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Synthesis, antibacterial and antitumor activity of methylpyridinium salts of pyridoxine functionalized 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles

Arthur A. Grigor'ev[a], Nikita V. Shtyrlin[b], Raylya R. Gabbasova[b], Marina I. Zeldi[b], Denis Yu. Grishaev[b], Oleg I. Gnezdilov[c], Konstantin V. Balakin[b,d], Oleg E. Nasakin[a], and Yurii G. Shtyrlin[b]

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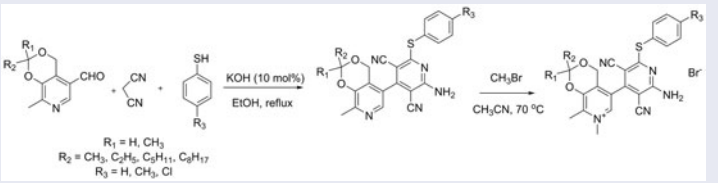
ABSTRACT

A library of 29 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles functionalized with a pyridoxine moiety was synthesized using a three-component one-pot reaction of aldehyde derivative of pyridoxine, malononitrile, and thiophenol. The obtained bipyridine structures were converted into methylpyridinium salts. Several compounds demonstrated expressed antibacterial activity with MICs (minimum inhibitory concentrations) in the range of 0.5–4 μg/mL against the three studied Gram-positive strains and 8–64 μg/mL against the Gram-negative *E. coli* strain, which was comparable or better than the activity of the reference antimicrobial agents. At the same time, all the synthesized compounds were inactive against the Gram-negative *P. aeruginosa*. Several compounds also demonstrated high cytotoxic activity against the studied tumor cells, but without selectivity for the normal HSF (human foreskin fibroblast) cells. Despite the preliminary character of the performed biological studies, the obtained results make the obtained structural chemotype a promising starting point for the design of physiologically active compounds.

GRAPHICAL ABSTRACT



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2-Amino-6-sulfanylpyridine-3,5-dicarbonitriles; antibacterial activity; antitumor activity; pyridoxine; quaternary ammonium salts

Introduction

Variously substituted 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles (general structure **I**, Fig. 1) represent an interesting structural chemotype with promising biological activities.

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Supplemental data for this article can be accessed on the publisher's website.

They have been described as potent antimicrobial[1] and antitumor[2] agents, inhibitors of tyrosine kinases,[3] modulators of androgen receptor function,[4] inhibitors of DNA methyltransferase 1[5], agents for the treatment of prion diseases,[6] and agonists of adenosine receptors.[7,8] One of the most known agents is Capadenoson (**II**, Fig. 1), a highly potent selective partial adenosine A1 receptor agonist ($EC_{50} = 0.1$ nM), which has completed a phase-II clinical trial at Bayer for use in patients with atrial fibrillation.[8a] Its close structural analog, Neladenoson bialanate, an oral partial A1 receptor agonist, is in phase-II clinical development for the treatment of patients with chronic heart failure.[8b]

The structural diversity of the 2-amino-6-sulfanylpyridine-3,5-dicarbonitrile pharmacophore can be achieved by chemical modification of positions 2, 4 and 6 of the studied molecular scaffold **I**. Of particular interest is position 4 which can be occupied by various R_3-substituents for modification of biological activity profile. The most actively investigated type of R_3-substituents are variously substituted phenyl rings (for example, Capadenoson **II**). At the same time, different aryl, heteroaryl and alkyl substituted derivatives can also possess interesting biological activities. Thus, 4-(2-naphtyl)-substituted compounds have been reported as potent antitumor agents.[2] 4-(2-Furyl)- and 4-(2-thienyl)-substituted derivatives demonstrate expressed activity against replication of the infectious prion isoform (PrPSc).[6b,6c] Bioisosteric 4-(3-pyridyl)-[7d] and 4-(1*H*-pyrazol-3-yl)-[7e] substituted analogs of Capadenoson have been described as highly potent adenosine A1 agonists selective over A2A and A2B receptors. 4-Ethyl-substituted agents are potent inhibitors of DNA methyltransferase 1, potential antitumor agents.[5] To complete the picture, 4-unsubstituted compounds ($R_3 = H$) are able to selectively suppress recombinant human Plk-1 tyrosine kinase activity and display antiproliferative activity against non-small lung cancer A549 cells.[9]

These promising pharmacological results encouraged us to develop novel synthetic routes to various derivatives of 2-amino-6-sulfanylpyridine-3,5-dicarbonitrile, including their heterocyclic analogs.[10] As a continuation of this work, in the present paper, we report a novel structural chemotype **III** (Fig. 1) in which the substituent at position 4 of the pyridine-3,5-dicarbonitrile scaffold represents a methylpyridinium derivative of pyridoxine.

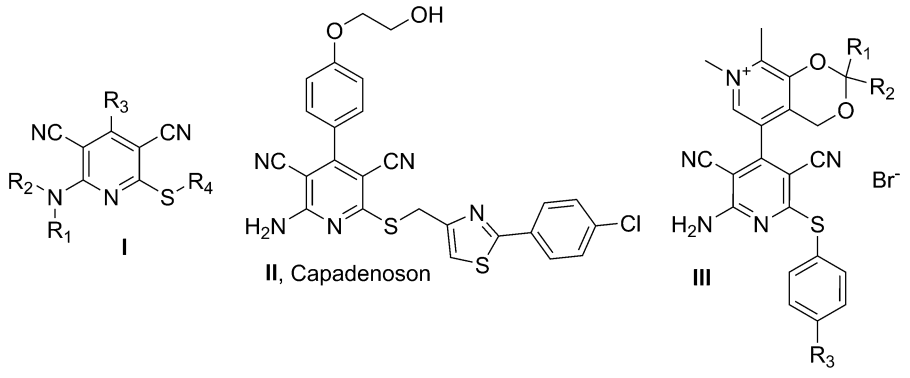


Figure 1. General formula of 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles (I); example of a selective partial adenosine A1 receptor agonist Capadenoson which has completed a phase II clinical trial (II); compounds synthesized in this work (III).

From the "pyridoxine-centric" point of view, the obtained compounds **III** represent 5-heteroaryl substituted derivatives of pyridoxine (vitamin B6), a key cofactor of many important enzymes. In our group, we have systematically studied chemistry and biology of the physiologically active pyridoxine derivatives. In particular, we have described a wide series of 5-substituted pyridoxine derivatives, including 5-alkenyl-substituted compounds[11], as well as ammonium and phosphonium salts[12], which possess promising pharmacological potential as antibacterial and antitumor drugs. We have also reported N-methylpyridoxinium salts with potent anticholinesterase activity.[13]

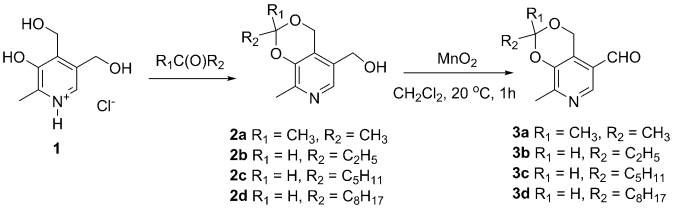
The mentioned examples demonstrate the excellent potential of 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles and 5-substituted pyridoxines (including their ammonium salts) in the design of novel pharmacological agents. Molecules **III** synthesized in this work contain both pharmacophore motifs that make the designed hybrid structures a promising object for biological and pharmacological studies. The obtained compounds have been tested *in vitro* for their ability to inhibit growth of Gram-positive and Gram-negative bacterial pathogens, as well as tumor cells.

Results and discussion

Chemistry

A good synthetic accessibility of various 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles favors to their active synthetic and biological studies. The target compounds can be obtained using a three-component one-pot reaction of aldehyde, malononitrile, and thiol actively studied by Evdokimov et al.[14] The reaction is typically catalyzed by bases[15], but examples of successful use of Lewis acids[16], boronic acid[17], ionic liquids[18], nanoparticles of various composition[19] and organometallic compounds[20] as catalysts have been reported. The reaction can also be promoted by microwaves[21] and ultrasound.[22]

As the key aldehyde reagents for the multicomponent reaction, we used aldehyde derivatives of pyridoxine with various substituents at the acetal carbon atom, that were prepared from pyridoxine hydrochloride **1** in two synthetic stages (Scheme 1). At the first step, the cyclic ketal **2a** and acetals **2 b** and **2d** were obtained according to a literature procedure by reaction of **1** with the corresponding carbonyl reagents in the presence of acid.[12a,23] Acetal **2c** was obtained by reaction of **1** with *n*-hexanal in the presence of *para*-toluenesulphonic acid under reflux. The variation of substituents at the acetal carbon atom makes it possible to vary compound's lipophilicity and thus investigate the lipophilicity-activity relationships. Compounds **2a-d** were then treated with MnO_2 in chloroform to obtain aldehydes **3a-d** in good yields (72–86%).

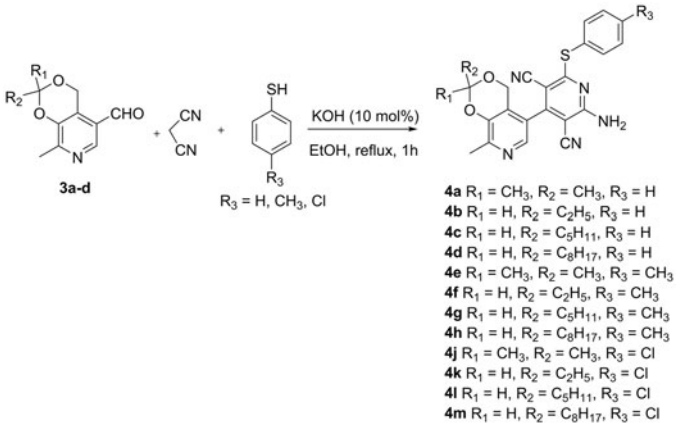


Scheme 1. Synthesis of aldehydes **3a-d**.

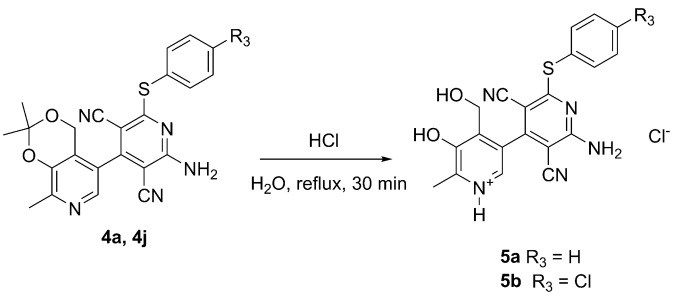
At the next stage, aldehydes **3a-d** were introduced into a multicomponent reaction with 2 molar equivalents of malononitrile and 1 molar equivalent of three commercially available thiophenols (thiophenol, *p*-thiocresol, 4-chlorothiophenol). It should be noted that in this type of multicomponent reactions, only aromatic thiols can be used. The use of triethylamine, in accordance with the method developed by Evdokimov et al.[14], led to the target compounds in low yields (5-10%). The use of 10% (mol.) KOH reported by Khan et al.[24] was more successful and led to the target pyridine-3,5-dicarbonitriles **4a-m** in 25–40% yield (Scheme 2). The reaction also resulted in a number of side products, which were problematic to separate and identify. All further attempts to increase the reaction yields by varying the experimental conditions (time, temperature, base nature) were unsuccessful.

Compounds **4a-m** appeared to be completely insoluble in water, and therefore it was problematic to perform their biological studies in aqueous media. In an attempt to improve solubility, ketals **4a** and **4j** with different R_3-substituents were treated with aqueous HCl to deprotect the hydroxyl groups of the pyridoxine part (Scheme 3). The resulting diols **5a,b** were obtained in 85% and 77% yields, respectively, as stable crystalline substances. Unfortunately, the deprotection did not significantly improve the solubility in water, and compounds **5a,b** was still problematic to evaluate in biological tests. In view of very poor solubility of compounds **5a,b** with $R_3 = H$ and Cl, we did not synthesize the third possible diol with a more lipophilic R_3-substituent ($R_3 = CH_3$).

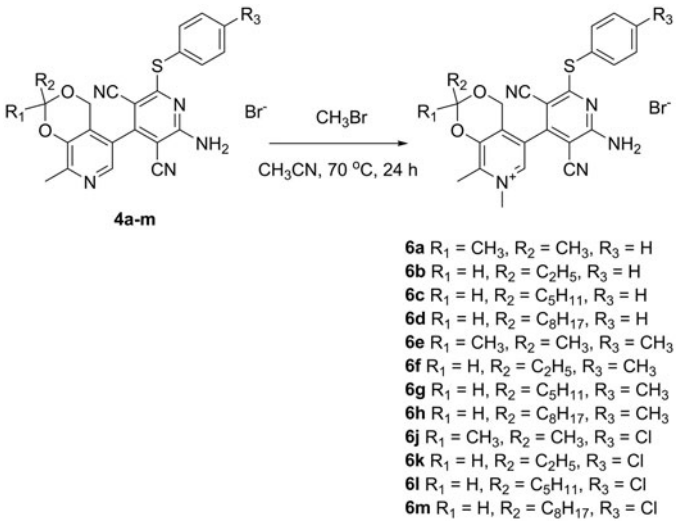
It is widely known that many quaternary ammonium compounds (QACs) possess expressed antibacterial properties.[25] In our previous works, we have reported several chemotypes of pyridoxine-based QACs with potent antimicrobial activity.[12] To increase solubility and enhance the antimicrobial potential of the obtained structures, we converted compounds **4a-m** into the corresponding pyridinium salts (Scheme 4). The reaction of **4a-m** with methyl bromide led to selective methylation of the pyridoxine nitrogen atom to give compounds **6a-m** in 50–70% yields. The chemoselective character of this reaction can be unequivocally confirmed by ^{1}H NMR spectral data which demonstrate an expressed downfield shift of signals from protons belonging to 2- and 6-substituents of the pyridoxine ring adjacent to the endocyclic N1 atom. Thus, as a result of the conversion of compounds **4a-m** to **6a-m**, we observed a shift from



Scheme 2. Synthesis of compounds **4a-m**.



Scheme 3. Synthesis of compounds **5a,b** with deprotected hydroxyl groups.

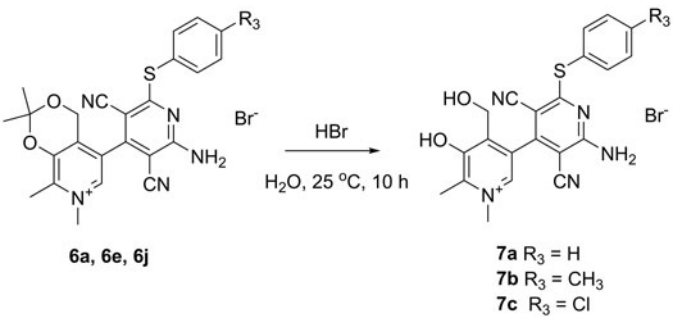


Scheme 4. Synthesis of methylpyridinium salts **6a-m**.

2.40–2.42 ppm to 2.68–2.71 ppm for the three protons of 2-methyl group, and a shift from 7.96–8.04 ppm to 8.96–9.02 ppm for the aromatic proton at position 6. Similar observations were reported in our recent work in which we obtained N-methylated pyridoxine derivatives.[13] At the same time, we did not observe any changes in ^{13}C NMR signals from carbon atoms of the pyridine-3,5-dicarbonitrile ring, and this fact indicated that the endocyclic nitrogen atom of this fragment remained intact during the methylation reaction. Interestingly, even a prolonged treatment with an excess of ethyl bromide or decyl bromide under reflux did not result in the corresponding pyridinium slats, probably, due to steric hindrance.

At the final synthetic step, three compounds **6a**, **6e** and **6j** with different R_3-substituents were treated with aqueous HBr to deprotect the hydroxyl groups of the pyridoxine part (Scheme 5). The resulting diols **7a-c** were obtained in good yields (70–75%) as stable crystalline substances.

The developed synthetic route to compounds **4-7** is straightforward and well reproducible. The obtained pyridinium salts **6a-m** and **7a-c** were soluble enough in the water that allowed us to evaluate their antibacterial and antitumor activity.



Scheme 5. Synthesis of compounds **7a–c** with deprotected hydroxyl groups.

Table 1. *In vitro* antimicrobial activity of compounds **6a-m** and **7a-c**.

	MICs (μg/mL)					
	Gram-positive bacteria			Gram-negative bacteria		
Compounds	*S. aureus* ATCC 29213	*S. epidermidis* (clinical isolate)	*B. subtilis 168*	*E. coli* ???? 25922	*P. aeruginosa* ATCC 27853	AlogPS
6a	>64	>64	>64	>64	>64	0.34 ± 1.5
6b	64	32	64	>64	>64	0.35 ± 1.5
6c	4	4	4	32	>64	0.98 ± 1.5
6d	2	1	1	>64	>64	1.18 ± 1.5
6e	>64	>64	>64	>64	>64	0.51 ± 1.5
6f	32	16	16	64	>64	0.53 ± 1.5
6g	4	4	4	>64	>64	1.2 ± 1.5
6h	2	0.5	1	8	>64	1.6 ± 1.5
6j	>64	>64	>64	>64	>64	0.69 ± 1.5
6k	32	32	32	64	>64	0.7 ± 1.5
6l	4	2	4	32	>64	1.4 ± 1.5
6m	2	2	2	32	>64	1.6 ± 1.5
7a	>64	>64	>64	>64	>64	−0.32 ± 1.5
7b	>64	>64	>64	>64	>64	−0.19 ± 1.5
7c	>64	>64	>64	>64	>64	−0.02 ± 1.5
Miramistin	4	2	2	32	16	4.5 ± 1.5
Benzalkonium chloride	2	2	32	4	64	–

In vitro antibacterial activity

The antibacterial activity of 15 new synthesized compounds was evaluated against three Gram-positive and two Gram-negative bacterial strains. Table 1 shows the MIC values of the tested compounds **6a-m** and **7a-c**. The lipophilicity of the synthesized ammonium salts was expressed in terms of their partition coefficient values (logP) calculated using AlogPS algorithm.[26]

As reference drugs, two widely used antiseptic quaternary ammonium compounds, miramistin and benzalkonium chloride, were used. Due to broad-spectrum efficacy, substantivity for the skin, and low irritation, benzalkonium chloride belongs to the most widely used biocides in antiseptic products, in particular in handwashing and oral products but also as disinfectants and preservatives.[27] Miramistin, which has been developed in the 1980s in the former USSR as an antiseptic for the treatment of skin of cosmonauts at orbital stations[28], is one of the most popular antimicrobial agents on

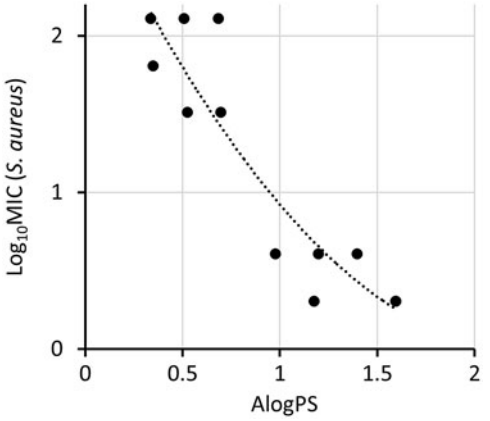


Figure 2. Log10MIC (*S. aureus*) versus calculated AlogPS relationship for compounds **6a-m**. A polynomial trend line is shown. For three inactive compounds **6a**, **6e** and **6j** (MICs > 64 μg/mL), MICs were accepted equal to 128 μg/mL.

the pharmaceutical market of the Russian Federation. Five bacterial test organisms, *S. aureus, S. epidermidis, B, subtilis, E. coli* and *P. aeruginosa* were selected as the most common Gram-positive and Gram-negative hospital pathogens.[27]

At least five compounds demonstrated expressed antibacterial activity with MICs in the range of 0.5–4 μg/mL against the three studied Gram-positive strains and 8–64 μg/mL against the Gram-negative *E. coli* strain, which was comparable or better than the activity of the reference agents. However, all the obtained compounds were inactive against the Gram-negative *P. aeruginosa*. The decreased activity of against Gram-negative pathogens is in agreement with literature data on cationic disinfectants (for example,[25]).

The antibacterial activity was positively correlated with the length of the substituents at the acetal carbon atom (octyl > pentyl > ethyl), reaching a maximum for octyl substituent (MIC 0.5–4 μg/mL for compounds **6d**, **6h,** and **6m**). In agreement with this observation, a strong correlation between the lipophilicity and antimicrobial activity of quaternary ammonium pyridoxine derivatives was found. Figure 2 demonstrates the relationships between the experimental MICs (expressed in a decimal logarithm scale) for *S. aureus* and calculated AlogPS values for the studied quaternary ammonium pyridoxine derivatives **6a-m**. Similar relationships were found for the other studied bacterial strains. It can be suggested that the found relationships reflect the important features of the active compounds essential for their effective interaction with the hydrophobic membrane core of bacterial cells.

Compounds **7a-c** with the deprotected hydroxyl groups were inactive in this experiment. This result might be explained by their hydrophilic nature (AlogPS in the range of $-0.02 \div -0.32$).

In vitro cytotoxicity

The synthesized compounds were then studied for antitumor activity and cytotoxicity *in vitro* (Table 2). The study of antitumor activity was carried out on breast

Table 2. Antitumor activity and cytotoxicity *in vitro* of compounds **6a-m** and **7a-c**.

	IC_{50} (μM)			
Compounds	MCF-7	SNB-19	HCT-116	HSF
6a	141.5	106.4	91.3	272.4
6b	27.7	35.5	8.3	11.2
6c	6.9	13.9	8	7
6d	2.8	5.1	2.8	2.8
6e	99.2	99.7	66.1	264.1
6f	17.0	22.2	4.1	14.6
6g	3.8	13.2	6.6	3.4
6h	14.4	9.4	6.1	15.7
6j	138.8	101.5	161.4	241.9
6k	8.4	13.8	8.1	7.5
6l	5.6	13.6	4.2	4.4
6m	2.3	10.3	3.4	3.6
7a	119.4	96.2	121.6	266
7b	130.2	91.8	94.4	268.1
7c	15.7	37.6	15.7	16.2
Doxorubicin	0.2	0.1	0.1	0.9

adenocarcinoma MCF-7, glioblastoma SNB-19, and human colon colorectal carcinoma HCT-116 cells. Cytotoxicity was studied on primary human foreskin fibroblast (HSF) cells. Doxorubicin, one of the most widely used cytostatic agents, was used as a reference drug.

Several compounds (for example, **6d**, **6 g**, **6m**) demonstrated a relatively high antitumor activity against the studied tumor cells in a low-micromolar concentration range. However, their activity was at least one order of magnitude lower than that of Doxorubicin. In addition, their cytotoxicity against the normal HSF cells was comparable to antitumor activity thus suggesting poor selectivity of their action and high *in vivo* toxicity.

In general, the obtained results demonstrate the interesting pharmacological potential of the described novel chemotype. Several compounds can be considered as antimicrobial agents active against Gram-positive bacterial pathogens.

Conclusions

In conclusion, we have synthesized a diverse library of 29 new pyridoxine functionalized 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles and studied their antibacterial activity and cytotoxicity *in vitro*. The synthetic route is based on a three-component one-pot reaction of the corresponding aldehyde, malononitrile and thiol reactants, that leads to the target bipyridine scaffold in moderate-to-good yields. To increase solubility and to enhance the antimicrobial potential of the obtained structures, they were regioselectively converted into methylpyridinium salts. Mild acidic hydrolysis led to the corresponding compounds with deprotected hydroxyl groups. To the best of our knowledge, the obtained structures represent a first reported example of 5-aryl-substituted pyridoxine derivatives. In the primary biological experiments, several compounds demonstrated a potent antibacterial activity, which was more expressed with respect to the studied Gram-positive strains compared to Gram-negative pathogens. A strong correlation between the lipophilicity and antimicrobial activity of the obtained compounds was

observed. Several compounds also demonstrated high antitumor activity against the studied tumor cells, though without any selectivity against the normal HSF cells.

Despite the preliminary character of the performed biological studies, the described active compounds can be considered as a starting point for the development of new antibacterial therapies. In a more general sense, the presence of two strong pharmacophore motifs in the designed hybrid structures makes them a highly interesting object for further biological and pharmacological studies. From one hand, they belong to 4-heteroaryl substituted analogs of 2-amino-6-sulfanylpyridine-3,5-dicarbonitriles that have been reported as potent antitumor agents,[2] inhibitors of replication of the infectious prion isoform (PrPSc),[6b,6c] and adenosine A1 agonists.[7d,7e] From the other hand, they represent 5-substituted derivatives of pyridoxine that possess promising pharmacological potential as antibacterial and antitumor drugs.[11,12]

Experimental

All reagents were obtained from commercial sources and were used without further purification unless otherwise stated.

Acetals and ketals **2a, 2 b** and **2d** were synthesized according to literature procedures.[12a,23] Compound **3a** was synthesized according to a literature procedure.[29] ^{1}H and ^{13}C NMR spectra were recorded on a "Bruker AVANCE 400" instrument at operating frequencies 400.17 and 100.62 MHz, respectively. Chemical shifts were measured with reference to the residual protons of the solvent (DMSO-d_6, ^{1}H, 2.50 ppm, ^{13}C, 39.52 ppm; $CDCl_3$, ^{1}H, 7.26 ppm, ^{13}C, 77.16 ppm). Coupling constants (*J*) are given in Hertz (Hz). The following abbreviations are used to describe coupling: s - singlet; d - doublet; t - triplet; m - multiplet; q - quartet, br s - broad singlet, AB - AB system. Melting points were determined using a Stanford Research Systems MPA-100 OptiMelt melting point apparatus and are uncorrected. For TLC analysis, silica gel plates from Sorbfil (Krasnodar, Russia) were used with UV light (t, 254 nm/365 nm) or iron (III) chloride as developing agent. Column chromatography was performed on silica gel (60–200 mesh) from Acros. All reactions were carried out in flasks.

High-resolution mass spectroscopy mass spectra were obtained on a quadrupole time-offlight (t, qTOF) AB Sciex Triple TOF 5600 mass spectrometer using turbo-ion spray source (nebulizer gas nitrogen, a positive ionization polarity, needle voltage 5500 V). Recording of the spectra was performed in "TOF MS" mode with collision energy 10 eV, declustering potential 100 eV and with resolution more than 30,000 full-width half-maximum. Samples with the analyte concentration 5 µmol/L were prepared by dissolving the test compounds in a mixture of methanol (HPLC-UV Grade, LabScan) and water (LC-MS Grade, Panreac) in 1:1 ratio.

Synthesis of 5-hydroxymethyl-8-methyl-2-pentyl-4H-[1, 3]dioxino[4,5-c]pyridine (2c)

n-Hexanal (0.78 ml, 9.6 mmol) and *p*-toluenesulfonic acid monohydrate (0.93 g, 4.9 mmol) were added to a suspension of compound **1** (1.00 g, 4.9 mmol) in 120 mL of benzene. The reaction mixture was refluxed with a Dean-Stark trap for 7 h. Then the

solvent was evaporated under reduced pressure, and the residue was neutralized with aqueous solution of $NaHCO_3$. The precipitate was collected by filtration, washed with benzene and dried. Yield 75% (0.91 g), white solid, mp 60-64 °C. ^{1}H NMR ($CDCl_3$) δ: 0.88 (*t*, 3 H, $^3J_{HH} = 7.2$ Hz, $\underline{CH_3}CH_2CH_2CH_2CH_2$), 1.31–1.34 (*m*, 4 H, $CH_3\underline{CH_2CH_2}CH_2CH_2$), 1.47–1.51 (*m*, 2 H, $CH_3CH_2CH_2\underline{CH_2}CH_2$), 1.81–1.85 (*m*, 2 H, $CH_3CH_2CH_2CH_2\underline{CH_2}$), 2.35 (*s*, 3 H, CH_3), 4.47 (*s*, 2 H, $\underline{CH_2}OH$), 4.95 (*s*, 2 H, CH_2O), 4.96 (*t*, 1 H, $^3J_{HH} = 5.1$ Hz, $\underline{CH}C_5H_{11}$), 7.82 (*s*, 1 H, $CH_{Pyr.}$). ^{13}C NMR ($CDCl_3$) δ: 14.04, 17.89, 22.59, 23.29, 31.59, 34.28, 59.82, 64.25, 100.15, 128.08, 130.39, 138.67, 146.85, 148.00. ESI-HRMS *m/z*: 252.1589 $[M+H]^+$ (calculated for $[C_{14}H_{22}NO_3]^+$ - 252.1594).

General procedure for the synthesis of compounds 3b-d

Activated MnO_2 (6.52 g, 75.0 mmol) was added to a solution of the corresponding pyridoxine acetal **2b-d** (5.00 mmol) in 100 ml of dichloromethane, and the reaction mixture was stirred at 20 °C for 1 h. The formed precipitate was filtered off, and the solvent was evaporated in vacuo. The product was purified by column chromatography on silica gel (eluent ethyl acetate). Analytical characteristics of compounds **3c,d** are described in the Electronic Supplementary Material.

2 -Ethyl-8-methyl-4H-[1, 3]dioxino[4,5-c]pyridine-5-carbaldehyde (3b)

The general procedure was followed using compound **2 b** (1.05 g, 5.0 mmol). Yield 72% (0.75 g), yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.07 (*t*, 3H,$^3J_{HH} = 7.5$ Hz, $CHCH_2\underline{CH_3}$), 1.85 – 1.94 (*m*, 2 H, $CH\underline{CH_2}CH_3$), 2.51 (*s*, 3H, CH_3), 4.95 (*t*, 1H, $^3J_{HH} = 5.1$ Hz, $\underline{CH}CH_2CH_3$), 5.14, 5.28 (AB, 2 H, $^2J_{HH} = 18.0$ Hz, CH_2O), 8.46 (*s*, 1H, CH), 10.00 (*s*, 1H, C(O)H). ^{13}C NMR ($CDCl_3$) δ: 7.86, 19.52, 27.53, 65.87, 100.79, 126.41, 128.53, 147.08, 148.45, 154.10, 191.93. HRMS-ESI: 208.0968 $[M+H]^+$ (calculated for $[C_{11}H_{14}NO_3]^+$ -208.0968).

General procedure for the synthesis of compounds 4a-m

A mixture of the corresponding aldehyde **3a-d** (1 mol. equiv.), malononitrile (2 mol. equiv.), KOH (0.1 mol. equiv.), thiol (1 mol. equiv.) and ethanol (10 ml) was placed into a 25 mL round bottom flask fitted with reflux condenser. The reaction mixture was refluxed for approximately 1 h until a clear solution was obtained. Then, the reaction mixture was slowly cooled to 2 °C. The solid product was collected by filtration, washed with ethanol and dried. Analytical characteristics of compounds **4c-m** are described in the Electronic Supplementary Material.

2 -Amino-6-(phenylthio)-4-(2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridin-5-yl)pyridine-3,5-dicarbonitrile (4a)

The general procedure was followed using compound **3a** (638 mg, 3.0 mmol), malononitrile (407 mg, 6.0 mmol), KOH (17 mg, 0.3 mmol) and benzenethiol (339 mg, 3.0 mmol). Yield 30% (400 mg), yellow solid, mp 126-128 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 1.55 (s, 6 H, $2CH_3$), 2.40 (*s*, 3 H, CH_{3Pyr}), 4,72 (*s*, 2 H, CH_2O), 7.50–7,62 (*m*, 5 H,

C_6H_{5Ar}), 7.97 (br.s, 2 H, NH_2), 8.03 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 18.58, 24.17, 24.44, 58.15, 87.82, 93.73, 100.56, 114.64 (CN), 114.99 (CN), 124.53, 124.75, 127.00, 129.55, 129.83, 134.84, 138.51, 145.34, 148.90, 153.28, 159.48, 166.23. ESI-HRMS *m/z*: 430.1338 $[M+H]^+$ (calculated for $[C_{23}H_{20}N_5O_2S]^+$ - 430.1332).

2 -Amino-4-(2-ethyl-8-methyl-4H-[1, 3]dioxino[4,5-c]pyridin-5-yl)-6-(phenylthio)pyridine-3,5-dicarbonitrile (4b)

The general procedure was followed using compound **3 b** (697 mg, 3.4 mmol), malononitrile (444 mg, 6.8 mmol), KOH (19 mg, 0.3 mmol) and benzenethiol (370 mg, 3.4 mmol). Yield 30% (430 mg), pale yellow solid, mp 112–114 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 1.03 (*t*, 3 H, $^3J_{HH}$=6.88 Hz, $\underline{CH_3}CH_2$), 1.82–1.87 (*m*, 2 H, $CH_3\underline{CH_2}$), 2.42 (*s*, 3 H, CH_{3Pyr}), 4.70, 4.84 (AB, 2 H, $^2J_{HH}$ = 16.26 Hz, CH_2O), 5.16 (*t*, 1 H, $^3J_{HH}$=5.00 Hz, CH), 7.48–7.63 (*m*, 5 H, C_6H_{5Ar}), 7.97 (br.s, 2 H, NH_2), 8.04 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 7.64, 18.40, 26.90, 63.47, 87.82, 93.82, 100.92, 114.50 (CN), 114.90 (CN), 124.90, 125.97, 126.99, 129.52, 129.81, 134.81, 139.07, 146.88, 148.44, 153.13, 159.58, 166.12. ESI-HRMS *m/z*: 430.1338 $[M+H]^+$ (calculated for $[C_{23}H_{20}N_5O_2S]^+$ - 430.1332).

General procedure for the synthesis of compounds 5a,b

A mixture of the corresponding compound **4** (0.5 mmol) and 0.4 ml of concentrated HCl in 10 ml of water was refluxed for 30 min. Then the reaction mixture was slowly cooled to 2 °C. The solid product was collected by filtration, washed with water and dried. Analytical characteristics of compound **5 b** are described in the Electronic Supplementary Material.

2 ′-Amino-5-hydroxy-4-(hydroxymethyl)-6-methyl-6′-(phenylthio)-[3,4′-bipyridine]-3′,5′-dicarbonitrile (5a)

Yield 85% (180 mg), pale yellow solid, mp 198–202 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 2.64 (*s*, 3 H, CH_{3Pyr}), 4.68 (*s*, 2 H, CH_2O), 7.49–7.62 (*m*, 5 H, C_6H_{5Ar}), 7.93 (br.s, 2 H, NH_2), 8.35 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 16.83, 56.48, 88.30, 94.34, 114.65 (CN), 115.01 (CN), 126.97, 129.58, 129.86, 133.82, 134.84, 136.71, 146.01, 150.91, 152.05, 159.14, 165.45. ESI-HRMS m/z: 390.1025 $[M-Cl]^+$ (calculated for $[C_{20}H_{16}N_5O_2S]^+$ - 390.1019).

Preparation of methyl bromide solution in acetonitrile

Potassium bromide (12.39 g, 104.1 mmol) and water (0.34 ml, 18.9 mmol) were added to dimethyl sulfate (11.88 ml, 125.4 mmol), and the reaction mixture was heated at 160 °C for 4 h. Evolved methyl bromide was absorbed by 200 ml of cold acetonitrile. Methyl bromide concentration was determined by an increase in mass of the acetonitrile solution.

General procedure for the synthesis of compounds 6a-m

The corresponding compound **4a-m** (0.1–0.7 mmol) was added to a solution of methyl bromide (5.0 mmol) in acetonitrile (10 mL). and the reaction mixture was heated at 70°C for 24 h. Then, the solvent was evaporated under reduced pressure, and the residue was washed with diethyl ether. The precipitate was collected by filtration, washed with diethyl ether and dried. Analytical characteristics of compound **6c-m** are described in the Electronic Supplementary Material.

5 -(2-Amino-3,5-dicyano-6-(phenylthio)pyridin-4-yl)-2,2,7,8-tetramethyl-4H-[1, 3]dioxino[4,5-c]pyridin-7-ium bromide (6a)

The general procedure was followed using compound **4a** (270 mg, 0.6 mmol). Yield 64% (210 mg), gray solid, mp 162–167 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 1.63 (s, 6 H, 2CH_3), 2.69 (*s*, 3 H, CH_{3Pyr}), 4.30 (*s*, 3 H, CH_3-N), 5,03 (*s*, 2 H, CH_2O), 7.46–7,63 (*m*, 5 H, C_6H_{5Ar}), 8.22 (br.s, 2 H, NH_2), 8.98 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 16.71, 24.45, 24.65, 47.03, 58.97, 88.08, 93.63, 103.70, 114.56 (CN), 114.97 (CN), 126.98, 127.64, 130.11, 130.52, 130.52, 133.62, 135.33, 136.36, 149.05, 149.19, 149.27, 159.76, 167.31. ESI-HRMS *m/z*: 444.1494 $[M\text{-}Br]^+$ (calculated for $[C_{24}H_{22}N_5O_2S]^+$ - 444.1489).

5 -(2-Amino-3,5-dicyano-6-(phenylthio)pyridin-4-yl)-2-ethyl-7,8-dimethyl-4H-[1, 3]dioxino[4,5-c]pyridin-7-ium bromide (6b)

The general procedure was followed using compound **4 b** (200 mg, 0.4 mmol), Yield 61% (149 mg), light green solid, mp 200-205 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 1.05 (*t*, 3 H, $^{3}J_{HH} = 7.50$, $\underline{CH_3}CH_2$), 1.89–1.96 (*m*, 2 H, $CH_3\underline{CH_2}$), 2.71 (*s*, 3 H, CH_{3Pyr}), 4.31 (*s*, 3 H, CH_3-N), 5,09 (*s*, 2 H, CH_2O), 5.42 (*t*, 1 H, $^{3}J_{HH} = 5.00$, CH), 7.51–7.63 (*m*, 5 H, C_6H_{5Ar}), 8.20 (br.s, 2 H, NH_2), 8.99 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 7.34, 13.19, 26.52, 46.56, 63.67, 87.53, 93.09, 102.56, 113.96 (CN), 114.33 (CN), 126.48, 127.31, 130.03, 134.70, 134.88, 136.41, 138.78, 148.13, 148.70, 150.04, 159.25, 166.73. ESI-HRMS m/z: 444.1494 $[M\text{-}Br]^+$ (calculated for $[C_{24}H_{22}N_5O_2S]^+$ - 444.1489).

General procedure for the synthesis of compounds 7a-c

A mixture of the corresponding compound **6** (0.3 mmol) and 0.4 ml of concentrated HBr in 10 ml of water was stirred at 25°C for 10 h. Then, the solvent was evaporated under reduced pressure, and the residue was washed with chloroform and dried. Analytical characteristics of compounds **7b,c** are described in the Electronic Supplementary Material.

2 '-Amino-3',5'-dicyano-5-hydroxy-4-(hydroxymethyl)-1,6-dimethyl-6'-(phenylthio)-[3,4'-bipyridin]-1-ium bromide (7a)

Yield 75% (109 mg), yellow solid, mp 161–164 °C (decomp.). ^{1}H NMR (DMSO-d_6) δ: 2.73 (*s*, 3 H, CH_{3Pyr}), 4.29 (*s*, 3 H, CH_3-N), 4.76 (*s*, 2 H, CH_2O), 7.51 – 7,61 (*m*, 5 H, C_6H_{5Ar}), 8.03 (br.s, 2 H, NH_2), 8.86 (*s*, 1 H, CH_{pyr}). ^{13}C NMR (DMSO-d_6) δ: 14.10,

46.52, 56.96, 88.04, 93.95, 114.40 (CN), 114.79 (CN), 126.71, 129.64, 129.98, 134.72, 136.72, 143.47, 147.75, 152.02, 152.91, 159.07, 165.79. ESI-HRMS *m/z*: 404.1181 $[M\text{-}Br]^+$ (calculated for $[C_{21}H_{18}N_5O_2S]^+$ - 404.1176).

Antibacterial activity

The antibacterial activity of the obtained compounds was evaluated on three Gram-positive (*Staphylococcus aureus* ATCC® 29213™, *Staphylococcus epidermidis* (clinical isolate), *Bacillus subtilis* 168) and two Gram-negative bacteria (*Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™). MICs were determined by using the broth microdilution method in 96-well plates. Two-fold serial dilutions of the test samples in 10% dimethyl sulfoxide (DMSO) were prepared to obtain a final test range of 64–0.5 μg/mL. Two microliters of each dilution and 200 μL of test organism (s, $2–9 \times 10^4$ cfu mL^{-1}) in the LB-broth medium were then dispensed into each well. The MIC was defined as the lowest concentration of compound at which no visible growth could be seen after 24 h of cultivation at 37 °C. All experiments were carried out in triplicate.

In vitro cytotoxicity studies

Breast adenocarcinoma MCF-7 (ATCC HTB-22), glioblastoma SNB-19 (ATCC CRL-2219), colorectal carcinoma HCT-116 (GSM136288) and primary human foreskin fibroblast cells (HSF) isolated from the skin explant according to conventional protocol were cultured in α-MEM (PanEko, Russia) supplemented with 10% fetal bovine serum (PAA, Australia), L-glutamine, and 1% penicillin-streptomycin at 37 C in a 5% humidified CO_2 atmosphere in air. Cells were removed from the culture substrate by treatment with trypsin-EDTA with subsequent inactivation of trypsin by adding α-MEM-containing serum. Cell suspensions were precipitated by centrifugation at 500 g and pellet was resuspended in phosphate-buffered saline (PBS). Cell viability and density were measured in a Neubauer chamber by using 0.4% solution of trypan blue. Suspensions with the amount of viable cells no less than 90% were used in the experiments. The effect of synthesized compounds on the proliferation of the cells was measured by MTT-assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Promega). The cells were seeded in a 96-well plate at a concentration of 2000 cells per well and were cultured in supplemented α-MeM (180 μL) according to the standard culturing conditions for 1 day. The cell viability was measured at 72 h after treatment with the test agent (20 μL). Then, the medium was exchanged for the supplemented medium (80 μL). MTT solution (5 mg mL^{-1}) was added to a volume of 20 μL in each well and incubated for 3.5 h. Then the solution was removed and DMSO (100 μL) was added to solubilize the formazan crystals. After 10 min, absorbance was measured at 555 nm (the reference wavelength 650 nm) by a TECAN plate reader (Switzerland). The results were presented as the percent ratio to the control sample treated with PBS. For each compound tested, the IC_{50} values ($\mu < 0.05$) were generated from the dose-response curves for each cell line.

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