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Research paper

Structure based design, synthesis and in vitro antitumour activity of tiazofurin stereoisomers with nitrogen functions at the C-2′ or C-3′ positions

Vesna Kojić [a], Mirjana Popsavin [b, *], Saša Spaić [b], Dimitar Jakimov [a], Ivana Kovačević [b], Miloš Svirčev [b], Lidija Aleksić [a], Bojana Srećo Zelenović [b], Velimir Popsavin [b, c]

[a] Oncology Institute of Vojvodina, Faculty of Medicine, University of Novi Sad, Put Dr Goldmana 4, 21204, Sremska Kamenica, Serbia
[b] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, Novi Sad, Serbia
[c] Serbian Academy of Sciences and Arts, Knez Mihajlova 35, 11000, Belgrade, Serbia

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ABSTRACT

Three novel tiazofurin analogues having D-*arabino* stereochemistry and nitrogen functionalities at the C-2′ position (**5**–**7**) have been designed and synthesized in multistep sequences, starting from D-glucose. The known D-*xylo* stereoisomer of **1** (compound **2**) along with two new analogues bearing nitrogen functions at the C-3′ (**3** and **4**) has also been synthesized from the same sugar precursor. The synthetic sequence consisted of the following three stages: (i) the multistep synthesis of suitably protected pentofuranosyl cyanides, (ii) the construction of ethyl thiazole-4-carboxylate part by cyclocondensation of thus obtained glycofuranosyl cyanides with L-cysteine ethyl ester followed by dehydrogenation, and (iii) the final transformation of the ethyl thiazole-4-carboxylates into the target tiazofurin analogues using the esters ammonolysis. The tiazofurin analogues were evaluated for their antitumour activities in cell-culture-based assays. Compounds **3**, **4** (D-*xylo*) and **7** (D-*arabino*), showed remarkable antitumour activities, with IC_{50} values in the range of 4–7 nM. Preliminary structure-activity relationship allowed identification of two analogues with antiproliferative activities exceeding that of the parent compound **1** for several orders of magnitude (e.g. **4**: 1354-fold against Raji, **7**: 309-fold against K562). Flow cytometry data and Western blot analysis suggested that cytotoxic effects of D-*xylo* stereoisomers in the culture of K562 cells caused changes in the cell cycle distribution, as well as the induction of apoptosis in caspase-dependent way. The increase of apoptotic cells percentage in treated samples is also confirmed with fluorescent double-staining method. Genotoxicity testing showed that the analogues with the *xylo*-configuration (**2**–**4**) are far less genotoxic than tiazofurin.

1. Introduction

C-Nucleosides represent a class of nucleoside analogues of significant biological interest. Their chemistry and biomedicinal potential has been reviewed [1–5]. *C*-Nucleoside tiazofurin (**1**, Fig. 1) and its selenium analogue selenazofurin (**1a**) are potent antitumour agents [6,7]. They are prodrugs, which are metabolically activated by a two-step sequence comprised from an initial phosphorylation to the corresponding 5′-monophosphates followed by their subsequent conversion to analogues of NAD (Scheme S1 in the Supplementary data), namely, thiazole-4-carboxamide adenine dinucleotide (TAD) and selenazole-4-carboxamide adenine dinucleotide (SAD). Both TAD and SAD potently inhibit inosine 5′-monophosphate dehydrogenase (IMPDH) that results in cancer cell death [6–9]. Due to its severe toxicity, selenazofurin was abolished from clinical testing, but tiazofurin proceeded to phase III clinical trials in patients with chronic myelogenous leukemia [6]. This led to the approval of **1** as an orphan drug for the treatment of patients with chronic myelogenous leukemia in blast crisis [6]. However, the lack of specificity and occasional toxicity found for tiazofurin during clinical trials [10–12] prompted the search for structural analogues, especially those with variations in the furanose ring. In this context, analogues with D-*xylo* and D-*arabino* configurations have

* Corresponding author.
E-mail address: mirjana.popsavin@dh.uns.ac.rs (M. Popsavin).

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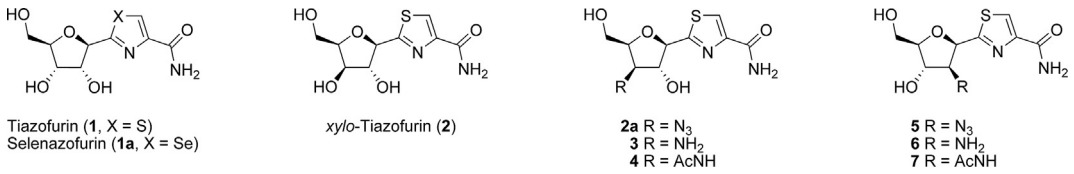


Fig. 1. Structures of tiazofurin (**1**, X = S), selenazofurin (**1a**, X = Se), *xylo*-tiazofurin (**2**), and analogues **2a**–**7**.

been prepared [13]. Their structural features [14] and antitumour properties [15] have been studied, but no significant antitumour activity was recorded in the cell assays. In the preliminary communication [16] we have described a novel synthesis of D-*xylo*-tiazofurin (**2**), as well as the re-investigation of its antiproliferative activity against a panel of human malignant cells. A remarkable cytotoxicity was recorded in the culture of several tumour cell lines, including K562 cells. Additionally, a D-*xylo*-*C*-nucleoside containing pyrrolo[2,1-f] [1,2,4]triazin-4-amine has been recently synthesized and shown to exhibit a strong antiproliferative activity against a panel of tumour cell lines [17].

Based upon these observations, we wanted to prepare new D-*xylo* (**2**–**4**) and D-*arabino* tiazofurin analogues (**5**–**7**) with nitrogen functionalities at the C-2′ or C-3′ position (N_3, NH_2 and NHAc). Furthermore, some D-*ribo*-tiazofurin analogues with similar nitrogen functions at the C-2′ position have shown interesting activities in the cultures of certain tumour cells [18]. Finally, we were intrigued by the possibility of applying a molecular design approach using the available X-ray crystallographic data of human enzyme IMPDH (type II) in complex with SAD (PDB code 1B3O) [19] to produce novel IMPDH inhibitors.

Herein, we wish to report structure based design and full details of the synthesis of D-*xylo* tiazofurin (**2**) [16], its isosteres **3** [20] and **4** [21], as well as the attempted synthesis of azido derivative **2a**. We also disclosed the synthesis of three novel tiazofurin analogues having D-*arabino* stereochemistry and nitrogen functionalities at the C-2′ position (**5**–**7**). Their effects onto the proliferation of some malignant cell lines will also be discussed in details.

2. Results and discussion

2.1. Chemistry

Our approach to design of novel inhibitors of IMPDH included an initial inspection of the active site within the X-ray crystal structure of the enzyme containing the bound inhibitor (SAD), which suggested that it may be possible to change of the $C_{2'}$–$C_{3'}$ sugar portion of the ligand whilst still retaining binding due to the presence of the ligand/protein contacts within the remaining part of the molecule (Fig. 2).

The active site was further analyzed using the Discovery Studio Visualizer (DSV) [22] molecular design programme. This revealed the presence of a proton donating region within the binding cavity (Fig. 2, the central pink region) which was not being utilized for binding of the ligand. Further analysis using DSV indicated that the binding of the SAD analogues could be increased by placing the proton acceptor groups above the $C_{2'}$–$C_{3'}$ sugar region. This means that D-*xylo* and D-*arabino* stereoisomers of tiazofurin, with proton acceptor functions at the C-2′ or C-3′ positions can bind to the enzyme by means of additional attractive interactions, which would result in stronger antitumour effects. It was further assumed that analogues **2**–**7** can be metabolically converted to the corresponding SAD analogues **8a** or **9a** (Scheme S2 in the Supplementary data), which can be bound to the active centre of the enzyme. To check this assumption we performed molecular docking of

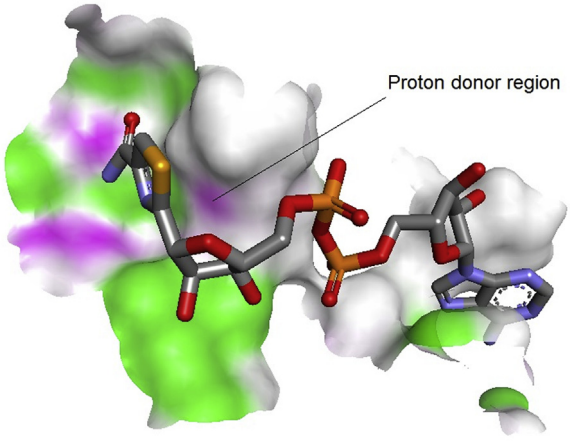


Fig. 2. SAD in the active site of IMPDH (binding site of NAD+) with regions suitable for the formation of H-bonds (purple: proton donor regions, green: proton acceptor regions). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

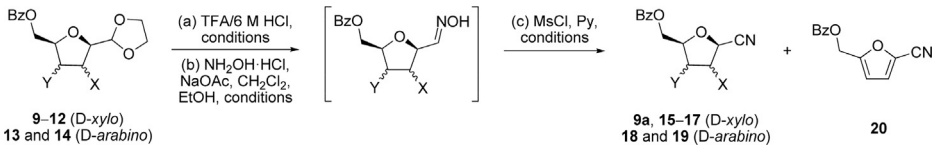
compound **8a** to the NAD^+ binding site of human type II IMPDH (1B3O). Conformationally rigid ligand **8a** (see Fig. S2 in the Supplementary data) and two neighbouring subunits of IMPDH with five flexible amino acid residues in the active site (Asp274, Thr252, Ser275, Ser276 and Met325) were used as input files for molecular docking. Indeed, the docking analysis revealed the existence of an additional hydrogen bond between the amino group at the C-3′ position of the ligand and NH group of Ser276 from the peptide backbone, which additionally stabilizes the potential inhibitor **8a** in the active site (for details see, the Supplementary data). These results encouraged us to synthesize analogues **2**–**7** and to evaluate their antitumour properties.

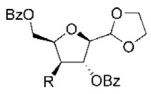
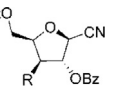
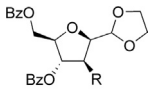
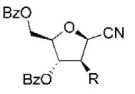
Our strategy for the synthesis of target *C*-nucleosides **2**–**7** involves the preparation of suitably protected pentofuranosyl cyanides **9b** and **15**–**19** (Table 1), followed by their cyclocondensation with L-cysteine ethyl ester, and by dehydrogenation [23] whereby the protected *C*-nucleosides should be obtained. The final step of the sequences will include ester ammonolysis of intermediary ethyl thiazole-4-carboxylates into the corresponding tiazofurin analogues **2**–**7**. The preparation of initial intermediates **9**–**13** is shown in Scheme 1.

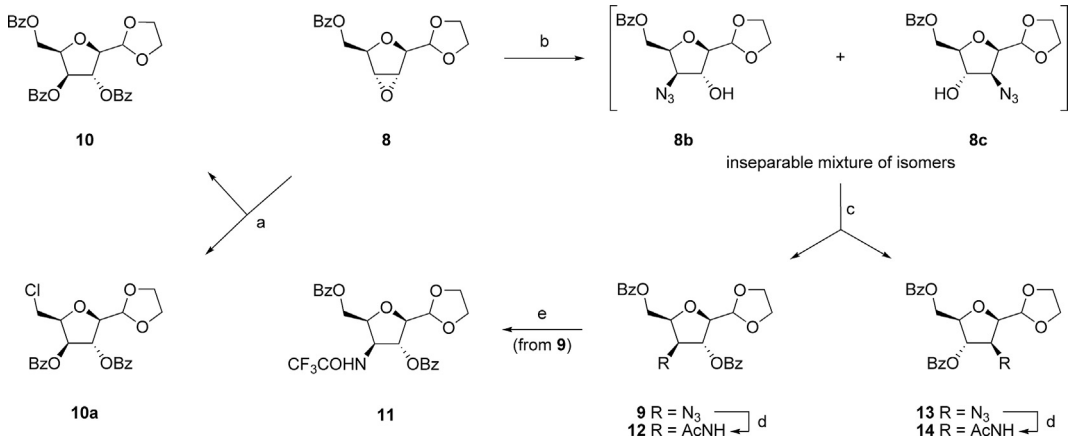
Since this synthesis is described in detail in our preliminary communication [20], it will not be further commented. However, the corresponding experimental details are given in the Supplementary data.

Preparation the desired pentofuranosyl cyanides **15**–**19** is shown in Table 1. It is a three-step sequence that commenced with the hydrolytic removal of acetal ring followed by subsequent aldehyde oximination and the final oxime dehydration. In addition to the required pentofuranosyl cyanides, this sequence provides a variable quantity of furan **20**, mainly formed as a side product during the oxime dehydration reactions. Unfortunately, in the case of D-*gulo* isomer **9** (entry 1) elimination product **20** was obtained as

Table 1
Preparation of 2,5-anhydro-D-aldononitriles **15**–**19**.



Entry	Starting compound		Conditions	Products (yield, %)	
1	 D-*xylo*	**9** R = N_3	(a) +4 °C, 24 h, rt, 24 h; (b) rt, 5 h; (c) −18 °C, 0.5 h, then rt, 2 h.	 D-*xylo*	**9b** R = N_3 (0) **20** (30)
2		**10** R = OBz	(a) +4 °C, 168 h; (b) rt, 2 h; (c) −15 °C, 45 min, then rt, 2 h.		**15** R = OBz (59), **20** (5)
3		**11** R = $NHCOCF_3$	(a) +4 °C, 70 h; (b) rt, 2 h; (c) −18 °C, 144 h, then rt, 1 h.		**16** R = $NHCOCF_3$ (59) **20** (4)
4		**12** R = NHAc	(a) +4 °C, 168 h; (b) rt, 2 h, +4 °C, 70 h; (c) −15 °C, 0.5 h, then rt, 2 h.		**17** R = NHAc (79), **20** (traces)
5	 D-*arabino*	**13** R = N_3	(a) +4 °C, 161 h; (b) rt, 6 h; (c) −15 °C, 45 min, then rt, 2 h.	 D-*arabino*	**18** R = N_3 (41) **20** (6)
6		**14** R = NHAc	(a) +4 °C, 140 h; (b) rt, 2 h; (c) −15 °C, 0.5 h, then rt, 2 h.		**19** R = NHAc (45) **20** (5)



Scheme 1. Reagents and conditions: (a) (i) $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 46 h; (ii) BzCl, Py, rt, 4 days, 36% of **10**, 29% of **10a**; (b) NaN_3, DMSO, 108−112 °C, 26 h; (c) BzCl, Py, rt, 24 h, 36% of **13** (from **8**), 44.5% of **9** (from **8**); (d) H_2−PtO_2, Ac_2O, AcOH, rt, 21 h for **9**, 57% of **12**, 48 h for 13, 56% of **14**; (e) (i) H_2−Pd/C, EtOH, rt, 24 h; (ii) TFAA, Py, CH_2Cl_2, −10 °C for 0.5 h, then +4 °C for 72 h, 62%.

the only reaction product. No desired nitrile **9b** could be detected in the reaction mixture. Due to the unfavourable outcome of this reaction, we were forced to temporarily abandon the synthesis and biological testing of isostere **2a**. The remaining reactions (entries 2−6) took place successfully giving generally good yields of the desired nitriles **15** (59%), **16** (59%), **17** (79%), **18** (41%) and **19** (45%).

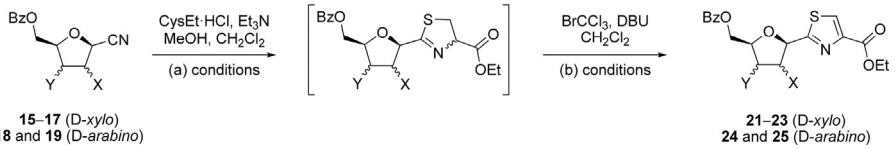
Having obtained desired 2,5-anhydro-D-aldononitriles **15**–**19**, we next focused on their conversion to protected *C*-nucleosides **21**–**25** (Table 2) using the two-step sequence that includes cyclocondensation of glycofuranosyl cyanides with L-cysteine ethyl ester, followed by dehydrogenation of the resulting thiazolines with $BrCCl_3$ in the presence of DBU. This procedure provided acceptable yields of protected *C*-nucleosides **21** (31%), **22** (39%), **23** (50%), **24** (56%) and **25** (58%). Arabinofuranosyl nitriles **18** and **19** gave somewhat higher yields of the corresponding C-nucleosides **24** and **25** (entries 4 and 5), when compared with xylofuranosyl series (entries 1 and 2). Although generally lower, the yields of xylofuranosyl derivatives **21** and **22** are acceptable.

The protected *C*-nucleosides **22** and **23** were alternatively prepared using a two-step sequence comprised from previous conversion of nitriles **16** and **17** into the corresponding xylofuranosyl thioamides, followed by their subsequent cyclocondensation with ethyl bromopyruvate using a modified Hantzsch thiazole synthesis protocol [24] (for details see the Supplementary data). The desired intermediates **22** and **23** were obtained in similar overall yields, independently of procedures used for their preparation.

The final transformation of the ethyl thiazole-4-carboxylates **21**–**25** into the corresponding thiazole-4-carboxamides **2**–**5** and **7** are shown in Table 3. The ammonolysis of D-*xylo* isomers **21** and **23** (entries 1 and 3) and D-*arabino* isomers **24** and **25** (entries 4 and 5) with saturated ammonia in MeOH selectively cleaved only ester groups to afford good yields of target *C*-nucleosides **2** (89%), **4** (85%), **5** (85%) and **7** (58%). The acetamido functionalities in both compounds **23** and **25** remained unchanged under these reaction conditions (entries 3 and 5). However, the amide C−N bond in trifluoroacetamido derivative **22** was easily cleaved under the applied ammonolysis conditions, to afford the corresponding 3-amino derivative **3** in 69% yield (entry 2).

The isomeric 2′-amino *C*-nucleoside **6** was prepared starting

Table 2
Conversion of 2,5-anhydro-D-aldononitriles **15**–**19** to protected *C*-nucleosides **21**–**25**.



15–**17** (D-*xylo*)
18 and **19** (D-*arabino*)

21–**23** (D-*xylo*)
24 and **25** (D-*arabino*)

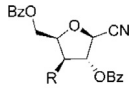
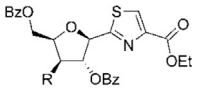
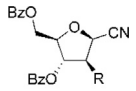
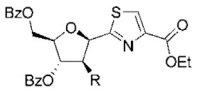
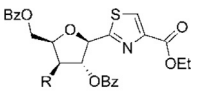
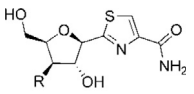
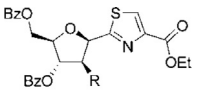
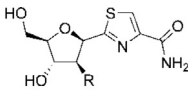
Entry	Starting compound		Conditions	Product (yield, %)	
1	 D-*xylo*	**15** R = OBz	(a) rt, 2 h; (b) 0 °C, 5 h, +4 °C, 66 h.	 D-*xylo*	**21** R = OBz (31)
2		**16** R = NHCOCF$_3$	(a) rt, 3.5 h; (b) 0 °C, 5 h, +4 °C, 24 h.		**22** R = NHCOCF$_3$ (39)
3		**17** R = NHAc	(a) rt, 4 h; (b) 0 °C 5 h, +4 °C, 24 h.		**23** R = NHAc (50)
4	 D-*arabino*	**18** R = N_3	(a) rt, 4 h; (b) 0 °C, 5 h, +4 °C, 43 h.	 D-*arabino*	**24** R = N_3 (56)
5		**19** R = NHAc	(a) rt, 2 h; (b) +4 °C, 16 h.		**25** R = NHAc (58)

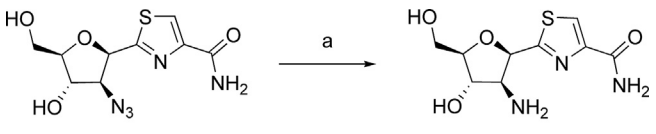
Table 3
Conversion of protected *C*-nucleosides **21**–**25** to tiazofurin analogues **2**–**5** and **7**.

21–**23** (D-*xylo*)
24 and **25** (D-*arabino*)

2–**4** (D-*xylo*)
5 and **7** (D-*arabino*)

Entry	Starting compound		conditions	Products (yield, %)	
1	 D-*xylo*	**21** R = OBz	rt, 6 days	 D-*xylo*	**2** R = OH (89)
2		**22** R = NHCOCF$_3$	rt, 8 days		**3** R = NH_2 (69)
3		**23** R = NHAc	rt, 8 days		**4** R = NHAc (85)
4	 D-*arabino*	**24** R = N_3	rt, 4 days	 D-*arabino*	**5** R = N_3 (85)
5		**25** R = NHAc	rt, 5 days		**7** R = NHAc (58)

from 2′-azido-2′-deoxy *C*-nucleoside **5**. Thus, catalytic hydrogenation of **5** over 10% Pd/C, gave the desired amino derivative **6** in 82% yield (Scheme 2).



5 **6**

Scheme 2. Reagents and conditions: (a) H_2—Pd/C, EtOH, rt, 48 h, 82%.

2.2. Antiproliferative activities and SAR

Cytotoxicities of synthesized analogues were recorded in the cultures of six human tumour cell lines (K562, HL-60, Jurkat, Raji, HT-29 and HeLa) and a single normal cell line (MRC-5) employing MTT assay [25]. The cells were treated with test compounds for 72 h [26]. The usage of MRC-5 cells serves to demonstrate the selectivity of synthesized compounds toward tumour cells. Doxorubicin (DOX) and tiazofurin (**1**) were used as reference compounds. The results are presented in Table 4.

D-*xylo*-Tiazofurin (**2**) was more potent than DOX against two cell lines (K562, 2-fold; HL-60, 7-fold) and more active than tiazofurin

Table 4
In vitro cytotoxicity of tiazofurin (**1**) and analogues **2**–**7** after 72 h.

Compounds	IC_{50} (μM)[a]						
	K562	HL-60	Jurkat	Raji	HT-29	HeLa	MRC-5
1 (tiazofurin)	1.89	0.19	0.04	5.28	0.26	3.82	0.36
2	0.12[b]	0.13[b]	0.53[b]	3.11[b]	>100	0.08[b]	>100[b]
3	0.035	9.39	0.0036	3.85	0.005	0.012	>100
4	0.34	3.77	0.0072	0.0039	0.26	>100	42.35
5	6.28	7.55	>100	40.52	2.48	29.87	>100
6	1.97	1.02	0.04	1.03	23.45	1.03	>100
7	0.0061	12.86	>100	2.56	>100	>100	3.21
DOX	0.25	0.92	0.03	2.98	0.15	0.065	0.10

[a] IC_{50} is the concentration of compound required to inhibit the cell growth by 50% compared to an untreated control. Values are means of three independent experiments. Coefficients of variation were less than 10%.
[b] Taken from Ref. [16].

(**1**) against four cell lines (K562, HL-60, Raji, HeLa; between 2 and 48 fold). Amino derivative **3** exhibited highly potent activities toward K562, Jurkat, HT-29 and HeLa cells in low nanomolar range being more potent than both DOX (5–30 fold) and **1** (11–318 fold). It is worth pointing out that cytotoxicity of compound **3** towards Jurkat cells was the highest recorded in this assay (IC_{50} 3.6 nM). Acetamido-derivative **4** is more potent inhibitor of growth of two and three tumour cell lines compared to DOX and to tiazofurin, respectively. It showed particularly potent activity in the culture of T-cells leukemia (Jurkat, IC_{50} 7.2 nM) exhibiting the 5- and 4-fold higher potency than **1** and DOX, respectively. This compound was remarkably potent toward Burkitt's lymphoma (Raji, IC_{50} 3.9 nM) being 1353- and 764-fold more active than lead **1** and DOX, respectively. D-*arabino*-Derivative **5** showed the modest activity toward K562, HL-60 and HT-29 cells. Compound **6** was the most cytotoxic against Jurkat cells where it exhibited the similar activity as DOX and lead **1**. Acetamido derivative **7** exhibited a low nanomolar activity towards myelogenous leukemia cells (K562, IC_{50} 6.1 nM), with 309-fold higher potency than **1** and 40-fold higher potency than DOX. Moreover, compound **7** was more active than DOX and **1** in the culture of Burkitt's lymphoma cells.

Conclusively, it is important to highlight that in contrast to tiazofurin (**1**) and DOX, compounds **2**, **3**, **5** and **6** are not toxic against normal foetal fibroblasts (MRC-5), while compound **4** showed modest activity against MRC-5 cells (IC_{50} 42.35 μM). This could imply their potential selectivity toward tumour cells, but it remains to be further investigated toward wider panel of normal cells. Only compound **7** is toxic against these cells (IC_{50} 3.21 μM), being almost 9-fold higher than **1** (IC_{50} 0.36 μM) and 32-fold higher than DOX (IC_{50} 0.1 μM).

The next step in our work was establishing correlations between the structures of synthesized compounds and their antitumour activity in an attempt to identify structural features beneficial for potency. In order to investigate the influence of configuration on activity, first we compared activity of tiazofurin (**1**) with *xylo* analogues **2**–**4** (see the Supplementary data for details, Fig. 1Sa). The results showed that compounds with *xylo*-configuration **2**–**4** were more active than **1** toward the majority of tumour cell lines. However, comparison of IC_{50} values of tiazofurin (**1**) and *arabino* analogues **5**–**7** (Fig. S1b) showed that change to *arabino*-configuration lead to decrease of potency. These results indicate that *xylo*-configuration is more beneficial for antiproliferative activity.

Further, we considered substituent influence at C-3′ of *xylo*-series (Fig. S1c) and at C-2′ of *arabino*-derivatives (Fig. S1d) on antitumour activity. The data revealed that *xylo* compounds bearing amino (**3**) and acetamido (**4**) group at C-3′ were more active than corresponding compound with OH group (**2**) against at least 50% of cell lines, and amino-derivative **3** was more potent than NHAc analogue **4** toward majority of investigated tumour cell lines. Similarly, in the *arabino* series, the presence of amino group at C-2′ influenced increase in potency compared to 2′-azido (**5**) and 2′-NHAc (**7**) derivatives. Based upon these observations, it appears that the amino group has the most beneficial effects on antitumour properties of this type of *C*-nucleosides.

2.3. Cell cycle analysis

The cell cycle profile of exponentially growing K562 cells treated with synthesized compounds for 24 and 72 h was analyzed by flow cytometry in cells stained with propidium iodide. Untreated cells served as a control. The results are presented in Fig. 3.

In order to determine the antiproliferative effect of synthesized analogues of tiazofurin, their effect on the cell cycle of K562 cells was studied using flow cytometry.

All compounds of *xylo*-configuration reduce the percentage of cells in the G2/M phase; after 72 h, this percentage is similar to controls and 2–2.4 times greater than the values obtained for tiazofurin (**1**). Compounds **2** and **3** induce significant apoptosis of K562 cells (subG1 fraction), as well as tiazofurin (**1**), after 72 h (Fig. 3). The formation of the subG1 peak originates from a population of cells with fragmented DNA molecules. This hypodiploid DNA content is the basic characteristic of the cells that entered the apoptosis, and the population of these cells is clearly distinguished in the form of subG1 peak on an one-parameter histogram of the cell cycle.

After exposure of the K562 cells to compounds of *arabino*-configuration (**5**, **6** and **7**) for 72 h, all the investigated compounds and tiazofurin (**1**) increase the percentage of apoptotic cells (subG1 peak) with a reduced percentage of cells in G0/G1 phase relative to control, while the percentage of cells in the S phase is almost the same as after treatment with tiazofurin (**1**). The percentage of cells in the G2/M phase for all investigated compounds, other than tiazofurin, is similar to control (Fig. 3).

2.4. Detection of apoptosis

Percentage of apoptotic, necrotic and live cells was determined with flow cytometry after staining of treated cells with Annexin V-FITC/PI and the results are shown in Fig. S3 (see also Table S2 in the Supplementary data).

The apoptotic response, presented as a percentage of specific apoptosis (Fig. 3B), shows that analogues **3**, **6** and **7** after 72 h multiply the percentage of Annexin-V positive K562 cells compared to tiazofurin. Analogue **3** was the most active (34.37%), while **6** and **7** caused a 2-fold higher percentage of specific apoptosis than tiazofurin (**1**).

The percentage of specific necrosis after 72 h for analogues **2**, **3** and **4** was between 14% and 21%, while for other analogues it was slightly lower (6–10%) with respect to tiazofurin (Fig. 3B).

2.5. Western blot analysis

Western blot analysis of samples treated with compounds **2**–**7** (Table 5 and Fig. S6 in the Supplementary data) shows that analogue **5** after 72 h most reduced the expression of the anti-apoptotic Bcl-2 protein relative to control and to **1**, while the analogues **2**, **3**, **4**, **6** and **7** increased the total amount of Bcl-2 protein relative to control. When compared to tiazofurin, the expression of Bcl-2 is higher only for analogues **2** and **6**.

The expression of BAX protein was increased after 72 h treatment by all the tested substances. These results are consistent with measured increase in specific apoptosis after 72 h.

The expression level of Caspase 3 precursor and its active subunit in cells exposed to the action of tiazofurin and its analogues **2**–**7** were determined to verify whether these compounds induced apoptosis by activating the enzyme Caspase 3. Western blot analysis showed the expression of Caspase 3 precursor in K562 cells treated with all of the examined analogues (**2**–**7**). However, the expression level of Caspase 3 active subunit was different, depending on the nature of analogues. A lower effect on the Caspase 3 content in cells after 72-h treatment was observed in tested substances **4** and **7**.

Western blot analysis showed that the proteolytic cleavage of PARP protein in K562 cells after treatment with all analogues depends on the analogue structure. Among others, the analogue **3** (D-*xylo*-configuration) induces the lowest PARP cleavage.

2.6. Double fluorescent staining

Morphological features present in apoptotic cells can be

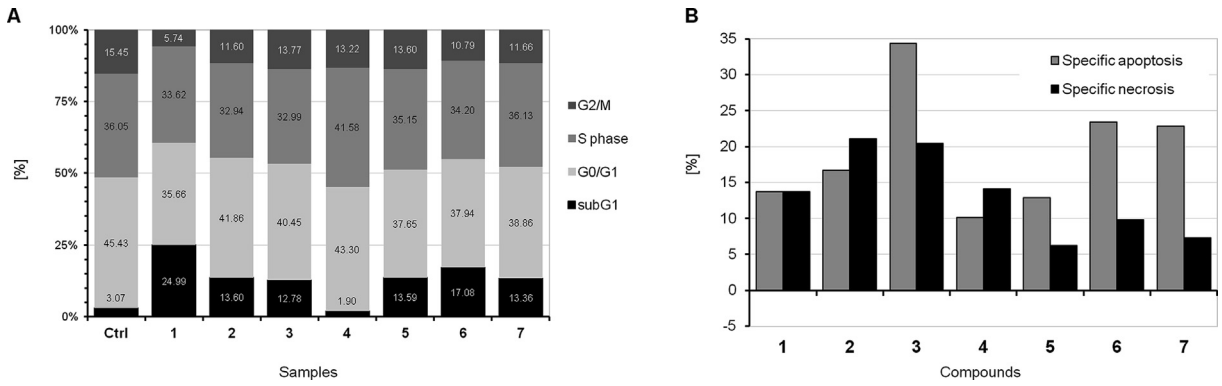


Fig. 3. (A) Results of cell cycle phases distribution for control sample (Ctrl) and samples treated with analogues **1**–**7** for 72 h, obtained by flow cytometry. (B) Specific apoptosis and necrosis calculated from the results obtained by Annexin-V flow cytometry after cells treatment for 72 h.

Table 5
Results of Western blot analysis.

	% of control (72 h)[a]					
	Bcl-2	BAX	Caspase3 precursor (32 kDa)	Caspase3 active subunit (18 kDa)	PARP (112 kDa)	PARP (85 kDa)
control	100.00	100.00	100.00	100.00	100.00	100.00
1	344.33	338.37	1302.79	89.69	299.68	214.99
2	436.51	180.18	4433.80	230.09	665.40	552.68
3	164.90	240.62	1465.64	172.46	84.76	104.95
4	274.16	126.68	432.96	87.05	336.99	364.31
5	35.23	186.25	3222.35	156.25	355.91	169.74
6	414.66	193.00	1144.41	153.73	387.73	570.91
7	149.59	141.43	777.09	59.32	477.15	534.18

[a] For data recorded after 24-h of cells treatment see the Supplementary data.

visualized by staining with appropriate selective dyes. The loss of membrane integrity of cells is detectable with fluorescent double staining, when one of the fluorescent dyes enters the cell freely (acridine orange) and the other (ethidium bromide) is excluded. In late apoptosis, both dyes entered the cell and the nuclei are stained orange or red.

Presence of apoptotic cells was evident in all samples (Fig. S5 in the Supplementary data). Induced apoptotic changes by tested compounds were quantified and compared by the ratio of the red and green colour intensity. The results are presented in Fig. 4A.

The obtained results confirm that tested compounds induce apoptotic changes in K562 cells. Tiazofurin (**1**) has the highest impact. Among the tested analogues of D-*xylo* configuration, compound **2** was the most active.

2.7. Comet assay

Finally, we used the alkaline method of comet assay [29] to evaluate DNA damage of K562 cells after treatment with synthesized compounds. Comet assay is based on electrophoresis of nucleic acids from a single cells immobilized in agarose layer, where the DNA fragments cross a distance in an electric field depending on their mass, leaving a trail (comet) after staining with fluorescent dyes. Comet Tail Moment is taken as a parameter for evaluating DNA fragmentation.

Results of the comet assay for samples treated with the tested derivatives are shown in Fig. 4B. Tiazofurin (**1**) was the most genotoxic compound to the K562 sells. Analogues of D-*xylo* configuration (**2**–**4**) caused less than half damage to the DNA molecule than tiazofurin.

In addition to assessing genotoxicity, the results of comet assay can be considered also as an indicator of cell apoptosis, since the DNA fragmentation and degradation is one of the stages in this complex process.

3. Conclusions

Six novel tiazofurin mimics (**2**–**7**) bearing nitrogen functionalities at the C-2′ or C-3′ position have been designed and synthesized in multistep sequences starting from D-glucose. The synthetic strategy to targets **2**–**7** assumed the initial multistep synthesis of suitably protected pentofuranosyl cyanides, the construction of ethyl thiazole-4-carboxylate ring by cyclocondensation of thus obtained glycofuranosyl cyanides with L-cysteine ethyl ester followed by dehydrogenation. Final exposure of the resulting protected *C*-nucleosides to methanolic ammonia provided targets **2**–**7** in good yields.

Synthesized compounds were evaluated for their antiproliferative activity against a panel of human malignant cell lines. The most active compounds that displayed low nanomolar activities from D-*xylo* series are: 3′-amino derivative **3** (IC_{50} 0.0036 μM, Jurkat and 0.005 μM, HT-29) and 3′-acetamido derivative **4** (IC_{50} 0.0039 μM, Raji and 0.0072 μM, Jurkat). The only D-*arabino* isomer that exhibited a nanomolar activity against K562 cells is 2′-acetamido derivative **7** (IC_{50} 0.0061 μM). Unfortunately, this compound showed a potent cytotoxicity against the normal cell line, MRC-5. A brief SAR analysis has shown that D-*xylo* configuration increases antiproliferative activity, while D-*arabino* stereochemistry reduces the activity of the analogues against the majority of cell lines under evaluation. All tested compounds increased the percentage of apoptotic cells, which is confirmed with the consistent results from flow cytometric cell cycle analysis, Western blot analysis of apoptotic protein expression and fluorescent double-staining method. Genotoxicity testing showed that the analogues of D-*xylo* configuration (**2**–**4**) are far less genotoxic than tiazofurin.

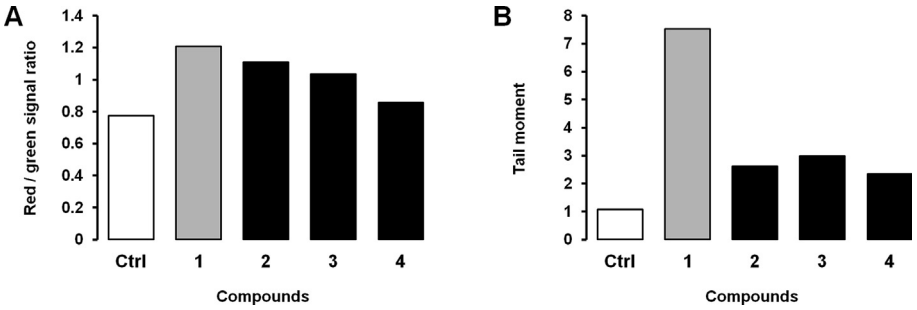


Fig. 4. (A) Results of the double-fluorescence test for control sample (Ctrl) and samples treated with derivatives **1**–**4** for 72 h, obtained by analysis of red and green channel density ratio in the *ImageJ* computer program. (B) The quantified values of the comet tail moment for control (Ctrl) and treated samples (**1**–**4**, 72 h) obtained by the *CometScore* computer program.

Due to their potent nanomolar activities, compounds **3**,**4** and **7** have serious potential as possible antitumour drugs. This is especially true for analogue **3**, which is completely inactive against the normal cell line (MRC-5). However, in order to prove such a claim, additional in vivo tests must be done, followed by detailed clinical trials.

4. Experimental section

For general experimental procedures and for the preparation of initial intermediates **9**—**14**, see the Supplementary data.

*4.1. General procedure for the synthesis of glycofuranosyl cyanides **15**—**19***

A solution of acetal **9**—**14** (1 equiv) in a 4:1 mixture of TFA/6 M HCl (0.2—0.3 M) was kept at +4 °C for 2—7 days. The mixture was concentrated to a third of the initial volume and poured into saturated aq $NaHCO_3$. The aqueous solution was rendered alkaline with solid $NaHCO_3$ to pH 8—9 and extracted with CH_2Cl_2. The combined extracts were washed successively with saturated aq $NaHCO_3$ and water, dried and evaporated. The remaining crude aldehydes were immediately dissolved in a mixture of EtOH/CH_2Cl_2 (0.2—0.3 M) and treated with NaOAc (~3 equiv) and $NH_2OH \times HCl$ (~2 equiv) while stirring at room temperature for 2—6 h. The mixture was evaporated and the residue distributed between water and CH_2Cl_2. The organic layer was separated and the aqueous phase extracted with CH_2Cl_2. The combined organic solutions were washed with water, dried and evaporated to afford crude oximes, as mixtures of the corresponding *E*- and *Z*-isomers. To a cooled (−15 to −18 °C) and stirred solution of crude oximes in anhydrous pyridine (~0.2 M) was added dropwise during 0.5 h a cold solution of MsCl (~4 equiv) in dry pyridine (1 M). The mixture was first stirred at −15 °C for 0.5 h, then at room temperature for 2 h and then poured into a 1:1 mixture of ice and concentrated HCl (pH ~ 2). The emulsion was extracted with CH_2Cl_2, the combined extracts were washed with water, saturated aq $NaHCO_3$ and again with water. The extract was dried and evaporated, and the residue was purified by flash column chromatography or by preparative TLC.

*4.1.1. (2,3,5-Tri-O-benzoyl-β-D-xylofuranosyl)cyanide (**15**)*

Acetal **10** (0.450 g, 0.93 mmol) was converted into crude **15** according to the above general procedure. Pure **15** (0.114 g, 59%), was isolated by flash column chromatography (4:1 cyclohexane/Me_2CO) as a colourless oil, $[\alpha]_D^{23} = +34.6$ (*c* 0.2; $CHCl_3$), $R_f = 0.55$ (9:1 toluene/EtOAc).

IR (film): ν_{max} 1725 (C=O), 1263 (C—O).

^{1}H NMR (250 MHz, $CDCl_3$): δ 4.69 (d, 2 H, $J_{4,5} = 5.9$ Hz, 2 × H-5), 4.88 (m, 1 H, $J_{4,5} = 5.9$, $J_{3,4} = 3.9$ Hz, H-4), 4.97 (br s, 1 H, H-1), 5.79 (d, 1 H, $J_{2,3} = 3.7$ Hz, H-2), 6.01 (dd, 1 H, $J_{2,3} = 3.7$, $J_{3,4} = 3.9$ Hz, H-3), 7.37—8.19 (m, 15 H, 3 × Ph).

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 61.75 (C-5), 70.69 (C-1), 74.97 (C-2), 79.96 (C-3), 80.39 (C-4), 115.82 (C≡N), 127.89, 128.02, 128.34, 128.46, 128.66, 129.23, 129.68, 129.92, 130.11, 133.23, 133.99 and 134.16 (3 × Ph), 164.59, 164.73 and 165.95 (3 × PhC=O).

HRMS (ESI): *m/z* 472.1380 (M^++H), calcd for $C_{27}H_{22}NO_7$: 472.1391.

A minor amount of unreacted starting compound **10** (0.0475 g, 11%) was recovered.

*4.1.2. (2,5-Di-O-benzoyl-3-deoxy-3-trifluoroacetamido-β-D-xylofuranosyl)cyanide (**16**)*

Acetal **11** (0.450 g, 0.93 mmol) was converted into crude **16** according to the above general procedure. Pure **16** (0.114 g, 59%), was isolated by flash column chromatography (4:1 cyclohexane/Me_2CO) as a colourless oil, $[\alpha]_D^{23} = +20.5$ (*c* 1.3, $CHCl_3$), $R_f = 0.55$ (20:1 CH_2Cl_2/EtOAc).

IR (film): ν_{max} 3330 (NH), 1726 (C=O, ester), 1662 (amide I band), 1554 (amide II band).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.63 (dd, 1 H, $J_{5a,5b} = 12.6$, $J_{4,5a} = 4.0$ Hz, H-5a), 4.72 (dd, 1 H, $J_{5a,5b} = 12.6$, $J_{4,5b} = 4.2$ Hz, H-5b), 4.79—4.85 (m, 2 H, $J_{1,2} = 3.8$, $J_{3,4} = 5.5$ Hz, H-1 and H-4), 5.00 (ddd, 1 H, $J_{3,NH} = 7.6$, $J_{2,3} = 3.8$, $J_{3,4} = 5.5$ Hz, H-3), 5.75 (t, 1 H, $J_{1,2} = J_{2,3} = 3.8$ Hz, H-2), 7.44—8.16 (m, 10 H, 2 × Ph), 7.76 (d, 1 H, $J_{3,NH} = 7.6$ Hz, NH).

^{13}C NMR (100 MHz, $CDCl_3$): δ 56.2 (C-3), 61.8 (C-5), 70.0 (C-1), 78.5 (C-4), 80.0 (C-3), 115.4 (q, *C*F_3, $^1J_{C,F} = 287.6$ Hz), 116.0 (C≡N), 127.6, 128.7, 128.72, 128.8, 129.0, 129.8, 130.1, 133.8, 134.4 (2 × Ph), 157.8 (q, $^2J_{C,F} = 38.6$ Hz, *C*OCF_3), 165.5 and 166.2 (2 × PhC=O).

LRMS (CI): *m/z* 463 (M^++H).

*4.1.3. (3-Acetamido-2,5-di-O-benzoyl-3-deoxy-β-D-xylofuranosyl) cyanide (**17**)*

Compound **12** (0.620 g, 1.39 mmol) was converted into crude **17** according to the above general procedure. Pure **17** (0.409 g, 72% from 3 steps, 79% calculated to reacted **12**), was isolated by flash chromatography (7:3 toluene/EtOAc) as a colourless oil, $[\alpha]_D^{23} = +20.7$ (*c* 2.0, $CHCl_3$), $R_f = 0.54$ (7:3 toluene/EtOAc).

IR (film): ν_{max} 3283 (NH), 1723 (C=O, ester), 1661 (amide I band), 1541 (amide II band).

^{1}H NMR (250 MHz, $CDCl_3$): δ 2.04 (s, 3 H, CH_3CO), 4.61 (m, 2 H, 2 × H-5), 4.72 (m, 2 H, $J_{1,2} = 3.4$, $J_{3,4} = 5.2$ Hz, H-2 and H-4), 5.02 (m, 1 H, $J_{1,2} = 3.4$ Hz, $J_{3,NH} = 7.9$ Hz, H-4), 5.66 (t, 1 H, $J_{1,2} = J_{2,3} = 3.4$ Hz, H-3), 6.98 (d, 1 H, $J_{3,NH} = 7.9$ Hz, NH), 7.39—8.14 (m, 10 H, 2 × Ph).

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 22.8 (*C*H_3CO), 55.3 (C-3), 62.4 (C-5), 70.1 (C-1), 79.1 (C-4), 80.7 (C-2), 116.4 (C≡N), 127.9, 128.1, 128.4, 129.1, 129.6, 129.8, 133.3 and 134.0 (2 × Ph), 165.3 and 166.0 (2 × PhC=O), 170.8 (NHCOCH$_3$).

HRMS (ESI): *m/z* 409.1389 (M^++H), calcd for $C_{22}H_{20}N_2O_6$: 409.1394.

A minor amount of unreacted starting compound **12** (0.054 g, 9%) was recovered.

4.1.4. (2-Azido-3,5-di-O-benzoyl-2-deoxy-β-D-arabinofuranosyl) cyanide (**18**)

Acetal derivative **13** (1.362 g, 3.10 mmol) was converted into crude **18** according to the above general procedure. Pure product **18** (0.464 g, 41% calculated to reacted **13**) was isolated by preparative TLC (49:1 toluene/EtOAc, developed twice) as colourless crystals, mp 110 °C (CH_2Cl_2/hexane), $[\alpha]_D^{23} = -20.7$ (*c* 0.5, $CHCl_3$), $R_f = 0.59$ (9:1 toluene/EtOAc).

IR (KBr): ν_{max} 2262 (C≡N), 2148 (N_3), 1721 (C=O).

^{1}H NMR (250 MHz, $CHCl_3$): δ 4.48 (m, 1 H, H-4), 4.55 (dd, 1 H, $J_{1,2} = 5.1$, $J_{2,3} = 2.4$ Hz, H-2), 4.61 (dd, 1 H, $J_{5a,5b} = 12.0$, $J_{4,5a} = 5.3$ Hz, H-5a), 4.67 (dd, 1 H, $J_{5a,5b} = 12.0$, $J_{4,5b} = 5.2$ Hz, H-5b), 4.96 (d, 1 H, $J_{1,2} = 5.1$ Hz, H-1), 5.54 (dd, 1 H, $J_{3,4} = 3.4$ Hz, $J_{2,3} = 2.4$ Hz, H-4), 7.38–8.16 (m, 10 H, 2 × Ph).

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 63.0 (C-5), 66.1 (C-2), 69.2 (C-1), 77.8 (C-3), 82.1 (C-4), 114.2 (C≡N), 120.0, 128.2, 128.3, 128.5, 129.6, 129.65, 133.1 and 133.9 (2 × Ph), 165.1 and 165.9 (2 × Ph*C*=O).

HRMS (ESI): *m/z* 393.1212 (M^++H), calcd for $C_{20}H_{17}N_4O_5$: 393.1194.

Anal. Found: C, 60.54; H, 4.14; N, 14.13. Calcd for $C_{20}H_{16}N_4O_5$: C, 61.22; H, 4.11; N, 14.28.

A minor amount of unreacted starting compound **13** (0.089 g, 6.5%) was recovered.

4.1.5. (2-Acetamido-2-deoxy-3,5-di-O-benzoyl-β-D-arabinofuranosyl)cyanide (**19**)

Compound **14** (0.413 g, 0.91 mmol) was converted into crude **19** according to the above general procedure. Pure **19** (0.166 g, 45% from **14**) was obtained after purification by flash column chromatography (8:3 light petroleum/Me_2CO) as a colourless syrup. Crystallization from CH_2Cl_2/hexane gave an analytical sample **19**, as colourless crystals, mp 113–114 °C, $[\alpha]_D^{23} = -18.5$ (*c* 1.3, $CHCl_3$), $R_f = 0.54$ (1:1 toluene/EtOAc).

IR (film): ν_{max} 3282 (NH), 1731 (C=O, ester), 1667 (amide I band), 1539 (amide II band).

^{1}H NMR (400 MHz, $CDCl_3$): δ 2.00 (s, 3 H, C*H*$_3$CO), 4.49 (m, 1 H, $J_{4,5} = 6.8$ Hz, H-5), 4.63 (dd, 1 H, $J_{5,6a} = 4.6$, $J_{6a,6b} = 12.3$, Hz H-6a), 4.67 (dd, 1 H, $J_{6a,6b} = 12.3$, $J_{5,6b} = 4.2$ Hz, H-6b), 4.89 (m, 1 H, $J_{3,NH} = 6.3$, $J_{3,4} = 6.8$, $J_{2,3} = 6.9$ Hz, H-3), 5.27 (d, 1 H, $J_{2,3} = 6.8$ Hz, H-2), 5.64 (t, 1 H, $J_{3,4} = 6.8$, $J_{2,3} = 6.8$ Hz, H-4), 7.15 (bs, 1 H, NH), 7.34–8.10 (m, 10 H, 2 × Ph).

^{13}C NMR (100 MHz, $CDCl_3$): δ 22.8 (*C*H$_3$CO), 56.7 (C-3), 63.2 (C-6), 69.6 (C-2), 75.6 (C-4), 80.1 (C-5), 115.7 (C≡N), 128.2, 128.5, 128.7, 129.3, 129.8, 129.9, 133.5 and 134.1 (2 × Ph), 166.2 and 166.3 (2 × Ph*C*=O), 171.2 (NH*C*OCH$_3$).

Anal. Found: C, 62.99; H, 4.95; N, 6.63. Calcd for $C_{22}H_{20}N_2O_6 \times 0.5H_2O$: C, 63.25; H, 5.03; N, 6.71.

4.2. General procedure for the conversion of glycofuranosyl cyanides **15**–**19** to the protected C-nucleosides **21**–**25**

To a solution of glycofuranosyl cyanides **15**–**19** (1 equiv) in a mixture of absolute MeOH and CH_2Cl_2 (0.04–0.05 M) was added L-cysteine ethyl ester hydrochloride (1.5–1.8 eq) and dry Et_3N (1.5–1.8 eq). The mixture was stirred at room temperature (2–4 h) and then evaporated. The residue was distributed between CH_2Cl_2 and water, the organic layer was separated washed with saturated aq $NaHCO_3$ and brine, then dried and evaporated. To a cooled (0 °C) and stirred solution of crude thiazolines in dry CH_2Cl_2 (0.1 M) were added DBU (~2 eq) and $BrCCl_3$ (~1.2 eq). The solution was left at +4 °C until the starting materials were consumed (TLC). The mixture was evaporated, and the residue was purified by flash column chromatography or by preparative TLC.

4.2.1. Ethyl 2-(2,3,5-tri-O-benzoyl-β-D-xylofuranosyl)thiazole-4-carboxylate (**21**)

Compound **15** (0.2850 g, 0.60 mmol) was converted to crude **21** (0.3532 g) according to the above general procedure. Purification by preparative TLC (17:3 toluene/EtOAc) gave pure **21** (0.112 g, 31% from **15**) as a colourless oil, $[\alpha]_D^{23} = +19.20$ (*c* 1.0, $CHCl_3$), $R_f = 0.25$ (23:2 toluene/EtOAc).

IR (film): ν_{max} 1724 (C=O), 1262 (C–O).

^{1}H NMR (250 MHz, $CDCl_3$): δ 1.36 (t, 3 H, $J = 7.2$ Hz, CH_2CH_3), 4.37 (q, 2 H, $J = 7.2$ Hz, CH_2CH_3), 4.69 (dd, 1 H, $J_{5'a,5'b} = 11.7$, $J_{4',5'a} = 5.1$ Hz, H-5′a), 4.78 (dd, 1 H, $J_{5'a,5'b} = 11.7$, $J_{4',5'b} = 6.4$ Hz, H-5′b), 4.93 (m, 1 H, H-4′), 5.69 (d, 1 H, $J_{1',2'} = 1.4$ Hz, H-1′), 5.90 (d, 1 H, $J_{3',4'} = 3.3$ Hz, H-3′), 5.98 (d, 1 H, $J_{1',2'} = 1.4$ Hz, H-2′), 7.29–8.14 (m, 15 H, 3 × Ph), 8.21 (s, 1 H, H-5). NOE contact: H-1′ and H-4′.

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.2 (CH_2CH_3), 61.4 (CH_2CH_3), 62.12 (C-5′), 76.0 (C-3′), 79.9 (C-4′), 81.8 (C-2′), 82.9 (C-1′), 127.7 (C-5), 128.3, 128.32, 128.5, 128.7, 129.3, 129.6, 129.7, 129.8, 133.1, 133.6 and 133.6 (3 × Ph), 147.7 (C-2), 161.1 (C-4), 164.4, 164.5 and 166.0 (3 × Ph*C*=O), 170.92 (*C*O$_2$Et).

HRMS (ESI): *m/z* 602.1471 (M^++H), calcd for $C_{32}H_{28}NO_9S$: 602.1479.

4.2.2. Ethyl 2-(2,5-di-O-benzoyl-3-deoxy-3-trifluoroacetamido-β-D-xylofuranosyl)thiazole-4-carboxylate (**22**)

Compound **16** (0.020 g, 0.04 mmol) was converted in to crude **22** according to the above general procedure. Pure product **22** (0.010 g, 39%) was isolated by preparative TLC (100:3 CH_2Cl_2/EtOAc, developed twice, elution with EtOAc), as a colourless oil, $[\alpha]_D^{23} = +25.0$ (*c* 0.1, $CHCl_3$), $R_f = 0.37$ (100:3 CH_2Cl_2/EtOAc).

IR (film): ν_{max} 3240 (NH), 1721 (C=O), 1602 (amide I band), 1554 (amide II band).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.42 (t, 3 H, $J = 7.1$ Hz, CH_2CH_3), 4.46 (q, 2 H, CH_2CH_3), 4.57 (dd, 1 H, $J_{4',5'a} = 7.4$, $J_{5'a,5'b} = 11.9$ Hz, H-5′a), 4.68 (dd, 1 H, $J_{4',5'b} = 4.8$ Hz, H-5′b), 4.90 (m, 1 H, $J_{3',4'} = 4.1$ Hz, H-4′), 5.19 (dd, 1 H, $J_{3',NH} = 9.3$ Hz, H-3′), 5.37 (d, 1 H, $J_{1',2'} = 0.9$ Hz, H-1′), 5.66 (d, 1 H, H-2′), 7.38–8.12 (m, 10 H, 2 × Ph), 8.24 (s, 1 H, H-5), 9.23 (d, 1 H, $J_{3',NH} = 9.3$ Hz, NHCOCF$_3$).

NOE contact: H-2′ and NH, H-5′ and NH.

^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2 (*C*H$_3$CH$_2$), 55.8 (C-3′), 61.7 (CH_2CH_3), 62.1 (C-5′), 80.6 (C-4′), 81.4 (C-1′), 82.0 (C-2′), 115.7 (q, $^1J_{C,F} = 286.8$ Hz, CF_3), 128.4 (C-5′) 128.3, 128.7, 129.4, 129.8, 130.0, 133.2 and 134.1 (2 × Ph), 148.4 (C-2), 157.4 (q, $^2J_{C,F} = 40.0$ Hz, *C*OCF$_3$), 160.6 (C-4), 165.5 and 166.1 (2 × Ph*C*=O), 167.4 (*C*O$_2$Et).

HRMS (ESI): *m/z* 593.1195 (M^++H), calcd for $C_{27}H_{23}F_3N_2O_8S$: 593.1206.

4.2.3. Ethyl 2-(3-acetamido-2,5-di-O-benzoyl-3-deoxy-β-D-xylofuranosyl)thiazole-4-carboxylate (**23**)

Compound **17** (0.020 g, 0.04 mmol) was converted in to crude **23** according to the above general procedure. Pure product **23** (0.013 g, 50%) was isolated by flash column chromatography (3:2 toluene/EtOAc as a colourless oil, $[\alpha]_D^{23} = -19.8$ (*c* 2.3, $CHCl_3$), $R_f = 0.45$ (3:2 toluene/EtOAc).

IR (film): ν_{max} 3320 (NH), 1717 (C=O), 1669 (amide I band), 1543 (amide II band).

^{1}H NMR (250 MHz, $CDCl_3$): δ 1.41 (t, 3 H, $J = 7.3$ Hz, CH_2CH_3), 2.20 (s, 3 H, C*H*$_3$CO), 4.43 (q, 2 H, CH_2CH_3), 4.55 (dd, 1 H, $J_{4',5'a} = 7.0$ Hz, $J_{5'a,5'b} = 12.0$ Hz, H-5′a), 4.69 (dd, 1 H, $J_{4',5'b} = 3.8$, $J_{5'a,5'b} = 12.0$ Hz, H-5′b), 4.84 (m, 1 H, $J_{3',4'} = 3.1$ Hz, H-4′), 5.17 (ddd, 1 H, $J_{2',3'} = 1.2$, $J_{3',NH} = 9.5$ Hz, H-3′), 5.31 (d, 1 H, $J_{1',2'} = 0.9$ Hz, H-1′), 5.52 (bs, 1 H, H-2′), 6.58 (d, 1 H, NH), 7.25–8.01 (m, 10 H, 2 × Ph),

8.19 (s, 1 H, H-5), 8.63 (2 × s, 1 H each, NH_2).

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.4 ($CO_2CH_2CH_3$), 23.1 (CH_3CO), 55.2 (C-3′), 61.6 (CH_2CH_3), 63.3 (C-5′), 81.1 (C-4′), 81.3 (C-1′), 82.7 (C-2′), 128.0 (C-5), 128.4, 128.6, 129.7, 129.72, 129.8, 133.4 and 133.8 (2 × Ph), 147.7 (C-2), 160.5 (C-4), 165.6 and 166.2 (2 × Ph*C*=O), 167.8 and 170.6 ($NHCOCH_3$ and $CO_2CH_2CH_3$).

LRMS (FAB): *m/z* 561.8 (M^++Na).

HRMS (ESI): *m/z* 561.1305 (M^++Na), calcd for $C_{27}H_{26}N_2NaO_8S$: 561.1308.

4.2.4. Ethyl 2-(2-azido-3,5-di-O-benzoyl-2-deoxy-β-D-arabinofuranosyl)thiazole-4-carboxylate (**24**)

Compound **18** (0.384 g, 0.98 mmol) was converted into crude **24** according to the above general procedure. Purification by preparative TLC (17:3 toluene/EtOAc) gave pure **24** (0.286 g, 56% from **18**) as a colourless oil, $[\alpha]_D^{23} = -6.7$ (*c* 2.9, $CHCl_3$), $R_f = 0.43$ (17:3 toluene/EtOAc).

IR (film): ν_{max} 2112 (N_3), 1724 (C=O), 1270 (C–O).

^{1}H NMR (250 MHz, $CHCl_3$): δ 1.35 (t, 3 H, J = 7.1 Hz, CH_2CH_3), 4.38 (q, 2 H, J = 7.1 Hz, CH_2CH_3), 4.55 (ddd, 1 H, $J_{4',5'a}$ = 5.1, $J_{4',5'b}$ = 5.3, $J_{3',4'}$ = 2.0 Hz, H-4′), 4.62 (d, 1 H, $J_{1',2'}$ = 4.0 Hz, H-2′), 4.67 (dd, 1 H, $J_{4',5'a}$ = 5.1, $J_{5'a,5'b}$ = 11.6 Hz, H-5′a), 4.74 (dd, 1 H, $J_{4',5'b}$ = 5.3, $J_{5'a,5'b}$ = 11.6 Hz, H-5′b), 5.51 (d, 1 H, $J_{3',4'}$ = 1.8 Hz, H-3′), 5.68 (d, 1 H, $J_{1',2'}$ = 3.9 Hz, H-1′), 7.30–8.15 (m, 10 H, 2 × Ph), 8.21 (s, 1 H, H-5).

^{13}C NMR (62.9 MHz, $CDCl_3$): δ 14.2 (CH_2CH_3), 61.3 (CH_2CH_3), 63.4 (C-5′), 67.8 (C-2′), 79.2 (C-3′), 80.5 (C-1′), 82.6 (C-4′), 128.2 (C-5), 128.4, 128.6, 128.9, 129.5, 129.6, 133.0 and 133.7 (2 × Ph), 146.8 (C-2), 160.9 (C-4), 165.1 and 166.0 (2 × Ph*C*=O), 166.6 ($CO_2CH_2CH_3$).

HRMS (ESI): *m/z* 523.1277 (M^++H), calcd for $C_{25}H_{23}N_4O_7S$: 523.1282.

4.2.5. Ethyl 2-(2-acetamido-3,5-di-O-benzoyl-2-deoxy-β-D-arabinofuranosyl)thiazole-4-carboxylate (**25**)

Compound **19** (0.157 g, 0.38 mmol) was converted into crude **25** according to the above general procedure. Pure **25** (0.120 g, 58% from **19**) was obtained after purification on a column of flash silica (2:1 light petroleum/Me_2CO) in the form of colourless oil, $[\alpha]_D^{23} = -40.8$ (*c* 1.2, $CHCl_3$), $R_f = 0.19$ (2:1 light petroleum/Me_2CO).

IR (film): ν_{max} 1722 (C=O), 1602 (amide I band), 1537 (amide II band).

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.41 (t, 3 H, J = 7.1 Hz, CH_2CH_3), 1.70 (s, 3 H, CH_3CO), 4.43 (q, 2 H, J = 7.1 Hz, CH_2CH_3), 4.49 (m, 1 H, $J_{4',5a}$ = 4.5 Hz, $J_{4',5b'}$ = 3.9, H-4′), 4.79 (dd, 1 H, $J_{5a',5b'}$ = 12.2, $J_{4',5a}$ = 4.5 Hz, H-5′a), 4.84 (dd, 1 H, $J_{5a',5b'}$ = 12.2, $J_{4',5b'}$ = 3.9 Hz, H-5′b), 5.21 (ddd, 1 H, $J_{1',2'}$ = 5.2, $J_{2',\ NH}$ = 8.7, $J_{2',3'}$ = 3.9 Hz, H-2′), 5.51 (t, 1 H, $J_{2',3'} = J_{3',4'}$ = 3.9 Hz H-3′), 5.65 (d, 1 H, $J_{1',2'}$ = 5.2 Hz, H-1′), 6.52 (d, 1 H, $J_{2',\ NH}$ = 8.7 Hz, NH), 7.42–8.08 (m, 10 H, 2 × Ph), 8.16 (s, 1 H, H-5). NOE contact: H-1′ and H-4′, H-3′ and H-5′.

NMR (100 MHz, $CDCl_3$): δ 14.3 (CH_2CH_3), 22.8 (CH_3CO), 57.0 (C-2′), 61.6 (CH_2CH_3), 63.6 (C-5′), 78.4 (C-1′), 78.8 (C-3′), 81.9 (C-4′), 127.9 (C-5), 128.4, 128.6, 128.7, 128.8, 129.6, 129.7, 129.8, 129.9, 133.4 and 133.8 (2 × Ph), 147.6 (C-2), 161.0 (C-4), 165.9 and 166.2 (2 × Ph*C*=O), 166.9 and 169.8 ($NHCOCH_3$ and $CO_2CH_2CH_3$).

LRMS (CI): *m/z* 539 (M^++H).

HRMS (ESI): *m/z* 539.1490 (M^++H), calcd for $C_{27}H_{27}N_2O_8S$: 539.1488.

4.3. General procedure for the preparation of C-nucleosides **2**–**5** and **7**

A solution of protected thiazole **21**–**25** (1 equiv) in saturated methanolic ammonia was kept at room temperature for 4–8 days, then evaporated and the residue was purified on a column of silica gel or by preparative TLC.

4.3.1. 2-(β-D-Xylofuranosyl)thiazole-4-carboxamide (**2**)

Compound **21** (0.0445 g, 0.082 mmol) was converted to crude **2** according to the above general procedure. Pure **2** (0.0192 g, 89%) was isolated by preparative TLC (5:2 $CHCl_3$/MeOH, elution with 1:1 EtOAc-^{i}PrOH) as a colourless oil, $[\alpha]_D^{23} = -5.3$ (*c* 0.5; MeOH), $R_f = 0.18$ (5:1 $CHCl_3$/MeOH).

IR (Nujol): ν_{max} 1666 (amide I band), 1592 (amide II band).

^{1}H NMR (250 MHz, methanol-d_4): δ 3.90 (dd, 1 H, $J_{4',5'a}$ = 6.1, $J_{5'a,5'b}$ = 11.7 Hz, H-5′a), 3.95 (dd, 1 H, $J_{4',5'b}$ = 5.0, $J_{5'a,5'b}$ = 11.8 Hz, H-5′b), 4.15 (dd, 1 H, $J_{2',3'}$ = 1.7, $J_{3',4'}$ = 3.6 Hz, H-3′) 4.30 (ddd, 1 H, $J_{3',4'}$ = 3.7, $J_{4',5'a}$ = 6.1, $J_{4',5'b}$ = 5.0 Hz, H-4′), 4.37 (t, 1 H, $J_{1',2'}$ = 1.9, $J_{2',3'}$ = 1.9 Hz, H-2′), 5.04 (d, 1 H, $J_{1',2'}$ = 2.1 Hz, H-1′), 8.13 (s, 1 H, H-5).

^{13}C NMR (62.9 MHz, methanol-d_4): δ 61.8 (C-5′), 78.6 (C-3′), 84.5 (C-2′ and C-4′), 86.4 (C-1′), 125.6 (C-5), 150.5 (C-2), 165.9 (C-4), 174.9 ($CONH_2$).

HRMS (ESI): *m/z* 261.0536 (M^++H), calcd for $C_9H_{13}N_2O_5S$: 261.0540.

4.3.2. 2-(3-Amino-3-deoxy-β-D-xylofuranosyl)thiazole-4-carboxamide (**3**)

Compound **22** (0.018 g, 0.003 mmol) was converted to crude **3** according to the above general procedure. Pure **3** (0.006 g, 69%) was isolated by preparative TLC (5:1 $CHCl_3$/MeOH, elution with 1:1 EtOAc-^{i}PrOH) as a colourless oil, $[\alpha]_D^{23} = -0.82$ (*c* 0.24, MeOH), $R_f = 0.17$ (2:1 $CHCl_3$/MeOH).

IR (film): ν_{max} 3344 (OH, NH_2), 1669 (amide I band), 1592 (amide II band).

^{1}H NMR (400 MHz, methanol-d_4): δ 3.44 (dd, 1 H, $J_{2',3'}$ = 5.2, $J_{3',4'}$ = 6.4 Hz, H-3′), 3.80 (dd, 1 H, $J_{4',5'a}$ = 4.7, $J_{5a',5'b}$ = 12.0 Hz, H-5′a), 3.85 (dd, 1 H, $J_{4',5'b}$ = 4.3 Hz, $J_{5a',5'b}$ = 11.9 Hz, H-5′b), 4.20 (pt, 1 H, J = 5.2 Hz, H-2′), 4.35 (m, 1 H, $J_{3',4'}$ = 6.4 Hz, $J_{4',5'}$ = 4.5 Hz, H-4′), 4.95 (d, 1 H, $J_{1',2'}$ = 5.5 Hz, H-1′), 8.18 (s, 1 H, H-5).

^{13}C NMR (100 MHz, methanol-d_4): δ 61.5 (C-3′), 62.1 (C-5′), 82.6 (C-4′), 84.2 (C-1′), 84.5 (C-2′), 125.7 (C-5), 150.9 (C-2), 165.7 (C-4), 173.4 ($CONH_2$).

HRMS (ESI): *m/z* 260.0703 (M^++H), calcd for $C_9H_{13}N_3O_4S$: 260.0705.

4.3.3. 2-(3-Acetamido-3-deoxy-β-D-xylofuranosyl)thiazole-4-carboxamide (**4**)

Compound **23** (0.065 g, 0.24 mmol) was converted to crude **4** according to the above general procedure. Pure **4** (0.031 g, 85%) was obtained after flash column chromatography (7:3 EtOAc/Pr^{i}OH) as a colourless oil, $[\alpha]_D^{23} = -3.9$ (*c* 1.3, MeOH), $R_f = 0.24$ (5:1 $CHCl_3$/MeOH).

IR (film): ν_{max} 3334 (OH, NH), 1668 (amide I band), 1590 (amide II band).

^{1}H NMR (250 MHz, methanol-d_4): δ 1.97 (s, 3 H, CH_3CO), 3.68 (dd, 1 H, $J_{4',5'a}$ = 4.6, $J_{5'a,5'b}$ = 12.3 Hz, H-5′a), 3.79 (dd, 1 H, $J_{5'a,5'b}$ = 12.3, $J_{4',5'b}$ = 3.5 Hz, H-5′b), 4.36 (t, 1 H, $J_{1',2'} = J_{2',3'}$ = 5.7 Hz, H-2′), 4.40 (m, 1 H, $J_{3',4'}$ = 6.6 Hz, H-4′), 4.47 (dd, 1 H, $J_{3',4'}$ = 6.6, $J_{2',3'}$ = 5.7 Hz, H-3′), 4.98 (d, 1 H, $J_{1',2'}$ = 5.7 Hz, H-1′), 8.23 (s, 1 H, H-5).

^{13}C NMR (62.9 MHz, methanol-d_4): δ 22.5 (CH_3CO), 60.0 (C-3′), 61.9 (C-5′), 81.2 (C-4′), 81.8 (C-2′), 83.8 (C-1′), 125.9 (C-5), 150.8 (C-2), 165.7 (C-4), 172.6 ($CONH_2$), 173.8 ($NHCOCH_3$).

Anal. Found: C, 44.02; H, 5.18; N, 13.67; S, 10.43. Calcd for $C_{11}H_{15}N_3O_5S$: C, 43.85; H, 5.02; N, 13.95; S, 10.64.

4.3.4. 2-(2-Azido-2-deoxy-β-D-arabinofuranosyl)thiazole-4-carboxamide (**5**)

Compound **24** (0.286 g, 0.55 mmol) was converted to crude **5** according to the above general procedure. Pure **5** (0.133 g, 85%) was isolated by preparative TLC (5:1 $CHCl_3$/MeOH, elution with 1:1

EtOAc/^{i}PrOH) as a colourless oil, $[\alpha]_D^{23} = +84.6$ (*c* 0.6, MeOH), $R_f = 0.42$ (5:1 $CHCl_3$/MeOH).

IR (film): ν_{max} 2110 (N_3), 1664 (amide I band), 1588 (amide II band).

^{1}H NMR (250 MHz, methanol-d_4): δ 3.74 (d, 2 H, $J_{4',5'} = 5.8$ Hz, 2 × H-5′), 3.97 (m, 1 H, $J_{3',4'} = 3.8$, $J_{4',5'} = 5.9$ Hz, H-4′), 4.21 (dd, 1 H, $J_{2',3'} = 2.6$, $J_{3',4'} = 3.5$ Hz, H-3′), 4.34 (dd, 1 H, $J_{1',2'} = 4.8$, $J_{2',3'} = 2.4$ Hz, H-2′), 5.54 (d, 1 H, $J_{1',2'} = 4.8$ Hz, H-1′), 8.25 (s, 1 H, H-5).

^{13}C NMR (62.9 MHz, methanol-d_4): δ 63.2 (C-5′), 71.9 (C-2′), 77.7 (C-3′), 81.0 (C-1′), 88.1 (C-4′), 126.2 (C-5), 150.7 (C-2), 165.6 (C-4), 170.1 (*C*ONH$_2$).

HRMS (ESI): *m/z* 286.0599 (M^++H), calcd for $C_9H_{12}N_5O_4S$: 286.0604.

Anal. Found: C, 38.10; H, 3.94; N, 24.68; S, 11.17. Calcd for $C_9H_{11}N_5O_4S$: C, 37.89; H, 3.89; N, 24.55; S, 11.24.

4.3.5. 2-(2-Acetamido-β-D-arabinofuranosyl)thiazole-4-carboxamide (**7**).

Compound **25** (0.0293 g, 0.054 mmol) was converted to crude **7** according to the above general procedure. Pure **7** (0.0095 g, 58%) was isolated by preparative TLC (5:1 $CHCl_3$/MeOH, elution with 5:1 $CHCl_3$/MeOH) as a colourless oil, $[\alpha]_D^{23} = -132.7$ (*c* 0.7, MeOH), $R_f = 0.23$ (5:1 $CHCl_3$/MeOH).

IR (film): ν_{max} 3445 (OH, NH), 1652 (amide I band), 1558 (amide II band).

^{1}H NMR (400 MHz, methanol-d_4): δ 1.76 (s, 3 H, *C*H$_3$CO), 3.82 (dd, 1 H, $J_{5'a,5'b} = 12.0$, $J_{4',5'} = 3.8$ Hz, H-5′a), 3.94 (dd, 1 H, $J_{5'a,5'b} = 12.0$, $J_{4',5'} = 2.4$ Hz, H-5′b), 3.98 (m, 1 H, $J_{3',4'} = 4.5$ Hz, H-4′), 4.18 (t, 1 H, $J_{2',3'} = 4.3$, $J_{3',4'} = 4.5$ Hz, H-3′), 4.63 (dd, 1 H, $J_{1',2'} = 5.8$, $J_{2',3'} = 4.3$ Hz, H-2′), 5.51 (d, 1 H, $J_{1',2'} = 5.8$ Hz, H-1′), 5.82 and 6.12 (2 × bs, the minor OH signals remained after partial exchange with methanol-d_4), 7.53, 7.93 and 8.18 (3 × bs, the minor NH_2 and NH signals remained after partial exchange with methanol-d_4), 8.20 (s, 1 H, H-5). NOE contact: C*H*$_3$CO and H-5′.

^{13}C NMR (100 MHz, methanol-d_4): δ 22.4 (*C*H$_3$CO), 61.1 (C-2′), 62.6 (C-5′), 76.9 (C-3′), 80.4 (C-1′), 87.2 (C-4′), 125.8 (C-5), 150.7 (C-2), 165.7 (C-4), 170.4 (*C*ONH$_2$), 172.7 (NH*C*OCH$_3$).

LRMS (CI): *m/z* 302 (M^++H).

HRMS (ESI): *m/z* 324.0628 (M^++Na), calcd for $C_{11}H_{15}N_3NaO_5S$: 324.0630.

4.4. 2-(2-Amino-2-deoxy-β-D-arabinofuranosyl)thiazole-4-carboxamide (**6**)

A solution of azido derivative **5** (0.133 g, 0.47 mmol) in absolute EtOH (14 mL) was hydrogenated over 10% Pd/C (0.043 g) for 2 days at room temperature and normal pressure of H_2, then filtered and the catalyst washed with MeOH. The organic solutions were combined and evaporated and the residue was purified by preparative TLC (5 plates, 1:2 $CHCl_3$/MeOH, eluted with ^{i}PrOH). Pure **6** (0.099 g, 82%) was obtained as a colourless syrup, $[\alpha]_D^{23} = +61.2$ (*c* 0.3, MeOH), $R_f = 0.30$ (1:1 $CHCl_3$/MeOH).

IR (film): ν_{max} 3334 (OH, NH_2), 1666 (amide I band), 1559 (amide II band).

^{1}H NMR (250 MHz, methanol-d_4): δ 3.59 (dd, 1 H, $J_{1',2'} = 4.8$, $J_{2',3'} = 2.4$ Hz, H-2′), 3.76 (dd, 1 H, $J_{4',5'a} = 3.8$, $J_{5'a,5'b} = 12.0$ Hz, H-5′a), 3.87 (dd, 1 H, $J_{4',5'b} = 3.0$, $J_{5'a,5'b} = 12.0$ Hz, H-5′b), 3.99 (m, 1 H, $J_{3',4'} = 3.4$, $J_{4',5'a} = 3.8$, $J_{5'a,5'b} = 12.0$, Hz, H-4′), 4.08 (dd, 1 H, $J_{3',4'} = 3.4$, $J_{2',3'} = 2.4$ Hz, H-3′), 5.39 (d, 1 H, $J_{1',2'} = 4.9$ Hz, H-1′), 8.20 (s, 1 H, H-5).

^{13}C NMR (62.9 MHz, methanol-d_4): δ 62.0 (C-5′), 62.9 (C-2′), 79.9 (C-3′), 81.9 (C-1′), 88.6 (C-4′), 125.8 (C-5), 151.0 (C-2), 165.8 (C-4), 170.9 (*C*ONH$_2$).

HRMS (ESI): *m/z* 260.0696 (M^++H), calcd for $C_9H_{14}N_3O_4S$: 260.0699.

4.5. MTT assay

The colorimetric MTT assay was carried out following the reported procedure [25]. The cytotoxicity of tested compounds was examined using six human tumour cell lines: K562 (ATCC CCL 243, chronic myeloid leukemia), HL-60 (ATCC CCL 240, promyelocytic leukemia), Jurkat (ATCC CCL 1435, T cell leukemia), Raji (ATCC CCL 86, Burkitt's lymphoma), HT-29 (ATCC HTB38, colorectal adenocarcinoma) and HeLa (ATCC CCL2, human cervix adenocarcinoma), as well as one normal human cell line MRC-5 (ATCC CCL 171, lung fibroblast).

4.6. Cell cycle analysis

Cell cycle analysis of K562 cell line was performed on FACS Calibur E440 (Becton Dickinson) flow cytometer following the reported procedure [27].

4.7. Detection of apoptosis

Apoptosis of K562 cells was evaluated with an FACS Calibur E440 (Becton Dickinson) flow cytometer according to the reported procedure [27]. Results were presented as percent of Annexin V positive gated cells. Specific apoptosis and necrosis were calculated according to Bender et al. [28].

4.8. Western blot analysis

Western blot analysis was performed after treatment of K562 cells with analogues **2**–**7** according to the procedure described in the literature [27].

4.9. Double fluorescent staining

We used acridine orange and ethidium bromide staining method for discrimination of cells on the basis of cell membrane integrity. The K562 cells were seeded for double fluorescence staining test and the experimental samples were incubated with tested compounds at their IC_{50} concentrations, for 72 h, along with untreated cells (control). After staining, cells were collected, placed on clean microscope slides with cover slips and immediately examined on a fluorescence microscope. Microphotographs with the fluorescent signal were analyzed in the *ImageJ* computer program (NIH Image, http://imagej.nih.gov), measuring density of separated red and green colour channels, which ratio is chosen as a parameter for comparison of samples. Detailed experimental procedure is given in the Supplementary data.

4.10. Comet assay

For the comet assay, K562 cells were seeded and treated with IC_{50} concentrations of tested compounds during 72 h in an incubator. Untreated cells were used as a control sample. A modified version of the alkaline Comet assay [29] was performed to evaluate DNA damage (single and double breaks). Cells were immobilized on slides in agarose layers, prepared according to the protocol and exposed to electric field in horizontal electrophoresis unit. Washed and dried slides were then stained with ethidium bromide fluorescent dye and analyzed using a fluorescence microscope. A series of microphotographs were taken by a digital camera and 50 comets per sample were analyzed by *CometScore* computer program. The mean value of the parameter “Tail Moment" was selected to compare the samples. Complete experimental procedure used for the comet assay is described in the Supplementary data.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.111712.

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