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Research paper

Synthesis and biofilm formation reduction of pyrazole-4-carboxamide derivatives in some *Staphylococcus aureus* strains

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ABSTRACT

The ability of several *N*-phenyl-1*H*-pyrazole-4-carboxamide derivatives and other pyrazoles opportunely modified at the positions 3, 4 and 5, to reduce the formation of the biofilm in some *Staphylococcus aureus* strains (ATCC 29213, ATCC 25923 and ATCC 6538) were investigated. All the tested compounds were able, although to a different extent, to reduce the biofilm formation of the three bacterial strains considered. Among these, the 1-(2,5-dichlorophenyl)-5-methyl-*N*-phenyl-1*H*-pyrazole-4-carboxamide **14** resulted as the best inhibitor of biofilm formation showing an IC_{50} ranging from 2.3 to 32 μM, against all the three strains of *S. aureus*. Compound **14** also shows a good protective effect *in vivo* by improving the survival of wax moth larva (*Galleria mellonella*) infected with *S. aureus* ATCC 29213. These findings indicate that **14d** is a potential lead compound for the development of new anti-virulence agents against *S. aureus* infections.

1. Introduction

Bacterial biofilm formation is one of the common cause of persistent infection and represents a major health problem as it plays an important role in device-related infections such as prosthetic valves, catheters, orthopedic devices etc. Furthermore, biofilms are responsible for persistent infections due to *Pseudomonas aeruginosa*, *Escherichia coli*, *Mycobacterium tuberculosis* and *Streptococcus mutans* [1]. Such and other medically relevant pathogens are responsible for biofilm associated disease, such as cystic fibrosis lung infection (*P. aeruginosa*, *Burkholderia cepacia*), burn wound and trauma infection (*Acinetobacter baumanii*), urinary and gastrointestinal infection (*E. coli*), respiratory infection (*Bordetella pertussis*), catheters and lung infections (*Staphylococcus aureus*), nosocomial sepsis and catheter infection (*Staphylococcus epidermidis*) and dental caries (*S. mutans*) [2], very difficult to be treated by conventional antibiotics [3].

The correlation between the biofilm formation and the bacterial persistence has made necessary the development of new potential therapeutic approaches which combine the current antibiotic therapy with drugs targeting biofilm formation [1,4,5].

Biofilms are surface bacterial aggregates encased in a synthesized hydrated matrix, the formation of which require the following four stages: attachment of cells to a surface, formation of micro colonies, maturation of the micro colonies into an established biofilm, and dispersal of the bacteria from the biofilm [6]. Three principal strategies have been developed to counteract biofilm formation or target different biofilm developmental stages: inhibition of the adhesion of bacteria to living or non-living surfaces [7], disruption of biofilm architecture during the maturation process, inhibition of quorum sensing (QS).

The aryl rhodanine derivatives [8], the cis-2-decenoic acid [9], and the halogenated furanone derivatives [10] respectively are examples of these three strategies. Other examples of bacterial biofilm formation inhibitors, for which the mechanism of action remain to be elucidated, are the 5-alkylidenethiophen-2(5*H*)-ones [11], the pyrazole-1-carbothioamides [12], the 1,3,5-triazine-pyrazole conjugates [13], the chromen-pyrazole-pyrimidines [14], and the 4-diazo-pyrazoles [15,16]. Furthermore, the antibiofilm activity, through diguanylate cyclase inhibition, for compounds bearing the

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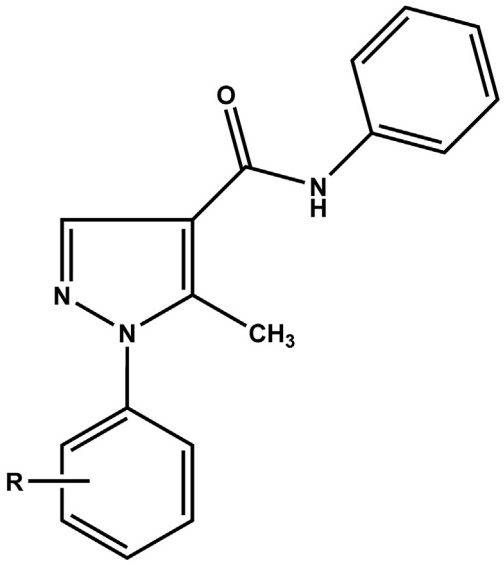
N-phenyl-1-carboxamide moiety was described [17].

Prompted by these observations and in combination with our researches on pyrazole nucleus [15,16,18–23], we synthesized pyrazole derivatives of type **1**, bearing at the position 4 the *N*-phenylcarboxamide moiety, with the aim to evaluate their anti-biofilm activity (Fig. 1).

Furthermore, to study the influence of substituents on the biofilm formation inhibitory activity, pyrazoles opportunely modified at the positions 3, 4 and 5 and, in particular, the ethyl 5-amino-1-phenyl-1*H*-pyrazole-4-carboxylate **2**, the ethyl 5-amino-1-(pyridin-2-yl)-1*H*-pyrazole-4-carboxylate **3**, the ethyl 5-amino-1-(4-sulfamoylphenyl)-1*H*-pyrazole-4-carboxylate **4**, the ethyl 5-amino-1-methyl-1*H*-pyrazole-4-carboxylate **5**, the ethyl 5-amino-1*H*-pyrazole-4-carboxylate **6**, the ethyl 5-amino-3-methyl-1*H*-pyrazole-4-carboxylate **7** and the 3-methyl-*N*,1-diphenyl-1*H*-pyrazole-5-carboxamides **8a,b** (Fig. 2) were considered.

Compounds of type **1** and **8a,b** were synthesized, pyrazoles **2**-**7** have been largely used in the past as starting material in our laboratories and they were available with us [23–27].

All derivatives, thus, obtained were tested for both the planktonic growth inhibitory activity and the inhibition of biofilm formation against the following reference bacterial strains: *S. aureus* ATCC 6538, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213. Finally, the antivirulence activity and the toxicity of the most active compound were also evaluated *in vivo* by the wax moth larva model (*Galleria melonella*, Lepidoptera: Pyralidae). Insects are considered good model to study the effect of different substances and also the host pathogens interaction [28]. *G. melonella* is a model widely used for studies concerning different aspects of host-entomopathogen interactions but only recently was considered a suitable host for assessing the *in vivo* efficacy of antimicrobial agents against human pathogens [29]. In addition, this animal model is very useful considering that it is a preliminary and alternative infection model that generate *in vivo* data quickly and inexpensively. Three essential features make it useful for this purpose. First, the microbial virulence is similar in the wax moth larva and mammalians; second, the wax moth model can be run at the human core body temperature of 37 °C and, finally, an accurate inoculum of the pathogen can be injected directly into the host's body [29].



R = H, 3-Cl, 4-Cl, 2,5-Cl_2, 3-NO_2, 4-CF_3,

1

Fig. 1. Structure of pyrazoles of type **1**.

2. Results and discussion

2.1. Chemistry

The synthesis of 1-(2-R^1-3-R^2-4-R^3-5-R^4-phenyl)-5-methyl-*N*-phenyl-1*H*-pyrazole-4-carboxamide derivatives **1a-c,e-h** was achieved according to the Scheme 1.

A mixture of ethyl acetoacetate **9** and aniline **10** was reacted in a microwave oven for 5 min at 480 W to obtain a mixture of 3-oxo-*N*-phenylbutanamide **11a** [30] and 1,3-diphenylurea **11b** [31] which was separated by flash chromatograpy [32]. Compound **11a** was then refluxed with acetic anhydride and triethyl orthoformate for 24 h to give pure 2-(ethoxymethylene)-3-oxo-*N*-phenyl-butanamide **12**. Compound **12** was finally treated with the appropriate phenylhydrazine **13a-h**. As outlined in the Scheme 1, when compound **12** was treated with the phenylhydrazines **13a-c,e-h**, pyrazoles **1a-c,e-h** were isolated as principal products. When compound **12** was treated with the 2,5-dichlorophenylhydrazine **13d**, the only compound isolated was the 2-((2-(2,5-dichlorophenyl)hydrazinyl)methylene)-3-oxo-*N*-phenyl-butanamide **14d** (Scheme1) [33].

Compound **1d** was finally obtained by fusion of **14d** at 170 °C for 15 min (Scheme 2).

The 3-methyl-*N*,1-diphenyl-1*H*-pyrazole-5-carboxamides **8a,b** were synthesized as shown in Scheme 3.

The intermediate ethyl acetopyruvate **17** was obtained in good yield by adding, in an ice bath, stirred ethanolic solution of sodium, a solution of acetone **15** and diethyl oxalate **16**. Treatment of **17** with phenyl hydrazine in acetic acid under magnetic stirring for 24 h yield a 1:1.5 mixture of the two isomers ethyl 3-methyl-1-phenyl-1*H*-pyrazole-5-carboxylate **18a** and ethyl 5-methyl-1-phenyl-1*H*-pyrazole-3-carboxylate **18b** which were separated by flash chromatography [32]. Compounds **18a,b** are known [34] and this allow us to differentiate the two isomers by means of melting point, IR and ^{1}H NMR. Compounds **18a,b** were then hydrolysed with an alcoholic solution of potassium hydroxide to give the corresponding acids **19a,b** and finally treated with thionyl chloride to give **20a,b**. Condensation of aniline with **20a,b** in chloroform under reflux for 6 h afforded the expected 3-methyl-*N*,1-diphenyl-1*H*-pyrazole-5-carboxamides **8a,b**.

The structures of the new compounds were determined by analytical and spectroscopic measurements. In particular, all derivatives **1a-h** and **8a,b** showed signals attributable to aromatic protons in the range 6.93–8.44 δ and a singlet in the range 7.98–9.26 δ, exchangeable with D_2O, for the amidic NH. Derivatives **1a-h** and **8a,b** showed also a singlet in the range 2.40–2.73 δ attributable to the 5-methyl or 3-methyl pyrazole hydrogens.

^{1}H NMR spectrum of compound **14d** showed how, in solution (DMSO), two tautomeric forms are present (Fig. 3).

Tautomer A showed two singlets at 11.84 δ and 8.82 δ and a doublet at 11.32, exchangeable with D_2O, for NHs. The split as doublet at 11.32 δ of NH is due to the coupling with the adjacent=CH (doublet at 8.23 δ). After exchange with D_2O the signal at 11.32 δ disappears and the doublet at 8.23 δ collapse into a single signal. Tautomer B instead, showed three singlets at 16.47 δ, 11.15 δ and 9.89 δ, exchangeable with D_2O, for NHs and OH, as well as a

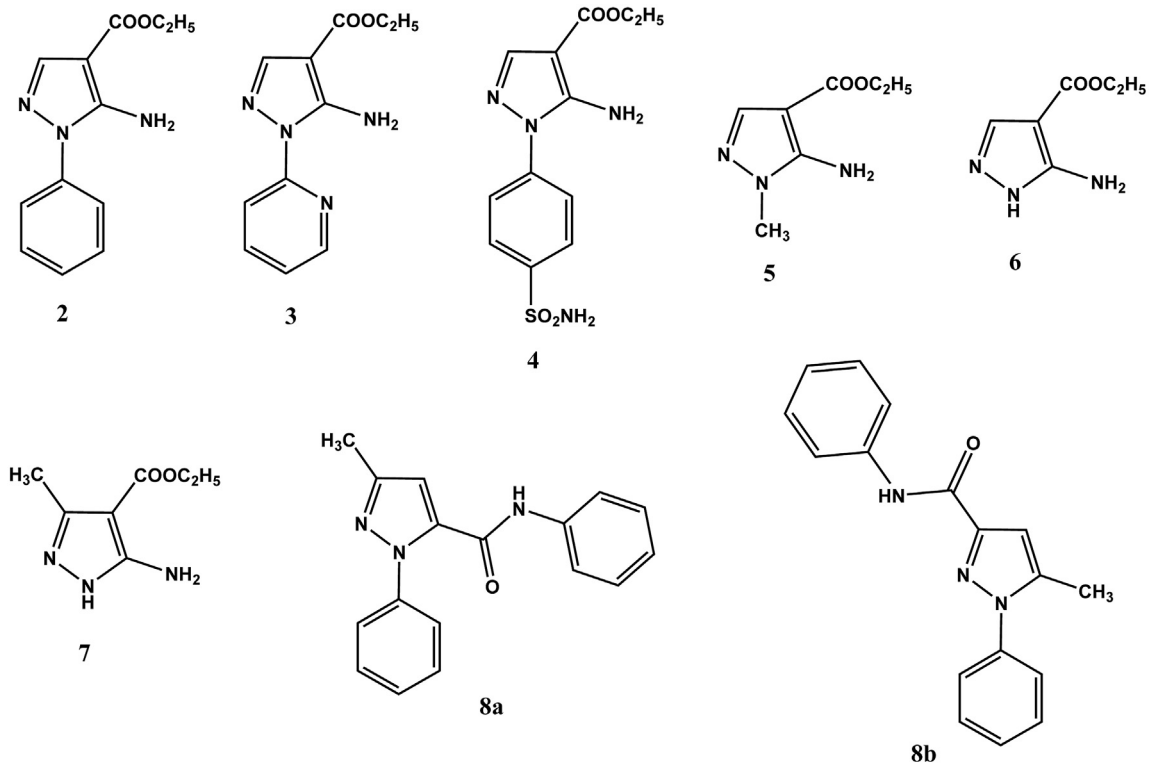
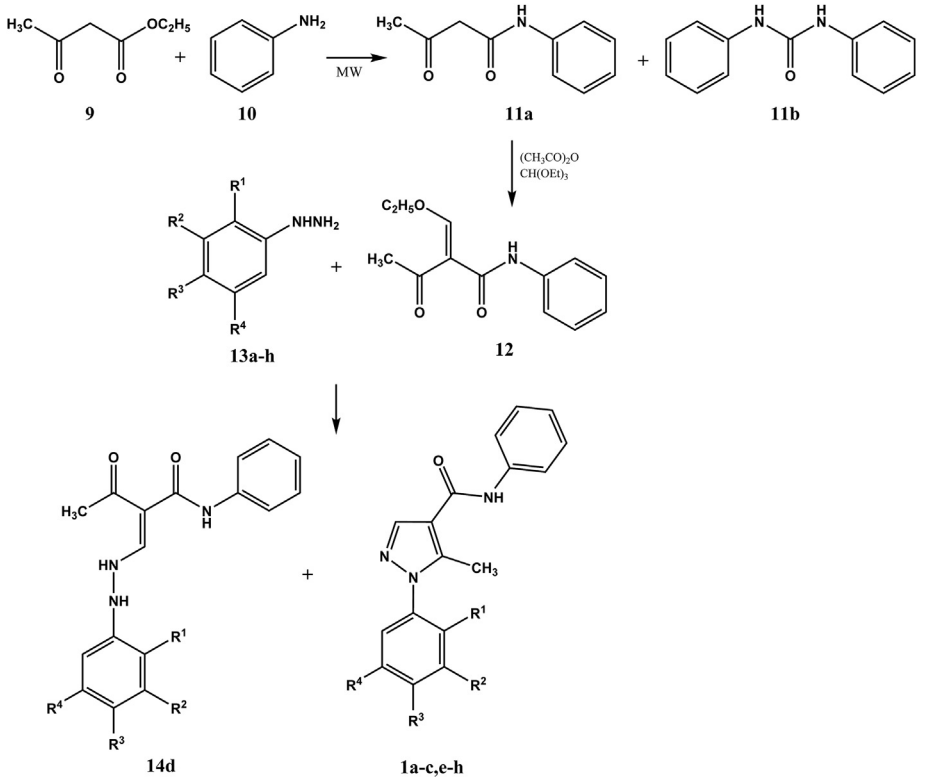


Fig. 2. Structures of pyrazoles **2-7** and **8a,b**.



a R^1=H, R^2=H, R^3=H, R^4=H
b R^1=H, R^2= Cl, R^3=H, R^4=H
c R^1=H, R^2=H, R^3=Cl, R^4= H
d R^1=Cl, R^2=H, R^3=H, R^4= Cl
e R^1=H, R^2=NO_2, R^3=H, R^4=H
f R^1=H, R^2=H, R^3=NO_2, R^4=H
g R^1=H, R^2= H, R^3=CF_3, R^4=H
h R^1=F, R^2=H, R^3=H, R^4= F

Scheme 1. Synthetic pathway for the formation of 1-(2-R_1-3-R_2-4-R_3-5-R_4-phenyl)-5-methyl-*N*-phenyl-1H-pyrazole-4-carboxamide derivatives **1a-c,e-h** and **14d**.

singlet at 8.59 δ attributable to =CH. Finally, both tautomers showed a singlet, attributable to the methyl group, one at 2.37 δ (tautomers A), the other 2.02 δ (tautomer B); by these last signals, an approximate percentage of the two form was calculated (tautomer A ~86% and tautomer B ~14%).

2.2. Biology

The compounds **1a-h**, **8a,b** and **14d** were tested for their ability to interfere with the biofilm formation in *S. aureus* ATCC 6538, *S. aureus* ATCC 25913 and *S. aureus* ATCC 29223 bacterial strains, the results are shown in Table 1.

Previously to the investigation of the effect of the compounds **1a-h**, **8a,b** and **14d** on biofilm formation inhibition, their inhibitory activity on the planktonic growth of above mentioned strains was evaluated by determining the minimum inhibitory concentration (MIC). This preliminary assay is essential to determine if any possible observed effects caused by the compounds on bacterial biofilm formation are specific to biofilm formation or could be related to a possible decline in the population of bacteria due to biocidal activity.

None of the tested compounds caused any significant planktonic growth inhibition at 250 μM. In view of the results obtained for planktonic growth inhibition, we can assume that the effect of the compounds on biofilm inhibition is not related to their biocidal activity. Moreover, drugs could target the early steps of bacterial adhesion, essential for the establishment of infections and colonization, without affecting the bacterial growth, imposing a low selection pressure and thus, avoiding development of resistance.

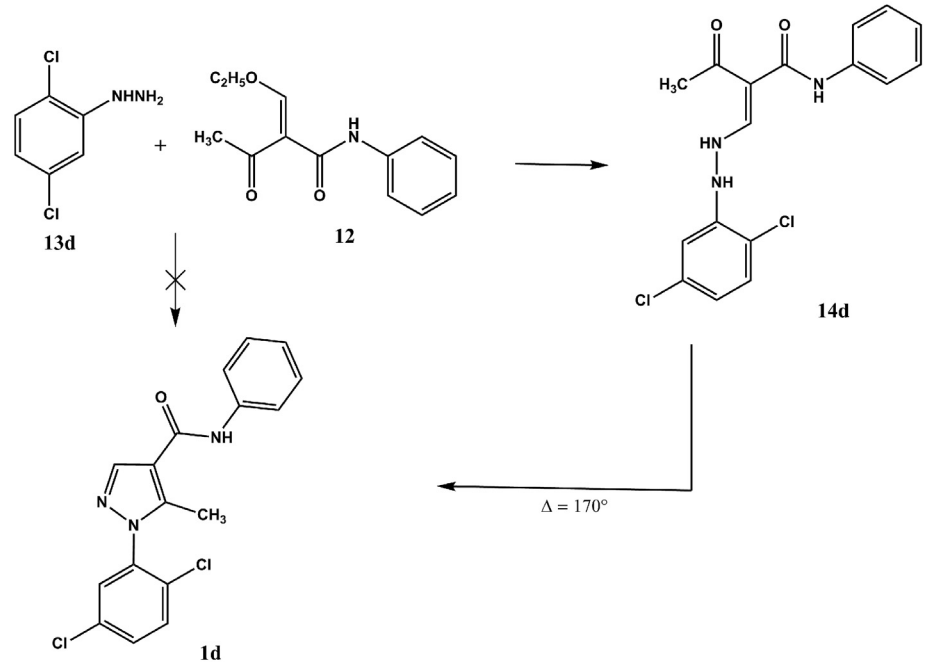
The data reported in Table 1 showed that all the tested compounds (**1a-h**, **8a,b** and **14d**) were able, although to a different extent, to reduce the biofilm formation of the three bacterial strains considered. Among these, compounds **1c,e,h** and **14d** showed the best activity against all the three strains of *S. aureus* with **1c** and **14d** being the most active. In general, the ability to reduce the staphylococcal biofilm formation of our compounds is in the range 2.3–434 μM in agreement with the activity of numerous inhibitors of biofilm formation reported in the literature such as analogs of rubolides: 1.3–12.4 μM (0.6–5.7 μg/mL) [35], analogs of oroidin: 20–90 μM [36], pyrimido chromen-2-ones: 4.13–194 μM (2.34–75 μg/mL) [14] and triazine-pyrazole coniugates: 23.39–190 μM (15.62–125 μg/mL) [13].

In particular, data of Table 1 revealed how the best activity was showed by the open structure of the intermediate **14d** respect to *N*-phenylpyrazoles **1a-h** and **8a,b**. This is in agreement with the results obtained by us for new series of phenylhydrazinylidene derivatives of type **21-23,** [37,38] structurally related to **14d** (Fig. 4). The activity in compounds **21-23** is due to the presence of a phenylhydrazinylidene moiety which is related to the phenylhydrazinylmethylene group of **14d**.

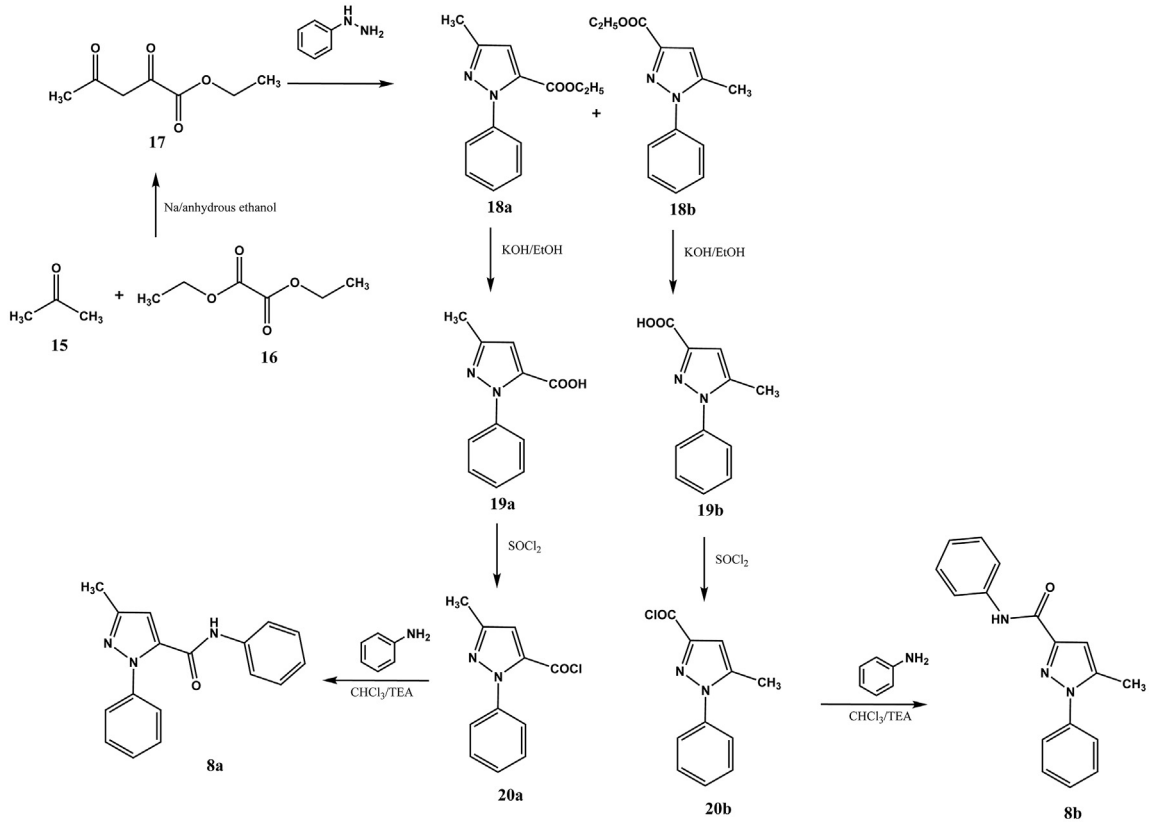
Table 1
Ability of **1a-h**, **8a,b** and **14** to reduce the biofilm formation of *S. aureus* 6538, *S. aureus* 25913 and *S. aureus* 29223 bacterial strains.

Compound	IC_{50} (μM)		
	S. a. 6538	*S. a.* 25923	*S. a.* 29213
2	95 ± 2	222 ± 2	34 ± 1
3	91 ± 2	102 ± 3	31 ± 2
4	142 ± 2	77 ± 2	28 ± 1
5	ns	159 ± 3	152 ± 2
6	85 ± 2	213 ± 2	83 ± 1
7	82 ± 1	378 ± 4	62 ± 3
1a	77 ± 2	112 ± 3	309 ± 4
1b	103 ± 2	161 ± 2	169 ± 3
1c	30 ± 1	552 ± 1	38 ± 2
1d	ns	92 ± 3	52 ± 2
1e	90 ± 1	64 ± 1	46 ± 1
1f	55 ± 1	434 ± 1	127 ± 1
1g	ns	64 ± 2	46 ± 2
1h	81 ± 1	61 ± 4	89 ± 1
8a	54 ± 1	74 ± 1	123 ± 2
8b	64 ± 4	76 ± 2	54 ± 2
14d	32 ± 1	2.3 ± 0.1	2.5 ± 0.2

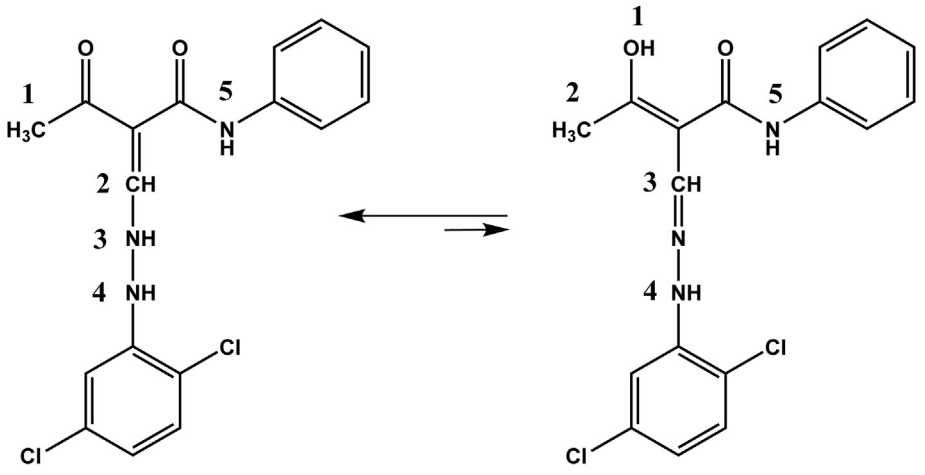
Values are the mean of at least three independent determinations. ns = not significant.



Scheme 2. Synthetic pathway to obtain the 1-(2,5-dichlorophenyl)-5-methyl-*N*-phenyl-1H-pyrazole-4-carboxamide **1d**.



Scheme 3. Synthetic pathway to obtain the 3-methyl-N,1-diphenyl-1H-pyrazole-5-carboxamides **8a,b**.



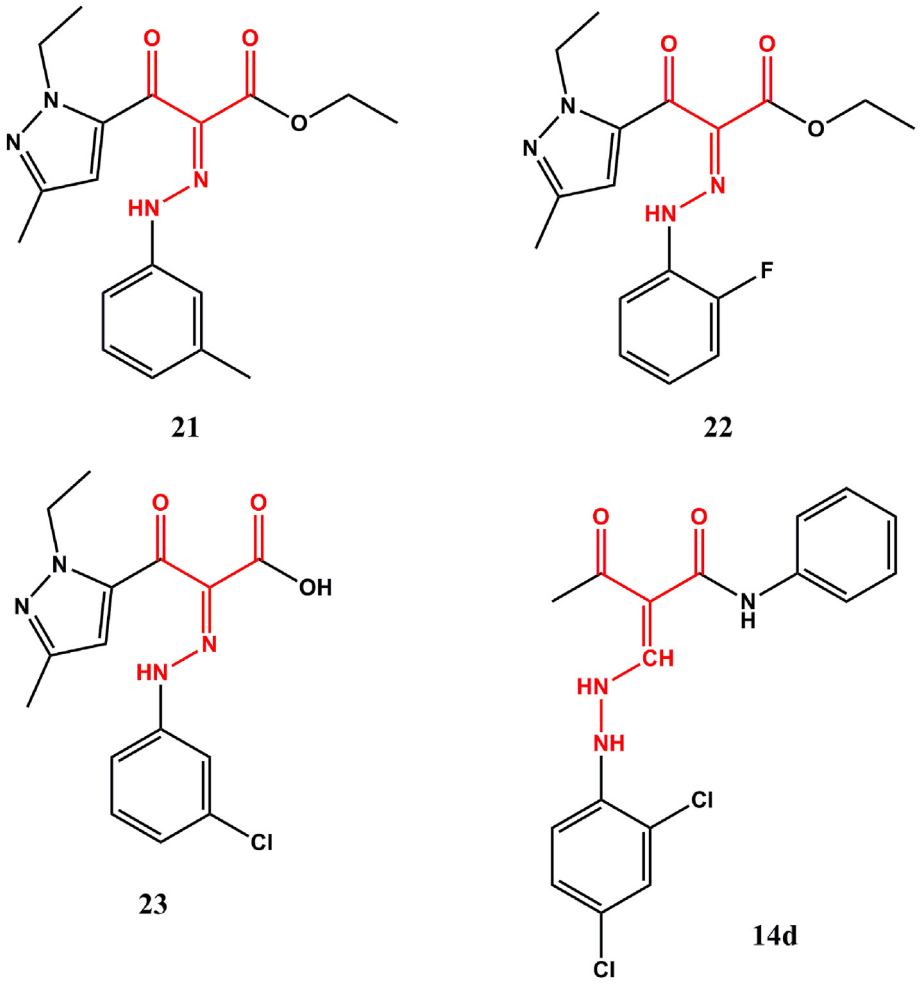
A (~86%)

B (~14%)

1: 2.73, s
2: 8.23 d, J = 10.8 Hz
3: 11,32 d, J = 10.8 Hz
4: 8.82, s
5: 11,84, s

1: 16,74, s
2: 2,02, s
3: 8,59, s
4: 9.89, s
5: 11,15, s

Fig. 3. Tautomeric forms of **14d** and differences in ^{1}H NMR signals.



Comp.	S. aureus 6538	S. aureus 25923	S. aureus 29213
21	43	10	1.7
22	0.9	42	13
23	11	20	16
14d	32	2.3	2.5

Fig. 4. Antibiofilm activity (IC_{50}, μM) of **14d** and related compounds **21-23** [37].

A comparation of the antibiofilm activities of these compounds show the good influence of the phenylhydrazinylmethylene group which generally leads to a more active compound (**14d**) respect to compounds **21-23** [38], especially against *S. aureus* 25913 and *S. aureus* 29223 bacterial strains.

On the basis of such results, the antivirulence activity of the most active compound **14d** was also evaluated *in vivo* by the wax moth larva model (*G. mellonella*).

The survival of wax moth larvae were evaluated on five groups of larvae: untreated larvae; uninfected larvae treated with the solvent used to dissolve the tested compound (PBS and 3% DMSO); uninfected larvae treated with the solvents used to dissolve the tested compound (PBS and 3% DMSO) plus the compound **14d**; larvae pre-treated with the solvent (PBS and 3% DMSO) 1 h before the injection of live bacteria; and larvae pre-treated with 1 mg/kg of **14d** 1 h before the inoculum of live bacteria.

Fig. 5 shows the effect of the compound **14d** on the survival of the larvae of *G. melonella*. No mortality was detected in the untreated larvae. Significant statistical differences were recorded in the infected larvae compared with all the other groups. In addition, statistical differences were recorded also between the two infected groups with *S. aureus* highlighting that the compound **14d** had a protective role for the larvae.

As it can be seen from Fig. 5, the survival after 24 h of infected *G. mellonella* larvae was increased by a 1.6 factor when compound **14d** was injected 1 h before the inoculation of bacteria thus proving a good protective effect and the reduction of the bacterial virulence exerted by the tested compound. The low toxicity of compound **14d** was also demonstrated considering that there is a slightly lower survival percentage difference between uninfected larvae and larvae treated with **14d**. No sub-lethal effects were recorded for the larvae injected with the compound **14d**. In fact, all larvae that were kept in starvation, lost weight and no statistical differences were recorded among the groups ($df = 3,68$, $p = 0.7119$). In addition, the time to the pupation and formation of the chrysalis was in average 7 days (±1 day) and no statistical

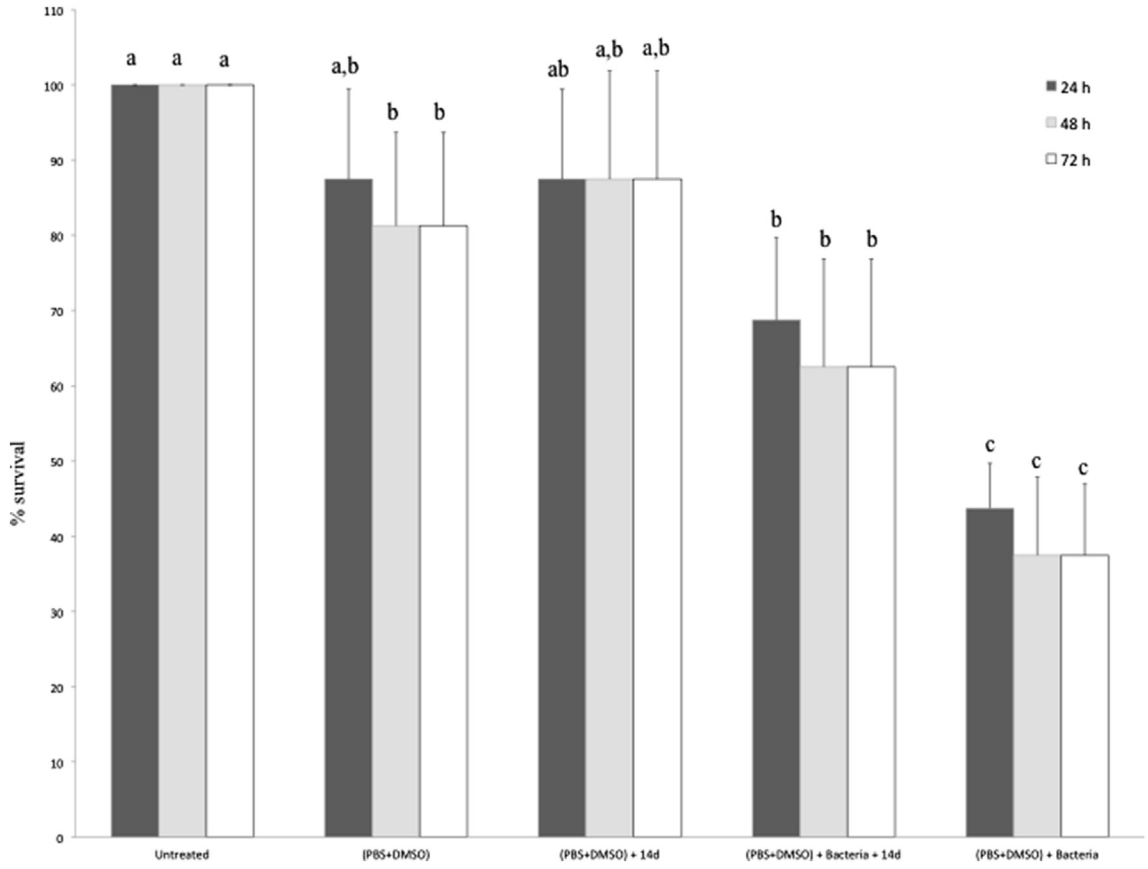


Fig. 5. Effect of pre-treatment with a single dose (1 mg/kg) of compound **14d** on survival of *Galleria mellonella* larvae at 37 °C infected with *S.aureus* ATCC 29213 Survival was checked at 24, 48 and 72 h (h) after injection. Results are given as mean (n = 3 replicates) with standard deviation. Data entries with different letter are statistically different at post hoc Tukey's *t*-test for multiple comparisons within each character ($P < 0.05$).

differences were recorded among the three groups that did not receive the bacterial inoculum.

3. Conclusion

Based on the literature on anti-biofilm activity possessed by compounds in which the core is a pyrazole nucleus, the pyrazolo derivatives **2-7**, **1a-c,e-h**, **14d** and **8a,b** were synthesized and evaluated for their ability to inhibit the biofilm formation of three strains of *S. aureus*. The synthesized compounds resulted in active interference with biofilm formation of all the tested bacterial strains. The most active compound in preventing biofilm formation of the three bacterial strains considered, was not a pyrazole derivative but the intermediate 3-oxo-*N*-phenyl-2-((2-phenylhydrazinyl)methylene) butanamide **14d**. In the preliminary *in vivo* experiment, we observed that compound **14d**, also showed an interesting protective effect at 1 mg/kg increasing the survival of wax moth larvae infected with *S.aureus* ATCC 29231 strain. Moreover, compound **14d** had no detrimental effect on larvae as no differences were recorded in the variation of weight or in the time pass from the later larva instar to chrysalis. In this study, we demonstrated that the compound **14d** did not affect the bacterial growth but targeted the virulence of the tested pathogen inhibiting the biofilm formation *in vitro* and improving the survival of infected larvae when administered prior the infection. These observation are very encouraging. On the basis of results obtained, compound **14d** can be considered a good candidate as lead, useful for further developments as antivirulence agent.

4. Experimental section

4.1. Chemistry

4.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F_{254}, 0.2 mm). 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, with compound as a solid in a KBr disc. ^{1}H and ^{13}C NMR spectra (300 and 75.43 MHz respectively) were obtained using a Bruker AC-E 300 MHz spectrometer (tetramethylsilane as internal standard): chemical shifts are expressed in δ values (ppm). Merck silica gel (Kiesegel 60/230-400 mesh) was used for flash chromatography columns.

Exact mass measurements were carried out by a high resolution Orbitrap mass spectrometer (Q-Exactive by Thermo Fisher Scientific) via direct infusion through an electrospray interface (HESI) operating in negative ionization mode using the following operation parameters: flow rate 5 μL/min; electrospray voltage −3.1 KV; capillary temperature: 320 °C; sheath gas: 6 (arbitrary units); resolution 35000.

Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and were within ±0.4% of the theoretical values. Yields refer to purified products and are not optimized. The names of the products were obtained using the ACD/I-Lab Web

service (ACD/IUPAC Name Free 8.05).

*4.1.2. Synthesis of the 3-oxo-N-phenylbutanamide **11a***

An equimolar mixture (38 mmol) of ethyl acetoacetate **9** and aniline **10** was irradiated at 480 W for 5 min in a Anton Paar Multiwave 3000 microwave oven. After this time the reaction mixture was concentrated under vacuum to give a white solid which was washed with diethyl ether then chromatographed by flash chromatography [32]: external diameter of the column 4.5 cm, silica gel (0.040–0.063 mm, 600 g), ethyl acetate/cyclohexane 6:4 as eluent; fractions 50 ml each.

The initial twenty fractions were discarded, fractions 21-32 were evaporated under reduced pressure to give pure 1,3-diphenylurea **11b** (1.81 g), and fractions 33-56 were evaporated under reduced pressure to give pure 3-oxo-*N*-phenylbutanamide **11a** (yields 30%). Spectroscopic and physical data of **11a** was identical (mp, IR, ^{1}H NMR) with the reported data [30].

*4.1.3. Synthesis of the 2-(ethoxymethylene)-3-oxo-N-phenylbutanamide **12***

A mixture of 3-oxo-*N*-phenylbutanamide **11** (5.64 mmol), triethyl orthoformate (7 mmol) and acetic anhydride (16.92 mmol) was placed under reflux for 90 min. The reaction mixture was then evaporated in a rotavapor and, after cooling, by addition of a few drops of ethanol, the 2-(ethoxymethylene)-3-oxo-*N*-phenylbutanamide **12** precipitates as brown solid. The solid was finally purified by crystallization with hexane/toluene (1:1). Derivative **12** is known but no characterization was found.

2-(ethoxymethylene)-3-oxo-N-phenylbutanamide (12): yields 30%, mp 87–88 °C, I.R (KBr) cm^{-1} 3071 (NH), 1956 (C=C), 1618 (CO); ^{1}H NMR (DMSO) δ 1.47 (t, 3H, CH_3); 2.51 (s, 3H, CH_3); 4.38 (q, 2H, CH_2); 7.06–7.63 (a set of signals, 5H, aromatic protons); 8.47 (s, 1H, CH); 11.04 (s, 1H, NH; exchangeable with D_2O).

*4.1.4. General procedure for preparation of 1-(2-R^1-3-R^2-4-R^3-5-R^4-phenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamides **1a-c,e-h***

Derivatives **1a-c,e-h** were obtained by placing at reflux 1.71 mmol of 2-(ethoxymethylene)-3-oxo-*N*-phenylbutanamide **12** in 8.5 mL of anhydrous ethanol with 1.71 mmol of the appropriate phenylhydrazine **13a-c,e-h** for a variable time of 1–48 h as reported in Table 2. After this time, The solid which separated off were collected and purificated as reported in Table 2 to give pure **1b**, **1c**, **1e**, **1g** and **1h**.

Derivatives **1a** and **1f** are known [39] and their spectroscopic and physical data were identical with the reported data.

1-(3-chlorophenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamide (1b): yields 23%, mp 97–100°, I.R (KBr) cm^{-1} 3307, (NH), 1667 (CO); ^{1}H NMR (Acetone) δ 2.67 (s, 3H, CH_3); 7.06–8.21 (a set of signals, 10H, aromatic protons, pyrazole H-3); 9.20 (s, 1H, NH; exchangeable with D_2O). Anal. Calc. for $C_{17}H_{14}ClN_3O$: C, 65.49%; H, 4.53%; N, 13.48%. Found: C, 65.50%; H, 4.51%; N, 13.51.

1-(4-chlorophenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamide (1c): Compound **1c** was known (CAS number 1134899-50-5), but no references were found. Yields: 68%, mp 190–194 °C, I.R (KBr) cm^{-1} 3269, (NH), 1640 (CO); ^{1}H NMR (Acetone) δ 2.64 (s, 3H, CH_3); 7.08–8.20 (a set of signals, 10H, aromatic protons, pyrazole H-3); 9.18 (s, 1H, NH; exchangeable with D_2O). ^{13}C NMR(δ) (Acetone) 12.22 (CH_3), 117.17 (C_{Ar}), 120.81 (2XCH_{Ar}), 124.21 (CH_{Ar}), 127.86 (2XCH_{Ar}), 129.47 (2XCH_{Ar}), 130.19 (2XCH_{Ar}), 134.49 (C_{Ar}), 139.02 (C_{Ar}), 139.86 (CH_{Ar}), 140.40 (C_{Ar}), 143.47 (C_{Ar}), 162.47 (CO). Anal. Calc. for $C_{17}H_{14}ClN_3O$: C, 65.49%; H, 4.53%; N, 13.48%.Found: C, 65.66%; H, 4.65%; N, 13.37.

5-methyl-1-(3-nitrophenyl)-N-phenyl-1H-pyrazole-4-carbossamide (1e): yields 51%, mp 184–187°, I.R (KBr) cm^{-1} 3418, (NH), 1661 (CO); ^{1}H NMR (Acetone) δ 2.73 (s, 3H, CH_3); 7.07–8.44 (a set of signals, 10H, aromatic protons, pyrazole H-3); 9.26 (s, 1H, NH; exchangeable with D_2O). Anal. Calc. for $C_{17}H_{14}N_4O_3$: C, 63.35%; H, 4.38%; N, 17.38%. Found: C, 63.49%; H, 4.12%; N, 17.20.

5-methyl-N-phenyl-1-(4-(trifluoromethyl)phenyl)-1H-pyrazole-4-carboxamide (1g): yields 68%, mp 155–159°, I.R (KBr) cm^{-1} 3304, (br, NH), 1630 (CO); ^{1}H NMR (Acetone) δ 2.71 (s, 3H, CH_3); 7.06–8.25 (a set of signals, 10H, aromatic protons, pyrazole H-3); 9.23 (s, 1H, NH; exchangeable with D_2O). Anal. Calc. for $C_{18}H_{14}F_3N_3O$: C, 62.61%; H, 4.09%; N, 12.17%. Found: C, 62.73%; H, 3.92%; N, 12.00.

1-(2,5-difluorophenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamide (1h): yields 50%, mp 115–117°, I.R (KBr) cm^{-1} 3307, (NH), 1667 (CO); ^{1}H NMR ($CDCl_3$) δ 2.53 (s, 3H, CH_3); 7.14–7.65 (a set of signals, 9H, aromatic protons, pyrazole H-3); 7.98 (s, 1H, NH; exchangeable with D_2O). Anal. Calc. for $C_{17}H_{13}F_2N_3O$: C, 65.17%; H, 4.18%; N, 13.41%. Found: C, 65.31%; H, 4.37%; N, 13.14.

*4.1.5. Preparation of 2-((2-(2,5-dichlorophenyl)hydrazinyl)methylene)-3-oxo-N-phenylbutanamide **14d***

Derivative **14d** was obtained by placing at reflux, 1.71 mmol of 2-(ethoxymethylene)-3-oxo-*N*-phenylbutanamide **12** in 8.5 mL of anhydrous ethanol with 1.71 mmol of the 2,5-dichlorophenylhydrazine **13d** for 24 h. After this time, the solvent was removed under reduced pressure and the compound **14d**, are thus obtained and purified by crystallization from ethanol; derivative **14d** is known [33] but no characterization was found. ^{1}H NMR spectrum showed that compound **14d** is a mixture of two tautomers (Fig. 3). Owing to the prevalence of tautomer A in the mixture, the intensity of signals relative to tautomer B were negligible in ^{13}C NMR spectrum.

2-((2-(2,5-Dichlorophenyl)hydrazinyl)methylene)-3-oxo-N-phenylbutanamide (14d): yields 79%, mp 145–147 °C, I.R (KBr) cm^{-1} 3322, 3198-3045 (NH), 1658-1563 (CO); ^{1}H NMR (tautomeric mixture, Acetone) δ: tautomer A 2.33 (s, 3H, CH_3); 6.83–7.63 (a set of signals, 8H, aromatic protons); 8.23 (d, 1H, =CH); 8.82 (s, 1H, NH; exchangeable with D_2O), 11.32 (d, 1H, NH; exchangeable with D_2O), 11.84 (s, 1H, NH; exchangeable with D_2O); tautomer B 2.28 (s, 3H, CH_3); 6.83–7.63 (a set of signals, 8H, aromatic protons); 8.59 (s,

Table 2
Time of reaction and purification methods for **1a-c,e-h**.

Comp.	Time of reaction	Purification method
1a	6 h	Crystallized from ethanol
1b	24 h	Crystallized first from ethyl acetate then from ethanol
1c	3 h	Crystallized from ethanol
1e	48 h	Crystallized first from ethanol then purified by preparative TLC(chloroform as eluent)
1f	24 h	Crystallized from ethyl acetate
1g	1 h	Crystallized first from ethyl acetate/petroleum ether then from ethanol
1h	24 h	Washed with ethyl acetate

1H, =CH); 9.89 (s, 1H, NH; exchangeable with D_2O), 11.55 (s, 1H, NH; exchangeable with D_2O), 16.46 (s, 1H, OH; exchangeable with D_2O). ^{13}C NMR(δ) (Acetone) tautomer A 26.70 (CH_3), 103.05 (C_{Ar}), 114.16 (CH_{Ar}), 117.65 (C_{Ar}), 120.61 (2XCH_{Ar}), 121.87 (CH_{Ar}), 124.25 (CH_{Ar}), 129.70 (2XCH_{Ar}), 131.68 (CH_{Ar}), 134.04 (C_{Ar}), 139.51 (C_{Ar}), 146.10 (C_{Ar}), 164.62 (CH_{Ar}), 167.55 (CO), 197.74 (CO). Anal. Calc. for $C_{17}H_{15}Cl_2N_3O_2$: C, 56.06%; H, 4.15%; N, 11.54%. Found: C, 56.36%; H, 3.99%; N, 11.26. HR-MS (TOF, HESI): *m/z* calc. for $C_{17}H_{15}Cl_2N_3O_2$: [M] 363.0514; found [M+1] 362.0468.

4.1.6. Preparation of 1-(2,5-dichlorophenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamide (1d)

The 2-((2-(2,5-Dichlorophenyl)hydrazinyl)methylene)-3-oxo-*N*-phenylbutanamide **14d** (mg 750, 2 mmol) was fused a 170 °C for 15 min. After cooling, the solid which was obtained was crystallized from ethanol to give pure **1d**.

1-(2,5-dichlorophenyl)-5-methyl-N-phenyl-1H-pyrazole-4-carboxamide (1d): yields 83%, mp 145–147°, I.R (KBr) cm^{-1} 3063, (NH), 1645 (CO); ^1H NMR (Acetone) δ 2.47 (s, 3H, CH_3); 7.09–8.42 (a set of signals, 9H, aromatic protons and pyrazole H-3); 9.23 (s, 1H, NH; exchangeable with D_2O). Anal. Calc. for $C_{17}H_{13}Cl_2N_3O$: C, 58.98%; H, 3.78%; N, 12.14%. Found: C, 59.06%; H, 3.56%; N, 12.34.

4.1.7. Synthesis of the ethyl acetopyruvate (17) [40]

To an ice bath cooled solution of sodium (0.217 mol) in anhydrous ethanol(125 mL), a solution of acetone **15** (200 mmol) and diethyl oxalate **16** (200 mmol) was slowly added. The reaction mixture was allowed to stir for 4 h keeping the temperature below 10 °C. Subsequently the mixture was filtered, dissolved in cold water and acidified to pH 3 with sulfuric acid (20%). After the acidification the solution was extracted three times with dichloromethane (each 50 mL), the extracts collected and evaporated under reduced pressure to give the ethyl acetopyruvate **17** (yields 60%).

4.1.8. Synthesis of the ethyl 3-methyl-1-phenyl-1H-pyrazole-5-carboxylate 18a and ethyl 5-methyl-1-phenyl-1H-pyrazole-3-carboxylate 18b

An ice bath cooled solution of 63 mmol ethyl acetopyruvate **17** in 23 mL of acetic acid was treated under magnetic stirring with 63 mmol of phenylhydrazine. The solution was put under reflux for 24 h. After this time, the solution was added of water (69 mL) and extracted four times with diethyl ether (each 30 mL). The ethereal fractions were collected, washed four times with a 10% solution of sodium bicarbonate (each 12 mL), dried with anhydrous sodium sulphate then concentrated under reduced pressure to obtaining a mixture of ethyl 3-methyl-1-phenyl-1*H*-pyrazole-5-carboxylate **18a** and ethyl 5-methyl-1-phenyl-1*H*-pyrazole-3-carboxylate **18b** as oil (7.70 g) which was separated by flash chromatography [32]: external diameter of the column 5.0 cm, silica gel (0.040–0.063 mm, 137 g), ethyl acetate/cycloexane 3:7 as eluent; fractions 55 ml each. The initial four fractions were discarded, fractions 5-7 were evaporated under reduced pressure to give pure ethyl 3-methyl-1-phenyl-1H-pyrazole-5-carboxylate **18a** (1.81 g), fraction 8 contain a mixture of **18a** and **18b** (0.5 g), and finally fractions 9-13 were evaporated under reduced pressure to give pure ethyl 5-methyl-1-phenyl-1H-pyrazole-3-carboxylate **18b** (2.71 g). Spectroscopic and physical data of **18a** and **18b** were identical with the reported data [34].

4.1.9. Synthesis of 3-methyl-1-phenyl-1H-pyrazole-5-carboxylic acid (19a) and 5-methyl-1-phenyl-1H-pyrazole-3- carboxylic acid (19b)

A solution of **18a,b** (0.0169 mol) in 6.50 mL of ethanol and 1.5 g of KOH in 1.1 mL of water were put under reflux for 30 min. After this time the ethanol was removed under reduced pressure, and the residue was dissolved in water (3.3 m). The solution was acidified with HCl, filtered and dried to give acids **19a,b**. Spectroscopic and physical data of **19a** and **19b** were identical with the reported data for 3-methyl-1-phenyl-1*H*-pyrazole-5-carboxylic acid [41], and ethyl 5-methyl-1-phenyl-1*H*-pyrazole-3-carboxylic acid [34].

4.1.10. Synthesis of 3-methyl-1-phenyl-1H-pyrazole-5-carbonyl chloride (20a) and 5-methyl-1-phenyl-1H-pyrazole-3-carbonyl chloride (20b)

Compounds **19a,b** were refluxed for 5 h with 15 mL of thionyl chloride. After this time, the mixture was evaporated under reduced pressure to give the appropriate chlorides **20a,b** that were used crude for the next reaction.

4.1.11. Synthesis of 3-methyl-N,1-diphenyl-1H-pyrazole-5-carboxamide (8a) and 5-methyl-N,1-diphenyl-1H-pyrazole-3-carboxamide (8b)

The crude liquid residue of **20a,b** coming from the previous reaction was dissolved in anhydrous chloroform (135 mL) and refluxed for 6 h with 0.0296 mol of aniline. After the first hour of reflux, five portion of triethylamine, 2 mL, 1 mL, 0.5 mL, 0.5 mL and 0.3 mL each, were added at intervals of 1 h. The mixture was evaporated under reduced pressure and the obtained residue was washed three times with water (50 mL). Then 10 mL of ethanol was added and the solution was finally evaporated to dryness. The residue was then crystallized first twice with ethyl acetate and then with ethanol to give pure the 3-methyl-*N*,1-diphenyl-1*H*-pyrazole-5-carboxamide **8a** and 3-methyl-*N*,1-diphenyl-1*H*-pyrazole-5-carboxamide **8b**.

3-Methyl-N,1-diphenyl-1H-pyrazole-5-carboxamide (8a): yields 95%, mp 128–1131°, I.R (KBr) cm^{-1} 3381, (NH), 1676 (CO); ^1H NMR ($CDCl_3$) δ 2.34 (s, 3H, CH_3); 6.62 (s, 1H, pyrazole H-4); 7.12–7.45 (a set of signals, 10H, aromatic protons); 7.62 (s, 1H, NH; exchangeable with D_2O). ^{13}C NMR(δ) ($CDCl_3$) 13.43 (CH_3), 109.21 (CH_{Ar}), 120.02 (CH_{Ar}), 124.88 (2XCH_{Ar}), 125.08 (CH_{Ar}), 128.42 (CH_{Ar}), 129.07 (3XCH_{Ar}), 137.16 (CH_{Ar}), 137.56 (C_{Ar}), 139.68 (C_{Ar}), 149.13 (C_{Ar}), 157.63 (CO). Anal. Calc. for $C_{17}H_{15}N_3O$: C, 73.63%; H, 5.45%; N, 15.15%. Found: C, 73.36%; H, 5.69%; N, 15.29.

5-Methyl-N,1-diphenyl-1H-pyrazole-3-carboxamide (8b): yields 98%, mp 105–106°, I.R (KBr) cm^{-1} 3381, (NH), 1689 (CO); ^1H NMR ($CDCl_3$) δ 2.73 (s, 3H, CH_3); 6.83–7.71 (a set of signals, 11H, aromatic protons, pyrazole H-4); 8.77 (s, 1H, NH; exchangeable with D_2O). ^{13}C NMR(δ) ($CDCl_3$) 12.48 (CH_3), 107.59 (CH_{Ar}), 119.67 (2XCH_{Ar}), 123.96 (CH_{Ar}), 125.24 (2XCH_{Ar}), 128.68 (CH_{Ar}), 128.99 (2XCH_{Ar}), 129.35 (2XCH_{Ar}), 137.94 (C_{Ar}), 139.10 (C_{Ar}), 141.38 (C_{Ar}), 146.62 (C_{Ar}), 159.22 (CO). Anal. Calc. for $C_{17}H_{15}N_3O$: C, 73.63%; H, 5.45%; N, 15.15%. Found: C, 73.96%; H, 5.80%; N, 15.47.

4.2. Biology

4.2.1. Microbial strains

The staphylococcal reference strains used were: *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 29213, known for its ability to form a biofilm.

4.2.2 Antibacterial activity

MICs were determined by a micro-method described previously [42].

4.2.3. 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 ~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 2 mL of sterile phosphate-buffered saline (PBS) and stained with 2 mL of crystal violet 0.1% v/v for 30 min. The excess stain was removed by placing the plates under running tap water.

Crystal-violet stained adherent bacteria in each well were re-dissolved to homogeneity in 2 mL of ethanol, and the optical density (OD) was read at 600 nm. Each assay was performed in triplicate and repeated at least twice.

4.2.4. Biofilm prevention assay

Procedure described above was used to evaluate the activity of compounds in preventing biofilm formation. Polystyrene 24-well tissue culture plates were filled with 2 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 formation assay.

By comparing the average OD 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:

$$\text{Inhibition } (\%) = \frac{OD_{600} \text{ growth control} - OD_{600} \text{ sample}}{OD_{600} \text{ growth control}} \times 100 \quad (1)$$

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

4.2.5. Insect

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.

4.2.6. Pre-treatment of larvae with antivirulence agents

To evaluate the property of compound **14d** to protect against subsequent *S. aureus* ATCC 29213 infection, a single treatment dose of 1 mg/kg of the compound was given to each larva 1 h before the inoculation of 10 μL of live bacteria (2.8×10^6 cfu). In the experiments there were two negative (uninfected) group controls: one untreated and the other was injected with PBS containing 3% DMSO, the same solvents used to dissolve the tested compound. Three replicates of four larvae for each group were disposed. A positive group control was pre-treated with PBS containing 3% DMSO and infected as previously seen for the tested compound **14d**. Inoculations were given into alternate prolegs moving up the body toward the head such that each proleg was injected only once. Moreover an untouched group of larvae (called untreated) was also used as control.

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 **14d**, the variation in larval weight and the time pass from the inoculum at the later larval stage to the pupation were also checked. For safety reasons only larvae without bacterial inoculum were used for this bioassay.

4.2.7. Statistical analysis

For survival and weight variation of *G. mellonella* larvae data analysis, collected 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 analysed 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).

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.2016.07.030. These data include MOL files and InChiKeys of the most important compounds described in this article.

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