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Isolation and semi-synthesis of enterocin analogues as novel antibiotics

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Declaration

I hereby declare that this thesis entitled 'Isolation and semi-synthesis of enterocin analogues as novel antibiotics' represents my own work entirely and has not previously been submitted for a degree or diploma to any other university or tertiary institution. In addition, I certify that all information sources and literature used are clearly indicated in the thesis.

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Michael S. Cowled Date

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Last but not least, I thank my partner as well as my parents, Scott and Cherryl, for all their love, support and encouragement throughout the year.

Abstract

Enterocin, a uniquely-structured polyketide, was first isolated in 1976 from two soil *Streptomyces* with reported broad-spectrum bacteriostatic activity. In this thesis, the chemical and biological properties of enterocin were re-investigated for its suitability as a next-generation antibiotic scaffold. Importantly, enterocin was found to be less stable than previously reported, undergoing a facile intramolecular rearrangement to a novel polycyclic ketal isomer bereft of antibacterial activity. Enterocin also undergoes further, undocumented degradation under neutral and basic conditions in a range of solvents at room temperature. Semi-synthetic derivatisation of enterocin at its carbonyl and secondary hydroxy positions yielded five new analogues with improved stabilities, albeit with reduced antibacterial activities. These analogues may have applications as pro-drugs to increase the antibiotic bioavailability and half-life. Furthermore, a microbial biodiscovery study was conducted to investigate the co-metabolite profile of an enterocin-producing culture, *Streptomyces* sp. MST-MA9095. Six α-pyrones related to enterocin were isolated from the culture on an optimised culture medium, four of which showed moderate antibacterial activities against *Micrococcus luteus*. Preliminary investigations into the mode of action of enterocin suggest that its true ecological role may not be as an antibiotic, but rather as a first-in-class broad-spectrum regulator of secondary metabolite production of other microorganisms.

Table of Contents

1. Introduction

1.1. Multidrug Resistance and the Need for New Antibiotics

Antibiotic resistance has been on the rise for the last three decades, with bacteria able to transfer this resistance easily through rapid replication and horizontal gene transfer mechanisms.[1] To further compound this issue, there has also been a decrease in the development of new antibacterial agents.[2] Because of this, there is a need for new antibiotics as the ability to treat bacterial infections proves increasingly more challenging. For instance, in 2014, the U.S. reported more than 55,000 deaths attributable to pneumonia and influenza infections representing 2.1% of the total deaths to the population.[3]

Most bacterial infections that show resistance to a single class of antibiotic can be easily treated by an antibiotic of a different class. It is when this is not the case, with the emergence of so-called "superbugs", that the infection is much more difficult to treat. Superbugs are those microorganisms that have accumulated resistances to multiple antibiotics, and hence infections caused by such superbugs result in higher morbidity and mortality rates.[4] In a 2016 report by the World Health Organization (WHO), for example, multidrug-resistant (MDR) tuberculosis in children resulted in an approximately 10% incidence of death (Figure 1.1a). Hence, there is a need for antibiotics with novel mechanisms of action that can treat such infections.

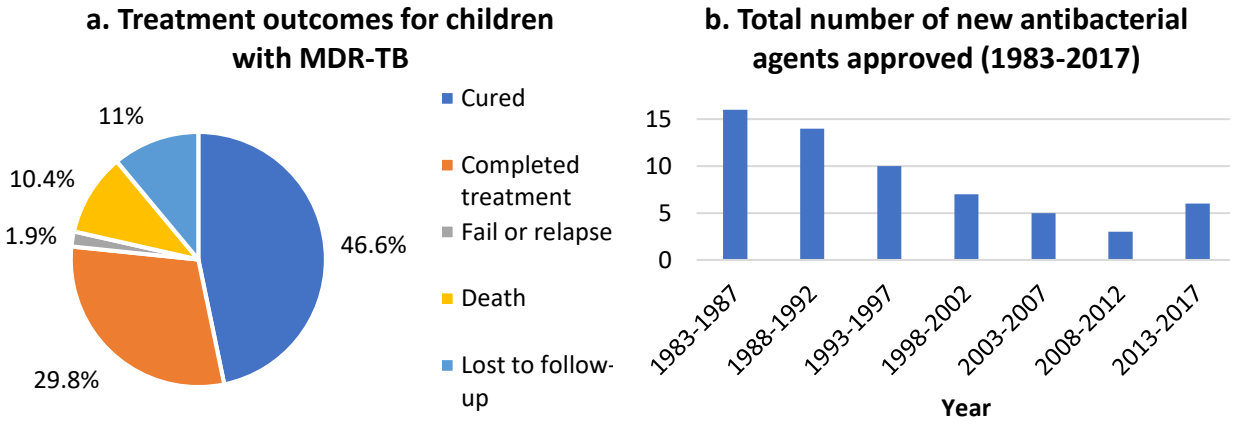


Figure 1.1: a. Treatment outcomes for children with MDR tuberculosis (adapted from WHO treatment guidelines for drug-resistant tuberculosis 2016 update).[5] **b.** Antibacterial agents approved 1983–2007. Adapted by permission from Nature Publishing Group: [Nature Biotechnology] (6), copyright 2016.[6-8]

1.2. Natural Products as Leads for Antibiotic Drug Discovery

Natural products are structurally diverse chemical entities biosynthesised inside living organisms typically in response to their environment and interactions with other organisms. Some of these chemical entities are produced as defence mechanisms against other organisms and hence harbour unique antibiotic potential. The first recognised instance of this was made in 1870 with the growth of a *Penicillium* mould on oranges and jam.[9] This led to a series of investigations by researchers at St Mary's Hospital in London which ultimately led to the fortunate discovery by Alexander Fleming in 1928. Fleming, who was also working at St. Mary's Hospital, returned from his holidays to observe a strange region of growth inhibition pertaining to a *Penicillium* mould contaminant on colonies of a staphylococcal variant, which had likely wafted through the air from the laboratory below him.[10] This accidental discovery ultimately led to the development of the most historically important antibiotic, penicillin.

This discovery initiated what is now known as the "Golden Era" of antibiotic discovery. This period (1940s to 1960s) saw the discovery of many of the antibiotic classes that are in use today.[11] This involved primarily the screening of soil-derived actinomycetes. Continuing this age of discovery was the development of new diving techniques in the 1970s, which allowed for the uncovering of unique marine organisms and their chemical products.[12] However, many of the natural products identified as antibiotics during this time of plenty were shelved and not further investigated due to there being so many other lead compounds to explore with less toxicity or more impressive bioactivities.

However, from the 1980s, the rate of discovery of new antibiotic classes was on the decline, prompting drug discovery towards a new era. This era focussed on the high-throughput screening (HTS) of large chemical libraries generated from a new approach to chemical synthesis known as combinatorial chemistry. Underpinning the beginning of the HTS era was the development of solid-phase peptide synthesis by Merrifield in the early 1960s,[13] which enabled efficient separation of synthetic products and reagents with simple washing steps. Such simplicity enabled one to perform several simultaneous chemical transformations all in high yielding reactions. Additionally, split-and-pool synthesis, introduced in the early 1990s, allowed for even more chemical diversity to be synthesised.[14] This gave rise to the generation of combinatorial chemistry libraries, often with hundreds of thousands to millions of chemical entities – more

limited by the ability to assay these compounds than to enumerate – all exploring a narrow portion of the vast chemical space in hopes of generating new drug leads.[15] Unfortunately, whilst the number of chemical entities was high, the compounds generated lacked the structural diversity and complexity often present in natural products. Only a potential three *de novo* chemical entities have arisen from the use of combinatorial chemistry, the first being sorafenib, which was approved in 2005 as a drug by the U.S. Food and Drug Administration (FDA).[16-17] This highlights the limited success of combinatorial chemistry as a drug discovery tool.

Bioactive natural products tend to have complexity in their chemical structures in the form of polycycles, heteroatoms and multiple stereochemical centres.[18] To add molecular complexity to combinatorial chemistry libraries, diversity-oriented synthesis (DOS) was developed. DOS introduces "diversity" into the synthesised compounds through varying the functional groups, stereochemical centres and distinct molecular skeletons used.[19] Additionally, molecular transformations are performed on previously identified molecular scaffolds that have been shown to result in unique bioactivities. An example of this is the work conducted by Park and his team, who constructed 22 unique scaffolds from a benzopyran core.[20] This benzopyran core compound was shown to exhibit activity against a human cancer cell line. Using DOS, biologically active molecules were synthesised with a range of similar IC_{50} values to the benzopyran scaffold.

Since the decline of the Golden Era of antibiotics, there has been a decreased rate in the development of new antibacterial agents, with only a single antibiotic approved by the FDA in 2016.[21] This decrease, as indicated in Figure 1.1b above, has prompted re-exploration of natural products, including those still yet to be discovered from previously uncultivable microorganisms, and those that were overlooked during the busy Golden Era of antibiotic discovery.

1.2.1. Revival of Forgotten Antibiotics

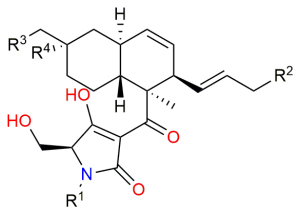
The revival of forgotten antibiotics is a potential strategy for dealing with multidrug resistance by identifying molecules operating via novel mechanisms of action. An example of an antibiotic that has been revived is daptomycin, which is a cyclic lipopeptide discovered in the 1980s by researchers at Eli Lily with good bactericidal activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MIC_{90} = 1 μg/mL) on a comparable level to vancomycin (MIC_{90} = 0.5 μg/mL).[22-23] However, due to the drug's toxicity in skeletal muscle, it was

shelved and not further developed. It was not until 1997 that the drug was revisited and it was eventually FDA-approved in 2003 for the treatment of MRSA infections, with a new dosing regimen to combat the toxicity effects of the drug. Daptomycin has gone on to generate annual revenues of over $1-billion since its approval for Cubist Pharmaceuticals.[24] It has been estimated that over 1,000,000 patients with Gram-positive infections have been successfully treated with daptomycin.[25] Merck has since acquired this antibiotic pipeline for $9.5-billion.[26] The recognition that known but unexploited antibiotic classes might offer advantages in a multi-drug resistant world has gained widespread scientific and commercial appeal.[27]

1.2.2. Isolation and Discovery of Biologically Active Natural Products

Microorganisms can be cultivated on a variety of different media to optimise the production of their secondary metabolomes.[28] Alternatively, microorganisms can also be co-cultured to enhance the production of novel antimicrobial natural products, after all, adventitious co-culture was how penicillin came to be discovered by Fleming. This is because microorganisms do not exist alone in the natural environment, typically diverse species are competing for food and resources, responses triggered by complex and poorly understood signalling mechanisms.[29]

The significance of co-culturing techniques in the isolation of novel natural product analogues can be demonstrated by the co-culturing of a fungus *Fusarium palliodoroseum* with the bacterium *Saccharacopolyspora erythraea*.[30] Three new analogues of tetramic acid were isolated along with the related known antibacterial analogue, equisetin, and non-biologically active ophiosetin. The newly isolated analogues, *N*-demethylophioseten, pallidorosetin A and pallidorosetin B, were all reported to be inactive against the Gram-positive bacteria tested against (*Bacillus subtilis* and *Staphylococcus aureus*) (Figure 1.2). This confirmed the lack of activity observed in ophiosetin as both *N*-demethylphioseten and pallidorosetin B possess a hydroxy group at C-16, which is absent from the biologically active equisetin. Whilst pallidorosetin A does not contain a hydroxy group at C-16 (R^3), it does contain an additional hydroxy group at C-8 (R^4), perhaps indicating that polarity plays a role in the biological activity of equisetin.[31] Whilst no biologically active analogues were isolated in this study, the analogues that were isolated provided useful information regarding the structure-activity relationships of equisetin, providing a framework for the generation of semi-synthetic and biosynthetic analogues.



Antibiotic	R^1	R^2	R^3	R^4
Equisetin	CH_3	H	H	H
Ophiosetin	CH_3	H	OH	H
N-demethylophiosetin	H	H	OH	H
Pallidorosetin A	CH_3	H	H	OH
Pallidorosetin B	CH_3	OH	OH	H

Figure 1.2: Structures of isolated analogues of tetramic acid by co-culturing *Fusarium pallidoroseum* with *Saccharopolyspora erythraea*.

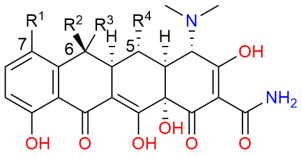
The microorganisms from which natural products with biological activity have been isolated are often re-explored using new strains and different culturing conditions to enhance the production of new co-metabolites to the originally isolated natural product. There are many examples of this approach in the literature, with one being the isolation of platensimycin, and later platencin, from two different strains of *Streptomyces platensis.*[32-33] Both natural products display selective inhibition of the FabH enzyme in *S. aureus*, responsible for the biosynthesis of the essential fatty acids needed for microorganism's survival.

Nature offers enormous chemical diversity with >250,000 natural products recorded in the CRC Dictionary of Natural Products (DNP).[34] However, only 1% of microorganisms are thought to be cultivable using the current suite of culturing techniques and conditions.[35] Hence, there is vast chemical diversity yet to be explored. In 2010, the use of so-called "iChips" was demonstrated to culture previously uncultivable bacteria in their unique environments.[36] Teixobactin is a new antibiotic identified using this iChip technology with excellent activity against Gram-positive pathogens.[37] It is also useful in the treatment of drug-resistant infections due to its novel mechanism of action.

1.2.3. Semi-Synthesis of Natural Product Analogues

Semi-synthetic modification of biologically active natural products is used to improve the biological activity, overcome resistance, decrease toxicity and improve the pharmacokinetics/ pharmacodynamics of the antibiotic.[38] In semi-synthesis, a structural motif is recognised through structure-activity relationship (SAR) studies that can be modified with no loss of biological activity. With the widespread production of penicillin-degrading β-lactamases in *S. aureus* by 1944,[39] the first semi-synthetic derivative of penicillin, methicillin, was generated to overcome this evolving threat.

It was initially believed that semi-synthesis yielded antibiotics with decreased biological activities. There was, however, others that opposed this view and continued semi-synthesis in the hopes of generating more potent antibiotics. The discovery of tetracycline *via* the semi-synthetic modification of chlortetracycline is particularly noteworthy in the history of antibiotic discovery due to its non-compliance with the above widely-accepted view (Figure 1.3). Palladium-catalysed hydrogenation of chlortetracycline yielded tetracycline which showed greater biological activity, solubility and pharmacological characteristics than the original natural product.[40] About 10 years after the class was first discovered, it was shown that the 6-OH group on tetracycline and other natural tetracycline products, oxytetracycline and 6-demethyltetracycline, could be removed by palladium-catalysed hydrogenation.[41] The resulting dehydroxy derivatives were more stable than the original compounds whilst still retaining similar biological activities. This enabled further derivatisations that were not previously possible due to the inherent acid and base sensitivities of the tetracycline compounds, leading to the eventual discovery of minocycline. Minocycline was derived from 6-deoxy-6-demethyltetracycline with an electrophilic aromatic substitution at C-7.[42] Minocycline exhibits a broader spectrum of activity than other tetracyclines, as well as activity against tetracycline-resistant staphylococci.



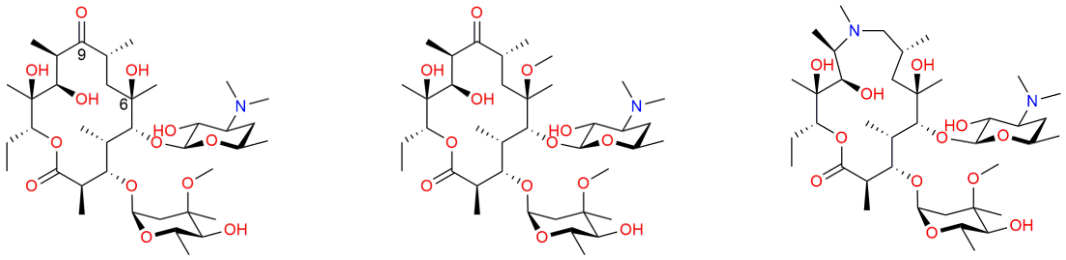
Antibiotic	**R^1**	**R^2**	**R^3**	**R^4**
Chlortetracycline	Cl	CH_3	OH	H
Tetracycline	H	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
6-Demethyltetracycline	H	H	OH	H
6-Deoxy-6-demethyltetracycline	H	H	H	H
Minocycline	$N(CH_3)_2$	H	H	H
Doxycycline	H	CH_3	OH	OH

Figure 1.3: Structures of semi-synthetic analogues of tetracycline.

One semi-synthetic derivative of tetracycline that is particularly interesting is doxycycline. Doxycycline is another example of an old antibiotic that has found new life in the treatment of new types of infections. First produced as a semi-synthetic derivative of tetracycline in 1966, doxycycline showed similar bioactivity to that of tetracycline in the treatment of various infections, including cholera.[43] Despite this similarity, the pharmacokinetics of doxycycline were shown to be inferior to those of tetracycline, which has a longer serum half-life.[44] Doxycycline, however, has better activity than tetracycline against enterococci, specifically vancomycin-resistant enterococci (VRE).[45] In addition, doxycycline has been shown to be useful in the

treatment of some tetracycline-resistant microorganisms,[46] and has been given recommended clinical dosages following a review of the literature with comparisons to other penicillin and tetracycline antibiotics, for various VRE-associated infections.[47]

Another notable example of the use of semi-synthesis is that of erythromycin (Figure 1.4). Erythromycin is the first macrolide antibiotic, discovered in 1949 from the soil fungus *Saccharopolyspora erythrea* with excellent activity against Gram-positive bacteria.[48] However, erythromycin was shown to be unstable at low pH conditions giving it poor oral bioavailability and uncomfortable side effects such as stomach pain.[49] Stability studies revealed that intramolecular cyclisation was occurring involving the 6-OH group and C-9 ketone giving a range of anhydrohemiketal and spiroketal decomposition products (Figure 1.5). To minimise this limitation, semi-synthetic methylation of this 6-OH group was conducted, yielding clarithromycin as an acid-stable analogue with enhanced biological activity compared to erythromycin.[50] Following on from this, in the 1980s, the stability issue was completely overcome with the removal of the C-9 ketone itself. This was achieved through oxime formation and a Beckmann rearrangement to yield an iminoether intermediate, which was subsequently reduced by hydrogenation to the methylamino derivative azithromycin.[51] Azithromycin has excellent acid stability and oral bioavailability whilst retaining the bioactivity of the original antibiotic. It has now gone on to become one of the most prescribed antibiotics in the U.S., taking the top spot in 2011 with 54.1 million prescriptions.[52]



Erythromycin Clarithromycin Azithromycin

Figure 1.4: Structures of erythromycin and its semi-synthetic analogue azithromycin.

Figure 1.5: Structures of acid-catalysed decomposition products of erythromycin.

1.2.4. Biosynthesis of Natural Product Analogues

Chemical diversity surrounding a biologically active natural product can also be enhanced through precursor-directed biosynthesis and mutasynthesis. Precursor-directed biosynthesis relies on feeding the organism with unnatural precursor molecules that may be incorporated into the natural product scaffold enzymatically.[53] Derivatisation of polyketides using precursor-directed biosynthesis is generally limited to the incorporation of unnatural starter units and even then the biosynthetic enzymes must be able to demonstrate some degree of substrate flexibility from the natural starter unit.[54] Furthermore, there is the inevitable competition of the natural substrate with the unnatural substrate, thereby reducing the production of the desired analogues. To overcome some of these limitations, mutasynthesis is often used in tandem with precursor-directed biosynthesis, whereby the production of the natural substrate is blocked so that the unnatural substrate uptake is improved.[55] Generally, mutasynthesis relies on the use of molecular biology techniques such as gene silencing, insertions and deletions to manipulate the biosynthesis of a natural product. It was first used over 50 years ago to produce analogues of neomycin in a *Streptomyces fradiae*,[56] and has become a staple in the development of second generation analogues within many antibiotic classes.

Erythromycin has been at the centre of many precursor-directed biosynthesis studies,[57-59] due to the complexity present in performing semi-synthetic transformations on the polyketide natural product. One study produced biosynthetic analogues of erythromycin through the introduction of synthetic substrates to an engineered polyketide synthase (PKS) mutant generating a series of 6-deoxyerythronolide B (6-dEB) derivatives (Figure 1.6).[57] This mutant blocked the production of the natural substrate so that the production of unnatural erythromycin analogues was favoured. The 6-dEB derivatives were subsequently oxidised at C-6 and glycosylated at C-3 and C-5, resulting in the desired analogues of erythromycin.

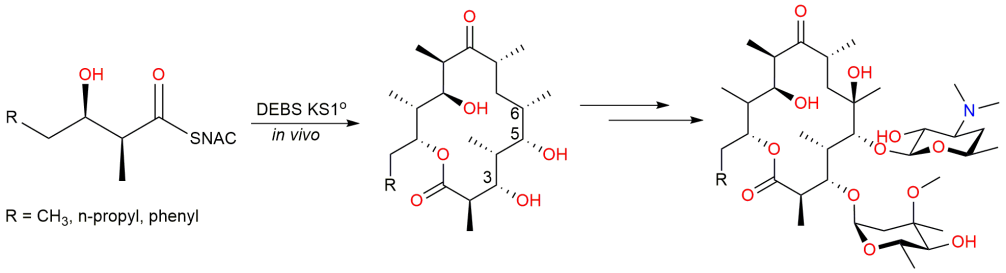


Figure 1.6: Precursor-directed biosynthesis of erythromycin analogues from synthetic substrates featuring *N*-acetyl cysteamine (SNAC) groups via a deoxyerythronolide B synthase (DEBS) *in vivo*.

1.3. Enterocin as a Forgotten Antibiotic with a Unique Chemical Structure

1.3.1. The Discovery of Enterocin and Related Enterocin Analogues

Enterocin (**1**), sometimes referred to as vulgamycin,[60] (Figure 1.7) was first isolated and characterised in 1976 from two species of soil *Streptomyces: S. candidus* and *S. viridochromogenes*,[61-62] and later that year from *S. hygroscopicus*.[60] Enterocin (**1**) was shown to possess bacteriostatic activity against both Gram-positive and Gram-negative bacteria,[62] cytotoxicity against HeLa cells[63] and moderate herbicidal activity,[64] but no activity against fungi and yeast. The antibiotic activity reported was suggested to be non-dilutable in nature.[62] Its chemical structure was identified by both X-ray crystallography of a *m*-bromobenzoyl derivative at its secondary hydroxy group on C-5,[61] and NMR spectroscopy.[60]

Enterocin (**1**) was later isolated from a marine ascidian of the genus *Didemnum,*[65] the first report of **1** from a non-microbial species, suggesting that a producer of **1** resides on or exists symbiotically with the marine organism. In addition to **1**, the Western Australian researchers isolated deoxyenterocin (**2**) and two novel lipoester analogues containing long fatty acid chains off of the secondary hydroxy group on C-5, 5-*O*-arachidylenterocin (**3**) and 5-*O*-behenylenterocin (**4**) (Figure 1.7). Compound **2** was shown to inhibit *Sarcina lutea*, *S. aureus*, *Klebsiella pnuemoniae*, and *Vibrio percolans* at a concentration of 0.5 mg/mL;[66] however, **3** and **4** have yet to be investigated for biological activity.

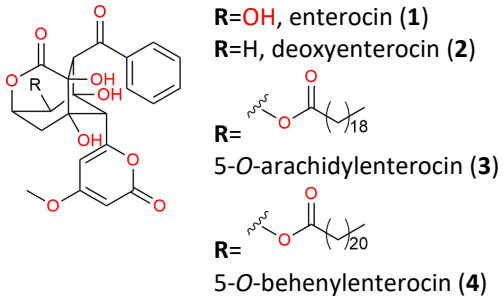
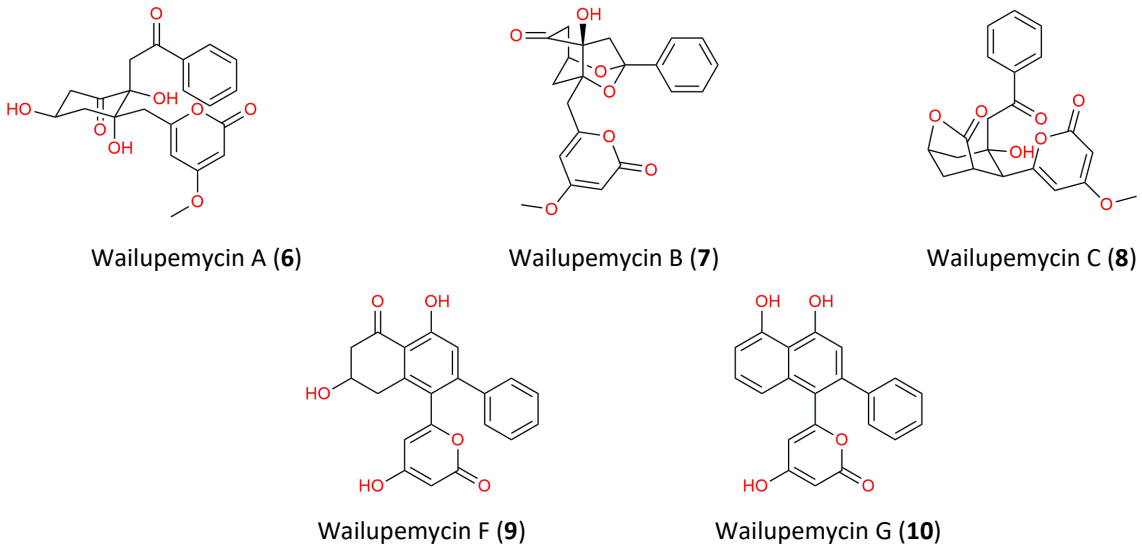


Figure 1.7: Structure of enterocin and isolated enterocin analogues deoxyenterocin, 5-*O*-arachidylenterocin, and 5-*O*-behenylenterocin.

Further analogues of **1** were isolated from a Hawaiian marine-derived *Streptomyces*, at a similar point in time.[67] One of the isolated compounds, 3-*epi*-5-deoxyenterocin (**5**), is an epimer of **2**, differing only in its configuration at C-3. This epimer showed antimicrobial activity against *Escherichia coli* in a paper disc diffusion assay. The other three compounds isolated from this

marine microorganism are a related set of compounds to **1** known as wailupemycins A (**6**), B (**7**) and C (**8**) (Figure 1.8). These compounds share the same α-pyrone system as **1** and were suggested to be derived from the same biosynthetic pathway.[68] Only one out of the three wailupemycins, **6**, showed evidence of antimicrobial activity in the paper disc diffusion assay carried out.



Wailupemycin A (**6**) Wailupemycin B (**7**) Wailupemycin C (**8**)

Wailupemycin F (**9**) Wailupemycin G (**10**)

Figure 1.8: Structures of wailupemycins A, B, C, F and G as co-metabolites of enterocin.

Enterocin (**1**) is a polyketide featuring a caged tricyclic, non-aromatic core biosynthetically-derived from a type II PKS.[68] Polyketides are a structurally-diverse set of natural products often featuring unique polyether, polyphenol, and polyester functionalities derived from chains of carboxylic acid units.[69] These diverse functionalities provide polyketides with a range of biological activities with wide-spread use as antibiotics, antitumour agents and immunosuppressants.[70] Whilst such complex structures are a significant challenge for total organic synthesis, polyketides are comparatively biosynthesised from small carboxylic acids units of mainly acetate and propionate in simple Claisen condensation reactions.[69] Type II PKSs contain multiple enzymes that generate poly-β-keto intermediates, which condense further to polyphenolic compounds.[71]

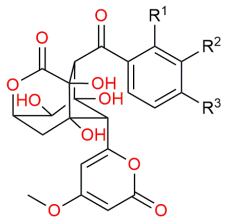
The structure of enterocin (**1**) differs from most type II PKS products, with the absence of the typical polyaromatic structure, indicative of tetracycline (Figure 1.3).[72] Rather, the tricyclic non-aromatic core of **1** was thought to be derived from an unusual Favorskii-type rearrangement,[60] something that had been observed similarly in the biosynthesis of aspyrone.[73] Further distinguishing **1** as an unusual polyketide product, **1** accepts benzoate, rather than

acetate, as a starter unit for biosynthesis.[60] The presence of benzoic acid starter units in metabolic pathways is very rare, with only one other example known: the biosynthesis of soraphen A.[74]

More recently, efforts have begun on the total synthesis of **1**, with initial investigations into a possible *meta*-photocycloaddition reaction to produce its tricyclic core.[75] However, the reaction lacked the ability to stereoselectively produce the highly oxygenated tricyclic structure required to synthesise **1**, and hence an alternate route was developed to generate the oxygenated lactone component.[76] A complete total synthesis of **1** has yet to be reported; however, the total synthesis of the structurally-related wailupemycin B (**7**) has been successfully achieved.[77]

1.3.2. Precursor-Directed Biosynthesis of Enterocin Analogues

The first precursor-directed biosynthesis study of enterocin (**1**) analogues was reported in 1985, utilising fluorobenzoic acid precursors in the fermentation broth,[78] thereby altering the phenyl substituent on **1**. Fluorinated analogues are useful for exploring bioactivity due to the near-isosteric nature of fluorine and hydrogen, and the highly energetic bond that results from electronegative fluorine atoms. The fluorophenyl analogues of **1** (Figure 1.9) exhibited no observable antibacterial activity against the tested microorganisms except for *Micrococcus luteus*, with *p*-fluoroenterocin (**13**) showing greater activity than **1**. However, in general, fluoro-substitution of the phenyl substituent did not have a significant influence on the bioactivity of **1**. Importantly, the results of this study confirmed that the enterocin (**1**) PKS can accept unnatural starter units.



Compound	R^1	R^2	R^3
Enterocin (**1**)	H	H	H
o-Fluoroenterocin (**11**)	F	H	H
m-Fluoroenterocin (**12**)	H	F	H
p-Fluoroenterocin (**13**)	H	H	F
3',4'-Difluoroenterocin (**14**)	H	F	F

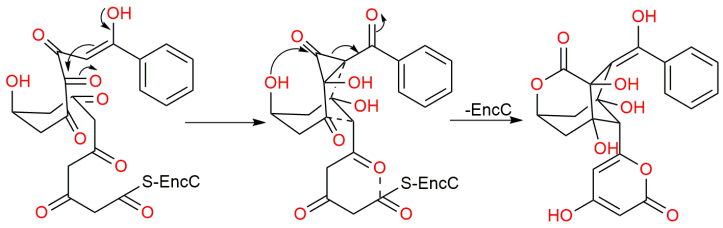
Figure 1.9: Structures of precursor-directed biosynthetic fluorinated analogues of enterocin.

Much of the research involving the biosynthesis of **1** and the respective *enc* cluster of encoding genes was carried out by Bradley Moore and his group starting from 2000.[68, 79-80] At the time of its discovery, the *enc* gene cluster was shown to encode significantly greater natural diversity than expected from type II PKS gene sets.[80] Notably, the *enc* gene cluster is missing the cyclases responsible for acting as chaperones for the PKS in cyclising linear polyketides into

aromatic structures.[68] Cyclisation of **1** is further inhibited by a Favorskii-type rearrangement at the linear octaketide stage.

As part of the initial biosynthetic studies by Moore *et al.*, a new wailupemycin, wailupemycin D, was isolated from *S. maritimus*.[79] Not long after, two isomers, wailupemycin E and wailupemycin F (**9**) as well as the dehydro derivative of **9**, wailupemycin G (**10**), were also isolated from *S. maritimus*.[81] Wailupemycin D-G all share a highly aromatic backbone more in line with those expected for type II PKS products. Hence, it was hypothesised that the biosynthesis of wailupemycins D-G occurs before the Favorskii-type cyclisation that is indicative of **1** and the other wailupemycins. In addition, it was thought that a single decarboxylation step determines whether production of wailupemycins is favoured over enterocins.

The unusual Favorskii-type reaction was later shown to be catalysed by the flavoprotein EncM, which is also responsible for the two aldol condensations and two heterocycle forming reactions that lead to desmethyl-5-deoxyenterocin (**15**) and deoxyenterocin (**2**) formation.[82] Figure 1.10 shows mechanistically how the dual oxidation and Favorskii-type reactions involve the intramolecular nucleophilic attack from a secondary hydroxy group.[83] The final step in the biosynthesis of **1** is the hydroxylation of **2** at C-5 with the cytochrome P-450 EncR.[80]



Desmethyl-5-deoxyenterocin (**15**)

Figure 1.10: Mechanism for the dual oxidation and Favorskii-type reaction catalysed by EncM.[83]

Enterocin (**1**) was later shown to be produced in a non-*Streptomyces* bacterium, with its isolation in a species of *Salinispora* in 2015.[84] The authors of this study went on to use the marine actinomycete to heterologously express the biosynthetic pathway of **1** in two different *Streptomyces* host strains. This investigation, however, yielded no new co-metabolites of **1**.

1.3.3. Mutasynthesis of Enterocin Analogues

In relation to the 1985 precursor-directed biosynthesis study of **1** using fluorophenyl starter units, Moore *et al.* continued on from this in 2003 using a mutasynthetic approach. This

body of work delivered a series of mono-substituted benzoates and heteroaromatic carboxylates to an ΔencP mutant.[85] EncP was previously identified by the group to be responsible for the natural production of the benzoyl-CoA starter unit, and hence disruption to the gene would result in the removal of the natural background production of expected products.[86] Not all aryl acids were incorporated into the biosynthesised analogues, with some incorporated into just the enterocins or wailupemycins, some to both and some not at all. This suggested that there was a level or selectivity for starter units at some point in the *enc* biosynthetic cluster.

With investigations into the *enc* cluster of genes culminating, Moore *et al.* showed the first total enzymatic biosynthesis of **1**.[87] This would allow for an alternative to mutasynthesis for the generation of novel analogues of **1**. In 2009, 18 novel analogues of deoxyenterocin (**2**), wailupemycin F (**9**) and wailupemycin G (**10**) were generated *ex vivo* by altering the concentrations of various enzymes in the biosynthetic pathway and using different aryl acids.[88] No bioassay data were collected for this group of analogues.

1.4. Aims and Scope of Study

In this study, enterocin has been re-investigated for its potential use as a next-generation antibiotic scaffold. Whilst the biosynthesis of enterocin has been studied extensively, the chemical and biological properties of this unique antibiotic has been largely ignored. In this thesis, the stability of this unusual structural scaffold has been investigated and semi-synthetic studies undertaken to key structural features pivotal to maintaining the polycyclic core undertaken. Further, the thesis explores enterocin as a component of a microbial co-metabolite profile to gain an insight into how this metabolite is used in Nature. The study is undertaken with a known high-producing enterocin strain, *Streptomyces* sp. MST-MA9095, cultivated on various media to identify known and novel co-metabolites of enterocin. These co-metabolites isolated and semi-synthetic analogues were tested as antibiotics using *Micrococcus luteus* as a model bacterium to identify structure-activity relationships to aid the discovery of more efficient “next-gen” analogues. Finally, the biological mode of action of enterocin was investigated to gain an insight into the mode of action of enterocin.

2. The Stability of Enterocin

2.1. Introduction

In their original paper, Miyairi *et al.* noted that enterocin (**1**) was stable at room temperature under both acidic and neutral conditions, but labile under basic conditions.[62] However, the authors did not describe or further investigate the decomposition products. In this project, initial studies revealed that **1** is fundamentally unstable and undergoes decomposition in a variety of solvents and conditions that have not been previously reported in the literature. This chapter describes the studies performed to explore and characterise the stability of **1** in different solvents, temperatures, and pHs. The resulting decomposition products were characterised to reveal a unique scaffold generated by a base-catalysed re-arrangement. The biological activities of the decomposition products of **1** were investigated in Chapter 5, whilst Chapter 3 discusses the semi-synthetic strategies utilised to stabilise the enterocin pharmacophore.

2.2. The Rearrangement of Enterocin

Enterocin (**1**) was initially observed to change in DMSO-d_6 during acquisition of 2D-NMR spectra over a 15-hour period, with the gradual loss of existing and formation of resonances belonging to a new product. The formation of a second product was confirmed by HPLC and the product named, isoenterocin (**16**). The formation of **16** was enhanced in DMF, with almost quantitative yields obtained after 48 h.

Isoenterocin (**16**) was recovered from DMF on C_{18} silica and isolated by semi-preparative HPLC as a yellow oil. HR-ESI(−)-MS analysis of **16** revealed an ion ($[M-H]^-$ *m/z* 443.0981) indicative of a molecular formula of $C_{22}H_{20}O_{10}$ requiring 13 double bond equivalents (DBEs). The molecular formula of **16** is identical to that of **1**, with similarities in its UV spectrum (λ_{max} 206 and 284 nm) suggesting that **16** is an isomer of **1**. However, the UV spectrum of **16** differs from that of **1** with the absence of a peak at λ_{max} 250 nm, suggesting the loss of the C-2 carbonyl group. The ^{13}C NMR data for **16** also indicated a loss of the carbonyl group at δ_C 194.9 when compared to **1** and the appearance of a new signal at δ_C 112.0 suggesting the presence of a ketal. In the ^1H NMR spectrum **16**, the signal corresponding to the hydrogen on chiral carbon C-3 (δ_H 3.09) had undergone a significant chemical shift change compared to **1** (δ_H 4.46), providing evidence for the changed electronic environment adjacent to this carbon. A change in configuration at C-3 was suggested

by the presence of ROESY correlations between H-3 and H-9 and between H-3 and 4-OH (see Figure 2.1 and Supplementary Material, Table 10.1).

The appearance of a new secondary hydroxy group on C-6 was evident from the HMBC correlations from 6-OH (δ_H 4.86) to C-5, C-6 and C-7. Furthermore, none of the remaining three hydroxy groups showed the same HMBC correlations to C-4, C-5 and C-6 that were present in **1**, suggesting the loss of 5-OH in **16**. The COSY correlation between H-5 and 5-OH in **1** was missing in **16** with a new COSY correlation between H-6 and 6-OH. Additionally, the assignment of C-5 and C-6 were supported by COSY correlations from the methylene protons on C-7. Whilst both H-5 and H-6 showed COSY correlations with H-7a/b, only H-5 showed a correlation to H-9, confirming the assignment of these atoms. The *J* couplings of H-5 for both **16** and **1** were also examined. The ^{1}H signal for H-5 in **16** was a doublet showing a coupling of 5.4 Hz to H-6, while H-5 in **1** was a doublet of doublets with a coupling to both H-6 (4.6 Hz) and 6-OH (5.7 Hz). Therefore, analysis of 2D NMR spectra led to the assignment of all protons and carbons in isoenterocin (**16**) and secured the structure.

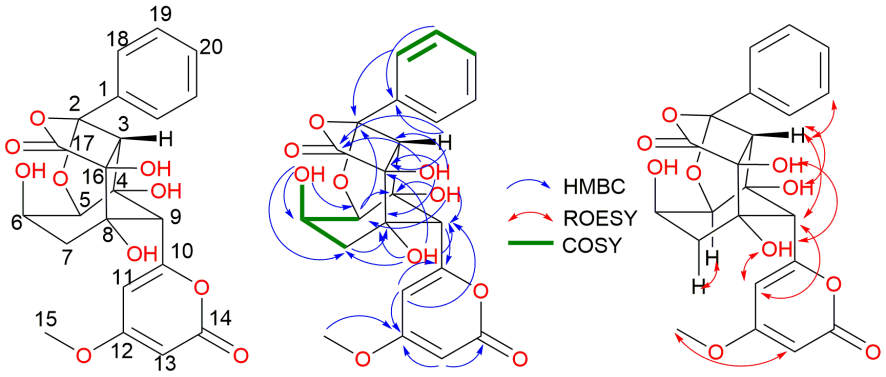


Figure 2.1: Key 2D NMR correlations observed in isoenterocin (**16**)

Isoenterocin (**16**) was postulated to result from the following rearrangement mechanism (Figure 2.2), undergoing an initial epimerisation to 3-*epi*-enterocin (**17**) followed by intramolecular nucleophilic attack from the secondary hydroxy group (5-OH) to the ketone group at C-2 to form a hemiketal, followed by opening of the lactone ring to give the new, more-stable product, **16**. Compound **16** has not been previously reported in the literature.

To confirm the proposed rearrangement mechanism shown below, efforts were made to isolate the intermediary compounds, **17** and its hemiketal. Despite observing two other isomers of enterocin in methanol that could be related to the aforementioned compounds, their isolation

was not possible and it is believed that their presence is solvent-dependent, resulting in conversion to either **1** or **16** during HPLC.

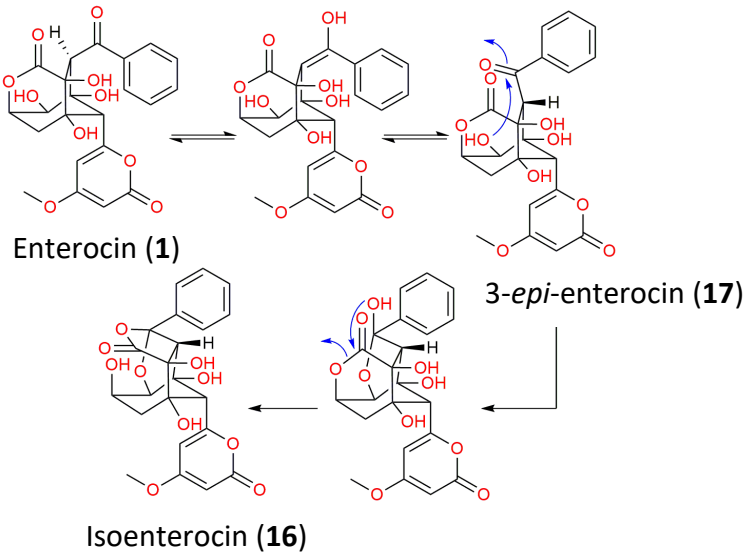


Figure 2.2: Proposed mechanism for the rearrangement of enterocin to isoenterocin.

The proposed rearrangement reaction was supported by examination of deoxyenterocin (**2**), which was missing the secondary hydroxy group responsible for the rearrangement evident in **1**. By acquiring 2D NMR spectra in a similar manner to **1**, it was observed that **2** did not form an intramolecular rearrangement product analogous to **16**, but rather converted partially to *epi*-3-deoxyenterocin (**5**). The presence of **5** was confirmed with reference to the literature,[67] and full HMBC, COSY and ROESY correlations are included in the Digital Supplementary Information (Table S1.1, Figures S1.3-S1.4). The conversion of **2** to **5** gives precedent for the formation of 3-*epi*-enterocin (**17**).

The structure of **16** was also confirmed computationally by correlating the density functional theory (DFT)-calculated isotropic shielding of each carbon atom against the experimentally obtained ^{13}C NMR chemical shifts. A similar method utilising residual dipolar couplings (RDCs) in anisotropic media was reported earlier this year,[89] and has also been demonstrated to assign the configuration of the chiral carbon (5-OH) on enterocin (**1**).[90] Here, the same method was applied to confirm the configuration of C-3. Firstly, **1** and 3-*epi*-enterocin (**17**) were energy-minimised (MOE2016.08; molecular mechanics, MMFF94x) and subjected to a conformational search (MOE2016.08; low-mode molecular dynamics, MMFF94x). The lowest energy geometries were further optimised (Turbomole 7.1; DFT//B3LYP/def2-TZVPP) and the

isotropic shieldings calculated for the global minimum energy structures using time-dependent DFT (TD-DFT//BP86/TZVPP). Figure 2.3 demonstrates that the experimental ^{13}C NMR chemical shifts correlate more closely with the calculated shieldings for **1** and not **17** as can be seen by the large residual (>10) for C-3 (δ_C 53.0) in **17** compared to **1**. Similarly, conformational searches, energy minimisation and DFT calculations for isoenterocin (**16**) gave a good match for the observed ^{13}C chemical shifts, providing further support for the assigned structure.

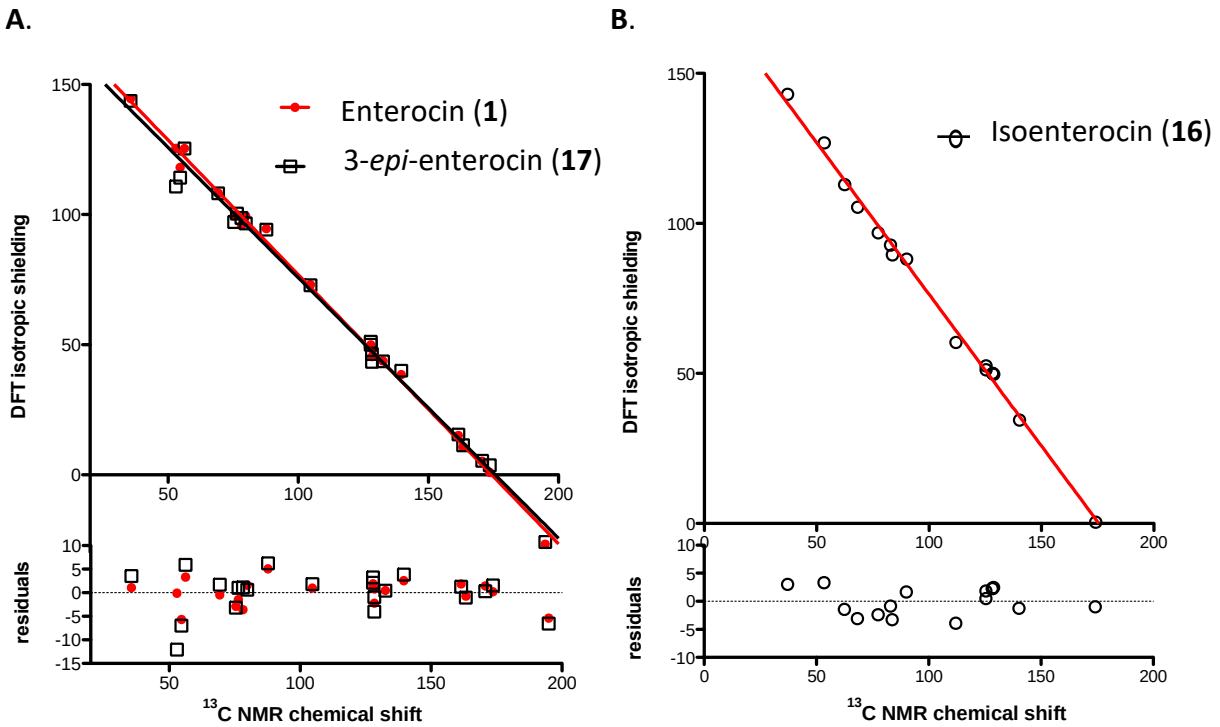


Figure 2.3: Plot of the experimental ^{13}C NMR chemical shifts versus the DFT isotropic shieldings for: **A.** enterocin (R^2=0.9479) and 3-*epi*-enterocin (R^2=0.9110) against the ^{13}C NMR chemical shifts of **1** and **B.** isoenterocin (R^2=0.9958).

2.3. Solvent Stability Study of Enterocin

Given the epimerisation step in the proposed mechanistic pathway for the rearrangement of **1**, the stability of **1** was investigated in a range of different conditions related to solvent (acetonitrile or methanol), temperature (room temperature or 60 °C) and pH (neutral, 5 mM H_2SO_4 or 10 mM KOH final concentration). All experiments were conducted using 0.1 mg enterocin dissolved in 100 μL of solvent, with the addition of acid or base containing no more than 10 μL of water to give the final concentrations listed. No water was added to the neutral solutions. The solvents selected aimed to differentiate protic and aprotic rearrangement mechanisms under neutral conditions.

The results of this study showed that enterocin (**1**) was stable under acidic conditions in both solvents and temperatures and unstable under basic conditions, especially at the elevated temperatures (see Supplementary Information, Section 10.4). The presence of a low concentration of acid likely prevents epimerisation from occurring. Under neutral conditions at room temperature, it was observed that only acetonitrile resulted in conversion of **1** to isoenterocin (**16**). At 60 °C, the use of methanol as a solvent resulted in the formation of a more polar isomer (t_R = 3.19 min) of **1** in addition to **16**. Identification of these isomers was conducted by LCMS whereby the retention times and UV profiles were compared to those corresponding to **1** (t_R = 3.58 min) and **16** (t_R = 3.90 min) following NMR characterisation in DMSO-d_6.

The above stability study was also repeated for deoxyenterocin (**2**). The conversion of **2** was limited to the formation of 3-*epi*-deoxyenterocin (**5**) under neutral conditions in both methanol and acetonitrile as was observed in DMSO-d_6. As expected, the formation of this epimer was reduced in acidic conditions, providing further evidence for the initial epimer formation for **1** in the proposed rearrangement mechanism to **16**.

Further information regarding the stability of **1** was obtained from a more comprehensive solvent stability study. Enterocin (**1**) was dissolved in various solvents at room temperature and the solutions were analysed by LCMS at regular intervals over a 19-day period, with the temperature increased to 40 °C at day 14. The solvents **1** was tested in (and hence was soluble in) include: water, acetone, methanol, ethanol, DMSO, dimethylformamide, pyridine, tetrahydrofuran, dioxane, acetonitrile and ethyl acetate. Enterocin (**1**) was insoluble in toluene, chloroform and dichloromethane, and hence no decomposition was observed in these solvents. The percentage decomposition was estimated by UV comparison of the new products compared to **1** remaining, and this decomposition is shown over time in Figure 2.4.

Of the solvents tested, only THF, dioxane and ethyl acetate, resulted in no decomposition of **1** over the 19-day period. The remaining solvents, however, resulted in varying degrees of decomposition, as shown in Figure 2.4. Where instability was observed in the solvents tested, the major product produced was **16**. However, two other isomers of **1** were additionally observed in the cases of methanol and ethanol. The exception to this was when water was used as the solvent. At room temperature, **1** was stable in water with <1% decomposition after 14 days. After

increasing the temperature to 40 °C, however, decomposition became readily apparent with the formation of a decomposition product with a *m/z* of 463, which is 18 Da heavier than **1**, suggesting the addition of water. Unfortunately, this decomposition product could not be isolated in significant yields. The most significant conversion occurred in DMF which resulted in almost quantitative formation of **16** (as determined by NMR spectroscopy) after 6 days. This could potentially be due to the presence of trace amounts of dimethylamine in the solvent.[91]

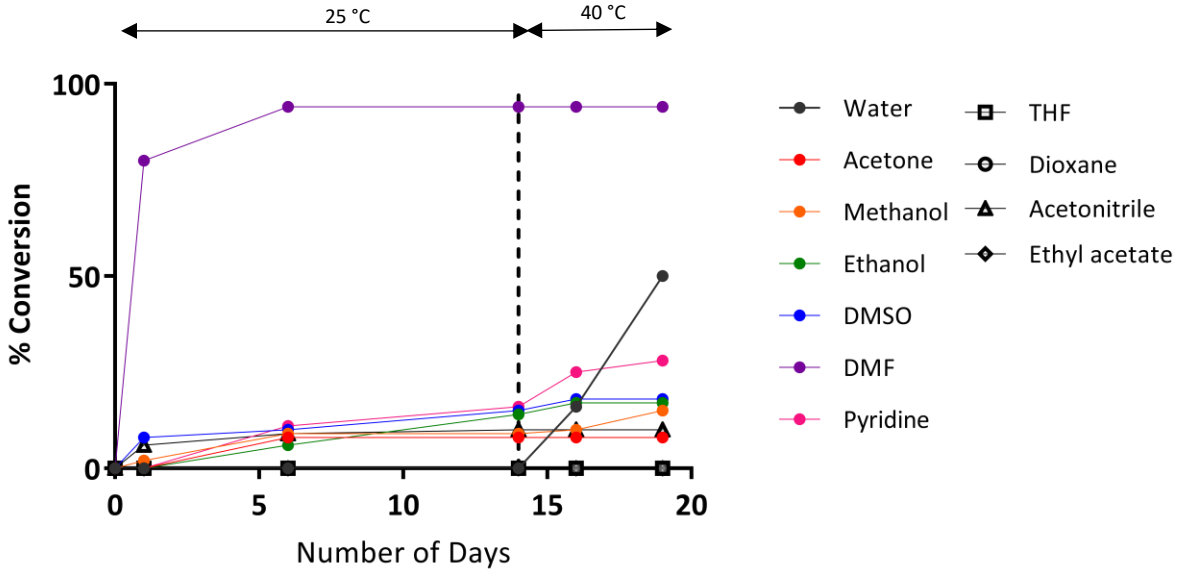


Figure 2.4: Stability of enterocin (**1**) in a variety of solvents over time at room temperature.

2.4. Summary

Enterocin was shown to undergo a unique rearrangement to form a novel scaffold, isoenterocin, *via* the formation of 3-*epi*-enterocin and subsequently the hemiketal of 3-*epi*-enterocin in a novel rearrangement mechanism. This rearrangement was supported by comparison with deoxyenterocin, which lacking the secondary 5-OH moiety cannot re-arrange but nonetheless is capable of forming 3-*epi*-deoyenterocin. Epimer formation was also supported through an initial solvent stability study, which compared the stability of enterocin in acetonitrile (aprotic) and methanol (protic) solvent; mildly acidic, alkaline or neutral conditions; as well as its stability at 60 °C compared to room temperature. Enterocin was shown to be most stable in mildly acidic conditions. A more extensive solvent stability study showed enterocin to be highly unstable in DMF with almost quantitative conversion to isoenterocin after 1 day. On the other hand, enterocin was shown to be stable in water, ethyl acetate, THF and dioxane under standard laboratory conditions. The main chemical instabilities of enterocin relate to its nucleophilic 5-OH and its electrophilic C-2 ketone sites.

3. Semi-synthesis of Enterocin Analogues

3.1. Introduction

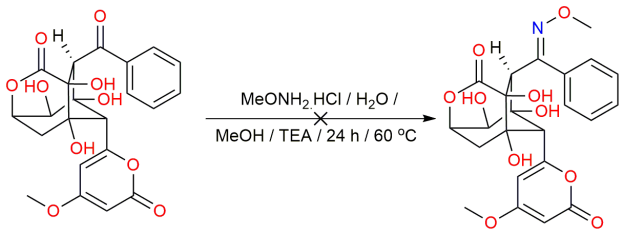
In the previous chapter, enterocin (**1**) was shown to be unstable, undergoing a facile rearrangement to isoenterocin (**16**) from the intramolecular attack of the nucleophilic secondary hydroxy group (5-OH) to the electrophilic carbonyl group (C-2). In this chapter, semi-synthetic modification of **1** was explored at both these sites to assess whether **1** could be stabilised and whether this impacted the bioactivity of the compound. Additionally, derivatives at the 5-OH site were further explored as potential prodrugs, which was inspired by the previous reports of 5-*O*-arachidylenterocin (**3**) and 5-*O*-behenylenterocin (**4**) from a Western Australian marine ascidian.[65]

3.2. Semi-synthetic Derivatives of Enterocin

3.2.1. Semi-synthetic Modification of Enterocin at its C-2 site

In Chapter 1, erythromycin was identified as an antibiotic with an instability relating to its ketone group; hence signifying a similar instability to that observed in **1**. As described, this was overcome through derivatisation of the ketone group to an oxime, followed by Beckmann rearrangement and hydrogenation to a methylamino derivative. This section describes a similar synthetic approach to stabilise the ketone group on enterocin.

Initially, the C-2 carbonyl on **1** was converted to a methyl oxime by reacting **1** with a large excess of methoxylamine hydrochloride in methanol/water (1:1) with triethylamine as the base as depicted by the reaction scheme in Figure 3.1.[92]



1 **18**

Figure 3.1: Reaction scheme for the preparation of enterocin-(*Z*)-2-*O*-methyloxime (**18**)

Rather than the expected product, **18**, 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) was isolated as a white solid. HR-ESI(−)-MS analysis of **19** revealed an ion ($[M-H]^-$ *m/z* 427.1243) indicative of a molecular formula of $C_{23}H_{23}O_{10}N$. The ^1H and ^{13}C NMR data for **19** and **1** were also

very similar. However, **19** contained an additional methoxy group (δ_H 3.80, δ_C 61.6) and did not contain a signal corresponding with the C-2 ketone of **1**, δ_C 194.9 (see Figure 3.2 and Supplementary Material, Table 10.3). The C-2 carbon was more shielded in comparison to **1** with a chemical shift at δ_C 153.1, which is characteristic of an imine. The position of this oxime group was confirmed to be on C-2 with a HMBC correlation from H-21 to C-2.

Detailed analysis of the ROESY spectrum of **19** (Figure 3.2) suggested an inversion of configuration at C-3, with correlations between H-3 and the two spatially close 16-OH and 8-OH groups. Like in 3-*epi*-deoxyenterocin (**5**), there is a large chemical shift change of H-3 from δ_H 4.46 in **1** to δ_H 3.64 in **19**. Furthermore, the imino alkene was determined to have a (Z) configuration due to the presence of a single signal in the ^{1}H NMR spectrum and a single ROESY correlation of H-21 to H-18. Therefore, analysis of 2D NMR spectra led to the assignment of all protons and carbons in **19** and secured the structure.

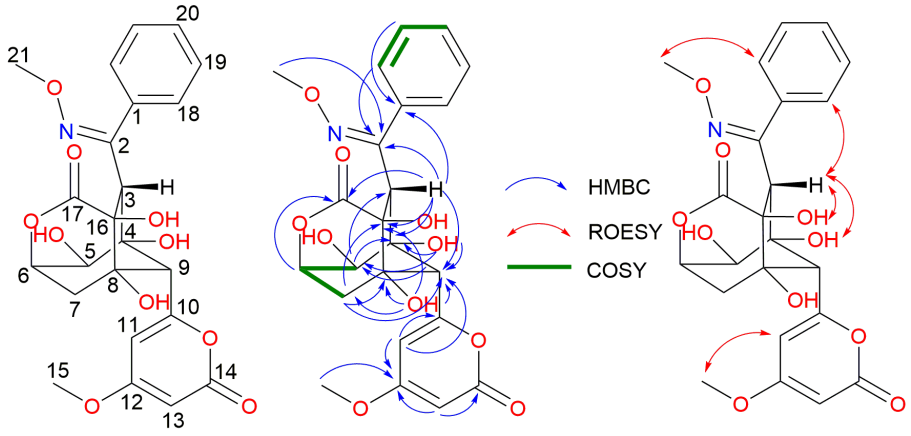


Figure 3.2: Key 2D NMR correlations observed in 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**)

Whilst retention of the configuration of the C-3 carbon was preferred, the basic conditions of the reaction encouraged epimerisation of C-3 leading to 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) as the major product. Interestingly, as the desired product, enterocin-(*Z*)-2-*O*-methyloxime (**18**), was not observed by LCMS, it can be inferred that nucleophilic attack of the C-2 carbonyl is favoured when 3-*epi*-enterocin (**17**) is formed first. This provides further evidence for epimerisation of C-3 as the first step in the formation of isoenterocin (**16**) from **1**.

3.2.2. Semi-synthetic Modification of Enterocin at its 5-OH site

Aside from derivatising the electrophilic carbonyl group on **1**, derivatisation at the nucleophilic secondary hydroxy group, 5-OH, was also employed in an attempt to stabilise the molecule. As observed in deoxyenterocin (**2**), protecting or eliminating this hydroxy group should limit the ability of **1** to rearrange; ideally only forming the C-3 epimer. Another reason for the generation of 5-OH esters of **1** was inspired by Nature, with the previous isolation of 5-*O*-arachidylenterocin (**3**) and 5-*O*-behenylenterocin (**4**), featuring C_{20} and C_{22} fatty acid chains. It was hypothesised that these esters were produced in Nature to act as prodrugs whereby the long side chain helps to stabilise **1** which is subsequently hydrolysed to the active molecule at the target location. Furthermore, these fatty acid chains may make **1** more non-polar for persisting the marine environment.

Esterification at the 5-OH site should be preferred over the three other tertiary hydroxy groups due to the enhanced nucleophilicity of secondary hydroxy groups. To esterify **1**, two different methods were employed: i. reaction with an anhydride, ii. Steglich esterification with DCC and a carboxylic acid. The reaction schemes used to esterify **1** are shown in Figure 3.3. Reactions with acid chlorides were also trialled but were unsuccessful in all cases. Even with the excess addition of acid chloride, LCMS indicated that the expected product was only produced to a minor extent, with the starting material recovered close to 100%.

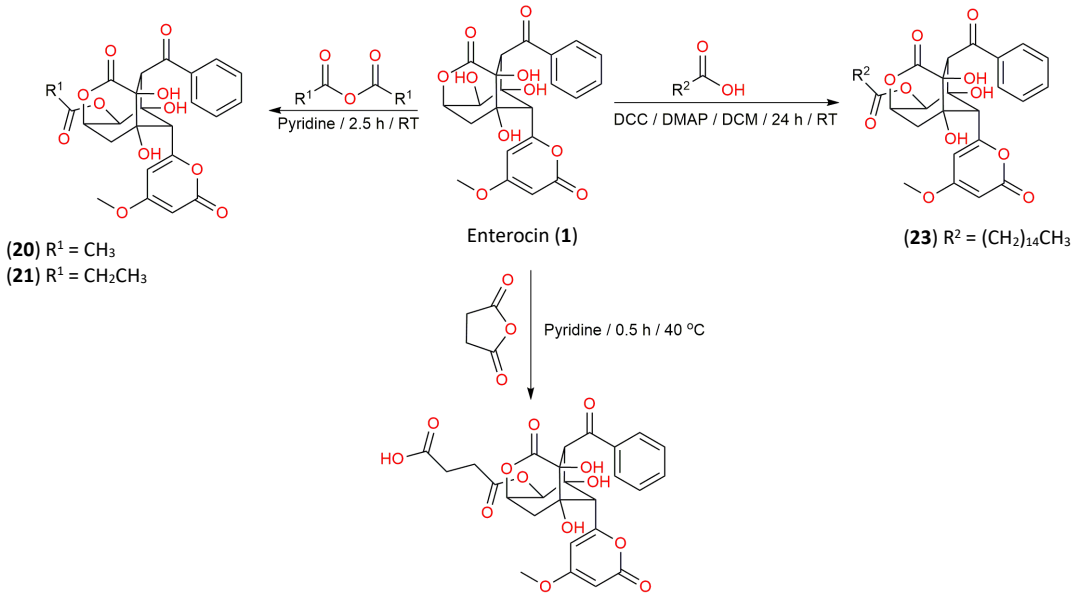


Figure 3.3: Reaction schemes for the preparation of enterocin analogues.

3.2.2.1. 5-O-Acetylenterocin (20)

Acetic anhydride was added to a chilled solution of **1** in pyridine to afford 5-*O*-acetylenterocin (**20**). After purification, **20** was obtained as a white solid and its structure confirmed by NMR (see Figure 3.4 and Supplementary Material, Table 10.3). HR-ESI(−)-MS analysis of **20** revealed an ion ($[M-H]^-$ *m/z* 485.1083) indicative of a molecular formula of $C_{24}H_{22}O_{11}$. The ^{1}H and ^{13}C NMR data for **20** and **1** were very similar. However, **20** only showed three signals corresponding to hydroxy groups in its ^{1}H NMR spectrum rather than the four present in **1**. Additionally, **20** has new signals corresponding with a methyl group (δ_H 2.11, δ_C 20.9) and an ester carbonyl (δ_C 169.8), providing further evidence for the successful addition of an acetyl group.

The position of this acetyl group was determined to be on C-5 since H-5 showed a HMBC correlation to C-21. The chemical shift of H-5 (δ_H 5.73) was also more deshielded in **20** compared to **1** (δ_H 4.46), further highlighting the addition of an acetyl group on C-5. The ROESY correlation between H-3 and H_3-22 further confirms this position due to their proximity in space. Despite using pyridine in the reaction, epimerisation of C-3 was not observed with no distinctive ROESY correlations like that observed for 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) nor the typical large chemical shift change of H-3 evident in 3-*epi*-deoxyenterocin (**5**). The lack of epimerisation, here, is likely a result of the anhydrous conditions used. Therefore, analysis of 2D NMR spectra led to the assignment of all protons and carbons in 5-*O*-acetylenterocin (**20**) and secured the structure.

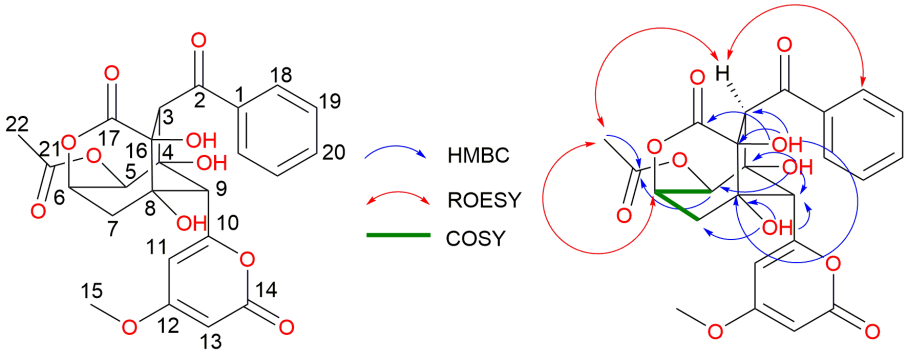


Figure 3.4: Key 2D NMR correlations observed in 5-*O*-acetylenterocin (**20**)

3.2.2.2. 5-O-Propionylenterocin (21)

Propionic anhydride was added to a chilled solution of **1** in pyridine to afford 5-*O*-propionylenterocin (**21**). After purification, **21** was obtained as a white solid and its structure

confirmed by NMR (see Figure 3.5 and Supplementary Material, Table 10.4). HR-ESI(−)-MS analysis of **21** revealed an ion ([M−H]$^-$ *m/z* 499.1241) indicative of a molecular formula of $C_{25}H_{24}O_{11}$. The ^{1}H and ^{13}C NMR data for **21** and **1** were also very similar. As for 5-*O*-acetylenterocin (**20**), **21** only showed three signals corresponding to hydroxy groups in its ^{1}H NMR spectrum as well as having new signals corresponding with a propyl group at C-22 (δ_H 2.43, δ_C 26.8) and C-23 (δ_H 1.04, δ_C 8.8), and an ester carbonyl (δ_C 172.9), providing further evidence for the successful addition of a propionyl group. The position of the propionyl group was determined in a similar manner as conducted for **20**. Therefore, analysis of 2D NMR spectra led to the assignment of all protons and carbons in 5-*O*-propionylenterocin (**21**) and secured the structure.

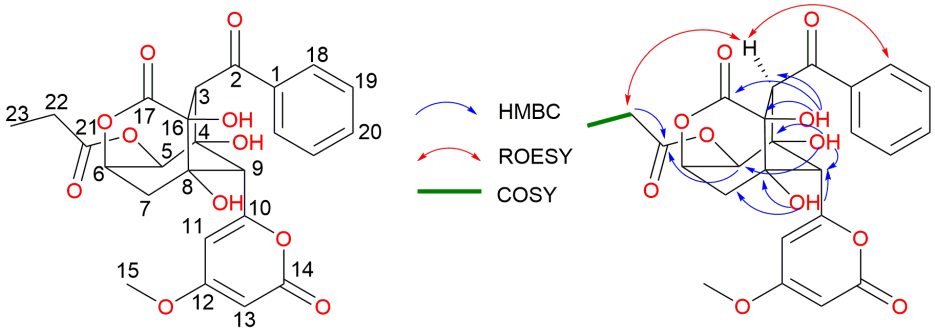


Figure 3.5: Key 2D NMR correlations observed in 5-*O*-propionylenterocin (**21**)

3.2.2.3. 5-O-Carboxypropionylenterocin (22)

Succinic anhydride was added to a solution of **1** in pyridine to afford 5-*O*-carboxypropionylenterocin (**22**). After purification, **22** was obtained as white needles and its structure confirmed by NMR (see Figure 3.6 and Supplementary Material, Table 10.5). HR-ESI(−)-MS analysis of **22** revealed an ion ([M−H]$^-$ *m/z* 543.1142) indicative of a molecular formula of $C_{26}H_{24}O_{13}$. The ^{1}H and ^{13}C NMR data for **22** and **1** were also very similar. As for 5-*O*-acetylenterocin (**20**), **22** only showed three signals corresponding to hydroxy groups in its ^{1}H NMR spectrum as well as having additional signals corresponding to H-22 (δ_H 2.47, δ_C 28.7) and H-23 (δ_H 2.63, δ_C 28.7), a carboxylic acid (δ_H 12.23, δ_C 171.6) and an ester carbonyl (δ_C 173.0). These additional signals corresponded with those expected for **22** and did not indicate any further intramolecular reaction of the new carboxylic acid functional group. Unfortunately, this signal did not show any diagnostic correlations to other atoms in **22**. The protons on C-23, however, showed ROESY correlations to both H-3 and H-5 and a HMBC correlation to C-24. The position of C-21, however,

could not be confirmed due to the absence of HMBC correlations to C-21 from protons on the enterocin core structure.

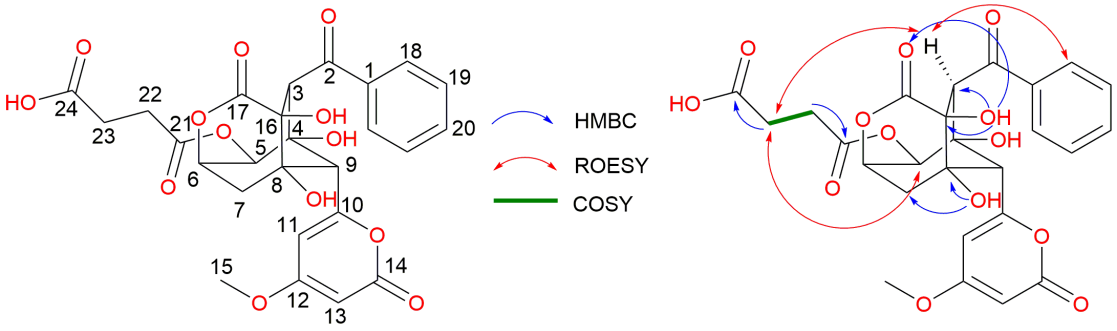


Figure 3.6: Key 2D NMR correlations observed in 5-*O*-carboxypropionylenterocin (**22**)

*3.2.2.4. 5-O-Palmitylenterocin (**23**)*

Palmitic acid was added to a solution of **1** in dichloromethane with the coupling reagent, DCC, and DMAP catalyst. After purification, 5-*O*-palmitylenterocin (**23**) was obtained as a white solid and its structure confirmed by NMR (see Figure 3.7 and Supplementary Material, Table 10.6). HR-ESI(−)-MS analysis of **23** revealed an ion ($[M-H]^-$ *m/z* 681.3279) indicative of a molecular formula of $C_{38}H_{50}O_{11}$. The ^1H and ^{13}C NMR data for **23** and **1** were also very similar. As for 5-*O*-acetylenterocin (**20**), compound **23** only showed three signals corresponding to hydroxy groups in its ^1H NMR spectrum as well as new signals corresponding to the addition of a palmityl group. These include C_α-22 at (δ_H 2.39, δ_C 33.5), C-23-C-35 (δ_H 1.23, δ_C 29.0) and C-36 (δ_H 0.85, δ_C 13.9). The position of the palmityl group was determined in a similar manner to **20**. Therefore, analysis of 2D NMR spectra led to the assignment of all protons and carbons in 5-*O*-palmitylenterocin (**23**) and secured the structure.

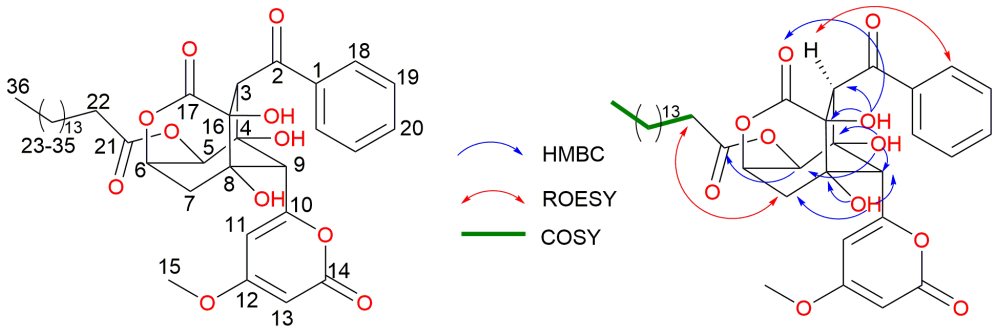


Figure 3.7: Key 2D NMR correlations observed in 5-*O*-palmitylenterocin (**23**)

3.3. Solvent Stability Study of Semi-Synthetic Enterocin Analogues

The stability study conducted in Section 2.3 was also repeated for the two semisynthetic esters of **1**, 5-*O*-acetylenterocin (**20**) and 5-*O*-propionylenterocin (**21**) (see Supplementary Material Section 10.4). The two semisynthetic esters of **1** showed improved stability when compared to **1**, with **21** being the most stable. Both analogues however, were still highly unstable in basic conditions. Interestingly, both esters were hydrolysed at room temperature in the protic solvent methanol but not in the aprotic solvent acetonitrile, thus avoiding the formation of isoenterocin (**16**) that was shown to occur for **1**. The two esters were also relatively stable under acidic conditions at room temperature but not at 60 °C, hydrolysing back to **1**. Hydrolysis was not observed in acetonitrile at 60 °C for either ester, showing their enhanced stability over **1**. This improved stability highlights the possibility for these esters to act as prodrugs, with hydrolysis to the active drug only occurring under protic conditions, with inhibition of **16** formation under aprotic conditions. Compounds **20** and **21** also inhibited the rearrangement of **1** to **16** in DMF, a conversion that occurred in almost quantitative yields after 48 h. This further supports the possibility of these esters of **1** being used as prodrugs.

3.4. Summary

This chapter described the semi-synthesis of five enterocin analogues as a means of stabilising the natural compound. The structures of all derivatives were confirmed by 2D NMR spectroscopy and HR-MS. Derivatisation of the 5-OH site of enterocin led to the production of more stable versions of the compound, giving precedent for the biosynthetic esters of enterocin produced in Nature. The biological activity of these analogues will be useful to further understand enterocin's pharmacophore and whether inhibition of the degradation of enterocin has impacts on its antibacterial activity. The bioactivity of these analogues was examined in Chapter 5.

4. Optimisation of Cultivation Conditions for *Streptomyces* sp.

4.1. Introduction

This chapter describes the isolation of new co-metabolites of enterocin through analysis of the metabolic profiles of *Streptomyces* sp. MST-MA9095 on different culture media. Since supplementation with different media components can result in the biosynthesis of different secondary metabolites,[93] an optimisation was performed to identify the effects of different media. On this basis, one medium was selected for large-scale cultivation, yielding three new metabolites in addition to previously reported compounds. Further exploration of the chemical classes isolated was conducted with a short semi-synthetic study.

4.2. Optimisation of Culture Media for *Streptomyces* sp. MST-MA9095

The Gram-positive bacterium *Streptomyces* sp. MST-MA9095 was isolated by MST from a macro marine sponge sample collected at Sorrento Pier, Port Phillip Heads, Sorrento, VIC, Australia. To optimise the cultivation of the microorganism, various grains (cracked wheat (BL); basmati rice (BS); jasmine rice (JR); and pearl barley (PB)), agars (glycerol casein (AC); glucose, yeast extract, malt extract and soluble starch (GYMS); International *Streptomyces* protocol (ISP2); Bennett's medium modified (MS); and oatmeal (OA)), and liquids (AC; GYMS; ISP2; OA; and N-Z-amine (NZAM)) were inoculated with the resulting suspension of the culture. All media were prepared according to the recipes outlined in Supplementary Material Section 10.1.

4.3. Co-metabolite Profile for *Streptomyces* sp. on Different Media

Growth of *Streptomyces sp.* varied with culture media as shown in Figure 4.1. Subsamples were taken from all culture media at 7, 10 and 14 days of growth.

Figure 4.1: Growth of *Streptomyces sp.* after 7 days at 28 °C on: **A.** agars (top: AC, GYMS; bottom: ISP2, MS, OA); **B.** liquids (AC, GYMS, ISP2, NZAM, OA); **C.** grains (BL, BS, JR, PB).

The co-metabolite profiles of *Streptomyces sp.* MST-MA9095 were compared across the different media over a fourteen-day period to assess the production of related co-metabolites to enterocin (**1**). Figure 4.2 A shows a representative HPLC trace for PB media after 14 days. PB media showed the greatest diversity in produced co-metabolites related to **1** as identified through similarities in their UV spectra, a composite of the UV maxima of the two isolated chromophores, benzoate and pyrone, to give highly characteristic maxima at λ_{max} 250 and 283 nm. These co-metabolites belonged to two main classes of natural products: wailupemycins and germicidins. Figure 4.2 B compares the diversity in co-metabolites produced on all grains at Day 14. The production of **1** co-metabolites was the greatest on the grain media tested. MST-MA9095 did not grow as well in both liquid and agar media and hence the production of **1** co-metabolites were minimal. In the media tested, the production of **1** co-metabolites were higher at Day 10 than at Day 7 and were not significantly higher at Day 14.

The production of secondary metabolites on BL media at Day 14 was the lowest of all the grain media tested with 30-50% lower production compared to on PB. Whilst cultivation on BS and JR media provided increased production of deoxyenterocin (**2**), wailupemycin F (**9**) and wailupemycin G (**10**), the production of minor secondary metabolites was 40-80% lower than that demonstrated on PB. PB media was selected as the optimal medium for large-scale cultivation due to the higher abundance of secondary metabolite production.

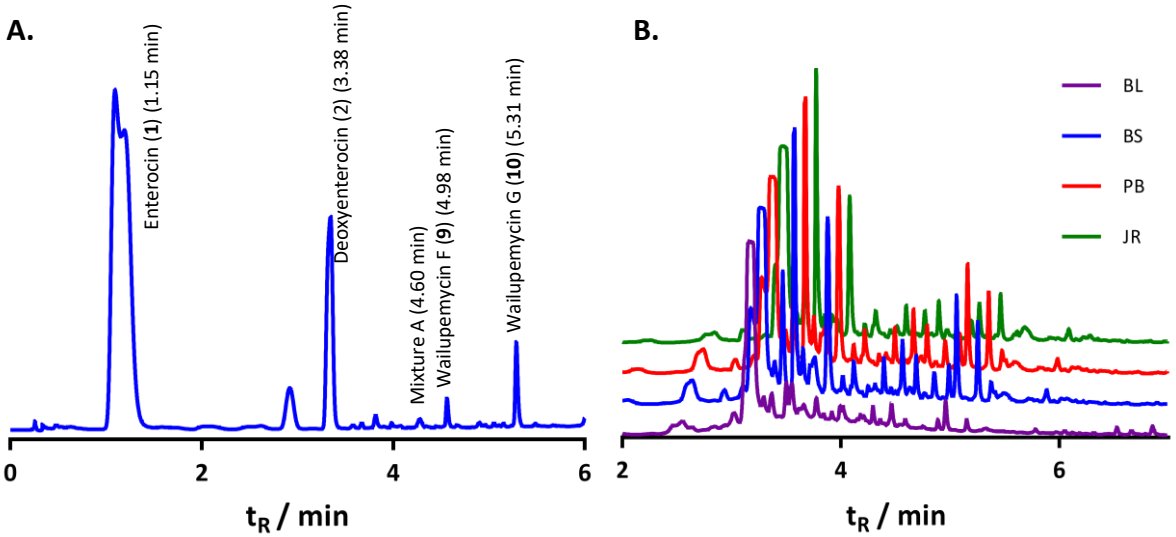


Figure 4.2: A. HPLC trace at 210 nm for a crude extract of MST-MA9095 after cultivation on PB for 10 days. Mixture A includes *O*-methylgermicidin D, germicidin A, *p*-hydroxywailupemycin F, and *p*-hydroxywailupemycin G. **B.** HPLC traces at 210 nm for methanol extracts of MST-MA9095 grown on all grain media for 14 days. Note: HPLC traces for **A.** and **B.** used different columns.

4.4. Characterisation of Co-metabolites of Enterocin in *Streptomyces* sp.

4.4.1. *p*-Hydroxywailupemycin F (24)

p-Hydroxywailupemycin F (**24**) was isolated as a yellow oil. HR-ESI(−)-MS analysis of **24** revealed an ion ([M−H]$^-$ *m/z* 379.0822) indicative of a molecular formula of $C_{21}H_{16}O_7$ requiring 14 DBEs. The molecular mass of **24** was 16 Da more than that of reported compound wailupemycin F (**9**) (molecular formula $C_{21}H_{16}O_6$), which suggested that **24** was a hydroxylated derivative of **9**. The ^{1}H and ^{13}C NMR data for **24** and **9** were also very similar. However, **24** had an additional signal in its ^{1}H NMR spectrum at δ_H 9.66 providing further evidence for the presence of a hydroxy group.

In the ^{1}H NMR spectrum, wailupemycin F (**9**) showed three coupled aromatic ^{1}H signals with a ratio of 2:2:1 indicating the presence of a mono-substituted phenyl ring. In *p*-hydroxywailupemycin F (**24**), however, only two coupled aromatic ^{1}H signals with a ratio of 2:2 was observed. *Para*-substitution was confirmed by the doublet appearance of these two aromatic signals at δ_H 7.07 and δ_H 6.73 (see Supplementary Material, Table 10.7). The configuration of C-9 has not been previously reported. A Mosher ester analysis will be employed in the future to determine the configuration at this site.[94] Therefore, analysis of 2D NMR spectra, as well as the data correlation with those of wailupemycin F (**9**) led to the assignment of protons and carbons in *p*-hydroxywailupemycin F (**24**) and secured the structure.

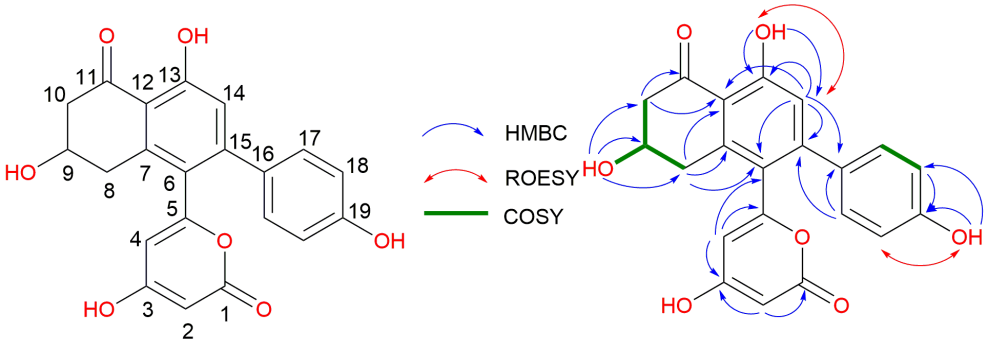


Figure 4.3: Key 2D NMR correlations observed in *p*-hydroxywailupemycin F (**24**)

4.4.2. *p*-Hydroxywailupemycin G (25)

p-Hydroxywailupemycin G (**25**) was isolated as a yellow oil. HR-ESI(−)-MS analysis of **25** revealed an ion ([M−H]$^-$ *m/z* 361.0716) indicative of a molecular formula of $C_{21}H_{14}O_6$ requiring 15 DBEs. The molecular mass of **25** was 16 Da more than that of reported compound wailupemycin G (**10**) (molecular formula $C_{21}H_{14}O_5$), which suggested that **25** was a hydroxylated derivative of **10**. The ^{1}H and ^{13}C NMR data for **25** and **10** were also very similar. However, **25** had an additional signal

in its ^{1}H NMR spectrum at δ_H 9.57, similarly deshielded as for *p*-hydroxywailupemycin F (**24**), providing further evidence for the presence of a hydroxy group.

In the ^{1}H NMR spectrum, wailupemycin G (**10**) showed three coupled aromatic ^{1}H signals with a ratio of 2:2:1 indicating the presence of a mono-substituted phenyl ring. In *p*-hydroxywailupemycin G (**25**), however, only two coupled aromatic ^{1}H signals with a ratio of 2:2 was observed. *Para*-substitution was confirmed by the doublet appearance of these two aromatic signals at δ_H 6.75 and δ_H 7.10 (see Supplementary Material, Table 10.9). Therefore, analysis of 2D NMR spectra, as well as the data correlation with those of wailupemycin G (**10**) led to the assignment of protons and carbons in *p*-hydroxywailupemycin G (**25**) and secured the structure.

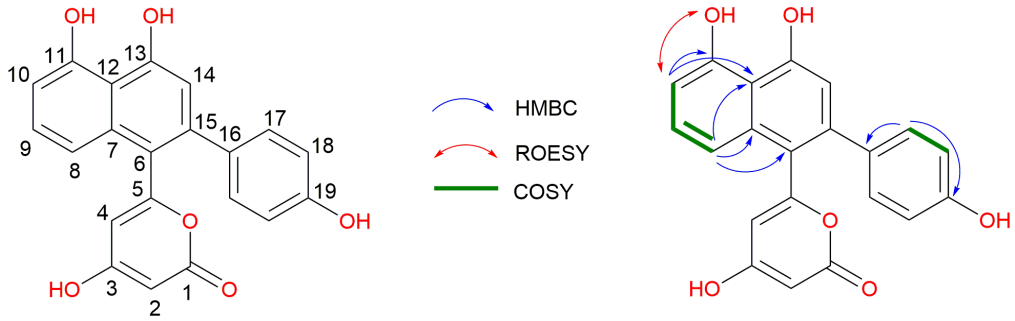


Figure 4.4: Key 2D NMR correlations observed in *p*-hydroxywailupemycin G (**25**)

4.4.3. *O*-Methylgermicidin D (26)

O-Methylgermicidin D (**26**) was isolated as a white solid along with the coeluting known compound, germicidin A (**27**), in a 1:2 ratio. HR-ESI(+)-MS analysis of **26** revealed an ion ($[M+H]^+$ *m/z* 183.1021) indicative of a molecular formula of $C_{10}H_{14}O_3$ requiring 4 double bond equivalents (DBEs). The molecular mass of **26** was 14 Da more than that of germicidin D (**30**) (molecular formula $C_9H_{12}O_3$) suggesting methyl-substitution. Despite germicidin B (**30**) and germicidin C (**29**) also sharing the same molecular formula as **26**, neither possess a methoxy group, as evident in the ^{1}H NMR spectrum for **26**. Compound **26** also has the same two alkyl substitutions on its 2-pyrone at C-2 and C-5 as **30**, differing to that of **27**.

Due to the scarcity of **30**, compound **27** was used a model compound for comparison of spectroscopic data. The reported NMR spectra for **30** were obtained in $CDCl_3$, whereas the NMR spectra for **26** were acquired in DMSO-d_6. Apart from the differences in alkyl substitution, the ^{1}H and ^{13}C NMR data for **26** and **27** were very similar. The main difference was the replacement of a hydroxy group at δ_H 11.19 with a methoxy group at δ_H 3.94 (see Supplementary Material, Table

10.8). Therefore, analysis of 2D NMR spectra, as well as the data correlation with those of germicidin A (**27**) led to the assignment of protons and carbons in *O*-methylgermicidin D (**26**) and secured the structure.

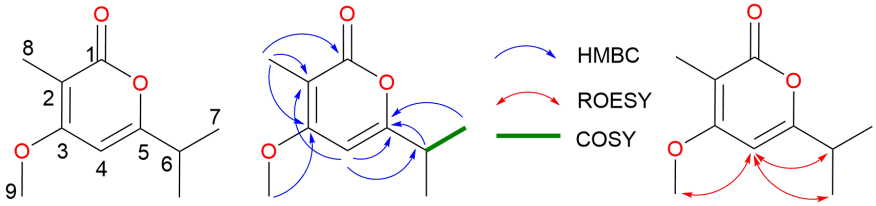


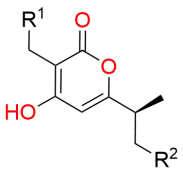
Figure 4.5: Key 2D NMR correlations observed in *O*-methylgermicidin D (**26**)

4.5. Isolation of Previously Reported Compounds

Wailupemycin F (**9**), wailupemycin G (**10**), and germicidin A (**27**) were also isolated as known compounds and characterised spectroscopically with reference to the literature.[81, 95] Additionally, two other impure compounds were isolated in this study that have yet to be characterised.

4.6. Methylation of α-pyrones

In the following chapter, *O*-methylgermicidin D (**26**) was shown to exhibit high antibacterial activity against both *Micrococcus luteus*, whereas the germicidin A (**27**) component of the mixture was shown to be inactive. Because germicidin D (**30**) was absent from the culture and not readily available from the MST library of compounds, germicidins A-C (**27**-**29**) were used as model compounds for methylation (Figure 4.7). Methylation was initially trialled on a model α-pyrone system, 4-hydroxy-6-methyl-*2H*-pyran-2-one, using stoichiometric equivalents of iodomethane with triethylamine in acetonitrile.[96] These reaction conditions, however, did not yield the desired product, nor did the addition of excess iodomethane. The literature precedent for this reaction used bromomethane rather than iodomethane and may hence be a contributor to the unsuccessful nature of the reaction.



Germicidin A (**27**)	$R^1 = CH_3$	$R^2 = CH_3$
Germicidin B (**28**)	$R^1 = CH_3$	$R^2 = H$
Germicidin C (**29**)	$R^1 = H$	$R^2 = CH_3$
Germicidin D (**30**)	$R^1 = H$	$R^2 = H$

Figure 4.6: Structures of germicidin analogues.

Next, methylation of the α-pyrone was trialled using excess methanol and 2 drops of concentrated sulfuric acid as the catalyst.[97] This produced the desired product after heating at

60 °C for 3 days. These conditions were repeated for pure **27**, and a mixture of two related α-pyrones with a molecular weight of 182 (presumably **28** and **29**) purified from an enriched fraction of germicidins. However, whilst the methylation of **27** was possible, the methylation products of **28** and **29** was not successfully produced in significant yields.

4.6.1. *O*-Methylgermicidin A (31)

O-Methylgermicidin A (**31**) was isolated as a colourless oil. HR-ESI(+)-MS analysis of **31** revealed an ion ($[M+H]^+$ *m/z* 211.1331) indicative of a molecular formula of $C_{12}H_{18}O_3$ requiring 4 DBEs. The mass of **31** was 14 Da more than that of germicidin A (**27**) (molecular formula $C_{11}H_{16}O_3$) indicating methyl-substitution. The ^1H and ^{13}C NMR data for **31** and **27** was also very similar. However, **31** had an additional signal in its ^1H NMR at δ_H 3.89, providing further evidence for the presence of a methoxy group, in place of a signal at δ_H 11.19 as present in **27**, representing the loss of a hydroxy group (see Figure 4.7 and Supplementary Material, Table 10.10). Therefore, analysis of 2D NMR spectra, as well as the data correlation with those of germicidin A (**27**) led to the assignment of protons and carbons in *O*-methylgermicidin A (**31**) and secured the structure.

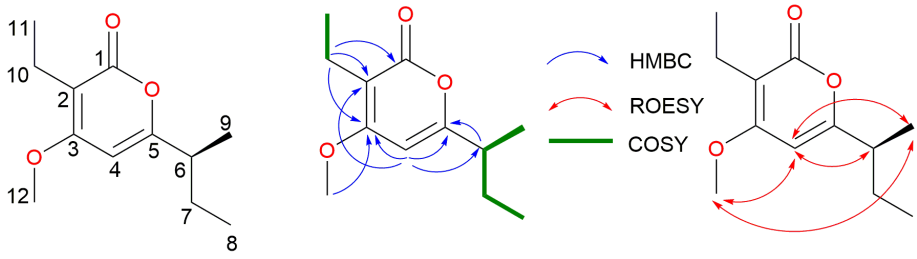


Figure 4.7: Key 2D NMR correlations observed in *O*-methylgermicidin A (**31**)

4.7. Summary

An optimisation of culture media for the enhanced production of enterocin co-metabolites was performed with large-scale cultivation conducted on *Streptomyces* sp. MST-MA9095 on pearl barley (PB) media for 10 days. Three new compounds were isolated, *p*-hydroxywailupemycin F, *p*-hydroxywailupemycin G and *O*-methylgermicidin D, in addition to three previously reported compounds. Though the metabolites isolated comprised three structural scaffolds, collectively they belonged to a single chemical class, α-pyrones. This unusual coincidence of co-metabolites is explored further in Chapters 5 and 6.

5. The Biological Activity of Enterocin and Related Analogues

5.1. Introduction

This chapter describes the intrinsic activity and selectivity of enterocin (**1**), related analogues and co-metabolites as antibacterial agents. Existing literature presents a confusing *in vitro* bioassay proposition with previous researchers highlighting a "non-dilutable" behaviour for the antibacterial activity of enterocin; electing instead to perform disc diffusion assays with 4 mg loads.[62] In dilution bioassays, **1** has only shown antibiotic activity against a single microorganism, *Micrococcus luteus*[78], and for this purpose, *M. luteus* was found to be a very useful model microorganism for the comparison of antibiotic activity. All biological assays described herein were performed and analysed by the candidate.

5.2. Antibacterial Assay for Enterocin

Consistent with other researchers, enterocin (**1**) was found to be an "enigmatic" antibacterial agent with disc diffusion assays showing no activity against the Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) microorganisms tested with **1** (100 μg). Microtitre dilution assays against these microorganisms also demonstrated no activity for **1** (250 = μg/mL). On the other hand, **1** was confirmed to be active against *M. luteus* in both biological assays performed (MIC = 31 μg/mL, 60% growth inhibition, zone of inhibition = 39.5 ± 0.5 mm). Deoxyenterocin (**2**), also showed biological activity against *M. luteus* (MIC 47 = μg/mL, 40% growth inhibition, zone of inhibition = 38 ± 1 mm). The activity of both compounds was absent after 48 hours suggesting that their inherent instabilities prevented them from retaining concentrations above their high MIC values.

Concurrently, other biological experiments were conducted to explore the potential mode of action of enterocin. One such study was a synergism study using **1** (62.5 μg/mL) to supplement antibiotic standards in microtitre dilution assays against *B. subtilis, E. coli* and *S. aureus.* In some cases, combinations of drugs can have enhanced antibacterial activity due to having different mechanisms of action than if they were administered separately.[98] Because of this, antibiotic synergism is often used as a strategy against antibiotic resistant infections. For both *B. subtilis* and *E. coli*, gentamicin and polymyxin B were used as antibiotic standards, whereas gentamicin and clarithromycin were used against *S. aureus*. No evidence of synergism by **1** was observed for

the antibiotics used against *E. coli* and *S. aureus*. However, **1** showed a positive enhancement of antibacterial activity when added to polymyxin B, but not gentamicin, against *B. subtilis.* The activity of polymyxin B (MIC = 0.31 μg/mL) supplemented with **1** showed a 2 to 4-fold potency increase in bactericidal activity (MIC = 0.08-0.16 μg/mL). To complement this finding, a dose-response assay was performed for polymyxin B against *B. subtilis* supplemented with **1** (31.25-500 μg/mL). No dose-response was observed with significant changes in the concentration of **1**, despite all supplements providing a synergistic antibacterial effect with polymyxin B.

To confirm whether **1** was acting as a synergist *in situ* by the producing culture *Streptomyces* sp. MST-MA9095, **1** (62.5 μg/mL) was added to two-fold diluted wells of wailupemycin F (**9**) (250-0.12 μg/mL) in a similar manner to polymyxin B above. Similarly, a 2-fold potency increase was observed against *M. luteus* after 48 hours. The production of synergistic co-metabolites is not an uncommon microbial defence mechanism.[99]

5.3. Antibacterial Activity of Enterocin Analogues

All semi-synthetic analogues of **1** showed lower antibacterial activity compared to **1** as shown in Table 5.1. Furthermore, bioactivity decreased with longer ester chain lengths as can be seen with the difference in bioactivity between the shorter 5-*O*-acetylenterocin (**20**) and 5-*O*-propionylenterocin (**21**) to the longer ester chain lengths of 5-*O*-carboxypropionylenterocin (**22**) and 5-*O*-palmitylenterocin (**23**). Evidently, the presence of a hydroxy group at C-5 is important but not essential (as is the case for deoxyenterocin) for the biological activity of enterocin. The negative charge on **22** and the increased non-polarity of **23** were also shown to be insignificant factors to the **1** pharmacophore. Whilst 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) showed a reduction in antibacterial activity compared to **1**, it was not determinable whether the chemical modification or the epimerisation was responsible.

The rearrangement product of **1**, isoenterocin (**16**) showed a loss of activity compared to **1** against *M. luteus*, indicating that some or all of the disrupted elements of the **1** pharmacophore were essential for bioactivity. In particular, the loss of the secondary hydroxy group, 5-OH, as well as the change in configuration at C-3 were shown to result in reduced activity compared to **1** as shown by the semi-synthetic analogues. However, disruption of the lactone ring on **1** may also be a contributing factor to the complete loss of activity.

The biosynthetic analogues of **1** showed more promise as antibiotics than **1** itself, suggesting that the activity of **1** and its analogues stems from the presence of an α-pyrone moiety. In particular, the wailupemycins tested had bactericidal activity. with MICs <10 μg/mL in all cases except for wailupemycin G (**10**). Wailupemycin F (**9**) was the most active of the wailupemycins tested with an MIC of 1.1 μM. The presence of a *para*-substituted hydroxy group, as is the case of *p*-hydroxywailupemycin F (**24**), decreases the activity of the molecule. This may suggest that polarity plays a significant role in the biological activity of these molecules. In contrast to **1**, the antibacterial activity of the wailupemycins did not show evidence of depletion over a 48-hour period, suggesting an improved stability.

The new germicidin, *O*-methylgermicidin D (**26**), tested as a mixture with 2 parts germicidin A (**27**), showed the greatest activity out of all the analogues tested with an MIC of 1.1 μM. A mixture was tested here due to the coelution of the two compounds during separation. Germicidin A (**27**) was also tested separately and not shown to be a contributing factor towards the bioactivity of the mixture. In order to explain the activity observed for **26,** *O*-methylgermicidin A (**31**) was used as a model compound due to the scarcity of germicidin D (**30**). Compound **31** was shown to be more active than **27** against *M. luteus*, however, this activity is insignificant (MIC = 120 μM) and does not sufficiently explain the antibacterial activity observed for **26.**

Table 5.1: MIC values for enterocin analogues against *M. luteus*

Compound	MIC (μM)
Enterocin (**1**)	7.0
Deoxyenterocin (**2**)	11
Isoenterocin (**16**)	>50
3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**)	26
5-*O*-Acetylenterocin (**20**)	26
5-*O*-Propionylenterocin (**21**)	25
5-*O*-Carboxypropionylenterocin (**22**)	46
5-*O*-Palmitylenterocin (**23**)	>35
Wailupemycin F (**9**)	1.1
Wailupemycin G (**10**)	>70
p-Hydroxywailupemycin F (**24**)	2.1
p-Hydroxywailupemycin G (**25**)	2.2
Germicidin A (**27**)	>125
Germicidin A/*O*-methylgermicidin D (**26**) (2:1)	1.1
O-Methylgermicidin A (**31**)	120
Gentamicin	1.7

5.4. Cytotoxicity and Selectivity Assay of Enterocin and Related Analogues

Enterocin (**1**), deoxyenterocin (**2**), 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**), wailupemycin F (**9**), wailupemycin G (**10**), *p*-hydroxywailupemycin F (**24**) and *p*-hydroxywailupemycin G (**25**) were tested against mouse NS-1 myeloma cells for cytotoxicity against myeloma cells and against neonatal foreskin fibroblast (NFF) cells for cytotoxicity against mammalian cells. Whilst enterocin (**1**), deoxyenterocin (**2**), 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) and wailupemycin G (**10**) were inactive against the tested cell lines, the remaining wailupemycins showed moderate activity against both of the cell lines (Table 5.2). The cytotoxic compounds tested were also twice as selective for NFF cells than NS-1 cells. The selectivity ratios also indicate that the compounds tested were an order of magnitude more selective for bacteria over mammalian cells.

Table 5.2: LD_{99} values for enterocin analogues against NS-1 and NFF cell lines.

Compound	**LD_{99} (μM)**		**Selectivity Ratio**	
	NS-1	**NFF**	**NS-1/NFF**	**NS-1/MIC-*M. luteus***
Enterocin (**1**)	>50	>50	-	-
Deoxyenterocin (**2**)	>50	>50	-	-
3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**)	>50	>50	-	-
Wailupemycin F (**9**)	8.6	4.3	2.0	7.8
Wailupemycin G (**10**)	>70	>70	-	-
p-Hydroxywailupemycin F (**24**)	17	8.2	2.1	8.3
p-Hydroxywailupemycin G (**25**)	17	8.2	2.1	7.7
Mitomycin C	0.93	-	-	-

5.5. Summary

Enterocin was confirmed to exhibit weak, yet selective, antibacterial activity against *M. luteus*, with all related semi-synthetic compounds showing reduced activity compared to enterocin. This demonstrates the ineffectiveness of structural modification on the enterocin pharmacophore to produce better antibiotics. On the other hand, the biosynthetically related compounds demonstrated significantly improved activities over enterocin with a similar selectivity. Importantly, the bioassay data strongly supports the hypothesis that in Nature enterocin is not driving the antibacterial activity of the producing *Streptomyces* but acts to augment the activity of the other co-metabolites, as exemplified by its synergism with wailupemycin F. This leaves unaddressed the puzzle of why the microorganism invests so much effort into producing high levels of a structurally unique secondary metabolite. In Nature, enterocin likely demonstrates a different ecological role which was further explored in the following chapter.

6. Mode of Actions Studies for Enterocin and Related α-Pyrones

6.1. Introduction

Enterocin (**1**) and related analogues exhibited only weak bacteriostatic activity. The biosynthetic complexity and high levels of production of **1** appear inconsistent with its activity as an antibiotic leading to the hypothesis that **1** may possess an alternative primary ecological role. Often in Nature, an organism will produce multiple evolutionary-derived antimicrobial compounds to ensure protective overlap of biological activity in a broader range of microorganisms.[100] With the synergism observed between **1** and wailupemycin F (**9**), it is highly possible that these α-pyrones may also share a common regulatory role to the germicidin co-metabolites isolated from *Streptomyces* sp. MST-MA9095.

6.2. Interactions of an Enterocin-Producing *Streptomyces*

The mode of action of **1** was explored by first investigating the interactions of the enterocin-producer *Streptomyces* sp. MST-MA9095 by co-culturing with other *Streptomyces*. Figure 6.1 shows HPLC traces for the methanol extracts derived from four strains of *Streptomyces* sp. co-cultured with MST-MA9095. Each of the *Streptomyces* selected was a high and/or talented producer strain producing unrelated known secondary metabolites. Compound **1** was detected in the agar subsamples taken from the co-cultured microorganism closest to MST-MA9095, with decreasing or undetectable concentrations in the subsamples collected further from the interface of the two cultures.

In some cases, minor morphological changes were observed in either MST-MA9095 or the co-cultured microorganism with metabolite production also shown to be affected as evident in the differences to the control HPLC trace in each case. The effect demonstrated through this co-culture experiment was that MST-MA9095 suppressed the secondary metabolism of the other species. It was hypothesised that this observation was a result of its interaction with **1**. While there are many examples of metabolite induction in the literature, there is only a single example of a secondary metabolite selectively inhibiting the metabolites of another species. Thus, the *Streptomyces* metabolite, aflastatin A, has been reported to inhibit the production of aflatoxin by aflatoxigenic fungi.[101] To show that **1** was involved in the suppression of metabolite production, treatment with **1** (25 μg) was also applied to the microorganism in a disc-diffusion format.

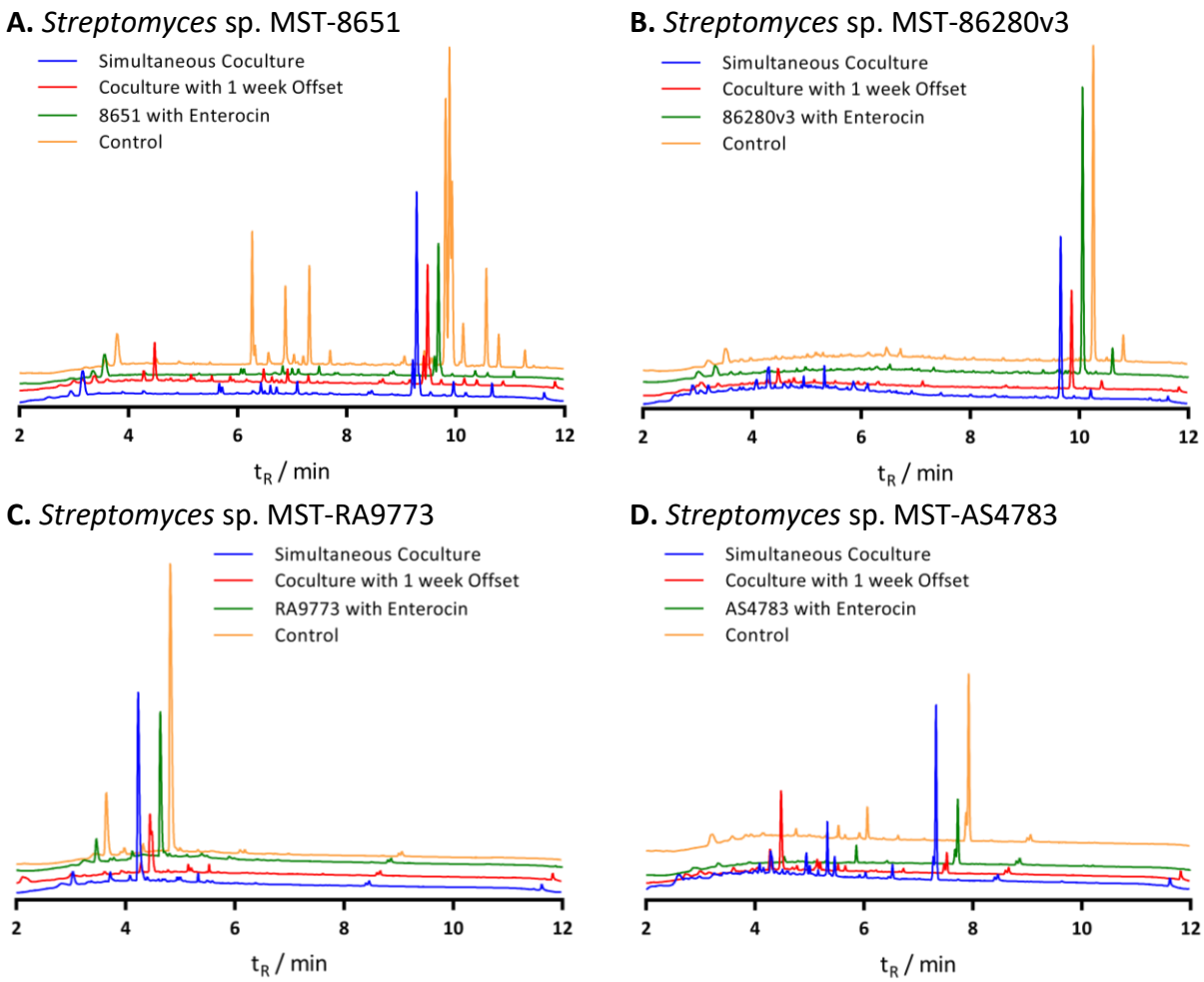


Figure 6.1: HPLC traces for the methanol extracts of **A.** *Streptomyces* sp. MST-8651 (piericidin A producer); **B.** *Streptomyces* sp. MST-86280v3 (oligomycin producer); **C.** *Streptomyces* sp. MST-RA9773 (staurosporine producer); **D.** *Streptomyces* sp. MST-AS4783 (aureothin producer) with 0.2 min offset in retention time and 20 mAU offset in absorbance. In each case, the control trace represents the microorganism of interest grown in isolation and extracted after 1 week; the simultaneous co-culture trace represents an extract of the microorganism of interest co-cultured against MST-MA9095 after 1 week; the co-culture with 1 week offset trace represents an extract of the microorganism of interest grown for 1 week in co-culture against MST-MA9095 that had advantageously grown for 1 week prior to co-culture; the trace with enterocin (**1**) represents an extract of the microorganism of interest after 1 week supplemented initially with **1** (25 μg).

The most notable suppression of metabolism was observed for *Streptomyces* sp. MST-8651 (piericidin A producer). Metabolite production for t_R = 5.5-7 min was almost completely suppressed, whilst the more non-polar metabolites, piericidin A (t_R = 9.3 min) and drimentines A, B, C, and D (t_R = 9.5-11 min) were suppressed only to a moderate extent.[102-103] Interestingly, **1** was shown to have the same effect as that observed for the co-culture assay, suggesting that **1** is the signalling molecule responsible for the observed suppression of metabolite production.

In most cases, the suppression of metabolite production in the microorganism of interest was greater when grown against MST-MA9095 that had been given an advantageous 1 week head-start. This is likely due to the marked increase in **1** and wailupemycin diffused across the agar plate. In *Streptomyces* sp. MST-86280v3, the production of oligomycin A (M = 791, t_R = 9.64 min) was significantly reduced when co-cultured against MST-MA9095. Whilst the addition of **1** only resulted in a minor suppression of the metabolite production, the concentration of **1** used (25 μg) may not have been sufficient to cause the level of suppression observed in the co-culture assay. This difference may well reflect the limited stability of **1** as a pure metabolite, a problem overcome by the steady-state production achieved by a viable and growing culture.

In *Streptomyces* sp. MST-RA9773, the production of staurosporine (M = 466, t_R = 4.22 min) was moderately suppressed when co-cultured against MST-MA9095 and when treated with **1** (25 μg). This suppression was more significant when co-cultured against. MST-MA9095 with a 1 week head-start in growth. In *Streptomyces* sp. MST-AS4383, an interesting case of aureothin (M= 397, t_R = 7.34 min) suppression was indicated. When co-cultured against MST-MA9095, the production of aureothin was unaffected; however, when MST-MA9095 was allowed to grow for 1 week in advance, the production of aureothin was almost completely suppressed. Compound **1** was also shown to result in the suppression of aureothin production.

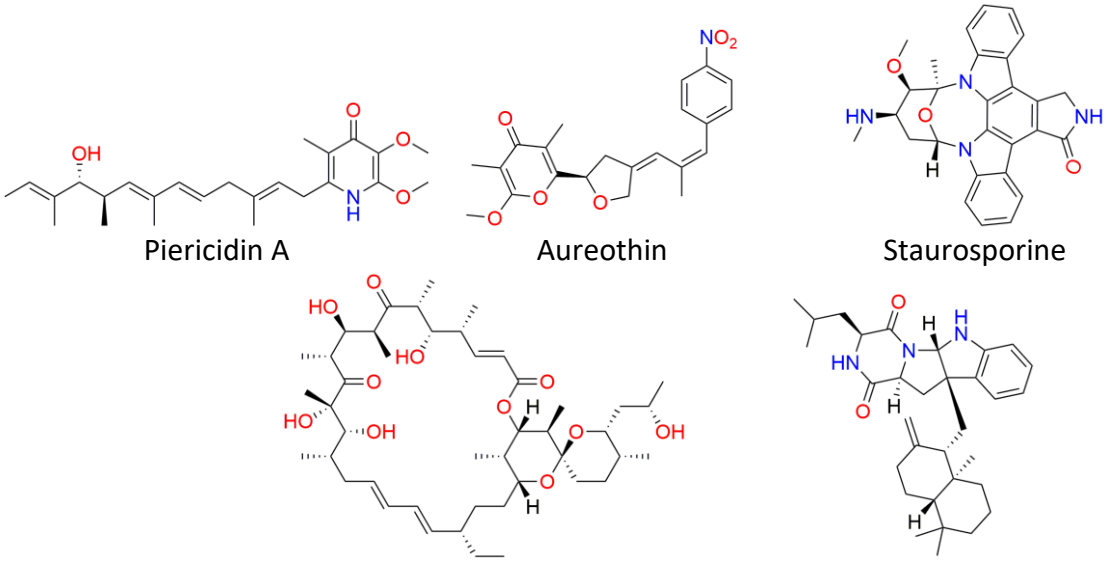


Figure 6.2: Structures of secondary metabolites affected by co-cultivation with MST-MA9095.

The morphological and metabolic changes observed after 7 days growth were not exclusive to the microorganism co-cultured with MST-MA9095, suggesting both microorganisms

were competing and/or coexisting synergistically through chemical signalling molecules. The most notable examples were co-cultures of MST-MA9095 with *Streptomyces* sp. MST-AS4783 and *B. subtilis* MST-AS5674. In both cases, sporulation in MST-MA9095 was turned off (Figure 6.3). The metabolic profile of the non-sporulating MST-MA9095, however, suggested an enhanced production of wailupemycins as a response to the introduced stress.

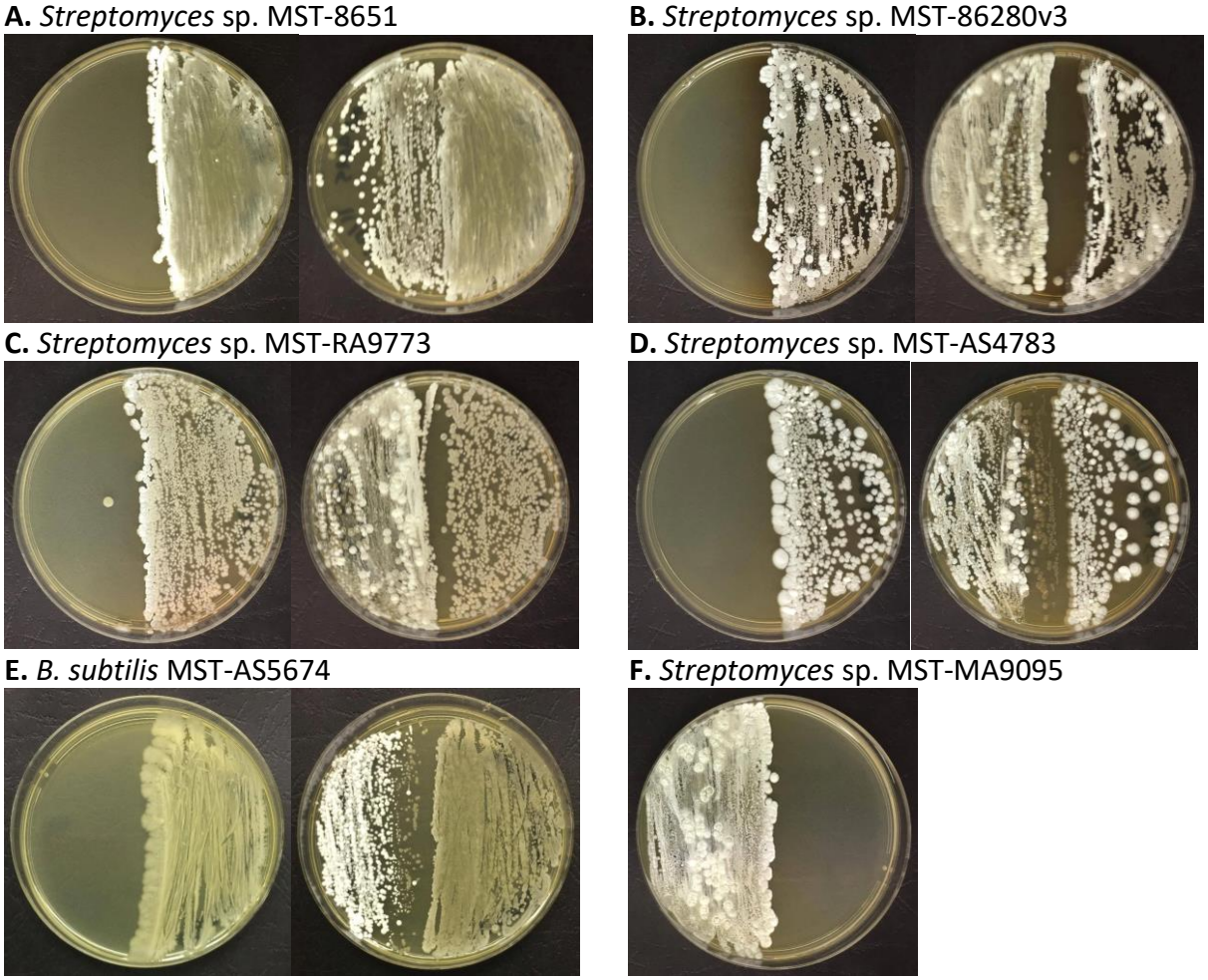


Figure 6.3: Images of co-culture assays after 7 days growth. For each microorganism, the left image corresponds with the microorganism grown by itself (control) and the right image corresponds with an assay of the microorganism of interest (right-side) co-cultured against MST-MA9095 (left-side).

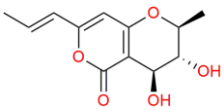
6.3. α-Pyrones as Regulators of Metabolism

Enterocin (**1**) and its related biological analogues all possess a distinct α-pyrone ring system with C-4 and C-6 substitutions. This substitution pattern is common for α-pyrones due to the reactivity of the carbonyl to hydroxide ions and the induced resonance stability substitutions at C-4 and C-6 contribute to the aromaticity of its intermediates.[104] Natural products with α-pyrone

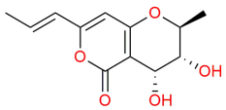
substituents are generally considered medicinally important with a range of antitumour,[105] antibacterial and antifungal biological activities.[106]

More recently, however, α-pyrones have been attributed as signalling molecules in bacteria at the nanomolar concentration range.[107] Quorum sensing in bacteria regulates a diverse range of physiological responses including sporulation, biofilm formation, symbiosis and antibiotic production.[108] The germicidins have been demonstrated to act as autoregulators of sporulation.[95, 109] An earlier Japanese paper reported a physiological response – antibiotic production and/or sporulation – for ≈50% of all *Streptomyces* interactions when co-cultured.[110] In this section, the potential mode of action of **1** was explored.

Compound **1** was shown to suppress metabolism to varying degrees in all the microorganisms tested. A subsequent study was conducted to test whether such suppression of metabolism was also caused by other α-pyrones. These assays were performed in a disc-diffusion format in a similar manner to the above supplementation of **1**. The microorganisms tested against include *Streptomyces* sp. AS4437 (teleocidin-producer) and *Streptomyces* sp. 86280v3 (oligomycin-producer). Six α-pyrones were tested of which included enterocin (**1**), germicidin A (**27**), wailupemycin F (**9**), wailupemycin G (**10**), as well as radicinol (**32**) and *epi*-radicinol (**33**), which are polycyclic fungal metabolites isolated from *Curvularia lunata* MST-FP2369 (Figure 6.4). The addition of **32** and **33**, here, was aimed at determining whether such metabolite suppression could be caused by more structurally-diverse α-pyrones.



Radicinol (**32**)



epi-Radicinol (**33**)

Figure 6.4: Structures of radicinol (**32**) and *epi*-radicinol (**33**) as isolated from *Curvularia lunata*.

The addition of the α-pyrones resulted in variable levels of metabolite suppression in both *Streptomyces* sp. AS4437 and *Streptomyces* sp. MST-86280v3 (Figure 6.5). For *Streptomyces* sp. AS4437, the metabolite at t_R = 2.7 min was the most affected, with ≈50% reduced production when supplemented **32** and **33**. The remaining metabolites were less significantly suppressed.

For the oligomycin-producing microorganism, *Streptomyces* sp. MST-86280v3, minor suppression of oligomycin A (t_R = 9.64 min) was observed for **1**, **9**, and **27** but not **10**. More

significant suppression of this metabolite was observed for **32** and **33** with a 43% and 67% reduction respectively.

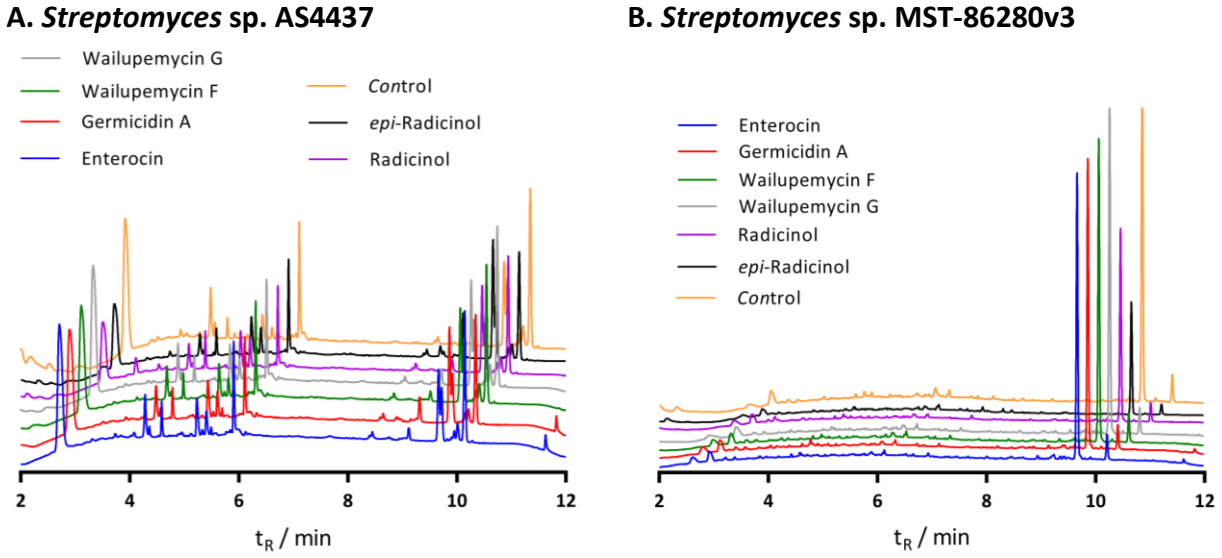


Figure 6.5: HPLC traces for the methanol extracts of the α-pyrone-treated **A.** *Streptomyces* sp. AS4437 (teleocidin-producer), and **B.** *Streptomyces* sp. 86280v3 (oligomycin-producer), with 0.2 min offset in retention time and 20 mAU offset in absorbance.

The suppression of metabolites in the above cultures with the addition of the α-pyrones tested was significantly less than the effect observed for the co-culture assays. This is likely due to the low amount of compound used in the treatments (25 μg). After the 7-day incubation, the discs were extracted with methanol to determine whether any compound remained. No compound remained in all cases and hence was likely metabolised by the microorganism during the duration of the experiment. Hence, this type of disc diffusion assay is not the ideal format for this type of experiment. Future experiments will involve optimisation of the treatment dose as well as observing whether a daily application of the treatment is required. Other assay formats will also be investigated.

6.4. Summary

The mode of action studies performed suggest that enterocin has a profound broad-spectrum effect both *in situ* and *in* vitro on the biosynthetic routes of many classes of secondary metabolism of other microorganisms. This effect was shared by other structurally unrelated α-pyrones, with the greatest suppression of metabolism observed for radicinol and *epi*-radicinol. These studies suggest the potential of a new approach to pathogen control by suppression of metabolites. In particular, those secondary metabolites may play a role as virulence factors and in pathogenesis.

7. Conclusions and Future Directions

Antibiotic resistance is a world-wide threat to human health. New antibiotics with novel mechanisms of action are urgently needed to deal with the rise of difficult-to-treat infections. The reinvestigation of forgotten antibiotics is one promising route that medicinal chemists explore to find new lead antibiotics.

In this study, the chemical and biological limitations of enterocin were investigated to assess its suitability as a next-generation antibiotic scaffold. In this thesis, it was found that enterocin possesses an inherent instability whereby it rearranges to an inactive ketal isomer. Inhibition of this rearrangement was possible through derivatisation of the secondary hydroxy group on enterocin. The acetyl and propionyl esters were formed through reaction of enterocin with acetic and propionic anhydrides in pyridine respectively, which resulted in more stable molecules that could be used as potential prodrugs. A carboxypropionyl ester of enterocin was formed through reaction with succinic anhydride in pyridine to provide increased solubility and a handle for further chemical modification. A palmityl ester of enterocin was formed through Steglich esterification with palmitic acid and DCC to enable the evaluation of the potential of highly hydrophobic prodrugs. Esters at the 5-OH site on enterocin with increased chain lengths and/or the addition of a negative charge were both shown to be ineffective means of altering the enterocin pharmacophore, with the major disruption of activity evident through the removal of the 5-OH on enterocin as indicated by the potency decrease of deoxyenterocin. The methyl oxime of enterocin was obtained by semi-synthetic modification of the C-2 ketone on enterocin by reacting enterocin with methoxylamine hydrochloride under basic conditions.

Further biosynthetic analogues of enterocin were obtained through optimisation of *Streptomyces* sp. MST-MA9095. In this study, six co-metabolites were isolated from the wailupemycin and germicidin classes of natural products. Both classes share an α-pyrone moiety in common with enterocin. The isolated wailupemycins, were superior antibiotics to enterocin, showing a ten-fold increase in potency against *M. luteus*. *O*-methylgermicidin D (as a 1:2 mixture with germicidin A) also demonstrated excellent activity against *M. luteus* (1.1 μM), however, this will have to be confirmed through isolation and subsequent *O*-methylation of germicidin D.

The mode of action of enterocin was also explored in this study. It was observed that when an enterocin-producing microorganism (*Streptomyces* sp. MST-MA9095) was cultured against other *Streptomyces*, their co-metabolite production was inhibited. Enterocin was demonstrated to be one of the molecules responsible for this observed suppression of metabolite production. Other related α-pyrones were also shown to have similar effects on metabolite production with radicinol and *epi*-radicinol resulting in the most significant changes. The suppression of metabolism indicated here demonstrates a novel mechanism of microbial control that can be harnessed for the development of next-generation antibiotics.

Future experiments will be conducted to determine whether a more consistent dose of these α-pyrones is required to more significantly affect metabolite production. The dose concentrations will also be altered to determine if a particular concentration range is optimal. An alternative biological assay will also be investigated as a substitute for the disc diffusion assay employed in this study. Future experiments will involve the identification of potential binding partners for these α-pyrones with radicinol and *epi*-radicinol chosen as lead compounds for further derivatisation studies as potential next-generation antibiotics.

8. Experimental

8.1. General Experimental Details

^{1}H NMR and ^{13}C NMR spectra were recorded in 5 mm Pyrex tubes (Wilmad, USA) on either a Bruker AVIIIHD 500 MHz NMR spectrometer or Bruker AVII 600 MHz NMR spectrometer. All spectra were obtained at 25 °C, processed using Bruker Topspin 3.5 software and referenced to residual solvent (DMSO-d_6 δ_H 2.50/ δ_C 39.5 ppm). The following abbreviations are used to describe the NMR data – singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of doublets of doublets (ddd), multiplet (m), and broad (br). High resolution electrospray ionisation mass spectra (HRESIMS) were obtained on a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) by direct infusion. Electrospray ionisation mass spectra (ESIMS) were acquired on an Agilent 1260 UHPLC coupled to an Agilent 6130B single quadrapole mass detector. UV-Vis spectroscopy was performed on a CARY 1BIO UV-Visible Spectrophotometer between 800 and 200 nm and a 60 nm/min scan rate. Polarimetry was performed on a JASCO P-1010 polarimeter with a sodium D-line filter (589 nm) and 10 mm x 10 mm cell.

The progress of all reactions was monitored with TLC or HPLC. TLC was performed by using Merck Kieselgel 60 F254 plates and viewed under ultraviolet light (254 nm and 365 nm). Analytical HPLC was conducted on a gradient Agilent 1260 Infinity quaternary HPLC system with G4212B diode array detector on an Agilent Poroshell 120 EC-C_{18} (50 × 4.6 mm, 2.7 µm) column with a flow rate of 1 mL/min and gradients between 10-100% acetonitrile/water (0.005% TFA) over 8.33 min. All compounds were purified on preparative or semi-preparative HPLC systems. Semi-preparative HPLC was performed on a gradient Agilent 1260 Infinity quaternary HPLC system with a G4212B diode array detector on an Agilent Zorbax SB-C_{18} (250 × 9.4 mm, 5 µm) column with a flow rate of 4.18 mL/min and gradients between 10-100% acetonitrile/water under neutral conditions over 25-40 min. Preparative HPLC was performed on a gradient Shimadzu HPLC system consisting of two LC-8A preparative liquid pumps with a static mixer, SPD-M10AVP diode array detector and SCL-10AVP system controller with a standard Rheodyne injection port. An Alltima C_{18} column (150 × 22 mm, 5 µm; Grace Discovery) column was used with a flow rate of 20 mL/min eluted isocratically between 30-50% acetonitrile/water under neutral conditions over 15-25 min. Solvents were removed under reduced pressure using a Büchi Rotavapor R-200, a KNF lab vacuum

pump, a Huber unichiller set to 5 °C and Büchi B-490 heating bath set to a temperature of 40 °C. All chemicals and reagents unless otherwise stated were received from Sigma-Aldrich (St. Louis, U.S.A) and used without further purification.

8.2. Culture Media Optimisation

A suspension of *Streptomyces* sp. MST-MA9095 was prepared by recovery onto ISP2 agar one-week prior to media optimisation, followed by suspension of 1.5 cm^2 slices in 150 mL of sterilised distilled water with stirring at 400 rpm for 1 h. Culture media optimisation of *Streptomyces* sp. MST-MA9095 was performed on a range of media including grains (cracked wheat (BL); basmati rice (BS); jasmine rice (JR); and pearl barley (PB)), agars (glycerol casein (AC); glucose, yeast extract, malt extract and soluble starch (GYMS); International *Streptomyces* protocol (ISP2); Bennett's medium modified (MS); and oatmeal (OA)), and liquids (AC; GYMS; ISP2; OA; and N-Z-amine (NZAM)). All media were prepared according to the recipes outlined in the Supplementary Material (see Section 10.1). The grains were prepared by hydration in water (25-35 mL in 250 mL flasks) during sterilisation at 121 °C for 40 min. All media were inoculated with the culture at 28 °C for 7-14 days. The liquid suspensions were prepared by inoculating sterilised distilled water (150 mL) with circular slices (≈1.5 cm diameter) of the culture agar which was swirled for 1-2 h at 400 rpm. The agar plates (15 mL) were dispensed into 9 cm sterile plastic plates and cooled to room temperature before use. The plates were inoculated by sterile culture loop from a 7 to 10-day old actively growing mother plate. The cultures were subsampled (≈5 g for grains, 1 mL for liquids and 2 circular slices (≈1.5 cm diameter) of the agars) and extracted with methanol (2 mL) for a minimum of 1 h on a wrist shaker, centrifuged (16000 × g) for 5 min and the resulting supernatants analysed by LCMS. Co-culture experiments used single agar disks as subsamples for analysis. The major and minor metabolites were analysed by HPLC retention time, UV-Vis spectroscopy and mass spectrometry.

8.3. Cultivation, extraction and fractionation of *Streptomyces* sp.

Cultivation, extraction and fractionation of *Streptomyces* sp. was carried out with the assistance of our collaborators at MST. The culture of *Streptomyces* sp. MA9095 was inoculated onto 60 × 250 mL Erlenmeyer flasks each containing 48 g of pearl barley (PB). The flasks were incubated at 28 °C for 10 days. The media and conditions were those identified for the optimal production of enterocin co-metabolites. The culture was extracted with acetone (2 × 2 L), and the combined

organic extracts evaporated under vacuum to give an aqueous residue. The resulting residue was partitioned against ethyl acetate (2 × 2 L) and dried to give a crude extract (20.185 g). The crude extract (AL17.16-ME) was then fractionated on silica with chloroform and methanol, resulting in six fractions. Fraction 1 (369 mg) was fractionated further by preparative-HPLC isocratically at 55% acetonitrile/water with 0.01% TFA resulting in an unidentified pigment and a pyrone mixture. The pyrone mixture (51.93 mg) was purified further by preparative-HPLC isocratically at 35% acetonitrile/water with 0.01% TFA to afford a 1:2 mixture of *O*-methylgermicidin D (**26**) and germicidin A (**27**) (t_R = 4.74 min, 2.59 mg). Fraction 4 was recrystallised in methanol to yield pure enterocin (**1**) (3.716 g). The resulting non-polar mother-liquor was purified by semi-preparative HPLC isocratically at 25% acetonitrile/water under neutral conditions to afford pure wailupemycin G (**10**) (t_R = 5.26 min, 18.45 mg). A crude fraction also isolated along with **10** was further purified by preparative HPLC isocratically at 45% acetonitrile/water with 0.01% TFA to give pure wailupemycin F (**9**) (t_R = 5.02 min, 12.14 mg). Fraction 5 (1.231 g) was further purified by preparative HPLC with a gradient between 25-40% acetonitrile/water with 0.01% TFA to afford pure *p*-hydroxywailupemycin G (**25**) (t_R = 4.47 min, 10.81 mg). In a similar manner to Fraction 4, pure *p*-hydroxywailupemycin F (**24**) (t_R = 4.42 min, 5.10 mg) was isolated from a fraction acquired alongside **25,** which was further purified by preparative HPLC isocratically at 35% acetonitrile/water with 0.01% TFA.

8.4. Chemical Synthesis

8.4.1. Semi-synthetic Analogues of Enterocin

*8.4.1.1. Isoenterocin (**16**)*

To enterocin (53.2 mg, 0.120 mmol) dissolved in acetonitrile (2.5 mL), potassium hydroxide (1M; 25 μmol) was added. The reaction mixture changed from yellow to colourless almost instantly. The reaction was allowed to stir at room temperature overnight. The reaction mixture was evaporated to 1 mL under nitrogen, and diluted with water to 1.5 mL before purifying by preparative HPLC isocratically at 30% MeCN:H_2O to yield isoenterocin (**16**) as a yellow oil (t_R = 8.351 min, 5.79 mg, 11% yield). UV (MeOH) λ_{max} (log ε) 206 (4.41), 284 (3.75) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 443.0981 (calculated for $C_{22}H_{19}O_{10}^-$ 443.0984). ^1H and ^{13}C NMR, see Table 10.1.

*8.4.1.1. 3-epi-enterocin-(Z)-2-O-methyloxime (**19**)*

To enterocin (52.6 mg, 0.118 mmol) dissolved in methanol/water (1:1, 2.5 mL), triethylamine (20 μL, 0.143 mmol, 1.2 equiv.) and methoxylamine hydrochloride (41 mg, 0.491 mmol, 4.1 equiv.) were added. The reaction mixture was stirred at 60 °C for 3 days and subsequently evaporated to dryness. The crude mixture was purified by preparative HPLC isocratically at MeCN:H_2O (30%) to yield 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) as a pure white powder (t_R = 9.4 min, 7.4 mg, 14% yield). [α_D^{20} = +1] (*c* = 0.17, MeOH). UV (MeOH) λ_{max} (log ε) 206 (4.45), 282 (3.80) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 472.1243 (calculated for $C_{23}H_{22}O_{10}N^-$ 472.1249). ^1H and ^{13}C NMR, see Table 10.2.

*8.4.1.2. 5-O-acetylenterocin (**20**)*

To a solution of enterocin (49.6 mg, 0.112 mmol) in pyridine (2.5 mL) on ice, acetic anhydride (125 μL, 1.16 mmol, 10 equiv.) was added dropwise over 1 min. The reaction was allowed to stir at room temperature for 2.5 h (monitored by HPLC) and was recovered from pyridine on C_{18} silica in methanol. Methanol was removed under vacuum. The crude mixture was purified by preparative HPLC isocratically at 50% MeCN:H_2O to yield 5-*O*-acetylenterocin (**20**) as a pure white powder (t_R = 6.07 min, 15.8 mg, 29% yield). [α_D^{20} = −1.51] (*c* = 0.15, MeOH). UV (MeOH) λ_{max} (log ε) 206 (4.59), 250 (4.20), 283 (4.02) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 485.1083 (calculated for $C_{24}H_{21}O_{11}^-$ 485.1089). ^1H and ^{13}C NMR, see Table 10.3.

*8.4.1.3. 5-O-propionylenterocin (**21**)*

To a solution of enterocin (46.7 mg, 0.105 mmol) in pyridine (2.5 mL) on ice, propionic anhydride (125 μL, 0.946 mmol, 9 equiv.) was added dropwise over 1 min. The reaction was allowed to stir at room temperature for 2.5 h (monitored by HPLC) and recovered from pyridine on C_{18} silica in methanol. Methanol was removed under vacuum. The crude mixture was purified by preparative HPLC isocratically at 50% MeCN:H_2O to yield 5-*O*-propionylenterocin (**21**) as a pure white powder (t_R = 7.56 min, 22.05 mg, 39% yield). [α_D^{20} = -2] (*c* = 0.15, MeOH). UV (MeOH). λ_{max} (log ε) 207 (4.54), 250 (4.19), 283 (4.01) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 499.1241 (calculated for $C_{25}H_{23}O_{11}^-$ 499.1246). ^1H and ^{13}C NMR, see Table 10.4.

*8.4.1.4. 5-O-carboxypropionylenterocin (**22**)*

To enterocin (10.0 mg, 0.023 mmol), a solution of succinic anhydride (6.76 mg, 0.067 mmol, 3 equiv.) in pyridine (2 mL) was added. The reaction mixture was stirred at 40 °C for 30 min

(monitored by TLC), filtered and reduced to dryness under nitrogen. The crude mixture was purified by semi-preparative HPLC using a 35-min gradient of MeCN:H_2O (15-95%) with 0.005% TFA modifier to yield 5-*O*-carboxypropionylenterocin (**22**) as pure white needles (t_R = 29.0 min, 1.2 mg, 12% yield). [α_D^{20} = +1] (*c* = 0.26, MeOH). UV (MeOH) λ_{max} (log ε) 204 (4.38), 250 (3.90), 283 (3.75) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 543.1142 (calculated for $C_{26}H_{23}O_{13}^-$ 543.1144). ^1H and ^{13}C NMR, see Table 10.5.

*8.4.1.5. 5-O-palmitylenterocin (**23**)*

To enterocin (10.0 mg, 0.023 mmol), DMAP (0.14 mg, 0.001 mmol, 0.05 equiv.) and a solution of palmitic acid (28.8 mg, 0.112 mmol, 5 equiv.) in dry dichloromethane (0.5 mL) was added. The resulting solution was chilled to 5 °C and a solution of DCC (9.2 mg, 0.045 mmol, 2 equiv.) in dry DCM (1.5 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. This was then extracted with ethyl acetate (2 × 1 mL), washed with water (2 × 1 mL), dried over anhydrous sodium sulfate and reduced to dryness under nitrogen. The crude mixture was purified by semi-preparative HPLC MeCN:H_2O (10-90%) with 0.005% TFA modifier to yield 5-*O*-palmitylenterocin (**23**) as a pure white powder (t_R = 32.7 min, 2.5 mg, 16% yield). UV (MeOH) λ_{max} (log ε) 204 (4.56), 250 (4.12), 283 (3.94) nm. HR-ESI(−)-MS *m/z* $[M-H]^-$ 681.3279 (calculated for $C_{38}H_{49}O_{11}^-$ 681.3280). ^1H and ^{13}C NMR, see Table 10.6.

8.4.2. Semi-synthetic Analogues of Germicidins

*8.4.2.1. O-Methylgermicidin A (**31**)*

To germicidin A (18.71 mg, 0.095 mmol) dissolved in methanol (10 mL), conc. sulfuric acid (2 drops) was added. The reaction mixture was allowed to sit at 60 °C for 7 days, before recovering from acid on C_{18} silica in acetonitrile. The crude mixture was purified by preparative HPLC isocratically at MeCN:H_2O (60%) to yield *O*-methylgermicidin A (**31**) as a colourless oil (t_R = 9.7 min, 0.86 mg, 4% yield). HR-ESI(+)-MS *m/z* $[M+H]^+$ 211.1331 (calculated for $C_{12}H_{19}O_3^+$ 211.1329). ^1H and ^{13}C NMR, see Table 10.10.

8.5. Preparation of Microtitre plate for Bioassay

The compounds used in this study were dissolved in DMSO to give stocks with a concentration of 25 mg mL^{-1}. From each of these stock solutions, 20 μL was transferred to the first column of a 96-well microtitre plate in rows B to G, replacing row A and H with 20 μL of DMSO to act as positive

and negative method controls respectively. This was then two-fold serially diluted across the 12 columns of the plate. Daughter plates were prepared by transferring 2 μL of each well to a fresh plate. To all wells in this plate, resazurin (10 μL, 120 μg/mL) was added as an indicator compound, which is converted from purple to light pink in the presence of metabolically active cells. Nutrient agar media (190 μL) was then added to each well, except for row H, resulting in a 100-fold dilution of the concentration in each well. After dilution, the final concentration in each well ranged from 250-0.12 μg mL^{-1} in 0.1% DMSO. The absorbance of each well was measured using a Spectromax plate reader at 605 nm and MIC and LD_{99} values determined visually.

8.5.1. Antibacterial assay

The antibacterial activity of the compounds used in this study were evaluated against the Gram-positive bacterium *Micrococcus luteus*. Gentamicin (BioAustralis®, code: BIA-G1563, purity: >95% by HPLC, 1.7 μM) was used as a positive control. A bacterial suspension (50 mL in a 250 mL Erlenmeyer flask) was prepared in nutrient media by cultivation for 24 h at 28 °C, swirling at 250 rpm. The suspensions were diluted with sterile water to standardise to give an absorbance of 0.01. To each well of the prepared microtitre plates, 10 μL of the standardised solutions was added. Row H was substituted with nutrient media instead to act as the negative control. The plates were incubated at 28 °C for 24 h during which the negative control wells change colour from purple to light pink. Experiments were performed in duplicate.

8.5.2. Cytotoxicity assay

For the selected analogues of enterocin, cytotoxicity in myeloma cells was evaluated using NS-1 (ATCC TIB-18) mouse myeloma cells, and cytotoxicity in mammalian cells was evaluated using Neonatal Foreskin Fibroblast (NFF) cells. These cells were cultured in Dulbecco's Modified Eagle IS Medium (DMEM) +10% fetal bovine serum (FBS) +1% penicillin/streptomycin (Life Technologies) and incubated at 37 °C (5% CO_2) for 72 h. Mitomycin C (BioAustralis®, code: BIA-M1183, purity: >98% by HPLC, 0.93 μM) was used as a positive control against NS-1 cells. To standardise the concentration used, cells were counted under the microscope after staining with erythrocin B and diluted to the required concentration. Once standardised, the cells (190 μL, 50,000 cells/mL (NS-1), 25,000 cells/mL (NFF)) were loaded in 96-well microtitre plates with resazurin (10 μL, 120 μg/mL) and the test compound (2 μL, 250-0.12 μg/mL) and incubated for72 h at 37 °C (5% CO_2). The experiments were performed in triplicate.

9. References

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10. Supplementary Material

10.1. Bacterial Media Recipes

Glycerol Casein Agar (AC)				***Trace element solution**		
Ingredient	*Supplier*	*Quantity*	*Unit*	*Ingredient*	*Quantity*	*Unit*
Glycerol	CS	30	g	$CaCl_2.2H_2O$	3	g
Casein peptone	Amyl	2	g	$FeC_6O_7H_5$	1	g
K_2HPO_4	CS	1	g	$MnSO_4$	0.2	g
NaCl	CS	1	g	$ZnCl_2$	0.1	g
$MgSO_4$	AnalaR	0.5	g	$CuSO_4.5H_2O$	0.025	g
Trace element solution*		5	mL	$Na_2B_4O_7.2H_2O$	0.02	g
Distilled H_2O		1000	mL	$CoCl_2$	0.004	g
Bacteriological agar[a]	Amyl	20	g	$Na_2MoO_4.2H_2O$	0.01	g
Autoclave				Distilled H_2O	1000	mL
				Filter sterilise		

Glucose Yeast Extract Starch (GYMS)				**ISP2 Agar (ISP2)**			
Ingredient	*Supplier*	*Quantity*	*Unit*	*Ingredient*	*Supplier*	*Quantity*	*Unit*
Yeast extract	Difco	4	g	Yeast extract	Difco	4	g
Malt extract	Difco	10	g	Malt extract	Difco	10	g
Glucose	CB	4	g	Glucose	CB	4	g
Distilled H_2O (pH adjusted to 7.3)		1000	mL	Distilled H_2O (pH adjusted to 7.3)		1000	mL
Bacteriological agar[a]	Amyl	20	g	Bacteriological agar[a]	Amyl	20	g
Soluble starch	Difco	20	g	Autoclave			
$CaCO_3$	UA	2	g				
Autoclave							

Oatmeal Agar[b] (OA)				**Oatmeal Liquid[b] (OA)**			
Ingredient	*Supplier*	*Quantity*	*Unit*	*Ingredient*	*Supplier*	*Quantity*	*Unit*
Distilled H_2O		1000	mL	Oat Flakes	Coles	20	g
Oatmeal agar	Amyl	72.5	g	$CaCO_3$	Univar	1	g
Autoclave				Trace element solution*		5	mL
				Distilled H_2O		1000	mL
				Autoclave			

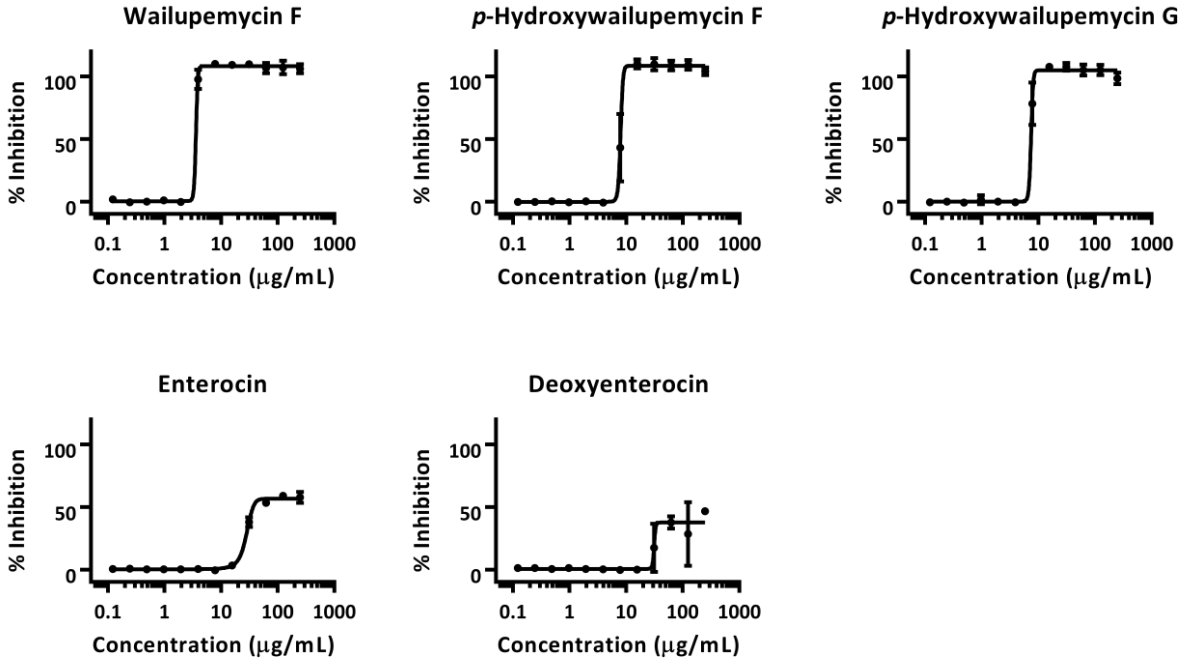
Modified Bennett's Agar (MSA)				**N-Z Amine Agar (NZAM)**			
Ingredient	*Supplier*	*Quantity*	*Unit*	*Ingredient*	*Supplier*	*Quantity*	*Unit*
Yeast extract	Difco	2	g	Yeast extract	Difco	4	g
Beef extract	Difco	1.6	g	N-Z-Amine	SA	10	g
Casitone	Difco	4	g	Glucose	Amyl	4	g
Glycerol	AnalaR	20	g	Distilled H_2O		1000	mL
Bacteriological agar[a]	Amyl	20	g	Bacteriological agar[a]	Amyl	20	g
Soytone	Amyl	10	g				
Distilled H_2O (pH adjusted to 7.0) Autoclave		1000	mL				

[a]Omit agar for liquid media

[b]Oatmeal agar and liquid differ in more than just the addition of agar.

Abbreviations: CB – Country Brewers, CS – Chem-Supply, SA – Sigma Aldrich, UA – Univar Ajax

10.2. Antibacterial activity of enterocin analogues against *M. luteus*



10.3. Tabulated NMR Data

Table 10.1: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for isoenterocin (**16**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		140.2			
2		112.0			
3	3.09, d (0.7)	68.2	1, 4, 5, 8, 16, 17	5	4-OH, 9, 18
4		83.7			
5	4.58, d (5.4)	90.0	2, 3, 4, 6, 7	3, 6, 6-OH, 9, 7a	6, 7a
6	3.97, ddd (15.1, 9.6, 5.4)	62.4		5, 7a/b	5, 7a
7a	2.24, dd (15.1, 9.6)	37.1	5, 6, 8, 16	5, 6, 7b	5, 6, 7b
7b	1.49, dd (15.1, 9.6)		6, 8, 9, 16	6, 7a, 9	7a
8		77.4			
9	3.60, s	53.4	4, 5, 7, 8, 9, 10, 11	5, 7b, 11	3, 4-OH, 8-OH, 11
10		160.9			
11	6.35, dd (2.2, 1.4)	102.0	9, 10, 12, 13	9, 13	8-OH, 9
12		170.5			
13	5.61, m	88.1	11, 12, 14	11	15
14		163.1			
15	3.81, s	56.3	12		13
16		82.9			
17		174.1			
18	7.53, d (7.0)	125.4	2, 18, 20		3
19	7.45, dd (7.2, 7.0)	128.3	1, 19		
20	7.40, t (7.2)	128.8	18		
4-OH	6.11, s		3, 4, 5		3, 8-OH, 9, 16-OH
6-OH	4.86, d (5.9)		5, 6, 7	6	6
8-OH	5.63, m		7, 8, 9, 16		4-OH, 9, 11, 16-OH
16-OH	6.69, s		3, 8, 16, 17		4-OH, 8-OH

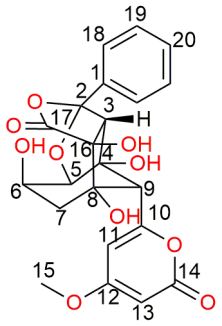


Table 10.2: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for 3-*epi*-enterocin-(*Z*)-2-*O*-methyloxime (**19**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		136.0			
2		153.1			
3	3.64, s	55.4	1, 2, 4, 5, 8, 9, 10, 16, 17		4-OH, 16-OH, 18
4		78.7			
5	4.40, dd (4.6, 4.6)	69.1	2, 3, 4, 6	6	5-OH, 6, 7a
6	4.59, ddd (4.6, 3.0, 2.8)	75.2	3, 5, 7, 17	5, 7a/b	5, 7a/b
7a	2.37, dd (14.5, 3.0)	35.5	8, 9, 16	6, 7b	5, 6, 7b
7b	1.67, ddd (14.5, 2.8, 2.5)		5, 8, 9	6, 7a	6, 7a
8		75.7			
9	4.25, br s	55.6	5		
10		161.4			
11	5.94, br s	104.6	10, 13	13	
12		170.5			
13	5.61, d (2.3)	87.7	11, 12, 14	11	15
14		163.3			
15	3.81, s	56.2	11, 12, 13		13
16		80.0			
17		173.9			
18	7.41, d (7.9)	128.4	2, 19	19	3, 21
19	7.35, dd (7.9, 7.4)	127.9	1, 20	18, 20	
20	7.30, t (7.4)	127.4	18, 19	19	
21	3.80, s	61.6	2		18
4-OH	5.29, s		4, 5, 9		3
5-OH	5.57[a]				
8-OH	5.71, s		7, 8, 9, 16		
16-OH	5.57[a]				3

[a] – Overlapping

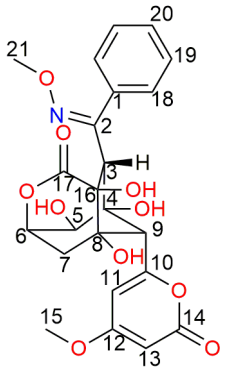


Table 10.3: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 5-*O*-acetylenterocin (**20**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		139.3			
2		193.9			
3	4.40, s	54.1	2, 4, 5, 8, 9, 20, 16, 17		4-OH, 5, 18, 22
4		76.7			
5	5.73, d (4.4)	71.8	3, 4, 6, 21	6, 9	3, 6, 7a
6	4.83 ddd (4.4, 3.0, 2.9)	72.4	3, 5, 17	5, 7a/b	5, 7a/b
7a	2.38, dd (14.7, 3.0)	35.4	8, 9, 16	6, 7b	5, 8-OH, 11
7b	1.74, ddd (14.7, 2.9, 2.6)		6, 8, 9	6, 7a, 9	5, 8-OH, 16-OH
8		76.3			
9	4.77, d (2.6)	54.7	4, 5, 8, 10, 11	5, 7b, 11	4-OH, 8-OH, 16-OH, 11, 18
10		160.6			
11	6.31, d (2.3)	105.0	9, 10, 12, 13	9, 13, 15	7a, 9, 15
12		170.6			
13	5.65, d (2.3)	88.0	11, 12, 14	11, 15	15
14		163.1			
15	3.84, s	56.3	12	11, 13	11, 13
16		79.9			
17		173.3			
18	7.79, d (7.3)	128.6	2, 19, 20	19, 20	3, 4-OH, 9, 16-OH
19	7.54, dd (7.4, 7.3)	127.8	18, 20	18, 20	
20	7.62, t (7.4)	132.8	19	18, 19	
21		169.8			
22	2.11, s	20.9	21		3, 4-OH
4-OH	5.93, s		4, 5, 9		3, 9, 18, 22
8-OH	6.08, s		7, 8, 9		7a/b, 9
16-OH	6.11, s		3, 8, 16, 17		7b, 9, 18

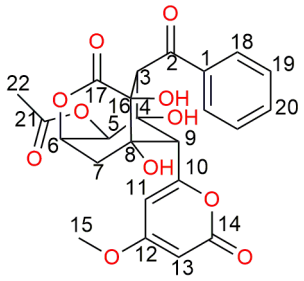


Table 10.4: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 5-*O*-propionylenterocin (**21**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		139.3			
2		193.9			
3	4.42, s	54.0	2, 4, 5, 8, 9, 16, 17		4-OH, 18, 22
4		76.8			
5	5.75, d (4.5)	71.7	2, 3, 4, 21	6	4-OH, 6, 11
6	4.83, ddd (4.5, 3.1, 2.8)	72.4	5, 8, 17	5, 7a/b	5, 7a/b
7a	2.83, dd (14.5, 3.1)	35.4	8, 9, 16	6, 7b	5, 6, 7b, 8-OH, 11
7b	1.73, ddd (14.5, 2.8, 2.6)		5, 6, 8, 9	6, 7a, 9	6, 7a, 8-OH
8		76.3			
9	4.76, d (2.6)	54.7	4, 5, 7, 8, 10	7b, 11	4-OH, 8-OH, 11
10		160.6			
11	6.32, d (2.3)	105.0	10, 12, 13	9, 13	5, 7a, 8-OH, 9, 15
12		170.6			
13	5.65, d (2.3)	88.0	11, 12, 14	11, 15	15
14		163.1			
15	3.84, s	56.3	12	13	11, 15
16		79.9			
17		173.3			
18	7.78, d (7.2)	127.8	2, 18, 20	19, 20	3
19	7.54, dd (7.4, 7.2)	128.6	1, 18	18, 20	
20	7.63, t (7.4)	132.8	19	18, 19	
21		172.9			
22	2.43, q (7.5)	26.8	21, 23	23	3, 4-OH, 23
23	1.04, t (7.5)	8.8	21, 22	22	22
4-OH	5.91, s		4, 5, 9		3, 5, 8, 22
8-OH	6.09, s		7, 8, 9		7a/b, 9, 11
16-OH	6.11, s		3, 16, 17		

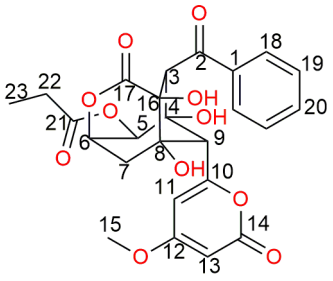


Table 10.5: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for 5-*O*-carboxypropionylenterocin (**22**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		139.3			
2		193.8			
3	4.42, s	53.9	2, 5, 8, 9, 16, 17	9	18, 23
4		76.8			
5	5.75, d (4.4)	72.0	3, 4	6, 7a	7a, 11, 23
6	4.80, ddd (4.4, 3.0, 2.8)	72.4		5	5, 7a/b, 11
7a	2.37, dd (14.7, 3.0)	35.5	8, 16	6, 7b	5, 6
7b	1.74, ddd (14.7, 8.2, 2.6)		5, 8, 9	7a, 9	6
8		76.3			
9	4.76, d (2.6)	54.7	5, 7, 8, 10, 11	3, 7b	11
10		160.6			
11	6.31, d (2.2)	105.0	9, 10, 12, 13	13	5, 6, 9, 15
12		170.5			
13	5.65, d (2.2)	87.9	11, 12, 14	11	11, 15
14		163.0			
15	3.84, s	56.3	12		11, 13
16		79.9			
17		173.2			
18	7.79, d (7.9)	127.8	2, 20	19	3
19	7.54, dd (7.9, 7.3)	128.5	1, 18	18, 20	
20	7.63, t (7.3)	132.7	19	19	
21		173.0			
22	2.47, m	28.9	21, 23, 24	23	4-OH
23	2.63, m	28.7	21, 22, 24	22	3, 4-OH, 5
24		171.6			
4-OH	5.97, br s				22-23
8-OH	6.08[a], br s		7, 8		
16-OH	6.07[a], br s		3, 16, 17		
24-COOH	12.23, br s				

[a] – Assignments interchangeable

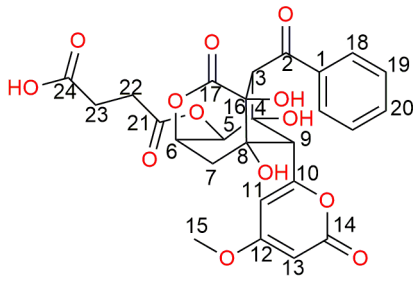


Table 10.6: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for 5-*O*-palmitylenterocin (**23**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		139.3			
2		193.9			
3	4.41, s	54.0	2, 4, 5, 8, 9, 16, 17		18
4		76.8			
5	5.73, d (4.5)	71.7	3, 4, 6, 21	6	4-OH, 6, 7a, 11
6	4.83, m	72.4	4	5, 7a/b	5
7a	2.39, m	35.4	8, 9, 16	6, 7b	5, 8-OH
7b	1.74, ddd (14.8, 2.8, 2.6)			6, 7a, 9	8-OH, 22
8		76.3			
9	4.75, d (2.6)	54.8	4, 5, 7, 10, 11	7a	4-OH, 8-OH, 11, 16-OH
10		160.6			
11	6.31, d (2.3)	105.0	9, 10, 12, 13	13	5, 8-OH, 9, 15
12		170.6			
13	5.65, d (2.3)	88.0	11, 12	11	15
14		163.1			
15	3.84, s	56.3	12		11, 13
16		79.9			
17		173.2			
18	7.78, d (7.2)	127.8	2, 20	19	3
19	7.53, dd (7.5, 7.2)	128.5	1, 20	18, 20	
20	7.63, t (7.5)	132.8	18	19	
21		172.1			
22	2.39, m	33.5	21, 23-35		7b, 23-35
23-35	1.23, m	29.0	36	36	36
36	0.85, t (7.0)	13.9	23-35	23-35	23-35
4-OH	5.89, s		4, 5, 9		5, 9
8-OH	6.08, s		7, 8, 9		7a/b, 9, 11
16-OH	6.10, s		3, 16, 17		9

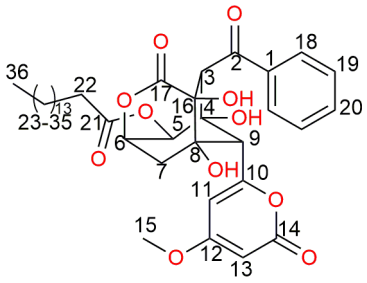
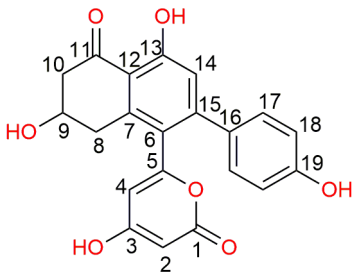


Table 10.7: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for *p*-hydroxywailupemycin F (**24**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		164.0			
2	5.22, m	89.2	1, 3, 4	4	
3		170.2			
4	5.79, m	105.5	2, 5, 6	2	
5		160.4			
6		123.0			
7		142.6			
8a	3.04, dd (16.7, 2.9)	35.8	6, 7, 9, 10, 12	8b, 9	
8b	2.79, dd (16.7, 6.1)		6, 7, 9, 10, 12	8a, 9	
9	4.30, m	64.3		8a/b, 9-OH, 10a/b	9-OH
10a	2.97, dd (16.7, 2.9)	46.3	8, 9, 11	9, 10b	
10b	2.70, dd (16.7, 6.8)		8, 9, 11, 12	9, 10a	
11		204.6			
12		115.1			
13		162.0			
14	6.79, s	115.8	5, 6, 11, 12, 13, 16		13-OH
15		149.6			
16		129.4			
17	7.07, d (8.6)	129.8	15, 16, 18, 19	18	
18	6.73, d (8.6)	115.2	16, 17, 19	17	19-OH
19		115.6			
3-OH	11.82, br s				
9-OH	5.22, m				9
13-OH	12.70, s		13, 14	9	14
19-OH	9.66, s		18, 19		18

Table 10.8: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for *O*-methylgermicidin D (**26**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		164.6			
2		98.6			
3		165.9			
4	6.44, s	92.8	2, 5, 6		6, 7, 9
5		168.3			
6	2.81, sept (6.9)	32.6	5, 7	7	7
7	1.21, d (6.9)	20.0	5, 6	6	6
8	1.80, s	8.4	1, 2, 3		
9	3.94, s	56.7	3		4



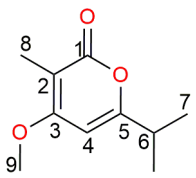
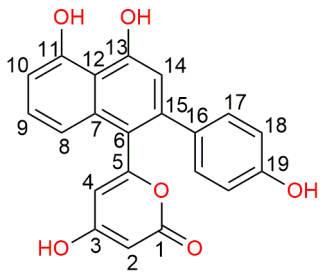


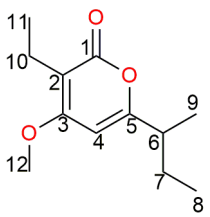
Table 10.9: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for *p*-hydroxywailupemycin G (**25**) in DMSO-d_6

Position	δ_H, mult. (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		164.0			
2	5.82, d (2.1)	105.5	1, 4, 5, 6	4	3-OH, 4
3		170.0			
4	5.31, d (2.1)	89.2	1, 2, 3	2	2, 3-OH
5		161.5			
6		119.2			
7		135.1			
8	7.05, dd (8.5, 0.9)	116.0	6, 10, 12	9	
9	7.36, dd (8.5, 7.6)	128.6	7, 11	8, 10	
10	6.81, dd (7.6, 0.9)	109.0	8, 12	9	11-OH
11		154.4			
12		113.1			
13		155.7			
14	6.73, m	109.7	6, 12, 13, 15		13-OH
15		130.7			
16		141.1			
17	6.75, d (8.3)	115.1	16, 18, 19	18	
18	7.10, d (8.3)	129.7		17	
19		156.9			
3-OH	11.65, br s		2, 3, 4		2, 4
11-OH	11.35, br s				10
13-OH	11.12, br s				14
19-OH	9.57, br s				

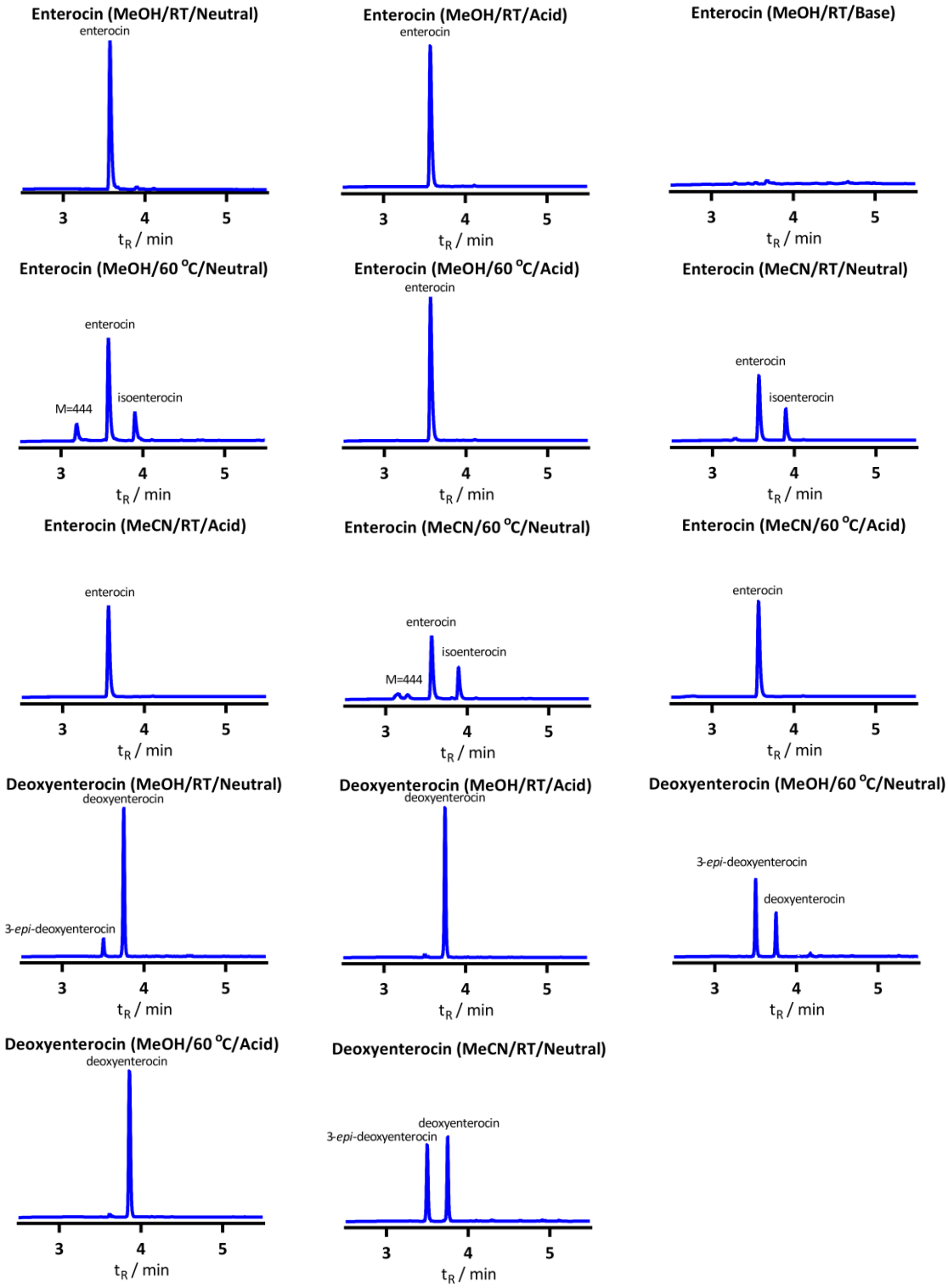
Table 10.10: ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for *O*-methylgermicidin A (**31**) in DMSO-d_6

Position	δ_H, mult (J / Hz)	δ_C	^{1}H-^{13}C HMBC	COSY	ROESY
1		164.0			
2		104.8			
3		165.9			
4	6.42, s	94.1	2, 3, 5, 6	12	6, 9, 12
5		167.5			
6	2.54 m	40.0	4, 5, 7, 8, 9	7, 9	4, 7b
7a	1.60, m	27.0	5, 6, 8, 9	6, 7b, 8	7b, 8, 9
7b	1.50, m		5, 6, 8, 9	6, 7a,8	6, 7a, 8, 9
8	0.84, t (7.5)	11.4	6, 7	7a/b	7a/b
9	1.16, d (7.0)	17.7	5, 6, 7	6	4, 7a/b
10	2.29, q (7.4)	16.1	1, 2, 3, 11	11	11
11	0.95, t (7.4)	12.4	2, 10	10	10
12	3.89, s	56.7	5, 6, 8, 9	4	4, 9

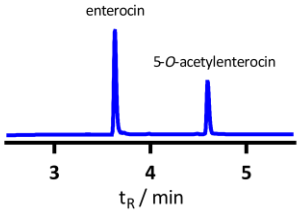




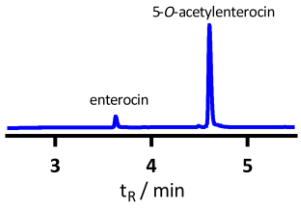
10.4. Key HPLC traces showing degradation of enterocin, and related analogues



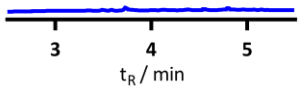
5-*O*-Acetylenterocin (MeOH/RT/Neutral)



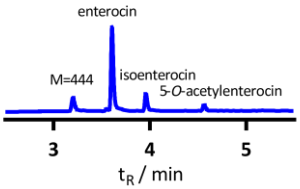
5-*O*-Acetylenterocin (MeOH/RT/Acid)



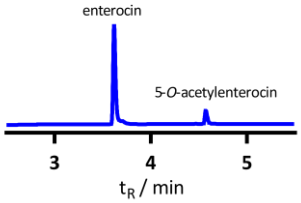
5-*O*-Acetylenterocin (MeOH/RT/Base)



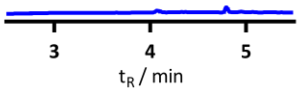
5-*O*-Acetylenterocin (MeOH/60 °C/Neutral)



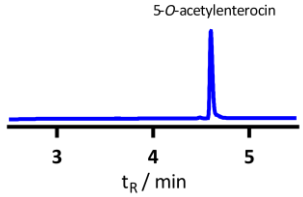
5-*O*-Acetylenterocin (MeOH/60 °C/Acid)



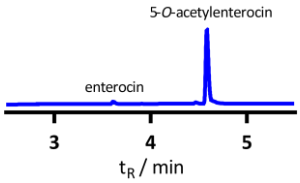
5-*O*-Acetylenterocin (MeOH/60 °C/Base)



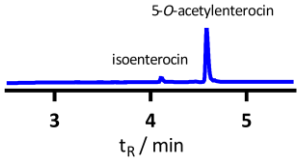
5-*O*-Acetylenterocin (MeCN/RT/Neutral)



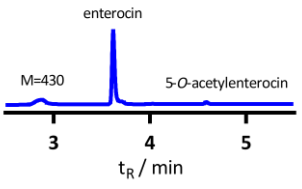
5-*O*-Acetylenterocin (MeCN/RT/Acid)



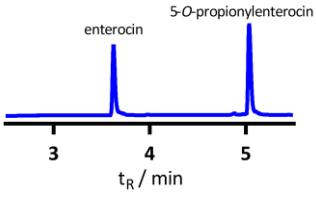
5-*O*-Acetylenterocin (MeCN/60 °C/Neutral)



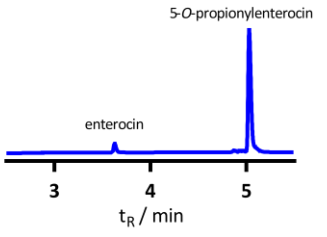
5-*O*-Acetylenterocin (MeCN/60 °C/Acid)



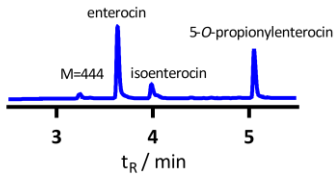
5-*O*-Propionylenterocin (MeOH/RT/Neutral)



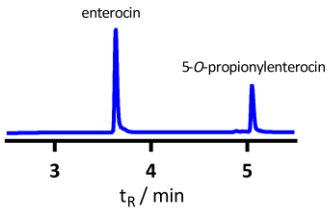
5-*O*-Propionylenterocin (MeOH/RT/Acid)



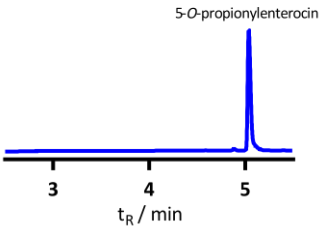
5-*O*-Propionylenterocin (MeOH/60 °C/Neutral)



5-*O*-Propionylenterocin (MeOH/60 °C/Acid)



5-*O*-Propionylenterocin (MeCN/RT/Neutral)



10.5. Digital Supplementary Material

Macquarie University MRes thesis policy dictates a maximum of 12 pages of Supplementary Material. To view ^{1}H and ^{13}C NMR spectra for all compounds, and DFT calculations, please visit Digital Supplementary Material:

http://cbms.mq.edu.au/~apiggott/mres/CowledDigitalSupplementary.pdf