**Design, chemical synthesis and antiviral evaluation of 2’-deoxy-2’-fluoro-2’-*C*-methyl-4’-thionucleosides**

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**Abstract**

Nucleoside analogues represent an historically accomplished class of antiviral drug. Notwithstanding this, new molecular scaffolds are required to overcome their limitations and evolve pharmacophore space within this established field. Herein we develop concise synthetic access to a new 2’-deoxy-2’-fluoro-2’-*C*-methyl-4’-thionucleoside chemotype, including the ProTide form of the uridine analogue. Biological evaluation of these materials in the Hepatitis C replicon assay shows little activity for the canonical pyrimidine forms, but the phosphoramidate of 2’-deoxy-2’-fluoro-2’-*C*-methyl-b-d-4’-thiouridine has an EC50 of 2.99 mM. Direct comparison to the established Hepatitis C drug Sofosbuvir shows a 100-fold drop in activity upon substituting the furanose chalcogen; the reasons for this are as yet unclear.

Nucleoside analogues constitute an essential class of antiviral drug. They are routinely used in therapeutic regimens against HSV (Acyclovir and Ganciclovir), HIV (Tenofovir, Zidovudine, Abacavir, Emtricitabine and Lamivudine), HBV (Entecavir, Tenofovi, Adefovir, Lamivudine and Telbivudine) and HCV (Sofosbuvir).[1,2] Most recently, Remdesivir and Molnupiravir have been authorised for treatment of SARS-Cov-19.[3,4]Despite their success as medicinal agents,[5] research to overcome issues surrounding their pharmacokinetic and pharmacological profiles is continually required. Modification of nucleoside analogues for structure-activity-relationship studies often focuses on the ribose ring, using both chemical and enzymatic approaches.[4,6–9] Substitution of furanose oxygen to access and evaluate 4’-thionucleosides is also of particular interest.[10–12] In this regard, we recently reported a scalable and chromatography-free route to thioribose building blocks (Figure 1a) *via* an open-chain oxime derivative with retention of the C4 d-ribo configuration.[13] Herein we template this approach onto a privileged nucleoside analogue scaffold, a 2-deoxy-2-fluoro-2-*C*-methyl system, thereby creating a novel 4’-thionucleoside chemotype for biological evaluation (Figure 1b). Importantly, this allows for direct comparison of the commercial antiviral drug, Sofosbuvir, with its 4’-thio analogue.



**Figure 1:**a) Previous work establishing 4-thioribose building block synthesis and subsequent access to 4’-thio and 4’-sulfinyl nucleoside analogues b) This work templating thionucleoside synthesis onto the established 2’-deoxy-2’-fluoro-2’-*C*-methyl nucleoside chemotype.

**Synthesis of a 2-deoxy-2-fluoro-2-*C*-methyl-4-thioribose building block**

A scalable and reliable method for the synthesis of a key building block, 1-*O*-acetyl-3,5-di-*O*-benzoyl-2-deoxy-2-fluoro-2-*C*-methyl-1-(4-thio-d-ribofuranose) **7**, was developed from commercially available lactone **1** (Scheme 1). The approach utilised a methodology previously reported by our group,[13] adopting a double inversion strategy at C4 within an open-chain oxime derivative, used to facilitate sulfur insertion with net retention of the d-ribo configuration.

**Scheme 1.** (a) Li(O*t*Bu)3AlH, THF, -10 °C, 92% (b) H2N-OMe·HCl, Et3N, pyridinium *p*-toluenesulfonate, MeCN, H2O, rt (c) 2,4,5-Trichlorobenzenesulfonyl chloride, *N*-methylimidazole, MeCN, rt, 56%, 2 steps (d) LiBr, DMF, 80 °C, 53% (e) (i) Glyoxylic acid, MeCN, 70 °C (ii) NaSH·H2O, DMF, H2O, 0 °C, 61%, 1/3 , α/β, 20% recovery of **5** (f) DMAP, Ac2O, Et3N, CH2Cl2, rt, 71%, 3/1, α/β; Ar = 2,4,5-trichlorophenyl.

Starting from lactone **1**, reduction to the corresponding hemiacetal was effected *via* treatment with Li(OtBu)3AlH, delivering **2** as an anomeric mixture (2/1, α/β) in 92% yield and on 20 g scale.[14] Hemiacetal **2** was next treated with H2N-OMe, Et3N and pyridinium *p*-toluenesulfonate to furnish the corresponding oxime **3** as a 13/1 mixture of E/Z diastereoisomers isomers. This material was used crude with the C4-hydroxyl group converted to 2,4,5-trichlorobenzenesulfonate ester **4**. This process was also scaled to 20 g, furnishing **4** in 56% yield over two steps. A trichlorobenzenesulfonate ester was selected over other, more common leaving groups (e.g., Ts, Ms) as its use by us had previously enabled facile isolation of the sulfonyl ester *via* trituration.[13] This simplified purification on large scale, however trituration here was less successful (low isolated yields of around 30%) and column chromatography was instead used to purify **4**.

Inversion of configuration at the C4 stereocentre using SN2 bromination was evaluated next. The choice of reaction solvent and temperature proved important; no reaction occurred when 2-butanone was used, despite it having been used successfully on related substrates.[13] Use of DMF proved effective, however, little reaction occurred at temperatures below 80 °C and above 100 °C significant decomposition of **4** was observed. Hence, 80 °C was selected as the optimal temperature, furnishing the C4-(*S*)-bromide **5** in a moderate yield of 53%. 13C NMR showed an expected upfield shift for C4 of the major diastereoisomer, from δC = 80.4 ppm in **4** to δC = 46.3 ppm in **5**.

Hydrolysis of oxime **5** using glyoxylic acid in MeCN at 70 °C delivered an intermediate aldehyde which was used immediately for C4 sulfur insertion and ring closing to afford thiohemiacetal **6** in 61% yield over the two steps. Finally, anomeric acetate **7** was prepared *via* treatment of **6** with Ac2O and Et3N, delivering **7** in 71% yield with a 3/1 preference for the a-anomer.

In summary, a novel 2-fluoro-2-*C*-methyl-4-thioribose derivative **7** was furnished in seven steps and 12% overall yield from commercially available ribonolactone **1**. The synthetic route was scalable, enabling preparation of multi-gram final quantities of **7** and confirming a suitable platform for the preparation of the corresponding nucleoside derivatives.

**Synthesis of 2’-deoxy-2’-fluoro-2’-*C*-methyl-4’-thionucleosides**

With an accessible route to multi-gram quantities of **7** established, the corresponding uridine and cytidine 4’-thionucleosides were synthesised (Scheme 2). Glycosylation of uracil with **7** was achieved using modified Vorbrüggen conditions,[13,15] delivering **8** as an inseparable anomeric mixture in 54% yield (2/1, α/β). The anomers could by identified through differences in their H1’-F coupling constants; a larger 1,2-*trans* H1’-F coupling (3*J*H1’-F = 22.5 Hz) was evident for the major α-anomer and a smaller 1,2-*cis* H1’-F coupling (3*J*H1’-F = 14.2 Hz) for the minor β-form. Deprotection of **8** with 7M NH3 in MeOH furnished the free nucleoside **9** in 67% yield. The anomers were separated using preparative HPLC to deliver **9-β** in 15% yield and **9-a** in 26% yield. Selective 1D NOESY could not confirm the anomeric assignment of **9-β**, however, for **9-α** a clear correlation from H1’ to H3’was evident, alongsideno observable correlation between H1’ and H4’, supporting this anomeric assignment (see SI, Figure S3).

**Scheme 2.**(a) (i) Uracil, HMDS, pyridine, reflux (ii) **7**, TMSOTf, MeCN, reflux, 54%, 2/1, a/b (b) (i) *N*4-Benzoyl cytosine, HMDS, pyridine, reflux (ii) **7**, TMSOTf, MeCN, reflux, 41%, 1.6/1, a/b, 36% recovery of **7** (c) (i)7M NH3, MeOH, rt, 67% crude, 2/1 a/b, 16% recovery of **8** (ii) Prep. HPLC: **9-b**, 15% and **9-a** 26% (d) (i)7M NH3, MeOH, rt, quant. crude, 1/9 a/b, (ii) Prep. HPLC: **11-b**, 87% and **11-a** 10%

The same modified Vorbrüggen conditions were then employed to glycosylate *N*4-benzoyl cytosine with **7**, delivering **10** as an anomeric mixture in 41% yield and with 36% recovery of **7**. Nucleoside **10** was furnished with preference for the a-anomer (1.6/1, α/β), similarly to **8** (2/1, α/β). Overall though, low diastereoselectivity was observed in glycosidating these C2’-modified substrates with pyrimidine nucleobases. Direct preparative HPLC separation of mixture **10** proved challenging and a prior partial separation, using fractional precipitation, gave diastereomerically enriched quantities of each of the protected anomers (4% of 1/9, α/β of **10**; 3% of **10-α**; 27% of mixture remaining). Subsequent treatment of a 1/9, α/β mixture of **10** with 7M NH3 MeOH delivered **11-α** and **11-β** in 10% and 87% yields respectively, following preparative HPLC. Characterisation of the anomers was again confirmed using selective 1D NOESY (see SI Figure S3). Alternatively, the initial anomeric mixture of **10** could be deprotected and separated *via* preparative HPLC, but this approach required several rounds of purification to deliver acceptable anomeric purity and the prior fractional precipitation was favoured.

Finally, a synthesis of the phosphoramidate ProTide form of **9-β** was completed in order to enable direct comparison to the HCV nucleoside analogue drug Sofosbuvir, which contains a 2’-b-Me-substituent and phosphoramidate modifications as integral elements of its scaffold. Treatment of nucleoside **9-β** with *t*BuMgCl, followed by addition of the commercially available pentafluorophenyl phosphoramidate reagent, furnished **12** as a single diastereoisomer (31P NMR for **12**: singlet at δP 3.81 ppm, Scheme 3). Solubility of **9-β** in THF was low, and significant quantities (63%) of **9-β** were recovered from the reaction mixture. Exploration of alternative solvents (DMF or 1,4-dioxane) proved fruitless, with no reaction being observed. Following an initial purification of crude **12** *via* flash column chromatography, an analytically pure sample was obtained in 18% overall yield, following preparative HPLC.

**Scheme 3.**(a) *t*BuMgCl, *N*-[(*S*)-(2,3,4,5,6-pentafluorophenoxy)-phenoxyphosphinyl]-1-methylethyl ester-d-alanine, THF, -20 °C to rt, 18%, 63% recovery of **9-b**.

Final compound purities for **9-a**, **9-b**, **11-a**, **11-b** and **12** were confirmed as >95% by HPLC (see SI Figure S4). These materials were next evaluated in an HCV replicon assay,[16] alongside the series of pyrimidine 4’-thio and 4’-sulfinyl nucleoside analogues, **13**-**20**, reported previously (Table 1).[13]

Compound **12** was a low mM inhibitor in the HCV replicon assay (EC50 = 2. 99 mM) but was less active than Sofosbuvir (EC50= 0.03 mM, Table 1, entries 1–2). Neither of the a- or b- anomers of **9** or **11** were active below 100 mM (Table 1, entries 3–6), suggesting that for this 2’-deoxy-2’-fluoro-2’-*C*-methyl-4’-thio chemotype, the phosphoramidate form is required for antiviral activity, presumably due to a lack of recognition by cellular kinases. This is in line with results described recently by Liotta and co-workers, for a related 2-*C*-methyl-4-thio scaffold,[17] which also showed low mM antiviral activity as the prodrug form (EC50 = 2.10 mM).

4’-Thioarabinocytidine **16**,[18] which previously demonstrated cytotoxicity (CC50 = 0.19 uM in human primary glioblastoma cells),[13,19] also showed activity here against HCV (Table 1, entry 10). This was similar to results reported by Yoshimura and colleagues for their series of 4’-thioarabino purines and pyrimidines,[20] and demonstrates an interesting interplay between antiviral and anticancer activity for 4’-thioarabinocytidine. Other 4’-thionucleosides (**13**, **14** and **17**) also showed mM activity (Table 1, entries 7, 8 and 11), whilst having previously demonstrated no observable cytotoxicity in cancer cells. [13] Finally, of the 4’-sulfinyl analogues evaluated (**18**–**20)**, compound **20**, a 4’-sulfinyl gemcitabine variant, showed low μM activity (Table 1, entry 12). None of the analogues evaluated here showed cytotoxicity below 100 μM.

**Table 1.** Evaluation of 4’-thionucleosides. To generate a full dose-responsive curve for the active compounds, the antiviral activity and cytotoxicity experiments were performed at 9 points of 3-fold serial dilutions, in duplicate (See SI Figure S2.1 for dose-response curves for **12** and **16**). Diastereomeric identity at S for compounds **18**-**20** is unconfirmed. Diastereomeric ratios at S are: 1/1 for **18**, 2.5/1 for **19** and 4/1 for **20**.

|  |  |  |  |
| --- | --- | --- | --- |
| Entry | Compound | EC50 (*μ*M) | CC50 (*μ*M) |
| 1 | Sofosbuvir | 0.03 | > 5.00 |
| 2 | **12** | 2.99 | > 100 |
| 3 | **9-a** | > 100 | > 100 |
| 4 | **9-b** | > 100 | > 100 |
| 5 | **11-a** | > 100 | > 100 |
| 6 | **11-b** | > 100 | > 100 |
| 7 | **13** | 65.9 | > 100 |
| 8 | **14** | 50.0 | > 100 |
| 9 | **15** | > 100 | > 100 |
| 10 | **16** | 0.16 | > 100 |
| 11 | **17** | 22.5 | > 100 |
| 12 | **18** | > 100 | > 100 |
| 13 | **19** | > 100 | > 100 |
| 14 | **20** | 21.8 | > 100 |

A 2-deoxy-2-fluoro-2-*C*-methyl-4-thioribofuranose building block was successfully prepared on gram-scale starting from commercial material in 12% yield over seven steps. Modified Vorbrüggen conditions then granted entry to the pyrimidine nucleoside analogue forms and from there to a phosphoramidate ProTide of the b-uridine. Evaluation of these materials in an HCV replicon assay showed that replacement of furanose oxygen with sulphur (or sulfinyl) appears, within the nucleoside analogue classes examined, to have a deleterious effect upon biological activity; a direct comparison of the 4’-thio congener with the anti-HCV drug sofosbuvir demonstrated an approximately 100-fold drop in activity. This suggests that the templating of this structural modification onto established nucleoside analogue scaffolds does not present an enhanced biological activity profile, at least for gemcitabine and sofosbuvir. The case for thiocytarabine is more positive, with activity observed in both antiviral and anticancer cell lines and we will report further on this and other prodrug forms of such 4’-thionucleosides in due course.

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