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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 1736–1740

New thiopyrazolo[3,4-*d*]pyrimidine derivatives as anti-mycobacterial agents

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Received 27 November 2006; revised 14 December 2006; accepted 16 December 2006
Available online 22 December 2006

Abstract—The multiple parallel synthesis of a series of N,S-bis-alkylated thiopyrazolo[3,4-*d*]pyrimidines, based on sequential S- then N-alkylation, is reported. These compounds showed significant anti-mycobacterial activity (MICs down to $\leqslant 2$ μg/ml) and their potential as significant drug-like leads is substantiated through cytotoxicity evaluation and in silico profiling.

Synthetic drugs for treating tuberculosis (TB) have been available for over half a century, but incidences of the disease continue to rise world-wide. In 2004, the last year for which statistics are available, it is estimated that 24,500 people developed active disease and close to 5500 died from TB every day.[1] Co-infection with HIV is driving the increase in incidence[2] and the cause of death in 31% of AIDS patients in Africa can be attributed to TB.[3,4] When coupled with the emergence of multi-drug resistant strains of *Mycobacterium tuberculosis* (MDR-TB), the scale of the problem is amplified. It is now more than a decade since the WHO declared TB 'a global health emergency'.[1] The need for new drugs to extend the range of TB treatment options is hence acute; in particular, new chemical entities[5] with novel mechanisms of action are required.[6]

Purine-containing molecules are ubiquitous in nature and are found as components of nucleosides, nucleotides, co-factors and signalling molecules. A significant proportion of any genome codes for proteins that recognise purine-containing ligands (e.g., kinases, DNA and RNA polymerases, ATPases, GTPases, purine receptors). Unsurprisingly, structural analogues of purines have proved attractive templates for drug discovery programmes, leading to a number of significant synthetic drugs (e.g., 6-thiopurine for leukaemia,[7] acyclovir as an anti-viral[8] and allopurinol for treatment of gout[9]). Recently, the purine core has been exploited in the synthesis of protein kinase inhibitors,[10–13] inhibitors of carbohydrate[14] and estrogen[15] sulfotransferases, as well as in compounds displaying osteogenesis-inducing activity in stem cells.[16] Compounds of this generic nature (**1**–**5**) have also been reported to display anti-mycobacterial activity (Fig. 1).[17–20]

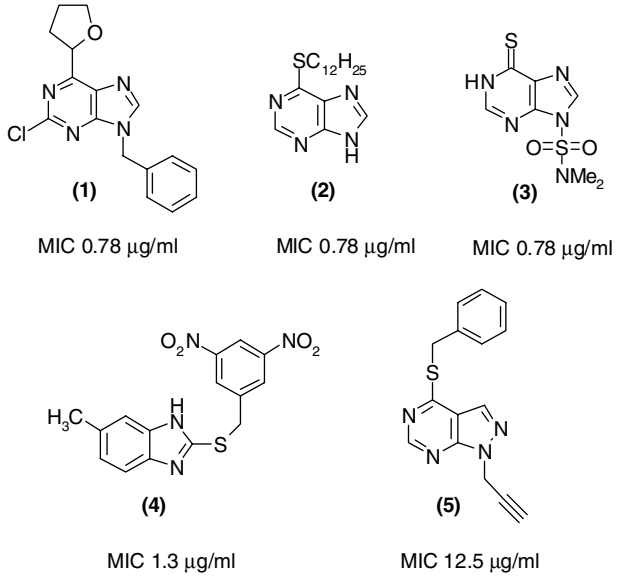

Figure 1. Purine-related anti-mycobacterials: (**1**),[17] (**2**),[18] (**3**),[19](**4**),[20] (**5**).[25]

Keywords: Anti-mycobacterial; Anti-tubercular; Thiopyrazolopyrimidines; SAR.
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doi:10.1016/j.bmcl.2006.12.066

The isomeric pyrazolo[3,4-*d*]pyrimidine nucleus has attracted comparatively little attention, though it has been exploited in inhibitors of Src protein kinases,[21] EGF receptor tyrosine kinases,[22] cyclic AMP phosphodiesterases[23] and *Staphylococcus aureus* DNA polymerase III.[24] Interestingly, such a compound reported as an intermediate in the synthesis of prospective HIV replication inhibitors showed modest anti-mycobacterial activity (compound **5**; MIC 12.5 μg/ml).[25] In addition, the often present alkylsulfanyl moiety has been previously reported as important for anti-mycobacterial activity.[26] This result prompted us to explore the thiopyrazolo[3,4-*d*]pyrimidine template (TPP) in our search for new anti-mycobacterial agents, given an encouraging level of potency and straightforward syntheses via sequential S- then N-alkylation of a commercial heterocycle.

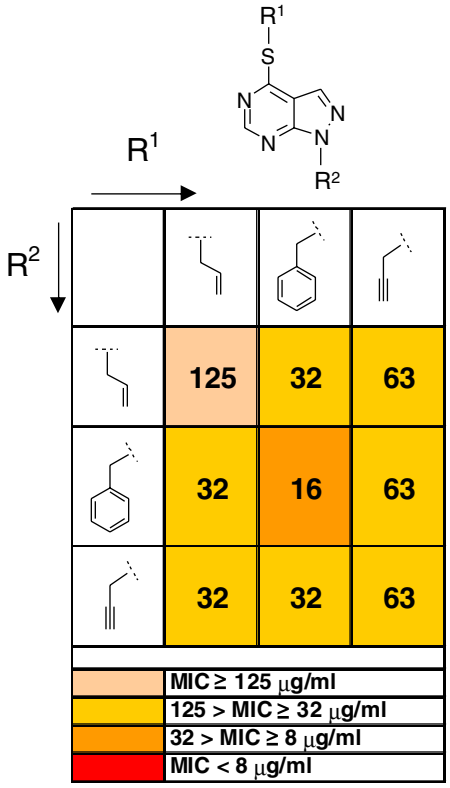
To explore the potential of this observation, we first constructed a 3×3 array around this hit. Synthetically, the S-alkylation reactions of commercially available 1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidine-4-thione with the requisite alkyl bromides were readily accomplished in good yields (60–90%) using hydroxide resin in DMF.[27] The subsequent 1-N-alkylation reactions (employing alkyl bromides with triethylamine or K_2CO_3 in DMF) proved somewhat capricious, but yields of the desired compounds varying from 25% to 80% were ultimately achieved. In spite of these issues, the requisite compounds were produced and purified in parallel on a 100 mg scale.[28,29] Compounds from this first iteration were assayed against *M. tuberculosis* $H_{37}R_v$; MIC data are recorded in Table 1.[30]

Whilst in our hands compound **5** (R_1 = benzyl; R_2 = propargyl) was marginally less active (MIC 32 μg/ml vs 12.5), activity was established with all nine compounds. In a second iteration, a set of 11 prospective S/N-substituents was selected to construct a bigger array; however, to lessen the synthetic burden, we employed stepwise logic in our synthetic execution with the 11×11 matrix of possible combinations. Thus, of the 121 possible structures this presents, an initial sub-set of 40 compounds was synthesized by restricting the choice of either the S- or N-substituent to one of two groups (allyl or benzyl) and exploiting all 11 options at the second site (Fig. 2). These initial sets of compounds, plus the mono-alkylated intermediates, were assayed to identify preferred S-(R^1) and N-(R^2) substituents, with a view to focusing SAR work in subsequent iterations, without having to make all 121 elements of the matrix.

Compounds from this second iteration were also assayed against *M. tuberculosis* $H_{37}R_v$; MIC data are recorded in Table 2.[30] Analysis of the partially filled rows and columns of this matrix enabled us to select a focused set of 3×3 R^1 and R^2 groups for the second iteration, displayed in the inner box of Table 2.

The results obtained provided evidence to vindicate our approach. An examination of the proportion of compounds with an MIC $\leqslant 8$ μg/ml generated by the 'blind synthesis' (main entries, Table 1) versus the 'oriented synthesis' (central table entries, Table 2) shows an improvement from 5% at or below the 8 μg/ml threshold to 33% in the second iteration. Pleasingly, one compound (R^1 = 4-chlorobenzyl; R^2 = 3-pyridylmethylene)[29] showed an MIC $\leqslant 2$ μg/ml. Subsequent re-testing showed this compound to have an MIC between 0.5 and 1 μg/ml.

Table 1. MIC data[30] for an initial set of nine 4-thiopyrazolopyrimidine derivatives against *Mycobacterium tuberculosis* $H_{37}R_v$



These compounds are based around literature hit **5**.[25]

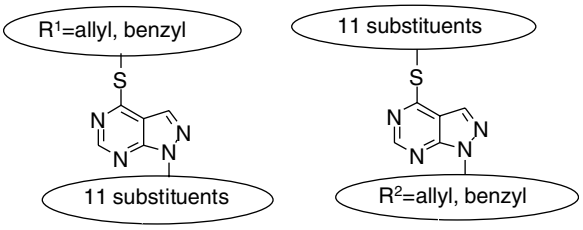
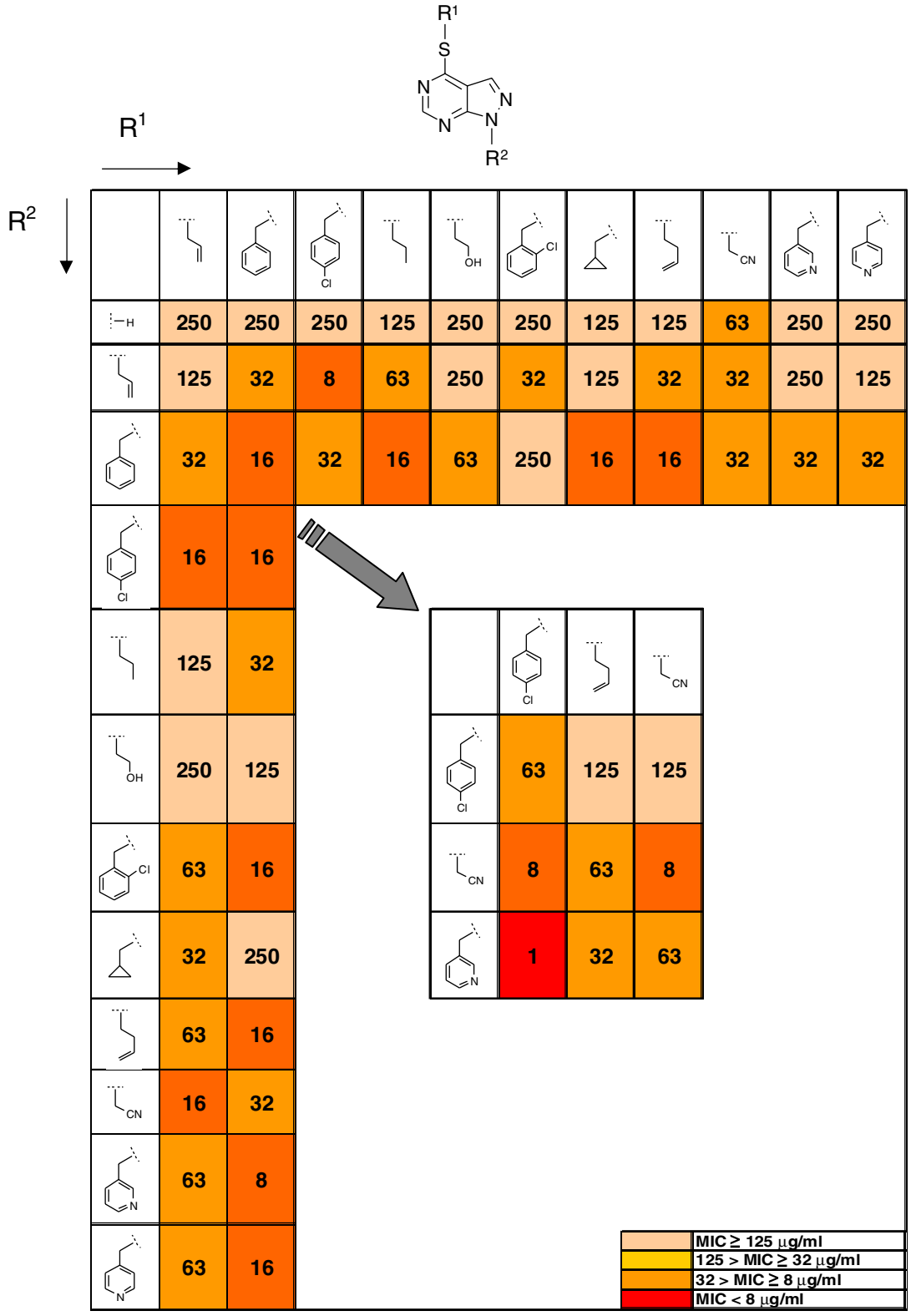


Figure 2. Strategy for development of initial SAR data.

In this exercise, we were able to substantiate the originally reported TPP as a genuine hit and improved on it, whilst delineating some SAR. It is interesting to compare the presence of electron poor aromatic rings in our more active molecules with the reported QSAR study based on electron withdrawing properties of substituents on *S*-benzyl thiopyridine derivatives[31] and alkylthiochloropyrimidines.[32] Clearly, any future work should first focus on *S*-benzyl analogues in order to explore electron withdrawing substituents, their preferred positioning, thus enabling possible QSAR studies.

In order to substantiate these molecules as robust leads, which might warrant a significant programme of medic-

Table 2. MIC data for 4-thiopyrazolopyrimidinez derivatives against *Mycobacterium tuberculosis* $H_{37}R_v$



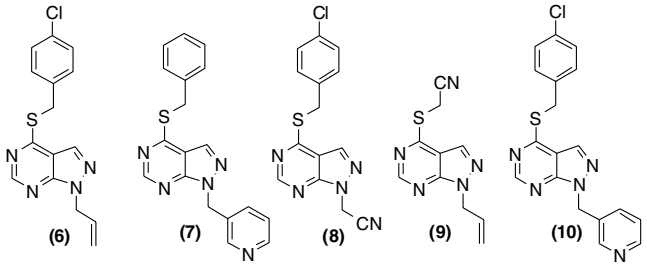
The central table entries represent compounds with R^1 and R^2 groups selected for a subsequent round of optimisation.

Table 3. In silico prediction of parameters associated with bioavailability for all compounds possessing MICs <8 μg/ml

Compound	H-bond donors	H-bond acceptors	M_w	*C*log *P*	Lipinski compliant?	Rotatable bonds	PSA (Å^2)	Veber compliant?	*C*log *D* at pH 7.4	*C*log *D* at pH 6.5	Topliss score	Bioavailability (Topliss)
6	0	4	316.8	3.72	Yes	5	68.9	Yes	2.2	2.2	4.07	Class 4
7	0	5	333.4	2.56	Yes	5	81.8	Yes	2.56	2.56	3.98	Class 3
8	0	5	315.7	2.20	Yes	5	92.7	Yes	3.72	3.72	3.41	Class 3
9	0	5	230.2	2.45	Yes	5	92.7	Yes	3.16	3.16	4.01	Class 4
10	0	5	367.8	3.16	Yes	5	81.8	Yes	3.11	3.11	3.82	Class 3

Only 2 of the 18 Topliss assessment criteria calculated are included in the table.

Table 4. Cytotoxicity and anti-mycobacterial activity of hit compounds. Isoniazid (INH), Rifampicin (RIF) and Ethambutol (ETH) were employed as controls in the MIC assays



Compound	Tox 50 HepG2 (μg/mL)	MIC $H_{37}R_v$ (μg/mL)
6	>20	8
7	>40	8
8	2	8
9	4	8
10	>40	0.5–1
INH*	—	0.25
RIF*	—	0.016
ETH*		2.5

inal chemistry, we generated cytotoxicity data (Tox 50) on the most potent compounds in a HepG2 cell line and generated in silico predictions of the likely oral pharmacokinetic profile. Encouragingly, three of the five hit molecules were shown non-cytotoxic at the highest concentration assayed (Table 4). The in silico predictors also indicated a strong likelihood of good oral bioavailability;[35] through the generation of predictors based on the calculated parameters described by Lipinski,[33] Veber[34] and Topliss[35] (Table 3).

In summary, *N*-,*S*-di-alkyl 4-thio-1*H*-pyrazolo[3,4-*d*]pyrimidines have been investigated as anti-mycobacterial agents. The best compound exhibited activity in vitro that is comparable to clinically successful drugs (e.g., ethambutol MIC 2 μg/ml) coupled with no cytotoxicity against a HepG2 cell line. The SAR developed herein, coupled with favourable pharmacokinetic predictions, substantiate these molecules as a significant lead series in the search for new anti-tubercular agents.

Acknowledgments

We thank the GlaxoSmithKline Action TB Initiative for financial support and María Jesús Almela for generating the cytotoxicity data.

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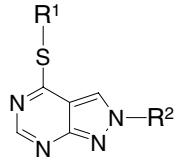
27. *Typical procedure for thiol alkylation:* TPP (1 equiv) was dissolved in dry DMF (10 ml/g of substrate), ion exchange resin (Dowex AG 1-X8, OH^- form; 1 g per 100 mg of substrate) was added and the mixture was stirred gently for 5 min. The alkylating agent (1.2 mol equiv) was then added and the mixture was stirred until LC–MS showed the reaction to be complete.[26] The reaction was quenched by the addition of aqueous NH_3 (5 mol equiv), filtered to remove the resin and concentrated in vacuo. The crude product obtained was preabsorbed onto silica (5 g) and then eluted in series with a prepacked silica column, eluted with a gradient of $0 \rightarrow 70\%$ EtOAc in cyclohexane, to give the S-alkylated product. *Typical procedure for N-alkylation*: The substrate (1 equiv) was dissolved in dry DMF (10 ml/g of substrate), base (Et_3N, 3 mol equiv, or K_2CO_3, 5 mol equiv) was added and the mixture was stirred for 5 min. The alkylating agent (3 mol equiv) was then added and the mixture was heated at reflux under N_2 for 16 h. The reaction was quenched by the addition of aqueous NH_3 (5 mol equiv), filtered to remove the resin and concentrated in vacuo. The bis-alkylated product was purified as described above.
28. The isomeric 2-alkyl-4-(alkylthio)-2*H*-pyrazolo[3,4-*d*]pyrimidines were sometimes significant by-products in these syntheses.



2-alkyl-4-(alkylthio)-2H-pyrazolo[3,4-d]pyrimidine

The regioisomers are easily distinguishable by virtue of NOE signals between the heterocycle H-3 proton and N-2 substituents. Such signals are not evident for the N-1-substituted isomer.
29. All compounds showed at least $\geqslant 95\%$ purity by HPLC and had satisfactory MS and NMR data, for example, Compound **10** 4-{[(4-chlorophenyl)methyl]thio}-1-(3-pyridinylmethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine: δ_H (DMSO-d_6) 8.86 (1H, s), 8.56 (1H, d, $J = 1.8$ Hz), 8.50 (1H, dd, $J = 4.8$, 1.8 Hz), 8.38 (1H, s), 7.63 (1H, m), 7.51 (2H, d, $J = 8.1$ Hz), 7.38 (2H, d, $J = 8.1$ Hz), 7.34 (1H, m), 5.69 (2H, s), 4.69 (2 H, s). δ_C (DMSO-d_6) 164.6, 155.0, 149.8, 149.7, 137.0, 136.2, 132.9, 131.8, 129.2, 124.4, 48.4, 31.8. Mass spectrum, expected: $C_{18}H_{15}ClN_5S^+$ 368.0737, found 368.0736.
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