Synthesis and Antiviral Evaluation of N-Carboxamidine-Substituted Analogues of 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine Hydrochloride

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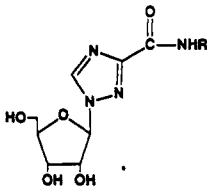
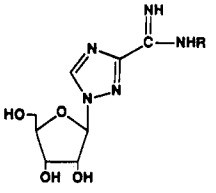
Ten, hitherto unreported, analogues of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (**2a**, ribamidine) and methyl carboximidate **5** have been synthesized. These include the *N*-cyano (**2b**), *N*-alkyl (**2c–e**), *N*-amino acid (**2f–h**), *N,N'*-disubstituted (**6**, **7a,b**), and the *N*-methylated carboxamide (**1f**) analogues of ribavirin. In addition, a new facile synthesis of carboxamidine **2a** was also developed. All compounds were evaluated for biological activity against the following RNA viruses: Punta Toro (PT) and sandfly fever (SF) viruses (bunyaviruses); Japanese encephalitis (JE), yellow fever (YF), and dengue-4 viruses (flaviviruses); parainfluenza type 3 (PIV3), respiratory syncytial virus (RSV), and measles viruses (paramyxoviruses); influenza A and influenza B viruses (orthomyxoviruses); Venezuelan equine encephalomyelitis virus (VEE, alphavirus); human immunodeficiency virus type-1 (HIV-1, lentivirus); the DNA-containing vaccinia (VV) virus (poxvirus); and adeno type 5 (Ad5) viruses. All of the compounds except for **2b** and **7a,b** exhibited activity against the bunyaviruses such as that observed with **2a**; however, higher IC_{50} values were generally observed. Glycine analogue **2f** showed activity in PT-virus-infected mice in terms of increased survivors and decreased markers of viral pathogenicity. Carboxamidine **2a**, carboximidate **5**, and dimethyl amidine **6** exhibited activity against dengue type-4 virus. Monomethyl amidine **2c** demonstrated activity against RSV, PIV3, and, to a lesser extent, influenza A and B. Activity of **2c** generally required higher IC_{50} values than unsubstituted **2a**. The latter exhibited hitherto unreported activity against RSV; therapeutic indices for **2a** against RSV and PIV3 were >64 and >21. No substantial in vitro activity was observed for any of the compounds tested against Ad5, measles, JE, YF, VEE, or HIV-1. In addition, evidence is presented which argues in favor of a distinct antiviral mechanism of action for carboxamidines, e.g. **6**, in contrast to a role as a carboxamide precursor.

Ribavirin, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (**1a**), and its 3-carboxamidine hydrochloride analogue **2a** are broad-spectrum antiviral agents which were





1a: R = H
1b: R = $-CH_2-CONH_2$
1c: R = $-CH(CONH_2)-CH_2-CONH_2$
1d: R = $-CH(CONH_2)-(CH_2)_2-CONH_2$
1e: R = $-CH(CONH_2)-CH_2-Ph$
1f: R = CH_3

2a: R = H (HCl)
2b: R = CN
2c: R = CH_3 (HCl)
2d: R = $-(CH_2)_3CH_3$
2e: R = $-(CH_2)_7CH_3$
2f: R = $-CH_2-COOH$ (Gly)
2g: R = $-CH(COOH)-CH_2-CONH_2$ (Asp)
2h: R = $-CH(COOH)-CH_2-CH_2-CONH_2$ (Gln)

synthesized and developed nearly concurrently.[1,2] Both compounds possess efficacy against a broad array of DNA and RNA viruses,[2-5] are known inhibitors of inosine monophosphate dehydrogenase (IMP) after adenosine-kinase dependent conversion to nucleotides, and, in general, possess similar mechanisms of action.[6,7] In addition to IMP dehydrogenase, **2a** is an effective competitive inhibitor of purine nucleoside phosphorylase.[6] A ribavirin analogue, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (**3**, EICAR)[8,9] has recently been reported to

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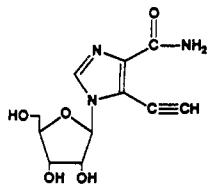

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possess a similar in vitro spectrum of antiviral activity with greater potency but lesser selectivity when compared to that of **1a**.[10] In vivo studies demonstrate that ribavirin is generally effective at lower doses than is carboxamidine **2a**. For example, ribavirin was shown to be significantly active against lethal influenza A_2 virus infections in mice down to 37.5 mg/kg per day whereas the carboxamidine was active only at 75 mg/kg per day.[2] It has been suggested that the superior antiviral activity exhibited by ribavirin (as compared to the carboxamidine) may be reflected by the steric and hydrogen-bonding properties of the 3-substituent on the triazole nucleoside.[2] Therefore, the glycinamide **1b**, aspartic acid diamide **1c**, glutamic acid diamide **1d** and phenylalaninamide **1e** derivatives of ribavirin were synthesized.[11] However, these compounds were devoid of significant antiviral or antitumor activity. As part of an ongoing program of synthesis of nucleosides as potential anti-RNA-viral agents, a series of derivatives of carboxamidine **2a** conjugated to alkyl, cyano, or amino acids at the amidine nitrogen were synthesized and evaluated for antiviral (RNA) activity. Since kilogram quantities of amidine **2a** were required for advanced studies, a facile synthesis based upon that previously described[12] was also developed and is discussed in this paper.

Chemistry

The reaction sequences for the preparation of target compounds **2a–h**, **5**, **6**, and **7a,b** are outlined in Scheme I. Methyl carboximidate **5** served as a starting material for most reactions and was prepared from the acetylated carbonitrile **4** by treatment with sodium methoxide in methanol.[12,13] The *N*-cyano analogue **2b** was prepared from carboximidate **5** by treatment with cyanamide in sodium methoxide followed by acidification. Monosubstituted amidines may be prepared by the condensation of imidates with primary amines[14] or amino acids[14,15] to give N-substituted amidines. Amidines conjugated to amino acids may exhibit facilitated cellular transport or uptake. Therefore, the amino acids glycine (Gly), glutamine (Gln), and asparagine (Asp) were condensed with methyl carboximidate **5** in methanol at 35–50 °C to give the N-substituted amidines **2f** (Gly), **2g** (Asp), and **2h** (Gln). Analytical and NMR spectral data indicated that **2f–h** were obtained as zwitterions. Coupling of a hydrophobic "tail" onto a hydrophilic, biologically-active molecule can often affect its biological properties. Thus, methylamine hydrochloride, *n*-butylamine, and *n*-octylamine were condensed with imidate **5** in methanol to produce the mono-*N*-methyl hydrochloride (**2c**), *n*-butyl (**2d**), and *n*-octyl (**2e**) alkylated carboxamidines. Methylation at both amidine nitrogens was accomplished by treating carboxamidine **2a** with excess methylamine in anhydrous methanol in a reaction bomb heated to 60 °C for 6 days to produce **6** as a hydrated hydrochloride salt. In order to determine the effect of conjugating the amidine moiety of **2a** within the skeletal framework of a 6-membered ring, the 1,4,5,6-tetrahydrohydropyrimidine hydrochlorides **7a,b** were prepared from carboxamidine **2a** by treatment with 1,3-diaminopropane (or its 2-hydroxy analogue) in refluxing absolute ethanol.[16]

Stability Studies Leading to 1f. Amidines and their N-substituted analogues undergo hydrolysis at various rates and could thus serve as potential precursors of carboxamides.[17] Studies were undertaken to evaluate the stability of carboxamidine **2a**, dimethylamidine **6**, and glycine conjugate **2f** to hydrolysis. The rate of conversion of carboxamidine hydrochloride **2a** to ribavirin **1a** in D_2O (referenced to TSP) was monitored by quantitation of the ^{1}H-NMR absorptions of the C_5-H (proton) at δ 8.91 and 8.77 for **2a** and **1a**, respectively, as well as by observing the appearance of the carbonyl ($CONH_2$) absorption at δ 165.5 in the ^{13}C-NMR spectrum of **1a**. No interconversion to ribavirin was observed within 2 weeks, 12% conversion being observed after 7 weeks. Thin-layer chromatographic studies revealed the *N,N'*-dimethyl analogue **6** to be stable to hydrolysis in pH 7.4 phosphate buffer solution at room temperature for 1, 5, and 8 h. However after 24 h, TLC showed complete conversion of **6** (R_f = 0.45) to the *N*-methyl carboxamide derivative *N*-methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (**1f**, *N*-methylribavirin, R_f = 0.69). This conversion was confirmed by FAB-mass spectrometric (FAB/MS) analysis of the lyophilized crude reaction mixture which showed (M + 1) = 259 corresponding to the methyl carboxamide as opposed to (M + 1) = 272 corresponding to the *N,N'*-dimethyl amidine starting material. Since the sample of **1f** obtained by hydrolysis represented a hitherto unreported analogue of ribavirin, it was independently synthesized and characterized by treating carboxamidine **2a** with ethanolic methylamine. Similarly, glycine analogue **2f** was converted quantitatively to ribavirin **1a** in 1 h in phosphate-buffered saline (pH 7.4).

Recently, it became necessary to prepare kilogram quantities of the carboxamidine **2a**-HCl. The Pinner synthesis involves treating a nitrile with dry ethanolic

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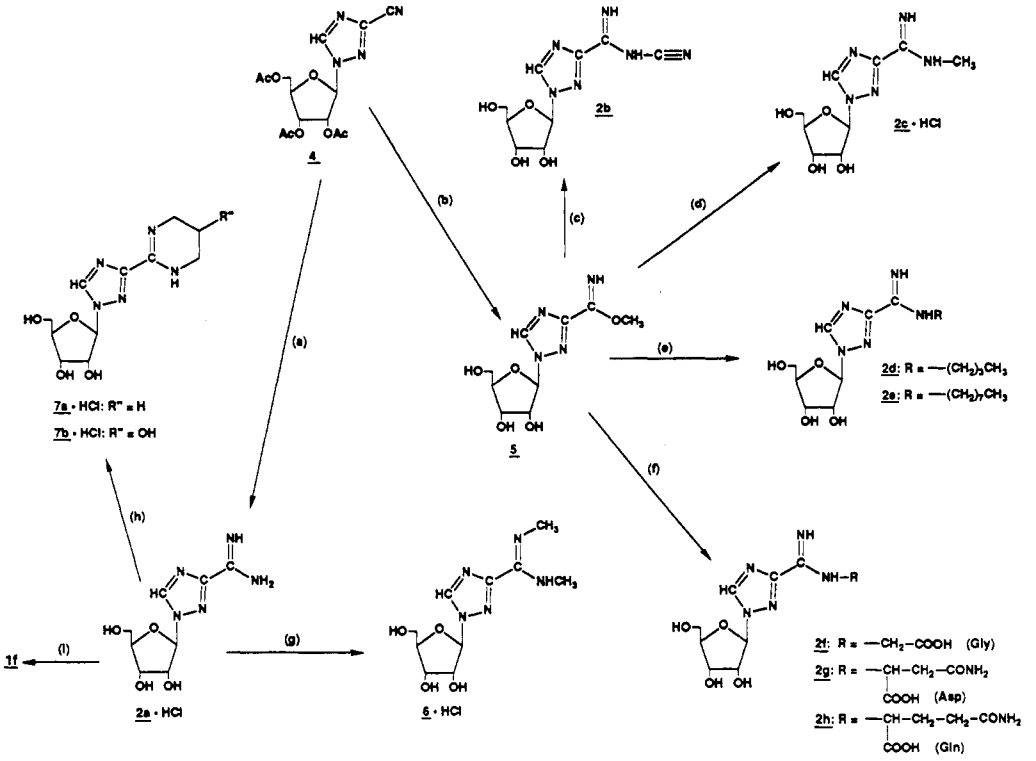
Scheme I.[a] Synthesis of Carboxamidine Analogues Evaluated in This Study



[a] (a) (1) $NaOCH_3/CH_3OH$, 25 °C, (2) NH_4Cl, reflux; (b) $NaOCH_3/CH_3OH$, 25 °C; (c) H_2NCN, $NaOCH_3$, CH_3OH, 25 °C; (d) CH_3NH_2, HCl, CH_3OH, reflux; (e) $CH_3(CH_2)_3NH_2$ or $CH_3(CH_2)_7NH_2$, CH_3OH, 25 °C for 5 d or 35 °C for 24 h; (f) glycine, CH_3OH, 50 °C, 1 h, or asparagine or glutamine, CH_3OH, 35 °C, 2 d; (g) CH_3NH_2 (1), EtOH, bomb, 60 °C, 6 d; (h) $(H_2NCH_2)_2CHR$ (R = H, OH), absolute EtOH, reflux; (i) CH_3NH_2 (40% aqueous), EtOH, reflux.

hydrogen chloride to give the imidate followed by ammonia or an amine in absolute ethanol to yield the amidine.[13,17] The preparation of kilogram quantities of carboxamidine **2a** by treatment of the methyl carboximidate **5** with a saturated methanolic ammonia solution in a pressure bottle containing ammonium chloride has been described.[12] The use of ammonia in a pressure bottle can be eliminated by treating the methyl carboximidate **5** (prepared but not isolated from the reaction of acetylated cyanotriazole **4** with methanolic sodium methoxide followed by neutralization with ion-exchange resin) with anhydrous ammonium chloride in refluxing methanol. Carboxamidine hydrochloride **2a** was thus prepared in 86% yield and characterized by ^1H and ^{13}C NMR[18,19] in both DMSO-d_6 and D_2O. The ^1H- and ^{13}C-NMR spectra of the carboxamidine **2a** exhibited marked solvent dependence. Changing solvent from DMSO-d_6 to D_2O shifted all proton absorptions to lower field by 0.25–0.37 ppm except that for the C-5-triazole proton, which was shifted upfield from δ 9.21 to 8.91 ppm. Similarly, all ^{13}C absorptions appeared at lower field by 2.0–3.5 ppm in D_2O.

Antiviral Activity

In Vitro Studies. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (**2a**), its N-cyano (**2b**), *N*-alkyl (**2c–e**), and *N*-amino acid (**2f–h**) analogues, the *N,N'*-disubstituted analogues **6**, **7a,b**, methyl carboximidate **5**, and *N*-methyl ribavirin derivative **1f** were evaluated in vitro to determine their inhibitory properties against the following RNA viruses: flaviviruses (family Flaviviridae) Japanese encephalitis (JE), yellow fever (YF), and dengue type-4 viruses; phleboviruses (family Bunyaviridae) Punta Toro (PT) and sandfly fever-Sicilian (SF) viruses; the alphavirus (family Togaviridae) Venezuelan equine encephalomyelitis (VEE) virus; paramyxoviruses (family Paramyxoviridae) respiratory syncytial (RSV), measles, and parainfluenza type 3 (PIV3) viruses; orthomyxoviruses (family Orthomyxoviridae) influenza A and influenza B viruses; and the lentivirus human immunodeficiency virus type 1 (HIV-1). Activity against the DNA-containing vaccinia virus (VV, Poxviridae) and adenotype 5 (Ad5, Adenoviridae) virus was also evaluated. The antiviral quantitative MTT assay[20–25] was used to

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Table I. In Vitro Antiviral (RNA) Evaluation of Carboxamidine Analogues

	sandfly fever[a]			Punta Toro[a]			other[a,b]		
compd	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}
1f	>320	inactive[c]		>320	inactive		–	–	–
							dengue-4 virus[b]		
2a	800	36	22	752	83	9.0	250	100	2.5
							respiratory syncytial virus		
							>1000	16	>64
							influenza A and influenza B virus		
							>1000	48	>20
							vaccinia virus[a]		
							>320	59	9
							dengue-4 virus[b]		
2b	>1000	inactive		>1000	inactive		>250	250	>1
							respiratory syncytial virus		
2c	>1000	104	9.6	>1000	250	>4.0	>1000	24	>42
							parainfluenza type 3 virus		
							>1000	125	>8
							influenza A virus		
							>1000	63	>16
							vaccinia virus[a]		
							>320	198	>1.6
2d	>1000	73	13.7	660	41	16	–	–	–
2e	>1000	339	>3	>1000	inactive		–	–	–
2f	>3200	566	>5.66	>1000	*d*		–	–	–
2g	>3200	484	>6.6	>3200	1600	>2.0	–	–	–
2h	>3200	547	5.9	>3200	2690	>1.2	–	–	–
							dengue-4 virus[b]		
5	840	98	8.6	744	201	3.7	>250	76	>3.3
							dengue-4 virus[b]		
6	>1000	94	>10.0	2900	181	16.1	>250	162	>1.5
							vaccinia virus[a]		
							>1000	184	>5.4
7a	>3200	inactive		>3200	inactive		–	–	–
7b	>1000	inactive		>1000	inactive		–	–	–

[a] TI (therapeutic index) was calculated as the ratio of TC_{50} (the concentration of test compound in μg/mL which was cytotoxic to 50% of uninfected cells) and IC_{50} (the concentration of test compound in μg/mL which inhibited 50% of virus-induced cytotoxicity) as obtained by the MTT assay. [b] IC_{50} measured by plaque reduction; TC_{50} measured by cytopathic effect. [c] Inactive at the maximum nontoxic concentration. [d] Viral cytopathic effect reduced 25%–49% only.

determine the 50% inhibition (IC_{50}) of virus-induced cell death, except for dengue virus, where activity was determined by a plaque-reduction assay.[21,22,26] Paramyxo- and orthomyxoviruses were assayed by inhibition of virus-induced syncytia formation using ribavirin as the positive control.[27,28] The concentration of test compound which was cytotoxic to 50% of uninfected cells (TC_{50}) was also determined, as was the therapeutic index, TI_{50}, a ratio of these two values (TC_{50}/IC_{50}).

Ribavirin exhibits a broad spectrum of antiviral activity against DNA viruses such as vaccinia virus (VV) and RNA viruses.[1,3] Against RNA viruses such as those described above, ribavirin has demonstrated exceptional in vitro and in vivo efficacy against bunyaviruses,[4,7,29] arenaviruses,[30] paramyxo- and orthomyxoviruses.[27,28] However, its activity against the flaviviruses (JE and YF) and alphavirus (VEE) is 10–100-fold less in vitro, and in vivo activity is not demonstrable.[30,31] The carboxamidine analogue of ribavirin (**2a**) demonstrates a similar spectrum of activity[2] with exceptional in vitro and in vivo activity against Punta Toro,[32] parainfluenza,[2] and influenza[2] A_2 viruses. In the current study, **2a** (a) showed activity against VV but was inactive against Ad5 virus; (b) was inactive against HIV-1 and measles viruses; (c) exhibited good activity against RSV, influenza A, and influenza B viruses (as demonstrated by therapeutic indices of >64, >20, and >20, TC_{50} values of >1000 μg/mL, and IC_{50} values of 16, 48, and 48 μg/mL, respectively); (d) was marginally active against the JE, YF, and VEE viruses, inhibiting viral cytopathic effect (CPE) by 25–40% at 320 μg/mL; (e) was cytotoxic to Vero cells at 1000 μg/mL; (f) was active against dengue-4 virus, exhibiting 99% plaque reduction at concentrations of 100 and 250 μg/mL, although **2a** was cytotoxic to MK-2 cells (these being more sensitive to cytoxicity than are Vero

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cells) at the higher (250 μg/mL) concentration; and (g) reinforced its efficacy against PIV3, PT, and SF viruses.[2,30] A therapeutic index of >21 was indicative of PIV3 virus inhibition (50%) at a concentration of **2a** corresponding to 48 μg/mL. SF- and PT-virus-induced CPE were inhibited by 100% and 50–100%, at drug concentrations of 100 and 100–320 μg/mL, respectively. In general, comparisons of IC_{50} values of ribavirin and carboxamidine **2a** against bunyaviruses reveal that higher IC_{50} values are required for **2a** to achieve similar antiviral efficacy. This pattern is duplicated against paramyxo- and orthomyxoviruses. In these experiments, IC_{50} values for ribavirin against PIV3, RSV, and influenza A and B viruses were (in μg/mL) 6, 6, 6, and 12, corresponding to therapeutic indices of 167, 167, 167, and 83, respectively.

The in vitro activity of the compounds studied is listed in Table I. No substantial in vitro activity was observed for any of the compounds tested against flaviviruses, an alphavirus, or HIV-1. Substitution of a cyano substituent at the amidine nitrogen to give **2b** eliminated all in vitro activity observed for carboxamidine **2a**. Incorporation of the amidine moiety within a tetrahydropyrimidine ring (as in **7a,b**) gave similar results. In the case of **7a,b**, the presence of a second ring at the 3-position may induce conformational changes and thereby preclude phosphorylation of the nucleoside. Imidate **5** exhibited significant in vivo anticancer activity[12] against murine leukemia L1210. However, its antiviral (RNA) activity was limited to the bunyaviruses PT and SF and dengue-4 virus. In each case, higher IC_{50} values were observed in comparison to those of ribavirin. Activity against PT and SF viruses was observed at 320 μg/mL with accompanying Vero cell toxicity at 1000 μg/mL. Significant host cell (MK-2) toxicity was observed at 250 μg/mL in the dengue virus assay. Imidate **5** was inactive against HIV, VV, JE, YF, and VEE. Substitution of an N-amino acid such as glycine (**2f**), asparagine (**2g**), or glutamine (**2h**) at the carboxamidino carbon (C=N) eliminated or significantly decreased all in vitro activity; the sole residual activity being against the bunyaviruses PT and SF viruses. The asparagine and glutamine analogues **2g** and **2h** exhibited reductions in bunyaviral (PT and SF) cytopathic effects (CPE) of 70–90% and 50–70%, respectively. This activity was observed only at high nontoxic concentrations of 1000–3200 μg/mL; IC_{50} values were considerably higher than those observed for **2a**. The 3-*N*-acetoxycarboxamidine analogue **2f** (glycine) produced 40% and 70% reductions in PT- and SF-viral-induced CPE, respectively. These effects were observed only at drug concentrations of at least 1000 μg/mL. High IC_{50} values (180–250 μg/mL) indicated marginal (at best) activity against PIV3, RSV, influenza A, and influenza B viruses; no activity was observed against Ad5 and measles viruses. Since ribavirin is active against the para- and orthomyxoviruses, the inactivity shown by **2f** argues against its hydrolysis to ribavirin under the conditions of in vitro evaluation. Glycyl analogue **2f** (R_f = 0.5) is nearly quantitatively converted into ribavirin (as determined by TLC, R_f = 0.88) within 1 h in phosphate-buffered saline (pH 7.4). Therefore, definitive statements regarding structure/activity of **2f** (and most likely **2g,h**) may be uncertain in nature even though appropriate precautions were taken during biological evaluation.

Methyl substitution at the amidine nitrogen produced **2c**-HCl. This replacement (a) eliminated the activity exhibited by **2a** against dengue-4 virus, **2c** being inactive at 100 and 250 μg/mL and cytotoxic to MK-2 cells at the higher concentration; (b) paralleled the inactivity of **2a** against measles and Ad5 viruses; (c) resulted in marginal (<50% CPE reduction) activities against JE, YF, and VEE viruses at nontoxic concentrations (1000 μg/mL); (d) produced activity against bunyaviruses PT and SF and the DNA vaccinia virus, though requiring 3-fold higher IC_{50} values than those for the unsubstituted amidine **2a**; and (e) paralleled the activity of **2a** against RSV, PIV3, influenza A, and influenza B viruses, although higher doses of **2c** were required to achieve the same antiviral effect (IC_{50}). Therapeutic indices for **2c** against RSV, PIV3, and influenza A and B viruses were >42, >8, >16 and >6; in each case, the TC_{50} value was >1000 μg/mL and IC_{50} values were 24, 125, 63, and 188, respectively. These data also indicate that an increase in the dose of **2c** by a factor of 0.5–2.5 is required to achieve the same antiviral effect as for unsubstituted **2a**. Substitution of an *n*-butyl chain in **2d** in place of a carboxamidine hydrogen in **2a** resulted in retention of the bunyavirus (PT, SF) activity of **2d** at concentrations (100 μg/mL) comparable to that for **2a**. However, also in contrast to **2a**, **2d** was inactive against dengue-4 virus. Marginal activity, as evidenced by 25–49% reduction in CPE, was observed for **2d** against VEE and JE viruses at 320 μg/mL; **2d** was inactive against YF, VV, and HIV-1 viruses. Substitution of an *n*-octyl chain resulted in a completely inactive compound (**2e**) except for slight residual activity against SF virus at elevated IC_{50} values. Dialkylation (methylation) at each amidine nitrogen as in **6**-HCl, markedly enhanced the activity against dengue-4 virus over the inactive monomethyl analogue **2c**. At the nontoxic concentration of 250 μg/mL, 99% plaque reduction was observed for **6**; unsubstituted amidine **2a** produced similar activity at 100 μg/mL but was cytotoxic at 250 μg/mL. In general, the in vitro activity of **6** paralleled that of **2a**, except that higher IC_{50} values were observed for **6**. No activity was observed against the other flaviviruses, JE and YF, and adeno type 5 virus; marginal activity (<50% CPE reduction) was observed against VEE at 320 μg/mL; however, 60–100% CPE reduction was observed at 320 μg/mL against the bunyaviruses PT and SF and vaccinia virus. Doses of **6** required to achieve similar activities to carboxamidine **2a** were 3-fold higher. In contrast to **2a**, dimethyl **6** was ineffective against RSV, PIV3, and influenza B, showing only marginal activity against influenza A (IC_{50} = 188 μg/mL). Cytotoxicity was enhanced in Vero, HEp2, and A549 cells, with TC_{50} values corresponding to 375 μg/mL.

It can be argued that most of the carboxamidine analogues in this study serve merely as precursors of ribavirin, the latter arising by hydrolysis. While we have determined that **2a** and **6** are stable to aqueous hydrolysis for hours (**2f** may be less stable), conditions of in vitro and in vivo evaluations differ. The preceding antiviral data for the dimethyl analogue **6** provides evidence for the existence of an independent mechanism of action for carboxamidines such as **2a**[6] resulting in the antiviral activities observed in contrast to the role of amidines merely serving as prodrugs of carboxamides such as ribavirin. Hydrolysis of the *N,N'*-dimethyl carboxamidine **6** would be expected to produce the *N*-methyl carboxamide analogue (**1f**) of ribavirin. In vitro evaluation of **1f** revealed no antiviral activity nor cytotoxicity (to Vero, HEp2, MDCK, or A549 cells at 1000 μg/mL) against JE, YF, SF, PT, VEE, RSV, PIV3, measles, influenza A and B, Ad5, or VV viruses. Against dengue-4 virus, ribavirin **1a** was active and nontoxic at 50–100 μg/mL while *N*-methyl **1f** was inactive and cytotoxic to MK-2 cells at 100 μg/mL, in clear contrast to the activity of **6**. Thus, it is reasonable to conclude that the *N,N'*-dimethyl carboxamidine functionality of **6** is

biologically responsible for the activity against dengue-4 virus and that in situ hydrolysis is not a major factor.

In Vivo Studies. Ribavirin[29] and its carboxamidine analogue[32] **2a** have demonstrated consistent and potent inhibitory effects against PT virus, both in vitro and in a mouse model in which the principal pathogenic process involved hepatitis. Efficacy was measured in terms of survivor numbers and survival times. In addition, viral pathogenicity and the development of infectious virus in sera and livers were correlated with the degree of hepatic icterus (liver score) and with elevations in serum glutamic oxalic and pyruvic acid transaminases, SGOT and SGPT, respectively. *N*-Methyl and glycyl-substituted carboxamidines **2c** and **2f**, the *N,N'*-dimethyl analogue **6**, and the tetrahydropyrimidine **7a** were evaluated in PTV-infected mice. All (except **7a**) were administered in doses of 1200, 600, and 300 mg/kg per day, subcutaneously (sc) in saline, twice daily for 4 days (b.i.d. × 4), beginning 4 h pre virus inoculation. The dimethyl derivative **6** was ineffective at any dose used in the study. The monomethyl analogue **2c** was marginally effective at the highest nontoxic dose (1200 mg), producing slight increases in mean survival times and decreased levels of SGOT, SGPT, and mean liver virus titers. *N*-Glycyl **2f** produced a 90% survival rate (cf. 30% survival in the control mice) when administered at a dose (1200 mg) approaching its maximum tolerated dose (MTD). Significant reductions in mean liver score and SGOT and SGPT levels and moderate decreases in liver and serum virus titers were observed. It is not possible to ascribe these results to the activity of **2f** since it is known that **2f** is converted to ribavirin **1a** in phosphate-buffered saline in ca. 1 h. Administration of 1,4,5,6-tetrahydropyrimidine **7a** (sc, b.i.d. × 5) at nontoxic doses of 600, 300, 150, and 75 mg/kg per day resulted only in slight increases in mean survival times at 300- and 150-mg dose levels. Under similar conditions, ribavirin (administered at 75 mg/kg per day) and carboxamidine **2a** (administered at 62.5 and 125 mg/kg per day) produced 100% survival for >21 days. The MTD values for ribavirin and **2a** are 100 and 1000 mg/kg per day, respectively.[32]

Experimental Section

Chemistry. All solvents were distilled before use and dried when necessary, and all chemicals were reagent grade. Evaporations were conducted at bath temperatures ≤30 °C with a Büchi rotary evaporator under water aspirator or mechanical oil pump vacuum. Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Results agreed within ±0.4% of the theoretical values. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian XL200 spectrometer with an ADVANCE data system operating at 200 and 50.3 MHz, respectively. Additional ^{1}H-NMR spectra were recorded on a Nicolet NT300WB spectrometer with a 1280 data system operating at 300 MHz. Standard Varian COSY was used for proton–proton connectivity determination. Mass spectral data were obtained employing a MAT 312 mass spectrometer. Chemical shifts are expressed in parts per million referenced to tetramethylsilane (^{1}H NMR) or dimethyl sulfoxide at 39.7 ppm (^{13}C NMR). Coupling constants (*J*) are recorded in hertz. IR spectra were recorded using a Perkin-Elmer Model 1310 spectrophotometer as Nujol mulls or KBr pellets. TLC was performed on Woelm F silica gel sheets (254/366) with detection of products under a short-wavelength UV lamp and/or spraying with 40% methanolic H_2SO_4 and charring.

***N*-Methyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (1f).** A 95% ethanolic solution (20 mL) of carboxamidine **2a** (250 mg, 1 mmol) and 40% aqueous methylamine (8 mL, 229 mmol) was refluxed for 36 h then evaporated in vacuo. Thin-layer chromatography of the residue (1:1:0.5 $CHCl_3$/MeOH/HOAc, R_f = 0.80) indicated quantitative conversion to **1f** (320 mg), which was crystallized from ethanol and dried over P_2O_5: MS (FAB) m/z = 259 (M + 1); IR (KBr) ν 3320, 1668, 1530, 1505, 1460, 1350, 1230, 1100 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.88 (s, 1 H, C_5-H), 8.47 (d, 1 H, NH), 5.81 (d, 1 H, J = 4.0 Hz, H-1'), 5.65–5.59 (d, 1 H, 2'-OH), 5.36–5.21 (d, 1 H, 3'-OH), 4.93 (t, 1 H, 5'-OH), 4.36 (m, 1 H, H-2'), 4.14 (m, 1 H, H-3'), 3.96 (m, 1 H, H-4'), 3.64, 3.49 (m, 2 H, H-5'), 2.78 (d, 3 H, NHCH_3). Anal. ($C_9H_{14}N_4O_5$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamidine Hydrochloride (2a). Cyanotriazole derivative **4** (704 g, 2 mol) was suspended in methanol (12 L) in a 22-L round-bottomed flask. Sodium methoxide (45 g) was slowly introduced and the reaction mixture was stirred for 2 h at 25 °C, pH 8.5–9.0. When thin-layer chromatography (6:1 CH_2Cl_2/MeOH, R_f = 0.2) indicated complete deacetylation of the ribose ring as well as imidate formation, the sodium ions in solution were neutralized by the addition of Amberlite IR 120 H+ ion-exchange resin with stirring. The mixture was filtered and anhydrous ammonium chloride (107 g, 2 mol) was added to the filtrate. The stirred mixture was gently refluxed for 2 h. Upon completion (by TLC) of amidine formation, charcoal (15 g) was added. The mixture was stirred for 15 min then filtered through a Celite pad. The filtrate was concentrated in vacuo to one-third its volume and left overnight. The crystallized product **2a**-HCl was collected by filtration, and the mother liquor concentrated further to provide a second crop. The two batches were combined and recrystallized from methanol to give 480 g (85.8%) of pure amidine hydrochloride **2a**. This sample was identical in all respects with an authentic sample.[2]

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-cyanocarboxamidine (2b). Cyanamide (1.8 g, 42.6 mmol) and a molar solution of freshly-prepared methanolic sodium methoxide (42.6 mL) were successively added to a methanolic solution of methyl 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboximidate[11] (**5**) (3.95 g, 15.2 mmol). The resulting solution was stirred at room temperature for 6 h, acidified with acetic acid to pH 4, and concentrated to dryness in vacuo. The residue was chromatographed over silica gel (flash chromatography) with 10% methanol in dichloromethane as eluent to yield **2b** as an amorphous solid (1.63 g, 40%): ^{1}H NMR (DMSO-d_6) δ 8.80–9.10 (2 s, 3 H, amidine NH, C-5-H), 5.85 (d, 1 H, J = 3.1 Hz, H-1'), 4.89, 5.20, 5.61 (t, d, d, 3 H, OH), 4.35 (d, 1 H, H-2'), 4.16 (d, 1 H, H-3'), 3.96 (m, 1 H, H-4'), 3.57 (m, 2 H, H-5'); ^{13}C NMR (DMSO-d_6) δ 61.75, 70.42, 75.07, 86.06, 92.50, 116.41, 146.23, 155.61, 160.83. Anal. ($C_9H_{12}N_6O_4$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-methylcarboxamidine Hydrochloride (2c). Methyl carboximidate **5** (2 g, 7.75 mmol) was combined with methylamine hydrochloride (0.53 g, 7.75 mmol) followed by the addition of methanol (40 mL). The stirred solution was heated at gentle reflux until TLC monitoring (6:1 CH_2Cl_2/MeOH, product R_f = 0.09) indicated completion of the reaction. The solvent was evaporated in vacuo to yield a solid foam which was dissolved in ethanol (15 mL). The ethanolic solution was concentrated in vacuo until a semisolid product appeared. Trituration of the mixture with 4:1 ether/ethanol (50 mL) gave *N*-methyl carboxamidine hydrochloride **2c** (2.0 g, 89%): mp 175–177 °C; IR (KBr) 3420–3060 (br), 1690, 1630, 1450, 1050, 1025, 900 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.1 (brs, 1 H, NH), 9.7 (brs, 2 H, NH), 9.23 (s, 1 H, H-5), 5.93 (d, 1 H, J = 3.67 Hz, H-1'), 5.79 (d, 1 H, J = 5.36 Hz, 2'-OH), 5.32 (d, 1 H, J = 5.53 Hz, 3'-OH), 5.10 (t, 1 H, J = 5.31 Hz, 5'-OH), 4.44 (dd, 1 H, J = 4.62, 4.13, 4.62 Hz, H-2'), 4.23 (dd, 1 H, J = 5.1, 5.0, 5.13 Hz, H-3'), 4.03 (dd, 1 H, J = 4.3, 4.5, 4.4 Hz, H-4'), 3.73–3.45 (m, 2 H, H-5'), 3.077 (s, 3 H, CH_3); ^{13}C NMR δ 153.21 (C=N), 152.27 (C-3-triazole), 146.33 (C-5-triazole), 92.52 (C-1'), 85.99 (C-4'), 74.80 (C-2'), 69.94 (C-3'), 61.01 (C-5'), 29.6 (CH_3). Anal. ($C_9H_{16}O_4N_5Cl$) C, H, N, Cl.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-*n*-butylcarboxamidine (2d). A solution of methyl imidate **5** (0.3 g, 1.2 mmol) and *n*-butylamine (100 mg, 1.37 mmol) in anhydrous methanol (40 mL) was flushed with argon, sealed, and stirred at room temperature for 5 days. The solvent was removed in vacuo and the residue was stirred overnight with anhydrous ether (50 mL). The ether was decanted and the procedure repeated with a fresh portion of anhydrous ether. Removal of the solvent in vacuo gave **2d** as a glassy solid: 316 mg (87%); mp 63–66 °C; ^{1}H NMR (DMSO-d_6) δ 8.86 (s, 1 H, H-5), 5.80 (d, 1 H, J = 3.9 Hz, H-1'), 5.8 and 5.0 (brs, 5 H, OH, NH), 4.356 (dd, 1 H, J = 3.8, 4.9 Hz, H-2'), 4.147 (dd, 1 H, J = 4.9, 3.8 Hz, H-3'), 3.955 (dt, 1 H, J =

4.0, 5.1 Hz, H-4'), 3.63 (d, AB, 1 H, J = 4.1, 12.0 Hz, H-5'$_a$), 3.51 (d, AB, 1 H, J = 5.0, 12.0 Hz, H-5'$_b$), 3.27 (d, AB, 1 H, J = 7.0, 13.0 Hz, H-1''$_a$), 3.23 (d, AB, 1 H, J = 7.2, 13.0 Hz, H-1''$_b$); 1.55 (pentuplet, 2 H, J = 7.3 Hz, H-2''), 1.32 (sextet, 2 H, J = 7.4 Hz, H-3''), 0.90 (t, 3 H, J = 7.2 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ 157.35 (C=N), 153.4 (C-3-triazole), 144.94 (C-5-triazole), 91.85 (C-1'), 85.54 (C-4'), 74.55 (C-2'), 69.99 (C-3'), 61.31 (C-5'), 40.78 (C-1''), 30.99 (C-2''), 19.83 (C-3''), 13.79 (CH_3). Anal. ($C_{12}H_{21}N_5O_4 \cdot 0.3H_2O$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-*n*-octylcarboxamidine (2e). The procedure for the synthesis of **2d** was followed, yielding **2e** as white crystals: mp 86–89 °C; ^{1}H NMR (DMSO-d_6) δ 8.85 (s, 1, H-5), 6.5–7.1 (brs, 2 H, NH), 5.80 (d, 1 H, J = 3.9 Hz, H-1'), 5.6, 5.3, 4.9 (3 × brs, 3 × 1 H, 3 × OH), 4.35 (dd, 1 H, J = 3.9, 4.8 Hz, H-2'), 4.15 (dd, 1 H, J = 4.9, 5.1 Hz, H-3'), 3.95 (dt, 1 H, J = 4.1, 5.0 Hz, H-4'), 3.64 (dab, 1 H, J = 3.9, 12.0, H-5'$_a$), 3.51 (dab, 1 H, J = 4.9, 12.0, H-5'$_b$), 3.23 (t, 2 H, J = 7.1 Hz, H-1''), 1.54 (m, 2 H, H-2''), 1.26 (m, 10 H, H-3''–H-7''), 0.85 (t, 3 H, J = 7.0 Hz, CH_3); ^{13}C NMR (DMSO-d_6) δ 157.5 (C=N), 153.3 (C-3-triazole), 144.9 (C-5-triazole), 91.8 (C-1'), 85.4 (C-4'), 74.4 (C-2'), 69.9 (C-3'), 61.2 (C-5'), 41.0 (C-1''), 31.3, 28.9, 28.9, 28.7, 26.7, 22.1, 13.9 (based on octyl nitrite). Anal. ($C_{16}H_{29}N_5O_4$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-acetoxycarboxamidine (2f, Gly). The procedure for the synthesis of **2g** was followed except for reaction time (1 h) and temperature (50 °C) to give **2f** (TLC in 7:3 acetonitrile/0.1 M NH_4Cl, R_f = 0.26) as white crystals: >90%; mp 190–192 °C; ^{1}H NMR (DMSO-d_6) δ 9.2 (brs, 3 H, NH), 9.16 (s, 1 H, H-5), 5.91 (d, 1 H, J = 3.5 Hz, H-1'), 6.1, 5.6, 5.1 (3 × brs, 3 × 1 H, 3 × OH), 4.397 (dd, 1 H, J = 3.5, 4.8 Hz, H-2'), 4.19 (dd, 1 H, J = 5.1, 4.8 Hz, H-3'), 3.99 (ddd, 1 H, J = 4.8, 5.1, 3.9 Hz, H-4'), 3.71 (s, 2 H, –CH_2–), 3.67 (d, AB, 1 H, J = 3.9, 12.1 Hz, H-5'$_a$), 3.54 (d, AB, 1 H, J = 4.8, 12.1 Hz, H-5'$_b$); ^{13}C NMR (DMSO-d_6) δ 167.09 (COOH), 152.56 and 151.33 (C=N and C-3-triazole, unassigned), 146.18 (C-5-triazole), 92.55 (C-1'), 85.81 (C-4'), 74.74 (C-2'), 69.73 (C-3'), 61.02 (C-5'), 46.20 (CH_2). Anal. ($C_{10}H_{15}N_5O_6$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-(α-carboxy-3'-propionamido)carboxamidine (2g, Asp). A solution of imidate **5** (300 mg, 1.2 mmol) and asparagine (159 mg, 1.2 mmol) in anhydrous methanol (50 mL) was flushed with argon, sealed, and stirred at room temperature for 5 days. The solvent was removed in vacuo and the residue was stirred overnight with anhydrous ether (50 mL). The ether was decanted and the procedure repeated three to five times with fresh portions of anhydrous ether. Removal of the solvent in vacuo gave **2g** (424 mg, >95%) as white crystals: mp 151–153 °C; ^{1}H NMR (DMSO-d_6) δ 9.14 (s, 1 H, H-5), 9.10 (brs, 3 H, NH), 7.77 and 7.10 (each d, 2 H, J = 1.7, 1.7 Hz, $CONH_2$), 5.90 (d, 1 H, J = 3.5 Hz, H-1'), 5.9, 5.5, 5.0 (3 × brs, 3 H, 3 × OH), 4.39 (dd, 1 H, J = 3.5, 4.8 Hz, H-2'), 4.21 (dd, 1 H, J = 3.8, 7.1 Hz, C*H* methine, H-1''), 4.17 (dd, 1 H, J = 5.4, 4.7 Hz, H-3'), 3.99 (ddd, 1 H, J = 3.8, 4.8, 5.4 Hz, H-4'), 3.66 (d, AB, 1 H, J = 3.8, 12.1 Hz, H-5'$_a$), 3.54 (d, AB, 1 H, J = 4.7, 12.1 Hz, H-5'$_b$), 2.86 (d, AB, 1 H, J = 3.7, 16.4 Hz, H-2''$_a$), 2.67 (d, AB, 1 H, J = 7.2, 16.5, H-2''$_b$); ^{13}C NMR (DMSO-d_6) δ 174.00 ($CONH_2$), 169.16 (COOH), 152.72 and 151.49 (C=N and C-3-triazole, unassigned), 146.14 (C-5-triazole), 92.57 (C-1'), 85.78 (C-4'), 74.73 (C-2'), 69.70 (C-3'), 61.00 (C-5'), 53.93 (C-1''), 37.50 (C-2''). Anal. ($C_{12}H_{18}N_6O_7$) C, H, N.

1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-(α-carboxy-4'-butyramido)carboxamidine (2h, Gln) or *N*-[(1-β-D-Ribofuranosyl-1,2,4-triazol-3-yl)iminomethyl]glutamine. The procedure for the preparation of **2g** was followed, allowing imidate **5** (220 mg, 0.85 mmol) and L-glutamine (125 mg, 0.85 mmol) to react for 7 days, producing **2h** as white crystals (305 mg, mp 209–210 °C) is essentially quantitative yield: ^{1}H NMR (DMSO-d_6) δ 9.5–8.8 (brs, 2 H, =NH and –NH–), 9.12 (s, 1 H, H-5), 7.55, 6.91 (2 s, 2 H, $CONH_2$), 5.89 (d, 1 H, J = 3.9 Hz, H-1'), 5.7, 5.3, 5.0 (3 brs, 3 H, 2',3',5'-OH), 4.39 (t, 1 H, J = 4.0, 5.2 Hz, H-2'), 4.17 (t, 1 H, J = 4.5, 5.0 Hz, H-3'), 3.99 (dd, 1 H, J = 4.4, 12 Hz, H-4'), 3.88 (m, 1 H, C*H*COOH), 3.65 (t, 1 H, J = 4.2, 5.0 Hz, H-5'), 3.54 (m, 1 H, H-5'), 2.38, 2.17 (2 m, 2 H, CH_2CH_2CO), 1.85 (m, 2 H, CH_2CH_2CO); ^{13}C NMR (DMSO-d_6) δ 174.79 ($CONH_2$), 170.29 (COOH), 152.52 (C-3-triazole), 150.93 (C=NH), 146.00 (C-5-triazole), 92.53 (C-1'), 85.71 (C-4'), 74.67 (C-2'), 69.59 (C-3'), 60.86 (C-5'), 55.70 (NHCHCOOH), 30.73 (CH_2CONH_2), 26.31 (–$CHCH_2CH_2$–). Anal. ($C_{13}H_{20}N_6O_7$) C, H, N.

3-Cyano-2',3',5'-tri-*O*-acetyl-1-β-D-ribofuranosyl-1,2,4-triazole (4). This compound was prepared as previously described.[12]

Methyl 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboximidate (5). This compound was prepared as previously described[12] and was identical in all respects with an authentic sample.

***N*,*N*'-Dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine Hydrochloride (6).** Anhydrous ethanol (40 mL) was cooled to –20 °C in the glass liner of a stainless steel bomb. Liquid methylamine was then bubbled into the cooled solution until a total volume of approximately 90 mL was obtained. To this solution was added 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (**2a**-HCl) (3.0 g, 12 mmol) in portions. The reaction mixture/glass liner was transferred to the reaction bomb, the bomb was sealed and the mixture was heated to 60 °C with stirring for 6 days. After thin-layer chromatography (1:1:0.5 $CHCl_3$/CH_3OH/HOAc) had indicated that the reaction had gone to completion giving a single component, the solvent was removed in vacuo and the residue was dried in vacuo over P_2O_5 to give a hygroscopic, light brown foam (3.0 g, 92.3%). Attempts to obtain a melting point using a nitrogen-filled glove box led to the observations that the crystals became gummy at 60 °C with melting occurring at 95–115 °C: MS (FAB) m/z = 272 (M + 1); IR (KBr) ν 3320 (broad), 3100, 2945, 1660, 1600, 1500 cm^{-1}; ^{1}H NMR (DMSO-d_6 + TFA-d) δ 9.92 (brs, 1 H, N*H*CH_3), 7.90 (brs, 2 H, 2',3'-OH), 5.97 (d, 1 H, H-1'), 4.42 (t, 1 H, H-2'), 4.20 (t, 1 H, H-3'), 4.02 (m, 1 H, H-4'), 3.59 (d, 1 H, 5'-OH), 3.54 (m, 2 H, H-5'), 3.28 (s, 3 H, =NCH_3), 3.02 (s, 3 H, NHCH_3); ^{13}C NMR (DMSO-d_6 + TFA-d) δ 152.02 and 153.70 (C-3, NC=N), 145.82 (d, C-5), 92.55 (d, C-1'), 85.91 (d, C-4'), 74.93 (d, C-2'), 69.98 (d, C-3'), 61.01 (t, C-5'), 32.18 (d, =NCH_3), 29.61 (d, $NHCH_3$). Anal. ($C_{10}H_{17}N_5O_4 \cdot 1.2H_2O \cdot 0.5EtOH \cdot HCl$) C, H, N.

Hydrolysis Studies of 2f and 6. 1-β-D-Ribofuranosyl-1,2,4-triazole-3-*N*-acetoxycarboxamidine (**2f**) (6 mg) and *N*,*N*'-dimethyl-1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamidine hydrochloride (**6**) (200 mg) were added to 3 and 5 mL, respectively, of a pH 7.4 phosphate buffer solution at room temperature. The solutions were stirred and monitored by thin-layer chromatography (mobile phase, 1:1:0.5 $CHCl_3$/CH_3OH/HOAc) over a 1–24-h period. Hydrolysis products were determined by TLC comparison with products ribavirin (R_f = 0.88) and *N*-methyl analogue **1f** (R_f = 0.80).

1-(β-D-Ribofuranosyl)-3-(1,4,5,6-tetrahydropyrimidin-2-yl)-1,2,4-triazole Hydrochloride (7a). Carboxamidine hydrochloride **2a** (3 g, 10.8 mmol), absolute ethanol (15 mL), and freshly-distilled 1,3-diaminopropane (0.82 g, 11 mmol) were heated at reflux for 2–8 h. The course of the reaction was monitored by TLC in ethyl acetate/*n*-propanol/water (4:2:1) or acetonitrile/0.1 M NH_4Cl (7:3), and in addition, the odor of evolved ammonia was evident as the reaction progressed. When the reaction was complete (as determined by TLC and the absence of ammonia), the reaction mixture was cooled to room temperature and decanted from any residual oil. Solvent was removed in vacuo and the residue was crystallized from dry methanol at 0 °C. Crystallization was also induced by trituration of the methanolic solution with dry diethyl ether. Crystals were filtered under nitrogen and dried in vacuo to produce tetrahydropyrimidine analogue **7a** (2.62 g, 76%): mp 123–125 °C; IR (KBr) 1634, 1676 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 10.06 (brs, 1 H, NH), 9.20 (s, 1 H, H-5), 5.91 (d, J = 3.58 Hz, H-1'), 5.75, 5.31, 5.04 (brs, 3 H, OH), 4.40 (t, 1 H, J = 4.13 Hz, H-2'), 4.20 (t, 1 H, J = 4.92 Hz, H-3'), 4.01 (dd, 1 H, J = 9.04, 4.53 Hz, H-4'), 3.59 (ddd, 2 H, J = 3.8, 4.8, 12.1 Hz, H-5'), 3.48 (t, 4 H, H-3'',5''), 1.97 (quintet, 2 H, J = 4.7 Hz, H-4''); ^{13}C NMR δ 151.99 (d, J_{CNCH} = 11.9 Hz, C-3-triazole), 149.44 (d, J = 48 Hz, C(=N)NH), 146.3 (d, J = 216.6 Hz, C-5-triazole), 92.44 (d, J_{CH} = 170.1 Hz, C-1'), 85.95 (d, J = 147.8 Hz, C-4'), 74.81 (d, J = 150.3 Hz, C-2'), 69.98 (d, J = 147.8 Hz, C-3'), 61.08 (t, J = 139 Hz, C-5'), 38.56 (t, J = 144.3 Hz, C-3''), 17.66 (t, J = 133.1 Hz, C-4''). Anal. ($C_{11}H_{17}N_5O_4 \cdot HCl \cdot H_2O$) C, H, N.

3-(5-Hydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-1-β-D-ribofuranosyl-1,2,4-triazole Hydrochloride (7b). Hydroxy-substituted tetrahydropyrimidine **7b** was prepared from carboxamidine **2a** (3.5 g, 12.57 mmol) and 2-hydroxy-1,3-diaminopropane (freshly-distilled, 1.18 g, 13 mmol) in absolute ethanol

(17 mL) as described for **7a**. In this manner, **7b** was obtained (3.56 g, 80%): mp 102–106 °C; IR (KBr) 1630, 1670 cm^{-1}; ^1H NMR (DMSO-d_6) δ 10.0 (brs, 1 H, NH), 9.20 (s, 1 H, H-5), 5.922 (d, 1 H, J = 3.66 Hz, H-1′), 5.75 and 5.05 (brs, OH), 4.410 (t, 1 H, J = 3.8 Hz, H-2′), 4.247 (t, 1 H, J = 2.8 Hz, H-4″), 4.205 (t, 1 H, J = 5.0 Hz, H-3′), 4.006 (q, 1 H, J = 4.24 Hz, H-4′), 3.66 (dd, 1 H, J = 3.54, 3.33 Hz, H-5′$_a$), 3.53 (dd, 3 H, H-3″$_a$, H-5″$_a$, H-5′$_b$), 3.384 (dd, 2 H, J = 3.17 Hz, H-3″$_b$, H-5″$_b$); ^{13}C NMR δ 151.99 (d, J = 11.9 Hz, C-3-triazole), 149.2 (s, C(=N)NH), 146.5 (d, J_{CH} = 217.5 Hz, C-5-triazole), 92.5 (d, J = 168.7 Hz, C-1′), 86.01 (d, J = 149.4 Hz, C-4′), 74.88 (d, J = 152.6 Hz, C-2′), 70.08 (d, J = 148.9 Hz, C-3′), 61.22 (td, J = 141.2, 3 Hz, C-5′), 56.6 (dd, J = 150.5, 4 Hz, C-4″), 45.0 (td, J = 144, 3 Hz, C-3″). Anal. ($C_{11}H_{17}N_5O_5$·HCl·H_2O) C, H, N.

In Vitro Antiviral and Cytotoxicity Assays. Compounds were evaluated for antiviral efficacy against the following viruses (viral strain): (a) Japanese encephalitis virus, JE (Nakayama); (b) yellow fever virus, YF (Ashibi); (c) sandfly fever virus, SF (Sicilian); (d) Punta Toro virus, PT (Adames); (e) Venezuelan equine encephalomyelitis virus, VEE (Trinidad donkey); (f) vaccinia virus, VV (Lederle vaccine); (g) dengue type-4 (Caribbean) virus; (h) human immunodeficiency virus type 1, HIV-1; (i–n) adenovirus type 5, respiratory syncytial virus, parainfluenza virus type 3, measles virus, and influenza A and influenza B viruses as described.[27,28] The in vitro antiviral and cytotoxic effects of a test compound were measured[20] either (a) by observing inhibition of viral cytopathic effect[21–23] using an MTT assay (JE, YF, SF, PT, VEE, VV, and HIV-1 viruses[24,25]) or (b) by a general plaque-reduction assay (dengue virus[26]). All assays were carried out in Vero cells except for the use of MT-2 and CEM cells in the HIV-1 assay.[24,25]

A general plaque-reduction assay[26,30,31] was used to test for antiviral activity of candidate compounds against dengue virus. Each drug to be tested is dissolved in appropriate diluent (DMSO, ethanol), brought to twice the highest concentration to be tested in cell culture maintainance medium (Hank's basal salt solution: Hepes containing 2% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 10 μg/mL streptomycin), and sterilized by filtration. Five 2-fold dilutions of 2× drug are prepared in cell culture medium. For each drug, 12 wells of a 24-well plastic tissue culture plate containing confluent monolayer cultures of LLCMK2 cells are used. Six wells are infected by removal of growth medium and addition of 100 mL of dengue-4 virus (Caribbean strain) containing 50–100 plaque-forming units. The remaining six wells receive medium without virus. After adsorption for 1 h, 0.5 mL each of 2× drug is added to duplicate (infected and control) wells. Medium without drug is added as a control. Cultures are then overlaid with 2.5% agarose in nutrient medium and incubated for 6 days, at which time they are stained by addition of 2 mL of 5% neutral red. Wells are decanted after 4 h and plaques counted. The IC_{50} is determined as the concentration of drug reducing plaques by 50% over the untreated control, while the minimum toxic concentration (MTC) is estimated visually by inspection of uninfected drug-treated wells.

Basic measurements and definitions used throughout these studies include (a) 50% cellular toxic concentration, TC_{50}, the drug concentration (μg/mL) that reduces the cell number and their metabolic activity by 50% as compared to the viability of uninfected control cells in duplicate test wells in the MTT assay; (b) 50% viral inhibitory concentration, IC_{50}, the drug concentration (μg/mL) at which 50% reduction of viral cytopathic effect (CPE) is observed in triplicate test wells; the therapeutic (or antiviral) index, TI_{50}, a value proportional to the overall in vitro activity calculated as a ratio of TC_{50}/IC_{50}. It is a single drug concentration measurement of the relative anticellular and antiviral effectiveness of a compound during the same test and time period. All in vitro MTT assay results given represent an average of two to six individual test results.

In Vivo Evaluation in the Murine Punta Toro Model. Compounds were evaluated in Punta Toro virus-infected mice as previously described for ribavirin and carboxamidine **2a**.[29,32]

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