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Discovery of a potent phenolic N^1-benzylidene–pyridinecarboxamidrazone selective against Gram-positive bacteria

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Abstract—As part of a study into antimycobacterial compounds a set of phenolic N^1-benzylidene–pyridinecarboxamidrazones was prepared and evaluated. This report describes the unexpected discovery of a potent compound with a pronounced selectivity for Gram-positive bacteria over Gram-negative micro-organisms. In addition, this compound is active against various drug-resistant Gram-positive bacteria.

Certain N^1-benzylidene–pyridinecarboxamidrazones are known to have antimycobacterial activity and this constitutes most of the published work for these compounds in the antimicrobial area.[1–6] Four compounds of this type were also published by Mamolo and Vio.[7] These were inactive against a panel of (non-myco)bacteria apart from one compound, a 2,4-dimethylphenyl derivative, which exhibited activity against various Gram-positive bacteria in the MIC range 5–10 μg mL^{-1}. Most of the published compounds of this type that have been examined for their antimicrobial activity contain benzylidene moieties substituted with relatively non-polar functionalities (halogen, alkyl and alkoxy) and very little indeed by way of hydrogen bond donor functionality. Therefore, in order to explore this neglected area for possible bioactivity we prepared and screened a set of phenolic N^1-benzylidene–heteroarylcarboxamidrazones.

The compounds were prepared by condensation of the appropriate aldehyde and pyridine-, pyrazine- or quinolyl-heteroarylcarboxamidrazone[6] (Scheme 1 and Table 1). The latter building blocks were prepared by the action of ethanolic hydrazine hydrate upon the corresponding cyano compounds. The benzaldehydes were all commercially available apart from those used to prepare compounds **7** and **10**. The precursor to **7**, 2-hydroxy-3,5-dimethylbenzaldehyde, was prepared from 2,4-dimethylphenol and paraformaldehyde according to the method proposed by Casiraghi et al.[8] In addition, 2-hydroxy-3,5-di-*tert*-butylbenzaldehyde was acetylated with acetic anhydride to give the precursor to **10**. The benzylidene compounds were obtained as solids which usually precipitated from the reaction mixture. These were recrystallised where appropriate. Characterisation of the key compound is given.[9]

The MIC for each compound was measured against a panel of ten clinical isolate methicillin-resistant strains of *Staphylococcus aureus* using an agar dilution method[10] (Mueller–Hinton agar) by means of a multipoint inoculator delivering 10^4 colony-forming units per spot. The MIC was defined as the lowest concentration inhibiting growth after incubation at 37 °C for 18 h. For two selected cases a second panel of Gram-positive organisms was also investigated. See Table 2 for the full set of organisms. This comprised three methicillin-sensitive *Staphylacoccus aureus* strains, two *Enterococcus faecium* strains, and seven strains of *Enterococcus faecalis* (including five clinical isolates). A selection of eight different Gram-negative bacteria was also tested, to investigate the possibility of any broad-spectrum activity. One compound was also screened against a panel of vancomycin-resistant enterococci using the multipoint inoculator approach described above. Seventeen of the compounds (**1**–**6**, **11**–**21**) were also screened against

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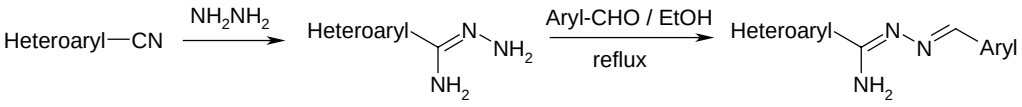
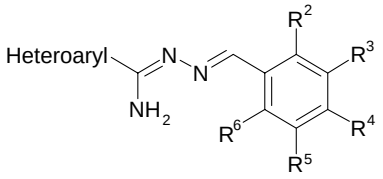


Scheme 1. Preparation of N^1-benzylidene-heteroarylcarboxamidrazones.

Table 1. Structures of the compounds examined in this study and the range of MIC values obtained from a panel of 10 MRSA strains



Compound	Heteroaryl	R^2	R^3	R^4	R^5	R^6	MRSA MIC (μg mL^{-1})	Calcd Log P[a]
1	4-Pyridyl	OH	*t*-Butyl	H	*t*-Butyl	H	2–4	5.1
2	2-Pyridyl	OH	*t*-Butyl	H	*t*-Butyl	H	X	5.5
3	3-Pyridyl	OH	*t*-Butyl	H	*t*-Butyl	H	X	5.1
4	2-Pyrazinyl	OH	*t*-Butyl	H	*t*-Butyl	H	X	4.2
5	2-Quinolyl	OH	*t*-Butyl	H	*t*-Butyl	H	X	6.9
7	4-Pyridyl	OH	CH_3	H	CH_3	H	X	2.8
8	4-Pyridyl	OH	H	H	H	H	X	1.9
9	4-Pyridyl	OH	Iodo	H	Iodo	H	10–20	4.4
10	4-Pyridyl	OCH_3	*t*-Butyl	H	*t*-Butyl	H	X	5.1
11	4-Pyridyl	$OCOCH_3$	*t*-Butyl	H	*t*-Butyl	H	4–8	4.9
12	4-Pyridyl	OH	OH	H	H	H	X	1.6
13	4-Pyridyl	H	OH	OH	H	H	X	1.6
14	4-Pyridyl	OH	OH	OH	H	H	30–40	1.3
15	4-Pyridyl	OH	H	OH	OH	H	20–30	1.3
16	4-Pyridyl	OH	H	OH	H	OH	X	1.3
17	4-Pyridyl	OH	OCH_3	H	H	H	X	1.6
18	4-Pyridyl	OH	H	OCH_3	H	H	X	1.6
19	4-Pyridyl	OH	H	H	NO_2	H	20–30	1.8
20	4-Pyridyl	OH	OCH_3	H	NO_2	H	X	1.6
21	4-Pyridyl	OH	NO_2	H	Br	H	X	2.6
22	4-Pyridyl	OH	Br	H	NO_2	H	X	2.6

X, inactive.

[a] Log P calculated using CAChe WorkSystem Pro version 6.1.10 (Fujitsu Ltd).

Mycobacterium fortuitum (NCTC 10394). Each compound was tested for a zone of inhibition on Columbia agar plates, supplemented with horse blood, against *M. fortuitum* (reference strain NCTC 10394). In addition, the compounds were screened at a single concentration of 32 μg mL^{-1} in Middlebrook 7H9 broth, supplemented with glycerol and Middlebrook ADC enrichment. If a zone of inhibition, or activity at 32 μg mL^{-1}, was observed, the MIC for that compound was measured in broth. Two compounds were screened for inhibition of *Mycobacterium tuberculosis* $H_{37}Rn$ (ATCC 27294) in BACTEC 12B medium[11] conducted by the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF), Southern Research Institute, 2000, 9th Ave., Birmingham, AL.

One compound in particular (**1**, Table 1) gave an intriguing antimicrobial activity profile and it is from that perspective that the results are presented. The compound in question contained a 4-pyridyl moiety and a phenolic hydroxyl at the 2′-position as well as two bulky lipophilic *tert*-butyl substituents. It exhibited the greatest potency against the MRSA panel of all the compounds tested. Any structural variation made to this compound proved to be detrimental to its antimicrobial activity. Thus, the simple isomeric variation of substituting the 4-pyridyl group of **1** for 2-pyridyl, 3-pyridyl (compounds **2** and **3**) resulted in loss of activity. Going further and replacing the 4-pyridyl group by pyrazinyl or 2-quinolyl (compounds **4** and **5**, respectively) similarly abolished the antimicrobial activity. Moving to the benzylidene fragment, compound **6**, an isomer of **1**, different only by virtue of the position of the hydroxyl group, was effectively inactive (MIC > 256 μg mL^{-1}). Maintaining the same substitution pattern in the benzylidene portion but reducing the size of the alkyl substituents from *tert*-butyl to methyl (compound **7**) similarly abolished the antimicrobial activity as did removal of the alkyl groups altogether (compound **8**). Some activity was regained, however, by replacement of the alkyl groups by iodine atoms, which are approximately the same size as a methyl group (compound **9**, MIC 10–20 μg mL^{-1}). Blocking the phenolic hydroxyl group by conversion to the methyl ether (compound **10**) negat-

Table 2. Expanded panel of organisms exposed to compounds **1** and **6**, and vancomycin

Organism	Reference	**1**	**6**	Vancomycin
S. aureus	NCTC 6571	2–4	>256	0.05
S. aureus	NCTC 10788	2–4	X	0.12
S. aureus	Cowan 1	2–4	X	0.25
MRSA	Isolate 1	2–4	X	0.12
MRSA	Isolate 2	2–4	X	0.12
MRSA	Isolate 3	2–4	>256	0.12
MRSA	Isolate 4	2–4	X	0.12
MRSA	Isolate 5	2–4	X	0.12
MRSA	Isolate 6	2–4	X	0.12
MRSA	Isolate 7	2–4	X	0.12
MRSA	Isolate 7	2–4	X	0.12
MRSA	Isolate 8	2–4	X	0.12
MRSA	Isolate 10	2–4	X	0.12
E. faecium	ACTCC 10541	2–4	X	0.12
E. faecium	NCTC 7171	2–4	X	0.12
E. faecalis	NCTC 5957	2–4	X	0.25
E. faecalis	Isolate 1	2–4	X	0.12
E. faecalis	Isolate 2	2–4	X	0.25
E. faecalis	Isolate 3	2–4	X	0.25
E. faecalis	Isolate 4	2–4	X	16
E. faecalis	Isolate 5	2–4	X	2
S. epidemisis	NCTC 11047	2–4	X	8
S. epidemisis	Isolate 1	2–4	X	6
S. haemolyticus	Isolate 1	2–4	X	8
E. coli	W3110 R−	X	X	>16
E. coli	W3110 R+	X	X	>16
K. pneumoniae	Strain 327	X	X	>16
S. marcesens	Strain 4444	X	X	>16
P. auriginosa	NCTC 6749	X	X	>16
S. maltophilia	NCTC 10257	X	X	>16
E. cloacae	NCTC 11582	X	X	>16
B. bronchiseptica	NCTC 8344	X	X	>16

X, inactive.

ed the activity completely, indicating that the phenolic hydroxyl is vital to antimicrobial activity. Moderate activity was observed for compounds **14**, **15**, and **19**, which lacked the alkyl substituents but contained instead a tri-hydroxy motif or a 4-nitrophenol moiety.

The dataset is too small and too polarised in terms of activity to be able to present a computational rationalisation of the biological activity. Attempts were made to construct a quantitative structure activity relationship from these data using the program TSAR 3D (Version 3.3, Oxford Molecular Ltd). The calculated properties molecular volume, surface area, ellipsoidal volume, inertia moments, dipole moment, dipole moment components, Log*P*, lipole, lipole components, molar refractivity and the Verloop substituent parameters were considered along with various connectivity indices. No meaningful equation connecting anti-MRSA activity with calculated properties could be derived. A visual inspection of the data did reveal some partial trends. If the assumption can be made that the optimum Log*P* value is that found for compound **1** (5.1), then certain conclusions may be made. For example, it can be seen from the MRSA MIC values for the isomeric compounds **2**, **3** and **6** that the optimum position for the pyridyl nitrogen is the 4-position and that the best place for the benzylidene hydroxyl is the 2′-position. Similarly, reducing the size of the alkyl substituents as in **7** (3′, 5′-dimethyl) depresses the Log*P* by 2.7 units. Removing the alkyl groups altogether as in **8** shaves a further 0.9 units from the Log*P* value. In both cases, the anti-MRSA activity vanished. The Log*P* of **8** is increased to 4.4 by the introduction of iodo substituents at the 3′- and 5′-positions as in **9**. This was rewarded by a return of some anti-MRSA activity. It cannot be ruled out at this point, however, that it was the introduction of mildly electron-withdrawing groups in place of the hydrogens or electron-releasing methyl groups which engendered the biological activity rather than the change in Log*P* value. Blocking the phenolic group of **1** as a methyl ether (**10**) or an acetate (**11**) resulted in only small changes to the Log*P* value. In the former case, the anti-MRSA activity was abolished, whereas for the latter the activity was only slightly diminished. It may be, however, that **11** was simply acting as a prodrug of **1** under the conditions of the assay and that there was hydrolysis of the acetyl group in vivo, thus regenerating the potent compound **1**. Though not conclusive, it is highly probable that a free hydroxyl in the 2′-position is essential for the activity of compound **1**. The remaining compounds (**12**–**22**) lack alkyl substituents and are much less lipophilic than **1,** falling in the Log*P* range 1.3–2.6. Moderate activity was seen for two of the polyhydroxylated compounds, **14** and **15**. It may be that these two compounds acted through a different mechanism, perhaps through a catechol-based chelation of essential metal ions, though that cannot be confirmed at this time. Moderate activity was also

observed when the inactive phenolic compound **8** was converted to **19** by addition of a nitro group para to the phenolic OH. Bearing in mind the difference of 3.6 Log *P* units between **19** and the most active compound **1** it is probable that the activity observed for **19** is via a different mechanism. The presence of the strongly electron-withdrawing nitro group para to the hydroxyl increases the acidity of the phenol. It is not proved, however, that this is essential for the observed activity since the same can be said for the inactive compounds **20**–**22** where the nitro group is either ortho or para to the hydroxyl.

Compounds **1** and **6** were tested against an expanded panel of organisms. This comprised 24 Gram-positive and 8 Gram-negative organisms. The results are presented in Table 2. Compound **6** was essentially inactive across this range of organisms. In contrast, compound **1** exhibited good activity against all of the Gram-positive organisms but was inactive against all of the Gram-negative strains. The structures of the two bacterial cell walls are very different. Gram-positive bacteria, such as staphylococci and enterococci, have a single, very thick cell wall, consisting largely of peptidoglycan. Gram-negative bacteria, such as *Eschericha coli*, have a very thin inner membrane consisting of only 1–5% peptidoglycan, surrounded by an outer membrane consisting of a large amount of lipoproteins and lipopolysaccharides. It is this difference in structure that may account for the selectivity of **1** between the two bacterial types, particularly if the mode of action of this compound is associated with peptidoglycan or its synthesis.

It is tempting to speculate that the antimicrobial mechanism of action of compound **1** might be connected with its potential metal-chelating properties. The data, however, are inconsistent on this point. Compound **1** has a 4-pyridyl ring. Substitution by 2-pyridyl or 3-pyridyl abolishes the antimicrobial activity. This suggests that the if the mechanism of action is anything to do with metal chelation then it does not involve the heteroaryl ring. This is owing to the fact that only the 2-pyridyl case has a 1,4-disposition of nitrogen atoms suitable for the formation of a five-membered chelate with a metal ion involving the pyridyl nitrogen. This has been exemplified in a related case for copper (II).[12] Using the remainder of the structure it is possible to form a six-membered chelate using the motif of the ortho-hydroxyl of the benzylidene substituent of compound **1** and the adjacent chain nitrogen.[13] If this is the source of the antimicrobial activity for **1**, then it is very surprising that no activity is seen for **2** and **3**, which are simply isomeric in the pyridyl part. Similarly, there are a further eleven compounds in Table 1 which contain the same 1,5-O,N arrangement but which show no antimicrobial activity.

Since **1** was active against all strains of MRSA as well as all other Gram-positive bacteria, it was tested further against some vancomycin-resistant enterococci. It can be seen from Table 3 that the high level of activity was retained. Mycobacteria do not fit perfectly into the pigeonholes of Gram-positive and Gram-negative. Thus, in order to investigate activity against this class of organisms, 17 of the compounds (**1**–**6**, **11**–**21**) were also screened against *M. fortuitum* (NCTC 10394). It was noted that a compound, which produced a larger zone of inhibition, did not necessarily give a higher MIC reading. This suggested that different compounds permeated the agar to different extents. Since compounds of high lipophilicity may not permeate through the hydrophilic agar, testing of substances in broth at a single concentration of 32 μg mL^{-1}, was also carried out. If a zone of inhibition, or activity at 32 μg mL^{-1} was observed, the MIC for that compound was measured in broth. Compounds **1** and **6** were screened against *M. tuberculosis* H_{37}Rv. All members of this subset of compounds were inactive against *M. fortutium*. Compound **6** exhibited weak activity against *M. tuberculosis* (75% inhibition at 6.25 μg mL^{-1}). The isomeric compound **1**, however, was far more active (100% inhibition at 6.25 μg mL^{-1} and a MIC of 6.25 μg mL^{-1}.)

Table 3. MIC values for compound **1** and vancomycin against a panel of vancomycin-resistant enterococci

Organism	**1** MIC (μg mL^{-1})	Vancomycin MIC (μg mL^{-1})
3001562	2–4	4–8
3002043	2–4	4–8
3002066	2–4	4–8
3005323	2–4	>16
3005426	2–4	4–8
3005353	2–4	>16
3102095	2–4	>16

In light of the promising activity of compound **1** against *M. tuberculosis* and all the Gram-positive bacterial tested, including drug-resistant strains, this compound and its synthetic precursors, pyridine-4-carboxamidrazone and 3,5-di-*tert*-butyl-2-hydroxybenzaldehyde, were investigated for their toxicity towards human white blood cells following procedures previously published for related compounds.[14–16] The anti-tuberculosis drug isoniazid was included for comparison. The in vitro assay used human mononuclear leucocytes (MNL, white blood cells) which were incubated with the test compound for 1 h and then subsequently incubated in toxin-free media for 18 h; cell death was determined by trypan blue exclusion. All test compounds were incubated at a concentration of 1 mM apart from compound **1**, which was limited to 0.5 mM owing to its lesser solubility. The results are shown in Table 4. Compound **1** was found to be very toxic to leucocytes, causing lysis of the cells during the course of the experiment. Compound **8**, which lacks the two *tert*-butyl groups, was only mildly

Table 4. Results of direct leucocyte toxicity testing

Compound	Concn (mM)	% leucocyte death
DMSO (control)	—	7.4 ± 1.1
Isoniazid	1	12.4 ± 2.8
Pyridine-4-carboxamidrazone	1	13.8 ± 0.4
3,5-Di-*tert*-butyl-2-hydroxybenzaldehyde	1	32.7 ± 3.2
1	0.5	100
8	1	11.7 ± 1.2

toxic in comparison, indicating that the alkyl groups may be implicated in the toxicity of **1**.

In conclusion, sporadic antimicrobial activity was encountered in the set of phenolic N^1-benzylidene–heteroarylcarboxamidrazones prepared and tested. The most active member was compound **1**. This proved to be consistently more potent across a panel of Gram-positive bacteria, including drug-resistant strains, than any of its structural isomers or very close structural analogues. Compound **1** was also active against *M. tuberculosis* but inactive against Gram-negative bacteria. Its mechanism of action is not clear at this point and the possibility that it is via metal chelation cannot be substantiated by the data presented. Compound **1** was also found to be a highly cytotoxic compound, which prevented any further study of the compound as a potential antimicrobial drug. It has, nevertheless, provided the basis for ongoing studies which are focussed upon the preparation of potent antimicrobial compounds but with better therapeutic ratios.

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9. Characterisation of compound **1**, N^1-[3,5-di-(*tert*-butyl)-2-hydroxybenzylidene]-pyridine-2-carboxamidrazone. Recrystallised from methanol/40–60 petroleum ether to give a yellow solid, 68% yield. R_f [EtOAc]: 0.48 (single spot). ^{1}H NMR (DMSO-d_6 ; 250 MHz): 1.27 (s, 9H, CMe_3), 1.42 (s, 9H, CMe_3), 7.18 (br s, 2H, NH_2), 7.30 (d, 1H, J = 2.4 Hz, 4′H), 7.34 (d, 1H, J = 2.3 Hz, 6′H), 7.87 (d, 2H, J = 6.1 Hz, Pyr-H3 and H5), 8.67–8.69 (overlapping m, 3H, =CHAr and Pyr-H2 and H6), 11.60 (br s, 1H, OH) ppm. ^{13}C NMR ($CDCl_3$-d_6): 29.4 (CMe_3), 31.4 (CMe_3), 34.1 (CMe_3), 35.0 (CMe_3), 117.4 (C1′), 120.6 (C3 and C5), 126.5 (C4′), 127.2 (C6′), 136.3 (C3′), 141.2 (C4), 150.3 (C2 and C6), 154.3 (C5′), 156.1 (C2′), 162.4 (C8) ppm. IR (KBr disc): 3468 (ν_{as} NH_2), 3282 (ν_s NH_2), 3250–3000 (ν OH, overlapping ν Ar-CH), 2954 (ν satd CH), 2865 (ν satd CH), 1633 (ν C=N), 1610 (ν skeletal Ar or Pyr), 1595 (ν skeletal Ar or Pyr), 1534 (ν skeletal Pyr), 1463 (ν skeletal Ar or Pyr), 1436 (ν skeletal Ar or Pyr), 1374, 1247, 1178, 1070 (ν C–N), 997, 968, 877, 818 (γ CH, 4-Pyr), 746 (β ring, 4-Pyr), 713, 642 cm^{-1}. APCI-MS (+ve) *m/z*: 353 $(M+H)^+$. Mp corrected: 156.7–158.0 °C. CHN Analysis, % m/m (% calculated/% found): C, 71.56/71.70; H, 8.01/7.96; N, 15.89/16.01.
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