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ARTICLE

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Rapid spectrophotometric detection for optimized production of landomycins and characterization of their therapeutic potential

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Abstract

Microbial-derived natural products remain a major source of structurally diverse bioactive compounds and chemical scaffolds that have the potential as new therapeutics to target drug-resistant pathogens and cancers. In particular, genome mining has revealed the vast number of cryptic or low-yield biosynthetic gene clusters in the genus *Streptomyces*. However, low natural product yields—improvements to which have been hindered by the lack of high throughput methods—have slowed the discovery and development of many potential therapeutics. Here, we describe our efforts to improve yields of landomycins—angucycline family polyketides under investigation as cancer therapeutics—by a genetically modified *Streptomyces cyanogenus* 136. After simplifying the extraction process from *S. cyanogenus* cultures, we identified a wavelength at which the major landomycin products are absorbed in culture extracts, which we used to systematically explore culture medium compositions to improve total landomycin titers. Through correlational analysis, we simplified the culture optimization process by identifying an alternative wavelength at which culture supernatants absorb yet is representative of total landomycin titers. Using the subsequently improved sample throughput, we explored landomycin production during the culturing process to further increase landomycin yield and reduce culture time. Testing the antimicrobial activity of the isolated landomycins, we report broad inhibition of Gram-positive bacteria, inhibition of fungi by landomycinone, and broad landomycin resistance by Gram-negative bacteria that is likely mediated by the exclusion of landomycins by the bacterial membrane. Finally, the anticancer activity of the isolated landomycins against A549 lung carcinoma cells agrees with previous reports on other cell lines that glycan chain length correlates with activity. Given the prevalence of natural products produced by *Streptomyces*, as well as the light-absorbing moieties common

Abbreviations: A-Lan, anhydrolandomycinone; BGC, biosynthetic gene cluster; Co30, soytone glucose medium + 30 μM $CoCl_2$; LaA, landomycin A; LaB, landomycin B; LaD, landomycin D; LaE, landomycin E; Lan, landomycinone; LaR, landomycin R; Sc136, *S. cyanogenus* 136; Sc92a, *S. cyanogenus* 136+pOOB92a; SG, soytone glucose medium; Trace, soytone glucose medium + 1× trace metals solution.

to bioactive natural products and their metabolic precursors, our method is relevant to improving the yields of other natural products of interest.

KEYWORDS
angucycline, chemotherapeutic, media optimization, natural product, polyketide, *Streptomyces*

1 | INTRODUCTION

Advances in higher throughput and more sensitive technologies for the identification of natural products and biosynthetic gene clusters (BGCs)—genes that encode enzymatic pathways responsible for natural product biosynthesis—have revealed a vast landscape of natural biochemical diversity (Lee et al., 2020; Liu et al., 2021). Natural products, especially those with complex polycyclic structures, continue to serve as promising new drug leads to treat several relevant health threats, such as multidrug-resistant cancers and antibiotic-resistant microbial pathogens (Harvey et al., 2015; Patridge et al., 2016; Pham et al., 2019). Though biosynthesis is a catalytically efficient method to produce natural products, BGC expression is often poor and pathway activation mechanisms remain indeterminant. Additionally, while efforts to develop chemical synthesis methods have met with significant success, they are often expensive, time-consuming, and low-yielding, and thus are better suited for semi-synthetic methods that start with biologically derived chemical scaffolds (Li & Trost, 2008). Thus, the production of sufficient bioactive natural products remains a major hurdle that limits their eventual translation to clinical applications.

Of the thousands of identified microbial BGCs, the genus *Streptomyces* encodes the greatest number and highest density, producing numerous bioactive compounds and relevant chemical scaffolds (Belknap et al., 2020; Chung et al., 2021; Ward & Allenby, 2018). Among these, landomycins—polyketides that belong to the angucycline family—exhibit bioactivity via diverse and potentially novel mechanism(s) of action, making them promising therapeutics to target drug-resistant phenotypes (Crow et al., 1999; Henkel et al., 1990; Panchuk et al., 2017; Terenzi et al., 2021). Landomycin BGCs have only been directly identified in three genomic samples, though a fourth is presumed due to landomycin detection in culture extracts (Feng et al., 2011; Matselyukh et al., 2016; Peng et al., 2018; Westrich et al., 1999). Landomycin A (LaA) and landomycin E (LaE) are the most widely studied landomycins, and both possess the widest reported spectrum of anticancer activities among the landomycins (Kharel et al., 2012). The structures of LaA and LaE only differ in the composition of their glycan chain. LaA has the longest glycan of naturally occurring landomycins, a hexasaccharide composed of a repeated trisaccharide unit of two D-olivoses and a single L-rhodinose, while LaE, contains only a single trisaccharide unit (Henkel et al., 1990; Kharel et al., 2012; Weber et al., 1994). Very limited data is available regarding the antimicrobial spectra of landomycins, with only a few studies reporting activity against one or two sensitive organisms.

Streptomyces cyanogenus 136 (Sc136) is the only known natural source of LaA (Hrab et al., 2021). Though meticulous and innovative methods have demonstrated the feasibility of chemically synthesizing the complex benz[*a*]anthracene core, hexasaccharide glycan, complete LaA molecule, and a variety of non-natural landomycins, these methods are not suitable for the production of landomycins on the scale required for drug development (Bugaut et al., 2010; Guo & Sulikowski, 1998; Lee et al., 2019; Roush & Bennett, 2000; Roush & Neitz, 2004; Tanaka et al., 2010; Yalamanchili et al., 2019; Yang & Yu, 2016; Yang et al., 2011; Yu & Wang, 2002). Alternatively, control over the biosynthetic regulon via genetic interventions and optimization of culture conditions have proven quite successful at improving yield or altering dominant products (Luzhetskyy et al., 2005; Von Mulert et al., 2004; Shaaban, Srinivasan, et al., 2011; Shaaban, Stamatkin, et al., 2011; Westrich et al., 1999). In particular, modifications to the major pathway regulators *adpA* and *bldA* have improved landomycin titers—though modifications to pathway gene expression can alter the composition of landomycin products compared to wildtype Sc136 (Gessner et al., 2015; Myronovskyi et al., 2016; Yushchuk et al., 2018). Of particular note is recent work that showed that complementation of Sc136 with a functional copy of the transcription factor *adpA* from *S. ghanaensis* ($adpA_{gh}$) improved LaA yields by more than fivefold and rendered Sc136 capable of synthesizing LaA in a variety of media (Yushchuk et al., 2018). Still, additional improvements to increase the yield as well as methods that facilitate the purification of landomycins will aid in their potential for future clinical testing and modification into next-generation, semi-synthetic analogs unavailable via biosynthesis.

Here, we describe the development and utilization of spectrophotometric methods to optimize production and the characterization of the antimicrobial and anticancer activity of the major landomycin products of an $adpA_{gh}$ expressing Sc136 strain (Sc92a). To this end, we characterized the major landomycin products of Sc92a, improved extraction, and fractionation conditions, and identified a wavelength of light (265 nm; A265) at which all of these products absorb. Using A265 as an estimate for total landomycin production, we systematically explored the impact of media components on landomycin titers. We further simplified and increased the throughput of this method by correlating the absorbance measurements from culture extracts to those of supernatants, ultimately identifying a wavelength (345 nm) at which the culture supernatant absorbance is considerably more representative of landomycin titers than the optimal absorbance wavelengths identified for culture extracts and purified products. Of the microbes tested, we found that Gram-positive bacteria are most sensitive to landomycins, and fungi

are inhibited by landomycinone, although mostly at concentrations that are unlikely to be clinically relevant. Gram-negative organisms are resistant to landomycins yet could be sensitized by disrupting the outer membrane. Consistent with prior studies, we confirm that the landomycins exhibit potent anticancer activity against lung carcinoma cells (A549), indicating the future potential to develop landomycins into effective chemotherapeutic candidates.

2 | MATERIALS AND METHODS

2.1 | Culture conditions

The strains used in this study are listed in Supporting Information S1: Table 1. *S. cyanogenus* was grown in TSB for general propagation. *Bacillus subtilis*, *Listeria seeligeri*, *Lactococcus cremoris*, *Enterococcus faecium*, *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* ser. Typhimurium was cultured in Brain Heart Infusion (BHI) broth. *Saccharomyces cerevisiae* and *Candida albicans* were cultured in 2 × YPD (40 g/L casein peptone, 20 g/L yeast extract, 20 g/L glucose, 100 mg/L adenine). For solid media growth, 15 g/L agar was added to BHI or 2 × YPD. All cultures were grown at 250 rpm shaking and 37°C, except for *S. cyanogenus*, *L. cremoris*, *S. cerevisiae*, and *C. albicans*, which were grown at 30°C.

2.2 | Landomycin production and mycelia fractionation

Initial *S. cyanogenus* propagation and landomycin production conditions were kindly provided by personal communication from Prof. Bohdan Ostash (Ivan Franko Lviv National University). *S. cyanogenus* 136 + pOOB92a (a.k.a. Sc92a) frozen stock was streaked on soy mannitol agar (SMA; 20 g/L soy flour, 20 g/L mannitol, 15 g/L agar, pH to 8.0) and grown for 48 h. Colonies with a red-brown halo were used for pre-culture. A 1–2 cm^2 piece of agar with visibly grown colonies was cut from the plate and added to 30 mL of soytone-glucose (SG) medium (20 g/L glucose, 10 g/L phytone peptone, 2 g/L $CaCO_3$, pH to 7.0; autoclave; add 1.9 mg/L $CoCl_2.6H_2O$) in a 250 mL flask and grown for 48 h as pre-culture. 2.5 mL of pre-culture was used to inoculate each of the 10 × 500 mL flasks with 100 mL SG and grown for 48 h as the production culture. SG was modified as required for medium optimization by adding or replacing components before autoclaving (nitrogen sources), or adding concentrated stocks after autoclaving (glucose, metal ions). The culture solids were pelleted by centrifugation (4500×*g* for 10 min) and the supernatant decanted, buffered to pH 7.0, and set aside for extraction. The cell-mycelium and culture solids pellet were then resuspended in PBS, vortexed vigorously to homogenize, and centrifuged (4500×*g* for 5 min) again. The supernatant was decanted and set aside for extraction. The pellets were again resuspended to 35 mL total volume in PBS and vortexed vigorously. The homogenized samples were then centrifuged for 1–1.5 min at 4500×*g* to selectively pellet the cell-mycelium fraction while retaining the culture solids in suspension to decant for extraction. Resuspension, vortexing, and centrifugation steps were repeated until the pellet was composed only of a beige-gray cell-mycelium pellet, and the supernatant was clear.

2.3 | Isolation of pure landomycins

Initial isolation methods were determined from previous works and adapted as needed (Henkel et al., 1990; Weber et al., 1994). The supernatant fraction (~1 L) from the *S. cyanogenus* 136 + pOOB92a was extracted once with 1 L and twice with 500 mL ethyl acetate. The 500 mL culture solids suspension was extracted with three 1 L, followed by two 500 mL volumes of ethyl acetate. The combined extract was evaporated down to solid crude material and put under a high vacuum to ensure dryness.

The crude red solid (904.2 mg) was dissolved in about 5 mL of 7% MeOH/$CHCl_3$ solution and loaded onto a Yamazen column (180 g of silica) equilibrated for 2 min at 3% MeOH/$CHCl_3$. The system used was an Automated flash column chromatography (normal phase) Smart Flash EPCLC W-Prep 2XY Dual Channel Automated Flash Chromatography System with an additional ELSD detector, provided by Yamazen Corporation. The column was run at 4% MeOH/$CHCl_3$ for 20 min, then increased over 2 min to 10% MeOH/$CHCl_3$ to run for an additional 15 min. This column yielded fractions I (pink/purple solid), II (red solid), and III (orange/red solid). Fraction I was evaporated to dryness, then separated further by size exclusion chromatography with 40 g LH-20 Sephadex (Sigma-Aldrich), in 50% MeOH/$CHCl_3$ (2 × 50 cm) to yield pure landomycinone (6.7 mg) and 5,6-anhydrolandomycinone (0.3 mg). Fraction II was evaporated to dryness, then precipitated from a concentrated chloroform solution into pentane, giving pure landomycin A (423.1 mg). Fraction III was evaporated to dryness, then separated further with 40 g LH-20 Sephadex in MeOH (2 × 50 cm) to yield landomycin B and landomycin D. After rotary evaporation, landomycin B (39.2 mg) was precipitated from a concentrated chloroform solution into pentane. Landomycin D (9.6 mg) was precipitated from a concentrated ethyl acetate solution into pentane. Products were visualized on TLC using UV and staining with a 5% aqueous sulfuric acid solution. The purity of all landomycin species was confirmed by a normal phase Elite LaChrom Hitachi HPLC (90% EtOAc in Hexanes). NMR spectra were recorded on a Bruker Avance III NMR spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C-NMR. Mass spectrometry data was collected on a low-resolution Finnigan LTQ ESI-MS (Thermo Fisher Scientific). See Supporting Information for more details. All solvents used were HPLC grade (Thermo Fisher Scientific).

2.4 | Landomycin standard curves

All standard curves were generated by diluting landomycin stocks (10 mg/mL in DMSO) 10× into Dulbecco's PBS (DPBS, pH 7.0; Corning), followed by threefold serial dilutions into DPBS 10%

DMSO, and performing an absorbance spectral scan (200–1000 nm; 5 nm increments) using a Spectramax M3 (Molecular Devices).

2.5 | Spectrophotometric detection of landomycins from culture extracts and supernatants

The absorbance of culture supernatants was determined by pelleting culture solids from the landomycin production cultures and diluting the resulting supernatant 2× into DPBS or 10× into HEPES buffer (100 mM, pH 7.0). Extraction of landomycin fractions for spectrophotometric determination of relative concentrations was performed by adding 300–500 µL aliquots of landomycin culture (supernatant, cells, and mycelia) to an equal volume of chloroform. Samples were then vortexed for 30 s and centrifuged to separate fractions. Samples were vortexed and centrifuged for a second time and a 100 µL aliquot of the chloroform extract was removed from the lower fraction. Samples were dried at room temperature under vacuum for approximately 30 min using an Eppendorf Vacufuge and stored at −80°C. Immediately before recording absorbance readings, samples were resuspended in 100 µL DMSO and diluted 10× into DPBS. Spectrophotometric scans from 200 to 800 nm at 5 nm intervals were performed for all supernatant and extract samples.

2.6 | General A549 cell culture

Human non-small cell lung cancer (NSCLC) cells (A549; CCL-185) from ATCC were cultured in F-12K medium (ATCC) with 10% (v/v) fetal bovine serum (Neuromics). Cultures of A549 cells were incubated at 37°C, 95% humidity, and 5% CO_2 atmosphere. Cells were passaged every three to four population doubling times when their concentration was approximately 4×10^6 cells mL^{-1} (approximately 3 days between each passage). All experiments were performed using cultures that had been passaged less than 20 times.

2.7 | A549 viability assay

A549 cells were seeded at 10,000 cells per well (200 µL) in 96-well tissue culture-treated plates (CellTreat) (t = 0 h). 20 mM concentrated stocks of landomycins and doxorubicin hydrochloride (Tocris Biosciences) were made in DMSO immediately before addition to cells. Concentrated stocks to 20 mM were diluted to make working stocks that were diluted to 0–100 µM in a cell culture medium with a final DMSO concentration of 0.5% (v/v). At 24 h, the medium was aspirated from each well, washed with 100 µL PBS, and 0–100 µM of product of interest was added to each well in complete media (supplemented with a final concentration of 0.5% v/v DMSO) and incubated cells for 24 h. This concentration of DMSO afforded no change in viability when assessed without the drug present and compared to the 0 µM sample (>95% cell viability with 0.05% DMSO). Then, the medium was aspirated, washed cells with PBS, and trypsinized cells using 100 µL of 0.25% trypsin/EDTA (Gibco) to release the cells from the surface of the plate. Trypsin/EDTA was neutralized with an equal amount (100 µL) of complete media. Cells were pelleted (300×*g*, 8 min), and washed with 100 µL PBS. Three stains were used to classify the viability of cells: Hoechst 33342 to identify nuclei, and Annexin V-FITC, and Ethidium homodimer I to label viable, apoptotic, and necrotic cells, respectively. Hundred micrometer concentrated stocks of Ethidium homodimer I and Hoechst 33342 were prepared in PBS. Then, 5 µM working solutions of Annexin V-FITC (BioLegend), Ethidium homodimer I (Biotium), and Hoechst 33342 (Thermo Fisher Scientific) were prepared in 1× Annexin V Binding Buffer (100 mM HEPES, 25 mM $CaCl_2$, 1.4 M NaCl; BioLegend). To analyze cells, 50 µL of Annexin V-FITC, Ethidium homodimer I, and Hoechst 33342 working solutions were added to each well containing a washed cell pellet. An additional three wells received each of the single dyes for compensation controls. Samples were incubated in the well plate for 10 min (37°C, 5% CO_2). Before plate analysis via flow cytometry, 200 µL of 1× Binding Buffer was added to each well. All wells were analyzed using a Guava easyCyte 12HT flow cytometer (Cytek). Different laser/filter combinations were used for each dye (Supporting Information S1: Table 2). Gain controls were adjusted for GRN-B, RED-B, and BLU-V channels to 1.00 to accommodate the dyes used (Supporting Information S1: Table 3), and YEL-B (emission filter 575/25) was also adjusted to 1.00 to accommodate the natural fluorescence of doxorubicin hydrochloride (ex/em: 470/560).

2.8 | Agar well diffusion assay

Landomycin stock solutions were made by resuspending purified landomycin solid fractions at 10 mg/mL (LaD, LaB, LaA, Lan, and A-Lan) in DMSO. Stock solutions were stored at −20°C. Overnight cultures of each strain were diluted 50× into fresh medium and 100 µL was spread-plated on the appropriate solid agar medium. Agar plates were allowed to dry and circular wells were excised. Working solutions of landomycin were made by diluting concentrated stocks to 250 ppm (µg/mL) into PBS pH 7.0, and 100 µL of a working solution was added to an agar well. Plates were imaged after overnight incubation at the appropriate temperature.

2.9 | Microbial MIC assay

Overnight cultures were subcultured 1:1000 (bacteria) or 1:500 (fungi) into fresh media containing 250 µg/mL landomycin (diluted from 10 mg/mL stocks), or 2.5% (v/v) DMSO (control) and aliquoted into 96-well plates. Subcultures were serially diluted twofold into

fresh medium with 2.5% DMSO to ensure consistent vehicle concentration. Plates were sealed with parafilm to prevent culture evaporation and grown in a Biotek Epoch 2 microplate reader (37°C, continuous linear shaking at 731 cpm) for 24–48 h, with an absorbance reading at 600 nm taken every 5 min. Due to the change in absorbance caused by the likely breakdown of the landomycins, cultures were blanked at each time point using BHI media with an equivalent concentration of each landomycin.

2.10 | Statistics

Standard linear regression was performed using GraphPad Prism and Microsoft Excel. Ordinary one-way ANOVA and correlational analysis (Pearson's *r*) were performed using GraphPad Prism. See SI for a description of nonlinear regression used for MIC calculation.

3 | RESULTS

3.1 | An improved extraction and isolation protocol for landomycin products

Landomycin production by *Streptomyces* is commonly performed in a three-step process: (i) differentiation on agar plates, (ii) preculturing, and (iii) production culturing (Figure 1a) (Weber et al., 1994). Landomycins are then extracted from production cultures using a biphasic extraction, and then individual compounds are isolated using traditional separation methods. Though landomycins can be extracted quite simply from the supernatant, this approach sacrifices much of the landomycin produced (>80%), which remains trapped in culture solids. Previous reports of extraction from total cultures (cells + mycelia + solids + supernatant) have been reported to produce higher yields. However, our initial efforts produced greasy, poorly crystallized solids that retained a considerable amount of contaminant (Shaaban, Srinivasan, et al., 2011; Weber et al., 1994). To improve product quality and reduce the complexity of separations, we developed a simple method to fractionate the colored culture solids, likely densely packed with secondary metabolites such as landomycins, from the remaining cells and/or mycelia. We found that the size and/or density difference between cells/mycelia and culture solids were sufficient to fractionate samples by differential centrifugation. Successive rounds of vortexing and pelleting generated two distinct layers of well-separated solids: (i) a white-tan layer (cells + mycelium) on the bottom and (ii) a red-brown layer (culture solids) on the top (Figure 1a, Figure S1). Using the extracts from the supernatant and culture solids fractions, we simplified the previously reported fractionation process to a single normal phase silica column and a precipitation step to obtain 390–430 mg/L LaA at high purity (>97%). In total, we identified five major products in Sc92a cultures: anhydrolandomycinone (A-Lan), landomycinone (Lan), landomycin A (LaA), landomycin B (LaB), and landomycin D (LaD) (Figure 1a–c).

3.2 | Optimized media formulations enhance landomycin production

The electron orbitals of polyketides are frequently oriented such that electrons can absorb light in the near UV (>200 nm) to near infrared (<800 nm) range, which is detectable by standard UV-vis spectroscopy. More specifically, LaA production by Sc136 has been estimated by measuring the absorbance of culture extracts at 445 nm (Yushchuk et al., 2018). We, therefore, explored the applicability of a similar spectrophotometric approach to detect the other soluble landomycin products that we identified in culture extracts (Lan, LaA, LaB, and LaD). To validate this approach, we acquired absorbance spectra of the purified compounds from 200 to 800 nm (Figure 1d). We found a wide absorbance peak from approximately 350–550 nm, with a maximum at 450 nm, which was specific to the glycosylated forms of landomycin (LaA, LaB, and LaD). Conversely, Lan absorbed well from 575 to 650 nm, while the glycosylated forms had negligible absorbance in this range. All compounds absorbed well in the UV range, with peak maxima at 230 and 260 nm, suggesting absorbances near these wavelengths could be effective determinants of total Lan and LaA/B/D production. We generated standard curves using linear regression for Lan, LaA, LaB, and LaD at 265 nm and found strong goodness of fit for all compounds ($R^2 \geq 0.99$) (A265; Figure 1e).

Integration of *adpA* in Sc136 alters the conditional expression of the landomycin biosynthetic pathway in diverse media. This alleviates the dependence on the standard SG production medium for landomycin production and presents an opportunity to improve production by optimizing the medium composition. SG is composed of a soybean-derived peptone (soytone or phytone peptone), glucose, calcium carbonate, and cobalt, though the causal components of SG essential to landomycin production are unknown (Weber et al., 1994). Accordingly, we utilized our newly identified correlation between A265 and the concentration of the major landomycin products to identify modified SG compositions that improve landomycin yield from Sc92a cultures.

We first investigated the effect of cobalt concentration on landomycin production (Figure 2a). SG medium contains 0.001 g/L (7.7 μM) cobalt, yet we found that increasing cobalt to 30 μM increased total landomycins by ~twofold. Increasing cobalt concentration further to 100 μM resulted in a drop in landomycin production, and cultures turned a dark brown–black, which is frequently associated with poor yields that are thought to be due to the degradation of landomycins. Using the improved 30 μM cobalt concentration in the SG base medium (Co30), we next investigated the effect of glucose concentration on landomycin production (Figure 2b). Relatively high glucose concentrations (2.0%) are stipulated as necessary for good landomycin production. However, glucose is also known to frequently repress secondary metabolism while nutrients are abundant. We found that glucose is required at a minimum concentration of 1.0% (w/v) to obtain maximum landomycin titers, and there was no detriment to further increasing its concentration, indicating that production is not glucose-inhibited or limited (Figure 2c). Maintaining 2.0% glucose, we then investigated different complex nitrogen sources but found that they all resulted in

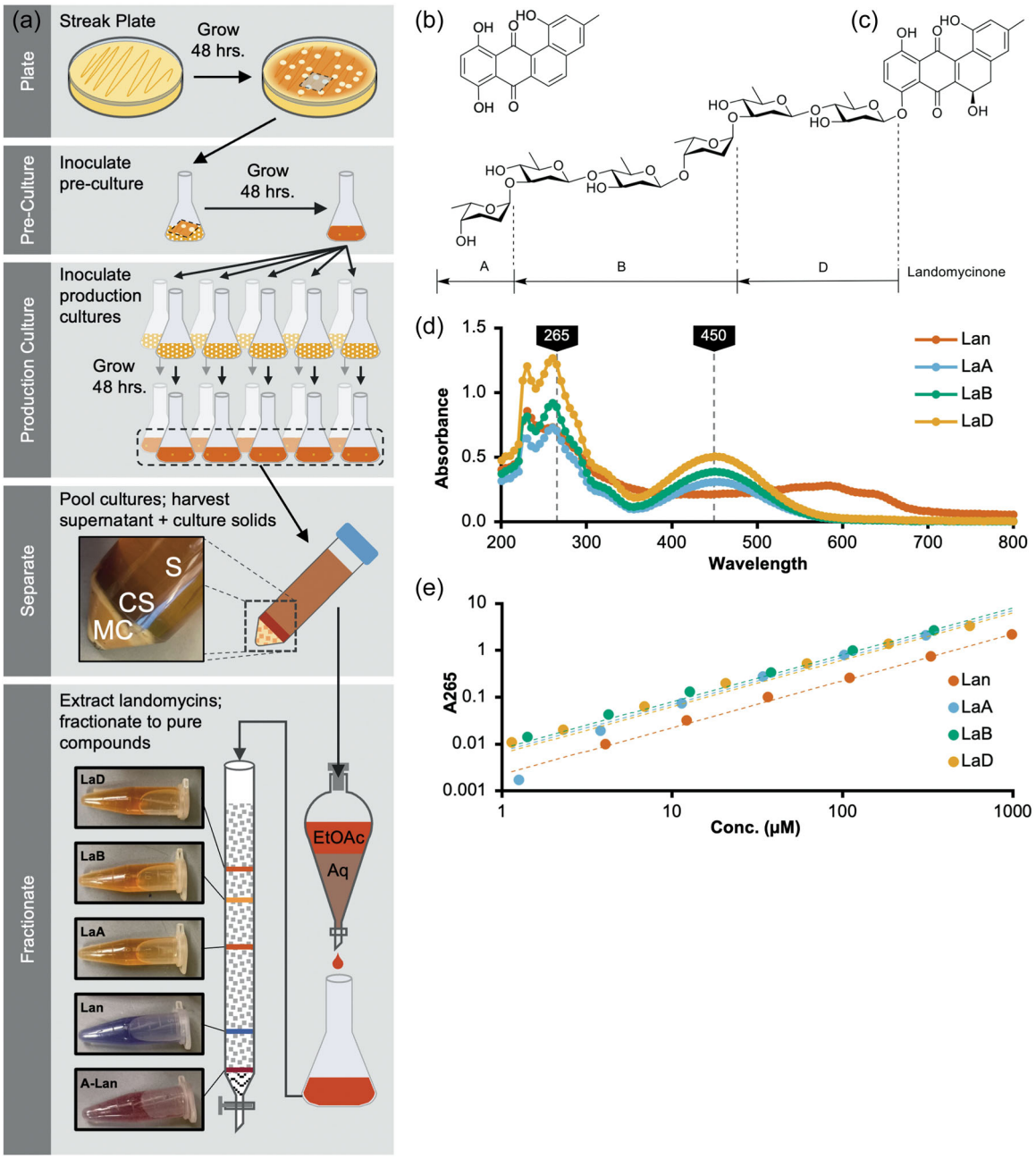


FIGURE 1 Landomycin production method and major products of *Streptomyces cyanogenus* 136 + pOOB92a. (a) The major steps of landomycin production include (i) differentiating Sc136 on SMA agar, (ii) inoculating a pre-culture using an agar plug, and (iii) subculturing the pre-culture into production cultures. Culture supernatants (S) and (CS) solids are then separated from cells and mycelium (MC) and landomycins are extracted from the aqueous using ethyl acetate (EtOAc), and fractionated on a silica column. (b, c) Structures of five major products, including (i) anhydrolandomycinone (A-Lan), (ii) landomycinone (Lan), (iii) landomycin A (LaA), (iv) landomycin B (LaB), and (v) landomycin D (LaD). (d) Absorbance spectra of purified Lan, LaA, LaB, and LaD. Absorbance peaks at 265 and 450 nm are labeled. Readings were taken every 5 nm from 200 to 800 nm. (e) Standard curves and absorbance spectra for Lan, LaA, LaB, and LaD demonstrating the linear relationship of absorbance to compound concentration ($R^2 \geq 0.99$ for all trendlines).

a drastic reduction in landomycin titer. This result suggests that specific components of the phytone peptone are necessary to achieve maximum landomycin yields. Finally, since previous studies have had success in replacing cobalt with other divalent metal ions, we tested the suitability of a commercially available trace metals mixture (trace) as well as a previously reported minimal trace metals solution (T2) (Figure 2d, Figure S2A) (Yushchuk et al., 2018). While we found that T2 decreased landomycin production, trace increased it by ~50% when compared to the Co30 base medium.

To determine the ions responsible for inducing landomycin production in the trace, we evaluated the impact of six of the dominant ions in trace (Zn^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, and Cu^{2+}) on

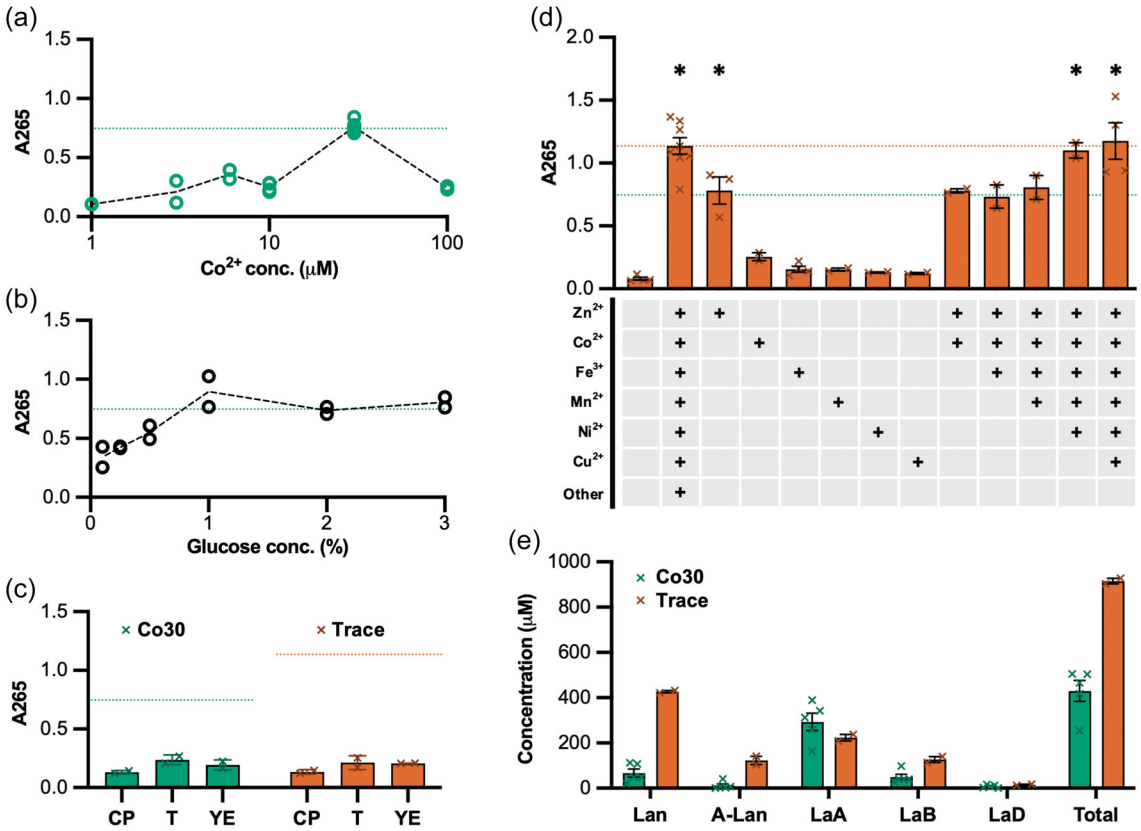


FIGURE 2 Optimization of landomycin production medium. Chloroform extractions of cultures with modified media compositions were tested to determine the impact on landomycin production. (a) Effect of cobalt concentration on landomycin production in SG medium. Concentrations tested include 1, 3, 6, 10, 30, and 100 μM. (b) Effect of glucose concentration on landomycin production in SG medium with 30 μM Co. Concentrations tested include 0.1%, 0.25%, 0.5%, 1%, 2%, and 3% (w/v) glucose. (c) Effect of replacing phytone peptone with casein peptone (CP), tryptone (T), or yeast extract (YE) in the presence of 30 μM Co (green) or trace metals solution (orange). (d) Impact of trace metal composition in SG medium on landomycin production. Metal ions were added as the following salts from 1000x stocks (final concentrations given): 10 μM $ZnCl_2$, 2 μM $CoCl_2$, 50 μM $FeCl_3$, 10 μM $MnCl_2$, 2 μM $NiCl_2$, 2 μM $CuSO_4$. Other indicates the use of a commercial trace metals solution which additionally contains 2 μM Na_2MoO_4, 2 μM Na_2SeO_3, 20 μM $CaCl_2$, and 2 μM B(OH)3. ∗ denotes samples with means significantly different from all other unlabeled means and not significantly different from one another, as determined by ordinary one-way ANOVA and Tukey multiple comparison test ($p < 0.05$). (e) Landomycin yield from 1 L production cultures of Co30 (green) or trace (orange) media. Error bars represent SEM. SG, soytone-glucose.

landomycin titers (Figure 2d). We found that Zn^{2+} was the major inducer of landomycin production in the trace mixture, achieving equivalent landomycin production at 10 μM as Co^{2+} did at 30 μM. Though the other individual ions yielded more modest landomycin titers, the addition of any one of the metals was an improvement over the base medium, suggesting all six cations can activate the landomycin biosynthetic pathway. We then explored the additive effect of the metal ions by successively adding metal ions to the medium in an order based on their individual effect. Addition of (i) Co^{2+}, (ii) Co^{2+} and Fe^{3+}, or (iii) Co^{2+}, Fe^{3+}, and Mn^{2+} to Zn^{2+} had no additional benefit relative to Zn^{2+} alone, while the addition of Co^{2+}, Fe^{3+}, Mn^{2+}, and Ni^{2+} yielded landomycins equivalent to the trace mixture. These observations suggest that a minimal solution of Zn^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, and Ni^{2+} is sufficient to achieve landomycin yields equivalent to that of the complete trace mix. Growth in 30 μM Zn^{2+}, Mn^{2+}, Ni^{2+}, or Cu^{2+}, did not improve yield further, nor did the addition of 30 μM Co^{2+}, 20 μM Zn^{2+} or 30 μM Co^{2+}, and 20 μM Zn^{2+} in combination with trace (Figure S2B–C). To validate that our improved production conditions are relevant at scale, we characterized the relative product yields of 1 L production cultures of Co30 and trace. Trace produced more than double the total landomycin products compared to Co30 (915 vs. 430 μM). However, trace produced less total and relative LaA, the pathway end-product, compared to Co30 (223 vs. 293 μM; 24% vs. 68% total products), and far more of the intermediates or byproducts Lan, LaB, and A-Lan (427 vs. 68, 127 vs. 49, and 123 vs. 12 μM, respectively).

3.3 | A rapid spectrophotometric approach to quantifying landomycins helps shorten production runs and improve yield

Although the A265 of culture extracts proved to be an effective descriptor of landomycin titer, the requirement of an extraction step

limited sample throughput for production optimization. When we plotted the absorbance (200–800 nm) of the supernatants of each of the 55 media compositions for which we had previously evaluated the A265 of culture extracts, we found that the peaks identified in the purified landomycins or culture extracts (265 and 450 nm) were masked (Figure 3a). To identify a more descriptive wavelength of landomycin titer in culture supernatants, we used linear regression to evaluate the correlation of the A265 of culture extracts to the absorbance of culture supernatants at every 5 nm interval (200–800 nm) for the 55 media compositions tested previously (Figure 3b,a inset). When we plotted the R^2 for the resulting trendline at each supernatant wavelength, A265 and A450 of culture supernatants indeed proved to be very poor predictors of landomycin titer ($R^2 = 0.4$ and 0.3, respectively). The maximum R^2 was identified at 345 nm, with a value of 0.78, suggesting it is a good predictor of the extract A265, and thus total landomycin titer. When we plotted A265 of culture extracts versus A345 of culture supernatants, we were able to identify specific outlier sets within our data that likely resulted from different background media absorbances, including those in which glucose was added post-autoclaving (carbon) and those with different peptone sources (nitrogen) (Figure 3c). When we excluded these data from our analyses, the remaining 42 conditions retained a Pearson's correlation coefficient of 0.97 ($p < 0.0001$), and the trendline had an R^2 of 0.93, both indicating A345 is an effective measure of total landomycin titer. We further validated the effectiveness of A345 of culture supernatants as a measure of landomycin production by directly measuring total landomycin production in production cultures by LC-MS at different time points during a production run (Figure 3d).

Utilizing the increase in sample throughput provided by supernatant A345, we investigated the optimal subculture time point and production culture endpoint (Figure 3e). Starting at the pre-culture step, we subcultured pre-cultures into new production cultures every 12 h, while monitoring landomycin production. We found that using a protocol that we named "Faster", we could reduce the pre-culture from 48 to 12 h, while extending the production culture from 48 to 60 h, thereby reducing total production by 24 h, without any loss in landomycin production. We also developed a protocol that we named "More", where the production culture from a 12 h pre-culture was extended to 84 h, resulting in a further 32% increase in landomycin production compared to the starting condition (control; 48 h pre-culture, 48 h production culture).

3.4 | Landomycins exhibit antibacterial and antifungal activities

Although landomycins are commonly reported to exert antimicrobial activity, the breadth of their activity spectra is poorly described (Lai et al., 2021; Matseliukh et al., 1998; Yushchuk et al., 2021). Additionally, the different landomycins, complex and impure mixtures, and/or qualitative metrics used in these studies limit comparison between them. We screened the five major landomycins found in production cultures for their antimicrobial activity against a diverse array of microbes (five Gram-positive, three Gram-negative, and two fungi) using an agar well diffusion assay. We found that LaA was active against all Gram-positive bacteria, while Lan, LaB, and LaD inhibited at least three of the five Gram-positive strains tested (Figure 4a). Lan also inhibited the growth of the fungi *S. cerevisiae* and *C. albicans*. A-Lan was not active against any of the organisms tested, although this may be a result of the compound precipitating out when added to the medium. None of the landomycins inhibited the growth of Gram-negative bacteria. Given the broad antimicrobial activity, we found landomycins had against Gram-positive bacteria and fungi, as well as the well-characterized anticancer activity of many landomycins, we postulated that the complete absence of growth inhibition seen with any Gram-negative bacterium may be due to the impermeability of the outer membrane. To investigate this hypothesis, we assessed whether permeabilization of the outer membrane using EDTA could sensitize Gram-negative bacteria to LaD (Brown & Richards, 1965). We initially determined non-inhibitory concentrations of EDTA for each Gram-negative strain (Figure S6). The addition of 1 mM EDTA to the growth medium was not inhibitory for *S. typhimurium* or *E. coli*, but it was partially inhibitory to *P. aeruginosa*. As hypothesized, the addition of 1 mM EDTA sensitized both *S. typhimurium* and *E. coli* to LaD, as evidenced by the delay in initiation of detectable growth from 4 to 12 h (Figure 4b). The growth delay for *P. aeruginosa* was less drastic due to the partial toxicity of EDTA. To better validate the bioactivity of the active landomycins, we also ran MIC assays on the landomycin-sensitive organisms *E. faecium*, *L. seeligeri*, *S. aureus*, *B. subtilis*, *S. cerevisiae*, and *C. albicans* (Figures S4 and S5). The activity of erythromycin on *B. subtilis* was used as a control to validate our MIC method (MIC = 133 ng/mL). Although we found these compounds frequently delayed growth, similar to our findings for LaD activity in the presence of EDTA for Gram-negative organisms, most compounds eventually permitted some microbial growth at the 24 h time point used to calculate the MIC (Figure S5 and Figure 4c). Only LaD and Lan completely inhibited *B. subtilis* and *S. cerevisiae* growth, respectively, at sub-50-μM concentrations. Overall, these five landomycins appear to have limited antimicrobial applications but show promise as starting scaffolds to synthesize the next generation of improved landomycins.

3.5 | Landomycins exhibit anticancer activity

We tested the efficacy of each of the landomycin products identified against the human NSCLC cell line (A549-CCL-185; A549), using doxorubicin hydrochloride (DOX) as a positive control. After 24 h of incubation with each compound of interest, we analyzed the A549 cells using flow cytometry. To determine the efficacy of each compound against A549 cells, we normalized to the negative control (0 μM), fit curves to each data set (Figure 5a), and determined LC_{50} values for each drug of interest (Figure 5b). These results indicate that while LaA is not as potent as DOX, it was the most potent member of the products of interest. For landomycins containing

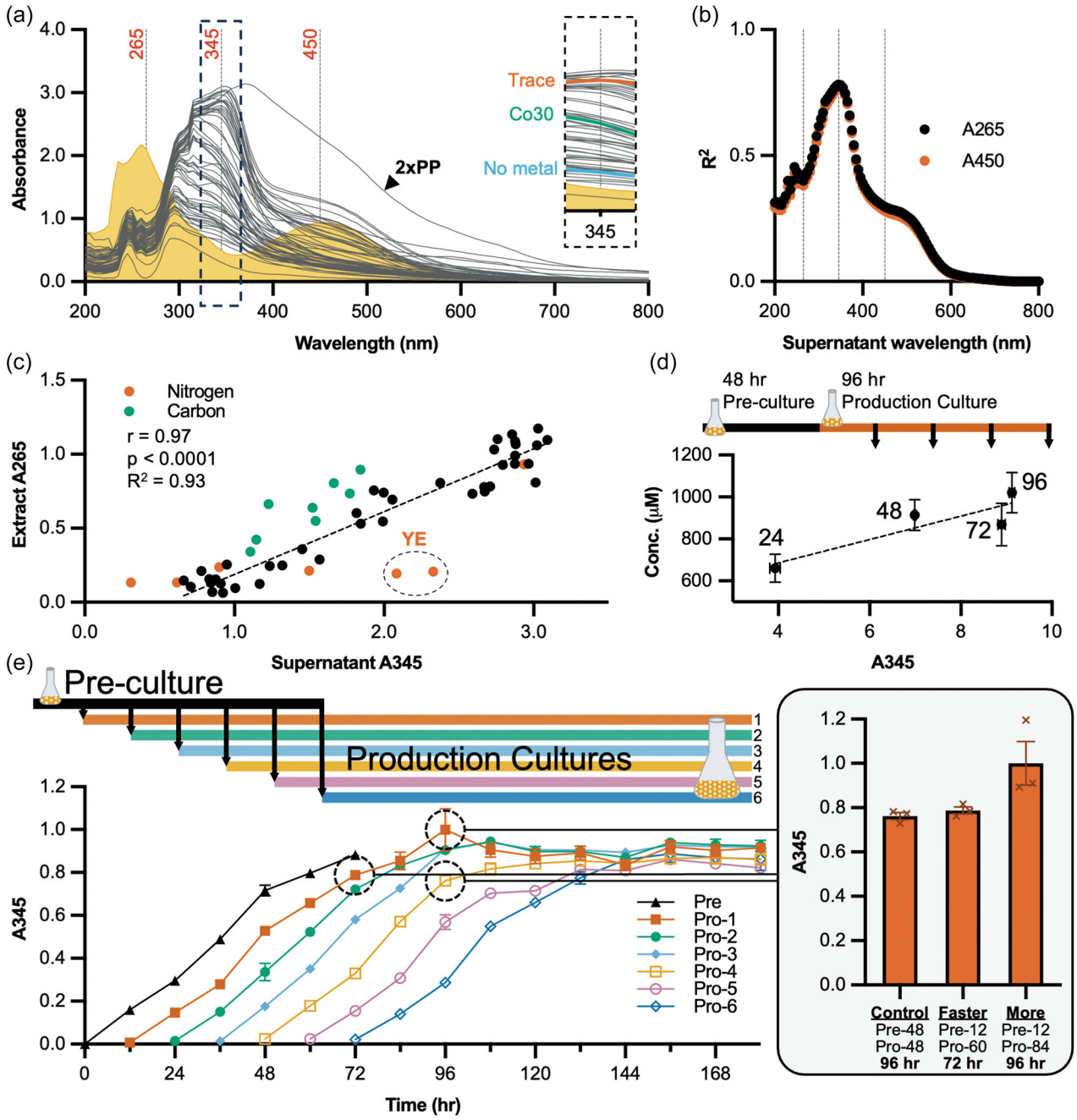
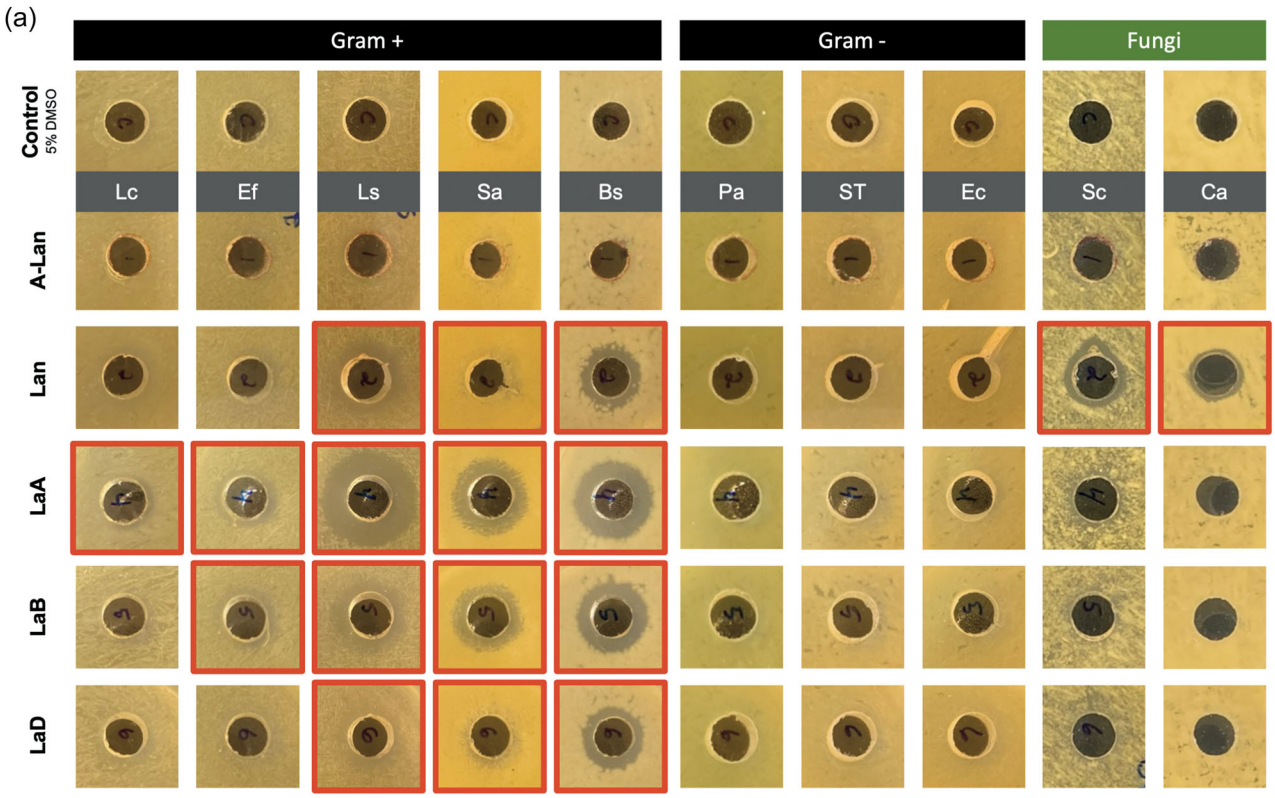
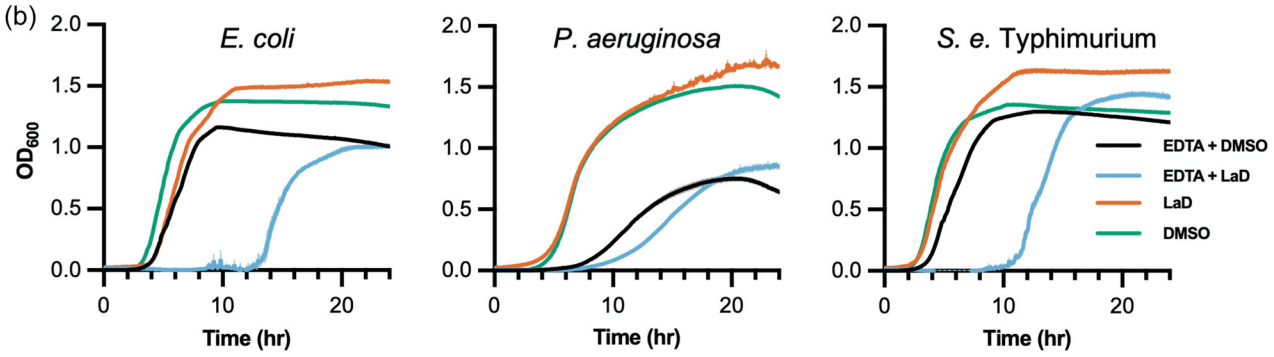


FIGURE 3 Detection of relative landomycin production in culture supernatants. (a) Average absorbance spectrum of the supernatant of 55 distinct production conditions tested for method optimization (gray lines). The absorbance spectrum of purified LaA (0.1 mg/mL) is shown in yellow. Vertical dashed lines indicate 265, 345, and 450 nm wavelengths. The spectrum of SG containing 2x phytone peptone (2xPP) is indicated by the label and black arrow. The inset depicts 345 nm with trace, Co30, and No metal conditions labeled in orange, green, and blue, respectively. (b) R^2 values for linear regression analysis of supernatant absorbance at the given wavelength relative to the extract absorbance at 265 nm (black circles) or 450 nm (orange circles). Vertical dashed lines indicate 265 (R^2 approximately 0.4), 345 (the maximum; $R^2 = 0.78$), and 450 nm (R^2 approximately 0.3) wavelengths. (c) Plot of extract absorbance at 265 nm versus supernatant absorbance at 345 nm. Samples from media containing different nitrogen and carbon sources are orange and green, respectively. Yeast extract samples are encircled and labeled YE. Nitrogen and carbon samples were removed from correlational and linear regression analysis due to apparent non-correlational clustering thought to result from the different base media absorbance. Pearson's correlation coefficients and R^2 of the final 42 conditions are 0.965 ($p < 0.0001$) and 0.93, respectively. (d) Linear regression analysis of total landomycin concentration determined by LC-MS relative to A345 of culture time-course with samples taken every 24 h from production cultures ($R^2 = 0.78$). Arrows from production culture indicate sampling time points. (e) Time-course analysis of pre-culture and production culture landomycin production determined by A345. Production cultures were set every 12 h from the pre-culture, and A345 was measured every 12 h ($n = 3$). The bar plot depicts the magnitude of two different improved production methods ("Faster" and "More"), compared to the starting condition (control), which decreased production time by 24 h or increased yield by 32%. SG, soytone-glucose.

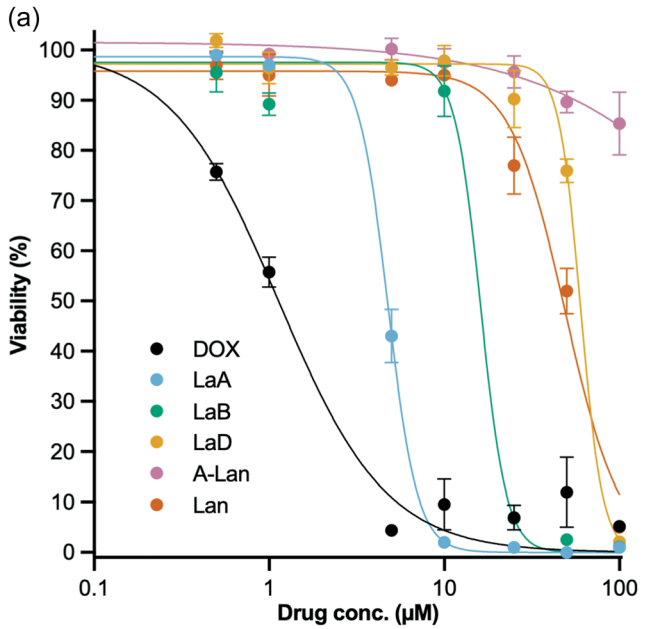




(c)

Organism	Lan	LaA	LaB	LaD
B. subtilis	172	>230	>257	<6.5
S. aureus	478	152	193	113
E. faecium	>739	>230	>257	418
L. seeligeri	179	>230	>257	78
C. albicans	116	>230	>257	>418
S. cerevisiae	45	107	170	83

FIGURE 4 Antimicrobial spectrum of landomycin products of *Streptomyces cyanogenus* 136 + pOOB92a. (a) Inhibitory spectrum of anhydrolandomycinone (A-Lan), landomycinone (Lan), landomycin A (LaA), landomycin B (LaB), and landomycin D (LaD), against: *Lactococcus cremoris* (Lc), *Enterococcus faecium* (Ef), *Listeria seeligeri* (Ls), *Staphylococcus aureus* (Sa), *Bacillus subtilis* (Bs), *Pseudomonas aeruginosa* (Pa), *Salmonella enterica* ser. Typhimurium (ST), *E. coli* (Ec), *Saccharomyces cerevisiae* (Sc), and *Candida albicans* (Ca) as determined by agar well diffusion assay. 100 µL of 250 µg/mL of each compound were added per agar well; the control contained only DMSO (5% v/v). Wells, where inhibition of growth was visible, are outlined with a bold red border. (b) Growth curves of *E. coli*, *P. aeruginosa*, and *S. typhimurium* in the presence of 250 µg/mL LaD with and without the addition of 1 mM EDTA. DMSO and EDTA + DMSO are provided as controls; growth inhibition by LaD required EDTA in Gram-negative organisms. (c) Minimum inhibitory concentration values (µM) were calculated from growth inhibition curves (Figure S5) for organisms sensitive to at least one landomycin.



(b)

Compound	LC_{50} ± SEM (μM)
Doxorubicin	1.1 ± 0.2
Landomycin A	4.8 ± 0.1
Landomycin B	16.1 ± 1.4
Landomycin D	59.9 ± 2.9
Landomycinone	48.9 ± 2.5
Anhydrolandomycinone	>100

FIGURE 5 Viability of non-small cell lung carcinoma (A549) cells treated with landomycins. (a) Dose-response curves for A549 cells treated with 0–100 μM DOX, landomycin A, landomycin B, landomycin D, landomycinone, and anhydrolandomycinone for 24 h before analysis via flow cytometry. We report the average percent viability ($n = 3$), normalized to the 0 μM sample. Error bars represent the SEM. We fit each data point to a nonlinear [inhibitor] versus response curve. (b) Tabulated LC50 values from dose-response curves.

deoxyoligosaccharides, LaA was the most active, while LaD was the least active. Additionally, we found that Lan was one of the least active landomycins in this assay, while A-Lan was inactive up to 100 μM.

4 | DISCUSSION

Unspecified production/induction conditions and low yield are major obstacles to the translation of natural products into viable therapeutics and chemical goods. Similarly, complicated separations add considerable costs and require the use of large amounts of energy and/or harmful solvents. Landomycins are not unique in this regard, and the lack of studies performed on this promising scaffold can be attributed, in part, to the difficulty in obtaining the molecule(s) of interest. To this end, we developed a spectrophotometric method using culture extracts to screen media compositions that reached LaA concentrations as high as 390 μM and a total polyketide concentration of 915 μM. By simplifying this method through the substitution of culture extracts with culture supernatants, we rapidly increased sample throughput, allowing us to monitor landomycin titers during production and across multiple cultures. Compared to other relatively high throughput analytical methods such as UPLC and RapidFire, which can accommodate analysis of hundreds of samples, the spectrophotometric method proposed here is faster, low-cost, and offers real-time production information that immediately actionable (Dueñas et al., 2023; Vervoort et al., 2021). While the detection of natural products by UV-vis spectroscopy is context-dependent and may not be applicable to all products of interest and media conditions, the underlying methodology that we developed—finding a universal absorbance distinct to pure compounds of interest and correlating that to an equally representative wavelength in the culture supernatant—is high throughput, low-cost, and can be easily extended to other natural products that absorb light in the UV-vis range. Furthermore, by developing a differential centrifugation method to isolate landomycin-rich culture solids, we reduced the typically grueling purification method for LaA to a single normal phase silica column and precipitation step to yield >97% pure LaA (Henkel et al., 1990; Shaaban, Srinivasan, et al., 2011). This application should also serve to simplify the isolation of other natural products expressed in the culture solids of *Streptomyces*.

Of the landomycins investigated in this work (LaA, LaB, LaD, and Lan), all compounds generally inhibited the growth of Gram-positive bacteria, Lan inhibited the growth of fungi, and none of the compounds were active against Gram-negative bacteria without the addition of EDTA. LaA and LaB had the broadest spectrum of activity against Gram-positive bacteria in the agar well diffusion assay, but they generally had high MICs. Only LaD and Lan had <50 μM MIC when treating *B. subtilis* and *S. cerevisiae*, respectively. Interestingly, LaA, LaB, and LaD only differ structurally by the length of the glycan chain, yet these subtle differences resulted in vastly different antimicrobial activities. In another study, landomycin R (LaR)—an anhydro-landomycin with a disaccharide glycan—was more inhibitory than landomycin Q, which only differs from LaR by an extra rhodinose on the glycan chain (Lai et al., 2021). Similarly, LaR only differs from LaD by the loss of an alcohol group and subsequent formation of a carbon–carbon double bond in the polyketide core, yet we found LaD to be far less active against *S. aureus* than was previously found for LaR (7 and 14 vs. 104 μM, respectively). Together, these findings better elucidate some of the activity-defining moieties of landomycins. In particular, the length and composition of the glycan chain are key to the bioactivity of landomycins and provide targets for future structure-function studies and synthetic modifications intended to improve potency and/or spectrum.

A common feature of many landomycins is their color (Figure 1a), and during the course of the MIC assay, we noticed that the orange (LaA, LaB, LaD) or dark blue (Lan) tinted media changed color to a

darker, brown-black (Figure S7). Even the aseptic media underwent this transformation. Given the underlying physical properties determinant of color, we hypothesize that a chemical reaction that alters the bonding in landomycin structures to be the likely cause. This suggests that the landomycins in this study are not particularly stable under the conditions tested, which could account for the transient growth inhibition found for many of the MIC assay samples. In fact, previous mechanistic investigations into the bioactivity of LaE noted both the rapid generation and subsequent decline of reactive oxygen species (ROS) in cancer cells, as well as the rapid generation and subsequent quenching of a fluorescent Michael adduct with biothiols, predicted to be mediated by the oxygen-dependent removal of the glycan (Panchuk et al., 2017; Terenzi et al., 2021). Taken with our findings, this suggests stabilization of the landomycin backbone and glycan may be an effective strategy to generally improve the bioactivity of landomycins. Identification of the probable degradation products could further direct chemical modifications that improve landomycin stability and should be considered an important step in engineering a next generation of semi-synthetic landomycin therapeutics.

LaA and LaE are the most studied landomycins for their chemotherapeutic potential, which are reported to be mediated by inhibition of DNA synthesis, and/or oxidative stress to mitochondria via the rapid generation of ROS (Crow et al., 1999; Korynevska et al., 2007; Lehka et al., 2015; Panchuk et al., 2017; Terenzi et al., 2021). We found the LC_{50} of LaA to be 4.8 μM, which is within the range previously reported by the NCI for A549 cells (LC_{50} = 0.8 and ≥10 μM), and near the reported IC_{50} (Reinhold et al., 2012; Shankavaram et al., 2009; Shoemaker, 2006; Zhang et al., 2021). Concerning the broader activity trend of the glycated landomycins tested, we found an inverse relationship between oligosaccharide chain length and LD_{50} (LaA<LaB<LaD). This trend has been identified in other cell lines, which suggests that longer glycan chains generally enhance landomycin anticancer activity, and glycan composition can heavily impact activity (Shaaban, Srinivasan, et al., 2011; Shaaban, Stamatkin, et al., 2011). Though the aglycone Lan was previously reported to have an IC_{50} below LaA for this cell line, we found Lan to have reduced efficacy, and similar activity to LaD (Zhang et al., 2021). Interestingly, by substituting the more common but less informative, binary viability methods with a multiplexed, fluorescent dye assay, we identified that landomycin-treated A549 cells undergo late-stage apoptosis, compared to DOX-treated cells that exhibit cellular necrosis (Supporting Information S1: Table 4). Indeed, investigations into the mechanism of LaE bioactivity on Jurkat T-cell leukemia cells have noted that while both landomycins and DOX result in ROS generation, they may travel different paths to their common fate (Korynevska et al., 2007; Panchuk, 2011; Panchuk et al., 2017). Taken together, and given our improved production of the polyketide core, landomycins are promising chemotherapeutic candidates. Our results provide further guidance towards the designing and testing of new, semi-synthetic landomycins that better elucidate the relevant structural features by which landomycins mediate their cytotoxic effects, thereby improving candidate efficacy.

AUTHOR CONTRIBUTIONS

Todd C. Chappell: Conceptualization; formal analysis; investigation; methodology; data visualization; writing—original and revised draft. **Kathleen G. Maiello, Allison J. Tierney, Karin Yanagi, and Jessica A. Lee**: Formal analysis; investigation; methodology; data visualization; writing—editing and review. **Kyongbum Lee**: funding acquisition; supervision; writing—review. **Charles R. Mace, Clay S. Bennett, and Nikhil U. Nair**: Conceptualization; funding acquisition; supervision; writing—review and editing.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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