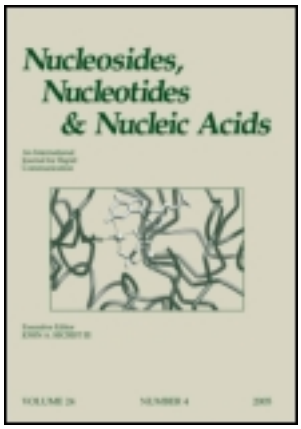

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▫ *Based on the discovery of β-D-2′-deoxy-2′-fluorocytidine as a potent anti-hepatitis C virus (HCV) agent, a series of β-D- and L-2′-deoxy-2′-fluororibonucleosides with modifications at 5 and/or 4 positions were synthesized and evaluated for their in vitro activity against HCV and bovine viral diarrhea virus (BVDV). The introduction of the 2′-fluoro group was achieved by either fluorination of 2,2′-anhydronucleosides with hydrogen fluoride-pyridine or potassium fluoride, or a fluorination of arabinonucleosides with DAST. Among the 27 analogues synthesized, only the 5-fluoro compounds, namely β-D-2′-deoxy-2′,5-difluorocytidine (5), had anti-HCV activity in the subgenomic HCV replicon cell line, and inhibitory activity against ribosomal RNA. As β-D-N^4-hydroxycytidine (NHC) had previously shown potent anti-HCV activity, the two functionalities of the N^4-hydroxyl and the 2′-fluoro were combined into one molecule, yielding β-D-2′-deoxy-2′-fluoro-N^4-hydroxycytidine (**12**). However, this nucleoside showed neither anti-HCV activity nor toxicity. All the L-forms of the analogues were devoid of anti-HCV activity. None of the compounds showed anti-BVDV activity, suggesting that the BVDV system cannot reliably predict anti-HCV activity in vitro.*

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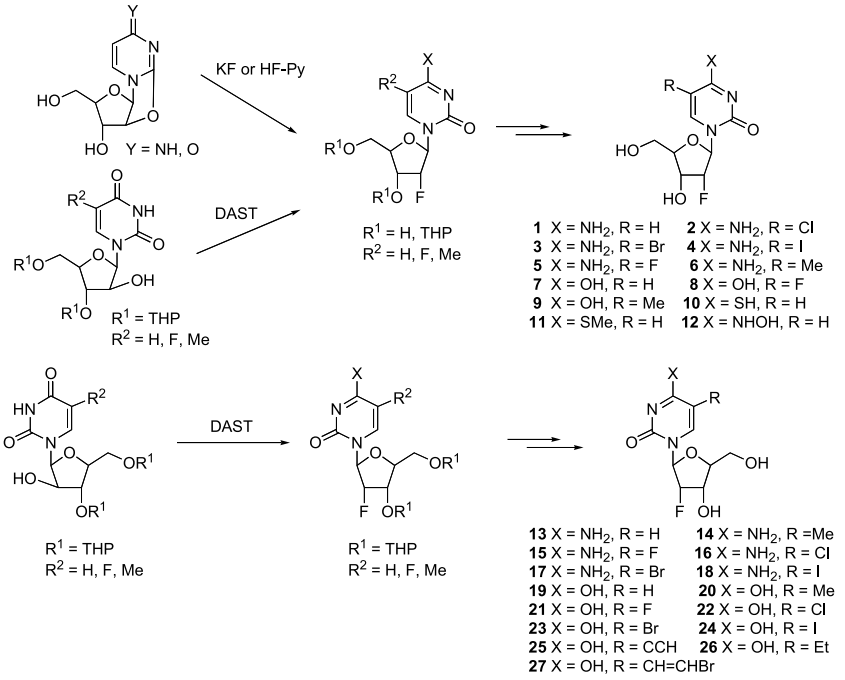
INTRODUCTION

HCV is an important pathogen affecting nearly 170 million people worldwide. HCV infections become chronic in about 50% of cases, and about 20% of these chronically infected patients develop liver cirrhosis that can lead to hepatocellular carcinoma. The current therapies, based on interferon-alpha (IFN-α) alone or combination with ribavirin, are only moderately effective. Therefore, there is a need for more effective anti-HCV agents. Recently, we reported that a sugar-fluorinated nucleoside, β-D-2′-deoxy-2′-fluorocytidine (**1**), had potent anti-HCV activity.[1] Based on the activity of this compound, a series of β-D- and L-analogues were synthesized and evaluated against HCV in the HCV subgenomic replicon system and BVDV. In addition, our earlier discovery of a base-modified nucleoside, NHC, possessing potent anti-HCV activity,[2] prompted us to combine these two features in one molecule. Herein, we report the synthesis and the biological evaluation of several β-D- and L-2′-deoxy-2′-fluororibonucleosides.

RESULTS AND DISCUSSION

Two main approaches have been widely used for the synthesis of 2′-deoxy-2′-fluororibonucleosides: 1) fluorination of anhydronucleosides with hydrogen fluoride or potassium fluoride, and 2) fluorination of arabinonucleosides with diethylaminosulfur trifluoride (DAST) or with tetrabutylammonium fluoride (TBAF) via a sulfonate intermediate.



SCHEME 1 Synthesis of β-D- and L-2′-deoxy-2′-fluororibonucleosides.

We utilized the direct fluorination approach for the preparation of β-D-2′-deoxy-2′-fluorouridine (**2**) and D-2′-deoxy-2′-fluorocytidine (**1**) from their anhydronucleosides by HF-pyridine or KF, as the literature reported.[3,4] The halogenation of **1** gave 5-halogenated analogues **2–4**. For the preparation of 5-fluoro and 5-methyl substituted analogues, DAST fluorination was adopted. Thus, 5-substituted 3′,5-THP-protected arabinonucleosides were treated with DAST, resulting in the protected 2′-deoxy-2′-fluoronucleosides. After deprotection and/or amination, 5-substituted D-2′-deoxy-2′-fluoronucleosides **5 6** were obtained (Scheme 1).

β-D-2′-Deoxy-2′-fluoro-N^4-hydroxycytidine (**12**) was synthesized from β-D-2′-deoxy-2′-fluorouridine (**7**) by acetylation, sulfonation, hydroxyamination, and deprotection (Scheme 1). 4-Thio analogue **10** and 4-methylthio analogue **11** were synthesized by thioation and methylation, as described in the literature.[5] Similarly, β-D-2′-deoxy-2′-fluoroadenosine (**28**) was prepared using DAST fluorination, following a literature procedure.[6]

All the L-series 2′-deoxy-2′-fluororibonucleosides were synthesized by DAST fluorination. The corresponding arabinonucleosides were prepared either by Holy's method,[7] or Vorbrüggen sugar-base condensation. After fluorination, the resulting nucleosides were aminated and/or deprotected to give compounds **13–15** and **19–21**, while the 5-halogenated (Cl, Br, I) nucleosides **16–18** and **22–24** were prepared by halogenation of the corresponding nucleosides. 5-Ethynyl nucleoside **25** was synthesized from 5-iodouridine nucleosides via palladium-mediated reaction, and hydrogenation of **25** resulted in 5-ethyl analogue **26** (Scheme 1). β-L-5-Bromovinyl-2′-deoxy-2′-fluorouridine (**27**) was prepared by a published procedure.[8]

The synthesized 2′-deoxy-2′-fluororibonucleosides were evaluated in BVDV and HCV subgenomic replicon RNA-containing Huh7 cells, as described previously.[2,9] Briefly, for the BVDV assay, Madin-Darby bovine kidney (MDBK) cells were infected with cpBVDV (NADL strain) in DMEM/F12 media in the presence or absence of test compounds. After a 3-day incubation, viral RNA was extracted and analyzed using quantitative real-time RT-PCR (Q-RT-PCR). For the HCV replicon assay, HCV subgenomic replicon RNA-containing Huh7 cells were incubated in the presence or absence of tested compounds for 4 days. After

TABLE 1 Anti-BVDV and Anti-HCV Activity of 2′-Deoxy-2′-Fluororibonucleosides In Vitro

Compound	Configuration	Base	4-Substitution	5-Substitution	EC_{90} (μM) BVDV	EC_{90} (μM) HCV	CC_{50} (μM) rRNA
1	D	C			>100	5.6	>100
5	D	C		F	ND	9.35	<1
11	D	U	SCH_3		>100	>100	8.7
12	D	C	NHOH		ND	>100	>100
NHC					5.4	5.0	>100
ribavirin					1.5	≥100	14.4

ND: not determined; EC_{90}: effective concentration required for reducing the HCV RNA or BVDV levels by 90% in 96 or 72 h; CC_{50}: cytotoxic concentration required for reducing the rRNA levels by 50% in 96 h.

incubation, total cellular RNA was extracted. Replicon RNA and an internal control were amplified in a single-step multiplex RT-PCR protocol, as described previously.[9] Recombinant interferon alfa-2a (for HCV) and ribavirin (for BVDV) were used as positive controls in these experiments. The results using some nucleoside analogues are summarized in Table 1 (the rest of the nucleosides showed EC_{50} and CC_{50} values over 100 μM).

All the synthesized nucleosides showed no inhibitory activity against BVDV, and all the L-series nucleosides showed no anti-HCV activity. In the D-series, among 5-substituted cytidine analogues, only β-D-2′-deoxy-2′,5-difluorocytidine (**5**) exhibited anti-HCV activity and inhibitory activity against ribosomal RNA. The 5-chloro, 5-bromo, 5-iodo, and 5-methyl substituted 2′-deoxy-2′-fluorocytidine analogues showed no anti-HCV activity. Uridine analogues β-D-2′-deoxy-2′-fluorouridine, β-D-2′-deoxy-2,5′-difluorouridine, and β-D-2′-fluorothymidine were not active against HCV. Replacement of the amino with a thiol group at the 4-position also resulted in an inactive compound **10**. However, the 4-methylthio analogue **11** demonstrated inhibition of ribosomal RNA. Surprisingly, the N^4-hydroxylamino analogue (**12**) showed neither activity against HCV nor inhibitory activity to ribosomal RNA.

β-D-2′-Deoxy-2′-fluororibonucleoside analogues are known to be active against some RNA viruses. Several analogues of this class showed high activity against influenza A and B, and parainfluenza 1.[10] Also, β-D-2′-deoxy-2′-fluorocytidine has demonstrated inhibitory activity against herpes simplex virus type 1 and 2, pseudorabies and equine abortion virus.[11] It is surprising that our findings indicate that β-D-2′-deoxy-2′-fluororibonucleosides possess anti-HCV activity, as some earlier works on HCV polymerase concluded that in order to be recognized by HCV RNA dependent RNA polymerase, a ribonucleoside was needed.[12] The discovery that β-D-2′-deoxy-2′-fluorocytidine possesses potent anti-HCV activity suggests that the 2′-fluoro instead of the 2′-hydroxyl group is recognized by the HCV RNA polymerase. In terms of Van der Vaal radii, the fluorine atom is closer to a hydrogen atom than a hydroxyl group. However, the fluorine atom may mimic the hydroxyl group in terms of electronegativity and the ability to form a hydrogen bond. The conformational study of 2′-fluorinated nucleosides also suggests that 2′-deoxy-2′-fluororibonucleosides are more like ribonucleosides than 2′-deoxyribonucleosides, since it has been confirmed that both 2′-deoxy-2′-fluororibonucleosides and ribonucleosides adopt a 3′-endo conformation.[13,14] However, the fact that the anti-HCV activity of β-D-2′-deoxy-2′-fluorocytidine can be abolished by the addition of 2′-deoxycytidine, not by cytidine[1] demonstrates that 2′-deoxy-2′-fluororibonucleosides are recognized as 2′-deoxynucleosides in at least one step in the metabolic pathway in HCV replicon cells.

It seems that the anti-HCV activity resides with the β-D-nucleosides. To the best of our knowledge, no L-enantiomer has been reported to possess any specific anti-HCV activity. As more L-nucleosides are evaluated against HCV in vitro, this hypothesis will be further tested.

In summary, a series of β-D- and L-2′-deoxy-2′-fluororibonucleosides were synthesized and evaluated for in vitro anti-HCV and anti-BVDV activity, as well as their inhibition of ribosomal RNA. The study revealed that β-D-2′-deoxy-2′,5-difluorocytidine showed lower anti-HCV potency and higher toxicity against ribosomal RNA than β-D-2′-deoxy-2′-fluorocytidine. All of the other 5-modified β-D-nucleosides were not active against HCV, and all the L-series compounds were devoid of anti-HCV activity. The 4-methylthio analogue exhibited inhibitory activity against ribosomal RNA. None of the tested compounds demonstrated anti-BVDV activity. Surprisingly, β-D-2′-deoxy-2′-fluoro-N^4-hydroxylcytidine was neither active nor toxic to liver cells.

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