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

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NOEDS Experiments

In particular, the irradiation of $H_{1'}$ (6.01 ppm) in compound **14d**, chosen as a reference compound, induced a positive NOE effect on the methyl of the acetyl group (2.13 ppm) and on $H_{4'}$ (4.44 ppm); the irradiation of $H_{2'}$ (5.29 ppm) gave rise to strong positive NOE effect for $H_{3'a}$ (2.21 ppm). Moreover, irradiation of $H_{4'}$ produced a strong enhancement for $H_{3'b}$ (2.44 ppm) and a weak positive effect for $H_{1'}$, while irradiation of $H_{3'b}$ produced a NOE effect other than on $H_{3'a}$, and $H_{4'}$ also on $H_{1'}$. These results are clearly indicative of *cis* relationship between $H_{1'}$ and $H_{4'}$ (Figure 1).

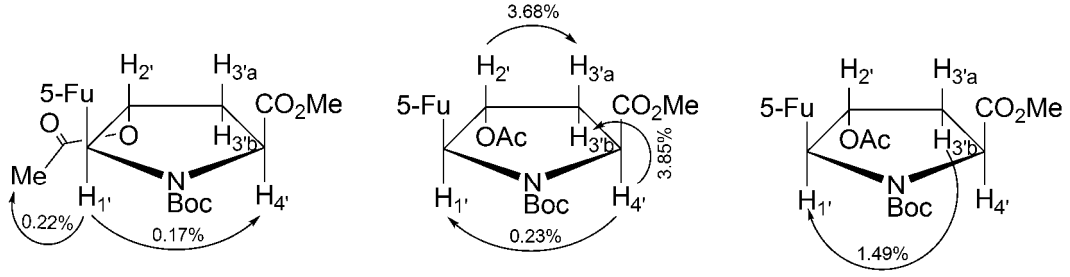

Figure 1S. NOE effects for compound **14d**.

Explanation of diastereofacial differentiation in the *N*-glycosylation reaction

Preliminary theoretical calculations, at AM1 semiempirical level, showed that there is not anchimeric effect of the acetyl group at position 2. In addition, the *t*-butyl carbamate adopted a transoid orientation with the *t*-butyl group affecting the two diasterofaces in the same way, and consequently is the AcO group which decided the preferential attack for the opposite face thus leading to one isomer. On the other hand, in the case of ethylcarbamate the carbonyl group adopted a non-planar orientation with respect to the C=N double bond positioning the ethyl group by the opposite face to the AcO group.

Under these conditions both diastereofaces presented similar steric hindrance and a mixture of isomers is obtained.

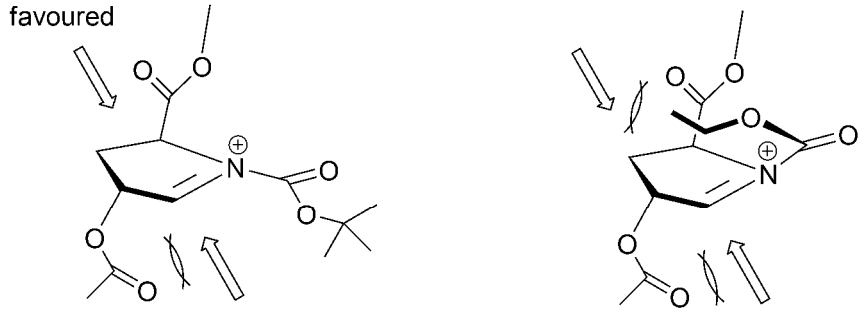

Figure 2S. Steric hindrance for cationic form of compounds **12** and **20**, respectively.

Experimental Section

Melting points were measured on a Koffler apparatus and are uncorrected. ^{1}H NMR spectra were measured on a 500 MHz Varian Unity Inova instrument in $CDCl_3$, CD_3OD and D_2O as solvents. Chemical shifts are in ppm (δ) from TMS as internal standard. NOE difference spectra were obtained by subtracting alternatively right-off-resonance free induction decays (FIDS) from right-on-resonance induced FIDS. MS spectra were measured with a JEOL JMS-D 300 spectrometer. Elemental analyses were performed on a Perkin-Elmer 240B microanalyzer. Merck silica gel 60H and RP-18, 40–63 μm colomn (12 × 75 mm) were used for preparative short-column chromatography. The purity of all compounds was tested by combustion analysis and in any case results ≥ 95%.

1-*tert*-Butyl-2-methyl-(2*S*,4*R*,5*RS*)-4{[*tert*-butyl(dimethyl)silyl]-oxy}-5-hydroxy-pyrrolidine-1,2-dicarboxylates (10). To a –78 °C solution of 1-*tert*-butyl-2-methyl-(2*S*,4*R*)-4-{[*tert*-butyl(dimethyl)silyl]oxy}-5-oxopyrrolidine-1,2-dicarboxylate (**9**)[1] (3.6 g, 9.66 mmol) in anhydrous THF (90 mL) was slowly added dropwise Lithium triethyl borohydrate (4.83 mL, 1 M in THF, 0.51 g, 4.83 mmol) under a nitrogen atmosphere. The reaction mixture was stirred for 1 h, then hydride (0.72 g, 6.76 mmol) was further added. After 45 min, the reaction was quenched with water (160 mL) and the organic solvent was evaporated. The aqueous phase was extracted with ethyl acetate (3 × 160 mL), dried over anhydrous sodium sulfate, filtered and concentrated in vacuum to afford a mixture of dicarboxylates **10** as yellow oil (4.5 g, 12.1 mmol, 80% global yield) in a 4:1 ratio. Major isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.01 (s, 6H), 0.81 (s, 9H), 1.41 (s, 9H), 2.00−2.11 (m, 2H), 3.68 (s, 3H), 4.28−4.43 (m, 3H), 5.18 (s, 1H). Minor isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.04 (s, 6H), 0.78 (s, 9H), 1.34 (s, 9H), 2.08−2.19 (m, 2H), 3.66 (s, 3H), 4.08 (m, 1H), 4.17−4.27 (m, 3H), 5.08 (s, 1H).

1-*tert*-Butyl-2-methyl-(2*S*,4*R*,5*RS*)-4,5-dihydroxypyrrolidine-1,2-dicarboxylates (11). To a 0° C solution of **10** (3.1 g, 8.25 mmol) in anhydrous THF (60 mL), was added dropwise, at room temperature, tetrabutyl ammonium fluoride (1.0 M in THF, 2.16 g, 8.25 mmol). The mixture was stirred at room temperature for 2 h and the reaction was quenched with water (118 mL). Then, the mixture was extracted with ethyl acetate (3 × 118 mL). The combined organic phases were washed with brine (3 × 118 mL), dried over anhydrous sodium sulfate and evaporated to dryness to give an epimeric mixture of **11** as yellow oil (2.73 g, 10.4 mmol, 79% global yield). Major isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.41 (s, 9H), 2.17−2.23 (m, 2H), 3.72 (s, 3H), 4.08−4.11 (m, 1H,), 4.30−4.37 (m, 3H), 4.83−4.86 (m, 1H). Minor isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.39 (s, 9H), 2.24−2.37 (m, 2H), 3.70 (s, 3H), 4.012−4.16 (m, 2H,), 4.30−4.37 (m, 1H), 4.88−4.92 (m, 1H).

***1-tert*-Butyl-2-methyl-(2*S*,4*R*,5*RS*)-4,5-*bis*(acetyloxy)-pyrrolidine-1,2-dicarboxylates (12).** To a solution of dicarboxylates **11** (2.16 g, 8.25 mmol) in anhydrous CH_2Cl_2 (60 mL) was added dropwise acetic anhydride (8.42 g, 82.5 mmol) and then triethylamine (16.7 g, 165 mmol). The mixture was stirred for 12 h at room temperature. The solution was washed with water (3 × 60 mL), dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography on silica gel using ethyl acetate/cyclohexane 25:75 as eluent to afford a mixture of acetylated compounds **12** as

yellow oil (3.8 g, 11.0 mmol, 75% global yield). Major isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.36 (s, 9H), 1.94 (s, 3H), 2.03 (s, 3H), 2.06−2.31 (m, 2H), 3.68 (s, 3H), 4.36 (m, 1H), 5.28 (m, 1H), 6.54−6.65 (m, 1H). Minor isomer: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.39 (s, 9H), 2.06 (s, 3H), 2.09 (s, 3H), 2.20−2.35 (m, 2H), 3.72 (s, 3H), 4.39 (m, 1H), 5.05 (m, 1H), 6.39 (m, 1H).

Experimental procedure for the nucleosidation reaction with Uracil (13a), Thymine (13b) and Cytosine (13c). A suspension of nucleobases **13a**–**c** (uracil 62 mg, thymine 70 mg, cytosine 61 mg, 0.55 mmol) in anhydrous acetonitrile (4 mL) was treated with *N*,*O*-bis-trimetylsilylacetamide (0.45 g, 2.23 mmol) under an atmosphere of nitrogen and refluxed for 15 min under vigorous stirring. To the clear solution obtained was added a solution of dicarboxylate **12** (0.16 g, 0.46 mmol) in anhydrous acetonitrile (4 mL) and then trimethylsilyltriflate (0.15 g, 0.69 mmol), dropwise. The mixture was refluxed for 1 h. After being cooled at 0 °C, the solution was neutralized by careful addition of saturated aqueous sodium bicarbonate, and then it was concentrated in vacuum. To the crude was added dichloromethane (15 mL), and the organic phase was separated, washed with water (2 × 10 mL), dried over sodium sulfate, filtered, and evaporated to dryness. The residue was purified by flash chromatography on silica gel and then by HPLC (flow 3.5 mL/min) in *n*-hexane.

1-*tert*-Butyl-2-methyl-(2*S*,4*R*,5*R*)-4-(acetyloxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1-(2*H*)-yl)pyrrolidine-1,2-dicarboxylate (14a). Eluent mixture: ethyl acetate/cyclohexane 45:55. R_f = 0.10; HPLC purification: isopropanol/n-hexane 25:75, λ = 270 nm, R_t = 6.98 min. White solid: mp = 194−195 °C (0.147 g, 0.37 mmol, 80% yield); $[\alpha]_D^{25}$ = –28,0 (*c* = 0,61, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz, 60 °C): δ 1.38 (s, 9H), 2.07 (s, 3H), 2.80 (ddd, 1H, *J* = 5.1, 8.3, 14.0 Hz), 2.40 (ddd, 1H, *J* = 2.8, 7.4, 14.0 Hz), 3.79 (s, 3H), 4.46 (t, 1H, *J* = 8.0 Hz), 5.24 (m, 1H), 5.73 (d, 1H, *J* = 8.1 Hz), 5.99 (d, 1H, *J* = 0.8 Hz), 8.22 (d, 1H, *J* = 8.1 Hz), 9.21 (bs, 1H, NH). ^{13}C NMR ($CDCl_3$, 125 MHz, 60 °C): δ 20.6, 28.0, 33.2, 52.6, 58.5, 76.0, 77.3, 100.1, 102.2, 139.8, 150.5, 152.6, 163.1, 169.3, 172.8. MS (ES^+) *m/z* (MH^+): 398. Anal. calcd. for $C_{17}H_{23}N_3O_8$: C, 51.38; H, 5.83; N, 10.57. Found: C, 51.21; H, 5.84; N, 10.55.

1-*tert*-Butyl-2-metyl-(2*S*,4*R*,5*R*)-4-(acetyloxy)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1-(2*H*)-yl)-pyrrolidine-1,2-dicarboxylate (14b). Eluent mixture: methanol/chloroform 2:98, R_f = 0.21, HPLC purification: isopropanol/*n*-hexane 13:87, λ = 270 nm, R_t = 11.48 min. White solid: mp = 92−93 °C (152 g, 0.37 mmol, 80% yield); $[\alpha]_D^{25}$ = –24.65 (*c* = 0.71, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz, 50 °C): δ 1.40 (s, 9H), 1.96 (d, 3H, *J* = 1.2 Hz), 2.10 (s, 3H), 2.23 (ddd, 1H, *J* = 5.3, 8.6, 14.4 Hz), 2.41 (ddd, 1H, *J* = 3.1, 7.7, 14.4 Hz), 3.83 (s, 3H), 4.50 (dd, 1H, *J* = 7.7, 8.6 Hz), 5.25 (ddd, 1H, *J* = 2.6, 3.1, 5.3 Hz), 6.01 (d, 1H, *J* = 2.6 Hz), 8.15 (q, 1H, *J* = 1.2 Hz), 8.58 (bs, 1H, NH). ^{13}C NMR ($CDCl_3$, 125 MHz, 50 °C): δ 12.5, 20.8, 28.0, 33.1, 52.7, 58.3, 76.1, 77.1, 97.6, 110.8, 135.7, 150.5, 152.7, 163.6, 169.4, 172.8. MS (ES^+) *m/z* (MH^+): 412. Anal. calcd. for $C_{18}H_{25}N_3O_8$: C, 52.53; H, 6.12; N, 10.21. Found: C, 52.41; H, 6.13; N, 10.24.

1-*tert*-Butyl-2-methyl-(2*S*,4*R*,5*R*)-4-(acetyloxy)-5-(4-amino-2-oxopyrimidin-1-(2*H*)-yl)-pyrrolidine-1,2-dicarboxylate (14c). Eluent mixture: methanol/dichloromethane 4:96 to 15:85, R_f = 0.1. White solid: mp = 171−172 °C (0.147 g, 0.37 mmol, 80% yield); $[\alpha]_D^{25}$ = +1.88 (*c* = 0.80, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.39 (s, 9H), 2.04 (dd, 1H, *J* = 9.7, 14.3 Hz), 2.11 (s, 3H), 2.41 (dd, 1H, *J* = 7.2, 14.3Hz), 3.75 (s, 3H), 4.49 (dd, 1H, *J* = 7.2, 9.7 Hz), 5.27 (m, 1H), 4.31 (m, 1H), 6.03 (m, 1H), 8.29 (d, 1H, *J* = 7.4 Hz) 9.18 (bs, 2H, NH_2). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 21.0, 27.9, 32.8, 52.7, 58.3, 75.1, 76.6, 82.6, 94.9, 140.4, 153.1, 156.0, 166.1, 169.5, 172.7. MS (ES^+) *m/z* (MH^+): 397. Anal. calcd. for $C_{17}H_{24}N_4O_7$: C, 51,52.; H, 6.10; N, 14.13. Found: C, 51.39; H, 6.09; N, 14.15.

1-*tert*-Butyl-2-methyl-(2*S*,4*R*,5*R*)-4-(acetyloxy)-5-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1-(2*H*)-yl)pyrrolidine-1,2-dicarboxylate (14d). To a stirred mixture of 5-fluorouracil (**13d**) (20 mg, 0.19 mmol), and 1,2-dicarboxylates **12** (60 mg, 0.185 mmol) in anhydrous CH_2Cl_2 (5 mL) was added, dropwise, at room temperature and under a nitrogen atmosphere, *N*,*O-bis*-trimethylsylilacetamide (90 mg, 0.43 mmol). After 2 h of stirring at room temperature, the clear solution was cooled at 0 °C and tin tetrachloride (50 mg, 0.19 mmol) was added. The mixture was warmed to room temperature, left to stir overnight and, finally, poured slowly into a mixture of cold saturated aqueous sodium bicarbonate (5 mL) and chloroform (10 mL). The resulting emulsion was separated by filtration through a pad of Celite,

the aqueous layer was extracted further with ethyl acetate (3 × 10 mL), and the combined organic layers were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by flash chromatography using ethyl acetate/cyclohexane 30:70 as eluent, (R_f = 0.2), and then by HPLC: isopropanol/*n*-hexane 8:92, (λ = 270 nm, R_t = 7.91 min) to afford **14d** as white solid: mp = 96−98 °C (623 mg, 0.15 mmol, 80% yield); $[\alpha]_D^{25}$ = –21.86 (*c* = 0.53, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz, 50 °C): δ 1.43 (s, 9H), 2.13 (s, 3H), 2.21 (m, 1H, $H_{3'a}$), 2.44 (m, 1H, $H_{3'b}$), 3.85 (s, 3H), 4.50 (m, 1H, $H_{4'}$), 5.29 (m, 1H, $H_{2'}$), 6.01 (m, 1H, $H_{1'}$), 8.54 (d, 1H, *J* = 6.5 Hz, H_6), 9.57 (1H, NH). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 20.8, 28.0, 32.7, 53.0, 58.0, 74.0, 75.8, 94.0, 124.3, 139.7, 141.6, 149.3, 152.5, 156.8, 162.0, 169.5, 172.9. MS (ES^+) *m/z* (MH^+): 416. Anal. calcd. for $C_{17}H_{22}N_3O_8F$: C, 49.16; H, 5.34; N, 10.12. Found: C, 48.95; H, 5.36; N, 10.14.

(2*S*,4*R*)-1-Ethyl-2-methyl-4-(*tert*-butyldimethylsilyloxy)pyrrolidine-1,2-dicarboxylate (17). To a vigorously stirred solution of **16**[2] (4.1 g, 18.7 mmol) and imidazole (2.8 g, 41.1 mmol) in anhydrous dichloromethane (20 mL) at 0 °C was added *tert*-butyldimethylsilyl chloride (3.1 g, 20.6 mmol), dropwise, in anhydrous dichloromethane (20 mL). The mixture was stirred at room temperature overnight and the reaction was quenched with water (25 mL). The aqueous layer was extracted and the organic phase was washed with brine (3 × 30 mL), dried over anhydrous sodium sulfate, filtered and evaporated in vacuum to give **17** as yellow oil (6.1 g, 18.14 mmol, 97% yield).^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.01 (s, 6H), 0.82 (s, 9H), 1.39 (t, 3H, *J* = 6.9 Hz), 1.96 (m, 1H), 2.09 (m, 1H), 3.29 (m, 1H), 3.53 (m, 1H), 3.65 (s, 3H), 4.32 (q, 2H, *J* = 6.9 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 4.6, 17.9, 25.6, 28.3, 38.9, 51.9, 54.5, 54.8, 69.7, 78.0, 159.8, 173.9. MS (ES^+) *m/z* (MH^+): 332. Anal. calcd. for $C_{15}H_{29}SiNO_5$: C, 54.35; H, 8.82; N, 4.23. Found: C, 54.28; H, 8.84; N, 4.26.

(2*S*,4*R*)-1-Ethyl-2-methyl-4-(*terz*-butyldimethylsilyloxy)-5-oxopyrrolidine-1,2-dicarboxylate (18). In the three neck bottom flask containing $NaIO_4$ (9.73 g, 45.5 mmol), was added water (165 mL) and the mixture was stirred until complete salt dissolution (about 1.5 h). To the aqueous solution was added RuO_2●H_2O (0.46 g, 3.46 mmol) under protection of nitrogen. The mixture was stirred at room temperature for 30 min and the solution of **17** (6.1 g, 18.2 mmol) in ethyl acetate (70 mL) was added in one portion. After 4 h was added a second portion of $NaIO_4$ (11.76 g, 54.6 mmol). The solution was stirred at room temperature for 16 h, remaining yellowish during the reaction. The resulting mixture was then diluted with ethyl acetate and filtered through a pad of Celite. The organic layer was washed with saturated $NaHSO_3$ (3 × 60 mL), which resulted in precipitation of black rutenium. The precipitate was filtered off through a pad of Celite. The organic layer was then washed with brine, filtered through Celite and dried over sodium sulfate. Evaporation of solvent gave **18** as clear oil (4.78 g, 13.83 mmol, 76% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.13 (s, 3H), 0.18 (s, 3H), 0.89 (s, 9H), 1.34 (m, 3H), 2.32 (m, 2H), 3.80 (s, 3H), 4.13 (m, 3H), 4.63 (m, 1H,). MS (ES^+) *m/z* (MH^+) 346. Anal. calcd. for $C_{15}H_{27}SiNO_6$: C, 52.15; H, 7.88; N, 4.05. Found: C, 51.99; H, 7.90; N, 4.09.

(2*S*,4*R*,5*RS*)-1-Ethyl-2-methyl-4-(*tert*-butyldimethylsilyloxy)-5-hydroxypyrrolidine-1,2-dicarboxylates (19). To a –78 °C solution of **18** (4.8 g, 13.75 mmol) in anhydrous THF (128 mL) was added lithium triethylborohydride (6.45 mL, 1M in THF, 0.5 g, 6.9 mmol) under a nitrogen atmosphere. After 1 h a second portion of hydride (9.6 mL, 1 M in THF, 0.7 g, 9.6 mmol) was added to the cooled solution. The mixture was then stirred at –78 °C for 1 h and the reaction was quenched with water (80 mL) at –78 °C. After the mixture was warmed to room temperature, the organic solvent was evaporated and the mixture was extracted with ethyl acetate (3 × 160 mL). The combined organic phases were washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated in vacuum to obtain dicarboxylates **19** as clear oil in isomeric mixture 1:1 ratio (3.68 g, 10.59 mmol, 77% global yield). Isomer A: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.05 (s, 6H), 0.84 (s, 9H), 1.20 (m, 3H), 1.81 (m, 1H), 2.19 (m, 1H), 3.46 (s, 4H), 3.72 (m, 2H), 4.07−4.14 (m, 2H), 5.20 (m, 1H). Isomer B: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 0.07 (s, 6H), 0.88 (s, 9H), 1.27 (m, 3H), 1.66 (m, 1H), 2.02 (m, 1H), 3.45 (s, 4H), 3.73 (m, 2H), 4.07−4.14 (m, 2H), 5.20 (m, 1H).

(2*S*,4*R*,5*RS*)-1-Ethyl-2-methyl-4,5-diacetyloxypyrrolidine-1,2-dicarboxylates (20). To the solution of **19** (3.2 g, 9.1 mmol) in anhydrous THF (66 mL) was added dropwise at room temperature tetrabutyl ammonium fluoride (9.1 mL, 1.0 M in THF, 9.1 mmol). Stirring was continued for 2 h and then acetic

anhydride (50 mL) was added under a nitrogen atmosphere. The reaction was stirred overnight at room temperature. After removal of the solvent to dryness, the resulting residue was purified by flash chromatography using ethyl acetate/cyclohexane 25:75 (R_f = 0.18) as eluent to afford isomers **20** (1.85 g, 5.82 mmol, 64% global yield) as yellow oil in mixture 1:1 ratio. Isomer A: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.13 (m, 3H), 1.95 (s, 3H), 2.07 (s, 3H); 2.30 (m, 2H), 3.75 (s, 3H), 4.29−4.35 (m, 2H), 4.39 (m, 1H), 5.36 (m, 1H), 6.70 (m, 1H). Isomer B: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.20 (m, 3H), 1.99 (m, 6H), 2.4 (m, 1H); 2.50 (m, 1H), 3.77 (s, 3H), 4.37−4.43 (m, 2H), 4.46 (m, 1H), 5.10 (m, 1H), 6.40 (m, 1H).
To a stirred mixture of 5-fluorouracil (**13d**) (0.41 g, 3.15 mmol) and epimeric mixture of **20** (1.0 g, 3.15 mmol) in anhydrous CH_2Cl_2 (60 mL) was added, dropwise, *N*,*O*-bis-(trimethylsilyl)acetamide (1.76 mL, 7.24 mmol). After 2 h of stirring at room temperature, the clear solution was cooled at 0 °C and tin tetrachloride (3.15 mmol, 0.37 mL) was added. The reaction mixture was warmed to room temperature, left to stir overnight, and, finally, slowly poured into a cold mixture of 5% sodium bicarbonate solution (8 mL). The resulting emulsion was separated by filtration through Celite, the aqueous layer was extracted further with ethyl acetate (3 × 10 mL), and the combined organic layers were dried over anhydrous sodium sulfate. After filtration and removal of the solvent under vacuum, the raw product was subjected to silica gel flash chromatography (EtOAc/cyclohexane 1:1) to afford 1.18 g (2.83 mmol, 90% yield) of a not separated mixture of **21** and **22** in a 1:1 ratio which was used, without further purification, in the next step. Compound **21**: ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.25 (t, 3H, *J* = 7.2 Hz), 2.17 (s, 3H), 2.09 (m, 1H), 2.49 (m, 1H), 3.85 (s, 3H), 4.2 (q, 2H, *J* = 7.2 Hz), 4.54 (m, 1H), 5.30 (m, 1H), 6.03 (m, 1H), 8.52 (m, 1H), 9.18 (bs, 1H, NH). Compound **22**: ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 1.24 (t, 3H, *J* = 7.2 Hz), 1.96 (s, 3H), 2.21 (m, 1H), 2.48 (m, 1H), 3.78 (s, 3H), 4.16 (q, 2H, *J* = 7.2 Hz), 4.55 (m, 1H), 5.29 (m, 1H), 5.99 (m, 1H), 8.55 (m, 1H), 9.23 (bs, 1H, NH).

***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).** ^{1}H NMR (CD_3OD, 500 MHz): *δ* 1.39 (s, 9H), 1.98 (ddd, 1H, *J* = 4.3, 7.4, 13.4 Hz, $H_{3'a}$), 2.20 (ddd, 1H, *J* = 5.5, 7.3, 13.4 Hz, $H_{3'b}$), 3.66 (dd, 1H, *J* = 1.3, 11.5 Hz, $H_{4''a}$), 4.03 (dddd, 1H, *J* = 1.3, 2.6, 7.3, 7.4 Hz, $H_{4'}$), 4.18 (dd, 1H, *J* = 2.6, 11.5 Hz, $H_{4''b}$), 4.31 (ddd, 1H, *J* = 2.6, 4.3, 5.5 Hz, $H_{2'}$), 5.65 (d, 1H, *J* = 7.9 Hz, H_5), 5.82 (d, 1H, *J* = 2.6 Hz, $H_{1'}$), 8.31 (d, 1H, *J* = 7.9 Hz, H_6). ^{13}C NMR (CD_3OD, 125 MHz): *δ* 28.4, 34.0, 60.6, 62.4, 75.0, 78.2, 82.6, 101.9, 142.7, 152.6, 155.8, 166.4. MS (ES^+) *m/z* (MH^+): 328. Anal. calcd. for $C_{14}H_{21}N_3O_6$: C, 51.37; H, 6.47; N, 12.84. Found: C, 51.19; H, 6.49; N, 12.80.

***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).** ^{1}H NMR (CD_3OD, 500 MHz): *δ* 1.38 (s, 9H), 1.85 (s, 3H), 1.97 (m, 1H, $H_{3'a}$), 2.23 (ddd, 1H, *J* = 5.6, 7.5, 13.3 Hz, $H_{3'b}$), 3.62 (dd, 1H, *J* = 1.0, 11.4 Hz, $H_{4''a}$), 4.03 (m, 1H, $H_{4'}$), 4.23 (dd, 1H, *J* = 2.9, 11.4 Hz, $H_{4''b}$), 4.29 (m, 1H, $H_{2'}$), 5.81 (d, 1H, *J* = 2.9 Hz, $H_{1'}$), 8.23 (s, 1H, H_6). ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 12.5, 28.5, 34.0, 60.6, 62.3, 75.1, 78.1, 82.6, 110.7, 138.6, 147.0, 152.9, 166.8. MS (ES^+) *m/z* (MH^+): 342. Anal. calcd. for $C_{15}H_{23}N_3O_6$: C, 52.78; H, 6.79; N, 12.31. Found: C, 52.61; H, 6.81; N, 12.32.

***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).** ^{1}H NMR (CD_3OD, 500 MHz): *δ* 1.29 (s, 9H), 1.92 (m, 1H, $H_{3'a}$), 2.15 (ddd, 1H, *J* = 5.0, 9.5, 11.5 Hz, $H_{3'b}$), 3.65 (d, 1H, *J* = 11.5 Hz, $H_{4''a}$), 4.06 (m, 1H, $H_{2'}$), 4.21 (m, 1H, $H_{4'}$), 4.27 (m, 1H, $H_{4''b}$), 5.83 (m, 2H, $H_{1'}$ and H_5), 8.28 (d, 1H, *J* = 7.0, H_6). ^{13}C NMR (CD_3OD, 125 MHz): *δ* 28.5, 35.6, 61.0, 61.9, 75.0, 80.2, 82.4, 95.1, 143.0, 156.0, 158.7, 167.7. MS (ES^+) *m/z* (MH^+): 327. Anal. calcd. for $C_{14}H_{22}N_4O_5$: C, 51.52; H, 6.79; N, 17.17, Found: C, 51.47; H, 6.81; N, 17.21.

***tert*-Butyl-(2*R*,3*R*,5*S*)-2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)-3-hydroxy-5-(hydroxymethyl)pyrrolidine-1-carboxylate (15d).** ^{1}H NMR (CD_3OD, 500 MHz): *δ* 1.40 (s, 9H), 1.97 (ddd, 1H, *J* = 4.3, 7.9, 13.6 Hz, $H_{3'a}$), 2.23 (ddd, 1H, *J* = 5.5, 7.9, 13.6 Hz, $H_{3'b}$), 3.58 (dd, 1H, *J* = 0.6,

11.6 Hz, $H_{4''a}$), 4.03 (ddd, 1H, J = 0.6, 2.8, 7.9 Hz, $H_{4'}$), 4.25 (dd, 1H, J = 2.8, 11.6 Hz, $H_{4''b}$), 4,30 (dd, 1H, J = 4.3, 5.5 Hz, $H_{2'}$), 5.81 (s, 1H, $H_{1'}$), 8.71 (d, 1H, J = 6.2 Hz, H_6). ^{13}C NMR (CD_3OD, 125 MHz): δ 28.4, 34.0, 60.0, 62.1, 75.0, 78.4, 82.7, 127.0, 139.3, 143.9, 151.2, 159.4, 163.4, 163.9. MS (ES^+) *m/z* (MH^+): 346. Anal. calcd. for $C_{14}H_{20}N_3O_6F$: C, 48.69; H, 5.84; N, 12.17. Found: C, 48.51; H, 5.88; N, 12.20.

(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). ^{1}H NMR (D_2O, 500 MHz): δ 1.11 (t, 3H, J = 7.0 Hz), 1.95 (m, 1H, $H_{3'a}$), 2.25 (m, 1H, $H_{3'b}$), 3.95 (m, 2H, $H_{4''}$), 4.09 (q, 2H, J = 7.0 Hz), 4.22 (m, 1H, $H_{2'}$), 4.32 (m, 1H, $H_{4'}$), 5.73 (m, 1H, $H_{1'}$), 8.83 (m, 1H, H_6). ^{13}C NMR (D_2O, 125 MHz): δ 16.1, 36.9, 63.8, 65.1, 66.0, 76.6, 79.7, 127.6, 142.1, 149.8, 159.2, 161.5. MS (ES^+) *m/z* (MH^+) 318. Anal. calcd. for: $C_{12}H_{16}N_3O_6F$: C, 45.43; H, 5.08; N, 13.24; Found: C, 45.39; H, 5.06; N, 13.26.

(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). ^{1}H NMR (D_2O, 500 MHz): δ 1.12 (t, 3H, J = 7.0 Hz), 2.00 (m, 1H, $H_{3'a}$), 2.12 (m, 1H, $H_{3'b}$), 3.70 (m, 2H, $H_{4''}$), 4.03 (m, 1H, $H_{4'}$), 4.16 (d, 2H, J = 7.0 Hz), 4.31 (m, 1H, $H_{2'}$), 5.76 (m, 1H, $H_{1'}$), 7.99 (m, 1H, H_6). ^{13}C NMR (D_2O, 125 MHz): δ 16.0, 34.7, 61.6, 63.5, 65.5, 73.2, 75.91, 126.9, 142.0, 147.3, 157.8, 159.9. MS (ES^+) *m/z* (MH^+) 318. Anal. calcd. for: $C_{12}H_{16}N_3O_6F$: C, 45.43; H, 5.08; N, 13.24; Found: C, 45.37; H, 5.10; N, 13.27.

Biological assays

Cell culture. Huh-7 cells, originally obtained from Ralf Bartenschlager (University of Mainz, Mainz, Germany), were grown in Dulbecco's modified minimal essential medium (D-MEM, EuroClone, Pero, Italy), supplemented with 10% fetal bovine serum (FBS, Life Technologies, Paisley, Scotland, UK). Huh-7-derived HBI10A cells expressing an HCV subgenomic replicon, have been previously described.[3] They were grown as described for Huh-7 cells, but the medium was supplemented with the addition of 0.8 mg of neomycin sulfate (G418, Life Technologies). Cells were passaged 1:5 twice a week using 1X trypsin-EDTA.

Anti-hepatitis C virus assay. The effect of compounds on HCV viral replication was monitored in HBI10A cells by a cell-enzyme-linked immunosorbent assay, as previously described.[4] Briefly, HBI10A cells, either treated with different concentrations of the compounds or control diluent, were assayed for NS3 protein expression with the anti-NS3 10E5/24 MAb. Compounds were dissolved in DMSO (Sigma Chemicals CO., St Louis , MO) and serially diluted in D-MEM in a way that DMSO concentration was never higher than 1%. Final concentrations of the compounds were from 10^3 to 10^{-6} μM. The assay was performed in triplicate. As a positive control, IFN-α at concentrations ranging from 0.01 to 0.008 U/mL, was utilized. The inhibitor concentration that reduced by 50% the expression of NS3 (IC_{50}) was calculated by fitting the data to the Hill equation: fraction inhibition = $1 - (A_i - b)/(A_0 - b) = [I]^n/([I]^n + IC_{50})$, where A_i is the absorbance value of HBI10A cells supplemented with the appropriate compound (I) concentration, A_0 is the absorbance value of HBI10A cells incubated with control diluent, b is the absorbance value of Huh-7 cells plated at the same density in the same microliter plates and incubated with control diluent, and n is the Hill coefficient. The IC_{50} values were calculated according to the best-fit curve, y value versus log x, where y is the value of the examined function and x is the drug concentration.

Cytotoxicity assay. Cytotoxicity of the compounds on HBI10A cells was detected by a MTS assay. HBI10A cells were seeded at 1×10^4 cell/100 μL of D-MEM + 5% FBS in a 96 well plate and after 4 h incubation at 37 °C cells the compounds were added at the final concentration of 10^3,10^2, 10 and 1 μM. After 20 h incubation at 37 °C, 20 μL of MTS solution, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] were added to each well. Samples were then incubated for a further 4 h at 37 °C before the reaction was stopped through the addition of 20 μL SDS 10% SDS and the absorbance was measured at 492 nm. Each condition was analysed in triplicate.

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