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Katedra organické a jaderné chemie

Charles University in Prague, Faculty of Science
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Příloha disertační práce
Appendix of the Ph.D. Thesis

Syntéza a studium reaktivity a biologické aktivity C5 substituovaných analog uracilu

Synthesis, reactivity and biological activity of C5 substituted uracil analogues

RNDr. Lucie Brulíková

Školitel/Supervisor: prof. RNDr. Antonín Holý, DrSc., Dr.hc. mult.
Školitel-konzultant/Supervisor-consultant: doc. RNDr. Jan Hlaváč, Ph.D.

Praha, 2011

1. Statement of Department 1.
2. Statement of Department 2.
3. Full-text article: Bioorganic and Medicinal Chemistry Letters **2007**, *17*, 6647-6650 (with co-author statement).
4. Full-text article: European Journal of Medicinal Chemistry **2010**, *45*, 3588-3594 (with co-author statement).
5. Full-text article: Bioorganic and Medicinal Chemistry **2010**, 18, 4702-4710 (with co-author statements).

Stanovisko vedoucího pracoviště k předložené disertační práci

RNDr. Lucie Brulíkové

RNDr. Lucie Brulíková vypracovávala svou disertační práci na našem pracovišti v letech 2005-2010. Během této doby se postupně zdokonalovala ve všech technikách souvisejících s organickou syntézou a analýzou organických sloučenin za pomoci instrumentálních metod. Díky tomu se v průběhu řešení problematiky disertační práce stala rovněž platnou pedagogickou posilou při výuce studentů v praktických cvičeních a později i při vedení studentů vykonávajících na naší katedře bakalářskou práci. RNDr. Brulíková se rovněž podílela na vedení seminářů z organické chemie a na organizaci nově zavedeného studia Bioorganické chemie, které naše katedra garantuje. Aktivně se zapojovala do ostatních činností katedry, jako byly exkurze studentů ze středních škol, popularizační či propagační akce.

Během svého působení na naší katedře přistupovala Dr. Brulíková ke své práci vždy aktivně a zodpovědně, projevovala ve velké míře svou samostatnost a dále snahu a ochotu podílet se na chodu pracoviště a pomáhat studentům a mladším kolegům v jejich práci.

Na základě výše uvedených skutečností se domnívám, že RNDr. Lucie Brulíková je dostatečně vyzrálou vědeckou osobností a proto vydávám kladné stanovisko k zahájení všech procedur vedoucích k udělení titulu PhD.

V Olomouci dne 14. 2. 2010

Doc. RNDr. Jan Hlaváč, Ph.D.

SYDDANSK UNIVERSITET
UNIVERSITY OF SOUTHERN DENMARK

Odense, Denmark, 11 January, 2011

To whom it might concern

I hereby confirm that Lucie Brulikova (Spacilova) has worked 6 months (September 2007 – March 2008) as a guest Ph.D student in my research group at the Department of Physics and Chemistry, University of Southern Denmark, joining the Marie Curie *NAC-DRUG* Training Site.

In the stay in my group, she worked with organic synthesis of modified nucleosides and nucleotides focused on RNA-targeting and click chemistry.

With regards

Poul Nielsen

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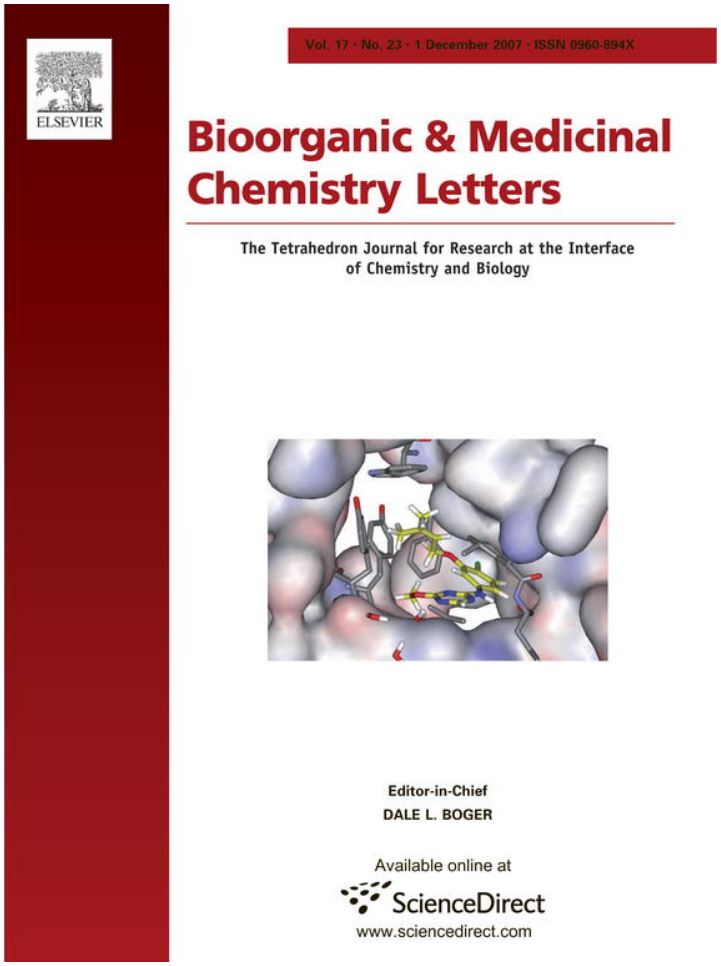

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Bioorganic & Medicinal Chemistry Letters

Synthesis and cytotoxic activity of various 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils in their racemic form

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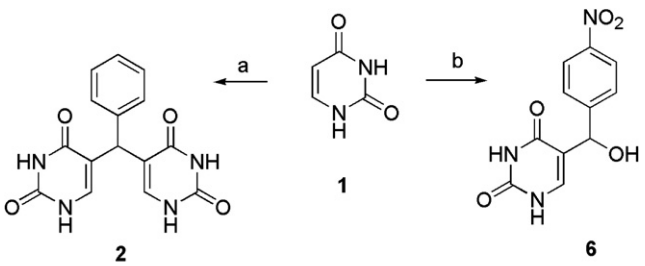
Abstract—The preparation of various 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils with alkyl chain lengths C_1–C_{12} is described. The synthesis is based on the preparation of 5-[chloro-(4-nitro-phenyl)-methyl]-uracil and subsequent substitution of chlorine with appropriate alcohols. The resulting ethers were tested for their cytotoxic activity in vitro against five cancer cell lines. The compounds were less active in lung resistance protein expressing cell lines, suggesting the involvement of this multidrug resistant protein in control of the biological activity. Cytotoxic substances induced rapid inhibition of DNA and modulation of RNA synthesis followed by induction of apoptosis. The data indicate that the biological activity of 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils depends on the alkyl chain length.

Compounds derived from 5-alkyluracil are well known for their biological activity. Some of them, for example, have been described as suitable agents for treating various diseases caused by excessive cell proliferation, such as in the treatment of various cancers[1] or treatment of proliferative diseases mediated by second messengers.[2] The most potent 5-alkyluracils inhibited the proliferation of leukemia, lymphoma, and solid tumor-derived cell lines at micromolar concentrations.[3] Our research is focused on derivatives of 5-alkoxyuracils with anticancer activity. In this paper we describe the synthesis and cytotoxic activity of 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils having various alkyl chain lengths.

The reaction of uracil with benzaldehyde is reported to afford the appropriate derivative **2**.[4] If 4-nitrobenzaldehyde is used instead, 5-[hydroxy-(4-nitro-phenyl)-methyl]-uracil **6** results as is described in the same publication (Scheme 1).

In our hands the reaction does not lead to compound **6**, but rather to a mixture of 5-[chloro-(4-nitro-phenyl)-methyl]-uracil **3** and 5,5′-(4-nitrophenyl)-methyl-bis-1*H*-pyrimidine-2,4-dione **4** instead. We successfully tried to find reaction conditions for selective preparation of each derivative (Scheme 2).

Derivative **4** has been prepared recently by nitration of 5,5′-phenylmethylene-bis-uracil and patented in the class of anti-ictogenic or anti-epileptogenic agents.[5]



a) benzaldehyde, conc. HCl, reflux, 2h;
b) 4-nitrobenzaldehyde, conc. HCl, 60°C, then reflux

Scheme 1. Reaction of uracil with benzaldehyde and p-nitrobenzaldehyde according to Ref. 4.

Keywords: Uracil; DNA; RNA; Apoptosis; Cytotoxic; Anticancer activity.
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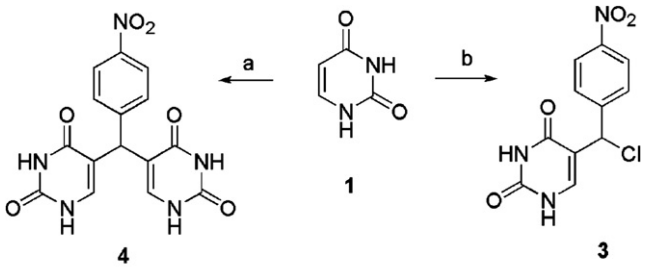


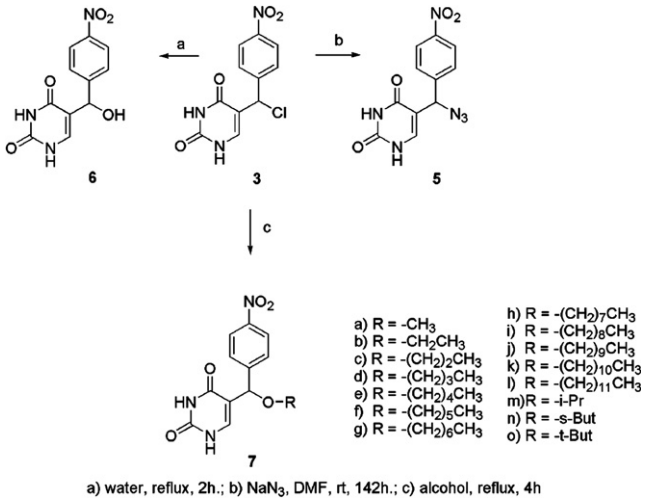
Scheme 2. Reaction of uracil with *p*-nitrobenzaldehyde.

The chlorine atom of derivative **3** is very reactive. When treated with sodium azide the appropriate azide **5** results, with DMF/water the alcohol **6** is obtained and with alcohols the desired ethers **7** are formed (Scheme 3).

The prepared compounds **4**–**7** were tested for their cytotoxic activity against five tumor cell lines under in vitro conditions. Compounds **4**, **5** were found to be quite inactive with IC_{50} above 100 μM. However, the activity of first prepared ethers differed. For this reason, we decided to prepare a series of alkoxyderivatives **7** with various alkyl chain lengths. Compounds with longer alkyl chains (heptyl **7g** to dodecyl **7l**) exhibited relatively higher cytotoxic activity in CEM and K562-Tax leukemia cells. In these lines, the only exception in the activity trend is derivative **7f** (Fig. 1).

In cell lines A549, CEM-DNR-B, and K-562, the cytotoxic activity also increases with increasing chain length (7-9 carbons) and then decreases. Again, derivative **7f** exhibits exceptions in the activity trend (Fig. 2).

Branching of alkyls reduces the activity, because compounds **7m**, **n**, and **o** are quite inactive.



Scheme 3. Preparation of 5-[(alkoxy-(4-nitrophenyl)-methyl]-uracil 7 and other derivatives.

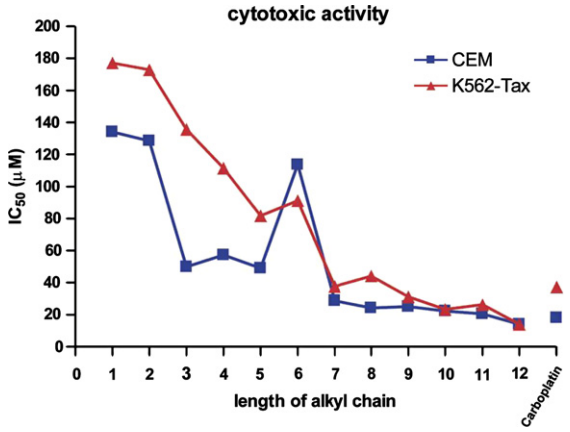


Figure 1. Cytotoxic activity of compounds **7a**–**7j** and control agent (carboplatin) as a function of chain length in LRP negative CEM and K562-Tax leukemia cell lines.

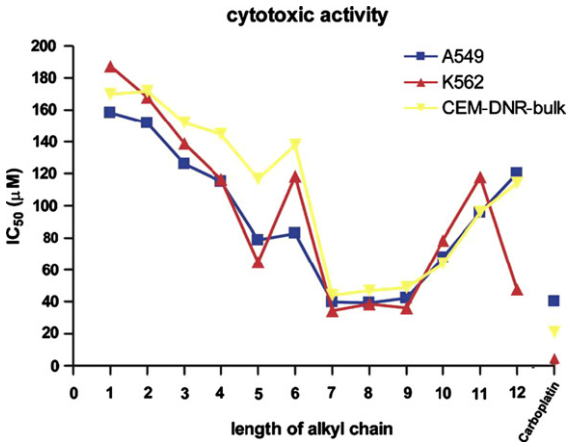


Figure 2. Cytotoxic activity of compounds **7a**–**7j** and control agent (carboplatin) related to chain length in LRP positive A549, CEM-DNRB, and K562 cell lines.

For better understanding of the biological uniqueness of compound **7f** we have studied its activity in more detailed analysis of the cell cycle, apoptosis, DNA and RNA synthesis using CEM T-lymphoblastic leukemia cells.[6,7] The potency of compound **7f** was compared to those of the two most potent substances **7g** and **7h**, whose alkyl chain lengths differ only by one or two carbon lengths, respectively. Analyses were performed at equiactive concentrations corresponding to 1× IC_{50} and 5× IC_{50}.

Interestingly, the cytotoxic activity of the alkoxyderivatives **7f–h** was accompanied by rapid inhibition of DNA synthesis at concentration 5× IC_{50} (Fig. 3).

However, at concentration 1× IC_{50} inhibition of DNA synthesis is significant only for derivative **7f**. Moreover, there was an apparent increase of RNA synthesis in compounds **7g** and **7h** but inhibition in **7f** at 1× IC_{50}, while in 5× IC_{50} the total RNA synthesis was inhibited in cells treated with any of the compounds. Based on these data we hypothesize that the transient increase of

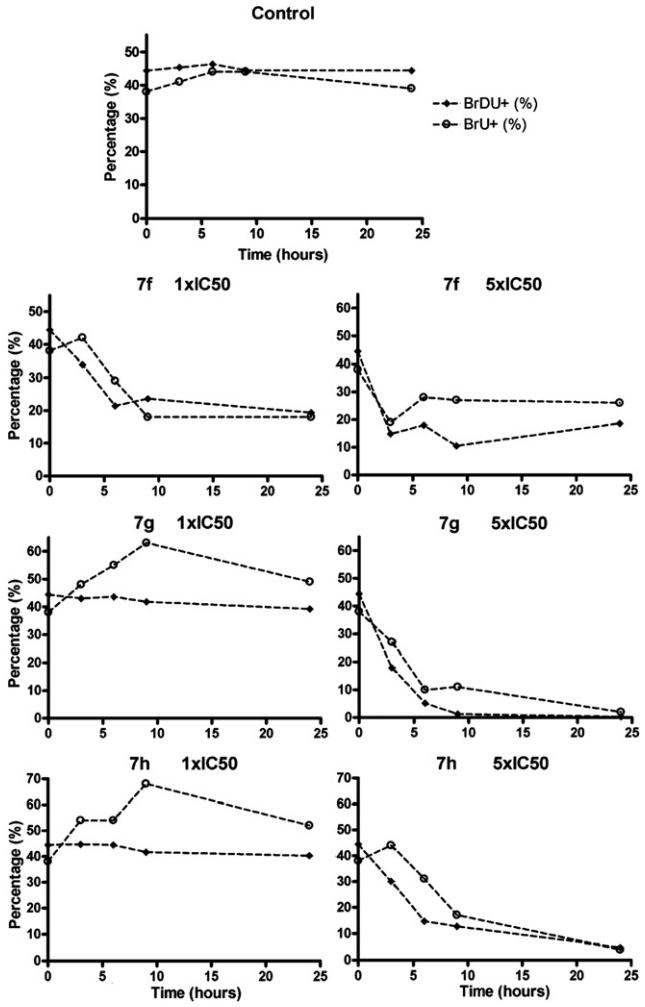


Figure 3. Summary of RNA/DNA analysis for CEM cancer cell line treated with compounds **7f–h**. Data are expressed as a percentage of positive cells in the total cellular population. [6,7]

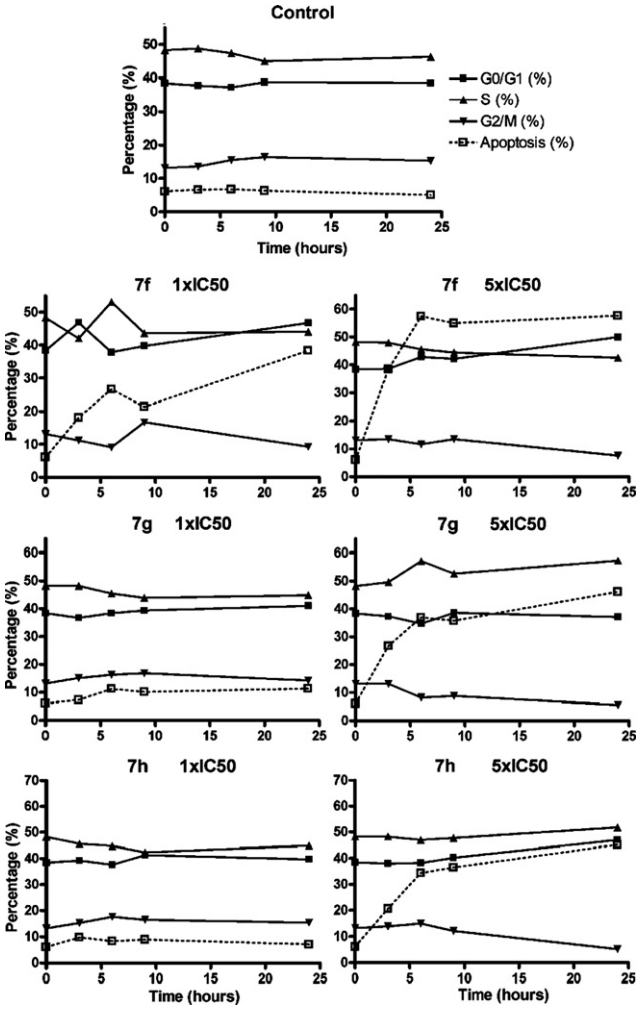


Figure 4. Summary of conventional cell cycle and apoptosis analysis for CEM cancer cell line treated with compounds **7f–h**. Data are expressed as a percentage of cells with corresponding DNA content in the total cellular population. [6,7]

RNA synthesis at 1× IC_{50} in cells treated with **7g** and **7h** is due to compensatory mechanisms, for example, treated cells are compensating insufficiency of macromolecules via increased RNA biosynthesis.

All three compounds **7f–h** induced apoptosis at 5× IC_{50} concentrations, but did not cause any significant cell cycle alterations in treated CEM cells (Fig. 4). Interestingly, at concentration 1× IC_{50} only **7f** caused significant apoptosis within 24 h. This finding rather contrasts with the lower cytotoxic potency of compound **7f** in 3-day cytotoxic MTT assay compared to structures **7g**, **7h** and suggests that chemical or metabolic stability of **7f** could explain the incoherent biological behavior of the compound.

Cell lines A549, CEM-DNR-B, and K-562 were generally more resistant to this class of compounds. Indeed, cell lines A549, CEM-DNR-B, and K-562, express consistently significant concentrations of lung resistance protein (LRP) but not other multidrug resistance associated proteins compared to CEM and K562-Tax cells.[8] Thus, we hypothesize that the cytotoxic activity of 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils is controlled by the multidrug resistance associated protein LRP. Interestingly, the cytotoxic activity of substances **7g** and **7h** in contrast to derivative **7f** is active in LRP negative, but p-glycoprotein (PgP) positive K562-Tax cells. This means that they were not influenced by expression of multidrug resistance associated protein PgP, suggesting activity of compounds in PgP overexpressing tumors.[8] However, the cytotoxic potency of all compounds was reduced in CEM-DNR-bulk cells, which are, in addition to LRP positivity, characterized by overexpression of the multidrug resistance protein 1 (MRP) and decreased expression of topoisomerase IIα gene.[8] These data suggest that compounds **7f–h** are transported via LRP and may, directly or indirectly, target topoisomerase IIα. The involvement of MRP1 dependent transportation is not clear, since derivatives were relatively potent in another MRP1 positive cell line A549.[8]

In conclusion, the synthesized derivatives of 5-[(azido-(4-nitrophenyl)-methyl]-uracil (**5**), 5-[hydroxy-(4-nitro-

phenyl)-methyl]-1H-pyrimidine-2,4-dione (**6**), 5,5′-(4-nitrophenyl)-methyl-bis-1H-pyrimidine-2,4-dione (**4**) as well as the 5-[(alkoxy-(4-nitrophenyl)-methyl]-uracil (**7**) substituted with low alkyl chain did not exhibit any significant cytotoxic activity against cancer lines. Activity increases with chain lengths continuously in lines CEM and K562-Tax. In ethers **7** with longer chains (from nonyl to undecyl), the activity decreases in LRP positive cell lines A549, K562, and CEM-DNR-B. Compounds **7f–h**, which were studied in the DNA/RNA synthesis inhibition test, exhibited similar activity in concentration 5× IC_{50}, where the synthesis of both nucleic acids was inhibited. Inhibition of de novo DNA and RNA synthesis is the most probable reason for rapid induction of apoptosis in treated cells.

At the concentration 1× IC_{50}, only compound **7f** effectively inhibited both synthesis of DNA and RNA and induced apoptosis. However, the structure was relatively inactive in a 3-day cytotoxic assay. The exceptional behavior of the hexylderivative **7f** may be caused by chemical or metabolic stability or differential affinity to the multidrug resistance associated proteins. Compounds **7g**, **7h** left the DNA synthesis uninfluenced, but caused an increase in RNA synthesis. This may be caused by promotion of compensatory mechanisms. Therefore, the apoptosis at this concentration was not apparent within the first 24 h of treatment.

The bulky alcohols used to form ethers **7m–o** did not enhance the activity to any noticeable extent.

The activity of individual enantiomers and nucleosides derived from derivatives **7** is now under intensive examination and will be published separately.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.09.022.

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Co-author statement in connection to the PhD thesis made by:

Lucie Brulíková

Paper/manuscript (authors, title, journal):
Spáčilová, L.; Džubák, P.; Hajdúch, M.; Křupková, S.; Hradil, P.; Hlaváč, J. "Synthesis and Cytotoxic Activity of Various 5-[Alkoxy-(4-nitro-phenyl)-methyl]-uracils in their racemic form" *Bioorganic and Medicinal Chemistry Letters* **2007**, 17, 6647-6650.

The undersigned who is
- ☒ corresponding author
- ☐ co-author

on the paper/manuscript above, hereby confirms that Lucie Brulíková has contributed to the work as stated below:

1. Intellectual input:

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: _____

2. Experimental results (indicate contribution to individual figures, tables and supplementary data):

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: Lucie Brulíková performed the syntheses of all compounds presented in the above mentioned paper.

3. Writing process:

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: _____

JAN HLAVÁČ

Written Name

Signature

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Original article

Synthesis of 5-[alkoxy-(4-nitro-phenyl)-methyl]-uridines and study of their cytotoxic activity

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ABSTRACT

A series of uridine analogues modified at the 5-position with the 5-[alkoxy-(4-nitrophenyl)-methyl] moiety was synthesized. Nucleosides were formed as a mixture of two diastereoisomers, which were separated and tested for their cytotoxic activity in vitro against different cancer cell lines and for antimicrobial activity. Relationships between structure and the above mentioned activities were studied. The cytotoxic activity was slightly increased in some cases by transformation of bases to nucleosides. Depending on the length of the alkyl chain increased cytotoxic and antimicrobial activity were noted. The cytotoxic activity of the nucleosides was not due to cell cycle alterations, DNA and/or RNA synthesis.

1. Introduction

Modern synthesis in the field of drug discovery is focused on several types of derivatives. Nucleoside analogues, as potential inhibitors of nucleic acid metabolism, play a significant role in this area. In this context, we could mention as examples, the reverse transcriptase inhibitors as potential AIDS therapies [1,2] as well as inhibitors of thymidylate synthase used for the treatment of leukemias or solid tumors [3,4]. Since the discovery of the anticancer agent 5-fluorouracil, which has been used against cancer for about 40 years, or drugs possessing antiviral activity, such as 5-ethyl-2′-deoxyuridine, investigation of the chemistry of pyrimidine nucleosides modified at the 5 position has grown extensively. The synthesis of alkoxy-uracil derivatives [5–13] and their use as sensitive and valuable markers for studies on DNA oxidation damage, has been described [14]. Also, another 5-modified uracil – 5,5′-(4-nitrophenyl)-methyl-bis-1*H*-pyrimidine 2,4-dione - has been shown to possess significant biological properties and is patented in the class of anti-ictogenic or anti-epileptogenic agents [15].

Our recent interest was centered on the 5 position of pyrimidine nucleobases as potential anticancer agents. We modified the above mentioned alkoxy-uracils by introducing a nitro-phenyl moiety and reported the synthesis of 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracils **3a**–**3o** (Scheme 1) and studied their in vitro anticancer activity against five tumor cell lines under in-vitro conditions [16].

2. Results and discussion

2.1. Chemistry

Despite finding an interesting relationship between structure and cytotoxic activity, the values of IC_{50} remained beyond micromolar concentrations. Consequently, we decided to convert these structures into the ribonucleosides in order to improve solubility and increase their possibility to interact with the enzymes responsible for nucleic acids transformation and/or biosynthesis.

Now we have selected derivatives **3f**–**3i** with the highest activity and transformed them into the corresponding nucleosides. The Vorbrüggen method [17] – as the most widely used reaction for the preparation of ribonucleosides - was used. This reaction utilizes silylated nucleobases and strong Lewis acids.

The synthesis was initiated by silylation of the starting compounds **3f**–**3i** (Scheme 2). Silylated uracils reacted with

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[1] Both authors contributed equally to the work.

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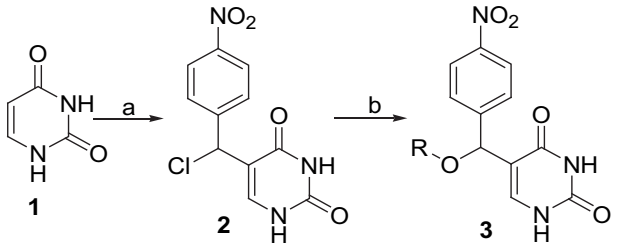


a) 4-nitrobenzaldehyde, conc. HCl, reflux, 4h
b) alcohol, reflux, 4h

a) R = -CH_3
b) R = -CH_2CH_3
c) R = -$(CH_2)_2CH_3$
d) R = -$(CH_2)_3CH_3$
e) R = -$(CH_2)_4CH_3$
f) R = -$(CH_2)_5CH_3$
g) R = -$(CH_2)_6CH_3$
h) R = -$(CH_2)_7CH_3$
i) R = -$(CH_2)_8CH_3$
j) R = -$(CH_2)_9CH_3$
k) R = -$(CH_2)_{10}CH_3$
l) R = -$(CH_2)_{11}CH_3$
m) R = -i-Pr
n) R = -s-But
o) R = -t-But

Scheme 1. Preparation of 5-[alkoxy-(4-nitro)-methyl]-uracils **3a**–**3o** [16].

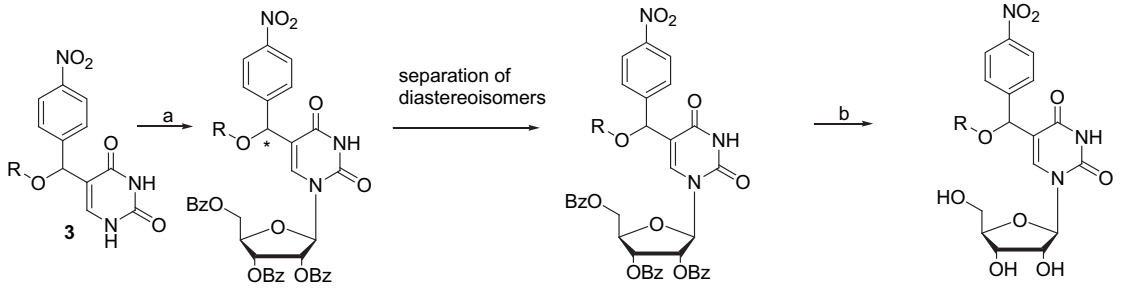
protected sugar in the presence of 1.1 equivalents of TMSOTf at room temperature to afford benzoylated ribonucleosides **4f**–**4i** that were formed as a mixture of two diastereoisomers.

The separation of diastereoisomers was not simple due to the similar properties of both isomers. The isomeric structures from each mixture **4f**–**4i**, however, had almost the same R_f and it was therefore very difficult to separate them in amounts sufficient for all studies and analyses. This separation was very sensitive to the presence of water and size of column. Furthermore, a carefully chosen gradient of the mobile phase was one of the most influencial factors. Nevertheless, we have found an acceptable mobile phase and conditions for separation by silica gel column chromatography. Two diastereoisomers **5f**–**5i** and **6f**–**6i** from each mixture **4f**–**4i** were isolated using methanol in chloroform (0–5%).

Treatment of ribonucleosides **5f**–**5i** and **6f**–**6i** with methanolic ammonia at room temperature afforded the nucleosides **7f**–**7i** and **8f**–**8i**.

2.2. Cytotoxic activity

The prepared nucleosides **7f**–**7i** and **8f**–**8i** were tested under in-vitro conditions for their cytotoxic activity against cancer cell lines including drug sensitive (CEM and K-562) as well as drug resistant (CEM-DNR-B and K-562 TAX) cell lines and A549 cells as representative of solid tumors (Table 1) [18,19]. Cytotoxic activity was analyzed in comparison to purine based anticancer agents 6-thioguanine and fludarabine. Although pyrimidine derivative 5-fluorouracil is structurally more related to our compounds, it was not used as a comparative agent due to low activity in hematopoietic tumors, Figs. 1–5 show the activity-chain length dependence. Compounds with longer alkyl chains exhibited a trend to higher cytotoxicity in all tested cell lines. Nucleosides are not always more potent than the free bases. The most active compounds **7h**, **7i** and **8h**, **8i** were tested for their effect on the cell cycle alterations including $pH3^{Ser10}$ positive mitotic cells, apoptosis, DNA and RNA synthesis inhibition (Table 2) in treated versus control CEM T-lymphoblastic leukemia cells. Analyses were performed at equiactive concentrations corresponding to 1× IC50 or 5× IC50 in a 24 h treatment interval. However, in these assays we did not observe significant changes in cell distribution during the cell cycle or differences in percentage of mitotic cells based on the monitoring of $pH3^{Ser10}$ positivity. All tested compounds provided massive induction of apoptosis at 5× IC50 which was accompanied by complete inhibition of DNA and RNA synthesis. However, at a concentration of 1× IC50 we observed only small differences in DNA/RNA synthetic activity suggesting that nucleic acid biosynthesis is not a primary target of our compounds. On the contrary, control agents, 6-thioguanine and fludarabine, inhibited DNA and/or RNA synthesis even at low cytotoxic concentrations (Table 2). These data correspond to our previous study on the free bases of tested compounds [16].



4
f) R = -$(CH_2)_5$-CH_3
g) R = -$(CH_2)_6$-CH_3
h) R = -$(CH_2)_7$-CH_3
i) R = -$(CH_2)_8$-CH_3

5f) R = -$(CH_2)_5$-CH_3 ; $[\alpha]_D^{25}$ **+151.9**
5g) R = -$(CH_2)_6$-CH_3 ; $[\alpha]_D^{25}$ **+32.7**
5h) R = -$(CH_2)_7$-CH_3 ; $[\alpha]_D^{25}$ **+38.5**
5i) R = -$(CH_2)_8$-CH_3; $[\alpha]_D^{25}$ **+90.2**
6f) R = -$(CH_2)_5$-CH_3 ; $[\alpha]_D^{25}$ **+213.9**
6g) R = -$(CH_2)_6$-CH_3 ; $[\alpha]_D^{25}$ **+48.6**
6h) R = -$(CH_2)_7$-CH_3 ; $[\alpha]_D^{25}$ **+56.8**
6i) R = -$(CH_2)_8$-CH_3 ; $[\alpha]_D^{25}$ **+56.4**

7 (+)-isomers
8 (-)-isomers

f) R = -$(CH_2)_5$-CH_3
g) R = -$(CH_2)_6$-CH_3
h) R = -$(CH_2)_7$-CH_3
i) R = -$(CH_2)_8$-CH_3

a) hexamethyldisilazane, $(NH4)_2SO_4$, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, trimethylsilyl trifluoro-methanesulfonate, anhydrous 1,2-dichloroethane, RT, 2 days;
b) MeOH/NH_3, RT, 6 days

Scheme 2. Preparation of nucleosides **7f**–**7i** and **8f**–**8i**.

Table 1
Cytotoxic activity of standard anticancer agents and derivatives **7f**–**7i** and **8f**–**8i** on human malignant cell lines of different tissue origin and drug resistance profile.

Compounds	R	A 549	CEM	IC_{50} (μM) CEM-DNR-B	K 562	K562-TAX
6-thioguanine		2.82 ± 1.2	2.98 ± 0.8	9.47 ± 1.2	1.54 ± 0.07	3.9 ± 0.56
fludarabine		47.44 ± 9.0	19.49 ± 0.9	1.01 ± 0.34	267 ± 22.5	0.26 ± 0.15
7f	hexyl	121.3 ± 23.4	39.8 ± 1.9	131.0 ± 7.1	58.4 ± 3.1	141.4 ± 14.3
7g	heptyl	39.8 ± 13.5	12.8 ± 3.5	103.7 ± 9.8	33.6 ± 1.8	42.8 ± 3.6
7h	oktyl	38.7 ± 11.0	11.4 ± 1.7	34.6 ± 2.4	15.1 ± 2.8	33.1 ± 3.4
7i	nonyl	23.1 ± 11.0	7.9 ± 1.4	32.3 ± 3.0	10.9 ± 1.4	14.6 ± 3.5
8f	hexyl	156.0 ± 22.0	45.5 ± 16.5	145.8 ± 12.6	64.5 ± 12.3	136.9 ± 14.3
8g	heptyl	69.9 ± 35.8	29.0 ± 4.9	95.6 ± 12.1	36.7 ± 2.2	52.3 ± 4.4
8h	oktyl	39.7 ± 10.3	16.9 ± 5.1	36.6 ± 3.1	24.7 ± 7.7	34.4 ± 2.7
8i	nonyl	29.1 ± 11.1	10.0 ± 1.0	33.8 ± 2.8	13.0 ± 1.1	23.9 ± 5.8

2.3. Antimicrobial activity

The compounds were also tested for their antimicrobial activity (see Table 3) against standard reference gram-positive and gram-negative bacterial strains (*Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 3953, *Escherichia coli* CCM 3954 and *Pseudomonas aeruginosa* CCM 3955) from the Czech Collection of Microorganisms (CCM, Faculty of Science, Masaryk University Brno), and against gram-positive and gram-negative bacteria obtained from clinical material of patients treated at University Hospital in Olomouc (methicillin resistant *S. aureus* – MRSA, *Staphylococcus haemolyticus*, *E. coli* and *P. aeruginosa*) with resistance to fluoroquinolones used in clinical practice. Only the octyl and nonyl derivatives **7h**, **8h** and **7i**, **8i** showed slight activity against *E. faecalis* CCM 4224, *S. aureus* CCM 3953, *S. aureus* MRSA and *S. haemolyticus*.

3. Conclusion

In conclusion, insertion of a ribose moiety into nucleosides derived from 5-[alkoxy-(4-nitro-phenyl)-methyl]-uracil did not bring about a significant increase of cytotoxic activity against cancer lines in comparison to the analogous free bases. The activity of these nucleosides was higher in drug sensitive than in drug resistant cancer lines and was independent of the chirality of the molecule. In contrast to the free bases, the nucleosides exhibited only weak activity against several bacterial strains suggesting specific target(s) in eukaryotic cells.

Because the activity increases with chain length in both anticancer as well as antimicrobial activity, derivatives with longer chains might be promising for future study of their activity and mechanism of action.

4. Experimental section

4.1. Chemistry

Melting points were determined on a Boetius stage and are uncorrected. ^{1}H NMR spectra were measured in DMSO-d_6 at 300 K on a Bruker Avance 300 spectrometer (300 MHz with TMS as an internal standard; chemical shifts are reported in ppm, and coupling constants in Hz). Mass spectrometric experiments were performed using a triple quadrupole mass spectrometer TSQ Quantum Access and chromatographic analysis were performed using ultra-high pressure liquid chromatograph Accela (both from Thermo Scientific, San Jose, CA, USA). HPLC experiments were performed using Dionex liquid chromatograph (P 680 HPLC Pump, PDA-100 Photodiode Array Detector). Preparative chromatography was performed with using of Sepacore chromatography system (Büchi). Optical rotations were recorded at the Sodium D line with a polarimeter at room temperature.

4.1.1. 2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl-5-[hexyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**4f**), (**5f**), (**6f**)

5-[Hexyl-(4-nitro-phenyl)-methyl]-1*H*-pyrimidine-2,4-dione (**3f**) (600 mg, 1.7273 mmol) was heated in hexamethyldisilazane (15 ml) at 140 °C with $(NH_4)_2SO_4$ (approximately 5 mg) for 8 h. After that it was evaporated and residue was dissolved in anhydrous 1,2-dichloroethane (20 ml). To this solution, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (871.4 mg, 1.7273 mmol) and trimethylsilyl trifluoro-methanesulfonate (345 μl, 1.9061 mmol) were added. This mixture was stirred at room temperature for 2 days, washed with water (20 ml) and ethyl acetate (60 ml), dried over sodium sulphate, filtered and evaporated to dryness. Yield of

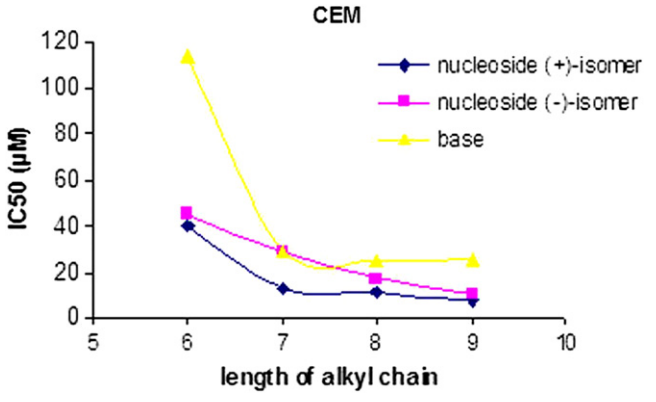


Fig. 1. Cytotoxic activity of compounds **3f**–**3i**, **6f**–**6i** and **7f**–**7i** as a function of chain length in the CEM cell line.

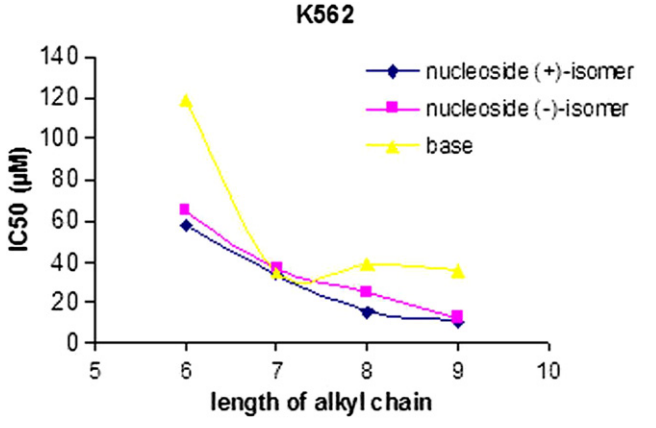


Fig. 2. Cytotoxic activity of compounds **3f**–**3i**, **7f**–**7i** and **8f**–**8i** as a function of chain length in the K562 cell line.

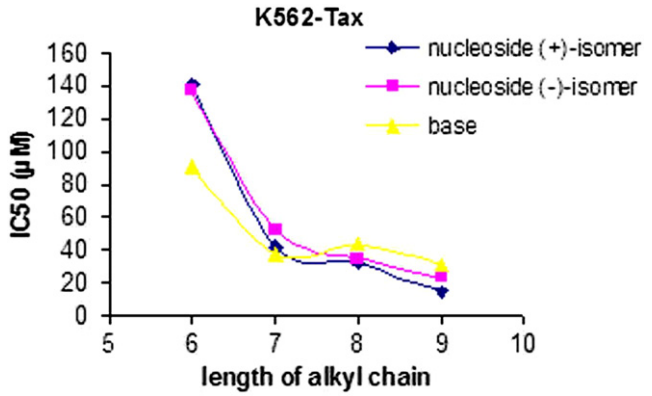


Fig. 3. Cytotoxic activity of compounds **3f**–**3i**, **7f**–**7i** and **8f**–**8i** as a function of chain length in the K562-TAX cell line.

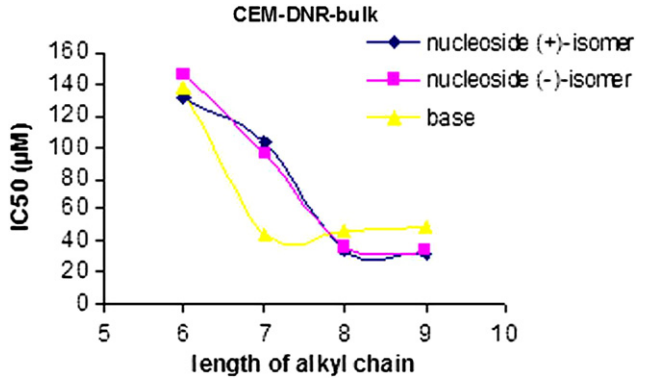


Fig. 5. Cytotoxic activity of compounds **3f**–**3i**, **7f**–**7i** and **8f**–**8i** as a function of chain length in CEM-DNR-B cell line.

crude product (**4f**) (mixture of diastereoisomers) 852.3 mg (62%). Two diastereoisomers (**5f**) and (**6f**) were isolated in this order from the mixture by silica gel column chromatography using methanol in chloroform (0–5%).

*4.1.1.1. Isomer (**5f**).* Yield 189.3 mg (14%), m.p. 75–77 °C, $[\alpha]_D^{25}$ + 151.9 (c 0.027, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.76–0.81 (m, 3H, CH_3); 1.12–1.26 (m, 6H, CH_2); 1.37–1.47 (m, 2H, CH_2); 3.23–3.44 (m, 2H, CH_2); 4.63–4.65 (m, 2H, H-5′); 4.77–4.81 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.94–6.02 (m, 2H, H-2′, H-3′); 6.25 (d, 1H, H-1′, J = 4.2 Hz); 7.41–7.51 (m, 6H, Ph); 7.57 (d, 2H, Ph, J = 8.7 Hz); 7.61–7.69 (m, 3H, Ph); 7.82 (s, 1H, Ph); 7.86 (d, 2H, Ph, J = 7.2 Hz); 7.93 (d, 2H, Ph, J = 7.2 Hz); 8.02 (d, 2H, Ph, J = 7.2 Hz); 8.15 (d, 2H, Ph, J = 8.7 Hz); 11.63 (s, 1H, NH). MS m/z Calc. for $C_{43}H_{41}N_3O_{12}$: 791.82, found 790.25 $[M - H]^-$.

*4.1.1.2. Isomer (**6f**).* Yield 167.2 mg (12%), m.p. 69–72 °C, $[\alpha]_D^{25}$ + 213.9 (c 0.018, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.81 (t, 3H, CH_3, J = 7.2 Hz); 1.19–1.26 (m, 6H, CH_2); 1.42–1.50 (m, 2H, CH_2); 3.23–3.45 (m, 2H, CH_2); 4.62–4.68 (m, 2H, H-5′); 4.73–4.78 (m, 1H, H-4′); 5.33 (s, 1H, CH); 5.95–6.02 (m, 2H, H-2′, H-3′); 6.21 (d, 1H, H-1′, J = 3.6 Hz); 7.42–7.53 (m, 6H, Ph); 7.58–7.68 (m, 5H, Ph); 7.79 (s, 1H, Ph); 7.88–7.91 (m, 4H, Ph); 8.02 (d, 2H, Ph, J = 6.9 Hz); 8.15 (d, 2H, Ph, J = 8.7 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{43}H_{41}N_3O_{12}$: 791.82, found 790.26 $[M - H]^-$.

*4.1.2. 2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl-5-[heptyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**4g**), (**5g**), (**6g**)*

5-[Heptyl-(4-nitro-phenyl)-methyl]-1*H*-pyrimidine-2,4-dione (**3g**) (600 mg, 1.6602 mmol) was heated in hexamethyldisilazane (15 ml) at 140 °C with $(NH_4)_2SO_4$ (approximately 5 mg) for 8 h. After that it was evaporated and residue was dissolved in anhydrous 1,2-dichloroethane (20 ml). To this solution, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (837.6 mg, 1.6603 mmol) and trimethylsilyl trifluoro-methanesulfonate (331 μl, 1.8288 mmol) were added. This mixture was stirred at room temperature for 2 days, washed with water (20 ml) and ethyl acetate (60 ml), dried over sodium sulphate, filtered and evaporated to dryness. Yield of crude product (**4g**) (mixture of diastereoisomers) 954.5 mg (71%). Two diastereoisomers (**5g**) and (**6g**) were isolated in this order from the mixture by silica gel column chromatography using methanol in chloroform (0–5%).

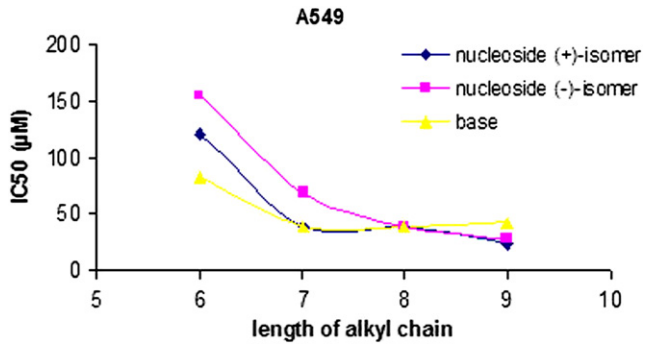


Fig. 4. Cytotoxic activity of compounds **3f**–**3i**, **7f**–**7i** and **8f**–**8i** as a function of chain length in the A549 cell line.

*4.1.2.1. Isomer (**5g**).* Yield 372.7 mg (28%), m.p. 72–74 °C, $[\alpha]_D^{25}$ + 32.7 (c 0.11, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.77–0.82 (m, 3H, CH_3); 1.20–1.23 (m, 8H, CH_2); 1.38–1.46 (m, 2H, CH_2); 3.25–3.44 (m, 2H, CH_2); 4.64–4.65 (m, 2H, H-5′); 4.77–4.81 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.94–6.01 (m, 2H, H-2′, H-3′); 6.25 (d, 1H, H-1′, J = 4.5 Hz); 7.41–7.51 (m, 6H, Ph); 7.56–7.59 (m, 2H, Ph); 7.62–7.69 (m, 3H, Ph); 7.82–7.87 (m, 3H, Ph); 7.91–7.94 (m, 2H, Ph); 8.00–8.03 (m, 2H, Ph); 8.14 (d, 2H, Ph, J = 9.0 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{44}H_{43}N_3O_{12}$: 805.85, found 804.25 $[M - H]^-$.

*4.1.2.2. Isomer (**6g**).* Yield 361.5 mg (27%), m.p. 65–66 °C, $[\alpha]_D^{25}$ + 48.6 (c 0.074, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.78–0.82 (m, 3H, CH_3); 1.19–1.26 (m, 8H, CH_2); 1.42–1.52 (m, 2H, CH_2); 3.25–3.45 (m, 2H, CH_2); 4.62–4.65 (m, 2H, H-5′); 4.73–4.78 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.95–6.02 (m, 2H, H-2′, H-3′); 6.21 (d, 1H, H-1′, J = 3.9 Hz); 7.42–7.52 (m, 6H, Ph); 7.58–7.68 (m, 5H, Ph); 7.80 (s, 1H, Ph); 7.87–7.91 (m, 4H, Ph); 8.00–8.03 (m, 2H, Ph); 8.15 (d, 2H, Ph, J = 8.7 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{44}H_{43}N_3O_{12}$: 805.85, found 804.25 $[M - H]^-$.

*4.1.3. 2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl-5-[oktyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**4h**), (**5h**), (**6h**)*

5-[Oktyl-(4-nitro-phenyl)-methyl]-1*H*-pyrimidine-2,4-dione (**3h**) (600 mg, 1.5982 mmol) was heated in hexamethyldisilazane (15 ml) at 140 °C with $(NH_4)_2SO_4$ (approximately 5 mg) for 8 h. After that it was evaporated and residue was dissolved in anhydrous 1,2-dichloroethane (20 ml). To this solution, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (806.3 mg, 1.5982 mmol) and trimethylsilyl trifluoro-methanesulfonate (319 μl, 1.7625 mmol) were added. This mixture was stirred at room temperature for 2 days, washed with water (20 ml) and ethyl acetate (60 ml), dried over sodium sulphate, filtered and evaporated to dryness. Yield of crude product (**4h**) (mixture of diastereoisomers) 842.3 mg (67%). Two diastereoisomers (**5h**) and (**6h**) were isolated in this order from the mixture by silica gel column chromatography using methanol in chloroform (0–5%).

Table 2
Summary of conventional cell cycle, apoptosis, RNA/DNA – BrU/BrDU analysis of CEM leukemia cell line treated with standard anticancer drugs or the most potent compounds **7h**, **7i** and **8h**, **8i**. Data are expressed as a percentage of positive cells in the total cell population.

Compounds	Apoptosis (%)	G0/G1 (%)	S (%)	G2/M (%)	BrU+ (%)	BrDU+ (%)	$pH3^{Ser10}$+ (%)
Control	3	44.87	44.37	10.76	47.59	58.56	1.35
6-thioguanine 1× IC50	11.27	43.42	49.39	7.19	45.84	35.32	1.21
6-thioguanin**e** 5× IC50	18.69	43.58	46.47	9.95	48.93	19.82	0.43
fludarabine 1× IC50	62.1	39.66	40.84	19.5	7.62	14.12	0.28
fludarabine 5× IC50	74.84	42.11	52.94	4.95	8.92	10.37	0.01
7h 1× IC50	7	42.36	47.81	9.83	58.1	46.03	2.05
7h 5× IC50	76	ND	ND	ND	1.44	6.01	0.03
7i 1× IC50	4	40.95	47.81	11.25	60.22	52.17	1.66
7i 5× IC50	83	ND	ND	ND	0.8	2.95	0.25
8h 1× IC50	5	40.02	48.56	11.33	56.18	48.73	1.68
8h 5× IC50	71	ND	ND	ND	1.61	6.67	0.01
8i 1× IC50	4	43.38	47.79	8.83	57.26	47.38	1.74
8i 5× IC50	83	ND	ND	ND	1.9	0.95	0.04

*4.1.3.1. Isomer (**5h**).* Yield 260.0 mg (21%), m.p. 69–70 °C, $[\alpha]_D^{25}$ + 38.5 (c 0.091, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.81 (t, 3H, CH_3, J = 7.2 Hz); 1.16–1.23 (m, 10H, CH_2); 1.37–1.46 (m, 2H, CH_2); 3.22–3.44 (m, 2H, CH_2); 4.64–4.65 (m, 2H, H-5′); 4.76–4.81 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.94–6.01 (m, 2H, H-2′, H-3′); 6.25 (d, 1H, H-1′, J = 4.5 Hz); 7.41–7.51 (m, 6H, Ph); 7.57 (d, 2H, Ph, J = 8.7 Hz); 7.62–7.68 (m, 3H, Ph); 7.82 (s, 1H, Ph); 7.86 (d, 2H, Ph, J = 7.2 Hz); 7.93 (d, 2H, Ph, J = 7.2 Hz); 8.02 (d, 2H, Ph, J = 7.2 Hz); 8.14 (d, 2H, Ph, J = 8.7 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{45}H_{45}N_3O_{12}$: 819.87, found 818.27[M − H]$^-$.

*4.1.3.2. Isomer (**6h**).* Yield 205.0 mg (16%), m.p. 60–62 °C, $[\alpha]_D^{25}$ + 56.8 (c 0.059, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.78–0.83 (m, 3H, CH_3); 1.18–1.26 (m, 10H, CH_2); 1.41–1.52 (m, 2H, CH_2); 3.25–3.45 (m, 2H, CH_2); 4.62–4.65 (m, 2H, H-5′); 4.73–4.78 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.95–6.02 (m, 2H, H-2′, H-3′); 6.22 (d, 1H, H-1′, J = 3.6 Hz); 7.42–7.52 (m, 6H, Ph); 7.56–7.68 (m, 5H, Ph); 7.80 (s, 1H, Ph); 7.88–7.91 (m, 4H, Ph); 8.01 (d, 2H, Ph, J = 7.5 Hz); 8.14 (d, 2H, Ph, J = 8.7 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{45}H_{45}N_3O_{12}$: 819.87, found 818.29 [M − H]$^-$.

*4.1.4. 2′,3′,5′-tri-O-benzoyl-β-D-ribofuranosyl-5-[nonyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**4i**), (**5i**), (**6i**)*

5-[Nonyl-(4-nitro-phenyl)-methyl]-1*H*-pyrimidine-2,4-dione (**3i**) (600 mg, 1.5406 mmol) was heated in hexamethyldisilazane (15 ml) at 140 °C with $(NH_4)_2SO_4$ (approximately 5 mg) for 8 h. After that it was evaporated and residue was dissolved in anhydrous 1,2-dichloroethane (20 ml). To this solution, 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (777.2 mg, 1.5406 mmol) and trimethylsilyl trifluoro-methanesulfonate (307 μl, 1.6962 mmol) were added. This mixture was stirred at room temperature for 2 days, washed with water (20 ml) and ethyl acetate (60 ml), dried over sodium sulphate, filtered and evaporated to dryness. Yield of crude product (**4i**) (mixture of diastereoisomers) 721.3 mg (54%). Two diastereoisomers (**5i**) and (**6i**) were isolated in this order from the mixture by silica gel column chromatography using methanol in chloroform (0–5%).

Table 3
Antimicrobial activity of compounds **7h**–**i** and **8h**–**i** [MIC (μM)].

Compounds	*Enterococcus faecalis* CCM 4224	*Staphylococcus aureus* CCM 3953	*Staphylococcus aureus* (MRSA)	*Staphylococcus haemolyticus*
7h	200	100	100	200
7i	50	50	50	100
8h	200	100	100	200
8i	50	50	50	100

*4.1.4.1. Isomer (**5i**).* Yield 207.7 mg (16%), m.p. 62–63 °C, $[\alpha]_D^{25}$ + 90.2 (c 0.041, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.82 (t, 3H, CH_3, J = 6.9 Hz); 1.16–1.24 (m, 12H, CH_2); 1.37–1.47 (m, 2H, CH_2); 3.23–3.44 (m, 2H, CH_2); 4.64–4.65 (m, 2H, H-5′); 4.77–4.81 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.94–6.01 (m, 2H, H-2′, H-3′); 6.26 (d, 1H, H-1′, J = 4.5 Hz); 7.41–7.51 (m, 6H, Ph); 7.57 (d, 2H, Ph, J = 8.7 Hz); 7.62–7.69 (m, 3H, Ph); 7.82–7.87 (m, 3H, Ph); 7.93 (d, 2H, Ph, J = 6.9 Hz); 8.02 (d, 2H, Ph, J = 7.2 Hz); 8.14 (d, 2H, Ph, J = 8.7 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{46}H_{47}N_3O_{12}$: 833.90, found 832.39 [M − H]$^-$.

*4.1.4.2. Isomer (**6i**).* Yield 213.2 mg (16%), m.p. 54–55 °C, $[\alpha]_D^{25}$ + 56.4 (c 0.078, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.81 (t, 3H, CH_3, J = 6.9 Hz); 1.18–1.24 (m, 12H, CH_2); 1.42–1.51 (m, 2H, CH_2); 3.25–3.46 (m, 2H, CH_2); 4.62–4.64 (m, 2H, H-5′); 4.73–4.77 (m, 1H, H-4′); 5.34 (s, 1H, CH); 5.95–6.02 (m, 2H, H-2′, H-3′); 6.22 (d, 1H, H-1′, J = 3.6 Hz); 7.42–7.52 (m, 6H, Ph); 7.58–7.68 (m, 5H, Ph); 7.80 (s, 1H, Ph); 7.87–7.91 (m, 4H, Ph); 8.01 (d, 2H, Ph, J = 8.4 Hz); 8.14 (d, 2H, Ph, J = 9.0 Hz); 11.64 (s, 1H, NH). MS m/z Calc. for $C_{46}H_{47}N_3O_{12}$: 833.90, found 832.40 [M − H]$^-$.

*4.1.5. General procedure for preparation of β-D-ribofuranosyl-5-[alkoxy-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-diones (**7**,**8**)*

Nucleosides **5f**–**5i** and **6f**–**6i** were dissolved in MeOH/NH_3 solution (7 ml) and stirred at room temperature for 6 days. Then the mixture was evaporated, co-evaporated with methanol and purified by silica gel column chromatography using $CHCl_3$/MeOH (9/0.5).

*4.1.5.1. (+)-β-D-ribofuranosyl-5-[hexyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**7f**).* Nucleoside **7f** was prepared from **5f** (171.8 mg, 0.2170 mmol) according to general procedure.

Yield 83.1 mg (80%), m.p. 72–74 °C, $[\alpha]_D^{25}$ + 55.2 (c 0.077, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, J = 7.2 Hz); 1.23–1.35 (m, 6H, CH_2); 1.49–1.58 (m, 2H, CH_2); 3.41–3.48 (m, 2H, CH_2); 3.50–3.64 (m, 2H, H-5′); 3.88–3.98 (m, 3H, H-2′, H-3′, H-4′); 5.01 (t, 1H, 5′-OH, J = 4.8 Hz); 5.06 (d, 1H, 3′-OH, J = 4.2 Hz); 5.35–5.38 (m, 2H, 2′-OH, CH); 5.78 (d, 1H, H-1′, J = 5.4 Hz); 7.62 (d, 2H, Ph, J = 9.0 Hz); 7.98 (s, 1H, Ph); 8.18 (d, 2H, Ph, J = 8.7 Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{22}H_{29}N_3O_9$: 479.49, found 478.21 [M − H]$^-$.

*4.1.5.2. (−)-β-D-ribofuranosyl-5-[hexyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**8f**).* Nucleoside **8f** was prepared from **6f** (113.5 mg, 0.1433 mmol) according to general procedure.

Yield 60.1 mg (88%), m.p. 68–69 °C, $[\alpha]_D^{25}$ − 39.0 (c 0.39, $CHCl_3$); ^{1}H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, J = 6.9 Hz); 1.25–1.36 (m, 6H, CH_2); 1.49–1.58 (m, 2H, CH_2); 3.42–3.50 (m, 2H, CH_2); 3.52–3.56 (m, 2H, H-5′); 3.84–3.86 (m, 1H, H-4′); 3.95–3.99 (m, 1H, H-3′); 4.02–4.08 (m, 1H, H-2′); 5.02 (t, 1H,

5′-OH, $J = 4.5$ Hz); 5.10 (d, 1H, 3′-OH, $J = 5.1$ Hz); 5.39 (s, 1H, CH); 5.42 (d, 1H, 2′-OH, $J = 5.4$ Hz); 5.81 (d, 1H, H-1′, $J = 5.1$ Hz); 7.61 (d, 2H, Ph, $J = 8.7$ Hz); 7.94 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 8.7$ Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{22}H_{29}N_3O_9$: 479.49, found 478.25 $[M - H]^-$.

*4.1.5.3. (+)-β-D-ribofuranosyl-5-[heptyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**7g**).* Nucleoside **7g** was prepared from **5g** (207.6 mg, 0.2576 mmol) according to general procedure.

Yield 95.7 mg (75%), m.p. 63–65 °C, $[\alpha]_D^{25} + 7.5$ (c 0.30, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 7.2$ Hz); 1.23–1.32 (m, 8H, CH_2); 1.49–1.58 (m, 2H, CH_2); 3.35–3.48 (m, 2H, CH_2); 3.51–3.64 (m, 2H, H-5′); 3.88–3.98 (m, 3H, H-2′, H-3′, H-4′); 4.99–5.02 (m, 1H, 5′-OH); 5.06 (bs, 1H, 3′-OH); 5.35–5.38 (m, 2H, 2′-OH, CH); 5.79 (d, 1H, H-1′, $J = 4.8$ Hz); 7.63 (d, 2H, Ph, $J = 8.7$ Hz); 7.98 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 8.7$ Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{23}H_{31}N_3O_9$: 493.52, found 492.31 $[M - H]^-$.

*4.1.5.4. (−)-β-D-ribofuranosyl-5-[heptyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**8g**).* Nucleoside **8g** was prepared from **6g** (232.4 mg, 0.2884 mmol) according to general procedure.

Yield 107.5 mg (76%), m.p. 71–72 °C, $[\alpha]_D^{25} - 40.0$ (c 0.30, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 6.9$ Hz); 1.23–1.32 (m, 8H, CH_2); 1.50–1.59 (m, 2H, CH_2); 3.47–3.52 (m, 2H, CH_2); 3.54–3.60 (m, 2H, H-5′); 3.84–3.87 (m, 1H, H-4′); 3.95–3.99 (m, 1H, H-3′); 4.02–4.08 (m, 1H, H-2′); 5.00 (t, 1H, 5′-OH, $J = 4.8$ Hz); 5.09 (d, 1H, 3′-OH, $J = 4.8$ Hz); 5.39–5.41 (m, 2H, CH, 2′-OH); 5.80 (d, 1H, H-1′, $J = 5.1$ Hz); 7.62 (d, 2H, Ph, $J = 8.7$ Hz); 7.94 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 8.7$ Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{23}H_{31}N_3O_9$: 493.52, found 492.22 $[M - H]^-$.

*4.1.5.5. (+)-β-D-ribofuranosyl-5-[oktyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**7h**).* Nucleoside **7h** was prepared from **5h** (228.3 mg, 0.2785 mmol) according to general procedure.

Yield 96.0 mg (68%), m.p. 67–69 °C, $[\alpha]_D^{25} + 9.5$ (c 0.32, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 7.2$ Hz); 1.22–1.31 (m, 10H, CH_2); 1.49–1.58 (m, 2H, CH_2); 3.43–3.48 (m, 2H, CH_2); 3.54–3.65 (m, 2H, H-5′); 3.86–3.98 (m, 3H, H-2′, H-3′, H-4′); 5.00 (t, 1H, 5′-OH, $J = 5.1$ Hz); 5.06 (m, 1H, 3′-OH); 5.36 (d, 1H, 2′-OH, $J = 5.1$ Hz); 5.38 (s, 1H, CH); 5.78 (d, 1H, H-1′, $J = 4.8$ Hz); 7.63 (d, 2H, Ph, $J = 8.7$ Hz); 7.98 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 8.7$ Hz); 11.43 (s, 1H, NH). MS m/z Calc. for $C_{24}H_{33}N_3O_9$: 507.55, found 506.23 $[M - H]^-$.

*4.1.5.6. (−)-β-D-ribofuranosyl-5-[oktyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**8h**).* Nucleoside **8h** was prepared from **6h** (175.0 mg, 0.2134 mmol) according to general procedure.

Yield 76.9 mg (71%), m.p. 62–64 °C, $[\alpha]_D^{25} - 36.0$ (c 0.38, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 7.2$ Hz); 1.23–1.32 (m, 10H, CH_2); 1.49–1.57 (m, 2H, CH_2); 3.42–3.49 (m, 2H, CH_2); 3.52–3.56 (m, 2H, H-5′); 3.83–3.87 (m, 1H, H-4′); 3.95–3.99 (m, 1H, H-3′); 4.02–4.08 (m, 1H, H-2′); 5.02 (t, 1H, 5′-OH, $J = 4.5$ Hz); 5.10 (d, 1H, 3′-OH, $J = 4.8$ Hz); 5.38 (s, 1H, CH); 5.41 (d, 1H, 2′-OH, $J = 5.1$ Hz); 5.80 (d, 1H, H-1′, $J = 5.1$ Hz); 7.61 (d, 2H, Ph, $J = 8.7$ Hz); 7.95 (s, 1H, Ph); 8.18 (d, 2H, Ph, $J = 8.7$ Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{24}H_{33}N_3O_9$: 507.55, found 506.28 $[M - H]^-$.

*4.1.5.7. (+)-β-D-ribofuranosyl-5-[nonyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**7i**).* Nucleoside **7i** was prepared from **5i** (180.7 mg, 0.2167 mmol) according to general procedure.

Yield 89.0 mg (79%), m.p. 62–64 °C, $[\alpha]_D^{25} + 7.7$ (c 0.26, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 6.9$ Hz); 1.22–1.33 (m, 12H, CH_2); 1.48–1.57 (m, 2H, CH_2); 3.42–3.50 (m, 2H, CH_2); 3.54–3.64 (m, 2H, H-5′); 3.88–3.98 (m, 3H, H-2′, H-3′, H-4′); 5.02 (t, 1H, 5′-OH, $J = 4.8$ Hz); 5.07 (d, 1H, 3′-OH, $J = 4.5$ Hz); 5.37–5.38 (m, 2H, 2′-OH, CH); 5.77 (d, 1H, H-1′, $J = 4.8$ Hz); 7.62 (d, 2H, Ph, $J = 8.7$ Hz); 7.99 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 8.7$ Hz); 11.44 (s, 1H, NH). MS m/z Calc. for $C_{25}H_{35}N_3O_9$: 521.57, found 520.30 $[M - H]^-$.

*4.1.5.8. (−)-β-D-ribofuranosyl-5-[nonyl-(4-nitro-phenyl)-methyl]-pyrimidine-2,4-dione (**8i**).* Nucleoside **8i** was prepared from **6i** (186.0 mg, 0.2230 mmol) according to general procedure.

Yield 84.4 mg (73%), m.p. 63–64 °C, $[\alpha]_D^{25} - 32.2$ (c 0.38, $CHCl_3$); ^1H NMR (DMSO-d_6): δ 0.84 (t, 3H, CH_3, $J = 6.9$ Hz); 1.22–1.31 (m, 12H, CH_2); 1.49–1.58 (m, 2H, CH_2); 3.44–3.47 (m, 2H, CH_2); 3.52–3.56 (m, 2H, H-5′); 3.85–3.86 (m, 1H, H-4′); 3.95–3.99 (m, 1H, H-3′); 4.02–4.08 (m, 1H, H-2′); 5.02 (t, 1H, 5′-OH, $J = 4.2$ Hz); 5.10 (d, 1H, 3′-OH, $J = 5.1$ Hz); 5.38 (s, 1H, CH); 5.42 (d, 1H, 2′-OH, $J = 5.7$ Hz); 5.79 (d, 1H, H-1′, $J = 5.1$ Hz); 7.61 (d, 2H, Ph, $J = 9.0$ Hz); 7.94 (s, 1H, Ph); 8.19 (d, 2H, Ph, $J = 9.0$ Hz); 11.43 (s, 1H, NH). MS m/z Calc. for $C_{25}H_{35}N_3O_9$: 521.57, found 520.26 $[M - H]^-$.

4.2. Biological activity

4.2.1. Cell lines

CEM, A549, and K562 cell lines were purchased from the American Tissue Culture Collection (ATTC). Paclitaxel/daunorubicin resistant sublines of K562/CEM cells were prepared and characterized in our laboratories. The human T-lymphoblastic leukemia cell line, CEM, was used for routine screening of compounds [19] The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

4.2.2. Cytotoxicity assay

Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30,000 cells/well based on cell growth characteristics). Cells were added by pipette (80 μL) into 96-well microtiter plates. Inoculates were allowed a pre-incubation period of 24 h at 37 °C and 5% CO_2 for stabilisation. Four-fold dilutions, in 20-μL aliquots, of the intended test concentration were added at time zero to the microtiter plate wells.

All tested compounds were dissolved in 10% DMSO and concentrations were examined in quadruplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 mL) of the MTT stock solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period the formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH = 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumour cell inhibitory concentration (IC) was calculated using the following equation: $IC = (OD_{drug\text{-}exposed\ well}/\text{mean}\ OD_{control\ wells}) \times 100\%$. The IC_{50} value, the drug concentration lethal to 50% of the tumour cells, was calculated from appropriate dose-response curves.

4.2.3. Apoptosis and cell cycle analysis by FACS

CEM cells were treated with appropriate compound at concentrations corresponding to 1× and 5× IC50 values (107/537 μM, 29/144 μM and 26/130 μM) for 3, 6, 9 and 24 h. Following the incubation cells were pelleted, washed in PBS and fixed with ice-cold 70% ethanol overnight at −20 °C. Low molecular weight apoptotic DNA was extracted in citrate buffer and RNA was cleaved by RNAse (0.5 mg/ml). The DNA was stained by propidium iodide

(0.1 mg/ml), and the cells were analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).

4.2.4. BrdU incorporation and cell cycle analysis [2]

CEM cells were treated with appropriate compound at concentrations corresponding to 1× and 5× IC50 values (107/537 μM, 29/144 μM and 26/130 μM) for 3, 6, 9 and 24 h. The cultures were fed a pulse of 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min at 37 °C before harvesting. The cells were collected, washed with PBS and fixed with ice-cold 70% ethanol overnight. Then cells were washed with PBS, and resuspended in 2 M aq HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, pH 5, the cells were harvested by centrifugation and washed with PBS containing 0.5% Tween-20 and 1% BSA. They were then stained with primary monoclonal anti-BrdU antibody (1 μg/ml) (Exbio, s.r.o., Prague, Czech Republic) for 30 min at room temperature, following the incubation cells were washed with PBS and stained for 30 min at room temperature with the secondary mouse IgG-FITC antibody (4 μg/ml) (Sigma Chemical Co., Prague, Czech Republic). The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/ml) and RNAse A (0.5 mg/ml) for 1 h at room temperature in the dark and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).

4.2.5. BrU incorporation, flow cytometric analysis of RNA synthesis [3]

The cellular synthesis of RNA was analyzed by flow cytometry, based on in vitro incorporation of the RNA precursor 5′-bromouridine (BrU), followed by its immunocytochemical detection with suitable cross reacting BrDU antibody. CEM cells were treated with derivatives at concentrations corresponding to 1× and 5× IC50 values for 3, 6, 9 and 24 h. The cultures were fed a pulse of 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 min at 37 °C before harvesting. The cells were collected, washed with PBS and fixed 15 min with 1% paraformaldehyde, 0.05% NP-40 in PBS at room temperature with rotation, the fixed cells were stored in the paraformaldehyde solution at 4 °C at least overnight before staining. Cells were washed once in cold 1% glycine in PBS to quench autofluorescence and then once in PBS. They were then stained with primary monoclonal anti-BrdU antibody (1 μg/ml) (Exbio, s.r.o., Prague, Czech Republic) for 30 min at room temperature, following the incubation cells were washed with PBS and stained for 30 min at room temperature with the secondary mouse IgG-FITC antibody (4 μg/ml) (Sigma Chemical Co., Prague, Czech Republic). Then cells were fixed 15 min with 1% paraformaldehyde, 0.05% NP-40 in PBS at room temperature with rotation, washed once in cold 1% glycine in PBS. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/ml) and RNAse A (0.5 mg/ml) for 1 h at room temperature in the dark and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).

4.2.6. Antimicrobial activity

Antimicrobial activity was determined by the using of the minimum inhibitory concentration as the lowest concentration of the test substance that inhibited the growth of the bacterial strain after incubation for 24h in a thermostat at 37 °C.

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- ☒ corresponding author
- ☐ co-author

on the paper/manuscript above, hereby confirms that Lucie Brulíková has contributed to the work as stated below:

1. Intellectual input:

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: _____

2. Experimental results (indicate contribution to individual figures, tables and supplementary data):

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: Lucie Brulíková performed the syntheses of all compounds presented in the above mentioned paper.

3. Writing process:

☐ less than 25% ☐ 25 - 50 % X 50 - 75% ☐ 75 - 100%

Comments: _____

JAN HLAVÁČ

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Efficient RNA-targeting by the introduction of aromatic stacking in the duplex major groove via 5-(1-phenyl-1,2,3-triazol-4-yl)-2′-deoxyuridines

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ABSTRACT

Three pyrimidine nucleosides with differently substituted phenyltriazoles attached to the 5-position were prepared by Cu(I)-assisted azide–alkyne cycloadditions (CuAAC) and incorporated into oligonucleotides. Efficient π–π-stacking between two or more phenyltriazoles in the major groove was found to increase the thermal stability of a DNA:RNA duplex significantly. The best stacking, and most stable duplex, was obtained by a sulfonamide substituted derivative.

1. Introduction

The nucleic acid duplex constitutes an excellent scaffold for chemically designed supramolecular chemistry.[1] The duplex is formed between complementary oligonucleotide sequences on the basis of selective hydrogen-bonding and strong π–π-stacking of the nucleobases. With the aim of targeting RNA-sequences by synthetic oligonucleotides, following the so-called antisense approach,[2] the idea of synthetically increasing the stability of the duplex by increasing the stacking has been approached in several ways including synthetic nucleobases with larger ring systems.[3] A major example is a tricyclic phenoxazine replacing a cytosine and increasing the thermal stability of a DNA:RNA duplex with up to 5 °C per modification.[4] Furthermore, the 5-position of pyrimidine nucleosides has been functionalised with the propyn-1-yl group[5] as well as with five-membered heterocycles,[6] and these modifications have been found to increase the duplex stability via increased π–π-stacking.

In our former study, we followed the concept of Click Chemistry[7] and studied the Cu(I)-catalysed azide–alkyne cycloadditions (CuAAC)[8] performed on 5-ethynyl-2′-deoxyuridine (**1**, Scheme 1).[9] This building block has been used for Click Chemistry conjugation of various moieties to DNA[10,11] and leads to the positioning of a triazole in the major groove of the duplex. We found that one triazole, either unsubstituted or substituted with a phenyl or a benzyl group, in general leads to decreased duplex stability, whereas four consecutive incorporations lead to significant duplex stabilisation of a DNA:RNA duplex.[9] Hence, a 9-mer duplex with four triazoles in the centre (replacing the 5-methyl groups of the bold thymidines in the duplex 5′-dGTG**TTTT**GC:3′-rCA-CAAAACG) displayed an increase in melting temperature of 14 °C as compared to the unmodified duplex, and a further 7 °C increase was obtained by phenyl substituted triazoles (**X** in Scheme 1). Modelling demonstrated (1) a clear preference for a coplanar orientation between the pyrimidine and the triazole with the C5 of the triazole oriented towards the O4 of the uracil (via a C–H···O interaction), and (2) a significant intrastrand π–π-stacking between the triazoles in the duplex involving to some degree also the phenyl groups. In combination with CD-spectroscopy, it was also shown that the duplexes are driven towards A- or A/B-type like duplexes by the introduction of the π–π-stacking triazoles.[9]

In the present study, we explore the scope of this stacking effect of phenyltriazoles concerning (1) the degree of modification in the duplex necessary to obtain the stabilisation, and (2) the effect of hydrophilic substituents in combination with electron-donating and withdrawing properties. We therefore decided to introduce a phenol and a sulfonamide (**Y** and **Z**, respectively, Scheme 1). To our best knowledge, this is at the same time the first introduction

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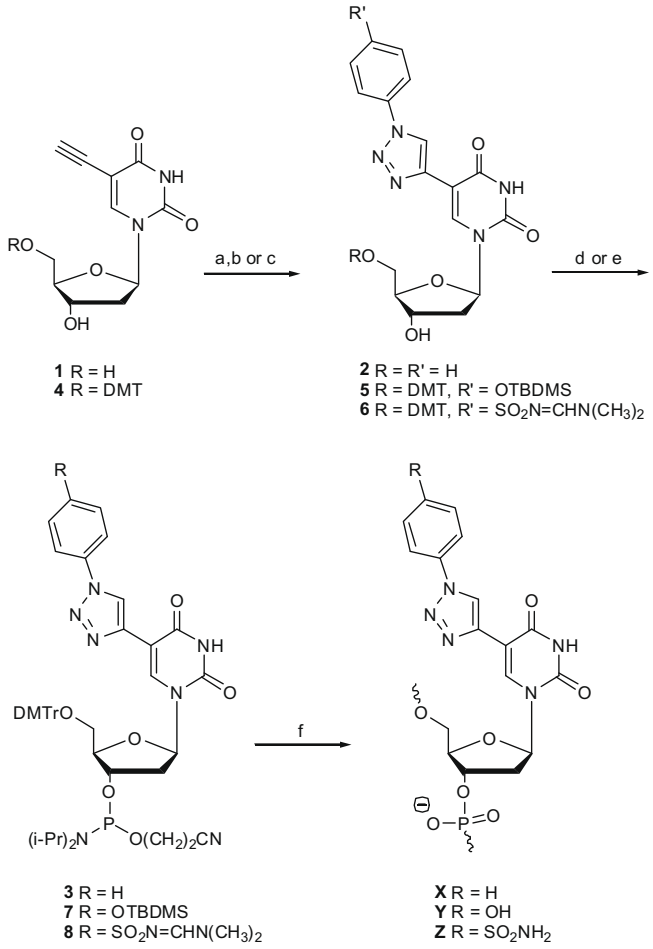


Scheme 1. Reagents: (a) Ref. 9: PhBr, NaN_3, CuI, Na ascorbate, EtOH, H_2O, MW, **2** 68%; (b) azide **10**, CuI, Na ascorbate, pyridine, EtOH, H_2O, **5** 78%; (c) azide **12**, $CuSO_4$, Na ascorbate, *t*-BuOH, H_2O, THF, pyridine, **6** 88%; (d) Ref. 9: (i) DMT–Cl, pyridine, CH_3CN; (ii) $NC(CH_2)_2OPClN(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, **3** 42%; (e) $NC(CH_2)_2OPClN(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, **7** 74%, **8** 67%; (f) automated DNA synthesis. DMT = 4,4′-dimethoxytrityl, TBDMS = *t*-butyldimethylsilyl.

of a sulfonamide into DNA. Ultimately, these aromatic nucleosides are extremely simple building blocks for the design of oligonucleotides with improved and selective hybridisation to complementary RNA and hereby an interesting potential in antisense therapeutics.[2]

2. Results and discussion

2.1. Chemical synthesis

In our first study, we prepared the triazole containing pyrimidine nucleosides directly from the unprotected 5-ethynyl-2′-deoxyuridine **1** using sodium azide, aryl or alkyl halides, and an in situ azidation/cycloaddition protocol (Scheme 1).[9] Each nucleoside, including **2**, was hereafter protected at the 5′-position by the 4,4′-dimethoxytrityl (DMT) group and converted to 3′-*O*-phosphoramidites in order to afford building blocks suitable for incorporation into oligonucleotides using standard automated solid phase DNA synthesis. By the phosphoramidite **3**, oligonucleotides containing the 5-(1-phenyl-1,2,3-triazol-4-yl)-2′-deoxyuridine moiety **X** were obtained.[9] For the present study, we obtained the best results by performing the cycloaddition reactions on the 5′-*O*-DMT-protected 5-ethynyl-2′-deoxyuridine **4** using azides that were prepared and isolated from suitable building blocks (Scheme 2). For introducing a phenol moiety, the protected azide **10** was prepared by direct silylation of the known *p*-azidophenol **9**, which has been prepared from *p*-bromophenol.[12] The TBDMS-group was chosen as an appropriately base-sensitive protection for the phenol group. For introducing a sulfonamide moiety, the known *p*-azidobenzensulfonamide **11**, made by a diazotation of *p*-aminobenzensulfonamide,[13] was protected with the equally base-sensitive dimethylamidine group to give **12**. The azides were reacted with **4** in the Cu(I)-catalysed cycloaddition to give the two protected triazole nucleosides **5** and **6** in good yields (Scheme 1). These were converted to the corresponding phophoramidites **7** and **8**, respectively.

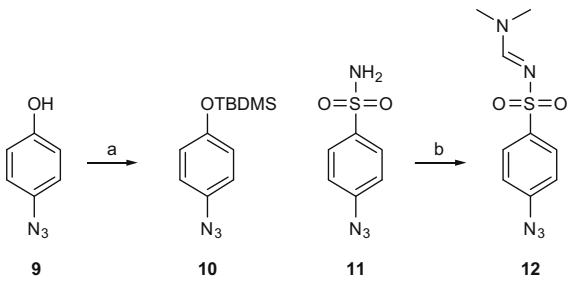
The phosphoramidites **3**, **7** and **8** were successfully incorporated into oligodeoxynucleotides using automated solid phase synthesis with tetrazole as the activator and extended coupling times for the modified phosphoramidites. After completion of the synthesis, the oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia. This treatment also removed all protecting groups including the silyl protection of the phenol and the amidine protection of the sulfonamide giving the incorporated monomers **Y** and **Z**, respectively, (Scheme 1). The three monomers **X**, **Y** and **Z** were incorporated into the same series of 9-mer oligonucleotides **ON1–ON6** (Tables 1 and 2). The constitution and purity of these were controlled by MALDI-MS and RP-HPLC, respectively.

2.2. Hybridisation studies

The series of oligonucleotides for this study was chosen with the purpose of finding the minimum of modification needed for duplex stabilisation. Hence, the 9-mer oligonucleotides with one and four incorporations of **X** in the centre, **ON1** and **ON6**, were taken from our former study,[9] whereas **ON2–ON5** represent different positions of a single modification as well as two or three consecutive incorporations of **X**. The same series **ON1–ON6** was hereafter prepared with both **Y** and **Z** (Table 1).

The hybridisation studies of the oligonucleotides were performed by UV-spectroscopy. For determining concentrations, extinction coefficients for oligonucleotides were determined by standard methods using extinction coefficients for the single nucleotides. For the modified monomers **X** and **Y**, the extinction coefficients at 260 nm for the deprotected phenyl[9] and hydroxyphenyltriazole nucleosides were determined by UV-measurements. Ab initio calculations of UV-spectra for a series of derivatives of **X** indicated no increased absorption at 260 nm for **Z** compared to **X** and therefore a similar extinction coefficient was assumed. In order to secure a concentration of 1.5 μM for the duplexes, however, a practical 50% lower coefficient for monomer **Z** was applied in the hybridisation experiments.

The oligonucleotides were mixed with the complementary DNA and RNA-sequences and the melting temperatures (T_m) of the resulting duplexes were determined. Table 1 shows the results



Scheme 2. Reagents: (a) TBDMS–Cl, DMAP, pyridine, CH_3CN, 90%; (b) DMF, $POCl_3$, 73%.

Table 1
Hybridisation data for DNA:DNA duplexes[a]

		T_m (ΔT_m/mod.)/(°C)[b]		
		B = X	**Y**	**Z**
ON1	5′-dGTG T**B**T TGC	28.0[c] (−5.0)	28.5 (−4.5)	28.0 (−5.0)
ON2	5′-dGTG **B**TT TGC	30.0 (−3.0)	29.5 (−3.5)	29.0 (−4.0)
ON3	5′-dGTG **BB**T TGC	30.0 (−1.5)	29.0 (−2.0)	31.5 (−0.8)
ON4	5′-dGTG T**BB** TGC	30.0 (−1.5)	28.5 (−2.3)	30.5 (−1.3)
ON5	5′-dGTG **BBB** TGC	30.0 (−1.0)	29.0 (−1.3)	33.0 (0.0)
ON6	5′-dGTG **BBB B**GC	32.0[c] (−0.3)	29.0 (−1.0)	35.5 (+0.7)

[a] Target sequence 5′-dGCA AAA CAC.
[b] Melting temperatures (T_m values/°C) obtained from the maxima of the first derivatives of the melting curves (A_{260} vs temperature) recorded in a medium salt buffer (Na_2HPO_4 (5 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.5 μM concentrations of each strand. In brackets the changes in melting temperature for each modification **B** (ΔT_m/mod./°C) as compared to the unmodified reference duplex (T_m = 33.0 °C).
[c] Data taken from Ref. 9.

Table 2
Hybridisation data for DNA:RNA duplexes[a]

		T_m (ΔT_m/mod.)/°C[b]		
		B = X	**Y**	**Z**
ON1	5′-dGTG T**B**T TGC	29.0[c] (−2.0)	30.5 (−0.5)	30.0 (−1.0)
ON2	5′-dGTG **B**TT TGC	30.0 (−1.0)	29.0 (−2.0)	29.0 (−2.0)
ON3	5′-dGTG **BB**T TGC	35.0 (+2.0)	37.0 (+3.0)	35.5 (+2.3)
ON4	5′-dGTG T**BB** TGC	37.5 (+3.3)	39.0 (+4.0)	39.0 (+4.0)
ON5	5′-dGTG **BBB** TGC	43.0 (+4.0)	45.0 (+4.7)	46.0 (+5.0)
ON6	5′-dGTG **BBB B**GC	51.5[c] (+5.1)	51.0 (+5.0)	55.5 (+6.1)
Mismatch sequences[d]				
ON6	5′-dGTG **BBB B**GC 3′-rCAC ACA ACG	24.0[c] (−27.5)	26.5 (−24.5)	nt[e]
ON6	5′-dGTG **BBB B**GC 3′-rCAC AGA ACG	42.0[c] (−9.5)	41.0 (−10.0)	46.0 (−9.5)
ON6	5′-dGTG **BBB B**GC 3′-rCAC AUA ACG	31.0[c] (−20.5)	29.0 (−22.0)	*nt*[e]

[a] Matched target sequence 5′-rGCA AAA CAC.
[b] Melting temperatures (T_m values/°C) obtained from the maxima of the first derivatives of the melting curves (A_{260} vs temperature) recorded in a medium salt buffer (Na_2HPO_4 (5 mM), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.5 μM concentrations of each strand. In brackets the changes in melting temperature for each modification **B** (ΔT_m/mod./°C) as compared to the unmodified reference duplex (T_m = 31.0 °C).
[c] Data taken from Ref. 9.
[d] Mismatch studies, in brackets the changes in melting temperature as compared to the matched duplex **ON6**:RNA.
[e] No transition observed.

obtained with modified DNA:DNA duplexes. A single incorporation of either of the modified monomers **X**, **Y** or **Z** once in the centre of the duplex, **ON1**, lead to a significant decrease in duplex stability as validated by T_m's around 5 °C lower than for the unmodified duplex. The same was observed for **ON2** although the decreases in T_m were somewhat smaller. With the second incorporation of the modified monomers, in **ON3** and **ON4**, the relative destabilisation was even less pronounced, especially in the case of the sulfonamide **Z** (ΔT_m = −0.8 °C per mod.). The same trend continued with three incorporations, **ON5**, and with four modified nucleoside monomers, **ON6**, the decrease in duplex stability was fully compensated by the stacking of the modified nucleobases. Hence, the duplex stabilities were similar to the unmodified duplex, varying from a small decrease with the phenol **Y** (ΔT_m = −1.0 °C per mod.) to a small increase with the sulfonamide **Z** (ΔT_m = +0.7 °C per mod.).

Table 2 shows the hybridisation data of the modified DNA:RNA duplexes. A single incorporation of either **X**, **Y** or **Z**, in **ON1** or **ON2**, lead to decreases in thermal stability that were somewhat smaller than with DNA:DNA (ΔT_m's between −0.5 and −2.0 °C). This picture changed significantly by the introduction of the second modified nucleoside. **ON3** displayed significantly increased duplex stabilities, and in the slightly different sequence **ON4**, even further increases were observed with ΔT_m's up to +4 °C per modification. The tendency continued by three and four incorporations in **ON5** and **ON6** revealing further relative increases in duplex stability with ΔT_m's up to +6.1 °C per modification for the four sulfonamide substituted phenyltriazoles **Z**. Interestingly, the hyperchromicity observed for the melting of a duplex generally decreased by the numbers of monomer **Z** but not in the case of monomers **X** and **Y**. Nevertheless, the melting transitions were clearly determined.

Comparing the data for the modified DNA:RNA duplexes in a different way, the introduction of the second phenyltriazole moiety on the top of the first gave an increase in T_m of 5 °C (compare **ON3** with **ON2**, Table 2) or even 8.5 °C (compare **ON4** with **ON1**) for **X**. The third incorporation of **X** gave an increase in T_m of 5.5 or 8 °C (compare **ON5** with **ON3** or **ON4**), and the fourth incorporation of **X** gave a further increase in T_m of 8.5 °C (compare **ON6** with **ON5**). The corresponding increases in T_m for the phenol moiety **Y** were +8/8.5° for the second, +6/+8 °C for the third and +6 °C for the fourth incorporation. For the sulfonamide **Z**, the increases in T_m were +6.5/9° for the second, +7/+10.5 °C for the third and +9.5 °C for the fourth incorporation.

The remarkable RNA recognition was further investigated by mismatch studies. Hence, **ON6** with either **X**, **Y** or **Z** was mixed with RNA-sequences containing a single central mismatch, and the melting temperatures of the mismatched duplexes were determined (Table 2). In most cases, fine mismatch discrimination was observed as indicated by the large decreases in T_m relative to the matched duplexes formed by **ON6**. A to C mismatches were perfectly discriminated by **X** and **Y** (ΔT_m values of −27.5 and −24.5 °C, respectively), whereas no mismatched duplex by **Z** could be detected. Similar results were observed for the A to U mismatches, whereas the discrimination of the A to G mismatches was slightly smaller for all the three modifications **X**–**Z** (ΔT_m's around −10 °C). This is however similar to unmodified DNA:RNA duplexes, where the A to G mismatch is the most stable of the three.

The fact that two mismatched duplexes formed by **ON6**-**Z** were not detectable by UV-spectroscopy was puzzling but seemed associated with the generally low hyperchromicity observed for the DNA:RNA duplexes with **Z**. With the local denaturation induced in the middle of the duplex by a mismatch the transition might be undetectable by the UV-methodology.

2.3. Circular dichroism spectroscopy

To further study the influence of π–π-stacking and the stepwise increasing modification on the duplex structure, circular dichroism (CD) spectroscopy was applied. It is well known that DNA:DNA duplexes adopt a B-type form in solution, whereas RNA:RNA duplexes adopt an A-type and DNA:RNA duplexes intermediate A/B-type structures. A- and B-type duplexes are known to display distinctly different CD spectra. A-type duplexes give an intense negative band at ~210 nm and a positive band at ~260 nm, whereas B-type

duplexes give a negative band at ~250 nm and positive bands at ~220 and ~280 nm. For this study the unmodified DNA:DNA duplex was taken as a standard for the B-type, and the CD-spectrum (Fig. 1) clearly displayed the B-type characteristics. The DNA:RNA duplex showed the expected intermediate A/B-type with some clear A-type characteristics. The single incorporation of monomer **X** in **ON2** indicated that the DNA:DNA duplex retained its inherent B-type form (Fig. 1). This is similar to what was observed for **ON1-X** in our first study.[9] However, the double incorporation of **X** in **ON3** and **ON4** resulted in two slightly different CD-curves. **ON3** resembled **ON2**, whereas **ON4** showed a small shift of the band at 280 nm towards 275 nm. Upon the triple incorporation of **X** in **ON5** we observed a further small shift towards the A/B-type helical form with a beginning shoulder at 265 nm and a decreasing negative band at 250 nm. This is fully in accordance with our previous observations for **ON6-X**.[9]

For the DNA:RNA hybrid duplex, only small changes in the CD-spectra were observed by the introduction of **X** (Fig. 2). Hence, all curves were similar to the one obtained from the unmodified DNA:RNA duplex indicating that the modifications are not changing the overall A/B-type duplex structure. This is consistent with our first study where both **ON1-X** and **ON6-X** displayed similar CD-curves with RNA.[9]

In the case of the sulfonamide modification **Z**, the CD-curves revealed somewhat different observations. For the DNA:DNA duplex, the single modification in **ON1** demonstrated almost no changes in the CD-spectrum, whereas the spectrum for **ON2** demonstrated a lower band at 280 nm and a beginning shoulder at 265 nm (Fig. 3). With the two modifications in **ON3** and **ON4**, this trend continued with the largest changes observed for **ON3**. In other works, both the number of modifications and the sequence context influenced the gradual change in duplex structure. With the three modifications in **ON5**, an even larger band at 265 nm was seen indicating the expected shift towards an A/B-type duplex but also other changes in the CD curve were seen. With **ON6**, the positive band has moved backwards to 275 nm, and the negative band at 250 nm has become very small.

For the DNA:RNA duplexes containing the sulfonamide **Z**, all CD-spectra indicated that the modified duplexes had an A/B-type hybrid duplex conformation not differing significantly from the unmodified DNA:RNA duplex (Fig. 4). With the increasing number of modifications, however, the intensity of the negative band at 250 nm was decreasing and moving towards 235 nm, and a new small positive band appeared at 310 nm.

2.4. Molecular modelling

The DNA:RNA hybrid duplexes formed by the oligonucleotides with two and four consecutive incorporations of monomer **X**, **Y** and **Z** (**ON3** and **ON6**) were built in MACROMODEL[14] and studied in molecular dynamics simulations. The initial hybrid structures were built in the B-type duplex conformation and the incorporated monomers were subjected to a Monte Carlo conformational search verifying the C5 (pyrimidine)–C4 (triazole) bond previously studied via ab initio calculations.[9] The obtained lowest energy structure was then subjected to a 5 ns molecular dynamics simulation during which 500 structures were sampled. These 500 structures were subsequently minimised, and the local minimum structure obtained was used for further analysis. Models of the resulting modified duplexes are shown in Figures 5 and 6.

The DNA:RNA duplexes with four consecutive incorporations of either **X**,[9] **Y** and **Z** (Fig. 5) were found to be A/B-type duplexes with almost perfect stacking between both triazoles and phenyl moieties. However, some differences were observed. In the duplex with the phenol moieties **Y**, the aromatic rings seems to bend slightly away towards the 5′-end, perhaps from a repulsion between the hydroxy groups. The duplex with the sulfonamide monomer **Z**, on the other hand, demonstrated a perfect stacking of the aromatic rings and apparently a consistent organisation between the neighbouring sulfonamide groups. Furthermore, some distortion in the duplex inclination was observed in all three cases but most pronounced with **Z**. Thus, a short 2.8 Å distance consistent with hydrogen-bonding was observed between the second sulfonamide group from the 5′-end in **ON6-Z** and the 2′-hydroxyl group of the 5′-terminal cytosine in the complementary RNA strand. This seemed to be forcing the duplex to bend, although, the Watson–Crick base pairing was conserved.

Also the DNA:RNA duplexes with two consecutive incorporations of either **X**, **Y** and **Z** (Fig. 6) were found to be A/B-type duplexes. Stacking between the aromatic systems are observed in

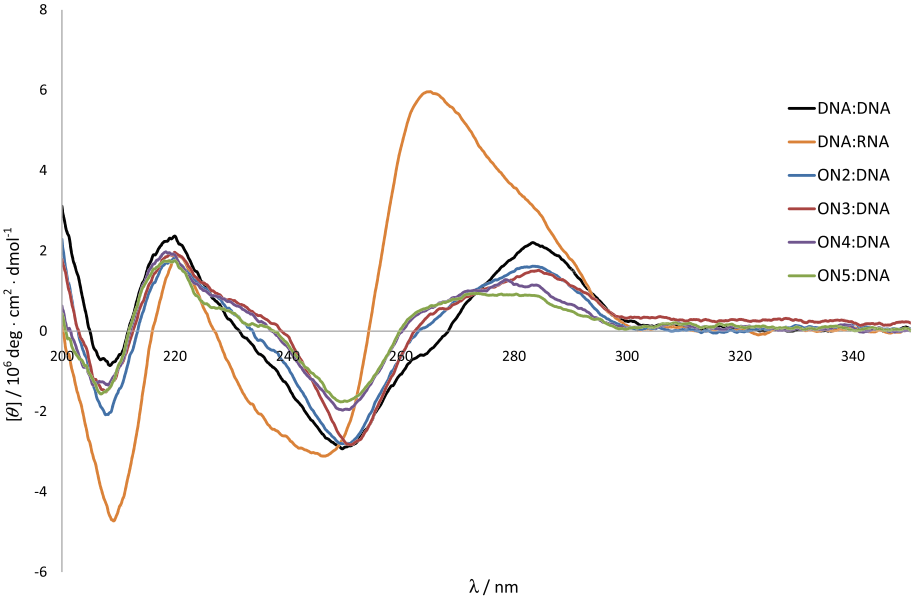


Figure 1. CD spectra of the DNA:DNA duplexes containing one to three incorporations of **X**.

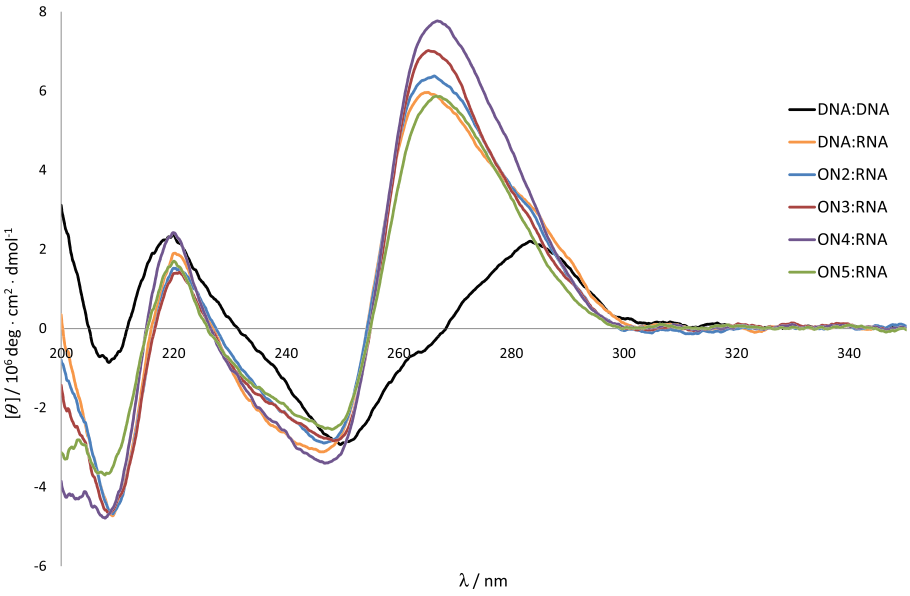


Figure 2. CD spectra of the DNA:RNA duplexes containing one to three incorporations of **X**.

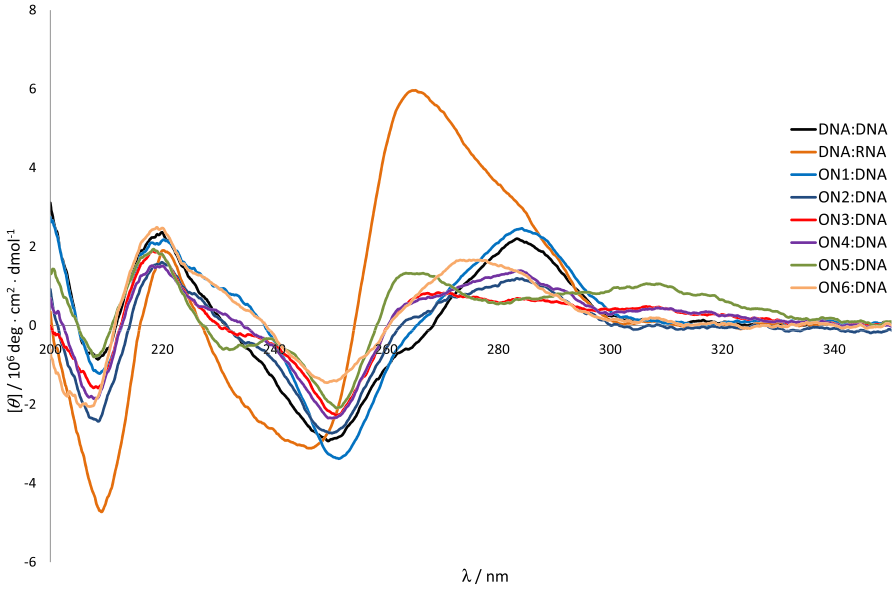


Figure 3. CD spectra of the DNA:DNA duplexes containing one to four incorporations of **Z**.

all three cases, although the two modified nucleobases were tilted out of the nucleobase plane pointing towards the 3′-end of the strand. This is less pronounced with the two monomers **X** but in the 3′-modification of the two, the co-planarity between the two heterocycles appeared to be lost. For monomer **Y**, this tilting of the modifications towards the 3′-end was even more pronounced, although no changes in the co-planarity were observed. An almost similar structure is seen with monomer **Z**.

3. Discussion

In the present study we have demonstrated the simple and efficient synthesis of two new nucleoside building blocks following the CuAAC method. Clearly, 5-ethynyl-2′-deoxyuridine, **1**, constitutes an obvious substrate for the easy preparation of various triazoles, and click chemistry is hereby a convenient method for introducing aromatic stacking in the major groove of nucleic acid duplexes.

The hybridisation data for the oligonucleotides of the current study followed the trend of our first study demonstrating that one incorporation of **X** lead to decreased stability of both DNA:DNA and DNA:RNA duplexes, whereas four consecutive incorporations of **X** demonstrate a net neutral effect on the stability of the DNA:DNA duplex and a massive increase in stability of the DNA:RNA duplex. Herein, we have demonstrated that the increase in duplex stability graduates by the number of modifications, and that two incorporations were enough to see the major part of the effect. The reason for the significant decrease in duplex stability obtained with single modifications with **X** might be found in the hydrophobicity of the phenyl group and the distortion in hydration of the duplex. This can, however, be

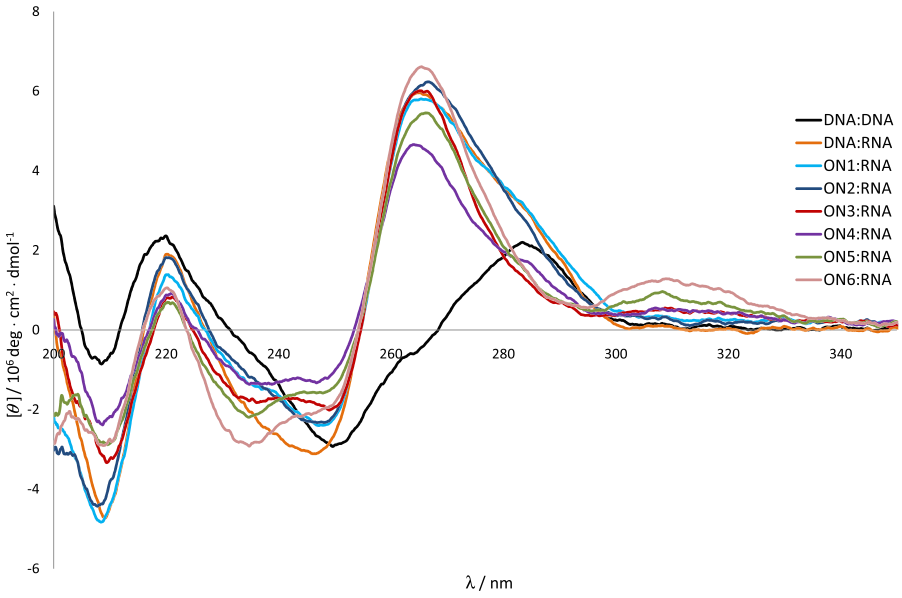


Figure 4. CD spectra of the DNA:RNA duplexes containing one to four incorporations of **Z**.

completely counterbalanced by the stacking of two phenyltriazole moieties as indicated by the very large increase in thermal stability of DNA:RNA duplexes when going from **ON1** and **ON2** to **ON3** and **ON4**. Also in the DNA:DNA duplexes some compensation by the second modification was clearly seen. The third and fourth consecutive incorporation of **X** lead to further relative increase in stabilisation up to an increase in thermal stability of the DNA:RNA duplex of 5 °C for each modification. The observation that only two consecutive incorporations of **X** were enough to give significant duplex stabilisation is very important for practical RNA-targeting, as the number of possible target sequences increases dramatically. Future studies will show, whether triazoles attached to other nucleobases can demonstrate the same duplex stabilisation by stacking with **X** hereby open the access to even more potential target RNA-sequences.

The two new derivatives **Y** and **Z** displayed almost similar influence on duplex stability as **X** indicating only small influence from the substituents on the distal phenyl position. In the DNA:DNA duplexes, the phenol **Y** seems to give the largest decreases in duplex stability, whereas the partial compensation obtained with consecutive incorporations seems to be most pronounced with the sulfonamide **Z**. In the DNA:RNA duplexes, the only obvious difference between **X**, **Y** and **Z** was that the stabilising effect of stacking three or four building blocks is even more pronounced with **Z** ending with the most stable duplex of the entire study with a T_m of 55.5 °C corresponding to a gain in thermal stability of +6.1 °C per modification.

When considering duplex structures as studied by modelling and CD-spectroscopy, some differences between the three modifications were indicated supporting the hybridisation data. The sulfonamide modification **Z** seems to have the largest impact on duplex structure of the three, probably due to the most efficient stacking behaviour. The modelling data indicated some bending of the DNA:RNA duplex with 4 × **Z** due to a hydrogen-bonding interaction across the major groove, and the CD-spectrum supported some deviation from the standard A/B-type duplex.

The current study demonstrated the value of π–π-stacking for obtaining duplexes with increased thermal stability. The effect of stacking was strongest in the DNA:RNA duplexes as compared to DNA:DNA duplexes, which might be due to the A/B-type duplex form being shorter and more compact than the B-type duplex. In the modified DNA:DNA, the increasing number of modifications and hereby the increasing stacking in the major groove was followed by a shift in duplex structure towards an A/B-type form as demonstrated by CD-spectroscopy. In the DNA:RNA duplex, the A/B-type was more or less retained when the modifications were introduced.

With the specific goal of targeting RNA, oligonucleotides containing enlarged bi- or tricyclic nucleobases or aromatic substituents on the nucleobases have been approached before.[4,6] In the case of the phenoxazine cytosine analogue, increased effect by the number of consecutive incorporations due to stacking in the major groove has also been demonstrated.[4] Nevertheless, the nucleoside monomers **X**, **Y** and **Z** from the current study demonstrates that this stacking effect can be obtained with simpler aromatic moieties obtained by straightforward click chemistry. This simple approach opens the possibility for a large variation of different entities in the major groove and with a much wider range of potential target sequences. The 5-(1,2,3-triazole-4-yl)pyrimidine nucleosides can therefore be important future building blocks for the development of antisense oligonucleotides.

4. Conclusions

From the easily available 5-ethynyl-2′-deoxyuridine, three simple nucleic acid building blocks introducing triazoles into the major groove of nucleic acid duplexes have been obtained. The stacking of triazoles, and the aromatic substituents attached, in the major groove leads to very stable DNA:RNA duplexes—the most stable containing a distal sulfonamide moiety. Efficient RNA-targeting, and hereby therapeutic potential, can be obtained with oligonucleotides containing only two consecutive incorporations of the triazoles.

5. Experimental section

All commercial reagents were used as supplied. Reactions were carried out under argon or nitrogen when anhydrous solvents were used. Column chromatography was performed with Silica Gel 60 (particle size 0.040–0.063 μm, Merck). NMR spectra

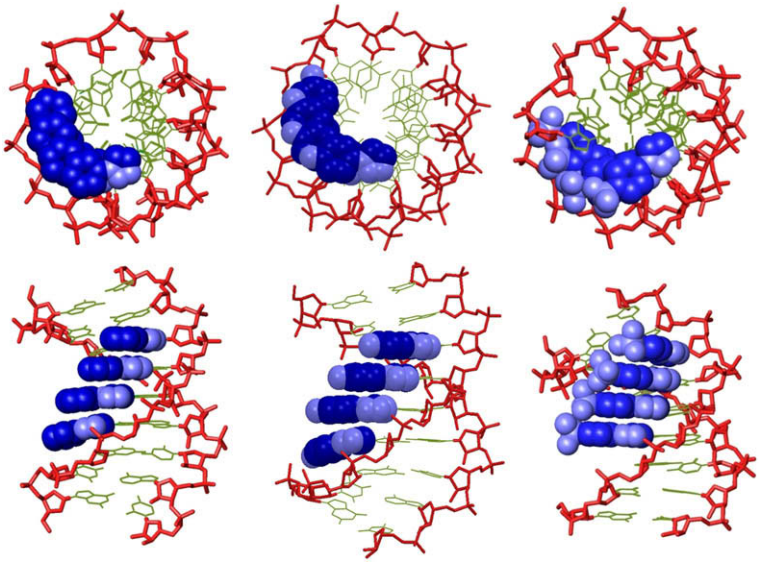


Figure 5. Modelling structures of modified DNA:RNA duplexes containing four consecutive incorporations of **X**, **Y** or **Z**. From left to right: **ON6**-**X**:RNA, **ON6**-**Y**:RNA, **ON6**-**Z**:RNA. Blue: 5-substituents with the heteroatoms in light blue; red: backbone; green: nucleobases.

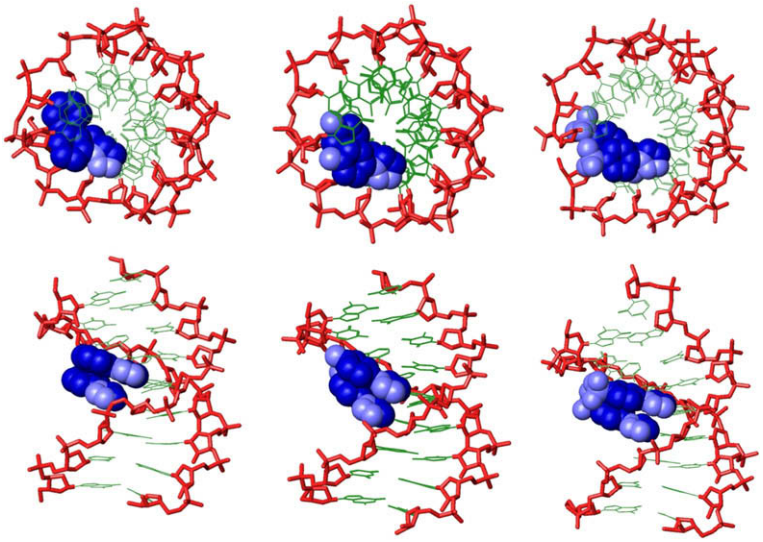


Figure 6. Modelling structures of modified DNA:RNA duplexes containing two consecutive incorporations of **X**, **Y** or **Z**. From left to right: **ON3**-**X**:RNA, **ON3**-**Y**:RNA, **ON3**-**Z**:RNA. Blue: 5-substituents with the heteroatoms in light blue; red: backbone; green: nucleobases.

were recorded on a Varian Gemini 2000 spectrometer or a Bruker Advance III 400 spectrometer. Values for δ are in ppm relative to tetramethylsilane as an internal standard or 85% H_3PO_4 as an external standard. Assignments of NMR-signals when given are based on 2D spectra and follow standard nucleoside convention. ESI mass spectra as well as accurate mass determinations were performed on a Thermo Finnigan TSQ 700 spectrometer. Microwave heated reactions were performed on an Emrys™ Creator.

5.1. Synthesis of 5-(1-(4-(*tert*-butyldimethylsilyloxy)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4′-dimethoxytrityl)-2′-deoxyuridine (5)

To a solution of nucleoside **4** (485 mg, 0.88 mmol) and the azide **10** in ethanol and water (10 mL, 7:3, v/v) was added CuI (108 mg, 0.57 mmol), sodium ascorbate (281 mg, 1.42 mmol) and pyridine (3 mL). The mixture was stirred at rt for 4.5 h and then concentrated under reduced pressure. The residue was co-evaporated with toluene (2 × 10 mL), and methanol (10 mL), and then purified by column chromatography (0–10% CH_3OH in CH_2Cl_2) to give the nucleoside **5** (550 mg, 78%) as a white foam. R_f 0.3 (5% MeOH in CH_2Cl_2). ^{1}H NMR (DMSO-d_6; 300 MHz) δ 11.81 (s, 1H, NH), 8.72 (s, 1H, triazole-H), 8.40 (s, 1H, H-6), 7.77 (d, 2H, J = 8.9 Hz, Ar), 7.39 (m, 2H, Ar), 7.29–7.21 (m, 6H, Ar), 7.15 (m, 1H, Ar), 7.04 (d, 2H, J = 8.9 Hz, Ar), 6.82 (dd, 4H, J = 8.7, 1.5 Hz, Ar), 6.21 (t, 1H, J = 6.3 Hz, H-1′), 5.37 (d, 1H, J = 4.5 Hz, 3′-OH), 4.23 (m, 1H, H-3′), 3.98 (m, 1H, H-4′), 3.68 (s, 3H, OCH_3), 3.67 (s, 3H, OCH_3), 3.25–3.22 (m, 2H, H-5′), 2.33–2.27 (m, 2H, H-2′), 0.98 (s, 9H, $(CH_3)_3C$), 0.24 (s, 6H, $(CH_3)_2Si$). ^{13}C NMR (DMSO-d_6; 75 MHz) δ 161.1 (C-4), 158.0, 155.3 (Ar), 149.7 (C-2), 149.4, 144.8 (Ar), 136.2, 135.5, 135.4 (C-6, Ar), 130.7 (C-4 triazole), 129.7, 129.6, 127.7, 127.6, 126.5, 121.9, 120.8 (Ar), 120.1 (C-5 triazole), 113.1 (Ar), 104.8 (C-5), 85.7 (C-4′), 85.7 (Ar_3C), 85.3 (C-1′), 70.4 (C-3′), 63.6 (C-5′), 54.9 (OCH_3), 39.5 (C-2′), 25.5 ($(CH_3)_3C$), 17.9 ($(CH_3)_3C$), −4.6

($(CH_3)_2Si$). HiRes ESI MS *m/z* (M+Na) found/calcd 826.3213/826.3243.

5.2. Synthesis of 5-(1-(4-(*N*-((dimethylamino)methylidene)aminosulfonyl)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4′-dimethoxytrityl)-2′-deoxyuridine (6)

To a suspension of the nucleoside **4** (300 mg, 0.54 mmol), the azide **12** (178 mg, 0.70 mmol), sodium ascorbate (65 mg, 0.32 mmol) and $CuSO_4{\cdot}5H_2O$ (25 mg, 0.1 mmol) in H_2O/*t*-BuOH (8 mL, 1:1, v/v) was added THF (1 mL) and pyridine (0.25 mL). The resulting clear solution was stirred at rt for 14 h, and then diluted with CH_2Cl_2 (50 mL) and brine (30 mL). The phases were separated, and the organic phase was washed with a saturated aqueous solution of $NaHCO_3$ (30 mL). The combined aqueous phase was extracted with EtOAc (2 × 30 mL), and the combined organic phase was dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by column chromatography (0–8% MeOH in CH_2Cl_2) to afford the nucleoside **6** (220 mg, 87%) as a white foam. R_f 0.3 (5% *i*-PrOH in $CHCl_3$). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.70 (br s, 1H, NH), 8.55 (br s, 1H, HC=N), 8.17 (s, 1H, triazole-H), 8.06 (m, 2H, Ar), 7.90 (d, 2H, *J* = 8.0 Hz, Ar), 7.40 (d, 2H, *J* = 7.6 Hz, Ar), 7.33–7.12 (m, 8H, Ar, H-6), 6.82 (m, 4H, Ar), 6.32 (t, 1H, *J* = 6.0 Hz, H-1′), 4.45 (br s, 1H, H-3′), 4.07 (m, 1H, H-4′), 3.74 (s, 6H, OCH_3), 3.45–3.29 (m, 2H, H-5′), 3.16 (s, 3H, CH_3), 3.05 (s, 3H, CH_3), 2.48 (m, 1H, H-2′), 2.31 (m, 1H, H-2′). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.9, 158.58, 158.56, 144.6, 142.4, 135.7, 135.6, 130.1, 130.1, 128.3, 128.1, 127.9, 126.9, 120.2, 113.3, 86.9, 85.9, 77.2, 72.5, 63.6, 55.2, 41.6, 35.7. HiRes ESI MS *m/z* (M+Na) found/calcd 830.2610/830.2578.

5.3. Synthesis of 5-(1-(4-(*tert*-butyldimethylsilyloxy)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4-dimethoxytrityl)-3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphinyl)-2′-deoxyuridine (7)

The nucleoside **5** (213 mg, 0.26 mmol) was dried by the coevaporation with anhydrous CH_2Cl_2 (2 × 5 mL) and dissolved in anhydrous CH_2Cl_2 (5.5 mL). DIPEA (0.23 mL, 1.33 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropyl-phosphoramidochloridite (177 μL, 0.79 mmol) were added, and the mixture was stirred at rt for 1 h, 15 min. The solution was diluted with CH_2Cl_2 (25 mL) and washed with brine (25 mL) and water (25 mL). The organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (2–5% acetone in CH_2Cl_2) to give the product **7** (196 mg, 74%) as a white foam. R_f 0.8 (10% acetone in CH_2Cl_2). ^{31}P NMR (DMSO-d_6, 121.5 MHz) δ 148.6, 148.3. HiRes ESI MS *m/z* (M+Na) found/calcd 1026.4308/1026.4321).

5.4. Synthesis of 5-(1-(4-(*N*-((dimethylamino)methylidene)aminosulfonyl)phenyl)-1,2,3-triazol-4-yl)-5′-(4,4-dimethoxytrityl)-3′-*O*-(*P*-(2-cyanoethoxy)-*N*,*N*-diisopropylamino-phosphinyl)-2′-deoxyuridine (8)

The nucleoside **6** (300 mg, 0.37 mmol) was dried by the coevaporation with anhydrous CH_2Cl_2 (2 × 10 mL) and dissolved in anhydrous CH_2Cl_2 (10 mL). DIPEA (0.25 mL, 1.4 mmol) and 2-cyanoethyl-*N*,*N*′-diisopropyl-phosphoramidochloridite (0.25 mL, 1.1 mmol) were added, and the reaction mixture was stirred at rt for 4 h. The solution was diluted with CH_2Cl_2 (25 ml) and washed with a 5% aqueous solution of $NaHCO_3$ (2 × 10 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 10 mL) and the combined organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by column chromatography (0–96% EtOAc in petroleum ether) to give the phosphoramidite **8** (250 mg, 67%) as a white foam. R_f 0.5 (7.5% *i*-PrOH in $CHCl_3$). ^{31}P NMR ($CDCl_3$, 162 MHz) δ 149.1, 148.7. HiRes ESI MS *m/z* (M+Na) found/calcd 1030.3629/1030.3657.

5.5. Synthesis of *tert*-butyldimethylsilyl 4-azidophenylether (10)

A solution of the azide **9** (1.12 g, 8.27 mmol), *tert*-butyldimethylsilyl chloride (3.74 g, 24.8 mmol) and DMAP (0.5 g, 4.09 mmol) in a mixture of pyridine and acetonitrile (32 mL, 1:1, v/v) was stirred at rt for 48 h. The mixture was concentrated under reduced pressure and the residue was co-evaporated with toluene (2 × 20 mL) and methanol (10 mL). The residue was purified by column chromatography (CH_2Cl_2) to give the azide **10** (1.86 g, 90%) as a yellow liquid. R_f 0.9 (CH_2Cl_2). IR (KBr) 2122.4 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz) δ 7.01 (m, 2H, Ar), 6.89 (m, 2H, Ar), 0.94 (s, 9H, $(CH_3)_3C$), 0.17 (s, 6H, $(CH_3)_2Si$). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 152.5, 132.3, 121.2, 120.2 (Ar), 25.5 ($(CH_3)_3C$), 17.9 ($(CH_3)_3C$), −4.7 ($(CH_3)_2Si$). HiRes ESI MS *m/z* (M+Na) found/calcd 272.1184/272.1190.

5.6. Synthesis of *N*-dimethylaminomethylidene-4-azidobenzenesulfonamide (12)

To a cold stirred solution of $POCl_3$ (0.98 mL, 10.6 mmol) in dimethylformamide (20 mL) was added the azide **11** (1.05 g, 5.30 mmol). The reaction mixture was stirred at rt for 3 h, and then poured into cold water and neutralised with saturated aqueous ammonia. The formed precipitate was isolated, washed with water (100 mL) and dried to afford the product **12** (0.98 g, 73%) as white solid. R_f 0.4 (40% EtOAc in petroleum ether). Mp 155–156 °C. ^1H NMR (DMSO-d_6, 400 MHz) δ 8.21 (s, 1H, HC=N), 7.78 (d, 2H, *J* = 8.4 Hz, Ar), 7.24 (d, 2H, *J* = 8.4 Hz, Ar), 3.14 (s, 3H, CH_3), 2.90 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 159.7, 142.8, 139.4, 127.8, 119.4 (Ar, C=N), 40.8, 35.0 (CH_3).

5.7. Synthesis of oligodeoxynucleotides

Oligonucleotide synthesis was carried out on an automated DNA synthesiser following the phosphoramidite approach. Synthesis of oligonucleotides **ON1–ON6** (**X–Z**) was performed on a 0.2 μmol scale by using the amidites **3**, **7** and **8** as well as the corresponding commercial 2-cyanoethyl phosphoramidites of the natural 2′-deoxynucleosides. The synthesis followed the regular protocol for the DNA synthesiser. For compound **3**, **7** and **8**, a prolonged coupling time of 20 min was used. 1*H*-Tetrazole was used as the activator and coupling yields for all 2-cyanoethyl phosphoramidites were 95–99.8%. The 5′-*O*-DMT-ON oligonucleotides were removed from the solid support by treatment with concentrated aqueous ammonia at 55 °C for 16 h, which also removed the protecting groups. The oligonucleotides were purified by reversed-phase HPLC on a Waters 600 system using a X_{terra} prep MS C_{18}; 10 μm; 7.8 × 150 mm column; buffer A: 0,05 M triethyl ammonium acetate pH 7.4. Buffer B: MeCN/H_2O (1:1). Program used: 2 min 100% A, 100–30% A over 38 min, 10 min 100% B, 10 min 100% A. All oligonucleotides were detritylated by treatment with an 80% aqueous solution of acetic acid for 20 min, quenched with a aqueous solution of sodium acetate (3 M, 15 μL) and then added sodium perchlorate (5 M, 15 μL) followed by acetone (1 mL). The pure oligonucleotides precipitated overnight at −20 °C. After centrifugation 12,000 rpm, 10 min at 4 °C, the supernatant was removed and the pellet washed with cold acetone (2 × 1 mL) and dried for 30 min under reduced pressure, and dissolved in pure water (500 μL). The concentration was determined by UV at 260 nm, and the purity confirmed by IC analysis. MALDI-TOF-MS $[M-H]^-$ gave the following results (calcd/found): **ON2-X** (2864.9/

2865.4); **ON3-X** (2994.0/2992.0); **ON4-X** (2994.0/ 2995.3); **ON5-X** (3124.5/3123.9); **ON1-Y** (2880.9/2878.0); **ON2-Y** (2881.3/2876.5); **ON3-Y** (3026.9/3024.7); **ON4-Y** (3026.0/3024.1); **ON5-Y** (3173.0/ 3171.7); **ON6-Y** (3316.0/ 3315.8); **ON1-Z** (2943.0/2941.8); **ON2-Z** (2943.0/2943.5); **ON3-Z** (3151.0/3146.0); **ON4-Z** (3151.0/)3147.0; **ON5-Z** (3526.8/3522.4); **ON6-Z** (3567.0/3567.0).

5.8. Thermal denaturation experiments

Extinction coefficients of the modified oligonucleotides were estimated from a standard method but calibrated by the micromolar extinction coefficients of the monomeric compounds **X**, **Y** and dT, which were estimated from their UV-spectra (dT: $\varepsilon_{260} = 8.5$, **X**: $\varepsilon_{260} = 7.8$, **Y**: $\varepsilon_{260} = 12.6$), and **Z**, which was estimated by ab initio calculated oscillator strength to be $\varepsilon_{260} \sim 8.0$; practically we used a $\varepsilon_{260} \sim 5.0$. UV melting experiments were thereafter carried out on a UV spectrometer. Samples were dissolved in a medium salt buffer containing 2.5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 100 mM NaCl, and 0.1 mM EDTA at pH 7.0 with 1.5 μM concentrations of the two complementary sequences. The increase in absorbance at 260 nm as a function of time was recorded while the temperature was increased linearly from 5 to 60 or 75 °C at a rate of 0.5 or 1.0 °C/min by means of a Peltier temperature programmer. The melting temperature was determined as the local maximum of the first derivatives of the absorbance versus temperature curve. The melting curves were found to be reversible. All determinations are averages of at least duplicates within ±0.5 °C.

5.9. Circular dichroism spectroscopy

CD-spectra were obtained at 5 °C using the same medium salt buffer as in the UV melting experiments with 1.5 μM concentrations of the two complementary sequences.

5.10. Molecular modelling

The duplexes investigated (**ON3-X–Z** and **ON6-X–Z**) were built with a standard B-type helical geometry within the MACROMODEL V9.1 suite of programs.[14] In all the calculations the phosphodiester backbone charge was neutralised with sodium ions, placed 3.0 Å from the negatively charged oxygen atoms in the plane described by the phosphorus and the non-bridging oxygen atoms. The sodium–oxygen distances were restrained to 3.0 Å by a force constant of 418 kJ/mol Å^2. The modifications **X**, **Y** and **Z** were each subjected to a Monte Carlo conformational search[15] rotating the C5 (nucleobase)—C4 (triazole) bond and the N1 (triazole)—C1 (phenyl) bond to generate 1000 structures, which were minimised to identify the lowest energy structure. The lowest energy structure obtained from the Monte Carlo search was hereafter subjected to a 5 ns molecular dynamics simulation (simulation temperature 300 K, time step 2.2 fs, SHAKE all bonds to hydrogen), during which 500 structures were sampled and subsequently minimised. The duplex structures were minimised using the Polak–Ribiere Conjugate Gradient Method, the all-atom AMBER force field[16,17] and GB/SA solvation model[18] as implemented in MACROMODEL V9.1. Nonbonded interactions were treated with extended cut-offs (van der Waals 8.0 Å and electrostatics 20.0 Å). Oscillator strength calculations for monomer **Z** were performed using a Hartree–Fock water model with the aug-pc-1 basis set in GAUSSIAN 3.[19]

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Co-author statement in connection to the PhD thesis made by:

Lucie Brulíková

Paper/manuscript (authors, title, journal):
Nicolai Krog Andersen, Navneet Chandak, Lucie Brulíková, Pawan Kumar, Michael Dalager Jensen, Frank Jensen, Pawan K. Sharma and Poul Nielsen: "Efficient RNA-targeting by the introduction of aromatic stacking in the duplex major groove via 5-(1-phenyl-1,2,3-triazol-4-yl)-2′-deoxyuridines" *Bioorganic and Medicinal Chemistry* **2010**, 18, 4702-4710.

The undersigned who is
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on the paper/manuscript above, hereby confirms that Lucie Brulíková has contributed to the work as stated below:

1. Intellectual input:

☐ less than 25% X 25 - 50 % ☐ 50 - 75% ☐ 75 - 100%

Comments: _____

2. Experimental results (indicate contribution to individual figures, tables and supplementary data):

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Comments: Lucie Brulíková performed the syntheses of compounds **5, 7** and **10** and prepared two oligonucleotides **ON1-Y** and **ON6-Y**, whose synthesis was further repeated by main author.

3. Writing process:

X less than 25% ☐ 25 - 50 % ☐ 50 - 75% ☐ 75 - 100%

Comments: _____

Poul Nielsen
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Lucie Brulíková

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