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New Ianthelliformisamine Derivatives as Antibiotic Enhancers against Resistant Gram-Negative Bacteria

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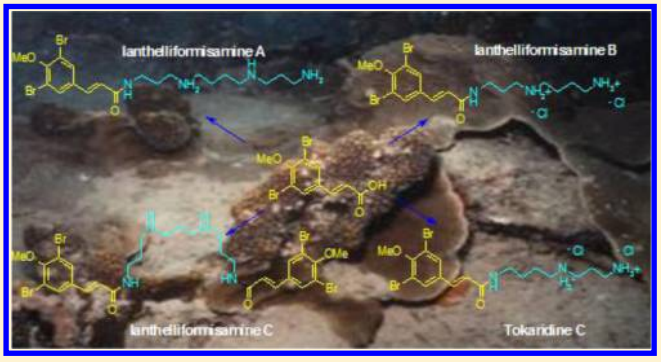
Supporting Information

ABSTRACT: A series consisting of ianthelliformisamimes A, B, and C as well as its synthetic analogues was prepared in high chemical yield, from 27 to 91%, using peptide coupling as the key step, and the compounds were evaluated for their in vitro antibiotic enhancer properties against resistant Gram-negative bacteria and clinical isolates. The mechanism of action of one of these derivatives against *Pseudomonas aeruginosa* when combined with doxycycline was precisely evaluated utilizing bioluminescence to measure ATP efflux and fluorescence to evaluate membrane depolarization.



1. INTRODUCTION

Because of their increased use for the treatment of numerous bacterial infections during recent decades, antibiotics represent one of the biggest healthcare blockbusters by decreasing both morbidity and mortality. Nevertheless, this success has led to inappropriate prescribing, thus increasing the number of antibiotic-resistant bacteria. Because of development costs and low profitability, novel antibiotic compounds are lacking in the commercial market.[1] The emergence of Gram-negative multidrug-resistant (MDR) bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, has prompted efforts to develop new classes of antibiotics to overcome this problem. The global strategy of identifying compounds that can circumvent the MDR phenotype is very promising because one molecule could potentially enhance the activity of numerous antibiotics. Furthermore, the bacterial membrane, which forms an effective barrier to many types of antibiotics,[2] represents an appealing target because it is highly conserved among most strains of Gram-negative bacteria. Thus, resistance to membrane-active antibiotics would require a major change in membrane structure, which would influence the permeability barrier and increase susceptibility to hydrophobic antibiotics. Most responses to Gram-negative bacteria can be attributed to the major components of the outer membrane, namely, lipopolysaccharides (LPS) and their lipid A anchor.[3−8]

The permeability barrier of the outer membrane is due to the cross-bridging between lipid A molecules and calcium or magnesium divalent cations.[3] Thus, cationic peptides[9] and polyamines,[10] which can weaken the binding sites of divalent cations, can lead to the disruption of the outer membrane organization, increasing its permeability. In this context, the use of compounds targeting the membranes of Gram-negative bacteria and enhancing the sensitivity of bacteria to hydrophobic antibiotics represents an attractive approach for the development of antibacterial agents because they are not expected to promote resistance. The presence of a polyamino moiety is crucial for achieving high antimicrobial activities.[10] We recently reported the synthesis of substituted polyamino geranic acid molecules containing a terpene core and various polyamino groups and investigated their use as potent activators of various common antibiotics against several Gram-negative bacteria strains that exhibit a MDR phenotype.[11] Additionally, in 2012, Xu et al. reported the isolation of ianthelliormisamines A−C, antibacterial bromotyrosine-derived metabolites from the marine sponge, *Suberea ianthelliformis* (Figure 1).[12]

Here, we describe the design, synthesis, and biological activity of new ianthelliformisamine derivatives as antibiotic enhancers against resistant Gram-negative bacteria. We also analyze the original mechanism of action of this class of derivatives against Gram-negative bacteria.

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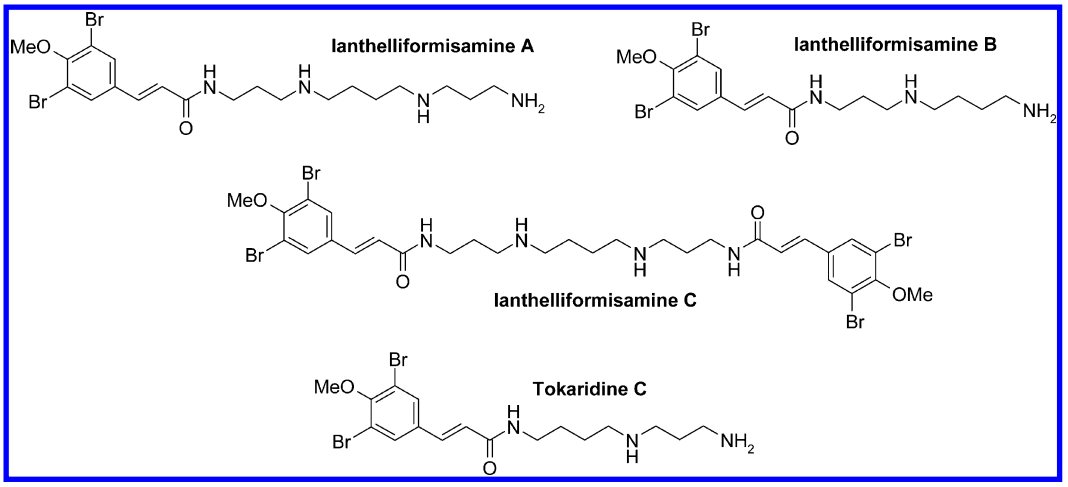
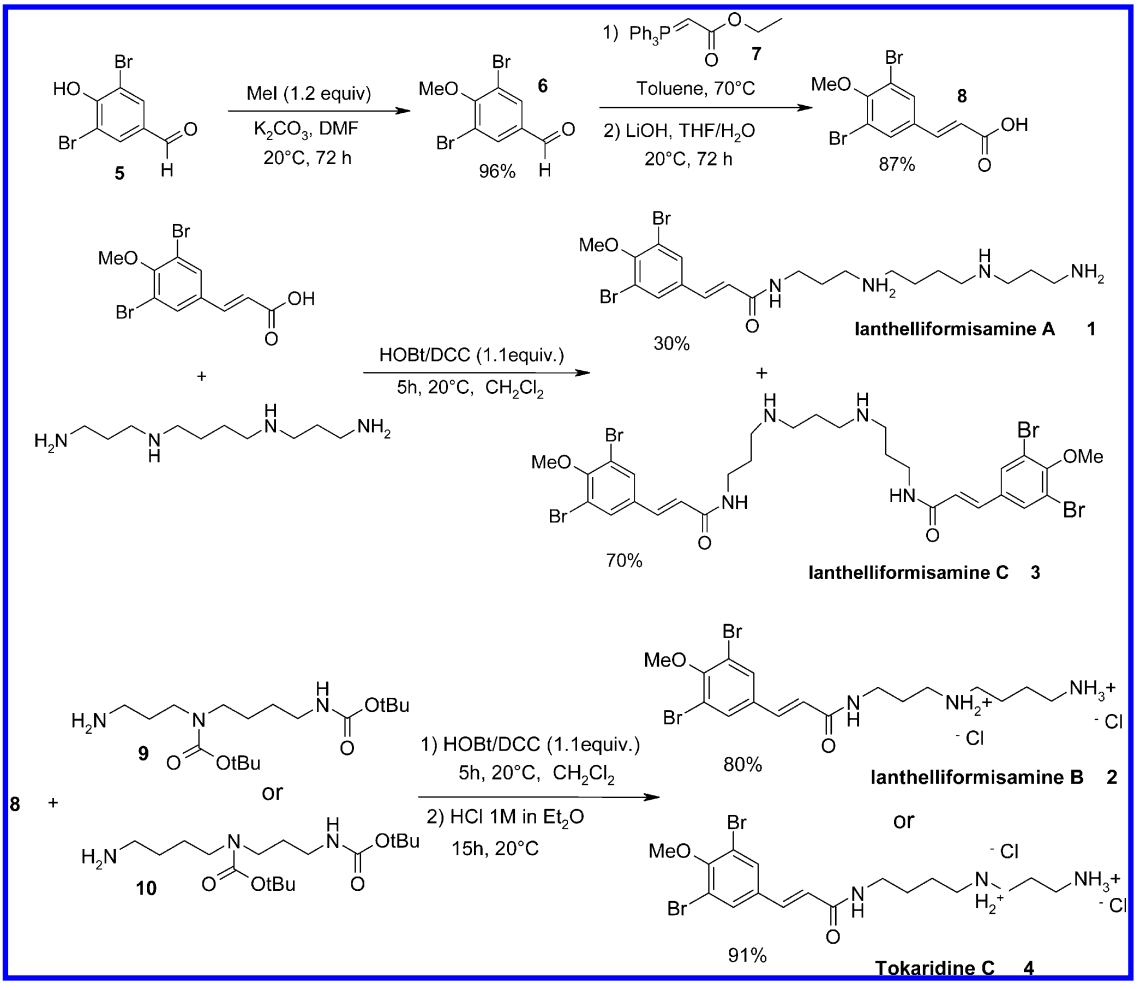


Figure 1. Structure of natural ianthelliformisamine derivatives.

Scheme 1. Synthesis of Ianthelliformisamine A–C and Tokaridine C



2. RESULTS AND DISCUSSION

Because of the novel chemical structure of ianthelliformisamines A–C and tokaridine C, we decided to synthesize these natural products (Scheme 1).

The common precursor for these compounds is the use of (*E*)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid, which is easily prepared in a three-step synthesis from 3,5-dibromo-4-hydroxybenzaldehyde (**5**) in 84% overall yield; this route involves a Wittig reaction with ethoxymethylidenetriphenylphosphorane (**7**) as the key step. Subsequent coupling involving a dicyclohexylcarbodiimide (DCC)/HOBt reagent afforded ianthelliformisamine A–C (**1**–**3**) and tokaridine C (**4**) in isolated yields varying from 32 to 80%. Notably, all analytical data are in accordance with those previously reported, thus confirming the assigned structures of these isolated marine metabolites. This chemical approach was successfully utilized for the preparation of various non-natural ianthelliformisamine

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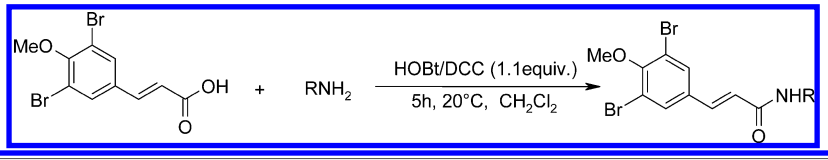
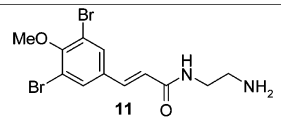
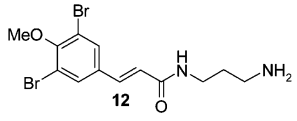
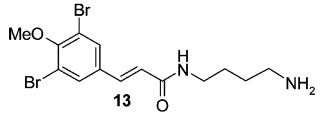
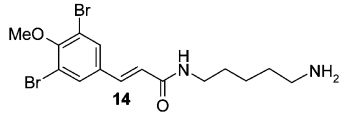
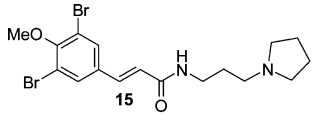
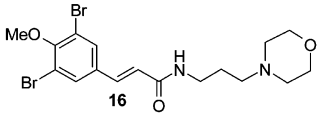
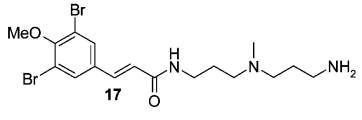
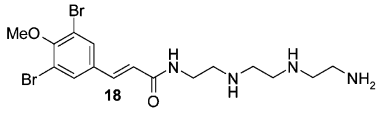
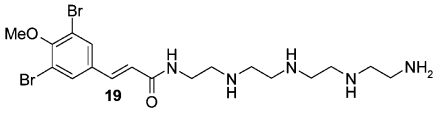
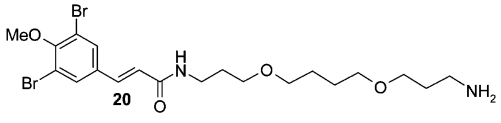
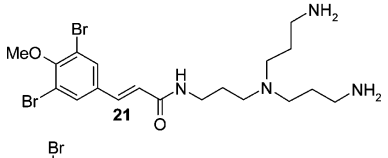
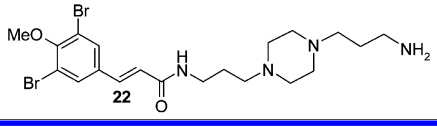
Table 1. New Ianthelliformisamine Derivatives 11−22



RNH_2	Product	Yield (%)[a]
1,2-Ethylenediamine	 **11**	27
1,3-Propanediamine	 **12**	60
Putrescine	 **13**	85
Cadaverine	 **14**	26
N-(3-Aminopropyl) pyrrolidine	 **15**	64
N-(3-Aminopropyl) morpholine	 **16**	84
3,3'-Diamino-N-methyl dipropylamine	 **17**	80
Triethylenetetramine	 **18**	26
Tetraethylenepentamine	 **19**	56
1,4-Bis(3-aminopropoxy) butane	 **20**	29
Tris(3-aminopropyl)amine	 **21**	51
1,4-Bis(3-aminopropyl) piperazine	 **22**	36

[a]Isolated yield.

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derivatives by varying the structure of the polyamines involved (Table 1).

Adducts **11**–**22** were obtained in good to excellent yield, varying from 27 to 85%, depending on the nature of the polyamine.

Infections caused by MDR Gram-negative bacteria are increasing worldwide,[13,14] and the emergence of bacteria resistant to all classes of antibiotics (namely, pandrug-resistant (PDR) bacteria) appears as a frightening end point in the development of antimicrobial resistance.[15,16] Despite the small number of strains of such bacteria, there is great concern in the medical community because clinicians have no options for treating patients with PDR bacterial infections. Recently, numerous clinical reports confirmed that Gram-negative bacteria have developed resistance to polymyxins,[17,18] which have become widely used in several countries as the last therapeutic barrier against PDR Gram-negative bacteria.[19] Thus, we investigated the potency of our new class of derivatives against two Gram-negative bacteria, *P. aeruginosa* and *K. pneumoniae*, that are commonly observed in hospitals.

First, the minimum inhibitory concentrations (MICs) of molecules **1**–**4** and **11**–**22** were evaluated against Gram-positive and Gram-negative bacterial strains to determine the concentrations to which each strain could be exposed to produce direct antibacterial activity.

As summarized in Table 2, all compounds demonstrated MICs over 200 μM except for the natural derivative, ianthelliformisamine C (**3**), which presented MICs varying from 12.5 to 100 μg/mL depending on the Gram-positive and Gram-negative bacterial strains.[12]

Table 2. MIC of Antibiotics and Compounds 1–4 and 11–22 against Various Bacterial Strains

	MIC μM (μg/mL)			
compound	EA289	SA DSM 799	PAO1	KPC2 ST258
chloramphenicol	>200	ND	>200	ND
cefepime	52 (25)	ND	26 (12.5)	ND
doxycycline	56 (25)	<3	112 (50)	ND
1	>200	>200	200	>200
2	100	>200	>200	>200
3	**100**	**12.5**	**25**	**12.5**
4	>200	200	200	>200
11	>200	>200	>200	>200
12	>200	>200	100	>200
13	>200	>200	>200	>200
14	>200	>200	>200	>200
15	>200	>200	>200	>200
16	>200	>200	>200	>200
17	>200	>200	200	>200
18	>200	>200	>200	>200
19	200	50	100	>200
20	>200	>200	>200	>200
21	>200	50	100	>200
22	>200	**6.25**	>200	>200

As previously described, the development of a chemosensitizing agent to increase the internal antibiotic concentration in resistant strains is an attractive approach to circumvent bacterial resistance. Thus, we envisioned the use of our ianthelliformisamine derivatives in combination with classic antibiotics to bypass the mechanistic and enzymatic barriers that reduce the intracellular concentrations of active antibacterial drugs. In a preliminary experiment, doxycycline (2 μg/mL, a concentration corresponding to its pharmacokinetic properties in humans[20]) was used to determine the concentrations of our products that could achieve this effect. At this concentration of doxycycline, natural derivatives **2**–**4** at 3.12–12.5 μM are able to restore the activity of doxycycline against *Enterobacter aerogenes* EA289, *P. aeruginosa* PAO1, and *K. pneumoniae* KPC2 ST258. Moreover, non-natural compounds **17**, **19**, and **21** were highly efficient in the same range of concentrations (Table 3).

Table 3. Concentration of Ianthelliformisamine Derivatives 1–4 and 11–22 Necessary To Restore Doxycycline Activity (2 μg/mL) against EA289, PAO1, and KPC2 ST258 Gram-Negative Bacterial Strains

	concentration of ianthelliformisamine derivative used (μM)		
compound	EA289	PAO1	KPC2 ST258
1	>100	12.5	>100
2	**25**	**12.5**	**3.12**
3	**12.5**	**3.12**	**12.5**
4	**6.25**	**3.12**	**3.12**
11	>100	>100	50
12	>100	100	100
13	>100	>100	>100
14	>100	>100	6.25
15	>100	25	>100
16	>100	>100	>100
17	**12.5**	**6.25**	**3.12**
18	>100	>100	>100
19	**12.5**	**6.25**	**3.12**
20	>100	>100	>100
21	**6.25**	**3.12**	**3.12**
22	>100	>100	>100

We also observed that after the MIC of doxycycline was decreased by treatment with a compound that later it was also particularly active with cefepime. Thus, we identified two groups of compounds, one consisting of derivatives **1**, **11**–**16**, **18**, **20**, and **22**, which displayed weak or no activity, and a second group consisting of compounds **2**–**4**, **17**, **19**, and **21**, which increased the antibiotic susceptibility effectively against PAO1. Similar dose-dependent effects were observed for ianthelliformisamine derivatives **1**–**4** and **11**–**22** in combination with doxycycline against *P. aeruginosa* PA01 (Figure 2).

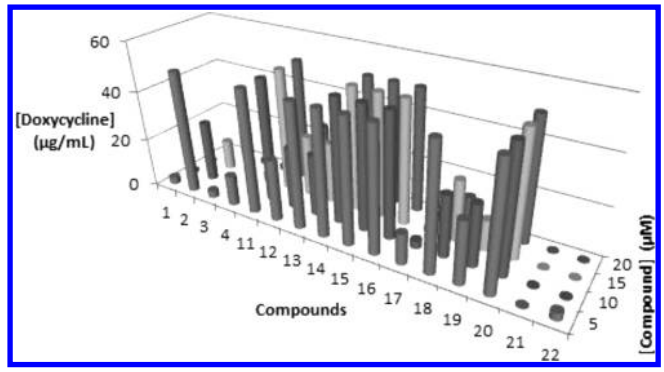

Figure 2. Dose-dependent effect of ianthelliformisamine derivatives **1**–**4** and **11**–**22** in combination with doxycycline against *P. aeruginosa* PA01.

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Notably, the chloramphenicol activity was improved in the presence of high concentrations of these derivatives, whereas the effect on cefepime resistance was stronger at lower concentrations of these later (Table 4).

Table 4. Concentration of the Select Ianthelliformisamine Derivatives Required To Restore Chloramphenicol, Doxycycline, and Cefepime Activity (2 μg/mL) against the PAO1 Bacterial Strain and Their Associated Cytotoxicity

	P. aeruginosa PAO1			IC_{50} (μM)	
compound	CHL[a]	DOX[a]	FEP[a]	CHO	fibroblasts
2	200	12.5	0.8	1665	129
3	12.5	**3.12**	**1.6**	663	237
4	100	**3.12**	**3.125**	108	33
17	200	6.25	6.25	174	24
19	100	6.25	6.25	573	237
21	100	**3.12**	**0.4**	846	345

[a]CHL, choramphenicol; DOX, doxycycline; FEP, cefepime.

Compound **21** was subsequently applied to restore doxycycline activity against clinical MDR isolates of *P. aeruginosa* (Figure 3). Five strains remained insensitive to

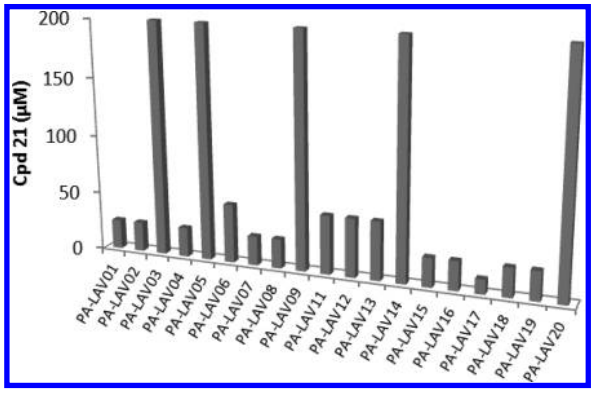

Figure 3. Concentration of ianthelliformisamine derivative **21** used to restore doxycycline activity (2 μg/mL) against *P. aeruginosa* clinical isolates.

compound **21**, and all of these strains were resistant to gentamycin. Some strains of *P. aeruginosa* exhibit aminoglycoside resistance related to a transport defect or membrane impermeabilization. This mechanism is most probably chromosomally mediated, and it results in cross-reactivity to all aminoglycosides, which could explain the ineffectiveness of compound **21** against these five strains.[21,22] All other strains presented similar antibiograms (data not shown), providing good to moderate results at a concentration of derivative **21** (12.5−50 μM) necessary to restore doxycycline activity at 2 μg/mL.

Moreover, nondetrimental cytotoxicities against Chinese hamster ovary cells (CHO) and human fibroblasts, with IC_{50} values up to 100 μM, were noted for the best derivatives, suggesting their potential therapeutic use for circumventing MDR.

From a mechanistic point of view, even if these compounds exhibit low antibacterial activity, their synergism with different antibiotics against Gram-negative bacteria remains questionable. Thus, two main modes of action are envisioned: (i) permeabilization and/or disruption of the outer membrane of the bacteria and (ii) efflux pump inhibition. Recently, Katsu et al. investigated the structure−activity relationship between the outer membrane of Gram-negative bacteria and original polyamines such as naphthylacetylspermine and methoctramine.[23] These studies demonstrated that the presence of lipophilic moieties as well as a number of amino groups on polyamines was important for permeabilization.[23]

Thus, the effect of **21** on the intracellular pool of bacterial ATP was determined by using a bioluminescence method. Thus, the external concentration of ATP was used as an indicator of the dose-dependent permeabilizing effect of **21**. For *P. aeruginosa*, no ATP release was observed (less than 16% of maximal efflux) using 2 mM compound **21** (Figure 4).

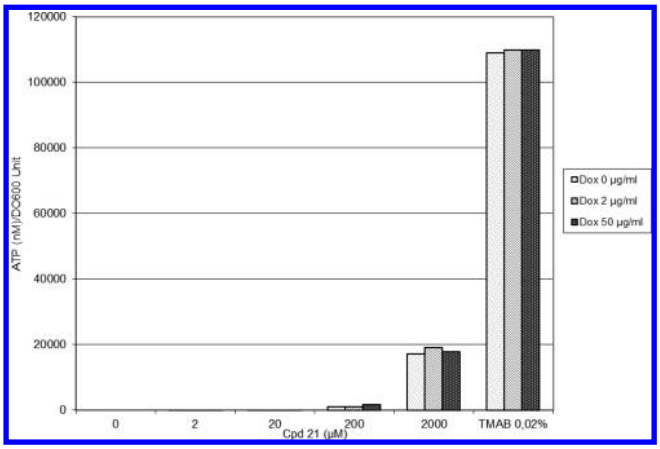

Figure 4. Effect of compound **21** on ATP efflux in *P. aeruginosa* (PAO1) in the presence or absence of doxycycline.

Furthermore, no ATP efflux was observed using a synergistic combination of **21** and doxycycline, even at high concentrations. Thus, the integrity of the outer membrane is not affected. Next, nitrocefin, a chromogenic β-lactam that is efficiently hydrolyzed by periplasmic β-lactamases, was used to measure the cell outer membrane integrity and to determine if our compounds possess outer membrane-permeabilizing activity.[24,25] As shown in Figure 5, even at a high concentration

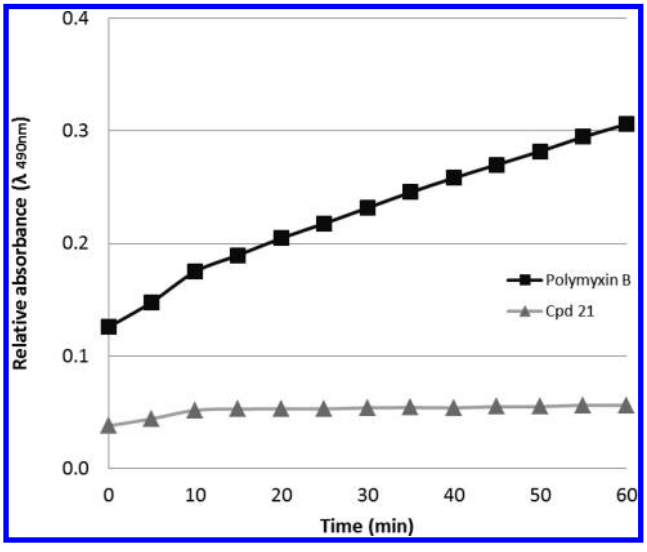

Figure 5. Effect of polymyxin B and compound **21** (500 μM) on nitrocefin hydrolysis in the periplasmic space of *P. aeruginosa* (PA01).

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(500 μM), the efflux pump inhibitor **21** did not increase the rate of nitrocefin hydrolysis compared to that of the untreated control. Lower concentrations were also tested under the same conditions and resulted in a similar response (see the Supporting Information). In contrast, polymyxin B (PMB) treatment dramatically increased the rate of nitrocefin hydrolysis.

Efflux pumps can expel a wide variety of compounds from bacterial cells, including antibiotics and dyes. In this context, the transport measurement of a dye, known as a substrate, can be used to directly monitor the function of efflux pumps. Treatment with 200 μM **21** resulted in dose-dependent depolarization of the PAO1 membrane, as indicated by a rapid and important increase in relative fluorescence units (ΔRFU values); 60% of the maximal RFU was attained in less than 3 min (Figure 6), indicating disruption of the proton

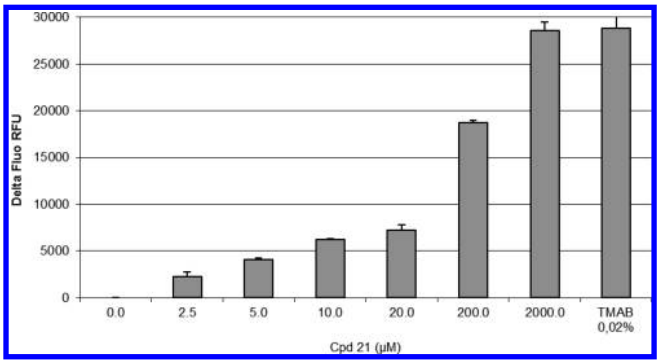

Figure 6. Dose-dependent depolarization of the bacterial membrane of PAO1 in the presence of compound **21**.

gradient. Efflux pumps use energy generated by the proton gradient across the inner membrane for drug extrusion. Taken together, these data indicate that changes in the transmembrane electrical potential in *P. aeruginosa* are not correlated with the permeabilization of cell membranes by ianthelliformisamines, suggesting altered proton homeostasis. Finally, ianthelliformisamines that disrupt the proton gradient may be considered to be efflux pump inhibitors.

3. CONCLUSIONS

An original chemical strategy was developed to produce new ianthelliformisamine derivatives in moderate to good yield.[26] Among the synthesized derivatives, compounds **2**–**4**, **17**, **19**, and **21** dramatically affected the antibiotic susceptibility of *E. aerogenes*, *P. aeruginosa*, and *K. pneumoniae* MDR strains. This efficiency was correlated with the inhibition of a dye transport, suggesting that these molecules can act on the activity of drug transporters. Studies are now under current investigation to elucidate whether this restoration of antibiotic susceptibility occurs via direct interaction of the molecule with the efflux pump or by deenergization of the efflux pump later. Finally, these derivatives may prove to be useful for studying the importance of efflux in acquired and natural resistance to antibiotics in Gram-negative bacteria.

4. EXPERIMENTAL SECTION

4.1. Materials. All solvents were purified according to previously reported procedures, and the reagents used are commercially available. Methanol, ethyl acetate, and dichloromethane were purchased from Fisher Scientific and used without further purification. Column chromatography was performed using Merck silica gel (70–230 mesh). ^{1}H and ^{13}C NMR spectra were recorded in MeOD using a Bruker AC 300 or AC 400 spectrometer (abbreviations: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet). Tetramethylsilane was used as the internal standard. All chemical shifts are presented in parts per million (ppm). Mass spectroscopy analysis was performed using the Spectropole (Analytical Laboratory) at the University Paul Cézanne (Marseille). The purity of the compounds was verified by analytical HPLC (C18 column, eluent CH_3CN/water/TFA, 2.3 mL/min) with a PDA detector from 210 to 310 nm. All compounds showed a purity greater than 95%, as determined by analytical HPLC-PDA at 214 and 254 nm.

4.2. Preparation of Compounds 1–4 and 11–22. *Synthesis of 3,5-Dibromo-4-methoxybenzaldehyde* ***6****.* To a stirred suspension of 3,5-dibromo-4-hydroxybenzaldehyde (5.1 g, 18.2 mmol) and K_2CO_3 in DMF (15 mL) was added iodomethane (1.4 mL, 22.7 mmol), and the resulting mixture was stirred at room temperature for 72 h. Then, the mixture was diluted with EtOAc (200 mL) and washed with water (2 × 60 mL) and brine (2 × 60 mL). The organic layers were combined and dried (Na_2SO_4), and the solvents were removed in vacuo to afford pure 3,5-dibromo-4-methoxybenzaldehyde (5.1 g, 96%) as a pale yellow solid. ^{1}H NMR (400 MHz, MeOD): δ 9.86 (s, 1H), 8.03 (s, 2H), 3.97 (s, 3H). ^{13}C NMR (75 MHz, MeOD): δ 188.33, 159.08, 34.19, 133.87, 119.26, 60.84.

Synthesis of (E)-Ethyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate. A solution of 3,5-dibromo-4-methoxybenzaldehyde (900 mg, 3.1 mmol) and ethoxymethylidenetriphenylphosphorane (1.4 g, 4 mmol) in toluene (20 mL) was heated at 70 °C for 15 h. The mixture was evaporated to dryness, and the resulting residue was purified by column chromatography (eluent 100% Et_2O) to yield pure (*E*)-ethyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate (only the (*E*) isomer was obtained; 1.01 g, 91%). ^{1}H NMR (400 MHz, MeOD): δ 7.66 (s, 2H), 7.5 (d, J = 15.8 Hz, 1H), 6.36 (d, J = 15.8 Hz, 1H), 4.26 (q, J = 7.3 Hz, 2H), 3.91 (s, 3H), 1.33 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, MeOD): δ 166.1, 155.35, 140.82, 133.09, 131.89, 120.10, 118.63, 60.68, 14.21.

Synthesis of (E)-3-(3,5-Dibromo-4-methoxyphenyl)acrylic Acid. To a solution of (*E*)-ethyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate (350 mg, 0.97 mmol) in a mixture of THF/H_2O (7 mL/2 mL) was added LiOH·H_2O (160 mg, 3.9 mmol), and the solution was stirred at room temperature for 72 h. The mixture was acidified with concentrated HCl (approximately 0.3 mL) until pH 1, and the aqueous layer was extracted with EtOAc (3 × 15 mL). The organic layers were combined, washed with brine (20 mL), and dried ($MgSO_4$), and the solvent was removed in vacuo. The pure desired compound was obtained as a white solid (310 mg, 96%). ^{1}H NMR (400 MHz, MeOD): δ 7.69 (s, 2H), 7.60 (d, J = 15.8 Hz, 1H), 6.37 (d, J = 15.8 Hz, 1H), 3.92 (s, 3H).

Synthesis of Ethoxymethylidenetriphenylphosphorane ***7****.* A solution of triphenylphosphine (4.93 g, 18.8 mmol) and ethyl chloroacetate (2 mL, 18.7 mmol) in toluene (40 mL) was stirred at 110 °C for 72 h. The precipitate was filtered and washed with ethyl acetate (2 × 15 mL) to yield 8.8 g of a white solid (pure bromophosphonium salt; 100%). ^{1}H NMR (400 MHz, MeOD): δ 7.96–7.65 (m, 15H), 5.66 (d, J = 14 Hz, 2H), 4.11 (q, J = 7.2 Hz, 2H), 1.04 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, MeOD): δ 164.38, 164.33, 134.86, 133.77, 133.63, 130.07, 129.89, 118.46, 117.28, 62.43, 32.84, 32.10, 20.73, 13.89. ^{31}P NMR: 20.85. To a solution of the previous bromophosphonium salt (4.2 g, 9.8 mmol) was added 2 M NaOH (200 mL). The mixture was vigorously shaken several times and extracted with CH_2Cl_2 (2 × 100 mL). The organic layers were combined, washed with water (200 mL) and brine (200 mL), and dried ($MgSO_4$), and the solvent removed in vacuo. The desired phosphonium ylide was obtained as a white powder (2.8 g, 82%). ^{1}H NMR (400 MHz, MeOD): δ 7.69–7.62 (m, 6H), 7.56–7.42 (m, 9H), 3.96 (m, 2H), 2.88 (s, 1H), 1.06 (m, 3H). ^{13}C NMR (75 MHz, MeOD): δ 132.98, 132.95, 131.83, 128.69, 128.53, 57.76, 30.87, 29.21, 14.75. ^{31}P NMR: 17.59.

Preparation of Ianthelliformisamine A ***1*** *and C* ***3****.* To a solution of (*E*)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (200 mg, 0.6 mmol) in CH_2Cl_2 (15 mL) were added HOBt (86 mg, 0.66 mmol)

and DCC (135 mg, 0.65 mmol). After stirring at room temperature for 5 h, the precipitate was filtered and washed with CH_2Cl_2. Spermine (135 mg, 0.64 mmol) in CH_2Cl_2 (2 mL) was added to the filtrate, and the resulting solution was stirred at room temperature for 15 h. Evaporation of solvents in vacuo afforded an oil that was purified by column chromatography (eluent CH_2Cl_2/MeOH/NH_4OH, 7:3:1) to afford monoalkylated ianthelliformisamine A (**1**) (100 mg, 30%) and ianthelliformisamine C (**3**) as a side product (180 mg, 70%).

Ianthelliformisamine C ***3***. ^{1}H NMR (400 MHz, MeOD): δ 7.67 (s, 4H), 7.28 (d, J = 15.8 Hz, 2H), 6.46 (d, J = 15.8 Hz, 2H), 3.77 (s, 6H), 3.36−3.18 (m, 4H), 2.65−2.49 (m, 8H), 1.7−1.2 (m, 8H). ^{13}C NMR (75 MHz, MeOD): δ 168.80, 157.11, 139.01, 136.14, 133.83, 124.79, 120.38, 62.10, 50.54, 48.18, 39.08, 35.60, 26.90. MS (ESI) $C_{30}H_{38}N_4O_4Br_4$ m/z 839.0 (100%, ($M + H^+$)), 837.0 (100%, ($M - H^-$)).

Ianthelliformisamine A ***1***. ^{1}H NMR (400 MHz, MeOD): δ 7.83 (s, 2H), 7.42 (d, J = 15.8 Hz, 1H), 6.61 (d, J = 15.8 Hz, 1H), 3.90 (s, 3H), 2.94−2.78 (m, 12 H), 1.90−1.85 (m, 4H), 1.47−1.70 (m, 4H). ^{13}C NMR (75 MHz, MeOD): δ 168.96, 157.12, 138.99, 136.23, 133.89, 124.92, 120.37, 62.11, 47.96, 47.55, 40.14, 38.68, 29.21, 28.85, 27.25, 26.87. MS (ESI) $C_{20}H_{32}N_4O_2Br_2$ m/z 521.1 (100%, ($M + H^+$)), 519.1 (100%, ($M - H^-$)).

Preparation of Ianthelliformisamine B Hydrochloride Salt ***2***. To a solution of (*E*)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (150 mg, 0.45 mmol) in CH_2Cl_2 (10 mL) were added HOBt (65 mg, 0.48 mmol) and DCC (100 mg, 0.47 mmol). After stirring at room temperature for 1 h, the white precipitate was filtered, washed with CH_2Cl_2 (2 mL), and {4-[3-amino-propyl)-*tert*-butoxycarbonyl-amino]-butyl}-carbamic acid *tert*-butyl ester (160 mg, 0.46 mmol) in CH_2Cl_2 (2 mL) was added to the filtrate. After 3 h, the solution was evaporated to dryness, and the crude residue (240 mg, 80%) was used in the next step.

BOC-protected ianthelliformisamine B (50 mg, 0.15 mmol) was dissolved in MeOH (1 mL), and 1 M HCl in Et_2O (1 mL) was added. After stirring at room temperature for 15 h, a white precipitate appeared, and Et_2O (10 mL) was added to complete the precipitation of ianthelliformisamine B hydrochloride salt **3**. This compound was then filtered to yield the desired compound as a white solid (40 mg, 100%). ^{1}H NMR (400 MHz, D_2O): δ 7.44 (s, 2H), 7.01 (d, J = 15.8 Hz, 1H), 6.25 (d, J = 15.8 Hz, 1H), 3.73 (s, 3H), 3.29 (m, 2H), 3.01−2.97 (m, 6H), 1.71−1.63 (m, 6H). ^{13}C NMR (75 MHz, D_2O): δ 168.10, 153.88, 137.98, 133.24, 131.83, 121.44, 117.74, 60.88, 46.93, 45.16, 38.76, 36.35, 25.64, 23.88, 22.79. MS (ESI) $C_{17}H_{27}N_3O_2Cl_2Br_2$ m/z 464.1 (100%, ($M + H^+$)), 462.0 (100%, ($M - H^-$)).

Preparation of {4-[3-Amino-propyl)-tert-butoxycarbonyl-amino]-butyl}-carbamic acid tert-Butyl Ester ***9***. *Synthesis of tert-Butyl-4-aminobutylcarbamate.* To a 0 °C solution of 1,4-diaminobutane (12 mL, 119.4 mmol) in chloroform (100 mL) was added *tert*-butyl dicarbonate (5 mL, 21.7 mmol) dropwise over 1.5 h. After stirring at room temperature for 15 h, the solvents were removed in vacuo, water was added (150 mL), and the insoluble bis-substituted product was removed by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 100 mL), and the organic layers were combined, washed with brine (100 mL), and dried over $MgSO_4$; the solvents were removed in vacuo to afford a pale yellow oil (4.2 g, 100%). ^{1}H NMR (400 MHz, MeOD): δ 4.72 (s large, 1H), 3.09 (t, J = 6.2 Hz, 2H), 2.68 (t, J = 7.0 Hz, 2H), 1.52−1.4 (m, 13H). ^{13}C NMR (75 MHz, MeOD): δ 155.81, 41.53, 30.64, 28.15.

Synthesis of [4-(2-Cyano-ethylamino)-butyl]-carbamic Acid tert-Butyl Ester. To a stirred suspension of *tert*-butyl-4-aminobutylcarbamate (2 g, 10.6 mmol) and K_2CO_3 (5 g, 36.1 mmol) in acetonitrile (50 mL) was added 3-bromopropionitrile (0.9 mL, 10.8 mmol), and the mixture was stirred at 50 °C for 15 h. The solvents were removed in vacuo, and the crude oil was purified by column chromatography (40 g SiO_2, eluent MeOH/CH_2Cl_2, 9:1) to yield the desired compound as a colorless oil (820 mg, 32%). ^{1}H NMR (400 MHz, MeOD): δ 4.81 (s large, 1H), 3.06 (m, 2H), 2.86 (t, J = 6.7 Hz, 2H), 2.60 (t, J = 6.4 Hz, 2H), 2.46 (t, J = 6.5 Hz, 2H), 1.48−1.38 (m, 13H). ^{13}C NMR (75 MHz, MeOD): δ 155.88, 118.58, 78.84, 48.55, 44.86, 40.19, 28.27, 27.08, 18.53.

Synthesis of (4-tert-Butoxycarbonylamino-butyl)-(2-cyano-ethyl)-carbamic Acid tert-Butyl Ester. To a 0 °C solution of [4-(2-cyano-ethylamino)-butyl]-carbamic acid *tert*-butyl ester (750 mg, 3.1 mmol) in CH_2Cl_2 (15 mL) was added a solution of *tert*-butyl dicarbonate (0.85 mL, 3.7 mmol) in CH_2Cl_2 (10 mL) dropwise. After stirring at room temperature for 15 h, the solvents were removed in vacuo, and the crude residue was dissolved in CH_2Cl_2 (50 mL) and washed with 10% Na_2CO_3 (2 × 30 mL) and brine (30 mL). The organic layer was dried ($MgSO_4$), and the solvent was removed to afford a colorless oil (1 g, 94%). ^{1}H NMR (400 MHz, MeOD): δ 4.64 (s large, 1H), 3.41 (t, J = 6.7 Hz, 2H), 3.23 (t, J = 6.8 Hz, 2H), 3.09−3.07 (m, 2H), 2.57 (s large, 2H), 1.51−1.39 (m, 22 H). ^{13}C NMR (75 MHz, MeOD): δ 155.91, 146.62, 118.22, 80.37, 78.95, 53.33, 39.88, 28.28, 27.27, 25.78, 25.36.

Synthesis of {4-[3-Amino-propyl)-tert-butoxycarbonyl-amino]-butyl}-carbamic Acid tert-Butyl Ester. A 0 °C solution of (4-*tert*-butoxycarbonylamino-butyl)-(2-cyano-ethyl)-carbamic acid *tert*-butyl ester (1 g, 2.9 mmol) in distilled Et_2O (20 mL) was carefully added to a suspension of $LiAlH_4$ (480 mg, 12.6 mmol) in distilled Et_2O (20 mL). After stirring at 0 °C for 3 h, 5% NaOH (10 mL) was added dropwise, and the white precipitate was removed by filtration and washed with EtOAC (20 mL). The aqueous layer was extracted with EtOAc (2 × 20 mL), and the organic layers were combined, washed with brine (2 × 10 mL), and dried ($MgSO_4$); the solvent was removed in vacuo to afford the desired compound as a colorless oil (760 mg, 75% yield). ^{1}H NMR (400 MHz, MeOD): δ 4.73 (s large, 1H), 3.18−3.03 (m, 6H), 2.61 (t, J = 6.7 Hz, 2H), 1.44−1.36 (m, 24H). ^{13}C NMR (75 MHz, MeOD): δ 155.87, 155.55, 79.16, 46.36, 40.02, 28.25, 27.26, 25.51.

Preparation of Tokaridine C Hydrochloride Salt ***4***. To a solution of (*E*)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (152 mg, 0.45 mmol) in CH_2Cl_2 (10 mL) were added HOBt (67 mg, 0.48 mmol) and DCC (102 mg, 0.47 mmol). After stirring at room temperature for 1 h, the white precipitate was removed by filtration and washed with CH_2Cl_2 (2 mL). A solution of (4-amino-butyl)-(3-*tert*-butoxycarbonylamino-propyl)-carbamic acid *tert*-butyl ester (158 mg, 0.45 mmol) in CH_2Cl_2 (2 mL) was added to the filtrate. After 3 h, the solution was evaporated to dryness, and the crude residue (275 mg, 91%) was used in the next step.

BOC-protected tokaridine C (100 mg, 0.07 mmol) dissolved in MeOH (1 mL) and 1 M HCl in Et_2O (1 mL) were added. After stirring at room temperature for 15 h, a white precipitate appeared, and Et_2O (10 mL) was added to completely precipitate the tokaridine C hydrochloride salt. This suspension was then filtered to yield the desired compound as a white solid (120 mg, 100%). ^{1}H NMR (400 MHz, D_2O): δ 7.56 (s, 2H), 7.12 (d, J = 15.8 Hz, 1H), 6.35 (d, J = 15.8 Hz, 1H), 3.84 (s, 3H), 3.30 (t, J = 6.8 Hz, 2H), 3.13−3.06 (m, 6H), 2.10−2.04 (m, 2H), 1.72−1.62 (m, 4H). ^{13}C NMR (75 MHz, D_2O): δ 167.81, 153.88, 137.46, 133.40, 131.84, 121.82, 117.78, 60.94, 47.38, 44.46, 38.83, 36.57, 25.63, 23.73, 23.08. MS (ESI) $C_{17}H_{25}N_3O_2Br_2$ m/z 464.0 (100%, ($M + H^+$)), 462.3 (100%, ($M - H^-$)).

Preparation of (4-Amino-butyl)-(3-tert-butoxycarbonylamino-propyl)-carbamic Acid tert-Butyl Ester ***10***. *Synthesis of tert-Butyl-3-aminopropylcarbamate.* To a 0 °C solution of 1,4-diaminopropane (10 mL, 120 mmol) in chloroform (100 mL) was added *tert*-butyl dicarbonate (5 mL, 21.7 mmol) in chloroform (100 mL) dropwise over 1.5 h. After stirring at room temperature for 15 h, the solvents were removed in vacuo, water was added (150 mL), and the insoluble bis-substituted product was removed by filtration. The filtrate was extracted with Et_2O (3 × 100 mL), the organic layers were combined, washed with brine (100 mL), and dried ($MgSO_4$), and the solvents were removed in vacuo to afford the desired product as a pale yellow oil (90% yield (3.4 g)). ^{1}H NMR (400 MHz, MeOD): δ 4.90 (s large, 1H), 3.20 (q, J = 6.3 Hz, 2H), 2.74 (t, J = 6.5 Hz, 2H), 1.59 (m, 2H), 1.43 (s, 9H).

Synthesis of [3-(3-Cyano-propylamino)-propyl]-carbamic Acid tert-Butyl Ester. To a stirred suspension of *tert*-butyl-3-amino-propylcarbamate (1.5 g, 8.6 mmol) and K_2CO_3 (4 g, 28.9 mmol) in acetonitrile (50 mL) was added 3-bromopropionitrile (0.9 mL, 9

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mmol), and the mixture was stirred at 50 °C for 15 h. The solvents were removed in vacuo, and the crude oil (100%) was used without further purification for the next step. ^{1}H NMR (400 MHz, MeOD): δ 5.10 (s large, 1H), 3.20 (m, 2H), 2.72 (t, J = 6.5 Hz, 2H), 2.65 (t, J = 6.5 Hz, 2H), 2.44 (t, J = 7.3 Hz, 2H), 1.80 (m, 2H), 1.63 (t, J = 6.5 Hz, 2H), 1.42 (s, 9H). ^{13}C NMR (75 MHz, MeOD): δ 155.97, 119.61, 47.73, 47.13, 38.76, 29.66, 28.22, 25.54, 14.70.

Synthesis of (3-[tert-Butoxycarbonyl-(3-cyano-propyl)-amino]-propyl)-carbamic Acid tert-Butyl Ester. To a 0 °C solution of [3-(3-cyano-propylamino)-propyl]-carbamic acid *tert*-butyl ester (2.05 g, 8.5 mmol) in CH_2Cl_2 (40 mL) was added *tert*-butyl dicarbonate (1.9 mL, 8.2 mmol) in CH_2Cl_2 (20 mL) dropwise. After stirring at room temperature for 15 h, the solvents were removed in vacuo, and the crude residue was dissolved in CH_2Cl_2 (100 mL) and washed with 10% Na_2CO_3 (2 × 50 mL) and brine (50 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed to afford a colorless oil (2.8 g, 96%). ^{1}H NMR (400 MHz, MeOD): δ 3.28 (t, J = 7 Hz, 4H), 3.10 (m, 2H), 2.34 (t, J = 7.3 Hz, 2H), 1.88 (t, J = 6.8 Hz, 2H), 1.69−1.61 (m, 2H), 1.51−1.43 (m, 18H). ^{13}C NMR (75 MHz, MeOD): δ 161.15, 155.88, 105.85, 85.06, 80.18, 79.01, 53.34, 52.02, 45.47, 37.41, 30.61, 28.27, 27.29, 22.99, 15.87, 14.74, 14.59.

Synthesis of (4-Amino-butyl)-(3-tert-butoxycarbonylamino-propyl)-carbamic Acid tert-Butyl Ester. A 0 °C solution of (4-*tert*-butoxycarbonylamino-butyl)-(2-cyano-ethyl)-carbamic acid *tert*-butyl ester (2.8 g, 8.2 mmol) in distilled Et_2O (50 mL) was carefully added to a suspension of $LiAlH_4$ (1.3 g, 34.2 mmol) in distilled Et_2O (50 mL). After stirring at 0 °C for 3 h, 5% NaOH (30 mL) was added dropwise, and the white precipitate was isolated by filtration and washed with EtOAC (20 mL). The aqueous layer was extracted with EtOAc (2 × 50 mL), and the organic layers were combined, washed with brine (2 × 30 mL), and dried over $MgSO_4$; the solvent was removed in vacuo to afford the desired compound as a colorless oil (2.2 g, 78%). ^{1}H NMR (400 MHz, MeOD): δ 3.25−3.09 (m, 6H), 2.72−2.69 (m, 2H), 2.03 (br s, 2H), 1.68−1.44 (m, 18H). ^{13}C NMR (75 MHz, MeOD): δ 155.97, 79.39, 46.75, 43.72, 37.52, 30.86, 25.90, 28.35.

*General Procedure for the Synthesis of Compounds **11**−**22**.* To a solution of **8** (100 mg, 0.3 mmol) in CH_2Cl_2 (5 mL) were added HOBt (45 mg, 1.1 equiv) and DCC (70 mg, 1.1 equiv). After stirring at room temperature for 1 h, the white precipitate was removed by filtration, and a solution of amine (1.1 equiv) in dichloromethane (2 mL) was added to the filtrate. The reaction was continued for 5−12 h at room temperature, and the solvents were removed in vacuo to yield a crude residue that was purified by column chromatography.

*(E)-N-(2-Amino-ethyl)-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **11**.* ^{1}H NMR (MeOD, 300 MHz): δ 7.82 (s, 2H), 7.43−7.39 (d, J = 15.6 Hz, 1H), 6.61−6.57 (d, J = 15.6 Hz, 1H), 3.90 (s, 3H), 3.41−3.37 (t, J = 6.3 Hz, 2H), 2.83−2.80 (t, J = 7.3 Hz, 2H). ^{13}C NMR (MeOD, 75 MHz): δ 169.03, 157.16, 139.08, 136.23, 133.87, 124.81, 120.39, 62.09, 43.87, 42.79. MS (ESI) $C_{12}H_{14}N_2O_2Br_2$ m/z 379.0 (100%, (M + H$^+$)), 376.9 (100%, (M − H$^-$)).

*(E)-N-(3-Amino-propyl)-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **12**.* ^{1}H NMR (MeOD, 300 MHz): δ 7.81 (s, 2H), 7.42−7.38 (d, J = 15.8 Hz, 1H), 6.59−6.55 (d, J = 15.8 Hz, 1H), 3.90 (s, 3H), 3.40−3.37 (t, J = 7.0 Hz, 2H), 2.73−2.70 (t, J = 7.0 Hz, 2H), 1.75−1.72 (M, J = 7.0 Hz, 2H). ^{13}C NMR (MeOD, 75 MHz): δ 168.77, 157.1,1, 138.96, 136.20, 133.82, 124.77, 120.38, 62.09, 40.54, 38.76, 33.99. MS (ESI) $C_{13}H_{16}N_2O_2Br_2$ m/z 393.0 (100%, (M + H$^+$)), 391.0 (100%, (M − H^{--})).

*(E)-N-(4-Amino-butyl)-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **13**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.80 (s, 2H), 7.41−7.37 (d, J = 15.6 Hz, 1H), 6.59−6.55 (d, J = 15.6 Hz, 1H), 3.89 (s, 3H), 3.36 (m, 2H), 2.71−2.67 (t, J = 7.0 Hz, 2H), 1.62−1.55 (m, 4H). ^{13}C NMR (MeOD, 75 MHz): δ 168.51, 156.99, 138.77, 136.17, 133.76, 124.92, 120.33, 62.09, 42.95, 41.27, 31.77, 28.63. MS (ESI) $C_{14}H_{18}N_2O_2Br_2$ m/z 407.0 (100%, (M + H$^+$)), 405.0 (100%, (M − H^{--})).

*(E)-N-(5-Amino-pentyl)-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **14**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.81 (s, 2H), 7.41−7.37 (d, J = 15.6 Hz, 1H), 6.60−6.56 (d, J = 15.6 Hz, 1H), 3.90 (s, 3H), 3.35−3.30 (m, 2H), 2.76−2.72 (t, J = 7.3 Hz, 2H), 1.63−1.56 (m, 4H), 1.46−1.41 (m, 2H). ^{13}C NMR (MeOD, 75 MHz): δ 168.61, 156.99, 138.82, 163.17, 133.78, 124.89, 120.32, 62.11, 42.76, 41.34, 32.84, 30.93, 25.99. MS (ESI) $C_{15}H_{20}N_2O_2Br_2$ m/z 421.0 (100%, (M + H$^+$)), 419.0 (100%, (M − H^{--})).

*(E)-3-(3,5-Dibromo-4-methoxy-phenyl)-N-(3-pyrrolidin-1-yl-propyl)-acrylamide **15**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.81 (s, 2H), 7.42−7.38 (d, J = 15.8 Hz, 1H), 6.59−6.55 (d, J = 15.8 Hz, 1H), 3.90 (s, 3H), 3.38−3.34 (t, J = 7.0 Hz, 2H), 2.61−2.55 (m, 6H), 1.86−1.82 (m, 6H). ^{13}C NMR (MeOD, 75 MHz): δ 168.55, 157.10, 138.89, 136.22, 133.81, 124.86, 120.37, 62.08, 55.85, 39.82, 30.41, 25.03. MS (ESI) $C_{17}H_{22}N_2O_2Br_2$ m/z 446.9 (100%, (M + H$^+$)), 445.2 (100%, (M − H^{--})).

*(E)-3-(3,5-Dibromo-4-methoxy-phenyl)-N-(3-morpholin-4-yl-propyl)-acrylamide **16**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.69 (s, 2H), 7.30−7.26 (d, J = 15.6 Hz,1), 6.47−6.43 (d, J = 15.6 Hz, 1H), 3.78 (s, 3H), 3.62−3.60 (t, J = 4.5 Hz, 4H), 3.27−3.24 (t, J = 6.8 Hz, 2H), 2.42 (m, 4H), 2.38−2.34 (t, J = 7.3 Hz, 2H), 1.70−1.66 (t, J = 7.0 Hz, 2H). ^{13}C NMR (MeOD, 75 MHz): δ 168.55, 157.02, 138.87, 136.14, 133.80, 124.85, 120.33, 68.15, 62.09, 58.11, 55.32, 39.57, 27.11. MS (ESI) $C_{17}H_{22}N_2O_3Br_2$ m/z 463.1 (100%, (M + H$^+$)), 461.0 (100%, (M − H^{--})).

*(E)-N-{3-[(3-Amino-propyl)-methylamino]-propyl}-3-(3,5-dibromo-4-methoxy-phenyl)-phenyl)-acrylamide **17**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.80 (s, 2H), 7.42−7.37 (d, J = 15.7 Hz, 1H), 6.59−6.54 (d, J = 15.7 Hz, 1H), 3.89 (s, 3H), 2.73−2.68 (t, J = 6.8 Hz, 2H), 2.48−2.44 (m, 4H), 2.27 (s, 3H), 1.80−1.66 (m, 4H). ^{13}C NMR (MeOD, 75 MHz): δ 168.56, 157.05, 138.81, 136,19, 133.75, 124.87, 120.32, 62.06, 57.23, 43.08, 41.70, 39.74, 30.99, 28.57. MS (ESI) $C_{17}H_{25}N_3O_2Br_2$ m/z 464.1 (100%, (M + H$^+$)), 462.0 (100%, (M − H^{--})).

*(E)-N-{2-[2-(2-Amino-ethylamino)-ethylamino]-ethyl}-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **18**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.82 (s, 2H), 7.43−7.39 (d, J = 15.8 Hz, 1H), 6.62−6.58 (d, J = 15.8 Hz, 1H), 3.90 (s, 3H), 3.47 (t, J = 7.3 Hz, 2H), 2.85−2.74 (m, 10H). ^{13}C NMR (MeOD, 75 MHz): δ 168.99, 157.13, 139.07, 136.21, 133.87, 124.84, 120.37, 62.12, 51.97, 42.12, 41.2, 34.86, 27.12, 26.84. MS (ESI) $C_{16}H_{24}N_4O_2Br_2$ m/z 465.1 (100%, (M + H$^+$)), 463.0 (100%, (M − H^{--})).

*(E)-N-(2-{2-[2-(2-Amino-ethylamino)-ethylamino]-ethylamino}-ethyl)-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **19**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.83 (s, 2H), 7.43−7.39 (d, J = 15.6 Hz, 1H), 6.61−6.58 (d, J = 15.6 Hz, 1H), 3.90 (s, 3H), 3.48−3.44 (t, J = 6.3 Hz, 2H), 2.82−2.61 (m, 14H). ^{13}C NMR (MeOD, 75 MHz): δ 168.82, 157.09, 138.94, 136.23, 133.85, 124.94, 120.37, 62.12, 53.27, 42.53, 35.58, 34.87, 26.89. MS (ESI) $C_{18}H_{29}N_5O_2Br_2$ m/z 508.1(100%, (M + H$^+$)), 506.2 (100%, (M − H^{--})).

*(E)-N-{3-[4-(3-Amino-propoxy)-butoxy]-propyl}-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **20**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.68 (s, 2H), 7.29−7.25 (d, J = 15.8 Hz, 1H), 6.47−6.43 (d, J = 15.8 Hz, 1H), 3.77 (s, 3H), 3.54−3.46 (m, 8H), 3.38 (t, J = 7.0 Hz, 2H), 2.64−2.60 (t, J = 7.0 Hz, 2H), 1.73−1.70 (m, 2H), 163−1.59 (m, 2H), 1.53−1.51 (m, 4H). ^{13}C NMR (MeOD, 75 MHz): δ 168.59, 157.12, 138.84, 136.29, 133.83, 124.97, 120.39, 72.66, 70.89, 70.28, 62.09, 40.99, 38.99, 34.38, 31.41, 28.43. MS (ESI) $C_{20}H_{30}N_2O_4Br_2$ m/z 523.0 (100%, (M + H$^+$)), 521.2 (100%, (M − H^{--})).

*(E)-N-{3-[Bis(3-amino-propyl)-amino]-propyl}-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **21**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.82 (s, 2H), 7.42−7.38 (d, J = 15.8 Hz, 1H), 6.60−6.56 (d, J = 15.8 Hz, 1H), 3.90 (s, 3H), 2.73−2.69 (m, 4H), 2.56−2.51 (m, 8H), 1.70−1.63 (m, 6H). ^{13}C NMR (MeOD, 75 MHz): δ 168.56, 157.11, 138.86, 136.26, 133.82, 124.94, 120.39, 62.10, 53.71, 53.38, 41.84, 39.86, 31.22, 28.49. MS (ESI) $C_{19}H_{30}N_4O_2Br_2$ m/z 507.1 (100%, (M + H$^+$)), 505.1 (100%, (M − H^{--})).

*(E)-N-{3-[4-(3-Amino-propyl)-piperazin-1-yl]-propyl}-3-(3,5-dibromo-4-methoxy-phenyl)-acrylamide **22**.* ^{1}H NMR (MeOD, 400 MHz): δ 7.80 (s, 2H), 7.40−7.36 (d, J = 15.6 Hz, 1H), 6.59−6.55 (d, J = 15.6 Hz, 1H), 3.89 (s, 3H), 2.89−2.87 (m, 2H), 2.55−2.48 (m, 14H), 1.78 (m, 4H). ^{13}C NMR (MeOD, 75 MHz): δ 168.62, 157.14, 138.89, 136.25, 133.83, 124.93, 120.39, 62.10, 57.93, 54.76, 54.68, 41.41, 39.84, 28.24, 28.08.

4.3. Bacterial Strains. Four bacterial strains were used in this study: *S. aureus* DSM799, *P. aeruginosa* PAO1 (ATCC 15692), *E. aerogenes* EA289, a Kan derivative of the MDR clinical isolate EA27,[27] and *K. pneumoniae* KPC2 ST258 (Poland). *P. aeruginosa* clinical isolates (PA-LAV) were obtained from E. Garnotel (Laveran Hospital). Strains were maintained at −80 °C in 15% (v/v) glycerol for cryoprotection. Bacteria were routinely grown in Mueller−Hinton (MH) broth at 37 °C.

4.4. Antibiotics. Chloramphenicol and cefepime were purchased from Sigma (St. Quentin Fallavier, France). Doxycycline was purchased from TCI Europe. All antibiotics were dissolved in dimethyl sulfoxide (DMSO), and DMSO concentrations up to 5% v/v did not detrimentally affect bacterial growth.

4.5. Antibiotic Susceptibility Testing. The susceptibility of bacterial strains to antibiotics and compounds was determined in microplates using the standard broth dilution method in accordance with the recommendations of the Commité de l'AntibioGramme de la Société Française de Microbiologie (CA-SFM).[28] Briefly, the MICs were determined with an inoculum of 10^5 CFU in 200 μL of MH broth containing 2-fold serial dilutions of each drug. The MIC was defined as the lowest concentration of drug that completely inhibited visible growth after incubation for 18 h at 37 °C. To determine all MICs, the measurements were independently repeated at least three times.

4.6. Determination of the MICs of Antibiotics in the Presence of Synergizing Compounds. Briefly, restoring enhancer concentrations was determined with an inoculum of 10^5 CFU in 200 μL of MH broth containing 2-fold serial dilutions of each derivative in the presence of the desired antibiotic (chloramphenicol, doxycycline, or cefepime; 2 μg/mL). The lowest concentration of ianthelliformisamine derivative that completely inhibited visible growth after incubation for 18 h at 37 °C was determined. These measurements were independently repeated at least three times.

4.7. Measurement of ATP Release. Solutions of compound **21** in doubly distilled water were prepared at different concentrations. A suspension of growing bacteria in MH broth was prepared and incubated at 37 °C. Nine hundred microliters of this suspension was added to 100 μL of compound **21** to obtain the indicated final concentration of **21** in the presence or absence of doxycycline. After 5 min, an aliquot of 100 μL of this mixture was collected and vortexed for 1 s. Fifty microliters of luciferin−luciferase reagent (Yelen, France) was immediately added, and the luminescent signal was quantified using a Lucy luminometer (Yelen, France) for 5 s. The ATP concentration was quantified by the addition of an internal standard. Maximum ATP release was obtained with a 0.02% solution of tetramethylammonium bromide (TMAB).

4.8. Membrane Depolarization Assays. Bacteria were grown in MH broth for 24 h at 37 °C and centrifuged at 10 000 rpm at 20 °C. The supernatant was discarded, and the bacteria were washed twice with buffered sucrose solution (250 mM) and magnesium sulfate solution (5 mM). The fluorescent dye, 3,3-diethylthiodicarbodyanine iodide, was added to a final concentration of 3 μM, and it was allowed to penetrate into bacterial membranes during a 1 h incubation at 37 °C. Compound **21** was then added at different concentrations. Fluorescence measurements were performed using a Jobin Yvon Fluoromax 3 spectrofluorometer with slit widths of 5/5 nm. The relative corrected fluorescence (RCF) was recorded at time intervals of 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 min. The maximum RCF was considered to be that recorded with a pure solution of the fluorescent dye in buffer (3 μM).

4.9. Nitrocefin Hydrolysis Assay. Outer membrane permeabilization was measured using nitrocefin as a chromogenic substrate of periplasmic β-lactamase. Ten milliliters of MH broth was inoculated with 0.1 mL of an overnight culture of PA01 and grown at 37 °C until the OD600 reached 0.5. The remaining steps were performed at room temperature. Cells were recovered by centrifugation (4000 rpm for 20 min) and washed once in 20 mM potassium phosphate buffer (pH 7.2) containing $MgCl_2$ (1 mM). After a second centrifugation, the pellet was resuspended and adjusted to a OD600 of 0.5. Then, 50 μL of either polymyxin B (positive control) or compound **21** was added to 100 μL of the cell suspension to obtain a final concentration varying from 0.98 to 500 μM. Fifty microliters of nitrocefin was then added to obtain a final concentration of 50 μg/mL. Nitrocefin hydrolysis was monitored spectrophotometrically by measuring the increase in absorbance at 490 nm. Assays were performed in 96-well plates using a M200 Pro Tecan spectrophotometer.

4.10. Cytotoxicity toward Chinese Hamster Ovary Cells and Normal Human Fibroblasts. CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France) and human fibroblasts (Clinisciences, Paris, France) were maintained in McCoy's 5A and DMEM media, respectively, supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 (U/mL)/10 μg/mL penicillin/streptomycin. They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cell lines were seeded in 96-well plates and incubated overnight. Various concentrations of compounds were incorporated in triplicate cultures, and the cells were incubated at 37 °C for 24 h. At the end of the incubation period, cells were submitted to three successive washes in phosphate buffer saline (PBS) and incubated in PBS containing 10% WST-1 for an additional 30 min. Cell viability was evaluated by measuring WST-1 absorbance at 450 nm in a microplate spectrophotometer. The results are expressed as the percentage of cell viability compared to that of the control (culture medium only), which corresponded to 100% cell viability. Dose−response curves were calculated by nonlinear regression analysis using TableCurve V2 software. The inhibitory concentration 50% (IC_{50}) was defined as the concentration of saponin that induced a 50% decrease in cell viability.

■ ASSOCIATED CONTENT

Supporting Information

Analytical data of all compounds and biological test systems. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PDR, pandrug resistance; TMAB, tetramethylammonium bromide

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