Brief Articles

Stereoselective Synthesis and Biological Evaluations of Novel 3′-Deoxy-4′-azaribonucleosides as Inhibitors of Hepatitis C Virus RNA Replication

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3′-Deoxy-4′-azaribonucleosides (**15a**−**d**) were synthesized starting from the commercially available (4*R*)-*trans*-4-hydroxy-L-proline **7**. From biological evaluations, **15b** and **15d** emerged as potent inhibitors of HCV replication on a replicon assay. These findings demonstrate that synthesized pyrrolidine nucleosides represent a new template for antiviral or other biological studies and could be considered for novel combination therapy against HCV infection using nucleoside inhibitors and non-nucleoside inhibitors of HCV NS5B.

Introduction

Hepatitis C virus infection constitutes one of the major problems for public health. Current estimates suggest that HCV[a] infects 3.5% of the world population, and fatalities associated with HCV infection result from chronic liver disease and hepatocellular carcinoma.[1,2] HCV infection is also responsible for the majority of liver transplants currently performed.[3]

Current therapies, based on the combination of ribavirin and pegylated interferon-α, have improved the sustained response rates for patients infected with HCV genotypes 2 and 3 but suffer from high cost and relapse rates, and the success rate for current therapy is <50% in genotype 1 patients.[4,5] Thus, the development of new therapies for the treatment of HCV infection is an intensive area of research.[6]

A rational approach for the treatment of this pathology is the design of new agents that can specifically act against viral enzymes. HCV encodes a series of viral proteins such as the NS2/3 autoprotease, the NS3 serine protease/NTPase/helicase, and the NS5B RNA-dependent RNA polymerase.[7] The NS5B RNA-dependent RNA polymerase is essential for viral genome replication and therefore represents a good target in the search of new potential drugs.[8]

Different RNA polymerase inhibitors have been reported in literature; these compounds can be classified as nucleoside inhibitors and non-nucleoside inhibitors.[9] Nucleoside and nucleotide analogues of DNA or RNA substrates can inhibit HCV replication by acting as chain terminators, viral mutagens, or simple competitive inhibitors. Although the biological activity of NIs is strictly dependent on their conversion to the corresponding triphosphates, they possess advantages over the NNIs in the mode of action and, acting by chain termination, they bind in the highly conserved active-site region of HCV NS5B, and thus, they have a high likelihood of affecting RNA replication for all HCV genotypes.[10]

Different nucleoside analogues, structurally modified both on the heterocyclic base and on the sugar moiety, have been reported as new anti-HCV agents. The best results have been achieved by small modifications at 2′ and 4′ positions of the ribose nucleus such as the introduction of a methyl group at $C_{2'}$ or an azido group at $C_{4'}$ (Figure 1).[11]

In addition, new templates of 4′-oxonucleosides, such as 4′-thio,[12] 4′-carbo,[13] and 4′-seleno[14] analogues, were also synthesized, but they did not exhibit significant anti-HCV activity. Thus, as part of our efforts toward novel templates for the development of new therapeutic agents, we turned our attention to 4′-aza-nucleosides. Literature data report that nucleosides containing pyrrolidine and 4-hydroxyproline rings possess substantial similarity to products that contain the tetrahydrofuran ring.[15] Thus, small geometric perturbations could significantly influence the antiviral activity of these compounds.

On the basis of these considerations, we report in this paper the first results on the synthesis and the biological evaluation of the novel template, the 3′-deoxy-4′-azaribonucleosides. The synthesized compounds have been shown to be HCV inhibitors in a cell-based replicon assay at nanomolar order with no or low toxicity.

Results and Discussion

Chemistry. The synthetic approach toward 3′-deoxy-4′-azaribonucleosides **15a**−**d**, reported in Scheme 1, starts from

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[a] Abbreviations: AM1, Austin model 1; Boc, *tert*-butoxycarbonyl; CC_{50}, cytotoxic concentration required to cause 50% toxicity detected by MTS assay; HB 1, heparin-binding protein 1; HCV, hepatitis C virus; IC_{50}, inhibitory concentration that reduces by 50% the viral replication in replicon assay; IFN, interferon; $LiBEt_3H$, lithium triethylborohydride; MTS, [3-(5,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium]; NIs, nucleoside inhibitors; NNIs, non-nucleoside inhibitors; NS2, nonstructural protein 2; NS3, nonstructural protein 3; NS5B, nonstructural protein 5B; NTPase, nucleoside 5′-triphosphatase; TBAF tetrabutylammonium fluoride; TBDMSCl, *tert*-butyldimethylsilyl chloride.

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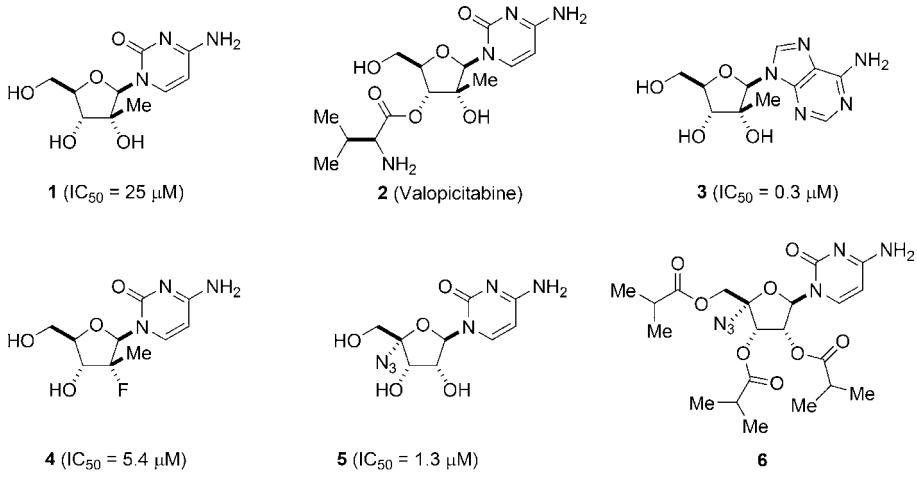
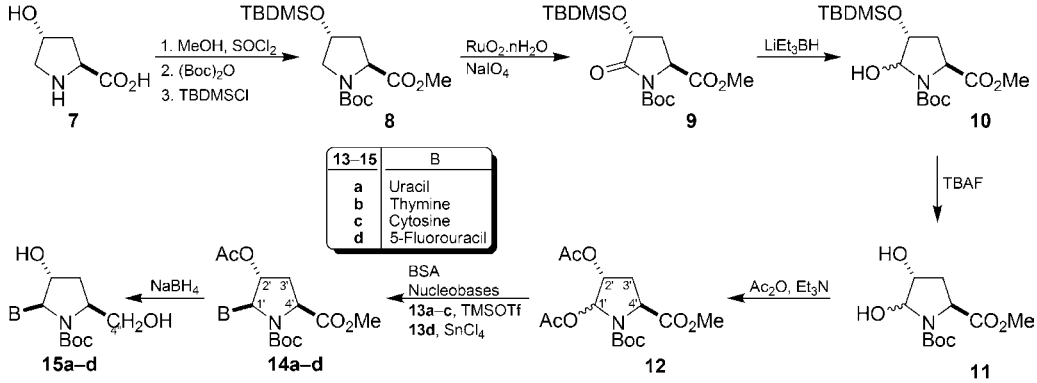
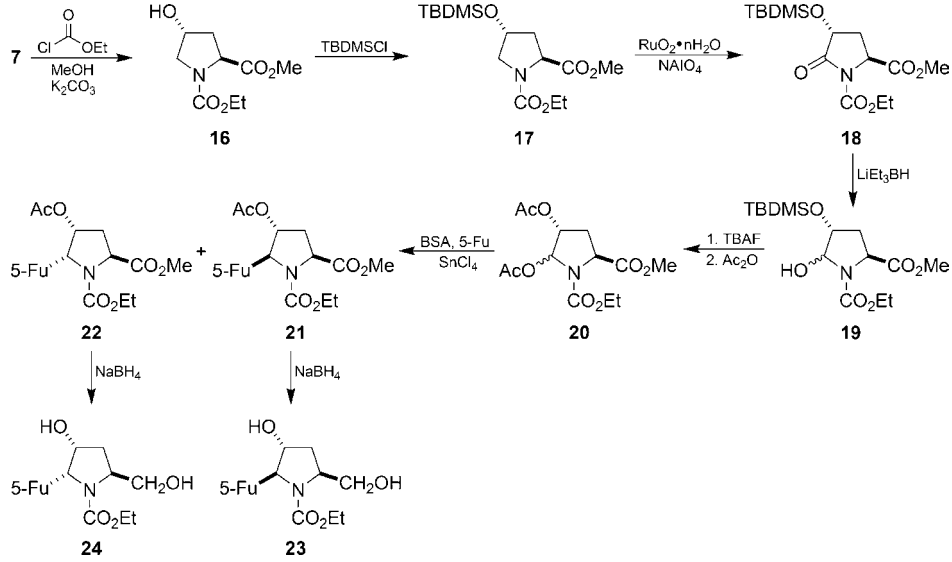


Figure 1. Representative examples of nucleoside inhibitors.

Scheme 1. Synthesis of 3′-Deoxy-4′-azaribonucleosides **15**



Scheme 2. Synthesis of 3′-Deoxy-4′-azaribonucleosides **23** and **24**



(4*R*)-*trans*-4-hydroxy-L-proline **7**, which was converted into **9**[16] and reduced with $LiBEt_3H$ to a 4:1 mixture of hemiaminals **10**. Further reaction with TBAF and subsequently with Ac_2O afforded the di-*O*-acetyl derivatives **12**. Nucleosidation with silylated pyrimidine nucleobases **13a**−**d** gave compounds **14a**−**d** as single diastereomers.

The relative configurational assignments for **14a**−**d** were determined on the basis of ^1H NMR and NOE experiments (see Supporting Information). Finally, the target 3′-deoxy-4′-aza-nucleosides **15a**−**d** in enantiomerically pure form were obtained by reduction with $NaBH_4$ of **14a**−**d**.

3′-Deoxy-4′-azaribonucleosides **23** and **24**, containing the ethoxycarbonyl group at the nitrogen atom of pyrrolidine ring, were obtained by the procedure described in Scheme 2. Thus, **7** was reacted with ethyl chloroformate in methanol[17] and subsequently protected with TBDMSCl, giving **17**. Oxidation of **17** with $RuO_2/NaIO_4$, followed by reduction with $LiBEt_3H$ and reaction with TBAF and Ac_2O, afforded **20**. Reaction of

Table 1. Inhibitory Concentration (IC_{50}) of Azaribonucleosides on HCV RNA Replication in a Subgenomic Replicon Harbored in HB-1 Cells and Cytotoxycity (CC_{50}) As Measured by MTS Assay

compd	HCV replicon IC_{50} (μM)	MTS CC_{50} (μM)
15a	40.6	>1000
15b	0.018	>1000
15c	96	>1000
15d	0.00035	623
23	4.8	>1000
24	>1000	200
IFN	0.01−0.008 U	>100

20 with silylated fluorouracil yielded a nearly equimolar mixture of pyrrolidine derivatives β-**21** and α-**22**, which, reduced with $NaBH_4$, gave the corresponding pure azaribonucleosides **23** and **24** (Scheme 2).

Interestingly, the difference between the alkyl groups of the carbamate moiety (ethyl vs *tert*-butyl) exerted a complete diastereofacial differentiation in the N-glycosylation reaction. Whereas the *tert*-butyl carbamate only led to one isomer, the ethyl group led to a mixture of isomers. The explanation, as supported by preliminary AM1 semiempirical calculations, could be found in a different conformational disposition of both groups, which conditions the attack of the nucleophile on the iminium ion intermediate (see Supporting Information).

Biological Evaluation. The synthesized 3′-deoxy-4′-azaribonucleosides **15a**−**d**, **23**, and **24** have been tested in a cell-based subgenomic HCV replicon assay[18] to evaluate their ability to inhibit HCV RNA replication (Table 1). In addition, cytotoxicity, expressed as CC_{50}, was also evaluated with a MTS based assay.[19] The screening of all the compounds was performed up to the fixed concentration of 10^3 μM and compared with IFN.

As shown in Table 1, all the modified nucleosides with a cis relationship between the hydroxymethyl group and the nucleobase, such as compounds **15a**−**d** and **23**, are endowed with a relevant antiviral activity while showing low or no toxicity. Compound **24**, which possesses a trans configuration not present in natural nucleosides, shows no antiviral activity. The biological activity of the evaluated compounds is strictly correlated to their bonded nucleobase, in the order 5-fluorouracil > thymine > uracil > cytosine. The potency of the 5-substituted pyrimidines increases when the hydrogen atom is replaced by a methyl or fluoride group. Thus, the more active compound **15d**, which possesses a 5-fluorouracil moiety as nucleobase, shows an IC_{50} in the nanomolar range (0.35 nM), even if it possesses a moderate level of toxicity. The toxicity seems to be also related to the substituent bonded at the nitrogen atom: when the Boc group in **15d** is substituted by an ethoxycarbonyl group, as in **23**, a decreased toxicity is observed. Despite a drastic reduction in the antiviral activity, the selective index of compound **23** is still good ($CC_{50}/IC_{50} \geq 208$). To gain a better understanding of the mechanism of action of these nucleosides, triphosphates of compounds **15d** and **23** are necessary to evaluate the inhibition of HCV NS5B-mediated RNA replication. However, the synthesis of triphosphates by literature methods requires the use of acidic conditions that are not compatible with the acidic labile substituents present at the nitrogen atom. Thus, the mechanism of action of these compounds cannot be elucidated in detail, but by considering that the α-anomer is completely inactive and the close relation between the active β-anomers and the natural ribonucleosides, we hypothesize that these compounds act as nucleoside analogues at the active site of the NS5B polymerase.

Conclusions

In summary, we have accomplished the stereoselective synthesis of novel 3′-deoxy-4′-azaribonucleosides, starting from the chiral template (4*R*)-*trans*-4-hydroxy-L-proline, together with their biological evaluations. From these investigations, thymine and 5-fluouracil derivatives emerged as potent inhibitors of HCV replication on a replicon assay. Considering that few different classes of nucleoside analogues have been shown to inhibit HCV replication, the reported results seem to be relevant to discover new potent and safe antiviral agents possessing a nucleoside structure. The findings reported here demonstrate that the synthesized pyrrolidine nucleosides represent a new template for antiviral or other biological studies that at the moment are largely lacking. Particularly, similar to what occurs in HIV infection, this new class of compounds could be considered for novel combination therapy against HCV infection using NIs and NNIs of HCV NS5B.

Experimental Section

The purity of all the compounds was tested by combustion analysis, and it is ≥95%.

General Procedure for Reduction of Nucleosides 15a−d. To a stirred solution of **14a**−**d** (0.11 mmol) in a 1:1 methanol/dioxane mixture (10 mL), $NaBH_4$ (40 mg, 1.05 mmol) was added at 0 °C, and the obtained suspension was stirred overnight at room temperature. At the end of this time the solvent was removed and the residue was extracted with ethyl acetate (2 × 5 mL). The collected organic phases, dried over sodium sulfate, gave after evaporation of the solvent at reduced pressure a sticky oil, which was purified by flash chromatography on silica gel.

***tert*-Butyl-(2*R*,3*R*,5*S*)-2-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (15a).** Eluent mixture: methanol/chloroform 10:90, $R_f = 0.2$. White solid: mp = 177−179 °C (27.5 mg, 0.084 mmol, 76% yield); $[\alpha]_D^{25}$ −87.8 (*c* 0.71, CH_3OH).

***tert*-Butyl-(2*R*,3*R*,5*S*)-3-hydroxy-5-(hydroxymethyl)-2-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)pyrrolidine-1-carboxylate (15b).** Eluent mixture: methanol/chloroform 7:93, $R_f = 0.13$. White solid: mp = 119−122 °C (28.7 mg, 0.084 mmol, 76% yield); $[\alpha]_D^{25}$ −80.0 (*c* 0.2, CH_3OH).

***tert*-Butyl-(2*R*,3*R*,5*S*)-2-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (15c).** Eluent mixture: methanol/chloroform 15:85, $R_f = 0.14$. White solid: mp = 140−141 °C (29 mg, 0.084 mmol, 76% yield); $[\alpha]_D^{25}$ −35,34 (*c* 0.57, CH_3OH).

***tert*-Butyl-(2*R*,3*R*,5*S*)-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (15d).** Eluent mixture: methanol/chloroform 10:90, R_f = 0.21. White solid: mp = 60−61 °C (27.4 mg, 0.084 mmol, 76% yield); $[\alpha]_D^{25}$ −52.63 (*c* 0.38, CH_3OH).

(2*R*,3*R*,5*S*)-Ethyl-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (23) and (2*S*,3*R*,5*S*)-Ethyl-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (24). To a stirred solution of **21** and **22** (1.0 g; 2.58 mmol) in a 1:1 methanol/dioxane (50 mL), $NaBH_4$ (0.98 g, 25.8 mmol) was added at 0 °C. The obtained mixture was further stirred for 5 h. At the end of this time, the solvent was removed and the residue was subjected to flash chromatography on RP-18 reverse phase using water as eluent. The first eluted product was identified as **23** (0.33 g, 1.05 mmol, 40% yield); white solid, mp = 56−58 °C. The second eluted product was identified as **24** (0.32 g, 1.05 mmol, 40% yield); white solid, mp = 55−57 °C.

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Supporting Information Available: Experimental conditions and spectral data of intermediates **10**−**12**, **14a**−**d**, **17**−**22**; spectral data of target compounds **15a**−**d**, **23**, and **24**; NOEDS for **14d**; explanation for diastereoselective N-glycosylation reaction; details of cell culture, antihepatitis C virus, and cytotoxicity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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