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6-*N*-Acyltriciribine Analogues: Structure−Activity Relationship between Acyl Carbon Chain Length and Activity against HIV-1

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Triciribine (TCN) and triciribine-5′-monophosphate (TCN-P) are active against HIV-1 at submicromolar concentrations. In an effort to improve and better understand this activity, we have conducted a structure−activity relationship study to explore the tolerance of TCN to structural modifications at the 6-position. A number of 6-*N*-acyltriciribine analogues were synthesized and evaluated for antiviral activity and cytotoxicity. The cytotoxicity of these compounds was minimal in three human cell lines (KB, CEM-SS cells, and human foreskin fibroblasts (HFF)). The compounds were marginally active or inactive against herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV). In contrast, most of the compounds exhibited moderate to high activity against human immunodeficiency virus type 1 (HIV-1), IC_{50}'s = 0.03 to 1 μM. This structure−activity relationship study identified the *N*-heptanoyl group as having the optimal carbon chain length. This compound was as active against HIV-1 as TCN and TCN-P. Reverse phase HPLC of extracts from uninfected cells treated with 6-*N*-acyltriciribines detected sufficient TCN-P to account for anti-HIV activity thereby suggesting a prodrug effect. Studies in an adenosine kinase deficient cell line showed that the 6-*N*-acyl derivative was not phosphorylated directly but first was metabolized to triciribine which then was converted to TCN-P.

Introduction

The tricyclic nucleoside triciribine (TCN) was synthesized by Schram and Townsend in 1971.[1] Early studies revealed that TCN is converted intracellularly to triciribine-5′-monophosphate (TCN-P) by adenosine kinase.[2−4] Unlike other nucleoside analogues, TCN is metabolized only to the monophosphate and not to the di- or triphosphate forms.[5] TCN is also not incorporated into nucleic acids.[6] Although the exact mechanism by which it inhibits the growth of certain cell lines is unknown, TCN-P inhibits DNA and protein synthesis in these cells.[6,7] Due to poor water solubility and difficulty in formulating TCN for clinical use, triciribine-5′-monophosphate (TCN-P) was synthesized[8] as a water soluble prodrug of TCN.[9]

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.[10] These studies also found no cross resistance to TCN or TCN-P in AZT- or TIBO-resistant HIV strains,[10] suggesting that TCN and TCN-P have an entirely different mode of action than AZT and TIBO. We also found that triciribine has no activity against the HIV encoded enzymes, reverse transcriptase, RNase H, integrase, and protease.[11] Furthermore, cytotoxicity such as that observed in murine L1210 cells appears to be highly cell line specific and was not observed in human cell lines used to propagate HIV and human cytomegalovirus (HCMV).[10] Even though TCN was not very cytotoxic in these cell lines, it must be phosphorylated to TCN-P to be active against HIV-1.[12] The antiviral mechanism of action of TCN has yet to be elucidated fully, but it involves viral accessory proteins.[13]

As part of our ongoing research with triciribine,[10−12,14−19] we have been conducting systematic structure−activity relationship studies to determine the tolerance of triciribine to structural changes and how they affect phosphorylation and activity against HIV-1.[18,19] The current study was designed to explore the effect that acylation of the 6-amino group would have on activity against HIV-1. The results uncovered an interesting structure−activity relationship indicating that these compounds act as prodrugs of TCN. Herein, we describe the synthesis and structure−activity relationship of these 6-*N*-acyltriciribine analogues.

Results and Discussion

All 6-*N*-acyltriciribine analogues were synthesized from triciribine, as illustrated in Scheme 1. Selective acetylation of the hydroxyl groups to give 6-amino-4-methyl-8-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)pyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (**1**) was accomplished with 3.3 equiv of acetic anhydride in dry pyridine at room temperature for 4 h. Once the carbohydrate moiety of TCN was acetylated, the 6-amino group was acylated with the desired acyl chloride to give intermediates **2a**−**2m**. Subsequent deprotection with methanolic ammonia afforded the 6-*N*-acyltriciribine analogues (**3a**−**3m**), where compounds **3a**−**3j** have straight chain alkyl acyl

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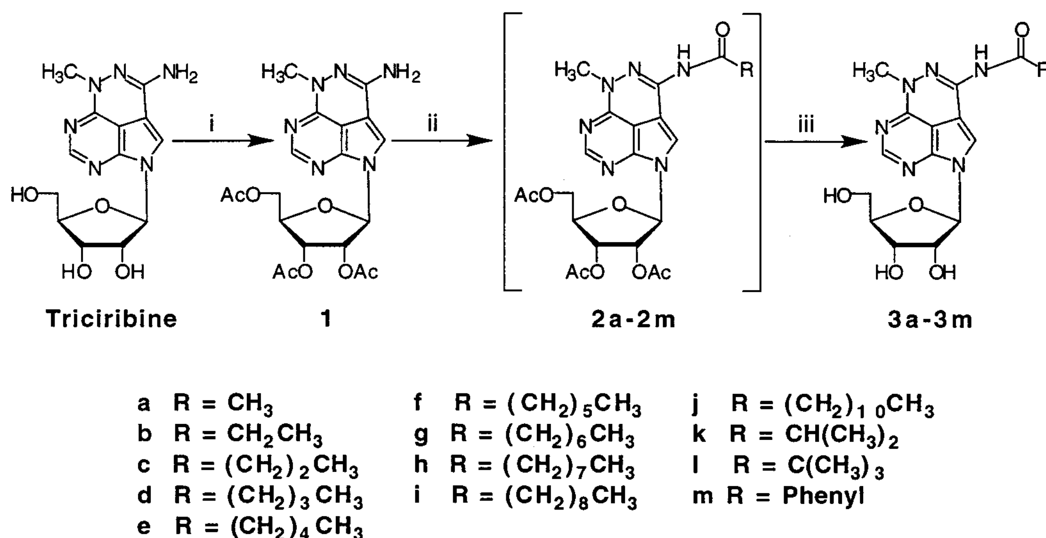
Scheme 1. Synthesis of 6-*N*-Acyltriciribine[a]



[a] Reagents: (i) Ac_2O, pyridine; (ii) acyl chlorides; (iii) NH_3, MeOH.

Table 1. Antiviral Activity and Cytotoxicity of 6-*N*-Acyltriciribine Analogues

		50% inhibitory concentration (μM)					
		antiviral activity[a]			cytotoxicity[b]		
compound	R	HIV-1 RT	HSV-1 ELISA	HCMV plaque	KB growth	HFF visual	CEM-SS visual
TCN[c]		0.04	23	2.5	>100	100	>100
TCN-P[c]		0.04	20	0.8	10	19	>1.0
3a	CH_3	6.8[d]	>100	>100	>100	>100	>100[d]
3b	CH_2CH_3	8.4[d]	>100	40	>100	100	>100[d]
3c	$(CH_2)_2CH_3$	0.50[d]	>100	10	100	100	>100[d]
3d	$(CH_2)_3CH_3$	0.80	>100	0.45	30	>100	32
3e	$(CH_2)_4CH_3$	0.35	>100	8.8	40	100	45
3f	$(CH_2)_5CH_3$	0.035	>100	11	30	32	32
3g	$(CH_2)_6CH_3$	0.1	>100	12	40	21	32
3h	$(CH_2)_7CH_3$	0.8	>100	12	65	21	32
3i	$(CH_2)_8CH_3$	0.25	>100	12	40	21	32
3j	$(CH_2)_{10}CH_3$	1	45	12	75	21	32
3k	$CH(CH_3)_2$	8	>100	>100	>100	>100	>100
3l	$C(CH_3)_3$	>100	>100	>100	>100	>100	>100
3m	phenyl	>100	>100	>100	>100	>100	>100

[a] Antiviral activity was determined using the amount of reverse transcriptase (RT) activity in culture supernatants in triplicate for HIV-1, an ELISA assay in quadruplicate for HSV-1, and a plaque assay in duplicate for HCMV as described in the text. [b] Inhibition of KB cell growth was measured as described in the text in quadruplicate assays. Visual cytotoxicity was scored on uninfected HFF and CEM-SS cells not affected by virus in HCMV plaque and HIV RT assays. [c] Averages of replicate experiments as published previously in ref 10. [d] Average IC_{50} from two experiments.

groups, from 2 to 12 carbons, compounds **3k** and **3l** have branched chain alkyl acyl groups, and compound **3m** has an aromatic acyl group. Each compound was evaluated for activity against three viruses (HIV-1, HSV-1, and HCMV) and for cytotoxicity in three human cell lines (KB, HFF, and CEM-SS cells) (Table 1).

TCN, TCN-P, and compounds **3a**−**3m** were tested for activity against HIV-1 in an assay that employed reverse transcriptase (RT) in culture supernatants as a marker for the virus. Table 1 presents the results and shows that all compounds except **3l** and **3m** were active against HIV and that there was a relationship between the 6-*N*-acyl chain length and activity against HIV. A comparison of the activity of the analogues to the activity of TCN clearly revealed that increasing the carbon chain length of the acyl group increased activty until the chain length reached seven carbons (**3f**) (Figure 1). This established that compound **3f**, with a heptanoyl group (seven-carbon straight chain alkyl acyl group) on the 6-amino group of triciribine, had the optimum carbon chain length for activity against HIV-1. Interestingly, the IC_{50} of compound **3f** was the same as that of TCN and TCN-P, suggesting that compound **3f** is either inherently as active as TCN and TCN-P against HIV-1 or that compound **3f** is a prodrug of TCN or TCN-P.

The 6-*N*-acyltriciribine analogues also were evaluated against HSV-1 in an ELISA and HCMV in a plaque reduction assay. For each compound, no inhibition of HSV-1 was observed or it was found that inhibition of HSV-1 occurred at concentrations that were cytotoxic (Table 1). Compounds **3a**, **3b**, **3k**, **3l**, and **3m** also were weakly active or inactive against HCMV, even at concentrations as high as 100 μM (Table 1). In contrast, compounds **3c**−**3j** had activity against HCMV (IC_{50} = 0.45−12 μM) similar to that of TCN and TCN-P (IC_{50} = 2.5 and 0.8 μM, respectively) (Table 1).

Cytotoxicity of all the 6-*N*-acyltriciribine analogues was determined in three human cell lines (KB, HFF, and CEM-SS cells). Compounds **3a**, **3b**, **3c**, **3k**, **3l**, and

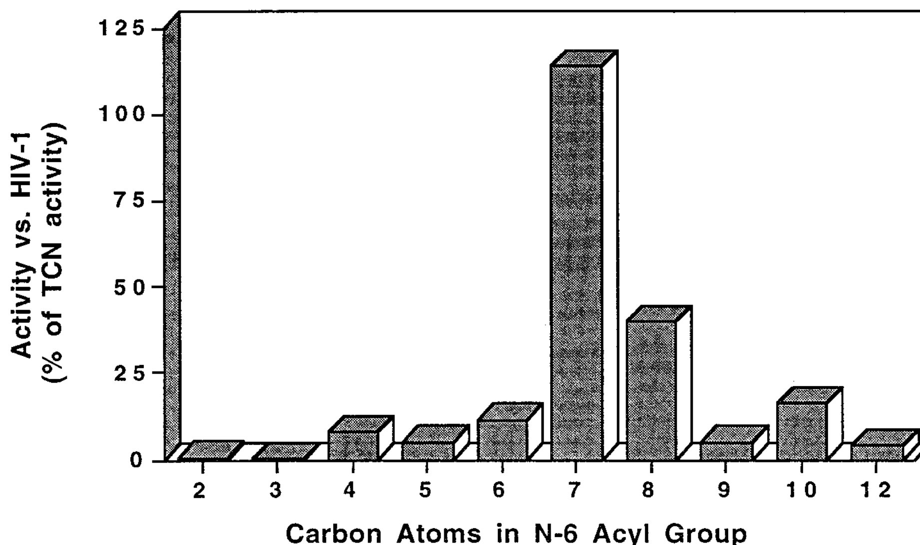


Figure 1. Relationship between number of carbon atoms in *N*-acyl group of TCN analogues and % of TCN activity against HIV. Activity data are plotted as percentage of IC_{50} of analogue compared to IC_{50} of TCN as positive control in the same experiment.

Table 2. Intracellular Phosphorylation of TCN and 6-*N*-Acyltriciribine Analogues in Uninfected Cells

compound	extracellular concentration (μM)	incubation time (h)	intracellular monophosphate concentration (μM)[a]
		CEM-SS Cells[b]	
TCN	10	5	286
	100	5	323[c]
	100	5	318[d]
3d	100	24	710
3f	10	24	225
3f	100	5	850[d]
3h	10	24	243
		AA-2 Cells[b]	
TCN	100	5	9[d]
3f	100	5	48[d]

[a] Nucleotide extractions and quantitation by HPLC was performed as described in the text. [b] Lymphoblastoid cells used for propagation of HIV-1. AA-2 cells have approximately 1−3% of adenosine kinase and deoxycytidine kinase of CEM-SS cells. [c] Control data for TCN phosphorylation previously published in ref 12. [d] Average from two experiments including experiments in which HPLC peaks were analyzed by mass spectrometry.

3m were not cytotoxic at concentrations as high as 100 μM (Table 1), and compounds **3d**−**3j** showed marginal to minimal toxicity to each cell line (IC_{50} = 21−100 μM) (Table 1), thereby establishing that the activity against HIV was not a manifestation of cytotoxicity.

To determine whether these compounds were inherently active or a prodrug of TCN, compounds **3d**, **3f**, and **3h** were incubated with CEM-SS cells for 24 h. Intracellular nucleotides were extracted, separated, and quantitated by reverse phase HPLC. A peak in the HPLC chromatograph coinciding with TCN-P was observed in each case, strongly indicating that compounds **3d**, **3f**, and **3h** were metabolized to TCN-P (Table 2). The amount of intracellular TCN-P detected was sufficient to account for activity against HIV.

To explore the possibility that the 6-*N*-acyl derivatives were acting as prodrugs, TCN and **3f** were incubated with uninfected CEM-SS cells and AA-2 cells−a cell line with 1−3% wild-type adenosine kinase (AK) and deoxycytidine kinase (dCK) activity. Analysis of cell extracts by HPLC/mass spectrometry revealed the presence of high concentrations of TCN-P in CEM-SS cells when either TCN or **3f** were incubated with CEM-SS cells. In fact, higher concentrations of TCN-P were detected in cultures incubated with **3f** (Table 2), suggesting that **3f** entered cells more readily than TCN. In contrast, when the two compounds were incubated with AA-2 cells in which TCN is inactive against HIV-1,[12] the amount of TCN-P found in cell extracts was only about 3 to 6% of that found in CEM-SS cells, indicating that phosphorylation of each compound was required for activity. No phosphorylated product of **3f**, per se, was detected by either ultraviolet absorption or mass spectrometry of the HPLC effluent in any experiment, strongly indicating that **3f** was deacylated to TCN which then was phosphorylated to TCN-P.

On this basis, it appears that these compounds act as prodrugs of TCN. This observation is important for devising a new strategy for the delivery of TCN. We envision that these compounds could be formulated in an oil-based vehicle and delivered orally, as a hydrophobic prodrug, instead of intravenously, as the hydrophilic prodrug TCN-P. Further studies are needed to confirm this hypothesis. Regardless, this structure−activity relationship has identified the heptanoyl group as having the optimal carbon chain length needed to obtain activity against HIV-1 equal to TCN and TCN-P.

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 Devices Mel-Temp melting point apparatus. Thin-layer chromatography used Analtech GHLF SiO_2 prescored plates. TLC plates were developed using a chloroform:methanol (9:1) mixture and visualized under ultraviolet light (254 nm). E. Merck silica gel (230−400 mesh) was used for gravity or flash column chromatography. Proton nuclear magnetic resonance (^1H 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 (ppm) downfield from tetramethylsilane as the internal standard. UV spectra were obtained with a Kontron UVIKON 860 ultraviolet spectrometer. Elemental analysis were performed by the Analytical Laboratory, Department of Chemistry, University of Michigan, Ann Arbor, MI.

6-Amino-4-methyl-8-(2,3,5-tri-*O*-acetyl-*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (1). Acetic anhydride (0.34 g, 0.31 mL, 3.3 mmol) was added to a solution of triciribine (320 mg, 1 mmol) and dry pyridine (10 mL), and the mixture was stirred for 4 h at room temperature. The solvent was removed under vacuum at 60 °C, and the oily residue was coevaporated with toluene (2 × 20 mL) and ethanol (2 × 20 mL). The resulting brown oil was eluted from a silica gel column (2.5 cm × 12 cm) using a solution of methanol:chloroform (1:99). Fractions of 10 mL were collected, and the UV containing fractions (TLC, 1:9 methanol:chloroform) with R_f values of 0.62 were combined and evaporated to give a red oil. (It was observed that the fractions containing the product eluted from the column as a clear and colorless solution, but became light red in color upon exposure to air). Yield = 0.39 g (87%); R_f = 0.62 (TLC, 1:9 methanol:chloroform); ^{1}H NMR ($CDCl_3$) δ 8.24 (1H, s, H-2), 6.70 (1H, s, H-7), 6.21 (1H, d, H-1′), 5.77 (1H, t), 5.56 (1H, dd), 4.45 (2H, bs, NH_2), 4.40 (3H, m), 3.51 (3H, s), 2.12 (3H, s), 2.10 (3H, s), 2.04 (3H, s).

General Procedures for the Synthesis of 6-*N*-Acyltriciribine Analogues. Method 1. The acyl chloride (5 mmol) was added to a solution of compound **1** (0.22 g, 0.5 mmol) in dry pyridine (40 mL) and stirred for 2 h at room temperature. The reaction mixture was diluted with ethyl acetate (150 mL), extracted with 3 N HCl (3 × 125 mL), a saturated solution of aqueous sodium bicarbonate (2 × 125 mL), and brine (125 mL), and dried over magnesium sulfate. The magnesium sulfate was removed by filtration, and the solvent was removed under vacuum. The residue, presumed to be intermediate **2**, was dissolved in methanol (20 mL) and transferred to a glass pressure bottle. Methanolic ammonia (100 mL), saturated at 0 °C, was added, and the sealed reaction vessel was stirred for 18 h at room temperature. The reaction vessel was cooled to 0 °C and then opened, and the solvent was evaporated to dryness. The residue was adsorbed onto silica gel and eluted from a silica gel column (2.5 cm (d) × 12 cm (h)) using a solvent system of 1:19 methanol:chloroform. Fractions of 10 mL were collected, and the UV containing fractions (TLC, 1:9 methanol: chloroform) with the same R_f values were combined and evaporated. The yellow residue was recrystallized from a hot mixture of ethyl acetate:hexane. The solid was collected and dried under vacuum (water aspirator) for 18 h at 80 °C.

Method 2. The acyl chloride (3.2 mmol) was added to a solution of compound **1** (0.73 g, 1.6 mmol) in dry pyridine (10 mL) and stirred for 18 h at room temperature. The solvent was removed under vacuum, and the residue was coevaporated with toluene (2 × 20 mL). The residue, presumed to be intermediate **2**, was dissolved in methanol (20 mL) and transferred to a glass pressure bottle. Methanolysis and workup as described for method 1 above was followed except for a different column size. The resulting yellow residue was either heated at reflux temperature in dichloromethane for 1 h or recrystallized or both. The solid was collected and dried under vacuum (water aspirator) for 18 h at 80 °C.

6-Acetamido-4-methyl-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3a). Compound **3a** was synthesized by method 2 using acetyl chloride (0.25 g, 0.23 mL, 3.2 mmol). The column-purified product was recrystallized from acetone. Yield = 0.47 g (81%); R_f = 0.21; mp 200−202 °C; UV [λ_{max} (ϵ)] (pH 1) 286 (11643), 278 (10906), 238 (17989), (pH 7) 287 (12577), (pH 11) 285 (15354); ^{1}H NMR (DMSO-d_6) δ 10.47 (1H, bs, NH), 8.10 (1H, s, H-2), 7.20 (1H, s, H-7), 5.84 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.48 (1H, q, H-4′), 4.10 (1H, m), 3.95 (1H, m), 3.56 (2H, m, H-5′), 3.35 (3H, s, NCH_3), 2.07 (3H, s, CH_3). Anal. ($C_{15}H_{18}N_6O_5$) C, H, N.

4-Methyl-6-propionylamido-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3b). Compound **3b** was synthesized by method 1 using propionyl chloride (0.46 g, 0.43 mL, 5 mmol). Yield = 0.10 g (55%); R_f = 0.30; mp 235 °C dec; ^{1}H NMR (DMSO-d_6) δ 10.41 (1H, bs, NH), 8.09 (1H, s, H-2), 7.21 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, q, CH_2), 1.06 (3H, t, CH_3). Anal. ($C_{16}H_{20}N_6O_5$) C, H, N.

6-Butyrylamido-4-methyl-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3c). Compound **3c** was synthesized by method 1 using butyryl chloride (0.53 g, 0.52 mL, 5 mmol). Yield = 0.10 g (54%); R_f = 0.35; mp 145 °C dec; ^{1}H NMR (DMSO-d_6) δ 10.41 (1H, bs, NH), 8.09 (1H, s, H-2), 7.21 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.60 (2H, m, CH_2), 0.93 (3H, t, CH_3). Anal. ($C_{17}H_{22}N_6O_5$) C, H, N.

4-Methyl-6-pentanoylamido-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3d). Compound **3d** was synthesized by method 2 using valeryl chloride (0.39 g, 0.38 mL, 3.2 mmol). The column purified product was recrystallized from acetone. Yield = 0.21 g (33%); R_f = 0.39; mp 236−238 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (2H, m, CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{18}H_{24}N_6O_5$) C, H, N.

6-Hexanoylamido-4-methyl-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3e). Compound **3e** was synthesized by method 2 using hexanoyl chloride (0.43 g, 0.45 mL, 3.2 mmol). The column purified product was heated in dichloromethane, at reflux temperature, for 1 h. Yield = 0.24 g (36%); R_f = 0.40; mp 216−218 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (4H, m, 2 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{19}H_{26}N_6O_5$) C, H, N.

6-Heptanoylamido-4-methyl-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3f). Compound **3f** was synthesized by method 2 using heptanoyl chloride (0.48 g, 0.5 mL, 3.2 mmol). The column purified product was heated in dichloromethane, at reflux temperature, for 1 h. Yield = 0.27 g (39%); R_f = 0.44; mp 164−166 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (6H, m, 3 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{20}H_{28}N_6O_5$) C, H, N.

4-Methyl-6-octanoylamido-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3g). Compound **3g** was synthesized by method 2 using octanoyl chloride (0.52 g, 0.55 mL, 3.2 mmol). The column purified product was heated in dichloromethane, at reflux temperature, for 1 h. Yield = 0.16 g (23%); R_f = 0.48; mp 170−172 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (8H, m, 4 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{21}H_{30}N_6O_5$) C, H, N.

4-Methyl-6-nonanoylamido-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3h). Compound **3h** was synthesized by method 2 using nonanoyl chloride (0.57 g, 0.58 mL, 3.2 mmol). The column purified product was recrystallized from hot acetone. Yield = 0.10 g (14%); R_f = 0.52; mp 170−172 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (10H, m, 5 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{22}H_{32}N_6O_5$) C, H, N.

6-Decanoylamido-4-methyl-8-(*β*-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3i). Compound **3i** was synthesized by method 2 using decanoyl chloride (0.61 g, 0.66 mL, 3.2 mmol). The column purified product was recrystallized from hot acetone. Yield = 0.22 g (29%); R_f = 0.55; mp 171−173 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m,

2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (12H, m, 6 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{23}H_{34}N_6O_5$) C, H, N.

6-Dodecanoylamido-4-methyl-8-(β-D-ribofuranosyl)-pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3j). Compound **3j** was synthesized by method 2 using lauroyl chloride (0.70 g, 0.74 mL, 3.2 mmol). The column purified product was recrystallized from hot acetone. Yield = 0.21 g (26%); R_f = 0.60; mp 176−178 °C; ^{1}H NMR (DMSO-d_6) δ 10.43 (1H, bs, NH), 8.09 (1H, s, H-2), 7.19 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.35 (2H, t, CH_2), 1.57 (2H, m, CH_2), 1.27 (16H, m, 8 × CH_2), 0.85 (3H, t, CH_3). Anal. ($C_{25}H_{38}N_6O_5$) C, H, N.

6-Isobutyrylamido-4-methyl-8-(β-D-ribofuranosyl)pyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3k). Compound **3k** was synthesized by method 1 using isobutyryl chloride (0.53 g, 0.52 mL, 5 mmol). Yield = 0.12 g (63%); R_f = 0.38; mp 186−188 °C; ^{1}H NMR (DMSO-d_6) δ 10.41 (1H, bs, NH), 8.09 (1H, s, H-2), 7.21 (1H, s, H-7), 5.83 (1H, d, H-1′), 5.40 (2H, m, 2 × OH), 5.20 (1H, d, OH), 4.49 (1H, m), 4.08 (1H, m), 3.95 (1H, m), 3.55 (2H, m, H-5′), 3.43 (3H, s, NCH_3), 2.72 (1H, m, CH), 1.10 (6H, d, 2 × CH_3). Anal. ($C_{17}H_{22}N_6O_5$) C, H, N.

4-Methyl-8-(β-D-ribofuranosyl)-6-trimethylacetylamidopyrrolo[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3l). Compound **3l** was synthesized by method 1 using trimethylacetyl chloride (0.60 g, 0.62 mL, 5 mmol). Yield = 0.11 g (55%); R_f = 0.51; mp 209−211 °C; ^{1}H NMR (DMSO-d_6) δ 9.91 (1H, bs, NH), 8.11 (1H, s, H-2), 7.08 (1H, s, H-7), 5.85 (1H, d, H-1′), 5.42 (2H, m, 2 × OH), 5.18 (1H, d, OH), 4.49 (1H, m), 4.10 (1H, m), 3.95 (1H, m), 3.50 (2H, m, H-5′), 3.48 (3H, s, NCH_3), 1.23 (9H, s, 3 × CH_3). Anal. ($C_{18}H_{24}N_6O_5$·0.25EtOAc) C, H, N.

6-Benzoylamido-4-methyl-8-(β-D-ribofuranosyl)pyrrolo-[4,3,2-*de*]pyrimido[4,5-*c*]pyridazine (3m). Compound **3m** was synthesized by method 1 using benzoyl chloride (0.70 g, 0.58 mL, 5 mmol). The column purified product was recrystallized from a hot mixture of ethyl acetate:hexane. Yield = 0.10 g (47%); R_f = 0.52; mp 265 °C dec; ^{1}H NMR (DMSO-d_6) δ 10.90 (1H, bs, NH), 8.14 (1H, s, H-2), 8.00 (2H, d), 7.60 (3H, m), 7.16 (1H, s, H-7), 5.85 (1H, d, H-1′), 5.36 (2H, m, 2 × OH), 5.18 (1H, d, OH), 4.49 (1H, m), 4.10 (1H, m), 3.94 (1H, m), 3.55 (2H, m, H-5′), 3.48 (3H, s, NCH_3). Anal. ($C_{20}H_{20}N_6O_5$) C, H, N.

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.[20] 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-SS and AA-2 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. CEM-SS cells were from Dr. P. Nara;[21,22] AA-2 cells were from Dr. M. Hershfield. AA-2 cells are a subclone of AA cells derived from the WIL-2 human splenic EBV+ B lymphoblastoid line. The AA-2 subclone was selected for resistance to 6-methylmercaptopurine ribonucleoside and arabinosylcytosine to give cells with <1−3% wild-type AK dCK activity.[23] Cells were passaged twice weekly at 1:10 dilutions using RPMI 1640 with 10% fetal calf serum supplemented with nonessential amino acids and 1 mM pyruvate.

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.[10,24] To evaluate the activities of compounds in cells acutely infected with HIV, reverse transcriptase (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) per 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. One-tenth milliliter 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 μM to 0.14 μM. After 6 days 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.[25]

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 plaque forming units (pfu) of HCMV per well using the procedures detailed earlier.[20] Following virus adsorption, compounds dissolved in MEM(E) containing 0.5% methyl cellulose and 5% fetal calf serum 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 were 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)[26] was employed to detect HSV-1. Briefly, 96-well cluster dishes were planted with 10 000 BSC-1 cells per well in 200 μL per 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 fixed, 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 stopped 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.[20] (*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.[27] Briefly, 96-well cluster dishes were planted with KB cells at 5000 cells per 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, fixed with 95% ethanol, and stained with 0.1% crystal violet. Acidified ethanol was added, and plates 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. Fifty percent 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 and AA-2 cells were grown in suspension culture with concentrations of compounds given in Table 2 for 5 to 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.[28] 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 trioctylamine was added to the tube. The mixture was vortexed for 5 or more seconds 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.[9] or by ion pair reverse phase HPLC as described by Walseth et al.[29] 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 with TCN, analysis was performed using anion exchange HPLC, but superior resolution of monophosphates was achieved by reverse phase HPLC; consequently it was adopted in subsequent experiments with TCN and TCN-P. Ion pair reverse phase chromatography was performed on a 3.9 × 300 mm μBondapak C18 column (Waters). The solvent was 5 mM tetrabutylammonium hydroxide in 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 retention time of unknown peaks to retention time of a TCN-P standard and on the ratio of 290/254 nm due to the absorbance maximum of TCN and TCN-P at 290 nm.[9]

Solvent conditions were changed for the analysis of the TCN analogues (**3d**, **3f**, and **3h**). The ion pairing reagent was removed because these conditions resulted in a strong retention of these analogues (**3d**, **3f**, and **3h**) on the column. Following equilibration with dilute formic acid (pH 2.5), a linear gradient was used at a flow rate of 1 mL/min with a 20 min elution time beginning with formic acid (pH 2.5) and ending with 50% methanol in formic acid (pH 2.5) followed by a 15 min isocratic elution with the final solution. These conditions also were employed in a separate study in which electrospray mass spectrometry was performed to identify peaks using a Finnigan LCQ mass spectrometer interfaced with a Hitachi HPLC.

Intracellular concentrations were calculated based upon the integrated area under HPLC peaks of TCN-P, conversion of area to nanomoles of TCN-P based upon the area under TCN-P peaks from standards of known concentration, and conversion of concentration from nanomoles/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 hemocytometer, measurement of the diameter of 10−20 representative cells, and assumption of spherical geometry.

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References

(1) Schram, K. H.; Townsend, L. B. The synthesis of 6-amino-4-methyl-8-(beta-D-ribofuranosyl)(4-H,8-H)pyrrolo[4,3,2-de]pyrimido-[4,5-c]pyridazine, A new tricyclic nucleoside. *Tetrahedron Lett.* **1971**, *49*, 4757−4760.

(2) Roti Roti, L. W.; Roti Roti, J. L.; Townsend, L. B. Studies on the mechanism of cytotoxicity of tricyclic nucleoside NSC#154020 in L1210 cells. *Proc. Am. Assoc. Cancer Res.* **1978**, *19*, 40.

(3) Schweinsberg, P. D.; Smith, R. G.; Loo, T. L. Identification of the metabolites of an antitumor tricyclic nucleoside (NSC-154020). *Biochem. Pharmacol.* **1981**, *30*, 2521−2526.

(4) Wotring, L. L.; Townsend, L. B.; Crabtree, G. W.; Parks, R. E., Jr. A possible role for ecto-5′-nucleotidase in cytotoxicity and intracellular nucleotide formation from the tricyclic nucleoside-5′-phosphate (TCN-P). *Proc. Am. Assoc. Cancer Res.* **1981**, *22*, 257.

(5) Wotring, L. L.; Passiatore, J. E.; Roti Roti, J. L.; Hudson, J. L.; Townsend, L. B. Effects of the tricyclic nucleoside 6-amino-4-methyl-8-(beta-D- ribofuranosyl)pyrrolo[4,3,2-de]pyrimido[4,5-c]pyridazine on the viability and cell cycle distribution of L1210 cells in vitro. *Cancer Res.* **1985**, *45*, 6355−6361.

(6) Wotring, L. L.; Townsend, L. B.; Jones, L. M.; Borysko, K. Z.; Gildersleeve, D. L.; Parker, W. B. Dual mechanisms of inhibition of DNA synthesis by triciribine. *Cancer Res.* **1990**, *50*, 4891−4899.

(7) Moore, E. C.; Hurlbert, R. B.; Boss, G. R.; Massia, S. P. Inhibition of two enzymes in de novo purine nucleotide synthesis by triciribine phosphate (TCN-P). *Biochem. Pharmacol.* **1989**, *38*, 4045−4051.

(8) Townsend, L. B.; Lewis, A. F.; Roti Roti, L. W. Synthesis of 6-amino-4-methyl-8-(beta-D-ribofuranosyl)pyrrolo[4,3,2-de]pyrimido[4,5-c]pyridazine-5′-phosphate as a novel compound and its utility against L-1210 mouse leukemia. United States. Patent Number: 4,123,524. October 31, 1978.

(9) Wotring, L. L.; Crabtree, G. W.; Edwards, N. L.; Parks, R. E., Jr.; Townsend, L. B. Mechanism of activation of triciribine phosphate (TCN-P) as a prodrug form of TCN. *Cancer Treat. Rep.* **1986**, *70*, 491−497.

(10) Kucera, L. S.; Iyer, N. P.; Puckett, S. H.; Buckheit, R. W., Jr.; Westbrook, L.; Toyer, B. R.; White, E. L.; Germany-Decker, J. M.; Shannon, W. M.; Chen, R. C.; Nassiri, M. R.; Shipman, C.; Townsend, L. B.; Drach, J. C. Activity of triciribine and triciribine-5′-monophosphate against human immunodeficiency virus types 1 and 2. *AIDS Res. Hum. Retroviruses.* **1993**, *9*, 307−314.

(11) Ptak, R. G.; Jacobson, J. G.; Borysko, K. Z.; Townsend, L. B.; Drach, J. C. Triciribine inhibits HIV nuclear import and requires phosphorylation by cellular enzymes for activity. 5th Conf. Retroviruses & Opportunistic Infections. Chicago, IL. 1998. abst. 354.

(12) Ptak, R. G.; Borysko, K. Z.; Porcari, A. R.; Buthod, J. L.; Holland, L. E.; Shipman, C.; Townsend, L. B.; Drach, J. C. Phosphorylation of triciribine is necessary for activity against HIV-1. *AIDS Res. Human Retroviruses.* **1998**, *14*, 1315−1322.

(13) Ptak, R. G.; Drach, J. C.; Jacobson, J. G.; Townsend, L. B.; Stup, T. L.; Russell, J.; Buckheit, R. W. HIV-1 Regulatory Proteins: Targets of Triciribine? 6th Conf. Retroviruses & Opportunistic Infections. Chicago, IL. February, 1999. abst. 622.

(14) Drach, J. C.; Shipman, C.; Ptak, R. G.; Borysko, K. Z.; Jacobson, J. G.; Nassiri, M. R.; Wotring, L. L.; Porcari, A. R.; Townsend, L. B. Activity of triciribine (TCN) against HIV-1 requires cellular phosphorylation and inhibition of a viral gene product. 36th Interscience Conf. Antimicrob. Agents Chemother. New Orleans, LA. 1996. abst. 2099.

(15) Ickes, D. E.; Shipman, C.; Drach, J. C. Antiviral activity of triciribine and triciribine monophosphate against human herpesvirus type 6. Seventh International Conference on Antiviral Research. Charleston, SC. 1994.

(16) Musherure, P. R.; Breitenbach, J. M.; Aseltine, K. R.; Shipman, C.; Drach, J. C. Antiviral activity of triciribine (TCN) in combination with phosphonoformate (PFA) on human herpesvirus type 6 (HHV-6). AADR Meeting. San Antonio, TX. 1995. abst. 391.

(17) Nassiri, M. R.; Achim, C. L.; Martinez, A. J.; Wiley, C. A.; Drach, J. C.; Townsend, L. B. Antiretroviral activity of TCN-P in HIV-infected Human Macrophages. Eleventh International Conference on Antiviral Research. San Diego, CA. 1998.

(18) Porcari, A. R.; Drach, J. C.; Wotring, L. L.; Townsend, L. B. The design, synthesis and evaluation of a series of acyclic triciribine analogues for their antiviral and antineoplastic activity. 213th National Meeting of the American Chemical Society. San Francisco, CA. 1997. abst. MEDI 269.

(19) Porcari, A. R.; Drach, J. C.; Wotring, L. L.; Townsend, L. B. Synthesis and structure−activity relationship of a series of deoxy triciribine analogues against HIV and murine L1210 cells. 214th National Meeting of the American Chemical Society. Las Vegas, NV. 1997. abst. MEDI 181.

(20) Turk, S. R.; Shipman, C., Jr.; Nassiri, M. R.; Genzlinger, G.; Krawczyk, S. H.; Townsend, L. B.; Drach, J. C. Pyrrolo[2,3-d]-pyrimidine nucleosides as inhibitors of human cytomegalovirus. *Antimicrob. Agents Chemother.* **1987**, *31*, 544−550.
(21) Nara, P. L.; Hatch, W. C.; Dunlop, N. M.; Robey, W. G.; Fischinger, P. J. Simple, rapid quantitative, syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res. Hum. Retroviruses.* **1987**. *3*, 283−302,
(22) Nara, P. L.; Fischinger, P. J. Quantitative infectivity assay for HIV-1 and -2. *Nature* **1988**, *332*, 469−470.
(23) Chaffee, S.; Leeds, J. M.; Matthews, T. J.; Weinhold, K. J.; Skinner, M.; Bolognesi, D. P.; Hershfield, M. S. Phenotypic variation in the response to the human immunodeficiency virus among derivatives of the CEM T and WIL-2 B cell lines. *J. Exp. Med.* **1988**, *168*, 605−621.
(24) Kucera, L. S.; Iyer, N.; Leake, E.; Raben, A.; Modest, E. J.; Daniel, L. W.; Piantadosi, C. Novel membrane-interactive ether lipid analogues that inhibit infectious HIV-1 production and induce defective virus formation. *AIDS Res. Hum. Retroviruses.* **1990**, *6*, 491−501.
(25) White, E. L.; Buckheit, R. W.; Ross, L. J.; Germany, J. M.; Andries, K.; Pauwels, R.; Janssen, P. A. J.; Shannon, W. M.; Chirigos, M. A. A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates. *Antiviral Res.* **1991**, *16*, 257−266.
(26) Prichard, M. N.; Shipman, C. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res.* **1990**, *14*, 181−206.
(27) Prichard, M. N.; Prichard, L. E.; Baguley, W. A.; Nassiri, M. R.; Shipman, C. Three-dimensional analysis of the synergistic cytotoxicity of ganciclovir and zidovudine. *Antimicrob. Agents Chemother.* **1991**, *35*, 1060−1065.
(28) Pogolotti, A. L.; Santi, D. V. High-pressure liquid chromatography-Ultraviolet analysis of intracellular nucleotides. *Anal. Biochem.* **1982**, *126*, 335−345.
(29) Walseth, T. F.; Graff, G.; Moos, M. C.; Goldberg, N. D. Separation of 5′-ribonucleoside monophosphates by ion-pair reverse-phase high-performance liquid chromatography. *Anal. Biochem.* **1980**, *107*, 240−245.

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