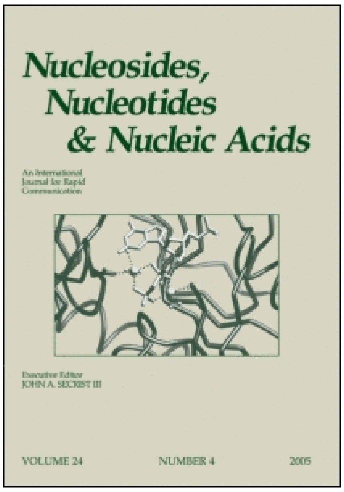

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Synthesis and Antiviral Evaluation of Some Novel Tricyclic Pyrazolo[3,4-*b*]indole Nucleosides[†]

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ABSTRACT

Novel pyrazolo[3,4-*b*]indole nucleoside analogs were synthesized from the corresponding 3-formyl-2-chloroindole and 3-cyano-2-chloroindole nucleosides by treatment with hydrazine. Very few examples of pyrazolo[3,4-*b*]indole heterocycles have been published in the literature and this is the first synthesis of nucleoside analogs containing this heterocycle. These new pyrazolo[3,4-*b*]indole nucleosides were active against human cytomegalovirus and herpes simplex virus type 1, but this activity was not well separated from cytotoxicity.

Key Words: Pyrazolo[3,4-*b*]indole; Nucleoside analogs; Antiviral.

[†] In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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INTRODUCTION

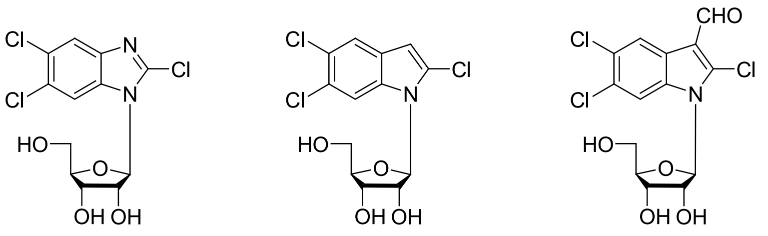
Human cytomegalovirus (HCMV) is an opportunistic infection which is endemic in both the industrialized and developing countries.[1] Although healthy persons seldom exhibit HCMV-related pathologies, immunocompromised individuals can show a wide array of symptoms including retinitis and gastritis in AIDS patients, organ rejection in transplant recipients, and birth defects in neonates.[2] The five currently licensed HCMV treatments, ganciclovir (GCV),[3] valganciclovir,[4] cidofovir,[5] foscarnet,[6] and fomivirsen,[7] all suffer from limitations including toxicity and poor bioavailability. The search for compounds with good antiviral activity and fewer limitations has led our laboratory to synthesize a number of different nucleoside analogs, including 2,5,6-trichloro-1-(β-D-ribofuranosyl)benzimidazole (TCRB, **1**, Fig. 1).[8] TCRB was found to be a potent inhibitor of HCMV replication, but it was unstable in vivo due to glycosidic bond cleavage.[9] Subsequent investigations in our laboratory have focused on the synthesis of compounds which maintain the activity of TCRB, but are less susceptible to glycosidic bond cleavage.

Indole nucleoside analogs should be less susceptible to glycosidic bond cleavage because, in contrast to the benzimidazole nucleosides, the 3-position of the indole nucleosides cannot be protonated. The indole nucleoside 2,5,6-trichloro-1-(β-D-ribofuranosyl)indole (TCRI, **2**, Fig. 1) was synthesized as an analog of TCRB, but was found to be devoid of antiviral activity.[10] In contrast, the 3-formylindole nucleoside **3**, was found to be both a potent and selective inhibitor of HCMV replication in antiviral assays.[11]

The chemical versatility of the formyl group of nucleoside analog **3** prompted us to initiate a program to expand the structure–activity relationship (SAR) among this series of compounds. Our initial investigations led us to react **3** with various substituted hydrazine derivatives in order to synthesize a series of substituted hydrazones.[11] We now describe the synthesis and antiviral evaluation of selected tricyclic pyrazolo[3,4-*b*]indole nucleoside analogs derived from an unexpected cyclization.

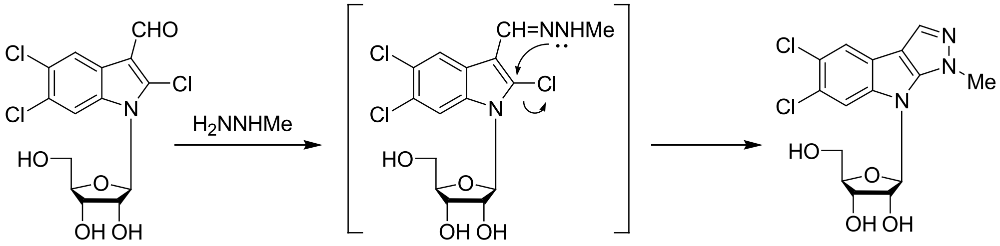
RESULTS AND DISCUSSION

Condensation of aromatic aldehydes with hydrazine derivatives generally proceeds under mild conditions to produce substituted or unsubstituted hydrazones in good

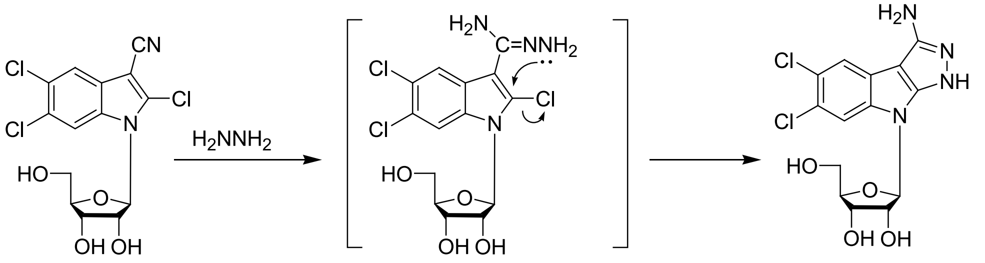


TCRB, 1 **TCRI, 2** **3**

Figure 1. Previously synthesized benzimidazole and indole nucleosides.



Scheme 1. Hydrazone **4** cyclizes to provide a pyrazolo[3,4-*b*]indole nucleoside.



Scheme 2. Synthesis of the 3-aminopyrazolo[3,4-*b*]indole **8** proceeds as expected.

yields. It was initially expected that the addition of methylhydrazine to the nucleoside analog **3** would provide the hydrazone **4** (Scheme 1). However, the physical data obtained for the product of this condensation did not match that which we had expected for the hydrazone **4**. Especially diagnostic was the result obtained from mass spectrometry. The product lacked the requisite mass expected for compound **4**, and the isotope distribution confirmed that the product contained only two chlorine atoms rather than the expected three. Proton NMR confirmed that no exchangeable –NH proton remained in the molecule.

Although we did not initially expect a displacement of the 2-chloro substituent of the indole ring, this is apparently what had occurred. We propose that **4** was initially formed but that the subsequent cyclization occurred so rapidly that the hydrazone could not be isolated. The ease with which the cyclization occurred was especially surprising given that several other hydrazones have already been synthesized from **3** in our laboratory[11] without the analogous displacement of the 2-chloro substituent of the indole. There have been very few pyrazolo[3,4-*b*]indoles reported in the literature. To date, only nine examples[a] have been reported,[12–14] none of which are nucleosides.

In order to further document this unexpected reaction, we elected to utilize a 2-chloroindole nucleoside with a different substituent at the 3-position, and determine

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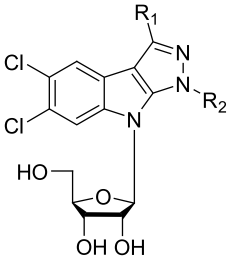
[a] The pyrazolo[3,4-*b*]indoles reported in Ref. [13] were synthesized by a dipolar cycloaddition reaction and may actually be pyrazolo[4,3-*b*]indoles.

Table 1. Antiviral activity and cytotoxicity of synthesized compounds.



			50% inhibitory concentration (μM)			
			Antiviral		Cytotoxicity	
No.	R_1	R_2	HCMV plaque[a]	HSV-1 ELISA[b]	Visual[c]	Growth[c]
5	–H	–Me	0.7	50	10	20
8	$-NH_2$	–H	4.0	7.0	5.0	4.5
TCRB[e]			2.9	102	238	210
GCV[f]			7.4	3.5	>100[d]	>100[d]

[a] Plaque reduction assays were performed in duplicate wells as described in the text.
[b] Compounds were assayed by ELISA in quadruplicate wells.
[c] Visual cytotoxicity was scored on HFF cells at the time of HCMV plaque enumeration; inhibition of KB cell growth was determined in triplicate wells as described in the text.
[d] > 100 indicates an IC_{50} greater than the highest concentration tested.
[e] Data for TCRB published previously as compound **9** in reference 8.
[f] Averages from 108, 33, and 3 experiments, respectively using GCV.

whether the pattern of reactivity was similar. After a review of the different substituents that could be effective, we decided that a carboxamidrazone would likely undergo a similar cyclization to provide the analogous 3-aminopyrazolo[3,4-*b*]indole nucleosides. The 3-cyanoindole nucleoside **6**[10] was treated with hydrazine hydrate to provide the expected pyrazolo[3,4-*b*]indole nucleoside **8** (Scheme 2) in excellent yield.

As was the case for pyrazolo[3,4-*b*]indole **5**, mass spectrometry was particularly useful for the structural determination of compound **8**. The isotope pattern again indicated that only two chlorine atoms remained in the product. The proton NMR was also consistent with compound **8**, showing only three exchangeable –NH protons, not four as would be expected for an uncyclized product. One of these protons, located at 8.68 ppm, is also much farther downfield than would be expected for the uncyclized compound **7**.

The compounds were assayed for antiviral activity against both HCMV and HSV-1 (Table 1), and for cytotoxicity. These newly synthesized pyrazolo[3,4-*b*]indole nucleosides were cytotoxic in low micromolar concentrations, and the antiviral effects were not well separated from the toxicity of the compounds, apart from the activity of **5** against HCMV.

EXPERIMENTAL

General Procedures. Melting points were taken on a Mel–Temp apparatus, and are uncorrected. NMR spectra were obtained on a Bruker DRX500 spectrometer;

chemical shift values were determined relative to an internal tetramethylsilane standard. Mass spectrometry was performed at the University of Michigan Department of Chemistry facility. Elemental analysis was performed at the University of Michigan Chemistry Department elemental analysis facility. Thin layer chromatography was performed on silica gel GHLF plates from Analtech (Newark, DE). Chromatograms were visualized under UV light at 254 nm. Column chromatography was performed on Silica gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany) with the specified column size and solvent system. Evaporation of all solvents was accomplished under reduced pressure, at less than 40°C. All reagents were obtained from commercial sources or were synthesized from literature procedures, and were used without further purification.

5,6-Dichloro-1-methyl-8-(β-D-ribofuranosyl)pyrazolo[3,4-*b*]indole (5). 2,5,6-Trichloro-3-formyl-1-(β-D-ribofuranosyl)indole[11] (**3**, 82 mg, 0.22 mmol) was dissolved in 4 mL of methanol to which was added 0.5 mL of methylhydrazine. The solution was stirred at room temperature for 16 h, then the solvent was removed under vacuum. The residue was suspended in 20 mL of brine and extracted with EtOAc (2 × 20 mL). The combined organic extracts were dried over $MgSO_4$, filtered and evaporated to yield a yellow oil. The oil was subjected to column chromatography (40 × 350 mm) on C18-reverse phase silica gel with 75% MeOH/water. Fractions containing product (UV detection) were pooled and evaporated to yield 29 mg (33%) of **5** as a pale yellow solid: mp 135–138°C; R_f 0.6 (20% MeOH/$CHCl_3$); ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.25 (s, 1H), 7.94 (s, 1H), 7.73 (s, 1H), 5.95 (d, 1H), 5.38 (d, 1H, D_2O exch.), 5.25 (m, 2H, D_2O exch.), 4.21 (q, 1H), 4.13 (m, 1H), 4.05 (s, 3H), 3.97 (m, 1H), 3.74 (m, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 146.23, 139.94, 128.98, 123.94, 122.80, 120.16, 119.24, 115.28, 109.15, 88.91, 85.71, 71.94, 68.75, 60.89, 37.81. HRMS (ES) m/z calcd. for $C_{15}H_{15}Cl_2N_3O_4 \cdot Na \cdot MeOH$ 426.0599, found 426.0593. Anal calcd for $C_{15}H_{15}Cl_2N_3O_4$: C, 48.40; H, 4.06; N, 11.29. Found: C, 48.13; H, 4.16; N, 11.12.

5,6-Dichloro-3-amino-8-(β-D-ribofuranosyl)pyrazolo[3,4-b]indole (8). 2,5,6-Trichloro-3-cyano-1-(β-D-ribofuranosyl)indole[11] (**6**, 102 mg, 0.27 mmol) was dissolved in hydrazine hydrate (2 mL), and the resulting solution was stirred at room temperature for 30 min. The suspension thus obtained was diluted with water (8 mL), cooled to 0°C; and the suspension filtered and the solids rinsed with water. The solids were recrystallized from MeOH/H_2O to yield 91 mg (90%) of **8** as a white crystalline solid: mp 198–201°C; R_f 0.3 (20% MeOH/$CHCl_3$); ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.68 (s, 1H, D_2O exch.), 7.76 (s, 1H), 7.27 (s, 1H), 5.81 (d, 1H), 5.78 (b, 1H, D_2O exch.), 5.31–5.28 (m, 2H, D_2O exch.), 4.84 (s, 2H, D_2O exch.), 4.27 (q, 1H), 4.07 (s, 1H), 4.00 (s, 1H), 3.68 (q, 2H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 155.50, 131.99, 129.00, 123.60, 121.60, 116.91, 115.45, 111.96, 88.32, 85.95, 71.06, 69.95, 63.28, 60.78. HRMS (EI) m/z calcd. for $C_{14}H_{14}Cl_2N_4O_4$ 372.0392, found 372.0397. Anal calcd for $C_{14}H_{14}Cl_2N_4O_4 \cdot 1/4\ H_2O$: C, 44.52; H, 3.87; N, 14.83. Found: C, 44.63; H, 4.10; N, 14.72.

Biological Evaluation

Cell Culture and Virological Procedures. The routine growth and passage of KB, BSC-1, and HFF cells was performed in monolayer cultures as routinely employed by our laboratories.[15,16]

The Towne strain, plaque–purified isolate P_o, of HCMV was kindly provided by Dr. Mark Stinski, University of Iowa. The KOS strain of HSV-1 was used in most experiments and was provided by Dr. Sandra K. Weller, University of Connecticut. Stock HCMV was prepared by infecting HFF cells at a multiplicity of infection (m.o.i.) of < 0.01 plaque-forming units (p.f.u.) per cell as detailed previously.[16] High titer HSV-1 stocks were prepared by infecting KB cells at an m.o.i. of < 0.1 also as detailed previously.[16] Virus titers were determined using monolayer cultures of HFF cells for HCMV and monolayer cultures of BSC-1 cells for HSV-1 as described earlier.[17] Briefly, HFF or BSC-1 cells were planted in 96-well cluster dishes and incubated overnight at 37°C. The next day cultures were inoculated with HCMV or HSV-1 and serially diluted 1:3 across the remaining eleven columns of the 96-well plate. After virus adsorption the inoculum was replaced with fresh medium and cultures were incubated for seven days for HCMV, two or three days for HSV-1. Plaques were enumerated in wells having the dilution which gave 5 to 20 plaques per well and virus titers were calculated based on the dilution used.

Antiviral Assays. HCMV was quantified by a plaque–reduction assay. HFF cells in 24-well cluster dishes were infected with approximately 100 p.f.u. of HCMV per cm^2 cell sheet. Following virus adsorption, the compounds (prepared as 10 mg/mL stock solutions in DMSO) were diluted with growth medium and were added to duplicate wells in four to eight selected concentrations. After incubation at 37°C for 10 days, cell sheets were fixed, stained with crystal violet and microscopic plaques enumerated as described previously.[16] 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 was quantified by an ELISA developed in our laboratory.[18] Briefly, 96-well cluster dishes were planted with 10,000 BSC-1 cells per well. After overnight incubation at 37°C, selected drug concentrations in quadruplicate and HSV-1 at a concentration of 100 p.f.u./well were added. Following a 3-day incubation at 37°C, the medium was removed, and the plates were treated as described previously,[18] and the 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. Two different assays were used for routine cytotoxicity testing. 1) Cytotoxicity produced in stationary HFF cells was determined by microscopic inspection of cells not affected by the virus used in plaque assays.[16] 2) 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.[19] Briefly, 96-well cluster dishes were planted with KB cells at 3000–5000 cells per well. After overnight incubation at 37°C, test compound was added in quadruplicate at six to eight concentrations. Plates were incubated at 37°C for 48 h, stained with 0.1% crystal violet, and read at 570 nm as described previously.[19]

Data Analysis. Dose response relationships were used to quantitate drug effects by linear regression of the percent inhibition of parameters derived in the preceding assays against $\log_{10}$ drug concentrations. Fifty percent inhibitory concentrations (IC_{50}'s) were calculated from the linear portions of the regression lines. Samples containing positive controls (acyclovir for HSV-1, GCV for HCMV, and 2-acetylpyridine thiosemicarbazone for cytotoxicity) were used in all assays.

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