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An analogue of AICAR with dual inhibitory activity against WNV and HCV NTPase/helicase: Synthesis and in vitro screening of 4-carbamoyl-5-(4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl) imidazole-1-β-D-ribofuranoside

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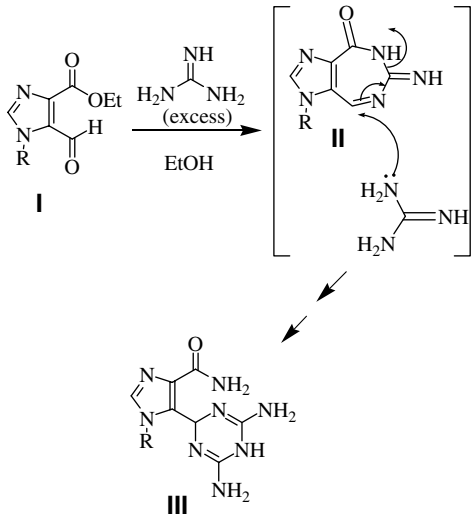
Abstract—The title compound (**4**) was synthesized by the reaction of ethyl 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-formylimidazole-4-carboxylate with excess guanidine in ethanol at reflux. Compound **4** was evaluated in vitro against NTPases/helicases of four different viruses of the *Flaviviridae* family, including the West Nile virus (WNV), hepatititis C virus (HCV), dengue virus (DENV), and the Japanese encephalitis virus (JEV), employing both an RNA and a DNA substrate. The compound showed activity against NTPase/helicase of WNV and HCV with an IC_{50} of 23 and 37 μM, respectively, when a DNA substrate was employed, while no activity was observed when an RNA substrate was used. There was no activity against the NTPase/helicase of either DENV or JEV irrespective of whether an RNA or a DNA substrate was employed. Considering that *Flaviviridae* are RNA viruses, the observed absence of activity against an RNA substrate, but the presence of activity against a DNA substrate is intriguing and somewhat surprising. The preliminary studies show that compound **4** does not form a tight complex with either an RNA or a DNA substrate, suggesting that its mechanism of action may involve direct interaction with the enzyme.

We have recently reported[1] the synthesis of novel imidazole analogues (III) from the corresponding imidazoles (I), employing an interesting functional group transformation[2] mediated by guanidine (Scheme 1). We also reported that compound III (R = deoxyribosyl) exhibited a potent in vitro inhibitory activity against the West Nile Virus (WNV) NTPase/helicase when an RNA substrate was employed, but no activity with a DNA substrate.[1] Since WNV is an RNA virus, the observed inactivity with a DNA substrate was not too surprising, but instead gave further impetus to synthesize and screen the ribose analogue of III (R = ribosyl) against not only WNV[3–5] but also a few other similarly dreadful viruses belonging to the same *Flaviviridae* family, which are of current global health threat, including the hepati-



Scheme 1.

Keywords: AICAR analogue; Synthesis; In vitro screening; Inhibition of *Flaviviridae* NTPase/helicase; West Nile virus (WNV) and hepatitis C virus (HCV).
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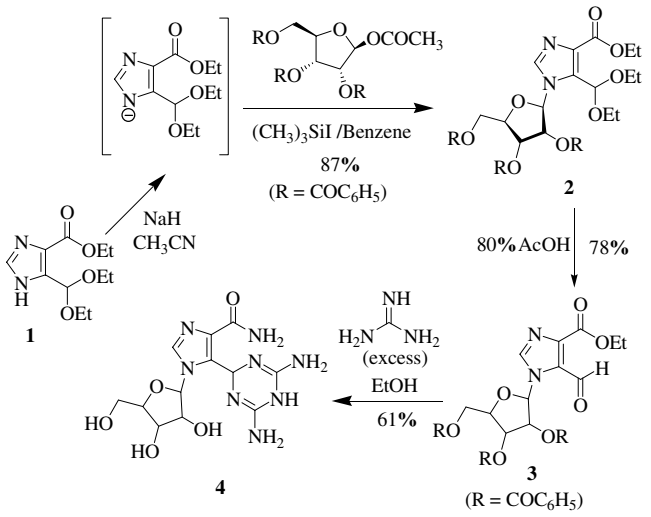

Scheme 2.

tis C virus (HCV),[6–11] dengue virus (DENV),[12–16] and the Japanese encephalitis virus (JEV).[17–20]

Synthesis of the target nucleoside **4** (Scheme 2) commenced from ethyl 5-diethoxymethylimidazole-4-carboxylate **1**.[1,21,22] The latter was converted into its sodium salt using sodium hydride in acetonitrile and was further reacted with 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl-1-iodide under standard conditions of glycosylation (acetonitrile/50 °C/2 h).[21,23] The iodide itself was prepared by reaction of 1-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose and iodotrimethylsilane at room temperature in benzene.[24] The glycosylation procedure is known to be stereospecific giving predominantly the β-anomeric product.[24,25] The product ethyl 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-(diethoxymethyl)imidazole-4-carboxylate (**2**) was isolated in 87% yield. The acetal **2** was reacted with 80% aqueous acetic acid to obtain the corresponding carboxaldehyde **3** in 78% yield. The reaction of the latter with excess guanidine in ethanol at reflux for 12 h provided the target nucleoside **4**. All intermediates as well as the final compound were fully characterized by spectroscopic and microanalytical data.[26] Compound **4** can be considered as an analogue of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an important biosynthetic precursor of purine nucleosides, with a diaminodihydrotriazine substituent replacing the 5-amino group of AICAR.

The NTPases/helicases from four closely related *Flaviviridae*, including the West Nile virus (WNV), hepatitis C virus, Japanese encephalitis virus (JEV), and dengue virus (DENV), were employed for the helicase inhibition studies. The WNV NTPase/helicase was isolated and purified from the cell culture medium harvested from virus-infected Vero E6 cells as described by us previously.[27–30] The NTPase/helicase domains of HCV,[31,32] JEV,[33] and DENV[34] NS3 were expressed in *Escherichia coli* and purified according to the protocol for the HCV enzyme, which we reported earlier.[27] 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, employing the protocol as described.[27,28] 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.[27,28] The anti-helicase activity of Nucleoside **4** against the mentioned four different NTPases/helicases is listed in Table 1 employing a DNA substrate.

Table 1. Inhibitory effect of nucleoside **4** against the helicase activity of WNV, HCV, JEV, and DENV NTPases/helicases, using a DNA substrate[a]

WNV IC_{50} (μM)[b]	HCV IC_{50} (μM)[b]	JEV IC_{50} (μM)[b]	DENV IC_{50} (μM)[b]
23	37	>500	>430

[a] The helicase activity was determined as a function of increasing concentrations of the compound in the presence of ATP adjusted to the respective K_M values equal to 9.5 μM, 105 μM, 4.2 μM, and 165 μM WNV, HCV, JEV, and DENV NTPase/helicase, and 4.7 pM DNA substrate.

[b] The inhibitory effect of the compound was expressed as the concentration at which 50% of the unwinding activity was observed. The helicase activity of the enzyme measured in the absence of the compound was referred to as 100%. The term IC_{50} is defined as the concentration of the compound required for 50% inhibition of enzyme activity.

However, no inhibition could be detected when the same experiments were repeated using an RNA substrate. As *Flaviviridae* are RNA viruses, the observed results are intriguing, especially considering that the 2′-deoxyribose analogue of **4** (i.e., **III**; R = deoxyribosyl in Scheme 1) has shown activity against the NTPase/helicase of WNV with an RNA substrate, but no activity against a DNA substrate as we reported recently.[1] The significance and implications of the observed contrasting results between the ribose analogue **4** and its 2′-deoxy counterpart with respect to an RNA or DNA substrate of viral helicase are not clear at the moment.

Since the observed antiviral activity of **4** against NTPase/helicase is very specific to the type of nucleic acid (DNA vs RNA) substrate employed, we wondered if the mechanism of action of **4** is dependent upon its ability to form a tight complex with a DNA substrate but not with RNA. There are many documented reports demonstrating non-covalent, tight-binding interactions of analogues of nucleobases, nucleosides, and nucleotides, which simply bind to major or minor grooves of DNA or RNA double helices.[35,36] We ourselves have recently reported such interactions with some ring-expanded nucleoside (REN) analogues that were found to form tight complexes with DNA or RNA substrates of viral helicases, as evidenced by the observed complete stability of the complexes in the presence of 0.5% sodium dodecyl sulfate (SDS) as well as by the observed severe hindrance to migration of the DNA or RNA substrates in TBE–polyacrylamide gel electrophoresis in the presence of REN analogues with or without the enzyme

being present.[27,28] By contrast, and to our surprise, no such tight complex formation was observed with either a DNA or an RNA substrate when the same experiments were conducted using the nucleoside analogue **4**. This observation is indeed exciting as it implies that the compound must be interacting directly with the enzyme. The observed difference in activity resulting from the different types of substrates employed may well be due either to the differential conformational effects exerted by the enzyme–substrate complex upon subsequent binding of the inhibitor to the protein or due to those caused by the enzyme–inhibitor complex upon eventual binding of a DNA or an RNA substrate. These speculations, however, are only tentative at this point.

Acknowledgments

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26. Experimental. Preparation and physicochemical properties of the compounds are as follows: *Ethyl 5-diethoxymethyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl) imidazole-4-carboxylate (**2**)*.
Step 1: Preparation of sodium salt of ethyl 5-diethoxymethyl-imidazole-4-carboxylate: To a solution of ethyl 5-diethoxymethylimidazole-4-carboxylate (0.267 g, 1 mmol) in dry acetonitrile (10 mL), sodium hydride (80 mg, 2 mmol) was added and the mixture was stirred at room temperature under nitrogen atmosphere for 30 min.
Step 2: Reaction of sodium salt of ethyl 5-diethoxymethyl-imidazole-4-carboxylate with 1-*O*-acetyl-2,3,5-*O*-benzoyl-β-D-ribofuranosyl iodide: To a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (0.567 g, 1 mmol) in dry benzene (5 mL), iodotrimethylsilane (0.225 mL, 1.6 mmol) was added dropwise. The mixture was swirled for 10 min at room temperature and then it was added dropwise to a stirred suspension of the above sodium salt of ethyl 5-diethoxymethylimidazole-4-carboxylate. The temperature of the reaction mixture was gradually raised to 50 °C and was stirred for 2 h. The reaction mixture was then brought to room temperature, acetonitrile was removed under vacuum, and the residue was purified by column chromatography using hexanes/ethyl acetate (3:1) as an eluting solvent. Appropriate fractions were pooled and evaporated under vacuum. Gummy residue was crystallized from benzene-pet ether to obtain colorless, flaky solid. Yield 0.595 g, 87%; mp 43–45 °C, $R_f = 0.21$ (hexanes/ethyl acetate 3:1); IR, 1718, 1703, 1577, 1501 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.1 (m, 7H, Ar–H and imidazole CH), 7.59–7.29 (m, 9H, Ar–H), 6.82 (d, $J = 3.9$ Hz, 1H, 1′-H), 6.37 (s, 1H, CH), 5.60 (m, 2H, 2′-H, and 3′-H), 4.8–4.4 (m, 3H, 4′-, 5′-, and 5″-H), 4.40 (q, $J = 1.8$ Hz, 2H), 3.85–3.45 (m, 4H, $2CH_2$, 1.39 (t, $J = 6.9$ Hz, 3H, CH_3), 1.19 (t, $J = 7.2$ Hz, 3H, CH_3), 1.02 (t, $J = 6.9$ Hz, 3H, CH_3); HRMS (FAB) Calcd for $C_{37}H_{38}N_2O_{11}$, 687.2554 (MH^+); observed *m/z* 687.2562 (MH^+).
*Ethyl 5-formyl-1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl) imidazole-4-carboxylate (**3**)*. A solution of ethyl 5-diethoxymethyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)imidazole-4-carboxylate (0.515 g 0.75 mmol) in 80% aqueous acetic acid (5 mL) was stirred at room temperature for 15 h. The solution was then poured into ice-water (25 mL) and the precipitated solid was extracted with chloroform (3 × 50 mL). The chloroform layer was washed with water (2 × 25 mL) and dried over anhyd sodium sulfate. The solvent was removed under vacuum and the residue was purified by flash chromatography on silica gel, using hexanes/ethyl acetate (2:1) as an eluting solvent. Appropriate fractions were pooled and evaporated under vacuum. The residue was triturated with methanol to obtain a white solid. Yield 0.360 g, 78%; mp 167–169 °C, $R_f = 0.34$ (hexanes/ethyl acetate 2:1); IR 1729, 1706, 1532, 1480 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 10.5 (s, 1H, CHO), 8.17 (s, 1H, imidazole CH), 8.08–7.90 (m, 6H,

Ar–H), 7.62–7.32 (m, 9H, Ar–H), 6.92 (d, $J = 4.5$ Hz, 1H, 1′-H), 5.88-5.76 (m, 2H, 2′-H, and 3′-H), 4.89–4.41 (m, 3H, 4′-, 5′- and 5″-H), 4.40 (q, $J = 7.2$ Hz, 2H), 1.42 (t, $J = 6.9$ Hz, 3H, CH_3); HRMS (FAB) Calcd for $C_{33}H_{28}N_2O_{10}$, 613.1822 (MH^+); observed *m/z* 613.1828 (MH^+).

*5-(4,6-Diamino-2,5-dihydro-1,3,5-triazin-2-yl)-1-(β-D-ribofuranosyl-)imidazole-4-carboxamide (**4**)*. Guanidine hydrochloride (0.192 g, 2 mmol) was neutralized with a solution of sodium (46 mg, 2 mmol) in anhyd ethanol (5 mL) by stirring at 5 °C for 30 min. The precipitated sodium chloride was removed through filtration and the filtrate was added to a solution of ethyl 5-formyl-1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)imidazole-4-carboxylate (0.307 g, 0.5 mmol) in dry ethanol (10 mL). The reaction mixture was heated at reflux for 12 h. The reaction mixture was cooled, mixed with flash silica gel (2 g), and the solvent was evaporated. The residue was purified by flash chromatography, using a mixture of chloroform/methanol/ammonium hydroxide (1:1:0.25) as an eluting solvent. The appropriate fractions were pooled and the solvents were removed under vacuum to obtain a colorless solid. Yield 0.108 g, 61%; mp 144–146 °C, $R_f = 0.24$ (chloroform/methanol/ammonium hydroxide, 1:1:0.25); IR 3341, 3305, 3296, 1647, 1621 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.03 (s, 1H, imidazole CH), 7.44 (s, 1H, CONH), 7.21 (s, 1H, CONH), 6.5 (s, 1H, CH), 6.92 (d, $J = 5.1$ Hz, 1H, 1′-H), 6.10–5.90 (br s, 2H, 2OH), 5.55–4.90 (br s, 5H, OH+ 2 NH_2), 4.10–3.81 (m, 2H, 2′-H, and 3′-H), 3.62–3.38 (m, 3H, 4′-, 5′- and 5″-H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 164.9, 161.0, 159.6, 134.4, 131.5, 126.2, 87.9, 83.5, 77.9, 71.1, 60.8, 57.9; HRMS (FAB) Calcd for $C_{12}H_{18}N_8O_5$, 355.1401 (MH^+); observed *m/z* 355.1418 (MH^+).

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