Contents lists available at ScienceDirect





European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

# Discovery of novel N<sup>4</sup>-alkylcytidines as promising antimicrobial agents

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## ARTICLE INFO

Article history: Received 9 October 2020 Received in revised form 18 November 2020 Accepted 12 January 2021 Available online 1 February 2021

Keywords: Nucleoside Cytidine Antifungal Antibacterial Preservation

# ABSTRACT

The emergence of drug-resistant strains of pathogenic microorganisms necessitates the creation of new drugs. In order to find new compounds that effectively inhibit the growth of pathogenic bacteria and fungi, we synthesized a set of N<sup>4</sup>-derivatives of cytidine, 2'-deoxycytidine and 5-metyl-2'-deoxycytidine bearing extended N<sup>4</sup>-alkyl and N<sup>4</sup>-phenylalkyl groups. The derivatives demonstrate activity against a number of Gram-positive bacteria, including *Mycobacterium smegmatis* (MIC = 24–200  $\mu$ M) and *Staphylococcus aureus* (MIC = 50–200  $\mu$ M), comparable with the activities of some antibiotics in medical use. The most promising compound appeared to be N<sup>4</sup>-dodecyl-5-metyl-2'-deoxycytidine **4h** with activities of 24 and 48  $\mu$ M against *M. smegmatis* and *S. aureus*, respectively, and high inhibitory activity of 0.5 mM against filamentous fungi that can, among other things, damage works of art, such as tempera painting. Noteworthy, some of other synthesized compounds are active against fungal growth with the inhibitory concentration in the range of 0.5–3 mM.

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## 1. Introduction

Microorganisms are closely tied to all aspects of human life, bringing both benefit and harm. A fundamentally new level of healthcare has been reached with the introduction of antibiotics into medical practice. As a result, the mortality rate from infectious diseases has decreased drastically. Unfortunately, microbial resistance to most classes of antibiotics has been an emerging challenge ever since. Every new antibiotic introduced to therapeutic use so far has eventually promoted a rapid spread of drug resistance in microorganisms towards the respective antibiotic, a direct consequence of antibiotic intake by the pathogens [1]. This leads to an

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ever-increasing proportion of resistant strains in relation to total pathogen populations, gradually rendering antibiotic therapy ineffective [2]. According to the World Health Organization (WHO), current situation with multiple drug resistance poses a serious threat to human society and continues to worsen each year [3]. It is believed that humanity is entering a post-antibiotic era, when even common infections or minor injuries can be life-threatening [4,5]. In this light, one of the most important tasks of modern medicinal chemistry is to find new types of compounds that would inhibit pathogen growth and become effective antibacterial, antiviral and antifungal drugs [6].

The idea of using analogues and derivatives of nucleic acid components (i.e. nucleic bases, nucleosides and nucleotides) as medicine was proposed in the middle of the XX century. At present, analogues and derivatives of nucleic acid components are important elements of anticancer, antiviral [4,7-9] and antifungal therapy [9-11]. Many of the antiviral and antifungal nucleoside drugs

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that have been in common practice for many decades also appear to be effective antagonists of bacterial growth [4].

Systematic studies of antibacterial (primarily antimycobacterial) as well as antifungal activity of nucleoside derivatives have been conducted over the past two decades; several reviews have been published on modified nucleosides that suppress the growth of bacteria [9,12–15]. 5-Modified pyrimidine nucleosides with extended substituents introduced through alkynyl, hydroxymethyl or triazolyl moiety were shown to exhibit good inhibitory activity *in vitro* against *Mycobacterium tuberculosis, M. avium* and *M. bovis* [9,14–18]. Even today, the exact mechanism of action of these compounds remains unclear. However, it was demonstrated that the cell wall of *M. tuberculosis* is lysed in the presence of 5-modified 2'-deoxyuridines or 5-modified 5'-norcarbocyclic pyrimidine nucleosides with extended substituents [19,20].

In addition to medical use, antimicrobial compounds (natural antibiotics and xenobiotics) have found application in different areas of human activity, such as food industry, agriculture, and so on [21]. Nowadays, an alternative application of nucleoside derivatives is being thoroughly researched: their use as antifungal agents also has a good potential [9–11].

We suggest that N<sup>4</sup>-modified cytidine derivatives bearing extended lipophilic alkyl or phenylalkyl substituents could exhibit antibacterial and/or antifungal activity in the same way it was observed for 5-modified uridine derivatives [14-20,22].

The goal of this work was to synthetize a set of new  $N^4$ -modified cytidine derivatives bearing extended  $N^4$ -alkyl and  $N^4$ -phenylalkyl groups as potential microorganism growth inhibitors as well as to evaluate their toxicity to biological objects along with their antibacterial and antifungal activity towards a wide range of microorganisms. As test objects, we used various clinical isolates and bacteria strains provided by the Gause Institute of New Antibiotics and the collection of degrader filamentous fungi isolated from ancient Russian tempera painting from the State Tretyakov Gallery collection.

### 2. Results and discussion

## 2.1. Chemistry

Synthesis of some N<sup>4</sup>-modified pyrimidine nucleosides was reported in a number of previous works [23–26]. Some of them demonstrated antiviral activity against the human immunodeficiency viruses (HIV-1, HIV-2), the herpes virus (HSV-1) [23,24] and the hepatitis B virus (HBV) [25,26], but their antibacterial and/or antifungal activities have not been reported. In our study, we present evidence of antimicrobial activities of N<sup>4</sup>-modified derivatives of cytidine and 2'-deoxycytidine.

The synthesis of  $N^4$ -modified cytidine derivatives bearing extended  $N^4$ -alkyl and  $N^4$ -phenylalkyl groups was performed using the method of Divakar and Reese [27] in its later modification [28], by coupling 3',5'-diacetyl-2'-deoxyuridines (1a), 3',5'-diacetylthymidines, (1b) or 2',3',5'-triacetyluridines (1c) with 1,2,4-triazole and 2-chlorophenyl dichlorophosphate in pyridine to obtain C<sup>4</sup>triazolyl derivatives followed by their condensation with alkyl- or phenylalkyl amines (Scheme 1). The reaction of C<sup>4</sup>-triazolyl derivatives with alkylamines was accompanied by the partial removal of O-acetyl protecting groups. Therefore, in order to completely remove the protective groups, the cleavage was carried out using ammonia solution (controlled by TLC). The isolation of N<sup>4</sup>-alkylcytidines proved difficult due to their chromatographic mobilities being close to those of the starting amines.

N<sup>4</sup>-Modified cytidines with extended N<sup>4</sup>-alkyl and N<sup>4</sup>-phenylalkyl substituents are poorly soluble in water, the fact that significantly complicates their study. To solve this problem, we applied the prodrugs strategy, which is often used to optimize the pharmacological and physicochemical properties of drugs, and thus improve their solubility and pharmacokinetic features and decrease their toxicity [29].

Previously, we synthesized a number of water-soluble forms of modified pyrimidine 2'-deoxynucleosides [30,31]. They turned out to be at least two orders of magnitude more soluble than the starting compounds. They also had lower cytotoxicity and exhibited activity against a number of Gram-positive bacteria. Similarly, in the current work, we've introduced triethylene glycol residues at the 3' position of the carbohydrate fragment of the most active compounds 4g and 4h to obtain the 3'-O-[(8-hydroxy-3,6dioxaoctyloxy)carbonyl]-N<sup>4</sup>-alkyl-5-methyl-2'-deoxycytidines **4n** and **40** using the method described in Refs. [30,32] as a basis. Namely, we first protected the 5' hydroxyl of thymidine 5 with the synthesized TBDMS-group, then 3'-0-(8-hydroxy-3,6dioxaoctyloxycarbonyl)-5'-O-(tert-butyldimethylsilyl)thymidine 7 by sequential treatment of **6** with *N*,*N*'-carbonylditriazole and triethylene glycol followed by protection of the  $\omega$ -hydroxyl group of the glycol fragment with the TBDMS-group and then used the (3'-O-(8-tert-butyldimethylsilyloxy-3,6synthon 8 dioxaoctyloxycarbonyl)-5'-O-(*tert*-butyl)dimethylsilylthymidine) to obtain N<sup>4</sup>-modified 5-methyl-2'-deoxycytidines **4n** and **4o** (Scheme 2).

As a result, we obtained a representative library (Table 1) of N<sup>4</sup>substituted cytidine derivatives.

## 2.2. Chemical and enzymatic stability

The chemical and enzymatic stability of 3'-O-carbonyl triethylene glycol derivatives of N<sup>4</sup>-alkyl-5-methyl-2'-deoxycytidines **4n** and **4o** was assessed using the method described in Refs. [30,31], thus yielding estimates of their half-life ( $\tau_{1/2}$ ). The enzymatic hydrolysis of compounds **4n** and **4o** was performed in human blood serum. The compounds were hydrolyzed with  $\tau_{1/2} \ge 24$  h, slightly exceeding the half-life optimal for prodrug forms [33]. The hydrolysis yields the only parent nucleosides **4j** and **4h**. On the other hand, these compounds were stable in buffer solutions at two pH values (2.2 and 7.5) for more than a week, and only slightly hydrolyzed at pH 9.0. This indicates that the studied compounds were chemically stable and their hydrolysis in human blood serum was almost exclusively enzymatic.

## 2.3. Cytotoxicity

The cytotoxicity of the synthesized compounds (CD<sub>50</sub>) was estimated by MTT assay [34] in A549 cell line (human pulmonary adenocarcinoma) (Table 3). The compounds demonstrated cytotoxic activity at concentrations of 80–200  $\mu$ M. The cytotoxicity of the glycol derivatives **4n** and **4o** (90  $\mu$ M) was 2 times higher than that of the parent nucleosides **4g** and **4h** (about 160  $\mu$ M). This is probably due to the better solubility of compounds **4n** and **4o** in aqueous media.

## 2.4. Bacterial growth inhibition

Antibacterial effect of the obtained compounds was studied by their ability to inhibit the growth of a number of microorganisms *in vitro* using the same method as in our previous works [30,35]. The antibacterial activities were tested against such Gram-positive bacteria as: *Bacillus subtilis,* meticillin-resistant *Staphylococcus aureus* strain MRSA (MRSA strains are wide-spread and cause nosocomial infections that resist the modern antibiotic therapy); streptococcus-like bacteria *Leuconostoc mesenteroides* (strain distinguished by a high native resistance to glycopeptide antibiotics



Scheme: 1. Synthesis of compounds 4a-m. Reagents and conditions: *i*) 1H-1,2,4-triazole, 2-chlorophenyl dichlorophosphate, Py, r.t., 16 h; *ii*) R–NH<sub>2</sub>, dioxane, r.t., 16 h; *iii*) NH<sub>3</sub>/H<sub>2</sub>O, dioxane, r.t., 16 h.



Scheme: 2. Reagents and conditions: *i*: TBDMSCI, Py, r.t.; *ii*: CDT, DMF, (37 °C); *iii*: triethylene glycol, dioxane, (37 °C), 16 h; *iv*: 1*H*-1,2,4-triazole, 2-chlorophenyl dichlorophosphate, Py, r.t., 16 h; *v*: R–NH<sub>2</sub>, dioxane, r.t., 16 h; *vi*: Bu<sub>4</sub>NF·3H<sub>2</sub>O, dioxane, r.t.

Table 1Obtained N4-alkylcytidines.



N≏	R	$\mathbb{R}^1$	$\mathbb{R}^2$	R <sup>3</sup>	N≏	R	$\mathbb{R}^1$	$\mathbb{R}^2$	R <sup>3</sup>
4a	C8H17	Н	Н	ОН	4i	$C_2H_4 - C_6H_5$	CH₃	Н	ОН
4b	$C_{10}H_{21}$	Н	Н	OH	4j	$C_4H_8 - C_6H_5$	Н	Н	OH
4c	$C_{12}H_{25}$	Н	Н	OH	4k	C <sub>8</sub> H <sub>17</sub>	Н	OH	OH
4d	$C_{14}H_{29}$	Н	Н	OH	41	$C_{10}H_{21}$	Н	OH	OH
4e	$C_{6}H_{13}$	$CH_3$	Н	OH	4m	$(C_2H_4O)_3CH_3$	$CH_3$	Н	OH
4f	C <sub>8</sub> H <sub>17</sub>	$CH_3$	Н	OH	4n	C <sub>10</sub> H <sub>21</sub>	$CH_3$	Н	$OC(O)(OC_2H_4)_3OH$
4g	$C_{10}H_{21}$	$CH_3$	Н	OH	40	C <sub>12</sub> H <sub>25</sub>	$CH_3$	Н	$OC(O)(OC_2H_4)_3OH$
4h	$C_{12}H_{25}$	$CH_3$	Н	OH					

of the vancomycin group, which often appear effective towards pathogenic bacteria with multidrug resistance) and *Micrococcus luteus* NCTC 8340; mycobacteria: two strains of *Mycobacterium smegmatis* (which are used for the preliminary assessment of the activity followed by the analysis of promising compounds against the strains of the causative agent of tuberculosis, i.e. *Mycobacterium tuberculosis*); Gram-negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa* (opportunistic human pathogen causing difficulty in the treatment of nosocomial infection).

The compounds under investigation demonstrate activity

against Gram-positive bacteria as well as mycobacteria (Table 3), and there is no activity towards Gram-negative microorganisms.

2'-Deoxynucleosides had a higher activity than the respective ribo derivatives with the same alkyl substituent. Moreover, in most cases, N<sup>4</sup>-modified 5-methyl derivatives of 2'-deoxycytidine had a slightly greater inhibitory effect than the compounds without such methyl group at the C-5 position of the cytosine residue. The data obtained are comparable with the activities of a number of antibiotics in medical use. For example, MIC of widely used first line antibiotics against *M. tuberculosis* are: 800  $\mu$ M of pyrazinamide (one of the first line antituberculosis drugs) [36] and 50  $\mu$ M of amikacin (Table 3).

Substituting linear alkyl residues for phenylalkyl residues at the N<sup>4</sup> position of the nucleobase did not lead to an increase in antimicrobial activity. For instance, compounds **4i** and **4j** did not demonstrate any inhibitory activity. This might be explained by the low solubility of such nucleosides in aqueous solutions. E.g., a compound is expected to be poorly soluble if it features an extended linear substituent at N<sup>4</sup> (**4d**). On the other hand, the introduction of the hydrophylic triethylene glycol fragment at N<sup>4</sup> of 5-methyl-2'-deoxycytidines (**4k**) resulted in high water solubility of the compound, but this also led to a complete loss of the antibacterial action.

The 3'-O-carbonyl triethylene glycol derivatives of N<sup>4</sup>-alkyl-5methyl-2'-deoxycytidines **4n** and **4o** demonstrated lower inhibitory effect than the parent nucleosides **4g** and **4h**; this difference is especially noticeable for the **4h/4o** pair. Since they also turned out to be twice as toxic, this modification did not lead to an improvement in the selectivity index.

Thus, among the studied compounds, nucleosides **4g** and, above all, **4h** are the most promising candidates for antibacterial agents.

#### Table 3

Minimal bacterial growth inhibitory concentration ( $\mu$ M) and cytotoxicity ( $\mu$ M).

Bacteria Compounds	Bacillus subtilis ATCC 6633	Leuconostoc mesenteroides VKPM B-4177	Mycobacterium smegmatis mc <sup>2</sup> 155	Mycobacterium smegmatis VKPM Ac-1339	Staphylococcus aureus FDA 209 P	Staphylococcus aureus INA 00761	Micrococcus luteus NCTC 8340	Cytotoxicity in A549 human cells
4 a, d-f, i-m	>200	>200	>200	>200	>200	>200	>200	90–200
4b	140	140	140	140	>140	>140	140	200
4c	70	70	70	70	70	70	70	100
4g	50	50	50	50	50	50	50	156
4h	24	24	24	24	48	48	24	158
4n	90	90	54	54	54	54	>45	90
40	68	68	68	68	50	50	50	90
Antibiotic	AN 7	VA>275	AN 50	AN 29	AN 3,4	AN 5	AN 1	
sensitivity <sup>a</sup>			CIP 15	CIP 12	CIP 3	CIP 12		
			INZ 29	INZ 2	OX 2.5	OX 80		
			RFP 5	RFP 10				

<sup>a</sup> Amikacin (AN), Ciprofloxacin (CIP), Isoniazid (INZ), Rifampicin (RFP), Oxacillin (OX), Vancomycin (VA).

Recently we demonstrated the destruction of the bacterial cell wall of *M. tuberculosis* H37Rv strain treated with 5-modified pyrimidine nucleosides with extended substituents using transmission electron microscopy (TEM), suggesting that the mechanism of action of these compounds may be related to their interactions with bacterial cell walls [28,29]. It is likely that N<sup>4</sup>-modified cytidines bearing extended N<sup>4</sup>-alkyl groups have the same mode of antibacterial action.

### 2.5. Acute and chronic toxicity

Acute and chronic toxicity of the most promising nucleoside **4h** were estimated by administering intraperitoneal injections of the compound in 30% Tween-80 in mice.

The compound was found to exhibit low toxicity: the  $LD_{50}$  of **4h** was found to be 270 mg/kg for acute administration, 215 mg/kg for subchronic administration and 200 mg/kg for the delayed effect of subchronic administration (Fig. 1). A decrease in subchronic  $LD_{50}$  of the compound **4h** compared to the respective value of acute toxicity by 26% indicates a cumulative toxic effect of the drug, without tolerance development.

#### 2.6. Fungi growth inhibition

Microbiological community plays a crucial role in the destruction of cultural heritage [37,38]. Microorganisms from various systematic groups (filamentous fungi especially) capable of damaging works of art, e.g., tempera painting, oil painting on canvas, have been extensively studied in recent years [39]. Traditional antiseptics used for tempera paintings have their limitations [40,41] and there is an urgent need for the development of novel modern antiseptics with broad-spectrum antimicrobial activity and high efficacy [41,42] which at the same time would be safe for paintings as well as the museum staff [38,43]. In this regard, the challenge is to develop a new generation of biocides, with a wide spectrum of action, inhibiting not only bacteria, but fungi as well, while not damaging the materials of the cultural heritage [38,44].

Recently, we isolated a set of filamentous fungi from Paintings of the Ancient Rus Halls, the State Tretyakov Gallery (STG), Russia, Moscow [38]. The antifungal activities of N<sup>4</sup>-modified cytidines and 5-fluorocytosine (5FC) as a positive control were evaluated against 10 representatives of filamentous fungi known to cause biodeterioration activity against organic materials used in tempera painting of 15–16th centuries and in restoration [38]. Five species were from the *Aspergillaceae* family (*Aspergillus versicolor* STG-25G, *A. creber* STG-57, *A. versicolor* STG-86, *Aspergillus sp.* STG-93W, *A. amoenus* STG-106); two species were from the *Cladosporiacea* family (*Cladosporium halotolerans* STG-52B, *C. parahalotolerans* STG-93B) and one representative for each of the families of *Pleosporaceae* (*Ulocladium chartarum* STG-36), *Cordycipitaceae* (*Simplicilium lamellicola* STG-96) and *Microascaceae* (*Microascus paisii* STG-103). These strains were dominant among all microbiome from STG, characterized by metagenomic profiling (SRA records Accession: PRJNA606688, https://www.ncbi.nlm.nih.gov/sra/PRJNA606688). In this regard, an important task for the preventive treatment of icons from STG is to seek out an inhibitor with a broad spectrum against these 10 fungi biodestructors. The toxicity effect was estimated quantitatively as the percentage of colony growth inhibition on CDA medium with an addition of N4-modified cytidines or 5FC according to the method described in Ref. [45] (the control medium contained no additions) (Fig. 2).

The sublethal dose of inoculum was evaluated by drop and dilution assay (Fig. 3A), as in Refs. [46,47]. The fungal growth inhibition (FGI) increases in series **4f**–**4g**–**4h** (Fig. 3B). The modification of **4g** to **4n** leads to a significant loss of inhibitory ability (Fig. 3). The strongest inhibition effect was observed for nucleoside **4h** (Figs. 2 and 3). The preliminary screening of antifungal activity was assessed in the range of 0.1 and 3.5 mM of compounds. To analyze the inhibition timescale of N<sup>4</sup>-modified cytidines or 5FC we used concentrations of 0.5 and 3 mM; measurements were taken every 3 days.

Fig. 4 shows the measurement results for the time range of 6–48 days, taken every 6 days. The strongest inhibition of fungi growth was demonstrated for 3 mM solution of 4h. However, after 24-30 days, the majority of strains from the Aspergillaceae family overcame the inhibition effected by the addition of 3 mM solution of **4h**. The genera most sensitive to any of the tested N<sup>4</sup>-modified cytidines were Cladosporium and Ulocladium. The dynamic of growth inhibition follows the same trend as FGI, with an increase in the series 4f-4g-4h. 5FC inhibited Cladosporiacea (STG-52B, STG-93B) and A. amoenus STG-106 at the same level as 4h. It also inhibited all other representatives of the Aspergillaceae family (STG-25G, STG-57, STG-86 and STG-93W) at the level between those of 4h and 4g, and was practically inactive against Pleosporaceae (STG-36) and Sordariomycetas (STG-96 and STG-103). N<sup>4</sup>-Modified cytidines also inhibited the growth of Aspergillus, slightly weaker than Cladosporium and Ulacladium; 5FC inhibited Aspergillaceae (STG-25G, STG-57, STG-86 and STG-93W) at the level slightly lower than that of 4g, and inhibited STG-106 at the level slightly higher than that of **4h**. The most resistant filamentous fungi among the tested species was Simplicillium STG-96, both to N<sup>4</sup>-modified cytidines and 5FC.

The antifungal activity of N<sup>4</sup>-modified 2'-deoxycytidines **4n** and **4f** demonstrated close inhibitory activity. Four of the 10 strains (STG-93W, STG-36, STG-96 and STG-106) demonstrated resistance



Fig. 1. Toxicity of the nucleoside 4h. A. Acute toxicity. B. Subchronic and long-term toxicity. Each solution contained 30% Tween-80 and a respective does of 4h. No toxicity was detected for negative control containing Tween-80 only (data not shown). Data are means  $\pm$  SD.



Fig. 2. Growth of fungi strains on CDA medium with the addition of 0.5 mM nucleoside 4h or without addition (control). 24 days after inoculation.

to 3 mM 5FC at early stages (6–18 days) (Fig. 4). During this period 3 mM **4h** completely inhibited growth of all the 10 strains. The inoculum dose used in determination of growth inhibition against control was  $5 \times 10^5$  Colony Forming Units (CFU). The majority of *Aspergillaceae* overcame the inhibitory effect of 3 mM **4h** at day 48. Using drop and dilution assay we demonstrated that an inoculum dose equal to or lower than  $5 \times 10^4$  CFU was completely inhibited by 3 mM **4h** at any given point. In this regard, the nucleoside **4h** can be perceived as a possible candidate for the preventive processing

of icons at the early stages of filamentous fungi infection (a test on mock-layers is required).

Our experiments on 10 filamentous fungi from various systematic groups demonstrated that the inhibitory effect of the studied N<sup>4</sup>-modified cytidines depends mainly on the nature and concentration of the inhibitor itself (Fig. 4). Among representatives of the 3 classes of *Ascomycota* (*Eurotiomycetes*, *Dothideomycetes* and *Sordariomycetes*), no species were identified that would exhibit specific resistance. In addition, 4 strains showed increased



**Fig. 3.** Growth of fungi strains on CDA medium with the addition of 0.5 mM 5FC, 0.5 mM nucleoside **4f-4h** and **4n**, or without addition (control). 6 days after inoculation. *A*. Drop and dilution assay. *B*. Growth inhibition (%). Data are means  $\pm$  SD, n = 3. ND, not detected.



Fig. 4. The dynamic of growth inhibition (%) for STG strains on CDA medium with 5FC, 4f-4h and 4n (all tested at concentrations of 0.5 mM and 3 mM). Data on 6, 12, 18, 24, 30, 36, 42 and 48 days after inoculation.

resistance to 5FC (STG-93W, STG-36, STG-96, STG-103), and 3 strains showed increased sensitivity to the same compound (STG-106, STG-52B, STG-93B). On the other hand, earlier for these 10 strains of filamentous fungi, we obtained a differential effect when studying the fungicidal properties of chitosan [48]. The highest sensitivity to chitosan was shown by representatives of the Cladosporiaceae family (STG-52B and STG-93B), the highest resistance was shown by a representative of the *Pleosporaceae* family (STG-36) and some representatives of Aspergillaceae (STG-93W and STG-106) [48]. These results correlate with the mechanism of action of chitosan on fungal cell membranes. Membranes of fungi with a high content of polyunsaturated fatty acids (such as linoleic acid, characteristic of Cladosporiaceae) are highly sensitive to chitosan, membranes with low fluidity, enriched with saturated fatty acids (such as stearic or palmitic, characteristic of Aspergillaceae), are resistant to chitosan. The demonstrated broad spectrum inhibition of filamentous fungi by N<sup>4</sup>-modified cytidines may be associated with some universal mechanism of action on fungi cell, which can be used for the development of a broad-spectrum antiseptic. We plan to investigate this in the future.

Filamentous fungi (or molds) are taxonomically diverse organisms from phyla *Zygomycota* and *Ascomycota* characterized by filamentous hyphae and their ability to produce airborne spores or conidia. Currently, more than 70 000 molds are known, some of them contain unique and unusual biochemical pathways. A number of products from those pathways (especially the secondary metabolite pathways) are used as important pharmaceuticals, including antibiotics, statins and immunosuppressors [38,49–52]. Furthermore, filamentous fungi have a variety of enzymatic activities leading to biodegradation of various materials, e.g., materials used in tempera painting [38]. Finally, the third group of enzymatic activities is associated with a variety of resistance mechanisms that are also unique to different groups [38,46]. In this regard, the new compounds, N<sup>4</sup>-modified cytidines, demonstrated a rather interesting effect against the studied fungi responsible for destroying tempera painting, exhibiting a broad-spectrum inhibitory activity. The toxic effect increased proportionally with the extension of the alkyl radical (from C8 to C12 length) in the range of the tested concentrations (0.5 mM and 3 mM).

## 3. Conclusions

In summary, we have employed a simple method for preparation of N<sup>4</sup>-modified cytidines bearing extended N<sup>4</sup>-alkyl or N<sup>4</sup>phenylalkyl groups. The compounds demonstrated activity against *M. smegmatis*, *S. aureus* and some other Gram-positive bacteria. The range of activity is comparable with that of some antibiotics in medical practice, compound **4h** with activities 24 and 48 uM towards *M. smegmatis* and *S. aureus*, respectively, being the best in the synthesized series. None of the obtained compounds are active against Gram-negative bacteria. Of most interest is the finding that N<sup>4</sup>-modified cytidines exhibit high inhibitory activity against filamentous fungi that can damage works of art, such as tempera painting. Sublethal doses of this type of antifungal inhibitory activity are in the range of 0.1–0.5 mM, and lethal doses are in the range of 0.5–3 mM; the most promising nucleosides are 4g and 4h. The latter completely blocks the growth of filamentous fungi at a concentration of 0.5 mM. The convenience of the method used to synthesize these N<sup>4</sup>-modified cytidines derivatives is that the reactions are carried out "in one pot" using inexpensive and readily available thymidine. This simplicity will make it possible to obtain novel series of effective inhibitors of bacterial and fungal replication.

#### 4. Experimental section

# 4.1. Reagents and equipment

Commercial reagents were purchased from Acros, Aldrich and Fluka. Column chromatography was performed on silica gel 60 0.040–0.063 mm (Merck, Germany). Thin laver chromatography was performed on silica gel 60 F<sub>254</sub> plates (Merck, Germany). NMR spectra were registered on an AMX III-400 spectrometer (Bruker BioSpin GmbH, Germany) with the working frequency of 400 MHz for <sup>1</sup>H NMR (Me<sub>4</sub>Si as an internal standard for organic solvents) and 101 MHz for <sup>13</sup>C NMR (with the carbon–proton interaction decoupling). The spectra of compounds 4c, 4e, 4h, 4k, 4m, 4o were registered on an AM-300 spectrometer (Bruker BioSpin GmbH, Germany) with the working frequency of 300 MHz for <sup>1</sup>H NMR and 75 MHz for <sup>13</sup>C NMR (with the carbon–proton interaction decoupling). UV spectra were recorded on a Perkin Elmer lambda 25 (Perkin Elmer, USA) in ethanol. High-resolution mass spectra were recorded on a Bruker Daltonics micrOTOF-Q II device by electrospray ionization mass spectrometry (ESI-MS). Measurements were carried out in positive ion mode in accordance with the previously applied conditions [29,35].

All research work with laboratory animals was carried out in accordance with generally accepted ethical standards for the treatment of animals, which comply with the rules adopted by the European Convention [53].

## 4.2. General method for the synthesis of compounds 4a-4m

2-Chlorophenyl dichlorophosphate (188 mg, 126 µl, 0.77 mmol) was added to a solution of acetyl protected 2'-deoxyuridine (1a), 5methyl-2'-deoxyuridine (1b) or uridine (1c) (0.5 mmol) and 1,2,4triazole (200 mg, 3 mmol) in anhydrous pyridine, cooled to 0 °C. The mixture was stirred for 20 h at room temperature, then evaporated. The residue was partitioned between chloroform and 0.5 M sodium bicarbonate, the chloroform layer was washed with water, dried over Na<sub>2</sub>SO<sub>4</sub>, evaporated and dissolved in anhydrous dioxane (3 ml). The corresponding 1-alkylamine (1 mmol) was added to a solution, cooled to 0 °C. The mixture was stirred for 20 h at room temperature, then 3 ml of conc. aq. ammonia solution were added and the mixture was stirred for 40 h at room temperature, then evaporated, the compounds were purified on a column of silica gel  $(2 \times 15 \text{ cm})$  in chloroform or ethyl acetate were isolated by column chromatography in CHCl<sub>3</sub> or ethyl acetate eluted with a gradient of ethanol in chloroform (0-15%) or in or ethyl acetate (0-10%), respectively. The target fractions were evaporated in a vacuum to give the expected compounds yields as colorless amorphous mass with the 60-79% yields.

4.2.1. N<sup>4</sup>-Octyl-2'-deoxycytidine (**4a**). Prepared according to the general procedure from 1a (156 mg) and octylamine (129 mg, 164 µl). Yield 132 mg (78%). UV:  $\lambda_{max}$  272 nm<sup>1</sup>H NMR (300 MHz, DMSO- $d_6$ )  $\delta$  0.86 (t, J = 6.7 Hz, 3H,  $-CH_2-CH_3$ ), 1.21–1.34 (m, 10H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>5</sub>-), 1.42-1.53 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.93 (ddd, J = 13.2, 7.5, 6.0 Hz, 1H, 2'-Ha), 2.10 (ddd, J = 13.2, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.17–3.27 (m, 2H, –NH–CH<sub>2</sub>-), 3.52 (ddd, *J* = 11.8, 5.3, 4.1 Hz, 1H, 5'-Hb), 3.57 (ddd, J = 11.8, 5.3, 3.8 Hz, 1H, 5'-Ha), 3.76 (ddd, J = 4.2, 3.7, 3.3 Hz, 1H, 4'-H), 4.20 (ddt, J = 6.0, 4.2, 3.4 Hz, 1H, 3'-H), 4.93 (t, J = 5.3 Hz, 1H, 5'-OH), 5.16 (d, J = 4.2 Hz, 1H, 3'-OH), 5.73 (d, J = 7.5 Hz, 1H, 5-H), 6.16 (dd, J = 7.5, 6.0 Hz, 1H, 1'-H), 7.63 (t, J = 5.5 Hz, 1H, N<sup>4</sup>-H), 7.71 (d, J = 7.5 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 13.89 (-CH<sub>2</sub>-<u>CH<sub>3</sub></u>), 22.05, 26.48, 28.55, 28.64, 28.73, 31.22 (-NH-CH2-(CH2)6-), 39.73 (2'-C), 40.28 (-NH-CH2-), 61.42 (5'-C), 70.46 (3'-C), 84.83 (1'-C), 87.15 (4'-C), 94.70 (5-C), 139.60 (6-C), 155.18 (2-C), 163.29 (4-C). HRMS (ESI) of C<sub>17</sub>H<sub>29</sub>N<sub>3</sub>O<sub>4</sub>, *m*/*z*: calcd [M+H]<sup>+</sup> 340.2231, found: 340.2246; calcd [M+Na]<sup>+</sup>

## 362.2050, found: 362.2063.

4.2.2. N<sup>4</sup>-Decyl-2'-deoxycytidine (**4b**). Prepared according to the general procedure from 1a (156 mg) and decylamine (157 mg, 200 µl). Yield 141 mg (77%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR (300 MHz, DMSO- $d_6$ )  $\delta$  0.85 (t, J = 6.7 Hz, 3H,  $-CH_2-\underline{CH}_3$ ), 1.20–1.31 (m, 14H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>7</sub>-), 1.41-1.53 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.93  $(ddd, I = 13.2, \overline{7.5}, \overline{6.0} \text{ Hz}, 1\text{H}, 2'-\text{Ha}), 2.10 (ddd, I = 13.2, \overline{6.0}, 3.1 \text{ Hz})$ 1H, 2'-Hb), 3.17–3.28 (m, 2H, –NH–CH<sub>2</sub>-), 3.53 (ddd, *J* = 11.9, 5.3, 4.1 Hz, 1H, 5'-Hb), 3.57 (ddd, *J* = 11.9, 5.3, 3.8 Hz, 1H, 5'-Ha), 3.77 (ddd, J = 4.2, 3.8, 3.0 Hz, 1H, 4'-H), 4.20 (ddt, J = 6.0, 4.2, 3.1 Hz, 1H, 3'-H), 4.94 (t, J = 5.3 Hz, 1H, 5'-OH), 5.17 (d, J = 4.2 Hz, 1H, 3'-OH), 5.74 (d, J = 7.5 Hz, 1H, 5-H), 6.16 (dd, J = 7.5, 5.9 Hz, 1H, 1'-H), 7.64 (t, J = 5.5 Hz, 1H, N<sup>4</sup>-H), 7.72 (d, J = 7.5 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 13.87 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.03, 26.44, 28.52, 28.64, 28.74, 28.91, 28.97, 31.23 (-NH-CH2-(CH2)8-), 39.71 (2'-C), 40.25 (-NH-CH<sub>2</sub>-), 61.39 (5'-C), 70.43 (3'-C), 84.79 (1'-C), 87.11 (4'-C), 94.63 (5-C), 139.56 (6-C), 155.11 (2-C), 163.24 (4-C). HRMS (ESI) of C<sub>19</sub>H<sub>33</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 368.2544, found: 368.2540; calcd [M+Na]<sup>+</sup> 390.2363, found: 390.2363.

4.2.3.  $N^4$ -Dodecyl-2'-deoxycytidine (**4c**). Prepared according to the general procedure from 1a (156 mg) and dodecylamine (185 mg). Yield 149 mg (75%). UV:  $\lambda_{max}$  274 nm. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 0.86 (t, *J* = 6.7 Hz, 3H, -CH<sub>2</sub>-CH<sub>3</sub>), 1.21-1.31 (m, 18H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>9</sub>-), 1.42-1.52 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.93 (ddd, J = 13.2, 7.5, 6.1 Hz, 1H, 2'-Ha), 2.10 (ddd, J = 13.2, 6.0, 3.1 Hz, 1H, 2'-Hb), 3.17–3.27 (m, 2H, –NH–CH<sub>2</sub>-), 3.52 (ddd, *J* = 11.7, 5.3, 4.1 Hz, 1H, 5'-Hb), 3.57 (ddd, *J* = 11.7, 5.3, 3.8 Hz, 1H, 5'-Ha), 3.76 (ddd, *J* = 4.2, 3.8, 3.0 Hz, 1H, 4'-H), 4.20 (ddt, *J* = 6.1, 4.2, 3.1 Hz, 1H, 3'-H), 4.93 (t, *J* = 5.3 Hz, 1H, 5'-OH), 5.16 (d, *J* = 4.2 Hz, 1H, 3'-OH), 5.73 (d, J = 7.5 Hz, 1H, 5-H), 6.16 (dd, J = 7.5, 5.9 Hz, 1H, 1'-H), 7.63 (t, I = 5.4 Hz, 1H, N<sup>4</sup>-H), 7.71 (d, I = 7.5 Hz, 1H, 6-H). <sup>13</sup>C NMR (75 MHz, DMSO-d<sub>6</sub>) δ 13.90 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.05, 26.46, 28.54, 28.67, 28.76, 28.97, 29.01, 31.26 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>10</sub>-), 39.71 (2'-C), 40.24 (-NH-CH<sub>2</sub>-), 61.40 (5'-C), 70.44 (3'-C), 84.78 (1'-C), 87.11 (4'-C), 94.61 (5-C), 139.60 (6-C), 155.07 (2-C), 163.23 (4-C). HRMS (ESI) of C<sub>21</sub>H<sub>37</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 396.2857: found: 396.2854; calcd [M+Na]<sup>+</sup> 418.2676, found: 418.2674.

4.2.4. N<sup>4</sup>-Tetradecyl-2'-deoxycytidine (**4d**). Prepared according to the general procedure from 1a (156 mg) and tetradecylamine (213 mg). Yield 176 mg (79%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  0.85 (t, J = 6.6 Hz, 3H,  $-CH_2-CH_3$ ), 1.20–1.30 (m, 22H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>11</sub>-), 1.41-1.50 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.92 (ddd, *J* = 13.3, 7.2, 6.0 Hz, 1H, 2'-Ha), 2.08 (ddd, *J* = 13.2, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.16–3.24 (m, 2H, –NH–CH<sub>2</sub>-), 3.51 (ddd, *J* = 11.7, 5.3, 4.1 Hz, 1H, 5'-Hb), 3.56 (ddd, *J* = 11.7, 5.3, 3.8 Hz, 1H, 5'-Ha), 3.75 (ddd, *J* = 4.1, 3.6, 3.0 Hz, 1H, 4'-H), 4.19 (ddt, *J* = 6.0, 4.2, 3.2 Hz, 1H, 3'-H), 4.94 (t, J = 5.3 Hz, 1H, 5'-OH), 5.17 (d, J = 4.2 Hz, 1H, 3'-OH), 5.73 (d, *J* = 7.5 Hz, 1H, 5-H), 6.15 (dd, *J* = 7.2, 6.2 Hz, 1H, 1'-H), 7.67 (t, J = 5.8 Hz, 1H, N<sup>4</sup>-H), 7.71 (d, J = 7.4 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 13.94 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.09, 26.50, 28.56, 28.71, 28.80, 29.02, 29.05, 31.30 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>12</sub>-), 39.74 (2'-C), 40.27 (-NH-CH2-), 61.41 (5'-C), 70.46 (3'-C), 84.81 (1'-C), 87.15 (4'-C), 94.66 (5-C), 139.64 (6-C), 155.03 (2-C), 163.17 (4-C). HRMS (ESI) of C<sub>23</sub>H<sub>41</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+Na]<sup>+</sup> 446.2989, found 446.2979.

4.2.5. N<sup>4</sup>-Hexyl-5-methyl-2'-deoxycytidine (**4e**). Prepared according to the general procedure from **1b** (163 mg) and hexylamine (101 mg, 138 µl). Yield 119 mg (73%). UV:  $\lambda_{max}$  272 nm. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>)  $\delta$  0.81 (t, J = 6.6 Hz, 3H,  $-CH_2$ -<u>CH</u><sub>3</sub>), 1.18–1.26 (m, 6H,  $-NH-(CH_2)_2$ -(<u>CH</u><sub>2</sub>)<sub>3</sub>-), 1.40–1.52 (m, 2H,  $-NH-CH_2$ -<u>CH</u><sub>2</sub>-), 1.80 (d, J = 1.0 Hz, 3H, 5-CH<sub>3</sub>), 1.91 (ddd, J = 13.3, 7.7, 6.0 Hz, 1H, 2'-Ha), 2.02 (ddd, J = 13.2, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.19–3.28 (m, 2H, -NH-<u>CH</u><sub>2</sub>-), 3.49 (ddd, J = 11.8, 5.3, 3.9 Hz, 1H, 5'-Hb), 3.55 (ddd, J = 11.8, 5.3, 3.9 Hz, 1H, 5'-Ha), 3.71 (td, J = 3.9, 3.1 Hz, 1H, 4'-H), 4.17 (ddt, J = 6.0, 4.2, 3.2 Hz, 1H, 3'-H), 4.93 (t, J = 5.3 Hz, 1H, 5'-OH), 5.12 (d, J = 4.2 Hz, 1H, 3'-OH), 6.14 (dd, J = 7.6, 5.9 Hz, 1H, 1'-H), 7.06 (t,

 $J = 5.7 \text{ Hz}, 1\text{H}, \text{N}^4 - \text{H}), 7.52 \text{ (d}, J = 1.2 \text{ Hz}, 1\text{H}, 6-\text{H}). {}^{13}\text{C} \text{ NMR} (75 \text{ MHz}, \text{DMSO-}d_6) \delta 13.11 (5-\text{CH}_3), 13.87 (-\text{CH}_2-\text{CH}_3), 22.04, 26.13, 28.56, 31.06 (-\text{NH}-(\text{CH}_2)_4-), 40.12 (2'-\text{C}), 40.21 (-\text{NH}-(\text{CH}_2-), 61.42 (5'-\text{C}), 70.46 (3'-\text{C}), 84.58 (1'-\text{C}), 87.06 (4'-\text{C}), 101.68 (5-\text{C}), 137.15 (6-\text{C}), 155.13 (2-\text{C}), 162.65 (4-\text{C}). \text{ HRMS (ESI) of } \text{C}_{16}\text{H}_{27}\text{N}_3\text{O}_4, \textit{m/z: calcd} \text{ [M+H]}^+ 326.2074, found 326.2063.$ 

4.2.6. N<sup>4</sup>-Octvl-5-methvl-2'-deoxvcvtidine (**4f**). Prepared according to the general procedure from **1b** (163 mg) and octvlamine (129 mg, 164  $\mu$ l). Yield 138 mg (78%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR  $(400 \text{ MHz}, \text{DMSO-}d_6) \delta 0.84 (t, I = 6.8 \text{ Hz}, 3\text{H}, -\text{CH}_2\text{-}\text{CH}_3), 1.21-1.30$ (m, 10H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>5</sub>-), 1.46-1.55 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.84 (s, 3H, 5-CH<sub>3</sub>), 1.95 (ddd, I = 13.3, 7.7, 6.0 Hz, 1H, 2'-Ha), 2.06 (ddd, J = 13.1, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.25-3.31 (m, 2H, -NH-CH<sub>2</sub>-), 3.54 (ddd, *J* = 12.0, 5.3, 3.9 Hz, 1H, 5'-Ha), 3.59 (ddd, *J* = 11.8, 5.5, 3.8 Hz, 1H, 5'-Hb), 3.75 (td, J = 3.9, 3.3 Hz, 1H, 4'-H), 4.21 (ddt, *J* = 6.0, 4.3, 3.3 Hz, 1H, 3'-H), 4.96 (t, *J* = 5.3 Hz, 1H, 5'-OH), 5.15 (d, J = 4.3 Hz, 1H, 3'-OH), 6.17 (dd, J = 7.6, 6.0 Hz, 1H, 1'-H), 7.10 (t, J = 5.7 Hz, 1H, N<sup>4</sup>-H), 7.56 (s, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 13.05 (5-CH<sub>3</sub>), 13.84 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.01, 26.43, 28.54, 28.61, 28.76, 31.19 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-), 40.05 (2'-C), 40.17 (-NH-CH<sub>2</sub>-), 61.39 (5'-C), 70.42 (3'-C), 84.56 (1'-C), 87.03 (4'-C), 101.63 (5-C), 137.09 (6-C), 155.09 (2-C), 162.61 (4-C). HRMS (ESI) of C<sub>18</sub>H<sub>31</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 354.2387, found 354.2383; calcd [M+Na]<sup>+</sup> 376.2207, found: 376.2204.

4.2.7. N<sup>4</sup>-Decyl-5-methyl-2'-deoxycytidine (4g). Prepared according to the general procedure from **1b** (163 mg) and decylamine (157 mg, 200  $\mu$ l). Yield 147 mg (77%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR  $(400 \text{ MHz}, \text{DMSO-}d_6) \delta 0.84 (t, I = 6.8 \text{ Hz}, 3\text{H}, -\text{CH}_2\text{-}\text{CH}_3), 1.21-1.28$ (m, 14H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>7</sub>-), 1.45-1.55 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.84 (d, J = 1.0 Hz, 3H,  $\overline{5-CH_3}$ ), 1.95 (ddd, J = 13.3, 7.8, 6.1 Hz,  $\overline{1H}$ , 2'-Ha), 2.06 (ddd, *J* = 13.1, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.25–3.31 (m, 2H, -NH-CH<sub>2</sub>-), 3.54 (ddd, *J* = 11.7, 5.3, 4.0 Hz, 1H, 5'-Ha), 3.59 (ddd, *J* = 11.7, 5.3, 3.8 Hz, 1H, 5'-Hb), 3.75 (ddd, *J* = 4.2, 3.7, 3.1 Hz, 1H, 4'-H), 4.21 (ddt, J = 6.0, 4.2, 3.2 Hz, 1H, 3'-H), 4.96 (t, J = 5.3 Hz, 1H, 5'-OH), 5.16 (d, J = 4.2 Hz, 1H, 3'-OH), 6.17 (dd, J = 7.6, 5.9 Hz, 1H, 1'-H), 7.09 (t, J = 5.7 Hz, 1H, N<sup>4</sup>-H), 7.56 (d, J = 1.2 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) δ 13.08 (5-CH<sub>3</sub>), 13.87 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.05, 26.45, 28.57, 28.67, 28.83, 28.94, 28.99, 31.26 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>8</sub>-), 40.14 (2'-C), 40.20 (-NH-CH2-), 61.42 (5'-C), 70.45 (3'-C), 84.59 (1'-C), 87.05 (4'-C), 101.66 (5-C), 137.11 (6-C), 155.12 (2-C), 162.64 (4-C). HRMS (ESI) of C<sub>20</sub>H<sub>35</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 382.2700, found 382.2707; calcd [M+Na]<sup>+</sup> 404.2520, found 404.2527.

4.2.8. N<sup>4</sup>-Dodecyl-5-methyl-2'-deoxycytidine (4h). Prepared according to the general procedure from 1b (163 mg) and dodecylamine (185 mg). Yield 147 mg (77%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR  $(300 \text{ MHz}, \text{DMSO-}d_6) \delta 0.86 (t, J = 6.8 \text{ Hz}, 3\text{H}, -\text{CH}_2-\frac{\text{CH}_3}{2}), 1.23-1.28$  $(m, 18H, -NH-(CH_2)_2-(CH_2)_9-), 1.47-1.56 (m, 2H, -NH-CH_2-CH_2-),$  $1.84 (d, J = 1.0 Hz, 3H, \overline{5-CH_3}), 1.95 (ddd, J = 13.2, 7.8, 6.0 Hz, 1H, 2'-$ Ha), 2.06 (ddd, *J* = 13.1, 6.0, 3.2 Hz, 1H, 2'-Hb), 3.19–3.32 (m, 2H, -NH-CH<sub>2</sub>-), 3.53 (ddd, *J* = 11.8, 5.3, 4.2 Hz, 1H, 5'-Hb), 3.59 (ddd, *I* = 11.7, 5.3, 3.9 Hz, 1H, 5'-Ha), 3.75 (ddd, *I* = 4.1, 3.6, 3.0 Hz, 1H, 4'-H), 4.21 (dddd, *J* = 6.0, 4.2, 3.2, 2.8 Hz, 1H, 3'-H), 4.97 (t, *J* = 5.3 Hz, 1H, 5'-OH), 5.16 (d, J = 4.2 Hz, 1H, 3'-OH), 6.18 (dd, J = 7.6, 6.0 Hz, 1H, 1'-H), 7.10 (t, J = 5.7 Hz, 1H, N<sup>4</sup>-H), 7.56 (d, J = 1.2 Hz, 1H, 6-H). <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 13.11 (5-CH<sub>3</sub>), 13.90 (-CH<sub>2</sub>-<u>CH<sub>3</sub>)</u>, 22.06, 26.45, 28.57, 28.68, 28.84, 29.00, 29.03, 31.27 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>10</sub>-), 40.11 (2'-C), 40.24 (-NH-CH<sub>2</sub>-), 61.40 (5'-C), 70.43 (3'-C), 84.58 (1'-C), 87.07 (4'-C), 101.65 (5-C), 137.18 (6-C), 154.93 (2-C), 162.50 (4-C). HRMS (ESI) of C<sub>21</sub>H<sub>37</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 410.3013, found 410.3016.

4.2.9.  $N^4$ -(2-Phenylethyl)-5-methyl-2'-deoxycytidine (**4i**). Prepared according to the general procedure from **1b** (163 mg) and 2-phenylethylamine (121 mg, 125 µl). Yield 147 mg (89%). UV:  $\lambda_{max}$  276 nm. <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  1.84 (d, J = 1.1 Hz, 3H, 5-CH<sub>3</sub>), 1.97 (ddd, J = 13.2, 7.6, 6.1 Hz, 1H, 2'-Ha), 2.08 (ddd,

 $J = 13.2, 5.9, 3.3 Hz, 1H, 2'-Hb), 2.84 (dd, J = 8.6, 6.5 Hz, 2H, -NH-CH<sub>2</sub>-QH<sub>2</sub>-), 3.51-3.56 (m, 2H, -NH-CH<sub>2</sub>-), 3.56-3.57 (m, 1H, 5'-Ha), 3.60 (ddd, J = 11.8, 5.3, 4.0 Hz, 1H, 5'-Hb), 3.76 (dd, J = 3.9, 3.0 Hz, 1H, 4'-H), 4.22 (dddd, J = 6.1, 4.3, 3.4, 3.0 Hz, 1H, 3'-H), 4.97 (t, J = 5.3 Hz, 1H, 5'-OH), 5.17 (d, J = 4.2 Hz, 1H, 3'-OH), 6.20 (dd, J = 7.6, 5.9 Hz, 1H, 1'-H), 7.16-7.20 (m, 1H, p-Ph), 7.20-7.22 (m, 2H, o-Ph), 7.22-7.26 (m, 1H, N<sup>4</sup>-H), 7.26-7.32 (m, 2H, m-Ph), 7.60 (d, J = 1.2 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>) <math>\delta$  13.04 (5-CH<sub>3</sub>), 34.53 (-NH-CH<sub>2</sub>-CH<sub>2</sub>-), 40.15 (2'-C), 41.73 (-NH-CH<sub>2</sub>-), 61.42 (5'-C), 70.45 (3'-C), 84.64 (1'-C), 87.10 (4'-C), 101.65 (5-C), 126.04 (p-Ph), 128.30 (o-Ph), 128.58 (m-Ph), 137.37 (6-C), 139.53 (i-Ph), 155.07 (2-C), 162.66 (4-C). HRMS (ESI) of C<sub>18</sub>H<sub>23</sub>N<sub>3</sub>O<sub>4</sub>, m/z: calcd [M+H]<sup>+</sup> 346.1761, found 346.1763; calcd [M+Na]<sup>+</sup> 368.1581, found: 368.1581.

4.2.10. N<sup>4</sup>-(4-Phenylbutyl)-2'-deoxycytidine (4j). Prepared according to the general procedure from 1a (156 mg) and 4phenylbutylamine (149 mg, 158  $\mu$ l). Yield 127 mg (71%). UV:  $\lambda_{max}$ 272 nm. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 1.45–1.54 (m, 2H, -CH<sub>2</sub>- $CH_2-C_6H_5$ ), 1.54–1.64 (m, 2H,  $-NH-CH_2-CH_2-$ ), 1.93 (ddd,  $J = \overline{13.3}$ , 7.6, 6.0 Hz, 1H, 2'-Ha), 2.10 (ddd, J = 13.2, 6.0, 3.2 Hz, 1H, 2'-Hb), 2.59 (t, J = 7.5 Hz, 2H, -CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 3.20–3.30 (m, 2H, -NH–CH<sub>2</sub>-), 3.53 (ddd, *J* = 11.8, 5.2, 4.0 Hz, 1H, 5'-Hb), 3.57 (ddd, *J* = 12.0, 5.2, 4.0 Hz, 1H, 5'-Ha), 3.77 (td, *J* = 4.0, 3.0 Hz, 1H, 4'-H), 4.20 (ddt, *J* = 6.0, 4.3, 3.2 Hz, 1H, 3'-H), 4.93 (t, J = 5.2 Hz, 1H, 5'-OH), 5.17 (d, J = 4.3 Hz, 1H, 3'-OH), 5.73 (d, J = 7.5 Hz, 1H, 5-H), 6.17 (t, J = 7.3, 6.2 Hz, 1H, 1'-H), 7.10-7.18 (m, 1H, p-Ph), 7.18-7.22 (m, 2H, o-Ph), 7.22-7.34 (m, 2H, *m*-Ph), 7.66 (t, J = 5.6 Hz, 1H, N<sup>4</sup>-H), 7.72 (d, J = 7.5 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, methanol- $d_4$ )  $\delta$  29.62 (-CH<sub>2</sub>-CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 29.90 (-NH-CH<sub>2</sub>-CH<sub>2</sub>-), 36.47 (-CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 41.39 (2'-C), 41.89 (-NH-CH<sub>2</sub>-), 62.86 (5'-C), 72.09 (3'-C), 87.41 (1'-C), 88.73 (4'-C), 97.06 (5-C), 126.74 (p-Ph), 129.30 (o-Ph), 129.42 (m-Ph), 140.90 (6-C), 143.54 (i-Ph), 158.62 (2-C), 165.40 (4-C). HRMS (ESI) of C<sub>19</sub>H<sub>25</sub>N<sub>3</sub>O<sub>4</sub>, *m/z*: calcd [M+H]<sup>+</sup> 360.1918, found 360.1929.

4.2.11. N<sup>4</sup>-Octylcytidine (**4k**). Prepared according to the general procedure from 1c (185 mg) and octylamine (129 mg, 164 µl). Yield 139 mg (78%). UV:  $\lambda_{max}$  272 nm. <sup>1</sup>H NMR (300 MHz, DMSO- $d_6$ )  $\delta$  0.86 (t, J = 6.7 Hz, 3H,  $-CH_2-CH_3$ ), 1.25–1.28 (m, 10H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>5</sub>-), 1.44-1.53 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 3.18–3.27 (m, 2H, –NH–<u>CH</u><sub>2</sub>-), 3.54 (ddd, *J* = 12.1, 5.3, 3.5 Hz, 1H, 5'-Ha), 3.66 (ddd, *J* = 12.0, 5.1, 3.1 Hz, 1H, 5'-Hb), 3.82 (ddd, *J* = 4.3, 3.5, 3.0 Hz, 1H, 4'-H), 3.89-3.99 (m, 2H, 2'-H + 3'-H), 4.94 (d, J = 4.8 Hz, 1H, 3'-OH), 5.01 (t, J = 5.2 Hz, 1H, 5'-OH), 5.24 (d, *J* = 4.7 Hz, 1H, 2′-OH), 5.72 (d, *J* = 7.5 Hz, 1H, 5-H), 5.77 (d, *J* = 3.5 Hz, 1H, 1'-H), 7.65 (t, J = 5.5 Hz, 1H, N<sup>4</sup>-H), 7.77 (d, J = 7.5 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 13.87 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.01, 26.43, 28.47, 28.60, 28.68, 31.18 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-), 39.83 (-NH-CH<sub>2</sub>-), 60.60 (5'-C), 69.41 (3'-C), 73.93 (2'-C), 84.07 (4'-C), 89.12 (1'-C), 94.61 (5-C), 140.14 (6-C), 155.05 (2-C), 162.91 (4-C). HRMS (ESI) of  $C_{17}H_{29}N_{3}O_{5}$ , *m/z*: calcd  $[M+H]^{+}$  356.2180, found 356.2174; calcd [M+H]<sup>+</sup> 378.1999, found 378.1999.

4.2.12.  $N^4$ -Decylcytidine (**4l**). Prepared according to the general procedure from **1c** (185 mg) and decylamine (157 mg, 200 µl). Yield 132 mg (78%). (**4l**). UV:  $\lambda_{max}$  272 nm. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  0.85 (t, J = 6.8 Hz, 3H,  $-CH_2-CH_3$ ), 1.23–1.28 (m, 14H,  $-NH-(CH_2)_2-(CH_2)_7-$ ), 1.44–1.50 (m, 2H,  $-NH-CH_2-CH_2-$ ), 3.18–3.24 (m, 2H,  $-NH-CH_2-$ ), 3.53 (ddd, J = 12.1, 5.4, 3.5 Hz, 1H, 5'-Ha), 3.64 (ddd, J = 12.1, 5.2, 3.1 Hz, 1H, 5'-Hb), 3.80 (ddd, J = 4.1, 3.5, 3.0 Hz, 1H, 4'-H), 3.89–3.96 (m, 2H, 2'-H + 3'-H), 4.92 (d, J = 5.2 Hz, 1H, 3'-OH), 4.99 (t, J = 5.2 Hz, 1H, 5'-OH), 5.23 (d, J = 5.1 Hz, 1H, 2'-OH), 5.71 (d, J = 7.5 Hz, 1H, 5'-OH), 5.75 (d, J = 3.7 Hz, 1H, 1'-H), 7.63 (t, J = 5.5 Hz, 1H, N<sup>4</sup>–H), 7.75 (d, J = 7.4 Hz, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  13.87 (-CH<sub>2</sub>-<u>CH<sub>3</sub>), 22.03, 26.45, 28.53, 28.64, 28.75, 28.92, 28.97, 31.23 (-NH-CH<sub>2</sub>-(<u>CH<sub>2</sub>)<sub>8</sub>-), 39.75 (-NH-CH<sub>2</sub>-), 60.64 (5'-C), 69.43 (3'-C), 73.93 (2'-C), 84.03 (4'-C), 89.19 (1'-C), 94.54 (5-C), 140.12 (6-C), 155.32 (2-C), 163.18 (4-C).</u></u>

HRMS (ESI) of  $C_{19}H_{33}N_3O_5$ , *m/z*: calcd  $[M+H]^+$  384.2493, found 384.2492; calcd  $[M+Na]^+$  406.2312, found 406.2311.

4.2.13.  $N^4$ -(3,6,9-Trioxadecyl)-5-methyl-2'-deoxycytidine (**4** m). Prepared according to the general procedure from 1b (163 mg, 0.5 mmol) and 1-amino-3,6,9-trioxadecane (106 mg, 0.65 mmol). Yield 132 mg (68%). UV:  $\lambda_{max}$  272 nm. <sup>1</sup>H NMR (300 MHz, DMSO- $d_6$ )  $\delta$  1.86 (d, I = 1.0 Hz, 3H, 5-CH<sub>3</sub>), 1.97 (ddd, I = 13.3, 7.6, 6.0 Hz, 1H, 2'-Ha), 2.09 (ddd, *J* = 13.1, 6.0, 3.3 Hz, 1H, 2'-Hb), 3.24 (s, 3H, -O-CH<sub>3</sub>), (m, 2H, -NH-CH<sub>2</sub>-), 3.44-3.56 (m, 3.39–3.44 10H. NH-CH2-CH2-O-CH2-CH2-O-CH2-CH2-O-), 3.56-3.59 (m, 1H, 5'-Hb), 3.61 (ddd, I = 11.8, 5.3, 3.8 Hz, 1H, 5'-Ha), 3.77 (ddd, I = 4.2, 53.7, 3.0 Hz, 1H, 4'-H), 4.23 (ddt, J = 6.0, 4.3, 3.3 Hz, 1H, 3'-H), 4.97 (t, *J* = 5.3 Hz, 1H, 5'-OH), 5.16 (d, *J* = 4.3 Hz, 1H, 3'-OH), 6.19 (dd, *J* = 7.5, 6.0 Hz, 1H, 1'-H), 7.11 (t, J = 5.3 Hz, 1H, N<sup>4</sup>-H), 7.61 (d, J = 1.1 Hz, 1H, 6-H). <sup>13</sup>C NMR (75 MHz, Acetonitrile-*d*<sub>3</sub>) δ 12.37 (5-CH<sub>3</sub>), 40.35 (2'-C), 40.40 (-NH-CH<sub>2</sub>-), 57.97 (-O-CH<sub>3</sub>), 61.83 (5'-C), 68.67, 69.98, 70.02, 70.10, 71.63 (NH-CH<sub>2</sub>-<u>CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O-CH<sub>2</sub>-O</u> ), 70.88 (3'-C), 86.25 (1'-C), 87.42 (4'-C), 102.55 (5-C), 138.11 (6-C), 156.25 (2-C), 163.48 (4-C). HRMS (ESI) of C17H29N3O7, m/z: calcd [M+H]<sup>+</sup> 388.2078, found 388.2080; calcd [M+Na]<sup>+</sup> 410.1898, found 410.1900.

## 4.3. 3'-O-(8-Hydroxy-3,6-dioxaoctyloxycarbonyl)-N<sup>4</sup>-alkyl-2'deoxycytidines (**4n-4o**)

## 4.3.1. 3'-O-(8-tert-Butyldimethylsilyloxy-3,6-

*dioxaoctyloxycarbonyl)-5'-O-(tert-butyldimethylsilyl)thymidine* (8)

5'-O-(tert-Butyldimethylsilyl)thymidine (**6**, 820 mg, 2.3 mmol) was dissolved in dry dimethylformamide (3 ml) and N.N'-carbonylditriazole was added (1.5 g, 0.84 mmol). The mixture was heated at 37 °C for 24 h. Then anhydrous triethylene glycol (1.65 g, 1.5 ml, 9.2 mmol) and dioxane (3 ml) were added. The mixture was heated at 37 °C for 24 h, and then solvents were evaporated. The product was extracted in (chloroform + hexane, 2:1 v/v) - water system, the organic layer was evaporated. 3'-O-(8-Hydroxy-3,6dioxaoctyloxycarbonyl)-5'-O-(tert-butyldimethylsilyl)thymidine (7) was isolated by column chromatography in chloroform:ethyl acetate:ethanol (5:5:0.1 v/v) eluting system to give 840 mg (73%). The product 7 (820 mg, 1.7 mmol) was dissolved in dry pyridine (5 ml) and tert-butyldimethylchlorosilane (453 mg, 3 mmol) was added. The mixture was stirred for 16 h at 4°C, then solvents were evaporated. The product 8 was isolated by column chromatography in chloroform:ethanol (20:1 v/v) eluting system to give 680 mg (49%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 0.05 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>((CH<sub>3</sub>)<sub>3</sub>C) Si-O-CH<sub>2</sub>-CH<sub>2</sub>-), 0.12 (s, 6H, 5'-OSi(C(CH<sub>3</sub>)<sub>3</sub>)(CH<sub>3</sub>)<sub>2</sub>), 0.88 (s, 9H, (CH<sub>3</sub>)<sub>2</sub>((CH<sub>3</sub>)<sub>3</sub>C)Si-O-CH<sub>2</sub>-CH<sub>2</sub>-), 0.92 5'-9H. (S.  $OSi(C(CH_3)_3)(CH_3)_2)$ , 1.91 (s, 3H, 5-CH<sub>3</sub>), 2.10 (ddd, J = 14.0, 8.8, 6.3 Hz, 1H, 2'-Ha), 2.49 (ddd, J = 13.9, 5.4, 1.2 Hz, 1H, 2'-Hb), 3.54 (t, I = 5.4 Hz, 2H, (CH<sub>3</sub>)<sub>2</sub>((CH<sub>3</sub>)<sub>3</sub>C)Si-O-CH<sub>2</sub>-CH<sub>2</sub>-), 3.65 (s, 4H, -CH<sub>2</sub>-0-CH<sub>2</sub>-CH<sub>2</sub>-0-CH<sub>2</sub>-), 3.74 (m, 4H. -CO3-CH2-CH2-+(CH<sub>3</sub>)<sub>2</sub>((CH<sub>3</sub>)<sub>3</sub>C)Si-O-CH<sub>2</sub>-CH<sub>2</sub>-), 3.89 (m, 1H, 5'-Hb), 3.92 (m, 1H, 5'-Ha), 4.18 (m, 1H, 4'-H), 4.29 (m, 2H, -CO<sub>3</sub>-CH<sub>2</sub>-), 5.15 (m, 1H, 3'-H), 6.34 (dd, J = 9.2, 5.3 Hz, 1H, 1'-H), 7.50 (s, 1H, 6-H), 10.79 (q, J = 1.3, 0.9 Hz, 1H, 3-NH).

#### 4.3.2. General method for the synthesis of compounds **4n-4o**

2-Chlorophenyl dichlorophosphate (188 mg, 126  $\mu$ l, 0.77 mmol) was added to a solution of 3'-O-(8-*tert*-butyldimethylsilyloxy-3,6-dioxaoctyloxycarbonyl)-5'-O-(*tert*-butyldimethylsilyl)thymidine (**8**, 324 mg, 0.5 mmol) and 1,2,4-triazole (200 mg, 3 mmol) in anhydrous pyridine, cooled to 0 °C. The mixture was stirred for 20 h at room temperature, with solvents evaporated afterwards. The residue was partitioned between chloroform and 0.5 M sodium bicarbonate, the chloroform layer was washed with water, dried over Na<sub>2</sub>SO<sub>4</sub>, the solvent was evaporated and the residue was

dissolved in anhydrous dioxane (3 ml). A corresponding 1alkylamine (1 mmol) was added to the dioxane solution, cooled to 0 °C. The mixture was stirred for 20 h at room temperature, then 3 ml of conc. aq. ammonia solution were added and the mixture was stirred for 40 h at room temperature. The solvents were evaporated: the compounds were purified on a silica gel column  $(2 \times 15 \text{ cm})$  in a gradient of concentrations of ethanol in chloroform (0-15%) or of ethanol in ethyl acetate (0-10%). The target fractions were evaporated in vacuum to give the expected compounds as an amorphous mass in each case. 3'-O-(8-tert-Butyldimethylsilyloxy-3,6-dioxaoctyloxycarbonyl)-5'-O-(tert-butyldimethylsilyl)- $N^4$ alkyl-5-methyl-2'-deoxycytidines were dissolved in dioxane (3 ml), then tetrabutylammonium fluoride trihydrate (380 mg, 1.2 mmol) was added. The mixture was stirred at 25 °C for 8 h and then the solvents were evaporated. The products (4n-4o) were isolated by column chromatography in a chloroform:ethanol (9:1 v/v) eluting

system. 4.3.2.1. 3'-O-(8-Hydroxy-3,6-dioxaoctyloxycarbonyl)-N<sup>4</sup>-decyl-2'deoxycytidines (4n). Prepared according to the general procedure from **8** and decylamine (1 mmol, 199 µl). Yield 175 mg (63%). UV:  $\lambda_{\text{max}}$  273 nm. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  0.85 (t, *J* = 6.8 Hz, 3H, -CH2-CH3), 1.23-1.29 (m, 14H, -NH-(CH2)2-(CH2)7-), 1.46-1.56  $(m, 2H, -NH-CH_2-CH_2-)$ , 1.84  $(d, J = 1.1 Hz, 3H, 5-CH_3)$ , 2.19 (ddd, J)*J* = 14.2, 8.8, 5.9 Hz, 1H, 2'-Ha), 2.28 (ddd, *J* = 14.0, 5.7, 1.8 Hz, 1H, 2'-Hb), 3.25-3.29 (m, 2H, -NH-CH<sub>2</sub>-), 3.40-3.65 (m, 12H, 5'-CH<sub>2</sub> + -CH<sub>2</sub>OC<sub>2</sub>H<sub>4</sub>OC<sub>2</sub>H<sub>4</sub>OH), 4.02 (td, J = 3.6, 1.8 Hz, 1H, 4'-H), 4.20-4.25 I = 5.9, 1.8 Hz, 1H, 3'-H), 5.15 (t, I = 5.3 Hz, 1H, 5'-OH), 6.17 (dd, I = 8.8, 5.6 Hz, 1H, 1'-H), 7.14 (t, I = 5.7 Hz, 1H, N<sup>4</sup>-H), 7.54-7.58 (m, 1H, 6-H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 13.09 (5-CH<sub>3</sub>), 13.88 (-CH2-CH3), 22.02, 26.41, 28.51, 28.63, 28.78, 28.90, 28.95, 31.23 (-NH-CH2-(CH2)8-), 36.96 (2'-C), 40.18 (-NH-CH2-), 60.18 (-CH2-CH<sub>2</sub>-OH), 61.37 (5'-C), 67.01, 68.06, 69.70, 69.74, 72.31 (-CO<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>OC<sub>2</sub>H<sub>4</sub>OCH<sub>2</sub>-CH<sub>2</sub>-OH), 78.62 (3'-C), 84.04 (1'-C), 84.60 (4'-C), 102.02 (5-C), 136.91 (6-C), 153.84 (-CO<sub>3</sub>-), 154.90 (2-C), 162.65 (4-C). HRMS (ESI) of C<sub>27</sub>H<sub>47</sub>N<sub>3</sub>O<sub>9</sub>, *m/z*: calcd [M+H]<sup>+</sup> 558.3385, found 558.3400.

4.3.2.2. 3'-O-(8-Hydroxy-3,6-dioxaoctyloxycarbonyl)-N<sup>4</sup>dodecyl-2'-deoxycytidines (40). Prepared according to the general procedure from 8 and dodecylamine (1 mmol, 185 mg). Yield 191 mg (65%). UV:  $\lambda_{max}$  273 nm. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 0.85 (t, J = 6.6 Hz, 3H, -CH<sub>2</sub>-<u>CH<sub>3</sub></u>), 1.21–1.30 (m, 18H, -NH-(CH<sub>2</sub>)<sub>2</sub>-(CH<sub>2</sub>)<sub>9</sub>-), 1.46-1.57 (m, 2H, -NH-CH<sub>2</sub>-CH<sub>2</sub>-), 1.86 (s, 3H, 5-CH<sub>3</sub>), 2.20 ( $\overline{d}dd$ , J = 14.1, 8.8, 5.8 Hz, 1H, 2'-Ha), 2.30 (ddd, J = 14.1, 5.8, 1.8 Hz, 1H, 2'-Hb), 3.26–3.33 (m, 2H, -NH–CH<sub>2</sub>-),  $3.40-3.66 \text{ (m, 12H, 5'-} \underline{CH}_2 + -\underline{CH}_2 O \underline{C}_2 \underline{H}_4 O \underline{C}_2 \underline{H}_4 O H), 4.04 \text{ (td, } J = 3.6,$ 1.8 Hz, 1H, 4'-H), 4.18–4.27 (m, 2H, –CO<sub>3</sub>-CH<sub>2</sub>-), 4.55 (t, *J* = 5.4 Hz, 1H, -CH<sub>2</sub>-CH<sub>2</sub>-OH), 5.15 (dt, J = 5.8, 1.8 Hz, 1H, 3'-H), 5.19 (t, I = 5.4 Hz, 1H, 5<sup>'-</sup>OH), 6.19 (dd, I = 8.8, 5.6 Hz, 1H, 1<sup>'</sup>-H), 7.19 (t, I = 5.6 Hz, 1H, N<sup>4</sup>-H), 7.59 (s, 1H, 6-H). <sup>13</sup>C NMR (75 MHz, DMSO- $d_6$ ) δ 13.10 (5-CH<sub>3</sub>), 13.86 (-CH<sub>2</sub>-CH<sub>3</sub>), 22.09, 26.51, 28.59, 28.73, 28.90, 29.05, 29.08, 31.31 (-NH-CH<sub>2</sub>-(CH<sub>2</sub>)<sub>10</sub>-), 37.12 (2'-C), 40.27 (-NH-CH<sub>2</sub>-), 60.25 (-CH<sub>2</sub>-CH<sub>2</sub>-OH), 61.43 (5'-C), 67.02, 68.13, 69.76, 69.81, 72.38 (-CO<sub>3</sub>-C<sub>2</sub>H<sub>4</sub>OC<sub>2</sub>H<sub>4</sub>OCH<sub>2</sub>-CH<sub>2</sub>-OH), 78.72 (3'-C), 84.18 (1'-C), 84.74 (4'-C), 102.15 (5-C), 136.90 (6-C), 153.90 (-CO<sub>3</sub>-), 155.08 (2-C), 162.72 (4-C). HRMS (ESI) of C<sub>29</sub>H<sub>51</sub>N<sub>3</sub>O<sub>9</sub>, m/z: calcd [M+H]<sup>+</sup> 586.3698, found 586.3717.

# 4.4. Chemical hydrolysis of compounds 4n and 4o

Compounds **4n** and **4o** (5 mg) were dissolved in dioxane (0.25 ml) and these aliquots were subjected to chemical hydrolysis. Each sample was added and incubated for 24 h at 37 °C in the three following media: 0.2 M glycine +0.2 M HCl buffer (pH 2.2); 0.1 M potassium phosphate buffer (pH 7.5, 0.25 ml); 0.2 M glycine + 0.2 M

NaOH buffer (pH 9.0). Aliquots were taken out and analyzed by TLC (in chloroform:ethanol 9:1 v/v).

## 4.5. Biological evaluation

## 4.5.1. Cytotoxic activity

The line of lung carcinoma A549 (ATCC № CCL-185) was obtained from the collection of the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences.

Adherent A549 cells being passaged were cultured in DMEM medium containing 10% fetal calf serum, 2 mM glutamine, at  $37^{\circ}$ C in an atmosphere of 5% CO<sub>2</sub> at a humidity of 90%. A suspension culture of Jurkat cells was cultured in medium RPMI containing 10% fetal calf serum, 2 mM glutamine, under the same conditions. Cytotoxicity of the compounds was determined by the MTT test [34]. The CC<sub>50</sub> value is the concentration of a compound required to inhibit 50% cell culture growth.

## 4.5.2. Acute and chronic toxicity of nucleoside 4h

Acute and chronic toxicity of nucleoside **4h** were estimated by intraperitoneally injections of compound into white mice (average weight of the mouse 30–35 g, 6 mice for each of the 8 doses of **4h**, 3 males and 3 females). To determine acute toxicity, nucleoside **4h** was dissolved in 30% aqueous solution of Tween-80 and injected into mice 0.2 ml intraperitoneally at doses of 80–400 mg/kg (0.2 ml of 30% Tween-80 used as a positive control) and the animals were observed for a week.

To determine the toxicity of nucleoside **4h** with subchronic administration, the drug was administered daily one time per day for 5 consecutive days at doses less than  $LD_{50}$  (<270 mg/kg) and the animals were observed for four weeks.

Statistical analysis was performed using the Statistics subpackage from *SciPy*.

# 4.5.3. Hydrolysis of compounds **4n** and **4o** in human blood serum

Human blood serum (100%, 99  $\mu$ l) was added to 100 mM solution of compounds **4n** and **4o** in DMSO (0.5  $\mu$ l), and the mixture was incubated at 37 °C. Aliquots were taken out after certain intervals, ethanol (40  $\mu$ l) was added, the mixture was kept for 20 min at -20 °C, and centrifuged. The supernatants were evaporated, the residues were dissolved in ethanol (50  $\mu$ l) and analyzed by HPLC or TLC.

## 4.5.4. In vitro antibacterial assay

4.5.4.1. Bacterial strains. The following test strains were used: Gram-positive bacteria Bacillus subtilis ATCC 6633, Staphylococcus aureus INA 00761 (MRSA), Leuconostoc mesenteroides VKPM B-4177; mycobacteria Mycobacterium smegmatis mc<sup>2</sup>155, and M. smegmatis VKPM Ac-1339; Gram-negative bacteria Pseudomonas aeruginosa ATCC 27853 and fungi Aspergillus niger and Saccharomyces cerevisiae INA 01129 from the collection of the Gause Institute of New Antibiotics.

4.5.4.2. In vitro study of the antibacterial effect. Test strains were incubated in modified Gause's nutrient medium N<sup>o</sup> 2. The level of infection with test cultures was  $10^6$  cells/ml. A compound being tested was dissolved in 50% aq. methanol. Ten volume percent of tested compound was added to the nutrient medium. Samples without the addition of substances, antibiotics in medical use (amikacin, ciprofloxacin, isoniazidi, rifampicin, oxacillin and vancomycin) and samples of medium supplemented with a mixture of solvents served as controls of the test culture growth. Fungal test cultures and *L. mesenteroides* were incubated at 28 °C, and all other strains were incubated at 37 °C.

# 4.5.5. Fungal growth inhibition

4.5.5.1. Fungi strains. The following fungi strains were used in the study: Aspergillus versicolor STG-25G (SRX7729174; MK260015.1), Ulocladium chartarum STG-36 (SRX7729176), Cladosporium halotolerans STG-52B (SRX7729178; MK258720.1), Aspergillus creber STG-57 (SRX7729180; MK266993.1), Aspergillus versicolor STG-86 Aspergillus (SRX7729182; MK262781.1), sp. STG-93W (SRX7729186), Cladosporium parahalotolerans STG-93B (SRX7729188; MK262909.1), Simplicilium lamellicola STG-96 STG-103 (SRX7729190; MK262921.1), Microascus paisii (SRX7729192), and Aspergillus amoenus STG-106 (SRX7729194; MK268342.1).

4.5.5.2. Fungal growth inhibition. The filamentous fungi cultivated on Czapek-Dox agar (CDA) medium (30 g/l sucrose, 2 g/l NaNO<sub>3</sub>, 1 g/ l K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>  $\times$  7H<sub>2</sub>O, 0.5 g/l KCl, 0.01 g/l FeSO<sub>4</sub>  $\times$  7H<sub>2</sub>O, 20 g/l agar, pH 7.0–7.4). To determine the toxicity effect of  $N^4$ modified cytidines on the growth of fungal cells the drop-dilution method was used with some modifications as described earlier [45–47]. Cells were collected from agar slants and diluted with 0.9% NaCl solution up to  $OD_{600} = 0.3$  (basic concentration), followed by serial tenfold dilutions with the same solvent. Then, 1.5  $\mu$ l of cell suspension was inoculated onto Petri dishes with CDA prepared with or without the addition of compounds, N<sup>4</sup>-modified cytidines or 5-fluorocytosine (5FC) for positive control in the concentration 0.5-3 mM. The inoculated plates were incubated for 48 days at 26°C. The inhibitory effects of the compounds were measured every three days after inoculation and evaluated by ratio of colony growth on CDA medium supplemented with N<sup>4</sup>-modified cytidines or 5FC to the control growth (CDA medium without any additions). To determine the percent of fungal growth inhibition (FGI) used the formula: FGI  $\% = [(Dc-Dt)/Dc] \times 100$ , where Dc indicates the colony diameter in control set, and Dt indicates the colony diameter in treatment set. The data recorded were measured in triplicate and repeated at least twice.

## **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgments

The synthesis, the cytotoxicity and antibacterial studies were supported by the Russian Foundation for Basic Research (RFBR, projects 20-04-00536 and 20-34-90056. The study of the antifungal effects was supported by the RFBR (project 17-29-04349). G.K. Nuraeva and I.A. Volkov are grateful to the Ministry of Science and Higher Education of the Russian Federation for support of the work on cultivation of filamentous fungi within the framework of the state contract no. 075-00337-20-03 (project identifier FSMG-2020-0007). Mass-spectrometry studies were supported by the Program of fundamental research for state academies for 2013–2020 (project identifier 01201363818).

The authors are grateful to Dr. R.A. Novikov (Engelhardt Institute of Molecular Biology RAS) for NMR investigations.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113212.

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