Anti-Staphylococcal Biofilm Activity of Miconazocitylium Bromide

Jérémie Tessier, Dr. Mahmood Golmohamadi, Kevin J. Wilkinson and Andreea R. Schmitzer*

Department of Chemistry, University of Montreal, PO Box 6128, Succursale Centre-Ville Montreal, QC, H3C 3J7, Canada.

* ar.schmitzer@umontreal.ca
1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethan-1-one (2). 2-Chloro-1-(2,4-dichlorophenyl)ethan-1-one (1) (1 g, 4.48 mmol) was added portion wise to a suspension of imidazole (1 g, 13.45 mmol) in CH₂Cl₂ (2 mL) at 0 °C. This mixture was allowed to warm to room temperature and stirred for 12 h before water was added. The aqueous layer was extracted three times with CH₂Cl₂, dried over Na₂SO₄ and purified using silica gel chromatography (5/95 MeOH/CH₂Cl₂) to afford the title compound (2) as a slightly brown solid (0.8 g, 3.22 mmol, 72 %).

**1H NMR (CDCl₃, 400 MHz):** δ 7.57 (d, J = 8.3 Hz, 2H), 7.51 (s, 1H), 7.38 (dd, J = 8.4, 1.9 Hz, 1H), 7.13 (s, 1H), 6.95 (s, 1H), 5.34 (s, 2H); **13C NMR (DMSO-d₆, 100 MHz):** δ 58.31, 122.62, 125.97, 126.96, 129.22, 131.48, 135.32, 139.64, 139.83, 194.77; HRMS: (ESI) calcd. for [M+]C₁₁H₈Cl₂N₂O: 254.0014, found 254.0028; IR (neat, cm⁻¹): 2929, 1715, 1584; Melting point: 169-171 °C.

1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol (3). To a solution of (2) (200 mg, 0.79 mmol) in CH₂Cl₂ (1 mL) was slowly added a suspension of NaBH₄ (45 mg, 1.19 mmol) in MeOH (1 mL). The mixture was stirred 2 hours at room temperature before water (1 mL) was added. The aqueous layer was extracted three times with CH₂Cl₂, dried over Na₂SO₄ and purified using silica gel chromatography (5/95 MeOH/CH₂Cl₂) to afford compound (3) as a slightly yellow solid (155 mg, 0.6 mmol, 77 %).

**1H NMR (CDCl₃, 400 MHz):** δ 7.56 (d, J = 8.2 Hz, 1H), 7.36 (d, J = 15.8 Hz, 2H), 7.28 (s, 1H), 6.89 (s, 1H), 6.78 (s, 1H), 6.54 (bs, 1H), 5.22 (d, J = 7.4 Hz, 1H), 4.20 (d, J = 13.9 Hz, 1H), 3.87 (dd, J = 13.8, 8.0 Hz, 1H); **13C NMR (CDCl₃, 101 MHz):** δ 57.62, 67.63, 120.35, 127.87, 128.84, 130.01, 130.12, 131.64, 133.56, 138.74, 139.87; (ESI) calcd. for [M+]C₁₁H₁₀Cl₂N₂O: 556.0170, found 556.0184; IR (neat, cm⁻¹): 3318, 2927, 1588; Melting point: 131-134 °C.

Miconazole, 1-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole (4). Commercially available 2,4-dichlorophenylmethanol (1 g, 5.65 mmol) was dissolved in CH₂Cl₂ (30 mL) and cooled to 0 °C. Et₃N (0.78 mL, 5.65 mmol) was added to this solution, followed by a dropwise addition of methanesulfonyl chloride (MsCl) (0.44 mL, 5.65 mmol). After the addition was complete, stirring was continued at 0 °C for 2 hours and the solvent was removed in *vacuo* to afford crude 2,4-dichlorophenylmesylate. A suspension of sodium hydride (15 mg, 0.36 mmol) in DMF (0.5 mL) was treated with a solution of (3) (100 mg, 0.33 mmol) in DMF (0.5 mL) at 0 °C and the resulting mixture was stirred
at 0 °C for 2 h before a solution of the crude 2,4-dichlorophenylmesylate (125 mg, 0.36 mmol) in DMF (0.5 mL) was added dropwise. The resulting mixture was stirred for 12 hours at room temperature before water (1 mL) was added. The aqueous layer was extracted three times with CH₂Cl₂, dried over Na₂SO₄ and purified using silica gel chromatography (6/4 EtOAc/hexanes) to afford miconazole as a white solid (118 mg, 0.29 mmol, 87 %).

\[ \text{H NMR (CDCl}_3, 400 MHz) \delta 7.48 (s, 2H), 7.40 – 7.28 (m, 4H), 7.23 (s, 1H), 7.06 (s, 1H), 6.93 (s, 1H), 5.05 (d, J = 3.2 Hz, 1H), 4.52 (d, J = 12.5 Hz, 1H), 4.38 (d, J = 12.5 Hz, 1H), 4.27 (d, J = 14.3 Hz, 1H), 4.20 – 4.04 (m, 1H); 13C NMR (CDCl₃, 50 MHz) \delta 57.6, 62.1, 67.6, 120.3, 127.2, 127.8, 128.8, 129.1, 129.3, 130.1, 130.6, 131.6, 133.1, 133.6, 134.6, 138.7, 139.8; (ESI) calcd. for [M+H]C₁₈H₁₄Cl₄N₂O: 413.9860, found 413.9849; IR (neat, cm⁻¹) 2968, 1590; Melting point: 183-184 °C.¹²

Cellular reactive oxygen species (ROS) detection.

**Cellular reactive oxygen species (ROS) detection.** Cells were incubated in LB medium at 37 °C for 12 h, rediluted in LB medium and incubated until an OD₆₀₀ₙₘₐₚ = 0.6 (early-log) was observed (2 h). Growth media was removed using centrifugation (1000 g; 10 min). The resulting cells were washed twice with an equal volume of phosphate buffer saline (PBS, pH= 7.4), resuspended in PBS containing 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) to provide a final working concentration of 25-50 μM in dye depending on the cell line and incubated at 37 °C (30-60 min.) with occasional shaking. At this point, the solution must be kept in the dark. The resulting cells were washed twice with PBS and diluted back to the desired concentration in PBS (OD₆₀₀ₙₘₐₚ 0.05-0.5). ROS detection assay were performed using fluorimetric assay (ex/em: 485/535 nm) in 96-well plates (150 μL) or quartz cuvette (3 mL).

ROS detection assay were performed using fluorimetric assay (ex/em: 485/535 nm) in 96-well plates (150 μL) or quartz cuvette (3 mL). Negative-control: a) DMSO (final concentration not exceeding 10 % volume) b) Unstained cells for autofluorescence in the green emission range. Positive-control: tert-butyl hydroperoxide (TBHP) to a final concentration of 100-250 μM.
**Figure S1.** Fluorescence intensity of compound (5c), miconazole (4), and BAC relative to tert-butyl hydroperoxide (100%) and DMSO (0%) in different cell lines (OD$_{600nm}$ = 0.1). Note: ROS production was not calculated for E. Coli (MG1655) since a very low intensity was observed with positive-control. All cell lines were tested with unstained cells for autofluorescence. *S. Aureus* ATCC 43300 and *E. Coli* SK037.

**Ortho-nitrophenyl-β-galactoside (ONPG) hydrolysis.**

*E.coli* (MG1655) cells were incubated in LB medium at 37 °C with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 10 μg/mL for 12 h. Cells were diluted in LB medium and incubated until a OD$_{600nm}$= 0.6 (early-log) was observed (2 h). Growth media was remove using centrifugation (1000 g; 10 min), the resulting cells were washed twice with an equal volume of phosphate buffer saline (PBS, pH= 7.4) and diluted back in PBS to the desired working concentration (OD$_{600nm}$= 0.1-0.5). Hydrolysis assay were performed using spectrometric assay (λ= 420 nm) in 96-well chambers (110 μL PBS, 15 μL ONPG (12.5 mM), 10 μL analogues in DMSO (1-100 mM), and 15 μL cells (OD$_{600nm}$= 0.1). Negative-control: DMSO (final concentration not exceeding 10% volume). Positive-control: 5 mL cells (OD$_{600nm}$= 0.1) were sonicated until a lysate (OD$_{600nm}$= 0) was obtained (10 x 1 min., 35 % amplitude).

Hydrolysis assay were performed using spectrometric assay (λ= 420 nm) in 96-well chambers (110 μL PBS, 15 μL ONPG (12.5 mM), 10 μL analogues in DMSO (1-100 mM), and 15 μL cells (OD$_{600nm}$= 0.1). Negative-control: DMSO (final concentration not exceeding 10 % volume). Positive-control: 5 mL cells (OD$_{600nm}$= 0.1) were sonicated until a lysate (OD$_{600nm}$= 0) was obtained (10 x 1 min., 35 % amplitude).
Figure S2. Membrane permeabilization induced by miconazole (4) at different concentrations. Assay performed with a bacterial population of $2.2 \times 10^7$ CFU mL$^{-1}$ ($OD_{600nm} = 0.1$).

Figure S3. Membrane permeabilization induced by miconazocytium bromide (5c) at different concentrations close to the MIC value. Assay performed with a bacterial population of $2.2 \times 10^7$ CFU mL$^{-1}$ ($OD_{600nm} = 0.1$).
Figure S4. Dose–response relationship between the concentration of (5c) and the ONPG hydrolysis rate for a bacterial population of $2.2 \times 10^7$ CFU mL$^{-1}$ ($OD_{600nm} = 0.1$).
Biofilm staining and confocal laser scanning microscopy (LSM) Analysis.

The images acquired by confocal laser microscope were processed through a segmentation algorithm (Fiji, ImageJ, Wayne Rasband National Institutes of Health) capable of separating the signals between the background and the foreground (sample). More precisely, the algorithm only considers the signals recorded in each pixel with value exceeding a threshold on the scale of gray tones that was established to distinguish a sample signal from the background noise. A variation (%) on the threshold value calculated by Fiji software was applied to better reflect the distinction of biofilm from background. The software calculated the surface area occupied by biofilm, by measuring the surface area occupied by the fluorescence foreground signal in both green and red channels. The mortality, then, calculated by dividing the surface area occupied in red channel to total surface area occupied by both green and red channels together.

Figure S5. *S. Aureus* biofilms were labeled with Live/Dead stains after 12 h incubation in growth media (LB broth) with a solution at 50 μM antimicrobials. a) Negative control (LB only). b) Positive control (70 % ethanol). c) Miconazole (4). d) Miconazoctylium bromide (5c). e) ≥ 50 μM BAC.
Figure S6. Preformed *S. aureus* biofilms treated with different concentration of BAC over 24 h monitored in 0.9 % NaCl solution. a) Negative control (DMSO only, after 24 h). b) Positive control (70 % ethanol). c) 330 μM (MIC after 5 min). d) 165 μM (1/2 x MIC after 5 min). e) 83 μM (1/4x MIC after 1 h). f) 41 μM (1/8x MIC after 24 h). g) 20 μM (1/8x MIC after 24 h).

REFERENCES

$^1$H NMR spectra
$^{13}\text{C} \text{ NMR Spectra}$