Research Article

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Future Medicinal Chemistry

N-Pyrazinylhydroxybenzamides as biologically active compounds: a hit-expansion study and antimicrobial evaluation

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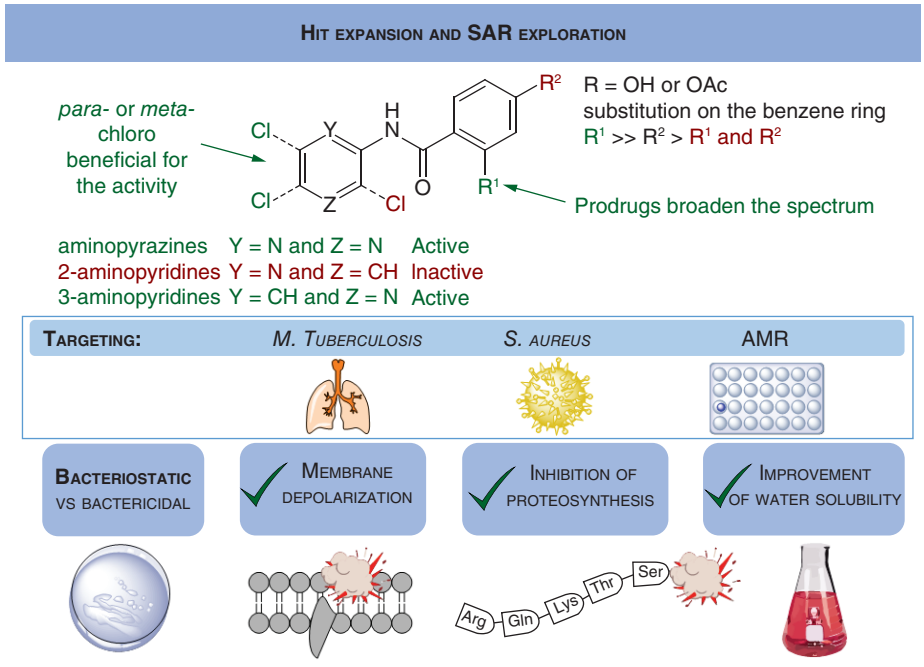
Background: The development of novel antimicrobial drugs is an essential part of combatting the uprising of antimicrobial resistance. Proper hit-to-lead development is crucially needed. **Methods & results:** We present a hit-expansion study of *N*-pyrazinyl- and *N*-pyridyl-hydroxybenzamides with a comprehensive determination of structure–activity relationships. The antimicrobial screening revealed high selectivity to staphylococci along with antimycobacterial activity with the best value of 6.25 μg/ml against *Mycobacterium tuberculosis* H37Rv. We proved an inhibition of proteosynthesis and a membrane depolarization of methicillin-resistant *Staphylococcus aureus*. **Conclusion:** Our results are a good starting point for further development of new antimicrobial compounds, where the next step would be tuning the potential between relatively nonspecific membrane depolarization effect and specific inhibition of proteosynthesis.

Graphical abstract:



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Keywords: antibacterial • antimicrobial resistance • antimycobacterial • hit expansion • macromolecular assay • membrane depolarization • minimum bactericidal concentration • structure–activity relationship • water solubility

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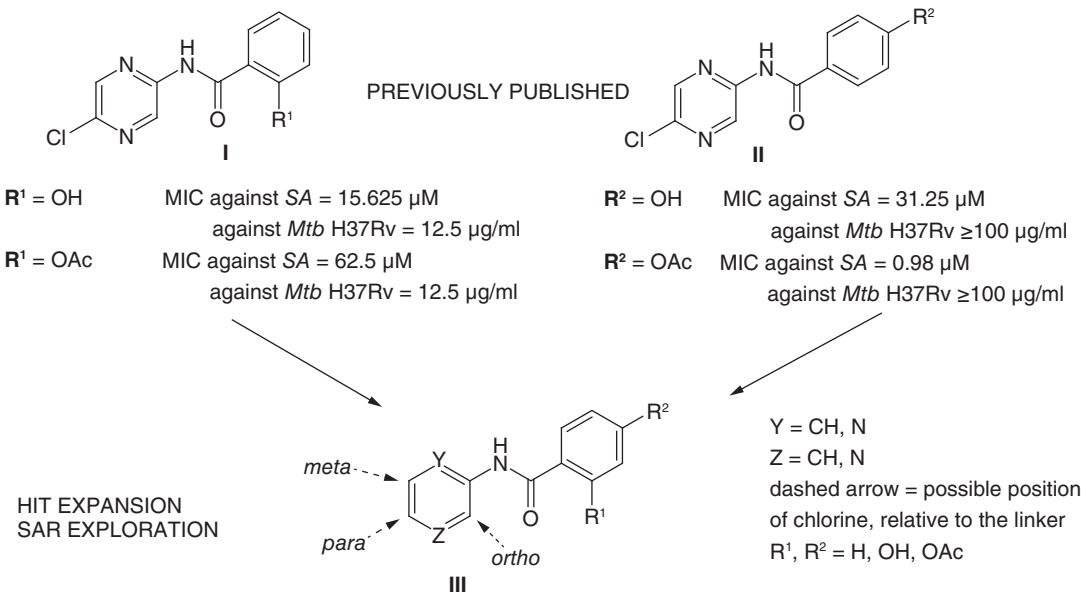


Figure 1. Design of the compounds.
MIC: Minimum inhibitory concentration; *Mtb: Mycobacterium tuberculosis*; *SA*: *Staphylococcus aureus*; SAR: Structure–activity relationship.

Infections caused by multidrug-resistant strains of bacteria are amongst the most dangerous global threats to public health with an unrivalled potential to escalate in the near future [1]. If no significant measures are taken, it is foreseen that the deaths from infections caused by resistant bacteria will reach 10 million in 2050, being at least on par with the deaths caused by all malignancies [1,2]. The development of new antimicrobial drugs has become of crucial importance [3]. Due to the phenomenon of cross-resistance or coresistance, novel compounds with an innovative mechanism of action are preferred over analogues (me-too drugs) of well-known antibiotics [4]. The WHO prioritized pathogens to guide the research and development of new compounds focusing on carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* family; methicillin-resistant *Staphylococcus aureus* (MRSA); multidrug-resistant *Mycobacterium tuberculosis (Mtb)* and others [5]. MRSA has become one of the leading causes of bacterial infections worldwide [6] and is perceived as a prototypical example of a pathogen causing healthcare-associated infections [7].

S. aureus (*SA*) belongs to a common microbiota of the skin or nasopharyngeal membranes (present approximately in 20–30% of adults), seldom causing any infection [8,9]. However, if the pathogen invades deeper into the host tissues or enters the bloodstream, serious or even fatal systemic infections can occur. Staphylococcal infections resistant to common penicillin antibiotics are usually treated with vancomycin [8–10]. However, strains of vancomycin-resistant *SA* were already isolated [11] and should not be overlooked and underestimated despite the rare occurrence [12].

Mtb, the causative agent of tuberculosis (TB), caused approximately 1.4 million deaths in 2021, ranking TB as the most frequent cause of death from infectious diseases [13]. Globally, the risk factors for TB epidemiology are immunocompromised patients, reinfections and multidrug resistance [14].

The development of new antimicrobial drugs has been critically inadequate. In 2020, only 30–40 new antimicrobial candidate drugs were in various phases of clinical testing. Moreover, the candidates targeting the WHO priority pathogens [5] are mainly derivatives (me-too drugs) of existing antibiotics [15] and therefore, prone to the risk of cross-resistance. Facing drug-resistant infections will require more intensive development and funding. Successful development requires novel compounds capable of hitting multiple targets, preferably with a new mechanism of action and proper hit-to-lead optimization [16]. The strengths and weaknesses of the hit must be completely described, as well as other important physicochemical properties [17].

In this paper, we present a structural hit-expansion study based on our previously published *N*-pyrazinylbenzamides (Figure 1; structures **I** and **II**) [18], which exerted promising activity against *SA* (structure

I and **II**) or *Mtb* (structure **I**). We have decided to prepare a series of compounds (**III**) around similar structural motifs to expand the knowledge of their structure–activity relationships (SARs) and provide additional biological activities with a focus on discovering a potential mechanism of action.

To explore the SARs, we decided to prepare various position isomers by altering the position of the chlorine (X) on the heterocycle combined with a variously substituted benzene ring by the hydroxy or acetoxy group (R^1 and R^2). A single change in the presence and position of the chlorine can lead to a dramatic change in the mechanism of action and biological activities. This can be demonstrated by the activity of 6-chloropyrazinoic acid as an inhibitor of mycobacterial aspartate decarboxylase [19] and 5-chloropyrazinamide inhibiting the activity of mycobacterial fatty acid synthase type I [20–22]. We also prepared various isosteres of the former structural motif by replacing the pyrazine ring with variously substituted pyridines (Y and Z). The isosteric change of pyrazine for pyridine can lead to compounds with improved antimicrobial activity [23]. The linker (heterocycle–NH–CO–Ph) was retained from our previous work [2318] because it reduced cytotoxicity compared with pyrazinecarboxamides (heterocycle–CO–NH–Ph) with inversed linker [24–26]. Combinations of 2-OH and 4-OH substitution on the benzene ring were drawn from previous publications [18,24–26]. 2-Hydroxy substitution can be found in salicylanilides with interesting antistaphylococcal activity [27,28]. 4-Hydroxy substitution is present in antimicrobial parabens.

Materials & methods

General

All chemicals (unless stated otherwise) were purchased from Merck (Darmstadt, Germany). The reaction progress and the purity of the final compounds were checked using Merck Silica 60 F254 TLC plates (Merck). The purification of the synthesized compounds was carried out on an automated flash chromatograph puriFlash XS420+ (Interchim, Montluçon, France) using original columns (spherical silica 30 μm, F0040 or F0080). The mixture of ethyl acetate and hexane was used as a mobile phase with elution and detection performed by ultraviolet-visible detector at a wavelength of 254 and 280 nm. NMR spectra were recorded on Varian VNMR S500 (Varian, CA, USA) at 500 MHz for ^{1}H and 125 MHz for ^{13}C or using Jeol JNM-ECG600 (JEOL, Tokyo, Japan), at 600 MHz for ^{1}H and 151 MHz for ^{13}C. The spectra were recorded in DMSO-d_6 at ambient temperature (unless stated otherwise). The chemical shifts as δ values in ppm. are indirectly referenced to tetramethylsilane via the solvent signal. Nicolet 6700 spectrometer (Thermo Scientific, MA, USA) was used to measure IR spectra using attenuated total reflectance on a germanium crystal. method. Elemental analysis was performed on Vario MICRO cube Element Analyzer (Elementar Analysensysteme, Hanau, Germany) with values given as percentages. The mass spectra in both positive and negative mode were measured using the Expression® Compact Mass Spectrometer (Advion, NY, USA) with a single-quad detector. Melting points of the synthesized compounds were measured in an open capillary on Stuart SMP30 melting point apparatus (Bibby Scientific Limited, Stone, UK) and are uncorrected. Yields are given as percentages and refer to the amount of chromatographically pure product obtained after all the purification steps. Log S values were calculated using ChemDraw 20.0 (PerkinElmer Informatics, MA, USA). The optimized structures were calculated by Molecular Operating Environment 2022.09 (Chemical Computing Group Inc., Montreal, Canada) and Gaussian 16 (revision C.01, Gaussian, Inc., CT, USA). Tested strains were purchased from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), from Belgian Coordinated Collections of Microorganisms (Antwerp, Belgium), or from the (American Type Culture Collection [ATCC]; VA, USA).

Synthesis

Compounds 1–30

A total of 4.5 mmol of the appropriate acetoxybenzoic acid was dispersed in 5 ml of dichloromethane (DCM). Then, 5 mmol of oxalyl chloride and two drops of *N*,*N*-dichloromethane were added. After the activation had finished (stopped bubbling and checked by TLC), the mixture was transferred dropwise to an ice-cooled solution of 3 mmol of various aminopyridines or aminopyrazines in 5 ml of DCM and 725 μl of pyridine (three equivalents). The resulting mixture was left to heat to room temperature and stirred for 24 h. After completion of the reaction (checked by TLC), the solvent was evaporated. The reaction mixture was purified by flash chromatography using a spherical silica column. The most efficient elution was gradient elution hexane:ethyl acetate (from 4:1 ratio to 1:2). Purification usually resulted in a mixture of acetoxy and hydroxy derivatives (partial hydrolysis on silica). The fractions were combined, and the acetoxy protection was cleaved with K_2CO_3 in ethanol boiling for 1 h. The mixture was filtered to remove K_2CO_3 and the filtrate was evaporated. The resulting crude product was dissolved

in EtOAc (100 ml) and extracted into water (100 ml) and 2 ml of triethylamine. The water layer was then acidified with 2 M HCl to pH 5 and cooled in a refrigerator for 3 h. The white precipitate was filtered and dried in an oven (90°C).

Acetylation: compounds 1Ac–29Ac

A total of 0.5 mmol of the respective hydroxyderivative was dissolved in 2 ml of acetic acid anhydride. Two drops of concentrated sulfuric acid were added. The solution was stirred continuously at room temperature for 1 h. The reaction mixture was then poured on ice and stirred until the ice melted. A white precipitate was formed and then filtered, washed with cold water several times and dried in an oven (90°C). The solid compound was triturated with hexane and resulted in a pure compound.

Biological methods

Macromolecular biosynthesis assay

MRSA ATCC 43300 grown on Tryptic Soy agar (Merck) was transferred into Tryptic Soy Broth (Merck) and cultured overnight. Completely defined medium/completely defined medium-Leu (for determination of protein synthesis) prepared according to literature was used for the preparation of log-phase culture of MRSA (approximately 2×10^7 colony forming units [CFU]). Tryptic Soy Broth culture was diluted with these media in a ratio 1:100 and cultured at 37°C for another 5 h. Then, 0.9 ml of suspensions were transferred into prewarmed glass tubes. All antimicrobials (vancomycin: 8 μg/ml; rifampicin: 0.064 μg/ml; ciprofloxacin: 1 μg/ml; chloramphenicol: 64 μg/ml; chlorhexidine: 4 μg/ml; compound **1**: 125 μg/ml; compound **5Ac**: 73 μg/ml) were added at the concentration equal to 4 × minimum inhibitory concentration (MIC) and mixed thoroughly. All standards were purchased from Merck. Untreated controls were incubated with an adequate volume of DMSO (Merck). [^{3}H]-labelled precursors (*N*-acetylglucosamine: 0.1 μCi/ml; uridine: 1 μCi/ml; thymidine: 1 μCi/ml; Leu: 3 μCi/ml; Hartmann Analytic, Braunsweig, Germany) were immediately added to corresponding tubes. Another incubation at 37°C for 2 h followed. Then, 0.5 ml aliquots were transferred into 2 ml Eppendorf tubes containing 1 ml of ice-cold 10% trichloroacetic acid (TCA; Merck), mixed thoroughly and placed on ice overnight to facilitate the precipitation. The precipitates were then washed once with 0.5 ml of 5% TCA/1.5 M NaCl followed by one-time washing with 0.5 ml of 5% TCA to remove free precursors. After the second wash, samples were solubilized in 0.5 ml of 0.1% SDS/0.1 M NaOH by vortexing at room temperature. The solubilized precipitates were transferred into scintillation tubes and thoroughly mixed with 2 ml of scintillation cocktail (Merck). The incorporated radioactivity was measured in counts per minute using a liquid scintillation analyzer TRI-CARB 2900TR (Perkin Elmer, MA, USA), and the results were expressed as a percentage of untreated controls. Data were analyzed using GraphPad Prism 9.0 software (GraphPad Software). One-way analysis of variance was used to determine the statistical significance (p-value) of differences in this *in vitro* assay.

Membrane depolarization assay

To evaluate the possible action of compounds **1** and **5Ac** on the staphylococcal cytoplasmic membrane (membrane depolarization), the fluorometric measurement of membrane potential using voltage-sensitive dye, $DiSC_3(5)$, was employed. The assay was carried out using the reference strain, MRSA ATCC 43300 and chlorhexidine dihydrochloride (CHX) as the positive control.

Briefly, MRSA was resuspended in cation-adjusted Mueller–Hinton broth and cultivated to the exponential (mid-log) phase. Bacteria were compacted by centrifugation (10,000× *g*, for 10 min at 24°C), washed, resuspended in 5 mM HEPES, 5 mM glucose, pH 7.2, and finally diluted to optical density (OD) ≈ 0.5 McFarland units. The voltage-sensitive dye, $DiSC_3(5)$, was then added to a final concentration of 0.5 μM (1% v/v DMSO). After a short incubation period (15 min), bacterial suspension was transferred to white polystyrene 96-well plate (200 μl/per well), and fluorescence quenching (λ_{Ex} = 620, λ_{Em} = 680, Synergy HTX Multi-Mode Microplate reader; BioTek, WA, USA) was monitored for 15 min (until a stable baseline was obtained). Depolarizing and tested compounds were added to wells in hexaplicates (with a final concentration of 1% v/v DMSO). Into wells serving as a positive control, the CHX in final concentration corresponding to 4 × MIC (4 μg/ml, 1% v/v DMSO) was added. The negative control was represented by untreated, stained bacterial cells in HEPES buffer (1% v/v DMSO). The impact of **1** and **5Ac** on the cytoplasmic membrane was revealed by the addition of these compounds in the final concentration corresponding to 4 × MIC (250 μM). The plate was rapidly placed back into the reader to continue monitoring fluorescence every 1 min for the next 15 min.

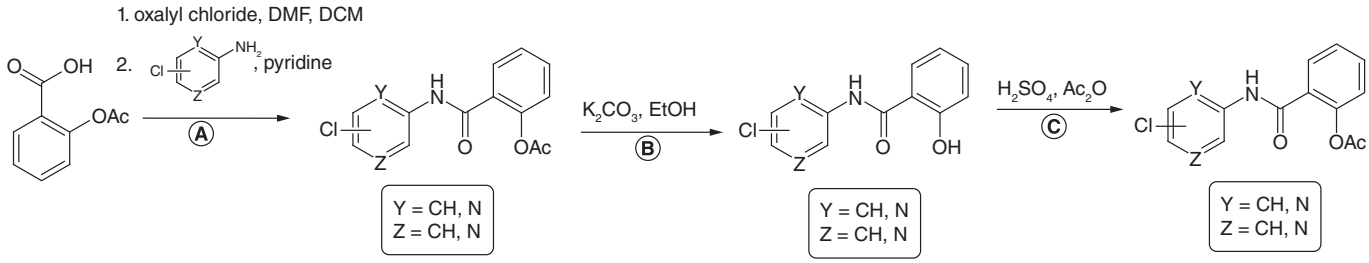


Figure 2. Representative scheme of the synthesis. (A) Acylation, **(B)** hydrolysis and **(C)** acetylation. Analogically used for 4-substituted and 2,4-disubstituted derivatives.
DCM: Dichloromethan; DMF: *N,N*-dimethylformamide.

Determination of bactericidal versus bacteriostatic activity

To distinguish between the bactericidal or bacteriostatic activity of compound **1** and compound **5Ac**, the strain MRSA ATCC 43300, microdilution method and subsequently spread plate technique for CFU evaluation were employed. Solutions of **1** and **5Ac** in cation-adjusted Mueller–Hinton broth with a final concentration ranging from 15.625 to 500 μM were prepared according to the procedure described in Supplementary materials (section on *in vitro* antibacterial evaluation). After incubation for 24 h, the MIC was evaluated and the representative aliquot from wells, where the 4 × MIC was registered, was taken, serially diluted, seeded and subcultured on Mueller–Hinton agar for 24 h in a humid atmosphere at 35 ± 2°C. Similarly, the initial bacterial inoculum was processed. After incubation, the number of CFU/ml was calculated.

Results & discussion

Synthesis

The parent hydroxy derivatives were prepared by acylation of different aminopyrazines or aminopyridines with the corresponding activated acetoxybenzoic acids. The carboxylic acids were activated by conversion to acyl chloride by the reaction with oxalyl chloride in the presence of DCM as the solvent and a catalytic amount of *N*,*N*-dimethylformamide. We experienced better yields using acetoxybenzoic acids with subsequent cleavage of the acetoxy group over acylation with nonprotected hydroxybenzoic acids. The acylation was performed in DCM with pyridine as a base at room temperature. The final compounds were obtained by multistep purification consisting of flash chromatography, cleavage of the protecting acetoxy group and extraction with modifications of pH (yield: 3–56%). The final acetylated compounds were prepared from sufficiently purified hydroxy derivatives by reaction with acetic anhydride and sulfuric acid as a catalyst. The reaction scheme can be found in Figure 2. Final compounds were characterized by melting point, ^{1}H NMR, ^{13}C NMR and MS spectra. Representative structural derivatives were additionally characterized by IR spectra. The purity was checked by elemental analysis. All analytical results were fully consistent with disclosed structures.

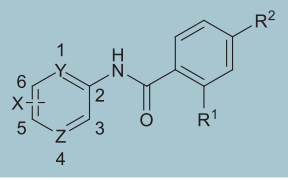
The typical ^{1}H NMR spectra measured in DMSO-d_6 contained carboxamide hydrogen signal observed as a singlet usually in the range 10.5–12.0 p.p.m., and phenolic hydrogen (if present) as a singlet usually in the range 10.0–11.5 p.p.m. The typical IR spectra contained amidic carbonyl stretching at 1651–1688 cm^{-1} and carbonyl from ester stretching at 1743–1769 cm^{-1} (acetylated derivatives).

Antibacterial activity

A microdilution method according to European Committee for Antimicrobial Susceptibility Testing [29–31] was used to evaluate the activity against a set of eight bacterial strains of clinical importance. The tested set contained *SA* subsp. *aureus* (*SA*), *Staphylococcus aureus* subsp. *aureus* methicillin-resistant (MRSA), *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *A. baumannii* and *P. aeruginosa*. The MIC values were expressed in μM. Due to lower solubility, the derivatives **6**, **6Ac** and **7** were tested up to the concentration 125 μM, whereas the other compounds were tested up to the concentration of 500 μM. For the methodology and the complete identification of tested strains, please see the Supplementary materials.

Only several compounds proved inhibitory activity at the tested concentrations against included strains of bacteria (Table 1) and the activity was generally low, typically ranging from 62.5 to 125 μM. The series was more

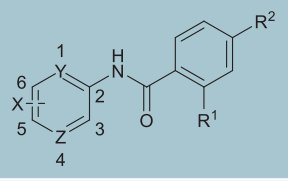
Table 1. Antibacterial activity expressed as MIC in μM.[†]



						Strain							
Code	Y	Z	R^1	R^2	X	*Staphylococcus aureus* subsp. *aureus*	*Staphylococcus aureus* subsp. *aureus* methicillin-resistant	*Staphylococcus epidermidis*	*Enterococcus faecalis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*
1	N	N	OH	H	5–Cl	**125**	**125**	**125**	>500	>500	>500	>500	>500
1Ac	N	N	OAc	H	5–Cl	**125**	**125**	**125**	**500**	**500**	**250**	**500**	>500
2	N	N	OH	H	6–Cl	**250**	**500**	**500**	**500**	>500	>500	>500	>500
2Ac	N	N	OAc	H	6–Cl	**125**	**125**	**62.5**	**250**	**500**	**250**	**250**	>500
3	N	N	OH	H	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
3Ac	N	N	OAc	H	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
4	N	N	OH	H	–	>500	>500	>500	>500	>500	>500	>500	>500
4Ac	N	N	OAc	H	–	>500	>500	>500	>500	>500	>500	>500	>500
5	N	N	H	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
5Ac	N	N	H	OAc	5–Cl	**62.5**	**125**	**125**	**500**	**500**	**250**	>500	>500
6	N	N	H	OH	6–Cl	>125	>125	>125	>125	>125	>125	>125	>125
6Ac	N	N	H	OAc	6–Cl	>125	>125	>125	>125	>125	>125	>125	>125
7	N	N	H	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
7Ac	N	N	H	OAc	3–Cl	>125	>125	125	>125	>125	>125	>125	>125
8	N	N	H	OH	–	>500	>500	>500	>500	>500	>500	>500	>500
8Ac	N	N	H	OAc	–	>500	>500	>500	>500	>500	>500	>500	>500
9	N	N	OH	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
9Ac	N	N	OAc	OAc	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
10	N	N	OH	OH	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
10Ac	N	N	OAc	OAc	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
11	N	N	OH	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
11Ac	N	N	OAc	OAc	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
12	N	N	OH	OH	–	>500	>500	>500	>500	>500	>500	>500	>500
13	N	CH	OH	H	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
13Ac	N	CH	OAc	H	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
14	CH	N	OH	H	6–Cl	**250**	**500**	>500	>500	>500	>500	>500	>500

[†]Bold text indicates signifcance of the result.

Table 1. Antibacterial activity expressed as MIC in μM.† (cont.).



						Strain							
Code	**Y**	**Z**	**R^1**	**R^2**	**X**	***Staphylococcus aureus* subsp. *aureus***	***Staphylococcus aureus* subsp. *aureus* methicillin-resistant**	***Staphylococcus epidermidis***	***Enterococcus faecalis***	***Escherichia coli***	***Klebsiella pneumoniae***	***Acinetobacter baumannii***	***Pseudomonas aeruginosa***
14Ac	CH	N	OAc	H	6–Cl	**125**	**125**	**125**	**500**	**250**	>500	>500	>500
15	N	CH	OH	H	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
15Ac	N	CH	OAc	H	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
16	CH	N	OH	H	5–Cl	**250**	**500**	**500**	>500	>500	>500	>500	>500
16Ac	CH	N	OAc	H	5–Cl	**250**	**500**	**500**	>500	500	>500	>500	>500
17	N	CH	OH	H	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
17Ac	N	CH	OAc	H	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
18	CH	N	OH	H	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
18Ac	CH	N	OAc	H	3–Cl	**125**	**500**	**250**	**500**	>500	**500**	>500	>500
19	N	CH	H	OH	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
19Ac	N	CH	H	OAc	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
20	CH	N	H	OH	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
20Ac	CH	N	H	OAc	6–Cl	>500	**500**	**500**	**500**	>500	>500	>500	>500
21	N	CH	H	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
21Ac	N	CH	H	OAc	5–Cl	>125	>125	>125	>125	>125	>125	>125	>125
22	CH	N	H	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
22Ac	CH	N	H	OAc	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
23	N	CH	H	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
23Ac	N	CH	H	OAc	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
24	CH	N	H	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
24Ac	CH	N	H	OAc	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
25	N	CH	OH	OH	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
25Ac	N	CH	OAc	OAc	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
26	CH	N	OH	OH	6–Cl	>500	>500	>500	>500	>500	>500	>500	>500
26Ac	CH	N	OAc	OAc	6–Cl	500	>500	>500	>500	>500	>500	>500	>500
27	N	CH	OH	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
27Ac	N	CH	OAc	OAc	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
28	CH	N	OH	OH	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
28Ac	CH	N	OAc	OAc	5–Cl	>500	>500	>500	>500	>500	>500	>500	>500
29	N	CH	OH	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
29Ac	N	CH	OAc	OAc	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
30	CH	N	OH	OH	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500
30Ac	CH	N	OAc	OAc	3–Cl	>500	>500	>500	>500	>500	>500	>500	>500

†Bold text indicates signifcance of the result.

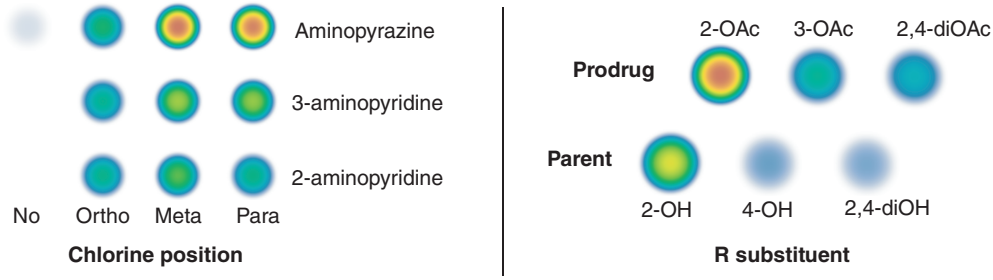


Figure 3. Visualization of structure–activity relationships for antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. The heat map represents the cumulative activity (reciprocal to MIC), from blue (lowest) to red (highest). See the online version of the manuscript for a colored version of the figure.
MIC: Minimum inhibitory concentration.

efficient against Gramm-positive bacteria bacteria, especially *Staphylococcus* species (*SA*, MRSA, *S. epidermidis*). The most active compound was compound **5Ac** with MIC = 62.5 μM against SA.

Regarding the chloro substitution on the heteroaromatic ring, derivatives of 6-Cl-pyrazine (chlorine in *meta* position relative to the linker) and derivatives of 5-Cl-pyrazine (*para* relative to the linker) exhibited similar activity. On the contrary, derivatives of 3-Cl-pyrazine (*ortho* relative to the linker) were inactive. The acetylation broadened the spectrum of activity to those other than staphylococcal strains (compare compounds **1**, **2** to **1Ac**, **2Ac**).

The antibacterial activity of pyridine derivatives was observed only among derivatives of 3-aminopyridine (Y = CH and Z = N, compounds **14**, **14Ac**, **16**, **16Ac** and **18Ac**). Their positional isomers, derivatives of 2-aminopyridine (Y = N and Z = CH) were inactive. This suggested the importance of the N4 of the former pyrazine core present in the original hit (**I**, **II**).

2,4-Dihydroxy substituted derivatives as well as their diacylated prodrugs were in all cases inactive against all tested strains (MIC >500 μM). Except for compound **5Ac** (one of the starting structures of our hit-expansion study), hydroxy and acetoxy substitution in position four of the benzene ring led in all cases to inactive compounds. The published activity of compound **5Ac** with a MIC of 0.98 μM against *SA* [18] was qualitatively confirmed but with a different MIC value. The difference in the MIC value between this and our previous study might be attributed to a different strain of *SA*.

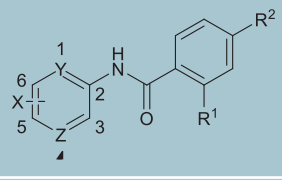
Antimycobacterial activity

Final compounds were screened for antimycobacterial activity using the microplate Alamar Blue assay [32,33] against *Mtb* H37Rv, avirulent strain *Mtb* H37Ra, fast-growing model organisms *Mycobacterium smegmatis* and *Mycolicibacterium aurum*, and nontuberculous mycobacteria *Mycobacterium avium* and *Mycobacterium kansasii*. The fast-growing *M. smegmatis* and *M. aurum* [34] (both recently reclassified into the genus *Mycolicibacterium* [35]) are commonly used as valid and safe nonpathogenic models for antimycobacterial research as an alternative to *Mtb* H37Rv [36–38]. The MIC values were measured in μg/ml. For full methodology, see Supplementary materials.

The following discussion will be focused on activity against *Mtb* H37Ra. In the following text, the position of the chlorine substituent is expressed relative to the linker (Figure 1). Generally, better activity was observed for derivatives of aminopyrazine compared with derivatives of aminopyridines (Figure 3). Comparing the pyridine derivatives, 3-aminopyridine derivatives (Y=CH and Z=N) achieved slightly better activity than 2-aminopyridines (Y=N and Z=CH). This supported the thesis about the importance of nitrogen in position four of the former pyrazine core (Z=N) as noted in the evaluation of SAR of antibacterial activity. The substitution of the heteroaromate (pyrazine, pyridine) by chlorine in positions *para* and *meta* relative to the position of the linker exerted almost identical activity, but the substitution by chlorine in position *ortho* decreased the activity throughout the whole tested series. The absence of the chlorine substitution led to an almost complete loss of activity.

The position of the hydroxy substitution on the benzene ring seemed to be crucial for *N*-pyrazinylbenzamides as well as for *N*-pyridylbenzamides. The higher activity with a broad antimycobacterial spectrum was achieved by 2-hydroxy substituted derivatives (Figure 3 & Table 2). The hydroxy substitution in position four led to a decrease (in the group of *N*-pyridylbenzamides) or complete loss (in the group of *N*-pyrazinylbenzamides) of antimycobacterial activity. The only exception was again compound **5**, one of our starting structures in our hit-expansion study, substituted with chlorine in the *para* position and its acetylated prodrug (**5Ac**). Interestingly,

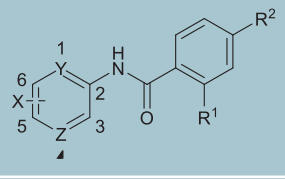
Table 2. Antimycobacterial activity expressed as minimum inhibitory concentration in μg/ml.



						Strain					
Code	Y	Z	R^1	R^2	X	*Mycobacterium tuberculosis* H37Ra	*Mycobacterium tuberculosis* H37Rv	*Mycobacterium kansasii*	*Mycolicibacterium avium*	*Mycobacterium aurum*	*Mycobacterium smegmatis*
1	N	N	OH	H	5–Cl	**15.625**	**12.5**	**7.81**	**15.625**	**15.625**	**7.81**
1Ac	N	N	OAc	H	5–Cl	**15.625**	>100	**1.98**	**7.81**	**15.625**	**15.625**
2	N	N	OH	H	6–Cl	**15.625**	**12.5**	**7.81**	**15.625**	**15.625**	**15.625**
2Ac	N	N	OAc	H	6–Cl	**7.81**	**12.5**	**7.81**	**7.81**	**15.625**	**7.81**
3	N	N	OH	H	3–Cl	**62.5**	>100	**62.5**	**62.5**	**62.5**	125
3Ac	N	N	OAc	H	3–Cl	**31.25**	100	**31.25**	**62.5**	**62.5**	250
4	N	N	OH	H	–	≥500	100	**15.625**	125	≥500	≥500
4Ac	N	N	OAc	H	–	250	100	**62.5**	125	500	≥500
5	N	N	H	OH	5–Cl	≥500	**50**	**15.625**	250	500	62.5
5Ac	N	N	H	OAc	5–Cl	**15.625**	**12.5**	**3.91**	**7.81**	**7.81**	**15.625**
6	N	N	H	OH	6–Cl	Insoluble	>100	Insoluble	Insoluble	Insoluble	Insoluble
6Ac	N	N	H	OAc	6–Cl	Insoluble	>100	Insoluble	Insoluble	Insoluble	Insoluble
7	N	N	H	OH	3–Cl	≥500	>100	125	≥500	≥500	≥500
7Ac	N	N	H	OAc	3–Cl	125	>100	125	≥500	250	500
8	N	N	H	OH	–	≥500	>100	≥500	≥500	≥500	≥500
8Ac	N	N	H	OAc	–	≥500	>100	125	≥500	≥500	≥500
9	N	N	OH	OH	5–Cl	≥500	**50**	**31.25**	**31.25**	≥500	≥500
9Ac	N	N	OAc	OAc	5–Cl	125	100	**31.25**	**62.5**	125	125
10	N	N	OH	OH	6–Cl	125	**50**	**62.5**	125	125	≥500
10Ac	N	N	OAc	OAc	6–Cl	125	**50**	**15.625**	**62.5**	125	250
11	N	N	OH	OH	3–Cl	250	>100	250	≥500	250	500
11Ac	N	N	OAc	OAc	3–Cl	125	>100	125	250	250	250
12	N	N	OH	OH	–	≥500	>100	≥500	≥500	250	≥500
12Ac	N	N	OAc	OAc	–	NA	NA	NA	NA	NA	NA
13	N	CH	OH	H	6–Cl	125	**50**	**7.81**	**31.25**	≥500	≥500
13Ac	N	CH	OAc	H	6–Cl	**15.625**	**50**	**7.81**	**15.625**	**31.25**	**31.25**
14	CH	N	OH	H	6–Cl	**31.25**	**50**	**15.625**	**15.625**	**31.25**	≥500
14Ac	CH	N	OAc	H	6–Cl	**15.625**	**50**	**7.81**	**31.25**	**31.25**	62.5
15	N	CH	OH	H	5–Cl	≥500	>100	**7.81**	≥500	≥500	≥500
15Ac	N	CH	OAc	H	5–Cl	250	>100	**7.81**	**7.81**	250	≥500
16	CH	N	OH	H	5–Cl	**31.25**	**12.5**	**15.625**	**31.25**	**31.25**	**62.5**
16Ac	CH	N	OAc	H	5–Cl	**31.25**	**50**	**15.625**	**31.25**	**62.5**	**62.5**
17	N	CH	OH	H	3–Cl	**31.25**	>100	**7.81**	**31.25**	**62.5**	250
17Ac	N	CH	OAc	H	3–Cl	125	**25**	**62.5**	**31.25**	**62.5**	250
18	CH	N	OH	H	3–Cl	**62.5**	**12.5**	**15.625**	**31.25**	125	≥500
18Ac	CH	N	OAc	H	3–Cl	**31.25**	**12.5**	**15.625**	**31.25**	**62.5**	**62.5**
19	N	CH	H	OH	6–Cl	≥500	>100	**3.91**	≥500	≥500	≥500
19Ac	N	CH	H	OAc	6–Cl	≥500	>100	**7.81**	≥500	≥500	≥500
20	CH	N	H	OH	6–Cl	≥500	**50**	**31.25**	≥500	125	**62.5**
20Ac	CH	N	H	OAc	6–Cl	125	**50**	**31.25**	125	125	250
21	N	CH	H	OH	5–Cl	**62.5**	**50**	**3.91**	**31.25**	250	250

Bold text indicates significant results. Results are considered significant, if the MIC is ≤ 62.5 μg/ml.
†Standard drug: isoniazid. Results for other standards can be found in Supplementary Table 1.
MIC: Minimum inhibitory concentration; NA: Not available.

Table 2. Antimycobacterial activity expressed as minimum inhibitory concentration in μg/ml (cont.).



						Strain					
Code	Y	Z	R^1	R^2	X	*Mycobacterium tuberculosis* H37Ra	*Mycobacterium tuberculosis* H37Rv	*Mycobacterium kansasii*	*Mycolicibacterium avium*	*Mycobacterium aurum*	*Mycobacterium smegmatis*
21Ac	N	CH	H	OAc	5–Cl	**62.5**	**50**	**7.81**	**62.5**	125	**31.25**
22	CH	N	H	OH	5–Cl	**62.5**	**50**	**15.625**	125	125	125
22Ac	CH	N	H	OAc	5–Cl	**62.5**	100	**15.625**	125	125	125
23	N	CH	H	OH	3–Cl	≥500	>100	250	≥500	500	≥500
23Ac	N	CH	H	OAc	3–Cl	500	>100	250	≥500	≥500	≥500
24	CH	N	H	OH	3–Cl	≥500	>100	125	125	≥500	≥500
24Ac	CH	N	H	OAc	3–Cl	≥500	>100	125	≥500	500	≥500
25	N	CH	OH	OH	6–Cl	≥500	>100	250	≥500	≥500	≥500
25Ac	N	CH	OAc	OAc	6–Cl	**62.5**	**50**	**15.625**	**62.5**	**62.5**	125
26	CH	N	OH	OH	6–Cl	≥500	**6.25**	500	≥500	≥500	≥500
26Ac	CH	N	OAc	OAc	6–Cl	250	**25**	**31.25**	250	**62.5**	≥500
27	N	CH	OH	OH	5–Cl	125	**25**	**3.91**	**31.25**	125	125
27Ac	N	CH	OAc	OAc	5–Cl	**62.5**	**50**	**3.91**	**62.5**	**62.5**	125
28	CH	N	OH	OH	5–Cl	≥500	>100	125	≥500	≥500	≥500
28Ac	CH	N	OAc	OAc	5–Cl	125	**25**	**31.25**	125	125	250
29	N	CH	OH	OH	3–Cl	125	**50**	**62.5**	125	250	≥500
29Ac	N	CH	OAc	OAc	3–Cl	125	**50**	**62.5**	125	250	≥500
30	CH	N	OH	OH	3–Cl	≥500	>100	≤≥500	≥500	≥500	≥500
30Ac	CH	N	OAc	OAc	3–Cl	125	>100	**62.5**	125	125	250
Isoniazid†	–	–	–	–	–	0.5	0.2	6.25	1000	3.91	31.25

Bold text indicates significant results. Results are considered significant, if the MIC is ≤ 62.5 μg/ml.
†Standard drug: isoniazid. Results for other standards can be found in Supplementary Table 1.
MIC: Minimum inhibitory concentration; NA: Not available.

altering the substitution of the chlorine to the *meta* position (compound **6** and **6Ac**) resulted in the compounds with no antimycobacterial activity. The loss of the activity of **6** and **6Ac** in comparison with their positional isomers (regarding chlorine) could be explained by decreased solubility in the water-based testing medium. Comparing compound **1** with its prodrug **A1** we might conclude that acetylation in this case led to a broadening of the spectrum and a significant increase of inhibition against the tested set.

2,4-Dihydroxy substitution exerted decreased activity with a narrower spectrum of antimycobacterial activity. The prodrug formation resulted in derivatives with a similar or slightly increased activity with few exceptions (**9**, **9Ac**, **26**, **26Ac**, **27**, **27Ac**). In the group of 2,4-dihydroxy substituted *N*-pyridylbenzamides we might generally see a worse correlation between the activity against *Mtb* H37Ra and *Mtb* H37Rv.

Most of the prepared *N*-pyrazinylbenzamides and *N*-pyridylbenzamides also proved activity against the virulent strain *Mtb* H37Rv with MIC in the range 12.5–50 μg/ml (Table 2). A satisfactory correlation was observed between the inhibitory activity against *Mtb* H37Rv and *Mtb* H37Ra except for a few compounds (**1Ac**, **3**, **3Ac**, **5**, **9**, **17**, **17Ac**, **20**, **26**, **26Ac**, **27** and **28Ac**). The most active compound against *Mtb* H37Rv was **26** (chlorine in *ortho* position, Y = CH, Z = N, $R^{1,2}$ = OH) with MIC = 6.25 μg/ml, but it must be noted that this compound lacked activity against other mycobacterial strains from the tested set. Several other candidates achieved comparable MIC = 12.5 μg/ml against *Mtb* H37Rv; these are compounds **1**, **2**, **2Ac**, **5Ac**, **16**, **18** and **18Ac.**

Regarding the activity against nontuberculous mycobacteria, the 2-hydroxy substituted compounds and their prodrugs proved broad-spectrum activity and mostly preserved activity through the tested strains. The most susceptible strain was *M. kansasii*, which was inhibited by the majority of the compounds with MIC in the range

3.91–31.25 μg/ml (Table 2). Some of the *N*-pyridylbenzamides had a narrower spectrum of antimycobacterial activity and missed activity against one or more mycobacterial strains from the tested set.

Antifungal activity

As a complementary screening, the final compounds were evaluated on activity against a set of eight fungal strains. A microdilution method according to European Committee for Antimicrobial Susceptibility Testing was used [29–31]. MIC values were expressed in μM. The derivatives **2**, **6**, **6Ac**, **13**, **18**, **19**, **19Ac**, **20** and **21Ac** were tested up to the concentration of 125 μM (solubility issues), and the rest were tested up to the concentration of 500 μM. For the results of internal quality control, see Supplementary Table 3. For the results, methodology and the complete list of tested strains, see the Supplementary materials.

Most of the tested compounds did not exert any inhibitory activity at the tested concentrations against the included strains of fungi and yeasts (Supplementary Table 4). Only compound A1 with MIC = 15.625 μM against *Trichophyton interdigitale* (*TI*; after 72 h incubation) proved interesting inhibitory activity. Compounds **1Ac**, **2**, **2Ac**, **13**, **13Ac**, **14Ac**, **15Ac** and **16** proved moderate activity against *TI* with MIC in the range 31.25–62.5 μM. Besides *TI*, only sporadic and weak activity (125–500 μM) was determined against the rest of the strains from the tested panel.

Investigation of the antistaphylococcal mechanism of action

Since our hit-expansion study did not bring significantly better antibacterial compounds in comparison with our starting structures (**I** and **II**), we took the original hits (newly synthesized and retested, now denoted as compounds **1** and **5Ac**) and attempted to get an insight into the mechanism of their antistaphylococcal action. The studies were performed with the reference strain of MRSA CCM 4750 (ATCC 43300).

Macromolecular biosynthesis assay

The macromolecular biosynthesis assay was performed according to the procedure described in literature [39] with slight modifications. The potential mechanism of action was investigated by a method based on the incorporation of [^{3}H] radioactively labelled precursors of biomolecules. Four biosynthetic pathways were investigated using four precursors, namely *N*-acetylglucosamine for peptidoglycan synthesis (Figure 4A), uridine for RNA synthesis (Figure 4B), thymidine for DNA synthesis (Figure 4C) and Leu for protein synthesis (Figure 4D). Five standards with a known mechanism of action were included (vancomycin: peptidoglycan; rifampicin: RNA; ciprofloxacin: DNA; chloramphenicol: protein synthesis; CHX: positive control). The results were read on the basis of radioactivity measured as counts per min with liquid scintillation counter TRI-CARB 2900TR (Perkin Elmer) and compared with untreated bacterial suspensions.

All standards proved their mechanism of action except for chloramphenicol, which did not achieve the expected decrease in protein synthesis. For compound **1**, we observed a decrease in the synthesis of peptidoglycan and RNA, but the effect did not reach statistical significance. On the contrary, the statistical significance criterion was met for the proteosynthesis inhibition effect of compound **1**. Compound **5Ac** exerted statistically insignificant effects on the examined biosynthetic pathways, although there was a mild effect on protein biosynthesis. It can be concluded that compound **1** can act as a compound affecting dominantly protein biosynthesis while compound **5Ac** acts probably nonspecifically with a slight affection to protein biosynthesis.

Membrane depolarization assay

The cytoplasmic membrane depolarization after exposure to tested compounds was monitored by a fluorometric measurement of membrane potential using reference strain, MRSA (ATCC 43300), voltage-sensitive dye, $DiSC_3(5)$ and CHX, as the positive control. The final concentration of $DiSC_3(5)$ corresponded to 0.5 μM. After 15 min of measurement (black arrow), compound **1** and **5Ac**, and positive control represented by CHX, in final concentrations corresponding to 4 × MIC, were added. As shown in Figure 5, a relatively high degree of membrane depolarization was registered after exposure of staphylococcal bacteria to both compounds **1** and **5Ac**. The increase of membrane depolarization, 2 min after exposure, corresponded to 36.54% for compound **1** and 42.16% for compound **5Ac**, compared with the positive control CHX (data normalized to the negative control).

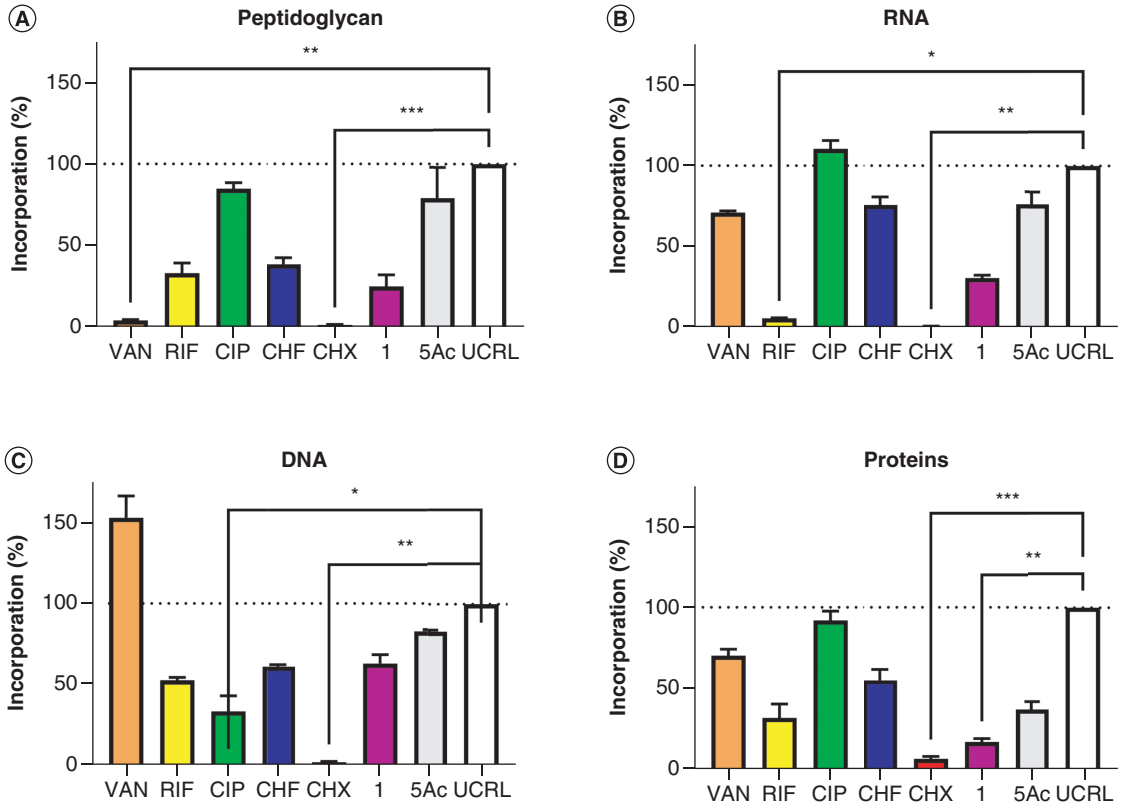


Figure 4. Results of macromolecular assay. Inhibition of biosynthetic pathway is indicated by lower incorporation of radioactively labelled precursors **(A)** [^{3}H] N-acetylglucosamine, **(B)** [^{3}H] uridine, **(C)** [^{3}H] thymidine and **(D)** [^{3}H] Leu by methicillin-resistant *Staphylococcus aureus* (ATCC 43300) strain treated for 2 h at 4 × MIC of VAN, RIF, CIP, CHF, CHX, compound **1** or **5Ac** and UCRL. Results are expressed as the percentage of untreated controls. The values shown are means of two independent experiments prepared in duplicates ± SEM. Dotted lines represent 100% incorporation of labelled macromolecules (UCRL). Significant reduction in biosynthetic pathway compared with UCRL is indicated by p-value where $p < 0.05$ was accepted as statistically significant determined by nonparametric one-way ANOVA test (Kruskal–Wallis test).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.
ANOVA: Analysis of variance; ATCC: American Type Collection Cultures; CHF: Chloramphenicol; CHX: Chlorhexidine; CIP: Ciprofloxacin; RIF: Rifampicin; SEM: Standard error of the mean; UCRL: Untreated control; VAN: Vancomycin.

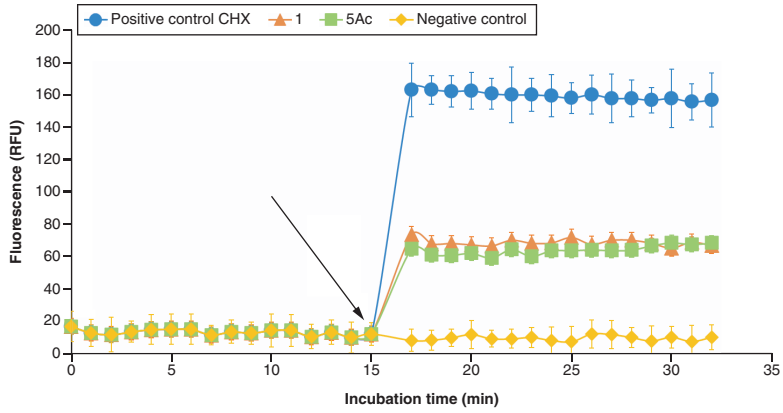


Figure 5. Membrane depolarization effect of compounds 1 and 5Ac on methicillin-resistant *Staphylococcus aureus*. Measured by $DiSC_3(5)$-based kinetic fluorescence. The graph depicts the mean and SD of six replicates. The black arrow indicates the time of addition of tested compounds or positive control.
CHX: Chlorhexidine; SD: Standard deviation; RFU: Relative fluorescence units.

Table 3. Comparison of experimentally determined solubility expressed as log S for chosen compounds.

Code	Solubility μg/ml		Method†	Experimental log S (mean ± SD)
	First run	Second run		
1	9.84	8.85	Kinetic	-4.43 ± 0.02
15	2.17	2.45	Kinetic	-5.03 ± 0.03
16	20.89	20.25	Kinetic	-4.08 ± 0.01
1Na	13581	–	Thermodynamic	-1.30

†For methodology, see Supplementary materials.

Determination of bactericidal versus bacteriostatic activity

Compounds **1** and **5Ac** were tested to distinguish between bactericidal and bacteriostatic activity on MRSA. The MIC of studied compounds was measured again to confirm previously determined values. The MIC against MRSA was 62.5 μM for both compounds after 24 h and 125 μM after 48 h. The values are in concordance with our previous measurement. Bacterial inoculum exposed to 4 × MIC of tested compounds was serially diluted, seeded and subcultured. After 24 of incubation, the number of CFU/ml was calculated. Minimum bactericidal concentration is defined as the lowest concentration of microbial agent that leads to a reduction of initial bacterial inoculum viability by ≥99.9% [40]. The antibacterial agent can be considered bactericidal if the minimum bactericidal concentration is lesser than or equal to 4 × MIC. The compound **5Ac** reduced CFU/ml by 87.84–90.91% and compound **1** by 90.91–93.33% compared with CFU/ml number of the initial bacterial inoculum. According to the abovementioned criterium, it can be concluded that both **1** and **5Ac** represent compounds with bacteriostatic action on MRSA.

Effect of isosteric exchange on water solubility

During our biological assays, we noticed that some final compounds were of decreased water solubility. The extent of the problem was not significant enough to negatively influence the determination of biological activities, with the exception of compounds **6** and **6Ac**, which could not be tested for antimycobacterial activity. One of the starting points of this study, compound **1**, two isosteric pyridines (compounds **15** and **16**) and sodium salt of compound **1** (**1Na**) were taken as model compounds to investigate how the isosteric exchange from pyrazine to pyridine will affect water solubility. The experimental solubility was performed according to the procedure described in literature [41] with slight modifications (for the method description, see the Supplementary materials) and used to calculate the experimental log S. The results are summarized in Table 3.

We focused on the correlation between experimental solubility and antimicrobial activity. Compound **1** was one of the most active compounds (MIC = 125 μM for MRSA and 12.5 μg/ml for *Mtb* H37Rv). One of its pyridine isosteres with N4 (compound **16**, Y= CH and Z= N) had around twice higher solubility in water. The compound proved comparable antimycobacterial activity (MIC = 12.5 μg/ml for *Mtb* H37Rv) and slightly worse antibacterial activity (MIC = 500 μM for MRSA). The other isosteric pyridine with N1 (compound **15**, Y=N and Z=CH) had almost four-times lower solubility than the title compound **1** and exerted no antimicrobial activity. Conversion of compound **1** to a salt (**1Na**: sodium salt of phenolate) significantly improved water solubility, but the antimicrobial activity remained the same (MIC = 125 μM to MRSA) as for the parent compound. We suggest that the compounds require log S > -4.5 to exert antimicrobial activity in our assay setup.

In silico prediction of 3D structure

Both for antibacterial and antibacterial activity, we observed that compounds with chlorine in *ortho* position to the linker were of significantly decreased or no activity compared with their *meta* or *para* isomers. Applying textbook rules, we expected that the presence of a sterically demanding chlorine atom on the neighboring position to the linker might force the noncoplanar conformation of the molecule. To confirm this presumption, we selected three positional pyrazine isomers (**1**, **2**, **3**) as model compounds and predicted their energetically most favorable conformation of their most abundant protomer (unionized phenolic group) on the B3LYP/cc-pVTZ level of theory. The conformation was evaluated by measuring the angle θ between the planes of the pyrazine and the benzene ring. As expected, the molecule of *para* (**1**) and *meta* (**2**) isomers were almost coplanar with θ <8°, whereas the *ortho* (**3**) isomer was in noncoplanar conformation with θ = 63°. Energetically minimized structures of positional isomers

can be seen in Supplementary Figure 1. We hypothesize that the distorted conformation of the *ortho*-chlorine derivatives of this paper might be unfavorable for binding to a hypothetic receptor.

Conclusion

In this hit-expansion study, we prepared 59 *N*-pyrazinyl- and *N*-pyridylhydroxybenzamides. For 54 compounds, this study is the first time to report an evaluation of antimicrobial activity. Best antibacterial activity was observed among *N*-pyrazinylbenzamides with high selectivity to *Staphylococcus* species. The isosteric exchange led to compounds with comparable activity (3-aminopyridine derivatives) or decreased activity (2-aminopyridine derivatives). Chloro substitution of the pyrazine or pyridine ring to *meta* or *para* position relative to the linker was preferred over *ortho* substitution. The inactivity of *ortho*-chlorine substituted derivatives was rationalized by *in silico* prediction of energetically most favorable conformations. The derivatives with 2-hydroxy substitution on the benzene ring exerted better antimicrobial activity and a broader spectrum compared with 4-hydroxy- or 2,4-dihydroxy. Most compounds exerted significant antimycobacterial activity with the best MIC value of 6.25 μg/ml against *Mtb* H37Rv. The spectrum of antimycobacterial activity included all tested strains of *Mycobacteria.* We revealed statistically significant inhibition of proteosynthesis in MRSA by compound **1**. Both compounds **1** and **5Ac** caused significant membrane depolarization of MRSA, but acted as bacteriostatic agents. Despite their depolarization effect, both compounds **1** and **5Ac** proved to be nontoxic on the human carcinoma (HepG2) cell line (IC_{50} = 155.30 μM for compound **1** and $IC_{50} \geq 500$ μM for compound **5Ac**) [18].

The water solubility of related positional isomers/isosteres differed up to the factor of ten, with derivatives of 3-aminopyridine being significantly more soluble than 2-aminopyridines and somewhat more soluble than derivatives of aminopyrazine. This might be a decisive point in frequently observed cases where the biological activities were similar among the pyrazine/pyridine isosteres.

Future perspective

Our results are a good starting point for further development toward new antimicrobial compounds, effective especially against drug-resistant staphylococci and mycobacteria. The next step would be tuning the potential of the future derivatives between relatively nonspecific membrane depolarization effect and specific inhibition of proteosynthesis. This would require determining the specific molecular mechanism by which proteosynthesis is affected. We believe that the nonspecific component of the antimicrobial effect can be convenient for antimicrobial treatment because it should decrease the probability of developing resistance to the compounds.

Summary points

- 59 compounds were synthesized and screened for antimicrobial activity to obtain comprehensive structure–activity relationships.
- The antibacterial activity was observed mainly among *N*-pyrazinylhydroxybenzamides with high selectivity to *Staphylococcus* species. The majority of compounds also exerted broad-spectrum antimycobacterial activity.
- The antistaphylococcal action of **1** and **5Ac** is based on a combination of inhibition of proteosynthesis and the nonspecific membrane depolarization effect.
- The compounds retained their activity against methicillin-resistant *Staphylococcus aureus*.
- Water solubility was improved in pyridine isosteres compared with original pyrazine derivatives.
- Promising perspective dwells in the potential of balancing between relatively nonspecific membrane depolarization effect and specific inhibition of proteosynthesis.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/fmc-2023-0189

Author contributions

M Kerda, P Šlechta and J Zitko designed the study. M Kerda and P Šlechta synthesized and purified the title compounds. O Janďourek, K Konečná and P Paterová performed the biological assays. P Hatoková performed solubility measurement. J Zitko performed *in silico* calculations. M Kerda, P Šlechta and J Zitko wrote the paper. All the authors have read and agreed to the published version of the manuscript.

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