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ORIGINAL RESEARCH ARTICLE

New derivatives of dipicolinic acid as metallo-β-lactamase NDM-1 inhibitors

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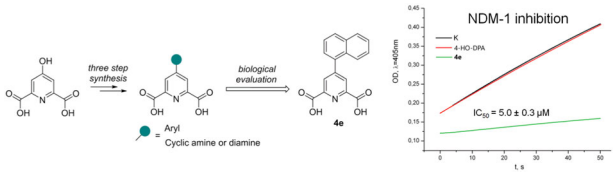
Abstract

Resistance to β-lactam antibiotics caused by β-lactamases such as New-Delhi lactamase (NDM-1) has become one of the major challenges in the current antimicrobial therapy. Pyridine-2,6-dicarboxylic acid (DPA) derivatives have been demonstrated to inhibit NDM-1 in a due to the interactions with Zn ion and amino acid residues of the enzyme's active site. In this study, a series of new 4-substituted DPA derivatives was synthesized. The SAR study has proven that the presence of a substituent at the 4-position of pyridine-2,6-dicarboxylic acid had a certain impact on the NDM-1 inhibitory. Some representatives, e.g., **4e** exhibited IC_{50} values against NDM-1 close to the previously reported hit-compound 4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid. The microdilution broth test confirmed an ability of derivative **4e** to increase susceptibility of NDM-1-producing *E. coli* strain and did not demonstrate cytotoxicity to eukaryotic cells. However, NDM-1 inhibition by 4-substituted derivatives dramatically dropped when Zn^{2+} was added. We observed a strong complexation of 4-modified derivatives with Zn^{2+} similar to unsubstituted pyridine-2,6-dicarboxylic acid. Taken together, a complexation mode of NDM-1 inhibition leading to potential off-target action on other metalloenzymes and low efficiency of structure optimization make DPA derivatives an unproductive scaffold for future development of clinically relevant metallo-β-lactamase inhibitors.



Keywords Pyridine-2,6-dicarboxylic acid · Antimicrobial resistance · Metallo-β-lactamase · NDM-1 · Chelating effect

Introduction

The rise of antimicrobial resistance (AMR) has become one of the most significant challenges in global healthcare in recent years. This issue strongly requires ongoing efforts to identify compounds with antimicrobial activity that also target multi-drug resistance (MDR) and pan-drug resistance in pathogens.

β-Lactam antibiotics account for more than 60% of antimicrobial drugs used in clinical practice due to their high efficacy and low toxicity, which provides a wide therapeutic window for prescribing drugs [1]. Such widespread use of β-lactam antibiotics, including carbapenems of last resort, causes the prompt increasing response of protective mechanisms in pathogens. One of the most significant AMR mechanisms involves the expression of genes encoding β-lactamase enzymes. Currently, the most concerning enzymes are extended-spectrum β-lactamases (ESBLs) and carbapenemases, including metallo-β-lactamases (MβLs). New Delhi metallo-β-lactamase

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(NDM-1) was isolated from *E. coli* and *K. pneumonia* and for the first time reported in 2009 by Walsh et al. [2]. NDM-1 has since been detected in a wide variety of bacterial microorganisms, with the most virulent among them being members of the *Enterobacterales* group, *P. aeruginosa*, and *A. baumannii* [3]. A distinguishing feature of NDM-1 compared to other clinically significant metallo-β-lactamases is the presence of a lipobox – a system that directs the protein to the inner leaflet of the outer membrane in Gram-negative bacteria [4]. This feature allows the enzyme to bind and cleave β-lactam antibiotics, including the last generation carbapenems, as soon as they enter the cell.

A combination of β-lactam antibiotics with inhibitors of β-lactamases, e.g., amoxicillin with clavulanic acid, ceftazidime with avibactam, meropenem, and vaborbactam, etc. represents the proven strategy which allows circumventing AMR and increasing susceptibility of pathogen microorganisms [5, 6]. A number of MβL inhibitors were discovered and exceptional representatives (taniborbactam, QPX7728) were involved in clinical trials giving some hope; nevertheless, there are no clinically approved drugs up to date.

Structure of MβL inhibitors comprises different organic molecular scaffolds. Aza-heterocyclic carboxylic acid derivatives (Fig. 1) take a special place among them due to a structural imitation of MβL's substrates [7–10]. Pyridine-2,6-dicarboxylic acid (DPA) was considered as one of the first potential MβL inhibitors due to well-known Zn^{2+} binding properties. Chen et al. synthesized a series of some 4-substituted DPA derivatives and demonstrated that 4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid inhibits metallo-β-lactamase NDM-1 with IC_{50} value of 0.32 μM in vitro and increases susceptibility of NDM-1-producing bacterial strains [11]. They demonstrated that aryl substituent at position 4 of DPA has a great impact on NDM-1 inhibition, while modification of even one carboxylic acid into amide derivatives led to the total drop of activity. Analysis of the published ternary complex NDM-1-Zn-inhibitor indicates that the enzyme has quite a large hydrophobic region around the 4-position of DPA-derived ligands. Taking into account potency of 4-aryl substituted ligands we decided to expand the series by introducing enlarged aryls, e.g., α- and β-naphthyl, previously unpublished aryls as *para*-tolyl, *para*-fluorophenyl, and so on. Further in silico structural insight at stability of the ternary complex NDM-1-Zn-inhibitor indicates that 3-amino group at the phenyl fragment of 4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid may produce ionic interaction and/or hydrogen bond with Asn220 residues in the active site. Thus, we introduced the lipophilic cyclic diamines because they bear salt-forming amino groups which promote compound's aqueous solubility and may produce additional interaction e.g., with Asn220 amino acid. Proposed DPA modifications represent a valuable impact for the next step of SAR analysis of NDM-1 ligands.

In this work, we have expanded the series of 4-substituted pyridine-2,6-dicarboxylic acid derivatives through an arylation reaction or an introduction of cyclic diamines. Inhibitory effects on NDM-1 as well as their ability to improve effectiveness of β-lactam antibiotics by the newly synthesized DPA analogs were evaluated.

Results and discussion

Synthesis

To improve NDM-1 inhibitory of DPA the hydrogen atom in the position 4 was substituted with various aryl radicals or cyclic amines and diamines. These residues presumably enhance [11] the complexation of the ligand in the NDM-1 binding site due to interactions with the side chains of amino acids. 4-Substituted derivatives of pyridine-2,6-dicarboxylic acid were synthesized from 4-hydroxypyridine-2,6-dicarboxylic acid (chelidamic acid, **1**) [11–13]. For the preparation a series of 4-arylpyridine-2,6-dicarboxylic acids by Suzuki cross-coupling, chelidamic acid **1** was initially converted into dimethyl 4-bromopyridine-2,6-dicarboxylate (**2**, Scheme 1). Alternatively, to provide the direct amination using nucleophilic substitution of 4-halogen atom in pyridine core the di-*tert*-butyl 4-

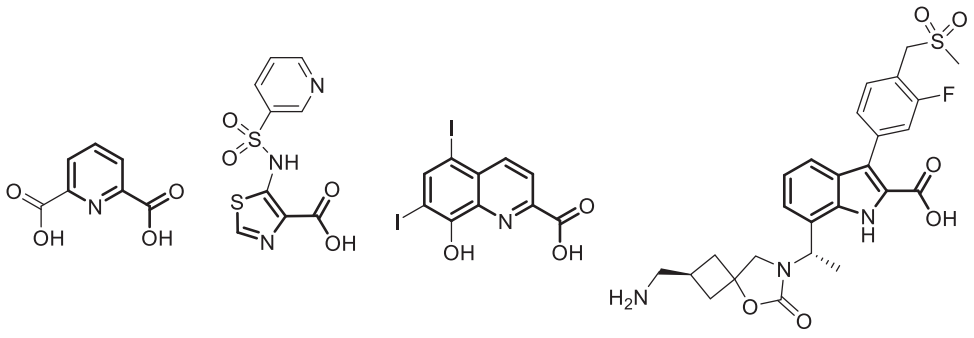
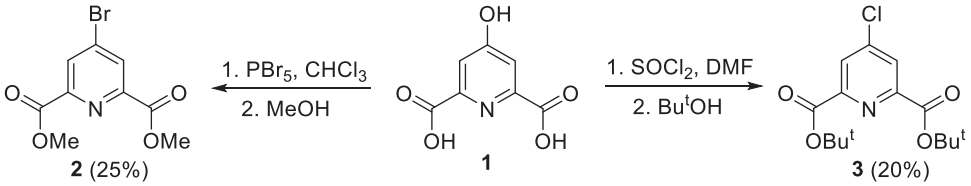


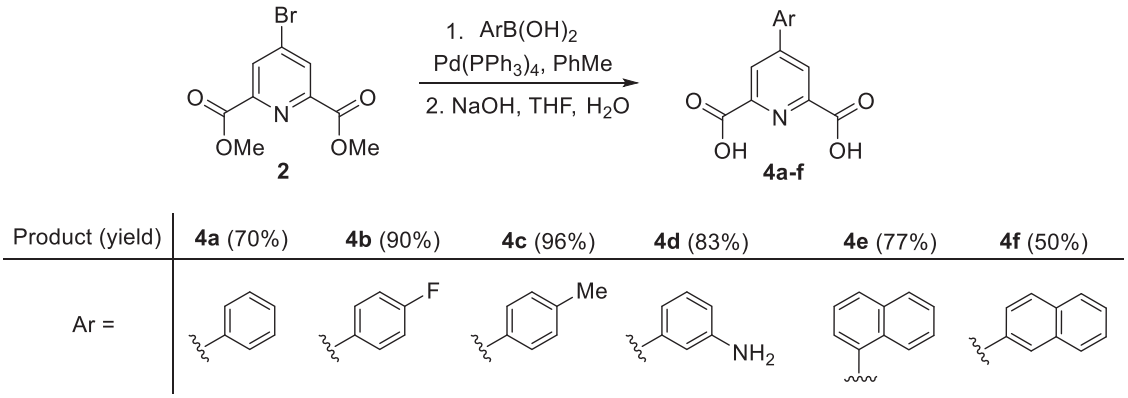
Fig. 1 Structures of metallo-β-lactamase NDM-1 inhibitors based on aza-heteroarene-carboxylic acids

Scheme 1



Synthesis of methyl (**2**) and *tert*-butyl (**3**) esters of 4-halopyridine-2,6-dicarboxylic acids

Scheme 2



Synthesis of 4-aryl-substituted pyridine-2,6-dicarboxylic acids **4a–f**

chloropyridine-2,6-dicarboxylate (**3**) was also synthesized from the acid **1** (Scheme 1).

4-Bromopyridine **2** was arylated via Suzuki-Miyaura cross-coupling using the corresponding boronic acids and $Pd(PPh_3)_4$ as the catalyst. The purification of intermediate 4-arylpyridine-2,6-diesters by column chromatography followed by alkali hydrolysis resulted in the 4-arylpyridine-2,6-dicarboxylic acids **4a–f** (Scheme 2). 3-Aminophenyl derivative **4d** was synthesized as reference compound which demonstrated the best results in previous study [11].

Derivative of 4-chloropyridine **3** was treated with secondary amines in DMSO in the presence of trimethylamine (TEA) yielding corresponding di-*tert*-butyl esters of 4-aminopyridine-2,6-dicarboxylic acids **5a–e**. Cleavage of the Boc-protecting group using trifluoroacetic acid gave 4-amino derivatives of pyridine-2,6-dicarboxylic acids **5a–e**. Of note, we were unsuccessful in attempts to provide nucleophilic substitution of chlorine atom of **3** with primary amines as butylamine or *N*-Boc-diaminoethane (Scheme 3).

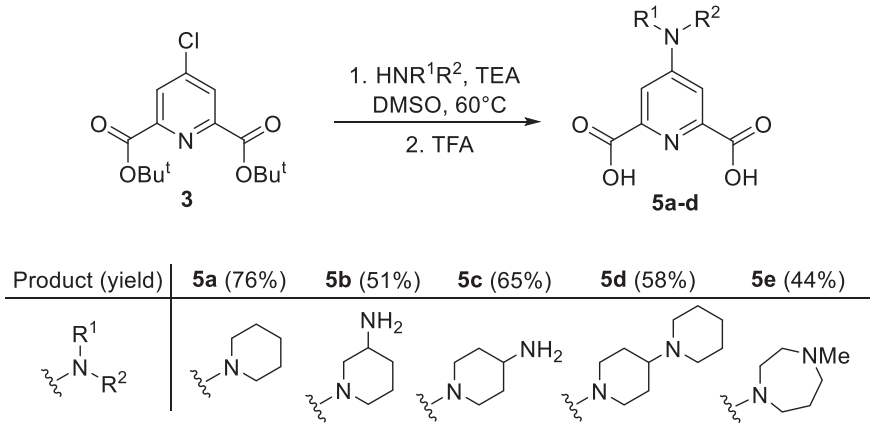
The structures of all new compounds were analyzed and confirmed by NMR and HRMS spectroscopy methods (Supporting Information, Fig. S1). The purity of the samples of acids **4a–f** and **5a–d** used for biological evaluation was ≥95%. Of note, solubility of 4-(4-aminopiperidin-1-yl) pyridine-2,6-dicarboxylic acid (**5c**) was extremely low, thus we were unable to perform biological investigation of **5c**.

In vitro inhibition of NDM-1 by new DPA derivatives

An ability of the prepared DPA derivatives to inhibit metallo-β-lactamase NDM-1 was studied in vitro using the enzymatic hydrolysis of the chromogenic substrate CENTA [14]. This photometric assay is based on a bathochromic shift in the absorption maximum from λ_{max} ~ 340 nm to 405 nm upon the cleavage of the β-lactam ring of CENTA. Compounds inhibiting this NDM-1-promoted reaction affect the rate of emergence of the 405 nm band. 4-Hydroxypyridine-2,6-dicarboxylic acid (**1**), DPA and 4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid (**4d**) were used as reference compounds.

Among the all tested compounds, nine representatives (except 4-hydroxy and 4-piperidinyl derivatives **1** and **5a**) demonstrated some inhibitory effect at a concentration of 50 μM on the Metallo-β-lactamase NDM-1, which is observed in a relative decrease of the initial rate of CENTA hydrolysis. Table 1 shows the calculated IC_{50} values of NDM-1 inhibition by DPA derivatives. Of note, activities of reference compounds **4d** and DPA weres less than previously published, probably due to different experimental conditions (e.g., chromogenic substrate CENTA instead chromacef). Replacement of 4-(3-aminophenyl) ring in **4d** with other aryl moieties (compounds **4a–c, e, f**) had no meaningful changes in inhibitory properties; the highest IC_{50} value was achieved when α-naphtyl was introduced. Substitution of 4-aryl fragment with cyclic diamines slightly

Scheme 3



Synthesis of substituted 4-aminopyridine-2,6-dicarboxylic acids **5a–e**

attenuates NDM-1 inhibition, while 3-aminopiperidine derivative **5c** is found to be same active as 4-(3-aminophenyl)pyridine **4d**. This data suggests either the calculated interaction of amino group of **4d** with Asn220 residue does not experimentally occurred or the rigid conformation of 4-piperidine cycle does not provide a qualitative ionic interaction and/or hydrogen bond.

Surprisingly, an addition of Zn^{2+} ions to the buffer dramatically drops the inhibitory ability of all DPA derivatives, including the reference 4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid (**4d**) which was claimed and as zinc-independent NDM-1 inhibitor. This indicates despite an addition of 4-substituents to DPA the mechanism of Metallo-β-lactamase inhibition mainly caused by the chelating effect of pyridine-2,6-dicarboxylic acid scaffold.

Study of complexation with Zn^{2+}

The loss of ability to inhibit metallo-*β*-lactamase NDM-1 by DPA derivatives in presence of Zn^{2+} ions prompted us to measure in vitro complexation. The binding ability of **4e** was assessed using UV-Vis spectroscopy through a titration method in aqueous solution, with $ZnSO_4$ serving as the source of Zn^{2+} ions. As demonstrated in the Fig. 2, the incremental addition of Zn^{2+} to a solution containing compound **4e** resulted in a hypsochromic shift of the absorption maximum from 365 nm to 320 nm. The isosbestic points observed on absorption spectra during of addition of Zn^{2+} suggests the preferential formation of a 2:1 (**4e**:Zn^{2+}) complex, with a K_D value of 0.3 μM, along with a minor formation of a 1:1 complex. Complexation of DPA with Zn^{2+} has the same order of magnitude: K_D for the $Zn(DPA)_2$ complex is 0.45 μM). This value is closely aligned with the K_D value which was independently established using potentiometric titration [15].

This result indicates that novel DPA derivatives exhibit markable Zn-binding capabilities in aqueous solutions. The dramatic decrease in compound's potency against NDM-1 upon the addition of physiological concentrations of Zn^{2+} most likely attributed to the chelating action of the ligand. This suggests that the sequence of amino acids in the NDM-1 active site might not significantly affect the bioactivity of these compounds and specific binding in the active site of NDM-1 was not observed.

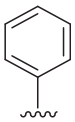
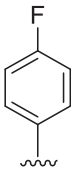
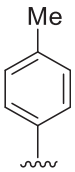
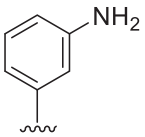
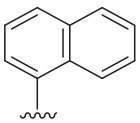
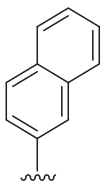
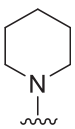
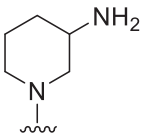
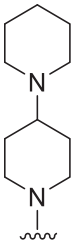
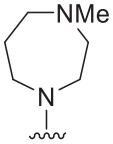
Microdilution broth minimum inhibitory concentrations

An ability of derivative **4e** to change the susceptibility of *E. coli* strain XL10-Gold producing metallo-*β*-lactamase NDM-1 [16] was studied in vitro by the broth microdilution method. The minimum inhibitory concentrations (MICs) in of meropenem and cefotaxime antibiotics were measured in the presence or absence of the inhibitor; EDTA was used as the positive control. DPA derivative **4e** decreased the MIC value of meropenem in a dose-dependent manner up to 8 times compared to the experiment with no NDM-1 inhibitor (Table 2). Of note, antibacterial activity of cephalosporin cefotaxime was not affected by the addition of **4e**, while EDTA greatly potentiated effectiveness both of the antibiotics. It can be proposed that the decrease in the MICs of carbapenem meropenem is due to the inhibition of MBLs by DPA derivative **4e**.

Conclusions

For systematic study of metallo-β-lactamase NDM-1 inhibitors we prepared and evaluated a series of new 4-aryl and 4-(piperidin-1-yl)dipicolinic acid derivatives. The designed compounds were prepared by Suzuki-Miyaura cross-coupling or nucleophilic substitution of halogen atoms using an appropriate ester 4-halopyridine-2,6-dicarboxylic acid. Inhibitory effects of new DPA derivatives towards metallo-

Table 1 IC_{50} values and relative initial rates (Vo(i)/Vo(K-)) of hydrolysis of chromogenic substrate CENTA by metallo-β-lactamase NDM-1 in presence of DPA derivatives

Entry	Compound	4-Substituent	Vo(i)/ Vo(K), %	IC_{50}, μM	IC_{50}, μM ($+Zn^{2+}$)
1	**DPA**		33 ± 3	11.0 ± 0.8	>50
2	**1**		100	>50	>50
3	**4a**		27 ± 2	9.0 ± 0.6	>50
4	**4b**		58 ± 4	8.0 ± 0.6	>50
5	**4c**		38 ± 3	7.0 ± 0.5	>50
6	**4d**		78 ± 6	9.0 ± 0.6	>50
7	**4e**		13 ± 1	5.0 ± 0.3	>50
8	**4f**		41 ± 3	8.0 ± 0.6	>50
9	**5a**		100	>50	>50
10	**5b**		69 ± 5	11.0 ± 0.8	>50
11	**5d**		75 ± 5	15 ± 1	>50
12	**5e**		78 ± 6	8.0 ± 0.6	>50

β-lactamase NDM-1 in enzymatic assay correspond to the low micromolar range of IC_{50} values. Structure-activity relationship completed in this work together with the previously gained data has proven that the presence of a substituent at the 4-position of DPA had a certain impact on the NDM-1 inhibitory. The most potent inhibitor 4-(naphth-1-yl)pyridine-2,6-dicarboxylic acid **4e** in the series improves susceptibility of *E. coli* XDL10-Gold producing metallo-β-lactamase NDM-1 as demonstrated in reducing the MIC of meropenem. All 4-aryl and 4-amino derivatives of pyridine-2,6-dicarboxylic acid **4a–f** and **5a–e** did not exhibit cytotoxicity ($IC_{50} > 100$ μM, MTT assay) towards human embryonic kidney HEK293 cells indicating good therapeutic index.

On the other hand, after a number of attempts for structure optimization of DPA, in particular by current replacement of 4-aryl with cyclic diamine, directions for future development of NDM-1 inhibitors based on pyridine-2,6-dicarboxylic remains to be unclear. Moreover, experimentally proven zinc complexation by derivative **4e** and a drop of NDM-1 inhibitory potentially lead to off-target action on other metalloenzymes by metal chelation. Taken together, from current point of view, pyridine-2,6-dicarboxylic acid derivatives cannot be considered as the productive scaffold for future development of clinically relevant metallo-β-lactamase inhibitors.

Experimental section

Instruments, general information, and synthetic procedures

Unless otherwise specified, all solvents and reagents were pursued from commercial suppliers as Merck, ABCR chemicals, etc., and used without further purification.

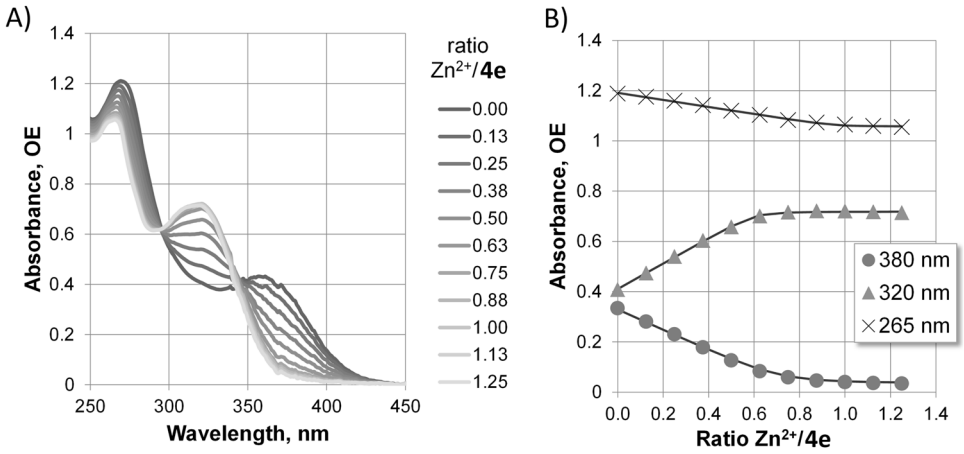


Fig. 2 **A** Changes in absorption spectra of **4e** due to addition of Zn^{2+} aqueous solution, concentrations: **4e**, 1.25×10^{-4} M $ZnSO_4$, 3×10^{-3} M. and (**B**) titration curves were plotted from the change in absorption on 265 nm (crosses), 320 nm (triangles) and 380 nm (circles)

Table 2 The effect of compound **4e** on susceptibility of *E. coli* XL10-Gold producing metallo-β-lactamase NDM-1 to meropenem and cefotaxime

Antibiotic	MIC, μg/mL				
	No inhibitor	Antibiotic/Cmpd 4e ratio			Antibiotic + EDTA (50 μM)
		1:2	1:1	2:1	
Meropenem	>128	16	32	64	0.125
Cefotaxime	128	64	64	64	0.06

Analytical TLC was carried out on Silica Gel F_{254} plates (Merck); for column chromatography was used SilicaGel Merck 60. NMR spectra were recorded on a Varian Mercury 400 Plus instrument operated at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) using $CDCl_3$ or DMSO-d_6 as solvents. High resolution mass spectra were recorded with electron spray ionization (ESI) on a Bruker Daltonics microOTOF-QII instrument. Analytical HPLC was performed on an LC-20AD chromatograph (Shimadzu, Kyoto, Japan) using a diode array UV detector and a Kromasil-100-C18 (4.6 × 250 mm) column with 5 μm particle size (AkzoNobel, Amsterdam, Netherlands) with injection volume of 20 μL at a flow rate of 1 mL/min in the following systems: system (1), A—H_3PO_4 (0.01 M) pH = 2.6, B—MeCN; system (2), A—$HCOONH_4$ (0.2%), B—MeCN. All products were vacuum dried at room temperature.

Synthesis of DPA derivatives

*Dimethyl 4-bromopyridine-2,6-dicarboxylate (**2**).* This compound was prepared from chelidamic acid (**1**) as previously described [12]. The yield of **2** is 186 mg (25%) as a yellow solid. Mp 153 °C (Mp 153–156 °C [17]). ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm: 8.46 (s, 2H, Py); 4.03 (s, 2H, $2CH_3$). HRMS (ESI) calculated for $C_{21}H_{15}N_2O_4$ $[M + H]^+$: 273.9709, found 273.9697.

*Di-tert-butyl 4-chloropyridine-2,6-dicarboxylate (**3**).* This compound was prepared from chelidamic acid (**1**) as previously described [13]. The yield of **3** is 755 mg (20%) as a light-yellow solid. Mp 91 °C (Mp 92–94 °C [18]). ^{1}H NMR (400 MHz, $CDCl_3$) δ, ppm: 8.14 (s, 2H, Py); 1.63 (s, 18H, Bu^t). HRMS (ESI) calculated for $C_{21}H_{15}N_2O_4$ $[M + H]^+$: 314.1154, found 314.1136.

General procedure for synthesis of compounds 4a–f

To a solution of dimethyl 4-bromopyridine-2,6-dicarboxylate (**2**, 136 mg, 0.5 mmol) in dioxane (2.5 mL) arylboronic acid (0.6 mmol) and potassium acetate (98 mg, 1.0 mmol) were added. The resulted suspension was stirred for 10 min under an inert atmosphere and $Pd(PPh_3)_4$ (116 mg, 0.1 mmol) was added. The reaction mixture was refluxed for 3 h (control by TLC) and concentrated under reduced pressure. The crude residue was purified by column chromatography (ethyl acetate/toluene, 0→15%) to give the corresponding ester. A solution of dimethyl 4-arylpyridine-2,6-dicarboxylate and aqueous (1 M) NaOH (9.0 mL, 9.0 mmol) in THF (2 mL per 1 mmol of ester) was stirred for 1 h at room temperature (control by TLC) and quenched by an addition of 4 M aqueous HCl to pH = 4. The precipitate was filtered off and dried under reduced pressure resulted in the title product **4**.

*4-Phenylpyridine-2,6-dicarboxylic acid (**4a**)* [11]. The yield of **4a** is 85 mg (70%) as a white solid. Mp > 250 °C. HPLC: t_R = 14.4 min (system 1, gradient B from 20% to 80% for 30 min), purity 99%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.45 (s, 2H, Py); 7.91 (d, 2H, J = 6.6 Hz, Ph); 7.52–7.58 (m, 3H, Ph). HRMS (ESI) calculated for $C_{13}H_9NO_4$ $[M + H]^+$: 244.0604, found: 244.0598.

*4-(4-Fluorophenyl)pyridine-2,6-dicarboxylic acid (**4b**).* The yield of **4b** is 117 mg (90%) as a white solid. Mp >250 °C. HPLC: t_R = 15.2 min (system 1, gradient B from 20% to 80% for 30 min), purity 100%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.43 (s, 2H, Py); 7.99 (s, 2H, Ph); 7.38

(s, 2H, Ph). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 165.7; 163.4 (d, $J = 247.7$ Hz); 149.2 (d, $J = 43.7$ Hz); 132.4; 129.7 (d, $J = 7.7$ Hz); 124.39; 116.4 (d, $J = 22.2$ Hz). HRMS (ESI) calculated for $C_{13}H_8FNO_4$ $[M + H]^+$: 262.0510, found: 262.0498.

*4-(4-Methylphenyl)pyridine-2,6-dicarboxylic acid (**4c**).* The yield of **4c** is 123 mg (96%) as a white solid. Mp > 250 °C. HPLC: $t_R = 17.1$ min (system 1, gradient B from 20% to 80% for 30 min), purity 98%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.41 (s, 2H, Py); 7.78 (d, 2H, $J = 7.9$ Hz, Ph); 7.34 (d, 2H, $J = 7.5$ Hz, Ph); 2.35 (s, 2H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 165.8; 149.9; 149.3; 140.0; 132.9; 130.1; 127.1; 124.0; 20.9. HRMS (ESI) calculated for $C_{14}H_{11}NO_4$ $[M + H]^+$: 258.0761, found: 258.0779.

*4-(3-Aminophenyl)pyridine-2,6-dicarboxylic acid (**4d**)* [11]. The yield of **4d** is 107 mg (83%) as a light-brown solid. Mp > 250 °C. HPLC: $t_R = 7.2$ min (system 1, gradient B from 20% to 80% for 30 min), purity 96%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.42 (s, 2H, Py); 7.19–7.16 (m, 1H, Ph); 7.01 (s, 1H, Ph); 6.94 (d, 1H, $J = 7.6$ Hz, Ph); 6.70 (d, 1H, $J = 7.6$ Hz, Ph). HRMS (ESI) calculated for $C_{13}H_{10}N_2O_4$ $[M + H]^+$: 259.0713, found: 259.0729.

*4-(1-Naphthyl)pyridine-2,6-dicarboxylic acid (**4e**)* [19]. The yield of **4e** is 113 mg (77%) as an off-white solid. Mp > 250 °C. HPLC: $t_R = 10.0$ min (system 1, gradient B from 40% to 90% for 30 min), purity 95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.19 (s, 2H, Py); 8.10–8.06 (m, 2H, napht); 7.78 (d, 2H, $J = 8.4$ Hz, napht); 7.67–7.57 (m, 4H, napht). HRMS (ESI) calculated for $C_{17}H_{12}NO_4$ $[M + H]^+$: 294.0761, found: 294.0770.

*4-(2-Naphthyl)pyridine-2,6-dicarboxylic acid (**4f**).* The yield of **4f** is 73 mg (50%) as a light-yellow solid. Mp > 250 °C. HPLC: $t_R = 10.8$ min (system 1, gradient B from 40% to 90% for 30 min), purity 96%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.64 (s, 2H, Py); 8.61 (s, 1H, napht); 8.13–8.10 (m, 2H, napht); 8.07–8.00 (m, 2H, napht); 7.63-7.60 (m, 2H, napht). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 165.6; 158.4 (d, $J = 39.4$ Hz); 149.6 (d, $J = 64.5$ Hz); 136.0; 133.5; 133.1 (d, $J = 19.2$ Hz); 129.2; 128.9; 127.6; 127.5; 127.1; 126.9; 124.7; 124.3. HRMS (ESI) calculated for $C_{17}H_{12}NO_4$ $[M + H]^+$: 294.0761, found: 294.0785.

General procedure for synthesis of compounds 5a–d

A solution of di-*tert*-butyl 4-chloropyridine-2,6-dicarboxylate (**3**, 157 mg, 0.5 mmol), triethylamine (312 µL, 2.25 mmol) and secondary amine (1.0 mmol) in DMSO was stirred at 60 °C for 2–4 h (control by TLC). The reaction mixture was diluted with water and the product was extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by column chromatography using 20% ethyl acetate in toluene. An intermediate *tert*-butyl ester was stirred in trifluoroacetic acid (4.5 mL per 1 mmol of ester) for 3 h and concentrated under reduced pressure. The product was purified by column chromatography using 0→10% methanol in chloroform to give the title product **5**.

*4-Piperidinylpyridine-2,6-dicarboxylic acid (**5a**)* [20]. The yield of **5a** is 95 mg (76%) as a beige solid. Mp > 250 °C. HPLC: $t_R = 16.3$ min (system 2, gradient B from 6% to 10% for 30 min), purity 100%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.56 (s, 2H, Py); 3.61–3.53 (m, 4H, $2CH_2$); 1.69–1.54 (m, 6H, $3CH_2$). HRMS (ESI) calculated for $C_{12}H_{14}N_2O_4$ $[M + H]^+$: 251.1026, found: 251.1035.

*4-(3-Aminopiperidine)pyridine-2,6-dicarboxylic acid trifluoroacetate (**5b**).* The yield of **5b** is 97 mg (51%) as a beige solid. Mp > 250 °C. HPLC: $t_R = 6.5$ min (system 2, gradient B from 5% to 40% for 30 min), purity 98%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 8.06 (br s, 3H, NH_3); 7.60 (s, 2H, Py); 4.02 (d, 1H, $J = 8.8$ Hz, CH_2); 3.81 (d, 1H, $J = 13.2$ Hz, CH_2); 3.24–3.13 (m, 3H, CH, CH_2); 2.03–1.96 (m, 1H, CH_2); 1.84–1.76 (m, 1H, CH_2); 1.63–1.53 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 165.5; 156.1; 147.9; 110.5; 65.0; 48.7; 46.1; 27.8; 21.8. HRMS (ESI) calculated for $C_{12}H_{16}N_3O_4$ $[M + H]^+$: 266.1135, found: 266.1192.

*4-(4-Aminopiperidine)pyridine-2,6-dicarboxylic acid trifluoroacetate (**5c**).* The yield of **5b** is 123 mg (65%) as a beige solid. Mp > 250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.82 (br s, 3H, NH_3); 7.52 (s, 2H, Py); 4.17 (d, 2H, $J = 13.3$ Hz, CH_2); 3.16-3.09 (m, CH_2); 2.79–2.73 (m, 1H, Hz, CH); 1.99–1.96 (m, 4H, $2CH_2$). HRMS (ESI) calculated for $C_{12}H_{16}N_3O_4$ $[M + H]^+$: 266.1135, found: 266.1150.

*4-(4-Piperidinylpiperidine)pyridine-2,6-dicarboxylic acid trifluoroacetate (**5d**).* The yield of **5b** is 130 mg (58%) as a beige solid. Mp > 250 °C. HPLC: $t_R = 11.1$ min (system 2, gradient B from 5% to 40% for 30 min), purity 97%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 9.51 (br s, 1H, NH); 7.62 (s, 2H, Py); 4.27 (d, 2H, $J = 12.7$ Hz, CH_2); 3.42–3.49 (m, 2H, CH_2); 3.05-2.93 (m, 4H, $2CH_2$); 2.54 (s, 1H, CH); 2.11 (d, 2H, $J = 11.0$ Hz, CH_2); 1.85–1.80 (m, 2H, CH_2); 1.67–1.65 (m, 5H, $3CH_2$); 1.40 (br s, 1H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 165.4; 155.9; 148.0; 110.4; 62.2; 49.1; 44.8; 25.1; 22.8; 21.5. HRMS (ESI) calculated for $C_{17}H_{24}N_3O_4$ $[M + H]^+$: 334.1761, found: 334.1685.

*4-(4-N-Methylhomopiperazinyl)pyridine-2,6-dicarboxylic acid trifluoroacetate (**5e**).* The yield of **5e** is 85 mg (44%) as a beige solid. Mp > 250 °C. HPLC: $t_R = 8.1$ min (system 2, gradient B from 5% to 40% for 30 min), purity 98%. ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: 7.42 (s, 2H, Py); 3.62-3.33 (m, 8H,); 2.83 (s, 3H, CH_3); 2.20 (br s, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: 164.3; 156.2; 147.1; 108.3; 55.0; 47.2; 43.6; 43.2; 27.9; 23.5. HRMS (ESI) calculated for $C_{13}H_{18}N_3O_4$ $[M + H]^+$: 280.1192, found: 280.1231.

Measuring the kinetic parameters and the inhibition constants of the recombinant metallo-β-lactamase NDM-1

Recombinant MBL NDM-1 was isolated and purified as described previously [21]. The NDM-1 activity was measured toward the chromogenic substrate CENTA at RT in 50 mM Na-phosphate buffer (pH 7.0) with or without zinc ions (50 μM) using a Shimadzu UV-1602 spectrophotometer. The initial CENTA concentration was 50 μM; all DPA derivatives were taken at 50 μM. The enzyme concentration in the assay was 20 nM. All measurements were repeated at least three times. The hydrolysis of CENTA was monitored by continuous recording of the absorbance at 405 nm ($\Delta\varepsilon = 6400\ M^{-1}cm^{-1}$) [14]. Screening of the inhibitory activity (IC_{50}) of DPA derivatives was carried out in 96-well microplates on a Multiscan FC spectrophotometer (Thermo Scientific) at 25 °C. 100 μL of a solution of the test compound was added to the wells of the plate; then 50 μL of the enzyme solution (1 nM final concentration) was added. [DPA derivatives] = 0.007, 0.02, 0.062, 0.185, 0.555, 0.167, and 5 μM (or 0.07, 0.2, 0.62, 1.85, 5.55, 16.7, 50 μM), depending on the inhibition power of tested compound. The reaction was initiated by adding 50 μL of CENTA substrate solution with a concentration of 400 μM. Optical absorbance readings at 405 nm were measured every 3 min for 30 min. All measurements were repeated three times. Δ optical density (30 min) we plotted in semilogariphmic concentration scale.

Study of complexation with Zn^{2+}

The absorbance was measured on Unico SQ-4802 Double Beam UV/Vis Spectrophotometer with 4.5 mL (10 mm) quartz cuvettes. DPA and derivative **4e** was dissolved in DMSO to initial concentration of 5.0×10^{-3} M and diluted with water to 1.25×10^{-4} M. The concentration of stock solution of $ZnSO_4$ in water was 1.88×10^{-3} M. To a solution of **4e** (3.0 mL) were added aliquots (25 μL) of the $ZnSO_4$ stock solution to prepare 0 – 1.2 eq. ratio over **4e**. Titration was performed by 11 measurements, and the binding constant (logβ) was calculated by fitting the change of UV-Vis absorbance with a 1:2 association model using the next equation [22]:

$$A = A0 + (Ab - A\mathbf{o}) \times \frac{(Zn + NX + Kd) - \sqrt{(Zn + NX + Kd)^2 - 4ZnNX}}{2NX}$$

X—chelator concentration, N—number of binding sites in molecule, (NX—concentration of binding sites), Zn—Zn^{2+} concentration, A0 —Absorbance of free chelator, Ab—Absorbance of bound chelator.

The binding constant (logβ, logarithm of equation constant of complex formation) for the interaction between **4e** and Zn^{2+} was found to be 6.5 (for unsubstituted DPA this meaning is 6.49). Ligand protonation effects were not taken into account, as the Ka_1 and Ka_2 (dissociation constant of DPA carboxylic groups) values for unsubstituted DPA are significantly higher than Kw (dissociation constant of water, 10^{-14}) [23], which means DPA and its derivatives exist in anionic form in aqueous solution. The error limit in β was less than 10%.

In vitro antibacterial activity

Strain of *E. coli* XL10-Gold harboring bla_{NDM-1} (PJET1,2-$_{NDM-1}$) kindly provided by Dr. Polina Starkova (Pediatric Research and Clinical Center for Infectious Diseases, Saint Petersburg, Russia) [14]. Strain was stored at −75 °C in Trypticase Soy broth (Becton, Dickinson, France) with 15% glycerol. Before the experiment, strain activated on Trypticase-Soy agar or broth (Beckton, Dickinson, France), containing 100 μg/mL ampicillin and incubated at 36 ± 1 °C for 16–24 h. Antimicrobial susceptibility test (MICs) was determined with 1:2, 1:1, and 2:1 antibiotic/inhibitor ratio or with 50 μM metal chelator agent ethylenediaminetetraacetic acid (EDTA) for zinc-limiting conditions and standard conditions described ISO 20776-1-2022.

Cell viability assay

HEK293 cell line was from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM medium (PanEco, Russia), supplemented with 5% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, 5% CO_2 in a humidified atmosphere. All studied cells were in the logarithmic phase. The 10 mM stock solutions of the compounds in DMSO were used. Serial dilutions in culture medium were prepared immediately before experiments. The cytotoxicity was determined in MTT-test [24].

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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