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Synthesis and antimicrobial activities of N^6-hydroxyagelasine analogs and revision of the structure of ageloximes

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ABSTRACT

(+)-N^6-Hydroxyagelasine D, the enantiomer of the proposed structure of (−)-ageloxime D, as well as N^6-hydroxyagelasine analogs were synthesized by selective N-7 alkylation of N^6-[*tert*-butyl(dimethyl)silyloxy]-9-methyl-9*H*-purin-6-amine in order to install the terpenoid side chain, followed by fluoride mediated removal of the TBDMS-protecting group. N^6-Hydroxyagelasine D and the analog carrying a geranylgeranyl side chain displayed profound antimicrobial activities against several pathogenic bacteria and protozoa and inhibited bacterial biofilm formation. However these compounds were also toxic towards mammalian fibroblast cells (MRC-5). The spectral data of N^6-hydroxyagelasine D did not match those reported for ageloxime D before. Hence, a revised structure of ageloxime D was proposed. Basic hydrolysis of agelasine D gave (+)-*N*-[4-amino-6-(methylamino) pyrimidin-5-yl]-*N*-copalylformamide, a compound with spectral data in full agreement with those reported for (−)-ageloxime D.

1. Introduction

Several bioactive secondary metabolites from marine sponges can be classified as purine-terpene hybrids. Important sub-classes are agelasines and ageloximes (Fig. 1). Agelasines are isolated from marine sponges of the genus *Agelas* and are associated with bioactivities like antimicrobial and cytotoxic effects as well as contractive responses of smooth muscles and inhibition of Na,K-ATPase.[1] Ageloximes, also isolated from *Agelas* sp., are claimed to be N^6-hydroxyagelasines. To date isolation of (−)-ageloxime B[2] from *A. mauritania* and (−)-ageloxime D[3] from *A. nakamurai* has been reported (Fig. 1). The ageloximes are named after the corresponding agelasines, purinium salts with a primary amino group in the purine 6-position.

(−)-Ageloxime D was reported to inhibit biofilm formation from *Staphylococcus epidermidis*, but did not inhibit the growth of the planktonic bacteria (MIC > 45 μM). (−)-Agelasine D, on the other hand, did not inhibit biofilm formation, but displayed potent growth inhibition against *S. epidermidis* (MIC < 0.09 μM),[3] indicating that the oxime substituent on C-6 in ageloximes is important for biofilm inhibition and at the same time reduces toxicity towards the bacteria. However, it was later reported that both (−)-ageloxime B and (−)-ageloxime D displayed antimicrobial activity against other microorganisms (*Cryptococcus neoformans* and *Staphylococcus aureus*; MICs ca. 10 μg/mL (ca. 23 μM, calculated from the Mw's of the revised structures).[2] Bacteria capable of forming biofilms are often less sensitive to antibiotics compared to planktonic organisms and there is a current need for efficient and non-toxic biofilm inhibitors.[4]

We have previously showed that synthetic analogs of agelasines, carrying a simpler terpenoid side chain (*i.e.* geranylgeranyl) and/or an alkoxy substituent at N^6 display just as high antimicrobial activities as the natural products.[5] Thus we wanted to develop an efficient synthetic route to ageloximes and analogs and compare their antimicrobial activity as well as ability to inhibit biofilm formation with the corresponding agelasines and N^6-alkoxyagelasines. As a continuance of our synthetic studies towards bioactive purine-terpene hybrids,[5,6] we now report the first synthesis of N^6-alkoxyagelasine analogs and their antimicrobial activities. We also propose a revised structure of ageloxime D.

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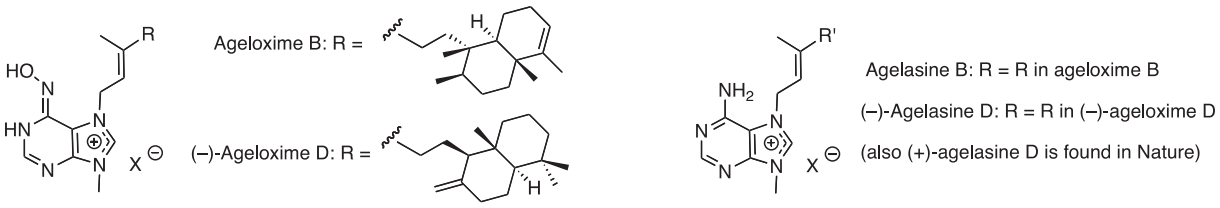
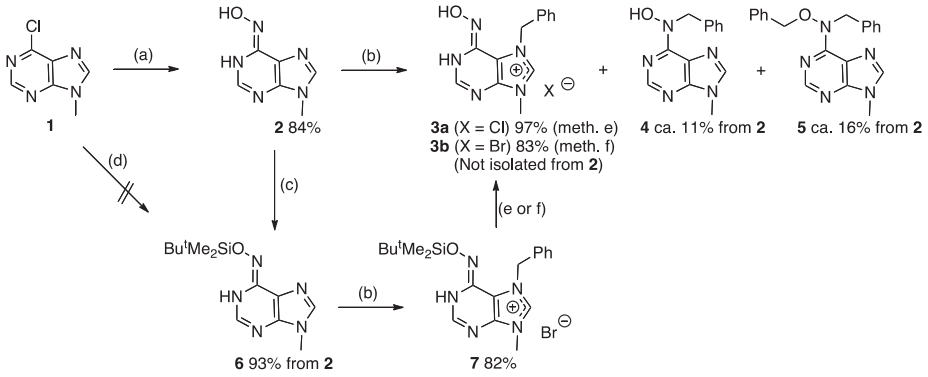


Fig. 1. Proposed structures of ageloximes B and D and structures of agelasine B and D.



Scheme 1. Reagents and conditions: (a) $HONH_2$·HCl, KOH, EtOH; (b) $PhCH_2Br$, DMA, 50 °C; (c) Bu^tMe_2SiCl, imidazole, DMF; (d) $Bu^tMe_2SiONH_2$, Et_3N, *n*-BuOH, Δ; (e) HCl(aq), MeOH, Δ; (f) NH_4F, MeCN, H_2O.

2. Results and discussion

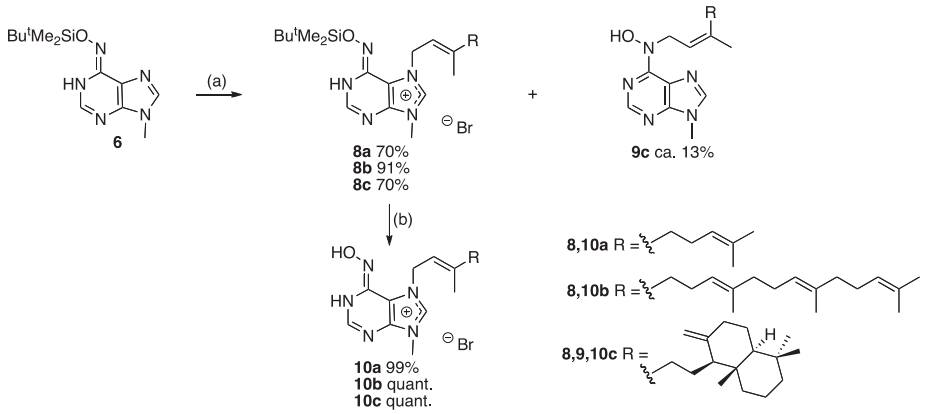
2.1. Chemistry

First we looked into methods for selective introduction of a side chain at N-7 on 6-hydroxylamino-9-methylpurine (**2**). Benzyl bromide was used as alkylating agent in these model reactions. Compound **2** was synthesized from the 6-chloropurine **1** by a modified procedure from literature (Scheme 1).[7] In this procedure, hydroxylamine was generated *in situ* by treating $HONH_2$·HCl with an excess of KOH in ethanol. However, we found that excess base resulted in partial hydrolysis of the starting material to give 9-methylhypoxanthine[8] whereby the desired product **2** could only be isolated in ca. 30% yield. When hydroxylamine was generated using exactly equal amounts of $HONH_2$·HCl and KOH, the oxime **2** was produced in 84% yield. We also attempted the substitution with commercially available hydroxylamine in water, but again hydrolysis of the starting material took place. Compound **2** existed as an almost 1:1 mixture of the imino (oxime; shown in Scheme 1) and amino (hydroxylamine) tautomer in DMSO-d_6 at ambient temperature. Benzylation of the oxime **2** resulted in at least three compounds; the desired product **3**, the N^6-benzylated isomer **4** and the dibenzylated compound **5**. Unfortunately we were not able to isolate the ageloxime analog **3** in pure form from this mixture. We have previously shown that a bulky alkoxy group at N^6 prevents alkylation of the exocyclic nitrogen,[6b] and we protected the hydroxyl group in compound **3** with the sterically demanding *tert*-butyldimethylsilyl (TBDMS) group to give the O-silylated derivative **6**. An attempt to synthesize compound **6** from chloropurine **1** by reaction with $Bu^tMe_2SiONH_2$ failed,[9] probably due to decomposition of the hydroxylamine derivate under reaction conditions normally used for similar reactions with alkoxyamines.[4] Compound **6** was selectively benzylated at N-7 and the O-protecting group was easily removed under acidic conditions to give the desired product **3a** in high yield. Unfortunately, acidic deprotection was more complicated when compounds with other N-7 side chains where the targets (*vide infra*), and we searched for other ways to deprotect the benzylpurinium salt **7**. TBAF mediated cleavage turned out to be sluggish and purification of the salt **3** was more complicated. CsF led to quantitative conversion into the target **3**, but removal of inorganic salts from the polar purinium salt **3** turned out to be difficult. NH_4F was initially introduced as an economical alternative to TBAF for cleavage of silyl ethers[10] and is often used for selective cleavage of *tert*-butyldiphenylsilyl ethers in the presence of TBDMS-ethers.[10,11] To our delight we found that the protecting group in compound **7** could be removed by treatment with one equivalent NH_4F in MeCN and water and pure target compound **3b** (bromide) was isolated in high yield after crystallization from MeCN. The reaction proceeded readily at ambient temperatures whereas most known examples of TBDMS ether cleavage using NH_4F have been performed at elevated temperatures (typically 40–75 °C).[12]

Next we synthesized ageloxime analogs **10** with terpenoid side chains on N-7 (Scheme 2). Compounds **10a–c** were easily formed by selective N-7 alkylation employing geranyl bromide, geranylgeranyl bromide or *anti*-copalyl bromide (the latter easily synthesized from commercially available (+)-manool via (+)-copalol/*anti*-copalol).[6a,6b] The N-alkylations could be performed at lower reaction temperatures and shorter reaction times compared to what has been used in related reactions before,[5,6a,6b,6e,6f,13] and in the syntheses of compounds **10b** and **10c** these milder reaction conditions were required in order to avoid unwanted side reactions. When compound **6** was reacted with *anti*-copalyl bromide for only 20 min at ambient temperature, ca. 13% of the desilylated N^6-substituted purine **9c** was formed together with the desired product **8c**. If the reaction mixture was allowed to stir over night, the yield of compound **8c** decreased (23%) and the yield of the by-product **9c** increased (ca. 34%). Also the desilylated product **10c** as well as unidentified compounds were observed. Compounds **9** were probably formed in minor amounts in the syntheses of compounds **8a** and **8b**, but were not isolated.

The N-7 benzylpurinium salt **7** was, as discussed above, quantitatively desilylated when treated with HCl in refluxing methanol for a couple of hours. Unfortunately, acidic deprotection of compounds **8** turned out to be more sluggish. For instance, when the copalyl derivative **8c** was reacted with acid in refluxing methanol for 70 h, the ratio between starting material **8c** and desired product **10c** was ca. 3:7 and minor amounts of an unknown product was observed. However, NH_4F mediated deprotections of compounds **8** gave all targets **10** in quantitative yields.

The structure of the target compound **10c** was established by NMR spectroscopy (Figs. 2 and 3, Table 1). The identity of the heterocyclic



Scheme 2. Reagents and conditions: (a) R-Br, DMA, r.t. or 50 °C; (b) NH_4F, MeCN, H_2O.

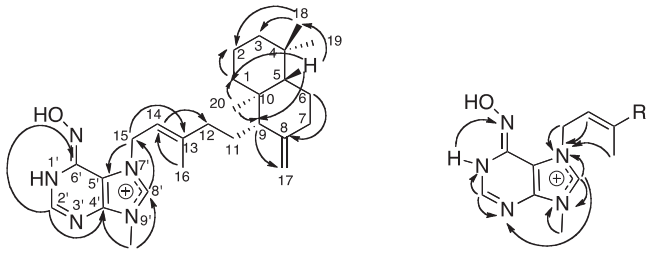


Fig. 2. Key correlations found in the ^{1}H-^{13}C HMBC spectrum (left) and ^{1}H-^{15}N HMBC spectrum (right) of compound **10c**.

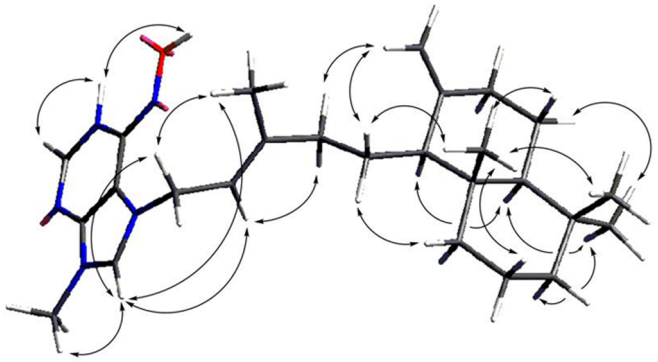


Fig. 3. Key correlations found in the 2D NOESY/1D SELNOE spectra of compound **10c**.

part of the molecule as well as the attachment of the side chains were determined from HMBC (^{1}H-^{13}C as well as ^{1}H-^{15}N) spectroscopy (Fig. 2) and the relative stereochemistry of the terpene side chain was established by 2D NOESY/1D SELNOE (Fig. 3). The heterocyclic NH was found to be localized at N-1 and the ^{13}C and ^{15}N NMR shifts of the atoms in the heterocycle were in good agreement with those reported before for 1*H*-imino tautomers of previously synthesized N^6-alkoxy agelasine analogs.[5,6b] Compounds **6**, **8** and **10** are all drawed as *syn* imines, since calculations and spectroscopic studies have shown the *syn* form of related molecules to be favored over the *anti* form.[14] Furthermore, SELNOE irradiating the NH showed that compound **10c** exists as *syn* imine in DMSO-d_6 (Fig. 3).

However, we were surprised to see that the NMR spectra of compound **10c** did not match those published for (−)-ageloxime D.[3] Literature ^{13}C NMR data (CD_3OD) are included in Table 1 for comparison. It was reported that ageloxime D exists as two tautomers in CD_3OD. Our ^{1}H NMR and ^{13}C NMR spectra of compound **10c** did only show one tautomer and looked substantially different from those published before, especially the signals from the heterocyclic part. One interesting difference was the signals for the purine H-8′/C-8′. The literature reported the H-8′ to appear at 7.95, but we could not see a signal for H-8′ in the CD_3OD ^{1}H NMR spectrum of compound **10c**. This indicated a very acidic proton at this site, being exchanged by deuterium. We also observed a triplet (^{13}C-^{2}H-coupling) for C-8′ in the ^{13}C NMR spectrum, not mentioned in the literature, which supported this observation. The acidity of H-8′ in the proposed imidazolium cation structure was also believed to be quite high. Furthermore, ageloxime B and D were reported to be protonated at N-9 in $CDCl_3$.[2,3] We found protonation of an imidazolium cation quite unlikely in a relatively weak acidic solution, and also that compound **10c** was almost insoluble in $CDCl_3$. Last but not least, the molecular ions reported for ageloxime B and D were *m/z* 440,[2,3] which corresponds to $C_{26}H_{40}N_5O+2$, whereas the molecular ion found for compound **10c** was 438 (M^+).

We suspected that the previously isolated ageloximes were in fact not N^6-hydroxyagelasines. Ageloxime D[2,3] seemed to have the same (enantiomeric) terpene side chain as our compound **10c**, but it was not likely that the heterocyclic parts were identical. Capon et al.[15] isolated formamides **12a** and **12b** (Fig. 4) and stated that they were artifacts formed during silica gel chromatography of crude sponge extracts containing the corresponding ageline A (agelasine F; **11a**) and ageline B (**11b**). Furthermore, they showed that ageline A and B could be converted to the corresponding formamides **12** when treated with aqueous base. Also other agelasines have been hydrolyzed to formamides using this protocol.[16]

Two formamidopyrimidines with terpene side chains, named axistatin 1 and 2 were isolated from the marine sponge *Agelas axifera* Hentschel.[17] The proposed structure of axistatin 2 (**12c**) is shown in Fig. 4. We found the NMR data reported for axistatin 2 and ageloxime B[2] to be essentially identical.* This led us to believe that the previously isolated ageloxime B has the same structure as axistatin 2 and that ageloxime D is the ring opened form of agelasine D. This would explain the molecular ion found for the ageloximes and the coupling between the CH_3N protons and the NH previously explained by N-9 protonation. Furthermore, presence of double NMR signals in CD_3OD could be explained by restricted rotation around the amide bond rather than the presence of two tautomers. The affected protons in the CD_3OD ^{1}H NMR spectrum[3] were those close to the formamide group earlier mistaken to be the purine H-8′. Thus we hydrolized our previously synthesized (+)-agelasine D (**11d**)[6a,6b] and obtained the formamide **12d** (Scheme 3). The spectral data for compound **12d** were identical with those previously reported for (−)-ageloxime D[3] and the optical rotation confirmed that this compound is the enatiomer of the previously reported (−)-ageloxime D. Thus we have also confirmed the absolute stereochemistry of the ageloxime reported by Hertiani et al. who just

* A table with literature NMR data of ageloxime B and axistatin 2 can be found in the Supplementary material.

Table 1
NMR data of compound **10c**. ^{13}C NMR data in CD_3OD reported for ageloxime D is included for comparison.

No.[a]	DMSO-d_6				CD_3OD			
	^{1}H NMR[b]		^{13}C NMR[c]	^{15}N NMR[d]	^{1}H NMR[b]		^{13}C NMR[c]	^{13}C NMR Ageloxime D[3]
	δ	Integration, multiplicity, *J* (Hz)	δ, DEPT		δ	Integration, multiplicity, *J* (Hz)	δ, DEPT	δ
1A, eq[e]	1.65–1.70	1H, m[f]	38.4, CH_2		1.72–1.77	1H, m[f]	40.3, CH_2	40.7
1B, ax[e]	0.88	1H, td, 3.8, 13.0			0.96	1H, td, 3.8, 13.0		
2A, ax[e]	1.48–1.54	1H, m[f]	18.8, CH_2		1.57–1.63	1H, m[f]	20.4, CH_2	20.4
2B, eq[e]	1.41–1.44	1H, m[f]			1.46–1.54	1H, m[f]		
3A, eq[e]	1.34	1H, br d, 12.9	41.6, CH_2		1.39–1.41	1H, m[f]	43.3, CH_2	43.3
3B, ax[e]	1.10	1H, td, 4.0, 13.4			1.19	1H, td, 4.0, 13.5		
4			33.2, C				34.5, C	40.2
5	0.99	1H, dd, 2.6, 12.6	54.7, CH		1.05	1H, dd, 2.7, 12.6	57.0, CH	56.8
6A, eq[e]	1.65–1.70	1H, m[f]	23.9, CH_2		1.72–1.77	1H, m[f]	25.6, CH_2	25.6
6B, ax[e]	1.22	1H, dq, 4.2, 12.9			1.31	1H, dq, 4.2, 12.9		
7A, eq[e]	2.33	1H, ddd, 3.4, 4.0, 12.7	37.6, CH_2		2.36	1H, ddd, 3.4, 4.0, 12.7	39.4, CH_2	39.4
7B, ax[e]	1.83–1.88	1H, m[f]			1.88	1H, ddd, 5.0, 13.0, 13.0		
8			148.0, C				149.7, C	149.8
9	1.48–1.54	1H, m[f]	55.1, CH		1.57–1.64	1H, m[f]	57.1, CH	58.3
10			39.2, C				40.7, C	34.5
11A	1.54–1.59	1H, m[f]	20.9, CH_2		1.65–1.70	1H, m[f]	22.5, CH_2	41.6
11B	1.41–1.44	1H, m[f]			1.46–1.54	1H, m[f]		
12A	2.11	1H, ddd, 4.0, 9.4, 14.0	37.7, CH_2		2.23	1H, ddd, 4.3, 9.1, 14.0	39.2, CH_2	25.6
12B	1.83–1.88	1H, m[f]			1.96	1H, dt, 8.1, 14.0		
13			144.1, C				147.2, C	145.4/144.4[g]
14	5.37	1H, qt, 1.0, 7.2	116.5, CH		5.43	1H, qt, 1.0, 7.2	117.4, CH	117.9/118.4
15A	4.98	1H, dd, 7.2, 14.8	47.1, CH_2		5.01–5.10	2H, m	49.0, CH_2	45.9/41.6
15B	4.94	1H, dd, 7.2, 14.8						
16	1.76	3H, d, 1.0	16.7, CH_3		1.84	3H, d, 1.0	17.0, CH_3	16.0/16.2
17A	4.81	1H, td, 1.1, 1.5	106.4, CH_2		4.82	1H, q, 1.5	107.0, CH_2	107.0
17B	4.48	1H, br s			4.52	1H, q 1.0		
18	0.83	3H, s	33.3, CH_3		0.87	3H, s	34.1, CH_3	34.1
19	0.75	3H, s	21.5, CH_3		0.81	3H, s	22.1, CH_3	20.4
20	0.61	3H, s	14.3, CH_3		0.69	3H, s	15.0, CH_3	15.1
1′ NH	11.00	1H, br s		−240.0	–[h]			
2′	7.79	1H, s	148.9, CH		7.74	1H, s	149.9, CH	157.7/157.3
3′				−179.5				
4′			140.7, C				142.5, C	161.5/160.6
5′			110.6, C				112.9, C	97.2/99.2
6′			136.9, C				137.9, C	162.0/160.6
6′-NOH	10.58	1H, br s		−105.5	–[h]			
7′				−208.5				
8′	9.26	1H, s	136.7, CH		–[h]		137.3, C[i]	165.9/166.5
9′				−218.5				
9′-CH_3	3.78	3H, s	31.6, CH_3				32.1	28.2

[a] For numbering of the atoms, see Fig. 2.
[b] ^{1}H NMR data were obtained at 600 MHz.
[c] ^{13}C NMR data were obtained at 150 MHz.
[d] ^{15}N NMR data were obtained at 60 MHz.
[e] Equatorial/axial positions in a (double) chair conformation, obtained by 2D NOESY/1D SELNOE.
[f] Unresolved or overlapping.
[g] Double set of signals were observed and explained by tautomery.[3]
[h] Exchangeable H not seen in CD_3OD.
[i] The carbon resonance appeared as a triplet.

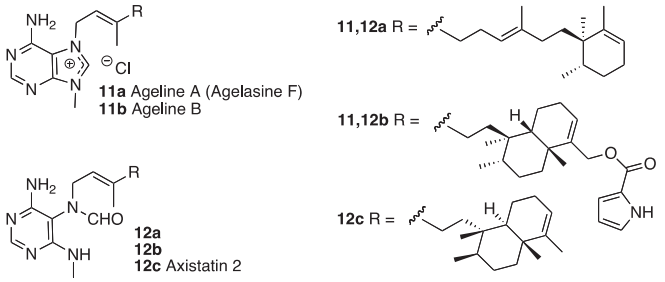


Fig. 4. Structures of agelasines **11** and ring opened derivatives/axistatins **12**.

assumed that the compound they named (−)-ageloxime D would have the same absolute stereochemistry as (−)-agelasine D.[3]

2.2. Biology

Even though the synthetic products **10** turned out not to be *ent*-ageloxime D and analogs, we chose to examine their activities against a variety of microorganisms (Table 2). The geranyl derivative **10a** was essentially inactive against bacteria (*S. aureus*, *Escherichia coli* and *Mycobacterium tuberculosis*) and yeast (*Candida albicans*) and showed only a modest inhibitory activity against protozoa (*Trypanosoma cruzi*, *T. brucei*, *T. rhodesiense* and *Leishmania infantum*). This is in line with our previous findings; a certain size of the lipophilic substituent on N-7 is required for antimicrobial activities of agelasine analogs.[5,18]

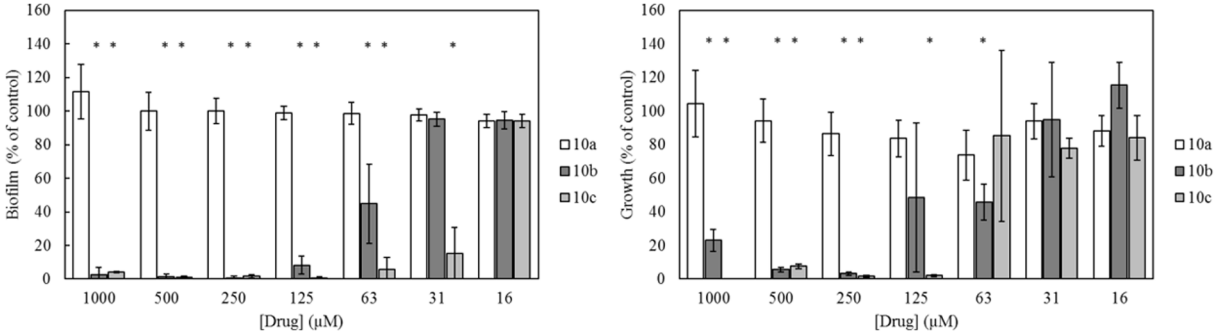
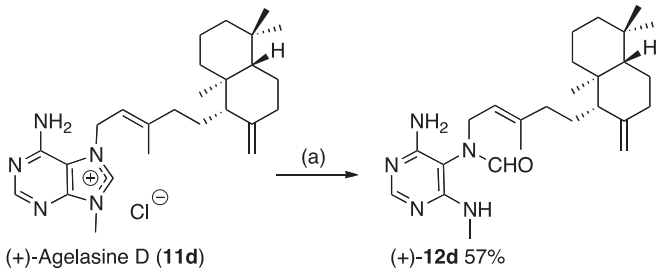


Fig. 5. Effect of compounds **10a**, **10b** and **10c** on biofilm (left) and growth (right) of *S. epidermidis*. Shown are averages and 95% confidence intervals of at least two independent experiments carried out in at least two technical replicates per experiment. * indicates statistical significant difference compared to the control (two-tailed students *t*-test, $p < 0.05$).



Scheme 3. Reagents and conditions: (a) $NaHCO_3$, H_2O.

Table 2
Antimicrobial activities of compounds **10**.

	Compound **10a**	Compound **10b**	Compound **10c**
S. aureus, IC_{50} (μM)[a]	> 64.0	1.89	1.84
E. coli, IC_{50} (μM)[b]	> 64.0	> 64.0	> 64.0
C. albicans, IC_{50} (μM)[c]	> 64.0	2.00	8.00
T. cruzi, IC_{50} (μM)[d]	11.5	0.54	0.53
T. brucei, IC_{50} (μM)[e]	28.0	2.01	2.01
T. rhodesiense, IC_{50} (μM)[f]	8.06	0.50	0.50
L. infantum IC_{50} (μM)[g]	20.3	2.16	2.38
M. tuberculosis MIC (μg/mL) MABA assay[h]	> 50.0	11.3	3.00
M. tuberculosis MIC (μg/mL) LORA assay[i]	> 50.0	15.3	5.50
MRC-5 IC_{50} (μM)[j]	> 64.0	1.90	1.59

[a] IC_{50} doxycycline 0.03 μM.
[b] IC_{50} doxycycline 0.58 μM.
[c] IC_{50} flucytosine 0.41 μM.
[d] IC_{50} benznidazol 2.69 μM.
[e] IC_{50} suramin 0.02 μM.
[f] IC_{50} suramine 0.03 μM.
[g] IC_{50} miltefosine 11.8 μM.
[h] MIC isoniazid 0.48 μg/mL.
[i] MIC isoniazid > 128 μg/mL.
[j] IC_{50} tamoxifen 11.23 μM.

Compounds **10b** and **10c**, containing larger lipophilic side chains, displayed good to very good activities towards all microorganisms examined except for *E. coli.* The IC_{50} or MIC values are comparable with those found for other agelasine analogs with a diterpenoid side chain. As also seen for related compounds, the N^6-hydroxyagelasine analogs **10b** and **10c** were not only toxic to microorganisms but also to mammalian MRC-5_{sv2} (human lung fibroblast) cells.[5,18]

The activity of compounds **10a**, **10b** and **10c** were also tested against biofilm formation and compared to the effect on growth under the same conditions. Compound **10a** did not have any significant effect on biofilm formation or growth of *S. epidermidis*, *Pseudomonas aeruginosa* or *E. coli*,[19] with the exception of a 50% reduction in biofilm formation for *P. aeruginosa* at the highest concentration tested (1 mM) (Fig. 6). These results correlate with the antibacterial activity of the compounds measured as reduced metabolic activity (Table 2). The N^6-hydroxyagelasine analogs **10b** and **10c** reduced biofilm formation of the Gram-positive bacterium *S. epidermidis* by 90% at 125 μM and 63 μM, respectively. In comparison, growth was reduced by 50% at 125 μM and 15% at 63 μM for **10b** and **10c**, respectively.

For the Gram-negative bacteria *P. aeruginosa* and *E. coli*, the effect on biofilm formation was less pronounced. For *P. aeruginosa* (Fig. 6), compounds **10b** and **10c** only inhibited biofilm formation by 40–50% at 250 μM and reached 60% and 70%, respectively at the highest concentration tested (1 mM). However, growth was only inhibited by 20–30% at this concentration.

Similarly, compounds **10b** and **10c** inhibited biofilm formation of *E. coli* by 70% at 500 μM, but only reduced growth by approximately 20% at this concentration (Fig. 7). These results indicate a small inhibitory effect of compounds **10b** and **10c** on biofilm formation. However, since the inhibition of biofilm formation is accompanied by a reduction in growth, it is not possible to conclude from these experiments whether it is a specific anti-biofilm effect or a more general growth mediated effect. In addition, the results (Table 2 and Fig. 5) suggest that Gram-positive bacteria are more susceptible to the antibacterial effects of the compounds.

3. Conclusions

We have synthesized (+)-N^6-hydroxyagelasine D and other N^6-hydroxyagelasine by selective N-7 alkylation of N^6-[*tert*-butyl(dimethyl) silyloxy]-9-methyl-9*H*-purin-6-amine followed by efficient cleavage of the silyl ether by NH_4F. However, the spectral data of synthetically prepared (+)-N^6-hydroxyagelasine D did not match those reported for the naturally occurring (−)-ageloxime D. Instead we found that when (+)-agelasine D was treated with aqueous base, ring opening of the imidazole ring took place to give a 4-amino-6-(methylamino)pyrimidin-5-ylformamide with spectral data essentially identical to those reported for (−)-ageloxime D before. This led us to propose a revised structure for (−)-ageloxime D. This pyrimidinylformamide may be found in *Agelas* sp. or alternatively the compound is an artifact from isolation of (−)-agelasine D. The work presented herein demonstrates the value of total synthesis in order to prove, or disprove, the structure of natural products.[20] N^6-Hydroxyagelasine analogs carrying a diterpenoid side chain at N-7, displayed toxicity towards both microorganisms as well as mammalian cells. These compounds also inhibited bacterial biofilm formation, but the data indicate that this effect is likely a result of the antibacterial properties of the compounds causing reduced bacterial growth rather than a specific anti-biofilm property.

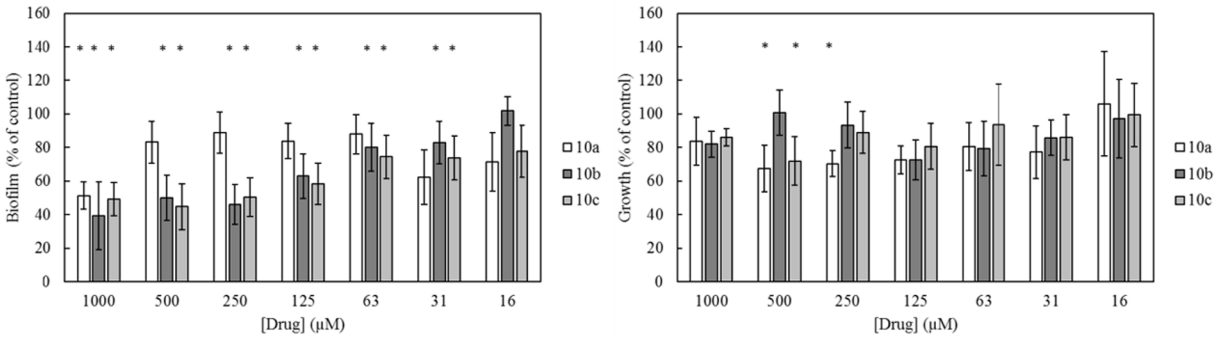


Fig. 6. Effect of compounds **10a**, **10b** and **10c** on biofilm (left) and growth (right) of *P. aeruginosa*. Shown are averages and 95% confidence intervals of at least two independent experiments carried out in at least two technical replicates per experiment. * indicates statistical significant difference compared to the control (two-tailed students *t*-test, $p < 0.05$).

4. Experimental section

4.1. Chemistry

^{1}H NMR spectra were recorded at 600 MHz on a Bruker AV 600 MHz or a Bruker AVII 600 MHz, at 400 MHz on a Bruker AVII 400 instrument. The decoupled ^{13}C NMR spectra were recorded at 150 or 100 MHz using the instruments mentioned above or at 125 MHz on a Bruker AVII 500 MHz. ^{15}N NMR spectra were recorded at 60 or 50 MHz using instruments mentioned above. ^{1}H chemical shift values are calibrated relative to internal CD_2HSOCD_3 at 2.49 ppm, CD_2HOD at 3.30 ppm or $CHCl_3$ at 7.24 ppm. ^{13}C chemical shift values are calibrated relative to internal $(CD_3)_2SO$ at 39.50 ppm, CD_3OD at 49.00 ppm or $CDCl_3$ at 77.00 ppm. ^{15}N chemical shift values are calibrated relative to external CH_3NO_2/DMSO-d_6 (9:1) at 0.00 ppm. Assignments of ^{1}H, ^{13}C and ^{15}N resonances are inferred from 1D ^{1}H NMR, 1D ^{13}C NMR, DEPT or APT, and 2D NMR (COSY, HMQC, HMBC and/or NOESY) spectroscopic data. ESI MS spectra were obtained on a Waters Micromass QTOF 2 W instrument. Melting points were determined on a Büchi Melting Point B-545 apparatus. Dry DMF were obtained from a solvent purification system, MB SPS-800 from MBraun, Garching, Germany. DMA was distilled over BaO prior to use, and *n*-BuOH was distilled over BaO and stored over 3 Å molecular sieves. Triethylamine was distilled from CaH_2 and stored over molecular sieves (3 Å). Flash chromatography was performed on silica gel Merck, Darmstadt, Germany (Merck No. 09385). Compounds available by literature methods: Geranyl bromide,[5a] geranylgeranyl bromide,[5a] 6-chloro-9-methyl-9*H*-purine (**1**),[21] *anti*-copalyl bromide,[6a,6b] (+)-agelasine D (**11d**).[6a,6b]

4.1.1. 6-Hydroxylamino-9-methyl-9H-purine (2)

A warm solution of KOH (3.270 g, 58.28 mmol) in EtOH (abs., 80 mL) was added to a boiling solution of $HONH_2$·HCl (4.035 g, 58.07 mmol) in EtOH (abs., 80 mL). Immediately upon mixing of the two solutions, precipitation of KCl was observed. The resulting mixture was stirred and maintained near the boiling point for an additional 10 min. The hot reaction mixture was filtered and 6-chloro-9-methyl-9*H*-purine (**1**) (1.241 g, 8.027 mmol) in EtOH (abs., 70 mL) was added to the hydroxylamine solution. The resulting solution was kept at reflux for 4 h. The reaction mixture was cooled to ambient temperature and the precipitated product was collected by filtration. The crystals were washed with EtOH (abs., 15 mL) and dried *in vacuo*; yield 992 mg (84%), colorless crystals, mp 233–234 °C. (lit.[8] 244 °C). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.96, 10.24 (s, 1H, OH taut. A and B), 9.49, 9.00 (s, H, NH taut. A and B), 8.25, 8.11, 7.71, 7.52 (s, 1H, H-2 and H-8, taut A and B), 3.73, 3.62 (br s, 3H, CH_3 taut. A and B); HRMS (ESI) found 166.0732, calcd for $C_6H_8N_5O^+$ 166.0723.

4.1.2. N^6-Benzyl-N^6-hydroxy-9-methyl-9H-purin-6-amine (4) and N^6-benzyl-N^6-benzyloxy-9-methyl-9H-purin-6-amine (5)

Compound **2** (81 mg, 0.49 mmol) was dissolved in dry DMA (8 mL) at ambient temperature under Ar atm before benzyl bromide (0.09 mL, 0.7 mmol) was added. The mixture was stirred at 50 °C for 18 h and evaporated *in vacuo*. The products were separated by flash chromatography eluting with 5–10% MeOH saturated with NH_3 in CH_2Cl_2; yield 13 mg (ca. 11%, contained 1–2 mol% DMA) of compound **4**, purple waxy material, and 29 mg (ca. 16%, contained ca. 17 mol% DMA) of compound **5**, pale greenish oil. **4**; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (s, 1H, H-2), 7.39 (s, 1H, H-8), 7.49–7.32 (s, 5H, Ph), 6.24 (s, 1H, OH), 5.32 (s, 2H, CH_2), 3.57 (s, 3H, CH_3); MS ESI found 240.2, calcd for $C_{13}H_{14}N_5^+$-OH 240.1. **5**; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.54 (s, 1H, H-8), 7.85 (s, 1H, H-2), 7.47–7.25 (m, 10*H*, Ph), 5.23 (s, 2H, NCH_2), 5.01 (s, 2H, OCH_2), 3.85 (s, 3H, NCH_3); HRMS (ESI) found 346.1683, calcd for $C_{20}H_{20}N_5O^+$ 346.1662. Spectral data were in good agreement with those reported before.[6b]

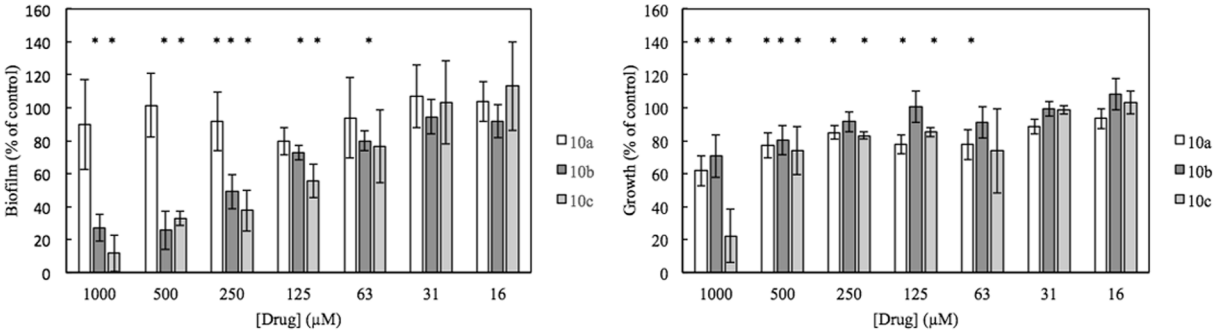


Fig. 7. Effect of compounds **10a**, **10b** and **10c** on biofilm (left) and growth (right) of *E. coli*. Shown are averages and 95% confidence intervals of at least two independent experiments carried out in at least two technical replicates per experiment. * indicates statistical significant difference compared to the control (two-tailed students *t*-test, $p < 0.05$).

*4.1.3. N^6-[tert-Butyl(dimethyl)silyloxy]-9-methyl-9H-purin-6-amine (**6**)*

A mixture of 6-hydroxylamino-9-methyl-9*H*-purine (**2**) (302 mg, 1.94 mmol), imidazole (329 mg, 4.84 mmol) and dry DMF (2.7 mL) was stirred under Ar atm before *tert*-butyldimethylsilyl chloride (307 mg, 2.03 mmol) was added. The reaction mixture was stirred at ambient temperature for 22 h, diluted with EtOAc (150 mL) and washed with water (3 × 30 mL) and brine (40 mL). The organic phase was dried (Na_2SO_4) and evaporated *in vacuo*; yield 317 mg (93%); colorless solid, mp 216–217 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.91 (br s, 1H, NH), 7.78 (s, 1H, H-8), 7.65 (s, 1H, H-2), 3.64 (s, 3H, NCH_3), 0.95 (s, 9H, Bu^t), 0.15 [s, 6H, $Si(CH_3)_2$]; ^{13}C NMR (100 MHz, DMSO-d_6) δ 144.3 (C-6), 144.1 (C-2), 141.6 (C-4), 138.8 (C-8), 118.1 (C-5), 29.5 (NCH_3), 26.2 (CH_3 in Bu^t), 18.0 (C in Bu^t), −5.0 [$Si(CH_3)_2$]; HRMS (ESI) found 280.1597, calcd for $C_{12}H_{22}N_5OSi^+$ 280.1588.

*4.1.4. 7-Benzyl-6-tert-butyl(dimethyl)silyloxyamino-9-methyl-7H-purinium bromide (**7**)*

Benzyl bromide (0.037 mL, 0.31 mmol) was added to a stirring solution of compound **6** (100 mg, 0.283 mmol) in dry DMA (5 mL) under Ar atm. The resulting mixture was stirred at ambient temperature for 1 h, and at 50 °C for 6 h and evaporated *in vacuo*. The crude product was purified by flash chromatography eluting with 5–10% MeOH in CH_2Cl_2; yield 104 mg (82%), colorless solid, mp 249–251 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (br s, 1H, NH), 9.33 (s, 1H, H-8), 7.86 (s, 1H, H-2), 7.39–7.34 (m, 5H, Ph), 5.65 (s, 2H, CH_2), 3.79 (s, 3H, NCH_3), 0.89 (s, 9H, Bu^t), 0.08 [s, 6H, $Si(CH_3)_2$]; ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.9 (C-2), 141.4 (C-4), 139.9 (C-6), 138.5 (C-8), 134.5 (C in Ph), 128.7 (CH in Ph), 128.4 (CH in Ph), 127.4 (CH in Ph), 110.6 (C-5), 52.1 (CH_2), 31.9 (NCH_3), 25.9 (CH_3 in Bu^t), 17.9 (C in Bu^t), −5.3 [$Si(CH_3)_2$]; HRMS (ESI) found 370.2057, calcd for $C_{19}H_{28}N_5OSi^+$ 370.2058.

*4.1.5. 7-Benzyl-6-hydroxylamine-9-methyl-9H-purin-7-ium chloride (**3a**)*

Compound **7** (127 mg, 0.283 mmol) was dissolved in methanol (2 mL) and 1 M HCl (aq) (1 drop) was added. The solution was stirred at reflux for 2.5 h, cooled to ambient temperature, washed with hexane (3 × 3 mL) and evaporated *in vacuo*; yield 92 mg (97%); colorless solid, mp 219–222 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.96 (br s, 1H, NH), 10.65 (s, 1H, OH), 9.39 (s, 1H, H-8), 7.82 (s, 1H, H-2), 7.41–7.37 (m, 5H, Ph), 5.63 (s, 2H, CH_2), 3.81 (s, 3H, NCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.9 (C-2), 140.8 (C-4), 137.5 (C-8), 136.6 (C-6), 134.5 (C in Ph), 128.88 (CH in Ph), 128.58 (CH in Ph), 127.92 (CH in Ph), 110.8 (C-5), 51.9 (CH_2), 31.79 (NCH_3); HRMS (ESI) found 256.1193, calcd for $C_{13}H_{14}N_5O^+$ 256.1193.

*4.1.6. 7-Benzyl-6-hydroxylamine-9-methyl-9H-purin-7-ium bromide (**3b**)*

Compound **7** (20.5 mg, 0.0453 mmol) was dissolved in MeCN-H_2O (9:1, 0.5 mL) and transferred to a vial containing NH_4F (1.3 mg, 0.035 mmol). Additional MeCN-H_2O (9:1, 0.5 mL) was used to transfer the starting material to the reaction vial. The clear and colorless solution stirred for 60 min at ambient temperature and was evaporated *in vacuo*. Dry MeCN (2 mL) was added and the resulting heterogeneous mixture was agitated by a spatula for 2 min while gently heating the solution with a heating gun. The crystals were allowed to settle by gravity (2 min) before the MeCN-phase was removed by a glass pipette. This process was repeated once more with MeCN (1 mL). The crystals were dried *in vacuo*; yield 12.7 mg (83% based on the amount of comp. **7**), colorless solid, mp 244–245 °C. Spectral data as reported for the chloride **3a** above.

*4.1.7. 6-(tert-Butyldimethylsilyloxyimino)-7-[(E)-3,7-dimethylocta-2,6-dienyl]-9-methyl-6,9-dihydro-1H-purin-7-ium bromide (**8a**)*

Geranyl bromide (265 mg, 1.12 mmol) was added dropwise over 1 min to a stirring solution of compound **6** (283 mg, 1.02 mmol) in dry DMA (10 mL) under Ar atm. The resulting mixture was stirred at ambient temperature for 10 min and at 50 °C for 18 h and evaporated *in vacuo*. The crude product was purified by flash chromatography eluting with 5–10% MeOH in CH_2Cl_2; yield 348 mg (70%), colorless solid, mp 196–197 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.90 (br s, 1H, NH), 9.37 (s, 1H, H-8 in purine), 7.92 (s, 1H, H-2 in purine), 5.49–5.46 (m, 1H, H-2), 5.07–5.03 (m, 1H, H-6), 4.97 (d, J = 7.0 Hz, 2H, H-1), 3.81 (s, 3H, NCH_3), 2.10–2.05 (m, 2H, H-5), 2.05–2.01 (m, 2H, H-4), 1.77 (s, 3H, CH_3 at C-3), 1.62 (s, 3H, H-8), 1.54 (s, 3H, CH_3 at C-7), 0.96 (s, 9H, Bu^t), 0.20 [s, 6H, $Si(CH_3)_2$]; ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.8 (C-2 in purine), 143.0 (C-3), 141.2 (C-4 in purine), 140.0 (C-6 in purine), 137.6 (C-8 in purine), 131.2 (C-7), 123.5 (C-6), 116.8 (C-2), 110.1 (C-5 in purine), 47.3 (C-1), 38.9 (C-4), 31.7 (NCH_3), 26.0 (CH_3 in Bu^t), 25.7 (C-5), 25.4 (C-8), 18.0 (C in Bu^t), 17.5 (CH_3 at C-7), 16.5 (CH_3 at C-3), −5.2 [$(Si(CH_3)_2$]; HRMS (ESI) found 416.2840, calcd for $C_{22}H_{38}N_5OSi^+$ 416.2840.

*4.1.8. 6-(tert-Butyldimethylsilyloxyimino)-9-methyl-7-[(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl]-6,9-dihydro-1H-purin-7-ium bromide (**8b**)*

Geranylgeranyl bromide (95 mg, 0.27 mmol) was dissolved in dry DMA (3 mL) under Ar atm and compound **6** (68 mg, 0.24 mmol) was added. The resulting mixture was stirred at ambient temperature for 1 h and evaporated *in vacuo*. The crude product was purified by flash chromatography eluting with 5–10% MeOH in CH_2Cl_2; yield 140 mg (91%), colorless solid, mp 170–171 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.88 (br s, 1H, NH), 9.34 (s, 1H, H-8 in purine), 7.91 (s, 1H, H-2 in purine), 5.46 (qt, J = 7.0 and 1.1, Hz, 1H, H-2), 5.05–5.02 (m, 3H, H-6, H-10, H-14), 4.96 (d, J = 7.0 Hz, 1H, H-1), 3.79 (s, 3H, NCH_3), 2.08–1.96 (m, 8H, H-4, H-5, H-9, H-13), 1.92–1.88 (m, 4H, H-8, H-12), 1.76 (s, 3H, CH_3 at C-3), 1.61 (s, 3H, H-16), 1.53 (s, 6H, CH_3 at C-7 and C-15), 1.52 (s, 3H, CH_3 at C-11) 0.95 (s, 9H, Bu^t), 0.18 [s, 6H, Si $(CH_3)_2$]; ^{13}C NMR (150 MHz, DMSO-d_6) δ 148.7 (C-2 in purine), 143.0 (C-3), 141.2 (C-4 in purine), 139.9 (C-6 in purine), 137.6 (C-8 in purine), 134.8 (C-7), 134.3 (C-11), 130.6 (C-15), 124.0 (C-14), 123.8 (C-10), 123.3 (C-6), 116.7 (C-2), 110.1 (C-5 in purine), 47.2 (C-1), 39.2 (2 × C, C-8 and C-12), 38.9 (C-4), 31.7 (NCH_3), 26.2 (2 × C, C-9 and C-13), 26.0 (CH_3 in Bu^t), 25.6 (C-5), 25.5 (C-16), 18.0 (C in Bu^t), 17.5 (CH_3 at C-15), 16.5 (CH_3 at C-3), 15.8 (2 × C, CH_3 at C-7 and C-11), −5.3 [$(Si(CH_3)_2$]; HRMS (ESI) 552.4090, calcd for $C_{32}H_{54}N_5O^+$ 552.4092.

*4.1.9. (+)-6-(tert-Butyldimethylsilyloxyimino)-9-methyl-7-{(E)-3-methyl-6-[(1S,4aS,8aS)-5,5,8a-trimethyl-2-methylenedecahydronaphthalen-1-yl] hex-2-enyl}-6,7-dihydro-1H-purin-9-ium bromide (**8c**) and (E)-N-{3-methyl-5-[(1S,4aS,8aS)-1,5,5,8a-tetramethyl-2-methylenedecahydronaphthalen-1-yl]pent-2-enyl}-N-(9-methyl-9H-purin-6-yl)hydroxylamine (**9c**)*

anti-Copalyl bromide (93 mg, 0.26 mmol) was dissolved in dry DMA (3.3 mL) under Ar atm and compound **6** (67 mg, 0.24 mmol) was added. The resulting mixture was stirred at ambient temperature for 20 min and evaporated *in vacuo*. The crude product was purified by flash chromatography eluting with 5–10% MeOH in CH_2Cl_2; yield 108 mg (70%) **8c**, colorless solid, and 15 mg (ca. 13%, contained ca. 30 mol% DMA) **9c**, waxy purple solid. **8c**; mp 194–196 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.89 (s, 1H, NH), 9.36 (s, 1H, H-8 in purine), 7.92 (s, 1H, H-2 in purine), 5.43 (qt, J = 7.2, 1.0 Hz, 1H, H-14), 5.01–4.91 (m, 2H, H-15), 4.79 (s, 1H, H-17_A), 4.47 (s, 1H, H-17_B), 3.80 (s, 3H, NCH_3), 2.32 (ddd, J = 12.7, 4.0, 3.4 Hz, 1H, H-7_A), 2.10 (ddd, J = 14.0, 9.4, 4.0 Hz, 1H, H-12_A), 1.88–1.78 (m, 2H, H-7_B, H-12_B), 1.75 (d, J = 1.0 Hz, 3H, H-16), 1.69–1.63 (m, 2H, H-1_A, H-6_A), 1.56–1.47 (m, 3H, H-11_A, H-2_A, H-9), 1.43–1.39 (m, 2H, H-2_B, H-11_B), 1.35 (br d, 1H, J = 12.9 Hz, H-3_A), 1.22 (dq, J = 12.9, 4.2 Hz, 1H, H-6_B), 1.09 (dt, J = 13.4, 4.0 Hz, 1H, H-3_B), 0.90–1.00 (m, 1H, H-5), 0.95 (s, 9H, Bu^t), 0.82–0.87 (m, 1H, H-1_B), 0.83 (s, 3H, H-18), 0.75 (s, 3H, H-19), 0.61 (s, 3H, H-20), 0.18 [s, 6H, Si $(CH_3)_2$]; ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.8 (C-2 in purine), 147.9 (C-8), 143.7 (C-13), 141.3 (C-4 in purine), 140.0 (C-6 in purine), 137.6 (C-8 in purine), 116.7 (C-14), 110.0 (C-5 in purine), 106.4 (C-17), 55.2 (C-9), 54.8 (C-5), 47.2 (C-15), 41.7 (C-3), 39.1 (C-10), 38.5 (C-1) 37.7

(C-12), 37.6 (C-7), 33.3 (C-18), 33.2 (C-4), 31.7 (NCH_3), 26.0 (CH_3 in Bu^t), 23.9 (C-6), 21.5 (C-19), 20.9 (C-11), 18.8 (C-2), 18.0 (C in Bu^t), 16.7 (C-16), 14.3 (C-20), −5.2 [$Si(CH_3)_2$]; ^{15}N NMR (50 MHz, DMSO-d_6) δ −239.5 (N-1), −218.5 (N-9), −208.5 (N-7), −176.0 (N-3), N^6 was hidden; HRMS (ESI) found 552.4092, calcd for $C_{32}H_{54}N_5OSi^+$ 552.4092, $[\alpha]_D^{25}$ = +4.2 (*c* 0.26 MeOH). **9c**; ^1H NMR (600 MHz, DMSO-d_6) δ 9.81 (br s, 1H, OH), 8.27 (s, 1H, H-2 in purine), 8.15 (s, 1H, H-8 in purine), 5.27–5.29 (m, 1H, H-14), 4.77–4.81 (m, 1H, H-15_A), 4.76 (br s, 1H, H-17_A), 4.61–4.65 (m, 1H, H-15_B), 4.44 (br s, 1H, H-17_B), 3.72 (s, 3H, NCH_3), 2.25 (br d, *J* = 11.7 Hz, 1H, H-7_A), 2.01–2.04 (m, 1H, H-12_A), 1.71–1.79 (m, 2H, H-12_B, H-7_B), 1.69 (s, 3H, H-16), 1.58–1.64 (m, 2H, H-1_A, H-6_A), 1.46–1.52 (m, 2H, H-11_A, H-9), 1.29–1.37 (m, 3H, H-2_B, H-3_A, H-11_B), 1.17 (q, *J* = 12.6 Hz, 1H, H-6_B), 1.05 (t, *J* = 12.5 Hz, 1H, H-3_B), 0.90 (d, *J* = 12.4 Hz, 1H, H-5), 0.81 (s, 3H, H-18), 0.73 (s, 3H, H-19), 0.58 (s, 3H, H-20); ^{13}C NMR (125 MHz, DMSO-d_6) δ 155.1 (C-6 in purine), 151.5 (C-2 in purine), 150.7 (C-4 in purine), 148.0 (C-8), 141.3 (C-8 in purine), 138.8 (C-13), 119.2 (C-14), 117.8 (C-5 in purine), 106.2 (C-17), 54.7 (C-9), 54.5 (C-5), 49.7 (C-15), 41.5 (C-3), 39.0 (C-10), 38.3 (C-1), 37.6 (C-12), 37.5 (C-7), 33.2 (C-18), 33.1 (C-4), 29.4 (NCH_3), 23.9 (C-6), 21.5 (C-19), 20.9 (C-11), 18.8 (C-2), 16.4 (C-16), 14.3 (C-20); ^{15}N NMR (50 MHz, DMSO-d_6) δ −229.6 (N-9), −152.2 (N-3), −148.6 (N-1), −136.8 (N-7), N^6 was hidden; HRMS (ESI) found 438.3227, calcd for $C_{26}H_{40}N_5O^+$ 438.3227.

*4.1.10. 7-[(E)-3,7-Dimethylocta-2,6-dienyl]-6-(hydroxyimino)-9-methyl-6,9-dihydro-1H-purin-7-ium bromide (**10a**)*

Compound **8a** (54 mg, 0.11 mmol) was dissolved in MeCN-H_2O (9:1, 1 mL) and transferred to a vial containing NH_4F (4 mg, 0.1 mmol) in MeCN-H_2O (9:1, 1 mL). Additional MeCN-H_2O (9:1, 0.5 mL) was used to transfer the starting material to the reaction vial. The resulting solution was stirred for 22 h at ambient temperature and evaporated *in vacuo*; yield 41 mg (99%), colorless solid, mp 187–188 °C (dec.). ^1H NMR (400 MHz, DMSO-d_6) δ 11.92 (br s, 1H, NH), 10.62 (s, 1H, OH), 9.22 (s, 1H, H-8 in purine), 7.80 (s, 1H, H-2 in purine), 5.44–5.41 (m, 1H, H-2), 5.08–5.05 (m, 1H, H-6), 4.97 (d, *J* = 7.1 Hz, 2H, H-1), 3.79 (s, 3H, NCH_3), 2.10–2.06 (m, 2H, H-5), 2.04–2.02 (m, 2H, H-4), 1.77 (s, 3H, CH_3 at C-3), 1.62 (s, 3H, H-8), 1.56 (s, 3H, CH_3 at C-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ 148.8 (C-2 in purine), 143.5 (C-3), 140.6 (C-4 in purine), 136.7 (C-6 in purine), 131.2 (C-7), 123.5 (C-6), 116.5 (C-2), 110.7 (C-5 in purine), 47.1 (C-1), 38.9 (C-4) 31.6 (NCH_3), 25.7 (C-5), 25.4 (C-8), 17.6 (CH_3 at C-7), 16.5 (CH_3 at C-3); ^{15}N NMR (50 MHz, DMSO-d_6) δ −240.0 (N-1), −218.4 (N-9), −209.2 (N-7), −179.4 (N-3), −105.5 (N^6); HRMS (ESI) found 302.1975, calcd for $C_{16}H_{24}N_5O^+$ 302.1975.

*4.1.11. 6-(Hydroxyimino)-9-methyl-7-[(2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenyl]-6,9-dihydro-1H-purin-7-ium bromide (**10b**)*

Compound **8b** (118 mg, 0.19 mmol) was dissolved in MeCN-H_2O (9:1, 4 mL) and NH_4F (6.9 mg, 0.19 mmol) was added as a powder. The vial previously containing NH_4F was rinsed with MeCN-H_2O (9:1, 2 mL). The resulting solution was stirred for 40 min and evaporated *in vacuo*; yield 99 mg (> 99%), colorless solid, mp 188–189 °C. ^1H NMR (600 MHz, DMSO-d_6) δ 11.70 (br s, 1H, NH), 9.25 (s, 1H, H-8 in purine), 7.78 (s, 1H, H-2 in purine), 5.41 (qt, *J* = 7.1, 1.1 Hz, 1H, H-2), 5.03–5.08 (m, 3H, H-6, H-10, H-14), 4.97 (d, *J* = 7.1 Hz, 1H, H-1), 3.78 (s, 3H, NCH_3), 2.10–2.07 (m, 2H, H-5), 2.05–2.02 (m, 2H, H-4), 2.00–1.96 (m, 4H, H-9, H-13), 1.91–1.89 (m, 4H, H-8, H-12), 1.77 (s, 3H, CH_3 at C-3), 1.62 (s, 3H, H-16), 1.54 (s, 3H, CH_3 at C-7), 1.53 (s, 3H, CH_3 at C-15), 1.52 (s, 3H, H-18); ^{13}C NMR (125 MHz, DMSO-d_6) δ 148.9 (C-2 in purine), 143.5 (C-3), 140.7 (C-4 in purine), 136.8 (C-6 in purine), 136.7 (C-8 in purine), 134.8 (C-7), 134.3 (C-11), 130.6 (C-15), 124.1 (C-14), 123.8 (C-10), 123.4 (C-6), 116.5 (C-2), 110.7 (C-5 in purine), 47.1 (C-1), 39.2 (C-12), 39.1 (C-8), 38.9 (C-4), 31.6 (NCH_3), 26.2 (C-13), 26.0 (C-9), 25.6 (C-5), 25.5 (C-16), 17.5 (CH_3 at C-15), 16.6 (CH_3 at C-3), 15.8 (CH_3 at C-7), 15.7 (CH_3 at C-11); ^{15}N NMR (50 MHz, DMSO-d_6) δ −239.9 (N-1), −218.4 (N-9), −208.7 (N-7), −178.5 (N-3), −105.5 (N^6); HRMS (ESI) 438.3226, calcd for $C_{26}H_{40}N_5O^+$ 438.3227.

*4.1.12. (+)-6-(Hydroxyimino)-9-methyl-7-{(E)-3-methyl-6-[(1S,4aS,8aS)-5,5,8a-trimethyl-2-methylenedecahydronaphthalen-1-yl]hex-2-enyl}-6,7-dihydro-1H-purin-9-ium bromide [(+)-N^6-Hydroxyagelasine D bromide, **10c**]*

Compound **8c** (108 mg, 0.171 mmol) was dissolved in MeCN-H_2O (9:1, 3 mL) and NH_4F (6.3 mg, 0.17 mmol) was added as a powder. The vial previously containing NH_4F was rinsed with MeCN-H_2O (9:1, 2 mL). The resulting solution was stirred for 1 h 30 min and evaporated *in vacuo*; yield 88.5 mg (> 99%), colorless solid, mp 188–189 °C. ^1H NMR, ^{13}C NMR and ^{15}N NMR data are presented in Table 1. HRMS (ESI) found 438.3228, calcd for $C_{26}H_{40}N_5O^+$ 438.3227; $[\alpha]_D^{20}$ = +6.2 (*c* 0.26 MeOH).

*4.1.13. N-[4-Amino-6-(methylamino)pyrimidin-5-yl]-N-{(E)-3-methyl-5-[(1S,4aS,8aS)-5,5,8a-trimethyl-2-methylenedecahydronaphthalen-1-yl]pent-2-enyl}formamide (**12d**)*

(+)-Agelasine D (**11d**) (25 mg, 0.050 mmol) was treated with 2 M $NaHCO_3$ solution (12.5 mL) for 20 min at ambient temperature, before the mixture was extracted with CH_2Cl_2 (3 × 50 mL), and evaporated *in vacuo*. The crude product was purified by flash chromatography eluting with 2–10% MeOH in CH_2Cl_2. Due to a low yield, the extraction was performed a second time on the basic water solution with CH_2Cl_2 (2 × 4 mL) yielding more product; total yield 13 mg (57%), pale yellow waxy solid. ^1H NMR (600 MHz, $CDCl_3$) δ 8.14 (s, 1H, H-2 in pyrimidine), 7.95 (s, 1H, NCHO), 5.30 (t, *J* = 7.7 Hz, 1H, H-14), 4.95 (br s, 2H, NH_2), 4.87 (br s, 1H, NH), 4.79 (s, 1H, H-17_A), 4.40 (s, 1H, H-17_B), 4.14–4.12 (m, 2H, H-15), 2.98–2.96 (m, 3H, NCH_3), 2.37–2.34 (m, 1H, H-7_A), 2.12–2.07 (m, 1H, H-12_A), 1.92 (ddd, *J* = 14.0, 13.4, 4.8 Hz, 1H, H-7_A), 1.76–1.67 (m, 3H, H-6_A, H-1_A, H-12_B), 1.60 (d, *J* = 5.0 Hz, 3H, H-16), 1.55–1.44 (m, 4H, H-9, H-11_A, H-2_A, H-2_B), 1.38–1.36 (m, 1H, H-3_A), 1.34–1.25 (m, 2H, H-11_B, 1H, H-6_B), 1.15 (ddd, *J* = 13.5, 13.4, 4.1 Hz, 13.4, 1H, H-3_B), 1.04 (dd, *J* = 12.7, 2.6 Hz, 1H, H-5), 0.93–0.96 (m, 1H, H-1_B), 0.85 (s, 3H, H-18), 0.77 (s, 3H, H-19), 0.64 (s, 3H, H-20); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.3 (NCHO), 160.5 (C-4 in pyrimidine), 158.9 (C-6 in pyrimidine), 156.4 (C-2 in pyrimidine), 148.5 (C-8), 144.2 (C-13), 117.0 (C-14), 106.2 (C-17), 99.3 (C-5 in pyrimidine), 56.4 (C-9), 55.5 (C-5), 42.1 (C-3), 41.4 (C-15), 39.7 (C-10), 39.1 (C-1), 38.6 (C-12), 38.3 (C-7), 33.6 (2×C, C-4, C-18), 28.1 (NCH_3), 24.4 (C-6), 21.9 (C-11), 21.7 (C-19), 19.4 (C-2), 16.4 (C-16), 14.5 (C-20); HRMS (ESI) found 440.3385, calcd for $C_{26}H_{42}N_5O^+$ 440.3384; $[\alpha]_D^{25}$ = +5.6 (*c* 0.50 MeOH). The spectral data and absolute value for optical rotation were in good agreement with those reported for (−)-ageloxime D before.[3]

4.2. Biology

4.2.1. Activity against M. tuberculosis

MICs against replicating and non-replicating *M. tuberculosis* H37Rv were determined using the Microplate Alamar Blue Assay and Low Oxygen Recovery Assay, respectively.[22]

4.2.2. Activity against S. aureus, E. coli, C. albicans, L. infantum, T. cruzi, T. brucei, T. rhodesiense and MRC-5 cells

4.2.2.1. Compounds and reagents. Compounds stock solutions were prepared in 100% DMSO at 20 mM. The compounds were serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further dilution in demineralized water to assure a final in-test DMSO concentration of < 1%.

4.2.2.2. Bacteria, fungi, parasite and cell cultures. *S. aureus* ATCC6538 and *E. coli* ATCC8739 were cultured in MHT (Mueller Hinton Broth) and maintained on TSA (Tryptone Soy Agar). *C. albicans* (azole

resistant) was cultured in RPMI-1640 medium supplemented with Mops buffer and glucose and maintained on PDA (Potato Dextrose Agar). *L. infantum* MHOM/MA(BE)/67 was maintained in the golden hamster. Amastigotes were collected from the spleen of an infected donor hamster using three centrifugation purification steps and spleen parasite burdens are assessed using the Stauber technique.[23] Primary peritoneal mouse macrophages were used as host cell and were collected 2 days after peritoneal stimulation with 2% potato starch suspension. *T. cruzi*, Tulahuen CL2 galactosidase strain (nifurtimox-sensitive),[24] was maintained on MRC-5_{sv2} (human lung fibroblast) cells in MEM medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% inactivated fetal calf serum. *T. b. brusei* Squib 427 strain (suramine-sensitive) *and T. b. rhodesiense* STIB-900 strain were maintained in Hirumi (HMI-9) medium, supplemented with 10% inactivated fetal calf serum. MRC-5_{sv2} cells were cultured in MEM + Earl's salts-medium, supplemented with L-glutamine, $NaHCO_3$, and 5% inactivated fetal calf serum. All cultures were conducted at 37 °C under an atmosphere of 5% CO_2.

4.2.2.3. Activity against S. aureus, E. coli, C. albicans and MRC-5 cells. The assays were performed at 37 °C in sterile 96-well microtiter plates, each well containing the aqueous compound (10 µL) dilution together with bacterial/fungal/MRC-5 inoculum (190 µL; 5×10^3 CFU/mL). The MRC-5 assay was performed under an atmosphere of 5% CO_2. The compounds were tested at 64, 16, 4, 1 and 0.25 µM. Bacterial/fungal/MRC-5 growth was compared to untreated-control wells (100% growth) and medium-control wells (0% cell growth). After 17 h (*S. aureus* and *E. coli*), 24 h (*C. albicans*) or 3 days (MRC-5) incubation, viability was assessed fluorimetrically after addition of resazurin. After 0.5 h (*S. aureus, E. coli* and *C. albicans*) or 4 h (MRC-5) at 37 °C, fluorescence was measured (λ_{ex} 550 nm, λ_{em} 590 nm). The results are expressed as % reduction in growth/viability compared to control wells and an IC_{50} (50% inhibitory concentration) was calculated. Doxycycline was used as positive control for *S. aureus* and *E. coli*, flucytosine for *C. albicans* and tamoxifen for MRC-5 cells.

4.2.2.4. Activity against L. infantum. The assay was performed at 37 °C under an atmosphere of 5% CO_2 in sterile 96-well microtiter plates, each well containing the aqueous compound (10 µL) dilution together with macrophage/parasite inoculum (190 µL; $3{\cdot}10^4$ cells + $4.5{\cdot}10^5$ parasites/well). The inoculum was prepared in RPMI-1640 medium, supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% inactivated fetal calf serum. The macrophages were infected after 48 h and the compounds were added after 2 h of infection. The compounds were tested at 64, 16, 4, 1 and 0.25 µM. Parasite multiplication was compared to untreated-infected controls and uninfected controls. After 5 days, parasite burdens (number of amastigotes/macrophage) were microscopically assessed after staining with a 10% Giemsa solution. The results were expressed as % reduction in parasite burden compared to control wells and an IC_{50} was calculated. Miltefosine was used as positive control.

4.2.2.5. Activity against T. cruzi. The assay was performed at 37 °C under an atmosphere of 5% CO_2 in sterile 96-well microtiter plates, each well containing the aqueous compound (10 µL) dilution together with MRC-5 cell/parasite inoculum (190 µL; $4{\cdot}10^3$ cells/well + $4{\cdot}10^4$ parasites/well). The compounds were tested at 64, 16, 4, 1 and 0.25 µM. Parasite growth was compared to untreated-infected controls and uninfected controls. After 7 days incubation, parasite burdens were assessed after adding the substrate CPRG [chlorophenolred β-D-galactopyranoside; 50 µL/well of a solution of CPRG (15.2 mg) and Nonidet (250 µL) in PBS (100 mL)]. The color change was measured spectrophotometrically at 540 nm after 4 h. The results were expressed as % reduction in parasite burden compared to control wells and an IC_{50} was calculated. Benznidazole was used as positive control.

4.2.2.6. Activity against T. brucei, and T. rhodesiense. The assay was performed at 37 °C under an atmosphere of 5% CO_2 in sterile 96-well microtiter plates, each well containing the aqueous compound (10 µL) dilution together with the parasite suspension [190 µL; 1.5×10^3 parasites/well (*T. brucei*) or 4×10^3 parasites/well (*T. rhodesiense*)]. The compounds were tested at 64, 16, 4, 1 and 0.25 µM. Parasite growth was compared to untreated-infected controls and uninfected controls. After 3 days incubation, parasite growth was assessed fluorimetrically after addition of resazurin [50 µL; 50 µg/mL in phosphate buffer] to each well. After 6 h (*T. rhodesiense*) or 24 h (*T. brucei*) at 37 °C, fluorescence was measured (λ_{ex} 550 nm, λ_{em} 590 nm). The results were expressed as % reduction in parasite growth/viability compared to control wells and an IC_{50} was calculated. Suramin was used as positive control.

4.2.3. Activity against biofilm formation of S. epidermidis, E. coli and P. aeruginosa.

Biofilm assays were optimized for each of the strains: *E. coli* (2006-22-1153-55-2),[19] *P. aeruginosa* (CCUG 56489) and *S. epidermidis* (ATCC 35984). The biofilm assays were performed in 96-well microtiter plates under ambient atmosphere in Brain Heart Infusion (BHI) medium (BD Biosciences) at 37 °C for 17 h (*S. epidermidis* and *P. aeruginosa*) or in LB broth without salt [LB^-; Yeast extract (10 g/L), Tryptone (5 g/L)] at 28 °C for 24 h (*E. coli*). Bacteria were grown overnight in shaken preculture (200 rpm) in BHI broth (*S. epidermidis, P. aeruginosa*) or LB broth (*E. coli*). For the biofilm assays, bacteria were diluted 1:100 in the indicated medium to give an approximate starting OD_{600} of 0.025. Two-fold serially diluted test compounds were added to the wells and biofilms were grown for the indicated optimized times before the medium was removed, the wells were washed 2 times with 0.9% NaCl solution and the biofilms were stained with safranin (0.1%) for 20 min. Following staining, the wells were washed 3 times with 0.9% NaCl solution and the biofilms were dissolved in 30% acetic acid for 10 min. Absorbance was measured at 530 nm using a Citation 3 Multi Cell Imaging Multi-Mode Reader (BioTek Instruments). Growth was recorded by measuring the OD_{600} of 50 µL of the growth medium. All experiments were performed at least three times and each condition was tested in duplicates in each experiment. In each experiment, the absorbance values were normalized against the untreated control sample and the effect of the different test compounds were reported as average percent biofilm formation compared to the untreated control with 95% confidence intervals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2019.01.002.

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