Ring-Expanded ("Fat") Nucleoside and Nucleotide Analogues Exhibit Potent in Vitro Activity against *Flaviviridae* NTPases/Helicases, Including Those of the West Nile Virus, Hepatitis C Virus, and Japanese Encephalitis Virus

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A series of ring-expanded ("fat") heterocycles, nucleoside and nucleotide analogues (RENs) containing the imidazo[4,5-*e*][1,3]diazepine ring system (**9**, **14**, **15**, **18**, **24-26**, **28**, **31**, and **33**) and imidazo[4,5-*e*][1,2,4]triazepine ring systems (**30b**, **30c**, **32**, and **34**), have been synthesized as potential inhibitors of NTPases/helicases of *Flaviviridae*, including the West Nile virus (WNV), hepatitis C virus (HCV), and Japanese encephalitis virus (JEV). An amino-terminal truncated form of human enzyme $Suv3_{(\Delta 1-159)}$ was also included in the study so as to assess the selectivity of RENs against the viral enzymes. The analogues of RENs included structural variations at position 1 of the heterocyclic base and contained changes in both the type of sugar moieties (ribo, 2′-deoxyribo, and acyclic sugars) and the mode of attachment (α versus β anomeric configuration) of those sugars to the heterocyclic base. The target RENs were biochemically screened separately against the helicase and ATPase activities of the viral NTPases/helicases. A number of RENs inhibited the viral helicase activity with IC_{50} values that ranged in micromolar concentrations and exhibited differential selectivity between the viral enzymes. In view of the observed tight complex between some nucleosides and RNA and/or DNA substrates of a helicase, the mechanism of action of RENs might involve their interaction with the appropriate substrate through binding to the major or minor groove of the double helix. The REN-5′-triphosphates, on the other hand, did not influence the above unwinding reaction, but instead exerted the inhibitory effect on the ATPase activity of the enzymes. The activity was found to be highly dependent upon the low concentration levels of the substrate ATP. 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. A tentative mechanistic model has been proposed to explain the observed results, which includes an additional allosteric binding site on the viral NTPases/helicases that can be occupied by nucleoside/nucleotide-type molecules such as RENs.

Introduction

The viruses of the *Flaviviridae* family are small, enveloped, spherical particles of 40 to 50 nm in diameter with single-stranded, positive sense RNA genomes.[1−3] The members of the *Flaviviridae* family could be classified into three genera: hepaciviruses, flaviviruses, and pestiviruses.[3,4] Recently, a virus related to the hepatitis C (HCV), the hepatitis G virus (HGV), formerly referred to as "GB-agent", was characterized.[5] The phylogenetic classification of the virus is, however, not established until now.[3] The hepaciviruses and flaviviruses are known to be the cause of severe encephalitic, hemorrhagic, hepatic, and febrile illnesses in humans.[3] The viral genome of the genera *Hepacivirus* and *Flavivirus* encodes a polyprotein of 3000 to 4000 amino acids. The NH_2-terminus of the polyprotein is processed into three structural proteins. The COOH-terminal part of the polyprotein of hepaciviruses is cleaved into in six mature nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and the polyprotein of the flaviviruses is processed into 7 proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (see Figure 1).[3,6−8]

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.[9−11] The catalytic domain of the chymotrypsin-like NS3 protease has been mapped to the NH_2-

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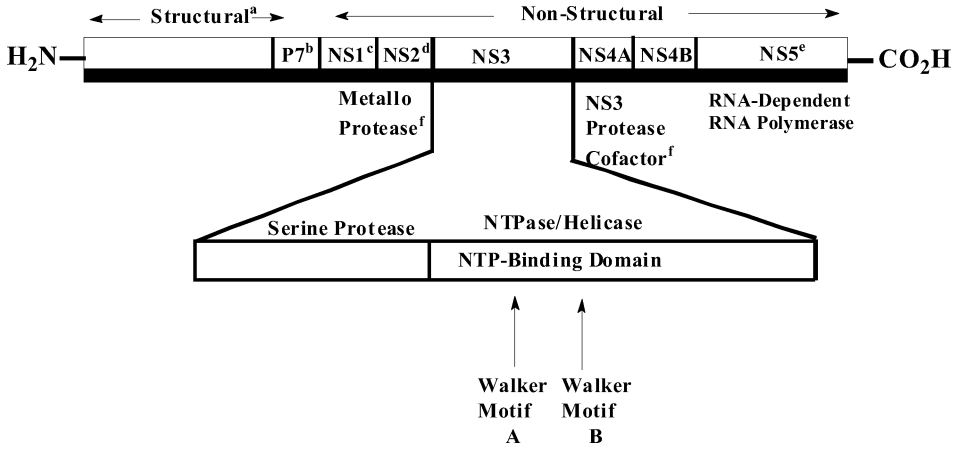
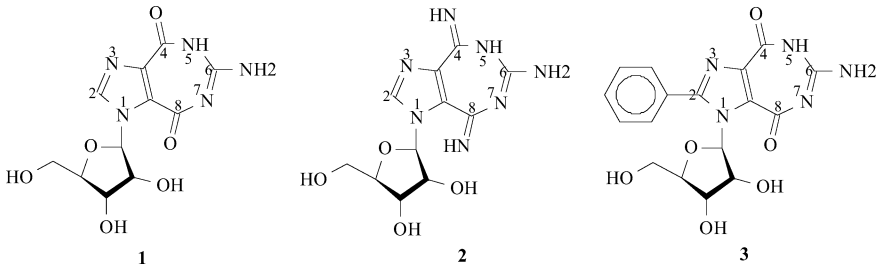


Figure 1. Simplified representation of the structure of hepaciviruses and flaviviruses polyprotein with the expanded NS3 region. The enzymatic activities associated with the nonstructural proteins are shown. The arrows indicate the position of the highly conserved motifs (Walker motifs A and B) within the NTPase/helicase molecule (see text). (a) The NH_2-terminal part of the polyprotein of the members of *Flavivirus* genus is processed into three structural proteins: a nucleocapsid protein (C), precursor membrane protein (pre-M), and one envelope protein (E); the polyprotein of hepaciviruses into nucleocapsid protein (C) and two envelope proteins (E1 and E2). (b) Peptide p7 of unknown function is encoded exclusively by hepaciviruses. (c) NS1 is encoded exclusively by flaviviruses. (d) NS2 of flaviviruses is processed into two proteins: NS2A and NS2B of unknown function. (e) NS5 of hepaciviruses is cleaved into NS5A and NS5B. The RNA polymerase activity is associated with the NS5B protein. (f) The functions are attributed to hepaciviruses.

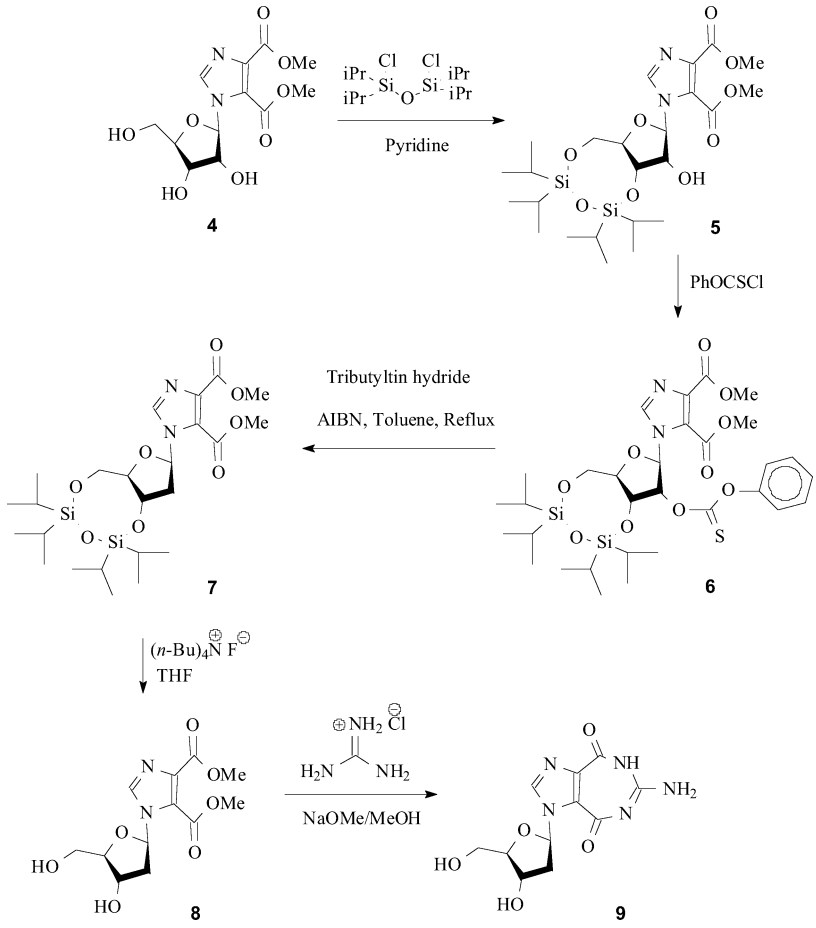
terminus region of the NS3, whereas the NTPase and the helicase activities are associated with the COOH-terminus of NS3.[8,9] Helicases are capable of unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together.[12,13] This unwinding activity is essential 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).[14,15] 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 ($IC_{50} = 30\ \mu M$).[16] A comparable inhibitory potency was also observed in tissue cultures of the virus.[16] Furthermore, closely related compounds that did not influence the helicase activity did not also exert any effect on the virus replication.[16]

Based on the solved crystal structure of HCV RNA NTPase/helicase and of DNA NTPase/helicases from *E. coli* and *Bacillus stearothermophilus*, two alternative mechanisms of the unwinding reaction have been postulated.[17−20] 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.[12,17] Alternatively, the energy could be transferred to the fork and used for disruption of the hydrogen bonds that keep the strands together.[12,17] 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,[21,22] (b) inhibition of NTPase activity by an allosteric mechanism,[21] and (c) inhibition of the coupling of NTP hydrolysis to unwinding reaction.[22] Additional mechanistic possibilities include interference in the interaction of helicase with its RNA or DNA substrate via (d) competitive blockade of substrate binding site[23] or by (e) inhibition of the unwinding by steric inhibition of translocation of the enzyme along the polynucleotide chain.[24] There are even more mechanistic possibilities by which the helicase activity could be inhibited. Binding studies of Porter et al. revealed a putative nucleoside-binding site within the HCV NTPase/helicase molecule.[25,26] The function and location of the second binding site remain unknown. Nevertheless, there is accumulating evidence that the NTPase and helicase activities of the viral super family II (SFII) enzymes might be modulated by occupation of these putative nucleoside-binding site. For example, ribavirin-5'-triphosphate (RTP), which is a potent, classical, competitive inhibitor of the NTPase activity of the West Nile virus (WNV) and hepatitis C virus (HCV) NTPase/helicases at lower ATP concentrations ($< K_m$), failed to inhibit the ATPase activity at higher ATP concentrations ($\gg K_m$), and instead, even stimulated the enzyme activity.[22,27] By contrast, the RTP inhibits moderately the helicase activity of both enzymes by a mechanism that is independent of the ATP concentrations.[27] The phenomenon results most probably from occupation of a second nucleoside binding site by RTP.[22] We present herein a class of nucleoside analogues, called ring-expanded (REN or "fat") nucleosides, which modulate, i.e. activate or inhibit, the unwinding reaction of some selected viral SFII NTPase/helicases. These modulatory effects are suggested to result from interaction of the compounds with RNA and/or DNA substrate of the respective helicase or directly with the enzyme itself. Although many of the compounds tested also affect the respective ATPase activity, it nevertheless appears to bear no significant consequence on the overall antihelicase activity.

Chart 1



Scheme 1



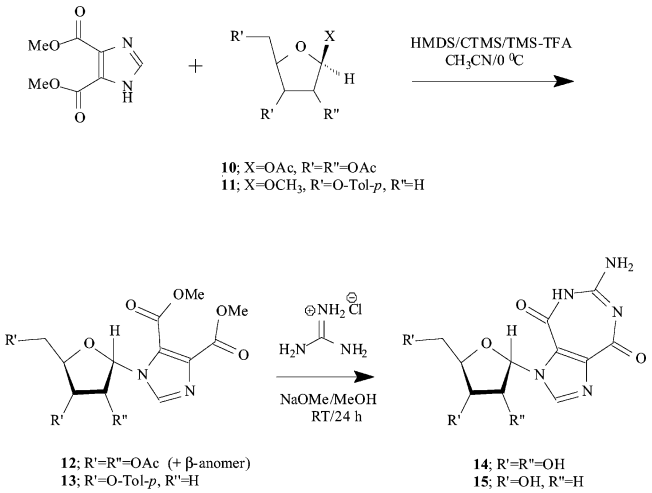
Chemistry

Encouraged by the broad spectrum antiviral activities of a number of imidazodiazepine and -triazepine nucleosides that we recently synthesized against hepatitis B, herpes, and respiratory viruses,[28−30] we desired to further explore these heterocyclic ring systems and their nucleoside/nucleotide derivatives against *Flaviviridae*. However, the parent compound, 6-aminoimidazo[4,5-*e*]-[1,3]diazepine-4,8-dione (**1**), which had earlier showed highly potent and selective antihepatitis B virus (anti-HBV) activity in cultured human hepatoblastoma 2.2.15 cells (IC_{50} = 0.13 μM),[29] totally failed to exhibit any activity against any of the *Flaviviridae* NTPase/helicase tested (IC_{50} > 500 μM) including the HCV, JEV, and WNV enzymes. Nevertheless, our prior experience suggested that even a minor structural variation could render **1** with a totally different biological activity. For example, the replacement of the two carbonyl (C═O) functionalities at positions 4 and 8 of **1** with the two imino (C═NH) groups of **2** resulted in a highly potent anticancer activity of the latter.[31] Likewise, the substitution at position 2 of **1** with a phenyl group as in **3**, transpired the latter into a compound of potent anti-rhino (IC_{50} = 6.7 μM) and antimeasles (IC_{50} = 0.5 μM) virus activity.[32]

A conspicuous locus of **1** that is viable for further structural modifications, but remained largely unexplored thus far, is position 1, where the sugar group is attached. Therefore, in exploring the potential anti-*Flaviviridae* activity of the ring system contained in **1**, we decided to focus on 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 2′-deoxy analogue of **1** was synthesized by deoxygenation of the imidazole riboside diester **4**[33−35] (Scheme 1), employing the Barton deoxygenation procedure,[36,37] before condensation with guanidine. Thus, the 3′,5′-positions of **4** were selectively protected with the Markiewicz reagent,[38] 1,3-dichloro-1,1,3,3-tetraisopropyldi-

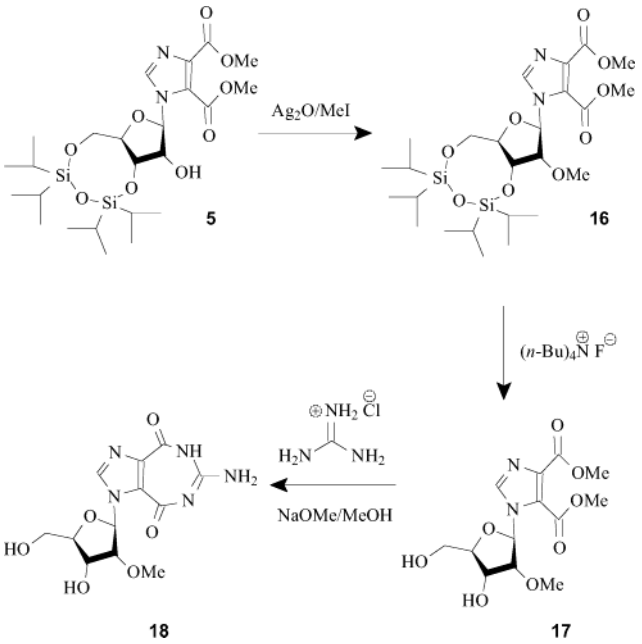
Scheme 2



siloxane, to form the corresponding 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) (TIPDS) derivative **5**. 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 **6**. Free radical-mediated Barton deoxygenation[36] 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 **7**. The silyl deprotection of the latter compound was achieved by treatment with tetra-*n*-butylammonium fluoride, which gave the desired 2′-deoxynucleosides **8**. Further ring-closure with guanidine afforded the target nucleoside **9**.

The respective α-anomers of nucleosides **1** (**14**) and **9** (**15**) were synthesized (Scheme 2) by condensation of the corresponding imidazole diester nucleosides **12** and **13** with guanidine. Nucleosides **12** and **13**, in turn, were prepared by the Vorbrüggen glycosylation[39] of methyl 4,5-imidazoledicarboxylate with either the *β*-acetoxy (**10** for **12**) or the *β*-methoxy (**11** for **13**) ribose analogues[40] with the appropriate sugar hydroxyl protection. The ratio of α: *β* anomers formed in the former case (**12**), based on the ^{1}H NMR integration of the product, was 3:2, while in the latter (**13**) it was almost exclusively the α-anomer. The observed relatively more downfield shift as well as the smaller coupling constant $J_{1',2'}$ of the anomeric proton in the *β*-anomer as compared those in the α are consistent with the reported general trend of ^{1}H NMR spectral patterns of α/*β*-anomers of a number of nucleoside analogues containing five-membered heterocyclic rings.[41] An interesting observation was that the two ester methyl groups in the α-anomer appeared as a singlet at *δ* 3.93, while they were slightly shifted upfield and appeared as two distinct peaks at *δ* 3.92 and 3.90 in the *β*-anomer. Another conspicuous result, which is consistent with the reported spectral pattern of five-membered heterocyclic nucleoside analogues,[41] is that the 2′- and 3′-*O*-acetyl methyl groups of the α-anomer appeared as two distinct singlets, with the 2′-*O*-acetyl methyl group appearing considerably upfield (*δ* = 2.15) from the signal of the 3′-*O*-acetyl methyl group (*δ* = 2.21), whereas in the *β*-anomer the same two signals appeared as a singlet at *δ* 2.16. In the case of

Scheme 3

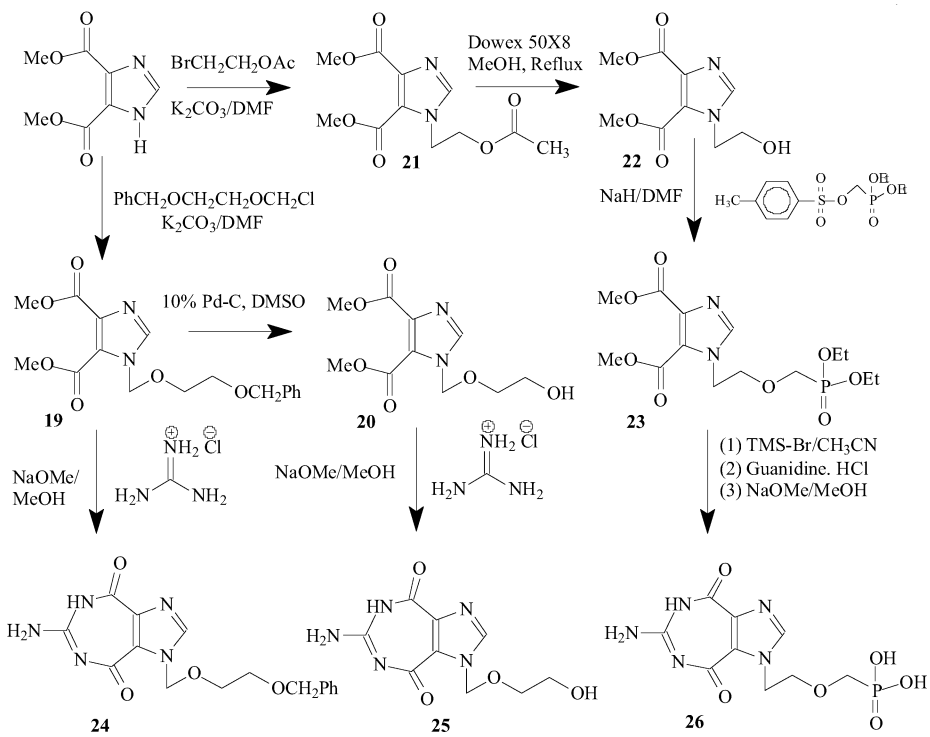


12, there was still no need to separate it from the corresponding *β*-anomer before condensation with guanidine as the pure product **14** precipitated out of the solution upon simple neutralization with methanolic hydrogen chloride, while leaving the corresponding *β*-anomer **1** in solution. The identities of the α-anomers **14** and **15** were established by comparison of their spectral data with those of the authentic samples of their respective *β*-anomers (**1** and **9**). It is to be noted that α-anomers of 2′-deoxynucleosides normally exhibit their H-1′ as a doublet of doublets with unequal coupling constants, one being significantly larger than the other. The fact that both **13** and **15** exhibited their respective H-1′ only as a doublet with J = 6.6 and 6.0 Hz, respectively, suggests that the second coupling constant must be near or equal to zero. This further implies a possible "N" (C2′-exo-C3′-endo) conformation for the sugar pucker in both, which would place their axial H-2′ farther away from H-1′, preventing any significant coupling between the two protons.

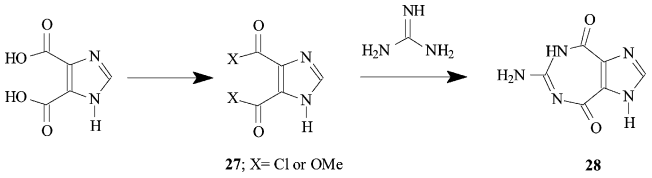
The 3′-*O*-methyl analogue of **1** (**18**, Scheme 3) was synthesized by sequential reactions of **5** with methyl iodide/silver oxide (to obtain **16**), tetra-*n*-butylammonium fluoride (to yield **17**), and condensation with guanidine.

The acyclic nucleosides **24** and **25**, along with a nucleoside phosphonate **26** (Scheme 4), were synthesized from the common precursor, methyl 4,5-imidazoledicarboxylate. Treatment of the latter with 1-benzyloxy-2-chloromethoxyethane, catalyzed by potassium carbonate in dimethylformamide, afforded **19**, which upon ring-closure with guanidine produced **24**. Catalytic hydrogenation of **19** with palladium/charcoal yelded **20**, which was further condensed with guanidine to afford **25**. The target acyclic nucleoside phosphonate **26** was obtained from the imidazole diester through sequential reactions involving (a) condensation with bromoethyl acetate, catalyzed by potassium carbonate/dimethylformamide (to obtain **21**), (b) hydrolysis with Dowex 50 WX8 resin in methanol at reflux (to obtain **22**), (c) treatment with diethyl *p*-toluenesulfonylmethanephos-

Scheme 4



Scheme 5



phonate in sodium hydride/dimethylformamide (to obtain **23**), (d) delalkylation with bromotrimethylsilane in acetonitrile, and condensation with guanidine.

The parent heterocyclic base of nucleoside **1** (**28**) was synthesized (Scheme 5) either by conversion of imidazole-4,5-dicarboxylic acid into the corresponding acid chloride (**27**; X = Cl)) by treatment with thionyl chloride, followed by condensation with guanidine, or by direct condensation of the imidazole diester (**27**; X = OMe) with guanidine.

The ring-expanded nucleoside-5′-triphosphates, **33** and **34** (Scheme 6), containing the 5:7-fused heterocyclic systems, imidazo[4,5-*e*][1,3]diazepine and imidazo[4,5-*e*][1,2,4]triazepine, respectively, were synthesized from the corresponding nucleosides **31** and **32**, employing the procedure of Ludwig.[42] The procedure is a one-pot process consisting of sequential operations involving (a) reaction with phosphorus oxychloride/trimethyl phosphate to form the 5′-monophosphate derivative, (b) treatment with bis(tri-*n*-butylammonium)pyrophos-

Scheme 6

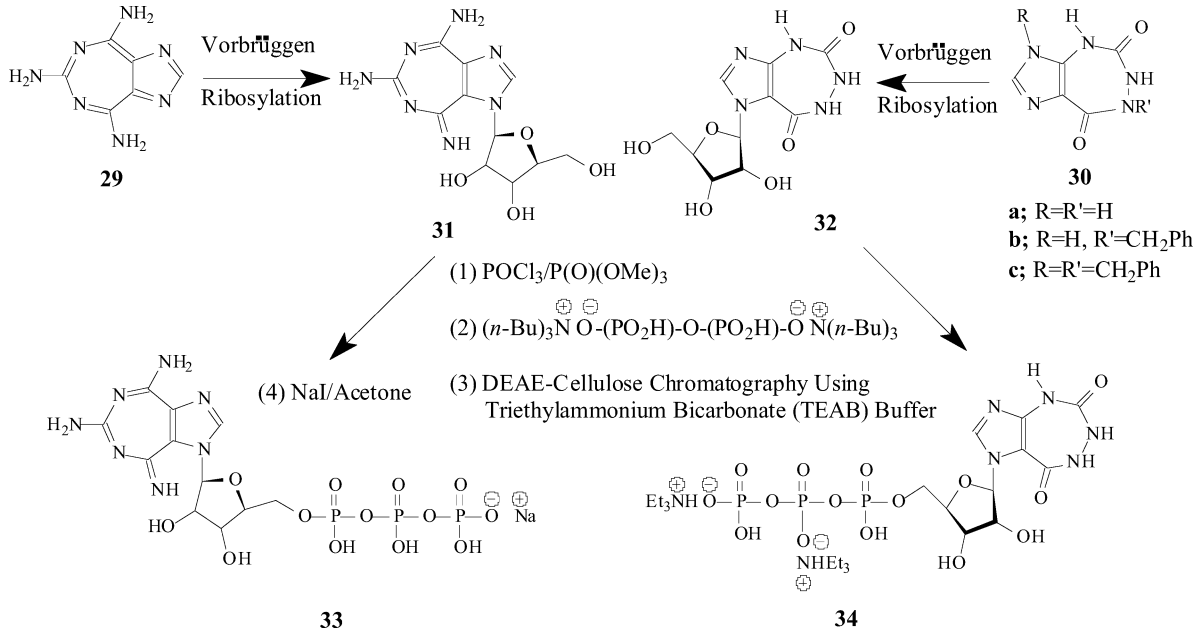


Table 1. Inhibitory or Activating Effect of Ring-Expanded Nucleoside (REN) Analogues against the Helicase Activity of WNV, HCV, and JEV NTPase/Helicases, Using a DNA Substrate. For Assessment of Selectivity, the Truncated Version of Human NTPase/Helicase $Suv3_{(\Delta 1-159)}$ Has Also Been Included in the Enzyme Inhibition Studies[a]

compd	WNV IC_{50} (μM)	HCV IC_{50} (μM)	JEV IC_{50} (μM)	$Suv3_{(\Delta 1-159)}$ IC_{50} (μM)
9	>500	activator: $ED_{200} = 100$	>500	>500
14	activator: $ED_{200} = 60$	activator: $ED_{200} = 150$	>500	>500
15	25	7	13	28
18	>500	activator: $ED_{200} = 350$	>500	>500
24	5.7	11	150	5
25	>500	>500	>500	ND
26	500	200	>500	>500
28	>500	>500	>500	ND
30b	1.3	>500	>500	>500
30c	3.5	>500	>500	>500
31	>500	>500	>500	>500
32	>500	>500	>500	>500
HMC-HO4	30	450	>500	>500

[a] The helicase activity was determined as a function of increasing concentrations of the compounds in the presence of ATP adjusted to 9.5 μM, 105 μM, 235 μM, and 4.2 μM for WNV, HCV, JEV, 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 Biochemistry 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 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 (μM) yielding 200% activity. N.D. = not determined. The results presented are representative of three independent experiments. The table includes also comparative studies performed with 1-(2′-*O*-methyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine-4,7(5*H*,6*H*)-dione (HMC-HO4) that we have, in part, reported previously.[16]

phate to yield the 5′-triphosphate, (c) purification by DEAE-cellulose chromatography on a DEAE-Sephadex A-25 column, using triethylammonium bicarbonate (TEAB) buffer to prepare the bis(triethylammonium) salt of the triphosphate, and (d) conversion of the latter, if necessary, into the corresponding sodium salt by treatment with sodium iodide in acetone. The products were purified and were characterized by ^1H and ^{31}P NMR spectral analyses as well as by high-resolution mass spectral data. The nucleosides **31** and **32**, in turn, were synthesized by Vorbrüggen ribosylation[39,40] of the respective heterocycles **29** and **30a**, as reported by us previously.[43,44]

Results and Discussion

The NTPases/helicases from three closely related *Flaviviridae*, including the West Nile virus (WNV), hepatitis C virus (HCV), and Japanese encephalitis virus (JEV), were chosen for the intended enzyme inhibition studies by a range of ring-expanded nucleosides/nucleotides (REN's). To assess the selectivity of the RENs-mediated inhibition, an enzyme from human source, namely, the NH_2-terminally truncated form of Suv3 protein [$Suv3_{(\Delta 1-159)}$], was also included in the study where necessary. 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 of the viral NTPases/helicases investigated (data not shown). 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.[27] The NTPase/helicase domains of HCV and JEV NS3 were expressed in *E. coli* and purified according to the literature procedure.[16] The NH_2-terminally truncated version of Suv3 NTPase/helicase was also 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 homogeneity 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 the 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. Since enzymes from different sources differ in their ATP requirement and its utilization in the strand-displacement reaction, all studies were carried out using amounts of enzymes possessing the same unwinding activity. Thus, the enzymes were calibrated with a DNA or RNA substrate that was unwound at ATP concentrations equal to the K_m value determined for the ATPase reaction.

The target compounds of Schemes 1−5 were screened against the helicase activity of three viral NTPase/helicases of WNV, HCV, and JEV, 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. 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}. 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

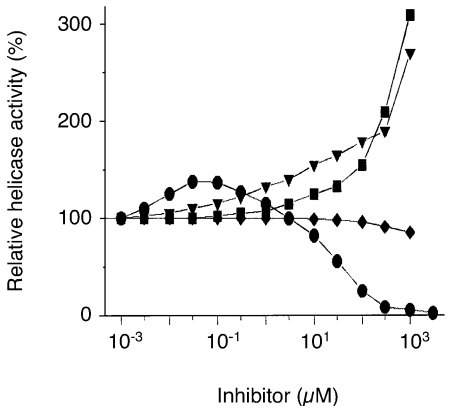


Figure 2. Comparison of the modulation of the helicase activity of HCV NTPase/helicase toward DNA substrate by ring-expanded nucleosides (RENs) **1**, **9**, **14**, and **15**. The helicase activity was determined as a function of increasing concentrations of RENs. Symbols: **1** (◆), **9** (■), **14** (▼), and **15** (●). The results presented are representative of three independent experiments.

be more convenient to compare the in vitro and in vivo data using either the IC_{50} or IC_{90} values.

The 2′-deoxy analogue of the parent REN **1**, (i.e., REN **9**, Scheme 1) which was earlier found inactive, was likewise found inactive (IC_{50} > 500 μM) against both WNV and JEV enzymes, but exhibited an interesting and surprising activating effect toward helicase activity of the HCV NTPase/helicase (see below).

The above results with **9** and **1** led us to further explore the importance of the configuration at the base−sugar junction, the anomeric carbon, in biochemical activity. So, we synthesized and screened compounds **14** and **15** (Scheme 2), which are the α-anomers of **1** and **9**, respectively. Compound **14**, the α-anomeric counterpart of the reference compound **1**, while being inactive against both JEV and the human enzyme, exhibited rather interesting activating effect against both HCV and WNV NTPase/helicase (see Figure 2). As mentioned earlier, a similar effect was observed with **9**. Compound **15**, by stark contrast to its β-anomeric counterpart **9**, exhibited potent antihelicase activity against all three NTPase/helicases (IC_{50} for HCV, WNV, and JEV were 7 μM, 25 μM, and 13 μM, respectively).

We have recently reported[16] observing such an activating (instead of inhibitory) effect of both WNV and HCV helicases, as with **9** and **14**, by a few imidazo[4,5-*d*][pyridazine nucleosides that we had synthesized. In addition, this activating effect was found to be considerably enhanced (WNV 170−180% and HCV 450−500%) by 2′-OMe analogue of the parent imidazopyridazine nucleoside with a β-anomeric configuration.[16] Therefore, to explore if this interesting observation made with imidazopyridazines would also be applicable to RENs, we synthesized the 2′-methoxy analogue of **1** (i.e., **18**, Scheme 3). Compound **18** did indeed exhibit the activating effect, but only against the HCV helicase, and not the other two.

We then wondered if the full sugar moiety necessary at all for the antihelicase activity or could it be substituted by a truncated form of the sugar such as acyclic nucleosides or nucleotides. To this end, we synthesized acyclic RENs **24** and **25** (analogues of the powerful antiherpes virus drug called acyclovir[45−48]), as well as a nucleoside phosphonate **26** (an analogue of the broad-spectrum antiviral drug called phosphonomethoxyethylguanine or PMEG;[49,50] see Scheme 4). Compound **24** was active against the helicase activity of both HCV (IC_{50} 11 μM) and WNV (IC_{50} 5.7 μM) enzymes, but was less effective against JEV NTPase/helicase (IC_{50} 150 μM). However, compound **25**, which lacked the terminal benzyl group of **24**, was totally devoid of activity against helicase activity of all three *Flaviviridae* enzymes including those of HCV, WNV, and JEV. The activity was also lost with the acyclic nucleotide analogue **26**. Therefore, it appears that the terminal benzyl group is highly critical for activity.

Table 2. Inhibitory Activity of Ring-Expanded Nucleoside (REN) Analogues against the Helicase Activity of the WNV, HCV, and JEV NTPases/Helicases, Using an RNA Substrate. For Assessment of Selectivity, the Truncated Version of Human Helicase $Suv3_{(\Delta 1-159)}$ Has Also Been Included in the Enzyme Inhibition Studies[a]

compd	WNV IC_{50} (μM)	HCV IC_{50} ((μM)	JEV IC_{50} ((μM)	$Suv3_{(\Delta 1-159)}$ IC_{50} ((μM)
14	220	12	4.5	3.5
15	5.7	>500	2	150
24	3.3	5.5	>500	6
28	220	200	>500	500
30b	>500	>500	>500	>500
30c	>500	>500	>500	>500

[a] The helicase activity was determined as described in the legend to Table 1 and under Biochemistry 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.

The varied results obtained with different sugar substitutions and their anomeric configurations led us to wonder if the sugar or the sugar-like moiety necessary at all for the antihelicase activity of RENs against *Flaviviridae*. To that end, we synthesized the parent heterocycle of both **1** and **9** (i.e., **28**) (see Scheme 5). Compound **28** was found to be practically devoid of activity. To explore if the observed marginal or lack of activity is limited to this particular ring system, we synthesized and screened heterocycles containing the imidazo[4,5-*e*][1,3]triazepine skeleton (**30**) (Scheme 6). While the parent compound **30a** was inactive, both **30b**, which has a benzyl group attached to the seven-membered ring, and **30c**, which has the benzyl groups attached to both the five- and seven-membered rings, were surprisingly both highly potent as well as selective against the helicase activity of WNV NTPase/helicase. These results suggested that a simple aralkyl group in place of a sugar moiety may suffice for an excellent activity profile in case of **30**.

Finally, since the above biochemical results were all obtained using a DNA substrate, and since the viruses in question are classified as RNA viruses, it was only logical to repeat the experiments using 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 most cases were significantly different from those obtained from a DNA substrate, at times to the point of totally opposite behavior. A case in point is the α-anomer **14** of the parent REN **1**. As mentioned under Introduction, compound **1**, a β-anomer**,** was totally inactive against all of the *Flaviviridae* NTPase/

helicases tested, whether with a DNA or an RNA substrate. Compound **14**, on the other hand, turned out to be an activator instead of an inhibitor of both WNV and HCV helicase activities and was inactive against both JEV and $Suv3_{(\Delta 1-159)}$ enzymes, all when a DNA substrate was used. A totally different scenario emerged when **14** was screened using an RNA substrate. It now strongly inhibited the unwinding activity of HCV (IC_{50} 12 μM), JEV (4.5 μM), as well as $Suv3_{(\Delta 1-159)}$ (3.5 μM) enzymes, and was also marginally active against WNV enzyme (220 μM).

Among all the RENs listed under Table 2, employing an RNA substrate, compound **15** has the best activity/selectivity profile against both WNV and JEV helicases as it minimally inhibited the human enzyme $Suv3_{(\Delta 1-159)}$ (IC_{50} 150 μM), while showing potent inhibition against the two viral enzymes in 2−5.7 μM range.

The above inhibition data of helicases with a variety of RENs employing either a DNA or an RNA substrate trigger an important, basic biochemical question: What is the mechanism of helicase inhibition by RENs? A close inspection of inhibition data outlined in Tables 1 and 2 reveal that there is no conspicuously single trend in activity that can be discerned outright. Apparently, there are considerable differences in activity between the ribose and deoxyribose sugar analogues, between analogues containing α- and β-anomeric configurations, between analogues equipped with full and half-sugar moieties, and between those with or without substitutions at the imidazole ring. While it is difficult to pinpoint a single operating mechanism to all of the RENs listed in Tables 1 and 2, one mechanism, although only tentative at this point, is worth consideration. The common molecular feature among all the structurally diverse RENs is that they resemble one or the other natural purine analogue. For example, the reference REN **1** as well as most of its analogues listed in Schemes 1−5, all contain carbonyl and amino substituents in the heterocyclic ring, and thus are close mimics of guanosine. In view of a number of documented reports demonstrating the noncovalent interactions of analogues of nucleobases, nucleosides, and nucleotides,[51,52] in particular as binders to major or minor grooves of duplex DNA or RNA, it is reasonable to consider an analogous mechanism for the RENs as well. Since 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, 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 helicases. Corroborating this notion of possible DNA and/or RNA binding of RENs is our observation that some compounds form a tight complexes with a DNA substrate that were completely stable in the presence of 0.5% sodium dodecyl sulfate (SDS).

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: (1) Do the above compounds of Table 1 inhibit the viral NTPase reaction as well? (2) If they do, is the inhibition of the NTPase reaction coupled to the observed inhibition of the unwinding (helicase) reaction by RENs?

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; 105 μM for HCV; 235 μM for JEV, 4.2 μM for $Suv3_{(\Delta 1-159)}$], 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.

Surprisingly, however, 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, 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.[53] Interestingly, the use of a 5′-triphosphate derivative of ribavirin transformed it into a highly potent, competitive inhibitor of ATPase activity of both HCV and WNV NTPases/helicases. To verify if a similar phenomenon would exist in the case of RENs, we synthesized the 5′-triphosphate derivatives, **33** and **34** (Scheme 6), of two RENs, **31** and **32**, respectively, whose synthesis we have reported earlier.[43,54] Neither the nucleoside analogues **31** and **32** nor their respective 5′-triphosphate derivatives **33** and **34** inhibited the unwinding activity of the *Flaviviridae* and Suv3 NTPase/helicases even up to concentration of 1.5 mM. When the ATPase activity of the WNV NTPase/helicase was determined as a function of increasing concentrations of RENs at ATP concentration equal to the K_m (9.5 μM) of the enzyme, neither the nucleoside **31** nor its heterocyclic aglycon **29** had any influencing effect. By contrast, the triphosphate derivative **33** significantly activated the ATP hydrolysis. The latter activating effect could be further enhanced by increasing the ATP concentration in the reaction mixture (see Figure 3). Interestingly yet, at reduced ATP concentrations that are below 1/10th of K_m of the enzyme, compound **33** exerted an inhibitory effect on the ATPase activity. Thus, for example, at an ATP concentration corresponding to 1×10^{-5} K_m, an IC_{50} value of 0.15 μM was measured.

An analogous observation was made when we investigated nucleoside **32** and its 5′-triphosphate derivative **34**. Compounds **32** and **34** did not influence the helicase activity of either HCV or WNV NTPase/helicase up to concentrations of 1.5 mM. On the other hand, when ATPase activity of the WNV enzyme was measured in the presence of ATP concentrations equal to the enzyme's K_m value, 300 μM concentration of **34** caused 2.5 to 3-fold stimulation of the hydrolytic activity of the enzyme. Higher concentrations of ATP in the reaction mixture caused further dramatic increase in stimulation of hydrolytic activity, for example, in the presence of

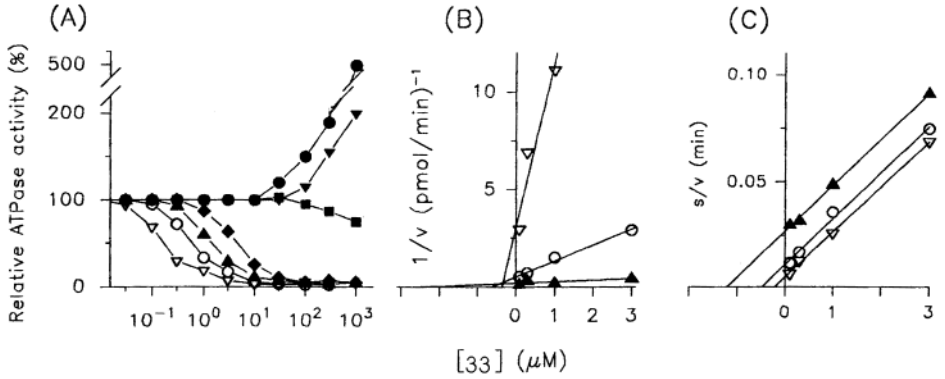


Figure 3. Modulation of the ATPase activity of the WNV NTPase/helicase by a ring-expanded nucleoside-5′-triphosphate (**33**) with variations in ATP concentration. (A) Investigation of the ATPase activity as a function of increasing concentrations of **33** in the presence of different ATP concentrations. The demonstrated assays were performed at ATP concentrations equal to (●) 95 μM; (▼) 9.5 μM; (■) 0.95 μM; (◆) 95 nM; (▲) 9.5 nM; (○) 0.95 nM; and (▽) 95 pM. (B and C) Kinetic analysis of the inhibition of the WNV ATPase activity by **33** at lower ATP concentrations. The kinetic data obtained at ATP concentrations equal to 9.5 nM, 0.95 nM, and 95 pM were replotted according to the methods of Dixon[61] (B) and Cornish−Bowden[62] (C). The results presented are an average of three independent experiments.

Table 3. Inhibitory Activity of Ring-Expanded Nucleoside (REN)-5'-triphosphates against the ATPase Activity of WNV, HCV, JEV, and Suv3$_{(\Delta 1-159)}$ NTPase/Helicase[a]

compd	WNV IC_{50} (μM)	HCV IC_{50} (μM)	JEV IC_{50} (μM)	Suv3$_{(\Delta 1-159)}$ IC_{50} (μM)
33	0.15 [0.38]	0.55 [0.2]	12 [3.5]	40 [15]
34	4 [2.9]	1.5 [1.7]	40 [15]	125 [45]

[a] The ATPase activity was determined as a function of increasing concentrations of the compounds in the presence of ATP adjusted to concentrations corresponding to 1×10^{-5} of K_m of each enzyme (95 pM, 1.05 nM, 2.35 nM, and 42 pM for WNV, HCV, JEV, and Suv3$_{(\Delta 1-159)}$ NTPase/helicase, respectively) for the determination of the IC_{50} values as well as to 1×10^{-5} of K_m and 1×10^{-4} of K_m for the determination of the respective K_i values (quoted in squared bracket). The ATPase 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 as described under Biochemical Methods in the Experimental Section. The ATPase activity of the enzyme measured in the absence of the compounds was referred to as 100%. The results presented are an average of three independent experiments.

100 μM ATP and 300 μM **34**, an increase of 1000−1100% ATPase activity was measured. Once again, as in the case of **33**, the inhibition of ATPase could be observed with lowered ATP concentrations, for example, to as low as 1×10^{-6} or lower of the enzyme's K_m value. The inhibitory effects of compounds **33** and **34** against ATPase activity of WNV, HCV, JEV, and Suv3$_{(\Delta 1-159)}$ NTPase/helicases are presented in Table 3.

The inspection of the above kinetic data reveals that the inhibition of the ATPase activity by the REN triphosphates **33** and **34** can be best approximated by a competitive inhibition mechanism with respect to ATP. The inhibition data, however, do not explain the mechanism of the observed activation or stimulation of the ATPase activity by **33** and **34** at higher ATP concentrations. The well-documented ATP binding studies of Porter et al.[25,26] together with our own kinetic data analyses suggest strongly the existence of a second nucleoside binding site within the SFII NTPases/helicases of *Flaviviridae*. One could speculate that the first one, equipped with Walker motifs [the short amino acid sequences interacting with β- and γ-phosphorus atom of nucleoside-5′-triphosphate (Walker A), and with Mg^{2+} or Mn^{2+} that are chelated by the β- and γ-phosphorus atom (Walker B)], which is known to be involved in the hydrolysis of NTPs, possesses significantly higher affinity for nucleoside-5′-triphosphates. By contrast, the second binding site has a much lower 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. In corroboration of this hypothesis are our preliminary binding studies that were performed employing the isolated domain I of HCV NTPase/helicase, which contains the first NTP binding site with Walker motifs. We have discovered that the well-known ATPase activator O^6-benzyl-N^7-chloroethylguanine or the helicase activator N^7-chloroethylguanine[27] is unable to displace either FSBA [5'-*O*-(4-fluorosulfonylbenzoyl)adenosine] or ATP that is bound to the enzyme.

The above REN-mediated inhibition and/or activation results, obtained separately against the helicase and ATPase activities of WNV, HCV, and JEV NTPases/helicases, prompt us to believe that the enzymes have an additional allosteric binding site where nucleoside/nucleotide-type molecules bind. Occupation of this site is largely responsible for modulation of NTPase and/or helicase. It is not clear at this point 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. Likewise, the REN-triphosphates exert their inhibitory effect on viral NTPase at low ATP concentrations by binding to the enzyme's NTP binding site (Walker motifs). However, at high concentrations of ATP, they 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 NTPases/helicases.

Conclusions

There exists more than one mechanism by which ring-expanded nucleosides and nucleotides modulate the activities of *Flaviviridae* NTPase/helicase, which, in this study, included the West Nile virus, the hepatitis C

virus, and the Japanese encephalitis virus enzymes. The specific modulation of viral enzyme activities that were investigated include the helicase and NTPase activities of WNV, HCV, and JEV helicases/NTPases. 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-, α-anomeric, and acyclic nucleoside analogues, as well as their heterocyclic aglycons, concerns either inhibition or activation of the viral helicase activity. In the case of inhibition, the mechanism of action might involve the interaction of the REN with a DNA or an RNA substrate of the helicase through binding to the major or minor groove of the double helix. The REN-5′-triphosphates, on the other hand, exert their inhibitory effect on the viral NTPase activity, but this activity is highly dependent upon the low concentration levels of the substrate ATP, preferably below 1/10th the K_m value of the enzyme. However, at higher concentrations of both ATP ($>10 \times K_m$) and the REN ($>500\ \mu$M), an activating effect of the enzyme was observed, which underwent dramatic enhancement (>1000%) by further increases in ATP concentration. The contemplated mechanistic model to explain the observed activating effect includes an additional allosteric binding site that can be occupied by nucleoside/nucleotide-type molecules including, but not limited to, the RENs. The occupation of this allosteric site is highly dependent upon the level of ATP (NTP) concentration in the reaction mixture.

A few additional points are worthy of comment. The three *Flaviviridae* enzymes investigated in this study, including WNV, HCV, and JEV, along with the truncated human enzyme $Suv3_{(\Delta 1-159)}$, all contain similar molecular structures with a close sequence homology of amino acids constituting their respective active sites. But this fact alone does not seem to be an obstacle to produce highly specific inhibitors for each of the four enzymes as they responded quite differently to various RENs tested. This is somewhat consistent with what is known with some other enzyme systems. For example, Szyszka, et al. [55] have developed a range of halogenated benzimidazoles and benzotriazoles that exhibit excellent discrimination between the ATP-binding sites of closely related casein kinases. In this context, ring-expanded nucleosides and nucleotides may represent a starting point for the development of highly selective inhibitors of the viral NTPases/helicases belonging to viral Super Family II (SFII). The site of binding of RENs on the viral enzymes could be an attractive target for the development of inhibitors, and therefore, mapping the three-dimensional structure of the enzyme binding site through a range of REN inhibitors and/or activators would be rewarding.

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, and to H_3PO_4 (external standard) for ^{31}P NMR spectra. 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).

Methyl 1-[(3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl))-β-D-ribofuranosyl]-4,5-imidazoledicarboxylate (5). To a solution of dry methyl 1-β-D-ribofuranosyl-4,5-imidazoledicarboxylate [56] (**4**) (500 mg, 1.58 mmol) in dry pyridine (10 mL) was added 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (550 mg, 1.75 mmol), and the mixture was stirred for 4 h at ambient temperature with protection from moisture. Volatile materials were evaporated in vacuo, and the residue was dissolved in chloroform. The chloroform solution was washed twice with cold water and dried over anhydrous sodium sulfate. The residue after evaporation was purified by silica gel flash chromatography, eluting with chloroform to give **5** as a colorless liquid, yield 800 mg (91%), R_f 0.38 (chloroform/methanol, 30:1); ^{1}H NMR ($CDCl_3$) δ 8.10 (s, 1H, imidazole), 6.11 (s, 1H, 1′-H), 4.44 (dd, 1H, J = 4.2 and 9.0 Hz, 3′-H), 4.26 (d, 1H, J = 13.5 Hz, 5′-H), 4.18 (d, 1H, J = 4.2 Hz, 2′-H), 4.16 (dd, 1H, J = 9.0 and 2.4 Hz, 4′-H), 4.03 (dd, 1H, J = 13.5 Hz, J = 2.4 Hz, 5′-H), 3.95 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 2.89 (brs, 1H, 2′-OH, exchangeable with D_2O), 1.05 (m, 28H, isopropyl groups); ^{13}C NMR ($CDCl_3$, 75.48 MHz) δ 12.45, 12.82, 12.90, and 13.35 (CHSi), 16.81, 16.87, 16.97 (2), 17.26, 17.26, 17.32, and 17.42 (CCH_3), 52.50 (OCH_3), 52.78 (OCH_3), 59.91 (C-5′), 68.43 (C-3′), 76.82 (C-2′), 81.87 (C-4′), 91.54 (C-1′), 122.83 (C-4 or 5), 137.26 (C-2), 138.57 (C-5 or 4), 160.26 (C═O), 162.91 (C═O). Anal. ($C_{24}H_{42}N_2O_9Si_2$). C, H, N.

Methyl 1-[(2′-*O*-Phenoxythiocarbonyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-ribofuranosyl)]-4,5-imidazoledicarboxylate (6). To a solution of **5** (560 mg, 1 mmol) and DMAP (250 mg, 2.05 mmol) in 15 mL of dry acetonitrile was added phenoxythiocarbonyl chloride (200 μL, 250 mg, 1.45 mmol). The solution was stirred for 16 h at ambient temperature, and evaporated to dryness in vacuo. The residue was purified by column chromatography (silica gel), eluting with chloroform to give a colorless oily product (660 mg, 95%). R_f 0.60, chloroform/methanol (30:1); ^{1}H NMR ($CDCl_3$) δ 8.16 (s, 1H, imidazole), 7.43 (t, 2H, J = 7.8 Hz, Ph_{meta}-H), 7.31 (t, 1H, J = 7.8 Hz, Ph_{para}-H), 7.13 (d, 2H, J = 7.8 Hz, Ph_{ortho}-H), 6.38(s, 1H, 1′-H), 6.01 (d, 1H, J = 4.5 Hz, 2′-H), 4.61 (dd, 1H, J = 4.5 and 9.3 Hz, 3′-H), 4.31 (d, 1H, J_{gem} = 13.5 Hz, 5′-H), 4.16 (dd, 1H, J = 9.3 and 2.4 Hz, 4′-H), 4.05 (dd, 1H, J_{gem} = 13.5 Hz, $J_{5',4'}$ = 2.4 Hz, 5′-H), 3.93 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 1.06 (m, 28H, isopropyl groups); ^{13}C NMR ($CDCl_3$, 75.48 MHz) δ 12.80 (CHSi), 12.91 (CHSi), 13.37 (CHSi), 13.49 (CHSi), 16.87 (CCH_3), 16.94 (CCH_3), 16.96 (CCH_3), 17.08 (CCH_3), 17.24 (CCH_3), 17.27 (CCH_3), 17.34 (CCH_3), 17.44 (CCH_3), 52.49 (OCH_3), 52.74 (OCH_3), 59.29 (C-5′), 67.69 (C-3′), 82.39 (C-4′), 85.10 (C-2′), 89.40 (C-1′), 121.68 (Ph-$C_{2,6}$), 126.69 (Ph-C_4), 129.57 (Ph-$C_{3,5}$), 137.13 (C-2), 138.93 (C-5 or 4), 139.24 (C-4 or 5), 153.47 (Ph-C_1), 159.79 (C═O), 162.76 (C═O), 193.70 (C═S). Anal. ($C_{31}H_{46}N_2O_{10}SSi_2$) C, H, N.

Methyl 1-[2′-Deoxy-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-erythropentofuranosyl]-4,5-imidazoledicarboxylate (7). A solution of **6** (695 mg, 1 mmol) and AIBN (32 mg, 0.2 mmol) in 30 mL of dry toluene was purged with nitrogen for 30 min. Tributylstannane (400 μL, 433 mg, 1.49 mmol) was added, and the solution was refluxed for 3 h. TLC showed a single spot with very slightly lower R_f value than that of starting material. The solvent was evaporated, and the pure product was obtained as a colorless liquid by silica gel column chromatography (hexane/ethyl acetate, 3:1) in almost quantitative yield. R_f 0.12, hexane/ethyl acetate (3:1); ^{1}H NMR ($CDCl_3$) δ 8.09 (s, 1H, imidazole), 6.33(dd, 1H, J = 7.4 and 1.2 Hz, 1′-H), 4.54 (dt, 1H, J = 10.2 and 7.4 Hz, 3′-H), 4.11 (dd, 1H, J_{gem} = 13.2 Hz, $J_{5',4'}$ = 2.4 Hz, 5′-H), 4.06 (dd, 1H, J_{gem} = 13.2 Hz, $J_{5',4'}$=3.0 Hz, 5′-H), 3.93 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.82 (dt, 1H, J = 8.2 and 2.6 Hz, 4′-H), 2.64 (ddd, 1H, J = 13.2, 10.2 and 7.4 Hz, 2′-H), 2.35 (ddd, 1H, J = 13.2, 7.4 and 1.2 Hz, 2′-H), 1.06 (m, 28H, isopropyl groups); ^{13}C NMR ($CDCl_3$, 75.48 MHz) δ 12.54 (CHSi), 12.94 (CHSi),

13.04 (CHSi), 13.43 (CHSi), 16.86 (CCH_3), 16.97 (CCH_3), 16.97 (CCH_3), 17.09 (CCH_3), 17.31 (CCH_3), 17.31 (CCH_3), 17.39 (CCH_3), 17.49 (CCH_3), 41.70 (C-2′), 52.34 (OCH_3), 52.50 (OCH_3), 60.48 (C-5′), 67.52 (C-3′), 85.25 (C-4′), 86.02 (C-1′), 136.41 (C-4 or 5), 137.12 (C-2), 138.41 (C-5 or 4), 160.37 (C=O), 163.04 (C=O). Anal. ($C_{24}H_{42}N_2O_8Si_2$) C, H, N.

Methyl 1-(2′-Deoxy-β-D-erythropentofuranosyl)-4,5-imidazoledicarboxylate (8). A solution of 1 M tetra-*n*-butylammonium fluoride in THF (2 mL, 2 mmol) was added to an ice-cold solution of **7** (543 mg, 1 mmol) in 10 mL of dry THF. The reaction was stopped after 45 min stirring at 0 °C. The solvent was evaporated in vacuo, and the pure product was obtained in 75% yield as a foam by silica gel column chromatography (chloroform/methanol, 10:1. R_f 0.20, chloroform/methanol (10:1); ^{1}H NMR ($CDCl_3$) δ 8.51 (s, 1H, imidazole), 6.38 (dd, 1H, J = 6.2 and 3.4 Hz, 1′-H), 4.82 (brs, 1H, OH, exchangeable with D_2O), 4.54 (dd 1H, J = 12.8 and 6.6 Hz, 3′-H), 4.15 (brs, 1H, OH, exchangeable with D_2O), 3.98 (m, 3H, 4′,5′-H), 3.91 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 2.56 (ddd, 1H, J = 13.6, 12.8 and 6.2 Hz, 2′-H), 2.35 (ddd, 1H, J = 13.6, 6.6 and 3.4 Hz, 2′-H); ^{13}C NMR ($CDCl_3$, 75.48 MHz) δ 42.49 (C-2′), 52.42 (OCH_3), 52.73 (OCH_3), 60.62 (C-5′), 68.91 (C− 3′), 87.03 (C-4′), 87.21 (C-1′), 136.50 (C-4 or 5), 136.86 (C-5 or 4), 137.68 (C-2), 160.40 (C=O), 163.05 (C=O). Anal. ($C_{12}H_{16}N_2O_7$) C, H, N.

6-Amino-1-(2′-deoxy-β-D-erythropentofuranosyl)-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (9). To a solution of **8** (150 mg, 0.5 mmol) and guanidine hydrochloride (240 mg, 2.5 mmol) in 30 mL of absolute methanol was added 1 mL of 25% (w/v) sodium methoxide solution in methanol. The reaction mixture was stirred for 24 h at room temperature, and then 1 g of silica gel was added. The resulting mixture was evaporated to dryness and applied to a silica gel column. The column was successively eluted with chloroform/methanol (10:1) and methanol. The proper fraction was collected and evaporated to afford **9** as a colorless solid, yield 110 mg (74.5%), mp >250 °C. ^{1}H NMR (DMSO-d_6) δ 10.65 (brs, 1H, NH, exchangeable with D_2O), 8.47 (s,1H, imidazole), 7.62 (brs, 1H, NH, exchangeable with D_2O), 6.65 (t, 1H, J = 5.7 Hz, 1′-H), 6.54 (brs, 1H, NH, exchangeable with D_2O), 5.27 (d, 1H, J = 4.5 Hz, 3′-OH, exchangeable with D_2O), 5.09 (t, 1H, J = 4.8 Hz, 5′-OH, exchangeable with D_2O), 4.24 (m, 1H, 4′-H), 3.84 (m, 1H, 3′-H), 3.60 (m, 2H, 5′-H), 2.18 (dt, 1H, J = 13.5 and 5.7 Hz, 2′-H), 2.02 (d, 1H, J = 13.5 Hz, 2′-H). HRMS (FAB) calcd for $C_{11}H_{14}N_5O_5$ (MH^+) *m/z* 296.0995, found, 296.0994. Anal. ($C_{11}H_{13}N_5O_5 \cdot 0.25H_2O$) C, H, N.

Methyl 1-(2′-Deoxy-3′,5′-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl)-4,5-imidazoledicarboxylate (13). A solution of methyl 4,5-imidazoledicarboxylate[35,36] (1.84 g, 10 mmol) and methyl 2-deoxy-3,5-di-*O*-*p*-toluoyl-β-D-erythropentofuranoside (**11**)[57] (3.84 g, 10 mmol) in dry acetonitrile (50 mL) was placed into a flame-dried 100 mL round-bottom flask. The solution was stirred in an ice bath for 10 min. Then, 1,1,1,3,3,3-hexamethyldisilazane (HMDS) (7 mL, 33 mmol), chlorotrimethylsilane (TMSCl) (4.5 mL, 36 mmol), and trifluoromethanesulfonic acid (TFMSA) (3 mL, 36 mmol) were consecutively added to the above solution. The resulting solution was stirred in an ice bath for 1 h. The reaction is complete by TLC analysis [silica gel plates, chloroform:methanol (30:1)]. The reaction mixture was evaporated to dryness in vacuo. The resulting residue was dissolved in chloroform and washed successively with saturated aqueous sodium bicarbonate and water. After being dried over anhydrous $MgSO_4$ and filtered, the chloroform solution was evaporated to dryness in vacuo. The residue was purified by column chromatography, eluting with chloroform to give pure **13** as a colorless oily product (4.6 g, 86%), R_f 0.83 [chloroform−methanol (10:1)]. ^{1}H NMR ($CDCl_3$) δ 7.97 (s, 1H, imidazole), 7.95 (d, 2H, J = 8.1 Hz, *o*-Ph), 7.62 (d, 2H, J = 8.1 Hz, *o*-Ph), 7.28 (d, 2H, J = 8.1 Hz, *m*-Ph), 7.20 (d, 2H, J = 8.1 Hz, *m*-Ph), 6.65 (d, 1H, J = 6.6 Hz, 1′-H), 5.65 (d, 1H, J = 6.6 Hz, 3′-H), 4.92 (t, 1H, J = 3.6 Hz, 4′-H), 4.58 (d, 2H, J = 3.6 Hz, 5′_H), 3.94 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 3.07 (dt, 1H, J = 15.6 and 6.6 Hz, 2′β-H), 2.57 (d, 1H, J = 15.6 Hz, 2′α-H), 2.44 (s, 3H, $PhCH_3$), 2.39 (s, 3H, $PhCH_3$); ^{13}C NMR ($CDCl_3$) δ 21.58 (2$PhCH_3$), 40.93 (C-2′), 52.23 (OCH_3), 52.48 (OCH_3), 63.99 (C-5′), 74.59 (C-3′), 85.52 (C-4′), 89.18 (C-1′), 129.28 (2 *m*-Ph), 129.33 (2 *m*-Ph), 129.59 (2 *o*-Ph), 129.68 (2 *o*-Ph), 136.47 (C-4 or 5), 137.11 (C-2), 138.58 (C-5 or 4), 144.16 (Ph-C_1), 144.47 (Ph-C_1), 160.48 (C=O), 162.87 (C=O), 165.76 (PhC=O), 166.02 (PhC=O). Anal. ($C_{28}H_{28}N_2O_9 \cdot 0.3CF_3SO_3H$) C, H, N.

6-Amino-1-(2′-deoxy-α-D-erythropentofuranosyl)-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (15). Guanidine hydrochloride (0.23 g, 2.4 mmol) was added to a solution of methyl 1-(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl)-4,5-imidazoledicarboxylate (**13**) (0.32 g, 0.6 mmol) in 30 mL of absolute methanol, containing 1 mL of 25% (w/v) sodium methoxide solution. The mixture was stirred at room temperature for 48 h. The reaction mixture was evaporated in vacuo to a small volume and then was isolated by preparative TLC on silica gel to give pure **15** as a white solid (150 mg, 85%), mp >250 °C, R_f 0.41 [chloroform:methanol:30% ammonium hydroxide (2:2:1)]; ^{1}H NMR (DMSO-d_6) δ 10.64 (brs, 1H, NH, exchangeable with D_2O), 8.13 (s, 1H, imidazole), 7.08 (brs, 1H, NH, exchangeable with D_2O), 6.67 (d, 1H, J = 6.0 Hz, 1′-H), 6.50 (brs, 1H, NH, exchangeable with D_2O), 5.09 (brs, 1H, OH, exchangeable with D_2O), 4.86 (brs, 1H, OH, exchangeable with D_2O), 4.26 (t, 1H, J = 4.5 Hz, 4′-H), 4.19 (d, 1H, J = 6.0 Hz, 3′-H), 3.41 (d, 2H, J = 4.5 Hz, 5′-H), 2.63 (dt, 1H, J = 14.4 and 6.0 Hz, 2′β-H), 2.00 (d, 1H, J = 14.4 Hz, 2′α-H); ^{13}C NMR (DMSO-d_6) δ 43.25 (C-2′), 62.13 (C-5′), 71.25 (C-3′), 89.06 (C-4′), 90.38 (C-1′), 140.41 (C-2, 3a, 4, 8, 8a), 153.30 (C-6); HRMS (FAB) calcd for $C_{11}H_{14}N_5O_5$ (MH^+) *m/z* 296.0995, found, 296.0992. Anal. (C11H13N5O5) C, H, N.

Methyl 1-[(2′-*O*-Methyl-3′,5′-*O*-(1,1,3,3,-tetraisopropyldisiloxane-1,3-diyl))-β-D-ribofuranosyl]-4,5-imidazoledicarboxylate (16). A mixture of methyl 1-[(3′,5′-*O*-(1,1,3,3,-tetraisopropyldisiloxane-1,3-diyl))-β-D-ribofuranosyl]-4,5-imidazoledicarboxylate (**5**) (560 mg, 1 mmol), Ag_2O (1.85 g, 8 mmol), and MeI (10 mL) was refluxed for 5 h. The mixture was diluted with Et_2O and was filtered over Celite. The filtrate was concentrated in vacuo, and the residue was purified by silica gel column chromatography, eluting with chloroform, to give **16** as a colorless oily product in quantitative yield, R_f 0.35 (hexane/ethyl acetate, 3:1); ^{1}H NMR ($CDCl_3$) 8.20 (s, 1H, imidazole), 6.02 (s, 1H, 1′-H), 4.42 (dd, 1H, J = 4.2 and 9.6 Hz, 3′-H), 4.28 (d, 1H, J_{gem} = 13.8 Hz, 5′-H), 4.16 (dd, 1H, J = 9.6 and 2.4 Hz, 4′-H), 4.00 (dd, 1H, J_{gem} = 13.8 Hz, $J_{5',4'}$ = 2.4 Hz, 5′-H), 3.94 (s, 3H, $COOCH_3$), 3.93 (s, 3H, $COOCH_3$), 3.78 (d, 1H, J = 4.2 Hz, 2′-H), 3.69 (s, 3H, OCH_3), 1.05 (m, 28H, isopropyl groups); ^{13}C NMR ($CDCl_3$, 75.48 MHz) 12.53 (CHSi), 12.90 (CHSi), 12.96 (CHSi), 13.46 (CHSi), 16.87 (CCH_3), 17.01 (CCH_3), 17.01 (CCH_3), 17.15 (CCH_3), 17.30 (CCH_3), 17.30 (CCH_3), 17.38 (CCH_3), 17.49 (CCH_3), 52.45 ($COOCH_3$), 52.62 ($COOCH_3$), 59.46 (OCH_3), 59.96 (C-5′), 68.66 (C-3′), 81.59 (C-4′), 84.99 (C-2′), 90.76 (C-1′), 122.84 (C-4 or 5), 137.55 (C-2), 138.57 (C-5 or 4), 160.23 (C=O), 163.06 (C=O). Anal. ($C_{25}H_{44}N_2O_9Si_2$) C, H, N.

Methyl 1-(2′-*O*-Methyl-β-D-ribofuranosyl)-4,5-imidazoledicarboxylate (17). A 1 M solution of tetra-*n*-butylammonium fluoride in THF (2 mL, 2 mmol) was added to an ice-cold solution of methyl 1-[(2′-*O*-methyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl))-β-D-ribofuranosyl]-4,5-imidazoledicarboxylate (**16**) (573 mg, 1 mmol) in 10 mL of dry THF. The reaction mixture was stirred for 45 min at 0 °C. The solvent was evaporated in vacuo, and the pure product **17** was obtained as a foam after silica gel column chromatography, eluting with a mixture of chloroform−methanol (20:1), 85% yield, R_f 0.29 (chloroform/methanol, 10:1); ^{1}H NMR ($CDCl_3$) 8.67 (s, 1H, imidazole), 6.17 (d, 1H, J = 2.1 Hz, 1′-H), 5.03 (brs, 1H, 3′-OH, exchangeable with D_2O), 4.47 (t, 1H, 5′-OH, exchangeable with D_2O), 4.12 (m, 2H, 2′,3′-H), 3.93 (s, 3H, $COOCH_3$), 3.91 (s, 3H, $COOCH_3$), 3.91 (m, 2H, 4′,5′-H), 3.60 (s, 3H, OCH_3), 3.32 (m, 1H, 5′-H); ^{13}C NMR ($CDCl_3$, 75.48 MHz) 52.31 ($COOCH_3$), 52.74 ($COOCH_3$), 59.10 (OCH_3), 60.03 (C-5′), 68.15 (C-3′), 84.85 (C-4′), 85.58 (C-2′), 88.83 (C-1′), 123.96 (C-4 or 5), 136.43 (C-5 or 4), 138.37 (C-2), 160.52 (C=O), 162.26 (C=O). Anal. ($C_{13}H_{18}N_2O_8$) C, H, N.

6-Amino-4,5-dihydro-8*H*-1-(2′-*O*-methyl-*β*-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (18). To a solution of methyl 1-(2′-*O*-methyl-*β*-D-ribofuranosyl)-4,5-imidazoledicarboxylate (0.33 g, 1 mmol) and guanidine hydrochloride (0.48 g, 5 mmol) in 50 mL of absolute methanol was added 2 mL of 25% sodium methoxide in methanol. The reaction mixture was stirred for 24 h at room temperature, and then 2 g of silica gel was added. The resulting mixture was evaporated to dryness and applied to a silica gel column. The column was successively eluted with chloroform/methanol (10:1) and methanol. The appropriate fractions were collected and evaporated to afford **18** as a colorless solid: 225 mg (69%), mp >250 °C; ^{1}H NMR (DMSO-d_6): δ 10.72 (brs, 1H, NH, exchangeable with D_2O), 8.69 (s,1H, imidazole), 7.66 (brs, 1H, NH, exchangeable with D_2O), 6.59 (brs, 1H, NH, exchangeable with D_2O), 6.50 (d, 1H, J = 1.8 Hz, 1′-H), 5.27 (t, 1H, J = 4.8 Hz, 5′-OH, exchangeable with D_2O),), 5.14 (d, 1H, J = 6.6 Hz, 3′-OH, exchangeable with D_2O), 4.17 (m, 1H, 2′-H), 3.95 (m, 1H, 3′-H), 3.90 (m, 1H, 4′-H), 3.77 (m, 1H, 5′-H$_1$), 3.59 (m, 1H, 5′-H$_2$), 3.45 (s, 3H, OCH_3); HRMS (FAB) calcd for $C_{12}H_{16}N_5O_6$ (MH^+) 326.1101, found, 326.1100. Anal. ($C_{12}H_{15}N_5O_6\cdot 0.75H_2O$) C, H, N.

Methyl 1-(2-Benzyloxyethoxymethyl)-4,5-imidazoledicarboxylate (19). A mixture of methyl 4,5-imidazoledicarboxylate (0.92 g, 5 mmol) and K_2CO_3 (0.7 g, 5 mmol) was stirred for 1 h at 80 °C. Then a solution of 1-benzyloxy-2-chloromethoxyethane (1.5 g, 7.5 mmol) in 20 mL of DMF was dropwise added, and the mixture was continued to stir for 24 h at 90 °C. The reaction mixture was evaporated under reduced pressure, and the residue was dissolved in 30 mL of water and then neutralized with 2 M hydrochloric acid. The solution was extracted with chloroform. The extract was evaporated in vacuo, and the residue was purified by silica gel column chromatography, eluting with chloroform to give **19** as a colorless liquid: 1.22 g (70%), R_f 0.31 (chloroform−methanol (30:1)). ^{1}H NMR ($CDCl_3$) δ 7.72 (s, 1H, imidazole), 7.24−7.35 (m, 5H, Ph), 5.66 (s, 2H, NCH_2O), 4.52 (s, 2H, $PhCH_2$), 3.92 (s, 3H, OCH_3), 3.91 (s, 2H, OCH_3), 3.63 (m, 4H, OCH_2CH_2O); ^{13}C NMR ($CDCl_3$) δ 52.16, 53.10, 69.06, 69.71, 73.25, 76.44, 127.58, 127.58, 127.66, 127.66, 128.32, 128.32, 137.65, 139.38, 139.47, 160.21, 162.45. Anal. (Calcd. for $C_{17}H_{20}N_2O_6$) C, H, N.

Methyl 1-(2-Hydroxyethoxymethyl)-4,5-imidazoledicarboxylate (20). Methyl 1-(2-benzyloxyethoxymethyl)-4,5-imidazoledicarboxylate (**19**) (1.74 g, 5 mmol) was dissolved in a mixture of 20 mL of methanol and 5 mL of glacial acetic acid, and the solution was transferred to a hydrogenation bottle. Palladium on activated carbon (10%) (1.74 g) was added, and the bottle was shaken in a Parr hydrogenation apparatus at 50 psi for 48 h. The reaction mixture was filtered through a pad of Celite. A TLC analysis showed that the reaction was complete. The filtrate was evaporated to dryness. The residue was purified by silica gel flash chromatography, eluting first with chloroform and then with chloroform−methanol (20:1) to give **20** as a pure waxy product (semisolid) (1.1 g, 85%), R_f 0.37 ($CHCl_3$−MeOH, 10:1); ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 1H, imidazole), 5.57 (s, 2H, NCH_2O), 4.70 (brs, 1H, OH, exchangeable with D_2O), 3.83 (s, 3H, OCH_3), 3.78 (s, 3H, OCH_3), 3.40 (m, 4H, OCH_2CH_2OH); ^{1}H NMR ($CDCl_3$) δ 7.75 (s, 1H, imidazole), 5.62 (s, 2H, NCH_2O), 3.90 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.69 (t, 2H, J = 4.8 Hz, OCH_2CH_2OH), 3.55 (t, 2H, J = 4.8 Hz, OCH_2CH_2OH), 2.80 (brs, 1H, OH, exchangeable with D_2O); ^{13}C NMR (DMSO-d_6) δ 52.05, 52.69, 59.75, 70.44, 75.84, 136.27, 140.85, 140.97, 160.05, 162.73. Anal. ($C_{10}H_{14}N_2O_6$) C, H, N.

Methyl 1-(2-Hydroxyethyl)-4,5-imidazoledicarboxylate (22). A mixture of methyl imidazole-4,5-dicarboxylate[35,36] (1.47 g, 8 mmol) and potassium carbonate (1.1 g, 8 mmol) in 40 mL of anhydrous DMF was stirred at 100 °C for 3 h, a solution of bromoethyl acetate (2.0 g, 12 mmol) in 10 mL of dry DMF was added to the above mixture, and the mixture was allowed to to stir for 48 h at 100 °C. The reaction mixture was evaporated to dryness in vacuo, and the residue was dissolved in water and neutralized with 1 M hydrochloric acid, followed by extraction with chloroform. The extract was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The resulting residue was purified by column chromatography on silica gel, eluting with chloroform to give pure methyl 1-(2-acetoxyethyl)-4,5-imidazoledicarboxylate (**21**) as a colorless oily product (1.62 g, 75%), R_f 0.73 (chloroform: methanol (10:1)). This compound was directly employed in the next step without further purification.

A mixture of the above methyl 1-(2-acetoxyethyl)-4,5-imidazoledicarboxylate (**21**) (1.5 g, 5.5 mmol) and Dowex 50WX8−200 ion-exchange resin (1.5 g) in 45 mL of methanol was refluxed with stirring for 48 h. The mixture was filtered, and the resin was washed with methanol. The combined filtrate was evaporated in vacuo, and the residue was recrystallized from methanol to afford **22** as white crystals (1.14 g, 91%): R_f 0.51 (chloroform:methanol (10:1)); ^{1}H NMR (DMSO-d_6) δ 8.07 (s, 1H, imidazole), 4.60 (t, 2H, J = 4.8 Hz, CH_2N), 4.39 (t, 2H, J = 4.8 Hz, OCH_2), 3.79 (s, 6H, OCH_3). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

Methyl 1-[(2-Diethoxyphosphonylmethoxy)ethyl]-4,5-imidazoledicarboxylate (23). A mixture of methyl 1-(2-hydroxyethyl)-4,5-imidazoledicarboxylate (**22**) (1.0 g, 4.38 mmol) and sodium hydride (60% suspension in oil) (0.18 g, 4.5 mmol) in 40 mL of anhydrous DMF was stirred for 30 min, a solution of diethyl *p*-toluenesulfonyloxymethanephosphonate[58] (1.5 g, 4.65 mmol) in 5 mL of dry DMF was added, and the reaction mixture was allowed to stir for 48 h. The reaction mixture was evaporated in vacuo. The residue was extracted with chloroform, and the chloroform layer was successively washed with 0.1 M hydrochloric acid and water. After being dried over anhydrous $MgSO_4$, the chloroform extract was evaporated to dryness in vacuo. The resulting residue was purified by column chromatography on silica gel, eluting with chloroform to give **23** as a colorless liquid (1.33 g, 80%): R_f 0.74 (chloroform: methanol (10:1)); ^{1}H NMR ($CDCl_3$) δ 7.67 (s, 1H, imidazole), 4.44 (t, 2H, J = 4.8 Hz, H-1′), 4.10 (dq, 4H, J_{P-OCH}=8.1 Hz, J = 6.9 Hz, CH_2CH_3), 3.92 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.88 (t, 2H, J = 4.8 Hz, H-2′), 3.75 (d, 2H, J_{P-CH} = 8.1 Hz, OCH_2P), 1.31 (t, 6H, J = 6.9 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$) δ 16.40 (s, CH_3), 16.48 (s, CH_3), 46.79 (s, C-1′), 52.17 (s, OCH_3), 52.40 (s, OCH_3), 62.33 (s, OCH_2CH_3), 62.41 (s, OCH_2-CH_3), 65.17 (d, $J_{P,C}$ = 166.4 Hz, OCH_2P), 71.50 (d, $J_{P,C-2'}$ = 9.9 Hz, C-2′), 137.08 (C-4 or 5), 139.81 (C-5 or 4), 140.63 (C-2), 160.44 (C=O), 162.74 (C=O); MS (EI) *m*/*z*: 379 (M + 1), 378 (M^+), 363 (M − 15), 347 (M − 31), 333 (M − 45), 319, 287, 257, 241, 225, 211 (100%), 193, 179, 165, 153, 139, 125, 95, 72; HRMS(FAB) calcd for $C_{14}H_{24}N_2O_8P$ (MH^+) 379.1270, found 379.1268. Anal. ($C_{14}H_{23}N_2O_8P\cdot H_2O$) C, H, N.

6-Amino-1-[(2-benzyloxyethoxy)methyl]-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (24). Guanidine hydrochloride (0.29 g, 3 mmol) was added to 3 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, and the filtrate was poured into a solution of pure methy l-benzyloxyethoxymethyl-4,5-imidazoledicarboxylate (**19**) (0.52 g, 1.5 mmol) in 10 mL of absolute methanol. The mixture was stirred at room temperature for 24−48 h, when a TLC analysis showed that the reaction was complete. The reaction mixture was evaporated to dryness in vacuo, and the residue was dissolved in water, neutralized with 2 M hydrochloric acid. The resulting precipitate was filtered to give the crude product (0.45 g, 87%) as a white solid which was recrystallized from a mixture of DMF and methanol to give a pure **24**: R_f 0.38 (chloroform−methanol−30% ammonium hydroxide (2:1:0.3)); mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 10.69 (brs, 1H, NH, exchangeable with D_2O), 8.25 (s, 1H, imidazole), 7.51 (brs, 1H, NH, exchangeable with D_2O), 7.25−7.32 (m, 5H, Ph), 6.63 (brs, 1H, NH, exchangeable with D_2O), 5.81 (s, 2H, NCH_2O), 4.42 (s, 2H, OCH_2Ph), 3.63 (t, 2H, J = 4.8 Hz, OCH_2CH_2OBn), 3.50 (t, 2H, J = 4.8 Hz,OCH_2CH_2OBn); ^{13}CNMR (DMSO-d_6) δ 68.15, 68.64, 71.96, 75.72, 127.35, 127.35, 127.52, 128.14, 128.14, 128.30, 138.22, 142.96, 142.99, 142.99, 143.01, 149.78. FAB-MS *m*/*z*: 344 (MH^+); EI-MS *m*/*z*

343, 315, 270, 240, 224, 209, 193, 180, 167, 164, 152, 138, 121, 107, 91 (100%), 79, 65. Anal. ($C_{16}H_{17}N_5O_4$) C, H, N.

6-Amino-1-[(2-hydroxyethoxy)methyl]-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (25). Guanidine hydrochloride (0.38 g, 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, and the filtrate was poured into a solution of methyl 1-(2-hydroxyethoxymethyl)-4,5-imidazoledicarboxylate (**20**) (0.52 g, 2 mmol) in 10 mL of absolute methanol. The mixture was stirred at room temperature for 48 h, when a TLC analysis showed the completion of reaction. The reaction mixture was evaporated to dryness in vacuo, and the residue was isolated by preparative TLC on silica gel plates to give pure **25** as a white solid: R_f 0.39 (chloroform−methanol−30% ammonium hydroxide (2:2:1)); 0.45 (acetonitrile:30% ammonium hydroxide (4:1); mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 8.25 (s, 1H, imidazole), 7.52 (brs, 1H, NH, exchangeable with D_2O), 7.09 (brs, 1H, NH, exchangeable with D_2O), 6.84 (brs, 1H, NH, exchangeable with D_2O), 5.79 (s, 2H, NCH_2O), 4.73 (brs, 1H, OH, exchangeable with D_2O), 3.37 (m, 4H, J = 4.8 Hz, OCH_2CH_2OH); ^{13}C NMR (DMSO-d_6) δ 59.92, 70.71, 75.98, 138.21, 143.21, 143.31, 143.31, 143.44, 149.87; FAB-MS m/z 254 (MH^+). Anal. ($C_9H_{11}N_5O_4 \cdot H_2O$) C, H, N.

6-Amino-1-[(2-phosphonylmethoxy)ethyl]-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (26). A solution of methyl 1-[(2-diethoxyphosphonylmethoxy)ethyl]-4,5-imidazoledicarboxylate (**23**) (0.48 g, 1.3 mmol) in 10 mL of dry acetonitrile was added bromotrimethylsilane (0.40 g, 2.6 mmol), and the mixture was stirred for 24 h at room temperature. After solvent evaporation, the residue was codistilled with acetonitrile (3 × 20 mL) and mixed with water (30 mL), and the mixture was adjusted to pH 8 with triethylamine. It was allowed to stand for 1 h and then evaporated in vacuo. The residue was codistilled with methanol (2 × 20 mL) and was purified by flash chromatography on silica gel, eluting successively with chloroform and chloroform−methanol (10:1). Appropriate fractions were pooled and evaporated to yield methyl 1-[(2-phosphonylmethoxy)ethyl]-4,5-imidazoledicarboxylate (precursor to **26**) as a colorless liquid (350 mg, 84%): R_f 0.13 (chloroform−methanol−30% ammonium hydroxide (2:1:0.3)); ^{1}H NMR (DMSO-d_6) δ 7.98 (s, 1H, imidazole), 5.64 (brs, 2H, OH, exchangeable with D_2O), 4.34 (t, 2H, J = 4.8 Hz, H-1′), 3.81 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.77 (t, 2H, J = 4.8 Hz, H-2′), 3.55 (d, 2H, J_{P-CH} = 8.4 Hz, OCH_2P); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ 46.41 (s, C-1′), 52.51 (s, OCH_3), 53.07 (s, OCH_3), 66.36 (d, $J_{P,C}$ = 160.2 Hz, OCH_2P), 71.09 (d, $J_{P,C-2'}$ = 10.2 Hz, C-2′), 135.32 (C-4 or 5), 141.43 (C-5 or 4), 141.43 (C-2), 160.45 (C=O), 162.95 (C=O); FABMS m/z 323 [MH^+]; HRMS (FAB) calcd for $C_{10}H_{16}N_2O_8P$ (MH^+) 323.0644, found 323.0649.

A solution of the above methyl 1-[(2-phosphonylmethoxy)-ethyl)]-4,5-imidazoledicarboxylate (0.32 g, 1 mmol) and guanidine hydrochloride (0.48 g, 5 mmol) in 50 mL of absolute methanol was stirred for 30 min at room temperature, and then 2 mL of 25% sodium methoxide solution in methanol was added. The resulting mixture was stirred for 48 h at room temperature. The formed precipitate was filtered and washed with anhydrous methanol to give disodium salt of **26** as a white solid (335 mg, 93%), mp >250 °C; ^{1}H NMR (DMSO-d_6 + D_2O) δ 8.17 (s, 1H, imidazole), 4.55 (t, 2H, J = 4.2 Hz, H-1′), 3.76 (t, 2H, J = 4.2 Hz, H-2′), 3.56 (d, 2H, J_{P-CH}=9.0 Hz, OCH_2P); ^{13}C NMR (DMSO-d_6 + D_2O, 75.5 MHz) δ 46.77 (s, C-1′), 66.34 (d, $J_{P,C}$ = 160.2 Hz, OCH_2P), 70.62 (d, $J_{P,C-2'}$ = 10.0 Hz, C-2′), 144.99 (C-2, 3a, 4, 8, 8a), 149.97 (C-6); FABMS m/z 362 [MH^+], 340, 318; HRMS(FAB) calcd for $C_9H_{11}N_5O_6Na_2P$ (MH^+) 362.0243, found 362.0240.

The above disodium salt of **26** was dissolved in water and neutralized with 2 M hydrochloric acid to give the free acid **26** as a white solid after filtering and washing with water, mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 10.65 (brs, 1H, NH, exchangeable with D_2O), 8.04 (s, 1H, imidazole), 7.44 (brs, 1H, NH, exchangeable with D_2O), 6.61 (brs, 1H, NH, exchangeable with D_2O), 4.53 (t, 2H, J = 4.2 Hz, H-1′), 3.74 (t, 2H, J = 4.2 Hz, H-2′), 3.52 (d, 2H, J_{P-CH} = 9.0 Hz, OCH_2P); ^{13}C NMR (DMSO-d_6, 75.5 MHz) δ 46.44 (s, C-1′), 66.81 (d, $J_{P,C}$ = 161.4 Hz, OCH_2P), 71.10 (d, $J_{P,C-2'}$ = 10.1 Hz, C-2′), 144.20 (C-2, 3a, 4, 8, 8a), 149.99 (C-6); FABMS m/z 318 [MH^+]; HRMS(FAB) calcd for $C_9H_{13}N_5O_6P$ (MH^+) 318.0604, found 318.0598. Anal. ($C_9H_{12}N_5O_6P$) C, H, N.

6-Amino-4,5-dihydro-8*H*-imidazo[4,5-*e*][1,3]diazepine-4,8-dione (28). Method A. In a flame dried three-necked round-bottomed flask, fitted with a $CaCl_2$ guard tube, was placed 4,5-imidazoledicarboxylic acid (2 g, 12.8 mmol). Thionyl chloride (10 mL, 0.137 mol) was introduced through a serum cap, and the reaction mixture was refluxed for 24 h. The product never appeared to completely go into solution. The reaction mixture was concentrated under anhydrous conditions, the residue was coevaporated with dry toluene three times, and then 20 mL of absolute methanol was added. A solution of free guanidine (12 mmol) [prepared from neutralization of guanidine hydrochloride (1.15 g, 12 mmol) with a solution of sodium methoxide, obtained from sodium (0.28 g, 12.17 mg·atom) dissolved in 15 mL of absolute methanol] was added to the reaction mixture and was heated to reflux for 24 h. The mixture was cooled in an ice bath and filtered to give the crude product **28**. The compound was recrystallized from water to give pure product as a white amorphous solid [The compound is insoluble in any organic solvent, but is soluble in hot water.]: yield 2.2 g (95%), mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 7.72 (s,1H, imidazole), 7.08 (brs, 4H, exchangeable with D_2O, NH); ^{13}C NMR (DMSO-d_6) δ 162.91, 157.99, 135.89, 135.68, 132.42.

Method B. Guanidine hydrochloride (0.19 g, 2 mmol) was added to 2 mL of 2.2 M sodium methoxide solution resulting from sodium (0.5 g) dissolved in 10 mL of absolute methanol. The mixture was stirred in an ice bath for 30 min. The precipitated sodium chloride was removed, and the filtrate was poured into a solution of methyl 4,5-imidazoledicarboxylate (0.27 g, 1.5 mmol) in 5 mL of absolute methanol. The mixture was stirred at room temperature for 24 h, refluxed for 3 h, cooled in ice bath, and filtered. The solid was washed with methanol and recrystallized from water to give **28** as a white solid: yield 0.25 g (93%), mp >250 °C; ^{1}H NMR (D_2O) δ 7.68 (s, 1H, imidazole); ^{13}C NMR (D_2O) δ 171.91, 163.24, 149.54, 149.35, 140.34.

General Method for the Synthesis of Nucleoside-5′-triphosphates, 33 and 34. To an ice-cold suspension of nucleoside **31**[43] or **32**[54] (0.2 mmol) in 0.7 mL of trimethyl phosphate was added 80 μL (0.85 mmol) of phosphorus oxychloride. The mixture was stirred at 0 °C for 5 h, until all precipitate had dissolved. Then a solution of bis(tri-*n*-butylammonium) pyrophosphate (782 mg,1.95 mmol) in 1.5 mL of dry DMF and 0.4 mL (1.95 mmol) of dry *tri*-*n*-butylamine was added, and the mixture was stirred in an ice-bath for 30 min. The reaction mixture was quenched by addition of triethylammonium hydrogen carbonate (TEAB) buffer (1 M, pH 7.5) so as to adjust the pH to about 7. The solution was extracted with EtOAc, and the aqueous layer (20 mL) was loaded onto a DEAE Sephadex A-25 column (40 × 2.5 cm) which was preequilibrated with 0.01 M triethylammonium bicarbonate (TEAB) buffer. The column was subsequently eluted with a linear gradient of TEAB buffer (0.01−1 M, 1200 mL). The fractions were monitored by TLC (2-propanol−concentrated NH_4OH−water, 7:1:2). Appropriate UV-absorbing fractions were pooled and evaporated.

4,6-Diamino-8-imino-8*H*-1-β-D-ribofuranosylimidazo-[4,5-*e*][1,3]diazepine-5′-triphosphate Monosodium Salt (33).[30] The residue obtained from DEAE cellulose chromatography was further purified by preparative TLC on Cellulose F plates, using a mixture of methanol−water (7:3) as the developing solvent. The product 5′-triphosphate obtained from the prep-TLC was dissolved in 3 mL of water and reprecipitated with 100 mg of NaI in 10 mL of acetone. The precipitated sodium salt was collected by centrifugation, washed with acetone, and dried over P_2O_5. ^{1}H NMR (D_2O) δ 8.5 (s, 1H, H-2), 6.1 (d, 1H, sugar H-1′), 4.48 (m, 1H, sugar H), 4.13 (m, 1H,

sugar H), 3.74 (m, 1H, sugar H), 3.53−3.21 (m, 2H, sugar 5′-H); ^{31}P NMR (D_2O) δ −6.41 (γ-P), −7.89 (α-P), −20.16 (β-P); UV (pH 7) λ_{max} 246 nm (ϵ 32 000); mass spectrum (FAB) *m/z* 572 (MH^+). The spectral data are consistent with those reported.[30]

5,8-Dioxo-5,6,7,8-tetrahydro-4*H*-1-β-D-ribofuranosylimidazo[4,5-*e*][1,2,4]triazepine-5′-triphosphate Bis(triethylammonium) Salt (34). The residual glassy material obtained from DEAE cellulose chromatography was directly employed for biological studies. ^{1}H NMR (D_2O) δ 8.44 (s, 1H, H-2), 6.24 (d, J = 3.7 Hz, 1H, H-1′), 4.53 (m, 1H, sugar H-2′), 4.40 (m, 1H, H-3′), 4.2 (m, 1H, H-4′), 3.69−3.39 (m, 2H, 5′-H); ^{31}P NMR (D_2O) δ −9.42 (α + γ-P), −22.06 (β-P); mass spectrum (FAB) *m/z* 726 (MH^+); HRMS (FAB) calcd for $C_{22}H_{47}N_7O_{15}P_3$ (MH^+) *m/z* 742.2343, found 742.2346; UV λ_{max} = 252 nm (ϵ = 31 200).

(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). [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 HCV, JEV, Suv3$_{(\Delta 1-159)}$, and WNV NTPase/Helicases. **The NTPase/helicase domain of HCV NS3** was expressed in *E. coli* and purified as previously described,[63,64] with some modifications. The bacteria were collected by centrifugation and disrupted by sonication in lysis buffer (100 mM Tris/HCl pH 7.5, 20% glycerol, 0.1% Triton X-100, 200 mM NaCl, 1 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 10 mM imidazole). Insoluble material was pelleted at 26 000*g* and the supernatant mixed with 3 mL nickel-charged resin (Qiagen) equilibrated with buffer containing 20 mM Tris/HCl pH 7.5, 10% glycerol, 0.05% Triton X-100, and 1 mM β-mercaptoethanol) for 12 h. The matrix was transferred to a column and washed with above buffer supplemented with 200 mM NaCl and 20 mM imidazole. Bound protein, eluted with 0.5 M imidazole in the same buffer, to a purity of 65−70%, was concentrated by ultrafiltration on a 30-kDa membrane and fractionated on a Superdex-200 column (Hi-Load; Amersham Pharmacia Biotech) equilibrated with TGT buffer (20 mM Tris/HCl pH 7.5, 10% glycerol, 0.05% Triton X-100, 1 mM EDTA, 1 mM β-mercaptoethanol). Fractions containing most of the ATPase and helicase activities (≫80%) were pooled and served to investigate its properties.

The JEV NTPase/helicase was expressed in *E. coli*[65] and purified according to the protocol for the HCV enzyme, as above.

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.[66] 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 as described above.

The WNV NTPase/helicase was purified from the cell culture medium of virus-infected Vero E6 cells as described previously,[27] 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, 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.[21,22] Briefly, assays were performed with 2 pmol of WNV, 0.5 pmol of HCV, 4 pmol of JEV, 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 was adjusted to concentrations indicated in the legends to figures. 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 WNV, 0.5 pmol HCV, 4 pmol JEV, 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 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, and ATP at concentrations indicated in figure legends. The reaction was conducted for 30 min at 30 °C and stopped by addition of 5 μL 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.[27] 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 was associated with the released strand and with the non-unwound substrate 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.[9] 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 described elsewhere.[22,27] The duplex DNA was electrophoresed on a 15% native TBE-polyacrylamide gel, visualized by autoradiography, and extracted as described previously.[27] The amount of DNA or RNA duplex used as substrates was determined by the ethidium bromide fluorescent quantitation method.[59]

Other Assays. Protein concentrations of preparations of the NTPase/helicases were determined on SDS/polyacrylamide gels as described previously.[60] Kinetic parameters were determined by nonlinear-regression analysis using ENZFITTER (BioSoft) and SIGMA PLOT (Jandel Corp.)

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