Potent Inhibition of NTPase/Helicase of the West Nile Virus by Ring-Expanded ("Fat") Nucleoside Analogues

Ning Zhang,[†] Huan-Ming Chen,[†] Verena Koch,[‡] Herbert Schmitz,[‡] Michal Minczuk,[§] Piotr Stepien,[§] Ali I. Fattom,[∥] Robert B. Naso,[∥] Kishna Kalicharran,[∥] Peter Borowski,*[,‡] and Ramachandra S. Hosmane*[,†]

Laboratory for Drug Design and Synthesis, Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250; Abteilung für Virologie, Bernhard-Nocht-Institut für Tropenmedizin, Bernhard-Nocht-Strasse 74, Hamburg, D-20359 Germany; Department of Genetics, University of Warsaw Pawinskiego 5A, 02-106, Poland; and W. W. Karakawa Microbial Pathogenesis Laboratory, Nabi Biopharmaceuticals, Rockville, Maryland 20852

Received June 9, 2003

A series of ring-expanded ("fat") nucleoside analogues (RENs) containing the 6-aminoimidazo-[4,5-*e*][1,3]diazepine-4,8-dione ring system have been synthesized and screened for inhibition of NTPase/helicase of the West Nile Virus (WNV). To assess the selectivity of RENs against the viral enzymes, a truncated form of human enzyme $Suv3_{(\Delta 1-159)}$ was also included in the study. Ring-expanded nucleosides **16**, **17**, and **19**, which possess the long C_{12}, C_{14}, and C_{18} side-chains, respectively, at position 6, as well as the ring-expanded heterocycle **39**, which contains aralkyl substitution at position 1, were all found to have excellent profiles of activity and selectivity toward the viral versus human enzymes against the West Nile Virus (IC_{50} ranging 1−10 μM). Compound **30**, while being an equally potent inhibitor of WNV, was found to be somewhat less selective, whereas compound **36**, which is an α-anomeric counterpart of **30**, exhibited potent and selective inhibition of WNV (IC_{50} 1−3 μM). The same compounds that showed potent inhibition of viral helicase activity completely failed to show any activity against the viral NTPase reaction even up to 500 μM. However, at concentrations >500 μM of RENs and the ATP concentrations >10 times the K_m value of the enzyme, a significant activation of NTPase activity was observed. This activating effect underwent further dramatic enhancement (>1000%) by further increases in ATP concentration in the reaction mixture, suggesting that the viral helicase and NTPase reactions are not coupled. A tentative mechanistic model has been proposed to explain the observed results.

Introduction

With a number of Science Focus and News Focus articles that have appeared in recent issues of Science magazine,[1−4] the West Nile Virus (WNV) is gaining wide attention in North America.[5−7] Nearly four years after the 1999 outbreak of the West Nile virus in New York City, which sickened 62 people, most of them elderly, and killed 7, the virus has been detected in more than 60 bird species and about a dozen mammals and has spread to almost every state and the District of Columbia, with more than 1300 human cases of WNV infection resulting in several dozen mortalities. West Nile virus is mainly a bird virus that is spread by mosquitoes. Humans and horses, as well as a dozen other mammals are its dead-end hosts. Crows are the virus's most conspicuous hosts as they have been dying en masse with WNV infection. Most humans infected with WNV don't even know it, or they experience only mild, flu-like symptoms. Those over 65 are especially vulnerable to WNV. Three months after the outbreak, 70% of the survivors still reported muscle weakness, 75% suffered from memory loss, 60% from confusion, and more than half could no longer live at home, although most were healthy, active, and lived normal lives before the WNV attack. Many of the patients end up with lingering neurological damage as often occurs with encephalitic infections. There are currently no drugs nor vaccines available to treat or prevent the disease. Although the drug ribavirin, a broad spectrum antiviral nucleoside possessing a triazole ring, was initially reported to halt the viral replication, the need to use very high doses of the drug proved too toxic to be clinically useful.

The West Nile virus belongs to the family of viruses called *Flaviviridae* that are small, enveloped, spherical particles of 40 to 50 nm in diameter with single-stranded, positive sense RNA genomes.[8−11] They are known to be the cause of severe encephalitic, hemorrhagic, hepatic, and febrile illnesses in humans. The viral genome encodes a polyprotein of 3000 to 4000 amino acids that is processed by proteases into three structural (C, prM, and E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[10−12] Among these proteins, the NS3 appears to be a promising target for antiviral agents because of the multiple enzymatic activities associated with this protein. NS3 exhibits serine protease, RNA-stimulated nucleoside triphosphatase (NTPase), and RNA helicase activities.[13−15] The catalytic domain of the chymot-

* The correspondence concerning organic synthesis should be addressed to the University of Maryland. Tel: 410-455-2520. Fax: 410-455-1148. E-mail: hosmane@umbc.edu. The correspondence concerning virology should be addressed to the Bernhard-Nocht-Institut. Tel: 040/42 818−458. Fax: 040/42 818−378. E-mail: borowski@bni.uni-hamburg.de.

[†] University of Maryland.

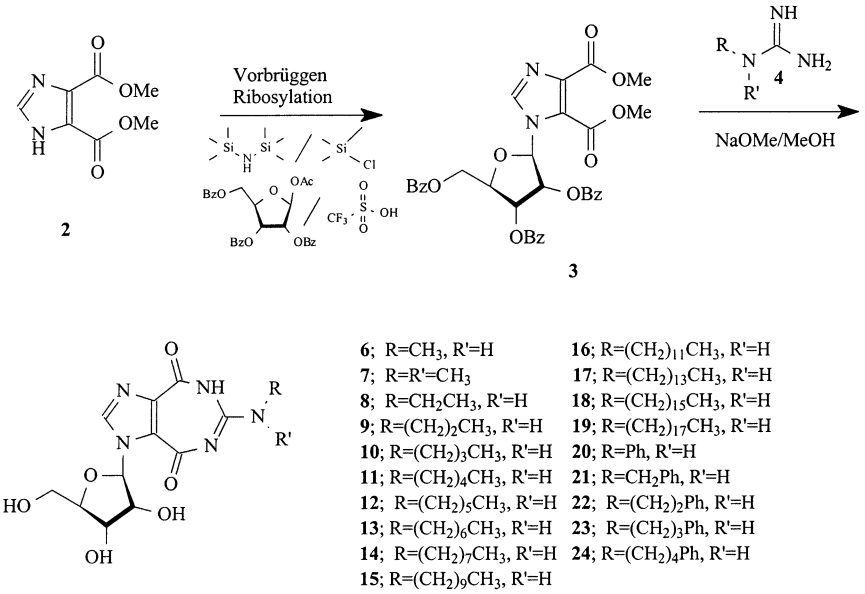
[‡] Bernhard-Nocht-Institut.

[§] University of Warsaw Pawinskiego 5A.

[∥] W. W. Karakawa Microbial Pathogenesis Laboratory.

10.1021/jm030277k CCC: $25.00
Published on Web 09/23/2003

Scheme 1



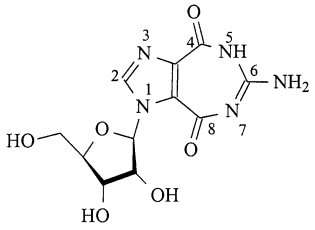
rypsin-like NS3 protease has been mapped to the NH_2-terminus region of the NS3, whereas the NTPase and the helicase activities are associated with the COOH-terminus of NS3.[12,13] Helicases are capable of unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together.[16,17] This unwinding activity is essential for the virus replication. Recently reported "knock out" experiments demonstrated unambiguously that the switch-off of the helicase activity abolishes the virus propagation of bovine diarrhea virus (BVDV) and of dengue fever virus (DENV).[18,19] According to the data, the inhibition of the helicase activity associated with NS3 protein may be an effective tool for reduction of virus replication. In a recent study, we have demonstrated that some imidazo-[4,5-*d*]pyridazine nucleosides act as inhibitors of WNV NTPase/helicase and reduce the unwinding activity of the enzyme with an IC_{50} value in the micromolar range.[20] A comparable inhibitory potency was also observed in tissue cultures of the virus.[20] Furthermore, closely related compounds that did not influence the helicase activity, did not also exert any effect on the virus replication.[20]

Two alternative mechanisms of RNA/DNA unwinding by NTPase/helicase have been postulated.[21−24] Both models predict that the enzymes bind and hydrolyze NTP by a well characterized NTP binding pocket. The energy released is used for the "march" of the enzyme along the DNA or RNA structures and the unwinding reaction results from capturing single strand (ss) regions which arise due to thermal fluctuations at the fork.[16,21] Alternatively, the energy could be transferred to the fork and used for disruption of the hydrogen bonds that keep the strands together.[16,21] Consistent with the proposed models, the following mechanisms of inhibition of the helicase activity could be considered: (a) inhibition of the NTPase activity by interference with NTP binding,[25,26] (b) inhibition of NTPase activity by an allosteric mechanism,[25] (c) inhibition of the coupling of NTP hydrolysis to unwinding reaction,[26] (d) interference in the interaction of helicase with its RNA or DNA substrate via competitive blockade of substrate binding site,[27] and (e) inhibition of unwinding by steric interference in translocation of the enzyme along the polynucleotide chain.[28] There are even more mechanistic possibilities by which the helicase activity could be inhibited.[29,30]

We present here a class of nucleoside analogues, called ring-expanded (REN or "fat") nucleosides, which are found to modulate, i.e., activate or inhibit, the unwinding reaction of WNV NTPase/helicase. Although many of the compounds tested also seem to affect the respective ATPase activity, it nevertheless appears to bear no significant consequence on the overall antihelicase activity.

Chemistry

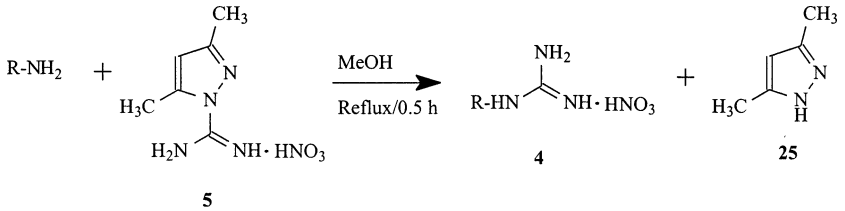
We have recently reported the in vitro antiviral activity of the parent ring-expanded nucleoside, 6-aminoimidazo[4,5-*e*][1,3]diazepine-4,8-dione (**1**), against NT-



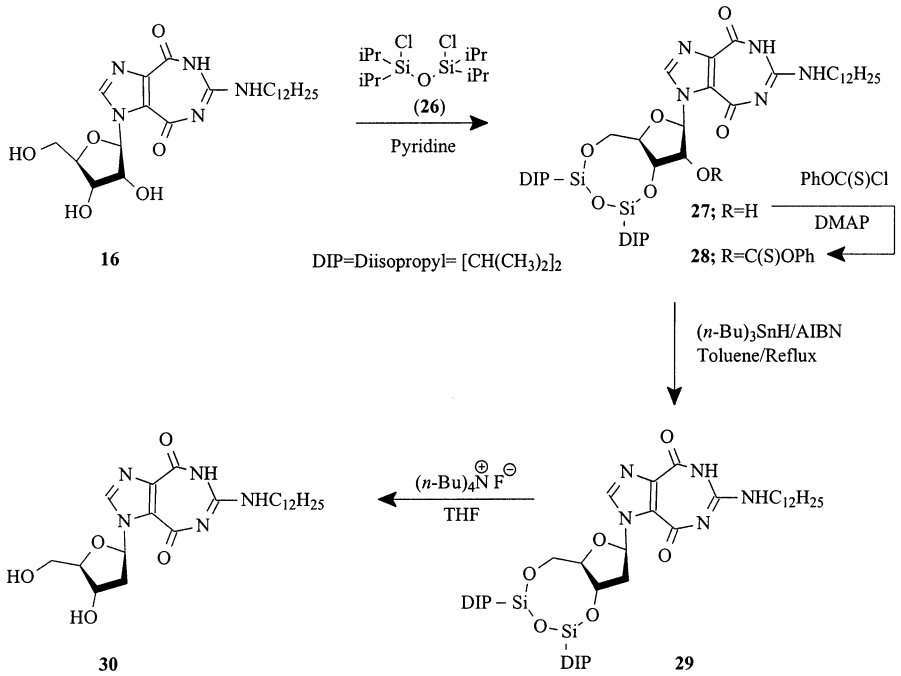
1

Pases/helicases of a family of *Flaviviridae*, including the hepatitis C virus (HCV), the West Nile Virus (WNV), and the Japanese encephalitis virus (JEV).[31] The observed promising activities of **1** prompted us to further explore the structure−activity relationship (SAR) of **1** against each of the above-mentioned viruses. In this particular study, we have focused on the West Nile virus. Two conspicuous loci of **1** that are viable for further structural modifications, but remained largely unexplored thus far, are position 6 and 1, where the amino and the sugar group are attached, respectively. Therefore, in exploring the potential anti-*Flaviviridae* activity of the ring system contained in **1**, we decided

Scheme 2



Scheme 3



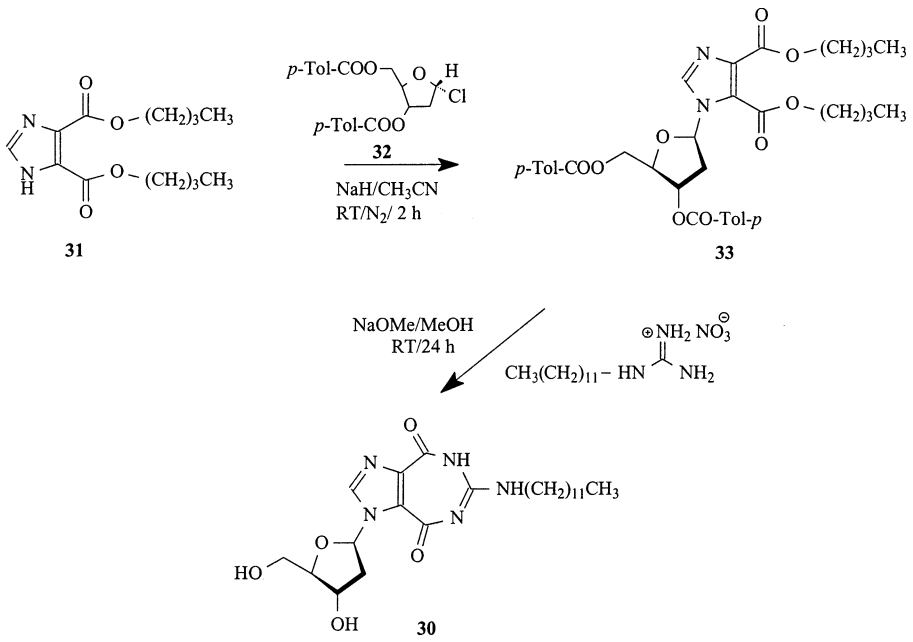
to focus on variation of substituents at position 6, along with variations of sugar moieties at position 1, including the type of sugar (ribo-, 2′-deoxyribo-, or acyclic) as well as its mode of attachment to the base (α or β configuration).

The general method for the synthesis of nucleosides (**6**−**24**) containing the parent structure **1** with different alkyl, aralkyl, and aromatic substituents at position 6 (see Scheme 1) involved Vorbrüggen ribosylation[32,33] of dimethyl imidazole-4,5-dicarboxylate (**2**),[34,35] followed by condensation of the resulting imidazole nucleoside (**3**) with the appropriately substituted guanidine derivatives (**4**). The products were fully characterized by elemental microanalyses, NMR, and mass spectral data. The necessary guanidine derivatives, when not commercially available, were synthesized using the general synthetic procedure[36] outlined in Scheme 2. The procedure consisted of reacting 3,5-dimethylpyrazole-1-carboxamidine nitrate (**5**) with the appropriately substituted amine in methanol at reflux for a brief period. We discovered that the use of methanol, instead of water as reported in the literature, greatly improves both yield and purity, in addition to significantly reducing the reaction time because of the increased solubility of most amines in methanol compared with water. Aromatic amines required more reaction time than aliphatic amines.

The 2′-deoxy analogue (**30**) of nucleoside **16** was synthesized by either (a) direct deoxygenation, employing the Barton deoxygenation procedure,[37,38] as outlined in Scheme 3, or by (b) base-catalyzed glycosylation of an imidazole diester with a pure, crystalline 1-α-chloro-2-deoxy-D-ribofuranosyl-3,5-bis(*p*-toluate), as outlined in Scheme 4. The deoxygenation procedure listed in Schemes 3 consisted of selectively protecting the 3′,5′-positions of the concerned nucleoside using the Markiewicz reagent,[39] 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, to form the corresponding 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) (TIPDS) derivative **27**. Further functionalization at O-2′ with phenyl chlorothionocarbonate (phenoxythiocarbonyl chloride), employing 4-(dimethylamino)pyridine (DMAP) as a catalyst, afforded the respective 3′,5′-*O*-TIPDS-protected 2′-*O*-(phenoxythiocarbonyl) ester **28**. Free radical-mediated Barton deoxygenation[37] with tributylstannane (tributyltin hydride), employing α,α′-azobis(isobutyronitrile) (AIBN) as a radical initiator in toluene at reflux, gave satisfactory conversion into the respective 3′,5′-TIPDS-protected-2′-deoxynucleoside **29**. The silyl deprotection of the latter compound was achieved by treatment with tetra-*n*-butylammonium fluoride, which gave the desired 2′-deoxynucleoside **30**.

The glycosylation procedure outlined in Scheme 4 warranted the use of a highly pure and crystalline sugar, 1-α-chloro-2-deoxy-D-ribofuranosyl-3,5-bis(*p*-toluate) (**32**),[40] strongly basic medium, as well as the appropriate imidazole diester in order to achieve both high yield and high anomeric purity. For example, the standard Vorbrüggen method of glycosylation of the dimethyl ester **2** with the above chloro sugar (**32**),

Scheme 4

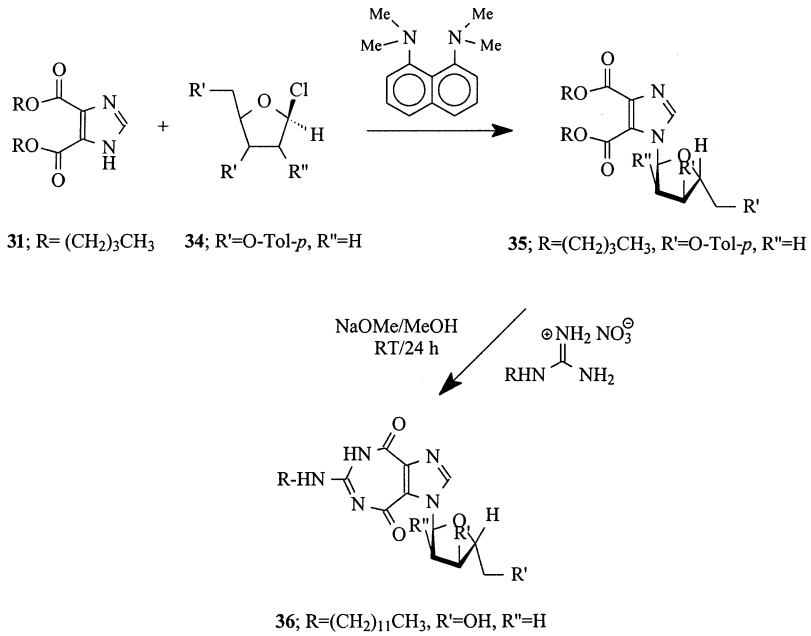


employing 1,1,1,3,3,3-hexamethyldisilazane (HMDS), chlorotrimethylsilane (CTMS) and trimethylsilyl triflate (TMSOTf) catalysis, only yielded a mixture of α and *β* nucleosides with a 2:1 (α:*β*) ratio. Direct base-catalyzed glycosylation without presilylation using a polar solvent such as DMF, acetonitrile, or pyridine only made the matter worse by formation of increasing amounts of the α anomer in the mixture (α:*β* ≥ 3:1). It was discovered that the use of even the purest form of the α-chloro sugar **32** for glycosylation under these conditions yielded a mixture of both α and *β* anomers of the nucleoside product, with predominance of the α-anomer in the mixture. Apparently, the α anomer of the chloro sugar (**32**) undergoes facile anomerization in a polar solvent such as DMF or acetonitrile and is mostly transformed into the thermodynamically more favored *β*-chloro anomer if allowed to stand for a long enough time. Since the displacement of the halide by the nucleobase is believed to proceed by an S_N2 mechanism, the resultant nucleoside product would have the undesired α anomeric configuration. It was, therefore, concluded that in order to form the desired *β*-nucleoside as the major or the exclusive product, the rate of glycosylation has to be significantly faster than the rate of anomerization. Thus, the problem was finally solved by (a) using a strong base such as sodium hydride, instead of bases such as proton sponge or triethylamine, for condensation, (b) portionwise addition of the α-chloro sugar to the suspension of the heterocyclic base and sodium hydride over a prolonged period of a couple of hours instead of just a few minutes, and (c) substitution of the dimethyl ester **2** with its dibutyl analogue **31** for better solubility. Indeed, this experimental modification yielded the desired *β*-anomer **33** in greater than 95% anomeric purity. The final ring-closure of **33** with *N*-dodecylguanidine yielded the target **30**, which was identical in all respects to that obtained by direct deoxygenation of the ribonucleoside **16** as outlined in Scheme 3. The overall yields of **30** by the two methods shown in Schemes 3 and 4 are 53% and 71%, respectively, suggesting that the glycosylation procedure is preferred over the direct deoxygenation of ribonucleoside both in terms of the small number of synthetic steps and the product yield.

The α anomer **36** of nucleoside **30** was synthesized (Scheme 5) by sequential reactions involving condensation of the imidazole diester **31**, in the presence of proton sponge, with the *β*-chloro sugar anomer **34**, generated in situ from the corresponding α-chlorosugar **32**, to obtain the desired α-anomer **35**, followed by ring-closure with *N*-dodecylguanidine. The distinction between α- and *β*-anomers was achieved by ^{1}H NMR and NOESY studies. The major difference between *β*- and α-anomers of the imidazole precursors **33** and **35**, respectively, lay in the absorption of the H-4′ signal which was 0.26 ppm (78 Hz) more downfield in the α-anomer relative to that of the *β*-anomer. Such an observation has been documented with other nucleoside analogues.[41] The major distinction between *β*- and α-anomers of the target RENs **30** and **36**, respectively, lay in the absorption of the imidazole proton H-2, which was 0.27 ppm (81 Hz) upfield in the α-anomer relative to that of the *β*-anomer. However, neither the difference in chemical shifts nor the pattern of coupling of H-1′ or H-2′ that are normally employed to distinguish the two anomers[41,42] were discriminatory enough in case of RENs to allow definitive assignments of their configurations. For example, while the *β*-anomer **30** revealed its H-1′ signal at *δ* 6.62 as a pseudo triplet with $J_{1',2'a}$. $J_{1',2'b}$ = 5.9 Hz, the corresponding α-anomer **36** revealed its H-1′ also at *δ* 6.62 but only as a doublet with J = 6.2 Hz. The α-anomers of 2′-deoxynucleosides normally exhibit their H-1′ as a doublet of doublets with unequal coupling constants, one being considerably larger than the other (e.g., 5−7 Hz vs 1−3 Hz). The fact that only a doublet with a coupling constant of 6.2 Hz was observed in **36** suggests that the second coupling constant must be near or equal to zero. This further implies the presence of a possible "north" (C2′-*exo*-C3′-*endo*) conformation for the sugar pucker of **36**, which would place its axial H-2′ farther away from H-1′, preventing effective coupling between the two protons.

The conclusive configurational assignments were

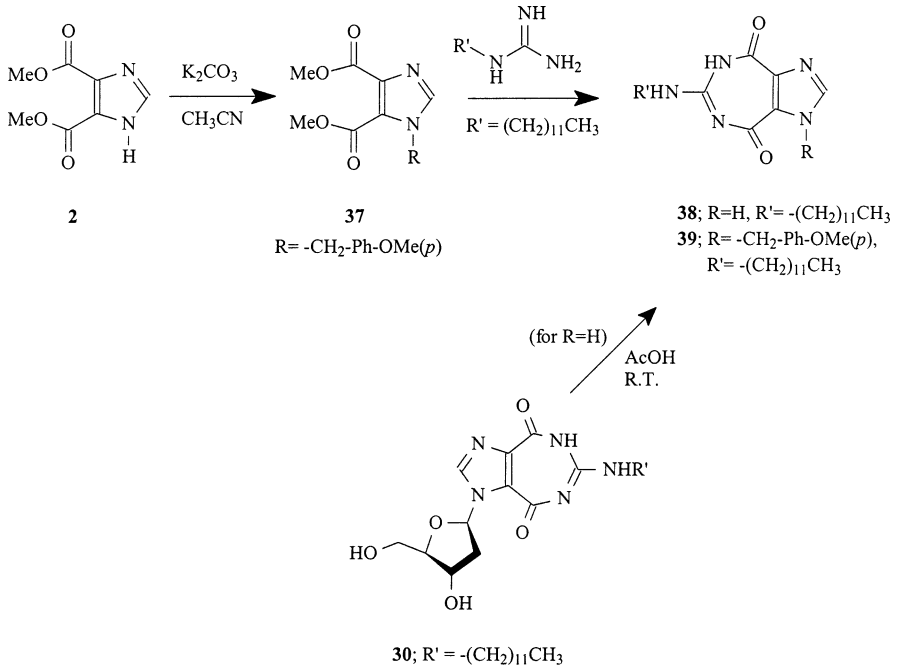
Scheme 5



31; R= $(CH_2)_3CH_3$ **34**; R'=O-Tol-*p*, R"=H **35**; R=$(CH_2)_3CH_3$, R'=O-Tol-*p*, R"=H

36; R=$(CH_2)_{11}CH_3$, R'=OH, R"=H

Scheme 6



2 **37** R= -CH_2-Ph-OMe(*p*) **38**; R=H, R'= -$(CH_2)_{11}CH_3$ **39**; R= -CH_2-Ph-OMe(*p*), R'= -$(CH_2)_{11}CH_3$

30; R' = -$(CH_2)_{11}CH_3$

finally made by two-dimensional nuclear Overhauser and exchange spectroscopy (NOESY). The energy-minimized structures of **30** and **36** by molecular modeling[43] studies clearly indicated the proximity of the imidazole H-2 of **36** with the sugar H-1′ (internuclear distance = 2.52 Å). By contrast, in the β-anomer **30**, the imidazole H-2 was considerably farther away from the H-1′ (distance = 3.79 Å) to exhibit any significant NOE. These predictions were borne out by the observed NOE correlations between H-2 and H-1′ in the α-anomer **36**, whereas no measurable correlation existed between the same set of protons in the corresponding β-anomer **30**.

The parent heterocyclic aglycon **38** (Scheme 6) of nucleoside **16** or **30** was prepared by deglycosylation of **30** by treatment with acetic acid. The 1-aralkyl derivative **39** of **38** was synthesized by condensation of the corresponding imidazole diester **37** with *N*-dodecylguanidine. The imidazole diester **37**, in turn, was prepared by alkylation of **2** with *p*-methoxybenzyl chloride, employing potassium carbonate in acetonitrile.

Results and Discussion

The NTPase/helicase from the West Nile virus was chosen for the intended enzyme inhibition studies by a range of ring-expanded nucleosides/nucleotides. To assess the selectivity of the RENs-mediated inhibition, an enzyme from human source, namely, the NH_2-terminally truncated form of Suv3 protein [Suv3(Δ_{1-159})], was also included in the study. The latter protein, which is commonly being referred to as putative RNA NTPase/helicase to date, displays considerable homologies with ATP- and RNA-binding sites (domains I and II) with

Table 1. Inhibitory (IC_{50})[a] or Activating (ED_{200}) Effect of Ring-Expanded Nucleoside (REN) Analogues against the Helicase Activity of the West Nile Virus (WNV) NTPase/Helicase, Using a DNA Substrate[b]

compound no.	WNV IC_{50} (μg/mL)[a]	Suv3IC_{50} (μg/mL)[a]
6−**14**	300 to >500	ND
15	30−100	>500
16	1−3	>500
17	3−10	300
18	250	>500
19	5.0	>500
20	activator: ED_{200} = 300	>500
21−**24**	>500	ND
30	3−10	3−5
36	3−10	250
38	>500	>500
39	5.0	>500

[a] The term IC_{50} is defined as the concentration of REN required for 50% inhibition of enzyme activity. The term ED_{200} reflects the effective dose of the compound yielding 200% activity. The helicase activity was determined as a function of increasing concentrations of the compounds in the presence of ATP adjusted to the K_m values of 9.5 μM and 4.2 μM for WNV and Suv3$_{(\Delta 1-159)}$ NTPase/helicase, respectively, and 4.7 pM of DNA substrate (concentration of nucleotide base). The substrate and released strand were separated in TBE polyacrylamide gel and visualized by exposition of dried gel onto X-ray film for 20 h. The parts of the gels corresponding to the released strand were excised, and the ^{32}P radioactivity was quantified as described under Biochemical Methods in the Experimental Section. The inhibitory potential of the compounds was expressed as the inhibitor concentration at which 50% of the unwinding activity was measured. The helicase activity of the enzyme measured in the absence of the compounds was referred to as 100%. The results presented are representative of three independent experiments. ND = not determined [b] For the assessment of selectivity, the truncated version of human helicase Suv3$_{(\Delta 1-159)}$ has also been included in the enzyme inhibition studies.

the viral NTPase/helicase.[44] The WNV NTPase/helicase was isolated and purified from the cell culture medium harvested from virus-infected Vero E6 cells according to our own procedure reported previously.[45] The NH_2-terminally truncated version of Suv3 NTPase/helicase was expressed in *E. coli* via PCR amplification of the appropriate human Suv3 cDNA coding for SuV3 protein with truncated 159 amino acids from the amino terminus, followed by cloning into NcoI and BamHI sites of pQE60 expression vector. The homogenity of final enzyme preparations was ascertained by SDS/polyacrylamide gel electrophoresis with Coomassie Blue staining.

The Helicase Reaction. To monitor the inhibitory potential of RENs toward WNV helicase reaction, we prepared the necessary DNA and RNA substrates consisting of two annealed DNA or RNA oligonucleotides. The unwinding activity was assessed by monitoring the release of the shorter labeled strand of the DNA or RNA duplex. Interestingly, the efficacy of unwinding was not affected by the nature of the substrate used. The helicase activity was calibrated with a DNA or RNA substrate that was unwound at an ATP concentration equal to the K_m value determined for the NTPase reaction.

The target compounds of Schemes 1−6 were screened against the helicase activity of WNV, and the results are summarized in Table 1. Although the inhibition constant K_i is the normal parameter used to express the enzyme inhibition, there are several reasons for presenting our results as IC_{50}, the concentration of the inhibitor required for 50% inhibition of the enzyme activity: (a) The inhibition of the helicase activity by RENs results from a mechanism that could only be speculated about. There is probably more than one target for RENs within the substrate−enzyme complex. Moreover, the inhibition appeared to be ATP-independent. Under these conditions one can only roughly and unreliably estimate the K_i. (b) Many of the RENs were previously screened in tissue culture systems, and the results were presented as IC_{50}.[46] To maintain the conformity as well as facile comparison, IC_{50} was considered the preferred unit for expression of activity. (c) The most potent RENs are currently being tested in vivo as antiviral agents, and therefore, it would be more convenient to compare the in vitro and in vivo data using either the IC_{50} or IC_{90} values.

The RENs of Scheme 1, compounds **6**−**24**, contained substituents at position-6, incorporating judicious incremental variations of length and bulk with alkyl or aralkyl groups. The results obtained for **6**−**14** and **21**−**24** were heterologous, with IC_{50} values ranging anywhere from 70 μg/mL to >500 μg/mL, indicating that they were, by and large, inactive by conventional standards. A notable exception, however, occurred with compound **20** containing an N^6-phenyl substituent, which exerted an activating effect on the WNV helicase activity. We have recently reported about a similar observation made with imidazo[4,5-*d*][pyridazine nucleosides.[20] On the other hand, RENs **16**−**19** containing an alkyl chain with 12 or more carbon atoms exhibited IC_{50} values ranging 1−10 μg/mL, with the only exception of **18**. Even more encouraging was the fact that they were all inactive against the human enzyme Suv3 (IC_{50} > 300 μg/mL), suggesting that the compounds are likely to be selective for the viral NTPase/helicase.

The favorable activity/selectivity profile of REN **16** against WNV helicase prompted us to test its 2′-deoxy analogue **30** (see Schemes 3 and 4). REN **30** also exhibited comparable antihelicase activity profile against WNV (IC_{50} 3−10 μg/mL), but unlike **16**, it also inhibited the human enzyme Suv3 with an IC_{50} value of 3−5 μg/mL, suggesting that it would be less discriminatory between viral and human NTPase/helicase.

The above results with **30** led us to explore the role of α/β-configuration at the base−sugar junction, the anomeric carbon, in biological activity. So, we synthesized and screened compound **36** (Scheme 5), which is the α-anomer of **30**. Compound **36**, like its β-anomeric counterpart **30**, exhibited potent and selective antihelicase activity against WNV NTPase/helicase (IC_{50} 3−10 μg/mL)

The varied results obtained with different sugar substitutions and their anomeric configurations left us to be confronted with one important final question concerning the modulation of WNV helicase reaction by RENs. Is the sugar or the sugarlike moiety necessary at all for the antihelicase activity of RENs? To address that question, we synthesized the parent heterocycle of **30** (i.e., **38**) (see Scheme 6). But compound **38** was found to be completely devoid of activity. We then synthesized an analogue of **38** that contained a *p*-methoxybenzyl group attached to the imidazole ring (i.e., **39**) in place of the sugar moiety. Surprisingly, compound **39** turned out to be both potent and selective against the helicase

Table 2. Inhibitory Activity (IC_{50})[a] of Ring-Expanded Nucleoside (REN) Analogues against the Helicase Activity of the West Nile Virus (WNV) NTPase/Helicase, Using an RNA Substrate[b]

compound no.	WNV IC_{50} (μg/mL)	Suv3 IC_{50} (μg/mL)
15	300	500
16	10	150
17	>500	30−100
18	=500	30
19	100	30
20	activator: $ED_{200} = 100$	>500
30	1.5	>500
36	100−200	150
38	>500	>500
39	1−3	>500

[a] The helicase activity was determined as described in the legend to Table 1 and under Biochemical Methods in the Experimental Section with the exception that 4.7 pM RNA duplex (concentration of nucleotide base) was used as a substrate. The term IC_{50} is defined as the concentration of REN required for 50% inhibition of enzyme activity. The results presented are representative of three independent experiments [b] For the assessment of selectivity, the truncated version of human helicase $Suv3_{(\Delta 1-159)}$ has also been included in the enzyme inhibition studies.

activity of WNV NTPase/helicase with an IC_{50} value of 5 μg/mL. These results suggest that a simple aralkyl group in place of a sugar moiety would not only suffice but also may be preferred for an excellent activity/selectivity profile.

Finally, the above biochemical results were all obtained using a DNA substrate. So, it was important to consider if the results would be any different with an RNA substrate. The data obtained with an RNA substrate for a few selected RENs are collected in Table 2. As is evident, the results in some cases were significantly different from those obtained from a DNA substrate. A case in point is REN **30**, which is 2−6 times more potent in inhibiting the WNV helicase activity with an RNA substrate as compared to a DNA substrate. In contrast, compound **16**, the ribose counterpart of **30**, was more active with a DNA substrate rather than an RNA substrate. Furthermore, compound **16** was found to be more selective toward WNV NTPase/helicase in comparison to the human enzyme Suv3 when a DNA substrate was employed, whereas the trend was reversed with **30** which was highly selective toward the viral enzyme only when an RNA substrate was employed.

The above antihelicase activity data raise an important biochemical question about the mechanism of activity of RENs. There appears to be no conspicuous or single trend in activity. As a matter of fact, considerable differences exist in activity between the ribose and deoxyribose sugar analogues, between analogues containing α and β-anomeric configurations, between those with or without substitutions at the imidazole ring, and between those with different length, bulk, and the type of substituents on the heterocyclic ring. Although speculation on the exact mechanism of action of RENs is not possible without further extensive studies, one likely mechanism involves their potential interactions with the DNA or RNA substrate of WNV helicase. This is based on a number of documented reports demonstrating the noncovalent interactions of analogues of nucleobases, nucleosides, and nucleotides,[47,48] in particular as binders to major or minor grooves of duplex DNA or RNA. In light of their structural similarity to purine bases, it is reasonable to consider an analogous mechanism for the RENs as well. It is also well established that the groove binding is greatly dependent and highly sensitive to the mode of substituents on the binder as well as the bounded. Thus, various RENs with different substitutions or topological structural features are expected to differently modulate their interactions with a DNA or an RNA substrate. Different modulations could potentially result in either the more stable or the less stable double-helix, thus accounting for the observed inhibitory or activating effect of different RENs on the unwinding activity of viral helicase. Corroborating the notion of possible DNA and/or RNA binding of RENs is our qualitative observation that compound **30** forms a tight complex with a DNA substrate that was completely stable in the presence of 0.5% sodium dodecyl sulfate (SDS). Although rigorous investigation on this subject is yet to be carried out, it was unmistakable to notice that the migration of the DNA substrate in the TBE-polyacrylamide gel electrophoresis was severely hindered in the presence of **30** whether in the presence or absence of the enzyme.

The NTPase Reaction. We then focused our attention on the NTPase reaction mediated by the viral enzymes. Our goal in this regard was 2-fold: (a) To investigate if the compounds of Table 1 inhibit the viral NTPase reaction as well, and (b) if they do, is the inhibition of the NTPase reaction coupled to the observed inhibition of the unwinding (helicase) reaction?

The viral NTPase activity was assessed by a standard assay that determines the amount of ^{33}Pi released from a [γ-^{33}P] ATP during the enzyme-mediated hydrolysis. The ATPase activity was measured as a function of increasing concentrations of the RENs investigated under the helicase tests described above. However, when investigated at ATP concentrations equal to the K_m values determined for the ATPase reaction of each of the viral NTPase/helicase [9.5 μM for WNV and 4.2 μM for Suv3 (Δ_{1-159})], none of the compounds inhibited the hydrolytic activity of the enzymes even up to the concentration of 0.5 mM. The successive lowering of concentration down to values corresponding to 1×10^{-5} of K_m of each enzyme also did not lead to any significant inhibition of the ATPase activity.

Interestingly, at concentrations >500 μM of RENs and $>10 \times K_m$ of ATP, an activation of the ATPase activity was observed. We had earlier made a similar observation on the activating effect of ATPase activity with ribavirin,[26,45] a nucleoside containing a 1,2,4-triazole ring with a carboxamide side chain, which is known to interact with nucleotide-binding sites of various enzymes.[49] The well-documented ATP binding studies of Porter et al.[29,30] suggest strongly the existence of a second nucleoside binding site within WNV NTPase/helicase. The first binding site is known to be equipped with Walker motifs [the short amino acid sequences interacting with β- and γ-phosphorus atoms of nucleoside-5′-triphosphate (Walker A), and with Mg^{2+} or Mn^{2+} that are chelated by the β- and γ-phosphorus atoms (Walker B)] and is known to be involved in the hydrolysis of NTPs. The speculation is that this site possesses significantly higher affinity for nucleoside-5′-triphosphates as compared to the second binding site, which has a much lower affinity and selectivity, and could be

occupied by a nucleotide, nucleoside, or even an aglycon of nucleoside. This second binding site fulfills a rather regulatory function with respect to the ATPase and/or helicase activity.

The above REN-mediated inhibition and/or activation results, obtained separately against the helicase and ATPase activities of WNV, prompt us to corroborate the speculation about the existence of an allosteric binding site on WNV NTPase/helicase where nucleoside/nucleotide-type molecules bind. Occupation of this site is largely responsible for modulation of WNV NTPase and/or helicase. It remains to be resolved as to whether this is a common site for both helicase and NTPase or whether there are two independent allosteric sites for the two enzymes. Most of the RENs that inhibit the viral helicase activity seem to exert their effect by binding to the major or minor groove of DNA or RNA substrate of helicase. However, at high concentrations of ATP, they seem to occupy the allosteric site and cause the enzyme activation instead. The presence of this allosteric binding site for RENs might also explain the seemingly uncoupled NTPase and helicase activities of the viral NTPase/helicase.

Conclusion

Ring-expanded nucleosides modulate the activities of the West Nile virus. The specific modulation explored in this study concerns the helicase and NTPase activities of the virus. The type of modulation exerted by RENs can either be an inhibition or activation. The mechanism of action of most of the ring-expanded ribo-, 2′-deoxyribo-, and α- and β-anomeric nucleoside analogues, as well as their heterocyclic aglycons, involves inhibition or activation of the viral helicase activity. In the case of inhibition, the mechanism may involve the interaction of RENs with a DNA or an RNA substrate of the helicase through binding to the major or minor groove of the double-helix. In the case of activation, the mechanism may involve an allosteric binding site that can be occupied by nucleoside/nucleotide-type molecules including, but not limited to RENs. The occupation of this allosteric site is dependent upon the high level of ATP (NTP) concentration in the reaction mixture. Many of the RENs tested were remarkably selective for the viral NTPase/helicase over the truncated human form of the enzyme Suv3, suggesting that the compounds may not be toxic to the host. In that regard, RENs may represent a starting point for the development of highly selective inhibitors of WNV NTPase/helicase.

Experimental Section

(A) Chemical Methods. The ^{1}H and ^{13}C NMR spectra were recorded on a General Electric QE-300 NMR spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The chemical shift data are reported with reference to Me_4Si (internal standard) for ^{1}H and ^{13}C NMR. The data are reported in the following format: Chemical shift, multiplicity (s = singlet, d = doublet, dt = double triplet, dd = double doublet, t = triplet, q = quartet, m = multiplet, br = broad, coupling constants, integration, and assignment). Elemental Microanalyses were performed by Atlantic Microlab, Inc., Norcross, GA. The mass spectra were recorded at the Mass Spectral Facility, Department of Biochemistry, Michigan State University. Thin-layer chromatography was performed on Merck Kieselgel 60 GF_{254} plates (0.2 mm thickness). Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Dry solvents were prepared as follows: Methanol was distilled from calcium hydride and was stored over molecular sieves (type 3 Å); methylene chloride was distilled from calcium hydride and was stored over molecular sieves (type 3 Å); dimethylformamide was dried over calcium oxide and then distilled under reduced pressure from calcium hydride and was subsequently stored over molecular sieves (type 3 Å). Starting materials were purchased from either Aldrich Chemical Co. or Lancaster. All solvents were reagent grade and were purchased from VWR Scientific. All yields reported are for dry compounds that require no further purification for use in other reactions.

General Method for the Synthesis of (Commercially Unavailable) Substituted Guanidines. A mixture of appropriate amine (4 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (**5**)[36] (0.830 g, 4 mmol) was refluxed in methanol (15 mL) for 2 h. The solution was rotary evaporated to dryness, and the residue was dried over P_2O_5 in vacuo. It was directly employed in the condensation reaction with **3** without further purification.

General Method for the Synthesis of Nucleosides 6−24. Hydrochloride, sulfate, or nitrate salt of guanidine (4 mmol) was added to 4 mL of 2.3 M sodium methoxide solution resulting from sodium (0.75 g) dissolved in 15 mL of absolute methanol. The mixture was stirred in an ice bath for 30 min. The precipitated sodium chloride was removed by filtration, and the filtrate was poured into a methanolic solution (20 mL) of methyl 1-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)imidazole-4,5-dicarboxylate (**3**)[50] (1 mmol), prepared by Vorbrüggen ribosylation[32,33] of imidazole-4,5-dicarboxylate.[34,35] The mixture was stirred at room temperature for 24−48 h and was monitored by frequent TLC analysis to check for the completion of reaction. The reaction mixture was filtered if necessary, and the clear filtrate was neutralized with 2 M hydrochloric acid. The resulting precipitate, if formed, was filtered and washed with water and methanol to give product. In cases where no precipitate formed, the solution was extracted with an appropriate organic solvent and the combined extracts were dried and evaporated to obtain the product. The latter was either recrystallized from an appropriate solvent or purified by flash chromatography on a silica gel column. The spectral and analytical data, along with solvent of recrystallization and/or solvent of elution for chromatography are collected here below.

4,5-Dihydro-8*H*-6-(*N*-methylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (6). Yield 93%, R_f = 0.09 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], mp 210 °C (dec); ^{1}H NMR (DMSO-d_6): δ 10.63 (brs, 1H, NH, exchangeable with D_2O), 8.54 (s, 1H, imidazole), 7.01 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.6 Hz, 1H, 1′-H), 5.47 (d, J = 5.1 Hz, 1H, OH, exchangeable with D_2O), 5.16 (t, J = 4.9 Hz, 1H, OH, exchangeable with D_2O), 5.07 (d, J = 5.5 Hz, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dd, J_1 = 12.1 Hz, J_2 = 4.0 Hz, 1H, 5′-H_1), 3.58 (dd, J_1 = 11.7 Hz, J_2 = 4.0 Hz, 1H, 5′-H_2), 2.75 (d, J = 4.0 Hz, 3H, CH_3). Anal. ($C_{12}H_{15}N_5O_6 \cdot H_2O$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*,*N*-dimethylamino)-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (7). Yield 44%, R_f = 0.17 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (5:1), and methanol, mp 215 °C; ^{1}H NMR (DMSO-d_6): δ 8.27 (s, 1H, imidazole), 6.47 (d, J = 3.3 Hz, 1H, 1′-H), 5.81 (d, J = 4.0 Hz, 1H, OH, exchangeable with D_2O), 5.17 (t, J = 4.9 Hz, 1H, OH, exchangeable with D_2O), 5.07 (d, J = 4.4 Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′-H), 3.91 (m, 1H, 4′-H), 3.76 (brs, 1H, NH, exchangeable with D_2O), 3.70 (td, J_1 = 12.6 Hz, J_2 = 3.8 Hz, 1H, 5′-H1), 3.58 (td, J_1 = 12.3 Hz, J_2 = 3.8 Hz, 1H, 5′-H_2), 3.02 (s, 6H, 2 CH_3). Anal. ($C_{13}H_{17}N_5O_6$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-ethyl)amino-1-(β-D-ribofuranosyl)-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (8). Yield 90%, R_f = 0.13 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol

(10:1), and methanol−water (4:1), mp 219 °C (dec); ^{1}H NMR (DMSO-d_6): δ 10.48 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.07 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.9 Hz, 1H, 1′-H), 5.50 (d, J = 4.8 Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, J = 4.8 Hz, 1H, OH, exchangeable with D_2O), 5.08 (d, J = 5.1 Hz, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dd, J_1 = 11.7 Hz, J_2 = 4.4 Hz, 1H, 5′-H$_1$), 3.58 (dd, J_1 = 11.9 Hz, J_2 = 4.2 Hz, 1H, 5′-H$_2$), 3.24 (m, 2H, CH_2); 1.09 (t, J = 7.1 Hz, 3H, CH_3). Anal. ($C_{13}H_{17}N_5O_6$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-propyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (9). Yield 47%, R_f = 0.25 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methanol (5:1), and methanol, mp 180 °C (dec); ^{1}H NMR (DMSO-d_6): δ 10.53 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.17 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.6 Hz, 1H, 1′-H), 5.50 (s, 1H, OH, exchangeable with D_2O), 5.18 (s, 1H, OH, exchangeable with D_2O), 5.08 (s, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (d, J = 10.6 Hz, 1H, 5′-H$_1$), 3.58 (d, J = 11.7 Hz, 1H, 5′-H$_2$), 3.17 (m, 2H, CH_2) 1.48 (m, 2H, CH_2), 0.88 (t, J = 7.3 Hz, 3H, CH_3). Anal. ($C_{14}H_{19}N_5O_6$•H_2O). C, H, N.

4,5-Dihydro-8*H*-6-(*N*-butyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (10). Yield 71%, R_f = 0.29 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methanol (5:1), and methanol, mp >250 °C; ^{1}H NMR (DMSO-d_6): δ 10.43 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.33 (d, J = 1.5 Hz, 1H, 1′-H), 5.49 (s, 1H, OH, exchangeable with D_2O), 5.18 (s, 1H, OH, exchangeable with D_2O), 5.07 (s, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′-H), 3.90 (m, 1H, 4′-H), 3.71 (m, 1H, 5′-H$_1$), 3.60 (m, 1H, 5′-H$_2$), 3.20 (m, 2H, CH_2) 1.44 (m, 2H, CH_2), 1.27 (m, 2H, CH_2), 0.85 (t, J = 6.6 Hz, 3H, CH_3). Anal. ($C_{15}H_{21}N_5O_6$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-pentyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (11). Yield 51%, R_f = 0.33 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methanol (5:1), and methanol, mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 10.44 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.07 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.6 Hz,1H, 1′-H), 5.49 (d, J = 4.8 Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, J = 4.9 Hz, 1H, OH, exchangeable with D_2O), 5.08 (d, J = 5.5 Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (ddd, J_1 = 12.0 Hz, J_2 = 4.9 Hz, J_3 = 3.0 Hz, 1H, 5′-H$_1$), 3.58 (ddd, J_1 = 12.2 Hz, J_2 = 4.5 Hz, J_3 = 3.2 Hz, 1H, 5′-H$_2$), 3.21 (m, 2H, CH_2) 1.48 (m, 2H, CH_2), 1.27 (m, 4H, CH_2CH_2) 0.86 (t, J = 6.8 Hz, 3H, CH_3). Anal. ($C_{16}H_{23}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(N-hexyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3] diazepine-4,8-dione (12). Yield: 36%, R_f = 0.35 [Chloroform:Methanol :Ammonium hydroxide (2:1:0.3)]; mp > 250 °C; ^{1}H NMR (DMSO-d_6): δ 10.47 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.2 Hz, 1H, 1′-H), 5.50 (d, J = 5.1 Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, J = 4.4 Hz, 1H, 5′-OH, exchangeable with D_2O), 5.08 (d, J = 4.4 Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dd, J_1 = 11.2 Hz, J_2 = 3.9 Hz, 1H, 5′-H$_1$), 3.57 (dd, J_1 = 12.1 Hz, J_2 = 3.3 Hz, 1H, 5′-H$_2$), 3.19 (m, 2H, −CH_2- group), 1.43 (m, 2H, −CH_2- group), 1.22 (m, 6H, -$(CH_2)_3$- chain), 0.86 (t, J = 5.9 Hz, 3H, CH_3- group). Anal. ($C_{17}H_{25}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-heptyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (13). Yield 36%, R_f = 0.36 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methanol (5:1), and methanol, mp 227 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.46 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 3.3 Hz, 1H, 1′-H), 5.47 (m, 1H, OH, exchangeable with D_2O), 5.16 (m, 1H, OH, exchangeable with D_2O), 5.08 (m, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.91 (m, 1H, 4′-H), 3.72 (m, 1H, 5′-H$_1$), 3.60 (m, 1H, 5′-H$_2$), 3.22 (m, 2H, CH_2) 1.47 (m, 2H, CH_2), 1.26 (m, 8H, $CH_2CH_2CH_2CH_2$) 0.85 (s, 3H, CH_3). Anal. ($C_{18}H_{27}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-octyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (14). Yield 29%, R_f = 0.37 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp 187 °C (dec); ^{1}H NMR (DMSO- d_6): δ 10.49 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.12 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 1.8 Hz, 1H, 1′-H), 5.49 (m, 1H, OH, exchangeable with D_2O), 5.18 (m, 1H, OH, exchangeable with D_2O), 5.08 (m, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (d, J = 12.5 Hz, 1H, 5′-H$_1$), 3.58 (d, J = 12.1 Hz, 1H, 5′-H$_2$), 3.21-(m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.24 (m,10H, $(CH_2)_5$), 0.84 (t, J = 6.4 Hz, 3H, CH_3). Anal. ($C_{19}H_{29}N_5O_6$•2/3H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-decyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (15). Yield 45%, R_f = 0.38 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), and methanol, mp 185 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.45 (brs, 1H, NH, exchangeable with D_2O), 8.54 (s, 1H, imidazole), 7.15 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.2 Hz, 1H, 1′-H), 5.50 (m, 1H, OH, exchangeable with D_2O), 5.18 (m, 1H, OH, exchangeable with D_2O), 5.08 (m, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (d, J = 11.7 Hz, 1H, 5′-H$_1$), 3.58 (d, J = 12.5 Hz, 1H, 5′-H$_2$), 3.20 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.22 (m, 14H, $(CH_2)_7$), 0.83 (t, J = 6.6 Hz, 3H, CH_3). Anal. ($C_{21}H_{33}N_5O_6$•H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (16). Yield 89%, R_f = 0.41 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), and methanol, mp 194 °C (dec); ^{1}H NMR (DMSO-d_6): δ 10.33 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.2 Hz, 1H, 1′-H), 5.50 (d, J = 3.1 Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, J = 4.8 Hz, 1H, 5′-OH, exchangeable with D_2O), 5.08 (d, J = 3.7 Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dd, J_1 = 8.4 Hz, J_2 = 4.0 Hz, 1H, 5′-H$_1$), 3.58 (dd, J_1 = 9.0 Hz, J_2 = 4.3 Hz, 1H, 5′-H$_2$), 3.20 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.22 (m,18H,$(CH_2)_9$), 0.83 (t, J = 6.2 Hz, 3H, CH_3). Anal. ($C_{23}H_{37}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-tetradecyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (17). Yield 75%, R_f = 0.42 [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), and methanol, mp 185 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.38 (brs, 1H, NH, exchangeable with D_2O), 8.54 (s, 1H, imidazole), 7.07 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, J = 2.2 Hz, 1H, 1′-H), 5.49 (d, J = 4.4 Hz, 1H, OH, exchangeable with D_2O), 5.17 (t, J = 4.9 Hz, 1H, 5′-OH, exchangeable with D_2O), 5.07 (d, J = 4.8 Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dt, J_1 = 12.1 Hz, J_2 = 3.4 Hz, 1H, 5′-H$_1$), 3.58 (dt, J_1 = 12.2 Hz, J_2 = 3.8 Hz, 1H, 5′-H$_2$), 3.20 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.22 (m, 22H, $(CH_2)_{11}$), 0.84 (t, J = 6.6 Hz, 3H, CH_3). Anal. ($C_{25}H_{41}N_5O_6$•0.75H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-hexadecyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (18). Yield 44%, $R_f = 0.45$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), and methanol, mp 191 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.41 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.07 (brs, 1H, NH, exchangeable with D_2O), 6.34 (d, $J = 2.6$ Hz, 1H, 1′-H), 5.49 (d, $J = 4.8$ Hz, 1H, OH, exchangeable with D_2O), 5.17 (t, $J = 4.9$ Hz, 1H, 5′-OH, exchangeable with D_2O), 5.07 (d, $J = 5.1$ Hz, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dt, $J_1 = 12.1$ Hz, $J_2 = 4.2$ Hz, 1H, 5′-H_1), 3.58 (dt, $J_1 = 12.8$ Hz, $J_2 = 3.9$ Hz, 1H, 5′-H_2), 3.20 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.23 (m, 26H, $(CH_2)_{13}$), 0.84 (t, $J = 6.6$ Hz, 3H, CH_3). Anal. ($C_{27}H_{45}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-octadecyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (19). Yield: 53%, $R_f = 0.50$ [chloroform:methanol:ammonium hydroxide (2:1: 0.3), mp 149 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.42 (brs, 1H, NH, exchangeable with D_2O), 8.56 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, $J = 2.6$ Hz, 1H, 1′-H), 5.49 (d, $J = 4.0$ Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, $J = 4.9$ Hz, 1H, 5′-OH, exchangeable with D_2O), 5.08 (d, $J = 4.8$ Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.71 (dt, $J_1 = 11.9$ Hz, $J_2 = 3.8$ Hz, 1H, 5′-H_1), 3.58 (dt, $J_1 = 12.3$ Hz, $J_2 = 3.7$ Hz, 1H, 5′-H_2), 3.20 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.22 (m, 26H, $(CH_2)_{15}$), 0.83 (t, $J = 6.6$ Hz, 3H, CH_3). Anal. ($C_{29}H_{49}N_5O_6$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-phenyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (20). Yield 42%, $R_f = 0.19$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp 201 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.58 (brs, 1H, NH, exchangeable with D_2O), 9.21 (brs, 1H, NH, exchangeable with D_2O), 8.62 (s, 1H, imidazole), 7.58 (t, $J = 7.7$ Hz, 2H, Phenyl), 7.35 (t, $J = 7.7$ Hz, 2H, Phenyl), 7.12 (t, $J = 7.0$ Hz, 1H, Phenyl), 6.32 (d, $J = 2.9$ Hz, 1H, 1′-H), 5.54 (d, $J = 5.1$ Hz, 1H, OH, exchangeable with D_2O), 5.19 (t, $J = 4.9$ Hz, 1H, 5′-OH, exchangeable with D_2O), 5.09 (d, $J = 5.5$ Hz, 1H, OH, exchangeable with D_2O), 4.08 (m, 2H, 2′- and 3′- H), 3.92 (m, 1H, 4′-H), 3.74 (dd, $J_1 = 9.2$ Hz, $J_2 = 2.9$ Hz, 1H, 5′-H_1), 3.59 (dd, $J_1 = 9.2$ Hz, $J_2 = 2.9$ Hz, 1H, 5′-H_2). Anal. ($C_{17}H_{17}N_5O_6$•1.25H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-benzyl)amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (21). Yield 44%, $R_f = 0.22$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 10.60 (brs, 1H, NH, exchangeable with D_2O), 8.58 (s, 1H, imidazole), 7.47 (brs, 1H, NH, exchangeable with D_2O), 7.33 (m, 5H, Phenyl), 6.36 (d, $J = 2.2$ Hz, 1H, 1′-H), 5.50 (s, 1H, OH, exchangeable with D_2O), 5.19 (m, 1H, OH, exchangeable with D_2O), 5.08 (m, 1H, OH, exchangeable with D_2O), 4.47 (m, 2H, CH_2), 4.06 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.73 (dd, $J_1 = 21.1$ Hz, $J_2 = 8.3$ Hz, 1H, 5′- H_1), 3.58 (dd, $J_1 = 18.1$ Hz, $J_2 = 6.1$ Hz, 1H, 5′-H_2). Anal. ($C_{18}H_{19}N_5O_6$•0.5H_2O). C, H, N.

4,5-Dihydro-8*H*-6-(*N*-(2-phenylethyl))amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (22). Yield 34%, $R_f = 0.24$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp >250 °C; ^{1}H NMR (DMSO- d_6) δ 10.57 (brs, 1H, NH, exchangeable with D_2O), 8.56 (s, 1H, imidazole), 7.30 (m, 5H, Phenyl), 7.11 (brs, 1H, NH, exchangeable with D_2O), 6.36 (d, $J = 2.6$ Hz, 1H, 1′-H), 5.51 (d, $J = 4.0$ Hz, 1H, OH, exchangeable with D_2O), 5.19 (m, 1H, OH, exchangeable with D_2O), 5.08 (d, $J = 3.3$ Hz, 1H, OH, exchangeable with D_2O), 4.06 (m, 2H, 2′- and 3′- H), 3.91 (m, 1H, 4′-H), 3.73 (dd, $J_1 = 12.8$ Hz, $J_2 = 2.6$ Hz, 1H, 5′-H_1), 3.58 (dd, $J_1 = 12.6$ Hz, $J_2 = 2.8$ Hz, 1H, 5′-H_2), 3.47 (m, 2H, CH_2), 2.76 (m, 2H, CH_2). Anal. ($C_{19}H_{21}N_5O_6$•2H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-(3-phenylpropyl))amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (23). Yield 28%, $R_f = 0.27$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp 178 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.35 (brs, 1H, NH, exchangeable with D2O), 8.54 (s, 1H, imidazole), 7.22 (m, 6H, Phenyl + NH, exchangeable with D_2O), 6.36 (d, $J = 2.3$ Hz, 1H, 1′-H), 5.51 (d, $J = 2.2$ Hz, 1H, OH, exchangeable with D_2O), 5.18 (t, $J = 6.2$ Hz, 1H, 5′-OH, exchangeable with D_2O), 5.09 (d, $J = 3.7$ Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′- H), 3.90 (m, 1H, 4′-H), 3.72 (dd, $J_1 = 10.9$ Hz, $J_2 = 2.4$ Hz, 1H, 5′-H_1), 3.57 (d, $J_1 = 12.1$ Hz, $J_2 = 2.6$ Hz, 1H, 5′-H_2), 3.22 (m, 2H, CH_2), 2.62 (m, 2H, CH_2), 1.79 (m, 2H, CH_2). Anal. ($C_{20}H_{23}N_5O_6$•0.5H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-(4-phenylbutyl))amino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (24). Yield 46%, $R_f = 0.29$ [chloroform:methanol:ammonium hydroxide (2:1:0.3)], purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform−methanol (10:1), chloroform−methano (5:1), and methanol, mp 178 °C (dec); ^{1}H NMR (DMSO-d_6) δ 10.45 (brs, 1H, NH, exchangeable with D_2O), 8.55 (s, 1H, imidazole), 7.20 (m, 6H, Ph + NH exchangeable with D_2O), 6.36 (d, $J = 1.5$ Hz, 1H, 1′-H), 5.49 (s, 1H, OH, exchangeable with D_2O), 5.18 (t, $J = 2.9$ Hz, 1H, OH, exchangeable with D_2O), 5.08 (d, $J = 4.4$ Hz, 1H, OH, exchangeable with D_2O), 4.05 (m, 2H, 2′- and 3′-H), 3.91 (m, 1H, 4′-H), 3.71 (m, 1H, 5′-H_1), 3.58 (d, $J = 10.6$ Hz, 1H, 5′-H_2), 3.23 (m, 2H, CH_2), 2.58 (m, 2H, CH_2), 1.53 (m, 4H, CH_2CH_2). Anal. ($C_{21}H_{25}N_5O_6$•H_2O) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-[(3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl))-β-D-ribofuranosyl]imidazo[4,5-*e*][1,3]diazepine-4,8-dione (27). Nucleoside **17** (208 mg, 0.43 mmol) dissolved in dry pyridine (5 mL), and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (0.14 mL, 0.45 mmol) was added. After stirring overnight at room temperature, a TLC analysis showed the completion of reaction. The reaction mixture was rotary evaporated to dryness, and the residue was washed with methanol and dried under vacuum to obtain **27** as a solid (91% yield), $R_f = 0.16$ [chloroform:methanol (30:1)], mp 200−202 °C; ^{1}H NMR (DMSO-d_6): δ 10.39 (brs, 1H, NH, exchangeable with D_2O), 8.22 (s, 1H, imidazole), 7.06 (brs, 1H, NH, exchangeable with D_2O), 6.27 (s, 1H, 1′-H), 5.79 (d, $J = 4.8$ Hz, 1H, 2′-OH, exchangeable with D_2O), 4.21 (d, $J = 13.6$ Hz, 2H, 5′-H), 4.10 (m, 2H, 3′-H, 4′-H), 3.93 (d, $J = 12.8$ Hz, 1H, 2′-H), 3.21 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 1.22 (s, 10H, 5 CH_2) 1.00 (m, 28H, 4 $(CH_3)_2CH$), 0.83 (t, $J = 6.6$ Hz, 3H, CH_3). Anal. ($C_{35}H_{63}N_5O_7Si_2$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-[(2′-*O*-phenoxythiocarbonyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl))-β-D-ribofuranosyl]imidazo[4,5-*e*][1,3]diazepine-4,8-dione (28). A mixture of **27** (177 mg, 0.25 mmol) and DMAP (62 mg, 0.51 mmol) in dry acetonitrile (4 mL) was stirred for 30 min in an ice-bath. Phenoxythiocarbonyl chloride (51μL, 0.37 mmol) was then added dropwise to the reaction mixture. The mixture was stirred at ambient temperature for 19 h. The reaction mixture was directly loaded on to a silica gel column, and the column was eluted successively with chloroform and chloroform: methanol (30:1). The appropriate UV-absorbing fractions were pooled and evaporated to give **28** as a colorless syrup (208 mg, 99%), $R_f = 0.28$ [chloroform: methanol (30:1). The syrup was dried over KOH pellets in a vacuum oven overnight. ^{1}H NMR ($CDCl_3$) δ 10.69 (brs, 1H, NH, exchangeable with D_2O), 8.35 (s, 1H, imidazole), 8.16 (brs, 1H, NH, exchangeable with D_2O), 7.35 (m, 5H, phenoxy), 6.21 (d, $J = 4.4$ Hz, 1H, 1′-H), 4.67 (m, 1H, 2′-H), 4.33 (m, 2H, 3′-H, 4′-H), 4.22 (dd, $J_1 = 9.5$ Hz, $J_2 = 1.5$ Hz, 1H, 5′-H_1), 4.08 (dd, $J_1 = 13.9$ Hz, $J_2 = 2.2$ Hz 1H, 5′-H_2) 3.41 (m, 2H, CH_2),

1.59 (m, 2H, CH_2), 1.24 (s, 18H, $(CH_2)_9$) 1.07 (m, 28H, 4 $(CH_3)_2$-CH), 0.87 (t, J = 6.6 Hz, 3H, CH_3). Anal. ($C_{42}H_{67}N_5O_8SSi_2$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-[(2′-deoxy-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl))-β-D-erythropentofuranosyl]imidazo[4,5-*e*][1,3]diazepine-4,8-dione (29). A mixture of **28** (331 mg, 0.39 mmol) and AIBN (15 mg) in dry toluene (30 mL) was purged with argon for 30 min. Tributyltin hydride (0.30 mL, 1.08 mmol) was added quickly to the reaction flask. It was refluxed for 24 h under argon protection. The solvent was evaporated and the pure product was obtained as a colorless liquid by silica gel column chromatography, eluting with a mixture of chloroform−methanol (60:1). The product was dried over KOH pellets in a vacuum oven overnight. Yield 209 mg (77%), R_f = 0.26 [chloroform: methanol (30:1)]; ^{1}H NMR ($CDCl_3$): δ 10.74 (brs, 1H, NH, exchangeable with D_2O), 8.31 (s, 1H, imidazole), 8.15 (brs, 1H, NH, exchangeable with D_2O), 6.71 (d, J = 7.0 Hz, 1H, 1′-H), 4.52 (m, 1H, 4′-H), 4.21 (d, J = 13.2 Hz, 1H, 5′-H_1), 4.06 (dd, J_1 = 13.2 Hz, J_2 = 2.6 Hz, 1H, 5′-H_2), 3.85 (d, J = 8.4 Hz, 1H, 3′-H), 3.44 (m, 2H, CH_2), 2.78 (m, 1H, 2′-H_1), 2.44 (dd, J_1 = 13.2 Hz, J_2 = 7.0 Hz, 1H, 2′-H_2), 1.60 (m, 2H, CH_2), 1.23 (m, 18H, $(CH_2)_9$) 1.02 (m, 28H, 4 $(CH_3)_2$CH), 0.88 (t, J = 6.6 Hz, 3H, CH_3). Anal. ($C_{35}H_{63}N_5O_6Si_2$•0.75CH_3OH) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-[(2′-deoxy-β-D-erythropentofuranosyl] imidazo[4,5-*e*][1,3]diazepine-4,8-dione (30). ***Method A:*** Compound **29** (0.133 g, 0.19 mmol) was dissolved in anhydrous THF (3 mL) and cooled to 0 °C. TBAF in THF (1 *M*, 0.38 mL) was added dropwise to the solution. After 40 min, the starting material disappeared and a new spot corresponding to the deoxynucleoside appeared as monitored by TLC. Methanol (2 mL) was added to the reaction mixture and was stirred overnight. Pure **30** (72 mg, 77%) was obtained by flash chromatography over silica gel by successive elutions with a mixture of chloroform−methanol, 30:1, chloroform−methanol, 10:1 and methanol, mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 10.50 (brs, 1H, NH, exchangeable with D_2O), 8.45 (s, 1H, imidazole), 7.14 (brs, 1H, NH, exchangeable with D_2O), 6.62 (t, J = 5.9 Hz, 1H, 1′-H), 5.27 (s, 1H, OH, exchangeable with D_2O), 5.08 (s, 1H, OH, exchangeable with D_2O), 4.25 (m, 1H, 4′-H), 3.84 (m, 1H, 3′-H_1), 3.60 (m, 2H, 5′-H), 3.21 (m, 2H, CH_2), 2.42 (m, 1H, 2′-H_1), 2.19 (m, 1H, 2′-H_2), 1.46 (m, 2H, CH_2), 1.22 (m,18H, $(CH_2)_9$), 0.83 (t, J = 6.4 Hz, 3H, CH_3). Anal. ($C_{23}H_{37}N_5O_5$•0.5H_2O) C, H, N.

Method B: A solution of dodecylamine (2.144 g, 11.34 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (**5**) (2.350 g, 11.34 mmol) in methanol (30 mL) was refluxed for 0.5 h. Solvent was evaporated under vacuum, and the residue was dried in a desiccator over P_2O_5 in a vacuum overnight. Without further purification, the residue was dissolved in anhydrous methanol (13 mL) in an ice-bath, and a solution of sodium methoxide (5.95 mL, 25wt % in methanol) was added. The reaction mixture was stirred for 0.5 h at 0 °C, and was directly filtered into a solution of **33** (2.284 g, 3.78 mmol) in anhydrous methanol (60 mL). The mixture was stirred overnight, the solvent was evaporated, and the residue was purified by silica gel flash chromatography, eluting successively with chloroform: methanol (30:1), chloroform:methanol (15:1), and methanol. Evaporation of the appropriate UV-absorbing fractions yielded **30** (1.3 g, 74%). The spectral data of this compound were superimposable with those of **30** obtained by Method A above.

Butyl 1-(2′-Deoxy-3′,5′-di-*O*-*p*-toluoyl-β-D-erythropentofuranosyl)-4,5-imidazoledicaboxylate (33). To a mixture of butyl 4,5-imidazoledicarboxylate (**31**)[51,52](1.59 g, 5.93 mmol) and NaH (268 mg, 6.70 mmol) in anhydrous acetonitrile (30 mL), was added portionwise pure 2- deoxy-3,5-di-*O*- *p*-toluoyl-α-D-erythropentofuaranosyl chloride (**32**) (1.538 g, 3.96 mmol) during a 2 h period under nitrogen atmosphere. The reaction mixture was stirred for an additional 1 h. The mixture was filtered in vacuo, and the filtrate was evaporated to dryness to obtain a syrup. The product was purified by silica gel flash chromatography, eluting with chloroform. Yield 2.34 g (95%), R_f = 0.25 (chloroform−methanol, 60:1); ^{1}H NMR ($CDCl_3$): δ 7.99 (s, 1H, imidazole), 7.94 (d, J = 2.6 Hz, 2H, aromatic), 7.84 (d, J = 3.3 Hz, 2H, aromatic), 7.24 (m, 4H, aromatic), 6.57 (dd, J_1 = 7.5 Hz, J_2 = 5.7 Hz 1H, 1′-H), 5.61 (m, 1H, 3′-H), 4.68 (m, 2H, 5′-H), 4.64 (m, 1H, 4′-H), 4.31 (m, 4H, CH_2), 2.98 (m, J_1 = 14.5 Hz, J_2 = 5.7 Hz J_3 = 2.0 Hz, 1H, 2′-H_1), 2.48 (m, 1H, 2′-H_2), 2.44 (s, 3H, *p*-CH_3Ph), 2.40 (s, 3H, *p*-CH_3Ph), 1.72 (m, 4H, CH_2), 1.43 (m, 4H, CH_2), 0.97 (t, J = 3.7 Hz, 3H, CH_3), 0.93 (t, J = 3.7 Hz, 3H, CH_3). Anal. ($C_{34}H_{40}N_2O_9$): C, H, N.

Butyl 1-(2′-Deoxy-3′,5′-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl)-4,5-imidazoledicarboxylate (35). 2-Deoxy-3,5-di-*O*- *p*-toluoyl-α-D-erythropentofuaranosyl chloride (**32**) (778 mg, 2.0 mmol) was dissolved in anhydrous $CHCl_3$ (10 mL), and the solution was slowly added via a syringe pump over a period of 2 h (generating **34** in situ) to a solution of butyl 4,5-imidazoledicarboxylate (**31**) (0.537 g, 2.0 mmol) and proton sponge (0.429 g, 2 mmol) in $CHCl_3$ (10 mL). The reaction mixture was stirred overnight. The reaction mixture was rotary evaporated, and the residue was purified by silica gel flash chromatography, eluting with chloroform. A syrup (0.761 g) was obtained after evaporation of the chloroform eluent. It was dried over P_2O_5 in vacuo overnight. Yield 62%, R_f = 0.25 (chloroform−methanol, 60:1); ^{1}H NMR ($CDCl_3$): δ 7.99 (s, 1H, imidazole), 7.95 (t, J = 4.0 Hz, 2H, aromatic), 7.62 (d, J = 4.0 Hz, 2H, aromatic), 7.24 (m, 4H, aromatic), 6.59 (d, J = 6.6 Hz, 1′-H), 5.65 (d, J = 6.2 Hz, 1H, 3′-H), 4.90 (m, 1H, 4′-H), 4.62 (m, 2H, 5′-H), 4.31 (m, 4H, CH_2), 3.03 (m, 1H, 2′-H_1), 2.59 (m, 1H, 2′-H_2), 2.44 (s, 3H, *p*-CH_3Ph), 2.38 (s, 3H, *p*-CH_3Ph), 1.72 (m, 4H, CH_2), 1.43 (m, 4H, CH_2), 0.95 (m, 6H, CH_3). Anal. ($C_{34}H_{40}N_2O_9$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-(2′-deoxy-α-D-erythropentofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (36). A mixture of dodecylamine (1.419 g, 7.5 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate[36] (1.554 g, 7.5 mmol) was refluxed in methanol (20 mL) for 2 h. The solvent was evaporated under vacuum, and the residue was dried in a desiccator over P_2O_5 in vacuo. Without further purification, it was dissolved in anhydrous methanol (10.0 mL), and the solution was cooled to 0 °C. A solution of sodium methoxide (25 wt % in methanol, 3.94 mL) was added, and the reaction mixture was kept in an ice-bath for 0.5 h. The reaction mixture was directly filtered into a solution of **35** (1.534 g, 2.5 mmol) in anhydrous methanol (40 mL). The reaction mixture was stirred overnight, and the solvent was rotary evaporated. The residue was purified by silica gel flash chromatography, eluting successively with chloroform−methanol (30:1), chloroform:methanol (15:1), and methanol. Yield 35%, R_f = 0.21 [chloroform:methanol:ammonium hydroxide (4:1:0.1)], mp > 250 °C; ^{1}H NMR (DMSO-d_6) δ 10.42 (brs, 1H, NH, exchangeable with D_2O), 8.19 (s, 1H, imidazole), 7.05 (brs, 1H, NH, exchangeable with D_2O), 6.62 (d, J = 6.2 Hz, 1H, 1′-H), 5.10 (d, J = 2.6 H, 1H, OH, exchangeable with D_2O), 4.87 (t, J = 5.7 Hz, 1H, 5′-OH, exchangeable with D_2O), 4.25 (m, 2H, 3′,4′-H), 3.43 (t, J = 4.9 Hz, 2H, 5′-$H_{1,2}$), 3.21 (m, 2H, CH_2), 2.64 (m, 1H, 2′-H_1), 2.01 (d, J = 14.3 Hz, 1H, 2′-H_2), 1.47 (m, 2H, CH_2), 1.23 (m,18H, $(CH_2)_9$), 0.84 (t, J = 6.2 Hz, 3H, CH_3). Anal. ($C_{23}H_{37}N_5O_5$•0.75H_2O) C, H, N.

Methyl 1-(*p*-Methoxybenzyl)imidazole-4,5-dicarboxylate (37). A mixture of methyl 4,5-imidazoledicarboxylate (**2**)[34,35] (921 mg, 5 mmol) and potassium carbonate (907 mg, 6.5 mmol) was stirred at room temperature in anhydrous CH_3-CN (30 mL), followed by the dropwise addition of *p*-methoxybenzyl chloride (0.84 mL, 6.0 mmol). The reaction mixture was stirred for 24 h and was then heated to 50 °C for an additional 72 h. The solvent was evaporated, and the residue was purified by silica gel flash chromatography, eluting with a mixture of chloroform−methanol (30:1). Evaporation of the appropriate UV-absorbing fractions yielded **37** as a solid (1.426 g, 94%), R_f = 0.26 (chloroform−methanol, 30:1), mp 66−67.5 °C; ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 1H, imidazole), 7.13 (d, J = 8.8 Hz, 2H, phenyl), 6.89 (d, J = 8.4 Hz, 2H, phenyl), 5.31 (s, 2H, CH_2Ph), 3.75 (s, 3H, CH_3OCO), 3.74 (s, 3H, CH_3OCO), 3.71 (s, 3H, *p*-CH_3OPh). Anal. ($C_{15}H_{16}N_2O_5$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)imidazo[4,5-*e*][1,3]-diazepine-4,8-dione (38). Compound **30** (232 mg) was dissolved in glacial acetic acid (45 mL) and stirred overnight at

room temperature. The solid (114 mg) that precipitated from the solution was filtered and dried over KOH pellets under vacuum. Yield, 66%, mp >250 °C. ^{1}H NMR (DMSO-d_6): δ 11.94 (brs, 1H, NH, exchangeable with D_2O), 10.42 (brs, 1H, NH, exchangeable with D_2O), 8.00 (s, 1H, imidazole), 7.01 (brs, 1H, NH, exchangeable with D_2O), 3.24 (m, 2H, CH_2 group), 1.47 (m, 2H, CH_2 group), 1.23 (m, 18 H, $(CH_2)_9$ chain), 0.84 (t, J = 6.6 Hz, 3H, CH_3 group); HRMS (FAB) calcd for $C_{18}H_{29}N_5O_2$ (MH^+) m/z 348.2399, found, 348.2401. Anal. ($C_{18}H_{29}N_5O_2 \cdot 0.5H_2O$) C, H, N.

4,5-Dihydro-8*H*-6-(*N*-dodecylamino)-1-(*p*-methoxybenzyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (39). A mixture of dodecylamine (0.851 g, 4.5 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (933 g, 4.5 mmol) was refluxed in methanol (15 mL) for 2 h. The solvent was evaporated under vacuum, and the residue was dried in a desiccator over P_2O_5 in a vacuum for two nights. Without purification, it was dissolved in anhydrous methanol (10.0 mL) and cooled to 0°C, and then a solution of sodium methoxide (25wt % in methanol, 2.27 mL) was added dropwise. The reaction mixture was kept in an ice-bath for 0.5 h. The reaction mixture was directly filtered into a solution of methyl 1-(*p*-methoxybenzyl)imidazole-4,5-dicarboxylate (**37**) (0.456 g) in anhydrous methanol (20 mL), and the solution was continued to stir at ambient temperature for 2 days. After evaporation of the solvent, the residue was purified by flash silica gel chromatography, eluting successively with a mixture of chloroform−methanol (30:1) and chloroform−methanol (10:1). After evaporation of the appropriate UV-absorbing fractions, compound **39** was collected as a solid (0.667 g, 95%), Rf = 0.30 (chloroform:methanol, 10:1), R_f = 0.79 (chloroform:methanol:ammonium hydroxide, 4:1:0.1), mp 274−276 °C; ^{1}H NMR (DMSO-d_6): δ 10.39 (brs, 1H, NH, exchangeable with D_2O), 8.22 (s, 1H, imidazole), 7.18 (d, J = 8.4 Hz, 2H, phenyl), 7.01 (brs, 1H, NH, exchangeable with D_2O), 6.87 (d, J = 8.8 Hz, 2H, phenyl), 5.53 (s, 2H, CH_2Ph), 3.70 (s, 3H, *p*-CH_3OPh), 3.17 (m, 2H, CH_2), 1.44 (m, 2H, CH_2), 1.22 (m,18H, $(CH_2)_9$), 0.83 (t, J = 6.4 Hz, 3H, CH_3). Anal. ($C_{26}H_{37}N_5O_3$) C, H, N.

(B) Biochemical Methods. Materials. DNA oligonucleotides were prepared by Dr. M. Schreiber (Bernhard Nocht Institute). RNA oligonucleotides were purchased from HHMI Biopolymer/Keck Foundation, Biotechnology Resource Laboratory, Yale University School of Medicine (New Haven, CT., USA). [γ-^{32}P]ATP (220 Tbq/mmol) and [γ-^{33}P]ATP (110 Tbq/mmol) were from Hartman Analytic. All other chemicals were obtained from Sigma.

Sources and Purification of Suv3$_{(\Delta 1-159)}$ and WNV NTPase/Helicases. NH_2-terminally truncated Suv3 NTPase/helicase (Suv3$_{(\Delta 1-159)}$) was expressed in *E. coli* as follows: a 1881 bp fragment of the human Suv3 cDNA coding for Suv3 protein truncated 159 aa from the amino terminus was amplified by PCR using the following primers: forward 5′−CAT GCC ATG GCG CCA TTT TTC TTG AGA CAT GCC−3′; reverse 5′−CTG GGA TCC GTC CGA ATC AGG TTC CTT C−3′ (purchased from SIGMA), and the pKK plasmid as a template (Dmochowska et al.[53]) The resulting fragment was cloned into NcoI and BamHI sites of the pQE60 expression vector (Qiagen). Sequences of both strands were verified, using an ABI Prism 377DNA Sequencer. The protein was purified according to the method established for the HCV enzyme.[54,55,58]

WNV NTPase/helicase was purified from the cell culture medium of virus-infected Vero E6 cells as described previously by Borowski et al.,[45] with some modifications. Briefly, the concentrated cell culture medium was mixed with 10 mL of Reactive Red 120 agarose (Sigma) equilibrated with TGT buffer for 4 h at 4 °C. The matrix was collected by sedimentation, transferred to a column, and washed with TGT buffer. Bound protein was eluted with 1 M KCl in the same buffer, concentrated by ultrafiltration on a 30-kDa membrane to a final volume of 2 mL, and subjected to gel exclusion chromatography on a Superdex-200 column. Fractions expressing ATPase and helicase activities were chromatographed again on Reactive Red 120 agarose (5 mL) as described above. The salt-eluted protein was precipitated with poly(ethylene glycol) (30% w/w), collected by centrifugation, solubilized with TGT buffer, and applied to a hydroxyapatite (HA-Ultrogel) column preequilibrated with TGT buffer. The column was washed with 10 mL of TGT buffer, and then with 2 mL of TGT buffer containing 1 M KCl, and again with 5 mL of TGT buffer. The NTPase/helicase was eluted with 1 mL of TGT buffer containing 50 mM KH_2PO_4, precipitated with PEG and dissolved in TGT buffer.

The final preparations of the enzymes were homogeneous, as demonstrated by Coomassie Blue staining of SDS/polyacrylamide gels (data not shown).

ATPase and Helicase Assays. The ATPase activity of the NTPase/helicases was determined as described previously.[25,26] Briefly, assays were performed with 2 pmol of WNV or 0.2 pmol of Suv3$_{(\Delta 1-159)}$ NTPase/helicase. The enzymes were incubated in a reaction mixture (final volume 25 μL) containing 20 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol, 0.01% Triton X-100, 0.1 mg/mL BSA, 25 nCi [γ-^{33}P]ATP, and ATP adjusted to concentrations indicated in the legends to tables. The reaction was conducted for 30 min at 30 °C and terminated by addition of 0.5 mL of activated charcoal (2 mg/mL). Following centrifugation at 10 000 × g for 10 min, 100 μL aliquots of the supernatant were removed and subjected to scintillation counting.

Helicase activity was tested with 2 pmol of WNV or 0.2 pmol of Suv3$_{(\Delta 1-159)}$ NTPase/helicase. Unwinding of the partially hybridized DNA or RNA substrate (4.7 pM of nucleotide base) was monitored in a reaction mixture (final volume 25 mL) containing 20 mM Tris/HCl, pH 7.5, 2 mM MgCl2, 1 mM β-mercaptoethanol, 10% glycerol, 0.01% Triton X 100, 0.1 mg/mL BSA and ATP at concentrations indicated in legends. The reaction was conducted for 30 min at 30 °C and stopped by addition of 5 mL of termination buffer (100 mM Tris/HCl, pH 7.5, 20 mM EDTA, 0.5% SDS, 0.1% Triton X-100, 25% glycerol, and 0.1% bromophenol blue). Samples were separated on a 15% Tris-borate-EDTA (TBE)-polyacrylamide gel containing 0.1% SDS [Borowski et al.[45]]. The gels were dried and exposed to Kodak X-ray films at −70 °C. The areas of the gels corresponding to the released strand and to the non-unwound substrate were cut out, and ^{32}P radioactivity was counted. Alternatively, the films were scanned, and the radioactivity associated with the released strand and with the non-unwound substrate was quantified with GelImage software (Amersham Pharmacia Biotech). The assays were carried out with the same activity of the enzyme, determined with the DNA substrate under conditions described above

Substrates for Helicase Reactions. The RNA substrate for the helicase assays consisted of two partially hybridized oligonucleotides with sequences as reported by Gallinari et al.[13] The DNA substrate was obtained by annealing two DNA oligonucleotides synthesized with sequences corresponding to the deoxynucleotide versions of the aforementioned RNA strands. The release strands (26-mer) were 5'-end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase (MBI, Fermentas) as recommended by the manufacturer. For the annealing reaction, the labeled oligonucleotide was combined at a molar ratio of 1:10 with the template strand (40-mer), denatured for 5 min at 96 °C, and slowly renatured as elsewhere described.[26,45] The duplex DNA was electrophoresed on a 15% native TBE-polyacrylamide gel, visualized by autoradiography, and extracted as described previously.[45] The amount of DNA or RNA duplex used as substrates was determined by the ethidium bromide fluorescent quantitation method.[56]

Other Assays. Protein concentrations of preparations of the NTPase/helicases were determined on SDS/polyacrylamide gels as described previously.[57] Kinetic parameters were determined by nonlinear regression analysis using ENZFITTER (BioSoft) and Sigma Plot (Jandel Corp.)

Acknowledgment. This paper is dedicated to R.S.H.'s Ph.D. mentor, advisor, and friend Dr. Stewart W. Schneller, Dean of Science and Mathematics, Auburn University, AL, on the occasion of his 61st birthday. The research was supported in part by a grant (#9RO1

AI55452) from the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health, Bethesda, Maryland (to R.S.H.), an unrestricted grant from Nabi, Rockville, MD (to R.S.H.), and a Biopharmaceuticals FP5RID grant (QLRT-2001-01079) (to P.B.).

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JM030277K