Novel Purification and Testing of the Antimicrobial Agent Moenomycin A Using Starbon® A300 and Enhancement of in vivo Efficacy with n-dodecyl-β-D-maltoside.

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Leon Erith[1], Rob McElroy[2] and Edward Taylor[3]

[1]Undergraduate Student Researcher
[2]Senior Lecturer in Sustainable Chemistry
[3]Associate Professor
School of Natural Sciences, Department of Life Sciences
University of Lincoln

Abstract

Antibiotic resistance is a growing global health crisis. This project aims to develop a new purification and delivery method for Moenomycin A, a novel antibiotic. The single-step purification method includes culturing Streptomyces viridosporus DSM 40746 on TSB media, purifying the extract and spent culture media utilising Starbon® A300 and measuring the purified extracts antimicrobial activity by quantifying its inhibitory effects on the growth of a Bacillus subtilis lawn. This project also addresses Moenomycin A's unfavourable binding with blood plasma proteins, a major limitation of Moenomycin A for clinical use. n-dodecyl-β-D-maltoside (DDM) and PEG400 were investigated to mitigate this binding in order to decrease Moenomycin A's minimum inhibitory concentration (MIC).

Keywords: Antibiotics, Multi-Drug Resistance (MDR), Moenomycin A, Novel Purification Methods, Starbon, *Streptomyces,* n-dodecyl-β-D-maltoside (DDM).

Introduction

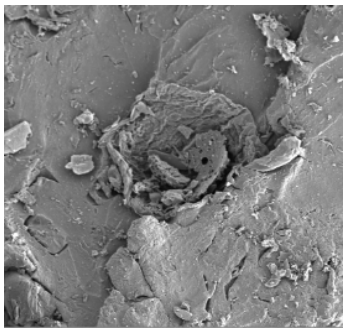
The Undergraduate Research Opportunities Scheme (UROS) at the University of Lincoln is a competitive bursary designed to actively engage undergraduate students in research across all disciplines. This scheme embodies the University's 'Student as Producer' approach to teaching and learning, providing undergraduates with invaluable hands-on research experience alongside academics, allowing them to take on research and author roles and gain insight into the higher education industry before graduation (University of Lincoln, 2024). This UROS-funded project will allow a deeper insight into

the field of antimicrobial development, a career option I am interested in, while learning valuable lab skills for future employment / 3rd year dissertation. The project further allows for a better understanding of the issues posed when working in a lab. A delivery of *Streptomyces viridosporus* was delayed until after the project's end date, forcing the project to pivot and work around these unforeseen issues.

Project Background

Antibiotic resistance is the method by which bacteria evolve immunity to certain antimicrobial agents (Benz and Hall, 2023), reducing their effectiveness. This poses a critical issue as bacterial infections that were previously easily treatable (such as tuberculosis, pneumonia and MRSA (Methicillin-resistant Staphylococcus aureus)) are becoming increasingly difficult to treat (Church and Mckillip, 2021). The increase in the prevalence of MDR (multi-drug resistant) bacteria highlights the importance of developing novel antimicrobial agents (Liu et al, 2022).

Moenomycin A is a phosphoglycolipid antibiotic first isolated from *Streptomyces* species in the 1960s (Wallhausser et al., 1965). It represents a promising avenue in the fight against bacterial infections, particularly those exhibiting resistance to conventional treatments. Moenomycin A is emerging as a means to combat certain types of antimicrobial resistance, as it has been used in animal husbandry for more than 50 years with no noticeable resistance arising (Chen et al., 2019; Lloyd et al., 2020). This growing demand for pure Moenomycin A underscores the necessity for efficient purification methods for experimental purposes.



Moenomycin A possesses a hydrophobic lipid tail, a structural feature that this project aims to exploit for the development of a single-step purification strategy utilising Starbons (*figure 1*) (Attard et al., 2022). Moenomycin A's poor pharmacokinetic properties are primarily attributed to its amphiphilic C25 isoprenoid lipid chain, which has historically prevented its clinical application in humans due to blood plasma protein binding. This project investigates DDM and PEG400 as a means to bypass this.

Figure 1. Scanning Electron Microscope image of Starbon P800 at 1000x, highlighting the presence of macro pores (original figure).

Literature Review

Early studies elucidated Moenomycin's potent activity against Gram-positive bacteria, including key pathogens such as *Staphylococcus aureus* (10 to 1000 times more potent than Vancomycin, a commonly used antibiotic) against Gram-positive bacteria, highlighting Moenomycin's high efficacy (Lloyd et al., 2020), while demonstrating

limited efficacy against Gram-negative organisms due to permeability barriers (Huber, 1979).

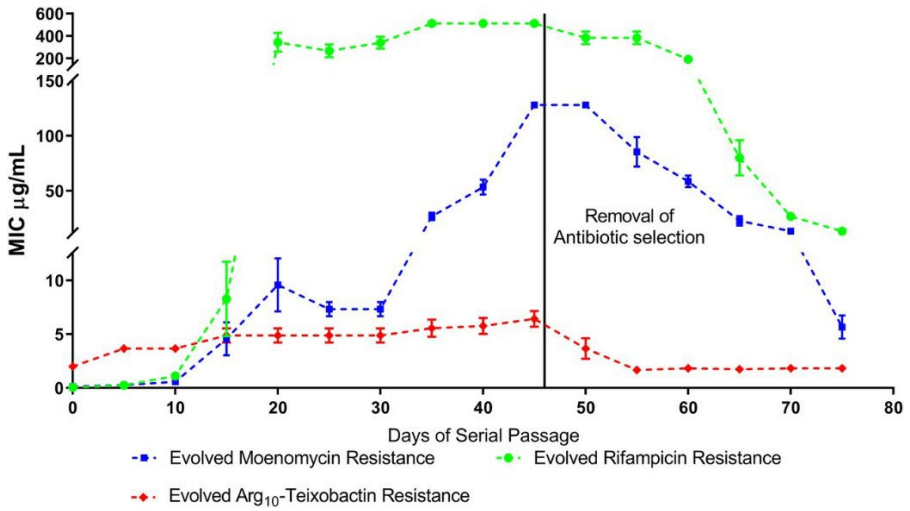


Figure 2. The development and loss of Arg10-teixobactin, Moenomycin A, and rifampicin resistance in *S. aureus* ATCC 33591 over a 75-day period. Antibiotic selective pressures were removed after 45 days (Lloyd et al., 2020).

Moenomycin A's resistance dynamics are between those of rifampicin and Arg10-Teixobactin (see *Figure 2)*. Evolved resistance to Moenomycin A is 8-fold less than the proportional increase in rifampicin resistance over the same 45-day period. From this, we can deduce that *S. aureus* evolves resistance much more slowly to Moenomycin than to rifampicin. When the selection pressure is removed, the resistance rapidly drops from the population (see *Figure 2)*. This suggests that the mutation giving rise to the resistance comes at a great metabolic cost to the organism; therefore, the establishment of an environmental resistance reservoir is less likely. This growing demand for pure Moenomycin A underscores the necessity for efficient purification methods for experimental purposes.

Moenomycin has a unique mechanism of action, targeting bacterial cell wall synthesis by inhibiting the bacterially ubiquitous GT5 transglycosylase enzymes involved in peptidoglycan polymerisation (*Figure 3*) (Walsh and Wencewicz, 2014; Lloyd et al., 2020), distinguishing it from many other classes of antibiotics.

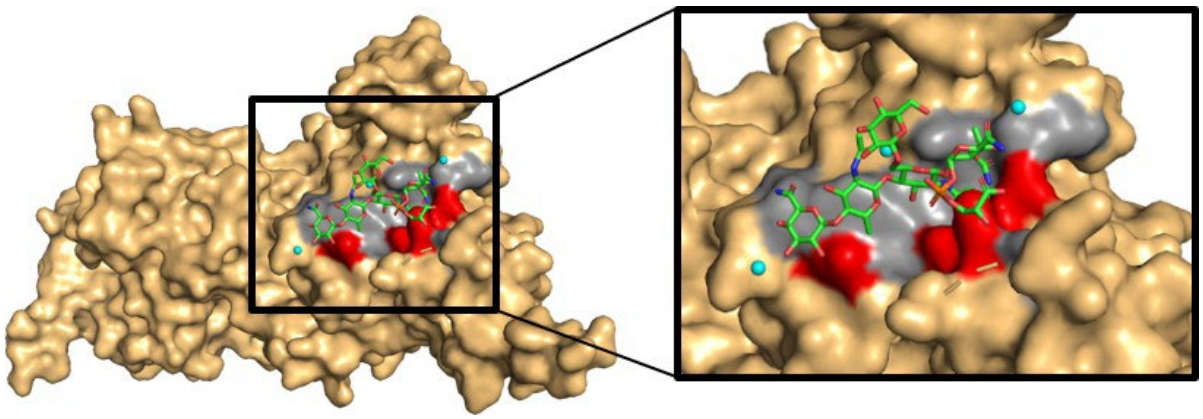


Figure 3. Moenomycin A (green) bound to *E. coli* PBP1b. Shown are key bonding amino acids (red), auxiliary amino acids (grey) and key water molecules (blue) (original figure using PDB structure 5HLD).

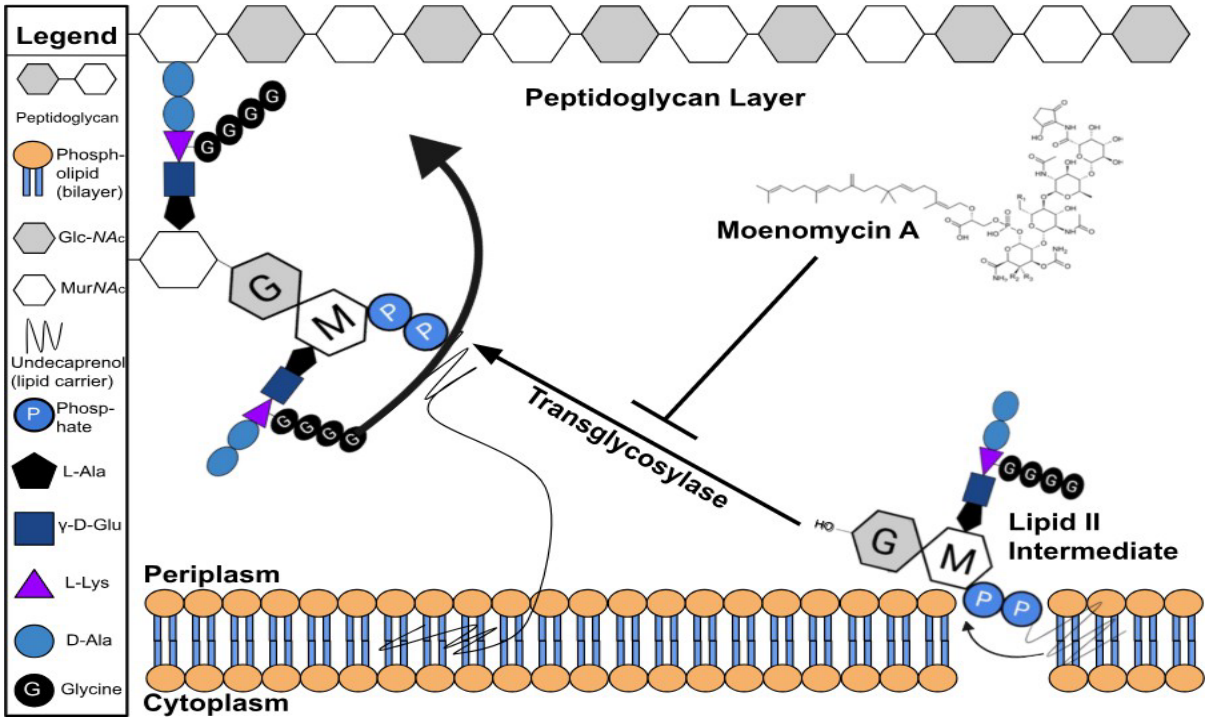


Figure 4. Moenomycin binds to class A penicillin-binding proteins (PBPs), inhibiting their transglycosylase activity (Cheng et al., 2008). This prevents the extension of the glycan chain to form a stable peptidoglycan layer. Moenomycin mimics and thus competes with the natural substrate of the enzyme (Walsh and Wencewicz, 2014). Original figure adapted from (Nakamura et al., 2013; Lloyd et al., 2020).

Methodology

A bioassay (using a 96-well plate to show the effect of antibiotics on bacterial growth) was performed with Moenomycin A concentrations ranging from 33.33 µg/ml to 0.03 µg/ml and Kanamycin from 16.66 µg/ml to 0.015 µg/ml, across columns 1 to 11. Column 12 served as a "No Antibiotic" growth control to confirm bacterial viability.

An intermediate row containing only LB Broth functioned as a sterility control, ensuring the medium wasn't contaminated. By observing the presence or absence of turbidity (bacterial growth), the Minimum Inhibitory Concentration (MIC) for each antibiotic was determined as the lowest concentration that inhibited visible growth.

An agar diffusion assay was used to ascertain the antimicrobial activity of Moenomycin A, Kanamycin, and Moenomycin A-induced Starbons (P300, P450, and P800). Three separate agar plates, inoculated with Bacillus subtilis, were incubated for 72 hours to observe zones of inhibition. Each plate contained three wells, into which 5 µL of 10 µg/mL Moenomycin A, 5 µL of 12.5 µg/mL Kanamycin and Moenomycin A-induced Starbons were loaded. The Starbons were prepared in an Eppendorf tube with 10 µL of 100 µg/mL Moenomycin A, followed by three sequential washes with 100 µL of deionised water and centrifuged at 13,000 rpm for 1 minute, with supernatant removal after each wash. The Starbons were then dried overnight in open Eppendorf tubes.

To establish a calibration curve for Moenomycin A, agar plates were prepared with a lawn of *Bacillus subtilis* (strain 168 (ATCC 23857)). Disks with 5µll of different concentrations of Moenomycin A (10 µg/ml to 0.0195 µg/ml). The plates were subsequently incubated for 24 hours. The diameters of the zones of inhibition around each well were measured, then plotted against the corresponding Moenomycin A concentrations to generate a calibration curve, which will enable the estimation of Moenomycin concentration in future agar diffusion assays.

To possibly mitigate the non-specific binding of Moenomycin A's amphiphilic lipid tail to blood plasma proteins, leading to loss of potency, two agents were incorporated into the assay system. MIC assays were conducted in a 96-well microplate, with each well containing 4% (w/v) Bovine Serum Albumin (BSA) to simulate physiological conditions (*in vivo*). Polyethylene Glycol 400 (PEG(400)), a hydrophilic polymer, was included at a final concentration of 5% (w/v) to assess its solubilising effect.

Additionally, the non-ionic detergent n-dodecyl-β-D-maltoside (DDM) was incorporated at a final concentration of approximately 2.45 mM (derived from 2.5 µL of a 10% w/v stock solution in a 200 µL well volume), double its critical micelle concentration (0.17 mM), to facilitate the encapsulation of Moenomycin A's hydrophobic moiety. DDM was then tested more thoroughly to determine its effects on the MIC of MOEA in the presence of BSA, and against both Gram-positive bacteria (*B.sub*) and Gram-negative bacteria (*E.coli*), also with Kanamycin as a control.

Results

The calculated MIC for Moenomycin was 0.26-0.065 µg/ml (*Figure 5*), within ranges given in the literature (0.43-0.07 µg/ml) (Cheng et al., 2008). The control exhibited no growth, showing no contamination.

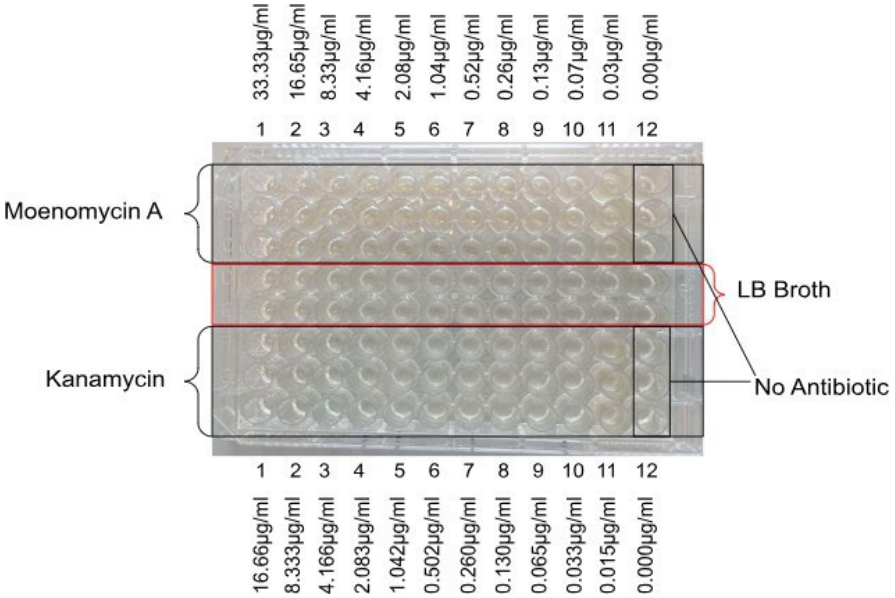


Figure 5. *B.subtilis* bioassay used to ascertain the Minimum Inhibitory Concentration (MIC) for Moenomycin A and Kanamycin (control), utilising a serial dilution.

The agar diffusion assay (*Figure 6*) revealed varying degrees of antimicrobial activity against *B.subtilis* for the Starbon formulations. Moenomycin A, applied at 10 µg/ml and Kanamycin at 12.5 µg/ml, produced a distinct zone of inhibition.

Moenomycin-induced Starbons (P300, P450, and P800) exhibited specific patterns of antimicrobial activity, with the size of zones of inhibition reflecting the amount of adsorbed Moenomycin from each Starbon type.

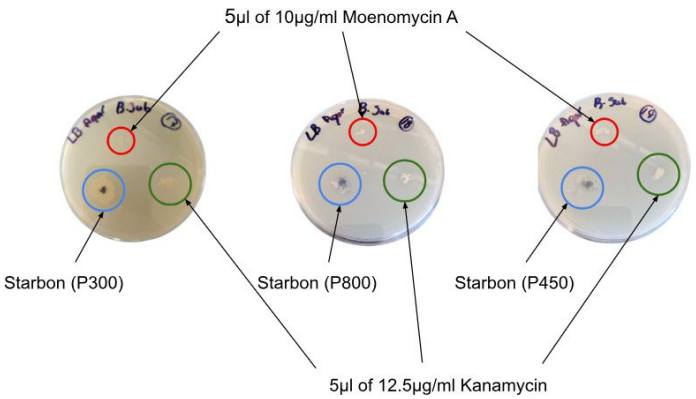


Figure 6. Agar diffusion plates showing the antibacterial properties of Starbons (P300, P450 and P800) with immobilised Moenomycin. Kanamycin and Moenomycin were used as controls.

The first calibration plate had a central disk with 5µl of 10µg/ml on a lawn of *B.sub*, yielding a zone of inhibition of 0.8cm (*Figure 7*). This was repeated with a serial dilution of Moenomycin down to 5µl of 0.156µg/ml in plate 10, which yielded a zone of inhibition of 0.4cm. The results of each plate were plotted to create a calibration curve, allowing estimation of Moenomycin concentration from the zone diameter (*Figure 8*).

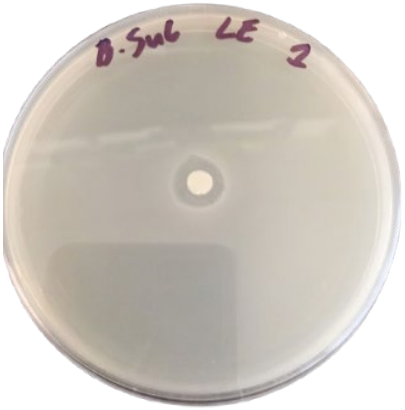


Figure 7. Zone of inhibition of *B.subtilis* created around a disk with 5µl of 10µg/ml Moenomycin A

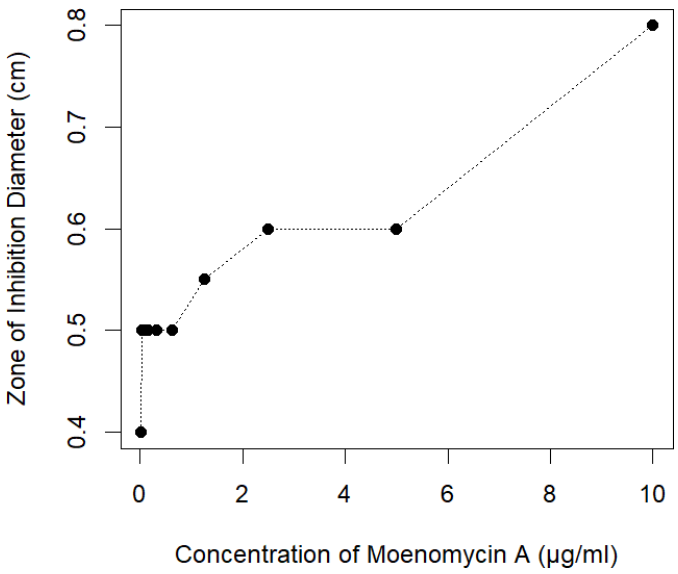


Figure 8. Inhibition diameters over serially diluted concentrations of Moenomycin allow for the creation of a calibration curve.

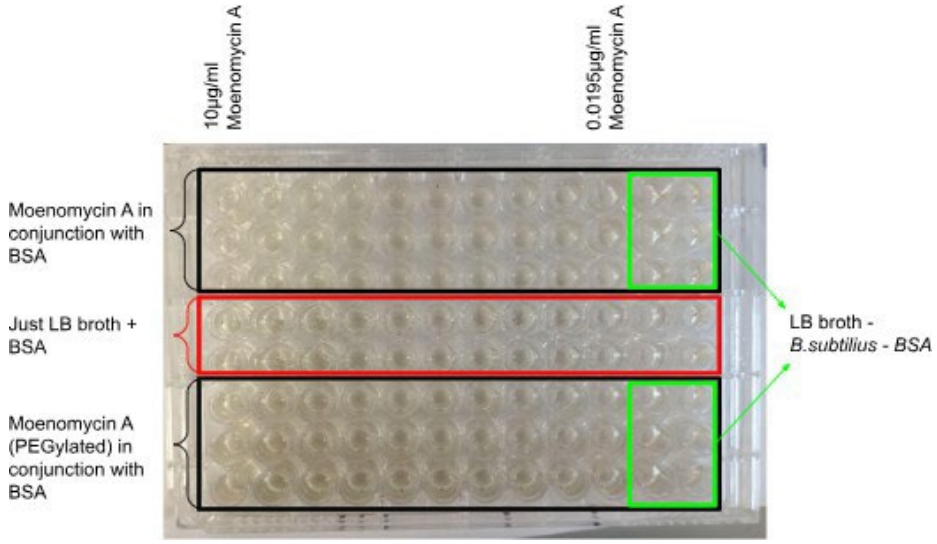


Figure 9. Moenomycin A alone exhibited growth, showing BSA has increased Moenomycin's MIC above the previously calculated MIC (*Figure 5)*. PEGylation had no effect on MIC. LB and BSA exhibited no growth, showing sterility. Green shows unimpeded growth.

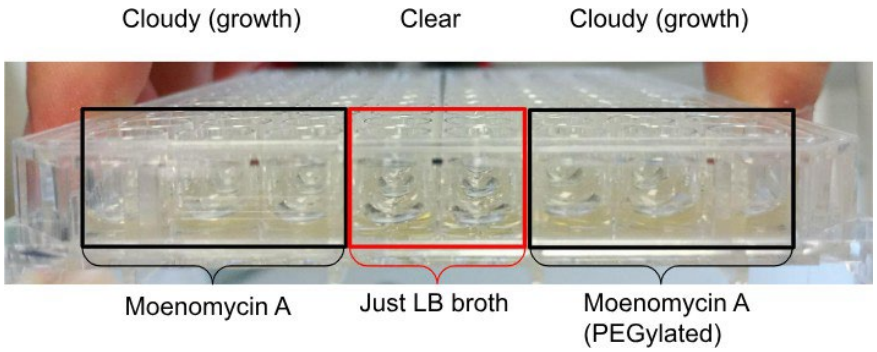


Figure 10. Moenomycin A (10µg/ml) did not impede bacterial growth; PEGylated Moenomycin gave identical results. LB + BSA had no growth (sterility control).

The inclusion of n-dodecyl-β-D-maltoside (DDM) significantly decreased Moenomycin's MIC in the presence of BSA, evidenced by complete inhibition of bacterial growth across the entire tested concentration range (*Figure 11*).

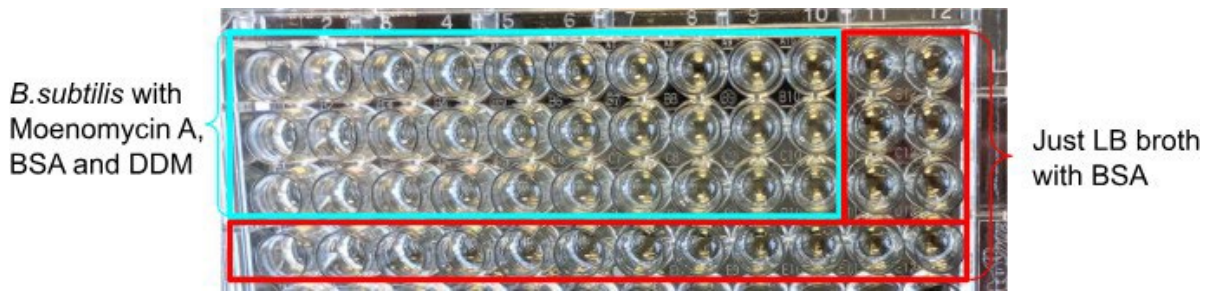


Figure 11. Broth microdilution plate showing *B. subtilis* MIC for Moenomycin with 4% (w/v) BSA and ~2.45 mM DDM (blue box). Red outlines are media sterility controls (LB broth with BSA). DDM significantly reduced the observed MIC.

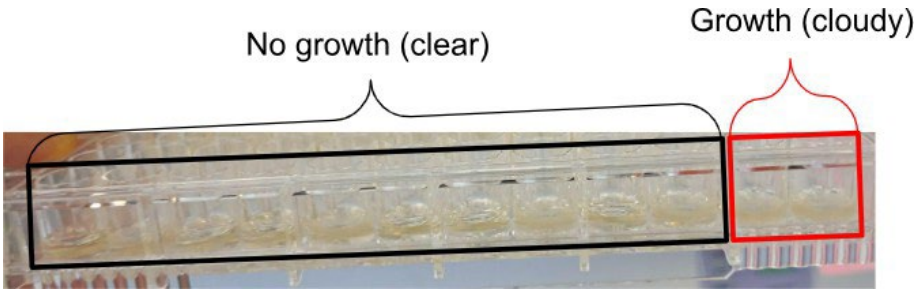


Figure 12. 96-well plate comparing Moenomycin + DDM (in the presence of BSA) (clear) and the control, showing normal unimpeded bacterial growth.

Plate 1

Moenomycin A Concentration	25µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/ml	1.563µg/ml	0.781µg/ml	0.390µg/ml	0.195µg/ml	0.098µg/ml	0.049µg/ml		
	1	2	3	4	5	6	7	8	9	10	11	12
Moenomycin A against *B.sub* in the presence of BSA — A	0.172	0.191	0.111	0.192	0.143	0.042	0.137	0.175	0.039	0.156	0.11	0.118
B	0.097	0.142	0.146	0.261	0.167	0.125	0.139	0.132	0.144	0.123	0.147	0.137
C	0.108	0.154	0.142	0.245	0.148	0.169	0.108	0.165	0.181	0.118	0.106	0.093
D	0.042	0.04	0.13	0.042	0.044	0.037	0.035	0.039	0.039	0.042	0.088	0.034
E	0.038	0.038	0.037	0.042	0.036	0.037	0.034	0.035	0.037	0.038	0.039	0.04
Moenomycin A / DDM against *B.sub* in the presence of BSA — F	0.054	0.084	0.097	0.116	0.102	0.08	0.077	0.073	0.079	0.084	0.107	0.104
G	0.056	0.06	0.089	0.064	0.073	0.083	0.085	0.078	0.066	0.079	0.102	0.093
H	0.06	0.054	0.07	0.078	0.058	0.054	0.051	0.059	0.066	0.058	0.105	0.117

B.sub + MH Broth + BSA | MH Broth + BSA | *B.sub* + MH Broth + BSA

Figure 13. Plate 1 illustrates that the MIC of Moenomycin against *B.subtilis* significantly increases in the presence of BSA; Moenomycin's antimicrobial activity is hindered by BSA, due to drug-protein binding, reducing the amount of free drug available. Conversely, cells F1-H10 demonstrate that pre-mixing Moenomycin with n-dodecyl β-D-maltoside (DDM) mitigates this BSA-induced MIC increase.

Plate 2

Moenomycin A Concentration	25µg/ml	12.5µg/ml	6.25µg/ml	3.125µg/ml	1.563µg/ml	0.781µg/ml	0.390µg/ml	0.195µg/ml	0.098µg/ml	0.049µg/ml		
	1	2	3	4	5	6	7	8	9	10	11	12
Moenomycin A against *E.coli* in the presence of BSA — A	0.132	0.086	0.088	0.1	0.098	0.099	0.09	0.094	0.104	0.131	0.162	0.175
B	0.125	0.098	0.085	0.102	0.091	0.085	0.099	0.081	0.091	0.112	0.153	0.138
C	0.175	0.18	0.167	0.112	0.182	0.126	0.124	0.118	0.141	0.272	0.213	0.068
D	0.034	0.033	0.039	0.036	0.034	0.043	0.035	0.037	0.041	0.033	0.033	0.033
E	0.068	0.035	0.036	0.034	0.035	0.036	0.036	0.037	0.038	0.036	0.035	0.036
Moenomycin A / DDM against *E.coli* in the presence of BSA — F	0.054	0.088	0.162	0.134	0.099	0.142	0.164	0.167	0.183	0.078	0.229	0.182
G	0.067	0.073	0.11	0.16	0.112	0.114	0.104	0.15	0.065	0.084	0.232	0.11
H	0.07	0.141	0.115	0.171	0.224	0.203	0.055	0.13	0.052	0.053	0.159	0.045

E.coli + MH Broth + BSA | MH Broth + BSA | *E.coli* + MH Broth + BSA

Figure 14. Plate 2 investigates the potential for DDM to enhance Moenomycin's efficacy against *E. coli*, a gram-negative bacterium. In cells F1-H3, lower absorbance values (indicating greater inhibition) are observed compared to when DDM is absent. This variability suggests that while DDM might have a positive impact on Moenomycin's

activity against gram-negative bacteria.

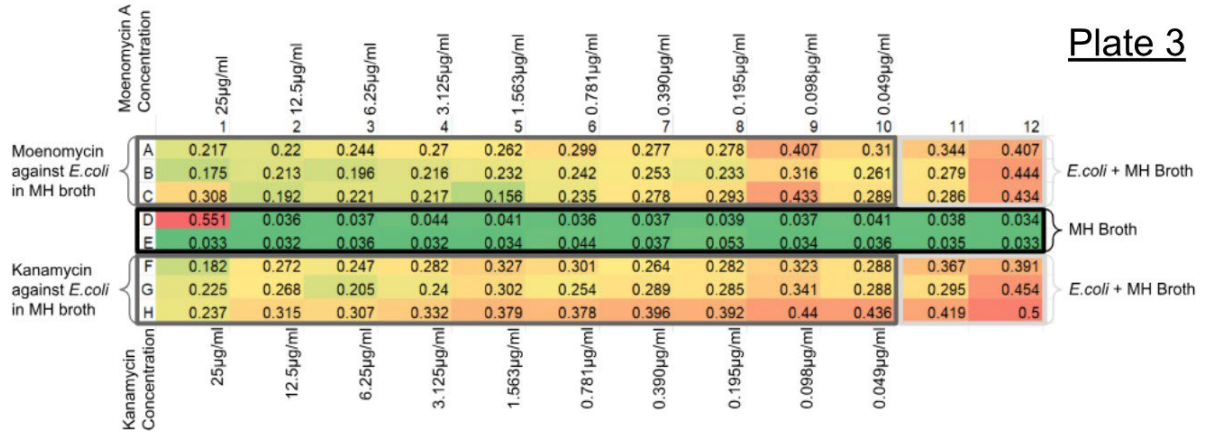


Figure 15. Plate 3 presents Moenomycin and Kanamycin against *E. coli*. Moenomycin has an inherently high MIC against the gram-negative bacterium,
E. coli. Comparing *Figure 15* to *Figure 14* (cells F1-H10), Moenomycin's MIC is significantly reduced when combined with DDM.

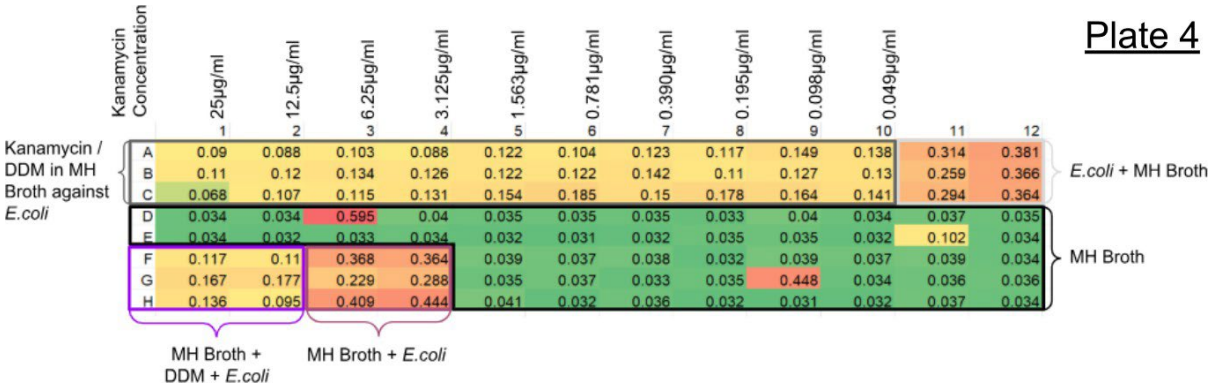


Figure 16. Plate 4. Comparing the MIC of Kanamycin (DDM) against *E. coli*. (Plate 3) (cells F1-H10) reveals a remarkable decrease in MIC when DDM is present, reducing it from approximately 3.125µg/ml to below 0.049µg/ml.

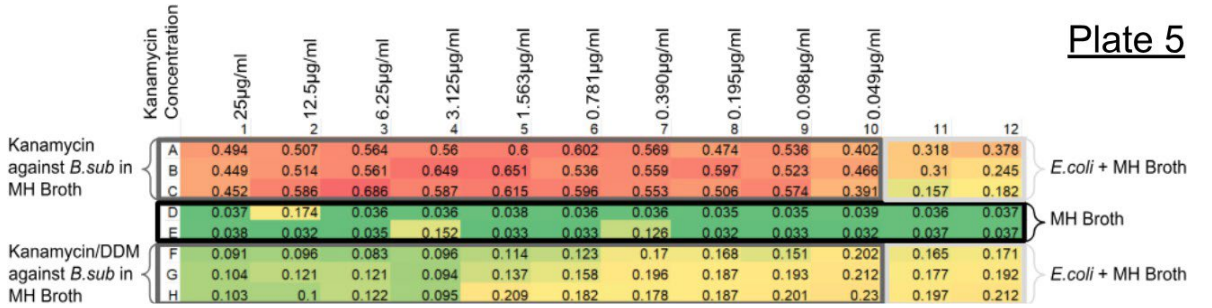


Figure 17. Plate 5. The addition of DDM to Kanamycin against *B. subtilis*. The concentration of Kanamycin used in cells A1-C10 was slightly lower than the MIC. Comparison with cells F1-H10 reveal that DDM has dramatically dropped the MIC from

approximately 35µg/ml to around 1.563µg/ml. This represents a 20-fold decrease in MIC.

UROS Experience

The Undergraduate Research Opportunity Scheme (UROS) at the University of Lincoln has provided me with an invaluable and comprehensive introduction to the realities of conducting scientific research. This process has been a very educational experience, allowing me to delve more into a disciplinary field I'm very interested in, antimicrobial research, and apply concepts in a practical laboratory setting. These practical skills will provide me with a significant advantage and a solid foundation when I begin my third-year dissertation, enabling me to approach my research with confidence and get started immediately.

Working in the lab with both academics and master's students has been very helpful. I have learnt valuable teamwork skills, collaborating with people from different academic course backgrounds, making for a vast range of knowledge for me to collaborate with (chemists/microbiologists).

When an unexpected delay in a bacterial strain delivery created a roadblock, I had to pivot the project and find an adjacent research avenue using readily available reagents. This experience taught me a valuable lesson about navigating unforeseen issues, typically encountered when working in a laboratory, and adapting to a project timeline.

Conclusion

From this project, we can conclude that Starbons (specifically P800) are effective at retaining Moenomycin A, making a single-step purification from *Streptomyces viridosporus* cell-free extract possible. Future work in this field should focus on refining this process by culturing *S.viridosporus* and optimising the methanol-based elution protocol. A separate, but significant finding, was the non-specific enhancement of antibiotic efficacy by n-dodecyl-β-D-maltoside (DDM). DDM mitigated Moenomycin's *in vivo* plasma protein binding and decreased the MIC against both gram-negative and positive bacteria, making Moenomycin a more promising candidate for intravenous use in humans. Kanamycin also had its MIC decreased, showing that DDM non-specifically decreases an antibiotic's MIC. Further research is needed to investigate DDM's mechanism of action (e.g., increasing cell wall fluidity or preventing aggregation), its effect on other antibiotics like penicillin and vancomycin, and DDM's potential to overcome cell wall-based immunity.

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