Research paper

Exploration of the antibiotic potentiating activity of indolglyoxylpolyamines

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ABSTRACT

A series of substituted di-indolglyoxylamido-spermine analogues were prepared and evaluated for intrinsic antimicrobial properties and the ability to enhance antibiotic action. As a compound class, intrinsic activity was typically observed towards Gram-positive bacteria and the fungus *Cryptococcus neoformans*, with notable exceptions being the 5-bromo- and 6-chloro-indole analogues which also exhibited modest activity (MIC 34−50 μM) towards the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*. Several analogues enhanced the activity of doxycycline towards the Gram-negative bacteria *Pseudomonas aeruginosa*, *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii*. Of particular note was the identification of five antibiotic enhancing analogues (5-Br, 7-F, 5-Me, 7-Me, 7-OMe) which also exhibited low to no cytotoxicity and red blood cell haemolytic properties. The mechanisms of action of the 5-Br and 7-F analogues were attributed to the ability to disrupt the integrity of, and depolarize, bacterial membranes.

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1. Introduction

Given the noted difficulties associated with the discovery and development of novel antibiotics [1−3], and particularly those with activity towards Gram-negative bacteria [4,5], an attractive approach for overcoming antimicrobial resistance is the identification of antibiotic adjuvants [6−8]. Such compounds, when co-dosed with antibiotics, have the potential to restore the action of an antibiotic towards bacteria that have developed resistance to that antibiotic. Beyond the classical adjuvant example, the β-lactamase inhibitor clavulanic acid co-dosed with the β-lactam antibiotic amoxicillin, a growing number of chemical scaffolds have been identified as being able to enhance antibiotic action [9,10].

Two such examples include the marine natural product ianthelliformisamine C **1** and 2-aminoimidazole analogues such as **2** (Fig. 1) the former which acts via bacterial membrane depolarization [11] while the latter compound class causes potentiation by disruption of the VraSR two-component system associated with cell wall biosynthesis [12].

We recently reported polyamine functionalised indolglyoxylamide **3** (Fig. 1) as a potentiator of antibiotic activity towards several examples of clinically important Gram-negative bacteria including *Pseudomonas aeruginosa* [13]. That same study provided insight into some of the structural elements required for potentiation, namely an intact polyamine core of suitable overall length. The biological activities of **3**, which also included intrinsic antibacterial activity towards Gram-positive bacteria and fungi, were attributed to the molecules ability to disrupt the integrity of, and depolarize, bacterial membranes. This ability to disrupt membranes also extended to mammalian cells, with **3** exhibiting cytotoxicity and also strong red blood cell haemolytic activities. We used **3** as a

ianthelliformisamine C (**1**)
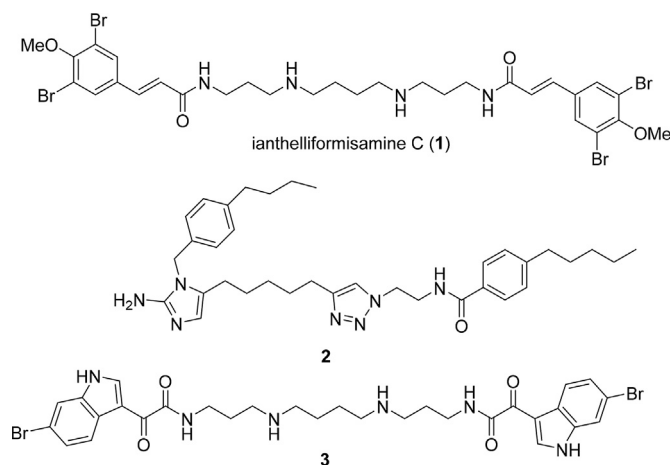
2

3

**Fig. 1.** Structures of antibiotic potentiators **1**–**3**.

starting point for a new study, reported herein, that explored the effect of variation of substitution on the indolglyoxylamide fragment in search for new potentiators that were devoid of cytotoxic/haemolytic liabilities.

2. Chemistry

A library of analogues of were prepared, exploring the influence of the 6-bromo substituent present in **3**. The method used for the synthesis of target compounds **4**–**19**, summarised in Scheme 1, was comprised of a three-step sequence starting with conversion of commercially available or previously reported substituted indoles to their corresponding 3-glyoxylchloride analogue. Subsequent reaction with the Boc-protected PA3-4-3 di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carboxylate) afforded Boc protected intermediates that were then directly subjected to Boc group deprotection (with TFA in CH_2Cl_2 or MeOH) to give tetramine diamides **4**–**19** as their di-TFA salts. Preparation of the 4-fluoroindole analogue by this glyoxylchloride method gave the desired product (**20**) in very low (<10%) yield — an alternative preparation via reaction of 2-(4-fluoro-1*H*-indol-3-yl)-2-oxoacetic acid [14] with spermine mediated by the coupling agent PyBOP in DMF afforded **20** in a marginally better yield of 14% (Scheme 2). The set of analogues was rounded off with des-bromo **21** and three methoxy derivatives (**22**–**24**) previously reported by us (Fig. 2) [15].

3. Results and discussion

We first evaluated the intrinsic antimicrobial activity of each compound against a range of Gram-positive (*Staphylococcus aureus* and *Staphylococcus intermedius*) and Gram-negative (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter baumannii*) bacteria and two fungal strains (*Candida albicans* and *Cryptococcus neoformans*) (Table 1). As with the original 6-bromoindole hit compound **3**, a number of the indolglyoxylamide analogues demonstrated some level of antimicrobial activity against Gram-positive bacteria and the fungus *Cryptococcus neoformans*. Notably analogues **4** (5-Br) and **10** (6-Cl) were the most potent, exhibiting broad spectrum antimicrobial activity against *S. aureus* (MIC 3.125–6.25 μM), *S. intermedius* (MIC 3.125 μM), *E. coli* (MIC 25–50 μM), *K. pneumoniae* (MIC 34.4–38.1 μM) and *C. neoformans* (MIC 2.2–4.8 μM). The results indicated that *C. neoformans* was the most susceptible test organism, with ten analogues exhibiting MIC <10 μM, while the compound class was only poorly active or inactive towards Gram-negative bacteria.
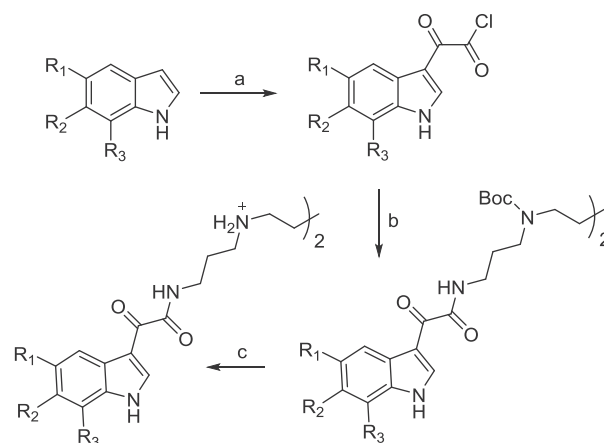
**4** $R_2 = R_3 = H, R_1 = Br$
5 $R_3 = H, R_1 = R_2 = Br$
6 $R_2 = R_3 = H, R_1 = F$
7 $R_1 = R_3 = H, R_2 = F$
8 $R_1 = R_2 = H, R_3 = F$
9 $R_2 = R_3 = H, R_1 = Cl$
10 $R_1 = R_3 = H, R_2 = Cl$
11 $R_1 = R_2 = H, R_3 = Cl$
12 $R_1 = R_3 = H, R_2 = CF_3$
13 $R_2 = R_3 = H, R_1 = CN$
14 $R_1 = R_3 = H, R_2 = CN$
15 $R_2 = R_3 = H, R_1 = COOCH_3$
16 $R_1 = R_3 = H, R_2 = COOCH_3$
17 $R_1 = R_2 = H, R_3 = COOCH_3$
18 $R_2 = R_3 = H, R_1 = CH_3$
19 $R_1 = R_2 = H, R_3 = CH_3$

Scheme 1. General synthesis of indolglyoxylamide analogues **4**–**19**. *Reagents and conditions*: a) Oxalyl chloride (2 eq.), Et_2O, 0 °C, 3 h; b) Di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (0.5 eq.), DIPEA (3 eq.), DMF, r.t., 48 h; c) TFA (0.2 mL), CH_2Cl_2/MeOH (2 mL), r.t., 3 h.
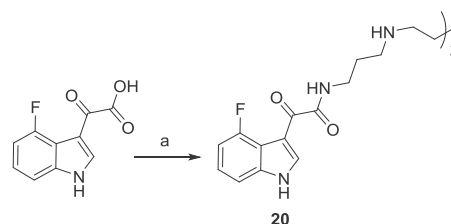
Scheme 2. Synthesis of 4-fluoroindolglyoxylamide analogue **20**. *Reagents and conditions*: a) PyBOP (1 eq.), triethylamine (2.5 eq.), DMF, N_2, r.t., 48 h.

The ability of compounds **3**–**24** to enhance the antibiotic activity of doxycycline against *P. aeruginosa* ATCC27853 was determined. These tests used a fixed concentration of doxycycline of 2 μg/mL (4.5 μM), which is twenty-fold lower than the intrinsic MIC [40 μg/mL (90 μM)] against this organism. Each of the test compounds were then evaluated at a range of concentrations varying from 3.125 to 50–100 μM, with the upper concentration dependent upon the compounds intrinsic MIC towards *P. aeruginosa*. From all the compounds examined (Table 2), 5-bromoindole **4** and 7-fluoroindole **8** analogues were the most effective potentiators (restoring the action of doxycycline at 3.125 μM), followed closely by 7-methoxy **24** (3.75 μM) with 6-chloroindole **10**, 7-methylcarboxylateindole **17**, 5-methylindole **18**, and 7-methylindole **19** analogues being equipotent (6.25 μM) to our original hit compound **3**.
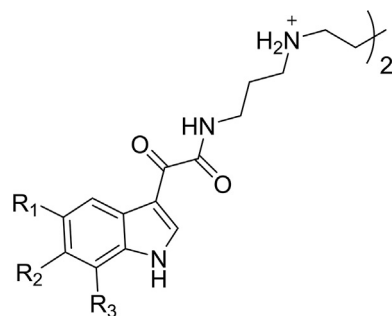
21 R$_1$ = R$_2$ = R$_3$ = H
22 R$_1$ = R$_3$ = H, R$_2$ = Br
23 R$_1$ = R$_3$ = H, R$_2$ = OMe
24 R$_1$ = R$_2$ = H, R$_3$ = OMe

Fig. 2. Des-bromo (**21**) and methoxy analogues **22**−**24** [15].

Table 1
Antimicrobial and antifungal activities of analogues **3**−**24**.

	MIC (μM)							
	S. a[a]	S. i[b]	P. a[c]	E. c[d]	K. p[e]	A. b[f]	C. a[g]	C. n[h]
3	6.25[i]	3.125[i]	100[i]	100[i]	>34[i]	>34[i]	17.2[i]	1.1[i]
4	3.125	3.125	50	25	34.4	>34.4	>34.4	2.2
5	25	n.t.[j]	100	50	>29.4	>29.4	>29.4	3.65
6	25	50	200	200	>39.6	>39.6	>39.6	9.9
7	100	>200	>200	>200	39.6	>39.6	>39.6	19.8
8	25	>200	200	200	>39.6	>39.6	>39.6	4.9
9	>200	n.t.	>100	>200	>38.0	>38.0	38.0	>38.0
10	6.25	3.125	100	50	38.1	38.1	38.1	4.8
11	50	n.t.	200	200	>38.1	>38.1	>38.1	19.1
12	50	50	200	100	35.2	>35.2	35.2	4.40
13	100	>200	>200	>200	>38.9	>38.9	>38.9	38.9
14	100	25	>200	200	>38.9	>38.9	>38.9	38.9
15	100	6.25	200	200	>36.0	>36.0	>36.0	>36.0
16	>200	12.5	>200	>200	>36.0	>36.0	>36.0	9.0
17	25	>200	200	200	>36.0	>36.0	>36.0	9.0
18	25	>200	100	200	>40.0	>40.0	>40.0	10.0
19	25	12.5	>200	>200	>40.0	>40.0	>40.0	10.0
20	>70	35	140	100	>55.1	>55.1	>55.1	>55.1
21	>100	>100	>100	>100	>41.4	>41.4	>41.4	10.3
22	100	50	>200	>200	>38.4	>38.4	>38.4	19.2
23	>100	>100	>100	>100	>38.4	>38.4	>38.4	19.2
24	15	n.t.	>200	>200	>38.4	>38.4	38.4	>38.4

[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 intermedius* 1051997 with streptomycin (MIC 10.7 μM) and chloramphenicol (MIC 3−6 μM) used as positive controls and values presented as the mean (n = 3).
[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.* [13].
[j] Not tested.

The spectrum of potentiation of the 5-bromoindole **4** and 7-methoxyindole **24** analogues was then evaluated for the antibiotics doxycycline, erythromycin, chloramphenicol and nalidixic acid against four Gram-negative ESKAPE pathogens *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *K. pneumoniae* ATCC13443 and *A. baumannii* AYE. In addition to original hit compound **3**, both compounds enhanced the action of doxycycline towards *E. coli* and *A. baumannii* (Table 3). Interestingly the two bromo analogues (6-bromo **3** and 5-bromo **4**) enhanced the action of doxycycline, and to a lesser degree nalidixic acid, towards *K. pneumoniae*.

The cytotoxicity of compounds **3**−**24** were evaluated against rat skeletal muscle (L6) and human embryonic kidney (HEK-293) cell lines. Other than the previously reported lead compound **3**, only the 5,6-dibromoindole (**5**) and 7-chloroindole (**11**) analogues exhibited cytotoxicity against L6 cells (IC$_{50}$ 6.03 and 7.47 μM, respectively) with all other compounds being weakly cytotoxic with IC$_{50}$ values ranging from 19.8 to >100 μM (Table 4). The HEK-293 cell line testing identified 5-chloroindole **9**, 6-trifluoromethylindole **12** and 7-methylcarboxylateindole **17** as also exhibiting cytotoxicity. Pronounced haemolytic activity towards rat red blood cells was observed for 6-bromoindole **3** with an HC$_{50}$ (concentration at which 50% of red blood cells were lysed) of 20 μM, and for 5,6-dibromoindole **5** (HC$_{50}$ 17 μM), with weaker activity observed for 5-bromoindole **4**, 6-chloroindole **10** and 7-methylcarboxylateindole **17** (Table 4). Modest to no haemolytic activities were observed for the other compounds in the library. Combined analysis of doxycycline potentiating activity with cytotoxicity and haemolytic data for the test compounds identified five analogues of particular interest (i.e. antibiotic potentiating with limited/no cytotoxicity and haemolytic properties): 5-bromoindole **4** [3.125 μM potentiation, weakly cytotoxic IC$_{50}$ 14.1−19.8 μM, and modestly haemolytic (34.7% haemolysis at 50 μM)], 7-fluoroindole **8** (3.125 μM, IC$_{50}$ 18.7−38.7 μM, 8.4% haemolysis at 50 μM), 5-methylindole **18** (6.25 μM, IC$_{50}$ 63.8 μM, 0.3% at 50 μM), 7-methylindole **19** (6.25 μM, IC$_{50}$ 59.6 μM, 0.1% at 50 μM) and 7-methoxyindole analogue **24** [3.75 μM, IC$_{50}$ 27−62 μM and not haemolytic (0% at 50 μM)].

Potential mechanisms of antibiotic potentiation include permeabilization of the outer membrane or membrane depolarization [9,10]. Compounds **4** and **8** were selected as the model compounds for these studies due to their abilities to potently enhance the action of antibiotics but with only limited cytotoxic/haemolytic properties. In the first set of studies, a bioluminescence method was used to detect extra-cellular concentrations of ATP that resulted from membrane permeabilization. Brief exposure (1 min) of either compound to bacterial cells induced dose-dependent increases in extracellular ATP levels in both *S. aureus* (Fig. 3 left) and, to a lesser extent, in *P. aeruginosa* (Fig. 3 right) suggesting both polyamine derivatives can disrupt the integrity of bacterial membranes. Both compounds were less effective than the known membrane disrupting steroidal-polyamine squalamine [16].

Further evidence for the ability of the test compounds to disrupt the outer bacterial membrane was determined in the Gram-negative bacterium *Enterobacter aerogenes* EA289 using a nitrocefin colorimetric assay. The basis of this assay is that membrane disruption leads to ingress of a chromogenic cephalosporin that acts as a substrate for periplasmic β-lactamase, whereupon β-lactam hydrolysis leads to a color change from yellow to red. As presented in Fig. 4, dose-dependent effect on the rate of nitrocefin hydrolysis was observed for both **4** and **8**, with the former being more potent.

Many drug efflux pumps function via an energy-dependent mechanism that makes use of membrane proton gradients [17,18]. Compounds **4** and **8** were investigated for their ability to act as disruptors of the bacterial transmembrane potential by using the membrane-potential-sensitive probe DiSC$_3$(5) which concentrates

Table 2
Doxycycline potentiation activity of analogues **3**–**24**.

Compound	Conc (μM) for potentiation[a]	Compound	Conc (μM) for potentiation[a]
3	6.25 [b]	**14**	50
4	3.125	**15**	25
5	25	**16**	25
6	12.5	**17**	6.25
7	25	**18**	6.25
8	3.125	**19**	6.25
9	14.9	**20**	35
10	6.25	**21**	>50
11	25	**22**	12.5
12	100	**23**	50
13	50	**24**	3.75

[a] Concentration (μM) required to restore doxycycline activity at 2 μg/mL (4.5 μM) against *P. aeruginosa* ATCC27853.
[b] Data taken from Li et al. [13].

Table 3
Antibiotic potentiating activity of analogues **3**, **4** and **24**.

Antibiotic	Compound	Concentration (μM) for potentiation[a]			
		P. aeruginosa[b]	*E. coli*[c]	*K. pneumoniae*[d]	*A. baumannii*[e]
Doxycycline	**3**	12.5 [f]	6.25 [f]	6.25 [f]	25 [f]
	4	6.25	1.68	13.4	6.72
	24	7.50	3.75	>200	15
Erythromycin	**3**	200 [f]	50 [f]	50 [f]	25 [f]
	4	>200	n.t. [g]	n.t.	n.t.
	24	100	>200	>200	>200
Chloramphenicol	**3**	>200 [f]	>200 [f]	200 [f]	>200 [f]
	4	26.9	n.t.	n.t.	n.t.
	24	30.0	>200	>200	>200
Nalidixic acid	**3**	50 [f]	>200 [f]	25 [f]	200 [f]
	4	>200	100	12.5	100
	24	60	>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 90 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* ATCC13443 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).
[f] Data taken from Li *et al.* [13].
[g] Not tested.

at the inner membrane level and quenches its own fluorescence. When a compound impairs the membrane potential, the dye is released leading to an increase in fluorescence. After 15 min incubation, modest membrane depolarization was observed against Gram-positive *S. aureus* (Fig. 5 left) and Gram-negative *P. aeruginosa* (Fig. 5 right) strains for both **4** and **8**.

Collectively the ATP release, nitrocefin hydrolysis and DiSC$_3$(5) membrane potential assay results suggest that the intrinsic antimicrobial activities of compounds **4** and **8** could be due to their abilities to disrupt bacterial membranes while their antibiotic potentiating activities may stem from their ability to depolarize membranes leading to efflux pump inhibition.

4. Conclusions

Our previous screening identified N^1,N^{14}-di-(6-bromoindolglyoxylamido)-spermine to be a potent enhancer of the action of doxycycline towards *P. aeruginosa* and that the polyamine core of the molecule was essential for activity. A limitation of the compound however was the observation of cytotoxicity and haemolytic properties. The present study has expanded this structure-activity relationship to include variation of substituents on the indolglyoxylamide head-group. Of note was the discovery of four analogues, bearing 7-methoxy, 7-fluoro, 7-methyl or 5-methyl indole substitution, that were either equipotent or more potent in their ability to enhance the action of doxycycline than the original hit compound. The relative location of these substituents appears to be a critical factor for potentiating activity. Of these four new potentiators, the 7-methoxy, 5-methyl and 7-methyl substituted variants were 3-10-fold less cytotoxic and had little or negligible haemolytic effect on red blood cells. Cell-based assays identified the 5-bromo and 7-fluoro analogues as being capable of permeabilizing bacterial membranes, likely having a bearing on their intrinsic antibacterial properties, while both were also capable of depolarizing membranes, a possible cause of their antibiotic potentiating activities. Current studies are directed towards the discovery of more potent antibiotic enhancers and their evaluation in *in vivo* models of infection.

5. Experimental

5.1. Chemistry: general methods

Infrared spectra were recorded on a PerkinElmer spectrometer

Table 4
Cytotoxic and haemolytic properties of analogues **3**–**24**.

Compound	Cytotoxicity		Haemolysis
	L6 [a] IC$_{50}$ (μM)	HEK-293 [b] CC$_{50}$ (μM)	% haemolysis at 50 μM (HC$_{50}$ μM) [c]
3	7.7 [d]	5.06 [e]	91 (20)
4	19.8	14.1	34.7
5	6.03	1.62	88.7 (17)
6	71.1	>39.6	0
7	69.1	12.5	0.3
8	38.7	18.7	8.4
9	19.8	4.73	0
10	33.2	19.1	11.6
11	7.47	31.6	1.1
12	31.4	3.36	3.2
13	37.1	>38.9	0
14	46.9	>38.9	0
15	41.8	>36.0	0
16	63.6	>36.0	0.6
17	34.4	3.78	15.0
18	63.8	>40.0	0.3
19	59.6	>40.0	0.1
20	167	>55	0.3
21	60 [d]	>41.5	0
22	56 [d]	>38.4	0
23	54 [d]	>38.4	0
24	62 [d]	27.1	0

[a] 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).
[b] Concentration of compound at 50% cytotoxicity on HEK293 human embryonic kidney cells and values presented as the mean (n = 2). Highest dose tested was 32 μg/mL.
[c] Percentage haemolysis detected at 540 nm at 50 μM concentration of compound with 0.1% Triton X-100 as the positive control (100% hemolysis) with values presented as the mean (n = 3). Where given the value in parenthesis is the concentration (μM) of compound required to induce 50% haemolysis.
[d] Data taken from Wang *et al.* [15].
[e] Data taken from Li *et al.* [13].
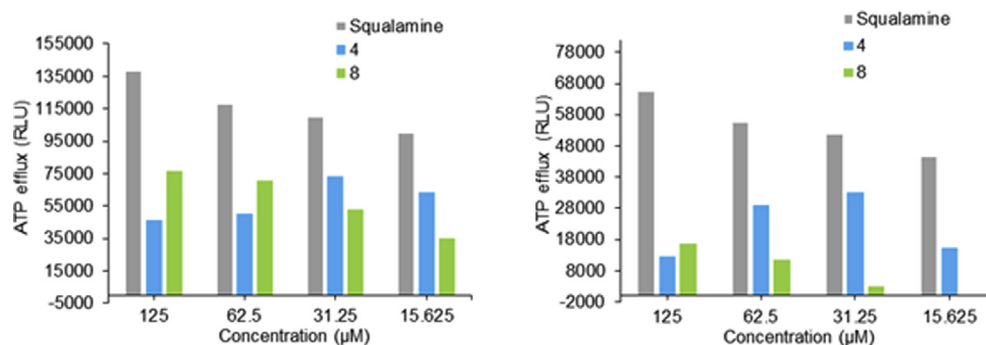
Fig. 3. Dose-dependent ATP release for *S. aureus* (left) and *P. aeruginosa* (right) exhibited by **4** and **8** with squalamine as the positive control.
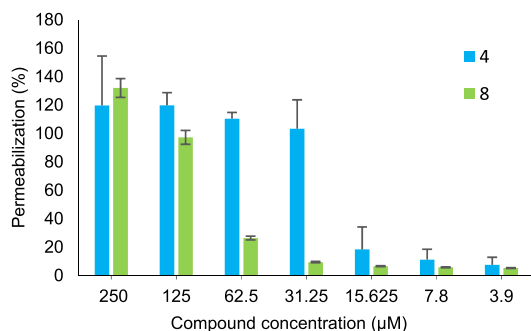
Fig. 4. Dose-dependent effect of **4** and **8** on the rate of nitrocefin hydrolysis in *E. aerogenes* EA289 resulting from outer membrane permeabilization.

100 Fourier Transform infrared spectrometer equipped with a universal ATR accessory. HRMS data were acquired on a Bruker micrOTOF QII spectrometer. NMR spectra were recorded on a Bruker Avance DRX-400 spectrometer operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C nuclei. Proto-deutero solvent signals were used as internal references (DMSO-d_6: δ_H 2.50, δ_C 39.52). 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. Flash column chromatography was carried out using either Davisil silica gel (40−60 μm) or Merck LiChroprep RP-8 (40−63 μm). Thin layer chromatography was conducted 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. Di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) [19], 5,6-dibromoindole [20], 2-(4-fluoro-1*H*-indol-3-yl)-2-oxoacetic acid [14], and **21**−**24** [15] were prepared according to a literature procedures.
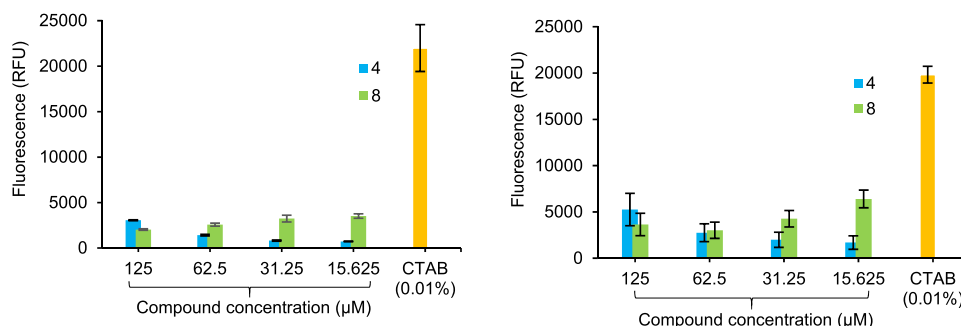
Fig. 5. Dose-dependent depolarization by **4** and **8** of the bacterial membranes of *S. aureus* (left) and *P. aeruginosa* (right) with cetyltrimethylammonium bromide (CTAB) (0.01%) as the positive control.

5.2. Synthesis of compounds

5.2.1. General procedure A — synthesis of substituted 3-indolglyoxylyl chlorides

To a solution of indole (1 equiv.) in anhydrous diethyl ether (12 mL) was added oxalyl chloride (1.15 equiv.) at 0 °C under N_2 atmosphere. The reaction mixture was stirred for 3 h before solvent removal under reduced pressure. The product was used in the next step without purification.

5.2.2. General procedure B — coupling of 3-indolglyoxylyl chlorides with Boc protected polyamine

To a solution of 3-indolglyoxylyl chloride (2 equiv.) in DMF (1 mL) was added DIPEA (6 equiv.) and di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (1 equiv.) in DMF (1 mL). Subsequently, the reaction mixture was stirred for 48 h before solvent removal under reduced pressure. The crude product was purified using silica gel flash column chromatography (3% MeOH:CH₂Cl₂).

5.2.3. General procedure C —Boc deprotection

A solution of *tert*-butyl-carbamate derivative in CH₂Cl₂ (2 mL) and TFA (0.2 mL) was stirred at room temperature under N_2 for 2 h followed by solvent removal under reduced pressure. The crude product was purified using C₈ reversed-phase flash column chromatography eluting with 0%−50% MeOH/H₂O (0.05% TFA) to afford the corresponding polyamine as a TFA salt.

5.2.3.1. N^1,N^4-Bis(3-(2-(5-bromo-1H-indol-3-yl)-2-oxoacetamido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**4**). Using general procedure A, reaction of 5-bromo-1*H*-indole (392 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(5-bromo-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using procedure B, a sub-sample of the glyoxylyl chloride (71.1 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(5-bromo-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow gum (36 mg, 38%). Using general procedure C, a sub-sample of this material (36 mg, 0.040 mmol) was deprotected to afford the di-TFA salt of **4** as a brown oil (36 mg, quant. yield) which required no further purification. R_f (MeOH/10% HCl, 7:3) 0.63; IR (ATR) ν_{max} 3152, 2981, 1671, 1623, 1501, 1430, 1199, 1130 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.49 (2H, br s, NH-1, NH-1′), 8.92 (2H, t, $J = 6.1$ Hz, NH-10, NH-10′), 8.80 (2H, d, $J = 3.3$ Hz, H-2, H-2′), 8.55 (4H, br s, NH₂-14, NH₂-14′), 8.35 (2H, d, $J = 1.9$ Hz, H-4, H-4′), 7.53 (2H, d, $J = 8.7$ Hz, H-7, H-7′), 7.42 (2H, dd, $J = 4.3$, 1.7 Hz, H-6, H-6′), 3.33−3.26 (4H, m, H₂-11, H₂-11′), 3.00−2.88 (8H, m, H₂-13, H₂-13′,

H₂-15, H₂-15′), 1.89−1.81 (4H, m, H₂-12, H₂-12′), 1.67−1.57 (4H, m, H₂-16, H₂-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.7 (C-8, C-8′), 163.4 (C-9, C-9′), 139.5 (C-2, C-2′), 135.1 (C-7a, C-7a′), 128.1 (C-3a, C-3a′), 126.2 (C-6, C-6′), 123.4 (C-4, C-4′), 115.4 (C-5, C-5′), 114.8 (C-7, C-7′), 111.6 (C-3, C-3′), 46.1 (C-15, C-15′), 44.8 (C-13, C-13′), 35.9 (C-11, C-11′), 25.7 (C-12, C-12′), 22.8 (C-16, C-16′); (+)-HRESIMS [M+Na]$^+$ m/z 723.0912 (calcd for C₃₀H₃₄^{79}Br₂N₆O₄Na, 723.0900), 725.0909 (calcd for C₃₀H₃₄^{79}Br^{81}BrN₆O₄Na, 725.0883), 727.0885 (calcd for C₃₀H₃₄^{81}Br₂N₆O₄Na, 727.0869).

5.2.3.2. N^1,N^4-Bis(3-(2-(5,6-dibromo-1H-indol-3-yl)-2-oxoacetamido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**5**). Using general procedure A, reaction of 5,6-dibromo-1*H*-indole [20] (207 mg, 0.75 mmol) and oxalyl chloride (0.087 mL, 0.90 mmol) afforded 2-(5,6-dibromo-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (105 mg, 0.289 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(5,6-dibromo-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow oil (39 mg, 41%). Using general procedure C, this material (39 mg, 0.037 mmol) was deprotected to afford the di-TFA salt of **5** as a red oil (25 mg, 78%). R_f (MeOH/10% HCl, 7:3) 0.15; IR (ATR) ν_{max} 3350, 3104, 2259, 1672, 1623, 1439, 1199, 1180, 1127 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.57 (2H, d, $J = 2.7$ Hz, NH-1, NH-1′), 8.95 (2H, t, $J = 5.9$ Hz, NH-10, NH-10′), 8.84 (2H, d, $J = 3.1$ Hz, H-2, H-2′), 8.61 (4H, br s, NH₂-14, NH₂-14′), 8.53 (2H, s, H-4, H-4′), 7.99 (2H, s, H-7, H-7′), 3.34−3.26 (4H, m, H₂-11, H₂-11′), 3.00−2.90 (8H, m, H₂-13, H₂-13′, H₂-15, H₂-15′), 1.91−1.81 (4H, m, H₂-12, H₂-12′), 1.67−1.60 (4H, m, H₂-16, H₂-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.6 (C-8, C-8′), 163.1 (C-9, C-9′), 140.4 (C-2, C-2′), 136.1 (C-7a, C-7a′), 127.1 (C-3a, C-3a′), 125.2 (C-4, C-4′), 117.6 (C-7, C-7′, C-5/C-6, C-5′/C-6′), 117.3 (C-5/C-6, C-5′/C-6′), 111.4 (C-3, C-3′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.6 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ m/z 856.9308 (calcd for C₃₀H₃₃^{79}Br₄N₆O₄, 856.9291), 858.9293 (calcd for C₃₀H₃₃^{79}Br₃^{81}BrN₆O₄, 858.9272), 860.9278 (calcd for C₃₀H₃₃^{79}Br₂^{81}Br₂N₆O₄, 860.9254), 862.9261 (calcd for C₃₀H₃₃^{79}Br^{81}Br₃N₆O₄, 862.9237), 864.9248 (calcd for C₃₀H₃₃^{81}Br₄N₆O₄, 864.9226).

5.2.3.3. N^1,N^4-Bis(3-(2-(5-fluoro-1H-indol-3-yl)-2-oxoacetamido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**6**). Using general procedure A, reaction of 5-fluoro-1*H*-indole (270 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(5-fluoro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (52 mg, 0.251 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA

(0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after silica gel column chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(5-fluoro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow oil (72 mg, 74%). Using general procedure C, a sub-sample of this material (37 mg, 0.047 mmol) was then deprotected to afford the di-TFA salt of **6** as a yellow oil (28 mg, 76%). R_f (MeOH/10% HCl, 7:3) 0.42; IR (ATR) ν_{max} 3384, 2973, 2109, 1702, 1647, 1368, 1231 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.39 (2H, br s, NH-1, NH-1′), 8.92 (2H, t, $J = 6.0$ Hz, NH-10, NH-10′), 8.81 (2H, d, $J = 3.2$ Hz, H-2, H-2′), 8.49 (4H, br s, NH$_2$-14, NH$_2$-14′), 7.89 (2H, dd, $J = 8.5, 2.6$ Hz, H-4, H-4′), 7.57 (2H, dd, $J = 8.9, 4.3$ Hz, H-7, H-7′), 7.13 (2H, ddd, $J = 9.2, 6.6, 2.7$ Hz, H-6, H-6′), 3.32–3.27 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.88 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.89–1.82 (4H, m, H$_2$-12, H$_2$-12′), 1.67–1.53 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.7 (C-8, C-8′), 163.6 (C-9, C-9′), 159.2 (d, $^1J_{CF} = 251.2$ Hz, C-5, C-5′), 158.8 (q, $^2J_{CF} = 25.5$ Hz, C-18, C-18′),139.9 (C-2, C-2′), 133.0 (C-7a, C-7a′), 127.0 (C-3a, C-3a′), 114.1 (d, $^3J_{CF} = 10.2$ Hz, C-7, C-7′), 112.3 (C-3, C-3′), 111.7 (d, $^2J_{CF} = 25.6$ Hz, C-6, C-6′), 106.3 (d, $^2J_{CF} = 25.5$ Hz, C-4, C-4′), 46.2 (C-15, C-15′), 44.9 (C-13, C-13′), 35.9 (C-11, C-11′), 25.8 (C-12, C-12′), 22.8 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ m/z 581.2682 (calcd for C$_{30}$H$_{35}$F$_2$N$_6$O$_4$, 581.2682).

*5.2.3.4. N^1,N^4-Bis(3-(2-(6-fluoro-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**7**).* Using general procedure A, reaction of 6-fluoro-1*H*-indole (270 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(6-fluoro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (52 mg, 0.251 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(6-fluoro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow gum (62 mg, 64%). Using general procedure C, a sub-sample of this material (26 mg, 0.033 mmol) was deprotected to afford the di-TFA salt of **7** as a yellow oil (16 mg, 65%). R_f (MeOH/10% HCl, 7:3) 0.41; IR (ATR) ν_{max} 2963, 2850, 1672, 1624, 1513, 1496, 1447, 1200, 1131 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.35 (2H, br s, NH-1, NH-1′), 8.91 (2H, t, $J = 6.1$ Hz, NH-10, NH-10′), 8.77 (2H, d, $J = 3.0$ Hz, H-2, H-2′), 8.57 (4H, br s, NH$_2$-14, NH$_2$-14′), 8.20 (2H, dd, $J = 8.7, 5.5$ Hz, H-4, H-4′), 7.35 (2H, dd, $J = 9.6, 2.4$ Hz, H-7, H-7′), 7.12 (2H, td, $J = 9.1, 2.3$ Hz, H-5, H-5′), 3.34–3.27 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.80 (4H, m, H$_2$-12, H$_2$-12′), 1.66–1.57 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.8 (C-8, C-8′), 163.5 (C-9, C-9′), 160.0 (d, $^1J_{CF} = 237.1$ Hz, C-6, C-6′), 158.5 (q, $^2J_{CF} = 34.5$ Hz, C-18, C-18′),139.3 (C-2, C-2′), 136.5 (d, $^3J_{CF} = 12.0$ Hz, C-7a, C-7a′), 122.9 (C-3a, C-3a′), 122.4 (d, $^3J_{CF} = 9.9$ Hz, C-4, C-4′), 112.1 (C-3, C-3′), 110.8 (d, $^2J_{CF} = 24.5$ Hz, C-5, C-5′), 99.1 (d, $^2J_{CF} = 26.0$ Hz, C-7, C-7′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.8 (C-11, C-11′), 25.7 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ m/z 581.2690 (calcd for C$_{30}$H$_{35}$F$_2$N$_6$O$_4$, 581.2682).

*5.2.3.5. N^1,N^4-Bis(3-(2-(7-fluoro-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**8**).* Using general procedure A, reaction of 7-fluoro-1*H*-indole (270 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(7-fluoro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (62 mg, 0.300 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(7-fluoro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow gum (62 mg, 64%). Using general procedure C, a sub-sample of this material

(39 mg, 0.050 mmol) was deprotected to afford the di-TFA salt of **8** as a brown oil (17 mg, 44%). R_f (MeOH/10% HCl, 7:3) 0.43; IR (ATR) ν_{max} 3250, 2840, 1667, 1606, 1438, 1200, 1176, 1126 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.90 (2H, d, $J = 7.6$ Hz, NH-1, NH-1′), 8.94 (2H, t, $J = 6.0$ Hz, NH-10, NH-10′), 8.78 (2H, d, $J = 3.1$ Hz, H-2, H-2′), 8.59 (4H, br s, NH$_2$-14, NH$_2$-14′), 8.04 (2H, d, $J = 8.1$ Hz, H-4, H-4′), 7.25 (2H, ddd, $J = 7.9, 7.9, 4.8$ Hz, H-5, H-5′), 7.13 (2H, dd, $J = 11.2, 7.9$ Hz, H-6, H-6′), 3.34–3.27 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.91–1.81 (4H, m, H$_2$-12, H$_2$-12′), 1.67–1.58 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.9 (C-8, C-8′), 163.4 (C-9, C-9′), 158.4 (q, $^2J_{CF} = 35.5$ Hz, C-18, C-18′), 149.2 (d, $^1J_{CF} = 245.7$ Hz, C-7, C-7′), 138.8 (C-2, C-2′), 129.8 (d, $^3J_{CF} = 4.3$ Hz, C-3a, C-3a′), 124.0 (d, $^2J_{CF} = 13.3$ Hz, C-7a, C-7a′), 123.4 (d, $^3J_{CF} = 5.9$ Hz, C-5, C-5′), 117.4 (C-4, C-4′), 112.8 (C-3, C-3′), 108.7 (d, $^2J_{CF} = 15.5$ Hz, C-6, C-6′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.6 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ m/z 581.2679 (calcd for C$_{30}$H$_{35}$F$_2$N$_6$O$_4$, 581.2682).

*5.2.3.6. N^1,N^4-Bis(3-(2-(5-chloro-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**9**).* Using general procedure A, reaction of 5-chloro-1*H*-indole (303 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(5-chloro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (60 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(5-chloro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow gum (10 mg, 10%). Using general procedure C, this material (10 mg, 0.012 mmol) was deprotected to afford the di-TFA salt of **9** as a brown oil (5.0 mg, 67%). R_f (MeOH/10% HCl, 7:3) 0.43; IR (ATR) ν_{max} 3374, 2977, 1676, 1426, 1132, 1034, 953, 817, 723 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.52 (2H, d, $J = 3.3$ Hz, NH-1, NH-1′), 8.92 (2H, t, $J = 5.5$ Hz, NH-10, NH-10′), 8.81 (2H, d, $J = 3.3$ Hz, H-2, H-2′), 8.66–8.58 (4H, m, NH$_2$-14, NH$_2$-14′), 8.20 (2H, d, $J = 2.0$ Hz, H-4, H-4′), 7.58 (2H, d, $J = 8.7$ Hz, H-7, H-7′), 7.30 (2H, dd, $J = 8.7, 2.0$ Hz, H-6, H-6′), 3.33–3.28 (4H, m, H$_2$-11, H$_2$-11′), 2.98–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.82 (4H, m, H$_2$-12, H$_2$-12′), 1.66–1.60 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.7 (C-8, C-8′), 163.4 (C-9, C-9′), 139.7 (C-2, C-2′), 134.8 (C-7a, C-7a′), 127.5 (C-3a, C-3a′/C-5, C-5′), 127.3 (C-3a, C-3a′/C-5, C-5′), 123.5 (C-6, C-6′), 120.3 (C-4, C-4′), 114.3 (C-7, C-7′), 111.7 (C-3, C-3′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.7 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+Na]$^+$ m/z 635.1902 (calcd for C$_{30}$H$_{34}^{35}$Cl$_2$N$_6$O$_4$Na, 635.1911), 637.1887 (calcd for C$_{30}$H$_{34}^{35}$Cl^{37}ClN$_6$O$_4$Na, 637.1890), 639.1852 (calcd for C$_{30}$H$_{34}^{37}$Cl$_2$N$_6$O$_4$Na, 639.1877).

*5.2.3.7. N^1,N^4-Bis(3-(2-(6-chloro-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (**10**).* Using general procedure A, reaction of 6-chloro-1*H*-indole (303 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(6-chloro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (60 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(6-chloro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow oil (53 mg, 55%). Using general procedure C, a sub-sample of this material (20 mg, 0.025 mmol) was deprotected to afford the di-TFA salt of **10** as a brown oil (15 mg, 75%). R_f (MeOH/10% HCl, 7:3) 0.67; IR (ATR) ν_{max} 3420, 2254, 1672, 1632, 1444, 1179, 1023, 1002 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.43 (2H, br s, NH-1, NH-1′), 8.94 (2H, t, $J = 6.1$ Hz, NH-10, NH-10′), 8.80 (2H, d, $J = 3.3$ Hz, H-2, H-2′),

8.59 (4H, m, NH$_2$-14, NH$_2$-14′), 8.20 (2H, d, J = 8.5 Hz, H-4, H-4′), 7.61 (2H, d, J = 1.8 Hz, H-7, H-7′), 7.29 (2H, dd, J = 8.5, 6.6 Hz, H-5, H-5′), 3.34–3.25 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.80 (4H, m, H$_2$-12, H$_2$-12′), 1.66–1.57 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.8 (C-8, C-8′), 163.4 (C-9, C-9′), 158.4 (q, J_{CF} = 36.1 Hz, C-17, C-17′), 139.4 (C-2, C-2′), 136.8 (C-7a, C-7a′), 128.0 (C-6, C-6′), 125.0 (C-3a, C-3a′), 122.9 (C-4, C-4′), 122.5 (C-5, C-5′), 112.5 (C-7, C-7′), 112.1 (C-3, C-3′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.8 (C-11, C-11′), 25.7 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ *m/z* 613.2070 (calcd for C$_{30}$H$_{35}^{35}$Cl$_2$N$_6$O$_4$, 613.2091), 615.2044 (calcd for C$_{30}$H$_{35}^{35}$Cl^{37}ClN$_6$O$_4$, 615.2070), 617.2023 (calcd for C$_{30}$H$_{35}^{37}$Cl$_2$N$_6$O$_4$, 617.2058).

5.2.3.8. N^1,N^4-Bis(3-(2-(7-chloro-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (11). Using general procedure A, reaction of 7-chloro-1*H*-indole (303 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(7-chloro-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (52 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(7-chloro-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow oil (53 mg, 55%). Using general procedure C, a sub-sample of this material (30 mg, 0.037 mmol) was deprotected to afford the di-TFA salt of **11** as a brown oil (20 mg, 65%). R_f (MeOH/10% HCl, 7:3) 0.48; IR (ATR) ν_{max} 2927, 1674, 1638, 1505, 1430, 1203, 1135, 1026, 835, 799, 722 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (2H, d, J = 3.3 Hz, NH-1, NH-1′), 8.95 (2H, t, J = 5.9 Hz, NH-10, NH-10′), 8.77 (2H, d, J = 3.3 Hz, H-2, H-2′), 8.55–8.50 (4H, m, NH$_2$-14, NH$_2$-14′), 8.19 (2H, d, J = 7.9 Hz, H-4, H-4′), 7.38 (2H, d, J = 7.9 Hz, H-6, H-6′), 7.28 (2H, t, J = 7.9 Hz, H-5, H-5′), 3.33–3.28 (4H, m, H$_2$-11, H$_2$-11′), 2.98–2.92 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.83 (4H, m, H$_2$-12, H$_2$-12′), 1.65–1.57 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.8 (C-8, C-8′), 163.3 (C-9, C-9′), 139.0 (C-2, C-2′), 133.2 (C-7a, C-7a′), 128.1 (C-3a, C-3a′), 123.8 (C-5, C-5′), 123.2 (C-6, C-6′), 120.2 (C-4, C-4′), 116.9 (C-7, C-7′), 112.9 (C-3, C-3′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.6 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+Na]$^+$ *m/z* 635.1898 (calcd for C$_{30}$H$_{34}^{35}$Cl$_2$N$_6$O$_4$Na, 635.1911), 637.1888 (calcd for C$_{30}$H$_{34}^{35}$Cl^{37}ClN$_6$O$_4$Na, 637.1890), 639.1869 (calcd for C$_{30}$H$_{34}^{37}$Cl$_2$N$_6$O$_4$Na, 639.1877).

5.2.3.9. N^1,N^4-Bis(3-(2-(6-trifluoromethyl-1H-indol-3-yl)-2-oxoacetamido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (12). Using general procedure A, reaction of 6-trifluoromethyl-1*H*-indole (278 mg, 1.5 mmol) and oxalyl chloride (0.146 mL, 1.7 mmol) afforded 2-(6-trifluoromethyl-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (68 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-oxo-2-(6-(trifluoromethyl)-1*H*-indol-3-yl)acetamido)propyl) carbamate) as a yellow oil (34 mg, 36%). Using general procedure C, a sub-sample of this material (24 mg, 0.027 mmol) was deprotected to afford the di-TFA salt of **12** as a brown oil (14 mg, 58%). R_f (MeOH/10% HCl, 7:3) 0.48; IR (ATR) ν_{max} 3017, 2957, 1671, 1628, 1494, 1329, 1199, 1109 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.67 (2H, br s, NH-1, NH-1′), 8.98–8.93 (4H, m, H-2, H-2′, NH-10, NH-10′), 8.56 (4H, br s, NH$_2$-14, NH$_2$-14′), 8.41 (2H, d, J = 8.4 Hz, H-4, H-4′), 7.83 (2H, s, H-7, H-7′), 7.59 (2H, d, J = 8.4 Hz, H-5, H-5′), 3.34–3.27 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.80 (4H, m, H$_2$-12, H$_2$-12′), 1.67–1.57 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 182.0 (C-8, C-8′), 163.3 (C-9, C-9′),

158.3 (q, $^2J_{CF}$ = 35.0 Hz, C-19, C-19′), 140.9 (C-2, C-2′), 135.4 (C-7a, C-7a′), 129.0 (C-3a, C-3a′), 124.1 (q, $^1J_{CF}$ = 257.3 Hz, C-17, C-17′), 123.6 (q, $^2J_{CF}$ = 31.7 Hz, C-6, C-6′), 122.0 (C-4, C-4′), 118.9 (C-5, C-5′), 112.1 (C-3, C-3′), 110.1 (C-7, C-7′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.7 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ *m/z* 681.2625 (calcd for C$_{32}$H$_{35}$F$_6$N$_6$O$_4$, 681.2618).

5.2.3.10. N^1,N^4-Bis(3-(2-(5-cyano-1H-indol-3-yl)-2-oxoacetamido) propyl) butane-1,4-diaminium 2,2,2-trifluoroacetate (13). Using general procedure A, reaction of 5-cyano-1*H*-indole (142 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded 2-(5-cyano-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (58 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(5-cyano-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a white gum (24 mg, 26%). Using general procedure C, a sub-sample of this material (16 mg, 0.020 mmol) was deprotected to afford the di-TFA salt of **13** as a brown oil (9.0 mg, 56%). R_f (MeOH/10% HCl, 7:3) 0.63; IR (ATR) ν_{max} 3312, 2964, 2225, 1732 1668, 1448, 1209, 1126, 1037 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.79 (2H, d, J = 2.5 Hz, NH-1, NH-1′), 8.98 (2H, t, J = 5.9 Hz, NH-10, NH-10′), 8.94 (2H, d, J = 2.5 Hz, H-2, H-2′), 8.59–8.54 (6H, m, NH$_2$-14, NH$_2$-14′, H-4, H-4′), 7.75 (2H, d, J = 8.5 Hz, H-7, H-7′), 7.67 (2H, dd, J = 8.5, 1.5 Hz, H-6, H-6′), 3.34–3.25 (4H, m, H$_2$-11, H$_2$-11′), 3.03–2.87 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.92–1.80 (4H, m, H$_2$-12, H$_2$-12′), 1.68–1.58 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.9 (C-8, C-8′), 163.0 (C-9, C-9′), 158.5 (q, J_{CF} = 34.0 Hz, C-19, C-19′), 140.7 (C-2, C-2′), 138.2 (C-7a, C-7a′), 126.4 (C-6, C-6′), 126.05 (C-3a, C-3a′/C-4, C-4′), 125.99 (C-3a, C-3a′/C-4, C-4′), 120.0 (C-17, C-17′), 114.2 (C-7, C-7′), 112.2 (C-3, C-3′), 104.8 (C-5, C-5′), 46.0 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.6 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ *m/z* 595.2766 (calcd for C$_{32}$H$_{35}$N$_8$O$_4$, 595.2776).

5.2.3.11. N^1,N^4-Bis(3-(2-(6-cyano-1H-indol-3-yl)-2-oxoacetamido) propyl) butane-1,4-diaminium 2,2,2-trifluoroacetate (14). Using general procedure A, reaction of 6-cyano-1*H*-indole (142 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded 2-(6-cyano-1*H*-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxyl chloride (58 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(6-cyano-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a white gum (43 mg, 45%). Using general procedure C, a sub-sample of this material (15 mg, 0.019 mmol) was deprotected to afford the di-TFA salt of **14** as a brown oil (10 mg, 66%). R_f (MeOH/10% HCl, 7:3) 0.57; IR (ATR) ν_{max} 3040, 2852, 2223, 1673, 1635, 1490, 1200, 1128, 1024 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.81 (2H, br s, NH-1, NH-1′), 9.00–8.93 (4H, m, H-2, H-2′, NH-10, NH-10′), 8.64 (4H, br s, NH$_2$-14, NH$_2$-14′), 8.36 (2H, d, J = 8.3 Hz, H-4, H-4′), 8.07 (2H, br s, H-7, H-7′), 7.63 (2H, dd, J = 8.4, 1.3 Hz, H-5, H-5′), 3.34–3.27 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.90 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.90–1.82 (4H, m, H$_2$-12, H$_2$-12′), 1.65–1.56 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.9 (C-8, C-8′), 163.2 (C-9, C-9′), 141.5 (C-2, C-2′), 135.3 (C-7a, C-7a′), 129.6 (C-3a, C-3a′), 125.5 (C-5, C-5′), 122.1 (C-4, C-4′), 119.7 (C-17, C-17′), 117.5 (C-7, C-7′), 112.2 (C-3, C-3′), 105.1 (C-6, C-6′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.6 (C-12, C-12′), 22.7 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ *m/z* 595.2772 (calcd for C$_{32}$H$_{35}$N$_8$O$_4$, 595.2776).

5.2.3.12. N^1,N^4-Bis(3-(2-(5-(methoxycarbonyl)-1H-indol-3-yl)-2-oxoacet amido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (15). Using general procedure A, reaction of 5-methyl-1H-indole carboxylate (176 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded methyl 3-(2-chloro-2-oxoacetyl)-1H-indole-5-carboxylate as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (77 mg, 0.289 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, dimethyl 3,3'-(7,12-bis(*tert*-butoxycarbonyl)-2,17-dioxo-3,7,12,16-tetraazaoctadecanedioyl) bis(1H-indole-5-carboxylate) as a yellow oil (36 mg, 38%). Using general procedure C, this material (36 mg, 0.042 mmol) was deprotected to afford the di-TFA salt of **15** as a brown oil (9.2 mg, 25%). R_f (MeOH/10% HCl, 7:3) 0.44; IR (ATR) ν_{max} 3345, 3040, 1677, 1619, 1497, 1433, 1200, 1111 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.61 (2H, s, NH-1, NH-1'), 8.95−8.89 (4H, m, H-4, H-4', NH-10, NH-10'), 8.87 (2H, d, J = 2.8 Hz, H-2, H-2'), 8.56 (4H, br s, NH$_2$-14, NH$_2$-14'), 7.89 (2H, dd, J = 8.5, 7.0 Hz, H-6, H-6'), 7.65 (2H, d, J = 8.5 Hz, H-7, H-7'), 3.88 (6H, s, H$_3$-18, H$_3$-18'), 3.36−3.28 (4H, m, H$_2$-11, H$_2$-11'), 3.00−2.90 (8H, m, H$_2$-13, H$_2$-13', H$_2$-15, H$_2$-15'), 1.91−1.82 (4H, m, H$_2$-12, H$_2$-12'), 1.67−1.58 (4H, m, H$_2$-16, H$_2$-16'); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 182.0 (C-8, C-8'), 166.8 (C-17, C-17'), 163.4 (C-9, C-9'), 140.2 (C-2, C-2'), 138.9 (C-7a, C-7a'), 125.8 (C-3a, C-3a'), 124.4 (C-6, C-6'), 123.9 (C-5, C-5'), 123.4 (C-4, C-4'), 112.8 (C-3, C-3', C-7, C-7'), 52.0 (C-18, C-18'), 46.1 (C-15, C-15'), 44.7 (C-13, C-13'), 35.9 (C-11, C-11'), 25.7 (C-12, C-12'), 22.7 (C-16, C-16'); (+)-HRESIMS [M+H]$^+$ m/z 661.2963 (calcd for C$_{34}$H$_{41}$N$_6$O$_8$, 661.2980).

5.2.3.13. N^1,N^4-Bis(3-(2-(6-(methoxycarbonyl)-1H-indol-3-yl)-2-oxoacet amido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (16). Using general procedure A, reaction of 6-methyl-1H-indole carboxylate (176 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded methyl 3-(2-chloro-2-oxoacetyl)-1H-indole-6-carboxylate as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (77 mg, 0.289 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, dimethyl 3,3'-(7,12-bis(*tert*-butoxycarbonyl)-2,17-dioxo-3,7,12,16-tetraazaoctadecanedioyl) bis(1H-indole-6-carboxylate) as a yellow oil (43 mg, 45%). After a repeated synthesis, this material (61 mg, 0.071 mmol) was deprotected to afford the di-TFA salt of **16** as a brown oil (59 mg, 97%). R_f (MeOH/10% HCl, 9:1) 0.87; IR (ATR) ν_{max} 3314, 2944, 2261, 1671, 1621, 1490, 1284, 1200, 1133 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.65 (2H, d, J = 2.7 Hz, NH-1, NH-1'), 8.98−8.90 (4H, m, H-2, H-2', NH-10, NH-10'), 8.62 (4H, br s, NH$_2$-14, NH$_2$-14'), 8.31 (2H, d, J = 8.3 Hz, H-4, H-4'), 8.18 (2H, d, J = 1.1 Hz, H-7, H-7'), 7.87 (2H, dd, J = 8.4, 7.0 Hz, H-5, H-5'), 3.88 (6H, s, H$_3$-18, H$_3$-18'), 3.34−3.27 (4H, m, H$_2$-11, H$_2$-11'), 3.00−2.90 (8H, m, H$_2$-13, H$_2$-13', H$_2$-15, H$_2$-15'), 1.92−1.82 (4H, m, H$_2$-12, H$_2$-12'), 1.67−1.58 (4H, m, H$_2$-16, H$_2$-16'); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.9 (C-8, C-8'), 166.6 (C-17, C-17'), 163.4 (C-9, C-9'), 141.1 (C-2, C-2'), 135.7 (C-7a, C-7a'), 129.9 (C-3a, C-3a'), 124.5 (C-6, C-6'), 123.2 (C-5, C-5'), 121.0 (C-4, C-4'), 114.3 (C-7, C-7'), 112.2 (C-3, C-3'), 52.0 (C-18, C-18'), 46.0 (C-15, C-15'), 44.7 (C-13, C-13'), 35.8 (C-11, C-11'), 25.6 (C-12, C-12'), 22.7 (C-16, C-16'); (+)-HRESIMS [M+H]$^+$ m/z 661.2959 (calcd for C$_{34}$H$_{41}$N$_6$O$_8$, 661.2980).

5.2.3.14. N^1,N^4-Bis(3-(2-(7-(methoxycarbonyl)-1H-indol-3-yl)-2-oxoacet amido)propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (17). Using general procedure A, reaction of 7-methyl-1H-indole carboxylate (176 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded methyl 3-(2-chloro-2-oxoacetyl)-1H-indole-7-carboxylate as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (77 mg, 0.289 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after chromatography, dimethyl 3,3'-(7,12-bis(*tert*-butoxycarbonyl)-2,17-dioxo-3,7,12,16-tetraazaoctadecanedioyl) bis(1H-indole-7-carboxylate) as a yellow oil (65 mg, 67%). Using general procedure C, a sub-sample of this material (60 mg, 0.070 mmol) was deprotected to afford the di-TFA salt of **17** as a brown oil (26 mg, 43%). R_f (MeOH/10% HCl, 7:3) 0.32; IR (ATR) ν_{max} 3321, 2958, 1670, 1625, 1505, 1280, 1202, 1130 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.17 (2H, s, NH-1, NH-1'), 8.96 (2H, t, J = 6.0 Hz, NH-10, NH-10'), 8.81 (2H, d, J = 3.2 Hz, H-2, H-2'), 8.58 (4H, br s, NH$_2$-14, NH$_2$-14'), 8.54 (2H, dd, J = 8.0, 0.7 Hz, H-4, H-4'), 7.92 (2H, dd, J = 7.4, 1.1 Hz, H-6, H-6'), 7.44−7.39 (2H, m, H-5, H-5'), 3.97 (6H, s, H$_3$-18, H$_3$-18'), 3.34−3.27 (4H, m, H$_2$-11, H$_2$-11'), 3.00−2.90 (8H, m, H$_2$-13, H$_2$-13', H$_2$-15, H$_2$-15'), 1.91−1.82 (4H, m, H$_2$-12, H$_2$-12'), 1.66−1.58 (4H, m, H$_2$-16, H$_2$-16'); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.9 (C-8, C-8'), 165.9 (C-17, C-17'), 163.2 (C-9, C-9'), 139.7 (C-2, C-2'), 134.4 (C-7a, C-7a'), 127.7 (C-3a, C-3a'), 126.9 (C-4, C-4'), 125.6 (C-6, C-6'), 122.5 (C-5, C-5'), 114.0 (C-7, C-7'), 112.0 (C-3, C-3'), 52.2 (C-18, C-18'), 46.1 (C-15, C-15'), 44.7 (C-13, C-13'), 35.9 (C-11, C-11'), 25.6 (C-12, C-12'), 22.7 (C-16, C-16'); (+)-HRESIMS [M+H]$^+$ m/z 661.2961 (calcd for C$_{34}$H$_{41}$N$_6$O$_8$, 661.2980).

5.2.3.15. N^1,N^4-Bis(3-(2-(5-methyl-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (18). Using general procedure A, reaction of 5-methyl-1H-indole (262 mg, 2 mmol) and oxalyl chloride (0.195 mL, 2.3 mmol) afforded 2-(5-methyl-1H-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (55.2 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford di-*tert*-butyl butane-1,4-diylbis((3-(2-(5-methyl-1H-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a white gum (40 mg, 42%). Using general procedure C, a sub-sample of this material (34 mg, 0.043 mmol) was deprotected to afford the di-TFA salt of **18** as a brown oil (34 mg, quant. yield). R_f (MeOH/10% HCl, 7:3) 0.65; IR (ATR) ν_{max} 3029, 1672, 1621, 1428, 1199, 1129, 1024, 1004 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.28 (2H, br s, NH-1, NH-1'), 8.87 (2H, t, J = 6.1 Hz, NH-10, NH-10'), 8.70 (2H, d, J = 3.3 Hz, H-2, H-2'), 8.67 (4H, m, NH$_2$-14, NH$_2$-14'), 8.04 (2H, s, H-4, H-4'), 7.42 (2H, d, J = 8.4 Hz, H-7, H-7'), 7.09 (2H, dd, J = 8.3, 7.2 Hz, H-6, H-6'), 3.32−3.27 (4H, m, H$_2$-11, H$_2$-11'), 2.98−2.91 (8H, m, H$_2$-13, H$_2$-13', H$_2$-15, H$_2$-15'), 2.42 (6H, s, H$_3$-17, H$_3$-17'), 1.90−1.83 (4H, m, H$_2$-12, H$_2$-12'), 1.65−1.61 (4H, m, H$_2$-16, H$_2$-16'); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.6 (C-8, C-8'), 163.9 (C-9, C-9'), 158.6 (q, J_{CF} = 35.7 Hz, C-19, C-19'), 138.4 (C-2, C-2'), 134.6 (C-7a, C-7a'), 131.5 (C-5, C-5'), 126.5 (C-3a, C-3a'), 124.9 (C-6, C-6'), 121.0 (C-4, C-4'), 112.2 (C-7, C-7'), 111.8 (C-3, C-3'), 46.0 (C-15, C-15'), 44.7 (C-13, C-13'), 35.8 (C-11, C-11'), 25.7 (C-12, C-12'), 22.7 (C-16, C-16'), 21.3 (C-17, C-17'); (+)-HRESIMS [M+H]$^+$ m/z 573.3174 (calcd for C$_{32}$H$_{41}$N$_6$O$_4$, 573.3184).

5.2.3.16. N^1,N^4-Bis(3-(2-(7-methyl-1H-indol-3-yl)-2-oxoacetamido) propyl)butane-1,4-diaminium 2,2,2-trifluoroacetate (19). Using general procedure A, reaction of 7-methyl-1H-indole (131 mg, 1 mmol) and oxalyl chloride (0.097 mL, 1.15 mmol) afforded 2-(7-methyl-1H-indol-3-yl)-2-oxoacetyl chloride as a yellow powder. Using general procedure B, a sub-sample of the glyoxylyl chloride (55.2 mg, 0.248 mmol) was reacted with di-*tert*-butyl butane-1,4-diylbis((3-aminopropyl)carbamate) (50 mg, 0.124 mmol) and DIPEA (0.130 mL, 0.744 mmol) in DMF (2 mL) to afford, after

chromatography, di-*tert*-butyl butane-1,4-diylbis((3-(2-(7-methyl-1*H*-indol-3-yl)-2-oxoacetamido)propyl)carbamate) as a yellow oil (30 mg, 32%). Using general procedure C, a sub-sample of this material (18 mg, 0.023 mmol) was deprotected to afford the di-TFA salt of **19** as a brown oil (12 mg, 65%). R_f (MeOH/10% HCl, 7:3) 0.40; IR (ATR) ν_{max} 3052, 1671, 1619, 1501, 1445, 1199, 1126, 1024, 1002 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.33 (2H, br s, NH-1, NH-1′), 8.89 (2H, t, $J = 5.7$ Hz, NH-10, NH-10′), 8.74 (2H, d, $J = 3.0$ Hz, H-2, H-2′), 8.58–8.46 (4H, m, NH$_2$-14, NH$_2$-14′), 8.06 (2H, d, $J = 7.8$ Hz, H-4, H-4′), 7.16 (2H, t, $J = 7.5$ Hz, H-5, H-5′), 7.07 (2H, d, $J = 7.2$ Hz, H-6, H-6′), 3.34–3.26 (4H, m, H$_2$-11, H$_2$-11′), 3.00–2.88 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 2.51 (6H, s, H$_3$-17, H$_3$-17′), 1.90–1.81 (4H, m, H$_2$-12, H$_2$-12′), 1.66–1.57 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.7 (C-8, C-8′), 122.8 (C-5, C-5′), 121.9 (C-7, C-7′), 118.8 (C-4, C-4′), 112.5 (C-3, C-3′), 46.1 (C-15, C-15′), 44.7 (C-13, C-13′), 35.8 (C-11, C-11′), 25.7 (C-12, C-12′), 22.7 (C-16, C-16′), 16.6 (C-17, C-17′); (+)-HRESIMS [M+H]$^+$ *m/z* 573.3170 (calcd for $C_{32}H_{41}N_6O_4$, 573.3184).

5.2.3.17. N,N'-((Butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl)) bis (2-(4-fluoro-1H-indol-3-yl)-2-oxoacetamide) (20). To a stirred solution of 2-(4-fluoro-1*H*-indol-3-yl)-2-oxoacetic acid [14] (38 mg, 0.18 mmol) and spermine (17 mg, 0.083 mmol) in DMF (1 mL) was added PyBOP (91 mg, 0.18 mmol) under N$_2$ atmosphere. Triethylamine (0.069 mL, 0.50 mmol) was then added and the reaction mixture was stirred for 48 h. Solvent was removed under reduced pressure to afford a crude product that was triturated with MeOH:H$_2$O (1 mL, 1:1) and the resulting solid was removed by filtration, washed and dried to afford **20** as the free base as a white powder (7.0 mg, 14%). R_f (MeOH/10% HCl, 7:3) 0.57; m.p > 250 °C; IR (ATR) ν_{max} 3274, 2951, 2742, 1614, 1501, 1427, 1312, 1258 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.54 (2H, br s, NH-1, NH-1′), 8.91 (2H, t, $J = 5.9$ Hz, NH-10, NH-10′), 8.68 (2H, s, H-2, H-2′), 7.37 (2H, d, $J = 8.0$ Hz, H-7, H-7′), 7.26 (2H, td, $J = 8.0$, 4.7 Hz, H-6, H-6′), 6.97 (2H, dd, $J = 11.0$, 7.9 Hz, H-5, H-5′), 3.33–3.26 (4H, m, H$_2$-11, H$_2$-11′), 2.94–2.87 (8H, m, H$_2$-13, H$_2$-13′, H$_2$-15, H$_2$-15′), 1.93–1.84 (4H, m, H$_2$-12, H$_2$-12′), 1.72–1.66 (4H, m, H$_2$-16, H$_2$-16′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 181.0 (C-8, C-8′), 164.4 (C-9, C-9′), 155.8 (d, $^1J_{CF} = 251.2$ Hz, C-4, C-4′), 139.5 (d, $^3J_{CF} = 11.3$ Hz, C-7a, C-7a′), 138.9 (C-2, C-2′), 124.5 (d, $^3J_{CF} = 7.0$ Hz, C-6, C-6′), 113.4 (d, $^2J_{CF} = 21.3$ Hz, C-3a, C-3a′), 111.8 (d, $^3J_{CF} = 6.1$ Hz, C-3, C-3′), 109.0 (C-7, C-7′), 108.0 (d, $^2J_{CF} = 21.7$ Hz, C-5, C-5′), 46.0 (C-15, C-15′), 44.7 (C-13, C-13′), 35.9 (C-11, C-11′), 25.7 (C-12, C-12′), 22.8 (C-16, C-16′); (+)-HRESIMS [M+H]$^+$ *m/z* 581.2667 (calcd for $C_{30}H_{35}F_2N_6O_4$, 581.2682).

5.3. Antimicrobial assays[11,21]

The susceptibility of bacterial strains *S. aureus* (ATCC25923), *S. intermedius* (1051997), *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). 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 *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 [21]. For antimicrobial assays, the tested strains were cultured in either Luria broth (LB) (In Vitro Technologies, USB75852), nutrient broth (NB) (Becton Dickson, 234000), or 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 0.008–32 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 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 0.008–32 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.

5.4. Determination of the MICs of antibiotics in the presence of synergizing compounds[11]

Briefly, restoring enhancer concentrations were determined with an inoculum of 5×10^5 CFU in 200 μL of MHB containing two-fold serial dilutions of each derivative in the presence of doxycycline at 2 μg/mL. 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.

5.5. Cytotoxicity assays[21,22]

The L6 cell line cytotoxicity assays were performed in 96-well microtiter plates, each well containing 100 μL of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4×10^4 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 [22].

HEK293 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. 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 °C, 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 °C 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.

5.6. Haemolytic assay

Haemolytic activities of the compounds were investigated by determining the hemoglobin release from erythrocyte suspensions of fresh Sprague Dawley (SD) rats blood. Fresh blood samples were obtained from the Vernon Jansen Unit (The University of Auckland) and stored in heparin-coated blood collection tubes. The blood cells were centrifuged at low speed for 5 min and the plasma removed. The blood cells were then washed with saline (10 mL of saline for 1 mL of blood) and re-suspended in saline 2% v/v. Re-suspended blood cells (100 μL) were added to the compounds, dissolved in DMSO (20 μL) and PBS buffer (80 μL), in a 96-well plate and incubated for 1 h at 37 °C. After incubation the plate was centrifuged for 5 min at 200 g, the supernatant (100 μL) was transferred into another plate and the haemolysis was measured with a microplate reader at absorbance 540 nm (A). DMSO in PBS buffer was used as the negative control (0% haemolysis) while 0.1% Triton X-100 was used as the positive control (100% haemolysis). The percentage haemolysis was calculated as ($A_{compound} - A_{negative\ control}$)/($A_{positive\ control} - A_{negative\ control}$)x100. All data corresponds to an average of three independent experiments. HC_{50} was determined from dose-response curves generated from 10 points in the 96-well plates.

5.7. Measurement of ATP efflux[11]

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. 90 μL of this suspension was added to 10 μL of squalamine solution and vortexed for 1 s. 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 5 s. ATP concentration was quantified by internal sample addition. A similar procedure was used for spermine (100 μg/mL), **4** and **8** (4 times the MIC).

5.8. Membrane depolarization assays[11]

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 compound **4** or **8** 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).

5.9. Nitrocefin hydrolysis assay[11]

Outer membrane permeabilization was measured using nitrocefin as a chromogenic substrate of periplasmic β-lactamase. Ten milliliters of MHB were inoculated with 0.1 mL of an overnight culture of 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 μM 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.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.ejmech.2019.111708. These data include MOL files and InChiKeys of the most important compounds described in this article.

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