Investigating the Biological Activity of Some Useful Heterocycles

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***Abstract*—**A series of aryl and alkyl pyrimido[1,2-a]benzimidazole derivatives was synthesized according to published procedures. All synthesized compounds were fully characterized by spectral and microanalytical data. The compounds (**1a**, **1b**, **2a**, **2b** and **2c**) were screened for their antimicrobial activity against Gram-positive and Gram-negative bacteria and only compound **2b** showed moderate to good activity with respect to minimum inhibitory concentrations and by disc diffusion assays. Apart from *Proteus vulgaris*, which showed resistance against all the compounds, *Enterobacter cloacae*, *Escherichia coli* and *Proteus mirabilis* were the most susceptible Gram-negative bacteria with MIC of 1 μg/ml, 4 μg/ml and 2 μg/ml respectively. On the other hand, *Staphylococcus epidermis, Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis and Mycobacterium smegmatis* all showed susceptibility with MIC's of 15.62 μg/ml, 31.25 μg/ml, 62.5 μg/ml, 3.9 μg/ml, 62.5 μg/ml and 7.81 μg/ml respectively.

***Keywords*—**2-aminobenzimidazole, antibacterial, double Michael addition, pyrimido[1, 2-a] benzimidazole,.

I. Introduction

Although antibiotics have long been part of our arsenal against microbial pathogens, recent discoveries point to a rather disturbing outlook with regards to antibiotic resistance, particularly with notorious pathogens like methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis* (Mtb). Moreover, through the misuse, mismanagement and delay in the diagnosis of TB, the disease can potentially progress to multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR TB) [1]. To complicate matters, only a handful of antimicrobials have been developed in the last couple of years and passed stringent clinical trials for public use. As such, drug resistance (even for microbial infections that were not viewed as problematic anymore) is on the increase. Novel efficacious antibiotics with less or no side effects are therefore required to add on and update existing therapies. A comprehensive review by Cantas *et al* [2] discusses a number of issues and advances with regard to the development of various antimicrobials (semi-synthetic, synthetic and natural products and their respective derivatives) which show a variety of bioactive profiles, ease of synthesis or isolation procedures and future prospects with respect to combating drug resistance.

As far as antimicrobial synthetic compounds are concerned, the last couple of years have witnessed the utility of 2-aminobenzimidazoles and their derivatives in the fight against bacterial pathogens and a variety of other non-communicable diseases. Commercially available drugs like omeprazole, candesartan, bendamustine, benoxaprofen (despite its suspension in the 80's) targeting diseases such as ulcers, hypertension, cancer and inflammation respectively, possess the benzimadazole nucleus [3].

While Shaaban *et al* managed to demonstrate that a pyrimido[1,2-a]benzimidazole derivative has weak anti-inflammatory activity [4], Shah *et al* synthesized a series of 1,4-dihydro pyrimido[1,2-a]benzimidazole derivatives which exhibited modest activity against *S. aureus, S. epidermidis, E. coli, P. aeruginosa* and the fungus *Aspergillus niger* [5]. Since a number of compounds bearing the benzimidazole scaffold have been shown to possess interestingly diverse biological activities, we were interested in evaluating the antibacterial activity of a series of pyrimido[1,2-a]benzimidazole derivatives so as to explore the significance of their aryl and alkyl moieties.

II. Materials and Methods

A. Reagents

All reagents used were of analytical grade and unless otherwise mentioned, they were all purchased from Sigma Aldrich. The bacterial strains were obtained from Davies diagnostics.

B. Bacteria

Twelve bacterial strains (Gram-positive bacteria: *Bacillus cereus* (ATCC10876), *B. subtilis* (ATCC19659), *Enterococcus faecalis* (ATCC13047), *Mycobacterium smegmatis* (MC^2155, kind gift from the Centre of Excellence in Biomedical TB Research, University of the Witwatersrand), *Staphylococcus epidermidis* (ATCC14990) and *S. aureus* (ATCC25923). Gram-negative bacteria: *Enterobacter cloacae* (ATCC13047), *Escherischia coli* (ATCC25922), *Klebsiella oxytoca*

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(ATCC8724), *K. pneumonia* (ATCC13882), *Proteus mirabilis* (ATCC7002) and *Pseudomonas aeruginosa* (ATCC27853) were cultured overnight in Mueller-Hinton broth at 25 °C; Merck Chemicals, SA). The turbidity of the culture solutions were adjusted to match a 0.5 McFarland standard within 15 minutes prior to antibacterial testing.

C. Antibacterial Testing

Antibacterial studies were initiated by the disc diffusion method as a means to evaluate the most potent alkyl and aryl pyrimido[1,2-a]benzimidazole derivatives. *In-vitro* antibacterial screening was carried out using a filter-paper disc-agar diffusion procedure [6]. Sterile filter-paper blank discs (6 mm) were impregnated with 1 mg of compounds **1a**, **1b**, **2a**, **2b**, and **2c**. The disks were air-dried while Muller-Hinton agar plates (prepared earlier) were inoculated with the test bacteria using a sterile cotton swab. Impregnated filter-paper discs (loaded with the same masses of the test compounds) were then placed on the surface of agar plates to allow for the diffusion of the compounds into the agar, the plates were incubated at 37 °C for 16 hrs.

Minimum inhibitory concentrations (MIC) of all the strains were determined by the broth microdilution assay [7]. The test compounds were accurately weighed and dissolved in DMSO to yield 512µg/ml. The dissolved compounds were then serially diluted in Mueller-Hinton broth till the lowest concentration of 1µg/ml. All dilutions were tested five-fold against each bacterial strain. 100µl of the bacterial suspension was mixed with 100µl of pre-diluted test compound in a 96 microwell plate and left to incubate overnight at 37 °C. 10µl of a 0.02% (w/v) tetrazolium sodium solution was added to each well and the plates were re-incubated for 2 hours. Visual change of the solution from blue to pink indicated that the bacteria were still alive. MIC was determined as the minimum concentration of compound where no colour change could be observed. The MIC of all strains tested were compared to two reference antibiotics (nalidixic acid and streptomycin sulphate). This was due to the fact that whilst streptomycin is a broad based antibiotic, nalidixic acid has been shown to be exclusively active against Gram-negative bacteria [8].

D. Synthesis of Aryl And Alkyl Pyrimido [1,2-A] Benzimidazole Derivatives [9]

2-amino-4-phenyl pyrimido[1,2-a]benzimidazoles were prepared by the Michael addition of 2-aminobenzimidazoles on phenylacetylene nitriles and acetylenic nitriles respectively, followed by cyclisation to give compounds **1a** and **1b**.

2-Amino-4-phenylpyrimido[1,2-a]benzimidazole (**1a**). Yield 1.97g (76%) colourless crystals (DMSO), mp 300°C; IR: ν/cm-1 3440, 3303 (NH2), 3054 (aryl. C-H), 1653 (C=N), 1600 (C=C), 1533 (NH2 def). NMR data: δH (DMSO-d6) 6.13 (1H, 6-H), 6.15 (1H, s, 3-H), 6.75 (1H, m, 7-H), 7.15 (1H, m, 8-H), 7.50 (1H, m, 9-H), 7.40 (2H, s, NH2 deuterium exchangeable), 7.63-7.69 (5H, m, Ph). δC (DMSO-d6) 99.6 (C-3), 112.5 (C-6), 117.6 (C-9), 118.7 (C-8), 123.9 (C-7), 127.7 (C-5a), 128.5 (Ph-C3, C5), 129.3 (Ph-C2, C6), 130.8 (Ph- C4), 132.6 (Ph-C1), 144.7 (C-9a), 147.7 (C-4), 153.6 (C-2), 160.5 (C-10a). MS: m/z (%) = 260 (M+, 100), 259 (23), 220 (18), 133 (8), 116 (4), 104 (5), 90 (9), 77 (5). Anal. Calcd. for C16H12N4: C, 73.85; H, 4.61; N, 21.54. Found: C, 73.41; H, 4.58; N, 21.22

2-Amino-7,8-dimethyl-4-phenylpyrimido[1,2-a]benzimidazole (**1b**). Yield 2.50g (87%), colourless crystals (DMSO), mp 300°C; IR: ν /cm-1 3442, 3301 (NH2), 3050 (aryl C-H), 1650 (C=N), 1600 (C=C), 1529 (NH2 def). NMR data: δH (DMSO-d6) 1.95 (3H, s, 7-CH3), 2.25 (3H, s, 8-CH3), 5.87 (1H, s, 6-H), 6.10 (1H, s, 3-H), 7.27 (2H, s, NH2 deuterium exchangeable), 7.65 (1H, s, 9-H), 7.59-7.70 (5H, m, Ph). δC (DMSO-d6) 19.84 (8-CH3), 20.06 (7-CH3), 98.91 (C-3), 112.94 (C-6), 117.94 (C-9), 126.29 (C-7), 126.51 (C-8), 128.39 (Ph-C3, C5), 129.06 (Ph- C2, C6), 130.50 (Ph-C4), 132.03 (C-5a), 132.61 (Ph-C1), 143.13 (C-9a), 147.50 (C-4), 153.50 (C-2), 161.5 (C-10a). MS: m/z (%) = 288 (M+, 100), 287 (34), 273 (15), 160 (6), 144 (7), 136 (6), 128 (5). Anal. Calcd. for C18H16N4: C, 75.00; H, 5.56; N, 19.44. Found: C, 74.86; H, 5.53; N, 19.15.

E. Synthesis of alkyl pyrimido[1,2-a]benzimidazole derivatives [10]

2-amino-4-(1-methylpropyl)pyrimido[1,2-a]benzimidazole was prepared from the reaction between 4-methylhexa-2,3-dienenitrile and 2-amino-benzimidazole in N,N-dimethylformamide under reflux conditions for 4 days.

2-amino-4-(1-methylpropyl)pyrimido[1, 2-a]benzimidazole (**2a**). Yield (4.30g, 90 %), mp 258 °C, IR: ν/cm-1 3300 (NH_2), 3150 (aryl. C-H), 1640 (C=N), 1640 (C=C), 1560 (NH_2 def). NMR data: ^{1}H NMR (DMSO-d_6) δ 3.52-3.66 (7H, m, 12-H); 6.22(1H, s, 3-H); 7.12(1H, dd, 7-H); 7.30(1H, dd, 8-H); 7.26(2H, s, NH_2 deuterium exchangable); 7.56(1H, d, 9-H); 7.88(1H, d, 6-H). 13C (DMSO-d_6) δ 34.8(C-12), 94.8(C-3), 113.7(C-7),117.3(C-8), 119.1(C-6), 129.6, (C-9) 144.6(C-4), 153.7(C-5a), 155.3(C-9a), 160.6(C-10a). MS: m/z (%) = 240(M+, 100), 255 (15), 221 (18), 211 (46), 209(21), 156 (15), 133 (11), 129 (10), 106(1), 90 (22), 44 (2), 41 (11). Anal. Calcd. for $C_{14}H_{16}N_4$: C, 70.00; H, 6.67; N, 23.33. Found: C, 69.97; H, 6.60; N, 23.36.

2-amino-4-(1-ethylpropyl)-7,8-dimethylpyrimido[1,2-a]benzimidazole (**2b**). Yield (92 %) white crystals. mp 307 °C, IR: ν/cm-1 3450, 3250 (NH2), 1650 (C=N), 1610 (C=C), 1560 (NH_2 def). NMR data: ^{1}H NMR (DMSO-d_6) 2.35(3H, s, 7-CH_3); 2.35(3H, s, 8-CH_3); 3.50-3.66(m, 12-H); 6.25(2H, s, 3-H); 7.30(1H, s, 9-H), 7.70(1H, s, 6-H) 13C (DMSO-d6) δ 19.7(C-7-CH_3), 19.9(C-8-CH_3), 40.9(C-12), 96.2(C-3), 114.4(C-6), 116.4(C-9), 125.6(C-7), 128.4(C-8), 132.9(C-2),139.4(C-4), 152.0(C-5a), 153.5(C-9a), 160.6(C-10a). MS: m/z (%) = 282(M+, 100), 253 (30), 238 (18), 237 (30), 223(11), 106 (2), 91 (10), 45 (5), 41(11), 36 (13), 29 (26), 27 (13). Anal. Calcd. for C17H22N4: C, 72.34; H, 7.80; N, 19.86. Found: C, 72.21; H, 7.86; N, 20.01.

2-Amino-4-(1,3-dimethylbutyl)pyrimido[1,2-a]benzimidazole (**2c**). Yield (75 %) white crystals mp 279 °C IR: ν/cm-1 3350, 3150 (NH_2), 1650 (C=N), 1575 (C=C), 1525 (NH2 def). NMR data: ^{1}H (DMSO-d_6) δ 2.48-2.51(m, 12-H); 6.21(1H, s, 3-H); 7.13(1H, dd, 7-H); 7.22(s, NH2); 7.30(dd, 1H, 8-H), 7.54(1H, d, 9-H), 7.81(1H,d, 6-H) ^{13}C (DMSO-d_6) δ 31.7 (C-12), 94.9(C-3), 113.9(C-7), 117.7(C-8), 119.4(C-6), 127.9(C-9), 144.8(C-4), 153.8(C-5a), 156.1(C-9a), 160.9(C-10a). MS: m/z

(%) = 268(M+, 100), 267 (4), 225 (4), 223 (3), 213(13), 212 (89), 211 (39), 210 (6), 209(16), 198 (4), 197 (7), 184 (6), 172 (5), 170 (4), 156 (7), 134(7), 133 (16), 105 (6), 92 (3), 90 (8), 83 (3), 57 (4), 44(20), 43 (5), 41 (8), 40 (11), 39 (11). Anal. Calcd. for C16H20N4: C, 71.61; H, 7.51; N, 20.87. Found: C, 71.63; H, 7.45; N, 20.64.

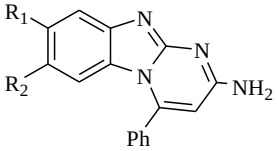
III. Results and Discussion

This series of derivatives was evaluated with the sole intention of probing its SAR profiles against clinically significant bacterial pathogens. Although the compounds had been earlier evaluated against *Staphylococcus aureus* and *Mycobacterium ranae* [10], it was not apparent whether the compounds would be active against a range of other medically important bacteria. Table 1 summarizes the results of compounds **1a, 1b, 2a, 2b** and **2c** against the test bacteria. The compounds were assessed for antibacterial activity and only compound **2b** possessed potent activities (compared to nalidixic acid) against 4 of the six gram-negative bacteria profiled (*Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa and Proteus mirabilis*). Moderate activities (in relation to streptomycin) were observed for the 5 gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Bacillus subtilis and Enterococcus faecalis*). The remaining compounds were inactive.

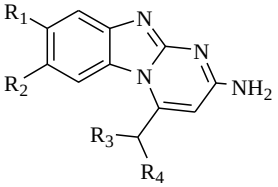
Table I: MIC OF PYRIMIDOBENZIMIDAZOLES

Bacterial strain	Zone of inhibition	**2b** MIC (µg/ml)	Streptomycin (µg/ml)	Nalidixic acid (µg/ml)
Bacillus cereus	20.2	64	32	
Bacillus subtilis	21.3	4	<4	
Enterococcus faecalis	21.0	64	128	
Enterobacter cloacae	9.4	1		16
Escherichia coli	18.3	4		>512
Klebsiella oxytoca	18.8	512		8
Klebsiella pneumoniae	18.8	64		64
Mycobacterium smegmatis	35.5	8	4	
Pseudomonas aeruginosa	11.5	2		>512
Proteus mirabilis	13.5	2		32
Staphylococcus aureus	21.5	32	8	
Staphylococcus epidermidis	22.8	16	8	

Although compounds **2a** and **2b** were initially tested against *Mycobacterium ranae* and *Staphylococcus aureus* and found to possess minor microbial activity [10], it is surprising to observe (Table I) that in this study, *S. aureus* and *M. smegmatis* were resistant to **2a**. This conflict could be clarified by re-evaluating **2a** against *M. ranae*, which we do not have in our possession. Another likelihood for the contradictory antimicrobial observations could arise from the fact that the *M. ranae* used by Asobo *et al* may have possibly been a *Mycobacterium fortuitum*, a fast growing strain having somewhat different growth patterns compared to *M. smegmatis*. Although this requires further studying, the differences in growth patterns could account for the observed conflicts in MIC's. In an in-depth study where it was shown that a vast number of fast growing Mycobacteria were initially incorrectly characterised, and in particular *M. fortuitum* was erroneously used to describe *M. ranae* [11].



1a: $R_1 = R_2 = H$
1b: $R_1 = R_2 = CH_3$



2a: $R_1 = R_2 = H$, $R_3 = Me$, $R_4 = Et$
2b: $R_1 = R_2 = Me$, $R_3 = R_4 = Et$
2c: $R_1 = R_2 = H$, $R_3 = Me$, $R_4 = i$-Bu

Fig. 1. Synthesis of 2-amino-4-phenylpyrimido[1, 2-a]benzimidazoles

IV. CONCLUSION

In an attempt to establish a platform and utility of benzimidazoles as potent antimicrobials, pyrimido[1,2-a]benzimidazole derivatives were resynthesized following published procedure and evaluated against some medically important Gram-positive and Gram-negative bacteria. The results revealed that only one compound (**2b**) with activating groups attached to the benzimidazole ring displayed strong antimicrobial activities and that alkyl and phenyl substituents on the pyrimidine ring did not significantly contribute to the bioactive properties of the test compounds. Compound **2b** may thus serve as a foundation for the synthesis of more potent antimicrobials. It would also be interesting to evaluate bioactive profiles of **1a, 1b, 2a** and **2c** encompassing a diverse group of electron donating and withdrawing groups around the benzimidazole ring as it seems this is a crucial pharmacophore.

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