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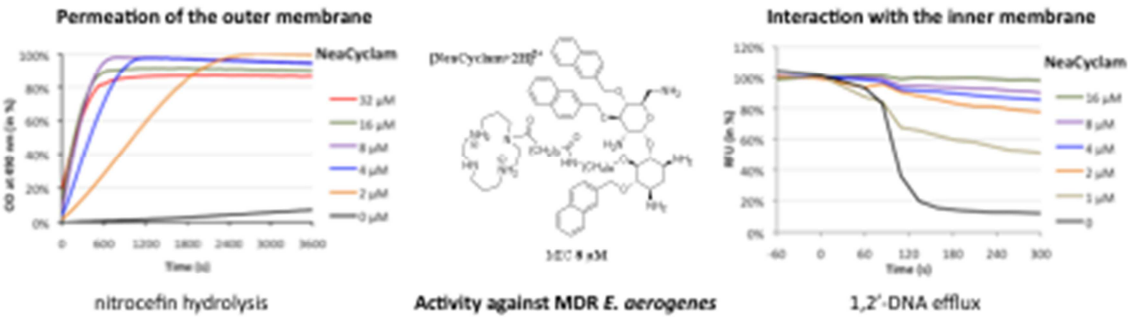

nitrocefin hydrolysis

Activity against MDR *E. aerogenes*

1,2'-DNA efflux

New Amphiphilic Neamine Conjugates Bearing a Metal Binding Motif Active Against MDR *E. aerogenes* Gram-negative Bacteria

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Author Contributions

J.M.P. and I.A. designed the research and wrote the manuscript. A.A. and R.A. synthezised and characterized the neamine conjugates. L.M., E.D. J.V. measured the antibiotic susceptibilities and performed nitrocefin hydrolysis as well as DNA efflux experiments. All authors have given approval to the final version of the manuscript.

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Abstract

Structure of bacterial envelope is one of the major factors contributing to Gram negative bacterial resistance. To develop new agents that target the bacterial membranes, we synthesized, by analogy with our previous peptide conjugates, new amphiphilic 3',4',6-trinaphthylmethylene neamines functionalized at position 5 through a short spacer by a chelating group, tris(2-pyridylmethyl)amine (TPA) and di-(picolyl)amine (DPA) and tetraazacyclotetradecane (Cyclam). ESI^+ mass spectrometry analyses showed that neither Zn(II)(NeaDPA) nor Cu(II)(NeaCyclam) were stable in the Mueller Hinton (MH) medium used for antibacterial assays. In contrast **Zn(NeaTPA)** was stable in the MH medium. Interestingly, in MH, the free ligand **NeaTPA** was found bound to zinc, the zinc salt being the most abundant salt in this medium. Thus, the antibacterial activities of all compounds were evaluated as free ligands against *E. coli* strains, wild type AG100 and *E. aerogenes* EA289 (a clinical MDR strain that overexpresses AcrAB-TolC efflux pump), as well as AG100A an AcrAB- *E. coli* strain and EA298 a TolC- derivative. **NeaCyclam** and **Zn(NeaTPA)** were by far the most efficient compounds active against resistant isolate EA289 with MICs in the range 16-4 and 4 µM, respectively, while usual antibiotics such as β-lactams and phenicols were inactive (MICs > 128) and ciprofloxacin was at 64 µM. **Zn(NeaTPA)** and **NeaCyclam** were shown to target and to permeabilize the outer membrane of EA289 by promoting the cleavage of nitrocefin by periplasmic β-lactamase. Moreover, all the neamine conjugates were able to block the efflux of 1,2'-dinaphthylamine in EA289, by acting on the efflux transporter located in the inner membrane. These membranotropic properties contribute to explain the activities of these neamine conjugates toward the MDR EA289 strain.

Keywords: Antibacterial activity, Gram-negative bacteria, Metal-binding groups, Membrane permeability, Multidrug resistant bacteria, Neamine derivatives.

1. Introduction

The widespread dissemination of Gram-negative bacterial pathogens resistant to available antibiotics requires the development of new classes of antibiotics with new modes of action [1,2]. The particular structure of Gram-negative cell wall is one of the major factors responsible for this resistance. Modifications of the outer membrane (OM) permeability reduce the uptake of both hydrophilic and hydrophobic compounds [3], while the efflux transporters, located in the two bacterial membranes, pump antibacterial agents out of the cell before they can reach their target [4]. As a result, the membrane barrier appears as a target of interest to circumvent drug resistance. The first drugs described to specifically target the membrane barrier were the naturally occurring antimicrobial peptides (AMPs) that are cationic and amphiphilic [5,6]. They interact with the negatively charged lipopolysaccharide (LPS) of the OM leading to membrane disruption and, eventually, they translocate across the cytoplasmic (inner) membrane to interact with cytoplasmic targets [7]. Recently it has been shown that grafting an ATCUN (Amino Terminal Cu and Ni) metal binding site on these AMPs improved their antibacterial efficiency [8-10]. This increase of activity was attributed to a dual action of these derivatives that, in addition to their usual mode of action, could promote the formation of radical oxygen species [9] and / or DNA cleavage [10]. In a related approach, we have developed tetra- and hexa-peptide analogues of AMPs with tris(2-pyridylmethyl)amine metal binding group that were more efficient than the parent peptides against *E. coli* and multidrug resistant (MDR) *E. aerogenes* bacterial strains [11]. They could also be used under a Co(III) or Zn(II) metalated form as drug carrier, but metalation was not absolutely required since the free system was readily linked to a metal cation in the culture medium used for bacterial growth [12]. However the efficiency of these compounds remained moderate and to improve it, we proposed to replace the moderatly active lipophilic cationic peptides by the more active lipophilic cationic neamines [13-19]. Indeed, recently, several groups have shown that converting the free hydroxyl groups of aminoglycosides into lipophilic ethers [13-18], or thioethers [19], while leaving the amines free, produced a new class of molecules active against a panel of Gram-negative bacteria. For instance, 3',4',6-trinaphthylmethylene neamine was active against susceptible and resistant Gram-negative bacteria. Décout's group has shown it was unable to bind to 16S rRNA but able to bind to LPS and to induce *P. aeruginosa* membrane depolarization, probably explaining its antibacterial activity [17].

Herein, and related to our previous work [11,12], we synthesized new neamine compounds, with amphiphilic character, coupled to several chelating groups, such as tris(2-pyridylmethyl)amine (TPA) and di-(picolyl)amine (DPA) and tetraazacyclotetradecane (Cyclam). Their antibacterial activities were evaluated against *Escherichia coli* wild type AG100 and *Enterobacter aerogenes* EA289 (a clinical MDR strain found in nosocomial infections that overexpresses AcrAB-TolC efflux pumps), as well as AG100A an AcrAB- *E. coli* derivative strain and EA298 a TolC- *E. aerogenes* derivative. Then, we evaluated their metal binding abilities in the Mueller Hinton (MH) medium used for bacterial growth and drug susceptibility determination. Then, to determine their membranotropic activity, the neamine conjugates were evaluated against EA289 for their ability to permeabilize the OM and to alter the efflux of 1,2'-dinaphthylamine (1,2'-DNA), a substrate of AcrB pump which is located in the inner membrane.

2. Results

2.1 Synthesis of neamine conjugates

3',4',6-trinaphthylmethylene neamine was previously prepared by Décout et *al.*[18]. Its membranotropic properties [17] make 3',4',6-trinaphthyl-methylene neamine a candidate of choice to introduce at position 5 of the neamine scaffold a chain and further a chelating group. Syntheses of all neamine conjugates are depicted in Scheme 1. Preparation of 3',4',6-trinaphthylmethylene neamine **1** was performed from neamine as previously described by Jackowski *et al.*[20]. The less reactive hydroxyl at position 5 of **1** (Scheme 1) was alkylated with 1,6-dibromohexane in DMF using NaH as base. Nucleophilic substitution of the bromide of **2** with sodium azide led to the azide **3** that was further reduced into the amine **4** with triphenyl phosphine. Compound **4** was the precursor to introduce the different chelating groups, TPA, DPA and Cyclam. They were grafted through a glutaryl spacer for Cyclam **5** (under its Boc protected form) and TPA **6**, and through a valeric one for DPA **7**. To assess the effect of the chelating group we also prepared the tri-benzylamine (TBA) derivative **8** equivalent to TPA but without any complexing ability. Syntheses of compounds **6**, **7** and **8** were described in our previous work [12]. Synthesis of the Cyclam derivative **5** was achieved in two steps (Scheme S1) from tri-*tert*-butyl 1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate as previously described by Fabbrizzi *et al*. [21]. After protecting three of the amine functions as *tert*-butyl carbamate, reaction of the last free amine with methyl 5-chloro-5-oxopentanoate followed by saponification with aqueous LiOH afforded the expected compound **5**. Coupling of the neamine derivative **4** with the acids **5**, **6**, **7** and **8** was achieved in DMF in the presence of DIEA as base and, HOAT and HATU as coupling agents. Then deprotection with TFA in CH_2Cl_2 afforded the various neamine conjugates bearing a Cyclam (**NeaCyclam**), a TPA (**NeaTPA**) and a DPA (**NeaDPA**) binding motif and the non-chelating TBA moiety (**NeaTBA**). Deprotected azide (**NeaN_3**) and deprotected amine (**NeaNH_2**) were also prepared for control biological assays.

Insert Scheme 1

2.2 Binding of a metal cation in the culture medium

Metallodrugs although promising are always assumed to be toxic and this leads to suspiciousness of pharmaceutical companies while highly efficient metallodrugs are already applied to therapy or diagnosis of diseases [22-24]. Using strong chelating metal-binding groups, our neamine conjugates could readily bind a metal cation in the rich MH medium broth used for bacterial growth in the antibacterial assays. TPA [25] and DPA [25] have a strong affinity for metal cations but decreasing in the order Cu(II) > Zn(II) > Fe(II). Cyclam with a *N*-substituted carboxamide binds efficiently copper [26]. While iron and zinc salts are present in high amounts in the MH medium, the content in copper salt is low [27,28]. In this regard, the best metal complexes candidates for this study are $[Zn(NeaTPA)]^{2+}$, $[Zn(NeaDPA)]^{2+}$ and $[Cu(NeaCyclam]^{2+}$ whose structures are shown in Scheme S2. So, we prepared zinc and copper complexes of our neamine conjugates by mixing the neutral free ligand with $Zn(ClO_4)_2$ in MeOH or $Cu(CF_3SO_3)_2$ in DMF. The three complexes, as well as the free ligands, were analyzed by

ESI^+ HRMS and their mass spectra effectively displayed the expected molecular peak. Then, we studied their stability upon dilution in MH at a concentration close to that used for the evaluation of their antibacterial activities. Concomitantly, we studied, under the same conditions, the direct binding of zinc or copper to the neamine conjugates in the MH broth. The mass spectra shown in Figure 1 and in Figures S1 and S2 as well as the analysis of the data listed in Table S1 revealed that $[Zn(NeaDPA)]^{2+}$ and $[Cu(NeaCyclam)]^{2+}$ dissociated upon incubation in MH. The only stable complex was $[Zn(NeaTPA)]^{2+}$ and, interestingly, the free **NeaTPA** ligand readily binds zinc upon incubation in the MH broth (Figure 1). As a result, we only tested the free ligands, bearing in mind that the species tested with **TPA** was **Zn(NeaTPA)**.

Insert Figure 1

2.3 Antibacterial activities of neamine conjugates against *E. coli* and *E. aerogenes* strains

Minimal inhibitory concentrations (MICs) of neamine conjugates were determined against isogenic strains: two *Escherichia coli*, the wild type AG100 and its derivative AG100A devoid of AcrAB efflux pump, and two *Enterobacter aerogenes*, EA289 a clinical MDR strain overexpressing the AcrAB-TolC efflux pumps and its *tol*C-derivative EA298 (Table 1). Neamine was not significantly active on strains that expressed efflux pumps with MIC in the range 32-16 µM and was inactive on strains devoid of efflux pump (MIC > 128 µM). The **$NeaN_3$** derivative used as control in our series exhibited activities similar to those of neamine on active efflux strains but was more efficient on efflux- AG100A and EA298. Among the neamine conjugates, **NeaTBA** with no metal binding ability was the less potent derivative. The other neamine conjugates showed similar activities against the parental strain and its efflux– derivative indicating that there were not well-recognized substrates of AcrAB pump. Their activity increased in the order: DPA < NH_2 < Cyclam ≈ TPA. It is worth noticing that MIC values obtained toward the resistant clinical strain (EA289) with **NeaTPA** and **NeaCyclam** were lower than those obtained with the usual antibiotics belonging to β-lactams, quinolones, and phenicol families (Table 1). Addition of sub-inhibitory concentration of polymyxin B nonapeptide (PMBN), a molecule known to permeabilize the membrane [29], did not significantly improve **NeaTBA** and **NeaDPA** activities (1 or 2 dilutions). All these data support a possible permeabilizing effect of the neamine conjugates and indicate that they are able to bypass the resistance mechanisms acting in this MDR strain.

Insert Table 1

2.4 Effect of neamine conjugates on the hydrolysis rate of nitrocefin.

The effect of the neamine conjugates on the OM permeability was analyzed using nitrocefin, a well-known substrate of the β-lactamase periplasmic enzyme [30] that has been previously used to assay the OM permeability during incubation with various compounds [31,32]. EA289 was incubated with different concentrations of neamine conjugates and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at 3.3 µM to prevent the efflux of molecules. After addition of nitrocefin, the OD at 490 nm was recorded for 1 h. An increase in the optical density (OD) indicated nitrocefin hydrolysis associated with its translocation across the OM. In the absence of neamine

conjugates, no significant variation of the absorbance was observed showing that nitrocefin did not pass the OM under these conditions. In contrast, addition of the neamine conjugates promoted a concentration-dependent increase of the rate of nitrocefin hydrolysis (Figure 2 and Figure S3). Among all neamine conjugates, **NeaCyclam** was the most efficient. While 90 % of nitrocefin was hydrolyzed after 50 min with 32 µM of **NeaTPA**, the reaction was complete with only 2 µM of **NeaCyclam** (Figure 2). Addition of **NeaDPA** or **NeaTBA** at 64 µM (Figure 2) promoted only 60% and 20% of nitrocefin hydrolysis, respectively. In the same assays, **$NeaNH_2$** (Figure S3) exhibited an intermediate profile between **NeaTPA** and **NeaCyclam.** Under the same conditions, we also assayed polymyxin B (PMB) (Figure 2), a well-known antibiotic that efficiently permeabilizes the bacterial OM [33]. A rapid and strong permeabilizing effect was observed with PMB at only 3 µM within 20 min, when only a weak action was noted with polymyxin B nonapeptide (PMBN) (Figure S3) known to exhibit a lower membranotropic action [34]. **NeaCyclam** with an activity profile quite close to that of PMB presented the higher permeabilizing effect. Finally, the activity range toward the bacterial OM was the following: **PMB** > **NeaCyclam** > **$NeaNH_2$** > **NeaTPA** >> **NeaDPA** > **NeaTBA** ≈ **PMBN**.

Insert Figure 2

2.5 Effect of neamine conjugates on the efflux of 1,2' DNA.

We investigated the effect of the neamine conjugates on the efflux pump activity using the fluorophore 1,2' DiNaphtyl-Amine (1,2'-DNA), a substrate of AcrAB-TolC system, which is non-fluorescent in aqueous solution but strongly fluorescent upon partitioning into the phospholipid bilayer [35]. EA289 overexpressing acrAB efflux pump, was pre-incubated in the presence of 1,2'-DNA and CCCP (CCCP collapses the proton motive force that energizes the efflux pump [4,36]). After centrifugation and resuspension in phosphate buffer, the release of 1,2'-DNA was assayed in the absence or in the presence of different concentrations of neamine conjugates. Since AcrAB pump, as other RND pumps, utilizes the Proton Motive Force (PMF) as an energy source driving the drug transport [37], we used both, as controls, phenylalanine arginine ß-naphthylamide (PAßN), an AcrAB inhibitor, and CCCP, an energy uncoupler [38]. Glucose was added to provide energy to efflux pumps [32] and start the expel (t = 0, Figure 3): the fluorescence intensity decrease was related to 1,2'-DNA efflux outside the bacterial membrane. The addition of neamine conjugates **NeaTPA, NeaDPA and NeaCyclam** was able to totally impair 1,2'-DNA efflux at around 8 and 16 µM (Figure 3) compared to the limited action of **NeaTBA** at 32 µM (Figure S4). PAßN was inefficient (Figure S4), but under the same conditions, CCCP blocked the efflux in the same range of concentrations (Figure 3) as neamine conjugates demonstrating that 1,2'-DNA efflux was dependent on the energy source. In addition, the respective profiles of the curves obtained with **NeaDPA** and **NeaCyclam** derivatives were only a little bit different from CCCP that supported an effect on the efflux transporter activity located in the inner membrane.

Insert Figure 3

3. Discussion

From the above-mentioned results, two of the neamine conjugates tested for their antibacterial activities, **NeaTPA** and **NeaCyclam** are highly effective against *E. coli* strain and resistant *E. aerogenes* strains. **NeaDPA** is less efficient than **NeaNH$_2$** while **NeaTBA** remains inactive. The antibacterial activities of the neamine conjugates follow quite closely their effect on the OM. Hence, they clearly affect the OM membrane stability by altering the permeability barrier as demonstrated by the increase of nitrocefin hydrolysis following the addition of **NeaTPA** or **NeaCyclam**, in contrast to the absence of any effect of **NeaTBA**. However, the difference in metal binding affinity of the neamine conjugates cannot by itself explain the variations of antibacterial activity, since the only stable complex in the biological conditions is $[Zn(NeaH_nTPA)]^{(n+2)+}$ (n being the number of positive charges arising from the neamine core, $1 \leq n \leq 4$). In contrast, Cyclam with a nitrogen substituted carboxamide remains highly efficient while binding relatively poorly Cu(II), since the copper complex is unstable in the MH broth. Thus, the effect on the OM seems to be modulated by the number of positive charge exhibited by the compounds. So, we calculated pKa of all the amines in each derivative using Discovery Studio software (Table S2). While pKa values of the aliphatic amine(s) in the metal binding groups are all around 6.6, those of the neamine core are clearly modulated by the nature of the nitrogen ligand. At pH 7.0, the tertiary amine of TBA and DPA in **NeaTBA** and **NeaDPA**, respectively, is mainly deprotonated, while the cyclam ring in **NeaCyclam** is partly protonated and can have an average charge of +1. Moreover, three of the neamine amines are protonated in **NeaCyclam** against two in **NeaTPA** and only one in **NeaDPA** and **NeaNH$_2$**, the others being partly protonated. Interaction with the OM is also modulated by the amphipatic nature of the neamine conjugates. The naphthyl residues of the neamine core, and the positive charge associated with the protonation of the NH_2 groups, facilitate the interaction with the lipids and the negatively charged phospholipid headgroups, respectively. The neamine conjugates bring additional positive charges. In that regard, incorporation of zinc in **NeaTPA** brings two additional positive charges (global charge +4), explaining its interaction with the OM as previously reported for other DPA [39,40] or phthalocyanine [41,42] zinc complexes. **NeaCyclam** is efficient as free ligand, since it exhibits 4 positive charges due to its protonation state, whereas **NeaDPA** brings only one charge. **NeaTBA** is the less efficient derivative, not only because of its protonation state, but also because the addition of three benzyl groups drastically impairs the hydrophobicity / hydrophilicity balance (calculated LogD (LogP at pH 7.0): **NeaTBA** 7.664, **NeaDPA** 4.286, **NeaTPA** 3.836, **NeaNH$_2$** 1.941, **NeaCyclam** -0.657).

All the neamine conjugates have a similar activity on the efflux and this property could be related to their overall protonation state in the more acidic periplasmic compartment (pH ≈ 6). The reserve of periplasmic protons is required for the antibiotic-H^+ antiport by the AcrB pump [4,43]. Overall protonation of the neamine conjugates could induce a decrease of free periplasmic protons under the threshold necessary for H^+ antiport flux that is required for an efficient AcrB activity, as previously reported for ethidium bromide extrusion [35].

4. Conclusion

To conclude, in this paper, we reported the synthesis and complete characterization of new amphiphilic neamine conjugates, the OH at position 5 of which was functionalized by tris(2-pyridylmethyl)amine (TPA) or di-

(picolyl)amine (DPA) or tetraazacyclotetradecane (Cyclam) via a short spacer. **NeaTPA** and **NeaCyclam** are the most potent antibacterial agents being active both against *E. coli* strains and a MDR *E. aerogenes* strain EA289, that has lost its susceptibility toward usual antibiotics. Clearly, the neamine core contributes to improve the activities relative to similar derivatives that we reported with tetra- or hexa-peptide analogues of AMPs [11,12]. In this series, the increase in activity relative to the precursor **NeaNH$_2$** is associated with an increase in the positive charges, due either to a high protonation state of the neamine core in **NeaCyclam**, or to a direct metallation of TPA in **NeaTPA** by Zn(II) cations available in the culture medium. The OM interaction and membranotropic action, evidenced by the increase of nitrocefin hydrolysis by periplasmic β-lactamase, could contribute to a significant antibacterial activity on MDR *E. aerogenes* EA289. Moreover all compounds are able to target the inner membrane by blocking the efflux of 1,2'-DNA.

5. Experimental

5.1 Chemical syntheses

All solvents and chemicals were purchased from SDS and Aldrich, respectively. DMF (CaH_2), MeOH (Mg turnings and iodine) and CH_3CN (CaH_2) were dried upon distillation over standard reagents indicated in brackets. ^{1}H NMR spectra were recorded on a Bruker ARX-250 spectrometer or on a Bruker Avance-500 spectrometer (Bruker, Wissembourg, France) and chemical shifts were reported in ppm downfield from TMS. Electrospray ionization (ESI) and HRMS mass spectrometry analyses were obtained using a Thermo Finnigan LCD Advantage spectrometer (Thermo Electron SAS, Courtaboeuf, France). Elemental analyses were carried out by microanalysis service at Gif-sur-Yvette CNRS.

Neamine was synthesized as previously described [44] with some modifications. Briefly, a concentrated HCl solution (25 mL, 12.1 N) was added to a MeOH solution (230 mL) of neomycin B (10.0 g, 10.4 mmol). The reaction was refluxed for 4 h before all reactants were completely consumed. After cooling to r.t., a light-yellow solution containing a white precipitate was observed. This later was filtered and washed with MeOH to give a first fraction of neamine. A second fraction was isolated by successive cycles of dissolutions of the concentrated light-yellow solution in water (3 mL) and precipitations in methanol (50 mL). This allowed obtaining the crude neamine product in 80% yield. ^{1}H NMR (250 MHz, D_2O) δ ppm 5.96 (d, J = 3.9 Hz, 1H), 4.10 – 3.96 (m, 3H), 3.73 (t, J = 8.9 Hz, 1H), 3.67 – 3.57 (m, 2H), 3.57 – 3.44 (m, 3H), 3.44 – 3.25 (m, 2H), 2.54 (dt, J = 12.5 & 4.4 Hz, 1H). 1.95 (q, J = 12.5 Hz, 1H). MS (ESI$^+$, *m/z*, H_2O): 322.9 (100%, $[M+H]^+$).

Compound 1 was prepared as previously described [20,45]. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.88 – 7.66 (m, 8H), 7.65 – 7.41 (m, 22H), 7.40 – 7.00 (m, 37H), 6.99 – 6.82 (m, 14H), 5.26 (s, 1H), 4.92 (d, J = 11.4 Hz, 1H), 4.88 (d, J = 11.0 Hz, 1H), 4.81 (d, J = 11.1 Hz, 1H), 4.78 (d, J = 11.1 Hz, 1H), 4.56 (d, J = 11.4 Hz, 1H), 4.45 (d, J = 11.0 Hz, 1H), 4.34 (br, 1H), 4.14 (m, 1H) 4.02 (br, 1H), 3.61(t, J = 9.5 Hz, 2H), 3.14 (t, J = 9.5 Hz, 1H), 3.02 (t, J = 9.5 Hz, 1H), 2.90 (m, 2H), 2.76 (br, 1H), 2.32 (m, 2H), 1.99 – 1.84 (m, 3H), 1.39 – 1.25 (m, 2H), 0.90 (m, 1H). MS (ESI$^+$, *m/z*, MeOH): 1712.3 (100%, $[M+H]^+$). El. anal. calcd. for $C_{121}H_{106}N_4O_6$: C, 84.88; H, 6.24; N, 3.27; found: C, 84.74; H, 6.12; N, 2.88.

Compound 2. To a DMF solution (20 mL) of compound **1** (1.95 g, 1.14 mmol) were successively added NaH (460 mg, 11.5 mmol) and 1,6-dibromohexane (1.86 mL, 11.3 mmol), and the mixture was stirred at 60 °C for 20 h. After evaporation of DMF and dissolution of the residue in CH_2Cl_2 (200 mL) the solution was washed twice (2 x 150 mL) with saturated aqueous NH_4Cl, dried over $MgSO_4$ and evaporated. The residue was purified over basic alumina gel using pentane/CH_2Cl_2 (70:30, *v*:*v*) as eluent to give compound **2** in 66% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.85 – 6.82 (m, 81H), 5.28 (s, 1H), 4.91 – 4.42 (m, 4H), 4.41 – 4.21 (m, 3H), 3.56 (m, 1H), 3.40 (m, 1H), 3.24 (m, 1H), 3.07 (m, 2H), 2.84 – 2.41 (m, 5H), 2.09 – 1.79 (m, 2H), 1.57 – 1.42 (m, 6H), 1.32 – 1.16 (m, 4H), 1.11 – 0.97 (m, 2H), 0.93 – 0.78 (m, 4H). MS (ESI$^+$, *m/z*, MeOH): 1875.2 (100%, $[M+H]^+$). El. anal. calcd. for $C_{127}H_{117}BrN_4O_6.1.5H_2O$: C, 80.19; H, 6.36; N, 2.95; found: C, 80.04; H, 6.50; N, 3.23.

Compound 3. Sodium azide (275 mg, 4.24 mmol) was added to a DMF solution (10 mL) of compound **2** (1.0 g, 0.53 mmol), and the mixture was stirred at r.t. for 20 h. After evaporating DMF, CH_2Cl_2 (300 mL) was added to the residue and the solution was washed twice (2 x 200 mL) with saturated aqueous NH_4Cl, dried over $MgSO_4$ and evaporated. The residue was purified over basic alumina gel using pentane/CH_2Cl_2 (70:30, *v*:*v*) as eluent to give compound **3** in 81% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.25 – 6.98 (m, 24H), 7.63 – 6.26 (m, 41H), 7.96 – 7.65 (m, 16H), 5.27 (s, 1H), 4.80 – 4.40 (m, 7H), 3.72 (m, 1H), 3.59 (m, 1H), 3.45 – 3.30 (m, 2H), 3.20 (m, 1H), 3.02 (m, 2H), 2.98 – 2.80 (m, 4H), 2.78 – 2.42 (m, 4H), 2.12 (m, 2H), 1.75 – 1.52 (m, 3H), 1.47 – 1.29 (m, 4H), 1.17 – 0.94 (m, 4H). MS (ESI$^+$, *m/z*, MeOH): 1837.3 (100%, $[M+H]^+$). El. anal. calcd. for $C_{127}H_{117}N_7O_6.0.6H_2O$: C, 82.53; H, 6.45; N, 5.31; found: C, 82.91; H, 6.46; N, 4.87.

Compound 4. Triphenyl phosphine (713 mg, 2.72 mmol) was added to a solution of compound **3** (1.0 g, 0.54 mmol) in a mixture of THF/water (95:5 *v*:*v*) (30 mL). Then the mixture was refluxed for 5 h. After evaporating the solvents, the residue was purified over basic alumina gel using CH_2Cl_2/ MeOH (98:2 *v*:*v*) as eluent to afford compound **4** in 71% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.76 – 7.55 (m, 12H), 7.54 – 7.25 (m, 32H), 7.24 – 7.07 (m, 15H), 7.05 – 6.84 (m, 22H), 4.85 – 4.33 (m, 5H), 4.31 – 4.17 (m, 3H), 3.49 (m, 1H), 3.33 (m, 1H), 3.17 (m, 1H), 2.98 (m, 1H), 2.80 – 2.54 (m, 4H), 2.53 – 2.42 (m, 2H), 2.41 – 2.19 (m, 3H), 2.01 – 1.72 (m, 2H), 1.63 – 1.35 (m, 5H), 1.28 – 1.13 (m, 3H), 1.11 – 0.98 (m, 3H), 0.96 – 0.70 (m, 4H) (in $CDCl_3$, 119H attributed including 4 NHTrt and 1 NH_2). HRMS (ESI$^+$, *m/z*, CH_2Cl_2): calcd for $C_{127}H_{120}N_5O_6$ 1811.9267 ($[M+H]^+$), found 1811.9282. El. anal. calcd. for $C_{127}H_{119}N_5O_6.2.6H_2O$: C, 82.09; H, 6.74; N, 3.77; found: C, 82.43; H, 6.69; N, 3.42.

General procedure for amine deprotection. To a CH_2Cl_2 solution (5 mL) kept at 0 °C of the amino protected compound (0.109 mmol) and anisole (250 µL) was added dropwise TFA (5 mL). After stirring the mixture at r.t. for 2 h, the solvents were evaporated and the product was isolated after precipitation into diethyl ether followed by centrifugation.

General procedure for coupling. To a DMF solution (2 mL) of compound **4** (0.055 mmol, 100 mg), compound **5**, **6, 7** or **8** (0.055 mmol), HOAT (0.0825 mmol, 11 mg) and HATU (0.0825 mmol, 31 mg) was added DIPEA (0.22 mmol, 38 µL) at 0 °C. After stirring for 3 h at r.t., addition of water to the DMF solution led to precipitation of the product that was washed with MeOH and used in the deprotection step without further purification.

NeaN$_3$. 81% yield (white powder) ^{1}H NMR (500 MHz, MeOD-d_4) δ ppm 7.88 – 7.77 (m, 9H), 7.76 – 7.69 (m, 3H), 7.52 – 7.39 (m, 9H), 5.52 (d, *J* = 3.3 Hz, 1H), 5.02 (s, 2H), 4.98 (d, *J* = 11.8 Hz, 1H), 4.94 (d, *J* = 11.5 Hz,

1H), 4.92 (d, *J* = 11.8 Hz, 1H), 4.86 (d, *J* = 11.5 Hz, 1H), 4.24 (ddd, *J* = 9.0, 7.8 & 3.0 Hz, 1H), 3.95 (d, *J* = 7.8 Hz, 1H), 3.72 (t, *J* = 8.9 Hz, 1H), 3.71 (m, 1H), 3.57 (t, *J* = 8.9 Hz, 1H), 3.54 (t, *J* = 7.8 Hz, 1H), 3.49 (t, *J* = 8.9 Hz, 1H), 3.27 (dd, *J* = 13.3 & 3.0 Hz, 1H), 3.19 (m, 1H), 3.18 (m, 1H), 3.17 (dd, *J* = 13.3 & 9.0 Hz, 1H), 3.11 (ddd, *J* = 12.4, 8.9 & 4.3 Hz, 1H), 3.06 (dd, *J* = 7.8 & 3.3 Hz, 1H), 2.86 (t, *J* = 6.7 Hz, 2H), 2.23 (dt, 12.4 & 4.3 Hz, 1H), 1.61 (q, 12.4 Hz, 1H), 1.47 – 1.32 (m, 2H), 1.18 (m, 2H), 1.03 (m, 1H), 0.92 – 0.95 (m, 3H) (53H attributed + 4 NH_2 = 61H) HRMS (ESI^+, m/z, MeOH): calcd. for $C_{51}H_{62}N_7O_6$ $[M+H]^+$ 868.4756, found 868.4752. El. anal. calcd. for $C_{51}H_{61}N_7O_6$.4.45CF_3COOH: C, 52.30; H, 4.80; N, 7.13; found: C, 52.76; H, 5.00; N, 6.64.

NeaNH$_2$. 73% yield (White powder) ^{1}H NMR (500 MHz, MeOD-d_4) δ ppm 7.90 – 7.82 (m, 6H), 7.82 – 7.75 (m, 4H), 7.73 (br, 2H), 7.53 – 7.47 (m, 7H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.37 (d, *J* = 8.5 Hz, 1H), 5.64 (d, *J* = 2.1 Hz, 1H), 5.07 (d, *J* = 11.8 Hz, 1H), 5.02 (d, *J* = 11.8 Hz, 1H), 4.85 (s, 2H), 4.82 (m, 1H), 4.78 (m, 1H), 4.62 (ddd, *J* = 10.8, 3.0 & 2.2 Hz, 1H), 4.31 (t, *J* = 4.0 Hz, 1H), 4.23 (dd, *J* = 10.2 & 9.2 Hz, 1H), 3.75 (t, *J* = 9.2 Hz, 1H), 3.73 (dd, *J* = 4.0 & 2.1 Hz, 1H), 3.71 (m, 2H), 3.70 (m, 1H), 3.67 (dd, *J* = 14.2 & 10.8 Hz, 1H), 3.60 (t, *J* = 9.2 Hz, 1H), 3.46 (ddd, *J* = 12.4, 10.2 & 4.4 Hz, 1H), 3.40 (ddd, *J* = 12.4, 9.2 & 4.4 Hz, 1H), 3.14 (dd, *J* = 14.2 & 2.2 Hz, 1H), 2.48 (dt, *J* = 12.4 & 4.4 Hz, 1H), 2.41 (dd, *J* = 8.4 & 7.2 Hz, 2H), 2.04 (q, *J* = 12.4 Hz, 1H), 1.45 (m, 1H), 1.36 (m, 1H), 1.28 – 1.20 (m, 2H), 1.10 – 0.97 (m, 2H), 0.87 – 0.78 (m, 2H) (53H attributed + 5 NH_2 = 63H). HRMS (ESI^+, *m/z*, MeOH): calcd. for $C_{51}H_{65}N_5O_6$ $[M+ 2H]^{2+}$ 421.7462, found 421.7473. El. anal. calcd. for $C_{51}H_{63}N_5O_6$.5.05CF_3COOH.0.35H_2O: C, 51.53; H, 4.87; N, 4.92; found: C, 51.66; H, 5.00; N, 4.79.

NeaCyclam. 50 % yield (White powder) ^{1}H NMR (500 MHz, MeOD-d_4) δ ppm 7.90 – 7.82 (m, 6H), 7.81 – 7.75 (m, 4H), 7.73 (br, 2H), 7.55 – 7.47 (m, 7H), 7.42 (d, *J* = 8.6 Hz, 1H), 7.37 (d, *J* = 8.5 Hz, 1H), 5.64 (d, *J* = 2.1 Hz, 1H), 5.08 (d, *J* = 11.7 Hz, 1H), 5.01 (d, *J* = 11.7 Hz, 1H), 4.85 (m, 1H), 4.77 (m, 1H), 4.82 (s, 2H), 4.62 (d, *J* = 9.9 Hz, 1H), 4.28 (dd, *J* = 5.9 & 3.6 Hz, 1H), 4.24 (dd, *J* = 10.5 & 9.3 Hz, 1H), 3.72 – 3.78 (m, 4 H), 3.68 – 3.70 (m, 2H), 3.58 – 3.60 (m, 3H), 3.43 – 3.46 (m, 3H), 3.40 (m, 1H), 3.30 (m, 2H), 3.20 (m, 2H), 3.14 – 3.17 (m, 4H), 3.11 (m, 1H), 2.88 (m, 2H), 2.84 (m, 2H), 2.78 (m, 2H), 2.50 (m, 1H), 2.34 (m, 2H), 2.15 (t, *J* = 7.2 Hz, 2H), 2.05 (q, *J* = 12.4 Hz, 1H), 2.00 (m, 2H), 1.82 – 1.85 (m, 4H), 1.54 – 1.40 (m, 2H), 1.21 – 1.14 (m, 2H), 1.13 – 1.06 (m, 2H), 0.97 (m, 2H) (79H attributed + 4 NH_2 + 1 NHCO + 3 NH Cyclam = 91H) . ^{13}C NMR (125.7 MHz, MeOD-d_4) δ ppm 22.63, 26.30, 26.86, 27.99, 29.61, 29.63, 30.36, 31.61, 33.85, 39.93, 40.35, 45.86, 46.80, 47.28, 48.64, 50.54, 50.92, 51.41, 73.55, 73.85, 74.66, 74.72, 74.74, 75.55, 80.04, 81.70, 84.83, 93.36, 117.18, 119.51, 126-130 (C not listed), 134.63, 134.80, 134.84, 134.89, 134.94, 163.20, 163.48. HRMS (ESI^+, *m/z*, MeOH) calcd. for $C_{66}H_{93}N_9O_8$ $[M+2H]^{2+}$ 569.8568, found 569.8562. El. anal. calcd. for $C_{66}H_{91}N_9O_8$.7.2CF_3COOH.2.5H_2O: C, 48.18; H, 5.19; N, 6.29; found: C, 48.25; H, 5.41; N, 6.14.

NeaTPA. 53% yield (white powder) ^{1}H NMR (500 MHz, MeOD-d_4): δ ppm 8.61 (d, *J* = 5.1 Hz, 2H), 7.90 – 7.83 (m, 6H), 7.82 (m, 1H), 7.80 – 7.76 (m, 6H), 7.74 – 7.71 (m, 2H), 7.61 (dd, *J* = 8.1 & 7.5 Hz, 1H), 7.53 – 7.49 (m, 5H), 7.47 (m, 2H), 7.46 – 7.44 (m, 2H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.41 (dd, *J* = 7.8 & 5.1 Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 1H), 7.09 (d, *J* = 7.4 Hz, 1H), 5.66 (d, J = 2.2 Hz, 1H), 5.07 (d, *J* = 11.6 Hz, 1H), 4.99 (d, *J* = 11.6 Hz, 1H), 4.85 (d, *J* = 11.5 Hz, 1H), 4.82 (s, 2H), 4.77 (d, *J* = 11.5 Hz, 1H), 4.64 (ddd, *J* = 10.4, 3.3 & 2.1 Hz, 1H), 4.31 (m, 5H), 4.24 (dd, *J* = 10.2 & 9.1 Hz, 1H), 4.11 (s, 2H), 3.78 (t, *J* = 9.1 Hz, 1H), 3.73 – 3.77 (m, 2H), 3.72 (m, 1H), 3.71 (dd, *J* = 13.9 & 10.4 Hz, 1H), 3.68 (m, 1H), 3.59 (t, *J* = 9.1 Hz, 1H), 3.45 (ddd, *J* = 12.5, 10.2 & 4.2 Hz, 1H),

3.41 (ddd, *J* = 12.5, 9.1 & 4.2 Hz, 1H), 3.13 (dd, *J* = 13.9 & 2.1 Hz, 1H), 2.94 (m, 1H), 2.83 (m, 1H), 2.50 (dt, J = 12.5 & 4.2 Hz, 1H), 2.41 (m, 2H), 2.19 (t, *J* = 7.7 Hz, 2H), 2.09 (q, *J* = 12.5 Hz, 1H), 1.93 (m, 2H), 1.52 – 1.39 (m, 2H), 1.20 (m, 1H), 1.12 – 1.21 (m, 2H), 1.06 (m, 1H), 0.98 (m, 2H) (76H attributed + 4 NH_2 + 2 NH = 86H). ^{13}C NMR (125.7 MHz, MeOD-d_4) δ ppm 23.03, 26.88, 27.82, 29.87, 30.29, 31.50, 36.45, 37.03, 39.69, 40.00, 48.34, 48.51, 48.64, 50.48, 50.81, 51.57, 60.50, 60.85, 73.85, 74.84, 74.99, 76.42, 81.78, 85.25, 124.55, 125.53, 126.71, 126.92, 127.33, 127.51, 127.54, 127.61, 127.77, 128.41, 128.6-129.8 (C not listed), 134.71, 134.80, 134.85, 135.43, 135.53, 136.51, 139.6, 140.24, 141.67, 175.22. HRMS (ESI$^+$, *m/z*, MeOH): calcd. for $C_{77}H_{88}N_{10}O_8$ $[M+2H]^{2+}$ 622.3388, found 622.3403. El. anal. calcd. for $C_{74}H_{86}N_{10}O_8$•5.8CF_3COOH.2.5H_2O: C, 52.73; H, 5.00; N, 7.18; found: C, 52.98; H, 5.03; N, 7.16.

NeaTBA. 74% yield (white powder) ^{1}H NMR (500 MHz, MeOD-d_4) δ ppm 8.58 (d, *J* = 7.1 Hz, 1H), 7.91 – 7.70 (m, 18H), 7.56 – 7.32 (m, 16H), 5.62 (d, *J* = 2.0 Hz, 1H), 5.07 (d, *J* = 11.7 Hz, 1H), 5.01 (d, *J* = 11.7 Hz, 1H), 4.82 (m, 1H), 4.79 (m, 2H), 4.76 (m, 1H), 4.60 (d, *J* = 10.4 Hz, 1H), 4.41 (br, 6H), 4.25 (t, *J* = 3.9 Hz, 1H), 4.21 (dd, *J* = 10.4 & 9.2 Hz, 1H), 3.79 – 3.72 (m, 2H), 3.68 – 3.71 (m, 2H), 3.65 (m, 1H), 3.64 (dd, *J* = 13.5 & 10.4 Hz, 1H), 3.58 (t, *J* = 9.2 Hz, 1H), 3.43 (m, 1H), 3.38 (m, 1H), 3.12 (m, 1H), 2.83 (t, *J* = 7.2 Hz, 2H), 2.46 (dt, *J* = 12.5 & 4.2 Hz, 1H), 2.08 (t, *J* = 7.3 Hz, 2H), 2.04 (q, *J* = 12.5 Hz, 1H), 1.72 (m, 2H), 1.54 (q, 7.3 Hz, 2H), 1.46 (m, 1H), 1.40 (m, 1H), 1.15 (m, 2H), 1.08 (m, 1H), 1.04 (m, 1H), 0.98 (m, 2H) (79H attributed + 4 NH_2 + 2 NH = 89H). ^{13}C NMR (125.7 MHz, MeOD-d_4) δ ppm 23.33, 26.71, 27.78, 29.03, 30.32, 30.86, 31.48, 36.46, 37.16, 40.04, 58.77, 58.86, 73.98, 74.80, 75.10, 76.35, 126-131 (C not listed), 134.64, 134.75, 134.79, 134.80, 134.85, 135.51, 136.61, 140.23, 173.97, 175.3. HRMS (ESI$^+$, *m/z*, MeOH): calcd. for $C_{77}H_{91}N_7O_8$ $[M+2H]^{2+}$ 620.8459, found 620.8452. El. anal. calcd. for $C_{77}H_{89}N_7O_8$.4.6CF_3COOH.2H_2O: C, 57.48; H, 5.46; N, 5.44; found: C, 57.30; H, 5.46; N, 5.56.

NeaDPA. 70% yield (white powder) ^{1}H NMR (500 MHz, MeOD-d_4) δ ppm 8.58 (d, *J* = 5.0 Hz, 2H), 7.90 – 7.83 (m, 6H), 7.82 (m, 2H), 7.80 – 7.76 (m, 4H), 7.74 – 7.71 (m, 2H), 7.53 – 7.47 (m, 7H), 7.46 – 7.44 (m, 2H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.38 (dd, *J* = 7.6 & 5.0 Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 1H), 5.62 (d, *J* = 2.1 Hz, 1H), 5.07 (d, *J* = 11.7 Hz, 1H), 5.01 (d, *J* = 11.7 Hz, 1H), 4.82 (m, 1H), 4.79 (m, 2H), 4.75 (m, 1H), 4.59 (m, 1H), 4.41 (s, 4H), 4.25 (t, *J* = 3.8 Hz, 1H), 4.21 (dd, *J* = 10.5 & 9.3 Hz, 1H), 3.78 – 3.73 (m, 2H), 3.70 (t, *J* = 9.2 Hz, 1H), 3.69 (m, 1H), 3.66 (m, 1H), 3.64 (dd, *J* = 13.9 & 10.5 Hz, 1H), 3.58 (t, *J* = 9.3 Hz, 1H), 3.42 (m, 1H), 3.38 (m, 1H), 3.12 – 3.09 (m, 2H), 3.13 (ddd, *J* = 13.9 & 1.9 Hz, 1H), 2.83 (t, *J* = 7.5 Hz, 2H), 2.46 (dt, *J* = 12.5 & 4.5 Hz, 1H), 2.08 (t, *J* = 7.3 Hz, 2H), 2.03 (q, *J* = 12.5 Hz, 1H), 1.71 (m, 2H), 1.54 (m, 2H), 1.46 (m, 1H), 1.37 (m, 1H), 1.20 – 1.12 (m, 2H), 1.08 – 1.02 (m, 2H), 0.96 – 0.89 (m, 2H) (73H attributed + 4 NH_2 + 1 NHCO = 82H). ^{13}C NMR (125.7 MHz, MeOD-d_4) δ ppm 23.95, 25.43, 26.81, 27.84, 27.97, 29.71, 30.39, 30.42, 31.65, 36.19, 39.95, 40.35, 51.49, 51.64, 55.98, 59.04, 73.65, 73.88, 74.67, 74.86, 76.31, 76.39, 81.92, 84.86, 84.91, 93.93, 125.37, 125.45, 126-129 (C not listed), 134.84, 134.88, 135.51, 136.78, 139.40, 150.37, 150.42, 163.12, 163.35, 175.13. HRMS (ESI$^+$, *m/z*, MeOH): calcd. for $C_{68}H_{83}O_8N_7$ $[M+H]^+$ 1123.6379, found 1123.6370. El. anal. calcd. for $C_{68}H_{82}N_8O_7$.5.2CF_3COOH.2.5H_2O: C, 53.46; H, 5.28; N, 6.36; found: C, 53.48; H, 5.27; N, 6.36.

5.2 Biological studies

Bacterial strains and growth

Two *Escherichia coli* (*E. coli*) strains and 2 *Enterobacter aerogenes* (*E. aerogenes*) strains were previously described [43,46]. Briefly, AG100 was an *E. coli* wild type strain and AG100A its AcrAB- derivative. EA289 was a Kan^s derivative of an *E. aerogenes* multi-drug resistant (MDR) clinical isolate overexpressing AcrAB-tolC efflux pumps, and EA298 its tolC- derivative. Strains were routinely grown at 37°C on Luria-Bertani agar or in MH broth, supplemented with kanamycin (50 µg.mL^{-1}) for AG100A and EA298.

Susceptibility determination

The minimal inhibitory concentrations (MICs) were determined by broth dilution method as previously described [36]. The different molecules were solubilized in DMSO, and under the used conditions, the final percentage of DMSO (≤ 0.5%) had no effect on the bacterial growth [11,12]. Susceptibilities were determined in 96-wells microplates with an inoculum of $2x10^5$ cfu in 200 µL of MH broth containing two-fold serial dilutions of each compounds. MICs were realized in the absence and in the presence of PMBN membrane permeabilizer, used at 51.2 mg/L (1/5 of its direct MIC previously determined). MIC was defined as the lowest concentration of each compound for which no visible growth was observed after 18 h of incubation at 37°C. MIC values were means of at least three independent experiments. Results were expressed in µM in order to have a direct comparison of biological activity.

Nitrocefin assay[31]

Bacteria were grown to the exponential phase (OD at 600 nm = 0.5), centrifuged 20 min at 4000 rpm at 20°C and washed in PPB buffer (K_2HPO_{44} 20 mM ; $MgCl_2$1mM; pH 7). The OD of the bacterial culture was then adjusted to 0.375 and 100 µL was added to 50 µL of two-fold serial dilutions of each molecule in PPB buffer at a final concentration of 2, 4, 8, 16, 32 and 64 µM. Tests were performed at 37°C in microplates of 96 wells. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added at 3.3 µM to prevent the efflux of molecules. Nitrocefin (50 µL at 0.2 mg / mL) was added at t = 0 and the OD at 490 nm was monitored for 1 h with the microplates lector TECAN infinite M200 Pro. Each test was performed in duplicate or triplicate.

Real time efflux assay [35]

After an incubation of 24 h at 37°C, bacteria were centrifuged for 20 min at 4000 rpm and 20°C; and washed in PPB buffer. The pellet was resuspended in PPB buffer and the OD at 600 nm was adjusted to 0.25. The fluorophore 1,2'-DiNaphtylAmine (1,2'-DNA) and the dissipater of energy Carbonyl Cyanide 3-ChloroPhenylhydrazone (CCCP) were added at 32 and 5 µM respectively. After an incubation time of 18 h at 37°C protected from light, the bacterial culture was washed in PPB and 99 µL were added to 1 µL of two-fold serial dilutions of each molecule at a final concentration of 0.5 to 128 µM. Tests were performed at 37°C in black flat bottom microplates of 96 half-wells (Greiner Bio-One). The fluorescence intensity (λ_{ex} 370 nm / λ_{em} 420 nm) was measured every 4 s during 6 min with the microplates lector TECAN infinite M200 Pro. Five µL of glucose at 1 M was added at cycle 20 (after about 100 s). Each test was performed in duplicate or triplicate.

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Abbreviations

HOAt, 1-hydroxy-7azabenzotriazole; HATU, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate. DIEA diisopropylethylamine. TBAF tetrabutylammonium fluoride

Supplementary data

Synthesis of intermediates – Scheme S1 synthesis of protected Cyclam - ESI^+ HRMRS of free ligands and complexes in MeOH and MH medium: Figure S1 NeaDPA and Zn^{II}(NeaDPA); Figure S2 NeaCyclam and Zn^{II}(NeaCyclam) – Structure of the complexes Scheme S2 –Table S1 analysis of the mass spectra - Microbiology : Figure S3 nitrocefin hydrolysis in the presence of **NeaTBA**, **NeaNH$_2$** and PMBN - Figure S4 : time-dependent 1,2'-DNA efflux curves of *E. aerogenes* EA289 in the presence of **NeaTBA** and PAβN - Table S2 : calculated pKa and logD of neamine derivatives - ^{1}H NMR of final free ligands: **NeaN$_3$**; **NeaNH$_2$**; **NeaTPA**; **NeaDPA**; **NeaCyclam**. This material is available free of charge at…...

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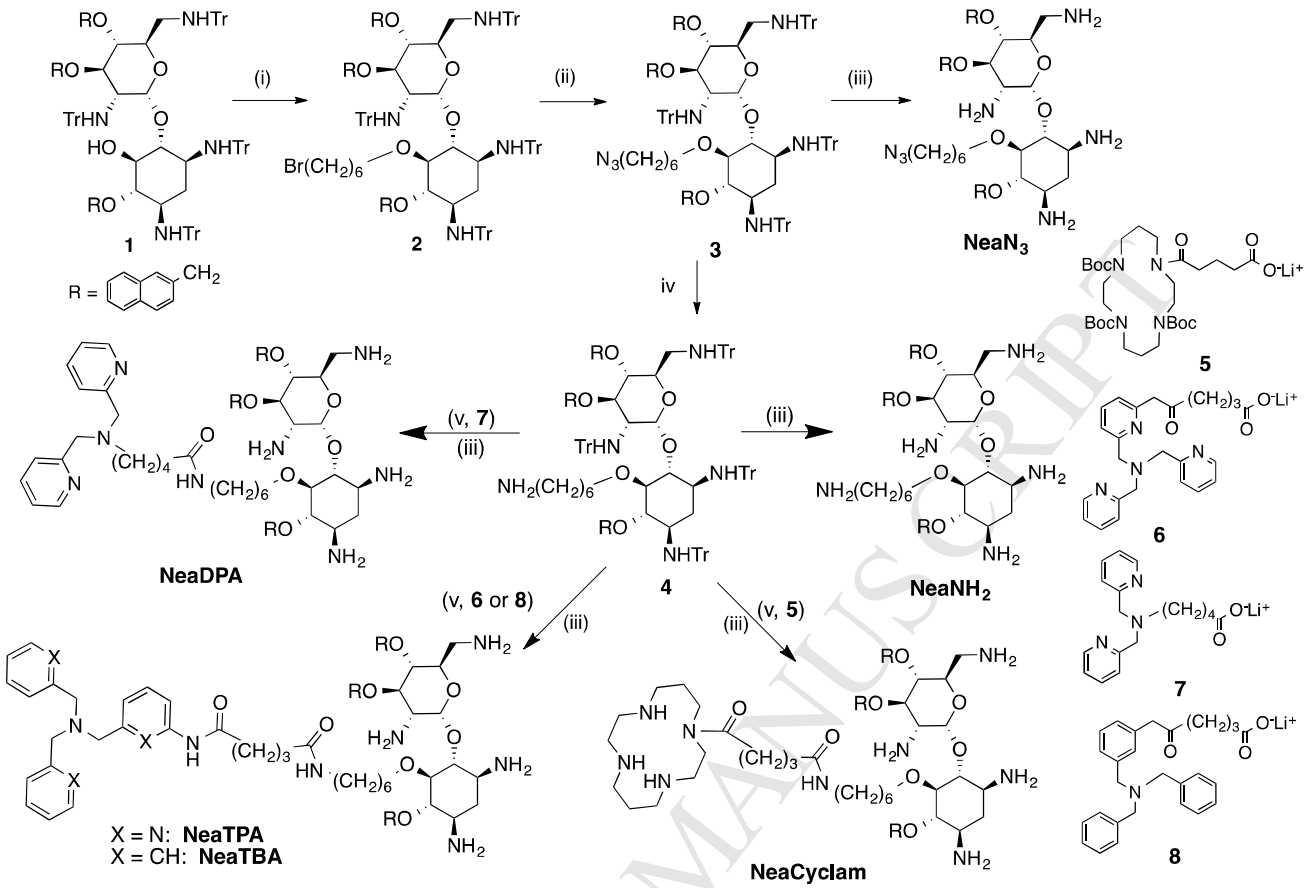

Scheme 1. Synthesis of neamine conjugates with metal binding groups.

(i) 1,6-dibromohexane, NaH, DMF, 60°C, 20 h (ii) NaN_3, DMF, r.t., 20 h (iii) TFA/CH_2Cl_2 (1:1), 0 °C, r.t., 2 h (iv) PPh_3, THF/water (95:5; v:v), reflux, 5 h. (v) HOAT, HATU, DIEA, DMF, r.t., 3 h.

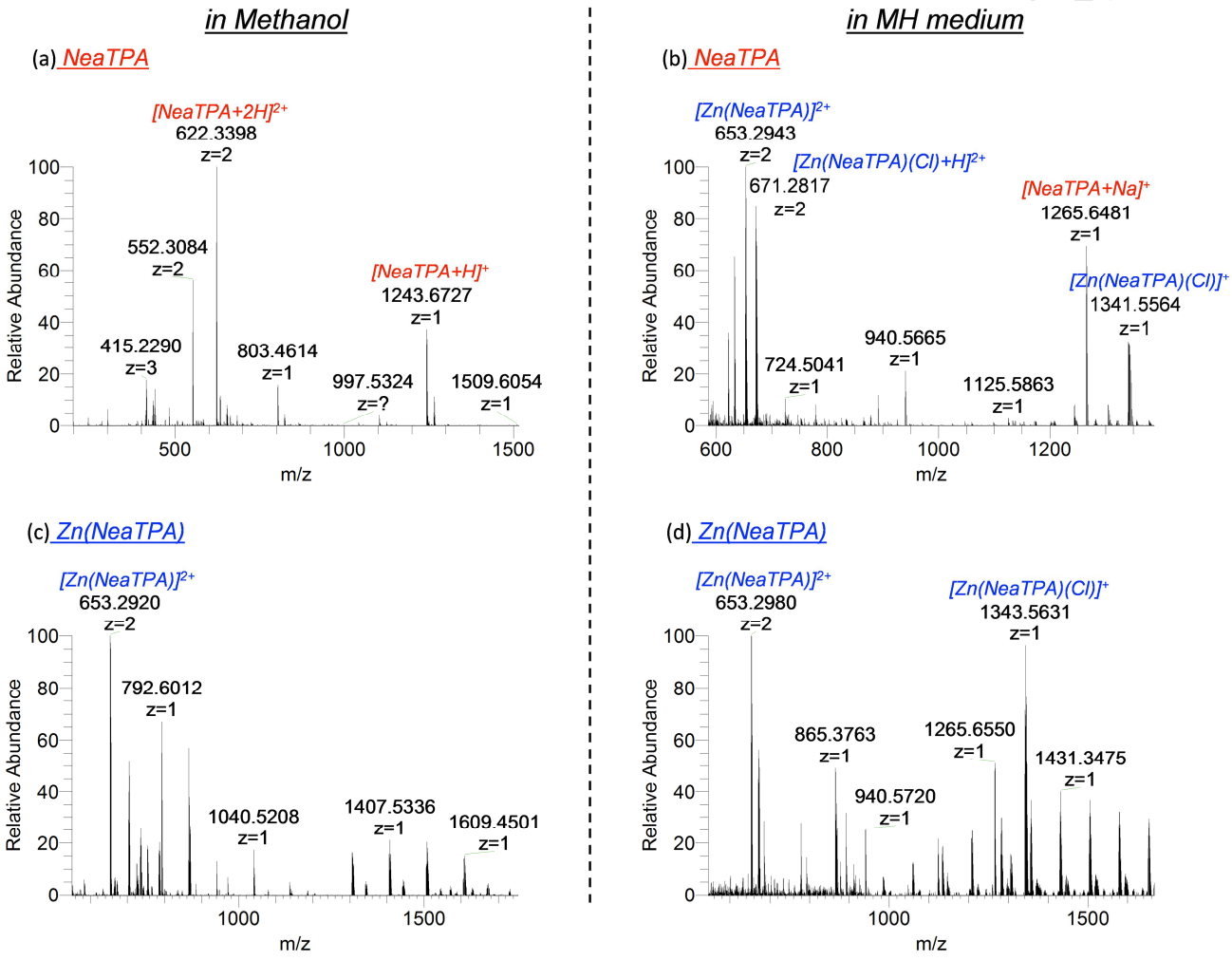

Figure 1. ESI$^+$ Mass spectra of NeaTPA and Zn(NeaTPA) complex in MeOH with or without further incubation in MH.

(a) HRMS of **NeaTPA** 50 µM in MeOH. (b) HRMS of **NeaTPA** 50 µM in MeOH after incubation in MH. (c) HRMS of **Zn(NeaTPA)** complex prepared from **NeaTPA** 50 µM and $Zn(ClO_4)_2$ 500 µM with 400 µM of DIPEA in MeOH. (d) HRMS of **Zn(NeaTPA)** complex prepared in MeOH and incubated at 50 µM in MH.

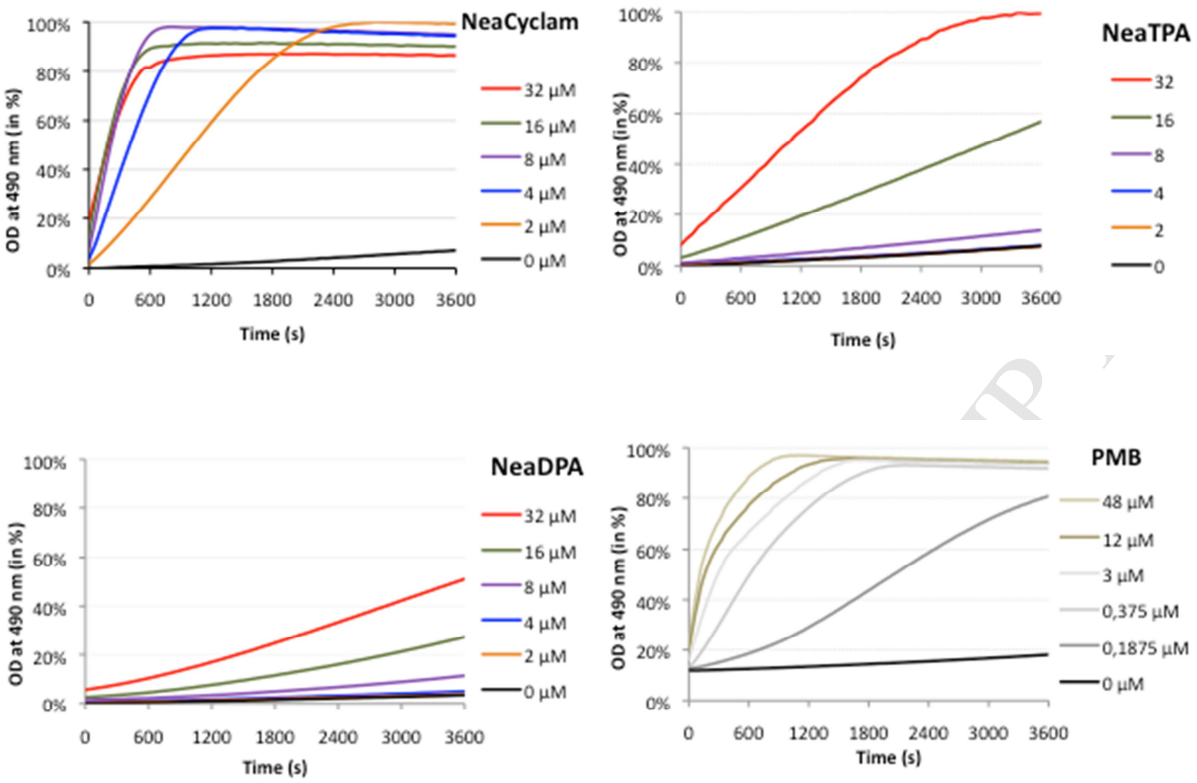

Figure 2: percentage of nitrocefin hydrolysis in *E. aerogenes* EA289

Percentage of the ODmax expected at 490 nm for complete hydrolysis of 50 µg/mL Nitrocefin for 1h - Curves at 0, 2, 4, 8, 16, 32 µM of neamine conjugates and PMB up to 48 µM.

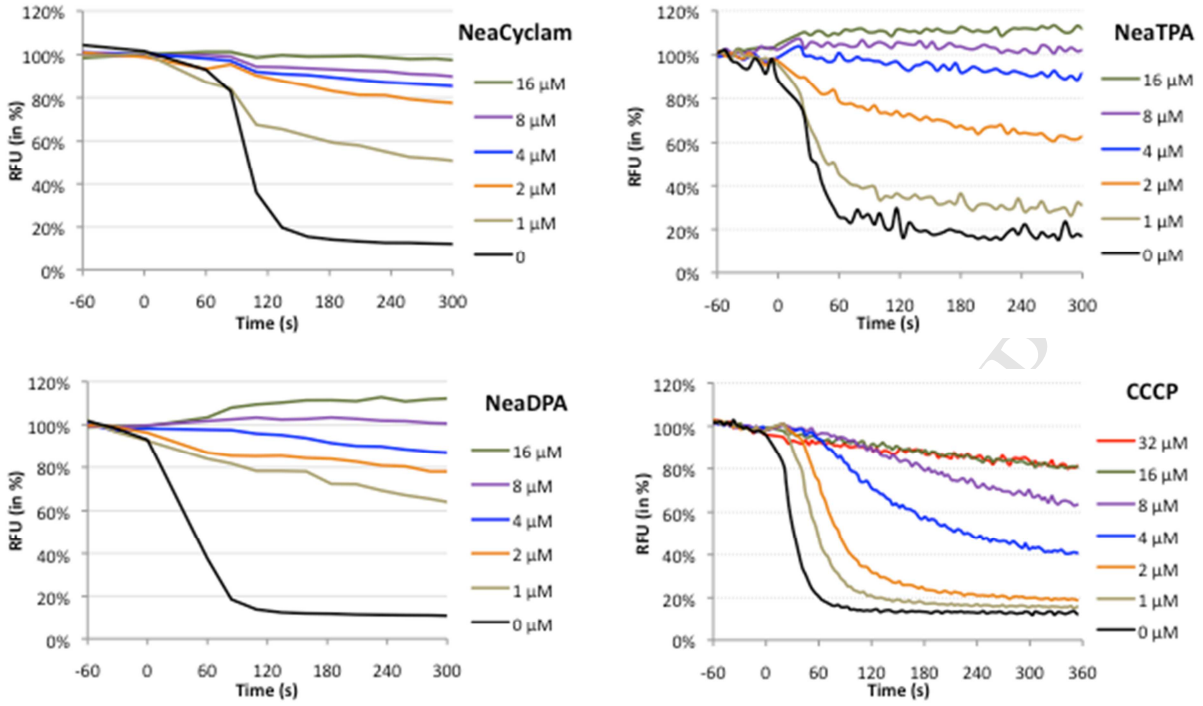

Figure 3: Representative 1,2'-DNA efflux curves of *E. aerogenes* EA289

Each neamine conjugate, as well as CCCP, was added at a final concentration of 0.5 to 16 or 32 µM. Tests were performed at 37°C. The fluorescence intensity (λ_{ex} 370 nm / λ_{em} 420 nm) was measured every 4 s during 5 min.

	CIP	FLE	CHL	CAZ	Neamine	$NeaN_3$	$NeaNH_2$	NeaCyclam	NeaTPA	NeaTBA	NeaDPA
AG100	0.125	0.5	8	0.5	32-16 (8)	32 (32)	8 (8)	4 (2)	4-2 (4-2)	128 (64-32)	16-8 (4)
AG100A	0.03	0.125	1	0.25	128 (128)	32 (16)	8 (4)	2 (2-1)	2 (2)	128-32 (32)	16-8 (4-2)
EA289	64	>128	>128	>128	32 (16)	16 (32-8)	16 (32-16)	16-4 (8)	4 (8-4)	128 (128)	16 (8)
EA298	8	16	32	>128	> 128 (> 128)	16 (32)	4 (8-4)	4-2 (2)	8-2 (2)	128-64 (64-32)	8 (8-4)

in parentheses values in the presence of PMBN 51.2 µg mL^{-1}

Table 1. Minimal Inhibitory concentrations in µM of neamine conjugates and tested antibiotics: CIP, ciprofloxacin; FLE, fleroxacin; CHL, chloramphenicol; CAZ, ceftazidime.

Highlights

• Synthesis of new amphiphilic neamine conjugates bearing a metal binding motif.

• Evaluation of their specific metal binding ability in the Mueller Hinton medium.

• Significant antibacterial activity against a multiresistant clinical isolate compared to usual antibiotic

• Determination of their membranotropic action: permeabilization of the outer membrane and action on the efflux transporter located in the inner membrane.