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Original article

Synthesis and anti-staphylococcal activity of new 4-diazopyrazole derivatives

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ABSTRACT

Several new 4-diazopyrazole derivatives **6a**–**g** and **9a**–**c** were obtained by the reaction of 1-(R-substituted-phenyl)-3-(1,3-dimethyl-1*H*-pyrazol-5-yl)ureas **5a**–**g** and *N*-(1,3-dimethyl-1*H*-pyrazol-5-yl)-2-(R-substituted-phenyl)acetamides **8a**–**c** respectively with a sevenfold excess of nitrous acid in acetic acid solution. The compounds were assayed for their activity against the *Staphylococcus aureus* reference strains ATCC 25923, ATCC 29213 and ATCC 6538, as well as six veterinary strains. The best anti-staphylococcal profile was showed by [(R-substituted-phenyl)acetyl](4-diazonio-1,3-dimethyl-1*H*-pyrazol-5-yl)azanides **9a**,**c**. Compound **9c** was also able at 3.1 μg mL^{-1} to inhibit of 45.7% the biofilm formation of the strains *S. aureus* ATCC 29213.

1. Introduction

Staphylococci have an extraordinary ability to acquire antibiotics resistance traits, and the rise of community and hospital-acquired methicillin resistant *Staphylococcus aureus* (MRSA) is a major health problem that has created a pressing need for novel therapeutic options [1].

The ability to form biofilms, bacterial communities able to grow on surfaces and surrounded by an extracellular polymeric substance (EPS) matrix, is probably the most important virulence factor of staphylococci in the development of the chronic and persistent form of some infectious diseases in humans, such as otitis media, osteomyelitis, endophthalmitis, urinary tract infections, acute septic arthritis, native valve endocarditis, burn or wound infections and cystic fibrosis associated infections (CF) [2–8].

In veterinary medicine, *Staphylococcus* spp. are involved in inflammatory diseases like mastitis. A correlation on the recurrence and persistence of the diseases and the capability of the bacterial strains to organise biofilm inside mammary glands has been reported [9].

Furthermore, staphylococcal biofilms are commonly isolated from device-related infections of medical relevance. In fact, *S. aureus* is an important cause of metal-biomaterial infections, while *Staphylococcus epidermidis* is seen more often in polymer-biomaterial associated infections [10]. Biofilm associated infections of indwelling medical devices are usually resolved after replacement of the device but involve a prolonged hospital stay and increased healthcare costs. The treatment of these kinds of infections is complicated because staphylococcal biofilms are typically highly resistant to conventional antibiotics [11] and considering also that increasing numbers of elderly patients require indwelling medical devices like artificial knees and hips, a new generation of anti-infective agents effective in the prevention or eradication of biofilms is needful [12].

The threat of staphylococcal strains resistant to most or all conventional antibiotics could be faced by developing new antibacterial agents with different chemical characteristics and mechanism of action with respect to current antibiotics.

Some of us previously reported the 4-diazopyrazoles **1** and **2** by reacting 5-benzamidopyrazoles with a sevenfold excess of nitrous acid in acetic acid media (see Fig. 1).

The obtained compounds showed antimicrobial activity against representative Gram-negative and Gram-positive bacteria. The highest microbial susceptibility was shown by Gram-positive bacteria, with minimum inhibitory concentration in the range 0.26–12.5 μg/mL [13,14]. Moreover, the most active compounds **1a**–**c** were active against five clinical methicillin-resistant *S. aureus* strains [13] in the range 2–8 μg/mL, and against standard staphylococcal strains such as *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 43866 and *S. epidermidis* RP62A in the range 1.5–12.5 μg/mL.

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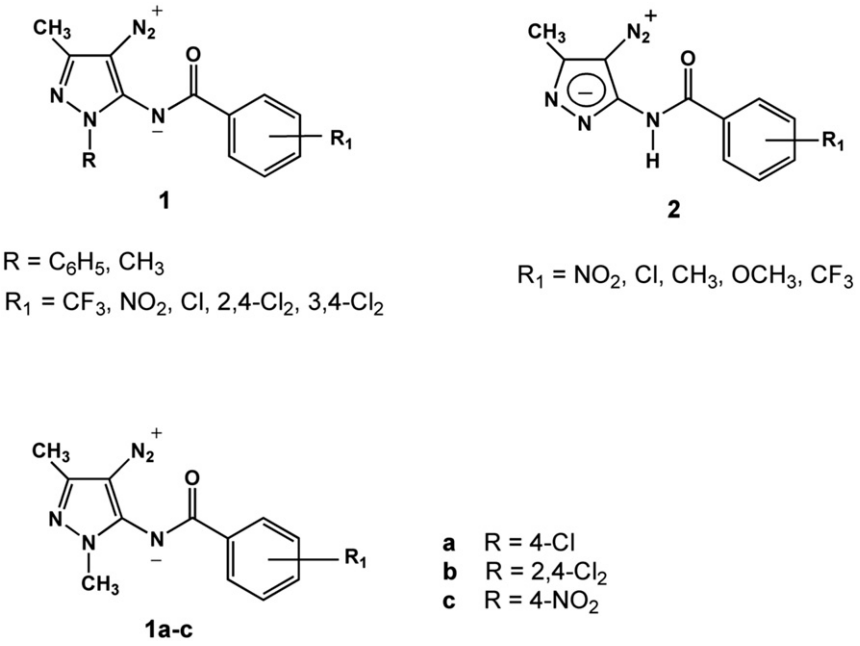


Fig. 1. Chemical structures of known 4-diazopyrazole derivatives.

Finally, compounds **1a**,**c** showed *in vitro* anti-biofilm activity when tested at 25 μg/mL against the above standard strains, showing inhibition percentages in the range 69.7–97.5%, whereas **1b** showed comparable activity only against the *S. epidermidis* RP62A [15].

As an extension of this previous work and with the aim to obtain more effective compounds, we thought to be of interest to synthesize compounds **6** and **9** which bear at the place of the phenyl ring of compounds **1** and **2** a phenylamino or phenylmethyl moiety respectively (see Fig. 2).

The new compounds show different structural features than **1** and **2** derivatives, such as increased distance between the rings, more potentiality for H bonds or hydrophobic interactions, which are all indicative of valuable chemical diversity than the reference compounds. The phenyl ring of these moieties is substituted with different groups or atom in order to obtain a good discrimination among the compounds as regards hydrophobic, electronic and steric effects. All the new compounds were tested against nine *S. aureus* strains, and the most active compounds were assayed for staphylococcal antibiofilm activity.

2. Results and discussion

2.1. Chemistry

The synthesis of the 4-diazoderivatives **6a**–**g** and **9a**–**c** has been obtained according to Schemes 1 and 2 (only a mesomeric form is indicated for compounds **9** and **6**).

In particular, the starting products 1-(1,3-dimethyl-1*H*-pyrazol-5-yl)-3-(R-substituted-phenyl)ureas **5a**–**g** and *N*-(1,3-dimethyl-1*H*-pyrazol-5-yl)-(R-substituted-phenyl)acetamides **8a**–**c** were obtained by reacting 1,3-dimethyl-1*H*-pyrazol-5-yl-amine **3** with phenylisocyanate derivatives **4a**–**g** or phenylacetylchloride derivatives **7a**–**c** respectively. The 4-diazoderivatives **6a**–**g** and **9a**–**c** were obtained by reacting the respective starting products **5a**–**g** and **8a**–**c** with nitrous acid in acetic acid solution. A nitro group was unexpectedly introduced at the 2-position of the phenyl ring of ureas **5e**–**g** affording **6e**–**g**.

The structures of all the new compounds were assigned on the basis of their satisfactory analytical as well as spectroscopic data. The IR spectra of compounds **6** showed absorption in the 2135–2161 cm^{-1} range, attributable to the diazo group, as well as in the 1624–1653 cm^{-1} one for the ureidic carbonyl group. For compounds **9a**–**c** the spectra showed bands at about 2160 cm^{-1} for the diazo group, and at about 1600 cm^{-1} for the carbonyl one. The low wave-number values for the carbonyl band are due to the contribution of the resonance form II to the structure of both bioisosters **6** and **9** (see Fig. 3).

The ^{1}H NMR spectra of compounds **6** showed, among the other signals, those for the two methyls linked to the pyrazole nucleus and for the phenyl moiety, whereas the signal for the pyrazole H(4) atom was lacking. In particular, for compounds **6e**,**f** the ^{1}H NMR spectra showed the typical pattern of a tri-substituted benzene. In order to discriminate between the 1,2,4 or 1,3,4 isomers, we compared the ^{1}H NMR spectra of **6e**,**f** with those of both 2-nitro-4-

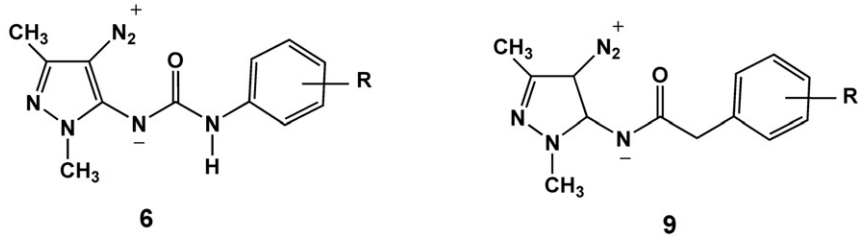
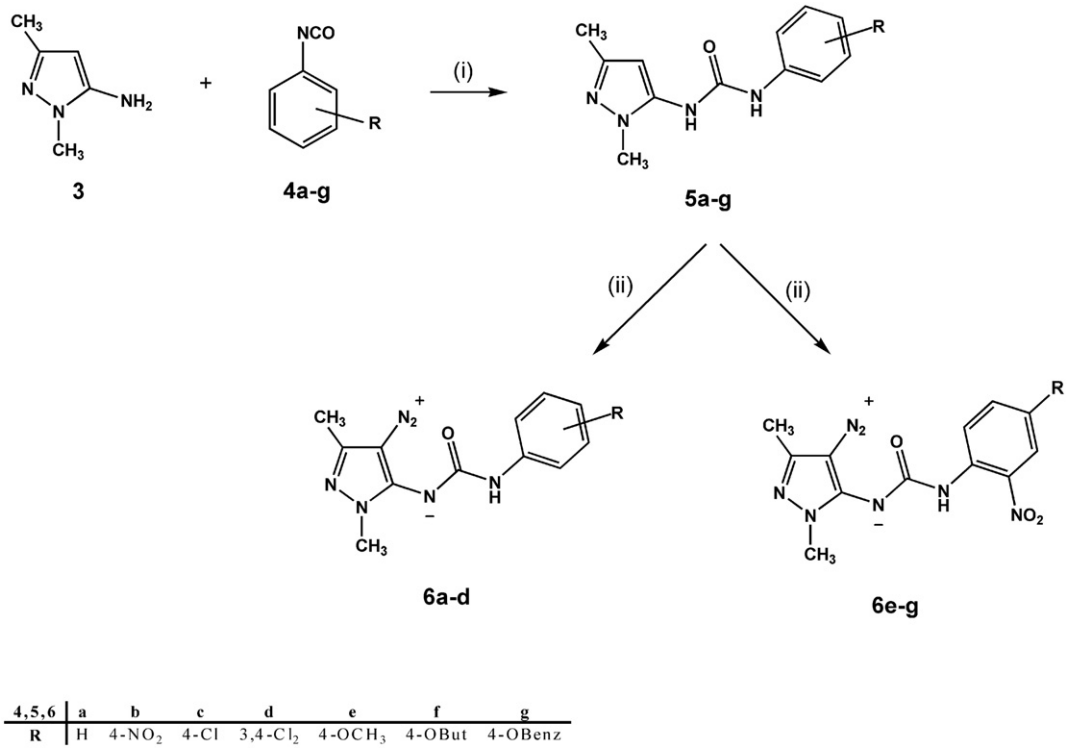


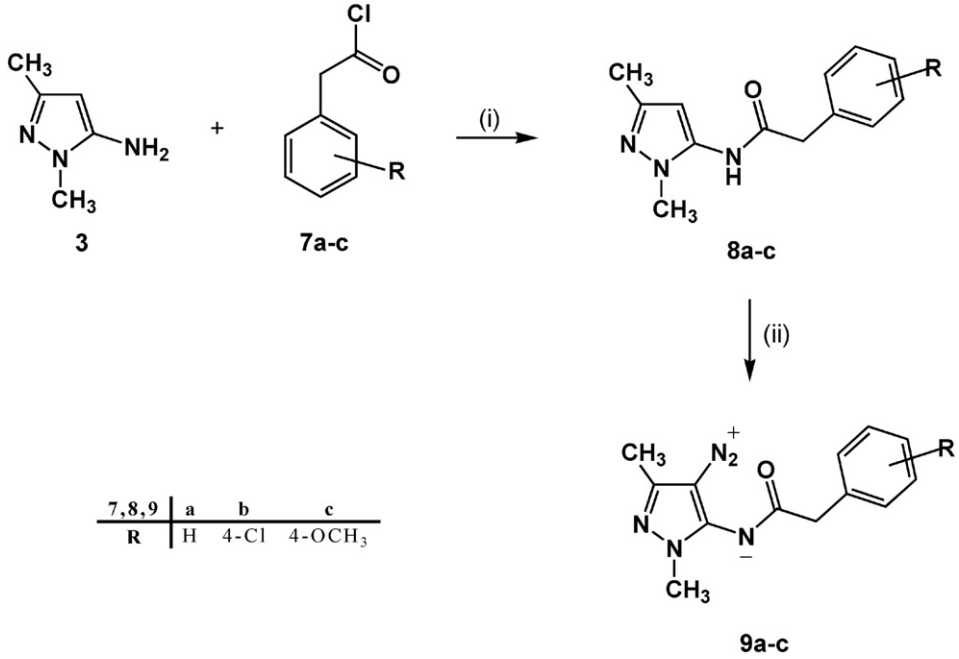
Fig. 2. Chemical structures of the new 4-diazopyrazole derivatives.



Scheme 1. Synthesis of [(R-phenyl)carbamoyl](4-diazonio-1,3-dimethyl-1*H*-pyrazol-5-yl)azanides **6a**–**g**. Reagents and conditions: (i) THF, r.t., 24 h; (ii) CH_3COOH/HCl 37%, KNO_2/H_2O, r.t., 24 h, away from light.

metoxyacetoanilide and 3-nitro-4-metoxyacetoanilide taken as reference models [16]. Compounds **6e**,**f** and 2-nitro-4-metoxyacetoanilide showed a similar aromatic signals pattern confirming the 1,2,4 substituted structure for **6e**,**f**. For compound **6g** comparison was not possible as benzyl signals superimposed on the tri-substituted benzene signals.

^{13}C NMR of some representative compounds confirmed the assigned structure and showed that the pyrazole C(4) atom in the 4-diazopyrazoles is quite shielded. The ^{13}C NMR spectra of **9a** and **9b**, taken as a example, showed the signal for the C(4) atom at 73.70 and 73.62 δ respectively, whereas for the corresponding ureas **8a**,**b** the C(4) resonances occurred at 99.56 and 99.88



Scheme 2. Synthesis of [(R-phenyl)acetyl](4-diazonio-1,3-dimethyl-1*H*-pyrazol-5-yl)azanides **9a**–**c**. Reagents and conditions: (i) dry $CHCl_3$, TEA, reflux, 5 h; (ii) CH_3COOH/HCl 37%, KNO_2/H_2O, r.t., 24 h, away from light.

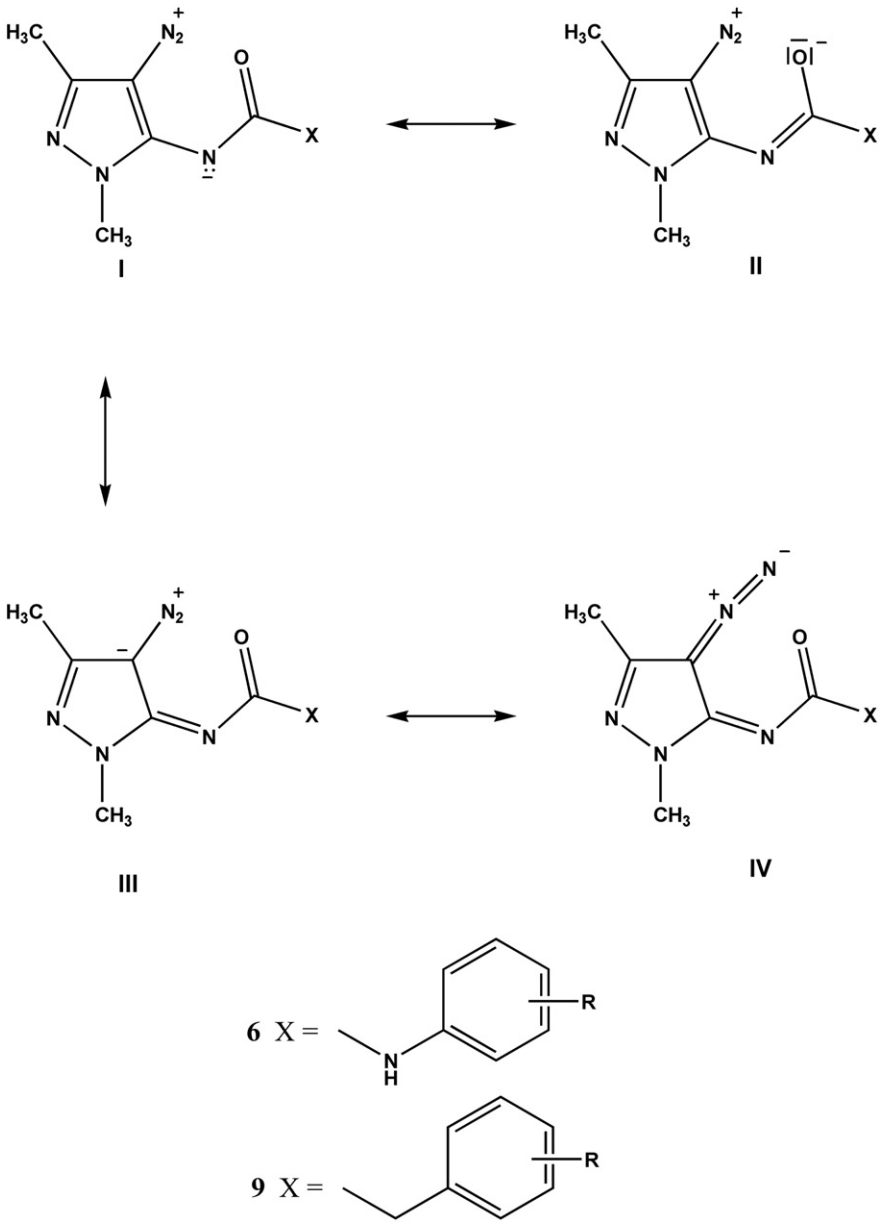


Fig. 3. Resonance forms for compounds **6a**–**h** and **9a**–**c**.

δ respectively, due to the lack of the 4-diazo group. The ^{13}C NMR data justify the resonance form III which together with the form II most closely represent the structure of this class of 4-diazopyrazoles.

2.2. Biological activity

Compounds **6a**–**g** and their bioisosters **9a**–**c**, in which a CH_2 group is in the place of an NH one, were primarily evaluated for their anti-staphylococcal activity against three reference strains of *S. aureus*, namely ATCC 25923, ATCC 29213, ATCC 6538, and six veterinary strains of the same species, namely 722, 708, 100, 723, 357, 712 (IZS bacterial collection, see Experimental section – Microbiology). The minimum inhibitory concentration (MIC) of the tested compounds as well as of rifampicin, used as a control, is reported in Table 1. The MIC value for rifampicin when tested against *S. aureus* ATCC 29213 is within the range of the literature data (0.06–0.008 μg mL^{-1}) [17].

Compounds **9a**–**c** resulted generally more active than the corresponding bioisosters **6a**,**c**,**e**. Possibly this is due to a higher lipophilicity of compounds **9a**–**c** than **6a**,**c**,**e**. The introduction of substituents in the phenyl ring of **9a** produced essentially a decrease of activity, despite their opposite electronic effects. As regards the compounds of series **6**, the most active were **6b**–**d**. These compounds bear substituents on the phenyl ring which exert an electron withdrawing effect (R_1 = 4-NO_2, 4-Cl, 3,4-Cl_2 respectively).

The anti-staphylococcal activity decreased significantly when the phenyl ring of compounds **6** bore an electron donating substituent together with the nitro group, such as for **6e**–**g**. As the nitro group should take an advantage for activity, due to its electron withdrawing properties (see above), we realized that the electron donating groups, namely OMe, OBz, OBu, afford a decreasing of activity.

A useful strategy to control staphylococcal biofilms is the inhibition of their growth on surfaces. With this aim in mind, the most

Table 1
Antistaphylococcal activity of **6a**–**g** and **9a**–**c** against *S. aureus* reference strains and field isolates of veterinary interest. Minimum inhibitory concentration MIC expressed in μg/mL. RIF = rifampicin.

Comp.	6a	6b	6c	6d	6e	6f	6g	9a	9b	9c	RIF
Reference strains											
ATCC 25923	12.5	6.2	6.2	6.2	>50.0	>50.0	>50.0	6.2	25.0	6.2	0.015
ATCC 29213	12.5	6.2	6.2	6.2	>50.0	>50.0	>50.0	3.1	25.0	6.2	0.015
ATCC 6538	12.5	6.2	25.0	6.2	>50.0	>50.0	>50.0	3.1	6.2	6.2	0.015
Isolates											
722	25.0	3.1	12.5	3.1	>50.0	>50.0	>50.0	6.2	12.5	6.2	0.6
708	25.0	3.1	25.0	6.2	>50.0	>50.0	>50.0	6.2	12.5	6.2	0.07
100	12.5	>100.0	6.2	3.1	>50.0	>50.0	>50.0	3.1	6.2	3.1	0.07
723	6.2	100.0	12.5	3.1	>50.0	>50.0	>50.0	3.1	3.1	6.2	1.25
357	6.2	3.1	6.2	6.2	>50.0	>50.0	>50.0	1.5	1.5	6.2	0.015
712	50.0	3.1	3.1	25.0	>50.0	>50.0	>50.0	1.5	50.0	25.0	0.3

active compounds against free living (planktonic) forms, **9a** and **9c**, were tested for their ability to prevent biofilm formation of two reference strains and of the best biofilm forming among the strains of animal origin, the *S. aureus* 708. Compound **9c** showed at 3.1 μg mL^{-1} an inhibition of 45.7, 38 and 25% against the strains *S. aureus* ATCC 29213, ATCC 25923 and 708 respectively. The above concentration is below the MICs determined against the mentioned planktonic strains. Compound **9a** showed an inhibition of 45.7% at sub-MIC concentration of 3.1 μg mL^{-1} against *S. aureus* 25923, but resulted scarcely effective in preventing the biofilm formation of the other two tested strains.

3. Conclusions

To summarize, we have synthesized new 4-diazopyrazole bioisosters **6a**–**g** and **9a**–**c** as an extension of our previous work in this field. Some of the new compounds showed a better anti-staphylococcal profile than the reference 4-diazo-5-benzamidopyrazoles **1**.

4. Experimental protocols

4.1. General

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, F_{254}, 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 Perkin Elmer Spectrum RXI FT-IR System spectrophotometer as solid in KBr disc. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 or $CDCl_3$ 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).

4.1.1. General procedure for the synthesis of 1-(R-substituted-phenyl)-3-(1,3-dimethyl-1H-pyrazol-5-yl)ureas (**5a**–**g**)

A solution of equimolar amounts (4.5 mmol) of 1,3-dimethyl-1*H*-pyrazol-5-amine **3** [18] and substituted phenylisocyanates **4a**–**g** in THF (12 mL) was stirred at room temperature for 24 h. The solid residue was filtered, washed with 1 mL of ethyl acetate, and then crystallized from a suitable solvent.

4.1.1.1. 1-(1,3-Dimethyl-1H-pyrazol-5-yl)-3-phenylurea (**5a**).

Yield: 32.3%; m.p.: 185–186 °C (ethyl acetate); IR (KBr) ν (cm^{-1}): 3268 (2 × NH), 1647 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.13 (s, 3H, C–CH_3), 3.63 (s, 3H, N–CH_3), 6.02 (s, 1H, pyrazole H-4), 7.00–7.51 (a set of signals, 5H, aromatic protons), 8.56 (s, 1H, NH, exchangeable), 8.88 (s, 1H, NH, exchangeable). Elemental analysis ($C_{12}H_{14}N_4O$) C, H, N.

4.1.1.2. 1-(1,3-Dimethyl-1H-pyrazol-5-yl)-3-(4-nitrophenyl)urea (**5b**).

Yield: 62.9%; m.p.: 226–230 °C (ethanol); IR (KBr) ν (cm^{-1}): 3352–3223 (2 × NH), 1733 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.10 (s, 3H, C–CH_3), 3.60 (s, 3H, N–CH_3), 6.00 (s, 1H, pyrazole H-4), 7.68–8.21 (a set of signals, 4H, aromatic protons), 8.84 (s, 1H, NH, exchangeable), 9.51 (s, 1H, NH, exchangeable). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.03 (CH_3), 35.22 (CH_3), 98.01 ($CH_{pyrazole}$), 118.08 (2 × CH_{Ar}), 125.57 (2 × CH_{Ar}), 137.17 (C_{Ar}), 141.68 (C_{Ar}), 146.02 (C_{Ar}), 151.92 (CO). Elemental analysis ($C_{12}H_{13}N_5O_3$) C, H, N.

4.1.1.3. 1-(4-Chlorophenyl)-3-(1,3-dimethyl-1H-pyrazol-5-yl)urea (**5c**).

Yield: 61.3%; m.p.: 219–221 °C (ethanol); IR (KBr) ν (cm^{-1}): 3270 (2 × NH), 1644 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.09 (s, 3H, C–CH_3), 3.58 (s, 3H, N–CH_3), 5.97 (s, 1H, pyrazole H-4), 7.31–7.51 (a set of signals, 4H, aromatic protons), 8.57 (s, 1H, NH, exchangeable), 9.01 (s, 1H, NH, exchangeable). Elemental analysis ($C_{12}H_{13}N_4OCl$) C, H, N.

4.1.1.4. 1-(3,4-Dichlorophenyl)-3-(1,3-dimethyl-1H-pyrazol-5-yl) urea (**5d**).

Yield: 49%; m.p.: 221–222 °C (ethyl acetate); IR (KBr) ν (cm^{-1}): 3265 (2 × NH), 1646 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.09 (s, 3H, C–CH_3), 3.58 (s, 3H, N–CH_3), 5.96 (s, 1H, pyrazole H-4), 7.33–7.87 (a set of signals, 3H, aromatic protons), 8.71 (s, 1H, NH, exchangeable), 9.13 (s, 1H, NH, exchangeable). ^{13}C NMR (DMSO-d_6) δ (ppm): 13.30 (CH_3), 34.45 (CH_3), 97.25 ($CH_{pyrazole}$), 118.14 (CH_{Ar}), 119.10 (CH_{Ar}), 123.11 (C_{Ar}), 130.26 (CH_{Ar}), 130.71 (C_{Ar}), 136.59 (C_{Ar}), 139.35 (C_{Ar}), 145.09 (C_{Ar}), 151.49 (CO). Elemental analysis ($C_{12}H_{12}N_4OCl_2$) C, H, N.

4.1.1.5. 1-(1,3-Dimethyl-1H-pyrazol-5-yl)-3-(4-methoxyphenyl)urea (**5e**).

Yield: 50.8%; m.p.: 209–210 °C (ethanol); IR (KBr) ν (cm^{-1}): 3269 (2 × NH), 1634 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.07 (s, 3H, C–CH_3), 3.56 (s, 3H, N–CH_3), 3.71 (s, 3H, O–CH_3), 5.93 (s, 1H, pyrazole H-4), 6.87–7.35 (a set of signals, 4H, aromatic protons), 8.40 (s, 1H, NH, exchangeable), 8.61 (s, 1H, NH, exchangeable). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.08 (CH_3), 35.16 (CH_3), 55.65 (O–CH_3), 97.35 ($CH_{pyrazole}$), 114.48 (2 × CH_{Ar}), 120.67 (2 × CH_{Ar}), 132.86 (C_{Ar}), 138.08 (C_{Ar}), 145.78 (C_{Ar}), 152.52 (C_{Ar}), 155.15 (CO). Elemental analysis ($C_{13}H_{16}N_4O_2$) C, H, N.

4.1.1.6. 1-(4-butoxyphenyl)-3-(1,3-dimethyl-1H-pyrazol-5-yl)urea (**5f**).

Yield: 36.7%; m.p.: 178–180 °C (ethanol); IR (KBr) ν (cm^{-1}): 3266 (2 × NH), 1645 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 0.92 (t, 3H,

CH_3), 1.42 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 2.07 (s, 3H, C–CH_3), 3.56 (s, 3H, N–CH_3), 3.90 (t, 2H, CH_2), 5.94 (s, 1H, pyrazole H-4), 6.83–7.34 (a set of signals, 4H, aromatic protons), 8.43 (s, 1H, NH, exchangeable), 8.63 (s, 1H, NH, exchangeable). ^{13}C NMR (DMSO-d_6) δ (ppm): 14.09 (CH_3), 14.13 (CH_3), 19.20 (CH_2), 31.27 (CH_2), 35.16 (CH_3), 67.75 (CH_2), 97.28 ($CH_{pyrazole}$), 115.06 (2 × CH_{Ar}), 120.62 (2 × CH_{Ar}), 132.74 (C_{Ar}), 138.08 (C_{Ar}), 145.77 (C_{Ar}), 152.48 (C_{Ar}), 154.55 (CO). Elemental analysis ($C_{16}H_{22}N_4O_2$) C, H, N.

*4.1.1.7. 1-[4-(Benzyloxy)phenyl]-3-(1,3-dimethyl-1H-pyrazol-5-yl) urea (**5g**).* Yield: 73.9%; m.p.: 204–206 °C (ethanol); IR (KBr) ν (cm^{-1}): 3274 (2 × NH), 1646 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.08 (s, 3H, C–CH_3), 3.57 (s, 3H, N–CH_3), 5.05 (s, 2H, O–CH_2), 5.95 (s, 1H, pyrazole H-4), 6.93–7.45 (a set of signals, 9H, aromatic protons), 8.45 (s, 1H, NH, exchangeable), 8.66 (s, 1H, NH, exchangeable). Elemental analysis ($C_{19}H_{20}N_4O_2$) C, H, N.

*4.1.2. General procedure for the synthesis of 2-(R-substituted-phenyl)-N-(1,3-dimethyl-1H-pyrazol-5-yl)acetamides (**8a**–**c**).*

A solution of equimolar amounts (9 mmol) of 1,3-dimethyl-1*H*-pyrazol-5-amine **3** [18] and substituted phenylacetylchlorides **7a**–**c** in dry $CHCl_3$ (30 mL) was refluxed for 5 h. After the first hour, triethylamine (1.25 mL, 9 mmol) was added in four portions (0.6, 0.3, 0.2, 0.15 mL respectively with intervals of 1 h between additions). The reaction mixture was evaporated under reduced pressure, the residue washed with cold water and crystallized from a suitable solvent.

*4.1.2.1. N-(1,3-Dimethyl-1H-pyrazol-5-yl)-2-phenylacetamide (**8a**).* Yield: 43.6%; m.p.: 87–90 °C (diethyl ether); IR (KBr) ν (cm^{-1}): 3252 (NH), 1666 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.06 (s, 3H, C–CH_3), 3.53 (s, 3H, N–CH_3), 3.67 (s, 2H, CH_2), 5.96 (s, 1H, pyrazolic H-4), 7.24–7.33 (a set of signals, 5H, aromatic protons), 10.07 (s, 1H, NH, exchangeable). ^{13}C NMR ($CDCl_3$) δ (ppm): 13.77 (CH_3), 34.94 (CH_3), 43.52 (CH_2), 99.56 ($CH_{pyrazole}$), 127.77 (CH_{Ar}), 129.22 (2 × CH_{Ar}), 129.30 (2 × CH_{Ar}), 134.08 (C_{Ar}), 135.53 (C_{Ar}), 147.24 (C_{Ar}), 169.73 (CO). Elemental analysis ($C_{13}H_{15}N_3O$) C, H, N.

*4.1.2.2. 2-(4-Chlorophenyl)-N-(1,3-dimethyl-1H-pyrazol-5-yl)acetamide (**8b**).* Yield: 11%; m.p.: 137–140 °C (ethyl acetate); IR (KBr) ν (cm^{-1}): 3255 (NH), 1664 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.06 (s, 3H, C–CH_3), 3.54 (s, 3H, N–CH_3), 3.68 (s, 2H, CH_2), 5.96 (s, 1H, pyrazolic H-4), 7.32–7.40 (a set of signals, 4H, aromatic protons), 10.07 (s, 1H, NH, exchangeable). ^{13}C NMR ($CDCl_3$) δ (ppm): 13.76 (CH_3), 35.07 (CH_3), 42.62 (CH_2), 99.88 ($CH_{pyrazole}$), 129.22 (2 × CH_{Ar}), 130.57 (2 × CH_{Ar}), 132.53 (C_{Ar}), 133.69 (C_{Ar}), 135.50 (C_{Ar}), 147.33 (C_{Ar}), 169.35 (CO). Elemental analysis ($C_{13}H_{14}N_3OCl$) C, H, N.

*4.1.2.3. N-(1,3-Dimethyl-1H-pyrazol-5-yl)-2-(4-methoxyphenyl) acetamide (**8c**).* Yield: 14.1%; m.p.: 112–115 °C (ethyl acetate); IR (KBr) ν (cm^{-1}): 3259 (NH), 1663 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.06 (s, 3H, C–CH_3), 3.53 (s, 3H, N–CH_3), 3.58 (s, 2H, CH_2), 3.72 (s, 3H, O–CH_3), 5.94 (s, 1H, pyrazolic H-4), 6.87–7.25 (a set of signals, 4H, aromatic protons), 9.99 (s, 1H, NH, exchangeable). ^{13}C NMR ($CDCl_3$) δ (ppm): 13.83 (CH_3), 34.97 (CH_3), 42.80 (CH_2), 55.34 (O–CH_3), 99.51 ($CH_{pyrazole}$), 114.73 (2 × CH_{Ar}), 125.94 (C_{Ar}), 130.52 (2 × CH_{Ar}), 135.42 (C_{Ar}), 147.31 (C_{Ar}), 159.25 (C_{Ar}), 169.89 (CO). Elemental analysis ($C_{14}H_{17}N_3O_2$) C, H, N.

*4.1.3. General procedure for the synthesis of [(R-substituted-phenyl) carbamoyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanides (**6a**–**d**), [(4-R-2-nitrophenyl)carbamoyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanides (**6e**–**f**), and of [(4-chlorophenyl) acetyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanides (**9a**–**c**).*

Aqueous 37% hydrochloric acid (1.98 mL) and then potassium nitrite (1.61 g, 18.9 mmol) dissolved in water (0.85 mL) were added at room temperature to a magnetically-stirred solution of 1-(R-substituted-phenyl)-3-(1,3-dimethyl-1*H*-pyrazol-5-yl)ureas (**5a**–**g**) or 2-(R-substituted-phenyl)-*N*-(1,3-dimethyl-1*H*-pyrazol-5-yl)acetamides (**8a**–**c**) (2.7 mmol) in glacial acetic acid (26.42 mL). The reaction mixture was then stirred overnight away from the light, after which it was filtered off and poured into 70 mL of cold water. Then, the solution was basified until pH 6 with aqueous concentrated NaOH and finally the solid product which separated was filtered and crystallized from a suitable solvent.

*4.1.3.1. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)(phenylcarbamoyl) azanide (**6a**).* Yield: 10.3%; m.p.: 152–154 °C (ethanol); IR (KBr) ν (cm^{-1}): 3261 (NH), 2138 (N_2^+), 1638 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, C–CH_3), 3.53 (s, 3H, N–CH_3), 6.88–7.63 (a set of signals, 5H, aromatic protons), 9.22 (s, 1H, NH, exchangeable). Elemental analysis ($C_{12}H_{12}N_6O$) C, H, N.

*4.1.3.2. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)[(4-nitrophenyl) carbamoyl]azanide (**6b**).* Yield: 87.1%; m.p.: 197–200 °C (ethanol); IR (KBr) ν (cm^{-1}): 3231 (NH), 2145 (N_2^+), 1644 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.31 (s, 3H, C–CH_3), 3.57 (s, 3H, N–CH_3), 7.84–8.16 (a set of signals, 4H, aromatic protons), 10.02 (s, 1H, NH, exchangeable). ^{13}C NMR (DMSO-d_6) δ (ppm): 11.98 (CH_3), 36.89 (CH_3), 72.10 (C_{Ar}), 118.00 (2 × CH_{Ar}), 125.17 (2 × CH_{Ar}), 141.94 (C_{Ar}), 144.90 (C_{Ar}), 146.99 (C_{Ar}), 146.61 (C_{Ar}), 153.32 (CO). Elemental analysis ($C_{12}H_{11}N_7O_3$) C, H, N.

*4.1.3.3. [(4-Chlorophenyl)carbamoyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanide (**6c**).* Yield: 23.5%; m.p.: 151–152 °C (ethanol); IR (KBr) ν (cm^{-1}): 3214 (NH), 2145 (N_2^+), 1638 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, C–CH_3), 3.53 (s, 3H, N–CH_3), 7.25–7.67 (a set of signals, 4H, aromatic protons), 9.42 (s, 1H, NH, exchangeable). Elemental analysis ($C_{12}H_{11}N_6OCl$) C, H, N.

*4.1.3.4. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)[(3,4-dichlorophenyl)carbamoyl]azanide (**6d**).* Yield: 17.7%; m.p.: 191–194 °C (ethanol); IR (KBr) ν (cm^{-1}): 3254 (NH), 2135 (N_2^+), 1638 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.30 (s, 3H, C–CH_3), 3.55 (s, 3H, N–CH_3), 7.44–7.98 (a set of signals, 3H, aromatic protons), 9.62 (s, 1H, NH, exchangeable). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.46 (CH_3), 35.02 (CH_3), 71.92 (C_{Ar}), 117.60 (2 × CH_{Ar}), 119.82 (2 × CH_{Ar}), 125.11 (C_{Ar}), 130.28 (CH_{Ar}), 132.54 (C_{Ar}), 139.41 (C_{Ar}), 144.82 (C_{Ar}), 154.95 (C_{Ar}), 161.23 (CO). Elemental analysis ($C_{12}H_{10}Cl_2N_6O_4$) C, H, N.

*4.1.3.5. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)[(4-methoxy-2-nitrophenyl)carbamoyl]azanide (**6e**).* Yield: 15.7%; m.p.: 151–152 °C (ethanol); IR (KBr) ν (cm^{-1}): 3411 (NH), 2161 (N_2^+), 1627 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, C–CH_3), 3.51 (s, 3H, N–CH_3), 3.80 (s, 3H, O–CH_3), 7.32 (dd, 1H, J = 2.1 Hz, 9.3 Hz), 7.51 (d, 1H, J = 2.1 Hz), 8.11 (d, 1H, J = 9.3 Hz), 9.54 (s, 1H, NH, exchangeable). Elemental analysis ($C_{13}H_{13}N_7O_4$) C, H, N.

*4.1.3.6. [(4-Butoxy-2-nitrophenyl)carbamoyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanide (**6f**).* Yield: 38.7%; m.p.: 124–125 °C (ethanol); IR (KBr) ν (cm^{-1}): 3404 (NH), 2154 (N_2^+), 1651 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 0.93 (t, 3H, CH_3), 1.43 (m, 2H, CH_2), 1.70 (m, 2H, CH_2), 2.28 (s, 3H, C–CH_3), 3.51 (s, 3H, N–CH_3), 4.01 (t, 2H, CH_2), 7.31 (dd, 1H, J = 3 Hz, 9.3 Hz), 7.48 (d, 1H, J = 3 Hz), 8.12 (d, 1H, J = 9.3 Hz), 9.53 (s, 1H, NH, exchangeable). Elemental analysis ($C_{16}H_{19}N_7O_4$) C, H, N.

*4.1.3.7. {[4-(Benzyloxy)-3-nitrophenyl]carbamoyl}(4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanide (**6g**).* Yield: 13.1%; m.p.: 195–197 °C (ethanol); IR (KBr) ν (cm^{-1}): 3399 (NH), 2141 (N_2^+), 1653 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.29 (s, 3H, C–CH_3), 3.51 (s, 3H, N–CH_3), 5.16 (s, 2H,

O–CH_2), 7.34–8.12 (a set of signals, 8H, aromatic protons), 9.53 (s, 1H, NH, exchangeable). Elemental analysis ($C_{19}H_{17}N_7O_4$) C, H, N.

*4.1.3.8. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)(phenylacetyl) azanide (**9a**).* Yield: 50.3%; m.p.: 112–114 °C (diethyl ether); IR (KBr) ν (cm^{-1}): 2160 (N_2^+), 1592 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, C–CH_3), 3.53 (s, 3H, N–CH_3), 3.63 (s, 2H, CH_2), 7.18–7.28 (a set of signals, 5H, aromatic protons). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.45 (CH_3), 35.30 (CH_3), 47.12 (CH_2), 73.70 ($CH_{pyrazole}$), 126.34 (CH_{Ar}), 128.30 (2 × CH_{Ar}), 129.43 (2 × CH_{Ar}), 137.16 (C_{Ar}), 145.35 (C_{Ar}), 154.74 (C_{Ar}), 181.35 (CO). Elemental analysis ($C_{13}H_{13}N_5O$) C, H, N.

*4.1.3.9. [(4-Chlorophenyl)acetyl](4-diazonio-1,3-dimethyl-1H-pyrazol-5-yl)azanide (**9b**).* Yield: 7.6%; m.p.: 100–104 °C (ethanol); IR (KBr) ν (cm^{-1}): 2154 (N_2^+), 1590 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.29 (s, 3H, C–CH_3), 3.49 (s, 3H, N–CH_3), 3.63 (s, 2H, CH_2), 7.31 (s, 4H, aromatic protons). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.33 (CH_3), 35.19 (CH_3), 46.37 (CH_2), 73.62 ($CH_{pyrazole}$), 128.30 (2 × CH_{Ar}), 130.78 (2 × CH_{Ar}), 132.12 (C_{Ar}), 135.74 (C_{Ar}), 145.22 (C_{Ar}), 154.89 (C_{Ar}), 181.80 (CO). Elemental analysis ($C_{13}H_{12}N_5OCl$) C, H, N.

*4.1.3.10. (4-Diazonio-1,3-dimethyl-1H-pyrazol-5-yl)[(4-methoxyphenyl) acetyl]azanide (**9c**).* Yield: 17.2%; m.p.: 100–105 °C (ethanol); IR (KBr) ν (cm^{-1}): 2160 (N_2^+), 1595 (CO); ^{1}H NMR (DMSO-d_6) δ (ppm): 2.28 (s, 3H, C–CH_3), 3.55 (s, 5H, N–CH_3, CH_2), 3.72 (s, 3H, O–CH_3), 6.81–7.53 (a set of signals, 4H, aromatic protons). ^{13}C NMR ($CDCl_3$) δ (ppm): 12.39 (CH_3), 35.20 (CH_3), 46.26 (CH_2), 55.25 (O–CH_3), 73.77 ($CH_{pyrazole}$), 113.74 (2 × CH_{Ar}), 128.62 (C_{Ar}), 130.37 (2 × CH_{Ar}), 131.19 (C_{Ar}), 145.11 (C_{Ar}), 158.19 (C_{Ar}), 182.87 (CO). Elemental analysis ($C_{14}H_{15}N_5O_2$) C, H, N.

4.2. Microbiology

4.2.1. Bacterial strains

The following reference strains were utilized: *S. aureus* ATCC 25923, *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538. Moreover, a group of six strains of *S. aureus* isolated from milk and food samples were also utilized. The isolates belong to the bacterial collection of IZS of Sicily [19].

4.2.2. Determination of MICs

Minimum inhibitory concentrations (MICs) against planktonic strains were determined by a broth dilution micro-method as previously described [20]. Briefly, a series of solutions with a range of concentrations from 50 to 0.001 μg mL^{-1} (obtained by twofold serial dilution) were made in Mueller Hinton broth (Merck) in a 96-well plate. To each well 10 μL of a bacterial suspension obtained from a 24 h culture containing ~10^6 cfu mL^{-1} was added. The plate was incubated at 37 °C for 24 h. After this time, the MIC values were determined by a microplate reader (ELX 800, Bio-Tek Instruments) as the lowest concentration of compound at which the optical density (OD) at 570 nm of the well was comparable to the negative control well (broth only).

4.2.3. Biofilm capability evaluation

All the staphylococcal 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. Polystyrene 24-well tissue culture plates were filled with 1 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 safranin 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. Safranin stained adherent bacteria in each well were re-dissolved to homogeneity in 1 mL of 30% v/v glacial acetic acid, and the OD was read at 492 nm. Each assay was performed in triplicate and repeated at least twice.

4.2.4. Biofilm prevention assay

The same safranin procedure was used to evaluate the activity of the tested compounds in preventing biofilm formation by adding directly sub-MIC concentrations of compounds to the bacterial suspension. Comparing the average optical density of the growth control wells with the sample wells the following formula was used to calculate the percentages of inhibition for the screening concentration of the compounds:

$$[(\text{OD growth control} - \text{OD sample})/\text{OD growth control}] \times 100.$$

All experiments were performed at least in triplicate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.09.041.

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