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#### Original article

## Synthesis and kinetic testing of new inhibitors for a metallo- $\beta$ -lactamase from *Klebsiella pneumonia* and *Pseudomonas aeruginosa*

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#### 1. Introduction

#### The $\beta$ -lactam antibiotics are crucial chemotherapeutics for the treatment of bacterial infections. In bacteria, the production of enzymes such as the serine- $\beta$ -lactamases (SBLs) and metallo- $\beta$ lactamases (MBLs) increasingly confers resistance to an ever broader range of common $\beta$ -lactam antibiotics such as penams. carbapenems and cephalosporins [1,2]. Thus, the development of an inhibitor for SBLs and MBLs is an attractive approach to maintain the usefulness of existing antibiotics. While there are efficient inhibitors for SBL activity available, e.g. clavulanic acid derivatives [3], no clinically useful antagonist of MBL activity has yet been reported. The MBLs are characterized by their $\alpha\beta\beta\alpha$ protein fold and the presence of one or two zinc (II) atoms in their active sites (at least one of these metal ions is required for catalysis [4-7]). The MBLs belong to the large family of binuclear metallohydrolases [8] and generally display a broad substrate profile, hydrolysing penams (e.g. benzylpenicillin), cephalosporins (e.g. cephalothin, cefoxitin) and carbapenems (e.g. meropenem and imipenem) [1,5]. Given this broad range of utilizable substrates, and the ease with which MBLs

#### ABSTRACT

There are currently no clinically useful inhibitors against metallo- $\beta$ -lactamases (MBLs), enzymes that confer resistance against a broad spectrum of commonly used antibiotics and that are produced by an increasing number of bacterial pathogens. New pyrrole derivatives were synthesized and assayed for their inhibitory effect on the catalytic activity of the IMP-1 MBL from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Six compounds tested (**3a-3c**, **5**, **7** and **8**) show micromolar inhibition constants ( $K_i$  values range from ~ 10 to 30  $\mu$ M). *In silico* docking was employed to investigate the binding mode of the strongest inhibitor, **3b**, in the active site of IMP-1. Implications for further improvements of binding efficiency and specificity are discussed.

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may be spread between bacterial species on plasmids, the development of new MBL inhibitors is very urgent [9,10].

It is observed from the literature that the pyrrole nucleus and pyrrolo[2,3-d]pyrimidines [11–16] have a variety of biological applications due to their antibacterial [14,17–25], antiviral [26–28], anticancer [29–35], anti-inflammatory [36,37] and anti-hyperglycemic [38,39] activities.

The aim of this work was to develop candidate MBL lead inhibitors through the synthesis, testing and *in silico* docking of compounds based on pyrrole. An IMP-1 MBL which occurs in both *Pseudomonas aeroginosa* and *Klebsiella pneumoniae* [40–43] was selected as a target since these pathogens are well known for recorded outbreaks of antibiotics-resistant bacterial infections in medical settings [44,45], and since this enzyme is relatively well studied (e.g. crystal structures have been reported [46]).

#### 2. Results and discussion

#### 2.1. Synthesis of lead compounds

The synthetic route [47,48] to compounds **1a–1d** is shown in Scheme 1. Condensation of benzoin with the appropriate primary aromatic amine in refluxing toluene gave the corresponding

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Scheme 1. Synthesis of compounds 1a-1d.

 $\alpha$ -aminoketone intermediates which were subsequently condensed, *in situ*, with malononitrile to afford compounds **1a** and **1b**.

Alternatively, refluxing a mixture of phenacyl bromide and primary aromatic amines in dry ethanol gave the respective  $\alpha$ -aminoketone intermediates which were condensed with malononitrile to afford compounds **1c** and **1d**.

Pyrroles **1a–1d** reacted with formic acid [49] to yield pyrrolo [2,3-d]pyrimidin-4(3H)-ones **2a–2d**, and these reacted with phosphorus oxychloride [50] to afford 4-chloro-pyrrolo[2,3-d] pyrimidines **3a–3d** which were converted to pyrrolo[2,3-d]pyrimidin-4(3H)-thiones **4a–4d** by reaction with thiourea in refluxing ethanol [51]. Preparation of pyrrolo[2,3-d]pyrimidin-4-ylidenemalononitrile **5** was accomplished by the reaction of **4b** with malononitrile in absolute ethanol [52] as revealed in Scheme 2.

On the other hand, synthesis of 4-hydrazino-pyrrolo[2,3-*d*] pyrimidines **6** was achieved by the reaction of **3a** with hydrazine hydrate [53] in absolute ethanol. *N*-Benzylidene-*N'*-pyrrolo[2,3-*d*] pyrimidin-4-yl-hydrazine **7** was obtained by the reaction [54] of **6** 

with bezaldehyde in absolute ethanol. Finally, pyrrolo[3,2-e][1,2,4] triazolo[4,3-c]pyrimidines **8**, **9** and **10** were obtained from **6** by reaction with carbon disulfide, formic acid and acetic anhydride, respectively, [48,54] as revealed in Scheme 3.

#### 2.2. Enzymatic inhibition assays

The inhibitory effects of the 11 compounds **1c**, **1d**, **2c**, **3a**–**3c**, **5**, **7**, **8**, **9** and **10** (Table 1) on the catalytic activity of IMP-1 were initially assessed by measuring enzyme activity in the absence and presence of 10  $\mu$ M of compound. A previously developed assay based on the chromogenic cephalosporin substrate CENTA (70  $\mu$ M) was used [43,55]. The hydrolysis of CENTA by IMP-1 releases the chromophore 4-nitrophenolate, the formation of which can be measured spectrophotometrically with a plate reader at  $\lambda = 405$  nm (at pH 7.0,  $\varepsilon = 6400$  M<sup>-1</sup> cm<sup>-1</sup>). The results are presented in Table 1.

Six compounds (**3a–3c**, **5b**, **7** and **8**) were of interest as they showed promising inhibition effects at a concentration of 10  $\mu$ M.



Scheme 2. Synthesis of compounds 2-5.



Scheme 3. Synthesis of compounds 6–10.

Therefore, they were selected for further analysis to determine their mode of inhibition and the magnitude of the corresponding inhibition constant(s) ( $K_i$  values).

As shown in Table 2, compound **3b** is the strongest inhibitor for IMP-1, acting in essence competitively with a  $K_{ic}$  of 12  $\mu$ M. However, the remaining compounds are also good inhibitors with inhibition constants ranging from 15  $\mu$ M to 33  $\mu$ M. For comparison, the  $K_{ic}$  of the known MBL inhibitor L-captopril is 12.5  $\mu$ M  $\pm$  2.4 [43], and those of compounds discovered from a fragment-based screening approach range from  $\sim 500 \ \mu\text{M}$  to  $\sim 1600 \ \mu\text{M}$  [43]. To investigate the potential binding mode of **3b** to IMP-1, in silico docking was employed (Fig. 1). The method employed was described in our previous work [43] with the exception that structures here were drawn in JME editor with hydrogen atoms added using the Dundee PRODRG 2 server (http://davapc1.bioch.dundee.ac.uk/prodrg/) [56]. Compound **3b** was docked into the active site of IMP-1 (Fig. 1); the model illustrates that **3b** binds within the active site occupying the groove parallel to the flexible loop and close to the two zinc atoms. The oxygen atom of the methoxy group of **3b** is located close to both metal ions in the active site (Zn1: 3.4 Å; Zn: 2.7 Å). Apart from these interactions with the metal ions hydrophobic interactions with residues in the flexible loop, notably residue Trp 64, may play an important role in enhancing the binding affinity of the inhibitor. The N3 atom of the pyrimidine ring in **3b** is oriented approximately 3.5 Å towards Glu 59 and binds to the oxygen atom of Glu 59 through a hydrogen bond.

#### 3. Conclusion

In the present study, we describe a straightforward and efficient synthesis of novel pyrrole and pyrrolo[*2*,*3*-*d*]pyrimidine derivatives as potential MBL inhibitors. The structure activity relationship (SAR) results indicate that the pyrrole derivatives **1c**, **1d** and the pyrrolo[*2*,*3*-*d*]pyrimidine **2c** have no activity. However, the introduction of a chloro group in **3a**–**3c** and the formation of the ary-lidine malononitrile **5** leads to pyrrolopyrimidine analogs that are rather potent inhibitors of MBL activity. Similarly, while the pyrrolo [*3*,*2*-*e*][*1*,*2*,*4*]triazolo[*4*,*3*-*c*]pyrimidines **9** and **10** have no effect on MBL activity, the addition of a thione group in position 3 of the pyrrolo[*3*,*2*-*e*][*1*,*2*,*4*]triazolo[*4*,*3*-*c*]pyrimidine moiety in compound

**8** results in activity derivative with considerable inhibitory effect. Finally, the benzylidene-pyrrolo[2,3-d]pyramidine-hydrazine **7** also inhibits MBL activity. The mode of inhibition is generally competitive and the flexible loop in the vicinity of the active site may play an important role in binding these lead compounds. In summary, pyrrole and pyrrolopyrimidine compounds are promising leads in the development of novel MBL inhibitors.

#### 4. Experimental

#### 4.1. Synthesis of lead compounds

All commercial chemicals used as starting materials and reagents in this study were purchased from Merck (Darmstadt, Germany) and were of reagent grade. All melting points were uncorrected and measured using Electro-thermal IA 9100 apparatus (Shimadzu, Japan); IR spectra were recorded as potassium bromide pellets on a Perkin-Elmer 1650 spectrophotometer (USA), Faculty of Science, Cairo University, Cairo, Egypt. 1H NMR spectra were determined on a Varian Mercury (300 MHz) spectrometer (Varian UK) and chemical shifts were expressed as ppm against TMS as internal reference (Faculty of Science, Cairo University, Cairo, Egypt). Mass spectra were recorded on 70 eV (EI Ms-QP 1000 EX, Shimadzu, Japan), Faculty of Science, Cairo University, Cairo, Egypt. Microanalyses were operated using Vario, Elmentar apparatus (Shimadzu, Japan), Organic Microanalysis Unit, Faculty of Science, Cairo University, Cairo, Egypt. Column Chromatography was performed on (Merck) Silica gel 60 (particle size 0.06-0.20 mm). All compounds prepared in this paper are new and confirmed with spectral data except 1b, 2b, 3b and 4b [47].

#### 4.1.1. General procedure for the synthesis of compounds 1a and 1b

A mixture of benzoin (2 g, 0.01 mol), aryl amine (0.01 mol) and conc. HCl (6–8 drops) in toluene (50 mL) was heated under reflux for 6 h and cooled. Malononitrile (0.66 g, 0.01 mol) was added, followed by a catalytic amount (1.5 mL) of pyridine portion wise and left to reflux until a solid was formed. The solvent was evaporated under reduced pressure and the residue was recrystallized from methanol to give compounds 1a-1b.

#### Table 1

| Percentage inhibition of IMP-1 MBL | 2 nM) at pH 7.0 by 11 | pyrrole derivatives using | 2 CENTA (70 μM) as substrate. |
|------------------------------------|-----------------------|---------------------------|-------------------------------|
|                                    |                       | 1.7                       |                               |

| Compound | Structure  | Inhibition % (10 µM) | Compound | Structure  | Inhibition % (10 $\mu M)$ |
|----------|--|----------------------|----------|--|---------------------------|
| 1c       | $Ph \xrightarrow{CN} NH_2$<br>$\bigcup_{CH_3}$   | 0                    | 5        | Ph<br>Ph<br>NH<br>NH<br>OCH <sub>3</sub>                   | 17                        |
| 1d       | Ph<br>N<br>NH <sub>2</sub><br>OCH <sub>3</sub>   | 0                    | 7        | Ph<br>Ph<br>N<br>N<br>$CH_3$                               | 13                        |
| 2c       | Ph<br>N<br>N<br>CH <sub>3</sub>  | 0                    | 8        | Ph $N$                 | 14                        |
| 3a       | Ph<br>Ph<br>N<br>CH <sub>3</sub>   | 10                   | 9        | Ph<br>Ph<br>N<br>N<br>CH <sub>3</sub>                      | 4                         |
| 3b       | Ph N N<br>Ph N N<br>OCH <sub>3</sub>   | 15                   | 10       | Ph<br>Ph<br>N<br>N<br>N<br>N<br>N<br>N<br>N<br>N<br>N<br>N | 7                         |
| 3c       | $\begin{array}{c} Cl \\ Ph \\ \\ \\ N \\ N$ | 9                    |          |  |                           |

4.1.1.1. 2-Amino-1-(4-methylphenyl)-4,5-diphenyl-1H-pyrrole-3carbonitrile (**1a**). Yield: 81%; m.p.: 193–194 °C; IR (KBr) v (cm<sup>-1</sup>): 3567, 3526 (NH<sub>2</sub>), 2205 (C $\equiv$ N); MS (EI) *m*/*z*: 349 (M<sup>+</sup>, 74.5%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.27 (s, 3H, CH<sub>3</sub>), 5.09 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O exchangeable), 6.5–7.9 (m, 14H, Ar–H); Anal. calcd. for C<sub>24</sub>H<sub>19</sub>N<sub>3</sub> (349.44): C, 82.52; H, 5.44; N, 12.03%. Found: C, 82.16; H, 5.81; N, 11.84%. 4.1.1.2. 2-Amino-1-(4-methoxyphenyl)-4,5-diphenyl-1H-pyrrole-3carbonitrile (**1b**). Yield: 93%; m.p.: 204–206 °C; IR (KBr)  $\cup$  (cm<sup>-1</sup>): 3526, 3659 (NH<sub>2</sub>), 2205 (C $\equiv$ N); 1246 (C–O); MS (EI) *m/z*: 365 (M<sup>+</sup>, 5.6%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.79 (s, 3H, OCH<sub>3</sub>), 5.1 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O exchangeable), 6.6–7.8 (m, 14H, Ar–H); Anal. calcd. for C<sub>24</sub>H<sub>19</sub>N<sub>3</sub>O (365.44): C, 78.90; H, 5.20; N, 11.51; O, 4.38%. Found: C, 78.58; H, 5.39; N, 11.22; O, 4.61%.

 Table 2

 Inhibition constants for the inhibibition of IMP-1 MBL by compounds 3a-3c, 5, 7 and 8.

| Compound | <i>K</i> <sub>ic</sub> (μM) | $K_{\rm iuc}$ ( $\mu$ M) |
|----------|-----------------------------|--------------------------|
| 3a       | $19\pm7$                    | $112\pm74$               |
| 3b       | $12 \pm 4$                  | $182\pm184$              |
| 3c       | $33\pm16$                   | $106\pm60$               |
| 5        | $29\pm7$                    | $225\pm139$              |
| 7        | $18\pm9$                    | -                        |
| 8        | $15\pm5$                    | -                        |

4.1.2. General procedure for the synthesis of compounds 1c and 1d

To a solution of phenacyl bromide (2 g, 0.01 mol) and aryl amine (0.01 mol) in ethanol (20 mL) was added a saturated solution of sodium bicarbonate (5 mL). The reaction mixture was kept at 70 °C for 1 h, cooled, then poured into cold water, filtered off and dried. The obtained product was dissolved in an appropriate amount of ethanol (20 mL) and then malononitrile (0.66 g, 0.01 mol) was added portion wise, followed by sodium ethoxide (0.01 mol) and left to reflux until a solid formed. The solvent was removed under reduced pressure and the residue was recrystallized from methanol to give **1c** and **1d**.

4.1.2.1. 2-*Amino*-1-(4-*methylphenyl*)-4-*phenyl*-1*H*-*pyrrole*-3-*carbonitrile* (**1***c*). Yield: 66%; m.p.: 173–175 °C; IR (KBr) v (cm<sup>-1</sup>): 3342, 3226 (NH<sub>2</sub>), 2212 (C $\equiv$ N); MS (EI) *m*/*z*: 273 (M<sup>+</sup>, 23%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.24 (s, 3H, CH<sub>3</sub>),4.3 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O exchangeable), 6.7–7.8 (m, 10H, Ar–H); Anal. calcd. for C<sub>18</sub>H<sub>15</sub>N<sub>3</sub> (273.34): C, 79.10; H, 5.53; N, 15.37%. Found: C, 81.03; H, 5.61; N, 15.65%.



**Fig. 1.** Surface view of the IMP-1 active site for the highest Autodock Vina score conformation of IMP-1 with **3b** docked into the active site. For clarity, Trp 64 of the flexible loop adjacent to the active site has been given a 'stick' representation. Atom colours are as follows: blue, nitrogen; red, oxygen; white, carbon (on IMP-1); green, carbon (on inhibitor); magenta, zinc active site metals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1.2.2. 2-Amino-1-(4-methoxyphenyl)-4-phenyl-1H-pyrrole-3carbonitrile (**1d**). Yield: 73%; m.p.: 164–166 °C; IR (KBr) v(cm<sup>-1</sup>): 3332, 3259 (NH<sub>2</sub>), 2210 (C $\equiv$ N); 1250 (C–O); MS (EI) *m/z*: 289 (M+, 18.2%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.41 (s, 3H, OCH<sub>3</sub>), 4.7 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O exchangeable), 6.8–7.9 (m, 10H, Ar–H); Anal. calcd. for C<sub>18</sub>H<sub>15</sub>N<sub>3</sub>O (289.34): C, 74.73; H, 6.27; N, 13.76; O, 5.53%. Found: C, 75.02; H, 6.39; N, 13.42; O, 5.69%.

#### 4.1.3. General procedure for the synthesis of compounds 2a-2d

Compounds **1a**–**d** (0.01 mol) in formic acid (20 mL, 85%) was refluxed for 8 h. The reaction mixture was cooled, poured onto icewater to give a precipitate which was filtered, dried, and recrystallized from ethanol to afford **2a**–**2d**.

#### 4.1.3.1. 7-(4-Methylphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyr-

*imidin-4*(3*H*)-*one* (**2a**). Yield: 82%; m.p.: 236–238 °C; IR (KBr) v (cm<sup>-1</sup>): 3182 (N–H), 1675 (C=O), 1595 (C=N) and disappearance of the CN group; MS (EI) *m/z*: 377 (M+, 10%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.4 (s, 3H, CH<sub>3</sub>), 6.9–7.5 (m, 14H, Ar–H), 8.1 (s, 1H, C2–H), 8.3 (s, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>25</sub>H<sub>19</sub>N<sub>3</sub>O (377.45): C, 79.58; H, 5.04; N, 11.14; O, 4.24%. Found: C, 79.38; H, 5.25; N, 10.86; O, 4.61%.

#### 4.1.3.2. 7-(4-Methoxyphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-one (**2b**). Yield: 79%; m.p.: 261–263 °C; IR (KBr) υ

(cm<sup>-1</sup>): 3130 (N−H), 1682 (C=O), 1587 (C=N), 1510 (C−O) and disappearance of the CN group; MS (EI) m/z: 393 (M<sup>+</sup>, 12.9%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.78 (s, 3H, OCH<sub>3</sub>), 6.7–8.3 (m, 14H, Ar−H), 8.8 (s, 1H, C2−H), 8.9 (s, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>25</sub>H<sub>19</sub>N<sub>3</sub>O<sub>2</sub> (393.45): C, 76.34; H, 4.83; N, 10.69; O, 8.13%. Found: C, 76.65; H, 5.21; N, 10.45; O, 7.85%.

#### 4.1.3.3. 7-(4-Methylphenyl)-5-phenyl-7H-pyrrolo[2,3-d]pyrimidin-

4(3*H*)-one (**2c**). Yield: 78%; m.p.: 182–184 °C; IR (KBr) v (cm<sup>-1</sup>): 3280 (N–H), 1671 (C=O), 1594 (C=N) and disappearance of the CN group; MS (EI) *m*/*z*: 301 (M<sup>+</sup>, 36%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.37 (s, 3H, CH<sub>3</sub>), 6.9–7.8 (m, 10H, Ar–H), 8.31 (s, 1H, C2–H), 8.5 (s, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>19</sub>H<sub>15</sub>N<sub>3</sub>O (301.35): C, 75.73; H, 5.02; N, 13.94; O, 5.31%. Found: C, 75.41; H, 4.85; N, 14.22; O, 4.99%.

4.1.3.4. 7-(4-Methoxyphenyl)-5-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-one (**2d**). Yield: 82%; m.p.: 189–191 °C; IR (KBr)  $\upsilon$  (cm<sup>-1</sup>): 3202 (N–H), 1687 (C=O), 1599 (C=N), 1503 (C–O) and disappearance of the CN group; MS (EI) *m*/*z*: 317 (M<sup>+</sup>, 25%); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.5 (s, 3H, OCH<sub>3</sub>), 6.8–7.9 (m, 10H, Ar–H), 8.2 (s, 1H, C2–H), 8.3 (s, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>19</sub>H<sub>15</sub>N<sub>3</sub>O<sub>2</sub> (317.35): C, 71.91; H, 4.76; N, 13.24; O, 10.08%. Found: C, 72.28; H, 5.01; N, 13.39; O, 10.35%.

#### 4.1.4. General procedure for the synthesis of compounds 3a-3d

Compounds 2a-d (0.01 mol) were refluxed in phosphorus oxychloride (30 mL) for 6 h. The solution was cooled and poured with stirring onto ice and the formed precipitated was filtered, washed several times with water, dried and recrystallized from ethanol to afford 3a-3d.

4.1.4.1. 4-Chloro-7-(4-methylphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d] pyrimidine (**3a**). Yield: 75%; m.p.: 236–238 °C; IR (KBr) v (cm<sup>-1</sup>): 1605 (C=N); MS (EI) *m*/*z*: 395 (M<sup>+</sup>, <sup>35</sup>Cl, 77%), 397 (M<sup>+</sup>+2, <sup>37</sup>Cl, 31%) <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.45 (s, 3H, CH<sub>3</sub>), 6.9–7.5 (m, 14H, Ar–H), 8.16 (s, 1H, C2–H); Anal. calcd. for C<sub>25</sub>H<sub>18</sub>ClN<sub>3</sub> (395.90): C, 75.95; H, 4.56; Cl, 8.86; N, 10.63%. Found: C, 75.61; H, 4.12; Cl, 8.49; N, 10.81%.

#### 4.1.4.2. 4-Chloro-7-(4-methoxyphenyl)-5,6-diphenyl-7H-pyrrolo

[2,3-*d*]*pyrimidine* (**3b**). Yield: 87%; m.p.: 209–211 °C; IR (KBr) v (cm<sup>-1</sup>): 1605 (C=N), 1226 (C–O); MS (EI) *m*/*z*: 411 (M<sup>+</sup>, <sup>35</sup>Cl, 40%), 413 (M<sup>+</sup>+2, <sup>37</sup>Cl, 14.5%) <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.65 (s, 3H, OCH<sub>3</sub>), 7.1–7.9 (m, 14H, Ar–H), 8.6 (s, 1H, C2–H); Anal. calcd. for C<sub>25</sub>H<sub>18</sub>ClN<sub>3</sub>O (411.89): C, 72.99; H, 4.38; Cl, 8.52; N, 10.22; O, 3.88%. Found: C, 73.24; H, 4.21; Cl, 8.48; N, 9.85; O, 3.62%.

# 4.1.4.3. 4-*Chloro-7-(4-methylphenyl)-5-phenyl-7H-pyrrolo*[2,3-*d*] *pyrimidine* (**3c**). Yield: 65%; m.p.: 193–195 °C; IR (KBr) v (cm<sup>-1</sup>): 1612 (C=N); MS (EI) m/z: 319 (M<sup>+</sup>, <sup>35</sup>Cl, 6%), 321 (M<sup>+</sup>+2, <sup>37</sup>Cl, 2.2%) <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz) $\delta$ (ppm): 2.27 (s, 3H, CH<sub>3</sub>), 6.9–7.8 (m, 10H, Ar–H), 8.23 (s, 1H, C2–H); Anal. calcd. for C<sub>19</sub>H<sub>14</sub>ClN<sub>3</sub> (319.80): C, 71.36; H, 4.41; Cl, 11.09; N, 13.14%. Found: C, 71.61; H, 4.72; Cl, 10.82; N, 12.99%.

4.1.4.4. 4-*Chloro-7-(4-methoxyphenyl)-5-phenyl-7H-pyrrolo*[2,3-*d*] *pyrimidine* (**3d**). Yield: 69%; m.p.: 198–200 °C; IR (KBr) v (cm<sup>-1</sup>): 1596 (C=N), 1217 (C–O); MS (EI) *m/z*: 335 (M<sup>+</sup>, <sup>35</sup>Cl, 35%), 337 (M<sup>+</sup>+2, <sup>37</sup>Cl, 11.8%) <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.5 (s, 3H, OCH<sub>3</sub>), 6.7–8.0 (m, 10H, Ar–H), 8.4 (s, 1H, C2–H); Anal. calcd. for C<sub>19</sub>H<sub>14</sub>ClN<sub>3</sub>O (335.80): C, 68.28; H, 5.16; Cl, 10.08; N, 11.94; O, 4.76% Found: C, 68.49; H, 4.88; Cl, 10.39; N, 12.21; O, 4.48%.

#### 4.1.5. General procedure for the synthesis of compounds 4a-4d

Compound **3a**–**d** (0.01 mol) and thiourea (1.5 g, 0.02 mol) was heated under reflux in dry ethanol (30 mL) for 4 h, cooled, poured onto ice-water, to give precipitate which was filtered off, dried, and recrystallized from methanol to give **4a**–**4d**.

4.1.5.1. 7-(4-Methylphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-thione (**4a**). Yield: 67%; m.p.: 214–216 °C; IR (KBr) v (cm<sup>-1</sup>): 3326 (N–H), 1645 (C=S), 1609 (C=N); MS (EI) *m*/*z*: 393 (M<sup>+</sup>, 14%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2,4 (s, 3H, CH<sub>3</sub>), 6.6–7.9 (m, 14H, Ar–H), 8.1 (s, 1H, C2–H), 9.3 (d, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>25</sub>H<sub>19</sub>N<sub>3</sub>S (393.51): C, 76.34; H, 4.83; S, 8.14; N, 10.69%. Found: C, 76.10; H, 5.12; S, 7.88; N, 10.47%.

#### 4.1.5.2. 7-(4-Methoxyphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-thione (**4b**). Yield: 89%; m.p.: 205–207 °C; IR (KBr) v(cm<sup>-1</sup>): 3368 (N–H), 1631 (C=S), 1586 (C=N), 1241 (C–O); MS (EI) m/z: 409 (M<sup>+</sup>, 6.5%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz) $\delta$ (ppm): 3.7 (s, 3H, OCH<sub>3</sub>), 6.8–7.8 (m, 14H, Ar–H), 8.0 (s, 1H, C2–H), 9.5 (d, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>25</sub>H<sub>19</sub>N<sub>3</sub>SO (409.51): C, 73.35; H, 4.65; S, 7.82; N, 10.27; O, 3.91%. Found: C, 73.41; H, 4.59; S, 7.59; N, 10.39; O, 4.22%.

#### 4.1.5.3. 7-(4-Methylphenyl)-5-phenyl-7H-pyrrolo[2,3-d]pyrimidin-

4(3*H*)-thione (**4c**). Yield: 63%; m.p.: 189–191 °C; IR (KBr) v (cm<sup>-1</sup>): 3293 (N–H), 1636 (C=S), 1595 (C=N); MS (EI) m/z: 317 (M<sup>+</sup>, 9%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.75 (s, 3H, CH<sub>3</sub>), 6.9–8.0 (m, 10H, Ar–H), 8.3 (s, 1H, C2–H), 9.2 (d, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>19</sub>H<sub>15</sub>N<sub>3</sub>S (317.42): C, 71.92; H, 4.73; S, 10.09; N, 13.25%. Found: C, 72.21; H, 4.36; S, 10.34; N, 13.57%.

4.1.5.4. 7-(4-Methoxyphenyl)-5-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4(3H)-thione (**4d**). Yield: 77%; m.p.: 176–178 °C; IR (KBr) v (cm<sup>-1</sup>): 3345 (N–H), 1652 (C=S), 1617 (C=N),1238 (C–O); MS (EI) *m*/*z*: 333 (M<sup>+</sup>, 24%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.54 (s, 3H, OCH<sub>3</sub>), 7.0–7.9 (m, 10H, Ar–H), 8.3 (s, 1H, C2–H), 8.8 (d,1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>19</sub>H<sub>15</sub>N<sub>3</sub>SO (333.41): C, 68.46; H, 4.50; S, 9.60; N, 12.61; O, 4.80%. Found: C, 68.75; H, 4.26; S, 9.82; N, 12.98; O, 4.54%.

## 4.1.6. 2-(7-(4-Methoxyphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d] pyrimidin-4-ylidene)-malononitrile (5)

Compound **4b** (4.09 g, 0.01 mol) and malononitrile (0.66 g, 0.01 mol) was heated under reflux in dry ethanol (30 mL) for 8 h, cooled, poured onto ice-water to give precipitate which was filtered off, dried, and recrystallized from methanol to give **5.** Yield: 67%; m.p.: 212–214 °C; IR (KBr) v (cm<sup>-1</sup>): 3325 (N–H), 2210 (C $\equiv$ N), 1546 (C $\equiv$ N), 1249 (C–O); MS (EI) *m*/*z*:441 (M<sup>+</sup>, 21%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 3.66 (s, 3H, OCH<sub>3</sub>), 6.6–7.9 (m, 14H, Ar–H), 8.65 (s, 1H, C2–H), 8.74 (s, 1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>28</sub>H<sub>19</sub>N<sub>5</sub>O (441.50): C, 76.18; H, 4.34; N, 15.86; O, 3.62%. Found: C, 76.35; H, 4.26; N, 15.98; O, 3.77%.

## 4.1.7. (7-(4-Methylphenyl)-5,6-diphenyl-7H-pyrrolo[2,3-d] pyrimidin-4-yl)-hydrazine (**6**)

Compound **3a** (3.95 g, 0.01 mol) and hydrazine hydrate (5 mL, 0.015 mol, 98%) was heated under reflux in dry ethanol (30 mL) for 4 h, cooled, poured onto ice-water to give precipitate which was filtered off, dried, and recrystallized from methanol to give **6**. Yield: 82%; m.p.: 204–206 °C; IR (KBr)  $\upsilon$  (cm<sup>-1</sup>): 3412, 3320 (NH<sub>2</sub>), 3237 (N–H), 1533 (C=N); MS (EI) *m/z*: 391 (M<sup>+</sup>, 34%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.46 (s, 3H, CH<sub>3</sub>), 4.9 (br s, 2H, NH<sub>2</sub>, D<sub>2</sub>O exchangeable), 6.7–7.9 (m, 15H, Ar–H, NH, D<sub>2</sub>O exchangeable), 8.3 (s, 1H, C2–H); Anal. calcd. for C<sub>25</sub>H<sub>21</sub>N<sub>5</sub> (391.48): C, 76.70; H, 5.41; N, 17.89%. Found: C, 76.93; H, 5.12; N, 17.56%.

#### 4.1.8. N-Benzylidene-N'-(7-(4-methylphenyl)-5,6-diphenyl-7Hpyrrolo[2,3-d]pyramidin-4-yl)-hydrazine (7)

Compound **6** (3.91 g, 0.01 mol) and benzaldehyde (3 mL) was heated under reflux in dry ethanol (30 mL) for 8 h, cooled, poured onto ice-water to give precipitate which was filtered off, dried, and recrystallized from methanol to give **7**. Yield: 32%; m.p.: 187–189 °C; IR (KBr) v (cm<sup>-1</sup>): 3323 (N–H), 1608 (C=N); MS (EI) *m*/*z*: 479 (M<sup>+</sup>, 37.9%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.54 (s, 3H, CH<sub>3</sub>), 5.12 (br s,1H, NH, D<sub>2</sub>O exchangeable), 7.0–7.9 (m, 19H, Ar–H), 8.26 (s, 1H, C2–H), 8.32 (s, 1H, CH); Anal. calcd. for C<sub>32</sub>H<sub>25</sub>N<sub>5</sub> (479.59): C, 80.14; H, 5.25; N, 14.60%. Found: C, 79.82; H, 5.61; N, 14.36%.

## 4.1.9. 7-(4–Methylphenyl)-8,9-diphenyl-7H-pyrrolo[3,2-e][1,2,4] triazolo[4,3-c]pyrimidine-3-thione (**8**)

Compound **6** (3.91 g, 0.01 mol) and carbon disulfide (10 mL) in absolute ethanol (30 mL) was heated under reflux for 3 h, cooled, poured onto ice-water to give a precipitate which was filtered off, dried, and recrystallized from ethanol to give **8**. Yield: 52%; m.p.: 190–192 °C; IR (KBr) v (cm<sup>-1</sup>): 3314 (N–H), 1652 (C=S), 1567 (C=N); MS (EI) *m*/*z*: 433 (M<sup>+</sup>, 13%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.36 (s, 3H, CH<sub>3</sub>), 6.8–7.5 (m, 14H, Ar–H), 8.89 (s, 1H, C5–H), 8.98 (br s,1H, NH, D<sub>2</sub>O exchangeable); Anal. calcd. for C<sub>26</sub>H<sub>19</sub>N<sub>5</sub>S (433.54): C, 72.03; H, 4.42; N, 16.15; S, 7.40%. Found: C, 71.85; H, 4.78; N, 15.83; S, 7.14%.

## 4.1.10. 7-(4-Methylphenyl)-8,9-diphenyl-7H-pyrrolo[3,2-e][1,2,4] triazolo[4,3-c] pyrimidine (**9**)

Compound **6** (3.91 g, 0.01 mol) was heated under reflux for 8 h in formic acid (20 ml, 85%), cooled, poured onto ice-water to give a precipitate which was filtered off, dried, and recrystallized from ethanol to give **9**. Yield: 64%; m.p.: 176–178 °C; IR (KBr) v (cm<sup>-1</sup>): 1613 (C=N); MS (EI) m/z: 401 (M<sup>+</sup>, 100%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.34 (s, 3H, CH<sub>3</sub>), 6.94–7.96 (m, 14H, Ar–H), 8.0 (s, 1H, C3–H), 8.9 (s, 1H, C5–H); Anal. calcd. for C<sub>26</sub>H<sub>19</sub>N<sub>5</sub> (401.47): C, 77.79; H, 4.77; N, 17.44%. Found: C, 78.03; H, 5.02; N, 17.81%.



**Fig. 2.** Determination of the inhibition constants for compound **3b** against IMP-1 MBL (2 nM) using CENTA as the substrate Inhibitor concentrations are indicated on the right side. The experimental data were fit to Eq. (1); error bars indicate standard errors.

#### 4.1.11. 3-Methyl-7-(4-methylphenyl)-8,9-diphenyl-7H-pyrrolo[3,2e][1,2,4]triazolo[4,3-c]pyrimidine (**10**)

Compound **6** (3.91 g, 0.01 mol) was heated under reflux for 5 h in acetic anhydride (30 ml), cooled, poured onto ice-water and neutralized with ammonia to give a precipitate which was filtered off, dried, and recrystallized from ethanol to give **10**. Yield: 59%; m.p.: 185–187 °C; IR (KBr) v (cm<sup>-1</sup>): 1598 (C=N); MS (EI) m/z: 415 (M<sup>+</sup>, 6.49%), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300 MHz)  $\delta$  (ppm): 2.21 (s, 3H, C3–CH<sub>3</sub>), 2.32 (s, 3H, Ar–CH<sub>3</sub>), 6.98–7.6 (m, 14H, Ar–H), 8.2 (s,1H, C5–H); Anal. calcd. for C<sub>27</sub>H<sub>21</sub>N<sub>5</sub> (415.50): C, 78.05; H, 5.09; N, 16.86%. Found: C, 78.35; H, 5.24; N, 17.03%.

#### 4.2. Enzyme expression and purification

The IMP-1 enzyme, lacking the first 21 signal peptide amino acid residues, was expressed and purified using a previously published procedure [43,57].

#### 4.3. Kinetic assays

Inhibition assays were performed in 96 well 400  $\mu$ L multi-titre plates using a UV/Vis multi-plate spectrophotometer. The assay was performed in HEPES buffer (50 mM HEPES, 0.1M NaCl, 100  $\mu$ M ZnCl2, pH 7.0). The substrate CENTA was synthesized according to previously published work [43,55] with final substrate concentrations ranging between 5 and 70  $\mu$ M. The final concentration of IMP-1 in the assay was 2 nM. A final concentration of 20  $\mu$ g/mL of bovine serum albumin (BSA) was added to the assay. Three different concentrations of the compounds **3a**–**3c**, **5**, **7** and **8** were used as illustrated in Fig. 2 for compound **3**. The inhibition data were analysed by non-linear regression using WinCurveFit (Kevin Raner Software) and Eq. (1), where  $V_{max}$  is the maximum rate,  $K_{M}$  the Michaelis constant, and  $K_{ic}$  and  $K_{iuc}$  the inhibition constants for the competitive and uncompetitive inhibition modes, respectively [58].

$$V = \frac{V_{\text{max}} [S]}{[S](1 + ([I]/K_{\text{inc}})) + K_{\text{M}}(1 + ([I]/K_{\text{ic}}))}$$
(1)

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