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Structure–activity relationship of 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts and 2*N*-substituted 4(5)-aryl-2-amino-1*H*-imidazoles as inhibitors of biofilm formation by *Salmonella* Typhimurium and *Pseudomonas aeruginosa*

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ABSTRACT

A library of 80 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts and 54 2*N*-substituted 4(5)-aryl-2-amino-1*H*-imidazoles was synthesized and tested for the antagonistic effect against biofilm formation by *Salmonella* Typhimurium and *Pseudomonas aeruginosa*. The nature of the substituent at the 1-position of the salts was found to have a major effect on their biofilm inhibitory activity. Salts with an intermediate length *n*-alkyl or cyclo-alkyl chain (C7–C10) substituted at the 1-position in general prevented the biofilm formation of both species at low micromolar concentrations, while salts with a shorter *n*-alkyl or cyclo-alkyl chain (C1–C5) or longer *n*-alkyl chain (C11–C14) were much less potent. Salts with a long cyclo-alkyl chain however were found to be strong biofilm inhibitors. Furthermore, we demonstrated the biofilm inhibitory potential of salts with certain aromatic substituents at the 1-position, such as piperonyl or 3-methoxyphenetyl. The activity of the 2-aminomidazoles was found to be dependent on the nature of the 2*N*-substituent. Compounds with a *n*-butyl, *iso*-butyl, *n*-pentyl, cyclopentyl or *n*-hexyl chain at the 2*N*-position have an improved activity as compared to their unsubstituted counterparts, whereas compounds with shorter 2*N*-alkyl chains do have a reduced activity and compounds with longer 2*N*-alkyl chains do have an effect that is dependent on the nature of the substitution pattern of the 4(5)-phenyl ring. Finally, we demonstrated that introduction of a 3-methoxyphenethyl or piperonyl group at the 2*N*-position of the imidazoles could also result in an enhanced biofilm inhibition.

1. Introduction

Bacteria are able to switch between a planktonic life style and a biofilm mode of growth, in which they live as structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.[1–3] As bacteria in biofilms are more resistant to challenges from predators, antibiotics, disinfectants and host immune systems,[2,4–8] serious problems and high costs have been associated with biofilms, both in medicine and industrial settings. According to the National Institutes of Health, more than 80% of microbial infections are related to biofilms.[9] Especially the use of indwelling medical devices comprises a high risk for the development of biofilm related infections.[2] This high prevalence of biofilm related infections is particularly problematic given the fact that bacteria in biofilms can be up to 1000-fold more resistant to antibiotics.[10,11] In industry, biofilms have been implicated, for example, in the contamination of installations in food industry, mild steel corrosion, decreased passage through pipelines by colonization of the interior of the pipes, and enhanced resistance of vessels by initiation of 'biofouling' on the vessel hulls. The yearly economic loss caused by 'biofouling' in the marine industry is estimated at $ 6.5 billion.[12]

One way to deal with the problem of biofilms is the development of small molecules that are able to prevent or destroy biofilm formation.[13–15] Only a few molecular scaffolds have been identified to date, among which the best-studied examples are (1) the halogenated furanones, which were originally isolated from the seaweed *Delisea pulchra*,[16–18] (2) analogues of the homoserine

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lactone signalling molecules[19] and (3) analogues of the sponge-derived marine natural alkaloids oroidin and bromoageliferin.[20–27] A particularly valuable approach is the development of small molecules that specifically target the biofilm formation in a non-toxic manner, as it is expected that resistance against these compounds will emerge much slower than against classical microbiocidal compounds. As a consequence, non-toxic biofilm inhibitors have the potential to be used in a preventive treatment of a wide diversity of industrial and medical surfaces. Furthermore, the potential to co-dose biofilm inhibitors and antibiotics for the treatment of biofilm infections is also an attractive option.[17]

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) and *Pseudomonas aeruginosa* are two well studied organisms in terms of biofilm formation. *Salmonella enterica* is worldwide one of the most important causes of foodborne infections. *Salmonella* is able to form biofilms (as reviewed by Steenackers et al.[28]) on a variety of both biotic surfaces (such as gallstones,[29] plant surfaces,[30] and epithelial cell layers[31]) and abiotic surfaces (such as concrete, plastics, glass, polystyrene, ...).[32,33] These biofilms are an important survival strategy in all stages of infection, from transmission to chronic infection. Severe non-typhoid *Salmonella* infections are commonly treated with fluoroquinolones and third-generation cephalosporins. Unfortunately, there are alarming reports concerning the development of resistance against these antibiotics,[34] underlining the urgent need of alternative anti-*Salmonella* strategies. Given the importance of biofilms in the spread of *Salmonella*, the development of *Salmonella* biofilm inhibitors seems a promising approach. *P. aeruginosa* is an ubiquitous Gram-negative microorganism found in many environments, such as soil and water. *P. aeruginosa* is also an opportunistic pathogen implicated in a myriad of infections. Patients with compromised host defenses, such as burn patients, HIV patients and cystic fibrosis patients (80% colonization rate of *P. aeruginosa*[35]) are particularly susceptible to *P. aeruginosa* infections.[36] *P. aeruginosa* biofilms have been related to recurrent ear infections, chronical bacterial prostatitis and lung infections in CF patients, the latter being extremely harmful as *P. aeruginosa* colonization and chronic lung infection is the major causative agent of morbidity and mortality in CF patients.[37–39] Moreover, *P. aeruginosa* can colonize as biofilms a variety of medical devices such as intravascular catheters and urinary catheters. Obviously there is an urgent need of agents that can prevent or eradicate *P. aeruginosa* biofilms on infected tissues and on medical devices.

Previously, we published a divergent synthesis of substituted 2-aminoimidazoles from 2-aminopyrimidines and α-bromocarbonyl compounds.[40,41] As described in Scheme 1, the cleavage of 1,2,3-trisubstituted imidazo[1,2-*a*]pyrimidinium salts with hydrazine or secondary amine leads to 1,4,5-trisubstituted 2-aminoimidazoles (pathway A), while the hydrazinolysis of 2-hydroxy-2,3-dihydro-imidazopyrimidinium salts results in formation of 2*N*-substituted 2-amino-1*H*-imidazoles (pathway B). Recently, we reported on the biofilm inhibitory activity of *N*1-substituted 5-phenyl-2-aminoimidazoles and their precursor imidazo[1,2-*a*]pyrimidinium salts,[42] which were synthesized via pathway A. A good correlation was found between the activity of the imidazo[1,2-*a*]pyrimidinium salts and their corresponding 2-aminoimidazoles, supporting the hypothesis that the imidazo[1,2-*a*]pyrimidinium salts could also in situ be cleaved by cellular nucleophiles to form the active 1-substituted 2-aminoimidazoles. In the same report we described the biofilm inhibitory activity of para-substituted 4(5)-phenyl-2-amino-1*H*-imidazoles and 4,5-distubstituted 2-amino-1*H*-imidazoles, which were synthesized via a different procedure.[42,43] In the present study, we focus on the biofilm inhibitory activity of compounds synthesized via pathway B of Scheme 1. We tested the influence of a series of 2-hydroxy-2-phenyl-2,3-dihydro-imidazopyrimidinium salts with alkyl, cyclo-alkyl and aromatic substituents at the 1-position and their corresponding 2*N*-substituted 2-aminoimidazoles on the biofilm formation of *Salmonella enterica* serovar Typhimurium and *P. aeruginosa* and we delineated a structure–activity relationship.

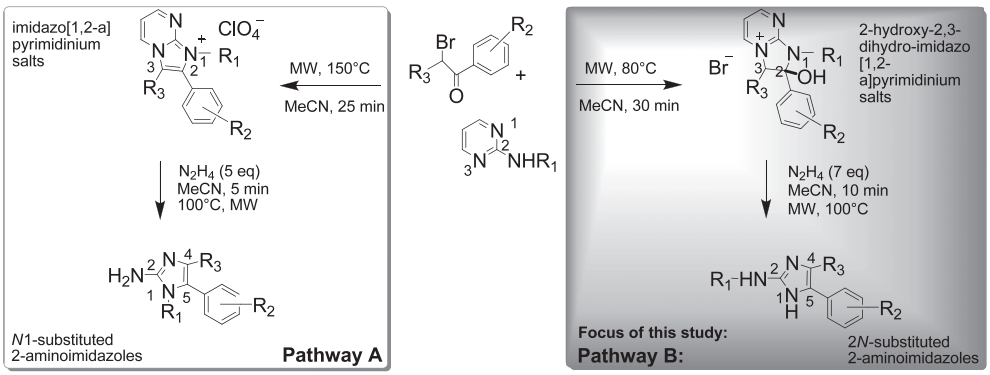
2. Results and discussion

2.1. 2-Hydroxy-2,3-dihydro-imidazopyrimidinium salts

A broad array of 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts were synthesized via pathway B of Scheme 1 and their ability to prevent the biofilm formation of *S.* Typhimurium and *P. aeruginosa* was tested. These molecules differ in the substitution pattern of the 2-phenyl ring and in the nature of the substituent at the 1-position, that is, *n*-alkyl, *iso*-alkyl, cyclo-alkyl or aromatic.

2.1.1. *n*-Alkyl or *iso*-alkyl substituents at 1-position

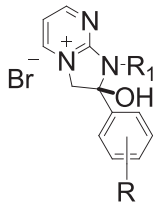
In first instance, a series of 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts with a broad variety of *n*-alkyl or *iso*-alkyl substituents at the 1-position, with lengths ranging from one carbon atom to 14 carbon atoms, was synthesized. The 2-phenyl group of these salts is either unsubstituted (compounds **1**–**14**), *para*-substituted with a chlorine atom (compounds **15**–**27**), a nitro group (compounds **28**–**36**), a fluorine atom (compounds **37**–**40**), a bromine atom (compounds **41**–**43**) or a methoxy group (compounds **45**–**47**), *meta*-substituted with a nitro group (Compound **44**) or 3,4-disubstituted with chlorine atoms (compounds **48**–**50**) (Table 1). Each compound was assayed for



Scheme 1. Divergent synthesis of substituted 2-aminoimidazoles from 2-aminopyrimidines and α-bromocarbonyl compounds.[41] (A) Synthesis of *N*1-substituted 2-aminoimidazoles from 2-aminopyrimidines via 1-substituted imidazo[1,2-*a*]pyrimidinium salts. (B) Synthesis of 2*N*-substituted 2-aminoimidazoles from 2-aminopyrimidines via 1-substituted 2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts.

the ability to inhibit *S.* Typhimurium ATCC14028 biofilm formation at 25 °C. As depicted in Figure 1A, a clear correlation was found between the length of the alkyl substituent and the biofilm inhibitory activity. In general the activity of the molecules with a short alkyl chain (C1, C2 and C3) was found to be very low (IC_{50} >400 μM). Within a series of molecules with the same substitution of the 2-phenyl ring, in general a gradual increase in biofilm inhibitory activity was observed by raising the length of the alkyl chain from

Table 1
Influence of 1-alkylated 2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts **1**–**50** on the biofilm formation of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



Compound	R1	R	*S.* Typhimurium				*P. aeruginosa*			
			IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]		IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]	
					IC_{50}[a]	40 μM			IC_{50}[a]	40 μM
1	Me	H	>800				>400			
2	*i*-Pr	H	540.5	420.4–695.0			>400			
3	*n*-Bu	H	409.5	348.4–481.3			>400			
4	*i*-Bu	H	131.2	64.0–268.9			289.4	148.3–564.6		
5	*n*-Pen	H	278.2	197.4–392.2			565.4	269.6–1186.0		
6	*n*-Hex	H	102.0	77.9–133.7			405.8	183.5–897.4		
7	*n*-Hep	H	23.4	19.6–27.8	o		25.5	16.5–39.4	+	
8	*n*-Oct	H	9.8	8.3–11.5	o		14.4	10.3–20.3	+	
9	*n*-Non	H	8.3	7.2–9.5	o		26.8	16.0–44.9	o	
10	*n*-Dec	H	15.0	13.4–16.8	o		29.9	20.1–44.4	o	
11	*n*-Und	H	24.6	20.6–29.4	o	o	>800[d]			
12	*n*-Dod	H	27.5	21.5–35.2	o	o	>800[d]			
13	*n*-Trd	H	81.6	70.5–94.6			>800[d]			
14	*n*-Tet	H	179.6	142.3–226.8			>800[d]			
15	Me	4-Cl	225.0	176.1–287.5			169.6	34.5–834.0		
16	*n*-Bu	4-Cl	29.7	23.6–37.3	–	–	24.9	15.9–39.1	–	–
17	*i*-Bu	4-Cl	31.4	21.1–46.6	–	–	83.4	60.2–115.6		
18	*n*-Pen	4-Cl	32.6	27.0–39.3	–	–	163.9	105.7–254.2	o	
19	*n*-Hex	4-Cl	13.7	11.5–16.3			79.8	29.9–213.0		
20	*n*-Hep	4-Cl	6.7	5.7–7.7			36.9	24.2–56.4		
21	*n*-Oct	4-Cl	7.0	6.1–8.0	o		15.7	10.6–23.3	o	
22	*n*-Non	4-Cl	18.3	15.3–22.0	o		20.2	13.5–30.2	o	
23	*n*-Dec	4-Cl	51.3	42.4–62.2	o		83.3	50.1–138.3	o	
24	*n*-Und	4-Cl	61.9	50.7–75.4			>800[d]			
25	*n*-Dod	4-Cl	122.1	101.7–146.5			>800			
26	*n*-Trd	4-Cl	316.1	240.9–414.7			>800			
27	*n*-Tet	4-Cl	>800				>800			
28	Me	4-NO_2	698.3	446.1–1093.0			>400			
29	Et	4-NO_2	578.8	432.9–774.0			>800			
30	*i*-Pr	4-NO_2	466.5	375.4–579.8			191.9	91.1–404.2		
31	*n*-Bu	4-NO_2	292.8	220.7–388.3			145.2	54.2–388.6		
32	*i*-Bu	4-NO_2	322.5	252.1–412.5			191.7	102.8–357.5		
33	*t*-Bu	4-NO_2	211.1	162.2–274.7			41.5	29.1–59.2		
34	*n*-Hex	4-NO_2	54.7	41.1–72.8	+		12.4	9.6–16.0	+	
35	*n*-Oct	4-NO_2	6.6	5.5–7.968	+		12.1	8.3–17.6	+	
36	*n*-Dec	4-NO_2	9.9	8.056–12.3	o		22.3	17.4–28.5	o	
37	Me	4-F	1825.0	526.1–6328.0			nd[c]			
38	*n*-Bu	4-F	163.0	136.8–194.2			nd			
39	*n*-Oct	4-F	7.6	6.334–9.058			nd			
40	*n*-Dec	4-F	20.0	14.9–27.0			nd			
41	*n*-Bu	4-Br	90.1	76.2–106.7			nd			
42	*i*-Bu	4-Br	5.2	3.2–8.6	–	+	6.3	3.7–10.6	–	–
43	*n*-Pen	4-Br	1.7	1.1–2.5	–	–	4.2	2.4–7.5	–	–
44	Me	3-NO_2	563.8	470.3–675.9			nd			
45	*n*-Bu	4-OMe	314.4	226.5–436.3			nd			
46	*n*-Oct	4-OMe	10.5	8.2–13.6			nd			
47	*n*-Dec	4-OMe	18.6	14.5–23.8	o		nd			
48	*i*-Bu	3,4-diCl	8.0	6.4–10.0	–	o	nd			
49	*n*-Oct	3,4-diCl	7.1	6.2–8.2			nd			
50	*n*-Dec	3,4-diCl	32.4	21.0–50.1			nd			

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.
[c] nd: not determined.
[d] Although IC_{50} >800 μM, the compound inhibits biofilm formation to a certain extent, but the dose response curve reaches a steady state level of 25–45% biofilm inhibition starting from concentrations between 25 and 50 μM.

1 to 8 carbon atoms. The compounds with an octyl substituent show a maximal activity with IC_{50} values in the range of 6–11 μM. By further raising the alkyl chain length from 8 to 14 carbon atoms, a gradual decrease in biofilm inhibitory activity was observed. For the molecules with a heptyl, octyl, nonyl and decyl side chain (which are the most active molecules), the nature of the substituent of the 2-phenyl group does not have a substantial effect on the biofilm inhibitory activity.

Next, we assayed the influence of a subset of the 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts (compounds **1**–**36**) for their ability to prevent the biofilm formation of *P. aeruginosa*. As represented in Table 1 and Figure 1B, a similar structure–activity relationship was found as in the case of *Salmonella* biofilm inhibition. Compounds with short alkyl substituents (C1–C6) in general only have a slight effect on the biofilm formation (IC_{50}'s >80 μM), while compounds with medium length side chains (C7–C10) have IC_{50} values between 20 and 40 μM and compounds with long side chains (C11–C14) have IC_{50} values higher than 800 μM. However, it should be mentioned that some of the compounds with long side chain (**11**–**14** and **24**) do reduce the biofilm formation to a certain extent, but their dose response curves reach a steady state level of 25–45% biofilm inhibition starting from concentrations between 25 and 50 μM.

Since the compounds with a *n*-octyl chain substituted at the 1-position have the highest activity against *Salmonella* biofilms and also have a high activity against *Pseudomonas* biofilms, we decided to synthesize the additional 1-octyl-2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts **51**–**56** with more variation in the substitution pattern of the 2-phenyl ring. As depicted in Table 2 these compounds generally inhibit biofilm formation of *S.* Typhimurium at low concentrations. However, no improved activity was observed in comparison with the previously described compounds (Table 1). The effect of the *n*-octyl substituted compounds **51**–**56** (Table 2) on the biofilm formation of *P. aeruginosa* strongly depends on the substitution pattern of the 2-phenyl ring, as some of the compounds (**51**, **52** and **56**) have low IC_{50} values (10–40 μM), while the other compounds only have a moderate activity (IC_{50} = 100–400 μM).

Finally, we determined the influence of some of the most active compounds on the planktonic growth of *S.* Typhimurium and *P. aeruginosa* in function of time. As depicted in Table 1, the tested compounds with a butyl or pentyl chain at the 1-position do not reduce the planktonic growth at concentrations equal to the IC_{50} for biofilm inhibition (except for compound **18**). This demonstrates the potential of the class of the 2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts as specific inhibitors of biofilm formation. However, all the tested compounds with alkyl side chains between 6 and 12 carbon atoms in length were found to strongly reduce or completely prevent the planktonic growth at concentrations equal to the IC_{50} for biofilm inhibition. This indicates that these compounds are more toxic and are likely to prevent biofilm formation by inhibiting the planktonic growth of the bacteria in the growth medium around the surface on which the biofilms is formed, that is, being toxic for planktonic cells.

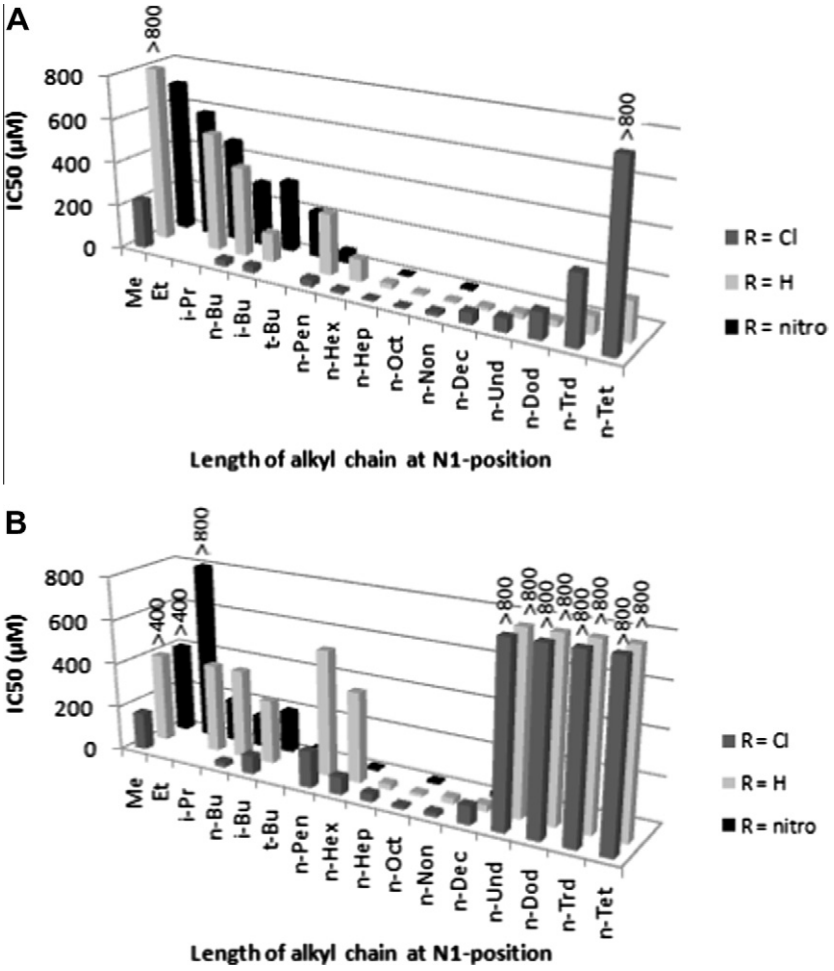
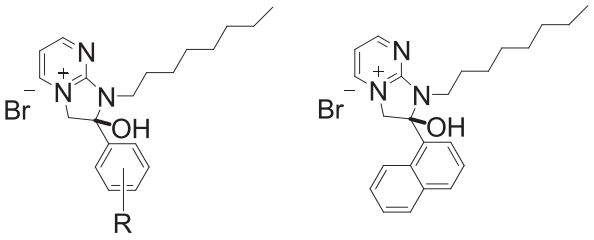


Figure 1. Effect of the length of the *n*-alkyl or iso-alkyl chain at the 1-position of 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts, substituted with Cl, H or nitro at the *para*-position of the 2-phenyl ring, on the IC_{50} (μM) for inhibition of biofilm formation of *S.* Typhimurium ATCC14028 (Graph **A**) and *P. aeruginosa* (Graph **B**).

2.1.2. Cyclo-alkyl substituents at 1-position

We synthesized and tested a series of 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts with a broad variety of cyclo-alkyl substituents at the 1-position, with lengths ranging from 3 to 12 carbon atoms (Table 3). We also tested one compound with an adamantyl group substituted at the 1-position (compound **70**). The 2-phenyl group of these salts was either unsubstituted (compounds **57**–**60**), or *para*-substituted with a chlorine atom (compounds **61**–**67**) or a nitro group (compounds **68**–**70**). Similarly to the structure–activity relationship delineated for the *n*-alkyl substituted salts, a gradual increase in the inhibitory activity against *Salmonella* biofilms was observed by raising the length of the cyclo-alkyl chain from 3 to 8 carbon atoms. However, in contrast to the salts with a *n*-dodecyl chain at the 1-position, the salts with a cyclo-dodecyl chain do have a very strong effect against *Salmonella* biofilms (IC_{50} values <6.25 μM). Also the adamantyl substituted compound **70** has a strong anti-*Salmonella* biofilm activity (IC_{50} = 9 μM). By analogy with the effect on

Table 2
Influence of *N*1-octyl 2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts **51**–**56** on the biofilm formation of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



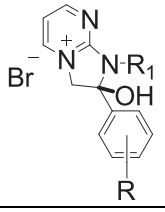
51 and **53-56**

52

Compound	R	*S.* Typhimurium		*P. aeruginosa*	
		IC_{50}[a] (μM)	95% confidence interval for IC_{50}	IC_{50}[a] (μM)	95% confidence interval for IC_{50}
51	4-SMe	~13.2[b]		36.5	30.1–44.3
52		14.7	13.4–16.2	15.1	10.2–22.2
53	4-(4′-NO_2Ph)	31	27.1–35.5	215.2	82.1–564.0
54	4-SO_2Me	60.3	42.9–84.7	99.9	66.8–149.4
55	4-(4′-Biphenyl)	~52.8		336.1	217.8–518.6
56	3-Br	7.1	6.2–8.2	29.0	20.6–40.8

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] ~: the IC_{50} value could not be determined accurately due to the steepness of the dose response curve.

Table 3
Influence of 1-cyclo-alkyl-2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts **57**–**70** on the biofilm formation of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



Compound	R1	R	*S.* Typhimurium				*P. aeruginosa*			
			IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]		IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]	
					IC_{50}[a]	80 μM			IC_{50}[a]	80 μM
57	*c*-Bu	H	493.0	352.7–689.1			>400			
58	*c*-Hex	H	139.0	100.6–192.0	+		22.4	10.5–47.9	–	+
59	*c*-Hep	H	84.2	72.8–97.3	–		191.3	83.9–436.5	–	
60	*c*-Oct	H	58.4	42.9–79.5	o	o	48.6	22.4–105.5	o	o
61	*c*-Pr	4-Cl	82.7	65.0–105.2			179.2	90.3–355.8		
62	*c*-Bu	4-Cl	120.8	94.1–155.2			371.5	273.4–504.6		
63	*c*-Pen	4-Cl	41.8	30.2–57.8	+	+	41.9	23.0–76.2	+	+
64	*c*-Hex	4-Cl	33.0	26.9–40.48	+	o	16.4	9.2–29.43	–	o
65	*c*-Hep	4-Cl	27.9	22.7–34.25	o		76.2	37.9–153.0	o	o
66	*c*-Oct	4-Cl	12.6	10.9–14.68	o		~22.7[c]		+	
67	*c*-Dod	4-Cl	5.6	4.8–6.715	o		6.8	5.6–8.2	o	
68	*c*-Pr	4-NO_2	125.1	88.5–177.0			20.4	10.6–39.0		
69	*c*-Dod	4-NO_2	<6.3		o		5.4	3.9–7.4	o	
70	Adamantyl	4-NO_2	8.8	6.8–11.4			14.4	10.4–20.1		

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.
[c] ~: the IC_{50} value could not be determined accurately due to the steepness of the dose response curve.

Salmonella, we observed that all the cyclo-alkyl substituted salts with a short side chain do only have a slight effect on the biofilm formation of *P. aeruginosa* (IC_{50}'s >150 μM), while the compounds with a medium length side chain have a stronger biofilm inhibitory activity. The salts with a cyclo-dodecyl chain do drastically reduce the *Pseudomonas* biofilm formation (IC_{50}'s ~7 μM), in sharp contrast to the compounds with a *n*-dodecyl side chain. Growth curve analysis revealed that some of the compounds have a specific effect on biofilm formation as they do not affect the planktonic growth at the IC_{50} for biofilm formation, that is, compound **59** in case of *Salmonella* biofilm inhibition and compounds **58**, **59** and **64** in case of *Pseudomonas* biofilm inhibition (Table 3). The other compounds tested do strongly decrease the planktonic growth of the bacteria at the IC_{50} for biofilm inhibition, which indicates that we cannot exclude the possibility that (part) of the biofilm inhibitory activity of the compounds is a consequence of the reduction of the amount of cells in the growth medium around the biofilm substrate.

2.1.3. Aromatic substituents at the 1-position

Finally, we synthesized and tested an array of 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts, which are substituted at the 1-position with a benzyl (compounds **71**–**73**), *para*-methoxybenzyl (compound **74**), 3-methoxyphenethyl (compounds **75**–**78**) or piperonyl group (compounds **79** and **80**) (Table 4). The compounds with a benzyl and *para*-methoxybenzyl substituent only inhibit the biofilm formation of *Salmonella* moderately (IC_{50} values between 36 and 212 μM), while the activity of the compounds with a 3-methoxyphenetyl group is dependent on the nature of the substitution pattern of the 2-phenyl ring. Indeed, compounds **75** and **76**, bearing respectively a *para*-nitro and *para*-fluoro substituted 2-phenyl ring, inhibit *Salmonella* biofilm formation only at moderate concentrations, while compounds **77** and **78**, bearing respectively a 3-dichlorobenzyl group and a [1,1′:4′,1″-terphenyl]-4-yl group at the 2-position have a strong activity (IC_{50}'s 7–12 μM). Compounds **79** and **80**, substituted with a piperonyl ring at the 2-position inhibit *Salmonella* biofilm formation at low to moderate concentrations (IC_{50}'s resp. 17 and 53 μM). Growth curve analysis revealed that compounds **73**, **79** and **80** do not affect the plantkonic growth of *Salmonella* at concentrations higher than the IC_{50} of biofilm inhibition. However, the most active compounds **77** and **78** have a toxic effect on *Salmonella* at concentrations equal to the IC_{50} for biofilm inhibition, implicating that we cannot exclude the possibility that part of the inhibitory effect against *Salmonella* biofilms is due to a reduction of the planktonic growth in the growth medium surrounding the surface on which the biofilms are formed.

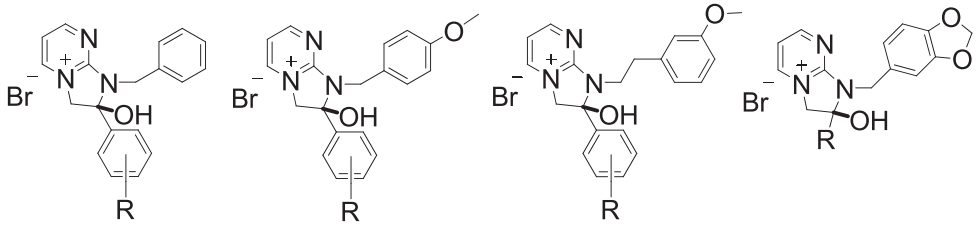
2.2. 2*N*-substituted 2-aminoimidazoles

In a previous publication, the biofilm inhibitory activity of the class of the 2*N*-unsubstituted 4(5)-aryl-2-amino-1*H*-imidazoles was described.[42] We also reported that introduction of a medium length *n*-alkyl or cyclo-alkyl chain at the *N*1-position of these imidazoles could drastically enhance the biofilm inhibitory activity. Also introduction of a benzyl or piperonyl group at the *N*1-position was described to enhance the biofilm inhibitory activity, although this effect is strongly dependent on the nature of the substitution pattern of the 4(5)-phenyl ring. These *N*1-substituted 2-aminoimidazoles were synthesized via synthesis pathway A of Scheme 1. In the present study, we aimed to investigate whether introduction of a *n*-alkyl, *iso*-alkyl, cyclo-alkyl or aromatic group at the 2*N*-position of the 4(5)-aryl-2-amino-1*H*-imidazoles could also enhance their biofilm inhibitory activity. Therefore a broad range of 2*N*-substituted 4(5)-aryl-2-amino-1*H*-imidazoles were synthesized by using pathway B (Scheme 1) and their activity was compared with the activity of the 2*N*-unsubstituted 4(5)-aryl-2-amino-1*H*-imidazoles.

2.2.1. *n*-Alkyl or iso-alkyl substituents at 2*N*-position

We synthesized and tested an array of 4(5)-aryl-2-aminoimidazoles 2*N*-substituted with either a short *n*- or *iso*-alkyl chain (C1–C5) or a *n*-octyl or *n*-nonyl chain. As depicted in Table 5, a broad diversity of 4(5)-aryl groups were included such as phenyl (compounds **81**–**84**), *para*-chlorophenyl (compounds **85**–**89**),

Table 4
Influence of 2-hydroxy-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts **71**–**80** with aromatic substituents at the *N*1-position on the biofilm formation of *S.* Typhimurium ATCC14028 at 25 °C

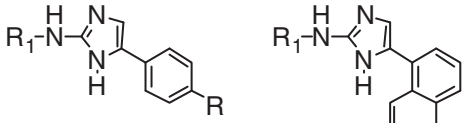


71-73 **74** **75-78** **79-80**

Compound	R	IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]		
				IC_{50}[a]	80 μM	40 μM
71	H	211.9	171.0–262.6			
72	4-NO_2	174.3	204.0–446.6			
73	4-I	36.7	28.5–47.21	–		–
74	4-Br	109.8	85.8–140.5			
75	4-NO_2	123.5	99.3–153.7			
76	4-F	105.2	70.9–155.9			
77	3,4-diCl	7.2	5.6–9.3	+		o
78	4-(4′-Biphenyl)	12.4	9.3–16.7	o		
79	4-FPh	17.2	12.1–24.5	–		–
80	3,4-MethylenedioxyPh	53.7	29.6–97.6	–	–	

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.

Table 5
Influence of 2N-alkylated 2-aminoimidazoles **81–117** on the biofilm formation and the planktonic growth of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



81-113 and **115-117**

114

Compound	R1	R	*S.* Typhimurium								*P. aeruginosa*							
			IC_{50} (μM)[a]	95% confidence interval for IC_{50}	Effect on growth at[b]						IC_{50} (μM)[a]	95% confidence interval for IC_{50}	Effect on growth at:[b]					
					IC_{50}[a]	200 μM	150 μM	80 μM	40 μM	20 μM			IC_{50}[a]	100 μM	80 μM	40 μM	20 μM	10 μM	5 μM
81	H	H	130.2	112.6–150.7	–		–				72.6	38.7–136.2	–		–				
82	*i*-Pr	H	>800								261.2	156.9–434.9							
83	*n*-Bu	H	25.3	23.3–27.6	–				–		31.8	21.3–47.3							
84	*i*-Bu	H	4.9	3.5–6.7	–				–		1.2	0.6–2.5	–						–
85	H	Cl	16.0	14.3–17.9	–				–		3.5	2.3–5.2	–						–
86	*i*-Bu	Cl	2.0	1.6–2.5	–				+	–	0.9	0.5–1.8	–						–
87	*n*-Pen	Cl	>400								~6.3[d,e]								
88	*n*-Oct	Cl	>400								~1.6[e]								
89	*n*-Non	Cl	>800								nd[c]								
90	H	NO_2	17.6	15.0–20.5	–				o		34.5	20.4–58.5	–	+		–			
91	Me	NO_2	170.4	113.3–256.2							56.1	31.4–100.3	–		–				
92	Et	NO_2	171.3	123.9–236.9							103.9	40.7–265.0							
93	*n*-Bu	NO_2	701.5	460.8–1068.0							>800								
94	*i*-Bu	NO_2	3.8	3.0–4.8	–				–		22.9	5.8–91.2	–						
95	*n*-Oct	NO_2	10.9	7.6–15.4	–			–			~25.0[e]								
96	H	F	84.4	69.7–102.3	–	–					15.0	8.6–26.2	–			–			
97	Me	F	101.7	85.7–120.6							25.2	21.1–30.2	–			–			
98	*n*-Bu	F	15.5	12.6–19.2	–			–			10.8	7.8–14.9	–			–			
99	*n*-Hex	F	>400								~12.5[e]								
100	*n*-Oct	F	>400								~3.1[e]								
101	H	Br	47.9	36.6–63.0							3.2	2.3–4.5	–			+		–	
102	*n*-Bu	Br	7.1	3.7–13.9	–					–	9.8	6.8–14.1	–				–		
103	*i*-Bu	Br	2.9	2.6–3.4	–				+	–	1.2	0.6–2.3	–						–
104	*n*-Pen	Br	3.1	2.0–5.0	–				+	+	10.2	2.9–35.9	–				–		
105	*n*-Oct	Br	1.9	1.1–3.4							>800								
106	H	4-OMe	119.7	106.9–134.1							186.3	136.2–254.8							
107	*n*-Bu	4-OMe	52.6	16.3–169.0	–			–			46.3	28.5–75.1	–		–				
108	*n*-Oct	4-OMe	~400[e]								>800								
109	Pr	3,4-diCl	10.9	9.6–12.4	–					–	27.7	11.7–65.4							
110	*n*-Pen	3,4-diCl	2.2	1.7–2.8							0.7	0.4–1.1	–						–
111	*n*-Oct	3,4-diCl	118.3[e]	71.7–195.2							>800								
112	Me	3,4-diF	601.9	288.1–1257.0							nd								
113	Me	4-OH	160.3	115.8–221.7							nd								
114	*n*-Oct		238.4[e]	132.1–430.3							>800								
115	H	4-SO_2Me	>800								>800								
116	*n*-Oct	4-SO_2Me	41.8	30.6–57.1	–			–			~3.1[e]		–				–		
117	*n*-Oct	3-Br	44.64	22.9–87.0	–			–			>800								

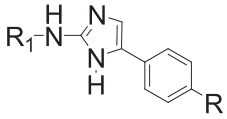
[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.
[c] nd: not determined.
[d] ~: the IC_{50} value could not be determined accurately due to the steepness of the dose response curve.
[e] The compound is not able to completely prevent biofilm formation, as the dose response curve either reaches a steady state level between 50% and 70% biofilm inhibition or shows a maximum between 50% and 70% biofilm inhibition and decreases again with higher compound concentrations.

para-nitrophenyl (compounds **90**–**95**), *para*-fluorophenyl (compounds **96**–**100**), *para*-bromophenyl (compounds **101–105**), *para*-methoxyphenyl (compounds **106–108**), 3,4-dichlorophenyl (compounds **109–111**), 3,4-difluorophenyl (compound **112**), *para*-hydroxyphenyl (compound **113**), naphthyl (compound **114**), *para*-methylsulphonylphenyl (**115**–**116**) and meta-bromophenyl (**117**). To be able to easily compare the activities of the 2*N*-substited and 2*N*-unsubstituted imidazoles, also the (previously described) activities of the 2*N*-unsubstituted imidazoles were included in Table 5 (compounds **81**, **85**, **90**, **96**, **101**, **106** and **115**). The 2-aminoimidazoles substituted at the 2*N*-position with a methyl, ethyl or (iso-)propyl were found to have lower activities as compared to their 2*N*-unsubstituted counterparts against both *Salmonella* and *Pseudomonas* biofilm formation, although they still show a moderate activity with IC_{50} values in the range of 10 μM (compound **107**) to 170 μM, except for compounds **82** and **112** which are almost completely inactive. On the other hand, the compounds with a *n*-butyl, *iso*-butyl or *n*-pentyl substitution at the 2*N*-position were found to be in general more active than their 2*N*-unsubstituted counterparts, with respect to both the *Salmonella* and *Pseudomonas* biofilm formation. The best activity was found for compound **110**, (bearing a 3,4-dichlorophenyl group at the 4(5)-position and a n-pentyl group at the 2*N*-position), which inhibits the biofilm formation of both *Salmonella* and *Pseudomonas* by 50% at concentrations below 2.5 μM. An exception is compound **87**, which inhibits *Salmonella* biofilm formation only at high concentrations (IC_{50} >400 μM). Furthermore this compound has a low IC_{50} value for *Pseudomonas* biofilm inhibition (IC_{50} ~6 μM), but is not able to prevent *Pseudomonas* biofilm formation by more than 50%. The effect of introduction of longer alkyl side chains (C6–C9) at the 2*N*-position was found to be strongly dependent on the substitution pattern of the 5-phenyl ring and the specific model organism studied. In case of *S.* Typhimurium, introduction of an octyl group at the 2*N*-position of compounds bearing a *para*-bromophenyl, *meta*-bromophenyl, *para*-nitrophenyl or *para*-methylsulphonyl group at the 5-position of the imidazole ring results in compounds with a high biofilm inhibitory activity (IC_{50} = 2–45 μM). On the other hand, introduction of an octyl group at compounds bearing a *para*-methoxyphenyl, naphtyl or 3,4-dichlorophenyl group at the 5-position results in compounds with much higher IC_{50} values (100–400 μM). Moreover, these compounds are not able to completely prevent biofilm formation at higher concentrations, as their dose response curves either reach a steady state level between 50% and 70% biofilm inhibition or show a maximum between 50% and 70% biofilm inhibition and decrease again with higher compound concentrations. Finally, the compounds with a *para*-chlorophenyl or *para*-fluorophenyl at the 5-position and hexyl, octyl or nonyl at the 2*N*-position had a very low or no biofilm inhibitory activity (IC_{50} >800 μM). In case of *P. aeruginosa*, introduction of an octyl chain at the 2*N*-position of imidazoles bearing 3,4-dichlorophenyl, naphtyl, *para*-methoxyphenyl or bromophenyl at the 5-position results in compounds that do not have an effect on the biofilm formation at the highest concentration tested (800 μM). On the other hand, the imidazoles with a chlorine atom, fluorine atom, nitro group or sulphonylmethyl group at the *para*-position of the 5-phenyl ring and a pentyl, hexyl or octyl chain at the 2*N*-position decrease the biofilm formation at low concentrations (IC_{50}'s 1.5–25 μM), however, they are not able to completely prevent biofilm formation as their dose response curves either reach a steady state level around 50% biofilm inhibition or show a maximum around 50% biofilm inhibition and decrease again with higher compound concentrations. Finally, we tested the effect of some of the most active imidazoles on the planktonic growth by growth curve analysis. Interestingly, all the imidazoles tested show a broad concentration range with only an effect on the biofilm formation and no effect on the planktonic growth, indicating that these compounds have potential to be used as selective biofilm inhibitors.

2.2.2. Cyclo-alkyl substituents at 2*N*-position

As described in the previous section, we found that the 2-aminoimidazoles substituted at the 2*N*-position with an intermediate length *n*-alkyl or *iso*-alkyl chain were very potent inhibitors of the biofilm formation of both *Salmonella* and *Pseudomonas*. In an attempt to even improve this biofilm inhibitory activity, we decided to synthesize an array of 2-aminoimidazoles substituted at the 2*N*-position with cyclo-alkyl groups with intermediate length, that is, cyclo-pentyl (compounds **118**–**122**) and cyclo-hexyl (compounds **123–126**). As depicted in Table 6, the compounds with a cyclo-pentyl chain at the 2*N*-positon have an improved

Table 6
Influence of 2*N*-cyclo-alkyl-2-aminoimidazoles **118**–**127** on the biofilm formation and the planktonic growth of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



Compound	R1	R	*S.* Typhimurium						*P. aeruginosa*					
			IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]				IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]			
					IC_{50}	80 μM	40 μM	20 μM			IC_{50}	80 μM	40 μM	20 μM
118	*c*-Pen	H	52.9	42.4–66.1	–	–			33.8	23.9–47.7	–		–	
119	*c*-Pen	4-Cl	4.4	4.0–4.8	–		–		13.5	9.0–20.5	–		–	
120	*c*-Pen	4-NO_2	11.8	7.9–17.7	–			–	20.2	12.3–33.1	–			
121	*c*-Pen	4-Br	12.1	6.5–22.5	–			–	7.2	4.4–12.0	–			–
122	*c*-Pen	3,4-diCl	5.7	3.8–8.5	–			–	7.9	4.2–14.8	–		–	
123	*c*-Hex	4-Cl	>800						~25[c,d]					
124	*c*-Hex	4-NO_2	36.7	29.7–45.3	–		–		~25[d]					
125	*c*-Hex	3-NO_2	629.5	274.6–1443.0					4.4	2.6–7.5	–		–	
126	*c*-Hex	4-OMe	~377.6						54.8	21.9–136.9	–	–		
127	*c*-Hex	4-SMe	191.3	129.1–283.6					15.8	9.5–26.1	–			–

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.
[c] ~: the IC_{50} value could not be determined accurately due to the steepness of the dose response curve.
[d] The compound is not able to completely prevent biofilm formation, as the dose response curve either reaches a steady state level at about 50% biofilm inhibition.

activity against *Salmonella* biofilms as compared to the 2*N*-unsubstituted compounds. However, no further improvement of the *Salmonella* biofilm inhibitory activity of the n-alkyl and iso-alkyl substituted imidazoles could be achieved. The compounds with a cyclo-pentyl chain also inhibit *Pseudomonas* biofilm formation at low to moderate concentrations (IC_{50}'s 7–35 μM), although their activities were not in all cases better than these of their unsubstituted counterparts. Interestingly, analogously to the 2*N*-*n*-alkyl substituted 2-aminoimidazoles, growth curve analysis revealed that all the 2*N*-cyclopentyl 2-aminozoles tested possess a concentration range with only biofilm inhibition and no effect on the planktonic growth, both in the case of *Salmonella* and *Pseudomonas*. The compounds 2*N*-substituted with a cyclo-hexyl, on the other hand, in general only show a moderate to low activity against *Salmonella* biofilm formation, with IC_{50} values between 50 and >800 μM, while they inhibit *Pseudomonas* biofilms at lower concentrations (IC_{50} 4–50 μM). Interestingly, all the 2-aminoimidazoles tested have a concentration range in which they specifically inhibit *Salmonella* and *Pseudomonas* biofilm formation, without affecting the planktonic growth (Table 6).

2.2.3. Aromatic substituents at 2*N*-position

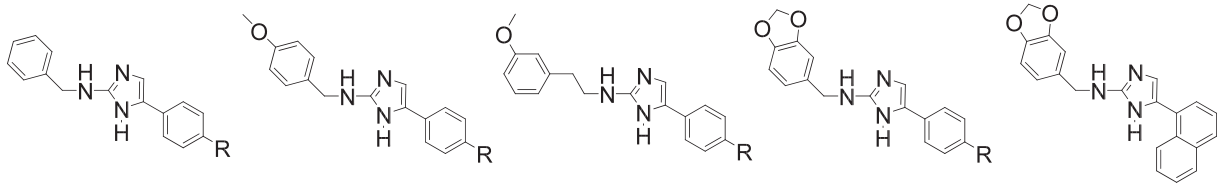
Finally we synthesized and tested an array of 4(5)-phenyl-2-aminoimidazoles, which are substituted at the 2*N*-position with a benzyl (compound **128**), *para*-methoxybenzyl (compound **129**), 3-methoxyphenethyl (compounds **130**–**132**) or piperonyl group (compounds **133**–**134**) (Table 7). Compound **128**, bearing a benzyl group, has a good activity against both *Salmonella* and *Pseudomonas* biofilm formation (IC_{50}'s ~30 μM), although these activities are lower than those of the 2*N*-unsubstituted counterpart. Compound **129**, bearing a *para*-methoxybenzyl group, has no activity. The activity of the compounds 2*N*-substituted with a 3-methoxyphenethyl group is clearly dependent on the substitution of the 4(5)-phenyl group, as compound **130**, with *para*-nitrophenyl group at the 4-position is more active than its unsubstituted counterpart, while compounds **131** and **132**, bearing respectively a 4,5-dichlorobenzyl group and a [1,1′:4′,1″-terphenyl]-4-yl group at the 4(5)-position, are inactive at the highest concentration tested (400 μM). Finally, the compounds with a 2*N*-piperonyl group (**133**–**134**) show a moderate activity both against *Salmonella* and *Pseudomonas* biofilm formation.

2.3. 2*N*-substituted 2-aminoimidazoles versus 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts

In the divergent chemical synthesis procedure, 2*N*-substituted 4(5)-aryl-2-aminoimidazoles are formed by cleavage of the 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts with a nucleophile such as hydrazine, under conventional heating or microwave irradiation (Scheme 1, pathway B). After cleavage, the 4(5)-aryl substituent and the 2*N*-substituent of the 2-aminoimidazole will be the same as respectively the 2-aryl substituent and the 1-substituent of the salt. Analogously, *N*1-substituted 2-aminoimidazoles are formed by cleavage of the 1-substituted imidazo[1,2-*a*]pyrimidinium perchlorate salts (Scheme 1, pathway A). In a previous publication, we described a correlation between the activity of the 1-substituted imidazo[1,2-*a*]pyrimidinium perchlorate salts and their corresponding *N*1-substituted 2-aminoimidazoles, supporting the hypothesis that these imidazo[1,2-*a*]pyrimidinium perchlorate salts could also in situ be degraded to the corresponding *N*1-substituted 2-aminoimidazoles, for example, by cellular nucleophiles.[42] To determine whether a similar correlation could be found between the biofilm inhibitory activity of the 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts and their corresponding 2*N*-substituted 2-aminoimidazoles, in this section we compare the structure–activity relationship of both classes of compounds.

The 2-aminoimidazoles substituted at the 2*N*-position with a methyl, ethyl or (iso-)propyl were found to have a low to moderate activity against the biofilm formation. This is essentially in agreement with the structure-activity relationship found for the 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts, although comparison of the IC_{50} values reveals that the activity of the imidazoles is in general a bit higher than the activity of the corresponding salts. Also the activity of the 2-aminoimidazoles substituted with an *n*-butyl, *iso*-butyl, *n*-pentyl and cyclo-pentyl group was in general found to be higher than the activity of the corresponding salts. The higher activity of the 2-aminoimidazoles compared to their precursor imidazopyrimidinium salts could possibly be explained by an incomplete in situ degradation of the salts to imidazoles. Therefore this difference in activity between salts and imidazoles does not have to lead to a rejection of the hypothesis that the biofilm inhibitory activity of the

Table 7
Influence of 2-aminoimidazoles **127**–**133** with aromatic substituents at the 2*N*-position on the biofilm formation of *S.* Typhimurium ATCC14028 and *P. aeruginosa* PA14 at 25 °C



128 **129** **130-132** **133** **134**

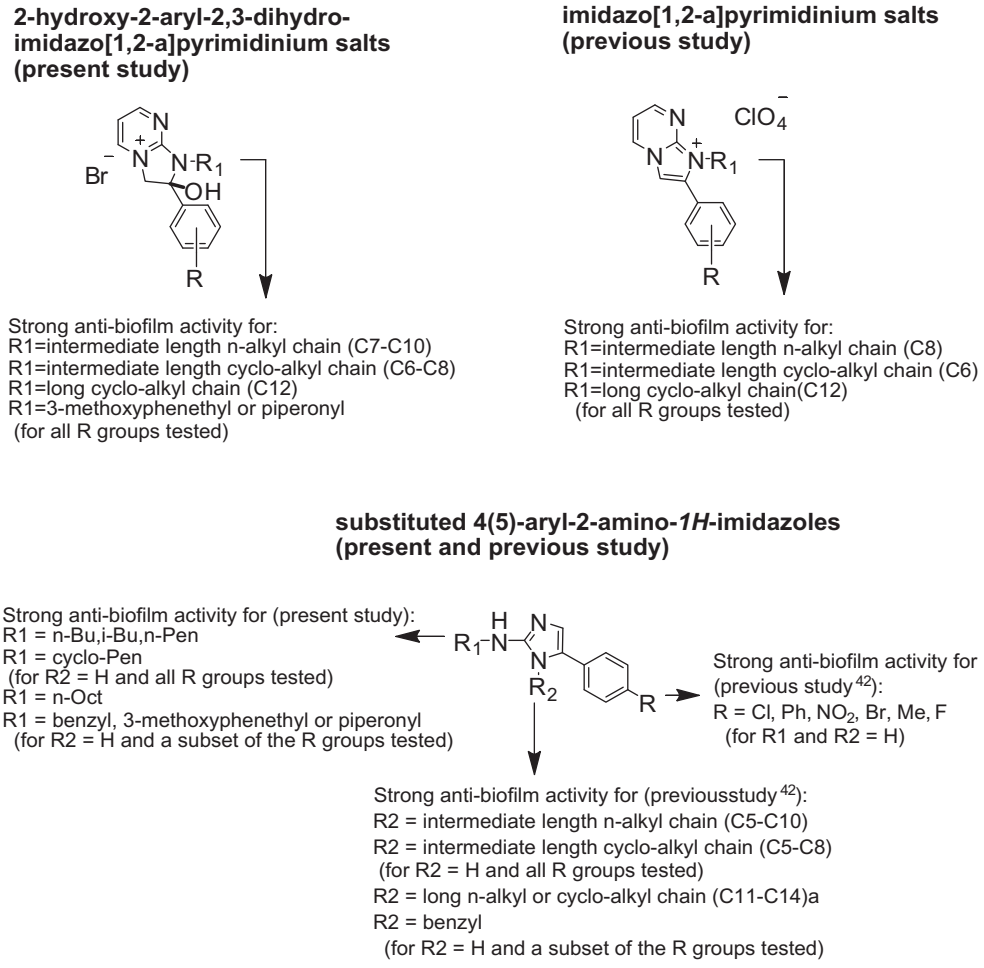
Compound	R	*S.* Typhimurium				*P. aeruginosa*			
		IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]		IC_{50}[a] (μM)	95% confidence interval for IC_{50}	Effect on growth at[b]	
				IC_{50}	40 μM			IC_{50}	40 μM
128	4-Cl	30.73	24.55–38.47	–	–	26.27	13.61–50.71	–	–
129	4-NO_2	>800				>800			
130	4-NO_2	19.07	11.85–30.67	–	–	3.704	1.913–7.172	–	–
131	3,4-diCl	>800				>800			
132	4-(4′-Biphenyl)	>800				>800			
133	4-F	66.59	53.29–83.21	–		455.5	296.8–699.1		
134		261	170.5–399.7			10.52	6.158–17.99		

[a] IC_{50}: concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] o: the planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; +: the planktonic growth is retarded when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; –: the planktonic growth is not or only slightly affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor; No symbol indicated: effect not determined.

imidazopyrimidinium salts is mediated by the 2-aminoimidazoles, formed by in situ cleavage of the salts. However, remarkably, some of the 2*N*-octyl 2-aminoimidazoles tested were found to have much higher IC_{50} values than their corresponding 1-octyl-2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts. Furthermore, the 2*N*-cylcohexyl 2-aminoimidazole **123** is much less active against *Salmonella* biofilms than its corresponding salt **64**. This discrepancy could be explained by the toxic effect of the salts at concentrations lower than the biofilm inhibitory concentrations of the corresponding imidazoles, which causes a non-specific inhibition of the biofilm formation by reducing the planktonic growth of the bacteria around the surface on which the biofilms are formed. Therefore, these data do not exclude the possibility that the biofilm inhibitory activity of the imidazopyrimidinium salts is mediated by the 2-aminoimidazoles, formed by in situ cleavage of the salts.

3. Conclusion

Previously, we reported the biofilm inhibitory activity of *N*1-substituted 5-aryl-2-aminoimidazoles and their precursor imidazo[1,2-a]pyrimidinium salts.[42] These compounds were synthesized by utilizing the first path of our divergent synthesis pathway towards 2-aminoimidazoles[40,41] The second path of this divergent pathway describes the synthesis of 2*N*-substituted 2-amino-1*H*-imidazoles and their precursor 1-substituted 2-hydroxy-2,3-dihydro-imidazopyrimidinium salts. In the present study, we investigated the potential of these 1-substituted 2-hydroxy-2,3-dihydro-imidazopyrimidinium salts and 2*N*-substituted 2-amino-1*H*-imidazoles as inhibitors of the biofilm formation by *S.* Typhimurium and *P. aeruginosa*. The results of the previous study and the present study are summarized in Scheme 2. We found that 2-hydroxy-2,3-dihydro-imidazopyrimidinium salts with intermediate length *n*-alkyl chains (C7–C10) substituted at the 1-position in general prevent the biofilm formation of both species at low micromolar concentrations (IC_{50}'s 5–50 μM). Salts with a shorter (C1–C5) or longer (C11–C14) *n*-alkyl chain at the 1-position were found to be much less potent. Consistent with this, we observed that salts with an intermediate length cyclo-alkyl chain are much more active against biofilm formation of both species as compared to the salts with a short cyclo-alkyl chain. However, remarkably, salts with a long cyclo-dodecyl side chain were found to have even better activities than salts with an intermediate length cyclo-alkyl side chain. Furthermore, we demonstrated the potential of 2-hydroxy-2,3-dihydro-imidazopyrimidinium salts with certain aromatic substituents at the 1-position, such as piperonyl or 3-methoxyphenetyl as inhibitors of *Salmonella* and *Pseudomonas* biofilm formation. In the framework of the 2-aminoimidazoles, we delineated that introduction of a butyl or pentyl side chain at the 2*N*-position of the 2-aminoimdazoles results in an enhanced biofilm inhibitory activity against both species, while introduction of a shorter *n*-alkyl chain reduces the biofilm inhibitory activity. The effect of introduction of longer n-alkyl chains, however, seems to be strongly dependent on the substitution pattern of the 5-phenyl ring and the bacterial species studied. Also introduction of a



Scheme 2. Overview of the substituents important for anti-biofilm activity of 2-hydroxy2-aryl-2,3-dihydroimidazo[1,2-*a*]pyrimidinium salts, imidazo[1,2-*a*]pyrimidinium salts and 4(5)-aryl-2-amino-1*H*-imidazoles.

cyclo-pentyl side chain in general results in an improved activity. Finally, we showed that introduction of a 3-methoxyphenethyl or piperonyl group at the 2*N*-position can also result in an enhanced biofilm inhibition, although this effect is also dependent on the nature of the substitution pattern of the 5-phenyl ring. Interestingly, growth curve analysis revealed that the 2-aminoimidazoles in general have a broad concentration range in which they specifically inhibit biofilm formation without affecting the planktonic growth, indicating that these compounds have potential to be used as selective biofilm inhibitors. As described in our previous publication,[42] we found a good correlation between the activity of the *N*1-substituted 4(5)-aryl-2-aminoimidazoles and their precursor imidazo[1,2-*a*]pyrimidinium salts, leading to the hypothesis that the salts could also in situ be cleaved by cellular nucleophiles to form the active 2-aminoimidazoles. However, in the present study no good correlation was found between the activity of some of the active 1-octyl-2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts (and 1-cyclohexyl-2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts) and their corresponding less efficient 2-aminoimidazoles. However, this could be explained by the toxic effect of the salts at concentrations lower than the biofilm inhibitory concentrations of the corresponding imidazoles, which causes a non-specific inhibition of the biofilm formation. Therefore this difference in activity between salts and imidazoles does not necessarily falsify the hypothesis that the biofilm inhibitory activity of the imidazopyrimidinium salts is mediated by the 2-aminoimidazoles, formed by in situ cleavage of the salts. In conclusion, the 2*N*-substituted 2-aminoimidazoles and 2-hydroxy-2-aryl-2,3-dihydro-imidazopyrimidinium salts of the present study are valuable candidates in the development of therapeutics and sanitizers for the combat of biofilm formation by *S.* Typhimurium, *P. aeruginosa* and possibly other pathogenic bacteria.

4. Experimental section

4.1. Chemistry

4.1.1. General experimental methods

Chemicals were purchased from commercial sources and used without further treatment. Melting points are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra at 75 with tetramethylsilane or solvent ($CDCl_3$, CD_3OD, DMSO-d_6) as internal standard (d ppm). The ion source temperature was 150–250 °C, as required. High-resolution EI-mass spectra were performed with a resolution of 10 000. The low-resolution spectra were obtained with a HP5989A MS instrument. For column chromatography 70–230 mesh silica gel was used. The purity of the compounds was checked by HPLC. All compounds were obtained with a purity >95%.

4.1.2. Microwave irradiation experiments

A multimode Milestone MicroSYNTH microwave reactor was used in the standard configuration as delivered, including proprietary software. All experiments were carried out in sealed microwave process vials (15, 30 and 50 mL). After completion of the reaction, the vial was cooled down to 25 °C via air jet cooling before opening. Reaction temperatures were monitored by an IR sensor on the outside wall of the reaction vial and a fibre-optic sensor inside the reaction vial.

4.1.3. 1-Substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts

All 1-substituted 2-hydroxy-2-aryl-2,3-dihydro-imidazo[1,2-*a*]pyrimidinium salts were synthesized by using previously described protocols.[41] A general procedure is described below (with compound **79** as an example):

In a 30 mL microwave vial were successively brought acetonitrile (15 mL), *N*-(1,3-benzodioxol-5-ylmethyl)pyrimidin-2-amine (1.15 g, 5 mmol), 4-fluorophenacylbromide (1.3 g, 6 mmol, 1.2 equiv), and a catalytic amount of4-dimethylaminopyridine (6 mg, 0.05 mmol). The reaction tube was sealed and irradiated in a microwave reactor at a ceiling temperature of 80 °C at 150 W maximum power for 30 min. After the reaction mixture was cooled with an air flow for 15 min, the precipitate was washed with acetone (25 mL), ether (20 mL) and dried in vacuum to afford **79** (1.98 g, 89% yield) as a white powder.

The analytical data of the new compounds are listed as Supplementary data.

4.1.4. 2*N*-Substituted 4(5)-aryl-2-amino-1*H*-imidazoles

All 2*N*-substituted 4(5)-aryl-2-amino-1*H*-imidazoles were synthesized by using previously described protocols.[41] A general procedure is described below (with compound **133** as an example): To a suspension of salt **79** (2 mmol) in acetonitrile (5 mL) was added hydrazine hydrate (0.7 mL, 14 mmol of a 64% solution, 7 equiv), and the mixture was irradiated in the sealed reaction tube for 10 min at a ceiling temperature of 100 °C at 150 W maximum power. After the mixture was cooled, hydrazine hydrate was evaporated with toluene (3 × 20 mL). The resulting residue was purified by column chromatography (silica gel; MeOH-DCM 1:4 v/v with 5% of 6 M NH3 in MeOH) to afford 2-amino-1*H*-imidazole **133** (473 mg, 76% yield) as an amorphous solid.

The analytical data of the new compounds are listed as Supplementary data.

4.2. Biological assays

4.2.1. Static peg assay for prevention of *Salmonella* Typhimurium and *Pseudomonas aeruginosa* biofilm formation[17]

The device used for biofilm formation is a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a microtiter plate lid with a peg hanging into each microtiter plate well (Nunc no. 269789).[44] Twofold serial dilutions of the compounds in 100 μl liquid broth (Tryptic Soy Broth diluted 1/20 (TSB 1/20)) per well were prepared in the microtiter plate (2 or 3 repeats per compound). Subsequently, an overnight culture of *S.* Typhimurium ATCC14028 (grown in Luria-Bertani medium[45]) or *P. aeruginosa* PA14 (grown in TSB) was diluted 1:100 into the respective liquid broth and 100 μl (~10^6 cells) was added to each well of the microtiter plate, resulting in a total amount of 200 μl medium per well. The pegged lid was placed on the microtiter plate and the plate was incubated for 24 h at 25 °C without shaking. During this incubation period biofilms were formed on the surface of the pegs. After 24 h, the optical density at 600 nm (OD_{600}) was measured for the planktonic cells in the microtiter plate using a VERSAmax microtiter plate reader (Molecular Devices). This gives a first indication of the effect of the compounds on the planktonic growth. For quantification of biofilm formation, the pegs were washed once in 200 μl phosphate buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 μl 0.1% (w/v) crystal violet in an isopropanol/methanol/PBS solution (v/v 1:1:18). Excess stain was rinsed off by placing the pegs in a 96-well plate filled with 200 μl distilled water per well. After the pegs were air dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid (200 μl). The OD_{570} of each well was measured using a VERSAmax microtiter plate reader (Molecular Devices). The IC_{50} value for each compound was determined from the concentration gradient by using the GraphPad software of Prism.

4.2.2. Bioscreen assay for measuring *Salmonella* Typhimurium and P. aeruginosa growth inhibition

The Bioscreen device (Oy Growth Curves Ab Ltd) was used for measuring the influence of the chemical compounds on the planktonic growth of *Salmonella* Typhimurium and *P. aeruginosa* PA14. The Bioscreen is a computer controlled incubator/reader/shaker that uses 10 × 10 well microtiter plates and measures light absorbance of each well at a specified wave length in function of time. An overnight culture of *S.* Typhimurium ATCC14028 (grown up in LB medium) or *P. aeruginosa* (grown up in TSB) was diluted 1:200 in liquid broth (TSB 1/20). 300 μl of the diluted overnight culture was added to each well of the 10 × 10 well microtiter plate. Subsequently, serial dilutions of the chemical compounds were prepared in DMSO or EtOH. 3 μl of each diluted stock solution was added to the wells (containing the 300 μl bacterial culture) in threefold. As a control 3 μl of the appropriate solvent was also added to the plate in three- or four-fold. The microtiter plate was incubated in the Bioscreen device at 25 °C for at least 24 h, with continuous medium shaking. The absorbance of each well was measured at 600 nm each 15 min. Excel was used to generate the growth curves for the treated wells and the untreated control wells.

The effect of each compound concentration on the planktonic growth was classified into one of the following categories:

(1) The planktonic growth is not or only slightly affected, indicated by the symbol '–'.
(2) The planktonic growth is retarded, indicated by the symbol '+'.
(3) The planktonic growth is completely or almost completely inhibited, indicated by the symbol 'o'.

The following criterium was used to decide between the first and the second category:

If the absorbance (measured at 600 nm) of the bacterial culture treated with the compound is at least 0.5 (for *Salmonella*) or 0.8 (for *Pseudomonas*) units lower than the absorbance of the untreated culture during four consecutive hours, then the effect on the planktonic growth is classified in category 2.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.026.

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