Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Microwave-Assisted One-Pot Synthesis and Anti-Biofilm Activity of 2-Amino-1*H*-imidazole/Triazole Conjugates

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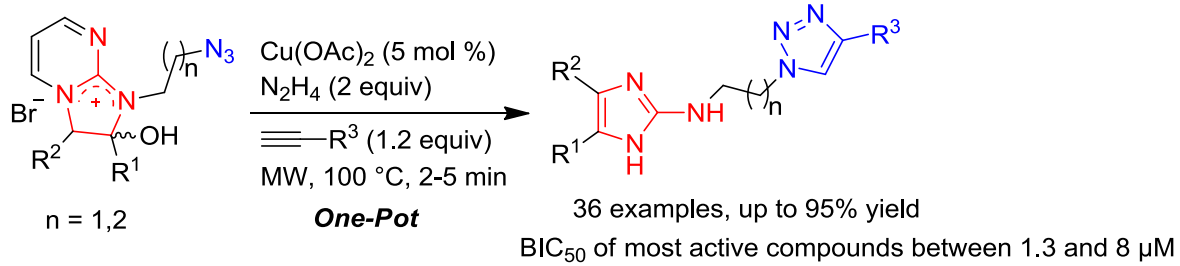
ABSTRACT

A microwave-assisted protocol was developed for the construction of 2-amino-1*H*-imidazole/triazole conjugates starting from the previously described 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts. The process involves a one-pot hydrazinolysis/Dimroth-rearrangement of these salts followed by a ligand-free copper nanoparticle-catalyzed azide-alkyne Huisgen cycloaddition. The 2-amino-1*H*-imidazole/triazole conjugates showed moderate to high preventive activity against biofilms of *S.* Typhimurium, *E. coli*, *P. aeruginosa* and *S. aureus*. The most active compounds had BIC_{50} values between 1.3 and 8 µM. A remarkable finding was that introduction of the triazole moiety in the side chain of 2-aminoimidazoles with a long (C_8-C_{13}) 2*N*-alkyl chain did drastically improve their activity. Conclusively, the 2-amino-1*H*-imidazole/triazole scaffold provides a lead structure for further design and development of novel biofilm inhibitors.



Introduction

The 2-amino-1*H*-imidazole (2-AI) structural motif is of particular interest within the realm of medicinal and bioorganic chemistry. Many compounds possessing this framework occur in nature and display a broad range of biological properties.[1] For example, among them are the potent modulators of the formation and dispersion of bacterial biofilms,[2] human β-secretase (BACE-1) inhibitors[3] and tubulin-binding agents.[4] Biofilms in particular account for more than 80% of all bacterial infections and are responsible for the mortality and morbidity of almost all cystic fibrosis patients.[1c] Moreover biofilms cause major problems in industrial and household settings.[2f] Given the biomedical and industrial prominence of biofilms, there have been significant efforts to discover potent compounds that modulate or/and inhibit the biofilm growth. In this vein, we have recently described the synthesis and anti-biofilm activity of diverse 5-aryl-substituted 2-AI (Fig. 1a).[2c-f] Moreover, it has recently been reported that 2-amino-1*H*-imidazole/triazole (2-AIT) conjugates, in which a triazole moiety is coupled to the 4(5)-position of the 2-AI-ring via an alkyl linker, inhibit and disperse both Gram-positive and Gram-negative bacterial biofilms through a non-microbiocidal mechanism (Fig. 1b).[5] The effect of coupling a triazole moiety to the exocyclic 2*N*-position of the 2AI-ring via an alkyl linkage, has however not been explored (Fig. 1c).

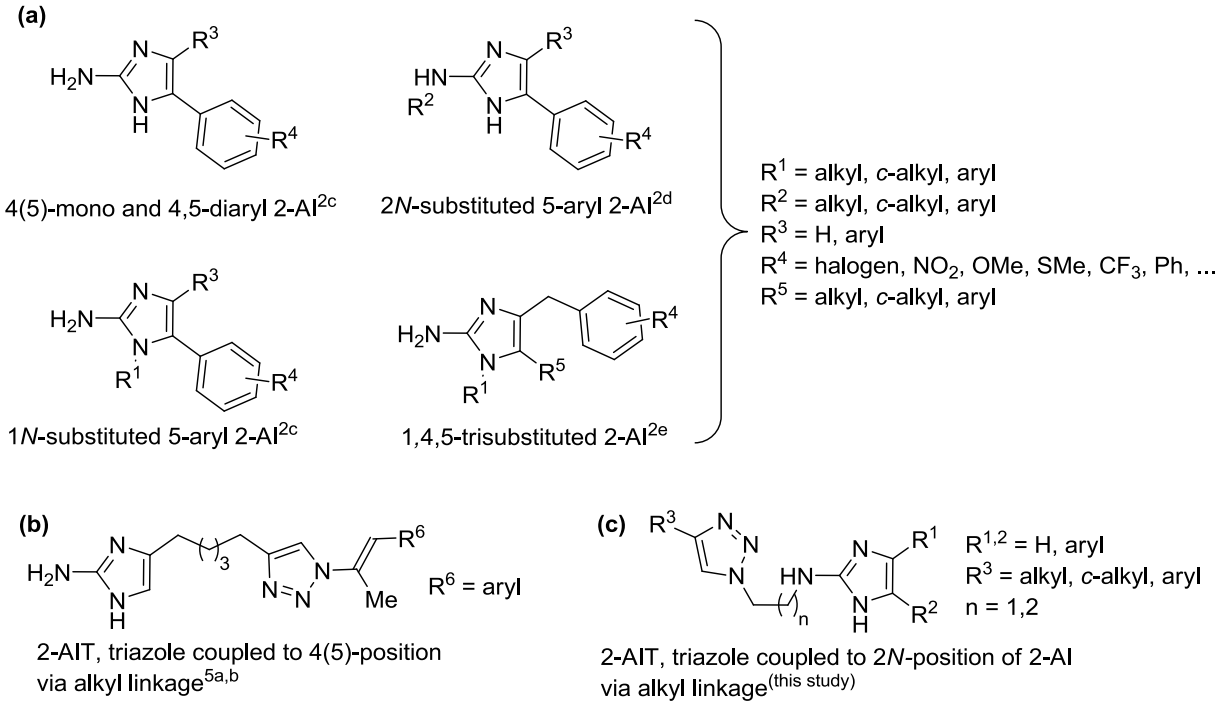


Figure 1. (a) Classes of 5-aryl-substituted 2-AI with anti-biofilm activity previously reported by our lab; (b) 2-amino-1*H*-imidazole/triazole (2-AIT) conjugates with anti-biofilm activity, reported by Melander *et al.*; (c) 2-amino-1*H*-imidazole/triazole conjugates investigated in the present study.

Despite the number of existing approaches to 2-AI, most of them involve long experimental procedures and the use of unstable precursors. There are only a few approaches that describe the direct synthesis of 2-AI. The earliest method involves condensation of α-aminocarbonyl compounds with cyanamide or their synthetic equivalents.[6,7] Other general applicable strategies are cyclocondensation of α-bromoketone with *N*-acetyl- or *N*-Boc-protected guanidine,[8] iminophosphorane-mediated cyclization of α-azido esters,[9] ammonolysis of 2-amino-1,3-oxazol-3-ium salts,[10] and the sequential functionalization of the 1,2-diprotected imidazole ring with different electrophiles.[11]

"Click chemistry", of which the CuAAC is the most important representative, has emerged as a fast and efficient approach to the synthesis of novel compounds with desired functionality employing selected "near perfect" reactions.[12,13] During the last years, a large number of protocols has been developed for this most studied and reliable ''click" reaction employing copper(I) complexes, copper on charcoal, solid phase-supported copper(I) catalysts and copper nanoparticles.[14]

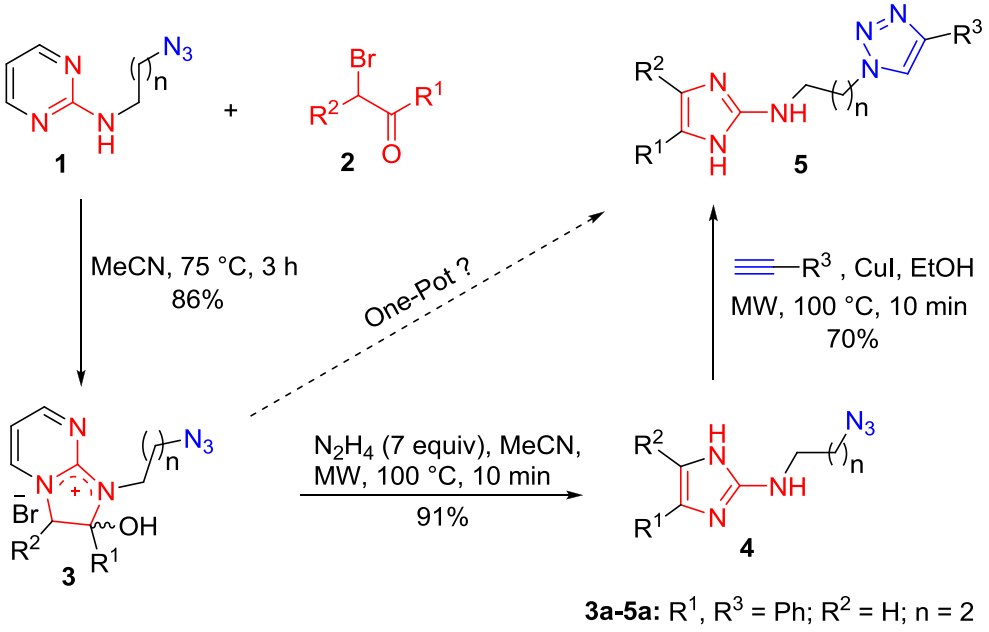
Herein, we report a rapid and highly efficient microwave-assisted one-pot synthesis of the 2-AIT framework, in which the triazole moiety is coupled to the 2*N*-position of the 2-AI ring via an alkyl linkage (fig 1c). The protocol starts from our previously described 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts, combining a Cu(I)-catalyzed azide-alkyne Huisgen cycloaddition (CuAAC) and a Dimroth-rearrangement.[15]The applicability of the protocol is demonstrated by the synthesis of a library of 36 compounds, of which we show the preventive anti-biofilm activity against Gram-negative and Gram-positive bacterial species, delineating a structure-activity relationship.

Results and discussion

Synthesis of 2-Amino-1*H*-imidazole/Triazole Conjugates

In order to develop a new strategy to the targeted 2-AIT **5**, we carefully examined the reaction conditions (Scheme 1). A mixture of *N*-(3-azidopropyl)pyrimidin-2-amine (**1a**) and phenacyl bromide (**2a**, R^1 = Ph, R^2 = H) in MeCN was heated at 75 °C for 3 h resulting in the formation of the 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salt **3a**. This salt smoothly underwent Dimroth-type rearrangement[15] upon treatment with 7 equiv of hydrazine hydrate yielding *N*-(3-azidopropyl)-1*H*-imidazol-2-amine **4a**. Subsequent CuAAC was performed upon treatment of 2-AI **4a** with phenylacetylene (1.5 equiv) and CuI (10 mol%) as catalyst under microwave irradiation at a ceiling temperature of 100 °C and a maximum power of 40 W for 10 min, delivering the desired 2-AIT **5a** (R^1, R^3 = Ph; R^2 = H; n = 2) in 70 % yield (Scheme 1).

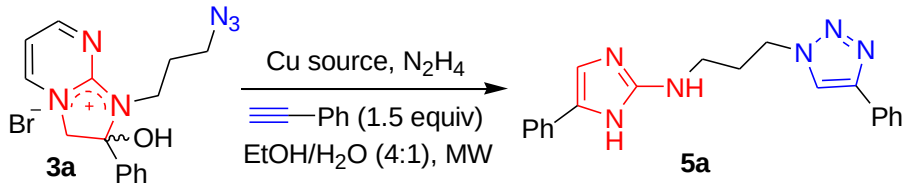
Scheme 1. Sequential synthesis of the 2-AIT framework.



Now we were keen to know whether the Dimroth rearrangement and the CuAAC could be run in a one-pot fashion, as we surmised that the required copper(0) nanocatalyst could be generated[14] *in situ* upon reduction of a Cu(II) salt by hydrazine. The procedure was evaluated employing hydroxy salt **3a** (R^1 = Ph, R^2 = H) and phenylacetylene as a model system (Table 1). To our satisfaction the reaction worked well when 2 equiv of hydrazine hydrate in combination with 10 mol% $Cu(OAc)_2$ were employed upon microwave irradiation at a ceiling temperature of 100 °C for 20 min (Table 1, entry 3). When only 1 equiv of hydrazine hydrate was used, the product was obtained in a moderate yield of 51 % (Table 1, entry 1), while increasing the amount of hydrazine hydrate did not influence the yield (Table 1, entry 2). The reaction time could be decreased to a mere 2 min while a further shortening resulted in an incomplete Dimroth rearrangement reaction (Table 1, entries 3-7). Also lowering the reaction temperature to 90 °C resulted in a decreased yield (Table 1, entry 9). Replacement of $Cu(OAc)_2$ with $CuSO_4$ gave the product in 67 % yield (Table 1, entry 11), while with Cu-powder (200 mesh) only trace amounts of the desired compound were observed (Table 1, entry 13). As expected no product was formed in the absence of catalyst (Table 1, entry 12). The optimal reaction conditions were achieved when a mixture of hydroxy salt **3a** (0.25 mmol), hydrazine hydrate (2 equiv), phenyl acetylene (1.5 equiv), $Cu(OAc)_2$ (5 mol%) in EtOH/H_2O (4:1; 1 mL) was irradiated for 2 min at a ceiling temperature of 100 °C applying a maximum power of 35 W. The desired compound **5a** was isolated in 90 % yield (Table 1, entry 8). When the reaction was performed at rt for 24 h, the product **5a** was obtained in very low yield (Table 1, entry 14). The novelty of this one-pot procedure is in the fact that the hydrazine is doing two totally different tasks in the same pot,

namely the decomposition of the pyrimidinium salt on the one hand, and the *in situ* generation of copper nanoparticles on the other hand. Moreover, to the best of our knowledge, the *in situ* generation of the required copper catalyst to catalyze the CuAAC-reaction ("click reaction") is unprecedented, and is a valuable addition to the arsenal of catalytic conditions for the CuAAC-reaction.

Table 1. Optimization of the one-pot synthesis of the 2-AIT.[a]



Entry	Time (min)	Temp. (°C)	Cu source, mol%	Yield (%)[b]
1[c]	20	100 °C	$Cu(OAc)_2$, 10	51
2[d]	20	100 °C	$Cu(OAc)_2$, 10	84
3	20	100 °C	$Cu(OAc)_2$, 10	86
4	10	100 °C	$Cu(OAc)_2$, 10	80
5	5	100 °C	$Cu(OAc)_2$, 10	90
6	2	100 °C	$Cu(OAc)_2$, 10	90
7	1	100 °C	$Cu(OAc)_2$, 10	53
8	**2**	**100 °C**	**$Cu(OAc)_2$, 5**	**90**
9	2	90 °C	$Cu(OAc)_2$, 5	73
10	2	100 °C	$Cu(OAc)_2$, 2	57
11	2	100 °C	$CuSO_4 \cdot 5H_2O$, 5	67
12	2	100 °C	-	-
13	2	100 °C	Cu-powder, 10	traces
14	24 h	rt	$Cu(OAc)_2$, 5	24

[a]All reactions were conducted on a 0.25 mmol scale of **3a**, applying hydrazine hydrate (2 equiv), phenylacetylene (1.5 equiv) in EtOH/H_2O (4:1, 1 mL) under microwave irradiation; the mixture was irradiated in a sealed tube at the indicated ceiling temperature and 35 W maximum power for the stipulated time; [b]isolated yields; [c]1.0 equiv of hydrazine hydrate was used; [d]5.0 equiv of hydrazine hydrate were used.

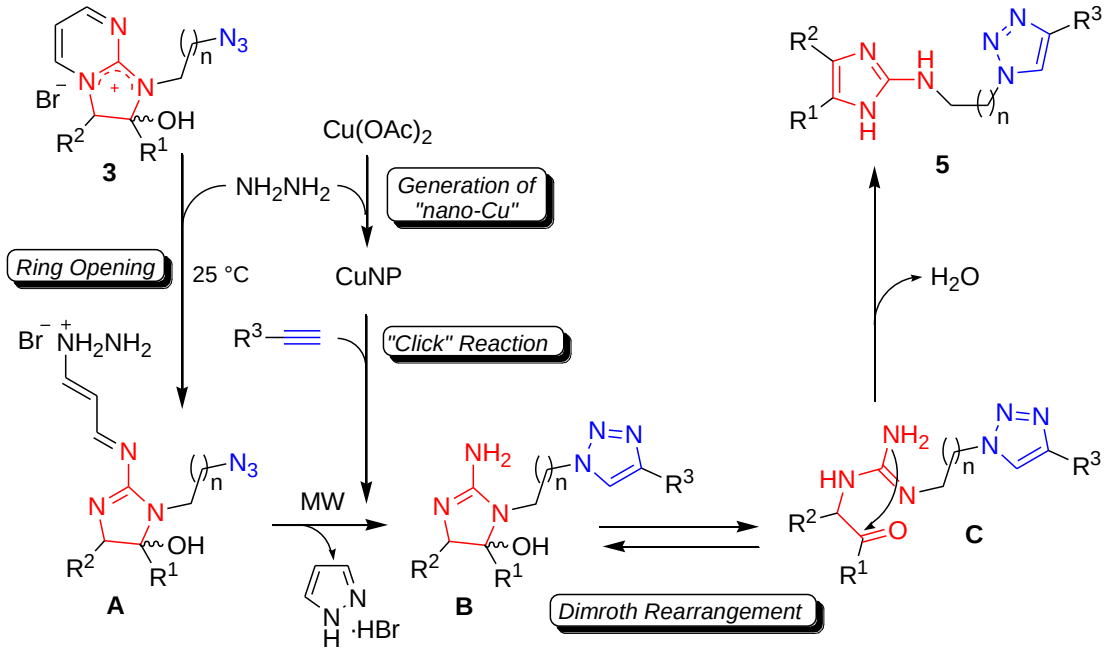
Encouraged by these findings, we explored the scope of this protocol. First, an array of hydroxy salts was prepared using our optimized conditions (Table 2). Special attention was given to the use of α-

bromoketones bearing halogenated phenyl substituents that were previously found to be crucial for the antibiofilm activity.[2c-d] In most cases, the reactions were run for 3 h in MeCN at 75 °C affording the hydroxy salts **3b-l** in good to excellent yields as white precipitates.

The thus prepared hydroxy salts **3b-m** (n = 1, 2) were reacted with different (hetero)aromatic and alkyl acetylenes (Table 2). All reactions were completed within 2 min delivering the desired compounds in good to excellent yields. However, in case of a morpholylamide substituent, lower yields were observed (Table 2, entry 22-23), probably due to hydrazinolysis of the starting hydroxy salts **3h** and **3i**. Remarkably, 4,5-disubstituted hydroxy salts **3l** and **3m** were successfully reacted, giving the desired products in 84 and 56% yield. Generally, the cycloaddition reaction proceeded efficiently with aliphatic and aromatic terminal acetylenes providing the corresponding 2-AIT **5** in good yields. Thus, a variety of substituted 2-AIT bearing an aromatic (Table 2, entry 1, 2, 9, 10, 11, 16, 17, 24), aliphatic (Table 2, entries 3-5, 12, 13, 22, 23, 25, 27), cyclic (Table 2, entries 7, 8, 14, 15, 20, 28) or heterocyclic (Table 2, entry 18) substituent at the C-4 position of the triazole ring, was obtained.

Regarding the mechanism of the transformation of the 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts **3** into **5**, we presume that the reaction proceeds via an unusual Dimroth-type rearrangement[15] (Scheme 2).

Scheme 2. Proposed mechanism for the one-pot formation of the 2-AIT framework.



In the first step at lower temperatures the 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salt **3** undergoes mild cleavage of the pyrimidinium ring via intermediate **A**, resulting in the generation of the pyrazole and 2-amino-5-hydroxyimidazolidine. Copper nanoparticles[14] generated by reduction of $Cu(OAc)_2$ with hydrazine hydrate at elevated temperature catalyse the CuAAC and generate intermediate **B**. The latter is in equilibrium with the open form **C**, which cyclizes to the more stable rearranged 2-AIT **5** at high temperature.

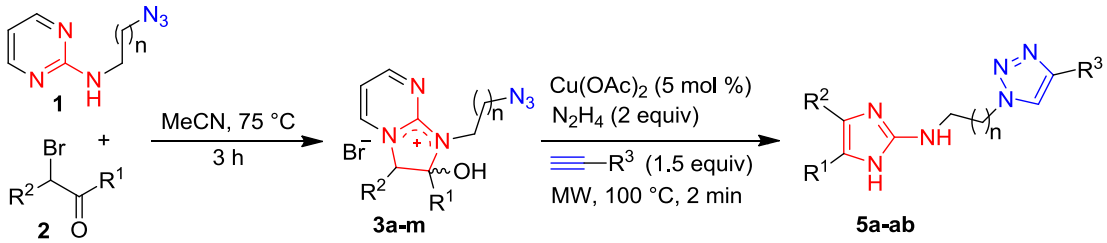
Anti-biofilm activity of 2-Amino-1*H*-imidazole/Triazole Conjugates and intermediates of their synthesis

Compounds **5a-ab** were assayed for their ability to prevent the biofilm formation of *Salmonella* Typhimurium ATCC14028 and *Pseudomonas aeruginosa* PA14 in TSB 1/20 at 25 °C, mimicking conditions outside the host. As indicated in Table 2 and Table S1, many of the compounds were active against both *Salmonella* and *Pseudomonas* with BIC_{50} values (i.e. IC_{50} for biofilm inhibition) in the range of 10-40 µM. Compounds **5g**, **5h**, **5s**, **5z** and **5ab** inhibited *Salmonella* biofilm formation even stronger, with BIC_{50} values between 2 and 8 µM. These activities are similar to those of the most potent *2N*-substituted 5-aryl-2-amimoimidazoles previously reported.[2d] To validate that the compounds are specific inhibitors of biofilm formation and not acting as bactericidal agents, also their effect on the planktonic growth was measured. IC_{50} values for planktonic growth inhibition are provided in Table 2, while effects on the growth curve are provided in Table S1. Most of the compounds are specific biofilm inhibitors with a broad difference between BIC_{50} and IC_{50} values. However, for some of the most active compounds, i.e. **5g** and **5s**, it cannot be excluded that the biofilm inhibition is at least partly due to killing the planktonic bacteria before biofilms could be established.

More detailed analysis of the data allows to draw a number of conclusions with regard to structure-activity relationship. (i) Comparison of compounds **5a**, **5c**, **5l**, **5v** with respectively **5b**, **5d**, **5m**,**5w** indicates that the length (n= 2 or 3) of the alkyl linker between triazole and 2-aminoimidazole does not markedly affect the activity. (ii) However, the activity is strongly dependent on the nature of the substituents at the 4(5)-position of the imidazole ring (R^1 and R^2). Indeed, compounds **5v** and **5w**, in which R^1 is a morpholinomethanone group, are not active at the highest concentration tested (400 µM), while compounds **5c**, **5d**, **5l** and **5m**, which have a halogenated phenyl group as R^1 (but are further identical to **5v** and **5w**), have BIC values around 50 µM. (iii) Also the substituent at the triazole ring (R^3) is of major importance as strong differences in activity were observed within series of compounds with the same R^1 and R^2 groups, i.e. compounds **5c**-**5k**, **5l**-**5s, 5t**-**5u** and **5y**-**5z**. (iv)

Remarkably, compound **5l** , in which R1 is 3,4-dichorophenyl and R^3 is propyl, has a good activity against both *Salmonella* and *Pseudomonas* biofilms. This while compound **A** (Figure 2), which has a side chain of the same length at the 2*N*-position of the imidazole, but lacks the triazole moiety, was previously shown to be inactive.[2d] Also, the compound **5t** in which R^1 is 4-fluorophenyl and R^3 is c-propyl, has a moderate activity against biofilms, while 4(5)-(4-fluorophenyl)-2-aminoimidazoles with long alkyl chain (>C5) at the 2*N*-position were previously shown to be inactive against *Salmonella* biofilms.[2d] This suggests that introduction of the triazole moiety in the long alkyl chains at the 2*N*-position of the 2-aminoimidazoles can increase their activity.

Table 2. Scope of the microwave-assisted one-pot synthesis of the 2-AIT framework and anti-biofilm activity against *S.* Typhimurium and *P. aeruginosa*.[a]



Entry	Compound	R^1	R^2	n	R^3	Yield of **3** (%)	Yield of **5** (%)	Anti-biofilm activity							
								Salmonella Typhimurium ATCC14028				*Pseudomonas aeruginosa* PA14			
								Compounds **3**		Compounds **5**		Compounds **3**		Compounds **5**	
								BIC_{50}[b] (μM)	IC_{50}[c] (μM)	BIC_{50} (μM)	IC_{50} (μM)	BIC_{50} (μM)	IC_{50} (μM)	BIC_{50} (μM)	IC_{50} (μM)
1	**3a, 5a**	Ph	H	2	Ph	83	91	71,7	>400	40,0	>400	23,5	365,5	19,0	>400
2	**3b, 5b**	Ph	H	1	Ph	89	84	142,6	>400	36,5	>400	29,3	>400	>400	>400
3	**3c, 5c**	4-BrC_6H_4	H	1	Pr	90	94	45,1	162,6	25,3	~ 94,5	43,6	>400	48,7	>400
4	**3d, 5d**	4-BrC_6H_4	H	2	Pr	83	75	19,5	>400	35,3	~ 96,3	63,3	133,1	27,2	>400
5	**3c, 5e**	4-BrC_6H_4	H	1	Hept	90	73			188,8	>400			>400	>400
6	**3d, 5f**	4-BrC_6H_4	H	2	$C(CH_3)_2(NH_2)$	83	75			30,9	~ 91,4			21,5	20,1
7	**3c, 5g**	4-BrC_6H_4	H	1	c-Pr	90	80			2,0	2,4			71,6	>400
8	**3c, 5h**	4-BrC_6H_4	H	1	c-Hex	90	71			8,4	18,5			12,5*	26,2
9	**3c, 5i**	4-BrC_6H_4	H	1	4-MeC_6H_4	90	73			>400	>400			>400	>400
10	**3d, 5j**	4-BrC_6H_4	H	2	4-pentylC_6H_4	83	80			>400	>400			>400	>400
11	**3c, 5k**	4-BrC_6H_4	H	1	4-$MeOC_6H_4$	90	66			91,2	>400			42,7	>400
12	**3e, 5l**	3,4-diClC_6H_3	H	1	Pr	75	85	15,3	>400	35,6	>400	46,6	196,0	27,4	>400
13	**3f, 5m**	3,4-diClC_6H_3	H	2	Pr	77	91	12,5	>400	31,8	60,9	50,5	120,3	24,4	>400
14	**3e, 5n**	3,4-diClC_6H_3	H	1	c-Hex	75	89			55,2	>400			~25*	>400

15	**3f, 5o**	3,4-diClC_6H_3	H	2	c-Hex	77	75			26,8	>400			>400	>400
16	**3f, 5p**	3,4-diClC_6H_3	H	2	4-*tert*BuC_6H_4	77	85			17,8	>400			~12,5*	>400
17	**3e, 5q**	3,4-diClC_6H_3	H	1	4-heptylC_6H_4	75	84			>800	>400			>400	>400
18	**3f, 5r**	3,4-diClC_6H_3	H	2	CH_2NMe	77	81			10,8	67,5			8,1	6,7
19	**3f, 5s**	3,4-diClC_6H_3	H	2	thiophen-3-yl	77	91			2,0	5,4			3,8**	>400
20	**3g, 5t**	4-FC_6H_4	H	1	c-Pr	69	80	172,8	>400	93,3	>400	19,0	>400	~50*	>400
21	**3g, 5u**	4-FC_6H_4	H	1	c-Pr-CH_2	69	68			128,3	>400			32,9	>400
22	**3h, 5v**	morpholino-methanone	H	1	Pr	76	39	>400	>400	>400	>400	332,8	>400	>400	>400
23	**3i, 5w**	morpholino-methanone	H	2	Pr	65	45	>400	>400	>400	>400	349,2	>400	>400	>400
24	**3j, 5x**	naphth-2-yl	H	1	4-BuC_6H_4	69	64	22,4	66,3	>400	>400	42,6	189,1	>400	>400
25	**3k, 5y**	$CHPh_2$	H	1	*tert*Bu	72	67	34,1	>400	30,9	~ 91,0	23,1	210,6	10,9	137,7
26	**3k,5z**	$CHPh_2$	H	1	c-Pen	81	68			8,4	>400			>400	>400
27	**3l, 5aa**	Ph	Ph	2	Hept	75	84	169,4	>400	10,8	>400	~200*	>400	>400	>400
28	**3m,5ab**	4-ClC_6H_4	4-Me C_6H_4	1	c-Pen	72	56	49,1	>400	6,5	>400	115,2	~ 384,6	>400	>400

[a]All reactions were conducted on a 0.25 mmol scale of **3b-m**, applying hydrazine hydrate (2 equiv), acetylene (1.5 equiv), $Cu(OAc)_2$ (5 mol%) in EtOH/H_2O (4:1) (1 mL); the mixture was irradiated in a sealed tube at a ceiling temperature of 100 °C and 35 W maximum power for 2 min; isolated yields are given.

[b]BIC_{50}: compound concentration at which the biofilm formation is inhibited with 50%; 95% confidence intervals are provided in Table S1.

[c]IC_{50}: compound concentration at which the planktonic growth is inhibited with 50%; 95% confidence intervals are provided in Table S1. Effect of the compounds on the planktonic growth curves are also provided in Table S2.

*The compound is not able to completely prevent biofilm formation, as the dose response curve reaches a steady state level at about 50% biofilm inhibition.

**With increasing concentrations, the dose response curve reaches a maximum of 90 % biofilm inhibition at a concentration of ~25 µM. At higher concentrations the % inhibition decreases again.

As indicated in Tables 2 and S1, also most of the 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts **3** (except for **3h** and **3j**), showed activity against both *Salmonella* and *Pseudomonas* biofilms, with BIC_{50} values between 10 to 150 µM (Table 2). None of the compounds (except for **3j**) had an effect on the planktonic growth at the BIC_{50}, indicating that they have a specific anti-biofilm activity. These compounds thus have a similar activity range and profile as the previously described 1-pentyl- and 1-hexyl-2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts, which have a side chain of the same length at the 1-position, but lack the azide function. As indicated in scheme **1**, 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts can be cleaved *in vitro* with a nucleophile such as hydrazine to yield 2-aminoimidazoles. We previously hypothesized that the activity of the 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts

could be explained by (a partial) *in situ* cleavage of these salts by cellular nucleophiles to form the active 2-aminoimidazoles. Consistently, compound B (Figure 2), which is formed after cleavage of compound **3m**, inhibits the biofilm formation of *Salmonella* and *Pseudomonas* at BIC_{50} values which are slightly lower than those of compound **3m**.

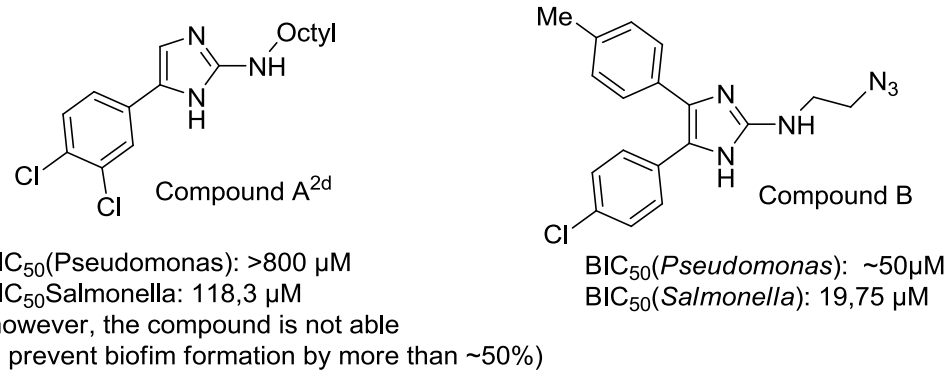


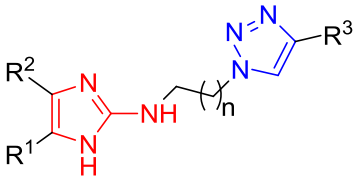
Figure 2. A. 2-octyl-4(5)-(m,p-dichlorophenyl)-2-aminoimidazole, previously reported to be inactive against *Salmonella* and *Pseudomonas* biofilms.[2d] B. 2*N*-(2-azidoethyl)-4-(p-chlorophenyl)-5-(p-methylphenyl)-2-aminoimidazole, formed after cleavage of compound **3m**.

Synthesis and anti-biofilm activity of 2-Amino-1*H*-Imidazole/Triazole Conjugates with alkyl chain substitutions at the triazole ring

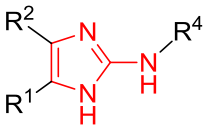
We previously reported that 5-phenyl-2-aminoimidazoles bearing a long alkyl chain (C6-C9) at the 2*N*-position in many cases have a low activity against biofilms, depending on the substitution pattern of the 5-phenyl ring and the model organism studied.[2d] The results above suggest that incorporation of a triazole moiety in this long alkyl chain can strongly enhance the activity. To further consolidate this finding, we synthesized an array of 2-AIT **5ac-aj** (n=1 or 2) in which R^3 ranges from butyl to heptyl, and compared their anti-biofilm activities with these of respectively 5-aryl-2-aminoimidazoles **6a-i**, bearing 2*N*-alkyl chains with the same total length. Next to *S.* Typhimurium and *P. aeruginosa*, also *Escherichia coli* and *Stapylococcus aureus* were included in these tests. As indicated in Tables 3 and S2, introduction of the triazole moiety makes the compounds in general (except for **5ae** and **5aj**) much more active (up to >100 times) against *S.* Typhimurium and *P. aeruginosa* biofilms. Most of the 2-AIT have a broad concentration range between BIC_{50} and IC_{50}, indicating that they specifically affect *Salmonella* and *Pseudomonas* biofilm formation. Incorporation of the triazole moiety makes the compounds also much more potent against *E. coli* (except for **5ae**). However, the concentration range between BIC_{50} and IC_{50} is in general much narrower. Finally, in case of *S. aureus*, the effect of introducing a triazole moiety is much less

pronounced. Both imidazoles and 2-AIT inhibit biofilm formation in an aspecific way, as *S. aureus* biofilm formation and planktonic growth are affected at similar concentrations.

Table 3. Effect of incorporation of a triazole moiety in the long 2*N*-alkyl chain of 5-aryl-2-aminoimidazoles on the anti-biofilm activity against *S.* Typhimurium, *E. coli*, *P. aeruginosa* and *S. aureus*.



5ac-aj



6a-h

compound	R^1	R^2	n	R^3	R^4	*S.* Typhimurium ATCC14028		*E. coli* TG1		*P. aeruginosa* PA14		*S. aureus* SH100	
						BIC_{50}[a] (µM)	IC_{50}[b] (µM)	BIC_{50} (µM)	IC_{50} (µM)	BIC_{50} (µM)	IC_{50} (µM)	BIC_{50} (µM)	IC_{50} (µM)
5ac	4-ClC_6H_4	H	2	Bu		23,9	56,9	12,2	19	5,0*	>400	~94,7	134,2
6a	4-ClC_6H_4	H			Dec	>400	>400	>400	>400	>400	>400	~ 200,8	236,0
5ad	4-FC_6H_4	H	2	Bu		186,9	>400	~100	340	3,7*	>400	304,3	390,8
6b	4-FC_6H_4	H			Dec	>400	>400	~ 199,5	~400	19,0**	>400	~75	~ 88,8
5ae	4-$OMeC_6H_4$	H	2	Bu		114,5	>400	71	~75	6,9*	159,7	~150	130,9
6c	4-$OMeC_6H_4$	H			Dec	49,70	>400	47,3	>400	>400	>400	~ 55,7	32,9
5af	3,4-diClC_6H_3	H	1	Bu		28,1	>400	6,7	~25	3,1	>400	~ 50,1	349,3
6d	3,4-diClC_6H_3	H			Non	38,2	>400	9,6	>400	14,9**	>400	~ 70,9	43,4
5ag	3,4-diClC_6H_3	H	2	Bu		41,5	>400	10,3	~25	6,6*	>400	45,7	125,4
6e	3,4-diClC_6H_3	H			Dec	46,5	>400	44,8	~50	>400	>400	~ 91,4	43,9
5ah	naphth-2-yl	H	1	Bu		137,2	>400	52,7	>400	3,6*	>400	>400	>400
6f	naphth-2-yl	H			Non	>400	>400	>400	>400	>400	>400	~201,9	440,8
5ai	naphth-2-yl	H	1	Pen		9,7	>400	13,2	~25	1,3*	>400	36,7	109,5
6g	naphth-2-yl	H			Dec	>400	>400	187,6	>400	>400	>400	~ 141,1	141,4
5aj	$CHPh_2$	H	1	Hept		31,9	>400	40,2	~100	37,4	>400	51,9	~ 51,0
6h	$CHPh_2$	H			Dodec	>400	>400	~200	>400	24,61*	>400	~ 55,0	42,1

[a]BIC_{50}: compound concentration at which the biofilm formation is inhibited with 50%; 95% confidence intervals are provided in Table S2.

[b]IC_{50}: compound concentration at which the planktonic growth is inhibited with 50%; 95% confidence intervals are provided in Table S2.

*With increasing concentrations, the dose response curve reaches a maximum of 70 to 90 % biofilm inhibition at a concentration between 6,25 and 50 µM. At higher concentrations the % inhibition decreases again.

**With increasing concentrations, the dose response curve reaches a maximum of 50 to 60 % biofilm inhibition at a concentration between 12,5 and 25 µM. At higher concentrations the % inhibition decreases again.

Experimental

General procedure for the preparation of 2-aminoimidazole-triazoles (2-AIT)

To a cooled (0 °C) solution of hydrazine hydrate (2 equiv) in EtOH (0.8 mL) was added a solution of $Cu(OAc)_2$ (5 mol%) in water (0.2 mL) and the mixture was stirred for 2 min at 0 °C. Then acetylene (1.5 equiv, 0.38 mmol) and hydroxy salt **3** (0.25 mmol) were added, the vial was sealed and the mixture was irradiated (microwaves) at a ceiling temperature of 100 °C applying a maximum power 35W for 2 min. After completion of the reaction, the solvent was removed under reduced pressure. The crude product was purified by column chromatography over silica gel using DCM/MeOH/NH_3 (7N soln. in MeOH) (96:3:1) as the eluent.

Static Peg Assay for Prevention of Biofilm Formation

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).[16] Two-fold serial dilutions of the compounds in 100 μL of liquid broth per well were prepared in the microtiter plate (two or three repeats per compound). For *S.* Typhimurium, *E. coli*, and *P. aeruginosa* Tryptic Soy Broth diluted 1/20 (TSB 1/20; BD Biosciences) was used, while for *S. aureus* undiluted TSB was used. Subsequently, overnight cultures (grown in Luria−Bertani medium)[17] of *S.* Typhimurium ATCC14028, *P. aeruginosa* PA14, *E. coli* TG1 and *S. aureus* TH1 were 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 of medium per well. The pegged lid was placed on the microtiter plate, and the plate was incubated for 24 h at 25°C (S. Typhimurium, *P. aeruginosa*, *E. coli*) or 48 h at 37°C (*S. aureus*) without shaking. During this incubation period, biofilms were formed on the surface of the pegs. After incubation, the optical density at 600 nm (OD_{600}) was measured for the planktonic cells in the microtiter plate using a microtiter plate reader (Multimode Synergy MX, Biotek). The IC_{50} value for planktonic growth inhibition was determined for each compound from the concentration gradient by using the GraphPad software of Prism. 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 of phosphate buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 μL of 0.1% (w/v) crystal violet in an 2-propanol/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 of 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 the Multimode Synergy MX, Biotek . The BIC_{50} value (i.e. IC50

for biofilm inhibition) for each compound was determined from the concentration gradient by using the GraphPad software of Prism.

The effect of the chemical compounds on the growth curve of *S.* Typhimurium and *P. aeruginosa* was assayed by using the Bioscreen device (Oy Growth Curves AB Ltd.) An overnight culture of *S.* Typhimurium ATCC14028 or *P. aeruginosa* was diluted 1:100 in liquid 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. Three μL of each diluted stock solution was added to the wells (containing the 300 μL of bacterial culture) in 3-fold. As a control, 3 μL of the appropriate solvent was also added to the plate in 3-fold. The microtiter plate was incubated in the Bioscreen device at 25 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 reduced, indicated by the symbol "+".

(3)The planktonic growth is completely or almost completely inhibited, indicated by the symbol "o".

Conclusions

In conclusion we have elaborated a microwave-assisted one-pot protocol for the generation of the 2-AIT framework starting from our previously described 2-hydroxy-2,3-dihydro-1*H*-imidazo[1,2-*a*]pyrimidin-4-ium salts. The process combines a copper nanoparticle-catalyzed azide-alkyne cycloaddition and a Dimroth-type rearrangement. The applicability of the protocol has been demonstrated by the synthesis of a library of 37 compounds. The synthesized 2-amino-1*H*-imidazole/triazole conjugates showed moderate to high inhibitory activity against biofilms of *S.* Typhimurium, *P. aeruginosa*, *E. coli* and *S. aureus*, which provides a lead structure for further design and development of novel biofilm inhibitors.

Acknowledgments

Support was provided by the Research Fund of the KU Leuven under grant BOF-IDO/11/008, by the Institute for the Promotion of Innovation through Science and Technology in Flanders under grant IWT-SBO 120050, and by the Fund for Scientific Research (FWO) – Flanders (Belgium). H.S. and D.E. are grateful to the Fund for Scientific Research (FWO) – Flanders (Belgium) for obtaining a postdoctoral fellowship. B.S. is grateful to Erasmus Mundus External Cooperation Window Lot 15

India (EMECW15) for obtaining a scholarship. U.K.S. is grateful to the Research Fund of KU Leuven for obtaining an F+ postdoctoral fellowship. T.T.T.T. is grateful to the 322-program of Vietnamese Government for obtaining a PhD-grant.

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