Synthesis and Evaluation of Water-Soluble 2-Aryl-1-Sulfonylpyrrolidine Derivatives as Bacterial Biofilm Formation Inhibitors


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The approach to the novel 1-[(2-aminoethyl)sulfonyl]-2-arylpyrrolidines via unique intramolecular cyclization/aza-Michael reactions of N-(4,4-diethoxybutyl)ethenesulfonamide have been developed, which benefits from high yields of target compounds, mild reaction conditions, usage of inexpensive and low-toxic reagents, and allows for wide variability in both amine and aryl moieties. Biotesting with whole-cell luminescent bacterial biosensors responding to DNA damage showed that all tested compounds are not genotoxic. Tested compounds differently affect the formation of biofilms by *Vibrio aquamarinus* DSM 26054. Some of the tested compounds were found to suppress the bacterial biofilms growth and thus are promising candidates for further studies.

Keywords: Sulfonamides, nitrogen heterocycles, Michael addition, biofilms, genotoxicity, synthesis design, biological activity.

Introduction

Although sulfonamide drugs are known for a long time, they still remain in the focus of medicinal chemistry.[1 – 3] Among a huge variety of biologically active compounds containing sulfonamide group, those having the pyrrolidine core are of a special interest. Such a sulfonamides are suggested for the treatment of thromboembolic[4] and neurodegenerative disorders, such as Huntington, Parkinson[5,6] and Alzheimer[d] diseases. There is also evidence of the ability of these compounds to inhibit matrix metalloproteinase 2 (MMP2).[8]

Recently, we have synthesized a series of novel 2-aryl-1-sulfonylpyrrolidine derivatives of general formula 1 (Figure 1).[9–11] Preliminary bioactivity experiments showed that some of these compounds are promising candidates for further optimization. The main disadvantage of these compounds appeared to be the low water solubility. Moreover, only simple alkyl and aryl substituents were present in the sulfonamide fragment of the molecules obtained so far, so there were limited possibilities of further modifications and fine tuning of their properties. Thus, we started to search a way to overcome these drawbacks.

Results and Discussion

It is well-known that the charged species possessing either positive or negative charge are usually well soluble in water, and ionization of appropriate functional group is widely used for drugs solubility enhancement.[12,13] Among the substituents capable of ionization, we chose the amine group as the most convenient and widely used one. Analysis of the known sulfonamide drugs revealed that many of them have an amine group attached to the aromatic ring...
Figure 1. Biologically active sulfonamide derivatives and attempt to enhance water solubility of compounds 1.

(Figure 1). However, in our case, the introduction of amine or amide group into the aromatic ring of sulfonamide fragment lead to the drastic decrease of solubility. Moreover, this group was found to have low basicity and thus be hardly ionizable. This is probably due to electron-withdrawing nature of sulfonyl fragment attached to the aromatic ring, which increases the conjugation between nitrogen lone pair and π-system of benzene fragment (Figure 1). Thus, we decided to replace the aryl fragment with polymethylene spacer, which does not lower basicity of amine group.

Compounds with activated double carbon-carbon bond are valuable intermediates in many carbon-carbon and carbon-heteroatom bond forming reactions.\(^{[14–16]}\) Among them, the aza-Michael reaction is known to be a convenient way to introduce amine functionality into the molecule.\(^{[17]}\) Therefore, the following approach to the target compounds was proposed (Scheme 1). The key stages of this approach are the unique intramolecular cyclization of N-(4,4-diethoxybutyl)sulfonamides in the presence of electron rich aromatics, as described by us previously\(^{[9–11]}\) and intermolecular aza-Michael reaction. The order of these stages could be changed, thus allowing routes A and B to the nitrogen-containing 1-sulfonylpyrrolidines 5.

First, we have synthesized the initial compound 2. According to the literature data, vinyl sulfonamides are easily obtained via reaction of 2-chloroethane-1-sulfonyl chloride with primary amines in the presence of bases.\(^{[18–21]}\) On the basis of these data, the reaction of 2-chloroethane-1-sulfonyl chloride with 4,4-diethoxybutane-1-amine was used for the synthesis of desired N-(4,4-diethoxybutyl)ethenesulfonamide (2). This reaction leads either to compound 2 or 2-ethoxy-1-(vinylsulfonyl)pyrrolidine (6) depending on the order of reagents addition (Scheme 2). The similar behavior was described by us previously for the reaction of 4,4-diethoxybutane-1-amine with other sulfonyl chlo-
rides. Compounds 2 and 6 were obtained in 82 and 73% yield, respectively.

Although the reaction of these compounds with various secondary amines led to the expected 2-(dialkylamino)ethane-1-sulfonamide derivatives 4 according to the route B (Scheme 1), the yields of them were rather low. Moreover, the reaction of sulfonamides 4 with phenols led to the unidentified mixture of products, from which no target compounds 5 could be isolated. Thus, we excluded the route B from further consideration.

Next, we studied the reaction of sulfonamide 2 and 2-ethoxypyrrolidine 6 with various phenols (Scheme 3).

The key question at this stage was to check whether the Michael addition of active C-nucleophile to carbon-carbon double bond in strongly acidic media is possible. To our delight, the vinyl fragment appeared to be quite stable under reaction conditions, allowing us to obtain 1-(vinylsulfonyl)pyrrolidines 3a–3d in good to high yields (Table 1, see Supporting Information for detailed characterization data and copies of NMR spectra).

In general, the reaction of N-(4,4-diethoxybutyl)ethenesulfonamide (2) with phenols results in lower yields of target compounds. This is probably due to the side reaction leading to the formation of diarylbutane derivatives as described by us earlier for N-(4,4-diethoxybutyl)urea derivatives.

In the case of resorcinol and pyrogallol, a mixture of diastereoisomeric disubstituted products (R,R/S,S)-3d,3e and (R,S/S,R)-3d,3e was obtained regardless of the reagents ratio. Interestingly, reaction of sulfonamide 2 appeared to proceed more stereoselectively compared with 2-ethoxypyrrolidine 6, allowing us to isolate one of the diastereoisomers in individual form. Unfortunately, we were not able to obtain crystals suitable for x-ray analysis and determine its absolute configuration. The structure of the compound 3b was confirmed by 1H-NMR data.

### Table 1. Synthesis of 1-(vinylsulfonyl)pyrrolidines 3

<table>
<thead>
<tr>
<th>No.</th>
<th>Ar Starting compound</th>
<th>Product</th>
<th>Yield [%][a]</th>
<th>dr[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>3a</td>
<td>98</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3a</td>
<td>34</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>3b</td>
<td>57</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>3b</td>
<td>46</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>3c</td>
<td>61</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>3c</td>
<td>77</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>3d</td>
<td>76</td>
<td>&lt;97:3</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>3d</td>
<td>58</td>
<td>&lt;99:1</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>3e</td>
<td>67</td>
<td>30:60</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>3e</td>
<td>34</td>
<td>&lt;99:1</td>
</tr>
</tbody>
</table>

[a] Isolated yield. [b] According to 1H-NMR data.
additionally proved by X-ray analysis (see Supporting Information).

Addition of amines to vinylsulfonamides generally does not require any catalysts or harsh reaction conditions.\[18,23 – 26\] Thus, we carried out the reaction of some secondary amines with pyrrolidine 4a by simply mixing the equimolar amounts of reagents in dichloromethane. The reaction proceeded smoothly at room temperature and afforded target compounds 7a–7f with good yields (Scheme 4). The only exception was the pyrrolidine 7c containing dibutylamine group, which was isolated in only 23% yield, probably due to its high solubility and losses during reaction workup (Table 2, characterization data and copies of NMR spectra are given in Supporting Information). Additionally, the hydrochlorides of compounds 7b–7f were obtained by dissolving appropriate pyrrolidine in a mixture of ethanol/hydrochloric acid. The structure of compound 7e has been additionally proved by X-ray data (see Supporting Information).

However, it turned out that more complex amines, as well as other NH-nucleophiles did not react with compound 4a under these conditions. The literature survey revealed that there is limited number of publications describing aza-Michael addition of nucleophiles other than simple amines to the vinylsulfonamides.\[27 – 37\] Thus, we optimized the reaction conditions using phenylhydrazine and pyrrolidine 4a as model compounds. The results are summarized in Table 3. The most optimal conditions appeared to be 10 mol-% triethylamine in refluxing ethanol/water mixture.

With this in hand, we further investigated reaction of pyrrolidine 4a with various NH-nucleophiles (Scheme 5). Acid hydrazides (Table 4, No. 1–3) and fluoroquinolones (Table 4, No. 4–6) reacted smoothly under these conditions giving 2-arylpyrrolidine deriva-

![Scheme 4. Reaction of 1-(vinylsulfonyl)pyrrolidine 4a with amines.](image)

![Scheme 5. Reaction of compound 4a with NH-nucleophiles.](image)

**Table 2.** Synthesis of pyrrolidines 7a–7f

<table>
<thead>
<tr>
<th>No.</th>
<th>Product</th>
<th>Yield [%][a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7a</td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>7b</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>7c</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>7d</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>7e</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>7f</td>
<td>75</td>
</tr>
</tbody>
</table>

[a] Isolated yield.

**Table 3.** Optimization of reaction conditions of phenylhydrazine and pyrrolidine 4a

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH$_2$Cl$_2$</td>
<td>–</td>
<td>20</td>
<td>12</td>
<td>–[b]</td>
</tr>
<tr>
<td>2</td>
<td>CH$_2$Cl$_2$</td>
<td>–</td>
<td>reflux</td>
<td>6</td>
<td>–[b]</td>
</tr>
<tr>
<td>3</td>
<td>MeCN</td>
<td>–</td>
<td>reflux</td>
<td>6</td>
<td>–[b]</td>
</tr>
<tr>
<td>4</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>–</td>
<td>reflux</td>
<td>6</td>
<td>–[b]</td>
</tr>
<tr>
<td>5</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>CuCl (10%)</td>
<td>reflux</td>
<td>6</td>
<td>25[c]</td>
</tr>
<tr>
<td>6</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>ZnCl$_2$ (10%)</td>
<td>reflux</td>
<td>6</td>
<td>30[c]</td>
</tr>
<tr>
<td>7</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>FeCl$_3$ (10%)</td>
<td>reflux</td>
<td>6</td>
<td>25[c]</td>
</tr>
<tr>
<td>8</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>DMAP (10%)</td>
<td>reflux</td>
<td>6</td>
<td>90[c]</td>
</tr>
<tr>
<td>9</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>Et$_3$N (10%)</td>
<td>reflux</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td>10</td>
<td>EtOH/H$_2$O (4:1)</td>
<td>Et$_3$N (100%)</td>
<td>reflux</td>
<td>6</td>
<td>90</td>
</tr>
</tbody>
</table>

[a] According to NMR data. [b] Reaction did not proceed. [c] The product was not separated from catalyst.
tives 7g–7l in good to excellent yields. Additionally, 1-sulfonylpyrrolidines 7m and 7n (Table 4, No. 7 and 8) bearing phosphoryl and acetal groups were obtained, which could be useful starting compounds for further modification. It should be noted that, in the latter cases, 1.1 equiv. of triethylamine were taken due to presence of acidic group in the reactant. Detailed characterization data and copies of NMR spectra for all synthesized compounds are given in the Supporting Information.

Next, we studied the influence of some novel water-soluble 2-aryl-1-sulfonylpyrrolidine derivatives on bacterial biofilms formation. The natural strains V. aquamarinus DSM 26054 were chosen as models due to its ability to actively form biofilms. Microbial biofilms are responsible for the etiology and pathogenesis of many acute and, especially, chronic bacterial infections in humans. It has been estimated that more than 80% of diseases of humans and animals are associated with the presence of stable bacterial communities enclosed in biofilms. At the same time, the drug resistance of bacteria living in biofilms is much higher than that of planktonic bacteria. Thus, one of the strategies for controlling pathogenic bacteria is to either inhibit biofilm formation or to degrade biofilms. The obtained biological activity results are summarized in Table 5.

Table 5. The effect of 2-aryl-1-sulfonylpyrrolidine derivatives on biofilm formation by V. aquamarinus DSM 26054

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Biofilm formation [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3a</td>
<td>+ 52% (1 × 10^{-4} M)[c]</td>
</tr>
<tr>
<td>2</td>
<td>7f·HCl</td>
<td>+ 47.9% (1 × 10^{-4} M)[c]</td>
</tr>
<tr>
<td>3</td>
<td>7h</td>
<td>+ 16.8% (1 × 10^{-4} M)[c]</td>
</tr>
<tr>
<td>4</td>
<td>7e</td>
<td>−13.9% (1 × 10^{-4} M)[c]</td>
</tr>
<tr>
<td>5</td>
<td>7d</td>
<td>−18.9% (1 × 10^{-6} M)[c]</td>
</tr>
<tr>
<td>6</td>
<td>7e·HCl</td>
<td>N/E</td>
</tr>
<tr>
<td>7</td>
<td>7d·HCl</td>
<td>N/E</td>
</tr>
<tr>
<td>8</td>
<td>7a</td>
<td>N/E</td>
</tr>
<tr>
<td>9</td>
<td>7a·HCl</td>
<td>N/E</td>
</tr>
<tr>
<td>10</td>
<td>7f</td>
<td>N/E</td>
</tr>
</tbody>
</table>

[a] The solutions of appropriate solvent in deionized H2O with the same concentration were used as control; eight replicates were done for each treatment and control. [b] (+)-Biofilm formation increased, (−)-biofilm formation reduced; N/E—no effect. [c] Differences compared to the control samples are statistically significant, t-criterion, p < 0.05.

According to the data obtained, compounds 7a, 7a·HCl, 7d·HCl, 7e·HCl and 7f (Table 5, No. 6–10) do not affect the biofilm formation significantly. The difference between them and control samples is either statistically unreliable or insignificant. The pyrrolidines 3a and 7f·HCl (Table 5, No. 1, 2) stimulate the formation of bacterial biofilm. The maximum increase in biofilm formation (+52%) is observed for compound 3a at the concentration of 1 × 10^{-4} M (Figure 2).
The activity of pyrrolidine 7h appeared to be concentration-dependent (Table 5, No. 3). Higher concentration of this compound stimulated the biofilm formation weakly (+16.8%), whereas at lower concentrations it was suppressed (−13.9%; Figure 3).

Although this result seems to be interesting from theoretical point of view, the multidirectional dose-dependent effects make compound 7h unsuitable for pharmacological application.

Pyrrolidines 7d and 7e suppress the formation of the biofilm by V. aquamarinus DSM 26054 strains at 1 × 10^{-4}M concentration (Table 5, No. 4, 5). Notably, in the presence of compound 7d, a statistically significant inhibition of biofilm growth was also recorded at a much lower concentration (1 × 10^{-6}M; Figure 4).

The genotoxicity of the synthesized compounds was evaluated using E. coli MG1655 (pRecA-lux) and E. coli MG1655 (pCoD-lux) strains. No bioluminescent response to DNA damage was detected in any case, indicating that compounds under study do not belong to the class of DNA damaging substances.

**Conclusions**

In conclusion, we have developed the approach to novel 1-[(2-aminoethyl)sulfonyl]-2-arylpyrrolidines via consecutive intramolecular cyclization/aza-Michael reactions of N-(4,4-diethoxybutyl)ethenedisulfonamide. The presented method benefits from high yields of target compounds, mild reaction conditions, usage of...
inexpensive and low-toxic reagents, and also allows for a wide variability in both amine and aryl moieties. The genotoxicity and influence on bacterial biofilm growth were tested for some of the synthesized compounds. All of the tested compounds were found to be non-genotoxic. The most promising candidates for further studies are compounds 7d and 7e possessing 2-(piperidin-1-yl)ethyl and 2-morpholinoethyl moieties at the sulfur atom, for which maximum suppression of bacterial biofilm growth was observed.

**Experimental Section**

**General**

All solvents were purified and dried according to standard procedures. IR spectra were obtained with a Bruker Vector 22 spectrometer. Melting points were determined in glass capillaries with a Stuart SMP 10 apparatus and are corrected. 1H-NMR spectra were recorded on a Bruker AVANCE 600 spectrometer (working frequency 600.1 MHz) using residual protons of deuterated solvent as reference. 13C-NMR spectra were recorded on a Bruker Avance 600 spectrometer (working frequency 150.9 MHz). 31P-NMR spectra were recorded on a Bruker MSL 400 spectrometer (working frequency 161.94 MHz). ESI-TOF-MS spectra were recorded on a Bruker AmazonX instrument. Elemental analysis was performed on Carlo Erba EA 1108 instrument.

**Chemicals**

All of the chemicals used were of analytical grade. Crystal violet and N-methyl-N-nitro-N-nitrosoguanidine were obtained from Sigma–Aldrich (USA). Ampicillin was obtained from Sintez (Russia). Test solutions were prepared in deionized water immediately before the tests. Rat liver microsomal enzymes (59 fraction) were from Moltox (USA).

The X-ray diffraction data for the crystals of 3b and 7e were collected on a Smart Apex II (3b) and KAPPA Apex (7e) automatic diffractometer using graphite monochromatic radiation. The structures were solved by direct methods and refined by full-matrix least-squares using the SHELXL97[44] program. All the non-hydrogen atoms, H(O) in 3b and H-atoms of water in 7e were refined with anisotropic atomic displacement parameters. H(O) atoms in 7e could not be identified, so they and H(C) atoms were constrained as riding atoms. All figures were made using the program OLEX2.[45] Crystallographic data (excluding structure factors) for the structure reported in this article have been deposited with the Cambridge Crystallographic Data Center (CCDC-1559736 (3b) and -1559735 (7e)).

**General Methods of Synthesis of 1-(Vinylsulfonyl)-pyrrolidines 3**

**Method A.** To a solution of N-(4,4-diethoxybutyl)-ethenesulfonamide (2; 0.40 g, 1.59 mmol) in dry benzene (10 mL), appropriate phenol (1.59 mmol, 0.80 mmol in the case of resorcinol and pyrogallol) and trifluoroacetic acid (0.12 mL, 1.59 mmol) were added. The mixture was stirred at room temperature for 12 h and the solvent was removed in vacuum. The residue was washed with diethyl ether, filtered off and dried in vacuum (0.01 Torr, r.t., 4 h) to give the target compound 3.

**Method B.** To a solution of 2-ethoxy-1-(vinylsulfon-yl)pyrrolidine (6; 0.33 g, 1.59 mmol) in dry benzene (10 mL), appropriate phenol (1.59 mmol, 0.80 mmol in the case of resorcinol and pyrogallol) and trifluoroacetic acid (0.12 mL, 1.59 mmol) were added. The mixture was stirred at room temperature for 12 h and the solvent was removed in vacuum. The residue was washed with diethyl ether, filtered off and dried in vacuum (0.01 Torr, r.t., 4 h) to give the target compound 3.

**4-Chloro-6-[1-(vinylsulfonfyl)pyrrolidin-2-yl]-benzene-1,3-diol (3a)**:

Yield: 0.16 g, 34% (method A); 0.47 g, 98% (method B). M.p. 154–157 °C. IR (KBr): 3364, 2935, 2873, 1638, 1578, 1547, 1534, 1458, 1426, 1412, 1378, 1332, 1301, 1278, 1144, 1598, 2875, 2985, 3423, 3483. 1H-NMR (600 MHz, (D6)DMSO): 1.68–1.80 (m, 2H, CHCH2), 2.02–2.11 (m, 1H, CH2), 3.23–3.31 (m, 1H, CH), 4.70–4.78 (m, 1H, CH), 6.09–6.18 (m, 2H, CH2), 6.49 (s, 1H, CHAr), 6.90 (dd, J = 16.4, 10.0, 1H, CH), 7.07 (s, 1H, CHAr), 9.62 (br. s, 1H, OH), 9.78 (br. s, 1H, OH). 13C-NMR (150 MHz, (D6)DMSO): 24.08, 34.16, 49.44, 58.38, 104.05, 109.57, 122.82, 127.79, 128.80, 132.73, 152.62, 153.47. ESI-TOF-MS: 326 ([M+Na]+). Anal. calc. for C12H12ClNO4S: C 47.45, H 4.65, Cl 11.67, N 4.61, S 10.56; found: C 47.64, H 4.49, Cl 11.90, N 4.56, S 10.68.

**General Method of Synthesis of Compounds 7a–7f**

To a suspension of 4-chloro-6-[1-(vinylsulfonfyl)pyrrolidin-2-yl]benzene-1,3-diol (3a; 0.4 g, 1.32 mmol) in dry CH2Cl2 (10 mL), appropriate amine was added (1.32 mmol, 0.66 mmol in the case of piperidine). The mixture was stirred at room temperature for 12 h. The
precipitate formed was filtered off, washed with diethyl ether and dried in vacuum (10 Torr, r.t., 2 h) to give target compound 7.

**General Method of Synthesis of Compounds 7g–7n**

To a suspension of 4-chloro-6-[1-(vinylsulfonyl)pyrroli
din-2-yl]benzene-1,3-diol (3a; 0.4 g, 1.32 mmol) in a mixture of ethanol/water (4 mL/1 mL), triethylamine (0.02 ml, 0.13 mmol; 0.2 ml, 1.45 mmol in the case of compounds 7j–7l) and appropriate NH-nucleophile (1.32 mmol) were added. The mixture was refluxed for 20 h. The precipitate formed was filtered off, washed with diethyl ether and dried in vacuum (10 Torr, r.t., 2 h) to give target compound 7.

4-Chloro-6-[1-[2-(hexylamino)ethyl]sulfonyl]-pyrroloidin-2-yl]benzene-1,3-diol (7a). Yield: 0.33 g, 61%. M.p. 113–115 °C. IR (KBr): 1129, 1596, 3378, 3424.

1H-NMR (600 MHz, (D6)DMSO): 0.85 (t, J = 6.1, 3H, CH3), 1.20–1.28 (m, 6H, CH2), 1.32–1.41 (m, 2H, CH2), 1.66–1.89 (m, 4H, CH2), 2.11–2.23 (m, 1H, CH), 2.30–2.35 (m, 1H, CH2), 2.64–2.70 (m, 1H, CH2), 2.78–2.90 (m, 3H, CH2), 3.13–3.21 (m, 2H, CH2), 4.86–4.94 (m, 1H, CH), 6.49 (s, 1H, CHA), 7.03 (s, 1H, CHA). 13C-NMR (150 MHz, (D6)DMSO): 14.36; 22.52; 24.12; 26.84; 29.65; 31.64; 34.17; 43.54; 48.49; 48.99; 49.12; 57.94; 103.91; 109.50; 122.88; 127.76; 152.45; 153.34. ESI-TOF-MS: 405 ([M + H]+). Anal. calc. for C38H38ClN2O5S: C 53.29, H 7.42, Cl 8.65, N 6.92, S 7.92. found: C 53.29, H 7.42, Cl 8.65, N 6.92, S 7.92.

Biological Studies

**Bacterial Strains and Cultivation Conditions.** E. coli MG1655 (pRecA-lux) and E. coli MG1655 (pCoD-lux) have been used for the evaluation of genotoxicity of the synthesized compounds. The strains were kindly furnished by I. V. Manukhov, Federal State Unitary Enterprise GosNIIGenetika.

Plasmids of lux-biosensors E. coli MG1655 (pRecA-lux) and E. coli MG1655 (pCoD-lux) contain a bioluminescence operon controlled by the SOS-promoters PcolD and PrecA. The SOS promoter PrecA was isolated from the E. coli genome; PcolD was isolated from the plasmid pCoD-EC23 containing the colD gene encoding the synthesis of ColD colicin. These promoters are only activated in the case of damage in DNA molecules. LuxCABGE genes isolated from the genome of entomopathogenic bioluminescent bacteria *Photobacteri um luminescens* ZM1 were used as reporter genes.

The strains of *Vibrio aquamarinus* DSM 26054 isolated by SFedU staff from the coastal waters of the Black Sea near the village of Abrau-Durso were used to study the biofilms formation.

The bacterial strains E. coli MG1655 (pRecA-lux) and E. coli MG1655 (pCoD-lux) were cultivated in Luria-Bertani (LB) medium containing 100 μg of ampicillin/mL. The cultures were grown under constant shaking to early exponential phase at 37 °C. Cells were used immediately for stress induction tests.

**Test System for Genotoxicity Evaluation**

The test compounds 3a, 7a·HCl, 7e, 7e·HCl, 7d and 7d·HCl were dissolved in ethanol to the concentration of 1×10−3 M. Further dilutions were prepared by adding deionized H2O. The control solutions were analogous dilutions of ethanol in deionized H2O.

The test compounds 7f, 7f·HCl and 7h were dissolved in DMSO/ethanol mixture (1:1) to the concentration of 5–10−3 M and diluted with DMSO/ethanol mixture (1:1) to obtain the concentration of 10−3 M. Further dilutions were prepared by adding deionized water. The control solutions were analogous dilutions of ethanol in deionized water.

Substance samples to be tested were added in 20-μl portions to wells of a 96-well microplate containing 180 μl of the culture. For control activation of the
PrecA and PcoID promoter, N-nitro-N-nitrosoguanidine was used.

Measurements were made with the help of a microplate luminometer Luminoskan Ascent (Thermo Fisher Scientific, USA). Numerical values of bioluminescence were expressed in relative luminescence units.

**Calculation**

The criterion of toxic influence is bioluminescence intensity change of the test object in the sample under study in comparison with the control sample.

The induction factor, I, was defined as the relation of luminescence intensity of a lux-biosensor suspension containing tested sample (Lc) to the luminescence intensity of a lux-biosensor control suspension (Lk): \( I = \frac{L_c}{L_k} \).

If at significant differences from control induction factor values were \( \leq 2 \), the detected genotoxic effect was evaluated as weak, and if they were in the range from 2 to 10 – as medium, above 10 – as strong. All the experiments were carried out three times independently.

Difference reliability of bioluminescence in experiment from control value was estimated by *t*-criterion with the help of Excel program. The conclusion about sample toxicity was made at \( p < 0.05 \).

**Test System for Biofilm Formation Evaluation**

To quantify the formation of biofilms, the crystal violet assay with some modifications was used.

*V. aquamarinus* DSM 26054 was cultivated for 24 h in LB medium supplemented with 3% NaCl in the Innova 40R shaker incubator (New Brunswick Scientific, USA) at 25 °C and 200 rpm. Then, a suspension of the daily culture of *V. aquamarinus* DSM 26054 was diluted with LB medium supplemented with 3% NaCl to the density of \( 1 \times 10^8 \) cells/ml.

The resulting suspension (180 µl) was added to the wells of a polystyrene microplate (Nuova Aptaca, Italy). To some of the wells, 20 µl of the test substances at various concentrations were added. Since solvents used could also influence the biofilm formation, 20 µl of the appropriate solvent was added to the other part of the wells at same dilutions. Eight replicates were done for each treatment and control. The microplate was covered with a lid and wrapped with a Parafilm (Bemis Company, Inc., USA).

After incubation at 25 °C for 72 h, biofilms were stained. The contents in the wells were removed by means of a dispenser. The wells were then carefully washed three times with 250 µl of sterile saline. The microplates were shaken to remove all non-adherent bacteria. Biofilms were fixed with 200 µl of 96% ethanol for 15 min. After the microplates were dried in the air, 200 µl of 0.5% crystal violet prepared according to Hooker (Gerhardt, 1984) were introduced into the wells. After 10 min, the dye was removed. The excess dye was removed by washing with water three times. After the microplates were air-dried, the dye in the wells bound to biofilms was dissolved with 200 µl of 96% ethanol. The extraction level (absorption) of crystal violet by ethanol was measured after 60 min at 570 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany) in optical density units (OD\(_{570}\)). The intensity of biofilm formation directly corresponds to the intensity of staining of the contents of the wells with the dye.

**Acknowledgements**

This work was supported by the Russian Science Foundation (Grant No. 16-13-10023).

**Author Contribution Statement**

Andrey V. Smolobochkin, Ekaterina A. Muravyeva, Liliya I. Vagapova and Irina R. Knyazeva performed the synthesis of compounds. Anastasiya V. Gildebrant and Ivan S. Sazykin performed biological studies. Julia K. Voronina performed the X-ray analysis. Marina A. Sazykina supervised the biological studies. Almir S. Gazizov designed the experiments, collected and analyzed the data, and wrote the article. Alexander R. Burilov and Michail A. Pudovik supervised the whole study.
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Received September 21, 2018
Accepted November 1, 2018