Supporting Information

D-amino acid derivatives as *in situ* probes for visualizing bacterial peptidoglycan biosynthesis

Yen-Pang Hsu, Garrett Booher, Alexander Egan, Waldemar Vollmer, Michael S. VanNieuwenhze*

*Corresponding author. E-mail: mvannieu@indiana.edu

This document contains the experimental protocols for the data presented in section 5 of the main text: **In vitro evidence of enzyme-mediated FDAA incorporation and potential FDAA applications for evaluating antibiotic inhibition activity**

FDAA (HADA) synthesis

HADA was prepared as described previously.[1] Briefly, to a 250 ml round bottom flask was added dimethylformamide (DMF), 7-Hydroxycoumarin-3-carboxylic acid, carbonyldiimidazole (CDI, 1.2 equiv.) and a stir bar. The flask was sealed with a septum, purged with nitrogen gas for 5 minutes and stirred for 3 hours at room temperature. Boc-D-diaminopropanoic acid (Boc-D-Dap, 2 equiv.) was then added to the flask, followed by overnight stirring at room temperature. The solvent was then removed *in vacuo*. The resulting product was dissolved in ethyl acetate and washed with 1M HCl, water, and brine solution. The collected organic layer was dried over $MgSO_4$ and filtered. After the solvent was removed *in vacuo*, the resulting product was subjected to 1:1 ratio of dichloromethane and trifluoroacetic acid, and stirred for 2 hours at room temperature for deprotection. The solvent was then removed *in vacuo*. The product was purified using HPLC (10-90 acetonitrile in H_2O over 10 minutes. RT: 6.5 min).

Cell culturing

Escherichia coli and *Bacillus subtilis* cells were streaked from -80°C freezer stocks (LB broth with 10% DMSO) onto LB agar plates and incubated at 37°C overnight. Single colonies from the overnight culture were transferred to liquid LB broth and incubated in a 37°C shaker. When their OD_{600} reached 0.4-0.5, the cultures were diluted with fresh LB broth (10X) and allowed to grow one more around to OD_{600} 0.2. The cells were then used for experiments.

FDAA labeling and antibiotic treatment

FDAA stock solutions were prepared in DMSO at a concentration of 100 mM. The stock solutions were stored in a -20°C freezer before use. Fresh antibiotic stock solutions were prepared right before the experiments in either water or DMSO at a concentration between 1 to 10 mg/ml. For the transpeptidase-inhibiting test of various antibiotics, 0.3 ml exponentially growing culture was added with antibiotic stock solutions to a final concentration of 2X MIC. After a 1-minute incubation at 37°C, HADA stock solution was added to the culture to a final concentration of 1 mM. The cell cultures were then incubated in a 37°C shaker for another 5 minutes. The cells were then fixed by directly adding 0.7 ml ice-cold ethanol (100%) and incubated on an ice-bath for 1 hour. The cells were then collected via centrifugation (9,000 g, 3 minutes), washed with 1X PBS, and resuspended into 1X PBS for subsequent imaging by fluorescence microscopy. Dose-dependent experiments with ampicillin were performed using the same protocol. Antibiotic MIC values used in this study: vancomycin 0.5 µg/ml; ampicillin 1 µg/ml; PC190723 1 µg/ml; kanamycin 1 µg/ml; and chloramphenicol 2 µg/ml.

Microscopy and data analyses

Fluorescence images were acquired using a Nikon Ti-E inverted microscope equipped with a 1.4 NA Plan Apo 60X oil objective and Andor iXon X3 EMCCD camera. NIS-Elements AR software was used for image acquisition. 24X50 mm coverslips (#1.5) were used as sample supports for the inverted microscope system. Cell samples were loaded onto the coverslips, and covered by an 8X8 mm wide, 2-mm thick PBS agar pad to flatten the cells. The coverslip-pad combination was placed onto a customized slide holder on the microscope with the pad facing upward. The HADA signal was obtained by using a 380-410 nm excitation filter (DAPI) and a 422-448 nm emission filter. Light source power, EM gain and exposure time were optimized based on the positive control sample (no antibiotic treatment) and kept identical throughout the experiment.

Quantitative measurement of FDAA intensity was achieved using FIJI and a plugin, MicrobeJ[2], where cells were identified in the phase contrast channel with a width limit from 0.3 to 2 µm and length above 1 µm. FDAA labeling intensity was then quantified and averaged (N> 100). GraphPad Prism 8 was used for statistical calculation (mean, standard deviation, the linear regression) and data plotting.

***In vitro* transpeptidase assays**

Lipid II substrate versions (mDAP and amidated mDAP versions) for *in vitro* transpeptidase assays were prepared as previously described.[3–5] The following proteins were prepared as previously described: *E. coli* PBP1A[6], *E. coli* LpoA[7], *E. coli* PBP1B[8] and *E. coli* LpoB[8]. Assays with radiolabelled lipid II substrate were performed in an *in vitro* detergent micelle system at the following conditions: 0.5 µM PBPs with 2 µM activator protein (Lpo proteins), in a buffer of 20 mM HEPES/NaOH pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1% DMSO, 0.05% Triton X-100 with 15 µM [14C]mDAP lipid II (1.5 nmol, ~10,000 dpm) was incubated for 1 h at 37°C with 500 µM of D-Ala (control) or FDAA as indicated. After reaction, samples were processed for HPLC analysis of the peptidoglycan product according to Biboy et al.[9] New peaks observed during the HPLC analysis of the peptidoglycan product were identified by mass spectrometry as described previously.[10]

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