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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

A new class of phenylhydrazinylidene derivatives as inhibitors of *Staphylococcus aureus* biofilm formation

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Abstract In the struggle against the emergence of the antibiotic resistance, new molecules targeting biofilm formation could be useful as adjuvant of conventional antibiotics. This study focused on a new class of 2-phenylhydrazinylidene derivatives as antivirulence agents. The compound **12e** showed interesting activities against biofilm formation of all tested *Staphylococcus aureus* strains with IC_{50} ranging from 1.7 to 43 μM; compounds **12f** and **13a** resulted strong inhibitors of *S. aureus* ATCC 6538 and ATCC 29213 biofilm formation with IC_{50} of 0.9 and 0.8 μM, respectively. A preliminary study on the mechanism of action was carried on evaluating the inhibition of sortase A transpeptidase. Compound **12e** resulted not to be toxic at 1 mg/ml by using an in vivo model (the wax moth larva model, *Galleria mellonella*).

Keywords Phenylhydrazinylidene derivatives · Antibiofilm agents · Sortase A · Antivirulence agents · Bacterial adhesion

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Introduction

The emergence of multidrug-resistant Gram-positive and Gram-negative bacteria continues to limit the clinical efficacy of most of the currently marketed antibiotics (Cascioferro *et al.*, 2015b). The World Health Organization, in a recent report on antimicrobial resistance in common bacterial pathogens, highlights that a post-antibiotic era may start the twenty-first century (WHO: http://www.who.int/drugresistance/documents/surveillancereport/en/). Furthermore, conventional antibiotics target planktonic (free living) bacterial cells, but may be ineffective against biofilm-associated infections due to the high-level multifactorial resistance provided by biofilm mode of growth (Yang *et al.*, 2012). The biofilm is a multistratified bacterial community that grows on a biological or artificial surface, where single cells are embedded in a self-made polymeric matrix. Biofilm-associated infections play a direct role in the failure of implanted medical devices like orthopedic prosthesis. The earlier removal of the device or/and additional surgical interventions remain the most effective means to treat biofilm-associated infections, with a consequent high increase in healthcare costs due to longer hospitalization periods and recurrent medical interventions (Donlan and Costerton, 2002).

Here, we focus on *Staphylococcus aureus*, a Gram-positive bacterium that may cause nosocomial infections such as bacteremia, endocarditis, osteomyelitis, pneumonia, and infections of the skin and soft tissues (Schaffer *et al.*, 2006; Diekema *et al.*, 2001). Methicillin-resistant *S. aureus* (MRSA) has been considered by the Antimicrobial Availability Task Force of the Infectious Diseases Society of America, as one of six high-priority dangerous ESKAPE pathogens (*Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*) (Chuang and

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Huang, 2013; Tenover and Goering, 2009; Talbot *et al.*, 2006).

Previously our research group synthesized a series of 2-phenylhydrazinylidenealkanoic derivatives (Maggio *et al.*, 2016) that showed inhibitory activity against sortase A (SrtA), a membrane transpeptidase responsible for the anchoring of surface proteins to the cell wall envelope of Gram-positive bacteria (Cascioferro *et al.*, 2014b, 2015a). These compounds also resulted to be able to reduce staphylococcal biofilm formation, and the most active derivatives, **1a,b**, are reported in Fig. 1, whereas inhibition data for sortase A and biofilm formation are showed in Table 1.

This work is concentrated on the synthesis of 11 novel 2-phenylhydrazinylidene derivatives, **12a–f** and **13a–e**, structurally related to compounds **1a,b** that bear the 5-methyl-1,2-oxazole-3-yl or the 1-ethyl-3-methyl-pyrazol-5-yl moiety at the place of the methyl group. These compounds, having no effect on *S. aureus* growth but interfering with biofilm formation, have a potential for the development as antivirulence agents (Cascioferro *et al.*, 2014a; Cascioferro, 2014) alternative or complementary to conventional antibiotics in the treatment of infections caused by this pathogen under biofilm form. The inhibition of the biofilm formation of the *S. aureus* reference strains ATCC 25923, ATCC 29213, and ATCC 6538 was investigated. We focused on inhibition of sortase A as a possible mechanism of action. Finally, the toxicity of the most active compound **12e** was also evaluated in vivo by the wax moth larva model (*Galleria melonella*, Lepidoptera: Pyralidae). Insects are considered a good model to study the toxicity of different substances. In addition this animal model is very useful considering that it is a preliminary and alternative toxicity model that generates in vivo data quickly and inexpensively (Desbois and Coote, 2011).

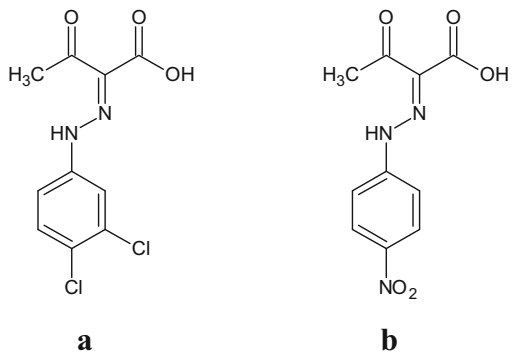


Fig. 1 2-Phenylhydrazinylidenealkanoic derivatives with inhibitory activity against Sortase A

Materials and methods

Chemistry

All commercial chemicals were purchased from Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Reaction progress was monitored by TLC on silica gel plates (Merck 60, F254, 0.2 mm) and visualization on TLC was achieved by UV light. Organic solutions were dried over Na_2SO_4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a PerkinElmer Spectrum RXI FTIR system spectrophotometer as solid in KBr disk. ^{1}H NMR spectra were recorded in $CDCl_3$ at 300.13 MHz, using a Bruker AC series 300-MHz spectrometer (tetramethylsilane as the internal standard): Chemical shifts are expressed in δ values (ppm). Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and are within ± 0.4 % of the theoretical values. Yields refer to products after crystallization. The name of the compounds was obtained using the ACD/I-Lab Web service (ACD/IUPAC Name Free 8.05).

*General procedure for preparation of 3-oxopropanoates (**11a,b**)*

To the appropriate acid derivative **7**, (Jackson *et al.*, 2012) **10** (0.021 mol) in 20 ml of THF, 3.6 g of *N,N′*-carbonyldiimidazol (CDI) was slowly added under stirring. After addition was complete, the solution was first magnetically stirred for 30 min at room temperature and then heated to 60 °C for 1 h. The reaction mixture was cooled to room temperature and treated with 2.3 g of $MgCl_2$ and 4.5 g of ethyl potassium malonate. After stirring (r.t.) for 3 h 15 ml of water followed by 5 ml of HCl 6 N was added. The mixture was then concentrated under reduced pressure, and the obtained white solid was collected by filtration, washed with water, and dried. Spectroscopical and physical data of compound **11a** are in accordance with those reported in the literature (Takagi *et al.*, 2008).

The ethyl 3-(1-ethyl-3-methyl-1H-pyrazol-5-yl)-3-oxopropanoate **11b** was used crude for the next reaction.

*General procedure for preparation of the ethyl-3-oxo-2-(2-phenylhydrazinylidene)propanoates (**12a–f**)*

A cold (0–10 °C) solution of the appropriate diazonium salts **3a–d** (2 mmol) was adjusted to pH 5 by saturated sodium acetate solution, and then a mixture of the proper 3-oxopropanoate **11a,b** in 25 ml of ethanol with 0.24 g of sodium acetate in 0.4 ml of water was slowly added. After addition was complete, the solution was magnetically stirred for

Table 1 Ability of **1a,b** to reduce the biofilm formation of *S. aureus* 6538, *S. aureus* 25923, and *S. aureus* 29213 bacterial strains and to inhibit SrtA, expressed as IC_{50} (μM)

Compound	IC_{50} (μM)			
	S. aureus 25923	*S. aureus* 29213	*S. aureus* 6538	SrtA
1a	91	1.6	39.3	50
1b	43.4	21	18.2	57

30 min at 0–10 °C and for 90 min at room temperature. Then 3 ml of water was added, and the product was collected by filtration, washed with water, and dried.

Ethyl 2-[2-(3,4-dichlorophenyl)hydrazinylidene]-3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoate (***12a***) Orange powder (EtOH); yield: 20 %; m.p.: 125 °C; IR (KBr) *ν*max 3136 (NH), 1678 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.36$ (t, 3H, CH_3), 2.52 (s, 3H, CH_3), 4.40 (q, 2H, CH_2), 6.42 (s, 1H oxazole H-4), 7.13–7.45 (3H, a set of signals, C_6H_3), 12.88 (s, 1H, exchangeable, NH).; ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 180.97$ (CO, C-3), 169.98 (C, C, isoxazole C-5), 163.33 (C, CO-OCH_2CH_3), 162.22 (C, isoxazole C-3), 141.18 (C, C-1′), 133.60 (C, C-2), 131.17 (CH, C-3′), 128.31 (C, C-5′), 127.12 (CH, C-4′), 117.70 (CH, C-6′), 115.28 (CH, C-2′), 102.26 (CH, isoxazole C-4), 61.85 (CH_2, O-CH_2CH_3), 14.00 (CH_3, O-CH_2CH_3), 12.30 (CH_3, isoxazole C-5); Anal. Calcd. for $C_{15}H_{13}Cl_2N_3O_4$: C, 48.67; H, 3.54; N, 11.35. Found C, 48.71; H, 3.68; N, 11.43.

Ethyl 3-(5-methyl-1,2-oxazol-3-yl)-2-[2-(2-nitrophenyl)hydrazinylidene]-3-oxopropanoate (***12b***) Bright yellow powder (EtOH); yield: 98 %; m.p.: 102–104 °C. IR (KBr) *ν*max 3144 (NH), 1687 (CO), 1666 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.38$ (t, 3H, CH_3), 2.54 (s, 3H, CH_3), 4.44 (q, 2H, CH_2), 6.49 (s, 1H oxazole H-4), 7.15–7.68 (4H, a set of signals, C_6H_4), 14.30 (s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 181.24$ (CO, C-3), 170.39 (C, isoxazole C-5), 162.04 (C, CO-OCH_2CH_3), 161.49 (C, isoxazole C-3), 138.55 (C, C-1′), 136.10 (CH, C-6′), 135.16 (C, C-2), 130.75 (C, C-2′), 125.84 (CH, C-3′), 123.33 (CH, C-4′), 118.00 (CH, C-5′), 102.14 (CH, isoxazole C-4), 62.26 (CH_2, O-CH_2 CH_3), 14.05 (CH_3, O-CH_2CH_3), 12.33 (CH_3, isoxazole C-5); Anal. Calcd. for $C_{15}H_{14}N_4O_6$: C, 52.03; H, 4.07; N, 16.18. Found C, 52.12; H, 4.09; N, 16.25.

Ethyl 2-[2-(2,6-difluorophenyl)hydrazinylidene]-3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoate (***12c***) Orange powder (EtOH); Yield: 48.6 %; m.p.: 104–105 °C; IR (KBr) *ν*max 3245 (NH), 1671 (CO), 1656 (CO) cm-1; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.43$ (t, 3H, CH_3), 2.51 (s, 3H, CH_3), 4.45 (q, 2H, CH_2), 6.54 (s, 1H oxazole H-4), 6.66–7.26 (3H, a set of signals, C_6H_3), 13.69 (s, 1H, exchangeable, NH); Anal. Calcd. for $C_{15}H_{13}F_2N_3O_4$: C, 53.42; H, 3.88; N, 12.46. Found C, 53.58; H, 3.94; N, 12.51.

Ethyl 3-(5-methyl-1,2-oxazol-3-yl)-2-[2-(4-nitrophenyl)hydrazinylidene]-3-oxopropanoate (***12d***) Orange powder (EtOH); yield: 99 %, m.p.: 100–102 °C; IR (KBr) *ν*max 3160 (NH), 1671 (CO) cm-1; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.38$ (t, 3H, CH_3), 2.56 (s, 3H, CH_3), 4.3841 (q, 2H, CH_2), 6.49 (s, 1H oxazole H-4), 7.42 (d, 2H, C_6H_4), 8.27 (d, 2H, C_6H_4), 12.96 (s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 180.99$ (CO, C-3), 170.43 (C, isoxazole C-5); 162.82 (C, CO-OCH_2CH_3), 162.02 (C, isoxazole C-3), 146.63 (C, C-1′), 144.07 (CH, C-4′), 129.03 (C, C-2), 125.76 (C, C-3′, 5′), 115.64 (CH, C-2′, 6′), 102.10 (CH, isoxazole C-4), 62.26 (CH_2, O-CH_2CH_3), 13.96 (CH_3, O-CH_2CH_3), 12.33 (CH_3, isoxazole C-5); Anal. Calcd. for $C_{15}H_{14}N_4O_6$: C, 52.03; H, 4.07; N, 16.18. Found C, 52.15; H, 4.14; N, 16.33.

Ethyl 2-[2-(3,4-dichlorophenyl)hydrazinylidene]-3-(1-ethyl-3-methyl-1H-pyrazol-5-yl)-3-oxopropanoate (***12e***) Pale yellow powder (EtOH); yield: 98 %, m.p.: 198–199 °C; IR (KBr) *ν*max 3188 (NH), 1721 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.36$ (t, 3H, CH_3), 1.46 (t, 3H, CH_3), 2.31 (s, 3H, CH_3), 4.37 (q, 2H, CH_2), 4.53 (q, 2H, CH_2), 6.49 (s, 1H pyrazole H-4), 7.04–7.42 (3H, a set of signals, C_6H_3), 12.61 (s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 179.04$ (CO, C-3), 163.47 (C, CO-OCH_2CH_3); 146.72 (C, pyrazole C-5), 141.44 (C, C-1′), 138.14 (C, C-2), 133.92 (C, pyrazole C-3), 131.18 (CH, C-5′), 128.38 (C, C-3′), 127.72 (C, C-4′), 116.94 (CH, C-2′), 114.56 (CH, C-6′), 113.51 (CH, pyrazole C-4), 61.86 (CH_2, O-CH_2CH_3), 47.18 (CH_2, N-CH_2CH_3), 15.88 (CH_3, N-CH_2CH_3), 14.02 (CH_3, O-CH_2CH_3), 13.34 (CH_3, pyrazole C-5); Anal. Calcd. for $C_{17}H_{18}Cl_2N_4O_3$: C, 51.40; H, 4.57; N, 14.10. Found C, 51.53; H, 4.61; N, 14.25.

Ethyl 2-[2-(2,6-difluorophenyl)hydrazinylidene]-3-(1-ethyl-3-methyl-1H-pyrazol-5-yl)-3-oxopropanoate (***12f***) Yellow powder (EtOH); yield: 41.2 %; m.p.: 102–103 °C; IR (KBr) *ν*max 3245 (NH), 1671 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.28$ (t, 3H, CH_3), 2.52 (s, 3H, CH_3), 4.38 (q, 2H, CH_2), 6.66 (s, 1H oxazole H-4), 7.53–8.31 (4H, a set of signals, C_6H_4), 12.26 (s, 1H, exchangeable, NH); Anal.

Calcd. for $C_{17}H_{18}F_2N_4O_3$: C, 56.04; H, 4.98; N, 15.38. Found C, 56.28; H, 5.03; N, 15.53.

*General procedure for preparation of the 3-oxo-2-(2-phenylhydrazinylidene)propanoic acids (**13a–e**)*

To a solution of 1.91 mmol of the appropriate esther derivative **12a–f** in 7 ml of ethanol, 1.68 ml of NaOH 5 N was added, and the reaction mixture was magnetically stirred at room temperature for 20 h. The sodium salt was then filtered under reduced pressure, washed with ethanol, and solubilized in water. This solution was treated with 5 N aqueous HCl affording the crude compound **13a–e**. The product was filtered and purified by crystallization.

2-[2-(3,4-Dichlorophenyl)hydrazinylidene]-3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoic acid (***13a***) Brownish orange powder (EtOH); yield: 48 %, m.p.: 201–202 °C IR (KBr) *v*max 3435 (OH), 3218 (NH), 1660 (CO), 1674 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 2.56$ (s, 3H, CH_3), 6.48 (s, 1H oxazole H-4), 7.26–7.67 (3H, a set of signals, C_6H_3), 13.31 (s, 1H, OH), 14.23 (s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 186.45$ (CO, C-3), 170.81 (C, isoxazole C-5); 165.29 (C, COOH), 160.39 (C, isoxazole C-3), 139.84 (C, C-1′), 134.32 (C, C-2), 131.54 (C, C-3′), 131.01 (CH, C-5′), 123.89 (C, C-4′), 119.24 (CH, C-2′), 116.69 (CH, C-6′), 103.04(C, isoxazole C-4), 12.26 (CH_3, isoxazole C-5); Anal. Calcd. for $C_{13}H_9Cl_2N_3O_4$: C, 45.64; H, 2.65; N, 12.28. Found C, 45.75; H, 2.94; N, 12.53.

3-(5-Methyl-1,2-oxazol-3-yl)-2-[2-(2-nitrophenyl)hydrazinylidene]-3-oxopropanoic acid (***13b***) Orange powder (EtOH). Yield: 50 %, m.p. >205 °C; IR (KBr) *v*max 3435 (OH), 3144 (NH), 1666 (CO), 1687 (CO) cm^{-1}; ^1H NMR (DMSO, 300 MHz): $\delta = 2.50$ (s, 3H, CH_3); 7.11–8.18 (4H, a set of signals, C_6H_4, s, 1H oxazole H-4); 14.55 (s, 1H, exchangeable, NH); 4.93 (s, 1H, exchangeable, OH); Anal. Calcd. for $C_{13}H_{10}N_4O_6$: C, 49.06; H, 3.17; N, 17.61. Found C, 49.15; H, 3.24; N, 17.73.

2-[2-(2,6-Difluorophenyl)hydrazinylidene]-3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoic acid (***13c***) Orange powder (EtOH); yield: 51 %, m.p.: 190–192 °C; mp 201–202 °C; IR (KBr) *v*max 3419 (OH), 3158 (NH), 1633 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 2.56$ (s, 3H, CH_3), 6.48 (s, 1H oxazole H-4), 7.26–7.67 (3H, a set of signals, C_6H_3), 13.31 (s, 1H, OH), 14.23 (s, 1H, exchangeable, NH); Anal. Calcd. for $C_{13}H_9F_2N_3O_4$: C, 50.49; H, 2.93; N, 13.59. Found C, 50.58; H, 2.97; N, 13.61.

3-(5-Methyl-1,2-oxazol-3-yl)-2-[2-(4-nitrophenyl)hydrazinylidene]-3-oxopropanoic acid (***13d***) Yellow powder (ethyl acetate) Yield: 20 %, m.p.: 210 °C; IR (KBr) *v*max 3418 (OH), 3232 (NH), 1711 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 2.50$ (s, 3H, CH_3); 6.63 (s, 1H oxazole H-4); 7.51–8.23 (4H, a set of signals, C_6H_4); 11.79 (s, 1H, exchangeable, OH); 12.47 (s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 186.61$ (CO, C-3), 171.03 (C, isoxazole C-5); 164.80 (C, COOH), 160.85 (C, isoxazole C-3), 145.76 (C, C-1′), 145.49 (C, C-4′), 125.83 (CH, C-3′, 5′), 124.59 (C, C-2), 117.63 (C, C-2′, 6′), 103.00 (CH, isoxazole C-4), 12.33 (CH_3, isoxazole C-5); Anal. Calcd. for $C_{13}H_{10}N_4O_6$: C, 49.06; H, 3.17; N, 17.61. Found C, 49.25; H, 3.31; N, 17.84.

2-[2-(3,4-Dichlorophenyl)hydrazinylidene]-3-(1-ethyl-3-methyl-1H-pyrazol-5-yl)-3-oxopropanoic acid (***13e***) Yellow orange (EtOH); yield: 78 %; m.p.: 131–133 °C; IR (KBr) *v*max 3654 (OH), 3401 (NH), 1662 (CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): $\delta = 1.45$ (t, 3H, CH_3); 2.36 (s, 3H, CH_3); 6.74 (s, 1H pyrazole H-4); 7.19–7.55 (3H, a set of signals, C_6H_3); 13.80–14.20 (s, 1H, exchangeable, OH, s, 1H, exchangeable, NH); ^{13}C NMR ($CDCl_3$, 300 MHz): $\delta = 184.06$ (CO, C-3), 165.71 (C, COOH); 147.22 (CH, pyrazole C-4), 140.34 (C, C-1′), 135.94 (C, C-2), 134.41 (C, pyrazole C-5), 131.60 (CH, C-5′), 130.46 (C, C-3′), 124.03 (C, C-4′), 118.60 (CH, C-2′), 116.19 (CH, C-6′), 114.93 (CH, pyrazole C-3), 47.63 (CH_2, N-CH_2CH_3), 15.97 (CH_3, N-CH_2CH_3), 13.28 (CH_3, pyrazole C-5); Anal. Calcd. for $C_{15}H_{14}Cl_2N_4O_3$: C, 48.80; H, 3.82; N, 15.18. Found C, 48.95; H, 3.94; N, 15.33.

Biology

Microbial strains

The staphylococcal reference strains used were: *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538.

Minimum inhibitory concentrations (MIC)

MICs were determined by a micromethod described previously (Schillaci *et al.*, 2008; Raimondi *et al.*, 2012).

Evaluation of biofilm formation

All the bacterial reference strains were tested for their ability to form biofilms. Briefly, bacteria were grown in tryptic soy broth (TSB, Sigma) containing 2 % glucose overnight at 37 °C in a shaking bath and then diluted 1:200 to a suspension with optical density (OD) of about 0.040 at 570 nm corresponding to $\sim 10^6$ CFU/ml. Polystyrene 24-well tissue culture plates were filled with 2 ml of diluted suspension and incubated for 24 h at 37 °C. Then, the wells were washed three times with 1 ml of sterile

phosphate-buffered saline (PBS) and stained with 1 ml of crystal violet 0.1 % v/v for 1 min. The excess stain was removed by placing the plates under running tap water.

Plates were dried overnight in inverted position at 37 °C. Crystal violet-stained adherent bacteria in each well were redissolved to homogeneity in 1 ml of ethanol, and the OD was read at 600 nm. Each assay was performed in triplicate and repeated at least twice (Schillaci *et al.*, 2010).

Biofilm prevention assay

Procedure described above was used to evaluate the activity of all the synthesized compounds in preventing biofilm formation. Polystyrene 24-well tissue culture plates were filled with 1 ml of diluted bacterial suspension (OD of about 0.040 at 570 nm), obtained and diluted as previously seen, and sub-MIC concentrations, ranging from 300 to 0.3 μM of each compound were directly added to the bacterial suspension at time zero and incubated at 37 °C for 24 h. After that time the wells were washed and stained with crystal violet as seen in biofilm forming assay. By comparing the average optical density of the growth in control wells with that in the sample wells, the following formula was used to calculate the percentages of inhibition for each concentration of the sample:

$$\text{Inhibition } (\%) = \frac{\text{OD}_{570} \text{ growth control} - \text{OD}_{570} \text{ sample}}{\text{OD}_{570} \text{ growth control}} \times 100$$

Each assay was performed in triplicate and assays were repeated at least twice.

Enzyme activity assay

Expression of recombinant sortase A

Recombinant and catalytically active sortase A with the N-terminal deletion of residues 1–59 and possessing C-terminal hexahistidine sequence (SrtAΔN59-6His) was used for the enzyme activity assay. SrtAΔN59-6His was prepared according to a slightly modified previously published method (Zhulenkovs *et al.*, 2014b). Briefly, after the IPTG induction of the *E. coli* BL21 (DE3)-transformed strain, the cell pellet was collected by centrifugation and resuspended in lysis buffer, and the recombinant protein was purified by affinity chromatography on a Ni–NTA column (Qiagen). The enzyme was eluted with an imidazole gradient, and the fractions containing the protein were further purified by the gel filtration. A Superdex 75 (GE Healthcare) column, which was equilibrated with 10 mM sodium phosphate (pH 7.0) buffer containing 100 mM NaCl and 1 mM DTT, was used for the final purification. The fractions containing the protein were collected and concentrated to 10 mg/ml. The purified protein was analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy and SDS-PAGE Coomassie blue staining.

*FRET-based screening of compounds **12a–f** and **13a–e***

All the compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and used for the IC50 determination. The compounds were screened at a single dose of 100 μM (1 % DMSO) in black 384-well plates (Greiner Bio-One). Two known sortase inhibitors, phenyl vinyl sulfone (Frankel *et al.*, 2004) and 1-(3,4-dichlorophenyl)-3-(dimethylamino)propan-1-one (Maresso *et al.*, 2007), were used as the positive controls. The inhibitory activity of all of the compounds was determined by quantifying the increase in fluorescence intensity upon cleavage of the FRET-peptide dabcyl-QALPETGEE-edans, which was used as the sortase substrate. A previously published method (Ton-That *et al.*, 1999) was used with slight modifications. Briefly, the reactions were performed in a volume of 100 μl containing 50 mM Tris–HCl, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.5, 10 μM *S. aureus* SrtA, 20 μM fluorescent peptide substrate dabcyl-QALPETGEE-edans, and the prescribed concentrations of the test compounds or positive controls.

The peptide substrate without the recombinant SrtA was incubated in the same manner and used as a negative control. The reactions were conducted for 24 h at 37 °C, and the fluorescence emitted with an excitation wavelength of 350 nm, and an emission wavelength of 495 nm after substrate cleavage was recorded.

Endpoint determination of product formation was used as a criterion for the primary screening. This determination was made by measuring the total product fluorescence 24 h after the initiation of the reaction. The relative inhibition activity was determined as $\%I = 100\ \% - (F_{\text{sample}}/F_{\text{control}} \times 100\ \%)$, where F_{sample} is the fluorescence intensity of the well containing the corresponding test compound and F_{control} is the fluorescence of the positive control reaction without inhibition.

For the IC_{50} determination, 10 μM *S. aureus* sortase A was preincubated in the reaction buffer with increasing concentrations of the inhibitory compounds (*x*–*y* μM) for 1 h at 37 °C prior to the addition of the dabcyl-QALPETGEE-edans substrate. The total fluorescence was recorded at 1-min intervals for 1 h, and the progress curves were constructed. The initial velocities of the biphasic reactions were obtained through nonlinear regression, as previously described (Zhulenkovs *et al.*, 2014a; Huang *et al.*, 2003). The IC_{50} values were determined by fitting

the obtained data to a default four-parameter variable-slope sigmoidal function in SigmaPlot 12.5 using a nonlinear least-squares algorithm.

Insects

Larvae of greater wax moth *G. mellonella* were reared on a natural diet honeybee nest debris at 30 °C in the dark. Last instar larvae of an average mass of in 521 mg (±110 mg) were selected for this study.

Evaluation of toxicity

To evaluate the toxicity of the compound **12e**, a single treatment dose of 1 mg/kg was given to each larva. In the experiments, there were two group controls: one untreated and the other was injected with PBS containing 3 % DMSO, and the same solvents were used to dissolve the tested compound. A sample group was treated with the compound **12e**. Three replicates of four larvae for each group were disposed. Injection of solvents or compound solution was given into alternate prolegs moving up the body toward the head such that each proleg was injected only once. Larvae were stored in Petri dishes in the dark at 37 °C for 72 h in dark conditions. Larvae were inspected every 24 h, and the survival rate and the weight was observed. Moreover, to detect potential deleterious effect of the compound **12e,** the variation in larval weight and the time pass from the inoculum at the later larval stage to the pupation were also checked.

Statistical analysis

For survival and weight variation of *G. mellonella* larvae data analysis, collated datasets were compared using analysis of variance (ANOVA) with homogeneity of variances performed using Cochran's test prior to ANOVA analysis. The statistical significances of the parameter analyzed were calculated by one-way ANOVA and Tukey's pairwise comparisons. Statistical significance was assessed by all applied tests ($P < 0.05$) using Statistica 6.0 (StatSoft, Tulsa, OK, USA).

Results and discussion

Chemistry

The phenylhydrazinylidene derivatives **12a–f** and **13a–e** were obtained as reported in scheme 1. In particular, the intermediate ethyl 3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoate **11a** was obtained by reacting diethyl ethanedioate **4** with propan-2-one, achieving the ethyl 2,4-dioxopentanoate **5** (Kamal *et al.*, 2014). Refluxing of the latter compound with hydroxylamine hydrochloride led to the formation of the ethyl 5-methyl-1,2-oxazole-3-carboxylate **6** (Baraldi *et al.*, 1982) which, in turn, was hydrolyzed to the corresponding acid **7** (Jackson *et al.*, 2012) that, finally, by reaction with ethyl potassium malonate lead to the obtaining of **11a** (Takagi *et al.*, 2008).

The starting product ethyl 3-(1-ethyl-3-methyl-1*H*-pyrazol-5-yl)-3-oxopropanoate **11b** was obtained by reacting the ethyl 2,4-dioxopentanoate **5** with hydrazine reaching initially the ethyl 3-methyl-1*H*-pyrazole-5-carboxylate **8**. The latter was ethylated obtaining **9** which was hydrolyzed to the corresponding acid **10**. Finally, the derivative **11b** was obtained by reacting **10** with ethyl potassium malonate.

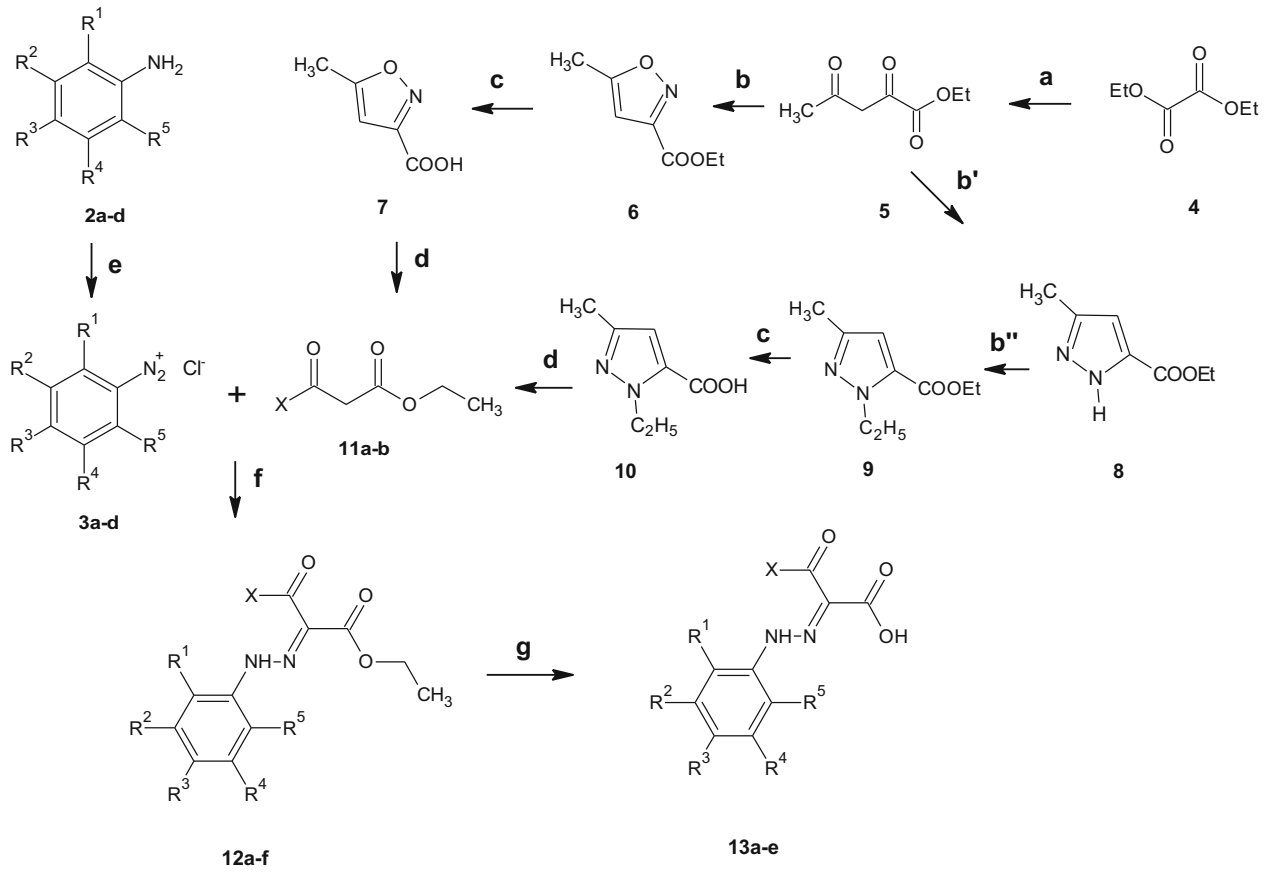
At this point, the diazonium salts **3a–d** afforded from the anilines **2a–d** were reacted with the ethyl propanoates **11a,b** to give the ester derivatives **12a–f** which, in turn, were transformed in the corresponding acids **13a–e** (Scheme 1).

The structures of the new compounds were determined by analytical and spectroscopic measurements. The IR spectra of compounds **12a–f** showed adsorptions in the range 1666–1685 cm^{-1} as distinct bands for the carbonyl groups. The ^1H NMR spectra of the above compounds confirmed the phenylhydrazinylidene structure as the showed the NH signal in the range 12.26–14.31 δ. The presence of electron withdrawing groups in the ortho positions of the phenyl ring produced a low field shift than the unsubstituted derivatives. The acid derivatives **13** produced in the IR spectra carbonyl bands in the 1660–1711 cm^{-1} region together with multiple bands in the 2500–3250 cm^{-1} for the OH and NH groups. The ^1H NMR spectra showed signal in the 13.2–15.2 δ which disappeared by addition of deuterium oxide, attributable to NH and OH groups. In the case of derivative **13b** COOH signal was observed at 4.93 δ superimposed with H_2O signal.

Biological activity

Compounds **12a–f** and **13a–e** were primarily evaluated for their antistaphylococcal activity against three reference strains of *S. aureus*, namely ATCC 25923, ATCC 29213, ATCC 6538. The minimum inhibitory concentration (MIC) of the tested compounds was evaluated, and the results showed the compounds have no effect on the microbial viability (MIC > 200 μM).

Esters **12a–f** and acids **13a–e** were also tested for their inhibitory activity on SrtA, utilizing the FRET technics (Table 2) (Sekar and Periasamy, 2003). Among the tested compounds, the ester derivative ethyl 2-[2-(2,6-difluorophenyl)hydrazinylidene]-3-(1-ethyl-3-methyl-1*H*-pyrazol-



2a R_1=H, R_2=H, R_3=Cl, R_4=Cl, R_5=H
2b R_1= NO_2, R_2= H, R_3=H, R_4=H, R_5=H
2c R_1=F, R_2=H, R_3=H, R_4=H, R_5=F
2d R_1=H, R_2=H, R_3= NO_2, R_4=H, R_5=H
3a R_1=H, R_2=H, R_3=Cl, R_4=Cl, R_5=H
3b R_1= NO_2, R_2= H, R_3=H, R_4=H, R_5=H
3c R_1=F, R_2=H, R_3=H, R_4=H, R_5=F
3d R_1=H, R_2=H, R_3= NO_2, R_4=H, R_5=H
11a X=5-methyl-1,2-oxazole-3-yl
11b X=1-ethyl-3-methyl-1H-pyrazole-5-yl
12a X=5-methyl-1,2-oxazole-3-yl, R_1=R_2=R_5=H, R_3=R_4=Cl,
12b X=5-methyl-1,2-oxazole-3-yl, R_1= NO_2, R_2= R_3= R_4= R_5= H,
12c X=5-methyl-1,2-oxazole-3-yl, R_1=R_5=F, R_2=R_3=R_4=H,
12d X=5-methyl-1,2-oxazole-3-yl, R_1= R_2= R_4=, R_5=H, R_3= NO_2,
12e X=1-ethyl-3-methyl-1H-pyrazole-5-yl, R_1=R_2=R_5=H, R_3=R_4=Cl,
12f X=1-ethyl-3-methyl-1H-pyrazole-5-yl, R_1=R_5=F, R_2=R_3=R_4=H,
13a X=5-methyl-1,2-oxazole-3-yl, R_1=R_2=R_5=H, R_3=R_4=Cl
13b X=5-methyl-1,2-oxazole-3-yl, R_1= NO_2, R_2= R_3= R_4= R_5= H
13c X=5-methyl-1,2-oxazole-3-yl, R_1=R_5=F, R_2=R_3=R_4=H
13d X=5-methyl-1,2-oxazole-3-yl, R_1= R_2= R_4=, R_5=H, R_3= NO_2,
13e X=1-ethyl-3-methyl-1H-pyrazole-5-yl, R_1=R_2=R_5=H, R_3=R_4=Cl

Scheme 1 Reagents and conditions: (*a*) EtONa, acetone, stirring 0–10 °C, 4 h; (*b*) EtOH, hydroxylamine hydrochloride, reflux, 3 h; (*b′*) Acetic acid, hydrazine monohydrochloride, reflux, 2 h; (*b″*) Diethyl sulfate, 150–160 °C, 2 h; (*c*) EtOH, NaOH (10 %), stirring, 10 h, HCl; (*d*) THF, CDI, $MgCl_2$, ethyl potassium malonate, stirring, rt, 4 h, (*e*) HCl 5 N, KNO_2, stirring, 0–5 °C; (*f*) EtOH, sodium acetate, stirring, 0–10 °C, 2 h; (*g*) EtOH, NaOH 5 N, stirring, rt, 20 h

5-yl)-3-oxopropanoate **12f** and the 2-[2-(2,6-difluorophenyl) hydrazinylidene]-3-(5-methyl-1,2-oxazol-3-yl)-3-oxopropanoic acid **13c** showed the best inhibitory activity on the enzyme with IC_{50} of 75 and 76.5 μM, respectively, better than that one shown by a known inhibitor as phenyl vinyl sulfone, PVS, (IC_{50} = 736 μM) (Frankel *et al.*, 2004) and very close to the IC_{50} of some previously reported analogues (submitted data).

Even if the compounds **12a–f** and **13a–e** were less active than derivatives **1a,b** against SrtA, they resulted, in some cases (Table 2), more potent as inhibitors of staphylococcal biofilm formation, the compounds **13a** and **12e** showed the best activity against *S.aureus* ATCC 29213 with an IC_{50}, respectively, of 0.8 and 1.7 μM. Interestingly, the compounds **13e** and **12f** interfere with biofilm formation of all the tested staphylococcal strains with IC_{50} ranging, respectively,

Table 2 Ability of **12a–f** and **13a–e** to reduce the biofilm formation of *S. aureus* 6538, *S. aureus* 25923 and *S. aureus* 29213 bacterial strains and to inhibit SrtA, expressed as IC_{50} (μM)

Compounds	*S. aureus ATCC* 25923	*S. aureus ATCC* 29213	*S. aureus* ATCC 6538	SrtA IC_{50}
12a	277	123	109	>200
12b	48	21	153	>200
12c	41	10	159	>200
12d	83	177	55	>200
12e	10	1.7	43	>200
12f	42	13	0.9	75
13a	85	0.8	53	>200
13b	64	52	506	195
13c	68	29	254	76.5
13d	231	26	88	>200
13e	20	16	11	>200
PVS	n.t.	n.t.	n.t.	736
1-(3,4-Dichlorophenyl)-3-dimethylamino)propan-1-one	n.t.	n.t.	n.t.	16.91

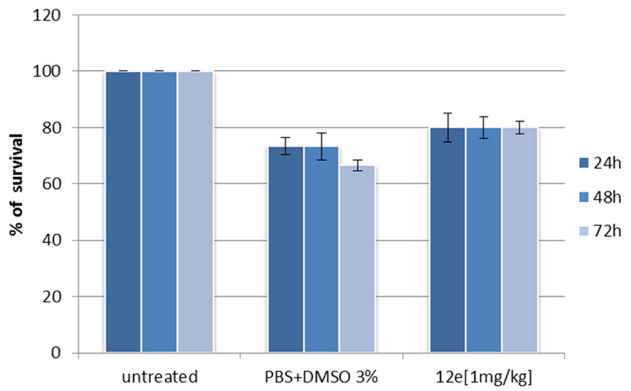


Fig. 2 Effect of treatment with a single dose (1 mg/kg) of compound **12e** on survival of *Galleria mellonella* larvae at 37 °C. Survival was checked at 24, 48, and 72 h after injection. Results are given as mean ($n = 3$ replicates) with standard deviation. Data entries with *different letter* are statistically different at post hoc Tukey's *t* test for multiple comparisons within each character ($P < 0.05$)

from 11 to 20 and from 0.9 to 42 μM. It is very difficult to correlate SrtA inhibition with the ability to interfere with biofilm formation; for example, the compound **12f** is a good inhibitor of SrtA and of staphylococcal biofilm formation, but the compound **13e,** despite its property to prevent biofilm formation, showed a not significant activity as SrtA inhibitor. However, we must stress that it is difficult to compare the data obtained with two completely different experimental models; in fact in the evaluation of antibiofilm activity, we use bacterial cells, but in the activity of inhibition of SrtA, a biochemical model.

The toxicity of the most active compound **12e** was also evaluated in in vivo model.

Derivative **12e,** which showed the best antibiofilm activity against two of the three tested strains of *S. aureus*, was tested by administering a single dose of 1 mg/kg dissolved in a mixture of PBS/DMSO (3 %) at time 0 to groups of 10 larvae and observing the effect after 24, 48, and 72 h. The toxicity is evaluated on the basis of survival percentage (see Fig. 2).

The comparison between survival percentage in the group of larvae treated with compound **12e** and the control group (injected with the used solvents only) indicates a lack of toxicity up to the examined dose and regimen under examination.

Conclusions

In this study we found that the activity of the 2-phenylhydrazinylidene derivatives is not directed against the planktonic form of staphylococcal strains but against biofilm formation.

In conclusion, this study demonstrated that these novel agents do not affect bacterial growth (MIC > 200 μM) but are biofilm inhibitors targeting a virulence mechanism like adhesion and biofilm formation of *S. aureus* strains.

With the aim to investigate the mechanism of action of these molecules, we evaluated the activity as SrtA inhibitors, and we found that there is no correlation between the activity on the biofilm formation and the inhibition of the transpeptidase even if it is very difficult to compare two so different experimental models (cellular and biochemical). Other possible mechanisms could be involved, e.g.,

quorum sensing inhibition, so a further and more detailed investigation is needed to clarify the mode of action.

In any case, the biological activity of such compounds is that typical of antivirulence agents because they do not target bacterial viability and they should impose a low selection pressure rendering less probable the development of resistance (Cascioferro *et al.*, 2014a). In addition, the absence of in vivo toxicity guarantees the active compound to be pushed forward for further studies.

In fact, the combination of antivirulence agents with current antibiotics offers potential for new therapeutic strategies for the treatment of chronic bacterial infections and could significantly impact on overcoming the problem of antibiotic resistance.

Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

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