Jarosław Sączewski*, Anna Kędzia and Aleksandra Jalińska

New derivatives of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one: synthesis, tautomerism, electronic structure and antibacterial activity

Abstract: The electronic structure and prototropic tautomerism of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) were studied theoretically with use of the B3LYP/6-31G* and ωB97X-D/6-31G* density functional methods and SM8 (H_2O, DMF) solvation models. Compound **1**, which is a weak acid with a pK_a of 6.9, undergoes regioselective alkylation and sulfonylation under basic reaction conditions to give a series of *N*1-substituted products **2a**–**i**. Later compounds were evaluated *in vitro* for antibacterial activity with the use of 68 strains of aerobic and anaerobic bacteria, including 12 reference strains.

Keywords: acidity; antibacterial activity; DFT quantum chemical calculations; isoxazolo[3,4-*b*]pyridin-3(1*H*)-one; *N*-alkylation; mesomerism.

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Introduction

It is well known that substituted 2,1-isoxazol-3-ones possess interesting pharmacological properties such as antibacterial [1–4], antitubercular [1], fungicidal [5, 6], antileukemic [4], antioxidant [3] and antiandrogenic [7] activities. They also act as inhibitors of tumor necrosis factor-α (TNF-α) [8] and inhibitors of p38 MAP kinases [9].

Recently, we have used 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) as a substrate for the fluorogenic tandem Mannich-electrophilic amination reaction with formaldehyde and secondary amines, which give rise to the formation of a new class of photostable fluorescent dyes with a [1, 2, 4]triazolo[3,4-*b*]pyridin-2-ium core structure [10–12].

In the present work, we describe the *N*-alkylation and *N*-sulfonylation reactions of **1** and antibacterial properties of the newly obtained isoxazolo[3,4-*b*]pyridin-3(1*H*)-one derivatives **2a**–**i**. To understand the observed regioselectivity of the above reactions, theoretical studies on tautomerism of **1** and electronic structure of the most stable tautomers 1*H*-oxo (**1-A**), 7*H*-oxo (**1-B**) have been carried out using the B3LYP/6-31G* and ωB97X-D/6-31G* density functional methods.

Results and discussion

Synthesis

4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) was synthesized according to a previously described procedure [13] with minor modification. As shown in Scheme 1, the condensation of *N*-hydroxy-3-(hydroxyamino)-3-iminopropanamide [14] with acetylacetone in the presence of piperidine resulted in a yellow-colored solution of salt **A** from which, upon neutralization with aqueous 10% HCl, the solid product **1** precipitated in good yield.

Previously, the exploration of chemical reactivity of compound **1** has revealed that acetylation and benzoylation take place exclusively at the *N*1 nitrogen atom [13, 15]. We have now found evidence that alkylation and sulfonylation of **1** also proceed regioselectively at the *N*1 nitrogen atom (Scheme 2). Due to poor solubility of **1** in aprotic organic solvents such as benzene or chloroform, the alkylation reaction was carried out in a mixture of chloroform and *N*,*N*-dimethylformamide (DMF) in the presence of triethylamine (Scheme 2). Pure products **2a**–**g** were isolated by means of a preparative, centrifugally accelerated, radial, thin-layer chromatography (chromatotron) in 62–84% yield. By contrast, the methylsulfonyl derivative **2h** was obtained by reacting **1** with methanesulfonyl chloride in aqueous 5% NaOH

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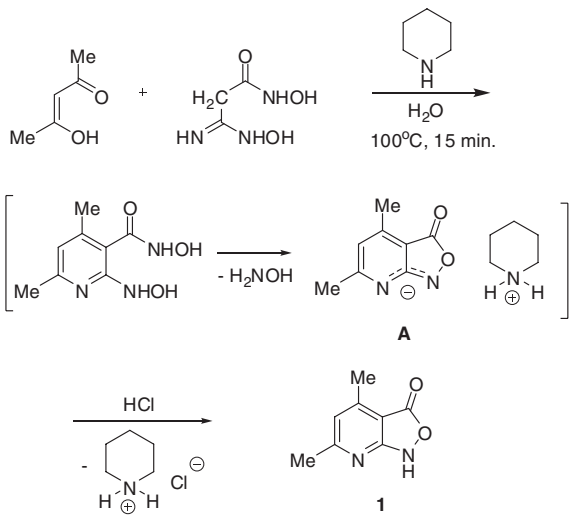
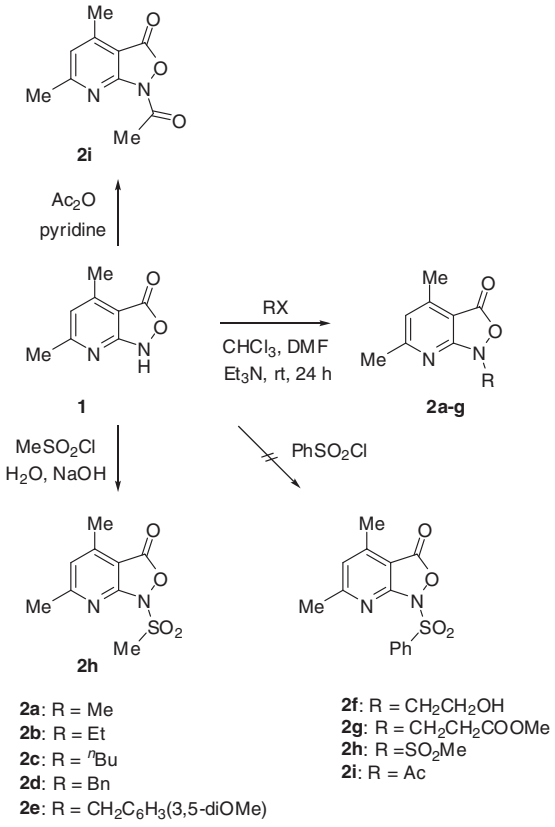


Scheme 1 Synthesis of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**).

solution, followed by chromatographic purification on silica gel. The acetyl derivative **2i**, which was required for biological tests, was synthesized according to the literature procedure [15].

The regioselectivity of the above reactions, that is, formation of *N*1-substituted products, has been confirmed by NMR measurements (lack of NOE between substituents at the *N*1 position and protons of 6-CH_3 group) and the previously reported single crystal X-ray diffraction analysis of compound **2i** [15].



Scheme 2 Alkylation, acetylation and sulfonylation of **1**.

pK_a, prototropic tautomerism and electronic structure of 1

Potentiometric titration of the NH acid **1** with use of 0.1 N NaOH has shown that its aqueous pK_a value is 6.9 (Figure 1), which indicates that it is more acidic than benzotriazole (pK_a = 8.38) [16], 1,2,3-triazole (pK_a = 9.26), 1,2,4-triazole (pK_a = 10.04) and significantly less acidic than tetrazole (pK_a = 4.89) [17]. Starting point of pH = 3 adjusted with 0.1 N HCl.

To obtain insight into the chemical properties of the isoxazolo[3,4-*b*]pyridin-3-one ring system, we have theoretically analyzed all three possible tautomeric forms: 1*H*-oxo (**1-A**), 7*H*-oxo (**1-B**) and hydroxy (**1-C**) (Figure 2) with use of the B3LYP/6-31G* and ωB97X-D/6-31G* density functional methods and SM8 (H_2O, DMF) solvation models [18–21]. It appears that in the gas phase the most stable is the 1*H*-oxo tautomer **1-A**, which also has the smallest dipole moment. However, the DFT calculations indicate that in a solution of high relative permittivity (H_2O and DMF) the 7*H*-oxo tautomer **1-B** is more stable than the 1*H*-oxo form **1-A** by approximately 1.5 kcal mol^{-1}. Therefore, according to the Boltzmann distribution, in solvents such as H_2O or DMF an estimated ratio of **1-A** to **1-B** may be 1:20, which is in agreement

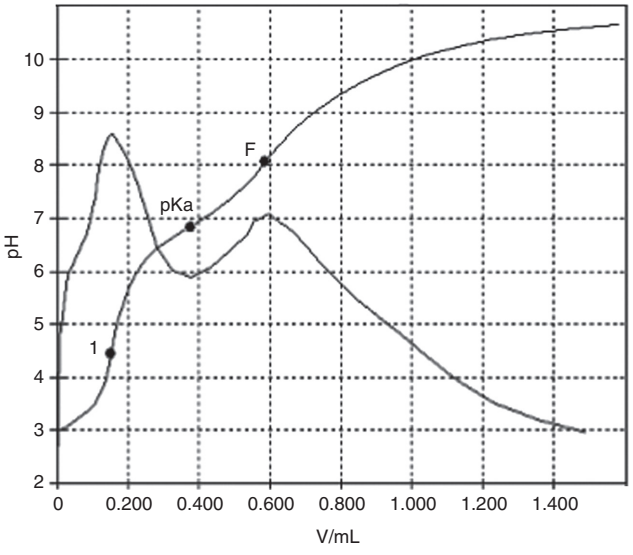
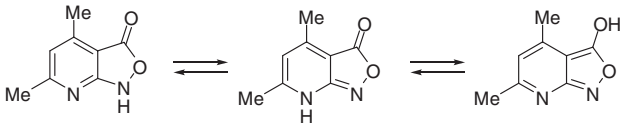
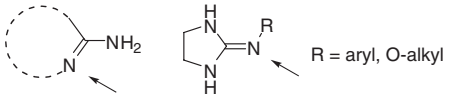


Figure 1 Potentiometric titration of compound **1** in water with use of 0.1 N NaOH.



	1-A	**1-B**	**1-C**
	1*H*-oxo	7*H*-oxo	hydroxy
vacuum	ΔH^o (μ)	ΔH^o (μ)	ΔH^o (μ)
B3LYP/6-31G*	0 (4.27)	5.8 (8.94)	18.0 (4.63)
ωB97X-D/6-31G*	0 (4.34)	6.2 (9.06)	18.1 (4.79)
H_2O			
B3LYP/6-31G*	1.8 (5.88)	0 (13.80)	16.6 (6.90)
ωB97X-D/6-31G*	1.8 (5.89)	0 (13.94)	17.2 (7.01)
DMF			
B3LYP/6-31G*	1.6 (5.23)	0 (12.72)	18.4 (6.56)
ωB97X-D/6-31G*	1.4 (5.28)	0 (12.86)	18.8 (6.67)

Figure 2 Relative enthalpies of formation $\Delta H°$ (kcal mol^{-1}, 298.15 K) and dipole moments μ (debye) of tautomeric forms **1-A**, **1-B** and **1-C** calculated using B3LYP and ωB97X-D density functional methods and SM8 (H_2O, DMF) solvation models [18, 19].



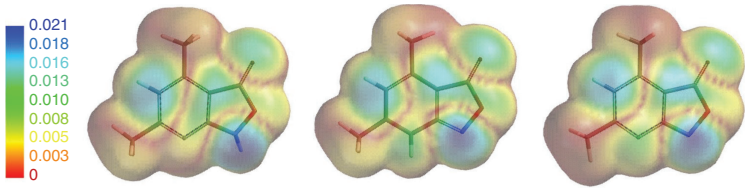
D **E**

Figure 3 Alkylation sites at heterocyclic amidines **D** and guanidines **E**.

with the following principles: (i) in polar solvents the dynamic equilibrium favors the more polar structure [22–25] and (ii) the most polar tautomer of a given compound is generally the one to be found in the solid state [23, 26]. According to our calculations, the hydroxy tautomer **1-C** is generally the least stable which, in turn, is in accordance with the previous examinations of tautomeric equilibrium for simple 5-hydroxyisoxazoles [27–29].

As shown in Figure 3, the alkylation reactions of both the heterocyclic amidine derivatives **D**, described as 1,3-dinucleophiles [30] or 'protomeric ambident nucleophiles' [31] and heterocyclic guanidines **E**, such as 2-aryliminoimidazolidines [32] or *O*-substituted imidazolidin-2-one oximes [33, 34], usually occur at a pyridine-type sp^2 hybridized endo- or exo-cyclic nitrogen atom.

To identify reactive sites at the amidine moiety of compound **1**, we have performed quantum-chemical calculations and molecular modeling, through which the electronic properties of the annular tautomers **1-A** and **1-B** as well as the anion **F** have been revealed. As shown in Figure 4, in all cases (**1-A**, **1-B** and **F**) the magnitude of the calculated charges are higher at the *N*7 nitrogen atoms. By contrast, the HOMO orbital density [√(e/au^3)] mapped on isodensity surface (0.002 e/au^3), corresponding to the molecular size and shape, is



	1-A		**1-B**		**F**	
ωB97X-D/6-31G*	1*H*-oxo		7*H*-oxo		anion	
	N7	N1	N7	N1	N7	N1
natural	-0.500	-0.314	-0.570	-0.222	-0.538	-0.281
electrostatic	-0.627	-0.385	-0.624	-0.410	-0.725	-0.501
Mulliken	-0.544	-0.377	-0.769	-0.297	-0.581	-0.359

Figure 4 The HOMO absolute values [√e/au^3] mapped on the isodensity surface (0.002 e/au^3) and *N*1/*N*7 atomic charges for the tautomers **1-A** (left) and **1-B** (middle) as well as the anion **F** (right) calculated with the ωB97X-D/6-31G* method.

Table 1 *In vitro* antibacterial activity of compounds **1**, **2a**,**b**, **2d**–**i** against anaerobic bacterial strains.

Bacteria (number of strains)	Minimum inhibitory concentration (MIC, μg mL^{-1})				
	Metronidazole	**1**	**2a**	**2b**	**2d**
Gram-positive cocci:					
Finegoldia magna (3)	≤0.4	200(2), 50	200, 100, 50	100, 12.5, <6.2	≥200
Parvimonas micra (2)	≤0.4	100	100	50, <6.2	200, 100
Peptostreptococcus anaerobius (1)	1.6	25	50	100	100
Gram-positive rods:					
Bifidobacterium breve (2)	50, 25	100	≥200	≥200	100, 50
Propionibacterium acnes (1)	>100	50	50	100	≥200
Propionibacterium granulosum (2)	≥100	100	200, 100	≥200	≥200
Gram-negative rods:					
Bacteroides fragilis (1)	<0.4	100	>200	>200	>200
Bacteroides uniformis (1)	<0.4	50	>200	>200	>200
Bacteroides ureolyticus (1)	1.6	50	200	>200	>200
Bacteroides vulgatus (1)	<0.4	>200	100	100	100
Prevotella levii (1)	<0.4	>200	100	200	>200
Prevotella loescheii (1)	<0.4	>200	>200	200	>200
Porphyromonas asaccharolytica (1)	<0.4	50	100	50	200
Porphyromonas gingivalis (1)	<0.4	50	100	100	200
Reference strains:					
Bacteroides fragilis ATCC 25285	<0.4	200	>200	>200	>200
Finegoldia magna ATCC 29328	<0.4	200	100	50	200
Peptostreptococcus anaerobius ATCC 27331	<0.4	100	100	100	200
Bifidobacterium breve ATCC 15700	50	200	200	≥200	200
Parabacteroides distasonis ATCC 8503	<0.4	>200	>200	>200	>200
Fusobacterium nucleatum ATCC 25586	<0.4	>200	>200	>200	>200
	2e	**2f**	**2g**	**2h**	**2i**
Gram-positive cocci:					
Finegoldia magna (3)	≥200	≥200	≥200, 100, 25	200, 100(2)	50, 12.5, <6.2
Parvimonas micra (2)	100, 25	50, 25	50, 25	25, 12.5	25, 12.5
Peptostreptococcus anaerobius (1)	100	100	50	100	25
Gram-positive rods:					
Bifidobacterium breve (2)	200, 100	100	100, 50	100, 50	12.5, <6.2
Propionibacterium acnes (1)	100	100	≥200	100	100
Propionibacterium granulosum (2)	200, 100	100	≥200	100	≥200
Gram-negative rods:					
Bacteroides fragilis (1)	>200	>200	50	>200	>200
Bacteroides uniformis (1)	>200	100	25	>200	>200
Bacteroides ureolyticus (1)	>200	>200	≥200	>200	>200
Bacteroides vulgatus (1)	100	200	50	100	100
Prevotella levii (1)	200	100	50	200	>200
Prevotella loescheii (1)	>200	≥200	>200	200	200
Porphyromonas asaccharolytica (1)	200	≥200	50	>200	200
Porphyromonas gingivalis (1)	>200	>200	>200	>200	>200
Reference strains:					
Bacteroides fragilis ATCC 25285	>200	>200	100	>200	200
Finegoldia magna ATCC 29328	>200	100	100	200	200
Peptostreptococcus anaerobius ATCC 27331	200	100	100	100	100
Bifidobacterium breve ATCC 15700	200	≥200	100	100	200
Parabacteroides distasonis ATCC 8503	>200	>200	>200	>200	>200
Fusobacterium nucleatum ATCC 25586	>200	>200	>200	>200	>200

greater at the vicinity of the isoxazolone *N*1 nitrogen atom than at the pyridine *N*7 atom. These results indicate that the orbital-controlled reactions of **1** take place exclusively because no *N*7-alkylation or acylation products resulting from electrostatically-controlled reaction were observed.

Table 2 *In vitro* antibacterial activity of compounds **1**, **2g**, **2f**, **2i** against aerobic bacterial strains.

Bacteria (number of strains)	Minimum inhibitory concentration (MIC, μg mL^{-1})				
	Amikacin	1	2g	2f	2i
Gram-positive cocci:					
Staphylococcus aureus (3)	≤6.2	≥200	100	100	≥200
Staphylococcus aureus (MRSA) (3)	≥200	≥200	100	≥200(2), 100	≥200
Staphylococcus epidermidis (2)	≤6.2	>200, 50	100, 50	100, 50	>200, 25
Streptococcus pyogenes (1)	50	100	100	100	>200
Streptococcus anginosus (2)	100, 50	100, 50	–	–	50
Enterococcus faecalis (2)	50, 25	50	100	100	200, 50
Gram-positive rods:					
Corynebacterium ulcerans (2)	50, 25	100	100	100	≥200, 100
Corynebacterium xerosis (1)	50	100	25	50	100
Gram-negative rods:					
Escherichia coli (2)	12.5, ≤6.2	100	≥200	≥200	≥200
Acinetobacter baumannii (1)	<6.2	>200	>200	100	>200
Citrobacter freundii (2)	≤6.2	200	>200, 100	200, 100	≥200
Klebsiella pneumoniae (2)	≤6.2	≥200	100	≥200, 100	≥200
Serratia marcescens (1)	<6.2	50	100	100	>200
Pseudomonas aeruginosa (2)	12.5	≥200	>200, 100	>200, 100	≥200
Pseudomonas stutzeri (2)	≤6.2	≥200	100	100	≥200
Reference strains:					
Staphylococcus aureus ATCC 25923	<6.2	>200	>200	>200	>200
Enterococcus faecalis ATCC 29212	25	100	>200	>200	>200
Corynebacterium xerosis ATCC 373	25	200	100	100	100
Klebsiella pneumoniae ATCC 13883	<6.2	>200	100	>200	>200
Acinetobacter baumannii ATCC 19606	<6.2	>200	>200	≥200	>200
Escherichia coli ATCC 25922	<6.2	200	>200	>200	>200

Antibacterial activity

The results of *in vitro* antibacterial activity of compounds **1**, **2a**,**b**, **2d**–**i** against anaerobic bacterial strains and compounds **1**, **2g**, **2f**, **2i** against aerobic bacterial strains are presented in Tables 1 and 2, respectively. The tested compounds show moderate potencies against anaerobic bacteria as they inhibit growth of 15–47% strains at concentrations in the range ≤6.2–100 μg/mL. The most potent methyl propanoate **2g** displays broad activity against 47% of bacterial strains, with minimal inhibitory concentration (MIC) values in the range 25–100 μg/mL. The unsubstituted 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) inhibits growth of 15 strains (44%) at concentrations in the range 25–100 μg/mL, whereas the remaining seven congeners exhibit relatively weaker biological activities: the methyl derivative **2a** is active against 13 bacterial strains (38%, MIC 50–100 μg/mL), **2b**, **2f** and **2h** show potent activity against 12 strains (35%, MIC ≤6.2–100 μg/mL) and compounds **2i** and **2e** inhibit growth of 11 (32%, MIC ≤6.2–50 μg/mL) and seven strains (21%, 25–100 μg/mL), respectively. The benzyl (**2d**) derivative proved to be the least active, whereas the butyl congener (**2c**) does not exert any antibacterial effect, probably due to its poor solubility. All tested compounds are more active against Gram-positive cocci (12–32% susceptible strains) than Gram-positive rods (3–15% susceptible strains). The most susceptible genera among Gram-positive cocci and Gram-positive rods are *Parvimonas micra* and *Bifidobacterium breve*, respectively. Among anaerobes, the following strains exhibit high resistance towards all tested compounds (MIC ≥200 μg/mL): *Parabacteroides distasonis* (1), *Prevotella bivia* (1), *Prevotella buccalis* (1), *Prevotella intermedia* (2), *Fusobacterium nucleatum* (2), *Fusobacterium necrophorum* (2), *Parabacteroides distasonis*, ATCC 8503 and *Fusobacterium nucleatum* ATCC 25586.

Aerobic bacterial strains exhibit susceptibility to only four tested compounds at the concentrations in the range of 25–100 μg/mL. The most potent derivative **2g** exhibits its activity against 22 (65%) bacterial strains, with MIC values in the range of 50–100 μg/mL. The unsubstituted and hydroxyethyl congeners **1** and **2f** inhibit growth of 13 (38%) and 20 strains (59%), respectively, at concentrations in the range of 25–100 μg/mL.

Gram-positive aerobic cocci and rods proved to be more susceptible than Gram-negative rods to the four tested

compounds. Among Gram-positive bacteria the *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Corynebacterium xerosis* are the most susceptible genera (MIC in the range 25–100 μg/mL). By contrast, *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) are susceptible to compounds **2g** and **2f**. Generally, the Gram-negative rods are most resistant to the genera tested as growths of only a few strains of *Citrobacter freundii*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas stutzeri* are inhibited at concentrations of 50–100 μg/mL. Among all the tested compounds, only **1**, **2f**, **2g** and **2i** are active against both aerobic and anaerobic bacterial strains. The most active compound in this category is the methyl propanoate **2g**, which inhibits 56% of the bacterial strains tested.

Compounds **2a**, **2h**, **2d** and **2i** were also evaluated for their cytotoxic activity [35, 36] against five human cancer cell lines: uterine cervical adenocarcinoma SISO, lung cancer LCLC and A-427, pancreas adenocarcinoma DAN-G and neural cell line RT-4. However, the tested compounds did not exhibit cytotoxic activity at concentrations of up to 20 μM.

Experimental

All chemicals were purchased from commercial sources and used without further purification. Silica gel chromatography was performed with use of silica gel 60 PF_{254} containing gypsum for preparative layer chromatography and chromatotron. ^1H and ^{13}C NMR data were obtained using 200 MHz or 50 MHz spectrometers, respectively. ^1H NMR data were internally referenced to TMS (0.0 ppm) or DMSO-d_6 (2.5 ppm); ^{13}C NMR spectra were referenced to $CDCl_3$ (77.23 ppm) or DMSO-d_6 (39.50 ppm). The IR spectra were recorded using KBr pellets.

4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (1)

N-Hydroxy-3-(hydroxyamino)-3-iminopropanamide [14] (1.3 g, 9.8 mmol), acetylacetone (1 mL, 9.8 mmol) and piperidine (1 mL, 9.8 mmol) were heated under reflux in water (30 mL) for 15 min. After cooling to room temperature the reaction mixture was acidified with 5% HCl and the resultant yellow precipitate was filtered off and washed with water (2 × 3 mL); yield 1.2 g (75%); mp 204–207°C (lit. mp 204°C [13]); IR: 3372, 2725, 1694, 1670, 1640, 1619, 1531, 1350, 1289, 1051, 1030, 980, 938, 823, 784 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.38 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 6.38 (s, 1H, CH), 13.00 (bs, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 16.7, 21.3, 100.8, 112.7, 155.9, 158.1, 159.9, 169.0; MS (ESI): *m/z* 163 $[M-1]^-$.

General procedure for compounds 2a–g

4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**), alkyl halide and triethylamine were allowed to react at room temperature as indicated below. After 12 h the mixture was concentrated under reduced pressure and the residue was treated with water (5 mL) and dichloromethane (10 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated. The crude compound was purified on a chromatotron eluting with the solvent indicated below.

1,4,6-Trimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2a) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.3 g, 1.83 mmol), methyl iodide (0.34 mL, 5.49 mmol) and triethylamine (0.51 mL, 3.66 mmol) were added to the mixture of chloroform (3 mL) and DMF (1 mL); eluent: petroleum ether-dichloromethane, 1:1, v/v; yield 0.16 g (49%); mp 72–75°C; IR: 2921, 1749, 1618, 1598, 1437, 11371, 1284, 1180, 1110, 1035, 1009, 869, 800 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.57 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 3.47 (s, 3H, CH_3), 6.81 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.5, 25.4, 40.1, 102.4, 121.2, 151.1, 166.6, 166.8, 168.6; MS (ESI): *m/z* 179 $[M+1]^+$. Anal. Calcd for $C_9H_{10}N_2O_2$: C, 60.66; H, 5.66; N, 15.72. Found: C, 60.51; H, 5.78; N, 15.56.

1-Ethyl-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2b) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.3 g, 1.83 mmol), ethyl iodide (0.29 mL, 3.66 mmol) and triethylamine (0.51 mL, 3.66 mmol) were added to the mixture of chloroform (3 mL) and DMF (1 mL); eluent: petroleum ether-dichloromethane, 1:1, v/v; yield 0.267 g (76%); mp 55–58°C; IR: 2982, 1758, 1613, 1371, 1278, 1172, 1082, 1018, 931, 845, 806, 737, 702, 613, 548, 501 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.26 (t, *J* = 7.0 Hz, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 3.87 (q, *J* = 7.0 Hz, 2H, CH_2), 6.79 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 11.7, 17.5, 25.5, 48.3, 102.8, 121.0, 150.9, 166.8 (two signals), 167.6; MS (ESI): *m/z* 193 $[M+1]^+$. Anal. Calcd for $C_{10}H_{12}N_2O_2$: C, 62.49; H, 6.29; N, 14.57. Found: C, 62.57; H, 6.33; N, 14.34.

1-Butyl-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2c) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.3 g, 1.83 mmol), butyl iodide (0.41 mL, 3.66 mmol) and triethylamine (0.51 mL, 3.66 mmol) were added to the mixture of chloroform (3 mL) and DMF (1 mL); eluent: petroleum ether-dichloromethane, 1:1, v/v; yield 0.25 g (62%); mp 22–25°C; IR: 2961, 2934, 2873, 1762, 1613, 1593, 1443, 1376, 1283, 1173, 1024, 802, 702, 612 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.95 (t, *J* = 7.0 Hz, 3H, CH_3), 1.37–1.48 (m, 2H, CH_2), 1.66–1.77 (m, 2H, CH_2), 2.56 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 3.79 (q, *J* = 7.0 Hz, 2H, CH_2), 6.78 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 14.2, 17.5, 20.4, 25.5, 29.0, 53.1, 102.3, 120.9, 151.0, 166.8 (two signals), 167.6; MS (ESI): *m/z* 221 $[M+1]^+$. Anal. Calcd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.72. Found: C, 65.64; H, 7.19; N, 12.48.

1-Benzyl-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2d) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.2 g, 1.22 mmol), benzyl bromide (0.16 mL, 1.34 mmol) and triethylamine (0.17 mL, 1.34 mmol) were added to the mixture of chloroform (3 mL) and DMF (1 mL); eluent: dichloromethane; yield 0.26 g (84%); mp 100–103°C; IR: 3030, 2950, 1761, 1612, 1592, 1434, 1376, 1174, 1031, 798, 760, 738, 703, 637 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.56 (s, 3H, CH_3), 2.61 (s, 3H, CH_3), 4.99 (s, 2H, CH_2), 6.80 (s, 1H, CH), 7.27–7.39 (m, 5H, CH); ^{13}C NMR ($CDCl_3$): δ 17.5, 25.5, 57.0, 103.0, 121.2, 128.7, 129.0, 129.7, 134.2, 151.0, 166.5, 166.8, 167.4; MS (ESI): *m/z* 255 $[M+1]^+$. Anal. Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.73; H, 5.76; N, 10.81.

1-(3,5-Dimethoxybenzyl)-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2e) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.2 g, 1.22 mmol), 3,5-dimethoxybenzyl bromide (0.28 mL, 1.34 mmol) and triethylamine (0.17 mL, 1.34 mmol) were added to DMF (3 mL); eluent: dichloromethane; yield 0.32 g (83%); mp 108–113°C; IR: 2947, 2843, 1761, 1601, 1474, 1452, 1438, 1393, 1375, 1354, 1296, 1276, 1206,

1170, 1161, 1068, 1054, 1032, 983, 962, 912, 837, 806, 743, 720 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.58 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 3.75 (s, 6H, CH_3), 4.92 (s, 2H, CH_2), 6.38 (s, 1H, CH), 6.52 (s, 2H, CH), 6.80 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.3, 25.2, 55.6, 56.8, 100.6, 102.8, 107.1, 121.0, 136.2, 150.9, 161.0, 166.2, 166.4, 167.0; MS (ESI): *m/z* 315 $[M+1]^+$. Anal. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.96; H, 5.77; N, 8.91. Found: C, 64.75; H, 6.16; N, 8.62.

1-(2-Hydroxyethyl)-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2f) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.1 g, 0.61 mmol), 2-bromoethanol (0.38 mL, 3.04 mmol) and triethylamine (0.17 mL, 1.22 mmol) were added to DMF (3 mL); eluent: ethyl acetate-dichloromethane, 5:95, v/v; yield 0.08 g (63%); mp 84–85°C; IR: 3489, 3044, 2923, 1743, 1613, 1590, 1445, 1407, 1373, 1359, 1277, 1180, 1073, 1028, 881, 801, 703, 614 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.56 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 3.87–3.92 (m, 2H, CH_2), 4.01–4.06 (m, 2H, CH_2), 6.82 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.6, 25.4, 56.4, 59.9, 102.4, 121.3, 151.7, 166.1, 166.6, 167.4; MS (ESI): *m/z* 209 $[M+1]^+$. Anal. Calcd for $C_{10}H_{12}N_2O_3$: C, 57.68; H, 5.81; N, 13.45. Found: C, 57.32; H, 6.08; N, 13.23.

Methyl 3-(4,6-dimethyl-3-oxoisoxazolo[3,4-*b*]pyridin-1(3*H*)-yl) propanoate (2g) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.1 g, 0.61 mmol), methyl 3-bromopropionate (0.20 mL, 1.83 mmol) and triethylamine (0.17 mL, 1.22 mmol) were added to DMF (3 mL); eluent: ethyl acetate-dichloromethane, 1:99, v/v; yield 0.12 g (79%); mp 33–34°C; IR: 3010, 2959, 2926, 1760, 1727, 1619, 1591, 1445, 1372, 1269, 1248, 1198, 1173, 1032, 1017, 803 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.56 (s, 3H, CH_3), 2.59 (s, 3H, CH_3), 2.77 (t, 2H, CH_2, J = 7.0 Hz), 3.70 (s, 3H, CH_3), 4.10 (t, 2H, CH_2, J = 7.0 Hz), 6.82 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.5, 25.5, 31.8, 49.1, 52.4, 102.7, 121.5, 151.1, 166.4, 167.0, 167.6, 171.7; MS (ESI): *m/z* 251 $[M+1]^+$. Anal. Calcd for $C_{12}H_{14}N_2O_4$: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.30; H, 5.96; N, 11.01.

4,6-Dimethyl-1-(methylsulfonyl)isoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2h) 4,6-Dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.2 g, 1.22 mmol), NaOH (0.6 g, 15 mmol) and methanesulfonyl chloride (0.45 mL, 5.8 mmol) were added to water (12 mL), and the resulting mixture was stirred at room temperature for 0.5 h and then extracted with dichloromethane (2 × 10 mL). The combined organic layers were dried over anhydrous sodium sulfate and concentrated to give crude compound **2i**, which was further purified by use of chromatotron; eluent: dichloromethane; yield 0.17 g (58%); mp 130–136°C; IR: 3020, 3010, 2927, 1783, 1762, 1618, 1585, 1387, 1364, 1334, 1321, 1284, 1243, 1181, 1172, 1033, 966, 815, 797, 759, 737, 697, 660, 607, 552, 514 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.67 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 3.36 (s, 3H, CH_3), 7.13 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.3, 25.5, 39.2, 105.1, 124.8, 151.7, 162.2, 164.4, 167.9; MS (ESI): *m/z* 243 $[M+1]^+$. Anal. Calcd for $C_9H_{10}N_2O_4S$: C, 44.62; H, 4.16; N, 11.56. Found: C, 44.37; H, 4.35; N, 11.47.

1-Acetyl-4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (2i) To the solution of 4,6-dimethylisoxazolo[3,4-*b*]pyridin-3(1*H*)-one (**1**) (0.2 g, 1.22 mmol) in pyridine (0.6 mL) acetic anhydride (0.4 mL, 4.24 mmol) was added and the resulting orange mixture was stirred at room temperature to discolor. The precipitated white solid was filtered and dried under reduced pressure; yield 0.17 g (68%); mp 188–194°C (lit. mp 195°C [13, 15]); IR: 3048, 2953, 1785, 1709, 1615, 1591, 1438, 1395, 1376, 1298, 1264, 1176, 1164, 1070, 1034, 887, 852, 791, 618, 604 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.62 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 6.98 (s, 1H, CH); ^{13}C NMR ($CDCl_3$): δ 17.7, 24.1, 25.8, 102.2, 122.8, 151.7, 157.2, 162.0, 163.2, 167.8; MS (ESI): *m/z* 207 $[M+1]^+$.

Antibacterial activity tests

The investigations included 28 strains of anaerobic bacteria and 28 strains of aerobic bacteria isolated from the oral cavity, respiratory system and intestinal tract as well as 12 reference strains. The anaerobes belonged to the following genera: *Finegoldia magna* (3), *Parvimonas micra* (2), *Peptostreptococcus anaerobius* (1), *Bifidobacterium breve* (2), *Propionibacterium acnes* (1), *Propionibacterium granulosum* (2), *Bacteroides fragilis* (1), *Bacteroides uniformis* (1), *Bacteroides ureolyticus* (1), *Bacteroides vulgatus* (1), *Parabacteroides distasonis* (1), *Prevotella bivia* (1), *Prevotella buccalis* (1), *Prevotella intermedia* (2), *Prevotella levii* (1), *Prevotella loescheii* (1), *Porphyromonas asaccharolytica* (1), *Porphyromonas gingivalis* (1), *Fusobacterium nucleatum* (2), *Fusobacterium necrophorum* (2) and following reference strains: *Bacteroides fragilis* ATCC 25285, *Parabacteroides distasonis* ATCC 8503, *Fusobacterium nucleatum* ATCC 25586, *Finegoldia magna* ATCC 29328, *Peptostreptococcus anaerobius* ATCC 27331, *Bifidobacterium breve* ATCC 15700. There were also the following aerobes used: *Staphylococcus aureus* (3), *Staphylococcus aureus* methicillin resistant (MRSA) (3), *Staphylococcus epidermidis* (2), *Streptococcus pyogenes* (1), *Streptococcus anginosus* (2), *Enterococcus faecalis* (2), *Corynebacterium ulcerans* (2), *Corynebacterium xerosis* (1), *Escherichia coli* (2), *Acinetobacter baumannii* (1), *Citrobacter freundii* (2), *Klebsiella pneumoniae* (2), *Serratia marcescens* (1), *Pseudomonas aeruginosa* (2), *Pseudomonas stutzeri* (2) and six reference strains: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Corynebacterium xerosis* ATCC 373, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922. The susceptibility of the anaerobic bacteria was determined by means of the plate dilution technique in Brucella agar, supplemented with 5% defibrinated sheep's blood [37, 38]. For aerobic bacteria experiments, the agar dilution technique with Mueller-Hinton agar was used [39, 40]. The derivatives were dissolved in 1 mL of DMSO immediately before the experiment. Sterile distilled water was used for further dilutions. The following concentrations of derivatives were used: 200, 100, 50, 25, 12.5 and 6.2 μg/mL. The inoculum containing 10^5 CFU/spot was applied to the appropriate agar plates with Steers replicator. For aerobes, the inoculated agar plates and agar plates without derivatives were incubated for 24 h at 37°C. For anaerobes, agar plates were incubated in anaerobic jars for 48 h at 37°C in 10% CO_2, 10% H_2 and 80% N_2 with palladium catalyst and indicator for anaerobiosis. The MIC was defined as the lowest concentration of the derivative that inhibited growth of the tested bacteria [39–41].

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