Development of screening assays and discovery of initial inhibitors of pneumococcal peptidoglycan deacetylase PgdA

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1 **Development of screening assays and discovery of initial inhibitors of pneumococcal**

2 **peptidoglycan deacetylase PgdA**

3

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1

1 **Abstract**

2

3 The essential cell wall peptidoglycan is the target of several components of the innate

4 immune system and its disruption results in lysis of invading bacteria. The pathogen

5 *Streptococcus pneumoniae* produces a peptidoglycan N-acetylglucosamine deacetylase,

6 PgdA, to modify the peptidoglycan structure. The activity of PgdA contributes to the

7 bacteria's resistance to lysozyme, which is an important antimicrobial factor of the human

8 innate immune system. In this study we report on the activity of PgdA against natural and

9 artificial substrates. We have also established a virtual high-throughput screening and a

10 new enzyme assay to search for compounds inhibiting PgdA. Two compounds with IC_{50}

11 values in the micromolar range have been identified and they could serve as leads for the

12 search of inhibitors of PgdA, an important pneumococcal virulence factor.

13

14 **Keywords:** peptidoglycan deacetylase; PgdA; lysozyme resistance; virtual screening**.**

15

16 **Abbreviations:**

17 PG, peptidoglycan; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid;

18 PgdA, peptidoglycan N-acetylglucosamine deacetylase A; IC_{50}, half maximal inhibitory

19 concentration; IPTG, Isopropyl-β-D-thiogalactopyranoside; CTAB,

20 cetyltrimethylammonium bromide; $GlcNAc_5$, N,N,N,N,N-pentaacetylchitopentaose;

21 pNPA, p-nitrophenyl acetate; αNA, α-napthyl acetate; 4MUA, 4-methylumbelliferyl

22 acetate; DMSO, Dimethylsulfoxide.

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1 **1. Introduction**

2

3 *Streptococcus pneumoniae* (the pneumococcus) is one of the most important human

4 pathogens and can cause infectious diseases like pneumonia, otitis media and meningitis

5 [1]. The encapsulated, Gram-positive bacterium has a lancet-shape morphology which is

6 maintained by the pneumococcal cell wall. A major element of the cell wall is the

7 peptidoglycan that protects the cell against the internal osmotic pressure. Peptidoglycan

8 consists of glycan chains of alternating β1,4-linked N-acetylglucosamine (GlcNAc) and N-

9 acetylmuramic acid (MurNAc) residues that are cross-linked by oligopeptides [2]. The

10 peptidoglycan hydrolase lysozyme is widely present in animals, plants, fungi and bacteria

11 and is an important bacteriolytic component of the human innate immune system.

12 Lysozyme hydrolyses the glycosidic bonds within the peptidoglycan glycan chains causing

13 disintegration of the peptidoglycan structure and thereby lysis of the cell. In *S. pneumoniae*

14 and other pathogens the glycan chains become modified shortly after their synthesis by O-

15 acetylation (of MurNAc) and de-N-acetylation (MurNAc and/or GlcNAc) [3-5]. These

16 modified glycan chains are poor substrates for lysozyme thereby contributing to the

17 resistance to this antimicrobial enzyme [6]. Peptidoglycan deacetylation is catalysed by the

18 peptidoglycan N-acetylglucosamine deacetylase A (PgdA) and affects 30-80% of the

19 GlcNAc residues. The *pgdA* gene was first identified in *S. pneumoniae* [5] and has since

20 been reported to be present in many other species including *Bacillus subtilis* [7], *Bacillus*

21 *cereus* [8], *Lactococcus lactis* [9], *Streptococcus mutans* [10], *Listeria monocytogenes* [11,

22 12] and *Enterococcus faecalis* [13]. Furthermore, zoonotic pathogens such as

23 *Streptococcus suis* [14] and *Streptococcus iniae* [15] that have a significant influence on

24 industrial livestock and aquaculture production have also been reported to have

25 peptidoglycan deacetylase. Mutants from human and animal pathogenic strains lacking the

26 peptidoglycan deacetylase gene are significantly impaired in their virulence [6, 11-15].

27 Interestingly, in *S. suis* the *pgdA* gene is expressed at low level under laboratory conditions

28 and is up-regulated during infection in the pig host organism [14].

29 The crystal structure of pneumococcal PgdA (*Sp*PgdA) has elucidated the catalytic

30 mechanism of peptidoglycan deacetylation and the kinetic parameters of *Sp*PgdA were

31 determined using $GlcNAc_3$ as a substrate [16]. The peptidoglycan deacetylases BC1960

32 and BC3618 from *B. cereus* are active against peptidoglycan of *B. subtilis* and *H. pylori*

3

1 [8]. More recently, PgdA from *L. monocytogenes* has been shown to be able to release

2 acetate from *Listeria* peptidoglycan and to deacetylate GlcNAc [12]. The growth and

3 morphology of the *L. monocytogenes pgdA* mutant resembled those of the parental strain.

4 However, the mutant was more susceptible to lysozyme or muramidases and was more

5 prone to EDTA- or Triton X-100-induced autolysis.

6 PgdA is a validated antibiotic target as demonstrated by the reduced virulence of mutant

7 strains of various pathogens [6, 11-15]. Thus, antibiotic inhibition of PgdA would enable

8 the host organism to lyse invading bacteria with lysozyme that is inactive against bacteria

9 with functional PgdA. However, to date no enzyme inhibitor has been published for this

10 protein. In this study we present evidence of an *in vitro* deacetylase activity of PgdA

11 against *S. pneumoniae* peptidoglycan and other substrates. We have developed a simple

12 microtiter-based activity assay with chromogenic substrates. We have used the assay to

13 screen for potential PgdA inhibitors selected by computational virtual-screening. Two of

14 the inhibitors identified had IC_{50} values in the micromolar range.

15

16 **2. Experimental procedures**

17

18 *2.1 Materials*

19

20 All Chemicals were purchased from Sigma (Munich, Germany) unless indicated otherwise.

21 96-well plates (ref. 655101) from Greiner bio-one (Frickenhausen, Germany) were used

22 for the screening assays. Cellosyl was provided by Hoechst AG, Frankfurt, Germany.

23

24 *2.2 Preparation of chemically acetylated, tritiated peptidoglycan*

25

26 Cell wall from *S. pneumoniae* strain R36A was prepared as described [6]. Twelve mg of

27 cell wall was suspended in 1.2 ml of water and stirred for 30 min at ambient temperature.

28 The sample was cooled with ice and 750 μl of saturated $NaHCO_3$ solution and 1 ml of 5%

29 freshly prepared, [^{3}H]-labelled acetic anhydride (total 25 mCi) were added and the sample

30 was stirred on ice for 30 min. After the addition of 0.5 ml of 5% unlabelled acetic

31 anhydride the sample was sirred for 30 min on ice and for 1 h at ambient temperature.

32 Thirty ml of water was added and the acetylated cell wall was collected by centrifugation

4

1   (25,000✕g, 30 min, 4°C), washed with 6 ✕ 30 ml of water and dried in a SpeedVac. To

2   remove wall teichoic acid 3.5 mg of [^{3}H]-labelled cell wall was incubated with 48%

3   hydrofluoric acid for 48 h at 4°C. The resulting tritiated peptidoglycan was washed as

4   described [6] and had a specific activity of 5.67×10^6 dpm/mg.

5

6   *2.3 Cloning of the pgdA gene*

7

8   Part of the *pgdA* gene (nucleotides 735-1389) was amplified from DNA from *S.*

9   *pneumoniae* R36A using standard polymerase chain reaction (PCR) procedures. The

10   following oligonucleotides (Biomers, Ulm, Germany) were used:

11   (PgdA735) TCA <u>CATATG</u> ATC CAA TCT TCG TAC TTA CTC and PgdA(1389) CCA

12   <u>GAATTC</u> GTC TGT CAA ATA TCT AAC CAG (NdeI and BamHI restriction sites

13   underlined).

14   The PCR products were digested with NdeI and BamHI (Fermentas, St. Leon-Rot,

15   Germany) and ligated into pET14b plasmid (Novagen, Darmstadt, Germany) digested with

16   the same restriction enzymes. This cloning strategy introduced an N-terminal His-tag.

17   After transformation into *E. coli* XL1-Blue the plasmid was isolated and transformed into

18   the expression strain *E. coli* BL21(DE3) pLysS resulting in strain BL21 (DE3) pLysS

19   pET14b-*pgdA*(735-1389). The product is a truncated PgdA version which lacks the signal

20   peptide and the N-terminal non-catalytic domain. It contains part of the middle domain and

21   the catalytic C-terminal domain and has the sequence MGSHHHHHHSSGLVPRGSHM-

22   PgdA$^{245-463}$ (numbers are according to amino acid sequence). Upon cleavage with thrombin

23   the recombinant protein has the sequence GSHM-PgdA$^{245-463}$ and is designated PgdA$^{245-463}$

24   in the following sections.

25

26   *2.4 Protein Purification*

27

28   BL21 (DE3) pLysS pET14b-*pgdA*(735-1389)was inoculated into 400 ml of LB medium

29   containing chloramphenicol (34 μg/ml) (Serva, Heidelberg, Germany) and ampicillin (50

30   μg/ml) (Boehringer, Mannheim, Germany) and incubated at 37°C to an A_{578} of 0.6. The

31   cells were harvested by centrifugation (20 min, 8300 g) and resuspended in 400 ml LB

32   medium. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration

5

1   of 0.4 mM to induce the expression of *pgdA*(735-1389) and the cells were incubated for a

2   further 3 h. The cells were harvested by centrifugation and resuspended in 25 mM

3   Tris/HCl buffer, pH 7.5, 100 mM NaCl (buffer 1).

4

5   DNase (0.5 - 1 mg) and 1 mM PMSF were added before cell disruption by French press

6   (AIC, Silverspring Maryland, USA) (700 psi). The soluble fraction was collected by

7   centrifugation (90 min, 29,600✕g) and was incubated with Co-affinity beads (Talon™,

8   Clonetech, Mountain View, USA) at 4°C for 2 h on a vertical rotating device at 4°C. The

9   beads were transferred to a column and washed with 20 mM Tris/HCl pH 7.5, 0.5 M NaCl,

10 10 mM imidazole followed by elution of the protein with 20 mM Tris/HCl pH 7.5, 0.5 M

11 NaCl, 200 mM imidazole. The protein was dialysed for 18 h against buffer 1 containing 10

12 units of Thrombin (Novagen, Germany) to remove the oligo-His-tag. The protein purity

13 and completeness of thrombin cleavage were verified by sodium dodecyl sulfate-

14 polyacrylamide gel electrophoresis (SDS-PAGE) (Supplemental material Figure 1).

15

16 *2.5 Enzyme assays*

17

18 Different substrates were used in assays with PgdA[245-463]. The samples contained 1 μM

19 enzyme and 5 μM $CoCl_2$ and had a total volume of 100 μl. The incubation temperature

20 was 37°C.

21 The enzyme activity was determined with chemically [3H]acetylated peptidoglycan (total

22 radioactivity used in each assay was 65000 dpm) from *S. pneumoniae* R36A in 25 mM

23 Tris-maleate, pH 5.0 (buffer A), 25 mM Tris/HCl, pH 7.0 (buffer B), 25 mM Tris/HCl, pH

24 8.0 (buffer C) or 25 mM sodium borate, pH 9.5 (buffer D). The reaction was terminated by

25 the addition of 100 μl 0.2 M HCl and 5 μl 1 M acetic acid. Acetic acid was extracted with

26 500 μl ethyl acetate and radioactivity in the organic phase was measured by scintillation

27 counting.

28 For testing the maximal release of acetate from the [3H]acetylated peptidoglycan from *S.*

29 *pneumoniae* R36A the reaction was performed in 25 mM Tris/HCl pH 7.0 in a total

30 volume of 400 μl. After 24 h one quarter of the sample was added to 100 μl 1%

31 cetyltrimethylammonium bromide (CTAB) and incubated for 20 min on ice to precipitate

32 the high-molecular weight peptidoglycan. The mixture was centrifuged (20 min, 16,000✕g)

6

1   and the radioactivity present in 100 µl of the supernatant was quantified by scintillation

2   counting. The remaining of the sample was split into 3 aliquots that received either another

3   1 µM of enzyme, 5 µl of peptidoglycan (13000 dpm/µl) or nothing. After 24 h of further

4   incubation the peptidoglycan was precipitated as described above and the radioactivity in

5   the supernatant was measured.

6   PgdA activity was also tested with cell wall, peptidoglycan and muropeptides from *S.*

7   *pneumoniae*; peptidoglycan and muropeptides from *E. coli,* cell wall from *S. aureus* and

8   from *S. carnosus*, and N,N,N,N,N-pentaacetylchitopentaose ($GlcNAc_5$) as possible

9   substrates. The incubation was performed in 25 mM Tris/HCl, 5 µM $CoCl_2$ pH 7.0.

10   Muropeptides and $GlcNAc_5$ were incubated for 2 h, while cell wall or peptidoglycan was

11   incubated for 16 h. The samples were analysed by HPLC and fractions collected were

12   analysed by mass spectrometry (see below). Cell wall, peptidoglycan and muropeptides

13   from *S. pneumoniae*, cell wall from *S. aureus* and *S. carnosus* were analysed by HPLC as

14   described [17]. Peptidoglycan and muropeptides from *E. coli*, the muropeptides from *S.*

15   *aureus* and $GlcNAc_5$ were analysed as described [18].

16

17   *2.6 Mass spectrometry*

18

19   HPLC fractions of muropeptides were desalted using ZipTip C18 pipette tips (Millipore,

20   Schwalbach, Germany). The samples were eluted with 4 µl 0.5% acetic acid and an aliquot

21   of 0.5 µl was applied on a metal well-plate coated with α-cyano-hydroxy-nitrocellulose.

22   MALDI-TOF analysis was performed on the Reflex III Mass Spectrometer (Bruker,

23   Bremen, Germany) using XACQ 4.0 software. The data was processed using the software

24   XMASS 5.1. Offline electrospray MS was performed as published [19].

25

26   *2.7 Determination of the kinetic parameters*

27

28   Samples contained 1 µM enzyme and 5 µM $CoCl_2$ and had a total reaction volume of 100

29   µl. The assay buffer was either 25 mM Tris/HCl pH 7.0 (buffer B) or 25 mM Tris/HCl pH

30   8.0 (buffer C). The kinetic parameters were determined with p-nitrophenyl acetate (pNPA)

31   and 4-methylumbelliferyl acetate (4MUA). The substrate concentrations ranged from 2 -

32   10 mM for *p*NPA and 0.2 - 1.0 mM for 4MUA, which is below the K_m values (Table 1),

7

1   due to their insufficient solubility. The assays were performed in buffer B, 10% DMSO in

2   a 96-well microtiter plate for 30 min at 37°C. The kinetic property of 4MUA was also

3   determined in buffer C. The reaction progress was monitored in a Spectra-Max 340 PC

4   (Molecular Devices, Sunnyvale, CA, USA)microplate reader at 405 nm for the *p*NPA

5   assay and at 354 nm for the 4MUA assay. The kinetic parameters K_m and V_{max} were

6   determined by fitting the data to the Michaelis Menten equation by non-linear regression

7   using the Prism 5 software (GraphPad Software, La Jolla, CA, USA).

8

9   *2.8 Compound screen*

10

11   The inhibitor screen was performed in 25 mM Tris/HCl pH 7.0 at 37°C for 15 min using 1

12   mM 4MUA as a substrate and 1 μM PgdA[245-463] enzyme with or without 1 mM compound

13   dissolved in DMSO. The total volume was 100 μl with a final concentration of 10%

14   DMSO. Some samples contained 0.01% Triton X-100. Reactions were monitored with the

15   BMG Labtech FluoStar Optima microtiter plate reader. The excitation wavelength was 355

16   nm and the emission wavelength was 460 nm.

17

18   *2.9 Computer-aided virtual high-throughput screening to identify potential inhibitors of*

19   *PgdA*

20

21   Computational part was done on workstation with 4 dual core AMD Opteron 2.0 GHz

22   processors, 16GB of RAM, 4 320GB hard drives in RAID10 array and nVidia GeForce

23   7900 graphic card. Workstation has Fedora 7 64bit installed.

24   For initial virtual screening the American National Cancer Institute (NCI) bank of 280000

25   compounds was selected. Due to the size of NCI bank of compounds, filtering procedure

26   was applied first to eliminate compounds with unwanted properties. Filtering was based on

27   simple molecular descriptors and was done with program Filter (OpenEye Scientific

28   Software Inc). Choice of descriptors was based loosely on Lipinski rule of five [20]:

29   molecular weight: 200-600 g/mol, number of ring systems: 1-5, number of hetero atoms: 4-

30   20, number of H-bond donors: 0-6, number of H-bond acceptors: 0-14; LogP: -3.0 – 4.0.

31   Additional filters were used to eliminate insoluble compounds (set to moderate), to

32   eliminate all the compounds with atoms other than H, C, N, O, F, S, Cl and Br, and to

8

1 eliminate all the compounds with reactive functional groups. Also a function developed by

2 Shoichet, implemented in the program Filter, that eliminates know aggregators and also

3 removes the predicted aggregators was used [21]. Using this function we anticipate

4 eliminating potential promiscuous inhibitors. Result of filtering was a new subset of

5 compounds containing roughly 55000 structures.

6 For docking experiment, FlexX 3.1. (BioSolveIT GmbH) [22] was used on the crystal

7 structure of PgdA from *S. pneumoniae* co-crystallized with acetate (PDB entry: 2C1G)

8 [16]. The active site was defined as an area of the enzyme within 7.5 Å from the co-

9 crystallized acetate. The co-crystallized zinc bound in zinc finger was left in the active site

10 and octahedral coordination was selected. Water 176 was defined as freely rotatable and

11 displaceable so that it can bridge H-bonds if possible, if not FlexX ignores it. Protonations

12 of amino acids and orientations of H-bond donors were setup manually. Since zinc ion is

13 an important part of the active site, docking experiment was set up in such manner that it

14 considers only the solutions that predict the binding of compounds with zinc ion. For base

15 placement Triangle Matching was used and the program generated maximally 200

16 solutions per iteration and 200 per fragmentation.

17 All of the 55000 compounds were then docked in the active site of PgdA and ranked

18 according to their scores. 40 of the best compounds were selected and ordered from NCI, 2

19 were out of stock and 38 compounds were tested *in vitro* (Supplemental material Table 1).

20 After obtaining *in vitro* results, a 3D similarity search was performed to obtain compounds

21 similar to the most active compound **2**. This time, the combined bank of compounds from

22 suppliers ChemBridge, Maybridge, Asinex and NCI, containing 1.8 million structures, was

23 used. 3D Similarity search was done with ROCS (OpenEye Scientific Software Inc.) [23].

24 Additionally, LIQUID plugin for PyMol [24] was used to visualize pharmacophoric

25 features of query compound **2** and only the compounds with the same features were

26 selected. Finally, 9 compounds (compounds **3**-**11**) with similar volume, shape and

27 pharmacophoric features as compound **2** were selected and purchased.

28

29 **3. Results**

30

31 *3.1 PgdA activity against the oligosaccharide GlcNAc$_5$*

32

9

1   We have purified from *E. coli* a recombinant, soluble version of PgdA, PgdA[245-463],

2   containing an N-terminal His$_6$-tag, by Co^{2+}-affinity chromatography. PgdA[245-463] consists

3   of the catalytic C-terminal domain and part of the middle domain. The oligohistidine tag

4   was removed by cleavage with thrombin (Figure S1). We found that PgdA[245-463] was able

5   to de-acetylate the GlcNAc$_5$ (Figure 1A) but not GlcNAc (not shown). GlcNAc$_5$ was

6   almost quantitatively converted to mono-, di- and tri-de-acetylated products as shown by

7   HPLC and MALDI-MS analysis of the products (Figure 1).

8

9   *3.2 PgdA activity against peptidoglycan and muropeptides*

10

11   We have next assayed the activity of PgdA against peptidoglycan or peptidoglycan

12   fragments (muropeptides) isolated from different bacterial species. The first substrate we

13   tested was peptidoglycan from wild-type *S. pneumoniae* that has been chemically N-

14   acetylated with ^{3}H-labelled acetic anhydride. PgdA[245-463] was able to release a small

15   fraction (~4%) of the radioactive acetate from this substrate (Figure 2). We would expect

16   to obtain a low percentage of radioactivity release from this substrate because (i) a large

17   proportion (30-50%) of naturally acetylated GlcNAc residues were not labelled and thus

18   their deacetylation is not detectable by the method, and (ii) the chemical acetylation most

19   likely also labels the primary amino groups in the peptide part that cannot be deacetylated

20   by PgdA. In addition, the low percentage of acetate release could be due to a limited

21   number of acetate residues being susceptible to the enzyme or due to the inactivation of the

22   enzyme during the period of the reaction. To test these possibilities we added either a

23   second aliquot of acetylated cell wall or more enzyme at the end of the reaction followed

24   by a second period of incubation. The addition of more enzyme could not release more

25   acetate, whereas more acetate was released upon addition of more peptidoglycan substrate

26   (Figure 2). We concluded that a limited fraction of GlcNAc residues in chemically

27   acetylated pneumococcal peptidoglycan is prone to deacetylation by PgdA.

28   Peptidoglycan from wild-type *S. pneumoniae* contains a fraction of de-acetylated

29   glucosamine residues. When the peptidoglycan was incubated with PgdA[245-463] the

30   percentage of deacetylation did not further increase indicating that the residual GlcNAc

31   residues are not accessible to the enzyme (Figure 3A). Interestingly, PgdA[245-463] was active

32   against peptidoglycan isolated from the *pgdA* mutant strain, which does not contain

10

1 glucosamine residues (Figure 3B, [5]), demonstrated by the presence of 7 - 21%

2 deacetylated muropeptides in the cellosyl-digested peptidoglycan (Figure 3B). MS and

3 MS/MS analysis confirmed that one of the muropeptides generated by PgdA corresponded

4 to the deacetylated version of the major monomeric muropeptide, Tri(deAc) (Figure 3D).

5 PgdA[245-463] was also active against peptidoglycan from *S. suis*, which has similar basic

6 structure as that from *S. pneumoniae*, but was inactive against peptidoglycans from *S.*

7 *aureus*, *S. carnosus* or *E. coli* that have different peptide structure than peptidoglycan from

8 *S. pneumoniae*. PgdA[245-463] was inactive against soluble muropeptides from the *S.*

9 *pneumoniae pgdA* mutant strain (Figure 3C) and from other species (not shown). These

10 data confirm that PgdA[245-463] has the ability to release *in vitro* a limited number of acetate

11 residues from pneumococcal peptidoglycan, but not from other species, and that it

12 deacetylates the high-molecular weight substrate but not small, soluble peptidoglycan

13 fragments. The limited activity of PgdA[245-463] against natural substrates was not due to the

14 absence of the N-terminal domain because a soluble version comprising the N-terminal

15 domain and lacking the signal peptide, PgdA[39-463], did not have higher activity than

16 PgdA[245-463] (data not shown).

17

18 *3.3 Enzyme activity against chromogenic substrates and development of an assay for*

19 *inhibitor screening*

20

21 Having demonstrated deacetylase activity against natural substrates we aimed to develop a

22 simple enzyme assay for screening of inhibitors. For this we have tested various possible

23 chromogenic substrates of PgdA. Indeed, we found that PgdA[245-463] released acetate from

24 p-nitrophenyl acetate (pNPA) or 4-methylumbelliferyl acetate (4MUA). The assays were

25 performed at pH 7.0 for all chromogenic substrates used. For 4MUA the assay was also

26 carried out at pH 8.0. The enzyme reaction for these chromogenic substrates followed the

27 steady-state kinetics described by the Michaelis-Menten equation. The kinetic parameters

28 K_m and V_{max} were determined by non-linear regression (Table 1). PgdA[245-463] had the

29 highest k_{cat}/K_m value with 4MUA (128.61 $M^{-1} s^{-1}$) compared to the pNPA. With 4MUA as

30 substrate the enzyme exhibited a ca. 6-fold higher k_{cat}/K_m value at a pH of 8.0 as compared

31 to pH 7.0.

32

11

1 *3.4 Discovery of a new potential inhibitor of PgdA by computational virtual screening and*

2 *in vitro assay*

3

4 Virtual screening was done on NCI bank of 280000 compounds which was filtered to

5 loosely comply with the Lipinski rule of five [20]. The remaining 55000 compounds were

6 then docked in PgdA active site using FlexX [22]. PgdA has well defined active site with

7 typical zinc finger. Zinc ion is complexed by two histidines (His326 and His330) and

8 aspartic acid (Asp276) (Figure 4A). This structural feature is conserved throughout CE-4

9 family of enzymes. In addition to amino acid residues mentioned above, water 176 and

10 acetate (product of the reaction catalyzed by PgdA) coordinate zinc in octahedral

11 coordination. Acid-base catalysis is performed by two charge relay pairs. One is formed by

12 Asp275 and Arg664 and the other by His417 and Asp 391. Zinc bound water 176 functions

13 as a nucleophile [16]. For docking experiment the active site was defined as an area of the

14 enzyme within 7.5 Å from the co-crystallized acetate. All 55000 pre-filtered compounds

15 were docked and 40 compounds with best scores were ordered from NCI. 38 available

16 compounds were tested in *in vitro* assay (Supplemental Table 1). Two compounds showed

17 promising inhibitory activities: compound **1** had an IC_{50} value of 638 μM and compound **2**

18 an IC_{50} value of 132 μM. We determined the inhibitory activity of compound **2** also in

19 presence of 0.01% of Triton X-100 and it remained unchanged confirming nonpromiscous

20 mode of inhibition [21].

21 Our docking study shows that compound **2** complexes zinc with the carboxylic and keto

22 functional groups (Figure 4B). The carboxylic group is placed at the same position as

23 acetate in the crystal structure 2C1G.pdb, while the keto group replaces water 176. The

24 keto group also forms an additional interaction with His417, while the aromatic hydroxyl

25 group forms a H-bond with Asp276.

26 In order to find additional inhibitors with structures related to compound **2**, 3D similarity

27 search was performed which involved the complete NCI bank of compounds and some

28 commercially available banks of compounds. 3D similarity search was done with the

29 program ROCS [23]. Results were then visualized with PyMol and only compounds that

30 comply to pharmacophoric model (Figure 4C) were selected. Nine structurally related

31 compounds were found; however, none of them exhibited better inhibitory activity than the

32 parent compound **2** (compounds **3**-**11**, Table 2).

12

1

4. Discussion

3

4 The peptidoglycan-specific sugar *N*-acetylmuramic acid is GlcNAc with a lactoyl residue

5 at C3 and so the poly-(GlcNAcMurNAc) glycan strand backbone of peptidoglycan is

6 chemically similar to linear oligo-GlcNAc chains, i.e. soluble N-acetylchitooligomers. It is

7 therefore not surprising that the peptidoglycan deacetylase PgdA is related to chitin

8 deacetylases and to nodulation factors that work on oligo-GlcNAc substrates. Moreover,

9 PgdA from *S. pneumoniae* has been shown to deacetylate GlcNAc$_3$ [16] and two homologs

10 from *B. cereus* were active against GlcNAc$_{2-6}$, with highest activity against GlcNAc$_4$ [8].

11 Both *B. cereus* enzymes were inactive against monomeric GlcNAc, whereas PgdA from *L.*

12 *monocytogenes* was capable of deacetylating GlcNAc as detected by mass spectrometry

13 analysis [11]. Our data confirmed an activity of *S. pneumoniae* PgdA against GlcNAc$_5$,

14 which was deacetylated to the mono-, di- and tri-deacetylated compounds by the enzyme,

15 and like in the case of the *B. cereus* enzymes we were unable to detect an activity against

16 monomeric GlcNAc. Together these data suggest that the *S. pneumoniae* and *B. cereus*

17 enzymes are capable of deacetylating only a limited number of GlcNAc residues in oligo-

18 GlcNAc chains but this might not be a general property of PgdA deacetylases.

19

20 There are few reports on the activity of PgdA enzymes on natural substrates. Two *B.*

21 *cereus* PgdA homologs BC1960 and BC3618 deacetylated the muropeptide GlcNAc-

22 MurNAc-L-Ala-D-Gln as well as peptidoglycan from *H. pylori* [8] although *H. pylori*

23 peptidoglycan contains different peptides than that of *B. cereus*. *S. pneumoniae* PgdA was

24 active against pneumococcal peptidoglycan, as shown in this work by the release of

25 radioactive acetate and by the HPLC/MS-detection of deacetylated muropeptides obtained

26 from a PgdA-treated peptidoglycan sample of a *pgdA* mutant strain. The extent of

27 deacetylation *in vitro* was lower than that observed in peptidoglycan from wt cells

28 indicating that the enzyme is more active in the cell, perhaps due to its membrane-location

29 in proximity of the newly made peptidoglycan or due to the stimulatory effects of cellular

30 components not present in the *in vitro* system. Interestingly, PgdA was neither active

31 against soluble peptidoglycan fragments, i.e. the muropeptides, nor against peptidoglycan

13

1 from other bacterial species. The structural basis for this specificity remains to be

2 determined.

3   PgdA had a higher catalytic efficiency at pH 8.0 as compared to pH 7.0 with

4 4MUA substrate (Table 1). Based on the suggested reaction mechanism for pneumococcal

5 PgdA [16] it is possible that the abstraction of a proton from the Zn^{2+}-bound water

6 molecule by Asp275, which initiates the reaction, is more efficient at pH 8.0 than at pH7.0,

7 but this hypothesis requires further testing. Notably, previous work showed different pH

8 optima for two peptidoglycan deacetylases from *B. cereus*. BC1960 has a pH optimum at

9 pH 6.0, BC3618 at pH 8.0 [8] and the reason for this difference is not known.

10   Virtual screening methods are increasingly used to identify potential new lead

11 compounds in drug discovery [25, 26] and have also been successfully used on

12 antibacterial targets [27]. To date no inhibitor of PgdA has been published. Using

13 computational structure-based virtual screening followed by biochemical evaluation, we

14 were able to identify two promising inhibitors of PgdA, compounds **1** and **2** (Table 2), with

15 IC$_{50}$ values of 584 μM and 130 μM, respectively. Our enzyme assays developed could also

16 be used for high-throughput screening. The more potent inhibitor **2** was selected as a

17 starting point for further computational similarity search in order to rapidly identify a

18 series of structurally related compounds. Although this similarity search did not yield more

19 potent inhibitors, it provided some insight into which structural elements are beneficial for

20 inhibition of PgdA within this series. Compounds **2**-**11** share a common 2-benzoylbenzoic

21 acid scaffold with different substituents on benzoyl part of the molecule and all except

22 compounds **3** and **9** show some inhibitory activity. Substitution on the position 4 of the

23 benzoyl part appears to be of significant importance for inhibitory activities of compounds

24 with diethylamino substituent (compound **2**) being the most promising. In order to find

25 inhibitors with improved PgdA inhibitory activities, we propose to use compound **2** as a

26 starting point for further medicinal chemistry efforts. We propose the synthesis of extended

27 series of structurally related compounds where 2-(4-(diethylamino)benzoyl)benzoic acid is

28 retained and different substituents are systematically introduced to various positions of

29 both aromatic rings.

30

31 **Acknowledgements**

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14

6

Supplemental material

8 The supplemental Figure S1 demonstrates completeness of thrombin cleavage of PgdA[245-463]. Supplemental Table 1 shows the effect of compounds on PgdA activity.

10

Figure Legends

12

Figure 1. Deacetylation of $GlcNAc_5$ by PgdA[245-463].

14 HPLC profiles (A, C) and MALDI mass spectra (positive ion mode) (B, D) of $GlcNAc_5$

15 (A, B) and its products upon incubation with PgdA[245-463] (C, D). (A) $GlcNAc_5$ eluted at 25

16 min (arrow 1). (B) Mass spectrum of $GlcNAc_5$ with the masses of the H^+ and Na^+ forms.

17 The numbers in brackets indicate the theoretical masses. (C) $GlcNAc_5$ was incubated with

18 PgdA[245-463] followed by HPLC analysis. The two new peaks (arrows 2 and 3) correspond

19 to deacetylated products of $GlcNAc_5$. (D) The combined fractions of peaks 2 and 3 (C)

20 were analysed by mass spectrometry. The PgdA[245-463] products lacked 1, 2 or 3 acetyl

21 groups (theoretical masses in brackets).

22

Figure 2. Release of radioactive acetate from pneumococcal cell wall by PgdA[245-463].

24 The time of incubation and the addition of more enzyme or substrate is indicated below the

25 columns. The values are mean ± SD of two independent samples. The total radioactivity

26 was 65000 dpm. PgdA[245-463] maximally released ∼4% of the total radioactivity of

27 chemically acetylated peptidoglycan.

28

Figure 3. Deacetylation of pneumococcal peptidoglycan by PgdA[245-463].

30 Peptidoglycan from *S. pneumoniae* wildtype (A), peptidoglycan from the *pgdA* mutant

31 strain (B) or muropeptides from the *pgdA* mutant strain (C) was incubated with or without

32 PgdA[245-463]. Peptidoglycan samples were digested with cellosyl, and all samples were

15

1   reduced with sodium borohydride and analysed by HPLC. (D) MS/MS spectra of the major

2   muropeptide Tri (GlcNAc-MurNAc-L-Ala-D-iGln-L-Lys) (top) and its deacetylated

3   version Tri(deAc) (bottom) obtained from HPLC fractions 1(Tri) and 2 [Tri(deAc)] of the

4   chromatograms shown in B. The mass difference corresponding to loss of either GlcNAc

5   or GlcN from the $[M-H_2O]H^+$ ion is shown on the right side. The insets show the structure

6   of the muropeptide and the determined neutral mass. The theoretical mass is given in

7   brackets.

8

9   **Figure 4.** Active site of PgdA.

10   (A) Only important amino acid residues of the active site of PgdA are shown as yellow

11   sticks. The rest of the enzyme is shown as blue cartoon. The zinc ion is shown as gray

12   sphere, water 176 is shown as red sphere and acetate is shown as green sticks. All relevant

13   interactions are shown as yellow dashes. (B) Compound **2** (green) docked in the active site

14   of PgdA. Interactions with active site amino acid residues and zinc ion are shown as green

15   dashes. (C) Pharmacophore model of structural features needed for inhibition of PgdA.

16   Regions A and B represent two aromatic rings. Region C represents functional groups that

17   can interact with the zinc ion and region D represents functional groups interacting with

18   Asp276.

19

16

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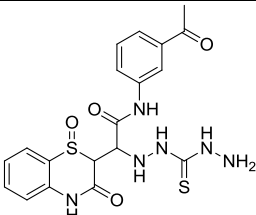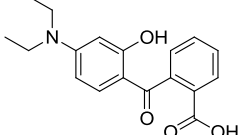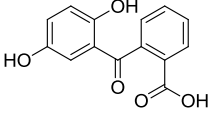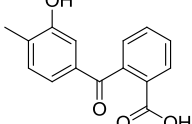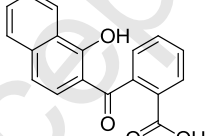**Table 1** ACCEPTED MANUSCRIPT
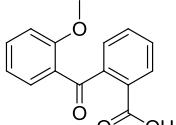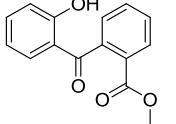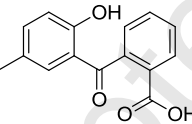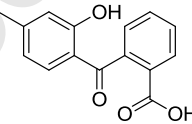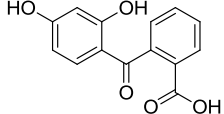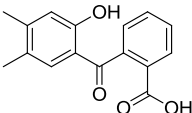
1   **Table 1.** Activity of PgdA$^{245-463}$ against artificial substrates.

Substrate (pH value)	K_m [mM]	V_{max} [µmol/min/mg]	k_{cat} [s^{-1}]	k_{cat}/K_m [M^{-1} s^{-1}]
pNPA	16.74 ± 7.11	1.11 ± 0.30	0.46 ± 0.12	28.8
4-MUA (pH 7.0)	2.27 ± 1.20	0.68 ± 0.27	0.28 ± 0.11	129
4-MUA (pH 8.0)	2.38 ± 0.67	3.95 ± 0.66	1.64 ± 0.27	710

2

1

Table 2

1   **Table 2.** Inhibitory effect of compounds on PgdA[245-463].

Compound[1]	Structure	% Residual enzyme activity at 1 mM	IC$_{50}$ (μM)
1 (NSC648612)		29±4.2	584±115
2 (NSC625797, CH5472223)[2]		7±1.4(5.9±0.2)[3]	130±20
3 (NSC37427)		123.0±0.4	
4 (NSC159284)		62.3±1.1	
5 (NSC338406)		66.4±0.5	
6 (NSC400732)		75.8±0.8	
7 (NSC407886)		62.5±0.9	
8 (CH5475540)		51.3±0.8	
9 (CH5626971)		93.8±1.1	
10 (CH5629492)		33.7±0.5	
11 (CH5944694)		53.0±0.2	

1

1   [1] Compounds with NSC numbers were obtained from NCI, compounds with CH numbers

2   were obtained from ChemBridge.

3   [2] Compound was obtained from two different suppliers.

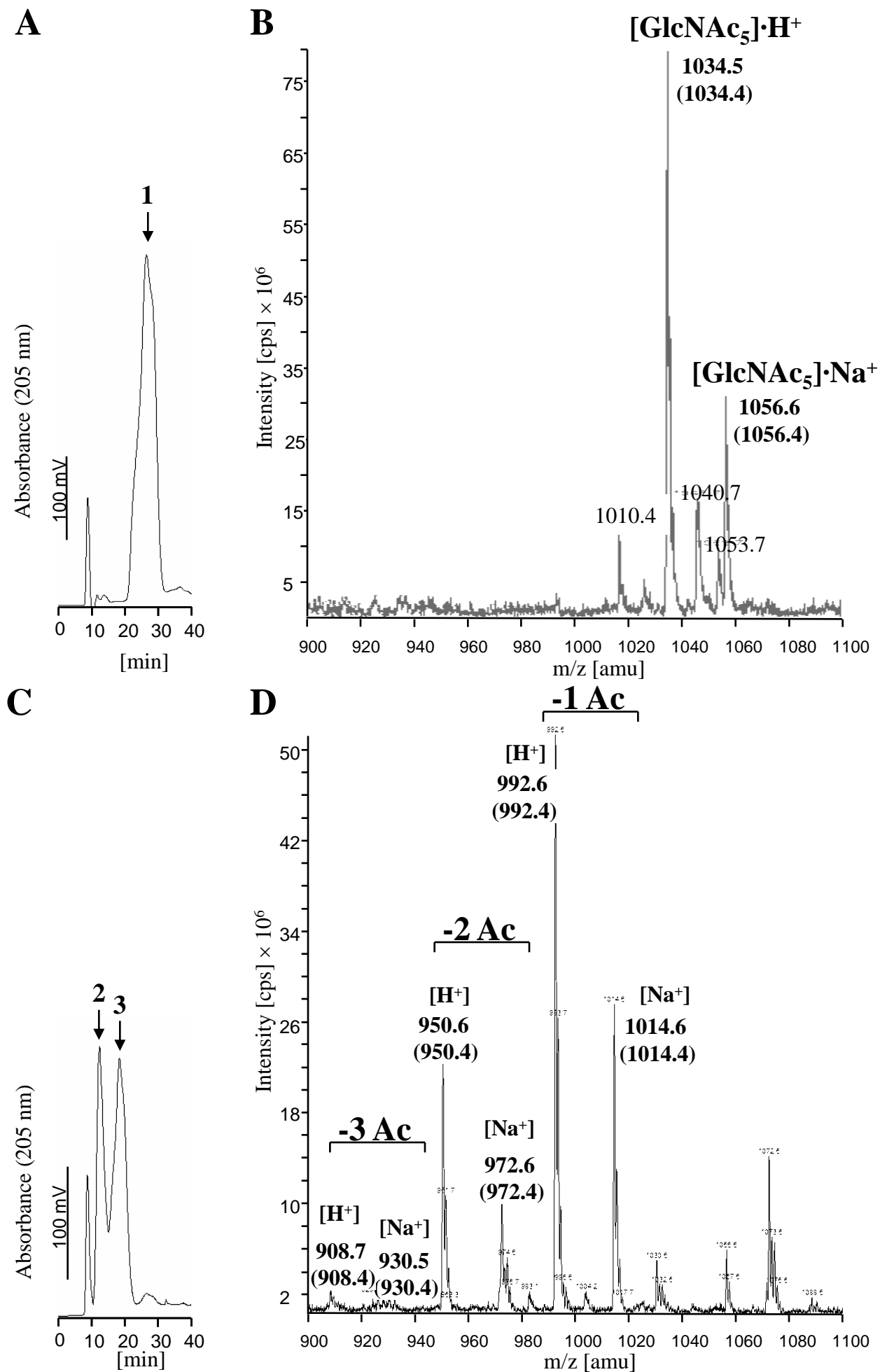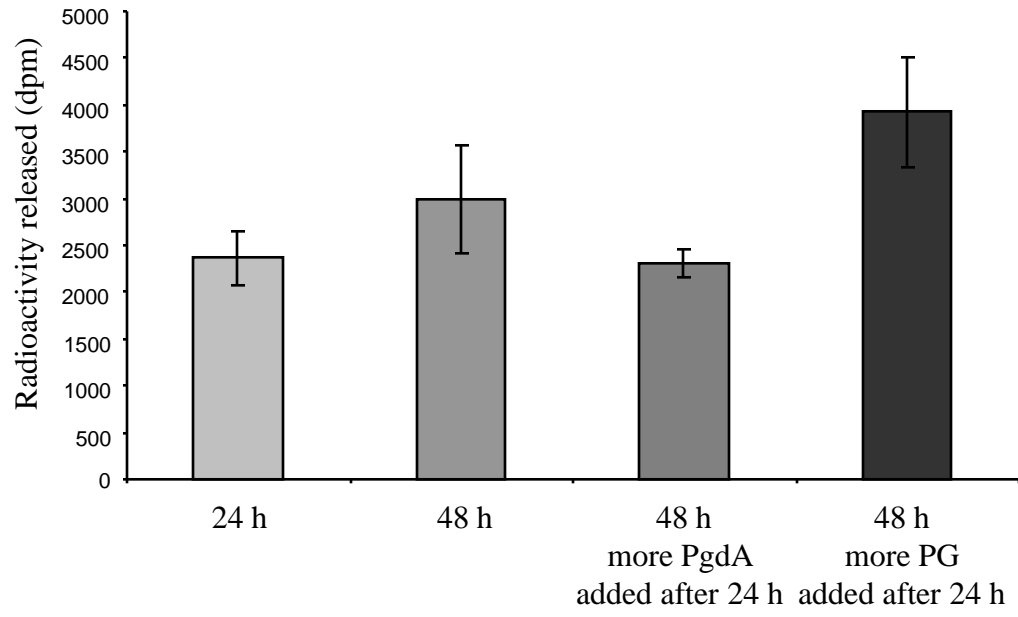4   [3] Number in brackets: assay contained 0.01% Triton X-100.

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Figure 1

Figure 2

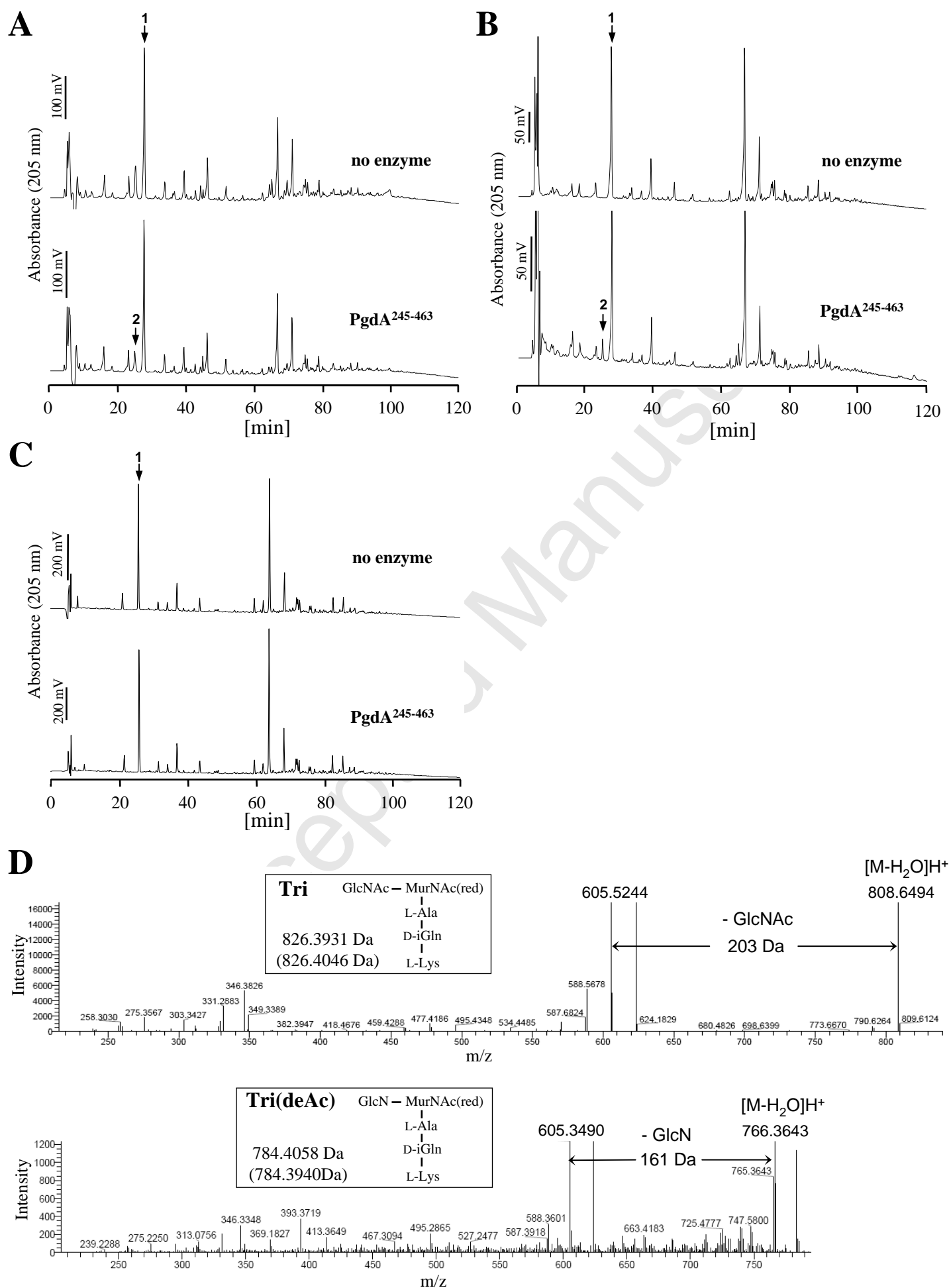Figure 3   ACCEPTED MANUSCRIPT

Figure 4

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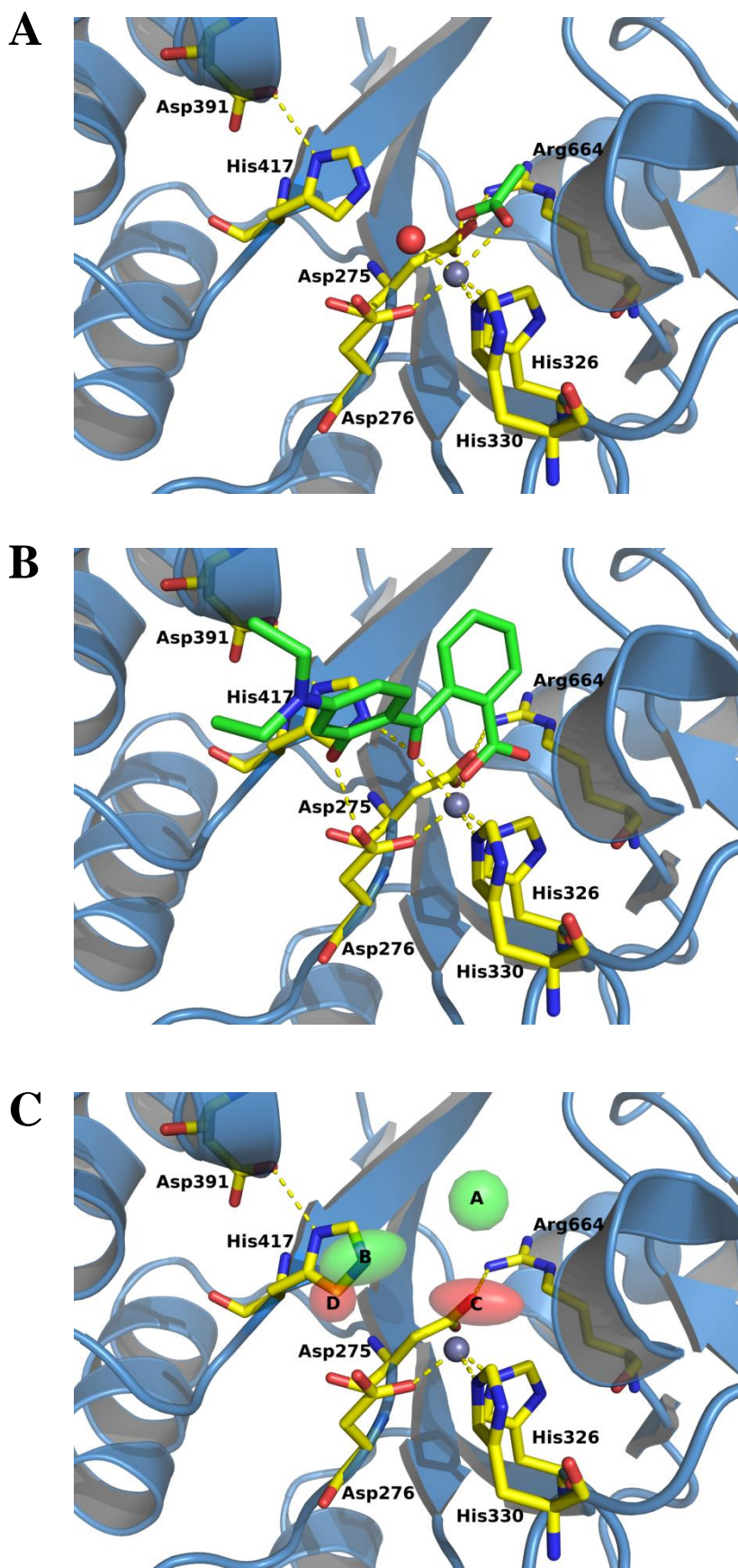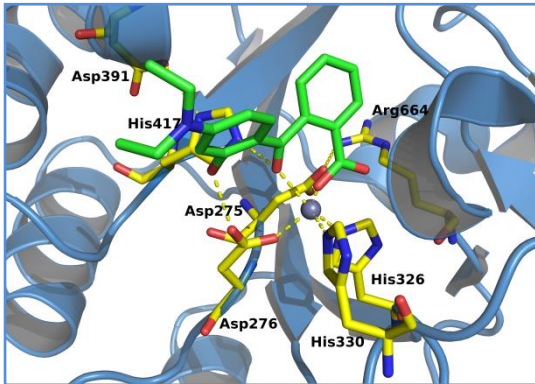
Inhibitory compounds of peptidoglycan deacetylase PgdA identified by virtual HTS and confirmed by novel enzyme assay.