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N-Arylcinnamamides as Anti-staphylococcal Agents

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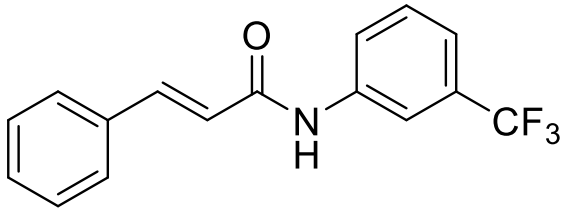
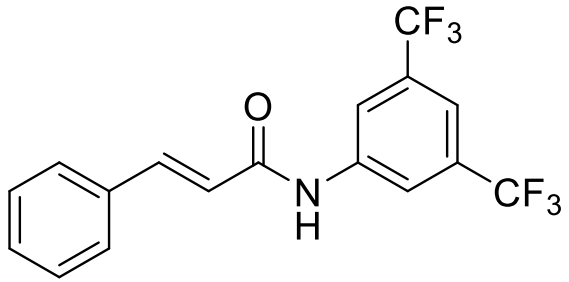
N-Arylcinnamamides as Anti-staphylococcal Agents

Graphical Abstract



compound **6**



compound **13**

MIC = 8 μg/mL against *S. aureus* ATCC 29213 and 3 MRSA strains
additivity/synergism with vancomycin, ciprofloxacin, tetracycline
bactericidal effects
inhibition of staphylococcal biofilm growth
disruption of mature biofilm in concentrations close to MICs

Abstract:
A series of 16 ring-substituted N-arylcinnamamides was synthetized and investigated for their antibacterial activity against *S. aureus* ATCC 29213 and 3 methicillin-resistant isolates. The microtitration dilution method was used for the determination of minimum inhibitory concentration (MIC). In addition, the most potent compounds were studied for their synergetic effect with clinically used antibacterial chemotherapeutics and ability to inhibit and degrade staphylococcal biofilm; besides, the dynamics of their antibacterial activity was characterized.
(2*E*)-*N*-[3,5-bis(Trifluoromethyl)phenyl]-3-phenylprop-2-enamide and (2*E*)-3-phenyl-*N*-[3-(trifluoromethyl)phenyl]prop-2-enamide showed the highest activities (MICs = 8 µg/mL) against all four staphylococcal strains. These compounds showed an activity against biofilm formation of *S. aureus ATCC* 29213 in concentrations close to MICs, and the disruptive effect on mature biofilm was observed. Both compounds showed abilities to increase the activity of clinically used antibiotics with different mechanisms of action (vancomycin, ciprofloxacin and tetracycline). In time-kill studies, a decrease of colony-forming units (CFU/mL) of >99% was observed after 8 h from the beginning of incubation.

Keywords: *Staphylococcus aureus*; biofilm; cinnamaldehyde; synergy; time-kill

Introduction

- As it is seen in Figure 1, the resistance of staphylococci to methicillin is still a current problem in Europe[1].
- Microbial biofilms have been associated with many chronic infections in humans.
- Despite this fact, there are still many countries with high level of resistance.
- Because of above mentioned facts, the development of new active and safe antibacterial drugs is still needed.

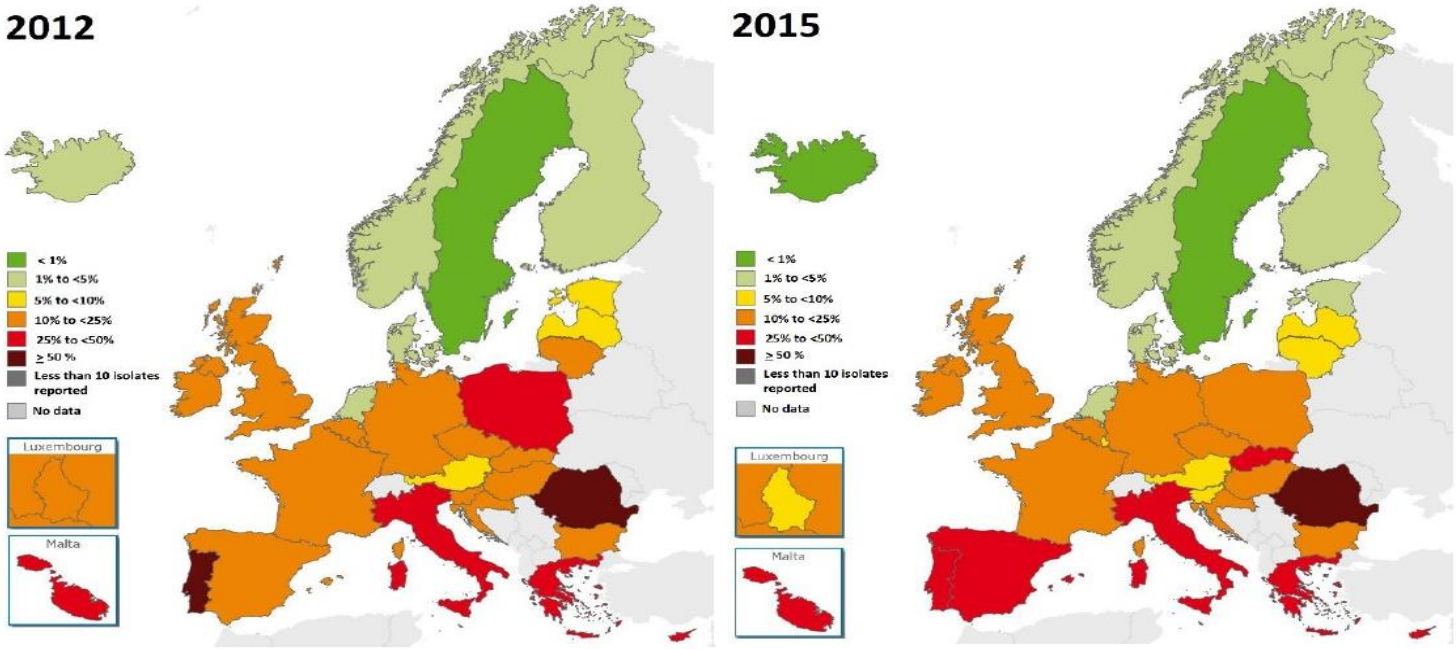


Figure 1: Percentage of methicillin-resistant isolates of *S. aureus* in Europe in 2012 and 2015.

1. WHO. Global Antimicrobial Resistance Surveillance System (GLASS) Report; HO Press: Geneva, Switzerland, 2017.

Introduction

- Derivatives of cinnamic acid show wide spectrum of pharmacological activities, such as anti-inflammatory, antioxidant, antifungal, antibacterial, antiviral[2].
- Cinnamamides are structurally close to naphtalencarboxamides, which were studied in recent years and proved antibacterial activity against many bacterial strains, including resistant isolates[3,4].
- Derivatives of cinnamic acid are also known as compounds that inhibit biofilm growing[5,6].

2. Pospisilova, S.; Kos, J.; Michnova, H.; Kapustikova, I.; Strharsky, T.; Oravec, M.; Moricz, A.M..; Bakonyi, J.; Kauerova, T.; Kollar, P.; Cizek, A. and Jampilek, J. Synthesis and spectrum of biological activities of novel N-arylcinnamamides. *Int. J. Mol. Sci*. **2018**, *19*, 2318.
3. Gonec, T.; Zadrazilova, I.; Nevin, E.; Kauerova, T.; Pesko, M.; Kos, J.; Oravec, M.; Kollar, P.; Coffey, A.; O'Mahony, J.; Cizek, A.; Kralova, K. and Jampilek, J. Synthesis and biological evaluation of N-alkoxyphenyl-3-hydroxynaphthalene-2-carbox- anilides. *Molecules* **2015**, *20*, 9767–9787.
4. Gonec, T.; Pospisilova, S.; Kauerova, T.; Kos, J.; Dohanosova, J.; Oravec, M.; Kollar, P.; Coffey, A.; Liptaj, T.; Cizek, A. and Jampilek, J. N-Alkoxyphenylhydroxynaphthalenecarboxamides and their antimycobacterial activity. *Molecules* **2016**, *21*, 1068.
5. De Vita, D.; Simonetti, G.; Pandolfi, F.; Costi, R.; Di Santo, R.; D'Auria, F.D.; Scipione, L. Exploring the antibiofilm activity of cinnamic acid derivatives in Candida albicans. *Bioorg. Med. Chem. Lett*. **2016**, *26*, 5931– 5935.
6. Budzynska, A.; Wieckowska-Szakiel, M.; Sadowska, B.; Kalemba, D.; Rozalska, B. Antibiofilm activity of selected plant essential oils and their major components. *Pol. J. Microbiol*. **2011**, *60*, 35–41.

Aims of study

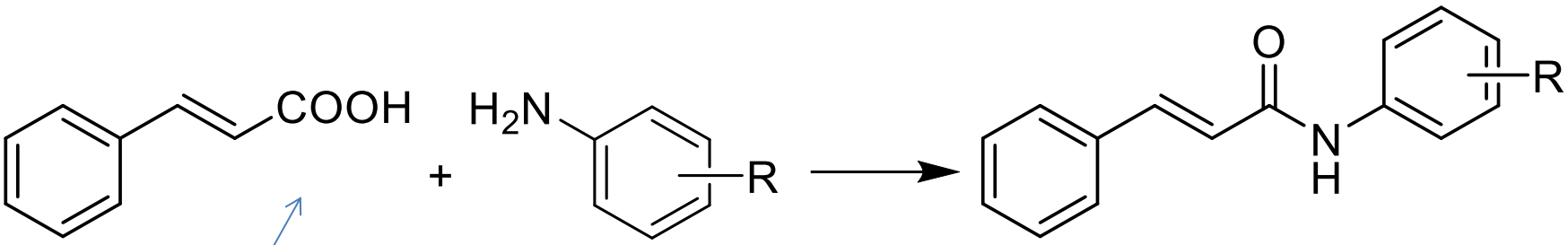
1. Evaluation of antibacterial activity of *N*-cinnamamides against *S. aureus* ATCC 29213 and 3 methicillin-resistant isolates.

2. Study of synergistic activity with commonly used antibacterial drugs.

3. Dynamics of antibacterial activity.

4. Ability of inhibition and disruption of the bacterial biofilm.

Synthesis



1. The carboxyl group of the cinnamic acid was activated by phosphorus trichloride.

2. Reaction with an appropriate ring-substituted aniline gave the final amide.

3. Reaction was was carried out under microwave irradiation in dry chlorobenzene.

4. The final products were recrystallized from ethanol.

Microorganisms

- *Staphylococcus aureus* ATCC 29213: reference strain, susceptible to methicillin, biofilm producer
- 3 methicillin-resistant isolates: MRSA 63718
  MRSA SA 630
  MRSA SA 3202[7]

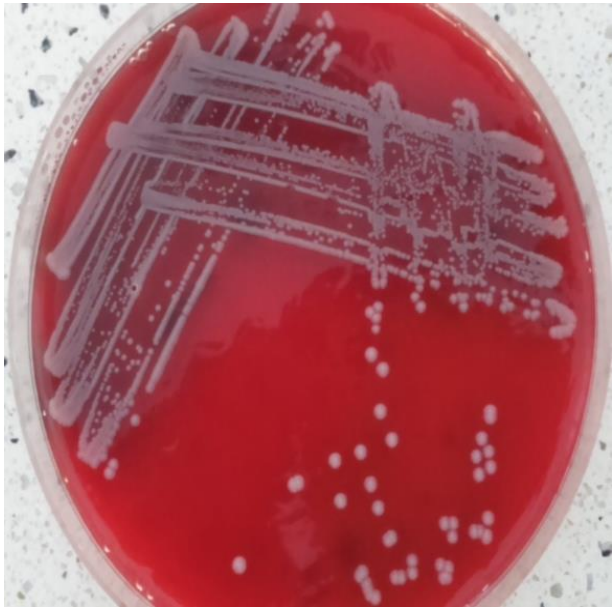


Figure 2: *Staphylococcus aureus* ATCC 29213.

7. Zadrazilova, I.; Pospisilova, S.; Pauk, K.; Imramovsky, A.; Vinsova, J.; Cizek, A.; Jampilek, J. In vitro bactericidal activity of 4- and 5-chloro-2-hydroxy-N-[1-oxo-1-(phenylamino)alkan-2-yl]benzamides against MRSA. *Biomed Res. Int.* **2015**, *2015*, 349534.

Evaluation of minimal inhibitory concentration

Method of broth microdilution in plates

- Compounds were diluted in Cation-adjusted Mueller-Hinton broth (CaMH) to reach concentrations 256–2 μg/mL, ciprofloxacin and ampicillin were used as reference drugs (Fig. 3).
- Plates were inoculated by multi-inoculator (Fig. 4).
- Final concentration of bacteria in wells was 10^5 CFU/mL.
- Tests were performed in triplicates.

Concentration of the compouds [μg/mL]	Tested compounds											
	1	2	3	4	5	6	7	8	9	10	CPX	AMP
A	256	256	256	256	256	256	256	256	256	256	8	16
B	128	128	128	128	128	128	128	128	128	128	4	8
C	64	64	64	64	64	64	64	64	64	64	2	4
D	32	32	32	32	32	32	32	32	32	32	1	2
E	16	16	16	16	16	16	16	16	16	16	0.5	1
F	8	8	8	8	8	8	8	8	8	8	0.25	0.5
G	4	4	4	4	4	4	4	4	4	4	0.125	0.25
H	2	2	2	2	2	2	2	2	2	2	GR	GR

Figure 3: Schema of dilution.

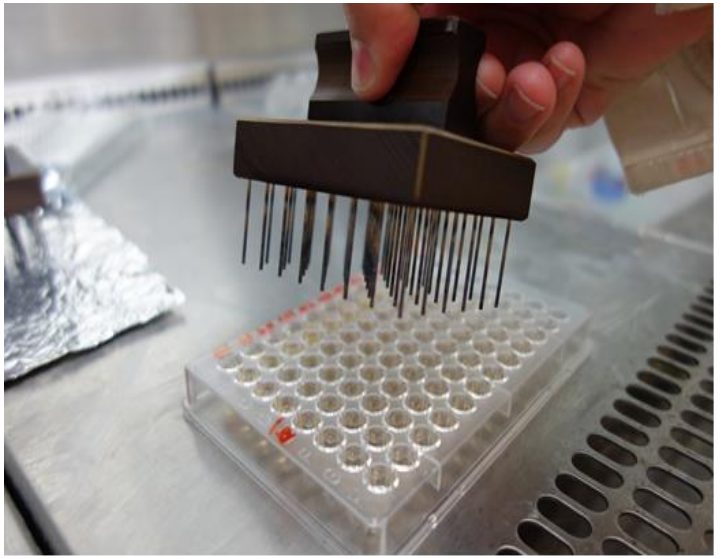


Figure 4: Inoculation by multi-inoculator.

Evaluation of synergistic activity

Method of fraction inhibitory concentrations

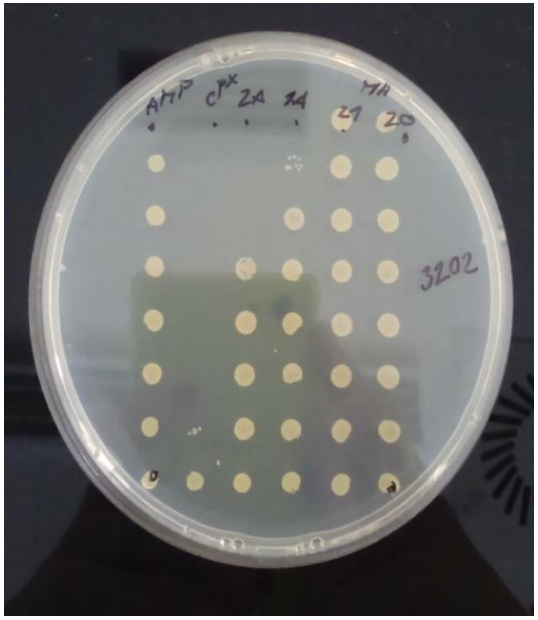
- Microdilution technique
- For all the wells of microtitration plates that corresponded to a MIC value, the sum of the FICs (ΣFIC) was calculated for each well, using the equation $\Sigma FIC = FIC_A + FIC_B = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MICs of drugs A and B alone and C_A and C_B are the concentrations of the drugs in the combination.

Synergy = $\Sigma FIC \leq 0.5$
Additivity = $0.5 < \Sigma FIC < 1$
Indifference = $1 \leq \Sigma FIC < 4$
Antagonism = $\Sigma FIC \geq 4$ [8,9]

8. Schwalbe, R.; Steele-Moore, L.; Goodwin, A.C. Antimicrobial Susceptibility Testing Protocols; CRC Press: Boca Raton, FL, USA, 2007.
9. Bonapace, C.R.; Bosso, J.A.; Friedrich, L.V.; White, R.L. Comparison of methods of interpretation of checkerboard synergy testing. *Diagn. Microbiol. Infect. Dis.* **2002**, 44, 363–366.

Time-kill assay

- Method for determining the dynamics of bactericidal activity.
- A compound is bactericidal, if MBC≤ 4× MIC.
- **Subcultivation of an aliquot on agar** was used as a pre-test for selecting compounds with bactericidal effect.



After incubation and evaluation of MICs, aliquots (10 µL) from the wells were transported to Mueller-Hinton agar by a multi-inoculator. The growth of less than 5 colonies meant a decrease of 99.9% of CFU/mL (bacteria concentration) compared to the starting inoculum = bactericidal effect[8].

8. Schwalbe, R.; Steele-Moore, L.; Goodwin, A.C. Antimicrobial Susceptibility Testing Protocols; CRC Press: Boca Raton, FL, USA, 2007.

Time-kill assay

- Compounds were tested in concentrations equal to 1× MIC, 2× MIC and 4× MIC against *S. aureus* ATCC 29213.
- Bacteria were cultivated statically in CaMH at 37 °C. Except the above mentioned concentrations, the control of growth without any antibacterial drug was used.
- Samples were taken and cultivated on agar plates in times 0, 4, 6, 8 and 24 h from the beginning of the incubation[8].
- Test was made in duplicates.
- Results were shown as graphs of the dependence of bacterial growth on time and concentration of the antibacterial compound.

8. Schwalbe, R.; Steele-Moore, L.; Goodwin, A.C. Antimicrobial Susceptibility Testing Protocols; CRC Press: Boca Raton, FL, USA, 2007.

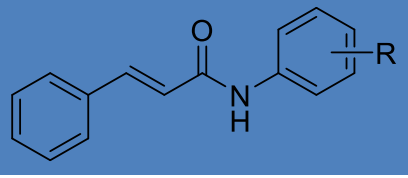
Inhibition of biofilm growth

- Compounds were diluted in Tryptic Soya Broth + 2% glucose to reach concentrations 256–2 µg/mL and inoculated by *S. aureus* ATCC 29213; final concentration of bacteria in the wells was 10^5 CFU/mL.
- Plates were incubated for 48 hours at 37 °C.
- After incubation, the content of the wells was removed, and the plates were washed three times with phosphate buffered saline (PBS).
- 125 µL of 0.1% crystal violet was added to each well and the plates were stained at the room temperature for 20 min.
- The content of the wells was removed, and the plates were washed three times with PBS.
- Coloured biofilm was taken off from the wells by 33% acetic acid.
- Absorbance at 595 nm was measured.
- The ability to inhibit biofilm formation was evaluated as a percentage inhibition of growth compared to the growth control.

Biofilm disruption

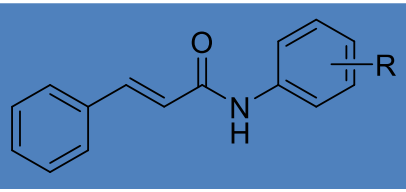
- Biofilm was cultivated in 96-well plate in Tryptic Soya Broth + 2% glucose at 37 °C for 48 hours.
- After cultivation, the content of the wells was removed and the plates were washed 3 times with PBS.
- Compounds were diluted in CaMH to reach concentrations 256–2 µg/mL .
- Plates were incubated at 37 °C for 24 hours.
- After incubation, the content of the wells was removed and the plates were washed 3 times with PBS.
- 100 µL of MTT solution (0.5 mg/mL) was added to each well and the plates were incubated at 37 °C for 1h.
- After incubation, the content of the wells was removed and the plates were washed once with PBS.
- Formazan crystals were dissolved with 17% sodium dodecyl sulfate in 40% dimethylformamide .
- Absorbance at 560 nm was measured.
- The ability to disrupt biofilm was evaluated as a percentage compared to the growth control.

Results – minimal inhibitory concentrations (1/2)



Comp.	R	MIC [µg/mL]			
		SA	MRSA 63782	MRSA SA 630	MRSA SA 3202
1	H	>256	>256	>256	>256
2	3-CH_3	>256	>256	>256	>256
3	4-CH_3	>256	>256	>256	>256
4	2-F	>256	>256	>256	>256
5	3-F	>256	>256	>256	>256
6	3-CF_3	8	8	8	8
7	2,5-CH_3	>256	>256	>256	>256
8	2,5-Cl	>256	>256	>256	>256
9	2,6-Cl	>256	>256	>256	>256

Results – minimal inhibitory concentrations (2/2)



Comp.	R	MIC [µg/mL]			
		SA	MRSA 63782	MRSA SA 630	MRSA SA 3202
10	3,4-Cl	128	256	128	256
11	3,5-Cl	128	256	64	128
12	2,6-Br	>256	>256	>256	>256
13	3,5-CF_3	8	8	8	8
14	2-F-5-Br	>256	>256	>256	>256
15	2-Br-5-F	>256	>256	>256	>256
16	2-Cl-5-CF_3	>256	>256	>256	>256
AMP	-	2	16	16	16
CPX	-	0.5	16	128	8

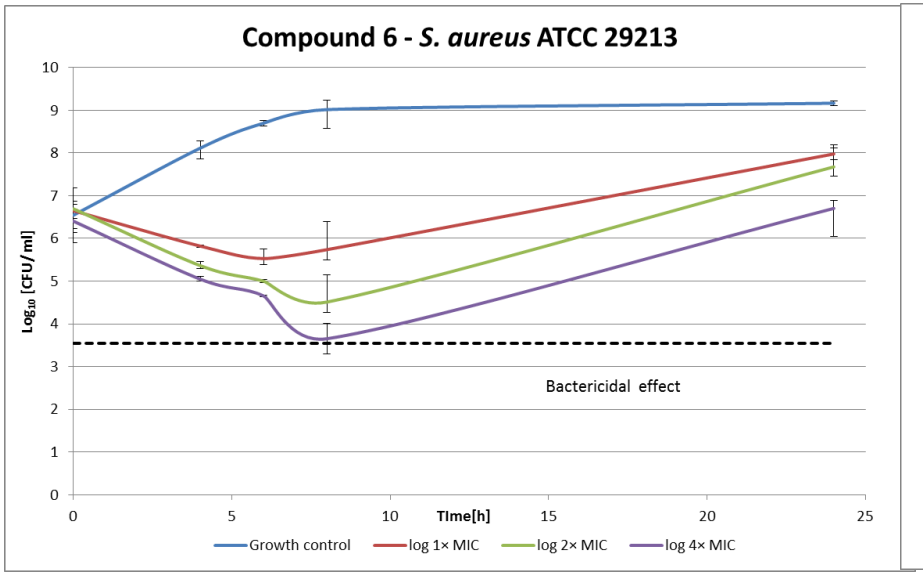
Results – synergistic effect

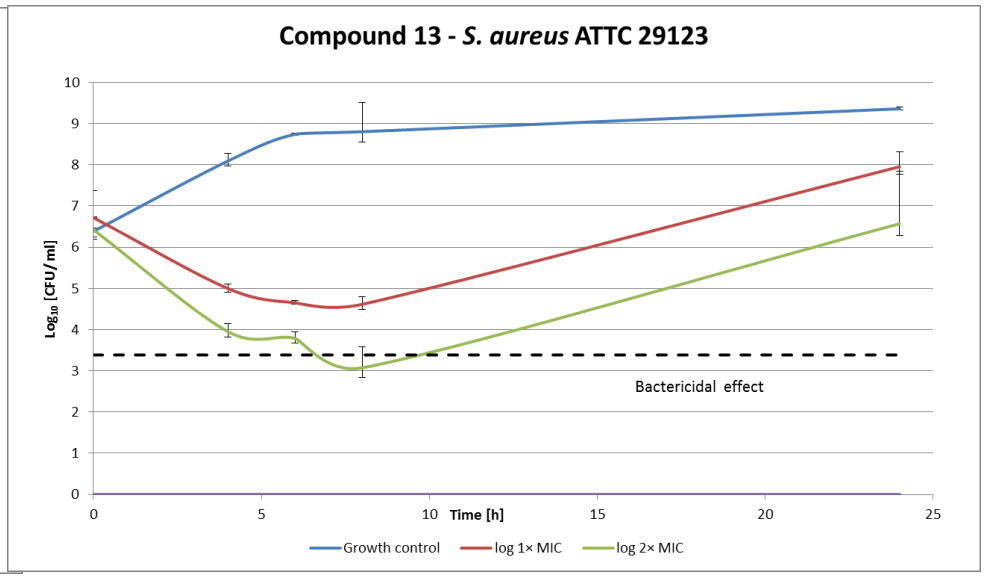
Isolate	Comb. of compds.	Separate MIC [µg/mL]	FIC index	Concentration [µg/mL] causing synergistic effect	Concentration [µg/mL] causing additive effect
MRSA 63718	6/TET	8/128	1.004–2.250	–	2/64; 8/32
	6/CPX	16/16	0.75–1.125	–	8/4; 4/8
	6/VAN	32/2	1.000–1.250	–	–
MRSA 3202	6/TET	16/64	1.002–1.25	–	–
	6/CPX	8/8	1.000–1.250	–	–
	6/VAN	8/1	0.750–1.256	–	4/0.25
	13/TET	32/64	0.500–1.125	8/16	16/16; 4/32; 2/64
	13/CPX	32/8	0.375–1.250	8/1	2/4
	13/VAN	32/1	0.750–1.25	–	16/0.25
MRSA SA 630	6/CPX	8/256	0.625–1.125	–	4/64; 1/128
	6/VAN	8/1	0.750–1.250	–	2/0.5
	13/CPX	8/256	0.375–1.004	2/32; 1/64	4/8
	13/VAN	4/1	0.562–1.250	–	0.25/5

Results – synergistic effect

- Both tested compounds **6** and **13** showed additivity with vancomycin against MRSA SA 630 and SA 3202.
- Compound **13** had synergistic effect with ciprofloxacin against both tested strains.
- The effect of derivative **13** was also synergistic with tetracycline against MRSA SA 3202. The rest of combinations with compound **13** had additive effect.
- Whereas compound **13** had a potential to increase the activity of all tested antibiotics, which have different mechanisms of actions and to which bacteria develop different resistance mechanisms, it can be expected that compound **13** acts by its own mechanism of action or increases the availability of the antibiotics by interaction with the membrane.

Results – dynamics of antibacterial activity

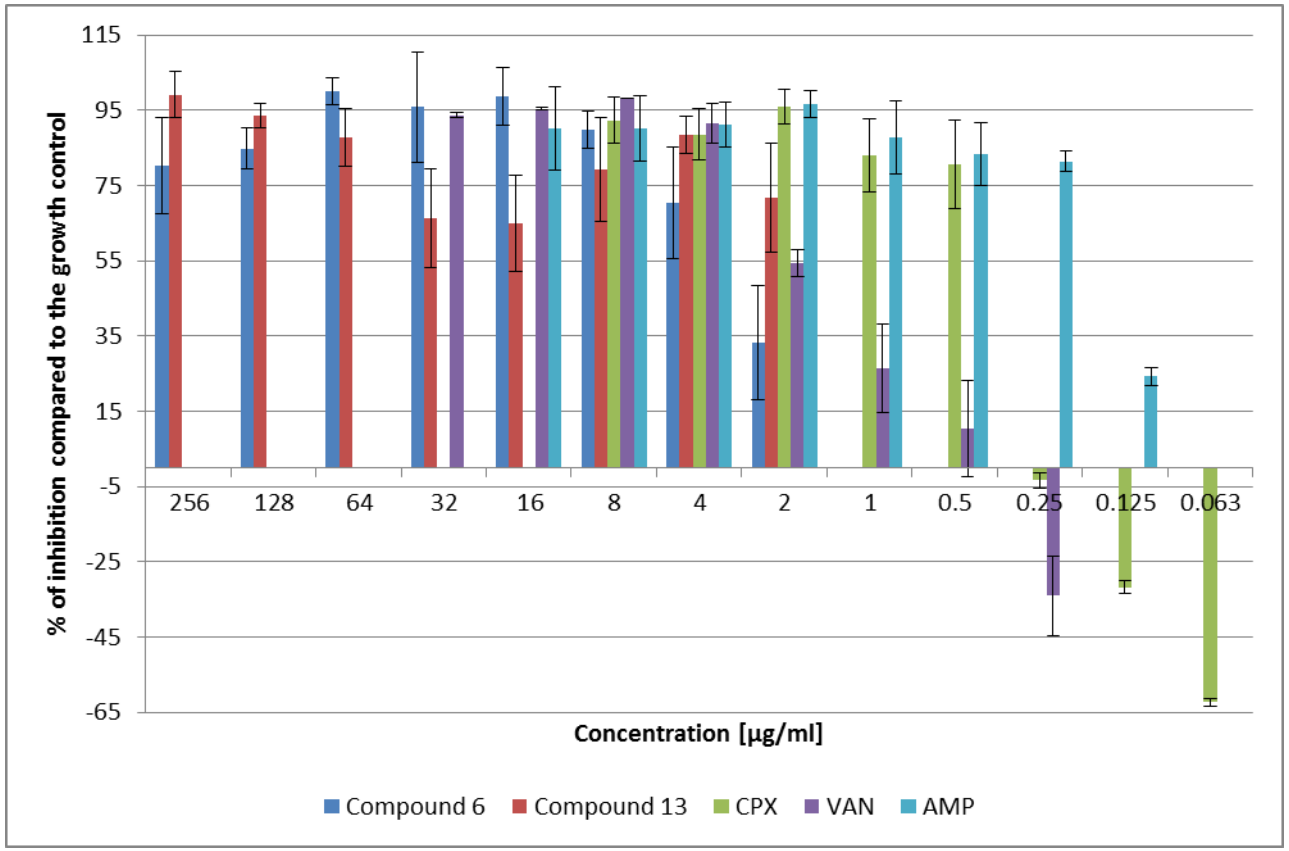




Both compounds showed concentration-dependent antibacterial activity:

- bactericidal in case of compound **13** (in concentration equal to 2×MIC after 8 h from incubation).
- very close to bactericidal level in case of compound **6**.

Results – inhibition of biofilm formation



Compound 6:

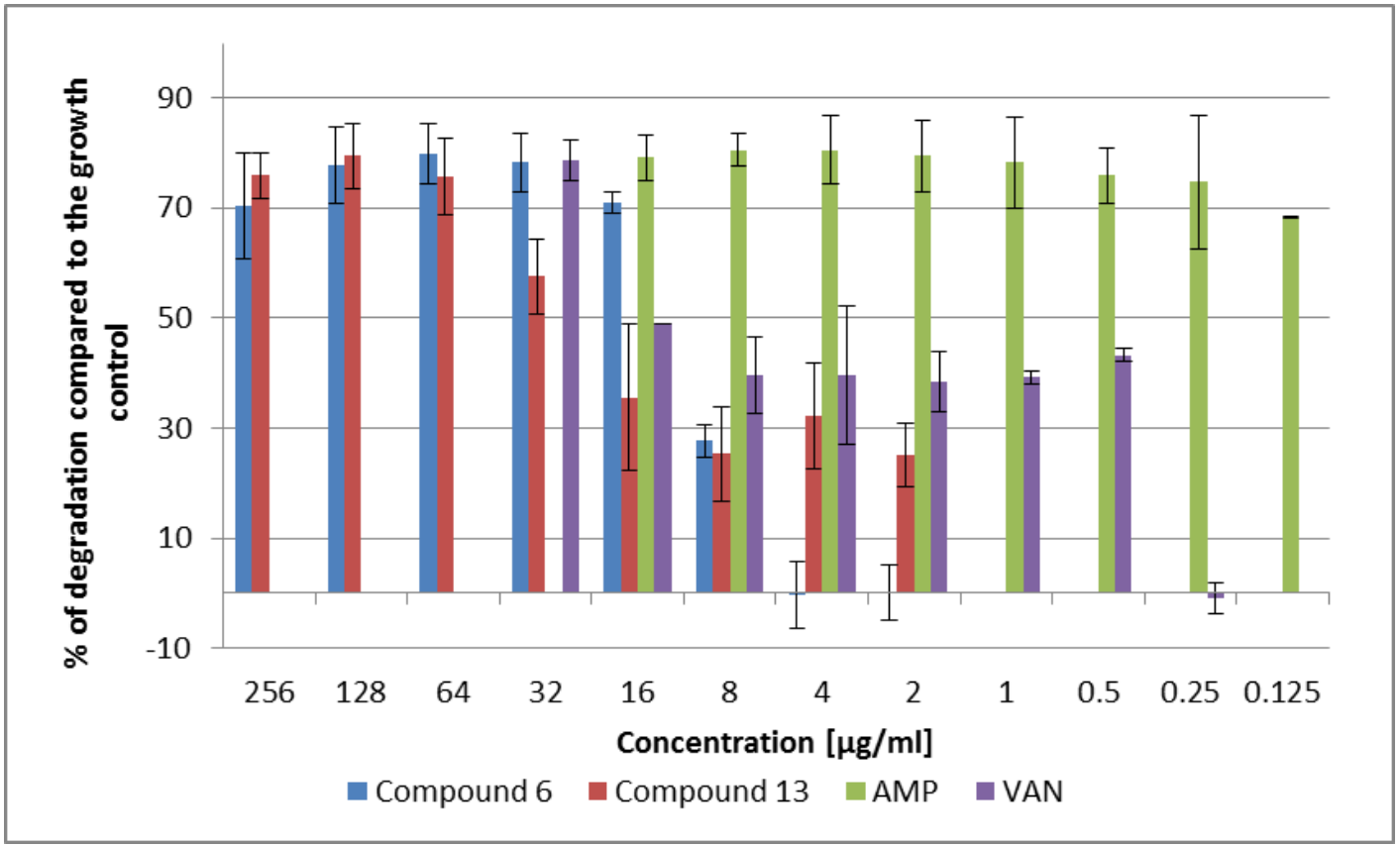
- The activity does not depend on concentration in concentrations above 8 µg/mL; only the highest concentration showed lower inhibition effect. This could be caused by the higher lipophilicity of the compound and potential formation of precipitates, which could decrease the antibacterial activity of the compound.
- MIC_{80} = 8 µg/mL

Compound 13

- Concentrations close to MIC against planktonic cells had the lowest inhibition activities against biofilm forming, and the activity increased for sub-MIC values. These conditions could be potentially toxic for planktonic cells, but they can induce biofilm formation [10].

10. Nuryastuti, T.; van der Mei, H.C.; Busscher, H.J.; Iravati, S.; Aman, A.T.; Krom, B.P. Effect of cinnamon oil on icaA expression and biofilm formation by *Staphylococcus epidermidis*. *Appl. Environ. Microbiol*. **2009**, *75*, 6850–6855.

Results – disruption of mature biofilm



- Disruptive activity showed similar trend as inhibitory activity.
- Interestingly, the disruptive activity of ampicillin does not depend on its concentration.
- $MBC_{(50)}$ for compound **6** was 8 µg/mL, for compound **13** was 32 µg/mL.

Conclusions

- Some derivatives of cinnamamides showed good antibacterial activity against *S. aureus* including methicillin-resistant strains
- Active compounds were substituted by electron-withdrawing substituents
- Compounds **6** and **13** were able to increase the antibacterial activity of clinically used antibiotics with different mechanism of actions, such as vancomycin, ciprofloxacin and tetracycline.
- Compounds **6** and **13** decreased colony-forming units (CFU/mL) by > 99%, which was observed after 8 h from the beginning of incubation.
- Compounds **6** and **13** inhibited the growth of staphylococcal biofilm and disrupted mature biofilm in concentrations close to MICs.
- Based on the above-mentioned observations, cinnamamide derivatives are promising compounds for future research.

Acknowledgments

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THANK YOU FOR YOUR ATTENTION