Brief Articles

Synthesis of N^3,5′-Cyclo-4-(β-D-ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridin-5-one, a Novel Compound with Anti-Hepatitis C Virus Activity

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A novel anti-hepatitis C virus (HCV) agent, N^3,5′-cyclo-4-(β-D-ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridinin-5-one, was identified, and the structure was confirmed by chemical synthesis from 2-hydroxy-5-nitropyridine.

Hepatitis C virus (HCV) is one of the most clinically important Flaviviridae infections affecting humans and is the second most common cause of viral hepatitis. Currently, nearly 2% of the U.S. population, and an estimated 170 million people worldwide, are HCV carriers.[1] HCV is an RNA virus, which replicates without involvement of DNA. Presently there is no universally effective treatment for this infection, and the only drugs available for treatment of chronic hepatitis C are various forms of alpha interferon (IFN-α), either alone or in combination with ribavirin.[2] However, the therapeutic value of these treatments has been compromised largely due to adverse effects,[2,3] which highlights the need for development of additional options for treatment.

Therefore, we embarked upon systematic investigation of ribonucleosides from our extensive chemical library of nucleosides in the hope that HCV hits may provide clues for the development of effective anti-HCV drugs. One of our programs was to synthesize various *base-modified* nucleosides and evaluate them for their activity in a replicon system.[4,5] None of the synthetic ribonucleosides with modified purine bases tested showed activity in our evaluation system. However, when we treated tribenzoylated 1-(β-D-ribofuranosyl)-2-oxo-5-nitropyridine (**1**, Scheme 1) with sodium azide in DMF, followed by saponification with NaOMe to a product, which we expected to be 4-(β-D-ribofuranosyl)-5-oxo-*vic*-triazolo[4,5-*b*]pyridine (**3**),[6] we found weak activity. Inactive preparations consisted only of 4-(β-D-ribofuranosyl)-5-oxo-*vic*-triazolo[4,5-*b*]pyridine (**3**),[6] but the active preparation was contaminated with a second component, which is less polar than **3** on TLC. Therefore, we focused our efforts on structural determination of the minor component, which was separated and purified by repeated silica gel column chromatography. This compound showed no H-6 signal of pyridine in its ^{1}H NMR spectrum, suggesting that it is a derivative of 5-oxo-*vic*-triazolo[4,5-*b*]pyridine. However, there were only two D_2O exchangeable doublets for 2′- and 3′-OH groups and no exchangeable triplet for 5′-OH. The chemical shifts of H-5′a and H-5′b were at δ 4.83 (dd, $J_{4',5'a} = 4$ and $J_{5'a,5'b} = 13.6$ Hz) and at δ 5.02 (apparent d, $J_{4',5'b} = 0$, $J_{5'a,5'b} = 13.6$ Hz). The large difference in chemical shifts and geminal coupling of H5′a and H5′b strongly suggest no free rotation about the C-4′−C-5′ bond. This was further supported by NOE difference spectra: irradiation of the resonances at 4.83 ppm gave enhancement of the signals at 4.60 and 5.02 ppm, whereas irradiation of the resonances at 5.02 ppm gave enhancement of the signals at 4.83, 4.60, and 3.98 ppm. In addition, the UV spectrum in water was similar to that of 4-methyl-5-oxo-*v*-triazolo[4,5-*b*]pyridine,[7] and the pattern did not change at pH 2 and pH 11, indicating no dissociable proton on the base. All these spectral characteristics are fully consistent with the novel cyclic compound **6**.[7] The elemental analyses ($C_{10}H_{10}N_4O_4$) and mass spectral data (HR−FAB MS obsd: *m/z* 249.0614, calcd for $C_{10}H_{10}N_4O_4$: *m/z* 249.0624 M − H$^-$) are also consistent with structure **6**.

We then investigated conditions to produce **6** more efficiently. The improved procedure that we tried was to start with 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-nitropyridin-2-one (**1**), which we obtained in 70% yield by condensation of the trimethylsilyated pyridine with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribofuranose under the Vorbrüggen's conditions. The ^{1}H NMR spectrum of the product was consistent with those that were previously reported.[6] The benzoyl protecting groups were removed with saturated ammonia in methanol, and after crystallization from water, 1-(β-D-ribofuranosyl)-5-nitropyridin-2-one (**2**) was obtained in 81% yield. Treatment of **2** with sodium azide in DMF at 110−120 °C for 12 h afforded the *vic*-triazolopyridine nucleoside **3** in 60% yield.

When **1** was directly treated with NaN_3, followed by de-O-benzoylation with NaOMe in MeOH, a mixture of **3** and **6** was produced. In this reaction, **3** was formed

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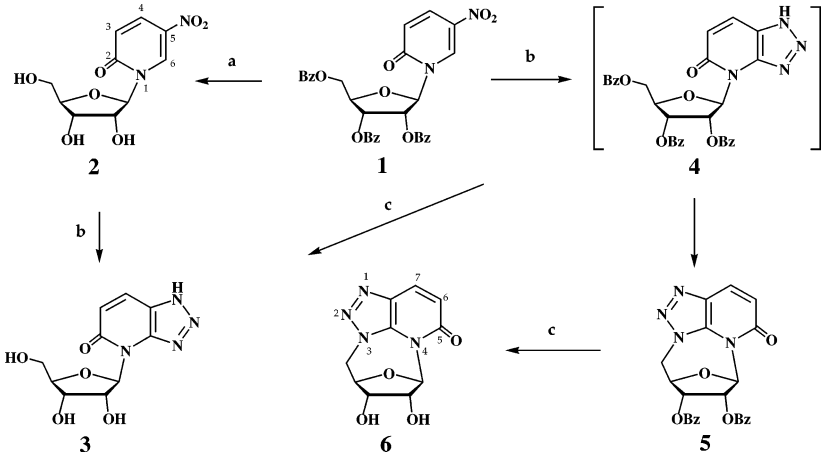
Scheme 1[a]



[a] Reagents and conditions: (a) NH_3/MeOH, rt; (b) NaN_3, DMF, (b′: at 110−120 °C, 12 h: from **2** to give **3**; b″: at 80−95 °C, 24 h, from **1** to **4**; b‴: at 110−120 °C, 48 h, from **1** to **5**); (c) 0.5 M NaOMe in MeOH, rt.

from **4** by solvolysis. Compound **6** could have been produced by two ways: after formation of **4** during NaN_3 treatment of **1**, the 5′-benzoyloxy group acted as a leaving group upon attack of N-3 on C-5′ leading to **5** which was then de-O-benzoylated; or intramolecular cyclization could have occurred between N-3 and C-5′during solvolysis of **4**. In the latter case two competing reactions occurred simultaneously; i.e., attack by solvent in the presence of base on the carbonyl carbon of the benzoyl group and intramolecular attack of N-3 on C-5′ with removal of the benzoyloxy group. We, therefore, studied the course of the reaction to find conditions that favor intramolecular reaction over solvolysis to convert **1** directly to **5**. We found that reaction of **1** with NaN_3 in DMF at 90 °C afforded only 4-(2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridin-5-one (**4**). However, we observed that by increasing the reaction temperature to 110 °C or higher, **4** was produced first then **5** was formed slowly as judged by TLC. The amount of **5** increased with concomitant decrease of **4**. Thus, after prolonged heating we were able to obtain **5** in 44% yield. Saponification of **5** with MeONa/MeOH furnished **6** in 62% yield (Scheme 1).

The antiviral activity of **6** was evaluated in the HCV subgenomic RNA replicon as previously described,[5] and the results are shown in Figure 1. Compound **6** had an anti-HCV effect with an EC_{50} (effective concentration to reduce the HCV RNA by 50%) of 19.7 μM after 96 h of compound exposure (Figure 1A). In addition to the antiviral effect, ribosomal RNA (which is a cellular marker for potential toxic side-effects) was also reduced (Figure 1A). To evaluate the specific antiviral effect of compound **6** over a longer exposure time, Replicon cells were kept in culture for 7 days, either in the presence (100 μM) or in absence of the compound (Figure 1B). These experiments showed that **6** caused a cytostatic effect at high concentration, since the treated cells did not proliferate with the same dynamics as the no-compound control. Concomitantly with the slower cell proliferation, a significant decrease in intracellular HCV RNA was observed.

In addition, an HCV RNA-dependent RNA polymerase (NS5B) assay was designed essentially as described.[8] Compound **6** showed no specific inhibitory activity against this recombinant NS5B enzyme (data not shown). Since there is no 5′-OH in compound **6**, it is not surprising that it does not appear to inhibit HCV−RNA-dependent RNA polymerase.

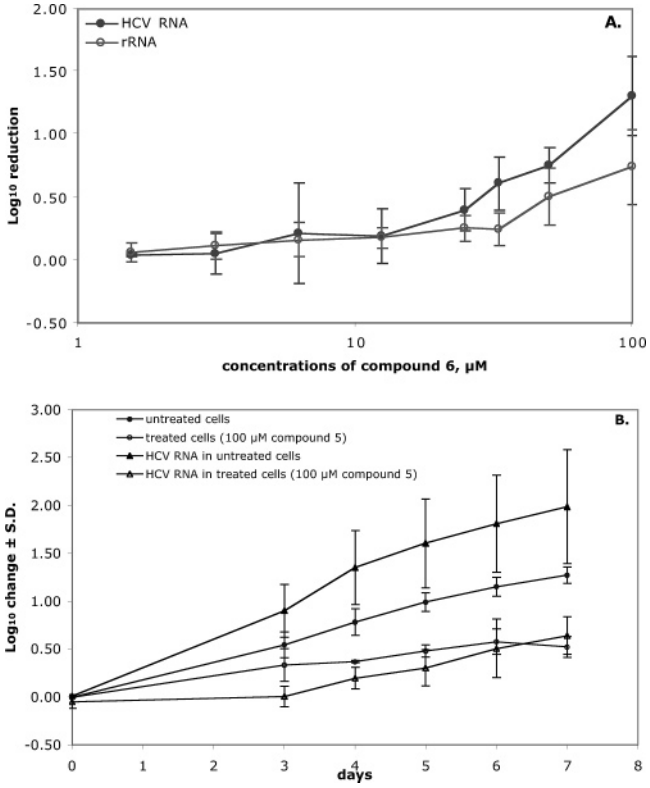


Figure 1. Antiviral activity of compound **6**. (A) Dose-dependent antiviral effect on HCV replicon RNA containing Huh-7 cells. Cells were seeded at 1000 cells per well in a 96-well plate in the presence of compound. After 96 h of incubation, replicon HCV RNA and rRNA levels were quantified by real time reverse transcription-PCR. (B) Comparison of the effects of compound **6** on cell growth and on HCV replicon dynamics over 7 days.

Compound **6** was tested against a range of other RNA viruses (Table 1). The compound was inactive and generally nontoxic (Table 2) with the exception of weak activity against influenza B virus with an EC_{50} of 23 μM.

In conclusion, we discovered and synthesized a novel compound **6** as a good lead for development of anti-HCV

Table 1. Antiviral Activity of Compound **6** When Tested against Various Viruses in Culture

virus	EC_{50}, μM	EC_{90}, μM
influenza a virus	>100	
influenza b virus	28	
respiratory syncytial virus	>100	
measles virus	>100	
rhinovirus	>100	
parainfluenza virus	>100	
pinchinde virus	>100	
VEE	>100	
yellow fever	>100	
West Nile	>100	
adenovirus type 1	>100	
punta toro A	>100	
hepatitis B virus	>100	>100
HCV replicon	19.7	79.8
BVDV	>100	>100
HIV	>100	>100
HSV type 1	>100	>100

agents. Compound **6** showed an interesting anti-hepatitis C effect in the HCV subgenomic RNA replicon. The mode of anti-HCV action is now the subject of further studies. In addition, structure−activity relationship studies aimed at optimizing the anti-HCV activity while reducing the cytostatic effect are underway.

Experimental Section

General. Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a Varian Unity Plus 400 spectrometer at room temperature, with tetramethylsilane as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm), and signals are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Values given for coupling constants are first order. UV spectra were recorded on a Varian CARY 50 Bio UV−visible spectrophotometer. Fast atom bombardment mass spectroscopy was performed by the Emory University Mass Spectrometry Center. TLC was performed on Uniplates (silica gel) purchased from Analtech Co., and column chromatography was performed using silica gel (60 Å) from Sorbent Technologies, Atlanta, GA. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-5-nitropyridine-2(1*H*)-one (1). 2-Hydroxy-5-nitropyridine (5.6 g, 40 mmol) in hexamethyldisilazane (80 mL) is refluxed with a catalytic amount of ammonium sulfate for 6 h in an argon atmosphere. Excess solvent is removed in vacuo and the residue dissolved in 1,2-dichloroethane (100 mL). To this solution are added 1-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (19 g, 37.6 mmol) in anhydrous 1,2-dichloroethane (100 mL) and 1 N solution of tin(IV) chloride in CH_2Cl_2 (7.68 mL), and the mixture was heated under reflux for 8 h. After being kept at room-temperature overnight, the reaction mixture is diluted with CH_2Cl_2, washed with saturated $NaHCO_3$ solution, and filtered through Celite. The organic layer was separated, washed with H_2O, dried, filtered, and concentrated to give a residue, which was crystallized from ethanol. The product **1** (15.53 g, 70%) was obtained as a solid. ^{1}H NMR (DMSO-d_6) δ 4.71 (m, 1H), 4.90 (m, 1H), 6.04 (m, 2H), 6.43 (d, J = 2.0 Hz, 1 H), 6.59 (d, J = 10.0 Hz, 1H), 7.40−8.02 (m, 15 H), 8.19 (dd, J = 3.2, 10.0 Hz, 1 H), 9.16 (d, J = 3.2 Hz, 1 H).[6]

1-(β-D-Ribofuranosyl)-5-nitropyrine-2(1*H*)-one (2). A mixture of **1** (100 mg, 0.17 mmol) and saturated MeOH/NH_3 (10 mL) was stirred at room temperature for 12 h. The mixture was concentrated to dryness, and the residue was triturated with EtOH to precipitate (**2**), which was collected and crystallized from water (37 mg, 81%) as a white solid. ^{1}H NMR (DMSO-d_6+D_2O) δ 3.64 (m, 1H), 3.90 (m, 1H), 4.01 (m, 3H), 5.89 (s, 1H), 6.48 (d, J = 10.4 Hz, 1 H), 8.12 (dd, J = 3.2, 10 Hz, 1H), 9.65 (d, J = 3.2 Hz, 1 H). This product was used directly in the next step without further purification.

4-(β-D-Ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridin-5-one (3). A mixture of **2** (54 mg, 0.2 mmol) and NaN_3 (20 mg, 0.3 mmol) in DMF (20 mL) was stirred at 110−120 °C for 12 h. The mixture was concentrated to dryness, and the residue was purified by silica gel column chromatography with 15% MeOH in CH_2Cl_2 to give **3** (32 mg, 60%) as a solid, ^{1}H NMR (DMSO-d_6) δ 3.53 (m, 1H), 3.66 (m, 1H), 3.88 (m, 1H), 4.16 (dd, J = 5.2, 9.2 Hz, 1 H), 4.79 (m, 1H), 5.04 (d, J = 5.2 Hz, 1H), 5.17 (d, J = 6 Hz, 1 H), 6.33 (d, J = 6 Hz, 1 H), 6.46 (d, J = 9.6 Hz, 1 H), 8.01 (d, J = 9.6 Hz, 1 H). Anal. Calcd for $C_{10}H_{12}N_4O_5$: C, 44.78; H, 4.51; N, 20.89; Found: C, 44.60; H, 4.53; N, 20.60.

4-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-*vic*-triazolo-[4,5-*b*]pyridin-5-one (4). To a solution of 1 (3.82 g, 6.5 mmol) in DMF (33 mL) was added NaN_3 (1.55 g, 23.8 mmol) under argon. The suspension was stirred at 80−95 °C for 24 h. The solvent was removed in vacuo, and 2 N HCl (12 mL) was added the residue. The mixture was extracted with CH_2Cl_2, dried (Na_2SO_4), and concentrated in vacuo. The oily residue was purified on a silica gel column with a stepwise gradient of EtOAc (0−20%)in CH_2Cl_2 to give 3.19 g (84%) of a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 4.57 (dd, J = 5.2, 12.4 Hz, 1H), 4.71 (dd, J = 3.6, 12.0 Hz, 1H), 4.79 (m, 1H), 6.23 (t, J = 8.0 Hz, 1 H), 6.33 (m, 1H), 6.60 (d, J = 10.0 Hz, 1H), 6.70 (s, 1H), 7.38−7.94 (m, 15 H), 8.10 (d, J = 10 Hz, 1 H).[6]

3,5′-Cyclo-3-(2,3-di-*O*-benzoyl-β-D-ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridin-5-one (5). A mixture of **1** (1 g, 1.71 mmol), NaN_3 (167 mg, 2.56 mmol), and DMF (28 mL) was stirred and heated at 110−120° C for 48 h. The solvent was removed in vacuo, and the residue purified by silica gel column chromatography with 20% EtOAc in hexanes to give **5** as a solid (343 mg, 44%). mp 232−233 °C (after crystallization from EtOAc/EtOH). ^{1}H NMR (DMSO-d_6) δ 5.00 (dd, J = 4.4 and 14 Hz, 1 H), 5.33 (m, 2 H), 5.79 (t, J = 4.8 Hz, 1 H), 5.88 (d, J = 5.2 Hz, 1 H), 6.44 (d, J = 9.6 Hz,1H), 6.75 (s, 1 H), 7.32−8.08 (m, 10 H), 8.14 (d, 1H, J = 9.6). HR−FAB MS Obsd; m/z 459.1321. Calcd for $C_{24}H_{19}N_4O_6$: m/z 459.1305 $(M + H)^+$. Anal.

Table 2. Cytotoxicity Results for Compound **6** in Various Cell Lines.

cell line	cell origination	CC_{50} (μM), MTS[a]	CC_{50} (μM), neutral red
HepG2	human hepatocellular carcinoma	74.4	
Huh-7	human hepatocellular carcinoma	49.6	
PBM	primary human peripheral blood mononuclear cells	>100	
Vero	African green monkey kidney	>100	>100
SK-MEL-28	human malignant melanoma	>100	
CEM	human T-cell lymphoma	13.8	
B-SC-1	African green monkey kidney		>100
MDCK	Madin Darby canine kidney		>100
A-549	human lung carcinoma		>100
CV-1	African green monkey kidney		>100
MA-104	African green monkey kidney		>100
LLC-MK2	rhesus monkey kidney		>100
KB	human epidermoid carcinoma of the nasopharnyx		>100

[a] MTS dye: Cell titer 96-cell proliferation assay (Promega, Madison, WI).

Calcd for $C_{24}H_{18}N_4O_3 \cdot 0.5\ H_2O$: C, 61.67; H, 4.10; N, 11.99; Found: C, 61.41; H, 4.10; N, 12.39.

3,5′-Cyclo-1-(β-D-ribofuranosyl)-*vic*-triazolo[4,5-*b*]pyridin-5-one (6). Compound **5** (160 mg, 0.35 mmol) was treated with 0.5 M NaOMe/MeOH at room temperature for 1 h. The mixture was neutralized with acetic acid, concentrated in vacuo to dryness, and the residue was purified by silica gel column chromatography with 5% MeOH in CH_2Cl_2 to give **6** (54 mg, 62%) as a white solid, mp 248−251 °C. ^{1}H NMR (DMSO-d_6) δ 3.98 (t, J = 4.8 Hz, 1 H, 2′-H), 4.14 (dd, J = 5.2 and 12.4 Hz, 1 H, 3′-H), 4.60 (t, J = 4.4 Hz, 1 H, 4′-H), 4.83 (dd, J = 4 and 13.6 Hz, 1 H, 5′-Ha), 5.02 (d, J = 13.6 Hz, 1 H, 5′-Hb), 5.39 (d, J = 7.2 Hz, 1 H, OH, D_2O exchangeable), 5.79 (d, J = 5.2 Hz, 1 H, OH, D_2O exchangeable), 6.21 (s, 1 H, 1′-H), 6.36 (d, J = 9.6 Hz, 1 H, 6-H), 8.05 (d, J = 9.6 Hz, 1 H, 7-H). ^{13}C NMR ((DMSO-d_6) δ 159.98, 136.55, 131.96, 129.64, 116.89, 93.22, 81.85, 74.02, 71.31, 54.90; HR−FAB MS Obsd; m/z 249.0614. Calcd for $C_{10}H_9N_4O_4$: m/z 249.0624 $(M-H)^-$. Anal. Calcd for $C_{10}H_{10}N_4O_4$: C, 48.00; H, 4.03; N, 22.39; Found: C, 48.10; H, 4.06; N, 22.45.

Antiviral Testing. HCV-replicon RNA-containing Huh7 cells (Clone A cells; Apath, LLC, St. Louis, MO) were kept in exponential growth in DMEM media (high glucose, no pyruvate) containing 10% fetal bovine serum, 1X nonessential amino acids, penicillin−streptomycin−glutamine (100 units/L, 100 μg/L, and 2.92 mg/L, respectively) and G418 (500 to 1000 μg/mL).[4] Antiviral assays were performed in the same media without G418. Cells were seeded in a 96-well plate at 1000 cells per well and test compounds were added immediately after seeding. After 96 h of incubation, total cellular RNA was isolated (Rneasy 96 kit, Qiagen, CA). Replicon RNA and an internal control (TaqMan Ribosomal RNA control Reagents, Applied Biosystems, CA) were amplified in a single-step multiplex RT-PCR protocol, as recommended by the manufacturer. The HCV primers and probe used have been described previously.[5]

To express the antiviral effectiveness of a compound, the threshold RT-PCR cycle of the test compound was subtracted from the average threshold RT-PCR cycle of the 'no drug' control (ΔCt_{HCV}). A ΔCt of 3.3 equals a 1-log reduction (i.e., EC_{90}) in replicon RNA levels. The cytotoxicity of the test compound was also determined by calculating the ΔCt_{rRNA} values. The $\Delta\Delta$Ct specificity parameter could then be introduced ($\Delta Ct_{HCV} - \Delta Ct_{rRNA}$), in which the levels of HCV RNA were normalized for the rRNA levels and are calibrated against the no drug control. Recombinant interferon alpha-2a (INF-α-2a; Roferon-A, Hoffman La Roche Inc, NJ) served as a positive control.

The neutral red and CPE (cytopathic effect) reduction assays for determining the antiviral activity and cytotoxicity were performed as described previously.[9] Cytotoxicty testing using MTS was performed as described previously.[10,11]

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