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Structure-activity relationships of pyrazole-4-carbodithioates as antibacterials against methicillin-resistant Staphylococcus aureus

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Structure-Activity Relationships of Pyrazole-4-carbodithioates as Antibacterials against Methicillin–Resistant *Staphylococcus aureus*

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Keywords: antibacterial, *Staphylococcus aureus*, MRSA, pyrazole-4-carbodithioate, MgrA

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of serious hospital-acquired infections and is responsible for significant morbidity and mortality in residential care facilities. New agents against MRSA are needed to combat rising resistance to current antibiotics. We recently reported 5-hydroxy-3-methyl-1-phenyl-1*H*-pyrazole-4-carbodithioate (HMPC) as a new bacteriostatic agent against MRSA that appears to act via a novel mechanism. Here, twenty nine analogs of HMPC were synthesized, their anti-MRSA structure-activity relationships evaluated and selectivity versus human HKC-8 cells determined. Minimum inhibitory concentrations (MIC) ranged from 0.5–64 μg/mL and up to 16-fold selectivity was achieved. The 4-carbodithioate function was found to be essential for activity but non-specific reactivity was ruled out as a contributor to antibacterial action. The study supports further work aimed at elucidating the molecular targets of this interesting new class of anti-MRSA agents.

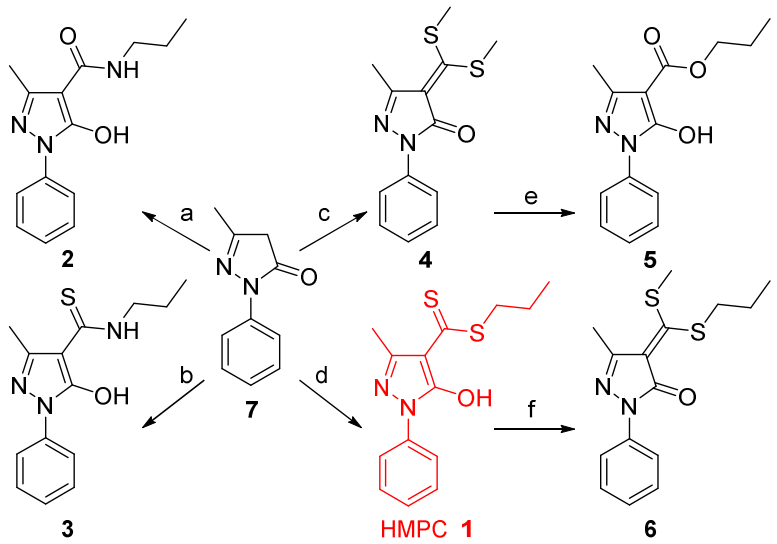
Methicillin-resistant Staphylococcus aureus (MRSA) is the most common cause of hospital-acquired infections[1,2] and a frequent source of skin and soft tissue infections in the North and Latin Americas, Europe and Asia.[3,4] In the USA, MRSA accounts for almost 60% of clinical *S. aureus* strains isolated from intensive care units[5] and it is widespread in residential care facilities.[6,7] Worryingly, pathogenic strains are also becoming more prevalent in the community (i.e. community-acquired MRSA).[8] Vancomycin has been used extensively for several decades to treat complicated *S. aureus* and other Gram-positive infections. However, concerns have been growing over the increasing minimum inhibitory concentration (MIC) of the drug against MRSA isolates,[9] and its use has other well-known shortcomings (e.g. nephrotoxicity, complex pharmacokinetics, requirement for slow intravenous infusion).[10]

It is evident that vancomycin's effectiveness will continue to wane in the coming years, thus compelling the discovery of effective new antibiotics against this major human pathogen.

We recently reported the discovery of 5-hydroxy-3-methyl-1-phenyl-1*H*-pyrazole-4-carbodithioate (HMPC) **1** (Scheme 1) as a new bacteriostatic agent against MRSA.[11] The compound showed the same minimum inhibitory concentration (MIC 4 μg/mL) against MRSA MW2 (lab strain) and six recent clinical isolates and was able to rescue *Caenorhabditis elegans* from an MRSA infection. Whole-genome sequencing of mutants resistant to **1** highlighted a role for the global defense regulator MgrA[12] in its mechanism and the compound displayed a *S. aureus* promoter-*lux* array luminescence profile distinct from all major classes of antibiotics. HMPC **1** appears to exert anti-staphylococcal effects through a novel, uncharacterized mechanism that involves an MgrA-mediated defense response. In the current report, we explored the structural requirements for anti-MRSA activity and eukaryotic cell selectivity in the pyrazole-4-carbodithioate class.

Initial efforts sought to understand the role of the 4-carbodithioate by replacing the group with amide **2**, thioamide **3** and ester **5** isosteres (Scheme 1). Analogs **2** and **3** were formed by quenching the enolate of pyrazolin-3-one **7** (generated using K_2CO_3) with n-propyl isocyanate and *n*-propyl isothiocyanate, respectively. Ester **5** was obtained by first generating ketene dithioacetal **4** from **7** using K_2CO_3, CS_2 and excess CH_3I via the reported method.[13] Subsequent reaction of **4** with sodium in n-PrOH at 80 °C delivered **5**. Compounds **2**, **3**, **5** and intermediate **4** all showed no activity against MRSA MW2 (MIC > 64 µg/mL), establishing the critical importance of the 4-carbodithioate function.

The role of the neighboring 3-OH substituent was investigated next but attempting to prepare the O-Me ether of **1** by treatment with K_2CO_3 and excess CH_3I instead delivered ketene dithioacetal **6** (44% yield). Compound **6** was also found to be inactive against MRSA (MIC > 128 µg/mL).



Scheme 1. Reagents and conditions: a. K_2CO_3, n-propyl isocyanate, DMF/benzene, rt, 56%; b. K_2CO_3, *n*-propyl isothiocyanate, DMF/benzene, 50 °C, 80% c. K_2CO_3, CS_2, CH_3I, DMF/benzene, 0 °C, 66%; d. *n*-BuLi, CS_2, *n*-bromopropane, THF, 0-25 °C, 52%,[14] e. $Na_{(s)}$, *n*-PrOH, 80 °C, 91%;[15] f. K_2CO_3, CH_3I, DMF 0-25 °C, 90%.

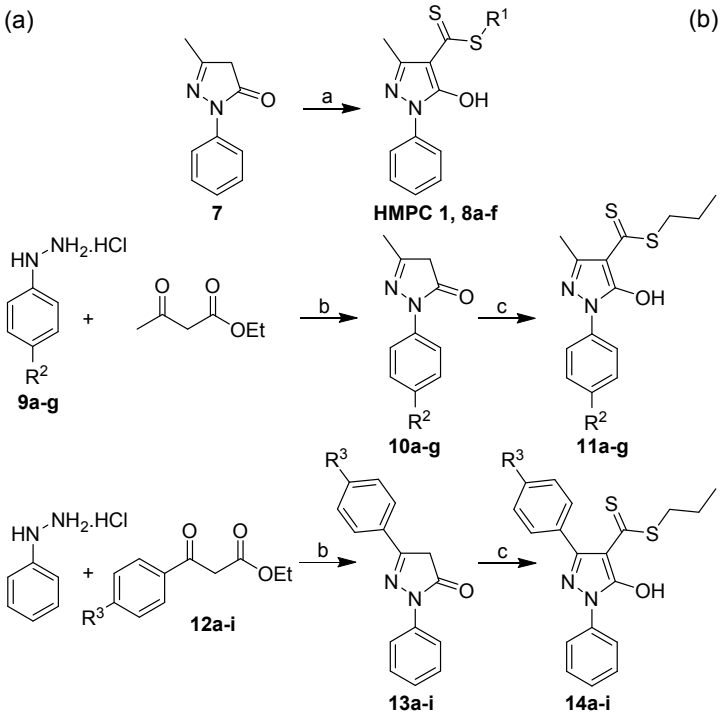
Replacement of the carbodithioate n-propyl chain with Me, Et, allyl, n-Bu, Bn and 3-pyridyl groups was explored next. Each of these was obtained by forming the enolate of 7 with n-butyllithium and successively quenching with CS_2 and the appropriate alkyl halide (Figure 1(a)). Shortening the chain to one carbon **8a** led to a 4-fold drop in potency, while Et derivative **8b** produced a 2-fold loss. Introduction of an alkene **8c** maintained or slightly reduced potency and extension by 1 carbon **8d** increased potency 2-fold. Addition of steric bulk and hydrophobicity with a benzyl group **8e** resulted in a 2-fold increase in activity, but a significant drop in potency occurred when a nitrogen atom was introduced into the benzylic substituent **8f** (MIC 16-32 mg/mL, Figure 1(b)).

In our previous report,[11] we showed that HMPC **1** does not cause hemolysis of human red blood cells at concentrations up to 64 μg/mL but is cytotoxic towards eukaryotic HKC-8 and HepG2 cells at concentrations around its MRSA MIC. Here, cytotoxicity of analogs **8a-e** was tested in HKC-8 cells and selectivity indices were calculated (SI = HKC-8 IC_{50}/MRSA-MW2 MIC, Figure 1(b)). No MRSA selectivity was observed for **8a**, **8b** or **8d** and modest selectivity (2-4 fold) was seen with allyl derivative **8c**. Benzylic derivative **8e** delivered the highest selectivity (8-16 fold) in this series.

A variety of halo, electron donating and electron withdrawing substituents were added to the 4-position of the pyrazole *N*-phenyl group. Commercially available 4-substituted phenylhydrazine.HCl salts **9a-g** were condensed with ethyl acetoacetate to form pyrazol-3-one intermediates **10a-g** in 25-87% yield. Treating ketones **10a-g** with n-butyllithium and quenching the enolates with CS_2 followed by n-bromopropane gave targets **11a-g** in 32-75% yield (Figure 1(a)).

Addition of a Me group **11a** gave no change in activity (relative to **1**) while introducing an electron donating methoxy group **11b** produced a 2-fold loss. Halogen

substituents **11c-e** gave slight increases in potency (2-4 fold), as did electron withdrawing cyano and nitro groups, with the *p*-NO_2-substituted analog **11g** showing the highest activity (MIC 0.5-1 μg/mL). Corresponding increases in eukaryotic cell cytotoxicity were observed though, with **11g** showing no MRSA selectivity (Figure 1(b)).



(b)

	R^1	R^2	R^3	MIC μg/mL	IC_{50} μg/mL	SI
1	n-Pr			4	4	1
8a	Me			16	16	1
8b	Et			8	8	1
8c	allyl			4-8	16-32	2-4
8d	n-Bu			2-4	4	1-2
8e	Bn			2-4	32	8-16
8f	3-pyridyl			16-32		
11a		Me		4		
11b		OMe		8		
11c		F		2-4		
11d		Cl		2-4		
11e		Br		1-2		
11f		CN		2		
11g		NO_2		0.5-1	0.5-1	1
14a			H	2	32	16
14b			Me	1-2	8	4-8
14c			OMe	2	8	4
14d			F	1	4	4
14e			Cl	0.5-1	4	4-8
14f			Br	0.5		
14g			CF_3	32-64		
14h			CN	2		
14i			NO_2	1	0.5	0.5

Figure 1. (a) Synthesis of pyrazole-4-carbodithioate analogs. Reagents and conditions: a. *n*-BuLi, CS_2, alkyl halide, THF, 0-25 °C, 59-84%; b. glacial acetic acid, EtOH, 45-50 °C, 19-90%; c. *n*-BuLi, CS_2, *n*-bromopropane, THF, 0-25 °C. (b) Antibacterial activities (MIC μg/mL) against MRSA-MW2, cytotoxicity towards HKC-8 cells (IC_{50} μg/mL) and selectivity indices (SI).

Replacement of the 3-methyl group of **1** with a phenyl ring was explored next. Condensation of phenylhydrazine.HCl with ethyl benzoylacetate **12a** produced pyrazol-3-one **13a**, which upon base treatment and successive quenching with CS_2 and n-bromopropane afforded 3-phenyl derivative **14a** in 90% yield. Compound **14a** showed a 2-fold increase in

MRSA potency and a 16-fold increase in selectivity. The promising selectivity obtained upon addition of the phenyl ring at the 3-position led to exploration of *para*-substituted analogs carrying halo, electron donating and electron withdrawing substituents. Condensation of *p*-substituted ethylbenzoylacetates **12b-i** with phenylhydrazine.HCl and appending n-propyl dithioate groups to the resulting ketones **13b-i** delivered target analogs **14b-i**. Addition of the Me group **14b** led to a slight increase in potency (MIC 1-2 μg/mL) relative to **1** but reduced selectivity. The methoxy group **14c** did not change activity against MRSA but selectivity was reduced. Larger increases in antibacterial potency were achieved with halo groups **14d-f** (MIC 0.5-1 μg/mL) but no improvements in selectivity were seen. A large drop in potency was observed with the CF_3 group **14g** (MIC 32-64 mg/mL), while other strongly electron withdrawing cyano **14h** and nitro **14i** substituents maintained activity but reduced selectivity.

We previously showed that treatment of a *S. aureus* promoter-lux array with HMPC **1** produces a unique luminescence profile (suggesting a unique mechanism of action), but some similarities to DNA-damaging agents and/or DNA replication inhibitors were noted.[11] This led to speculation that the anti-MRSA and apparent general cytotoxicity of **1** might arise from DNA binding. However, UV/vis experiments measuring the binding of **1** to calf thymus DNA and zone of growth inhibition disk measurements performed with **1** in the presence/absence of calf thymus or *S. aureus* genomic DNA appeared to rule this out. Nevertheless, the similar levels of HKC-8 toxicity observed in the current study with the majority of analogs of **1**, combined with the absolute requirement of a carbodithioate function for anti-MRSA activity, led us to examine more closely whether **1** (and hence the class) may exert effects through non-specific nucleic acid and/or protein reactivity.

The DNA-reactivity of **1** was probed by electrospray ionization mass spectrometry (ESI-MS) using a panel of single stranded, double stranded and G-quadruplex DNA oligonucleotides. When incubated with up to 10-fold excesses of HMPC **1** under a variety of

conditions, no evidence for any DNA:**1** adducts was observed (Supporting Information Figure S1). Similarly, no adducts were observed by ESI-MS when **1** was incubated with RNA oligonucleotides (data not shown).

The effects of HMPC **1** on DNA replication *in vitro* were explored next. In this assay, all of the enzymes, ancillary proteins, nucleotide precursors, DNA template and other molecular components required for duplication of circular bacterial DNA are present and able to effect replication in a cell-free environment.[16] Covalent reactivity with any of the reaction components would be expected to read out as inhibition of replication. However, only slight inhibition of replication was observed with **1** at concentrations > 160 μM, well above its MIC against MRSA (Supporting Information Figure S2). *In vitro* RNA transcription assays similarly showed no inhibition by **1** at relevant concentrations (data not shown). The absence of effects for **1** in these assays rules out non-specific nucleic acid or protein reactivity as a contributor to the anti-MRSA mechanism of the pyrazole-4-carbodithioate class.

In summary, pyrazole-4-carbodithioates are a new class of anti-MRSA agents that require the 4-carbodithioate function for activity. Non-specific covalent reactivity appears not to be part of the mechanism but intrinsic reactivity of the 4-carbodithioate may still play a role. We showed previously that MgrA-mediated defense responses are triggered by **1**.[11] MgrA is an oxidation-sensing mechanism used by MRSA to counter challenges of reactive oxygen and nitrogen species. Upon detecting these species, a unique cysteine residue (Cys12) located at the dimer interface of the protein is oxidized to cysteine sulfenic acid, causing dissociation of MgrA from DNA and initiation of signalling pathways that turn on antibiotic resistance.[17] We speculate that intracellular redox reactivity of the 4-carbodithioate function (aided by the neighboring OH group) triggers oxidative stress that leads to MgrA activation. Alternatively, metal chelation by the 4-carbodithioate and neighboring OH group may be involved.[18] While limited selectivity (maximum 16-fold) for MRSA over eukaryotic HKC-8

cells was achieved with the analogs explored here, further increases seem possible with a larger analog set. Studies to fully elucidate the anti-MRSA mechanism and identify the discrete intracellular targets using such selective analogs would undoubtedly prove insightful.

Author Contributions

H.M completed all synthetic chemistry. T.J completed all MRSA MIC and HKC-8 cytotoxicity measurements. Mass spectrometry studies were performed by C.K and replication assays by E.M and S.J. N.D, E.M and M.K directed the project. All authors contributed to writing the manuscript.

Notes

The authors declare no competing financial interests.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at XXXXX

References and notes

1. Kavanagh, K. T.; Abusalem, S. ; Calderon, L. E. The incidence of MRSA infections in the United States: is a more comprehensive tracking system needed? *Antimicrob. Resist. Infect. Control* 2017, 6; 34.
2. Casey, A. L.; Lambert, P. A.; Elliott, T. S. J. Staphylococci. *Int. J. Antimicrob. Agents.* 2007, 29 Suppl. 3, S23.
3. Moet, G. J.; Jones, R. N.; Biedenbach, D. J.; Stilwell, M. G.; Fritsche, T. R. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: Report from the SENTRY Antimicrobial Surveillance Program (1998–2004). *Diagn. Microbiol. Infect. Dis.* 2007, 57, 7.
4. Jean, S-S.; Hsueh, P-R. High burden of antimicrobial resistance in Asia. *Int. J. Antimicrob. Agents* 2011, 37, 291.
5. Noskin, G. A.; Rubin, R. J.; Schentag, J. J.; Kluytmans, J.; Hedblom, E. C.; Smulders, M.; Lapetina, E.; Gemmen, E. The burden of *Staphylococcus aureus* infections on hospitals in the United States. An analysis of the 2000 and 2001 nationwide inpatient sample database. *Arch. Intern. Med.* 2005, 165, 1756.
6. Kwok, K. O.; Read, J. M.; Tang, A.; Chen, H.; Riley, S.; Kam, K. M. A systematic review of transmission dynamic studies of methicillin-resistant *Staphylococcus aureus* in non-hospital residential facilities. *BMC Infect. Dis.* 2018, 18:188.
7. Dulon, M.; Haamann , F.; Peters , C.; Schablon, A.; Nienhaus, A. MRSA prevalence in european healthcare settings: a review. *BMC Infect. Dis.* 2011, 11:138.
8. Miller, L. G.; Diep, B. A. Colonization, fomites, and virulence: Rethinking the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.* 2008, 46, 752.

9. Sakoulas, G.; Moellering Jr, R. C. Increasing antibiotic resistance among methicillin resistant *Staphylococcus aureus* strains. *Clin. Infect. Dis.* 2008, 46 (Suppl 5), S360.

10. Savoldi, A.; Azzini, A. M.; Baur, D.; Tacconelli, E. Is there still a role for vancomycin in skin and soft-tissue infections? *Curr. Opin. Infect. Dis.* 2018, 31, 120.

11. Johnston, T.; Van Tyne, D.; Chen, R. F.; Fawzi, N. L.; Kwon, B.; Kelso, M. J.; Gilmore, M. S.; Mylonakis, E. Propyl-5-hydroxy-3-methyl-1-phenyl-1*H*-pyrazole-4-carbodithioate (HMPC): a new bacteriostatic agent against methicillin—resistant *Staphylococcus aureus. Sci. Rep.* 2018, 8: 7062, 1.

12. Sun, F.; Zhou, Lu.; Zhao, B-C.; Deng, X.; Cho, H.; Yi, C.; Jian, X.; Song, C-X.; Luan, C-H.; Bae, T.; Li, Z.; He, C. Targeting MgrA-Mediated Virulence Regulation in *Staphylococcus aureus*. *Chem. Biol.* 2011, 18, 1032.

13. Chauhan, S. M. S.; Junjappa, H. Ketene-S,S-acetals-V : The reactions of α-keto and α-cyanoketene-S,S-acetals with guanidine and thiourea: a new general synthesis of alkoxy-pyrimidines. *Tetrahedron* 1976, 32, 1779.

14. Maurelia, R.; León, G.; Oliva, A. The synthesis of 4-alkyldithioate-5-hydroxy-3-methyl-1-phenylpyrazoles. *Synth. Commun.* 1990, *20*, 477.

15. Tewari, A. K.; Srivastava, P.; Singh, V. P.; Singh, A.; Goel, R. K.; Mohan, C. G. Novel anti-inflammatory agents based on pyrazole based dimeric compounds; Design, synthesis, docking and in vivo activity. *Chem. Pharm. Bull.* 2010, *58*, 634.

16. Yin, Z.; Wang, Y.; Whittell, L. R.; Jergic, S.; Liu, M.; Harry, E. J.; Dixon, N. E.; Kelso, M. J.; Beck, J. L., Oakley, A. J. DNA Replication is the Target for the Antibacterial Effects of Non-Steroidal Anti-Inflammatory Drugs. *Chem. Biol.* 2014, 21, 481 – 487.

17. Chen, P. R.; Bae, T.; Williams, W. A.; Duguid, E. M.; Rice, P. A.; Schneewind, O.; He, C. An oxidation-sensing mechanism is used by the global regulator MgrA in *Staphylococcus aureus*. *Nat. Chem. Biol.* 2006, 2, 591.

18. Lopez, R.; Maurelia, R.; Leon, G.; Oliva, A. Uranium (VI) and copper (II) complexes of carboxylate, thiocarboxylate and dithiocarboxylate derivatives of 1-phenyl-3-methyl-2-pyrazolin-5-one. *Synth. React. Inorg. Met.-Org. Chem.* 1995, 25, 1155.