Supporting Information

α-Methylene-γ-butyrolactones attenuate *Staphylococcus aureus* virulence by inhibition of transcriptional regulation

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1) Materials

All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased from Sigma Aldrich or Acros Organics. For all reactions, only commercially available solvents of purissimum grade, dried over molecular sieve and stored under argon atmosphere were used. Solvents for chromatography and workup purposes were generally of reagent grade and purified before use by distillation. In all reactions, temperatures were measured externally. All experiments were carried out under argon.

Column chromatography was performed on Merck silica gel (Acros Organics 0.035–0.070 mm, mesh 60 Å).

¹H- and ¹³C-NMR spectra were recorded on a *Bruker Avance I 360* (360 MHz), a *Bruker Avance I* (500 MHz) or a *Bruker Avance III 500* (500 MHz) NMR-System and referenced to the residual proton and carbon signal of the deuterated solvent, respectively.

HR-ESI-MS, HR-LC-ESI-MS, HR-APCI-MS and HR-LC-APCI-MS mass spectra were recorded with a *Thermo Finnigan LTQ FT Ultra* coupled with a *Dionex UltiMate 3000* HPLC system. ESI-MS and LC-ESI-MS mass spectra were recorded with a *Thermo Finnigan LCQ ultrafleet* coupled with a *Dionex UltiMate 3000* HPLC system.

HPLC analysis was accomplished with a *Waters 2695 separations module*, an *X-Bridge*TM *C18 3.5* μ *m OBD*TM column (4.6 x 100 mm) and a Waters 2996 PDA detector.

HPLC separation was accomplished with a *Waters 2545 quaternary gradient module*, an *X*-*Bridge*TM *Prep C18 10 µm OBD*TM (50 x 250 mm), an *X*-*Bridge*TM *Prep C18 5 µm OBD*TM (30 x 150 mm) or an *YMC Triart C18 5 µm* column (10 x 250 mm), a *Waters 2998 PDA detector* and a *Waters Fraction Collector III*.

2) Synthesis of the γ -lactone probe library

3-(Trifluoromethanesulfonyloxy)-2-cyclohexen-1-one (11)¹



A solution of cyclohexane-1,3-dione (1.12 g, 10.0 mmol, 1.00 eq.) and pyridine (1.57 g, 1.6 mL, 20.0 mmol, 2.00 eq.) in dichloromethane (50 mL) was cooled to -78 °C. Trifluoromethanesulfonic anhydride (1 M in dichloromethane, 12.0 mL, 12.0 mmol, 1.20 eq.) was added slowly and the reaction mixture was stirred for 10 min at -78 °C. The reaction mixture was warmed to 0 °C and after consumption of the diketone (monitored by TLC) HCl_{aq} (1 M, 20 mL) was added. The reaction mixture was extracted with diethyl ether (3 x 50 mL), the organic layer was washed with saturated Na_2CO_{3aq} (100 mL), water (100 mL), dried over Na_2SO_4 , filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 10:1) to yield compound **11** (2.04 g, 8.35 mmol, 84%) as yellowish oil. Data is consistent with that reported in the literature.¹

 $R_{\rm f}$ (hexane/ethyl acetate = 10:1) = 0.16

 R_{f} (hexane/ethyl acetate = 5:1) = 0.31

¹**H-NMR** (500 MHz, CDCl₃) δ = 6.05 (s, 1 H, 2-H), 2.68 (td, ³*J*_{4,5} = 6.3 Hz, ⁴*J*_{2,4} = 1.2 Hz, 2 H, 4-H), 2.44 (t, ³*J*_{5,6} = 6.3 Hz, 2 H, 6-H), 2.12 (p, ³*J*_{4,5} = ³*J*_{5,6} = 6.3 Hz, 2 H, 5-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 197.6, 167.6, 119.3, 36.4, 28.6, 20.9.

Dec-9-yn-5-one (12)¹

To a solution of butylmagnesium bromide (1.82 M in toluene, 2.25 mL, 4.10 mmol, 1.00 eq.) in toluene (15 mL) was added compound **11** (1.10 g, 4.50 mmol, 1.10 eq.) in toluene (15 mL) at -78 °C. The reaction mixture was stirred for 10 min at -78 °C, 10 min at 0 °C, 30 min at room temperature and 30 min at 60 °C. Saturated NH₄Cl_{aq} (15 mL) was added and the reaction mixture was extracted with diethyl ether (3 x 30 mL). The organic layer was washed with water (30 mL), brine (30 mL), dried over Na₂SO₄, filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 20:1) to yield compound **12** (211 mg, 1.39 mmol, 34%) as colourless oil.

 $R_{\rm f}$ (hexane/ethyl acetate = 20:1) = 0.20

 $R_{\rm f}$ (hexane/ethyl acetate = 10:1) = 0.32

¹**H-NMR** (500 MHz, CDCl₃) $\delta = 2.54$ (t, ${}^{3}J_{6,7} = 7.2$ Hz, 2 H, 6-H), 2.40 (t, ${}^{3}J_{3,4} = 7.5$ Hz, 2 H, 4-H), 2.21 (td, ${}^{3}J_{7,8} = 6.9$ Hz, ${}^{4}J_{8,10} = 2.7$ Hz, 2 H, 8-H), 1.94 (t, ${}^{4}J_{8,10} = 2.7$ Hz, 1 H, 10-H), 1.78 (p, ${}^{3}J_{6,7} = {}^{3}J_{7,8} = 7.1$ Hz, 2 H, 7-H), 1.55 (h, ${}^{3}J_{2,3} = {}^{3}J_{3,4} = 7.5$ Hz, 2 H, 3-H), 1.30 (t, ${}^{3}J_{1,2} = {}^{3}J_{2,3} = 7.5$ Hz, 2 H, 2-H), 0.89 (t, ${}^{3}J_{1,2} = 7.5$ Hz, 3 H, 1-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 210.7, 83.8, 69.1, 42.8, 41.1, 26.1, 22.5, 22.4, 17.9, 14.0.

HRMS-ESI (m/z): $C_{10}H_{17}O^+$ [M+H]⁺, calc.: 153.12739, found: 153.12714, $\delta = 1.63$ ppm.

5-Butyl-3-methylene-5-(pent-4-yn-1-yl)dihydrofuran-2(3*H*)-one (1)²



Zinc dust (1.0 g) was activated by rinsing with 0.5 M HCl_{aq} (10 mL), then water (10 mL), anhydrous ethanol (10 mL), and diethyl ether (10 mL), then dried in vacuo (0.05 mbar at 150 °C) overnight and cooled to room temperature under argon atmosphere.

To a solution of compound **12** (100 mg, 0.657 mmol, 1.00 eq.) in THF (5 mL) was added freshly activated zinc dust (51.5 mg, 0.788 mmol, 1.20 eq.) and the mixture was stirred for 15 min at 37 °C. Ethyl 2-(bromomethyl)acrylate (109 μ L, 152 mg, 0.788 mmol, 1.20 eq.) in THF (5 mL) was added and the mixture stirred for 24 h at 37 °C. The reaction mixture was cooled to room temperature, HCl_{aq} (10% w/v, 2 mL) was added and the mixture was stirred for 30 min at room temperature. The solution was filtered and extracted with ethyl acetate (3 x 20 mL). The organic layer was washed with water (40 mL), brine (40 mL), dried over Na₂SO₄, filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 10:1) to yield racemic compound **1** (59.0 mg, 0.268 mmol, 41%) as colourless oil.

 $R_{\rm f}$ (hexane/ethyl acetate = 10:1) = 0.28

¹**H-NMR** (500 MHz, CDCl₃) δ = 6.21 (t, ⁴*J*_{4,6-*Z*} = 2.9 Hz, 1 H, 6-*Z*-H), 5.60 (t, ⁴*J*_{4,6-*E*} = 2.5 Hz, 1 H, 6-*E*-H), 2.69-2.80 (m, 1 H, 4-H), 2.22 (td, ³*J*_{8,9} = 6.9 Hz, ⁴*J*_{9,11} = 2.6 Hz, 2 H, 9-H), 1.96 (t, ⁴*J*_{9,11} = 2.6 Hz, 1 H, 11-H), 1.72-1.84 (m, 2 H, 7-H), 1.63-1.69 (m, 2 H, 12-H), 1.51-1.63 (m, 2 H, 8-H), 1.24-1.36 (m, 4 H, 13-H, 14-H), 0.90 (t, ³*J*_{14,15} = 7.0 Hz, 3 H, 15-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 170.0, 135.9, 122.1, 85.5, 83.7, 69.1, 39.2, 38.5, 37.6, 25.5, 23.0, 22.5, 18.7, 14.1.

HRMS-ESI (m/z): $C_{14}H_{21}O_2^+$ [M+H]⁺, calc.: 221.15361, found: 221.15353, $\delta = 0.34$ ppm.

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1-Phenylhept-6-yn-2-one (13)¹



To a solution of benzylmagnesium bromide (2.00 M in toluene, 3.67 mL, 7.34 mmol, 1.00 eq.) in toluene (15 mL) was added compound **11** (1.97 g, 8.07 mmol, 1.10 eq.) in toluene (15 mL) at -78 °C. The reaction mixture was stirred for 10 min at -78 °C, 10 min at 0 °C, 30 min at room temperature and 30 min at 60 °C. Saturated NH₄Cl_{aq} (15 mL) was added and the reaction mixture was extracted with diethyl ether (3 x 30 mL). The organic layer was washed with water (30 mL), brine (30 mL), dried over Na₂SO₄, filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 50:1 to 1:1) to yield compound **13** (61.2 mg, 0.329 mmol, 5%) as colourless oil.

 $R_{\rm f}$ (hexane/diethyl ether = 2:1) = 0.24

¹**H-NMR** (500 MHz, CDCl₃) δ = 7.33 (m, 2 H, 10-H), 7.26 (m, 1 H, 11-H), 7.21 (m, 2 H, 9-H), 3.70 (s, 2 H, 1-H), 2,61 (t, ${}^{3}J_{3,4}$ = 7.2 Hz, 2 H, 3-H), 2.19 (td, ${}^{3}J_{4,5}$ = 6.9 Hz, ${}^{4}J_{5,7}$ = 2.7 Hz, 2 H, 5-H), 1.93 (t, ${}^{4}J_{5,7}$ = 2.7 Hz, 1 H, 7-H), 1.77 (t, ${}^{3}J_{3,4}$ = ${}^{3}J_{4,5}$ = 7.0 Hz, 2 H, 4-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 207.9, 134.3, 129.5, 128.9, 127.2, 83.6, 69.1, 50.4, 40.5, 22.3, 17.8.

HRMS-ESI (m/z): $C_{13}H_{15}O^+$ [M+H]⁺, calc.: 187.11174, found: 187.11198, $\delta = 1.28$ ppm.

5-Benzyl-3-methylene-5-(pent-4-yn-1-yl)dihydrofuran-2(3*H*)-one (2)²



Zinc dust (1.0 g) was activated by rinsing with 0.5 M HCl_{aq} (10 mL), water (10 mL), anhydrous ethanol (10 mL), and diethyl ether (10 mL). The substance was then dried in vacuo (0.05 mbar at 150 °C) overnight and cooled to room temperature under argon atmosphere. To a solution of compound **13** (61.2 mg, 0.329 mmol, 1.00 eq.) in THF (2.5 mL) was added freshly activated zinc dust (25.8 mg, 0.394 mmol, 1.20 eq.) and the mixture was stirred for 15 min at 37 °C. Ethyl 2-(bromomethyl)acrylate (54.4 μ L, 76.1 mg, 0.394 mmol, 1.20 eq.) in THF (2.5 mL) was added and the mixture stirred for 2 h at 37 °C. The reaction mixture was cooled to room temperature, HCl_{aq} (10% w/v, 1 mL) was added and the mixture was stirred for 30 min at room temperature. The solution was filtered and extracted with ethyl acetate (3 x 10 mL). The organic layer was washed with water (20 mL), brine (20 mL), dried over Na₂SO₄, filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 7:1) to yield racemic compound **2** (40.0 mg, 0.157 mmol, 48%) as colourless oil.

 $R_{\rm f}$ (hexane/ethyl acetate = 7:1) = 0.26

¹**H-NMR** (500 MHz, CDCl₃) $\delta = 7.26-7.30$ (m, 2 H, 15-H), 7.19-7.26 (m, 3 H, 14-H, 16-H), 6.00 (t, ${}^{4}J_{4,6-Z} = 2.9$ Hz, 1 H, 6-Z-H), 5.40 (t, ${}^{4}J_{4,6-E} = 2.6$ Hz, 1 H, 6-E-H), 3.06 (d, ${}^{2}J_{12,12} =$ 14.0 Hz, 1 H, 12-H_a), 2.87 (d, ${}^{2}J_{12,12} = 14.1$ Hz, 1 H, 12-H_b), 2.85 (dt, ${}^{2}J_{4,4} = 17.2$ Hz, ${}^{4}J_{4,6-Z} =$ ${}^{4}J_{4,6-E} = 2.7$ Hz, 1 H, 4-H_a), 2.68 (dt, ${}^{2}J_{4,4} = 17.2$ Hz, ${}^{4}J_{4,6-Z} = {}^{4}J_{4,6-E} = 2.8$ Hz, 1 H, 4-H_b), 2.23 (td, ${}^{3}J_{8,9} = 6.9$ Hz, ${}^{4}J_{9,11} = 2.7$ Hz, 2 H, 9-H), 1.97 (t, ${}^{4}J_{9,11} = 2.7$ Hz, 1 H, 11-H), 1.77-1.92 (m, 2 H, 7-H), 1.64-1.71 (m, 2 H, 8-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 169.9, 135.4, 135.0, 130.9, 128.6, 127.2, 121.7, 84.9, 83.6, 69.2, 45.2, 39.3, 36.4, 22.5, 18.7.

HRMS-ESI (m/z): $C_{16}H_{17}O_2^+$ [M+H]⁺, calc.: 255.13796, found: 255.13801, $\delta = 0.22$ ppm.

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Ethyl 2-methyl-3-phenylacrylate (14)³



To a solution of benzaldehyde (531 mg, 510 μ L, 5.00 mmol, 1.00 eq.) in dichloromethane (15 mL) was added (carbethoxyethylidene)triphenylphosphorane (2.17 g, 6.00 mmol, 1.20 eq.) and the reaction mixture was stirred for 12 h at room temperature. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, hexane/diethyl ether = 3:1) to yield compound **14** (943 mg, 4.96 mmol, 99%) as colourless oil.

 $R_{\rm f}$ (hexane/diethyl ether, 3:1) = 0.41

¹**H-NMR** (500 MHz, CDCl₃) δ = 7.69 (q, ⁴*J*_{3,4} = 1.5 Hz, 1 H, 3-H), 7.37-7.41 (m, 4 H, 6-H, 7-H), 7.30-7.34 (m, 1 H, 8-H), 4.28 (q, ³*J*_{9,10} = 7.1 Hz, 2 H, 9-H), 2.12 (d, ⁴*J*_{3,4} = 1.5 Hz, 3 H, 4-H), 1.35 (t, ³*J*_{9,10} = 7.1 Hz, 3 H, 10-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 168.8, 138.8, 136.1, 129.8, 128.8, 128.5, 128.4, 61.0, 14.5, 14.2.

HRMS-ESI (m/z): $C_{12}H_{15}O_2^+$ [M+H]⁺, calc.: 191.10666, found: 191.10666, $\delta = 0.19$ ppm.

Ethyl 2-(bromomethyl)-3-phenylacrylate (15)³



To a solution of compound **14** (500 mg, 2.63 mmol, 1.00 eq.) in tetrachloromethane (35 mL) was added *N*-bromosuccinimide (NBS, 517 mg, 2.90 mmol, 1.10 eq.) and azobisisobutyronitrile (AIBN, 17.2 mg, 0.105 mmol, 0.0345 eq.) and the reaction mixture was stirred for 18 h under reflux. The reaction was cooled to room temperature and dichloromethane (35 mL) was added. The reaction mixture was washed with water (30 mL) and brine (30 mL), dried over Na₂SO₄ and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/diethyl ether = 5:1) to yield compound **15** (566 mg, 2.22 mmol, 84%) as colourless oil.

 $R_{\rm f}$ (hexane/diethyl ether, 5:1) = 0.32

ppm

¹**H-NMR** (500 MHz, CDCl₃) δ = 7.82 (s, 1 H, 3-H), 7.56-7.60 (m, 2 H, 6-H), 7.44-7.49 (m, 2 H, 7-H), 7.38-7.43 (m, 1 H, 8-H), 4.40 (s, 2 H, 4-H), 4.35 (q, ³*J*_{9,10} = 7.1 Hz, 2 H, 9-H), 1.38 (t, ³*J*_{9,10} = 7.1 Hz, 3 H, 10-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 166.3, 142.8, 134.5, 129.8, 129.7, 129.2, 129.0, 61.6, 27.0, 14.4.

HRMS-ESI (m/z): $C_{12}H_{14}^{79}BrO_2^+$ [M+H]⁺, calc.: 269.01717, found: 269.01696, $\delta = 0.79$ ppm

 $C_{12}H_{14}^{81}BrO_2^+$ [M+H]⁺, calc.: 271.01512, found: 271.01493, $\delta = 0.70$

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5-Hexyn-1-al (16)⁴

$$6 \xrightarrow{4} 2 \xrightarrow{2} 0$$

 $5 \xrightarrow{3} 1$

Dichloromethane (20 mL) was cooled to -78 °C, oxalyl chloride (1.65 g, 1.12 mL, 13.0 mmol, 2.50 eq.) was added and the reaction mixture was stirred for 5 min at -78 °C. Dimethyl sulfoxide (1.02 g, 923 μ L, 13.0 mmol, 2.50 eq.) was added and after gas evolution ceased, the reaction mixture was stirred for further 5 min at -78 °C. 5-Hexyn-1-ol (510 mg, 573 μ L, 5.20 mmol, 1.00 eq.) was added and the reaction mixture was stirred for 5 min at -78 °C. Subsequently, triethylamin (2.63 g, 3.63 mL, 26.0 mmol (5.00 eq.) was added and the reaction mixture was stirred for 15 min at -78 °C. The reaction mixture was warmed to room temperature, washed with HCl_{aq} (0.5 M, 15 mL), saturated NaHCO₃ (15 mL), dried over MgSO₄, filtered and solvents were evaporated under reduced pressure. The residue was purified by fractional vacuum distillation to yield compound **16** (1.02 g, 8.06 mmol, 62%) as colourless oil. Data is consistent with that reported in the literature.⁴

 $R_{\rm f}$ (hexane/diethyl ether, 4:1) = 0.45

¹**H-NMR** (500 MHz, CDCl₃) δ = 9.81 (s, 1 H, 1-H), 2.61 (td, ${}^{3}J_{2,3}$ = 7.2 Hz, ${}^{3}J_{1,2}$ = 1.4 Hz, 2 H, 2-H), 2.27 (td, ${}^{3}J_{3,4}$ = 6.9 Hz, ${}^{4}J_{4,6}$ = 2.7 Hz, 2 H, 4-H), 1.98 (t, ${}^{4}J_{4,6}$ = 2.7 Hz, 1 H, 6-H), 1.85 (p, ${}^{3}J_{2,3}$ = ${}^{3}J_{3,4}$ = 7.1 Hz, 2 H, 3-H).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 201.89, 83.31, 69.50, 42.67, 20.92, 17.91.

(4*S**,5*R** and 4*R**,5*R**)-3-methylene-5-(pent-4-yn-1-yl)-4-phenyldihydrofuran-2(3H)-one (3, 4)^{2, 5}



Zinc dust (1.0 g) was activated by rinsing with 0.5 M HCl_{aq} (10 mL), water (10 mL), anhydrous ethanol (10 mL), and diethyl ether (10 mL). The substance was then dried in vacuo (0.05 mbar at 150 °C) overnight and cooled to room temperature under argon atmosphere.

To a solution of compound **16** (45.2 mg, 0.470 mmol, 1.20 eq.) in THF (7 mL) was added freshly activated zinc dust (30.8 mg, 0.470 mmol, 1.20 eq.) and the mixture was stirred for 15 min at 37 °C. Compound **15** (100 mg, 0.392 mmol, 1.00 eq.) in THF (7 mL) was added and the mixture stirred for 15 h at 37 °C. The reaction mixture was cooled to room temperature, HCl_{aq} (10% w/v, 7 mL) was added and the mixture was stirred for 30 min at room temperature. The solution was filtered and extracted with ethyl acetate (3 x 30 mL). The organic layer was washed with water (60 mL), brine (60 mL), dried over Na₂SO₄, filtered and the solvents were evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 10:1 to 3:1) to yield racemic compounds **3** (17.7 mg, 0.074 mmol, 19%) and **4** (49.7 mg, 0.207 mmol, 53%) as colourless oils.

Diastereomer 3

 $R_{\rm f}$ (hexane/Et₂O, 3:1) = 0.46

¹**H-NMR** (500 MHz, CDCl₃) δ = 7.36-7.40 (m, 2 H, 14-H), 7.30-7.35 (m, 1 H, 15-H), 7.19-7.23 (m, 2 H, 13-H), 6.36 (d, ${}^{4}J_{4,6-Z}$ = 3.3 Hz, 1 H, 6-Z-H), 5.39 (d, ${}^{4}J_{4,6-E}$ = 2.8 Hz, 1 H, 6-*E*-H), 4.40 (td, ${}^{3}J_{4,5}$ = ${}^{3}J_{5,7a}$ = 7.9 Hz, ${}^{3}J_{5,7b}$ = 4.0 Hz, 1 H, 5-H), 3.79 (dt, ${}^{3}J_{4,5}$ = 6.8 Hz, ${}^{4}J_{4,6-Z}$ = ${}^{4}J_{4,6-E}$ = 3.2 Hz, 1 H, 4-H), 2.22 (td, ${}^{3}J_{8,9}$ = 6.9 Hz, ${}^{4}J_{9,11}$ = 2.6 Hz, 2 H, 9-H), 1.73-1.1.96 (m, 4 H, 7-H, 8-H_a, 11-H), 1.56-1.65 (m, 1 H, 8-H_b).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 169.8, 140.3, 139.0, 129.3, 128.5, 128.0, 123.9, 85.1, 83.5, 69.2, 52.8, 33.8, 24.4, 18.3.

HRMS-ESI (m/z): $C_{16}H_{17}O_2^+$ [M+H]⁺, calc.: 241.12231, found: 241.12211, $\delta = 0.80$ ppm.

Diastereomer 4

 R_{f} (hexane/Et₂O, 3:1) = 0.35

¹**H-NMR** (500 MHz, CDCl₃) $\delta = 7.33-7.37$ (m, 2 H, 14-H), 7.29-7.33 (m, 1 H, 15-H), 7.13-7.16 (m, 2 H, 13-H), 6.45 (d, ${}^{4}J_{4,6-Z} = 2.8$ Hz, 1 H, 6-Z-H), 5.62 (d, ${}^{4}J_{4,6-E} = 2.5$ Hz, 1 H, 6-E-H), 4.73 (ddd, ${}^{3}J_{5,7a} = 9.9$ Hz, ${}^{3}J_{4,5} = 7.9$ Hz, ${}^{3}J_{5,7b} = 3.8$ Hz, 1 H, 5-H), 4.37 (dt, ${}^{3}J_{4,5} = 7.8$ Hz, ${}^{4}J_{4,6-Z} = {}^{4}J_{4,6-E} = 2.6$ Hz, 1 H, 4-H), 2.04-2.16 (m, 2 H, 9-H), 1.86 (t, ${}^{4}J_{9,11} = 2.7$ Hz, 1 H, 11-H), 1.65-1.74 (m, 1 H, 8-H_a), 1.42-1.52 (m, 1 H, 8-H_b), 1.31-1.39 (m, 1 H, 7-H_a), 1.22-1.30 (m, 1 H, 7-H_b).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 170.4, 139.0, 137.5, 129.2, 128.9, 127.9, 124.6, 83.6, 81.3, 69.0, 49.5, 31.5, 24.8, 18.1.

HRMS-ESI (m/z): $C_{16}H_{17}O_2^+$ [M+H]⁺, calc.: 241.12231, found: 241.12211, $\delta = 0.80$ ppm.

(4R*,5R*)-3-methyl-5-pentyl-4-phenyldihydrofuran-2(3H)-one (10)



To a solution of compound **3** (100 mg, 0.406 mmol, 1.00 eq.) in ethanol (10 mL) was added 5% Pd on carbon (5.00 mg) and the mixture was stirred for 24 h at room temperature under hydrogen atmosphere. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, hexane/ethyl acetate = 10:1) to yield inseparable racemic diastereomers **10** (89.0 mg, 0.361 mmol, 89%) as colourless oil.

 R_{f} (hexane/EtOAc, 5:1) = 0..49

¹**H-NMR** (500 MHz, CDCl₃) δ = 7.37-7.42 (m, 2 H, 14-H, major diastereomer), 7.36-7.29 (m, 1.2 H, 15-H major, 14-H minor), 7.23-7.27 (m, 2.1 H, 13-H major, 15-H minor), 7.13-7.16 (m, 0.2 H, 13-H minor), 4.68-4.73 (m, 0.1 H, 5-H minor), 4.36-4.41 (m, 1 H, 5-H major), 3.37-3.42 (m, 0.1 H, 4-H minor), 2.99-3.07 (m, 0.1 H, 3-H minor), 2.77-2.86 (m, 2 H, 3-H major, 4-H major), 1.6-1.68 (m, 2.2 H, 7-H both), 1.45-1.56 (m, 1.1 H, 8_b-H both), 1.17-1.37 (m, 8.8 H both, 6-H both, 8_a-H both, 9-H both, 10-H both), 0.88-0.92 (m, 0.3 H, 11-H, minor), 0.82-0.87 (m, 3 H, 11-H, major).

¹³**C-NMR** (91 MHz, CDCl₃) δ = 179.35, 178.18, 138.33, 137.48, 129.25, 129.01, 128.02, 127.85, 127.59, 84.50, 84.15, 56.92, 50.62, 43.96, 39.00, 34.79, 33.67, 31.61, 25.59, 25.53, 22.63, 22.55, 14.10, 14.07, 13.41, 11.60.

HRMS-ESI (m/z): $C_{16}H_{23}O_2^+$ [M+H]⁺, calc.: 247.16926, found: 247.16920, $\delta = 0.22$ ppm.

3) NMR-Spectra















¹³C-NMR of **13**









¹³C-NMR of **14**















4) LC-HRMS-ESI-Spectra

LC-HRMS-ESI of 1











LC-HRMS-ESI of 10



5) In situ labeling

Bacteria were grown in lysogenic broth (LB, for *E. coli* strains) or in brain heart broth (BHB, for *S. aureus* strains) and a quantity equivalent with 1 mL of $OD_{600} = 4$ was harvested 1 h after reaching stationary phase by centrifugation (10 min, 4,000 g, 4 °C) for analytical and 5 mL for preparative studies, respectively. After washing with PBS, the cells were resuspended in 200 µL and 500 µL of PBS for analytical and preparative experiments. Unless indicated otherwise, bacteria were incubated for 2 h with varying concentrations of probe (1 µL in DMSO) at RT. Subsequently, the cells were washed three times with 1 mL PBS and lysed with a cell homogenizer (Precellys 24 and Precellys Glas-Kit 0.5 mm Small, *PEQLAB Biotechnologies GmbH*). Cell envelope and cytosol were separated by centrifugation (30 min, 21,000 g, 4 °C). For heat controls, the cells were lysed, the proteins were denatured with 2 µL of SDS (21.5 % in ddH₂O) at 96 °C for 6 min and cooled to RT before the probe was applied.⁶

6) Click reaction and analytical gel-based analysis

In case of analytical labelling, the click reaction was carried out with 44 μ L of proteome, so that after addition of all reagents a total volume of 50 μ L was reached. Both, cytosol fraction

and cell envelope fraction were analysed separately. Therefore, 1 μ L RhN₃ (5 mM in DMSO) was added to 44 μ L of proteome, followed by 1 μ L TCEP solution (53 mM in ddH₂O) and 3 μ L ligand TBTA (83 mM in DMSO/*tert*-butanol). Samples were gently vortexed and the cycloaddition was initiated by the addition of 1 μ L CuSO₄ solution (50 mM in ddH₂O). The reaction was incubated for 1 h at RT. For analytical gel electrophoresis, 50 μ L 2×SDS loading buffer were added and 50 μ L were applied on the gel. Roti[®]-Mark STANDARD (*Carl Roth GmbH & Co. KG*, for Coomassie staining) and BenchMarkTM Fluorescent Protein Standard (*Life Technologies Corp.*) were applied as markers to determine the protein mass. After application of the protein samples, the gels were developed for 4-5 h with 300 V. Fluorescence scans of SDS gels were performed with a *Fujifilm* Las-4000 luminescent image analyser containing a VRF43LMD3 lens and a 575DF20 filter.

7) Click reaction and preparative gel-based analysis

The cytosol fraction was used for preparative analysis. 3 µL trifunctional linker⁷ (10 mM in DMSO) was added to 500 µL labelled proteome, followed by 10 µL TCEP solution (53 mM in ddH₂O) and 30 µL ligand TBTA (83 mM in DMSO/tert-butanol). The samples were gently vortexed and the cycloaddition was initiated by the addition of 10 µL CuSO₄ solution (50 mM in ddH₂O). The reaction was allowed to proceed for 1 h at RT. Reactions for enrichment were carried out together with a control lacking the probe to compare the results of the biotinavidin enriched samples with the background of unspecific protein binding on avidin-agarose beads. The proteins were precipitated by addition of cold acetone (1 mL, -80 °C) and incubation for 18 h at -20 °C. Then the proteins were pelletized (15 min, 21,000 g, 4 °C) and the supernatant was discarded. The proteins were washed with prechilled methanol $(2 \times 200 \text{ mL}, -80 \text{ °C}, \text{ resuspension by sonication}, 5-10 \text{ sec}, 10 \% \text{ max}$. intensity; 15 min, 21,000 g, 4 °C). Subsequently, the pellet was dissolved at RT in 1 mL 0.4 % SDS in PBS by sonication and incubated under gentle mixing with 50 μ L of prewashed (3 × 1 mL 0.4 % SDS in PBS) avidin-agarose beads (avidin-agarose from egg white, 1.1 mg/mL in aqueous glycerol suspension, Sigma-Aldrich Co. LLC) for 2 h at RT. The beads were washed with 0.4 % SDS in PBS (3 \times 1 mL), aqueous urea (6 M, 2 \times 1 mL) and PBS (3 \times 1 mL). 50 μ L of 2 \times SDS loading buffer were added and the proteins were released for preparative SDS-PAGE by incubation for 6 min at 96 °C. The beads were pelletized (3 min, 21,000 g) and the supernatant was isolated and stored at -80 °C. The supernatant was applied on a preparative gel, and developed for 4-5 h (300 V). After gel electrophoresis, the bands were visualized using a Fujifilm Las-4000 luminescent image analyser containing a VRF43LMD3 lens and a 575DF20 filter. The observed bands were cut out, isolated and reduced to small pieces, prior to further processing.

8) Click reaction and preparative gel-free analysis

The cytosol fraction was used for preparative analysis. 3 µL trifunctional linker (10 mM in DMSO) were added to 500 µL labelled proteome, followed by 10 µL TCEP solution (53 mM in ddH₂O) and 30 µL TBTA (83 mM in DMSO/tert-butanol). The samples were gently vortexed and the cycloaddition was initiated by the addition of 10 µL CuSO₄ solution (50 mM in ddH_2O). The reaction was allowed to proceed for 1 h at RT. Reactions for enrichment were carried out together with a control lacking the probe to compare the results of the biotinavidin enriched samples with the background of unspecific protein binding on avidin-agarose beads. The proteins were precipitated by addition of cold acetone (1 mL, -80 °C) and incubation for 18 h at -20 °C. Then the proteins were pelletized (15 min, 21,000 g, 4 °C) and the supernatant was discarded. The proteins were washed with prechilled methanol $(2 \times 200 \text{ mL}, -80 \text{ °C}, \text{ resuspension by sonication}, 5-10 \text{ sec}, 10 \% \text{ max}$. intensity; 15 min, 21,000 g, 4 °C). Subsequently, the pellet was dissolved at RT in 1 mL 0.4 % SDS by sonication and incubated under gentle mixing with 50 μ L of prewashed (3 × 1 mL 0.4 % SDS) avidin-agarose beads (avidin-agarose from egg white, 1.1 mg/mL in aqueous glycerol suspension, Sigma-Aldrich Co. LLC) for 2 h at RT. The beads were washed with 0.4 % SDS in PBS (4 \times 1 mL), aqueous urea (6 M in ddH₂O, 4 \times 1 mL) and PBS (4 \times 1 mL). The beads were suspended in HEPES buffer (20 mM, pH = 7.5) containing urea (7 M) and thiourea (2 M). The proteins were reduced by addition of DTT (1 M in ddH_2O , 0.2 μ L, 45 min, RT) and then alkylated by addition of iodoacetamide (0.55 M in ddH₂O, 2 µL, 30 min, RT, in the dark). The reaction was quenched by addition of DTT (1 M in ddH₂O, 30 min, RT). The proteins were first digested with Lys-C (0.5 μ g/ μ L in 50 mM HEPES in ddH₂O, pH = 8, 1 μ L, 4 h in the dark, RT). The digest was then diluted with TEAB (50 mM in ddH₂O, 600 µL) and trypsin $(0.5 \ \mu g/\mu L$ in 50 mM acetic acid in ddH₂O, 1.5 μL) was added. After incubation for 18 h at 37 °C the reaction was stopped by addition of formic acid (4 µL). The beads were pelletized (1 min, 1000 g) and the supernatant was transferred into LoBind tube (*Eppendorf AG*). The beads were once washed with 0.1 % FA (100 µL, 1 min, 21,000 g) and the supernatant was transferred into the LoBind tube. The samples were desalted using Sep-Pak C18 1 cc Vac Cartridges (*Waters Corp.*). The cartridges were first equilibrated with ACN $(1 \times 1 \text{ mL})$, elution buffer (ACN/H₂O/FA = 80:19.5:0.5 v/v/v, 1×1 mL) and 0.1 % TFA (in ddH₂O, 1×1 mL). Then the sample was applied to the cartridge and the proteins were washed with 0.1 % TFA (in ddH₂O, 1 × 1 mL) and 0.5 % FA (in ddH₂O, 1 × 0.5 mL). The proteins were eluted into a LoBind tube (ACN/H₂O/FA = 80:19.5:0.5 v/v/v, 2 × 250 μ L) and concentrated *in vacuo* in a vacuum centrifuge (4 h, 1 mbar, RT). The remaining peptides were stored at -20 °C.

9) In gel digestion

The gel pieces corresponding to fluorescent bands were directly cut on the gel and washed with ddH₂O (100 µL, 15 min, 550 rpm, RT), MeCN/50 mM ammonium bicarbonate (200 µL, 15 min, 550 rpm, RT) and MeCN (100 µL, 10 min, 550 rpm, RT). The shrunken gel pieces were swollen in 50 mM ammonium bicarbonate (100 µL, 5 min, 550 rpm, RT), before additional MeCN (100 µL, 15 min, 550 rpm, RT) was added. The supernatant was removed and the gel pieces were again washed with MeCN (100 µL, 10 min, 550 rpm, RT) and then dried under vacuum in a centrifugal evaporator (15 min, 1 mbar, RT). The proteins were reduced by addition of DTT solution (10 mM in 50 mM ammonium bicarbonate, 100 µL, 45 min, 550 rpm, 56 °C) to the gel pieces. The pieces were then washed with MeCN (100 µL, 10 min, 550 rpm, RT), prior to alkylation with iodacetamide solution (55 mM in 50 mM ammonium bicarbonate, 100 µL, 30 min in the dark, 550 rpm, RT). The gel pieces were afterwards washed with MeCN/50 mM ammonium bicarbonate (100 µL, 15 min, 550 rpm, RT) and MeCN (100 µL, 10 min, 550 rpm, RT) and then dried in vacuo in a centrifugal evaporator (15 min, 1 mbar, RT). Digest solution was added (100 µL, 10 min, 4 °C then 37 °C, 300 rpm overnight). The next day the supernatant was transferred into a LoBind tube (Eppendorf AG) and 25 mM ammonium bicarbonate (100 µL, 15 min sonication, RT) was added to the gel pieces. Then MeCN (100 µL) was added and the sample sonicated for 15 min. The supernatant was then transferred into the same LoBind tube. 5 % FA (100 µL, 15 min sonication, RT) was added to the gel pieces, followed by additional MeCN (100 µL, 15 min sonication, RT). The supernatant was then transferred into the same LoBind tube and replaced by MeCN (100 µL, 15 min sonication, RT). The supernatant was transferred into the LoBind tube and the solvent was removed in vacuo in a vacuum centrifuge (4 h, 1 mbar, RT). The remaining peptides were stored at -80°C.⁸

10) Sample preparation for mass spectrometry

Centrifugal filters (modified Nylon, 0.45 μ m, low protein binding, *VWR International, LLC*) were pre-rinsed with ddH₂O (1 × 500 μ L, 13000 rpm, 1 min, RT), aqueous NaOH (1 × 500 μ L, 13000 rpm, 1 min, RT), ddH₂O (2 × 500 μ L, 13000 rpm, 1 min, RT) and 1 % FA

 $(1 \times 500 \ \mu\text{L}, 13000 \ \text{rpm}, 1 \ \text{min}, \text{RT})$. The peptides were dissolved in 1 % FA (20 $\mu\text{L}, 15 \ \text{min}$ sonication, RT) and added to the pre equilibrated filters and centrifuged (13000 rpm, 1 min, RT). The filtrate was transferred into a vial and stored at 4 °C until the measurement was performed.

11) Identification of binding sites

To analyse the binding sites of **3** and to identify the respective sites, recombinant proteins were incubated with a 10-fold excess of inhibitor for 30 min at RT. Unbound probe was removed during three buffer exchange steps with 25 mM ammonium hydrogen carbonate using size exclusion filters (5 kDa, *Sartorius Stedim Biotech S.A.*). Labelled proteins were digested overnight with trypsin or chymotrypsin (*Promega Corp.*), which were added at a ratio of 1:100 (w/w) of the protein amount. Acidifying the sample with trifluoroacetic acid to a final concentration of 0.5 % stopped the digest. Samples were filtered and directly used for MS analysis.

12) Mass spectrometry and bioinformatics

Measurements were performed using an Orbitrap XL coupled online to an Ultimate 3000 nano HPLC system (*Thermo Fisher Scientific Inc.*). Samples were loaded on a trap column and separated on a 15 cm C18 column (2 µm, 100 Å, *Thermo Fisher Scientific Inc.*) during a 50 min gradient from 5 to 30 % acetonitrile, 1 % formic acid. For protein identification either the five most intense ions of the full scan were fragmented using CID or a combination of CID of the three most intense ions and HCD of the two most intense ions was performed. The mass spectrometry data were searched using the SEQUEST algorithm against the corresponding databases via the Proteome Discoverer Software 1.3 (*Thermo Fisher Scientific Inc.*). Protein *N*-terminal acetylation, oxidation on methionine and additional masses for the specific inhibitors were added as variable modifications. Depending on the enzyme used for the digest, enzyme specificity was set to trypsin or chymotrypsin and mass tolerances of the precursor and fragment ions to 10 ppm and 0.5 Da, respectively. Only sites found with high confidence and localization probabilities better than 0.99 that were identified with the most spectra were considered as binding sites. Results are shown in Table S2 and S3.

13) Recombinant expression

The major hits of MS analysis were recombinantly expressed in *E. coli* as an internal control of the MS results by using the InvitrogenTM Gateway[®] Technology. Target genes were

amplified from the corresponding genomes by PCR with an AccuPrimeTM Pfx DNA Polymerase kit with 65 ng of genomic DNA, prepared by standard protocols. attB1 forward primer and attB2 reverse primer were designed to yield attB-PCR Products needed for Gatewav[®] Technology. PCR products were identified on agarose gels and gel bands were isolated and extracted with an E.Z.N.A.TM MicroEluteTM Gel Extraction Kit. Concentrations of DNA were measured by a Tecan Infinite[®] M200 PRO plate reader. 100 fmol of purified attB-PCR product and 50 fmol of attP-containing donor vector pDONR[™] 201 in TE buffer were used for *in vitro* BP recombination reaction with BP Clonase[™] II enzyme mix to yield the appropriate attL-containing entry clone. After transformation in chemically competent One Shot[®] TOP10 E. coli (Invitrogen), cells were plated on LB agar plates containing 25 µg mL⁻¹ kanamycin. Clones of transformed cells were selected and grown in kanamycin LB. Cells were harvested and plasmids were isolated using an E.Z.N.A.[™] Plasmid Mini Kit. The corresponding attB-containing expression clone was generated by in vitro LR recombination reaction of approx. 50 fmol of the attL-containing entry clone and 50 fmol of the attRcontaining destination vector pDest using LR Clonase[™] II enzyme mix in TE buffer. The expression clone was transformed in chemically competent BL21 E. coli cells (Novagen) and selected on LB agar plates containing 100 µg mL⁻¹ ampicillin. Validity of the clones was confirmed by plasmid sequence analysis. Recombinant clones were grown in ampicillin LB and target gene expression was induced with anhydrotetracyclin. The bacterial cell pellets were washed with PBS, resuspended in binding buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), lysed by French press and sonication. The protein was then purified with StrepTrapTM HP columns and stored in the corresponding buffer.

14) Primer for recombinant expression

MgrA, *S. aureus* NCTC 8325 Forward primer: 5'-ggggacaagtttgtacaaaaaagcaggctttatgtctgatcaacataatttaaaaga Reverse primer: 5'-ggggaccactttgtacaagaaagctgggtgttatttttcctttgtttcatcaaatg

SarA, S. aureus NCTC 8325 Forward primer: 5'-ggggacaagtttgtacaaaaagcaggctttatggcaattacaaaaatcaatgattg Reverse primer:

5`-ggggaccactttgtacaagaaagctgggtgttatagttcaatttcgttgtttgctt

SarR, S. aureus NCTC 8325

Forward primer:

5'-ggggacaagtttgtacaaaaagcaggctttatgagtaaaattaatgacattaatgattt Reverse primer:

5`-ggggaccactttgtacaagaaagctgggtgttaatttttaatgtattcttctaattctg

TrxA, S. aureus NCTC 8325

Forward primer:

5' - ggggacaagtttgtacaaaaaagcaggctttatggcaatcgtaaaagtaacaga

Reverse primer:

5' - ggggaccactttgtacaagaaagctgggtgttataaatgtttatctaaaacttcagcta

15) MTT assay for cytotoxicity determination

HeLa cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FCS (fetal calf serum). Cells were plated in 96 well flat-bottom plates (4,500 cells in 100 μ L medium for each well) and cultured for 24 h at 37 °C and 5% CO₂. Culture medium was removed and probes (DMSO stocks) were diluted 1:1,000 in new culture medium and added to cells. After 24 h incubation at 37 °C and 5% CO₂, MTT solution (20 μ L, 5mg/mL in PBS) was added followed by incubation for 2 h at 37 °C, 5% CO₂. The culture medium was removed and DMSO added (200 μ L) to dissolve the formazan salt (metabolic product of MTT). The resulting optical density was measured at 579 nm (background subtraction at 630 nm) by a TECAN Infinite M200 pro plate reader. All measurements were performed in triplicates and EC₅₀ values were calculated from curve fittings.

16) TrxA-Assay⁹

The TrxA-catalysed reduction of disulfide bonds within insulin was measured by a wellestablished assay (Holmgren, 1979). Insulin becomes therefore insoluble and the enzyme turnover can be followed by increasing turbidity. Insulin solutions were prepared by suspending insulin (50 mg, Sigma Aldrich) in 50 mM Tris-HCl (4 mL, pH 8.0) followed by addition of HCl_{aq} (1 M) until pH 2-3 and rapid addition of $NaOH_{aq}$ (1 M) until pH 8.0 was reached. Finally the volume was adjusted to 5 mL with water. The insulin stocks were stored at -20 °C. DTT in (100 mM in H₂O) was prepared fresh each day and was stored at 4 °C. TrxA buffer consisted of 100 mM KH₂PO₄ pH 7.0 and 2 mM EDTA. Measurements were done in 96 well plates at room temperature in a total volume of 200 μ L. Thioredoxin (170 μ M in TrxA buffer) was reduced by 10-fold molar excess of DTT solution for 1 h on ice and diluted in TrxA buffer to a final concentration of 1 μ M. 176 μ L of TrxA dilution were added to each well of a 96 well plate (triplicates) and increasing concentration of probe **3** (2 μ L in DMSO) was added. The plate was incubated for 2 h at room temperature. Insulin solution (20 μ L) and DTT solution (2 μ L) were added and the absorption at 650 nm was recorded on a Tecan Infinite® M200 PRO plate reader every 2 min for 30 min at room temperature. Each concentration was tested in at least three independent trials in triplicates.

17) MurA inhibition assay^{10, 11}

The inhibition of MurA1 and MurA2 by probe 3 was measured by examining the release of pyrophosphate from the MurA-catalysed enolpyruvyl transfer reaction with phosphoenolpyruvate (PEP) and UDP-N-acetylglucosamine (UDPAG) in a malachite green heptamolybdate assay. MurA1 and MurA2 from S. aureus NCTC 8325 were purified as described in "Recombinant Expression" and immediately desalted into 50 mM Hepes buffer pH 7.5. To MurA1 and MurA2 (5 µM and 1 µM in 60 µL 50 mM Hepes buffer pH 7.5) was added 1.2 µL probe 3 (DMSO solution) and the well plate was incubated 30 min at room temperature. 53.8 µL UDPAG-Solution (in 50 mM Hepes pH 7.5, final UDPAG concentration 1 mM) was added and the plate incubated for 10 min at room temperature. Then to each well was added 5 µL of 24 mM PEP (in 50 mM Hepes buffer pH 7.5, final PEP concentration 1 mM). The plate was shaken for 10 min, and allowed to sit at room temperature for 30 min. 20 µL from each well was withdrawn and mixed in another well plate with 80 µL of a 3:1 mixture of 0.045% malachite green in H₂O and 4.2% ammonium heptamolybdate in 4 M HCl. After 10 min, the absorption at 660 nm was read with a Tecan Infinite® M200 PRO plate reader. Each concentration was tested in at least three independent trials in triplicates.

18) Construction of NCTC8325 mutants¹²

We constructed *mgrA*, *sarA*, *sarR* and *sarA/sarR* mutants from strain NCTC8325 by phage ϕ 11 transduction of *mgrA*::*cat*, *sarA*::*kan* and *sarR*::*erm* as described previously. Colonies of interest were selected on BHI agar plates containing sodium citrate (10 g/ml) and the corresponding antibiotics (chloramphenicol 5 µg/ml, kanamycin 50 µg/mL or erythromycin 5

 μ g/mL). Mutations were confirmed by PCR reactions of the corresponding genes and their flanking regions.

19) Supporting tables and figures

Strain	Relevant Characteristics	Source or Reference
RN4220	Restriction-deficient transformation recipient	Kreiswirth et al. $(1983)^{13}$
RN6390	Laboratory strain related to 8325-4, rsbU	Novick (1993) ¹⁴
NCTC8325	Standard laboratory strain, rsbU	Novick (1967) ¹⁵
NCTC8325-4	NCTC8325 strain cured of three prophages	Novick (1967) ¹⁵
Mu50	Aminoglycosides and tetracycline resistant strain	Hiramatsu et al. (1997) ¹⁶
USA300 FPR3757	Clinically associated strain	LGS Standards (Wesel, Germany)
RN6390 $\Delta mgrA$	RN6390, mgrA::cat (Cm ^r)	Troung-Balduc et al. (2008) ¹⁷
PC1839	NCTC8325-4, <i>sarA::km</i> (Km ^r)	Chan & Foster (1998) ¹⁸
WA1396	NCTC8325-4, sarR::ermB (Em ^r)	Gustafsson & Oscarsson (2008) ¹⁹
WA1461	NCTC8325-4, <i>sarA::km</i> , <i>sarR::ermB</i> (Km ^r Em ^r)	Gustafsson & Oscarsson (2008) ²⁰
NCTC8325 $\Delta mgrA$	NCTC8325, mgr <i>A::cat</i> (Cm ^r)	this study
NCTC8325 $\Delta sarA$	NCTC8325, <i>sarA::km</i> (Km ^r)	this study
NCTC8325 $\Delta sarR$	NCTC8325, sarR::ermB (Em ^r)	this study
NCTC8325 $\Delta sarA/R$	NCTC8325, <i>sarA::km</i> , <i>sarR::ermB</i> (Km ^r Em ^r)	this study

Table S1. S. aureus strains used in this study.

 Cm^r , resistance to chloramphenicol; Em^r , resistance to erythromycin; Km^r , resistance to kanamycin; Tc^r , resistance to tetracyclin.

Protein		Protoin Description	Accession	Donligatos	Sequence	Unique	DSMg	Scores of individual				ΔΔε	MW
Stram	Abbreviation	Totem Description	Number	Replicates	Coverage	Peptides	1 51415	experiments		ments		AAS	141 44
Mu50	AacA-AphD	Bifunctional AAC/APH	P0A0C0	2	36	15	42	47.5	65.8	-	-	479	56.8
	Mur A 1	UDP-N-acetylglucosamine 1-	0021115	2	11	4	7	8.0	11.2			421	45.0
	MulAI	carboxyvinyltransferase 1	Q951115	2	11	4	/	0.0	11.5	-	-	421	45.0
	MurA2	UDP-N-acetylglucosamine 1-	P65456	4	53	14	81	873	102.7	274	30.8	419	45.0
		carboxyvinyltransferase 2											
	MgrA	HTH-type transcriptional regulator	Q99VT5	4	42	5	13	25.2	7.2	6.3	ni	147	17.1
	SarA	Transcriptional regulator	Q7A2W5	4	31	4	10	7.8	8.6	11.6	ni	124	14.7
	SarR	HTH-type transcriptional regulator	Q7A2M3	4	32	4	25	19.8	33.1	14.7	10.1	115	13.7
	TrxA	Thioredoxin	P0A0K4	4	36	3	10	9.6	16.8	5.4	ni	104	11.4
NCTC8325	MgrA	HTH-type transcriptional regulator	Q2G0B1	4	50	7	27	11.0	5.9	34.7	23.7	147	17.1
	SarA	Transcriptional regulator	Q2G2U9	4	31	4	10	6.0	13.3	9.2	ni	124	14.7
	SarR	HTH-type transcriptional regulator	Q9F0R1	4	46	5	43	29.5	51.6	43.7	13.5	115	13.7
	TrxA	Thioredoxin	Q2FZD2	4	48	4	18	11.9	21.4	22.2	ni	104	11.4
USA300	MgrA	Transcriptional regulator MarR family	Q2FIV3	2	31	5	9	5.9	21.3	-	-	147	17.1
	SarA	Transcriptional regulator	Q2FJ20	2	46	6	13	14.9	24.4	-	-	124	14.7
	SarR	HTH-type transcriptional regulator	Q2FEJ8	2	32	4	14	19.3	25.0	-	-	115	13.7
	TrxA	Thioredoxin	Q2FHT6	2	36	3	4	7.8	5.9	-	-	104	11.4

 Table S2. Proteins identified by gel-based analysis.

This list of shows strain, protein abbreviation, protein description, accession number, number of replicates, sequence coverage in percent, number of unique peptides, number of peptide spectral matches (PSM's), scores of individual experiments (-, no further experiment; ni, not identified), number of amino acids and molecular weight in kDa.

Table	S3 .	Proteins	identified	1 by	gel-free	analysis	in S.	aureus USA300.	
				/	LJ				

Protoin Description (Abbreviation)	Accession	n Sequence Unique Coverage Peptides		DSMg	Mean		Scores		Scores 20 µM Probe 3			AAs	MW
Protein Description (Addreviation)	Accession			r Sivis	Score	ore 5 µM probe 3		e 3					IVI VV
Thioredoxin (TrxA)	Q2FHT6	60	9	64	32,8	15,1	22,8	32,4	47,5	26,7	52,4	104	11,4
HTH-type transcriptional regulator (SarR)	Q2FEJ8	62	11	51	29,3	12,1	11,4	19,2	41,1	33,0	59,2	115	13,7
Iron-sulphur cluster repair protein (ScdA)	Q2FK11	44	11	39	21,7	11,7	7,1	13,2	40,3	23,0	34,6	224	25,5
Methylenetetrahydrofolate-tRNA-(uracil-5-)-	Q2FHI7	33	10	45	21,4	14,9	13,6	15,4	28,6	28,8	27,4	435	48,3
methyltransferase (TrmFO)													
Nitric oxide synthase oxygenase	Q2FFI1	43	13	46	20,3	20,5	31,1	13,2	25,1	25,1	6,8	358	41,7
Thioredoxin reductase	Q2FIM9	54	15	42	19,6	9,8	7,3	5,5	38,9	29,6	26,6	311	33,6
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (MurA)	Q2FF27	35	8	27	15,1	ni	9,9	10,4	5,5	15,4	34,3	421	45,0
Respiratory nitrate reductase, beta subunit	Q2FEA1	27	8	28	13,8	9,5	15,4	4,8	25,9	11,0	16,3	481	55,2
Staphylococcal accessory regulator (SarS)	Q2FKE7	22	7	26	12,3	8,0	8,8	5,5	17,4	23,7	10,3	250	29,9
Fructose-1,6-bisphosphatase class 3	Q2FDY9	15	8	19	12,0	ni	9,3	6,6	16,0	17,7	10,1	654	76,1
Glutathione peroxidase	Q2FHD4	46	6	25	11,9	5,1	13,3	5,7	16,0	24,9	6,4	158	18,1
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Q2FK15	33	4	17	11,0	5,5	5,0	ni	9,4	16,9	18,2	238	26,6
DNA-directed RNA polymerase subunit alpha	Q2FER5	25	5	16	11,0	ni	6,7	20,4	14,3	6,4	7,3	314	35,0
Transcriptional regulator, MarR family (MgrA)	Q2FIV3	37	6	20	10,1	11,8	4,8	5,2	ni	15,8	12,9	147	17,1
Pyridine nucleotide-disulfide oxidoreductase	Q2FGW2	38	8	14	8,1	5,7	ni	9,1	10,0	10,6	5,0	328	36,7
30S ribosomal protein S12	Q2FJ95	36	5	14	7,0	4,7	7,7	5,8	ni	9,2	7,7	137	15,3

This list shows protein description, accession number, protein abbreviation, sequence coverage in percent, number of unique peptides, number of peptide spectral matches (PSM's), mean score, scores of individual experiments (ni, not identified), number of amino acids and molecular weight in kDa. Only proteins which were identified in 5 out of 6 experiments and are not appearing in the DMSO controls are listed.

Protein	Labeled cysteine	Sequence of peptide	XCorr	Probability	Charge	MH+ [Da]	ΔM [ppm]
ManA	Crue 12	EQLC*FSLYNAQR	4.15	51.15	3	1711,815	0.33
MgrA	Cys12	EQLC*FSLYNAQR	2.70	69.25	2	1711,820	3.05
SarA	Cys9	INDC*FELLSMVTYADK	2.65	106.76	2	2101,994	3.82
SarR	Cys57	C*SEFKPYYLTK	3.72	57.95	2	1618,789	1.56

Table S4. Labeled cysteines in S. aureus NCTC8325 proteins MgrA, SarA and SarF
--

* 3 labeled cysteine



Scheme S1. Chemical structure of xanthatine and xanthatine inspired γ -lactone probes.



Figure S1. Hemolysis inhibition of *S. aureus* NCTC8325 by lactone probes 1 (A), 2 (B), 4 (C) and 10 (D). Each compound was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean. Data were fitted to the dose-response function $f(x) = A_1 + (A_2 - A_1) / (1 + 10^{(LOG(x_0-x))p)})$ with a variable Hill-slope given by parameter p.



Figure S2. A) MTT assay with lactone probes. Each compound was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean. Data were fitted to the dose-response function $f(x) = A_1 + (A_2 - A_1) / (1 + 10^{(LOG(x_0-x))p)})$ with a variable Hill-slope given by parameter p. B) EC₅₀ values of compounds **1-10** obtained by curve fitting.



Figure S3. A) *In situ* labeling of *S. aureus* NCTC8325 with 5 μ M probe 1-9. Names of proteins are listed on the left side (please refer to Table S2 for full names). hmw = high molecular weight; lmw = low molecular weight. B) *In situ* labeling of *S. aureus* NCTC8325 with higher concentrations of probe 3, 5 and 6 in order to compare lmw labeling pattern. C) *In situ* labeling of *S. aureus* NCTC8325 cell envelope fraction with 5 μ M probe 2, 3, 5 and 7.



Figure S4. *In situ* labeling of *S. aureus* NCTC8325, Mu50 and USA300 with lactone probes **1**, **2** and **3**. Names are listed on the left side (full names in Table S2). The resistance associated hmw protein is indicated on the gel.



Figure S5. Inhibition of MurA1- and MurA2-catalyzed enolpyruvyl transfer reaction by lactone probe **3**. Each concentration was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean. Data were fitted to the dose-response function $f(x) = A_1 + (A_2 - A_1) / (1 + 10^{(LOG(x_0-x))p})$ with a variable Hill-slope given by parameter p.



Figure S6. A) Growth and B) hemolysis-inhibition of *S. aureus* NCTC8325 by the antibiotic fosfomycin (FOS). Each compound was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean.



Figure S7. A) Recombinant expression and in *situ/vitro* labeling of target proteins with probe **3** (bi, before induction; i, after induction; ΔT , heat control of induced recombinant proteins).



Figure S8. Mass spectra of **3** labeled and digested SarA (A) and MgrA (C). Y-ions are shown in blue, b-ions in red. The fragmentation of the corresponding peptide is shown and the modified cysteine indicated with *.



Figure S9. Inhibition of TrxA-catalysed insulin reduction by lactone probe **3**. A) Increase in absorption caused by reduced and turbid insulin. Addition of **3** leads to a concentration dependent inhibition of the TrxA-catalysed reduction. B) The total absorption changes were plotted against the corresponding probe concentrations to determine the remaining TrxA-activity compared to the DMSO treated positive control (100% activity). Each concentration was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean. Data were fitted to the dose-response function $f(x) = A_1 + (A_2 - A_1) / (1 + 10^{(LOG(x_0-x))p})$ with a variable Hill-slope given by parameter p.



Figure S10. Electrophoretic mobility shift assay (EMSA) with *agr* promotor DNA and MgrA. The left part of the gel shows a concentration dependent analysis with anti-virulence compound 3, the right part displays a single concentration analysis with all other compounds. DNA = unbound fluorescent promotor DNA; MgrA-DNA = protein bound DNA.



Figure S11. Fluorescent SDS-gel of purified recombinant proteins (10 μ M) labeled with the corresponding lactone probes (10 μ M) and "clicked" to rhodamine azide.



Figure S12. Invasion of THP-1 cells by *S. aureus*. Addition of **3** leads to decreased invasion of THP-1 cells compared to the DMSO treated positive control (100% invasion). The experiment was done in two independent trials in triplicates; average values are shown and error bars display standard deviations from the mean.

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