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Tertiary amides of Salinomycin: A new group of antibacterial agents against *Bacillus anthracis* and methicillin-resistant *Staphylococcus epidermidis*

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ABSTRACT

For the first time, a series of tertiary amides of polyether antibiotic—Salinomycin have been obtained and screened for their antibacterial activity against different strains of bacteria, including *Bacillus anthracis* and clinical methicillin-resistant *Staphylococcus epidermidis* (MRSE). Moreover, biofilm inhibition of MRSE and genotoxicity tests against *Bacillus subtilis* have been performed. Our studies show that Salinomycin and its some derivatives are active against tested bacteria and exhibited definitely bacteriostatic, not bactericidal activity.

Salinomycin (**SAL**, Scheme 1), a polyether antibiotic isolated from *Streptomyces albus*, exhibits high activity against drug-sensitive and drug-resistant bacteria strains. For this reason, **SAL** is commonly used in veterinary medicine as a non-hormonal and growth-promoting agent. However, its activity against Gram-positive bacteria is much stronger than against Gram-negative bacteria. It can be explained by the greater complexity of the cell wall of latter type bacteria, which is impermeable to hydrophobic compounds, like **SAL**.[1]

The antibacterial activity of **SAL** results from the ability of this compound to form complexes with the metal cations, especially Na^+ and K^+, and to transport them across the cellular lipid bilayer. This leads to disruption of the intracellular pH, increase in the osmotic pressure and, consequently, to bacterial cell death. Recently, it has been proved that the transport of cations by the polyether ionophores takes place in accordance with the electroneutral or electrogenic mechanism.[2]

In addition to the antibacterial activity of **SAL** it has been also demonstrated in many reports that this antibiotic shows anti-parasitic, including anti-malarial and anti-coccidial, anti-fungal as well as anti-viral, including anti-HIV, activity.[1] On the other hand, in 2009 it was announced that **SAL** is about 100-fold more effective against breast cancer stem cells than the commonly used anticancer drug—*Paclitaxel*. It is worth noting that the anticancer activity of **SAL** is currently under clinical tests.[3] These studies have proved extremely potent anticancer properties of this compound and also demonstrated that **SAL** can be safely used by humans, without acute side effects.

Up to now no tertiary amide derivatives of **SAL** have been known. Other **SAL** derivatives, such as its esters and secondary amides, demonstrated a lot of interesting biological activities.[4] Recently, it has been shown that secondary amides and esters of **SAL** are active against several Gram-positive bacteria, including also methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. Furthermore, selected **SAL** derivatives have shown interesting anti-tubercular activity.[4]

We present here, for the first time, the efficient method for the synthesis of tertiary amides of **SAL** (Scheme 1). The structures of all these derivatives were characterized using different spectroscopic methods. The most important aim of this work was to determine the antibacterial activity of tertiary amides of **SAL** against different strains of Gram-negative and Gram-positive bacteria, such as clinical methicillin-resistant *S. epidermidis* (MRSE). Moreover, for the

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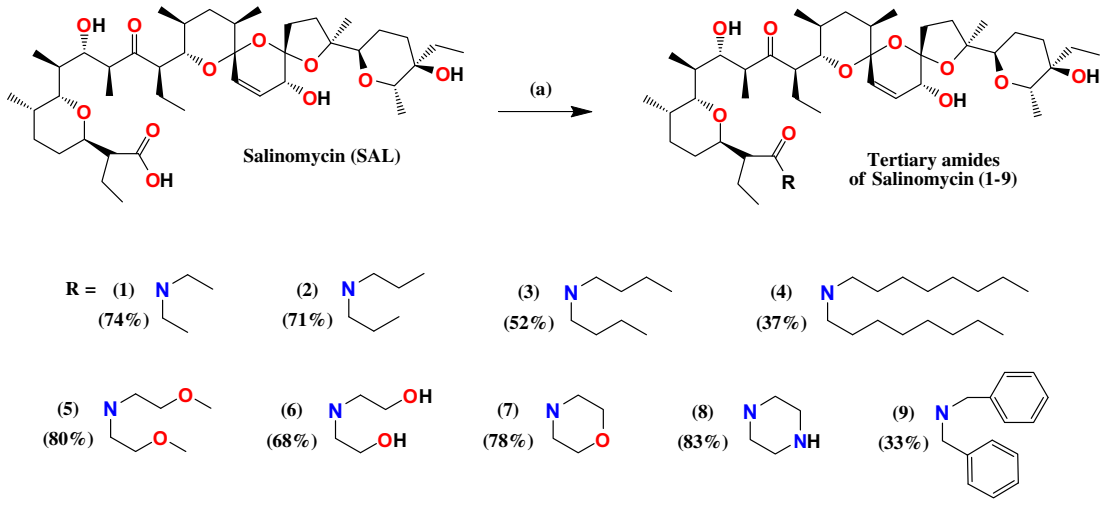

Scheme 1. Reagents and conditions: (a) **SAL** (1 equiv), DCC (1.2 equiv), HOBt (0.5 equiv), appropriate secondary amine (2.5 equiv), CH_2Cl_2/THF (3/1), 0 °C–1 h, then rt–24 h. Time for completion of the reaction at rt as indicated by TLC. Yield of isolated and purified products are given in the brackets.

first time **SAL** and its derivatives were tested against *Bacillus anthracis* and for their ability to inhibit biofilm formation by different *S. epidermidis* strains.

Genotoxicity is one of such main factors determining drug safety for it offers a reliable reference in evaluation of the carcinogenicity of drugs. It is, therefore, an important test of antibacterial agents prior to commercialization. Therefore, genotoxicity tests on *Bacillus subtilis* were performed and discussed.

The isolation procedure of the starting material for the synthesis, that is, **SAL** has been described by us previously.[5] Because **SAL** is very sensitive to acidic conditions and heating, the mild amidation reaction conditions were chosen. The nine tertiary amides of **SAL** were synthesized in the reaction between **SAL** and appropriate secondary amine in the presence of DCC (*N*,*N*′-dicyclohexylcarbodiimide) as a coupling agent and HOBt (1-hydroxybenzotriazole) as an activator of this reaction. All **SAL** derivatives can be easily purified by Dry Vacuum Column Chromatography,[6] using CH_2Cl_2/THF mixture as a mobile phase. This method gave tertiary amides of **SAL** in moderate to good yields (33–83%, Scheme 1). General procedure for the synthesis of **SAL** tertiary amides is described in detail in the Supplementary material.

To facilitate the structural activity relationship (SAR), nine secondary amines were chosen giving derivatives with different substituents, such as short aliphatic chains (diethylamide (**1**), dipropylamide (**2**), dibutylamide (**3**)), long aliphatic chains (dioctylamide (**4**)), aliphatic chains containing heteroatoms (bis(2-methoxyethyl)amide (**5**), diethanolamide (**6**)), heterocyclic ring (morpholine amide (**7**), piperazine amide (**8**)) and aromatic substituents (dibenzylamide (**9**)).

The purity and structures of **SAL** tertiary amides obtained were determined on the basis of elemental, FT-IR, NMR and ESI-MS analyses. The ^1H and ^{13}C NMR signals were assigned using one- as well as two-dimensional (^1H–^1H COSY, ^1H–^{13}C HETCOR and ^1H–^{13}C HMBC) spectra. The FT-IR, ^1H, ^{13}C and 2D NMR, as well as ESI-MS spectra of selected tertiary amides of **SAL** are included in the Supplementary material (Figs. S1–S7).

The evidence for the formation of **SAL** amides (**1**–**9**) is the presence of new characteristic bands in their FT-IR spectra (Fig. S1). In the spectrum of **SAL** a characteristic broad band arises with a maximum at 1713 cm^{-1} due to the overlapping of the ν(C=O) stretching vibrations of ketone and carboxylic groups. In the spectrum of its exemplary derivative **8** one additionally characteristic band is observed at 1628 cm^{-1}, which is assigned to the amide I band. In the spectra of the other **SAL** tertiary amides this intense band is observed in a narrow range of 1622–1634 cm^{-1}. Additionally, in the FT-IR spectra of compounds **1**–**9**, the intensity of the band at 1713 cm^{-1} decreased indicating that COOH group had been consumed during amide synthesis.

In the ^{13}C NMR spectra of **SAL** derivatives, a characteristic signal of carbon atom of the amide group is observed in a narrow range of 172.8–175.5 ppm. The signal of the carbon atom of the carboxyl group of chemically unmodified **SAL** is observed at 177.7 ppm. The exemplary ^1H, ^{13}C and 2D NMR spectra of selected tertiary amide of **SAL** are included in the Supplementary material (Figs. S2–S7).

Because the biological activity of **SAL** and its derivatives is strictly connected with their abilities of transporting monovalent metal cations through lipid bilayers, a very useful method for analysis of the ionophoretic properties of the obtained **SAL** amides is ESI-MS technique (Supplementary material, Fig. S8). The ESI MS spectra of the mixtures of respective **SAL** amides with the sodium perchlorate demonstrate that the amides similar to unmodified **SAL** can form exclusively 1:1 complexes with Na^+ cations.

The antibacterial activity of **SAL** and its derivatives were studied against standard Gram-positive and Gram-negative bacterial strains, such as *S. aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, as well as against hospital strains of MRSE. These activities are expressed by the values of two parameters: minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Five of the nine **SAL** derivatives showed very potent antibacterial activity and the results for these compounds are collected in Tables 1 and 2. The reference compound in these tests was ciprofloxacin—a well-known fluoroquinolone antibiotic. The detailed procedure for the determination as well as the results of the activity of all **SAL** tertiary amides obtained against standard bacterial strains are presented in the Supplementary material (Tables S1 and S2).

The antibacterial activity of **SAL** derivatives obtained is strictly related to the type of substituent in the amide moiety. As shown in Table 1, the most active against standard Gram-positive bacterial strains are amides with short aliphatic substituents, that is, **1** and **2** (MIC = 1–4 μg/ml), and this activity is comparable to that of the parental compound (MIC = 1–2 μg/ml). It has been shown that the antibacterial activity rapidly decreases with further increase in the carbon chain length (MIC = 8 μg/ml and MIC = 128–256 μg/ml for **3** and **4**, respectively). Amide **6** containing short chains with additional hydroxyl groups, in accordance with the above observations, also showed high antibacterial activity (MIC = 4 μg/ml).

Table 1
The activity of **SAL** and its the most active tertiary amides (**1**–**3**, **6** and **9**) against standard bacterial strains in comparison to the reference compound—ciprofloxacin. Data are given as MIC and MBC in μg/ml. In addition, in square brackets the values are given in μM[7,8]

Compound	**SAL**		**1**		**2**		**3**		**6**		**9**		Ciprofloxacin	
Strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	1 [1.33]	4 [5.33]	4 [4.96]	64 [79.40]	2 [2.40]	>256 [>306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	16 [17.20]	256 [275.27]	0.25 [0.76]	0.5 [0.54]
2	1 [1.33]	8 [10.65]	2 [2.48]	64 [79.40]	1 [1.20]	>256 [>306.95]	8 [9.28]	256 [296.98]	4 [4.77]	256 [305.49]	8 [8.60]	128 [137.63]	0.5 [0.54]	2 [6.04]
3	1 [1.33]	4 [5.33]	2 [2.48]	64 [79.40]	1 [1.20]	256 [306.95]	8 [9.28]	256 [296.98]	4 [4.77]	256 [305.49]	16 [17.20]	256 [275.27]	0.25 [0.76]	1 [3.02]
4	2 [2.66]	8 [10.65]	4 [4.96]	64 [79.40]	2 [2.40]	256 [306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	128 [152.74]	32 [34.41]	>256 [>275.27]	0.5 [0.54]	2 [6.04]
5	1 [1.33]	4 [5.33]	2 [2.48]	64 [79.40]	2 [2.40]	256 [306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	16 [17.20]	256 [275.27]	0.25 [0.76]	1 [3.02]
6	2 [2.66]	32 [42.61]	4 [4.96]	128 [158.81]	2 [2.40]	256 [306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	128 [152.74]	8 [8.60]	128 [137.63]	0.125 [0.38]	0.5 [0.54]
7	32 [42.61]	128 [170.44]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.008 [0.02]	0.0016 [0.005]
8	32 [42.61]	256 [340.88]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.008 [0.02]	0.0016 [0.005]
9	32 [42.61]	128 [170.44]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.008 [0.02]	0.0016 [0.005]
10	32 [42.61]	256 [340.88]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.125 [0.38]	0.25 [0.76]
11	64 [85.22]	256 [340.88]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.0625 [0.19]	0.125 [0.38]
12	32 [85.22]	128 [170.44]	>256 [>317.62]	>256 [>317.62]	>256 [>306.95]	>256 [>306.95]	>256 [>296.98]	>256 [>296.98]	>256 [>305.49]	>256 [>305.49]	>256 [>275.27]	>256 [>275.27]	0.125 [0.38]	0.25 [0.76]

Standard bacterial strains tested: Gram-positive bacteria: 1—*S. aureus* NCTC 4163; 2—*S. aureus* ATCC 25923; 3—*S. aureus* ATCC 6538; 4—*S. aureus* ATCC 29212; 5—*S. epidermidis* ATCC 12228; 6—*S. epidermidis* ATCC 35984; Gram-negative bacteria: 7—*E. coli* ATCC 10538; 8—*E. coli* ATCC 25922; 9—*E. coli* NCTC 8196; 10—*P. aeruginosa* ATCC 15442; 11—*P. aeruginosa* NCTC 6749; 12—*P. aeruginosa* ATCC 27853.

Table 2
The activity of **SAL** and its the most active tertiary amides (**1**–**3**, **6** and **9**) against planktonic cells of methicillin-resistant clinical strains of *Staphylococcus epidermidis* (MRSE) in comparison to the reference compound—ciprofloxacin. Data are given as MIC and MBC in μg/ml. In addition, in square brackets the values are given in μM[7,8]

Compound	**SAL**		**1**		**2**		**3**		**6**		**9**		Ciprofloxacin	
Strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
403/10	1 [1.33]	64 [85.22]	4 [4.96]	256 [317.62]	4 [4.80]	128 [153.48]	8 [9.28]	256 [296.98]	4 [4.77]	128 [152.74]	16 [17.20]	>256 [>275.27]	8 [24.17]	128 [96.68]
404/10	1 [1.33]	64 [85.22]	4 [4.96]	256 [317.62]	4 [4.80]	128 [153.48]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	32 [34.41]	>256 [>275.27]	32 [96.68]	>256 [>773.41]
405/10	2 [2.66]	128 [170.44]	8 [9.93]	256 [317.62]	8 [9.59]	128 [153.48]	4 [4.64]	128 [148.49]	2 [2.39]	128 [152.74]	8 [8.60]	128 [137.63]	2 [6.04]	128 [96.68]
407/10	4 [5.33]	128 [170.44]	4 [4.96]	128 [158.81]	4 [4.80]	256 [306.95]	8 [9.28]	256 [296.98]	4 [4.77]	128 [152.74]	16 [17.20]	256 [275.27]	64 [193.35]	>256 [>773.41]
409/10	1 [1.33]	32 [42.61]	4 [4.96]	256 [317.62]	8 [9.59]	128 [153.48]	16 [18.56]	>256 [>296.98]	8 [9.55]	256 [305.49]	16 [17.20]	256 [275.27]	2 [6.04]	64 [193.35]
422/10	2 [2.66]	64 [85.22]	8 [9.93]	256 [317.62]	8 [9.59]	128 [153.48]	8 [9.28]	256 [296.98]	2 [2.39]	128 [152.74]	16 [17.20]	256 [275.27]	2 [6.04]	64 [193.35]
424/10	2 [2.66]	128 [170.44]	8 [9.93]	256 [317.62]	8 [9.59]	256 [306.95]	8 [9.28]	256 [296.98]	4 [4.77]	128 [152.74]	16 [17.20]	256 [275.27]	8 [24.17]	256 [>773.41]
454/11	1 [1.33]	32 [42.61]	4 [4.96]	128 [158.81]	4 [4.80]	128 [153.48]	4 [4.64]	128 [148.49]	2 [2.39]	128 [152.74]	8 [8.60]	128 [137.63]	0.0625 [0.19]	0.25 [0.76]
455/11	2 [2.66]	64 [85.22]	8 [9.93]	256 [317.62]	8 [9.59]	256 [306.95]	16 [18.56]	256 [296.98]	4 [4.77]	256 [305.49]	32 [34.41]	256 [275.27]	32 [96.68]	>256 [>773.41]
457/11	2 [2.66]	32 [42.61]	4 [4.96]	128 [158.81]	8 [9.59]	256 [306.95]	4 [4.64]	256 [296.98]	4 [4.77]	256 [305.49]	16 [17.20]	256 [275.27]	32 [96.68]	>256 [>773.41]
458/11	1 [1.33]	32 [42.61]	4 [4.96]	128 [158.81]	8 [9.59]	256 [306.95]	4 [4.64]	256 [296.98]	4 [4.77]	128 [152.74]	16 [17.20]	>256 [>275.27]	32 [96.68]	>256 [>773.41]
459/11	1 [1.33]	32 [42.61]	4 [4.96]	256 [317.62]	4 [4.80]	128 [153.48]	4 [4.64]	256 [296.98]	4 [4.77]	256 [305.49]	16 [17.20]	256 [275.27]	32 [96.68]	>256 [>773.41]
460/11	1 [1.33]	32 [42.61]	8 [9.93]	256 [317.62]	8 [9.59]	256 [306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	32 [34.41]	>256 [>275.27]	0.125 [0.38]	0.5 [0.54]
461/11	4 [5.33]	128 [170.44]	4 [4.96]	128 [158.81]	4 [4.80]	128 [153.48]	8 [9.28]	>256 [>296.98]	4 [4.77]	128 [152.74]	32 [34.41]	>256 [>275.27]	0.25 [0.76]	1 [3.02]
469/11	1 [1.33]	32 [42.61]	8 [9.93]	256 [317.62]	4 [4.80]	128 [153.48]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	32 [34.41]	>256 [>275.27]	8 [24.17]	128 [386.71]
470/11	1 [1.33]	32 [42.61]	4 [4.96]	128 [158.81]	8 [9.59]	256 [306.95]	8 [9.28]	>256 [>296.98]	4 [4.77]	256 [305.49]	32 [34.41]	>256 [>275.27]	0.125 [0.38]	0.5 [0.54]

What is interesting, the activity dramatically decreased, when the hydroxyl groups had been replaced by methoxy groups (amide **5**, MIC = 64–128 μg/ml). Two heterocyclic tertiary amides **7** and **8** showed no significant activity in these tests (MIC = 128–256 μg/ml and MIC = 64–128 μg/ml for **7** and **8**, respectively). The exemplary aromatic derivative of **SAL** (compound **9**) showed moderate activity against standard strains of Gram-positive bacteria (MIC = 8–32 μg/ml). None of the compounds tested showed any activity against Gram-negative bacteria.

S. epidermidis, coagulase-negative staphylococcus, are comprised in the normal microflora of human mucous membranes and skin. They belong to the opportunistic microorganisms that are dangerous, especially for patients with weakened immune system. Particularly serious are infections in the post-operative patients observed as a result of the replacement surgery (i.e., joint implants) or cardiac and vascular device implantations. MRSE is the strain of *S. epidermidis*, that is, resistant to the antibacterial activity of methicillin as well as other β-lactam antibiotics.[9] Hospital strains of MRSE were isolated from blood of patients hospitalized in Warsaw Medical University Hospital. The results of the activity of all **SAL** tertiary amides obtained against MRSE are presented in the Supplementary material (Table S2). On the other hand, in Table 2 the results of the most anti-MRSE active derivatives in this study are collected.

As shown in Table 2, exactly the same **SAL** derivatives (**1**–**3**, **6**, **9**) were active against MRSE strains tested. The most anti-MRSE active in this study was compound **6** (MIC = 2–8 μg/ml), which indicates a preferential action of this compound against selected hospital bacteria. Moreover, the least sterically hindered compounds **1** and **2** were also characterized by a high anti-MRSE activity (MIC = 4–8 μg/ml in both cases). When the size of a substituent in the amide moiety was increased then the anti-MRSE activity of these derivatives decreased (MIC = 4–16 μg/ml and MIC = 8–32 μg/ml for **3** and **9**, respectively). The other tertiary amides tested exhibited relatively low activity (MIC = 64–256 μg/ml). It is worth noting that the anti-MRSE activity of **SAL** and its most active tertiary amides was in many cases much better than that of the reference compound, for example, the MIC value of ciprofloxacin against *S. epidermidis* 407/10 strain was about 32 μg/ml, but for compounds **1**, **2** and **6** was about 4 μg/ml. Moreover, after the conversion of these values to micromolar concentration (because **SAL** and its amides have high molecular weight) **SAL** derivatives were found nearly 40-times more anti-MRSE active than ciprofloxacin (MIC = 4.96 μM, 4.80 μM, 4.77 μM and 193.35 μM for **1**, **2**, **6** and ciprofloxacin, respectively).

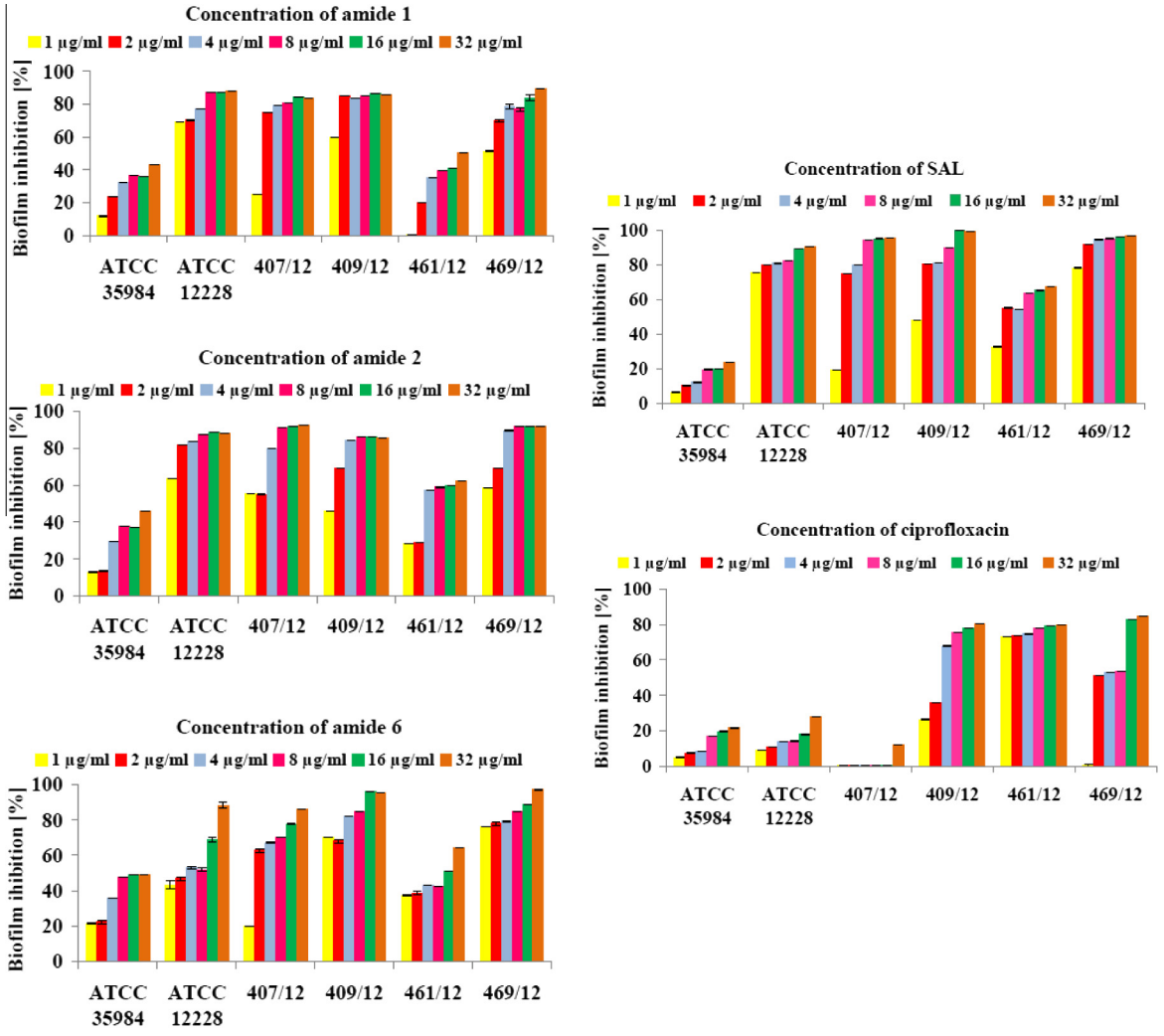

Figure 1. Effects of **SAL** and its the most active amide derivatives (**1**, **2** and **6**) on biofilm formation by standard *Staphylococcus epidermidis* (ATCC 35984 and ATCC 12228) and clinical methicillin-resistant *Staphylococcus epidermidis* strains (MRSE). The activity of reference compound—ciprofloxacin is also shown. All presented results are mean from experiments performed four times with a standard deviation.[11]

Table 3
The anti-*B. anthracis* activity of polyether ionophores (**MON-Na**, **LAS-Na**, **SAL-Na** and **SAL**) as well as the most antibacterial active tertiary amides of **SAL** (**1**–**3**, **6**, **9**) against *Bacillus anthracis* $34F_2$ strain. Data are given as MIC in μg/ml. In addition, in square brackets the values are given in μM[15]

Strain	MIC								
	Reference compounds				Tertiary amides of SAL				
	MON-Na	**LAS-Na**	**SAL-Na**	**SAL**	**1**	**2**	**3**	**6**	**9**
Bacillus anthracis $34F_2$	1 [1.44]	2 [3.26]	1 [1.29]	1 [1.33]	1 [1.24]	4 [4.80]	64 [74.25]	2 [2.39]	32 [34.41]

It is also worth noting large differences between the values of MIC as well as MBC parameters in Tables 1 and 2. For **SAL** and all tertiary amides obtained, the MIC values were always much lower than the corresponding MBC values. **SAL** and its active derivatives (**1**–**3**, **6** and **9**) applied in low concentrations (low MIC values) cause only inhibition of bacterial multiplication and growth, that is, a reversible bacteriostatic effect. However, in high concentrations (MBC values several or a few ten times higher than MIC) it brings irreversible changes and death of bacterial cells. The bactericidal effect is achieved only when MBC values are equal to or 2–4 times higher than MIC values. This clearly indicates that both parental antibiotic and all its derivatives are potent bacteriostatic, not bactericidal agents.

The main virulence factor of the staphylococcal strain is its ability to produce extracellular slime that promotes bacterial adhesion on various surfaces, such as tissues and medical devices. It initiates bacterial biofilm formation, a multilayer structure, consisting of polysaccharides, proteins, nucleic acids and water. Its resistance to anti-microbial agents (antibiotics, disinfectants) is much higher than that of planktonic cells.[10] Bacterial biofilm-formed infections are often chronic and difficult to fight. Therefore, it is important to search for new more effective anti-biofilm compounds.

Preliminary antibacterial studies have revealed that **SAL** and its three derivatives **1**, **2** and **6** show good activity against planktonic cells of clinical *S. epidermidis* strains (MIC = 1–8 μg/ml). For this reason, **SAL** and its tertiary amides **1**, **2** and **6** were additionally studied for their ability to inhibit bacterial film formation (Fig. 1). Four clinical methicillin-resistant and two reference *S. epidermidis* strains were used in the assay. The clinical strains were isolated from blood of hospitalized patients. *S. epidermidis* ATCC 35984 was used as a high biofilm-producer (positive control) and *S. epidermidis* ATCC 12228 was used as a low biofilm-producer (negative control). Ciprofloxacin was used as a reference antibacterial agent. The procedure for the determination of the anti-biofilm activity of **SAL** tertiary amides is described in detail in the Supplementary material.

Table 4
Genotoxicity of **SAL** and all its new derivatives in *rec*-assay. Concentration of tested compounds—256 μg per 9 mm disc. Data are given as diameter of growth inhibition zone (GIZ) in mm

Compound	Diameter of growth inhibition zones (GIZ)	
	Bacillus subtilis H17 (rec^+)	*Bacillus subtilis* M45 (rec^-)
SAL	31	32
1	26	26
2	28	26
3	29	30
4	um	um
5	13	14
6	28	29
7	um	um
8	14	14
9	21	20
NOQ*	13	24

* NOQ, 4-nitroquinoline *N*-oxide – a reference genotoxic agent, 2 μg per 9 mm disc; um – no growth inhibition zone observed.

There was no previous data concerning the influence of **SAL** and other polyether antibiotics on staphylococcal biofilm formation. Charlebois et al. in their study have shown the inhibitory effect of some ionophores (i.e., Salinomycin, monensin and narasin) on *Clostridium perfringens* biofilm formation.[12]

As shown in Figure 1, the biofilm formation by clinical strains was reduced from 50% to above 80% by **SAL** and its three derivatives **1**, **2** and **3** used in the concentration 4 μg/ml (MIC value for planktonic cells). In the presence of **SAL** and its derivatives, the produced biofilm was more susceptible to mechanical damage and removal of the dyeing process. What is interesting, the biofilm inhibitory activity of **SAL** and its amides **1**, **2** and **3** in most cases was higher than that of the reference compound—ciprofloxacin. Only for one methicillin-resistant *S. epidermidis* strain (461/12) the biofilm inhibition by ciprofloxacin was much higher than that by **SAL** and its derivatives. In other cases the biofilm inhibition of tested derivatives was better in comparison to that of the reference compound, especially in low concentrations (1–4 μg/ml). Biofilm inhibitory activity of **SAL** and its newly synthesized derivatives is a result of inhibition of multiplication of bacterial cells. In consequence, the compounds studied prevent biofilm formation and reduce its amount.

Because **SAL** and its tertiary amides showed high antibacterial activity described above, we wanted to check their activity against *Bacillus anthracis* $34F_2$ strain (Table 3). This type of Gram-postive bacteria is the etiologic agent of anthrax—a common disease of livestock and occasionally of humans.[13] *B. anthracis* is a long-known bacterial organism with a uniquely stable spore stage. Its stability and the lethal disease developed after inhalation of the spore made it a one of the most dangerous biological weapons. *B. anthracis* has been used in a few bioterrorist attacks.[14]

In this study **SAL**, Salinomycin sodium salt (**SAL-Na**) and two other polyether ionophores—monensin sodium salt (**MON-Na**) and lasalocid sodium salt (LAS-**Na**) were used as reference compounds. Sodium salts of monensin and lasalocid acid, similarly to **SAL**, are commonly used in veterinary medicine.[1] The detailed procedure for the determination and the results of the anti-*B. anthracis* activity of all **SAL** tertiary amides obtained are presented in the Supplementary material (Table S3).

As shown in Table 3, all tested polyether antibiotics were highly active against *B. anthracis* strain (MIC = 1 μg/ml for **MON-Na**, **SAL-Na** and **SAL** as well as MIC = 2 μg/ml for **LAS-Na**) and this activity was determined for the first time. Moreover, the most active **SAL** tertiary amides were again those with short aliphatic substituents, that is, **1**, **2** and **6**. Amide **3** with a slightly longer carbon chain (dibutyl) was much less anti-anthrax active (MIC = 64 μg/ml). Similarly, amide **9** with dibenzyl substituent showed also a lower activity in this test (MIC = 32 μg/ml).

DNA-damaging activity (genotoxicity) of **SAL** and its derivatives was tested by *rec*-assay using two genetically modified *Bacillus subtilis* strains: M45 (rec^-) and H17 (rec^+).[16] *B. subtilis* M45 is devoid of the recombinant-based DNA repair mechanism and is much more susceptible to genotoxic substances than *B. subtilis* H17 strain. Results of the genotoxicity test were estimated by comparing the diameter of the inhibition zone (growth inhibition zone,

GIZ) for the *B. subtilis* M45 (rec$^-$) strain with that observed for the *B. subtilis* H17 (rec$^+$) strain (Table 4). The detailed procedure for the determination of the genotoxicity of **SAL** tertiary amides is described in Supplementary material.

As shown in Table 4, there were no significant differences in the diameter of GIZ for both *B. subtilis* strains tested. It leads to the conclusion that all examined tertiary amides of **SAL** as well as a parental antibiotic are non-genotoxic compounds. A clear difference was observed in the reference compound—4-nitroquinoline *N*-oxide (NOQ), which confirmed the genotoxic properties of this agent.

To summarize, for the first time a simple and efficient method for the synthesis of tertiary amides of Salinomycin (**SAL**) has been described. All these derivatives were examined for their antibacterial activity against different Gram-positive and Gram-negative bacteria. It has been proved that parental antibiotic and its five amides, that is, diethyl (**1**), dipropyl (**2**), dibutyl (**3**), diethanol (**6**) and dibenzyl (**9**) amide, show potent antibacterial activity against standard bacterial strains tested. None of the tested compounds showed activity against Gram-negative bacteria. Our previous studies of the biological activity of series **SAL** secondary amides have also shown that amides containing short alkyl chain or containing aromatic substituent are the most active against Gram-positive bacteria.[4]

In addition, the tests carried out against methicillin-resistant *Staphylococcus epidermidis* (MRSE) strains clearly show that some of **SAL** derivatives are strongly active against these bacteria strains, especially in comparison to the reference compound used. In some cases this activity was about 40-times higher than that of ciprofloxacin, which suggest a preferential action of tertiary amides of **SAL** against MRSE strains. Large differences between the values of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) parameters indicated that both **SAL** and all its derivatives are potent bacteriostatic, not bactericidal agents. Furthermore, our investigation indicated that **SAL** as well as its three derivatives (**1**, **2** and **6**) exhibit high ability to inhibit biofilm formation by clinical methicillin-resistant *S. epidermidis* strains.

As proved by DNA-damaging activity (genotoxicity) tests, all tertiary amides of **SAL** examined as well as a parental antibiotic are non-genotoxic compounds. Our studies show that **SAL** and its derivatives can be potential antibacterial compounds capable of inhibition of bacteria growth, particularly effective against biofilm-forming bacteria such as *Staphylococci* on surfaces of biomaterials, such as tracheotomy tubes, implants, venous catheters or suitable for application as ear/eye/nose drops.

Moreover, for the first time the activities of **SAL**, its derivatives and two other polyether antibiotics—monensin as well as lasalocid acid against *Bacillus anthracis* have been documented. These tests clearly show that the anti-*B. anthracis* activity of all tested compounds is manifested at micromolar concentrations. These results are important because anthrax is an infectious fatal disease with epidemic potential. Nowadays, bioterrorism using *Bacillus anthracis* is a real possibility, and the search for a new and effective weapon to neutralize these microorganisms is a challenge for researchers. Our studies have shown that polyether ionophores and some amides of **SAL**, seem to be efficient tool against the potential use of *Bacillus anthracis* bacteria as a terrorist bioweapon.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.03.085. These data include MOL files and InChiKeys of the most important compounds described in this article.

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