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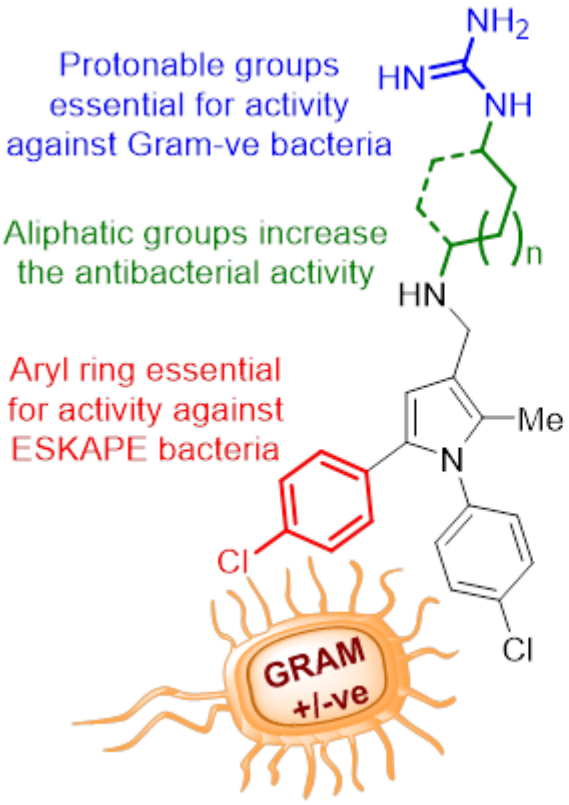

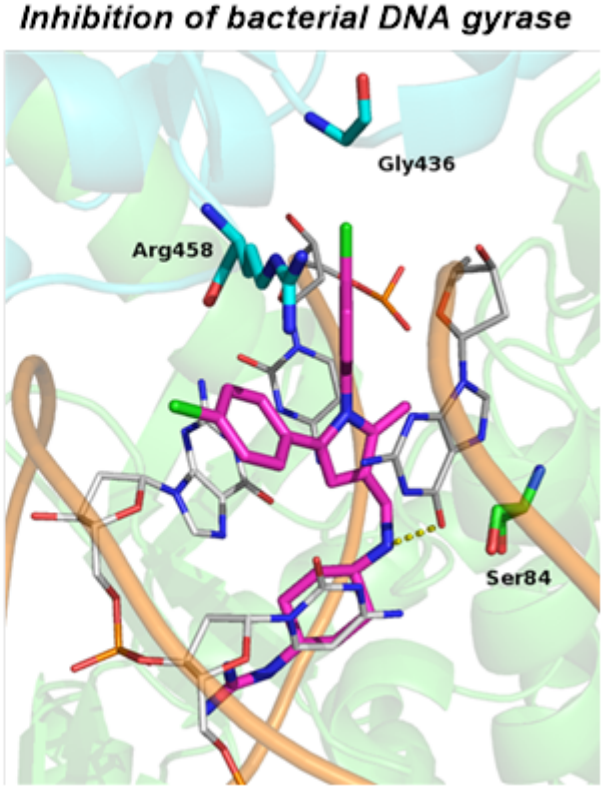

Switching on the activity of 1,5-diaryl-pyrrole derivatives against drug-resistant ESKAPE bacteria: structure-activity relationships and mode of action studies

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ABSTRACT

Antibiotic resistance represents a major threat worldwide. Gram-positive and Gram-negative opportunistic pathogens are becoming resistant to all known drugs mainly because of the overuse and misuse of these medications and the lack of new antibiotic development by the pharmaceutical industry. There is an urgent need to discover structurally innovative antibacterial agents for which no pre-existing resistance is known. This work describes the identification, synthesis and biological evaluation of a novel series of 1,5-diphenylpyrrole compounds active against a panel of ESKAPE bacteria. The new compounds show high activity against both wild type and drug-resistant Gram+ve and Gram-ve pathogens at concentrations similar or lower than levofloxacin. Microbiology studies revealed that the plausible target of the pyrrole derivatives is the bacterial DNA gyrase, with the pyrrole derivatives displaying similar inhibitory activity to levofloxacin against the wild type enzyme and retaining activity against the fluoroquinolone-resistant enzyme.

Keywords. Antimicrobial resistance, ESKAPE bacteria, drug resistance, pyrrole, DNA gyrase

1. Introduction

The treatment of infectious diseases, especially those caused by drug-resistant bacterial opportunistic pathogens, is becoming a major threat for the World Healthcare Systems [1,2]. For decades, bacterial infections have been successfully treated with a plethora of antibiotics developed mainly between the 1940s and the 1960s [3], so that at the end of the 1960s, many experts were confident that the war against bacterial infections was definitely won and that it was time to "*close the book on infectious diseases*" [4].

However, many decades after the first patients were treated with antibiotics, bacterial infections have again become a global threat due to the rapid emergence of drug resistant bacteria, which undermine the efficacy of available antibiotics worldwide. The antibiotic resistance crisis has been attributed mainly to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry, due to poor economic incentives and challenging regulatory requirements [5]. Antibiotic resistance is a serious threat to Global Public Health and the treatments available to date are often ineffective, especially against infections caused by Gram-negative bacteria.

Opportunistic pathogens such as the Gram-negative (Gram-ve) pathogens *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli* or the Gram-positive (Gram+ve) bacteria *Staphylococcus aureus* and *Enterococcus faecalis/faecium* are widespread around the world and are becoming resistant to all known drugs. *E. coli* infections in some parts of the world frequently resist antibiotic treatments with third generation cephalosporins [6], making many urinary tract infections, usually regarded as merely a nuisance, very difficult to treat. Many epidemic *K. pneumoniae* strains have become resistant to carbapenem antibiotics [7] as well as to fluoroquinolones [8]. Countries worldwide reported that half or more of *Staphylococcus* infections are resistant to methicillin (MRSA strains) [9,10] whilst infections caused by *P. aeruginosa* represent a global emergency due to the ability to rapidly develop resistance during the course of treating an infection [11,12].

In order to face up to the emergency of antibacterial resistance several simultaneous actions can be taken: 1) to reduce the misuse of antibiotics, 2) to develop alternatives to antibiotics or use antibiotics in combination, 3) to develop appropriate antibiotic dosing regimens that reduce the selection of resistance [13-18]. However, the best solution to the bacterial drug-resistance problem would be to discover and develop novel classes of antibiotics for which there is no pre-existing resistance among human bacterial pathogens [19].

The discovery of new antibiotics is a major challenge, and the rate of successfully discovering new and especially broad spectrum antibiotic hits is low [14,20]. With the aim to speed up the drug discovery process and to reduce the limitations of target-based high-throughput screening (HTS) protocols and compound collections, we recently adopted an alternative strategy, based on

the molecular hybridization of known drugs, to design new antitubercular drugs [21]. The *in-silico* hybridization of the old antitubercular pyrrole BM212 [22] with SQ109 [23], a new antitubercular drug currently undergoing clinical phase II trials, led to the discovery of the pyrrole **1** which showed potent activity against various mycobacteria, including multi drug-resistant (MDR) tuberculosis strains (Fig. 1). A structure-activity relationship (SAR) study revealed that the 2,5-dimethylpyrrole derivatives of **1** possess high antitubercular activity, whilst the 1,5-diphenylpyrrole analogues, such as **2**, were poorly active against the same mycobacterial strains.

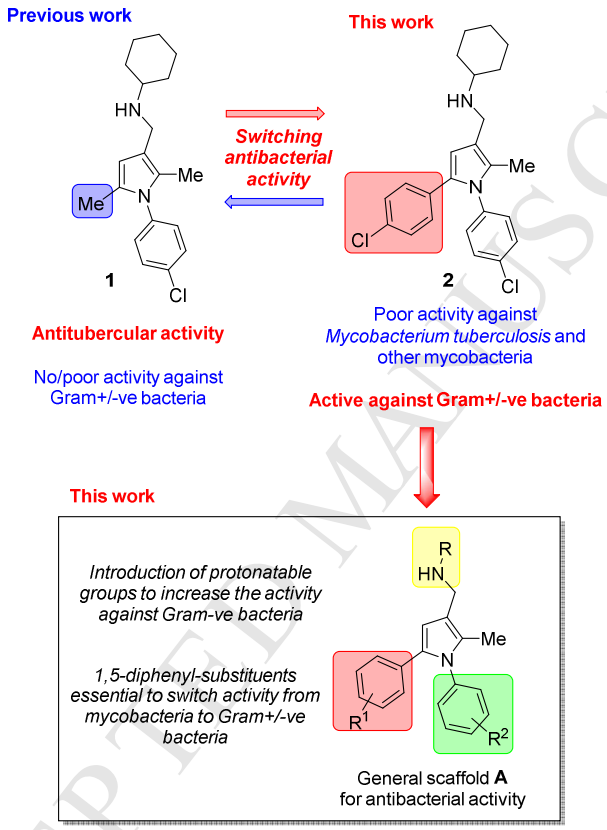


Fig. 1. Structure of pyrroles **1** and **2** and SAR evaluation plan.

Despite the phylogenetic and structural differences between mycobacteria and Gram+/-ve bacteria, we explored the antimicrobial spectrum of **1** against a panel of Gram+/-ve pathogens. Pyrrole **1** was screened against a selection of bacteria including MRSA strains, vancomycin resistant *Enterococcus*strains (VRE) and other Gram-ve microorganisms such as *P. aeruginosa*, *K. pneumoniae* and *E. coli*, but, disappointingly, it showed no or moderate inhibition of growth of various bacteria (Tables 2-3). On the other hand, the 1,5-diarylpyrrole **2** derivative, poorly active against mycobacteria, showed a very good antibacterial profile, being able to inhibit growth of a number of Gram+ve strains at micromolar concentrations. Initial studies [24] carried out on previously synthesised pyrrole derivatives clearly indicated that the replacement of the

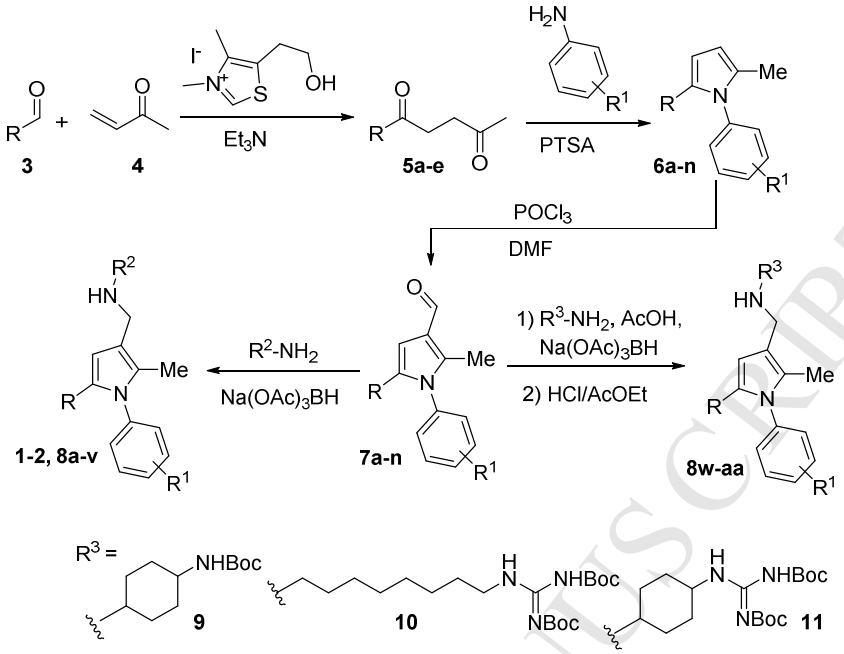
methyl group on C5 of the pyrrole **1** with a phenyl ring was a key element to switch the selectivity and the antimicrobial activity from mycobacteria to Gram+ve bacteria.

Herein, we describe the design, synthesis and discovery of a new series of 1,5-diarylpyrrole derivatives with general structure **A** (Fig. 1), highly active against both susceptible and drug-resistant Gram+/-ve bacteria. The chemical space around the pyrrole nucleus of **1-2** was explored and the key SAR responsible for the activity are disclosed. The bactericidal/bacteriostatic activity of the new compounds was also investigated. Finally, a mechanism of action for the new compounds is proposed as the new pyrroles proved to inhibit the bacterial DNA gyrase at concentrations similar to levofloxacin.

2. Results and discussion

2.1. Chemistry

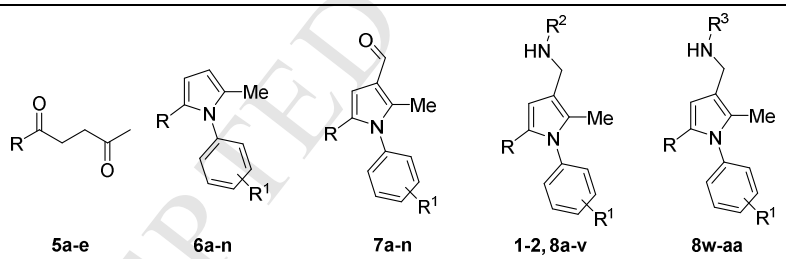
A library of pyrrole derivatives **8** bearing different substituents on the *N*-phenyl rings at N1 and C5 positions as well as different amine-side chains at C3, was synthesized (Scheme 1 and Table 1). The 2,5-hexandiones **5** were first obtained through Stetter reaction of different aldehydes **3** with methylvinyl ketone **4** [25]. Paal-Knorr condensation of diketones **5** with appropriate anilines led to pyrroles **6** [26] which were in turn formylated in the presence of $POCl_3$ and DMF to afford aldehydes **7**. Finally, the reductive amination of **7** with different amines in the presence of $Na(OAc)_3BH$ as reducing agent and stoichiometric AcOH led to desired pyrrole derivatives **8a-8v** in high yields. Derivatives **8w-aa** were synthesized from aldehydes **7** via a slightly modified reductive amination procedure using the Boc-protected amines **9-11**, followed by Boc cleavage with HCl/AcOEt. Pyrroles **1** and **2** were synthesized as previously reported [19].



Scheme 1. Synthesis of pyrroles **1**, **2** and **8a-aa**

Table 1.

Structures of pyrroles **1, 2** and **8a-aa**.



Diketones 5	Pyrroles 6 & Aldehydes 7	Pyrrole compounds 8	R	R^1	R^2 or R^3
5a	**6a, 7a**	**1**	Me	4-Cl	Cyclohexyl
5b	**6b, 7b**	**2**	4-Cl-Ph	4-Cl	Cyclohexyl
5a	**6a,7a**	**8a**	Me	4-Cl	Bn
5b	**6b, 7b**	**8b**	4-Cl-Ph	4-Cl	Bn
		8c	4-Cl-Ph	4-Cl	Cycloheptyl

		8d	4-Cl-Ph	4-Cl	2-adamantyl
		8e	4-Cl-Ph	4-Cl	Cyclohexylmethyl
		8f	4-Cl-Ph	4-Cl	iPr
		8g	4-Cl-Ph	4-Cl	α-methyl-Bn
		8h	4-Cl-Ph	4-Cl	$PhNHCH_2CH_2$
	6c, 7c	**8i**	4-Cl-Ph	4-F	Cyclohexyl
		8j	4-Cl-Ph	4-F	Cycloheptyl
	6d, 7d	**8k**	4-Cl-Ph	4-iPr	Cycloheptyl
	6e, 7e	**8l**	4-Cl-Ph	2,5-Me	Cyclohexyl
	6f, 7f	**8m**	4-Cl-Ph	3-F	Cyclohexyl
	6g, 7g	**8n**	4-Cl-Ph	2,4-Cl	Cyclohexyl
	6h, 7h	**8o**	4-Cl-Ph	4-MeO	Cyclohexyl
	6i, 7i	**8p**	4-Cl-Ph	$4\text{-}NO_2$	Cyclohexyl
	6j, 7j	**8q**	4-Cl-Ph	$2\text{-}CF_3$	Cyclohexyl
	6k, 7k	**8r**	4-Cl-Ph	4-OH	Cyclohexyl
5c	**6l, 7l**	**8s**	4-*i*Pr-Ph	4-Cl	Cyclohexyl
5d	**6m, 7m**	**8t**	4-F-Ph	4-Cl	Cyclohexyl
5e	**6n, 7n**	**8u**	4-^{t}Bu-Ph	4-Cl	Cyclohexyl
5b	**6b, 7b**	**8v**	4-Cl-Ph	4-Cl	4-OH-cyclohexyl
		8w	4-Cl-Ph	4-Cl	$4\text{-}NH_2$-cyclohexyl
		8x	4-Cl-Ph	4-Cl	Guanyl-$(CH_2)_8$-
		8y	4-Cl-Ph	4-Cl	4-guanidine-cyclohexyl
	6i, 7i	**8z**	4-Cl-Ph	$4\text{-}NO_2$	4-guanidine-cyclohexyl
5a	**6a, 7a**	**8aa**	Me	4-Cl	Guanyl-$(CH_2)_8$-

2.2. Biological evaluation and SAR analysis.

All the compounds were tested for their biological activity by determining the minimum inhibitory concentrations (MIC) against a panel of susceptible and drug-resistant Gram+ve and Gram-ve bacterial strains (ESKAPE) (Tables 2-3). The Gram+ve panel includes the methicillin-susceptible (NCTC 6571) and the methicillin-resistant EMRSA15 (NCTC 13616, containing the gyrA S84L and parC S80F mutations) and EMRSA16 (NCTC 13277, containing the gyrA S84F and parC S80F mutations) *S. aureus* as well as the vancomycin-susceptible (VSE) (NCTC 775) and vancomycin-resistant (VRE) (NCTC 12201 and NCTC 12204) *Enterococcus spp*. The Gram-ve panel includes drug susceptible and drug resistant strains of *K. pneumoniae* (NCTC 13368 and M6), *A. baumannii* (AYE and ATCC 17978), *P. aeruginosa* (PA01 and NCTC 13437) and *E. coli* (NCTC 12923).

2.2.1. Antibacterial activity against Gram+ve strains.

We first analysed the data arising from the screening of pyrroles **8a-aa** on Gram+ve bacteria. The results are reported in Table 2.

Table 2.

Antibacterial activity of pyrroles **1**, **2** and **8a-aa** against Gram+ve bacteria (MSSA, MRSA, VSE and VRE).

Cmpd	**MIC μg/mL**					
	MSSA	**MRSA**		**VSE**	**VRE**	
	NCTC 6571	**NCTC 13616**	**NCTC 13277**	**NCTC 775**	**NCTC 12201**	**NCTC 12204**
1	64	64	64	32	32	16
2	4	4	4	4	4	2
8a	>128	>128	>128	>128	>128	128
8b	8	8	4	4	4	ND
8c	4	4	4	4	2	2
8d	4	4	4	4	2	2
8e	4	4	4	2	4	2
8f	8	8	8-16	8	8	4

8g	4	4	4	2-4	2-4	2
8h	4-8	4-8	4-8	4	4	4-8
8i	8	8	8	4	4	4
8j	4	8	4	4	4	2
8k	4	4	4	2	2	2
8l	4	4	8	4	4	2
8m	8	8	8	8	4	4
8n	4	4	4	4	4	2
8o	16	16	16	8	8	8
8p	8	16	16	8	8	4
8q	4	8	8	4	4	2
8r	32	8-64	32	64	32	32
8s	4	4	4	4	4	2
8t	8	8	8	8	4	4
8u	4	4	4	4	2	2
8v	16	16	16	16	16	8
8w	8	8	8	8	8	4
8x	4	4	4	8	8	4
8y	2	2-4	4	8	16	4
8z	4	8	8	32	64	16
8aa	4	8	8	8-16	16	4-8
Levofloxacin	0.12	8	8	1	1	0.5

In agreement with preliminary results, the insertion of a second aryl ring at C5 on the pyrrole nucleus proved to be fundamental for the antibacterial activity. In fact, the 2,5-dimethyl pyrroles **1** and **8a**, both endowed with antitubercular activity, showed no or poor inhibition of growth of Gram+ve strains, whilst their corresponding 1,5-diaryl-pyrroles **2** and **8b** proved to be active with MIC values of 4-8 μg/mL against both drug susceptible and drug resistant *Staphylococcus*

and *Enterococcus* strains with a microbiology profile similar to levofloxacin. The presence of a cyclohexylamine (**2**) rather than a benzyl (**8b**) side chain at C3 of the pyrrole nucleus appears to be slightly beneficial to improve the activity against MSSA and MRSA strains. The replacement of the cyclohexyl ring in **2** with bulkier aliphatic groups (cycloheptyl, adamantyl and cyclohexylmethyl) led to **8c-e** which showed an MIC = 2 μg/mL against VRE bacteria similar or better than levofloxacin. A similar trend was observed with derivatives **8j-k** bearing a cycloheptylamine side chain. In general, the presence of a cyclic and bulky aliphatic amine side chain is beneficial in improving the activity, especially against VRE strains. As further corroboration of this observation, compound **8g**, a branched and bulkier analogue of **8b**, also showed a slightly improved antibacterial profile against VRE. On the other hand, the presence of a smaller or aromatic side chain on the pyrrole ring led to compounds **8f** and **8h** with lower activity against MSSA and MRSA strains. The compounds **8i-r** were synthesized with the aim to explore the influence on the antibacterial activity of different substituents on the *N*-phenyl ring. In general, all the compounds showed very good activity, especially against VRE strains. The presence of a halogen substituent or an alkyl group on the *N*-phenyl ring favors the improvement of the activity against MSSA and EMRSA (compounds **8j**, **8k**, **8n**, MIC = 4 μg/mL), whilst electron-withdrawing (**8p**) or electron-donating (**8o**, **8r**) substituents led to a decrease of activity. However, no electronic effect of the substituents on the phenyl ring was observed and compounds bearing electron-withdrawing or electron-donating groups showed similar antibacterial activity. Compounds **8s-u**, synthesized to explore the chemical space around the phenyl ring at C5, also showed good activity against VRE bacteria (MIC = 2 μg/mL). The cyclohexylamine side chain of **2** was finally replaced with more hydrophilic and polar groups in compounds **8v-aa**. Compounds **8v-w** bearing a diaminocyclohexyl or hydroxy-cyclohexylamine side chain at C3 showed lower activity than **2** against all Gram+ve bacteria, whilst the guanidine derivative **8y** showed a good activity against MSSA and MRSA strains (MIC = 2-4 μg/mL). Compound **8x** bearing an amino-octylguanidine chain showed good activity against MSSA and MRSA (MIC = 4 μg/mL), whilst the presence of a nitro substituent on the *N*-phenyl ring in **8z** proved to be detrimental. Finally, the 2,5-dimethylpyrrole **8aa** showed worse antibacterial activity than its 1,5-diaryl-pyrrole analogue **8w**, further confirming the key role of an aryl substituent on C5.

2.2.2. Antibacterial activity against Gram-ve strains

The pyrrole compounds **1**, **2** and **8a-aa** were then assayed against a panel of Gram-ve bacteria. Data are reported in Table 3 [27].

Table 3.

Antibacterial activity of pyrroles **1**, **2** and **8** against Gram-ve bacteria.

Cmpd	MIC μg/mL						
	K. pneumoniae		***A. baumannii***		***P. aeruginosa***		***E. coli***
	NCTC 13368	**M6**	**AYE**	**ATCC 17978**	**PA01**	**NCTC 13437**	**NCTC 12923**
1	>64	>64	>64	>64	>64	>64	-
2	>128	>128	128	64	>128	>128	>128
2+PMBN	>128	64	8	4	>128	32	-
8b	>128	>128	>128	>128	>128	>128	>128
8b+PMBN	>128	32	16	16	>128	>128	>128
8k	>128	>128	>128	32	>128	>128	>128
8k+PMBN	>128	>128	4	2	16	16	2
8k+PAβN	>128	>128	4	4	128	>128	8
8s	>128	>128	>128	32	>128	>128	>128
8s+PMBN	>128	128	8	2	8	32	2
8s+PAβN	>128	>128	8	8	>128	>128	16
8v	>128	>128	128	64	>128	>128	>128
8w	32	16	32	16	32	32	8
8x	32	16	32	8	128	64	8
8y	128	32	64-128	16	128	16	8
8y+PMBN	128	64	32	16	128	16	4
8y+PAβN	128	64	64	16	128	64	8
8z	>128	>128	>128	64	>128	>128	64
8aa	>128	128	64	16	>128	64	16
Levofloxacin	1	0.12	8	0.12	2	64	<0.12

The 2,5-dimethylpyrrole **1** and the 1,5-diaryl-pyrroles **2** and **8b** proved to be inactive against the whole panel of Gram-ve bacteria at the highest concentration tested (128 μg/mL). The low activity of these derivatives could be ascribable to two major factors, namely 1) the chemical structure of the pyrroles derivatives which prevents the binding to any bacterial target in Gram-ve bacteria, or 2) the low permeability of the Gram-ve outer membrane (OM) which prevents the pyrrole derivatives from reaching their molecular target. Thus, with the aim to understand if pyrrole compounds were simply unable to cross the OM, **2** and **8b** were assayed in combination with polymyxin B nonapeptide (PMBN). PMBN is a modification of polymixin B which lack the acyl chain important for antibiotic activity but is still able to permeabilize the outer membrane of Gram-ve bacteria and to allow lipophilic substances to penetrate inside the bacterial cell. As such, whilst it retains some antimicrobial activity, it is much less active than polymixin B. The conditions used for these experiments are selected such that the single PMBN concentration used for permeabilization is not antibacterial in its own right and indeed does not impact at all on the growth of the strains being tested. Interestingly, both **2** and **8b** showed improved antibacterial activity when used in combination with PMBN. In particular pyrrole**2** proved to be very active against *A. baumannii*, with MIC = 4-8 μg/mL and promisingly active against the MDR *P. aeruginosa* strain NCTC 13437 with MIC = 32 μg/mL. Pyrrole **8b** proved to be less active than **2**, also confirming the previous SAR observation that a cyclohexylamino side chain on the pyrrole ring is preferable to a benzylamino group. The experiments carried out with PMBN confirmed our initial hypothesis that the 1,5-diaryl-pyrroles **2** and **8b** are active against some Gram-ve species but cannot enter the cells due to the presence of the outer membrane barrier. The OM in Gram-ve bacteria has a major impact on the susceptibility of the microorganism to antibiotics which act on intracellular targets. Small hydrophilic antibiotics, such as β-lactams, use the porins to gain access to the cell interior, while macrolides and other hydrophobic drugs diffuse across the lipid bilayer [28]. Porins are large water-filled channels in the outer membrane of Gram-ve bacteria and they act as filters for hydrophobic compounds [29]. In general, small hydrophilic molecules and charged ions can be transported by diffusion through the porins [30]. Thus, we decided to decorate the pyrrole nucleus at C3 with different hydrophilic side chains bearing protonatable amine and guanidine moieties. Compound **8w**, designed as an analogue of **2** and containing an extra amine group on the cyclohexyl group, did show an improved antibacterial activity against the whole panel of Gram-ve bacteria with MIC = 8-32 μg/mL. Pyrrole **8y**, bearing a guanidine group on the cyclohexyl group also showed a good antibacterial profile especially against ATCC 17978 and NCTC 13437 (MIC = 16 μg/mL) and *E. coli* NCTC 12923 (MIC = 8 μg/mL). On the other hand, pyrrole **8v**, bearing a hydroxyl moiety on the cyclohexyl group, did not show any activity against Gram-ve bacteria, thus confirming the observation that a protonatable amine or guanidine moiety is essential to allow the pyrrole derivatives to cross the OM. Compound **8x** showed the best profile especially against *A. baumannii* and *E. coli* (MIC = 8 μg/mL). Finally, we compared the antibacterial activity of the 1,5-diaryl-pyrrole **8x** with the corresponding 2,5-dimethyl-pyrrole analogue **8aa**. The presence of a second phenyl ring on the pyrrole scaffold in **8x** proved again to be beneficial leading to an

increase of the antibacterial activity especially against *K. pneumoniae* (MIC = 16-32 μg/mL for **8x** versus MIC = >128 μg/mL for **8aa**). Derivatives **8k**, **8s** and **8y** were also tested in combination with PMBN and with the Resistance-Nodulation Division (RND) efflux pump inhibitor, phenylalanine-arginine β-naphthylamide (PAβN). Both **8k** and **8s** showed significantly improved MICs (4 to 128 fold potentiation) in the presence of PMBN in all strains tested, except the two *K. pneumoniae* strains where the MICs were unchanged. Both compounds also showed potentiation with the efflux pump inhibitor PAβN, in *A. baumannii* and *E. coli*, suggesting that they are efflux pump substrates in these species, but not in *K. pneumoniae* and *P. aeruginosa*. Compound **8y**, did not show any potentiation by PMBN or PAβN. This data further corroborated the observation that a hydrophilic moiety on the pyrrole scaffold is essential to allow the compound to penetrate the Gram-ve cells and dispatch their antibacterial activity.

2.3. Time kill assays and drug resistance

All the pyrroles **8** showed variable activity against Gram+/-ve strains. To determine if the compounds were bacteriostatic or bactericidal, a time kill assay was performed. Three pyrrole derivatives, namely **8k**, **8s** and **8y**, were selected on the basis of their antibacterial activities and chemical structures to carry out additional experiments**.** Compounds **8k**, **8s** and **8y** were added at 4 x MIC concentration to cultures of bacteria, and aliquots were taken at 0, 2, 4, 6 and 24 hour timepoints. Time kill assays demonstrated bacteriostatic modes of action at 4 x MIC concentrations for **8k, 8s** and **8y** in VRE at 24 hours, whilst in MRSA, **8k** and **8y** were bacteriostatic, but **8s** was observed to be bactericidal at 24 hours (Fig. 2). No resistant populations were generated during the 24 hour assay.

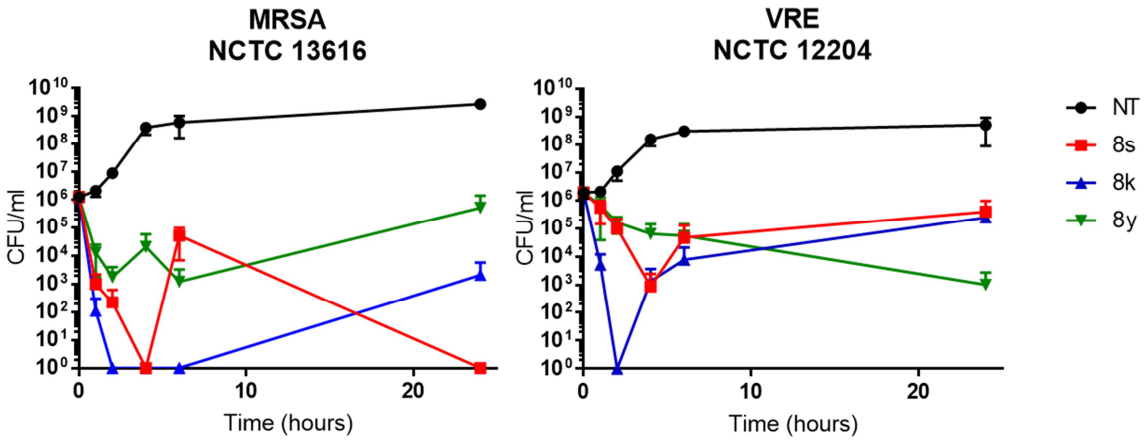


Fig. 2. Time kill analysis for **8k, 8s** and **8y** in MRSA and VRE strains. NT = no treatment control.

MRSA and VRE strains were also passaged in the presence of increasing concentrations of compounds to try to generate resistance or plated onto agar containing higher than MIC concentrations of compounds, with the aim to obtain genomic DNA for studies on the

mechanism of action of the new compounds. However, despite repeated attempts and the use of several methods to generate resistance, including broth and agar-based methods, no stable resistance emerged, whilst resistance was generated for the control antibiotic levofloxacin in both strains, using all methods. In the standard agar-based method, this equates to a frequency of $<7.6x10^{-8}$ and $<9.4x10^{-8}$ at 2xMIC for strains NCTC 13616 and NCTC 6521, respectively. In comparison, levofloxacin at 2xMIC had mutation frequencies of $1.87x10^{-5}$ and $7.08x10^{-6}$ for the same strains. Whilst this represents encouraging data, as it clearly shows the efficacy of 1,5-diphenyl-pyrroles against drug-resistant strains and low propensity to induce resistance, it should not be interpreted as being an indication that resistance will not emerge to these compounds. It also makes it difficult to use resistance emergence to generate target mutations that would help to define the mechanism of action.

2.4. Mechanism of action and computational studies

The pyrrole **2** is a structural analogue of the antitubercular agent BM212, which is known to inhibit the MmpL3 mycolic acid transporter protein in *M. tuberculosis*. However, this protein is not present in Gram+/-ve bacteria and thus a different mechanism of action had to be hypothesised. Recent literature showed that pyrrole derivatives often act as antibacterial agents through inhibition of bacterial DNA gyrase [31,32], and we were intrigued by the possibility that 1,5-diphenyl-pyrroles could act as DNA gyrase inhibitors as well.

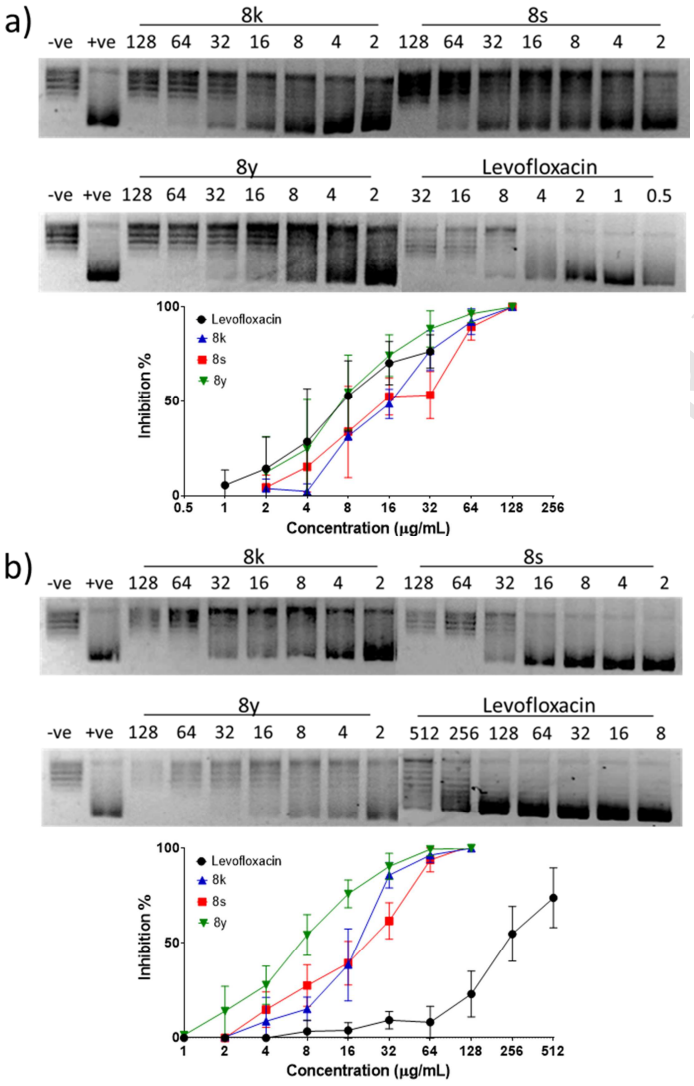


Fig. 3. Inhibition of the **a)** wild-type (*S. aureus* DNA Gyrase GyrA2B2 complex) enzyme and **b)** fluoroquinolone-resistant gyrase enzyme (*S. aureus* DNA Gyrase S84L) by **8k**, **8s** and **8y**. -ve = no enzyme added, +ve = enzyme added.

A biochemical assay to measure inhibition of the wild-type DNA gyrase enzyme (GyrA2B2 complex) from *S. aureus* was performed. Compounds **8k**, **8s** and **8y** displayed inhibitory activity against the wild-type enzyme from *S. aureus*. (Fig. 3, Table 4). In particular, pyrrole**8y** had a similar IC_{50} against the wild-type gyrase than levofloxacin, whilst **8k** was slightly less active. Because of the flat shape of the inhibitory curve for **8s**, an IC_{50} could not be calculated for this compound. The same assay was also repeated on the quinolone-resistant mutant (Ser84Leu) gyrase from *S. aureus*, which is present in most MRSA isolates [33,34]. Interestingly, unlike levofloxacin, which showed an increased IC_{50} of 196.9 µg/mL against the fluoroquinolone-

resistant enzyme, the three compounds **8k**, **8s** and **8y**maintained the high inhibitory activity observed against the wild type enzyme (Table 4). Fluoroquinolone antibiotics such as levofloxacin cannot bind to the S84L mutated DNA gyrase due to the mutational loss of the serine residue [35], but this does not appear to affect the ability of the pyrrole derivatives to bind to the enzyme. Most fluoroquinolones also bind to topoisomerase IV, but whilst levofloxacin maintained a very low IC_{50} against topoisomerase IV from *S. aureus*, compound **8y** had a much higher IC_{50} against this enzyme, suggesting a preference of the pyrrole derivatives to the gyrase enzyme over topoisomerase IV.

Table 4.

IC_{50} values of **8k**, **8s** and **8y**and levofloxacin (LVX) against DNA gyrase wild-type GyrA2B2 and mutant S84L, and topoisomerase IV. Students t-test analysis was performed for each compound compared to the levofloxacin control and the P value expressed in the table.

	DNA gyrase GyrA2B2			**DNA gyrase S84L**			**Topoisomerase IV**		
	IC_{50} (μg/mL)	**SD**	**P value**	**IC_{50} (μg/mL)**	**SD**	**P value**	**IC_{50} (μg/mL)**	**SD**	**P value**
8k	15.49	1.99	0.006	19.01	1.64	0.0011	ND	-	-
8s	NC	-	-	29.11	11.97	0.0016	ND	-	-
8y	7.48	3.13	0.55	7.23	1.26	0.009	28.8	1.47	<0.0001
LVX	8.71	0.95	-	196.9	36.59	-	3.65	0.19	-

NC = not calculable, ND = Not done, SD = Standard deviation

Since the crystal structures of the wild-type gyrases of *S. aureus* are available, we decided to further investigate and understand the exact mechanism of action of the novel pyrrole derivatives **8s**-**8y** through modelling studies. The DNA gyrase of *S. aureus* possess three potential binding sites for drugs, namely the quinolone binding site, to which levofloxacin binds, the Novel Bacterial Topoisomerase Inhibitor (NBTI) site and the ATP binding sites.[36] By docking studies, we decided to evaluate which site among the three would be more suitable for the binding of compounds **8s**-**8y**.

To study the quinolone site, we carried out by PLANTS [37] the superimposition of the studied compounds with moxifloxacin (PDB code 5CDQ) [36], QPT-1 (PDB code 5CDM) [38] and etoposide (PDB code 5CDN) [38]. The latter shared with the reported compounds an unrelated quinolone chemotype and a worthy activity against the quinolone-resistant GyrA S84L [39,40].

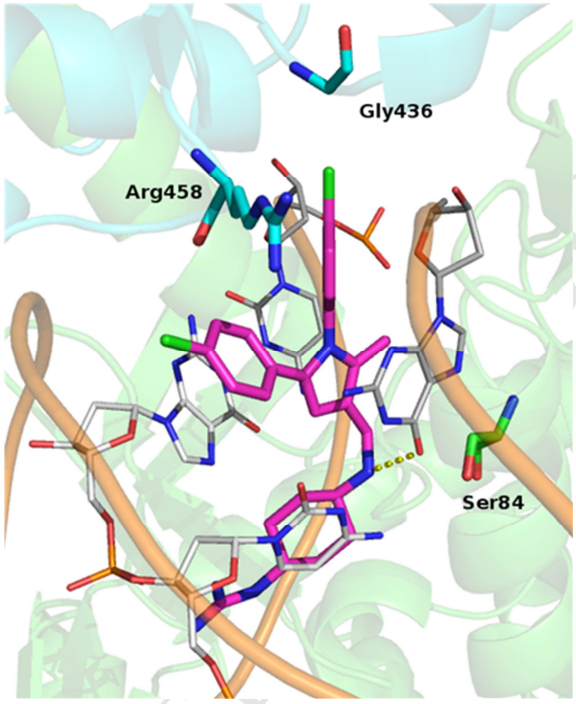


Fig. 4. PLANTS proposed binding mode of **8y** (magenta) for PDB 5CDM. The GyrA and GyrB subunits are reported in green and cyan cartoon, respectively. The DNA structure is depicted as orange cartoon, the nucleotides are in white lines. Residues involved in contacts with **8y** are reported as cyan stick. Ser84 is also showed as green stick. H-bond is depicted as a yellow dot line.

The reported compounds exhibited consistent binding modes for the studied crystal structures and showed good overlapping with the reference compounds (Supplementary data Fig. S1). Analyses of the docking results highlighted key interactions of derivative **8y**: (i) the pyrrole nucleus and the chlorophenyl ring on C5 made stacking interactions with the +1 and -1 bases at the DNA cleavage site [37]; (ii) the N1-chlorophenyl formed hydrophobic contacts with Gly436 and Arg458 side chains of the TOPRIM domain of the GyrB; (iii) the secondary amine and the cyclohexyl ring formed a H-bond and hydrophobic contacts, respectively, with the closest DNA bases; (iv) the guanidine group formed polar contacts with the DNA-sugar moiety (Fig. 4). Interestingly, the π–π interaction of the phenyl ring on C5 with the guanine nucleobase confirms

the key role of the second aromatic ring for the antibacterial activity. In fact, the dimethyl pyrrole analogues like **1**, **8a** or **8aa**, miss this extra binding, which may explain the loss of antibacterial activity.

All the compounds were also docked at the GyrA S84L enzyme. It was already reported that both QPT-1 and etoposide had better IC_{50} values for the mutated enzyme than for the wild type [39,40]. Indeed, the S48L mutation did not affect the binding mode. Furthermore, the hydrophobic Leu instead of the Ser was at bond distance to form further hydrophobic contacts. Similarly, the **8y**-**8s** binding modes were not affected by the mutation and also in these cases we observed hydrophobic contacts with the Leu84 (Supplementary data Fig. S2-S3). These results provide further confirmation of the proposed binding mode.

Docking studies of **8y**-**8s** at the NBTI site (PDB code 5BS3) were then performed [41]. In this case, the proposed binding modes were not consistent. Actually, the NBTI matched a well-defined pharmacophore model featured by a bulkier aromatic group, a linker bearing a basic nitrogen atom and an aromatic/hydrophobic moiety arranged in a linear molecular geometry [42]. Compounds **8y**-**8s** did not show any linear geometry and the most active derivative **8y** had the protonable nitrogen atom located far from the pharmacophore model. Based on these results, we suggest that **8y**-**8s** compounds do not bind the NBTI site.

Finally, the gyrase ATP site (PDB code 5D6Q) was investigated [43]. According to the reported structural requirements for binding to this site [44,45], a compound should possess at least a donor/acceptor pattern, a more or less sterically constrained linker and an acid or polar moiety [46-49]. The proposed binding modes of **8y**-**8s** did not match any feature of the pharmacophore model. These results also suggested that compounds **8y**-**8s** do not bind the ATP site.

As further confirmation to validate these results, we used the MM-GBSA method [50] to calculate the binding free energy of the **8y** best scored pose for each studied site (Supplementary data). The free energy calculation yielded the most favourable binding energy at the quinolone binding site, thus confirming, together with microbiology data, the mode of action of novel 1,5-diaryl-pyrroles.

2.5. Toxicity studies

The three lead compounds, **8y, 8k** and **8s**, were tested against the well validated cell-lines HeLa and HEK293 for toxicity to human cells. Compound **8y** offered a significant therapeutic window of around 5-15fold between the IC_{50} (11.67 - 18.9 µg/mL for the two cell-lines) and the peak activity against *S. aureus* (2 µg/mL). This was significantly greater than some of the other lead compounds (**8k** and **8s**) which showed IC_{50} values very close to the MIC (1.95 – 2.83 µg/mL and 2.4 – 3.0 µg/mL respectively). This difference in the cytotoxicity profiles for the three compounds, which have similar levels of activity against bacteria, suggests there is significant opportunity to further expand the therapeutic window through targeted modifications.

3. Conclusions

The identification of novel classes of antibacterial agents is a high priority in medicine due to the emergence of multi drug-resistant microorganisms. The vast majority of the antibiotics currently in use have been produced between 1930 and 1962 and since then almost exclusively analogues of existing classes have reached the market. The development of novel antibacterials, possibly with innovative chemical structures, with different modes of action and strongly active against drug-resistant rather than the wild-type bacteria, is highly desirable. Within this work, we have identified a new class of 1,5-diphenyl-pyrrole antibacterial compounds, designed using the antitubercular agent BM212 as template, through appropriate molecular decorations. The new compounds proved to be active against a wide panel of ESKAPE bacteria with activity similar or better than levofloxacin, while they possess only moderate antitubercular activity. Microbiology studies led to the identification of the plausible cellular target, namely the bacterial DNA gyrase. Interestingly, the pyrrole derivatives inhibit the DNA gyrase of *S. aureus* at concentrations similar than levofloxacin, and their activity is maintained against the mutated DNA gyrase form S84L, responsible for most fluoroquinolone resistance in *S. aureus.* Modelling studies pointed out that the 1,5-diphenyl-pyrroles bind the fluoroquinolones binding site in DNA gyrase, but the different chemical structures allow them to not be affected by the S84L mutation responsible for drug-resistance. Finally, when given at higher concentration than MIC (time kills and mutation frequencies) or when cultured at sub-MIC to above-MIC levels (serial-passage), the new pyrroles have not generated resistance in bacteria. This suggests that they are excellent scaffolds for new antibacterial agents that could be used alone or as part of novel combination therapies with existing antibiotics.

4. Experimental section

4.1. Chemistry

4.1.1. General

^{1}H-NMR and ^{13}C-NMR spectra were recorded with an Ascend 400 spectrometer Bruker, at room temperature (rt) operating at the frequencies indicated. Chemical shifts (δ) are in ppm, referenced to tetramethylsilane. Coupling constants (J) are reported in Hertz. Splitting patterns are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), sextet (sxt), broad (br) or some combination of them. Mass spectra (HRMS) were recorded at the EPSRC National Mass Spectrometry Service Centre on a Thermo Scientific LTQ Orbitrap XL Mass Spectrometer using low-resolution ESI or high-resolution nano ESI techniques. The purity of the compounds was assessed by reverse-phase liquid chromatography coupled with a mass spectrometer (Agilent series 1100 LC/MSD) with a UV detector at λ = 254 nm and an electrospray ionization source (ESI). HPLC analysis was carried out using a Perkin-Elmer 1100

HPLC system coupled with UV/Vis set to 254 nm. Mass spectra were acquired in positive mode scanning over the mass range of 50–1500. The following ion source parameters were used: drying gas flow, 9 mL/min; nebulize pressure, 40 psig; and drying gas temperature, 350 ºC. All target compounds possessed a purity of ≥ 95% as verified by HPLC analyses. TLC was performed using commercially available pre-coated plates and visualized with UV light at 254 nm; $KMnO_4$ was used to reveal the products. Flash column chromatography was carried out using Sigma Aldrich silica gel particle size, 40-63 µm particle size 60 Å. All reactions were conducted under a nitrogen atmosphere in oven-dried glassware unless stated otherwise. All solvents and commercially available reagents were used as received. The compound 2,5-hexandione **5a** was purchased from Sigma-Aldrich.

*4.1.2. Synthesis of differently-substituted 1,4-diketones **5***

Compounds **5b-e** were synthesized as described in the literature [22,57,58,59].

*4.1.3. General Procedure for the synthesis of pyrroles **6***

Pyrroles **6a-d, 6g, 6i, 6l, 6m** were synthesized according to a previously-described literature procedure [22,26,60,61].

The novel pyrrole compounds **6e, 6f, 6h, 6j, 6k, 6n** were prepared as followed.

The appropriate 1,4-pentanedione **5b**, **5e** (3.13 mmol) was dissolved in ethanol (5 mL) in a sealed glass tube equipped with a magnetic stirring bar. Then the suitable aniline (3.13 mmol) and *p*-toluensulfonic acid (0.22 mmol) were added. The tube was heated in the microwave reactor for 40 minutes (200W, internal temperature 180 °C, and internal pressure 200psi). After cooling down the reaction mixture was extracted with EtOAc (20 mL), washed once with a saturated solution of $NaHCO_3$ (10 mL) and twice with brine (20 mL). The organic layers were combined, dried over $MgSO_4$ and filtered. The filtrate was concentrated in vacuo and proved to be pure enough to be used in the next step without any further purification.

*4.1.3.1. 2-(4-chlorophenyl)-1-(2,5-dimethylphenyl)-5-methyl-1H-pyrrole (**6e**)*

Yield: 60 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (s, 2H), 7.10 (s, 1H), 7.08 - 7.05 (m, 2H), 7.01 (s, 1H), 7.00 - 6.97 (m, 1H), 6.41 - 6.37 (m, 1H), 6.10 (d, 1H, *J* = 3.7 Hz), 2.35 (s, 3H), 2.00 (s, 3H), 1.79 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.3, 136.5, 133.6, 132.7, 132.3, 131.8, 131.3, 130.8, 129.7, 129.4, 128.3, 128.0, 108.4, 107.4, 21.00, 16.9, 12.9 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{19}H_{18}ClN$, 296.1201; found, 296.1200.

*4.1.3.2. 2-(4-chlorophenyl)-1-(3-fluorophenyl)-5-methyl-1H-pyrrole (**6f**)*

Yield: 79 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 - 7.30 (m, 1H), 7.17 - 7.11 (m, 2H), 7.11 - 7.04 (m, 1H), 6.98 (d, 2H, *J* = 8.6 Hz), 6.94 (br d, 1H, *J* = 7.9 Hz), 6.92 - 6.87 (m, 1H), 6.35 (d, 1H, *J*

= 3.4 Hz), 6.10 (d, 1H, *J* = 2.6 Hz), 2.16 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$)δ163.9, 133.0, 132.1, 131.7 (d), 130.2 (d), 128.8, 128.3, 124.3 (d), 115.8 (d), 114.7 (d), 109.4, 108.1, 13.3 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{17}H_{13}ClFN$, 286.0793; found, 286.0786.

*4.1.3.3. 2-(4-chlorophenyl)-1-(4-methoxyphenyl)-5-methyl-1H-pyrrole (**6h**)*

Yield: 68 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, *J* = 8.8 Hz, 2H), 7.06 (d, *J* = 9 Hz, 2H), 6.99 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 9 Hz, 2H), 6.33 (d, *J* = 3.4 Hz, 2H), 6.07 (d, *J* = 3.5 Hz, 2H), 3.84 (s, 3H), 2.11 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.9, 133.1, 132.5, 132.2, 132.1, 131.5, 129.5, 128.9, 128.3, 114.4, 108.7, 107.5, 55.6, 13.3 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{18}H_{16}ClNO$, 298.0993; found, 298.0992.

*4.1.3.4. 2-(4-chlorophenyl)-5-methyl-1-(2-(trifluoromethyl)phenyl)-1H-pyrrole (**6j**)*

Yield: 44 %.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 - 7.90 (m, 1H), 7.55 (d, *J* = 8.6 Hz, 2H), 7.42 (d, *J* = 8.6 Hz, 1H), 7.32 (d, *J* = 8.6 Hz, 2H), 7.08 - 6.97 (m, 2H), 6.53 (d, *J* = 3.3 Hz, 1H), 6.06 (dd, J_1 = 3.2 Hz, J_2 = 1.0 Hz, 1H), 2.36 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.3, 151.2, 144.7, 141.8, 139.6, 135.0, 129.5, 128.9, 128.8, 128.2, 124.5, 122.8, 107.9, 106.5, 13.7 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{18}H_{13}ClF_3N$, 336.0761; found, 336.0760.

*4.1.3.5. 4-(2-(4-chlorophenyl)-5-methyl-1H-pyrrol-1-yl) phenol (**6k**)*

Yield: 72 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, *J* = 8.5 Hz, 2H), 7.02 – 6.97 (m, 4H), 6.82 (d, J = 8.6 Hz, 2H), 6.31 (d, J = 3.3 Hz, 1H), 6.07 (br. s, 1H), 4.93 (br. s, 1H), 2.11 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.0, 133.1, 132.5, 132.3, 132.2, 131.5, 129.7, 128.9, 128.3, 116.0, 108.8, 107.5, 13.3 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{17}H_{14}ClNO$, 284.0837; found, 284.0834.

*4.1.3.6. 2-(4-(tert-butyl)phenyl)-1-(4-chlorophenyl)-5-methyl-1H-pyrrole (**6n**)*

Yield: 80 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.8 Hz, 2H), 7.18 (d, *J* = 8.7 Hz, 2H), 7.11 (d, *J* = 8.8 Hz, 2H), 6.98 (d, *J* = 8.7 Hz, 2H), 6.32 (d, *J* = 3.4 Hz, 1H), 6.09 (dd, J_1 = 3.4 Hz, J_2 = 0.8 Hz, 1H), 2.13 (s, 3H), 1.27 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.8, 138.2, 134.3, 133.2, 131.4, 130.4, 129.8, 129.3, 127.5, 125.1, 108.8, 107.9, 34.5, 31.4, 13.4 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{21}H_{22}ClN$, 324.1514; found, 324.1515.

*4.1.4. General Procedure for the synthesis of Compounds **7*** [21]

$POCl_3$ (4 mmol) was added dropwise to a round-bottom flask containing ice cold DMF (5 mL) under N_2 atmosphere. After 15 min, a solution of the appropriate pyrrole **6a - n** (1 mmol) was added to the stirring solution. Then the reaction mixture was allowed to stir at 100 °C for 3 h. The reaction was monitored by TLC. After completion, the reaction was quenched with 10% w/v NaOH solution (20 mL). The reaction mixture was then diluted with EtOAc (10 mL) and washed

twice with EtOAc (10 mL) and once with brine (20 mL). The organic extracts were collected, dried over $MgSO_4$, filtered and concentrated in vacuo to give a crude that was then filtered through a pad of silica gel and evaporated under reduced pressure. The residue was then used in the next step without further purification.

*4.1.5. Synthesis of Pyrrole Derivatives **1-2**, **8a-b***

Pyrroles **1**, **2** and **8a-b** were synthesized according to literature [21].

*4.1.6. General Procedure for the synthesis of Pyrrole Derivatives **8c-v*** [21]

The appropriate aldehyde **7a-n** (1 mmol) was dissolved in 5 mL of THF in a round-bottom flask. AcOH (1 mmol) and the appropriate amine (1.2 mmol) were added to the mixture and allowed to stir at room temperature for 30 minutes before the addition of $NaB(AcO)_3H$ (3 mmol). The mixture was then allowed to stir at room temperature for 24 h. After completion, the reaction was quenched with 1 M NaOH solution (25 mL). The mixture was allowed to stir for a further 30 minutes, before the mixture was diluted with EtOAc (10 mL) and washed twice with EtOAc (10 mL) and once with brine (20 mL). The organic extracts were collected, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude residue was purified with flash chromatography (EtOAc/MeOH 9:1 v/v), affording the desired compounds **8c-v**.

*4.1.7. General Procedure for the synthesis of Pyrrole Derivatives **8w-aa*** [21]

The synthesis of the Boc-protected **8w-aa** compounds was performed according to General Procedure for the synthesis of Pyrrole Derivatives **8c-v**. Subsequently, the appropriate Boc-protected **8w-aa** (0.2 mmol) was placed in a sealed vial to which 3 mL of freshly prepared HCl/AcOEt solution was added. The mixture was stirred at room temperature for 24 h and then the solvent was removed under reduced pressure. The residue was washed several times with small portions of cold Et_2O affording the desired compounds **8w-aa** as HCl salts in quantitative yields.

4.1.7.1. N-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cycloheptanamine (**8c**)

Yield: 67 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.31 (m, 2H), 7.12 – 7.09 (m, 2H), 7.06 – 7.03 (m, 2H), 6.95 – 6.91 (m, 2H), 6.39 (s, 1H), 3.67 (s, 2H), 2.82 – 2.75 (m, 1H), 2.07 (s, 3H), 1.96 – 1.89 (m, 2H), 1.73 – 1.66 (m, 2H), 1.56 – 1.47 (m, 6H), 1.32 – 1.26 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.79, 133.53, 132.26, 131.75, 131.56, 129.80, 129.45, 129.18, 128.94, 128.40, 110.35, 58.92, 43.15, 34.65, 28.41, 24.59, 11.09 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{28}Cl_2N_2$, 427.1702; found, 427.1689.

*4.1.7.2. N-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)adamantan-2-amine (**8d**)*

Yield: 51 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.7 Hz, 2H), 7.11 (d, *J* = 8.5 Hz, 2H), 7.06 (d, *J* = 8.7 Hz, 2H), 6.94 (d, *J* = 8.5 Hz, 2H), 6.40 (s, 1H), 3.67 (s, 2H), 2.87 (br s, 1H), 2.09 (s, 3H), 2.05 (br s, 1H), 1.98 (br s, 2H), 1.89 – 1.86 (m, 3H), 1.80 – 1.73 (m, 6H), 1.54 – 1.51 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.83, 133.51, 132.17, 131.73, 131.63, 129.82, 129.46, 128.97, 128.41, 110.51, 61.76, 42.83, 38.09, 37.72, 31.98, 31.52, 29.85, 27.99, 27.78, 11.14 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{28}H_{30}Cl_2N_2$, 465.1859; found, 465.1847.

4.1.7.3. 1-(1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)-N-(cyclohexylmethyl)-methanamine ***(8e)***

Yield: 82 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, *J* = 8.6 Hz, 2H), 7.09 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 6.50 (s, 1H), 4.79 (br. s, 1H), 3.80 (s, 2H), 2.62 (d, 2H, *J* = 6.8 Hz), 2.08 (s, 3H), 1.87 – 1.84 (m, 2H), 1.74 – 1.63 (m, 6H), 1.30 – 1.23 (m, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.50, 133.71, 132.56, 131.93, 131.25, 130.40, 129.76, 129.49, 128.99, 128.38, 110.74, 54.64, 44.85, 36.61, 31.39, 26.46, 25.91, 11.21 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{28}Cl_2N_2$, 427.1702; found, 427.1688.

4.1.7.4. N-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)propan-2-amine (**8f**)

Yield: 37 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, 2H, *J* = 8.6 Hz), 7.09 (d, 2H, *J* = 8.7 Hz), 7.04 (d, *J* = 8.7 Hz, 2H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.39 (s, 1H), 3.68 (s, 2H), 2.99 – 2.93 (m, 1H), 2.78 (br s, 1H), 2.07 (s, 3H), 1.15 (d, *J* = 6.3 Hz, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.5, 133.7, 132.5, 131.9, 131.2, 130.4, 129.8, 129.5, 128.9, 128.4, 110.8, 48.4, 41.6, 21.1, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{21}H_{22}Cl_2N_2$, 373.1233; found, 373.1039.

4.1.7.4. N-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)-1-phenylethan-1-amine (**8g**)

Yield: 22 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.28 (m, 7H), 7.10 (d, *J* = 8.5 Hz, 2H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.92 (d, *J* = 8.4, 2H), 6.38 (s, 1H), 3.96 – 3.90 (m, 1H), 3.53 (s, 2H), 1.94 (s, 3H), 1.44 – 1.43 (m, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.7, 137.8, 133.5, 132.2, 131.7, 131.6, 129.8, 129.4, 129.2, 128.9, 128.6, 128.4, 127.1, 126.9, 119.9, 110.3, 58.2, 43.6, 24.7, 11.0 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{26}H_{24}Cl_2N_2$, 435.1389; found, 435.1192.

4.1.7.5. N-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)-N2-phenylethane-1,2-diamine (**8h**)

Yield: 26 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (d, *J* = 8.6 Hz, 2H), 7.16 – 7.08 (m, 4H), 6.95 (d, *J* = 8.6 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 6.68 (t, *J* = 7.3 Hz, 1H), 6.60 (d, *J* = 7.7 Hz, 2H), 6.43 (s, 1H), 3.86 (s, 2H), 3.42 – 3.39 (m, 2H), 3.09 – 3.06 (m, 2H), 2.02 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.6, 137.3, 133.9, 132.9, 132.2, 131.0, 129.7, 129.5, 129.4, 129.0, 128.5,

128.4, 117.6, 112.9, 110.6, 46.0, 43.8, 41.0, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{26}H_{25}Cl_2N_3$, 450.1498; found, 450.1489.

4.1.7.6. *N*-((5-(4-chlorophenyl)-1-(4-fluorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8i**)

Yield: 68 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 – 7.04 (m, 6H), 6.91 (d, *J* = 8.6 Hz, 2H), 6.48 (s, 1H), 3.78 (s, 2H), 2.74 – 2.69 (m, 1H), 2.05 (s, 3H), 1.79 – 1.76 (m, 2H), 1.66 – 1.62 (m, 2H), 1.37 – 1.22 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.1, 160.6, 135.1 (d), 132.6, 131.8, 131.4, 130.2 (d), 128.9, 128.3, 116.3, 116.1, 110.4, 56.2, 41.7, 32.0, 25.8, 25.0, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{26}ClFN_2$, 397.1841; found, 397.1832.

4.1.7.7. *N*-((5-(4-chlorophenyl)-1-(4-fluorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cycloheptanamine ***(8j)***

Yield: 65 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 – 7.04 (m, 6H), 6.90 (d, *J* = 8.7 Hz, 2H), 6.54 (s, 1H), 3.80 (s, 2H), 2.98- 2.92 (m, 1H), 2.06 (s, 3H), 1.77 – 1.63 (m, 5H), 1.56 – 1.54 (m, 4H), 1.48 – 1.39 (m, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.1, 160.6, 135.0 (d), 132.7, 131.9, 131.3, 130.2 (d), 128.9, 128.3, 116.4, 116.1, 110.6, 58.1, 41.7, 33.0, 28.1, 24.4, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{28}ClFN_2$, 411.1998; found, 411.1989.

4.1.7.8. *N-((5-(4-chlorophenyl)-1-(4-isopropylphenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cycloheptanamine* ***(8k)***

Yield: 72 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (d, *J* = 8.4 Hz, 2H), 7.04 (d, *J* = 8.9 Hz, 2H), 6.99 (d, *J* = 8.4 Hz, 2H), 6.91 (d, *J* = 8.9 Hz, 2H), 6.56 (s, 1H), 3.81 (s, 2H), 3.00 – 2.86 (m, 2H), 2.07 (s, 3H), 1.79 – 1.64 (m, 5H), 1.57 – 1.52 (m, 4H), 1.48 – 1.37 (m, 3H), 1.26 (s, 3H), 1.24 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.7, 136.5, 132.6, 131.6, 131.5, 131.0, 128.8, 128.3, 128.2, 127.2, 110.4, 58.0, 33.8, 32.8, 29.8, 28.1, 24.4, 24.1, 11.3 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{28}H_{35}ClN_2$, 435.2562; found, 435.2368.

4.1.7.9. *N-((5-(4-chlorophenyl)-1-(2,5-dimethylphenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine* ***(8l)***

Yield: 40 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 - 7.07 (m, 2 H), 7.04 - 7.09 (m, 3 H), 6.95 (d, *J* = 8.6, 2 H), 6.81 (s, 1 H), 4.01 (d, J = 3.2 Hz, 2 H), 2.99 - 2.94 (m, 1 H), 2.30 (s, 3 H), 2.25 - 2.17 (m, 2 H), 1.96 (s, 3 H), 1.84 - 1.77 (m, 2 H), 1.74 (s, 3 H), 1.70 - 1.60 (m, 4 H), 1.22 - 1.16 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ137.6, 136.7, 133.3, 132.7, 131.6, 131.5, 131.2, 130.8, 129.6, 129.5, 128.2, 128.0, 127.0, 111.0, 77.4, 55.0, 39.8, 29.4, 29.0, 24.9, 24.7, 20.8, 16.8, 10.7 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{26}H_{31}ClN_2$, 407.2249; found, 407.2056.

4.1.7.10. *N-((5-(4-chlorophenyl)-1-(3-fluorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine* ***(8m)***

Yield: 64 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 - 7.29 (m, 1H), 7.09 - 7.03 (m, 3H), 6.93 - 6.88 (m, 3H), 6.82 (dt, J_1 = 9.3 Hz, J_2 = 2.1 Hz, 1H), 6.58 (s, 1H), 3.81 (s, 2H), 2.82 - 2.75 (m, 1H), 2.12 (br. s, 1H), 2.09 (s, 3H), 1.82 - 1.76 (m, 2H), 1.65 - 1.60 (m, 1H), 1.50 - 1.40 (m, 2H), 1.30 - 1.20 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.0, 161.5, 140.5 (d), 132.7, 132.0, 131.2, 130.4 (d), 128.9, 128.4, 124.6 (d), 116.1, 115.1, 111.0, 55.8, 40.9, 31.1, 25.6, 24.9, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{26}ClFN_2$, 397.1841; found, 397.1828.

*4.1.7.11. N-((5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8n**)*

Yield: 57 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (d, J = 2.1 Hz, 1H), 7.28 (d, J = 2.2 Hz, 1H), 7.20 - 7.15 (dd, J_1 = 11.8 Hz, J_2 =8.6 Hz, 3H), 7.00 (d, J = 8.6 Hz, 2H), 6.72 (s, 1H), 4.03 - 3.95 (m, 2H), 2.93 (t, J = 11.2 Hz, 1H), 2.21 - 2.18 (m, 2H), 2.04 (s, 3H), 1.91 - 1.82 (m, 2H), 1.69 - 1.58 (m, 3H), 1.33 - 1.29 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ137.6, 135.3, 134.6, 132.7, 132.1, 131.9, 130.7, 130.5, 128.8, 128.6, 128.3, 123.2, 117.3, 110.9, 55.3, 39.9, 29.1, 25.0, 24.8, 10.7 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{25}Cl_3N_2$, 447.1156; found, 447.1157.

*4.1.7.12. N-((5-(4-chlorophenyl)-1-(4-methoxyphenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8o**)*

Yield: 60 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 8. Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 6.86 (d, J = 8.7 Hz, 2H), 6.46 (s, 1H), 3.96 (s, 2H), 3.82 (s, 3H), 2.96 - 2.91 (m, 1H), 2.03 (s, 3H), 1.85 - 1.71 (m, 4H), 1.65 - 1.59 (m, 2H), 1.52 - 1.45 (m, 2H), 1.21 - 1.11 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ159.2, 133.1, 132.5, 131.8, 131.4, 131.2, 129.6, 128.9, 128.3, 114.5, 110.6, 55.6, 55.1, 39.9, 29.2, 25.0, 24.7, 11.3 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{29}ClN_2O$, 409.2041; found, 409.1849.

*4.1.7.13. N-((5-(4-chlorophenyl)-2-methyl-1-(4-nitrophenyl)-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8p**)*

Yield: 68 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22 (d, J = 8.9 Hz, 2H), 7.25 (d, J = 8.8 Hz, 2H), 7.11 (d, J = 8.4 Hz, 2H), 6.89 (d, J = 8.4 Hz, 2H), 6.56 (s, 1H), 3.79 (s, 2H), 2.72 (t, J = unresolved, 1H), 2.13 (s, 3H), 2.10 - 2.05 (m, 2H), 1.82 - 1.75 (m, 2H), 1.67 - 1.60 (m, 1H), 1.42 - 1.15 (m, 5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.6, 144.6, 132.6, 132.4, 130.9, 129.9, 129.2, 129.1, 128.6, 124.6, 111.9, 56.2, 41.5, 31.9, 25.8, 25.0, 11.4 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{26}ClN_3O_2$, 424.1786; found, 424.1776.

*4.1.7.14. N-((5-(4-chlorophenyl)-2-methyl-1-(2-(trifluoromethyl)phenyl)-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8q**).*

Yield: 52 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, J = 7.7 Hz, 1H), 7.64 (t, J = 7.5 Hz, 1H), 7.55 (t, J = 7.5 Hz, 1H), 7.38 (d, J = 7.8 Hz, 1H), 7.05 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.7 Hz,

2H), 6.42 (s, 1H), 3.72 (s, 2H), 2.59 - 2.53 (m, 1H), 1.98 - 1.95 (m, 1H), 1.93 (s, 3H), 1.79 - 1.72 (m, 2H), 1.66 - 1.60 (m, 1H), 1.34 - 1.11 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.7, 133.3, 132.8, 132.4, 131.9, 131.6, 130.2, 129.2, 128.4, 128.3, 127.7 (d), 124.2, 121.5, 119.6, 109.9, 56.3, 42.6, 33.6, 33.3, 26.3, 25.2 (d), 10.6 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{26}ClF_3N_2$, 447.1809; found, 447.1807.

*4.1.7.15. 4-(5-(4-chlorophenyl)-3-((cyclohexylamino)methyl)-2-methyl-1H-pyrrol-1-yl)phenol (**8r**)*

Yield: 64 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (d, *J* = 8.5 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 6.86 - 6.80 (m, 4H), 6.65 (s, 1H), 3.96 (s, 2H), 2.99 (t, *J* = 10.7 Hz, 1H), 2.26 - 2.19 (m, 2H), 1.99 (s, 3H), 1.83 - 1.77 (m, 2H), 1.68 - 1.50 (m, 4H), 1.19 - 1.08 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.8, 133.1, 132.5, 131.8, 131.2, 130.5, 129.4, 128.9, 128.2, 116.2, 109.9, 60.6, 48.5, 33.3, 25.6, 25.0, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{27}ClN_2O$, 395.1885; found, 395.1884.

*4.1.7.16. N-((1-(4-chlorophenyl)-5-(4-isopropylphenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8s**)*

Yield 34 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (d, *J* = 8.5 Hz, 2H), 7.06 (d, *J* = 8.5 Hz, 2H), 6.99 (d, *J* = 8.5 Hz, 2H), 6.92 (d, *J* = 8.3 Hz, 2H), 6.46 (s, 1H), 3.79 (s, 2H), 2.85 - 2.77 (m, 1H), 2.75 - 2.67 (m, 1H), 2.08 (s, 3H), 1.81 - 1.74 (m, 2H), 1.71 - 1.48 (m, 2H), 1.42 - 1.27 (m, 4H), 1.24 - 1.22 (m, 1H), 1.18 (d, *J* = 6.9 Hz, 6H), 1.12 - 1.00 (m, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.7, 138.1, 133.8, 133.3, 130.4, 130.0, 129.3, 127.8, 126.3, 110.0, 56.1, 41.7, 33.8, 29.8, 25.9, 25.1, 24.0, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{27}H_{33}ClN_2$, 421.2405; found, 421.2391.

*4.1.7.17. N-((1-(4-chlorophenyl)-5-(4-fluorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8t**)*

Yield 53 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (d, *J* = 8.6 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.97 (dd, J_1 = 8.7 Hz, J_2 = 5.4 Hz, 2H), 6.83 (t, J_1 = 8.7 Hz, 2H), 6.36 (s, 1H), 3.73 (s, 2H), 2.66 - 2.57 (m, 1H), 2.07 (s, 3H), 2.03 - 1.95 (m, 2H), 1.85 (t, J_1 = 6.5 Hz, 1H), 1.80 - 1.74 (m, 2H), 1.67 - 1.60 (m, 1H), 1.38 - 1.29 (m, 2H), 1.21 - 1.14 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.6, 160.2, 137.9, 133.5, 132.6, 129.9, 29.6, 129.5, 129.4, 115.3, 115.1, 110.0, 56.6, 42.5, 33.1, 25.8, 25.2, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{26}ClFN_2$, 397.1841; found, 397.1651.

*4.1.7.18. N-((5-(4-(tert-butyl)phenyl)-1-(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexanamine (**8u**)*

Yield 86 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (d, *J* = 8.7 Hz, 2H), 7.15 (d, *J* = 8.5 Hz, 2H), 7.07 (d, *J* = 8.7 Hz, 2H), 6.94 (d, *J* = 8.5 Hz, 2H), 6.43 (s, 1H), 3.77 (s, 2H), 2.72 - 2.64 (m, 1H), 2.07 (s, 3H), 2.05 - 2.00 (m, 2H), 1.81 - 1.73 (m, 2H), 1.67 - 1.58 (m, 2H), 1.42 - 1.31 (m, 2H), 1.25 (s, 9H), 1.19 - 1.05 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.4, 149.2, 137.8, 134.1, 133.6, 130.0, 129.6, 129.4, 127.5, 125.2, 110.6, 55.3, 34.5, 31.4, 29.8, 25.2, 24.8, 11.5 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{28}H_{35}ClN_2$, 435.2562; found, 435.2553.

4.1.7.19. 4-(((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)amino)cyclohexan-1-ol ***(8v)***

Yield: 30 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 8.7 Hz, 2H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.91 (d, *J* = 8.6 Hz, 2H), 6.36 (s, 1H), 3.68 (s, 2H), 3.66 - 3.60 (m, 1H), 2.65 - 2.56 (m, 1H), 2.05 (s, 3H), 2.03 - 1.96 (m, 4H), 1.38 - 1.27 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.6, 133.6, 132.4, 131.8, 131.4, 129.7, 129.5, 128.9, 128.4, 119.0, 110.1, 55.7, 50.6, 42.9, 34.0, 30.8, 11.0 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{26}Cl_2N_2O$, 429.1495; found, 429.1488.

4.1.7.20. Boc-Protected 8w

Yield: 25 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 8.5 Hz, 2H), 7.10 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.6 Hz, 2H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.42 (s, 1H), 3.72 (s, 2H), 2.07 (s, 3H), 2.04 - 2.01 (m, 2H), 1.44 (s, 9H), 1.33 - 1.22 (m, 4H), 1.20 - 1.10 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.1, 144.2, 137.2, 133.4, 132.2, 129.4, 129.2, 128.8, 128.7, 128.2, 128.1, 120.5, 109.7, 79.1, 64.5, 57.6, 48.7, 31.8, 30.1, 28.2, 10.7 ppm. HRMS (m/z) $[M + H]^+$calcd for C29H35Cl2N3O2, 528.2179; found, 528.2178.

4.1.7.21. N^1-((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)cyclohexane-1,4-diamine ***(8w)***

Yield: 100 %. ^{1}H NMR (400 MHz, CD_3OD) δ 7.47 (d, *J* = 8.5 Hz, 2H), 7.19 (d, J_1 = 8.4 Hz, 2H), 7.15 (d, J_1 = 8.5 Hz, 2H), 7.04 (d, J_1 = 8.4 Hz, 2H), 6.55 (s, 1H), 4.19 (s, 2H), 2.42 - 2.35 (m, 2H), 2.24 - 2.18 (m, 2H), 2.16 (s, 3H), 1.72 - 1.60 (m, 6H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 138.6, 135.3, 134.8, 133.6, 133.2, 132.5, 131.2, 130.7, 130.4, 129.4, 112.3, 111.3, 56.0, 49.9, 42.3, 29.7, 28.1, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{24}H_{27}Cl_2N_3$, 428.1655; found, 428.1649.

4.1.7.22. Boc-Protected 8x

Yield: 52 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.5 (br. s, 1H), 8.30 - 8.26 (m, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.10 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.92 (d, *J* = 8.6 Hz, 2H), 6.40 (s, 1H), 3.70 (s, 2H), 3.41 - 3.36 (m, 2H), 2.71 (t, *J* = 7.3 Hz, 2H), 2.07 (s, 3H), 1.61 - 1.52 (m, 4H), 1.49 (d, *J* = 5 Hz, 18H), 1.35 - 1.30 (m, 8H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.8, 156.2, 153.5,

137.7, 133.6, 132.3, 131.8, 131.5, 129.8, 129.5, 128.9, 128.4, 110.5, 83.1, 79.4, 41.1, 29.5, 29.4, 29.1, 28.4, 28.2, 27.4, 26.9, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{37}H_{51}Cl_2N_5O_4$, 700.3391; found, 700.3386.

*4.1.7.23. 1-(8-(((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)amino)octyl)guanidine (**8x**)*

Yield: 100 %. ^{1}H NMR (400 MHz, CD_3OD) δ 7.46 (d, *J* = 8.5 Hz, 2H), 7.20 - 7.14 (m, 4H), 7.03 (d, *J* = 8.7 Hz, 2H), 6.51 (s, 1H), 4.13 (s, 2H), 3.17 (t, *J* = 7.0 Hz, 2H), 3.10 - 3.05 (m, 2H), 2.14 (s, 3H), 1.79 - 1.70 (m, 2H), 1.62 - 1.55 (m, 2H), 1.43 - 1.38 (m, 8H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 158.5, 138.5, 135.2, 134.6, 133.4, 133.2, 132.4, 131.3, 130.6, 130.5, 129.3, 112.1, 45.0, 42.6, 30.6, 30.3, 30.1, 29.9, 27.7, 27.6, 27.3, 11.5 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{27}H_{35}Cl_2N_5$, 500.2342; found, 500.2341.

4.1.7.24. Boc-Protected 8y

Yield: 43 %.^{1}H NMR (400 MHz, $CDCl_3$) δ 11.5 (br. s, 1H), 8.22 (d, *J* = 8.3 Hz, 1H), 7.33 (d, *J* = 8.6 Hz, 2H), 7.10 (d, *J* = 8.6 Hz, 2H), 7.04 (d, *J* = 8.6 Hz, 2H), 6.93 (d, *J* = 8.6 Hz, 2H), 6.34 (s, 1H), 4.07 - 3.92 (m, 1H), 3.67 (s, 2H), 2.59 - 2.51 (m, 1H), 2.13 - 2.07 (m, 2H), 2.06 (s, 3H), 2.02 - 1.97 (m, 2H), 1.48 (d, *J* = 5.6 Hz, 18H), 1.37 - 1.27 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.9, 155.5, 153.4, 137.8, 133.5, 132.3, 131.7, 131.6, 131.3, 129.8, 129.4, 128.9, 128.4, 110.2, 83.0, 79.2, 55.8, 48.9, 43.0, 31.8, 31.6, 28.4, 28.2, 11.0 ppm. HRMS (m/z) [M + H]+calcd for $C_{35}H_{45}Cl_2N_5O_4$, 670.2921; found, 670.2920.

*4.1.7.25. 1-(4-(((1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)methyl)amino)cyclohexyl)guanidine (**8y**)*

Yield: 100 %. ^{1}H NMR (400 MHz, CD_3OD) δ 7.46 (d, *J* = 8.5 Hz, 2H), 7.17 (d, *J* = 8.5 Hz, 2H), 7.16 (d, *J* = 8.4 Hz, 2H), 7.04 (d, *J* = 8.4 Hz, 2H), 6.58 (s, 1H), 4.18 (s, 2H), 3.56 - 3.45 (m, 1H), 3.29 - 3.21 (m, 1H), 2.37 - 2.29 (m, 2H), 2.16 (s, 3H), 2.14 -2.10 (m, 2H), 1.77 - 1.65 (m, 2H), 1.53 - 1.43 (m, 2H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 157.8, 138.6, 135.2, 134.6, 133.5, 133.2, 132.5, 131.3, 130.6, 130.4, 129.4, 111.4, 56.4, 50.6, 42.1, 31.4, 28.5, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{29}Cl_2N_5$, 470.1873; found, 470.1864.

4.1.7.26. Boc-Protected 8z

Yield: 56 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.47 (br. s, 1H), 8.22 (d, *J* = 8.9 Hz, 2H), 7.26 (d, J = 8.9 Hz, 2H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.60 (s, 1H), 4.07 - 3.98 (m, 1H), 3.89 (s, 2H), 2.91 - 2.82 (m, 1H), 2.21 - 2.15 (m, 2H), 2.11 (s, 3H), 1.79 - 1.62 (m, 2H), 1.47 (d, *J* = 6.2 Hz, 18H), 1.25 - 1.15 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.82, 155.47, 153.44, 146.86, 144.36, 133.05, 132.71, 131.45, 131.13, 130.56, 129.27, 129.18, 128.69,

124.72, 112.03, 83.25, 79.34, 54.31, 47.93, 40.20, 31.04, 30.42, 29.79, 28.40, 28.34, 28.19, 11.40 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{35}H_{45}ClN_6O_6$, 681.3162; found, 681.3141.

*4.1.7.27. 1-(4-(((5-(4-chlorophenyl)-2-methyl-1-(4-nitrophenyl)-1H-pyrrol-3-yl)methyl)amino)cyclohexyl)guanidine (**8z**)*

Yield: 100 %. ^{1}H NMR (400 MHz, CD_3OD) δ 8.31 (d, *J* = 8.9 Hz, 2H), 7.42 (d, *J* = 8.9 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 2H), 7.04 (d, *J* = 8.5 Hz, 2H), 6.63 (s, 1H), 4.20 (s, 2H), 3.56 - 3.46 (m, 1H), 3.29 - 3.22 (m, 1H), 2.38 - 2.30 (m, 2H), 2.21 (s, 3H), 2.149 - 2.12 (m, 2H), 1.78 - 1.66 (m, 2H), 1.56 - 1.43 (m, 2H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 157.8, 148.5, 145.4, 134.8, 133.8, 133.2, 132.2, 130.8, 130.6, 129.5, 125.7, 112.2, 56.5, 50.6, 42.1, 31.4, 28.6, 11.2 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{25}H_{29}ClN_6O_2$, 481.2113; found, 481.2098.

4.1.7.28. Boc-Protected 8aa

Yield: 24 %.^{1}H NMR (400 MHz, $CDCl_3$) δ 11.48 (br. s, 1H), 8.27 (br. s, 1H), 7.41 (d, *J* = 8.6 Hz, 2H), 7.09 (d, *J* = 8.5 Hz, 2H), 6.18 (s, 1H), 3.91 (s, 2H), 3.36 (q, *J* = 7 Hz, 2H), 2.83 (t, *J* = 8 Hz, 2H), 2.01 (s, 3H), 1.95 (s, 3H), 1.89 - 1.80 (m, 2H), 1.58 - 1.51 (m, 2H), 1.48 (d, *J* = 4.3 Hz, 18H), 1.35 - 1.19 (m, 8H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.8, 156.2, 153.4, 137.0, 134.1, 129.7, 129.6, 129.2, 128.9, 108.3, 83.1, 79.3, 45.6, 42.9, 41.0, 29.2, 29.1, 28.4, 28.2, 27.0, 26.9, 12.8, 11.1 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{32}H_{50}ClN_5O_4$, 604.3624; found, 604.3625.

*4.1.7.29. 1-(8-(((1-(4-chlorophenyl)-2,5-dimethyl-1H-pyrrol-3-yl)methyl)amino)octyl)guanidine (**8aa**)*

Yield: 99 %. ^{1}H NMR (400 MHz, CD_3OD) δ 7.55 (d, *J* = 8.6 Hz, 2H), 7.21 (d, *J* = 8.6 Hz, 2H), 6.05 (s, 1H), 4.03 (s, 2H), 3.18 (t, *J* = 7 Hz, 2H), 3.03 - 2.99 (m, 2H), 2.03 (s, 3H), 2.00 (s, 3H), 1.77 - 1.68 (m, 2H), 1.64 - 1.57 (m, 2H), 1.44 - 1.37 (m, 8H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 158.6, 138.4, 135.4, 131.0, 130.7, 130.5, 130.0, 110.3, 47.7, 44.7, 42.5, 30.1, 30.0, 29.8, 27.6, 27.2, 12.7, 10.7 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{22}H_{34}ClN_5$, 500.2342; found, 500.2340.

*4.1.8. Synthesis of the (trans-4-amino-cyclohexyl)-carbamic acid tert-butyl ester **9***

Trans-1,4-diaminocyclohexane (1.09 mmol) was added to a solution of Et_3N (1.09 mmol) in THF (5 mL) and the resulting mixture was cooled to 0 °C. Then, a solution of di-*tert*-butyl dicarbonate (4.38 mmol) in THF (5 mL) was added dropwise and the reaction was allowed to warm up to room temperature and subsequently stirred for 24 h. The reaction mixture was then diluted with water (10 mL) and washed twice with EtOAc (20 mL) and once with brine (10 mL). The organic extracts were collected and then dried over $MgSO_4$, filtered and concentrated under

reduced pressure. The crude residue was purified with flash chromatography (EtOAc/MeOH 9:1 v/v), affording the title compound as a white solid (180 mg).

Yield: 99 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.50 (br. s, 1H), 3.31 (br. s, 1H), 2.56 (br. s, 1H), 1.91 (br. s, 2H), 1.80 – 1.77 (m, 2H), 1.37 – 1.36 (m, 9H), 1.13 – 1.05 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.30, 79.03, 49.89, 49.20, 35.25, 32.14, 28.42 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{11}H_{22}N_2O_2$, 215.1754; found, 215.1751.

*4.1.9. Synthesis of the aminoguanidine **10**, **11***

Into a round bottom flask, the appropriate amine (3.06 mmol) and Et_3N (0.76 mmol) were dissolved in DCM (15 mL). Then a solution of 1,3-di-boc-2-trifluoromethylsulfonyl guanidine (0.76 mmol) in DCM (15 mL) was added dropwise and the resulting mixture was stirred at room temperature for 24 h. After completion, the reaction mixture was concentrated under reduced pressure to give a crude residue that was purified with flash chromatography (EtOAc/MeOH 9:1 v/v), affording the desired compounds **10** and **11** respectively as yellow and white foamy solids.

*4.1.9.1. 1-(8-aminooctyl)-2,3-di-boc-guanidine (**10**)*

Yield: 99 %. ^{1}H NMR (400 MHz, CD_3OD) δ 4.99 (br. s, 2H), 3.35 (t, *J* = 7.1 Hz, 2H), 2.77 (t, *J* = 7.5 Hz, 2H), 1.63 – 1.55 (m, 4H), 1.53 (s, 9H), 1.47 (s, 9H), 1.37 (br. s, 8H) ppm. ^{13}C NMR (100 MHz, CD_3OD) δ 164.47, 157.43, 154.19, 84.36, 80.18, 41.75, 41.57, 31.10, 30.20, 30.11, 30.01, 28.65, 28.31, 27.72, 27.59 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{19}H_{38}N_4O_4$, 387.2966; found, 387.2959.

*4.1.9.2. 1-(4-aminocyclohexyl)-2,3-di-boc-guanidine (**11**)*

Yield: 94 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (br. s, 1H), 8.19 (d, *J* = 8.2 Hz, 1H), 4.01 – 3.91 (m, 1H), 2.73 – 2.62 (m, 2H), 2.07 – 2.03 (m, 2H), 1.92 – 1.88 (m, 2H), 1.48 (d, *J* = 6.4 Hz, 18H), 1.30 – 1.20 (m, 4H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.93, 155.60, 153.44, 83.13, 79.30, 49.97, 48.44, 34.47, 31.62, 28.44, 28.22 ppm. HRMS (m/z) $[M + H]^+$calcd for $C_{17}H_{32}N_4O4$, 357.2496; found, 357.2491.

4.2. Computational chemistry and modelling

All molecular modelling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 14LTS. The Gyrase structures were downloaded from PDB [www.rcsb.org/.]: 5CDQ [38], 5CDM [38], 5CDN [38], 5D6Q [43], 5BS3 [41]. Hydrogen atoms were added to the protein, using Maestro protein preparation wizard and minimized [51], keeping all the heavy atoms fixed until rmsd gradient of 0.05 $kcal.mol^{-1}.Å^{-1}$ was reached. Ligand structures were built with Maestro and minimized using the MMFF94x force field until an rmsd gradient of 0.05 $kcal.mol^{-1}.Å^{-1}$ was reached. The docking simulations were carried out by PLANTS [37]. The centre of the docking grids was computed by the average of the coordinates of the co-crystallized

inhibitors. The GyrA S84L isoform was obtained by a single point mutation of the 5CDM structure. The mutated residue was minimized without constraints keeping the closest residues freeze. The MM-GBSA were computed with Prime by Maestro interface [52]. The images depicted in the manuscript were generated by Pymol [53].

4.3. Biology

4.3.1. Minimum inhibitory concentration (MIC)

MICs were performed according to a standard microbroth dilution method. Briefly, compounds were made up in DMSO stocks, diluted down in water and added to the plate in 2-fold dilution series, 100µL per well. Bacteria were grown overnight in tryptic soy broth (TSB) and back diluted to an optical density (OD) of 0.01. 100µL of bacterial culture was added to 100µL of compound in a 96 well plate and incubated overnight at 37 °C. OD readings were measured with a Fluorostar plate reader and the MIC defined as the lowest concentration where visible growth was not observed (<0.1 OD_{600}). Equivalent concentrations of DMSO caused no inhibition of growth. MICs were performed in duplicate.

4.3.2. Time-kill

Overnight cultures of bacteria were back-diluted to an OD of 0.01 and aliquoted into glass universal containers. Compounds were added at 4 x MIC concentration and samples were incubated at 37 °C, with shaking at 200rpm. Aliquots were taken at 0, 2, 4, 6 and 24 hour timepoints and Miles-Misra analysis was performed to determine the CFU / mL. A compound is defined as having a bactericidal mode of action if the kill is over 3 log, and bacteriostatic if it is 3 log or lower. Data are representative of three biological repeats.

4.3.3. Passaging to generate resistance

NCTC 13616 and NCTC 12204 were passaged in increasing concentrations of compound following two published methods. Firstly, overnight cultures were back diluted into 3 mL of TSB containing compounds or control antibiotics at 0.25 x MIC and incubated at 37 °C with shaking. After 48 hours, 30µL was taken and added to 3 mL TSB containing compounds at 0.5 x MIC. This was repeated with doubling concentrations of compound until a time when no growth was isolated [54,55]. Bacteria which grew in the highest concentrations were re-tested by MIC against the parent compound to identify if resistance had emerged. Passaging was carried out in duplicate. Secondly, repeat passaging was done following the method of Friedman *et al.* [56]. Briefly, an MIC was performed as above. After incubation of the MIC plate, bacteria from the well with the highest concentration of compound which displayed growth were isolated, diluted 1;1000 and another MIC was performed on these samples. This was repeated for 20 passages, in triplicate. Thirdly, resistant colonies on agar were generated by plating known quantities of exponentially growing bacteria onto agar plates containing doubling concentrations of

compounds at and above the MIC and incubating for 48 hours. Any colonies which grew at higher than MIC concentrations were picked and re-tested by microbroth MIC for increased resistance to compounds.

4.3.4. Gyrase inhibition assay

The ability of the compounds to inhibit the wild-type and mutant (S84L) gyrase enzyme and topoisomerase IV was assessed using the gyrase supercoiling kit (SAS4001) and topoisomerase relaxing kit (SAR4001) from Inspiralis (Norwich, UK) as per manufacturer's instructions. Briefly, the enzyme isolated from *S. aureus* was incubated at 37 $^{\circ}$C for 30 minutes with relaxed or supercoiled (pBR322) plasmid in the presence or absence of varying concentrations of compound. The reaction was stopped with STEB buffer and the enzyme and compounds removed from the DNA using chloroform:isoamyl alcohol (24:1). Samples were separated by centrifugation and the aqueous fraction was run on a 1% agarose gel. Gels were imaged using an ImageQuant LAS4000 and the supercoiled band was quantified using ImageQuant analysis software. IC_{50}s were calculated using GraphPad Prism with data from at least three independent replicates.

4.3.5. Cytotoxicity assay

HEK293 and HeLa cell-lines were purchased from EACC and maintained in Eagles Minimum Essential Media (EMEM) containing glutamine and supplemented with 10% fetal bovine serum and 1 x non-essential amino acids, at 37 $^{\circ}$C with 10 % CO_2. 100 µL of cells harvested from cultures of 70 – 80 % confluence, were plated out into tissue-culture treated polystyrene 96-well plates at cell counts of 1 x 10^5 cells/mL for HEK293 and 4 x 10^4 cells/mL for HeLa and incubated for 24 hours to allow the cells to adhere. Media was then gently removed and replaced with 100 µL of media containing compounds at doubling dilutions. Cells were incubated for a further 24 hours and then stained with 25 µL of the tetrazolium salt viability dye XTT, activated by addition of PMS. Cells were incubated with dye for 4 hours in the dark and absorbance was read using a plate reader at 450nm after shaking. Untreated cells were the 100% survival control and cells treated with 100 µL of water were the 0% survival controls. The IC_{50} was calculated using GraphPad Prism.

4.3.6. Statistics

Students t test was used to assess statistical significance between IC_{50}s. A significance of <0.05 was deemed significant.

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Appendix A. Supplementary data

Additional biological activity data of pyrroles on Gram+/-ve bacteria and computational data are reported in the Supplementary data.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

[¶]Co-first authors. These authors contributed equally.

Abbreviations

ESKAPE, acronym standing for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli*; NCTC, National Collection of Type Cultures; EMRSA, Epidemic meticillin-resistant *Staphylococcus aureus*; VSE, vancomycin susceptible enterococci; VRE, vancomycin resistant enterococci; PMBN, polymyxin B nonapeptide; RND, Resistance-Nodulation Division; PaβN phenylalanine-arginine β-naphthylamide; NBTI, Novel Bacterial Topoisomerase Inhibitor.

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- Novel 1,5-diphenyl-pyrrole derivatives were synthesized
- The new compounds are endowed with high antibacterial activity
- The phenyl substituents at N1 and C5 of the pyrroles is essential for activity
- Protonatable guanidine/amino moieties improve the activity against Gram-ve bacteria
- Bacterial DNA gyrase was identified as a plausible target