Potent Inhibitors Targeting Metallo-β-lactamases from the B1, B2 and B3 Subgroups: Promising Broad-spectrum Agents to Combat a Major Mechanism of Antibiotic Resistance

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Article

Potent Inhibitors Targeting Metallo-β-Lactamases from the B1, B2 and B3 Subgroups: Promising Broad-Spectrum Agents to Combat a Major Mechanism of Antibiotic Resistance

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Abstract: Metallo-β-lactamases (MBLs) are a group of Zn(II)-dependent enzymes that pose a major threat to global health. They are linked to an increasing number of multi-drug resistant bacterial pathogens, but no clinically useful inhibitor is yet available. Since β-lactam antibiotics, which are inactivated by MBLs, constitute ~65% of all antibiotics used to treat infections, the search for clinically relevant MBL inhibitors is urgent. Here, derivatives of a 2-amino-1-benzyl-4,5-diphenyl-1H-pyrrole-3-carbonitrile (**1a**) were synthesised and their inhibitory effects assessed against representatives of each of the three subgroups of MBLs (B1, B2, B3). Several compounds are potent inhibitors of each MBL tested, making them excellent candidates for the development of broad-spectrum drug leads. In particular, compound **5f** is highly potent across the MBL subfamilies, with K_i values in the low μM range. Furthermore, this compound also displays synergy in combination with antibiotics such as penicillin G, cefuroxime or meropenem. This molecule thus represents one of the most promising compounds developed yet to combat MBLs.

Keywords: antibiotic resistance; metallo-β-lactamases; metal-dependent enzymes; broad-spectrum inhibitors; inhibition assays; enzyme kinetics

1. Introduction

The discovery of antibiotics was one of the most important medical advances of the twentieth century, with β-lactams being a widely prescribed class of antibiotics. However, β-lactam resistance has become widespread and a serious threat to the effective treatment of common bacterial illnesses [1]. This is due to the overuse and misuse of antibiotics in medicine and the practice of using antibiotics as a preventative measure in the agriculture industry for animal feedstock [2–4]. Determinants of antibiotic resistance are typically found on transposable genomic elements and the spread of antibiotic resistance from urban environments to the natural world can occur through the process of horizontal gene transfer [3]. Antibiotic resistance is caused by various mechanisms, the most common being the secretion of β-lactamase enzymes produced by resistant bacteria. These enzymes catalyse the hydrolysis and deactivation of the essential β-lactam ring [5,6].

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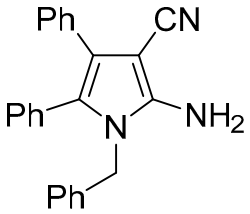
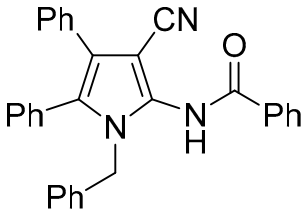
β-Lactamases can be divided into four classes (A, B, C and D) based on sequence homology, with each class differing in structure, mechanism of hydrolysis, and substrate specificity [5,6]. Class B β-lactamases are the metallo-β-lactamases (MBLs), zinc-dependent enzymes that are associated with many difficult-to-treat infections [7]. MBLs can be further subdivided into the B1, B2 and B3 subgroups based on primary sequence and distinguishing structural characteristics [5]. The MBLs in the majority of multidrug-resistant bacteria belong to the B1 subgroup [8]. A prominent representative is NDM-1 which, since its initial identification in 2008, has spread rapidly worldwide [9]. B2-type MBLs are rare but have attracted attention due to their unique selectivity for "last line" carbapenem substrates [10]. MBLs from the B3 subgroup have gained less attention, not least because the majority of these enzymes have been identified in environmental microorganisms [7,11–15]. The catalytic centres in the three MBL subgroups can accommodate up to two metal ions (Zn(II) in most cases) and are characterised by distinct sequence motifs. For the B1 subgroup the two Zn(II) binding sites (Zn1 and Zn2) use ligands His116, His118, His196 and Asp120, Cys221, His263, respectively (i.e., HHH/DCH). The corresponding motifs for the B2 and B3 subgroups are NHH/DCH and HHH/DHH, respectively [7]. Recently, atypical variations of the canonical active site motifs in B3-type MBLs have been reported (i.e., QHH/DHH, EHH/DHH and HRH/DQK) [7].

Bacteria that express MBLs are an increasingly significant threat to human health, and various approaches have been taken to discover new MBL inhibitors [16]. Although numerous compounds have been developed that inhibit these enzymes [17], none of them has yet reached clinical application [18]. McGeary et al. examined a series of pyrrole and pyrrolopyrimidine derivatives as MBL inhibitors against the B1-type MBL IMP-1 [19,20]. Of these, pyrrole **1a** (i.e., 2-amino-1-benzyl-4,5-diphenyl-1H-pyrrole-3-carbonitrile) was identified as a promising lead (K_{ic} = 21 μM), and further structure-activity relationship (SAR) studies revealed that acylation of the amino group (see **1b**) led to slightly increased potency against IMP-1 [21]. To assess their broad-spectrum activity, the most potent compounds were subsequently also tested against MBLs from the B2 (CphA) and B3 (AIM-1) subgroups. In particular, compound **1b** exhibited broad-spectrum inhibition, with K_i values in the low μM range for each of the three enzymes tested. This compound was thus used here as motivation for the synthesis of variants that were assessed for their efficiency in inhibiting representative MBLs from each of the three subgroups.



1A



1B

Due to the spread of multidrug-resistant (MDR) pathogenic bacteria, clinicians now have fewer options for effective anti-infective therapy, which is exacerbated by the slow rate of novel antibacterial drug discovery [22,23]. Antimicrobial synergy, in which two medications are administered in combination to have a greater-than-additive effect, allows the preservation of current antibiotics for use in treating MDR bacteria, even when they are resistant to one or both antibiotics individually. In this study, we thus also assessed possible synergetic effects of combinations of inhibitors designed here using the checkerboard array method [24,25]. This method, based on minimum inhibitory concentration (MIC) measurements, is one of the most widely used techniques to evaluate synergy effects *in vitro*.

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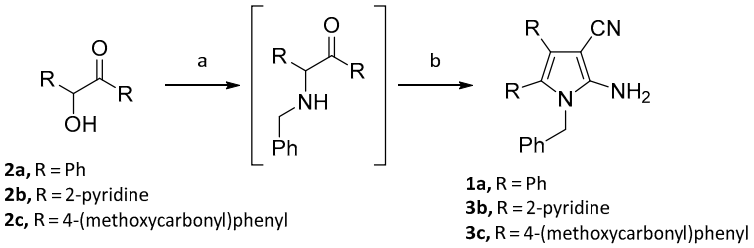
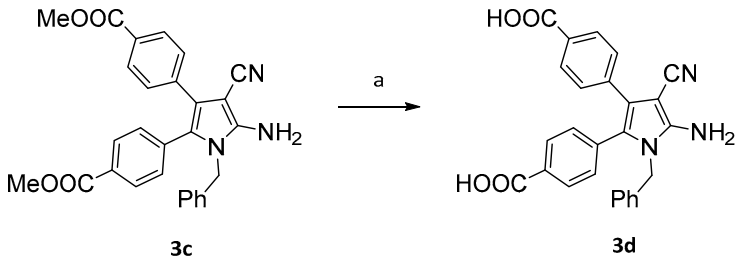
2. Results and Discussion

2.1. Syntheses of a series of derivatives of compounds 1a and 1b

To examine the impact of positively charged (pyridine; Scheme 1) and negatively charged (carboxylate; Scheme 2) aromatic rings on inhibitory activity, a series of analogues of **1a** were synthesised following the previously reported method using benzoin derivatives (**2a**-**c**) as the starting materials (Scheme 1) [21].

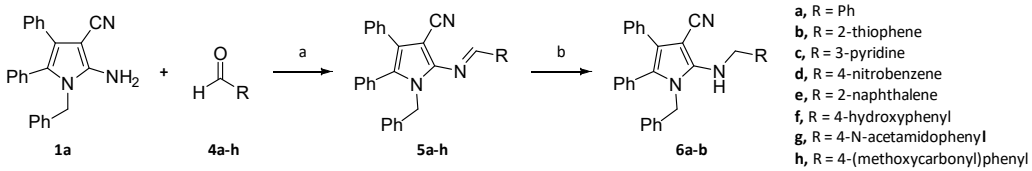


Scheme 1. Reagents and conditions: (a) benzylamine, trichloroacetic acid, toluene, reflux, 4-6 h. (b) malononitrile, pyridine, reflux, 24 h. **1a** (60%) **3b** (10%), **3c** (13%).



Scheme 2. Reagents and conditions: (a) NaOH, tetrahydrofuran, H2O, 24 h. (10%).

To investigate the importance of hydrogen bonding at the carbonyl oxygen of **1b**, an imine analogue (**5a**) was synthesised through the condensation of **1a** with benzaldehyde (**4a**), as outlined in Scheme 3. The imine was reduced to the secondary amine (**6a**) using sodium borohydride as the amine product was expected to be more stable than the imine, which are often prone to hydrolysis. However, the reduced product was found to have lower stability compared to the initial imine, partial oxidation back to the imine under aerobic conditions was observed in solution. This was likely due to the high level on conjugation between the imine and surrounding aromatic rings.



Scheme 3. Reagents and conditions: (a) trichloroacetic acid, toluene, reflux, 2-6 h. **5a** (85%), **5b** (46%), **5c** (72%), **5d** (75%), **5e** (88%), **5f** (80%), **5g** (67%), **5h** (62%). (b) NaBH4, ethanol, 24 h, rt. **6a** (85%), **6b** (80%).

As no significant decrease in potency was observed in initial assays for **5a** and **6a** compared to **1b** (see below), a series of imine derivatives (**5b**-**i**) were synthesised to further investigate the impact of polar functional groups at this position (Schemes 3 and 4). The derivatives synthesised included a thiophene analogue (**5b**) and a pyridine analogue (**5c**) to increase hydrophilicity while avoiding the introduction of additional steric bulk. In addition, a range of analogues were synthesised with various polar functional groups added at the para position (**5d**, **5f**-**i**). This specific position was

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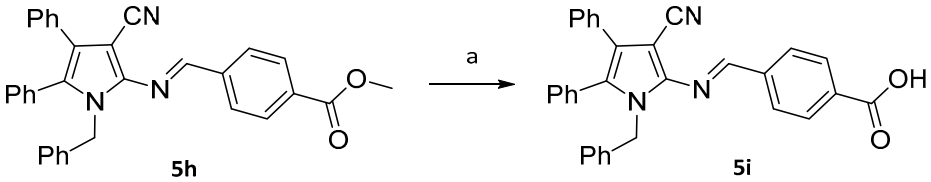
selected because previous studies indicated that an analogue of **1a** with a *p*-nitro benzamide was more potent than the corresponding *meta*-analogue [21]. All imine derivatives were synthesised in good yields, with the exception of the acetamide analogue (**5g**) which tended to degrade in solution back to the starting material. In the next section the inhibitory effects of these compounds on MBLs are described.



Scheme 4. Reagents and conditions: (a) NaOH, tetrahydrofuran, H_2O, 2 h, rt. (80%).

2.2. Assessment of the inhibitory effect using kinetic assays

In a first round of validation the compounds described in the previous section were assessed for their percentage inhibition of MBLs. Assays were performed with the B1-type MBL NDM-1 and the B3-type AIM-1. CphA, which is only active in the presence of one metal ion in the active site, but inhibited by the presence of a second, is also not as stable as the other two enzymes [10,16,26]. It was thus excluded from the initial screening process. Relevant data are presented in Table 1. Compound **1b** displays percentage inhibitions comparable to the values reported previously for **1a** [21,27]. The replacement of the phenyl moieties in positions 4 and 5 in **1a** by either 2-pyridine or 4-(methoxycarbonyl)phenyl groups (i.e., compounds **3b** and **3c**; Scheme 1) abolishes the inhibitory effect (no percentage inhibition observed). The demethylated form of **3c** (**3d**; Scheme 2) also has no inhibitory effect. In contrast, none of the modifications in the amino group of **1a** was significantly detrimental to the inhibitory effect of the resulting compounds, in agreement with the initial acylation study at this position that led to the synthesis of **1b** [21]. Percentage inhibitions range from ~10% to ~60%, and both enzymes are similarly impacted by most of the inhibitors tested.

Table 1. Percent inhibition of NDM-1 and AIM-1 activity in presence of 1 μM or 10 μM of the inhibitor, respectively. Data were collected in triplicates (n = 3).

Inhibitor	*NDM-1 (B1)*	*AIM-1 (B3)*
1b	47 ± 6	42 ± 2
5a	38 ± 2	42 ± 3
5b	57 ± 8	42 ± 3
5c	28 ± 4	11 ± 2
5d	45 ± 1	40 ± 3
5e	50 ± 3	26 ± 4
5f	49 ± 8	61 ± 3
5g	14 ± 3	10 ± 4
5h	27 ± 4	19 ± 6
5i	40 ± 7	39 ± 1
6a	41 ± 2	32 ± 3
6b	29 ± 4	25 ± 1

The complete loss in inhibitory activity for all compounds with modifications to the vicinal phenyl groups (i.e., **3b**-**d**) supports the predictions from previous docking studies, that hydrophobic interactions occur through these substituents [21]. The loss of activity may be due to the additional steric bulk on **3c** and **3d** or, in the case of 3**b**, the negative impact of hydrophilic pyridine functional groups on binding. Previous SAR studies placed methyl groups at this position and found that this resulted in poor activity against another B1-type MBL, IMP-1 [21]. Therefore, it is likely that a

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combination of hydrophobic and aromatic interactions facilitates effective inhibitor binding in this location.

There was little change in inhibitory activity between the benzamide **1b** and its respective imine (**5a**) and amine (**6a**) derivatives for both NDM-1 and AIM-1. This suggests that hydrogen bonding with the carbonyl oxygen is not of great importance for inhibitor binding. The thiophene (**5b**) and 4-hydroxyphenyl (**5f**) imine analogues were among the most potent inhibitors against both NDM-1 (57% and 49% inhibition, respectively) and AIM-1 (42% and 61% inhibition, respectively). Overall, the phenyl ring adjacent to the imine can tolerate a variety of modifications without substantially impacting inhibitory activity. Compounds with bulkier substituents, such as **5g** and **5h**, were the least active, while the pyridine imine (**5c**) also showed modest potency.

A subset of compounds (**1b**, **5a**, **6a**, **5b**, **5f** and **5h**) was selected for further evaluation to determine their K_i values and binding mode (Table 2). For NDM-1 and AIM-1, favourable experimental conditions enabled measurements of Michaelis-Menten parameters at various inhibitor concentrations. For CphA, a lack of enzyme stability and inhibition by metal ions rendered reproducible measurements challenging and hence only IC_{50} values are reported. Nonetheless, while no information about the mode of inhibitor binding could be gained for CphA, it was still possible to evaluate the broad-spectrum efficiency of the compounds in this study.

The majority of these compounds exhibit mixed-mode of inhibition in NDM-1 and AIM-1, suggesting that they can bind to either the enzyme alone or the enzyme-substrate complex. This has frequently been observed for inhibitors reported for various MBLs and may reflect the flexibility of their active sites [13,21,28]. The exception is **6a**, which only binds in an uncompetitive mode to NDM-1 but has a minimal inhibitory effect on CphA or AIM-1. This could potentially be due to the increased torsions about the single bond resulting in a more flexible molecule compared to the rigid amide or imine bonds, making it difficult for the compound to bind close to the catalytic metal centres in the active site. Since there is greater sequence and structure variability in the second (or outer) coordination sphere it may thus not be surprising that this compound may bind to only one of the enzymes selected in this study.

The inhibition of NDM-1 (K_{ic} = 9 μM, K_{iuc} = 5 μM) by **1b** is similar to that previously reported for the related B1-type MBL IMP-1 (K_{ic} = 11 μM, K_{iuc} = 4 μM; [21]). For both NDM-1 and AIM-1, compound **5a** is slightly more potent than **1b**, and compounds **5a**, **5b** and **5f** all have similar potency for their more effective binding mode (i.e., the competitive mode) with K_i values less than 5 μM. The uncompetitive binding affinity, which presumably does not include direct binding interactions with the catalytic metal ions in the active site, is more variable, possibly again a reflection of the inherent flexibility of the active site of MBLs [13].

In summary, compounds **5a**, **5b** and **5f** are promising broad-spectrum inhibitor candidates that are potent against representatives from each of the three MBL subgroups.

Table 2. Competitive (K_{ic}) and uncompetitive (K_{iuc}) inhibition constants for NDM-1 (using penicillin G as the substrate) and AIM-1 (cefuroxime substrate), and IC_{50} values for CphA (meropenem substrate). All values are expressed in micromolar (μM).

COMPOUND	NDM-1		CPHA	AIM-1	
	K_{ic}	K_{iuc}	IC_{50}	K_{ic}	K_{iuc}
1B	9 ± 14	5 ± 1	n/d	11 ± 3.0	19 ± 3
5A	3.2 ± 1.0	6 ± 1	75 ± 3	3.6 ± 0.5	11 ± 3
6A	-	2.7 ± 0.3	n/d	n/d	n/d
5B	1.7 ± 0.6	11 ± 6.3	57 ± 2	4.5 ± 1.0	35 ± 28
5F	1.3 ± 0.7	0.9 ± 0.1	45 ± 2	4.4 ± 0.4	4.7 ± 1.4
5H	34 ± 113	4.4 ± 0.8	n/d	6.4 ± 1.1	72 ± 37

2.3. Checkerboard sensitivity assays

Checkerboard sensitivity assays are a standard method used to compare the antimicrobial efficacy of two or more agents against a specific microorganism by combining the agents at various

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concentrations to assess their synergistic or antagonistic effects. The test is performed in a microtiter plate with serial dilutions of the antimicrobial agents, in this case a combination of antibiotics (penicillin G, cefuroxime and meropenem) and inhibitors (i.e., **1b**, **5a**, **5b** and **5f**), placed in rows and columns to form a checkerboard pattern. The fractional inhibitory concentration (FIC) index, which indicates the degree of interaction between the agents, is used to interpret the results. For example, an FIC index below 0.5 illustrates synergistic behaviour, while an index greater than 0.5 corresponds to antagonistic behaviour. Generally, checkerboard sensitivity assays are useful to optimise combination therapies and identify potential drug targets [25].

In this study *E. coli* cells were transformed with the genes encoding NDM-1, CphA or AIM-1 since these MBLs provide resistance to penicillin G, cefuroxime and meropenem (see also Materials and Methods section). *E. coli* cells transformed with the blaNDM-1 gene, when tested against the combination of the inhibitors (i.e., **1b**, **5a**, **5b** or **5f**) *versus* the selected antibiotics displayed synergy between all inhibitors and penicillin G and cefuroxime, but not meropenem (Table 3). CphA, on the other hand, displays synergistic sensitivity against all inhibitors and antibiotic combinations. For AIM-1 the situation is less clear-cut with synergy observed for compounds **5a** and **5f** in combination with each of the three antibiotics. However, compound **5b** only shows synergy in presence of penicillin G, while **1b** is not synergistic at all.

Checkerboard sensitivity assays help to determine the most effective drug combinations for targeted and effective treatment plans for bacterial infections. It is encouraging that a synergistic effect was observed here for representatives from each of the three major β-lactam antibiotic groups, and while none of the MBL inhibitors tested displays a synergistic effect for each combination tested, compound **5f** is the most promising molecule that emerged from this study as it displays both (i) potent inhibition of MBLs from each of the subgroups and (ii) synergy in all but one combination.

Table 3. Checkerboard sensitivity assays to evaluate possible synergistic effects. When the FIC index values are below 0.5 the effect is considered synergistic (S), when the FIC index values are above 0.5 the effect is considered antagonist (A). PenG, CXM and MEM are abbreviations for penicillin G, cefuroxime and meropenem.

Compounds	NDM-1			CphA			AIM-1		
	PenG	CXM	MEM	PenG	CXM	MEM	PenG	CXM	MEM
1b	S	S	A	S	S	S	A	A	A
5a	S	S	A	S	S	S	S	S	S
5b	S	S	A	S	S	S	S	A	A
5f	S	S	A	S	S	S	S	S	S

3. Conclusions

The development of broad-spectrum inhibitors that inhibit representatives from each of the three major subgroups of this family of enzymes has been an elusive goal for medicinal chemists and clinicians alike. The fact that there is still no clinically useful inhibitor available for any MBL and that β-lactam constitute approximately 65% of all antibiotics used to treat infections, combined with the observation that multi-drug resistant pathogens keep emerging at an alarming rate, make the search for such broad-spectrum inhibitors all the more urgent. The discovery of pyrrole derivatives (**1a** and **1b**) that potently inhibit representative enzymes from each of the three MBL subgroups was encouraging – relevant inhibition constants range from 2 to 20 μM for the different enzymes. Here, modi-fications to this molecule were introduced to elucidate structure-activity relationships, improve "drug-likeness", and improve inhibitory efficiency. Compound **5f** (Scheme 3) is highly potent across the MBL subfamilies. Its equilibrium inhibition constants (K_i values) are below 5 μM for NDM-1 (~1 μM) and AIM-1 (~4 μM), and while it was not possible to get accurate K_i values for the B2-representative CphA, an IC_{50} of 45 μM was measured (Table 2). Furthermore, the checkerboard sensitivity assays also indicated that this inhibitor operates synergistically with most antibiotics tested, except for *E. coli* strains that express the B1-type MBL NDM-1 (Table 3). This compound thus

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represents one of the most promising compounds developed to inhibit MBLs from each subgroup efficiently.

4. Materials and methods

4.1. Chemical synthesis for preparation of derivatives of compounds 1a and 1b

Compounds **1a** and **1b** were synthesised following a previously reported procedure [21]. Unless otherwise stated, reagents were obtained from commercial sources. Silica column chromatography was conducted with silica gel (0.040-0.063 mm, 230-400 mesh). Thin layer chromatography (TLC) was performed on pre-coated aluminium plates (Merck silica 60 F254). Melting points were obtained using an SRS Digimelt MPA161 apparatus. Infrared (IR) spectra were recorded using a Perkin Elmer FT-IR Spectrometer. ^{1}H and ^{13}C NMR were recorded on Bruker 300 MHz or 500 MHz machines. All shifts are reported in parts per million (ppm) and are referenced to the solvent residual peak ($CDCl_3$: ^{1}H = 7.26, ^{13}C = 77.0; DMSO-d6: ^{1}H = 2.50, ^{13}C = 40.0). Coupling constants (J) are reported in Hz. The following abbreviations are used, s=singlet, d=doublet, t=triplet, br=broad. High-resolution mass spectra were recorded on a Bruker MicroTOF-Q instrument.

4.2. Synthesis of compounds 3b-3d

3b: 2-Amino-1-benzyl-4,5-di(pyridin-2-yl)-1H-pyrrole-3-carbonitrile

2-Pyridoin (4.85 mmol, 1.04 g), benzylamine (4.85 mmol, 0.52 g, 531 µL), and a catalytic amount of trichloroacetic acid were combined in toluene (50 mL). The mixture was refluxed under argon using a Dean-Stark apparatus for 2 hours. The solution changed colour from orange to red. Malononitrile (4.85 mmol, 321 mg) and 3 drops of pyridine were added. The reaction was allowed to proceed overnight under the same conditions. The reaction mixture turned dark brown with a tarry precipitate at the bottom of the flask. The solvent was removed under reduced pressure, and the resulting reddish-brown residue was dissolved in ethyl acetate. The crude product was separated from the insoluble by-products via trituration, followed by purification by silica column chromatography (40-80% EtOAc in light petroleum). The purified product was then recrystallised from ethyl acetate to yield the title compound as light brown crystals (170 mg, 10%), m.p 183.0-183.7 ^{o}C, R*f* 0.53 (EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.99 (s, 2H, N**H**$_2$), 5.29 (s, 2H, C**H**$_2$), 7.07-7.15 (m, 6H, Ar-**H**), 7.21-7.31 (m, 4H, Ar-**H**), 7.43-7.51 (dtd, *J* = 25.5 & 7.7 & 1.8), 8.58-8.59 (m, 1H, Py-**H**), 8.59 (ddd, *J* = 5.2 & 1.7 & 0.7, 1H, Py-**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.15 (**C**H$_2$), 76.44 (**C**-CN), 116.89 (**C**≡N), 121.63 (Ar-**C**H), 122.15 (Ar-**C**H), 122.98 (Ar-**C**), 123.57 (Ar-**C**H), 125.45 (Ar-**C**), 126.49 (Ar-**C**H), 126.53 (Ar-**C**H), 127.80 (Ar-**C**H), 128.93 (Ar-**C**H), 136.01 (Ar-**C**H), 136.11 (Ar-**C**), 136.16 (Ar-**C**H), 147.08 (Ar-**C**H), 149.46 (Ar, C=N), 149.62 (Ar, C=N), 150.37 (Ar-**C**), 152.36 (Ar-**C**). HRMS, found: m/z 352.1556, $C_{22}H_{18}N_5^+$ requires 352.1557. IR υ_{max} (cm^{-1}): 3164 (N-H str), 2202 (C≡N), 1634, 1552, 1473, 1364, 1090, 991, 789, 736, 619.

3c: dimethyl 4,4'-(5-amino-1-benzyl-4-cyano-1H-pyrrole-2,3-diyl)dibenzoate

A mixture of 2c (0.4 g, 1.2 mmol), benzylamine (0.13 g, 1.2 mmol) and trichloroacetic acid (0.0098 g, 0.056 mmol) in dry toluene (10 mL) was heated under reflux and under argon using a Dean Stark apparatus for 3 h. A yellow solution was obtained. Malononitrile (0.080 g, 1.2 mmol) was then added to the reaction mixture, followed by 3 drops of pyridine. The mixture was heated to reflux under argon for a further 17 h. A colour change from yellow to brown was observed. The excess solvent was removed in vacuo to give a brown semi solid residue which was purified by silica gel column chromatography [EtOAc/light petroleum (20:80) → EtOAc/light petroleum (50:50)], followed by recrystallisation from ethyl acetate to yield the title compound (68 mg, 13 %). m.p: 172-173°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.88 (d, 6H, C(=O)OC**H**$_3$), 3.94 (br s, 2H, N**H**$_2$), 4.93 (br s, 2H, N-C**H**$_2$-Ph), 7.07 (d, 2H, J=7 Hz, Ar-C**H**), 7.17 (d, 2H, J=8 Hz, Ar-C**H**), 7.25-7.4 (m, 5H, Ar-C**H**), 7.90 (t, 4H, J=8 Hz, Ar-C**H**). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 48.6 (N-**C**H$_2$-Ph), 51.4 (2 X C(=O)O**C**H$_3$), 81.4 (**C**-CN), 111.3 (**C**N), 124.0 (**C**-Ph-COOMe), 125.7 (Ar-**C**H), 127.2 (**C**-Ph-COOMe), 127.8 (2 x Ar-**C**H), 128.1 (2 x Ar-**C**H), 128.3 (2 x Ar-**C**H), 130.1 (2 x Ar-**C**H), 136.1 (Ar-**C**H), 136.5 (Ar-**C**H), 137.2 (Ar-

CH), 165.9 (2 X C=O). IR ν_{max} (cm^{-1}): 3461, 3400, 3370, 2955, 2221, 1718, 1607, 1574, 1511, 1403, 1271, 1094, 815.

3d: 4,4'-(5-amino-1-benzyl-4-cyano-1H-pyrrole-2,3-diyl)dibenzoic acid

Compound 3c (70 mg, 0.2 mmol) was dissolved in tetrahydrofuran (10 mL) and a solution of sodium hydroxide (0.060 g, 2 mmol) was added. The mixture was stirred overnight at room temperature followed by the slow addition of 2 M HCl until pH=2. The product was extracted using ethyl acetate and the organic layer was dried with magnesium sulphate. The solvent was removed in vacuo to give the title compound as a yellow solid (57 mg, 10%). m.p: 220-222°C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.48 (2H, br s, N**H**$_2$), 4.87 (d, 2H, *J* = 6 Hz, N-C**H**$_2$-Ph), 7.08 (d, 2H, *J* = 8 Hz, Ar-C**H**), 7.18-7.34 (m, 5H, Ar-C**H**) 7.81 (d, 2H, *J* = 8 Hz, Ar-C**H**), 7.83 (d, 2H, *J* = 8 Hz, Ar-C**H**). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 42.5 (N-**C**H$_2$-Ph), 81.8 (**C**-CN), 111.7 (**C**N), 124.3 (**C**-Ph-COOH), 125.7 (Ar-**C**H), 127.6 (**C**-Ph-COOH), 127.8 (2 x Ar-**C**H), 128.1 (2 x Ar-**C**H), 128.3 (2 x Ar-**C**H), 130.2 (2 x Ar-**C**H), 136.1 (Ar-**C**H), 136.5 (Ar-**C**H), 137.2 (Ar-**C**H), 165.9 (2 X C=O). IR ν_{max} (cm^{-1}): 3449, 3400, 3370, 2955, 2221, 1720, 1607, 1574, 1511, 1403, 1271, 1094, 815.

4.3. General procedure for the preparation of imine derivatives 5a-5i

2-Amino-1-benzyl-4,5-diphenyl-1H-pyrrole-3-carbonitrile (**1a**) (1 eq), the corresponding aldehyde (1 eq), and a catalytic amount of trichloroacetic acid (TCA) were combined in toluene (10 mL). The mixture was refluxed for 2-6 h under argon using a Dean-Stark apparatus. The solvent was removed in vacuo and the crude product was purified by silica column chromatography or recrystallisation.

5a: 1-Benzyl-2-(benzylideneamino)-4,5-diphenyl-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (1 mmol, 349 mg) and benzaldehyde (1 mmol, 106 mg, 102 μL) were used. The crude product was obtained as an orange solid which was purified by silica column chromatography (10% EtOAc/light petroleum) to afford the title compound as yellow crystals (371 mg, 85%), m.p. 170.1-170.6°C, R*f* 0.72 (30% EtOAc/light petroleum). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.31 (s, 2H, C**H**$_2$), 6.90-6.92 (d, *J* = 7.4, 1H, Ar-**H**), 6.91-6.93 (d, *J* = 5.5, 1H, Ar-**H**) 7.07-7.11 (m, 2H, Ar-**H**), 7.18-7.36 (m, 11H, Ar-**H**), 7.42-7.52 (m, 3H, Ar-**H**), 7.89-7.92 (d, *J* = 7.9, 1H, Ar-**H**), 7.90-7.93 (d, *J* = 7.6, 1H, Ar-**H**) 9.30 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.06 (**C**H$_2$), 79.90 (**C**-CN), 117.72 (**C**≡N), 125.35 (Ar-**C**), 126.86 (Ar-**C**H), 126.91 (Ar-**C**H), 127.35 (Ar-**C**H), 128.24 (Ar-**C**H), 128.47 (Ar-**C**H), 128.52 (Ar-**C**H), 128.81 (Ar-**C**H), 128.98 (Ar-**C**H), 129.01 (Ar-**C**H), 129.17 (Ar-**C**H), 130.56 (Ar-**C**), 130.65 (Ar-**C**H), 131.21 (Ar-**C**H), 131.99 (Ar-**C**), 132.42 (Ar-**C**), 136.01 (Ar-**C**), 137.62 (Ar-**C**), 144.82 (N-**C**-N), 159.76 (N=**C**). ESI-MS: m/z 438.2 $[M + H]^+$. HRMS, found: m/z 438.1965, $C_{31}H_{24}N_3^+$ requires 438.1964. IR υ_{max} (cm^{-1}): 2205 (C≡N stretch), 1500 (N=C), 1494, 1450, 1335, 1207, 1073, 968, 765, 690, 590.

5b:1-Benzyl-4,5-diphenyl-2-((thiophen-2-ylmethylene)amino)-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (0.73 mmol, 257 mg) and 2-thiophenecarboxyaldehyde (0.73 mmol, 82 mg, 67 μL) were used to give an orange solid as the crude product. Recrystallisation from ethyl acetate yielded the title compound as yellow crystals (149 mg, 46%). m.p. 181.5-182.4 °C, R*f* 0.65 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.26 (s, 2H, C**H**$_2$), 6.91 (d, *J* = 6.6, 1H, Ar-**H**), 6.92 (d, *J* = 7.7, 1H, Ar-**H**) 7.10-7.12 (m, 2H, Ar-**H**), 7.13-7.15 (dd, *J* = 4.8 & 3.7, 1H, thiophene-**H**), 7.16-7.35 (m, 11H, Ar-**H**), 7.52-7.53 (dt, *J* = 5.0 & 1.0, 1H, thiophene-**H**), 7.56-7.57 (dd, *J* = 3.7 & 0.7, 1H, thiophene-**H**), 9.37 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.05 (**C**H$_2$Ph), 79.95 (**C**-CN), 117.82 (**C**≡N), 125.39 (Ar-**C**), 126.89 (Ar-**C**H), 127.28 (Ar-**C**H), 127.38 (Ar-**C**H), 128.26 (Ar-**C**H), 128.32 (Ar-**C**H), 128.43 (Ar-**C**H), 128.55 (Ar-**C**H), 128.59 (Ar-**C**H), 129.17 (Ar-**C**H), 130.53 (Ar-**C**H), 130.63 (Ar-**C**H), 131.27 (Ar-**C**H), 131.45 (Ar-**C**), 132.43 (Ar-**C**), 133.55 (Ar-**C**), 137.59 (Ar-**C**), 143.13 (N-**C**-N), 144.29 (CH-**C**-S), 152.09 (N=**C**H). HRMS, found: m/z 444.1527, $C_{29}H_{22}N_3S^+$ requires 444.1529. IR υ_{max} (cm^{-1}): 2210 (C≡N stretch), 1591 (C=N), 1470, 1425, 1344, 1198, 1022, 851, 758, 715, 689, 589.

5c: 1-Benzyl-4,5-diphenyl-2-((pyridin-3-ylmethylene)amino)-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (0.40 mmol, 139 mg) and 3-pyridinecarboxyaldehyde (0.40 mmol, 42 mg, 37 μL) were used. The crude product was obtained as a yellow-brown solid and was purified by silica column chromatography (10% → 30% EtOAc/light petroleum) yielding the title compound as a yellow powder (126 mg, 72%). m.p. 159.8-160.6 °C, R*f* 0.25 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz,

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$CDCl_3$) δ 5.31 (s, 2H, C**H**$_2$), 6.90 (d, *J* = 6.1, 1H, Ar-**H**), 6.91 (d, *J* = 7.5, 1H, Ar-**H**), 7.09-7.12 (m, 2H, Ar-**H**), 7.18-7.41 (m, 11H, Ar-**H**), 7.43 (br t, *J* = 5.5, 1H, N=CH-C**H**-CH pyridine), 8.16 (dt, *J* = 7.9 & 1.7, 1H, C-C**H**=CH pyridine), 8.69 (br s, 1H, N=C**H**-CH), 9.08 (br s, 1H, C=C**H**-N pyridine), 9.32 (s, 1H, N=C**H** imine). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.21 (**C**H$_2$), 80.59 (**C**-CN), 117.49 (**C**N), 123.82 (**C**H-CH-N pyridine), 125.80 (Ar-**C**), 126.67 (Ar-**C**H), 127.05 (Ar-**C**H), 127.45 (Ar-**C**H), 128.30 (Ar-**C**H), 128.56 (Ar-**C**H), 128.60 (Ar-**C**H), 128.70 (Ar-**C**H), 129.15 (Ar-**C**H), 130.27 (Ar-**C**), 131.12 (Ar-**C**H), 131.42 (Ar-**C**H), 131.79 (Ar-**C**), 132.14 (Ar-**C**), 135.29 (C-**C**H-CH pyridine), 137.46 (Ar-**C**), 143.77 (N-**C**-N), 150.45 (N-**C**H=C pyridine), 152.11 (N=**C**H-CH pyridine), 156.09 (N=**C**H imine). HRMS, found: m/z 439.1918, $C_{30}H_{23}N_4^+$ requires 439.1917. IR υ_{max} (cm^{-1}): 2211 (C≡N), 1616, 1569, 1471, 1342, 1205, 1023, 772, 690, 597.

5d: 1-Benzyl-2-((4-nitrobenzylidene)amino)-4,5-diphenyl-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (0.30 mmol, 116 mg) and 4-nitrobenzaldehyde (0.30 mmol, 50 mg) were used to yield the crude product as a reddish-brown solid. The crude product was dissolved in a minimum amount of hot chloroform and EtOAc was added until the product started to precipitate, yielding orange-red crystals (120 mg, 75%). m.p. 191.6-192.5 °C, R*f* 0.65 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.33 (s, 2H, C**H**$_2$), 6.91 (d, *J* = 7.0, 1H, Ar-**H**), 6.92 (d, *J* = 7.7, 1H, Ar-**H**), 7.09-7.12 (m, 2H, Ar-**H**), 7.18-7.38 (m, 11H, Ar-**H**), 8.01 (dt, *J* = 9.2 & 2.0, 2H, N=CH-C-C**H**), 8.28 (dt, *J* = 9.2 & 2.0, 2H, C**H**-C-NO$_2$), 9.38 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.30 (**C**H$_2$), 81.30 (**C**-CN), 117.35 (**C**N), 124.07 (**C**H-C-NO$_2$), 126.41 (Ar-**C**), 126.64 (Ar-**C**H), 127.22 (Ar-**C**H), 127.55 (Ar-**C**H), 128.35 (Ar-**C**H), 128.62 (Ar-**C**H), 128.66 (Ar-**C**H), 128.85 (Ar-**C**H), 129.15 (Ar-**C**H), 129.26 (Ar-**C**H), 130.09 (Ar-**C**), 131.05 (Ar-**C**H), 131.93 (Ar-**C**), 132.25 (Ar-**C**), 137.38 (Ar-**C**), 141.55 (N-**C**-N), 143.00 (N=CH-**C**), 155.455 (N=**C**H). HRMS found: m/z 483.1806, $C_{31}H_{23}N_4O_2^+$ requires 483.1816. IR υ_{max} (cm^{-1}): 2211 (C≡N), 1598 & 1576 (N-O asymmetric str), 1518, 1459, 1337 (N-O symmetric str), 1199, 1023, 849, 771, 694, 599.

5e: 1-Benzyl-2-((naphthalen-2-ylmethylene)amino)-4,5-diphenyl-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (0.29 mmol, 114 mg) and 2-napthaldehyde (0.29 mmol, 51 mg) were used, yielding the crude product as a yellow-brown solid. This was purified by silica column chromatography (5% → 15% EtOAc/light petroleum) to afford the title compound as dark yellow crystals (141 mg, 88%), m.p. 161.3-162.0 °C, R*f* 0.67 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.36 (s, 2H, C**H**$_2$), 6.95 (d, *J* = 6.6, 1H, Ar-**H**), 6.97 (d, *J* = 7.7, 1H, Ar-**H**), 7.10-7.13 (m, 2H, Ar-**H**), 7.18-7.37 (m, 11H, Ar-**H**), 7.51-7.59 (m, 2H, Nap-**H**), 7.85-7.95 (m, 3H, Nap-**H**), 8.11 (dd, *J* = 8.7 & 1.6, 1H, Nap-**H**), 8.25 (s, 1H, Nap-**H**), 9.27 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 47.11 (**C**H$_2$), 79.92 (**C**-CN), 117.83 (**C**N), 123.10 (Ar-**C**), 125.42 (Ar-**C**H), 126.77 (Ar-**C**H), 126.87 (Ar-**C**H), 126.97 (Ar-**C**H), 127.36 (Ar-**C**H), 127.89 (Ar-**C**H), 127.91 (Ar-**C**H), 128.24 (Ar-**C**H), 128.48 (Ar-**C**H), 128.53 (Ar-**C**H), 128.68 (Ar-**C**), 128.95 (Ar-**C**H), 129.18 (Ar-**C**H), 130.58 (Ar-**C**H), 130.73 (Ar-**C**), 131.21 (Ar-**C**H), 132.43 (Ar-**C**), 132.55 (Ar-**C**H), 133.06 (Ar-**C**), 133.75 (Ar-**C**), 135.27 (Ar-**C**), 137.66 (Ar-**C**), 144.87 (N-**C**-N=C), 159.56 (N=**C**). HRMS, found: m/z 488.2125, $C_{35}H_{26}N_3^+$ requires 488.2121. IR υ_{max} (cm^{-1}): 2204 (C≡N), 1599, 1469, 1329, 1209, 951, 819, 753, 707, 603.

5f:1-Benzyl-2-((4-hydroxybenzylidene)amino)-4,5-diphenyl-1H-pyrrole-3-carbonitrile

Pyrrole **1a** (0.41 mmol, 142 mg) and 4-hydroxybenzaldehyde (0.41 mmol, 50 mg) were used. A brownish yellow solution with a yellow precipitate was obtained. The solvent was removed under reduced pressure and the crude product washed with methanol, yielding the title compound as a yellow powder (148 mg, 80%). m.p. 237.9-239.3 °C, R*f* 0.28 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.25 (s, 2H, C**H**$_2$), 6.82 (d, *J* = 7.4, 1H, Ar-**H**), 6.84 (d, *J* = 8.1, 1H, Ar-**H**), 6.90 (dt, *J* = 9.4 & 2.0, 2H, C**H**-C-OH), 7.15-7.37 (m, 13H, Ar-**H**), 7.80 (d, *J* = 9.4 & 2.0, 2H, N=CH-C-C**H**), 9.00 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, DMSO-d_6) δ 45.86 (**C**H$_2$), 77.77 (**C**-CN), 115.57 (**C**H-C-OH), 116.81(**C**N), 123.06 (Ar-**C**), 125.93 (Ar-**C**H), 126.06 (Ar-**C**H), 126.39 (Ar-**C**H), 126.72 (Ar-**C**H), 127.80 (Ar-**C**H), 127.93 (Ar-**C**H), 128.05 (Ar-**C**H), 128.12 (Ar-**C**), 128.36 (Ar-**C**H), 129.13 (Ar-**C**), 129.55 (Ar-**C**), 130.50 (Ar-**C**H), 130.77 (Ar-**C**H), 131.92 (Ar-**C**), 136.89 (Ar-**C**), 145.87 (Ar-**C**), 160.57 (N=**C**H), 161.46 (**C**-OH). HRMS, found: m/z 454.1926, $C_{31}H_{24}N_3O^+$ requires 454.1914. IR υ_{max} (cm^{-1}): 3255 (OH str), 2211 (C≡N), 1601, 1571 (N=C), 1493, 1340, 1281, 1204, 1157, 835, 766, 590.

5g: N-(4-(((1-Benzyl-3-cyano-4,5-diphenyl-1H-pyrrol-2-yl)imino)methyl)phenyl) acetamide

Pyrrole **1a** (0.23 mmol, 80 mg) and 4-acetaminobenzaldehyde (0.23 mmol, 38 mg) were used. The crude product was purified by silica column chromatography (30% → 60% EtOAc/light petroleum)

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to yield the title compound as yellow crystals (76 mg, 67%) m.p. 218.9-219.2 °C, R*f* 0.69 (EtOAc). The product was unstable and degraded in solution. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.19 (s, 3H, C**H**$_3$), 5.28 (s, 2H, C**H**$_2$), 6.90 (d, *J* = 6.8, 1H, Ar-**H**), 6.91 (d, *J* = 8.7, 1H, Ar-**H**), 7.08-7.10 (m, 2H, Ar-**H**), 7.16-7.34 (m, 11H, Ar-**H**), 7.61 (d, *J* = 8.4, 2H, C**H**-C-NH), 7.84 (dt, *J* = 9.1 & 2.00, 2H, N=CH-C-C**H**), 9.20 (s, 1H, C**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.76 (**C**H$_3$), 47.06 (**C**H$_2$), 79.60 (**C**-CN), 117.91 (**C**≡N), 119.37 (**C**H-C-NH), 125.22 (Ar-**C**), 126.85 (Ar-**C**H), 126.93 (Ar-**C**H), 127.35 (Ar-**C**H), 128.23 (Ar-**C**H), 128.48 (Ar-**C**H), 128.50 (Ar-**C**H), 128.53 (Ar-**C**H), 129.16 (Ar-**C**H), 130.12 (Ar-**C**H), 130.51 (Ar-**C**), 130.60 (Ar-**C**), 131.15 (Ar-**C**), 130.60 (Ar-**C**H), 131.15 (Ar-**C**H), 131.22 (N=CH-**C**), 132.48 (Ar-**C**), 137.64 (Ar-**C**), 141.36 (Ar-**C**), 145.22 (**C**-NH), 158.90 (N=**C**H), 168.34 (**C**=O). HRMS, found: m/z 495.2155, $C_{33}H_{27}N_4O^+$ requires 495.2179. IR υ_{max} (cm^{-1}): 3322 & 3289 (NH str), 2214 (C≡N), 1588 (C=O str), 1580 (N=C), 1533, 1410, 1320, 1255, 1023, 828, 744, 690, 500.

5h: Methyl 4-(((1-benzyl-3-cyano-4,5-diphenyl-1H-pyrrol-2-yl)imino)methyl)benzoate

Pyrrole **1a** (0.26 mmol, 92 mg) and methyl 4-formylbenzoate (0.26 mmol, 44.8 mg) were used. The crude product was dissolved in a minimum amount of hot chloroform and EtOAc was added until the product started to precipitate, yielding the title compound as yellow crystals (86 mg, 62%) m.p. 178.-179.1 °C, R*f* 0.53 (30% EtOAc/light petroleum). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 3.94 (s, 3H, C**H**$_3$), 5.32 (s, 2H, C**H**$_2$), 6.91 (d, *J* = 6.9, 1H, Ar-**H**), 6.92 (d, *J* = 7.9, 1H, Ar-**H**), 7.10 (m, 2H, Ar-**H**), 7.18-7.36 (m, 11H, Ar-**H**), 7.95 (dt, *J* = 1.7 & 8.4, 2H, N=CH-C-C**H**), 8.10 (dt, *J* = 1.7 & 8.4, 2H, C**H**-C-COO-CH$_3$), 9.35 (s, 1H, N=C**H**). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ 47.19 (**C**H$_2$), 52.34 (**C**H$_3$), 80.59 (**C**-CN), 117.53 (**C**N), 125.89 (Ar-**C**H), 126.82 (Ar-**C**H), 127.03 (Ar-**C**H), 127.44 (Ar-**C**H), 128.28 (Ar-**C**H), 128.53 (Ar-**C**H), 128.58 (Ar-**C**H), 128.66 (Ar-**C**H), 128.68 (Ar-**C**H), 129.16 (Ar-**C**H), 129.97 (Ar-**C**H), 130.34 (Ar-**C**H), 131.14 (Ar-**C**), 131.44 (Ar-**C**), 132.18 (Ar-**C**), 132.64 (Ar-**C**), 137.50 (Ar-**C**), 139.87 (Ar-**C**), 143.88 (Ar-**C**), 157.72 (N=**C**), 166.45 (**C**=O). HRMS, found: m/z 496.1996, $C_{33}H_{26}N_3O_2^+$ requires 496.2020. IR υ_{max} (cm^{-1}): 2205 (C≡N), 1715 (C=O str), 1500 (C=N), 1452, 1274 (C-O str), 1104, 757, 712, 602.

5i: 4-(((1-Benzyl-3-cyano-4,5-diphenyl-1H-pyrrol-2-yl)imino)methyl)benzoic acid

The imine **5h** (0.24 mmol, 117 mg) was dissolved in tetrahydrofuran (15 mL). Sodium hydroxide (2.4 mmol, 95 mg) was dissolved in water (2 mL) and added to the tetrahydrofuran solution. The mixture was stirred at room temperature for 2 hrs. The reaction was acidified with 5% HCl, and the product was extracted with ethyl acetate (3 x 5 mL), washed with brine (3 x 5 mL), and dried with sodium sulfate. The crude product was then recrystallised from ethanol to give the title compound as orange crystals (91 mg, 80%), m.p. 331.5–131.7 °C, R*f* 0.59 (EtOAc). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 5.33 (s, 2H, C**H**$_2$), 6.85 (dd, *J* = 7.7 & 1.8, 2H, Ar-**H**), 7.14-7.40 (m, 17H, Ar-**H**), 9.25 (s, 1H, N=C**H**), 13.25 (br s, 1H, COO**H**). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ (ppm): 47.11 (**C**H$_2$), 80.17 (**C**-CN), 117.36 (**C**N), 125.08 (Ar-**C**), 126.98 (Ar-**C**H), 127.64 (Ar-**C**H), 127.81 (Ar-**C**H), 128.87 (Ar-**C**H), 128.98 (Ar-**C**H), 129.12 (Ar-**C**H), 129.35 (Ar-**C**H), 129.37 (Ar-**C**H), 129.41 (Ar-**C**), 130.24 (Ar-**C**H), 130.41 (Ar-**C**H), 131.47 (Ar-**C**), 131.56 (Ar-**C**), 132.56 (Ar-**C**), 134.24 (Ar-**C**H), 137.72 (Ar-**C**), 139.39 (**C**-N=C), 144.81 (N=**C**-H),160.01 (N=**C**)**,** 167.19 (**C**OOH). HRMS, found: m/z 480.1729, $C_{33}H_{22}N_3O_2^-$ requires 480.1718. IR υ_{max} (cm^{-1}): 2958-2530 (OH str), 2211 (C≡N), 1582 (COO$^-$), 1509 (N=C), 1405, 1279 (C=O str), 902, 855, 758, 598.

4.4. General Procedure for the reduction of imines to amines

The imine (1 eq) was suspended in ethanol (40 mL) and sodium borohydride (10 eq) was slowly added at room temperature. The reaction was allowed to proceed overnight. A colour change from yellow to off-white was observed. The solvent was removed in vacuo and the solid was re-dissolved in ethyl acetate, washed with water (2 x 10 mL) and brine (2 x 10 mL), dried with sodium sulfate and concentrated in vacuo to afford the product."

6a: 1-Benzyl-2-(benzylamino)-4,5-diphenyl-1H-pyrrole-3-carbonitrile

The imine (**5a**) (0.76 mmol, 333 mg) was used. The product was obtained as a sticky orange residue which dried to a glassy solid (170 mg, 50%), m.p. 97-101 °C, R*f* 0.63 (30% EtOAc/light petroleum). The product is unstable and slowly oxidises back to the imine in solution upon contact with the air. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.67 (br s, 1H, N**H**), 4.61 (s, 2H, N-C**H**$_2$), 4.88 (s, 2H, N-

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C**H**$_2$), 7.01-7.03 (d, *J* = 6.8, 2H, Ar-**H**), 7.12-7.44 (m, 18H, Ar-**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 46.97 (**C**H$_2$-Ph), 49.99 (NH-**C**H$_2$), 76.19 (**C**-CN), 118.34 (**C**≡N), 122.27 (Ar-**C**), 125.87 (Ar-**C**H), 126.04 (Ar-**C**), 126.41 (Ar-**C**H), 127.5 (Ar-**C**H), 127.76 (Ar-**C**H), 127.91 (Ar-**C**H), 128.00 (Ar-**C**), 128.12 (Ar-**C**H), 128.60 (Ar-**C**H), 128.65 (Ar-**C**H), 129.16 (Ar-**C**H), 129.21 (Ar-**C**H), 130.89 (Ar-**C**), 130.94 (Ar-**C**H), 133.05 (Ar-**C**), 136.27 (Ar-**C**), 138.25 (Ar-**C**). HRMS, found: m/z 440.2099, $C_{31}H_{26}N_3^+$ requires 440.2121. IR υ_{max} (cm^{-1}): 3334 (br, N-H str), 2187 (C≡N), 1709 (N-H bend), 1555, 1451, 1352, 1073, 1025, 918, 692.

6b: 1-Benzyl-4,5-diphenyl-2-((thiophen-2-ylmethyl)amino)-1H-pyrrole-3-carbonitrile

The imine (**5b**) (0.47 mmol, 210 mg) was used yielding the product as sticky yellow residue which dried to a glassy solid (171 g, 80%), m.p. 108.3-110.3 °C, R*f* 0.54 (30% EtOAc/light petroleum). The product is unstable and slowly oxidises back to the imine in solution upon contact with air. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 (s, 1H, N**H**), 4.78 (s, 2H, N-C**H**$_2$), 4.89 (s, 2H, N-C**H**$_2$), 6.92-6.94 (m, 2H, Ar-**H**), 7.02 (d, *J* = 6.5, 2H, Ar-**H**), 7.09-7.26 (m, 14H, Ar-**H**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 45.13 (**C**H$_2$-Ph), 47.02 (NH-**C**H$_2$), 77.55 (**C**-CN), 118.08 (**C**N), 122.37 (Ar-**C**), 125.20 (Ar-**C**H), 125.84 (Ar-**C**H), 125.96 (Ar-**C**H), 126.42 (Ar-**C**), 126.46 (Ar-**C**H), 126.90 (Ar-**CH**), 127.89 (Ar-**C**H), 128.06 (Ar-**C**H), 128.15 (Ar-**C**H), 128.60 (Ar-**C**H), 129.13 (Ar-**C**H), 129.19 (Ar-**C**H), 130.83 (Ar-**C**), 130.92 (Ar-**CH**), 132.98 (Ar-**C**), 136.27 (Ar-**C**), 141.38 (N-**C**-N), 145.37 (CH$_2$-**C**-S). HRMS, found: m/z 446.1660, $C_{29}H_{24}N_3S^+$ requires 446.1685. IR υ_{max} (cm^{-1}): 3355 (br, N-H str), 2203 (C≡N), 1715 (N-H bend), 1556, 1454, 1345, 1071, 1020, 847, 740, 693.

4.5. Enzyme activity and inhibition assays

NDM-1, CphA and AIM-1, MBLs representing the B1, B2 and B3 subgroups, respectively, were recombinantly expressed, purified and assayed as described previously [19–21]. Enzymatic activity assays were performed in aqueous buffer solutions with the following components: 10 mM HEPES, 75 μM $ZnCl_2$, pH 7.5. Enzyme activity was monitored spectrophotometrically using a Cary 60 Bio Varian US-Vis spectrophotometer measuring substrate depletion. Reaction rates (abs/min) were measured over 1 minute.

The effect of inhibitors was initially estimated by determining the percent inhibition using standard rate assays in the presence and absence of inhibitors; data were analysed using Equation 1. Inhibitors were solubilised in neat DMSO at 10 mM and then diluted to 1 mM using the activity assay buffer. To measure the reaction rates in the absence of inhibitors, DMSO was added to the reaction at the same concentration that would occur with the addition of inhibitor. For measurement of percentage inhibition conditions were as follows: NDM-1 (15 nM), penicillin G (300 μM), inhibitor (1 μM) and AIM-1 (3 nM), cefuroxime (70 μM), inhibitor (10 μM).

$$\%\ \mathbf{inhibition} = \frac{\mathbf{activity\ without\ inhibitor} - \mathbf{activity\ with\ inhibitor}}{\mathbf{activity\ without\ inhibitor}} \qquad \text{Equation 1}$$

To obtain accurate inhibition constants and information about the mode of inhibitor binding (competitive, uncompetitive) Michaelis-Menten curves were recorded in the absence of inhibitors and at three different inhibitor concentrations. The data were fit to Equation 2 [7,12,13,15,19–21,28–30], where V_{max} and K_m are the maximum velocity and Michaelis-Menten constant, respectively, and [S] and [I] are the concentration of substrate and inhibitor. K_{ic} and K_{iuc} are the competitive and uncompetitive inhibition constants.

$$\frac{1}{v} = \frac{1+\frac{[I]}{K_{iuc}}}{V_{max}} + \frac{K_M\left(\frac{[I]}{K_{ic}}\right)}{V_{max}[S]} \qquad \text{Equation 2}$$

4.6. Checkboard sensitivity test

Checkboard assays were used to evaluate the potential synergy of compounds **1b**, **5a**, **5b** and **5f** against penicillin G, meropenem and cefuroxime. The assays were performed to assess the effect of the inhibitors on bacterial growth [25]. The assays applied a combination of two compounds in increasing concentrations to provide an estimate of the Fractional Inhibitory Concentration Index

(FICI), defined as follows: S, synergy (FIC ≤ 0.5); and A, Antagonist (FIC > 0.5). A stock solution of the combined compounds was prepared in Mueller Hinton broth according to the manufacturer's specifications. The antibiotics penicillin G, meropenem and cefuroxime were diluted into the different wells at final concentrations of 0, 0.063, 0.125, 0.5, 1, 2, 4, 16, 32 µg/mL, and the inhibitors were diluted to final concentrations of 0, 0.12, 0.37, 1.11, 3.33, 8 and 10 µg/mL. *E. coli* BL21(DE3) cells were transformed with vector pET27b(+) containing the genes bla_{NDM-1}, bla_{CphA} or bla_{AIM-1} (encoding the enzymes NDM-1, CphA or AIM-1, respectively) and isolated colonies were used to prepare bacterial solutions in broth also containing 0.9% w/v of NaCl; the final cell concentration was 5x105 CFU mL^{-1} in a total volume of 1 mL. These solutions were then incubated at 37 °C for 18 hours, and the OD_{600} was measured. Wells with OD_{600} values less than 0.07 correlate to no growth upon visible inspection, while wells with OD_{600} values greater than 0.07 correspond to growth upon visual inspection. The lowest drug concentration at which bacterial growth is inhibited is defined as the minimum inhibitory concentration (MIC) for each inhibitor. The fractional inhibitory concentration (FIC) of each inhibitor is calculated in each well by dividing the amount of antibiotic present by the inhibitor's MIC, and the fractional inhibitory concentration index (FICI) is calculated by adding the two FICs [23].

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References

1. Fisher, J.F.; Meroueh, S.O.; Mobashery, S. Bacterial resistance to β-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem. Rev*. 2005, 105, 395-424, doi:10.1021/cr030102i.
2. Allen, H.K.; Donato, J.; Wang, H.; Cloud-Hansen, K.A.; Davies, J.; Handelsman, J. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol*. 2010, doi:10.1038/nrmicro2312.
3. Davies, J.; Davies, D. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev*. 2010, doi:10.1128/MMBR.00016-10.
4. Hawkey, P.M. Multidrug-resistant Gram-negative bacteria: a product of globalization. *J. Hosp. Infect*. 2015, 89, 241-247, doi:10.1016/j.jhin.2015.01.008.
5. Bush, K.; Jacoby, G.A. Updated Functional Classification of β-Lactamases. *Antimicrobial. Agents Chemother*. 2010, 54, 969-976, doi:10.1128/aac.01009-09.
6. Kapoor, G.; Saigal, S.; Elongavan, A. Action and resistance mechanisms of antibiotics: A guide for clinicians. *J. Anaesthesiol. Clin. Pharmacol*. 2017, 33, 300-305, doi:10.4103/joacp.JOACP349_15.
7. Pedroso, M.M.; Waite, D.W.; Melse, O.; Wilson, L.; Miti, N.; McGeary, R.P.; Antes, I.; Guddat, L.W.; Hugenholtz, P.; Schenk, G. Broad spectrum antibiotic-degrading metallo-β-lactamases are phylogenetically diverse. *Protein Cell*. 2020, 11, 613-617, doi:10.1007/s13238-020-00736-4.
8. Khan, A.U.; Maryam, L.; Zarrilli, R. Structure, Genetics and Worldwide Spread of New Delhi Metallo-β-lactamase (NDM): a threat to public health. *BMC Microbiol*. **2017**, *17*, 101, doi:10.1186/s12866-017-1012-8.
9. Moellering, R.C. NDM-1 — A Cause for Worldwide Concern. N. Eng. J. Med. 2010, 363, 2377-2379, doi:10.1056/NEJMp1011715.
10. Sun, Z.; Mehta, S.C.; Adamski, C.J.; Gibbs, R.A.; Palzkill, T. Deep Sequencing of Random Mutant Libraries Reveals the Active Site of the Narrow Specificity CphA Metallo-β-Lactamase is Fragile to Mutations. *Sci. Rep*. **2016**, *6*, doi:10.1038/srep33195.
11. Miraula, M.; Schenk, G.; Mitić, N. Promiscuous metallo-β-lactamases: MIM-1 and MIM-2 may play an essential role in quorum sensing networks. *J. Inorg. Biochem*. 2016, 162, 366-375, doi:10.1016/j.jinorgbio.2015.12.014.

12. Miraula, M.; Whitaker, J.J.; Schenk, G.; Mitić, N. β-Lactam antibiotic-degrading enzymes from non-pathogenic marine organisms: a potential threat to human health. *J. Inorg. Biochem*. 2015, 20, 639-651, doi:10.1007/s00775-015-1250-x.
13. Selleck, C.L., J. L., Harmer, J.; Guddat, L. W.; Mitić, N.; Helweh, W.; Ollis, D. L.; Craig, W. A.; Tierney, D. L.; Pedroso, M. M.; Schenk, G. AIM-1: An Antibiotic-Degrading Metallohydrolase That Displays Mechanistic Flexibility. *Chem. Eur. J*. 2016, 22, 17704-17714.
14. Pedroso, M.M.; Selleck, C.; Enculescu, C.; Harmer, J.R.; Mitić, N.; Craig, W.R.; Helweh, W.; Hugenholtz, P.; Tyson, G.W.; Tierney, D.L.; et al. Characterization of a highly efficient antibiotic-degrading metallo-β-lactamase obtained from an uncultured member of a permafrost community. *Metallomics* 2017, doi:10.1039/c7mt00195a.
15. Wilson, L.A.; Knaven, E.G.; Morris, M.T.; Pedroso, M.M.; Schofield, C.J.; Brück, T.; Moden, M.; Waite, D.W.; Hugenholtz, P.; Guddat, L.W.; Schenk G., SIE-1 from *Sphingobium indicum*, the first example of a B3 metallo-β-lactamase with an active site glutamic acid ligand. *Antimicrob. Agents. Chemther*. 2021, 65, 100015, doi:10.1128/aac.00936-21.
16. Bahr, G.; González, L.J.; Vila, A.J. Metallo-β-lactamases in the Age of Multidrug Resistance: From Structure and Mechanism to Evolution, Dissemination, and Inhibitor Design. *Chem. Rev*. 2021, doi:10.1021/acs.chemrev.1c00138.
17. McGeary, R.P..; Schenk, G.; Guddat, L.W. The Applications of Binuclear Metallohydrolases in Medicine: Recent Advances in the Design and Development of Novel Drug Leads for Purple Acid Phosphatases, Metallo-β-Lactamases and Arginases. *Eur. J. Med. Chem*. 2014, 76, 132-144, doi:10.1016/j.ejmech.2014.02.008.
18. Brem, J.; Van Berkel, S.S.; Zollman, D.; Lee, S.Y.; Gileadi, O.; McHugh, P.J.; Walsh, T.R.; McDonough, M.A.; Schofield, C.J. Structural basis of metallo-β-lactamase inhibition by captopril stereoisomers. *Antimicrob. Agents Chemother*. 2016, 60, 142-150, doi:10.1128/AAC.01335-15.
19. Hussein, W.M.; Fatahala, S.S.; Mohamed, Z.M.; McGeary, R.P.; Schenk, G.; Ollis, D.L.; Mohamed, M.S. Synthesis and Kinetic Testing of Tetrahydropyrimidine-2-thione and Pyrrole Derivatives as Inhibitors of the Metallo-β-lactamase from *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. *Chem. Biol. Drug. Des*. 2012, 80, 500-515, doi:10.1111/j.1747-0285.2012.01440.x.
20. Mohamed, M.S.; Hussein, W.M.; McGeary, R.P.; Vella, P.; Schenk, G.; Abd El-hameed, R.H. Synthesis and kinetic testing of new inhibitors for a metallo-β-lactamase from *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. *Eur. J. Med. Chem*. 2011, 46, 6075-6082, doi:10.1016/j.ejmech.2011.10.030.
21. McGeary, R.P.; Tan, D.T.C.; Selleck, C.; Monteiro Pedroso, M.; Sidjabat, H.E.; Schenk, G. Structure-activity relationship study and optimisation of 2-aminopyrrole-1-benzyl-4,5-diphenyl-1H-pyrrole-3-carbonitrile as a broad spectrum metallo-β-lactamase inhibitor. *Eur. J. Med. Chem.* 2017, 137, 351-364, doi:10.1016/j.ejmech.2017.05.061.
22. Spellberg, B. The future of antibiotics. *Crit. Care* 2014, 18, 228-228, doi:10.1186/cc13948.
23. Spellberg, B.; Guidos, R.; Gilbert, D.; Bradley, J.; Boucher, H.W.; Scheld, W.M.; Bartlett, J.G.; Edwards, J. The Epidemic of Antibiotic-Resistant Infections: A Call to Action for the Medical Community from the Infectious Diseases Society of America. *Clin. Infec. Dis.* 2008, 46, 155-164, doi:10.1086/524891.
24. Odds, F.C. Synergy, antagonism, and what the chequerboard puts between them. *J. Antimicrob. Chemother*. 2003, 52, 1-1, doi:10.1093/jac/dkg301.
25. Brennan-Krohn, T.; Kirby, J.E. Antimicrobial Synergy Testing by the Inkjet Printer-assisted Automated Checkerboard Array and the Manual Time-kill Method. *J. Visual. Exp*. 2019, doi:10.3791/58636.
26. Mitic, N.; Miraula, M.; Selleck, C.; Hadler, K.S.; Uribe, E.; Pedroso, M.M.; Schenk, G. Catalytic mechanisms of metallohydrolases containing two metal ions. *Adv. Protein Chem. Struct. Biol.* 2014, 97, 49-81, doi:10.1016/bs.apcsb.2014.07.002.
27. McGeary, R.P.; Tan, D.T.; Schenk, G. Progress toward inhibitors of metallo-β-lactamases. *Future Med. Chem*. 2017, 9, 673-691, doi:10.4155/fmc-2017-0007.
28. Arjomandi, O.K.; Hussein, W.M.; Vella, P.; Yusof, Y.; Sidjabat, H.E.; Schenk, G.; McGeary, R.P. Design, synthesis, and *in vitro* and biological evaluation of potent amino acid-derived thiol inhibitors of the metallo-β-lactamase IMP-1. *Eur. J. Med. Chem*. 2016, 114, 318-327, doi:10.1016/j.ejmech.2016.03.017.
29. Segel, I.H. *Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady-state enzyme systems*; John Wiley & Sons: United States of America, 1993; p. 985.

30. Valizadeh, M.; Schenk, G.; Nash, K.; Oddie, G.W.; Guddat, L.W.; Hume, D.A.; de Jersey, J.; Burke, T.R.; Hamilton, S Phosphotyrosyl Peptides and Analogues as Substrates and Inhibitors of Purple Acid Phosphatases. *Arch. Biochem. Biophys*. 2004, 424, 154-162, doi:10.1016/j.abb.2004.01.008.