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# Microwave-assisted synthesis of new environmentally friendly biodegradable and antibacterial heterocycles

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ΨThese authors jointly supervised this work.

## Abstract

The rise of bacterial resistance to common clinical antibiotics is calling for alternative approaches to synthesize new antibacterial drugs with high environmental biodegradability.

One such approach is the use of microwave-assisted synthesis, which is a powerful green synthetic technique used increasingly to accelerate organic reactions.

To discover new antibacterial compounds, we report the efficient synthesis of nineteen thiophene derivatives, five furan derivatives, and two thiazole derivatives, using Suzuki-Miyaura cross-coupling by microwave irradiation. Microwave irradiation conditions have reduced the reaction time, increased the yield and this method uses less organic solvent compared to conventional heating.

Several compounds showed broad antibacterial activity. One compound, in particular, demonstrated significant antibacterial activity against *Streptococcus pyogenes* with a minimal inhibitory concentration of 0.097 µg/mL and against *Salmonella enterica* with a minimal inhibitory concentration of 0.78 µg/mL. The biodegradability of two furan derivatives with the best antibacterial activities and their effects on environmental bacteria were studied using the closed bottle test. This test proved that the two compounds were readily biodegradable without toxic effects on environmental bacteria present in wastewater treatment plants.

Therefore our data demonstrated not only green synthesis but also environmental benignity due to the biodegradability of the novel antibacterials, which is a very desirable feature.

**Key-words:** Environmental biodegradability, closed bottle test, microwave-assisted synthesis, antibacterial, thiophenes, furans.

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## 1. Introduction

Antibiotics have revolutionized medicine, turning infections that were once lethal into uncomplicated diseases. Antibiotic resistance occurs when bacteria evolve to adapt to antibiotics, resulting in drug inefficiency and persistent infections with an increase of the risk of severe diseases and transmission. Unfortunately, misuse and overuse of existing antimicrobials in humans, animals and aquaculture are accelerating the development and spread of antibiotic resistance complicated by the fact that new antibiotics are discovered at an ever-decreasing rate (Bush, 2004; Economou and Gousia, 2015; Norrby et al., 2005).

After administration, non-metabolized antibiotics and their active metabolites can be excreted by animals or humans through urine and feces (Kim and Aga, 2007; Kümmerer et al., 2000; Sarmah et al., 2006). As a result, most antibiotics are prevalent in various environments (Javid et al., 2016; Kümmerer, 2009a, 2009b; Kümmerer and Henninger, 2003; Rodríguez-Rojas et al., 2013). Even trace amounts of antibiotics induce the development of antibacterial resistance by exerting selective pressure on environmental bacteria (Allen et al., 2010; Tello et al., 2012; Wang et al., 2018). This likely contributes to the global spread of antibiotic resistance genes and ultimately poses a major threat to the health and safety of aquatic and terrestrial organisms, as well as human beings (Tan and Shuai, 2015). We need global selectivity towards bacterial strains responsible for human diseases without disrupting microorganism populations present in water treatment plants. These observations thus highlight the urgent need for new antibacterial agents with high biodegradability.

Heterocyclic compounds, containing oxygen/sulfur/nitrogen atoms, have been of significant interest, because of their common applicability (Progress in Heterocyclic Chemistry, Volume 5 - 1st Edition, 1996). Among these heterocyclic compounds thiophenes have a special place, due to their synthetic accessibility and pharmacological properties, including, e.g., antibacterial (Alsayari et al., 2020; Boibessot et al., 2016; Nasr et al., 2014), antifungal (Ibrahim et al., 2015) and antitumor (Abdel-Rahman et al., 2017; Wang et al., 2013) activities. Furan compounds, obtained from synthetic or natural sources, have been attracting much interest, due to the wide range of pharmaceutical applications (De et al., 2015), including demonstrated antimicrobial activities (Erkuş et al., 2020; Long et al., 2019). Moreover, thiazoles have attracted continuing interest because of their numerous biological properties, such as anti-inflammatory (Sharma and Sawhney, 2010) and antibacterial (Mishra et al., 2017) activities. On the other hand, microwave-assisted synthesis is a growing synthetic approach among medicinal chemists and has proven to be more efficient in terms of reaction yield, reaction time, product purity and environmental friendliness for many reactions when compared to conventional thermal methods (Abbas et al., 2016; Abedinifar et al., 2020; Saad et al., 2011).

Motivated by these findings, we reported here an improved microwave-assisted synthesis of new thiophene, furan and thiazole derivatives in moderate to good yields. These compounds were evaluated for their antibacterial properties against selected clinical isolates, and the compounds with the best minimal inhibitory concentration (MIC) have been evaluated for their environmental biodegradability.

## 88 2. Experimental

### 89 2.1. Chemistry

90 All reagents were purchased from commercial suppliers (Acros, Sigma Aldrich, Alfa Aesar,  
91 and TCI) and were used without further purification. Nuclear Magnetic Resonance (NMR)  
92 spectra were recorded with a Bruker Avance 300 spectrometer (300 and 75 MHz for  $^1\text{H}$  and  $^{13}\text{C}$   
93 NMR, respectively) and Bruker Avance 400 spectrometer (376.5 for  $^{19}\text{F}$ ). Chemical shifts ( $\delta$ )  
94 and coupling constants ( $J$ ) are given in ppm and Hz, respectively, using residual solvent signals  
95 as reference for the  $^1\text{H}$  and  $^{13}\text{C}$ . The following abbreviations are used: s = singlet, d = doublet,  
96 t = triplet, q = quartet, br s = broad signal, dd = double doublet, dt = double of triplet, m =  
97 multiplet. High-resolution mass spectra (HRMS) were obtained by electrospray using a TOF  
98 analyzer Platform. Infrared (IR) spectra were obtained using a Jasco FT-IR 410 instrument as  
99 a thin film on NaCl disc as stated. Only structurally important peaks ( $\bar{\nu}$ ) are presented in  $\text{cm}^{-1}$ .  
100 Reactions were monitored with Merck Kieselgel 60F<sub>254</sub> precoated aluminum silica gel plates  
101 (0.25 mm thickness). Melting points were determined on a Stuart scientific SMP10 apparatus  
102 and are uncorrected. Flash chromatography was performed on a Grace Reveleris X2 using a 40  
103  $\mu\text{m}$  packed silica cartridge. Flash chromatography experiments were carried out on Silica Gel  
104 premium Rf grade (40-63  $\mu\text{m}$ ) or were performed on a Grace Reveleris X2 using a 40  $\mu\text{m}$   
105 packed silica cartridge. Ultra performance liquid chromatography (UPLC) analyses were  
106 obtained on the Waters Acquity H-Class using the following conditions: Waters Acquity BEH  
107 C18 column (1.7 $\mu\text{m}$ , 50 mm x 2.1 mm), 25 °C column temperature, 0.5 mL/min flow rate,  
108 photodiode array detection (TUV – 214nm) and the mobile phase consisted of a gradient of  
109 water and acetonitrile (each containing 0.1% of formic acid). High-performance liquid  
110 chromatography (HPLC) analyses were obtained on the Agilent Vwd 1100 Series using the  
111 following conditions: BDS Hypersil C18 column (1.7 $\mu\text{m}$ , 50 mm x 2.1 mm), 25 °C column  
112 temperature, 0.5 mL/min flow rate, photodiode array detection (TUV – 254nm), mobile phase  
113 consists of a gradient of water and acetonitrile (each containing 0.1% of trifluoroacetic acid).  
114 The purity of all synthetic compounds was determined by UPLC/HPLC analysis and was  
115 greater than 95% except for **8y**. The reaction was done in the monomode microwave Discover  
116 system from CEM (CEM MWaves SAS, Orsay, France) that has one magnetron (2450 MHz)  
117 with a maximum delivered power of 300 W in 1 W increments. The monomode microwave  
118 features an electromagnetic stirring with adjustable speeds (Teflon<sup>®</sup> coated stir bars are  
119 suitable), and direct control of temperature with the aid of a fiber-optic (FO).

120

#### 121 General experimental procedure

122 A 2 M solution of base (1.6 mL/mmol) was added to a commercial bromo-thiophene, bromo-  
123 thiazole or bromo-furan (1 eq), respectively, with corresponding boronic acid (1 eq to 1.5 eq)  
124 and Pd(PPh<sub>3</sub>)<sub>4</sub> (0.05 eq) in indicated solvent (2.5 mL/mmol). The reactor was placed into the  
125 synthesis monomode microwave oven cavity and carried out under microwave irradiation  
126 (300W) at 120 °C for 17 min and then at 130 °C for 3 min. The reaction mixture was stirred  
127 continuously during the reaction. The disappearance of starting materials was monitored by  
128 thin-layer chromatography (TLC). Once the reaction was finished, the mixture was extracted  
129 with 5x50 ml of ethyl acetate. The organic layers were washed with brine, dried with Na<sub>2</sub>SO<sub>4</sub>,  
130 filtered, and concentrated under pressure. The crude compound was purified by silica gel  
131 chromatography or flash chromatography, eluting petroleum ether/ ethyl acetate to generate the  
132 desired compound. Finally, the obtained powder was dissolved in a small volume of ethyl  
133 acetate and precipitated in cold pentane (-20 °C).

134 Selected spectra data. For all other data see the supplementary material section.

135 **2-(5-nitrofur-2-yl)phenol (8s)** Yellow powder. Yield: 39% (50 mg); mp: 218-220 °C;  $R_f$ :  
136 0.28 (PE/EtOAc: 7/3); IR (cm<sup>-1</sup>): 3446 (ν<sub>OH</sub>); <sup>1</sup>H NMR (DMSO - *d*<sub>6</sub>, 300 MHz) δ: 6.98 (td, 1H,  
137  $J_1 = 8.1$  Hz,  $J_2 = 1.2$  Hz); 7.02 (dd, 1H,  $J_1 = 8.4$  Hz,  $J_2 = 0.9$  Hz); 7.23 (d, 1H,  $J = 3.9$  Hz); 7.32  
138 (ddd, 1H,  $J_1 = 8.7$  Hz,  $J_2 = 7.2$  Hz,  $J_3 = 1.5$  Hz); 7.75 (dd, 1H,  $J_1 = 7.8$  Hz,  $J_2 = 1.8$  Hz); 7.78  
139 (d, 1H,  $J = 3.9$  Hz); 10.79 (br s, 1H, OH); <sup>13</sup>C NMR (DMSO - *d*<sub>6</sub>, 75 MHz) δ: 112.8 (CH); 114.5  
140 (C); 115.7 (CH); 116.6 (CH); 119.7 (CH); 126.2 (CH); 131.6 (CH); 150.4 (C); 154.0 (C, C-  
141 NO<sub>2</sub>); 155.8 (C, C-OH); UPLC:  $t_R$ : 2.29 min; purity: 100%; HRMS: [M + H]<sup>+</sup> calculated for  
142 C<sub>10</sub>H<sub>8</sub>NO<sub>4</sub>, 206.0453, found 206.0451 (mass error: -1.0 ppm).

143 **3-(5-nitrofur-2-yl)phenol (8t)** Yellow powder. Yield: 40% (53 mg); mp: 205-207 °C;  $R_f$ :  
144 0.31 (PE/EtOAc: 7/3); IR (cm<sup>-1</sup>): 3400 (ν<sub>OH</sub>); <sup>1</sup>H NMR (DMSO - *d*<sub>6</sub>, 300 MHz) δ: 6.89 (m, 1H);  
145 7.26 (m, 1H); 7.34 (m, 3H); 7.81 (d, 1H,  $J = 3.9$  Hz); 9.87 (br s, 1H, OH); <sup>13</sup>C NMR (DMSO -  
146 *d*<sub>6</sub>, 75 MHz) δ: 109.7 (CH); 111.4 (CH); 115.6 (CH); 116.2 (CH); 117.7 (CH); 128.7 (C); 130.5  
147 (CH); 151.1 (C); 156.1 (C, C-NO<sub>2</sub>); 158.0 (C, C-OH); UPLC:  $t_R$ : 2.15 min; purity: 100%;  
148 HRMS: [M + H]<sup>+</sup> calculated for C<sub>10</sub>H<sub>8</sub>NO<sub>4</sub>, 206.0453, found 206.0457 (mass error: 1.9 ppm).

149 **4-(5-nitrofur-2-yl)phenol (8u)** Yellow powder. Yield: 20% (63 mg); mp: 245-247 °C;  $R_f$ :  
150 0.42 (PE/EtOAc: 5/5); IR (cm<sup>-1</sup>): 3384 (ν<sub>OH</sub>); <sup>1</sup>H NMR (DMSO - *d*<sub>6</sub>, 300 MHz) δ: 6.91 (d, 2H,  
151  $J = 8.7$ Hz); 7.19 (d, 1H,  $J = 3.9$ Hz); 7.74 (d, 2H,  $J = 8.7$ Hz); 7.81 (d, 1H,  $J = 3.9$ Hz); 10.21 (br  
152 s, 1H, OH); <sup>13</sup>C NMR (DMSO - *d*<sub>6</sub>, 75 MHz) δ: 107.5 (CH); 116.2 (2-CH); 116.3 (CH); 118.6  
153 (C); 127.2 (2-CH); 150.7 (C); 157.2 (C, C-NO<sub>2</sub>); 159.8 (C, C-OH); UPLC:  $t_R$ : 2.21 min; purity:  
154 98%; HRMS: [M + H]<sup>+</sup> calculated for C<sub>10</sub>H<sub>8</sub>NO<sub>4</sub>, 206.0453, found 206.0453 (mass error:  
155 0.0 ppm).

156

## 157 **2.3 Biological evaluation**

### 158 **2.3.1 Disk Diffusion Assay**

159 The Kirby-Bauer disk diffusion susceptibility assay was used to determine the sensitivity of  
160 bacteria to selected compounds, according to the Clinical Laboratory Standards Institute (CLSI)  
161 guidelines (Wayne, 2012). See supplementary material for more details.

### 162 **2.3.2 Determination of the minimal inhibitory concentration and minimal bactericidal 163 concentration**

164 The MIC of the tested compounds was determined by the two-fold serial broth (lysogeny broth,  
165 Brain heart infusion or Todd Hewitt Broth medium) dilution method in 96 well plates (Fisher  
166 Scientific Co.), according to CLSI guidelines (Wayne, 2012). See supplementary material for  
167 more details.

### 168 **2.3.3 Biodegradability and toxicity assays following the closed bottle test**

169 The closed bottle test is known as one of the simplest tests to assess the biodegradability of low  
170 solubility or insoluble organic compounds (OECD, 1992). Following guidelines, the test was  
171 conducted in the dark in a proofer at 20 °C +/- 0.2 °C in closed bottle test flasks. The test was  
172 performed for 28 days as previously reported with compounds **8s,t** (Alexy et al., 2004; OECD,  
173 1992). See Table S2, See supplementary material for more details.

## 174 **3. Results and discussion**

### 175 **3.1 Synthesis of furan, thiazole, and thiophene derivatives 8a-y**

176 Previous results reported in our laboratory by Boibessot *et al.* showed that compound **8a** had  
177 promising antimicrobial activity (Boibessot et al., 2016). Compound **8a** was first synthesized  
178 using the Boibessot *et al.* method with Suzuki-Miyaura cross-coupling starting from 2-bromo-

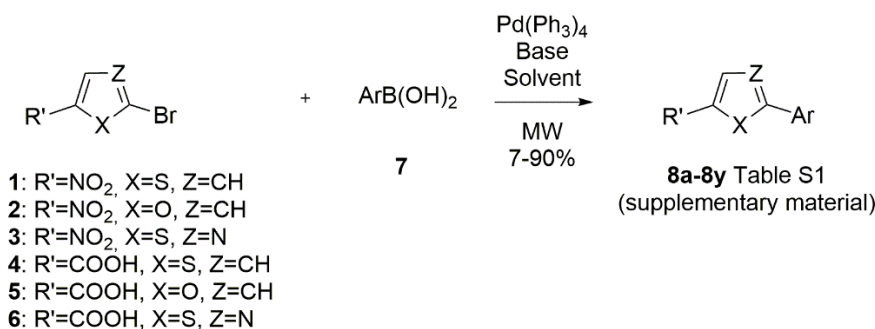
179 5-nitro-thiophene **1** and 2-hydroxy-phenylboronic acid in the presence of a 2 M solution of  
 180 potassium carbonate in hot dioxane. Compound **8a** was obtained by conventional heating  
 181 method with a 42% yield in 15 hours (Scheme 1, Table 1). Here we present improved synthetic  
 182 access to this series of compounds in moderate to good yields. To increase the chemical yield  
 183 and decrease the reaction time of molecule **8a**, microwave heating conditions were considered  
 184 (Scheme 1). Three microwave conditions were used and are summarized in Table 1. Initially,  
 185 compound **8a** was obtained with a 49% yield under stoichiometric conditions (Experiment 1)  
 186 in 10 minutes. This yield was significantly improved to 72% when the reaction time was slightly  
 187 increased to 20 min (Experiment 2). Finally, an excess of 2-hydroxy-phenylboronic acid  
 188 (Experiment 3) was used to obtain **8a** in 76% yield in 20 min.

189  
 190 **Table 1** Improved conditions for the synthesis of compound **8a**.

	Heating	Time	Yield	Comments
Previous work	Conventional heating	15 h	42%	Stoichiometric conditions
Experiment 1	Microwave	10 min	49%	Stoichiometric conditions
Experiment 2	Microwave	20 min	72%	Stoichiometric conditions
Experiment 3	Microwave	20 min	76%	1.5 eq of boronic acid

192  
 193 A total of 25 analogs of **8a** were synthesized using an improved Suzuki-Miyaura cross-coupling  
 194 reaction by microwave irradiation (see supplementary material for all spectra details).  
 195 Heterocycles **1-6** reacted with the corresponding boronic acids in the presence of a 2 M aqueous  
 196 solution of a base in the corresponding solvent. Compounds **8a-y** were obtained with yields  
 197 ranging from 7% to 90% after purification (Scheme 1). The synthesis of compound **10** was  
 198 detailed in the supplementary material.

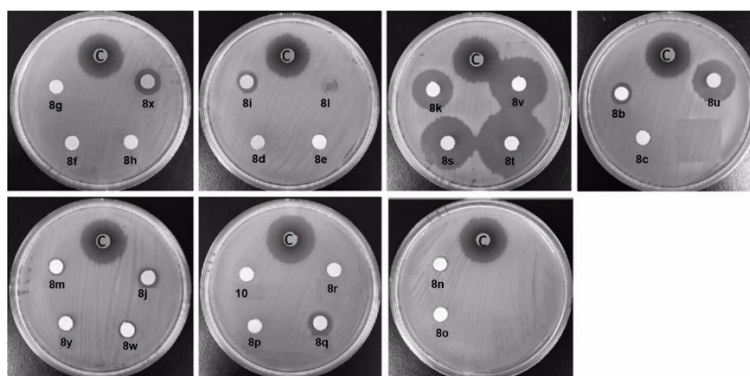
199  
 200 **Scheme 1** Synthesis of compounds **8a-8y** from bromo-heterocycles **1-6** and boronic acids **7**.  
 201 Reagents and conditions: Pd(PPh<sub>3</sub>)<sub>4</sub>, base and solvent using microwave heating, 7-90%. For  
 202 more details see supplementary material. The use of microwave heating renders the synthetic  
 203 pathway green compatible.



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207 **3.2 Antibacterial evaluation of furan, thiazole and thiophene derivatives 8a-y and 10**  
 208 Antibacterial activities of the 25 compounds were evaluated by a standard Kirby-Bauer disk  
 209 diffusion assay (Fig. 1). Each paper disc was impregnated with 100 µg of each of the  
 210 compounds, dried and placed on plates containing *B. subtilis* laboratory strain JH642. In this  
 211 assay, compounds diffuse out of the disk, building a gradient of compound surrounding the  
 212 disk. A lawn of bacteria is grown, and a clearing around a disk signals that the bacteria are  
 213 sensitive to growth inhibition or killing activity of the respective compound. Some clear level  
 214 of growth inhibition was observed for nine compounds such as **8b**, **8i-k**, **8q**, **8s-v** and **8x**, but  
 215 only compounds **8k** and **8s-v** showed inhibition rings similar to the control (ampicillin: 10  
 216 µg/disk). For some compounds, no growth inhibition was observed. It is worth noting that, eight  
 217 of the nine compounds with antimicrobial activity share a NO<sub>2</sub> group at position 2 of thiophene,  
 218 suggesting that this group is crucial for antimicrobial activity.

219  
 220 **Fig. 1** Evaluation of the antibacterial activity of 25 compounds on *B. subtilis* strain JH642 by  
 221 the Kirby-Bauer disk diffusion method. C is the positive control disk that contained 10 µg of  
 222 ampicillin. All other disks contain 100 µg of the indicated compound. The inhibitory activity  
 223 is represented by the dark circle of no bacterial growth around the disk.



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 226  
 227 The Kirby-Bauer assays are only semi-quantitative, since rings of inhibition are a function of  
 228 both, killing activity but also solubility of the compound. Thus, by using a quantitative serial  
 229 dilution assay, the MIC, as well as the minimal bactericidal concentration (MBC) of compounds  
 230 **8k**, **8s-t**, and **8v** were determined for eight different bacterial strains, and the results were  
 231 summarized in Table 2.

232  
 233 **Table 2** Minimal inhibitory concentration (MIC) and minimal bactericidal concentration  
 234 (MBC) result in eight bacterial strains of compounds **8a**, **8s**, and **8t**.

	<i>B. subtilis</i> (G +)	<i>S. aureus</i> (G +)	<i>B. anthracis</i> (G +)	<i>S. pyogenes</i> (G +)	<i>S. agalactiae</i> (G +)	<i>E. faecalis</i> (G +)	<i>S. enterica</i> (G -)	<i>E. coli</i> (G -)
<b>8s</b> MIC µg/mL	0.585	6.25	0.78	0.585	> 50	12.5	1.56	9.375
<b>8s</b> MBC	0.585	6.25	0.78	0.585	> 200	18.75	> 6.25	9.375

	μg/mL								
<b>8t</b>	MIC	0.146	1.56	0.097	0.097	25	3.125	0.78	3.125
	μg/mL								
	MBC	0.146	1.56	0.097	0.195	50	3.125	> 0.78	3.125
<b>8a</b>	MIC	7	16	7	10	16	32	32	32
	μg/mL								
	MBC	10	32	16	32	> 32	> 64	32	32
	μg/mL								

236

237 Molecules **8s,t** are analogs of molecule **8a** with a furan nucleus. Compound **8a** has antibacterial  
 238 activity with MICs ranging from 7 to 32 μg/mL and MBCs ranging from 10 to > 64 μg/mL.  
 239 The results obtained with the furan series **8s,t** clearly show antibacterial activity against Gram-  
 240 positive and Gram-negative bacteria. Their MICs range from 0.097 to 12.5 μg/mL and MBCs  
 241 range from 0.097 to 18.75 μg/mL (Table 2). These values are significantly higher than the  
 242 thiophene compound **8a**. None of the new molecules show improved activity against  
 243 *S. agalactiae*, when compared to compound **8a**. This suggests that either the new compounds  
 244 are not able to access their cellular target as easily in this strain, perhaps due to reduced  
 245 permeability, or otherwise, that the cellular target is more specific for compound **8a**, in contrast  
 246 to all other tested bacterial strains.

247

### 248 3.3 Biodegradability and toxicity of compounds **8s,t**

249 One of the easiest ways to readily measure the biodegradability of a molecule is the closed  
 250 bottle test described by the Organization for Economic Co-operation and Development  
 251 (OECD). This experiment allows the determination of chemical oxygen demand (COD) in a  
 252 mineral medium at low concentration of assay substance. To determine the biodegradability of  
 253 the structures, we focused on the compounds that have shown the best antibacterial activities  
 254 among the nitrofurans series: **8s** and **8t**.

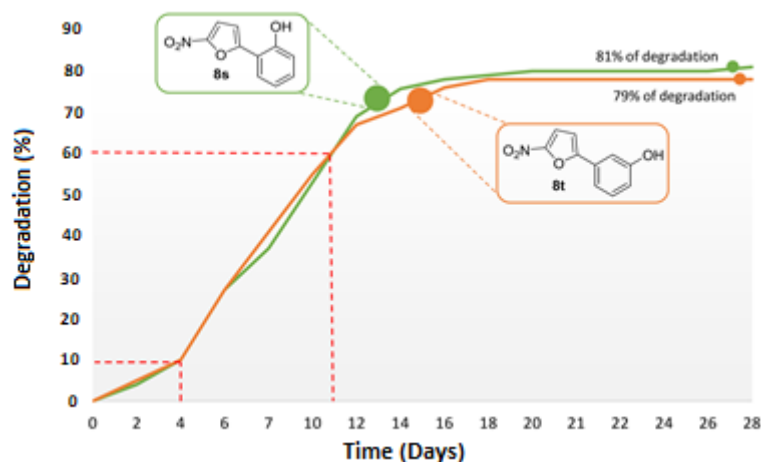
255

256 **Fig. 2** Percentage of degradation of compounds **8s** and **8t** over time. 10% biodegradability is  
 257 reached on day 4 and 60% on day 11. This result is represented by the dotted lines. The Green  
 258 curve corresponds to the **8s** degradability profile while the **8t** degradability profile is  
 259 represented by the orange curve. 81% biodegradability of **8s** and 79% biodegradability of **8t**  
 260 was achieved over the time of the experiment.

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Degradation experiments for compounds **8s** and **8t** are depicted in figure 2 and show that they are readily biodegradable. 60% of theoretical oxygen demand (ThOD) was consumed in ten days, once the ThOD reached 10% (between days 4 and 14 for **8s** and **8t**). According to OECD guidelines, the tested molecules proved to be nontoxic, since sodium acetate was degraded by more than 30% in toxicity control flasks within two weeks (Table S3, supplementary material). When sodium acetate is readily degraded in flasks in the presence of test substances, it demonstrates that the substances did not inhibit the biodegradation activity of the microorganism population. In summary, the closed bottle test assays suggest that compounds **8s** and **8t** have the desirable features of being non-toxic to the marine environment, while at the same time being readily biodegradable with a total degradation of 81% and 79% at day 28, respectively. More detailed studies on cultivatable bacteria to validate this observed absence of toxicity in an aquatic medium, however, are needed.

#### 4. Conclusion

We prepared a new series of thiophene, thiazole and furan derivatives using Suzuki-Miyaura cross-coupling by microwave irradiation. The use of microwaves allowed to reduce the reaction time and to improve significantly the yield of the reaction. All compounds were evaluated for antimicrobial activities against Gram-positive and Gram-negative bacterial strains. The furan derivative **8t** was the most potent compound against several Gram-positive pathogens with an impressive MIC of 0.097  $\mu\text{g/mL}$  against *B. anthracis* and *S. pyogenes*. It also inhibited Gram-negative strains with a MIC of 0.78  $\mu\text{g/mL}$  against *S. enterica*. Furthermore, compound **8t** proved to be readily biodegradable, while also showing to be non-toxic to aquatic microbial environments. In conclusion, our findings present an encouraging avenue for the further development of similar antibacterial compounds that have a suggested low impact on the environment and are thus expected to not easily contribute to a quick evolution of resistant strains in microbial communities.

292

**Supplementary Material** The online version contains supplementary material available at XX

#### Author contributions

ZB was responsible for the overall supervision, ZB, PM, HS, wrote the manuscript, MRH synthesized the different compounds, MOO, NF and HS performed the biological experiment,

296

297 VD and DB performed the biodegradability experiments. All authors approved the final version  
298 of the manuscript.

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307 **Availability of data and material** Supporting material for characterization of each synthesized  
308 compound are provided including NMR data and HRMS.

309 **Code availability Not applicable**

### 310 **Declarations**

311 **Conflict of interest** The authors declare that they have no conflict of interest.

312 **Ethical approval** Not applicable.

313 **Consent to participate** Not applicable.

314 **Consent for publication** All the authors have seen and approved the final version of the  
315 manuscript.

316

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