Synthesis and anticancer evaluation of 4'-thio and 4'-sulfinyl pyrimidine nucleoside analogues

Mieke Guinan[a,c], Ningwu Huang[b], Chris Hawes[a], Marcelo Lima[c,d], Mark Smith[b] and Gavin J. Miller[a,d]*

*g.j.miller@keele.ac.uk

[a] Lennard-Jones Laboratory, School of Chemical and Physical Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom.

[b] Riboscience LLC, 428 Oakmead Pkwy, Sunnyvale, CA 94085, USA.

[c] School of Life Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom.

[d] Centre for Glycoscience Research, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom.

1. Introduction

A significant proportion of current chemotherapeutic treatments for cancer involve the use of anti-metabolites, particularly modified nucleoside analogues that possess a capability to mimic native purine or pyrimidine nucleosides, which can disrupt metabolic and regulatory pathways.[1] Notwithstanding this significant medicinal capability, therapeutic intervention using nucleoside analogues is often limited by poor cellular uptake, low conversion to the active triphosphate metabolite, rapid degradation or clearance and development of resistance profiles in certain cell types.[2] Consequently, research activity in this field continues to develop syntheses for next generations of nucleoside analogues that can overcome these limitations and provide new therapeutic options.[3–5]

Within this context, 4'-thionucleosides, where the furanose ring oxygen is substituted with sulfur, have received attention from several academic and industrial groups since a first disclosure of 4'-thioadenosine in the early 1960's;[6,7] *Figure 1* highlights some key achievements in this area. Bioisosteric replacement of furanose oxygen with larger sulfur seeks to explore the biological effect, imparted through changes to furanose ring conformation and the hydrolytic stability of a thiohemiaminal glycosidic linkage.

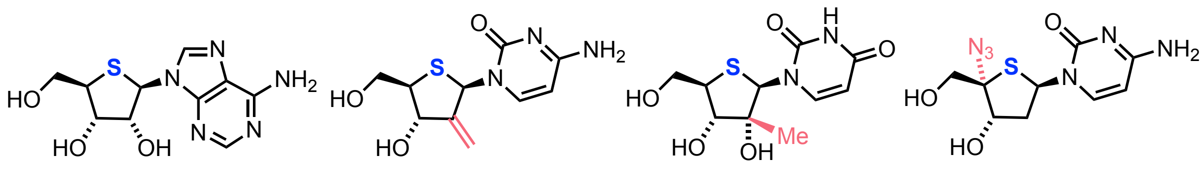


Figure 1: Historical and current examples of thionucleosides (blue sulphur) with additional ring substituent modifications (red).

Insertion of a sulfur atom to access thiofuranose components generally start from available chiral pool materials and the use of thiourea to convert 4,5-epoxides to 4,5-thiiranes,[8,9] alongside sodium sulfide-mediated formation of 2,5-bicyclic systems are notable examples of key intermediates developed to enable this.[10–12] Open chain systems have also been used,[13] for example, effecting double displacement of a 1,4-bismesylate with Na_2S to access 1-deoxy thiofuranoses,[14] and both C1 aldehyde and thioacetal oxidation levels have delivered access to anomeric thioglycosides.[15,16] Two main approaches have been adopted to install appropriate nucleobases; firstly thioglycosylation of a silylated nucleobase,[8,17] and, secondly a Pummerer-type reaction of the sulfoxide to condense with a corresponding nucleobase.[16,18] Hypervalent iodine reagents have also been used to effect a similar transformation.[19]

As part of a program exploring chemical synthesis approaches to next generation nucleoside scaffolds, we were interested in accessing appropriate 4′-thionucleosides and templating this bioisosteric replacement onto established chemotypes, a tactic recently implemented by Liotta and colleagues for 2′-*C*-methyl-4′-thionucleosides.[20] Herein we report our synthesis of 4′-thio and 4′-sulfinyl nucleosides; we targeted an open-chain C1-oxime to enable sulfur inclusion using displacement of an appropriate 4-position leaving group, with concomitant ring closure onto a C1 aldehyde. This was intended to provide a benchmark in then accessing related 2′-deoxy-2′,2′-*gem*-difluoro and D-*arabino* structures which, alongside recent examples 2′-deoxy-2′-fluoro and L-3′-deoxy-3′,3′-difluoro systems,[13,21,22] provides capability to more broadly study 4′-thionucleoside chemotypes and their structure activity relationships. These aims are highlighted in *Figure 2*.

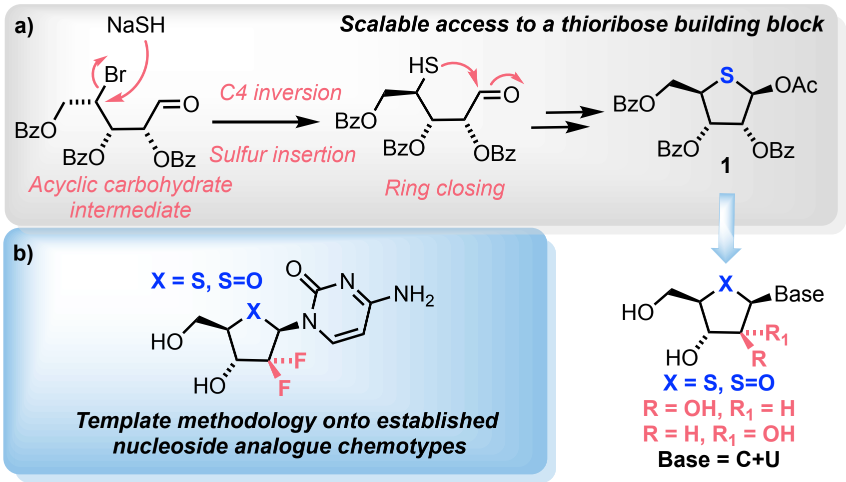
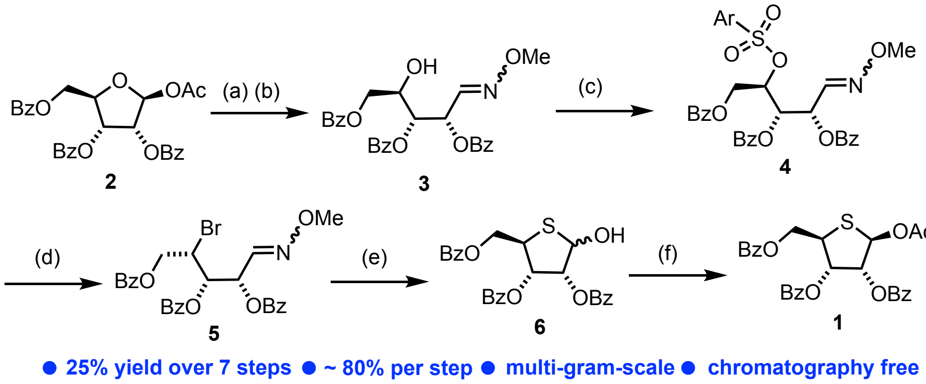


Figure 2: **a)** Strategy to access 4-thioribose derivative **1** as a key intermediate for 4′-thionucleoside synthesis and **b)** potential therefrom to template to established nucleoside analogue classes (blue box).

2. Results and Discussion

2.1. Synthesis of a 4-thioribose building block and extension to a 2-deoxy-2,2-difluoro-4-thioribose analogue

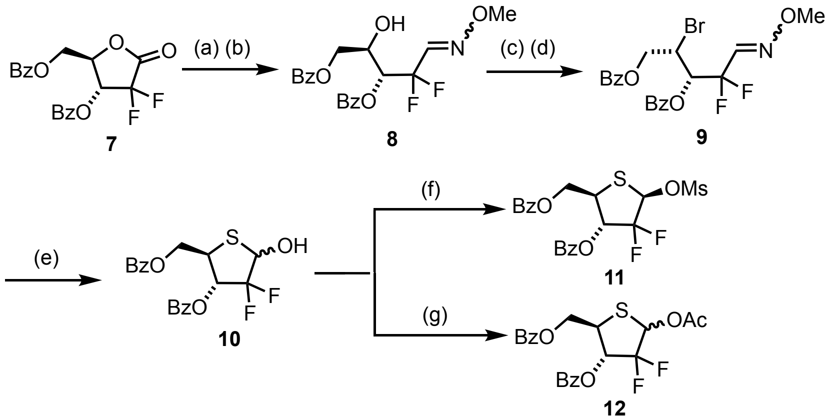
In order to access our intended series of thionucleoside derivatives, a scalable and reliable method for the synthesis of 4-thio-D-ribofuranose **1** was required (*Scheme 1*). Starting from commercially available **2**, the anomeric acetate was hydrolysed using $H_2O/BF_3{\cdot}OEt_2$ in 91% yield on 100 g scale. The resultant hemi-acetal was used crude and converted to the corresponding oxime **3** *via* treatment with *O*-methoxyhydroxylamine hydrochloride and Et_3N. This material was isolated as a mixture of oxime isomers (3/1 ratio) in 90% yield, again on multigram scale. Manipulation of the C4 position in **3** to facilitate conversion to the final thiofuranose, with retention of the C4-*ribo*-configuration, was explored next. Direct halogenation and inversion of stereochemistry at C4 using CBr_4 or Br_2 with PPh_3 proved unsuccessful, returning only starting material. Instead, the C4-OH in **3** was converted to an arylsulfonyl leaving group. Initially here we targeted a 4-nitrobenzenesulfonate, but persistent low yields (maximum obtained = 31%) and a requirement for chromatography led us to explore other options. An earlier report of a less common 2,3,5-tricholorobenzensulfonate proved fruitful,[23] delivering **4** in 72% yield. Purification of **4** was achieved using trituration from Et_2O, enabling easy isolation as a white solid. This material could be prepared in multigram amounts in 56% overall yield for three steps from **2** and required no column chromatography.



Scheme 1: (a) $BF_3{\cdot}Et_2O$, H_2O, MeCN, rt, 91%; (b) H_2N-OMe·HCl, Et_3N, MeOH, rt, 90%; (c) 2,4,5-Trichlorobenzenesulfonyl chloride, *N*-methylimidazole, MeCN, rt, 72%; (d) LiBr, butanone, 80 °C, 89% (e) (i) Glyoxylic acid, MeCN, 70 °C, 92%; (ii) $NaSH{\cdot}H_2O$, H_2O, DMF, 0 °C, 76%; (f) DMAP, Ac_2O, rt, 72% (β-only); Ar = 2,4,5-trichlorophenyl.

Nucleophilic displacement of the 4-sulfonyl group within **4** was explored using LiBr in a Finkelstein-type reaction. The use of DMI/THF as the solvent at 60 °C for 16 h resulted in a mixture of C4 diastereoisomers (3/1, *S*/*R,* see SI Figure S4), presumably forming *via* a second nucleophilic displacement of bromide **5** to give the unwanted the C4-*R* epimer. Similar issues surrounding unwanted C4 epimerisation were encountered by Codée and co-workers during their synthesis of 4-thiofuranosides.[14] Reducing the reaction time to 2 h in the same solvent system improved the diastereomeric outcome (10/1, *S*/*R*), however, this was accompanied by incomplete consumption of **4**. Switching solvent to 2-butanone and heating at 80 °C for 18 h delivered diastereomerically pure C4-bromide **5** in 89% yield. ^{13}C NMR analysis of **5** confirmed an upfield shift of C4 to δ_C 47.8 ppm, from δ_C 79.6 ppm. Treatment of bromide **5** with glyoxylic acid at 70 °C effected hydrolysis of the oxime to the corresponding aldehyde **in** 92% yield, which was used immediately for conversion to the desired 4-thiofuranose **6**. This proceeded *via* reaction with NaSH, effecting S_N2 displacement of the C4 bromide followed by ring closure onto the C1-aldehyde, obtaining **6** as a mixture of anomers in 76% yield. Crude **6** was treated with Ac_2O in pyridine with catalytic DMAP to obtain the acetylated product **1** as a mixture of anomers (4/1, β/α). Simple trituration from MeOH isolated the β-anomer of **1** exclusively in a satisfactory 50% yield from **5**, with analytical data confirming those reported previously for D-*ribo*thiofuranoses.[9] The development of this synthetic route to **1** required no column chromatography and was completed starting from 100 g of **2**, delivering **1** in an overall yield of 25% for the seven steps and in 25 g quantity.

The chemistry developed in *Scheme 1* was next mapped onto a 2-deoxy-2,2-*gem*-difluoro ribose system to access 4′-thiogemcitabine and analogues thereof (*Scheme 2*). Commercially available lactone **7** was manipulated in six steps to deliver 2-deoxy-2,2-difluoro-1-(4-thio-D-ribofuranose) **10**. Lactone **7** was first reduced using Li(O^{t}Bu)$_3$AlH to the hemiacetal in 95% crude yield and then converted directly to oxime **8** *via* reaction with *O*-methoxyhydroxylamine and Et_3N in the presence of pyridinium *p*-toluenesulfonate. Interestingly, no reaction to convert the intermediate hemi-acetal was observed in the absence of the pyridinium salt. Oxime **8** then underwent successive derivatisation at C4, first to the sulfonate ester and then nucleophilic substitution to yield bromide **9**. Finally, oxime hydrolysis was followed by incorporation of sulfur at C4 to restore the D-*ribo* configuration and subsequent cyclisation to thiohemiacetal **10**. This enabled access to multigram quantities of **10** in 6 steps and 56% overall yield from the commercial starting material.



Scheme 2: (a) $Li(O^tBu)_3AlH$, THF, 0 °C, 95%; (b) H_2N-OMe·HCl, Et_3N, MeCN, H_2O, pyridinium *p*-toluenesulfonate, rt, 90% (c) 2,4,5-Trichlorobenzenesulfonyl chloride, *N*-methylimidazole, MeCN, rt 75% (d) LiBr, butanone, 80 °C (e) (i) Glyoxylic acid, MeCN, 70 °C (ii) NaSH·H_2O, H_2O, DMF, 0 °C, 97% over 3 steps (f) MsCl, Et_3N, CH_2Cl_2, rt, 79% (53% for β-anomer only) (g) Ac_2O, Et_3N, CH_2Cl_2, rt, 88%.

The anomeric hydroxyl group in **10** was next converted to either mesylate **11** or acetate **12** as appropriate leaving groups to explore glycosylation of nucleobases. Treatment of **10** with MsCl and Et_3N (*Scheme 2*) afforded **11** in 79% yield as a 1/4, α/β mixture. The β-anomer was isolated by crystallisation from hot Et_2O and the resultant solid recrystallised by diffusion using CH_2Cl_2/hexanes to afford pure β-**11**. A sample of β-**11** was analysed by X-ray crystallography and confirmed the double stereochemical inversion at C4, which retained a D-*ribo* configuration (*Figure 3*). The anomeric mesylate and C5 were observed above the plane of the thiopentose ring, consistent with 1-β-D-*ribo* stereochemistry, with the ring adopting a C3-*endo* conformation in the solid state. Anomeric acetate **12** was accessed in 88% yield as a 3/2 mixture of anomers that, in our hands, proved inseparable by crystallisation or column chromatography.

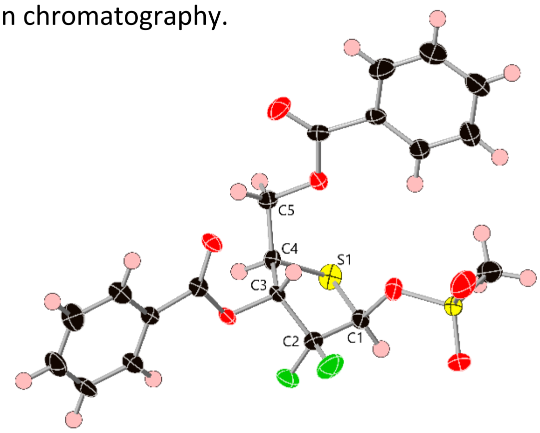
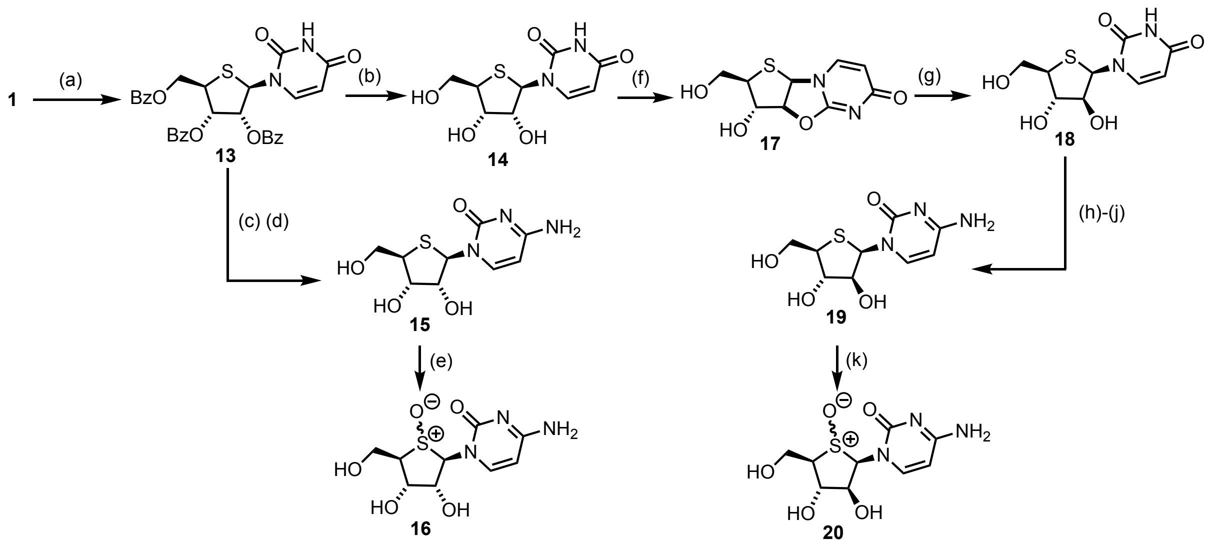


Figure 3: X-ray crystal structure of β-**11** with numbering scheme for core atoms. ADPs are rendered at 50% probability level and ring disorder on the C5 phenyl ester substituent is omitted for clarity. Atom colouring scheme: black = carbon, red = oxygen, green = fluorine, yellow = sulfur, pink = hydrogen.

2.2. Synthesis of 4'-thioribo- and 4'-thioarabino nucleosides

With access to multigram scale quantities of **1** a series of 4'-thio*ribo* and 4'-thio*arabino* nucleosides were targeted. A pathway to access these was proposed from 4'-thiouridine **14** (*Scheme*

3). Comparative literature for the synthesis of 2,'3',5'-tri-*O*-benzoyl protected uridine involved refluxing the reactants for 3 h in acetonitrile, following silyation of the nucleobase.[24] However, reaction under these conditions for **1** produced low yields of **13** (20-30%). By conducting the reaction just below the boiling point of MeCN, at 75 °C, the yield improved significantly, to 74. The β-anomer of **13** formed preferentially (^{1}H NMR of crude **13** indicated a 10/1, β/α ratio, [$^3J_{H1-H2}$ = 6.8 Hz for **β-13**]) and the reaction was scalable to 10 g.[25]



Scheme 3: (a) (i) Uracil, HMDS, reflux (ii) TMSOTf, MeCN, 75 °C, 74% (b) 7M NH_3 in MeOH, MeOH, 40 °C, 96% (c) $POCl_3$, 1,2,4-triazole, Et_3N, MeCN, rt (d) NH_4OH, 1,4-dioxane, rt, then 7M NH_3 in MeOH, MeOH, 40 °C, 77%, 2 steps from **13** (e) *m*-CPBA, H_2O, MeCN, 0 °C, 90%, 1/1 d.r. at S (f) $(PhO)_2CO$, $NaHCO_3$, DMF, 100 °C, 98% (g) KOH, EtOH, H_2O, rt, 90% (h) Ac_2O, DMAP, pyridine, rt, 82% (i) $POCl_3$, 1,2,4-triazole, Et_3N, MeCN, rt (j) 7M NH_3 in MeOH, MeOH, 120 °C, 70%, 2 steps from **18** (k) *m*-CPBA, H_2O, MeCN, 0 °C, 78%, 2.5/1 d.r. at S.

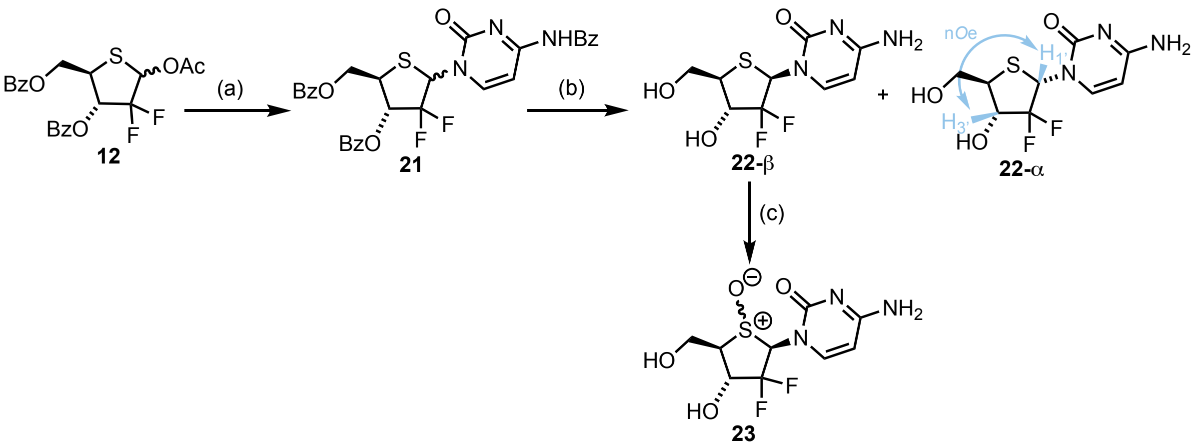
Global deprotection of **13** was completed using 7 M NH_3 in MeOH to obtain 4'-thiouridine **14** in 96% yield. This material was next converted to its cytosine form, first by treatment of **14** with $POCl_3$ and then 1,2,4-triazole to furnish the heterocyclic intermediate, before a final treatment with NH_3 in MeOH concomitantly substituted the triazole and removed the benzoyl protecting groups to give 4'-thiocytidine **15** in 77% yield over two steps.

4'-thiouridine **14** was also converted to its C2' epimer **18** *via* an intramolecular cyclisation of the C4-carbonyl, displacing a C2'-carbonate leaving group. This inverted the C2' stereochemistry to give β-1',2'-*syn*-bicycle **17** in 92% yield, with ^{13}C NMR confirming a chemical shift change of δ_C 152.3 ppm to δ_C 175.4 ppm for C2' in **17**. Subsequent ring opening of **17** was achieved by stirring with KOH in EtOH/H_2O, to give C2'-*arabino* configured **18** in almost quantitative yield. The observed ^{1}H NMR coupling constant between H2' and H3' for **18** ($^3J_{H2'-H3'}$ = 9.1 Hz) was significantly different than that observed for **14** ($^3J_{H2'-H3'}$ = 3.7 Hz), supporting inversion of stereochemistry at C2'. 4'-thio*arabino*uridine **18** was also converted to its cytosine form using the method previously established for **15**, delivering 4'-thio*arabino*cytidine **19** in 52% yield over three steps from **18**. Earlier efforts to directly convert **15** to **19** *via* a β-1',2'-*syn*-intermediate bi-cycle were unsuccessful; multiple reaction products were observed so this conversion was abandoned. Finally, the corresponding sulfoxides of **15** and **19** were prepared *via* treatment with 1.5 equivalents of *m*-CPBA, to afford novel **16** and **20** in 90% and 78% yields respectively. ^{13}C NMR analysis confirmed a significant downfield shift of C1' and C4' from δ_C 65.1

and 51.7 ppm in **16** to δ_C 73.1 and 65.7 ppm in **20** respectively. Attempted separation of the *S*-diastereoisomers by crystallisation and reverse phase preparative HPLC were unsuccessful.

2.3. Synthesis of 4'-thio and 4'-sulfinylgemcitabine

In order to access 2-deoxy-2,2-*gem*-difluorothionucleosides, we returned to anomeric acetate **12** and mesylate **11** to investigate nucleobase attachment. Prior reports suggested use of an anomeric mixture of **11** delivered the corresponding nucleosides as a 1/1, α/β mixture, regardless of the anomeric ratio of the starting mesyl glycoside.[10,26] In order to target 4'-thiogemcitabine, silylated *N*^{4}-benozyl cytosine was first glycosylated with donor **11**, using TMSOTf as activator. These conditions were unsuccessful (no product was isolated) and $SnCl_4$ was selected instead. Under these conditions the desired nucleoside was isolated, but in <10% yield. Accordingly, we switched glycosyl donor to anomeric acetate **12** and were pleased to observe an improved yield for glycosylation of silylated *N*^{4}-benozyl cytosine, delivering **21** in 28% yield, with 25% of **12** recovered. Expectedly, no diastereocontrol was observed for this glycosylation; **21** was isolated as a 1.1/1 anomeric mixture, requiring subsequent separation either using fractional precipitation or preparative HPLC. Following protecting group removal, anomer **22**-α was confirmed through selective 1D NOESY, where through-space dipolar interactions between H_1 and H_3 were observed. (*Scheme 4* and SI Figure S5). Anomer **22-**β was then oxidised at sulfur to afford the 4'-sulfinyl derivative of 4'-thiogemcitabine. Oxidation was confirmed through a significant downfield shift of C1' and C4' from δ_C 59.7 and 46.3 ppm respectively in **22-**β to δ_C 84.6 and 72.8 ppm respectively in **23**.



Scheme 4: (a) *N*^{4}-benzoyl cytosine, HMDS, pyridine, $SnCl_4$, DCE, reflux, 28% (25% returned **12**), 1/1, α/β, then fractional precipitation, 10% for **21-α**, 6% for **21-β** (b) From **21-α/β**, NH_3, MeOH, 40 °C, 96% crude, then preparative HPLC, 44% for **22**-α, 34% for **22**-β; From **21-β**, NH_3, MeOH, 63% (c) *m*-CPBA, H_2O, MeCN, 15%, 4/1 d.r. at S.

2.4. Biological Evaluation

Nucleoside analogues **15**, **16**, **19**, **20**, **22-β** and **23** were evaluated in cytotoxicity assays against human pancreatic cancer (PANC-1) and human primary glioblastoma (U87-MG) cells and their CC_{50} values compared to commercial arabinocytidine (Ara-C) and gemcitabine (*Table 1*). Only analogue **19** displayed measurable cytotoxicity, with a CC_{50} value of 0.59 μM in U87-MG cells and 12 μM in PANC-1 cells (Entry 3, *Table 1*); all other analogues had CC_{50} values >200 μM. The data for **19** were not immediately comparable to Ara-C or gemcitabine, as both displayed superior CC_{50} values in the cell lines evaluated (Entries 1 and 2, *Table 1*). These results suggest that a thio*arabino* chemotype may impart some degree of cytotoxicity, but that replacement of furanose oxygen with sufinyl is not tolerated.

Entry	Compound	CC_{50} (μM) U87-MG	CC_{50} (μM) PANC-1
1	**Ara-C**	0.19	0.43
2	**Gemcitabine**	0.01	0.21
3	**19**	0.59	12.0

Table 1: Cytotoxicity data for **19**, Ara-C and Gemcitabine in U87-MG and PANC-1 cells.

3. Conclusions

We have developed a scalable and column chromatography free synthesis of a protected 4-thioribose intermediate. This synthetic methodology has also been adapted for a multigram preparation of 2-deoxy-2-*gem*-difluoro 4-thioribose analogues. These building blocks enable entry to a range of pyrimidine-based 4'-thionucleoside analogues, including first examples of 4'-sulfinyl derivatives of *ribo*cytidine, Ara-C and gemcitabine.

From the compounds synthesised, 4'-thio*arabino*cytidine was shown to have moderate cytotoxicity towards U87-MG cells, but generally all the thionucleosides evaluated showed little cytotoxicity. This is not unexpected, if conversion to an active triphosphate form is limited by substrate tolerance within nucleotide-forming kinase pathways.[27] Further work to access monophosphate prodrug forms of these analogues is currently underway and will be reported in due course.

4. Acknowledgements

Keele University and Riboscience LLC are thanked for PhD studentship funding to M. G. UK Research and Innovation (UKRI, Future Leaders Fellowship, MR/T019522/1) are thanked for project grant funding to G. J. M.

5. References

1 L. P. Jordheim, D. Durantel, F. Zoulim and C. Dumontet, *Nat. Rev. Drug. Discov.*, 2013, 12, 447–464.

2 C. Galmarini, J. Mackey and C. Dumontet, *Leukemia*, 2001, 15, 875–890.

3 M. Guinan, C. Benckendorff, M. Smith and G. J. Miller, *Molecules*, 2020, 25, 2050.

4 M. Meanwell, S. M. Silverman, J. Lehmann, B. Adluri, Y. Wang, R. Cohen, L.-C. Campeau and R. Britton, *Science*, 2020, 369, 725–730.

5 G. J. Miller, *Science*, 2020, 369, 623.

6 E. J. Reist, D. E. Gueffroy and L. Goodman, *J. Am. Chem. Soc.*, 1964, 86, 5658–5663.

7 M. Betson, N. Allanson and P. Wainwright, *Org. Biomol. Chem.*, 2014, 12, 9291–9306.

8 D. Dukhan, E. Bosc, J. Peyronnet, R. Storer and G. Gosselin, *Nucleosides, Nucleotides Nucleic Acids*, 2005, 24, 577–580.

9 Z.-H. Sun and B. Wang, *J. Org. Chem.*, 2008, 73, 2462–2465.

10 Y. Yoshimura, K. Kitano, K. Yamada, H. Satoh, M. Watanabe, S. Miura, S. Sakata, T. Sasaki and A. Matsuda, *J. Org. Chem.*, 1997, 62, 3140–3152.

11 Y. Yoshimura, K. Kitano, H. Satoh, M. Watanabe, S. Miura, S. Sakata, T. Sasaki and A. Matsuda, *J. Org. Chem.*, 1996, 61, 822–823.

12 Y. Yoshimura, Y. Saito, Y. Natori and H. Wakamatsu, *Chem. Pharm. Bull.*, 2018, 66, 139–146.

13 S. Dostie, M. Prévost, P. Mochirian, K. Tanveer, N. Andrella, A. Rostami, G. Tambutet and Y. Guindon, *J. Org. Chem.*, 2016, 81, 10769–10790.

14 J. M. Madern, T. Hansen, E. R. van Rijssel, H. A. V. Kistemaker, S. van der Vorm, H. S. Overkleeft, G. A. van der Marel, D. V. Filippov and J. D. C. Codée, *J. Org. Chem.*, 2019, 84, 1218–1227.

15 I. Birtwistle, P. Maddocks, J. M. O'Callaghan and J. Warren, *Synth. Commun.*, 2001, 31, 3829–3838.

16 T. Naka, N. Minakawa, H. Abe, D. Kaga and A. Matsuda, *J. Am. Chem. Soc.*, 2000, 122, 7233–7243.

17 K. Haraguchi, H. Kumamoto, K. Konno, H. Yagi, Y. Tatano, Y. Odanaka, S. S. Matsubayashi, R. Snoeck and G. Andrei, *Tetrahedron*, 2019, 75, 4542–4555.

18 I. O'Neil* and K. Hamilton, *Synlett.*, 1992, 1992, 791–792.

19 N. Nishizono, R. Baba, C. Nakamura, K. Oda and M. Machida, *Org. Biomol. Chem.*, 2003, 1, 3692–3697.

20 Z. W. Dentmon, T. M. Kaiser and D. C. Liotta, *Molecules*, 2020, 25, 5165.

21 F. Zheng, X.-H. Zhang, X.-L. Qiu, X. Zhang and F.-L. Qing, *Org. Lett.*, 2006, 8, 6083–6086.

22 M. Takahashi, S. Daidouji, M. Shiro, N. Minakawa and A. Matsuda, *Tetrahedron*, 2008, 64, 4313–4324.

23 Nakamura, K.; Shimamura, S.; Imoto, J.; Takahashi, M.; Watanabe, K.; Wada, K.; Fujino, Y.; Matsumoto, T.; Takahashi, M.; Okada, H.; et al. Intermediate for Synthesis of 1-(2-Deoxy-2-Fluoro-4-Thio-Beta-D-Arabinofuranosyl) Cytosine, Intermediate for Synthesis of Thionucleoside, and Methods for Producing These Intermediates. EP 2,883,866 A1, August 13, 2013.

24 H. Shirouzu, H. Morita and M. Tsukamoto, *Tetrahedron*, 2014, 70, 3635–3639.

25 M. Guinan, D. Lynch, M. Smith and G. J. Miller, in *Carbohydrate Chemistry: Proven Synthetic Methods Volume 5*, CRC Press, 2020, pp. 227–232.

26 K. Brown, M. Dixey, A. Weymouth-Wilson and B. Linclau, *Carbohydr. Res.*, 2014, 387, 59–73.

27 N. L. Golitsina, F. T. Danehy, R. Fellows, E. Cretton-Scott and D. N. Standring, *Antivir. Res.*, 2010, 85, 470–481.