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Synthesis and evaluation of the quorum sensing inhibitory effect of substituted triazolyldihydrofuranones

Gilles Brackman [a,*], Martijn Risseeuw [b], Shari Celen [b], Paul Cos [c], Louis Maes [c], Hans J. Nelis [a], Serge Van Calenbergh [b], Tom Coenye [a]

[a] Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium
[b] Laboratory of Medicinal Chemistry, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium
[c] Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium

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ABSTRACT

Acylhomoserine lactone (AHL) analogues in which the amide function is replaced by a triazole group were synthesized and evaluated for their effect on quorum sensing (QS) and biofilm formation in *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*. In addition, the influence of the length of the acyl-mimicking chain was investigated. The compounds showed selectivity between two different AHL QS systems. 3-(1*H*-1,2,3-Triazol-1-yl)dihydrofuran-2(3*H*)-ones, in which the 4-substituent best resembled the acyl chain of the native AHL molecule exhibited significant QS agonistic and antagonistic activities. Replacing this aliphatic substituent by a phenyl-containing moiety resulted in active inhibitors of QS. The most active compounds showed biofilm inhibitory as well as biofilm eradicating activities in both test organisms.

1. Introduction

Bacteria monitor their population density using communication systems, referred to as quorum sensing (QS).[1] QS is based on the production and sensing of small signal molecules and typically a QS system consists of three components: signal molecules, signal synthases and signal receptors. At low population density, only low amounts of diffusible signal molecules are produced, provoking no effect. However, when population density reaches a 'quorum', signal molecules will bind to a receptor and this will trigger the induction or repression of the transcription of QS-regulated genes. Several pathogenic bacteria use QS for the control of virulence, biofilm formation and maturation, as well as many other cellular processes.[2,3] Interfering with QS systems has been proposed as a novel strategy to combat bacterial infections.[3,4]

Burkholderia cenocepacia and *Pseudomonas aeruginosa* are important human opportunistic pathogens, affecting amongst others cystic fibrosis patients and patients with impaired lung function.[5,6] Several *N*-acyl-homoserine lactone (AHL)-dependent QS systems have been reported in both species. In *B. cenocepacia*, CepI catalyzes the production of two AHLs, *N*-octanoyl-homoserine lactone (C8-HSL) (in high amounts) and *N*-hexanoyl-homoserine lactone (C6-HSL) (in lower amounts).[7] In addition, *B. cenocepacia* epidemic strains contain a second QS system, CciI/R. The major signal molecule in the latter is C6-HSL.[8] *P. aeruginosa* uses two AHL QS systems, designated LasI/R and RhlI/R. LasI and RhlI are involved in the synthesis of 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-homoserine lactone (C4-HSL), respectively, which are sensed by the LasR and RhlR receptors, respectively.[9] Although, each AHL system controls the regulation of specific genes, overlap and crosstalk exist between the different AHL QS systems in each species.

Several QS inhibitors (QSI) have been described over the last decades. Many studies have focused on developing analogues of the native AHL signal molecule in which the acyl side chain or the lactone moiety was modified.[10] Only a handful studies have focused on alterations of the central amide moiety.[11–13] In the present study, we investigated whether substitution of the central amide by a triazole ring, which may act as a functional, nonclassical bioisostere of an amide moiety, would affect QS in *B. cenocepacia* and *P. aeruginosa*. In addition, the length of the acyl-mimicking chain was varied in order to obtain more active inhibitors. Finally, the biological activity of the most active compounds was assessed by evaluating their effect on biofilms of both species.

2. Results and discussion

2.1. Design and synthesis

3-Azidodihydrofuran-2(3*H*)-one (**2**) was prepared from α-bromo-γ-butyrolactone (**1**) (Scheme 1). The different 3-(1,2,3-triazol-1-yl)dihydrofuran-2(3*H*)-one derivatives **3a–j** were prepared by

* Corresponding author. Tel.: +32 (0) 9 264 80 93; fax: +32 (0) 9 264 81 95.
E-mail address: Gilles.brackman@UGent.be (G. Brackman).

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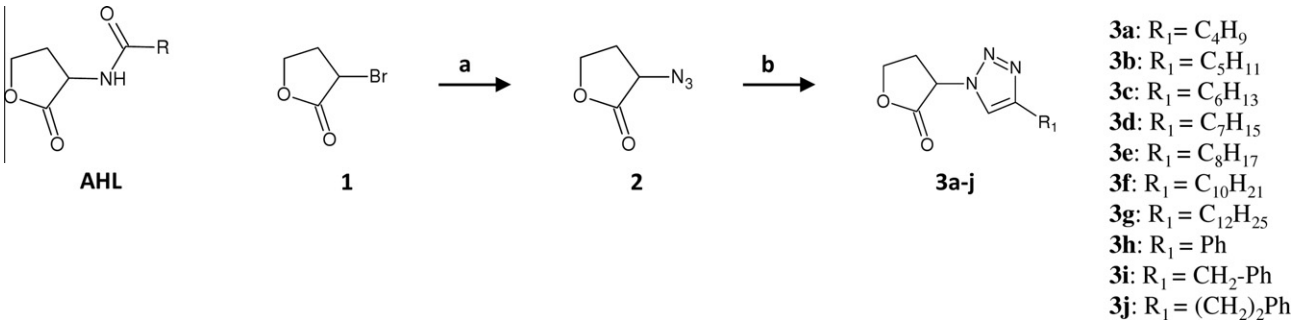

Scheme 1. General structure of an AHL molecule and Preparation of the different substituted triazol-1-yldihydrofuranones **3a–j**. Reagents and conditions: (a) NaN_3, acetone/H_2O, overnight; (b)$CuSO_4$/Na-ascorbate/t-BuOH/H_2O, 18–24 h, rt.

Table 1
MIC values (mM) for the strains tested

Compounds	MIC value for the different compounds (mM)			
	E. coli	*B. cenocepacia*	*P. aeruginosa*	
	JB523	LMG16656	PAO1	QSIS2
3a	5	5	5	5
3b	5	5	5	5
3c	5	5	5	5
3d	5	5	5	5
3e	5	2.5	2.5	2.5
3f	2.5	2.5	2.5	2.5
3g	2.5	2.5	2.5	2.5
3h	10	5	5	5
3i	5	5	5	5
3j	10	5	2.5	5

an Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between **2** and the appropriate alkyne. In the resulting target compounds, the central amide moiety of the AHL molecule is changed to a triazole, which, contrary to the native AHL molecules, will not be prone to microbial acylase degradation.[14] Triazoles have been employed in attempts to generate functional, nonclassical bioisosteres to substitute potentially labile functional groups (e.g., esters, amides) with heterocyclic rings which are able to mimic the same electrostatic potential maps. To probe the optimal sterical and functional requirements for LuxR binding Blackwell and co-workers evaluated a small library of non-natural AHLs, in particular analogues with aromatic groups on the side chains. These studies revealed that phenylpropionyl homoserine lactones and phenoxyacetyl homoserine lactones are significant AHL-based QSIs.[15,16] Also, the effectiveness of aromatic group substitution on the inhibition of the LuxR receptor was previously demonstrated.[12] It was speculated that the aromatic groups in these analogues might interact with aromatic residues in the AHL binding site of the LuxR receptor, possibly inhibiting dimerization.[12]

2.2. AHL QS inhibition

Minimal inhibitory concentrations (MICs) for all compounds were determined (Table 1). In all further experiments, the compounds were used in sub-inhibitory concentrations. The compounds were tested for their ability to decrease C6-HSL induced fluorescence production in an *Escherichia coli* JB523 biosensor strain containing a plasmid that couples *luxR-*P_{luxI} from *Vibrio fischeri* to a GFP reporter system. All compounds displayed a concentration dependent antagonistic activity (Table 2). The compounds were also tested for their ability to inhibit LasR-dependent QS in a *P. aeruginosa* QSIS2 assay. Compounds **3a–e** and **3h** did not display antagonistic activity at any concentration tested (0.1–1 mM) (Table 2). In contrast, **3f**, **3g**, **3i** and **3j** were moderately potent inhibitors of QS in the *P. aeruginosa* QSIS2 biosensor (Table 2). These results indicate that all compounds can inhibit the LuxR

Table 2
QS inhibitory effects on LuxR/LasR based QS

Compd	Concn (mM)	% Inhibition		Compd	Concn (mM)	% Inhibition	
		LuxR	LasR			LuxR	LasR
3a	0.1	12.7 ± 2.7	NS	**3f**	0.1	12.4 ± 2.7	7.0 ± 4.5
	0.25	19.9 ± 0.4	NS		0.25	22.3 ± 3.0	8.1 ± 6.2
	0.5	22.5 ± 4.3	NS		0.5	28.0 ± 8.0	15.0 ± 10.1
	1	27.9 ± 7.3	NS		1	31.9 ± 6.5	21.9 ± 13.3
3b	0.1	9.0 ± 1.4	NS	**3g**	0.1	NS	10.6 ± 1.3
	0.25	21.3 ± 2.0	NS		0.25	17.3 ± 3.4	12.8 ± 9.5
	0.5	27.5 ± 5.1	NS		0.5	23.7 ± 7.5	30.9 ± 13.0
	1	35.1 ± 0.9	NS		1	34.4 ± 10.8	36.5 ± 17.7
3c	0.1	11.6 ± 2.2	NS	**3h**	0.1	NS	NS
	0.25	19.9 ± 0.5	NS		0.25	19.7 ± 4.8	NS
	0.5	23.2 ± 4.1	NS		0.5	24.5 ± 3.2	NS
	1	34.3 ± 8.1	NS		1	26.9 ± 4.9	NS
3d	0.1	13.7 ± 3.9	NS	**3i**	0.1	6.3 ± 3.6	NS
	0.25	21.0 ± 2.0	NS		0.25	21.1 ± 1.6	NS
	0.5	34.6 ± 5.3	NS		0.5	29.6 ± 5.9	NS
	1	39.0 ± 3.2	NS		1	34.4 ± 7.4	49.9 ± 20.1
3e	0.1	34.1 ± 3.8	NS	**3j**	0.1	NS	15.8 ± 6.5
	0.25	41.0 ± 0.4	NS		0.25	19.6 ± 0.4	25.3 ± 8.7
	0.5	59.8 ± 11.3	NS		0.5	25.7 ± 5.4	35.2 ± 1.4
	1	71.6 ± 1.9	NS		1	29.2 ± 8.8	43.2 ± 12.8

The effect is expressed as% inhibition (±standard deviation). NS: not significant (p >0.05; independent sample T-test).

Table 3
QS activating effects on LuxR/LasR based QS

Compd	Concn (mM)	% Activation		Compd	Concn (mM)	% Activation	
		LuxR	LasR			LuxR	LasR
3a	0.1	NS	NS	**3f**	0.1	NS	32.1 ± 8.5
	0.25	NS	NS		0.25	NS	31.4 ± 11.1
	0.5	11.6 ± 4.2	NS		0.5	NS	38.0 ± 15.8
	1	17.3 ± 5.4	NS		1	NS	57.2 ± 19.7
3b	0.1	NS	NS	**3g**	0.1	NS	28.7 ± 9.9
	0.25	5.7 ± 1.5	NS		0.25	NS	32.0 ± 14.6
	0.5	16.3 ± 2.9	NS		0.5	NS	43.8 ± 6.2
	1	25.9 ± 4.2	NS		1	NS	45.5 ± 10.2
3c	0.1	NS	NS	**3h**	0.1	NS	NS
	0.25	10.0 ± 0.8	NS		0.25	NS	NS
	0.5	17.5 ± 3.3	NS		0.5	NS	14.4 ± 10.3
	1	32.7 ± 2.4	NS		1	12.8 ± 0.7	22.2 ± 25.2
3d	0.1	NS	NS	**3i**	0.1	NS	19.1 ± 5.7
	0.25	8.0 ± 2.2	NS		0.25	NS	31.1 ± 6.9
	0.5	12.2 ± 3.4	NS		0.5	NS	39.7 ± 11.1
	1	17.6 ± 2.8	NS		1	NS	67.5 ± 18.5
3e	0.1	NS	NS	**3j**	0.1	NS	39.1 ± 3.6
	0.25	NS	NS		0.25	NS	41.5 ± 5.6
	0.5	5.2 ± 1.2	19.4 ± 7.1		0.5	NS	67.2 ± 13.6
	1	10.6 ± 5.1	29.0 ± 6.0		1	NS	81.4 ± 2.1

The effect is expressed as% activation (±standard deviation). NS: not significant (p >0.05; independent sample T-test).

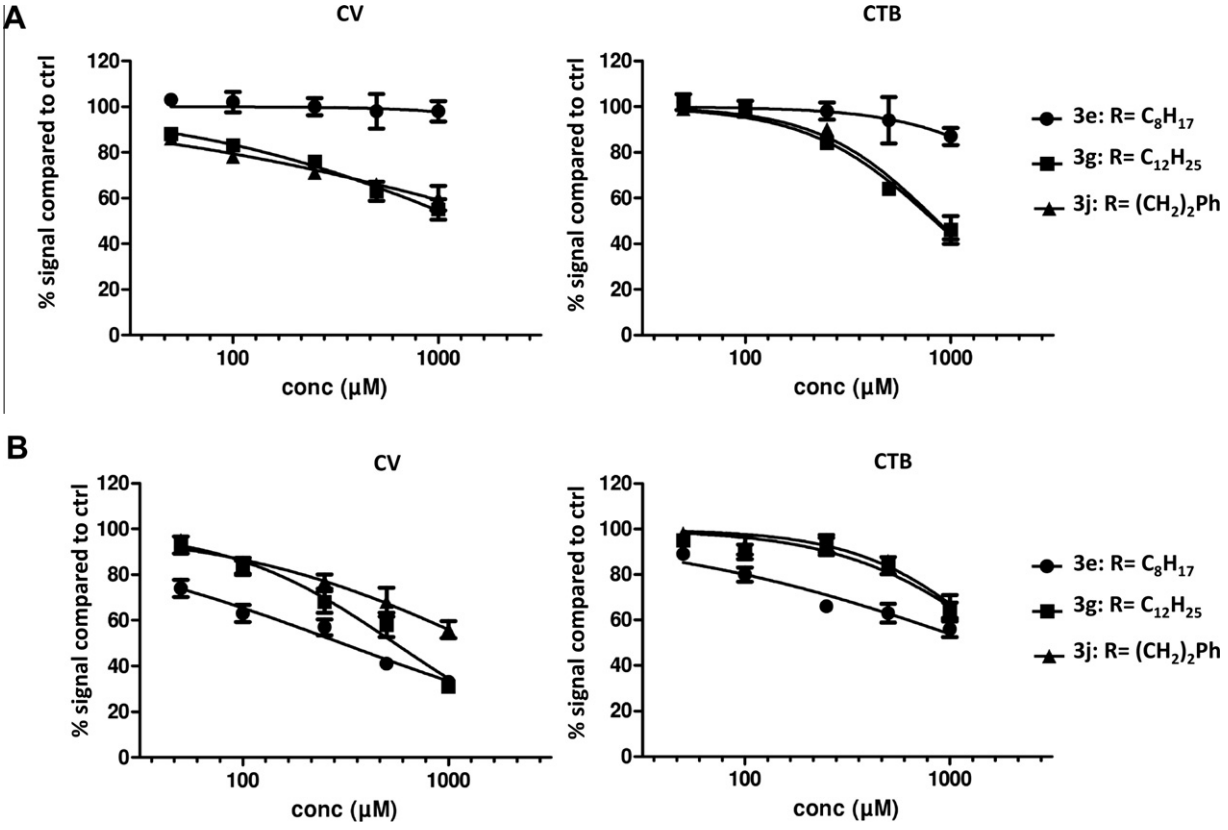

Figure 1. *Effect of triazol-1-yldihydrofuranones on biofilm formation. Pseudomonas aeruginosa* PAO1 (A) and *B. cenocepacia* LMG16656 (B) biofilms were formed in the presence of test compounds at different concentrations. Biomass was quantified by CV staining. Cell-viability was quantified by CTB staining. CV and CTB signals are presented as a percentage compared to 100% control biofilm formed in the absence of compounds.

QS system, while only compounds having a chain length of ten or twelve carbon atoms inhibit the LasR QS system. Although, the phenyl-substituted triazole compounds acted as active antagonists in both biosensors, compounds best resembling the native signal molecule were the most active. However, the IC_{50} value of the most active QS inhibitor (**3e**, IC_{50}: 307.7 ± 1.06 μM) found in this study was higher than IC_{50} values obtained for previously described AHL analogs (4–100 μM).[11–15]

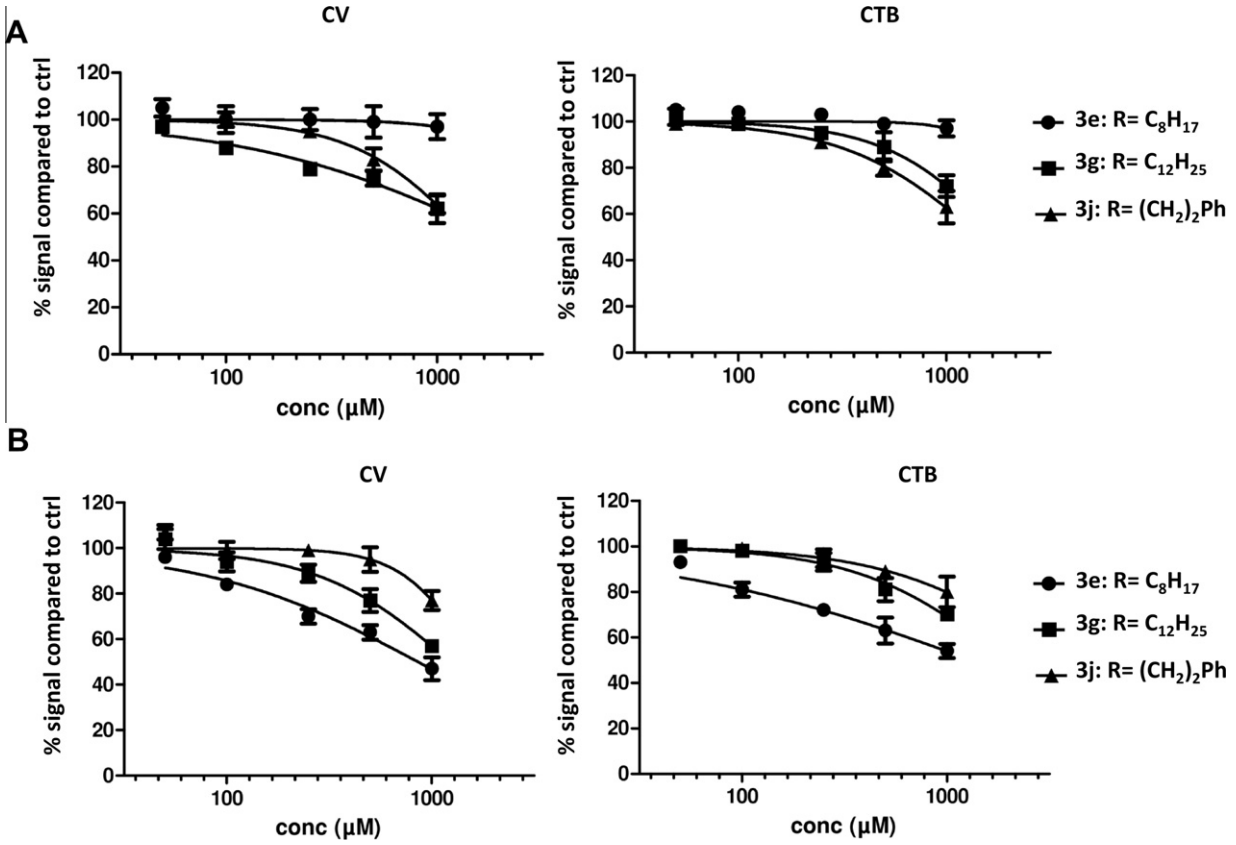

Figure 2. *Effect of triazol-1-yldihydrofuranones on 24 h old biofilms. Pseudomonas aeruginosa* PAO1 (A) and *B. cenocepacia* LMG16656 (B) biofilms were formed for 24 h in the absence of test compounds and subsequently treated with the test compounds for 24 h. Biomass was quantified by CV staining. Cell-viability was quantified by CTB staining. CV and CTB signals are presented as percentages compared to 100% control biofilm formed in the absence of compounds.

2.3. AHL QS activation

The compounds were also tested for their ability to induce QS in *E. coli* JB523 and *P. aeruginosa* QSIS2 biosensor in the absence of the cognate signal molecule. Compounds **3a–e** and **3h** displayed agonistic activities in at least one concentration in the *E. coli* JB523 biosensor (Table 3). In contrast, none of the other compounds activated QS in this biosensor. Compounds **3e–3j** Exhibited moderate agonistic activity in a *P. aeruginosa* QSIS2 biosensor in a concentration dependent way (Table 3). These results indicate that compounds with a relatively short acyl chain (4–8 carbons) act as agonists in a short-acyl chain sensitive biosensor, while compounds with relatively longer acyl chains (**3f–g**) or bulky substituents (**3i–j**) are incapable of activating QS in this biosensor. In contrast, compounds with a longer acyl-chain strongly activated QS in the *P. aeruginosa* QSIS2 biosensor, while compounds with shorter chain length were only moderate activators or were incapable of activating QS in the *P. aeruginosa* QSIS2 biosensor. Our data suggest that these compounds compete with the native AHL signal molecule for a similar binding pocket in the receptor. This indicates that, although a loss of activity is observed when replacing the amide with a triazole, the triazolyllactones are still capable of interacting with the receptor. Similar observations were previously made for sulphonamides and reverse-amide AHL analogues.[11,12]

2.4. Biofilm inhibitory and eradicating activities

A significant decrease in biofilm formation was observed when *B. cenocepacia* LMG16656 and *P. aeruginosa* PAO1 biofilms were formed in the presence of the most active QSI (Fig. 1). The use of **3j** (1 mM) resulted in approximately 40% reduction in biofilm biomass in both strains, while **3g** (1 mM) resulted in a decrease in biofilm biomass of 46 ± 14% and 70 ± 2% in *P. aeruginosa* PAO1 and *B. cenocepacia* LMG16656, respectively, (Fig. 1). Compound **3e** (1 mM) only affected biomass in *B. cenocepacia* LMG16656 (decrease of 68 ± 7%) (Fig. 1). Similar results were obtained using viability staining (Fig. 1). In addition, the compounds were also observed to affect mature biofilms. Compounds **3g** and **3j** (1 mM) yielded in a decrease of *P. aeruginosa* PAO1 biofilm biomass as well as in the number of viable cells (38 ± 19% and 28 ± 15% decrease for **3e** and a 36 ± 12% and 37 ± 22% decrease for **3j**, respectively) (Fig. 2). In addition, the use of three compounds reduced the biofilm biomass and the number of viable cells in *B. cenocepacia* LMG16656 (Fig. 2). These results suggest that the QS-inhibitory activity of our compounds is associated with an antibiofilm activity. This is not surprising since QS was previously shown to regulate biofilm formation in both species.[17–22] Finally an CC_{50} value of 0.67 ± 0.2 mM was found for compound **3g** in MRC-5 cells. In contrast, CC_{50} values for compounds **3e** and **3j** were much higher (>1 mM). This indicates that QS inhibitory effects and biofilm inhibitory and eradicating effects can be obtained using concentrations which are not cytotoxic.

3. Conclusion

Several AHL analogues in which the amide group is replaced by a triazole moiety were synthesized and evaluated for their effect on AHL-QS and biofilm inhibitory and eradicating activities in *B. cenocepacia* and *P. aeruginosa*. All compounds were capable of blocking C6-HSL-based QS, while only the short chain analogues were capable of activating QS in this system. In addition, only long

acyl-chain compounds affected 3-oxo-C12-HSL-based QS. Altering the acyl-side chain by phenyl-compounds resulted in active modulators of QS in both systems. Finally, the most active QSI exhibited strong inhibitory and eradicating activities against *B. cenocepacia* and *P. aeruginosa* biofilms.

4. Materials and methods

4.1. Strains and culture conditions

E. coli JB523[23] was grown aerobically on Luria Bertani (LB) agar (BD, Sparks, MD) supplemented with 100 μg/ml tetracycline and 0.4% NaCl. The QS inhibition selector *P. aeruginosa* QSIS2[24] was cultured aerobically in ABT minimal medium (AB medium, containing 2.5 mg/l of thiamine) supplemented with 0.5% glucose, 0.5% casamino acids and 80 μg/ml gentamicin. *B. cenocepacia* LMG16656 and *P. aeruginosa* PAO1 were cultured aerobically on tryptone soy agar (Oxoid, Hampshire, UK) or in Mueller Hinton medium (Oxoid). All strains were grown at 37 °C, with the exception of *E. coli* JB523 (30 °C).

4.2. General procedure for the synthesis of 2 and 3a–j

4.2.1. 3-Azidodihydrofuran-2(3*H*)-one (2)

α-Bromo-γ-butyrolactone (**1**) (925 μl, 10 mmol, 1 equiv) was dissolved in acetone (17 ml) and a solution of NaN_3 (3.25 g in 7 ml H_2O, 50 mmol, 5 equiv) was added. The resulting solution was stirred overnight. Acetone was removed by evaporation under reduced pressure. The resulting aqueous mixture was extracted with CH_2Cl_2 (2 × 50 mL) and the organic layers were combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The resulting oil was found to be pure by NMR analysis (and was used without further purification). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.42 (dt, 1H, *J* = 3.6 Hz and 8.9 Hz, H-3), 4.23–4.31 (m, 2H, H-3 and H-1), 2.50–2.61 (m, 1H, H-2), 2.10–2.24 (m, 1H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.6, 66.0, 56.8, 29.1

4.2.2. General procedure for the synthesis of 3a–j

To a solution of **2** (1 equiv) in a mixture of t-BuOH:H_2O, a 0.1 M aqueous solution of sodium ascorbate (15 mol %) and a 0.1 M aqueous solution of $CuSO_4$ (10 mol %) was added. To this mixture, 2 equiv of the appropriate alkyne was added and the reaction mixture was stirred at room temperature for 18–24 h. The reaction mixture was diluted with EtOAc and extracted with H_2O. The aqueous phase was extracted with EtOAc and the combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. After purification by column chromatography (CH_2Cl_2:MeOH 98.5:1.3), the desired triazoles **3a–j** were obtained in good yields.

4.2.3. 3a: 3-(4-Butyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.29 (t, 1H, *J* = 9.3 Hz, H-3), 4.64–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 6.9 Hz and 9.0 Hz, H-1), 2.92–3.12 (m, 2H, H-2), 2.74 (t, 2H, *J* = 7.7 Hz, H-4), 1.61–1.72 (m, 2H, H-5), 1.33–1.45 (m, 2H, H-6), 0.93 (t, 3H, *J* = 7.4 Hz, H-7); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 149.3, 120.9, 66.6, 57.7, 31.6, 29.5, 25.6, 22.5, 14.0; ESI-MS for $C_{10}H_{15}N_3O_2$ $[M-H]^+$. Found, 210.1244; Calcd, 210.1237 (Supplementary data).

4.2.4. 3b: 3-(4-Pentyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.30 (t, 1H, *J* = 9.2 Hz, H-3), 4.63–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 6.7 Hz and 9.1 Hz, H-1), 2.91–3.11 (m, 2H, H-2), 2.72 (t, 2H, *J* = 7.6 Hz, H-4), 1.63–1.73 (m, 2H, H-5), 1.31–1.37 (m, 4H, H-6 and H-7), 0.87–0.91 (m, 3H, H-8); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.4, 149.4, 120.9, 66.6, 57.7, 31.6, 29.5, 29.2, 25.8, 22.6, 14.2; ESI-MS for $C_{11}H_{17}N_3O_2$ $[M-H]^+$, Found, 224.1387; Calcd, 224.1394 (Supplementary data).

4.2.5. 3c: 3-(4-Hexyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.30 (t, 1H, *J* = 9.4 Hz, H-3), 4.63–4.70 (m, 1H, H-1), 4.48 (dt, 1H, *J* = 6.8 Hz and 9.0 Hz, H-1), 2.91–3.10 (m, 2H, H-2), 2.72 (t, 2H, *J* = 7.8 Hz, H-4), 1.61–1.71 (m, 2H, H-5), 1.24–1.40 (m, 6H, H-6, H-7 and H-8), 0.87 (t, 3H, *J* = 6.9 Hz, H9); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 149.3, 120.9, 66.5, 57.6, 31.7, 29.5, 29.4, 29.1, 25.8, 22.7, 14.2; ESI-MS for $C_{12}H_{19}N_3O_2$ $[M-H]^+$, Found, 238.1563; Calcd, 238.1550 (Supplementary data).

4.2.6. 3d: 3-(4-Heptyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.30 (t, 1H, *J* = 9.2 Hz, H-3), 4.64–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 6.8 Hz and 9.1 Hz, H-1), 2.91–3.11 (m, 2H, H-2), 2.72 (t, 2H, *J* = 7.8 Hz, H-4), 1.67 (quintet, 2H, *J* = 7.5 Hz, H-5), 1.28–1.38 (m, 8H, H-6, H-7, H-8 and H-9), 0.87 (t, 3H, *J* = 6.9 Hz, H-10); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 149.3, 120.8, 66.5, 57.6, 31.9, 29.5, 29.4, 29.2, 25.8, 22.8, 14.2; ESI-MS for $C_{13}H_{21}N_3O_2$ $[M-H]^+$, Found, 252.1719; Calcd, 252.1707 (Supplementary data).

4.2.7. 3e: 3-(4-Octyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.29 (t, 1H, *J* = 9.2 Hz, H-3), 4.64–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 6.8 Hz and 9.1 Hz, H-1), 2.93–3.12 (m, 2H, H-2), 2.73 (t, 2H, *J* = 7.8 Hz, H-4), 1.67 (quintet, 2H, *J* = 7.5 Hz, H-5), 1.25–1.38 (m, 10H, H-6, H-7, H-8, H-9 and H-10), 0.87 (t, 3H, *J* = 6.8 Hz, H-11); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2, 149.3, 120.8, 66.5, 57.6, 32.0, 29.47, 29.45, 29.44, 29.41, 29.3, 25.8, 22.8, 14.2; ESI-MS for $C_{14}H_{23}N_3O_2$ $[M-H]^+$, Found, 266.1877; Calcd, 266.1863 (Supplementary data).

4.2.8. 3f: 3-(4-Decyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.29 (t, 1H, *J* = 9.2 Hz, H-3), 4.64–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 6.7 Hz and 9.1 Hz, H-1), 2.91–3.12 (m, 2H, H-2), 2.72 (t, 2H, *J* = 7.6 Hz, H-4), 1.67 (quintet, 2H, *J* = 7.5 Hz, H-5), 1.20–1.40 (m, 14H, H-6, H-7, H-8, H-9, H-10, H-11 and H-12), 0.87 (t, 3H, *J* = 6.7 Hz, H-13); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 149.3, 120.8, 66.5, 57.6, 32.0, 29.74, 29.68, 29.51, 29.46, 29.45, 29.44, 29.41, 25.8, 22.8, 14.3; ESI-MS for $C_{16}H_{27}N_3O_2$ $[M-H]^+$, Found, 294.2180; Calcd, 294.2176 (Supplementary data).

4.2.9. 3g: 3-(4-Dodecyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.53 (s, 1H, triazole H), 5.29 (t, 1H, *J* = 9.2 Hz, H-3), 4.64–4.71 (m, 1H, H-1), 4.49 (dt, 1H, *J* = 7.0 Hz and 9.0 Hz, H-1), 2.92–3.12 (m, 2H, H-2), 2.72 (t, 2H, *J* = 7.6 Hz, H-4), 1.67 (quintet, 2H, *J* = 7.4 Hz, H-5), 1.23–1.38 (m, 18H, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13 and H-14), 0.87 (t, 3H, *J* = 6.7 Hz, H-15); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2, 149.3, 120.8, 66.5, 57.6, 32.1, 29.82, 29.80, 29.79, 29.7, 29.52, 29.50, 29.46, 29.45, 29.43, 25.8, 22.8, 14.3; ESI-MS for $C_{18}H_{31}N_3O_2$ $[M-H]^+$, Found, 322.2495; Calcd, 322.2489 (Supplementary data).

4.2.10. 3h: 3-(4-Phenyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.05 (s, 1H, arom. H), 7.81–7.86 (m, 2H, arom. H), 7.30–7.46 (m, 3H, arom. H), 5.39 (t, 1H, *J* = 9.3 Hz,

H-3), 4.66–4.73 (m, 1H, H-1), 4.51 (dt, 1H, J = 6.8 Hz and 9.1 Hz, H-1), 2.96–3.18 (m, 2H, H-2);); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.1, 148.5, 130.2, 129.0, 128.6, 126.0 119.9, 66.5, 57.8, 29.4; ESI-MS for $C_{12}H_{11}N_3O_2$ $[M-H]^+$, Found, 230.0941; Calcd, 230.0924 (Supplementary data).

4.2.11. 3i: 3-(4-Benzyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43 (s, 1H, arom. H), 7.19–7.34 (m, 5H, arom. H), 5.25 (t, 1H, J = 9.3 Hz, H-3), 4.60–4.67 (m, 1H, H-1), 4.45 (dt, 1H, J = 7.0 Hz and 9.0 Hz, H-1), 4.10 (s, 2H, H-4), 2.87–3.07 (m, 2H, H-2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2, 148.5, 138.9, 129.0, 128.9, 126.9, 121.9, 66.5, 57.7, 32.5, 29.5; ESI-MS for $C_{13}H_{13}N_3O_2$ $[M-H]^+$, Found, 244.1091; Calcd, 244.1081 (Supplementary data).

4.2.12. 3j: 3-(4-Phenethyl-1H-1,2,3-triazol-1yl)dihydrofuran-2(3*H*)-one

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40 (s, 1H, arom. H), 7.17–7.32 (m, 5H, arom. H), 5.27 (t, 1H, J = 9.4 Hz, H-3), 4.63–4.70 (m, 1H, H-1), 4.48 (dt, 1H, J = 7.1 Hz and 9.0 Hz, H-1), 2.90–3.10 (m, 6H, H-2, H-4 and H-5); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2, 148.1, 141.1, 128.61, 128.59, 126.3, 121.2, 66.4, 57.6, 35.5, 29.4, 27.6; ESI-MS for $C_{14}H_{15}N_3O_2$ $[M-H]^+$, Found, 258.1255; Calcd, 258.1237 (Supplementary data).

All final compounds are solids. Stock solution were made in DMSO (100 mM) and these were further diluted in MilliQ water before use. All compounds were soluble in DMSO. Solubility problems only occurred when we tried to obtain higher concentrations in water. Estimation of Log$P$, that is, logarithm of Partition Coefficient [n-Octanol/Water],[25] indeed indicate that the value for a triazole analogue is significantly higher than that of the corresponding amide, although the total polar surface area is comparable.

4.3. Determination of the MIC

MICs of the substituted triazolyldihydrofuranones were determined in triplicate according to the EUCAST broth microdilution protocol, using flat-bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland).[26] In brief, the inoculum was standardized to approximately 5×10^5 CFU/ml. The plates were incubated at 37 °C for 20 h, and the optical density at 590 nm was determined by using a multilabel microtiter plate reader (Envision; Perkin-Elmer LAS, Waltham, MA). The lowest concentration of compound for which the optical density was not different from that in the uninoculated control wells was recorded as the MIC.

4.4. QS inhibition/activation assays

E. coli JB523 contains the pJBA130 plasmid (expressing green fluorescent protein GFP in response to C6-HSL).[23] The *E. coli* JB523 QS assay was carried out as described previously.[18] In brief, an overnight culture of the reporter strain was diluted in fresh sterile LB medium containing 0.4% (w/v) NaCl to an OD_{590nm} = 0.1 and 100 μl of this cell suspension was added to the wells of a black 96-well MTP (Perkin-Elmer). Tetracycline was added in a final concentration of 100 μg/ml. Activation of QS was tested by addition of C6-HSL (5 μM) (Sigma) as a control or the substituted triazol-dihydrofuranones (100–1000 μM). Sterile MilliQ water served as a negative control. To study inhibition using *E. coli* JB523, compounds were added together with C6-HSL. The MTP was then incubated for 24 h at 30 °C and fluorescence from GFP expression was measured (excitation and emission wavelength of 485 and 510 nm, respectively) using a multilabel microtiter plate reader (Envision). The QS inhibition selector strain *QSIS2* was a *P. aeruginosa lasI rhlI* double mutant harboring pLasB-SacB1 encoding an AHL-inducible killing system. The *QSIS2* QS assay was conducted as previously described.[18] In brief, an overnight culture of the reporter strain was diluted in fresh sterile ABT medium to an OD_{590nm} = 0.1 and 50 μl of this cell suspension was added to the wells of a 96-well MTP. Fifty microliter LB supplemented with sucrose (56 mg/ml) was added to each well. Gentamicin was added in a concentration of 80 μg/ml. Activation of QS was tested by addition of 3-oxo-C12-HSL and C4-HSL (Sigma) (200 nm each) or the test compounds (100–1000 μM) to the wells. Sterile MilliQ water served as a negative control. To study inhibition, test compounds were added in 100–1000 μM concentrations together with the AHL compounds. The MTP was then incubated for 9 h at 37 °C and absorbance was measured at 450 nm using a multilabel microtiter plate reader (Envision). The difference in growth of the negative control (without addition of compound and without AHL) and that of the positive control (without addition of compound and with AHL) was set at 100%. Each compound was tested twelve times in each assay and each assay was repeated at least three times ($n \geqslant 36$).

4.5. Biofilm inhibitory and eradicating activities

Biofilms were formed as previously described[20] In brief, strains were grown overnight in MH, centrifuged, resuspended in double-concentrated MH (2 × MH) and diluted to an OD_{590nm} = 0.1 in 2 × MH. Fifty microliter of the diluted bacterial suspension was transferred to the wells of a round-bottom 96-well microtiter plate (TPP). Negative controls received 50 μl MilliQ. Positive controls received 50 μl of the test compound. Bacteria were allowed to adhere and grow without agitation for 4 h at 37 °C. After 4 h, plates were emptied and washed with sterile physiological saline (0.9% NaCl; PS). After this washing step, negative control wells were filled with 50 μl 2 × MH and 50 μl MilliQ. Other wells were filled with 50 μl 2 × MH and 50 μl of the test compounds and the plate was incubated for 20 h at 37 °C. To evaluate the effect on mature biofilms, control biofilms were formed in the absence of test compounds, as described above. After 24 h of biofilm formation, the medium was removed, the wells were rinsed with PS and fresh medium containing the compounds was added to the wells. The plates were then incubated for an additional 24 h at 37 °C. After biofilm formation or treatment of mature biofilms, the biomass was quantified by crystal violet (CV) staining, as described previously.[27] In brief, plates were rinsed with sterile PS, biofilms were fixed by adding 100 μl 99% methanol for 15 min, after which the methanol was removed and plates were air-dried. Biofilms were then stained with 100 μl CV (Pro-lab Diagnostics, Richmond Hill, ON, Canada). After 20 min, CV was removed and wells were filled with 150 μl 33% acetic acid (Sigma). The absorbance was measured at 590 nm using a multilabel microtiter plate reader (Envision). Quantification of the number of metabolically active cells in the biofilm was carried out using a resazurin-based assay.[27] In brief, wells were rinsed after 24 h biofilm formation and 100 μl PS was added, followed by the addition of 20 μl CellTiter-Blue (CTB) solution (Promega, Leiden, The Netherlands). After 60 min, fluorescence (excitation and emission filters of 535 and 590 nm) was measured using a multilabel microtiter plate reader (Envision). Each compound was tested twelve times in each assay and each assay was repeated at least two times ($n \geqslant 24$).

4.6. Investigation of the cytotoxicity

Cytotoxicity was tested on human simian virus 40-immortalized lung fibroblasts (MRC-5 SV_2 cells; European Collection of Cell Cultures, United Kingdom). MRC-5 cells were cultured in Earl's MEM (Gibco, UK) +5% FCSi. Assays were performed in 96-well microtiter plates, each well containing about 10^4 cells. After 3 days

incubation at 37 °C with 5% CO_2, cell viability was assessed fluorimetrically after addition of resazurin (excitation and emission wavelength of 550 and 590 nm, respectively). The results are expressed as% reduction in cell growth/viability compared to untreated control wells and a CC_{50} is determined.

4.7. Statistics

The normal distribution of the data was checked using the Shapiro–Wilk test. Normally distributed data were analyzed using an independent sample *T*-test. Statistical analyses were carried out using SPSS software, version 19.0 (SPSS, Chicago, IL, USA).

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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.bmc.2012.06.009. These data include MOL files and InChiKeys of the most important compounds described in this article.

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