Synthesis and Biological Activity of 2'-Deoxy-4'-thio Pyrimidine Nucleosides[1]

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2'-Deoxy-4'-thiocytidine (7β), 2'-deoxy-4'-thiouridine (9), and 4'-thiothymidine (10) have been synthesized and evaluated for cytotoxicity in vitro. All these compounds were cytotoxic to L1210, H-Ep-2, and CCRF-CEM cell lines. 4'-Thiothymidine was also active against herpes simplex 1 and human cytomegalovirus in cell culture.

Introduction

The importance of drug metabolism to anticancer and antiviral activity has been appreciated for some time. In particular for purines, pyrimidines, and their nucleosides both anabolic and catabolic events can be critical; even the relative rates can determine efficacy.[2] Evidence has accumulated over the past few years to support the premise that drug metabolism can be controlled by structural alterations in a way that can be beneficial to therapy.[3]

Carbocyclic nucleosides, where O-4' is replaced by a methylene unit, are known to be resistant to phosphorylase cleavage and have shown significant activity as both anticancer and antiviral agents.[3] Replacement of O-4' with sulfur has also led to some biologically active nucleosides.[4-9] The resistance of several 4'-thioribonucleosides to bacterial cleavage[5] and of 4'-thioinosine to cleavage by purine nucleoside phosphorylase[10] suggests that the replacement of the furanose ring oxygen with sulfur may also generally confer resistance to phosphorylases. No 2'-deoxy-4'-thioribonucleosides have been reported, but it is logical to assume that such nucleosides of both purines and pyrimidines would be resistant to phosphorolytic cleavage, given the similarity of cellular phosphorylases. Such metabolically stable nucleosides, if they are phosphorylated by cellular kinases to nucleotides capable of interfering selectively with DNA synthesis or being incorporated into DNA, might have useful biologic activity. Our initial efforts focused on the synthesis and biologic evaluation of 4'-thio analogues of the naturally occurring pyrimidine 2'-deoxyribonucleosides.

Chemistry

The methyl glycoside of 2-deoxy-4-thio-β-D-*erythro*-pentofuranose (1) was prepared from L-arabinose by the literature procedure of Bobek et al.[11] with several experimental modifications and was converted to its 3,5-di-*O*-toluoyl derivative 2 (Scheme I). Direct coupling of 2 with pyrimidine bases failed to provide the desired nucleosides. Several attempts to convert 2 into the more reactive glycosyl chloride using standard procedures as described in the literature for sugars having oxygen in the furanose ring were unsuccessful. It appeared from analysis of the reaction mixture that the chloro sugar in this series was too unstable to be isolated in appreciable yield. Experimental modification of conventional acetolysis conditions provided acetyl sugar 3 as a ~1:1 mixture of anomers stable for some time at room temperature, but reactive enough to provide the desired nucleosides. This anomeric mixture could be separated either by column chromatography or by fractional crystallization, but was generally used directly for coupling with pyrimidines. Trimethylsilyl triflate catalyzed coupling[12] of sugar 3 with uracil, thymine, and cytosine afforded the corresponding nucleosides (4, 5, and 6) as anomeric mixtures (β:α ratio ~1:1). Fractional crystallization of the thymidine (5) and uridine (4) analogues, but not the cytidine analogue (6), afforded pure anomers. Deprotection with sodium methoxide of compounds 4α, 4β, 5α, 5β, and 6α,β afforded 11, 9, 12, 10, and 7α,β, respectively. In order to obtain the pure β-anomer of the cytidine analogue, compound 4β was treated with 1,2,4-triazole and *p*-chlorophenyl phosphorodichloridate in pyridine to give intermediate 8, which was converted directly to compound 7β by sequential treatment with ammonium hydroxide and sodium methoxide.[13]

The assignments of the anomeric configurations of compounds 10 and 12 were made by NOE difference spectroscopy (Table I). As the table shows, compound 12 exhibits a 3.1% NOE of the H-4' signal on irradiation of H-6 of the thymine base but no enhancement of H-3'. This result demonstrates that H-4' and H-6 are on the same side of the sugar ring and establishes that compound 12 is the α-anomer. Further confirmation of the α-configuration of compound 12 was obtained by irradiation of

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Scheme I

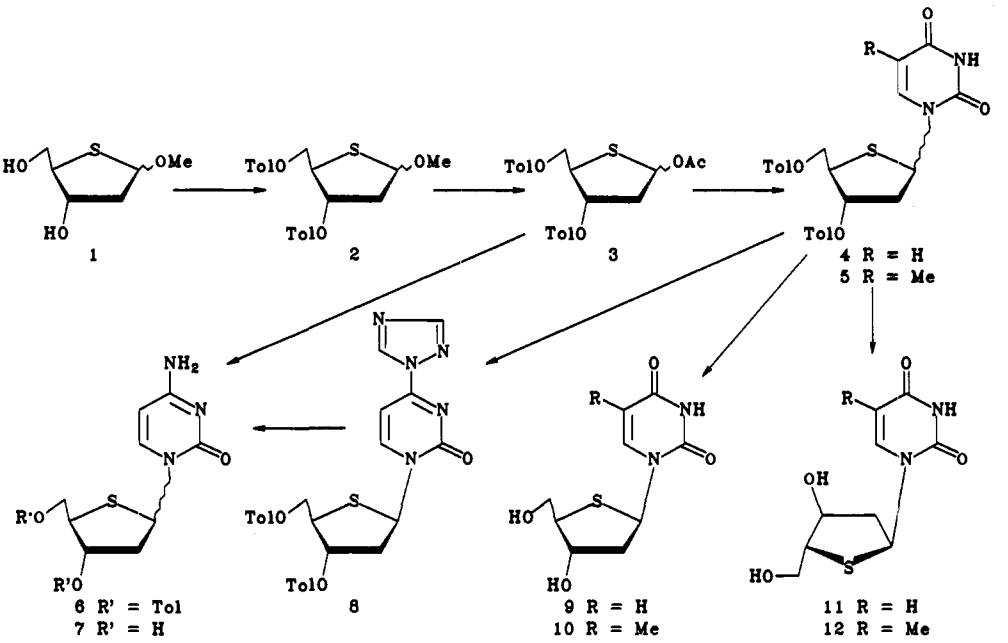

Table I

compd, atom irradiated	NOE data (%)							
	H-6	H-1′	H-3′	H-4′	H-2′[a]	H-2′[b]	5-CH_3	H-5′
12 (α), H-1′	1.2	–	1.0	*a*	*a*	3.9	*a*	*a*
12 (α), H-6	–	1.7	*a*	3.1	3.1	*a*	2.3	*a*
10 (β), H-6	–	1.6	1.0	*a*	*b*	4.1	2.4	*a*
10 (β), H-1′	1.0	–	*a*	*a*	2.8	*b*	*a*	*a*
10 (β), H-3′	1.8	*a*	–	3.1	*b*	2.4	*a*	0.9

[a] No detectable intensity enhancement (<0.5%). [b] Overlapping multiplets.

Table II. Chemical Shifts and Coupling Constants

compd	H-1′ δ (ppm), multiplicity	$^1J_{1'2'a}$ (Hz)	$J_{1'2'b}$ (Hz)	$J_{1'2'a} + J_{1'2'b}$ (Hz)	δ H-2′a (ppm)	δ H-2′b (ppm)	$\Delta\delta$ (H2′b–H2′a)
4β	6.61, dd	8.2	6.6	14.8	2.40	2.75	0.35
5β	6.66, dd	9.1	6.5	15.6	2.41	2.73	0.32
6β	6.69, dd	7.2	6.4	13.6	2.37	2.75	0.38
4α	6.43, dd	1.2	7.0	8.2	2.57	2.90	0.33
5α	6.46, dd	2.3	7.7	10.0	2.55	2.90	0.35
6α	6.45, dd	2.1	7.1	9.2	2.60	2.84	0.24
7β	6.34, dd	8.4	6.4	14.8	2.05	2.14	0.09
9 (β)	6.26, ψt	7.5	7.5	15.0	2.13	2.17	0.04
10 (β)	6.30, dd	8.5	6.7	15.2	2.14	2.19	0.05
7α	6.20, dd	4.1	8.1	12.2	1.98	2.44	0.46
11 (α)	6.14, dd	3.3	8.3	11.6	2.04	2.54	0.50
12 (α)	6.16, dd	4.4	8.2	12.6	2.05	2.49	0.44

H-1′, which resulted in a 1.0% enhancement of the H-3′ signal, demonstrating that these two hydrogens are on the same side of the sugar ring. On the other hand, compound **10** shows a 1.0% NOE of the H-3′ signal on irradiation of H-6 but no enhancement of H-4′. Also, irradiation of H-3′ results in a 1.8% enhancement of the H-6 signal. These results demonstrate that H-3′ and H-6 are on the same side of the sugar ring and that compound **10** is the β-anomer.[14]

The establishment of the anomeric configurations of compounds **10** and **12** allowed the assignments of the anomeric configurations of 5α and 5β, since compounds **10** and **12** were prepared from 5β and 5α, respectively. The NMR spectra of compounds **10**, 5β, **12**, and 5α were then examined, and several correlations were discovered that allow the assignment of the anomeric configurations of the other nucleosides (Table II). First, the peak width of H-1′ ($J_{1'2'a} + J_{1'2'b}$) for the β-anomers is greater than that for the α-anomers. The blocked β-anomers 4β–6β have H-1′ peak widths consistently larger than those of the α-anomers 4α–6α. The same trend is seen for the unblocked β-anomers (7β, **9**, and **10**) versus the corresponding α-anomers (7α, **11**, and **12**). Second, the chemical shifts of H-1′ for the blocked β-anomers are consistently downfield from those of the blocked α-anomers, and a similar trend can be seen for the unblocked compounds. Third, the difference in the chemical shift between H-2′a and H-2′b of the unblocked α-anomers is greater than that of the β-anomers. For the unblocked 2′-deoxy-4′-thioribopyrimidine nucleosides, the $\Delta\delta$ of H-2′a and H-2′b is between 0.04 and 0.09 ppm for the β-anomers (7β, **9**, **10**) and between 0.44 and 0.50 ppm for the α-anomers (7α, **11**, **12**).

(14) A further confirmation that **10** and **5** have the β configuration is derived from certain chemical transformations that will be reported later. Briefly, treatment of the 5′-*O*-trityl derivative of **10** with (diethylamido)sulfur trifluoride resulted in the formation of the 2,3′-cyclonucleoside, a result only possible from the β-anomer.

Table III. Cytotoxicity Data[a]

compd	IC_{50} (μM) L1210[b]	H-Ep-2[c]	CCRF-CEM[d]
7	1.0	<0.20	3.5
7β	1.2	<0.20	-
9	2.7	2.1	~4
10	0.12	0.14	0.67
11	na[e]	na[e]	-
12	66[f]	25[f]	-

[a] See ref 23 for details. [b] The concentration required to produce a 50% reduction in the rate of cell proliferation. [c] The concentration required to produce a 50% inhibition of clone formation. [d] The concentration required to inhibit cell growth to 50% of untreated controls. [e] No cytotoxicity at 160 μM, the highest level tested. [f] This cytotoxicity may be due to trace contamination by 10.

The differences in the chemical shifts between H-2'a and H-2'b of the blocked nucleosides 4–6 gave inconclusive results. Comparable differences in the α- and β-anomers of 2'-deoxyribonucleosides have been used extensively to assign anomeric configurations, in particular, the peak widths[15] and the Δδ of H-2'a and H-2'b.[16]

The carbon chemical shifts of 2'-deoxy-4'-thiopyrimidine nucleosides are very similar to those of the 2'-deoxyribopyrimidine nucleosides except for C-1' and C-4'. For example, chemical shifts of C-1' and C-4' of 4'-thiothymidine are upfield 23.6 and 28.1 ppm, respectively, relative to shifts of C-1' and C-4' of thymidine. This upfield shift in C-1' and C-4' would be expected, since sulfur is less electronegative than oxygen and therefore the sulfur is less deshielding than the oxygen.[17] Substitution of the less electronegative sulfur for the oxygen also leads to a slight decrease in one-bond carbon–hydrogen coupling constants.[18] For example, for C-1' $^1J_{C,H}$ is 161.7 Hz for 10 versus 168.5 Hz for thymidine.[19]

Information on the torsional angle,[20] χ, of the glycosidic bond may be derived from the magnitude of the three-bond coupling constant, $^3J_{C\text{-}2,H\text{-}1'}$. The basis for this use of the coupling constant is a graph of $^3J_{C\text{-}2,H\text{-}1'}$ vs the dihedral angle between C-2 and H-1'.[21,22] The $^3J_{C\text{-}2,H\text{-}1'}$ values for compounds 9, 11, 10, and 12 are 2.5, 2.4, 2.7, and 2.4 Hz, respectively. With a $^3J_{C\text{-}2,H\text{-}1'}$ of 2.5 Hz, the dihedral angle between C-2 and H-1' in these compounds is approximately ±40°. The torsional angle, χ, corresponding to a dihedral angle of 40° is −20° and to one of −40° is −100°, both values being in the anti range. The NOE data of compounds 10 and 12 also support the anti conformation in that irradiation of H-6 of 10 and 12 shows an enhancement of H-3' in 10 and of H-4' in 12, which would not be observed if the base adopted a syn conformation.

Table IV. The Effect of 4'-Thiothymidine on Yields of HCMV in MRC5 Cell Monolayer Cultures[a]

concn, (μM)	HCMV yield[b]	yield reductn[b]	drug cytotoxicity: gross morphology	drug cytotoxicity: MTT assay (% of control)
100	1.1	3.8	sl toxic	86
32	1.8	3.1	sl toxic	81
10	3.6	1.3	0	90
3.2	4.1	0.8	0	81
1.0	4.4	0.5		-
0	4.9	-		
32[c]	1.3	3.6	0	95

[a] MRC5 cells are infected with HCMV to give a multiplicity of infection of ca. 0.05 plaque-forming units/cell. Aliquots of drug solution are introduced into the cell cultures 1.5 h later (two virus-infected cultures and one uninfected control). A week postinfection the cells are examined microscopically for CPE and drug cytotoxicity. Drug toxicity is determined quantitatively by a method based on the reduction of the tetrazolium salt MTT (see ref 25). The infectious virus yields from the harvested test and virus control samples are determined by plaque assay in MRC5 cells grown in 12-well cluster plates. Inhibition of HCMV replication by the test compounds is determined by comparing the progeny virus yields in the drug-treated cultures with the progeny virus yields in the untreated, virus-infected controls. [b] Log_{10} plaque-forming units/mL. [c] Ganciclovir, positive control.

Biological Evaluations

4'-Thiothymidine (10) is the most cytotoxic of these thionucleosides to the three neoplastic cell lines employed, the murine leukemia L1210, human epidermoid carcinoma #2, and human T-cell leukemia CCRF-CEM, although the 2'-deoxycytidine (7β) and 2'-deoxyuridine (9) analogues are also quite cytotoxic (Table III). The α-anomer 12 was much less cytotoxic, and 11 was nontoxic at the highest concentration tested (160 μM). The cytotoxicities of 7, 9, and particularly 10 suggest that these compounds should be evaluated in animal models to determine if they are selectively toxic to neoplastic cells in vivo.

Evaluation of these nucleosides against herpes simplex virus 1 in Vero cells[24] showed that only 4'-thiothymidine

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had any activity, with a virus rating of 1.3, a minimum inhibitory concentration (MIC_{50}) of 0.8 μM, a maximum tolerated concentration (MTC) of 2.6 μM, and a therapeutic index (MTC/MIC_{50}) of 3.2. Its activity against human cytomegalovirus in MRC5 cells in monolayer culture was comparable to that of ganciclovir, the positive control, although 4′-thiothymidine was somewhat more toxic to MRC5 cells (Table IV). Despite the significant antiviral activity of 4′-thiothymidine (**10**) at tolerated doses in these slow-growing monkey and human cell lines, this nucleoside is unlikely to show in vivo activity at tolerated doses, because of its significant cytotoxicity to rapidly proliferating cell populations (Table III).

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator or by short-path distillation into a dry ice–acetone cooled receiver under high vacuum. Analytical samples were normally dried in vacuo over P_2O_5 at room temperature for 16 h. Analtech precoated (250 μm) silica gel G(F) plates were used for TLC analyses; the spots were detected by irradiation with a mineral light and/or by charring after spraying with saturated $(NH_4)_2SO_4$. All analytical samples were TLC homogeneous. Melting points were obtained with a Mel-Temp capillary melting point apparatus, and all melting points were uncorrected. Purifications by flash chromatography were carried out on Merck silica gel 60 (230–400 mesh) using the slurry method of column packing. The UV absorption spectra were determined in 0.1 N HCl (pH 1), pH 7 buffer, and 0.1 N NaOH (pH 13) with a Perkin-Elmer Lambda 9 spectrophotometer; the maxima are reported in nanometers ($\epsilon \times 10^{-3}$). The ^1H NMR spectra and the ^{13}C NMR spectra with tetramethylsilane as an internal reference were recorded with a Nicolet NT 300NB spectrometer operating at 300.635 MHz and 75.602 MHz, respectively. Chemical shifts (δ) quoted in the case of multiplets were measured from the approximate center. Where necessary, the chemical shift and coupling constant values for the non-first-order parts of the spectra were obtained from simulated spectra by employing the General Electric/Nicolet ITRACAL program for iterative analysis. The hydrogen-decoupled ^{13}C NMR spectra were assigned by comparison of the $J_{C,H}$ values obtained from the hydrogen-coupled ^{13}C NMR spectra, and when necessary, selective hydrogen decoupling was performed in order to confirm assignments. The NOE experiments were conducted on degassed solutions in Me_2SO-d_6. To minimize the effects of magnetic perturbations with the sample nonspinning, eight FIDs (free induction decays) were acquired with the decoupler set at a desired frequency, and eight FIDs were recorded with the decoupler off-resonance. The process was repeated until 400 FIDs had been accumulated. Subsequent subtraction of the two spectra afforded the net enhancement. The mass spectral data were obtained with a Varian-MAT 311A mass spectrometer in the fast-atom-bombardment mode. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

1-*O*-Acetyl-2-deoxy-4-thio-3,5-di-*O*-*p*-toluoyl-D-ribofuranose (3). To a solution of 1 (10 g, 60.97 mmol) in 250 mL of sieve-dried pyridine was added *p*-toluoyl chloride (23.57 g, 152.5 mmol) dropwise at 0–5 °C. The cooling bath was removed, and the reaction mixture was stirred for 10 h, at which time the reaction was found to be essentially complete as indicated by TLC analysis ($CHCl_3$–MeOH, 10:1). The reaction mixture was poured into an ice–water mixture (500 mL), stirred for 1 h, and then diluted with 500 mL of $CHCl_3$. The aqueous layer was extracted with $CHCl_3$ (2 × 100 mL). The combined organic extracts were washed with diluted sulfuric acid (200 mL), aqueous saturated sodium bicarbonate (2 × 200 mL), and water to neutrality, dried ($MgSO_4$), and evaporated to dryness. The residue was dissolved in $CHCl_3$ (200 mL), filtered through a 9.0 cm in diameter and 4 cm thick bed of silica gel, and washed with $CHCl_3$ (2 × 100 mL), and the filtrate was evaporated to dryness to afford crude **2** as a dark brown solid (24 g). This residue was dissolved in acetic anhydride (200 mL), glacial acetic acid (200 mL), and concentrated sulfuric acid (2.5 mL), and the mixture was warmed to 40 °C for 1 h. After cooling to 0 °C, anhydrous sodium acetate was added to neutralize the sulfuric acid. The resulting mixture was partitioned between 500 mL of water and 300 mL of $CHCl_3$. The aqueous phase was extracted with $CHCl_3$ (2 × 100 mL), and the combined $CHCl_3$ layers were evaporated to dryness in vacuo. Several portions of methanol were added and removed in vacuo to eliminate the last traces of acetic anhydride. The residue was purified on a flash column containing 100 g of silica gel and eluted with 6:1 cyclohexane–ethyl acetate, and appropriate fractions were combined and evaporated to give a white solid, which was crystallized from 95% ethanol to give **3**, 18.26 g (70% from **1**), as an α,β mixture: MS *z*/*e* 429 (M + 1); ^1H NMR (Me_2SO-d_6) δ 2.01 (s, 3, CH_3CO), 2.02 (s, 3, CH_3CO), 2.36 (s, 3, CH_3 of toluoyl group), 2.39 (s, 3, CH_3 of toluoyl group), 2.52–2.74 (m, 4, H-2), 3.92–4.06 (m, 2, H-4), 4.28–4.52 (m, 4, H-5), 5.64–5.74 (m, 2, H-3), 6.12 (dd, 1, H-1 of β, $J_{1,2a}$ = 3.3 Hz, $J_{1,2b}$ = 5.8 Hz), 6.20 (d, 1, H-1 of α, $J_{1,2}$ = 5.3 Hz), 7.24–7.36 (m, 8, H's of toluoyl group), 7.80–7.92 (m, 8, H's of toluoyl group). Anal. ($C_{23}H_{26}O_6S$) C, H, S.

1-(2-Deoxy-4-thio-3,5-di-*O*-toluoyl-β-D-*erythro*-pentofuranosyl)uracil (4β) and 1-(2-Deoxy-4-thio-3,5-di-*O*-toluoyl-α-D-*erythro*-pentofuranosyl)uracil (4α). To a suspension of **3** (428 mg, 1.0 mmol) and uracil (112.1 mg, 1.0 mmol) in anhydrous acetonitrile (30 mL) were added consecutively hexamethyldisilazane (HMDS, 161.5 mg, 1.0 mmol) and chlorotrimethylsilane (TMSCl, 108.6 mg, 1.0 mmol), and the mixture was stirred at room temperature for 0.5 h. The solution was cooled to −78 °C, trimethylsilyl trifluoromethanesulfonate (267 mg, 1.2 mmol) was added, and the reaction mixture was stirred at −78 °C for another 1 h, after which time the reaction was essentially complete. The reaction mixture was warmed to room temperature, concentrated to a small volume (5 mL), diluted with methylene chloride (ca. 50 mL), and then washed with water (15 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 a solid, which was crystallized from $CHCl_3$–dioxane to give pure 4β (185 mg, 38%): mp 182–184 °C; TLC (98:2 $CHCl_3$–MeOH) R_f 0.35; MS *z*/*e* 481 $(M + 1)^+$; ^1H NMR ($CDCl_3$) δ 2.40 (m, 1, H-2′a, $J_{1',2'a}$ = 8.2 Hz, $J_{2'a,3'}$ = 4.3 Hz), 2.42 (s, 6, CH_3's of toluoyl group), 2.75 (ddd, 1, H-2′b, $J_{1',2'b}$ = 6.6 Hz, $J_{2'b,3'}$ = 3.6 Hz, $J_{2'a,2'b}$ = 14.3 Hz), 4.00 (t of d, 1, H-4′, $J_{3',4'}$ = 2.8 Hz), 4.60 (d, 2, H-5′, $J_{4',5'}$ = 6.2 Hz), 5.69 (dd, 1, H-5, $J_{5,6}$ = 8.1 Hz, $J_{5,NH}$ = 2.2 Hz), 5.75 (m, 1, H-3′), 6.61 (dd, 1, H-1′), 7.26 (d, 4, H's of toluoyl group, *J* = 8.1 Hz), 7.80 (d, 1, H-6), 7.90–7.96 (m, 4, H's of toluoyl group), 8.86 (br s, 1, H-3); ^{13}C NMR ($CDCl_3$) δ 21.73 (CH_3 of toluoyl group), 40.15 (C-2′), 52.90 (C-4′), 61.33 (C-1′), 64.82 (C-5′), 76.80 (C-3′), 103.50 (C-5′), 126.28, 126.46, 129.17, 129.70, 129.77, 129.84 (toluoyl ring carbons), 140.01 (C-6), 144.42, 144.49 (toluoyl ring carbons), 150.40 (C-2), 162.39 (C-4), 165.57, 166.14 (carbonyl carbons of toluoyl).

Fractional crystallization of the mother liquor from EtOH–dioxane afforded 4α (192 mg, 40%): mp 118–120 °C; TLC (98:2 $CHCl_3$–MeOH) R_f 0.35; MS *z*/*e* 481 $(M + 1)^+$; ^1H NMR ($CDCl_3$) δ 2.41 (s, 6, CH_3's of toluoyl groups), 2.57 (m, 1, H-2′a, $J_{2'a,2'b}$ = 15.4 Hz), 2.90 (ddd, H-2b′, $J_{1',2'b}$ = 7.0 Hz, $J_{2'b,3'}$ = 5.4 Hz), 4.24 (t, 1, H-4′), 4.40 and 4.48 (m, 2, H-5′a, H-5′b, $J_{4',5'a}$ = 7.2 Hz, $J_{4',5'b}$ = 5.9 Hz, $J_{5a',5'b}$ = 11.5 Hz), 5.68–5.72 (m, 2, H-3′, H-5′), 6.43 (dd, 1, H-1′, $J_{1',2'a}$ = 1.2 Hz), 7.21–7.26 (m, 4, H's of toluoyl group), 7.79 (d, 2, H's of toluoyl group, *J* = 8.1 Hz), 7.96 (d, 2, H's of toluoyl group, *J* = 8.1 Hz), 8.12 (d, 2, H-6, $J_{5,6}$ = 8.2 Hz), 9.37 (s, 1, H-3); ^{13}C NMR ($CDCl_3$) δ 21.72 (CH_3 of toluoyl group), 42.15 (C-2′), 54.46 (C-4′), 63.55 (C-1′, *J* = 160.3 Hz), 64.96 (C-5′), 78.21 (C-3′), 101.94 (C-5), 126.03, 126.6, 129.24, 129.28, 129.73, 129.63 (toluoyl ring carbons), 141.74 (C-6), 144.14, 144.73 (toluoyl ring carbons), 150.71 (C-2), 163.06 (C-4), 165.36, 166.08 (carbonyl carbons of toluoyl group).

3′,5′-Di-*O*-toluoyl-4′-thiothymidine (5β) and 1-(2-Deoxy-4-thio-3,5-di-*O*-toluoyl-α-D-*erythro*-pentofuranosyl)thymine (5α). To a suspension of **3** (428 mg, 1.0 mmol) and thymine (126 mg, 1.0 mmol) in anhydrous acetonitrile (30 mL) were added

(25) Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, S.; Skehan, P.; Scudiero, D. A.; Monks, A.; Boyd, M. R. Comparison of In Vitro Anticancer-Drug-Screening Data Generated With A Tetrazolium Assay Versus a Protein Assay Against a Diverse Panel of Human Tumor Cell Lines. *J. Natl. Cancer Inst.* **1990**, *82*, 1113–1118.

consecutively hexamethyldisilazane (HMDS, 161.5 mg, 1.0 mmol) and chlorotrimethylsilane (TMSCl, 108.6 mg, 1.0 mmol), and the mixture was stirred at room temperature for 0.5 h. The resulting solution was cooled to −78 °C, trimethylsilyl trifluoromethanesulfonate (267 mg, 1.2 mmol) was added, and the reaction mixture was stirred at −78 °C for another 1.5 h, after which time the reaction was essentially complete. The reaction mixture was warmed to room temperature, concentrated to a small volume (5 mL), diluted with methylene chloride (ca. 50 mL), and then washed with water (15 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, 99:1) to afford a solid, which was crystallized from EtOH–$CHCl_3$ to give pure **5β** (173 mg, 35%): mp 178–182 °C; TLC (98:2 $CHCl_3$–MeOH) R_f 0.55; MS z/e 495 $(M + 1)^+$; ^1H NMR ($CDCl_3$) δ 1.78 (d, 3, 5-CH_3, $J_{6,5\text{-}CH_3}$ = 1.4 Hz), 2.42 (s, 3, CH_3 of toluoyl group), 2.43 (s, 3, CH_3 of toluoyl group), 2.41 (ddd, 1, H-2'a, $J_{1',2'a}$ = 9.1 Hz, $J_{2'a,3'}$ = 4.2 Hz), 2.73 (ddd, 1, H-2'b, $J_{2'a,2'b}$ = 14.0 Hz, $J_{1',2'b}$ = 6.5 Hz, $J_{2'b,3}$ = 2.9 Hz), 4.00 (t of d, 1, H-4', $J_{3',4'}$ = 2.1 Hz), 4.62 (d, 2, H-5', $J_{4',5'}$ = 6.2 Hz), 5.76 (m, 1, H-3'), 6.66 (dd, 1, H-1'), 7.26 (d, 4, H's of toluoyl group, J = 8.2 Hz), 7.56 (s, 1, H-6), 7.94 (d, 2, H's of toluoyl group, J = 8.2 Hz), 7.96 (d, 2, H's of toluoyl group, J = 8.2 Hz), 8.58 (br q, 1, H-3); ^{13}C NMR ($CDCl_3$) δ 12.41 (5-CH_3), 21.69, 21.72 (CH_3's of toluoyl group), 39.99 (C-2'), 53.13 (C-4'), 61.28 (C-1', $J_{C,H}$ = 163.3 Hz), 65.14 (C-5'), 77.13 (C-3'), 112.29 (C-5), 126.41, 126.58, 129.25, 129.35, 129.74, 129.87 (toluoyl ring carbons), 135.47 (C-6, $^1J_{C,H}$ = 176.5 Hz), 144.39, 144.48 (toluoyl ring carbons), 150.45 (C-2), 162.90 (C-4), 165.61, 166.18 (carbonyl carbons of toluoyl group).

Fractional crystallization of the mother liquor from EtOH–dioxane afforded **5α** (185 mg, 38%): mp 146–148 °C; TLC (98.2 $CHCl_3$–MeOH) R_f 0.55; MS z/e 495 $(M + 1)^+$; ^1H NMR ($CDCl_3$) δ 1.82 (d, 3, 5-CH_3, $J_{6,5\text{-}CH_3}$ = 1.3 Hz), 2.41 (s, 6, CH_3's of toluoyl group), 2.55 (d of t, 1, H-2'a, $J_{2'a,2'b}$ = 15.3 Hz, $J_{1',2'a}$ = 2.3 Hz, $J_{2'a,3'}$ = 1.7 Hz), 2.90 (ddd, 1, H-2'b, $J_{1',2'b}$ = 7.7 Hz, $J_{2'b,3'}$ = 5.0 Hz), 4.25 (m, 1, H-4'), 4.40 (A part of an ABX spin system, 1, H-5'a, $J_{5'a,5'b}$ = 11.5 Hz, $J_{4',5'a}$ = 7.7 Hz), 4.48 (B part of an ABX spin system, 1, H-5'b, $J_{4',5'b}$ = 6.0 Hz), 5.71 (m, 1, H-3'), 6.46 (dd, 1, H-1'), 7.26 (d, 4, H's of toluoyl group, J = 8.2 Hz), 7.80 (d, 2, H's of toluoyl group, J = 8.2 Hz), 7.86 (br q, 1, H-6), 7.96 (d, 2, H's of toluoyl group, J = 8.2 Hz), 8.82 (s, 1, H-3); ^{13}C NMR ($CDCl_3$) δ 12.67 (5-CH_3), 21.71 (CH_3 of toluoyl group), 42.05 (C-2'), 54.39 (C-4'), 62.97 (C-1', $^1J_{C,H}$ = 161.0 Hz), 65.06 (C-5'), 78.23 (C-3'), 110.47 (C-5), 126.18, 126.59 (toluoyl ring carbons), 129.23, 129.31, 129.57, 129.74 (toluoyl ring carbons), 137.25 (C-6), 144.14, 144.64 (toluoyl ring carbons), 150.60 (C-2), 163.35 (C-4), 165.40, 166.11 (carbonyl carbons of toluoyl group).

1-(2-Deoxy-4-thio-3,5-di-*O*-toluoyl-α,β-D-*erythro*-pentofuranosyl)cytosine (6). To a suspension of **3** (428 mg, 1.0 mmol) and cytosine (111.1 mg, 1.0 mmol) in anhydrous acetonitrile (25 mL) were added consecutively hexamethyldisilazane (HMDS, 161.6 mg, 1.0 mmol) and chlorotrimethylsilane (TMSCl, 108.6 mg, 1.0 mmol), and the mixture was stirred at room temperature for 0.5 h. This solution was 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 reaction 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 **6** (397 mg, 80%) as a colorless syrup: TLC (90:10 $CHCl_3$–MeOH) R_f 0.45; MS z/e 480 $(M + 1)^+$; ^1H NMR ($CDCl_3$) δ 2.36 (s, 6, CH_3's of toluoyl group), 2.39 (s, 6, CH_3's of toluoyl group), 2.33–2.41 (m, 1, H-2'a β), 2.60 (d of t, 1, H-2'a α, $J_{2'a,2'b}$ = 14.9 Hz), 2.75 (ddd, 1, H-2'b β, $J_{2'a,2'b}$ = 13.9 Hz, $J_{2'b,3'}$ = 3.7 Hz, $J_{1',2'b}$ = 6.4 Hz), 2.84 (ddd, H-2'b α, $J_{2'b,3'}$ = 4.9 Hz, $J_{1',2'b}$ = 7.1 Hz), 3.95 (t of d, 1, H-4' β, $J_{4',5'}$ = 6.8 Hz, $J_{3',4'}$ = 2.8 Hz), 4.18 (t of d, 1, H-4' α, $J_{4',5'a}$ = 7.4 Hz, $J_{4',5'b}$ = 6.1 Hz), 4.39 (A part of an ABX spin system, 1, H-5'a α, $J_{5'a,5'b}$ = 11.5 Hz), 4.46 (B part of an ABX spin system, 1, H-5'b α), 4.54 (d, 2, H-5' β), 5.65 (m, 1, H-3'α, $J_{2'a,3'}$ = 2.3 Hz, $J_{3',4'}$ = 2.9 Hz), 5.71 (m, 1, H-3' β), 5.76 (d, 1, H-5 α, $J_{5,6}$ = 7.5 Hz), 5.84 (d, 1, H-5 β, $J_{5,6}$ = 7.4 Hz), 6.45 (dd, 1, H-1' α, $J_{1',2'a}$ = 2.1 Hz, $J_{1',2'b}$ = 7.1 Hz), 6.69 (t, 1, H-1' β, $J_{1',2'a}$ = 7.2 Hz, $J_{1',2'b}$ = 6.4 Hz), 7.15–7.28 (m, 8, H's of toluoyl group), 7.73 (d, 2, H's of toluoyl group, J = 8.2 Hz), 7.89, (d, 1, H-6 β), 7.90–7.96 (m, 8, H's of toluoyl group), 8.14 (d, 1, H-6 α); ^{13}C NMR ($CDCl_3$) δ 21.70 (CH_3's of toluoyl group), 40.17 (C-2' β), 42.15 (C-2' α), 52.40 (C-4' β), 54.22 (C-4' α), 62.18 (C-1' β), 64.41 (C-1' α), 65.16 (C-5' α), 65.37 (C-5' β), 76.93 (C-3' β), 78.42 (C-3' α), 94.20 (C-4' α), 95.76 (C-4 β), 126.31, 126.47, 126.60, 126.69, 129.10, 129.19, 129.75, 129.84, 141.47, 143.08, 144.02, 144.20, 144.29, and 144.33 (toluoyl ring carbons and C-6 α and C-6 β), 156.26 (C-4 α), 156.08 (C-4 β), 165.40, 165.43, 165.63, 165.67, 166.15, and 166.21 (carbonyl carbons of toluoyl group and C-2 α and C-2 β).

1-(2-Deoxy-4-thio-α,β-D-*erythro*-pentofuranosyl)cytosine (7). A solution of **6** (298 mg, 0.6 mmol) in anhydrous MeOH (50 mL) was stirred at room temperature with a freshly prepared solution of sodium methoxide (65 mg, 1.2 mmol), in MeOH (10 mL). A TLC aliquot (2.5 h, $CHCl_3$–MeOH, 80:20) showed complete consumption of starting material. The solution was neutralized with Dowex 50W-X8 (H^+) ion-exchange resin, and the resin was filtered off, with MeOH washing. The filtrates were combined and evaporated to dryness, methyl *p*-toluate being removed at 50 °C/0.01 Torr. The residue failed to crystallize and was passed through a 3-cm bed of silica gel (elution with $CHCl_3$–MeOH, 4:1) to obtain **7** as an amorphous solid (123 mg, 85%): MS z/e 244 $(M + 1)$; ^1H NMR (Me_2SO-d_6) δ 1.98 (dt, 1, H-2'a α, $J_{2'a,2'b}$ = 14.3 Hz, $J_{1',2'a}$ = 4.1 Hz, $J_{2b',3}$ = 4.3 Hz), 2.05 and 2.14 (m, 2, H-2'a β, H-2'b β), 2.44 (m, 1, H-2'b β, $J_{1',2b'}$ = 8.1 Hz, $J_{2'b,3'}$ = 4.7 Hz), 3.22–3.59 (m, 6, H-4, H-5'), 4.26 (m, 1, H-3' α), 4.33 (m, 1, H-3' β), 5.00 (br t, 1, 5'-OH α), 5.11 (br t, 1, 5'-OH β), 5.22 (br d, 1, 3'-OH β, J = 3.5 Hz), 5.40 (br d, 1, 3'-OH α, J = 3.5 Hz), 5.73 (d, 1, H-5 α, J = 7.5 H), 5.77 (d, 1, H-5 β, J = 7.5 Hz), 6.20 (dd, 1, H-1' α, $J_{1',2'a}$ = 4.1 Hz, $J_{1',2'b}$ = 8.1 Hz), 6.34 (dd, 1, H-1' β, $J_{1',2'b}$ = 6.50 Hz, $J_{1',2'a}$ = 8.4 Hz), 7.04–7.18 (br s, 4, NH_2), 7.92 (d, 1, H-6 β), 8.18 (d, 1, H-6 α).

2'-Deoxy-4'-thiocytidine (7β). To a solution of compound **4β** (240 mg, 0.5 mmol) in 50 mL of pyridine were added 1,2,4-triazole (1.0 mmol) and *p*-chlorophenyl phosphorodichloridate (0.75 mmol), and the reaction mixture was stirred at room temperature for 3 days, during which time starting material was almost completely consumed. The reaction mixture was evaporated under reduced pressure to a syrup, which was then passed through a funnel having a 3-cm layer of silica gel (elution with $CHCl_3$–MeOH, 9:1) to afford crude **8** (300 mg, MS z/e 532 $(M + 1)^+$), which was treated with concentrated NH_4OH–dioxane (25 mL, 1:1) for 12 h at room temperature. Evaporation of the solvent and subsequent treatment of the residue with 2 equiv of NaOMe in MeOH (30 mL) for 2 h afforded crude compound **7β**, which was crystallized from ethanol to provide pure **7β** as an amorphous hygroscopic powder (75 mg, 62%): mp 129–132 °C; MS z/e 244 $(M + 1)^+$; ^1H NMR (Me_2SO-d_6) δ 2.05 and 2.14 (m, 2, H-2'a and H-2'b, $J_{2'a,3'}$ = 4.0 Hz, $J_{2'b,3'}$ = 3.7 Hz, $J_{1',2'a}$ = 8.4 Hz, $J_{1',2'b}$ = 6.4 Hz, $J_{2'a,2'b}$ = 13.2 Hz), 3.26 (t of d, 1, H-4', $J_{3',4'}$ = 3.0 Hz), 3.48 and 3.59 (m, 2, H-5'a, H-5'b), 4.33 (m, 1, H-3'), 5.15 (t, 1, 5'-OH, J = 5.4 Hz), 5.26 (d, 1, 3'-OH, J = 5.4 Hz), 5.78 (d, 1, H-5, $J_{5,6}$ = 7.5 Hz), 6.34 (dd, 1, H-1'), 7.12 (br s, 1, NH), 7.22 (br s, 1, NH), 7.93 (d, 1, H-6), spectrum also shows solvent impurities; ^{13}C NMR (Me_2SO-d_6) δ 41.64 (C-2'), 58.63, 60.36 (C-4', C-1'), 63.67 (C-5'), 73.33 (C-3'), 94.53 (C-5), 141.90 (C-6), 155.24 (C-2), 165.04 (C-4). Anal. ($C_9H_{13}NO_3S$) C, H, N, S.

2'-Deoxy-4'-thiouridine (9) and 1-(2-Deoxy-4-thio-α-D-*erythro*-pentofuranosyl)uracil (11). A solution of **4β** (175 mg, 0.36 mmol) in anhydrous MeOH (30 mL) was stirred at room temperature with a freshly prepared solution of sodium methoxide (30 mg, 0.72 mmol) in MeOH (6.5 mL). A TLC aliquot (2.5 h, $CHCl_3$–MeOH, 95:5) indicated complete reaction. The solution was rendered neutral with Dowex 50W-X8 (H^+) ion-exchange resin, the suspension was filtered, and the resin was washed with MeOH. The filtrates were combined and evaporated to dryness, and methyl *p*-toluate was removed at 50 °C/0.01 Torr. Crystallization of the residue from absolute ethanol gave pure **9** (76 mg, 85%): mp 186–188 °C; TLC (9:1 $CHCl_3$–MeOH) R_f 0.30; MS z/e 245 $(M + 1)^+$; UV λ_{max} (pH 1) 266 (9.88), (pH 7) 266 (9.76), (pH 13) 266 (8.08); ^1H NMR (Me_2SO-d_6) δ 2.13 and 2.17 (m, 2, H-2'a, H-2'b), 3.28 (t of d, 1, H-4', $J_{3',4'}$ = 2.3 Hz), 3.55 and 3.60 (m, 2, H-5'a, H-5'b, $J_{4',5'a}$ = 5.8 Hz, $J_{4',5'b}$ = 6.7 Hz), 4.35 (m, 1, H-3'), 5.18 (br s, 1, 5'-OH), 5.30 (br s, 1, 3'-OH), 5.67 (d, 1, H-5,

$J_{5,6}$ = 8.1 Hz), 6.26 (t, 1, H-1', $J_{1',2'}$ = 7.5 Hz), 7.98 (d, 1, H-6); ^{13}C NMR (Me_2SO-d_6) δ 41.25 (C-2'), 58.98 (C-4'), 60.11 (C-1', ^1J = 163.4 Hz), 63.47 (C-5'), 73.44 (C-3'), 102.13 (C-5, J = 175.9 Hz), 141.35 (C-6, J = 180.8 Hz), 150.62 (C-2), 162.74 (C-4). Anal. ($C_9H_{12}N_4O_2S$) C, H, N, S.

Compound 4α (262 mg) was deprotected in a similar manner as described for 4β to provide 11 (120 mg, 90%): mp 190–192 °C; TLC (9:1 $CHCl_3$–MeOH) R_f 0.30; MS z/e 245 (M + 1)$^+$; UV λ_{max} (pH 1) 266 (9.24), (pH 7) 266 (9.52), (pH 13) 265 (8.72); ^{1}H NMR (Me_2SO-d_6) δ 2.04 (dt, 1, H-2'a, $J_{2'a,2'b}$ = 14.3 Hz, $J_{1',2'a}$ = $J_{2'a,3'}$ = 3.3 Hz), 2.50 (ddd, 1, H-2'b, $J_{1',2'b}$ = 8.3 Hz, $J_{2'b,3'}$ = 4.6 Hz), 3.34 (m, 1, H-5'a, $J_{5'a,5'b}$ = 11.0 Hz, $J_{4',5'a}$ = 6.7 Hz), 3.44 (m, 1, H-5'b, $J_{4',5'b}$ = 6.7 Hz), 3.55 (t of d, 1, H-4', $J_{3',4'}$ = 2.7 Hz), 4.32 (m, 1, H-3'), 5.05 (br s, 1, OH), 5.49 (br s, 1, OH), 5.65 (d, 1, H-5, J = 8.1 Hz), 6.14 (dd, 1, H-1'), 8.26 (d, 1, H-6), 11.24 (br s, 1, H-3); ^{13}C NMR (Me_2SO-d_6) δ 42.16 (C-2'), 60.01 (C-4'), 60.96 (C-1', ^1J = 161.5 Hz), 63.57 (C-5'), 74.14 (C-3'), 101.16 (C-5, $^1J_{C,H}$ = 175.2 Hz), 142.92 (C-6, J_{CH} = 181.9 Hz), 150.7 (C-2), 162.98 (C-4). Anal. ($C_9H_{12}N_4O_2S$) C, H, N, S.

4'-Thiothymidine (10) and 1-(2-Deoxy-4-thio-α-D-*erythro*-pentofuranosyl)thymine (12). A solution of 5β (150 mg, 0.30 mmol) in anhydrous MeOH (30 mL) was stirred at room temperature with a freshly prepared solution of sodium methoxide (32.5 mg, 0.60 mmol) in MeOH (5 mL). A TLC aliquot (3 h, $CHCl_3$–MeOH, 95:5) showed complete consumption of starting material. The solution was rendered neutral with Dowex 50W-X8 (H^+) ion-exchange resin, and the resin was filtered off, with MeOH washing. The filtrates were combined and evaporated to dryness, and methyl p-toluate was removed at 50 °C/0.01 Torr. Crystallization of the residue from absolute EtOH gave pure 10 (61 mg, 78%): mp 213–215 °C; TLC (9:1 $CHCl_3$–MeOH) R_f 0.40; MS z/e 258 (M + 1); UV λ_{max} (pH 1) 272 (10.3), (pH 7) 272 (10.2), (pH 13) 271 (10.3); ^{1}H NMR (Me_2SO-d_6) δ 1.80 (d, 3, 5-CH_3, $J_{6,5\text{-}CH_3}$ = 1.1 Hz), 2.14 and 2.19 (m, 2, H-2'a, H-2'b, $J_{2'a,2'b}$ = 13.1 Hz, $J_{1',2'a}$ = 6.7 Hz, $J_{1',2'b}$ = 8.5 Hz, $J_{2'a,3'}$ = 3.4 Hz, $J_{2'b,3'}$ = 3.9 Hz), 3.28 (t of d, 1, H-4', $J_{3',4'}$ = 2.2 Hz), 3.52–3.67 (m, 2, H-5'), 4.38 (br s, 1, H-3'), 5.16 (br t, 1, 5'-OH), 5.24 (d, 1, 3'-OH, J = 3.7 Hz), 6.30 (dd, 1, H-1'), 7.81 (br q, 1, H-6), 11.32 (br s, 1, H-3); ^{13}C NMR (Me_2SO-d_6) δ 12.14 (5-CH_3), 40.98 (C-2'), 59.01 (C-4'), 59.93 (C-1', $J_{C,H}$ = 161.7), 63.51 (C-5'), 73.40 (C-3'), 109.82 (C-5), 136.70 (C-6, $^1J_{C,H}$ = 179.0 Hz), 150.60 (C-2), 163.37 (C-4). Anal. ($C_{10}H_{13}N_2O_4S$) C, H, N, S. H: calcd, 5.09; found, 5.56.

Compound 5α (100 mg, 0.20 mmol) was deprotected in a manner similar to that described for 5β to provide 12 (42 mg, 80% yield) (mp 205–207 °C): TLC (9:1 $CHCl_3$–MeOH) R_f 0.40; MS z/e 258 (M + 1)$^+$; UV λ_{max} (pH 1) 271 (10.5), (pH 7) 271 (10.5), (pH 13) 271 (8.64); ^{1}H NMR (Me_2SO-d_6) δ 1.80 (s, 1, 5-CH_3), 2.05 (d of t, 1, H-2'a, $J_{2'a,2'b}$ = 1.40 Hz, $J_{1',2'a}$ = $J_{2'a,3'}$ = 4.4 Hz), 2.49 (ddd, 1, H-2'b, $J_{1',2'b}$ = 8.2 Hz, $J_{2'b,3'}$ = 4.9 Hz), 3.33–3.38 (m, 1, H-5'a), 3.48–3.60 (m, 2, H-4', H-5'b), 4.26 (m, 1, H-3', $J_{3',4'}$ = 3.0 Hz), 4.99 (br s, 1, 5'-OH), 5.48 (br s, 1, 3'-OH), 6.16 (dd, 1, H-1'), 8.11 (s, 1, H-6), 11.24 (br s, 1, H-3); ^{13}C NMR (Me_2SO-d_6) δ 12.26 (C-5 CH_3), 42.03 (C-2'), 59.53 (C-4'), 59.77 (C-1', $^1J_{C,H}$ = 161.6 Hz), 63.57 (C-5'), 74.05 (C-3'), 108.94 (C-5), 138.28 (C-6, $J_{C,H}$ = 179.6 Hz), 150.62 (C-2), 163.47 (C-4). Anal. ($C_{10}H_{13}N_2O_4S$) C, H, N, S. H: calcd, 5.09; found, 5.54.

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Modeling Study of the Structure of the Macromolecular Antitumor Antibiotic Neocarzinostatin. Origin of the Stabilization of the Chromophore

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A three-dimensional structure of the apoprotein of neocarzinostatin (NCS) was built by using the actinoxanthin (AXN) crystal structure as template, and the subsequent favored-site search for the binding of the fragments (1–4 in Figure 7) of the chromophore (Figure 1) of NCS at the binding cleft led to a reasonable complex structure of apo-NCS and the chromophore (Figures 8 and 9), after refinement of the molecular mechanics program AMBER. The refined three-dimensional structure model of NCS shows the "Y"-shaped cleft of the binding site in which the bicyclic epoxy dienediyne part 4 and its substituents, the naphthoate 3, the amino sugar 2, and cyclic carbonate 1 moieties are nicely fitted. Contacts of the chromophore with the specific amino acid residues in the cleft indicate their contribution to the specific and high affinity binding through ionic interaction, hydrogen bonding, aromatic stacking, and van der Waals contact. Stabilization of the labile chromophore is likely due to the steric hindrance toward the reactive sites such as the C12 position as well as the epoxide, and, more interestingly, the stabilization interaction between the disulfide group (Cys37 and Cys47) and the acetylenic bond is also suggested.

Introduction

Neocarzinostatin (NCS) isolated from the culture filtrates of *Streptomyces carzinostaticus*[1] is a prominent member of the family of macromolecular antitumor antibiotics that include actinoxanthin (AXN)[2] and auromomycin (AUR).[3] NCS consists of an apoprotein composed of 113 amino acids and a non-amino acid chromophore (Figure 1). The isolated chromophore is extremely labile to heat, light, and higher pH (>6) and has been shown to carry the biological activity of the drug by dam-

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