Investigations into the biosynthesis and mode of action of methylenomycin antibiotics from *Streptomyces coelicolor* A 3(2)

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Contents

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy in Chemistry. It has been composed and written by the Author and has not been previously submitted for any degree.

The work presented in the thesis was carried out by the Author, except in the following cases:

- Cloning of methylenomycin resistance gene, *mmr*, to the plasmid pIJ86 was done by Dr. C. Corre prior to the start of this PhD
- The rearranged product of the epoxidized pre-methylenomycin C (compound **31** in chapter 2) was purified by Dr. C. Corre prior to the start of this PhD
- Scheme 2.3 in chapter 2 was proposed by Prof. G. L. Challis

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Gideon Aina Idowu

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Abbreviations

A – adenine

ACP – acyl carrier protein

Alamm – alanine minimal medium

apr – apramycin resistance gene

atm – atmosphere

ATP – adenosine triphosphate

BAC – bacterial artificial chromosome

BMM – Belitzky minimal medium

BPC – base peak chromatogram

C – cytosine

ca – circa

Cas9 – CRISPR assisted protein 9

CDA – calcium-dependent antibiotics

CoA – coenzyme A

COSY – correlation spectroscopy

CRISPR – clustered regularly interspaced short palindromic repeats

CTP – cytidine triphosphate

Dmp – diamino pimelic acid

DNA – deoxyribonucleic acid

DNAse – deoxyribonuclease

dNTPs – deoxynucleotide triphosphate

EGSC – *E. coli* genetic stock centre

EIC/s – extracted ion chromatogram/s

ESI – electrospray ionisation

FAS – fatty acid synthase

FAD/H_2 – flavin adenine dinucleotide/reduced form

G – guanine

Gcs – germicidin synthase

GTP – guanosine triphosphate

HGT – horizontal gene transfer

HMBC – heteronuclear multiple bond correlation

HPLC – high pressure liquid chromatography

HRMS – high resolution mass spectrometry

HSQC – heteronuclear single quantum correlation

IPTG – isopropyl β-D-1-thiogalactopyranoside

KAS II – β-ketoacyl-ACP synthase II

KAS III – β-ketoacyl-ACP synthase III

LC-MS – liquid chromatography – mass spectrometry

LPS – lipopolysaccharides

m/z – mass/charge ratio

MCAT – malonyl acyl carrier protein transacylase

MBC – minimum bactericidal concentration

MIC – minimum inhibitory concentration

Mm – methylenomycin

MMF – methylenomycin furan

NEB – new England biolabs

NAD/H – nicotinamide adenine dinucleotide/ reduced form

NADP – nicotinamide adenine dinucleotide phosphate

NMR – nuclear magnetic resonance

NRPS – non-ribosomal peptide synthase

O.D – optical density

ORF – open reading frame

PAM – protospacer adjacent motive

PBP – penicillin binding protein

PDB – protein data bank

PCP – peptidyl carrier protein

PCR – polymerase chain reaction

PEP – phosphoenol-pyruvic acid

PKS – polyketide synthetase

P-MmC – pre-methylenomycin C

P-MmCl – pre-methylenomycin C lactone

RNA – ribonucleic acid

RNAse – ribonuclease

rpm – revolutions per minute

SCP1 – *Streptomyces coelicolor* plasmid 1

SCP2 – *Streptomyces coelicolor* plasmid 2

SFM – soya flour mannitol medium

sgRNA – synthetic guide RNA

SMM – supplemented minimal medium

SMMS – supplemented minimal medium solid

SpCas9 – *Streptococcus pyogenes* Cas9

T – thiamine

TetR – tetracycline resistance protein

TIC/s – total ion chromatogram/s

TLC – thin-layer chromatography

TBE – Tris borate EDTA

UDP – uridine diphosphate

UTP – uridine triphosphate

UV – ultraviolet

Abstract

The genus *Streptomyces* is known to be responsible for the production of more than two-thirds of the world's antibiotics, through complex specialised metabolic pathways. However, given the high frequency of rediscovery of known antibiotics and the challenge of producing novel analogues *via* chemical synthesis, biosynthetic engineering has emerged as an attractive approach to optimising antibiotic natural products for clinical use. This technique utilises enzymes from antibiotic biosynthetic pathways to create novel antibiotic derivatives. However, its application requires an understanding of how antibiotics are biosynthesised.

This work is focused on the methylenomycin antibiotics produced by *Streptomyces coelicolor* A 3 (2), a model Actinobacterium. The cluster of genes directing methylenomycin production and its regulation are carried on the giant linear plasmid SCP1. The sequencing of the entire 356-kb SCP1 plasmid allowed bioinformatics analyses to be applied to the assignment of putative roles in methylenomycin biosynthesis for several of the enzymes encoded within the methylenomycin biosynthetic gene cluster. However, experimental evidence to support the proposed roles of several of these enzymes has yet to be obtained, while the roles of some of the proteins encoded by the cluster remain unclear.

Here, work towards understanding the biosynthesis as well as the mode of action of the methylenomycin antibiotics is reported. In particular, the roles of MmyO and MmyF in the epoxidation of methylenomycin C to produce methylenomycin A are demonstrated *via* feeding of methylenomycin C to a methylenomycin-resistant derivative of *S. coelicolor* M145 expressing *mmyO* and *mmyF*. A putative butenolide intermediate in the pathway, believed to derive from a MmyD-catalysed condensation of acetoacetyl-MmyA with a pentulose, was identified in *S. coelicolor* strains expressing the methylenomycin biosynthetic gene cluster. The pattern of incorporation of [U-^{13}C]-D-ribose into the putative butenolide intermediate was similar to that observed for methylenomycin C, indicating the former could indeed be a precursor to the latter.

A putative intermediate of the pathway, pre-methylenomycin C, accumulating in a *mmyE* mutant strain, and its lactone form, pre-methylenomycin C lactone, were shown to be 16 and 256 times, respectively, more potent than methylenomycin A, against methicillin-resistant *Staphylococcus aureus* (MRSA). Expression of the methylenomycin resistance determinant (*mmr*) in *Streptomyces* species also confers no resistance against these two putative intermediates unlike methylenomycin A, the final antibiotic product of the pathway. Investigations into the mechanism of action of methylenomycin antibiotics with luciferase and β-galactosidase pathway-specific promoter-reporter fusion strains strongly suggest that the methylenomycins exert their antibiotic effects in bacteria primarily by targeting the biosynthesis of cell wall peptidoglycan, consistent with their activities mainly against Gram-positive strains. This is the first report of the mode of action of methylenomycin family of antibiotics.

CHAPTER ONE

1. INTRODUCTION

1.1 Antibiotics and their importance in medicine

The term antibiotic literally means ‘against life’. It was first employed in biological abstracts in 1943 shortly after it was defined by Selman Waksman as a chemical substance produced by a microorganism and which, in dilute solutions, has the capacity to kill or inhibit the growth of other microorganisms.[1] This definition, although originally intended for substances isolated directly from microbial sources, is now also commonly used to refer to antimicrobial products of plant origin, those modified chemically after being isolated from microorganisms, and those produced *via* total chemical synthesis.

The first true antibiotic, penicillin (**1**) from *Penicillium notatum* (Fig.1.1), was discovered by Alexander Fleming in 1928, and was subsequently developed into a drug in the 1940s by Chain and co-workers.[2-4] That development revolutionised the

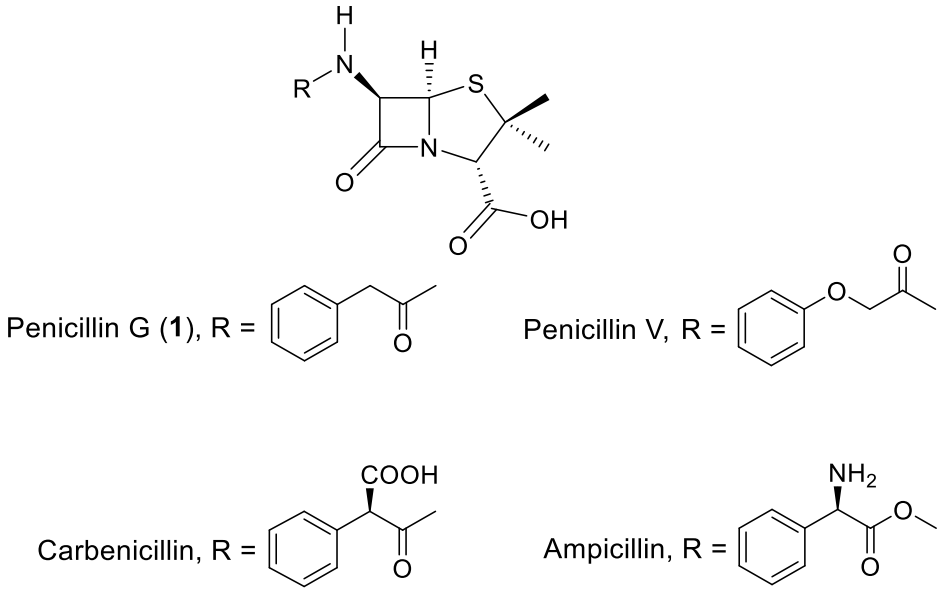


Fig.1.1: Structure of penicillin G (**1**) and some of its derivatives.

practice of medicine as many hitherto untreatable and deadly infectious diseases caused by *Streptococcus* and *Staphylococcus* strains were rapidly and effectively treated. Since then, the use of antibiotics in medicine has become widespread and almost indispensable. An estimated 100,000 to 200,000 tons of antibiotics are believed to be consumed world-wide on an annual basis.[5] A significant portion of these are used in veterinary medicine and agriculture for the treatment and prevention of infectious diseases, and as growth-promoting agents.[6,7]

The success and the excitement elicited by the introduction of penicillin prompted the search for substances with similar properties from a variety of sources.

1.2 Antibiotic producers

1.2.1 Actinobacteria are prolific producers of antibiotics and other bioactive compounds

Bacteria are broadly classified into two groups based on their response to a staining procedure developed by Christian Gram over a hundred years ago. The response reveals an underlying structural difference in the cell envelope of the two groups, with those retaining Gram's stain classified as Gram-positive while those that do not are termed Gram-negative.[8] The Gram-positive bacteria further include two major branches- those whose genomes contain a low percentage of Guanine and Cytosine (G + C), including the *Staphyloccocus*, *Bacillus* and *Streptococcus* genera; and those with a high G + C content, referred to as the Actinobacteria.[9]

Actinobacteria have been a very important source of bioactive natural products, including antibacterial, antifungal, antiviral, anti-hypertensive, antitumor, and immunosuppressant drugs.[10-12] This class of bacteria comprises a wide range of organisms, differing considerably in physiology, biochemistry, morphology and phylogeny. They range in morphological appearance from unicellular spheres, rods

and coccoids; to those with fragmenting hyphae and highly differentiated branched mycelium.[13-15] Actinobacteria consists of five orders: Actinomycetales, Bifidobacteriales, Acidimicrobiales, Coriobacteriales, and Rubrobacterales, based on 16S rRNA gene sequence comparisons.[35] The Actinomycetales order is further divided into 13 suborders of Actinomycineae, Actinopolysporineae, Streptomycineae, Micromonosporineae, Catenulisporineae, Corynebacterineae, Frankineae, Glycomycineae, Kineosporineae, Micrococcineae, Propionibacterineae, Pseudonocardineae, and Streptosporangineae.[16,35]

Of particular relevance to the current study is the suborder Streptomycineae which has only one family (Streptomycetaceae), subdivided into ten genera: *Streptomyces*, *Streptoverticillium*, *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasatoa*, *Kitasatospora*, *Microellobosporia*, and *Streptacidiphilus*.[35, 36] Of the genera under the Streptomycetaceae family, and in fact the entire Actinobacteria class, the contribution of the *Streptomyces* genus to the discovery of antibiotics cannot be over-emphasized. *Streptomyces* produce over 70% of antibiotics in clinical use today, either directly or as structurally – modified derivatives.[12,17-19] This productively unique group of bacteria mostly inhabit the soil and possess a characteristic earthy smell. Some species have also been found to exist in symbiotic relationships with plants, a situation where the antibiotics produced by *Streptomyces* protect the plant against invasion by disease agents while the *Streptomyces* benefit from nutrients coming from the plant's exudates.[9, 12, 26] *Streptomyces* exhibit a complex life cycle which reflects their adaptation to the soil and other environments. Single spores often germinate and grow into vegetative mycelium which further develops into branched aerial hyphae in a manner similar to moulds.[20-23] This enhances their ability to attach to, penetrate, and feed on plant and

invertebrate tissues in the soil *via* the secretion of cellulase and xylanase enzymes.[20] Depletion of nutrients within a given growth area leads to the formation of spores, enabling survival outside of the dead tissues and also serving as a means of dispersal or reproduction.[20, 22, 23] The onset of spores formation is also believed to coincide with specialised metabolite (e.g. antibiotic) production in many species of *Streptomyces* (Fig. 1.2).[24, 25]

(A) (B)

Fig. 1.2 (A): Aerial hyphae growth in *Streptomyces* species is temporally coupled to antibiotic production (Chater [20]). The antibiotic (in pink) protects the nutrients underneath from invasion by other microbes while ensuring a continued supply of the nutrients to the producer (B) *Streptomyces coelicolor* M145 sporulating on soya flour mannitol medium, SFM (Image obtained from the present study).

The first antibiotic to be isolated from *Streptomyces*, streptomycin (**2**) from *S. griseus*, was discovered by Selman Waksman in 1943, [27, 28] and soon became an effective drug for the treatment of tuberculosis. Researchers in the field of natural product discovery thus developed an interest in *Streptomyces* as sources of bioactive compounds and their efforts were handsomely rewarded with the discovery and introduction of many more antibiotic compounds with wide-ranging structural

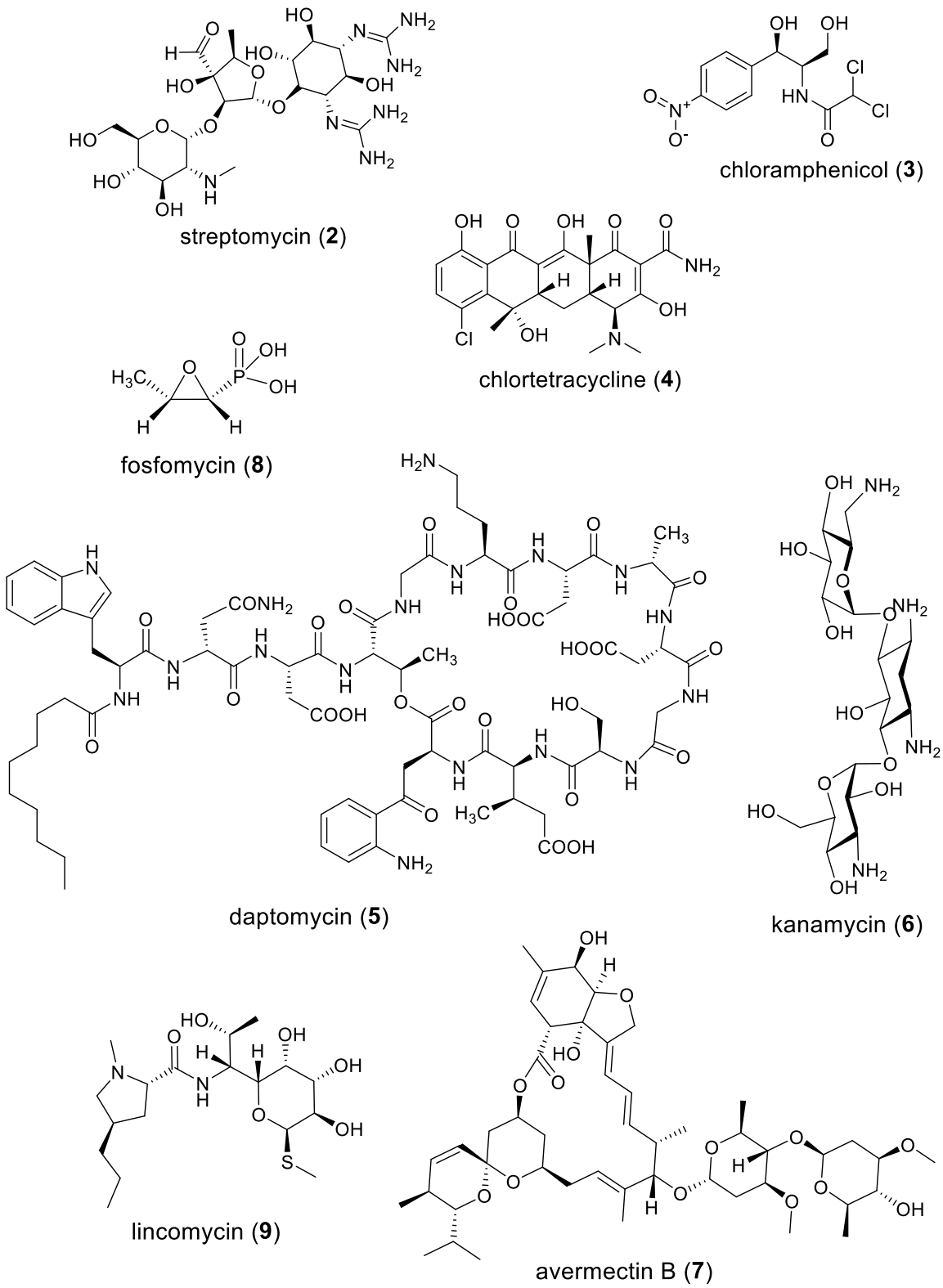


Fig. 1.3: Representative specialised metabolites produced by *Streptomyces* species.

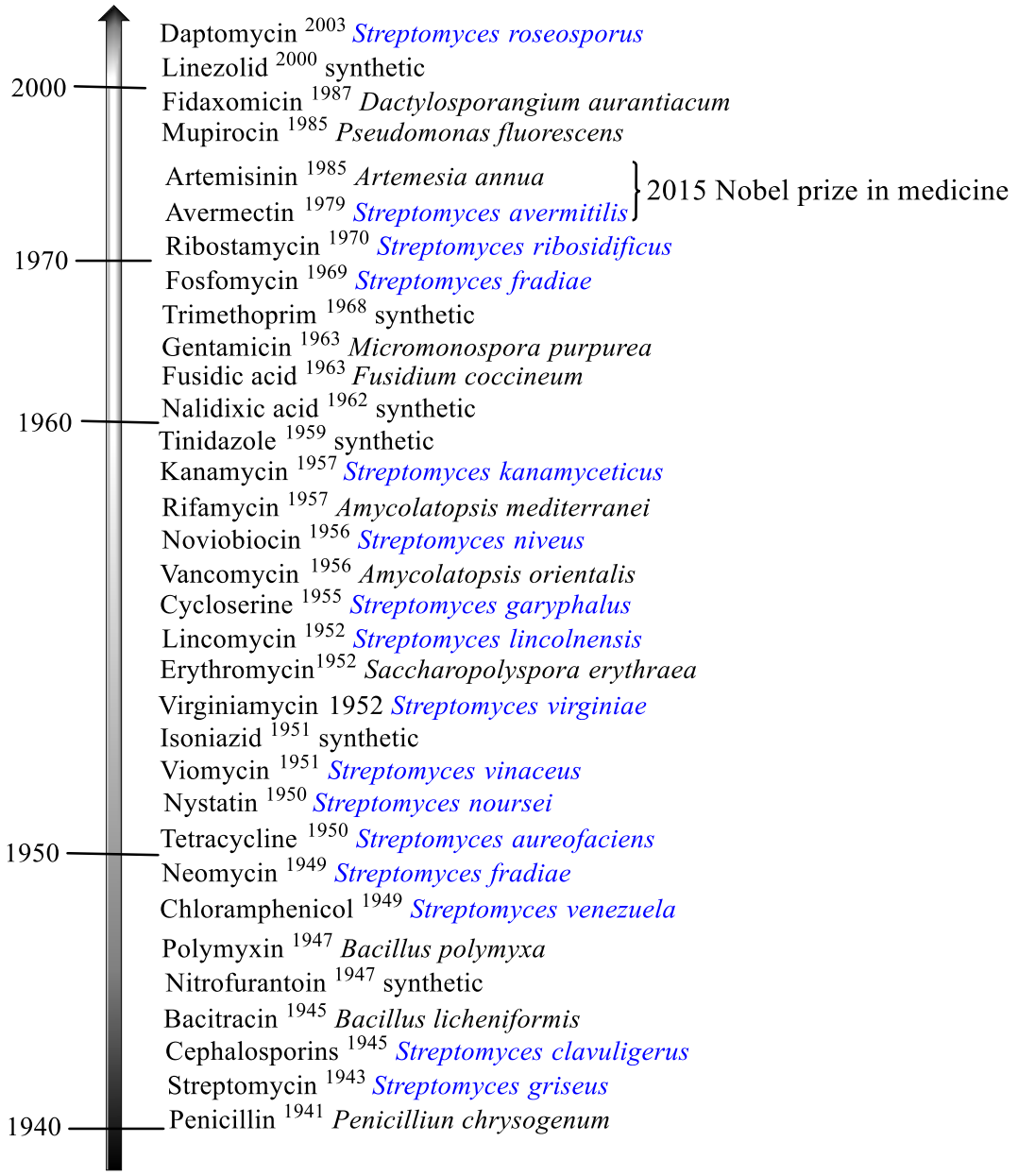


Fig. 1.4: Timeline of bioactive natural products discovery. The list (although not exhaustive) is intended to show the frequency of antibiotics discovery from *Streptomyces* species (in blue) compared to other sources. Cephalosporins were first discovered from *Acremonium chrysogenum* (formerly classified as *Cephalosporium*)[44,45]; penicillin was also first obtained from *Penicillium notatum* in 1928.[2,3]

features. Notable among the clinically used antibiotics produced by *Streptomyces* species are chloramphenicol (**3**) from *S. venezuelae*, chlortetracycline (**4**) from *S. aureofaciens* and daptomycin (**5**) from *S. roseosporus*.[29-34] Streptomyces species also

produce cephalosporins, noviobiocin, kanamycin (**6**), avermectin (**7**), fosfomycin (**8**), and lincomycin (**9**) (see Figs 1.3 and 1.4). It is noteworthy that Satoshi Omura and William Campbell were jointly awarded half the 2015 Nobel prize in medicine for their roles in the discovery and development of avermectin/ ivermectin, an important antiparasitic agent.[271]

Despite numerous modern genome-driven and molecular biology-inspired approaches for the discovery of novel S*treptomyces* specialised metabolites (section 1.5), new isolates of the genus from the environment also continue to contribute to the discovery of new antibiotics. Particularly noteworthy is the isolation of two antibiotic-producing endophytic *Streptomyces spp.* NRRL 30562 and NRRL 30566 in the Northern part of Australia. The former produces a family of antibiotics called the munumbicins while the later produces a quinoxaline group of antibiotics with highly potent activity against Gram-positive strains as well as the malarial parasite (*Plasmodium falciparum*). [37,38] These findings suggest that endophytic *Streptomyces*, and indeed other as yet unisolated *Streptomyces* species from various underexplored environmental niches, still offer some promise for the discovery of novel antibiotic scaffolds with potential therapeutic applications. This view is further underscored by the discovery of platensimycin (produced by *Streptomyces platensis* isolated from South Africa), introducing a unique structural class of antibiotics possessing an amino – dihydroxyl - benzoic acid moiety joined *via* an amide bond to a pentacylic terpenoid (Fig. 1.5). [39, 40]

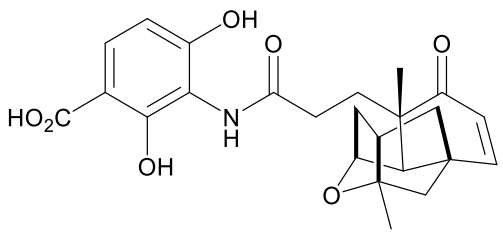
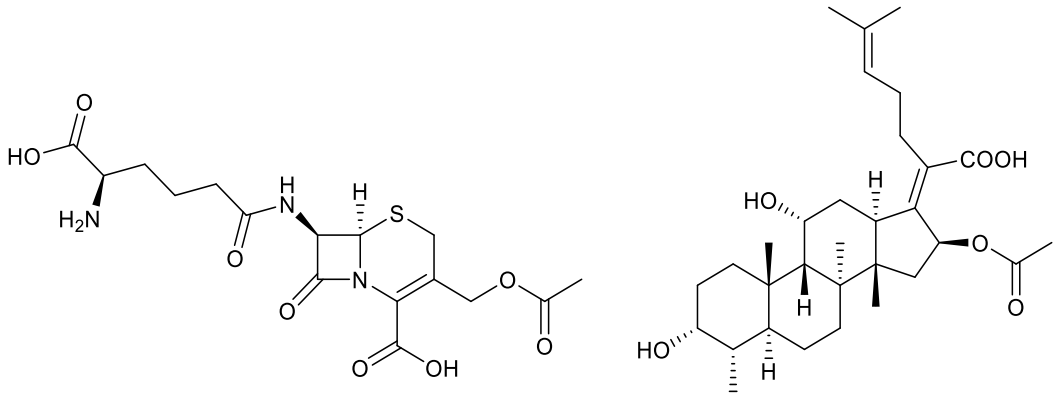


Fig.1.5: Structure of platensimycin (**10**), an antibiotic discovered in 2006 from *S. platensis.*

1.2.2 Other sources of antibiotics

The accidental discovery of penicillin from *Penicillium notatum* by Fleming did not mark the end of the road for fungi in terms of the discovery of antibacterial and other therapeutic natural products. Indeed, fungi account for an estimated 38 % of all bioactive compounds derived from microbial sources (unicellular bacteria, filamentous *Actinomycetales* and eukaryotic fungi).[41] Cephalosporins are another important group of antibiotics obtained from fungi (Fig.1.6). They were originally found to be produced by the fungus *Acremonium chrysogenum* (formerly called *Cephalosporium acremonium)* and are structurally related to penicillin due to the presence of a beta-lactam ring.[42-45] Fusidic acid, which has broad-spectrum activity against Gram – positive bacteria, was also obtained from a fungus (*Fusidium coccineum*) and has activity against most Gram-positive strains.[46, 47] It has been widely used topically in creams and dermatological products,[48, 49] as well as in the clinic for the treatment of systemic infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA).[50]



cephalosporin C (**11**) fusidic acid (**12**)

Fig 1.6: Structures of cephalosporin C and fusidic acid, antibiotics produced by fungi that were discovered after penicillin.

Apart from the prolific Actinobacteria, other groups of bacteria, especially *Pseudomonas*, Cyanobacteria and *Bacillus*, are known to produce an estimated 3,800 bioactive compounds, representing 17% of the bioactive natural products obtained so far from microbial origin (Table 1.1).[41] Mupirocin,[51] noscomin,[52] and bacitracin [53] are notable examples of antibiotics produced by these three groups respectively.

Table 1.1: Estimated number of antibiotics and other bioactive specialised metabolites from microbial origin (Adapted from Berdy [41])

Source	Antibiotics	Other bioactive metabolites	Total bioactive metabolites	Used in human therapy
Other bacteria	2900	900	3800	8 - 10
Actinobacteria	8700	1400	10100	70 - 75
Fungi	4900	3700	8600	13 - 15
Total	16500	6000	22500	91 - 100

Interestingly, antibiotic production is not restricted to microorganisms. Almost all forms of life have been reported to produce antimicrobials and other bioactive compounds. These include higher plants, invertebrates, worms, insects, fishes, reptiles, birds and mammals.[41] Unlike the compounds from bacteria and fungi, many of the products from the higher life forms have, however, not found applications in human or veterinary medicine.

1.3 Antibiotic classes and modes of action

Antibiotics exert their bacteriostatic or bactericidal effects on pathogens through a wide range of mechanisms. Antibiotics target and disrupt essential functions in bacterial cells. However, in order to be useful in the clinic, they must have no adverse effect on humans. Thus, based partly on their sources and chemical structures, and

more importantly, on the cellular functions which they disrupt, antibiotics can be classified as highlighted below.

1.3.1 Antibiotics inhibiting bacterial cell wall biosynthesis

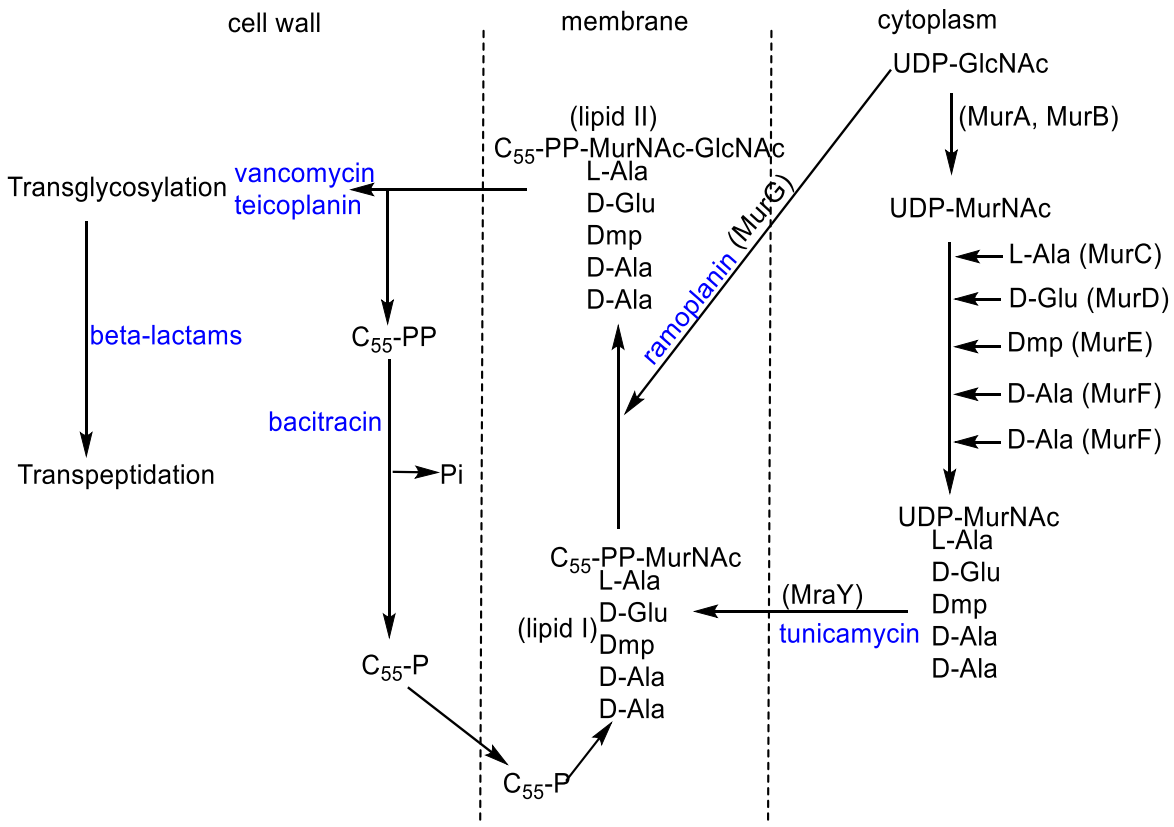
Antibiotics in this class disrupt different stages of the biosynthesis of peptidoglycan, a key component of the bacterial cell wall otherwise known as murein (Mur). Peptidoglycan is a polymer consisting of repeating disaccharide units comprising *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), which are cross-linked by short peptides.[54,55] The initial step in peptidoglycan biosynthesis is the conversion of UDP-GlcNAc to UDP-MurNAc through the actions of MurA and MurB. The UDP-MurNAc is then modified with a pentapeptide through the activities of the ligase enzymes (MurC,D,E,F) which sequentially add L-Ala, D-Glu, Dmp (diamino-pimelic acid), and D-Ala-D-Ala to give the hydrophilic product UDP-MurNAc pentapeptide.[56,57] This intermediate is linked by MraY to a poly-isoprenoid lipid carrier (undecaprenyl phosphate; C_{55}-P) to form lipid I, facilitating its export from the cytoplasm.[56,57]

Once lipid I is associated with the membrane, MurG (a membrane-associated glycosyl transferase) adds GlcNAc derived from UDP-GlcNAc to the developing precursor and leads to the formation of lipid II, regarded as the full monomer of peptidoglycan.[54,56] The lipid II is then translocated across the membrane to the extracellular surface where it serves as substrate for enzymes that polymerise it to form a linear peptidoglycan chain *via* transglycosylation, and cross-link adjacent polymers through the pentapeptide chains, thus incorporating newly biosynthesized polymers into the existing murein network (transpeptidation).[57-59]

Beta-lactam antibiotics, such as penicillins, cephalosporins and carbapenems, disrupt cell wall biosynthesis by irreversibly inhibiting the enzymes involved in

transpeptidation, the so-called penicillin binding proteins (PBPs), thereby preventing cross-linking of peptide side chains, ultimately leading to cell lysis.[60-62] Resistance to these antibiotics involves enzymatic cleavage of the beta-lactam by beta-lactamases.[63] Resistance to beta-lactam antibiotics may also occur by modification of the active site of the PBPs.[61]

The glycopeptide antibiotics (vancomycin and teicoplanin) exert their antibacterial action at the point of transglycosylation (scheme 1.1). Once the lipid II is translocated to the extracytoplasmic membrane, these compounds bind to its D-Ala-



Scheme 1.1: Overview of the steps in peptidoglycan biosynthesis. The point of action of tunicamycin [67], ramoplanin [68], bacitracin [69], glycopeptides and beta-lactams are indicated.

D-Ala terminus forming a stable and non-covalent complex. This sterically hinders the action of the peptidoglycan polymerases which are not able to transglycosylate the bulky antibiotic-substrate complex.[64-66]

Other antibiotics exist which interfere with earlier stages of peptidoglycan biosynthesis. The points of action of some of these antibiotics are also illustrated in scheme 1.1.

1.3.2 Antibiotics inhibiting DNA replication

Notable antibiotics inhibiting DNA replication in bacteria include the quinolones (particularly norfloxacin and ciprofloxacin, the clinically used fluorinated quinolone derivatives) and metronidazole. The quinolones act by inhibiting DNA gyrase and DNA topoisomerase IV, two enzymes essential for DNA replication. DNA gyrase controls DNA supercoiling, while topoisomerase IV functions in the separation of interlinked daughter chromosomes following DNA replication.[70] Normally, DNA gyrase functions by introducing a double-strand break into a region of duplex DNA and passing another region of duplex DNA through the break before rejoining the broken strands.[70,71] However, in the presence of a quinolone antibiotic, the DNA-bound gyrase is trapped as soon as the double strand break is generated, thereby preventing it from resealing the broken strands (Fig. 1.7). This causes damage to DNA by generating permanent double strand breaks, ultimately leading to cell death.[70,71] Topoisomerae IV is a homologue of DNA gyrase and uses a similar mechanism of double-strand breaking and passing of duplex DNA.[72,73] Resistance to quinolone antibiotics is associated with mutations in the *gyrA* and *gyrB* subunits of the DNA gyrase,[70] thereby preventing interaction with quinolones.

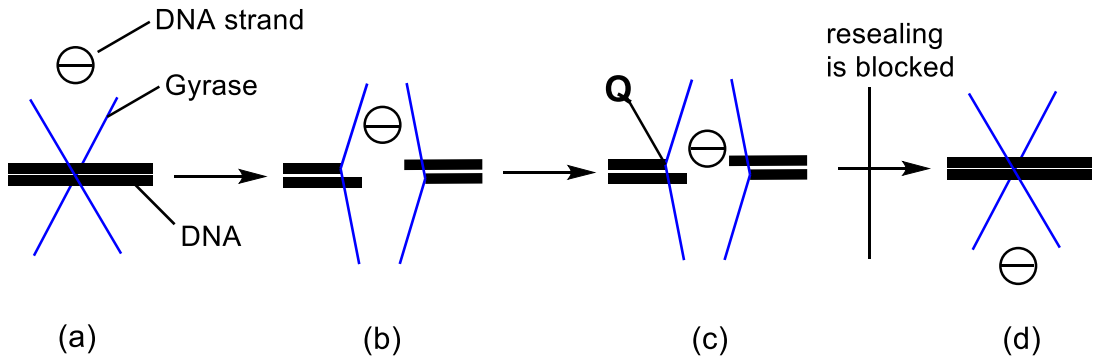


Fig. 1.7: Schematic representation of the interruption of gyrase action by a quinolone antibiotic: (a) DNA gyrase (in blue) binds DNA before strand passage (b) Gyrase undergoes a conformational change and DNA gate is opened *via* double-strand break. Another region of the same DNA molecule (shown in cross section as DNA strand) moves close to the DNA gate (c) Quinolone traps DNA gyrase and disrupt strand passage. This prevents the resealing and repair of the broken DNA that should occur at (d). Redrawn from Drlica and Zhao.[70]

In the case of metronidazole (a prodrug), the nitro-group of the compound is first reduced by an electron-transport ferredoxin pathway in anaerobic bacteria and protozoa. The reduced drug (a nitroso free radical) then binds non-specifically to bacterial DNA and causes high level DNA breakage and degradation (Fig. 1.8).[74,75]

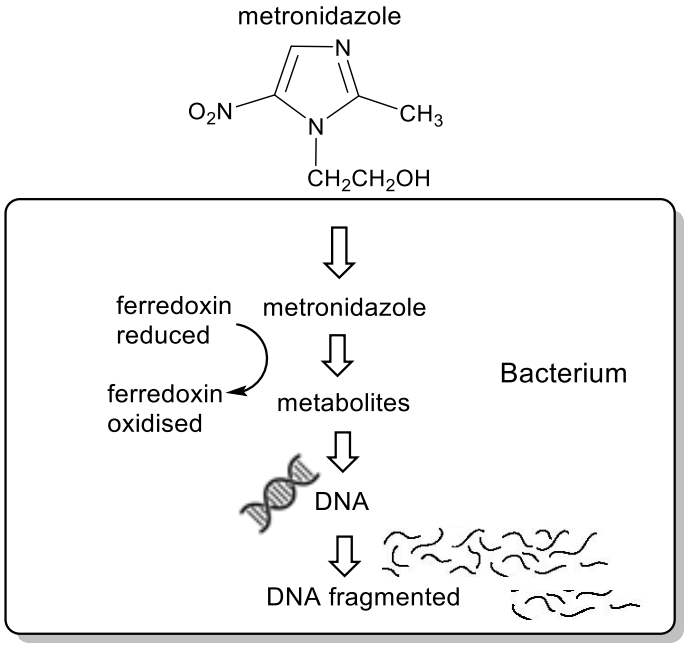


Fig. 1.8: Schematic depicting the mode of action of metronidazole, an antibiotic inhibiting replication. Redrawn from Neu and Gootz.[76]

1.3.3 Antibiotics inhibiting transcription

There are many antibiotics that inhibit transcription in bacteria, including rifampicin, fidaxomicin, streptoglydigin, and myxopyronin.[79] However, only rifampicin and fidaxomicin (Fig. 1.9) are used in the clinic. Rifampicin belongs to the rifamycin group of antibiotics produced by *Amycolatopsis mediterranei*.[77] Like other rifamycins, it disrupts transcription by binding strongly to the DNA-dependent RNA polymerase, a complex enzyme with an α_2, β, β', ω subunit structure.[77] Rifampicin acts at the transcription initiation stage by binding to the β subunit (Fig. 1.10), causing a conformational change that prevents the formation of phosphodiester bond, thereby blocking initiation of transcription or extension of a nascent RNA chain.[77,78] Resistance develops mainly due to mutation in the target polymerase and to a lesser extent by inactivation of the antibiotic.[77]

Fidaxomicin was recently approved for the treatment of *Clostridium difficile* infections. It inhibits RNA synthesis by binding to the DNA template-RNA polymerase complex, thereby preventing the separation of template DNA strands,[79] an essential prerequisite for transcription initiation.

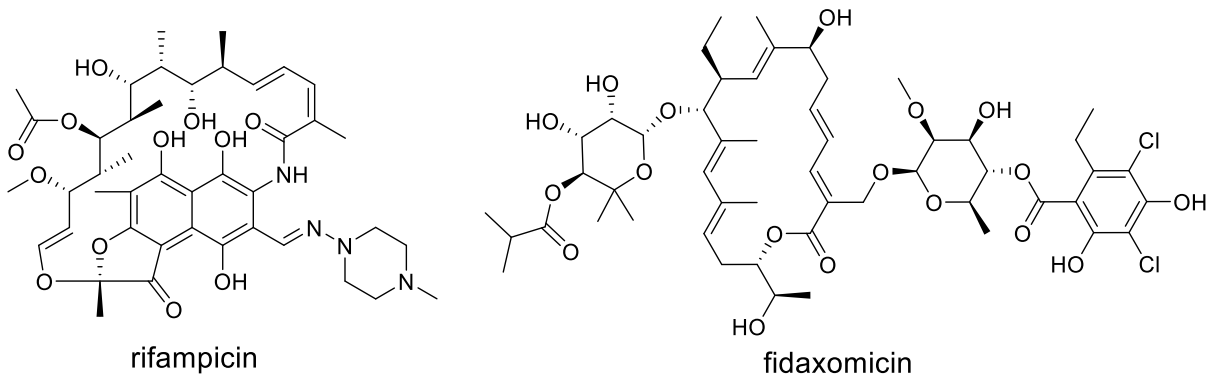
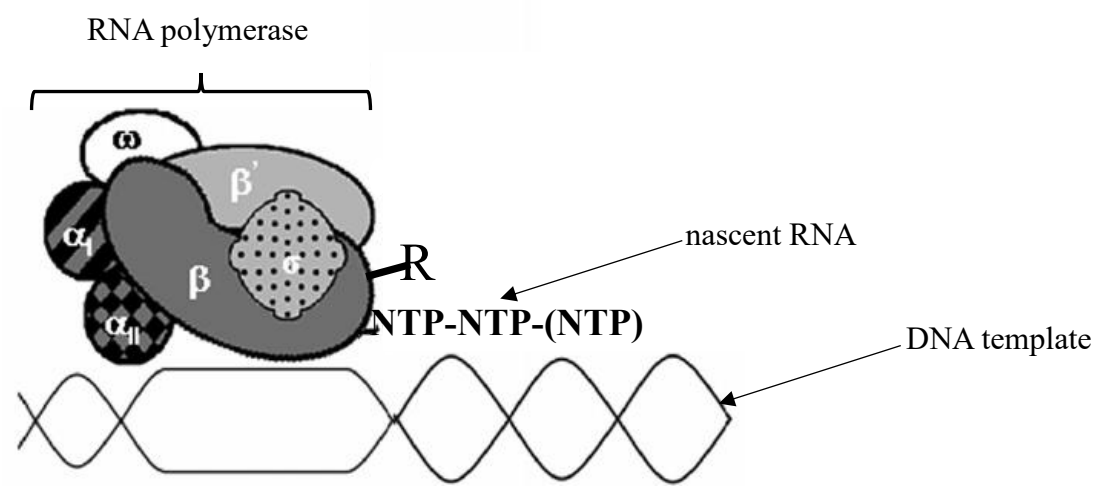


Fig. 1.9: Structures of rifampicin and fidaxomicin.

(A)



(B)

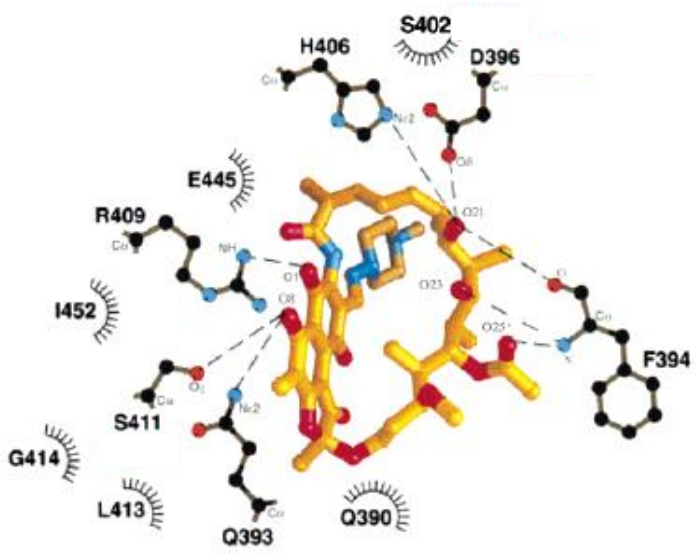


Fig. 1.10: (A) Schematic showing the point of action of rifampicin (**R**) at the start of RNA synthesis, after the separation of template DNA strands (B) Representation of the *Thermus aquaticus* RNA polymerase-rifampicin complex (PDB 1I6V), showing interaction of the antibiotic with amino acids in the β-subunit.[77,78] Residues depicted in dark ball and stick form interact with the antibiotic *via* hydrogen bonds (dashed lines); other residues interact *via* Van der Waals forces.

1.3.4 Antibiotics inhibiting translation

Many antibiotics target translation, owing to the nature and importance of this process in bacteria.[80,81] Translation is catalysed by ribosome and involves four key steps: initiation, elongation, termination and recycling.[82] Initiation step involves the

formation of 70S ribosome from small (30S) and large (50S) ribosomal subunits, and the positioning of an mRNA start codon and an initiator tRNA at the P-site in the 70S ribosome. The elongation cycle is the most common stage of translation inhibited by antibiotics. This involves the delivery of an aminoacylated tRNA (aa-tRNA) to the A-site of the ribosome by elongation factor Tu (EF-Tu). Peptide bond formation then occurs between the amino acid residues attached to tRNAs at the A and P-sites, causing the residue of peptide chain attached to the latter to be transferred to the former.[82] The tRNAs in the P and A sites of the ribosome are then translocated into

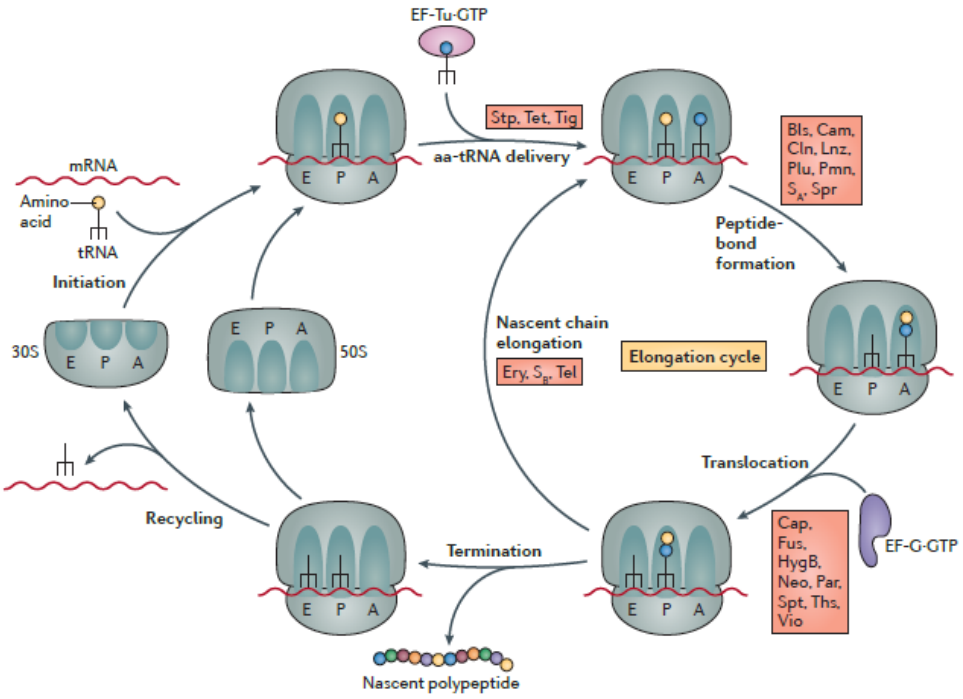


Fig. 1.11: Schematic depicting translation by the ribosome and the point at which various antibiotics disrupt the process. Ery (erythromycin), S_B (streptogramin B), Tel (telithromycin), Stp (streptomycin), Tet (tetracyclines), Tig (tigecycline), Bls (blasticidin), Cam (chloramphenicol), Cln (clindamycin), Lnz (linezolid), Plu (pleuromutilins), Pmn (puromycin), S_A (streptogramin A), Spr (sparsomycin), Cap (capreomycin), Vio (viomycin, **41**), HygB (hygromycin B), Neo (neomycin), Par (paromomycin), Fus (fusidic acid) and Spt (spectinomycin). Adapted from Wilson 2014.[82] E-, P-, and A- sites refer to exit, peptidyl, and aminoacyl sites respectively.

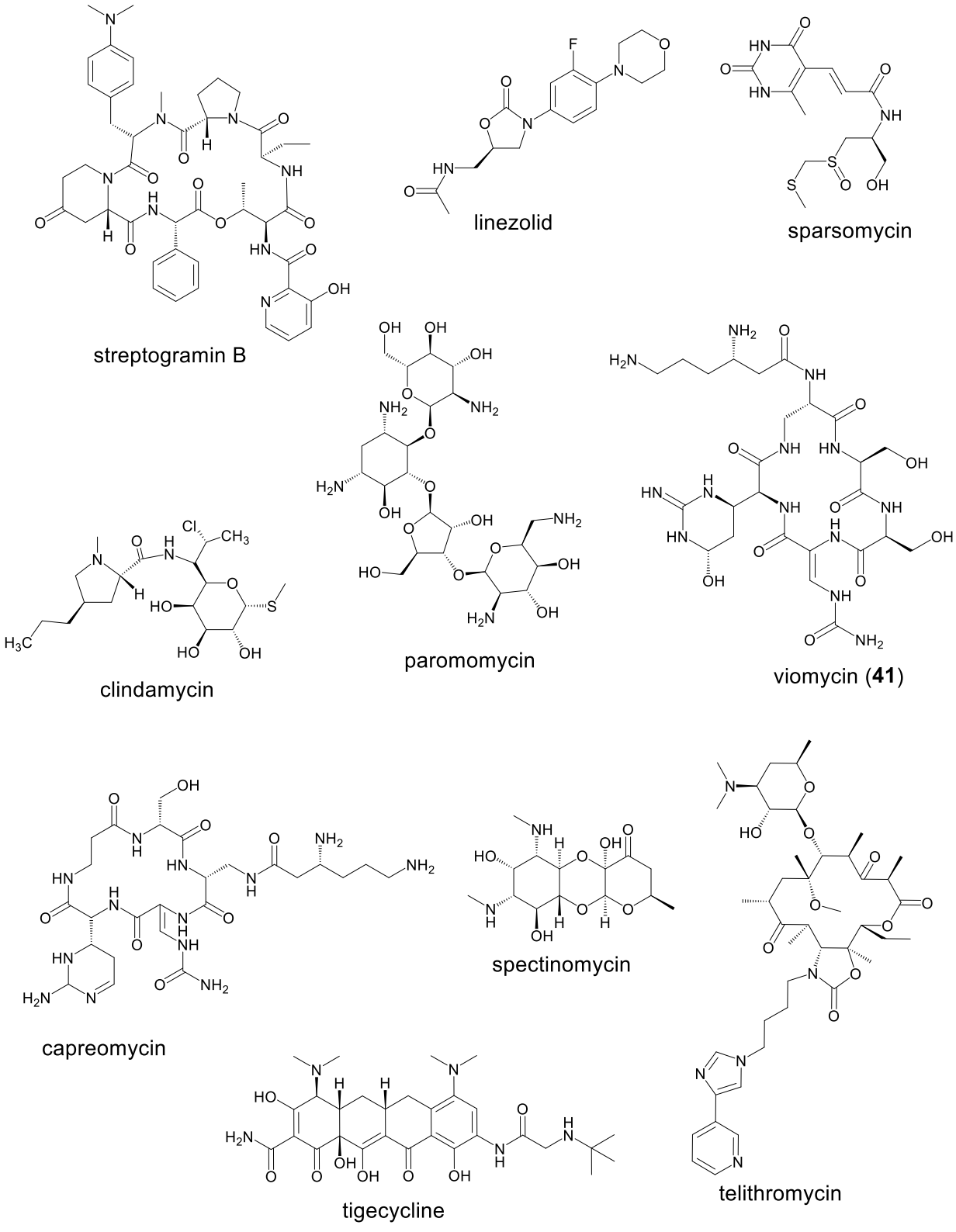


Fig. 1.12: Structures of some antibiotics inhibiting translation.

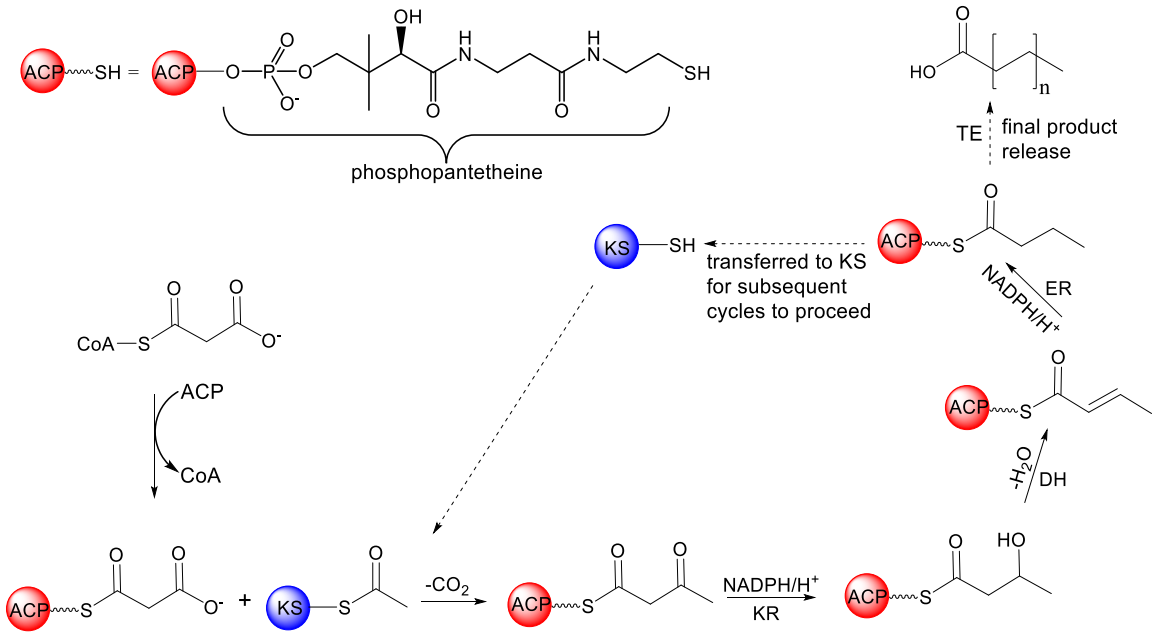
the E and P sites, respectively, to provide room in the A-site for a new incoming aa-tRNA, which is required for the next round of chain elongation.[83] The mRNA is recycled, while the nascent polypeptide chain is released into the cytoplasm at termination.

The points of action of various antibiotics targeting the elongation cycle during translation are illustrated in Fig. 1.11; the structures of some of these compounds are shown in Fig.1.12. As a specific example, viomycin (**41**) acts by stabilising the tRNA at the A-site in the ribosome, thereby preventing its translocation into the P-site and hindering the incoming aa-tRNA (i.e. blockage of translocation).[84]

1.3.5 Antibiotics inhibiting fatty acid biosynthesis

Fatty acids are important for the construction of bacterial membranes, and as component of the phospholipid bilayer.[85] They are biosynthesised on an acyl carrier protein (ACP) by the successive action of discrete components enzyme of the fatty acid synthase (FAS): ketoacyl synthase (KS), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER) and thioesterase (TE). In the first step of the biosynthesis, the ketoacyl synthase catalyses a decarboxylative condensation of a malonate with a starter acyl unit (usually an acetyl-CoA) to give an acetoacetyl-ACP (scheme 1.2). The β-keto group in this product is reduced by the ketoreductase, dehydrated by the dehydratase and finally reduced by the enoyl reductase to give a saturated chain longer than the starter acyl unit by two carbons. The cycle is repeated after the saturated chain is transferred to the ketosynthase, eventually leading to a chain of required length (14, 16, or 18 carbons),[118] which is finally hydrolysed by the thioesterase to give the free acid.

Notable antibiotics that inhibit fatty acid biosynthesis are thiolactomycin, triclosan and cerulenin, and the recently-discovered platencin (Fig. 1.13). Thiolactomycin mimics the malonate by forming strong hydrogen bond with histidine residues in the



Scheme 1.2: Fatty acid biosynthetic cycle. ACP: acyl carrier protein; KS: ketoacyl synthase; KR: ketoreductase; DH: dehydratase; ER: enoyl reductase; TE: thioesterase. n is number of cycles.

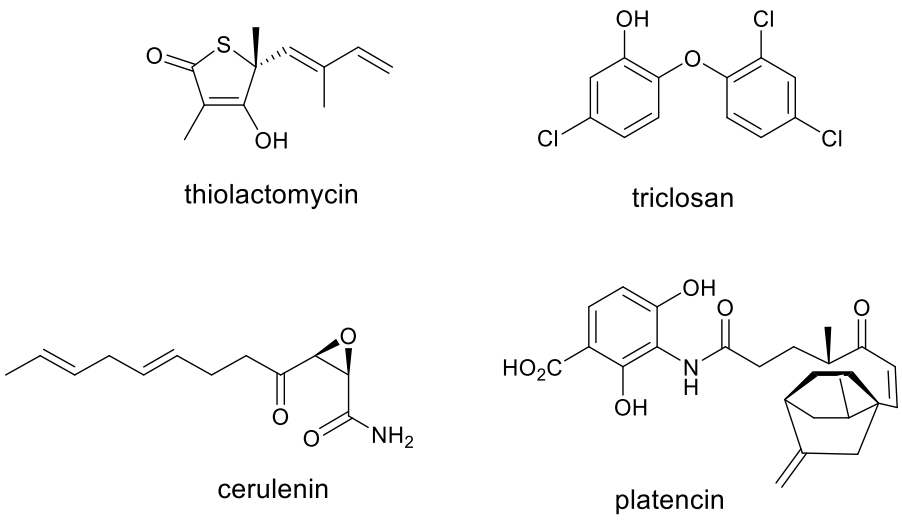


Fig. 1.13: Structures of some antibiotics inhibiting fatty acid biosynthesis in bacteria.

active site of the KS, FabB, thereby preventing the loading of malonate onto the enzyme.[86] Triclosan similarly binds to the active site of enoyl-(ACP) reductase in the fatty acid synthase.[87,88] Cerulenin inhibits the condensation step in fatty acid biosynthesis by reacting irreversibly with the active site cysteine residue in ketoacyl synthases.[89,90] Platencin uniquely inhibit both FabF and FabH, respectively beta-ketoacyl-ACP synthase II and III, thereby blocking chain elongation in fatty acid biosynthesis.[91]

1.4 The need to discover and develop new antibiotics

New antibiotics are required to find agents with improved pharmacological properties such as reduced toxicity, protection from rapid metabolism and excretion, and increased solubility and permeability.[93,94] Of greater urgency, however, is the need to find antibiotics to combat the currently high incidence of resistance by disease-causing pathogens to almost all classes of antibiotics. Recently, the world health organisation (WHO) published a list of bacterial strains which pose the greatest threat to human health and for which antibiotics are urgently required. Top on this priority list are carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae (e.g. *Escherichia coli* and *klebsiella pneumonia*); vancomycin-resistant *Enterococcus faecium*; and methicillin-resistant *Staphylococcus aureus*.[92] These strains are collectively termed the ESKAPE pathogens. Also infamous in terms of antibiotic resistance is *Mycobacterium tuberculosis*.[95, 96]

The traditional approach to discovering new antibiotics involves screening organisms from underexplored ecological niches for antimicrobial activity.

However, in recent years, this approach has frequently rediscovered known antibiotics rather than unveiling new ones.[97] While it is not impossible that novel antibiotic scaffolds could still be discovered by exploring unusual ecological niches (for example, the growing interests in endophytic and marine actinomycetes),[98-100] the techniques described in the following section are being proactively deployed to generate new antibiotics and other therapeutic agents.

1.5 Approaches for generating new antibiotics and other bioactive compounds

1.5.1 Exploitation of pathogen genomics to discover new antibiotic targets

The sequencing of the genomes of many pathogenic organisms has made a wealth of information available about them which is being exploited to identify novel targets for antibiotics.[113] Potential antibacterial targets are identified by bioinformatics and expression profiling of putative protein coding sequences. The essentiality of such coding sequences for cell survival is established *via* molecular genetic manipulation. Targets identified in this way are expressed and used in high-throughput screens for inhibitors, which are then tested for biological activity.[114-116] Such target-based screening has been used by Pfizer to discover three novel antibacterial leads which progressed into clinical trials.[117]

However, most of the compounds screened using this approach are often generated by total synthesis and very few antimicrobial leads are identified out of several thousands from library of compounds typically screened. Given that the majority of antibiotics which have found applications in the clinic are naturally-derived (see, for example, Fig. 1.4), many other biological approaches are focused on generating analogues of natural antibiotics *via* rational manipulation of the producing strains, and by chemical modifications.

1.5.2 Biosynthetic engineering

In biosynthetic engineering, enzymes from natural product biosynthetic pathways are harnessed to create novel antibiotic derivatives. For example, by developing an understanding of the enzymatic machinery for an antibiotic of interest, it is possible to exploit the substrate promiscuity of certain biosynthetic enzymes to produce antibiotic derivatives *via* feeding of substrate analogues to the producing organism.[105, 106] The production of penicillin V by addition of phenoxyacetate to penicillin producing fermentations [107-109] is a classic example of this approach (Fig.1.14). It is also possible to purify key enzymes in a biosynthetic pathway, and use them to produce new antibiotic derivatives by incubation with analogues of

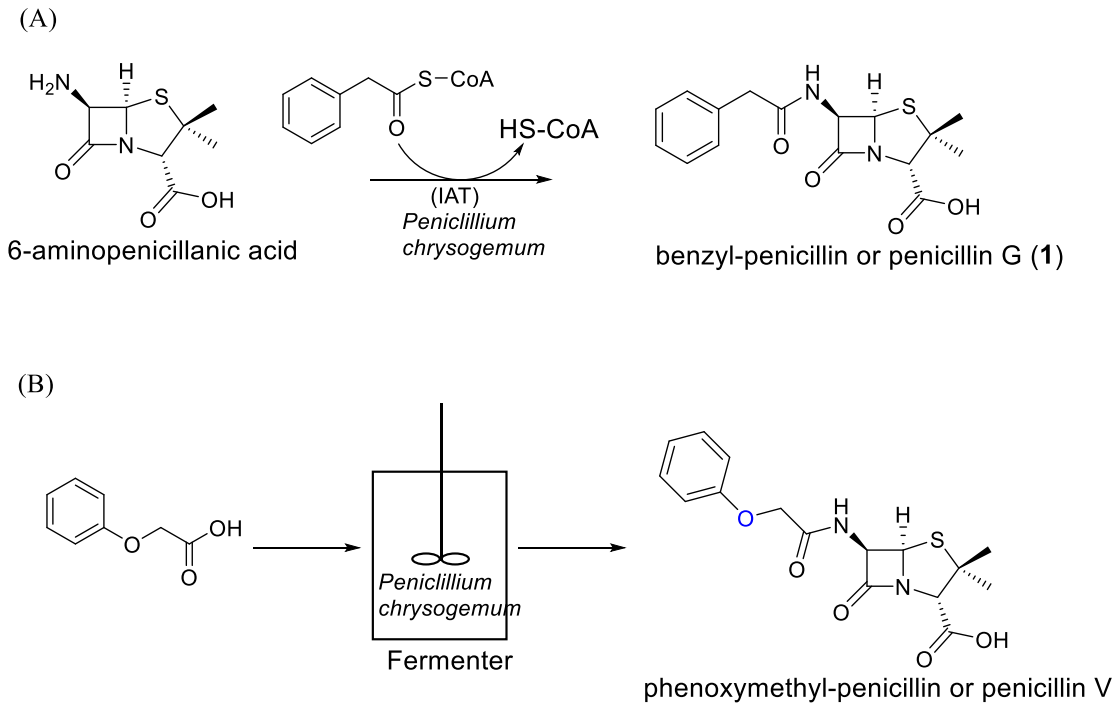


Fig.1.14:(A) Isopenicillin-*N*-acyltransferase (IAT)-catalysed coupling of phenylacetyl-CoA with 6-aminopenicillinic acid to give penicillin G (**1**), the natural antibiotic (B) Production of penicillin V, an industrially important analogue of **1**, by feeding phenoxyacetate as replacement for phenylacetyl-CoA during *Penicillium chrysogenum* fermentation.

intermediates in the pathway.[110] Isopenicillin-*N*-synthase (IPNS) was used in this manner to generate a variety of penicillin analogues *via* incubation with various derivatives of the natural substrate, L-amino adipyl-L-cysteine-D-valine.[110]

The technique of mutasynthesis is closely-related to the precursor-directed approach described above. In this case, the gene cluster for an antibiotic of interest is manipulated to block its biosynthesis. This may lead to the accumulation of biosynthetic intermediates which may possess antibiotic activity. Synthetic analogues of early biosynthetic intermediates can also be fed to the blocked mutants, resulting in novel antibiotic analogues.[111] Many applications of this approach, including to the vancomycin-type glycopeptides, have been reported.[112]

Polyketides are another group of natural products whose analogues have been generated by biosynthetic engineering. They include erythromycin A (**36**), amphotericin B, lovastatin and rapamycin (Fig. 1.15). Polyketides are assembled by large multi-domain enzymes, the polyketide synthases (PKSs), *via* condensation of simple precursors such as malonyl-CoA, propionyl-CoA and methylmalonyl-CoA, in a manner similar to the biosynthesis of fatty acids.[248]

One of the most studied polyketide natural product is the antibiotic erythromycin A, whose polyketide backbone, 6-deoxyerythronolide B (6-DEB), is assembled by 6-DEB synthase (DEBS). DEBS contains three bimodular polypeptides (DEBS 1, DEBS 2, and DEBS 3) which catalyse the biosynthesis of 6-DEB *via* stepwise decarboxylative Claisen condensation of a propionyl starter unit with six methyl-malonyl extender units (as illustrated in Fig. 1.16A).[249,269] A thioesterase domain at the *C*-terminal end of DEBS 3 then catalyses the release of the polyketide chain, with

concomitant macrolactone formation to give 6-DEB.[249] This is then hydroxylated at C-6 position by a P450 hydroxylase to give erythronolide B, followed by attachment

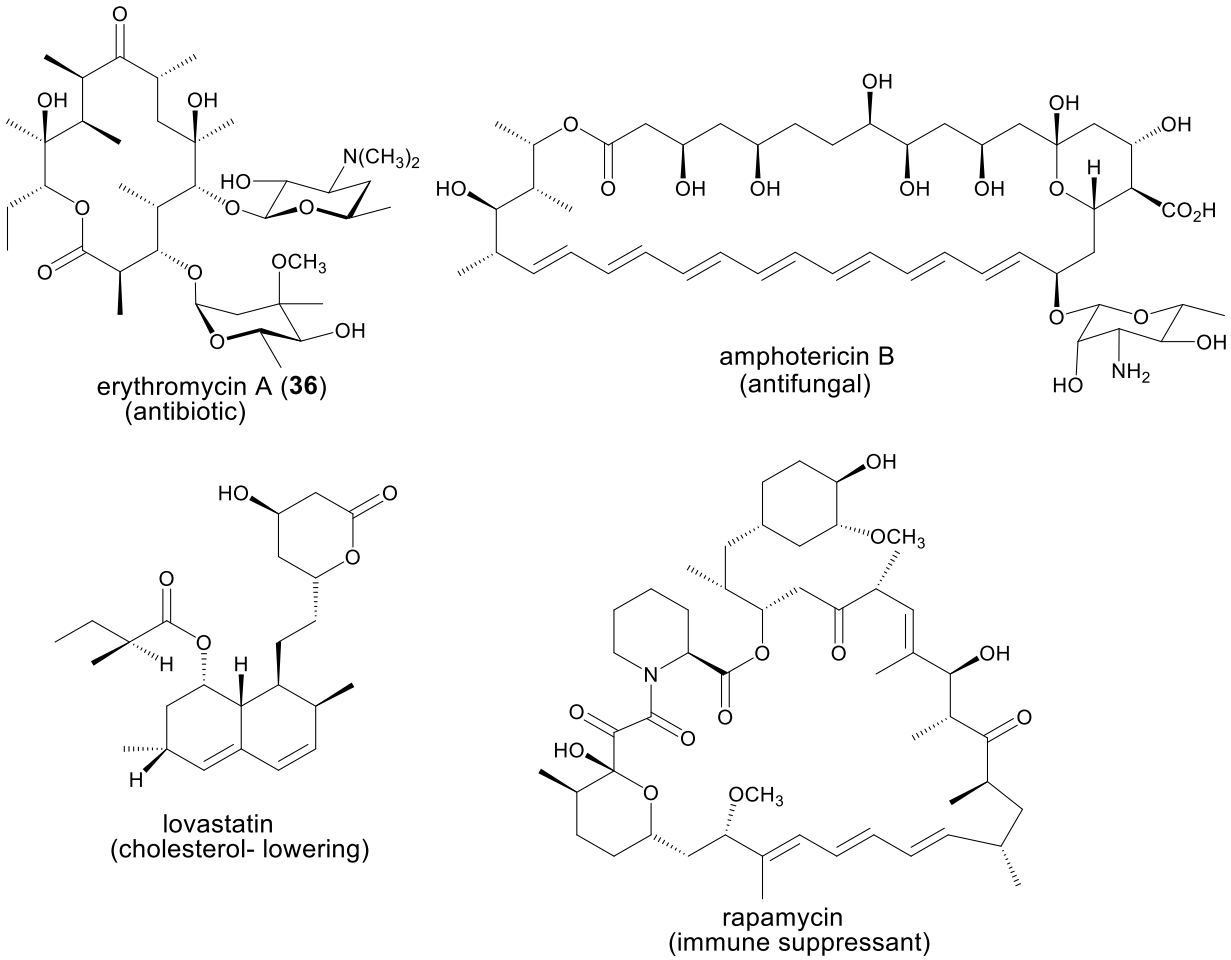


Fig. 1.15: Structures of some polyketide natural products.

of sugar moieties (L-mycarose to C-3 hyrodxyl group and D-desosamine to C-5 hydroxyl group). Further hydroxylation at C-12 and SAM-dependent methylation of the C-3 hydroxyl group of the mycarose unit will afford erythromycin A.[250, 270]

Organisation of many PKSs into domains and modules has made them particularly amenable to genetic manipulations *via* domain inactivation or replacement with domains from other PKSs, leading to the generation of novel analogues of polyketide

natural products.[248,267-268] This is exemplified by the substitution of the methylmalonyl-specific acyl transferase (AT) domain in DEBS module 2 with malo-

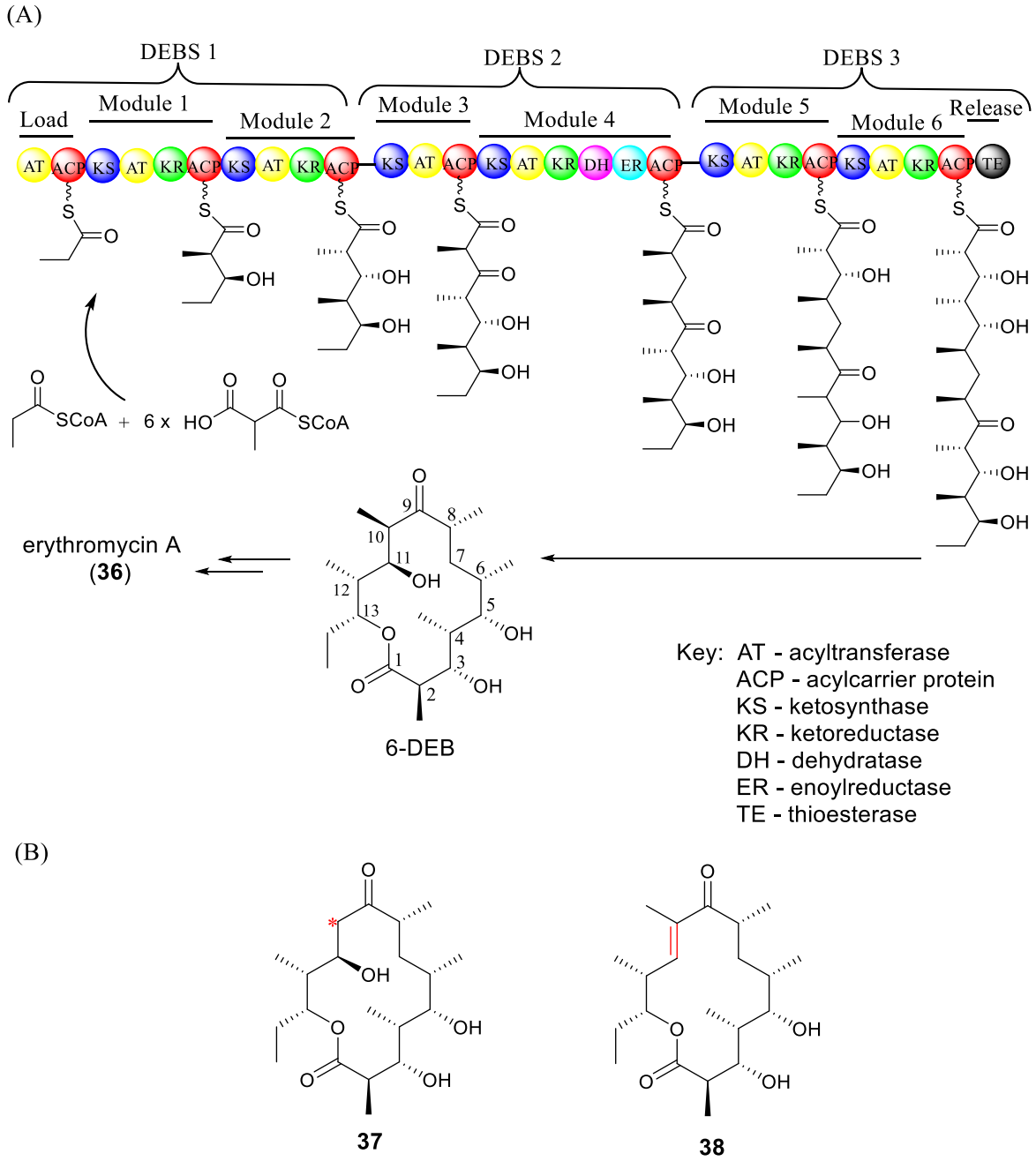


Fig. 1.16: (A) Biosynthesis of 6-DEB, the polyketide backbone of erythromycin A (B) Representative analogues of 6-DEB generated by biosynthetic engineering: **37** was generated by replacing the AT2 domain in DEBS with rapamycin AT2; **38** was generated by replacing KR2 of DEBS with (KR and DH) from rapamycin module 4.

nyl-specific AT domain from rapamycin PKS, leading to 10-desmethyl-6DEB (**37,** Fig. 1.16B). Similarly, 10,11-anhydro-6-DEB (**38**) was generated by replacing the ketoreductase in DEBS module 2 with ketoreductase plus dehydratase from the rapamycin cluster.[251] Loading modules may also be swapped between different PKSs to alter the selectivity of the starter acyl units.[250] These strategies have been used to generate numerous analogues of 6-DEB (and subsequently erythromycin), as well as analogues of many other polyketide natural products.

1.5.3 Activation of silent biosynthetic gene clusters

Microbial genome sequencing projects have led to the hypothesis that antibiotic-producing organisms have the capability to biosynthesize more bioactive products than they are currently known to produce.[122-124] For example, the *S. coelicolor*, *S. avermitilis*, *S. venezuelae* and *S. griseus* genome sequences contain, respectively, 25, 34, 31 and 34 specialised metabolite biosynthetic gene clusters, suggesting that they have the potential to assemble a significantly greater number of specialised metabolites than they have been shown to produce to date.[128-131] Methods are, therefore, being developed to identify the products of these so-called cryptic biosynthetic gene clusters, which have the potential to direct the production of novel specialised metabolites in antibiotic-producing organisms.[132]

Several of such strategies have been reported to yield novel natural compounds. The first is heterologous gene expression and comparative metabolite profiling, which involves the cloning of an entire cryptic biosynthetic gene cluster in a single cosmid or BAC vector, followed by expression in a heterologous host. LC-MS analysis is then used to identify metabolites present in the strain containing the cloned gene cluster

that are absent from the wild type organism (Fig.1.17).[133, 134] An example of a novel compound discovered using this strategy is CBS40 (Fig. 1.19).[135]

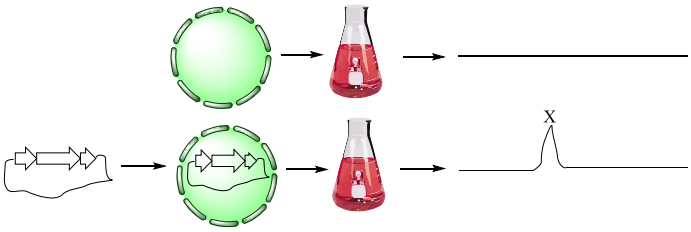


Fig. 1.17: Identification of the product of a cryptic biosynthetic gene cluster *via* expression in a heterologous host and comparative metabolite profiling. X is the likely product.

The second approach is gene knock out/comparative metabolic profiling. One or more genes believed to encode essential biosynthetic enzymes in a cryptic biosynthetic gene cluster are inactivated, and culture extracts from the wild type strain and the non-producing mutants are compared (Fig. 1.18). Metabolites present in the wild-type but absent in the mutant are likely products of the gene cluster, which can be purified and characterised.[134] This strategy was used to discover coelichelin in *Streptomyces coelicolor*.[136]

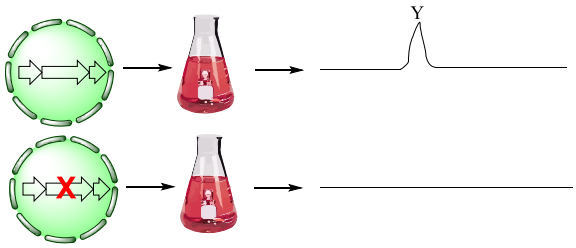


Fig. 1.18: Identification of the product of a cryptic biosynthetic gene cluster *via* gene knock out and comparative metabolite profiling. Y is the likely product.

The third approach involves manipulating the expression of putative pathway-specific activator genes within cryptic biosynthetic gene clusters. The activator gene is typically put under the control of a constitutive promoter, with the expectation that forcing expression of the activator will induce the expression of the biosynthetic

genes, thus trigerring metabolite production.[137] This strategy was used to discover the aspyridones in *Aspergillus nidulans*,[138] and the stambomycins in *S. ambofaciens*.[125] Closely related to the use pathway-specific activator is the deletion of pathway-specific transcriptional repressor genes. Such genes encode DNA-binding proteins which repress the expression of biosynthetic genes in a cluster. Thus, by identifying and inactivating a pathway-specific repressor gene, it is possible to turn on the production of metabolites encoded in a biosynthetic gene cluster. This approach was first successfully demonstrated with the discovery of gaburedins in *S. venezuelae*, by

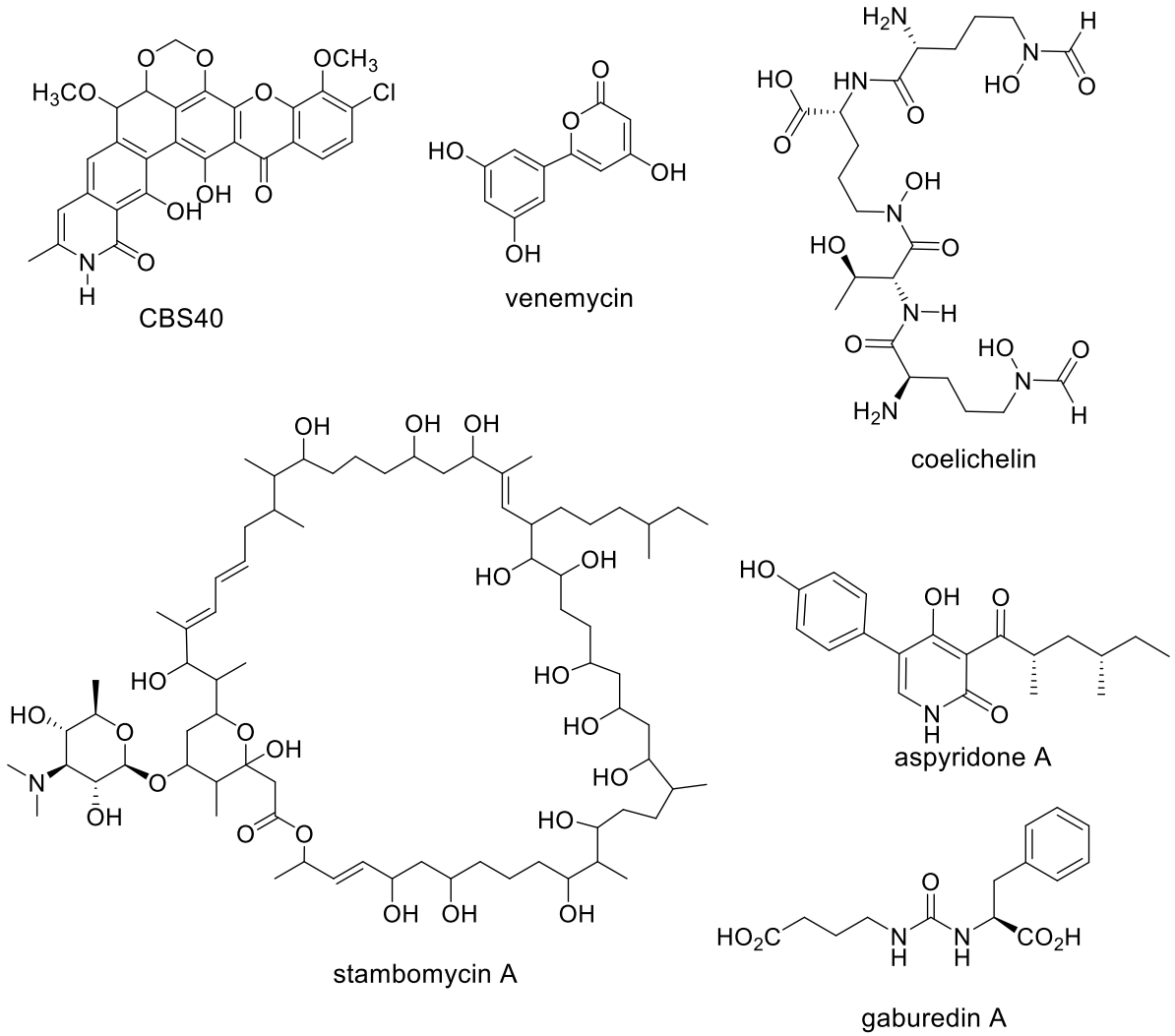


Fig. 1.19: Structures of some novel compounds discovered through activation of silent biosynthetic gene clusters.

inactivating *gbnR*, which encodes an ArpA – like transcriptional repressor.[126]

Two or more of the above approaches can also be combined, as exemplified by the discovery of venemycin *via* expression of the gene cluster in a heterologous host and manipulation of the pathway specific activator. The venemycin cluster, originally from *S. venezuelae*, was constitutively expressed in *S. coelicolor* host, together with *vemR*, the pathway-specific activator gene, leading to the production of venemycin and a monohalogenated derivative.[127]

1.5.4 Metagenomics-guided discovery

Metagenomics refers to the direct isolation and analysis of genetic material from natural microbial communities, bypassing the need to isolate microbes using traditional culturing methods.[139, 140] Antibiotic discovery using metagenomics-based approaches was inspired by the observation that only a small proportion of microbes can be cultivated in the laboratory; the vast majority in the environment (potentially harbouring novel antibiotic scaffolds) remain uncultivated.[141, 142]

DNA isolated from the environment is typically used for metagenomic library construction in a suitable host such as *E. coli*. Genes or gene clusters encoding enzymes potentially involved in antibiotic biosynthesis can then be identified either by homology-based screening, or phenotype/activity guided screening approaches, such as the appearance of zone of growth inhibition around clones, or the production of a coloured metabolite.[140, 143] Gene clusters identified by homology-based screening can be expressed in a heterologous host to identify the metabolic product.[144]Antibiotics discovered using metagenomics approaches include

turbomycins A and B,[145] and fasamycins A and B.[146] The unusual structures of the turbomycins (Fig. 1.20) were confirmed by synthesis and by single crystal X-ray diff-

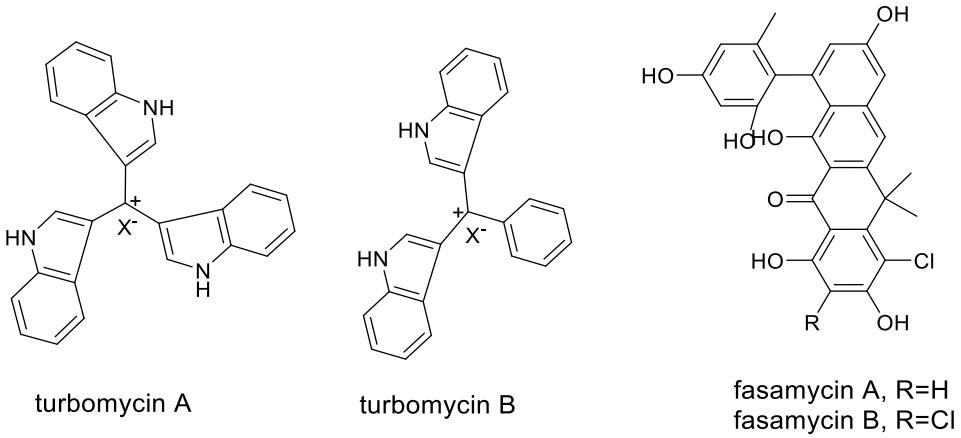


Fig.1.20: Structures of turbomycins and fasamycins, antibiotics discovered *via* metagenomics approach. X is counter ion.

raction experiments.[145] While isolation of DNA from the environment continues to show huge promise for the discovery of antibiotics from uncultivable bacteria, strategies are also being deployed to grow these bacteria and to uncover potential antimicrobial compounds encoded in their genomes. An interesting example was exemplified by the discovery of teixobactin, a non-ribosomal peptide synthetase (NRPS) - derived compound (Fig. 1.21). Teixobactin was produced by Gram-

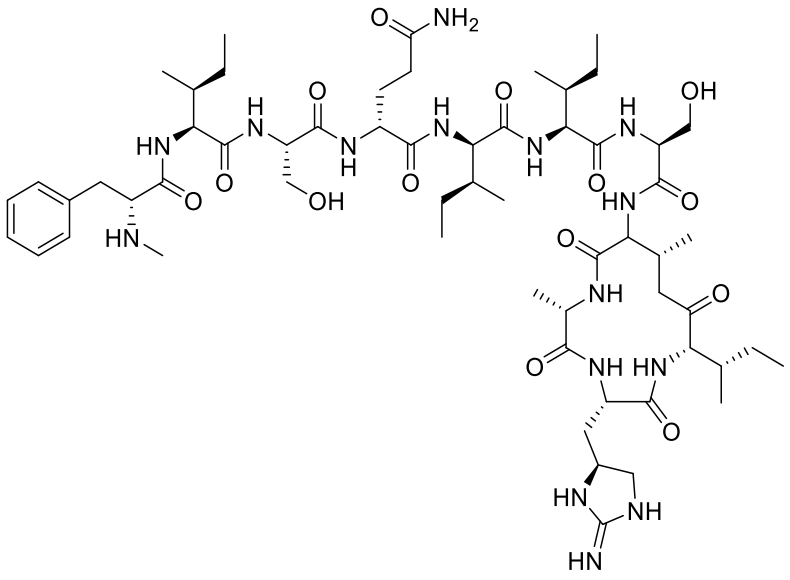


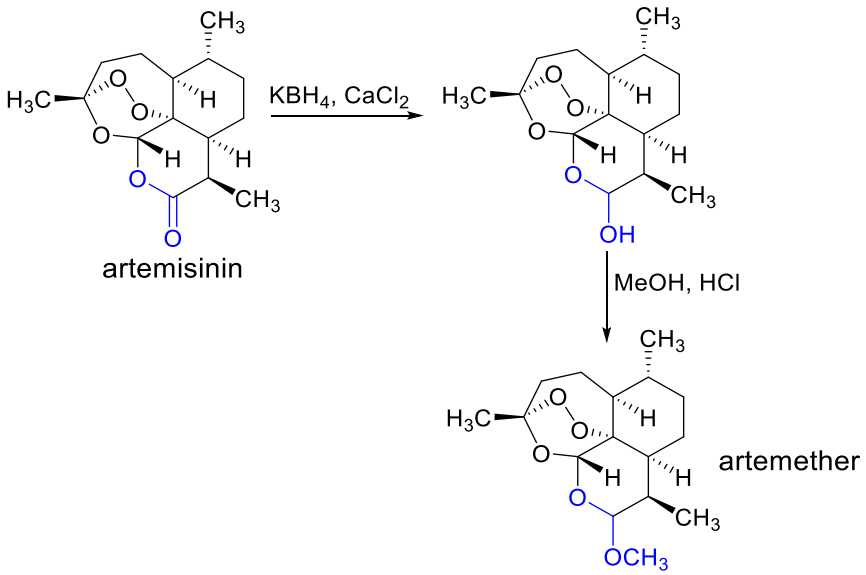
Fig. 1.21: Structure of teixobactin.

negative bacteria (*Eleftheria terrae*), cultured on a multi-channel chip device within the natural soil environment. This compound uniquely binds to both lipid II and lipid III (a precursor of cell wall teichoic acid).[101]

1.5.5 Semisynthesis

Semisynthetic antibiotics are developed *via* extensive studies of the chemistry, biochemistry, and pharmacology of naturally-derived antibiotics with a view to determining their structure-activity relationships. Chemical modifications are then applied to produce analogues with improved properties.[102]

Recently, the antimalarial drug artemether was developed by semi-synthetic modification of artemisinin, an unstable natural product, by conversion of its lactone to an acetal *via* reduction and subsequent methylation (Scheme 1. 3).[104] Artemisinin was originally discovered from extract of a plant, *Artemesia annua*, by Youyou Tu in 1985.[264,265] Tu was awarded half the 2015 Nobel prize in medicine for this discovery. However, artemisinin is only produced in low yield by *Artemesia annua*. Yo and co-workers subsequently engineered the production of artemisinin in yeast, *Saccharomy-*



Scheme 1.3: Route for semisynthesis of artemether, an important antimalarial drug, from the natural product artemisinin.

yces cerevisiae, leading to high titres of up to 100 mg/L, and paving the way for a large-scale industrial production of this important antimalarial agent.[266]

Another good example is in the development of cephalosporins into drugs. Whereas the natural product, cephalosporin C (**11**), has weak antibiotic activity against Gram-positive strains (MIC 25 to 100 µg/ml) and no activity against Gram-negative bacteria, several analogues with improved potency against Gram-positive bacteria and significant activity towards Gram-negative strains have been generated *via* chemical modification of the cephalosporin core structure.[44,103] This has culminated in the development of first, second, third, fourth and fifth generation cephalosporin antibio-

Table 1.2: Generations of cephalosporin antibiotics and their antimicrobial activity

Generation	Members	Active against
1st	cephalotin, cephaloridine, cephaloglycine, cephazoline, cephaprin, cefazolin and cephalexin	Gram-positive bacteria, especially *Staphylococci* and *Streptococci* species
2nd	cefamandole, cefoxitin, cefuroxime, cefaclor, cefadroxil, cefotetan, cefprozil and cefmetazole	Gram-negative *Bacilli* Slightly less active against Gram-positive cocci compared to the 1st generation
3rd	cefotaxime, cefoperazone, ceftazidime, ceftizoxime, cefsulodin, ceftriaxone, cefdinir, cefditoren, cefixime and cefpodoxime	Gram-positive cocci and Gram-negative *Haemophilus influenza*, Enterobacteria (e.g. *E. coli*, *K. pneumoniae*) that do not produce betalactamase. Ceftazidime is active against *Pseudomonas aeruginosa*
4th	notably cefepime and cefpirome	Gram-positive and Gram-negative strains (including *Pseudomonas* and betalactamase-producing bacteria)
5th	notably ceftaroline and ceftobiprole	methicillin-resistant *S. aureus* (MRSA), *K. pneumoniae*, *E. faecalis*. Also Gram-positive cocci and Gram-negative *Bacilli*. Not active against *Pseudomonas* species

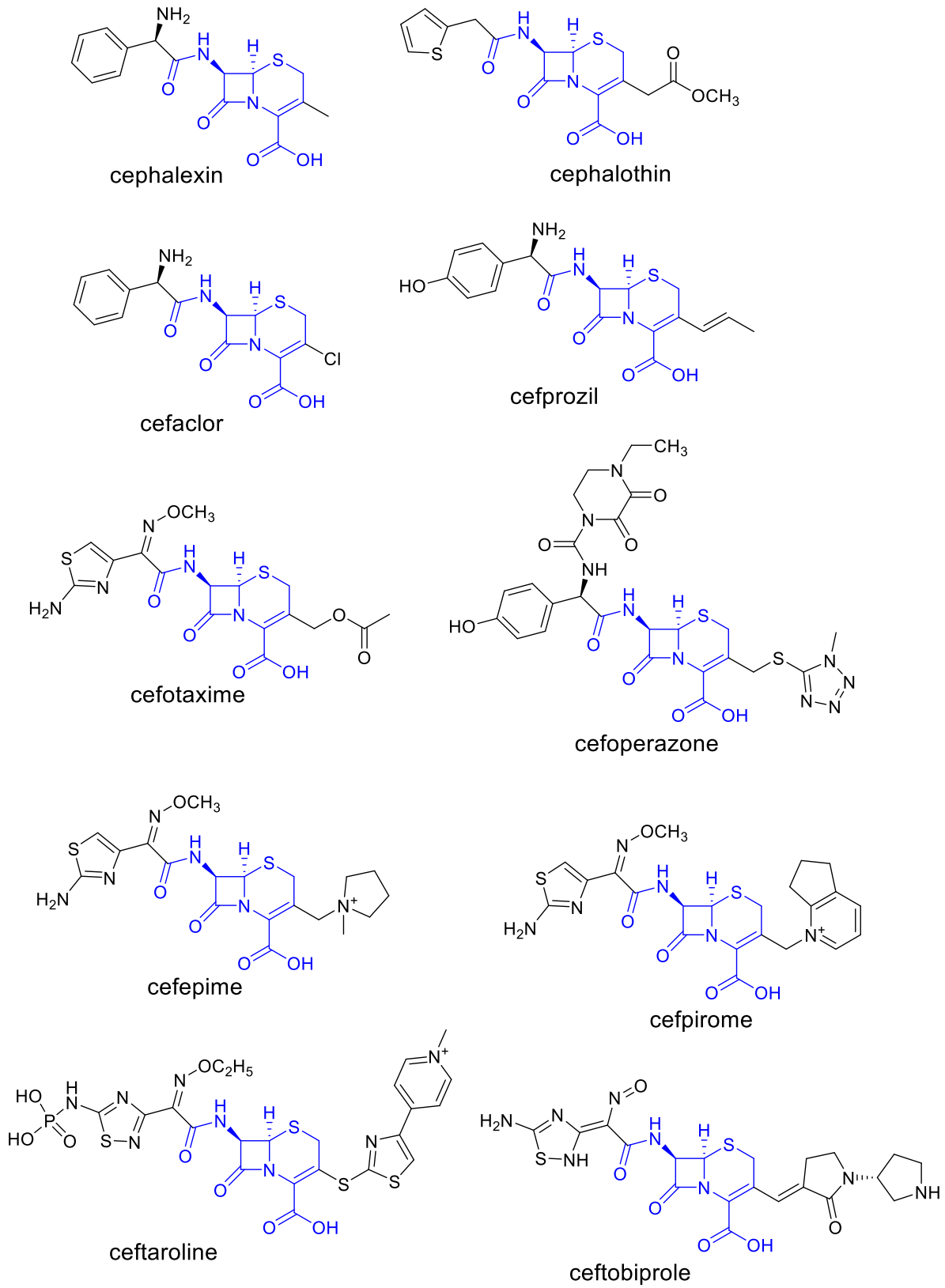


Fig. 1.22: Structures of selected 1st, 2nd, 3rd , 4th and 5th generation cephalosporin antibiotics. The common core structure derived from the natural product, cephalosporin C (**11**) is shown in blue.

tics (Table 1.2 and Figure 1.22)

1.5.6 Targeting non-multiplying bacteria

Non-multiplying and multiplying bacterial cells often exist simultaneously in a clinical infection.[119] However, most antibiotics in clinical use kill only multiplying cells, leaving behind the non-multiplying cells which serve as a pool from which multiplying bacteria can emerge again to cause recurrent disease.[113, 120-121] Thus, one strategy being pursued to combat antibiotic resistance in disease-causing pathogens is to develop drugs that can kill non-multiplying bacterial cells, in addition to inhibiting the growth of actively-dividing cells. HT61, developed by Helperby Therapeutics and currently in clinical trials, is an example of an antibiotic that kills non-multiplying bacteria, in particular *Staphylococcus aureus*.[119]

1.6 Antibiotics produced by *Streptomyces coelicolor* A 3(2)

Streptomyces coelicolor A 3(2) is a model Actinobacterium, widely used to study the genetic and biochemical basis for specialised metabolite production in Streptomycetes.[147-149] It was the first member of the genus to have its genome completely sequenced.[128] It produces many structurally-diverse antibiotics, including undecylprodigiosin (**13**) and streptorubin B (**35**), coelimycin A (**39**) and coelimycin P (**40**), actinorhodin (**14**), and calcium-dependent antibiotics (CDA, **15**) (Fig. 1.23), all of which are chromosomally determined. The biosynthesis of these antibiotics has been recently reviewed by Challis,[150] Hu *et al*,[151] and Okamoto *et al*.[152] *S. coelicolor* A 3(2) also produces the epoxy-cylcopentanone antibiotics: methylenomycin A (MmA, **16**) and methylenomycin B (MmB, **24**), as well as methylenomycin C (MmC, **17**), a common precursor to the two methylenomycin antibiotics.[150] MmA (**16**) and MmB (**24**) were first isolated from an Actinobacterium called *Streptomyces violaceo-*

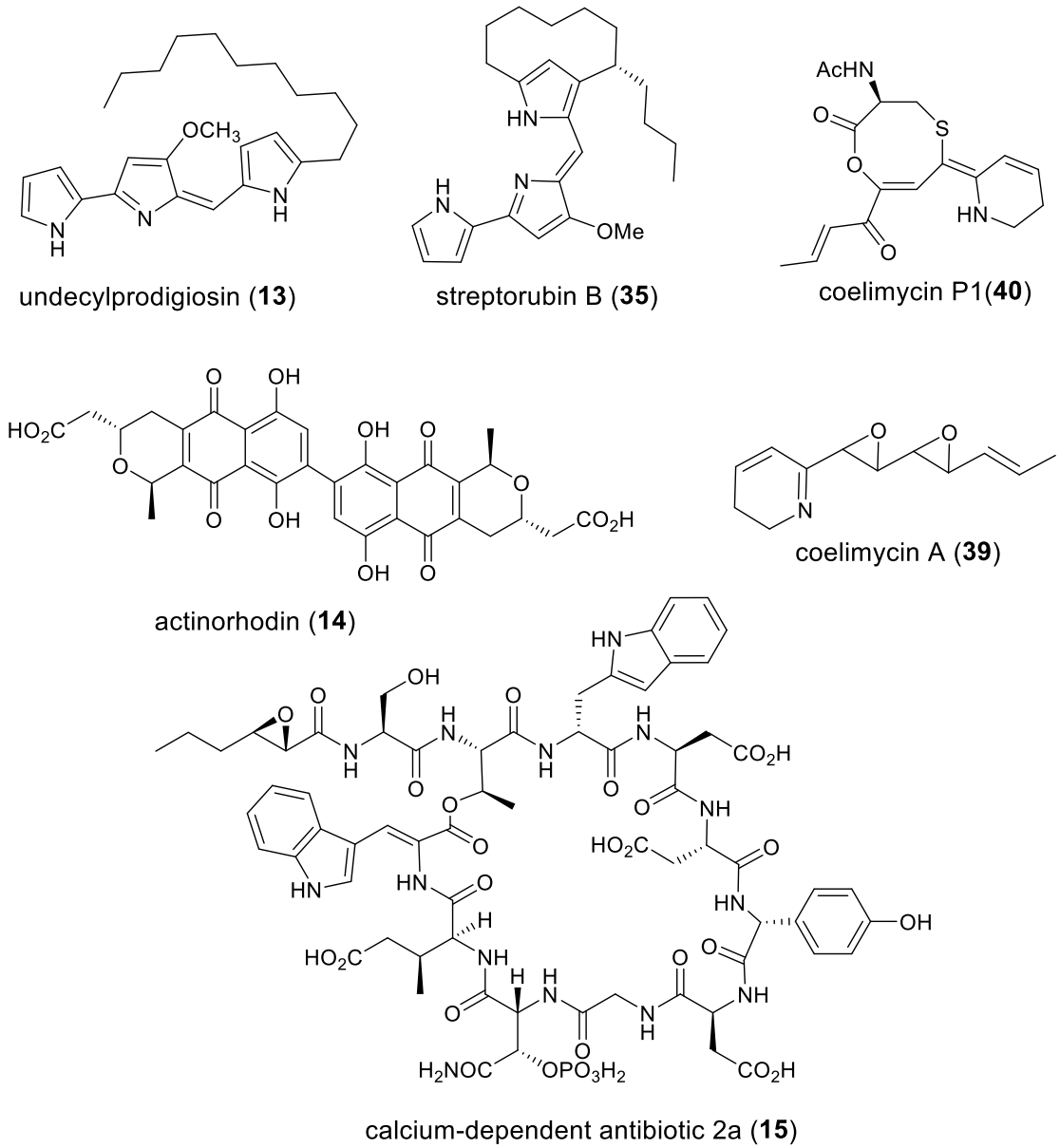


Fig. 1.23: Structures of representatives of the main groups of antibiotics produced by *Streptomyces coelicolor* A 3(2). Methylenomycins are shown in Fig. 1.24.

ruber in 1974 and were shown to exhibit a wide range of activity against many Gram-positive bacteria and some Gram-negative ones, with **16** being more potent.[261] Structure-activity study had revealed that the α,β – unsaturated ketone was the most essential feature for antibacterial activity in this compound, while the ketonic group at C-3 was a basic requirement for antifungal activity.[261]

In contrast to the chemical name of **16**, which is (1*S*,5*S*)-1,5-dimethyl-3-methylene-4-oxo-6-oxabicyclo[3.1.0]hexane-2-carboxylic acid, the compound was assigned a numbering scheme as shown in Fig. 1.24(A).[172, 262-263] The highly-functionalised nature of **16** was previously explored to generate semisynthetic derivatives (**42** – **49**, Fig. 1.24B), with consequent impact on its biological activity and toxicity. The dibrominated derivative (**43**) showed stronger antibacterial activity than MmA (**16**). The methyl ester derivatives (**42** and **44**) also showed stronger antibacterial and antifungal activities than **16**.[263] Substituting the methyl group of the ester with longer

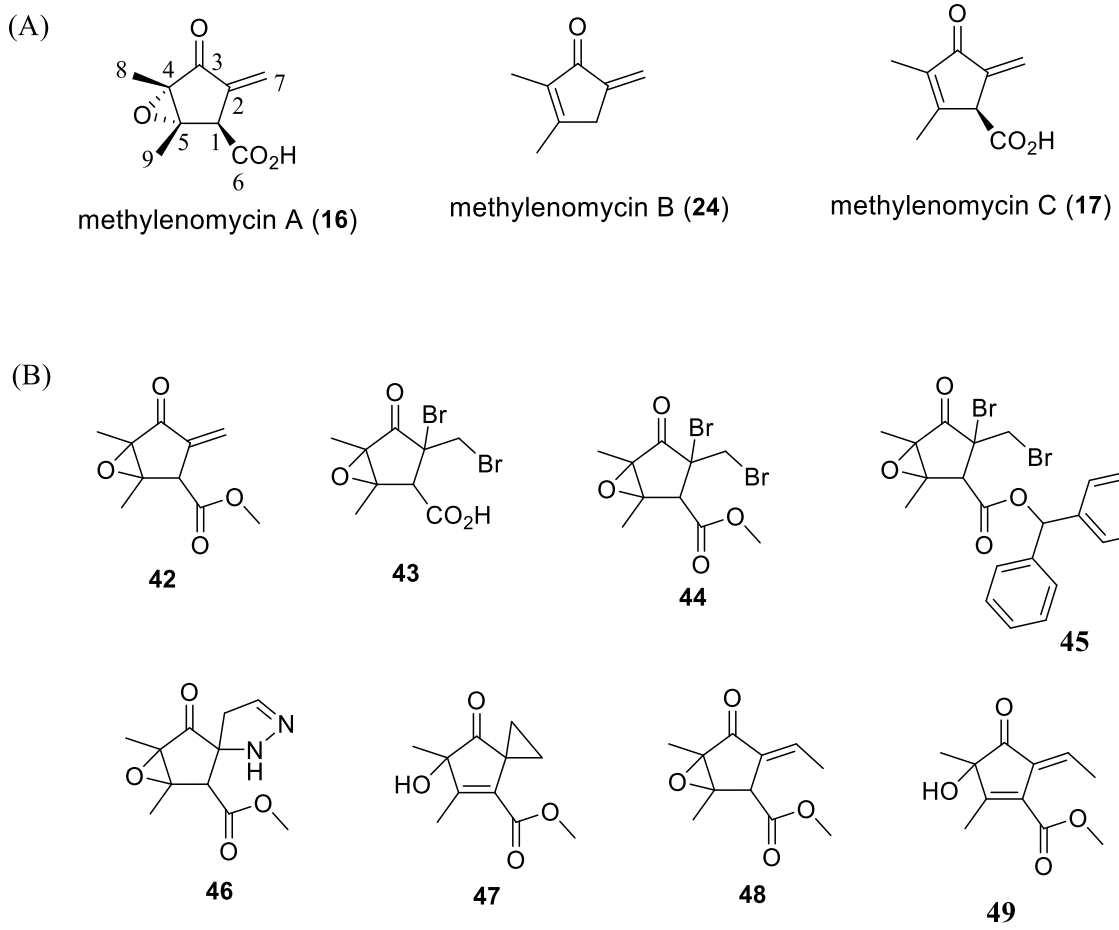


Fig. 1.24: (A) Structures of methylenomycin A (**16**), methylenomycin B (**24**) and their putative precursor, methylenomycin C (**17**). (B) Structures of some derivatives of methylenomycin A generated by chemical modifications.

alkyl chain significantly reduced the antimicrobial activity. Similarly, aromatic esters generated were not biologically active, except the dibromo - methylenomycin A - diphenyl ester (**45**).[263]

Replacing the C-2 double bond led to a complete loss of antibacterial activity in the semi-synthetic analogues, with the exception of the brominated derivatives and the 2-ethylidene derivative (**48**).[263] All of the semi-synthetic analogues with stronger antimicrobial activity than MmA (**16)** also had greater toxicity. Only the methyl ester (**42**) was slightly less toxic and had an improved antimicrobial activity.[263]

1.7 Regulation of methylenomycin production in *S. coelicolor* A 3(2)

All of the genetic elements required for the biosynthesis of the methylenomycin antibiotics and regulation are present within the 23kb *mmy* gene cluster, carried on a giant linear plasmid SCP1 of *S. coelicolor* (Fig. 1.25).[153 - 155] The complete sequence of the *mmy* cluster became available when the SCP1 plasmid was sequenced as part of the *S. coelicolor* genome sequencing project.[156] The cluster contains twenty-one genes, including seven (coloured in blue and green), encoding proteins that play various roles in transcriptional regulation.

The *mmr* gene encodes an efflux/export protein that confers methylenomycin resistance by exporting the antibiotics from the cell. To the left of this gene is *mmyJ*, which encodes a protein belonging to the ArsR family of transcriptional repressors that sense metal ions.[157] Recently, it was shown that MmyJ binds specifically to the intergenic region between *mmr* and *mmyJ* (containing the promoters for the two genes) and that binding of methylenomycin A to MmyJ causes it to be released from this operator.[158] This confirmed a previous suggestion that MmyJ controls its own expression, as well as that of the methylenomycin resistance determinant, *mmr*, prov-

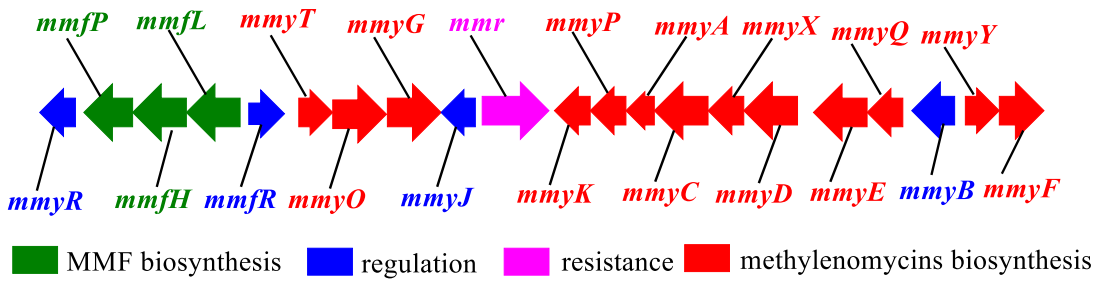


Fig. 1.25: Organisation of the methylenomycin biosynthetic gene cluster on SCP1 of *Streptomyces coelicolor* A 3(2). *mmfLHP* genes encoding the methylenomycin furan (MMF) biosynthetic enzymes are in green; Genes involved in regulation are in blue; the methylenomycin resistance gene, *mmr*, is in pink. Genes involved in methylenomycins biosynthesis are in red.

iding a mechanism for triggering *mmr* expression upon production of methylenomycin A.[159] The *mmyB* gene, located towards the right end of the cluster, encodes a transcription factor that activates expression of the methylenomycin biosynthetic genes.

At the left hand end of the cluster are five genes: *mmyR*, *mmfR*, and *mmfLHP*. Both *mmyR* and *mmfR* encode proteins belonging to the TetR superfamily of transcriptional repressors.[160, 161] Indeed, deletion of *mmyR* leads to methylenomycin overproduction, confirming that MmyR represses Mm biosynthesis.[161,162] Both MmyR and MmfR are similar in sequence to the *Streptomyces griseus* DNA-binding protein, ArpA (MmyR: 43% identity and 65% similarity; MmfR: 32% identity and 58% similarity).[167] ArpA binds to the promoter region of *adpA* which encodes the pathway-specific activator for streptomycin biosynthesis. Binding of A-factor (**18**), a gamma-butyrolactone signalling molecule, to ArpA causes it to be released from the *adpA* promoter, leading to expression of the streptomycin gene cluster.[163,164] Similar to ArpA, both MmyR and MmfR have been shown to bind to the promoter region of *mmyB*, the pathway

activator gene for methylenomycin production. These two transcription factors also bind to the *mmyR* promoter and the intergenic region between *mmfL* and *mmfR*.[165]

Like ArpA, MmfR (but not MmyR) is released from the *mmyR* and *mmyB* promoters, as well as the *mmfL-mmfR* intergenic region upon binding to diffusible signalling molecules.[168] These have been identified as a group of five 2- alkyl-4-hydroxymethylfuran-3-carboxylic acids, termed the methylenomycin furans (MMFs 1-5, **19**-**23**) (Fig. 1.26) and are the metabolic products of the enzymes encoded by the *mmfLHP* operon.[155] MMF biosynthesis involves MmfL-catalysed condensation of β-ketoacyl-ACP intermediates in fatty acid assembly with dihydroxyacetone phosphate, an intermediate in glycolysis. MmfP has been shown to catalyse dephosphorylation of the resulting phosphorylated butenolides, and MmfH is proposed to catalyse the rearrangement of the butenolides to give the MMFs (Sceheme 1.4).[150,166,167]

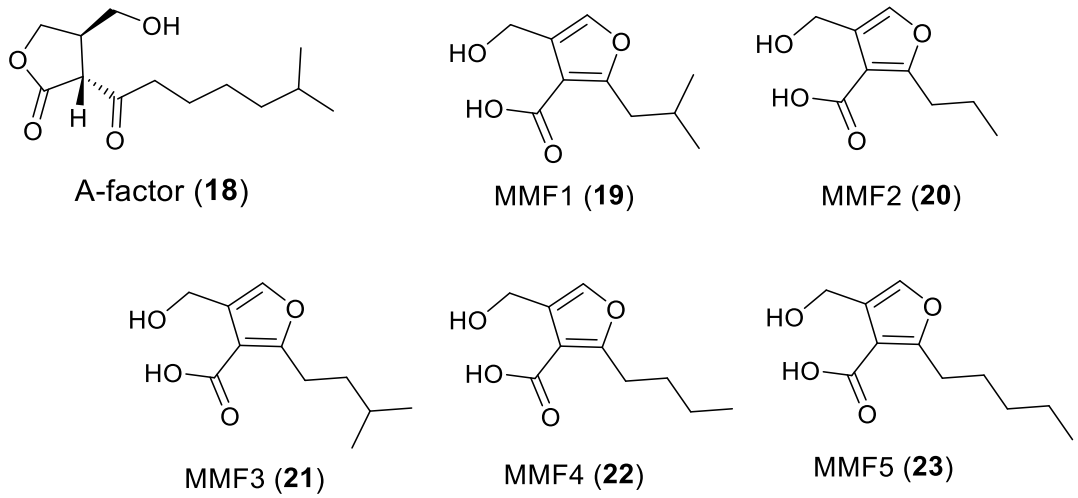
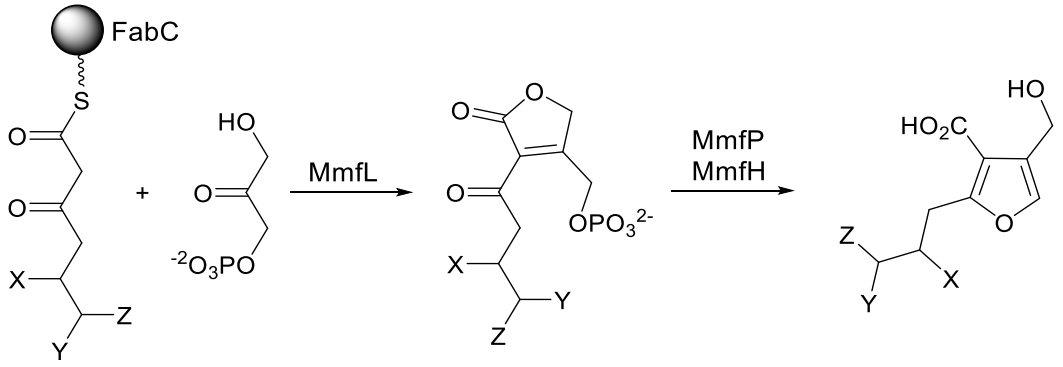


Fig. 1.26: Structures of A-factor (**18**), the signalling molecule that induces streptomycin production in *S. griseus*, and the methylenomycin furans (MMFs, **19**-**23**), which induce methylenomycin production in *S. coelicolor* A 3(2).



Scheme 1.4: Proposed pathway for methylenomycin furans (MMF) biosynthesis in *S. coelicolor*.[150] FabC is the acyl carrier protein from the fatty acid biosynthetic pathway. MMF1 (X=Me, Y=Z=H); MMF2 (X=Y=Z=H); MMF3 (X=H, Y=Z=Me); MMF4 (X=Y=H, Z=Me); MMF5 (X=Y=H, M=Et).

In the early stages of growth, the MmfR/MmyR repressor complex is proposed to bind to the operators upstream of *mmyB* and *mmyR*, and in the intergenic region of *mmfL* and *mmfR*. The expression of *mmyB* is proposed to be fully repressed, whereas the expression of *mmyR*, *mmfR* and *mmfL* is only partially blocked. This permits low levels of the MMFs to be produced and gradually accumulate.[165,167] As the extracellular, and subsequently, the intracellular levels of the MMFs increase, they begin to bind to MmfR, causing its release from the operators. This permits express-

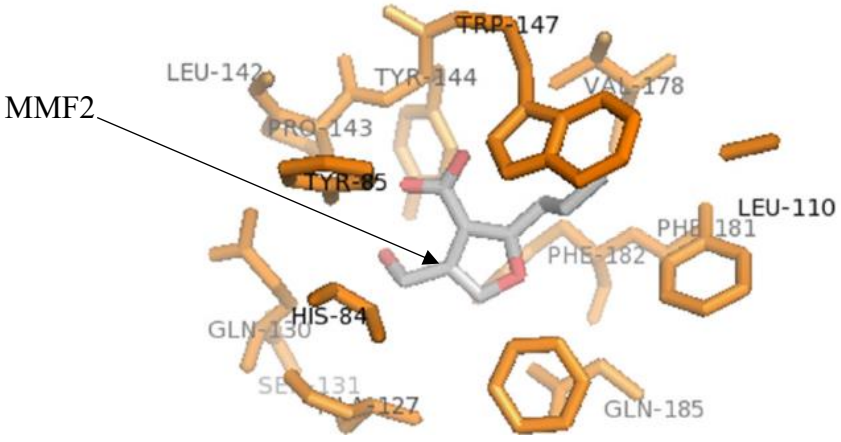


Fig. 1.27: Crystal structure of MmfR showing interaction with MMF2, **20**. Such interaction causes the protein to be released from the promoter region of *mmyB*, thereby switching on methylenomycins biosynthesis.

ion of *mmyB*, thereby switching on the expression of the genes involved in methylenomycin biosynthesis.[165] The X-ray crystal structure of MmfR bound to methylenomycin furan 2 (MMF2) has recently been solved (Fig. 1.27).[169]

1.8 Previous investigations into the biosynthesis of methylenomycin antibiotics

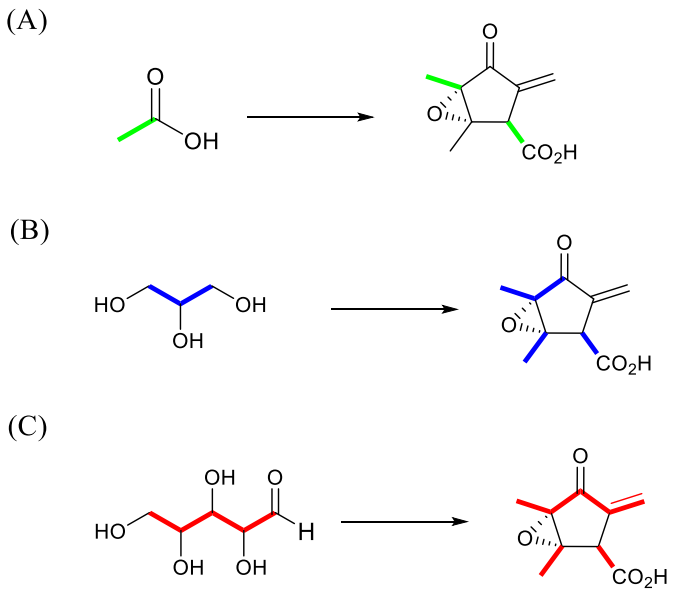
1.8.1 Incorporation of labelled putative precursors

Efforts to elucidate the biosynthetic pathway to methylenomycin A (**16**) began over three decades ago with incorporation experiments involving precursors. Intact incorporation of [1,2-$^{13}C_2$]acetate into C-4, C-8 and C-1, C-6 of **16** was reported (scheme 1.5A).[170] Moreover, radiolabelled methylenomycin C (**17**, also known as des-epoxy-4,5-didehydromethylenomycin A) was incorporated into methylenomycin A (**16**),[171] indicating that epoxidation of **17** is the final step in methylenomycin A biosynthesis. Methylenomycin B (**24**) appears to derive from decarboxylation of methylenomycin A (**16)**.

In a reinvestigation of the metabolic origins of the carbon atoms in the methylenomycins, Challis and Chater examined the incorporation of [U-^{13}C]glycerol in *S. coelicolor*. Using a combination ^{1}H, ^{13}C and HMBC NMR experiments, they were able to confirm the intact incorporation of the [U- ^{13}C]glycerol into C-3, C-4, and C-8 of **16**, as well as an indirect incorporation of a 2-carbon unit into the C-5, C-9 and C-1, C-6 portions of the molecule *via* catabolism of [U-^{13}C]glycerol to [1,2-^{13}C]acetyl-CoA (scheme 1.5B).[172]

A major advance towards understanding the biosynthesis of the methylenomycins was made when Corre and Challis [173] established the metabolic origin of C-2 and C-7 which was hitherto unknown. Feeding of [U- ^{13}C]glycerol to *Streptomyces lividans* 1326, with a cosmid containing the entire methylenomycin biosynthetic gene cluster

integrated into its chromosome, led to the labelling of **16** and **17**. Analysis of coupling data in the ^{13}C NMR spectra of these compounds confirmed that a 3-carbon precursor derived from a molecule of [U- ^{13}C]glycerol was incorporated intact into C-3, C-4, C-8 as observed previously. Interestingly, intact incorporation of a 5-carbon precursor derived from 2 molecules of [U- ^{13}C]glycerol into C-2, C-3, C-4, C-7 and C-8 was also observed. The incorporation of a 5-carbon precursor is explained based on the metabolism of [U-^{13}C]glycerol to [U-^{13}C]glyceraldehyde-3-phosphate (G3P), which is then converted through the pentose phosphate pathway to one or more of the following: [U-^{13}C]Xylulose-5-phosphate (Xu5P), [U-^{13}C]Ribulose -5-phosphate (Ru5P), and [U-^{13}C]Ribose-5-phosphate (R5P).[173] The intact incorporation of a pentulose into C-2, C-3, C-4, C-7 and C-8 of **16** and **17** was confirmed by feeding [U-^{13}C]-D-ribose (scheme 1.5C).[173]



Scheme 1.5: Pattern of incorporation of labelled precursors into methylenomycin A (**16**), observed following the feeding of : (A) [U- ^{13}C]acetate [170] (B) [U- ^{13}C]glycerol [172] (C) [U-^{13}C]D-ribose,[173] to *Streptomyces* spp. expressing the *mmy* cluster. The same incorporation pattern was observed for methylenomycin C (**17**).

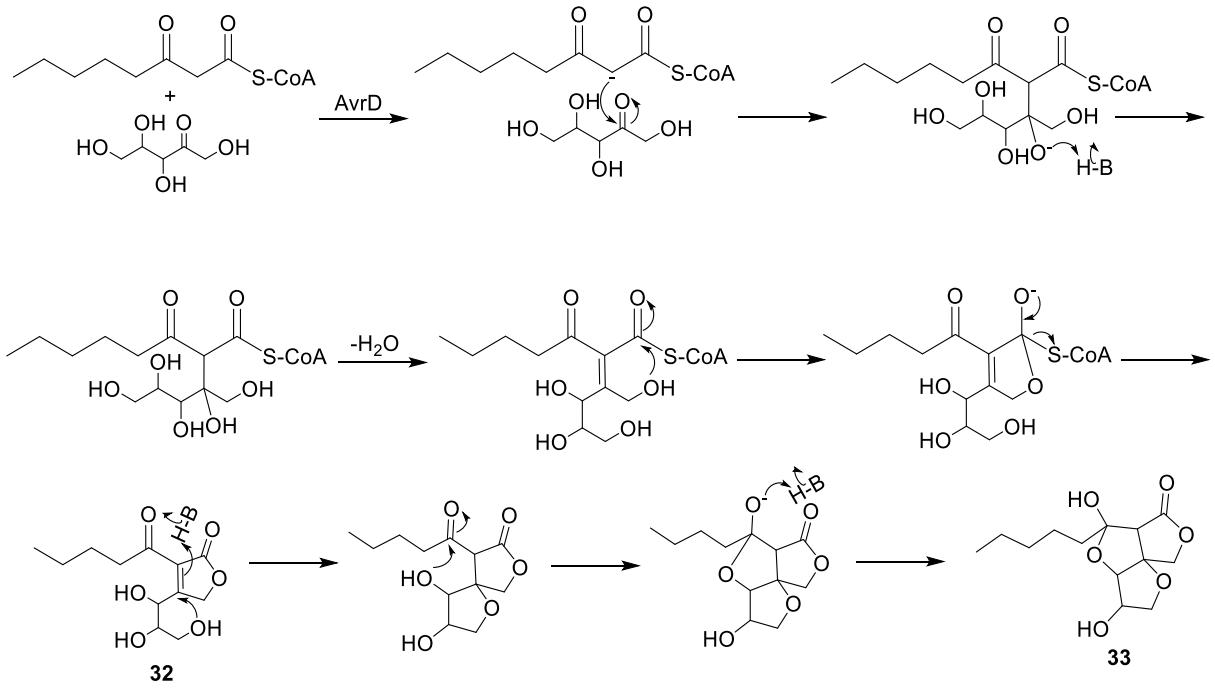
1.8.2 Proposed biosynthetic pathway for methylenomycin A (16)

Since the complete sequence of the *mmy* gene cluster became available,[156] attempts to further elucidate the biosynthetic pathway to the methylenomycins have focused on the assignment of plausible biosynthetic roles for the proteins encoded by each of the genes in the cluster. Comparison of the amino acid sequences of the proteins encoded by the genes in the cluster with proteins of known function have allowed plausible biosynthetic roles to be assigned to the majority of them (Table 1.3).

The *mmyD* gene within the methylenomycin cluster encodes a protein with 28% identity and 47% similarity over 333 amino acids to AvrD in *Pseudomonas*

Table.1.3: Putative functions of the proteins encoded by the methylenomycin gene cluster

Protein (aa)	UNIPROT	Homologue (% Identity)	Proposed function
MmyA (82)	Q7API2	RedQ *S. coelicolor* (33)	Acyl carrier protein
MmyB (313)	Q9ACS4	TR:Q9RDF4 *S. coelicolor*(43)	Mm pathway transcriptional activator
MmyC (332)	Q9JN82	FabH *E. coli* (39)	β-ketoacyl synthase III
MmyD (339)	Q7API4	AvrD *P. syringae* (28)	Butenolide synthase
MmyE (387)	Q7API5	PlmM *S.* sp. HK803 (32)	Flavin-dependent enoyl reductase
MmyF (174)	Q7API8	NtaB *C. heintzii* (40)	Flavin reductase, partner to monooxygenase
MmyG (393)	Q9ACS3	JadP *S. venezuelae* (29)	NAD(P)-dependent dehydrogenase
MmyJ (111)	Q7APH9	TR:O31480 *B. subtilis*(45)	AsR-type DNA-binding transcription factor
MmyK (206)	Q7API0	Adk *E. coli* (30)	Kinase
MmyO (373)	Q7APH8	LimB *R. erythropolis* (43)	Flavin-dependent monooxygenase
MmyP (232)	Q7API1	SsgB *S. griseus* (30)	Phosphatase
MmyQ (217)	Q7API6	NdpG *R. opacus* (30)	Coenzyme F-420-dependent reductase
MmyR (203)	Q7APH3	ArpA *S. griseus* (43)	DNA-binding receptor protein
MmyT (269)	Q9ACS2	MtmZ *S. argillaceus* (31)	Thioesterase
MmyX (234)	Q7API3	MmyK *S. coelicolor* (42)	Kinase
MmyY (153)	Q9JN76	JadX *S. venezuelae* (27)	Belongs to NTF2-like superfamily
MmfH (400)	Q7APH5	LndZ5 *S. globisporus* (42)	Flavin-dependent dehydrogenase
MmfL (353)	Q7APH6	BarX *S. virginiae* (34)	Butenolide synthase
MmfP (265)	Q7APH4	SsgB *S.griseus* (30)	Phosphatase
MmfR (214)	Q7APH7	ArpA *S. griseus* (32)	DNA-binding receptor protein
Mmr (475)	P11545	SW:P39886 *S. glaucescens* (32)	Methylenomycin resistance protein

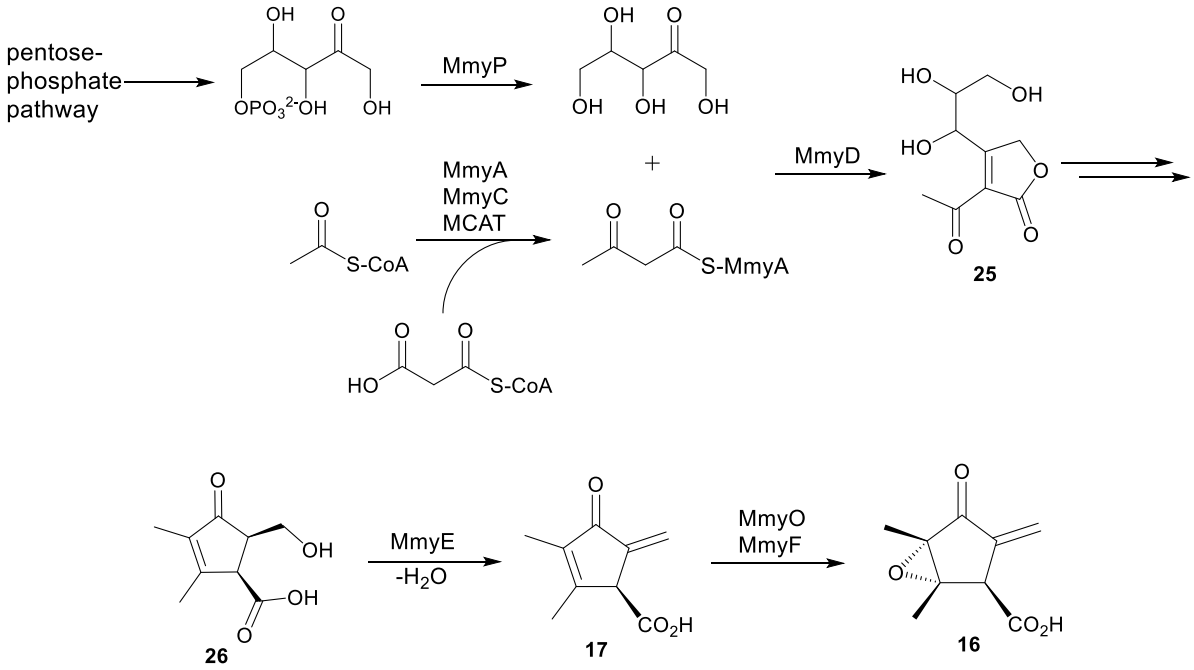


Scheme 1.6: AvrD-initiated condensation of β-ketooctanoyl CoA with xylulose to give syringolide 1 (**33**). Syringolide 2 has the alkyl side chain increased by 2 carbon units and is derived from β-ketodecanoyl CoA.

syringae.[173] AvrD catalyses the production of a group of compounds called syringolides, elicitors of plant hypersensitive response.[188,189] The biosynthesis of these compounds proceeds *via* AvrD-catalysed condensation of β-ketooctanoyl CoA or β-ketodecanoyl CoA with xylulose to form a key butenolide intermediate (**32**), which then rearranges to give the tricyclic ring system characteristic of the syringolides (scheme 1.6).[189]

Based on the similarity between MmyD and AvrD, it was proposed that MmyD could catalyse a similar condensation of a β-ketothioester ACP with a pentulose to give a butenolide intermediate (**25**) in methylenomycin pathway.[173] This hypothesis is consistent with the encoding, within the *mmy* cluster, of a ketoacyl synthase III, MmyC, and an acyl carrier protein, MmyA. MmyC could catalyse the decarboxylative condensation of malonyl-MmyA with acetyl-CoA to give the β- ketothioester ACP

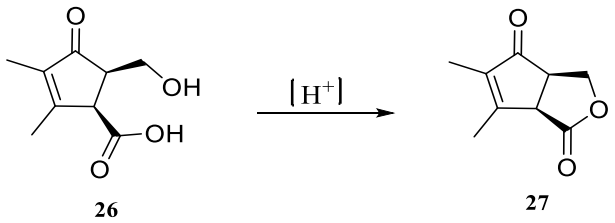
(acetoacetyl-MmyA), required by MmyD for condensation with a pentulose (Scheme 1.7).[173]



Scheme 1.7: Proposed pathway for methylenomycin A (**16**) biosynthesis in *S. coelicolor*.

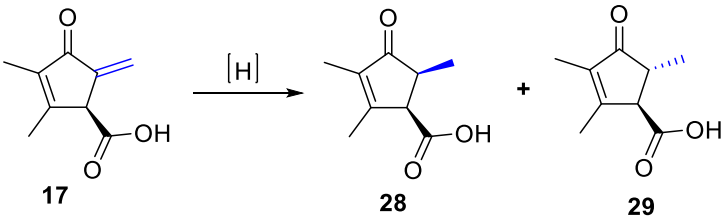
A post doc in the group, Christophe Corre, had previously generated *S. coelicolor* M145 strains in which putative methylenomycin biosynthetic genes have been individually deleted from the *mmy* cluster, carried on an integrative cosmid C73_787.[174] The transcriptional repressor, *mmyR*, was also disrupted in each of the constructs in order to obtain high titers of methylenomycin-related metabolites.[159] LC-MS analysis was carried out to examine the metabolite profiles of some of the transconjugant strains. Production of methylenomycin antibiotics was completely abolished in a mutant strain lacking *mmyD*, corroborating the hypothesis that MmyD catalyses a crucial early step leading to the butenolide intermediate (**25**) in the methylenomycin pathway.[174] A possible role for MmyE in forming the exomethylene function of the methylenomycins *via* dehydration of a recently-discovered putative intermediate, the pre-methylenomycin C (**26**), has also been proposed.[174] This

metabolite accumulates in a *mmyE* mutant and converts to pre-methylenomycin C lactone (**27**) under acidic conditions (scheme 1.8).



Scheme 1.8: Formation of pre-methlenomycin C lactone (**27**) from pre-methylenomycin C (**26**).

Abolition of the production of **16**, but not **17**, in mutants lacking *mmyO* or *mmyF* suggested the possible involvement of the enzymes encoded by these genes in the epoxidation of **17** to form **16**. MmyO shows similarity to $FADH_2$-dependent monooxygenases while MmyF is similar to NADH-dependent flavin reductases required for the supply of $FADH_2$ to flavin-dependent monooxygenases (Table 1.3). The involvement of molecular oxygen in formation of the epoxide group of **16** has also been demonstrated *via* incorporation of isotopically-labelled molecular oxygen ($^{18}O_2$).[174] In both *mmyO* and *mmyF* mutant, methylenomycin C (**17**) is metabolised to a mixture of diastereoisomers, methylenomycin D1 (**28**) and methylenomycin D2 (**29**), *via* the saturation of the C-2 double bond (scheme 1.9).



Scheme 1.9: Saturation of the exomethylene function at C-2 in methylenomycin C (**17)** leads to methylenomycin D1 (**28**) and methylenomycin D2 (**29**).

The function of MmyY, a nuclear transport factor (NTF2) encoded within the methylenomycin cluster, was investigated to some extent by O' Rourke *et al.*[155] In

this study, *mmyY* was replaced with an apramycin resistance cassette, and the resulting strain (J3994) was assessed for methylenomycin production on the basis of whether a zone of inhibition was visible when an agar plug inoculated with the strain was placed on a plate inoculated with a methylenomycin-sensitive *S. coelicolor* strain, J1501 (SCP1$^-$, SCP2$^-$). The absence of a zone of inhibition in the experiment (presumably due to the lack of methylenomycin production) led to the conclusion that MmyY probably plays role in methylenomycin biosynthesis.[155] However, the bioassay-based approach used in the study to evaluate the production of methylenomycins by the *mmyY* mutant did not provide the opportunity to detect levels of production below that required to inhibit growth of the indicator strain. It also did not provide an opportunity to identify any intermediates that might accumulate in the mutant that lack antimicrobial activity.

Of the 21 genes in the *mmy* cluster, eight have been shown to be involved in the regulation of methylenomycin production and biosynthesis of the MMF signalling molecules.[156, 165] A further seven genes have been assigned plausible roles in methylenomycin biosynthesis (Scheme 1.7), although direct evidence to support these roles is, in most cases, lacking. The functions of the remaining *mmy* genes (T, G, X, K, Q, Y) have yet to be unambiguously assigned. Experimental evidence is also lacking for the proposed butenolide intermediate (**25**) in methylenomycin biosynthesis.

1.9 Aims and objectives of this study

The aim of this PhD project was to investigate the biosynthesis and mode of action of methylenomycin antibiotics produced by *S. coelicolor* A 3(2). Elucidating the biosynthetic pathway to the methylenomycins could pave the way for a

bioengineering strategy to produce novel analogues, as well as providing insight into the biosynthesis of other structurally-related biologically-active compounds e.g. xanthocidin. The project builds on previous work in the group and seeks to achieve the following objectives:

- To obtain further evidence for the proposed role of MmyF and MmyO in the epoxidation of methylenomycin C to form methylenomycin A
- To identify and characterise biosynthetic intermediates accumulating in *mmyP*, *mmyT*, *mmyG*, *mmyX*, *mmyK*, *mmyQ*, and *mmyY* deletion mutants
- To investigate the role of a putative butenolide intermediate in methylenomycin biosynthesis
- To investigate the biological activity of biosynthetic intermediates and shunt metabolites in the methylenomycin pathway
- To investigate the mode of action of the methylenomycins

CHAPTER TWO

2. BIOSYNTHESIS OF METHYLENOMYCIN ANTIBIOTICS

2.1 Investigations into the roles of MmyO and MmyF

Early investigations into the biosynthesis of methylenomycin A (**16**) has suggested that epoxidation of methylenomycin C (**17**) is the last step in the biosynthesis.[170] As discussed in chapter one (section 1.8.2), deletion of *mmyF* or *mmyO* from the methylenomycin biosynthetic gene cluster led to accumulation of **16** in the corresponding *S. coelicolor* transconjugant strains, indicating that MmyF and MmyO both play roles in the epoxidation of **17** to form **16**. However, direct evidence for the roles of these proteins has not been obtained.

As a starting point for further investigations into the roles of MmyF and MmyO in the current study, the experiment showing accumulation of **16** in *mmyF* and *mmyO* mutants was repeated. The *S. coelicolor* strains W108 (M145/C73_787/Δ*mmyF*/*mmyR*::*apr*) and W100 (M145/C73_787/Δ*mmyO*/*mmyR*::*apr*) were grown on supplemented minimal medium solid (SMMS) for two days and the culture extracts were analysed by LC-ESI-MS. Figure 2.1 shows data from the analyses, confirming accumulation of **17** in the two mutant strains.

Previous attempts to obtain soluble recombinant MmyO and MmyF were unsuccessful, making it impossible to do an *in vitro* assay with the putative substrate, **17**. Thus, an *in vivo* alternative is considered which would involve feeding of **17** to an *S. coelicolor* W110 strain, in which both *mmyO* and *mmyF* are heterologously

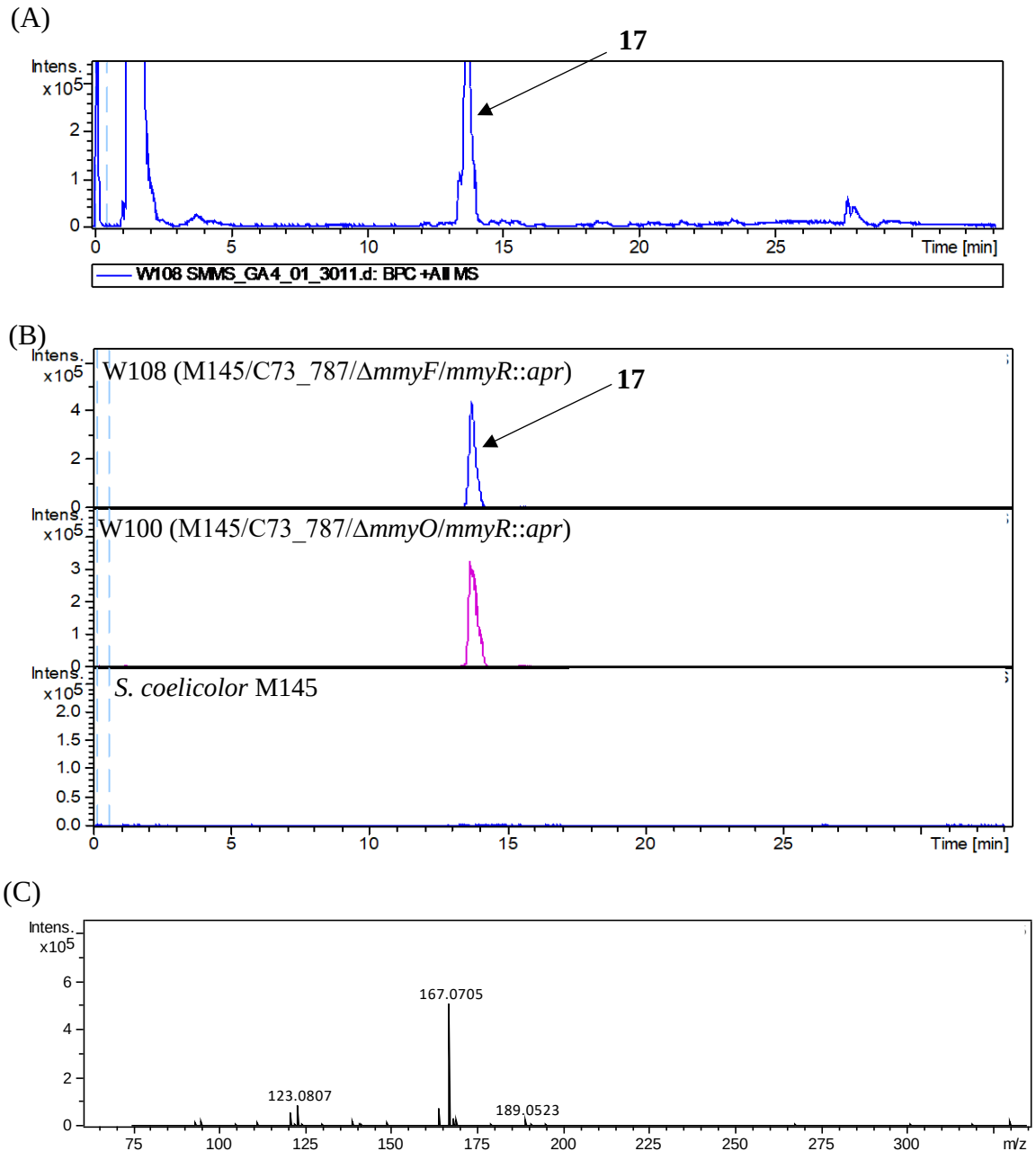


Figure 2.1: (A) Total ion chromatogram (TIC) showing methylenomycin C (**17**) and other metabolites present in *S. coelicolor* W108 crude extract (B) Extracted ion chromatograms (EICs) at *m/z* = 167.0700, corresponding to [M + H^+] for **17** following LC-MS analysis of extracts of *S. coelicolor* W108 and W100, and the heterologous host, *S. coelicolor* M145 (C) HR-MS data at 13.7 minutes showing *m/z* = 167.0705 and 123.0807, corresponding to [M + H^+] and [M + H^+ - CO_2] respectively for **17**.

expressed under a strong constitutive promoter *ermE** in a pOSV556 vector. Compound **17** has not previously been shown to have antimicrobial activity. However, direct feeding of **17** to W110 strain results in cell death, presumably because the onset

of the conversion of **17** to methylenomycin A (**16**) was suicidal to the heterologous *S. coelicolor* strain, which is susceptible to **16**. Thus, it was considered necessary to first generate a derivative of *S. coelicolor* W110 strain which is resistant to **16** . The construction of this strain is discussed in the next section.

2.1.1 Introduction of the methylenomycin resistance gene (*mmr*) into W110 and M145

S. coelicolor M145 is a derivative of the wild type strain A 3(2) which lacks the naturally occurring plasmids SCP2 and SCP1(containing the entire methylenomycin gene cluster).[9] *S. coelicolor* strain W110 (M145 carrying *mmyO* and *mmyF* genes) was constructed previously in the group and was kindly provided by Dr. C. Corre. The methylenomycin resistance gene, *mmr*, was previously cloned into a multicopy plasmid, pIJ86, under the control of a strong constitutive *ermE** promoter (Fig. 2.2). pIJ86 contains an *oriT* (for conjugal transfer) and origins of replication in *E. coli* and

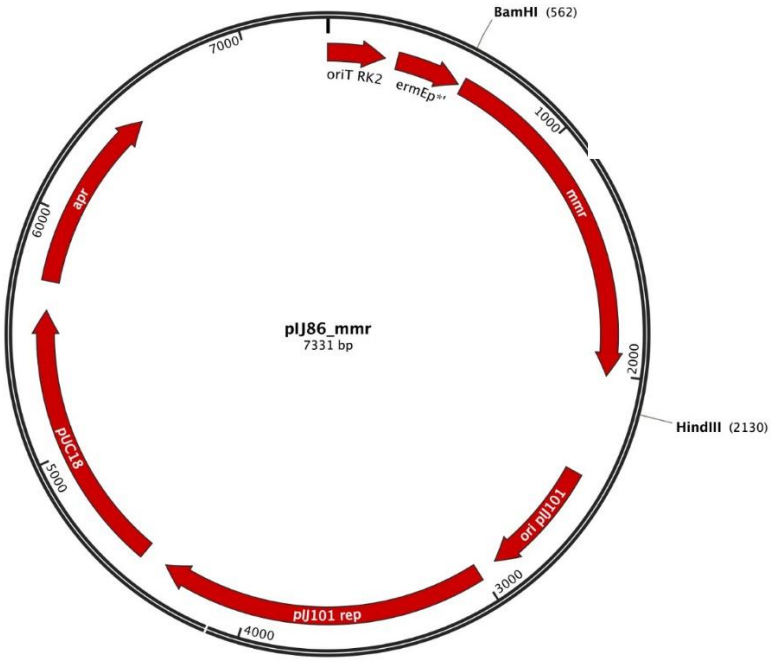


Fig. 2.2: Map of pIJ86 carrying the methylenomycin resistance gene, *mmr*.

Streptomyces species.[9] *E. coli* TOP10 carrying the pIJ86/*mmr* construct was provided by Dr. C. Corre. The construct pIJ86/*mmr* was purified from *E. coli* following standard procedures and the presence of the resistance gene was confirmed by polymerase chain reaction (PCR) (Figure 2.3).

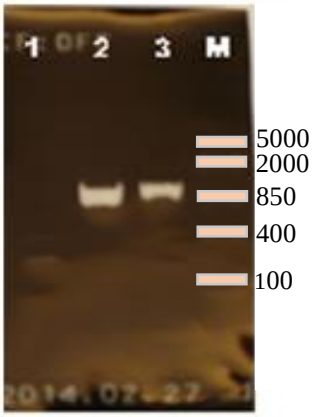


Figure 2.3: 1% gel from agarose electrophoresis separation following PCR amplification of *mmr*. 1- plasmid pIJ86, 2 & 3 - pIJ86/*mmr*, M- middle range DNA Ladder (bp). Expected size = 1.43 kb.

The purified pIJ86/*mmr* was used to transform a non-methylating *E. coli* ET12567/ pUZ8002. The plasmid DNA was then transferred into *S. coelicolor* W110 strain *via* conjugation with the transformed *E. coli* ET12567/pUZ8002, and apramycin-resistant colonies were selected to give strain W301 (M145/pOSV556/*mmyOF*/ pIJ86*mmr*) (methods section 5.11.3). Since control assays would require feeding of **17** to *S. coelicolor* M145, which also lacks SCP1 and is therefore sensitive to methylenomycin antibiotics, but does not contain *mmyO* and *mmyF*; the resistance gene was also introduced into M145 by conjugation, to give strain W302 (M145/pIJ86*mmr*).

2.1.2 Confirming methylenomycin resistance phenotype in W301 and W302

The newly generated *Streptomyces* strains W301 and W302 were both expected to have increased resistance to methylenomycin A (**16**), relative to their respective parent

strains W110 and M145. Firstly, the presence of *mmr* in the new strains was confirmed by PCR analysis. The strains were grown on SFM plates for four days and the colonies were treated with lysis buffer (section 5.11.4). The lysed colonies were then analysed by PCR using the same primers and conditions employed to confirm the presence of *mmr* DNA in the purified construct. Colonies of the parent strains W110 and M145 were also lysed and analysed simultaneously (Figure 2.4). As expected, a positive amplification band corresponding to the *mmr* gene (1.43 kb) was seen in colonies from the two new derivative strains, while the band was absent in the lysed samples from M145 and W110, thereby confirming the successful introduction of the *mmr* gene into the two strains.

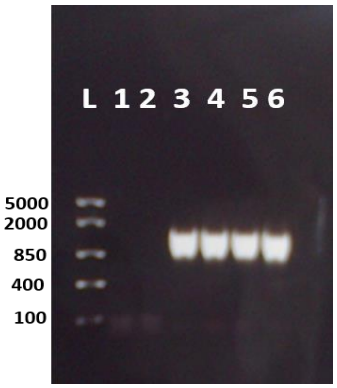


Figure 2.4: Gel from agarose electrophoresis separation following PCR amplification of *mmr* from lysed colonies of W110 (1), M145 (2), W301 (3 & 4), and W302 (5 & 6). L – middle range FastRuler^{TM} DNA Ladder. Expected size = 1.43 kb.

Following these results, bioassay experiments were conducted to demonstrate the methylenomycin-resistance phenotypes of the new strains. This was carried out by saturating filters with a concentrated solution of **16** (purified as discussed in section 2.1.4) and placing the filters in the centre of AlaMM pH 5 plates inoculated separately with spore suspensions of W301, W302, W110 and M145. Following incubation at 30 ^{o}C for four days, a zone of inhibition was visible in the region immediately

surrounding the filters in plates inoculated with M145 and W110, indicating sensitivity to **16**, while no zone of inhibition was seen in the plates inoculated with the derivative strains carrying the methylenomycin resistance gene, *mmr* (Figure 2.5). The experiments confirmed that the Mmr was produced in the derivative strains, thereby conferring resistance against methylenomycin A, **16**. The construction of these resistant derivatives, as well as the purification of **16** and **17** (discussed in the next sections) paved the way for feeding experiments to demonstrate the roles of MmyO and MmyF in the epoxidation of **17** to give **16**.

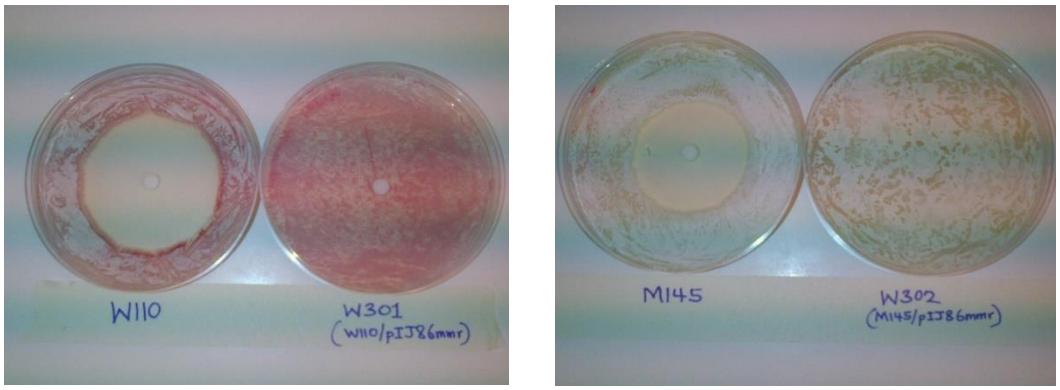


Figure 2.5: Growth inhibition of *S. coelicolor* strains W110 and M145 revealed sensitivity to methylenomycin A (left plates on each image). Introduction of methylenomycin resistance determinant, *mmr*, conferred resistance seen in W301 and W302 (Right plates on each image).

2.1.3 Purification of methylenomycin C (17)

The putative substrate for MmyO, methylenomycin C (**17**), accumulates in two mutant strains: *S. coelicolor* W108 (M145 with C73_787/Δ*mmyF*/*mmyR*::*apr*) and W100 (M145 with C73_787/Δ*mmyO*/*mmyR*::*apr*) (see Fig. 2.1). A litre of supplemented minimal medium solid (SMMS) was inoculated with spore suspension of strain W108 and incubated for 36 hours. Methylenomycin C (**17**) was purified from acidified ethyl acetate extract of this culture by preparative HPLC as detailed in the methods section

(5.14.1). The purified compound was subjected to ^{1}H, ^{13}C and 2D-NMR analyses and data obtained were consistent with the structure of **17**, as reported previously.[173]

2.1.4 Expression of the entire *mmy* gene cluster in heterologous hosts and purification of methylenomycin A (16)

An authentic standard of methylenomycin A (**16**) was also required. *S. coelicolor* strain W89 contains all the genes required for the regulation and production of methylenomycin antibiotics carried on the cosmid C73_787, except the transcriptional repressor, *mmyR*, which had been replaced with an apramycin resistance cassette.[174] This strain was expected to overproduce **16** and **17**. Although LC-MS analysis of supernatant and acidified culture extracts of W89 revealed that the strain indeed produced **16**, attempts to purify it from large cultures of this strain grown on SMMS were unsuccessful. This is because germicidins, another set of metabolites produced by the parent strain M145 under the same fermentation conditions,[136] were produced in much higher titers (Fig. 2.6) and completely obscured **16** in HPLC purification attempts. Thus, other heterologous hosts were considered for the expression of the methylenomycin gene cluster.

Two *Streptomyces* species were used as alternative hosts for the *mmy* cluster: *S. lividans*, a close genetic relation of *S. coelicolor* [176] and *S. albus*, widely used as heterologous host for the expression of secondary metabolite biosynthetic gene clusters.[177,178] *E. coli* ET12567/pUZ8002 was therefore transformed by the construct C73_787/*mmyR::apr* and subsequently conjugated with *S. lividans* TK24 and *S. albus* J1074 as detailed in the experimental section (5.11.3). The resultant strains, named W303 and W304 respectively for *S. albus* J1074 and *S. lividans* TK24 host strains,

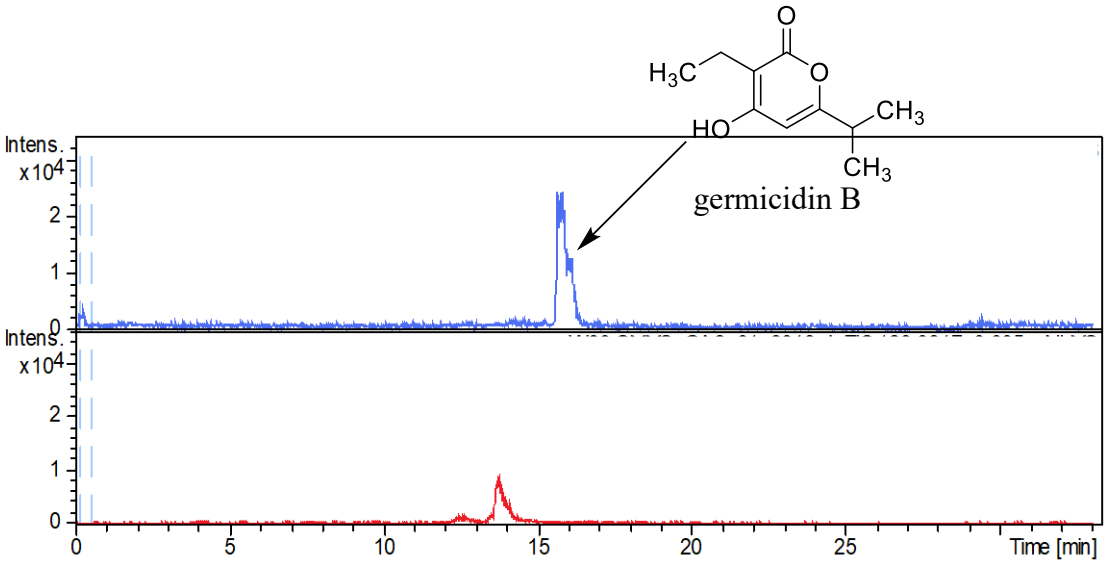


Fig. 2.6: LC-MS analysis of extract of *S. coelicolor* W89 (M145/C73_787/*mmyR*::*apr*), comparing EIC at *m/z* = 183.1181, corresponding to [M+H$^+$] for germicidin B (blue traces) with EIC at *m/z* = 183.0650 corresponding to [M+H$^+$] for methylenomycin A (**16**) (red traces).

were fermented on SMMS and their metabolic profile was examined. As expected, LC-ESI-HRMS of the acidified culture extracts of these strains revealed the presence of a peak at 14.5 minutes with *m/z* [M + H]$^+$ = 167.0705 corresponding to M = [$C_9H_{11}O_3$]$^+$, consistent with the protonated form of **17**, and a small peak at 15.1 minutes with *m/z* [M + H]$^+$ = 183.0654, corresponding to M = [$C_9H_{11}O_4$]$^+$ for **16** (Fig. 2.7). Compounds with *m/z* [M + H]$^+$ = 123.0807 corresponding to [$C_8H_{11}O$]$^+$ and [M + H]$^+$ = 139.0755 corresponding to [$C_8H_{11}O_2$]$^+$, resulting from spontaneous decarboxylation of **17** and **16** respectively were also observed.

In addition to the peaks corresponding to **16** and **17** in the transformed host strains, two prominent peaks were also found in W304 (*S. lividans* TK24/ C73_787/*mmyR*:: *apr*) at 16.7 and 17.3 minutes. These peaks had *m/z* values [M + H]$^+$ = 197.1172 and 183.1181 respectively, corresponding to molecular formulae of $C_{11}H_{17}O_3$ and $C_{10}H_{15}O_3$, previously assigned to germicidins A and B respectively.[136] Given that a

germicidin synthase (Gcs) with 99% identity to the *S. coelicolor* Gcs has been identified in *Streptomyces lividans*,[179,180] the additional metabolites produced by

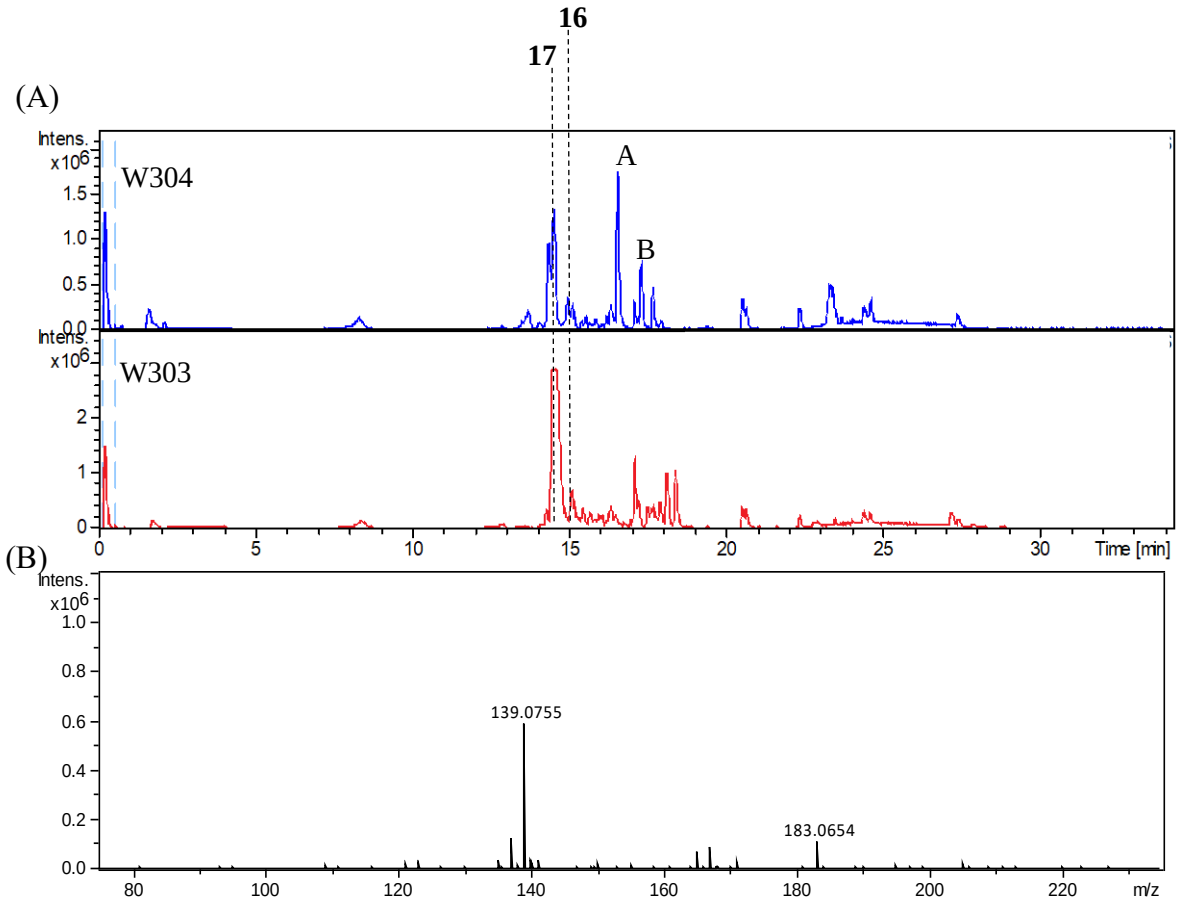


Fig. 2.7: LC-ESI-HRMS showing the production of **16** and **17** by *S.albus* and *S. lividans* transformed with the construct C73_787/*mmyR*::*apr* (A) TIC following extraction of W304 (*S. lividans* TK24/C73_787/*mmyR*::*apr*) and W303 (*S. albus* J1074/C73_787/*mmyR*::*apr*). Peaks labelled A and B gave exact *m/z* values and molecular formulae corresponding to germicidins A and B respectively (B) HR-MS data at 15.1 minutes showing *m/z* = 183.0654 and 139.0755 corresponding to $[M + H^+]$ and $[M + H^+ - CO_2]$ respectively for **16**.

W304 are proposed to be germicidins. Thus, to avoid similar challenges posed by the presence of germicidins in the attempted purification of **16** from *S. coelicolor* strainW89, W303 was chosen as the preferred strain for the purification. W303 was therefore fermented on SMMS and **16** (20 mg) was purified from acidified ethyl acetate extract of a litre culture as detailed in the methods section (5.14.2). The

compound was confirmed by ^{1}H and COSY NMR analysis and data obtained were consistent with those reported previously for **16**.[173]

2.1.5 Feeding of methylenomycin C (17) to *S. coelicolor* W301 confirms the roles of MmyO and MmyF in epoxide formation in methylenomycin A (16)

Strains W301 and W302 were inoculated over a semi-permeable membrane supported by Alamm medium (pH 5.0, allowing for better diffusion of methylenomycin metabolites). A solution of methylenomycin C (**17**) in DMSO was fed at low concentrations to W301 and W302 after an initial incubation at 30 ºC for 2 days. Following a further incubation for 4 days and extraction with methanol, LC-ESI-HRMS analyses of the W301 culture extract revealed the presence of a compound with the same *m/z* value (183.0652) and retention time (14.7 minutes) as the authentic standard of **16** (Fig. 2.8). Absence of this peak in the culture extract of W302 fed with

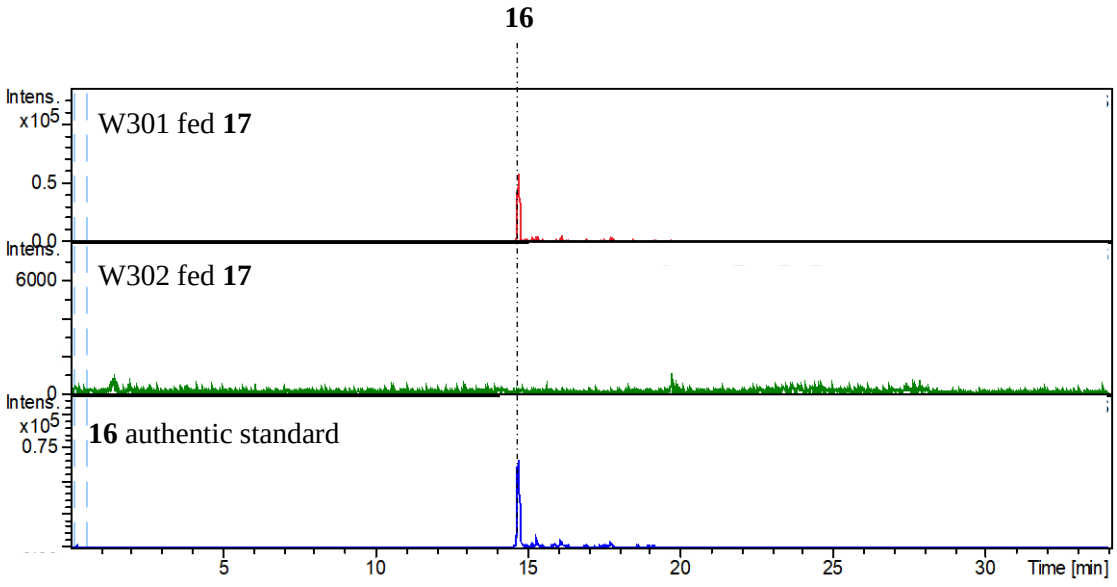
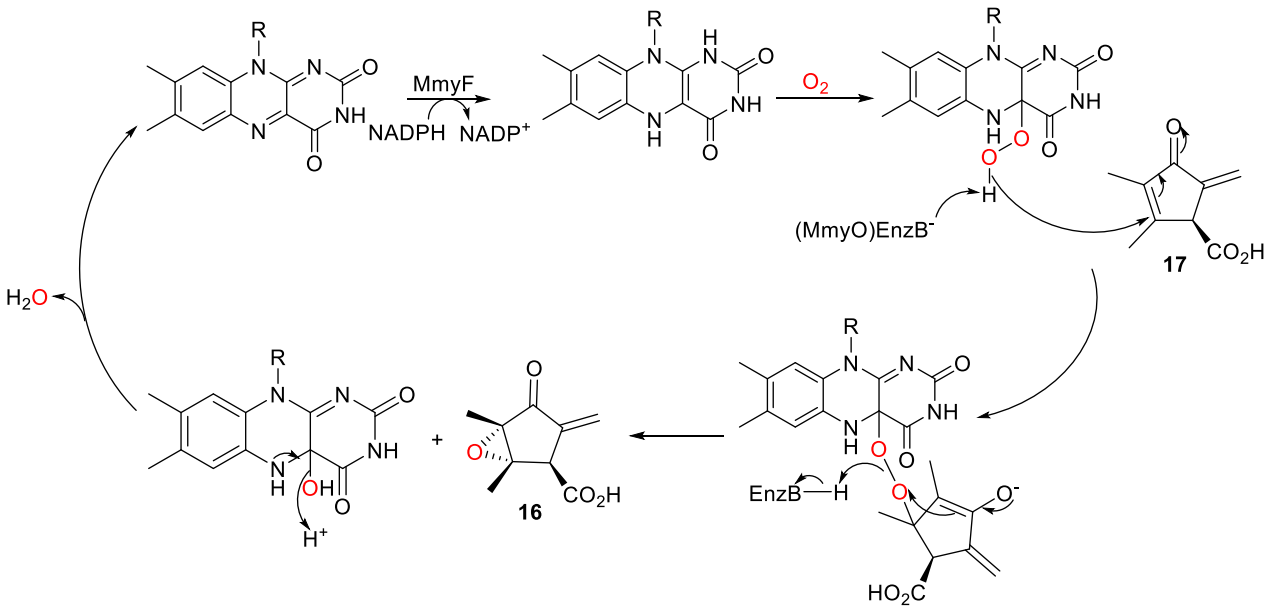


Figure 2.8: Extracted ion chromatograms (EIC) for *m/z* = 183.0650 ± 0.005 corresponding to [M + H^{+}] for **16** from LC-ESI-HRMS analysis of extracts of *S. coelicolor* W301 (M145/ pOSV556/*mmyOF*/pIJ86*mmr*) and W302 (M145/pIJ86*mmr*) fed with methylenomycin C, **17**.

17 clearly indicates that the presence of **16** in the W301 extract resulted from the activity of MmyO and MmyF. Feeding high concentrations of **17** (10x) to both strains resulted in cell death and no turnover was observed.

Based on the above results and on a previous experiment which has demonstrated the involvement of molecular oxygen in forming the epoxide function of **16**, a detailed mechanism may be proposed for the epoxidation of **17** to **16** (Scheme 2.1).



Scheme 2.1: Proposed mechanism for the epoxidation of methylenomycin C (**17**) to give methylenomycin A (**16**).

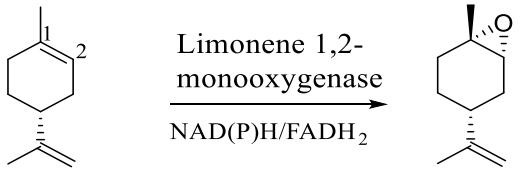
From bioinformatic analyses, MmyF is similar to NADPH- dependent flavin reductases known to be required for the supply of reduced flavin, $FADH_2$, to flavin-dependent monooxygenases.[181,182] MmyO shows a significant sequence similarity to LimB, a monooxygenase which catalyses $FADH_2$ –dependent epoxidation of the 1,2-double bond of limonene in *Rhodococcus erythropolis* using molecular oxygen as co-substrate (Scheme 2.2A).[183] We therefore propose that MmyO carries out the epoxidation of **17** to **16** using reduced flavin ($FADH_2$) generated by the proposed

flavin reductase, MmyF. The strict requirement for MmyF to supply the $FADH_2$ is evidenced by the fact that strain W108, although containing *mmyO* and all other genes coding for enzymes required to biosynthesize the methylenomycin compounds, but does not contain *mmyF*, could not produce **16**, leading to the accumulation of **17** (see Fig. 2.1).

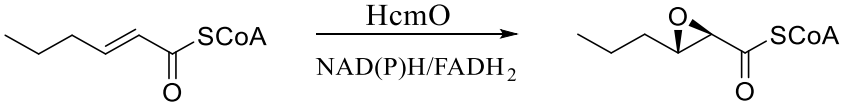
The mechanism is likely analogous to other flavin-dependent reactions and monooxygenases in which $FADH_2$ is generated *via* a hydride transfer from NAD(P)H to FAD. The reduced flavin then reacts with molecular oxygen *via* two one-electron transfers to generate a flavin hydroperoxide, *via* a semiquinone radical intermediate.[184, 204] Deprotonation of the hydroperoxide adduct by a base in the active site of the monooxygenase then produces a nucleophilic peroxide which can attack the β- Carbon in **17** to generate an enolate (scheme 2.1). The enolate then collapses to furnish **16** and a flavin hydroxide. Loss of a water molecule regenerates the oxidised flavin (FAD) which is again reduced by MmyF to continue the reaction cycle.

Although MmyO directly bears homology to the limonene-1,2-monooxygenase in *Rhodococcus erythropolis*, a mechanism similar to the one shown in scheme 2.1 for the epoxidation of **17** has also been proposed for the last step in the biosynthesis of the 2,3-epoxyhexanoyl side chain of the calcium-dependent antibiotics (CDA), produced by *Streptomyces coelicolor*. HcmO, a flavin-dependent monooxygenase encoded within the CDA cluster, catalyses epoxidation of the trans-hexenoyl-CoA intermediate in the pathway to give the 2,3-epoxyhexanoyl moiety.[185,150] (Scheme 2.2 B). Thus, the result obtained herein for MmyO is consistent with the reactions catalysed by similar monooxygenases in other pathways.

(A)



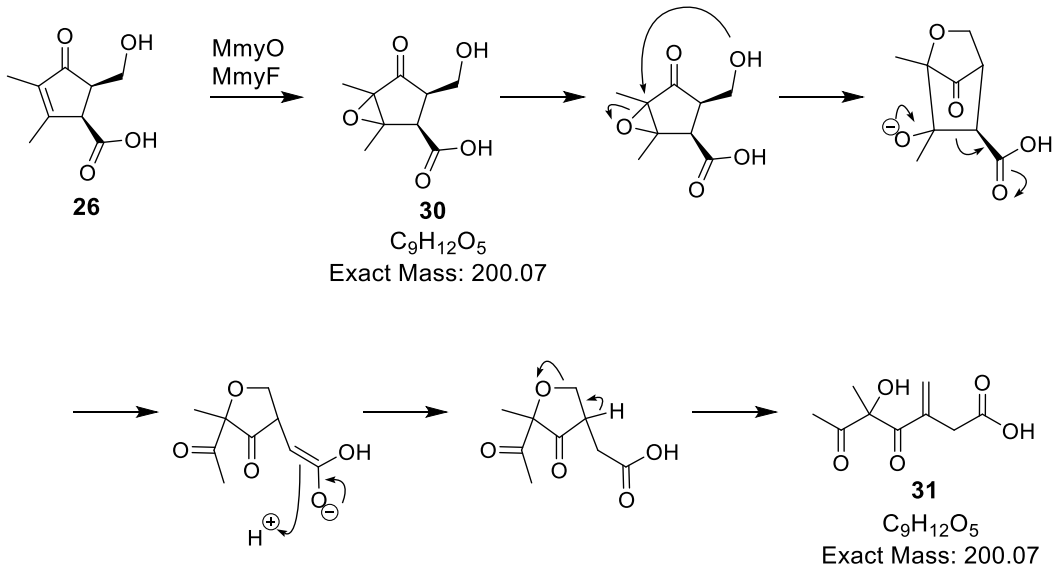
(B)



Scheme 2.2: Epoxidation of double bonds catalysed by monooxygenases similar to MmyO: (A) Conversion of limonene to limonene-1,2-epoxide (B) Formation of the epoxyhexanoyl moiety of calcium-dependent antibiotics.

2.2 Putative intermediates and shunt products of the methylenomycin pathway considered as substrate for MmyO

As discussed in the introduction (section 1.8.2), a putative intermediate of the methylenomycin pathway, pre-methylenomycin C (**26**), accumulates in a *mmyE* mutant strain. In addition to **26**, another metabolite, **31**, was also purified from the strain. This compound had the same *m/z* value and molecular formula as an epoxidized form of pre-methylenomycin C (**30**), but has a different structure. It was proposed that **26** could possibly be epoxidized by MmyF and MmyO to give **30**, which might then form **31** *via* a series of rearrangement steps (scheme 2. 3).[174] However, it has yet to be demonstrated that MmyF and MmyO are able to catalyse the epoxidation of **26** to form **30**. Indeed, **26** possesses the core cyclopentenone structure which appears crucial to the epoxidation mechanism proposed for methylenomycin C (**17**), and could potentially be a substrate for the monooxygenase (MmyO).



Scheme 2.3: Epoxidation of pre-methylenomycin C (**26**) by MmyO and MmyF is hypothesised to form **30**, which then undergoes a series of proposed rearrangement steps leading to **31** (scheme proposed by G. L. Challis [174]).

Pre-methylenomycin C lactone (**27**), methylenomycin D1 (**28**) and methylenomycin D2 (**29**), also possess the same cylopentenone structure as methylenomycin C (**17**) and are all used to probe the substrate promiscuity of MmyO in the current study. Likely products that may be formed if these compounds are epoxidised are shown in Fig. 2.9. Pre-methylenomycin C (**26**) and pre-methylenomycin C lactone (**27**) were purified from *S. coelicolor* W86 (M145/C73_787/Δ*mmyE*/*mmyR*::*apr*) as detailed in the methods section (5.14.3). Methylenomycin D1 (**28**) and methylenomycin D2 (**29**) were purified from *S. coelicolor* W108 (M145/C73_787/Δ*mmyF*/*mmyR*::*apr*) as detailed in the method section (5.14.4).

The compounds were fed to strain W301, and following extraction with methanol, all the extracts were analysed by LC-MS. The only potential substrate which resulted in an altered chromatogram when fed to W301 compared to W302 is pre-methylenomycin C (**26**) (Fig. 2.10). The new peak appearing at 14.2 minutes had an

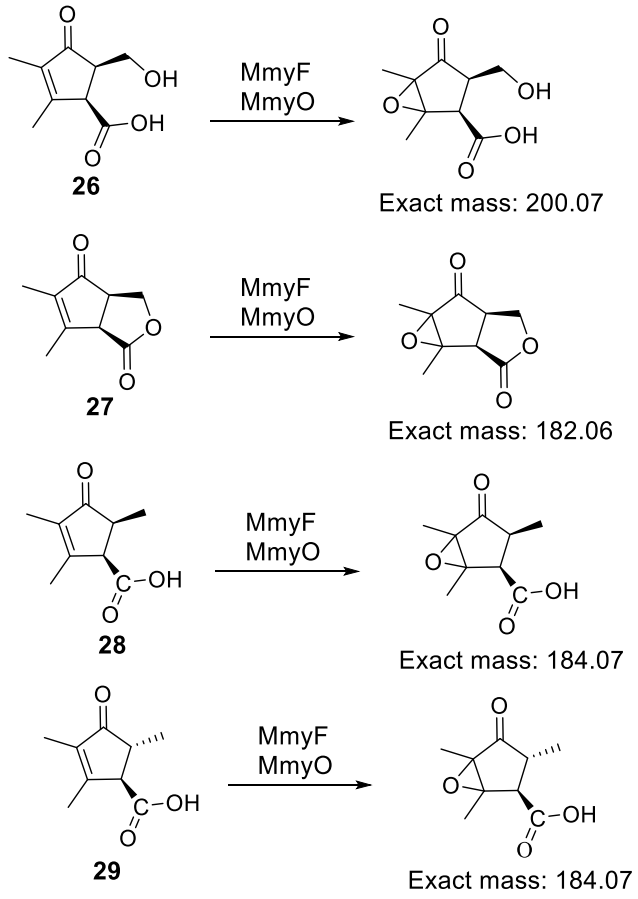


Fig. 2.9: Likely products of the epoxidation of **26**, **27**, **28** and **29**.

m/z [M + Na] = 223.0577 corresponding to $C_9H_{12}O_5Na$ and consistent with the sodiated form of **30**. This result suggests that MmyO and MmyF are also able to carry out the epoxidation of **26**, but not **27**, **28**, and **29**. Indeed, the new peak gave the same retention time and *m/z* value 223.0577 as compound **31**, present in the culture extract of *S. coelicolor* W86, carrying the cosmid C73_ 787, but with *mmyE* deleted (Fig. 2.11). The result indicates that **31** is indeed an an artefact of **30**, and it is formed spontaneously following the epoxidation of **26** to **30** by the joint actions of MmyF and MmyO in the W301 strain. Since **26** accumulates in the *S. coelicolor* W86, and both *mmyO* and *mmyF* are present in this strain; it follows that **26** is carried on to **30** following its formation in the strain, explaining why its artefact, **31**, was detectable in the culture extract.

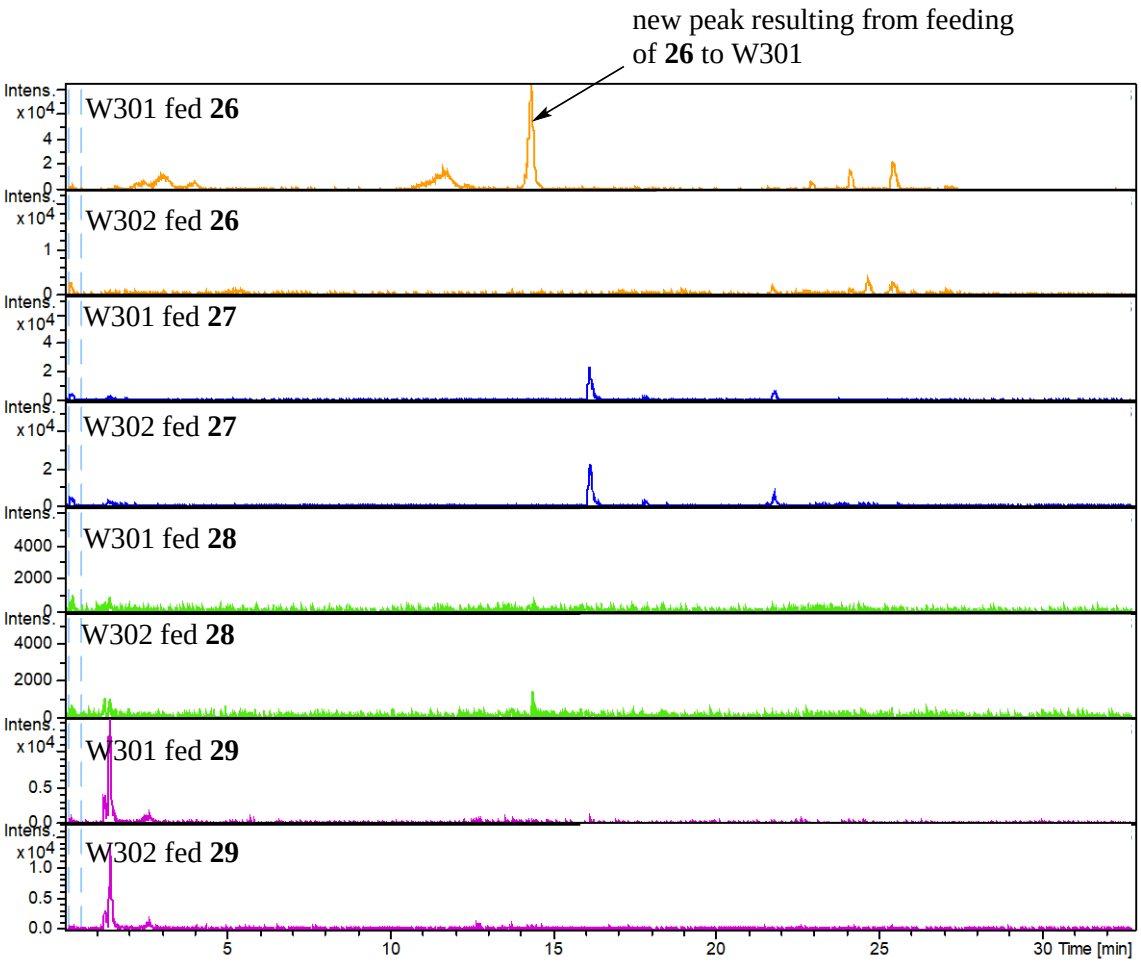
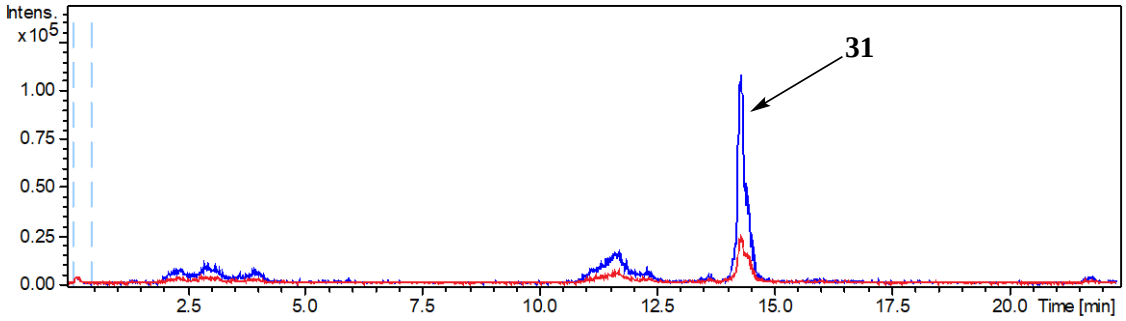


Fig. 2.10: Extracted ion chromatograms (EIC) from LC-MS analyses of extracts of W301 and W302 fed with **26**, **27**, **28** and **29**. Yellow: EIC at *m/z* = 201.07 and 223.07 corresponding to [M + H^+] and [M + Na^+] for possible epoxidized product of **26**. Blue: EIC at *m/z* = 183.06 and 205.06 corresponding to [M + H^+] and [M + Na^+] for possible epoxidized product of **27.** Green and Purple: EIC at *m/z* = 185.07 and 207.07 corresponding to [M + H^+] and [M + Na^+] for possible epoxidized product of **28** and **29**.

The inability of MmyF and MmyO to epoxidise **27**, **28** and **29** suggests that the monooxygenase (MmyO) is highly specific for methylenomycin C (**17**) and its putative precursor, pre-methylenomycin C (**26**). Simple synthetic analogues of methylenomycin C such as 2-cyclopentenone and 2-cyclohexenone were obtained commercially and also fed to W301, but no epoxidation of these compounds was observed. Rather, the compounds were likely degraded as they could not be detected in the culture extracts of the strain following incubation.

(A)



(B)

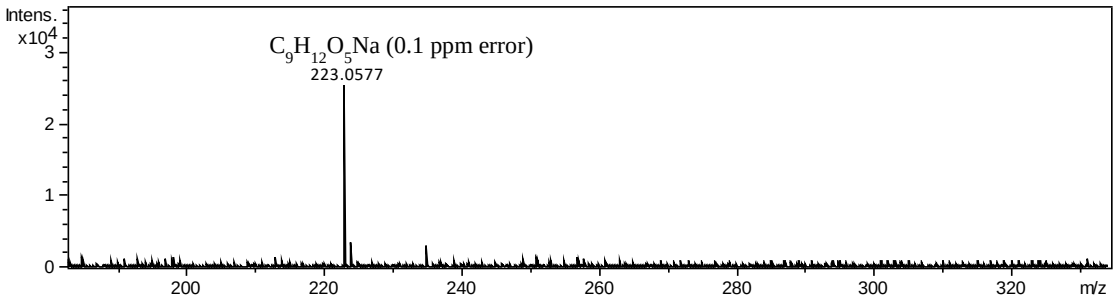


Fig. 2.11: (A) EICs for *m/z* = 223.0577 corresponding to [M + Na^+] for **31** from LC-MS analyses of extracts of *S. coelicolor* W86 (red traces) and W301 fed with **26** (blue traces) (B) HR-MS data at 14.3 minutes showing *m/z* = 223.0577 corresponding to [M + Na^+] for **31**, following LC-MS analysis of extracts from W301 fed **26**.

2.3 Metabolites profiling of *S. coelicolor* transconjugant strains lacking the genes: *mmyP, mmyG, mmyT, mmyK* or *mmyX*

As noted in the introduction, various *S. coelicolor* transconjugant strains lacking individual putative biosynthetic genes, and the transcriptional repressor, *mmyR* (replaced with an apramycin resistance gene), were previously constructed using the cosmid C73_787 which contains the entire methylenomycin biosynthetic gene cluster.[174] However, the metabolic profiles of double mutant strains: (Δ*mmyP*/*mmyR*::*apr*), (Δ*mmyG*/*mmyR*::*apr*), (Δ*mmyT*/*mmyR*::*apr*), (Δ*mmyK*/*mmyR*::*apr*) and (Δ*mmyX*/*mmyR*::*apr*) were not previously examined to assess the involvement of the

enzymes encoded by each of these *mmy* genes in the biosynthesis, and to characterise any intermediates which may accumulate in the double mutants.

Thus, the strains were fermented on SMMS and the culture extracts were analysed by LC-ESI-HRMS. Fig. 2.12 shows the metabolic profiles of the transconjugant strains, compared with those of the *mmyR* mutant and the heterologous host M145 fermented and analysed under the same conditions. Since the cosmid C73_787 includes the *mmfLHP* which code for the biosynthesis of the methylenomycin furan signalling molecules (and these genes have not been deleted in each of the mutant strains), they provide an opportunity, in the first instance, to confirm the integration of each of the mutated constructs into the M145 genome. Thus, each of the strains was expected to produce the methylenomycin furans. Indeed, methylenomycin furans (MMF1 and MMF4) were detected in all the double mutant strains as well as the *mmyR* only mutant.

More importantly, the data show that the production of methylenomycin antibiotics is completely abolished in the Δ*mmyX*/*mmyR*::*apr* double mutant strain, indicating that MmyX is essential for their biosynthesis. Since MmyX shows sequence similarity to a kinase, the result strongly points to a need for some intermediates in the methylenomycin pathway to be phosphorylated.

The data also show that methylenomycin production is not abolished in each of Δ*mmyG*/*mmyR*::*apr*, Δ*mmyK*/*mmyR*::*apr*, Δ*mmyP*/*mmyR*::*apr*, and Δ*mmyT*/*mmyR*::*apr* transconjugant strains, although the level of production is greatly reduced in the Δ*mmyP*/*mmyR*::*apr* and the Δ*mmyT*/*mmyR*::*apr* transconjugant strains.

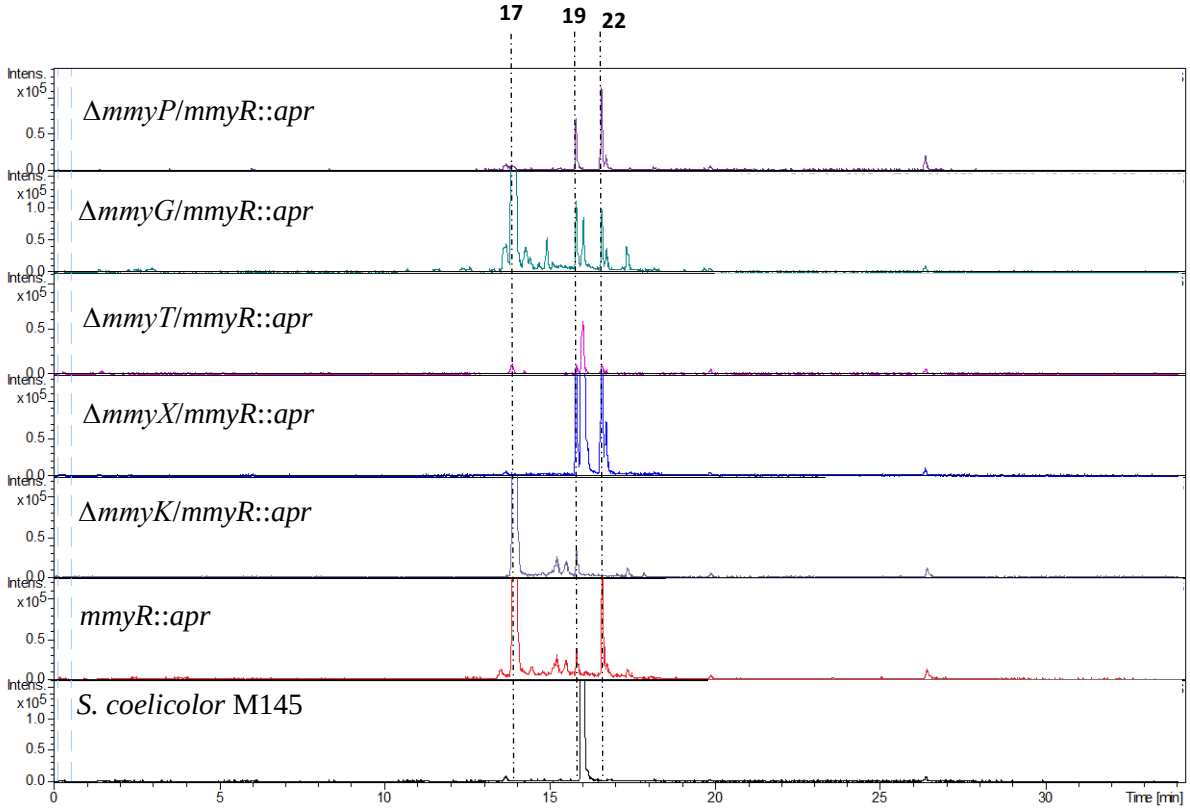


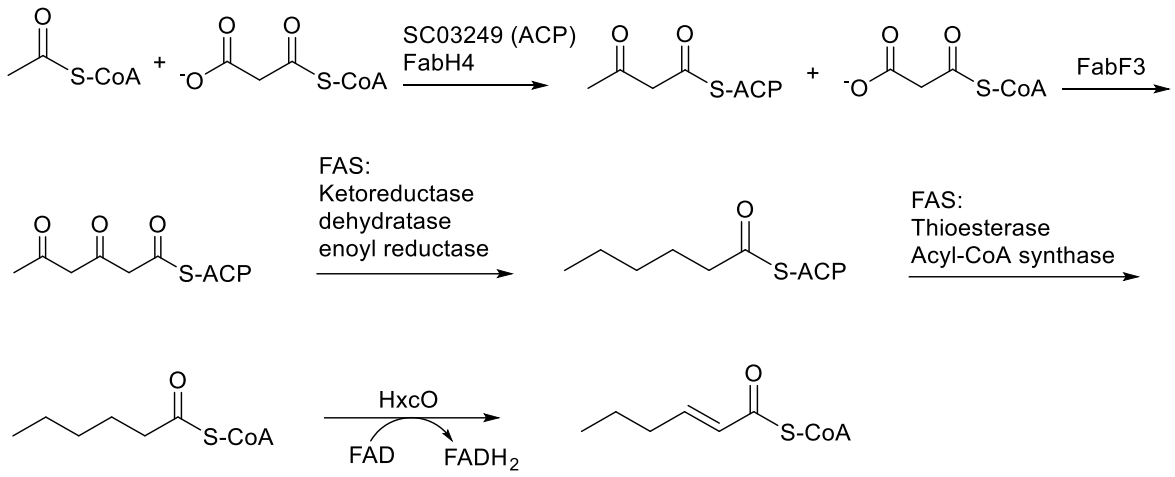
Fig. 2.12: EICs from LC-MS analyses of extracts of *S. coelicolor* transconjugants for *m/z* = 167.0700, corresponding to [M + H^+] for methylenomycin C (**17**), and *m/z* = 181.0850 corresponding to [M + H^+ - H_2O] for MMF1 (**19**) and MMF4 (**22**). Each strain contains the cosmid C73_787 with *mmyR* replaced with an apramycin resistance gene/ plus a putative methylenomycin biosynthetic gene deleted.

mmyG, *mmyK*, *mmyP* and *mmyT* respectively code for enzymes which have sequence homologies to a dehydrogenase/ketoreductase, kinase, phosphatase and a thioesterase. The results suggest that these enzymes are either not essential for the biosynthesis of methylenomycin antibiotics or that they are not very specific, in which case, enzymes with similar functions in the parent strain may perform their functions in their absence, accounting for why methylenomycins production is not aborted in the mutant strains.

The medium used for the production, SMMS, contains a high phosphate concentration, which has been reported to favour the production of methylenomycin compounds over the other antibiotics (prodiginnes, actinorhodins and CDAs)

produced by *Streptomyces coelicolor*.[159] None of these other antibiotics was detected in the LC-ESI-HRMS analysis of the culture extracts. It is therefore very unlikely that a "cross talk" between methylenomycin pathway and the pathways of these other antibiotics was responsible for sustaining methylenomycin production in the mutant strains. Rather, a set of compounds produced under the same fermentation conditions, and which were consistently detected in these transconjugant strains, are germicidins A and B7, biosynthesized by a type III polyketide synthase.[136] While bioinformatic analysis did not reveal homology between any of MmyG, MmyK, MmyP and MmyT and the enzymes encoded in the *S. coelicolor* M145 Gcs, responsible for germicidins production; the possibility of interactions between such a pathway and the methylenomycin pathway may not be completely ruled out.

The other possible reason for why methylenomycin production is not abolished in the mutant strains is that the pathway benefits from activity of enzymes involved in primary metabolism of the host strain. It has been noted that the pathway to many specialised metabolites of *S. coelicolor* (including CDAs, prodiginine and actinorhodin) rely on the fatty acid synthase (FAS) enzymes for some key steps in their biosynthesis.[186,187] A good example is the recruitment of the ketoreductase, dehydratase, and enoyl reductase from FAS to carry out the full reduction of a β,δ-diketo hexanoyl ACP intermediate of the CDA epoxyhexanoyl side chain, initially produced by FabF3, a ketoacyl synthase II encoded in the CDA cluster (Scheme 2.4).[185] The hexanoyl ACP generated is subsequently hydrolysed to an acid by a thioesterase from FAS, and converted to an hexanoyl CoA by an acyl-CoA synthase, also from FAS. The hexanoyl CoA is then desaturated by HxcO (a FAD-dependent



Scheme 2.4: Biosynthesis of the epoxyhexanoyl side chain of CDA initiated by SC03249 (an ACP), FabH4 (a KAS III) and FabF3 (a KAS II) encoded in the CDA cluster. Enzymes from primary metabolic fatty acid synthase (FAS) are employed to generate the fully reduced hexanoyl-CoA. The last step in this pathway (epoxidation) has been shown in scheme 2.2(B).

acyl-CoA oxidase/dehydrogenase) and epoxidized by HcmO (a flavin-dependent monooxygenase), both enzymes encoded in the CDA cluster. The biosynthesis of this moiety of the CDA thus reveal an interplay between enzymes encoded in the CDA cluter and those of the primary metabolic fatty acid synthase.

The pathway proposed for the biosynthesis of methylenomycin antibiotics already suggests that a malonyl-CoA acyl carrier protein transacylase (MCAT) derived from a primary metabolic FAS would catalyse the loading of a malonyl group onto MmyA, the ACP encoded in the methylenomycin gene cluster (see scheme 1.3). It is therefore possible that some FAS enzymes could also intervene downstream along the pathway in the absence of a *mmy* enzyme. The fact that the thioesterase, MmyT, is encoded in the methylenomycin cluster suggests a need for the MmyA-bound diketide in the methylenomycin pathway to first be hydrolysed prior to undergoing condensation with a pentulose. Thus, a thioesterase from a FAS may be responsible for this reaction in the absence of MmyT, explaining why methylenomycins were not completely abolished in the *mmyT* mutant strain. A ketoreductase encoded within a FAS may also

be acting in lieu of MmyG in the Δ*mmyG*/*mmyR*::*apr* double mutant strain, accounting for why methylenomycin production is fully retained in the strain.

The phosphatase, MmyP, might also be complemented by MmfP, another phosphatase encoded in the methylenomycin gene cluster. *mmfP* belongs to the operon of three genes required to biosynthesize the methylenomycin furans and has not been deleted in all the mutant strains. Similarly, the retainment of methylenomycin production in the Δ*mmyK*/*mmyR*::*apr* double mutant strain could be due to the presence of *mmyX*, whose product bears a 42% identity to MmyK (both kinases). As discussed earlier, methylenomycin production was abolished in the Δ*mmyX*/*mmyR*::*apr* double mutant strain, indicating that MmyX is essential for methylenomycins biosynthesis (Fig. 2.10). Thus, it appears MmyX may be sufficient for all the phosphorylation steps in the pathway.

However, no new methylenomycin-related intermediates were found in the transconjugant strains, except the Δ*mmyG*/*mmyR*::*apr* double mutant. This newly-identified compound is the focus of the next section.

2.4 Identification of a putative butenolide intermediate in the methylenomycin biosynthetic pathway

As noted in the introduction (section 1.8.2), the biosynthesis of the methylenomycins is proposed to proceed *via* a butenolide intermediate (**25**) formed by *mmyD*, a homologue of the *avrD* in *Pseudomonas syringae*. However, experimental evidence was lacking for this proposed intermediate.

In the LC-MS analysis of *S.coelicolor* transconjugants containing the *mmy* gene cluster, a compound was identified in the (Δ*mmyG*/*mmyR*::*apr*), (Δ*mmyO*/

mmyR::*apr*) and (Δ*mmyF*/*mmyR*::*apr*) strains. This compound eluted at *ca.* 15 mins and it is absent in the heterologous host, *S. coelicolor* M145 (Fig. 2.13). HR-MS data revealed that the compound shows an *m/z* $[M + Na]^+$ value of 239.0877 and molecular formula $C_9H_{12}O_6Na$ (4.2 ppm error), consistent with the sodiated form of the proposed butenolide intermediate. The compound fragments under the mass spectrometric conditions, losing a molecule of water to give *m/z* = 221.0416 corresponding to $C_9H_{10}O_5Na$ and *m/z* = 199.0595 corresponding to $C_9H_{11}O_5$. This in turn loses a molecule of water to give the prominent peak with *m/z* = 181.0493 corresponding to $C_9H_9O_4$ as shown in the figure. Fragmentation of the putative butenolide intermediate *via* the serial loss of water molecules is consistent with the proposed structure which has many easily-cleaved hydroxyl groups.

Interestingly, these ions were also detected in *S. albus* J1074 transformed with the cosmid containing the entire *mmy* gene cluster (except *mmyR*) but absent in wild type *S. albus* J1074 strain, indicating that the metabolite is a product of the *mmy* gene cluster. However, attempts to purify the compound from scaled-up cultures of the producing transconjugant strains was unsuccessful, due to the fact that the compound was present in much lower titers compared to other methylenomycin compounds produced by the strains.

Thus, in an effort to further investigate whether the compound is indeed related to the methylenomycins, [U-^{13}C]-D-ribose was fed to strain W108 (M145/C73_787/ Δ*mmyF*/*mmyR*::*apr*) grown in SMM liquid as detailed in the methods section (5.15.2). The heterologous host (M145), without methylenomycin gene cluster, was also grown

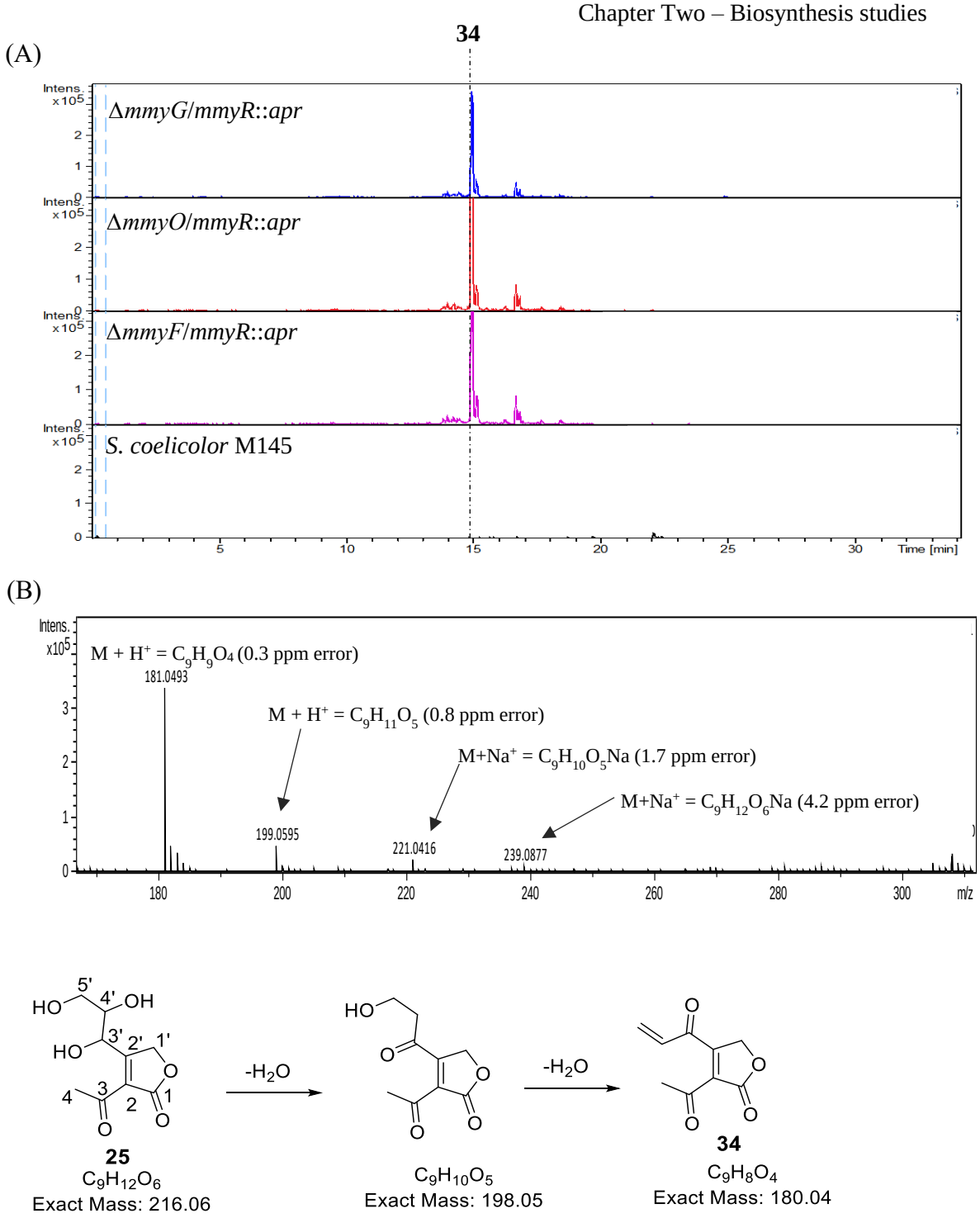


Fig. 2.13: LC-ESI-HRMS analyses showing the putative butenolide intermediate and its proposed fragment ions from extracts of *S. coelicolor* transconjugant strains lacking *mmyG*, *mmyO* or *mmyF* (A) EICs for *m/z* = 181.0493, corresponding to $C_9H_9O_4$ for **34** (B) HR-MS data showing *m/z* = 239.0877, the sodiated adduct of the proposed butenolide (**25**), and fragment ions resulting from loss of water molecules.

and fed in parallel. If the proposed butenolide (**25**) is a precursor of methylenomycin C (**17**), a similar pattern of incorporation of the labelled precursor would be expected

into **25** and **17**. Fig. 2.14 shows mass spectra data obtained from analysis of the culture extracts from the feeding experiments. The data revealed, in addition to *m/z* 167.07 normally obtained for **17**, the presence of peaks representing increases in *m/z* units of 2, 3, 4, 5, and 7. Similarly, a series of peaks with an increase in *m/z* of 2, 3, 4, 5 and 7 were also obtained for the *m/z* of 181.049, derived from the putative butenolide. These ions were completely absent when *S. coelicolor* M145 was fed with the ^{13}C-labelled D-ribose (bottom spectra Fig. 2.14).

The increases in *m/z* values observed for the putative butenolide (**25**) can be explained based, partly on the incorporation of various intermediates or combination of intermediates of the primary metabolic pentose-phosphate pathway and the interconnected glycolytic pathway, and on previous studies which have confirmed the positions of labelled 3-, 4-, and 5-carbon units into **16** and **17**.[172,173] The fed ^{13}C – labelled D-ribose is rapidly converted in the cytosol to [U-^{13}C]- ribose-5-phosphate, which is then isomerised *via* the pentose-phosphate pathway to [U-^{13}C]- ribulose-5-phosphate, itself in constant equilibrium with its epimer, [U-^{13}C]-xylulose-5-phosphate. Thus, the observed intact incorporation of a labelled 5-carbon precursor could have emanated from any of these three intermediates (Scheme 2.5, pink arrows).

Since the butenolide intermediate (**25**) is proposed to derive from a MmyD-catalysed condensation of a pentulose with acetoacetyl-MmyA,[173] it follows that the labelled 4-, 3-, and 2-carbon units incorporated separately into **25** must proceed *via* incorporation into a pentulose or the diketide, acetoacetate. Each of the transketolase steps in the pentose-phosphate pathway (the transfer of a 2-carbon fragment from [U-^{13}C]-xylulose-5-phosphate to [U-^{13}C]-ribose-5-phosphate and erythrose-4- phosphate)

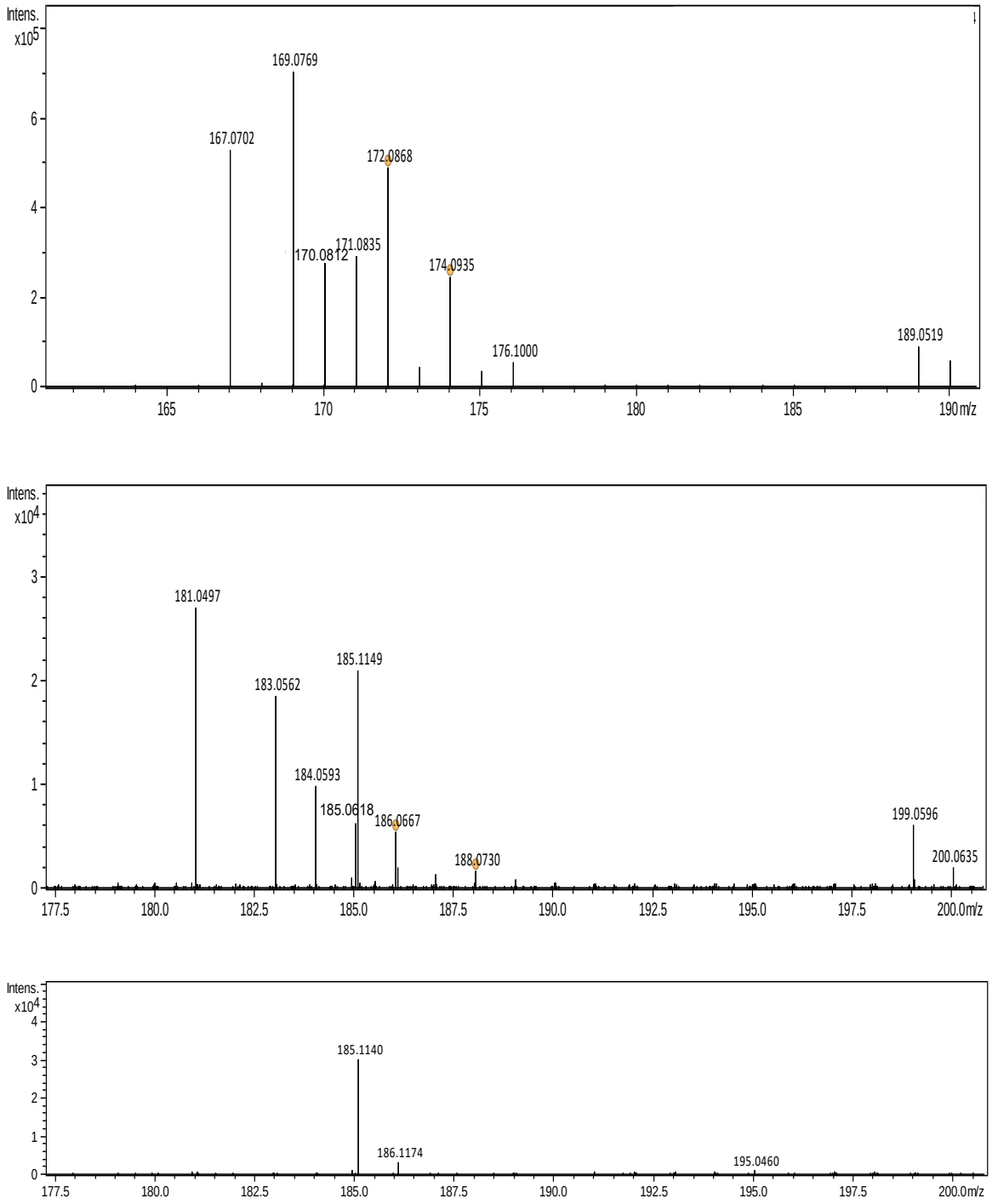
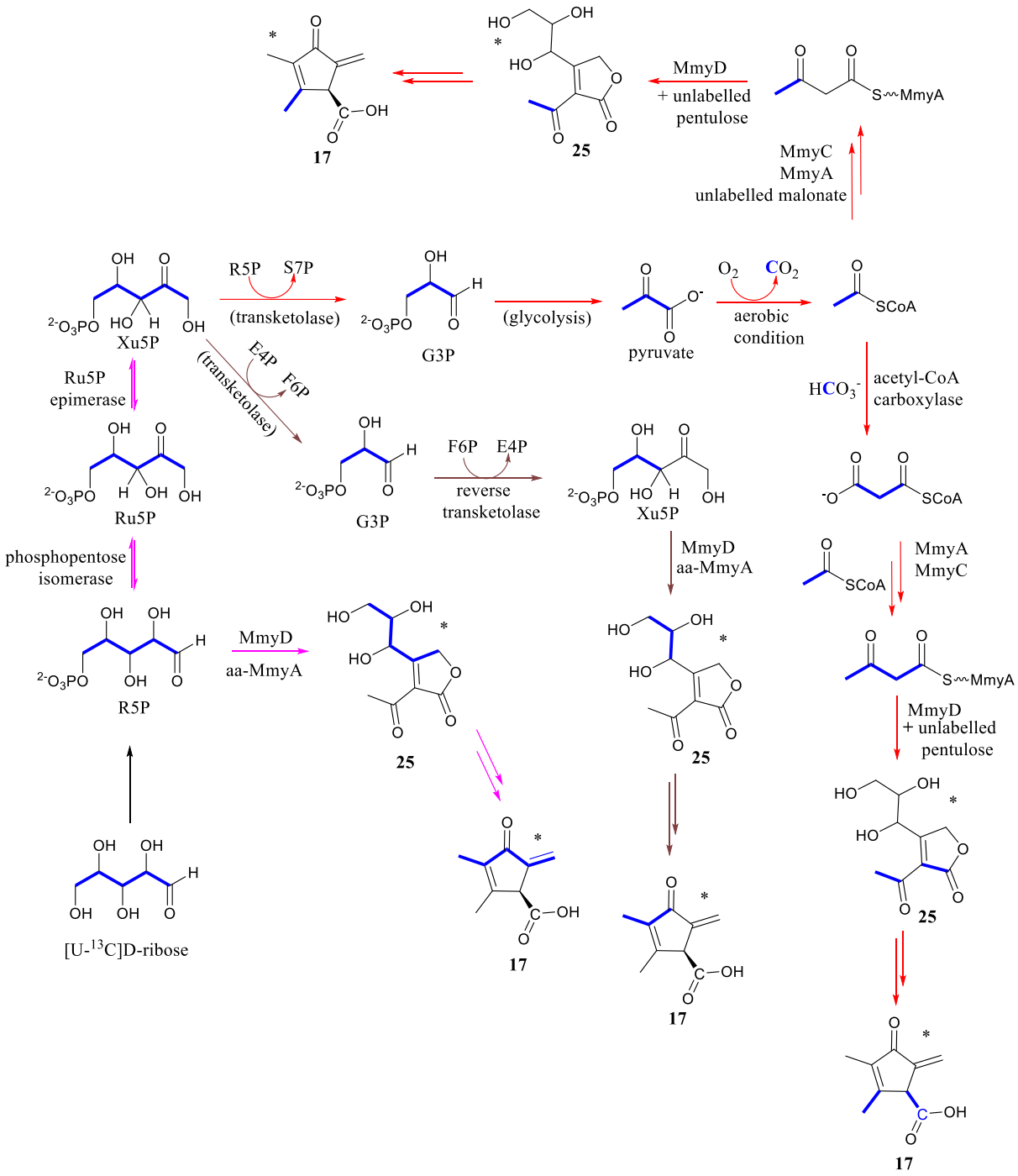


Fig. 2.14: H-MS data following LC-MS analyses of extracts of *S. coelicolor* W108 and M145 fed with [U-^{13}C]-D-ribose. Similar increases of *m/z* values by 2, 3, 4, 5, and 7 units were obtained with respect to methylenomycin C (167.07, top spectra) and the putative butenolide derivative (181.049, middle spectra). The ions were absent in the wild type M145 fed in parallel (bottom spectra). Peak with *m/z* 185.114 present in the bottom and the middle spectra is a metabolite of the wild type M145 and is unrelated to the methylenomycins. Results were reproducible in *S. coelicolor* W100 (M145/C73_787/Δ*mmyO*/*mmyR*::*apr*).



Scheme.2.5: Pathways for the incorporation of ^{13}C-labelled 5-carbon unit (pink arrows), 3-carbon unit (brown arrows), and 2- and 4-carbon units (red arrows) derived from [U-^{13}C]-D-ribose into the butenolide (**25**) and methylenomycin C (**17**). R5P: ribose-5-phosphate, Ru5P: ribulose-5-phosphate, Xu5P: xylulose-5-phosphate, G3P: glyceraldehyde-3-phosphate, E4P: erythrose-4-phosphate, 6FP: fructose-6-phosphate, S7P: seduheptulose-7-phosphate, aa-MmyA: unlabelled acetoacetyl-MmyA. ^{13}C labelling are denoted with bold blue bonds/atoms.* indicates the labelled compounds observed in the HR-MS analysis.

phosphate) will generate a molecule of [U-^{13}C]-glyceraldehyde-3-phosphate. This may then be combined with an unlabelled fructose-6-phosphate in a reverse transketolase step to give erythrose-4-phosphate and xylulose-5-phosphate, labelled only at C-3, C-4 and C-5 positions. MmyD-catalysed condensation of this partially-labelled xylulose-5-phosphate with unlabelled acetoacetyl-MmyA will afford **25** labelled only at C-3', C-4' and C-5', subsequently leading to methylenomycin C (**17**) labelled at C-3, C-4 and C-8 (scheme 2.5, brown arrows). This accounts for the observed incorporation of labelled 3-carbon unit into the putative butenolide (**25**).

[U-^{13}C]glyceraldehyde-3-phosphate is oxidised in the glycolytic pathway to [U-^{13}C]-pyruvate, which is further broken down under aerobic conditions to [U-^{13}C]-acetyl-CoA, with the release of $^{13}CO_2$. To account for the incorporation of a labelled 2-carbon unit into **25**, the [U-^{13}C]-acetyl-CoA would undergo a MmyC (KAS III) – catalysed Claisen condensation with an unlabelled malonyl-MmyA, giving acetoacetyl-MmyA that is labelled only at two carbon positions. This partly-labelled acetoacetyl-MmyA may then be condensed with a pentulose to give **25** labelled at C-3 and C-4, and subsequently **17** labelled at C-5 and C-9 (scheme 2.5 red arrows and top branching).

Increases in *m/z* values by 4 units observed in the metabolites correspond to the incorporation of 2 units of [U-^{13}C]-acetyl CoA. In this case, one unit of the [U-^{13}C]-acetyl CoA is first converted to [U-^{13}C]-malonyl CoA by acetyl CoA carboxylase, using the $^{13}CO_2$ released from pyruvate catabolism to form $H_2{}^{13}CO_3$ that provides the source of the carbon. The [U-^{13}C]-malonyl CoA formed is then loaded onto the ACP, MmyA, and condensed with [U-^{13}C]-acetyl CoA to give [U-^{13}C]-acetoacetyl-MmyA, a step catalysed by MmyC. The fully labelled acetoacetyl-MmyA may then be combined with a pentulose to give **25**, labelled at C-4, C-3 and C-2, C-1. This would

subsequently give **17** labelled at C-5, C-9 and C-1, C-6 (scheme 2.5, red arrows and downward branch). The observed increase in *m/z* values of the two metabolites by 7 units may result from the incorporation of a unit of [U-^{13}C]-glyceraldehyde 3-phosphate (*via* the partly labelled xylulose-5-phosphate route) plus 2 units of [U-^{13}C]-acetyl CoA as detailed above.

Similar incorporation patterns observed for methylenomycin C (**17**) and the putative butenolide fragment, coupled with the fact that the identified putative butenolide compound is absent in the heterologous host, strongly suggest that **17** (and other methylenomycin compounds) could indeed derive from this compound.

2.5 Overproduction of *mmyD* in *E. coli*

Cloning of the *mmyD* homologue, *avrD*, and overproduction in *E. coli* or *P. syringae* has been shown to lead to the production of syringolides 1 and 2. [188,189] To investigate whether overproduction of *mmyD* could also lead to the production of the butenolide intermediate (**25**) or other advanced intermediate of the methylenomycin pathway, the gene was amplified from the construct C73_787/*mmyR*::*apr* and cloned into pET151 under the control of an IPTG-inducible T7 promoter. The resulting construct, pGI003, was sequenced to confirm the presence of the *mmyD* gene in the right orientation, and was subsequently used to transform *E. coli* BL21*. The wild-type *E. coli* BL21* and the *E. coli* BL21* carrying pGI003 were then grown in M9 medium at 37 ^{o}C to an $O.D_{600nm}$ of 0.7, before IPTG was added to a final concentration of 0.5mM. The cultures were incubated overnight at 15 ^{o}C, followed by extraction with ethyl acetate. An additional control experiment in which *E. coli* BL21*/pIG003 was grown in parallel but without IPTG induction was also carried out.

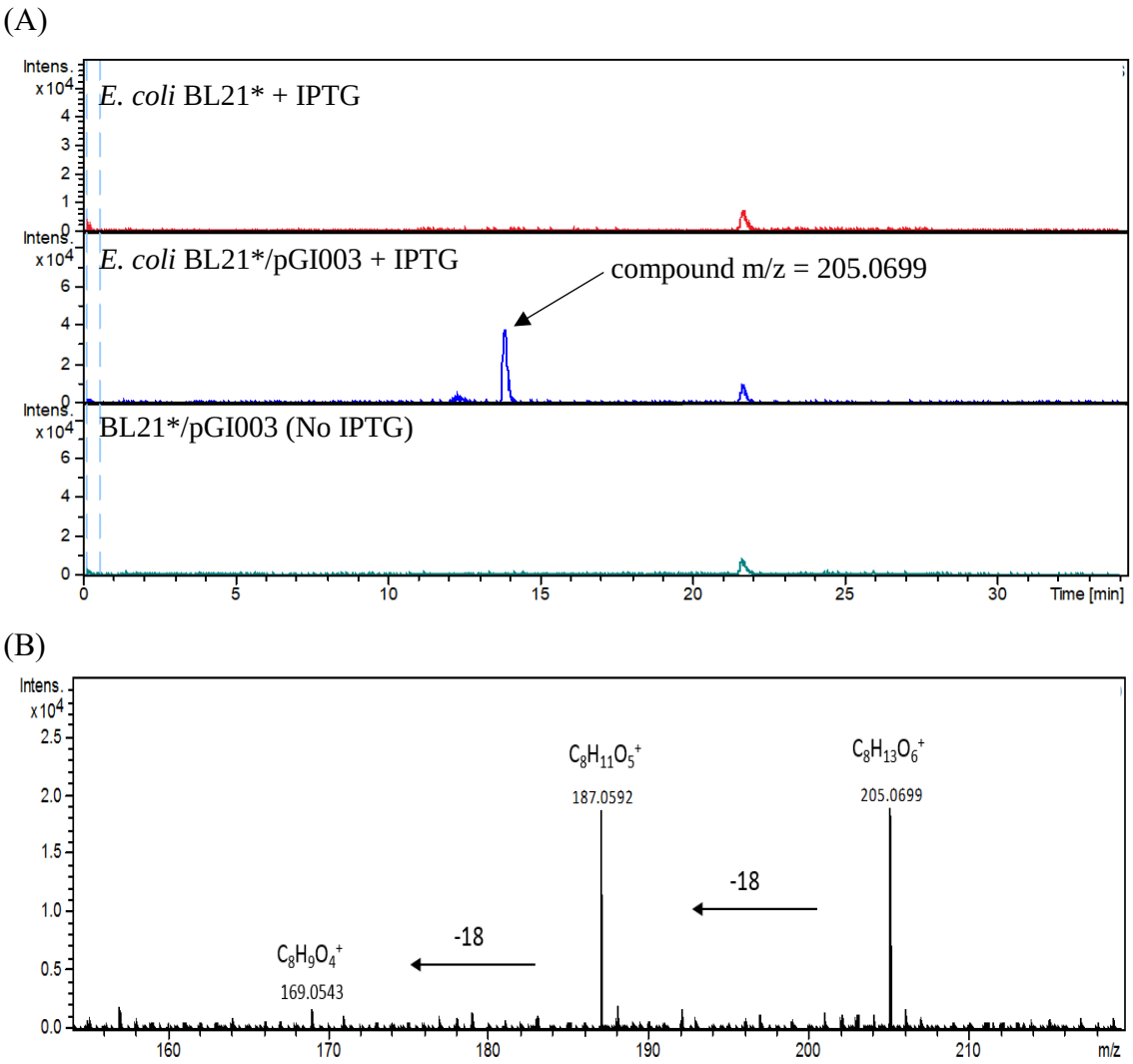


Fig. 2.15: LC-ESI-HRMS analyses of extracts from cultures of wild-type *E. coli* BL21* and *E. coli* BL21*/pGI003 strains (A) EICs at *m/z* = 205.0699 corresponding to $[C_8H_{13}O_6]^+$, produced only in the culture of *E. coli* BL21*/pGI003 to which IPTG was added (B) HR-MS data at 13.9 minutes showing the peak *m/z* 205.0699 and fragment ions resulting from loss of water molecules.

LC-MS analysis of the culture extracts revealed a compound with *m/z* $[M + H]^+$ of 205.0699, corresponding to $C_8H_{13}O_6^+$, produced in the presence of IPTG in the culture of BL21*/pGI003 (Fig. 2.15). The compound was absent in the induced culture of the wild-type *E. coli* BL21*, indicating that it forms due to *mmyD* overproduction in the strain carrying pGI003. The compound shows a serial loss of water molecules, similar to the putative butenolide (**25**) produced by the *S. coelicolor* transconjugant strains.

However, upon feeding of [U-^{13}C]D-ribose to a culture of BL21*/pGI003 containing IPTG and analysis of the extract by LC-ESI-MS, the result revealed increases in *m/z* value of the compound by only 2 and 6 mass units (Fig. 2.16), suggesting that the 8-carbon metabolite is derived from a 2-carbon and a 6-carbon precursor, in contrast to the incorporation patterns observed for the butenolide intermediate (**25**) and methylenomycin C (**17**).

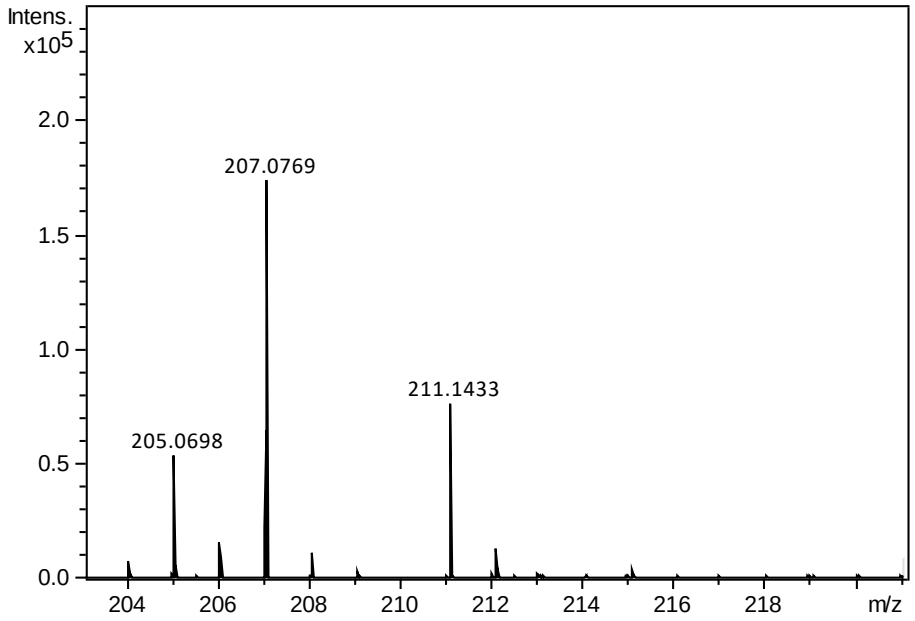


Fig. 2.16: HRMS data at 13.9 minutes following LC-MS analysis of extract of *E. coli* BL21*/pGI003 fed with [U-^{13}C]D-ribose. Two peaks representing increases in *m/z* value by approximately 2 and 6 units are seen in addition to the peak *m/z* 205.0698.

The results indicate that *mmyD* expression alone is not sufficient to produce **25** in *E. coli*, unlike its homologue, *avrD*, whose sole expression directly led to the production of the syringolides.[189] The results also suggest that the ketoacyl synthase III, MmyC (encoded in the methylenomycin cluster and required for the production of acetoacetyl-MmyA) is essential to the pathway and its action may not be substituted by KAS III from primary metabolic fatty acid synthase of *E. coli*, explaining why the butenolide (**25**) or other methylenomyin-related compound could not be detected in

this experiment. This may also account for why singular expression of *mmyD* in *S. coelicolor* (strain W117) or *S. albus* (strain W305) under the control of a strong constitutive promoter *ermE** did not lead to the production of any methylenomycin-related metabolite.

2.6 Construction of Δ*mmyQ* and Δ*mmyY* transconjugant strains *via In vitro* CRISPR-Cas9

In addition to the *mmy* enzymes discussed in the above sections, the cluster also encodes MmyQ, a coenzyme F420-dependent reductase, and MmyY, belonging to the superfamily of nuclear transport factor 2 (NTF2). *S. coelicolor* mutant strains lacking these individual genes were not previously constructed and it has not been investigated whether any methylenomycin intermediates accumulate in such mutants. These genes were therefore deleted to pave the way for metabolic profiling of the respective *S. coelicolor* transconjugant strains.

2.6.1 Principle of CRISPR-Cas9

The clustered regularly interspaced short palindromic repeats/CRISPR assisted protein (CRISPR/Cas) is a relatively new technique that exploits the innate immune abilities of bacteria to cleave foreign plasmids and viruses. This protective system has been reportedly found in about 40% of sequenced bacteria, with some having more than one CRISPR locus in their genome.[190,191]

Invading plasmids and DNA are first captured and integrated into the CRISPR locus as spacer sequences, separated by some repeat sequences which are highly conserved in the bacteria. These spacer sequences then function as templates to generate short CRISPR RNAs (crRNAs) which can pair with complementary DNA strand in the

invading molecule.[192] The CRISPR locus contains sequences for trans-activating RNAs (tracrRNAs) which forms a complex with the crRNAs and recruits it onto the Cas9 nuclease enzyme (Fig. 2.17).[192] The crRNA-tracrRNA complex then serves as a guide to direct the Cas9 nuclease to the target site on the foreign DNA where cleavage

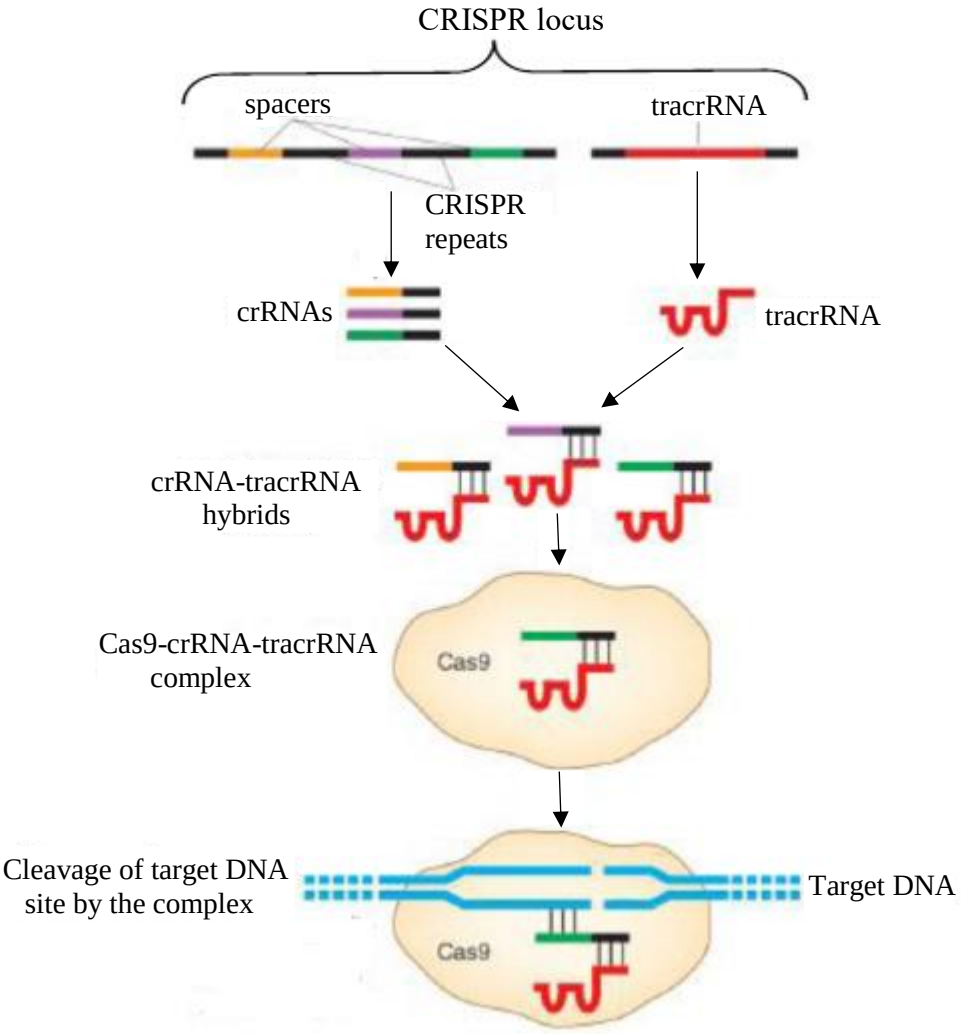


Fig. 2.17: Schematic depicting the mechanism of DNA cleavage by CRISPR/Cas9. CRISPR RNA (crRNA) generated using spacers from captured foreign DNA is combined with transactivating RNA (tracrRNA) from the CRISPR locus. The resulting hybrid associates with Cas9 nuclease and guides it to the target site on the invading DNA. Modified from Sander and Joung.[194]

occurs 3 or 4 base pairs upstream of a protospacer adjacent motif (PAM) sequence to give a double strand break.[193,194]

While the CRISPR/Cas system has been studied in a wide range of bacteria including *Escherichia coli*, *Solfolobus solfataricus*, *Haloquadratum walsbyi*, *Pseudomonas aeruginosa*, and many *Streptococcus* species,[195,196] the most widely used is the Cas9 nuclease derived from *Streptococcus pyogenes* (SpCas9). This cas9 nuclease has been shown to contain two catalytic domains, HNH and RuvC, for cleaving intruding DNA on the strand complementary to the crRNA and on the non-complementary strand respectively.[190,194] These nuclease domains are activated by the sequence: 5'-NGG-3', which is the PAM for the SpCas9 system and also provides a way to distinguish self from foreign DNA, preventing the cas9 from cutting within the *Streptococcus pyogenes*' CRISPR locus.[194]

The fact that *Streptomyces* genomes are rich in G and C nucleotides makes them particularly amenable to editing by SpCas9, as an 'NGG' sequence can be easily found near almost any region of interest.[192] 'N' in 'NGG' refers to any of the four nucleotide bases while 'G' is guanine.

In addition to its demonstrated use for targeting individual genes and genomic loci on bacterial chromosomes,[192,197] the Cas9 system of *Streptococcus pyogenes* has been used successfully to delete genes located on plasmids/cosmids in a variant of the technique regarded as *in vitro* CRISPR/Cas9-mediated editing (ICE).[198] This approach was employed in this study to delete *mmyQ* and *mmyY* from the cosmid C73_787/*mmyR*::*apr*.

2.6.2 sgRNA design and excision of *mmyQ* and *mmyY* by Cas9

Jinek and co-workers in 2012 made a major advance in CRISPR/Cas9 use when they showed that, instead of separate crRNAs and tracrRNA used naturally by bacteria, it is possible to fuse these two component RNA molecules into a single synthetic guide RNA (sgRNA), while still achieving excellent specificity of the target site.[199] Thus, with careful design of an approximately 20 nucleotide sequence upstream of an 'NGG' on a targeted sequence, it is possible to generate the required synthetic guide RNA (sgRNA) by extension PCR with the plasmid pCRISPomyces 2 as template.[192,203] Using the CRISPR design tool at (http://crispr.mit.edu), a pair of *ca.* 20 nucleotide sequences were selected to delete each of *mmyQ* and *mmyY* to generate in frame deletions. Fig. 2.18 shows the sequence of the primers designed and PCR amplification of the sgRNA first as DNA templates.

The generated DNA templates were transcribed to RNA (the actual sgRNAs) which were subsequently used to guide the Cas9 nuclease to delete *mmyQ* and *mmyY* on the C73_787/*mmyR*::*apr* cosmid (as detailed in the methods section 5.12.4). The Cas9 enzyme, in addition to its endonuclease activities, possesses exonuclease activity which trims the double strand break at the end without PAM of the non-complementary strand to give a sticky end, with up to 14 nucleotides randomly missing beginning from the cut site.[198, 200] This is undesirable as it may lead to unintended loss of sequences immediately 5' of the cut site in the cosmid and may affect the expression of elements encoded by the flanking sequences. Thus, the *mmyQ*-mutated and *mmyY*-mutated cosmids were repaired by treatment with T4 DNA polymerase to give blunt ends, which were subsequently self-ligated (as detailed in the methods section 5.12.5).

(A)

***mmyQ*-sgRNA-1 and *mmyQ*-sgRNA-2**

5′ GATCACTAATACGACTCACTATAGGGACGTACTGGTGTTGGTCATGTTTTAGAGCTAGAAATAGC 3′

5′ GATCACTAATACGACTCACTATAGGGTCGGCCGGAGTGAACATGTGGTTTTAGAGCTAGAAATAGC 3′

***mmyY*-sgRNA-1 and *mmyY*-sgRNA-2**

5′ GATCACTAATACGACTCACTATAGGGAGCTGTGCACCCGCATGGCGGTTTTAGAGCTAGAAATAGC 3′

5′ GATCACTAATACGACTCACTATAGGGCCGACGGGAACCGCCACATCGTTTTAGAGCTAGAAATAGC 3′

(B)

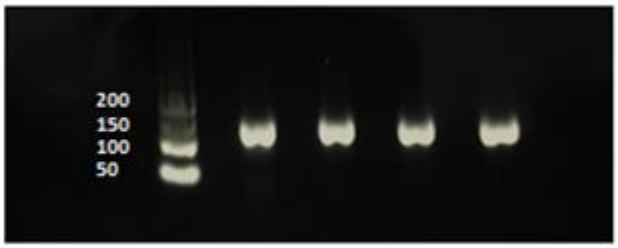


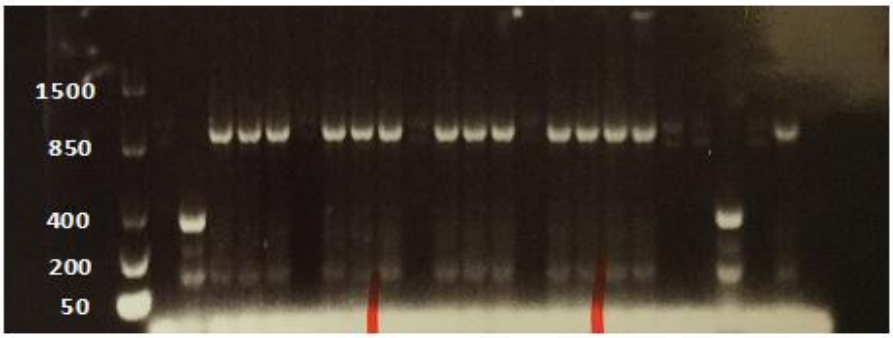
Fig. 2.18: Synthetic guide RNA (sgRNA) design and generation by extension PCR: (A) Sequences of the forward primers used to delete *mmyQ* and *mmyY*. Black: a protective sequence for the T7 promoter sequence (yellow). Green: the first and most essential half of the 6 nucleotide consensus initiation sequence for T7 promoter,[201, 202] the first base of which is the transcription start point. Blue: the 20 nucleotides crRNA sequence specific for each primer. Red: the sequence matching the plasmid (pCRISPomyces-2) segment flanking the trans-activating crRNA (tracrRNA) sequence. The reverse primer, sgRNA-rev, was the same for all the reactions (see methods section 5.12.1) (B) Gel from agarose electrophoresis separation following PCR amplification of the synthetic guide RNA (sgRNA) sequences as double–stranded DNA templates (Q1 and Q2 for *mmyQ*-sgRNA-1 and *mmyQ*-sgRNA-2 respectively, and Y1 and Y2 for *mmyY*-sgRNA-1 and *mmyY*-sgRNA-2 respectively).

Editing of cosmids by CRISPR/Cas9 would normally generate one or more copies of the mutated cosmid, in a mixture with many copies of the original cosmid. Thus, in order to identify the Δ*mmyQ* and Δ*mmyY* cosmids, aliquot from the ligation mixture was used to transform chemically-competent TOP10 *E. coli* cells, and colonies were selected on LB plates supplemented with apramycin. The colonies were passed through LB broth supplemented with apramycin at 37 ^{o}C overnight, and then served as templates in a PCR screening reaction with primers *mmyQ*-scrn-

fwd/*mmyQ*-scrn-rev and *mmyY*-scrn-fwd/*mmyY*-scrn-rev, annealing approximately 200 bp from the *mmyQ* and *mmyY* cut sites respectively (methods section 5.12.6). Fig. 2.19 shows the gel from electrophoretic separation of the PCR products, and the bands identifying the colonies containing the Δ*mmyQ* and Δ*mmyY* cosmids.

The mutated cosmids were purified by standard procedure from overnight cultures of the identified colonies and sequenced with the same pair of primers used for the screening in each case. Results from sequencing (shown in appendix 1) confirmed

(A)



(B)

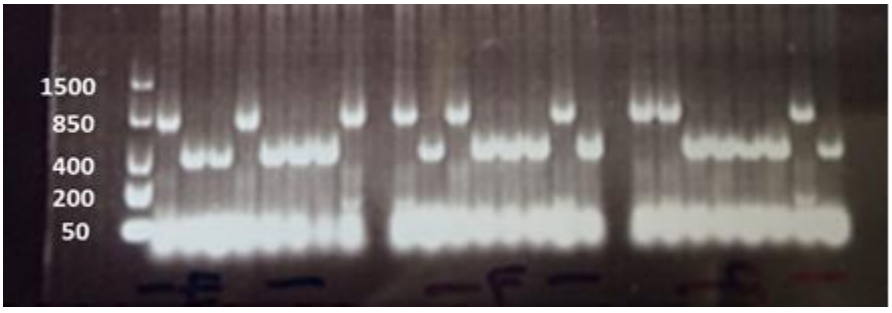


Fig. 2.19: gel from agarose electrophoresis separation following PCR amplification of *mmyQ* and *mmyY* in cultures of *E. coli* TOP10 transformed with CRISPR/Cas9-edited cosmids (A) Identification of C73_787/*mmyR*::*apr* cosmid lacking *mmyQ* (bands at *ca.* 400 bp). Copies of the original cosmid unedited by Cas9 are shown by bands appearing at *ca.* 1000 bp (B) Identification of C73_787/*mmyR*::*apr* cosmid lacking *mmyY* (bands at *ca.* 400 bp). Copies of the original cosmid unedited by Cas9 are shown by bands appearing at 850 bp.

the excision of *mmyQ* and *mmyY* on the cosmid while the flanking sequences remained intact, demonstrating the effectiveness of the CRISPR/Cas9 system in targeting only the intended DNA segment.

2.7 Transfer of the Δ*mmyQ* and Δ*mmyY* constructs to *S. coelicolor* M145 and LC-MS analysis of extracts from cultures of the resulting transconjugants

To examine whether MmyQ and MmyY are essential for the biosynthesis of the methylenomycin antibiotics, the Cas9-generated constructs, named pGI001 (for C73_787/*mmyR*::*apr*/Δ*mmyQ*) and pGI002 (for C73_787/*mmyR*::*apr*/Δ*mmyY*), were used to transform the non-methylating *E. coli* ET12567/pUZ8002. This was subsequently conjugated with *S. coelicolor* M145 to give W314 and W315 strains, lacking *mmyQ* and *mmyY* respectively. PCR analysis of the Δ*mmyQ* and Δ*mmyY* transconjugant colonies produced bands for *mmyD* (Fig. 2.20), encoding for the butenolide synthase essential for the methylenomycin pathway, thereby confirming the integration of the constructs into the M145 genome.

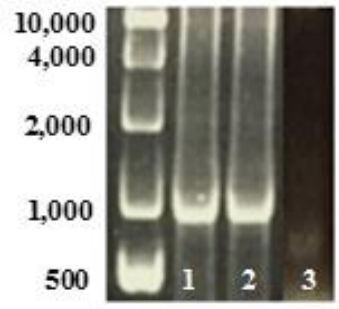


Fig. 2.20: gel from agarose electrophoresis separation following PCR amplification of *mmyD* in W314 (*S. coelicolor*/pGI001) and W315 (*S. coelicolor*/pGI002). 1- W314, 2- W315, 3- *S. coelicolor* M145, lacking the C73_787 cosmid. DNA size markers are in bp.

W314 and W315 strains were then fermented on SMMS and extracted with ethyl acetate. LC-ESI-HRMS analysis of extracts from the strains showed that only methylenomycin furan compounds could be detected in the extracts, while the

production of methylenomycin compounds was completely abolished (Fig. 2.21). Similar results were obtained with *S. albus* J1074 transformed with pGI001 and pGI002 (strains W316 and W317 respectively). The results suggest that both MmyQ and MmyY may play as yet unidentified roles in the biosynthesis of the methylenomycins. Indeed, possible steps involved in producing the methylemycin compounds from the putative butenolide intermediate (proposed in the concluding section 4.1.3) require the reduction of double bonds, and MmyQ, which is the only reductase encoded in the *mmy* cluster, may be required for these reductions. However, no methylenomycin-related intermediates were found to accumulate in the mutant strains, thus no further insight into their roles could be gained in the current study

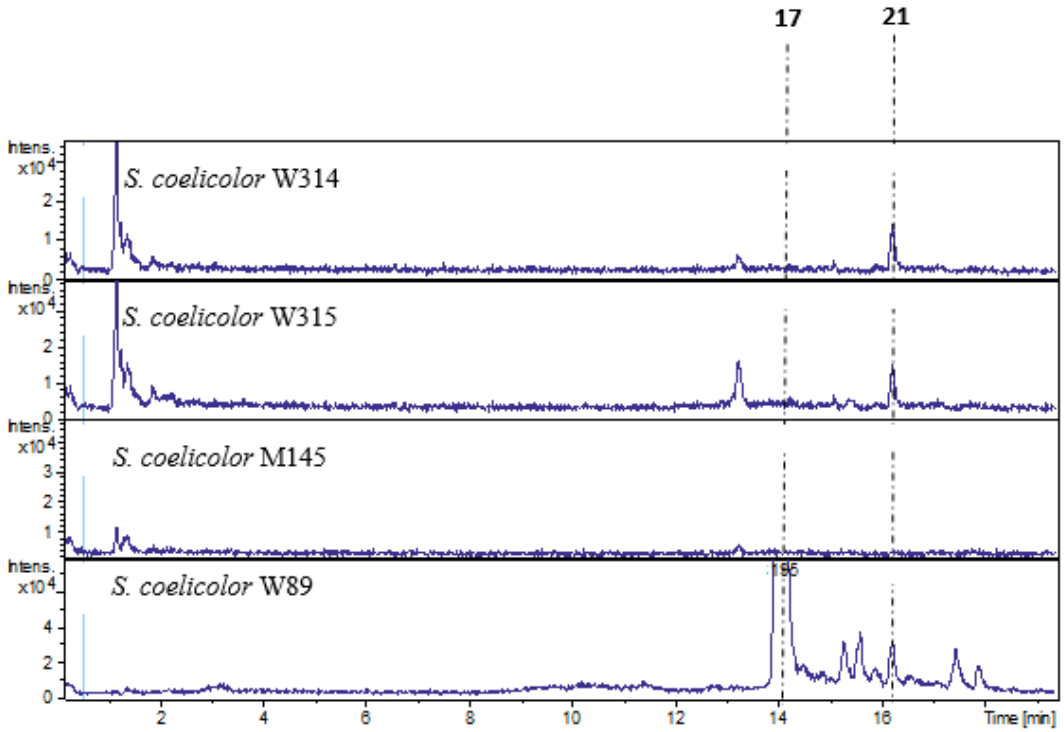


Fig. 2.21: EIC for *m/z* = 167.0700 corresponding to [M + H^+] for methylenomycin C (**17**) and *m/z* = 195.0800 corresponding to [M + H^+ - H_2O] for MMF3 (**21**), following LC-ESI-MS analysis of the culture extracts of *S. coelicolor* W314 (M145/pGI001), W315 (M145/pGI001), M145 and W89 (*mmyR* only mutant).

study. More work will be needed to demonstrate the role played by MmyQ in the biosynthesis of the methylenomycins, and that of MmyY (a nuclear transport protein) in the biosynthesis and/or regulation of the production of the methylenomy-cin antibiotics.

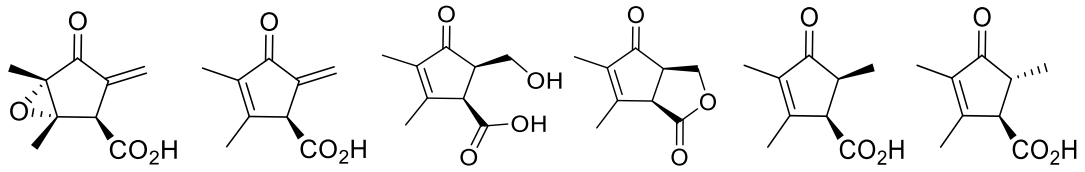
CHAPTER THREE

3. ANTIMICROBIAL ACTIVITIES AND MODE OF ACTION OF METHYLENOMYCINS AND INTERMEDIATES

3.1 Antimicrobial activities of the methylenomycins and intermediates

Methylenomycin A (MmA, **16**) had long been shown to exhibit activity against many Gram-positive bacterial strains and some Gram-negative ones, particularly the *Proteus* genus.[171] Efforts towards investigating the biosynthesis of this compound has led to the accumulation, in various *S. coelicolor* mutant strains, of intermediates and shunt products of the methylenomycin pathway. These include pre-methylenomycin C (P-MmC, **26**), pre-methylenomycin C lactone (P-MmCl, **27**), methylenomycin D1 (MmD1, **28**) and methylenomycin D2 (MmD2, **29**) (Fig. 3.1). As the biological activities of these newly characterised compounds had not been assessed, their antimicrobial properties were investigated as part of the current study. The penultimate compound of the pathway, methylenomycin C (MmC, **17**), was also investigated. The purification **26** and **27** is described in the methods section 5.14.3; purification of **28** and **29** is also described in section 5.14.4. Isolation of **17** was discussed in section 2.1.3 of chapter 2.

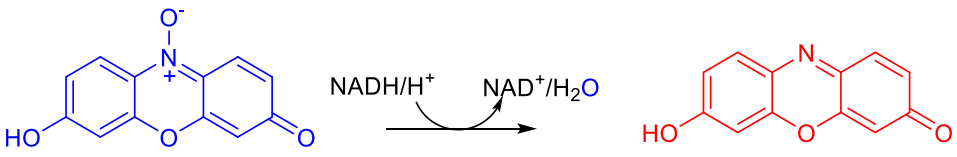
Activities of **17** and **26** – **29** were determined relative to the activity of MmA (**16**) against a range of Gram-positive and Gram-negative strains. The minimum inhibitory concentration (MIC) of each compound leading to no visible growth of the test strains was determined by a broth microdilution method in 96-well microtiter plates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (section 5.16.1).[205]



MmA (**16**) MmC (**17**) P-MmC (**26**) P-MmCl (**27**) MmD1 (**28**) MmD2 (**29**)

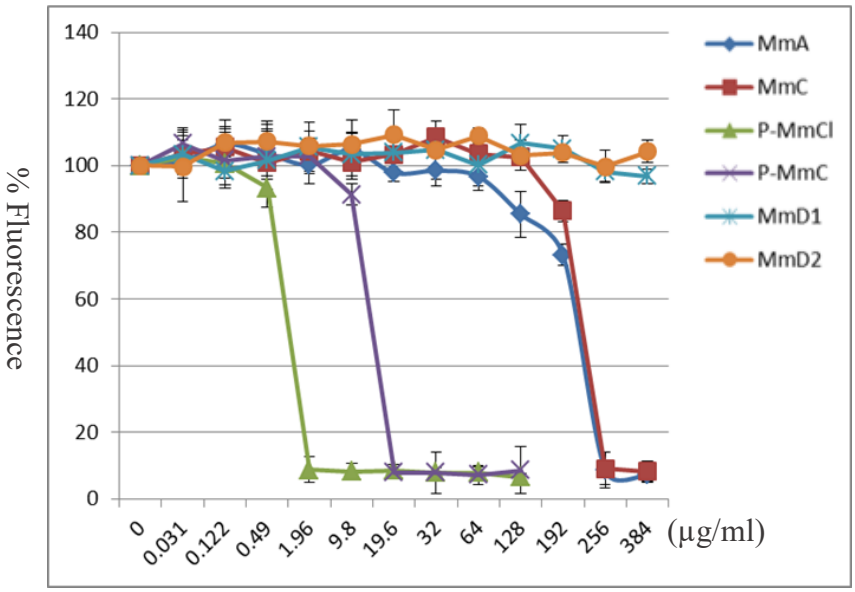
Fig. 3.1: Structures of methylenomycin A and intermediate/shunt products of the pathway investigated for their antimicrobial activities.

To determine the minimum bactericidal concentrations (MBC), a fluorescence-based microplate assay with AlamarBlue™ dye was employed.[206] Viable bacterial cells, due to the reducing environment maintained within their cytosols, irreversibly convert a non-fluorescent blue compound called resazurin (the active component of the AlamarBlue™) to a reduced and highly fluorescent red form, resorufin (Scheme 3.1).[207] The fluorescence generated is a measure of the amount of resorufin produced, which in turn depends on the population of viable cells in the culture. Incubation of test strains with increasing concentrations of an antimicrobial agent and addition of the AlamarBlue™ dye resulted in MBC values that correspond to a sharp drop in the Fluorescence-concentration curve. Fig. 3.2 exemplifies the plots obtained from AlamarBlue™ MBC assay for some Gram-positive strains and *Candida albicans*. Where necessary, additional tests were carried out within narrower concentration ranges. Complete killing of the strains was further confirmed by sub-culturing onto antibiotic-free agar plates and incubating for three days with no visible growth.

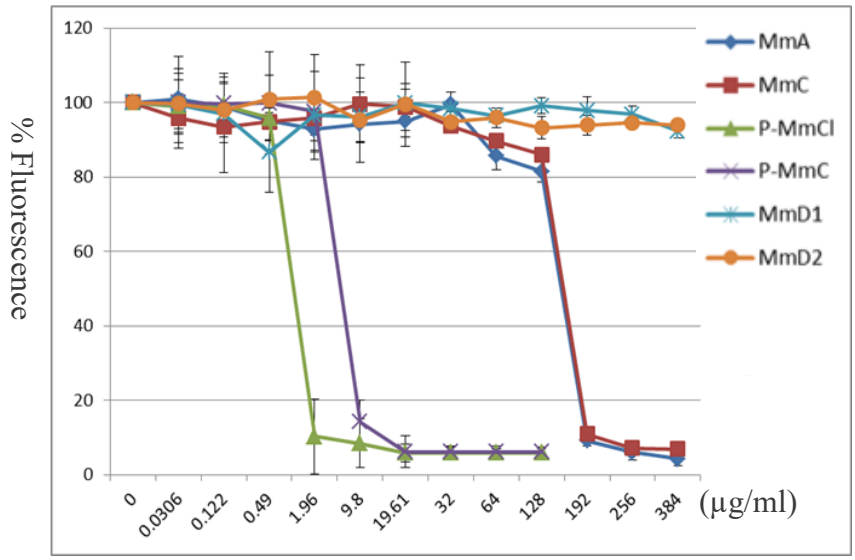


Scheme 3.1:Conversion of resazurin (the active component of AlamarBlue™ dye) to resorufin by viable cells.

Staphylococcus aureus R34



Bacillus subtilis M^T



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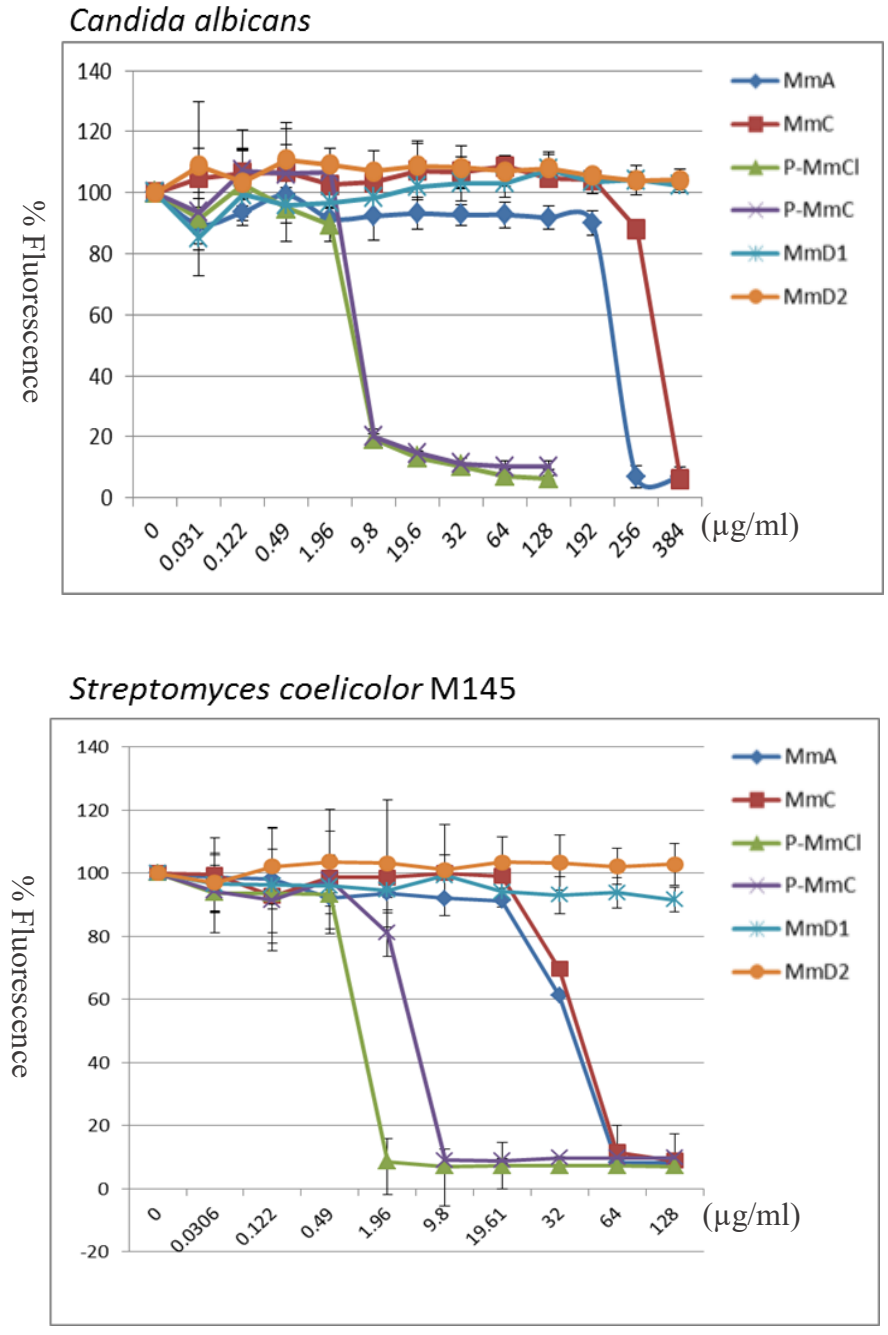


Fig. 3.2: MBC determination assays with AlamarBlue™ dye. The plots show dosage-dependent decrease in fluorescence due to cell death in test strains incubated with methylenomycin-related compounds. Vertical axis shows fluorescence values as % of drug-free controls; compound concentrations are in µg/ml. Data shown are representative of 12 replicates from 3 independent experiments.

Table 3.1 provides a summary of the final MIC and MBC values for all the compounds and strains investigated. Like MmA, all the intermediate/shunt compounds showed no activity against wild-type *E. coli*, *Pseudomonas*, *Klebsiella*, and other Gram-negative strains tested. MmD1 and MmD2 also showed no detectable antimicrobial activities up to a maximum of 512 μg/ml tested against the Gram-positive strains. However, MmC, not previously reported to have antibiotic activity, showed comparable efficacy to MmA in most of the Gram-positive strains, although both compounds only showed modest activity. Interestingly, the recently discovered intermediates, P-MmCl and P-MmC, showed much improved activities

Table 3.1: Activities of isolated methylenomycin intermediates/shunts compared to MmA

	MIC (MBC) in μg/ml					
Organism	MmA (**16**)	MmC (**17**)	P-MmCl (**27**)	P-MmC (**26**)	MmD1 (**28**)	MmD2 (**29**)
Gram-positive						
S. aureus DSM21979 (MRSA)	256(256)	512(512)	1(1)	16(16)	x	x
S. aureus R34	256(256)	256(256)	2(2)	16(16)	x	x
B. subtilis Marburg^T	192(192)	192(192)	1(1)	9.8(9.8)	x	x
S. coelicolor M145	64(64)	64(64)	1(1)	8(8)	x	x
S. albus J1074	256(256)	128(128)	0.5 (0.5)	0.5 (0.5)	x	x
E. faecium U0317	256(256)	512(512)	2(2)	32(64)	x	x
E. faecium 64/3	x	x	2(2)	8(16)	x	x
Yeast						
C. albicans SC 5314	256(256)	384(384)	9.8(64)	9.8(64)	x	x
Gram-negative						
E. coli SY327	-	-	-	-	-	-
P. aeruginosa DSM29239	-	-	-	-	-	-
K. pneumoniae DSM26371	-	-	-	-	-	-
S. plymuthica RVH1	-	-	-	-	-	-
R. mannitolilytica BCC1391	-	-	-	-	-	-
B. metallica DSM23519	-	-	-	-	-	-
B. ambifaria DSM16087	-	-	-	-	-	-

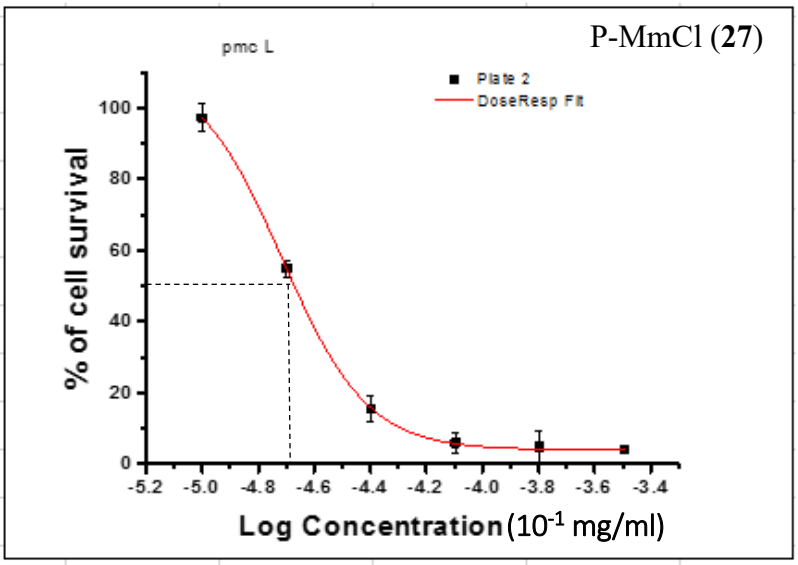
x (no activity up to 512μg/ml), - (no activity up to 128μg/ml)

compared to MmA and MmC. Whereas MmA and MmC were inactive against the methicillin-resistant *S. aureus* (MRSA) until a concentration of 256 and 512 µg/ml respectively, P-MmCl and P-MmC gave respective MICs of 1 µg/ml and 16 µg/ml against this pathogenic strain. P-MmCl and P-MmC are also very active against *Enterococci* strains, particularly the *E. faecium* strain 64/3 where these compounds gave MICs of 2 and 8 µg/ml respectively, representing at least 256 and 64 fold improved activities over MmA and MmC. Similar differences in potency between these two metabolites and MmA/MmC were also observed for the other Gram-positive strains and the yeast (*Candida albicans*).

The results indicate that P-MmCl is by far the most active of all methylenomycin-related compounds isolated to date. This compound showed greater potency against *Enteroccoci* species than vancomycin, an antibiotic that has been widely used for the treatment of enterococcal infections.[208] Whereas vancomycin only becomes bactericidal against *Enterococcus faecium* 64/3 and *Enterococcus faecium* U0317 at concentrations of 128 µg/ml (86 µM) and 64 µg/ml (43 µM) respectively, P-MmCl displayed MBC of 2 µg/ml (12 µM) against these two strains, representing up to a 7-fold improved activity compared to vancomycin. In addition, sequential passage of *E. faecium* 64/3 through increasing concentrations of vancomycin and P-MmCl over a period of 28 consecutive days (methods section 5.16.2) led to the generation of mutant colonies less susceptible to vancomycin (MIC 32 µg/ml), compared to the wild-type *E. faecium* 64/3 which is inhibited by vancomycin at 4 µg/ml. The same effect was not observed with P-MmCl where *E. faecium* 64/3 was still susceptible to 2 µg/ml after 28 days. The inability of the *E. faecium* 64/3 strain to develop resistance against P-MmCl over such a period of passage suggests a potential for this compound in the treatment of enterococcal infections.

However, tests of the activities of P-MmCl and P-MmC against ovarian cancer cells, shown by the dose-response plots in figure 3.3, reveal that P-MmCl gave IC50 corresponding to a final concentration of 4 µg/ml, while P-MmC gave IC50 corresponding to a final concentration of 12 µg/ml. The IC50 value for P-MmCl is very close to the concentrations at which the compound inhibits the pathogenic bacte-

(A)



(B)

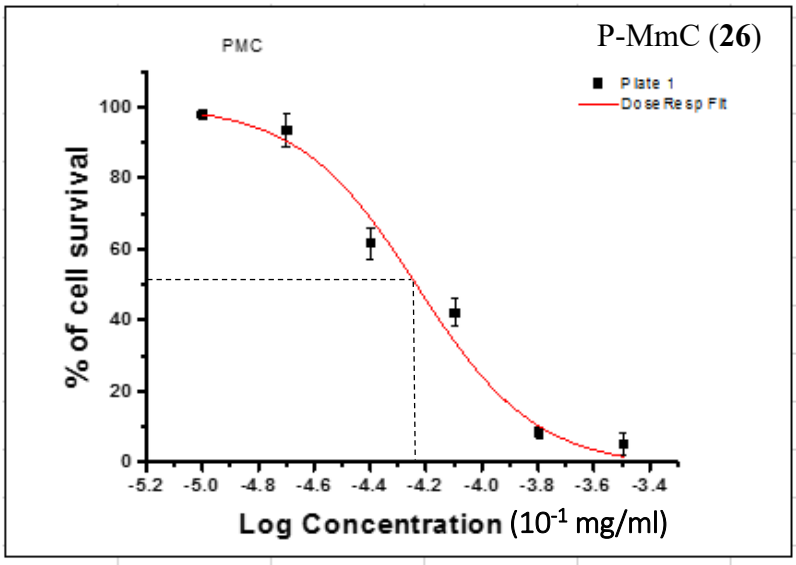


Fig. 3.3: Dose-response curves of activities of pre-methylenomycin C lactone (**27**) and pre-methylenomycin C (**26**) against ovarian cancer cell line. The concentration at which only 50% of the cells survive, IC50 (shown by broken lines), corresponds to a final concentration of 4 µg/ml for **27** (A) and 12 µg/ml for **26** (B) (Data kindly recorded by Isolda Canelon).

-rial strains. The IC50 for P-MmC is also within the range of concentrations at which the compound kills the Gram-positive strains. These results thus suggest that both P-MmCl and P-MmC may be cytotoxic at about the same concentrations as they are bactericidal.

3.2 Does *mmr* expression confer resistance against pre-methylenomycin C (26) and pre-methylenomycin C lactone (27)?

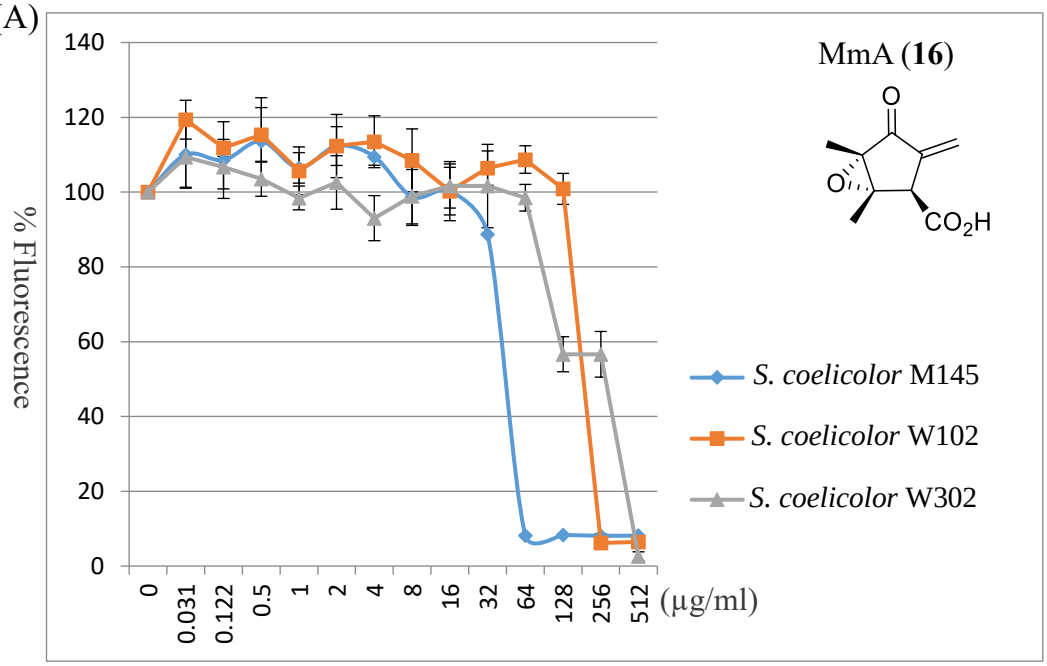
Antibiotic resistance genes present in antibiotic-producing strains can be transferred to other bacteria *via* horizontal gene transfer, HGT, often leading to the development of antibiotic resistance by otherwise susceptible pathogenic strains.[209, 210] As discussed in chapter 2 (section 2.1.2), expression of the methylenomycin resistance gene, *mmr*, was shown to confer resistance in the *S. coelicolor* M145 strain, enabling feeding experiment to be conducted with methylenomycin C (**17**), consequently leading to its turnover to MmA (**16)**. Given the much improved antimicrobial activity of P-MmC (**26**) and P-MmCl (**27**), it was interesting to investigate whether *mmr* could confer resistance against these potent methylenomycin derivatives.

Antibiotic activities of **26** and **27**, alongside those of **16** and **17**, were therefore investigated on *S. coelicolor* strains expressing multiple copies of *mmr* per cell and on strains expressing a single copy of the determinant, present on a cosmid integrated into the genome of *S. coelicolor* M145. W302 (M145/pIJ86/*mmr*) is a methylenomycin-resistant strain constructed by transforming *S. coelicolor* M145 (SCP1$^-$) with the construct pIJ86/*mmr*, in which the resistance gene is carried on the multicopy plasmid under the control of a strong constitutive promoter, *ermE** (section 2.1.1). W102 is an *S. coelicolor* M145 strain carrying the entire methylenomycin gene cluster except the transcriptional repressor gene, *mmyR*, and the putative kinase gene,

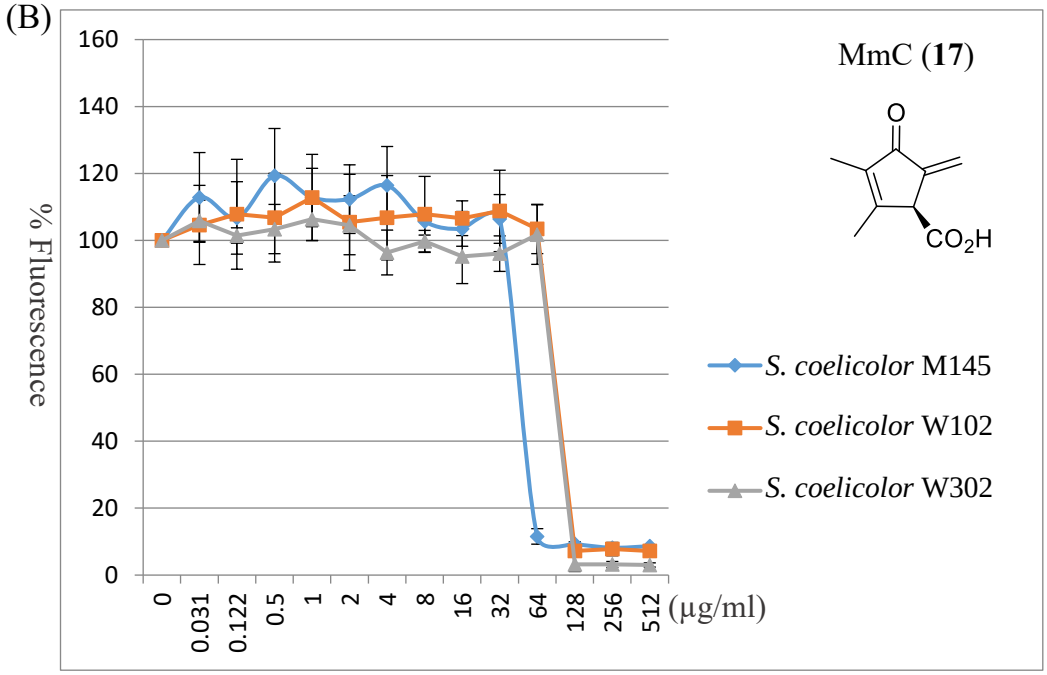
mmyX. While the lack of *mmyX* in W102 strain causes the abolition of methylenomycin production (as shown in section 2.3), it offers an *S. coelicolor* M145 strain with only one copy of the *mmr* gene per cell. The three strains M145, W102 (M145/C73_787/Δ*mmyX*/*mmyR*::*apr*) and W302 (M145/pIJ86/*mmr*) were therefore incubated overnight in Mueller-Hinton broth and diluted in the same medium to approximately 10^5 CFU/ml. The minimum concentration of **16**, **17**, **26**, and **27** required to kill each of the strains was then determined using the Alamar Blue™ assay.

Figure 3.4 (A to D respectively) shows fluorescence data obtained as a function of increasing concentrations of **16**, **17**, **27** and **26** incubated with the three *S. coelicolor* strains M145, W102 and W302. As expected, the data show that resistance is conferred against MmA (**16**) and MmC (**17**) in the *S. coelicolor* strains harbouring either the single copy or the multiple copies of the resistance gene. The MBC of **16** increased from 64 µg/ml in the wild type M145 to 256 µg/ml in W102 (M145/C73_787/Δ*mmyX*) and 512 µg/ml in W302 (M145/pIJ86/*mmr*) (Fig. 3.4A). In the case of **17**, the MBC only increased slightly from 64 µg/ml in M145 to 128 µg/ml in both the strains expressing *mmr* (Fig. 3.4B). Interestingly, however, neither strain M145/pIJ86*mmr* nor M145/C73_787/Δ*mmyX* required concentration of P-MmCl (**27**) and P-MmC (**26**) above that which killed the wild type strain M145 - both the transconjugant strains carrying the resistance determinant as well as the wild type *S. coelicolor* M145 were killed at approximately 0.5 µg/ml by **27** and at 8 µg/ml by **26** (Fig. 3.4C and D). The results indicate that *mmr* expression confers greater resistance against **16** than against **17**, while it has no effect on the activities of **26** and **27**.

(A)



(B)



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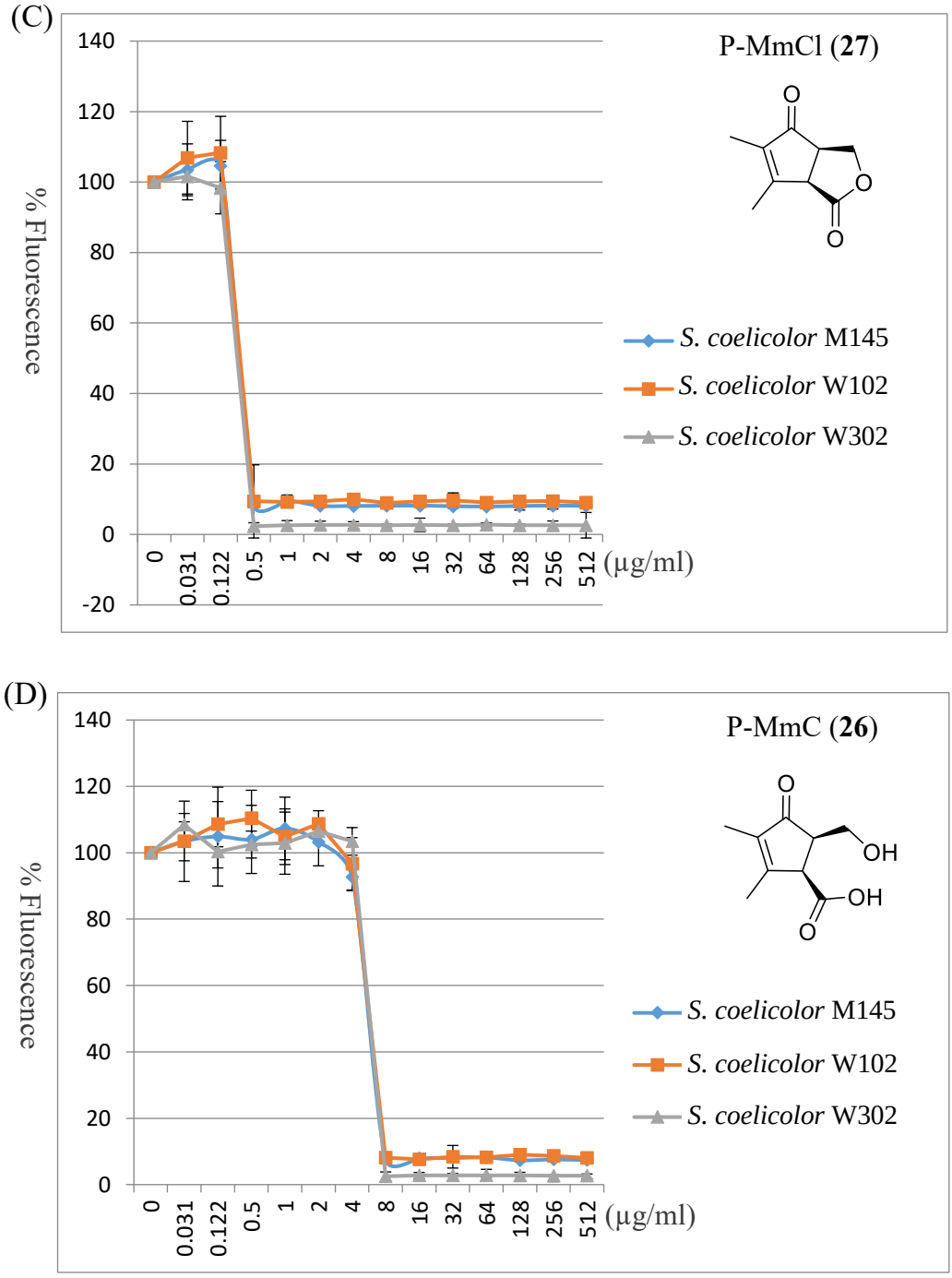


Fig. 3.4: Effect of expression of methylenomycin resistant determinant (*mmr*) on the activities of **16**, **17**, **27** and **26**. MBCs of **16** were raised significantly in strains harbouring *mmr*, compared to the wild type M145 (A); MBCs of **17** only increased slightly (B). MBC values of **27** and **26** were unaffected by *mmr* expression (C and D respectively). Data shown are averages of 9 replicates from 3 independent experiments. Error bars represent standard deviation values.

The indication that Mmr significantly reduced the antibiotic effect of **16** but only modestly affect the activity of **17** may probably explain why **17** is rapidly metabolised (*via* the saturation of the double bond at C-2) to the mixture of inactive diastereosiomers (MmD1, **28** and MmD2, **29**). Although **16** also possesses the same exomethylene function at C-2, no similar reduced compounds analogous to **28** and **29** (derived from **17)** were present in the extracts of the *S. coelicolor* strains overproducing **16**. Thus, the conversion of **17** to **28** and **29** may be an additional mechanism to prevent the antimicrobial effect of **17**, while Mmr may be sufficient to protect the strain against the activity of **16**, the final metabolite of the pathway.

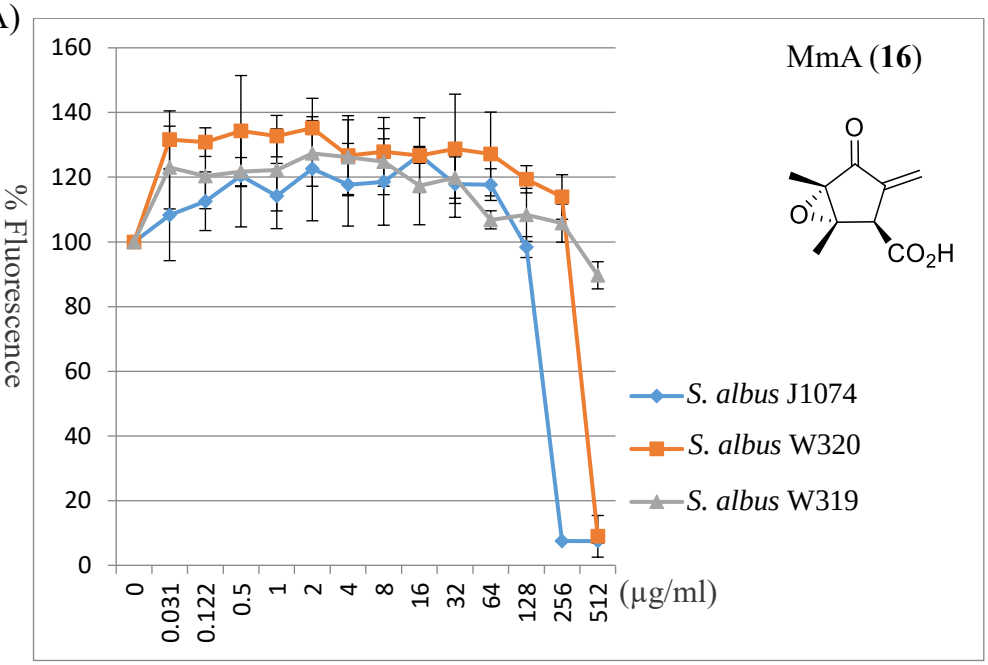
These results appear to be corroborated by a recent study within the group. It was previously proposed that MmyJ, an ArsR-like protein encoded within the methylenomycin cluster, could bind to the intergenic DNA sequence between *mmyJ* and *mmr* (containing the promoter region for the two genes), thereby preventing the transcription of *mmr*.[157, 159] Matt Lougher, a former PhD student in the Corre group, recently showed that MmyJ did bind to this intergenic region as envisaged. In addition, it was shown *via* electrophoretic mobility shift assay (EMSA) that **16** caused the full release of MmyJ from the MmyJ-DNA complex, and **17** caused partial release, while **27** had no effect on the MmyJ-DNA complex.[158] The study concluded that **16** was the main ligand sensed by MmyJ, indicating that the onset of production of **16** in *S. coelicolor* causes MmyJ to be released from the DNA (promoter region of *mmr*), eliciting expression of the methylenomycin resistance determinant to trigger the self-resistance mechanism of the producing strain.[158] The fact that **17** only causes partial release of MmyJ from DNA (i.e. not able to trigger the full expression of *mmr*) may be consistent with the result shown here which indicates that Mmr only confers partial

resistance against **17**. The results obtained with the strain carrying the construct pIJ86/*mmr*, where *mmyJ* is lacking, also suggest that the efflux protein, MmR, might bind and transport more specifically MmA (**16**) than MmC (**17**), while it does not transport P-MmC (**26**) and P-MmCl (**27**).

The complete inability of **27** to release MmyJ from DNA and cause the concomitant expression of *mmr* implies that the expression of this gene may not be the mechanism by which the producing strain protects itself against **27** (and probably **26**), explaining why Mmr could not confer resistance against **26** and **27** in the current study. Since **26** accumulates in isolable amounts in *S. coelicolor* (M145/C73_787/ Δ*mmyE*/*mmyR*::*apr*) and the strain is not killed in the process, the above results suggest that an alternative mechanism might be present in the producing strain to avoid suicide from the antibiotic effect of **26** (**27** is a semisynthetic derivative of **26** generated by treatment with acid).

More interestingly, the results suggest that a possible acquisition of the *mmr* gene by other bacterial strains, for instance *via* HGT, is not likely to confer resistance on them against these two potent methylenomycin-derived compounds. This was further demonstrated by transforming the wild type *S. albus* J1074 with the constructs pIJ86/*mmr* and C73_787/Δ*mmyX*, to give strain W319 (*S. albus* J1074/pIJ86/*mmr*), expressing multiple copies of the resistance gene, and W320 (*S. albus* J1074/C73_ 787/Δ*mmyX*), expressing a single copy of the gene per cell, respectively. Determination of MBCs of **16**, **17**, **26** and **27** on the strains revealed that the MBC of **16** was raised in the *S. albus* derivative strain expressing either multiple or single copy of the resistance gene, while that of **17** was raised only in the W319 strain (*S.albus*/pIJ86/*mmr*), expressing many copies of the gene (Fig. 3.5 (A) and (B)). In

(A)



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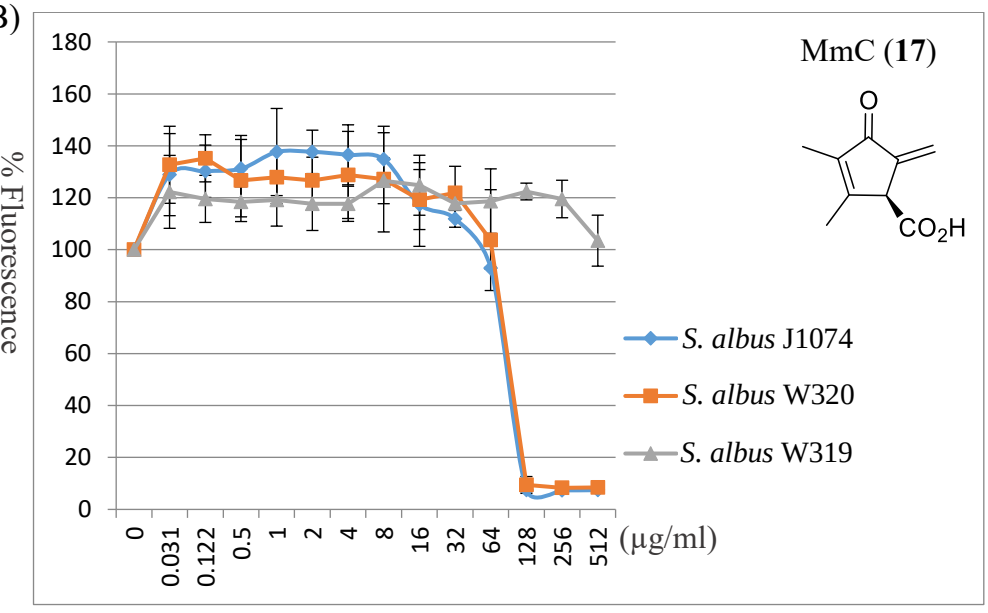

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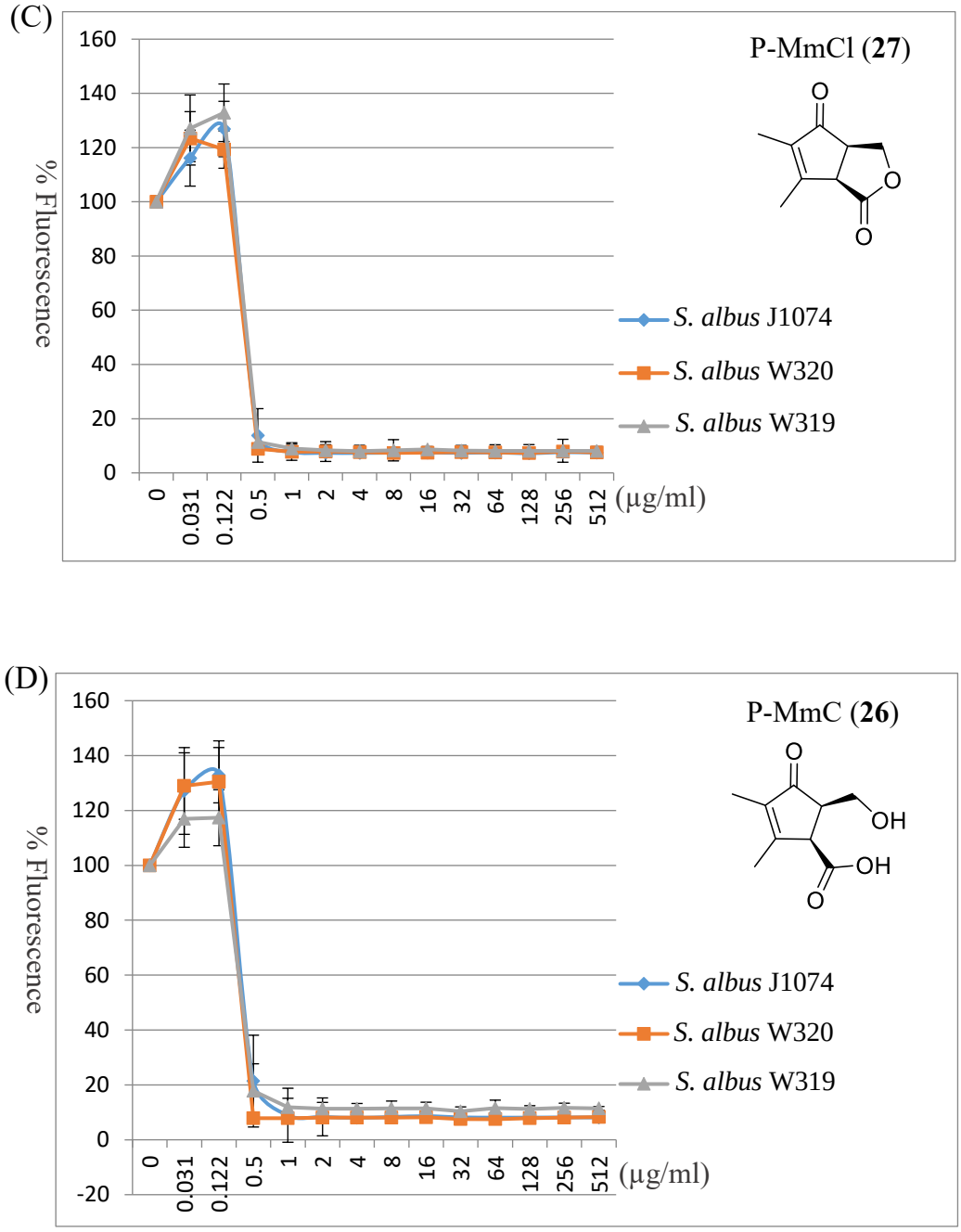


Fig. 3.5: Minimum bactericidal concentration (MBC) determinations for **16** (A), **17** (B), **27** (C) and **26** (D) against the wild-type *S. albus* J1074 and its derivative strains, W319 (*S. albus*/pIJ86/*mmr*) and W320 (*S. albus*/C73_ 787/Δ*mmyX*).

both cases, the result confirms that the expression of *mmr* conferred resistance against **16** and **17** on the otherwise susceptible *S. albus* J1074 strain, particularly in the derivative strain expressing multiple copies of the gene under the strong *ermE** promoter. However, the MBC of P-MmC (**26**) and P-MmCl (**27**) remained the same

(at 0.5 μg/ml) against the two derivative strains, as for the wild type *S. albus* J1074 (Fig. 3.5 (C) and (D)). These results demonstrate that the methylenomycin resistance protein, Mmr, has no effect on the antimicrobial activities of **26** and **27**, and strongly indicate that a possible acquisition of the corresponding gene by pathogenic bacteria may not confer immunity on them, against these two potent methylenomycin analogues.

3.3 Investigations into the mode of action of methylenomycin antibiotics

Despite its discovery over four decades ago, the mechanism of action of MmA (**16**) has not been investigated. Knowledge of the mode of action of antibiotics is generally desirable in view of rational drug design in which multi-hurdle drugs (incorporating moieties killing bacteria using different strategies) are developed, and in combinatorial therapies in which an auxiliary drug (acting differently from a main drug) is administered to enhance the effectiveness of a major drug.[213]

MmA was proposed to act as a DNA-alkylating agent, by forming covalent bonds to nitrogen or oxygen atoms in DNA bases, thereby inhibiting DNA replication in bacteria.[252] However, there is no experimental evidence to support this hypothesis. It is also inconsistent with the results from the current study (section 3.1) which have shown that MmC (**17**), pre-MmC (**26**) and pre-MmCl (**27**), all possess antimicrobial activities, despite lacking the epoxide functionality which may be critical to DNA binding in MmA (**16**). Thus, it was considered necessary to investigate the mode of action of **16**, along with those of the intermediates in the pathway which have shown antimicrobial activities in the current study. The mode of action study was carried out with two sets of promoter-reporter fusion strains: the first uses firefly luciferase as the reporter gene while the second incorporates a β-galactosidase gene. Further insights

were then obtained by testing the methylenomycin-related compounds against *E. coli* strains defective in the outer membrane permeability and in the efflux pump system.

3.3.1 Mode of action studies with firefly luciferase reporter strains

There are at least five major targets for antibacterial agents and they form the basis for the broad classification of the mechanism of action of antibiotics. These essential biosynthetic pathways/targets are DNA synthesis (or replication), RNA synthesis (or transcription), protein synthesis (or translation), cell wall biosynthesis and fatty acid biosynthesis.[12,260] Using the so-called reference compendium of antibiotic-triggered mRNA expression profiles, Freiberg and co-workers [211, 212] have identified specific promoter regions in *B. subtilis* which are selectively induced by antibiotics killing bacteria by inhibiting these individual pathways. The promoters of the genes *yorB*, *yvgS*, *yheI*, *ypuA*, and *fabHB* were identified as being specifically induced by substances inhibiting DNA synthesis, RNA synthesis, protein synthesis (translation), cell wall (including cell envelope stress) and fatty acids biosynthesis respectively.[211-213]

By cloning each of the identified promoter regions in front of a firefly luciferase reporter gene, Freiberg and co-workers generated promoter-reporter fusion constructs which were transformed into *Bacillus subtilis* 1S34. Each of the resulting strains thus constituted a biosensor for antibiotics interfering with one of the targets.[213] The biosensors have indeed been validated with a library of 14,000 compounds, including many antibiotics with well-defined mode of actions. Each biosensor was induced only by antibiotics that kill bacteria by inhibiting the respective pathway, while antibiotics with different mechanisms of action were not able to induce a given biosensor, or they generate signals below the set threshold.[213]

The methodology used followed the previously published assay procedure,[213] with only slight modifications (96-well plates were used in this study instead of 384-well plates due to template compatibility for luminescence instrumentation). All the *B. subtilis* strains carrying the different promoter-reporter fusion constructs, except *yheI*, were grown in liquid LB medium containing 5 µg/ml erythromycin to an O.D at 600 nm of 0.9 at 37 ^{o}C. The *yheI* biomarker was grown in Belitzky minimal medium (composition in section 5.6). The strains were then diluted to an O.D at 600 nm of 0.01 (*yorB*), 0.02 (*yvgS*), 0.1 (*yheI*), 0.02 (*ypuA*) and 0.25 (*fabHB*) and were kept at 4 ^{o}C overnight. Subsequently, 80 µl of each strain was incubated with 1 µl of methylenomycin compounds or 1 µl of a standard antibiotic, confirmed to be an inhibitor of the respective pathway and therefore serves as positive control for each strain. The standard antibiotics respectively inducing the *yorB*, *yvgS*, *yheI*, *ypuA* and *fabHB* were ciprofloxacin, rifampin, linezolid, vancomycin and cerulenin. Both the methylenomycin compounds and the standard compounds were dissolved in DMSO and were tested at three different sub-inhibitory concentration levels: 0.02, 0.08 and 0.3 µg/ml. Each strain was also incubated at the same time with 1 µl DMSO as the non-induced control. Citrate buffer containing luciferin was then added to each well and luminescence was measured (see details in the methods section 5.16.3). Fig 3.6 shows a sample of the plate used and the appearance of luminescence due to antibiotic-induction of the promoter in one of the biosensor strains.

The elicitation of luminescence in the assays may be explained by induction of the specific promoter in the biosensor strain (antibiotic-stress inducible), consequently leading to the strong expression of the firefly luciferase gene cloned downstream of each of the promoters. Once expressed, the resulting luciferase enzyme catalyses a reaction between molecular oxygen and luciferin, present in the citrate buffer, leading

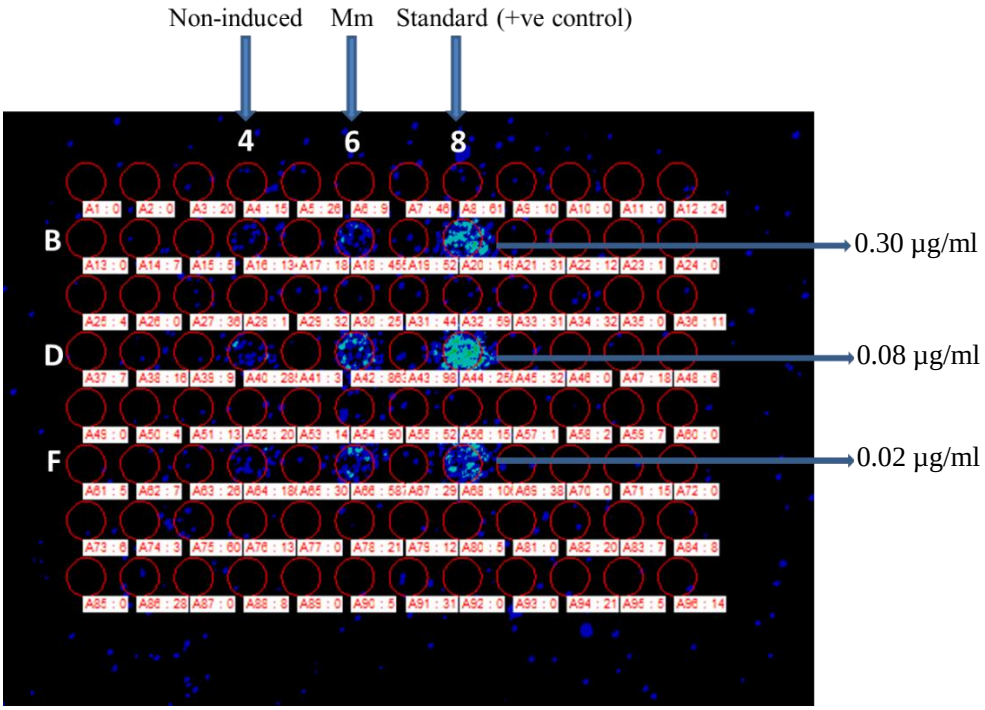
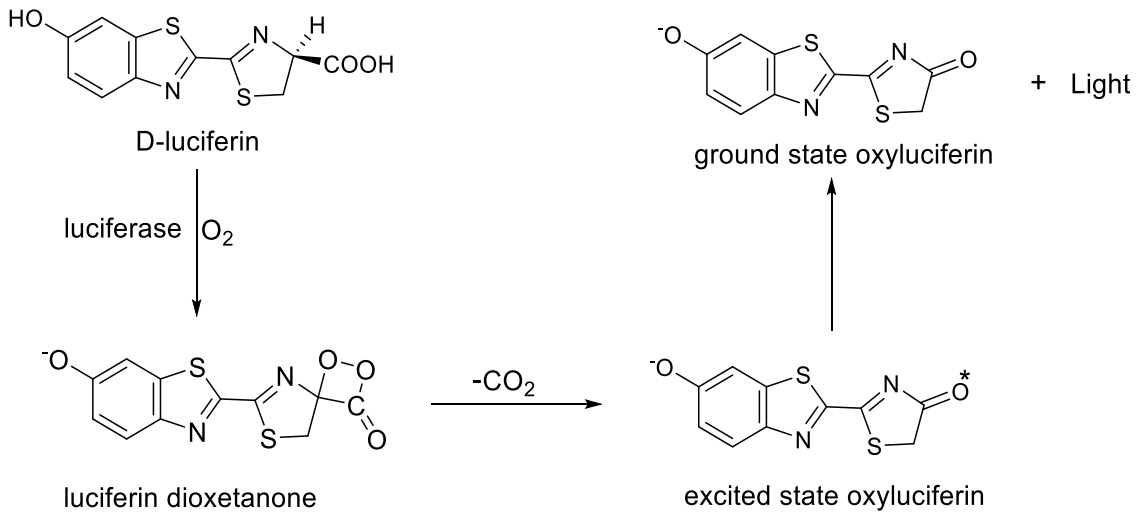


Fig. 3.6: Annotation of a sample of 96-well plate showing the bioluminescence produced by the *Bacillus subtilis* strains carrying the promoter-luciferase fusion constructs. Three samples in the column labelled 4 (B4, D4, F4) were non-induced controls, with only DMSO added to a biosensor strain. A methylenomycin compound (Mm) and a standard antibiotic were added respectively to the columns labelled 6 and 8 to give the final sub-inhibitory concentrations shown in the figure.

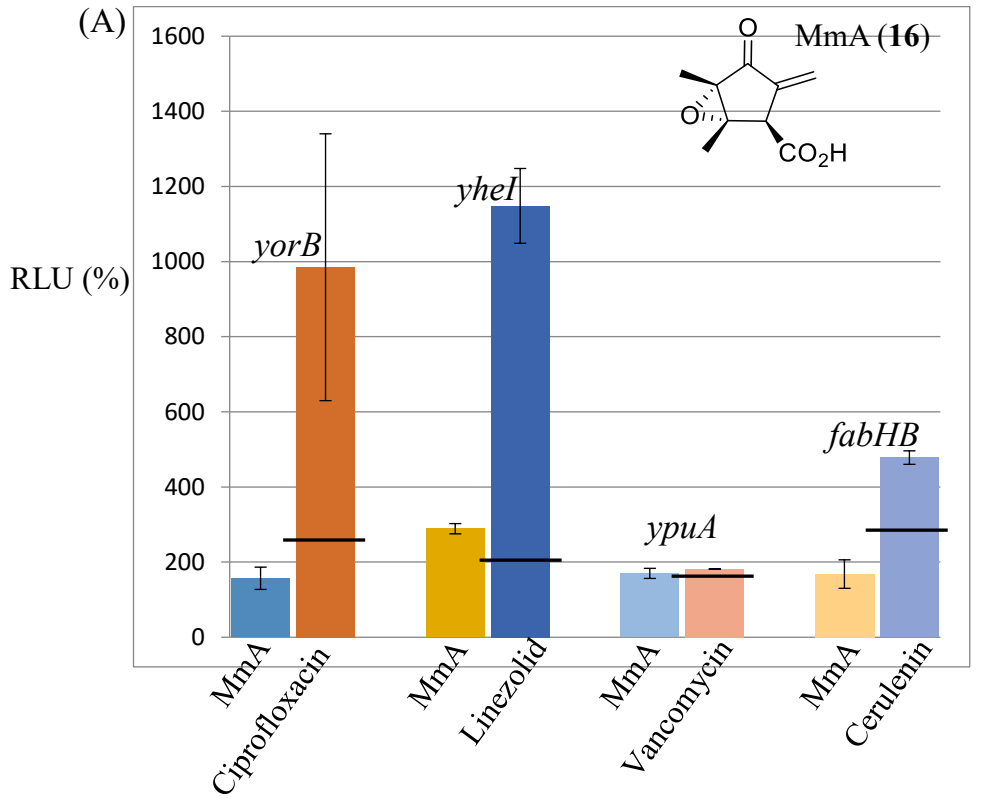
to the formation of a luciferin dioxetanone intermediate. This intermediate is highly energetic and unstable, due to the presence of a strained four-membered ring and a weak peroxide bond (scheme 3.2). Cleavage of this ring thus occurs spontaneously, with the release of energy which is efficiently directed towards the production of an electronically excited state in the resulting product, oxyluciferin.[253] Subsequent rapid relaxation of the excited state oxyluciferin to the ground state is accompanied by the release of excess energy as light photons, which is detected and measured in the assay. The greater a compound is able to induce a promoter, the more the luciferase enzyme is expressed, the more the amount of the energetic dioxetanone intermediate generated, and subsequently the higher the luminescence signal observed.

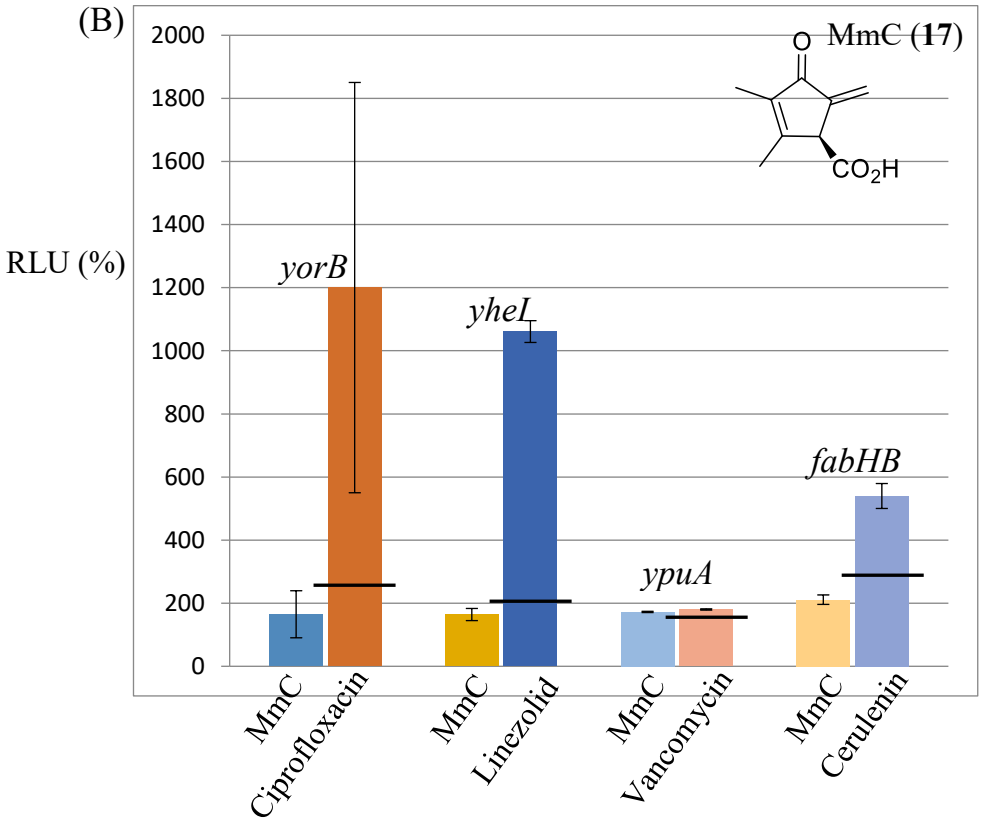


Scheme 3.2: Luciferase-catalysed reaction between molecular oxygen and luciferin, ultimately leading to the emission of light photons. (*) denotes an electronic excited state.

The decision of which of the major pathways (or biosensors) is ultimately inhibited by a test compound depends on comparison of the luminescence produced by the compound to that generated by standard antibiotics. Based on the assay development, a minimum signal of 250%, 200%, 200%, 170%, and 280% above the drug-free control is required respectively for *yorB*, *yvgS*, *yheI*, *ypuA*, and *fabHB* biosensors, in order for a test compound to be adjudged as an inhibitor of the given pathway.[213,214]

Luminescence data obtained with methylenomycin antimicrobial compounds and the standard antibiotics are presented in Figure 3.7. The data show that there is no significant induction of the *fabHB* and *yorB* biosensors by any of the four compounds, indicating that they do not inhibit fatty acid biosynthesis, or DNA replication as previously conceived. Three of the compounds (MmA, P-MmC and P-Mmcl) gene-





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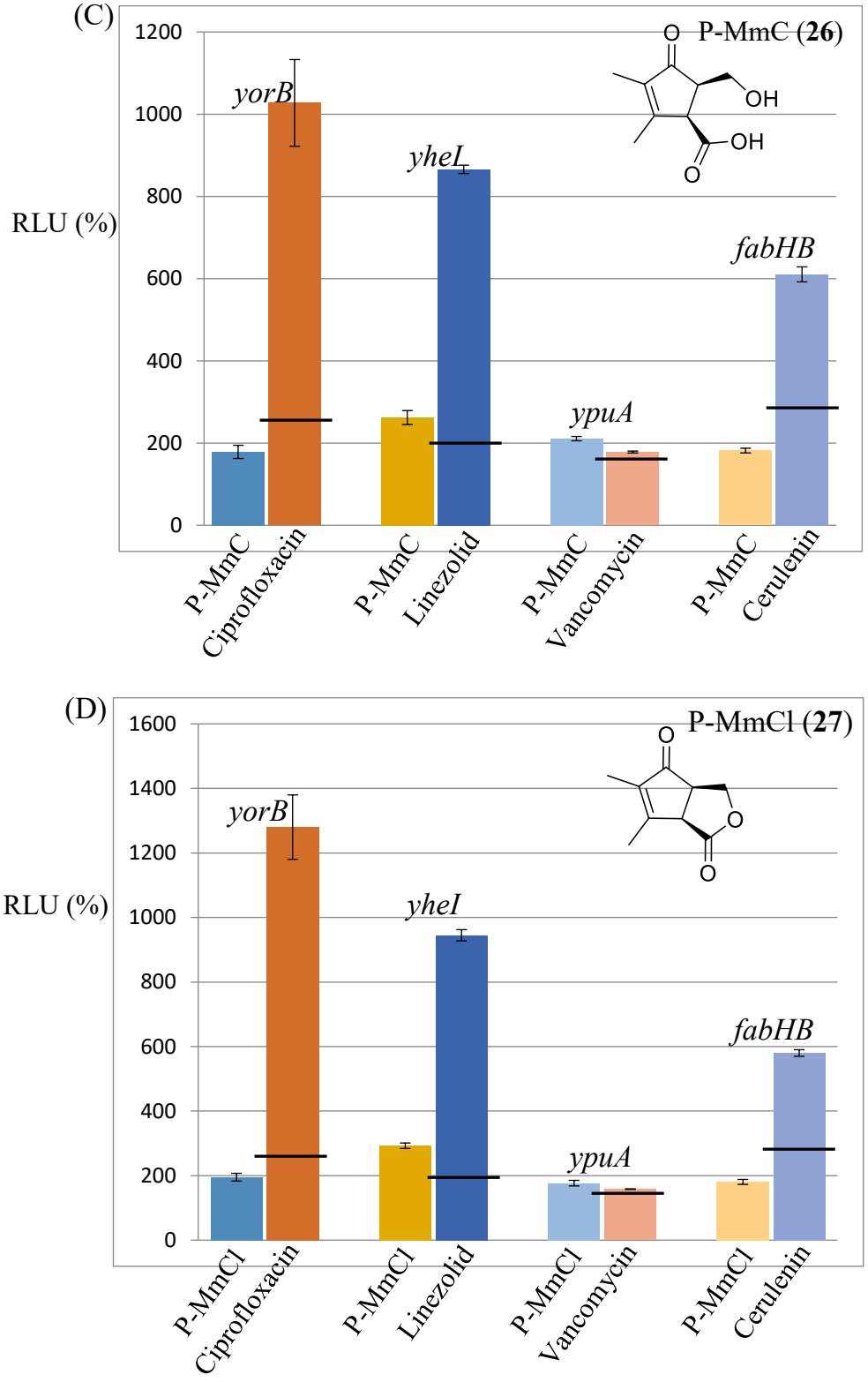


Fig. 3.7: Induction of the pathway-specific luciferase biosensors by MmA (A), MmC (B), P-MmC (C) and P-MmCl (D), compared with induction by the standard antibiotic for each pathway. Data shown are averages of 5 independent measurements calculated as % of the non-induced signal of the drug-free control. Black horizontal line cutting across each standard represents the minimum threshold that must be met by a test compound. (RLU – Relative Light Unit). Note: No data could be obtained for the *yvgs* promoter; the corresponding biosensor did not generate any response, even with the positive control.

rated signals in the *yheI* biosensor (marker of protein synthesis inhibition) above the 200% minimum threshold for a compound believed to be interfering with translation. However, the induction of the *yheI* biosensor by the compounds is far below that elicited by linezolid, the positive control for *yheI* inducers. Interestingly, the *ypuA* biosensor (marker of cell wall inhibition) was significantly induced by all the four compounds above the 170% threshold. The more potent P-MmC (**26**) and PMmCl (**27**) induced the promoter even more than vancomycin (the standard antibiotic for *ypuA* biosensor), suggesting that these compounds inhibit cell wall biosynthesis in bacteria.

In an attempt to further validate the results obtained with the bioactive methylenomycin compounds, further experiments were carried out with MmD1 which lacks antimicrobial activity. The result (shown in Fig. 3.8) revealed that MmD1 gave signal only approximately 180% above the drug-free control in *yheI* biosensor and 100% above the drug-free control in *ypuA* biosensor, compared to the required threshold of 200% and 170% respectively for these markers. The compound also did not induce the *yorB* and the *fabHB* markers up to the required minimum level.

The results thus indicate that the induction of *yheI* promoter (biosensor for protein synthesis inhibitors) and *ypuA* promoter (biosensor for cell wall biosynthesis inhibitors) by the active methylenomycin compounds is indeed a result of their antimicrobial effect. Further assays were then carried out as detailed in the next section.

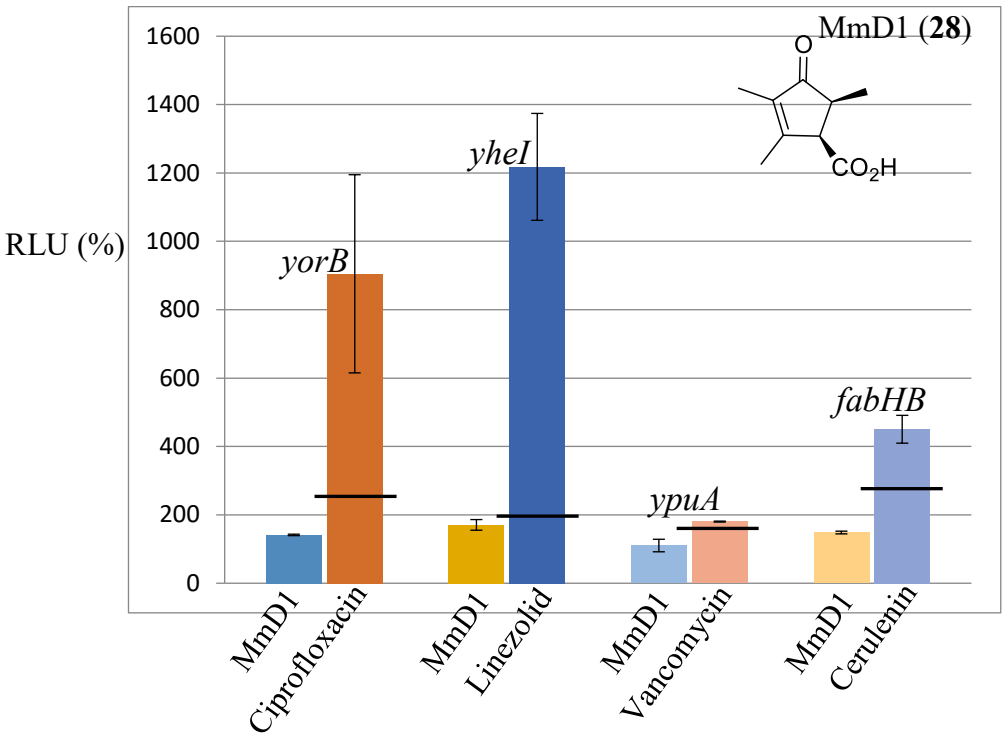


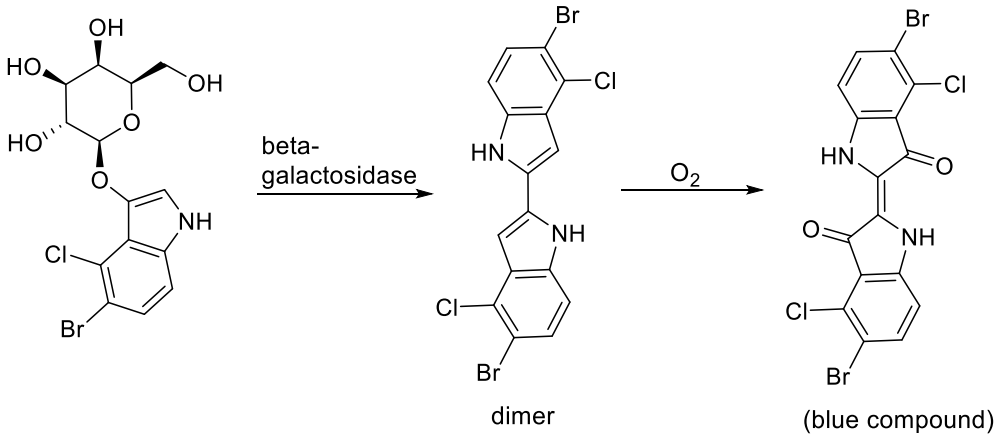
Fig. 3.8: Assay of methylenomycin D1 (MmD1) with the luciferase reporter strains. Signal generated in all the biosensors was below the minimum threshold. Data represent average of 4 independent measurements; error bars are standard deviation values associated with the measurements.

3.3.2 Mode of action studies with β-galactosidase reporter strains

β-galactosidase is a glycoside hydrolase enzyme that catalyses the cleavage of a β-glycosidic bond between a galactose and an organic moiety. This enzyme is normally present in cells where it functions to hydrolyse the disaccharide, lactose, into its component monosaccharides – glucose and galactose.[215,216] The β-galactosidase reporter strains used in this study contain the β-galactosidase gene (*lacZ*) cloned downstream of each of the promoter region of *yvgS*, *yheH*, *ypuA*, *yvgI* and *yjaX*, and are indicators for substances inhibiting RNA synthesis, translation, cell wall biosynthesis, lipid II biosynthesis, and fatty acid biosynthesis respectively.

The assay relies on the ability of the β-galactosidase to hydrolyse X-gal, a substrate consisting of a substituted indole moiety linked by a β-glycosidic bond to a galactose

(Scheme 3.3). β-galactosidase cleaves galactose off X-gal, leaving the remainder of the molecule which spontaneously dimerises, and is subsequently oxidised to give an insoluble blue product.[217] This only occurs in the assay if any of the pathway-specific promoters is induced by an antibiotic interacting with that pathway. Induction of a promoter causes β-galactosidase expression, consequently leading to the hydrolysis of X-gal present in the medium.



Scheme 3.3: Structure of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the insoluble dimer resulting from its hydrolysis by β-galactosidase.

Experiments were carried out by adding *Bacillus subtilis* strains carrying the different promoter-reporter fusion constructs to 1% nutrient agar containing X-gal (see experimental section 5.16.4 for details). A solution of a standard antibiotic or a methylenomycin compound (10 µl) was then placed on a filter in the middle of the plates, which were then incubated for 48 hours.

Figure 3.9 shows the plates with the standard antibiotics placed on the filters. As expected, a blue colouration is seen in each of the plates, indicating the induction of the respective promoters by the standard antibiotics. Figure 3.10 shows the results of the assay with methylenomcin antibiotics. As observed in the experiments with the luciferase reporter strains (section 3.3.1), the *ypuA* promoter (indicator of cell wall

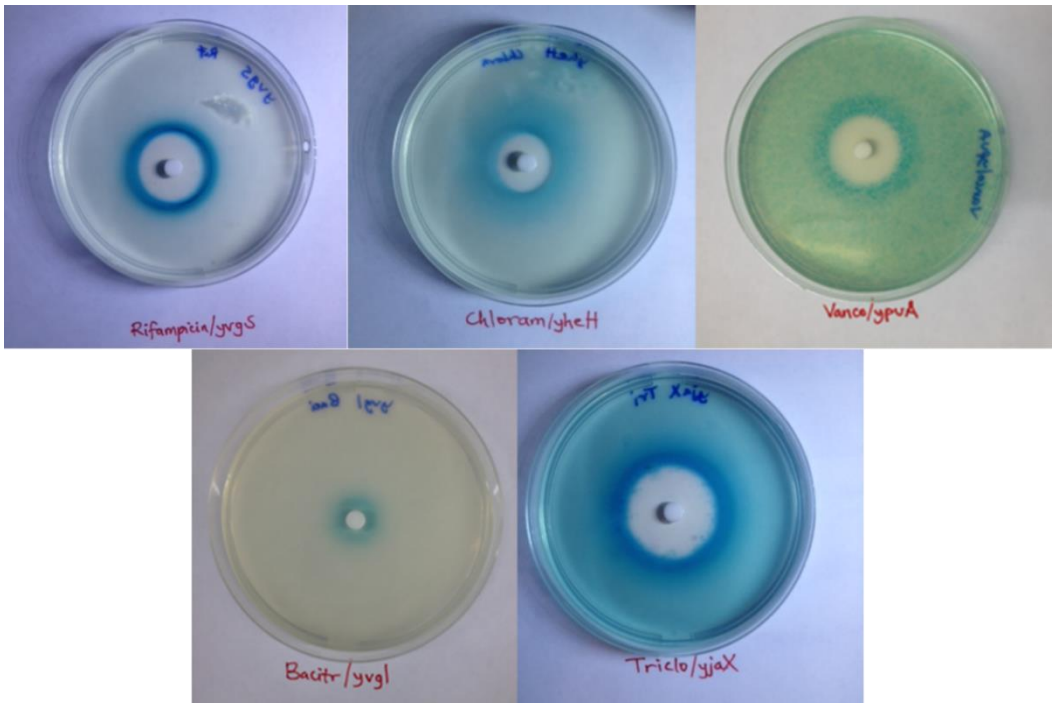
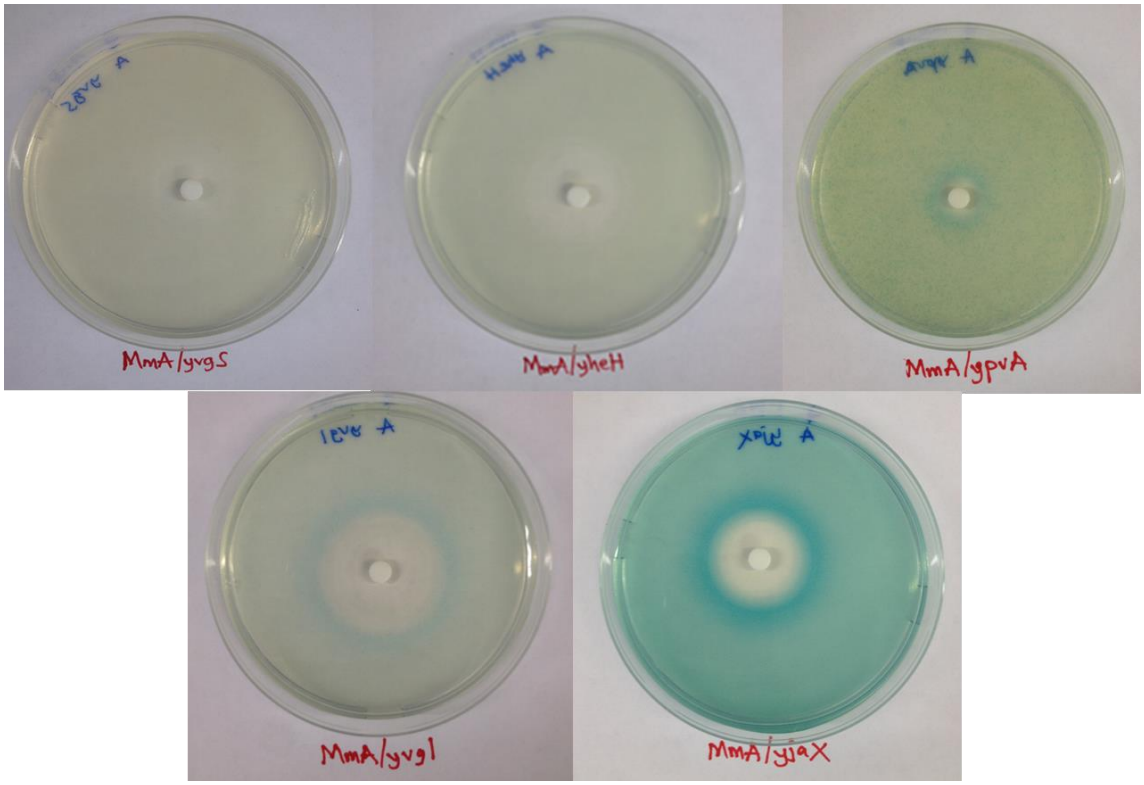


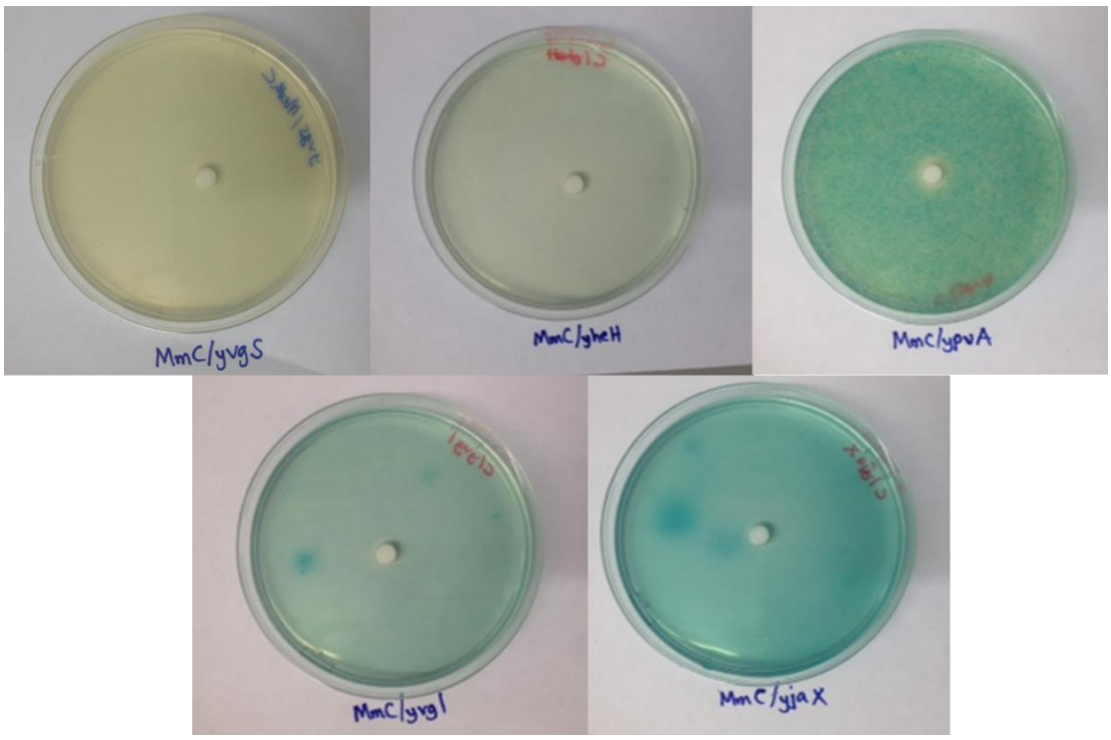
Fig 3.9: Induction of β-galactosidase in *B. subtilis* reporter strains by standard antibiotics: *yvgS* by rifampicin, *yheH* by chloramphenicol, *ypuA* by vancomycin, *yvgI* by bacitracin, and *yjaX* by triclosan.

biosynthesis inhibition) was induced by all the methylenomycin compounds. In addition, the *yvgI* promoter (marker of lipid II pathway inhibition) and the *yjaX* promoter (marker for fatty acid biosynthesis inhibition) were both induced by the methylenomycin compounds. While no connection is immediately apparent between cell wall biosynthesis and fatty acid biosynthesis, there is a direct relationship between cell wall biosynthesis and the lipid II pathway in bacteria. A key component of the cell wall is the polymer substance called peptidoglycan or murein, consisting of repeating units of *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), which are cross-linked by short peptides.[54] Peptidoglycan biosynthesis proceeds *via* a multi-step pathway starting with the enzymatic conversion of UDP-GlcNAc to UDP-MurNAc. The UDP-MurNAc is then modified by the successive

(A)



(B)



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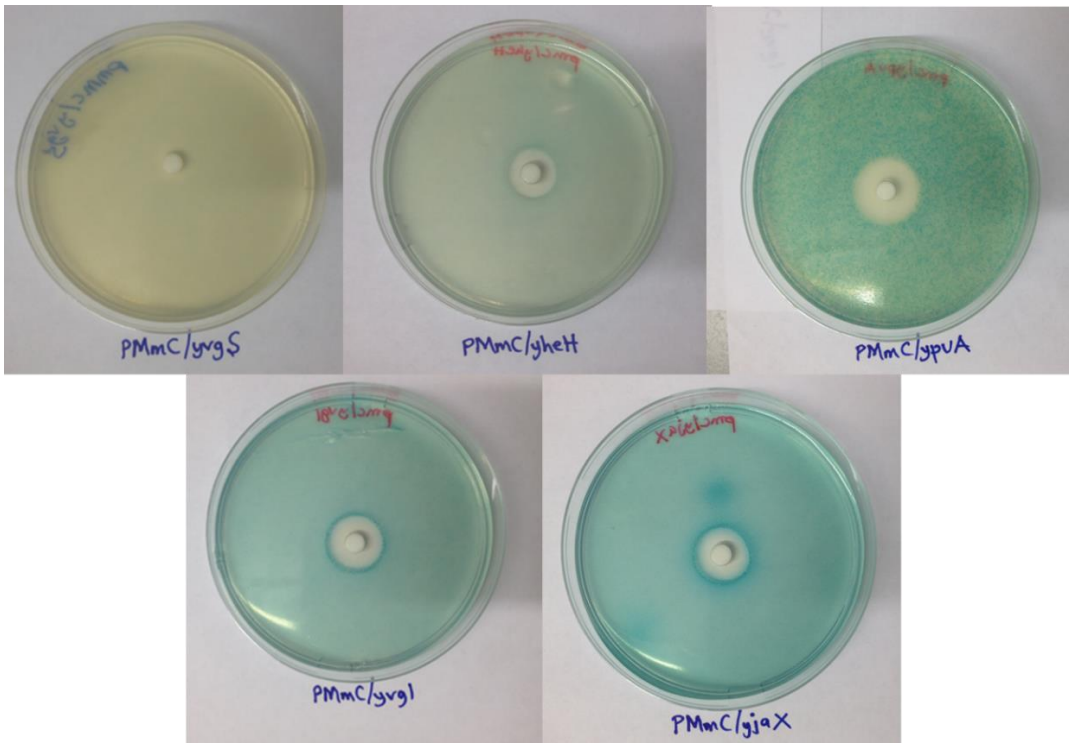
(C)



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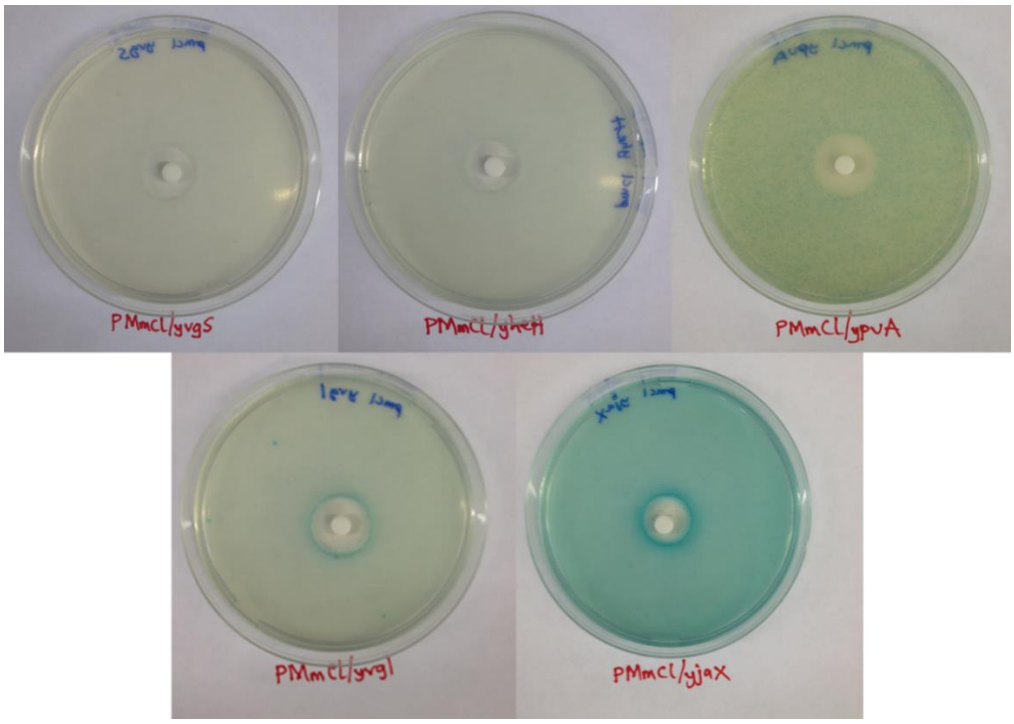


Fig. 3.10: Induction of β-galactosidase pathway-specific promoters by methylenomycin antibiotics: MmA (A), MmC (B), P-MmC (C), and P-MmCl (D).

addition of L-Ala, D-Glu, Dmp, D-Ala and D-Ala, catalysed respectively by ligase enzymes MurC, MurD, MurE and MurF.[56, 57] The resulting product, UDP-MurNAc pentapeptide, is then linked to a lipid carrier (C_{55}, undecaprenyl) by MraY to form lipid I (undecaprenyl-diphosphoryl-*N*-acetylmuramoyl-pentapeptide).[57] The so-called lipid II pathway is the conversion of lipid I *via* linkage to GlcNAc by MurG (a membrane-associated glycosyl transferase) to give undecaprenyl-diphosphoryl-*N*-acetylmuramoyl-(pentapeptide)-*N*-acetylglucosamine (lipid II, Fig. 3.11). This full monomer of the peptidoglycan is flipped out of the cell membrane and polymerised severally to form the linear chain of the peptidoglycan. The linear chains are then cross-linked with adjacent chains and into the existing murein network (see section 1.3.1, Scheme 1.1).[57-59] It is thus clear from the foregoing that the lipid II pathway is an integral part of the peptidoglycan biosynthesis. Hence, the induction of *yvgI* promoter (indicator for lipid II pathway inhibition) by methylenomycin compounds

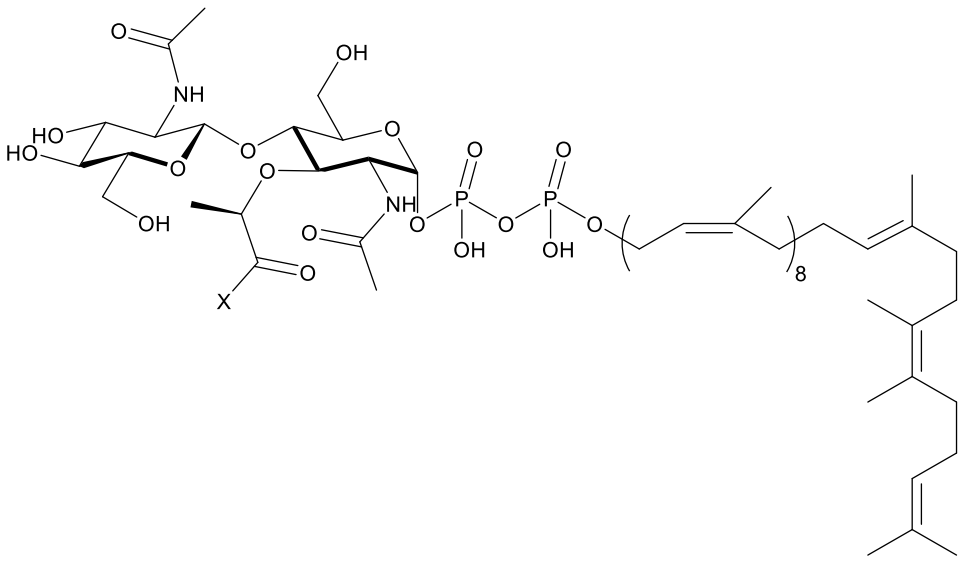


Fig.3.11: Structure of lipid II (undecaprenyl-diphosphoryl-*N*-acetylmuramoyl-(pentapeptide)-*N*-acetyl-glucosamine). X is the pentapeptide: L-Ala-D-Glu-Dmp-D-Ala-D-Ala.

is fully consistent with the observed induction of the *ypuA* promoters (indicator for cell wall biosynthesis inhibition) in both the luciferase and the β-galactosidase reporter systems.

Experiments performed with MmD1 (lacking antibiotic activity) showed that none of the pathway-specific promoters was induced (Fig. 3.12), indicating that the induction of the promoters observed for the active methylenomycin compounds was indeed due to their antibiotic effect. These results suggest that the methylenomycin antibiotics may act by inhibiting cell wall peptidoglycan biosynthesis in bacteria. This conclusion was further corroborated by results presented in the next section.

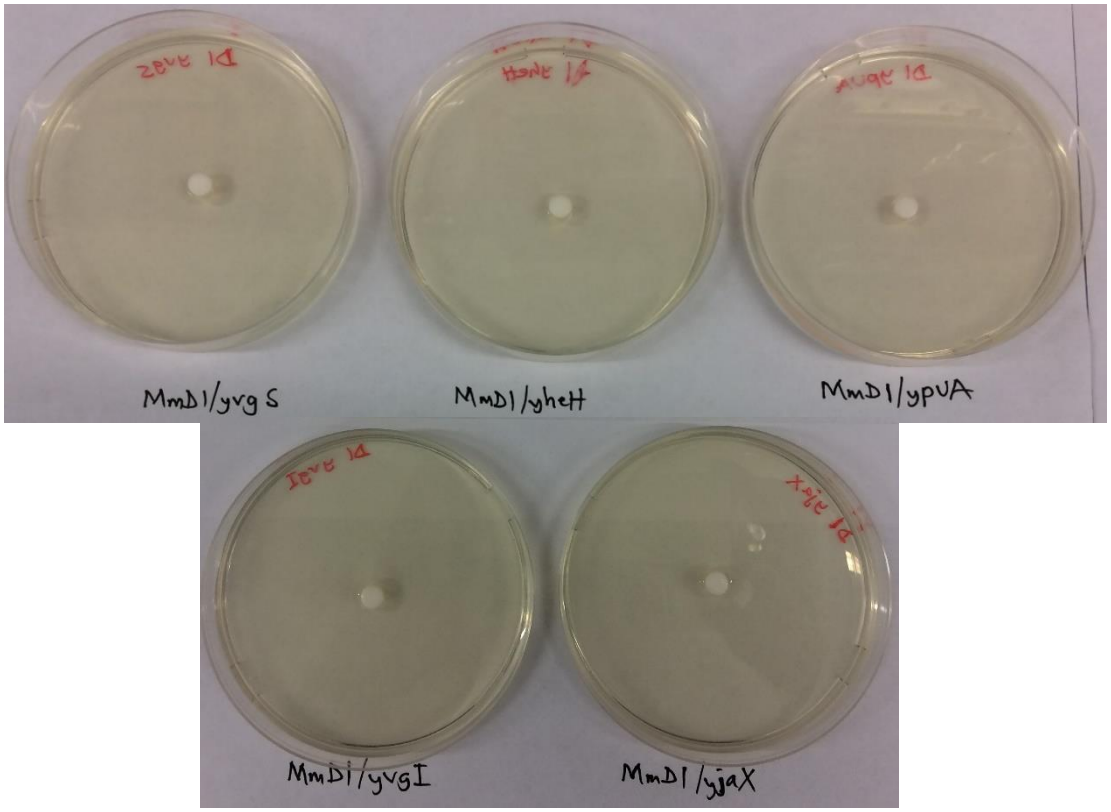


Fig. 3.12**:** Assay of β-galactosidase reporter strains with MmD1.

3.3.3 Activity of methylenomycin antibiotics against *E. coli* strains defective in outer membrane and in efflux pump system

Gram-negative bacterial strains are generally less susceptible to antimicrobial attacks compared to their Gram-positive counterparts.[218] This is due partly to the possession of an outer membrane which serves as the first line of defence and greatly limits the entrance of potentially harmful agents into the cell.[219,230] Given that the outer membrane is not able to completely exclude the intrusion of potentially harmful compounds,[220] the Gram-negative strains possess an additional defence mechanism in the efflux pump (Fig. 3.13), a system that expels harmful molecules such as antibiotics from the cell directly into the surrounding environment.[221,222] The final intracellular concentration of any harmful substance in Gram-negative strains is therefore determined by the net balance between the actions of the outer membrane which limits inflow and the efflux pump system which throws them out.[223] Thus, any defects in the functioning of the outer membrane and/or the efflux pump system may lead to a greater accumulation of harmful compounds in the cells, thereby causing increased sensitivity and cell death. A particular efflux pump in *E. coli* has been recognised to play a major role in the multiple-antibiotic-resistance (Mar) phenotype shown by the

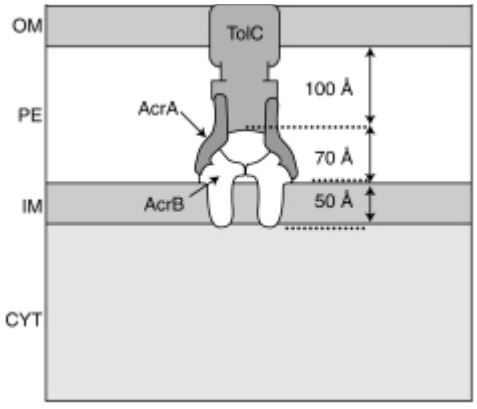


Fig. 3.13: Schematic representation of the Gram-negative cell envelope showing the protective outer membrane (OM) and efflux pump components (AcrA, AcrB and TolC). PE – periplasm; IM – inner membrane; CYT – cytoplasm. Adapted from Silhavy *et al*.[230]

organism.[224,225] This efflux pump comprises three important proteins: AcrA, AcrB and TolC.[226]

Methylenomycin compounds were shown to exert no antibiotic effect against an *E. coli* strain up to a concentration of 128 µg/ml (section 3.1). In order to further demonstrate that the methylenomycins act by inhibiting cell wall peptidoglycan biosynthesis as suggested by experiments with pathway-specific reporter strains (section 3.3.1 and 3.3.2), the compounds were tested against three *E. coli* strains, including strains with defects in the outer membrane barrier and in the efflux pump system. The first of the strains, N43, contains a mutation which prevents the expression of *acrA*, and is therefore defective in the efflux pump system.[227] The second strain, D22, contains mutation in the *lpxC* gene,[228] the product of which catalyses a key step in the biosynthesis of lipid A,[229] a crucial part of the outer membrane. The third strain (*E. coli* K-12) is a wild type strain and it is the parent for both N43 and D22. This strain has no known mutations and thus served as control for the experiment.

Given that N43 and D22 strains contain mutations which presumably compromise their defence mechanisms, it was expected that the methylenomycin compounds would exhibit improved potency against both N43 and D22, while having poor activity against the parent strain K-12. The minimum inhibitory concentrations (MICs) of methylenomycin antibiotics were therefore determined. MICs of vancomycin, chloramphenicol and triclosan were also determined in parallel.

Table 3.2 shows the results of the MICs determined against the three *E. coli* strains. As expected, all the methylenomycin compounds showed no activity against the wild type (K-12) up to a concentration of 128 µg/ml. The compounds were also not active

Table 3.2: Activities of methylenomycins and standard antibiotics against the wild type *E. coli* K-12 and its derivative mutant strains N43 and D22

Compound	MIC (μg/ml)*		
	E. coli K-12	*E. coli* N43	*E. coli* D22
MmA (**16**)	> 128	> 128	16
MmC (**17**)	> 128	> 128	64
P-MmC (**26**)	> 128	> 128	16
P-MmCl (**27**)	> 128	> 128	8
Vancomycin	> 128	> 128	8
Chloramphenicol	8	0.25	0.25
Triclosan	1	0.0625	0.0625

*Four independent determinations were carried out

against the mutant *E. coli* strain N43 up to 128 μg/ml, but all showed activity against *E. coli* D22 when compared to the parent strain K-12. These results were not anticipated as methylenomycin antibiotics were initially expected to show improved activity against both the strain N43 (defective in the efflux pump system) and D22 (defective in the outer membrane structure). The results therefore prompted some literature review into the organisation of cell envelopes in Gram-negative bacteria, and into the efflux pump mechanism, in particular with respect to the LpxC and the AcrA proteins which have been mutated in D22 and N43 strains respectively.

The cell envelope in Gram-negative organisms consists of three principal layers; the outer membrane, the periplasm containing a thin peptidoglycan layer, and the cytoplasmic or inner membrane, in the stated order.[230] The protective outer membrane is a distinguishing feature of the Gram-negative organisms and it is absent in the Gram-positive strains. The main component of the outer membrane which allows it to perform the protective function is a highly ordered and tightly bound structural material called lipopolysaccharide (LPS), present on the outermost leaflet of the

membrane.[231,232] The LPS is attached to the inner part of the outer membrane by a molecule called lipid A, consisting of a repeating disaccharide backbone, decorated with several fatty acid side chains (Fig. 3.14). The lipophilic fatty acid side chains of lipid A are actually responsible for anchoring the LPS to the inner part of the outer membrane.[229,233] The inner leaflet of the outer membrane are made of phospholipids which offer no protection to the cell. LpxC is required to catalyse an important step in lipid A biosynthesis (Scheme 3.4), but is non-functional in the *E. coli* D22 strain; lipid A production is therefore blocked in the strain, implying that the outer membrane is largely defective due to the lack of the protective LPS layer. This allowed methylenomycin compounds to gain access to the next layer in the cell envelope, the peptidoglycan, accounting for why they showed a much improved potency on the D22 strain.

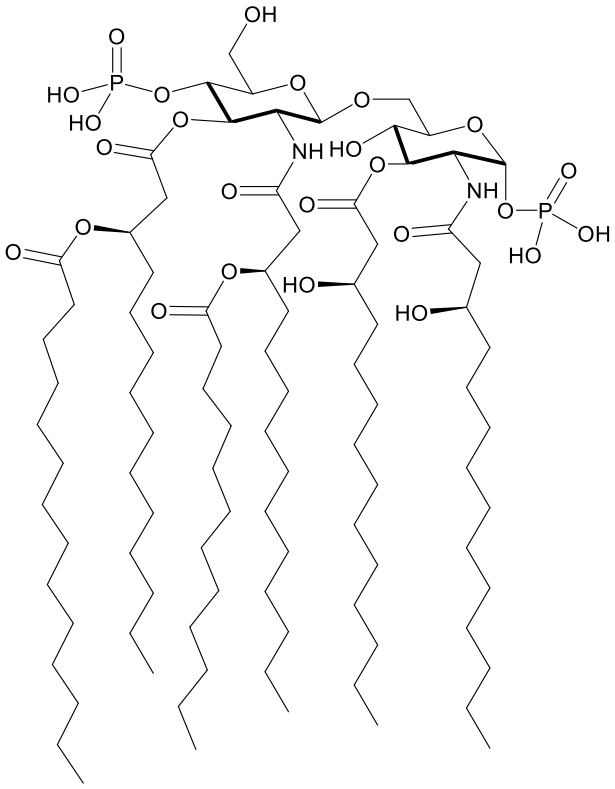
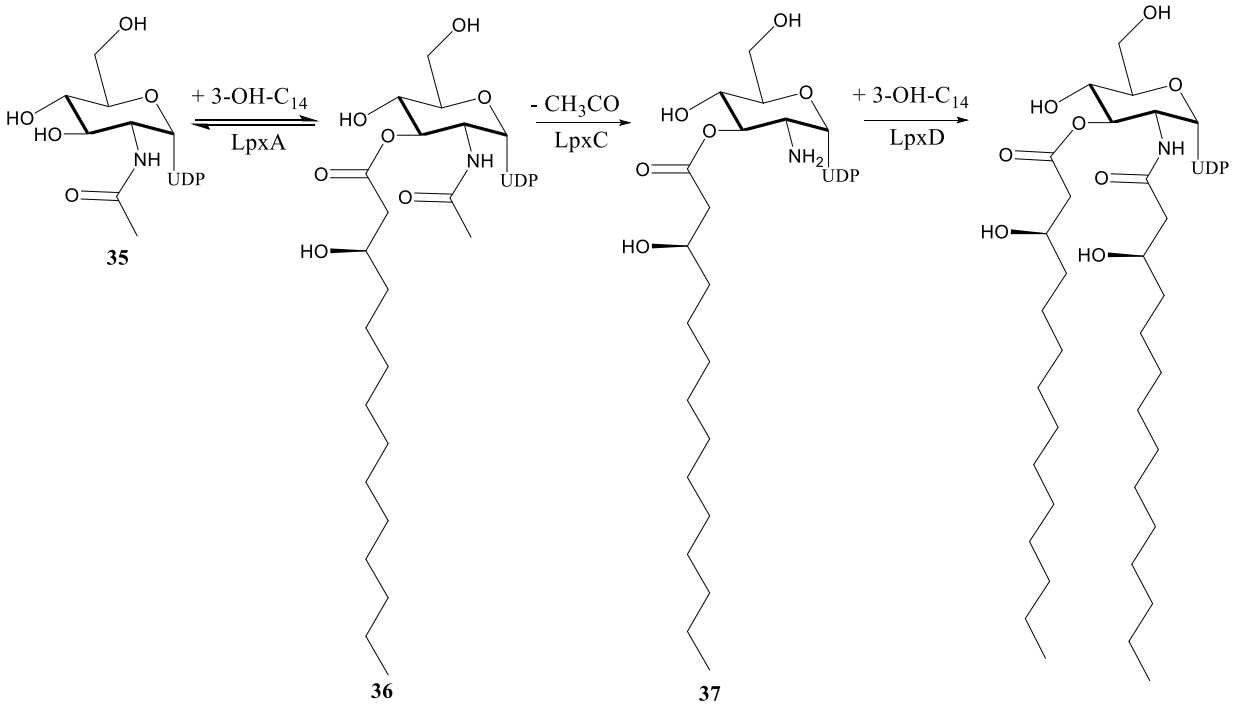


Fig. 3.14: Structure of *E. coli* lipid A.



Scheme 3.4: Early steps in the biosynthesis of lipid A: LpxC catalyses the deacetylation of UDP-3-O-acyl-GlcNAc (**36**) to give the amine compound (**37**) which is subsequently *N*-acylated by LpxD. UDP-D-GlcNAc (**35**) is a common precursor for biosynthesis of lipid A, lipopolysaccharides and peptidoglycan, all part of the Gram-negative cell envelope.

E. coli strain N43, on the other hand, has the outer membrane fully intact and the methylenomycin compounds could not gain access to the peptidoglycan layer. The only component of the Gram-negative defence cascade mutated in N43 is *acrA*, the product of which functions in the efflux pump system. Review into the mechanism of operation of the efflux pump system revealed that AcrA is an accessory protein located in the periplasm and serves as a bridge to allow the passage of harmful intruders from the cytoplasm to the outer membrane, from where they are subsequently ejected.[225,234]

The two other components of the efflux pump are *acrB* and *tolC*, and are not mutated in the N43 strain. AcrB is located in the cytoplasmic membrane and it is the actual efflux transporter removing unwanted substances from the cytoplasm. TolC is a small

outer membrane protein which provides the pore or an outlet through which inhibitors are finally extruded into the surrounding environment.[234 - 236] Given that TolC is the only component of the efflux pump located on the outer membrane, and has not been mutated in *E. coli* N43, the outer membrane remains intact in this strain. Thus, methylenomycin compounds were not able to gain access to the peptidoglycan layer, explaining why they showed no activity against the N43 strain. The same reason accounts for why vancomycin, an antibiotic targeting a peptidoglycan precursor, showed no activity against the N43 strain.

The results showed that both chloramphenicol and triclosan, antibiotics inhibiting translation and fatty acid biosynthesis respectively, showed increased activity on both *E. coli* strains N43 and D22, relative to the wild-type strain K-12. Defects in the outer membrane of strain D22 allowed these two antibiotics unhindered passage into the cell, leading to the increased potency which was observed. Mutation in *acrA* (i.e. lack of a functional perisplasmic bridge) in strain N43 also prevented the compounds from being extruded from the cell, leading to higher intracellular concentration of these compounds and the subsequent greater potency on the strain.

The results indicate that chloramphenicol and triclosan are able to infiltrate the outer membrane into the cytoplasm where they attack their target sites without being thrown out of the cell, whereas methylenomycins and vancomycins are not able to reach their target sites (peptidoglycan) in N43, as they were barred by the protective outer membrane which remained intact in this strain. Lack of a proper outer membrane in D22 allowed the methylenomycins and vancomycin access to the peptidoglycan layer, where they exert their antibiotic effects.

3.3.4 Final comments on the mode of action study

Antibiotic-induced cell death often occurs *via* inhibition of one of the key cellular functions as a primary mode of action. Methylenomycin antibiotics induced the *ypuA* and *yheI* promoters in the luciferase pathway-specific biosensors. These promoters are indicative of substances inhibiting cell wall biosynthesis and translation respectively. Experiments with the β-galactosidase reporter system also revealed that the compounds all induced the promoter for *ypuA*, as well as *yvgI* (indicative of lipid II biosynthesis inhibition) and *yjaX* (indicative of fatty acid biosynthesis inhibition).

Induction of the *ypuA* promoter (for cell wall biosynthesis) by methylenomycin antibiotics is consistent in both the luciferase and the β-galactosidase reporter systems. The results are also consistent with the observed induction of the biosensor for lipid II pathway, an intrinsic part of the peptidoglycan biosynthetic pathway. These results strongly suggest that the compounds may act by inhibiting cell wall peptidoglycan biosynthesis. This is in consonance with the activities of the methylenomycin compounds mainly against the Gram-positive bacterial strains (as demonstrated in section 3.1, table 3.1) and in agreement with previous reports that polyprenyl-coupled cell wall precursors, such as lipid II, are readily accessible on the outside of the Gram-positive bacteria, and represent an easy target for antibiotics.[238] The Gram-negative strains possess an outer membrane which serves as a protective layer for the cell, by preventing access to the peptidoglycan layer or precursors.[101] When the methylenomycin antibiotics were tested against *E. coli* strain D22 in which the protective outer membrane has been compromised, (due to mutation in the *lpxC*, essential for the biosynthesis of lipid A which serves as an anchor for LPS in the outer membrane), all methylenomycin compounds showed activity against the *E. coli* strain,

but not against the parent wild type strain containing an intact outer membrane. The result indicate that the lack of an integral outer membrane component in D22 allowed methylenomycin compounds access to the peptidoglycan, leading to the observed potency on the strain, in contrast to the wild type (K-12) in which the outer membrane was not defective. Interestingly, similar results as for the methylenomycin compounds were obtained for vancomycin (inhibiting cell wall peptidoglycan biosynthesis) against the various *E. coli* strains, but not for chloramphenicol and triclosan, antibiotics inhibiting translation and fatty acid biosynthesis respectively.

Vancomycin and the methylenomycin antibiotics showed no potency against *E. coli* strain N43, possessing an intact outer membrane but defective in the AcrA component of the efflux pump system which removes antibiotics from inside the cell. The lack of activity against *E. coli* N43 implies that the compounds did not affect the cytoplasmic content of the cells; hence, the lack of a functional efflux pump system in the strain did not, in any way, predispose or make the strain more susceptible to attack by vancomycin and the methylenomycin antibiotics. This is consistent with previous work which has shown that increased susceptibility of *E. coli* strains lacking *acrA* or *acrB* to antibiotics occurred due to the inability of the strains to expel the compounds, rather than by an increased permeability of the outer membrane [227](which would have allowed access to the inner parts of the cells starting with the peptidoglycan layer). In contrast to methylenomycins and vancomycin, the impaired efflux pump in the current study resulted in much improved potency of chloramphenicol and triclosan on strain N43 compared with the wild type K-12, indicating that these compounds indeed reached the cytoplasm but were not able to be pumped out. The foregoing strongly indicate that the primary site of action of the methylenomycin antibiotics in bacteria is the peptidoglycan and that the compounds

do not need to enter the cytoplasm in order to exert their antibiotic effects. Although the yeast cell envelope does not contain peptidoglycan, one of the main components of the cell wall is chitin, made up of polymers of *N*-acetyl-glucosamine (GlcNAc).[254,255] GlcNAc is also one of the two component monomers of the peptidoglycan in bacteria.[54] Thus, the chitin in yeast may represent the target for the methylenomycin metabolites, accounting for why they showed activity against the yeast, *Candida albicans*.

The above conclusions notwithstanding, the induction of promoters indicative of protein synthesis inhibition and fatty acid biosynthesis inhibition by methylenomycin compounds may not be totally discarded. This is because it is possible for antibiotics to interfere with other cellular functions in addition to their primary targets. For example, ciprofloxacin causes cell death primarily by inhibiting replication through the trapping of DNA-gyrase.[70,71] However, there have also been indications that the same compound may also disrupt cell membrane as a secondary mode of action.[239] The rifamycin group of antibiotics are also known to act primarily by inhibiting RNA synthesis *via* binding to RNA polymerase.[78,79] However, it has been observed that their hydroquinone moiety simultaneously interacts with DNA, thereby causing DNA damage.[240,241] The fact that the methylenomycin-related metabolites also inhibit mammalian (ovarian cancer) cells, which lack structures similar to the peptidoglycan in bacteria or the chitin in yeast,[256,257] suggests that there is an additional as yet unidentified target for these compounds in mammalian cells, which is responsible for their activity against ovarian cancer cell line.

CHAPTER FOUR

4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

4.1.1 MmyO and MmyF both play roles in the epoxidation of methylenomycin C (17) to give methylenomycin A (16)

The last step in the biosynthesis of methylenomycin A (**16**), the epoxidation of methylenomycin C (**17**), has been investigated in detail. Bioinformatic analyses of the various proteins encoded in the *mmy* cluster had implicated MmyO and MmyF in the epoxidation step. Individual deletion of either *mmyO* or *mmyF* from the *mmy* gene cluster also led to the accumulation of **17** in the mutant strains, [174] further corroborating the hypothesis that the product of these two genes might be responsible for the epoxidation reaction.

That hypothesis was investigated by transforming an *S. coelicolor* strain expressing both *mmyO* and *mmyF* with a multicopy plasmid carrying the methylenomycin resistance determinant (*mmr*); enabling feeding of methylenomycin C to the otherwise sensitive strain. Analysis of extract from the fed strain confirmed that methylenomycin C was converted to methylenomycin A as hypothesised, leading to a proposed mechanism for the epoxidation step. MmyO and MmyF showed sequence similarities to a monooxygenase and a flavin reductase (NADPH-dependent) respectively. MmyF could catalyse the reduction of FAD to $FADH_2$, which reacts with molecular oxygen to give flavin hydroperoxide. The flavin hydroperoxide is deprotonated to give a nucleophilic peroxide which attacks the C-5 position in **17** to ultimately furnish methylenomycin A (**16**) (see chapter 2, scheme 2.1).

Investigations into the substrate flexibility of the epoxidation reaction was also carried out. The result revealed that MmyO and MmyF were able to epoxidize methylenomycin C (**17**) and its putative precursor, pre-methylenomycin C (**26**), but not other methylenomycin-derived metabolites (**27**, **28** and **29**) which possess the same cyclopentenone structure. Simple synthetic analogues such as 2-cyclopentenone and 2-cyclohexenone were also not epoxidized, indicating that MmyO is highly specific for **17** and **26**.

4.1.2 Many *mmy* genes implicated in methylenomycin biosynthesis may not be essential to the pathway

LC-MS analyses of extracts of transconjugant *S. coelicolor* strains containing the entire *mmy* cluster but lacking the transcriptional repressor gene, *mmyR*, and a gene from a set of putative biosynthetic genes (*mmyP*, *mmyK*, *mmyT*, *mmyG*, *mmyX*, *mmyQ*, *mmyY*) were carried out. The results revealed that methylenomycin production was retained in the mutant strains lacking *mmyP*, *mmyK*, *mmyT*, or *mmyG*; indicating that the proteins encoded by these genes are not essential for methylenomycins biosynthesis. The results suggest the possibility of similar proteins from the *mmy* gene cluster, the pathway of another secondary metabolite in the host strain or the primary metabolic pathway (particularly the fatty acid synthase), acting in place of the *mmy* enzymes, accounting for why methylenomycin production was sustained in the mutants (see discussions in section 2.3)

Conversely, methylenomycin production was completely abolished in the transconjugant mutant strains lacking either of *mmyX* or *mmyQ*, indicating that MmyX and MmyQ are both essential for the biosynthesis. This points to a need for some phosphorylation and reduction steps in the methylenomycin pathway (MmyX and

MmyQ bear sequence similarities to a kinase and a reductase respectively). The fact that MmyK, also a kinase, is not required for methylenomycin biosynthesis may mean that MmyX is sufficient and able to do any required phosphorylation in the pathway, explaining why production was retained in the strain lacking *mmyK*, but containing *mmyX*.

While no new intermediates were found accumulating in isolable amounts in the mutant strains, the results provide an indication of the essential enzymes of the methylenomycin pathway i.e. the minimum required to biosynthesize methylenomycin A (**16**). These are likely to be the acyl carrier protein (MmyA), the ketoacyl synthase III (MmyC), the butenolide synthase (MmyD), the kinase (MmyX), the reductase (MmyQ), the enoyl reductase (MmyE), the flavin reductase (MmyF) and the monooxygenase (MmyO), in that order.

Although methylenomycins production was also abolished in the *mmyY* mutant, generated *via* CRISPR/Cas9 deletion in the current work, previous studies conducted with *jadX*,[258,259] a homologue of *mmyY* in *S. venezuelae*, suggest that the nuclear transport factor (NTF 2) proteins encoded by these genes may play as yet unidentified roles in transcriptional regulation or transport of intermediates (moieties), rather than catalysing enzymatic steps in the biosynthetic pathways.

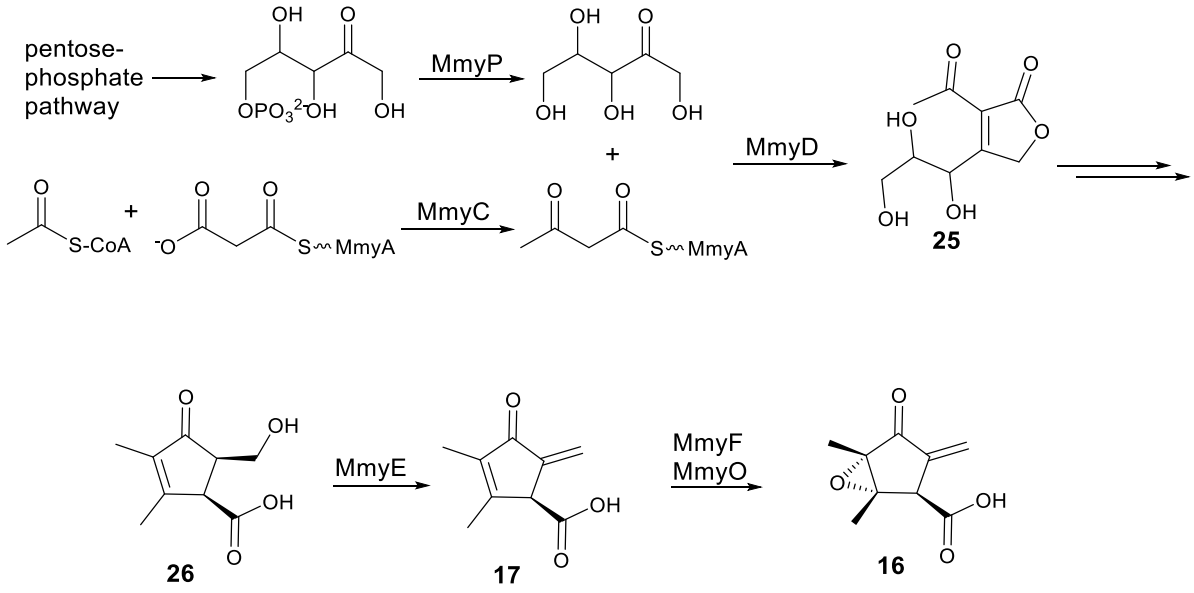
4.1.3 Putative butenolide intermediate (25) in the methylenomycin pathway was identified in some *S. coelicolor* transconjugant strains

MmyD, encoded within the methylenomycin cluster, shows 47% similarity to AvrD over 333 amino acids. AvrD catalyses the formation of a butenolide intermediate in the biosynthesis of syringolides.[189] The level of similarity between these proteins, coupled with the encoding of an ACP (MmyA) and a KAS III (MmyC) within *mmy*

cluster, that could facilitate the formation of a betaketothioester-ACP, led to the hypothesis that MmyD could catalyse the formation of a butenolide intermediate in the methylenomycin pathway, analogous to that formed by AvrD in the syringolides pathway.[173]

A compound consistent with the proposed butenolide (**25**) for the methylenomycin pathway has now been identified in some of the *S. coelicolor* transconjugants carrying the *mmy* cluster (section 2.4). Feeding of [U-^{13}C]D-ribose to the strains led to the incorporation of labelled 2-, 3-, 4- ,5- and 7-carbon units into the putative butenolide (**25**), and subsequently into methylenomycin C (**17**). While the details of the incorporation of labelled 3- and 5- carbon precursors were previously described for the methylenomycins,[173] pathways leading to the separate incorporation of a labelled 2-carbon precursor derived from acetyl-CoA and a 4-carbon precursor derived from two units of acetyl CoA have been proposed in this study for **25** and **17**. [U-^{13}C]D-ribose is metabolised in the cells to [U-^{13}C]ribose-5-phosphate, which is isomerised via the pentose-phosphate pathway to [U-^{13}C]ribulose-5-phosphate, also in equilibrium with its epimer, [U-^{13}C]xylulose-5-phosphate (Xu5P). The labelled Xu5P can then generate [U-^{13}C]glyceraldehyde-3-phosphate (G3P) *via* transketolase reactions of the pentose-phosphate pathway. The fully labelled G3P can enter glycolytic pathway where it is converted to [U-^{13}C]pyruvate, which is subsequently broken down aerobically to give [U-^{13}C]acetyl CoA, with the release of $^{13}CO_2$. The KAS III, MmyC, can then catalyse the decarboxylative condensation of [U-^{13}C]acetyl CoA with unlabelled malonyl-MmyA, to furnish acetoacetyl-MmyA, labelled only at two of the carbon positions (see chapter 2, scheme 2.5). MmyD-catalysed condensation of the partly labelled acetoacetyl-MmyA with a pentulose will furnish the putative butenolide (**25**) and subsequently methylenomycin C (**17**), both labelled

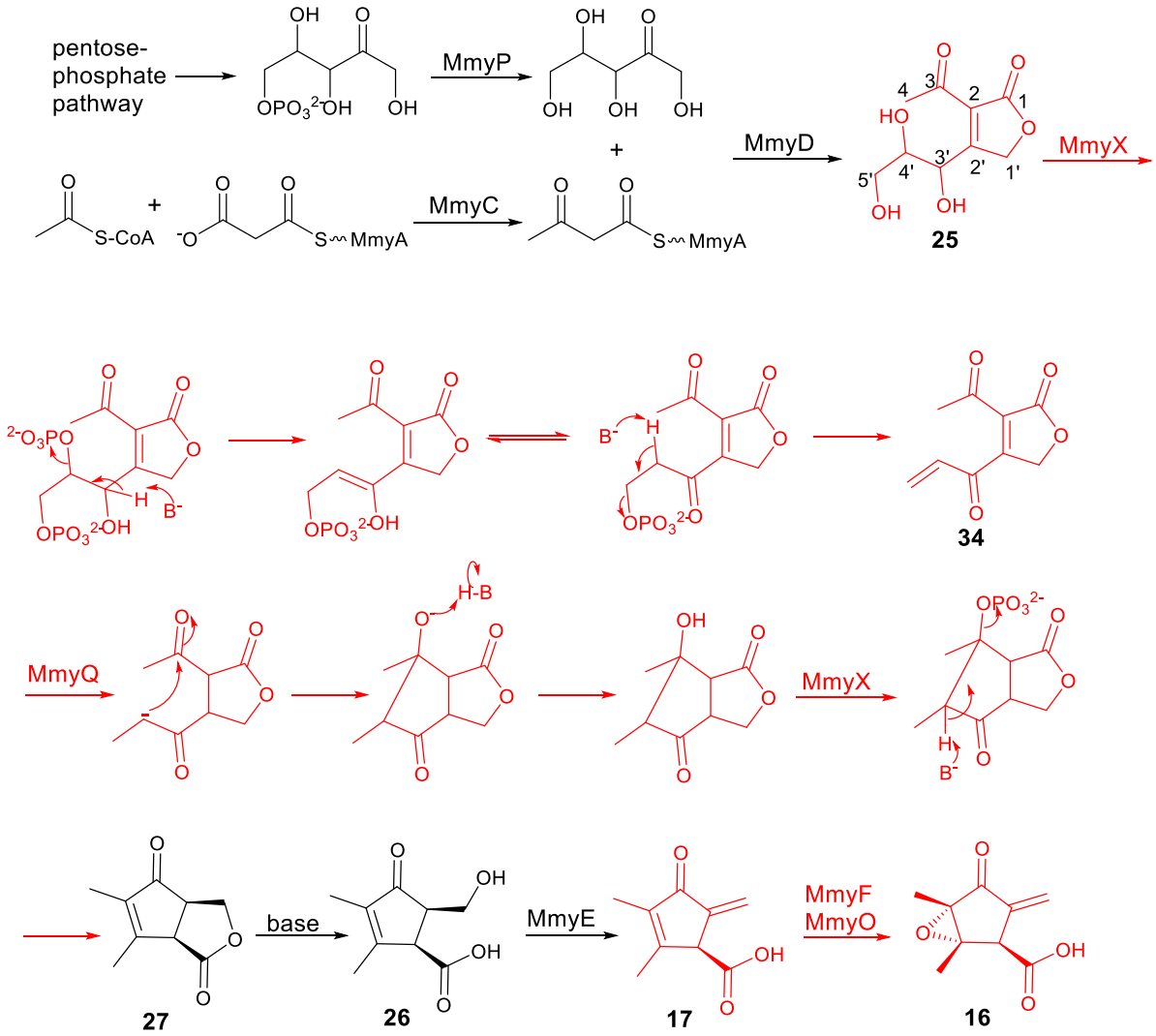
only at two corresponding carbon atoms. Incorporation of a labelled 4-carbon precursor into **25** and **17** results when the acetoacetyl-MmyA is fully labelled. This may proceed *via* conversion of [U-^{13}C]acetyl CoA to [U-^{13}C]malonyl CoA, catalysed by acetyl CoA carboxylase, using $H_2{}^{13}CO_3$ generated from the liberated $^{13}CO_2$ as the carbon source. MmyA and MmyC-mediated condensation of the [U-^{13}C]malonyl CoA and [U-^{13}C]acetyl CoA will then produce the fully labelled [U-^{13}C]acetoacetyl-MmyA, which is condensed with a pentulose by MmyD to give **25** and **17**, both labelled with four ^{13}C atoms as observed.



Scheme 4.1: Pathway proposed for methylenomycin A(**16**) biosynthesis prior to this study

The similar incorporation patterns observed for the putative butenolide (**25**) and methylenomycin C (**17**) indicate that the methylenomycins could indeed derive from **25**. Possible reactions to convert **25** to the more structurally-characterised advanced intermediates of the pathway (**27** and **26**) have therefore been proposed, leading to a revised pathway for methylenomycin A biosynthesis in *S. coelicolor* (Scheme 4.2, compared to scheme 4.1). Given that the deletion of *mmyX*, encoding a putative kinase, led to the complete abolition of methylenomycins biosynthesis (pointing to a

need for some phosphorylation in the pathway), the C-4' and C-5' hydroxyl groups of the butenolide (**25**) may be phosphorylated by MmyX. This would pave the way for loss water molecules, leading to an unsaturated intermediate (**34**), which would need



Scheme 4.2: A revised pathway for methylenomycin A (**16**) biosynthesis in *S. coelicolor*. Evidence was obtained for the butenolide (**25**) in this study. Possible reactions leading from **25** to **27** and **26** are proposed based on insights obtained from this study. The conversion of **16** to **17** by MmyO and MmyF has also been demonstrated unambiguously in this study.

to undergo reduction reactions. Again, *mmyQ* encodes a putative coenzyme F420-dependent reductase. The deletion of this gene, achieved *via* CRISPR/Cas9 excision in this study, also led to the complete abolition of methylenomycins production. Thus, MmyQ could possibly catalyse the reduction of the double bonds in **34**, using

hydrogen atoms derived from the reduced cofactor $F420H_2$. Intramolecular aldol condensation in the resulting intermediate, followed by the loss of a molecule of water, possibly mediated by MmyX, would afford pre-methylenomycin C (**26**) and the related lactone (**27**). Dehydration of **26** is proposed to give methylenomycin C (**17**), which is then epoxidized by MmyF and MmyO to give methylenomycin A (**16**), the final product of the pathway.

Expression of *mmyD* alone in *E. coli* was not sufficient to produce the butenolide intermediate in the methylenomycin pathway (**25**), unlike the expression of its homologue, *avrD*, which led to the production of syringolide compounds.[189] The results (discussed in section 2.5) suggest that the ketoacyl synthase III, MmyC, required for the formation of the diketide, acetoacete, is essential to the methylenomycin pathway and its action may not be substituted by other KAS III from *E. coli* fatty acid synthase.

4.1.4 Pre-methylenomycin C (26) and pre-methylenomycin C lactone (27) are much more potent antimicrobial agents than methylenomycin A

Investigations into the antimicrobial potentials of intermediates in methylenomycin pathway revealed that methylenomycin C, pre-methylenomycin C and pre-methylenomycin C lactone, all possess antimicrobial activities. While the activity of methylenomycin C compares with that of methylenomycin A (both only moderately potent), pre-methylenomycin C and pre-methylenomycin C lactone showed much improved activity against Gram-positive strains and yeast (*Candida albicans*), with the pre-methylenomycin C lactone being the most active of all the methylenomycin-related compounds.

Experiments investigating the effect of the methylenomycin resistance protein, Mmr, on the activities of these compounds revealed it to be most profound on the activity of methylenomycin A, and to a lesser extent on methylenomycin C (**17**). The results suggest an explanation for why **17** is rapidly metabolised to the mixture of inactive diastereoisomers (methylenomycin D1 (**28**) and methylenomycin D2, **29**), probably as an additional mechanism to resist the antimicrobial effect of methylenomycin C. Although methylenomycin A possesses the same exomethylene function which is saturated in methylenomycin C to give **28** and **29**, no reduced products analogous to **28** and **29** were found in the strains overproducing methylenomycin A, indicating that the methylenomycin resistance protein, Mmr, is sufficient to protect *S. coelicolor* against this metabolite. The potency of pre-methylenomycin C (**26**) and pre-methylenomycin C lactone (**27**) were not in any way reduced by *mmr* expression in *S. coelicolor* and *S. albus* strains. The results suggest that the likely acquisition of *mmr* by pathogenic strains, possibly *via* horizontal gene transfer, may not confer resistance on them against **26** and **27**.

Both pre-methylenomycin C (**26**) and pre-methylenomycin C lactone (**27**) were active against ovarian cancer cells at concentrations close to those at which they exert bactericidal effects on pathogenic Gram-positive strains, suggesting that these compounds might also be cytotoxic. This notwithstanding, the work demonstrated another example in which deletion of a gene within a biosynthetic gene cluster led to the production of analogues of an antibiotic with much improved potency.

4.1.5 Methylenomycin antibiotics may target cell wall peptidoglycan biosynthesis in bacteria

The mechanism of action of methylenomycin antibiotics, previously unknown, was investigated in the current study with two sets of *B. subtilis* inducible promoter-reporter systems which reveal the ability of antibiotics to interfere with specific essential pathways in bacteria.[213,214] Methylenomycin compounds induced the promoters indicative of inhibition of cell wall biosynthesis, lipid II biosynthesis, translation and fatty acid biosynthesis. While the induction of translation and fatty acid biosynthesis may not be entirely ruled out (since antibiotics sometimes interfere with more than one targets), only the induction of the promoter indicative of cell wall biosynthesis inhibition was consistent in the two promoter-reporter systems employed. This is also consistent with the induction of the promoter for the lipid II pathway, an integral part of the cell wall peptidoplycan biosynthesis,[54-57] and with the activities of the compounds mainly against the Gram-positive bacterial strains. Methylenomycin antibiotics mostly behaved like vancomycin, an antibiotic targeting the D-alanyl-D-alanine end of the pentapeptide precursor of the cell wall peptidoglycan,[64,65] suggesting they might similarly inhibit peptidoglycan biosynthesis.

Further evidence that methylenomycin-related metabolites target the peptidoglycan was obtained when the compounds showed activity against an *E. coli* strain with a defective outer membrane, indicating that they were able to gain access to the peptidoglycan layer. This is in contrast to the results obtained with the wild type *E. coli* strain, having the outer membrane intact, and to which the methylenomycin compounds showed no potency. Activity against the yeast, *C. albicans*, is likely due

to the presence of chitin, a key cell wall component biosynthesised from *N*-acetyl-glucosamine (GlcNAc),[254,255] which is also a precursor to the peptidoglycan in bacteria.[54] This is the first study to report the mode of action of methylenomycin antibiotics produced by *Streptomyces coelicolor* A 3(2).

4.2 Recommendations for future work

4.2.1 Role of MmyX and MmyQ in the biosynthesis of methylenomycins

Work presented herein suggests that both MmyX (a kinase) and MmyQ (a reductase) may play catalytic roles in the biosynthesis of methylenomycin compounds. However, no intermediates of the pathway were found accumulating in the mutant strains and further insights into their roles could not be gained in this study. It is therefore necessary to demonstrate the proposed roles for MmyX and MmyQ in the biosynthesis of methylenomycin antibiotics.

The corresponding genes may be cloned and overproduced in *E. coli* to possibly purify the recombinant proteins. Potential substrates from the methylenomycin pathway may then be chemically synthesised and incubated with these proteins to possibly establish their roles in the biosynthesis.

4.2.2 Chemical derivatisation of methylenomycins and intermediates

Efforts at investigating the biosynthesis of methylenomycin A (**16**) has recently uncovered putative intermediates: pre-methylenomycin C (**26**), pre-methylenomycin C lactone (**27**), methylenomycin D1 (**28**) and methylenomycin D2 (**29**),[174] all of which possess reactive functional groups (double bonds, ketones and carboxylic acid / lactone). As well as methylenomycin A and methylenomycin C, the reactivities of these compounds should be explored to generate novel semisynthetic derivatives,

which may then be investigated for possible biological activities. This might also lead to alteration or elimination of undesirable properties in the bioactive methylenomycin compounds, for instance, the cytotoxic nature of **26** and **27**. The *S. coelicolor* transconjugant strains producing the putative butenolide compound (**25**) may be cultured on media containing *N*-acetyl-cysteine, to possibly facilitate the isolation of this intermediate for further spectroscopic characterisation.

4.2.3 Chemical Synthesis of pre-methylenomycin C (26) and pre-methylenomycin C lactone (27)

While this is the first study to demonstrate the antimicrobial activities of **26** and **27**, a previous study has synthesized similar structures as intermediates in the attempted total synthesis of methylenomycin A.[242] It is worth synthesizing these compounds chemically to determine if they have the same strereochemistry as **26** and **27**, purified from the *mmyE* deletion mutant in this study. This may be interesting if the synthetic route proves to be simple, thereby providing a cheaper and faster source of these compounds. The potentials of **26** and **27** as anticancer agents should also be investigated and explored.

4.2.4 Mode of action of methylenomycin antibiotics

Experiments with *B. subtilis* promoter-reporter fusion strains and activities of the methylenomycins against the outer-membrane defective *E. coli* strain have strongly indicated that the compounds inhibit bacterial peptidoglycan biosynthesis. However, the final target in the biosynthesis is not yet known. For instance, vancomycin and bacitracin are two antibiotics inhibiting cell wall peptidoglycan biosynthesis: while the former binds to the pentapeptide terminus of the peptidoglycan precursor,[64,65] the later acts by inhibiting the enzyme required to dephosphorylate the undecaprenyl

diphosphate (C_{55}-PP) during peptidoglycan biosynthesis,[69] thereby blocking lipid I and subsequently lipid II production.

Methylenomycin compounds may therefore be incubated with reactions at different stages of the *in vitro* reconstituted peptidoglycan pathway. This would reveal the step/s or enzyme/s of the pathway that are inhibited by the methylenomycin antibiotics. The possible antagonisation of the activities of methylenomycin compounds by peptidoglycan precursors such as UDP-MurNAc-pentapeptide, lipid I and lipid II should also be investigated. This would reveal whether methylenomycin antibiotics inhibit peptidoglycan biosynthesis by binding directly to peptidoglycan intermediates, thereby blocking their conversion to the subsequent intermediates of the pathway. A possible combination of enzyme inhibition and precursor binding in the peptidoglycan biosynthesis may also not be ruled out.

CHAPTER FIVE

5. MATERIALS AND METHODS

5.1 General materials and equipment

Isolation of plasmid DNA from *E. coli* was performed with a GeneJet Plasmid Miniprep Kit (Thermo Scientific) following the manufacturer's instructions. Purification of DNA following agarose gel electrophoresis was performed with a GeneJet gel extraction kit (Thermo Scientific) according to the manufacturer's instructions. Polymerase chain reactions were performed using Taq polymerase from Fermentas (Lithuania) or High fidelity DNA polymerase from Roche (Germany), except where stated otherwise. Quick PCR screening of *E. coli* colonies were carried out with One Taq® Quick load® Master Mix PCR (New England Biolabs). All oligonucleotides were purchased from Sigma Aldrich.

E. coli strains were grown in Luria-Bertani (LB) medium obtained from Fisher BioReagents, unless where otherwise stated. Specialised media for *Streptomyces* cultures were prepared following published procedures,[9] and using reagents purchased from Difco and Becton, Dickinson and Co.

Erythromycin and chloramphenicol (from Duchefa Biochemie, Netherlands); rifampin (from Fischer Scientific); linezolid and triclosan (from Sigma Aldrich); ciprofloxacin, vancomycin, bacitracin and cerulenin (from VWR), were all used in microbiological assays. D-luciferin was purchased from Sigma Aldrich and X-gal was purchased from Applichem PanReac, Spain.

Optical density was measured using Biomate 3 spectrophotometer (Thermo Electron Corp.) and luminescence was measured using a charge-coupled device camera-based

luminescence detector (available in Life Sciences). A Bio-Rad Gene Pulser II with the Bio-Rad Pulse controller Plus was used for electroporation. An Eppendorf Mastercycler nexus GX2 or Eppendorf Mastercycler Personal was used for the PCR reactions. Centrifugation of samples was performed with Eppendorf Centrifuge 5804R or benchtop Eppendorf centrifuge 5424 (for 1.5ml samples). Concentration of DNA and RNA was measured on a NanoDrop ND-1000 spectrometer

All organic solvents were used as supplied (VWR or Sigma Aldrich). Solvents were evaporated from culture extracts using a Buchi Rotavapor R-200 equipped with a Buchi Vacuubrand pump. Flash column chromatography was performed with silica gel (pore size 60 Ǻ, particle size 40-63 μm)(Sigma Aldrich). Thin layer chromatography was carried out on aluminium plates pre-coated with Merck silica gel 60 F_{254} and visualised by UV light or potassium permanganate.

NMR spectra were recorded using Bruker DPX 300, 400 or 500MHz spectrometer and chemical shifts (δ) are reported in ppm with reference to the residual solvent peak.

5.2 Methods for HPLC and LC-MS analysis

5.2.1 Preparative HPLC

Methylenomycin compounds were separated from crude extracts of *Streptomyces* cultures by preparative HPLC, except where otherwise stated. Multiple injections of 100 μl solution of the crude extracts were performed, with water containing 0.1 % formic acid (solvent A) and acetonitrile/methanol containing 0.1% formic acid (solvent B) as eluents. The flow rate was maintained at 20 ml/min at a pressure of 400 atm. The elution program is as shown in Table 5.1. Fig. 5.1 also shows typical HPLC traces obtained from multiple runs during the purification of methylenomycin

compounds from extracts of *S. coelicolor* strains expressing the *mmy* gene cluster. Fractions collected for each compound were pooled, to give the purified compound in a mixture of water and acetonitrile/methanol containing 0.1% formic acid. The organic solvent was evaporated on a rotary evaporator and the left-over water was extracted with ethyl acetate. Alternatively, the mixture of water and organic solvent was evaporated completely and the flask was rinsed with ethyl acetate. The ethyl acetate was then evaporated and the residue was dried under high vacuum before further characterisations were carried out.

Table 5.1: Elution gradient for HPLC purification of methylenomycin compounds

Time (mins)	Solvent A (%)	Solvent B (%)
0	95	5
30	0	100
35	0	100
40	95	5

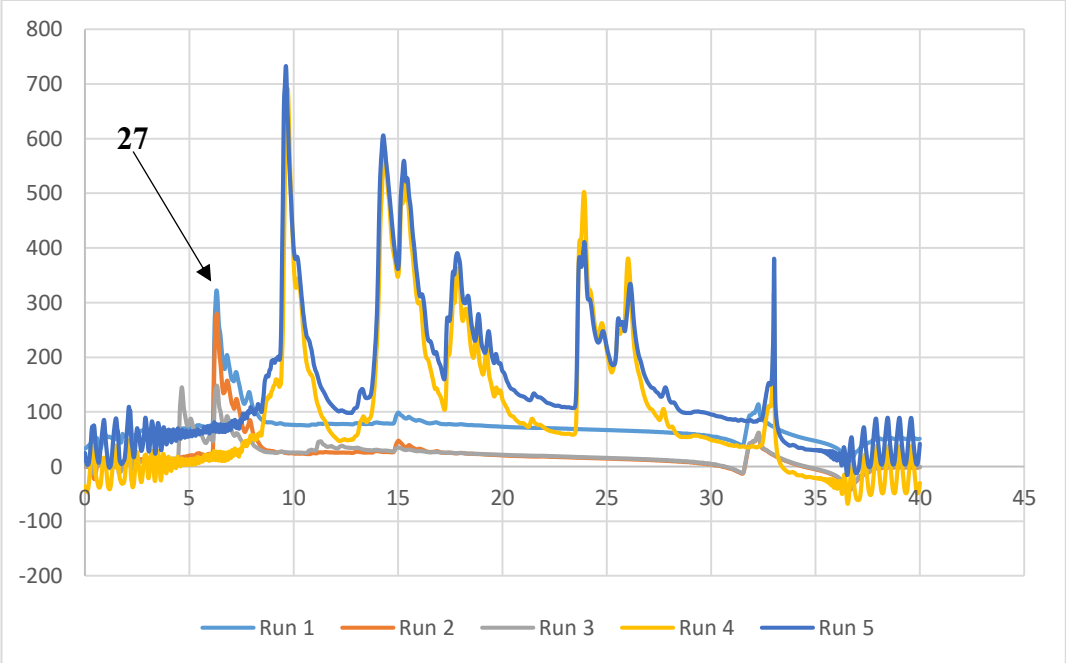


Fig. 5.1: HPLC traces from the purification of P-MmCl (**27**) from *S. coelicolor* W86 extract.

5.2.2 LC-MS analysis

Liquid Chromatography – Mass Spectrometric analyses were carried out using a reverse phase column (Agilent C18, 100 x 2.1 mm, 1.8 µm) connected to a Thermo Scientific Ultimate 3000 UHPLC^{+}. Samples (5 µl) were injected while the mobile phase flow rate was maintained at 0.2 ml/min at an operating pressure of approximately 350 atm. Separation of the metabolites was achieved using water containing 0.1% formic acid (solvent A) and acetonitrile or methanol containing 0.1% formic acid (solvent B) whilst in positive mode. For analysis in negative mode, ammonium hydroxide was added to the mobile phase solvents instead of formic acid. The method used is shown in Table 5.2. The outflow was routed to a Bruker (MaXis^{TM} Impact) High Resolution-Mass Spectrometer fitted with an electrospray ionisation (ESI) source operating in positive or negative mode. A scan of *m/z* range 50 to 1,500 was used in all analyses.

Table 5.2: Method for the separation of compounds during LC-MS analysis

Time (mins)	Solvent A (%)	Solvent B (%)
0	95	5
5	95	5
17	0	100
22	0	100
25	95	5
33	95	5

5.3 Culture conditions and sample preparation for analysis

S. coelicolor, *S. lividans* and *S. albus* wild-type strains and those expressing genes for methylenomycin production were cultured on Supplemented Minimal Medium Solid (SMMS) agar and were incubated at 30 ^{o}C for six days. The plates were frozen overnight, defrosted and the agar with the mycelia were transferred into syringes

plugged with cotton wool. The syringes were held tightly, through carefully cut-out portions on the lids, into plastic falcon tubes and were centrifuged at 2500 rpm for 10 minutes. 50 µl of the resulting filtrate samples were made up to 1 ml with methanol and desalted by passing through filters at 6000 rpm prior to analysing by LC-MS. Alternatively, the SMMS culture plates were adjusted to pH 2 with 2M HCl and left for about 30 mins. Equal volume of ethyl acetate was then added and the mixture was shaken for 1 hour before filtering. The filtrate was dried over magnesium sulphate and the solvent removed on a rotary evaporator. The dried residue was diluted appropriately with methanol, filtered as above and analysed by LC-MS.

5.4 Strains and Plasmids

5.4.1 *Escherichia coli* strains

E. coli strains used in this study are listed in Table 5.3

Table 5.3: *Escherichia coli* strains

Name	**Property**	**Source/Ref**
ET12567/pUZ8002	Non- methylating host for the transfer of DNA into Streptomyces species	Gust *et al*.[243]
K-12	Wild type *E. coli* having no known mutations	EGSC
N43	*acrA* mutant of K-12 strain	Ma *et al*.[227]
D22	*lpxC* mutant of K-12 strain	SN *et al.* [228]
BL21*	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)	Invitrogen
TOP10	F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80lacZΔM15 ΔlacX74 recA1 *ara*D139Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 nupG	Invitrogen

5.4.2 *Streptomyces* strains

Streptomyces strains used in this study are provided in Table 5.4

Table 5.4: *Streptomyces* Strains

Strains	Properties	Source/Reference
M145	*S. coelicolor* SCP1 minus , SCP2 minus	Kieser *et al.*[9]
W89	M145/C73_787/*mmyR*::*apr*	C. Corre
W86	M145/C73_787/Δ*mmyE*/*mmyR*::*apr*	C. Corre
W95	M145/C73_787/Δ*mmyD*/*mmyR*::a*pr*	C. Corre
W108	M145/C73_787/Δ*mmyF*/*mmyR*::*apr*	C. Corre
W100	M145/C73_787/Δ*mmyO*/*mmyR*::a*pr*	C. Corre
W102	M145/C73_787/Δ*mmyX*/*mmyR*::*apr*	C. Corre
W104	M145/C73_787/Δ*mmyG*/*mmyR*::*apr*	C. Corre
W105	M145/C73_787/Δ*mmyK*/*mmyR*::*apr*	C. Corre
W106	M145/C73_787/Δ*mmyP*/*mmyR*::*apr*	C. Corre
W107	M145/C73_787/Δ*mmyT*/*mmyR*::*apr*	C. Corre
W114	M145/pOSV556/*mmyE*	C. Corre
W117	M145/pOSV556/*mmyD*	C.Corre
W110	M145/pOSV556/*mmyOF*	C. Corre
W301	W110/pIJ86/*mmr*	This study
W302	M145/pIJ86/*mmr*	This study
W318	*S. albus*/pOSV556/*mmyOF*/pIJ86/*mmr*	This study
W319	*S. albus*/pIJ86/*mmr*	This study
W311	*S. albus*/pOSV556/*mmyE*	This study
W305	*S. albus*/pOSV556/*mmyD*	This study
W303	*S. albus*/C73_787/*mmyR*::*apr*	This study
W320	*S. albus*/C73_787/ Δ*mmyX*/*mmyR* ::apr	This study
W304	*S. lividans*/C73_787/*mmyR*::*apr*	This study
W314	M145/C73_787/Δ*mmyQ*/*mmyR*::*apr*	This study
W315	M145/C73_787/Δ*mmyY*/*mmyR*::*apr*	This study
W316	*S. albus*/C73_787/Δ*mmyQ*/*mmyR*::a*pr*	This study
W317	*S. albus*/C73_787/Δ*mmyY*/*mmyR*::*apr*	This study

5.4.3 Cosmids and plasmids

Table 5.5: Cosmids and plasmids used in this study

Plasmids/Constructs	properties	source
C73_787	Integrative cosmid containing the full methylenomycin gene cluster	Gust *et al.*[175]
C73_787/*mmyR*::apr	Construct to overexpress *mmy* genes	C. Corre
pGI001	C73_787/Δ*mmyQ*/*mmyR*::*apr*	This study
pGI002	C73_787/Δ*mmyY*/*mmyR*::*apr*	This study
pIJ86/*mmr*	Methylenomycin resistance determinant	C. Corre
pGI003	pET151/*mmyD*	This study

5.5 Primers

The primers used in this study are listed in Table 5.6.

Table 5.6: Primers

Primer	Sequence
mmr-fwd	5'ATGACCACTGTCCGAACAGG 3'
mmr-rev	5'TCAGGTGGCCGCCGTGACGG 3'
mmyQ-sgRNA-1	5'GATCACTAATACGACTCACTATAGGGACGTACTGGTGTTGGTCAT GTTTTAGAGCTAGAAATAGC 3'
mmyQ-sgRNA-2	5'GATCACTAATACGACTCACTATAGGGTCGGCCGGAGTGAACATGTG GTTTTAGAGCTAGAAATAGC 3'
mmyY-sgRNA-1	5'GATCACTAATACGACTCACTATAGGGAGCTGTGCACCCGCATGGCG GTTTTAGAGCTAGAAATAGC 3'
mmyY-sgRNA-2	5'GATCACTAATACGACTCACTATAGGGCCGACGGGAACCGCCACATC GTTTTAGAGCTAGAAATAGC 3'
sgRNA-rev	5'AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAG CCTTATTTTAACTTGCTAT 3'
mmyQ-scrn-fwd	5'TGCGGCTGGTGCCAGCTGAC 3'
mmyQ-scrn-rev	5'CCGGTTCTCCAGCGCCAGTG 3'
mmyY-scrn-fwd	5'CGTAACGGAGAGTTAAACCGG 3'
mmyY-scrn-rev	5'AGGGACACGGAAGCGAAGG 3'
mmyD-fwd	5'CACCATGCCAGTCAGCGGTTCCCTAT 3'
mmyD-rev	5'TCAAGGCAGGCGGTGGGCGAC 3'
T7-fwd	5´TAATACGACTCACTATAGGG-3´
T7-rev	5´TAGTTATTGCTCAGCGGTGG-3´

5.6 Culture Media

Unless ortherwise stated, all media were autoclaved after addition of all components.

5.6.1 SFM medium

Bacto agar	20 g/L
Mannitol	20 g/L
Soya Flour	20 g/L
Tap Water	to make up to 1 Litre

5.6.2 SMMS medium

Bacto agar	20 g/L

$MgSO_4$	5 mM
L-Alanine	30 mM
TES buffer pH 7.0	25 mM
Distilled Water	to make up to 1 Litre

Mixture of the above components was first autoclaved before the following were added:

Glucose	10 g/L (from 50 % stock)
NaH_2PO_4	5 mM
K_2HPO_4	5 mM
Trace elements	1 ml/L

Trace elements solution was made up of:

$ZnSO_4.7H_2O$	0.1 g/L
$FeSO4.7H_2O$	0.1 g/L
$MnSO_4.4H_2O$	0.1 g/L
$CaCl_2.2H_2O$	0.1 g/L
NaCl	0.1 g/L

5.6.3 LB medium

Bacto agar	15 g/L
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L
Water	to make up to 1 Litre

5.6.4 AlaMM medium (pH 5.0)

Bacto agar	15 g/L
L-Alanine	30 mM
K_2HPO_4	5 mM
$MgSO_4$	5 mM
Tap Water	to make up to 1 Litre
Glycerol	10 g/L (from 50% sterile stock)

Adjusted to pH 5.0 with 35% HCl

5.6.5 Belitzky minimal medium (BMM)

$(NH_4)_2SO4$	15 mM
$MgSO_4.7H_2O$	8 mM
KCl	27 mM
Tris Base	50 mM

Final pH adjusted to 7.5 with conc. HCl

5.6.6 Nutrient Agar medium

Beef Extract	3 g/L
Peptone	5 g/L
Agar	15 g/L

Made up with distilled water

5.6.7 Mueller Hinton Broth (MHB)

Casein acid hydrolysate	17.5 g/L
Beef extract	3.0 g/L
Starch	1.5 g/L

5.6.8 M92 medium

Trypticase Soy broth	30 g/L
Yeast Extract	3 g/L
Agar (When necessary)	15 g/L

Distilled water to make up to 1 litre

5.6.9 M9 medium

M9 salts (5x)	200 ml
Glucose (20%)	20 ml
$MgSO_4$ (1M)	2 ml
$CaCl_2$ (1M)	100 µl

Distilled water to make up to 1 litre

M9 salts (5x) was made up of:

$Na_2HPO_4.7H_2O$	64.0 g
KH_2PO_4	15.0 g
NaCl	2.5 g
NH_4Cl	5.0 g

Dissolved and made up to 1L with distilled water

5.7 Stock solutions

Table 5.7 lists the stock solutions used in this study.

Table 5.7: Stock solutions

stock solutions	components	solvent
TBE (1000 ml, 5x)	Tris base - 53.00 g Boric acid - 27.59 g EDTA - 10 mM, pH 8	Water
Fast screening Lysis buffer	Sucrose - 5.00 g KCl - 0.23 g NaOH - 200 mM (2.5 ml of 2 M) SDS - 0.5 % (1.25 ml of 10 % stock)	Water
Citrate buffer	Citric acid - 2.10 g Sodium citrate - 2.94 g	Water

5.8 Agarose gel electrophoretic analysis

Agarose gel electrophoresis was performed on Bio-Rad Power Pac 300. Except where stated otherwise, 100 ml of 1% agarose gel was prepared in 1x TBE buffer and 2 µl of GelRed DNA stain was added. Electrophoresis was carried out on 3 to 40 µl of PCR products for 50 minutes under 90 V. GeneRuler^{TM} 1 kb and/or middle range DNA ladder was used to provide size markers and allowed for the estimation of PCR products size when visualised under UV (BioDoc-It^{TM} 2UV Transilluminator) at 365 nm.

5.9 Spore stock preparation

Spores of *S. coelicolor, S. albus,* and *S. lividans* strains were propagated by inoculation on SFM agar containing 50 µg/ml apramycin or hygromycin, depending on the resistance marker carried by each strain. Following incubation for 7 days, 5 ml of sterile distilled water was added to each plate and a sterile spreader was used to scrape off the bacterial spores. The bacteria–laden water was filtered through glass wool, centrifuged at 4000 rpm for 10 mins and the supernatant was discarded. 400 µl of sterile distilled water and 600 µl of sterile 50 % glycerol were added and the content was mixed thoroughly before transferring into a sterile cryotube for storage at -80 ºC.

5.10 Preparation of electrocompetent *E. coli* cells

E. coli TOP10 was used in this study for maintaining and propagating plasmids and constructs, while *E. coli* ET12567/pUZ8002 (ET) served as the non-methylating host mediating the transfer of the constructs into *Streptomyces* by conjugation. 100 µl of the TOP10 or ET cells from – 80 ºC was used to inoculate 10 ml of LB (supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol in the case of *E. coli* ET)) and were incubated overnight in a 37 ºC shaker. The cells were refreshed the next day by inoculating 10ml LB with 100 µl of the overnight culture and incubating to an O.D $_{600}$ of 0.4. The cells were pelleted by centrifuging at 4,000 rpm for 5 mins and the supernatant was discarded. The pellets were then washed twice with 10 ml ice-cold 10 % glycerol and the supernatant discarded each time. The pellets were finally suspended in 100 µl 10 % glycerol and transferred into cryovials for storage at -80 ºC pending their use for electroporation.

5.11 Construction of *S. coelicolor* strains W301 and W302, respective methylenomycin-resistant derivatives of W110 and M145 strains

5.11.1 Purification of the plasmid pIJ86 carrying *mmr* gene

100 µl of *E. coli* TOP10 (carrying the DNA construct pIJ86/*mmr*) from a glycerol stock was added to 5ml of LB medium containing 50 µg/ml apramycin and was incubated at 37 ^{o}C, 180 rpm overnight. Plasmid DNA was purified from the cells and the presence of the methylenomyin resistance gene *mmr* was confirmed by PCR amplification using *mmr*-fwd and *mmr*-rev primers provided in Table 5.6 and the components and conditions outlined below. The PCR product was analysed by

Table 5.8: PCR mixture for amplifying *mmr*

Component of PCR	Volume (µl)
Purified plasmid DNA	2.0
Buffer with $MgCl_2$	5.0
dNTPs (10mM)	1.0
mmr-fwd (10µM)	1.5
mmr-rev (10µM)	1.5
Taq DNA polymerase	0.5
DMSO	5.0
Distilled water	33.5
TOTAL	50.0

Initial denaturation	94 ºC	5 min	
Denaturation	94 ºC	1 min	33x
Annealing	55 ºC	1 min	
Extension	72 ºC	1.5 min	
Final extension	72 ºC	10 min	
End and storage	4 ºC		

agarose gel electrophoresis, which showed a single band corresponding to the 1.4 kb *mmr*. The construct was kept at – 20 ºC pending further use.

5.11.2 Transformation of *E. coli* ET12567/pUZ8002

Purified plasmid pIJ86/*mmr* (2 µl) was added to 100 µl of electrocompetent *E. coli* ET12567/pUZ8002 cells and mixed gently before being transferred to an electroporation cuvette where 1.8 V was applied. 1 ml of ice-cold liquid LB medium was added and the mixture was incubated at 37 ºC, 180 rpm for 1 hour. It was thereafter spread over solid LB plates containing apramycin (50 µg/ml), kanamycin (50 µg/ml) and chloramphenicol (25 µg/ml). The plates were incubated at 37 ºC for 18 hours to generate individual colonies.

Transformation of the *E. coli* ET12567/pUZ8002 and *E. coli* TOP10 by the cosmid C73_787/*mmyR*::*apr* and its various mutated derivatives were also carried out in a similar manner. The selective plates however contained only apramycin in the case of TOP10 transformation.

5.11.3 Conjugation of *E. coli* ET12567/pUZ8002 with *Streptomyces* species

The procedure described here was used to conjugate ET12567/pUZ8002 (ET) carrying pIJ86/*mmr* (from section 5.11.2) with *S. coelicolor* strains W110, M145 and *S. albus* J1074, and also for the conjugation of the ET carrying the cosmid C73_787/*mmyR*::*apr* or any of its mutated derivatives with *S. coelicolor, S. albus* or *S. lividans* as required.

Single colonies of the *E. coli* ET12567/pUZ8002 carrying the required construct was inoculated into 5 ml LB medium containing kanamycin, chloramphenicol and apramycin. The culture was incubated overnight at 37 ^{o}C, and 100 µl was added to 10 ml LB containing the antibiotics and incubated for 5 hours ($O.D_{600}$ approximately 0.4). The cells were collected by centrifuging at 3000 rpm for 10 minutes, washed twice with liquid LB medium containing no antibiotics, and suspended in 400 µl of LB medium.

400µl of liquid LB medium was added to a 50 µl spore stock of each of the *Streptomyces* strains from -80 ^{o}C glycerol stocks. The cells were then heat-shocked at 55 ^{o}C for 10 minutes and were immediately transferred to an ice bath for 5 minutes. They were then mixed with the fresh cells of *E. coli* ET12567/pUZ8002 carrying the required construct. The mixture was centrifuged for 2 minutes at 10,000 rpm, half of the supernatant discarded, and the cells were resuspended in the remaining liquid. The resulting suspension was spread over three 25 ml solid SFM plates containing 100µl of 2.5 M $MgCl_2$ and incubated overnight at 30 ^{o}C. The plates were overlayed in the morning with 1ml of sterile water containing 25 µl of apramycin and 20 µl of nalidixic acid. The plates were then incubated at 30 ^{o}C for an additional 5 days.

Single colonies of the resulting *Streptomyces* transconjugants were picked and cultured on SFM plates containing apramycin and nalidixic acid. The inclusion of nalidixic acid ensured any residual *E. coli* that might be picked with the *Streptomyces* colonies were all eliminated. The plates were then incubated for 7 days and the spore stocks were generated. Strains W110 and M145 carrying the pIJ86/*mmr* construct were named W301 and W302 respectively. Table 5.3 provides the names of *Streptomyces* transconjugants carrying the cosmid C73_787 or its derivatives.

Integration of the cosmid into these *Streptomyces* species was confirmed by LC-MS analysis of the resulting transconjugants which showed that they produced methylenomycin antibiotics and/or the methylenomycin furans relative to their respective wild type strains. Genetic confirmation was obtained for strains W301 and W302 as detailed in section 5.11.4.

5.11.4 Colony PCR to confirm the presence of *mmr* in W301 and W302

W301 and W302 strains were inoculated on SFM agar plates and were incubated at 30 ^{o}C for 4 days. Wild type *S. coelicolor* M145 was also grown in parallel under the same conditions. Colonies of each of the strains were lysed using the following procedure: 1 loop of the spores was scrapped from the plates and 30 µl of fast screening lysis buffer was added. The mixture was incubated at 37 ^{o}C for 2 hours before adjusting to pH 8 with 1M HCl. The content was cooled on ice for 10 minutes and centrifuged at 13,200 rpm for 10 minutes. 20 µl of the resulting supernatant was diluted to 100 µl with sterile distilled water. 2 µl of this solution was analysed by PCR using the same primers and conditions outlined in section 5.11.1. The PCR products were analysed by agarose gel electrophoresis.

5.12 CRISPR/Cas9 deletion of *mmyQ* and *mmyY*

5.12.1 Generation of the synthetic guide sequence as DNA

sgRNA sequences required to delete *mmyQ* and *mmyY* were designed by searching for PAM (NGG) sequences near the beginning and the end of each gene. The CRISPR design tool at http://crispr.mit.edu was employed for the search and PAM sequences were selected that would lead to in-frame deletion of the genes. The 20 bp immediately upstream of the identified PAM sequences were then incorporated into the design of a primer which also contains the sequence for the T7 RNA promoter. The pairs of primers designed to respectively delete *mmyQ* and *mmyY* were *mmyQ*-sgRNA-1/*mmyQ*-sgRNA-2 and *mmyY*-sgRNA-1/*mmyY*-sgRNA-2 (Table 5.5), with the 20 bp guide sequence underlined in each case. Each of the primers served as the forward primer in the initial extension PCR to amplify the full synthetic RNA sequence as DNA templates. The reverse primer for each of the PCR reaction was

Table 5.9: PCR mixture and conditions for the generation of synthetic guide DNA templates for transcription to sgRNAs

Component of PCR	Volume (µl)
pCRISPOmyces 2 DNA	0.5
Forward primer (10µM)	2.0
Reverse primer (10µM)	2.0
Phusion® high-fidelity PCR mix (M0532S)	25.0
DMSO	1.5
RNAse-free water	19.0
TOTAL	50.0

Initial denaturation	98 °C	1 min	
Denaturation	98 °C	5 secs	35x
Annealing	58 °C	5 secs	
Extension	72 °C	8 secs	
Final extension	72 °C	8 mins	
End and storage	4 °C		

sgRNA-rev (Table 5.5), while the plasmid DNA pCRISPomyces 2 served as the template.[192] The conditions for the reaction are as detailed in Table 5.9. To confirm the presence of the expected synthetic guide sequence bands following the extension PCR, 1 µl of loading dye was added to 3 µl of the PCR products and were run on 2 % agarose gel at 90 V for 40 mins in TBE buffer and with super low range DNA ladder (50 bp) as marker.

5.12.2 *In vitro* transcription of the synthetic DNA templates to mRNA (sgRNA)

The PCR products from section 5.12.1 were purified directly with a GeneJet Gel extraction kit, omitting the steps for solubilising the gel, and the purified DNA was dissolved in 20 µl of RNAse/DNAse-free water. The transcription reaction was then set up as shown in Table 5.10 with HiScribe™ T7 high yield RNA synthesis kit (New England Biolabs) and incubated overnight at 37 °C.

The RNA generated was purified with MEGAclear™ RNA purification kit (Invitrogen) and eluted with 20 µl of RNAse/DNAse-free water to yield: *mmyQ*-sgRNA-1 (1,617 ng/µl), *mmyQ*-sgRNA-2 (1,467 ng/µl), *mmyY*-sgRNA-1 (719 ng/µl) and *mmyY*-sgRNA-2 (1,153 ng/µl).

Table 5.10 Reaction mixture for the transcription of synthetic guide DNA to RNA

Reaction component	Volume (µl)
RNAse/DNAse-free water	2
Reaction buffer (10x)	3
UTP (100mM)	3
ATP (100mM)	3
GTP (100mM)	3
CTP (100mM)	3
DNA template (66 – 100 ng/µl)	10
T7 RNA polymerase	3
TOTAL	30

5.12.3 Phenol/Chloroform purification of cosmid DNA for CRISPR/Cas9 work

E. coli TOP10 was transformed with the construct C73_787/*mmyR*::*apr* and 6 colonies were selected following overnight incubation at 37 ^{o}C on LB agar supplemented with 50 µg/ml apramycin. The selected colonies were transferred into liquid LB containing apramycin and grown overnight at 37 ^{o}C. The cosmid DNA was isolated from each overnight culture by miniprep and eluted with 20 µl of RNAse/DNAse-free water, then combined to give 120 µl crude cosmid. 80 µl of phenol-chloroform mix (Acros Organics) was added, and the mixture was shaken for 1 minute before centrifuging at 13,000 rpm for 5 mins. The supernatant (containing the cosmid DNA) was carefully transferred into a fresh tube and 50 µl of chloroform was added. The mixture was again shaken and centrifuged at 13,000 rpm for 5 minutes. 55 µl of the supernatant was transferred into a fresh tube, 33 µl of isopropanol was added, and the mixture was left on ice for about 10 mins to precipitate DNA. The mixture was then centrifuged as before and the supernatant was discarded. 200 µl of 70 % ethanol was added to the precipitate, and the mixture was shaken, centrifuged, and the supernatant discarded. The precipitate was allowed to dry in a flow hood for

2 hours before 20 µl of pre-warmed (50 ºC) RNAse/DNAse-free water was added to dissolve the DNA.

5.12.4 Excision of *mmyQ* and *mmyY* by Cas9 nuclease

In vitro CRISPR/Cas9-mediated reactions to delete *mmyQ* and *mmyY* were performed with Cas9 nuclease (New England Biolabs), and the prepared sgRNAs and the cosmid DNA, C73_787/*mmyR*::*apr*. The reactions were set up as detailed in Table 5.11. All components of the reaction (except the cosmid DNA) were first mixed and incubated at 26 ºC for 10 mins before the cosmid DNA was added. The reaction was then incubated at 37 ºC overnight.

The edited DNA was ethanol precipitated the next day by adding 5 µl of 3M sodium acetate and 150 µl of absolute ethanol. The mixture was kept on ice for 1 hour before centrifuging at 13,500 rpm for 15 mins. The supernatant was discarded and the precipitated DNA was washed with 150 µl of 70% ethanol and again centrifuged for 15mins. The DNA pellet was left to air dry for 3 hours and dissolved in 20 µl of water.

Table 5.11: Reaction components for CRISPR/Cas9 excision of *mmyQ* and *mmyY*

mmyQ		*mmyY*	
mmyQ-sgRNA-1	4.0 µl (6,468 ng)	*mmyY*-sgRNA-1	6.5 µl (4,674 ng)
mmyQ-sgRNA-2	4.5 µl (6,604 ng)	*mmyY*-sgRNA-2	4.0 µl (4,615 ng)
Cas9 nuclease	2.0 µl	Cas9 nuclease	2.0 µl
Cas9 buffer	3.0 µl	Cas9 buffer	3.0 µl
RNAse/DNAse-free water	14.5 µl	RNAse/DNAse-free water	12.5 µl
Cosmid DNA	2.0 µl (500ng)	Cosmid DNA	2.0 µl (500ng)
TOTAL	30.0 µl	TOTAL	30.0 µl

5.12.5 End repair and ligation of Cas9-editted cosmid C73_787/*mmyR*::apr

Since the SpCas9 nuclease has been shown to have exonuclease activity which trims the non-complementary strand following the double strand cleavage,[198,200] the cosmid DNA from the Cas9 reaction was repaired at the cleaved ends. To each of the 20 µl DNA solutions, 0.3 µl of dNTPs (10 mM), 3 µl of Buffer 2.1 (10x, New England biolabs), 6.2 µl of water and 0.5 µl of T4 DNA polymerase (New England biolabs) were added. The mixture was incubated at 12 ^{o}C for 15 mins and the polymerase was inactivated by heating at 75 ^{o}C for 20 mins.

The cosmid was then ligated directly by adding 3.3 µl of T4 DNA ligase buffer and 1.5 µl of T4 DNA ligase (New England biolabs) to the mixture. The content was incubated overnight at 16 ^{o}C and terminated at 65 ^{o}C for 10 mins.

5.12.6 Screening to identify *mmyQ* and *mmY*-mutated cosmids

50 µl electrocompetent TOP 10 *E. coli* cells were transformed with 13 µl of the mixture from ligation and colonies were selected on LB agar plates supplemented with 50 µg/ml apramycin following overnight incubation at 37 ^{o}C. Several individual colonies were picked and transferred into 100 µl of LB liquid (containing apramycin) in 96 well plates and incubated overnight in a 37 ^{o}C shaker. 2 µl of culture from cloudy wells (where the cells have grown actively) was used as template in the screening PCR reaction set up as shown in Table. 5.12. The screening primers for *mmyQ* mutation and *mmyY* mutation are the primers annealing 150 to 200 bp outside of the coding sequence for each gene and designated as *mmyQ*-scrn-fwd/*mmyQ*-scrn-rev and *mmyY*-scrn-fwd/*mmyY*-scrn-rev respectively (sequences provided in Table 5.5)

Table 5.12: PCR mixture and conditions for screening for Δ*mmyQ* and Δ*mmyY* constructs

Component of PCR	Volume (µl)
Template (*E. coli* culture or pure cosmid)	2
Forward primer (10µM)	1
Reverse primer (10µM)	1
One-taq® Quick load® PCR (NEB)	10
water	6
TOTAL	20

Initial denaturation	95 °C	4 mins	
Denaturation	95 °C	30 secs	35x
Annealing	58 °C	30 secs	
Extension	72 °C	1 min	
Final extension	72 °C	10 mins	
End and storage	4 °C		

The remaining culture from wells which gave the expected bands after agarose gel electrophoretic analysis of the PCR products was transferred into 10ml LB containing apramycin and grown overnight at 37 °C. The mutated constructs were then isolated from the overnight cultures by miniprep. The PCR was repeated using the isolated constructs directly as templates, followed by sequencing using the PCR screening primers for *mmyQ* and *mmyY* to confirm gene deletion.

E.coli ET 12567/pUZ8002 was transformed with the mutated constructs for onward transfer to *Streptomyces* spp. *via* conjugation (see section 5.11.3).

5.13 Cloning and expression of *mmyD* in *E. coli* BL21*

mmyD was amplified from the construct C73_787/*mmyR*::apr using Phusion high fidelity DNA polymerase and primers *mmyD*-fwd and *mmyD*-rev in Table 5.5. It was ligated into pET151 vector following guidelines provided in the Champion^{TM} pET TOPO® Expression manual.[244] 5 µl of the mixture from ligation was used to transform electrocompetent TOP10 *E. coli* cells and selection was done on LB plates supplemented with 100 µg/ml ampicillin. Colonies were grown overnight in liquid LB containing ampicillin and were screened by PCR using the *mmyD*-fwd primer and the T7 reverse primer (Table 5.5). Plasmid DNA was purified by miniprep from overnight cultures which gave the expected band in the PCR screening. The purified DNA was sequenced to confirm the presence of the cloned gene in the right orientation. The resulting construct pET151/*mmyD* was named pGI003.

E.coli BL21* was transformed with pGI003, single colonies were selected with ampicillin on LB plates, and passed through liquid LB overnight at 37 ^{o}C. 2 ml of the overnight culture was used to inoculate 100 ml of M9 medium and the mixture incubated at 37 ^{o}C until O.D of 0.7 at 600 nm. IPTG was then added to a final concentration of 0.5 mM to induce *mmyD* expression. The culture was further incubated overnight at 15 ^{o}C, centrifuged at 10,000 rpm for 10 mins and the supernatant was adjusted to pH 3 with concentrated HCl. The supernatant was extracted with 150 ml ethyl acetate, then dried and dissolved in methanol for LC-MS analysis. The wild type *E. coli* BL21* was grown simultaneously in M9 medium containing 0.5 mM IPTG and the extract analysed by LC-MS for comparison with the *E.coli* BL21*/pGI003 culture extract.

5.14 Purification of compounds

5.14.1 Purification of methylenomycin C (17)

Fourteen square petri plates, each containing 50 ml of SMMS medium, were inoculated with spore suspensions of *S. coelicolor* W108 (M145 with C73_787/*mmyR*::apr/Δ*mmyF*) and were incubated for 36 hours at 30 ^{o}C. The plates were then combined and acidified to pH 2 with 2M HCl solution prior to extraction with 800ml ethyl acetate. The organic extract was separated from the agar by filtration and was dried over magnesium sulphate. The solvent was removed under vacuum and the residue was dissolved in 2 ml of methanol. Methylenomycin C was purified from this solution by preparative HPLC (section 5.2.1). The retention time (R_T) was 6.5 minutes. ^{1}H and COSY NMR were consistent with those previously assigned for methylenomycin C (**17**).[173]

δ_H (400 MHz, $CDCl_3$), 1.84 (s, 3H, H-8), 2.14 (s, 3H, H-9), 4.10 (s, 1H, H-1), 5.67 (d, 1H, H-7b, J = 1.9 Hz), 6.22 (d, 1H, H-7a, J = 1.9 Hz) ppm; observed LC-HR-MS (positive mode) *m/z* = 167.0708 as $[M + H]^+$ (Calculated for $[C_9H_{11}O_3]^+$: 167.0703).

5.14.2 Purification of methylenomycin A (16) from strain W303

W303 (*S. albus*/C73_787 *mmyR*::*apr*) was inoculated on 18 plates of SMMS, each containing *ca.* 50 ml of the medium. The plates were incubated at 30 ^{o}C for 6 days, combined and acidified to pH 3 with 2M HCl before extraction with 1 litre of ethyl acetate. The agar was separated by filtration and the resulting organic extract was dried over magnesium sulphate. Ethyl acetate was then removed on a rotary evaporator and methylenomycin A (20 mg) was purified from the residue by flash column chromatography, R_f = 0.32 (silica, toluene/acetic acid 9:1). The purified

product was confirmed by ^{1}H and COSY NMR analysis and data obtained were consistent with methylenomycin A (**16**) as determined previously.[173]

δ_H (400 MHz, $CDCl_3$), 1.43 (s, 3H, H-8), 1.52 (s, 3H, H-9), 3.77 (s, 1H, H-1), 5.60 (d, 1H, H-7a, J = 1.5 Hz), 6.20 (d, 1H, H-7b, J = 1.5 Hz) ppm; observed LC-HR-MS (positive mode) *m/z* = 183.0650 as $[M + H]^+$ (Calculated for $[C_9H_{11}O_4]^+$: 183.0652).

5.14.3 Purification of pre-methylenomycin C lactone (27) and conversion to pre-methylenomycin C (26)

Pre-methylenomycin C lactone (**27**) was purified by flash column chromatography, R_f = 0.42 (silica, toluene/ethanol 9:1), from a 1-Litre SMMS culture of *S. coelicolor* strain W86 (M145 with C73_787/*mmyR*::apr/Δ*mmyE),* following the same procedure used to overproduce and purify methylenomycin A. Alternatively, the compound was purified from the crude extract by preparative HPLC method (R_t = 6.2 minutes).

The pre-methylenomycin C lactone was dissolved in $CDCl_3$ and ^{1}H and 2D-NMR experiments were performed. Data obtained were fully consistent with the the structure of **27** as assigned previously.[174]

δ_H (400 MHz, $CDCl_3$), 1.73 (s, 3H, H-8), 2.26 (s, 3H, H-9), 3.34 (d, 1H, H-2, J = 7.27 Hz), 3.66 (d, 1H, H-1, J = 7.28 Hz), 4.42 (dd, 1H, H-7b, J = 3.29 Hz), 4.57 (t, 1H, H-7a, J = 9.68 Hz) ppm, observed LC-HR-MS (positive mode) *m/z* = 167.0700 as $[M + H]^+$ (Calculated for $[C_9H_{11}O_3]^+$: 167.0703).

A 4 mg portion of the purified compound was dissolved in 1ml THF and hydrolysed to the free acid with 1ml of 10mM NaOH by stirring the mixture overnight at room temperature. The THF was evaporated and the remaining aqueous mixture was placed under high vacuum for several hours until all the liquid was removed to leave a

colourless residue of pre-methylenomycin C (**26**). The residue was dissolved in D_2O and ^1H and 2D-NMR experiments were carried out. Data obtained were fully consistent with those previously assigned for **26**. [174]

δ_H (400 MHz, D_2O), 1.62 (s, 3H, H-8), 2.01 (s, 3H, H-9), 2.80 (q, 1H, H-2, J= 6.6 Hz), 3.67 (d, 1H, H-1, J = 6.6 Hz), 3.79 (dd, 1H, H-7b, J= 5.90 Hz), 3.89 (dd, 1H, H-7a, J = 12.59 Hz) ppm, observed LC-HR-MS (positive mode) *m/z* = 167.0701 as $[M + H - H_2O]^+$ and 207.0629 as $[M + Na]^+$ (Calculated for $[C_9H_{11}O_3]^+$: 167.0703 and $[C_9H_{12}O_4Na]^+$: 207.0630 respectively)

5.14.4 Purification of methylenomycin D1 (28) and methylenomycin D2 (29)

Crude extract was obtained from *S. coelicolor* strain W108 (M145 with C73_787/*mmyR*::apr/Δ*mmyF*) following the same procedure as for methylenomycin C purification (section 5.14.1), with the exception that incubation was continued for 7 days. Methylenomycin D1 and D2 were first separated from the crude extract by flash column chromatography (silica, toluene/acetic acid 9:1). The solvent was evaporated under vacuum and the mixture of diastereoisomers was re-dissolved in 1.5 ml of methanol. The two compounds were thereafter separated by preparative HPLC (section 5.2.1).

Methylenomycin D1(**28**) eluted from the column at 5.9 minutes while methylenomycin D2 (**29**) eluted at 6.7 minutes. 2mg of methylenomycin D1 was finally obtained while methylenomycin D2 was 4mg. The compounds were dissolved in $CDCl_3$ and confirmed by NMR analysis as previously reported.[174]

Methylenomycin D1 (**28**): δ_H (400 MHz, $CDCl_3$), 1.25 (d, 3H, H-7, J = 7.48 Hz), 1.78 (s, 3H, H-8), 2.07 (s, 3H, H-9), 2.72 (m, 1H, H-2), 3.79 (d, 1H, H-1, J = 7.28 Hz) ppm; LC-HR-MS (positive mode) *m/z* = 169.0862 as $[M + H]^+$ (Calculated for $[C_9H_{13}O_3]^+$: 169.0859).

Methylenomycin D2 (**29**): δ_H (400 MHz, $CDCl_3$), 1.27 (d, 3H, H-7, J = 7.42 Hz), 1.78 (s, 3H, H-9), 2.11 (s, 3H, H-9), 2.66 (m, 1H, H-2), 3.21 (s, 1H, H-1) ppm; LC-HR-MS (positive mode) *m/z* = 169.0860 as $[M + H]^+$ (Calculated for $[C_9H_{13}O_3]^+$: 169.0859)

5.15 Feeding experiments

5.15.1 Feeding of methylenomycin C (17) to W301 and W302

70 µl each of *S. coelicolor* strains W301 (M145/pOSV556/*mmyOF*/pIJ86*mmr*) and W302 (M145/pIJ86*mmr*) from glycerol stock were used to inoculate Alamm (pH 5) plates containing sterile semi-permeable membrane. After 48 hours of incubation at 30 ^oC, 200 µl of a solution of methylenomycin C in DMSO (1 mg/ml) was fed in drops to each strain using a pipette. The plates were further incubated for 4 days before the cells were scrapped off the membrane and suspended in 20 ml of methanol in conical flasks. These were left for about 2 hours with intermittent shaking to dissolve compounds. 1ml of the extract was taken in each case and was passed through column centrifuge filters at 8,000 rpm prior analysis by LC-MS.

Feeding experiments with other methylenomycin C analogues/intermediates and strains were carried out similarly.

5.15.2 Feeding of ^{13}C-labelled D-ribose to *Streptomyces coelicolor* strains expressing *mmy* gene cluster

[U-^{13}C]-D-ribose was purchased from Cambridge Isotope Laboratories (Andover, USA). The supplemented minimal medium was modified for the feeding experiment

in order to observe significant incorporation of the labelled precursor: D-Glucose was reduced from 1 % to 0.2 % (w/v) and 0.2 % casamino acids was used instead of 0.25 % (w/v) L-Alanine.

25 ml of the modified SMM medium was inoculated separately with 100 µl of strain W108 (M145/C73_787/*mmyR*::apr/Δ*mmyF*) and W100 (M145/C73_787/*mmyR*:: apr/Δ*mmyO*), as well as the heterologous host M145, both directly from -80 ^{o}C glycerol stocks. After incubation at 30 ^{o}C for 6 hours in a 180 rpm shaker, 1ml of sterile water containing 25 mg of [U-^{13}C]-D-ribose was added to each flask. Incubation was continued for an additional 72 hours before the cultures were filtered and the filtrate adjusted to pH 3 with 2 M HCl. The filtrates were then extracted with an equal volume of ethyl acetate, dried on a rotary evaporator and dissolved in an appropriate amount of methanol for analysis by LC-MS.

5.16 Microbiological assays

5.16.1 Determination of the minimum inhibitory/bactericidal concentration (MIC/ MBC)

The minimum inhibitory concentrations (MICs) of methylenomycin compounds against Gram-positive and Gram-negative strains were determined by broth microdilution in 96-well microtiter plates according to the CLSI guidelines.[101,245] Cells growing in exponential phase were diluted to *ca.* 10^5 CFU/ml into cation-adjusted Mueller-Hinton broth (MHB) before addition of methylenomycin compounds to final concentrations ranging from 0.5 to 512 µg/ml in 100 µl culture aliquots. 50 times concentrated stocks of methylenomycin compounds to give the final concentrations upon dilution were prepared in DMSO and control experiments with only DMSO added to the wells were carried out in all cases. *Enterococci* strains were

grown and diluted with Medium 92. Strains were incubated for 20 hours (or up to 48 hours in the case of *Streptomyces* spp. and yeast) before visual inspection for growth. Minimum bactericidal concentrations (MBCs) were determined with the AlamarBlue™ fluorescence assay. 10 µl of the AlamarBlue™ dye was added to each well from the MIC plates and were further incubated for 2 hours. Fluorescence measurements were recorded at excitation wavelength (530 nm) and emission wavelength (590 nm) on a BioTek Synergy HT multi-detection microplate reader. MBCs correspond to the point at which sudden fall in the fluorescence-concentration curve was observed, and were further confirmed by sub-culturing wells at such concentration levels onto antibiotic-free agar plates and incubating for up to 3 days with no visible growth.

5.16.2 Development of resistant strains of *Enterococcus faecium* (64/3) *via* the sequential method

In order to investigate whether *E. faecium* (64/3) could develop resistance to pre-methylenomycin C and pre-methylenomycin C lactone, this strain was passed sequentially through increasing levels of the antibiotics beginning from sub-lethal concentrations. Vancomycin, an antibiotic to which *Enterococcus* strains often develop resistance, [246,247] was used as the control in a parallel experiment. The MICs of these compounds against *E. faecium* 64/3 were first determined as detailed in section 5.16.1. MIC-type assays were then set up with 0.25 x MIC, 0.5 x MIC, 1 x MIC, 2 x MIC and 4 x MIC final concentrations of the three compounds and incubated overnight.[101] Culture medium containing the highest concentration of each compound that allowed active growth of the bacterial cells was diluted 100 times with fresh M92 medium and the diluted cultures were used to re-set up the experiment which were again incubated overnight. This procedure continued for 28 consecutive days while

the concentration of compounds in the assay was consistently increased in instances where the strain was already able to survive the initial highest concentration of 4 x MIC.

At every 4 days during the 28-day lifetime of the run, 10 µl of culture from the highest concentration well which allows growth was streaked onto antibiotic-free M92 agar plates and grown overnight. 5 colonies were then selected from each plate, grown overnight in M92 broth and MIC of the strains was determined by the normal single step standard procedure. This was used to confirm whether the selected colonies had MICs above the parent *E. faecium* strain (resistance acquired).

5.16.3 Bioluminescence experiment with the luciferase reporter strains

Bacillus subtilis strains carrying the promoter regions of *yorB*, *yvgS*, *yheI*, *ypuA*, and *fabHB*, each fused to a firefly luciferase (reporter) gene, were originally constructed by Urban *et al*.[213] Erythromycin is used for routine growing of the strains while ciprofloxacin, rifampin, linezolid, vancomycin, and cerulenin are the standard antibiotics known to induce the *yorB*, *yvgS*, *yheI*, *ypuA*, and *fabHB* promoters respectively.

All methylenomycin compounds used for the assay were purified as detailed in the previous sections. Solutions of methylenomycin compounds and the standard antibiotics were prepared by dilution from an initial 1 mg/ml stock of each compound in DMSO.

The strains were incubated at 37 ^{o}C in liquid LB medium containing 5 µg/ml erythromycin to an O.D$_{600}$ of 0.9; only the *yheI* biosensor strain was grown in Belitzky minimal media (BMM) containing 5 µg/ml erythromycin to the same O.D value. The strains were then diluted with either LB or BMM as applicable to different O.D$_{600}$

values: 0.01, 0.02, 0.1, 0.02 and 0.25 for *yorB*, *yvgS*, *yheI*, *ypuA* and *fabHB* respectively, and were kept at 4 ^{o}C overnight. The following morning, 80 µl of each strain was incubated in a 96-well plate at 37 ^{o}C with 1 µl solution of methylenomycin compounds or the standard antibiotics from stock solutions of 25 µg/ml, 6.25 µg/ml and 1.56 µg/ml. Incubation times differ for the different strains and they were: 1 hr (*ypuA*), 1.5 hrs (*yvgS*), 3 hrs (*yorB* and *fabHB*) and 4 hrs (*yheI*).[213,214]

After incubation, 50 µl of 0.1M Citrate buffer containing 2 mM luciferin was added to each well and the plate was swirled gently before measuring Luminescence for 30 seconds.

5.16.4 Assay with the β-galactosidase reporter strains

β-galactosidase *Bacillus* reporter strains in which the promoter regions of *yvgS*, *yheH*, *yvgI*, *ypuA*, and *yjaX* were fused to the β-galactosidase gene were obtained from Demuris Limited, Newcastle, UK. Erythromycin antibiotic was used for routine growing and selection of the strains, while rifampicin, chloramphenicol, bacitracin, vancomycin and triclosan were used as the positive control antibiotics inducing the *yvgS*, *yheH*, *yvgI*, *ypuA*, and *yjaX* promoters respectively.

To carry out the assay, 1 % nutrient agar was prepared and supplemented with erythromycin and X-gal to 50 µg/ml and 100 µg/ml final concentrations respectively. 25 µl of each reporter strain was used to inoculate 25 ml of the nutrient agar while still warm, and the mixture was poured into petri dishes and allowed to solidify. A sterile filter was placed in the middle of each plate and 10 µl of a 3 mg/ml solution of a standard antibiotic or a methylenomycin compound (dissolved in DMSO) was placed on the filter. Control experiments with only DMSO placed on the filters were also set up simultaneously for each reporter strain. The plates were incubated for 48

hours at 27 ºC. A zone of inhibition with blue colouration around the circumference of inhibition indicated an induction of a given promoter.

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Appendices

Appendix 1: Data from sequencing of constructs pGI001 (C73_787/*mmyR*::*apr*/ Δ*mmyQ*) and pGI002 (C73_787/*mmyR*::*apr*/Δ*mmyY*)

Appendix 2: NMR spectra of purified methylenomycin compounds

Appendix 1

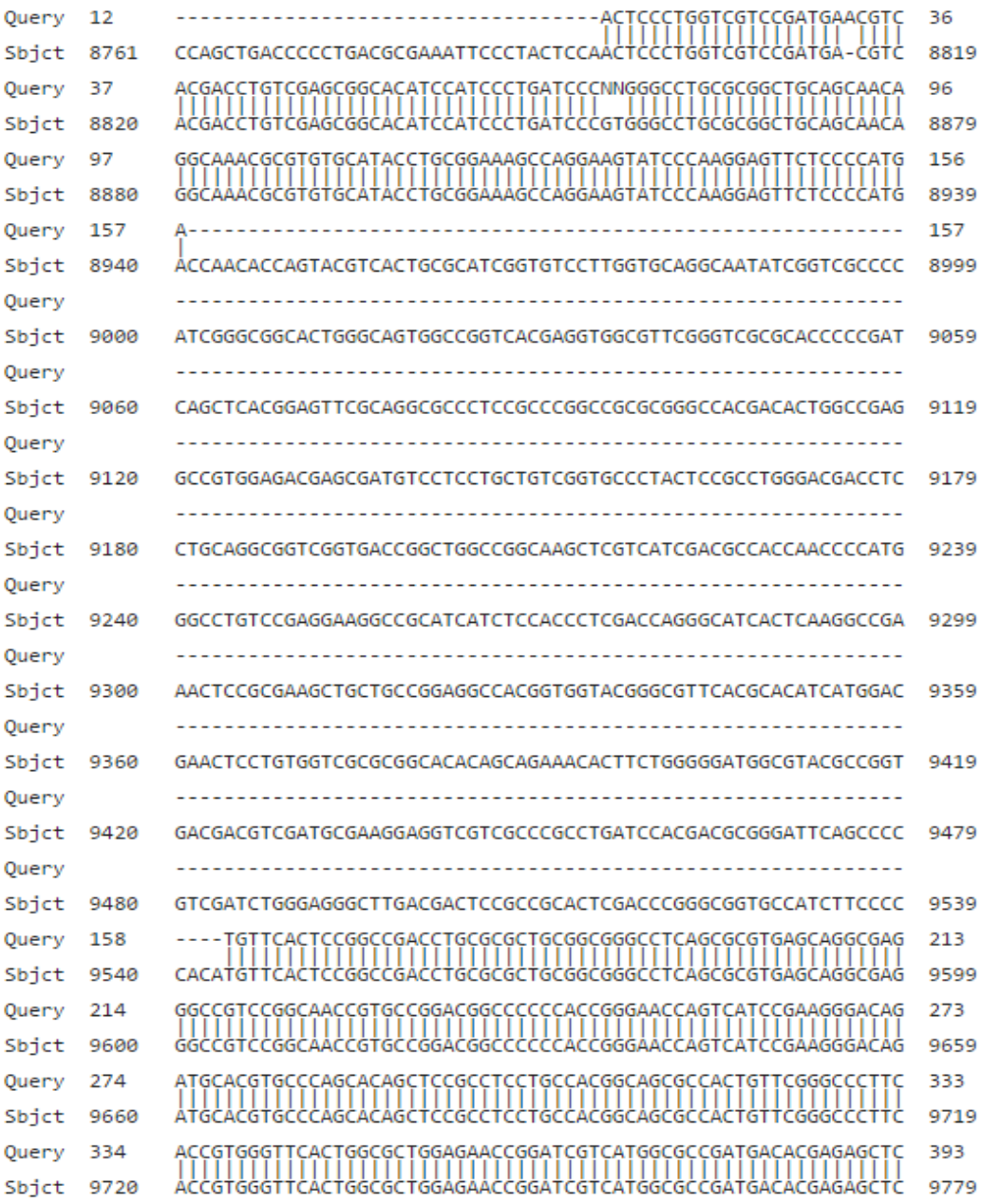

Fig. A1: Sequence alignment of the mutated construct C73_787/*mmyR*::*apr*/Δ*mmyQ* (Query) against the complete sequence of the C73_787/*mmyR*::*apr* cosmid (Subject). The missing nucleotides on the query sequence correspond to the 603 bp sequence of *mmyQ* which has been deleted by Cas9 editing.

Fig. A2: Sequence alignment of the mutated construct C73_787/*mmyR*::*apr*/Δ*mmyY* (Query) against the complete sequence of the C73_787/*mmyR*::*ap*r cosmid (Subject). The missing nucleotides on the query sequence correspond to the 375 bp sequence of *mmyY* which have been deleted by Cas9 editing.

Appendix 2: NMR spectra of purified methylenomycin compounds

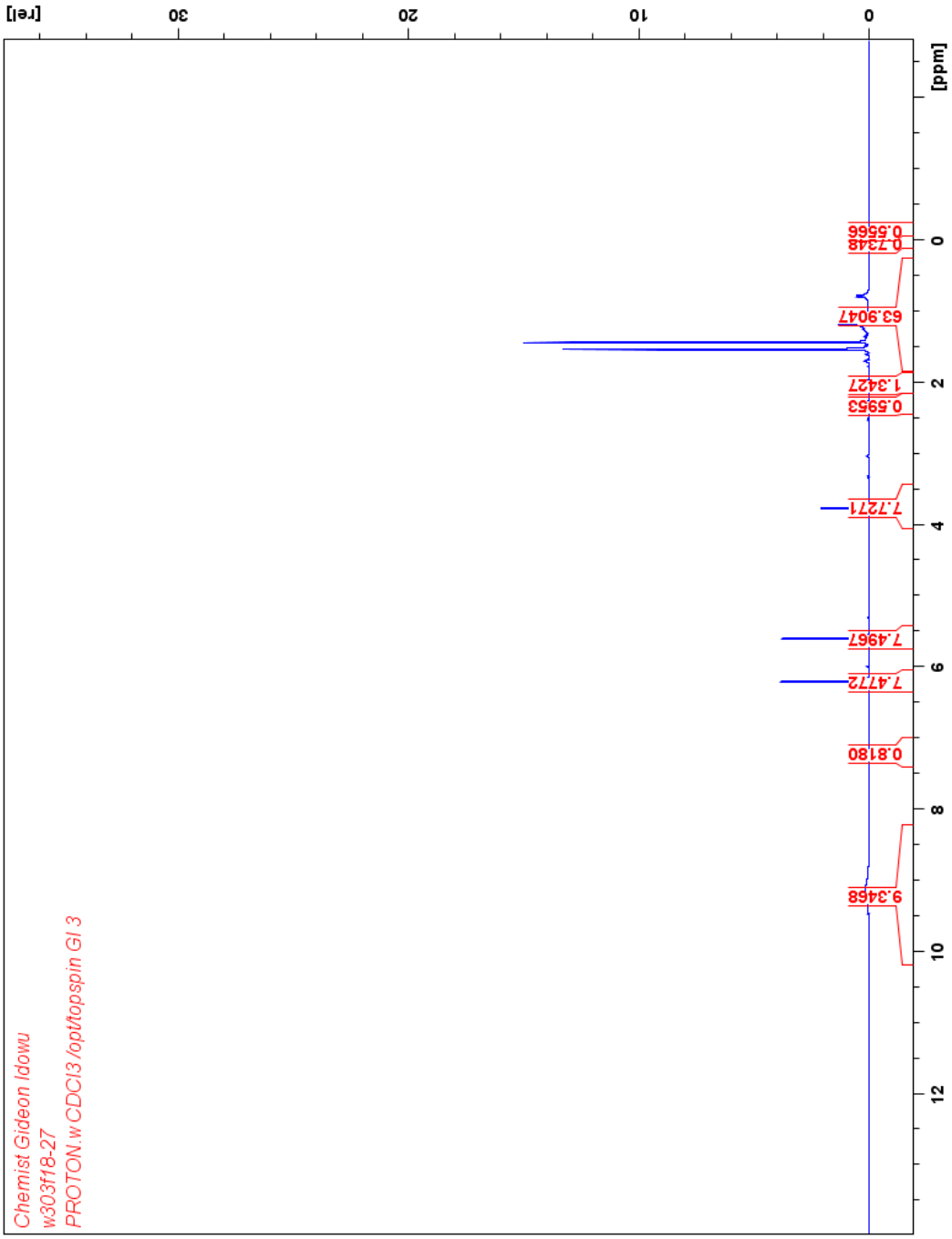

Fig. A3: ^{1}H NMR spectra of methylenomycin A (**16**)

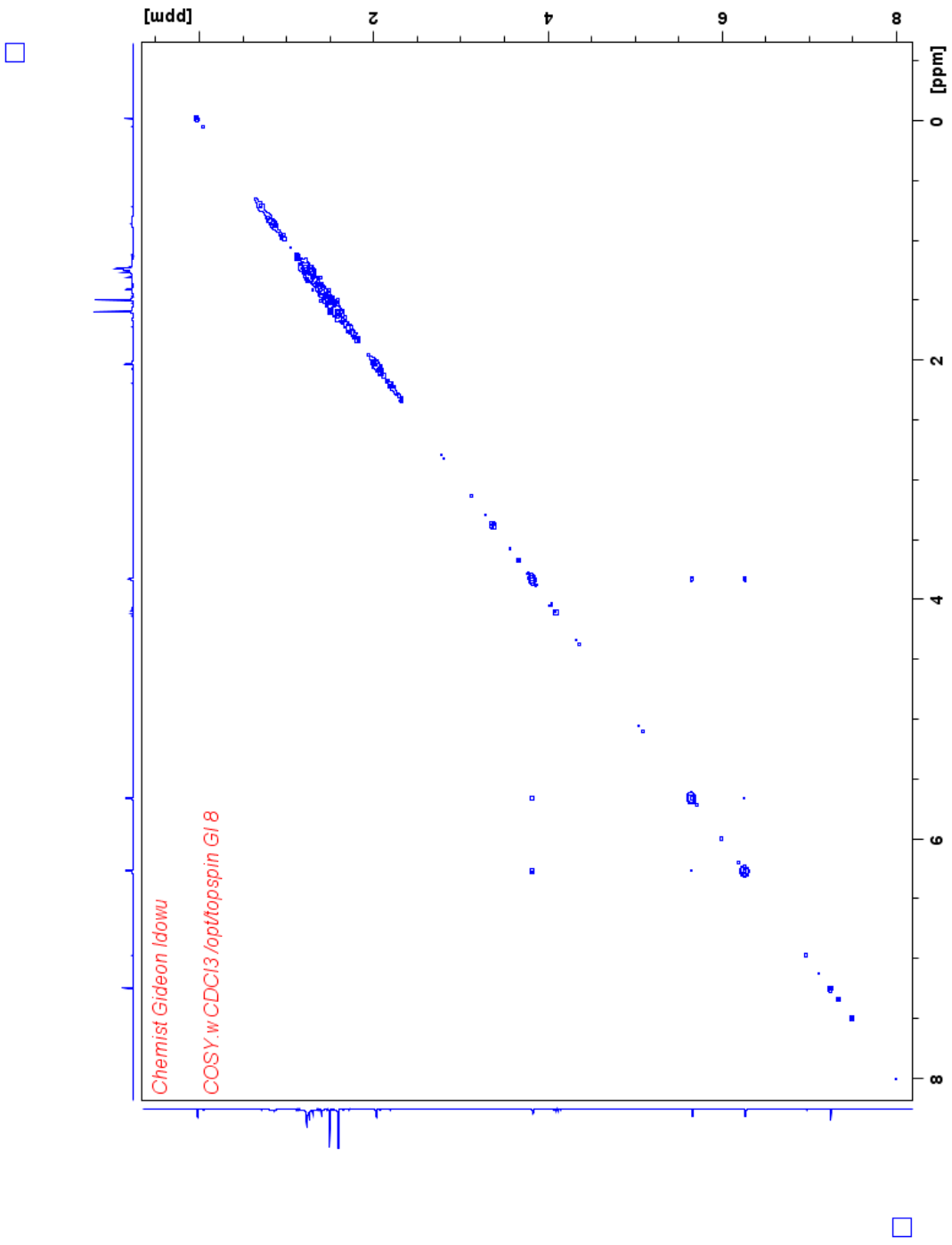

Fig. A4: COSY NMR spectra of methylenomycin A (**16**)

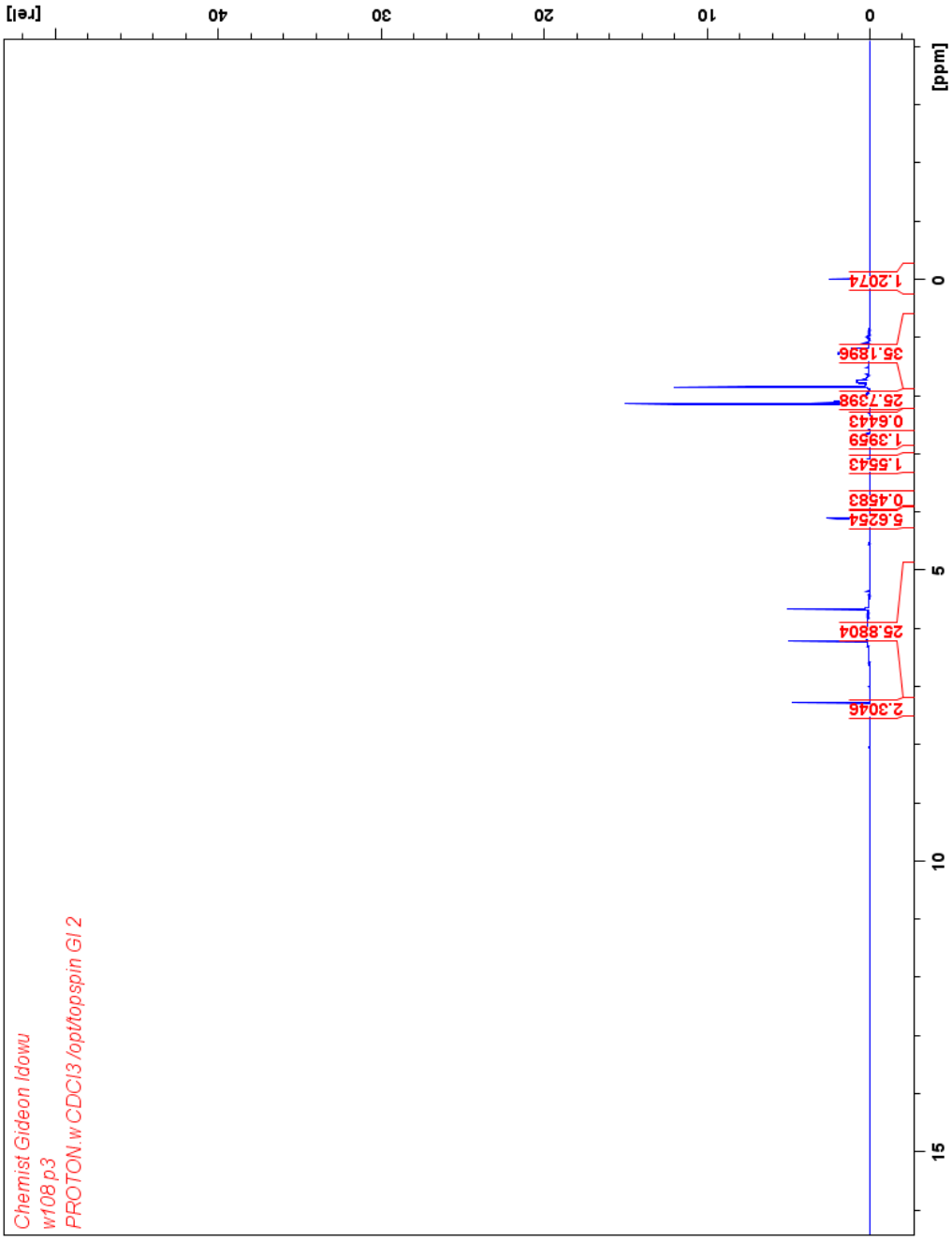

Fig. A5: ^{1}H NMR spectra of methylenomycin C (**17**)

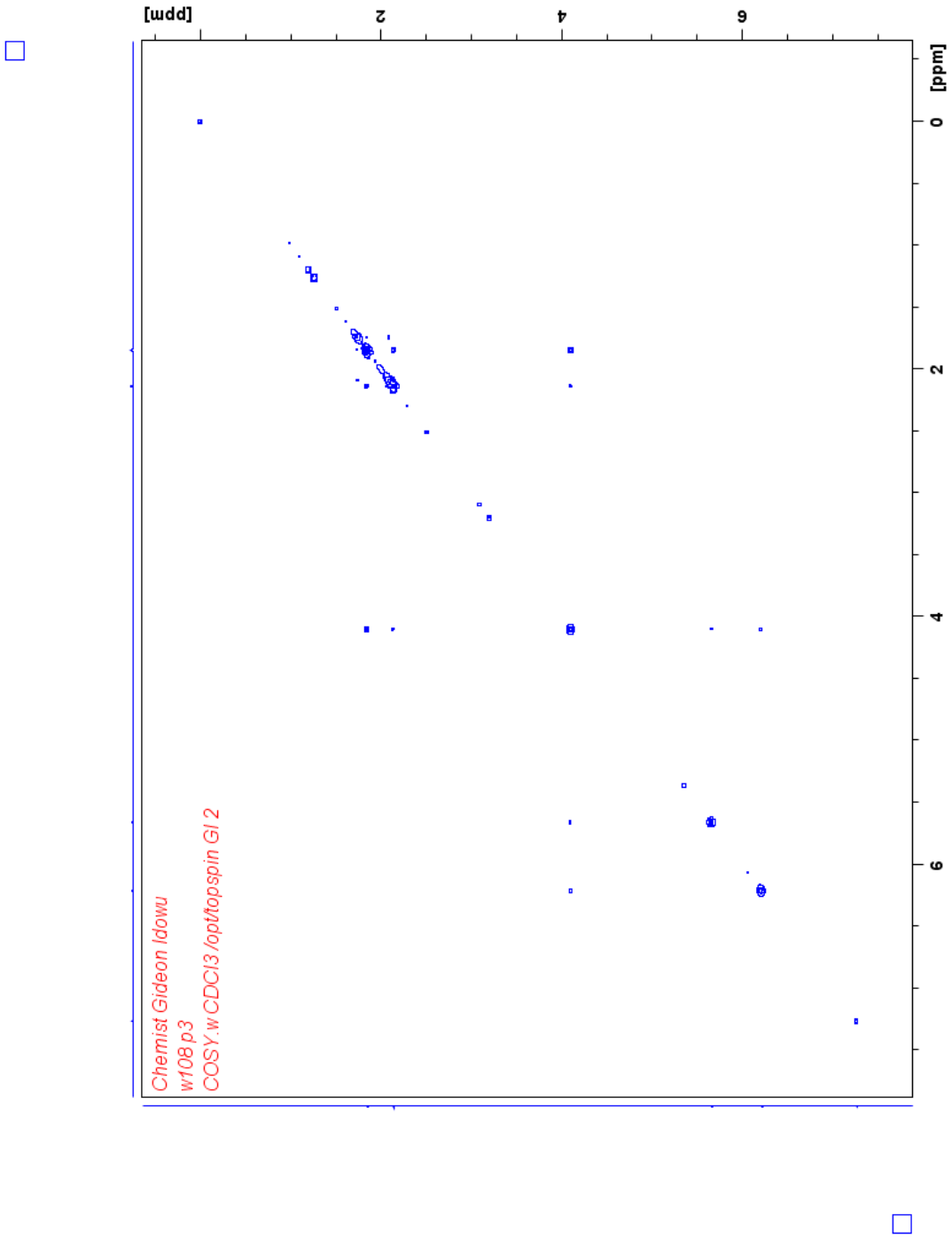

Fig. A6: COSY NMR spectra of methylenomycin C (**17**)

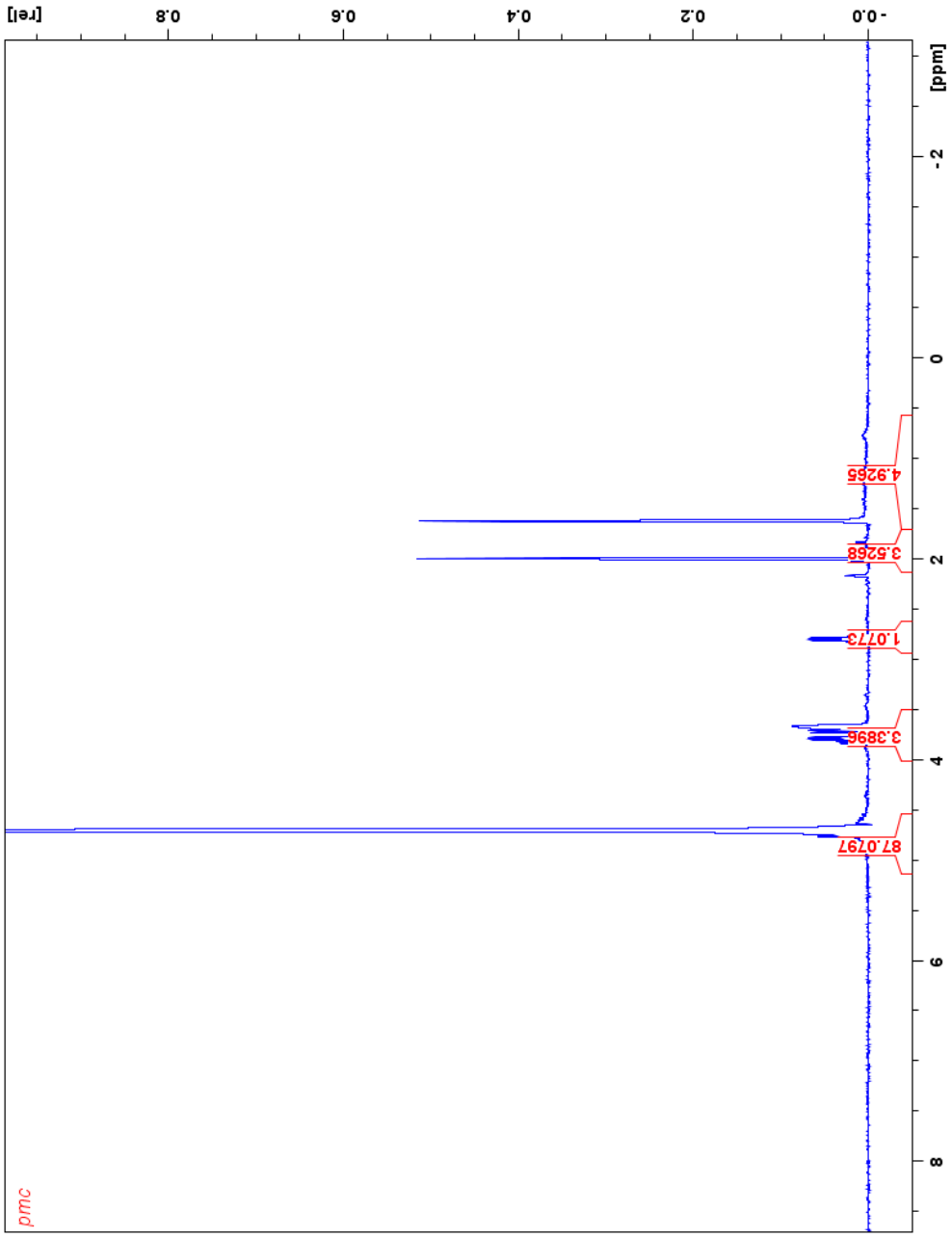

Fig. A7: ^{1}H NMR spectra of pre-methylenomycin C (**26**)

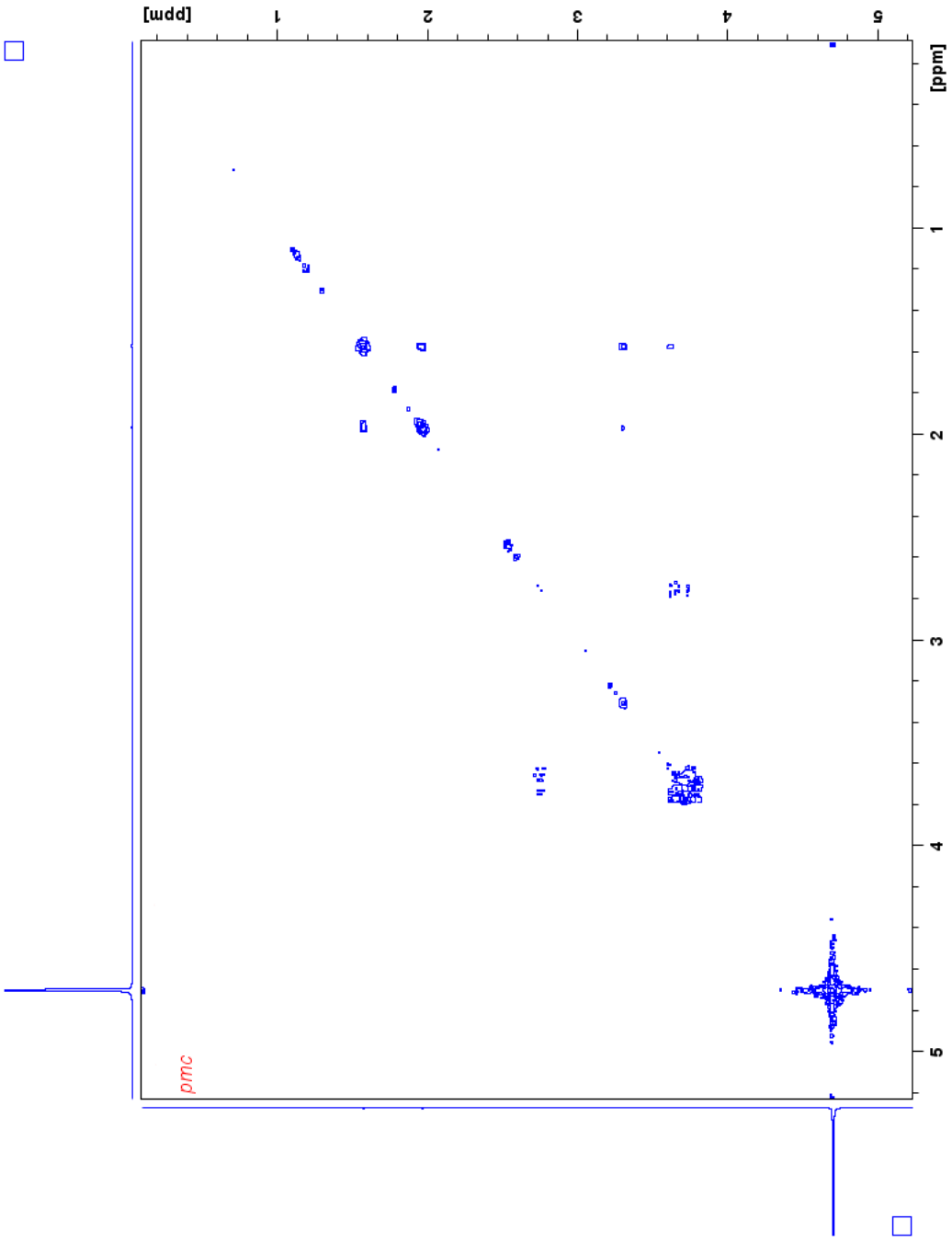

Fig. A8: COSY NMR spectra of pre-methylenomycin C (**26**)

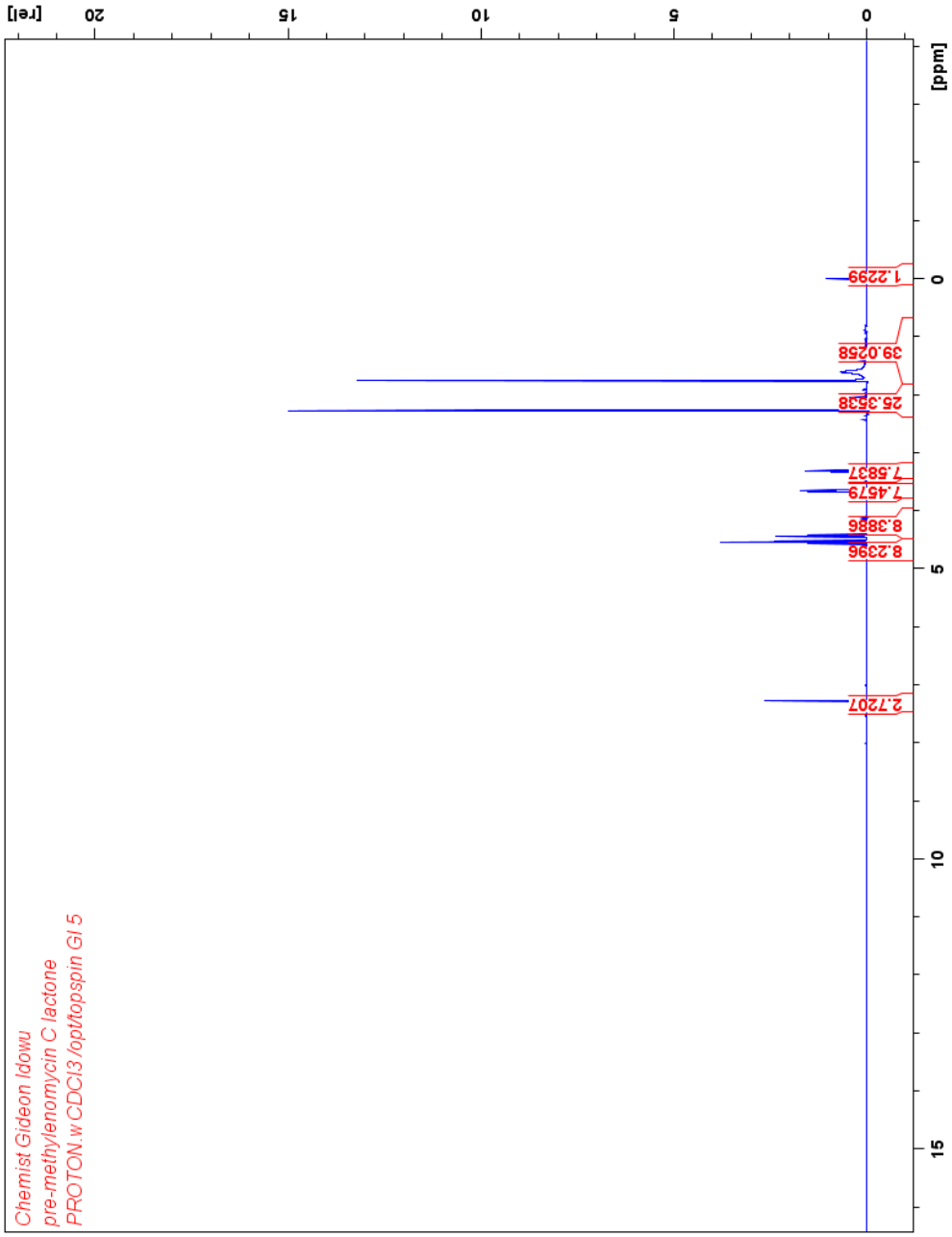

Fig. A9: ^{1}H NMR spectra of pre-methylenomycin C lactone (**27**)

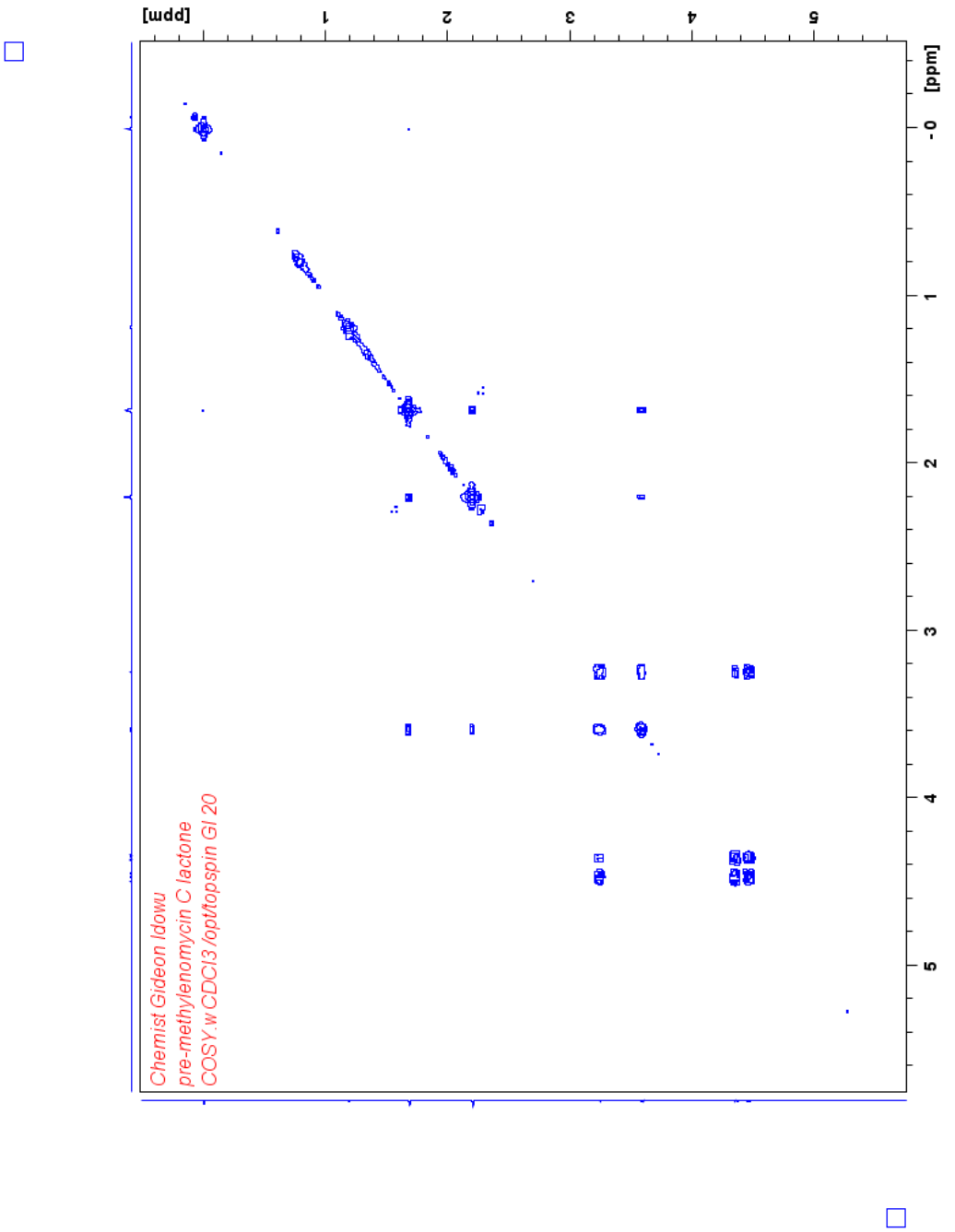

Fig. A10: COSY NMR spectra of pre-methylenomycin C lactone (**27**)

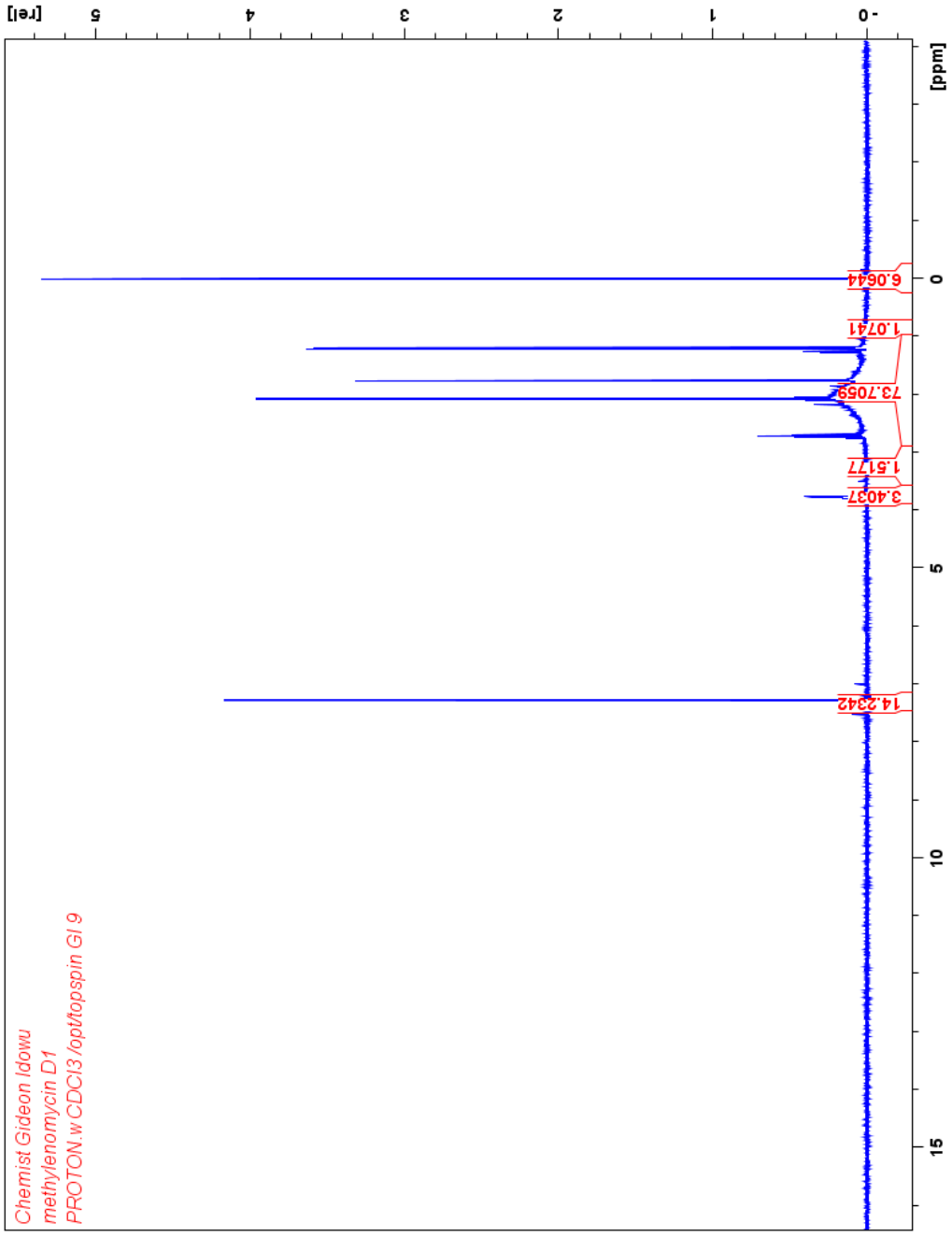

Fig. A11: ^{1}H NMR spectra of methylenomycin D1 (**28**)

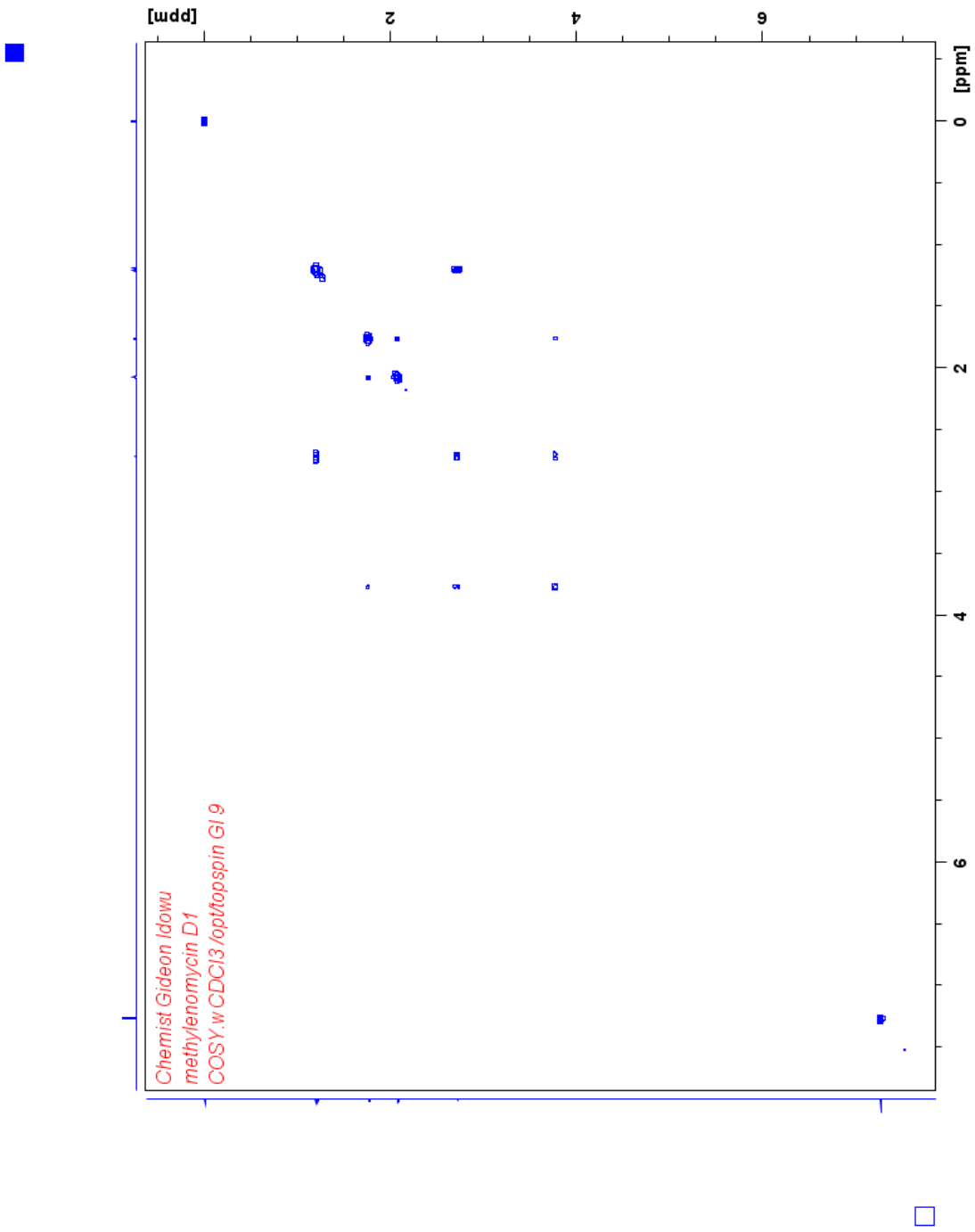

Fig. A12: COSY NMR spectra of methylenomycin D1 (**28**)

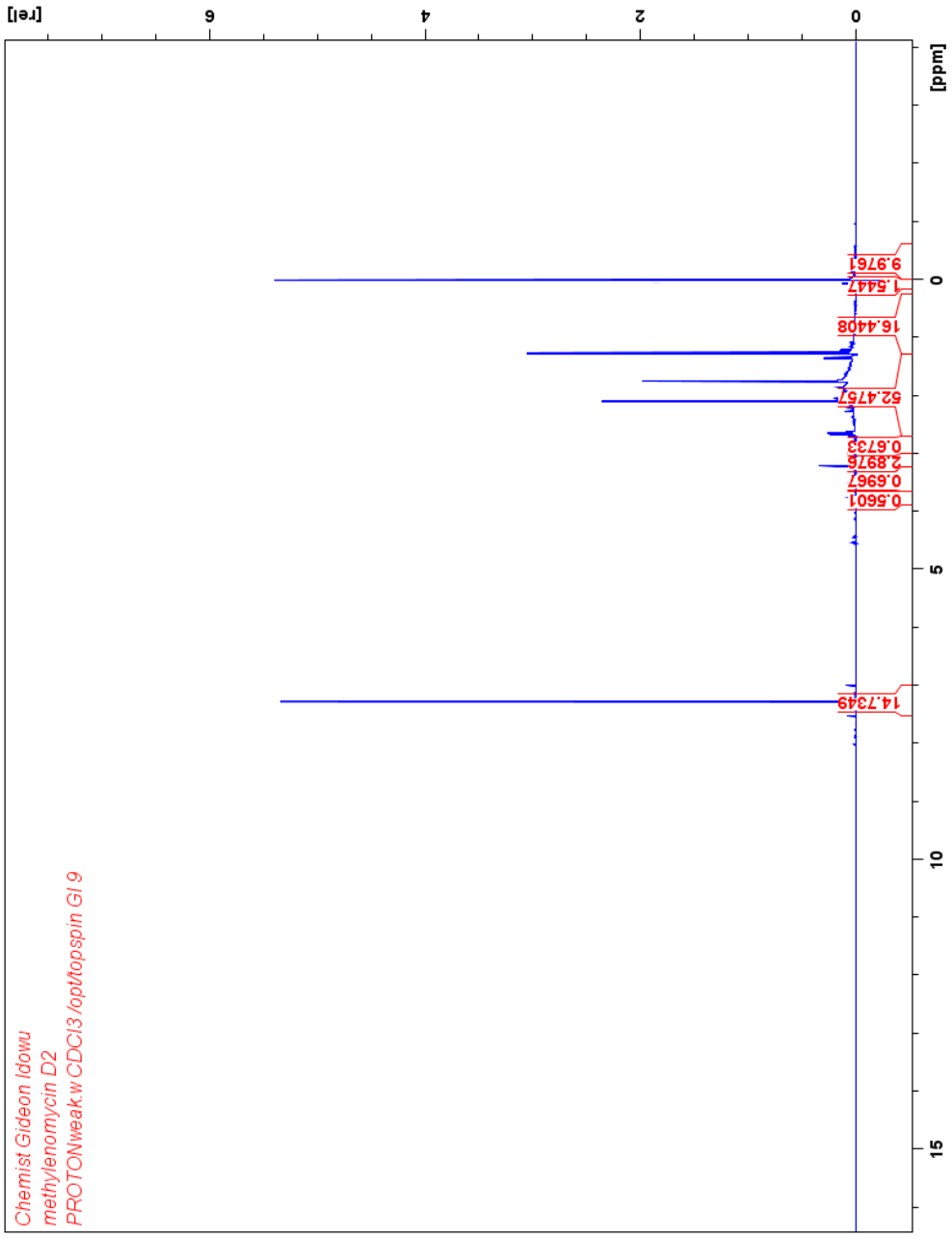

Fig. A13: ^{1}H NMR spectra of methylenomycin D2 (**29**)

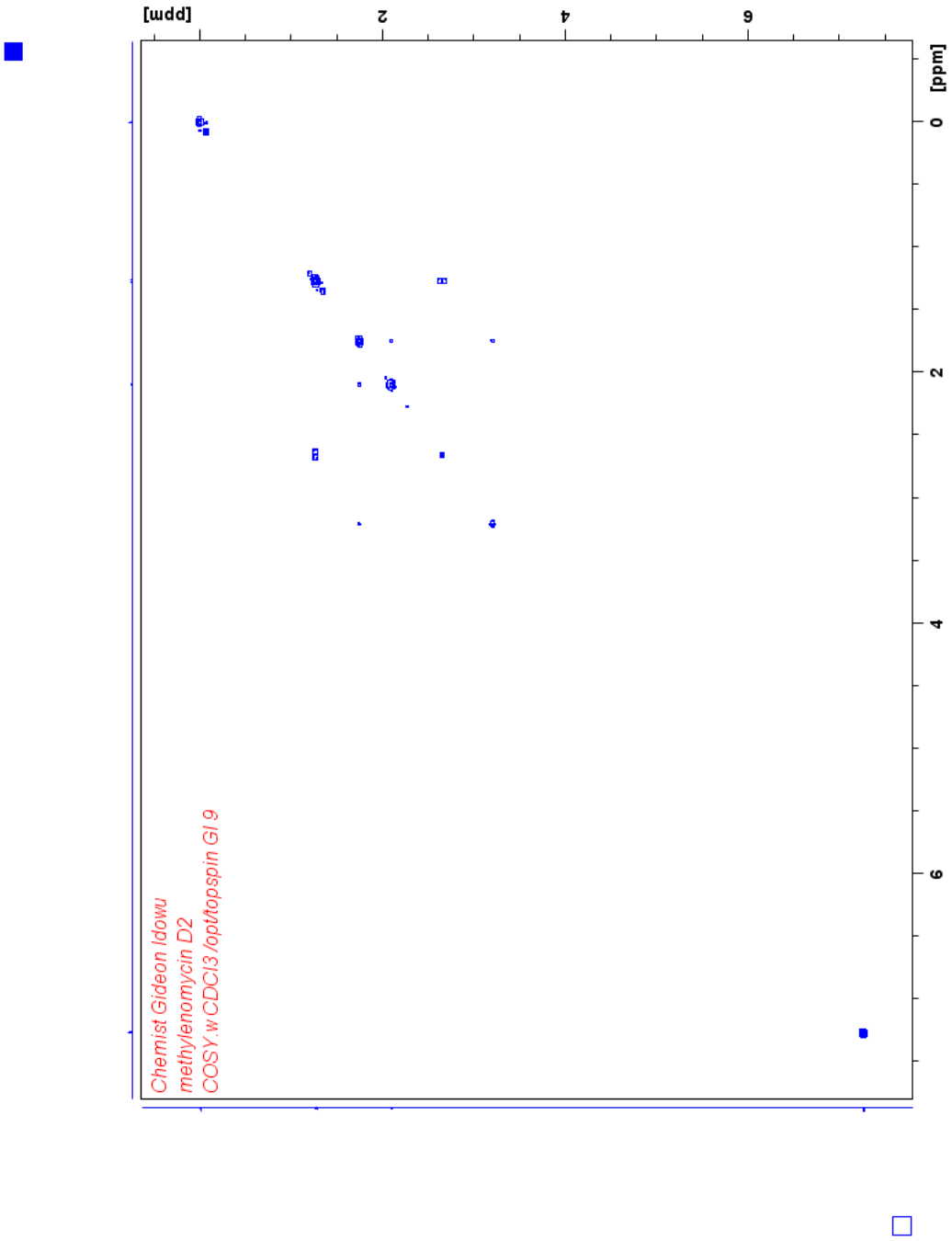

Fig. A14: COSY NMR spectra of methylenomycin D2 (**29**)