Supramolecular self-associating amphiphiles inhibit biofilm formation by the critical pathogens, *Pseudomonas aeruginosa* and *Candida albicans*

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Keywords: Amphiphile, Antibiofilm, Antifungal, Antimicrobial, Structure Activity Relationship, Supramolecular Chemistry

Abstract

In 2019, 4.95 million deaths were directly attributed to antimicrobial resistant bacterial infections globally. In addition, the mortality associated with fungal infections is estimated at 1.7 million annually, with many of these deaths attributed to species that are no longer susceptible to traditional therapeutic regimes. Herein, we demonstrate the use of a novel class of supramolecular self-associating amphiphilic (SSA) salts as antimicrobial agents against the critical pathogens *Pseudomonas aeruginosa* and *Candida albicans*. We also identify preliminary structure activity relationships for this class of compound that will aid the development of next-generation SSAs demonstrating enhanced antibiofilm activity. To gain insight into the possible mode of action for these agents, a series of microscopy studies were performed, taking advantage of the intrinsic fluorescent nature of benzothiazole substituted SSAs. Analysis of these data showed that the SSAs interact with the cell surface and that a benzothiazole containing SSA inhibits hyphal formation by *C. albicans*.

Introduction

The ability of microbes to reduce susceptibility to antimicrobial substances is well known, with increasing levels of antimicrobial resistance (AMR) reported for all pathogens.[1,2] In 2019, predictive statistical models estimated that approximately 4.95 million deaths recorded were directly related to AMR.[3,4] *Pseudomonas aeruginosa* is an opportunistic biofilm forming pathogen, often isolated from the lungs of patients diagnosed with cystic fibrosis. These infections are extremely difficult to eradicate and treat, resulting in extreme cases of morbidity and mortality. [5–7] In 2017, *P. aeruginosa* was declared as one of the top bacterial pathogens in the World Health Organisation (WHO) critical priority group.[8,9] Moreover, *P. aeruginosa* has been grouped with pathogens displaying multi-drug and extensively drug-resistant properties, referred to as the ESKAPE pathogens.[10–12]

Candida albicans is a polymorphic commensal yeast.[13] However, in immunocompromised individuals, *C. albicans* is an opportunistic pathogen, capable of causing a variety of infectious diseases, from cutaneous to deadly invasive systemic infections.[14] *C. albicans* related candidemia has been identified as a leading cause of hospital-acquired infections and bloodstream infections.[15–17] Recently, the WHO declared *C. albicans* as one of the top four fungal pathogens in its critical priority group,

demonstrating a significant unmet need for the development of novel therapeutic strategies to treat infections caused by this microorganism.[18] One of the contributing factors to this is the ability of this yeast to form biofilms through the production of an extracellular polymeric substance, which consists of a complex structured network of glycoproteins, carbohydrates, lipids and nucleic acids that directly contributes to pathogenic potential and fitness by providing antimicrobial protection.[19–21]

Notably, *C. albicans* is rarely identified and isolated alone. *C. albicans* and *P. aeruginosa* are the two most commonly co-occurring opportunistic pathogens that can colonise and infect humans.[22,23] Both *C. albicans* and *P. aeruginosa* utilize various virulence factors, including biofilm formation, to facilitate pathogenic potential and fitness. Furthermore, the co-inhabitation of these pathogens produces interkingdom polymicrobial biofilms. In such biofilms, the pathogens display complex interactions which facilitate enhanced antimicrobial resistance properties and promote chronic infections.[24–26] Therefore, novel antimicrobial strategies are required to address the global threat of polymicrobial biofilms.[27]

Novel antimicrobial strategies developed include the use of naturally derived antimicrobial peptides, which commonly display both hydrophobic and hydrophilic components, causing them to be classed as amphiphilic molecules.[28,30,31] Inspired by work in this area, and ongoing research to develop synthetic amphiphiles,[29,67] we have produced a novel class of compounds, known as supramolecular self-associating amphiphiles (SSAs), with the ability to self-assemble into higher order structures. These SSAs are amphiphilic salts, with the anionic component containing a hydrophobic functionality joined by a hydrogen bond donating/accepting (thio)urea functionality and alkyl group to a carboxylate or sulfonate moiety.[30,31] In polar organic solvents, such as dimethyl sulfoxide (DMSO), the SSA anionic component typically forms hydrogen bonded dimers. However, under aqueous conditions, the SSAs self-associate to form spherical aggregates, with hydrodynamic diameters between ~100-500 nm.[32] In specific examples, the addition of inorganic salts such as NaCl, followed by an annealing process, causes the formation of fibrous structures, resulting in a hydrogel.[33,41]

SSAs have been shown to act as antibacterial agents against clinically relevant planktonic Gram positive methicillin-resistant *Staphylococcus aureus* (MRSA) and

Gram negative *Escherichia coli*,[35] as well as potentiating compounds for traditional therapeutic agents against the ESKAPE pathogen *P. aeruginosa* and ovarian cancer cell lines.[36] In addition, they also show potential for development as drug delivery vehicles.[31,33] The use of these agents for such a wide variety of applications is attributed to their hypothesised mode of action, which includes the selective phospholipid membrane adhesion and permeation properties, which can be tuned based on stepwise variation in the modular molecular structure.[35,37,38]

Although, the potential for this class of compounds to transition into the clinical setting has been confirmed through preliminary and *in vivo* pharmacokinetic studies, their potential to inhibit growth of clinically relevant biofilms has not been addressed.[38] In the current study we aim to determine the ability of a library of SSAs to inhibit or eradicate biofilms of *P. aeruginosa*, *C. albicans* as well as polymicrobial biofilms.

Results and Discussion

SSAs **1**-**11** can be divided into four sub-categories based on the functionalities present within the molecular structure. SSAs in group 1 (SSAs **1**, **2**, **4**) contain a lipophilic trifluoromethylphenyl substituent alongside a urea moiety, while SSAs in group 2 (SSAs **5**, **6**, **8**) exchange the urea for a thiourea, which increases hydrogen bond donating acidity and lipophilicity, as well as decreases molecular planarity in comparison to the urea functionality. SSAs in group 3 (SSAs **9**-**11**) all contain an intrinsically fluorescent benzothiazole, increasing molecular aromaticity. The counter cation of most SSAs in this series of 11 compounds is weakly coordinating tetrabutylammonium (TBA). However, group 4 contains SSAs with different counter cations. SSAs **3** and **7** contain a pyridine and tetramethylammonium (TMA) cation, respectively. These cations contain a competitive hydrogen bond donating functionality and/or are considered strongly coordinating cations towards the corresponding anionic component of the SSA.[39]

The self-associative properties of SSAs **1**-**11** have been previously determined and are published alongside the characterisation of any self-associative structure formed. A summary of these data has been provided in Table 1, which also provides reference to the original work. Of the 11 SSAs incorporated within the scope of this work **1**, **2**, **4**-**6** and **8**-**11** all showed evidence of SSA anion dimerization events, stabilised through the formation of intermolecular hydrogen bonds in a DMSO-d_6 – 0.5% H_2O

environment. Comparison of the strength of complex formation was established through the elucidation of dimerization constants (K_{dim}), produced by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy dilution experiments. In general, the strength of SSA anion dimerization was found to increase with increasing hydrogen bond donor acidity, increasing anion basicity and removal of any intramolecular hydrogen bonding events. Correlation also exists between the increasing strength of SSA anion dimerization, SSA LogP values, critical micelle concentration (CMC – determined in an H_2O – 5% EtOH solution to aid SSA solubility) and antimicrobial efficacy against model Gram positive and model Gram negative bacteria.[35,40]

As SSAs have also been shown to self-assemble and arrive at the microbial surface as spherical aggregates under aqueous conditions, before forming a coating over the surface of bacteria and permeating through the cell membrane(s), it is also important within the context of the current work to consider some of the physical properties of these aggregates as they arrive at the microbial surface.[33] Here the results from intensity weighted dynamic light scattering studies (DLS) confirm the hydrodynamic diameter (d_H) of the spherical aggregates present at a set concentration after an annealing process, while zeta potential values confirm the stability of those same spherical aggregated species.

Table 1. Physicochemical data produced to characterise SSA self-association events in a H_2O:EtOH 95:5, to enable SSA solubility at higher concentrations, or a DMSO-d_6 – 0.5% H_2O solution (K_{dim} values only).[d1]

SSA	K_{dim} (M^{-1})	d_H (nm)	Zeta potential (mV)	CMC (mM)	Surface tension at CMC ($mN.m^{-1}$)	References
1	2.7 (± 0.3%)	164	-76	10.39	37.45	[40]
2	3.3 (± 1.0%)	122	-94	8.85	36.78	[40]
3	a	220	-28	198.42	36.16	[40]
4	41 (± 1.3%)	220	-37	11.21	39.33	[40]
5	0.2 (± 2.1%)	142	-34	6.12	42.24	[40]
6	2.6 (± 1.4%)	122	-38	5.61	33.59	[40]
7	a	b	-18.9	82.27	31.62	[35]
8	105 (± 0.7%)	164	-4	c	c	[40]
9	0.6 (± 1.1%)	295	-79	9.54	48.71	[32]
10	2.7 (± 0.3%)	122	-101	0.50	46.50	[32]
11	93 (± 1.3%)	127	-84.2	3.03	29.90	[41]

Aggregate stability and d_H were obtained via zeta potential and DLS measurements respectively, at a concentration of 5.56 mM and a temperature of 298 K, following an annealing process unless otherwise stated. The d_H of the aggregates listed were obtained from intensity distribution peak maxima. CMC was derived at approximately 291 K from surface tension measurements. Self-associative SSA anion dimerization constants (M^{-1}) calculated at 298 K. These constants were obtained from the fitting of ^{1}H NMR spectroscopy dilution data and refined to EK and CoEK models using Bindfit v0.5 (http://supramolecular.org/).[42,43] a = Presence of competitive SSA anion:cation interactions. b = Could not be determined due to lack of reproducibility. c = Could not be calculated due to compound insolubility. [d] Data represent mean ± standard error of the mean.

Antimicrobial activity

Potential antimicrobial activity of this library of 11 structurally related compounds was determined by measuring optical density (Figure 1). An OD_{595} value below that of the negative control value indicates potential antimicrobial activity at that concentration. It can be seen that certain SSAs displayed some level of inhibition against *P*. *aeruginosa* (Figure 1a) and *C. albicans* (Figure 1b) monomicrobial growth (consisting of both biofilm and planktonic cells).

Interestingly, the sulphonate containing SSAs from group 1 (i.e., SSA **1**, **2**), were able to inhibit growth of *P. aeruginosa* and *C. albicans* in monomicrobial settings, but was not effective at inhibiting polymicrobial growth, which is known to often be more resistant to antimicrobial compounds.[24-25] Substitution of the sulphonate with a carboxylate (as in SSA **4**) enhances the ability of SSA **1** to inhibit polymicrobial growth of this group of SSAs. This may be due to the increase in basicity of the anionic functionality, which increases the strength of SSA anion hydrogen bond donating/accepting propensity.

For SSAs in group 2, acyl chain length of the sulphonate containing SSAs (i.e., SSA **5**, **6**) may contribute towards specificity of the compounds for the different types of cells, with SSA **5**, containing an ethyl, being specific towards *P. aeruginosa* and SSA **6** (with a butyl) having increased activity against *C. albicans.* We hypothesise that this may be due to resultant changes in lipophilicity, which may influence the interaction between the thiourea-containing compounds in group 2 and the cell membranes. In addition, it has been reported that SSAs with an ethyl linker can form intramolecular hydrogen bonded monomeric units,[40] which will also influence the interaction and permeation properties of such molecules. This change in specificity is also reflected in the activity against polymicrobial biofilms, where the large *C. albicans* cells are the main contributors to OD of the biofilms. Interestingly, the substitution of the sulphonate for a carboxylate ion again allows SSA **8** to inhibit all the biofilms.

Exchanging the trifluorophenyl functionality for the benzothiazole group (SSA **9**, **10**, **11** in group 3) causes an increase in efficacy towards *C*. *albicans* monomicrobial biofilms. This may be due to the presence of enhanced aromatic stacking effects, caused by the presence of the benzothiazole functionality stabilising any molecular packing events, which may occur as the SSAs cross the cell membrane. Furthermore, when

comparing the efficacy of SSA **9**, SSA **10** and SSA **11**, it is apparent that the substitution of the sulfonate (SSA **10**) for a carboxylate (SSA **11**) also results in increased activity against the monomicrobial biofilms. However, this was not seen for SSA **9**, which we hypothesise to be due to the presence of the intermolecular hydrogen bond within the anionic component of this SSA, which deactivates the intermolecular self-associative properties for this molecule, [32] that we further propose are important for enhanced antibiofilm effects. This hypothesis is further supported when considering the increase in activity of SSA **11** compared to SSA **10**, as the carboxylate exhibits increased basicity over the analogous sulfonate containing SSAs, which, as shown by the comparative dimerization constants, (Table 1) enhances the intermolecular hydrogen bonding strength, for this class of compound. Importantly, these results contribute towards elucidation of the structure activity relationships (SAR) for this class of compound.

Based on these observations, five SSAs were selected for further study. These were: SSA **4** from group 1 (which exhibited the highest activity against polymicrobial biofilms), SSAs **5**, **6** and **8** from group 2 (which exhibited a level of specificity depending on acyl chain length) and SSA **10** (which exhibited high antifungal activity) from group 3). Due to solubility challenges, SSA **9** and **11** were not selected for further studies.

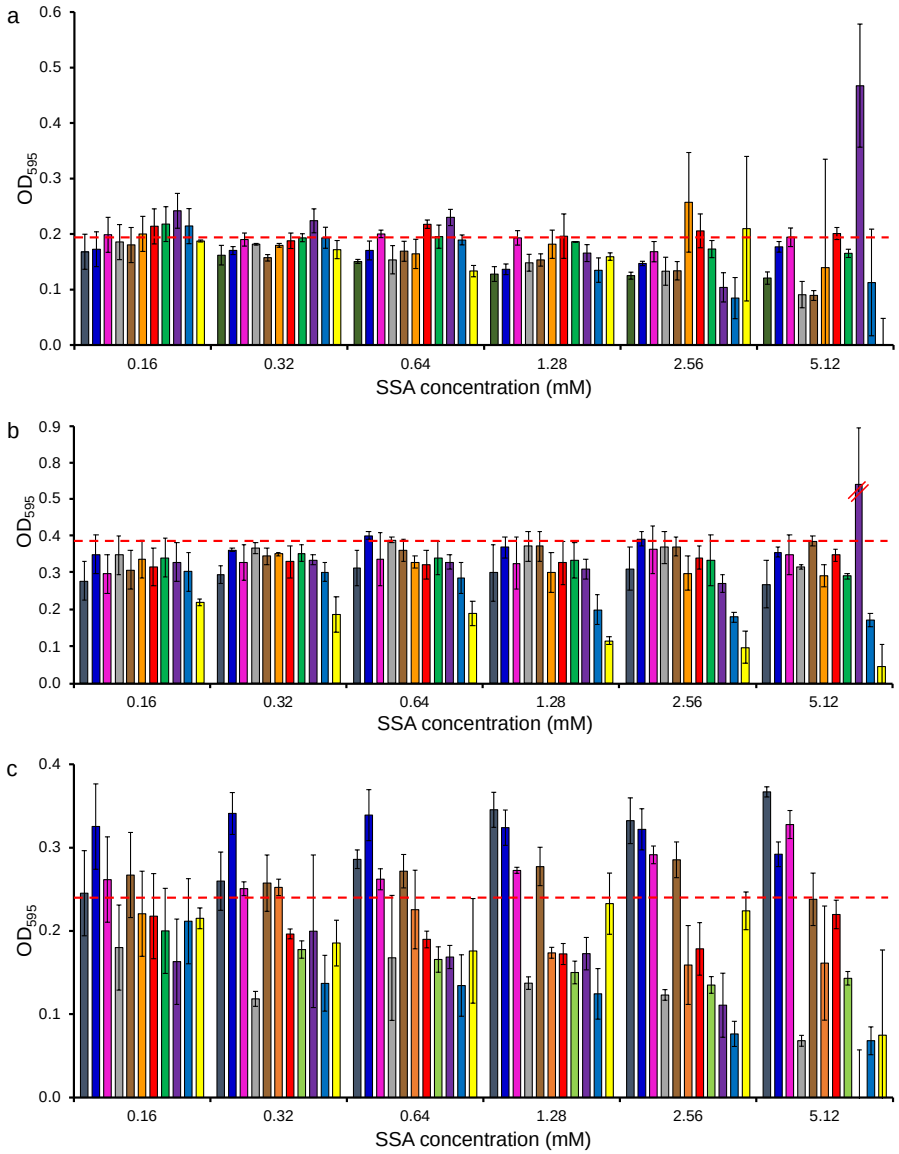

Figure 1. Optical density measurements of a) *P. aeruginosa* PAO1, b) *C. albicans* SC5314, and c) polymicrobial biofilms after 48 hours incubation at 37 °C with SSAs **1**-**11** (left to right) at increasing concentrations. A horizontal line value (HLV) displays the average negative control for *P. aeruginosa* with a value of 0.189, *C. albicans* with a value of 0.391, and the polymicrobial biofilm with a value of 0.243. Values are the mean of nine repetitions and the standard deviations are indicated by the error bars. SSA **1** = dark grey; SSA **2** = dark blue; SSA **3** = pink; SSA **4** = light grey; SSA **5** = brown; SSA **6** = orange; SSA **7** = red; SSA **8** = green; SSA **9** = purple; SSA **10** = light blue; SSA **11** = yellow.

SSA antibiofilm activity

Since optical density measurements, such as those undertaken to screen the SSAs for antimicrobial activity, cannot discriminate between planktonic cells and biofilms, an XTT assay was used to determine the activity of selected SSAs as antibiofilm agents.

Moreover, this metabolic activity assay is also able to determine the viability of cells contained within a biofilm.[44–46]

It can be seen that the selected SSAs could inhibit biofilm formation of *P. aeruginosa* between 25 and 70% (Figure 2a). Interestingly, most of the SSAs show a decrease in efficiency at the highest concentrations tested. This is particularly true for SSA **4** where the residual activity at 1.28 mM and 2.56 mM may be attributed to the activity of the cation. The structural similarity between SSA **5** and **6** indicates that the change in carbon chain length does not improve the compound efficiency against *P. aeruginosa*, however the substitution of the sulphonate for a carboxylate (SSA **8**) does increase the antibiofilm activity at lower concentrations. Interestingly, SSA **10** maintains a high level of inhibition (>50%) at all concentrations tested.

Most SSAs show a dose dependant response against *C. albicans* biofilm formation, with the highest percentage inhibition seen for SSA **6** and **10** (Figure 2b). The increased activity against *C. albicans* seen for SSA **6** compared to SSA **5** at 2.56 mM again indicates the importance of acyl chain length in the specificity of group 2 SSAs. Of those SSAs with antibiofilm activity (**6**, **8** and **10**), SSA **10** generally demonstrates the high activity across the range of concentrations tested. interestingly, this is the only SSA that shows the propensity for self-association in a way that results in the formation of different material morphologies, a propensity that may contribute to the increased activity observed in this instance.

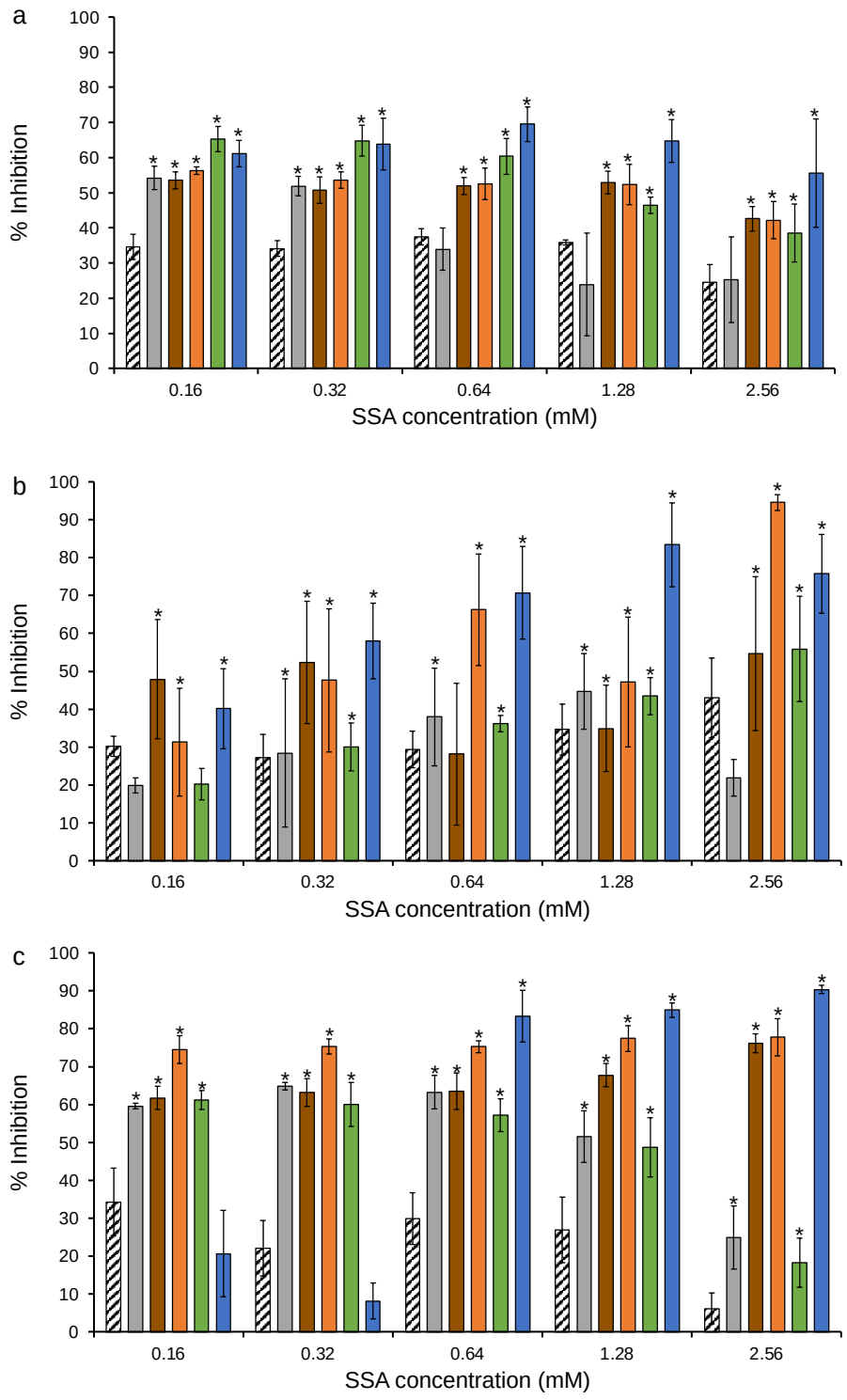

Figure 2. Percentage metabolic inhibitory action of TBA chloride and selected SSAs (**4**, **5**, **6**, **8** and **10**) against a) *P. aeruginosa*, b) *C. albicans*, and c) polymicrobial biofilms. Values represent mean (n = 5) of biological replicates and the standard deviations are indicated by error bars. TBA chloride = hatched pattern; SSA **4** = grey; SSA **5** = brown; SSA **6** = orange; SSA **8** = green; SSA **10** = blue. * indicates statistically significant inhibition when compared to TBA chloride at the corresponding concentration.

In addition, it is important to consider the complex interaction between *P. aeruginosa* and *C. albicans* when reviewing the percentage inhibition data of the polymicrobial biofilm (Figure 2c). Notably, the response profile of the polymicrobial biofilm shares similarities with both of the monomicrobial biofilms. SSA **4** and **8** indicate a dose dependent response similar to that of *P. aeruginosa*, whereas SSA **5** and **6** maintain inhibition at approximately 65% and 75% respectively, throughout the concentration ranges tested. SSA **10** shows significant inhibition at concentrations higher than 0.64 mM and is considered the best performing SSA out of this group, with the greatest activity against the range of biofilms tested.

Influence of SSAs on the morphology of biofilms cells

Figure 3-5 depicts SEM micrographs of *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms, cultivated for 48 hours in the presence and absence of selected SSAs **4**-**6**, **8** and **10**.

In the absence of SSAs the *P. aeruginosa* biofilm cells are smooth with some visible extracellular material (Figure 3a). Although treatment with any of the selected SSAs did not influence cellular morphology of *P. aeruginosa*, Figure 3b and 3c and 3d does indicate an increase in extracellular material of biofilms exposed to SSA **4** and SSA **5.** The extracellular matrix of a biofilm can be described as a complex organ continuously interacting with and responding to its immediate environment.[47] The production of extracellular material is directly correlated to the success of biofilm cells, providing spatial context for intercellular signalling events and thereby determining cellular behaviour, proliferation, migration and ultimately survival.[48] In line with this, it was observed that self-induced cell lysis promotes biofilm formation in response to external pressures.[49] This work showed that the presence of additional extracellular DNA contributed to biofilm formation and provided additional protection to antibiofilm substances. Thus, the presence of excess cellular material may be in response to cellular stress induced by these SSAs,[49,50] potentially indicating a specific stress response of *P. aeruginosa* to the presence of these compounds.

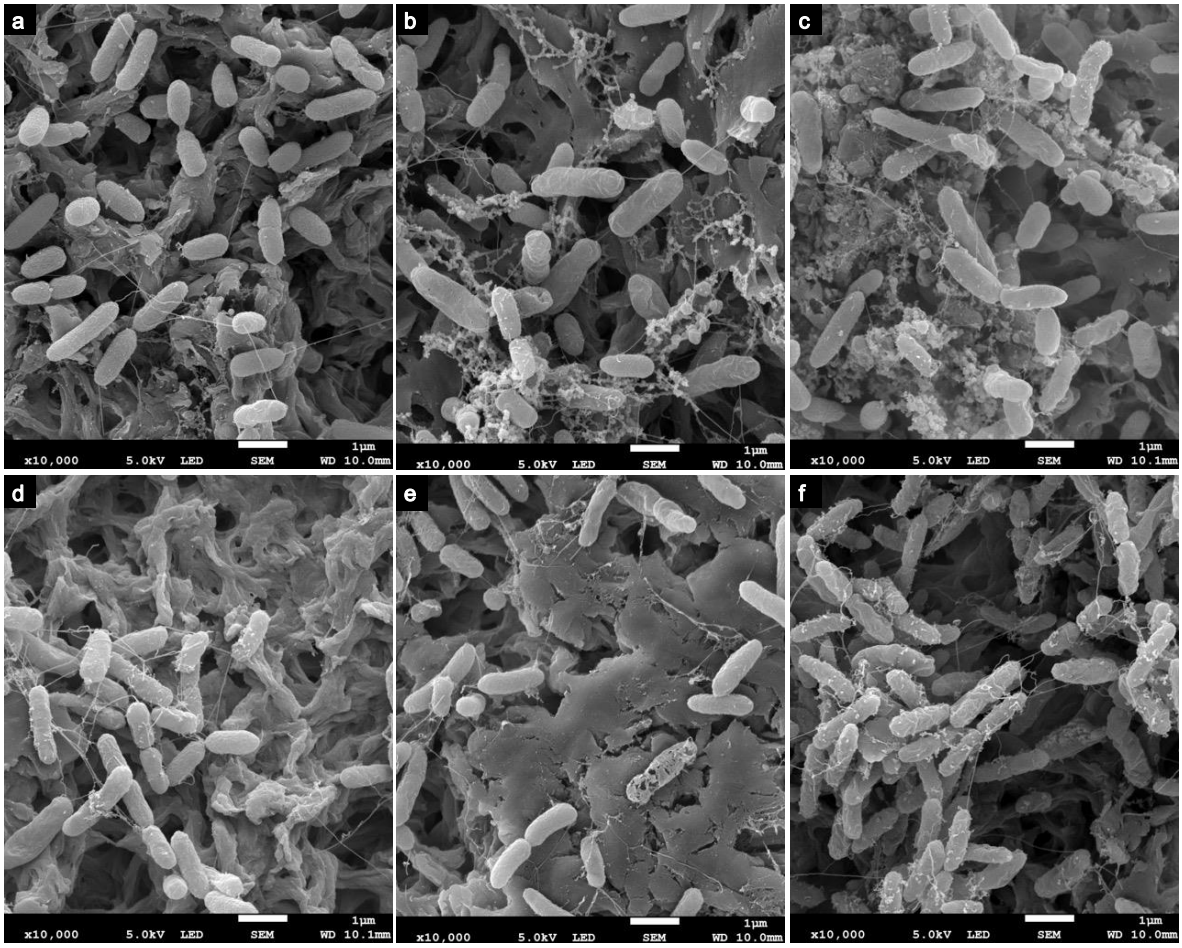

Figure 3. Scanning electron micrographs of *P. aeruginosa* PAO1 biofilms cultivated for 48 hours at 37 °C where a) control (untreated) biofilm and b) SSA **4**; c) SSA **5**; d) SSA **6**; e) SSA **8**; f) SSA **10** *P. aeruginosa* biofilms incubated with 2.56 mM, respectively.

In the absence of SSA the biofilm cells of *C. albicans* are smooth with visible hyphae (Figure 4a). No significant observable difference in the morphology of the biofilm is observed for cells exposed to the selected SSA from group 1 (SSA **4**) (Figure 4b) or group 2 (SSA **5**, **6** and **8**) (Figure 4c-e). However, the group 3 SSA, SSA **10**, caused a significant effect on the morphology of *C. albicans* biofilm cells (Figure 4f), inhibiting the production of hyphae. We hypothesise that this change in cellular morphology is due to the presence of the benzothiazole moiety in this SSA. Interestingly, there are also a series of other benzothiazole derivatives which have been shown to exhibit activity against fungi, including *C. albicans*.[51,52]

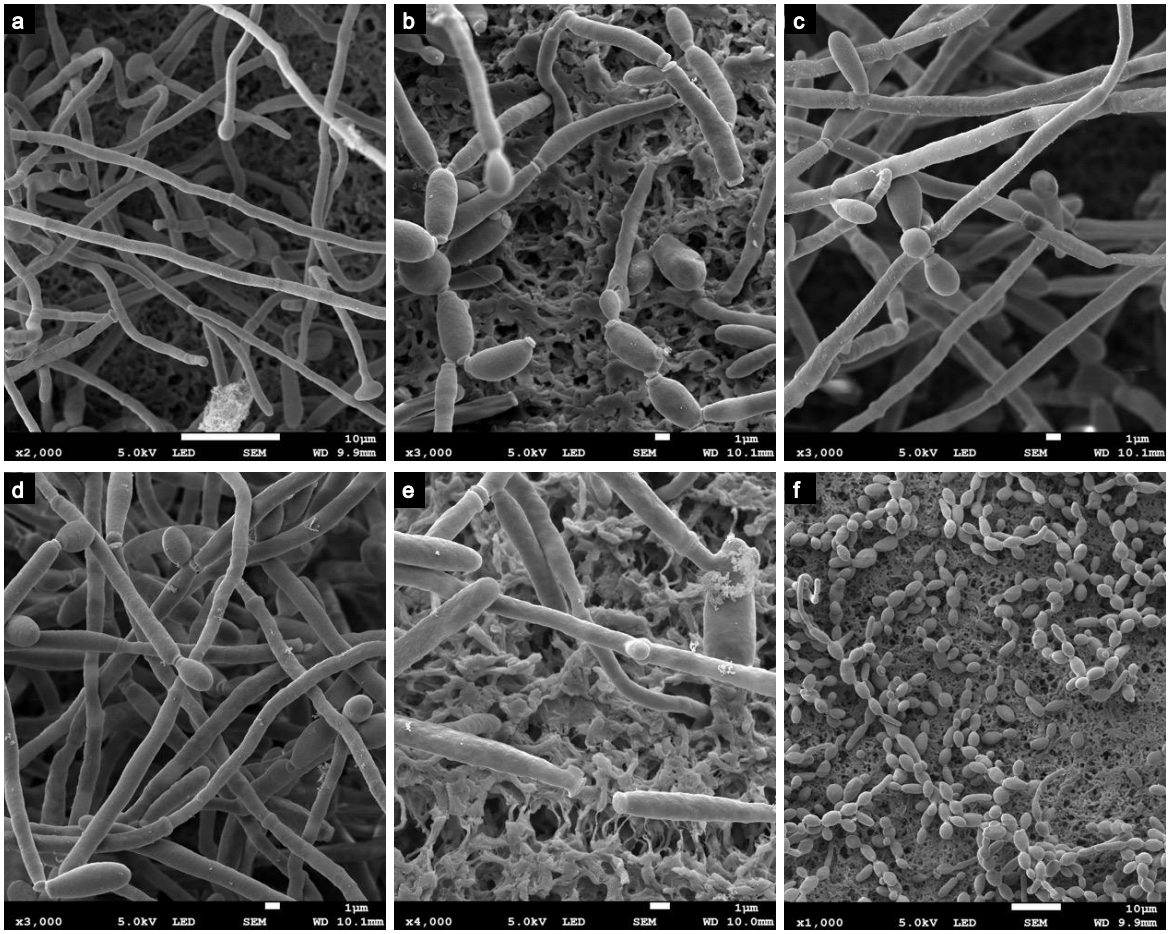

Figure 4. Scanning electron micrographs of *C. albicans* SC5314 biofilms cultivated for 48 hours at 37 °C where a) control (untreated) biofilm and b) SSA **4**; c) SSA **5**; d) SSA **6**; e) SSA **8**; f) SSA **10** *C. albicans* biofilms incubated with 2.56 mM, respectively

Figure 5 indicates the morphology of the polymicrobial biofilms exposed to the selected SSAs. In contrast to the *C*. *albicans* monomicrobial biofilms (Figure 4a), Figure 6a, which shows a polymicrobial biofilm containing both *C*. *albicans* and *P*. *aeruginosa*, only *C*. *albicans* yeast cells with very short hyphae are visible, indicating that *P*. *aeruginosa* inhibits hyphal formation *in vitro*.[25,53–56] Figure 5b indicates that SSA **4** does not cause any further morphological changes in the polymicrobial biofilm. However, exposure to SSA **5** completely inhibited hyphal growth in the polymicrobial biofilm (Figure 5c). This may be due to the combined effects of the SSA and *P. aeruginosa* as discussed above. Notably, yeast cells here also display possible cell surface modifications with rough surfaces, in comparison to the smooth surface of the microbes as shown in the control sample, Figure 4a. Figure 5d shows the morphological effect of SSA **6** on polymicrobial biofilms, where it is evident that filamentation of *C*. *albicans* is also considerably inhibited. Similar results are also seen

for SSA **8** (Figure 5e). From these results, it may be suggested that group 2 SSAs display selective morphological inhibitory action in a polymicrobial biofilm.

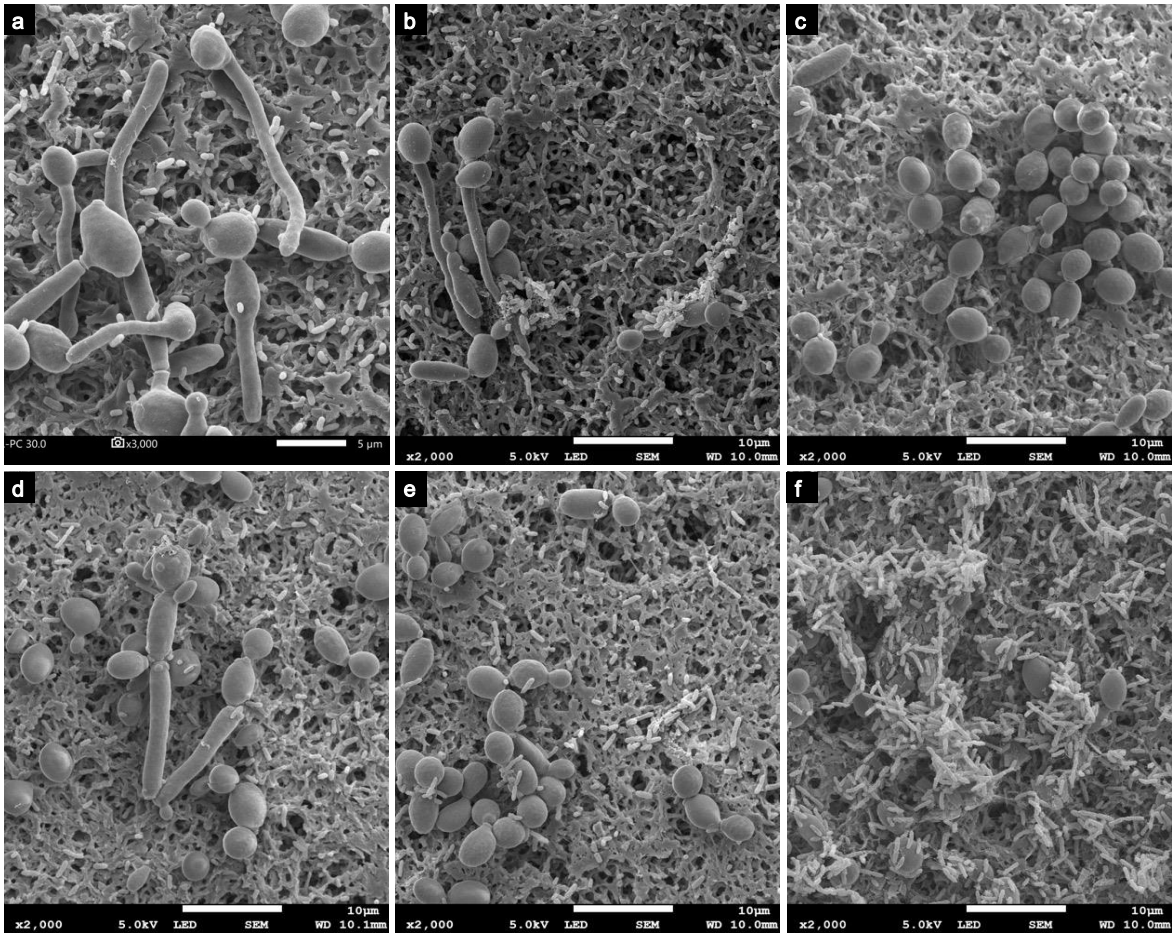

Figure 5. Scanning electron micrographs of *P. aeruginosa* PAO1 and *C. albicans* SC5314 polymicrobial biofilms cultivated for 48 hours at 37 °C where a) control (untreated) biofilm and b) SSA **4**; c) SSA **5**; d) SSA **6**; e) SSA **8**; f) SSA **10** *C. albicans* biofilms incubated with 2.56 mM, respectively

Although the group 3 SSA, SSA **10**, did not cause any significant morphological changes in the *P. aeruginosa* (Figure 3f) or polymicrobial biofilms (Figure 5f), an interesting effect was observed on the morphology of *C. albicans* biofilm cells (Figure 4f). From this micrograph, it is evident that pseudohyphae and hyphae are significantly inhibited, compared to the control and other SSA experimental groups. This unique morphological inhibitory action complements the observed metabolic inhibition and warrants further investigation. To further evaluate the ability of SSA **10** to inhibit *C. albicans* filamentation during biofilm formation, a second set of SEM studies were undertaken in which SSA **10** was introduced to the biofilms at a lower concentration of 1.28 mM. Interestingly, the lower concentration did not influence hyphal formation, in

contrast to the higher concentration of 2.56 mM (Figure 6), confirming this event to be SSA concentration dependent.

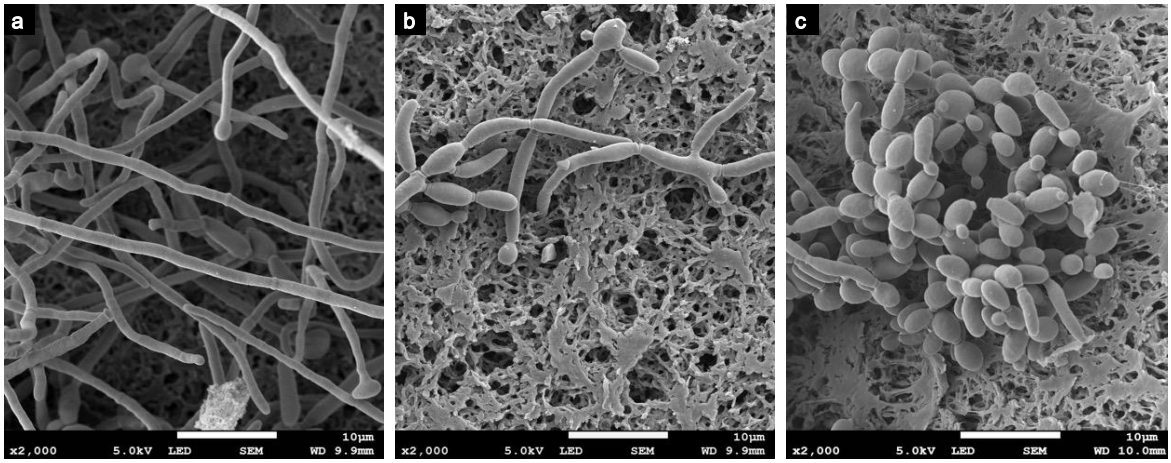

Figure 6. Scanning electron micrographs of *C. albicans* SC5314 biofilms cultivated at 37 °C for 48 hours where a) displays the control (untreated) biofilm; and biofilm cultivated in the presence of SSA **10** at b) 1.28 mM and c) 2.56 mM

Eradication of preformed biofilms

The complex tertiary composition of a biofilm extracellular matrix (EXM) avails various antimicrobial defence mechanisms through physical, electrostatic and microenvironment considerations.[57–59] These formidable resistance and defence mechanisms are employed at the attachment phase of biofilm formation and increase as the biofilm matures.[60] In line with this, when considering the application of SSAs against preformed biofilms, a decrease in antibiofilm activity is expected. We saw that none of the SSAs were able to eradicate pre-formed *P. aeruginosa* or polymicrobial biofilms, even after 120 minutes contact time (data not shown). However, limited eradication of *C. albicans* mature biofilms growth was seen after 60 minutes with most the SSAs tested, except SSA **10** (Figure 7a).

In order to further investigate the ability of SSAs to eradicate mature biofilms, an XTT assay was performed on *C. albicans* preformed biofilms. For all SSAs tested, the most significant results were seen after 120 minutes contact time, with SSA **5** and **6** from group 2 displaying the most significant results (Figure 7b). This confirms the ability of these SSAs to penetrate the EXM and affect the *C. albicans* cells within a mature biofilm to some extent.

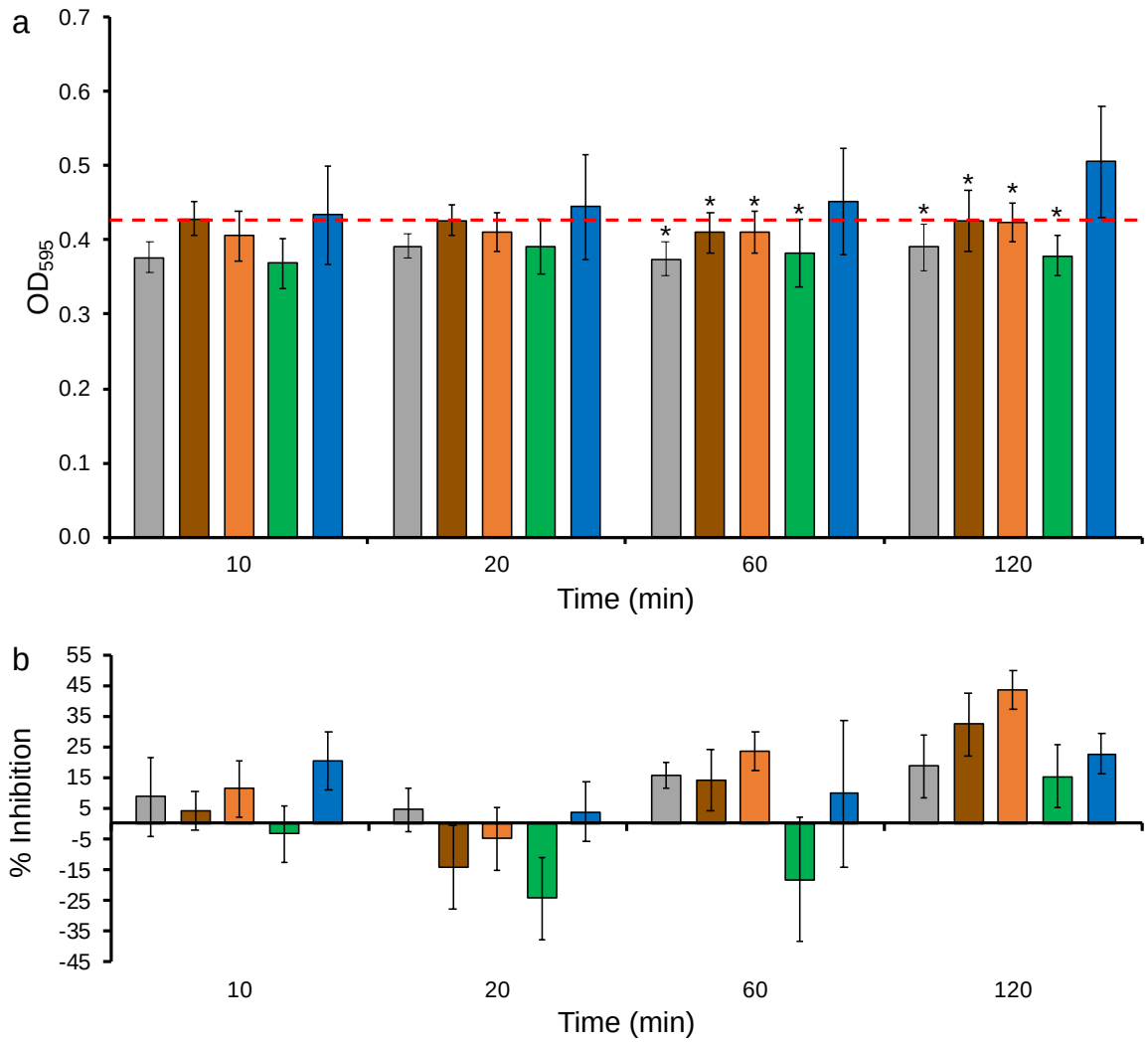

Figure 7. Graphs demonstrating the eradication of mature *C. albicans* SC5314 biofilms cultivated at 37 °C for 48 hours by SSAs employed at a concentration of 2.56 mM at different contact times. a) Optical density measurements; b) percentage inhibition of metabolic activity. Values are the mean of six repetitions and the standard deviations are indicated by the error bars. * $p < 0.002$. SSA **4** = grey; SSA **5** = brown; SSA **6** = orange; SSA **8** = green; SSA **10** = blue

SSA 10 interaction with the surface of *C. albicans* cells

Due to the inherent fluorescent properties of SSA **10**, combined with the fact that this is the first report of potential antifungal activity of SSAs, this compound was selected to investigate the interaction between the SSAs and *C. albicans* cells, using CLSM (Figure 8). As expected from Figure 4f, at 2.56 mM only yeast cells are present (Figure 8a,b), while at 1.28 mM, some filamentation can be observed (Figure 8c,d). From the fluorescence micrographs, it can be seen that SSA **10** can interact with the fungal cell surface at both concentrations tested (indicated by the blue fluorescence). Interestingly, a potential interaction between SSA **10** and the extracellular matrix of *C.*

albicans is observed (Figure 8d), which may explain the relative resistance of mature biofilms to these compounds.

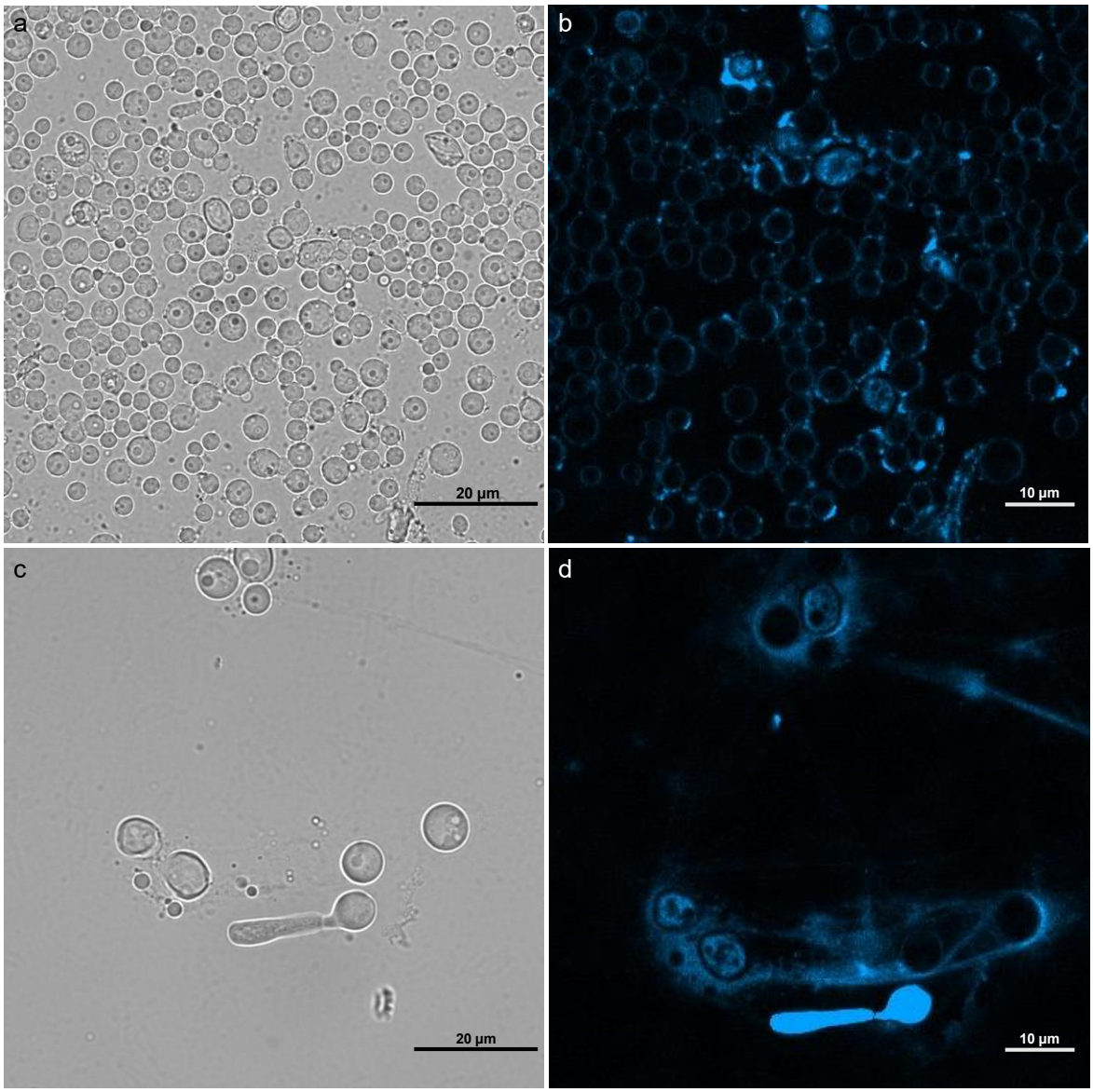

Figure 8. Interaction of SSA **10** with *C. albicans* SC5314. Light micrograph a) indicates a biofilm formed in the presence of SSA **10** at 2.56 mM. Micrograph b) indicates the blue fluorescence of SSA **10** on the yeast cell surfaces. Light micrograph c) indicates a biofilm cultivated in the presence of 1.28 mM SSA **10**. Micrograph d) indicates blue fluorescence of SSA **10** on the yeast cell surfaces, germ tube and interacting with the extracellular material

Conclusions

This study evaluated the potential action of novel SSA compounds against biofilms formed by the opportunistic pathogens *P. aeruginosa* and *C. albicans*. From this study it can be concluded that SSAs indeed have antibiofilm activity and may influence production of hyphae, an important virulence factor of *C. albicans*. Structure-activity

relationship (SAR) identification is an important tool to enable the informed design of ever more effective novel antimicrobial substances as it enables the identification of critical structural features that could inform rational design of antimicrobial compounds with improved activity.[61,62] From these data, it was determined that different functionalities of the SSAs, especially those involved in intra- and intermolecular hydrogen bonding, can influence the degree of inhibition as well as the specificity of the antibiofilm activity. In addition, increased hydrogen bond interactions as well as the presence of a benzothiazole group is important for antifungal antibiofilm formation activity. However, the tested SSAs are less effective against mature biofilms. This is not surprising since the production of complex EXM will change the environment and influence the ability of this molecule to interact with the cell. These data provide additional information regarding structural characteristics influencing antimicrobial activity in order to supplement the design and evolution of this novel class of compounds.

Traditionally, a limitation to the translation of amphiphilic agent technologies, such as SSAs, into the clinic is demonstration of enhanced haemolysis activity levels against erythrocytes (red blood cells). To assess the implication of SSA haemolysis activity on SSA clinical potential, we have determined the haemolytic activity for several SSAs, including **1**.[68] In addition, we have also conducted *in vivo* mouse model studies, in which **3** and **9** were introduced into mice intravenously, the SSA concentration within the mouse circulatory system (alongside SSA dissemination into lung, muscle and liver tissue) was then recorded with respect to time.[38] The results of these studies were found to support the potential for SSAs to be developed as therapeutic agents.

Experimental Section

SSA Stock Solution Preparation. Eleven SSAs (Figure 9) were synthesised from previously published methods. All compounds used are >99% pure by ^{1}H NMR spectroscopy analysis. Due to the chemical synthesis methods used, the presence of starting materials or by-products (at a combined concentration > 5% by weight) would be detected. For details, please see supplementary information. All SSA stock solutions were prepared in 25% ethanol in milli-Q water to obtain a final stock concentration of 25.6 mM.[63] The compounds were solubilized, while undergoing a

simultaneous annealing process in which the SSA solutions were cooled to room temperature after undergoing sonication in three cycles (Scientech Ultrasonic Cleaner) at 50 °C for 50 minutes. The solutions were stored sealed at room temperature until required for use.

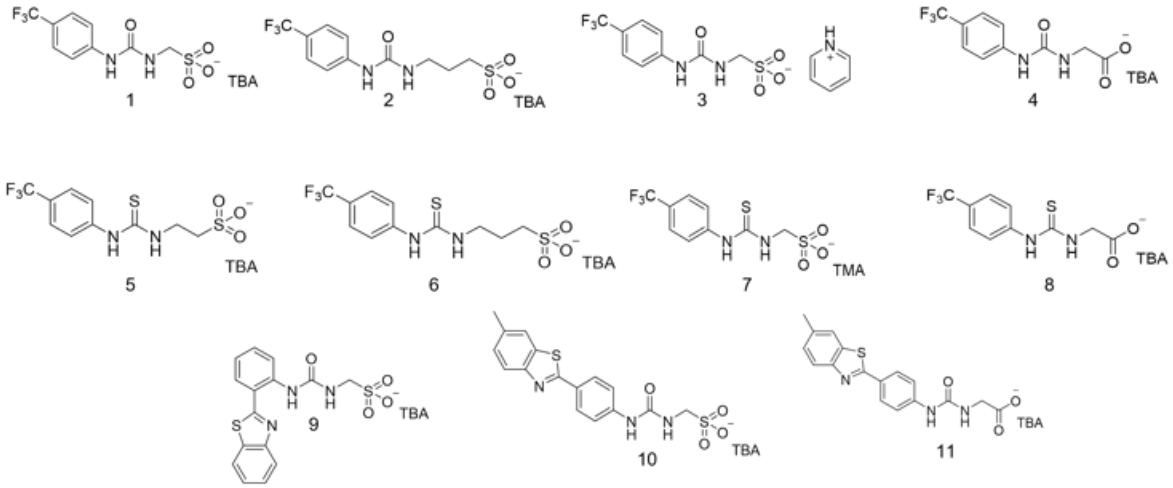

Figure 9. Chemical structures of SSAs **1**-**11**. TBA = tetrabutylammonium, TMA = tetramethylammonium

Strain Maintenance and Culture Conditions. The reference strains, *Pseudomonas aeruginosa* PAO1 and *Candida albicans* SC5314, were used in this study. The strains were stored at -80 °C in nutrient broth (NB) (1 $g.L^{-1}$ meat extract, 2 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ peptone, 8 $g.L^{-1}$ sodium chloride) supplemented with 25% and 15% (v/v) glycerol, respectively. Before each assay, the *P. aeruginosa* and *C. albicans* strains were cultured from the frozen stock onto nutrient agar (NA) (1 $g.L^{-1}$ malt extract, 2 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ peptone, 8 $g.L^{-1}$ sodium chloride, 20 $g.L^{-1}$ agar) and yeast malt extract (YM) agar (3 $g.L^{-1}$ malt extract, 3 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ glucose). For every experiment, a fresh (pre-inoculum) culture of *P. aeruginosa* was prepared by transferring a single colony from the maintained plates into 10 mL NB and incubating at 37 °C with shaking (150 revolutions per minute (RPM)) for 24 hours. Similarly, a fresh (pre-inoculum) culture of *C. albicans* was prepared into 5 mL yeast nitrogen base (YNB) broth (6.7 $g.L^{-1}$ YNB, 10 $g.L^{-1}$ glucose) and incubated at 30 °C for 24 hours. For all assays, filter-sterilized (0.22 µm nitrocellulose filter, ABLUO, GVS, United States of America) RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich, United Kingdom) at pH 7.0, was used.

SSA Antimicrobial Screening. The pre-inoculum was washed three times with phosphate-buffered saline (PBS) (0.2 $g.L^{-1}$ potassium chloride, 0.2 $g.L^{-1}$ potassium

dihydrogen phosphate, 1.15 $g.L^{-1}$ di-sodium hydrogen phosphate, 8 $g.L^{-1}$ sodium chloride) (Oxoid, United Kingdom) at pH 7.3 and standardised to an optical density (OD) OD_{595} of 0.5 for *P. aeruginosa* and 1 x 10^6 $cells.mL^{-1}$ for *C. albicans*, as described previously.[64–66] The standardised cell suspension was dispensed into 96-well flat-bottom culture (microtiter) plate (Greiner Bio-One, Germany) (250 µL total volume per well) together with a two-fold dilution series of each SSA to achieve a final concentration range of 0.08 - 2.56 mM. The microtiter plates were incubated for 48 hours at 37 °C and OD measurement (at a wavelength of 595 nm, OD_{595}) (EZ Read 800 Research, Biochrom, England) was performed. To cultivate polymicrobial biofilms, the methods described for the monomicrobial biofilm models were used. However, when standardising the cell solutions needed for the inoculation of a microtiter plate, *P. aeruginosa* and *C. albicans* cell suspensions were adjusted to an OD_{595} of 0.1 and 2 x 10^6 $cells.mL^{-1}$, respectively. Cell-free solvent controls and negative controls were included and the percentage inhibition, relative to the negative control, was calculated. These experiments were performed in technical triplicates and biological duplicates.

Metabolic Activity Assay for Inhibition of Biofilm Formation. *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms were cultivated for 48 hours at 37 °C as described previously in the presence of selected SSAs (final concentration of 2.56 mM). After incubation, an indirect and semi-quantitative measure of biofilm formation was performed, using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl-)-2H-tetrazolium-5-carboxanilide (XTT) colourimetric reduction assay as described using 1 $g.L^{-1}$ of filter sterilized (0.22 µm nitrocellulose filter) XTT salt (Sigma-Aldrich, United Kingdom), dissolved in PBS and supplemented with 1 mM menadione in acetone. The supernatant from the 96-well plates was discarded, the wells washed twice with 200 µL PBS before 50 µL of XTT-menadione solution was introduced to each well. The plates were incubated for 3 hours at 37 °C in the dark and the absorbance measured at 492 nm using a microtitre plate reader (EZ Read 800 Research, Biochrom, England). Furthermore, when considering the antimicrobial potential of quaternary ammonium compounds, such as TBA, the counter ion of the selected SSAs, the antibiofilm activity of TBA chloride was also evaluated. This was repeated five times. Additionally, appropriate controls were included for known antimicrobials, colistin and fluconazole, respectively. For details, please see supplementary information (Figures S13 and S14).

Scanning Electron Microscopy (SEM). Mono- and polymicrobial biofilms were prepared on a sterile polymer disc (Isopore 0.2 µm hydrophilic polycarbonate membrane disc, Merck, Germany) in wells of a flat bottom 6-well plate (Greiner Bio-One, Germany) with 2 mL RPMI-1640 medium containing selected SSAs (final concentration of 2.56 mM). After incubation, the polymer disc was aseptically removed and placed in primary fixative, 3% (v/v) glutardialdehyde (Merck, Germany) in phosphate buffer (pH 7.0), overnight. The biofilms were washed twice with PBS and fixed with a secondary fixative, 1% (v/v) osmium tetroxide (Merck, Germany), for 2 hours at room temperature, followed by a second wash step. The biofilms were dehydrated in an ethanol series (50% for 20 min, 70% for 20 min, 95% for 20 min, 100% for 1 hour - twice) and air dried in a desiccator. The biofilms were then subjected to critical point drying (Samdri-795 Critical Point Dryer, Tousimis, United States of America), coated with gold (EM ACE600 coating system, Leica, Austria) for 30 minutes and examined using a JSM-7800F Extreme-resolution Analytical Field Emission Scanning Electron Microscope (ZEISS, Germany).

Eradication of Preformed Biofilms. The efficacy of the SSAs against mature biofilms was also evaluated using a modified microtitre biofilm eradication assay. *C. albicans* biofilms were cultivated as previously described for 48 hours at 37 °C in a 96-well microtiter plate. After mature biofilm formation, selected SSAs (final concentration of 2.56 mM) were added to the biofilms, incubated for 10, 20, 60 and 120 minutes, and OD_{595} measured.

Confocal Laser Scanning Microscopy (CLSM). *C. albicans* biofilms were cultivated on a glass coverslip in a 6-well microtiter plate (Greiner Bio-One, Germany) in the presence of SSA **10** (final concentrations of 1.28 mM and 2.56 mM) for 48 hours at 37 °C. After incubation, the glass coverslip was fixed to a microscope slide and the biofilms were examined using a ZEISS LSM 900 with AiryScan 2 confocal laser scanning microscope (ZEISS, Germany) at an excitation/emission wavelength of 365/461 nm (using the DAPI filter setting). This was possible due to the inherent fluorescent properties of SSA **10**.

Statistical Analyses. For all quantitative experiments, averages and standard deviations were calculated. Data produced were analysed using a student t-test to

establish statistically significant differences between data sets. A *p*-value of ≤ 0.05 was considered significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Electronic Supplementary Information (ESI) on compound synthesis, ^{1}H NMR spectroscopy analysis and antimicrobial control tests (PDF).

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HJFS - performed biological experiments and wrote the initial draft of the manuscript

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KLFH – performed chemical synthesis and edited the manuscript

JRH and CHP – initiated and supervised the research and edited the final version of the manuscript

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AMR, antimicrobial resistance; CLSM, confocal laser scanning microscopy; CMC, critical micelle concentration; d_H, hydrodynamic diameter; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; EXM, extracellular matrix; HLV, horizontal line value; K_{dim}, dimerization constant; MRSA, methicillin resistant *Staphylococcus aureus*; NA, nutrient agar; NB, nutrient broth; NMR, nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance; OD, optical density; PBS, phosphate-buffered saline; SAR, structure activity relationship; SEM, scanning electron microscopy; SSA/s, self-associating amphiphile/s; TBA, tetrabutylammonium; TMA, tetramethylammonium; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl-)-2H-tetrazolium-5-carboxanilide; YM, yeast malt; YNB, yeast nitrogen base.

ACKNOWLEDGEMENTS

This work is based on the research supported in part by the National Research Foundation of South Africa (Grant Number MND200629538664 to HJFS and grant number 115566 to CHP). LJW and JRH would like to thank the UKRI (MR/T020415/1) for funding. KLFH would like to thank the University of Kent for funding.

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