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Norbornane-based Cationic Antimicrobial Peptidomimetics Targeting the Bacterial Membrane

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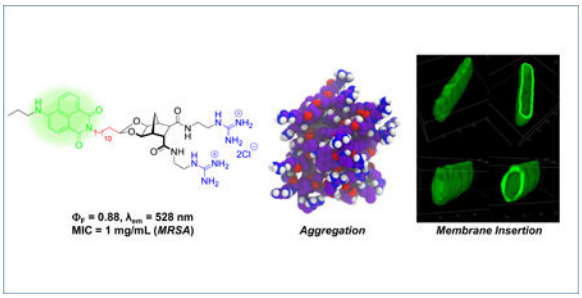
Abstract

The design, synthesis and evaluation of a small series of potent amphiphilic norbornane antibacterial agents has been performed (compound **10** MIC = 0.25 μg/mL against MRSA). Molecular modelling indicates rapid aggregation of this class of antibacterial agent prior to membrane association and insertion. Two fluorescent analogues (compound **29** with 4-amino-naphthalimide and **34** with 4-nitrobenz-2-oxa-1,3-diazole fluorophores) with good activity (MIC = 0.5 μg/mL against MRSA) were also constructed and confocal microscopy studies indicate that the primary site of interaction for this family of compounds is the bacterial membrane.

Graphical Abstract



Keywords

Antimicrobial; antibacterial; amphiphilic; fluorescence; naphthalimide; norbornane; microscopy

1. Introduction

There exists an urgent need to combat the emergence of multi-drug resistant (MDR) bacteria.[1–7] Positive steps have been taken to resolve the problem of bacterial resistance, and this issue has received significant media attention and high-priority recognition by leading health organisations including WHO, British Health and the IDSA.[5] A number of initiatives have been aimed at enticing pharmaceutical companies to re-establish antibacterial product development.[8] Nevertheless, the inevitability of bacterial resistance to any newly approved drug reinforces the need to have new classes of antibacterial agents in the development pipeline; and particularly new drugs that limit the potential for bacterial resistance.[9, 10]

Cationic antimicrobial peptides (CAMPs) are naturally occurring compounds that exhibit activity against most microbes including bacteria.[11, 12] The ability of CAMPs to adopt or be preorganised in a structurally amphiphilic manner, allows them to interact and ultimately perturb the bacterial membrane.[13, 14] Given their longstanding success in nature, it is widely thought that the development of bacterial resistance to these agents should be limited.[15, 16] The synthesis of peptidomimetics that also possess well-defined amphiphilic structure is therefore a logical step in the pursuit of new antibacterial agents and a number of research groups are currently active in this field.[17–20]

The rigid norbornane scaffold has previously been used to prepare preorganised anion hosts[21, 22] and in turn facially amphiphilic cationic peptidomimetics,[23–25] including bisethers (such as **1**, Figure 1)[24] and norbornane acetals (such as **2**),[23] with both classes active against Gram-positive and Gram-negative bacterial strains. The acetal family were the more potent with structure-activity relationship (SAR) studies indicating that a dicationic charge and a long hydrophobic component were essential for activity.[23]

In order to further probe the SAR of these compounds, we envisaged analogues of bisguanidinium **2** where the guanidine groups were further modified with, for example, a hydrophobic group. While a bisphenylguanidine norbornane has been previously reported,[25] the isolation of this product proved troublesome and as such we sought to devise a simpler and more robust synthetic route.

While it was originally hypothesised that the mode of action (MOA) of this norbornane class of compounds involved the membrane as the primary target, no experiments have been performed to establish this fact. Moreover, although the majority of CAMPs are membrane active there are many examples in which these peptides also have an intracellular target.[26] A combination of cellular targets is thought to lead to greater antibacterial activity as well as increasing the barrier to evolved resistance.[26]

Fluorescence microscopy has been used to help elucidate antibacterial MOA of several peptidomimetic compounds,[27, 28] and compounds that aren't inherently fluorescent can often be tagged with a small organic fluorophore with little impact on biological activity.[29] A plethora of small organic fluorescent compounds are available—including the 4-amino-1,8-naphthalimide[30] and 4-nitrobenz-2-oxa-1,3-diazole (NBD) fluorophores[31]—and these have been successfully used to provide an insight into important biological processes. Both of these fluorophores exhibit large Stokes shifts, high quantum yields and typically emit in the green portion of the UV-visible spectrum (495–570 nm).[32, 33]

This study outlines the synthesis and evaluation of six new norbornane-based antibacterial agents, including the most potent to date, and two fluorescent analogues that also possess excellent antibacterial activity. A combination of molecular modelling and fluorescence microscopy has been used to provide an insight into the MOA of this class of compounds.

2. Results and Discussion

2.1. Chemistry

Our preferred reagent for introducing the guanidine group was isothiourea **3**[34, 35] and this compound was used here as the initial starting point to generate further functionalised guanidinylating agents. Unfortunately, the direct alkylation of **3** using NaH and BnBr gave **4** in poor yield. Neverthless, using Mitsunobu conditions (BnOH, PPh_3, DIAD) benzylation was successful and **4** was isolated in excellent yield (95%, Scheme 1). Heating bisamines **5** and **8**[23] with guanidinylating agent **4** and Et_3N in CH_2Cl_2 gave the Boc-protected guanidines **6** and **9** respectively. The Boc-groups were then cleaved using methanolic HCl (generated from AcCl in MeOH) to give the desired guanidine hydrochloride salts **7** and **10**.

It is known that the anion recognition group is important to the success of many antimicrobial peptides (AMPs) due to their ability to interact with negatively charged phosphates on the outer bacterial membrane.[36–38] To date, we have investigated a number of different anion recognition groups with varying degrees of success.[23] A guanidiniocarbonylpyrrole (GCP, **11**) moiety has been reported by the Schmuck group[39] and has been shown to interact with anions that are part of large biological macromolecules.[40–42] In light of this work, we furnished bisamine **5** with the GCP group over two steps to give acyl guanidinium **13** (Scheme 2).

In order to further functionalise the hydrophobic component of the norbornane acetals, *N*-Boc-protected dodecanal **16** was desired. The synthesis of aldehyde **16** has been previously reported by Zeiler and co-workers where the oxidation of **14** was achieved using Swern conditions in 73% yield.[43] Unfortunately in our hands, and despite altering reaction

conditions, including the amount of oxalyl chloride, temperature and reaction duration, aldehyde **16** could only be isolated in a 35% yield after chromatographic purification (Table 1, entries 1–3). Additional studies using ^{1}H NMR indicated that aldehyde **16** degraded quickly; with the resonance assigned to the aldehyde (δ = 9.76 ppm) decreasing in size and relative integration over time. As such, another approach that could generate **16** rapidly, in high yields and without the need for a laborious column chromatography step was pursued.

Pyridinium chlorochromate (PCC) has been used extensively for the oxidation of primary alcohols to aldehydes.[44–46] Initially following the procedure described by Sanders and co-workers,[47] treating alcohol **14** with PCC (1.5 equiv.) gave aldehyde **16** in poor yield (6%), again, after chromatographic purification (Table 1, entry 4). However, using a larger excess of PCC (3.0 equiv.) the alcohol was consumed after 3 hours and **16** was isolated in 79% yield, but again chromatographic purification was required (Table 1, entry 5). Oxidation of **14** using TEMPO (0.2 equiv.) and (diacetoxyiodo)benzene, [48] afforded aldehyde **16** in 58% yield after a column chromatography step (Table 1, entry 6). By far the most successful approach was reduction of Weinreb amide **15** using $LiAlH_4$ to give aldehyde **16** in 95% yield after only two hours and with no requirement for chromatographic purification (Table 1, entry 7). Maintaining the reaction temperature at –78 °C prevented the formation of side-products and spectroscopically pure **16** was isolated after a simple extractive work-up.

Freshly prepared aldehyde **16** was reacted with diol **17** using previously established methodology[23] to give acetal **18** in a 78% yield (Scheme 3). The attachment of two guanidinium groups to the norbornane scaffold, using freshly prepared 2-[2,3-Bis(*tert*-butoxycarbonyl)guanidino]ethylamine (**22**),[34] was carried out over three steps.[24] Briefly, hydrolysis of the methyl esters gave dicarboxylic acid **19** in 88% yield. Standard EDCI/HOBt coupling conditions were employed to attach two units of **22** to the norbornane scaffold. Global deprotection using methanolic HCl gave the desired product **21** as the trihydrochloride salt.

In order to attach a fluorescent tag to the norbornane framework, Boc-protected amine **18** was deprotected to give amine **23** using the same methanolic HCl procedure described earlier. Condensation between amine **23** and 4-bromo-1,8-naphthalic anhydride **24** afforded the corresponding 4-bromo-1,8-naphthalimide **25** in near-quantitative yield (99%, Scheme 4). It should be noted that a trace amount of starting material **24** remained (as evidenced by ^{1}H NMR spectroscopy) but this was eventually removed using column chromatography in the next step. While amination of the 4-bromo position of naphthalimides can be achieved using thermal S_NAr conditions, concomitant amide formation could also occur at the methyl ester sites. As such, the method reported by Fleming and co-workers was followed to convert bromide **25** to the fluorescent 4-aminopropyl-1,8-naphthalimide using propylamine, in the presence of $Pd_2(dba)_3$ $CHCl_3$, xantphos and Cs_2CO_3 (3.0 equiv.) in $PhCH_3$ at 70 °C. [49] After chromatographic purification, fluorescent norbornane **26** was isolated in excellent yield (88%). The attachment of two guanidinium groups to the norbornane scaffold was achieved over three steps, following the previously described methodology, to give naphthalimide **29** (Scheme 4).

A similar protocol was used to construct the NBD analogue (Scheme 5). Aromatic substitution of commercially available NBC-Cl (**30**) with amine **23** proceeded smoothly to give the NBD-norbornane conjugate (**31**). After hydrolysis, amide coupling and Boc-deprotection, the desired NBD analogue (**34**) was isolated as the dihydrochloride salt.

2.2. Photophysical properties

All fluorescent analogues displayed properties typical of their respective fluorophores. Naphthalimide derivatives **28** and **29** exhibited absorption maxima at ~440 nm (Table 2) with corresponding emission maxima of ~530 nm in DMSO and 549 nm in H_2O with Stokes shifts in the range of 84–100 nm. Quantum yields for **28** and **29** in DMSO were high (0.89 and 0.88, respectively) whereas the quantum yield for **29** in H_2O was significantly reduced (Φ_f = 0.16). Similarly, the NBD analogues also displayed characteristic fluorescent behaviour in DMSO (λ_{abs} ~ 475 nm, λ_{ex} = 538 nm, Φ_f = 0.55) In H_2O a slight bathochromic shift in emission and decreased quantum yield was observed (λ_{ex} = 550 nm, Φ_f = 0.02). Nonetheless, the properties observed for **29** and **34** in H_2O were amenable for fluorescence microscopy.

2.3. Antimicrobial activity

The six new compounds were evaluated against a range of Gram-negative and Gram-positive bacteria. The principal means of assessing activity was by broth-micro dilution (BMD) assay, to ascertain the minimum inhibitory concentration (MIC), of each compound. For some analogues, disk diffusion assay (Kirby-Bauer), was also performed (see ESI for results). The new compounds exhibited excellent activity against a range of Gram-positive bacterial strains with MIC values comparable to Vancomycin (Table 3). The analogues containing the benzyl substituted guanidine groups (7, and 10) were the most active, with MIC values as low as 0.25 μg/mL; an improvement in activity compared to their previously synthesised non-substituted counterparts (no activity observed and one MIC value of 1μg/mL, respectively).[23] The tricationic analogue (**21**) only showed moderate activity (MIC = 8–16 μg/mL) despite the presence of an extra charged moiety; reinforcing the notion that structural amphiphilicity is required for antibacterial activity. Importantly, both naphthalimide **29** and NBD **34** were active (MIC value for both = 1 μg/mL against MRSA); comparable to the previously reported non-fluorescent norbornane 2 (MIC value of 2 μg/mL against MRSA).[23] This result suggests that the attachment of the largely hydrophobic fluorescent tag has minimal impact on the antibacterial activity of these compounds. Unfortunately, the GCP analogue (**13**) failed to show any activity at all. When these compounds were evaluated against Gram-negative bacterial strains only moderate or weak activity was noted, the best result was obtained for benzylguanidine **7** and NBD **34**; both with MIC values of 8 μg/mL against *Escherichia coli* (Table 3). Given that a number of groups have previously demonstrated the antifungal properties of CAMPs,[50, 51] compounds **7**, **29** and **34** were also evaluated against pathogenic fungal strains (Table S3). All compounds exhibited activity, highlighted by an MIC of 0.25 μg/mL for naphthalimide **29** against *Cryptococcus neoformans;* considerably more potent than the clinically used antifungal agent Fluconazole (MIC = 8 μg/mL, Table S3).

The cytotoxicity of most compounds when tested against human embryonic kidney cells (HEK293, Table 4) was modest, with CC_{50} values >50 μg/mL. While the highly active benzylguanidine **10** did show considerable toxicity the CC_{50} was still an order of magnitude above its MIC. Pleasingly, benzylgaunidine **7** and fluorescent analogues **29** and **34**, each of which exhibited excellent antibacterial activity against Gram-positive bacteria (MIC 1, 2 and 0.5 μg/mL respectively), showed CC_{50} values >50 μg/mL.

2.4 Molecular modelling

Molecular dynamics simulations were performed to identify potential interactions between the compounds and a model of a cell membrane from Gram-negative bacteria (see experimental for details regarding model membrane construction). The Gram-positive cell wall is comprised of a thick peptidoglycan layer whilst Gram-negative bacteria have a comparatively thin peptidoglycan layer within their cellular envelope (up to 90% versus 10%, respectively).[52, 53] Molecular modelling that incorporates peptidoglycans presents a significant challenge as the complete assembled structure of peptidoglycans is not yet fully understood.[54] As such, a Gram-negative bacterial cell membrane was chosen for this study to circumvent complications associated with peptidoglycans. The compounds modelled included the previously reported norbornanes bisguanidine (**2**) and bisamine (**8**)[23] as well as naphthalimide (**29**) and benzylguanidine (**7**). Two sets of simulations were performed; one set to determine if these compounds self-aggregate, and the second set to determine how they interact with the model bacterial cell wall.

Self-association was readily apparent and all compounds were found to aggregate to form clusters (Figure 2), a logical outcome given the amphiphilic nature of the compounds. The aggregation occurred relatively quickly, on the sub-microsecond timescale, and as such it is likely that, *in vivo*, a level of aggregation occurs prior to insertion into the lipid bilayer. Dimerisation, and even oligomerisation, to form the active antibacterial agent, is a feature of a number of antibiotics including Vancomycin,[55] Daptomycin[56] and a variety of cationic antimicrobial peptidomimetics.[57]

Additional computational studies were performed on bisguanidine (**2**), chosen as a representative example, to probe how this class of compounds interacts with bacterial cell membranes. An aggregate consisting of 28 molecules of **2** (see experimental section for the construction of this aggregate) was inserted into the aqueous (extracellular) portion of a Gram-negative bacterial inner membrane model (Figure 3, LHS). The $\mathbf{2_{28}}$ aggregate was partially absorbed into the membrane in each of the three replicate simulations and full absorption was expected were the simulations to be continued. The incorporation of the $\mathbf{2_{28}}$ aggregate into the membrane occurred in a series of steps. First, the aggregate contacted the membrane. The aggregate stayed attached at the same position of the membrane due to strong electrostatic interactions between the positively charged $\mathbf{2_{28}}$ aggregate and the negatively charged lipid molecules (DMPG). The aggregate maintained its integrity for a few hundred nanoseconds until individual molecules of **2** were drawn into the membrane (Figure 3, Centre). The aggregate then slowly broke down with additional individual molecules diffusing into the membrane (Figure 3, RHS). While no distinct pore formation was observed, some water molecules did penetrate into the membrane (see video in

supplementary material). These water molecules appeared to be stabilised inside the membrane by interactions with the charged guanidine moieties of **2**. In one replicate a significant amount of membrane curvature was evident when the intact aggregate contacted the membrane (see Figure S50). In all three replicates the interaction between the $\mathbf{2}_{28}$ aggregate and the model membrane followed an identical pattern that indicates this class of compound aggregates quickly *in vivo* prior to inserting into to the membrane, before slowly breaking apart. This series of events would likely decrease membrane stability, increase membrane permeability and ultimately lead to cell lysis—a known mode of action for CAMPs and mimics such as Colistin.[37]

2.5 Fluorescence microscopy

Confocal microscopy was performed to assess localisation of fluorescent compounds **29** and **34** in Gram-positive and Gram-negative bacteria. Two control fluorophores were also assessed, naphthalimide **35** and NBD **36** (Figure 4, see ESI for the full synthesis and characterisation of these controls).

Bacteria were incubated for 20 minutes with 10 μg/mL of the fluorescent compound. In Gram-positive *S. aureus* the staining pattern for both compounds **29** and **34** was similar with fluorescence observed throughout the cell but the intensity was greatest at the cell periphery (Figure 4B). Naphthalimide **35** localised throughout the cell and NBD **36** localised to the cell periphery; similar to norbornane compounds **29** and **34**, however the overall fluorescence was considerably less intense for the control compounds **35** and **36**. This decreased intensity is likely a result of weaker interactions with the bacterial membrane as both control compounds **35** and **36** lack the guanidine groups that act as anion recognition moieties targeting the phosphate groups present in the bacterial outer membrane.

When Gram-negative *E. coli* was incubated with **29** and **34**, a similar distribution within the cells was observed (Figure 4C). Once again, greater localisation occurred at the cell periphery, with distinct regions of higher concentration particularly evident when three dimensional images were taken (Figure 4D, also see ESI for associated video). The simple organic fluorophores used as controls (**35** and **36**) occasionally localised to similar regions, but with less consistency and intensity, than compounds **29** and **34**, again highlighting the role of the positively charged groups present on the norbornane-based compounds for cell wall interactions.

The use of molecular modelling and fluorescence microscopy indicate that this class of antibacterial agent likely aggregates rapidly prior to interacting with the bacterial cell membrane. Both the modelling outcomes and the fluorescence microscopy studies using naphthalimide **29** and NBD **34** clearly show the norbornane-based compounds localised on the bacterial membrane highlighting the important role of the cationic groups. Fluorescence microscopy experiments showed that naphthalimide **29** interacts with the membrane for both *S. aureus* and *E. coli*, however cell penetration was only observed in *S. aureus;* a potential cause for the increased antibacterial activity demonstrated by these compounds against Gram-positive bacterial species. Indeed, many AMPs such as the bacteriocin family, which includes colicins and lantibiotics,[58] demonstrate strain-specific antibacterial activity,

despite relying on a mode of action that primarily involves membrane interactions as the first step.[59, 60] With this in mind and comparing the outcomes to those of CAMPs in the literature,[61, 62] it is suggested that these compounds function as mimetics of cationic antimicrobial peptides.

3. Conclusions

In summary, a series of norbornane-based CAMP mimics were synthesised—two dibenzylguanidine substituted (**7** and **10**), one guanidiniocarbonylpyrrole-functionalised (**13**), one tricationic (**21**) and two fluorescently tagged, using the 4-amino naphthalimide and NBD fluorophores (**29** and **34**, respectively). Incorporation of the dibenzylguanidine groups enhanced the antibacterial activity of the compounds, albeit in the case of **10** some cytotoxicity was observed. The importance of retaining a structurally amphiphilic topology was reinforced as non-amphiphilic tricationic analogue **21** was considerably less active than its amphiphilic diatonic counterparts. Antibacterial activity of the fluorescent analogues was comparable to the previously described norbornane acetal **2** with each compound active against several strains of Gram-positive bacteria (MIC values as low as 0.5 μg/mL). Additionally, NBD **34** exhibited activity against several Gram-negative bacterial strains. This structurally amphiphilic class of low-molecular weight CAMP mimics are readily synthesised and possess promising antimicrobial activity.

4. Experimental

4.1. Chemistry

The following compounds and their respective precursors were prepared using literature methods and full reaction details can be found in the supplementary information; **3**,[34] **5**,[23] **8**,[23] **14**,[63] **17**,[24] **22**,[35] **35**[64, 65] and **36**.[66] Compound **11** was kindly donated by the Schmuck research group and its synthesis has been previously reported.[39]

4.1.1. *N,N'*-Bis(*tert*-butoxycarbonyl)-*N*-benzyl-*S*-methylisothiourea (4).[67]—

To a stirring solution of methylisothiourea **3** (1.04 g, 3.58 mmol) in anhydrous THF (12.8 mL) at ambient temperature under an inert atmosphere, was added Ph_3P (1.50 g, 5.73 mmol) followed by BnOH (490 μL, 4.65 mmol). The homogenous solution was cooled to 0 °C, before Diisopropyl azodicarboxylate (DIAD, 1.1 mL, 5.37 mmol) was added slowly. The yellow solution was warmed to ambient temperature and then heated to 66 °C for 16 h. The reaction mixture was concentrated under reduced pressure and the crude material was diluted with H_2O (10 mL) and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic phases were washed with brine (10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil which was stirred in pet. spirits (20 mL) at ambient temperature for 1 h. The resulting white precipitate was collected using vacuum filtration and discarded. The remaining filtrate was purified using column chromatography (10% EtOAc in pet. spirits) to

Supporting Information Available: Synthetic procedures for all known compounds and copies of NMR spectra (^1H and ^{13}C) for all new compounds. All bacterial strains tested, and all cytotoxicity data. Additional molecular modelling images. Fluorescence microscopy and molecular modelling videos can be supplied upon request. This material is available free of charge *via* the Internet at http://

give a light green oil (1.29 g, 95%). R_f = 0.38 (10% EtOAc in pet. spirits). ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.36–7.24 (5H, m, ArH), 4.78 (2H, s, CH_2), 2.28 (3H, s, CH_3), 1.52 (9H, s, *t*-Bu), 1.39 (9H, s, *t*-Bu). ^{13}C NMR (67.5 MHz, $CDC1_3$) δ 163.3, 158.1, 152.1, 137.5, 128.7, 128.5, 127.8, 127.6, 127.4 82.8, 81.9, 52.6, 28.2, 28.1, 15.7. HRMS (ESI, *m/z*) for $C_{19}H_{28}N_2O_4S$ $[M + Na]^+$ calc. 403.1662; found 403.1664.

4.1.2. *tert*-Butyl-[12-(methoxy[methyl]amino)-12-oxododecyl]carbamate (15).

—A mixture of carboxylic acid **40** (5.46 g, 17.30 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 4.02 g, 20.94 mmol), 1-hydroxybenzotriazole (HOBt, 241 mg, 1.78 mmol), *N,O*-dimethylhydroxylamine hydrochloride (2.05 g, 20.97 mmol), $CHCl_3$ (80 mL) and *N,N*-diisopropylethylamine (DIPEA, 15.4 mL, 86.5 mmol) was stirred at 65 °C for 24 h. The reaction was cooled to ambient temperature and the mixture was washed with 0.1 M HCl (40 mL), sat. $NaHCO_3$ (40 mL), brine (40 mL), then dried ($MgSO_4$), filtered and concentrated *in vacuo.* The crude material was purified using column chromatography (20–50% EtOAc in pet. spirits) to afford the title compound (5.40 g, 87%) as a colourless viscous oil. R_f = 0.56 (50% EtOAc in pet. spirits). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.51 (1H, br s, NH), 3.67 (3H, s, OCH_3), 3.17 (3H, s, NCH_3), 3.09–3.08 (2H, m, $NHCH_2$), 2.40 (2H, t, *J* = 7.5 Hz, CH_2CO), 1.65–1.57 (2H, m, CH_2), 1.46–1.39 (9H, m, *t*-Bu), 1.29–1.26 (16H, m, 8 × CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.9, 156.1, 79.2, 61.3, 40.8, 32.3, 32.0, 30.2, 29.64, 29.63, 29.58, 29.57, 29.5, 29.4, 28.6, 26.9, 24.8. HRMS (ESI, *m/z*) for $C_{19}H_{38}N_2O_4$ $[M + H]^+$ calc. 359.2904; found 359.2904.

4.1.3. *N*-(*tert*-Butoxycarbonyl)-12-aminododecanal (16).

Method A[43]: To the stirring solution of oxalyl chloride (140 μL, 1.60 mmol) in anhydrous CH_2Cl_2 (8 mL) at –78 °C, was added DMSO (230 μL, 3.19 mmol), alcohol **14** (400 mg, 1.33 mmol) in anhydrous CH_2Cl_2 (1.5 mL) and Et_3N (1 mL, 7.18 mmol) successively. Stirring was maintained under an inert atmosphere at –78 °C for 3 h before being warmed to ambient temperature and stirred for a further 16 h. The reaction was diluted with H_2O (20 mL) and extracted with Et_2O (2 × 20 mL). The combined organic phase was washed with brine (20 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The crude material was purified using column chromatography (10% EtOAc in pet. spirits) to give the title compound (140 mg, 35%) as a white solid.

Method B[47]: Alcohol **14** (1.04 g, 3.43 mmol) was added to a stirring mixture of neutral alumina (5.09 g, 50.0 mmol) and pyridinium chlorochromate (PCC, 2.18 g, 10.1 mmol) in CH_2Cl_2 (33 mL) and stirring was maintained at ambient temperature for 3 h. The solid material was removed by filtration (rinsing with CH_2Cl_2) and the filtrate was concentrated *in vacuo*. The crude material was purified using column chromatography (20% EtOAc in pet. spirits) to give the product (808 mg, 79%) as a white solid.

Method C[48]: Alcohol **14** (209 mg, 0.693 mmol) was stirred in CH_2Cl_2 (1.4 mL) at ambient temperature before (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 13 mg, 0.083 mmol) and (diacetoxyiodo)benzene (246 mg, 0.762 mmol) were added. The pale orange solution was stirred at ambient temperature for a further for 24 h before more TEMPO (13 mg, 0.083 mmol) was added. Stirring was maintained for another 24 h and the reaction was

then quenched with sat. Na_2SO_3 (8 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic phase was washed with sat. $NaHCO_3$ (5 mL), brine (5 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude material was purified using column chromatography (20% EtOAc in pet. spirits) to give the title compound (112 mg, 58%) as a white solid.

Method D[68]: A stirring solution of Weinreb amide **15** (994 mg, 2.77 mmol) in anhydrous THF (30 mL) was added $LiAlH_4$ (4.2 mL, 4.16 mmol, 1 M solution in Et_2O), at –78 °C under an inert atmosphere. Stirring was maintained for 2 h before the reaction was quenched with 1 M HCl (20 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phase was washed with brine (30 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a white solid (790 mg, 95%) that did not require further purification. R_f = 0.45 (20% EtOAc in pet. spirits). m.p. 51–53 °C (lit. 51 °C).[43] ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (1H, t, *J* = 1.8 Hz, CHO), 4.48 (1H, br s, NH), 3.09 (2H, t, *J* = 7.0 Hz, C*H*$_2$NHBoc), 2.41 (2H, dt, *J* = 7.4, 1.8 Hz, C*H*$_2$CHO), 1.65–1.59 (2H, m, CH_2), 1.47–1.44 (11H, m, CH_2, *t*-Bu), 1.29–1.26 (14H, m, 7 × CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 203.1, 156.2, 79.2, 44.1, 40.8, 30.2, 29.63, 29.60, 29.51, 29.46, 29.4, 29.3, 28.6, 26.9, 22.2. HRMS (ESI, *m/z*) for $C_{17}H_{33}NO_3$ $[M + Na]^+$ calc. 322.2353; found 322.2353.

4.1.4. 8-*endo*-9-*exo*-Di[2′-(2″,3″-bis-*tert*-butoxycarbonyl-3″-benzylguanidino)ethylcarbamoyl]-4-heptyl-3,5-dioxatricyclo [5.2.1.0^{2,6}] decane (6).—

A mixture of diamine **5** (80 mg, 0.165 mmol) and Et_3N (60 μL, 0.41 mmol) in CH_2Cl_2 (300 μL) was stirred for 30 min at ambient temperature before a solution of methylisothiourea **510** (130 mg, 0.341 mmol) in CH_2Cl_2 (900 μL) was added in one portion and the reaction was heated to 40 °C for 2 d. The reaction mixture was diluted with CH_2Cl_2 (10 mL), transferred to a separatory funnel and washed with H_2O (2 × 5 mL), brine (5 mL), dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* and the crude material was purified using flash column chromatography (20–70% EtOAc in pet. spirits) to give the title compound (53 mg, 30%) as a colourless viscous oil. R_f = 0.46 (70% EtOAc in pet spirits). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33–7.27 (10H, m, ArH), 4.79 (4H, br s, ArCH_2), 4.52 (1H, br s, H4), 4.10 (1H, br s, H6), 3.92 (1H, d, *J* = 5.5 Hz, H2), 3.33–3.21 (8H, m, 4 × NHC*H*$_2$), 2.87 (1H, app. t, *J* = 5.1 Hz, H8), 2.61 (1H, br s, H9), 2.57 (1H, br s, H7), 2.32 (1H, d, *J* = 5.9 Hz, H1), 1.76 (1H, d, *J* = 9.9 Hz, H10*a*), 1.61–1.25 (49H, m, 6 × CH_2, 4 × *t*-Bu, H10*s*), 0.87 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 172.6, 153.5 (2 × C), 138.0, 137.8, 128.7 (6 × C), 128.3, 128.2, 128.0, 127.8, 104.0, 83.3, 83.1, 81.5, 79.7 (2 × C), 78.7, 51.4, 51.3, 47.8, 44.6, 44.3, 43.2, 43.0, 39.6, 38.8, 33.0, 32.5, 31.9, 29.8, 29.6, 29.3, 28.33, 28.32, 28.21, 28.17, 24.3, 22.7, 14.2. HRMS (ESI, *m/z*) for $C_{57}H_{84}N_8O_{12}$ $[M + H]^+$ calc. 1075.6438; found 1075.6429.

4.1.5. 4-Heptyl-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-di[carboxamidoethyl-(3″- benzyl)guanidine] hydrogen chloride (7).—

To a stirring solution of Boc-protected guanidine **6** (40 mg, 0.04 mmol) and MeOH (370 μL) was added dropwise AcCl (70 μL, 0.987 mmol), and the reaction was stirred for 24 h at ambient temperature. The reaction was concentrated *in vacuo* and co-evaporated with MeOH (2 × 0.5 mL), to afford the title compound (25 mg, 93%) as a white solid. ^{1}H NMR (270 MHz,

CD_3OD) δ 7.40–7.30 (10H, m, ArH), 4.61 (1H, t, *J* = 4.6 Hz, H4), 4.42–4.41 (4H, m, 2 × $ArCH_2$), 4.03 (1H, d, *J* = 5.4 Hz, H2), 3.98 (1H, d, *J* = 5.4 Hz, H6), 3.41–3.34 (8H, m, 4 × $NHCH_2$), 3.21 (1H, app. t, *J* = 4.8 Hz, H8), 2.62–2.61 (2H, m, H1, H7), 2.45 (1H, br s, H9), 1.72 (1H, d, *J* = 10.2 Hz, H10*a*), 1.60–1.56 (2H, m, CH_2), 1.45 (1H, d, *J* = 10.2 Hz, H10*s*), 1.39–1.29 (10H, m, 5 × CH_2), 0.89 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR (67.5 MHz, CD_3OD) δ 176.7, 174.7, 157.7 (2 × C), 129.9 (4 × C), 129.0 (4 × C), 128.5 (2 × C), 128.4 (2 × C), 105.1, 82.8, 79.9, 47.7, 47.0, 46.2, 46.1, 45.1, 44.9, 42.4 (2 × C), 39.7, 39.6, 33.9, 32.9, 32.7, 30.6, 30.3, 25.2, 23.7, 14.4. HRMS (ESI, *m/z*) for $C_{37}H_{54}N_8O_4$ $[M + 2H]^{2+}$ calc. 338.2207; found 338.2208.

4.1.6. 8-*endo*-9-*exo*-Di[2′-(2″,3″-bis-*tert*-butoxycarbonyl-3″-benzylguanidino)ethylcarbamoyl]-4-pentadecyl-3,5-dioxatricyclo [5.2.1.$0^{2,6}$] decane (9).—

A mixture of diamine **8** (284 mg, 0.476 mmol) and Et_3N (30 μL, 0.21 mmol) in CH_2Cl_2 (2.5 mL) was stirred for 30 min at ambient temperature in a pressure vessel, before a solution of methylisothiourea **4** (431 mg, 1.13 mmol) in CH_2Cl_2 (2.5 mL) was added in one portion and the reaction was heated to 80 °C for 2 d. The reaction mixture was diluted with CH_2Cl_2 (10 mL), transferred to a separatory funnel and washed with H_2O (2 × 10 mL), brine (10 mL), dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* and the crude material was purified using flash column chromatography (50–70% EtOAc in pet. spirits) to give the title compound (83 mg, 14%) as a colourless viscous oil. R_f = 0.65 (70% EtOAc in pet spirits). ^1H NMR (270 MHz, $CDCl_3$) δ 7.35–7.26 (10H, m, ArH), 4.78 (4H, br s, $ArCH_2$), 4.52 (1H, br s, H4), 4.10 (1H, d, *J* = 9.8 Hz, H6), 3.92 (1H, d, *J* = 5.5 Hz, H2), 3.31–3.14 (8H, m, 4 × $NHCH_2$), 2.87 (1H, app. t, *J* = 5.1 Hz, H8), 2.61 (1H, br s, H9), 2.57 (1H, br s, H7), 2.32 (1H, d, *J* = 5.8 Hz, H1), 1.75 (1H, d, *J* = 9.8 Hz, H10*a*), 1.61–1.25 (61H, m, 12 × CH_2, 4 × *t*-Bu, H10*s*), 0.87 (3H, t, *J* = 7.0 Hz, CH_3). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 174.3, 172.6, 153.4 (2 × C), 138.0, 137.9, 128.7 (6 × C), 128.3 (2 × C), 127.8 (2 × C), 104.0, 83.3, 83.1, 81.5, 79.7, 78.8, 77.3, 51.4, 51.3, 47.8, 44.7, 44.3, 43.2, 43.0, 39.7, 38.8, 33.0, 32.5, 32.0, 29.80, 29.77, 29.71, 29.67, 29.5, 28.34 (3 × C), 28.32 (3 × C), 28.21, 28.17, 24.3, 22.8, 14.3.

4.1.7. 4-Pentadecyl-3,5-dioxatricyclo[5.2.1.$0^{2,6}$]decane-8-*endo*-9-*exo*-di[carboxamidoethyl-(3″-benzyl)guanidine] hydrogen chloride (10).—

To a stirring solution of Boc-protected guanidine **9** (101 mg, 0.085 mmol) and MeOH (5 mL) was added dropwise AcCl (250 μL, 3.52 mmol), and the reaction was stirred for 24 h at ambient temperature. The reaction was concentrated *in vacuo* and co-evaporated with MeOH (2 × 0.5 mL), to afford the title compound (54 mg, 74%) as a white solid. m.p. 174–180 °C (slow decomposition). ^1H NMR (270 MHz, CD_3OD) δ 7.38–7.31 (10H, m, ArH), 4.61 (1H, t, *J* = 4.6 Hz, H4), 4.47–4.41 (4H, m, 2 × $ArCH_2$), 4.04 (1H, d, *J* = 5.6 Hz, H2), 3.98 (1H, d, *J* = 5.6 Hz, H6), 3.40–3.30 (8H, m, 4 × $NHCH_2$), 3.21 (1H, app. t, *J* = 4.8 Hz, H8), 2.65–2.61 (2H, m, H1, H7), 2.46 (1H, br s, H9), 1.72 (1H, d, *J* = 10.2 Hz, H10*a*), 1.60–1.55 (2H, m, CH_2), 1.45 (1H, d, *J* = 10.2 Hz, H10*s*), 1.36–1.22 (24H, m, 12 × CH_2), 0.90 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR (67.5 MHz, CD_3OD) δ 176.7, 174.7, 157.7 (2 × C), 137.6, 129.9 (3 × C), 129.0 (4 × C), 128.5 (2 × C), 128.4 (2 × C), 105.1, 82.8, 80.0, 47.7, 46.9, 46.2, 46.1, 45.2, 44.9, 42.4 (2 × C), 39.7, 39.6, 33.9, 33.1, 32.7, 30.77, 30.75, 30.7 (2 × C), 30.64 (2 ×

C), 30.62, 30.5, 25.2, 23.7, 14.4. HRMS (ESI, *m/z*) for $C_{45}H_{70}N_8O_4$ $[M + 2H]^{2+}$ calc. 394.2833; found 394.2842.

4.1.8. 8-*endo*-9-*exo*-Di[2′-(5″-acetamido-1*H*-pyrrole-2″-*N*-*tert*-butoxycarbonyl-guanidiniocarbonyl)ethylcarbamoyl]-4-heptyl-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane (12).—

A solution of GCP **11** (104 mg, 0.116 mmol), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 163 mg, 0.313 mmol), 4-(dimethylamino)pyridine (DMAP, 8 mg, 0.07 mmol) and DMF (0.5 mL) was stirred at ambient temperature. To the homogenous yellow solution was added Et_3N (90 μL, 0.646 mmol) and the solution gradually turned to red and then eventually bright orange. To the stirred solution was added diamine **5** (56 mg, 0.116 mmol) and the biphasic mixture was stirred at 80 °C for 16 h. The reaction was quenched with H_2O (2 mL) and the white precipitate was collected using vacuum filtration. The crude material was stirred in 10% pet. spirits in CH_2Cl_2 for 1 h, cooled and then collected using vacuum filtration to afford the desired product (47 mg, 42%) as an off-white solid. R_f = 0.41 (89% CH_2Cl_2, 10% MeOH, 1% NH_4OH). m.p. 217–252 °C (slow decomposition). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 11.30–10.88 (4H, br s, 4 × NH), 9.34 (2H, br s, 2 × NH), 8.58 (2H, br s, 2 × NH), 8.40 (2H, br s, 2 × NH), 8.12–8.10 (2H, m, 2 × NH), 6.80–6.75 (4H, m, 4 × CH), 4.44 (1H, t, *J* = 4.3 Hz, H4), 3.84 (1H, d, *J* = 5.6 Hz, H6), 3.79 (1H, d, *J* = 5.6 Hz, H2), 3.36–3.27 (8H, m, 4 × CH_2), 3.14–3.09 (1H, m, H8), 2.50 (2H, m, H1, H7), 2.29 (1H, br s, H9), 1.45 (19H, br s, *t*-Bu, H10*a*), 1.21–1.08 (13H, m, 6 × CH_2, H10*s*), 0.85 (3H, t, *J* = 6.6 Hz, CH_3). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 173.1 (2 × C), 171.2 (2 × C), 159.8 (4 × C), 158.5 (2 × C), 129.7 (2 × C), 113.7 (2 × C), 111.7 (2 × C), 111.6 (2 × C), 102.9, 81.0, 78.2, 46.3, 44.3, 43.2, 42.7, 38.7 (2 × C), 38.4 (2 × C), 32.4, 31.2, 31.0, 29.0, 28.7, 27.8, 23.8, 22.1, 14.0.

4.1.9. 4-Heptyl-3,5-dioxatricydo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-di[carboxamidoethyl-(5″-acetamido-1-*H*-pyrrole-2″)-guamdiniocarbonyl] hydrogen chloride (13).—

To a stirring solution of Boc-protected guanidine **12** (93 mg, 0.096 mmol) and MeOH (2 mL) was added dropwise AcCl (280 μL, 3.94 mmol), and the reaction was stirred for 24 h at ambient temperature. The reaction was concentrated *in vacuo* and co-evaporated with MeOH (2 × 0.5 mL), to afford the title compound (79 mg, 99%) as a brown solid. m.p. 206–209 °C. ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 12.34–12.31 (2H, m, 2 × NH), 11.95–11.94 (2H, m, 2 × NH), 8.63–8.43 (8H, m, 8 × NH), 8.17–8.14 (2H, m, 2 × NH), 7.50 (2H, br s, 2 × CH), 6.84 (2H, br s, 2 × CH), 4.49 (1H, t, *J* = 4.7 Hz, H4), 3.87 (1H, d, *J* = 4.9 Hz, H6), 3.82 (1H, d, *J* = 4.9 Hz, H2), 3.75–3.63 (2H, br s, 2 × NH), 3.28–3.13 (8H, m, 4 × CH_2), 3.09 (1H, app. t, *J* = 4.8 Hz, H8), 2.50 (2H, m, H1, H7), 2.30 (1H, br s, H9), 1.50–1.45 (3H, m, CH_2, H10*a*), 1.28–1.10 (11H, m, 5 × CH_2, H10*s*), 0.85 (3H, t, *J* = 6.8 Hz, CH_3). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 173.1, 171.2, 159.6 (2 × C), 159.3 (2 × C), 155.4 (2 × C), 132.90, 132.87, 125.4 (2 × C), 115.9 (2 × C), 112.4 (2 × C), 102.9, 81.0, 78.2, 46.3, 44.4, 43.2, 42.7, 38.72, 38.71, 38.5, 38.3, 32.4, 31.2, 31.0, 28.9, 28.6, 23.8, 22.1, 14.0. HRMS (ESI, *m/z*) for $C_{35}H_{52}N_{12}O_8$ $[M + 2H]^{2+}$ calc. 384.2010; found 384.2019.

4.1.10. Dimethyl 4-[11′-(*tert*-butoxycarbonylammo)undecyl]-3,5-dioxatricydo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylate (18).—

A stirring suspension of diol **17** (323 mg, 1.32 mmol), TsOH H_2O (13 mg, 0.07 mmol), $MgSO_4$ (166

mg, 1.38 mmol) and PhMe (2.2 mL) was treated with aldehyde **16** (587 mg, 1.96 mmol) and heated to 110 °C for 3 h. Solid $MgSO_4$ was removed by filtration and the filtrate was diluted with EtOAc (20 mL), washed with H_2O (2 × 10 mL), brine (10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The crude material was purified using column chromatography (20% EtOAc in pet. spirits) to afford the title compound (542 mg, 78%) as a colourless oil. R_f = 0.44 (20% EtOAc in pet. spirits). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.65 (1H, t, *J* = 5.2 Hz, H4), 4.50 (1H, br s, NH), 4.02 (1H, d, *J* = 5.6 Hz, H2), 3.89 (1H, d, *J* = 5.6 Hz, H6), 3.70–3.69 (6H, m, 2 × Me), 3.22 (1H, app. t, *J* = 4.8 Hz, H8), 3.08 (2H, app. t, *J* = 7.0 Hz, C*H*$_2$NHBoc), 2.71 (1H, d, *J* = 4.8 Hz, H9), 2.64–2.63 (2H, m, H1, H7), 1.77 (1H, dd, *J* = 10.9, 1.4 Hz, H10*a*), 1.64–1.60 (2H, m, CH_2), 1.43 (9H, s, *t*-Bu), 1.39–1.24 (19H, m, 9 × CH_2, H10*s*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.1, 172.9, 156.1, 104.3, 81.4, 79.1, 78.9, 52.5, 52.3, 45.4, 45.1, 43.8, 43.4, 40.8, 32.9, 31.7, 30.2, 29.82, 29.78, 29.64, 29.59, 29.5, 29.4, 28.6, 26.9, 24.3. HRMS (ESI, *m/z*) for $C_{28}H_{47}NO_8$ $[M + Na]^+$ calc. 548.3194; found 548.3171.

4.1.11. 4-[11′-(*tert*-butoxycarbonylamino)undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylic acid (19).—

To the stirred solution of the ester **18** (27 mg, 0.05 mmol) in THF (210 μL), 2M NaOH (110 μL) was added and the reaction was stirred for 16 h at ambient temperature. The reaction was concentrated under reduced pressure and all organic-soluble impurities were extracted with CH_2Cl_2 (2 × 5 mL). The aqueous solution was acidified with sat. KH_2PO_4 (pH = 5), extracted with EtOAc (3 × 8 mL), dried ($MgSO_4$) and filtered to give a colourless oil (22 mg, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.68 (1H, t, *J* = 4.9 Hz, H4), 4.59 (1H, br s, NH), 4.04 (2H, s, H2, H6), 3.27 (1H, app. t, *J* = 5.2 Hz, H8), 3.07 (2H, br s, NHC*H*$_2$), 2.78 (1H, d, *J* = 4.3 Hz, H1), 2.73 (1H, br s, H7), 2.65 (1H, d, *J* = 5.2 Hz, H9), 1.82 (1H, d, *J* = 10.2 Hz, H10*a*), 1.66–1.62 (2H, m, CH_2), 1.51–1.25 (28H, m, 9 × CH_2, *t*-Bu, H10*s*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.4, 177.2, 104.4, 81.4, 79.5, 78.8, 45.3, 45.2, 43.7, 43.4, 40.8, 32.7, 31.9, 30.1, 29.8, 29.63, 29.56, 29.5 (2 × C), 29.4, 28.6, 26.9, 24.2. HRMS (ESI, *m/z*) for $C_{26}H_{43}NO_8$ $[M + H]^+$ calc. 498.3061; found 498.3054.

4.1.12. 8-*endo*-9-*exo*-Di[2′-(2″,3″-bis-*tert*-butoxycarbonylguanidino)ethylcarbamoyl]-4-[11′-(*tert*-butoxycarbonylamino)undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}] decane (20).—

A MW vial was charged with diacid **19** (21 mg, 0.004 mmol), EDCI (24 mg, 0.126 mmol), HOBt (1 mg, 0.01 mmol) and anhydrous $CHCl_3$ (210 μL) and was stirred at ambient temperature for 30 min. Amine **22** (38 mg, 0.13 mmol) was then added and the reaction was irradiated for 30 min at 50 °C. The resulting homogenous clear liquid was diluted with $CHCl_3$ (10 mL), washed with H_2O (2 × 5 mL), brine (5 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo* to afford a colourless oil that was purified using flash column chromatography (20% EtOAc in pet. spirits-EtOAc) to give the title compound (14 mg, 32%) as a colourless oil. R_f = 0.75 (EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.47–11.44 (2H, m, 2 × NH), 8.67 (1H, br s, NH), 8.56 (1H, br s, NH), 8.04 (1H, t, *J* = 4.1 Hz, NH), 6.92 (1H, br s, NH), 4.60 (1H, t, *J* = 4.8 Hz, H4), 4.50 (1H, br s, NH), 4.02 (1H, d, *J* = 5.7 Hz, H6), 3.95 (1H, d, *J* = 5.7 Hz, H2), 3.60–3.56 (4H, m, 2 × $NHCH_2$), 3.45–3.35 (4H, m, 2 × $NHCH_2$), 3.11–3.07 (2H, m, BocNHC*H*$_2$), 2.94 (1H, app. t, *J* = 5.3 Hz, H8), 2.70 (1H, d, *J*

= 4.1 Hz, H1), 2.57 (1H, br s, H7), 2.45 (1H, d, *J* = 5.3 Hz, H9), 1.77 (1H, d, *J* = 9.7 Hz, H10*a*), 1.62–1.57 (2H, m, CH_2), 1.50–1.43 (50H, m, 2 × CH_2, 5 × *t*-Bu, H10*s*), 1.36–1.24 (14H, m, 7 × CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.2, 172.1, 163.1, 162.7, 157.8, 157.0, 156.1, 153.2, 153.1, 104.1, 83.8, 83.6, 81.7, 80.1, 80.0, 79.1, 79.0, 47.8, 44.4, 44.2, 43.0, 42.1, 40.8, 40.4, 40.1, 40.0, 33.0, 32.5, 32.1, 30.2, 29.8, 29.7, 29.6, 29.4, 28.6, 28.38, 28.37, 28.20, 28.19, 27.0, 24.3, 22.8. HRMS (ESI, *m/z*) for $C_{52}H_{91}N_9O_{14}$ $[M + H]^+$ calc. 1066.6758; found 1066.6775.

4.1.13. 4-[11′-(Amino)undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxamidoethylguanidine hydrogen chloride (21).—To a stirring solution of Boc-protected guanidine **20** (14 mg, 0.01 mmol) and MeOH (1 mL) was added dropwise AcCl (30 μL, 0.422 mmol), and the reaction was stirred for 48 h at ambient temperature. The reaction was concentrated *in vacuo* and co-evaporated with MeOH (2 × 0.5 mL), to afford the title compound (9 mg, 99%) as a colourless oil. ^{1}H NMR (500 MHz, CD_3OD) 5 4.66 (1H, t, *J* = 4.6 Hz, H4), 4.05 (1H, d, *J* = 5.5 Hz, H6), 4.00 (1H, d, *J* = 5.5 Hz, H2), 3.37–3.26 (4H, m, 2 × NHCH_2), 3.21 (1H, app. t, *J* = 5.0 Hz, H8), 2.96–2.85 (6H, m, 3 × $NHCH_2$), 2.62–2.61 (2H, m, H1, H7), 2.45 (1H, br s,, H9), 1.73 (1H, d, *J* = 10.3 Hz, H10*a*), 1.64–1.58 (4H, m, 2 × CH_2), 1.48 (1H, d, *J* = 10.3 Hz, H10*s*), 1.40–1.29 (16H, m, 8 × CH_2). ^{13}C NMR (125 MHz, CD_3OD) δ 176.5, 174.6, 158.9 (2 × C), 105.1, 82.8, 80.0, 47.7, 47.0, 45.1, 44.9, 41.93, 41.88, 40.0, 39.7, 39.6, 33.9, 32.7, 30.8, 30.70, 30.67, 30.6, 30.5, 30.3, 29.2, 27.5, 25.2. HRMS (ESI, *m/z*) for $C_{27}H_{51}N_9O_4$ $[M + 3H]^{3+}$ calc. 189.4761; found 189.4766.

4.1.14. Dimethyl 4-(11′-aminoundecanyl)-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylate hydrogen chloride (23).—To a stirring solution of Boc-protected amine **18** (511 mg, 0.97 mmol) and MeOH (10 mL) was added dropwise AcCl (700 μL, 9.85 mmol), and the reaction was stirred for 24 h at ambient temperature. The reaction mixture was concentrated *in vacuo,* co-evaporated with MeOH (2 × 0.5 mL) and the title compound was isolated (444 mg, 99%) as an off-white sticky residue. ^{1}H NMR (500 MHz, CD_3OD) δ 4.66 (1H, t, *J* = 4.8 Hz, H4), 4.00 (1H, d, *J* = 5.6 Hz, H2), 3.90 (1H, d, *J* = 5.6 Hz, H6), 3.71 (3H, s, Me), 3.70 (3H, s, Me), 3.21 (1H, app. t, *J* = 5.0 Hz, H8), 2.91 (2H, app. t, *J* = 7.6 Hz, C$H_2$$NH_3$), 2.64–2.62 (2H, m, H1, H7), 2.56 (1H, br s, H9), 1.74 (1H, dd, *J* = 10.7, 1.5 Hz, H10*a*), 1.67–1.58 (4H, m, 2 × CH_2), 1.41–1.29 (17H, m, 8 × CH_2, H10*s*). ^{13}C NMR (125 MHz, CD_3OD) δ 175.4, 174.0, 105.2, 82.5, 80.0, 52.8, 52.6, 46.6, 46.2, 44.9, 44.5, 40.8, 33.8, 32.4, 30.62, 30.57 (3 × C), 30.5, 30.2, 28.6, 27.4, 25.2. HRMS (ESI, *m/z*) for $C_{23}H_{39}NO_6$ $[M + H]^+$ calc. 426.2850; found 426.2847.

4.1.15. Dimethyl 4-[11′-(6″-bromo-1″,3″-dioxo-1*H*-benzo[*de*]isoquinoline-2(3*H*))undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylate (25).—A 10 mL microwave vial was charged with 4-bromo-1,8-naphthalic anhydride (264 mg, 0.953 mmol), norbornane hydrochloride **23** (444 mg, 0.961 mmol), Et_3N (140 μL, 1.00 mmol) and EtOH (3 mL) and heated to 100 °C using microwave irradiation for 45 min. The orange slurry was diluted with H_2O (30 mL) and extracted with EtOAc (3 × 30 mL). The combined organic phase was washed with 0.1 M HCl (30 mL), sat. $NaHCO_3$ (30 mL), brine (30 mL), dried ($MgSO_4$), filtered and

concentrated *in vacuo* to give the title compound (649 mg, 99%) as an orange oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ 8.64 (1H, dd, *J* = 7.4, 1.2 Hz, H9″), 8.55 (1H, dd, *J* = 8.6, 1.2 Hz, H7″), 8.40 (1H, d, *J* = 7.8 Hz, H4″), 8.02 (1H, d, *J* = 7.8 Hz, H5″), 7.83 (1H, dd, *J* = 8.6, 7.4 Hz, H8″), 4.64 (1H, t, *J* = 4.9 Hz, H4), 4.18–4.12 (2H, m, H11′), 4.02 (1H, d, *J* = 5.5 Hz, H2), 3.89 (1H, d, *J* = 5.5 Hz, H6), 3.70 (6H, s, 2 × Me), 3.23 (1H, app. t, *J* = 4.9 Hz, H8), 2.71 (1H, d, *J* = 4.9 Hz, H9), 2.65–2.64 (2H, m, H1, H7), 1.78 (1H, dd, *J* = 11.0, 1.5 Hz, H10*s*), 1.74–1.58 (4H, m, H1′, H10′), 1.42–1.25 (17H, m, 8 × CH_2, H10*s*). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 174.1, 172.9, 163.74, 163.73, 133.3, 132.1, 131.3, 131.2, 130.8, 130.3, 129.1, 128.2, 123.3, 122.4, 104.3, 81.4, 78.9, 52.5, 52.3, 45.4, 45.2, 43.8, 43.4, 40.7, 32.9, 31.8, 29.7 (2 × C), 29.62, 29.60, 29.5 (2 × C), 28.2, 27.2, 24.3. HRMS (ESI, *m/z*) for $C_{35}H_{42}{}^{79}BrNO_8$ $[M + Na]^+$ calc. 706.1986; found 706.1972.

4.1.16. Dimethyl 4-[11′-(6″-propylamino-1″,3″-dioxo-1*H*-benzo[*de*]isoquinolme-2(3*H*))undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylate (26).—

A stirring solution of 4-bromo naphthalimide norbornane **25** (28 mg, 0.041 mmol), $Pd_2(dba)_3CHCl_3$ (2 mg, 0.002 mmol), Xantphos (1 mg, 0.002 mmol), propylamine (5 drops) and Cs_2CO_3 (40 mg, 0.123 mmol) in PhMe (410 μL) was heated at 70 °C for 24 h. The solvent was removed *in vacuo* and the resulting residue was loaded onto SiO_2 and purified using column chromatography (2% MeOH in 1:4 EtOAc in pet. spirits) to give the title compound (23 mg, 88%) as a yellow oil. R_f = 0.19 (2% MeOH in 1:4 EtOAc in pet. spirits). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (1H, dd, *J* = 7.4, 1.1 Hz, H9″), 8.45 (1H, d, *J* = 8.5 Hz, H4″), 8.08 (1H, dd, *J* = 8.4, 1.1 Hz, H7″), 7.61 (1H, dd, *J* = 8.4, 7.4 Hz, H8″), 6.72 (1H, d, *J* = 8.5 Hz, H5″), 5.26–5.25 (1H, m, NH), 4.65 (1H, t, *J* = 4.9 Hz, H4), 4.16–4.12 (2H, m, H11′), 4.02 (1H, d, *J* = 5.7 Hz, H2), 3.90 (1H, dd, *J* = 5.7, 1.4 Hz, H6), 3.70 (3H, s, Me), 3.69 (3H, s, Me), 3.40–3.35 (2H, m, NHCH_2), 3.22 (1H, app. t, *J* = 5.1 Hz, H8), 2.72 (1H, d, *J* = 4.9 Hz, H9), 2.65–2.64 (2H, m, H1, H7), 1.88–1.76 (3H, m, CH_2CH$_3$, H10*a*), 1.74–1.67 (2H, m, H10′), 1.64–1.59 (2H, m, H1′), 1.43–1.24 (17H, m, 8 × CH_2, H10*s*), 1.11 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1, 172.9, 164.8, 164.3, 149.5, 134.6, 131.2, 130.8, 125.8, 124.8, 123.4, 120.3, 110.5, 104.5, 104.4, 81.4, 78.9, 52.5, 52.3, 45.6, 45.4, 45.2, 43.8, 43.4, 40.4, 32.9, 31.8, 29.69, 29.67 (2 × C), 29.6 (2 × C), 29.5, 28.4, 27.3, 24.4, 22.4, 11.8. HRMS (ESI, *m/z*) for $C_{38}H_{50}N_2O_8$ $[M + H]^+$ calc. 663.3640; found 663.3648.

4.1.17. 4-[11′-(6″-Propylamino-1″,3″-dioxo-1*H*-benzo[*de*]isoquinoline-2(3*H*))undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}] decane-8-*endo*-9-*exo*-dicarboxylic acid (27).—

A biphasic solution of ester **26** (434 mg, 0.655 mmol) in 2 M NaOH/THF (1:4, 7 mL) was stirred at ambient temperature for 16 h. The reaction mixture was extracted with CH_2Cl_2 (2 × 15 mL) and the isolated aqueous phase was acidified to pH = 1 using 2 M HCl and extracted with EtOAc (3 × 15 mL). The combined organic phase was washed with brine (15 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the title compound (366 mg, 88%) as an orange solid. m.p. 94–95 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.56 (1H, dd, *J* = 7.5, 1.1 Hz, H9″), 8.44 (1H, d, *J* = 8.5 Hz, H4″), 8.08 (1H, dd, *J* = 8.5, 1.1 Hz, H7″), 7.58 (1H, dd, *J* = 8.5, 7.5 Hz, H8″), 6.69 (1H, d, *J* = 8.5 Hz, H5″), 4.68 (1H, t, *J* = 4.8 Hz, H4), 4.14–4.11 (2H, m, H11′), 4.05 (2H, br s, H2, H6), 3.37 (2H, app. t, *J* = 7.3 Hz, NHCH_2), 3.28 (1H, app. t, *J* = 5.2 Hz, H8), 2.79 (1H, d, *J* =

4.3 Hz, H7), 2.73 (1H, br s, H1), 2.66 (1H, d, *J* = 5.2 Hz, H9), 1.85–1.81 (3H, m, CH_2, H10*a*), 1.72–1.59 (4H, m, 2 × CH_2), 1.39–1.21 (17H, m, 8 × CH_2, H10*s*), 1.10 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) 5 178.1, 176.9, 164.9, 164.5, 149.7, 134.8, 131.4, 129.9, 126.0, 124.8, 123.2, 120.2, 110.1, 104.5, 104.4, 81.3, 78.8, 45.6, 45.2, 45.1, 43.7, 43.4, 40.5, 32.7, 31.9, 29.8, 29.64, 29.58, 29.55, 29.4 (2 × C), 28.4, 27.3, 24.2, 22.4, 11.8. HRMS (ESI, *m/z*) for $C_{36}H_{46}N_2O_8$ $[M - H]^-$ calc. 633.3181; found 633.3190.

4.1.18. 8-*endo*-9-*exo*-Di[2′-(2″,3″-bis-*tert*-butoxycarbonylguamdmo)ethylcarbamoyl]-4-[11′-(6″-propylamino-1″,3″-dioxo-1*H*-benzo[*de*]isoqumolme-2(3*H*))undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}] decane (28).—

A solution of diacid **27** (285 mg, 0.449 mmol), EDCI (260 mg, 1.35 mmol), HOBt (6 mg, 0.05 mmol), aminoethylguanidine **22** (423 mg, 1.40 mmol) and anhydrous DMF (6 mL) was stirred at ambient temperature for 2 d under an inert atmosphere. The reaction mixture was diluted with EtOAc (25 mL) and washed with H_2O (15 mL), brine (3 × 15 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give a yellow oil. The crude material was purified using column chromatography (EtOAc) to afford the title compound (444 mg, 82%) as a yellow solid. R_f = 0.50 (EtOAc). m.p. 98–99 °C. Φ_f = 0.89 (DMSO). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.48 (1H, br s, NH), 11.45 (1H, br s, NH), 8.63 (1H, t, *J* = 6.1 Hz, NH), 8.57 (1H, dd, *J* = 7.4, 1.0 Hz, H9″), 8.51 (1H, t, *J* = 5.8 Hz, NH), 8.45 (1H, d, *J* = 8.5 Hz, H4″), 8.09 (1H, dd, *J* = 8.5, 1.0 Hz, H7″), 8.01 (1H, t, *J* = 4.1 Hz, NH), 7.61 (1H, dd, *J* = 8.5, 7.4 Hz, H8″), 6.86 (1H, t, *J* = 5.3 Hz, NH), 6.72 (1H, d, *J* = 8.5 Hz, H5″), 5.29 (1H, t, *J* = 5.0 Hz, NH), 4.60 (1H, t, *J* = 4.9 Hz, H4), 4.15–4.12 (2H, m, H11′), 4.02 (1H, d, *J* = 5.5 Hz, H6), 3.94 (1H, d, *J* = 5.5 Hz, H2), 3.60–3.52 (4H, m, 2 × NHC*H*$_2$), 3.44–3.36 (6H, m, 3 × NHC*H*$_2$), 2.94 (1H, app. t, *J* = 5.2 Hz, H8), 2.78 (1H, br s, H7), 2.70 (1H, d, *J* = 4.1 Hz, H1), 2.44 (1H, d, *J* = 5.2 Hz, H9), 1.87–1.80 (2H, m, CH_2), 1.77 (1H, d, *J* = 9.5 Hz, H10*a*), 1.72–1.67 (2H, m, CH_2), 1.62–1.57 (2H, m, CH_2), 1.50–1.48 (37H, m, 4 × *t*-Bu, H10*s*), 1.42–1.24 (16H, m, 8 × CH_2), 1.10 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.2, 172.1, 164.8, 164.3, 163.5, 163.0, 157.9, 157.1, 153.2, 149.5, 134.6, 131.2, 129.9, 125.8, 124.8, 123.4, 120.3, 110.5, 104.5, 104.2, 83.7, 83.4, 81.6, 79.9, 79.7, 79.0, 47.8, 45.6, 44.30, 44.25, 42.3, 40.4, 40.2, 40.04, 39.97, 33.0, 32.5, 29.74, 29.71, 29.69, 29.66, 29.6, 28.40, 28.38, 28.3, 28.20, 28.19, 27.3, 24.4, 22.4, 11.8. HRMS (ESI, *m/z*) for $C_{62}H_{94}N_{10}O_{14}$ $[M + H]^+$ calc. 1203.7024; found 1203.7040.

4.1.19. 4-[11′-(6″-Propylamino-1″,3″-dioxo-1*H*-benzo[*de*]isoquinoline-2(3*H*))undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxamidoethylguanidine hydrogen chloride (29).—

To a stirring solution of Boc-protected guanidine **28** (87 mg, 0.072 mmol) and MeOH (720 μL) was added dropwise AcCl (200 μL, 2.81 mmol), and the reaction was stirred for 2 d at ambient temperature. The reaction mixture was concentrated *in vacuo*, co-evaporated with MeOH (2 × 0.5 mL) and the title compound was isolated (61 mg, 97%) as an off-white sticky residue. m.p. 122–159 °C (slow decomposition). Φ_f = 0.88 (DMSO) and 0.16 (H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 8.55 (1H, dd, *J* = 8.5, 1.1 Hz, H9″), 8.50 (1H, dd, *J* = 7.5, 1.1 Hz, H7″), 8.35 (1H, d, *J* = 8.7 Hz, H4″), 7.63 (1H, dd, *J* = 8.5, 7.5 Hz, H8″), 6.78 (1H, d, *J* = 8.7 Hz, H5″), 4.65 (1H, t, *J* = 4.7 Hz, H4), 4.12–4.09 (2H, m, H11′), 4.05 (1H, d, *J* = 5.7 Hz, H6), 3.99 (1H, d, *J* = 5.7 Hz, H2), 3.43–3.28 (10H, m, 5 × NHC*H*$_2$), 3.23 (1H, app. t, *J* = 5.1 Hz,

H8), 2.62–2.61 (2H, m, H1, H7), 2.44 (1H, br s, H9), 1.85–1.78 (2H, m, CH_2), 1.73 (1H, d, *J*= 10.0 Hz, H10*a*), 1.70–1.66 (2H, m, CH_2), 1.61–1.57 (2H, m, CH_2), 1.47 (1H, d, *J*= 10.0 Hz, H10*s*), 1.40–1.28 (16H, m, 8 × CH_2), 1.07 (3H, t, *J* = 7.4 Hz, CH_3). ^{13}C NMR (125 MHz, CD_3OD) δ 176.5, 174.6, 166.3, 165.8, 158.9 (2 × C), 152.8, 136.1, 132.2, 131.3, 129.4, 125.4, 123.4, 121.9, 109.1, 105.11, 105.09, 82.8, 79.9, 47.8, 46.8, 46.2, 45.1, 44.9, 42.0, 41.9, 41.0, 39.7, 39.6, 33.9, 32.7, 30.6 (4 × C), 30.5, 30.4, 29.1, 28.1, 25.2, 22.7, 11.9. HRMS (ESI, *m/z*) for $C_{42}H_{62}N_{10}O_6$ $[M + 2H]^{2+}$ calc. 402.2500; found 402.2506.

4.1.20. Dimethyl 4-[11′-(7″-nitrobenzo[*c*][1,2,5]oxadiazol-4″-amino)undecyl]-3,5-dioxatricyclo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylate (31).—A solution of norbornane hydrochloride **23** (371 mg, 0.803 mmol), 4-nitrobenz-2-oxa-1,3-diazole chloride (160 mg, 0.803 mmol) and Et_3N (170 μL, 1.22 mmol) in MeOH (8 mL) was stirred at ambient temperature for 24 h. The reaction mixture was concentrated under reduced pressure then diluted with EtOAc (20 mL). The organic phase was washed with 0.1 M HCl (10 mL), sat. $NaHCO_3$ (10 mL), brine (10 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The orange residue was purified using column chromatography (10% EtOAc in CH_2Cl_2) to give the title compound (320 mg, 68%) as a dark orange viscous oil. R_f = 0.9 (10% EtOAc in CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (1H, d, *J*= 8.6 Hz, H6″), 6.33 (1H, t, *J*= 4.7 Hz, NH), 6.17 (1H, d, *J*= 8.6 Hz, H5″), 4.65 (1H, t, *J*= 4.8 Hz, H4), 4.02 (1H, d, *J*= 5.6 Hz, H6), 3.94 (1H, d, *J*= 5.6 Hz, H2), 3.70 (3H, s, Me), 3.69 (3H, s, Me), 3.50–3.46, (2H, m, H11′), 3.22 (1H, app. t, *J*= 5.2 Hz, H8), 2.71 (1H, d, *J*= 4.5 Hz, H7), 2.65–2.64 (2H, m, H1, H9), 1.83–1.75 (3H, m, H10′, H10*a*), 1.64–1.60 (2H, m, H1′), 1.48–1.42 (2H, m, H9′), 1.39–1.26 (15H, m, H10*s*, 7 × CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.1, 172.9, 144.4, 144.1, 144.0, 136.7, 123.9, 104.3, 98.6, 81.3, 78.9, 52.5, 52.3, 45.4, 45.1, 44.1, 43.7, 43.4, 32.9, 31.7, 29.60, 29.57, 29.5 (3 × C), 29.3, 28.6, 27.1, 24.3. HRMS (ESI, *m/z*) for $C_{29}H_{40}N_4O_9$ $[M + H]^+$ calc. 589.2868; found 589.2883.

4.1.21. 4-[11′-(7″-Nitrobenzo[*c*] [1,2,5]oxadiazol-4″-amino)undecyl]-3,5-dioxatricydo[5.2.1.0^{2,6}]decane-8-*endo*-9-*exo*-dicarboxylic acid (32).—A biphasic solution of ester **31** (259 mg, 0.440 mmol) in 2 M NaOH/THF (1:4, 3 mL) was stirred at ambient temperature for 16 h. The reaction mixture was extracted with CH_2Cl_2 (2 × 5 mL) and the aqueous phase was isolated and acidified to pH = 1 using 2 M HCl and then extracted with EtOAc (3 × 15 mL). The combined organic phase was washed with brine (15 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the title compound (193 mg, 78%) as an orange solid. m.p. 98–99 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.55 (1H, t, *J*= 5.3 Hz, NH), 8.50 (1H, d, *J*= 9.1 Hz, H6″), 6.40 (1H, d, *J*= 9.1 Hz, H5″), 4.61 (1H, t, *J* = 4.8 Hz, H4), 3.96 (1H, d, *J*= 5.8 Hz, H6), 3.88 (1H, d, *J*= 5.8 Hz, H2), 3.45–3.44, (2H, m, H11′), 3.00 (1H, app. t, *J*= 5.5 Hz, H8), 2.52 (1H, d, *J*= 4.6 Hz, H7), 2.45 (1H, br s, H1), 2.40 (1H, d, *J*= 5.5 Hz, H9), 1.70–1.63 (2H, m, H10′), 1.59 (1H, d, *J*= 9.6 Hz, H10*a*), 1.54–1.49 (2H, m, H1'), 1.37–1.19 (17H, m, H10s, 8 × CH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.6, 173.3, 145.2, 144.4, 144.2, 138.0, 120.5, 103.2, 99.1, 80.7, 78.2, 45.0, 44.4, 43.4, 43.2, 42.6, 32.3, 31.2, 28.9 (5 × C), 28.7, 27.6, 26.4, 23.7. HRMS (ESI, *m/z*) for $C_{27}H_{36}N_4O_9$ $[M – H]^–$ calc. 559.2410; found 559.2424.

4.1.22. 8-*endo*-9-*exo*-Di[2″-(2″,3″-bis-*tert*-butoxycarbonylguanidino)ethykarbamoyl]-4-[11′-(7″-mtrobenzo[*c*][1,2,5]oxadiazol-4″-amino)undecyl]-3,5-dioxatricydo[5.2.1.$0^{2,6}$]decane (33).—

A solution of diacid **32** (200 mg, 0.357 mmol), EDCI (222 mg, 1.16 mmol), HOBt (5 mg, 0.04 mmol), aminoethylguanidine **22** (339 mg, 1.12 mmol) and anhydrous DMF (5.1 mL) was stirred at ambient temperature for 3 d under an inert atmosphere. The reaction mixture was diluted with EtOAc (25 mL) and washed with H_2O (15 mL), brine (3 × 15 mL), dried ($MgSO_4$), filtered and concentrated *in vacuo.* The crude material was purified using flash column chromatography (50% EtOAc in CH_2Cl_2) and the title compound (263 mg, 65%) was isolated as an orange solid. R_f = 0.3 (50% EtOAc in CH_2Cl_2). m.p. 117–124 °C (slow decomposition). Φ_f = 0.55 (DMSO). ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.48 (1H, br s, NH), 11.44 (1H, br s, NH), 8.64 (1H, t, *J* = 6.1 Hz, NH), 8.51–8.49 (2H, m, H6″, NH), 8.03 (1H, t, *J* = 4.1 Hz, NH), 6.87 (1H, t, *J* = 5.2 Hz, NH), 6.32 (1H, t, *J* = 5.2 Hz, NH), 6.17 (1H, d, *J* = 8.7 Hz, H5″), 4.61 (1H, t, *J* = 4.7 Hz, H4), 4.04 (1H, d, *J* = 5.6 Hz, H6), 3.95 (1H, d, *J* = 5.6 Hz, H2), 3.60–3.35 (10H, m, 5 × $NHCH_2$), 2.93 (1H, app. t, *J* = 5.2 Hz, H8), 2.70 (1H, d, *J* = 4.1 Hz, H1), 2.58 (1H, br s, H7), 2.45 (1H, d, *J* = 5.2 Hz, H9), 1.83–1.76 (3H, m, H10′, H10*a*), 1.61–1.58 (2H, m, H1′), 1.53–1.43 (37H, m, H10*s*, 4 × *t*-Bu), 1.38–1.27 (16H, m, 8 × CH_2). ^{13}C NMR (125 MHz, $CDCl_3$) 5 174.2, 172.0, 163.5, 163.0, 157.9, 157.1, 153.2 (2 × C), 144.4, 144.04, 144.00, 136.6, 124.1, 104.1, 98.6, 83.7, 83.4, 81.7, 79.9, 79.7, 79.0, 47.9, 44.3, 44.2, 44.1, 43.0, 42.3, 40.2, 40.1, 40.0, 32.9, 32.5, 29.61, 29.56, 29.54, 29.51, 29.48, 29.3, 28.7, 28.41, 28.39, 28.21, 28.20, 27.1, 24.2. HRMS (ESI, *m/z*) for $C_{53}H_{84}N_{12}O_{15}$ $[M + H]^+$ calc. 1129.6252; found 1129.6265.

4.1.23. 4-[11′-(7″-Nitrobenzo[*c*][1,2,5]oxadiazol-4″-amino)undecyl]-3,5-dioxatricyclo[5.2.1.$0^{2,6}$]decane-8-*endo*-9-*exo*-dicarboxamidoethylguanidine hydrogen chloride (34).—

To a stirring solution of Boc-protected guanidine **33** (89 mg, 0.079 mmol) and MeOH (790 μL) was added dropwise AcCl (230 μL, 3.23 mmol), and the reaction was stirred for 2 d at ambient temperature. The reaction mixture was concentrated *in vacuo*, co-evaporated with MeOH (2 × 0.5 mL) and the title compound was isolated (60 mg, 95%) as an orange solid. m.p. 121–124 °C. Φ_f = 0.55 (DMSO) and 0.02 (H_2O). ^{1}H NMR (500 MHz, CD_3OD) δ 8.53 (1H, d, *J* = 8.8 Hz, H6″), 6.35 (1H, d, *J* = 8.8 Hz, H5″), 4.64 (1H, t, *J* = 4.7 Hz, H4), 4.04 (1H, d, *J* = 5.6 Hz, H6), 3.99 (1H, d, *J* = 5.6 Hz, H2), 3.55–3.52 (2H, m, H11′), 3.40–3.29, (8H, m, 4 × CH_2NH), 3.22 (1H, app. t, *J*= 5.0 Hz, H8), 2.62–2.61 (2H, m, H1, H7), 2.44 (1H, br s, H9), 1.80–1.75 (2H, m, H10′), 1.72 (1H, d, *J* = 10.4 Hz, H10*a*), 1.59–1.55 (2H, m, H1'), 1.48–1.28 (17H, m, H10*s*, 8 × CH_2). ^{13}C NMR (125 MHz, CD_3OD) δ 176.5, 174.6, 158.8 (2 × C), 146.7, 145.9, 145.6, 138.6, 122.9, 105.1, 99.5, 82.8, 79.9, 47.7, 46.9, 45.1, 44.9, 44.7, 42.0, 41.9, 39.7, 39.6, 33.9, 32.7, 30.61, 30.57, 30.51 (2 × C), 30.49, 30.3, 29.2, 28.0, 25.2. HRMS (ESI, *m/z*) for $C_{33}H_{52}N_{12}O_7$ $[M + 2H]^{2+}$ calc. 365.2114; found 365.2115.

4.2. Disk Diffusion Assay.

A stock solution of 10 mg/mL was made for each compound under observation using DMSO as a solvent. Each stock solution was then diluted 1:2 to bring the concentration to 5 mg/mL in DMSO. The diluted solutions were then filter-sterilised using a 0.2-μm nylon filter, and 10 μL of the 5 mg/mL stock was pipetted onto a blank disk (i.e. 50 μg/disk; Oxoid

Limited, Hampshire, UK). Suspensions of all bacterial isolates were adjusted to a 0.5 McFarland standard (in 0.9% NaCl) before they were swabbed onto nutrient agar plates. The controls used were a 10 μg Colistin disk (sulphate, Oxoid), 10 μL of DMSO and a plate swabbed with saline from the dispenser used.

4.3. Minimum Inhibitory Concentration (MIC) determination.

Bacteria were obtained either from American Type Culture Collection (ATCC; Manassas, VA, USA) or Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) as listed in Table S1. Bacteria were cultured in Nutrient broth (NB; Bacto Laboratories, catalogue No. 234000) or Mueller-Hinton broth (MHB; Bacto Laboratories, catalogue No. 211443) at 37 °C overnight with shaking (~180 RPM). A sample of each culture was diluted 50-fold in fresh MHB and incubated at 37 °C for 1.5–3 h with shaking (~180 RPM). Compound stock solutions were prepared as 10 mg/mL in DMSO and Colistin was dissolved in milli-Q water at 5.12 mg/mL. The compounds, at twice the final desired concentration, were serially diluted 2-fold across the wells of 96–well plates (Non-Binding Surface (NBS), Corning, catalogue No. 3641). Mid-log phase bacterial cultures (after 1.5–3 h incubation) were diluted to give a final cell density of 5×10^5 colony forming units (CFU)/mL when adding 50 μL to each well giving a final compound concentration range of 32 μg/mL to 0.015 μg/mL (DMSO ≤ 1%). MICs were determined visually after 18–20 h of incubation at 37 °C, with the MIC defined as the lowest compound concentration at which no bacterial growth was visible. Determined MIC values are the result of two independent experiments of n = 2, giving a final dataset of n = 4.

4.4. Cytotoxicity evaluation.

HEK293 (ATCC CRL-1573, human embryonic kidney) cells were seeded as 3000 cells per well in a 384-well plate in DMEM medium (GIBCO-Invitrogen #11995–073), in which 10% of FBS was added. Cells were incubated for 24 h at 37 °C, 5% CO_2 to allow cells to attach to the plates. A concentration series of compounds was then added into each well. The cells were incubated with the compounds for 24 h at 37 °C, 5% CO_2. After the incubation, 10 μM resazurin (dissolved in PBS) was added to each well. The plates were then incubated for 2 h at 37 °C, 5% CO_2. The fluorescence intensity was read using Polarstar Omega with excitation/emission 560/590. The data was analysed by Prism software. Results are presented as the average percentage of control ± SD for each set of duplicate wells.

4.5. Microscopy.

Escherichia coli (ATCC© 25922™, USA) and *Staphylococcus aureus* (*Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 43300™ MRSA), USA) were grown in 50 mL of Luria-Bertani (LB) broth at 37 °C with gentle shaking overnight. *S. aureus* and *E. coli* were incubated with for 20 min at 37 °C with 10 μg/mL of fluorescent compound (**29, 34**, **35** or **36**) in LB broth. For time dependent assessment, *E. coli* were incubated with 10 μg/mL of compound **34** for 5 min, 30 min, 1 h, 2 h, 4 h and 18 h. *S. aureus* were fixed in 4% paraformaldehyde for 20 min before being wet mounted onto slides for visualisation. Imaging was performed on a Nikon A1+ confocal microscope (Nikon, Japan) using a 60× object with excitation at 488 nm and emissions detected at 500–550 nm by a GaAsP PMT

detector. 3D reconstruction and rendering were performed using NIS elements software (Nikon, Japan).

4.6. Modelling.

GROMACS version 2016.1 molecular dynamics package[69] in conjunction with the GROMOS 54A7 force field[70] was used for all MD simulations. Water was represented explicitly using the simple point charge (SPC) model.[71] Parameters for norbornane molecules prepared in the study were determined by the Automated Topology Builder.[72] Each system was simulated under periodic boundary conditions in a rectangular simulation box with a timestep of 2 fs. The temperature of the system was maintained by coupling each component of the system to an external temperature bath at 310 K with a coupling constant of τ_T = 0.1 ps using a velocity rescaling thermostat. The pressure was maintained at 1 bar by weakly coupling the system to a semi-isotropic pressure bath using an isothermal compressibility of 4.5×10^{-5} bar^{-1} and a coupling constant of τ_P = 0.5 ps. During the simulations, the length of all bonds in all non-water molecules were constrained using the LINCS algorithm.[73] The SETTLE algorithm[74] was used to constrain the geometry of water molecules. Electrostatic interactions were calculated using particle mesh Ewald summation and nonbonded interactions were calculated with a cut-off of 1.0 nm. Both were updated each timestep. All images and videos were prepared in VMD.[74]

Aggregation simulations were conducted on bisamine (**8**), bisguanidine (**2**), benzylguanidine (**7**) and naphthalimide (**29**) species. Each simulation system consisted of 10 molecules of norbornane based compound, 20 chloride ions and 15,000 water molecules. Solute molecules were randomly positioned in a 9 × 9 × 9 nm box, to which water was then added. 1000 steps of energy minimisation, then 500 ps under an NPT ensemble was simulated for each species. Finally 300 ns, 400 ns, 200 ns, 200 ns long simulations was conducted for **8**, **2**, **7** and **29** respectively using an NVT ensemble until a single aggregate formed. To construct the aggregate for use in the bilayer simulations, the same procedure was followed on a system comprising of 200 molecules of **2**, 400 chloride ions and 30,000 water molecules in a 15 × 15 × 15 box. The NVT ensemble was simulated for 100 ns.

The starting coordinates of the model membrane used in the bilayer simulations were taken from Anandan *et al.*[75]. This represents the inner membrane of a Gram-negative bacteria. The outer membrane was not modelled as the structure of the peptidoglycan layer remains elusive. The model membrane is composed of 80% (408 molecules) of 1-palmitoyl-2-palmitoleyl-*sn*-glycero-3-phophoethanolamine (PPoPE) and 20% (104 molecules) of 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac)-glycerol (DMPG). The membrane in Anandan *et al.* contained both D and L-enantiomers of the DMPG head group; this was corrected in this study so that only the naturally occurring L-enantiomer was modelled. The model also contained 31,457 water molecules, 260 sodium ions and 156 chloride ions. After 5000 steps of energy minimisation, the system was equilibration using an NPT ensemble for 100 ns.

The largest aggregate of **2** (28 molecules), was placed with random orientation in the water surrounding the model membrane. The addition of more water molecules was necessary to accommodate the aggregate; *ca.* 11,500 additional water molecules were added, expanding the system size in the direction normal to the bilayer plane. Three replicates were then

energy minimised for 10000 steps each, then were simulated using an NPT ensemble. The first replicate was simulated for 780 ns; the second and third replicates were simulated for 500 ns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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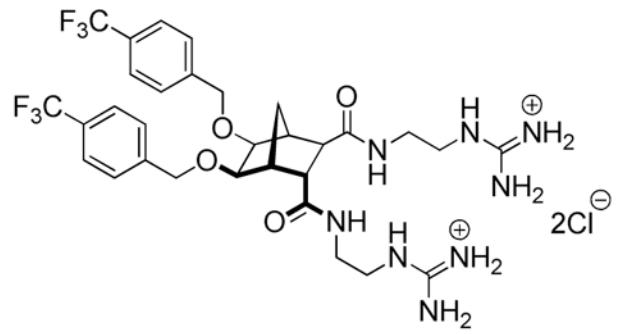
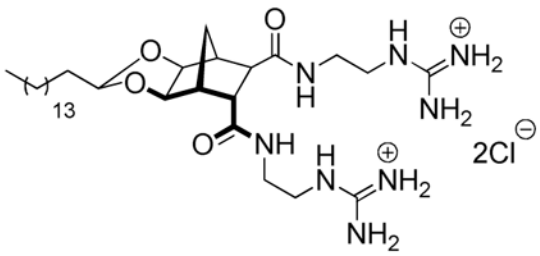
Highlights

- Antibacterial norbornane based amphiphiles with MIC as low as 0.25 μg/mL.
- Fluorescent microscopy of fluorophore-tagged analogues shows membrane localisation.
- Molecular modelling indicates rapid aggregation prior to membrane insertion.



1

MIC = 8 μg/mL (MRSA, *E.coli* & *P.aeruginosa*)



2

MIC = 1 μg/mL (MRSA)

Figure 1:
Previously reported norbornane-based antibacterial agents.[23, 24]

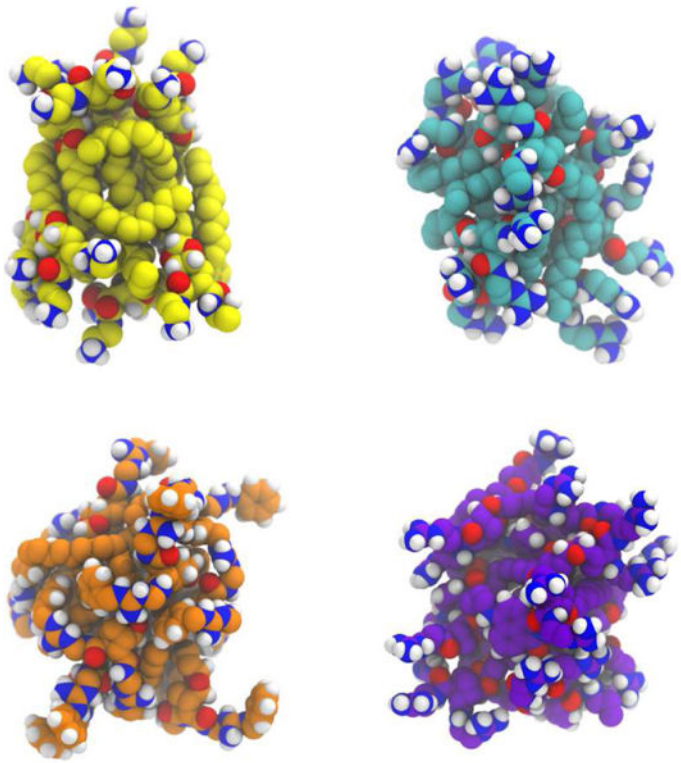


Figure 2:

A snapshot of the assembled ten molecule aggregates. Yellow, cyan, orange and purple represent the carbon atoms of the bisamine (**8**), bisguanidine (**2**), benzylguanidine (**7**) and naphthalimide (**29**) species respectively. Nitrogen is coloured blue, oxygen = red, hydrogen = white). Water molecules and Cl^- have been omitted for clarity.

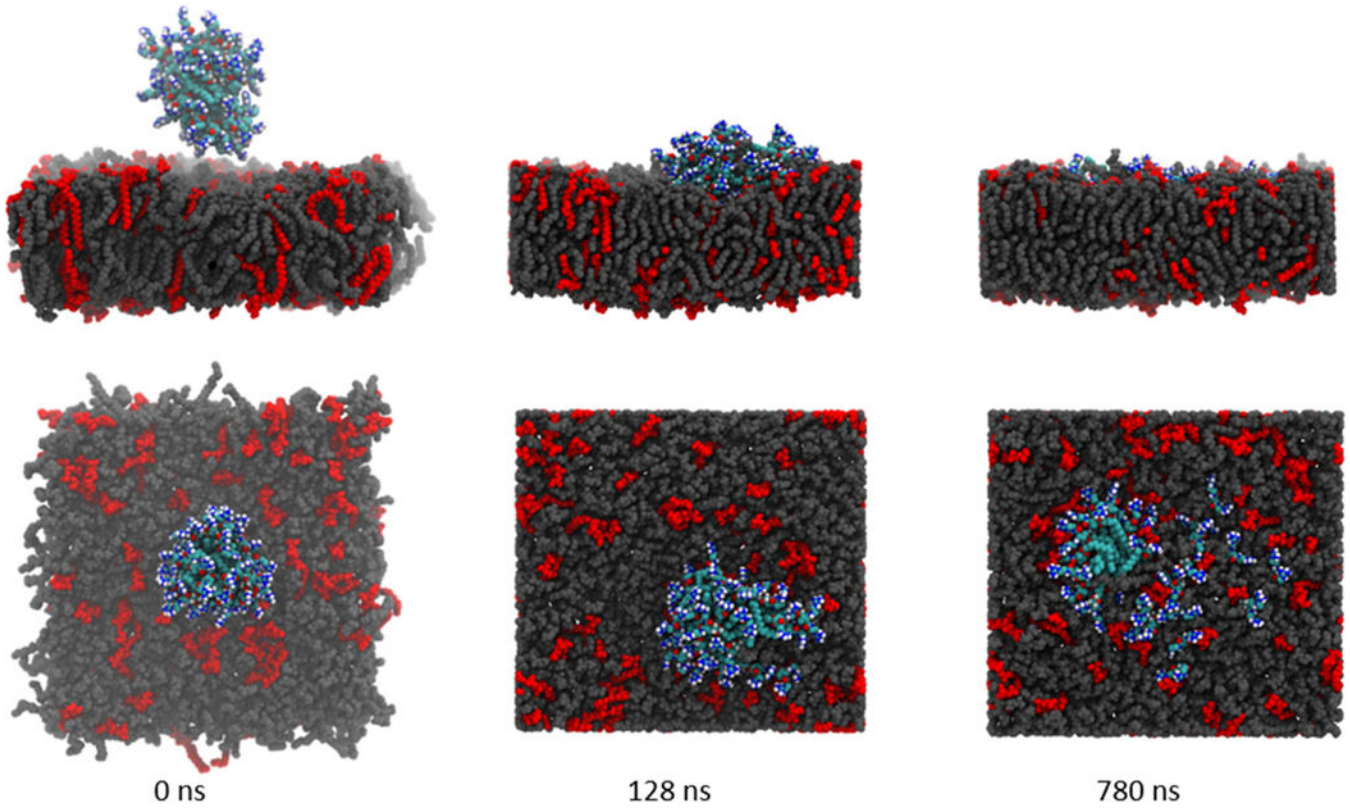


Figure 3:

Simulation snapshots highlighting the insertion/absorption of bisguanidine aggregate ($\mathbf{2_{28}}$) into a model membrane. LHS: Simulation begins with the aggregate deliberately positioned in the "extracellular environment" above the model membrane. Centre: membrane contact established and lipid absorption underway. RHS: simulation end with aggregate embedded in the membrane and some dispersion evident. Lipid molecules are coloured grey (PPoPE) and red (DMPG), **2** is coloured by element (carbon = cyan, nitrogen = blue, oxygen = red and hydrogen = white). Water molecules, Na^+ and Cl^- have been omitted for clarity. A video of the membrane insertion process is available in the electronic supplementary information.

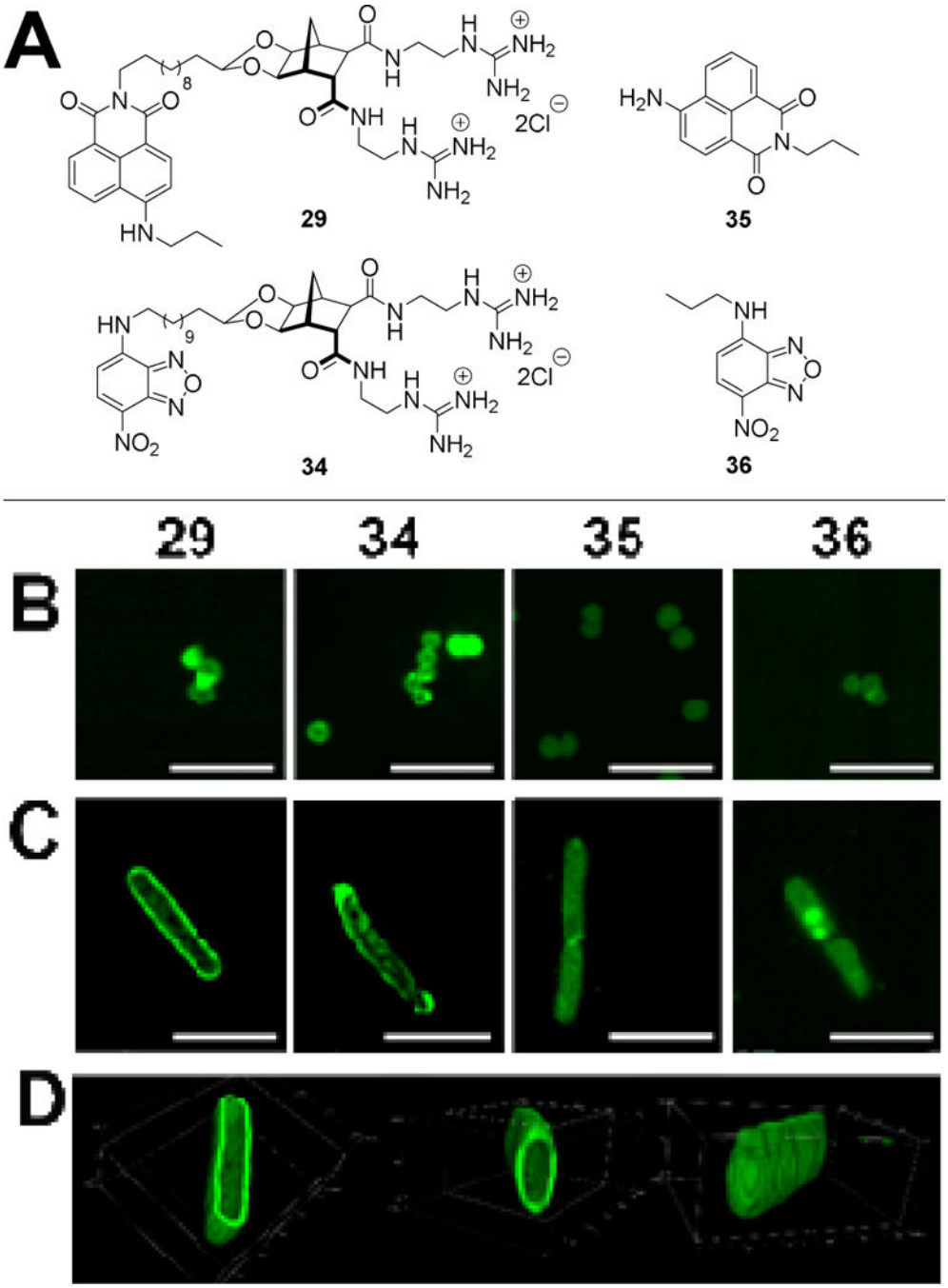
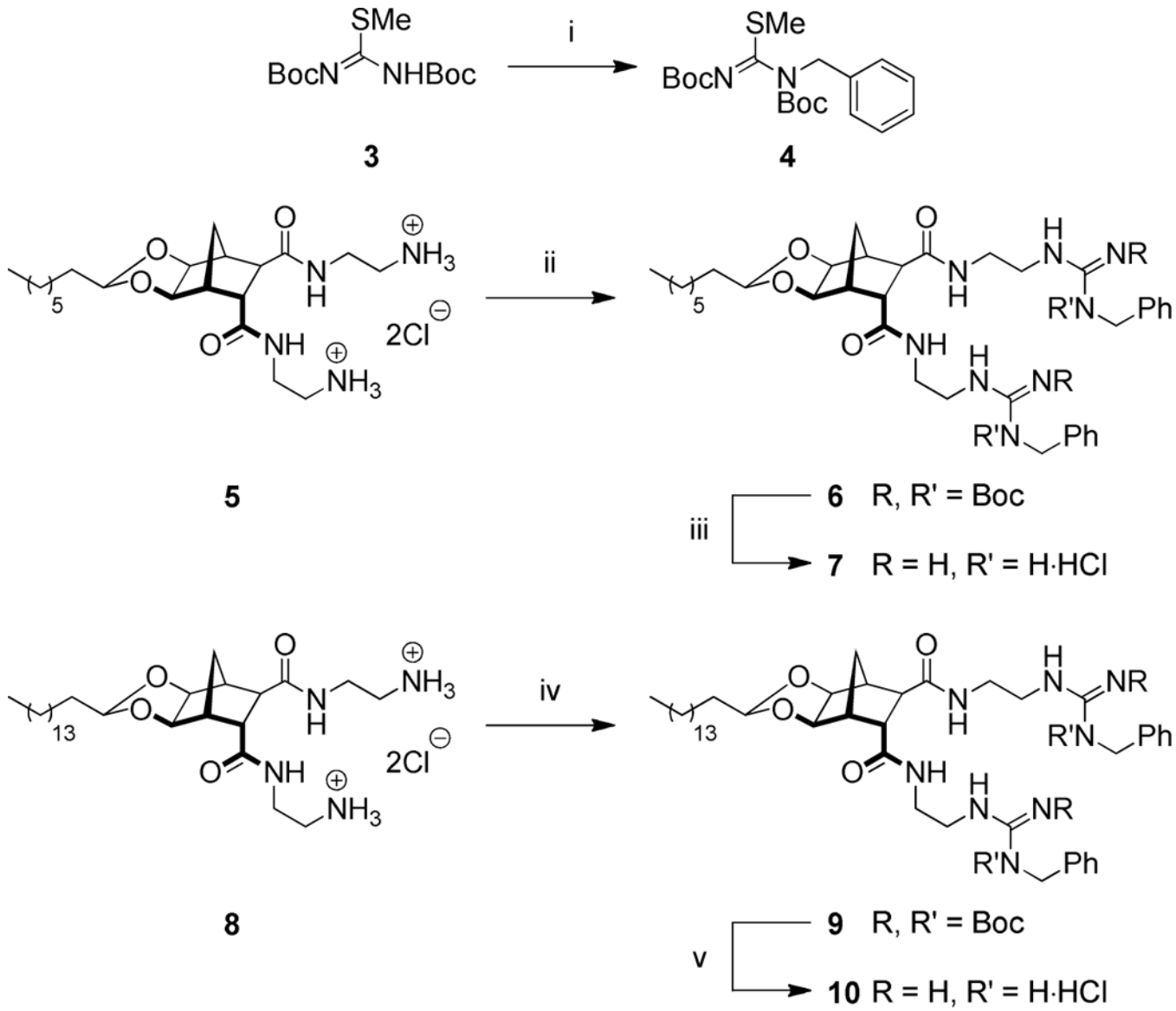


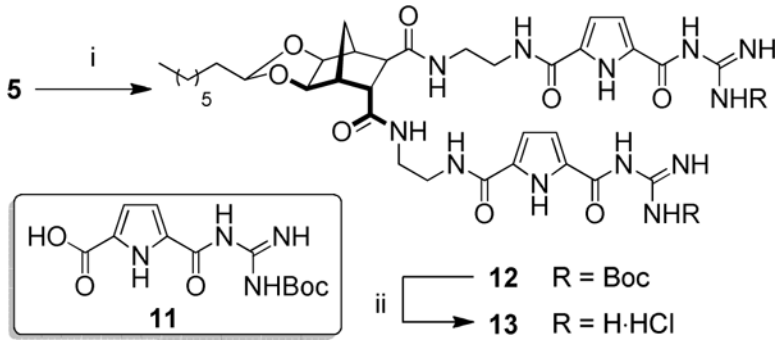
Figure 4:

(A) Structure of compounds used in microscopy studies: Norbornanes **29** and **34** plus controls **35** and **36**. (B–C) Localisation of compounds in bacteria. Confocal micrographs showing localisation of compounds in (B) *S. aureus MRSA* and (C) *E. coli.* Scale bar = 5 μm. (D) 3D rendering showing cell wall localisation of compound **29** in *E. coli.* Box dimensions x:y:z 5.4 μm:4.0 μm:2.3 μm.



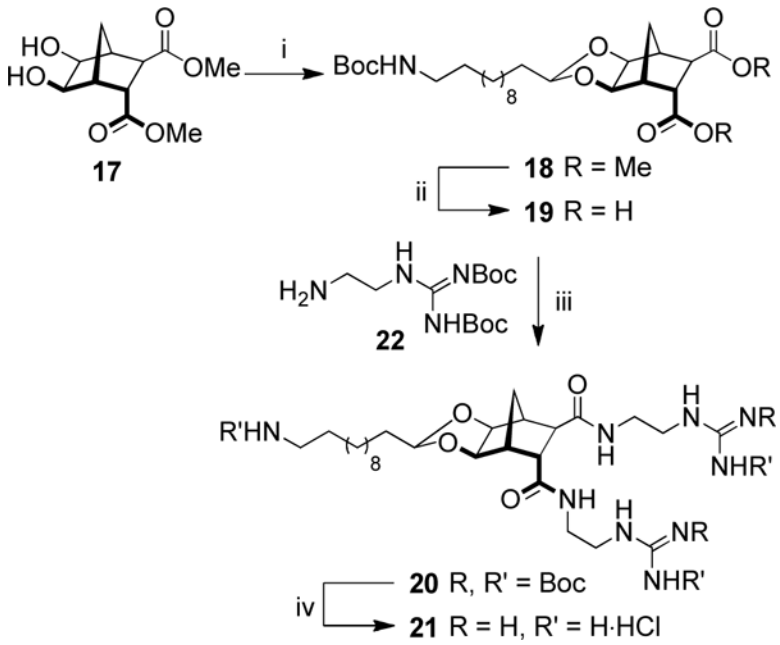
Scheme 1:

Reagents and conditions: (i) BnOH, DIAD, Ph_3P, THF, 66 °C, 16 h, 95%; (ii) **4**, Et_3N, CH_2Cl_2, 40 °C, 2 d, 30%; (iii) AcCl, MeOH, 21 °C, 24 h, 93%; (iv) **4**, Et_3N, CH_2Cl_2, 80 °C, 2 d, 14%; (v) AcCl, MeOH, 21 °C, 24 h, 74%;



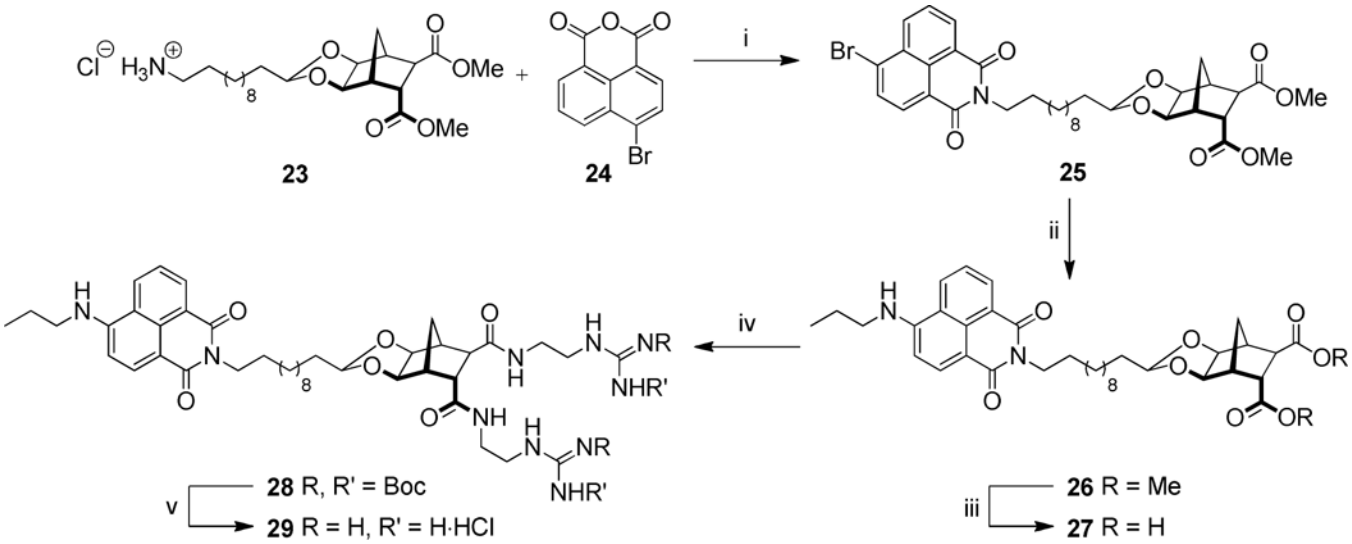
Scheme 2:

Reagents and conditions: (i) **11**, PyBOP, DMAP, DMF, 80 °C, 16 h, 42%; (ii) AcCl, MeOH, 21 °C, 24 h, 99%.



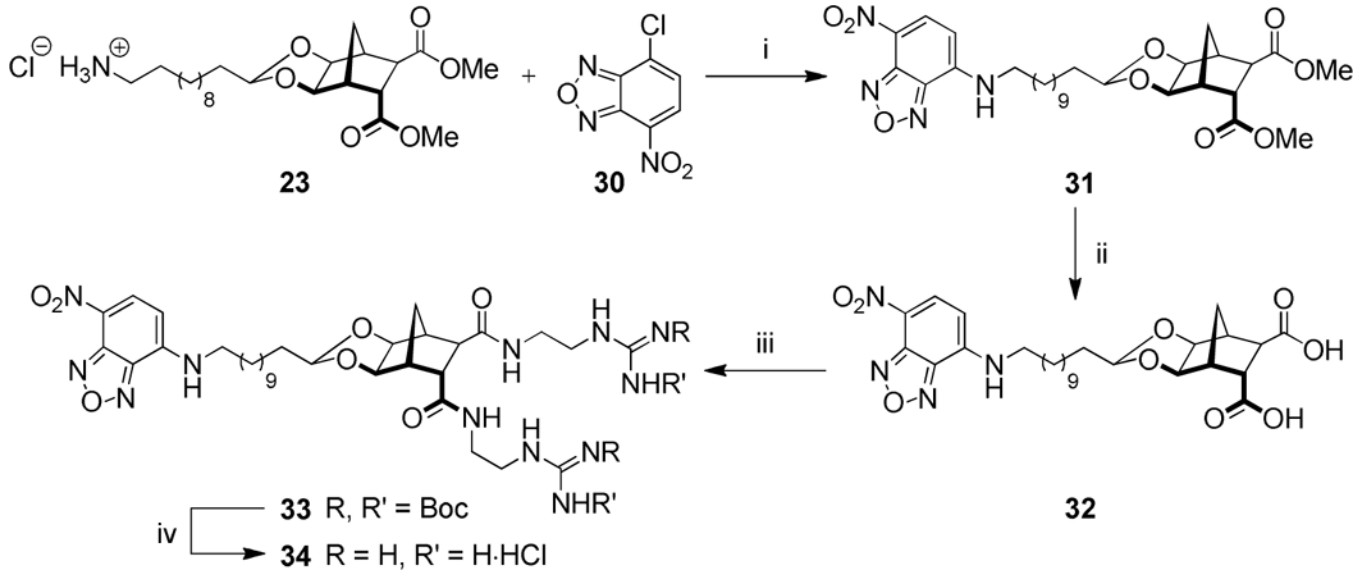
Scheme 3:

(i) **16**, $TsOH \cdot H_2O$, $MgSO_4$, PhMe, 110 °C, 3 h, 78%; (ii) NaOH, THF/H_2O, 21 °C, 16 h, 88%; (iii) **22**, EDCI, HOBt, $CHCl_3$, 50 °C, 30 min, 32%; (iv) AcCl, MeOH, 21 °C, 2 d, 99%.



Scheme 4:

Reagents and conditions: (i) Et_3N, EtOH, MW: 100 °C, 45 min, 99%; (ii) Propylamine, $Pd_2(dba)_3CHCl_3$, Xantphos, Cs_2CO_3, PhMe, 70 °C, 24 h, 88%; (iii) NaOH, THF/H_2O, 21 °C, 16 h, 88%; (iv) **22**, EDCI, HOBt, DMF, 21 °C, 2 d, 82%; (v) AcCl, MeOH, 21 °C, 2 d, 97%.

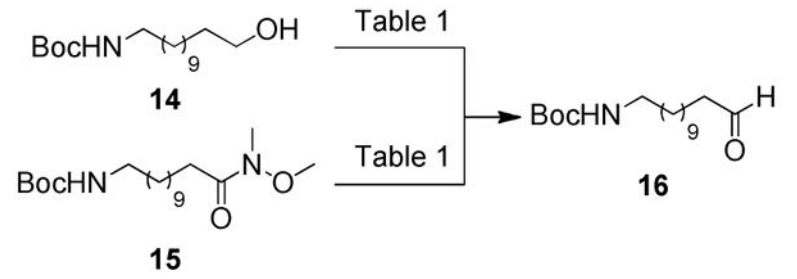


Scheme 5:

Reagents and conditions: (i) Et_3N, MeOH, 21 °C, 24 h, 68%; (ii) NaOH, THF/H_2O, 21 °C, 16 h, 78%; (iii) **22**, EDCI, HOBt, DMF, 21 °C, 3 d, 65%; (iv) AcCl, MeOH, 21 °C, 2 d, 95%.

Table 1:

Optimised synthesis of aldehyde **16**.



Entry	S.M.[a]	Redox agent (equiv.)	Temp. (°C)	Time (h)	Yield (%)
1[b]	**14**	$(COCl_2)_2$ (1.2)[c]	21	24	35
2	**14**	$(COCl_2)_2$ (1.2)[c]	–78	4	18
3	**14**	$(COCl_2)_2$ (2.4)[c]	–78	3	20
4	**14**	PCC (1.5)	21	3	6
5	**14**	PCC (3.0)	21	3	79
6	**14**	TEMPO (0.2)	21	48	58
7	**15**	$LiAlH_4$ (1.5)	–78	2	95

[a] S.M. = starting material

[b] Stirred at –78 °C for 3 h before warming to 21 °C and stirred for a further 21 h

[c] DMSO (5.0 equiv.) used

Table 2:

Photophysical properties

Compound	Solvent	λ_{abs} (nm)	λ_{em} (nm)	Stokes shift (nm)	Φ_f
28	DMSO	442	526	84	0.89
29	DMSO	437	528	91	0.88
29	H_2O	449	549	100	0.16
33	DMSO	475	538	63	0.55
34	DMSO	472	538	66	0.55
34	H_2O	475	550	75	0.02

Table 3:

MIC values (μg/mL)

Bacterial strain	7	10	13	21	29	34	*COL*[a]	*VAN*[b]
A. baumannii ATCC 19606	>32	>128	>128	>32	>32	**32**	0.06	NT[c]
P. aeruginosa ATCC 27853	32	>128	>128	>32	>32	**32**	0.25	NT
K. pneumoniae ATCC 700603	>32	>128	>128	>32	>32	>32	0.03	NT
E. coli ATCC 25922	**8**	>128	>128	>32	>32	**8**	0.06	NT
S. aureus ATCC 43300	**2**	**0.25**	>128	**16**	**1**	**1**	NT	1
S. aureus NARSA NRS17	**1**	**0.25**	>128	**16**	**4**	**1**	NT	4
S. aureus NARSA NRS1	**2**	**0.5**	>128	**8**	**4**	**4**	NT	4
S. aureus NARSA VRS 10	**1**	**0.5**	128	**8**	**2**	**0.5**	NT	>64
S. pneumoniae ATCC 7000677	**2**	**1**	>128	**8**	**1**	**1**	NT	1

[a] COL = Colistin sulphate

[b] VAN = Vancomycin

[c] NT = Not tested

Table 4:

Cytotoxicity (CC_{50}) against HEK293 cells (μg/mL)

	7	**10**	**13**	**21**	**29**	**34**	**TAM**[a]
CC_{50}	>50	7.8	>100	>50	>50	>50	13

[a]TAM = Tamoxifen