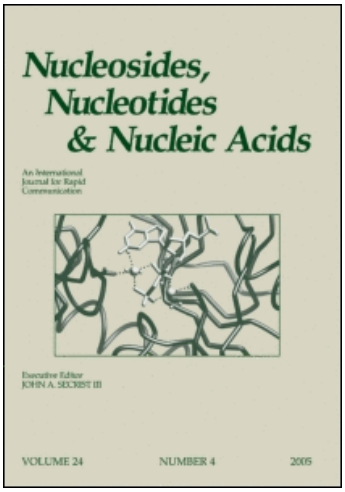

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SYNTHESIS OF 2′-*N*-FORMAMIDO NUCLEOSIDES AND BIOLOGICAL EVALUATION

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□ *The 2′-*N*-formamide derivatives of adenosine, cytidine, and 9-β-*D*-arabinofuranosyladenine were synthesized and tested (as triphosphate) for their substrate capacities for the HCV NS5B polymerase.*

Keywords 2′-*N*-formamide derivatives; HCV drugs; RNA viruses; anti HCV nucleosides; antiviral drug design

INTRODUCTION

The discovery of the hepatitis C virus[1] has activated research groups in industry and academia to develop antiviral compounds targeting RNA viruses. The current therapy uses a combination of pegylated interferon alpha and ribavirin, but its effectiveness is limited and side effects may be severe.[2] The research on potential hepatitis C virus (HCV) drugs has been boosted by the development of in vitro and in vivo test systems to screen compound.[3] As far as nucleoside chemistry is concerned, the most attractive target to develop anti-HCV nucleosides is the NS5B RNA dependent RNA polymerase, which plays an essential role in viral replication and which has no equivalent in mammalian cells.[4] The approach itself, that is, selecting a modified nucleoside that is phosphorylated in the cell and, as a triphosphate, inhibits the polymerase, is common in the field of antiviral drug design. So far, only a limited number of nucleoside analogues has been found to inhibit the HCV RNA dependent RNA polymerase.[5] Most of these

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nucleoside analogues carry a 3′-hydroxyl group, which means that they are most probably not absolute chain terminators. The presence of a substituent at either the 2′- or the 4′-position is apparently sufficient to inhibit chain elongation resulting in an antiviral effect. The specific recognition of the 2′-position of nucleosides by the RNA dependent RNA polymerase is required to distinguish between incoming nucleoside triphosphates (2′-OH) and incoming deoxynucleoside triphosphates (2′-OH). 2′-OMe and 2′-deoxy-2′-fluoro2′-C-methylnucleosides as triphosphates have been identified as inhibitors of the NS5B polymerase.[5a,h] We have synthesized 2′-N-formamido nucleosides and their triphosphates and we have evaluated these triphosphates as potential inhibitors of NS5B. By formylating the 2′-NH_2 group of 2′-amino-2′-deoxynucleosides, the basic amino group is converted into a slightly acidic 2′-substituent (pK_a of formamide: 23.5[6]), however, with considerable steric bulk.

RESULTS AND DISCUSSION

The 2′-formamido nucleosides and their triphosphates are very poor described in literature. Only 2′-formamido-2′-deoxyuridine was prepared by formylation of protected 2′-amino-2′-deoxyuridine with acetic formic anhydride in moderate yield.[7]

The synthesis of 2′-*N*-formamido-2′-deoxyadenosine (**IV**) is based on the procedure of Robins.[8] We used Markiewicz's disiloxane group[9] to regiospecifically mask the 5′- and 3′-hydroxyl groups of *ara*-A, leaving the 2′-hydroxyl group free for sulfonylation (triflation) (see Figure 1). Transient 2′-triflation with trifluoromethanesulfonyl chloride followed by S_N2 displacement at C2′ using LiN_3 gave the 2′-azidonucleoside in good yield which was reduced to the 2′-amino derivative. To avoid a possible formylation of exocyclic aminogroup of adenine nucleobase with acetic formic anhydride the 2′-amino group was formylated using ammonium formate[10] as a more selective formylating reagent. Deprotection with $Et_3N.HF$/NMP gave the desired *N*-formamido compound (**IV**) in 60% yield. 2′-*N*-Formamido-2′-deoxy-*ara*-adenosine (**V**) was synthesized in a similar way starting from adenosine.

The synthesis of 2′-*N*-formamido-2′-deoxycytosine (**VI**) is based on the procedure described earlier.[11,12] Uridine was converted into its 2,2′-*O*-anhydroderivative.[13] The interaction of this compound with sodium azide in the presence of dibenzo-18-crown-6 leads to the formation of 2′-azido-2′-deoxyuridine which is protected at the 5′- and 3′-hydroxyl groups with a disiloxane group.[9] The uridine base was converted into cytosine according to the published procedure[11] and azidonucleoside was reduced into the amino-derivative using a Staudinger reduction.[14] The selective

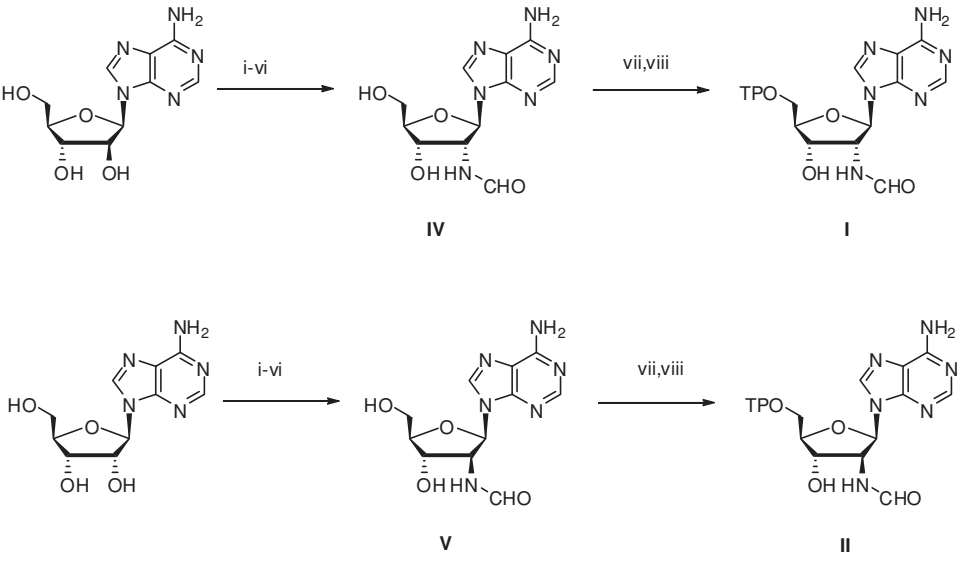

FIGURE 1 Synthesis of 2′-deoxy-2′-formamidonucleosides (**IV, V**) and its triphosphates (**I, II**). Reagents and conditions: (i) TIPS-Cl/Pyr; (ii) CF_3SO_2Cl, DMAP/DCM, 0°C; (iii) LiN_3, DMF; (iv) H2, 5% Pd(C); (v)$HCOONH_4$, MeCN; (vi) $Et_3N.HF$/THF; (vii) $POCl_3$, $PO(OMe)_3$; (viii) $(Bu_3NH)_4P_2O_7$, Bu_3N/TEAB.

aminogroup *N*-formylation using ammonium formate[10] followed by deprotection gave the desired *N*-formamido compound (**VI**) in 75% yield.

To obtain triphosphates (**I–III**) the one pot synthesis of Ludwig[15] as the most straightforward method for the 5′-selective phosphorylation of nucleosides (**IV–VI**) was applied.

The synthetic nucleoside triphosphate analogues were tested on their substrate capacities for the HCV NS5B polymerase. In order to function as an inhibitor of a polymerase, a compound has to be accepted as a substrate by the polymerase and has to be incorporated in an elongating primer

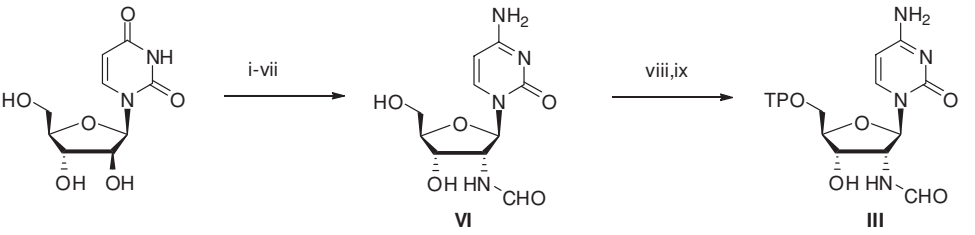

FIGURE 2 Synthesis of 2′-deoxy-2′-formamido-β-D-ribofuranosylcytidine (**VI**) and its triphosphate (**III**). (i) $(PhO)_2CO$, 120°C; (ii) NaN_3, dibenzo-18-crown-6; (iii) TIPS-Cl, Py; (iv) $POCl_3$, 1,2,4-triazole, NH_3; (v) PPh_3, H_20-THF; (vi) $HCOONH_4$, MeCN; (vii) $Et_3N.HF$/NMP; (viii) POCl3, $PO(OMe)_3$; (ix) $(Bu_3NH)_4P_2O_7$, Bu_3N/TEAB.

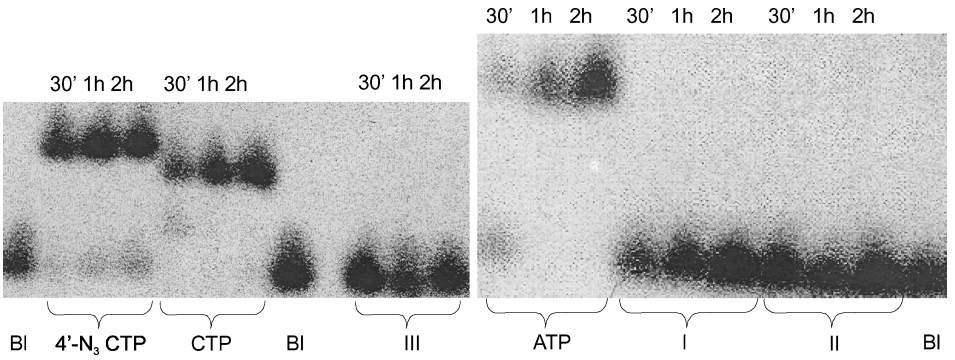

FIGURE 3 Incorporation of the nucleotide analogues into an RNA duplex by the HCV NS5B polymerase after 30 minutes, 1 hour, and 2 hours at 30°C. The blanc reaction was carried out without the addition of triphosphate analogues. [NTP*] = 1 mM; [HCV NS5B pol] = 3.8 μM.

strand hybridized to a template sequence. The single nucleotide incorporation (SNI) assay was used to check the substrate capacities of the nucleoside triphosphate analogues for the NS5B polymerase. In the SNI assay used, a dinucleotide acts as the priming agent for the synthesis. Consequently, the capacity of the compounds to interact during the transition from the initiation to the elongation process of the RNA synthesis was checked.The molecules were tested for their interference at a very early stage of the viral replication. 4′-Azido-cytidine 5′-*O*-triphosphate was used as a reference compound as it is known to be an efficient substrate of the NS5B polymerase (enzyme IC50 being 0.04 μM).[5d] The assay was carried out with the Δ55 variant of the NS5B polymerase. None of the three compounds was accepted by the enzyme as a substrate.

CONCLUSIONS

Ammonium formate is a convenient selective reagent for the N-formylation of 2′-amino-2′-deoxy nucleosides. As triphosphate, the synthesized nucleosides were, however, not substrates for HCV NS5B polymerase. In the past, many compounds were tested for their potential to inhibit the HCV polymerase, but only a few exhibited activity. The number of structural classes that can interfere with the enzyme function, seems to be limited.

It remains, however, important to continue exploring this polymerase as a target for modified nucleoside triphosphates. The work, as presented here, may contribute to a better understanding of the SAR for nucleotides in the polymerase field.

TABLE 1 Overview of the RNA primer and template sequences used in the NS5B single nucleotide incorporation assay

Nucleotide analogue tested	Primer and template sequences used
	5′-GC-3′
A	3′-CG**U**AAAAAAAC-5′
C	3′-CG**G**AAAAAAAC-5′

EXPERIMENTAL

General

For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware under N_2. Reagents and solvents were provided by Acros (Geel, Belgium), Sigma-Aldrich (Bornem, Belgium), or Pharma Waldhof (Dusseldorf, Germany). TLC: Precoated aluminium sheets (Fluka silica gel/TLC cards, 254 nm); the spots were visualized with UV light. Column chromatography (CC): ICN silica gel 63–200 60 P. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra: a Bruker Avance 300-MHz, or a Bruker Avance 500-MHz spectrometer (Bruker, Brussels, Belgium). For sake of clarity, NMR signals of nucleoside-sugar-moiety H- and C-atoms are indicated with primes. Exact mass measurements were performed on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface; samples were infused in i-PrOH/H_2O 1 : 1 at 3 ml/min.

The GC dinucleotide primer was obtained from Sigma-Aldrich. All template sequences were ordered from Eurogentec. An overview of the primer and template sequences is given in Table 1.

Typical Procedure for the Synthesis of 2′-Deoxy-2′-formamidonucleosides

To a solution of 2′-amino-2′-deoxy-3′,5′-*O*-(1,1,3,3-tetraisopropyl disiloxane-1,3-diyl) nucleoside (1 mmol) in dry acetonitrile (5 mL) was added anhydrous ammonium formate (1.5 mmol) and the resulting mixture was heated at 95°C (bath temperature) for 11 hours. Acetonitrile was removed under reduced pressure. The residue was diluted with ethyl acetate (20 mL) and washed with water (2 × 10 mL). The organic layer was dried over anhydrous Na_2SO_4 and then concentrated to dryness. N-Methyl-2-pyrrolidinone (1800 μl), triethylamine (900 μl, 6.47 mmol) and triethylamine tris-hydrofluoride (1200 μl, 7.37 mmol) was added and the reaction mixture was stirred at RT for 30 min. The reaction mixture was diluted with water (20 mL) and washed with dichloromethane (3 × 25

mL) The aqueous layer was concentrated using a rotary evaporator. The crude product was purified by column chromatography on silica gel with EtOH/$CHCl_3$ (1:3 to 1:1).

2′-Deoxy-2′-formamido-β-D-ribofuranosyladenine. ^{1}H NMR (D_2O): 8.30 (s, 1H, H8), 8.19 (s, 1H, H2), 7.95 (s, 1H, H-CO), 6.01 (d, J = 8.2 Hz, 1H, H1′), 5.01 (dd, J = 8.0,5.7 Hz, 1H, H2′)4.41 (dd, J = 1.9,5.7 Hz, 1H, H3′), 4.26 (m, 1H, H4′), 3.82 (m, 2H, H5′). ^{13}C NMR (D_2O) δ: 164.4(CHO), 149.2, 149.1(C2, C6), 148.3(C4), 141.6(C8), 119.0(C5), 87.4, 87.3 (C1′, C4′), 70.4(C3′), 61.6(C5′), 54.4(C2′). HRMS calcd for $C_{11}H_{14}N_6O_4$, [MH^+] 295.1155, found, 295.1149.

2′-Deoxy-2′-formamido-arabinofuranosyladenine. ^{1}H NMR (D_2O): 8.22 (s, 1H, H8), 8.10 (s, 1H, H2), 7.79 (s, 1H, H-CO), 7.24 (s, 2H, NH_2), 6.32 (d, J = 6.8 Hz, 1H, H1′), 5.61 (d, J = 6.0, 1H,OH_3′), 5.24(t, J = 5.2, 1H, OH_5′), 4.65 (m, H2′)4.41 (m, 1H, H3′), 4.42 (m, 1H, H4′), 3.62–3.84 (m, 3H, H4, H5′H5″). ^{13}C NMR (DMSO-d6) δ: 161.3(CHO), 155.9(C6), 152.3(C2), 149.1(C4), 139.6(C8), 118.5(C5), 83.2, 81.8 (C1′, C4′), 70.7(C3′), 59.7(C5′), 56.5(C2′). HRMS calcd for $C_{11}H_{14}N_6O_4$, [MH^+] 295.1155, found, 295.1150.

2′-Deoxy-2′-formamido-β-D-ribofuranosylcytidine. ^{1}H NMR (D_2O): 8.02 (s, 1H, CHO), 7.75 (d, 1H, J = 7.6 Hz, H6), 6.00 (m, 2H, H5 and H1′), 4.49 (dd, J = 7.6, 6.0 Hz, 1H, H2′), 4.27 (dd, J = 6.0, 2.6 Hz, 1H, H4′), 4,09 (dd, J = 7.6,2.6 Hz, 1H, H3′), 3.75 (m, 2H, H5′, H5″). ^{13}C NMR (D_2O) δ: 165.6 (C4), 164.4 (CHO), 157.2(C2), 141.4(C6), 96.85 (C5), 87.4, 86.07 (C4′ and C1′), 70.4 (C3′), 61.3 (C5′), 54.(C2′). HRMS calcd for $C_{10}H_{14}N_4O_5$, [MH^+] 271.1042, found 271.1038.

Typical Procedure for the Synthesis of 2′-deoxy-2′-Formamidonucleoside 5′-O-Triphosphate Tetra(triethylammonium) Salts

After lyophilisation overnight the unprotected nucleoside was dissolved in trimethylphosphate (10 μl/mg nucleoside) and cooled to 0°C. Under stirring phosphorous oxychloride (2 equivalents) was added. The mixture is kept at 0°C for several hours and followed up with a 2-propanol, NH_4OH, water (6:3:1) TLC system. Subsequently 10 equivalents of tri-(tetra-n-butylammonium)hydrogenpyrophosphate (1 M solution in DMF in the presence of 0.3 M Bu_3N) was slowly added under argon. After two minutes of vigorously stirring, the solution is pipetted into a 1 M TEAB solution at pH 7.5. The mixture was stirred for 40 minutes, the solvent was removed and the residue was lyophilized. Purification of the triphosphates was performed on a Source 15Q ion exchange column (Amersham Biosciences, Diegem, Belgium) using a TEAB gradient from 0 to 0.5 M in 30 minutes.

2′-Deoxy-2′-formamido-β-D-ribofuranosyladenine5′-O-triphosphate tetra (triethyl-ammonium) salt. ^{31}P NMR (D_2O): -9.00 (d, γ-P); −11.30 (d, α-P);

−22.83 (t, β-P). HRMS calcd for $C_{11}H_{17}N_6O_{13}P_3$ [MH^-] 532.9994, found 532.9996.

2′-Deoxy-2′-formamido-β-D-ribofuranosylcytidine 5′-O-triphosphate tetra (triethylammonium) salt. ^{31}P NMR (D_2O): −10.25 (d, γ-P); −11.54 (d, α-P); -23.16 (t, β-P). HRMS, calcd for $C_{10}H_{17}N_4O_{14}P_3$, [MH^-] 508.9881, found 508.9887.

2′-Deoxy-2′-formamido-arabinofuranosyladenine 5′-O-triphosphate tetra (triethyl-ammonium) salt. ^{31}P NMR (D_2O): −10.90 (d, γ-P); −11.52 (d, α-P); −23.36 (t, β-P). HRMS calcd for $C_{11}H_{17}N_6O_{13}P_3$, [MH^-] 532.9994, found 532.9996.

^{33}P-Labelling of the primer. An unlabelled primer (100 pmol) was mixed with ^{33}P-γ-ATP (2.5 μl of a 370 MBq/ml (10 mCi/ml) solution, Perkin Elmer), 45 units of T4 polynucleotide kinase (Amersham Biosciences), kinase buffer, and water. After incubation of the mixture at 37°C for 1 hour, the kinase was inactivated by heating the solution at 75°C for 10 minutes. A 4 μM 5′-labelled solution of primer in water was obtained.

HCV NS5B Polymerase reaction. After labelling the 5′-end of the primer strand the oligonucleotide was added to a cold solution of the primer sequence, hybridized to a template strand and mixed with reaction buffer (1× concentrate: 50 mM HEPES (pH 7.3), 5 mM $MgCl_2$ and 10 mM DTT). The polymerase was added and the solution (2× concentrate) was put on an electric heating block at 30°C. A solution of nucleoside triphosphate (2× concentrate) was added and the reaction was again incubated at 30°C. Final concentrations were 20 μM for the primer and the template strand, 1 mM for the triphosphate analogue and 3.8 μM for the polymerase. After 30 minutes, 1 hour, and 2 hours an aliquot of the reaction mixture was removed and mixed with a double volume of gel loading solution containing 90% formamide and 0.05% of both the colouring agents bromofenolblue and xylenecyanol. Analysis of the reaction was carried out through loading of a 2 μl sample on a 25% polyacrylamide gel in a TBE buffer containing 7 M urea, 100 mM Tris–borate, 2.5 mM EDTA running buffer, pH 8.3. By applying a power of 70W until the lowest blue dye was 70% down on the gel the non-extended primer was separated from the extended primer. The gel was exposed to a phosphor screen overnight after which the screen is scanned with a phosphorimager. The percentage of incorporation was determined by integration using the Optiquant image analysis software (Perkin Elmer, Zaventem, Belgium).

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