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Antimicrobial Activity of Non-natural Prodigiosenes

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E. Marchal,[a] Md. I. Uddin,[a] D. A. Smithen,[a] C. L.A. Hawco,[a] M. Lanteigne,[b] D. P. Overy,[b] R. G. Kerr,[b,c] and A. Thompson*[a]

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[a]Department of Chemistry, Dalhousie University, Halifax, NS, B3H 4R2, Canada; [b]Department of Chemistry, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI, C1A 4P3, Canada; [c]Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PEI, C1A 4P3, Canada

*Tel: (+1)902-494-3305; Fax: (+1)902-494-1310 ; E-mail: Alison.Thompson@dal.ca

Tripyrrolic prodigiosenes, derivatives of the natural product prodigiosin, have been produced via multi-step synthesis beginning with 2-formyl pyrroles bearing various functionalities at the 4-position. Two tin complexes are also reported, and these feature a prodigiosene ligand bearing a conjugated benzyl-ester. Antimicrobial activities of prodigiosenes are evaluated against Gram positive and Gram negative bacterial strains, as well as a yeast.

With the discovery of original classes of antibiotics being extremely rare over the last four decades,[1] our modern search for antibiotics to fight resistant and multi-resistant strains has never been more important. Indeed the majority of known antibacterial classes were discovered between the 1940's and 1960's and only five new classes have been introduced for human use since 1989.[2-4] As such, most contemporary antibiotics are structural variations of established antibacterial classes, or sub-classes of antibacterial drugs: for example carbapenems, cephalosporins and monobactams are subclasses of the beta-lactam/penicillin class.[5, 6] Resistant and multi-resistant organisms[7, 8] arise from the pressure exerted by the use of antibiotics and are at the origin of nosocomial and community-acquired infection.[9] Methicillin-resistant *Staphylococcus aureus* (MRSA),[10] vancomycin-resistant enterococci (VRE)[11] and penicillin-resistant *Streptococcus pneumoniae* (PRSP)[12] are the most importunate, and efforts have focused on finding new treatments or combination therapies against these pathogens.[13, 14] However, with the release of new antibiotic drugs in decline,[3] the quest to find authentic antibacterial classes is unwaveringly critical. Nature provides the best source for antibiotics: indeed, of the 90 new antibacterial drugs approved by the FDA between 1981 and 2002, only 19 were totally synthetic drugs – the others were natural compounds or modified natural compounds.[15] Continuing in this vein, we herein report our work investigating the antimicrobial properties of analogues of prodigiosin, a tripyrrolic natural product.

Prodigiosin (**1**) is a naturally occurring red pigment[16, 17] produced by certain strains of *Serratia marcescens* (Fig. 1).[18] Despite the antibacterial,[16, 19-25] antiprotozoal[26-28] and antifungal[29] properties of the natural product, its toxicity prevents its use as a clinical antibiotic.[30] To our knowledge synthetic analogues of prodigiosin, termed prodigiosenes,[31] have not been evaluated for their antimicrobial activities despite the fact that derivatives have been assessed for a variety of other biological activities including immunosuppressive,[32] antimalarial[33] and anti-cancer[34-38] effects. Given the antimicrobial activity of the natural product,[16, 19-25] we believe that derivatives based on this molecular scaffold have potential in the discovery of new drugs with unique antibiotic properties. To begin assessing the utility of synthetic prodigiosenes as potential antimicrobial agents, we prepared a series of analogues modified at the C-ring.[37-39] We then screened their anti-microbial properties against a panel of Gram-positive and Gram-negative bacteria, as well as the yeast Candida albicans *(C. albicans)* to assess the breadth of their activity. The results are reported against clinical antimicrobial agents and the natural product prodigiosin,[40] all used as controls.

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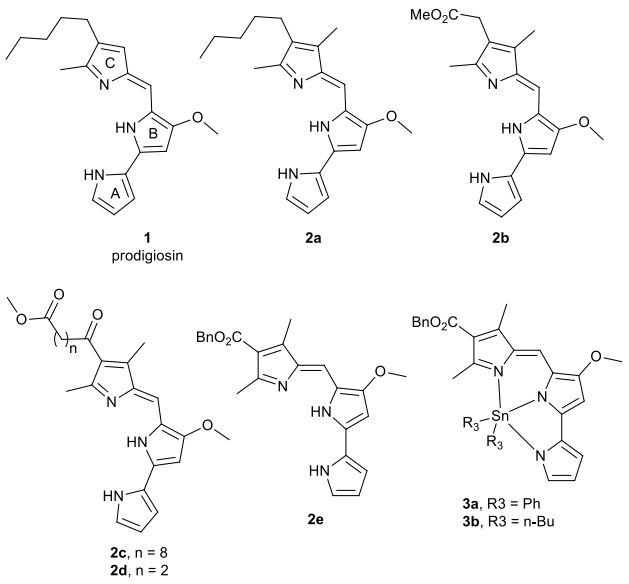
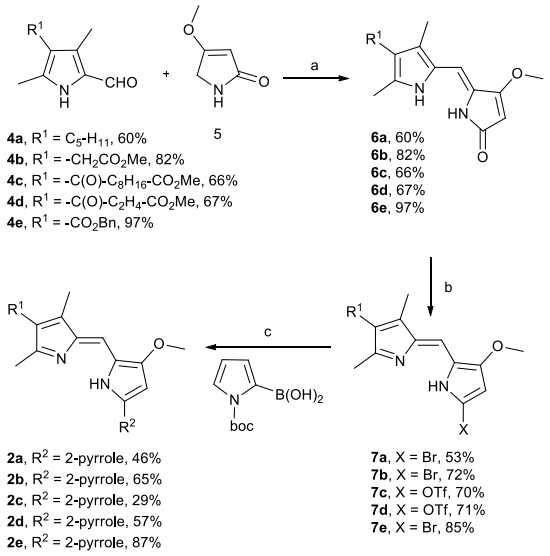


Fig. 1 Natural product prodigiosin (**1**), synthetic prodigiosenes (**2**) and prodigiosene tin complexes (**3**).

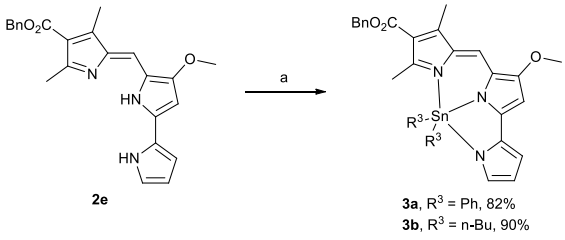
Cognizant of the various circuitous routes by which total syntheses of prodigiosin have been achieved,[41-44] our strategy originated by incorporating design features to facilitate the preparation of a range of analogues via intermediates that are both stable and easily accessible. In this regard, our synthetic prodigiosenes all bear an extra methyl group at the C-ring *cf.* the natural product, allowing an easier and shorter synthesis (Fig. 1) without significant impact upon the core structure.[45] This feature enables the incorporation of Knorr-type pyrroles into the retrosynthetic approach; compounds that are extremely facile to prepare on a large scale and through which a wide range of functionality, steric effects and electronic characteristics may be introduced. Furthermore, the integration of a conjugated carbonyl group increases the stability of intermediates throughout the synthetic route and opens the door to accessing a wide range of functionalized C-ring analogues.[37-39, 46]

For this study, prodigiosenes were selected so as to survey the influence of substituents upon the corresponding antimicrobial activity. Thus, prodigiosene **2a** differs from the natural compound **1** by just an extra methyl group at the C-ring. Compound **2b** features a pendant ethanoate instead of the pentyl alkyl chain of the natural product. The influence of conjugated stabilizing groups at the C-ring upon antimicrobial activity was evaluated with **2c-e**. Finally, in order to map the importance of the N-H groups of pyrroles, two tin complexes (**3a**, **b**) of the ester **2e** were also evaluated for their antimicrobial activity, particularly given their improved toxicity profile compared to the natural product **1** (acute systemic toxicity in mice of 4 mg/kg for prodigiosin **1**, yet 200 mg/kg for **3a** and 400 mg/kg for **3b**).[47]

Prodigiosenes **2a-e** were prepared following literature routes that enabled isolation of the five prodigiosenes via a common strategy (Scheme 1).[37, 39, 45, 48] All syntheses began with the preparation of a 2-formyl pyrrole (**4**), with the appropriate functionality pre-installed at the 4-position. Condensation of each 2-formyl pyrrole (**4)** and 4-methoxy-3-pyrrolin-2-one (**5**) provided the desired dipyrrinones (**6**). After triflation or bromination to generate an activated dipyrrin, a Pd-catalyzed Suzuki-Miyaura coupling reaction using 1-*N*-Boc-pyrrole-2-boronic acid rendered the expected prodigiosenes **2a-e**. Tin complexation was achieved by reacting methanolic solutions of the prodigiosene **2e** with either diphenyltin oxide or dibutyltin oxide at reflux temperature, furnishing the prodigiosenes complexes **3a** and **3b** in good yields (Scheme 2).[47] Complexation with tin was chosen as tin-prodigiosene adducts have been demonstrated to exhibit considerably improved toxicology profiles over their parent ligands.[47]



Scheme 1 Synthesis of prodigiosenes **2**: a) TMSOTf, Et_3N, DCM, then HCl, or KOH, THF, H_2O, 60 °C, then MeOH, H_2SO_4; b) Tf_2O, DCM, 0 °C, or $POBr_3$, DCM, R.T.; c) $Pd(PPh_3)_4$, LiCl, Na_2CO_3, DME, 85 °C.



Scheme 2 Preparation of tin complexes of prodigiosenes: a) Ph_2SnO or n-Bu_2SnO, MeOH, 65 °C, 18 h.

Using microbroth antibacterial assays prodigiosin (**1**), prodigiosenes **2a-e** and the prodigiosene complexes **3a,b** were screened for their ability to inhibit the growth of three Gram-positive bacteria [methicillin-resistant *Staphylococcus aureus* (*MRSA), Staphylococcus warneri (S. warneri),* vancomycin-resistant *Entrococcus faecium (VRE)*, Fig. 2], two Gram-negative

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bacteria [*Proteus vulgaris* (*P. vulgaris), Pseudomonas aeruginosa* (*P. aeruginosa)*] and the yeast *C. albicans* (Fig. 3). The concentration where half the growth inhibition was observed (IC_{50}), as well as the minimal concentrations where complete inhibition of growth occurred (MIC), were determined using optical density measurements after 22 h incubation at 37 °C. For these assays, each of the drugs was tested at twelve different concentrations varying from 256 μg/mL to 0.0625 μg/mL. Control experiments against each strain were carried out with known antimicrobial agents: vancomycin for *MRSA* and *S. warneri*; rifampicin for *VRE*; ciproflaxin for *P. vulgaris*; gentamicin for *P. aeruginosa*; and nystatin for *C. albicans*.

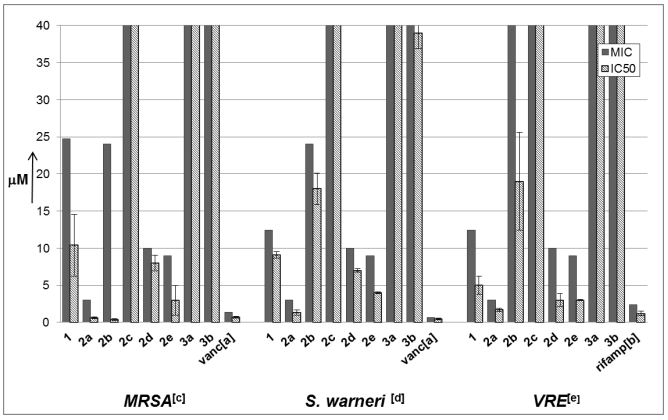

Fig. 2 Antimicrobial assays. IC_{50}: 50% inhibitory concentration (μM), MIC: minimal inhibitory concentration (μM), average of six replicates, bars represent standard error; [a] vancomycin, [b] rifampicin, [c] methicillin-resistant *Staphylococcus aureus*; [d] *Staphylococcus warneri*; [e] Vancomycin-resistant *Entrococcus faecium.*

As demonstrated in Figure 2, the natural compound prodigiosin **1** exhibits modest activity against Gram-positive bacteria (*MRSA*, *S. warneri* and *VRE*). However, prodigiosin exhibits almost no activity against Gram-negative bacteria (*P. vulgaris* and *P. aeruginosa*, Fig. 3) or *C. albicans* (Fig. 3). This selectivity for Gram-positive bacteria is in agreement with previous reports.[16, 20, 24]

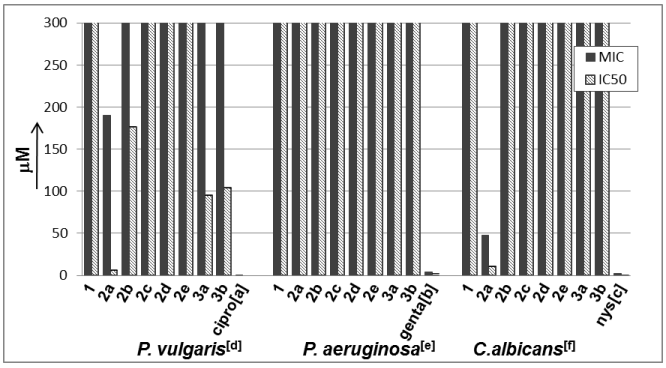

Fig. 3 Antimicrobial assays. IC_{50}: 50% inhibitory concentration (μM), MIC: minimal inhibitory concentration (μM), average of six replicates, bars represent standard error; [a] ciprofloxacin MIC = 0.2 μM, IC_{50} = nd; [b] gentamycin; [c] nystatin; [d] *Proteus vulgaris*; [e] *Pseudomonas aeruginosa*; and [f] *Candida albicans*.

By consideration of the ≈ three-fold (or more) increase in growth inhibition and cell death activity of **2a** cf. **1**, it appears that methylation at the C-ring of prodigiosin improves the antibacterial properties of prodigiosenes. Indeed, the analogue **2a** exhibits significantly better activities against Gram-positive bacteria than prodigiosin itself (Fig. 2), with MIC and IC_{50} values similar to the control antibiotics vancomycin and rifampicin (*MRSA* IC_{50} = 0.6 for **2a**, 0.7 for vancomycin; *S. warneri* IC_{50} = 1.3 for **2a**, 0.5 for vancomycin; *VRE* IC_{50} = 1.7 for **2a**, 1.2 for rifampicin). In contrast to the natural product, **2a** is somewhat active against the Gram-negative bacteria *P. vulgaris* (Fig. 3). Compound **2a** also exhibited interesting activity against the yeast *C. albicans*.

Although **2b** and **2c** exhibited modest antimicrobial activity, compounds **2d** and **2e** exhibited increased activity against Gram-positive bacteria compared to the natural compound (Fig. 2). These results show that the presence of neither a conjugated carbonyl moiety nor a pendant carboxylate group, courtesy of the C-ring in our prodigiosenes, is detrimental to activity agsinst Gram-positive bacteria. Interestingly compound **2c**, bearing merely a longer alkyl chain (n = 8) cf. compound **2d** (n = 2), exhibited almost no activity against the three Gram-positive strains studied, indicating that increased lipophilicity decreases the antibacterial properties of prodigiosenes. The tin complexes **3a** and **3b** exhibited much lower activity compared to the parent ligand **2e**. This suggests that the pyrrolic N–*H* moieties of the prodigiosenes play an important role in the prodigiosene mechanism of action against Gram-positive bacteria. Compounds **2b-e** did not exhibit significant activity against the Gram-negative bacteria evaluated, nor the fungal agent (Fig. 3).

We then evaluated the toxicity of our prodigiosenes and tin complexes against human keratinocyte and fibroblast cells (Fig. 4), so as to gain a sense of the utility of our best compounds: for clinical use, a good therapeutic window would effect excellent antimicrobial activity at a dosage that did not result in significant damage to human cells. Via IC_{50} and MIC values, the activity of our prodigiosenes and tin complexes, as well as the natural

product prodigiosin, was compared to that of zinc pyrithione, a FDA-approved antibacterial agent that is used ubiquitously in anti-dandruff shampoo but is toxic by ingestion.[49] The methylated analogue **2a** that previously showed broad-spectrum Gram-positive antibiotic activity, as well as a significant inhibition of *C. albicans*, exhibited also a detrimental activity against keratinocytes and fibroblasts with lower MIC and IC_{50} values than prodigiosin itself. However, analogues **2d** and **2e** that also demonstrated interesting activity against Gram-positive bacteria (Fig. 2), look more promising: **2d** exhibited almost no activity against keratinocytes and thus heralds potential for topical application; **2e** also exhibited a promising therapeutic window with a selectivity index of 11.7 when looking at keratinocytes, and 4.8 when looking at fibroblasts (**2e** MIC = 9.1 μM for *MRSA*, *S.warneri* and *VRE*; SI = cell IC_{50}/pathogen MIC).

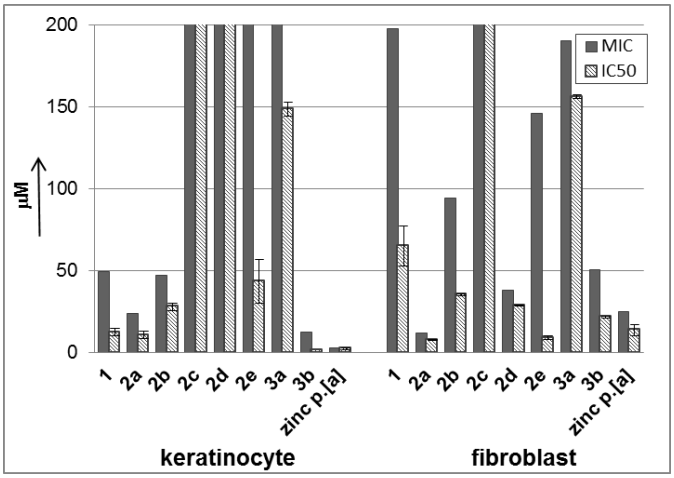

Fig. 4. Cytotoxicity assays. IC_{50}: 50% inhibitory concentration (μM), MIC: minimal inhibitory concentration (μM), average of three replicates, bars represent standard error; [a] zinc pyrithione.

Conclusions

This is, to our knowledge, the first report of the antibacterial activity of prodigiosenes. This preliminary study demonstrates the importance of the N–H functionality of the pyrrolic rings of prodigiosene in order to obtain consequent antibacterial activity against Gram-positive bacteria. Lipophilicity seems also to be a prejudicial parameter. Prodigiosenes **2a**, **2d** and **2e** showed good activity against Gram-positive bacteria at low concentration. The methylated analogue of prodigiosin **2a**, because of its residual toxicity against the keratinocyte and fibroblast human cells, cannot be considered as a promising antibiotic drug. However, compounds **2d** and **2e**, both incorporating a carbonyl group in lieu of the pentyl chain of the natural product, exhibit an interesting therapeutic window for potential use as antibiotics. These findings would be critical for our future design of prodigiosin-like antimicrobial agents.

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Electronic Supplementary Information (ESI) available: Procedures for biological assays and data for microbroth antimicrobial assays. See DOI: 10.1039/c000000x/

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