Discovery of 4,6-bis((E)-benzylidene)hydrazinyl)pyrimidin-2-Amine with Antibiotic Activity


Robenidine (E)-N-(E)-1-(4-chlorophenyl)ethylidene)-2-(1-(4-chlorophenyl)ethylidene)hydrazine-1-carboximidhydrazide displays methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococci (VRE) MICs of 2 μg mL⁻¹. Herein we describe the structure-activity relationship development of a novel series of guanidine to 2-aminopyrimidine isosteres that ameliorate the low levels of mammalian cytotoxicity in the lead compound while retaining good antibiotic activity. Removal of the 2-NH₂ pyrimidine moiety renders these analogues inactive. Introduction of a central 2-NH₂ triazine moiety saw a 10-fold activity reduction. Phenyl to cyclohexyl isosteres were inactive. The 4-BrPh and 4-CH₃Ph with MIC values of 2 and 4 μg mL⁻¹, against MRSA and VRE respectively, are promising candidates for future development.

Introduction

Bacteria resistant to polymyxin have been reported, this marks the advent of an era where bacteria resistant to all current antibiotics have been observed.[1] The importance of developing new antibiotics has been highlighted by the World Health Organization, the Centre for Disease Control, the Infectious Disease Society of America and the European Centre for Disease Control.[2–5] The drive to produce novel antibiotics has received a global call over a significant threat to human life by bacteria with current estimates citing >50,000 deaths in the USA and Europe alone as a consequence of antibiotic resistance.[4,5]

Of the antibiotics brought to market in the past 30 years, most have been derivatives of existing drugs.[3,6] These next generation antibiotics are typically a response to resistance emerging to the prior generation. It is unclear how long this cycle of next generation – resistance – new generation antibiotics within the same class of compounds can be perpetuated. Of equal concern is that the Food and Drug Administration (FDA) only approved one new antibiotic in 2015, Avycaz® (avibactam/ceftazidime) for the treatment of complicated intra-abdominal infections.[7] This lack of innovation, and investment, has meant that a number of multidrug resistant bacterial strains, particularly the “ESKAPE” pathogens: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species, are extremely challenging to treat and in some cases require complex antibiotic cocktails.[3,5–10]

Our critical reliance on antibiotics has engendered government initiatives and global strategies to rejuvenate the antibiotic pipeline, such as the Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) initiative and “The 10 × 20 Initiative” seek to combat this crisis and has the ambitious target of ten new antibacterial drugs by 2020.[11–13] Whilst these ambitious targets have stimulated a resurgence in antibacterial research at the academic level, this research has failed to translate into new antibiotics with novel mechanisms of action.[12,14–16] Of particular concern is the lack of efficacious compounds which treat Gram-negative bacteria, owing to the poor drug penetration of the outer membrane and the efficient efflux systems widespread within this group of microbes, making these pathogens extremely challenging to treat.[10] Both the Infectious Diseases Society of America and the European Centre for Disease Control have announced that only a handful of potential drugs which target Gram-negative bacteria in clinical trials offer significant benefits over current clinically used antibiotics.[15,18] Clearly there is a pressing need to develop new antibiotic classes, especially those with lower inherent resistance susceptibility.[15–23]

We recently reported the development of robenidine based analogues with antibiotic activity against clinically relevant strains of MRSA and VRE.[24–26] These prior efforts included the identification and biological evaluation of a pyrimidine based robenidine analogue.[24] Herein we explore the structure activity relationship data and design characteristics that led to the
identification of a novel guanidine bioisostere,\textsuperscript{27–30} and the
discovery of a family of benzylidenehydrazylpyrimidin-2-amines
displaying modest to good levels of antibiotic activity against
MRSA and VRE.

**Results and Discussion**

Our earlier studies revealed that 1, displayed good levels of
activity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) with MIC
values of 2 μg mL\(^{-1}\) against both bacteria.\textsuperscript{24,25} However, some
of our parent robenidine analogues displayed moderate levels
mammalian cell cytotoxicity.\textsuperscript{26} We were thus keen to explore
possible isosteric modifications that would enable retention or
enhancement of antibiotic activity while ameliorating this low
level of cytotoxicity further.

We envisaged that replacement of the central guanidine
core could be accomplished through the installation of a
diaminopyrimidine nucleus with retention of the key binding
features of the lead, 1 (Figure 1). As such we targeted the
development of a small focused library of diaminopyrimidine
based analogues of 1 (Scheme 1).

![Figure 1. Guanidine based lead, 1, with MIC values of 2 μg mL\(^{-1}\) against both MRSA and VRE.](image)

**Scheme 1.** Reagents and Conditions: i) EtOH, reflux, 16 h.

In a typical synthesis 4,6-dihydrazinylpyrimidine 2 was
refluxed with a phenone and/or aldehydes 3a–c for 16 h, which
after reaction work up (see experimental) gave pyrimidines 4–6
in good (4, 68%) to excellent yields (6, 91%). These analogues
were screened for activity against the Gram-positive MRSA and
VRE and the Gram-negative *E. coli* and *Pseudomonas*. These
data are presented in Table 1. The antibiotic activity screening
was conducted in Luria Bertani (LB) broth as the robenidine has
been shown to chelate Ca\(^{2+}\) ions.\textsuperscript{28} It is not known, nor
explored here, if all robenidine analogues do so. The use of LB
broth ensured assay to assay comparison consistency. In

**Table 1.** Inhibition of MRSA, VRE, *E. coli* and *Pseudomonas* growth by pyrimidines 4–6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R'</th>
<th>R''</th>
<th>MIC mode (\mu g mL^{-1}) at 24 h</th>
<th>MRSA</th>
<th>VRE</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>4-Cl</td>
<td>Me</td>
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<td>128</td>
<td>–</td>
<td>–</td>
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<tr>
<td>5</td>
<td>4-Cl</td>
<td>CH(_3)OH</td>
<td>–</td>
<td>128</td>
<td>–</td>
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<tr>
<td>6</td>
<td>4-Cl</td>
<td>H</td>
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</table>

\(a\) MIC value among all observations that occurs at the greatest frequency, \(b\) no activity at 128 μg mL\(^{-1}\) compound concentration.

As such we synthesised a focused library based on the 2-
amino pyrimidine core using the same approach as outlined in
Scheme 1 commencing from pyrimidine-2,4,6-triamine. As antici-
patated the condensation of aldehydes and phenones
occurred exclusively at the 4,6-amino moieties to afford
analogues 7–23 (see Table 2 for detail), which were subse-
quently screened for activity against MRSA, VRE, *E. coli* and
*Pseudomonas*. No Gram-negative activity was observed, Gram-
positive data are presented in Table 2.

Examination of the antibacterial data presented in Table 2
reveals good levels of activity with the aminopyrimidine isostere
7 of our initial lead 1 retaining high levels of activity against
MRSA (4 μg mL\(^{-1}\)) and VRE (8 μg mL\(^{-1}\)). This activity was
enhanced through the introduction of a 4-Br 9 or a 4-CH\(_3\),

![Figure 2.](image)

**Figure 2.** A. Superimposition of 1 and 4; and B. Superimposition of 1 and 7, showing the introduction of the exocyclic NH moiety capable of the similar H-bonding interactions as the central guanidine NH of 1.
moiety 13. In this Library, good tolerance for a 4-substituent was noted with 7–13, and 16–18 returning MIC values <64 μg mL⁻¹ against MRSA or VRE or both bacteria. Only bulky groups appear to be disfavoured with the 4-t-Bu 14, 4-Ph 15 and 4-OCH₃ 19 analogues inactive. In most cases where activity was observed, each analogue was more potent against either MRSA or VRE, e.g. 12 with an MRSA MIC of 4 μg mL⁻¹, but inactive against VRE (MIC >128 μg mL⁻¹). Introduction of the acetyl moiety 21 effectively removed all antibiotic activity whereas the replacement of the phenyl moiety with a cyclohexyl moiety 22 retained modest activity against MRSA and VRE. Introduction of a methyl moiety at the hydrazone carbon (C–N–NH), with 23, afforded good MRSA activity (MIC 8 μg mL⁻¹), but only modest VRE activity (MIC 64 μg mL⁻¹). In all cases no Gram-negative activity was observed.

We have reported a more detailed in vivo biochemical evaluation of the aminopyrimidine isostere analogue 13, wherein we noted that this compound displayed potent bactericidal activity against Streptococcus pneumoniae and Staphylococcus aureus by disrupting the cell membrane potential. Critically this guanidine to aminopyrimidine isosteric modification gave analogues with lower levels of mammalian cell toxicity (3.5-fold less toxic to MCF-7 (breast), Hel299 (lung) and MDBK (kidney) tumour cell lines relative to 1); low metabolic degradation rates in human and mouse liver microsomes; high plasma concentrations after 5 mg/kg i.v. dosing, and low plasma clearance rates in mice relative to the guanidine equivalent analogue.²⁵

Having successfully introduced an aminopyrimidine guanidine isostere as the core linker with retention, and modest potency enhancement with a reduction in cytotoxicity, we explored further modifications through the installation of 1,3,5-triazine moiety through the synthesis of 24–32. These analogues were synthesised as per Scheme 1 from 1,3,5-triazin-2-amine and screened for antibiotic activity as before and these data are presented in Table 3.

Despite the promising activity observed with the equivalent aminopyrimidine analogues (Table 2), the installation of the 1,3,5-triazin-2-amine moiety essentially abolished antibiotic activity with only 2-OH 24, 4-CF₃ 27 and 4-Br 29 displaying modest levels of activity with MIC values of 16–64 μg mL⁻¹. Even in these instances’ activity was only observed against either MRSA or VRE, but not both Gram positive bacteria. No Gram-negative activity was observed.

### Table 2. Inhibition of MRSA and VRE growth by aminopyrimidines 7–23.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R¹</th>
<th>MIC mode (μg mL⁻¹) at 24h</th>
<th>Compound</th>
<th>R¹</th>
<th>MIC mode (μg mL⁻¹) at 24h</th>
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<tbody>
<tr>
<td>R²</td>
<td>MRSA</td>
<td>VRE</td>
<td>R²</td>
<td>MRSA</td>
<td>VRE</td>
</tr>
<tr>
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<td>H</td>
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<td>15</td>
<td>H</td>
<td>–</td>
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</tr>
</tbody>
</table>

* MIC value among all observations that occurs at the greatest frequency. * no activity at 128 μg mL⁻¹ compound concentration.
Increasing the complexity and/or the number of substituents on the phenyl moiety had mixed outcomes (these data are presented in Table 4). A phosphate ester 33 was inactive, whereas di- and tri-OH substitution was only effective with the 2,3-di-OH 37 (8 μg mL⁻¹) against both MRSA and VRE and the 2,4-di-OH 39 (64 μg mL⁻¹) against only MRSA. Naphthyl substituted 40 and 41 returned good activity at 8/64 and 8/32 μg mL⁻¹ against MRSA and VRE, respectively. This suggests a preference for aromatic moieties in this region, noting that the 4-t-Bu 14 and cyclohexyl 22 were inactive. Introduction of a spacer unit between the phenyl and guanidine moieties with 42 and 43 was detrimental to activity, especially against VRE with both analogues inactive. Taking this into consideration we next evaluated the introduction of a simple pyridyl moiety with 44–47. Favourable outcomes were noted with 44 and 45 with a marked preference for the 3-pyridyl moiety in effecting antibiotic activity with 44 more active than 2-pyridyl 45. The 4-pyridyl 46 showed low VRE activity and the 4-Cl-3-pyridyl 47 was inactive.

### Conclusions

Attempts to introduce a 4,6-dihydrazinopyrimidine moiety as a guanidine bioisostere to robenidine based antibiotic lead compounds were unsuccessful with the pyrimidine analogue 4 inactive whereas the parent guanidine lead 1 displayed an MIC of 2 μg mL⁻¹ against both MRSA and VRE bacterial strains. Examination of the potential 3-dimenstional conformation of 1 and 4 highlighted the probable lack of a key hydrogen bonding moiety – the guanidine NH, which potentially explained the abrogation of activity. Subsequent use of a 2-aminopyrimidine afforded a library of guanidine to 2-aminopyrimidine isosteres that returned good to excellent MIC values against both MRSA and VRE. Of most note were the 4-BrPh 9 and 4-CH₃Ph 13 with MIC values of 2 and 4 μg mL⁻¹ against MRSA and VRE respectively. The presence of the pendant aromatic ring was critical to activity, the equivalent cyclohexyl analogue, 22, was inactive. The central nature of the 2-aminopyrimidine isostere brooked limited modification with all exemplars 24–32 of the triazine bioisosteres effectively inactive or showing only low levels of activity against either MRSA (24, MIC 16 μg mL⁻¹) or VRE (29, 32 μg mL⁻¹).

The introduction of more complex aromatic moieties in conjunction with the 2-aminopyrimidine moiety had mixed outcomes. Of the OH substituted analogues only the 2,3-di-OH 37 showed noteworthy activity with a MRSA and VRE MIC of 8 μg mL⁻¹. Bulky aromatic systems were tolerated with the 1- and 2-naphthyl 40 and 41 8 μg mL⁻¹ active against MRSA, with reduced activity against VRE. The introduction of a spacer between the aromatic moiety and 2-aminopyrimidine with 42 and 43 was better tolerated with MRSA than VRE, with good to modest activity. A heteroatom, viz the pyridyl analogues 44–46, showed moderate to good activity in the absence of a 4-substituent, with the 4-Cl-3-pyridyl analogue inactive. No analogue displayed Gram-negative activity against the E. coli and Ps. Aeruginosa strains examined.

A more extensive biochemical evaluation of aminopyrimidine, 13, was consistent with this class of compounds being promising chemical leads for on-going medicinal chemistry development. Combined these data suggest that 9 and 13 are excellent candidates for further development, and we will report on this in due course.
Experimental

Chemistry – General Methods

All reagents were purchased from Sigma-Aldrich, AK Scientific, Matrix Scientific or Lancaster Synthesis and were used without purification. All solvents were re-distilled from glass prior to use.

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker Advance™ AMX 400 at 400.13 and 100.62 MHz, respectively and Advance™ AMX 600 at 600.21 and 150.92 MHz, respectively. Chemical shifts ($\delta$) are reported in parts per million (ppm) measured relative to the internal standards. Coupling constants ($J$) are expressed in hertz (Hz). Mass spectra were recorded on a Shimadzu LCMS 2010 EV and Agilent 6100 series single quadrupole LCMS using a mobile phase of 1 : 1 acetonitrile : H$_2$O with 0.1 % formic acid. The University of Wollongong, Australia, Mass Spectrometry User resource & Research Facility analysed samples for High Resolution Mass Spectrometry. The spectra were acquired on the VG Autospec-oa-tof tandem high resolution mass spectrometer using CI (chemical ionization), with methane as the carrier gas and PFK (perfluorokerosene) as the reference. HRMS Analytical HPLC traces were obtained using a Shimadzu system possessing a SIL-20A auto-sampler, dual LC-20AP pumps, CBM-20A bus module, CTO-20A column heater, and a SPD-20A UV/vis detector. This system was fitted with an Alltima™ C$_{18}$ 5 μm 150 mm x 4.6 mm column with solvent A: 0.06% trifluoroacetic acid (TFA) in water and solvent B: 0.06% TFA in CH$_3$CN–H$_2$O (90:10). In each case HPLC traces were acquired at a flow rate of 2.0 mL min$^{-1}$, gradient 10–100 (%B), over 15.0 min, with detection at 220 nm and 254 nm. All samples returned satisfactory analyses. Compound purity was confirmed by a combination of LC-MS (HPLC), micro and/or high resolution mass spectrometry and NMR analysis. All analogues are $\geq$ 95 % purity.

Melting points were recorded on a Büchi Melting Point M-565 instrument. IR spectra were recorded on a PerkinElmer Spectrum Two™ FTIR Spectrometer with the UATR accessories. Thin layer chromatography (TLC) was performed on Merck 60 F254 pre-coated aluminium plates with a thickness of 0.2 mm. Column chromatography was performed under ‘flash’ conditions on Merck silica gel 60 (230–400 mesh).

Microbiology

Antimicrobial Agents

Robenidine (1, NCL812) was provided by Neoculi Pty. Ltd. Ampicillin used in this study for quality control of susceptibility testing was sourced from Sigma Aldrich.
**Bacterial Isolates**

Isolates used in initial screening assay were sourced as follows: SCCmec type IV MRSA (n = 2), VRE (n = 2), multidrug-resistant *E. coli* (n = 2) and *P. aeruginosa* (n = 2) clinical isolates were kindly provided by Prof Mary Barton, University of South Australia. MSSA strains of *S. aureus* ATCC 25923 and 29213 were obtained from the American Type Culture Collection together with *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

**Susceptibility Testing**

The MIC of all isolates was determined using a slightly modified microdilution method according to the CLSI guidelines as follows: Luria Bertani (LB) broth was used instead of CAMHB as it has been previously shown that 1 can chelate calcium ions. In addition, the antimicrobial dilutions of all analogues were completed in 100% DMSO, with 1 μL added to each well in the challenge plate, as the compounds are hydrophobic. The assay was performed in a total volume of 100 μL with test concentration increasing 2-fold from 0.25 μg/mL to 128 μg/mL in 96 well plates. MIC tests involving ampicillin were performed according to CLSI guidelines in CAMHB. Plates were incubated for 24 hours at 35 ± 2 °C before determination of the MIC.

Control reference strains, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, were tested against the test and control antimicrobials to ensure MIC values were within range according to CLSI documents.

**Synthesis**

4,6-Bis(2-((E)-1-(4-chlorophenyl)ethylidene)hydrazinyl)pyrimidin-2-amine (7)

A suspension of 2-amino-4,6-dihydrizinylpyrimidine (65 mg, 0.465 mmol) and 4-chloroacetophenone (182 mg, 1.175 mmol, 2.53 eq.) in EtOH (25 mL) was heated at reflux for 16 h. After this time, the condenser was removed and the solution concentrated to afford the pyrimidine (131 mg, 68%) as an off-white amorphous solid. MP 251–252 °C.

1H NMR (DMSO-δ) δ 10.17 (s, 2H), 8.24 (s, 1H), 7.83 (d, J = 8.6 Hz, 4H), 7.50 (d, J = 8.6 Hz, 4H), 6.97 (s, 1H), 2.32 (s, 6H); 13C NMR (DMSO-δ) δ 162.5, 157.4, 145.0, 137.5, 133.2, 128.4, 127.3, 83.2, 13.4; MS: LRMS 412.65; HRMS calculated for M+H: C_{28}H_{21}Cl_{2}N_{8}, 413.1043; found 413.1049.

4,6-Bis(2-((E)-1-(4-chlorobenzylidene)hydrazinyl)pyrimidin-2-amine (6)

A suspension of 4,6-dihydrizinylpyrimidine (146 mg, 1.042 mmol) and 4-chlorobenzaldehyde (365 mg, 2.599 mmol, 2.49 eq.) in EtOH (20 mL) was heated at reflux for 16 h. Upon cooling to 40 °C the resulting precipitate was collected and washed with EtO (30 mL) to afford the pyrimidine (374 mg, 93%) as a white amorphous solid. MP 350°C (Decomp).

1H NMR (DMSO-δ) δ 11.20 (s, 2H), 8.17 (s, 1H), 8.09 (s, 2H), 7.72 (d, J = 8.5 Hz, 4H), 7.54 (d, J = 8.5 Hz, 4H), 6.83 (s, 1H); 13C NMR (DMSO-δ) δ 161.6, 157.8, 140.4, 133.8, 133.5, 129.0, 127.9, 81.4; MS: LRMS ESI + ve 385 (M+1); HRMS calculated for M+H: C_{28}H_{20}Br_{2}N_{6}, 487.9911; found 487.9830.

4,6-Bis(2-((E)-1-(4-chlorobenzylidene)ethylidene)hydrazinyl)pyrimidin-2-amine (8)

A suspension of 4,6-dihydrizinylpyrimidine (67 mg, 0.434 mmol) and 4-chlorobenzaldehyde (199 mg, 1.414 mmol, 3.26 eq.) in EtOH (25 mL) was heated at reflux for 16 h. After this time, the condenser was removed and the solution concentrated to approx. 1 mL and the resulting precipitate filtered hot and washed with EtO (10 mL) to afford the aminopyrimidine (43 mg, 25%) as an off-white amorphous powder. MP 275°C (Decomp).

1H NMR (DMSO-δ) δ 10.70 (s, 2H), 8.02 (s, 2H), 7.67 (d, J = 8.4 Hz, 4H), 7.52 (d, J = 8.4 Hz, 4H), 6.28 (s, 1H), 5.85 (s, 2H); 13C NMR (DMSO-δ) δ 162.7, 162.6, 138.7, 134.1, 133.1, 128.9, 127.6, 73.5; MS: LRMS 399.8; HRMS calculated for M+H: C_{28}H_{20}Cl_{2}N_{6}, 400.0839; found 400.0844.

4,6-Bis(2-((E)-1-(4-chlorobenzylidene)ethylidene)hydrazinyl)pyrimidin-2-amine (9)

A suspension of 2-amino-4,6-dihydrizinylpyrimidine (88 mg, 0.568 mmol) and 2-chlorobenzaldehyde (0.15 mL, 190 mg, 1.3 mmol, 2.3 eq.) in EtOH (25 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature, diluted with EtO (30 mL) and concentrated in vacuo to ca. 5 mL before collecting the precipitate to afford the pyrimidine (24 mg, 11%) as an off-white powder. MP 244–246°C.

1H NMR (DMSO-δ) δ 10.91 (s, 2H), 8.41 (s, 2H), 7.98 (d, J = 7.5 Hz, 2H), 7.50–7.35 (m, 6H), 6.34 (s, 1H), 5.93 (s, 2H); 13C NMR (DMSO-δ) δ 162.75, 162.68, 136.0, 132.4, 131.9, 130.1, 129.7, 126.7, 73.7; MS: LRMS ESI + ve 400.1 (M+1); HRMS calculated for M+H: C_{28}H_{20}Br_{2}N_{6}, 400.0839; found 400.0840.

4,6-Bis(2-((E)-1-(4-bromobenzylidene)ethylidene)hydrazinyl)pyrimidin-2-amine (10)

A suspension of 2-amino-4,6-dihydrizinylpyrimidine (66 mg, 0.423 mmol) and 4-bromobenzaldehyde (186 mg, 1.005 mmol, 2.38 eq.) in EtOH (4 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature before collecting precipitate and washing with ice cold EtO (10 mL) and EtO (10 mL) to afford the pyrimidine (133 mg, 64%) as a white crystalline solid. MP 274°C (Decomp).

1H NMR (DMSO-δ) δ 10.71 (s, 2H), 8.00 (s, 2H), 7.63 (dd, J = 24.3, 8.6 Hz, 8H), 6.27 (s, 1H), 5.86 (s, 2H); 13C NMR (DMSO-δ) δ 162.7, 162.6, 138.8, 134.5, 131.8, 127.9, 121.7, 73.5; MS: LRMS ESI + ve 488.1 (M+1); HRMS calculated for M+H: C_{28}H_{20}Br_{2}N_{6}, 487.9828; found 487.9830.
4,6-Bis(2-((E)-4-fluorobenzylidene)hydrazinyl)pyrimidin-2-amine (10)

A suspension of 2-amino-4,6-dihydrazinopyrimidine (109 mg, 0.704 mmol) and 4-fluorobenzaldehyde (0.16 mL, 180 mg, 1.5 mmol, 2.13 eq.) in EtOH (10 mL) was heated at reflux for 16 h. The reaction mixture was filtered hot, washing with Et$_2$O (10 mL), to afford the pyrimidine (110 mg, 42%) as a tan powder. MP 262°C (Decomp).

$^1$H NMR (DMSO-$_d_6$) δ 10.61 (s, 2H), 8.03 (s, 2H), 7.70 (dd, J = 8.8, 5.6 Hz, 4H), 7.29 (t, J = 8.8 Hz, 4H), 6.27 (s, 1H), 5.82 (s, 2H); $^{13}$C NMR (DMSO-$_d_6$) δ 162.8, 162.6, 162.3 (d, J = 246.0 Hz), 138.9, 131.8 (d, J = 30.0 Hz), 128.0 (d, J = 8.3 Hz), 115.9 (d, J = 21.8 Hz), 73.4. $^{19}$F NMR (376 MHz, DMSO-$_d_6$) δ −112.57. MS: LRMS ESI +ve 368.2 (M+1); HRMS calculated for M+H: C$_{23}$H$_{25}$F$_3$N$_5$, 368.1430; found 368.1431.

4,6-Bis(2-((E)-4-(trifluoromethyl)benzylidene)hydrazinyl)pyrimidin-2-amine (11)

A suspension of 2-amino-4,6-dihydrazinopyrimidine (112 mg, 0.7224 mmol) and 4-(trifluoromethyl)benzaldehyde (0.21 mL, 270 mg, 1.5 mmol, 2.08 eq.) in EtOH (11 mL) was heated at reflux for 16 h. The reaction mixture was concentrated using a compressed air stream before suspending the resulting crude material in Et$_2$O (10 mL) and collected, washing with Et$_2$O (10 mL), to afford the pyrimidine (19 mg, 5%) as a brown powder. MP 261°C (Decomp).

$^1$H NMR (DMSO-$_d_6$) δ 10.89 (s, 2H), 8.11 (s, 2H), 7.84 (dd, J = 20.5, 8.4 Hz, 8H), 6.34 (s, 1H), 5.93 (s, 2H); $^{13}$C NMR (DMSO-$_d_6$) δ 162.7, 162.6, 139.1, 138.4, 128.4 (q, J = 31 Hz), 126.9 (q, J = 263 Hz) * $^{12}$6.5, 125.8 (q, J = 3.8 Hz)*, 74.1. * poorly resolved quartet. $^{19}$F NMR (376 MHz, DMSO-$_d_6$) δ −60.90. MS: LRMS ESI +ve 468.2 (M+1); HRMS calculated for M+H: C$_{22}$H$_{23}$F$_3$N$_5$, 468.1366; found 468.1371.

4,6-Bis(2-((E)-benzylidene)hydrazinyl)pyrimidin-2-amine (12)

To a slurry of 2-amino-4,6-dihydrazinopyrimidine (49 mg, 0.316 mmol) and benzaldehyde (0.100 mL, 104 mg, 0.980 mmol, 3.10 eq.) was added EtOH (10 mL) and the solution heated at reflux for 16 h. Upon cooling the resulting precipitate was collected, washing with Et$_2$O (5 mL) to afford the target compound (23 mg, 22%) as a white powder. MP 242–244°C.

$^1$H NMR (DMSO-$_d_6$) δ 10.60 (s, 2H), 8.04 (s, 2H), 7.66 (d, J = 7.5 Hz, 4H), 7.45 (t, J = 7.1 Hz, 4H, 7.38–7.34 (m, 2H), 6.30 (s, 1H), 5.82 (s, 2H). $^{13}$C NMR (DMSO-$_d_6$) δ 163.3, 163.1, 140.5, 135.7, 132.9, 129.2, 126.5, 73.9; MS: LRMS ESI +ve 484.2 (M+1); HRMS calculated for M+H: C$_{22}$H$_{25}$N$_5$, 484.2244; found 484.2248.

4,4’-((1E,1E)-[(2-amino pyrimidin-2-yl)-1-ylidene]bis(methanylylidene))diphenol (16)

A suspension of 2-amino-4,6-dihydrazinopyrimidine (70 mg, 0.454 mmol) and 4-hydroxybenzaldehyde (140 mg, 1.149 mmol, 2.53 eq.) in EtOH (3 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with Et$_2$O (25 mL) to afford the pyrimidine (91 mg, 55%) as an off-white powder. MP 298°C (Decomp).

$^1$H NMR (CDCl$_3$) δ 10.31 (s, 2H), 9.74 (s, 2H), 7.94 (s, 2H), 7.48 (d, J = 8.6 Hz, 4H), 6.83 (d, J = 8.6 Hz, 4H), 6.20 (s, 1H), 5.70 (s, 2H); $^{13}$C NMR (CDCl$_3$) δ 162.7, 162.5, 158.3, 140.5, 127.7, 126.7, 126.6, 73.5; MS: LRMS ESI +ve 364.2 (M+1); HRMS calculated for M+H: C$_{22}$H$_{25}$NO$_2$, 364.1516; found 364.1519.

3,3’-((1E,1E)-[(2-amino pyrimidin-2-yl)-1-ylidene]bis(methanylylidene))diphenol (17)

A suspension of 2-amino-4,6-dihydrazinopyrimidine (66 mg, 0.427 mmol) and 3-hydroxybenzaldehyde (207 mg, 1.693 mmol, 3.96 eq.) in EtOH (3 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with Et$_2$O (25 mL) to afford the pyrimidine (19 mg, 12%) as a white powder. MP 256°C (Decomp).

$^1$H NMR (DMSO-$_d_6$) δ 10.51 (s, 2H), 9.55 (s, 2H), 7.95 (s, 2H), 7.22 (t, J = 7.9 Hz, 2H), 7.11–7.04 (m, 4H), 6.76 (d, J = 8.4 Hz, 2H), 6.23 (s, 1H), 5.80 (s, 2H). $^{13}$C NMR (DMSO-$_d_6$) δ 162.8, 162.6, 157.7, 140.4, 136.4,
A suspension of 2-amino-4,6-dihydrazinopyrimidine (66 mg, 0.425 mmol) and 2-hydroxybenzaldehyde (110 mg, 0.900 mmol) was heated at reflux for 62 h, whilst protecting the flask from light. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with EtO (25 mL) to afford the pyrimidine (65 mg, 42%) as a white powder. 

To a refluxing solution of 2-amino-4,6-dihydrazinopyrimidine (19) (0.5439 mmol) and N-(4-formylphenyl)acetamide (210 mg, 0.673 mmol, 2.27 eq.) in EtOH (5 mL) was heated at reflux for 6 h. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with EtO(O2) (10 mL), to afford the pyrimidine (65 mg, 40%) as a white powder. 

A suspension of 2-amino-4,6-dihydrazinopyrimidine (145 mg, 0.94 mmol) and cyclohexanecarboxaldehyde (264 mg, 0.25 mL, 2.2 eq.) in EtOH (3 mL) was subject to microwave irradiation for 20 minutes at 120°C. The reaction was concentrated in vacuo before column chromatography (hexanes:EtOAc gradient). The resulting solid was collected and slurred with EtO (10 mL) to afford the pyrimidine (83 mg, 40%) as a tan powder. 

HRMS calculated for M + H: C18H10N2O6, 344.2127; found 344.2126.

A suspension of 2-amino-4,6-dihydrazinopyrimidine (145 mg, 0.94 mmol) and 2-hydroxybenzaldehyde (110 mg, 0.900 mmol) was heated at reflux for 62 h, whilst protecting the flask from light. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with EtO (25 mL) to afford the pyrimidine (65 mg, 42%) as a white powder. 

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HRMS calculated for M + H: C18H10N2O6, 344.2127; found 344.2126.

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HRMS calculated for M + H: C18H10N2O6, 344.2127; found 344.2126.
the triazine (151 mg, 48%) as a pale pink powder. MP 264 °C (Decomp).

H NMR (DMSO-\(d_6\)) \(\delta\) 9.63 (s, 1H), 7.81 (d, \(J = 8.6\) Hz, 2H), 7.45 (d, \(J = 8.6\) Hz, 2H), 6.73 (s, 2H), 2.28 (s, 3H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 167.9, 165.3, 146.5, 137.6, 133.2, 128.2, 127.8, 13.5; MS: LRMS ESI + ve 429.1 (M + 1); HRMS calculated for M + H: \(C_{19}H_{17}Cl_{2}N_9\) 429.1104; found 429.1108.

3,3\'-((1E,1'E)-(6-amino-1,3,5-triazine-2,4-diyl)bis(hydrazin-2-yl-1-ylidene))bis(methanylylidene)diphenol (26)

A suspension of 2-amino-4,6-dihydrazino-1,3,5-triazine (48 mg, 0.305 mmol) and 3-hydroxybenzaldehyde (161 mg, 1.321 mmol, 4.33 eq.) in EtOH (3 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature before collecting the precipitate, washing with Et\(_2\)O (20 mL), to afford the triazine (24 mg, 21%) as a white powder. MP 306 °C (Decomp).

H NMR (DMSO-\(d_6\)) \(\delta\) 10.75 (s, 1H), 9.55 (s, 1H), 8.04 (s, 1H), 7.21 (t, \(J = 7.8\) Hz, 1H), 7.06 (s, 1H), 7.02 (d, \(J = 7.6\) Hz, 1H), 6.84–6.57 (m, 2H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 167.4, 164.6, 157.6, 142.6, 136.4, 129.7, 117.9, 116.4, 112.5; MS: LRMS ESI – ve 363.1 (M – 1); HRMS calculated for M + H: \(C_{19}H_{17}N_9O_2\) 365.1469; found 365.1472.

4,6-Bis((E)-(4-formylphenyl)phosphoryl)benzaldehyde (30)

A suspension of 4,6-dihydroxybenzaldehyde (0.368 mmol) and 4-bromobenzaldehyde (0.943 mmol, 2.62 mmol, 2.2 eq.) in EtOH (20 mL) was heated at reflux for 6 h. After cooling, the emulsified mixture was diluted with Et\(_2\)O (15 mL) and hexanes (10 mL) and the resulting precipitate was collected afford the triazine (32 mg, 39%) as a white powder. MP 120 °C (Slow Decom).

H NMR (DMSO-\(d_6\)) \(\delta\) 10.34 (br s, 2H), 7.27 (d, \(J = 5.7\) Hz, 2H), 7.46–7.33 (m, 3H), 6.76 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 167.4, 164.6, 142.3, 135.1, 129.0, 128.7, 126.4; MS: LRMS ESI + ve 333.2 (M + 1); HRMS calculated for M + H: \(C_{19}H_{17}N_9O_2\) 333.1571; found 333.1572.

4,6-Bis((E)-cyclohexylmethylene)hydrazoneyl)-1,3,5-triazin-2-amine (31)

A suspension of 2-amino-4,6-dihydrazino-1,3,5-triazine (59 mg, 0.380 mmol) and cyclohexanecarboxaldehyde (0.10 mL, 93 mg, 0.83 mmol, 2.2 eq.) in EtOH (4 mL) was heated at reflux for 16 h. The reaction was concentrated in vacuo before dilution with Et\(_2\)O (10 mL) and hexanes (10 mL) and the resulting precipitate was collected afford the triazine (52 mg, 39%) as a white powder. MP 120 °C (Decomp).

H NMR (DMSO-\(d_6\)) \(\delta\) 10.84 (s, 1H), 7.63 (d, \(J = 7.4\) Hz, 2H), 7.46–7.33 (m, 3H), 6.76 (s, 1H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 167.4, 164.6, 142.3, 135.1, 129.0, 128.7, 126.4; MS: LRMS ESI – ve 333.2 (M + 1); HRMS calculated for M + H: \(C_{19}H_{17}N_9O_2\) 333.2510; found 334.2516.

4,6-Bis((E)-4-methylbenzylidene)hydrazoneyl)-1,3,5-triazin-2-amine (32)

A suspension of 2-amino-4,6-dihydrazino-1,3,5-triazine (49 mg, 0.311 mmol) and 4-methylbenzaldehyde (0.10 mL, 100 mg, 0.832 mmol, 2.67 eq.) in EtOH (4 mL) was heated at reflux for 16 h. The reaction mixture was cooled to ambient temperature before collecting the yellow precipitate, washing with Et\(_2\)O (20 mL) to afford the pyridimine (67 mg, 60%) as a yellow powder. MP 318 °C (Decomp).

H NMR (DMSO-\(d_6\)) \(\delta\) 10.75 (s, 1H), 7.52 (d, \(J = 8.1\) Hz, 2H), 7.24 (d, \(J = 8.0\) Hz, 2H), 6.71 (s, 1H), 2.33 (s, 3H); \(^{13}\)C NMR (DMSO-\(d_6\)) \(\delta\) 167.4, 164.6, 142.4, 138.6, 132.4, 129.3, 126.4, 21.0; MS: LRMS ESI – ve 361.2 (M + 1); HRMS calculated for M + H: \(C_{19}H_{17}N_9O_2\) 361.1884; found 361.1887.

4-(E)-(2-(2-amino-6-(2-(E)-4-(diethoxyphosphoryl)oxy)benzylidene)hydrazinyl)pyrimidin-4-yl)hydrazono)methyl)phenyl diethyl phosphate (33)

Diethyl (4-formylphenyl)phosphate: To a stirring suspension of 4-hydroxybenzaldehyde (412 mg, 3.372 mmol) in \(CH_2Cl_2\) (10 mL) was
added diethyl phosphorochloridate (0.53 mL, 640 mg, 3.7 mmol, 1.1 eq.) followed by triethylamine (0.50 mL, 360 mg, 3.6 mmol, 1.1 eq.). The solution was stirred at ambient temperature for 16 h before being diluted with H2O (10 mL), 1 M NaOH (10 mL) and CH2Cl2 (30 mL). The organics were partitioned and washed with 1 M HCl (20 mL) and 1 M NaOH (20 mL) before drying over MgSO4 and concentrating in vacuo to afford the phosphate ester (555 mg, 64%) as a colourless oil.

To a suspension of 2-amino-4,6-dihydroxyazopyrimidine (151 mg, 0.9729 mmol) in THF (16 mL) was added diethyl (4-formylphenyl) phosphate (555 mg, 2.148 mmol, 2.21 eq.) and the solution heated at reflux for 48 h. The cooled reaction mixture was filtered to remove unreacted 2-amino-4,6-dihydroxyazopyrimidine and resulting filtrate was concentrated over a stream of compressed air. The resulting crude material was triturated with EtOAc (10 mL) to afford the pyrimidinyl phosphate (117 mg, 19%) as an orange/brown powder. MP 235 °C (Decomp).

1H NMR (DMSO-d6) δ 10.81 (s, 2H), 8.06 (s, 2H), 7.71 (d, J = 8.5 Hz, 4H), 7.29 (d, J = 8.1 Hz, 4H), 6.24 (s, 1H), 4.17 (dd, J = 14.2, 7.1 Hz, 8H), 1.28 (td, J = 7.1, 0.6 Hz, 12H); 13C NMR (DMSO-d6) δ 139.7, 127.8, 120.4, 120.4, 73.1, 64.44, 64.38, 15.93, 15.87; 19F NMR (162 MHz, DMSO-d6) δ −6.54. MS: LRMS ESI + ve 636.3 (M + 1); HRMS calculated for M + H: C20H19N8O8F6, found 636.2100.

4.4'-(1E,1'2)-(2-aminoazopyrimidine-4,6-diyldi)bis (hydrazidin-2-yl-1-yldiene)bis(methylnylidene)bis (benzene-1,2-diol) (36)

2-Amino-4,6-dihydroxyazopyrimidine (122 mg, 0.7843 mmol) and 3,4-dihydroxybenzaldehyde (253 mg, 1.828 mmol, 2.32 eq.) were suspended in EtOH (10 mL) and heated at reflux for 16 h. The resulting solution was concentrated to ca 5 mL by boiling at ambient pressure and the resulting precipitate was filtered hot, washing with Et2O (20 mL) to afford the bis-hydrazone (725 mg, 71%) as a tan powder. MP > 400 °C (Discolours to Black).

3.3'-(1E,1'2)-(2-aminoazopyrimidine-4,6-diyldi)bis (hydrazidin-2-yl-1-yldiene)bis(methylnylidene)bis (benzene-1,2-diol) (37)

A suspension of 2-amino-4,6-dihydroxyazopyrimidine (200 mg, 1.286 mmol) and 2,3-dihydroxybenzaldehyde (516 mg, 3.738 mmol, 2.91 eq.) in EtOH (10 mL) was heated at reflux for 16 h. The resulting yellow precipitate was collected and washed with EtO2 (20 mL) to afford the bis-hydrazone (405 mg, 61%) as a yellow powder. MP 256 °C (Decomp).

5.5'-(1E,1'2)-(2-aminoazopyrimidine-4,6-diyldi)bis (hydrazidin-2-yl-1-yldiene)bis(methylnylidene)bis (benzene-1,2,3-triol) (38)

2-Amino-4,6-dihydroxyazopyrimidine (371 mg, 2.387 mmol) and 3,4,5-trihydroxybenzaldehyde (939 mg, 6.093 mmol, 2.55 eq.) were suspended in EtOH (20 mL) and heated at reflux for 16 h. The resulting suspension was filtered hot, washing with EtO2 (50 mL) to afford the bis-hydrazone (725 mg, 71%) as a pale yellow powder. > 400 °C (Discolours to Black).

5.5'-(1E,1'2)-(2-aminoazopyrimidine-4,6-diyldi)bis (hydrazidin-2-yl-1-yldiene)bis(methylnylidene)bis (benzene-1,2,3-triol) (38)

A suspension of 2-amino-4,6-dihydroxyazopyrimidine (301 mg, 1.940 mmol) and 2,4-dihydroxybenzaldehyde (601 mg, 4.350 mmol, 2.24 eq.) in EtOH (52 mL) was heated at reflux for 16 h. The resulting precipitate was collected, washed with EtOH (10 mL) and Et2O (25 mL) and dried to afford the bis-hydrazone (450 mg, 59%) as a tan powder. MP 270 °C (Decomp).

1H NMR (DMSO-d6) δ 10.47 (s, 4H), 9.81 (s, 2H), 8.14 (s, 2H), 7.29 (d, J = 8.4 Hz, 2H), 6.40–6.24 (m, 4H), 5.78 (s, 3H); 13C NMR (DMSO-d6) δ
2-Amino-4,6-dihydrazinopyrimidine (104 mg, 0.6715 mmol) and 1-naphthaldehyde (0.20 mL, 230 mg, 1.5 mmol, 2.2 eq.) were suspended in EtOH (10 mL) and heated at reflux for 3 h. The resulting suspension was filtered hot and the resulting material washed with Et₂O (20 mL) to afford the bis-hydrazone (83 mg, 29%) as a white powder. MP 226 °C (decomp).

1 H NMR (DMSO-d₆) δ 10.75 (s, 2H), 8.83–8.78 (m, 2H), 8.74 (s, 2H), 8.05–8.00 (m, 2H), 7.98 (d, J = 8.2 Hz, 2H), 7.92 (d, J = 7.0 Hz, 2H), 7.67–7.53 (m, 6H), 6.51 (s, 1H), 5.92 (s, 2H); 13C NMR (DMSO-d₆) δ 162.9, 162.7, 139.7, 133.7, 130.5, 129.9, 128.8, 126.9, 126.1, 126.0, 125.6, 124.0, 73.5; MS: LRMS ESI + ve 342.2 (M + 1); HRMS calculated for M + H: C₁₁H₁₁N₂O, 342.1931; found 342.1936.

4,6-Bis-(2-(E)-naphthalen-1-ylmethylene)hydrazinyl) pyrimidin-2-amine (41)

To a suspension of 2-amino-4,6-dihydrazinopyrimidine (170 mg, 1.094 mmol) and 1-naphthaldehyde (382 mg, 2.447 mmol, 2.24 eq.) were suspended in EtOH (7.5 mL) and heated at reflux for 16 h. The resulting suspension was diluted with EtOH (10 mL), boiled and filtered hot. The resulting material was slurried with EtOH (2 × 10 mL), washed with Et₂O (2 × 10 mL) to afford the bis-hydrazone (285.8 mg, 61%) as a yellow powder. MP 160–161 °C (decomp).

1 H NMR (DMSO-d₆) δ 10.82 (s, 2H), 8.80 (d, J = 1.6 Hz, 2H), 8.54 (dd, J = 4.7, 1.5 Hz, 2H), 8.11–8.03 (m, 4H), 7.46 (dd, J = 7.9, 4.8 Hz, 2H), 6.33 (s, 1H), 5.91 (s, 2H); 13C NMR (DMSO-d₆) δ 162.7, 162.6, 149.4, 147.7, 137.2, 132.6, 131.0, 124.0, 73.6; MS: LRMS 333.7; HRMS calculated for M + H: C₁₁H₁₁N₂O, 334.1523; found 334.1530.

4,6-Bis-(2-(E)-naphthalen-2-ylmethylene)hydrazinyl) pyrimidin-2-amine (44)

To a suspension of 2-amino-4,6-dihydrazinopyrimidine (280 mg, 1.339 mmol) in EtOH (50 mL) was added 3-pyrimidinecarbaldehyde (0.30 mL, 340 mg, 3.2 mmol, 2.4 eq.) and the solution heated at reflux for 16 h. The resulting suspension was filtered hot, washing with Et₂O (20 mL) to afford the bis-hydrazone (111 mg, 25%) an off-white powder. MP 280 °C (decomp).

1 H NMR (DMSO-d₆) δ 10.82 (s, 2H), 8.80 (d, J = 1.6 Hz, 2H), 8.54 (dd, J = 4.7, 1.5 Hz, 2H), 8.11–8.03 (m, 4H), 7.46 (dd, J = 7.9, 4.8 Hz, 2H), 6.33 (s, 1H), 5.91 (s, 2H); 13C NMR (DMSO-d₆) δ 162.7, 162.6, 149.4, 147.7, 137.2, 132.6, 131.0, 124.0, 73.6; MS: LRMS 333.7; HRMS calculated for M + H: C₁₁H₁₁N₂O, 334.1523; found 334.1532.
to afford the hydrazone (414 mg, 60%) as an off white powder. MP 243 °C (Decomp).

$^1$H NMR (DMSO-d$_6$) $\delta$ 10.92 (s, 2H), 8.61 (d, $J$ = 1.7 Hz, 2H), 8.14 (dd, $J$ = 8.3, 2.0 Hz, 2H), 8.05 (s, 2H), 7.59 (d, $J$ = 8.3 Hz, 2H), 6.30 (s, 1H), 5.93 (s, 2H); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 162.7, 162.6, 149.7, 147.9, 135.9, 130.6, 124.6, 73.6; MS: LRMS 402; HRMS calculated for M + H$: C_{16}H_{21}Cl$_2$N$_4$: 402.0744; found 402.0750.

Acknowledgements

This research was supported by a Linkage Project grant from the Australian Research Council in collaboration with Neoculi Pty Ltd (ARC LP110200770).

Conflict of Interest

Dr Stephen Page is Director of Neoculi Pty Ltd who funded this study

Keywords: Aminopyrimidines · antibacterial activity · robenidine · drugs discovery


Manuscript received: November 4, 2018
Revised manuscript received: April 15, 2019