Design, Synthesis, and Antiviral Activity of Certain 2,5,6-Trihalo-1-(β-D-ribofuranosyl)benzimidazoles

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A new series of 2-substituted 5,6-dichlorobenzimidazole ribonucleosides has been synthesized and tested for activity against two human herpes viruses and for cytotoxicity. 2,5,6-Trichloro-1-(β-D-ribofuranosyl)benzimidazole (TCRB) was prepared by ribosylation of the heterocycle 2,5,6-trichlorobenzimidazole followed by a removal of the protecting groups. The 2-bromo derivative (BDCRB) was made in a similar fashion from 2-bromo-5,6-dichlorobenzimidazole. In contrast, the 2-iodo derivative presented a more difficult problem since the appropriate heterocycle was unavailable. This prompted us to prepare the 2-amino derivative followed by nonaqueous diazotization and removal of the blocking groups. Biological evaluation revealed marked differences in the activities of these compounds and the closely related known compound 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB). DRB was weakly active against both human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1), (IC_{50}'s = 42 and 30 μM, respectively) but was cytotoxic to uninfected human foreskin fibroblasts and KB cells in the same dose range. Similar results were obtained with the heterocycle 2,5,6-trichlorobenzimidazole. In marked contrast, the ribonucleoside of 2,5,6-trichlorobenzimidazole (TCRB) was active against HCMV (IC_{50} = 2.9 μM, plaque assay; IC_{90} = 1.4 μM, yield assay) but only weakly active against HSV-1 (IC_{50} = 102 μM, plaque assay). Little to no cytotoxicity was observed in HFF and KB cells at concentrations up to 100 μM. By changing the substituent at the 2-position from chlorine to bromine (BDCRB), a 4-fold increase in activity against HCMV was observed without any significant increase in cytotoxicity. In contrast, the 2-I and 2-NH_2 derivatives were only weakly active against HCMV and HSV-1 with activity not well-separated from cytotoxicity. These data establish that for maximum activity against HCMV with separation from cytotoxicity, ribose is preferred at the 1-position and that Cl or Br is apparently preferred at the 2-position. The activity and selectivity of both TCRB and BDCRB were better than that observed with either ganciclovir or foscarnet.

Introduction

Human cytomegalovirus (HCMV) is a human herpes virus which has a high order of genome sequence complexity and a narrow host range.[1] Although HCMV is innocuous in the immunocompetent individual, it is a significant pathogen in neonates and immunocompromised individuals.[2] Bone marrow[3] and organ transplant[4] patients as well as individuals with acquired immune deficiency syndrome (AIDS)[5] are vulnerable to the effects of this virus. Several organ systems, especially the lungs[6] and retina,[7] are especially susceptible and can be severely compromised. Currently, ganciclovir and foscarnet (Figure 1) are the only FDA-approved drugs for the treatment of HCMV infections. Both drugs, however, have poor oral bioavailability; the use of ganciclovir can lead to granulocytopenia,[8] and therapy with foscarnet may produce renal dysfunction.[9] Moreover virus strains resistant to each of these drugs may be emerging.[10] Consequently, there is a need for better, more potent and selective antiviral drugs to treat HCMV infections.

As a part of our search for new antiviral drugs, we have been exploring pyrrolo[2,3-*d*]pyrimidine[11] and benzimidazole nucleosides as potential inhibitors of HCMV. The current study details our work with certain key halogenated benzimidazole nucleosides. In 1954, Tamm and co-workers first reported the synthesis and antiviral activity of halogenated benzimidazole nucleosides.[12,13] They found that 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB) has multiple biological activities including activity against RNA[13] and DNA[14] viruses. DRB inhibits viral[15] and cellular[16] RNA synthesis, most likely as a consequence of inhibiting cellular RNA polymerase II.[17] DRB also is an inhibitor of casein kinase[18] and DNA topoisomerase II[19] and is an interferon inducer.[20] Thus DRB is a nucleoside that affects multiple cellular processes, and consequently its antiviral activity is poorly separated from cytotoxicity resulting in little potential as an antiviral drug.[13,21]

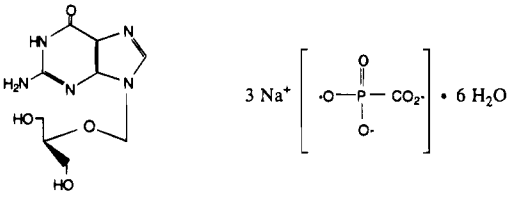

Figure 1. Structures of ganciclovir and foscarnet.

In order to improve the antiviral profile of DRB, various synthetic modifications have been performed on the parent structure. With one exception,[22] DRB derivatives modified in the benzene moiety with different

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halogens[23] or halogens at different positions have shown weaker antiviral activity than DRB itself. Most sugar-modified analogs also have been less active than DRB as antiviral agents[12,24] except for the xylo and arabino analogs which were active against HCMV and/or varicella zoster virus but at concentrations not well-separated from cytotoxicity.[25]

The studies by Tamm and co-workers in the 1950s and 1960s prompted us to synthesize a series of 2-substituted benzimidazole ribonucleosides as potential anticancer agents.[26] Their lack of activity in anticancer tests[27] and low cytotoxicity[28] led us to examine the 2-chloro derivative of DRB [2,5,6-trichloro-1-(β-D-ribofuranosyl)benzimidazole (TCRB)] for activity against HCMV. In a series of preliminary reports, we have described the activity of TCRB and its 2-Br analog (BDCRB) against HCMV.[29] Both compounds act by a unique mechanism which does not involve inhibition of DNA synthesis but does involve inhibition of DNA processing.[30] Apart from these recent studies of ours and studies on related analogs,[31,32] we know of no work on the activity of 2,5,6-trihalogenated benzimidazole nucleosides against HCMV. In this study we describe the synthesis and antiviral activity of lead compounds which establish certain 2,5,6-trihalogenated benzimidazole nucleosides as potent and selective inhibitors of HCMV replication.

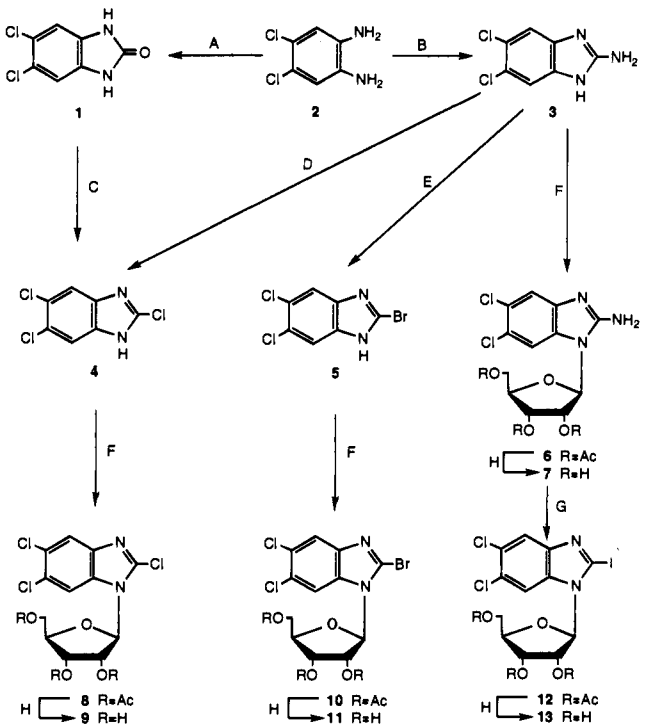
Results and Discussion

Chemistry. In order to carry out the necessary studies, we needed to synthesize large quantities of 2,5,6-trichlorobenzimidazole and its riboside TCRB. The synthesis of 2,5,6-trichlorobenzimidazole (**4**) was initially accomplished[33] in our laboratory by chlorination of the corresponding 5,6-dichlorobenzimidazol-2-one[34] (**1**) with phosphorus oxychloride (Scheme 1); however, this approach provided very low and capricious yields. This prompted us to consider other approaches which might provide a more efficient method of synthesizing 2,5,6-trichlorobenzimidazole. The approach we envisaged involved a ring closure of commercially available 4,5-dichloro-*o*-phenylenediamine (**2**) with cyanogen bromide to give the corresponding 2-amino-5,6-dichlorobenzimidazole (**3**) which would then be diazotized in the presence of cupric chloride to afford 2,5,6-trichlorobenzimidazole (**4**).

The cyclization of *o*-phenylenediamines with cyanogen bromide is a well-known method[35] for synthesizing various 2-aminobenzimidazoles.[36] In fact, 2-amino-5,6-dichlorobenzimidazole (**3**) originally was synthesized by this route as a potential antimalarial[36c] agent but only in a reported yield of 22%. We now have used this basic approach, with some modifications, to obtain the versatile 2-amino-5,6-dichlorobenzimidazole (**3**) in very high yield (>98%). The conversion of **3** into **4** has been accomplished and has provided **4** in consistent yields.

Silylation of 2,5,6-trichlorobenzimidazole (**4**) with bis(trimethylsilyl)acetamide[38] (BSA) was followed by ribosylation with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (TAR) in the presence of trimethylsilyl trifluoromethanesulfonate[39] (TMSOTf) to yield the desired 2,5,6-trichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**8**) with negligible amounts of the α-anomer. The acetyl protecting groups on **8** were removed with methanolic ammonia to afford the target compound TCRB (**9**; Scheme 1).

Scheme 1[a]

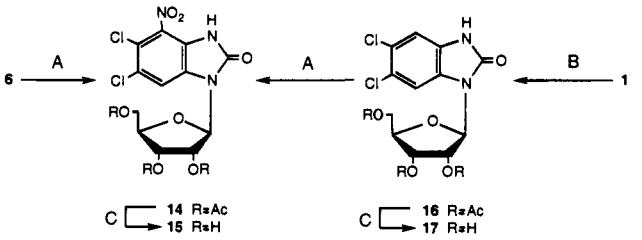


[a] (A) Urea, amyl alcohol; (B) CNBr, MeOH; (C) $POCl_3$, HCl; (D) HCl, NaONO, $CuCl_2$; (E) HBr, NaONO, CuBr; (F) (1) bis(trimethylsilyl)acetamide/CH_3CN, (2) 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (TAR), trimethylsilyl trifluoromethanesulfonate; (G) isoamyl nitrite/CH_2I_2; (H) NH_3/MeOH.

2-Bromo-5,6-dichlorobenzimidazole (**5**) was synthesized in a similar fashion to that described for 2,5,6-trichlorobenzimidazole. Thus, 2-amino-5,6-dichlorobenzimidazole (**3**) was diazotized with sodium nitrite in aqueous HBr, and the diazonium salt was decomposed in the presence of cuprous bromide to give **5**. 2-Bromo-5,6-dichlorobenzimidazole (**5**) was then silylated with BSA and subsequently ribosylated with TAR in the presence of TMSOTf, affording 2-bromo-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**10**). 2-Bromo-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (**11**, BDCRB) was obtained upon removal of the acetyl protecting groups. We then found that 5,6-dichloro-2-iodobenzimidazole was not accessible by a Sandmeyer reaction, and this prompted us to initiate an alternative route for the synthesis of 5,6-dichloro-2-iodo-1-β-D-ribofuranosylbenzimidazole (**13**, IDCRB).

The deamination–halogenation of purine nucleosides with alkyl nitrites in halogenated solvents has been reported to occur at the 6-position[40] and the 2-position[41] to provide various halogenated purine nucleosides. Although the synthesis of several 2-halogenated benzimidazole nucleosides has been reported,[26,37,42] including TCRB and BDCRB, the nonaqueous diazotization of benzimidazole nucleosides to afford the corresponding 2-halogenated nucleosides has only recently been described.[31] In order to investigate the nonaqueous diazotization of a 2-amino 5,6-disubstituted benzimidazole riboside using a tertiary alkyl nitrite, we were required to prepare the previously unreported 2-amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole.[31] This was accomplished by silylation of compound **3** followed by ribosylation with TAR to furnish **6** in 51% yield. The treatment of **6** with 10 equiv of *tert*-butyl nitrite in CH_2I_2 under anhydrous conditions for

Scheme 2[a]



[a] (A) *tert*-Butyl nitrite/CH_2I_2; (B) (1) bis(trimethylsilyl)acetamide/CH_3CN, (2) 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (TAR), trimethylsilyl trifluoromethanesulfonate; (C) NH_3/MeOH.

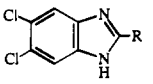
24 h at 110 °C gave (after solvent removal via a Kugelrohr apparatus and column chromatography) a bright-yellow product. A mass spectrum (FAB^+) of the product identified a molecular ion at *m/z* 506 instead of the expected *m/z* 571 for 5,6-dichloro-2-iodo-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**12**). The structure of this unexpected product (*m/z* 506) was subsequently elucidated from spectral data[31] and chemical studies to be 5,6-dichloro-4-nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazol-2-one (**14**; Scheme 2).

The mechanism of this reaction was established by isolating some of the key intermediates, e.g., 5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazol-2-one (**16**). Compound **16** was also prepared[31] by ribosylation of the known 5,6-dichlorobenzimidazol-2-one[34] (**1**), which confirmed the structural assignment and provided further proof for its role as an intermediate. 4,5-Dichloro-1,2-phenylenediamine (**2**) was ring-closed with urea to yield 5,6-dichlorobenzimidazol-2-one (**1**). Ribosylation of this compound furnished compound **16**, and this was followed by deprotection to give 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazol-2-one (**17**). Ribosylation at the oxygen atom of the heterocycle was ruled out on the basis of ^1H and ^{13}C NMR. The ^1H NMR spectrum revealed two separate peaks in the aromatic region (1 H each) and only one broad peak (1 H) at δ 9.83 (N-*H*, D_2O exchangeable). The carbon spectrum revealed the presence of seven unequivalent resonances in the aromatic region which was consistent with an asymmetrically ribosylated 5,6-dichlorobenzimidazol-2-one. When **16** was treated with 10 equiv of *tert*-butyl nitrite in diiodomethane at 100 °C, a complete conversion to compound **14** was observed within 2 h without the detection of any other intermediates. Compound **14** was isolated from this reaction in 86% yield after recrystallization. Removal of the protecting groups from **14** furnished a good yield of compound **15**.

It was subsequently found that the desired 2-iodo compound could be obtained when a primary alkyl nitrite was used in the diazotization reaction. The treatment of compound **6** with either amyl or isoamyl nitrite under the same reaction conditions gave only the expected 5,6-dichloro-2-iodo-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**12**) in 55% or 63% yield, respectively. The desired compound 5,6-dichloro-2-iodo-1-(β-D-ribofuranosyl)benzimidazole (**13**, IDCRB) was obtained by removal of the protecting groups from **12** with methanolic ammonia.

Biology. As part of our program to discover antiviral drugs, we have examined certain benzimidazoles and their nucleosides for activity against HCMV. The heterocycles were active against HCMV and more potent than ganciclovir and foscarnet in plaque reduction assays. They were weakly active or inactive against HSV-1 (Table 1). The heterocycles were not studied further, however, because their activity against HCMV was not well-separated from cytotoxicity in uninfected cells. The most active 2-Cl analog **4** produced cytotoxicity in uninfected human foreskin fibroblasts (HFF cells) at concentrations near those required for antiviral activity.

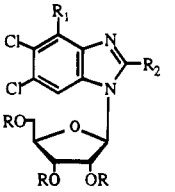
Table 1. Antiviral Activity and Cytotoxicity of 5,6-Dichlorobenzimidazoles



		50% or 90% inhibitory concentration (μM)				
		antiviral activity[a]				
		HCMV		HSV-1[b]	cytotoxicity[c]	
compd	R	plaque	yield	ELISA	visual	growth
3[e]	NH_2	100		>100[d]	100	>100
4[e]	Cl	6.4		51	24	19
5[e]	Br	12			21	56
foscarnet[f]		39 ± 26			>100	
ganciclovir	(DHPG)[g]	7.4 ± 6.5	1.6 ± 1.2	3.5 ± 2.1	>100	>100

[a] Plaque and yield reduction assays were performed in duplicate as described in the text. Results from plaque assays are reported as IC_{50}'s, those for yield reduction experiments as IC_{90}'s. [b] The plaque assay was used to determine the activity of DHPG against HSV-1; all other compounds were assayed by ELISA in quadruplicate wells. [c] Visual cytotoxicity was scored on HFF cells at time of HCMV plaque enumeration. Inhibition of KB cell growth was determined as described in the text in quadruplicate assays. Results are presented as IC_{50}'s. [d] >100 indicates IC_{50} or IC_{90} not reached at the noted (highest) concentration tested. [e] Average derived from two to four experiments for each parameter studied. [f] Average ± standard deviation from 15 experiments. [g] Average ± standard deviation from 108, 33, and 3 experiments, respectively.

Table 2. Antiviral Activity and Cytotoxicity of 5,6-Dichloro-1-(β-ribofuranosyl)benzimidazoles



				50% or 90% inhibitory concentration (μM)				
				antiviral activity[a]				
				HCMV		HSV-1[b]	cytotoxicity[c]	
compd	R_1	R_2	R	plaque	yield	ELISA	visual	growth
DRB[e,f]	H	H	H	42	19	30	24	36
7	H	NH_2	H	40	8	50	32	40
8	H	Cl	Ac	2.0	0.2		100	>100[d]
9[e]	H	Cl	H	2.9	1.4	102	238	210
10	H	Br	Ac	1.5	0.2	>100	100	25
11[e]	H	Br	H	0.7	0.2	130	118	>100
13[e]	H	I	H	30	26	21	118	23
15	NO_2	OH	H	>100		>100	100	
17	H	OH	H	>100			100	

[a] See Table 1 for footnotes a–d. [e] Averages derived from three to six experiments for each parameter studied. [f] Compound referred to as DRB by Tamm and co-workers.[12]

Evaluation of the ribonucleosides produced significantly different results. 2,5,6-Trichloro-1-β-D-ribofuranosylbenzimidazole (**9**, TCRB) was a potent inhibitor of HCMV in a plaque reduction assay with activity well separated from cytotoxicity (Table 2). Likewise, analysis of the 2-Br derivative **11** (BDCRB) and the tri-*O*-acetyl derivatives of both **9** and **11** (compounds **8**, **10**) revealed potent activity against HCMV at noncytotoxic concentrations. BDCRB was the most active in both plaque and yield reduction experiments. Preliminary studies showed that the acetyl protecting groups on **8** and **10** were hydrolyzed in cell culture medium presumably by serum esterases (data not presented), thereby

suggesting that the ribonucleosides were the active compounds.

Results from yield reduction assays established more firmly the activity and selectivity of TCRB and BDCRB. Both compounds reduced HCMV titers by 10^4-10^5-fold at concentrations that were not toxic to HFF or KB cells. BDCRB was the most potent, giving 10^4-10^5-fold titer reductions at 3–10 μM; 32 μM TCRB was required to give similar activity compared to 100 μM for ganciclovir.

In contrast to the potent and selective activity of TCRB and BDCRB, the 2-unsubstituted compound (termed DRB by Tamm and colleagues[12-14]) was only weakly active against HCMV with little separation between antiviral activity and cytotoxicity (Table 2). Similar results have been reported[25] recently for several DRB analogs. Unlike TCRB and BDCRB, DRB was weakly active against HSV-1 at cytotoxic concentrations. Thus the incorporation of Cl or Br at the 2-position of DRB dramatically improved the therapeutic index of these halogenated benzimidazole ribonucleosides. The presence of a ribosyl moiety at the 1-position proved to be very important because the activity of 2,5,6-trichlorobenzimidazole (**4**) was very similar to that of DRB and not to that of TCRB.

Since the activities of TCRB and BDCRB were so different from those of DRB, we explored the influence of other substituents at the 2-position and their possible contribution to antiviral activity. The 2-iodo derivative **13** was not as active against HCMV and also not as selective as **9** and **11** as demonstrated by an increase in activity toward HSV-1 and by increased cytotoxicity in KB cells (Table 2). The 2-amino derivative **7** was more active against HCMV in the yield assay but less so than the 2-Cl and 2-Br compounds and was less selective. Evaluation of compounds **15** and **17** established that a 2-one functionality completely abolished activity against HCMV and HSV-1 (Table 2). Together these observations indicate that the requirements for the substituent at the 2-position are extremely narrow and specific. A slight increase in the lipophilicity and polarizability of the halogen leads to considerably different antiviral properties. Thus only TCRB and BDCRB (and their esters) demonstrated antiviral properties superior to those of ganciclovir and foscarnet.

Experimental Section

General Chemical Procedures. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Silica gel 60 230–400 mesh (E. Merck, Darmstadt, West Germany) was used for chromatography. Thin layer chromatography (TLC) was performed on prescored SilicAR 7GF plates (Analtech, Newark, DE). TLC plates were developed in the following solvent systems: system 1, 2% MeOH/$CHCl_3$; system 2, 10% MeOH/$CHCl_3$; system 3, 50% EtOAc/hexane. Compounds were visualized by illuminating under UV light (254 nm) or spraying with 20% methanolic sulfuric acid followed by charring on a hot plate. Evaporations were carried out under reduced pressure (water aspirator) with the bath temperature below 40 °C, unless specified otherwise. IR spectra were obtained on a Nicolet 5DXB FT-IR spectrophotometer. UV spectra were performed on a Hewlett-Packard 8450-A UV/vis spectrophotometer. Nuclear magnetic resonance (NMR) spectra were determined at 360 MHz with a Bruker WP 360 SY. The chemical shift values are expressed in δ values (ppm) relative to the standard chemical shift of the solvent DMSO-d_6. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

5,6-Dichlorobenzimidazol-2-one (1). A mixture of 4,5-dichloro-1,2-phenylenediamine (**2**; 100 g, 0.56 mol) and urea (70 g, 1.17 mol) was heated at reflux in amyl alcohol (2 L) for 9 h. After cooling the mixture to room temperature, the mixture was vacuum filtered and the isolated material dissolved in aqueous NaOH (50/50). The basic solution was filtered through a bed of Celite and the filtrate neutralized with concentrated HCl. The neutralized mixture was cooled and filtered to give 92.5 g (81%) of a white powder. Mp: >325 °C (lit.[34] mp >340 °C). ^1H NMR (DMSO-d_6): δ 10.92 (s, 2 H, D_2O exchangeable, N-*H*), 7.08 (s, 2 H, Ar-*H*).

2-Amino-5,6-dichlorobenzimidazole (3). 4,5-Dichloro-1,2-phenylenediamine (**2**; 100 g, 0.565 mol) was added slowly to 1200 mL of MeOH, 1200 mL of H_2O, and CNBr (112 mL, 0.565 mol from a 5 M solution in CH_3CN). The mixture was stirred overnight at room temperature. TLC analysis (developed in 10% MeOH/$CHCl_3$) showed a near complete conversion of starting material to product. The MeOH was removed from solution by evaporation under reduced pressure. A liquid–liquid extraction with ethyl acetate was then performed to remove organic byproducts. To the aqueous layer was added a saturated aqueous solution of $NaHCO_3$, causing the product to precipitate. The product was collected by vacuum filtration and dried *in vacuo* to a constant weight. The product was recrystallized from MeOH/H_2O, giving 2-amino-5,6-dichlorobenzimidazole in 98% yield (108.03 g). Mp: 250–251 °C (lit.[36c] mp 260–262 °C dec). ^1H NMR (DMSO-d_6): δ 10.88 (s, 1 H, D_2O exchangeable, N-*H*), 7.24 (s, 2 H, Ar-*H*), 6.51 (s, 2 H, D_2O exchangeable, NH_2). ^{13}C NMR (DMSO-d_6): δ 157.17, 138.81, 120.79, 112.37. GC–MS: *m/z* 201. UV λ_{max} (nm) ($\epsilon \times 10^4$): pH 7, 228 (1.039), 257 (0.467), 301 (0.948); pH 1, 225 (1.743), 292 (1.338); pH 11, 228 (1.560), 248 (0.724), 299 (1.041). Anal. ($C_7H_5Cl_2N_3$) C, H, N.

2,5,6-Trichlorobenzimidazole (4). An aqueous solution (20 mL) of $NaNO_2$ (1.035 g, 0.015 mol) was added to a saturated aqueous solution of $CuCl_2$ (15 mL). After 5 min, 2-amino-5,6-dichlorobenzimidazole (**3**; 935 mg, 5 mmol) was slowly added to the aqueous mixture, and the mixture was allowed to stir at room temperature for 2 h. The mixture was heated on a steam bath for an additional 1 h. This was followed by liquid–liquid extraction of the reaction mixture with ethyl acetate (4 × 50 mL). The organic layer was then washed with brine (50 mL) and dried over $MgSO_4$. The organic layer was concentrated to ca. 5 mL and purified on a silica gel column (3.5 × 15 cm) using 2% MeOH/$CHCl_3$ to elute the compound. Fractions were collected (20 mL), and those having a UV-absorbing compound ($R_f = 0.61$, solvent system 2) were pooled and treated with charcoal (300 mg). The charcoal was removed by filtration, and the filtrate was concentrated to dryness. The resulting compound was recrystallized from MeOH to give 2,5,6-trichlorobenzimidazole in 41% yield (2.71 g). Mp: 245–246 °C (lit.[33] mp 233–234 °C dec). ^1H NMR (DMSO-d_6): δ 13.70 (s, 1 H, D_2O exchangeable, N-*H*), 7.80 (s, 2 H, Ar-*H*). ^{13}C NMR (DMSO-d_6): δ 141.25, 137.94, 125.26, 116.15. MS (GC–MS): *m/z* 220. UV λ_{max} (nm) ($\epsilon \times 10^4$): pH 7, 252 (0.011), 259 (0.029), 288 (0.334), 298 (0.462); pH 1, 224 (0.780), 251 (0.274), 287 (0.687), 296 (0.729); pH 11, 227 (1.216), 293 (0.793), 300 (0.769). Anal. ($C_7H_3Cl_3N_2$) C, H, N.

2-Bromo-5,6-dichlorobenzimidazole (5). 2-Amino-5,6-dichlorobenzimidazole (**3**; 3 g, 16 mmol) was suspended in 150 mL of water and dissolved upon the addition of 2 mL HBr. Sodium nitrite (3.3 g, 55 mmol) was then added, and the mixture was stirred at room temperature for 1 h. Excess CuBr was then added, and the mixture was heated on a steam bath for 1 h. The aqueous solution was extracted with ethyl acetate (3 × 100 mL). The organic extracts were dried over $MgSO_4$, concentrated, and crystallized from ethyl ether to give 1.13 g (26%) of 2-bromo-5,6-dichlorobenzimidazole. Mp: 233–234 °C. ^1H NMR (DMSO-d_6): δ 13.62 (s, 1 H, N-*H*), 7.81 (s, 2 H, Ar-*H*). GC–MS: *m/z* 266. Anal. ($C_7H_3BrCl_2N_2$) C, H, N.

2-Amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (6). 2-Amino-5,6-dichlorobenzimidazole (**3**; 3 g, 16 mmol) was dissolved in dry acetonitrile (150 mL) and the mixture stirred in an inert atmosphere at 60 °C. BSA (4.37 mL, 17 mmol) was added, and the mixture was stirred for 10 min. 1,2,3,5-Tetra-*O*-acetylribofuranose (5.09 g, 16 mmol) and TMSOTf (3.29 mL, 17 mmol) were added to the clear solution, and the mixture was heated at reflux for

16 h. The mixture was concentrated under reduced pressure, separated on a silica gel column (9.5 × 17 cm) and eluted with 2% MeOH/$CHCl_3$ to yield 3.74 g (51%) of 2-amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole as a foam. Mp: 88–89 °C. ^1H NMR ($CDCl_3$): δ 7.42 (s, 1 H, C_7-*H*), 7.27 (s, 1 H, C_4-*H*), 5.87 (d, 1 H, 1′-*H*, *J* = 6.8 Hz), 5.58 (s, 2 H, Ar-N*H*$_2$), 5.47 (t, 1 H, 2′-*H*, *J* = 8.7 Hz), 5.37 (dd, 1 H, 3′-*H*, *J* = 4.1 Hz), 4.48 (dm, 2 H, 5′-*H*), 4.35 (m, 1 H, 4′-*H*), 2.17 (s, 3 H, acetyl), 2.15 (s, 3 H, acetyl), 1.99 (s, 3 H, acetyl). ^{13}C NMR ($CDCl_3$): δ 170.04, 169.56, 169.11, 154.57, 141.64, 132.53, 125.90, 123.45, 117.72, 109.49, 85.78, 80.99, 70.95, 69.83, 62.91, 20.74, 20.54, 20.20. MS (FAB^+): *m/z* 460.

2-Amino-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (7). 2-Amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimadazole (**6**; 125 mg, 0.3 mmol) was treated with a methanolic ammonia solution for 16 h at ambient temperature. The mixture was concentrated under reduced pressure, and the remaining residue was applied to a silica gel column (3.5 × 6 cm) and eluted with 5% MeOH/$CHCl_3$. A compound with R_f = 0.1 (solvent system 2) was isolated and recrystallized from water to give a mossy white material (42 mg, 46% yield). Mp: 136–137 °C. ^1H NMR (DMSO-d_6): δ 7.68 (s, 1 H, C_7-*H*), 7.29 (s, 1 H, C_4-*H*), 6.92 (bs, 2 H, NH_2), 5.72 (d, 1 H, 1′-*H*, *J* = 7.5 Hz) 5.52 (t, 1 H, 5′-*OH*, *J* = 4.1 Hz), 5.25 (d, 1 H, 2′-*OH*, *J* = 7.6 Hz), 5.21 (d, 1 H, 3′-*OH*, *J* = 3.9 Hz), 4.30 (q, 1 H, 2′-*H*, *J* = 6.5 Hz), 4.07 (m, 1 H, 3′-*H*), 3.96 (m, 1 H, 4′-*H*), 3.66 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 155.97, 143.03, 133.05, 122.80, 119.67, 115.20, 110.41, 87.63, 85.51, 71.24, 69.83, 60.86. MS (FAB^+): *m/z* 333. UV λ_{max} (nm) (ε × 10^4): pH 7, 259 (0.254), 303 (0.353); pH 1, 238 (0.451), 293 (0.491); pH 11, 257 (0.373), 300 (0.408). Anal. ($C_{12}H_{13}Cl_2N_3O_4$·0.5H_2O) C, H, N.

2,5,6-Trichloro-1-β-D-ribofuranosylbenzimidazole (9, TCRB). 2,5,6-Trichlorobenzimidazole (**4**; 700 mg, 3.2 mmol) was dissolved in acetonitrile, and BSA (1 mL, 3.8 mmol) was added. The mixture was heated at 75 °C for 20 min and then allowed to cool to ambient temperature. TMSOTf (1 mL, 5.1 mmol) and 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (1.9 g, 3.2 mmol) were added, and the mixture was allowed to stir at room temperature for 16 h. The acetonitrile was removed under reduced pressure, and the protected nucleoside was separated on a silica gel column (3.5 × 10 cm), eluting with chloroform and collecting 20 mL fractions. Those fractions having a UV-absorbing spot that also charred under H_2SO_4 were pooled and concentrated to a foam. The foam was then recrystallized from methanol, giving 1.04 g (68%) of **8** as light-yellow crystals. ^1H NMR ($CDCl_3$): δ 7.78 (s, 1 H, C_7-*H*), 7.77 (s, 1 H, C_4-*H*), 6.15 (d, 1 H, 1′-*H*, *J* = 7.1 Hz), 5.45 (m, 2 H, 2′-*H*, 3′-*H*), 4.47 (dm, 2 H, 5′-*H*), 4.38 (m, 1 H, 4′-*H*), 2.29 (s, 3 H, acetyl), 2.16 (s, 3 H, acetyl), 2.03 (s, 3 H, acetyl). ^{13}C NMR ($CDCl_3$): δ 170.31, 169.57, 169.10, 141.55, 141.32, 131.86, 128.06, 128.02, 121.12, 113.04, 86.83, 80.72, 70.95, 69.49, 62.90, 21.00, 20.53, 20.16.

The acetyl protecting groups were removed by overnight treatment with methanolic ammonia. The nucleoside was separated on a column using 50% EtOAc/hexane and then 10% MeOH/$CHCl_3$. The isolated compound was recrystallized from methanol four times to achieve an analytically pure sample. Yield: 74%. Mp: 185–186 °C (lit.[26] mp 188–189 °C). TLC: R_f = 0.20 (solvent system 2). ^1H NMR (DMSO-d_6): δ 8.55 (s, 1 H, C_7-*H*), 7.96 (s, 1 H, C_4-*H*), 5.57 (d, 1 H, 1′-*H*, *J* = 7.8 Hz), 5.49 (d, 1 H, 2′-O*H*, *J* = 6.4 Hz), 5.41 (t, 1 H, 5′-O*H*, *J* = 4.7 Hz), 5.28 (d, 1 H, 3′-O*H*, *J* = 4.3 Hz), 4.40 (q, 1 H, 2′-*H*, *J* = 5.9 Hz), 4.12 (m, 1 H, 3′-*H*), 4.00 (m, 1 H, 4′-*H*), 3.68 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 142.16, 140.96, 132.30, 125.97, 125.77, 120.04, 114.93, 89.16, 86.47, 71.70, 69.80, 61.08. MS (FAB^+): *m/z* 353. UV λ_{max} (nm) (ε × 10^4): pH 7, 229 (1.942), 253 (0.641), 288 (0.549), 298 (0.591); pH 1, 226 (1.384), 253 (0.500), 288 (0.522), 297 (0.496); pH 11, 228 (1.200), 253 (0.478), 288 (0.461), 297 (0.489). Anal. ($C_{12}H_{11}Cl_3N_2O_4$) C, H, N.

2-Bromo-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (11, BDCRB). 2-Bromo-5,6-dichlorobenzimidazole (**5**; 1 g, 3.8 mmol) was dissolved in dry acetonitrile (150 mL) and stirred in an inert atmosphere at 60 °C. BSA (1.03 mL, 4.2 mmol) was added, and the mixture was stirred for 10 min. 1,2,3,5-Tetra-*O*-acetyl-β-D-ribofuranose (1.21 g, 3.8 mmol) and TMSOTf (0.81 mL, 4.2 mmol) were added to the clear solution, and the mixture was allowed to stir at 60 °C for 1 h. The mixture was then allowed to stir at room temperature for an additional 16 h. The mixture was concentrated under reduced pressure and separated on a silica gel column to give the protected 2-bromo-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**10**). ^1H NMR ($CDCl_3$): δ 7.81 (s, 1 H, C_7-*H*), 7.78 (s, 1 H, C_4-*H*), 6.17 (d, 1 H, 1′-*H*, *J* = 7.4 Hz), 5.48 (m, 1 H, 2′-*H*), 5.43 (m, 1 H, 3′-*H*), 4.46 (m, 2 H, 5′-*H*), 4.38 (m, 1 H, 4′-*H*), 2.29 (s, 3 H, acetyl), 2.16 (s, 3 H, acetyl), 2.02 (s, 3 H, acetyl). ^{13}C NMR ($CDCl_3$): δ 170.26, 169.50, 168.99, 142.97, 132.32, 130.71, 128.09, 128.06, 121.09, 112.98, 88.09, 80.76, 71.01, 69.50, 62.87, 20.97, 20.49, 20.13. The protected nucleoside was stirred for 16 h at room temperature in a methanolic ammonia solution. The mixture was then concentrated and suspended in methanol (3 × 25 mL) to give 2-bromo-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (11) in 37% yield. Mp: 168–169 °C. ^1H NMR (DMSO-d_6): δ 8.56 (s, 1 H, C_7-*H*), 7.95 (s, 1 H, C_4-*H*), 5.87 (d, 1 H, 1′-*H*, *J* = 7.9 Hz), 5.45 (d, 1 H, 2′-O*H*, *J* = 6.5 Hz), 5.40 (t, 1 H, 5′-O*H*, *J* = 4.5 Hz), 5.27 (d, 1 H, 3′-O*H*, *J* = 4.2 Hz), 4.41 (q, 1 H, 2′-*H*, *J* = 5.9 Hz), 4.11 (m, 1 H, 3′-*H*), 3.99 (m, 1 H, 4′-*H*), 3.69 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 142.57, 132.60, 132.57, 125.76, 119.86, 114.73, 90.21, 86.35, 71.55, 69.76, 61.05. MS (FAB): *m/z* 399. UV λ_{max} (nm) (ε × 10^4): pH 7, 229 (1.338), 255 (1.259), 289 (1.170) 299 (1.227); pH 1, 227 (0.977), 255 (0.704), 289 (0.783), 299 (0.823); pH 11, 228 (2.255), 255 (1.119), 289 (1.053), 299 (1.071). Anal. ($C_{12}H_{11}BrCl_2N_2O_4$) C, H, N.

2-Iodo-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (12). 2-Amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**6**; 50 mg, 0.1 mmol) was dissolved in diiodomethane (15 mL). Amyl nitrite (0.13 mL, 1 mmol) was added, and the reaction mixture was heated at 85 °C for 40 min while under an inert atmosphere. The mixture was concentrated under reduced pressure (Kugelrohr apparatus), and the remaining residue was applied to a silica gel column (3.5 × 8 cm). The column was eluted with chloroform, and 20 mL fractions were collected. Those fractions having a UV-absorbing spot that also charred under H_2SO_4 were pooled and concentrated to dryness. The remaining residue was recrystallized from methanol to give 36 mg (63%) of 2-iodo-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-benzimidazole (**12**). ^1H NMR ($CDCl_3$): δ 7.81 (s, 1 H, C_7-*H*), 7.75 (s, 1 H, C_4-*H*), 6.07 (d, 1 H, 1′-*H*, *J* = 7.0 Hz), 5.43 (m, 2 H, 2′-*H*, 3′-*H*), 4.47 (dm, 2 H, 5′-*H*), 4.35 (m, 1 H, 4′-*H*), 2.27 (s, 3 H, acetyl), 2.14 (s, 3 H, acetyl), 2.01 (s, 3 H, acetyl). ^{13}C NMR ($CDCl_3$): δ 170.25, 169.50, 168.95, 145.43, 132.27, 127.68, 120.72, 112.53, 103.82, 90.03, 80.61, 70.89, 69.31, 62.79, 20.89, 20.39, 20.07. MS (FAB): *m/z* 445. MS (DCI with ammonia): *m/z* 571. MS (EI, 70 eV): *m/z* 570.

2-Iodo-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (13, IDCRB). 2-Iodo-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazole (**12**; 50 mg, 0.1 mmol) was treated with a methanolic ammonia solution for 16 h at ambient temperature. The mixture was concentrated under reduced pressure, and the remaining residue was applied to a silica gel column (3.5 × 4 cm) and eluted with 2% MeOH/$CHCl_3$ to give 40 mg (90%) of 2-iodo-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (**13**). The compound was recrystallized from methanol. Mp: 180–182 °C. ^1H NMR (DMSO-d_6): δ 8.52 (s, 1 H, C_7-*H*), 7.89 (s, 1 H, C_4-*H*), 5.82 (d, 1 H, 1′-H, *J* = 7.7 Hz), 5.37 (m, 2 H, 2′-O*H*, 5′-O*H*), 5.23 (d, 1 H, 3′-O*H*, *J* = 4.4 Hz), 4.40 (q, 1 H, 2′-*H*, *J* = 6.2 Hz), 4.12 (m, 1 H, 3′-*H*), 4.07 (m, 1 H, 4′-*H*), 3.71 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 145.33, 132.51, 125.26, 125.18, 119.51, 114.22, 110.32, 92.11, 86.22, 71.47, 69.99, 61.11. UV λ_{max} (nm) (ε × 10^4): pH 7, 230 (2.181), 259 (0.961), 292 (1.233), 302 (1.357); pH 1, 230 (1.522), 258 (0.423), 294 (1.339), 303 (1.393); pH 11, 228 (2.492), 258 (0.881), 292 (1.108), 302 (1.188). MS (FAB): *m/z* 444. Anal. ($C_{12}H_{11}Cl_2IN_2O_4$·1.5CH_3OH) C, H, N.

5,6-Dichloro-4-nitro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazol-2-one (14). Method A: 2-Amino-5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuransosyl)benzimidazole (**6**; 50 mg) was purged with N_2 and treated with diiodomethane (10 mL). The mixture was stirred and heated in an oil bath (110 °C). *tert*-Butyl nitrite (10 equiv) was added,

and the reaction mixture was heated at 100 °C for 24 h. Excess diiodomethane and *tert*-butyl nitrite were removed via a Kugelrohr apparatus, and the remaining residue was suspended in $CHCl_3$ (5 mL) and added to the top of a silica gel column. The column was eluted with $CHCl_3$ (to elute traces of CH_2I_2) and then with 2% MeOH/$CHCl_3$ to remove the bright-yellow material. Yield: 33% from **6**. Mp: 186–187 °C. IR (KBr): 1542 cm^{-1}. ^1H NMR (DMSO-d_6): δ 10.05 (s, 1 H, N-*H*), 7.56 (s, 1 H, Ar-*H*), 5.96 (d, 1 H, 1′-*H*, J = 6.59 Hz), 5.65 (t, 1 H, 2′-*H*), 5.46 (dd, 1 H, 3′-*H*), 4.41 (dm, 2 H, 5′-*H*), 4.35 (m, 1 H, 4′-*H*), 2.20 (s, 3 H), 2.15 (s, 3 H), 2.05 (s, 3 H). ^{13}C NMR (DMSO-d_6): δ 170.37, 169.84, 169.74, 152.70, 131.11, 128.63, 127.93, 125.07, 120.53, 114.90, 84.85, 80.13, 70.37, 69.94, 63.14, 20.89, 20.49, 20.34. MS (EI with DCI probe): *m/z* 506.

Method B: The procedure is the same as that described for **6** to **14** except that **16** was used instead of **6** and the reaction time was 2 h instead of 24 h. The product was identical with the product isolated from the same reaction conditions when using compound **6** as the starting material.

5,6-Dichloro-4-nitro-1-β-D-ribofuranosylbenzimidazol-2-one (15). Compound **14** was treated with methanolic ammonia and the mixture stirred overnight at room temperature to give a quantitative yield of **15** which was recrystallized from methanol. Mp: 238–240 °C. ^1H NMR (DMSO-d_6): δ 9.0 (s, 1 H, N*H*), 8.28 (s, 1 H, C_7-*H*), 5.7 (d, 1 H, 1′*H*), 5.27 (m, 2 H, 2′-O*H*, 5′-O*H*), 5.13 (d, 1 H, 3′-O*H*), 4.41 (q, 1 H, 2′-*H*), 4.09 (m, 1 H, 3′-*H*), 3.9 (m, 1 H, 4′-*H*), 3.62 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 153.39, 131.1, 129.6, 124.68, 124.23, 115.9, 114.8, 80.08, 85.5, 70.05, 69.89, 61.26. MS (FAB): *m/z* 380. Anal. ($C_{12}H_{11}Cl_2N_3O_7$) C, H, N.

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazol-2-one (17). A solution of 5,6-dichlorobenzimidazol-2-one (**1**; 3 g, 14.8 mmol) and bis(trimethylsilyl)acetamide (4.03 mL, 16.3 mmol) in acetonitrile (100 mL) was heated at 70 °C. After 5 min, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (4.7 g, 14.8 mmol) and TMSOTf (3.14 mL, 16.3 mmol) were added, and the mixture was kept at 70 °C for 1 h and then for an additional 16 h at room temperature. The mixture was concentrated under reduced pressure, and the remaining residue was suspended in a sodium bicarbonate-saturated aqueous solution (50 mL) and extracted with chloroform (3 × 150 mL). The chloroform extracts were dried over $MgSO_4$ and concentrated to dryness, and the remaining material was recrystallized from MeOH to give 3.57 g (52%) of 5,6-dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazol-2-one (**16**). Mp: 177–178 °C. ^1H NMR ($CDCl_3$): δ 10.37 (s, 1 H, N-*H*), 7.33 (s, 1 H, C_7-*H*), 7.19 (s, 1 H, C_4-*H*), 5.97 (d, 1 H, 1′-*H*, J = 6.8 Hz), 5.73 (t, 1 H, 2′-*H*), 5.49 (dd, 1 H, 3′-*H*), 4.41 (m, 3 H, 4′-*H*, 5′-*H*), 2.21 (s, 3 H), 2.14 (s, 3 H), 2.04 (s, 3 H). ^{13}C NMR ($CDCl_3$): δ 170.51, 169.68, 169.58, 154.64, 127.59, 127.36, 126.38, 125.31, 111.61, 111.34, 84.48, 79.67, 70.16, 69.96, 63.14, 20.92, 20.53, 20.37. MS (EI with DCI probe): *m/z* 460.

5,6-Dichloro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)benzimidazol-2-one (**16**; 3 g, 6.5 mmol) was treated with a methanolic ammonia solution at room temperature for 16 h. After removing the solvent under reduced pressure, the nucleoside was purified on a silica gel column (3.5 × 14 cm) using 2% MeOH/$CHCl_3$ to elute the compound and afford 1.87 g (86%) of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol-2-one (**17**). Mp: 246–247 °C. ^1H NMR (DMSO-d_6): δ 9.83 (s, 1 H, N-*H*, D_2O exchangeable), 7.87 (s, 1 H, C_7-*H*), 7.18 (s, 1 H, C_4-*H*), 5.66 (d, 1 H, 1′-*H*, J = 7.6 Hz), 5.19 (d, 1 H, 2′-O*H*, J = 6.2 Hz), 5.14 (t, 1 H, 5′-O*H*, J = 4.7 Hz), 5.07 (d, 1 H, 3′-O*H*, J = 4.2 Hz), 4.43 (q, 1 H, 2′-*H*, J = 6.1 Hz), 4.07 (m, 1 H, 3′-*H*), 3.86 (m, 1 H, 4′-*H*), 3.60 (m, 2 H, 5′-*H*). ^{13}C NMR (DMSO-d_6): δ 153.91, 128.77, 128.28, 123.48, 122.74, 112.08, 110.15, 85.80, 85.17, 70.14, 69.65, 61.48. MS (FAB) *m/z*: 335. UV λ_{max} (nm) (ε × 10^4): pH 7, 224 (0.415), 298 (0.236); pH 1, 218 (1.021); pH 11, 225 (0.829), 302 (0.242). Anal. ($C_{12}H_{12}Cl_2N_2O_5$) C, H, N.

Cell Culture Procedures. The routine growth and passage of KB, BSC-1, and HFF 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 serum or 10% fetal bovine serum (HFF cells). The sodium bicarbonate concentration was varied to meet the buffering capacity required. Cells were passaged at 1:2–1:10 dilutions according to conventional procedures by using 0.05% trypsin plus 0.02% EDTA in a HEPES-buffered salt solution as described previously.[43]

Virological Procedures. The Towne strain, plaque-purified isolate P_o, of HCMV was kindly provided by Dr. Mark Stinski, University of Iowa, IA. The KOS strain of HSV-1 was used in most experiments and was provided by Dr. Sandra K. Weller, University of Connecticut, CT. Stock HCMV was prepared by infecting HFF cells at a multiplicity of infection (MOI) of <0.01 plaque-forming units (PFU)/cell. Cell growth medium was changed every 4 days until cytopathology was evident in all cells (ca. 21 days). Supernatant fluids were retained as the virus stock. High-titer HSV-1 stocks were prepared by infecting KB cells at an MOI of <0.1 as detailed previously.[43] 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.[44] Briefly, HFF or BSC-1 cells were planted as described above in 96-well cluster dishes and incubated overnight at 37 °C in a humidified 3% CO_2–97% air atmosphere. The next day cultures were inoculated with HCMV or HSV-1 and serially diluted 1:3 across the remaining 11 columns of the 96-well plate. Cultures were incubated at 37 °C for 2 h to permit virus adsorption, and then virus innoculum was replaced with 0.2 mL of fresh medium. Cultures were incubated for 7 days for HCMV and 2 or 3 days for HSV-1, medium was removed, and the cell sheets were stained with 0.1% crystal violet in 20% methanol. Plaques were enumerated under 20-fold magnification in wells having the dilution which gave 5–20 plaques/well. Virus titers were calculated according to the following formula: their (PFU/mL) = number of plaques × 5 × 3^n, where n represents the nth dilution of the virus used to infect the well in which plaques were enumerated.

HCMV Plaque Reduction Assay. HFF cells in 24-well cluster dishes were infected with approximately 100 PFU of HCMV/cm^2 of cell sheet using the procedures detailed above. 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 7–10 days, cell sheets were fixed and stained with crystal violet and microscopic plaques enumerated as described above. 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.

HCMV Yield Assay. The procedure devised by us[44] was used for yield reduction assays. HFF cells were planted as described above in 96-well cluster dishes at a concentration of 12 500 cells/well and incubated overnight. The next day, medium was shaken out, and the cultures were inoculated with HCMV at a MOI of 0.5–1 PFU/cell. After virus adsorption, innoculum was replaced with 0.2 mL of fresh medium containing test compounds. The first row of 12 wells was left undisturbed and served as virus controls. Each well in the second row received an additional 0.1 mL of MEM(E) containing 5% serum, antibiotics, and test compound at 3 times the desired final concentration. The contents of the 12 wells were mixed by repeated pipetting and then serially diluted 1:3 along the remaining wells. In this manner, six compounds could be tested in duplicate on a single plate with concentrations ranging nearly 1000-fold between the highest and lowest dilutions (100–0.14 μM, for example). Plates were incubated at 37 °C for 7 days and then subjected to one cycle of freezing at −76 °C and thawing at 37 °C to disrupt the cells. Aliquots from each of the eight wells of a given column were transferred to the first column of a fresh 96-well monolayer culture of HFF cells. Contents were mixed and serially diluted 1:3 across the remaining 11 columns of the secondary plate. Each column of the original primary plate was diluted across a separate plate in this manner. Cultures were incubated, plaques were enumerated, and titers were calculated as described above.

HSV-1 ELISA. An ELISA developed in our laboratories[45] was employed to detect HSV-1. Ninety-six-well cluster dishes were planted with 10 000 BSC-1 cells/well in 200 μL/well MEM(E) plus 10% calf serum. After overnight incubation at 37 °C, selected drug concentrations in quadruplicate and

HSV-1 at a concentration of 100 PFU/well were added. Following a 3–5-day incubation at 37 °C, medium was removed and plates were blocked with 200 μL/well 10% calf serum and 0.05% Tween 80 in HEPES-buffered saline HBS[46] (HBS-T). After 30 min, the blocking agent was removed, and the wells were rinsed with HBS-T. Horse radish peroxidase-conjugated rabbit anti-HSV-1 antibody in HBS was added and incubated on a rocker for 1 h at room temperature. Following removal of the antibody-containing solution, plates were rinsed four times with HBS-T and then developed by adding 150 μL/well of 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. Two different assays were used to explore cytotoxicity of selected compounds using methods we have detailed previously. (i) Cytotoxicity produced in stationary HFF cells was determined by microscopic inspection of cells not affected by the virus used in plaque assays.[43] (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.[47] Briefly, 96-well cluster dishes were planted with KB cells at 3000–5000 cells/well, in a total volume of 200 μL/well MEM(E) plus 10% calf serum. 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 in a CO_2 incubator and rinsed with 200 μL of HBS and the cells fixed by adding 200 μL of 95% ethanol to all plates; 100 μL/well 0.1% crystal violet was added, incubated for 5 min at room temperature, and rinsed four times with tap distilled water; 100 μL/well of 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. Fifty percent inhibitory (IC_{50}) concentrations were calculated from the regression lines. Samples containing positive controls (acyclovir for HSV-1, ganciclovir for HCMV, and 2-acetylpyridine thiosemicarbazone for cytotoxicity) were used in all assays. Results from sets of assays were rejected if inhibition by the positive control deviated from its mean response by $>\pm 1.5$ standard deviations.

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