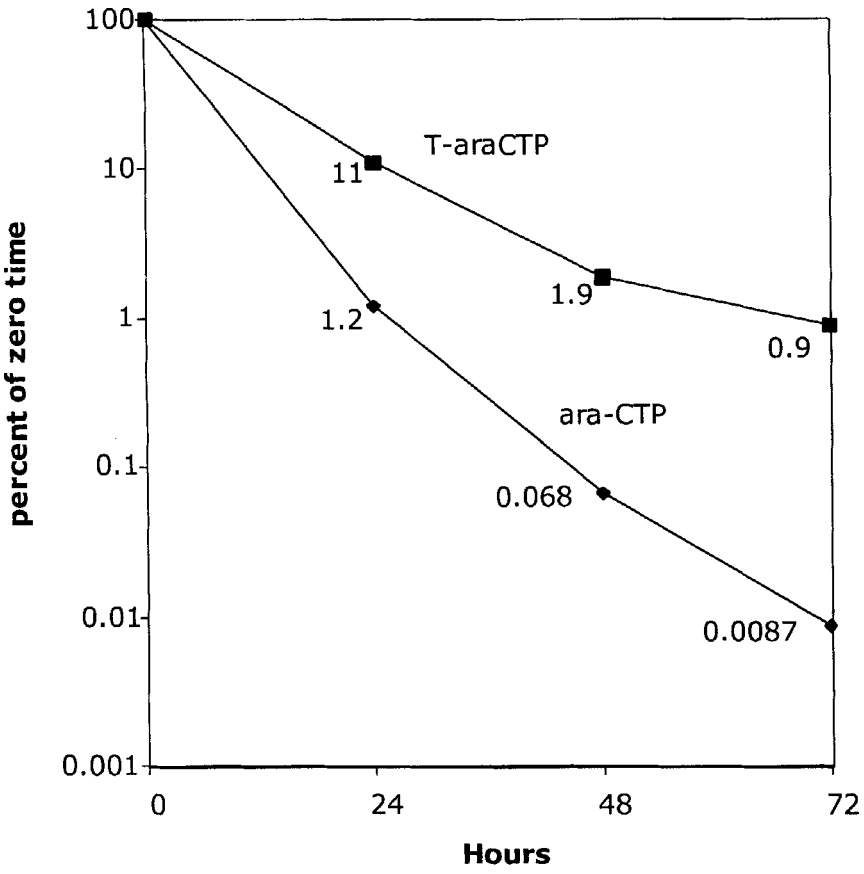
Melting points were determined on a Mel-Temp apparatus and are uncorrected. ^1H NMR spectra were recorded on a Nicolet NT-300 NB spectrometer operating at 300.635 MHz (^1H). Chemical shifts are expressed in parts per million downfield from

Table 2. Response of SC HCT-116 Colon Tumor to Treatment

Treatment					Tumor Regression				
Agent	Dosage (mg/kg/dose)	Rt	Schedule	Non-Specific Deaths/ Total	Number of Partial	Number of Complete	Tumor Free Surv/ Total	Days to 2 Doubling	Days Delay (T-C)
Control		ip	q4h x 3 days 6-14				0/12	8.9	
1-(4-Thio-β-D-arabinofuranosyl)cytosine	20	ip	q4h x 3 days 6-14	0/6	0	6	6/6	>52.0	>43.1
	13.3	ip	q4h x 3 days 6-14	0/6	0	6	5/6	>52.0	>43.1
Ara-C	13.3	ip	q4h x 3 days 6-14	0/6	0	0	0/6	12.2	3.3
	9	ip	q4h x 3 days 6-14	0/6	0	0	0/6	10.9	2.0

Table 3. Response of SC CAKI-1 Renal Tumor to Treatment

Treatment					Tumor Regression				
Agent	Dosage (mg/kg/dose)	Rt	Schedule	Non-Specific Deaths/ Total	Number of Partial	Number of Complete	Tumor Free Surv/ Total	Days to 2 Doubling	Days Delay (T-C)
Control		ip	q4h x 3 days 7-15				0/12	9.5	
1-(4-Thio-β-D-arabinofuranosyl)cytosine	16	ip	q4h x 3 days 7-15	1/6	0	5	0/6	59.8	50.3
	8	ip	q4h x 3 days 7-15	0/6	1	4	0/6	50.7	41.2
Ara-C	20	ip	q4h x 3 days 7-15	5/6	0	0	0/6	15.7	6.2 (Toxic)
	13.3	ip	q4h x 3 days 7-15	0/6	0	0	0/6	20.5	11.0



After incubation of CEM cells for 1 hour with either 5 nM [5-^{3}H]araC or 200 nM[5-^{3}H]T-araC, the cells were collected, washed with fresh medium, and resuspended in fresh medium that did not contain radiolabeled nucleosides. Samples were collected at various times after the cells were resuspended in fresh medium, and the amount of radioactivity in the 5'-triphosphate peak was determined using SAX HPLC. In this experiment there was 0.639 pmoles araCTP/10^6 cells and 0.246 pmoles of %-araCTP after the 1 hour incubation with radiolabeled compound.

Figure 1. Retention of araCTP and T-araCTP in CEM cells

tetramethylsilane. The NOE experiments were conducted on a degassed solution of DMSO-d_6. To minimize the effects of magnetic perturbations with the sample non-spinning, eight FID's were acquired with the decoupler set to a desired frequency and eight FID's were recorded with the decoupler off resonance. The process was repeated until 800 FID's had been acquired. UV absorption spectra were determined with a

Perkin-Elmer λ 9 spectrometer by dissolving each compound in methanol or water and diluting 10-fold with 0.1 N HCl, pH 7 buffer, or 0.1 N NaOH. Numbers in parentheses are extinction coefficients (x 10^{-3}), sh = shoulder. Microanalyses were performed by Atlantic Microlab, Inc. (Atlanta, GA) or the Spectroscopic and Analytical Laboratory of Southern Research Institute. Analytical results indicated by elemental symbols were within ± 0.4% of the theoretical values. Mass spectra were recorded on a Varian/MAT 311A double-focusing mass spectrometer in the fast atom bombardment (FAB) mode. HPLC analyses were carried out on a Hewlett-Packard HP1084B liquid chromatograph with a Waters Associates μBondapak C_{18} column (3.9 mm x 30 cm) and UV monitoring (254 nm). Flash chromatographic separations were carried out by using 230-400 mesh silica gel from E. Merck. TLC was carried out on Analtech precoated (250 μm) silica gel (GF) plates. Ion exchange chromatography was done on Dowex 50WX8-200 ion exchange resin.

Cell Culture Cytotoxicity Data. All cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum, sodium bicarbonate, and 2 mM L-glutamine. For *in vitro* evaluation of the sensitivity of these cell lines to 4'-thio-ara-C and ara-C, cells were plated in 96-well microtiter plates and then were exposed continuously to various concentrations of the compounds for 72 h at 37°C. Cell viability was measured using either the neutral red assay (absorbance read at 550 nm) or the sulforhodamine B assay (absorbance read at 570 nm). The background absorbance mean was subtracted from the data followed by conversion to percent of control. The drug concentrations producing survival just above and below the 50% level were used in a linear regression analysis to calculate the IC_{50}.

1-(2,3,5-Tri-*O*-benzyl-4-thio-α,β-D-arabinofuranosyl)cytosine (3). To a suspension of 1-*O*-acetyl 2,3,5-tri-*O*-benzyl-4-thio-D-arabinofuranose (**1**, 478 mg, 1 mmol) and cytosine (111.0 mg, 1 mmol) in anhydrous acetonitrile (25 mmol) were added consecutively hexamethyldisilazane (HMDS, 162 mg, 1 mmol) and chlorotrimethylsilane (TMSCl, 434 mg, 4 mmol). The mixture was stirred at room temperature for 0.5 h then cooled to -78 °C. Trimethylsilyl trifluoromethanesulfonate (267 mg, 1.2 mmol) was added, and the

resulting solution was stirred at -78 °C for another 2.5 h, after which time the reaction was essentially complete. The mixture was warmed to room temperature, concentrated to a small volume (5 mL), diluted with methylene chloride (50 mL) and then washed with water (20 mL) followed by saturated sodium bicarbonate and water. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was purified by chromatography over silica gel (50 g. elution with $CHCl_3$/MeOH 98:2) to afford **3** (412 mg, 77.5%) as a colorless syrup TLC (95:5 $CHCl_3$/MeOH) R_f 0.65; as 2:1 α,β mixture (HPLC); MS z/e 536 $(M+Li)^+$. ^1H NMR ($CDCl_3$) 8.22 (d, 1H, H-6_β, J = 7.6); 8.05(d, 1H, H-6_α, $J_{5,6}$ = 7.5 Hz); 7.38-7.09 (m, 30H, aromatic H's); 6.65 (d, 1H, H-1'$_\beta$, $J_{1',2'}$ = 5.7 Hz); 6.36 (d, 1H, H-1'$_\alpha$, $J_{1',2'}$ = 1.2 Hz); 5.44 (d, 1H, H-5_α); 5.26 (d, 1H, H-5_β); 4.97-4.33 (overlapping multiplets, 12H, $C_6H_5CH_2$), 4.26 (dd, 1H H-2'$_\beta$, $J_{2',3'}$ = 6.8 Hz); 4.22 (m, 1H, H-3'$_\alpha$, $J_{3',4'}$ = 1 Hz); 4.16 (dd, 1H, H-3'$_\beta$, $J_{3',4'}$ = 6.4 Hz); 4.13 (m, 1H, H-2'$_\alpha$, $J_{2',3'}$ = 1.7 Hz); 3.91 (m, 1H, H-4'$_\alpha$); 3.78 (m, 1H, H-5'$_\alpha$); 3.73-3.63 (m, 2H, H-5'$_\beta$); 3.55 (m, 1H, H-5'$_\alpha$); 3.46 (m, 1H, H-4'$_\beta$).

1-(4-Thio-α,β-D-arabinofuranosyl) cytosine (4). To a 1 M solution of boron trichloride in dry dichloromethane (7 mL, 7 mmol) cooled to -78 °C was added dropwise over a period of 30 min a solution of compound **3** (265 mg, 0.5 mmol) in dry dichloromethane (10 mL). Stirring was continued overnight at -20 °C. The solvent was removed in vacuo and the residue was coevaporated with dichloromethane (4 x 20 mL). The residue was neutralized with saturated $NaHCO_3$ (25 mL) and the solution was extracted with chloroform (15 mL). The aqueous layer was applied to a cation exchange (H^+) column that was eluted with water to remove salts, further elution with 1N NH_4OH provided the desired compound **4** (110 mg, 85%), as 2:1 α,β mixture (HPLC), MS z/e 260$(M+H)^+$. ^1H NMR (Me_2SO-d_6) 7.94 (d, 1H, H-6_β, $J_{5,6}$ = 7.5 Hz); 7.90 (d, 1H, H-6_α, $J_{5,6}$ = 7.5 Hz); 7.17-7.03 (overlapping bs, 2H, NH's); 6.33 (d, 1H, H-1'$_\beta$, $J_{1',2'}$ = 4.6 Hz); 5.86 (d, 1H, H-1'$_\alpha$, $J_{1',2'}$ = 7.3 Hz); 5.77 (d, 1H, H-5); 5.70 (d, 1H, H-5); 5.61 and 5.59 (overlapping doublets, 2H, 2'-OH$_\beta$, $J_{2',2'\text{-OH}}$ = 5.1 Hz, 2'-OH , $J_{2',2'\text{-OH}}$ = 5.9 Hz); 5.47 (d, 1H, 3'-OH$_\alpha$, $J_{3',3'\text{-OH}}$ = 5.1 Hz); 5.38 (d, 1H, 3'-OH$_\beta$, J 3',3'-OH = 4.2 Hz); 5.08 (t, 1H, 5'-OH$_\beta$, $J_{5',5'\text{-OH}}$ = 5.4 Hz); 4.90 (t, 1H, 5'-OH$_\alpha$, $J_{5',5'\text{-OH}}$ = 5.2 Hz); 4.00-3.93 (m, 3H, H-2'$_\alpha$, H-2'$_\beta$, H-3'$_\beta$); 3.86-3.76 (m, 2H,

H-5'$_\alpha$ and H-5'$_\beta$); 3.67-3.55 (m, 2H, H-5'$_\beta$ + H-3'$_\alpha$); 3.49-3.33 (m, 2H, H-4'$_\alpha$, H-5'$_\alpha$); 3.17 (m, 1H, H-4'$_\beta$).

1-(5-*O*-dimethoxytrityl-4-thio-β-D-arabinofuranosyl) cytosine (5). To a solution of compound **4** (100 mg, 0.38 mmol) in dry pyridine (10 mL) was added 4,4'-dimethoxytrityl chloride (135 mg, 0.6 mmol). The solution was stirred for 2 h at room temperature, then evaporated to dryness. The crude residue was dissolved in ethyl acetate (20 mL) and washed with water and evaporated to dryness to afford a solid which was purified on silica gel column ($CHCl_3$/MeOH, 98:2) to obtain pure compound **5** (64 mg, 30% overall, 90% based on a 2:1 α/β mixture of **4**). MS z/e 568.3 $(M+Li)^+$. ^{1}H NMR (Me_2SO-d_6) 7.77 (d, 1H, H-6, $J_{5,6}$ = 7.5 Hz); 7.42-7.23 (m, 9H, aromatic H's); 7.17 (bs, 1, NH); 7.05 (bs, 1, NH); 6.91-6.88 (m, 4H, aromatic H's); 6.36 (d, 1H, H-1', $J_{1',2'}$ = 4.8 Hz); 5.65 (d, 1H, H-5); 5.76 (d, 1H, 2'-OH, $J_{2',2'\text{-OH}}$ = 4.6 Hz); 5.43 (d, 1H, 3'-OH, $J_{3',3'\text{-OH}}$ = 3.3 Hz); 3.98-3.91 (m, 2H, H-2', H-3'); 3.75 (s, 6, OCH_3); 3.39-3.25 (m, 3H, H-4', H-5').

1-(4-Thio-β-D-arabinofuranosyl)cytosine (4'-thio-ara-C) (6). Compound **5** (90 mg, 0.16 mmol) was treated with trifluoroacetic acid (22 mg) in chloroform (5 mL) at room temperature for 10 min, then neutralized with aq. $NaHCO_3$. The aqueous layer was applied to a cation exchange column which was eluted first with water to remove salt and finally with 1N NH_4OH to afford compound **6** (35 mg, 85%); mp 218-220 °C (lit[9] 221-222 °C); MS z/e 260$(M+H)^+$. ^{1}H NMR (Me_2SO-d_6) 7.94 (d, 1H, H-6, $J_{5,6}$ = 7.5 Hz); 7.12 (bs, 1H, NH); 7.04 (bs, 1H, NH); 6.33 (d, 1H, H-1', $J_{1',2'}$ = 4.6 Hz); 5.70 (d, 1H, H-5); 5.61 (bd, 1H, 2'-OH, $J_{2',2'\text{-OH}}$ = 3.1 Hz); 5.38 (bd, 1H, 3'-OH, $J_{3',3'\text{-OH}}$ = 3.5 Hz); 5.08 (bt, 1H, 5'-OH, $J_{5',5'\text{-OH}}$ = 4.9 Hz); 4.00-3.93 (m, 2H, H-2', H-3'); 3.78 (m, 1H, H-5'$_a$); 3.61 (m, 1, H-5'$_b$); 3.16 (m, 1, H-4'). Anal. calcd for $C_9H_{13}N_3O_4S$: C, 41.69; H, 5.05; N, 16.21. Found: C, 41.62; H, 5.11; N, 16.23.

1-(2,3,5-Tri-*O*-benzyl-4-thio-β-D-arabinofuranosyl)uracil (7). To a suspension of uracil (1.39 g, 12.4 mmol) in dry acetonitrile (70 mL) was added BSA (5.25 g, 25.8 mmol), and the mixture was refluxed for 3 h. The solution was cooled to room temperature, and a solution of thiosugar **2** (2.5 g, 4.60 mmol) in acetonitrile (20 mL) and

powdered molecular sieves (4A 2.22g) were added. The suspension was stirred at room temperature for 10 min, and then NBS (1.09 g, 6.1 mmol) was added. The resulting suspension was stirred at 50 to 55 °C for 18 h, after which time TLC ($CHCl_3$/EtOAc, 9:1) indicated the absence of sugar **2**. The suspension was filtered, and the solids were washed with EtOAc. The filtrate and washings were evaporated to dryness to afford crude compound **7**. HPLC and ^1H NMR showed a mixture of α:β, 1:1.15. This material was applied to a column of silica gel ($CHCl_3$/EtOAc 95:5), and the faster moving β anomer **7** was isolated as amorphous solid. (890mg, 36.5%); MS 536.8 (M+Li). ^1H NMR ($CDCl_3$) 8.20 (bd, 2H, H-6, H-3), 7.30 (m, 15H, aromatic CH's of benzyls), 6.32 (d, 1H, H-1', J=2Hz), 5.20 (dd, 2H, H-5, J=3 and 8Hz), 4.55 (m, 6H, CH2's of benzyls), 4.20 (m, 2H, H-2' and H-3'), 3.70 (m, 2H, H-5'a, H-5'b), 3.45 (m, 1H, H-4').

1-(2,3,5-Tri-*O*-benzyl-4-thio-β-D-arabinofuranosyl) cytosine (3β). To a solution of **7** (2.5 g, 4.71 mmol) in dry acetonitrile (70 mL) was added 4-DMAP (122 mg, 1.0 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (4.2 g, 13.9 mmol). This solution was cooled to 5 °C and triethylamine (1.9 mL) was added dropwise over 20 min. The stirring was continued at 5 °C for 30 min and then at room temperature overnight. TLC (cyclohexane/EtOAc, 85:15) showed complete consumption of starting material. The reaction mixture was evaporated, dissolved in EtOAc, and filtered through silica gel that was then washed with more EtOAc. The combined filtrate and wash was evaporated to give a yellow-colored crude product (**8,** 3 g) that was not very stable and was used directly. Crude product **8** was dissolved in acetonitrile (50 mL) and ammonium hydroxide (50 mL) was added. The solution was stirred overnight at room temperature, and TLC ($CHCl_3$/MeOH, 95:5) showed complete consumption of starting material. After evaporation of solvent, the crude residue was purified on a silica gel column ($CHCl_3$/MeOH, 97:3) to give compound **3β** (125g, 50%); MS z/e 536 $(M+Li)^+$. ^1H NMR ($CDCl_3$) 8.22 (d, 1H, H-6_β, J = 7.6); 7.38-7.09 (m, 15H, aromatic H's); 6.65 (d, 1H, H-1', $J_{1',2'}$ = 5.7 Hz); 5.44 (d, 1H, H-5); 4.97-4.33 (overlapping multiplets, 6H, $C_6H_5CH_2$), 4.26 (dd, 1H H-2', $J_{2',3'}$ = 6.8 Hz); 4.16 (dd, 1H, H-3', $J_{3',4'}$ = 6.4 Hz); 3.73-3.63 (m, 2H, H-5'); 3.46 (m, 1H, H-4').

Acknowledgment. This investigation was supported by NIH Grant No. CA34200. We gratefully acknowledge the data received from the following laboratories at Southern Research Institute: Ms. Sheila Campbell (HPLC analyses), Mr. Jerry Frye (large-scale synthesis), and the Molecular Spectroscopy Laboratory (spectral data).

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