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Inhibitory Effect on Biofilm Formation of Pathogenic Bacteria Induced by Rubrolide Lactam Analogues

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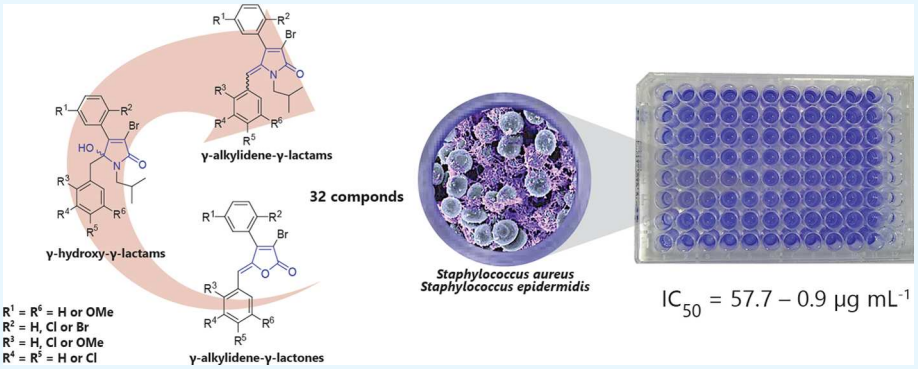
Supporting Information



ABSTRACT: A series of 32 novel potential bacterial biofilm inhibitor analogues of rubrolides and their lactam derivatives were synthesized. The compounds were prepared and tested against *Staphylococcus aureus* and *Staphylococcus epidermidis*. In general, γ-alkylidene-γ-lactams are more active than the corresponding γ-alkylidene-γ-lactones, and the two derivatives are more effective in inhibiting bacterial planktonic growth (IC_{50} of 1.8 and 5.6 μg mL^{-1}) than natural rubrolides. Compounds showing little effect on planktonic growth were assayed for their ability to inhibit biofilm formation. As biofilm inhibitors, none of the new compounds were effective against *S. epidermidis*, whereas the three *Z*-lactam derivatives were very active against *S. aureus* (IC_{50} = 0.9−3.3 μg mL^{-1}). The strong antibiofilm formation activity displayed by some γ-alkylidene-γ-lactams indicates that this may represent a promising class of compounds for the development of novel antimicrobial agents against clinically relevant Gram-positive bacteria.

1. INTRODUCTION

Community-acquired infections caused by antimicrobial resistance to antibiotics have become a great concern for public heath worldwide.[1] "Superbugs", such as MRSA, have been responsible for approximately the same number of deaths in the United States as AIDS, viral hepatitis, and tuberculosis combined.[2] In 2004, the Infectious Disease Society of America reported that the decline in discovery and development of new antibiotics was leading to a public health crisis.[3] In the United States, more than 50% of bacterial strains, isolated from patients in intensive care units, were resistant to at least one antibiotic. Globally, the picture is worse, with >80% of isolates being resistant.[4]

Because of the increasing number of multidrug-resistant bacteria, many common infections, considered easy to treat in the past, have become very difficult to manage, resulting in an increasing number of hospitalization of patients and even death.[5] In this scenario, the emergence of resistant strains of the *Staphyloccocus aureus* and *Staphylococcus epidermidis*, both Gram-positive bacteria associated with severe nosocomial infections, has attracted a lot of attention.[5,6] Multidrug-resistant *S. aureus* strains are highly virulent, being the causative agent of fatal necrotizing pneumonia,[6] whereas *S. epidermidis* infections, despite not commonly resulting in death, are often related to the development of important chronic diseases and increased hospitalization.[7] Failure of current drugs to treat such bacterial infections has turned the search for new effective and efficacious antimicrobial agents

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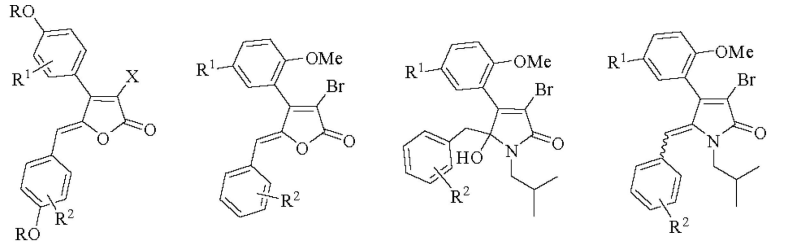
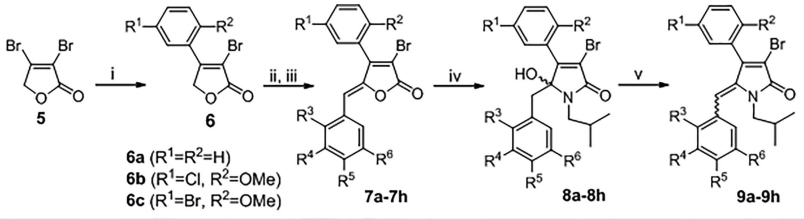


Figure 1. General structure of natural rubrolides (**1**), rubrolides analogues (**2**), and lactam derivatives (**3**, **4**).

Scheme 1. Synthesis of Rubrolide Analogues and γ-Hydroxy-γ-lactam and γ-Alkylidene-γ-lactam Derivatives[a]



R1	R2	R3	R4	R5	R6	Compound (yield %)			Z:E[a]
H	H	H	O-CH2O		H	**7a** (78)	**8a** (85)	**9a** (81)	79:21
H	H	OMe	H	H	OMe	**7b** (70)	**8b** (88)	**9b** (94)	54:46
H	H	Cl	H	F	H	**7c** (58)	**8c** (91)	**9c** (83)	53:47
H	H	Cl	H	H	H	**7d** (60)	**8d** (83)	**9d** (88)	61:39
H	H	H	Cl	H	H	**7e** (62)	**8e** (87)	**9e** (80)	58:42
H	H	H	H	Cl	H	**7f** (73)	**8f** (91)	**9f** (83)	60:40
OMe	Cl	OMe	H	H	OMe	**7g** (79)	**8g** (80)	**9g** (84)	58:42
OMe	Br	OMe	H	H	OMe	**7h** (72)	**8h** (90)	**9h** (91)	92:08

[a]Proportions of the isomers **9*E*** and **9*X*** by NMR experiments. Reagents and conditions: (i) Boronic acid, $AsPh_3$, $PdCl_2(MeCN)_2$, Ag_2O, THF, 65 °C, 24 h; (ii) Aldehyde, TBDMSOTf, DIPEA, DCM; (iii) DBU, reflux; (iv) Isobutylamine, DCM, 0 °C, 23 h; (v) *p*-TsOH, CHCl3, reflux, 2 h.

into a major public health and pharmaceutical issue. In this context, the use of natural compounds with described antimicrobial activities as models for the discovery and development of novel therapeutic agents has become a major research strategy.[8,9] Compounds, which show antimicrobial activity, can be used as tools for finding new resistant pathways and modes of action. Marine organisms produce a great variety of natural metabolites with a range of pharmacological properties.[10,11] Among such structurally diverse metabolites, a group of γ-alkylidenebutenolides known as rubrolides (**1**, Figure 1) have shown the ability to inhibit the growth of Gram-positive bacteria and are now being considered as promising models for the development of novel antibacterial drugs.[12−15]

As examples, rubrolides A, B, and C, isolated from *Ritterella rubra*, were capable of inhibiting *S. aureus* and *Bacillus subtilis* growth with an MIC ranging from 2 to 11 μg/disc.[12] Rubrolides E and F, 3′-bromorubrolide E and F, 3″-bromorubrolide F, and 3′,3″-bromorubrolide E, isolated from *Synoicum* sp., have demonstrated inhibitory activity against *S. epidermidis* (IC_{50} from 21 to 98 μM) and MRSA (IC_{50} from 82 to 1006 μM).[13] Rubrolides A, J, P, and Q, isolated from *Pseudodistoma antiboja*, also showed activity against various Gram-positive bacteria, including *S. aureus* (MIC from 3.1 to >50 μg mL^{-1}) and *S. epidermidis* (MIC from 0.8 to 50 μg mL^{-1}).[14] Several other brominated butenolides, structurally related to the natural products called fimbrolides, have shown to be efficient inhibitors of bacterial biofilm formation.[16,17] Biofilms are organized bacterial communities encapsulated in a complex matrix of macromolecules, including carbohydrates, proteins, and DNA, secreted by the bacteria. It has been estimated that 65−80% of all microbial biomass exists in biofilm form and that more than 80% of all microbial infections are biofilm-related. Within biofilms, bacteria are capable of resisting antibiotics at concentrations up to a thousand times higher than those necessary to kill their planktonic counterparts.[18,19] Therefore, the development of new compounds able to inhibit bacterial biofilm formation appears as an important new strategy to control bacterial infection, complementary to the use of antibiotics. Moreover, biofilm-inhibiting compounds have the advantage over conventional antibiotics that they avoid the development of bacterial resistance to drug, because they target bacterial virulence instead of viability.

In view of the potential of natural γ-alkylidenebutenolides to act as models for the development of novel antimicrobial agents, our research group has been working on the synthesis and biological evaluation of analogues of natural butenolides, including rubrolides.[20−22] Results demonstrated that rubrolide analogues and the corresponding lactam derivatives (**2**−**4**, Figure 1) were capable of inhibiting the formation of biofilm by both *S. aureus* and *S. epidermides*.[21,22] Other butyrolactams derived from mucochloric and mucobromic acids have shown

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Table 1. IC_{50} of Rubrolides Analogues and Lactam Derivatives against *S. aureus* and *S. epidermidis* Growth

	IC_{50} ($\mu g\ mL^{-1}$)			IC_{50}[a] ($\mu g\ mL^{-1}$)			IC_{50} ($\mu g\ mL^{-1}$)			IC_{50} ($\mu g\ mL^{-1}$)	
compound	S.a.[b]	S.e.[b]	compound	S.a.[b]	S.e.[b]	compound[c]	S.a.[b]	S.e.[b]	compound	S.a.[b]	S.e.[b]
7a	>87.5	80.7	**8a**	nc[d]	55.0	**9a**	46.4	21.4	**(Z)9a**	5.6	nc[d]
7b	71.6	63.3	**8b**	78.5	25.4	**9b**	59.4	6	**(Z)9b**	1.8	nc[d]
7c	69.9	nc[d]	**8c**	80	86.4	**9c**	57.4	54.1	**(Z)9c**	34.0	nc[d]
7d	69	47.4	**8d**	>87.5	58.9	**9d**	78.2	72	**(Z)9d**	44.3	nc[d]
7e	82.1	79.8	**8e**	80.3	68.5	**9e**	57.6	80.5	**(Z)9e**	58.0	nc[d]
7f	41.4	>87.5	**8f**	85.7	79.1	**9f**	41.4	79.3	**(Z)9f**	68.4	nc[d]
7g	nc[d]	43.4	**8g**	18.9	>87.5	**9g**	70.7	46.5	**(Z)9g**	>87.5	nc[d]
7h	13.8	76.3	**8h**	55.9	53.3	**9h**	45.8	32.1	**(Z)9h**	76.7	nc[d]

[a]IC_{50}: concentration of compound needed to inhibit bacterial growth by 50%. [b]*S. aureus* (S.a.) and *S. epidermidis* (*S.e.*). [c]Diastereoisomeric mixture of *E* and *Z* isomers. [d]Not calculated (nc): the IC_{50} was not possible to be calculated because of irregular dose−response values obtained for the tested compound.

to effectively inhibit the quorum sensing process in *Pseudomonas aeruginosa*.[23]

From a medicinal chemistry perspective, aiming the discovery of new antimicrobial compounds, understanding of the exact set of properties that make small molecules effective against Gram-negative and Gram-positive cells is of great value. For Gram-negative cells, the general trend is that bacteria tend to be more susceptible to hydrophilic compounds. Typically, small hydrophilic molecules, including the most important antibiotics in clinical use, cross the Gram-negative outer membrane through the water-filled channels provided by porins.[24−28] In the case of Gram-positive cells, because of their different cell wall architecture from Gram-negative cell walls, which contain an outer less permeable polar membrane and promiscuous efflux pumps, the chemical properties of known antimicrobial compounds are different, and currently, there is insufficient data to make a meaningful metanalysis.[29]

Although studies of biofilm inhibitors have led to the discovery of several active molecules,[30] further investigation on the relationship between the activity and the properties of any class of antibacterial compounds is valuable and will help develop an understanding of the mechanism/mode of action and enhance further drug design efforts.

In this context, to get a better insight into the SAR and the physicochemical properties of rubrolides, we herein report the synthesis of 32 new analogues of such natural products and the results of their activities on the biofilm formation of *S. aureus* and *S. epidermidis*.

2. RESULTS AND DISCUSSION

The rubrolide analogues and the corresponding derived lactams were prepared using an experimental procedure previously reported[22,31] as illustrated in Scheme 1. Briefly, lactone **5**, easily obtained from mucobromic acid, was converted to compounds **6a−c** by Suzuki−Miyaura cross-coupling with various boronic acids, employing $PdCl_2(MeCN)_2$ in catalytic amounts. As before, these reactions were highly regioselective, resulting exclusively in 4-aryl-substituted products with yields ranging from 43 to 61%.

The conversion of intermediates **6a−c** into the corresponding products **7a−h** was carried out by their reaction with aromatic aldehydes using TBDMSOTf and DIPEA.[32] The TBS-protected aldol intermediate form was not isolated but treated with DBU under reflux conditions, followed by HCl (3 mol L^{-1}), to afford the γ-alkylidenebutenolides **7a−h** with yields ranging from 58 to 79% (Scheme 1). As already observed in our previous studies on the synthesis of similar compounds,[20−22,32,33] this step was highly stereoselective, producing the required products (**7a−7h**) as *Z* isomers.

The γ-hydroxy-γ-lactams (**8a−h**) were obtained in good yields (Scheme 1) by reacting compounds **7a−h** with excess isobutylamine.[21,22] Further treatment of γ-hydroxy-γ-lactams **8a−h** with *p*-toluenesulfonic acid in chloroform under reflux conditions, generated compounds **9a−h** as *Z*/*E* diastereosomeric mixtures, with yields ranging from 80 to 94% (Scheme 1). The ratio of the *Z*/*E* isomers was obtained by the analyses of the ^1H NMR spectra of isolated products. After a careful silica gel column chromatography fractionation, pure samples of the *Z* isomers were obtained for biological assays (see the Supporting Information for experimental procedure).

For this new series of rubrolide analogues (**7a−h**), we have prepared six compounds bearing a simple phenyl group attached to the β-carbon and two derivatives with the groups 5-chloro-2-methoxyphenyl and 5-bromo-2-methoxyphenyl at the same position. In our previous work,[21,22] we found that some of the most potent derivatives had halogen or methoxy groups at the benzylidene ring. In the present study, we have also included a variety of such groups (Cl, F, and OMe) at different positions on this second aromatic ring. For comparison purposes, we have made the lactams using isobutylamine in previous studies.[21,22]

All newly synthesized compounds were tested against *S. aureus* and *S. epidermidis*. Because we have found no conclusive tendency in terms of the activity of the *Z*/*E* isomers[21,22] and because of the isomerization of the *E* into *Z*, we have tested compounds **9a−h** as a mixture in the proportion isolated and also the corresponding pure more stable **(Z)9a−h** isomers.

In the bacterial assays, compounds were tested for their activity against planktonic growth of *S. aureus* and *S. epidermidis* before biofilm quantification. Bacterial strains were grown statically for 20 h at 37 °C in TSB. Polystyrene 96 well microtiter plates were then inoculated with bacterial suspension, previously diluted to 10^8 CFU mL^{-1} in TSB supplemented with 4% sucrose (w/v) and 3.5% (v/v) DMSO. A total of 10 different concentrations of each compound were obtained by serial dilution (87.5, 43.8, 21.9, 10.9, 5.5, 2.73, 1.37, 0.68, 0.34, and 0.17 $\mu g\ mL^{-1}$). Microtiter plates were incubated for 20 h at 37 °C in a humidified chamber, and bacterial growth was quantified by optical density at 630 nm using a microplate reader, to access eventual growth inhibition (see the Supporting Information for experimental detail).

Because the objective of this work was to evaluate the antibiofilm activities of the newly synthesized compounds, preliminary assays were performed to establish the bacterial

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inoculum that would result in the formation of the most robust biofilm under the experimental conditions, in the absence of planktonic inhibition. These preliminary assays indicated that the ideal bacterial counts resulting in the most robust biofilm was 10^8 UFC mL^{-1}.

Biofilm production and quantifications were performed by colorimetric assay as described previously.[34,35] In short, after planktonic growth quantification, bacterial suspensions were discarded from the 96 well plates prepared as described above, and the wells were washed three times with distilled water to remove any nonadherent bacteria. Next, 200 μL of 0.1% (w/v) crystal violet solution was added to each well, and the remaining biofilm was left to stain for 15 min at room temperature. The wells were then thoroughly washed five times with distilled water to remove excess crystal violet and allowed to dry for 15 min at 37 °C. Finally, 200 μL of 1% sodium dodecyl sulfate was added to the wells, and the crystal violet stain in the biofilm was allowed to solubilize for 15 min at room temperature. Biofilm was quantified by measuring the absorbance of the solubilized crystal violet at 595 nm on a microtiter plate reader.

Results for planktonic growth inhibition assays are presented in the Supporting Information (Tables S1 and S2). At the highest concentration tested (87.5 μg mL^{-1}), all compounds inhibited planktonic growth of both *S. aureus* (from 35 to 92%) and *S. epidermides* (from 10 to 91%). Almost all compounds showed a dose−response effect against growth of both bacteria, thus allowing the determination of their IC_{50} (Table 1).

The IC_{50} values shown on Table 1 clearly indicate that, although all compounds were active against planktonic growth, most of them displayed low activity against bacterial viability. For *S. aureus*, among all 32 compounds, only **(*Z*)9a** and **(*Z*)9b** strongly inhibited the bacterial growth (IC_{50} = 5.6 and 1.8 μg mL^{-1}, respectively), with most of the remaining compounds displaying IC_{50} > 40 μg mL^{-1} and **7h** and **8g** showing moderate activities (IC_{50} = 13.8 and 18.9 μg mL^{-1}, respectively).

Similarly, for *S. epidermidis*, most of the compounds had very little effect on planktonic growth, with most of them presenting IC_{50} > 40 μg mL^{-1}. Compound **9b** was the only one to display high activity against bacterial viability (IC_{50} = 6.0 μg mL^{-1}), whereas compound **9a** had a moderate effect on planktonic growth (IC_{50} = 21.4 μg mL^{-1}). For compounds **(*Z*)9a−h**, it was not possible to calculate the IC_{50} values, because all compounds caused less than 50% inhibition of the planktonic growth at the highest concentration tested (Table 1).

All these results are in agreement with our previous studies[21,22] that demonstrated the low inhibitory effect of most rubrolide analogues and their derived lactams on bacterial planktonic growth. Despite this general trend, comparing the IC_{50} values obtained for compounds **7−9** to those reported by Sikorska et al.,[13] it is clear that several synthetic compounds reported here are more potent against the same bacteria species than several natural rubrolides. For example, when tested against *S. epidermidis*, IC_{50} obtained in these studies for compound **9b** was 15.9 μM while the reported value[15] for the most active natural rubrolide E was 21 μM.

To identify any potential correlation between activity and physicochemical properties, clogP and polar surface area (tPSA) calculations were carried out.[36] The data obtained (see Table S3, Supporting Information) showed that, for the lactones, the clogP was in the range 4.47−5.42, suggesting that the molecules are very lipophilic. There is a crude trend where increasing clogP shows increasing potency. No trend was found when comparing the tPSA and potency for the rubrolide analogues described in this paper.

The hydroxylated compounds **8a−h** were found to be more polar and with a larger tPSA. The tPSA is generally low (<75) over all the analogues, and clogP is slightly high if we consider Lipinski's rule of five.[37] However, in general, molecules **8a−h** are less potent, and this is probably due to a change in their tridimensional shape. Lactams **9a−h** are generally more lipophilic than the corresponding lactones **8a−h** with lower tPSA. Overall, no trend can be seen from such data.

Having demonstrated that most compounds had little effect on the planktonic growth of *S. aureus* and *S. epidermidis*, biofilm inhibition assays were carried out with all compounds (Supporting Information, Tables S4 and S5). For such assays, a maximum concentration of 44.8 μg mL^{-1} for each compound was used, because at this concentration, the planktonic inhibition growth was <20%, established as the cutoff limit for growth inhibition.[21,22]

Results showed that, against both bacterial strains tested, the effect on biofilm inhibition was not concentration-dependent, therefore preventing calculations of IC_{50} values for most of the compounds. Such behavior was also observed in a previous study,[21] which, for comparative purposes, we present here the biofilm inhibition effect of all compounds at the concentration of 44 μg mL^{-1} (Figures 2 and 3).

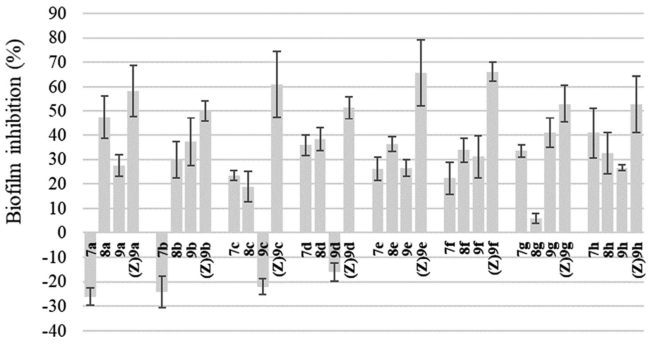

Figure 2. Effect of compounds **7a−h**, **8a−h**, **9a−h**, and **(*Z*)9a−h** at 44 μg mL^{-1} on the formation of *S. aureus* biofilm. Compounds **(*Z*)9a−h** show IC_{50} = 0.9−71.4 μg mL^{-1}.

When tested against *S. epidermidis*, none of the compounds were able to inhibit biofilm formation. Conversely, it is interesting to note that some compounds, rather than inhibit,

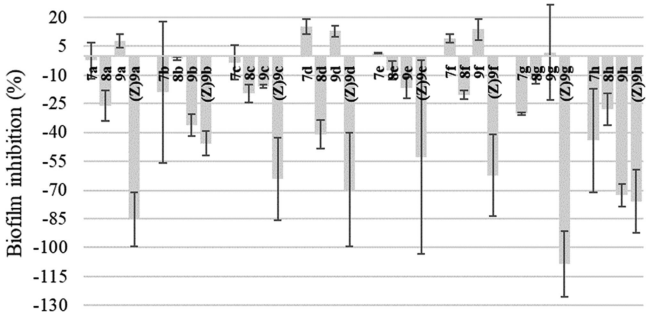

Figure 3. Effect of compounds **7a−h**, **8a−h**, **9a−h**, and **(*Z*)9a−h** at 44 μg mL^{-1} on the formation of *S. epidermidis* biofilm.

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induced biofilm formation, especially those of series (*Z*)**9a**−**h**, where compound (*Z*)**9g** caused 109% increase in biofilm formation and (*Z*)**9a** and (*Z*)**9h** induced biofilm formation by 85 and 76%, respectively. We had previously already identified rubrolide analogues capable of enhancing *Staphylococcus* biofilm formation[21] at subinhibitory concentrations, probably because of interference in QS control via luxS repression.[38] Regarding the inhibition of *S. aureus* biofilm, it is worth noting that five compounds presented $IC_{50} < 10\ \mu g\ mL^{-1}$, indicating a significantly high activity against biofilm formation.

Data presented in Figures 2 and 3 indicate that several compounds were active against *S. aureus* biofilm formation at 44.8 $\mu g\ mL^{-1}$, but except for compounds (*Z*)**9a**−**h**, in most cases the inhibition effect was lower than 50%. The IC_{50} calculated for compounds (*Z*)**9a**−**h** are shown in Table 2.

Table 2. IC_{50} of Compounds (*Z*)9a−h on the Formation of *S. aureus* Biofilm

compound	IC_{50} ($\mu g\ mL^{-1}$)
(*Z*)**9a**	6.3 ± 0.7
(*Z*)**9b**	57.7 ± 5.1
(*Z*)**9c**	3.3 ± 1.4
(*Z*)**9d**	12.8 ± 0.04
(*Z*)**9e**	0.9 ± 0.5
(*Z*)**9f**	1.3 ± 1.4
(*Z*)**9g**	10.0 ± 2.3
(*Z*)**9h**	29.2 ± 1.7

The two most active compounds, (*Z*)**9e** and (*Z*)**9f**, have a chlorine atom at the *meta*- and *para*-positions. The analogue (*Z*)**9d** with a chlorine at the *ortho*-position was less active, but further inclusion of a fluorine at the position *para* (compound presented $IC_{50} < 10\ \mu g\ mL^{-1}$) resulted in an improvement in the inhibitory activity.

As previously reported,[21] the *Z* isomers were among the least active compounds against *S. aureus* and *S. epidermidis* biofilms. In that study, the four most active compounds corresponded to two γ-hydroxy-γ-lactams (**3a**, **3b**) and two *E*-alkylidene-γ-lactams (**4a**, **4b**). The most potent compound against *S. aureus* previously reported was **3b**, a γ-hydroxy-γ-lactam with an IC_{50} of 3.7 $\mu g\ mL^{-1}$, which is four times less potent than compound (*Z*)**9e**. The most potent against *S. epidermidis* was an *E*-alkylidene-γ-lactam, with an IC_{50} of 12.2 $\mu g\ mL^{-1}$ (**4b**).

Results herein reported present a further advancement in this area, because we describe new compounds that are more active than the previously reported ones of the same class and we also demonstrate that the substitution on the β-phenyl ring is not a requirement for the activity. Contrary to previous reports, we found that the more stable *Z* isomer is more active than the *E*−*Z* mixture of isomers. In the current case, because of difficulties in the isolation process and also because of spontaneous isomerization to the *Z* form, we were unable to obtain pure samples of isomers *E* for the biological evaluation. Considering the biological activities observed for the isomeric mixtures **9a**−**h**, we propose that the *E* isomers are less active than the corresponding *Z* isomers. The less active compounds were those bearing two methoxy groups.

In conclusion, we found that lactams derived from rubrolide analogues are more active against *S. aureus* and *S. epidermidis* and that the substitution on the benzylidene ring can affect the potency of the compounds. The most active compounds reported here, with IC_{50} on the lower micromolar range, are among the most active rubrolide derivatives so far reported against the microorganisms studied and that further structural modifications may equally lead to production of more potent compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02334.

Detailed experimental procedure and full characterization data for the compounds, including NMR spectra (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
SAR	structure−activity relationship
TBDMSOTf	*tert*-butyldimethylsilyl trifluoromethanesulfonate
DIPEA	*N*-ethyl-*N*-isopropylpropan-2-amine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
QS	quorum sensing
TSB	tryptic soy broth

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