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# The effect of deoxyfluorination and *O*-acylation on the cytotoxicity of *N*-acetyl-D-gluco- and D-galactosamine hemiacetals†

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Fully acetylated deoxyfluorinated hexosamine analogues and non-fluorinated 3,4,6-tri-*O*-acylated *N*-acetyl-hexosamine hemiacetals have previously been shown to display moderate anti-proliferative activity. We prepared a set of deoxyfluorinated GlcNAc and GalNAc hemiacetals that comprised both features: *O*-acylation at the non-anomeric positions with an acetyl, propionyl and butanoyl group, and deoxyfluorination at selected positions. Determination of the *in vitro* cytotoxicity towards the MDA-MB-231 breast cancer and HEK-293 cell lines showed that deoxyfluorination enhanced cytotoxicity in most analogues. Increasing the ester alkyl chain length had a variable effect on the cytotoxicity of fluoro analogues, which contrasted with non-fluorinated hemiacetals where butanoyl derivatives had always higher cytotoxicity than acetates. Reaction with 2-phenylethanethiol indicated that the recently described *S*-glycomodification is an unlikely cause of cytotoxicity.

## Introduction

Fluorinated carbohydrates have been long recognized as mechanistic probes and inhibitors for carbohydrate-processing enzymes and carbohydrate-binding proteins.[1] The similarity between fluorine and a hydroxyl group or hydrogen with respect to size has also stimulated research into the potential of fluorosugars to act as glycan chain modifiers by inhibiting carbohydrate-processing enzymes and/or by affecting the balance between carbohydrate metabolites.[1,2] While this research identified several potent metabolic inhibitors,[3–8] notably acetylated derivatives of (3*R*)-3-fluoro-*N*-acetylneuraminic acid[3,4] and 2-deoxy-2-fluoro-L-fucose,[3,5] it also revealed that some fluorosugars exhibited cytotoxicity, which complicated their use for remodelling cell-surface glycans, but opened the possibility of therapeutic use.[2] For example, fully acetylated derivatives of 3-deoxy-3-fluoro-*N*-acetyl-D-glucosamine (3F-$Ac_3$-GlcNAc **1**),[9] 4-deoxy-4-fluoro-*N*-acetyl-D-glucosamine (4F-$Ac_3$-GlcNAc **2**),[10] 4-deoxy-4-fluoro-*N*-acetyl-D-galactosamine (4F-$Ac_3$-GalNAc **3**),[10] and 4,6-dideoxy-4,6-difluoro-*N*-acetyl-D-galactosamine (4,6-diF-$Ac_2$-GalNAc **4**)[10] displayed moderate cytotoxicity against L1210 murine leukaemia cells ($IC_{50}$ 24–35 μM, Fig. 1). Their non-acetylated counterparts were inactive due to poor cellular permeability. Fully acetylated 6,6-difluoro-L-fucose (6,6-diF-$Ac_4$-Fuc **5**) and 6,6,6-trifluoro-L-fucose (6,6,6-triF-$Ac_4$-Fuc **6**) inhibited proliferation of the human colon cancer cells HCT116 ($IC_{50}$ 43 μM and 58 μM, respectively, Fig. 1).[11]

The cytotoxicity depended on the acetylation pattern.[8] For example, fully acetylated **2** showed very low cytotoxicity towards the human prostate cancer cell line PC-3 ($IC_{50}$ 277 μM). However, its hemiacetal (or lactol) derivative **7** with an unprotected anomeric hydroxyl (C1-OH) was cytotoxic ($IC_{50}$ 61 μM) whereas 1,3-di-*O*-acetylated derivative **8** and fully de-*O*-acetylated derivative **9** were inactive (Fig. 1, the inset).

We clearly observed enhancement of cytotoxicity towards PC-3 and ovarian A2780 cell lines when going from fully acetylated 3F-GalNAc analogue **10** to its hemiacetal **13** (Fig. 1). The cytotoxicity enhancement for hemiacetals **11** and **12** relative to their acetylated counterparts **1** and **3**, respectively, occurred with A2780 cells for **11** and PC-3 cells for **12**.[12] Lactol **7** was more cytotoxic to ovarian A2780 cells than fully acetylated **2** was.[12]

These results were consistent with the findings of the Yarema group who showed that non-fluorinated 3,4,6-tri-*O*-butanoyl derivatives of *N*-acetyl-D-glucosamine (GlcNAc) and

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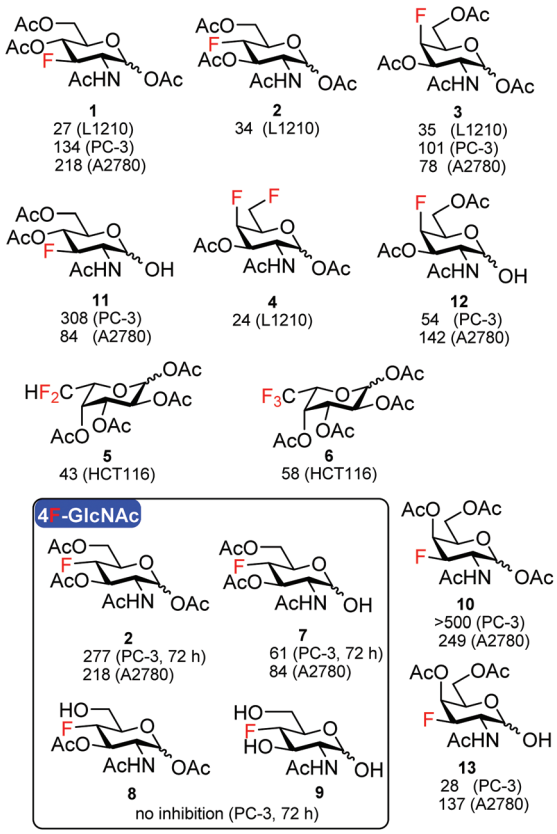



**Fig. 1** The structures and cytotoxicity expressed as $IC_{50}$ [μM] of cytotoxic acetylated fluorinated hexoses.[8,10–12] Cytotoxicity against PC-3 and A2780 cells were determined using 24 h exposure if not otherwise indicated.

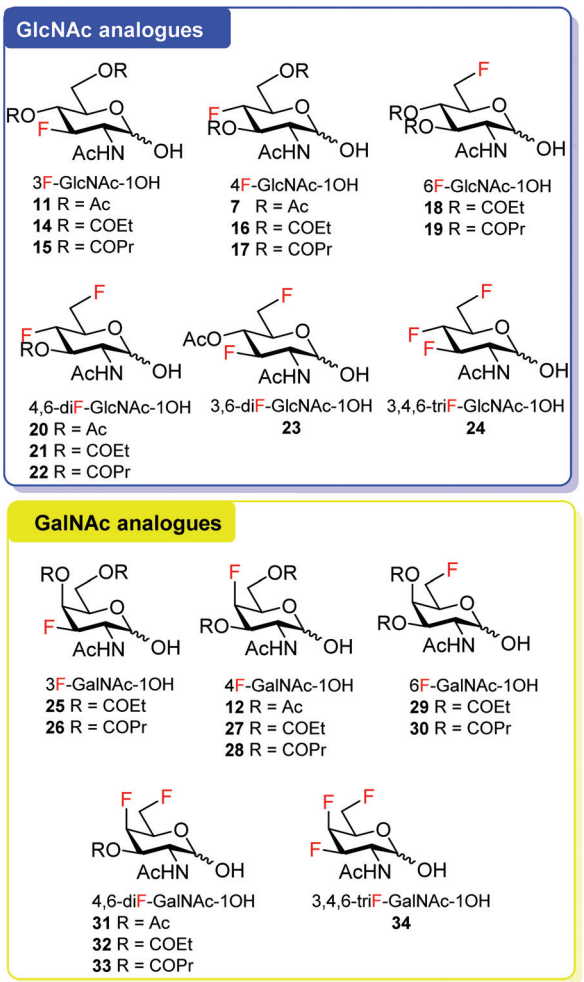



**Fig. 2** Structures of the target deoxyfluorinated analogues.

*N*-acetyl-D-mannosamine (ManNAc) hemiacetals were more cytotoxic toward MDA-MB-231 breast cancer cells and Jurkat cells (at the concentration range approximately 30–50 μM, $IC_{50}$ values were not given) than their fully *O*-butanoylated counterparts.[13] The corresponding 1,3,4-tri-*O*-butanoyl derivatives having an unprotected primary (O6) hydroxyl group were non-toxic. Lower cytotoxicity of the acetylated ManNAc hemiacetal relative to its butanoylated analogue was attributed to diminished cell membrane permeability.[13] Ensuing studies by the Yarema group confirmed the importance of the 3,4,6-tri-*O*-acyl pattern for the antiproliferative effect of non-fluorinated hexosamine hemiacetals.[14–16]

These results motivated us to investigate more systematically the cytotoxicity of hexosamine analogues that would combine structural features contributing to *in vitro* cytotoxicity: (1) deoxyfluorination, (2) an unprotected anomeric hydroxyl group, and (3) protection of the non-anomeric OH groups with short C2–C4 fatty acid esters. We hypothesized that by combining these structural features we might obtain fluoro analogues possessing cytotoxic activity in low micromolar range with potential utility in the development of cancer chemotherapy.

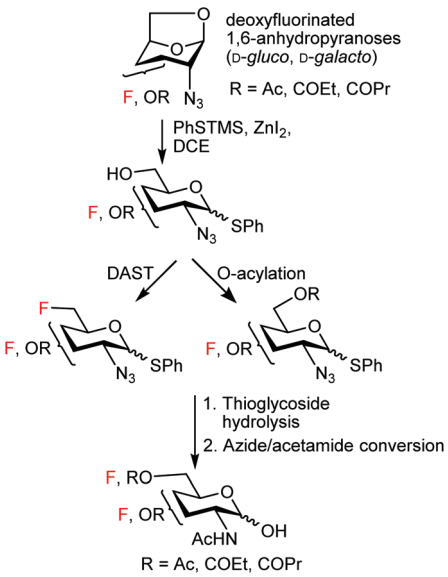
Therefore, we prepared a panel of mono-, di-, and trifluorinated GlcNAc and GalNAc analogues with an unprotected anomeric hydroxyl group and the remaining hydroxyl groups protected with acetyl, propionyl, or butanoyl (butyryl) esters (Fig. 2). We determined the *in vitro* cytotoxicity of these deoxyfluorinated hexosamine hemiacetals using the cancerous MDA-MB-231 cell line and non-cancerous HEK-293 cell line and compared it with the cytotoxicity of the corresponding acetylated and butanoylated non-fluorinated hemiacetals. Deoxyfluorination enhanced cytotoxicity in the majority of cases but it remained lower than that of the cisplatin benchmark. The influence of the ester alkyl chain length differed from the trend reported for non-fluorinated derivatives. The possibility that the cytotoxicity resulted from the recently described reaction of GlcNAc hemiacetals with the thiol group of cysteine residues[17] was investigated by reaction with 2-phenylethanethiol.

## Results

Apart from fluorine introduction, the main synthetic challenge was orthogonal protection of the anomeric position. Building on our previous results,[12,18,19] we designed a synthesis that relies on the use of a thiophenyl moiety for the temporary pro-

tection of the anomeric position and an azide as a masked 2-acetamido group. Synthesis of the majority of the target analogues follows the approach in Scheme 1. The C-2 azido group and C-3 and C-4 fluorine substituents have been introduced using 1,6-anhydropyranose chemistry as we previously described.[12] Opening of the internal acetal by treatment with phenyl trimethylsilyl sulfide/$ZnI_2$ introduced the thiophenyl moiety at the anomeric carbon and liberated the C-6 hydroxyl for acylation or deoxyfluorination.[18,20] Standard hydrolysis of the thioglycosidic bond and transformation of the azido group into an acetamido functionality completed the synthesis. The 6-fluoro analogues were prepared by modification of reported methods.[21,22] Selection of the deoxyfluorination and *O*-acylation patterns was partly the matter of synthetic convenience. We prepared 3-, 4-, and 6-monodeoxyfluorinated, 3,6- and 4,6-difluorinated, and 3,4,6-trifluorinated analogues shown in Fig. 2.

The synthesis is detailed in Scheme 2. The starting deoxyfluorinated 1,6-anhydro-2-azido-pyranoses **35–38** were prepared as previously described.[12] The *O*-acylation of the 3- and 4-positions provided compounds **39–49** (Scheme 2a). Cleavage of the internal acetal and installation of the thiophenyl moiety at C1 on reaction with PhSTMS/$ZnI_2$[19,20,23] yielded intermediates **50–60** as a mixture of anomers (Scheme 2b). 3-*O*-Acyl *gluco*-derivatives **53–55** produced a relatively high proportion of the α-anomer (α/β = 5.3 : 1, 4.5 : 1 and 3.8 : 1 for acetyl, propionyl and butyryl, respectively). Comparable α-selectivity (α/β = 6 : 1) was also reported by Demchenko *et al.* for a non-fluorinated counterpart of **53** ($R^2$ = OBn) and might indicate a remote participation of the O3-acyl in the acetal cleavage.[24] We attempted chromatographic separation of the thioglycoside anomers resulting from 1,6-anhydro bridge cleavage because the β-anomer was expected to undergo unwanted thiophenyl migration from C1 to C6 on reaction with diethylaminosulfur trifluoride (DAST) in the next step as reported by Lin *et al.*[25] The separation was possible for the *galacto*-configured products **56–60** and 4-fluoro *gluco*-derivatives **53–55**, giving fractions enriched in the α-anomer (α/β ≈ 10 : 1) but failed for 3-fluoro *gluco*-derivatives **50–52**.



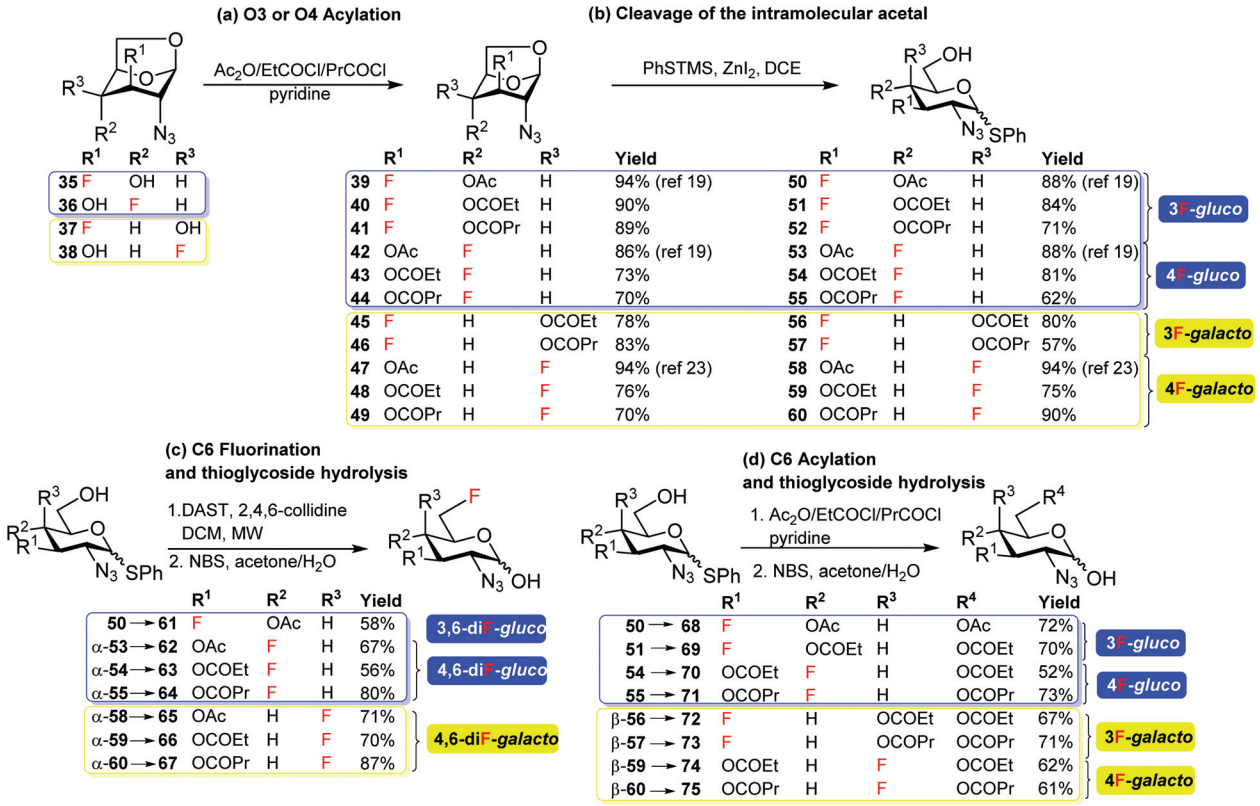

**Scheme 1** An overview of the synthesis of 3F, 4F, 3,6-diF, and 4,6-diF analogues.

The opening of the intramolecular acetal by reaction with PhSTMS was accompanied by the formation of low quantities (≤11% by $^{19}F$ NMR analysis) of side-products detectable by TLC very near the spot of the phenyl thioglycoside. Some of them (see ESI,† the synthesis of compounds **54–56**, and **59**) resulted from the pyranose ring-contraction discussed in our previous report.[26] Some of the side-products were only partially separable by chromatography, but were removed *via* thioglycoside hydrolysis in the subsequent steps.

The primary C6 hydroxyl of hexopyranoses **50**, **53–55**, and **58–60** was deoxyfluorinated by treatment with DAST under microwave conditions.[27] Treatment of the resulting difluoro derivatives with NBS in acetone/water effected thioglycoside hydrolysis and yielded hemiacetals **61–67** (Scheme 2c). The product of DAST-mediated migration of the β-configured thiophenyl moiety was not formed from the β-anomer of **50** in accordance with our previous findings about the O4-benzylated variant of **50** ($R^2$ = OBn).[26] As thioglycosides **53–55** and **58–60** were subjected to deoxyfluorination as the α-anomers only, thiophenyl migration could not occur. Thioglycosides **50**, **51**, **54–57** and **59–60** were O6-acylated and then hydrolysed at C1 to afford hemiacetals **68–75** (Scheme 2d). Hemiacetals **61–75** were subjected to azide/acetamide transformation by treatment with thioacetic acid in pyridine[28] to produce the target 2-acetamido hemiacetals **11**, **14**, **16–17**, **20–23**, **25–28** and **31–33** (Scheme 3). The products of this reaction were occasionally contaminated by traces of the anomeric *O*-acetates (see below formation of **87**/**88**, Scheme 4b) that were removed by chromatography and/or recrystallization.

Reversing the order of the last two steps and conducting azide/acetamide transformation prior to thioglycoside hydrolysis (as with thioglycosides **51** and **52**, Scheme 4a) was inconvenient because hydrolysis of the thiophenyl moiety vicinal to C2 acetamide in intermediates **76** and **77** was sluggish and incomplete as judged by thin-layer chromatography and the moderate yields (Scheme 4a).

The approach outlined in Scheme 1 was initially adopted also for the preparation of 6-fluoro D-*gluco* analogues. Thus, known 2-azido-4-*O*-benzyl-1,6-anhydropyranose **78**[29,30] (Scheme 4b) was O3-acylated, de-*O*-benzylated by treatment with $NaBrO_3/Na_2S_2O_4$,[31] and the liberated C4 hydroxyl was acylated to give di-*O*-acyl derivatives **81** and **82**. Compounds **81** and **82** were converted to hemiacetals **85** and **86** *via* thioglycosides **83** and **84** by reaction with PhSTMS, followed by deoxyfluorination at C6,[32] and hydrolysis at the anomeric position (Scheme 4b). The final step, however, was problematic because azide/acetamide transformation gave expected hemiacetals **18** and **19** in mixture with O1-acetylated products **87** and **88**, respectively, which were difficult to separate chromatographically. The structures of the inseparable C1-OAc by-products are supported by NMR assignment (see ESI†) and also by the



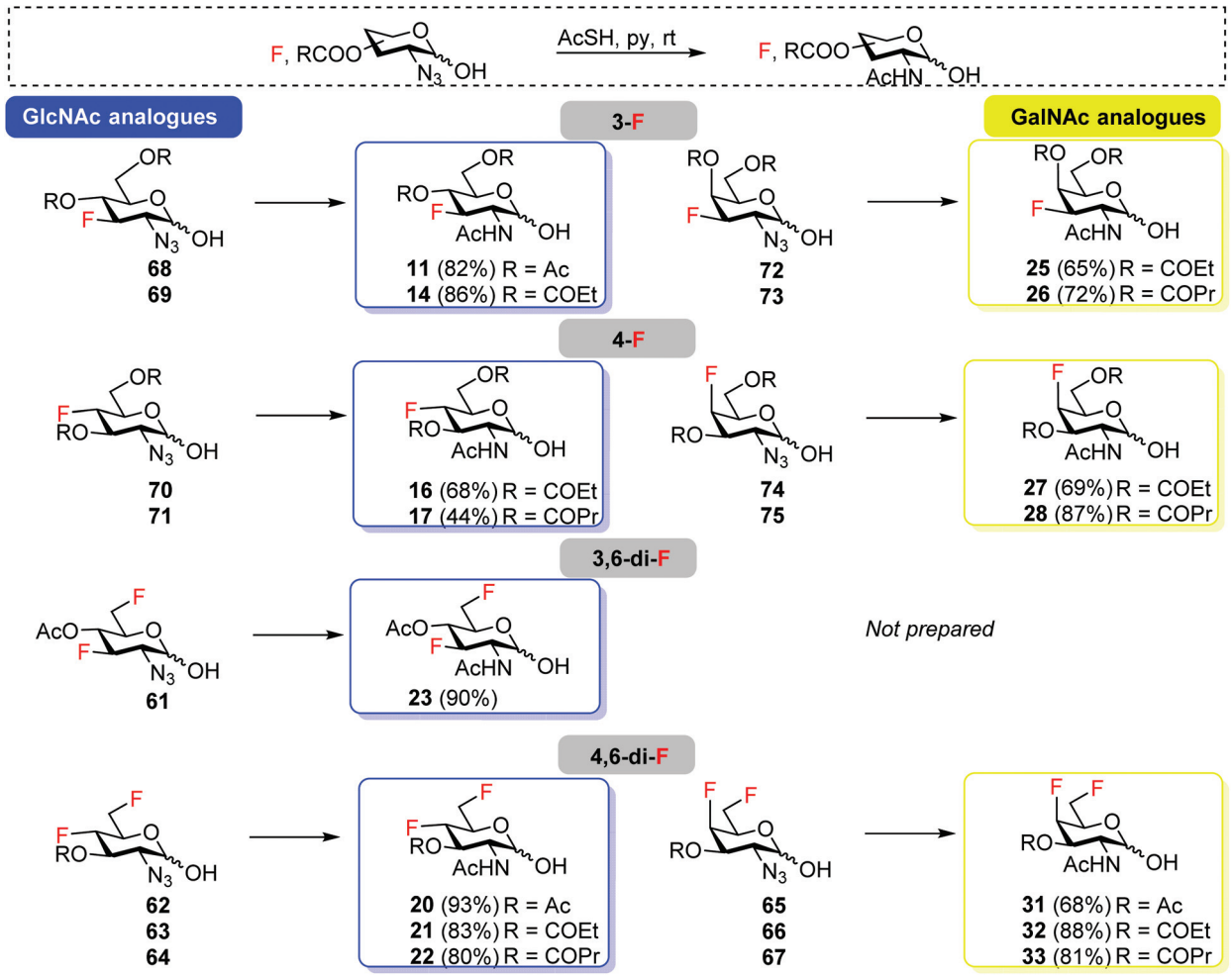

Scheme 2 Synthesis of deoxyfluorinated 2-azido precursors.

finding that a sample of compound **18** was converted to compound **87** by acetylation in pyridine.

Although the anomeric acetates **87** and **88** could probably be converted to desired hemiacetals by established methods,[33] we investigated an alternative route, in which reaction with thioacetic acid was avoided, and the anomeric centre was protected as a benzyl glycoside (Scheme 5A). Readily available benzyl 2-acetamido-D-glucopyranoside **89**[34] was *tert*-butyldiphenylsilylated at O6 and benzoylated at O3 and O4 in one step to give fully protected glucosamine **90**. Acid-catalysed desilylation[35] provided alcohol **91**. Compound **91** was first deoxyfluorinated under microwave conditions and the resulting 6-fluoro analogue **92** subjected to debenzoylation under Zemplén conditions to yield diol **93**. *O*-Acylation and hydrogenolytic debenzylation provided the desired target fluoro analogues **18** and **19** (Scheme 5A). Very recently, butane-2,3-diacetal protection was effectively applied to shorten a similar reaction sequence.[36] The 6-fluoro D-galacto analogues **29** and **30** were obtained from readily available benzyl 2-acetamido-galactoside **94**[37] by the synthetic sequence comprising deoxyfluorination with DAST to produce **95**, subsequent hydrolysis of the isopropylidene ketal to diol **96**, *O*-acylation, and hydrogenolytic debenzylation to yield hemiacetals **29** and **30** (Scheme 5B). A similar approach was utilized to prepare non-fluorinated derivatives used as reference compounds for the cytotoxicity study. Hence, known benzyl 2-acetamido-glucoside **89**[34] and 2-acetamido-galactoside **101**[38] were acylated at all available hydroxyls to produce the corresponding esters. Hydrogenolytic debenzylation afforded the target non-fluorinated acylated hemiacetals **99**, **100**, **102**, and **103**. (Scheme 5C) Monofluorinated acetylated derivatives **7** and **12** and trifluorinated analogues **24** and **34** were prepared by published procedures.[12,26]

The *in vitro* cytotoxicity of fluorinated analogues towards the MDA-MB-231 cell line derived from human triple negative breast adenocarcinoma and the human embryonic kidney HEK-293 cell line[39,40] was determined using MTT cell viability assay after 72 h treatment (Table 1) and expressed as $IC_{50}$ values. The MDA-MB-231 cell line was chosen because Yarema *et al.* reported its sensitivity to acylated hexosamine hemiacetals.[13] The HEK-293 cell line was chosen to investigate the effects of fluoro analogues on non-cancerous cells. Fluorinated analogues showed cytotoxicity in the range 15–92 μM, indicating that all of our analogues were less cytotoxic than the cisplatin benchmark (entry 29). Furthermore, there was no selectivity for cancerous MDA-MB-231 cells over HEK-293 cells. In fact, the HEK-293 cell line was often more sensitive to GlcNAc analogues than the MDA-MB-231 line was (*e.g.*, entries 13–15). Increasing the length of the ester alkyl chain increased the cytotoxicity of non-fluorinated hexosamines in accordance with the literature data (entries 1 *vs.* 2 and 16 *vs.* 17). However, this trend did not translate to fluorinated analogues because we did not observe the expected positive correlation between the length of the ester alkyl chain and cytotoxicity. The effect on cytotoxicity going from acetyl to propionyl esters depended




**Scheme 3** $N_3$ to NHAc transformation to obtain target fluoro analogues **11**, **14**, **16–17**, **20–23**, **25–28** and **31–33**.

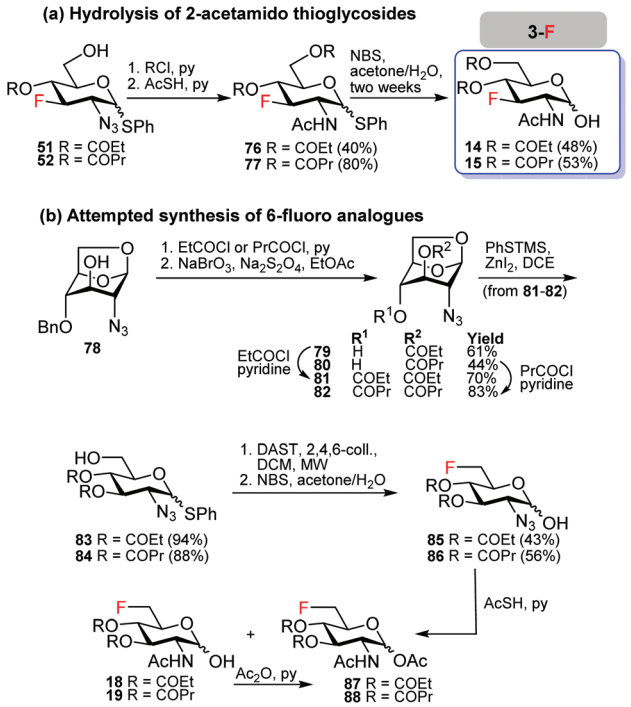
on the cell line (entries 12 *vs.* 13) and configuration (entries 6 and 7 *vs.* 20 and 21). Butanoylation, however, often decreased cytotoxicity, particularly in the MDA-MB-231 line (entries 7 *vs.* 8, 13 *vs.* 14, 18 *vs.* 19, 23 *vs.* 24, and 26 *vs.* 27).

Deoxyfluorination usually enhanced cytotoxicity relative to non-fluorinated hemiacetals, especially for fluorinated GalNAc analogues—all of these had lower $IC_{50}$ values than their non-fluorinated counterparts **102** and **103** (entries 16 and 17 *vs.* 18–28). The increase in cytotoxicity upon fluorination was pronounced for $Ac_3$-GalNAc-1-OH that was almost non-toxic in the MDA-MB-231 line (entry 16). The contribution of fluorination at the 4-position to cytotoxicity was usually superior to fluorination at the 6-position (entries 7 *vs.* 9, 21 *vs.* 23, and 22 *vs.* 24) and quite frequently also to fluorination at the 3-position (entries 12 *vs.* 11, 6 *vs.* 3, 22 *vs.* 19). Increasing the number of fluorine substituents improved cytotoxicity in about a half of the cases, and trifluorinated (entries 15 and 28) and some 4,6-difluorinated (*e.g.* entries 12 and 26) analogues belonged to the most cytotoxic compounds.

Since per-*O*-butanoylated ManNAc reduced cell migration,[41] we determined the inhibitory effect of analogues **15**, **20**, **30** and **32** on migration ability of MDA-MB-231 cells using a scratch assay.[42] Compounds **20** and **32** were chosen because of their good cytotoxicity whereas compounds **15** and **30** represented *O*-butanoyl analogues. None of the tested compounds exhibited anti-migratory properties in the scratch assay (data not shown).

We attempted to simulate membrane permeation of compounds **20–22** (4,6-diF-GlcNAc analogues), **31–33** (4,6-diF-GalNAc analogues), and **99** and **102** (acetylated non-fluorinated analogues) using the COSMOPerm[43] approach. Dipalmitoylphosphatidylcholine (DPPC) and dioleoylphosphatidylcholine (DOPC) were used as common model membranes. No clear correlation between the measured cytotoxicity and calculated permeation coefficients $\log P_{\mathrm{erm}}$ was found. The molecules tend to permeate freely through the chosen model membranes considering the free permeation threshold approximately $\log P_{\mathrm{erm}} = -8$ (see ESI† for details).

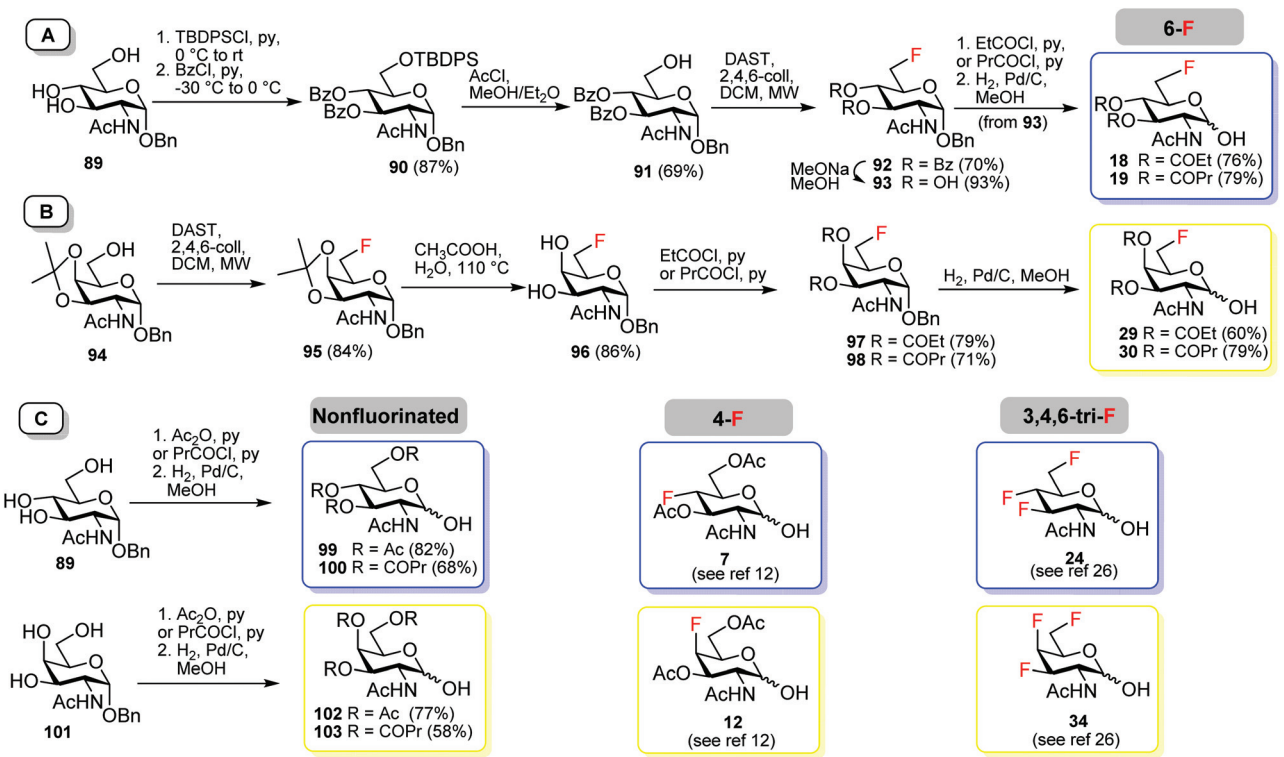
Qin *et al.* have recently suggested that 3,4,6-tri-*O*-acetylated GlcNAc non-specifically and non-enzymatically reacts with the thiol group of cysteine residues to produce proteins covalently labelled with thiolated amino sugars.[17] Only cysteine residues




Scheme 4 (a) Slow hydrolysis of 2-acetamido-thioglycosides **76** and **77**; (b) attempted synthesis of 6-fluoro analogues **18** and **19**.

located in a basic microenvironment of proteins rich in lysine residues are likely to react. This reaction, which was termed *S*-glyco-modification, was modelled by incubation of hemiacetal **99** with 2-phenylethanethiol under alkaline conditions (aqueous $Na_2CO_3$) for 1 h (Scheme 6A).[17] Following acetylation, a mixture of 3-deoxy-3-thio amino sugars was isolated in which compounds **104–106** were identified by NMR.[17] These products resulted from a conjugate addition of 2-phenylethanethiol to enals **108** and **109** followed by closure of the pyranose or furanose ring. Enals **108** and **109** interconvert by acetyl migration and were formed by E1cB elimination of acetic acid from the open chain form **107** of lactol **99**. In addition, base-catalysed deacetylation of **99** proceeded in parallel with elimination.[17]

We hypothesized that *S*-glyco-modification of proteins may contribute to the mechanisms by which our fluoro analogues exerted their cytotoxicity. However, cytotoxic difluorinated analogue **20** reacted with 2-phenylethanethiol much more slowly than **99** under conditions described in ref. 17, and no products were detectable by TLC after 1 h (Scheme 6B, conditions (a)). After 24 h, the NMR analysis indicated deacetylated analogue **112** as the main component together with a low quantity of the expected elimination-addition products **110** and **111** and other unidentified products (Scheme 6B, conditions (b)). Using sodium methanolate in methanol accelerated the formation of the thiolated products **110** and **111** (Scheme 6B, conditions (c)) so that they could be isolated in about 90% and 60% purity, respectively. Attempts to induce formation of **110** or **111** using excess $Et_3N$ in THF failed, most likely because the

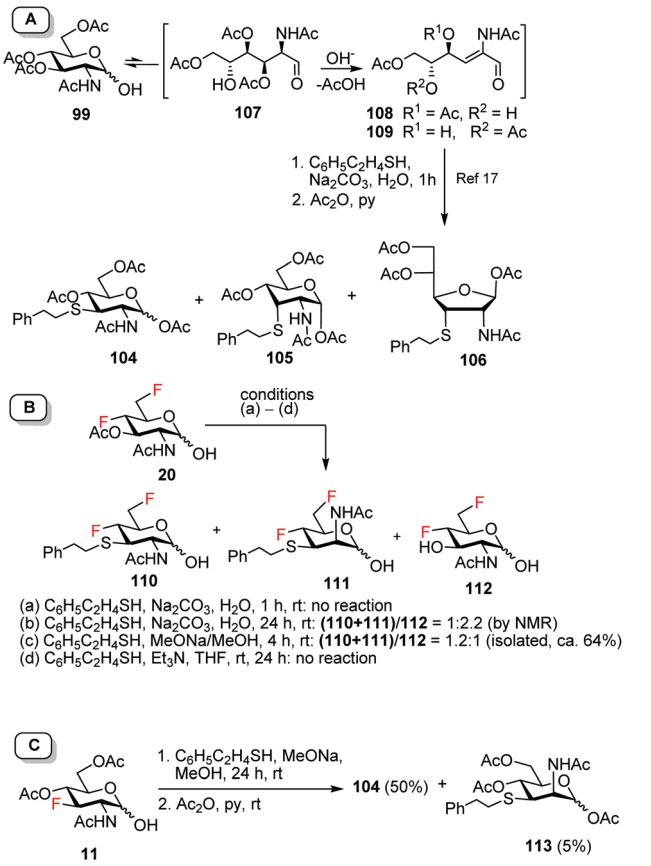



Scheme 5 (A) Synthesis of 6F-GlcNAc analogues **18** and **19**; (B) synthesis of 6F-GalNAc analogues **29** and **30**; (C) synthesis of non-fluorinated derivatives **99**, **100**, **102**, and **103**, and structures of the remaining fluorinated target analogues **7**, **12**, **24** and **34** prepared by known procedures.[12,26]

**Table 1** Cytotoxicity towards MDA-MB-231 and HEK-293 cell lines expressed as $IC_{50}$ [μM], obtained after 72 h treatment using the MTT assay

| Entry | Analogue | Substitution pattern | MDA-MB-231 | HEK-293 |
|---|---|---|---|---|
| **GlcNAc analogues** | | | | |
| 1 | **99** | $Ac_3$-GlcNAc-1-OH | 96 ± 10 | 61 ± 10 |
| 2 | **100** | $Bu_3$-GlcNAc-1-OH[a] | 49 ± 9 | 42 ± 2 |
| 3 | **11** | 3**F**-$Ac_2$-GlcNAc-1-OH | 49 ± 9 | 51 ± 7 |
| 4 | **14** | 3**F**-$Pr_2$-GlcNAc-1-OH[b] | 50 ± 7 | 39 ± 6 |
| 5 | **15** | 3**F**-$Bu_2$-GlcNAc-1-OH | 46 ± 6 | 21 ± 3 |
| 6 | **7** | 4**F**-$Ac_2$-GlcNAc-1-OH | 28 ± 7 | 30 ± 8 |
| 7 | **16** | 4**F**-$Pr_2$-GlcNAc-1-OH | 49 ± 2 | 27 ± 5 |
| 8 | **17** | 4**F**-$Bu_2$-GlcNAc-1-OH | 74 ± 5 | 44 ± 10 |
| 9 | **18** | 6**F**-$Pr_2$-GlcNAc-1-OH | 61 ± 13 | 46 ± 5 |
| 10 | **19** | 6**F**-$Bu_2$-GlcNAc-1-OH | 55 ± 11 | 52 ± 10 |
| 11 | **23** | 3,6-$\mathbf{F}_2$-Ac-GlcNAc-1-OH | 48 ± 9 | 47 ± 7 |
| 12 | **20** | 4,6-$\mathbf{F}_2$-Ac-GlcNAc-1-OH | 25 ± 10 | 33 ± 16 |
| 13 | **21** | 4,6-$\mathbf{F}_2$-Pr-GlcNAc-1-OH | 41 ± 7 | 17 ± 5 |
| 14 | **22** | 4,6-$\mathbf{F}_2$-Bu-GlcNAc-1-OH | 54 ± 3 | 19 ± 3 |
| 15 | **24** | 3,4,6-$\mathbf{F}_3$-GlcNAc-1-OH | 29 ± 7 | 15 ± 8 |
| **GalNAc analogues** | | | | |
| 16 | **102** | $Ac_3$-GalNAc-1-OH | 414 ± 130 | 123 ± 26 |
| 17 | **103** | $Bu_3$-GalNAc-1-OH | 136 ± 40 | 69 ± 17 |
| 18 | **25** | 3**F**-$Pr_2$-GalNAc-1-OH | 37 ± 13 | 35 ± 4 |
| 19 | **26** | 3**F**-$Bu_2$-GalNAc-1-OH | 61 ± 13 | 34 ± 6 |
| 20 | **12** | 4**F**-$Ac_2$-GalNAc-1-OH | 38 ± 4 | 50 ± 11 |
| 21 | **27** | 4**F**-$Pr_2$-GalNAc-1-OH | 27 ± 6 | 40 ± 4 |
| 22 | **28** | 4**F**-$Bu_2$-GalNAc-1-OH | 38 ± 3 | 42 ± 9 |
| 23 | **29** | 6**F**-$Pr_2$-GalNAc-1-OH | 60 ± 9 | 38 ± 5 |
| 24 | **30** | 6**F**-$Bu_2$-GalNAc-1-OH | 92 ± 12 | 43 ± 11 |
| 25 | **31** | 4,6-$\mathbf{F}_2$-Ac-GalNAc-1-OH | 61 ± 5 | 59 ± 11 |
| 26 | **32** | 4,6-$\mathbf{F}_2$-Pr-GalNAc-1-OH | 20 ± 4 | 30 ± 6 |
| 27 | **33** | 4,6-$\mathbf{F}_2$-Bu-GalNAc-1-OH | 54 ± 8 | 37 ± 12 |
| 28 | **34** | 3,4,6-$\mathbf{F}_3$-GalNAc-1-OH | 28 ± 3 | 26 ± 2 |
| 29 | **cisPt** | | 3.7 ± 0.6 | 3.8 ± 0.5 |

[a] Bu = butanoyl. [b] Pr = propionyl (in Table 1 only).




**Scheme 6** Reaction of 2-phenylethanethiol with (A) non-fluorinated hemiacetal **99**,[17] (B) 4,6-difluorinated analogue **20**, (C) 3-fluorinated analogue **11**.

interconversion between the cyclic and open-chain forms was very slow under aprotic conditions.

Acylated sugars are hydrolyzed by cytoplasmic esterases and the *S*-glyco-modification of proteins can only proceed as long as the ester at the 3-position remains intact because it functions as the leaving group in the elimination step. Fluorine at the 3-position can also act as a leaving group and, unlike an ester, cannot be readily hydrolyzed. Accordingly, reaction of 3-fluoro analogue **11** with the MeONa/MeOH/2-phenylethanethiol and subsequent acetylation mostly yielded the expected thiolated product **104** together with a low amount of *manno*-configured product **113**, both arising from elimination of HF and subsequent addition of 2-phenylethanethiol (Scheme 6C). Formation of thiolated *N*-acetyl-mannosamine derivatives **111** and **113** could also result from a base-catalysed C2 epimerization of glucosamine products **110** and **104**, respectively.[44,45] NMR analysis did not detect signals of previously identified compounds **105** and **106**.[17]

## Discussion

Derivatives based on the hexosamine scaffold display an array of useful bioactivities depending on the scaffold stereochemistry and protecting group pattern.[14,46–51] Replacing an ester with a fluorine was shown to bring new biological activity and ameliorate pharmacokinetic properties of hexosamines.[7,8,52–55] A subset of hexosamine hemiacetals carrying short fatty acid esters (C2 to C4) at the 3-, 4- and 6-positions and an amide at the 2-position are cytotoxic; some of them have other antitumor properties including antimetastatic effects.[41] The effect of fluorination on cytotoxicity of these derivatives have not yet been systematically studied except for 4-fluoro GlcNAc.[8,53] This is the first comparative study of cytotoxicity using a panel of deoxyfluorinated hexosamine lactols.[56] The use of 1,6-anhydropyranose chemistry for regio- and stereoselective installation of azide and fluorine at C2–C4 and a thiophenyl group at C1[12,18,19,57] was adapted in this report for the synthesis of selectively fluorinated, ester-protected hexosamine hemiacetals. This methodology is flexible and can be adjusted for the regioselective synthesis of fluorinated hexosamines carrying different acyl groups at preselected positions (including *N*-acyl) because these groups were installed sequentially at different stages of the synthesis. It should be noted that intermediate fluorinated 2-azido thioglycosides and hemiacetals obtained by this methodology are also valuable glycosyl donors for the synthesis of

glycoconjugates containing fluorinated glucosamine and galactosamine.[19,58]

Fluorination of 3,4,6-*O*-acylated GlcNAc/GalNAc moderately enhanced their cytotoxicity except for essentially non-cytotoxic 3,4,6-*O*-acetylated GalNAc where the increase in cytotoxicity upon fluorination was pronounced. The most cytotoxic fluoro analogues had $IC_{50}$ values approximately five-fold higher than cisplatin did. Although cisplatin was more cytotoxic in the tested cell lines, it also causes severe adverse effects in chemotherapy and has to be administered in low doses while fluoro sugars might be tolerated at higher concentrations. *In vivo* experiment will be necessary to investigate this possibility.

We confirmed that for non-fluorinated analogues, increasing the ester alkyl chain length from acetate to butanoate resulted in a parallel increase in cytotoxicity in accordance with the literature.[16] Improved efficiency of longer alkyl chains was attributed to improved diffusion through the cell membrane due to higher lipophilicity.[16] However, this trend did not operate for our fluorinated analogues and was reversed in the case of 4-fluoro GlcNAc and 4,6-difluoro GlcNAc analogues using MDA-MB-231 cell line (Table 1, entries 6–8 and 12–14). Recently Stephenson *et al.* observed an even more pronounced reversal of cytotoxicity of acetylated, propionylated, and butanoylated 4-fluoro GlcNAc hemiacetals **7**, **16** and **17**, respectively, against human neurons at 100 μM.[53] The authors suggested that excessive lipophilicity added by longer alkyls might have slowed down the diffusion from cell membrane to cytoplasm although it remains unclear why this argument should not also apply to non-fluorinated hemiacetals. Unlike neurons, astrocytes displayed good cell viability at a concentration of 100 μM of 4-fluoro GlcNAc analogue **7**.[53] This suggests that higher susceptibility of the HEK-293 cell line relative to MDA-MB-231 cells to the cytotoxic effect of our GlcNAc analogues could result from a close similarity between HEK-293 cells and neuronal cells.[39,59]

The 3,4,6-*O*-acetylated *N*-acyl-hexosamines can react with thiol groups of cysteine residues[17,49,60] by an elimination-addition mechanism (*S*-glyco-modification) as long as the 3-position remains *O*-acetylated.[17] An increased cysteine modification by 3,4,6-*O*-acetylated *N*-azidoacetyl-galactosamine ($Ac_3$-GalNAz) relative to the corresponding per-*O*-acetate ($Ac_4$-GalNAz) was reported to correlate with the increased cytotoxicity of $Ac_3$-GalNAz compared to $Ac_4$-GalNAz suggesting that *S*-glyco-modification was responsible for the cell death.[49] However, for the fluoro analogues tested in this work the extent of *S*-glyco-modification did not correlate with cytotoxicity provided that the reaction with 2-phenylethanethiol correctly modelled this process. The cytotoxic difluoro analogue **20** reacted with 2-phenylethanethiol sluggishly whereas the less cytotoxic non-fluorinated counterpart **99** was significantly more reactive. In addition, 3-fluoro analogue **11** was predominantly converted to 3-thio-products and therefore should be more cytotoxic than compound **20** that mostly gave a deacetylated product, whereas the opposite was true. Alternatively, compound **11** might have been quickly removed by cellular metabolism once the O4 and O6 esters were hydrolysed before it could react with cysteine thiol groups. Fluorinated sugars can indeed be metabolized. For example, 4-fluoro GlcNAc was converted to nucleotide sugar 4-fluoro-UDP-GlcNAc inside cells,[8] and Keenan *et al.* reported anomeric phosphorylation of some mono and multi-fluorinated hexopyranoses by the action of anomeric kinases.[61]

The lack of correlation between *in vitro* modelled *S*-glyco-modification and growth inhibition suggests that other mechanisms may contribute to the cytotoxicity of fluoro analogues. They can include the effect of fluorination on the membrane permeability or the rate at which the esters are hydrolyzed by cytosolic esterases. Furthermore, fluorinated hexosamines or products of their enzymatic conversions can trigger mechanisms leading to cell death by inhibiting key carbohydrate-processing enzymes or other critical targets.

## Conclusions

A set of 3,4,6-*O*-acylated mono-, di- and trifluorinated GlcNAc/GalNAc lactols has been prepared. The synthetic methodology allows for the synthesis of regioselectively protected multifluorinated 2-azido-thioglycosides having potential in the synthesis of fluorinated glycoconjugates. Cell-based assay showed that deoxyfluorination of acylated GlcNAc/GalNAc hemiacetals generated compounds cytotoxic against the MDA-MB-231 and HEK-293 cell lines, suggesting that they may also inhibit tumour growth *in vivo*. A follow-up *in vivo* study can decide whether the lower *in vitro* cytotoxicity compared to cisplatin is compensated by better tolerability. Model reaction with 2-phenylethanethiol indicated that cysteine modification is an unlikely mechanism leading to cell death. Glycoproteomic analysis is required to confirm this hypothesis. Future research can also focus on the investigation of the influence of fluorination on the lipophilicity and the possible correlation between the lipophilicity, membrane permeability and cytotoxicity using a $^{19}F$ NMR-based method for lipophilicity measurement.[62] Furthermore, $^{19}F$ NMR methods may be adapted to determine the rate of transmembrane transport of fluorinated analogues and give insight into their transformations inside cells.[63–67]

## Author contributions

Conceptualization: J.K. and V.H.; organic synthesis: V.H., J.K. and M.K.; spectroscopic characterization: L.C. and P.C.; tests of cytotoxicity: P.V. and R.H.; computation M.B.; article writing: J.K. with input from all authors.

## Conflicts of interest

There are no conflicts to declare.

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