225 nm (15 000), 246 (9850), 323 (7200), 335 sh (6600).

A sample for analysis was recrystallized from CH_3OH–EtOAc and dried over P_2O_5 at 65 °C under vacuum for 3 h, mp 185 °C.

Anal. Calcd for $C_{18}H_{26}N_4O_8S$: C, 47.15; H, 5.72; N, 12.21. Found: C, 47.13; H, 5.71; N, 12.19.

1,2-Diamino-4-β-D-ribofuranosylpyrazin-2-onium Chloride (6) An aqueous solution of 200 mg of the mesitylate **5** was applied to an AG-1 [Cl^-] column (28 × 250 nm) which was then eluted with H_2O to obtain the chloride salt **6**. Removal of the solvent under reduced pressure and crystallization of the residue from CH_3OH (c) afforded **6**: yield 110 mg (100%); mp >180 °C dec; R_f (silica gel; CH_3OH) 0.30; NMR (Me_2SO-d_6) δ 9.3 (s, 2, exch, NNH_2), 7.4 (d, 1, J = 6.4 Hz, CH), 7.0 (d, 1, J = 6.4 Hz, CH), 6.87 (s, 2, exch, CNH_2), 5.88 (d, 1, J = 2.8 Hz, $C_{1'}$ H), 5.56 (d, 1, exch, OH), 5.29–5.24 (m, 1, exch, OH), 3.96 (m, 3, $C_{2'}$, $C_{3'}$, $C_{4'}$ H), 3.66 (s, 2, $C_{5'}$ H_2); UV λ_{max} at pH 3, 220 nm (ε 9100), 240 sh (6200), 313 (8200), 325 sh (5100); at pH 11, 247 nm (ε 8900), 322 (6800), 335 sh (6400), 350 sh (4000).

A sample for elemental analysis was dried under vacuum at 65 °C for 3 h over P_2O_5.

Anal. Calcd for $C_9H_{15}N_4O_5Cl$: C, 36.68; H, 5.13; N, 19.01. Found: C, 36.46; H, 5.05; N, 18.65.

1-Amino-4-β-D-ribofuranosylpyrazine-2,3-dione (10). A solution of 200 mg (0.82 mmol) of the *N*-aminopyrazinium nucleoside **6** in 4 mL of 0.1 M phosphate buffer (pH 7.4) was incubated in a sealed flask at 37 °C overnight. The dark brown reaction mixture was reduced to dryness under reduced pressure. The residue was dissolved in CH_3OH, and the solution was applied to a 20 × 180 mm dry silica gel column; CH_2Cl_2–CH_3OH (1:1) eluted one component, and CH_3OH removed a small amount of unreacted **6**. The first fraction was recrystallized from CH_3OH–EtOAc: yield 100 mg (47%); mp >120 °C grad dec; IR 3400 (s), 2980 (m), 1680 (s), 1640 (s), 1600 (s) cm^{-1}; NMR (Me_2SO-d_6) δ 6.90 (d, 1, J = 6.4 Hz, CH), 6.65 (d, 1, J = 6.4 Hz, CH), 5.94 (s, 2, exch, NH_2), 5.90 (d, 1, J = 3.7 Hz, $C_{1'}$ H), 5.45 (d, 1, J = 4.3, exch, OH), 5.17–5.07 (m, 2, exch, 2 OH), 3.98–3.89 (m, 3, $C_{2'}$, $C_{3'}$, $C_{4'}$ H), 3.60 (m, $C_{5'}$ H_2); UV λ_{max} at pH 7, 219 nm (ε 9000), 240 sh (5100), 310 (6700), 323 sh (6100), 338 sh (3300).

A sample dried over P_2O_5 at 120 °C under vacuum melted and then resolidified to yellow crystals. This did not alter the NMR spectrum of the sample.

Anal. Calcd for $C_9H_{13}N_3O_6 \cdot 0.5H_2O$: C, 41.34; H, 5.10; N, 16.07. Found: C, 41.26; H, 5.04; N, 16.11.

Cytotoxicity Studies. Cytotoxicity was determined by the inhibition of L1210 murine leukemia cell growth in culture. Cells in the logarithmic phase of growth were harvested, resuspended in RPMI 1640 medium (Grand Island Biochemical Co., Grand Island, NY), 10% with respect to fetal bovine serum (Flow Laboratories, Rockville, MD), and dispensed into 16 × 125 mm culture tubes (final volume: 5.0 mL). A starting density of 17.5 × 10^4 cells/mL permitted 6–7 population doublings during 72 h. A solution (5 × 10^{-3} M) of compound was prepared in phosphate-buffered saline, pH 7.4, and filter was sterilized with a 0.22 μM Swinnex filter (Millipore Corp., Bedford, MA). Following filtration, serial dilutions were made with medium and serum before the analogue was added to the culture tubes (final concentration range: 5 × 10^{-5} to 1 × 10^{-6} M). The growth at 37 °C in control (no analogue) and drug-treated cultures was monitored every 24 h with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

Cytidine Deaminase Studies. Cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) purified from BD_2F_1 mouse kidney was assayed both spectrophotometrically and chromatographically by separation of substrate and product on an ion-exchange column.[27] AG-50-X-8 cation-exchange resin (Bio-Rad) was used to separate labeled compounds, as described.[31] For the present studies, reaction solutions containing 0.15 mM substrate and 10 μg (0.2 unit/mg) of enzyme in a final volume of 0.4 mL of 10 mM (pH 7.6) Tris-HCl buffer containing 5 mM dithiothreitol were incubated at 37 °C for 30 min.

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Registry No. 1, 55321-99-8; **2**, 43029-19-2; **3**, 83831-20-3; 4, 83831-21-4; 4 (isopropylidene deriv), 83831-22-5; **5**, 83831-24-7; **6**, 83831-25-8; **10**, 83831-26-9; glyoxal sodium bisulfite, 517-21-5; aminomalonamide, 62009-47-6; 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, 6974-32-9.

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Syntheses and Antitumor Activity of 2-Deoxyribofuranosides of 3-Deazaguanine

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Synthesis of 2-deoxyribofuranosides of 3-deazaguanine (IX–XII) has been achieved by a base-catalyzed ring closure of appropriate 2-deoxyribofuranosides of methyl 5(4)-(cyanomethyl)imidazole-4(5)-carboxalate (IV–VII). The separation of isomers and anomers were accomplished by column chromatography and HPLC. The site of glycosidic linkage and the anomeric configurations were established on the basis of C-13 and proton magnetic resonance spectroscopy, as well as UV absorption characteristics. Preliminary results of the antitumor activity of these derivatives, in vitro and in vivo, are described.

The synthesis of 3-deazaguanine[1,2] [6-aminoimidazo[4,5-*c*]pyridin-4(5*H*)-one (DG)] its ribosides[1,3] [6-amino-1-β-D-ribofuranosylimidazo[4,5-*c*]pyridin-4(5*H*)-one (9-DGR) and 6-amino-3-β-D-ribofuranosylimidazo[4,5-*c*]-pyridin-4(5*H*)-one (7-DGR)], arabinosides[4] [6-amino-1-β-D-arabinofuranosylimidazo[4,5-*c*]pyridin-4(5*H*)-one (9-araDG) and 6-amino-3-β-D-arabinofuranosylimidazo[4,5-*c*]-pyridin-4(5*H*)-one (7-araDG)], and related derivatives[5] have been described. DG and 9-DGR have been reported to exhibit a broad spectrum antiviral activity against both RNA and DNA viruses, in vitro.[5b,6] DG was also active against influenza types A and B and parainfluenza type I virus infection in mice.[5b,6] DG inhibited the growth of

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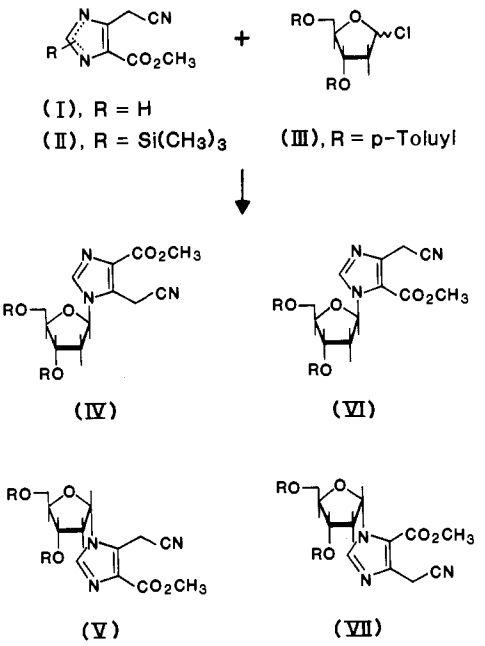
Escherichia coli B in vitro but did not exhibit any antibacterial activity in vivo,[5b,7,8] whereas 7-DGR showed antibacterial activity against several Gram-negative strains without any apparent toxicity to the host. It is interesting to note that 9-DGR was inactive under these conditions. The antibacterial activity of 7-DGR has been ascribed to its selective cleavage to DG in *E. coli* B infected cells.[9] Our studies have shown that DG is a potent inhibitor of a number of slow and rapid growing murine solid tumors, especially those that are models for postoperative breast cancer.[10] It has been proposed that antitumor activity of DG may be a consequence of its incorporation into tumor-cell DNA.[11] These observations suggested the need for the synthesis of 2-deoxyribose nucleosides of DG as potentially useful antineoplastic and/or antibacterial agents. In this paper,[12] we report the synthesis and biological evaluation of 6-amino-1-(2′-deoxy-β-D-ribofuranosyl)imidazo[4,5-*c*]pyridin-4(5*H*)-one (2′-deoxy-3-deazaguanosine, 9-DGdR, IX), its α anomer (9-DGdR-α, X), as well as 6-amino-3-(2′-deoxy-β-D-ribofuranosyl)-imidazo[4,5-*c*]pyridin-4(5*H*)-one (7-DGdR, XI) and its α anomer (7-DGdR-α, XII).

Chemistry. The approaches utilized for the synthesis of these compounds were similar to the procedure used earlier for the synthesis of 3-deazaguanosine.[1,3] The key intermediate, methyl 5(4)-(cyanomethyl)imidazole-4(5)-carboxylate (I) was synthesized from dimethyl acetonedicarboxylate according to the procedure of Robins et al.[13] Compound I was silylated with hexamethyldisilazane, and the silylated product (II) was reacted with 2-deoxy-3,5-di-*O*-*p*-toluyl-D-*erythro*-pentosyl chloride[14] (III) under a variety of conditions. In the first set of reactions, equimolar amounts of compounds II and III (dissolved in a solvent) were stirred under anhydrous conditions, either at ambient temperature (in dichloroethane) or under reflux (in benzene), for various intervals of time. In another set of experiments, II and III were fused at high temperature, either in the presence or in the absence of an acid catalyst. In the third set of conditions, clear solutions of II and III were mixed and allowed to react at ambient temperature in the presence of different amounts of anhydrous $SnCl_4$. Under all of these conditions (Scheme I), deoxyribosylation had occurred at both ring nitrogens of the imidazole (II)

Scheme I



and produced a mixture of positional isomers. The ratio of N-1 isomer [methyl 5-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β- and -α-D-*erythro*-pentosyl)imidazole-4-carboxylate, IV and V] to N_3 isomer [methyl 4-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β- and -α-D-*erythro*-pentosyl)imidazole-5-carboxylate, VI and VII] varied, depending upon the conditions used. The first two sets of reaction conditions favored the formation of N-3 isomer (VI and VII), and the yield for both of the isomers ranged between 25 and 36%. When the reaction were performed in the presence of $SnCl_4$, the formation of the desired N-1 isomer was favored, and the overall yield was improved. Thus, treatment of 1 equiv each of II and III, in the presence of 0.35 and 0.70 molar equiv of $SnCl_4$ afforded an equal ratio of N-1 (IV and V) and N-3 (VI and VII) isomers in 45 and 63% yield, respectively. Further increase of $SnCl_4$ to 1.4 equiv produced a 2:1 ratio of N-1 to N-3 isomer, and an improved total yield of 71% was obtained. Further increases in the amounts of $SnCl_4$ resulted in the precipitation of the aglycon (compound II) and precluded any reaction. It may be noted that under similar reaction conditions when protected ribose was reacted with II, N-1 isomer was formed exclusively.[1] This regiospecific ribosylation was explained on the basis of the interaction of silylated imidazole (II) with $SnCl_4$ in a fashion that facilitated the reaction at N-1. Although we were unable to synthesize the N-1 isomer exclusively, it is clear that the use of $SnCl_4$ has resulted in increased N-1 deoxyribosylation.

The exclusive formation of β-nucleoside during the synthesis of ribofuranosides and glycopyranosides is due to the direct effect of the adjacent 2′-*O*-acyl group through an acyloxonium ion intermediate.[15] However, in the case of 2′-deoxy-D-*erythro*-pentofuranosyl nucleosides, various factors, including solvent, temperature, protecting group, etc., seem to influence the anomeric ratios of the prod-

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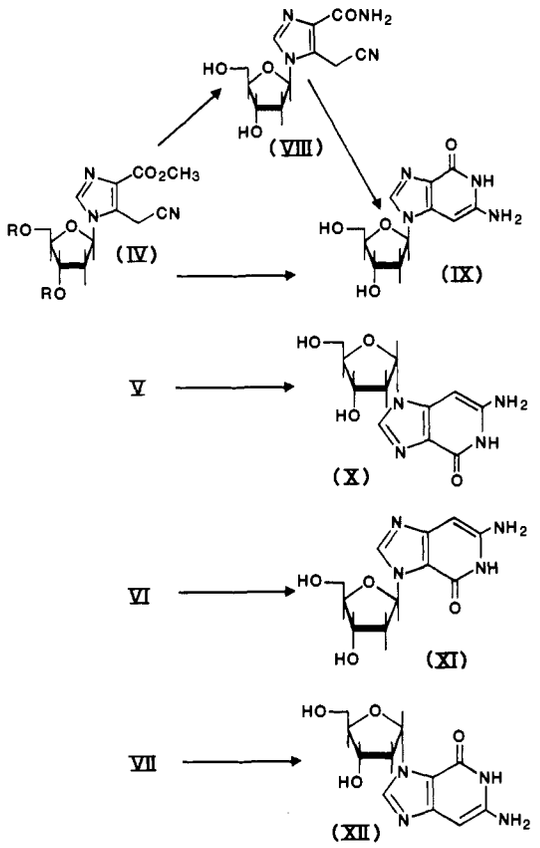
Scheme II



ucts.[16] It has been reported that a protecting group may influence the control of anomeric formation either through steric or participating group effects.[17] In our studies, the predominant formation of β-nucleosides (IV and VI) could be explained due to the participation of the 3′-*O*-toluyl group in producing the six-membered acyloxonium ion, which facilitated the formation of β-nucleosides.

Purification of the reaction products was tedious and was accomplished by multistep silica gel column chromatography. The separation of N-1 and N-3 isomers was achieved by a silica gel column chromatography and was based upon the observation that the N-1 isomer, being more polar, was adsorbed more tightly onto the column. Each isomer, being a mixture of α and β anomers, was further resolved by preparative high-performance liquid chromatography to obtain pure anomers. In a typical experiment, in which 1.4 molar equiv of $SnCl_4$ was used for glycosidation reaction, the yields of isolated pure products were found to be: IV, 39%; V, 8%; VI, 21%; VII, 3%. Under these conditions, the N-1 isomer and β anomer in each of the positional isomers were predominantly formed.

Base-catalyzed cyclization of each of the imidazole nucleosides produced the corresponding 3-deazaguanine 2′-deoxyribofuranoside (Scheme II). Thus, treatment of the blocked imidazole nucleoside IV with anhydrous liquid ammonium (20 h, 110 °C) gave 2′-deoxy-3-deazaguanosine (IX) (in 47% yield). Alternatively, the reaction was stopped after 4 h and the intermediate, 5-(cyanomethyl)-1-(2′-deoxy-β-D-ribofuranosyl)imidazole-4-carboxamide (VIII), was isolated by silica gel column chromatography in 44% yield. (This reaction, unlike the corresponding imidazole riboside, contained a 12% impurity of IX.) The cyclization of VIII to IX proceeded smoothly (61% yield). However, unlike 3-deazaguanosine,[1] product IX could not be crystallized and was purified by column chromatography. Similarly, the corresponding α-anomer V, as well as its positional isomers (VI and VII), were heated with liquid ammonia, and the reaction mixture was refluxed with aqueous Na_2CO_3–ethanol to affect the ring closure to provide the corresponding products X–XII in good yields.

Table I. ^{13}C NMR Chemical Shifts of Imidazole Ring Carbon Atoms for the Base Anion I and Its Nucleosides IV–VII

	chemical shift,[a] ppm		
compd	C-2	C-4	C-5
I	142.9	124.2	135.8
IV	138.0 (136.5)	126.6 (126.8)	129.3 (129.4)
V	137.5 (136.5)	127.4 (126.8)	128.6 (129.4)
VI	136.8 (136.5)	118.1 (117.8)	137.2 (138.4)
VII	136.9 (136.5)	116.9 (117.8)	137.8 (138.4)

[a] Values in parentheses are predicted chemical shifts calculated according to the rules in ref 19b.

The site of the deoxyribosylation and the anomeric configuration at carbon-1′ of the imidazole nucleosides IV–VII (and, therefore, of the corresponding cyclized products IX–XII) were established by ^1H and ^{13}C NMR spectroscopy. It has been demonstrated that the site of alkylation or glycosidation in both five- and six-membered heterocycles can be determined by ^{13}C NMR spectroscopy.[18] The assignments were made on the basis of the α- and β-substitution shifts observed when the neutral species are compared with the anion of the parent base.[19] It has been reported that when the N-1 position was substituted in an imidazole, carbon-4, being β, would exhibit a downfield (2–3 ppm) chemical shift, and carbon-5, being α, would exhibit an upfield (6–7 ppm) chemical shift when compared to the base anion. In contrast, when N-3 of an imidazole was substituted, carbon-4, being α, would exhibit an upfield (6–7 pm) chemical shift and carbon-5, being β, would exhibit a downfield (2–3 ppm) chemical shift in comparison to the base anion.[19c] Table I gives the pertinent carbon-13 chemical shifts of the anion of methyl 5-(cyanomethyl)imidazole-4-carboxylate (I) and its deoxyribosylated products (IV–VII). The chemical-shift values for carbon-2 in all the four products are upfield (5–6 ppm) when compared with the base anion. A comparison of the chemical shifts values of carbon-4 and carbon-5 of the products shows that the compounds IV and V exhibit a downfield chemical shift (2–3 ppm) for carbon-4 and an upfield chemical shift (6–7 ppm) for carbon-5, whereas compounds VI and VII, in contrast, exhibit an upfield chemical shift (6–7 ppm) for carbon-4 and a downfield chemical shift (1–2 ppm) for carbon-5. These results lead us to the assignment of N-1 deoxyriboside to IV and V and

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Table II. Effect of 3-Deazaguanine and Its Derivatives on the Growth of Leukemia L1210 Cells in Culture

drug	ED_{50},[a] μM
3-deazaguanine	11 (±0.23)
9-DGdR (IX)	9 (±0.21)
7-DGdR (XI)	950 (±4.00)
9-DGR	7 (±0.11)
7-DGR	750 (±8.00)

[a] Effective dose 50 is the concentration of drug in the culture media that produced 50% inhibition of the leukemia cell growth (48-h incubation period) as compared to the untreated controls. The mean plus or minus standard errors are presented in parentheses.

N-3 deoxyriboside to VI and VII.

Further support for the assignment of the structure of the imidazole deoxynucleosides IV–VII was obtained when the anisotropic effect of an ester carbonyl group, in the aglycon moiety, on the chemical shift of the anomeric proton of the deoxyribose was considered. The method has been used successfully to ascertain the ribosylation site in various types of nucleosides.[18a,20] The comparison of ^1H NMR spectra of compounds IV–VII revealed that when the deoxyribose was attached to the nitrogen atom adjacent to the carbonyl function, as in VI and VII, an expected downfield shift of the anomeric proton (0.21–0.24 ppm) was observed as compared with the respective positional isomers IV and V.

The anomeric configurations of the cyclized products IX–XII were determined from their ^1H NMR spectra. The patterns of the anomeric proton ($H_{1'}$) signals followed the convention observed with other 2'-deoxyribonucleosides.[21] Thus, compound IX exhibited a pseudotriplet centered at 6.1 ppm (peak width 13.5 Hz), indicating a β configuration, while compound X gave a quartet centered at 6.0 ppm (peak width 10.5 Hz), indicating it to be the α anomer. Similarly, compound XI gave a pseudotriplet at 6.68 ppm (peak width 13.0 Hz), and compound XII exhibited a quartet at 6.55 ppm (peak width 10.2 Hz), indicating the compounds to be β and α anomers, respectively. Peak widths of these compounds were equally characteristic and provided a basis for the assignments of β (IX, XI) and α (X, XII) configuration.

The UV absorption characteristics of compounds IX–XII were compared with the UV data of 9-DGR and 7-DGR.[1,3] The UV maxima of compounds IX and X were identical with 9-DGR [λ_{max} (pH 7) 270 and 298 nm], and the UV maxima of compounds XI and XII were identical with 7-DGR [λ_{max} (pH 7) 258 and 317 nm], confirming the structural assignments of these products.

Biological Studies. In cell culture studies (Table II), 9-DGdR (IX) was a somewhat better inhibitor of the growth of leukemia L1210 than 3-deazaguanine. The 7-deoxyribosyl derivative XI, as expected, was about 100-fold less active than IX. In animal model studies (Table III), IX at 80 mg/kg (nine daily treatments) caused a 69% inhibition of the growth of C3H mammary adenocarcinoma 16/C. The tumor growth inhibition is significant but less than that caused by optimal treatments with 3-deazaguanine.[11] Further studies are needed to fully establish optimal treatment regimens and the spectrum of the antitumor activity of this compound.

Experimental Section

NMR spectra were recorded on Varian EM-390 and XL-100 spectrometers with Me_3Si as an internal standard. Ultraviolet spectra were determined on a Cary 118c UV spectrophotometer, and IR spectra were taken on a Beckman 4210 spectrometer. Melting points were determined on a Buchi SMP-20 capillary melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel plates (1 B2-F Bakerflex or Kieselgel 60 F-254) in ascending fashion, and the values of the solvents in various solvent systems are volume to volume. Preparative TLC was accomplished on 2-mm silica gel F-254 (E. Merck) plates. Column chromatography was performed on silica gel 60 (70–230 mesh) EM reagent material. Analytical and semipreparative HPLC was carried out on Micromeretic instrument 7000B. Altex pump 110A equipped with preparative head was used for solvent delivery to lobar columns. Elemental analysis were performed by Galbraith Laboratories, Inc., Knoxville, TN, and are within ±0.4% of the required values.

Methyl 5-(Cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β- and -α-D-*erythro*-pentosyl)imidazole-4-carboxylate (IV and V, respectively) and Methyl 4-(Cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β- and -α-D-*erythro*-pentosyl)imidazole-5-carboxylate (VI and VII). Method A. A mixture of methyl 5(4)-(cyanomethyl)imidazole-4(5)-carboxylate (I; 6.6 g, 40 mmol), hexamethyldisilazane (100 mL), and ammonium sulfate (0.25 g) was refluxed for 4 h under anhydrous conditions. Excess of hexamethyldisilazane was removed under reduced pressure to provide trimethylsilyl derivative II as a brownish oil. The oil was dissolved in acetonitrile (200 mL) and added to a solution of 2-deoxy-3,5-di-*O*-*p*-toluyl-D-*erythro*-pentosyl chloride (III; 15.6 g, 40 mmol) in dichloroethane (300 mL). The reaction mixture was stirred for 10 min before the addition of anhydrous $SnCl_4$ (6.6 mL, 56 mmol) and left at ambient temperature for 18 h. Chromatography [TLC, silica gel, $CHCl_3$–hexane, (2:1) and $CHCl_3$–methanol (4:1)] of an aliquot (treated with MeOH) revealed almost complete conversion of starting base and sugar to a number of new products. The reaction mixture was evaporated, and the residue was redissolved in $CHCl_3$ (600 mL) and treated with a cold saturated Na_2CO_3 solution (200 mL). The mixture was filtered through Celite, and the residue was washed with $CHCl_3$ (3 × 100 mL). The combined $CHCl_3$ extract was washed with H_2O (1 × 100 mL), dried (Na_2SO_4), and filtered, and the filtrate was evaporated under reduced pressure to obtain a pale product (22.0 g, >100%). This material was a mixture of compounds IV–VII, as well as some sugar residues.

Purification of Isomers and Anomers. The crude product was evaporated with silica gel (50 g), and the residue was added on a dry silica gel (500 g) column. The column was eluted successively with hexane (2000 mL), hexane–ethyl acetate (3:1, 4000 mL), hexane–ethyl acetate (1:1, 4000 mL), and finally with hexane–ethyl acetate–MeOH (10:10:1, 5000 mL). The eluates were collected as 20-mL fractions, which were monitored by UV absorption. The UV-absorbing fractions were pooled and analyzed. The elution pattern followed from relatively nonpolar sugar residues to more polar imidazole base (I). The anomeric mixture of methyl 4-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-D-*erythro*-pentosyl)imidazole-5-carboxylate (VI and VII) came off the column with hexane–ethyl acetate (1:1, white foam, 5.2 g); MeOH (5%) was added to the above solvent mixture to elute the more polar anomeric mixture of compounds IV and V (pale yellow foam, 9.5 g). The overall yield was over 70% (both the isomers).

The isomers were further chromatographed for their respective α- and β-anomer separation. The anomeric mixture of IV and V (1.0 g) was fractionated on a prepacked Lobar LiChroprep Si 60 (particle size 40–63 μm, EM Reagents) column (preequilibrated with hexane). The column was eluted with a gradient of hexane–ethyl acetate (2:1 to 1:1). The appropriate fractions were pooled and evaporated to obtain methyl 5-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β-D-*erythro*-pentosyl)imidazole-4-carboxylate (IV) as white foamy solid (0.83 g) [mp 60–62 °C; UV λ_{max} ($CHCl_3$) 244 nm (ε 37 078); ^1H NMR ($CDCl_3$) δ 2.38 (s, 6 $2CH_3$), 3.88 (s, 3, OCH_3), 4.55 (s, 2, CH_2), 6.37 (d, 1, J = 7 Hz, $H_{1'}$), 7.25 (m, 4 Ph), 7.71 (s, 1 C_2 H), 7.81 (m, 4, Ph). Anal. ($C_{28}H_{27}N_3O_7$) C, H,

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Table III. Effect of 3-Deazaguanine and Its Derivatives on the Growth of C3H Mammary Adenocarcinoma 16/C (sc) in B6C3F$_1$ Female Mice

treatment ip	tumor wt, g (day 17)	Δ wt (day 9)	animals with palpable tumors (day 17)	tumor growth inhibn, %
controls, 0.2 M phosphate buffer 0.2 mL, qd (1-9)	3.60	+1.70	10/10	
3-deazaguanine 80 mg/kg, qd (1-9)	0.25	+0.06	5/6	93.1
9-DGdR (IX) 40 mg/kg, qd (1-9)	2.70	+0.58	6/6	25.0
80 mg/kg, qd (1-9)	1.09	+1.20	6/6	69.7
7-DGdR (XI) 50 mg/kg, qd (1-9)	2.95	+0.72	6/6	18.1
100 mg/kg, qd	3.50	+0.95	6/6	2.8

N] as well as its α anomer, namely, methyl 5-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-α-D-*erythro*-pentosyl)imidazole-4-carboxylate (V), as a white solid (0.17 g) [mp 58–60 °C UV λ_{max} ($CHCl_3$) 245 nm (ε 33 605); ^{1}H NMR ($CDCl_3$) δ 2.39 and 2.40 (s, 6, $2CH_3$), 3.86 (s, 3, OCH_3), 4.61 (s, 2, CH_2), 6.22 (m, 1, J = 5 Hz, $H_{1'}$) 7.2 (m, 4, Ph), 7.71 (s, 1, C_2 H), 7.84 (m, 4, Ph). Anal. ($C_{28}H_{27}N_3O_7$) C, H, N].

Similarly, the anomeric mixture of the other isomer (VI and VII, 1.5 g) was chromatographed on a column (3 × 45 cm) dry packed with polygosil 60 (particle size 25–40 μm) silica gel (Macherey–Nagel) (equilibrated with hexane overnight). The column was eluted with a gradient of hexane–ethyl acetate (6:1 to 3:1) to obtain pure methyl 4-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-β-D-*erythro*-pentosyl)imidazole-5-carboxylate (VI) as white crystalline product (1.31 g) [mp 144–147 °C; UV λ_{max} ($CHCl_3$) 247 nm (ε 37 854); ^{1}H NMR ($CDCl_3$) λ_{max} 2.39 and 2.41 (s, 6, $2CH_3$), 3.90 (s, 3, OCH_3), 4.45 (s, 2, CH_2), 6.73 (d, 1, J = 8 Hz, $H_{1'}$), 7.30 (m, 4, Ph), 7.55 (s, 1, C_2 H) and 7.91 (m, 4, Ph). Anal. ($C_{28}H_{27}N_3O_7$) C, H, N] as well as methyl 4-(cyanomethyl)-1-(3,5-di-*O*-*p*-toluyl-α-D-*erythro*-pentosyl)imidazole-5-carboxylate (VII) as crystalline solid (0.19 g) [mp 133–135 °C; UV λ_{max} ($CHCl_3$) 246 nm (ε 35 517); ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 6, $2CH_3$), 3.92 (s, 3, OCH_3), 4.60 (s, 2, CH_2), 6.62 (d, 1, J = 6 Hz, $H_{1'}$), 7.28 (m, 4, Ph), 7.80 (s, 1, C_2 H), 7.98 (m, 4, Ph). Anal. ($C_{28}H_{27}N_3O_7$) C, H, N.

Under similar deoxyribosylation reaction conditions, equimolar quantities of II and III were reacted in the presence of either 0.35 or 0.70 molar equiv of $SnCl_4$. The reaction products were resolved by column chromatography, for the positional isomers as described above. In each reaction the formation of N-1 to N-3 isomer was similar (1:1 ratio) with 45 and 63% yields, respectively.

Method B. Silylated methyl 5(4)-(cyanomethyl)imidazole-4-(5)carboxylate (II, 10 mmol) was stirred under (anhydrous conditions) with 2-deoxy-3,5-di-*O*-*p*-toluyl-D-*erythro*-pentosyl chloride (10 mmol) in dichloroethane (250 mL) for 72 h at ambient temperature. The reaction dissolved in $CHCl_3$ (30 mL) and subjected to column chromatography as detailed in method A to provide purified N-1 (IV and V, 0.20 g) and N_3 isomers (VI and VII, 1.68 g) in 36% yield.

In another set of experiments silylated II (10 mmol) was refluxed with equimolar amounts of halogenose III in benzene (300 mL) for 24 h. The reaction products were purified as described above to obtain N-1 (0.22 g) and N-3 isomers (1.07 g) in 25% yield.

Method C. 5(4)-(Cyanomethyl)imidazole-4(5)carboxylate (1.00 g, 6 mmol) was silylated, mixed with halogenose III (2.30 g, 6 mmol), and heated with stirring (170–180 °C) until a clear melt was obtained. experiments were performed in the absence or in the presence of bis(*p*-nitrophenyl) phosphate (0.02 g), under reduced pressure (30 min). The reaction mixtures were allowed to cool to ambient temperature and were extracted with $CHCl_3$ (3 × 25 mL). Each reaction mixture was purified by column chromatography as described above to obtain pure N-1 (0.27 g) and N-3 (0.51 g) isomers in 25% yield.

5-(Cyanomethyl)-1-(2′-deoxy-β-D-ribofuranosyl)-imidazole-4-carboxamide (VIII). Compound IV (2.50 g, 5 mmol) was heated (110 °C, oil bath temperature) with liquid ammonia (80 mL) in a sealed stainless-steel bomb for 4 h. The ammonia was allowed to evaporate at room temperature and the residue was kept under vacuum overnight to remove the last traces of ammonia. Thin-layer chromatography [silica gel, $CHCl_3$–CH_3OH (4:1)] showed no component corresponding to the starting material but a considerable amount (15–20%) of 2′-deoxyribofuranosyl-3-deazaguanine (IX) was formed. The dark-colored residue was extracted with hot ether (6 × 50 mL) to remove *p*-toluamide, and the residue was dissolved in MeOH, adsorbed on silica gel (25 g), and placed on a silica gel (250 g) column in $CHCl_3$. The column was eluted with $CHCl_3$–MeOH (4:1), and the appropriate UV-absorbing fractions were pooled and evaporated under vacuum to a small volume and kept in a refrigerator overnight. The crystallized product was filtered to obtain 0.58 g (44%) of VIII: mp 164–167 °C; IR (KBr) 2256 (w, C≡N), 1660 (s, C=O) cm^{-1}; UV λ_{max} (MeOH) 234 nm (ε 8650); ^{1}H NMR (D_2O) δ 4.22 (s, 2, CH_2), 6.1 (t, 1, J = 5 Hz, $H_{1'}$), 7.88 (s, 1, C_2H).

6-Amino-1-(2′-deoxy-β-D-ribofuranosyl)imidazole[4,5-*c*]-pyridin-4(5*H*)-one (2′-Deoxy-3-deazaguanosine, IX). Method A. A mixture of VIII (1.3 g, 5 mmol), 10% aqueous Na_2CO_3 (20 mL), and ethanol (10 mL) was heated under reflux for 40 min, and the pale solution was filtered. The product could not be crystallized. The reaction mixture was treated with Dowex-50 (H^+) and filtered, and the resin was washed with water (2 × 5 mL). The washings and the filtrate were combined, evaporated to dryness under vacuum, and coevaporated with MeOH (3 × 10 mL). The residue (dissolved in MeOH) was adsorbed on silica gel (20 g) and added to a silica gel column (300 g) packed in $CHCl_3$–MeOH (4:1). Elution with a gradient, of $CHCl_3$–MeOH (4:1) to MeOH, provided the pure nucleoside IX as the major UV-absorbing peak. The peak was pooled and evaporated to obtain a beige solid (0.80 g, 61%): mp >250 °C; UV λ_{max} (pH 7) 271 and 301 nm (ε 10 500 and 8400); UV λ_{max} (pH 1) 284 and 310 nm (ε 10 900 and 6600); UV λ_{max} (pH 12) 274 and 296 (sh) nm (ε 10 500 and 8500); ^{1}H NMR (Me_2SO-d_6) δ 5.62 (s, 1, C_7 H), 5.80 (br, 2, NH_2), 6.10 (t, 1, J = 6.5 Hz, $H_{1'}$, peak width = 13.5 Hz), 7.92 (s, 1, C_2 H) and 10.42 (br, 1, NH). Anal. $C_{11}H_{14}N_4O_4 \cdot 1.5H_2O$) C, H, N.

Method B. Compound IV (5.1 g, 10 mmol) and liquid ammonia (110 mL) were stirred and heated (120 °C, bath temperature) in a stainless-steel bomb for 20 h. The ammonia was allowed to evaporate at room temperature, and the dark residue was left under high vacuum overnight to remove the last traces of ammonia. The residue was extracted with ether (6 × 50 mL) to remove the bulk of *p*-toluamide, and the remaining reside was chromatographed as described in method A. Appropriate fractions were pooled, and the solution was evaporated to obtain 1.25 g (47%) of pure product IX. This material was idential with 2′-deoxy-3-deazaguanosine prepared by method A.

6-Amino-1-(2′-deoxy-α-D-ribofuranosyl)imidazo[4,5-*c*]-pyridin-4(5*H*)-one (X). Compound V (0.34 g, 0.65 mmol) was heated (110 °C bath temperature) with liquid ammonia (10 mL, 6 h) in a stainless-steel bomb. Then the ammonia was evaporated, the residue was suspended in 10% aqueous Na_2CO_3 (4 mL) and ethanol (2 mL), and the mixture was heated under reflux for 30 min. The product was purified as described in method A for IX to obtain 0.055 g (32%) of X: mp >250 °C; UV λ_{max} (pH 7) 270 and 301 nm (ε 10 600 and 8550); UV λ_{max} (pH 12) 273 and 299 (sh) nm (ε 10 600 and 8500); UV λ_{max} (pH 1) 284 and 312 nm (ε 11 050 and 6700); ^{1}H NMR (D_2O) δ 8.15 (s, 1, C_2 H), 6.00 (q, 1, peak width 10.5 Hz) and 5.72 (s, 1, C_7 H).

6-Amino-3-(2′-deoxy-β-D-ribofuranosyl)imidazo[4,5-*c*]-pyridin-4(5*H*)-one (XI). Compound VI (6.2 g, 12 mmol) was

heated with anhydrous ammonia (80 mL) for 18 h in a stainless-steel bomb. The next day the ammonia was evaporated, and the residue was refluxed with ethanolic aqueous Na_2CO_3. The product was purified by silica gel column chromatography as described above to obtain 1.35 g (42%) of XI as a pale green powder: mp >250 °C; UV λ_{max} (pH 7) 259 and 316 nm (ϵ 6150 and 7200); UV λ_{max} (pH 12) 259 and 316 nm (ϵ 6100 and 7000); UV λ_{max} (pH 1) 278 and 317 (ϵ 10970 and 5700); ^{1}H NMR (Me_2SO-d_6) δ 8.20 (s, 1, C_2 H), 6.68 (t, 1, J = 6 Hz, $H_{1'}$, peak width = 13 Hz) and 5.50 (s, 1, C_7 H). Anal. ($C_{11}H_{14}N_4O_4 \cdot H_2O$) C, H, N.

6-Amino-3-(2′-deoxy-α-D-ribofuranosyl)imidazo[4,5-*c*]pyridin-4(5*H*)-one (XII). Compound VII (0.25 g, 0.5 mmol) was cyclized as described for XI above. The product (XII) was purified by preparative TLC [2-mm silica gel, developed in $CHCl_3$–MeOH (2:1)] to obtain 0.048 g (47%) of XII as a beige foam: UV λ_{max} (pH 7) 258 and 314 nm; UV λ_{max} (pH 12) 257 and 313 nm; UV λ_{max} (pH 1) 277 and 314 nm; ^{1}H NMR (D_2O) δ 8.18 (s, 1, C_2 H), 6.55 (q, 1, peak width = 10.2 Hz) and 5.77 (s, 1, C_7 H).

Cell Culture. Leukemia L1210 cells were obtained from Associated Biomedic Systems, Inc., Buffalo, NY. The cells were maintained in RPMI-1640 media containing 10% fetal calf serum and 1% (v/v) penicillin–streptomycin (supplies were obtained from Grand Island Biological Co., Grand Island, NY) in a carbon dioxide incubator at 37 °C. The cells had a doubling time of 8–10 h. Our methods for drug screening have been described previously.[22]

Animal Model Studies. All animals were obtained through C. R. Reeder, Division of Mammalian Genetics and Animal Production, National Cancer Institute, Bethesda, MD. The tumor line, C3H mammary adenocarcinoma 16/C, was obtained from Dr. T. Corbett, Southern Research Institute, Birmingham, AL, and maintained (lung-passed) in C3H female mice according to the method of Corbett et al.[23] For drug testing, the tumors (2- to 4-mm^3 freshly harvested fragments) were transplanted (subcutaneously) in 18- to 20-g B6C3F$_1$ female mice on day 0. The following day, the animals were randomized into various treatment groups. The drugs were dissolved in 0.2 M phosphate buffer and administered by intraperitoneal injections. The tumor weights were obtained by measuring the length (l) and width (w) of each tumor with a calliper and using the conventional $(l + w^2)/2$ formula.

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Registry No. I, 56039-06-6; II (isomer 1), 56596-91-9; II (isomer 2), 58459-35-1; III (α), 83587-57-9; III (β), 83587-65-9; IV, 83587-58-0; V, 83587-59-1; VI, 83603-90-1; VII, 83587-60-4; VIII, 83587-61-5; IX, 961-07-9; X, 83587-62-6; XII, 83587-63-7; XIII, 83587-64-8.

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A Synthetic Approach to Poly(γ-glutamyl) Conjugates of Methotrexate

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Methotrexate poly(γ-L-glutamate)s bearing two and three glutamate units above that present in methotrexate have been synthesized by extension of a previously described route used to synthesize the lower conjugate bearing one added glutamate unit. Key steps in the sequence are the peptide coupling of *N*-[4-[[(benzyloxy)carbonyl]-methylamino]benzoyl]-L-glutamic acid α-benzyl ester (**5**) with oligo(γ-L-glutamate) benzyl esters, removal of blocking groups by catalytic hydrogenolysis, and introduction of the (2,4-diamino-6-pteridinyl)methyl grouping by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide. Elaboration of the required oligo(γ-L-glutamate) chain was achieved one unit at a time, beginning with the coupling of L-glutamic acid dibenzyl ester with [(*tert*-butyloxy)carbonyl]-L-glutamic acid α-benzyl ester (**7**), followed by selective removal of the *tert*-butyloxycarbonyl grouping and another coupling step with **5** or **7** as required. Diphenylphosphoryl azide was used as the coupling reagent in each conversion producing a peptide linkage.

Intracellular conversion of methotrexate (MTX) to poly(γ-glutamyl) peptide derivatives has been shown to occur in a variety of animal and human tissues.[1-9] The poly(γ-glutamate)s are known to inhibit dihydrofolate reductase as strongly as MTX itself[2b,9-11] and also to inhibit thymidylate synthetase,[12] but many aspects of their biochemical actions remain to be elucidated. Current studies on their identification, extent of in vivo synthesis, transport characteristics, and role in antifolate activity led to a need for pure reference samples of authentic MTX poly(γ-glutamate)s. We describe in this paper a synthetic approach that allows unequivocal syntheses of these compounds.

The approach is based on syntheses of α- and γ-substituted peptides and amides of MTX, which involved the

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