Deoxy Sugar Analogues of Triciribine: Correlation of Antiviral and Antiproliferative Activity with Intracellular Phosphorylation

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Triciribine (TCN) and triciribine monophosphate (TCN-P) have antiviral and antineoplastic activity at low micromolar or submicromolar concentrations. In an effort to improve and better understand this activity, we have conducted a structure−activity relationship study to explore requirements for the number of hydroxyl groups on the ribosyl moiety for biological activity. 2′-Deoxytriciribine (2′-dTCN), 3′-deoxytriciribine (3′-dTCN), 2′,3′-epoxytriciribine (2′,3′-epoxyTCN), 2′,3′-dideoxy-2′,3′-didehydrotriciribine (2′,3′-d4TCN), and 2′,3′-dideoxytriciribine (2′,3′-ddTCN) were synthesized and evaluated for activity against human immunodeficiency virus (HIV-1), herpes simplex virus type 1 (HSV-1), and human cytomegalovirus (HCMV). Antiproliferative activity of the compounds also was tested in murine L1210 cells and three human tumor cell lines. All compounds were either less active than TCN and TCN-P or inactive at the highest concentration tested (100 μM) in both antiviral and antiproliferative assays. Reverse-phase HPLC of extracts from uninfected cells treated with the deoxytriciribine analogues only detected the conversion of 3′-dTCN and 2′,3′-ddTCN to their respective monophosphates. Therefore, either the deoxytriciribine analogues were not transported across the cell membrane or, more likely, they were not substrates for a nucleoside kinase or phosphotransferase. We have concluded that the hydroxyl groups on the ribosyl ring system of TCN and TCN-P must be intact in order to obtain significant antiviral and antineoplastic activity.

Introduction

Triciribine (TCN) is a tricyclic nucleoside that was first synthesized by Schram and Townsend in 1971.[1] Initial testing of triciribine and the water-soluble prodrug, triciribine-5′-monophosphate[2] (TCN-P), against murine leukemic L1210 cells revealed their potential as antineoplastic agents. This discovery led to extensive in vitro[3−17] and in vivo[18−22] studies of TCN and TCN-P as novel antineoplastic agents. Phase I clinical trials were completed with TCN-P,[23−29] and it was advanced to phase II studies as a potential antineoplastic agent.[27,29−32]

Early studies revealed that TCN is converted intracellularly to TCN-P by adenosine kinase.[4,6,7] Phosphorylation is essential for antineoplastic activity as demonstrated by the absence of growth inhibition when adenosine kinase-deficient cells were treated with TCN.[3,11,12] The activity of TCN-P also requires adenosine kinase because extracellular TCN-P is a charged species and does not cross the cell membrane, and therefore it must be dephosphorylated to TCN by extracellular phosphatases or cellular ecto-5′-nucleotidase and rephosphorylated to TCN-P by intracellular adenosine kinase.[7,12] Unlike other nucleoside analogues, TCN is metabolized only to the monophosphate and not to di- or triphosphate forms.[9] Furthermore, no incorporation of TCN into nucleic acids has been observed.[16] TCN-P inhibits both DNA and protein synthesis,[15,16] but the exact mechanism is unknown.

More recently, we have found that TCN and TCN-P are selective and potent inhibitors of HIV-1 and HIV-2 in acutely and persistently infected cells.[33] These studies also found no cross-resistance to TCN or TCN-P in HIV strains resistant to the reverse transcriptase (RT) inhibitors AZT and TIBO[33] suggesting that TCN and TCN-P have a different mode of action than AZT and TIBO. Furthermore, cytotoxicity such as that observed in murine L1210 cells appears to be highly cell line-specific[3] and was not observed in human cell lines used to propagate HIV and human cytomegalovirus (HCMV).[33] Even though TCN was not very cytotoxic in these cell lines, it must be phosphorylated to TCN-P to be active against HIV-1.[34] Studies on the antiviral mechanism of action of TCN are currently underway and suggest inhibition of the function of an HIV accessory protein.[35]

Since 1971, the only analogues of TCN to have been synthesized and evaluated have been 7-azaTCN analogues,[36−40] which were found[39] to be virtually inactive due to their inability to be phosphorylated. This prompted us to initiate specific structure−activity relationship studies to further investigate the structural requirements and mode of action of TCN and TCN-P. In a series of studies designed to specifically explore the structural requirements for the sugar moiety at the N-8 position

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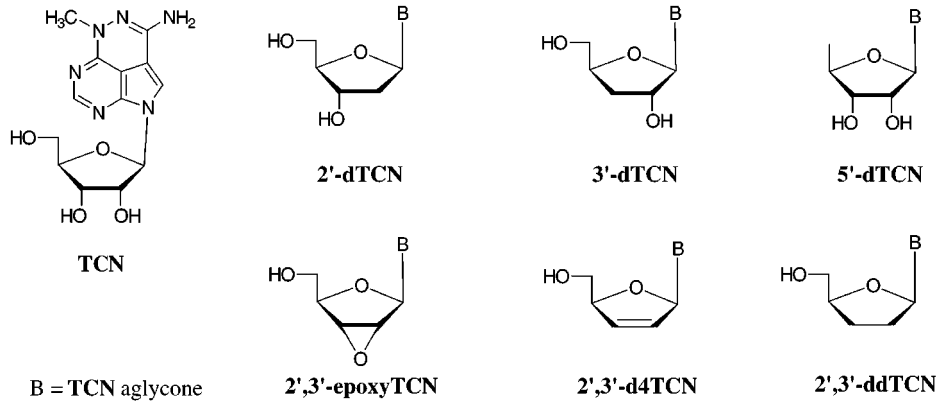
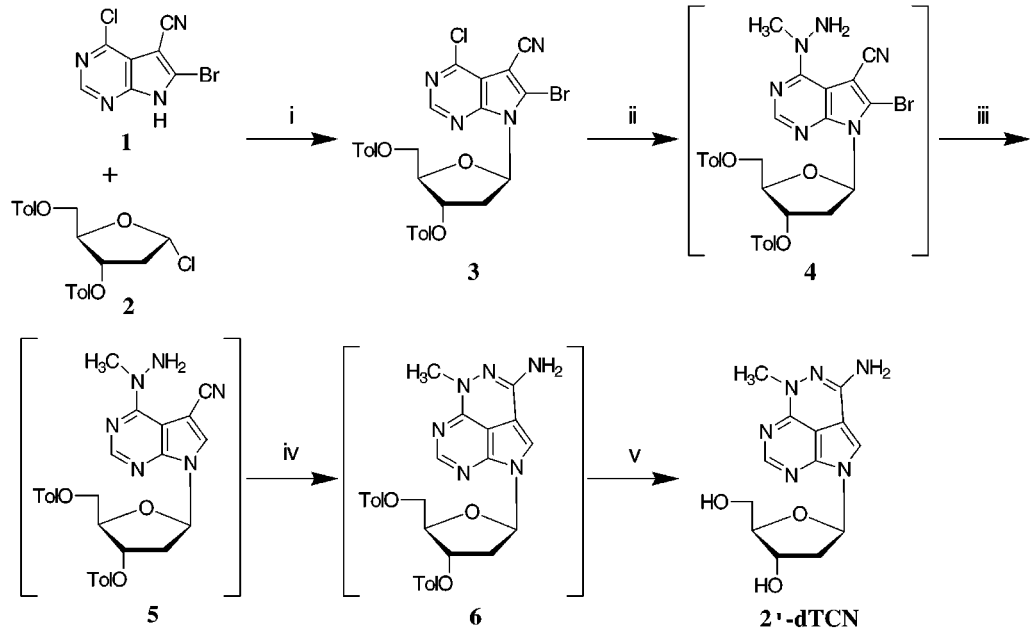
Chart 1. Deoxy Analogues of Triciribine (TCN)



Scheme 1. Synthesis of 2′-Deoxytriciribine (2′-dTCN)[a]



[a] Reagents: (i) NaH, CH_3CN; (ii) NH_2NHCH_3, EtOH, $CHCl_3$; (iii) 10% Pd−C, 50 psi H_2, EtOH, EtOAc, 1 N NH_4OH; (iv) concd HCl, EtOH, reflux; (v) NH_3, MeOH.

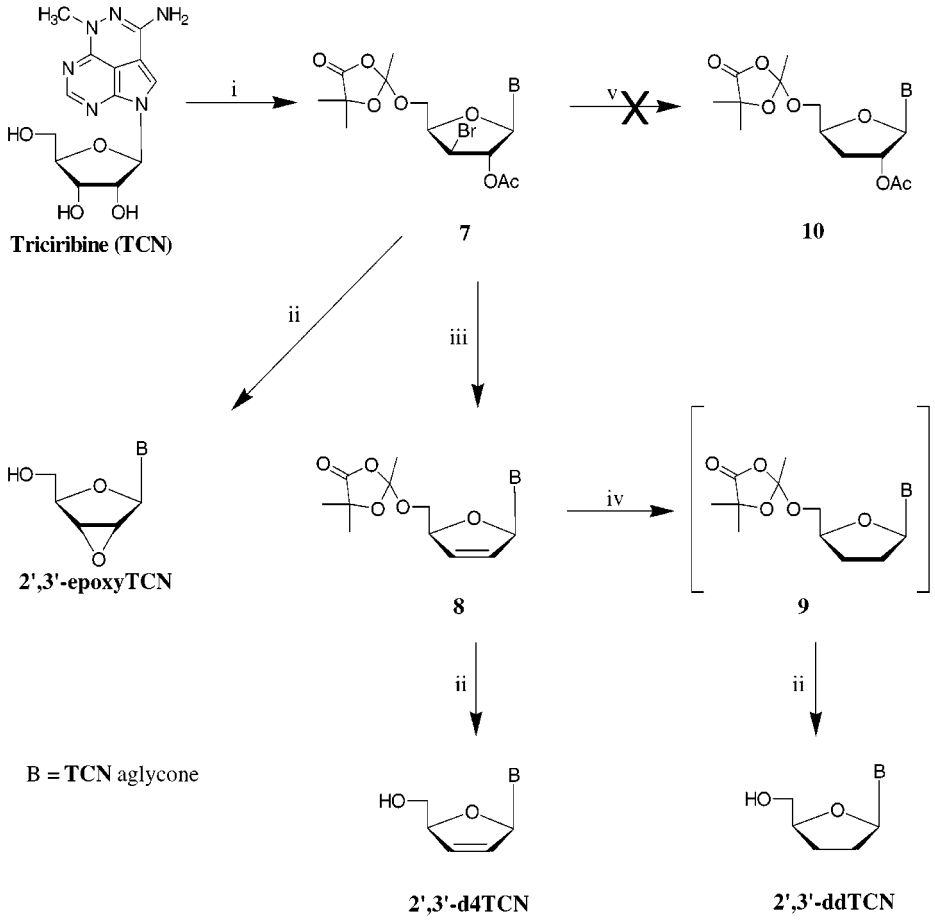
of TCN for biological activity, our first study explored the requirements for rigidity of the ribosyl moiety. That study established that a disruption of the rigidity of the ribosyl moiety adversely affected phosphorylation and therefore biological activity.[41]

This article describes the second study in this series and was designed to explore the hydroxyl requirements of the ribosyl moiety at the N-8 position of TCN. A systematic removal of the hydroxyl groups while maintaining the rigidity of the furan ring system should determine which individual and which combination of hydroxyl groups are necessary for TCN to be phosphorylated and active against selected viruses and cancer cell lines. We designed most of these analogues to maintain the relative availability and position of the 5′-hydroxyl group due to the obvious importance of the 5′-hydroxyl group to phosphorylation and activity. On the basis of this rationale, 2′-deoxytriciribine (2′-dTCN), 3′-deoxytriciribine (3′-dTCN), and 2′,3′-epoxytriciribine (2′,3′-epoxyTCN) were synthesized as monodeoxy analogues of triciribine and 2′,3′-dideoxy-2′,3′-didehydrotriciribine (2′,3′-d4TCN) and 2′,3′-dideoxytriciribine (2′,3′-ddTCN) were synthesized as dideoxy analogues of triciribine (Chart 1). We also elected to synthesize 5′-deoxytriciribine (5′-dTCN) to unequivocally confirm the fact that TCN requires phosphorylation to TCN-P in order to exhibit biological activity. Biological analysis of these analogues has provided an important insight into the hydroxyl requirements of TCN with regards to phosphorylation and in vitro activity against selected viruses and cancer cell lines.

Results and Discussion

6-Amino-8-(2-deoxy-β-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′-deoxytriciribine, 2′-dTCN) was synthesized from 6-bromo-4-chloro-5-cyanopyrrolo[2,3-*d*]pyrimidine (**1**), as illustrated in Scheme 1. The sodium salt of 6-bromo-4-chloro-5-cyanopyrrolo[2,3-*d*]pyrimidine[42] (**1**), formed by treating compound **1** with sodium hydride, was glycosylated with 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranose[43] (**2**) to afford 6-bromo-4-chloro-5-cyano-7-(2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine[44] (**3**). The reaction of **3** with methylhydrazine gave 6-bromo-5-cyano-7-(2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-ribofuranosyl)-4-(1-methylhydrazino)pyrrolo[2,3-*d*]pyrimidine (**4**). De-

Scheme 2. Synthesis of 2′,3′-Epoxytriciribine (2′,3′-epoxyTCN), 2′,3′-Dideoxy-2′,3′-didehydrotriciribine (2′,3′-d4TCN), and 2′,3′-Dideoxytriciribine (2′,3′-ddTCN)[a]



[a] Reagents: (i) 2-acetoxyisobutyryl bromide, CH_3CN; (ii) NH_3, MeOH; (iii) Zn−Cu couple, DMF, AcOH; (iv) 40 psi H_2, 10% Pd−C, EtOH, EtOAc; (v) Bu_3SnH, AIBN, THF.

bromination of intermediate **4** with 10% palladium on charcoal under 50 psi of hydrogen gas afforded 5-cyano-7-(2-deoxy-3,5-di-*O*-*p*-toluoyl-*β*-D-ribofuranosyl)-4-(1-methylhydrazino)pyrrolo[2,3-*d*]pyrimidine (**5**). Ring annulation of intermediate **5** to form 6-amino-8-(2-deoxy-3,5-di-*O*-*p*-toluoyl-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**6**) was accomplished in ethanol, under acidic conditions, at reflux temperature. Deprotection of intermediate **6** with methanolic ammonia afforded 6-amino-8-(2-deoxy-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′-deoxytriciribine, 2′-dTCN) in 25% overall yield from compound **1**.

The reaction of triciribine with an excess of 2-acetoxyisobutyryl bromide in acetonitrile at room temperature furnished a good yield of 8-[2-*O*-acetyl-3-bromo-3-deoxy-5-*O*-(2,5,5-trimethyl-1,3-dioxolan-4-one-2-yl)-*β*-D-xylofuranosyl]-6-amino-4-methylpyrrolo[4,3,2-*de*]pyrimido-[4,5-*c*]pyridazine (**7**) (Scheme 2). 6-Amino-8-(2,3-epoxy-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′,3′-epoxytriciribine**,** 2′,3′-epoxyTCN), 6-amino-8-(2,3-dideoxy-2,3-didehydro-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′,3′-dideoxy-2′,3′-didehydrotriciribine, 2′,3′-d4TCN), and 6-amino-8-(2,3-dideoxy-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′,3′-dideoxytriciribine, 2′,3′-ddTCN) were all synthesized from this common intermediate (**7**), as illustrated in Scheme 2.

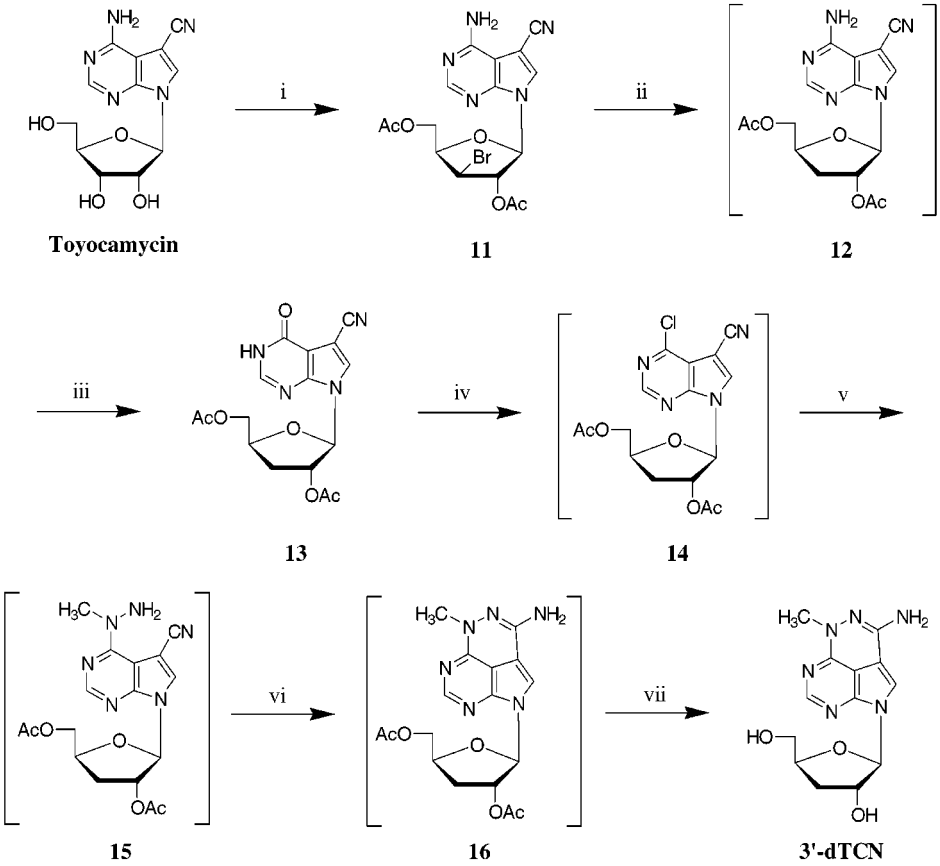
A 47% yield of 2′,3′-epoxytriciribine (2′,3′-epoxyTCN) was synthesized, using similar procedures as described with adenosine,[45] by sealing compound **7** in a pressure bottle with methanolic ammonia, saturated at 0 °C, for 18 h at room temperature (Scheme 2).

2′,3′-Dideoxy-2′,3′-didehydrotriciribine (2′,3′-d4TCN) was synthesized from compound **7**, by first introducing a double bond between the 2′- and 3′-carbons with a zinc copper couple to give compound **8**, using a similar procedure as described with toyocamycin.[46] Subsequent deprotection of compound **8** with methanolic ammonia gave 2′,3′-dideoxy-2′,3′-didehydrotriciribine (2′,3′-d4TCN) in a 57% yield from compound **7** (Scheme 2).

2′,3′-Dideoxytriciribine (2′,3′-ddTCN) was synthesized by first reducing the double bond in compound **8** with 10% palladium on charcoal in a mixture of ethyl acetate and ethanol, under 40 psi of hydrogen gas, to give the intermediate **9**. Subsequent deprotection of **9** with methanolic ammonia afforded 2′,3′-dideoxytriciribine (2′,3′-ddTCN) in a 73% yield from compound **8** (Scheme 2). Several unsuccessful attempts were made to synthesize 6-amino-8-(3-deoxy-*β*-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3′-deoxytriciribine, 3′-dTCN) from compound **7** using tributyltin hydride and 2,2′-azobis[isobutyronitrile] (AIBN) (Scheme 2).

Spectral analysis (^{1}H NMR) of what should have been compound **10** revealed that the methyl protons of the

Scheme 3. Synthesis of 3′-Deoxytriciribine (3′-dTCN)[a]

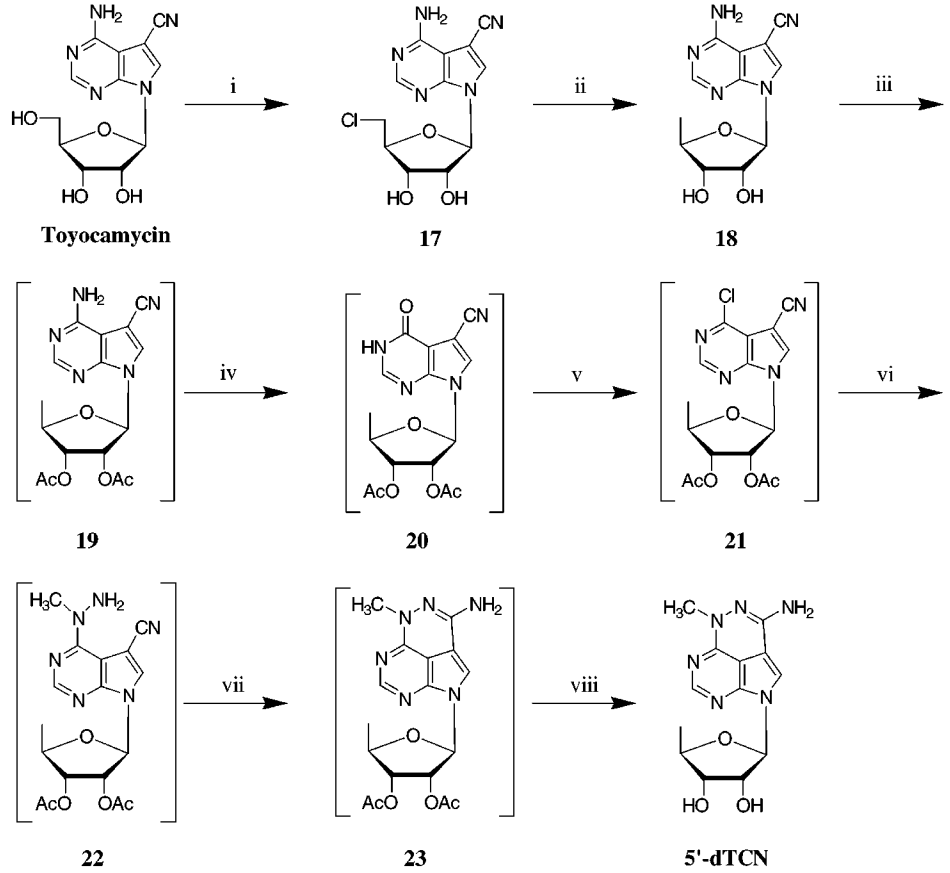


[a] Reagents: (i) ref 46; (ii) Bu_3SnH, AIBN, THF; (iii) $NaNO_2$, H_2O, AcOH; (iv) $POCl_3$; (v) NH_2NHCH_3, EtOH; (vi) concd HCl, EtOH, reflux; (vii) NH_3, MeOH.

2′-*O*-acetyl group and the characteristic multiplets for the 3′-protons, between δ 2.0 and 3.0, were missing. Subsequent deprotection of this intermediate with methanolic ammonia, saturated at 0 °C, afforded 2′,3′-epoxytriciribine instead of 3′-deoxytriciribine. Therefore, we assumed that excessive steric bulk on the α-face of the furan ring system prevented a radical reduction of the 3′-bromo group by the bulky reducing agent, tributyltin hydride. Unfortunately, we were unable to remove the 2,5,5-trimethyl-1,3-dioxolan-4-one protecting group as described in the literature.[46] Therefore, we synthesized 3′-deoxytriciribine from toyocamycin using the route illustrated in Scheme 3. Sequentially treating toyocamycin with 2-acetoxyisobutyryl bromide, methanol, and acetic anhydride afforded 4-amino-5-cyano-7-(3-bromo-3-deoxy-2,5-di-*O*-acetyl-β-D-xylofuranosyl)pyrrolo[2,3-*d*]pyrimidine[46] (**11**). A removal of the 3′-bromo group on compound **11** with tributyltin hydride and ′AIBN in tetrahydrofuran (THF) at reflux temperature for 18 h was successful and gave 4-amino-5-cyano-7-(3-deoxy-2,5-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine (**12**). Diazotization of **12** with sodium nitrite in aqueous acetic acid at 65 °C over 2.0 h gave 5-cyano-7-(3-deoxy-2,5-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-4-one (**13**). Chlorination of compound **13** was accomplished with phosphorus oxychloride ($POCl_3$) at reflux temperature to give 4-chloro-5-cyano-7-(3-deoxy-2,5-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine **(14)**. Displacement of the 4-chloro group from **14** with methylhydrazine in a mixture of ethanol and chloroform gave 5-cyano-7-(3-deoxy-2,5-di-*O*-acetyl-β-D-ribofuranosyl)-4-(1-methylhydrazino)pyrrolo[2,3-*d*]-pyrimidine (**15**) after 2 h. Ring annulation of **15** was accomplished in ethanol at reflux temperature for 3 h, under acidic conditions using concentrated HCl to give 6-amino-8-(3-deoxy-2,5-di-*O*-acetyl-β-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**16**). Deprotection of **16** with methanolic ammonia afforded 3′-deoxytriciribine (3′-dTCN).

6-Amino-8-(5-deoxy-β-D-ribofuranosyl)-4-methylpyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (5′-deoxytriciribine, 5′-dTCN) was synthesized by treating toyocamycin with thionyl chloride in hexamethylphosphoramide (HMPA) to afford 4-amino-5-cyano-7-(5-chloro-5-deoxy-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine[47] (**17**) (Scheme 4). Treatment of compound **17** with tributyltin hydride and AIBN in THF at reflux temperature for 18 h gave 4-amino-5-cyano-7-(5-deoxy-β-D-ribofuranosyl)pyrrolo-[2,3-*d*]pyrimidine[47] (**18**, 5′-deoxytoyocamycin). Acetylation of compound **18** with acetic anhydride in pyridine afforded 4-amino-5-cyano-7-(5-deoxy-2,3-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine (**19**), which was diazotized with sodium nitrite in aqueous acetic acid at 85 °C for 1.5 h to give 5-cyano-7-(5-deoxy-2,3-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-4-one (**20**). Chlorination of **20** was accomplished with $POCl_3$ at reflux temperature to give 4-chloro-5-cyano-7-(5-deoxy-2,3-di-*O*-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine (**21**). Displacement of the 4-chloro group of intermediate **21** with methylhydrazine in a mixture of ethanol and chloroform gave 5-cyano-7-(5-deoxy-2,3-di-*O*-acetyl-β-D-ribofuranosyl)-4-(1-methylhydrazino)pyrrolo-

Scheme 4. Synthesis of 5′-Deoxytriciribine (5′-dTCN)[a]



[a] Reagents: (i) $SOCl_2$, HMPA; (ii) Bu_3SnH, AIBN, THF; (iii) Ac_2O, pyridine; (iv) $NaNO_2$, H_2O, AcOH; (v) $POCl_3$; (vi) NH_2NHCH_3, EtOH; (vii) concd HCl, EtOH, reflux; (viii) NH_3, MeOH.

Table 1. Antiproliferative and Antiviral Activity of Deoxy Analogues of TCN

	50% inhibitory concentration (μM)						
	antiproliferative activity in murine L1210 cells[a]	cytotoxicity in human cells[b]			antiviral activity[c]		
compound	cytotoxicity	KB growth	HFF visual	CEM-SS visual	HIV-1 RT	HSV-1 ELISA	HCMV plaque
TCN[d]	0.035	>100	100	>100	0.04	23	2.5
TCN-P[d]	0.025	10	19	>1.0	0.04	20	0.8
2′-dTCN	>100	>100	>100	>100[e]	>100[e]	>100	>100
3′-dTCN	2.2	16[e]	100	100[e]	>100[e]	6.0[e]	>100
5′-dTCN	>100	>100[e]	>100	>100[e]	>100[e]	>100	>100
2′,3′-epoxyTCN	NT[f]	>100	>100	>100	>100	65	>100
2′,3′-d4TCN	>100	>100	>100	>100[e]	100[e]	>100	>100
2′,3′-ddTCN	25	>100[e]	>100	>100[e]	21[e]	>100	>100

[a] Antiproliferative activity was determined as described in the text and is reported as the concentration required to reduce murine L1210 cell growth rate to 50% of control rate. [b] Inhibition of KB cell growth was measured as described in the text in triplicate. Visual cytotoxicity was scored on uninfected HFF and CEM-SS cells used in HCMV plaque and HIV-1 RT assays. [c] Antiviral activity was determined using an ELISA in quadruplicate for HSV-1, a plaque assay in duplicate for HCMV, and amount of RT activity in culture supernatants in triplicate for HIV-1 as described in the text. [d] Data published previously in ref 33. [e] Average from two or three separate experiments. [f] NT = not tested.

[2,3-*d*]pyrimidine (**22**). Ring annulation of **22** was accomplished in ethanol at reflux temperature for 3 h, under acidic conditions using concentrated HCl to give 6-amino-8-(5-deoxy-2,3-di-*O*-acetyl-β-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**23**). Deprotection of **23** with methanolic ammonia afforded 5′-deoxytriciribine (5′-dTCN).

These TCN analogues (2′-dTCN, 3′-dTCN, 5′-dTCN, 2′,3′-d4TCN, and 2′,3′-ddTCN) were evaluated in an assay that uses growth inhibition of murine L1210 cells as an indicator of cytotoxicity. After 4 days of incubation with L1210 cells, inhibition of cell proliferation (cytotoxicity) was not observed for 2′-dTCN, 5′-dTCN, and 2′,3′-d4TCN at concentrations as high as 100 μM (Table 1). Only 3′-dTCN and 2′,3′-ddTCN were cytotoxic in L1210 cells, IC_{50} = 2.2 and 25 μM, respectively. In the three human cell lines, KB cells, human foreskin fibroblasts (HFF), and CEM-SS cells, only 3′-dTCN was cytotoxic (IC_{50} = 16 μM) in KB cells (Table 1). Thus, in contrast to TCN and TCN-P, which are potent inhibitors of L1210 cell growth, none of the deoxy analogues inhibited the growth of the murine or human cells tested.

The six target compounds also were evaluated for activity against three viruses. Activity against HIV-1 was measured in an assay that employed reverse tran-

Table 2. Intracellular Phosphorylation of DeoxyTCN Analogues in Uninfected CEM-SS Cells

compound	extracellular concn (μM)	incubation time (h)	intracellular monophosphate concn (μM)[a]
TCN[b]	10	5	286[c]
	100	5	323
	330	5	775[c]
TCN[b]	10	12	176[c]
	100	12	307[c]
	330	12	390[c]
2′-dTCN	100	24	<3−5[d]
3′-dTCN	100	24	276[c]
5′-dTCN	100	24	<3−5[d]
2′,3′-epoxyTCN	100	24	<3−5[d]
2′,3′-d4TCN	100	24	<3−5[d]
2′,3′-ddTCN	100	24	45[c]

[a] Nucleotide extractions and quantitation by HPLC were performed as described in the text. [b] Control data for TCN phosphorylation previously published in ref 34. [c] Average from two to five experiments. [d] Not detected; limit of detection was 3−5 μM.

scriptase (RT) in culture supernatants as a marker for HIV-1. Neither 2′-dTCN, 3′-dTCN, 5′-dTCN, 2′,3′-epoxyTCN, nor 2′,3′-d4TCN significantly reduced the amount of RT activity in the culture supernatants of CEM-SS cells acutely infected with HIV-1 (Table 1) thereby establishing that these five analogues had no activity against HIV-1. Under the same conditions, 2′,3′-ddTCN gave an IC_{50} = 21 μM, suggesting that this analogue was active against HIV, but at a small fraction of the activity of TCN and TCN-P (IC_{50} of each = 0.04 μM).

The deoxy analogues were also evaluated against HSV-1 in an ELISA and HCMV in a plaque assay. With the exception of 3′-dTCN, all of the analogues also were inactive, or nearly so, against HSV-1 (Table 1). Activity of 3′-dTCN (IC_{50} = 6.0 μM) against HSV-1 is puzzling in light of the weak activity of TCN and TCN-P and the inactivity of the other analogues. This activity could be related to or a manifestation of the cytotoxicity seen in L1210 and KB cells and to the fact that it is metabolized to its 5′-monophosphate (see below). No activity against HCMV was observed for any of these deoxyTCN analogues, even though TCN and TCN-P are active (Table 1).

Given the results of the biological assays, the lack of activity of the deoxy derivatives could be due to the inability of these molecules to be phosphorylated to an active species, or if the phosphorylated metabolites are formed, it might be due to the hydroxyl groups of the ribosyl ring system being necessary for activity. Therefore the phosphorylation of the deoxyTCN analogues was studied. The compounds were incubated at a concentration of 100 μM with CEM-SS cells for 24 h, and intracellular nucleotides were extracted, separated, and quantified by reverse-phase HPLC conditions that also separate TCN-P from TCN. Data in Table 2 show that in contrast to TCN, intracellular monophosphate concentrations of 2′-dTCN, 5′-dTCN, 2′,3′-epoxyTCN, and 2′,3′-d4TCN were not detected. However, the monophosphate forms of 3′-dTCN and 2′,3′-ddTCN were detected. This observation correlates with the antiproliferative and antiviral activity of these compounds (Table 1) and supports the idea that these analogues need to be metabolized to the monophosphate in order to exhibit antiproliferative or antiviral activity. Furthermore, the lack of antiproliferative and antiviral activity exhibited by 5′-dTCN unequivocally confirms the fact that TCN requires phosphorylation to TCN-P in order to exhibit biological activity.[3,11,12,34]

Experimental Section

General Procedures. Reaction mixtures were evaporated at temperatures less than 60 °C under reduced pressure (water aspirator) using a Buchi R-151 rotary evaporator. Melting points (uncorrected) were obtained on a Laboratory Devises Mel-Temp melting point apparatus. Thin-layer chromatography used Analtech GHLF SiO_2 prescored plates. Developed TLC plates were visualized under ultraviolet light (254 nm). E. Merck silica gel (230−400 mesh) was used for gravity or flash column chromatography. Proton magnetic resonance (^{1}H NMR) spectra were obtained with a Brucker Avance DPX 300 or DRX 500 spectrometer (solutions in $CDCl_3$ or DMSO-d_6) with the chemical shifts reported in parts per million downfield from tetramethylsilane as the internal standard. UV spectra were obtained with a Kontron UVIKON 860 ultraviolet spectrometer. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of Michigan, Ann Arbor, MI.

6-Amino-8-(2-deoxy-β-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′-Deoxytriciribine, 2′-dTCN). Sodium hydride (60% in oil, 470 mg, 11.7 mmol) was added to a stirred suspension of 6-bromo-4-chloro-5-cyanopyrrolo[2,3-*d*]pyrimidine[48] (**1**) (2.0 g, 7.8 mmol) in dry acetonitrile (150 mL) at room temperature under argon. After 1 h, 1-chloro-2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-ribofuranose[43] (**2**) (3.0 g, 7.8 mmol) was added and the reaction mixture was allowed to stir at room temperature for 3 h, at which time TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.65 (1:3 ethyl acetate:hexane). The solvent was removed and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (7 cm (d) × 9 cm (h)) using a mixture of ethyl acetate:hexane (1:3) as the eluting solvent system. Fractions of 25 mL were collected and the fractions containing spots on TLC with an R_f value of 0.65 (1:3 ethyl acetate:hexane) were combined and evaporated to dryness to give 2.90 g of a gummy white solid, presumed to be 6-bromo-4-chloro-5-cyano-7-(2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine[44] (**3**). Without further purification, this intermediate (**3**) was dissolved in a mixture of ethanol (40 mL), chloroform (40 mL), and methylhydrazine (0.49 g, 0.35 mL, 10 mmol) and the reaction mixture was stirred for 18 h at room temperature. TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.35 (1:3 ethyl acetate:hexane). The solvent was removed and the residue, presumed to be compound **4**, was dissolved in a mixture of ethyl acetate (100 mL), ethanol (100 mL), and 1 N ammonium hydroxide (5 mL). Palladium on charcoal (10%, 300 mg) was added and the mixture was placed under 50 psi of hydrogen gas and shaken on a Parr hydrogenator for 12 h at room temperature. TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.39 (1:1 ethyl acetate:hexane). The palladium on charcoal was removed by filtration through Celite, the solvent was removed under vacuum, and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (5 cm (d) × 12 cm (h)) using a mixture of ethyl acetate:hexane (1:1) as the eluting solvent. Fractions of 25 mL were collected and the fractions containing spots on TLC with an R_f value of 0.39 (1:1 ethyl acetate:hexane) were combined and evaporated to dryness to give 1.38 g of a white solid, presumed to be compound **5**. Without further purification, this intermediate (**5**) was suspended in a mixture of ethanol (100 mL) and 1 drop of concentrated HCl and heated at reflux temperature for 12 h. TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.19 (1:1 ethyl acetate:hexane). The solvent was removed and the residue, presumed to be compound **6**, was suspended in methanolic ammonia (100 mL), saturated at 0 °C, and sealed in a pressure bottle, and the reaction mixture was stirred at

room temperature for 18 h. TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.21 (9:1 chloroform:methanol). The solvent was removed under vacuum; the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (3 cm (d) × 12 cm (h)) using a mixture of chloroform:methanol (9:1) as the eluting solvent system. Fractions of 10 mL were collected and the fractions containing spots on TLC with an R_f value of 0.21 (9:1 chloroform: methanol) were combined and evaporated to dryness to give 0.60 g (25% overall yield from compound **1**) of 2′-deoxytricir-ibine (2′-dTCN) as a light brown solid: R_f = 0.21 (9:1 chloroform:methanol); mp 123−125 °C effervesced, 181−183 °C melted; UV [λ_{max} (ϵ)] (pH 1) 286 (10239), 278 (10239), (pH 7) 293 (11456), (pH 11) 289 (14370); ^{1}H NMR (DMSO-d_6) δ 8.01 (1H, s, CH-2), 7.07 (1H, s, H-7), 6.30 (1H, pseudo t, H-1′), 6.24 (2H, s, NH_2), 5.36 (1H, t, OH), 5.31 (1H, d, OH), 4.38 (1H, m), 3.86 (1H, m), 3.52 (2H, m, H-5′), 3.37 (3H, s, CH_3), 2.58 (1H, m, H-2′), 2.25 (1H, m, H-2′). Anal. ($C_{13}H_{16}N_6O_3$·0.25H_2O) C, H, N.

8-[2-*O*-Acetyl-3-bromo-3-deoxy-5-*O*-(2,5,5-trimethyl-1,3-dioxolan-4-one-2-yl)-*β*-D-xylofuranosyl]-6-amino-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (7). 2-Acetoxyisobutyryl bromide (7.52 g, 36 mmol, 5.3 mL) was added to a stirred suspension of triciribine (0.96 g, 3 mmol) in acetonitrile (40 mL), under argon at room temperature. After 18 h, the reaction mixture was poured into a saturated solution of sodium bicarbonate (100 mL) and carefully extracted with ethyl acetate (2 × 75 mL). The aqueous layer was removed and discarded and the ethyl acetate layer was washed with a saturated solution of sodium bicarbonate (100 mL) and a brine solution (100 mL) and then dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the solvent was removed under vacuum to yield 1.13 g (71%) of compound **7**. The product was used without further purification: R_f = 0.60 (9:1 chloroform:methanol); ^{1}H NMR (DMSO-d_6) δ 8.25 (1H, s, H-2), 7.11 (1H, s, H-7), 6.28 (1H, d, H-1′), 5.71 (1H, m), 4.54 (6H, m, NH_2, 2 × H-5′, 2H), 3.55 (3H, s, CH_3), 2.15 (3H, s, CH_3), 2.08 (3H, s, CH_3), 1.60 (3H, s, CH_3), 1.58 (3H, s, CH_3).

8-[2,3-Epoxy-5-*O*-(2,5,5-trimethyl-1,3-dioxolan-4-one-2-yl)-*β*-D-ribofuranosyl]-6-amino-4-methylpyrrolo[4,3,2-*de*]-pyrimido[4,5-*c*]pyridazine (2′,3′-Epoxytriciribine, 2′,3′-epoxyTCN). Methanolic ammonia (200 mL), saturated at 0 °C, was added to a pressure bottle containing **7** (0.83 g, 1.5 mmol). The sealed reaction vessel was warmed to room temperature and the mixture was stirred for 18 h. The solvent was removed under vacuum and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (3 cm (d) × 12 cm (h)) using a mixture of chloroform:methanol (19:1) as the eluting solvent. Fractions of 10 mL were collected and the fractions containing spots on TLC with an R_f value of 0.18 (9:1 chloroform:methanol) were combined and evaporated to dry-ness to give 0.21 g (47% yield) of 2′,3′-epoxyTCN as a light brown solid: R_f = 0.18 (9:1 chloroform:methanol); mp 245 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.07 (1H, s, CH-2), 7.08 (1H, s, H-7), 6.29 (2H, s, NH_2), 6.20 (1H, s, H-1′), 5.06 (1H, bs, OH), 4.42 (1H, d), 4.24 (1H, d), 4.13 (1H, t, H-4′), 3.50 (2H, m, H-5′), 3.39 (3H, s, CH_3). Anal. ($C_{13}H_{14}N_6O_3$·0.5H_2O) C, H, N.

6-Amino-8-(2,3-dideoxy-2,3-didehydro-*β*-D-ribofurano-syl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]py-ridazine (2′,3′-Didehydro-2′,3′-dideoxytriciribine, 2′,3′-d4TCN). Zinc−copper couple (4 g) was added to a solution of **7** (1.5 g, 2.7 mmol), *N*,*N*-dimethylformamide (10 mL), and glacial acetic acid (1 mL) and stirred for 2 h. The reaction mixture was filtered through Celite and the Celite was washed with ethyl acetate (50 mL). The filtrate was diluted with ethyl acetate (50 mL) and extracted with water (50 mL). The aqueous layer was extracted with ethyl acetate (3 × 50 mL) and then discarded. The ethyl acetate layers were combined and washed with a saturated solution of sodium bicarbonate (20 mL), water (2 × 20 mL), and brine (20 mL) and then dried over magnesium sulfate. After removing the magnesium sulfate by filtration, the solvent was evaporated to give 800 mg (71% yield) of compound **8:** R_f = 0.47 (9:1 chloroform: methanol); ^{1}H NMR (DMSO-d_6) δ 8.06 (1H, s, H-2), 6.97 (1H, s), 6.91 (1H, s), 6.46 (1H, d), 6.22 (1H, d), 6.20 (2H, bs, NH_2), 4.97 (1H, s), 4.30 (1H, m), 4.02 (1H, m), 3.38 (3H, s, CH_3), 1.94 (3H, s, CH_3), 1.41 (6H, s, 2 × CH_3).

Methanolic ammonia (200 mL), saturated at 0 °C, was added to a pressure bottle containing compound **8** (1.33 g, 2.4 mmol). The sealed reaction vessel was warmed to room temperature and the mixture was stirred for 18 h. The solvent was removed under vacuum and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (3 cm (d) × 12 cm (h)) using a mixture of chloroform:methanol (9:1) as the eluting solvent system. Fractions of 10 mL were collected and the fractions containing spots on TLC with an R_f value of 0.32 (9:1 chloroform: methanol) were combined and evaporated to dryness. The residue was recrystallized in a hot mixture of ethyl acetate and hexane to give 0.55 g (80% yield) of 2′,3′-d4TCN as a light brown solid: R_f = 0.32 (9:1 chloroform:methanol); mp 65 °C shrank, 126 °C effervesced, 165 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.04 (1H, s, H-2), 6.96 (2H, s, H-7 and H-1′), 6.49 (1H, m, H-2′), 6.24 (2H, bs, NH_2), 6.12 (1H, m, H-3′), 5.10 (1H, t, OH), 4.84 (1H, m, H-4′), 3.50 (2H, m, H-5′), 3.38 (3H, s, CH_3). Anal. ($C_{13}H_{14}N_6O_2$) C, H, N.

6-Amino-8-(2,3-dideoxy-*β*-D-ribofuranosyl)-4-methylpyr-rolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (2′,3′-Dideox-ytriciribine, 2′,3′-ddTCN). Compound **8** (0.80 g, 1.9 mmol) was dissolved in a mixture of ethyl acetate (50 mL) and ethanol (50 mL) along with 10% palladium on charcoal (100 mg) and shaken on a Parr hydrogenator under 40 psi of hydrogen gas for 1 h at room temperature. TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.48 (9:1 chloroform:methanol). The palladium on charcoal was removed by filtration through Celite and the solvent was removed under vacuum. The residue, presumed to be inter-mediate **9**, was transferred to a glass pressure bottle. Metha-nolic ammonia (200 mL), saturated at 0 °C, was added to the pressure bottle, the sealed pressure bottle was warmed to room temperature, and the mixture was stirred for 24 h. The solvent was removed and the residue was recrystallized in a hot mixture of ethyl acetate and hexane to give 0.40 g (73% yield from compound **8**) of 2′,3′-ddTCN as a light brown solid: R_f = 0.36 (9:1 chloroform:methanol); mp 50 °C shrank, 127−130 °C melted; ^{1}H NMR (DMSO-d_6) δ 8.03 (1H, s, H-2), 7.08 (1H, s, H-7), 6.22 (2H, bs, NH_2), 6.17 (1H, t, H-1′), 5.12 (1H, t, OH), 4.10 (1H, m, H-4′), 3.50 (2H, m, H-5′), 3.38 (3H, s, CH_3), 2.37 (2H, m, H-2′or H-3′), 2.05 (2H, m, H-2′or H-3′). Anal. ($C_{13}H_{16}N_6O_2$·0.75H_2O) C, H, N.

6-Amino-8-(3-deoxy-*β*-D-ribofuranosyl)-4-methylpyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3′-Deoxytriciribine, 3′-dTCN). 4-Amino-5-cyano-7-(3-bromo-3-deoxy-2,5-di-*O*-acetyl-*β*-D-xylofuranosyl)pyrrolo[2,3-*d*]pyrimidine[46] (**11**) (3.20 g, 7.30 mmol) and AIBN (1 g) were sealed in a dry 500-mL round-bottom flask, purged with argon. Dry tetrahydrofuran (150 mL) was added, via syringe, and the mixture was stirred for 15 min before tributyltin hydride (9.0 g, 9.0 mL, 31 mmol) was added to the reaction mixture, via syringe, and stirred for 10 min. The flask was fitted with a condenser and the reaction mixture was heated at reflux temperature, under argon, for 18 h. The solvent was removed under vacuum and the oily residue was diluted with petroleum ether (300 mL). The resulting suspension was stirred for 6 h and stored at −5 °C for 48 h before the residue was collected by filtration. Upon collection, the white residue, presumed to be compound **12** with an R_f value of 0.73 (9:1 chloroform:methanol), began to absorb water from the atmosphere and become sticky so without further purification, it was added to water (150 mL) and the mixture was heated to 60 °C before glacial acetic acid (20 mL) was added. Sodium nitrite (9.0 g, 130 mmol) was added, in six portions, over a period of 30 min. After 1.5 h, TLC showed the disappearance of starting material and the appearance of a new spot at R_f = 0.63 (9:1 chloroform:methanol). The solvent was removed and the light yellow solid was extracted with ethyl acetate (150 mL) and a saturated solution of sodium

bicarbonate (90 mL). The aqueous layer was washed with ethyl acetate (2 × 50 mL) and then discarded. The ethyl acetate layers were combined and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the ethyl acetate was evaporated under vacuum to give 1.40 g (53% yield from compound **11**) of compound **13** as a white solid: $R_f = 0.63$ (9:1 chloroform:methanol); ^{1}H NMR (DMSO-d_6) δ 12.56 (1H, bs, NH), 8.34 (1H, s), 8.11 (1H, s), 6.21 (1H, s), 5.45 (1H, m), 4.51 (1H, m), 4.22 (2H, m), 2.21 (1H, m), 2.10 (3H, s), 2.02 (3H, s), 1.98 (1H, m).

Without further purification or characterization, **13** (1.2 g, 3.30 mmol) was dissolved in phosphorus oxychloride (25 mL) and heated at reflux temperature for 1 h. The cooled solution was poured over ice water and shaken vigorously until all the phosphorus oxychloride was destroyed, leaving a precipitate, presumed to be compound **14**, suspended in the water. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.95$ (9:1 chloroform:methanol). The precipitate was collected by filtration and air-dried for 18 h. Without further purification or characterization, methylhydrazine (0.11 g, 0.13 μL, 2.4 mmol) was added to a solution of **14** (0.76 g, 2.0 mmol) in a mixture of ethanol (40 mL) and chloroform (20 mL) and stirred for 2 h. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.81$ (9:1 chloroform:methanol). The solvent was removed under vacuum and the yellow residue, presumed to be compound **15**, was dissolved in a mixture of ethanol (40 mL) and 1 drop of concentrated HCl. The solution was heated at reflux temperature for 3 h, at which time TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.54$ (9:1 chloroform:methanol). The solvent was removed under vacuum and the residue, presumed to be compound **16**, was dissolved in methanol (20 mL) and transferred to a glass pressure bottle. Methanolic ammonia (180 mL), saturated at 0 °C, was added to the pressure bottle containing **16** and the bottle was sealed. The sealed reaction vessel was warmed to room temperature and the mixture was stirred for 18 h. The solvent was removed under vacuum and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (3 cm (d) × 12 cm (h)) using a mixture of chloroform:methanol (19:1) as the eluting solvent system. Fractions of 10 mL were collected and the fractions containing spots on TLC with an R_f value of 0.21 (9:1 chloroform:methanol) were combined and evaporated to dryness. The residue was recrystallized from a hot mixture of methanol:ethyl acetate:hexane to give 0.27 g (44% yield from intermediate **14**) of 3′-dTCN as a light brown solid: $R_f = 0.21$ (9:1 chloroform:methanol); mp 237 °C dec; ^{1}H NMR (DMSO-d_6) δ 8.03 (1H, s, CH-2), 7.05 (1H, s, H-7), 6.24 (2H, s, NH_2), 5.81 (1H, d, H-1′), 5.63 (1H, d, OH), 5.23 (1H, bs, OH), 4.52 (1H, m), 4.35 (1H, m), 3.60 (2H, m), 3.37 (3H, s, NCH_3), 2.00 (1H, m, H-3′), 2.15 (1H, m, H-3′). Anal. ($C_{13}H_{16}N_6O_3 \cdot 0.75H_2O$) C, H, N.

6-Amino-8-(5-deoxy-β-D-ribofuranosyl)-4-methylpyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (5′-Deoxytriciribine, 5′-dTCN). Toyocamycin[49] (4.40 g, 15 mmol) was added to a solution of thionyl chloride (6 mL) and hexamethylphosphoramide (40 mL) previously cooled to 0 °C in an ice bath. The mixture was allowed to warm to room temperature, under argon, and then stirred for 18 h. The red solution was poured over ice water (200 mL) to obtain a precipitate. Concentrated ammonium hydroxide was added to the reaction mixture to dissolve the precipitate at pH 7 and reprecipitate the product at pH 9. The precipitate was collected by vacuum filtration and the wet solid was washed with water (100 mL) and dried under vacuum at 60 °C for 3 days to yield 3.6 g (77%) of 5′-chloro-5′-deoxytoyocamycin (**17**): $R_f = 0.65$ (9:1 chloroform:methanol); ^{1}H NMR (DMSO-d_6) δ 8.42 (1H, s), 8.24 (1H, s), 6.92 (2H, bs), 6.12 (1H, d), 5.61 (1H, d, OH), 5.48 (1H, d, OH), 4.51 (1H, q), 4.10 (2H, m), 3.91 (2H, m).

Without further purification, compound **17** (2.0 g, 6.4 mmol) and AIBN (0.5 g, 3 mmol) were sealed in a dry 500-mL round-bottom flask, purged with argon. Dry tetrahydrofuran (100 mL) was added, via syringe, and the mixture was stirred for 15 min before tributyltin hydride (8 g, 8 mL, 27.5 mmol) was added to the reaction mixture, via syringe. The flask was fitted with a condenser and the reaction mixture was heated at reflux temperature, under argon, for 18 h. The solvent was removed under vacuum and the oily residue was diluted with petroleum ether (300 mL). The resulting suspension was stirred for 30 min before the residue was collected by filtration and dried under vacuum at 60 °C for 18 h to give 1.42 mg (81% yield) of 5′-deoxytoyocamycin (**18**): $R_f = 0.50$ (9:1 chloroform:methanol); mp 144−147 °C shrank, 186−188 °C melted (lit.[47] mp 187−188); ^{1}H NMR (DMSO-d_6) δ 8.39 (1H, s), 8.22 (1H, s), 6.87 (2H, bs), 6.03 (1H, d), 5.45 (1H, d, OH), 5.16 (1H, d, OH), 4.39 (1H, q), 3.93 (2H, m), 1.27 (3H, d).

Without further purification, compound **18** (900 mg, 3.27 mmol) was dissolved in a mixture of acetic anhydride (1.34 g, 1.30 mL, 13.08 mmol) and pyridine (50 mL) and the mixture was stirred for 6 h, under argon. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.72$ (9:1 chloroform:methanol). The reaction mixture was evaporated, under vacuum, at 60 °C and the residue was coevaporated with toluene (2 × 50 mL). The resulting solid, presumably compound **19,** was suspended in water (50 mL) and the suspension was heated to 60 °C before glacial acetic acid (5 mL) was added. Sodium nitrite (0.96 g, 14 mmol) was added, in six portions, over 1 h. The reaction mixture was then heated to 85 °C for 1.5 h, upon which the suspension became a solution. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.54$ (9:1 chloroform:methanol). The solvent was removed and the light yellow solid was extracted with ethyl acetate (50 mL) and a saturated solution of sodium bicarbonate (30 mL). The aqueous layer was washed with ethyl acetate (2 × 50 mL) and then discarded. The ethyl acetate layers were combined and dried over magnesium sulfate. The magnesium sulfate was removed by filtration and the ethyl acetate was evaporated under vacuum to give a yellow solid, presumed to be compound **20**. Without further purification or characterization, **20** was dissolved in phosphorus oxychloride (10 mL) and heated at reflux temperature for 30 min. The cooled solution was poured over ice water and shaken vigorously until all the phosphorus oxychloride was destroyed, leaving a light yellow precipitate, presumed to be compound **21**, suspended in the water. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.97$ (9:1 chloroform:methanol). The precipitate was collected by filtration and air-dried for 18 h. Without further purification or characterization, methylhydrazine (60 mg, 70 μL, 1.32 mmol) was added to a solution of **21** (400 mg, 1.1 mmol), ethanol (40 mL), and chloroform (20 mL) and the mixture was stirred for 2 h. TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.82$ (9:1 chloroform:methanol). The solvent was removed under vacuum and the yellow residue, presumed to be compound **22**, was dissolved in a mixture of ethanol (40 mL) and 1 drop of concentrated HCl. The solution was heated at reflux temperature for 3 h, at which time TLC showed the disappearance of starting material and the appearance of a new spot at $R_f = 0.51$ (9:1 chloroform:methanol). The solvent was removed under vacuum and the residue, presumed to be compound **23**, was dissolved in methanol (20 mL) and transferred to a glass pressure bottle. Methanolic ammonia (180 mL), saturated at 0 °C, was added to the pressure bottle containing **23** and the bottle was sealed. The sealed reaction vessel was warmed to room temperature and the mixture was stirred for 18 h. The solvent was removed under vacuum and the residue was adsorbed onto silica gel and applied to the top of a silica gel column. The residue was eluted from the column (3 cm (d) × 12 cm (h)) using a mixture of chloroform:methanol (19:1) as the eluting solvent system. Fractions of 10 mL were collected and the fractions containing spots on TLC with an R_f value of 0.30 (9:1 chloroform:methanol) were combined and evaporated to dryness. The residue was recrystallized from a hot mixture of methanol:ethyl acetate:hexane to give 0.19 g (57% yield from **21**) of 5′-dTCN as a light brown solid: $R_f = 0.30$ (9:1 chloroform:methanol); mp 114−

116 °C; ^{1}H NMR (DMSO-d_6) δ 8.04 (1H, s, CH-2), 7.01 (1H, s, H-7), 6.20 (2H, s, NH_2), 5.81 (1H, d, H-1′), 5.43 (1H, d, OH), 5.16 (1H, bs, OH), 4.39 (1H, q, H-4′), 3.90 (2H, m), 3.37 (3H, s, NCH_3), 1.29 (3H, d, CH_3). Anal. ($C_{13}H_{16}N_6O_3 \cdot 0.5H_2O$) C, H, N.

In Vitro Antiproliferative Studies. The in vitro cytotoxicity against murine L1210 leukemic cells was determined in a cell growth assay described previously.[50] L1210 cells were grown in Fischer's medium supplemented with 10% heat-inactivated (56 °C, 30 min) horse serum and subcultured by serial dilution. Growth rates were calculated from determinations of the number of cells at 0, 24, 48, 72, and 96 h in the presence of selected concentrations of the test compound. The 50% inhibitory concentration (IC_{50}) was defined as the concentration required to decrease the growth rate to 50% of the untreated control cells. Growth rate was calculated from the slope of a semilogarithmic plot of cell number against time for the treated culture as a percent of the control.

In Vitro Antiviral Studies. Cell culture procedures: The routine growth and passage of KB, HFF, and BSC-1 cells was performed in monolayer cultures using minimal essential medium (MEM) with either Hanks salts [MEM(H)] or Earle salts [MEM(E)] supplemented with 10% calf or fetal calf serum as detailed previously.[51] The sodium bicarbonate concentration was varied to meet the buffering capacity required. Cells were passaged at 1:2 to 1:10 dilutions according to conventional procedures by using 0.05% trypsin plus 0.02% EDTA in a HEPES buffered salt solution. CEM cells were passaged twice weekly at 1:10 dilutions using RPMI 1640 with 10% fetal calf serum.

HIV-1 assay: The HIV strain III_B producer cell line $H9III_B$ was obtained through the courtesy of Dr. R. C. Gallo. HIV strain III_B was propagated in CEM-SS cells as described previously by Kucera et al.[33,52] To evaluate the activities of compounds in cells acutely infected with HIV, RT was employed as a marker for HIV-1. CEM-SS cells were infected at a multiplicity of infection of approximately 0.001 plaque-forming units (pfu)/cell with strain III_B of HIV-1 in a minimal volume of stock virus in growth medium. Cultures were incubated at 37 °C for 2 h to permit virus adsorption and then diluted to 5×10^5 cells/mL with RPMI 1640 containing 10% fetal bovine serum; 0.1 mL was then added to each well of a 96-well cluster dish which had been pretreated with poly-L-lysine. Fresh medium (0.1 mL with 10% fetal bovine serum) containing test compounds in twice the desired final concentration was added to triplicate wells at seven concentrations ranging from 100 to 0.14 μM. After 6 days of incubation, supernatant samples were taken and the amount of RT activity was measured by the incorporation of [^{3}H]dTTP into acid-insoluble material using the assay described by White et al.[53]

HCMV plaque assay: The Towne strain, plaque-purified isolate P_o, of HCMV was kindly provided by Dr. Mark Stinski, University of Iowa. HFF cells in 24-well cluster dishes were infected with approximately 100 pfu of HCMV/well using the procedures detailed earlier.[51] Following virus adsorption, compounds dissolved in growth medium were added to duplicate wells in four to eight selected concentrations. After incubation at 37 °C for 8−10 days, cell sheets were fixed and stained with crystal violet and microscopic plaques enumerated. Drug effects were calculated as a percentage of reduction in number of plaques in the presence of each drug concentration compared to the number observed in the absence of drug.

HSV-1 ELISA: An enzyme-linked immunosorbent assay (ELISA)[54] was employed to detect HSV-1. Briefly, 96-well cluster dishes were planted with 10 000 BSC-1 cells/well in 200 μL/well of MEM(E) plus 10% calf serum. After overnight incubation at 37 °C, selected drug concentrations in triplicate and HSV-1 (KOS strain kindly provided by Dr. Sandra K. Weller, University of Connecticut) at a concentration of 100 pfu/well were added. Following a 3-day incubation at 37 °C, medium was removed, plates were blocked and rinsed, and horseradish peroxidase conjugated rabbit anti-HSV-1 antibody was added. Following removal of the antibody-containing solution, plates were rinsed and then developed by adding a solution of tetramethylbenzidine as substrate. The reaction was terminated with H_2SO_4, and absorbance was read at 450 and 570 nm. Drug effects were calculated as a percentage of the reduction in absorbance in the presence of each drug concentration compared to absorbance obtained with virus in the absence of drug.

Cytotoxicity assays: Several different assays were used to explore the cytotoxicity of selected compounds using methods we have detailed previously. (i) Cytotoxicity produced in stationary HFF cells and in CEM-SS cells was determined by microscopic inspection of cells not affected by the virus used in the respective assays.[51] (ii) The effect of compounds during two population doublings of KB cells was determined by crystal violet staining and spectrophotometric quantitation of dye eluted from stained cells as described earlier.[55] Briefly, 96-well cluster dishes were planted with KB cells at 5000 cells/well. After overnight incubation at 37 °C, test compound was added in triplicate at eight concentrations. Plates were incubated at 37 °C for 48 h in a CO_2 incubator, rinsed, fixed with 95% ethanol, and stained with 0.1% crystal violet. Acidified ethanol was added, and plates were read at 570 nm in a spectrophotometer designed to read 96-well ELISA assay plates.

Data Analysis. Dose−response relationships were constructed by linearly regressing the percent inhibition of parameters derived in the preceding sections against log drug concentrations. 50% Inhibitory (IC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls [acyclovir, ganciclovir, and zidovudine (AZT), respectively] for HSV-1, HCMV, and HIV were used in all assays.

Extraction Procedures for Chromatographic Analysis. CEM-SS cells were grown in suspension culture with concentrations of compounds given in Table 2 for 5−24 h. Cells were harvested by centrifugation (250*g*, 5 min) at 25 °C, the resulting cell pellet was resuspended in 10 mL of cold Puck's saline with glucose (Gibco), and the cell suspension was again centrifuged (250*g*, 5 min). The extraction procedures used to obtain the aqueous phase of supernatant were previously described.[56] Briefly, nucleotides were extracted by vortexing the resulting cell pellet in 0.4 mL of 0.6 N TCA (4 °C) which was kept on ice for 15 min. This suspension was transferred to a microfuge tube and microfuged for 30 s at 12000*g* (4 °C). The supernatant was carefully removed to an Eppendorf tube, volume was measured, and an equal amount of ice-cold Freon containing 0.5 M TOA (trioctylamine) was added to the tube. The mixture was vortexed for 5 s or more and then microfuged for 30 s at 12000*g* (4 °C). The lower phase was removed by aspiration and discarded. The aqueous phase was frozen at −76 °C for high-performance liquid chromatography (HPLC) analysis.

Nucleotide Analysis. TCN-P was separated and quantitated by either anion-exchange HPLC as detailed by Wotring et al.[12] or ion pair reverse-phase HPLC as described by Walseth et al.[57] A Spectra-Physics system was employed consisting of a model SP8800 ternary pump, a SP8500 dynamic mixer, a SP8780 autosampler, and a SP8490 variable wavelength detector. Peaks were integrated on a model SP4270 integrator. A Compaq model 386 computer with WINner 386 software (Spectra-Physics) was used for system and data management.

In an initial experiment, analysis was performed using anion-exchange HPLC but superior resolution of monophosphates was achieved by reverse-phase HPLC; consequently it was adopted in all subsequent experiments. Ion pair reverse-phase chromatography was performed on a 3.9 × 300 mm μBondapak C18 column (Waters). The solvent was 5 mM tetrabutylammonium hydroxide (TBA), 5% methanol adjusted to pH 2.5 with formic acid. Separation of nucleotide monophosphates was carried out at a flow rate of 1 mL/min with dual wavelength detection set at 254 and 290 nm. TCN-P metabolite identification was based on comparison of unknown peaks to retention time of TCN-P standard and on the ratio

of 290/254 nm due to the absorbance maximum of TCN and TCN-P at 290 nm.[12]

Intracellular concentrations were calculated based upon the integrated area under HPLC peaks of TCN-P, conversion of area to nmol of TCN-P based upon the area under TCN-P peaks from standards of known concentration, and conversion of concentration from nmol/10^6 cells to micromolarity based upon a cell volume of 1.9 pL/cell. Volumes of CEM-SS cells were determined by microscopic examination of cells in a hemacytometer, measurement of the diameter of 10−20 representative cells, and assumption of spherical geometry.

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