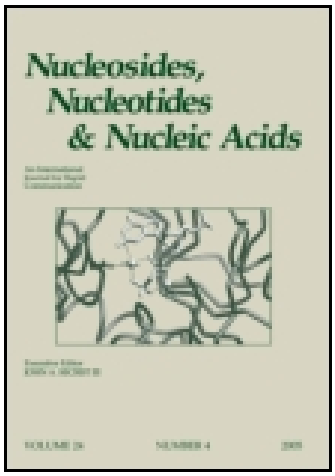

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A Novel Imidazole Nucleoside Containing a Diaminodihydro-S-triazine as a Substituent: Inhibitory Activity Against the West Nile Virus NTPase/Helicase

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A NOVEL IMIDAZOLE NUCLEOSIDE CONTAINING A DIAMINODIHYDRO-*S*-TRIAZINE AS A SUBSTITUENT: Inhibitory Activity Against the West Nile Virus NTPase/Helicase

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□ *The attempted synthesis of a ring-expanded guanosine (***1***) containing the imidazo[4,5-e][1,3]diazepine ring system by condensation of 1-(2′-deoxy-β-D-erythropentofuranosyl)-4-ethoxycarbonylimidazole-5-carbaldehyde (***2***) with guanidine resulted in the formation of an unexpected product, 1-(2′-deoxy-β-D-erythropentofuranosyl)-5-(2,4-diamino-3,6-dihydro-1,3,5-triazin-6-yl)imidazole-4-carboxamide (***7***). The structure as well as the pathway of formation of* **7** *was corroborated by isolation of the intermediate, followed by its conversion to the product. Nucleoside* **7** *showed promising in vitro anti-helicase activity against the West Nile virus NTPase/helicase with an IC_{50} of 3-10 μg/mL.*

Keywords Imidazole; s-Triazine; Nucleoside; West Nile Virus; Inhibition; Rearrangement; Ring-expanded nucleoside

In honor and celebration of the life and career of John A. Montgomery.

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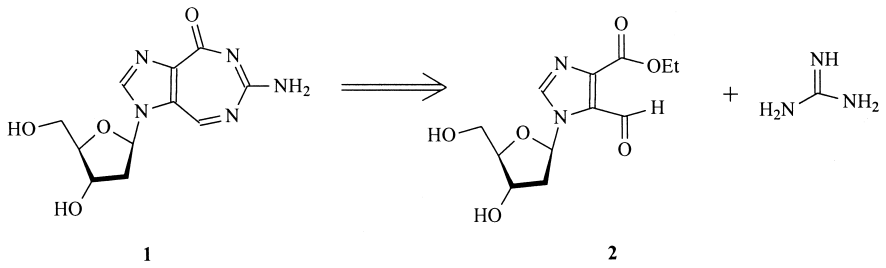
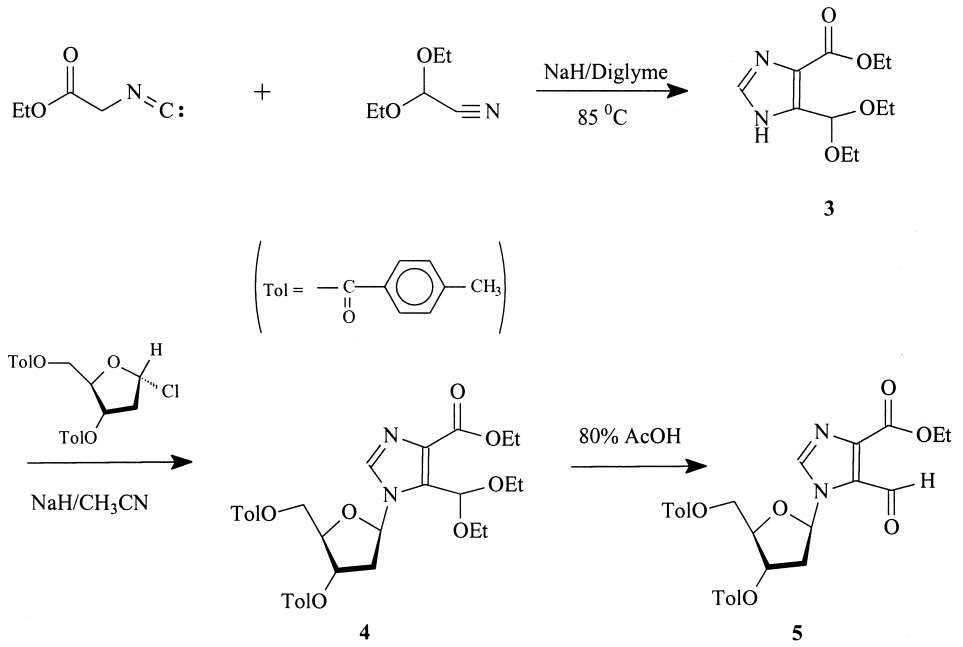


SCHEME 1

INTRODUCTION

We have outlined a broad program to synthesize and biologically screen a variety of ring-expanded ("fat") nucleosides and nucleotides as a potential and proven source of antiviral and anticancer agents.[1] As part of this program, we recently set out to synthesize a ring-expanded 2′-deoxyguanosine analogue (**1**) (Scheme 1) by condensation of the imidazole nucleoside **2** with guanidine. To this end, compound **5** was prepared as a protected precursor to **2**, using a literature procedure for the corresponding methyl ester,[2,3] starting from ethyl 5-diethoxymethyl-1*H*-imidazole-4-carboxylate (**3**), as outlined in Scheme 2. The condensation of diethoxyacetonitrile with



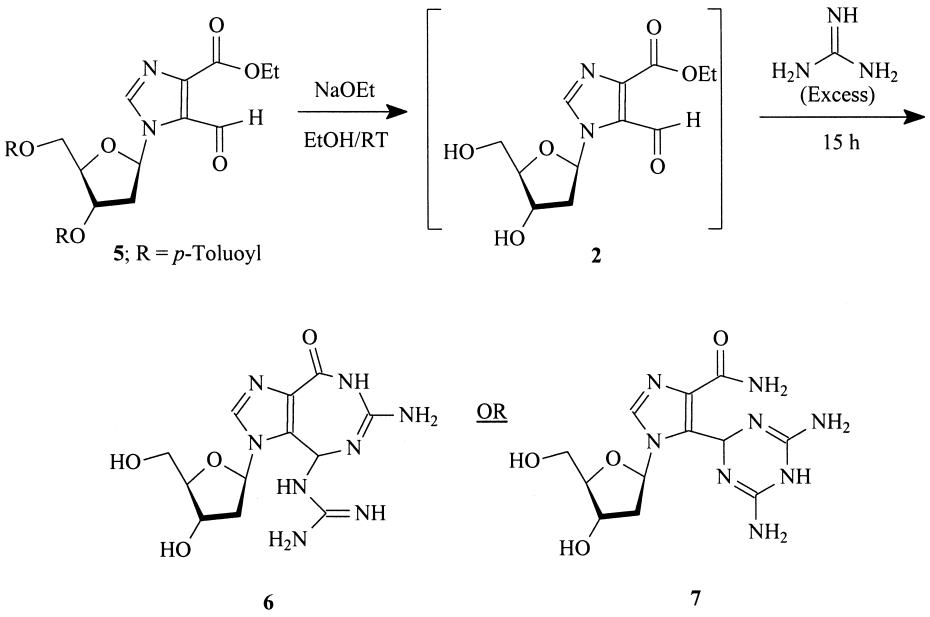
SCHEME 2

ethyl isocyanate, in the presence of sodium hydride in diglyme at 85°C, provided **3** in 56% yield. The glycosylation of **3** with 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl chloride,[4,5] catalyzed by sodium hydride, produced the sugar-protected nucleoside **4** in 46% yield after purification by flash chromatography on a silica gel column. The ^{1}H and ^{13}C NMR spectral data as well as TLC analyses suggested that the product was a single isomer. The sodium salt glycosylation procedure is known to be stereospecific and is expected to produce mainly the β-anomer.[2,5,6] The β-anomeric structure of **4** was corroborated by the observed splitting pattern, a doublet of doublets with nearly equal coupling constants ($J = 5.4$ and 5.7 Hz), of the anomeric proton of **4** at δ 6.69 in its ^{1}H NMR spectrum. The two-dimensional ^{1}H-^{1}H NOE spectroscopy (NOESY) of **4** provided the confirmatory evidence for the β-configuration by revealing NOE between H-1′ and H-4′. The observed splitting mode and the coupling constants are generally considered a diagnostic of β-anomeric configuration of nucleosides.[7] The regioisomeric structure of **4**, as assigned, was confirmed by the nuclear Overhauser effect (NOE) studies of the product aldehyde (**5**) formed by hydrolysis of the acetal group of **4**. The hydrolysis was accomplished by reaction of **4** with 80% aqueous acetic acid at room temperature for 22 h.

The two-dimensional ^{1}H-^{1}H NOE spectroscopy (NOESY) of **5** revealed the presence of NOE between its aldehydic group and the sugar H-1′. The observed NOE is not possible if the sugar moiety were to be attached to the other N atom of the imidazole ring.

When compound **5** was reacted with excess guanidine and sodium ethoxide in ethanol at room temperature for 15 hours, a new product was obtained, which moved slower than the starting material on a silica gel plate (Scheme 3). The ^{1}H NMR, elemental microanalyses, and the mass spectral data of the chromatographically purified product showed that the compound had incorporated two molecules of guanidine in its structure instead of one. Both structures **6** and **7** were consistent with the acquired spectral and microanalytical data, and their formation by the reaction of **5** with guanidine could be rationalized by the two reaction pathways outlined in Scheme 4.

The structure of the product was confirmed as **7** by the following reaction sequences. Compound **4** was reacted with ammonia in ethanol in a stainless steel vessel at 125°C for 36 h to obtain the corresponding carboxamide **8** with its free hydroxyl groups (Scheme 5). An attempted conversion of the acetal groups of **8** into the corresponding carboxaldehyde, however, resulted in concurrent deglycosylation to produce the heterocycle **9**. Therefore, the two sugar hydroxy groups were reprotected by reaction with toluoyl chloride in pyridine to obtain **10**. The latter, upon treatment with 80% acetic acid at room temperature for several hours, gave the desired vinylogous aldehyde-amide **11**. The reaction of **11** with excess guanidine in

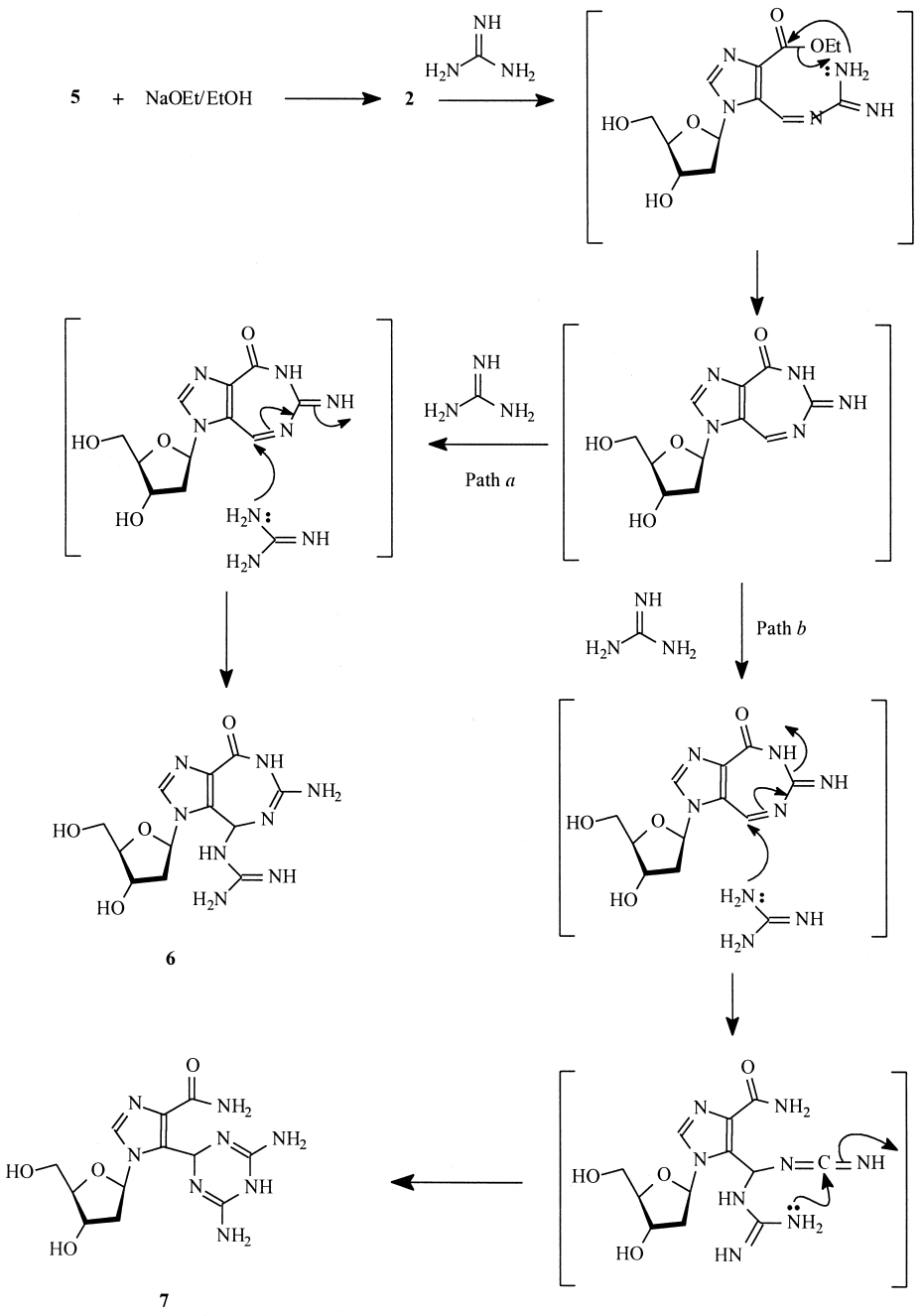


SCHEME 3

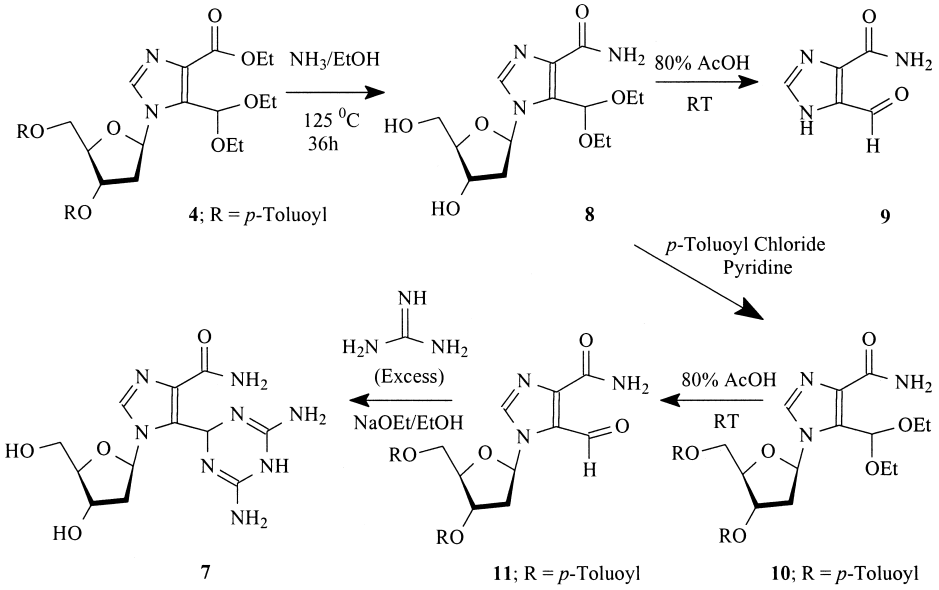
a solution of sodium ethoxide/ethanol afforded **7**, which was identical in all respects to the product formed by the reaction of ester **5** with excess guanidine.

The above results raise another mechanistic possibility for the formation of **7** from **5**, in which the latter's carboxaldehyde group would react with excess guanidine to form the triazine ring first, as in **10** (Scheme 6). A molecule of ammonia released during the formation of **10** would subsequently react to produce **7**. While this reaction pathway could not be completely ruled out, it appears less likely in view of the lack of formation of equally plausible other products such as **11**, especially when there is a large molar excess of guanidine in the reaction mixture as compared to the transiently formed ammonia. Finally, in an attempt to prepare the target **1**, intermediate **2** was reacted with one equivalent of guanidine in anhydrous ethanol. Unfortunately, the reaction produced an intractable mixture of products that were difficult to separate and purify. The use of a base, change of solvent, or increase or decrease in reaction temperature did not improve the situation. Therefore, it appears that the targeted ring-expanded guanosine (**1**) has limited existence and stability.

Although our attempts to synthesize the desired **1** was unsuccessful, the product **7**, obtained easily from condensation of **5** or **2** with excess guanidine, possessed novel structural characteristics worthy of further exploration, especially for its biological potential. In that context, nucleoside **7**



SCHEME 4



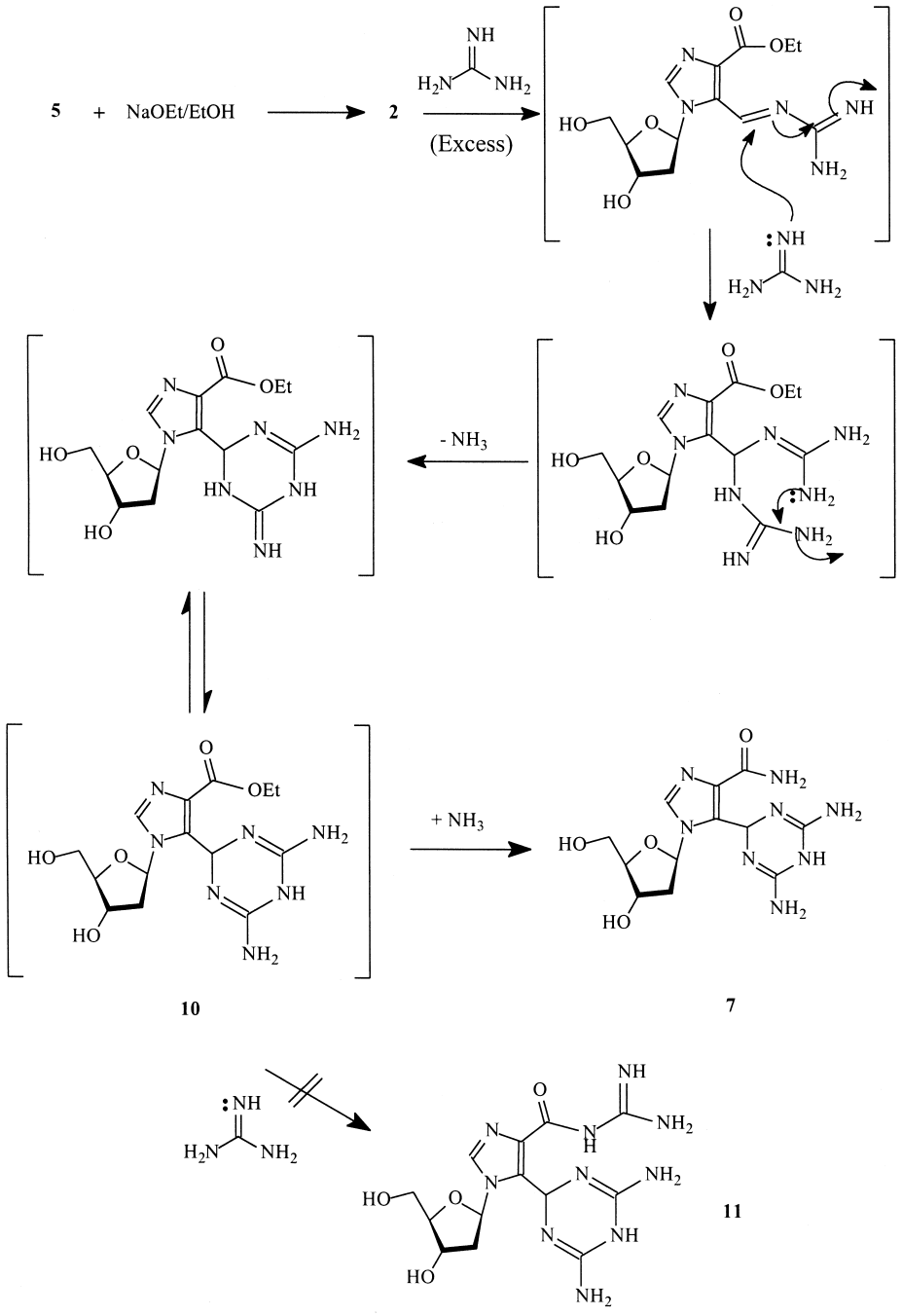
SCHEME 5

became an attractive choice for screening against the West Nile virus (WNV), a topic of our current research focus.[5,8]

Nucleoside **7** was screened for antihelicase activity of WNV NTPase/helicase harvested from the virus-infected Vero E6 cells, employing established protocols.[5] The compound was tested against both RNA and DNA substrates consisting of two annealed RNA or DNA oligonucleotides. The unwinding activity of the enzyme was assessed by monitoring the release of the shorter labeled strand of the RNA or DNA duplex. The helicase activity was calibrated with an RNA or a DNA substrate that was unwound at an ATP concentration equal to the K_M value determined for the NTPase reaction. Compound **7** exhibited helicase inhibitory activity against WNV NTPase/helicase with an IC_{50} value of 3–10 μg/mL when an RNA substrate was employed. However, no inhibition could be detected when the same experiment was repeated using a DNA substrate. As WNV is an RNA virus, this result is not totally surprising. In that regard, a ribose analogue of **7** may carry a better prospect for WNV inhibition than **7** itself, and such an endeavor is currently in progress.

EXPERIMENTAL

The ^{1}H and ^{13}C NMR spectra were recorded on a General Electric QE-300 or a JEOL-400 NMR spectrometer, operating at 300 or 400 MHZ for ^{1}H and 75 or 100 MHz for ^{13}C, respectively. The data are reported in the



SCHEME 6

following format: Chemical shift (all relative to Me_4Si), multiplicity (s = singlet, d = doublet, dt = double triplet, dd = double doublet, t = triplet, q = quartet, m = multiplet, b = broad), coupling constants, integration and assignment. Thin-layer chromatography was performed on Merck Kieselgel 60 F_{254} (0.2 mm thickness). Flash column chromatography was performed using 32–63 mesh silica gel. Elemental microanalyses were performed by Atlantic Microlab, Inc., Norcross, Georgia. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Mass spectra were recorded either at the Mass Spectral Facility, Department of Biochemistry, Michigan State University, East Lansing, MI or the University of Maryland, College Park, Maryland. Anhydrous solvents were purchased and used without further drying. All alcohols were dried over sodium metal, distilled, and stored over molecular sieves.

Ethyl 5-Diethoxymethylimidazole-4-carboxylate (3). To a stirred suspension of 60% sodium hydride (2.7 g) in freshly distilled diglyme (100 mL), a solution of diethoxyacetonitrile (6.45 g, 0.05 mol) and ethyl isocyanoacetate (5.65 g, 0.05 mol) in diglyme (75 mL) was added slowly with ice cooling under nitrogen atmosphere. The solution was heated at 80°C for 5 h and left overnight at room temperature. The reaction mixture was quenched with saturated aqueous ammonium chloride solution and extracted with dichloromethane for three times. The combined organic extracts were dried over anhydrous sodium sulfate. The solid obtained after removal of solvent under reduced pressure was purified by flash chromatography over silica gel. The pure product obtained by elution with ethyl acetate was finally crystallized from a mixture of dichloromethane-petroleum ether (20:3) as colorless needles. Yield 6.82 g, 56%; mp 138°C; Rf = 0.34 (chloroform:methanol, 10:1); IR, 1710, 1570, 1505, 1447, 1179, 1142 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 12.50 (s, 1H, NH), 7.79 (s, 1H, imidazole CH), 6.10 (s, 1H, acetal CH), 4.31 (q, $J = 6.9$ Hz, 2H, ester CH_2), 3.72–3.50 (m, 4H, 2 CH_2, acetal CH_2), 1.29 (t, $J = 6.9$ Hz, 3H, ester CH_3), 1.12 (t, $J = 6.9$ Hz, 6H, 2 acetal CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) 161.91, 137.00, 135.87, 126.25, 94.73, 62.20, 59.94, 14.49, 13.74; MS (FAB) *m/z* 243 (MH^+). Anal. Calcd for $C_{11}H_{18}N_2O_4$: C, 54.53; H, 7.49; N, 11.56. Found: C, 54.53, H, 7.44, N, 11.50.

Ethyl 5-Diethoxymethyl-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropentofuranosyl]imidazole-4-carboxylate (4). To a solution of ethyl 5-diethoxymethylimidazole-4-carboxylate (**3**) (2.42 g, 0.01 mol) in anhydrous acetonitrile (75 mL) was added sodium hydride (60% in oil, 1.0 g, 25 mmol), and the mixture was stirred at room temperature under nitrogen atmosphere for 30 min. To this mixture, 2-deoxy-3,5-di-*O*-*p*-toluoyl-α-D-erythropentofuranosyl chloride (3.88 g, 0.01 mol) was added portion-wise over a period of 1 h. After the addition was complete, the reaction mixture

was allowed to stir at room temperature for 2 h. It was then filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on a silica gel column using a mixture of dichloromethane: methanol (99:1) as an eluant. Appropriate fractions were pooled and evaporated under reduced pressure to obtain a gummy residue. Yield: 2.71 g, 46%; Rf 0.40 (chloroform:methanol, 50:2); IR 1716, 1611, 1446, 1267, 1177, 1096 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.93–7.87 (m, 4H, Ar-H), 7.81 (s, 1H, imidazole CH), 7.26–7.20 (m, 4H, Ar-H), 6.69 (dd, $J = 5.4$ and 5.7 Hz ,1H, 1′-H,), 6.37 (s, 1H, C*H*$(OEt)_2$), 5.59 (m, 1H, 3′-H), 4.73 (m, 2H, 4′, 5′-H), 4.68 (m, 1H, 5″-H), 4.36 (q, $J = 7.2$ Hz, 2H, ester CH_2), 3.86–3.48 (m, 4H, 2 CH_2, acetal CH_2), 2.90–2.87 (m, 1H, 2′-H), 2.50–2.42 (m, 2 H, 2″-H), 2.41 (s, 3H, Ar-CH_3), 2.38 (s, 3H, Ar-CH_3), 1.38 (t, 3H, ester CH_3), 1.22–1.14 (m, 6H, 2 acetal CH_3; Mass (FAB), *m/z* 595.60 (MH^+); Anal. Calcd for $C_{32}H_{38}N_2O_9$: C, 64.63; H, 6.44; N, 4.71. Found: C, 64.64, H, 6.22, N, 4.70.

Ethyl 5-Formyl-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropentofuranosyl]imidazole-4-carboxylate (5). A solution of ester-acetal **4** (2.37 g, 4 mmol) in 80% aqueous acetic acid (10 mL) was stirred at room temperature for 22 h. The reaction mixture was poured into ice water and the precipitated solid was extracted with dichloromethane. The organic extract was dried over anhydrous sodium sulfate. The residue obtained after removal of the solvent was purified by silica gel flash chromatography using a mixture of dichloromethane-methanol (98:2) as the eluant. Appropriate fractions were pooled and evaporated under reduced pressure. The residue was triturated with methanol to get pure ester-aldhehyde **5**. Yield 1.15 g, 55%; mp 125–127°C; Rf = 0.44 (dichloromethane:methanol, 99:1); IR 1723, 1651, 1611, 1537, 1485, 1265, 1084 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 10.42 (s, 1H, CHO), 8.03 (s, 1H, imidazole CH), 7.89 (d, $J = 6.8$ Hz, 2H, Ar-H), 7.76 (d, $J = 6.68$ Hz, 2H, Ar-H), 7.20 (d, $J = 8.72$ Hz, 2H, Ar-H), 7.14 (d, $J = 7.36$ Hz, 2H, Ar-H), 6.67 (t, $J = 6.4$ Hz, 1H, 1′-H), 5.51 (s, 1H, 3′-H), 4.69–4.59 (m, 3H, 4′, 5′, and 5″-H), 4.40 (q, $J = 6.88$ Hz, 2H, ester CH_2), 3.01–2.96 (m, 1H, 2′-H), 2.36 (s, 3H, Ar-CH_3), 2.32 (s, 3H, Ar-CH_3), 2.28–2.23 (m, 1 H, 2″-H), 1.36 (t, $J = 6.88$ Hz, 3H, ester CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) 182.84, 166.21, 166.07, 161.89, 144.68, 144.45, 141.98, 138.27, 131.45, 129.94, 129.68, 129.50, 129.40, 126.43, 126.33, 88.53, 83.91, 74.53, 63.81, 61.89, 40.97, 21.85, 21.78, 14.37. Mass Spectrum (FAB) *m/z* 521.50 (MH^+); Anal. Calcd for $C_{28}H_{28}N_2O_8$: C, 64.61; H, 5.42; N, 5.38. Found: C, 64.50, H, 5.43, N, 5.35.

5-(4,6-Diamino-2,5-dihydro-1,3,5-triazin-2-yl)-1-(2′-deoxy-β-D-erythropentofuranosyl)imidazole-4-carboxamide (7). *Method A:* Guanidine hydrochloride (0.38 g, 0.006 mol) was neutralized with a solution of sodium (0.32 g, 0.02 mol) in anhydrous ethanol (10 mL) at ice cold temperature. This guanidine solution was added to the aldehyde **5** or **2** (0.0015 mol) in anhy-

drous ethanol (10 mL). This reaction mixture was stirred at room temperature under nitrogen atmosphere for 15 h. TLC showed the completion of reaction (chloroform-methanol-ammonium hydroxide (1:1:0.5). Solvent was evaporated and the residue was purified by silica gel flash chromatography, using a mixture of chloroform-methanol-ammonium hydroxide (2:1:0.25); Yield 0.32 g, 63%; mp 106°C, Rf = 0.39 (chloroform:methanol:ammonium hydroxide, 1:1:0.25); IR, 3302, m152, 3092, 1654, 1620, 1585, 1518, 1268 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.12 (s, 1H, imidazole CH), 7.90 (s, 1H, NH, exchangeable with D_2O), 7.52 (s, 2H, NH_2, exchangeable with D_2O), 7.10–7.40 (brs, 4H, NH2+NH2, exchangeable with D_2O), 6.80 (s, 1H, triazine CH), 6.24 (t, J = 6.0 Hz, 1H, 1′-H), 5.10–5.50 (brs, 2H, OH+OH, exchangeable with D_2O), 3.75 (m, 2H, 5′H, 5″-H), 2.35–2.18 (m, 2 H, 2′- and 2″-H); ^{13}C NMR(DMSO-d_6, 75 MHz); 164.44, 158.52, 158.32, 136.10, 134.29, 128.95, 87.59, 85.39, 69.48, 60.76, 55.54, 42.39; Mass (FAB) *m/z* 339.30 (MH^+); Anal. Calcd for $C_{12}H_{18}N_8O_4$. 0.25 H_2O: C, 42.02; H, 5.39; N, 32.68. Found: C, 42.05, H, 5.74, N, 32.31.

Method B: Guanidine hydrochloride (20 mg, 0.2 mmol) was neutralized with a solution of sodium (5 mg, 0.2 mmol) in ethanol (2 mL) at ice-cold temperature. Separated sodium chloride was removed by filtration and the guanidine solution was added to the aldehyde **11** (25 mg, 0.05 mmol) in ethanol (3 mL). This reaction mixture was stirred at room temperature under nitrogen atmosphere for 15 h. TLC (cholorform-methanol-ammonium hydroxide, (1:1:0.5) showed the completion of reaction. Solvent was evaporated and the residue was purified by silica gel flash chromatography, eluting with a mixture of cholorform-methanol-ammonium hydroxide, (2:1:0.25). The melting point and chromatographic mobility (mp 106°C, Rf = 0.39) as well as the spectral data of the compound were identical with those of **7** obtained by Method A above.

5-Diethoxymethyl-1-(2′-deoxy-β-D-erythropentofuranosyl)imidazole-4-carboxamide (8). Compound **4** (0.59 g, 0.001 mol) was added to a saturated ethanolic ammonia solution (5 mL) in a stainless steel pressure vessel. The vessel was heated at 125°C in an oil bath for 36 h. It was cooled to room temperature, solvent was evaporated and the residue was purified by silica gel flash chromatography, eluting first with a mixture of cholroform: methanol (99:1) to remove *p*-toluamide, followed by elution with a mixture of chloroform:methanol (10:1) to obtain **8** as a hygroscopic product. Yield 245 mg (73%); Rf 0.21 (9:1 chloroform:methanol); ^1H NMR (DMSO-d_6, 300 MHz) δ 8.03 (s, 1H, imidazole CH), 7.38 (s, 2H, NH_2), 6.54 (t, J = 6.8 Hz, 1H, 1′H,), 6.43 (s, 1H, acetal CH), 5.9 (s, 1H, OH), 5.18–5.08 (m, 1H, 4′H), 4.55–4.43 (m, 1H, OH), 3.96–3.70 (m, 3H, 4′-, 5′-, and 5″-H), 3.52–3.46 (m, 4H, acetal CH_2), 2.65–2.54 (m, 1H, 2′H), 2.35–2.24 (m, 1H, 2″H), 1.20–1.15 (m, 6H, 2 acetal CH_3); Mass (FAB), *m/z* 330 (MH^+).

5-Formylimidazole-4-carboxamide (9). Compound **8** (0.110 g, 0.33 mmol) was added to 1 mL of 80% aqueous acetic acid, and the mixture was stirred at room temperature for 16 h. Then it was poured into ice-cold water. Separated solid was filtered and washed with water. Yield 39 mg, 84%; mp $>$ 300°C; Rf 0.23 (4:1 chloroform:methanol); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.50 (s, 1H, NH), 10.17 (s, 1H, CHO), 7.97 (s, 1H, imidazole CH), 7.58 (s, 2H, NH_2); ^{13}C NMR (DMSO-d_6, 75 MHz); 183.49, 163.37, 139.60, 134.25, 132.08; Mass (FAB), *m/z* 140 (MH^+); Anal. Calcd for $C_5H_5N_3O_2$. 0.125 CH_3OH: C, 42.97; H, 3.84; N, 29.34. Found: C, 42.67, H, 3.68, N, 29.38.

5-Diethoxymethyl-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropentofuranosyl]imidazole-4-carboxamide (10). To a mixture of compound **8** (0.083 g, 0.25 mmol) in anhydrous pyridine (5 mL), was added *p*-toluoyl chloride (0.097 g, 0. 63 mmol) at ice cold temperature. The reaction mixture was slowly brought to 50°C and stirred for 2 h at this temperature. It was then kept at room temperature for 12 h. The reaction mixture was poured onto ice-cold water and extracted with ether. Ether layer was washed with water, and dried over anhydrous sodium sulfate. The evaporation of ether gave a gummy residue, which was purified by silica gel flash chromatography, eluting with a mixture of 99:1 chloroform:methanol. Yield 0.078 g, 55%; Rf 0.25 (98:2 chloroform:methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.75 (s, 2H, NH_2), 8.12–7.87 (m, 4H, ArCH), 7.81 (s, 1H, imidazole CH), 7.26–7.20 (m, 4H, ArCH), 6.47 (t, J = 6.8 Hz, 1H, 1′H,), 6.09 (s, 1H, acetal CH), 5.60–5.54 (m, 1H, 3′H), 4.68–4.52 (m, 3H, 4′-, 5′-, and 5″-H), 3.79–3.50 (m, 4H, 2 CH_2, 2.94–2.72 (m, 1H, 2′-, and 2″-H), 2.44 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 1.21–1.16 (m, 6H, 2 CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) 167.86, 163.34, 163.26, 143.51, 137.64, 136.92, 134.33, 129.84, 128.85, 95.81, 90.26, 86.68, 71.43, 71.32, 62.30, 61.68, 21.59, 21.41, 15.53, 15.39; Mass (FAB), *m/z* 566 (MH^+).

5-Formyl-1-[(2′-deoxy-3′,5′-di-*O*-*p*-toluoyl)-β-D-erythropentofuranosyl] imidazole-4-carboxamide (11). A mixture of compound **10** (0.062 g, 0.11 mmol) and 0.5 mL of 80% aqueous acetic acid was stirred at room temperature for 22 h. Then the reaction mixture was poured into ice cold water. It was extracted with dichloromethane (3 $\times$ 10 mL), the extracts were washed with water and dried over sodium sulfate. Evaporation of solvent gave a semi solid, which was purified through flash chromatography over silica gel, eluting with a mixture of 99:1 chlorform:methanol. Yield 0.032 g, 58%. Rf 0.42 (50:2 chloroform:methanol); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 10.01 (s, 1H, CHO) 8.2 (s, 2H, NH_2), 7.98–7.90 (m, 4H, ArCH), 7.87 (s, 1H, imidazole CH), 7.29–7.19 (m, 4H, ArCH), 6.68 (t, J = 6.8 Hz, 1H, 1′H,), 5.57–5.53 (m, 1H, 3′-H), 4.73–4.45 (m, 3H, 4′-, 5′-, and 5″-H), 3.15–2.81 (m,

1H, 2′- and 2″-H), 2.46 (s, 3H, CH_3), 2.43 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) 184.41, 166.35, 165.76, 163.44, 144.08, 143.87, 140.75, 137.16, 131.57, 128.45, 128.20, 128.11, 128.00, 126.45, 126.34, 87.83, 83.98, 74.77, 64.06, 61.96, 41.34, 22.26, 22.03; Mass (FAB), *m/z* 492 (MH^+).

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, Connecticut). [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.

The WNV NTPase/helicase was purified from the cell culture medium of virus-infected Vero E6 cells as described previously,[9] with some modifications. Briefly, the concentrated cell culture medium was mixed with 10 mL 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 polyethylene glycol (30% w/w), collected by centrifugation, solubilized with TGT buffer, and applied to a hydroxyapatite (HA-Ultragel) column preequilibrated with TGT buffer. The column was washed with 10 mL TGT buffer, then with 2 mL TGT buffer containing 1 M KCl, and again with 5 mL TGT buffer. The NTPase/helicase was eluted with 1 mL TGT buffer containing 50 mM KH_2PO_4, precipitated with PEG and dissolved in TGT buffer. The final preparations of the enzymes were homogenous, as demonstrated by Coomassie Blue staining of SDS/polyacrylamide gels.

Helicase activity was tested with 2 pmol WNV 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 9.5 μM ATP. The reaction was conducted for 30 min at 30°C and stopped by addition of 5 mL 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.[9] 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 counted.

Alternatively, the films were scanned and the radioactivity 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 Reaction

The RNA substrate for the helicase assays consisted of two partially hybridized oligonucleotides with sequences as reported by Gallinari et al.[10] 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.[9,11] The duplex DNA was electrophoresed on a 15% native TBE-polyacrylamide gel, visualized by autoradiography and extracted as described previously.[11] The amount of DNA or RNA duplex used as substrates was determined by the ethidium bromide fluorescent quantitation method.[12]

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