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FULL PAPER

Spermine derivatives of indole-3-carboxylic acid, indole-3-acetic acid and indole-3-acrylic acid as Gram-negative antibiotic adjuvants

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Abstract: The discovery of new antibiotic adjuvants is an attractive option for overcoming antimicrobial resistance. We have previously reported the discovery of a bis-6-bromoindolglyoxylamide derivative of spermine as being able to enhance the action of antibiotics against Gram-negative bacteria but suffers from being cytotoxic and red-blood cell haemolytic. A series of analogues was prepared exploring variation of the indolglyoxylamide unit, to include indole-3-acrylic, indole-3-acetic and indole-3-carboxylate units, and evaluated for antibiotic enhancing properties against a range of Gram-negative bacteria, and for intrinsic antimicrobial, cytotoxic and haemolytic properties. Two spermine derivatives, bearing 5-bromo-indole-3-acetic acid (**17**) and 5-methoxy-indole-3-acrylic acid (**14**) end groups were found to exhibit good to moderate antibiotic adjuvant activities for doxycycline towards the Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*, but with more modest intrinsic antimicrobial activity and greatly reduced cytotoxic and haemolytic properties. The mechanism of action of the latter derivative identified its ability to disrupt the outer membranes of bacteria and to inhibit the AcrAB-TolC efflux pump directly or by inhibiting the proton gradient.

Introduction

With the continual emergence of bacteria that are resistant to many common antibiotics, there is a well-documented need for the discovery and development of new antibacterial therapies.[1-3] With the development of new antibiotics being a difficult and, in the case of Gram-negative antibacterials, unforthcoming process,[4-5] there is an increasing effort directed towards the discovery of adjuvants (enhancers, potentiators) that can restore the action of antibiotics towards drug-resistant bacteria.[6-11] Antibiotic adjuvants in current clinical use (e.g. Augmentin, combination of amoxicillin and clavulanic acid) achieve their effects by inhibiting the resistance mechanism. An alternative pathway to enhancing antibiotic action is to increase drug uptake through the bacterial membrane. To avoid further undue selective drug resistance pressure, antibiotic adjuvants should preferably lack their own antibiotic activity.[12]

Marine natural products represent an excellent resource for the discovery of new biologically active therapeutic lead-like compounds.[13] One such scaffold is the indole ring, of which over 1500 examples have been reported from the marine environment.[14] A diverse array of biological activities have been observed for these compounds including anti-infective,[15] the control of depression and anxiety,[16] cytotoxicity and antibacterial [17] properties. Our screening for natural products and natural product-like compounds for the ability to enhance the action of doxycycline against the Gram negative bacterium *Pseudomonas aeruginosa* identified the 6-bromoindoleglyoxylamide-polyamine **1** (Figure 1), a synthetic compound related to a reported marine natural product,[18] as an antibiotic adjuvant and intrinsic antimicrobial agent that unfortunately also suffers from being cytotoxic and haemolytic.[19]

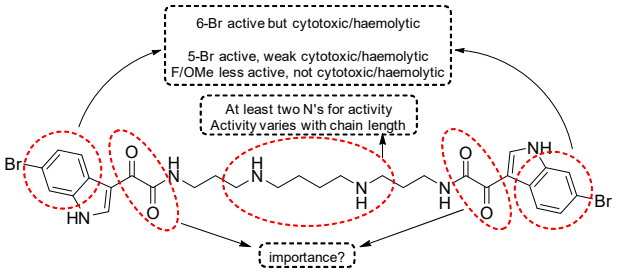


Figure 1. Structure of polyamine derivative **1** with summary of SAR for adjuvant activities.

Further exploration of **1** identified the 5-bromo analogue to be a more potent enhancer of antibiotic action towards Gram-negative

bacteria, and to be less cytotoxic and haemolytic than the original 6-bromoindole hit compound.[20] Other substituted variants, including 5-/6-fluoro and 5-/6-methoxy analogues exhibited less effective enhancement of antibiotic action but were notable for the absence of cytotoxic and haemolytic properties (Figure 1). In an attempt to ascertain the importance of the glyoxylamide moiety of **1** on biological activity, we have prepared a set of indole-3-acrylic acid, indole-3-acetic acid and indole-3-carboxylate analogues that maintain the more biologically active spermine core[19] and that contain variation at the indole 5- and 6- positions with bromo, fluoro or methoxy substituents. Herein we report these preliminary data, exploring the effect of structure on intrinsic antimicrobial properties, the ability to enhance the action of doxycycline and other antibiotics against *Pseudomonas aeruginosa* and other Gram-negative bacteria and evaluate the ability of one of the enhancers to influence bacterial membrane permeability and polarization and to the inhibit AcrAB-TolC efflux pump.

Results and Discussion

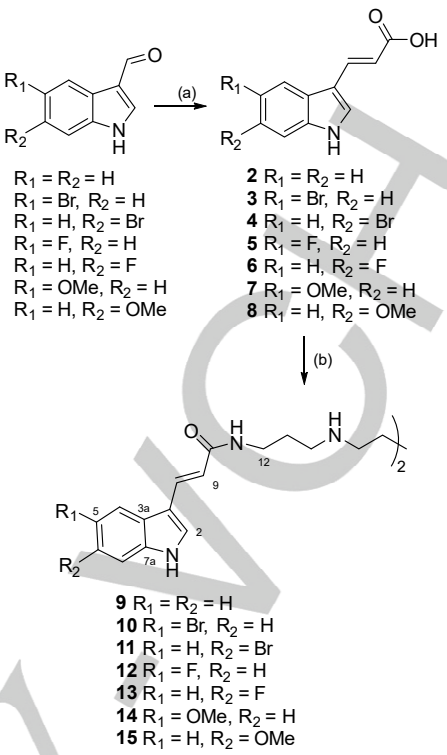
Synthesis of polyamine derivatives 9–20

Synthesis of the acrylamide series commenced with the preparation of suitably substituted indole-3-acrylic acids from the corresponding indole-3-carbaldehydes. Commercially available indole-3-carbaldehyde and the 5-bromo, 6-bromo, 5-fluoro, 6-fluoro, 5-methoxy and 6-methoxy substituted analogues were reacted with malonic acid and piperidine in pyridine at 80 °C for 6 h to afford the respective indole-3-acrylic acids **2**–**8** after purification (Scheme 1). Subsequent incubation of indole-3-acrylic acids with the peptide coupling agent 1,1'-carbonyldiimidazole (CDI) for 3 h followed by addition of spermine and reaction for a further 72 h afforded the desired acrylamide analogues **9**–**15**.

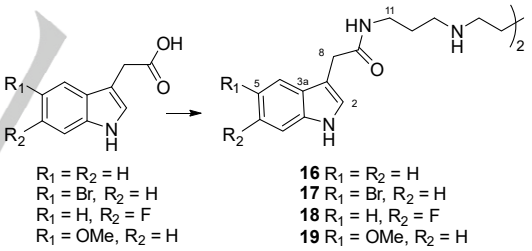
The acetamide analogues **16**–**19** were synthesized in a similar manner, by reaction of the appropriate indole-3-acetic acid with CDI for 3 h followed by reaction with spermine for 72 h (Scheme 2). A single example of an indolcarboxamide analogue (**20**) was prepared in a similar manner starting from 5-bromoindole-3-carboxylic acid (Scheme 3).

Biological activities

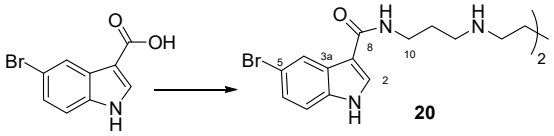
The intrinsic antimicrobial activity of each compound was evaluated against a panel of Gram-positive (*Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA)) and Gram-negative (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii*) bacteria and two fungal strains (*Candida albicans* and *Cryptococcus neoformans*) (Table 1). Several of the analogues exhibited antimicrobial activity towards Gram-positive bacteria and the fungus *Cryptococcus neoformans*, with 5-bromocarboxamide **20** exhibiting strong activity against *S. aureus* (MIC 4.8 μM), *S. aureus* MRSA (MIC 12.4 μM) and *C. neoformans* (MIC 12.4 μM). Similar selectivity's were observed for **10**, **11** and to a less active extent **12** and **13**. As noted previously for indole-linked polyamine derivatives, *C. neoformans* was particularly susceptible, with notable potency observed for the **10**–**13** analogues (MIC 2.9–6.9 μM). Little to no activity was observed towards Gram-negative bacteria.



Scheme 1. Reagents and conditions: (a) Malonic acid (1.5 eq.), piperidine, pyridine, 80 °C, 6 h, **2** 34 %, **3** 53 %, **4** 54 %, **5** 60 %, **6** 68 %, **7** 48 %, **8** 35 %; (b) spermine (0.5 eq.), CDI (1 eq.), DMF, r.t., 72 h, **9** 62 %, **10** 65 %, **11** 60 %, **12** 76 %, **13** 54 %, **14** 75 %, **15** 72 %.



Scheme 2. Reagents and conditions: Spermine (0.5 eq.), CDI (1 eq.), DMF, r.t., 72 h, **16** 42 %, **17** 38 %, **18** 14 %, **19** 33 %.



Scheme 3. Reagents and conditions: Spermine (0.5 eq.), CDI (1 eq.), DMF, r.t., 72 h, 28%.

In particular, it is relevant to note the exceptionally weak activity (MIC 100 to >200 μM) of the series of compounds towards *Pseudomonas aeruginosa* ATCC27853.

The set of compounds were next evaluated for the ability to enhance the antibiotic activity of doxycycline towards *P.*

aeruginosa ATCC27853 (Table 2). The assay used a fixed concentration of doxycycline [2 μg/mL (4.5 μM)] which is ineffective as the intrinsic MIC of doxycycline towards this bacterium was twenty-fold higher [MIC 40 μg/mL (90 μM)]. Two-fold serial dilutions of the test compounds covering a concentration range of 3.125 to 200 μM were examined for the ability to completely inhibit visible growth of the bacterium. Halogen-substituted compounds were found to be more effective than either their methoxyl or non-substituted variants, with brominated analogues (**10**, **11**, **17**, **20**, 3.12–6.25 μM) being more effective again than the fluorine-containing analogues (**12**, **13**, **18**, 6.25–50 μM). The indole-3-carboxamide analogue **20** was identified as the most effective enhancer of doxycycline activity towards *P. aeruginosa* in the compound test set.

Table 1. Antimicrobial and antifungal activities (MIC, μM) of compounds **1**, **9**–**20**.

Cmpd	*S. aureus*[a]	*S. aureus MRSA*[b]	*P. aeruginosa*[c]	*E. coli*[d]	*K. pneumoniae*[e]	*A. baumannii*[f]	*C. albicans*[g]	*C. neoformans*[h]
1	6.25[i]	2.16	100[i]	100[i]	>34[i] [j]	>34[i] [j]	17.2[i]	1.1[i]
9	50	59	>200[k]	200	>59[j]	>59[j]	>59[j]	14.8
10	12.5	11.4	100	50	>46[j]	>46[j]	45.8	5.7
11	12.5	11.4	200	50	>46[j]	>46[j]	45.8	2.9
12	50	27.7	>200[k]	200	>55[j]	>55[j]	55.5	6.9
13	25	27.7	200	200	>55[j]	>55[j]	55.5	6.9
14	100	>53[j]	>200[k]	>200[k]	>53[j]	>53[j]	>53[j]	13.3
15	100	>53[j]	>200[k]	>200[k]	>53[j]	>53[j]	53.3	13.3
16	200	>62[j]	>200[k]	>200[k]	>62[j]	>62[j]	>62[j]	>62[j]
17	25	47.4	200	200	>48[j]	>48[j]	>48[j]	11.9
18	100	>58[j]	>200[k]	>200[k]	>58[j]	>58[j]	>58[j]	>58[j]
19	200	>55[j]	>200[k]	>200[k]	>55[j]	>55[j]	>55[j]	>55[j]
20	4.8	12.4	200	200	>50[j]	>50[j]	49.5	12.4

[a] *Staphylococcus aureus* ATCC25923 with streptomycin (MIC 21.5 μM) and chloramphenicol (MIC 1.5–3 μM) used as positive controls and values presented as the mean (n = 3). [b] *Staphylococcus aureus* ATCC43300 (MRSA) with vancomycin (MIC 1 μg/mL) used as a positive control and values presented as the mean (n = 2). [c] *Pseudomonas aeruginosa* ATCC27853 with streptomycin (MIC 21.5 μM) and colistin (MIC 1 μM) used as positive controls and values presented as the mean (n = 3). [d] *Escherichia coli* ATCC25922 with streptomycin (MIC 21.5 μM) and colistin (MIC 2 μM) used as positive controls and values presented as the mean (n = 3). [e] *Klebsiella pneumoniae* ATCC700603 with colistin (MIC 0.25 μg/mL) as a positive control and values presented as the mean (n = 2). [f] *Acinetobacter baumannii* ATCC19606 with colistin (MIC 0.25 μg/mL) as a positive control and values presented as the mean (n = 2). [g] *Candida albicans* ATCC90028 with fluconazole (MIC 0.125 μg/mL) as a positive control and values presented as the mean (n = 2). [h] *Cryptococcus neoformans* ATCC208821 with fluconazole (MIC 8 μg/mL) as a positive control and values presented as the mean (n = 2). [i] Data taken from Li *et. al* [19] [j] Not active at a single dose test of 32 μg/mL. [k] Not active at a single dose test of 200 μM.

Next, four of the more active doxycycline/*P. aeruginosa* enhancers, **11**, **14**, **17** and **20**, were evaluated for their abilities to restore the action of an expanded set of antibiotics (doxycycline, erythromycin, chloramphenicol and nalidixic acid) against four Gram-negative ESKAPE[21] pathogens (*P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii*) (Table 3).

In addition to *P. aeruginosa*, all four analogues were generally able to strongly enhance the action of doxycycline towards Gram-negative bacteria (1.56–12.5 μM), with less effectiveness observed for **20** towards *K. pneumoniae* (100 μM), for **14** towards *E. coli* (25 μM), and for **11**, **14** and **17** towards *A. baumannii* (25–50 μM). Three analogues were good enhancers of nalidixic acid against *P. aeruginosa*, and in the cases of **11** and **17** also exhibiting enhanced activity against *K. pneumoniae*.

The antibiotic enhancing properties of **17** were further explored in a dose-dependent study, where enhancing activity was determined against the same four pathogens but this time in the presence of increasing concentrations (2, 4 or 8 μg/mL) of antibiotics (Table 4). In the presence of doxycycline and nalidixic acid, **17** demonstrated dose-dependent antibiotic enhancement, with notable effectiveness for doxycycline against *P. aeruginosa*, *E. coli* and *K. pneumoniae* and for nalidixic acid against *P. aeruginosa* and *K. pneumoniae*. Even at the highest test dose (8 μg/mL) limited or no enhancement was observed in the presence of erythromycin or chloramphenicol.

Table 2. Doxycycline potentiating activity of compounds **1**, **9**–**20**.

Compound	Potentiation[a]	Compound	Potentiation[a]
1[b]	6.25[b]	**15**	50
9	25	**16**	50
10	6.25	**17**	6.25
11	6.25	**18**	50
12	6.25	**19**	200
13	12.5	**20**	3.125
14	12.5		

[a] Concentration (μM) required to restore doxycycline activity at 2 μg/mL (4.5 μM) against *P. aeruginosa* ATCC27853. MIC of doxycycline alone is 40 μg/mL (90 μM). Values presented as the mean (n = 3). [b] Data for compound **1** taken from Li *et. al* [19].

Low levels of cytotoxicity, towards the rat skeletal muscle (L6) and human embryonic kidney (HEK-293) cell lines (IC_{50} / CC_{50} values >46 μM), and weak human red blood cell haemolytic properties (HC_{10} >50 μM) were observed for the compound set. When compared to the cytotoxicity observed for the original 6-bromoindole-glyoxylamido lead compound (**1**) in the series, the results clearly identify the indole-3-acrylic acid, indole-3-acetic acid and indole-3-carboxylic analogues as being more selective as antimicrobial agents with little to no effect on mammalian cells and red blood cell membranes.

Table 3. Antibiotic potentiating activity of analogues **11**, **14**, **17** and **20**.

Antibiotic	Compound	Potentiation[a]			
		P. aeruginosa[b]	*E. coli*[c]	*K. pneumoniae*[d]	*A. baumannii*[e]
Doxycycline	**11**	6.25	3.125	6.25	25
	14	12.5	25	6.25	25
	17	6.25	3.125	6.25	50
	20	3.125	1.56	100	6.25
Erythromycin	**11**	100	50	100	100
	14	>200	100	>200	50
	17	50	100	100	50
	20	100	100	200	100
Chloramphenicol	**11**	200	100	200	100
	14	100	200	>200	>200
	17	200	200	50	200
	20	12.5	>200	200	200
Nalidixic acid	**11**	12.5	100	12.5	100
	14	200	100	>200	>200
	17	6.25	200	12.5	200
	20	12.5	200	200	200

[a] Concentration (μM) of compound required to restore antibiotics activity at 2 μg/mL concentration of antibiotic. [b] *Pseudomonas aeruginosa* ATCC27853 against doxycycline (MIC 50 μM), erythromycin (MIC >200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3). [c] *Escherichia coli* ATCC25922 against doxycycline (MIC 25 μM) erythromycin (MIC >200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3). [d] *Klebsiella pneumoniae* ST258 against doxycycline (MIC 25 μM) erythromycin (MIC >200 μM), chloramphenicol (MIC 50 μM) and nalidixic acid (MIC 100 μM) and values presented as the mean (n = 3). [e] *Acinetobacter baumannii* AYE against doxycycline (MIC 12.5 μM) erythromycin (MIC 200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3).

Previous studies have noted the ability of antimicrobial polyamine analogues to disrupt the outer membrane of bacteria, resulting in permeabilisation and increased antibiotic influx leading to cell death.[10][22][23] To examine whether such effects are applicable to the current set of compounds, we selected **14**, being non-cytotoxic, non-haemolytic, as exhibiting limited intrinsic antibacterial properties and as being a moderate antibiotic adjuvant, for further study. A bioluminescence method was used to determine the effect of **14** on the intracellular pool of bacterial ATP – the detection of extra-cellular ATP was used as a reporter reflecting the permeabilising effect of the compound. After a 1 sec exposure to the test compound, rapid dose-dependent intracellular ATP leakage from *S. aureus* was observed over a 5 sec period. The response was lower than that observed for the positive control, squalamine (Figure 2). Significantly lower ATP efflux was observed for *P. aeruginosa*, both in the presence and absence of doxycycline. This suggests that a disruption of the membrane barrier occurs in a weak manner but is sufficient to enhance the entrance of doxycycline into the cytoplasm.

Next, **14** was investigated for its ability to alter the outer membrane integrity of *Enterobacter aerogenes* (EA289) using a chromogenic β-lactam (nitrocefin) colorimetric assay where the rate of nitrocefin hydrolysis by periplasmic β-lactamases is a measure of outer membrane permeabilisation. As shown in Figure 3, **14** demonstrated a dose-dependent effect, identifying it to be capable of permeabilising the outer membrane of Gram-negative bacteria at high concentrations.

We next investigated the ability of **14** to disrupt transmembrane potential, an energy source for drug efflux pumps.[10] Use was made of the membrane-potential-sensitive probe $DiSC_3(5)$, which when preloaded into bacteria, concentrates in the inner membrane and self-quenches its fluorescence. If the test compound effects membrane depolarisation, the dye is released and fluorescence increases. In a 15 min incubation experiment, with compound doses ranging from 15.625–125 μM, only weak levels of membrane depolarisation were observed for both of the test bacteria, *S. aureus* (Figure 4, upper) and *P. aeruginosa* (PAO1) (Figure 4, lower). These results are in stark contrast to the ability of the original hit compound **1**, which we have previously found to exhibit potent ability to depolarise bacterial membranes.[19]

Table 4. Dose-dependent antibiotic potentiating activity of **17**.

Antibiotic	Conc. [a]	Potentiation[b]			
		P. aeruginosa[c]	*E. coli*[d]	*K. pneumoniae*[e]	*A. baumannii*[f]
Doxycycline	2	6.25	3.125	6.25	>50
	4	3.125	1.56	3.125	>50
	8	1.56	0.78	0.78	50
Erythromycin	2	50	>50	>50	50
	4	50	50	>50	n.t.[g]
	8	50	50	50	n.t. [g]
Chloramphenicol	2	>50	>50	50	>50
	4	50	>50	25	>50
	8	50	>50	12.5	>50
Nalidixic acid	2	6.25	>50	12.5	>50
	4	3.125	>50	3.125	n.t. [g]
	8	1.56	>50	0.78	n.t. [g]

[a] Concentration of antibiotic used, in μg/mL. [b] Concentration (μM) of compound required to restore antibiotic activity at varying concentrations of antibiotic. [c] *Pseudomonas aeruginosa* ATCC27853 against doxycycline (MIC 50 μM), erythromycin (MIC >200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3). [d] *Escherichia coli* ATCC25922 against doxycycline (MIC 25 μM) erythromycin (MIC >200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3). [e] *Klebsiella pneumoniae* ST258 against doxycycline (MIC 25 μM) erythromycin (MIC >200 μM), chloramphenicol (MIC 50 μM) and nalidixic acid (MIC 100 μM) and values presented as the mean (n = 3). [f] *Acinetobacter baumannii* AYE against doxycycline (MIC 12.5 μM) erythromycin (MIC 200 μM), chloramphenicol (MIC >200 μM) and nalidixic acid (MIC >200 μM) and values presented as the mean (n = 3). [g] Not tested.

To specifically address whether derivative **14** could inhibit efflux pumps, we investigated its ability to alter the transport of the fluorescent AcrAB-TolC efflux pump substrate 1,2'-dinaphthylamine (1,2'-diNA) in EA289 bacteria. This bacterial species overexpresses the AcrAB-Tolc C pump which uses the proton gradient across the inner membrane as an energy source. By loading EA289 bacteria with the 1,2'-diNA dye and de-energization by adding carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the bacteria are rendered fluorescent. Bacteria were then incubated with and without **14** at different concentrations (16–256 μM) before addition of glucose as an energy source. In the absence of **14**, a rapid decrease of the fluorescence (i.e. active transport of the dye) was observed (Figure 5, purple line) whereas in the presence of **14**, significant dose-dependent inhibition was observed resulting in retention of the dye at all test concentrations (Figure 5, blue lines). These results suggest that this class of compounds may act as an inhibitor of the AcrAB-TolC efflux pump directly or by inhibiting the proton gradient.

Taken together these results identify the methoxyindoleacrylamide **14** to be an antibiotic adjuvant, that can increase the permeability of bacterial outer membranes and inhibit the AcrAB-TolC efflux pump, but with little to no membrane depolarisation.

Conclusion

In our search for new examples of antibiotic enhancers, we have previously reported that 6-bromoindolglyoxylamide **1** can enhance the activity of doxycycline towards a panel of Gram-negative bacteria, including *P. aeruginosa*, but unfortunately the compound also exhibits cytotoxicity and is haemolytic. A targeted series of analogues of the hit, identified that changing substituents on the indole ring system and varying the length of the polyamine core could afford antibiotic adjuvants that had reduced levels of cytotoxicity and haemolytic properties. To explore further variation on the indole moiety, analogues bearing indole-3-carboxylic acid, indole-3-acetic acid and indole-3-acrylic acid residues were synthesised and evaluated for antibiotic enhancing activity. Examples of each compound class were found to be as potent, or more so, as enhancers of doxycycline towards a range of Gram-negative bacteria compared to **1**. Gratifyingly, the new adjuvants were found to be non-cytotoxic towards two mammalian cell lines and non-haemolytic towards human red blood cells, highlighting their selectivity and identifying them as leads for further exploration. Mechanistically, **14** targets bacterial membrane integrity and also inhibits drug efflux, both effects presumably leading to heightened antibiotic ingress and retention, and subsequent cell death. The finding that several of the analogues, including **14**, can act as an antibiotic enhancer but with attenuated intrinsic antimicrobial activities, suggests the indole-3-acrylamido-polyamine scaffold could be approaching that of a true adjuvant.[12]

Experimental Section

General methods

Infrared spectra were recorded on a Perkin-Elmer spectrometer 100 Fourier Transform infrared spectrometer equipped with a universal ATR accessory. Melting points were determined on a Reichert-Hofler block and are uncorrected. Mass spectra were acquired on a Bruker micrOTOF Q II spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 298 K on Bruker AC300, AVANCE 400 or 500 spectrometer using standard pulse sequences. Proto-deutero solvent signals were used as internal references (DMSO-d_6: δ_H 2.50, δ_C 39.52; $CDCl_3$: δ_H 7.26, δ_C 77.16). For ^{1}H NMR, the data are quoted as position (δ), relative integral, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant (*J*, Hz), and assignment to the atom. The ^{13}C NMR data are quoted as position (δ), and assignment to the atom. Silica gel column chromatography was carried out using Davisil silica gel (40–60 μm) or Merck silica gel (15–40 μm). Column chromatography was also conducted on Merck Diol bonded silica (40–63 μm) and Luknova amino-bonded silica (50 μm). Gel filtration flash chromatography was carried out on Sephadex LH-20 (Pharmacia). Thin layer chromatography was conducted on Merck DC-plastikfolien Kieselgel 60 F254 plates or on Merck DC Kieselgel 60 RP-18 F254S plates. All solvents used were of analytical grade or better and/or purified according to standard procedures. Chemical reagents used were purchased from standard chemical suppliers and used as purchased. Indole-3-acrylic acid (**2**) was prepared according to a literature procedure.[24]

Table 5. Cytotoxicity (IC_{50}, μM) and haemolytic activity (HC_{10}, μM) of analogues **1**, **9**–**20**.

Compound	Cytotoxicity		Haemolysis[a]
	L6[b]	HEK-293[c]	HC_{10}
1	7.7 [d]	5.06 [d]	>34 [d][e]
9	102	>59[e]	>59 [e]
10	67.4	>46 [e]	>46 [e]
11	78.9	>46 [e]	>46 [e]
12	70.2	>55 [e]	>55 [e]
13	56.4	>55 [e]	>55 [e]
14	93.2	>53[e]	>53 [e]
15	100	>53 [e]	>53 [e]
16	95.9	n.t. [f]	n.t. [f]
17	59.8	>48 [e]	>48 [e]
18	79.3	n.t. [f]	n.t. [f]
19	78.9	n.t. [f]	n.t. [f]
20	58.9	>50 [e]	>50 [e]

[a] Concentration (HC_{10}, μM) of compound at 10% haemolytic activity on human red blood cells and values presented as the mean (n=2). Melittin was the positive control (HC_{10} 2.7 μg/mL). [b] L6 rat skeletal myoblast cell line with podophyllotoxin as the positive control (IC_{50} 0.018 μM) and values presented as the mean (n = 2). [c] Concentration of compound at 50% cytotoxicity on HEK-293 human embryonic kidney cells and values presented as the mean (n=2). Tamoxifen was the positive control (IC_{50} 9 μg/mL, 24μM). [d] Data taken from Li *et. al* [19] [e] Not active at a single dose test of 32 μg/mL. [f] Not tested.

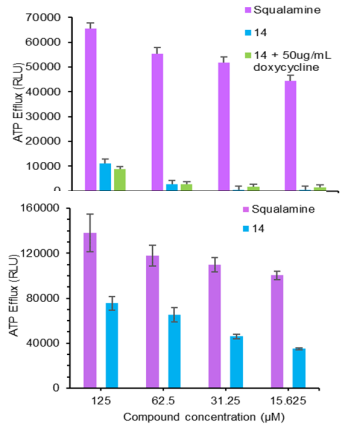


Figure 2. Dose-dependent ATP release for *S. aureus* (upper) and *P. aeruginosa* (lower) exhibited by **14** alone or in combination with doxycycline (50 μg/mL) with squalamine as the positive control.

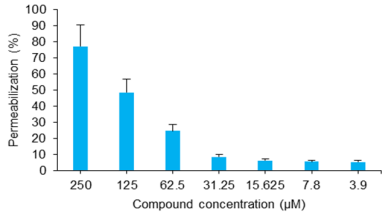


Figure 3. Dose-dependent effect of **14** on the rate of nitrocefin hydrolysis in *E. aerogenes* EA289.

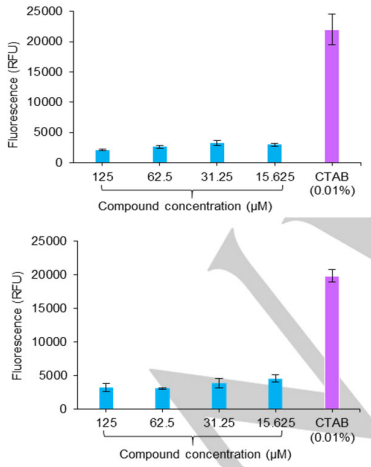


Figure 4. Depolarisation of the bacterial membrane of *S. aureus* (upper) and *P. aeruginosa* (lower) exhibited by **14** with cetyltrimethylammonium bromide (CTAB) (0.01%) as the positive control.

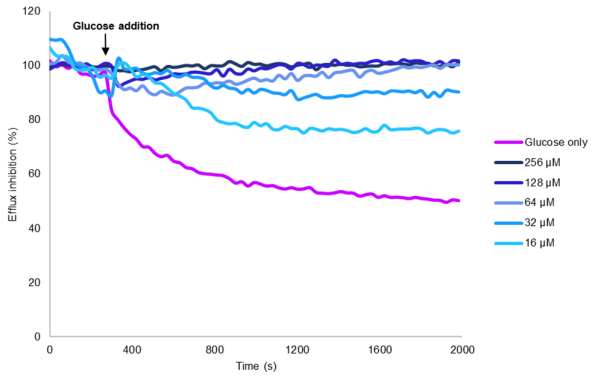


Figure 5. Dose-dependent inhibition by **14** of glucose-triggered 1,2'-diNA release via efflux pumps in *E. aerogenes* EA289.

General procedure A – Acrylic acid formation

To a stirred solution of indole-3-carbaldehyde (1 eq.) and malonic acid (1.5 eq.) in pyridine was added piperidine. The reaction mixture was allowed to stir at 80 °C for 6 h under N_2 atmosphere. Upon cooling, the crude reaction product was concentrated under reduced pressure and subjected to purification by silica gel column chromatography (EtOAc/*n*-hexane, 1:1) to afford the corresponding indole acrylic acid.

General procedure B – Coupling with spermine

To a stirred solution of indole acid (2 eq.) in DMF (1 mL) was added CDI (2 eq.) and stirred at room temperature for 3 h under N_2 atmosphere. Spermine (1 eq.) in DMF (1 mL) was then added and the reaction mixture was stirred for a further 72 h. Solvent was removed under reduced pressure and the crude product subjected to purification by amino-bonded silica gel (CH_2Cl_2/MeOH, 9:1) and diol-bonded silica gel (CH_2Cl_2/MeOH, 9:1) column chromatography to afford indole acrylamide, acetamide and carboxamide analogues.

(*E*)-3-(5-Bromo-1*H*-indol-3-yl)acrylic acid (3): Following general procedure A, 5-bromoindole-3-carbaldehyde (0.783 g, 3.2 mmol) was reacted with malonic acid (0.502 g, 4.8 mmol) and piperidine (100 μL) in pyridine (20 mL) to afford the title compound as a white solid (0.450 g, 53%). R_f (EtOAc) 0.57; m.p 227.4–228.2 °C; IR (ATR) ν_{max} 3222, 2901, 2532, 1640, 1611, 1421, 1231, 791, 701 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.92 (1H, s, OH), 11.90 (1H, br s, NH-1), 7.99 (1H, d, *J* = 1.5 Hz, H-4), 7.97 (1H, d, *J* = 2.2 Hz, H-2), 7.79 (1H, d, *J* = 16.1 Hz, H-8), 7.43 (1H, d, *J* = 8.5 Hz, H-7), 7.32 (1H, dd, *J* = 8.5, 1.5 Hz, H-6), 6.31 (1H, d, *J* = 16.1 Hz, H-9); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.4 (C-10), 137.6 (C-8), 136.0 (C-7a), 132.1 (C-2), 126.8 (C-3a), 124.9 (C-6), 121.9 (C-4), 114.3 (C-7), 113.4 (C-5), 113.0 (C-9), 111.3 (C-3); (–)-HRESIMS $[M–H]^-$ *m/z* 263.9668 (calcd for $C_{11}H_7{}^{79}BrNO_2$, 263.9666), 265.9649 (calcd for $C_{11}H_7{}^{81}BrNO_2$, 265.9646).

(*E*)-3-(6-Bromo-1*H*-indol-3-yl)acrylic acid (4): Following general procedure A, 6-bromoindole-3-carbaldehyde (0.200 g, 0.893 mmol) was reacted with malonic acid (0.139 g, 1.34 mmol) and piperidine (30 μL) in pyridine (6 mL) to afford the title compound as a pale yellow solid (0.130 g, 54%). R_f (EtOAc) 0.57; m.p 233.3–234.3 °C; IR (ATR) ν_{max} 3253, 2971, 2528, 1637, 1609, 1568, 955 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 11.82 (1H, br s, NH-1), 7.93 (1H, s, H-2), 7.81 (1H, d, *J* = 8.5 Hz, H-4), 7.77 (1H, d, *J* = 16.1 Hz, H-8), 7.64 (1H, d, *J* = 1.8 Hz, H-7), 7.27 (1H, dd, *J* = 8.5, 1.8 Hz, H-5), 6.31 (1H, d, *J* = 16.1 Hz, H-9), not observed (OH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.4 (C-10), 138.2 (C-7a), 137.7 (C-8), 131.8 (C-2), 124.0 (C-3a), 123.5 (C-5), 121.5 (C-4), 115.0 (C-6), 114.9 (C-7), 113.3 (C-9), 111.8 (C-3); (+)-HRESIMS $[M+Na]^+$ *m/z* 287.9626 (calcd for

$C_{11}H_8{}^{79}BrNNaO_2$, 287.9631), 289.9614 (calcd for $C_{11}H_8{}^{81}BrNNaO_2$, 289.9611).

(*E*)-3-(5-Fluoro-1*H*-indol-3-yl)acrylic acid (5): Following general procedure A, 5-fluoroindole-3-carbaldehyde (0.200 g, 1.23 mmol) was reacted with malonic acid (0.193 g, 1.85 mmol) and piperidine (40 µL) in pyridine (8 mL) to afford the title compound as a yellow solid (0.152 g, 60%). R_f (EtOAc) 0.37; m.p 208.5–209.7 °C; IR (ATR) ν_{max} 3257, 2964, 2843, 1634, 1611, 1424, 1243, 670 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.88 (1H, s, OH), 11.81 (1H, br s, NH-1), 7.98 (1H, d, J = 2.9 Hz, H-2), 7.78 (1H, d, J = 16.0 Hz, H-8), 7.60 (1H, dd, J = 10.2, 2.5 Hz, H-4), 7.46 (1H, dd, J = 8.8, 4.7 Hz, H-7), 7.05 (1H, ddd, J = 9.1, 9.1, 2.5 Hz, H-6), 6.28 (1H, d, J = 16.0 Hz, H-9); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.5 (C-10), 157.9 (d, $^1J_{FC}$ = 233.5 Hz, C-5), 137.9 (C-8), 133.9 (C-7a), 132.6 (C-2), 125.3 (d, $^3J_{FC}$ = 9.8 Hz, C-3a), 113.4 (d, $^3J_{FC}$ = 8.5 Hz, C-7), 112.5 (C-9), 111.8 (d, $^4J_{FC}$ = 5.1 Hz, C-3), 110.4 (d, $^2J_{FC}$ = 25.8, C-6), 104.8 (d, $^2J_{FC}$ = 24.2 Hz, C-4); (+)-HRESIMS $[M+Na]^+$ *m/z* 228.0435 (calcd for $C_{11}H_8FNNaO_2$, 228.0431).

(*E*)-3-(6-Fluoro-1*H*-indol-3-yl)acrylic acid (6): Following general procedure A, 6-fluoroindole-3-carbaldehyde (0.200 g, 1.23 mmol) was reacted with malonic acid (0.193 g, 1.85 mmol) and piperidine (40 µL) in pyridine (8 mL) to afford the title compound as a yellow solid (0.172 g, 68%). R_f (EtOAc) 0.69; m.p 198.5–199.7 °C; IR (ATR) ν_{max} 3279, 2934, 2867, 1637, 1611, 1420, 1231, 669 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.90 (1H, s, OH), 11.75 (1H, br s, NH-1), 7.90 (1H, d, J = 2.7 Hz, H-2), 7.85 (1H, dd, J = 8.8, 5.2 Hz, H-4), 7.78 (1H, d, J = 16.0 Hz, H-8), 7.25 (1H, dd, J = 9.8, 2.4 Hz, H-7), 7.00 (1H, ddd, J = 9.6, 9.6, 2.4 Hz, H-5), 6.31 (1H, d, J = 16.0 Hz, H-9); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.5 (C-10), 159.1 (d, $^1J_{FC}$ = 236.0 Hz, C-6), 138.0 (C-8), 137.4 (d, $^3J_{FC}$ = 12.5 Hz, C-7a), 131.8 (C-2), 121.8 (C-3a), 120.9 (d, $^3J_{FC}$ = 10.1 Hz, C-4), 112.7 (C-9), 111.8 (C-3), 109.0 (d, $^2J_{FC}$ = 24.1 Hz, C-5), 98.4 (d, $^2J_{FC}$ = 25.3, C-7); (+)-HRESIMS $[M+Na]^+$ *m/z* 228.0425 (calcd for $C_{11}H_8FNNaO_2$, 228.0431).

(*E*)-3-(5-Methoxy-1*H*-indol-3-yl)acrylic acid (7): Following general procedure A, 5-methoxyindole-3-carbaldehyde (0.200 g, 1.14 mmol) was reacted with malonic acid (0.178 g, 1.71 mmol) and piperidine (40 µL) in pyridine (7 mL) to afford the title compound as a white solid (0.118 g, 48%). R_f (EtOAc) 0.86; m.p 208.7–210.1 °C; IR (ATR) ν_{max} 3427, 3123, 2953, 1652, 1596, 1407, 1216, 670 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.81 (1H, s, OH), 11.59 (1H, br s, NH-1), 7.85 (1H, d, J = 2.8 Hz, H-2), 7.80 (1H, d, J = 15.9 Hz, H-8), 7.35 (1H, d, J = 8.8 Hz, H-7), 7.26 (1H, d, J = 2.4 Hz, H-4), 6.84 (1H, dd, J = 8.8, 2.4 Hz, H-6), 6.24 (1H, d, J = 15.9 Hz, H-9), 3.82 (3H, s, 5-OMe); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.6 (C-10), 154.7 (C-5), 138.5 (C-8), 132.1 (C-7a), 131.3 (C-2), 125.6 (C-3a), 113.0 (C-7), 112.52 (C-6), 112.48 (C-3), 111.5 (C-9), 101.6 (C-4), 55.4 (OMe); (+)-HRESIMS $[M+Na]^+$ *m/z* 240.0634 (calcd for $C_{12}H_{11}NNaO_3$, 240.0631).

(*E*)-3-(6-Methoxy-1*H*-indol-3-yl)acrylic acid (8): Following general procedure A, 6-methoxyindole-3-carbaldehyde (0.200 g, 1.14 mmol) was reacted with malonic acid (0.178 g, 1.71 mmol) and piperidine (40 µL) in pyridine (7 mL) to afford the title compound as a pale yellow solid (0.086 g, 35%). R_f (EtOAc) 0.60; m.p 186.6–187.5 °C; IR (ATR) ν_{max} 3427, 3383, 2944, 2836, 1655, 1599, 1403, 1228, 664 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.81 (1H, s, OH), 11.50 (1H, br s, NH-1), 7.75 (1H, d, J = 2.7 Hz, H-2), 7.74 (1H, d, J = 15.9 Hz, H-8), 7.71 (1H, d, J = 8.7 Hz, H-4), 6.95 (1H, d, J = 2.3 Hz, H-7), 6.80 (1H, dd, J = 8.7, 2.3 Hz, H-5), 6.24 (1H, d, J = 15.9 Hz, H-9), 3.79 (3H, s, 5-OMe); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 168.6 (C-10), 156.1 (C-6), 138.6 (C-8), 138.3 (C-7a), 130.5 (C-2), 120.4 (C-4), 119.0 (C-3a), 111.8 (C-3, C-9), 110.5 (C-5), 95.4 (C-7), 55.2 (OMe); (+)-HRESIMS $[M+Na]^+$ *m/z* 240.0637 (calcd for $C_{12}H_{11}NNaO_3$, 240.0631).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(1*H*-indol-3-yl)acrylamide) (9): Following general procedure B, reaction of (*E*)-3-(1*H*-indol-3-yl)acrylic acid (**2**) (0.069 g, 0.367 mmol) was reacted with CDI (0.060 g, 0.367 mmol) and spermine (0.038 g, 0.185 mmol) afforded the title compound as a yellow gum (0.062 g, 62%). R_f (MeOH/10% HCl, 7:3) 0.59; IR (ATR) ν_{max} 3412, 3254, 2928, 2863, 1654, 1605, 1438, 1023, 747 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.59 (2H, br s, NH-1), 7.99 (2H, t, J = 5.5 Hz, NH-11), 7.89 (2H, d, J = 7.3 Hz, H-4), 7.73 (2H, s, H-2), 7.61 (2H, d, J = 15.9 Hz, H-8), 7.45 (2H, d, J = 7.3 Hz, H-7), 7.17 (2H, ddd, J = 18.5, 7.3, 1.0 Hz, H-6), 7.14 (2H, ddd, J = 18.5, 7.3, 1.0 Hz, H-5), 6.62 (2H, d, J = 15.9 Hz, H-9), 3.28–3.21 (4H, m, H_2-12), 2.65–2.51 (8H, m, H_2-14, H_2-16), 1.69–1.58 (4H, m, H_2-13) 1.53–1.45 (4H, m, H_2-17), not observed (NH-15); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.4 (C-10), 137.4 (C-7a), 132.8 (C-8), 130.1 (C-2), 124.9 (C-3a), 122.1 (C-6), 120.2 (C-5), 119.9 (C-4), 116.3 (C-9), 112.2 (C-3 or C-7), 112.1 (C-7 or C-3), 48.6 (C-16), 46.3 (C-14), 36.6 (C-12), 29.0 (C-13), 26.7 (C-17); (+)-HRESIMS $[M+Na]^+$ *m/z* 563.3099 (calcd for $C_{32}H_{40}N_6NaO_2$, 563.3105).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(5-bromo-1*H*-indol-3-yl)acrylamide) (10): Following general procedure B, reaction of (*E*)-3-(5-bromo-1*H*-indol-3-yl)acrylic acid (**3**) (0.076 g, 0.286 mmol) was reacted with CDI (0.046 g, 0.286 mmol) and spermine (0.029 g, 0.143 mmol) afforded the title compound as a yellow gum (0.065 g, 65%). R_f (MeOH/10% HCl, 7:3) 0.14; IR (ATR) ν_{max} 3388, 3261, 2932, 2857, 1650, 1600, 1455, 1276, 1023, 760 cm^{-1};^1H NMR (DMSO-d_6, 400 MHz) δ 8.03 (2H, d, J = 1.7 Hz, H-4), 7.94 (2H, t, J = 5.0 Hz, NH-11), 7.80 (2H, s, H-2), 7.55 (2H, d, J = 16.0 Hz, H-8), 7.42 (2H, d, J = 8.7 Hz, H-7), 7.31 (2H, dd, J = 8.7, 1.7 Hz, H-6), 6.57 (2H, d, J = 16.0 Hz, H-9), 3.25–3.18 (4H, m, H_2-12), 2.55–2.45 (8H, m, H_2-14, H_2-16), 1.63–1.54 (4H, m, H_2-13), 1.45–1.37 (4H, m, H_2-17), not observed (NH-1, NH-15); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.1 (C-10), 136.1 (C-7a), 132.1 (C-8), 131.6 (C-2), 126.5 (C-3a), 124.7 (C-6), 122.1 (C-4), 117.0 (C-9), 114.3 (C-7), 113.1 (C-5), 111.8 (C-3), 49.5 (C-16), 47.1 (C-14), 36.9 (C-12), 29.8 (C-13), 27.6 (C-17); (–)-HRESIMS $[M–H]^-$ *m/z* 695.1377 (calcd for $C_{32}H_{37}{}^{79}Br_2N_6O_2$, 695.1350), 697.1377 (calcd for $C_{32}H_{37}{}^{79}Br^{81}BrN_6O_2$, 697.1332), 699.1352 (calcd for $C_{32}H_{37}{}^{81}Br_2N_6O_2$, 699.1320).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(6-bromo-1*H*-indol-3-yl)acrylamide) (11): Following general procedure B, reaction of (*E*)-3-(6-bromo-1*H*-indol-3-yl)acrylic acid (**4**) (0.076 g, 0.286 mmol) was reacted with CDI (0.046 g, 0.286 mmol) and spermine (0.029 g, 0.143 mmol) afforded the title compound as a yellow gum (0.060 g, 60%). R_f (MeOH/10% HCl, 7:3) 0.26; IR (ATR) ν_{max} 3391, 3265, 2933, 2863, 1652, 1605, 1450, 1254, 1001, 759 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.69 (NH-1), 8.00 (2H, t, J = 5.5 Hz, NH-11), 7.82 (2H, d, J = 8.6 Hz, H-4), 7.79 (2H, s, H-2), 7.65 (2H, d, J = 1.7 Hz, H-7), 7.58 (2H, d, J = 15.9 Hz, H-8), 7.29 (2H, dd, J = 8.6, 1.7 Hz, H-5), 6.61 (2H, d, J = 15.9 Hz, H-9), 3.28–3.19 (4H, m, H_2-12), 2.67–2.54 (8H, m, H_2-14, H_2-16), 1.70–1.60 (4H, m, H_2-13), 1.54–1.46 (4H, m, H_2-17); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.2 (C-10), 138.2 (C-7a), 132.2 (C-8), 130.8 (C-2), 123.9 (C-3a), 123.0 (C-5), 121.5 (C-4), 117.1 (C-9), 114.9 (C-6 or C-7), 114.8 (C-7 or C-6), 112.2 (C-3), 48.4 (C-16), 46.1 (C-14), 36.5 (C-12), 28.8 (C-13), 26.5 (C-17); (+)-HRESIMS $[M+Na]^+$ *m/z* 719.1339 (calcd for $C_{32}H_{38}{}^{79}Br_2N_6NaO_2$, 719.1315), 721.1334 (calcd for $C_{32}H_{38}{}^{79}Br^{81}BrN_6NaO_2$, 721.1297), 723.1316 (calcd for $C_{32}H_{38}{}^{81}Br_2N_6NaO_2$, 723.1284).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(5-fluoro-1*H*-indol-3-yl)acrylamide) (12): Following general procedure B, reaction of (*E*)-3-(5-fluoro-1*H*-indol-3-yl)acrylic acid (**5**) (0.071 g, 0.347 mmol) was reacted with CDI (0.056 g, 0.347 mmol) and spermine (0.035 g, 0.173 mmol) afforded the title compound as a yellow gum (0.076 g, 76%). R_f (MeOH/10% HCl, 7:3) 0.56; IR (ATR) ν_{max} 3240, 3167, 3052, 2930, 1654, 1607, 1471, 1275, 1005, 757 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.65 (2H, br s, NH-1), 7.92 (2H, t, J = 5.6 Hz, NH-11), 7.82 (2H, s, H-2), 7.63 (2H, dd, J = 10.3, 2.4 Hz, H-4), 7.58 (2H, d, J = 15.9 Hz, H-8), 7.46 (2H, dd, J = 9.1, 4.4 Hz, H-7), 7.06 (2H, ddd, J = 9.1, 9.1, 2.4 Hz, H-6), 6.58 (2H, d, J = 15.9 Hz, H-9), 3.27–3.19 (4H, m, H_2-12), 2.64–2.51 (8H, m, H_2-14, H_2-16), 1.67–1.59 (4H, m, H_2-13), 1.52–1.43 (4H, m, H_2-17); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.3 (C-10), 157.7 (d, $^1J_{FC}$ = 233.3 Hz, C-5), 134.0 (C-7a), 132.3 (C-8), 131.9 (C-2), 125.0 (d, $^3J_{FC}$ = 10.0 Hz, C-3a), 116.5 (C-9), 113.3 (d, $^3J_{FC}$ = 10.3 Hz, C-7), 112.2 (d, $^4J_{FC}$ = 4.1 Hz, C-3), 110.2 (d, $^2J_{FC}$ = 26.0 Hz, C-6), 104.9 (d, $^2J_{FC}$ = 24.2 Hz, C-

4), 48.7 (C-16), 46.9 (C-14), 36.6 (C-12), 29.0 (C-13), 26.7 (C-17); (–)-HRESIMS $[M–H]^-$ *m/z* 575.2930 (calcd for $C_{32}H_{37}F_2N_6O_2$, 575.2952).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(6-fluoro-1*H*-indol-3-yl)acrylamide) (13): Following general procedure B, reaction of (*E*)-3-(6-fluoro-1*H*-indol-3-yl)acrylic acid (**6**) (0.071 g, 0.347 mmol) was reacted with CDI (0.056 g, 0.347 mmol) and spermine (0.035 g, 0.173 mmol) afforded the title compound as a yellow gum (0.054 g, 54%). R_f (MeOH/10% HCl, 7:3) 0.54; IR (ATR) ν_{max} 3245, 2926, 2855, 1653, 1607, 1453, 1235, 1005, 758 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.61 (2H, br s, NH-1), 7.95 (2H, t, *J* = 5.5 Hz, NH-11), 7.87 (2H, dd, *J* = 8.9, 5.4 Hz, H-4), 7.75 (2H, s, H-2), 7.56 (2H, d, *J* = 15.9 Hz, H-8), 7.25 (2H, dd, *J* = 9.8, 2.4 Hz, H-7), 7.03 (2H, ddd, *J* = 9.8, 8.9, 2.4 Hz, H-5), 6.62 (2H, d, *J* = 15.9 Hz, H-9), 3.26–3.18 (4H, m, H_2-12), 2.60–2.51 (8H, m, H_2-14, H_2-16), 1.65–1.54 (4H, m, H_2-13), 1.49–1.42 (4H, m, H_2-17); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.2 (C-10), 159.0 (d, $^1J_{FC}$ = 236.8 Hz, C-6), 137.4 (d, $^3J_{FC}$ = 12.0 Hz, C-7a), 132.3 (C-8), 130.6 (C-2), 121.7 (C-3a), 120.8 (d, $^3J_{FC}$ = 9.3 Hz, C-4), 116.8 (C-9), 112.2 (C-3), 108.5 (d, $^2J_{FC}$ = 24.5 Hz, C-5), 98.3 (d, $^2J_{FC}$ = 25.2 Hz, C-7), 48.9 (C-16), 46.5 (C-14), 36.7 (C-12), 29.2 (C-13), 27.0 (C-17); (–)-HRESIMS $[M–H]^-$ *m/z* 575.2943 (calcd for $C_{32}H_{37}F_2N_6O_2$, 575.2952).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(5-methoxy-1*H*-indol-3-yl)acrylamide) (14): Following general procedure B, reaction of (*E*)-3-(5-methoxy-1*H*-indol-3-yl)acrylic acid (**7**) (0.072 g, 0.330 mmol) was reacted with CDI (0.054 g, 0.330 mmol) and spermine (0.033 g, 0.165 mmol) afforded the title compound as a yellow gum (0.075 g, 75%). R_f (MeOH/10% HCl, 7:3) 0.60; IR (ATR) ν_{max} 3244, 3043, 2935, 1651, 1603, 1481, 1218, 1023, 758 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.43 (2H, br s, NH-1), 7.95 (2H, t, *J* = 5.6 Hz, NH-11), 7.70 (2H, s, H-2), 7.58 (2H, d, *J* = 15.8 Hz, H-8), 7.36 (2H, d, *J* = 8.7 Hz, H-7), 7.35 (2H, d, *J* = 2.2 Hz, H-4), 6.86 (2H, dd, *J* = 8.7, 2.2 Hz, H-6), 6.53 (2H, d, *J* = 15.8 Hz, H-9), 3.84 (6H, s, 5-OMe), 3.27–3.19 (4H, m, H_2-12), 2.58–2.49 (8H, m, H_2-14, H_2-16), 1.65–1.55 (4H, m, H_2-13), 1.48–1.40 (4H, m, H_2-17); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.4 (C-10), 154.4 (C-5), 132.9 (C-8), 132.4 (C-7a), 130.5 (C-2), 125.4 (C-3a), 115.7 (C-9), 112.8 (C-7), 111.8 (C-3 or C-6), 111.6 (C-6 or C-3), 102.6 (C-4), 55.6 (OMe), 49.0 (C-16), 46.7 (C-14), 36.7 (C-12), 29.4 (C-13), 27.1 (C-17); (–)-HRESIMS $[M–H]^-$ *m/z* 599.3361 (calcd for $C_{34}H_{43}N_6O_4$, 599.3351).

(2*E*,2'*E*)-*N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(6-methoxy-1*H*-indol-3-yl)acrylamide) (15): Following general procedure B, reaction of (*E*)-3-(6-methoxy-1*H*-indol-3-yl)acrylic acid (**8**) (0.072 g, 0.330 mmol) was reacted with CDI (0.054 g, 0.330 mmol) and spermine (0.033 g, 0.165 mmol) afforded the title compound as a yellow gum (0.072 g, 72%). R_f (MeOH/10% HCl, 7:3) 0.66; IR (ATR) ν_{max} 3202, 2929, 2836, 1651, 1604, 1524, 1162, 1023, 757 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.36 (2H, br s, NH-1), 7.91 (2H, t, *J* = 5.6 Hz, NH-11), 7.76 (2H, d, *J* = 8.8 Hz, H-4), 7.60 (2H, s, H-2), 7.55 (2H, d, *J* = 15.7 Hz, H-8), 6.95 (2H, d, *J* = 2.2 Hz, H-7), 6.80 (2H, dd, *J* = 8.8, 2.2 Hz, H-5), 6.56 (2H, d, *J* = 15.7 Hz, H-9), 3.80 (6H, s, 5-OMe), 3.25–3.18 (4H, m, H_2-12), 2.58–2.49 (8H, m, H_2-14, H_2-16), 1.65–1.54 (4H, m, H_2-13), 1.47–1.39 (4H, m, H_2-17); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 166.4 (C-10), 156.0 (C-6), 138.3 (C-7a), 132.8 (C-8), 129.1 (C-2), 120.5 (C-4), 119.0 (C-3a), 116.1 (C-9), 112.2 (C-3), 110.1 (C-5), 95.3 (C-7), 55.2 (OMe), 49.1 (C-16), 46.7 (C-14), 36.7 (C-12), 29.4 (C-13), 27.2 (C-17); (–)-HRESIMS $[M–H]^-$ *m/z* 599.3352 (calcd for $C_{34}H_{43}N_6O_4$, 599.3351).

***N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(2-(1*H*-indol-3-yl)acetamide) (16):** Following general procedure B, reaction of indole-3-acetic acid (0.050 g, 0.29 mmol) was reacted with CDI (0.046 g, 0.29 mmol) and spermine (0.029 g, 0.14 mmol) afforded the title compound as a pale yellow oil (0.030 g, 42%). R_f (CH_2Cl_2/MeOH, 9:1) 0.28; IR (ATR) ν_{max} 3250, 3059, 2931, 1643, 1553, 1401, 1341, 1229, 1049, 1024, 1008, 745 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.89 (2H, br s, NH-1), 7.93 (2H, t, *J* = 5.5 Hz, NH-10), 7.53 (2H, d, *J* = 8.0 Hz, H-4), 7.33 (2H, d, *J* = 8.0 Hz, H-7), 7.17 (2H, d, *J* = 2.0 Hz, H-2), 7.05 (2H, td, *J* = 6.9, 0.9 Hz, H-6), 6.96 (2H, td, *J* = 6.9, 0.9 Hz, H-5), 3.48 (4H, s, H_2-8), 3.08 (4H, dt, *J* = 6.3, 6.0 Hz, H_2-11), 2.52–2.48 (4H, m, H_2-13), 2.46–2.43 (4H, m, H_2-15), 1.58–1.51 (4H, m, H_2-12), 1.40–1.37 (4H, m, H_2-16); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.7 (C-9), 136.1 (C-7a), 127.2 (C-3a), 123.8 (C-2), 120.9 (C-6), 118.6 (C-4), 118.2 (C-5), 111.3 (C-7), 108.9 (C-3), 48.4 (C-15), 46.1 (C-13), 36.7 (C-11), 32.8 (C-8), 28.5 (C-12), 26.4 (C-16); (+)-HRESIMS $[M+H]^+$ *m/z* 517.3283 (calcd for $C_{30}H_{41}N_6O_2$, 517.3286).

***N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(2-(5-bromo-1*H*-indol-3-yl)acetamide) (17):** Following general procedure B, reaction of 5-bromoindole-3-acetic acid (0.050 g, 0.20 mmol) was reacted with CDI (0.032 g, 0.20 mmol) and spermine (0.020 g, 0.10 mmol) afforded the title compound as a pale yellow gum (0.025 g, 38%). R_f (CH_2Cl_2/MeOH, 9:1) 0.44; IR (ATR) ν_{max} 3271, 2932, 1647, 1554, 1453, 1228, 1036, 953, 883, 796 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.12 (2H, br s, NH-1), 8.01 (2H, t, *J* = 5.2 Hz, NH-10), 7.74 (2H, d, *J* = 1.9 Hz, H-4), 7.31 (2H, d, *J* = 8.6 Hz, H-7), 7.23 (2H, s, H-2), 7.16 (2H, dd, *J* = 8.6, 1.9 Hz, H-6), 3.46 (4H, s, H_2-8), 3.08 (4H, dt, *J* = 6.2, 5.9 Hz, H_2-11), 2.51–2.47 (4H, m, H_2-13), 2.47–2.44 (4H, m, H_2-15), 1.58–1.51 (4H, m, H_2-12), 1.40–1.37 (4H, m, H_2-16); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.5 (C-9), 134.8 (C-7a), 129.1 (C-3a), 125.5 (C-2), 123.3 (C-6), 121.1 (C-4), 113.3 (C-7), 111.0 (C-5), 108.9 (C-3), 48.5 (C-15), 46.1 (C-13), 36.7 (C-11), 32.6 (C-8), 28.6 (C-12), 26.5 (C-16); (+)-HRESIMS $[M+H]^+$ *m/z* 673.1497 (calcd for $C_{30}H_{39}{}^{79}Br_2N_6O_2$, 673.1496), 675.1478 (calcd for $C_{30}H_{39}{}^{79}Br^{81}BrN_6O_2$, 675.1578), 677.1460 (calcd for $C_{30}H_{39}{}^{81}Br_2N_6O_2$, 677.1464).

***N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(2-(6-fluoro-1*H*-indol-3-yl)acetamide) (18):** Following general procedure B, reaction of 6-fluoroindole-3-acetic acid (0.050 g, 0.26 mmol) was reacted with CDI (0.042 g, 0.26 mmol) and spermine (0.026 g, 0.13 mmol) afforded the title compound as a pale yellow gum (0.010 g, 14%). R_f (CH_2Cl_2/MeOH, 9:1) 0.42; IR (ATR) ν_{max} 3430, 2975, 1679, 1420, 1268, 1130, 952 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.94 (2H, br s, NH-1), 7.92 (2H, br s, NH-10), 7.51 (2H, dd, *J* = 8.4, 5.4 H, H-4), 7.16 (2H, s, H-2), 7.10 (2H, dd, *J* = 10.5, 2.5 Hz, H-7), 6.82 (2H, ddd, *J* = 10.5, 8.4, 2.5 Hz, H-5), 3.46 (4H, s, H_2-8), 3.07 (4H, dt, *J* = 6.4, 5.9 Hz, H_2-11), 2.52–2.48 (4H, m, H_2-13), 2.48–2.42 (4H, m, H_2-15), 1.54–1.49 (4H, m, H_2-12), 1.39–1.36 (4H, m, H_2-16); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.5 (C-9), 158.8 (d, $^1J_{FC}$ = 236.9 Hz, C-6), 135.9 (d, $^3J_{FC}$ = 12.7 Hz, C-7a), 124.3 (d, $^4J_{FC}$ = 3 Hz, C-3a), 124.1 (C-2), 119.6 (d, $^3J_{FC}$ = 10.5 Hz, C-4), 109.2 (C-3), 106.7 (d, $^2J_{FC}$ = 24.2 Hz, C-5), 97.2 (d, $^2J_{FC}$ = 25.5 Hz, C-7), 48.6 (C-15), 46.3 (C-13), 36.7 (C-11), 32.7 (C-8), 28.8 (C-12), 26.7 (C-16); (+)-HRESIMS $[M+H]^+$ *m/z* 553.3090 (calcd for $C_{30}H_{39}F_2N_6O_2$, 553.3097).

***N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(2-(5-methoxy-1*H*-indol-3-yl)acetamide) (19):** Following general procedure B, reaction of 5-methoxyindole-3-acetic acid (0.050 g, 0.24 mmol) was reacted with CDI (0.040 g, 0.24 mmol) and spermine (0.025 g, 0.12 mmol) afforded the title compound as a pale yellow gum (0.023 g, 33%). R_f (CH_2Cl_2/MeOH, 9:1) 0.14; IR (ATR) ν_{max} 3392, 3272, 2972, 2940, 1645, 1560, 1487, 1439, 1406, 1216, 1165, 953 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 10.73 (2H, br s, NH-1), 7.93 (2H t, *J* = 5.7 Hz, NH-10), 7.22 (2H, d, *J* = 8.8 Hz, H-7), 7.13 (2H, d, *J* = 1.8 Hz, H-2), 7.04 (2H, d, *J* = 2.5 Hz, H-4), 6.70 (2H, dd, *J* = 8.8, 2.5 Hz, H-6), 3.74 (6H, s, 5-OMe), 3.44 (4H, s, H_2-8), 3.08 (4H, dt, *J* = 6.1, 6.1 Hz, H_2-11), 2.51–2.47 (4H, m, H_2-13), 2.44–2.41 (4H, m, H_2-15), 1.57–1.51 (4H, m, H_2-12), 1.38–1.35 (4H, m, H_2-16); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.7 (C-9), 153.0 (C-5), 131.3 (C-7a), 127.5 (C-3a), 124.4 (C-2), 111.9 (C-7), 111.0 (C-6), 108.7 (C-3), 100.5 (C-4), 55.3 (OMe), 48.5 (C-15), 46.2 (C-13), 36.7 (C-11), 32.9 (C-8), 28.7 (C-12), 26.5 (C-16); (+)-HRESIMS $[M+H]^+$ *m/z* 577.3501 (calcd for $C_{32}H_{45}N_6O_4$, 577.3497).

***N*,*N*'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(5-bromo-1*H*-indole-3-carboxamide) (20):** Following general procedure B, 5-bromoindole-3-carboxylic acid (0.050 g, 0.24 mmol) was reacted with CDI (0.040 g, 0.24 mmol) and spermine (0.025 g, 0.12 mmol) in DMF for 72 hours. Purification by column chromatography afforded the title compound as a pale brown oil (0.019 g, 28 %). R_f (CH_2Cl_2/MeOH, 9:1) 0.22; IR (ATR) ν_{max} 3263, 2927, 1627, 1553, 1457, 1379, 1225, 1142, 952,

803, 713 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 11.96 (2H, br s, NH-1), 8.31 (2H, br s, H-4), 8.18–8.14 (2H, m, NH-9), 8.09 (2H, br s, H-2), 7.42 (2H, d, *J* = 8.6 Hz, H-7), 7.26 (2H, d, *J* = 8.6 Hz, H-6), 3.33–3.29 (4H, m, H_2-10), 2.67–2.62 (4H, m, H_2-12), 2.61–2.57 (4H, m, H_2-14), 1.74–1.69 (4H, m, H_2–11), 1.53–1.49 (4H, m, H_2-15); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.2 (C-8), 134.8 (C-7a), 129.0 (C-2), 127.9 (C-3a), 124.2 (C-6), 123.1 (C-4), 113.9 (C-7), 113.0 (C-5), 110.2 (C-2), 48.3 (C-14), 46.1 (C-12), 36.4 (C-10), 28.6 (C-11), 26.2 (C-15); (+)-HRESIMS $[M+H]^+$ *m/z* 645.1158 (calcd for $C_{28}H_{35}{}^{79}Br_2N_6O_2$, 645.1183), 647.1139 (calcd for $C_{28}H_{35}{}^{79}Br^{81}BrN_6O_2$, 647.1164), 649.1130 (calcd for $C_{28}H_{35}{}^{81}Br_2N_6O_2$, 649.1150).

Biological evaluation

Antimicrobial assays: The susceptibility of bacterial strains *S. aureus* (ATCC25923), *E. coli* (ATCC25922) and *P. aeruginosa* (ATCC27853) to antibiotics and compounds was determined in microplates using the standard broth dilution method in accordance with the recommendations of the Comité de l'AntibioGramme de la Société Française de Microbiologie (CA-SFM) as previously described.[23] Briefly, the minimal inhibitory concentrations (MICs) were determined with an inoculum of 10^5 CFU in 200 µL of Mueller-Hinton broth (MHB) containing two-fold serial dilutions of each drug. The MIC was defined as the lowest concentration of drug that completely inhibited visible growth after incubation for 18 h at 37 °C. To determine all MICs, the measurements were independently repeated in triplicate.

Additional antimicrobial evaluation against *Staphylococcus aureus* MRSA (ATCC43300), *Klebsiella pneumoniae* (ATCC700603), *Acinetobacter baumannii* (ATCC19606), *Candida albicans* (ATCC90028), and *Cryptococcus neoformans* (ATCC208821) was undertaken at the Community for Open Antimicrobial Drug Discovery at The University of Queensland (Australia) according to their standard protocols.[25] For antimicrobial assays, the tested strains were cultured in either Luria broth (LB) (In Vitro Technologies, USB75852), nutrient broth (NB) (Becton Dickson, 234000), or cation-adjusted MHB at 37 °C overnight. A sample of culture was then diluted 40-fold in fresh MHB and incubated at 37 °C for 1.5−2 h. The compounds were serially diluted 2-fold across the wells of 96-well plates (Corning 3641, nonbinding surface), with compound concentrations ranging from 0.015 to 64 µg/mL, plated in duplicate. The resultant mid log phase cultures were diluted to the final concentration of 1 × 10^6 CFU/mL; then, 50 µL was added to each well of the compound containing plates giving a final compound concentration range of 0.008 to 32 µg/mL and a cell density of 5 × 10^5 CFU/mL. All plates were then covered and incubated at 37 °C for 18 h. Resazurin was added at 0.001% final concentration to each well and incubated for 2 h before MICs were read by eye.

For the antifungal assay, fungi strains were cultured for 3 days on yeast extract-peptone dextrose (YPD) agar at 30 °C. A yeast suspension of 1 × 10^6 to 5 × 10^6 CFU/mL was prepared from five colonies. These stock suspensions were diluted with yeast nitrogen base (YNB) (Becton Dickinson, 233520) broth to a final concentration of 2.5 × 10^3 CFU/mL. The compounds were serially diluted 2-fold across the wells of 96-well plates (Corning 3641, nonbinding surface), with compound concentrations ranging from 0.015 to 64 µg/mL and final volumes of 50 µL, plated in duplicate. Then, 50 µL of the fungi suspension that was previously prepared in YNB broth to the final concentration of 2.5 × 10^3 CFU/mL was added to each well of the compound-containing plates, giving a final compound concentration range of 0.008 to 32 µg/mL. Plates were covered and incubated at 35 °C for 36 h without shaking. *C. albicans* MICs were determined by measuring the absorbance at OD_{530}. For *C. neoformans*, resazurin was added at 0.006% final concentration to each well and incubated for a further 3 h before MICs were determined by measuring the absorbance at $OD_{570-600}$.

Colistin and vancomycin were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as a positive fungal inhibitor standard for *C. albicans* and *C. neoformans*. The antibiotics were provided in 4 concentrations, with 2 above and 2 below its MIC value, and plated into the first 8 wells of column 23 of the 384-well NBS plates. The quality control (QC) of the assays was determined by the antimicrobial controls and the Z'-factor (using positive and negative controls). Each plate was deemed to fulfil the quality criteria (pass QC), if the Z'-factor was above 0.4, and the antimicrobial standards showed full range of activity, with full growth inhibition at their highest concentration, and no growth inhibition at their lowest concentration.

Determination of the MICs of antibiotics in the presence of synergising compounds: Briefly, restoring enhancer concentrations were determined with an inoculum of 5x10^5 CFU in 200 µL of Mueller-Hinton Broth (MHB) containing two-fold serial dilutions of each derivative in the presence of doxycycline at 2 µg/mL.[23] The lowest concentration of the polyamine adjuvant that completely inhibited visible growth after incubation for 18 h at 37 °C was determined. These measurements were independently repeated in triplicate.

Cytotoxicity assays: The L6 cell line cytotoxicity assays were carried out exactly as previously described.[26] Briefly, each well of a 96-well microplate contained 100 µL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 x 10^4 L6 cells (a primary cell line derived from rat skeletal myoblasts).Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue solution (10 µL) was then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the reference drug used.

In the case of the HEK-293 cytotoxicity assay, cells were counted manually in a Neubauer haemocytometer and plated at a density of 5,000 cells/well into each well of the 384-well plates containing the 25x (2 µL) concentrated compounds.[25] The medium used was Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated together with the compounds for 20 h at 37 ºC, 5% CO_2. To measure cytotoxicity, 5 µL (equals 100 µM final) of resazurin was added to each well after incubation, and incubated for further 3 h at 37 ºC with 5% CO_2. After final incubation fluorescence intensity was measured as Fex 560/10 nm, em 590/10 nm ($F_{560/590}$) using a Tecan M1000 Pro monochromator plate reader. CC_{50} values (concentration at 50% cytotoxicity) were calculated by normalizing the fluorescence readout, with 74 µg/mL tamoxifen as negative control (0%) and normal cell growth as positive control (100%). The concentration-dependent percentage cytotoxicity was fitted to a dose response function (using Pipeline Pilot) and CC_{50} values determined.

Haemolytic assay: [25] Human whole blood was washed three times with 3 volumes of 0.9% NaCl and then resuspended in same to a concentration of 0.5 × 10^8 cells/mL, as determined by manual cell count in a Neubauer haemocytometer. The washed cells were then added to the 384-well compound-containing plates for a final volume of 50 µL. After a 10 min shake on a plate shaker the plates were then incubated for 1 h at 37 °C. After incubation, the plates were centrifuged at 1,000 g for 10 min to pellet cells and debris, 25 µL of the supernatant was then transferred to a polystyrene 384-well assay plate. Haemolysis was determined by measuring the supernatant absorbance at 405 mm (OD_{405}). The absorbance was measured using a Tecan M1000 Pro monochromator plate reader. HC_{10} and HC_{50} (concentration at 10% and 50% haemolysis, respectively) were calculated by curve fitting the inhibition values *vs.* log(concentration) using a sigmoidal dose-response function with variable fitting values for top, bottom and slope.

Measurement of ATP efflux: [23] Squalamine solutions were prepared in doubly distilled water at different concentrations. A suspension of growing *S. aureus* or *P. aeruginosa* PAO1 to be studied in MHB was prepared and incubated at 37°C. An aliquot of 90 µL of this suspension was added to 10 µL of squalamine solution and vortexed for 1 sec. Luciferin-luciferase reagent (Yelen, France; 50 µL) was immediately added to the precedent mix and luminescent signal quantified with an Infinite M200 microplate reader (Tecan) for five sec. ATP concentration was quantified by internal sample addition. A similar procedure was used for **14** (4 times the MIC).

Membrane depolarisation assays: [23] Bacteria were grown in MHB for 24 h at 37°C and centrifuged at 10,000 rpm at 20°C. The supernatant was discarded, and the bacteria were washed twice with buffered sucrose solution (250 mM) and magnesium sulfate solution (5 mM). The fluorescent dye 3,3′-diethylthiacarbocyanine iodide was added to a final concentration of 3 µM, and it was allowed to penetrate into bacterial membranes during 1 h of incubation at 37°C. Bacteria were then washed to remove the unbound dye before adding the compound at different concentrations. Fluorescence measurements were performed using a Jobin Yvon Fluoromax 3 spectrofluorometer with slit widths of 5/5 nm. The maximum fluorescence was recorded with a pure solution of the fluorescent dye in buffer (3 µM).

Nitrocefin hydrolysis assay: [23] Outer membrane permeabilisation was measured using nitrocefin as a chromogenic substrate of periplasmic β-lactamase. MHB (10 mL) was inoculated with 0.1 mL of an overnight culture of *Enterobacter aerogenes* (EA289) bacteria and grown at 37°C until the OD_{600} reached 0.5. The remaining steps were performed at room temperature. Cells were recovered by centrifugation (4,000 rpm for 20 min) and washed once in 20 mM potassium phosphate buffer (pH 7.2) containing $MgCl_2$ (1 mM). After a second centrifugation, the pellet was re-suspended and adjusted to OD_{600} of 0.5. Then, 50 µL of the desired compound were added to 100 µL of the cell suspension to obtain a final concentration varying from 3.9 to 250 µM. Fifty microliters of nitrocefin were then added to obtain a final concentration of 50 µg/mL. Nitrocefin hydrolysis was monitored spectrophotometrically by measuring the increase in absorbance at 490 nm. Assays were performed in 96-well plates using a M200 Pro Tecan spectrophotometer.

Glucose-triggered 1,2'-diNA efflux assays: [27] Bacteria were grown to stationary phase, collected by centrifugation, and resuspended to OD_{600}=0.25 in PPB (20 mM, pH 7.2) supplemented with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 5 µM), and incubated overnight with 1,2'-dinaphthylamine (1,2'-diNA, 32 µM) at 37°C. Before addition of compound **14** (100 µM), the cells were washed with phosphate buffer. Glucose (50 mM) was added after 300 s to initiate bacterial energization. Release of membrane-incorporated 1,2'-diNA was followed by monitoring the fluorescence (λ_{ex}=370 nm; λ_{em}=420 nm) every 30 s at 37°C in an Infinite M200 Pro plate reader. Assays were performed in 96-well plates (half area, black with solid bottom, 100 µL per well).

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Keywords: Indole • polyamine • adjuvant • enhancer • antibiotic

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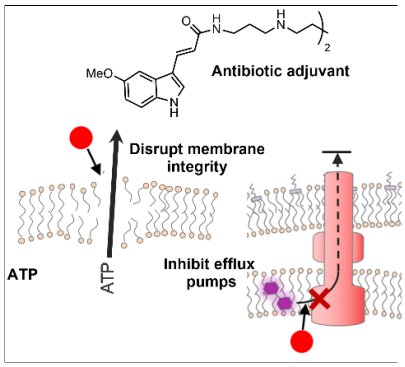

Antibiotic adjuvant: Indole-3-acrylamido-spermine conjugates were identified as being able to enhance the action of doxycycline towards Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Escherichia coli* and with attenuated cytotoxic and haemolytic properties. Mechanism of action experiments suggest enhancement derives from the disruption of bacterial membrane integrity and inhibition of efflux pumps.