httpss://doi.org/10.2478/acph-2024-0024

Original research paper

Synthesis and biochemical evaluation of new 3-amido-4-substituted monocyclic ß-lactams as inhibitors of penicillin-binding protein(s)

KATARINA GRABRIJAN[1]
NIKA STRAŠEK BENEDIK[1]
ALEN KRAJNC[1]
KRIŠTOF BOZOVIČAR[2]
DAMIJAN KNEZ[1]
MATIC PROJ[1]
IRENA ZDOVC[3]
IZIDOR SOSIČ[1]
CARLOS CONTRERAS-MARTEL[4]
ANDRÉA DESSEN[4]
MARTINA HRAST RAMBAHER[1]
STANISLAV GOBEC[1,*]

[1] *The Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana, Slovenia*

[2] *The Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Ljubljana, 1000 Ljubljana, Slovenia*

[3] *Institu te of Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia*

[4] *University Grenoble Alpes, CNRS, CEA, Institut de Biologie Structurale (IBS), Bacterial Pathogenesis Group, F-38044 Grenoble, France*

*****Correspondence: stanislav.gobec@ffa.uni-lj.si

ABSTRACT

In the final phases of bacterial cell wall synthesis, penicillin-binding proteins (PBPs) catalyze the cross-linking of peptidoglycan. For many decades, effective and non-toxic β-lactam antibiotics have been successfully used as mimetics of the D-Ala-D-Ala moiety of the natural substrate and employed as irreversible inhibitors of PBPs. In the years following their discovery, the emergence of resistant bacteria led to a decline in their clinical efficacy. Using Staudinger cycloaddition, we synthesized a focused library of novel monocyclic β-lactams in which different substituents were introduced at the C4 position of the β-lactam ring, at the C3 amino position, and at the N1 lactam nitrogen. In biochemical assays, the compounds were evaluated for their inhibitory effect on the model enzyme PBP1b from *Streptococcus pneumoniae*. Upon investigation of the antibacterial activity of the newly prepared compounds against ESKAPE pathogens, some compounds showed moderate inhibition. We also examined their reactivity and selectivity in a biochemical assay with other enzymes that have a catalytic serine in the active site, such as human cholinesterases, where they also showed no inhibitory activity, highlighting their specificity for bacterial targets. These compounds form the basis for further work on new monocyclic β-lactams with improved antibacterial activity.

Keywords: antibacterial agents, monocyclic-β-lactams, penicillin-binding proteins, covalent inhibitors, transpeptidase

Accepted June 3, 2024
Published online June 4, 2024

INTRODUCTION

Antimicrobial resistance is emerging as a major threat to global health (1). The World Health Organization (WHO) has published a list of bacteria for which new antibiotics are urgently needed. Of particular significance are ESKAPE pathogens, which are often multi-drug resistant: *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., *Enterococcus faecium* and *Staphylococcus aureus* (2). Most of the clinically

used antibiotics target the biosynthesis of peptidoglycan, the main component of the bacterial cell wall, which is present in both Gram-positive and Gram-negative bacteria (3). The bacterial cell wall gives bacteria their shape and rigidity, maintains proper osmotic pressure, and allows them to survive in a hypotonic environment by protecting them from lysis (4). Bacterial cell wall biosynthesis occurs in three phases, taking place in the cytoplasm, the inner membrane, and finally in the periplasm. Several successive steps in the cytoplasm lead to the formation of the monomeric building block precursor UDP-*N*-acetylmuramic acid pentapeptide, which, upon association with *N*-acetylglucosamine, leads to the generation of lipid II, the main peptidoglycan building block. After transport across the membrane, lipid II is incorporated into the peptidoglycan through the action of penicillin-binding proteins (PBPs) and SEDS (shape, elongation, division, and sporulation family proteins (5). PBPs catalyze two sequential steps, namely, the polymerization of glycan strands (transglycosylation), and stem peptide cross-linking (transpeptidation), forming a three-dimensional network (Fig. 1) (6–10). Inhibition or deregulation of peptidoglycan biosynthesis often lead to impaired cell growth, shape defects, cell lysis and death.

Since 1941, when penicillin was first used in humans, β-lactam antibiotics have remained the largest and most important structural class of antibiotics worldwide (11–13). β-lactams (*i.e.*, penicillins, cephalosporins, carbapenems and monobactams), whose core structure mimics the terminal D-Ala-D-Ala unit of the native enzyme substrate, act as irreversible inhibitors of the cross-linking reactions between the nascent peptidoglycan chains during the final stages of peptidoglycan biosynthesis (14) *via* acylation of catalytic serine residues in the active sites of transpeptidase and carboxypeptidases, including PBPs. The hydroxyl group of a serine residue reacts with the lactam carbonyl resulting in the opening of ring. The resulting acyl-enzyme complex is stable, and its hydrolysis proceeds very slowly (15, 16). Monocyclic β-lactams are four-membered cyclic amides with an oxo group at a second position of the ring and various substitutions at the amide nitrogen (N1), at the C3 carbon adjacent to the carbonyl group, and at the C4 carbon adjacent to the nitrogen (17). Based on the N1 substituents, they are divided into several classes (monobactams, monosulfactams, oxamazines, thiamazines, monocarbams and nocardins), which also determine their chemical reactivity (18). The first monocyclic β-lactam, nocardicin A, was discovered in 1976 in the bacterium *Nocardia uniformis*. Subsequently, sulfazecin and isosulfazecin were isolated from *Pseudomonas* strains (19). The first synthetic monocyclic β-lactam with antibacterial activity in clinical use was aztreonam (Fig. 2) (20). Recently, ancremonam (Fig. 2), which has shown potent activity against

Enterobacteriaceae and is also stable against serine β-lactamases, has completed the second phase of clinical trials (21–23). The second promising new monocyclic β-lactam is AIC499 developed by AiCuris (Fig. 2). Monocyclic β-lactams mainly target PBP3 and have a limited spectrum of activity against Gram-negative bacteria, including *Pseudomonas aeruginosa*, and good stability against β-lactamases. Particularly important is their stability against metalo-β-lactamases (24–29). Although β-lactams are relatively non-toxic, as already shown by Fleming, there is the possibility of a rare idiosyncratic immune reaction to penicillins; however, there is no cross-reactivity to aztreonam (30).

Amongst several bacterial resistance mechanisms, such as, *e.g.*, PBP mutations or the expression or alterations of porins, the production of β-lactam-hydrolyzing enzymes (*i.e.*, β-lactamases) remains the clinically most important mode of resistance to this structural class (31–34). Pharmaceutical industry research in this area is inadequate because new antibacterial agents are likely to be classified as "reserve" antibiotics in the WHO's AWaRe classification (35), making them even less economically viable; solutions are being sought in public-private partnerships and government programs such as IMI, CARB-X and the AMR Action Fund – (36, 37)

Monocyclic β-lactams, which can be assembled synthetically, are active against some ESKAPE pathogens, and currently appear to resist metallo-β-lactamase-mediated hydrolysis (34). Herein, we present the synthesis and biological evaluation of a focused library of novel 3-amido-4-substituted monocyclic β-lactams. Analogues bearing various aliphatic, aromatic, and heteroaromatic substituents at the C4 position, and amides with various carboxylic acids from known antibiotics at the C3 amino group were prepared. We also incorporated various substitutions at N1 in the lactam ring that affect the reactivity of the ring. The compounds were evaluated for their inhibition of PBP1b from the human pathogen *S. pneumoniae* (38), and for their antibacterial activity against a panel of Gram-positive and Gram-negative bacteria. To further characterize the series, the compounds were evaluated for their aqueous stability, initial chemical reactivity with the cysteine surrogate, and activity against other serine and cysteine hydrolases.

EXPERIMENTAL

General chemistry

Chemicals and solvents were from commercial sources (Sigma Aldrich, USA; Acros Organics, Belgium; TCI, Japan; Fluorochem, UK and Apollo Scientific, UK) and were used as supplied. Dry tetrahydrofuran was prepared by distillation from a mixture of sodium and benzophenone.

Reactions were monitored by thin-layer chromatography (TLC) on 0.25-mm silica gel $60F_{254}$ plates (Merck KGaA, Germany). Flash column chromatography was performed on silica gel 60 (Merck KGaA, particle size 0.040–0.063 mm) using the indicated solvents in each individual synthetic step. Yields are given for purified products.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400 (USA) spectrometer at 295 K in commercially available deuterated solvents (as indicated) with TMS as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS. Coupling constants (*J*) are given in Hertz (Hz), and splitting patterns are given as follows: s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; m, multiplet. Mass spectra were recorded using an ADVION Expression CMSL mass spectrometer (Advion Inc., USA) with ESI ionization. High-resolution mass spectra were obtained with the Exactive™ Plus Orbitrap mass spectrometer with ESI ionization (Thermo Fisher Scientific Inc., USA).

Full experimental procedures (including analytical data) are available in the Supplementary materials.

Expression and purification of S. pneumoniae *PBP1b*

A vector expressing PBP1b from *S. pneumoniae* (pGEX-GST-PBP1b) was used to transform chemically competent *Escherichia coli* NiCo21(DE3) (New England Biolabs, USA), as previously described (39, 40). Cells were cultured at 37 °C and shaken at 250 rpm in LB broth containing 100 µg mL^{-1} ampicillin until an $OD_{600} \cong 1$ was achieved. Expression was induced by addition of 1 mmol L^{-1} IPTG and cultured at 16 °C for additional 20 hours. Cells were harvested by centrifugation (10 min, 3000×*g*, 4 °C), and cell pellets were stored at –80 °C until purification. Cell pellet was resuspended in buffer A (50 mmol L^{-1} Tris × HCl, 200 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} DTT, pH 8.0) and lysed on ice by sonication. Cell debris was removed by centrifugation for 30 min (16,000×*g*, 4 °C, repeated twice). The cleared lysate was loaded onto a two interconnected 1-mL GSTrap HP columns (Cytiva, USA), which were pre-equilibrated with buffer A. The column was washed with buffer A and the protein was eluted with buffer B (50 mmol L^{-1} Tris, 200 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA,

pH 8.0, 1 mmol L^{-1} DTT, 10 mmol L^{-1} reduced glutathione). Eluted PBP1b was transferred to buffer C (50 mmol L^{-1} HEPES, 100 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 10 % glycerol, pH 7.0) by buffer exchange. The protein was concentrated with a 50-kDa molecular mass cut-off filter (Amicon® Ultra-4 Centrifugal Filter Unit, Merck KGaA), aliquoted, frozen in liquid nitrogen, and stored at –80 °C. Protein purity was assessed using SDS-PAGE, and the concentration was determined fluorometrically using Invitrogen Qubit (Thermo Fisher).

PBP1b inhibition assay using Ellman reagent

Inhibition of *S. pneumoniae* PBP1b was measured spectrophotometrically by measuring the formation of 2-nitro-5-thiobenzoate anion (TNB^{2-}) during the reaction; residual activities were determined based on the ability of a potential inhibitor to prevent hydrolysis of the substrate analogue thioester 2-{[(benzoyl-D-alanyl)-thio]-acetic acid} as described previously (40). PBP1b (0.4 µmol L^{-1}) was incubated with the compound (final concentration 100 µmol L^{-1}) in 10 mmol L^{-1} sodium phosphate buffer (pH 7.0) in the presence of 100 mmol L^{-1} D-alanine, 0.01 mg mL^{-1} BSA, and 0.01 % Triton X-100 for 60 minutes at 25 °C. After preincubation, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman reagent) and thioester were added to initiate the reaction and reach a final concentration of 1 mmol L^{-1} and 5 mmol L^{-1}, resp. The final volume of the reaction mixture was 150 µL. Triton X-100 was added to minimize the detection of false positives (promiscuous inhibitors). The initial rate of thioester hydrolysis was determined by measuring absorbance at 412 nm for 30 min using a 96-well microtiter plate using BioTek Synergy H4 Hybrid microplate reader (BioTek Instruments, USA). The same assay was performed in the absence of the inhibitor (1 %, *V/V*, DMSO).

Aztreonam, which completely inhibits PBP1b [RA (at 500 µmol L^{-1}) = 1.4 ± 0.1 %; IC_{50} (60 min pre-incubation) = 1.2 ± 0.1 µmol L^{-1}], was used as a positive control. All experiments were performed in triplicate. The ratio of the reaction rate with inhibitor to the reaction rate without it, expressed as a percentage, gives the residual activity {RA (%) = $[(v_i - b)/(v_o - b)] \times 100$}, where b is the blank value for the initial rate of spontaneous hydrolysis of the thioester in the presence of the inhibitor and in the absence of PBP1b. IC_{50} values were determined by measuring the reaction rates at seven different inhibitor concentrations using a non-linear regression (four-parameter model) applied in GraphPad Prism 9.0.2 (GraphPad Inc, USA).

PBP1b inhibition assay with BOCILLIN FL

A complementary assay to measure inhibition of *S. pneumoniae* PBP1b with BOCILLIN FL was also used (41). Fluorescence anisotropy was measured using 60 nmol L^{-1} purified

PBP1b, 30 nmol L^{-1} BOCILLIN FL in 100 mmol L^{-1} sodium phosphate buffer pH 7.0 containing 0.01 % Triton X-100 to reduce promiscuous inhibitor detection and protein binding to the plate. The assay was performed in triplicate in a volume of 50 µL in black flat-bottom, 384-well microplates at 30 °C. The change in fluorescence anisotropy was measured using a Biotek Synergy H4 Hybrid microplate reader with polarizing filters at excitation λ = 482 nm and emission λ = 530 nm and calculated using the following equation: fluorescence anisotropy (FA) = $(F_{para} - F_{perp})/(F_{para} + 2F_{perp})$, where F_{para} is the fluorescence intensity parallel to the excitation plane and F_{perp} is the fluorescence intensity perpendicular to the excitation plane. Residual activities were determined by preincubating the test compound (100 µmol L^{-1}) and the protein for 1 h at 30 °C before initiating the reaction by adding BOCILLIN FL. To determine the residual activity, the change in FA after 30 minutes was compared to the uninhibited (1 %, *V/V*, DMSO) control.

Inhibition of cholinesterases

The inhibitory potency of the compounds toward hBChE and hAChE was determined by the Ellman method according to the procedure described previously (42). Briefly, stock solutions of the compounds in DMSO containing DTNB and ChEs (final concentrations: 370 µmol L^{-1} DTNB, 1 nmol L^{-1} or 50 pmol L^{-1} recombinant hBChE, or recombinant hAChE, resp.) were incubated in 0.1 mol L^{-1} sodium phosphate pH 8.0 for 60 min at 20 °C. Reactions were started by adding the substrate (final concentration equal to 500 µmol L^{-1} butyrylthiocholine iodide or acetylthiocholine iodide for hBChE and hAChE, resp.). The final DMSO concentration was always 1 % (*V/V*). The increase in absorbance at 412 nm was monitored for 2 minutes using a 96-well microplate reader (BioTek Synergy H4, BioTek). The initial velocities in the presence (v_i) and absence (v_o) of the test compounds were calculated. The inhibitory effect was expressed as residual activity, corresponding to RA (%) = $(v_i / v_o) \times 100$.

Inhibition of 3CL^{pro}

The enzymatic activity of 3CL^{pro} was measured by a kinetic assay using the fluorogenic FRET substrate DABCYL-KTSAVLQSGFRKME-EDANS (CPC Scientific, USA). Experiments were performed in assay buffer containing 50 mmol L^{-1} Tris-HCl pH 7.3, 1 mmol L^{-1} EDTA, 0.05 % Triton X-114. Briefly, compounds were pre-incubated at a concentration of 100 µmol L^{-1} with 3CL^{pro} for 30 min at 30 °C. The reaction was started by addition of substrate, and the increase in fluorescence intensity was measured using a Synergy H4 microplate reader (BioTek) at λ_{ex} = 360/40 nm and λ_{em} = 440/40 nm. The final

concentrations were as follows: compound, 100 µmol L^{-1}; substrate, 20 µmol L^{-1}; $3CL^{pro}$, 50 nmol L^{-1}; DMSO, 10 % (*V/V*). For the control experiments, the compound was replaced by DMSO. For the determination of "b" (blank), the enzyme was replaced by Tris-HCl buffer. The initial velocities (v) were calculated from the linear trends obtained, each measurement being performed in duplicate. The inhibitory potency was expressed as the residual activity RA = $(v_i - b)/(v_o - b)$, where v_i is the velocity in the presence of the test compound, and v_0 is the control velocity in the presence of DMSO. To check for spectral interference, absorbance at the excitation and emission wavelengths and autofluorescence were determined for the active compounds in buffer solution. Boceprevir and carmofur (at 100 µmol L^{-1} concentration in the assay) were used as positive controls with a residual activity of 4.8 ± 0.5 and 34.8 ± 2.8 %, resp.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (*MIC*s) were determined by the broth microdilution method in 96-well U plates according to CLSI guidelines and European Committee on Antimicrobial Susceptibility Testing recommendations (44, 45). Suspensions of specific bacterial strains (*S. aureus* ATCC 29213, *E. coli* ATCC 25922, *K. pneumoniae RDK 070A* (ATCC 51503), *P. aeruginosa* RDK 184 (ATCC 15442), *E. faecalis* ATCC 29212, *E. coli* N43 (CGSC no. 5583) and *E. coli* D22) (CGSC no. 5163) corresponding to the 0.5-McFarland turbidity standard were diluted with cation-adjusted Mueller-Hinton broth with TES to yield an end inoculum of 5×10^5 CFU mL^{-1} for the assay. The compounds, dissolved in DMSO, and the bacterial inoculum were mixed and incubated at 35 °C for 18 – 24 hours. The *MIC* values were determined by visual inspection as the lowest dilution of the compounds that did not exhibit turbidity. Tetracycline was used as a positive control on each test plate. All experiments were performed in duplicate.

Thiol reactivity assay

The thiol reactivity of the compounds with DTNB was determined according to a previously published protocol (46, 47). TNB^{2-} anion was prepared *in situ* from DTNB and tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The reaction was performed in a 96-well microplate at 37 °C with a final volume of 300 µL buffer (20 mmol L^{-1} sodium phosphate buffer, 150 mmol L^{-1} NaCl, pH 7.4) containing 100 µmol L^{-1} compound, 100 µmol L^{-1} TCEP, 25 µmol L^{-1} DTNB, and 1 % (*V/V*) DMSO. The plate was incubated at 37 °C in a plate reader (Synergy H4, BioTek), and absorbance was recorded at 412 nm every 5 minutes for 12 hours.

Each compound was measured in duplicate, and a parallel experiment was performed without DTNB to determine the background absorbance of the compound, which was then subtracted from each measurement. The second-order rate constant k was calculated using the equation: $ln\frac{[A][B_0]}{[B][A_0]} = k([A_0] - [B_0])t$, where $[A_0]$ and $[B_0]$ are the initial concentrations of the compound and TNB^{2-}, resp., and [A] and [B] represent the remaining concentrations of TNB^{2-} and the compound as a function of time. Iodoacetamide was used as a positive control.

Buffer stability assay

The stability of the compounds in phosphate buffer was measured at different pH values (at pH 7.0 and pH 8.0) as described previously (56). Stock solutions of the investigated compounds were prepared in DMSO. The final concentration of each studied compound in 10 mmol L^{-1} phosphate buffer was 50 µmmol L^{-1}, with 5 % (*V/V*) DMSO. The reaction was carried out in a 96-well microplate. The plate was incubated at 37 °C in a plate reader (Synergy H4, BioTek) for 120–240 min. Absorbance values were measured in sweep mode after 0, 15, 30, 60, 120, 180 and 240 min using a discontinuous kinetic procedure in Gen5 software (BioTek). The time required to read the entire 96-well plate was 3 min. To determine the baseline, the compound solution was replaced with pure DMSO and subtracted from each reading. The relative absorbance difference between the first time point and 240 min at the most responsive wavelength was calculated. If the relative absorbance difference for the compound in the buffer was below 0.1, between 0.1–0.2, and above 0.2, the compound was classified as stable, intermediate and unstable, resp.

RESULTS AND DISCUSSION

Chemical synthesis

In a previous publication, we described our initial development and optimization efforts to prepare a range of desired 3-amino-4-substituted monocyclic ß-lactam intermediates using Staudinger cycloaddition (48). In our quest to obtain biologically active compounds, further derivatization at the C3 position and the activation of the N1 position were envisaged. Primarily, *N*-sulfonation (18) was used as the most common activation method to obtain N1 activated monocyclic β-lactam derivatives, as summarized in Scheme 1. Briefly, C-3 pthalimido N1–$SO_3^-TBA^+$ monocylic β-lactam analogues **1**–**8** (detailed structures shown in Supplementary Table SI) were prepared from corresponding 2,4-dimethoxybenzyl (DMB) N1 protected β-lactams by sulfonation with an excess of SO_3×DMF complex in anhydrous

dimethylformamide at room temperature (49). In some cases, the sulfonation reaction took several days, despite a large excess of reagent added. Once the reaction was complete as monitored by TLC analysis, the sulfonated intermediates were isolated in the form of tetrabutylammonium (TBA) salts. Note that in the case of free amino analogue **5**, an extra synthetic manipulation was necessary to obtain the compound (catalytic hydrogenation of nitro compound **4**; see supplementary information for more details). We then turned our attention to the preparation of novel monocyclic β-lactams with diverse substituents at the C4 position and different aminothiazolemethoxime (ATMO) side-chains. We have used two different approaches, starting from intermediates with either Boc or Fmoc protecting groups (Scheme 2). To obtain the intermediates with the desired aromatic 4-substitutions on the monocyclic β-lactam core, appropriate imines were first prepared by condensation of dimethoxybenzylamine with aromatic aldehydes in dichloromethane at room temperature, using an excess of anhydrous sodium sulphate as a drying agent (**9**–**12**). Subsequent Staudinger cycloaddition of imines with activated *N*-phthaloylglycine (*i.e.*, in its acyl chloride form) afforded monocylic β-lactams **13–16** which were easily isolated in high purity by precipitation or flash column chromatography. Since deprotection of the phthalimido (Phth) group generally requires relatively harsh conditions, we opted to remove it in the next step and replace it with carbamate protecting groups, which can be more easily removed. To this end, the Phth protecting group was cleaved with methylhydrazine and the resultant *C*3 free amine directly protected with *tert*-butyloxycarbonyl (Boc, compounds **17–18**) or fluorenylmethoxycarbonyl (Fmoc, compounds **19–20**) protecting group. The resultant monocyclic β-lactams were then subjected to the oxidative cleavage of the N1–DMB moiety with cerium ammonium nitrate under mild conditions, to afford the desired N1–H building blocks **21–24**. For the Boc-protected intermediates **21** and **22**, the Boc protecting group was removed in high yields by trifluoroacetic acid with triethylsilane used as a scavenging agent. With the free C3 aminoazetidin-2-ones in hand, we initially tried to couple the selected ATMO side-chains with the aid of the common amide coupling reagents, such as, *e.g.*, HATU or TBTU. Because these efforts were unproductive we turned our attention to the coupling reactions between *N*-hydroxysuccinimide (NHS) ester modified ATMO side-chains and amines under basic conditions.

Surprisingly, when we subjected crude amines **21** and **22** to conditions featuring an NHS-activated ATMOs in DMF at 70 °C, a diverse set of amide compounds **25**–**28** was obtained. Next, the treatment with an excess of SO_3-DMF complex furnished the desired *N*-sulfonated β-lactams **29** and **30** as TBA salts. In the case of the Fmoc-protected intermediates **23**–**24**, we

first formed N1–$SO_3^-TBA^+$ monocyclic β-lactams **31**–**32**, and subsequently removed the Fmoc group under basic conditions. Coupling of crude amines with NHS-activated ATMO side-chains yielded analogues **33–37**. Additionally, the less hydrophilic 2,6-dimethoxyphenylacetamido side-chain of methicillin was also incorporated in analogue **38**. For all TBA salts, the last step we envisaged was a cation exchange (to obtain the corresponding sodium salts) using Dowex® resin (50WX8, Na^+ form). Unfortunately, except for the methicillin analogue **38** and compound **39**, the conversions of N1–$SO_3^-TBA^+$ β-lactams to the corresponding Na^+ form were unsuccessful, as the compounds likely remained bound to the Dowex® cation exchange resin, and could not be successfully washed off. Hence, this step must be optimized in the future synthetic efforts. Note that under the conditions used the products of the Staudinger [2+2]-cycloaddition reaction were isolated as *cis*-isomers, since electron-withdrawing groups on the imine facilitate a direct ring closure (in turn, this also leads to better yields); the *cis*-configuration of the newly synthesized monocyclic β-lactams was deduced from the corresponding ^{1}H NMR coupling constants of the β-lactam ring hydrogens H3 and H4 (see sample spectra in supporting information); for *cis*-β-lactams $J_{3–4} \sim 5–6$ Hz and for *trans*-β-lactams $J_{3–4} \sim 2$ Hz, consistent with the literature data (15).

In the case of monocyclic β-lactams with aliphatic substituents at the C4 position (*i.e.*, Scheme 3), the synthesis was more challenging. Although we attempted to prepare several different analogues with aliphatic substituents (*e.g.*, cyclohexane, cyclopropyl, *iso*propyl …), only the C4 cyclohexane analogue **45** was successfuly synthesised. Whilst the synthetic conditions used were largely similar to the ones described earlier, we were careful to execute: (*i*) the imine condensation reactions on ice and use them directly without evaporation of the solvent (due to increased reactivity of aliphatic aldehydes), and (*ii*) the cycloaddition reaction at room temperature to afford a monocyclic β-lactam. Since the deprotection of the N1–DMB protecting group in analogues bearing aliphatic side-chains at C4-position were unproductive, we elected to utilise benzyl (Bn) group for N1 protection instead; the key N1-benzyl intermediate **42** was therefore synthesized in three steps starting from commercially available cyclohexanecarbaldehyde. Ammonia-free Birch reduction of **42** gave the desired intermediate **43** in excellent yield and with almost no by-products. Following the Boc cleavage, coupling with NHS-activated ATMO side-chain and subsequent N1-sulfonation of **44**, the *N*-sulfonated monocyclic β-lactam **45** was isolated as a TBA salt.

Biological and reactivity evaluation of monocyclic β-lactams

Compounds of interest were first tested on PBP1b in a biochemical assay on microtiter plates using a thioester analogue of the substrate (S2d) and detection with Ellman reagent at λ_{ABS} = 412 nm (40). The first series of N1-activated monobactams we tested were TBA salts of C3–phthalimido *N*-sulfonated β-lactams **1–8** (Supplementary material, Table SI). Expectedly, these compounds did not show any inhibition of PBP1b. Then, the second series featuring fully functionalised novel analogues (Table I) were evaluated in the same assay. Compound **38** with a 2,6-dimethoxybenzoic acid (*i.e.*, 'methicillin mimic') substitution at the C3 position was also not active. However, other monocyclic β-lactams (*e.g.*, **29**–**30**, **33**–**36** and **45**) with various ATMOs attached at the C3 position showed modest PBP1b inhibitory activity. Overall, the IC_{50} values measured after one hour of pre-incubation with the enzyme showed 5- to 15-fold less potential than positive control (*i.e.*, aztreonam, IC_{50} = 1.3 ± 0.4 μmol L^{-1}) for compounds **30, 34** and **45** (IC_{50}s = 6.4 μmol L^{-1}, 14.8 μmol L^{-1} and 18.7 μmol L^{-1}, resp.), while the IC_{50}s of the remaining analogues were about 60-fold higher. The less soluble cyano derivative **33** was also not very active at a concentration of 100 μmol L^{-1}, exhibiting only ~50 % resuidual activity in both PBP1b inhibition assays.

The thioester assay was developed for inhibitors that follow a two-step (reversible bonding - K_I, covalent bond formation - k_{inact}) covalent binding mechanism with turnover corresponding to the mechanism of β-lactam inhibition (3). To confirm the results of the first inhibition assay by measuring hydrolysis of the thioester substrate analogue S2d (which could be problematic for other mechanism models because detection is indirect by measuring TNB^{2-}), we decided to additionally perform a fluorescence anisotropy assay (FA) as a secondary biochemical evaluation model (51). Whilst we were only able to determine the residual activity (RA) of our monocyclic β-lactam compounds in the FA assay, the results generally showed the same trend as observed previously in the thioester assay.

The selectivity and/or reactivity of the synthesised monocyclic β-lactams was then evaluated on other in-house available enzymes (Supplementary Table SII) which employ catalytic serine, *i.e.*, human butyryl-/acetyl-cholinesterase (hBChE and hAChE, resp.) or cysteine residue, *i.e.*, SARS-CoV-2 main protease, *i.e.*, $3CL^{pro}$ (42). Suprisingly, ATMO-functionalised monocyclic β-lactams did not inhibit human serine enzymes, suggesting they are likely selective for their bacterial targets. Only some compounds (*e.g.*, **2**, **3**, **4** and **8**), all possesing a phthalimido moiety at C3 position, indicated some minimal inhibitory activity in these assays (note that C3 phthalimido intermediates do not inhibit PBP). Next, we wanted to confirm that the detected PBP1b inhibitors were not false positives. Based on our experiences

with detection of false positives due to reactivity with the thiol substrate (Ellman reagent), we tested the prepared compounds in the thiol reactivity assay and successfully confirmed that they are not non-specifically thiol reactive (46). Furthermore, we confirmed the stability of final compounds in the phosphate buffer at different pH or assay conditions (52, 53). For this purpose, we determined the stability in buffers at different pH values and found that the C3 phthalimido monobactams **1**–**8** were unstable even at neutral pH, while on the other hand, the fully funtionalised monocyclic β-lactams appeared stable in the phosphate buffer (see supplementary Table SIII).

Antibacterial activity

We evaluated the antibacterial activity of the monocyclic β-lactams on selected Gram-negative *Acinetobacter baumannii* 8C6 GES-14 (strain obtained from a European reference laboratory, EURL-AMR, DTU, Copenhagen, Denmark) as a reference strain for process control, *Klebsiella pneumoniae* RDK 070A (ATCC 51503), *Pseudomonas aeruginosa* RDK 184 (DSM 939; ATCC 15442)) and *Escherichia coli* (ATCC 25922), and Gram-positive *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* DRK 057 (ATCC 29212) bacteria. In addition to the potency of the inhibitors against bacterial enzymes, the efficacy in the whole cell assays depends primarily on the success of the uptake of the inhibitors by Gram-positive and Gram-negative bacteria. This depends on the physicochemical properties of the compounds and their ability to be substrates for efflux pumps. Therefore, in order to investigate their uptake profiles, we also determined the antibacterial activity of the compounds using two mutant strains of *E. coli* N43 (CGSC 5583) and *E. coli* D22 (CGSC no. 5163), the first lacking an AcrAB efflux pump in its outer cell membrane and the second having a mutation in the *lpxC* gene that increases membrane permeability. Reference drug was aztreonam.

The C3-phtalimido protected intermediates **1–8** were inactive, as expected. However, while C3-amido monocyclic β-lactams showed no significant antibacterial activity against Gram-positive wild-type bacteria, moderate antibacterial activity was observed against some Gram-negative wild-type bacteria (*i.e.*, *K. pneumoniae* and *E. coli*, *MIC*s ~ 32–64 μg mL^{-1}, see Table II). Morover, when the same set of compounds was evaluated against the two mutant strains, interesting results were obtained. While C3-phthalimido analogues **1–8** and the methicillin analogue **38** remained inactive, the N1-sulfonate activated analogues (*e.g.*, **29, 30** and **33**–**36**) exhibited moderate antibacterial activity particulary against the *E. coli* N43 mutant strain with a deleted efflux pump, with *MIC*s ranging from 1–32 μg mL^{-1} (indicating that these compounds could be AcrA membrane efflux pump substrates), and to a lesser extent in the

strain with a mutation in the *lpxC* gene of *E. coli* D22 (*MIC*s 4–32 μg mL^{-1}), compared to wild-type *E. coli* which was essentially not inhibited. Overall, in the case of the mutant strains, monocyclic β-lactam analogue **36** was particulary active (*MIC*s 1–4 μg mL^{-1}), while some other compounds (**29**, **33**, **34**, **45**) also exhibited notable antibacterial activity.

CONCLUSIONS

In this study, we successfully prepared a series of novel C3/C4 substituted *N*-sulfonated monocyclic β-lactams and evaluated them *in vitro*. Incorporation of C3 aminothiazole side-chains improved the activity of this structural class, as expected based on our previous studies. While none of the compounds were active against a representative Gram-positive strain (*S. aureus*), methoxime and the aminooxy-2-methylpropanoic acid derivatives **30** and **35** showed moderate activity against some Gram-negative bacteria (*K. pneumoniae* and *E. coli* strains). The lack of significant *in vitro* activity of newly developed compounds can likely be attributed to sterically bulkier and highly lipophilic substituents at the C4 position, if compared to aztreonam, which bacteria can expell using efflux pump activity (as proven in the case of mutant strains where our novel compounds exibited good *MIC* values). Overall, the results provide a clear scope for futher medicinal chemistry optimization of the C4-substituted monocyclic β-lactam class towards analogues which will not be hampered by the activity of efflux pumps, will be sufficently lipophilic to cross the bacterial outer membrane and will exert potent bactericidal effect on Gram-negative pathogens. Lastly, since β-lactamase-mediated hydrolysis is an important resistance mechanism for this structural class of antibiotics, we will also aim to incorporate β-lactamase testing into our future research efforts. In the follow-up of this work, we will therefore try not only to further optimize the antibacterial activity and cellular permeability of the best-performing compounds, but will also simultaneously assess their susceptibility towards clinically relevant β-lactamases to enable even more informative drug discovery and optimisation process.

Abbreviations, acronyms, symbols.- ATMO - aminothiazolemethoxime, CAN - cerium ammonium nitrate, Bn - benzyl, Boc - *tert*-butyloxycarbonyl, DMB - 2,4-dimethoxybenzyl, Fmoc - fluorenylmethoxycarbonyl, HATU - 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate, hAChE - human acetylcholinesterase, hBChE - human butyrylcholinesterase, LiHDMS - lithium hexadimethylsilazane, *MIC* - minimum inhibitory concentration, NHS - *N*-hydroxysuccinimide, PBP - penicillin-binding protein, Ptht - phtalimido, RA -

residual activity, TBA - tetrabutylammonium, TBTU - 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate

Acknowledgements. - This research was funded by the Slovenian Research and Innovation Agency (ARIS), Research Core Funding P1-0208, Project N1-0169, Project Z1-4405 and a young researcher grant to K. G. We thank David Roper laboratory (University of Warwick, UK) for providing *E. coli* N43 and *E. coli* D22 mutant strains.

Conflict of interest. - The authors declare no competing financial interest in connection with this manuscript.

Supplementary data. - In the supporting information, synthetic procedures and spectroscopic data of all compounds described in the manuscript are available. Additionally, selected biochemical and biophysical data are also available.

Author contributions. - Supervision, SG; conceptualization, KG, NSB and SG; synthesis, KG and NSB; biochemical and biophysical experiments, KG, DK, MHR, MP, KB, IS; CCM and AD provided plasmid and enzyme; data analysis, AK and KG; writing, original draft preparation, KG and AK; writing, review and editing, AK, MHR and SG. All authors have read and agreed to the published version of the manuscript.

*ORCID*s. - KATARINA GRABRIJAN (0000-0002-6478-7696), NIKA STRAŠEK BENEDIK, ALEN KRAJNC (ORCID: 0000-0001-7822-1944), KRIŠTOF BOZOVIČAR (0000-0003-0025-1734), DAMIJAN KNEZ (0000-0001-9917-1384), MATIC PROJ (0000-0003-4043-9686), IRENA ZDOVC (0000-0002-5419-2779), IZIDOR SOSIČ (0000-0002-3370-4587), CARLOS CONTRERAS-MARTEL (0000-0003-1151-5766), ANDRÉA DESSEN (0000-0001-6487-4020), MARTINA HRAST RAMBAHER (0000-0003-0488-2445), STANISLAV GOBEC (0000-0002-9678-3083)

REFERENCES

1. C. J. Murray, K. S. Ikuta, F. Sharara, L. Swetschinski, G. Robles Aguilar, A. Gray, C. Han, C. Bisignano, P. Rao, E. Wool, *et al.*, Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, *Lancet* **399**(10325) (2022) 629–655; https://doi.org/10.1016/S0140-6736(21)02724-0

2. World Health Organization, *2020 Antibacterial Agents in Clinical and Preclinical Development: An Overview and Analysis*, WHO, Geneva, April 15, 2021; https://www.who.int/publications-detail-redirect/9789240021303; last access May 25, 2022

3. A. Zervosen, E. Sauvage, J. M. Frère, P. Charlier and A. Luxen, Development of new drugs for an old target – the penicillin binding proteins, *Molecules* **17**(11) (2012) 12478–12505; https://doi.org/10.3390/molecules171112478

4. P. J. Matteï, D. Neves and A. Dessen, Bridging cell wall biosynthesis and bacterial morphogenesis, *Curr. Opin. Struct. Biol.* **20**(6) (2010) 749–755; https://doi.org/10.1016/j.sbi.2010.09.014

5. A. J. Meeske, E. P. Riley, W. P. Robins, T. Uehara, J. J. Mekalanos, D. Kahne, S. Walker, A. C. Kruse, T. G. Bernhardt and D. Z. Rudner, SEDS proteins are a widespread family of bacterial cell wall polymerases, *Nature* **537**(7622) (2016) 634–638; https://doi.org/10.1038/nature19331

6. J. F. Fisher, S. O. Meroueh and S. Mobashery, Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity, *Chem. Rev.* **105**(2) (2005) 395–424; https://doi.org/10.1021/cr030102i

7. E. Sauvage and M. Terrak, Glycosyltransferases and transpeptidases/penicillin-binding proteins: valuable targets for new antibacterials, *Antibiotics* **5**(1) (2016) Article ID 12 (27 pages); https://doi.org/10.3390/antibiotics5010012

8. E. Sauvage, F. Kerff, M. Terrak, J. A. Ayala and P. Charlier, The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis, *FEMS Microbiol. Rev.* **32**(2) (2008) 234–258; https://doi.org/10.1111/j.1574-6976.2008.00105.x

9. P. Macheboeuf, C. Contreras-Martel, V. Job, O. Dideberg and A. Dessen, Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes, *FEMS Microbiol. Rev.* **30**(5) (2006) 673–691; https://doi.org/10.1111/j.1574-6976.2006.00024.x

10. W. Vollmer, D. Blanot and M. A. de Pedro, Peptidoglycan structure and architecture, *FEMS Microbiol. Rev.* **32**(2) (2008) 149–167; https://doi.org/10.1111/j.1574-6976.2007.00094.x

11. A. Fleming, On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*, *Bull. World Health Organ*. **79**(8) (2001) 780–790.

12. E. P. Abraham, E. Chain, C. M. Fletcher, A. D. Gardner, N. G. Heatley, M. A. Jennings and H. W. Florey, Further observations on penicillin, *Lancet* **238**(6155) (1941) 177–189; https://doi.org/10.1016/S0140-6736(00)72122-2

13. World Health Organization, *WHO Report on Surveillance of Antibiotic Consumption*, WHO, Geneva, July 21, 2019; https://www.who.int/publications-detail-redirect/who-report-on-surveillance-of-antibiotic-consumption; last access June 4, 2022

14. G. Patrick, *An Introduction to Medicinal Chemistry*, Oxford University Press, 6th ed., Oxford 2017

15. L. M. Lima, B. N. M. da Silva, G. Barbosa and E. J. Barreiro, β-lactam antibiotics: an overview from a medicinal chemistry perspective, *Eur. J. Med. Chem.* **208** (2020) Article ID 112829 (21 pages); https://doi.org/10.1016/j.ejmech.2020.112829

16. A. Zapun, C. Contreras-Martel and T. Vernet, Penicillin-binding proteins and β-lactam resistance, *FEMS Microbiol. Rev.* **32**(2) (2008) 361–385; https://doi.org/10.1111/j.1574-6976.2007.00095.x

17. S. Deketelaere, T. Van Nguyen, C. V. Stevens and M. D'hooghe, Synthetic approaches toward monocyclic 3-amino-β-lactams, *ChemistryOpen* **6**(3) (2017) 301–319; https://doi.org/10.1002/open.201700051

18. L. Decuyper, M. Jukič, I. Sosič, A. Žula, M. D'hooghe and S. Gobec, Antibacterial and β-lactamase inhibitory activity of monocyclic β-lactams, *Med. Res. Rev.*, **38**(2) (2018) 426–503; https://doi.org/10.1002/med.21443

19. D. Braga and G. Lackner, One ring to fight them all: the sulfazecin story, *Cell Chem. Biol.* **24**(1) (2017) 1–2; https://doi.org/10.1016/j.chembiol.2017.01.001

20. R. B. Sykes and D. P. Bonner, Aztreonam: the first monobactam, *Am. J. Med.* **78**(2) (1985) 2–10; https://doi.org/10.1016/0002-9343(85)90196-2

21. F. Reck, A. Bermingham, J. Blais, V. Capka, T. Cariaga, A. Casarez, R. Colvin, C. R. Dean, A. Fekete, W. Gong, E. Growcott, H. Guo, A. K. Jones, C. Li, F. Li, X. Lin, M. Lindvall, S. Lopez, D. McKenney, L. Metzger, H. E. Moser, R. Prathapam, D. Rasper, P. Rudewicz, V. Sethuraman, X. Shen, J. Shaul, R. L. Simmons, K. Tashiro, D. Tang, M. Tjandra, N. Turner, T. Uehara, C. Vitt, S. Whitebread, A. Yifru, X. Zang and Q. Zhu, Optimization of novel monobactams with activity against carbapenem-resistant *Enterobacteriaceae* – identification of LYS228, *Bioorg. Med. Chem. Lett.* **28**(4) (2018) 748–755; https://doi.org/10.1016/j.bmcl.2018.01.006

22. Z. Fei, Q. Wu, W. Gong, P. Fu, C. Li, X. Wang, Y. Han, B. Li, L. Li, B. Wu, Y. Zhao, J. Li, W. Zhu, W. Qiu, J. Guo, J. Zhou, Y. Li, M. Villa and C. Ming Cheung, Process development for the synthesis of a monobactam antibiotic—LYS228, *Org. Process Res. Dev.* **24**(3) (2020) 363–370; https://doi.org/10.1021/acs.oprd.9b00330

23. J. Blais, S. Lopez, C. Li, A. Ruzin, S. Ranjitkar, C. R. Dean, J. A. Leeds, A. Casarez, R. L. Simmons and F. Reck, *In vitro* activity of LYS228, a novel monobactam antibiotic, against multidrug-resistant *Enterobacteriaceae*, *Antimicrob. Agents Chemother.* **62**(10) (2018) e00552-18 (10 pages); https://doi.org10.1128/AAC.00552-18

24. K. Grabrijan, N. Strašek and S. Gobec, Monocyclic beta–lactams for therapeutic uses: a patent overview (2010–2020), *Expert Opin. Ther. Pat.* **31**(3) (2020) 247–266; https://doi.org/10.1080/13543776.2021.1865919

25. L. Decuyper, S. Deketelaere, L. Vanparys, M. Jukič, I. Sosič, E. Sauvage, A. M. Amoroso, O. Verlaine, B. Joris, S. Gobec and M. D'hooghe, In silico design and enantioselective synthesis of functionalized monocyclic 3-amino-1-carboxymethyl-β-lactams as inhibitors of penicillin-binding proteins of resistant bacteria, *Chem. Eur. J.* **24**(57) (2018) 15254–15266; https://doi.org/10.1002/chem.201801868

26. M. F. Brown, M. J. Mitton-Fry, J. T. Arcari, R. Barham, J. Casavant, B. S. Gerstenberger, S. Han, J. R. Hardink, T. M. Harris, T. Hoang, M. D. Huband, M. S. Lall, M. M. Lemmon, C. Li, J. Lin, S. P. McCurdy, E. McElroy, C. McPherson, E. S. Marr, J. P. Mueller, L. Mullins, A. A. Nikitenko, M. C. Noe, J. Penzien, M. S. Plummer, B. P. Schuff, V. Shanmugasundaram, J. T. Starr, J. Sun, A. Tomaras, J. A. Young, R. P. Zaniewski, Pyridone-conjugated monobactam antibiotics with gram-negative activity, *J. Med. Chem.* **56**(13) (2013) 5541–5552; https://doi.org/10.1021/jm400560z

27. Z. W. Li, X. Lu, Y. X. Wang, X. X. Hu, H. G. Fu, L. M. Gao, X. F. You, S. Tang, D. Q. Song, Synthesis and antibacterial evaluation against resistant gram-negative bacteria of monobactams bearing various substituents on oxime residue, *Bioorg. Chem.* **94** (2020) Article ID 103487 (12 pages); https://doi.org/10.1016/j.bioorg.2019.103487

28. L. Tan, Y. Tao, T. Wang, F. Zou, S. Zhang, Q. Kou, A. Niu, Q. Chen, W. Chu, X. Chen, H. Wang and Y. Yang, Discovery of novel pyridone-conjugated monosulfactams as potent and broad-spectrum antibiotics for multidrug-resistant gram-negative infections, *J. Med. Chem.* **60**(7) (2017) 2669–2684; https://doi.org/10.1021/acs.jmedchem.6b01261

29. Q. Kou, T. Wang, F. Zou, S. Zhang, Q. Chen and Y. Yang, Design, synthesis and biological evaluation of C(4) substituted monobactams as antibacterial agents against multidrug-resistant gram-negative bacteria, *Eur. J. Med. Chem.* **151** (2018) 98–109; https://doi.org/10.1016/j.ejmech.2018.03.058

30. G. Patriarca, D. Schiavino, C. Lombardo, G. Altomonte, M. Decinti, A. Buonomo and E. Nucera, Tolerability of aztreonam in patients with IgE-mediated hypersensitivity to beta-lactams, *Int. J. Immunopathol. Pharmacol.* **21**(2) (2008) 375–379; https://doi.org/10.1177/039463200802100215

31. W. C. Reygaert, An overview of the antimicrobial resistance mechanisms of bacteria, *AIMS Microbiol.* **4**(3) (2018) 482–501; https://doi.org/10.3934/microbiol.2018.3.482

32. B. R. da Cunha, L. P. Fonseca and C. R. C. Calado, Antibiotic discovery: where have we come from, where do we go? *Antibiotics* **8**(2) (2019) Article ID 45 (21 pages); https://doi.org/10.3390/antibiotics8020045

33. R. Tommasi, D. G. Brown, G. K. Walkup, J. L. Manchester and A. A. Miller, ESKAPEing the labyrinth of antibacterial discovery, *Nat. Rev. Drug Discov.* **14** (2015) 529–542; https://doi.org/10.1038/nrd4572

34. M. Lakemeyer, W. Zhao, F. A. Mandl, P. Hammann and S. A. Sieber, Thinking outside the box—novel antibacterials to tackle the resistance crisis, *Angew. Chem., Int. Ed.* **57**(44) (2018) 14440–14475; https://doi.org/10.1002/anie.201804971

35. World Health Organization, *2021 AWaRe Classification*, WHO, Geneva, September 30, 2021; https://www.who.int/publications-detail-redirect/2021-aware-classification; last access October 22, 2022

36. M. S. Butler, V. Gigante, H. Sati, S. Paulin, L. Al-Sulaiman, J. H. Rex, P. Fernandes, C. A. Arias, M. Paul, G. E. Thwaites, L. Czaplewski, R. A. Alm, C. Lienhardt, M. Spigelman, L. L. Silver, N. Ohmagari, R. Kozlov, S. Harbarth and P. Beyer, Analysis of the clinical pipeline of treatments for drug-resistant bacterial infections: despite progress, more action is needed, *Antimicrob. Agents Chemother*. **66**(3) (2022) e01991-21 (20 pages); https://doi.org/10.1128/aac.01991-21

37. S. M. Bhavnani, K. M. Krause and P. G. Ambrose, A broken antibiotic market: review of strategies to incentivize drug development, *Open Forum Infect. Dis.* **7**(7) (2020) ofaa083 (6 pages); https://doi.org10.1093/ofid/ofaa083

38. P. Macheboeuf, A. M. Di Guilmi, V. Job, T. Vernet, O. Dideberg and A. Dessen, Active site restructuring regulates ligand recognition in class A penicillin-binding proteins, *Proc. Natl. Acad. Sci. USA* **102**(3) (2005) 577–582; https://doi.org/10.1073/pnas.0407186102

39. A. M. di Guilmi, A. Dessen, O. Dideberg and T. Vernet, Functional characterization of penicillin-binding protein 1b from *Streptococcus pneumoniae*, *J. Bacteriol.* **185**(5) (2003) 1650–1658; https://doi.org/10.1128/JB.185.5.1650-1658.2003

40. C. Contreras-Martel, A. Amoroso, E. C. Y. Woon, A. Zervosen, S. Inglis, A. Martins, O. Verlaine, A. M. Rydzik, V. Job, A. Luxen, B. Joris, C. J. Schofield and A. Dessen, Structure-guided design of cell wall biosynthesis inhibitors that overcome β-lactam resistance in *Staphylococcus aureus* (MRSA), *ACS Chem. Biol.* **6**(9) (2011) 943–951; https://doi.org/10.1021/cb2001846

41. H. Newman, A.Krajnc, D. Bellini, C. J. Eyermann, G. A. Boyle, N. G. Paterson, K. E. McAuley, R. Lesniak, M. Gangar, F. von Delft, J. Brem, K. Chibale, C. J. Schofield and C. G. Dowson, High-throughput crystallography reveals boron-containing inhibitors of a penicillin-binding protein with di- and tri-covalent binding modes, *J. Med. Chem.* **64**(15) (2021) 11379–11394; https://doi.org/10.1021/acs.jmedchem.1c00717

42. A. Meden, D. Knez, N. Malikowska-Racia, X. Brazzolotto, F. Nachon, J. Svete, K. Sałat, U. Grošelj and S. Gobec, Structure-activity relationship study of tryptophan-based butyrylcholinesterase inhibitors, *Eur. J. Med. Chem.* **208** (2020) Article ID 112766 (21 pages); https://doi.org/0.1016/j.ejmech.2020.112766

615 43. M. Proj, M. Hrast, D. Knez, K. Bozovičar, K. Grabrijan, A. Meden, S. Gobec and R. Frlan,
616 Fragment-sized thiazoles in fragment-based drug discovery campaigns: friend or foe?, *ACS Med.*
617 *Chem. Lett.* **13**(12) (2022) 1905–1910; https://doi.org10.1021/acsmedchemlett.2c00429

618 44. Clinical and Laboratory Standards Institute, *M07 - Methods for Dilution Antimicrobial*
619 *Susceptibility Tests for Bacteria That Grow Aerobi*cally *(11th ed*.), CLSI, Wayne (PA) USA, January
620 2018; https://clsi.org/media/1928/m07ed11_sample.pdf; last access March 17, 2022

621 45. European Committee on Antimicrobial Susceptibility Testing, *EUCAST: Clinical Breakpoints and*
622 *Dosing of Antibiotics*, EUCAST (v 12.0, January 2022);
623 https://www.eucast.org/clinical_breakpoints; last access March 17, 2022

624 46. M. Proj, D. Knez, I. Sosič and S. Gobec, Redox active or thiol reactive? Optimization of rapid
625 screens to identify less evident nuisance compounds, *Drug Discov. Today* **27**(6) (2022) 1733–1742;
626 https://doi.org/10.1016/j.drudis.2022.03.008

627 47. E. Resnick, A. Bradley, J. Gan, A. Douangamath, T. Krojer, R. Sethi, P. P. Geurink, A. Aimon, G.
628 Amitai, D. Bellini, J. Bennett, M. Fairhead, O. Fedorov, R. Gabizon, J. Gan, J. Guo, A. Plotnikov,
629 N. Reznik, G. F. Ruda, L. Díaz-Sáez, V. M. Straub, T. Szommer, S. Velupillai, D. Zaidman, Y.
630 Zhang, A. R. Coker, C. G. Dowson, H. M. Barr, C. Wang, K. V. M. Huber, P. E. Brennan, H. Ovaa,
631 F. von Delft and N. London, Rapid covalent-probe discovery by electrophile-fragment screening, *J.*
632 *Am. Chem. Soc.* **141**(22) (2019) 8951–8968; https://doi.org/10.1021/jacs.9b02822

633 48. K. Grabrijan, N. Strašek and S. Gobec, Synthesis of 3-amino-4-substituted monocyclic ß-lactams -
634 Important structural motifs in medicinal chemistry, *Int. J. Mol. Sci.* **23**(1) (2022) Article ID 360 (26
635 pages); https://doi.org/10.3390/ijms23010360

636 49. S. Carosso, R. Liu, P. A. Miller, S. J. Hecker, T. Glinka and M. J. Miller, Methodology for
637 monobactam diversification: Syntheses and studies of 4-thiomethyl substituted β-lactams with
638 activity against Gram-negative bacteria, including carbapenemase producing *Acinetobacter*
639 *baumannii*, *J. Med. Chem.* **60**(21) (2017) 8933–8944;
640 https://doi.org/10.1021/acs.jmedchem.7b01164

641 50. T. Lupia, C. Pallotto, S. Corcione, L. Boglione and F. G. De Rosa, Ceftobiprole perspective: Current
642 and potential future indications, *Antibiotics* **10**(2) (2021) Article ID 170 (11 pages);
643 https://doi.org/10.3390/antibiotics10020170

644 51. A. B. Shapiro, R. F. Gu, N. Gao, S. Livchak and J. Thresher, Continuous fluorescence anisotropy-
645 based assay of BOCILLIN FL penicillin reaction with penicillin binding protein 3, *Anal. Biochem.*
646 **439**(1) (2013) 37–43; https://doi.org/10.1016/j.ab.2013.04.009

52. J. S. Martin, C. J. MacKenzie, D. Fletcher and I. H. Gilbert, Characterising covalent warhead reactivity, *Bioorg. Med. Chem.* **27**(10) (2019) 2066–2074; https://doi.org/10.1016/j.bmc.2019.04.002

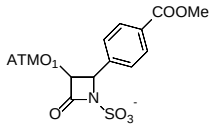
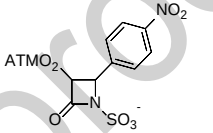
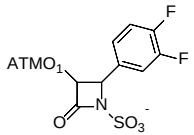
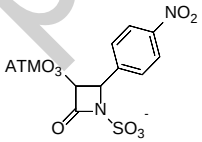
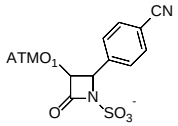
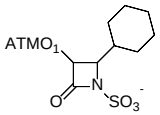
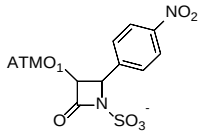
53. A. Urbach, G. Dive, B. Tinant, V. Duval and J. Marchand-Brynaert, Large ring 1,3-bridged 2-azetidinones: Experimental and theoretical studies, *Eur. J. Med. Chem.* **44**(5) (2009) 2071–2080; https://doi.org/10.1016/j.ejmech.2008.10.016

54. A. H. Delcour, Outer membrane permeability and antibiotic resistance, *Biochim. Biophys. Acta - Proteins Proteomics* **1794**(5) (2009) 808–816; https://doi.org/10.1016/j.bbapap.2008.11.005

55. H. Nikaido, Molecular basis of bacterial outer membrane permeability revisited, *Microbiol. Mol. Biol. Rev.* **67**(4) (2003) 593–656; https://doi.org/10.1128/MMBR.67.4.593-656.2003

56. M. Proj, N. Strašek, S. Pajk, D. Knez and I. Sosič, Tunable heteroaromatic nitriles for selective bioorthogonal click reaction with cysteine, *Bioconjugate Chem* **34**(7) (2023) 1271–1281, https://doi.org/10.1021/acs.bioconjchem.3c00163

663 *Table I. Residual activities of novel* cis-*C3/C4 functionalised* N-*sulfonated β-lactams isolated as TBA$^+$ salts*[a]

Compd. No.	Structure	PBP1b RA (%) or IC_{50} (μmol L^{-1})	PBP1b BOCILLIN FL RA (%)	Compd. No.	Structure	PBP1b RA (%) or IC_{50} (μmol L^{-1})	PBP1b BOCILLIN FL RA (%)
29		28.0 ± 1.7 μmol L^{-1}	32 ± 6	**35**		62.5 ± 0.1 μmol L^{-1}	26 ± 4
30		6.4 ± 1.9 μmol L^{-1}	0.5 ± 2	**36**		28.3 ± 0.5 μmol L^{-1}	23 ± 4
33		49.5 ± 3.8 %	46 ± 8	**45**		18.7 ± 0.4 μmol L^{-1}	0.5 ± 1
34		14.8 ± 0.2 μmol L^{-1}	15 ± 3		Aztreonam	1.3 ± 0.4 μmol L^{-1}	

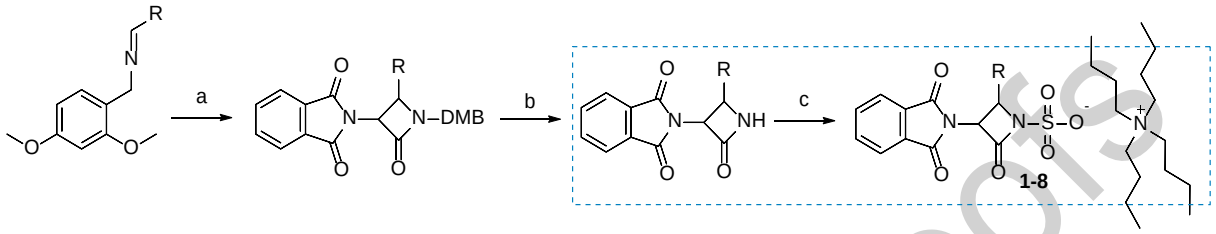
664 [a]Residual activities were determined at a concentration of 100 μmol L^{-1} of the tested compounds in the assay after 60 min of pre-incubation with the enzyme.
665 $ATMO_1$ - 2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetate, $ATMO_2$ - 2-(2-aminothiazol-4-yl)-2-(((1-(*tert*-butoxy)-2-methyl-1-oxopropan-2-yl)oxy)imino)acetate,
666 $ATMO_3$ - 2-(5-amino-1,2,4-thiadiazol-3-yl)-2-(ethoxyimino)acetate
667

Table II. Antibacterial activities of novel cis-*C3/C4 functionalised* N-*sulfonated β-lactams isolated as TBA*$^+$ *salts on selected wild-type and mutant Gram-positive and Gram-negative bacterial strains*

Compd. No.	*S. aureus*	*E. faecalis*	*A. baumannii*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*	*E. coli N43*[a]	*E. coli D22*[b]
	Gram positive					Gram negative		
29	>128	>128	>128	>128	>128	>128	8	16
30	>128	64	>128	64	>128	64	8	16
33	>128	>128	>128	>128	>128	>128	16	32
34	>128	>128	>128	>128	>128	>128	8	32
35	>128	>128	>128	32	>128	32	32	32
36	>128	>128	>128	>128	>128	>128	1	4
38	>128	/	/	/	/	>128	>128	>128
45	>128	>128	>128	>128	>128	>128	16	32
Aztreonam	>128	>128	16	<1	2	<1	<1	<1

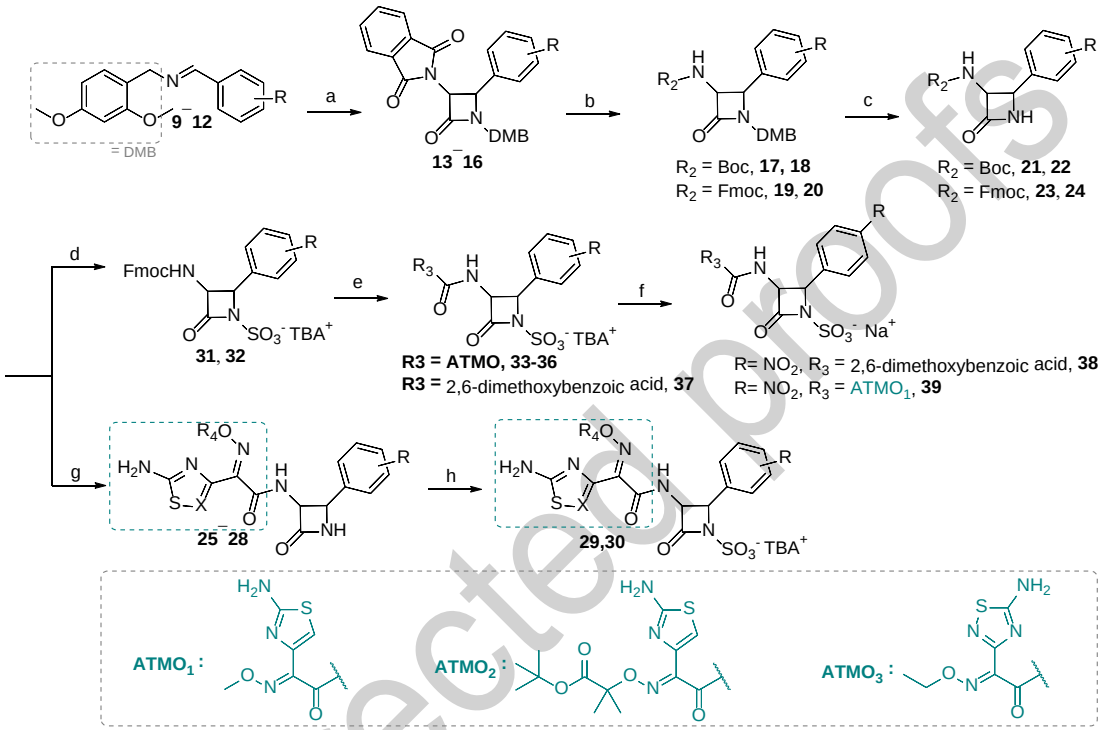
[a]Strain with an *acrA* knockout (cell membrane efflux pump).

[b]Strain with a mutation in the *lpxC* gene that increases membrane permeability.



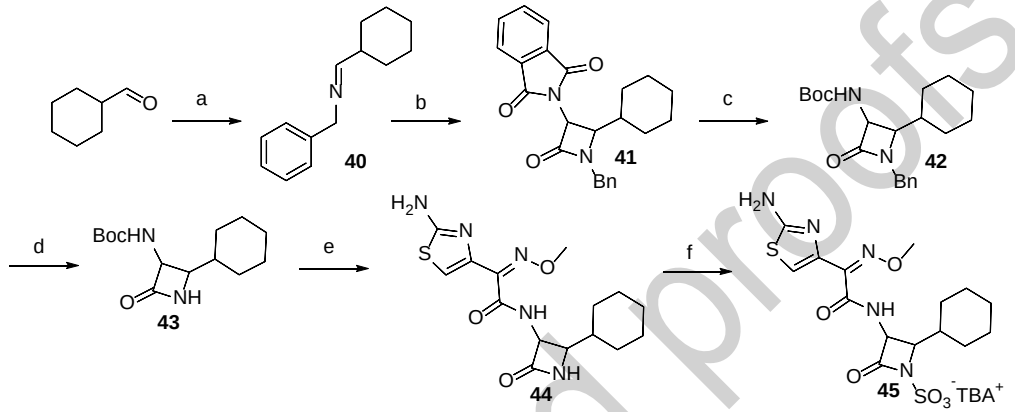
Scheme 1

Reagents and conditions: a) 2-(1,3-dioxoisoindolin-2-yl)acetyl chloride, Et_3N, toluene, 70°C; b) $(NH_4)_2[Ce(NO_3)_6$, CH_3CN/H_2O, -10°C; c) $SO_3\times DMF$, DMF, K_2HPO_4, nBu_4NHSO_4



Scheme 2

Reagents and conditions: a) 2-(1,3-dioxoisoindolin-2-yl)acetyl chloride, Et_3N, toluene, 70 °C; b) CH_3NHNH_2, CH_2Cl_2, rt, then fluorenylmethyloxycarbonyl chloride, *N*,*N*-diisopropylethylamine, CH_3CN, 0 °C or Boc_2O, Et_3N, CH_3OH, rt; c) $(NH_4)_2[Ce(NO_3)_6$, CH_3CN/H_2O, -10 °C; d) SO_3×DMF, DMF, then $K_2HPO_4^-$, nBu_4NHSO_4; e) Et_3N, CH_3CN, rt, then NHS-activated carboxylic acid side-chain, DMF, 70 °C; f) Dowex® (50WX8, Na^+ form), THF/H_2O, rt; g) trifluoroacetic acid, triethylsilane, CH_2Cl_2, 0 °C, then NHS-activated carboxylic acid sidechain, DMF, 70 °C; h) SO_3×DMF, DMF, then $K_2HPO_4^-$, nBu_4NHSO_4; i) Dowex® (50WX8, Na^+ form), THF/H_2O, rt



Scheme 3

Reagents and conditions: a) benzylamine, Na_2SO_4, CH_2Cl_2, rt; b) 2-(1,3-dioxoisoindolin-2-yl)acetyl chloride, Et_3N, toluene, 70 °C; c) CH_3NHNH_2, CH_2Cl_2, rt, then Boc_2O, Et_3N, CH_3OH, rt; d) SD Super Fine™ (sodium 25 %, *m*/*m*, dispersion in mineral oil), 15-crown-5, *iso*propyl alcohol, THF, 0 °C; e) trifluoroacetic acid, triethylsilane, CH_2Cl_2, 0 °C, then NHS-activated carboxylic acid sidechain, DMF, 70 °C; f) SO_3×DMF, DMF, then $K_2HPO_4^-$, *n*Bu_4NHSO_4

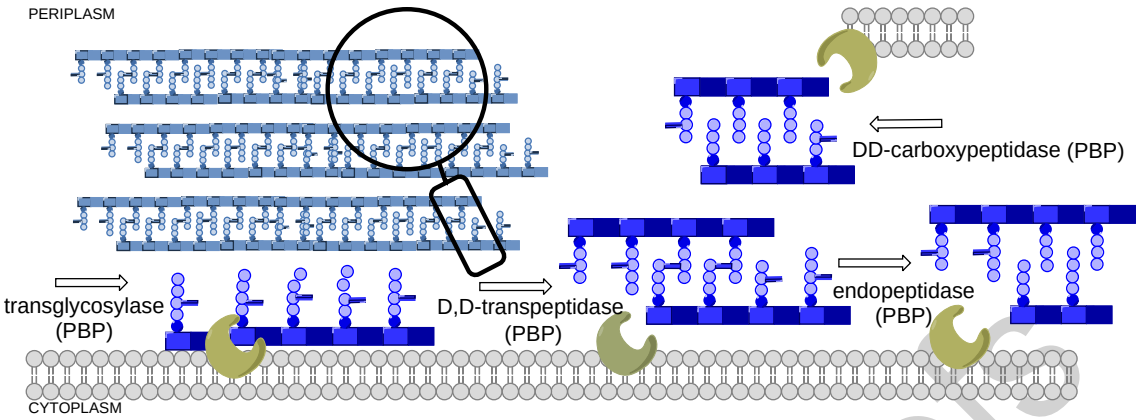
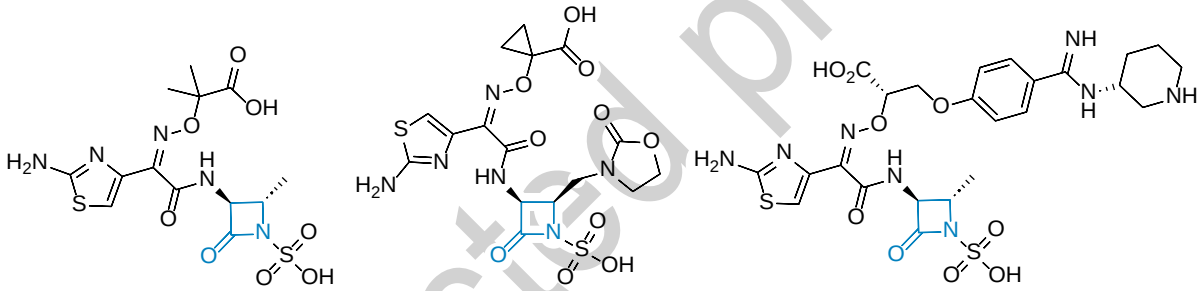


Fig. 1. The versatile roles of penicillin-binding proteins (PBPs) in the biosynthesis of peptidoglycan.



Aztreonam
The only monobactam in clinical use

Ancremonam
(LYS228/BOS228)

Cpd AIC499
(in development by AiCuris)

Fig. 2. Selected examples of monocyclic β-lactam compounds (β-lactam ring is highligthed in blue).