ISSN 0027-1314, Moscow University Chemistry Bulletin, 2008, Vol. 63, No. 2, pp. 89–93. © Allerton Press, Inc., 2008.
Original Russian Text © N.A. Golubeva, A.V. Ivanov, M.A. Ivanov, O.A. Batyunina, A.V. Shipitsyn, V.L. Tunitskaya, L.A. Aleksandrova, 2008, published in Vestnik Moskovskogo Universiteta. Khimiya, 2008, No. 2, pp. 112–116.

New Nucleoside Analogues and Their 5'-Triphosphates: Synthesis and Biological Properties

N. A. Golubeva[a], A. V. Ivanov[a, b], M. A. Ivanov[a], O. A. Batyunina[a], A. V. Shipitsyn[a], V. L. Tunitskaya[a], and L. A. Aleksandrova[a]

[a] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, Moscow, 119991 Russia*
e-mail: ala2004@zmail.ru

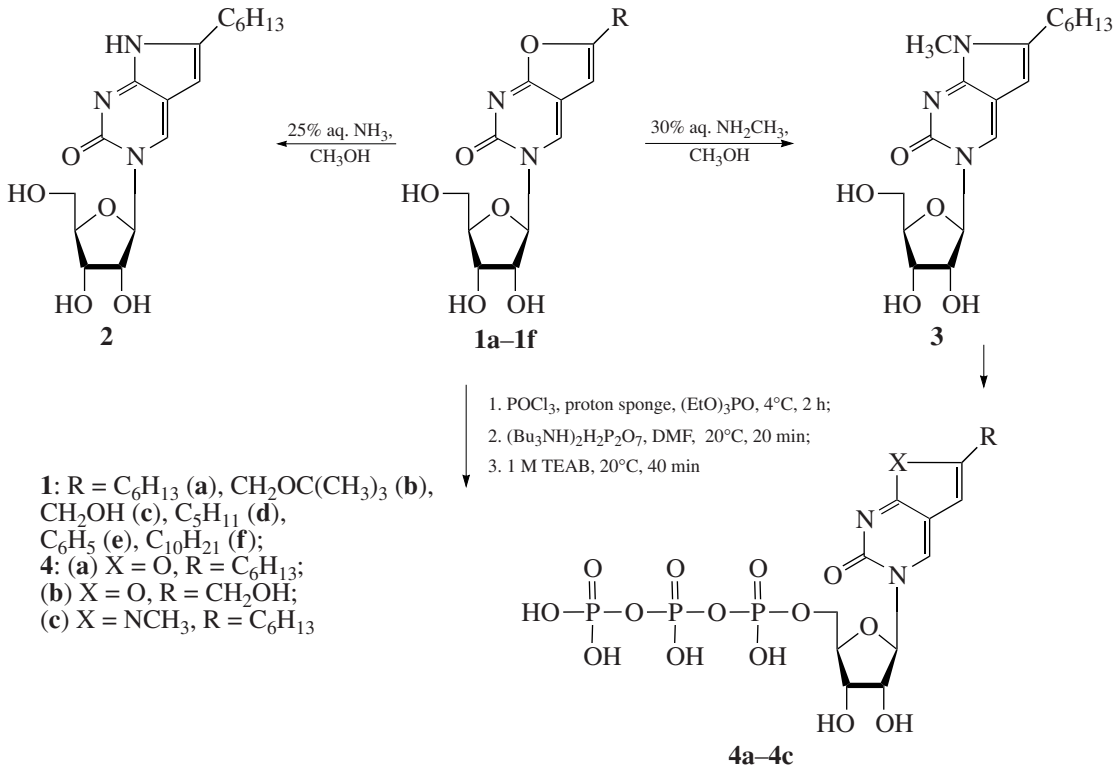
[b] *Centre for Medical Studies in Russia, University of Oslo, ul. Vavilova 34/5, Moscow, 119991 Russia*

Received November 23, 2007

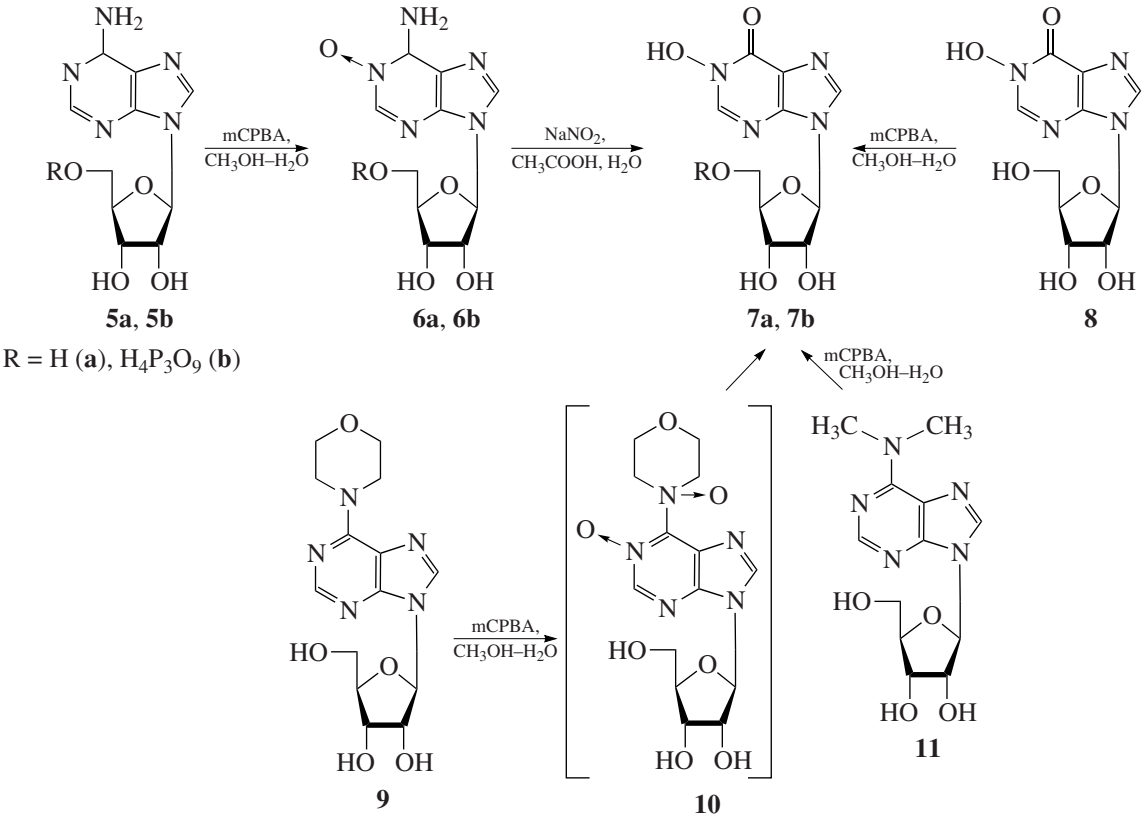
Abstract—Bicyclic furano- and pyrrolo[2,3-*d*]pyrimidine nucleosides and purine nucleosides modified at the N^1 atom and/or the 6-position have been synthesized. Among the tested nontoxic bicyclic nucleosides and N^6-carboxyalkyladenosines, only furo[2,3-*d*]pyrimidine with the $C_{10}H_{21}$ substituent and N^6-carboxymethyladenosine exhibit moderate anti-HCV activity in the virus replicon system and N^1-hydroxyinosine exhibits high anti-HCV activity and significant cytotoxicity. The corresponding 5'-triphosphates have been synthesized and studied as substrates/inhibitors of HCV enzymes: NS5B protein (RNA-dependent RNA polymerase) and NS3 protein (NTP-dependent RNA helicase).

DOI: 10.3103/S0027131408020090

Hepatitis C virus (HCV) is one of the most dangerous infections [1, 2]. Currently, only a few types of HCV replication inhibitors are known, which makes the search for new anti-HCV agents an important task. This work deals with the synthesis of new analogues of ribonucleosides, tests for their activity as inhibitors of HCV reproduction in the HCV replicon system, and the synthesis of 5'-triphosphates of the synthesized nucleo-



Scheme 1.



Scheme 2.

sides and study of them as substrates/inhibitors of nucleotide-dependent HCV enzymes: proteins NS5B (RNA-dependent RNA polymerase) and NS3 (NTP-dependent RNA helicase).

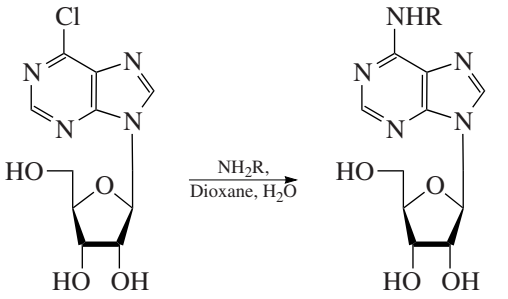
We developed a scheme of synthesis of N^1-hydroxyinosine (**7a**) in preparative amounts by means of oxidation of commercially available adenosine (**5a**) by *m*-chloroperbenzoic acid [7] with subsequent deamination of adenosine N^1-oxide (**6a**) by sodium nitrite in an acid medium (Scheme 1) with 45% yield (for two stages). Previously, N^1-hydroxyinosine (**7a**) was obtained by oxidation of inosine (**8**), N^6-morpholinylpurineriboside (**9**), or N^6,N^6-dimethyladenosine (**11**) by *m*-chloroperbenzoic acid (yields 24, 10, and <5%, respectively) [8]. The formation of N^1-hydroxyinosine (**7a**) can be explained by either consecutive or simultaneous oxidation at the N^1 position and the N^6 exocyclic purine group to form intermediate compound **10**.

N^6-Substituted adenosine derivatives are well documented [9]; however, their analogues with an anionic group (zwitterionic structure) have not been described. We synthesized N^6-carboxyalkyladenosines (**13a**–**13c**) by the reaction of 6-chloropurineriboside (**12**) with the corresponding amino acid in the presence of N-ethyldiisopropylamine (Schemes 1, 2). In a nonaqueous medium, the reaction does not take place. However, in 20% aqueous dioxane as the solvent, compounds **13a**–**13c** were obtained in almost quantitative yields.

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded in D_2O on a Bruker AMXIII-400 spectrometer operating at 400, 133, and 162 MHz, respectively. Sodium 3-trimethylsilyl-1-propanesulfonate was used as the internal reference. TLC analysis was carried out on Kieselgel 60 F_{254} plates in different systems. UV spectra were recorded as solutions in water (pH 7) on a Shimadzu UV-2401 PC spectrophotometer.

The HCV replicon was cultured and its relative titer was determined as described in [10]. The cytotoxicity of the compounds in *Huh7* cells was determined with the use of MTT (Sigma, United States) [10].

N^1-Hydroxyinosine (7a). To a solution of 2.68 g (1 mmol) of adenosine in 50 mL of 50% aqueous methanol, 3.44 g (2 mmol) of *m*-chloroperbenzoic acid was added under stirring. After 24 h, another 3.44 g (2 mmol) of *m*-chloroperbenzoic acid was added, and the mixture was stirred for 24 h. The reaction mixture was concentrated in vacuo, dissolved in water, and extracted with ethyl acetate. The aqueous fraction was purified by reverse-phase chromatography on a LiChroprep RP-18 column (50 × 400 mm) and lyophilized from water to give 2 g of adenosine N^1-oxide (**6a**). To a solution of 2 g (0.7 mmol) of **6a** in 50 mL of water,



12 **13a–13c**

R = CH_2COOH (**a**), CH_2CH_2COOH (**b**), $CH(CH_3)COOH$ (**c**)

Scheme 3.

11 mL (2 mmol) of acetic acid and 13.8 g (2 mmol) of sodium nitrite were added. The reaction mixture was stirred for two days; then 6.9 g (1 mmol) of sodium nitrite was added; and the resulting mixture was stirred for three days, evaporated in vacuo, and purified by reverse-phase chromatography on a LiChroprep RP-18 column (50 × 400 mm). The product was lyophilized from water. The total yield was 1.28 g (45%) (the physicochemical characteristics were the same as reported in [8]).

N⁶-Carboxyalkyladenosines (13a–13c). To a solution of 50 mg (0.17 mmol) of 6-chloropurineriboside (**12**) in 1 mL of aqueous dioxane, 300 μL (1.74 mmol) of N-ethyldiisopropylamine and 78 mg (0.87 mmol) of β-alanine, 65 mg (0.87 mmol) of glycine, or 78 mg (0.87 mmol) of alanine were added. The mixture was kept for two days at 37°C and then evaporated in vacuo. The residue was dissolved in 1 mL of water and purified by reverse-phase chromatography on a LiChroprep RP-18 column (20 × 200 mm) in a linear gradient of methanol concentration (0 ⟶ 20%, V = 400 mL) in 0.02 M aqueous NH_4HCO_3. The products were lyophilized from water. The yield was 95%.

N⁶-(2-Carboxyethyl)adenosine (13a). UV: λ_{max} = 267 nm (ε = 16100). ^{1}H NMR (δ, ppm; SCC, Hz): 8.09 and 8.01 (both s, 2H, H-2 and H-8); 5.87 (d, 1H, H-1', J = 5.9); 4.63 (t, 1H, H-2', J = 5.6); 4.31 (~t, 1H, H-3', J = 5.6); 4.16 (~q, 1H, H-4', J = 2.8); 3.81 (dd, 1H, H-5'a, J = 2.8 and 12.9); 3.72 (dd, 1H, H-5'b, J = 3.6 and 12.9); 3.56 (br s, 2H, CH_2N); 2.46 (t, 2H, CH_2COOH, J = 6.9). ^{13}C NMR (δ, ppm): 178.62 (COOH), 159.14 (C-6), 149.54 (C-4), 153.52 (C-2), 140.93 (C-8), 119.61 (C-5), 89.46 (C-1'), 86.75 (C-4'), 74.88 (C-3'), 71.70 (C-2'), 62.64 (C-5'), 37.84 (C–COOH and C–NH).

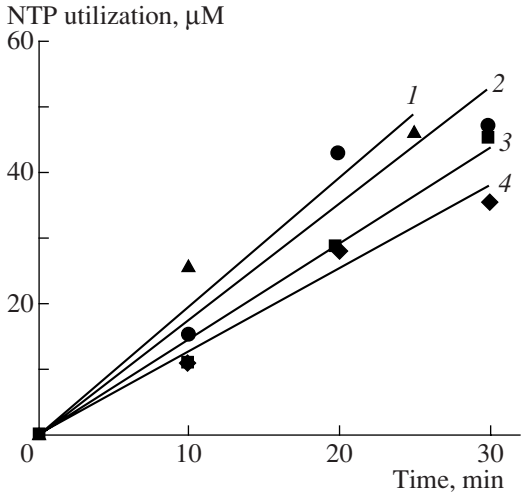
N⁶-Carboxymethyladenosine (13b). UV: λ_{max} = 266 nm (ε = 15300). ^{1}H NMR (δ, ppm; SCC, Hz): 8.14 and 8.03 (both s, 2H, H-2 and H-8); 5.91 (d, 1H, H-1', J = 5.9); ~4.8 (the signal of H-2' is partially overlapped by the signal of HOD); 4.33 (~t, 1H, H-3', J = 4.2); 4.18 (~q, 1H, H-4', J = 3.1); 3.97 (br s, 2H, CH_2N); 3.83 (dd, 1H, H-5'a, J = 2.7 and 12.9); 3.74 (dd, 1H, H-5'b, J = 3.7 and 12.9).^{13}C NMR (δ, ppm): 173.49 (COOH), 159.25 (C-6); 149.81 (C-4), 152.90 (C-2), 140.47 (C-8), 119.65 (C-5), 88.87 (C-1'), 86.13 (C-4'), 74.19 (C-3'), 71.03 (C-2'), 61.98 (C-5'), 44.93 (C–N).

N⁶-(1-Carboxyethyl)adenosine (13c). UV: λ_{max} = 268 nm (ε = 14800). ^{1}H NMR (δ, ppm; SCC, Hz): 8.11 and 8.01 (both s, 2H, H-2 and H-8); 5.89 (d, 1H, H-1', J = 5.9); ~4.8 (the signal of H-2' is partially overlapped by the signal of HOD); 4.32 (~t, 2H, H-3' and CH, J = 4.2); 4.18 (~q, 1H, H-4', J = 2.8); 3.83 (dd, 1H, H-5'a, J = 2.5 and 12.9); 3.74 (dd, 1H, H-5'b, J = 3.6 and 12.9); 1.41 (d, 3H, CH_3, J = 7.2). ^{13}C NMR (δ, ppm): 176.42 (COOH), 159.13 (C-6), 149.61 (C-4), 152.76 (C-2), 140.18 (C-8), 119.51 (C-5), 88.73 (C-1'), 85.94 (C-4'), 74.11 (C-3'), 70.89 (C-2'), 61.83 (C-5'), 52.18 (C–N), 18.38 (CH_3).

Cytotoxicity in *Huh7* cells and the antiviral effect of the synthesized compounds in the HCV replicon system

Compound	LC_{50}, μM	IC_{50}, μM
1a–**1e**, **2**, **3**	>500	Inactive
1f	>500	30
7a	20	2–3
13a	100	30–50
13b, **13c**	>500	Inactive
2'MeC*	>200	1.23
4'N_3C*	>33	1.28

* Taken from [11]; LC_{50} is the concentration of a compound that is lethal to 50% of cultured cells; IC_{50} is the concentration of a compound that inhibits virus replication by half.



Hydrolysis of bicyclic nucleoside triphosphates and UTP catalyzed by NTP-dependent RNA helicase: (*1*) UTP, (*2*) **4b**, (*3*) **4c**, and (*4*) **4a**. The compounds (200 μM were incubated for 5–30 min with 0.2 μg of protein NS3 at 37°C in 50 mM Tris–HCl (pH 7.5), 25 mM NaCl, 3 mM $MgCl_2$, and 1.5% (v/v) of glycerol. The products were analyzed by HPLC (Lichrosorb-NH2 column, 4 × 150 mm, 6 μm) or TLC.

N^1-hydroxyinosine 5'-triphosphate (7b) was synthesized from adenosine 5'-triphosphate (**5b**) by the method described above for the nucleoside. UV: λ_{max} = 232 nm (ε = 35 000), 261 nm (ε = 8500). ^{1}H NMR (δ, ppm; SCC, Hz): 8.48 and 8.46 (both s, 2H, H-2 and H-8); 6.01 (d, 1H, H-1', *J* = 4.6); ~4.8 (the signal of H-2' is partially overlapped by the signal of HOD); 4.46 (br s, 1H, H-3'), 4.26 (br s, 1H, H-4'); 4.12 (m, 2H, H-5'). ^{31}P NMR (δ, ppm): –6.44 (br s, 1P, P_α), –10.64 (s, 1P, P_γ), –21.50 (br s, 1P, P_β).

Bicyclic furo[2,3-*d*]pyrimidine nucleosides (**1a**–**1f**) were synthesized by the reaction of 5-iodouridine with terminal alkynes in the presence of catalytic amounts of Pd(0) and CuI [3, 4] by analogy with the method suggested for the synthesis of corresponding 2'-deoxynucleosides [5, 6]. Treatment of nucleoside **1a** with aqueous alcohol solutions of ammonia or methylamine (Scheme 1) led to pyrrolo- and N^7-methylpyrrolo[2,3-*d*]pyrimidine nucleosides (**2** and **3**), respectively [3, 4].

The synthesized nucleosides were tested as potential inhibitors of HCV replication in the virus replicon system [10] (table). Compounds **1**–**3**, **13b**, and **13c** were inactive in this system and exhibited no cytotoxicity in concentrations up to 500 μM in human hepatocyte cells *Huh7*. Only bicyclic nucleoside **1f**, containing the $C_{10}H_{12}$ substituent, and N^6-carboxymethyladenosine **13a** exhibited moderate anti-HCV activity. In the series of N^6 derivatives (**13**), only N^6-carboxymethyladenosine (**13a**) exhibited moderate cytotoxicity. N^1-Hydroxyinosine (**7a**) exhibited high anti-HCV activity and high cytotoxicity in *Huh7* cell culture.

The mechanism of the antiviral action of most nucleoside analogues involves their enzymatic transformation into nucleoside 5'-triphosphates with their subsequent incorporation into the 3' end of viral DNA or RNA, which inhibits virus replication [12, 13]. In the search for the possible target for the nucleosides, we synthesized a series of 5'-triphosphates and studied their substrate specificity with respect to HCV enzymes: proteins NS5B and NS3.

5'-Triphosphates **4a**–**4c** were synthesized using the method described in [14] (Scheme 1) by the action of $POCl_3$ in triethyl phosphate in the presence of proton sponge followed by condensation with tributylammonium pyrophosphate. The yields were 5–14% [3, 4]. N^1-Hydroxyinosine 5'-triphosphate (**7b**) was obtained in two stages by oxidation of the commercially available adenosine 5'-triphosphate (**5b**) followed by deamination of adenosine N^1-oxide 5'-triphosphate (**6b**) (Scheme 2).

Bicyclic nucleoside 5'-triphosphates **4a**–**4c** were not recognized by RNA polymerase of HCB and were extremely weak inhibitors of the ATPase reaction catalyzed by HCV NTPase; however, they turned out to be rather efficient NTPase substrates (figure). Their activity is only slightly lower than the activity of natural UTP [3, 4]. The difference in hydrolysis rates could be responsible for the low pseudo-inhibitory activity of these compounds. At the same time, N^1-hydroxyinosine 5'-triphosphate (**7b**) noticeably inhibited the ATPase reaction catalyzed by HCV NTPase (*Ki* 109 μM).

The above data point to the need for further study of the structure–anti-HCV activity relationship for this group of compounds in order to more exactly assess the prospects for their use as HCV inhibitors.

ACKNOWLEDGMENTS

We are grateful to A.O. Kuzyakin (Institute of Molecular Biology, RAS) for supplying HCV protein NS3.

This work was supported by the program of the Presidium of the RAS on molecular and cell biology and by the Russian Foundation for Basic Research (project nos. 05-04-49492, 05-04-49500, and 04-04-49621).

REFERENCES

1. Wasley, A. and Alter, M.J., *Semin. Liver Dis.*, 2000, vol. 20, p. 1.
2. Rosen, H.R. and Gretch, D.R., *Mol. Med. Today*, 1999, vol. 5, p. 393.
3. Alexandrova, L.A., Ivanov, M.A., Victorova, L.S., and Kukhanova, M.K., Nucleosides, Nucleotides Nucleic Acids, 2007 (in press).
4. Ivanov, M.A., Ivanov, A.V., Krasnitskaya, I.A., et al., *Biorg. Khim.*, 2007 ((in press).
5. Rubins, M.J. and Barr, P.J., *J. Org. Chem.,* 1983, vol. 48, p. 1854.

6. McGuigan, C., Yarnold, C.J., Jones, G., et al., *J. Med. Chem.*, 1999, vol. 42, p. 4479.

7. Kwong, C.D. Krauth, C.A., et al., *Nucleosides Nucleotides*, 1998, vol. 17, p. 1409.

8. Khandazhinskaya, A.L., Shirokova, E.A., Shipitsin, A.V., et al., *Collect. Czech. Chem. Commun.*, 2006, vol. 71, p. 1107.

9. Gao, Z.G., Blaustein, J.B., Gross, A.S., et al., *Biochem. Pharmacol.*, 2003, vol. 65, p. 1675.

10. Lohmann, V., Korner, F., Herian, U., et al., *Science*, 1999, vol. 285, p. 110.

11. Klumpp, K., Leveque, V., Le Pogam, S., et al., *J. Biol. Chem.*, 2006, vol. 281, p. 3793.

12. De Clercq, E., *Biochim. Biophys. Acta*, 2002, vol. 1587, p. 258.

13. De Clercq, E., *Nat. Rev. Drug Discov.*, 2002, vol. 1, p. 13.

14. Kovacs, T. and Otvos, L., *Tetrahedron Lett.*, 1988, vol. 29, p. 4525.