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Isolation of Fidaxomicin and Shunt Metabolites from *Actinoplanes deccanensis*

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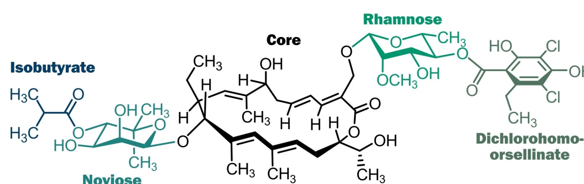
A protocol for the isolation of the antibiotic fidaxomicin (Fdx) from *Actinoplanes deccanensis* and the isolation of shunt metabolites from *A. deccanensis fdxG2*⁻ is reported. We constructed the mutant strain *A. deccanensis fdxG2*⁻ by genetic manipulation which enabled the isolation of shunt metabolites as useful starting points for semisynthetic analogues of Fdx. Furthermore, a synthetic protocol for the conversion of complex *A. deccanensis fdxG2*⁻ extracts into the single compound FdxG2-OH *via* methanolysis is presented. This synthetic procedure is complemented by images and practical notes. Full structure assignment is given in the SI and the characterization data files are published to aid experimentalists. The protocol is also suitable as an undergraduate laboratory project. We hope to facilitate research into new Fdx derivatives through the availability of this procedure.

Keywords: Natural Products, Antibiotics, Metabolic Engineering, Natural Product Isolation, Chemical Education.

Introduction

The natural product antibiotic Fidaxomicin (**1**, Fdx) was discovered in 1972 and is in clinical use for the treatment of *Clostridioides difficile* infections since 2011.[1–5] A first case of clinically observed resistance emerged recently, a fate that befalls most antibiotics.[6,7] This resistance could be overcome by structural modification of Fdx *via* site-selective approaches. The complex structure of Fdx consists of an 18-membered macrolactone core that is decorated with a modified D-noviose and a rhamnoside-dichlorohomoorsellinate fragment (*Figure 1*).[8] So far, selective modification of the individual constituents of Fdx

The Antibiotic Fidaxomicin



Fidaxomicin (**1**, Fdx)
Also known as: lipiarmycin A3, clostomicin B1, tiacumicin B
This work:
Isolated yield from *Actinoplanes deccanensis*: **633-705 mg/L**

Figure 1. The antibiotic Fidaxomicin can be isolated from *A. deccanensis* (DSM 43806) in high yields.

+ These authors contributed equally.

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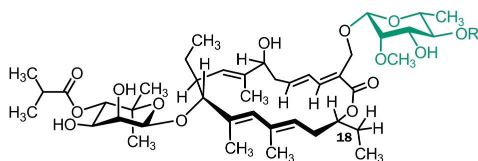
has been the focus of Fdx derivatisation campaigns.[9–13]

The accessibility of truncated analogues missing one glycosyl moiety could lead to new opportunities for semi-synthesis. Introducing new substituents to the shunt metabolite scaffold would modulate the antibiotic activity and physicochemical properties of the resulting derivatives. Interrupting the biosynthetic pathway of a natural product is an attractive strategy to generate these shunt metabolites.[14,15] Zhang and co-workers elucidated the biosynthesis of Fdx and obtained several Fdx shunt metabolites.[16–20] For an overview of Fdx biosynthesis and a summary of known shunt metabolites see reference [17], the biosynthetic gene cluster of Fdx is described in detail in reference [16]. Fdx analogues lacking one or both glycosides have been obtained from glycosyltransferase knock-out strains. Therefore, we targeted the isolation of shunt metabolites lacking the rhamnoside-dichlorohomoorsellinate moiety as a basis for the development of new Fdx antibiotics (*Figure 2*).

We provide a detailed and illustrated protocol for the isolation of Fdx from *A. deccanensis* WT and the isolation of shunt metabolites from the glycosyltransferase knock-out strain *A. deccanensis fdxG2*$^-$. We lower the barrier for researchers to access these valuable molecules and facilitate research towards highly needed new Fdx antibiotics. In addition, we believe that this may also be an attractive project for practical teaching courses in the natural sciences. This was verified in the form of a three-week research project as part of the 2nd year organic laboratory course of the chemistry bachelor at the University of Zurich.

Disruption of Biosynthesis

FdxG2 Glycosyl Transferase installs Rhamnose



***fdxG2*⁻ Strain produces Shunt Metabolites**

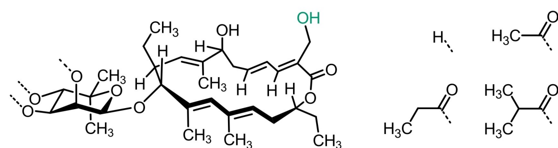


Figure 2. Shunt metabolites that lack the rhamnoside-dichlorohomoorsellinate fragment are obtained by disrupting the *fdxG2* gene encoding the glycosyltransferase FdxG2. The metabolites also lack the C18 hydroxy group, since it is installed later in the biosynthesis.[16,20]

Results and Discussion

A genetically tractable producer organism was sought in order to enable the construction of a knock-out strain capable of producing the desired shunt metabolites. Fdx is produced by the microorganisms *Actinoplanes deccanensis*,[1] *Micomonospora echinospora* subsp. *armeniaca* subsp. nov.,[21,22] *Catellatospora* sp. Bp3323-81,[23] and *Dactylosporangium aurantiacum* subsp. *hamdenensis*.[24,25] Since it has been isolated from different organisms, Fdx is also known in the literature under other names such as lipiarmycin A3,[1–3,23] clostomicin B1,[21–22] and tiacumicin B.[24–25] In our hands, *D. aurantiacum* was found to be very difficult to cultivate. Instead, we turned to the genetically tractable actinobacterium *A. deccanensis*.[26–28] Previous studies and patents describe cultivation of *A. deccanensis* and genetically engineered *A. deccanensis* strains, resulting in Fdx titers of 65.6 mg/L (not isolated),[29] 130 mg/L (not isolated),[30] 619 mg/L (not isolated),[28] 900 mg/L (not isolated),[31] and $>$1500 mg/L (crude).[32] We optimized media composition and growth parameters and found that temperature, flask shape and size, and time were the most influential parameters. Following our optimized protocol, we isolated Fdx in 633–705 mg/L from *A. deccanensis* WT (DSM 43806).

For the elucidation of the Fdx biosynthesis, Zhang and co-workers constructed several mutant *D. aurantiacum* strains, including *D. aurantiacum* subsp. *hamdenensis* Δ*tiaG2*. From this glycosyltransferase knock-out strain, they isolated shunt metabolites lacking the rhamnoside-dichlorohomoorsellinate moiety: FdxG2-OH (**2**, 71.2 mg/L), FdxG2-OAc (**3**, 11.2 mg/L), FdxG2-OPr (**4**, 8.8 mg/L), and FdxG2-O*i*Bu (**5**, 29.3 mg/L). These shunt metabolites additionally lack the C18 hydroxy group, as it is installed later in the biosynthesis (*Figure 2*).[16,20] Our goal was to construct an equivalent *A. deccanensis* strain and optimise growth conditions to obtain these shunt metabolites in higher yields. We constructed the glycosyltransferase knock-out strain *A. deccanensis fdxG2*$^-$ through introduction of the suicide vector pKC1132-KOfdxG2, containing a disrupted *fdxG2* gene, into *A. deccanensis* WT. A two-step allelic exchange led to incorporation into the genome, resulting in the desired strain *A. deccanensis fdxG2*$^-$ (*Figure 3*). A detailed protocol for the genetic

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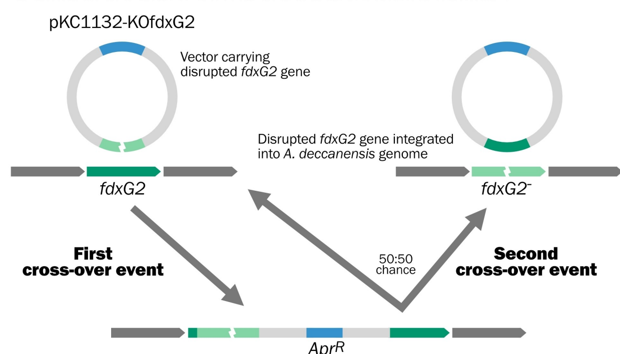


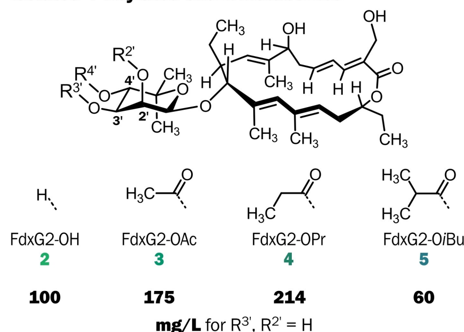
Figure 3. Genetic manipulation of *A. deccanensis.*

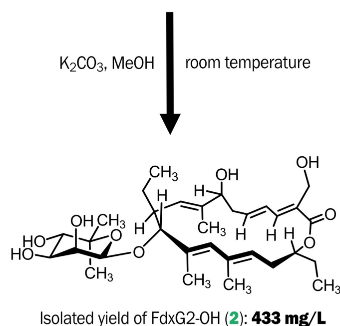
manipulation can be found in the *Supporting Information.*

We successfully obtained the four shunt metabolites from cultures of *A. deccanensis fdxG2*⁻: FdxG2-OH (**2**, 100 mg/L), FdxG2-OAc (**3**, 175 mg/L), FdxG2-OPr (**4**, 214 mg/L), and FdxG2-O*i*Bu (**5**, 60 mg/L). Their structures were confirmed by 2-dimensional NMR spectroscopy (see *Supplementary Tables 1 & 2, Supplementary Figure 1*). The total amount of shunt metabolites isolated from *A. deccanensis fdxG2*⁻ exceeds the yields from *D. aurantiacum* subsp. *hamdenensis ΔtiaG2* more than 4-fold.

Although high titers of the shunt metabolites were produced, the presence of three different acyl groups (acetyl, propionyl, isobutyryl) and their 2′- and 3′-isomers (*Figure 4*, minor peaks) leads to a complex culture extract. These secondary metabolites of varying ester moiety or attachment point are also observed in WT producers of Fdx, although in much smaller quantities relative to Fdx.[17,25] The relative increase of these isomers compared to the WT may be caused by promiscuity of the acyltransferase depending on its substrate as well as changes to the metabolic state of the mutant cells. The presence of these isomers drastically complicates purification and reduces isolated yields of the respective metabolites. To facilitate methodology development and synthetic derivatisation, large amounts of material are required. Upon hydrolysis, all ester isomers of the extract would converge into the unacylated FdxG2-OH. Therefore, we treated the unpurified extract from *A. deccanensis fdxG2*⁻ with K_2CO_3 in methanol, which led to complete deacylation of the extract within 5 minutes at room

Isolation of Shunt Metabolites from *A. deccanensis fdxG2*⁻





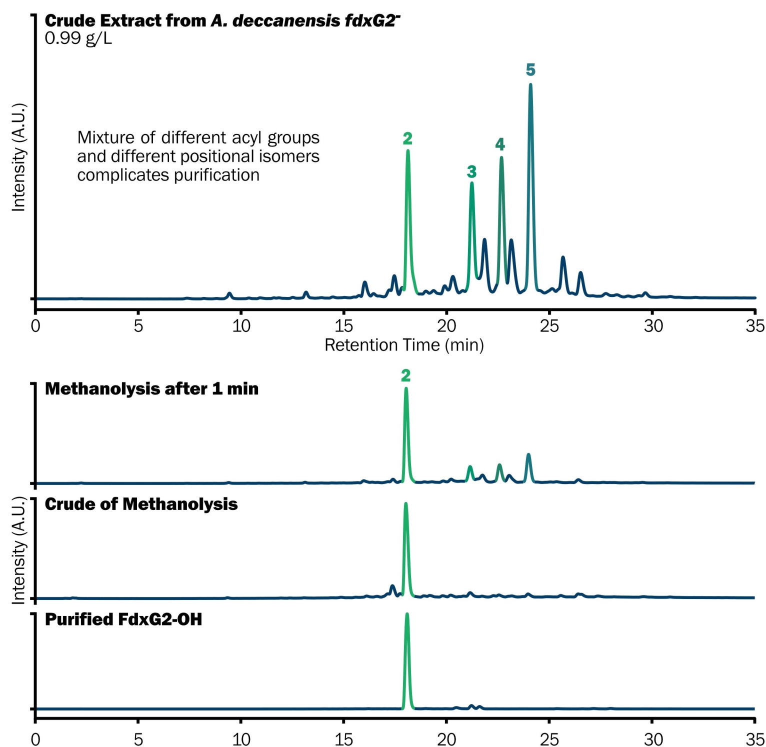


Figure 4. Isolation of shunt metabolites from complex extract of *A. deccanensis fdxG2*⁻. Methanolysis converts most extract components to FdxG2-OH. UV detection at 270 nm, using gradient elution (5–95 % MeCN in water, 0.1 % formic acid, over 30 min).

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temperature (*Figure 4*). After isolation, this gave FdxG2-OH in 433 mg/L, providing practically useful amounts of material while greatly simplifying purification.

Conclusion

We have established a robust protocol for the isolation of Fdx from *A. deccanensis* WT in high yields of 633–705 mg/L. After constructing the glycosyltransferase knock-out strain *A. deccanensis fdxG2*$^{-}$, the shunt metabolites FdxG2-OH (**2**, 100 mg/L), FdxG2-OAc (**3**, 175 mg/L), FdxG2-OPr (**4**, 214 mg/L), and FdxG2-O*i*Bu (**5**, 60 mg/L) were obtained. To facilitate purification, we methanolized the crude extract to obtain FdxG2-OH in an increased yield of 433 mg/L. The cultivation and isolation of secondary metabolites from *A. deccanensis* was also carried out as a three-week research project in the 2nd year practical course of the chemistry bachelor's program at the University of Zurich, demonstrating the suitability of this protocol to become a part of a chemical education curriculum. Furthermore, the facile access to Fdx and its shunt metabolites will accelerate the development of next-generation Fdx antibiotics.

Isolation of Secondary Metabolites from *A. deccanensis*

General Considerations

Actinoplanes deccanensis is an organism that should be handled according to Biosafety Level 1 (BSL1). All individuals handling this strain need to be trained in the handling of risk group 1 organisms and proper disposal of waste.

Prior to handling of chemicals, a risk assessment and hazard analysis should be carried out. The laboratory equipment, safety measures, and personal protective equipment need to be appropriate for each procedure.

The media and cultivation procedure for *A. deccanensis* given here and in the *Supporting Information* differ slightly. The procedure given in the manuscript was optimized to obtain high yields of Fdx and its shunt metabolites and should be used for this purpose. The procedure in the *Supporting Information* was used during the genetic manipulation of *A. deccanensis.*

Preparation of Media (Figure 5)

Soy-Mannitol Agar (SM)

20.0 g D-mannitol (*Fluka*)
20.0 g soybean flour (*Sigma*)
20.0 g agar (*Sigma*)

Ingredients were dissolved in milliQ water (1 L) and autoclaved.

V-Medium for Production

3.0 g meat extract (*Sigma*)
5.0 g tryptone enzymatic digest from casein (*Sigma*)
5.0 g yeast extract (*Sigma*)
1.0 g sucrose (*Sigma*)[33]
24.0 g soluble starch (*Roth*)
4.0 g calcium malate (*Himedia*)

Ingredients were dissolved in milliQ water and autoclaved (1 L). The autoclaved medium is not entirely transparent and contains a white precipitate.

E-Medium for Production

4.0 g meat extract (Sigma)

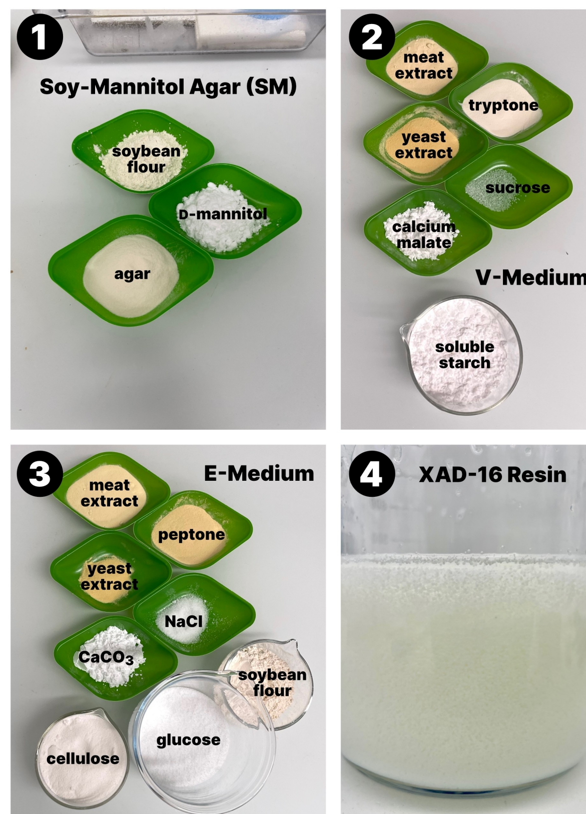

Figure 5. 1) Components of Soy-Mannitol Agar (SM). 2) Components of the V-Medium used for the pre-culture. 3) Components of the E-Medium used for the production culture. 4) Suspension of XAD-16 resin in milliQ water.

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4.0 g peptone (Sigma)
1.0 g yeast extract (Sigma)
2.5 g NaCl (Sigma)
10.0 g soybean flour (Sigma)
5.0 g CaCO3 (Sigma)
20.0 g cellulose (Sigma)[34]
50.0 g glucose (Sigma)

Ingredients except glucose were dissolved in milliQ water (900 mL). The pH was adjusted to 7.6 using NaOH (1 M aq.). Glucose (50 g) was dissolved in milliQ water (100 mL) and sterile-filtered with an 0.2 μm syringe-filter and added to the medium after autoclave treatment.

XAD-16 Resin

To Amberlyst XAD-16 (100 g, *Sigma*) was added milliQ (300 mL) water. After stirring for 30 min, the water was decanted and MeOH (300 mL) was added. After 30 min the MeOH was removed by filtration and the resin was extensively washed with water. The obtained resin was suspended in milliQ water (200 mL, 50 % w/v) and the mixture autoclaved (*Figure 5-4*).

Agar Plates

Heated, liquid Soy-Mannitol agar (SM) was poured into a Petri dish (92×117 mm, *Thermo*). After setting of the agar, *Actinoplanes deccanensis* WT (DSM 43806; ATCC 21983) or *Actinoplanes deccanensis fdxG2*$^-$ (*Note:* A detailed procedure for the genetic manipulation of *Actinoplanes deccanensis* can be found in the *Supporting Information)* from frozen glycerol stock was distributed on the plate using an inoculation loop. The plates were sealed with parafilm. The plates were incubated upside-down at 30 °C for 10–13 days (until significant growth was observed, see *Figure 6-1* and *6-2*). The plates were stored at 8 °C for up to one month.

Pre-Culture

Three small squares (ca. 1 cm^2) of the agar plate were cut out with a sterile loop and inoculated into three 100 mL baffled Erlenmeyer flasks each containing V-Medium (20 mL, *Note: Due to large amounts of precipitate in the media, the bottle should be agitated before dispensing into the culture flasks to ensure a homogenous suspension*). The bacteria were grown at 28 °C in a rotary shaker at 200 rpm for 56 h. Then, the contents of the three flasks were combined to obtain a homogenous pre-culture.

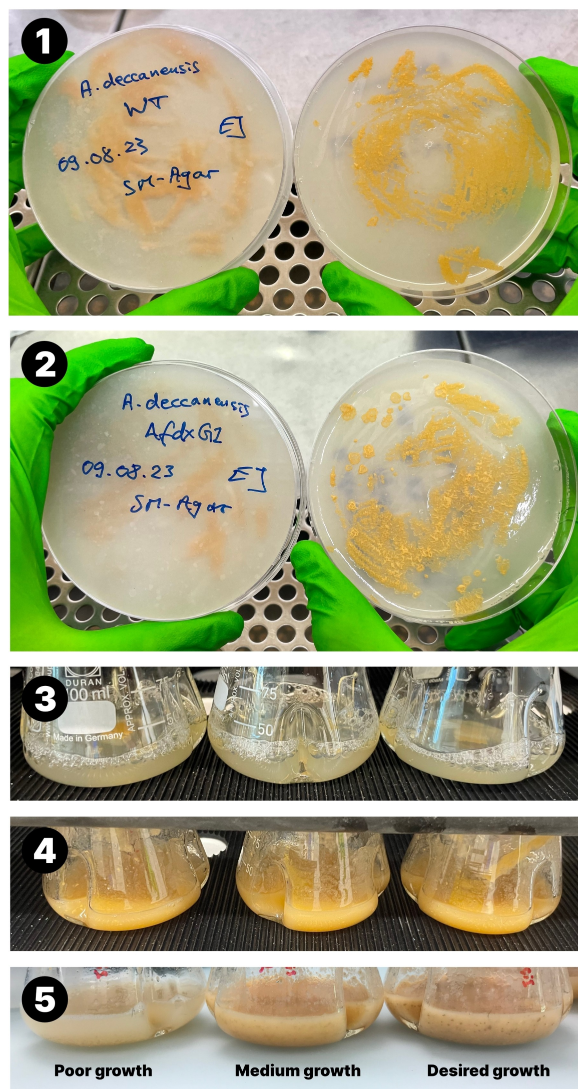

Figure 6. 1) Growth of *A. deccanensis* WT on SM-Agar after 13 days at 30 °C. 2) Growth of *A. deccanensis fdxG2*$^-$ on SM-Agar after 13 days at 30 °C. 3) Pre-culture just after inoculation. 4) Pre-culture after 56 h at 28 °C and 200 rpm. 5) Exemplary production cultures showing poor, medium, and desired growth. Dark coloured XAD-16 resin and thick culture consistency are indicative of high secondary metabolite titers.

Production Culture

Eight 250 mL baffled Erlenmeyer flasks each containing E-medium (50 mL, *Note: Due to large amounts of precipitate in the media, the bottle should be agitated before dispensing into the culture flasks to ensure a homogenous suspension*) and XAD-16 (5 mL, 50 % w/v, *Note: To ensure an even distribution of the resin, the bottle should be agitated before dispensing into the culture flasks to ensure a homogenous suspension*) were each inoculated with 5 mL of the combined pre-

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culture (*Note: 5 mL Serological pipettes are convenient to dispense the pre-cultures*). The bacteria were grown at 28 °C at 200 rpm for 7 days. Typically, metabolites production plateaued after 6–8 days and began to decrease from day 8. If desired, the production of secondary metabolites can be tracked by HPLC (see following section).

Tracking of Secondary Metabolite Production

To track production progress, 1 mL was taken aseptically from each flask on up to five days. The samples were centrifuged at 21130 rcf (*Eppendorf* Centrifuge 5424 R) for 2–6 min. The supernatant was discarded, and the pellet was resuspended in 500 μL MeOH with a toothpick. The resulting suspension was vortexed for 15 sec. The samples were centrifuged at 21130 rcf (*Eppendorf* Centrifuge 5424 R) for 2–6 min and 400 μL of the supernatant were filtered through a 4 mm syringe filter (PTFE (hydrophilic), pore size: 0.22 μm obtained from *BGB Analytik AG*) into HPLC vials. The vials were each spiked with internal standard (20 μL, 0.5 mg/mL caffeine in MeOH). The samples were analysed by UHPLC-MS using the UV chromatogram at 270 nm. The secondary metabolite production was tracked by comparing the integration of the caffeine peak against the desired metabolite.

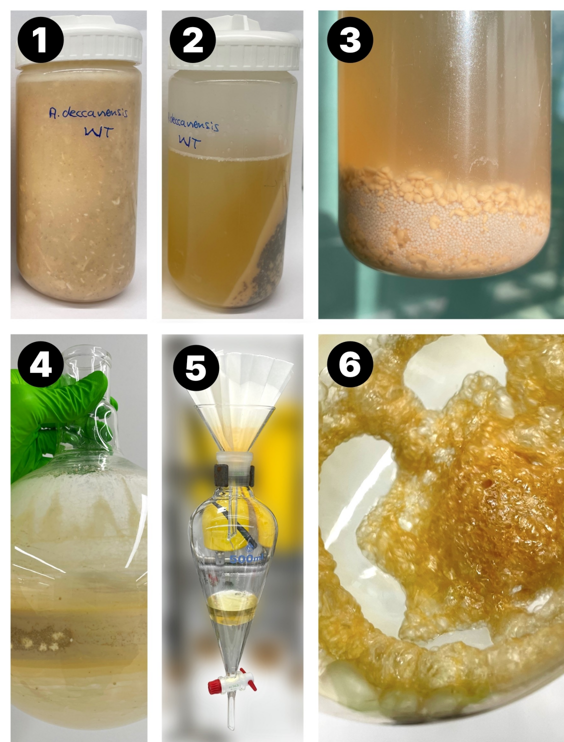

Figure 7. 1) Combined production cultures. 2) Culture after centrifugation, supernatant is discarded. 3) Biomass and XAD-16 resin after *n*-butanol extraction. Decolouration of resin indicates successful extraction. 4) Concentrated *n*-butanol extract. 5) Filtration and aqueous wash of EtOAc extract. 6) Concentrated EtOAc extract from *A. deccanensis* WT.

Extraction

The contents of the Erlenmeyer flasks containing the production cultures were combined and the flasks were washed with water. The combined cultures were centrifuged at 4500 rpm (*Hermle* Z 446 K) for 3 min. The supernatant was discarded, and the resulting pellet stirred in *n*-butanol (300 mL *n*-butanol per 500 mL centrifuge bottle) overnight. The mixture was centrifuged at 4500 rpm (*Hermle* Z 446 K) for 3 min, the supernatant collected, and the residue washed with *n*-butanol (2×50 mL). The organic extract was concentrated to dryness *in vacuo* at 45 °C. The resulting residue was suspended in EtOAc (150 mL) under sonication and filtered into a separating funnel. The organic layer was washed with water (2×50 mL), brine (sat. aq. solution of NaCl, 50 mL), dried over sodium sulfate, and concentrated *in vacuo* at 40 °C to yield for *A. deccanensis* WT a brown foam (990 mg) and for *A. deccanensis fdxG2*$^-$ a brown oil (475 mg) (see *Figure 7*).

Purification of Fidaxomicin from A. deccanensis *WT Extract*

The extract from *A. deccanensis* WT (990 mg) was dissolved in MeCN (8 ml) and eluted through an SPE cartridge (Discovery® DSC-18 SPE Tube 52604-U) and the filtrate was concentrated *in vacuo* at 40 °C. The resulting residue was dissolved in MeCN (3.5 mL) and purified by preparative RP-HPLC ([Gemini NX, C18, 5 μ, 110 Å, 250 mm×21.2 mm, solvent A: H_2O + 0.1 % HCOOH, solvent B: MeCN + 0.1 % HCOOH, 20 mL/min; LC time program (min – %B): 50 % isocratic], 155 mg of mixture per run) to yield, after lyophilisation, fidaxomicin (**1**, t_R = 27.0 min, 304 mg, 0.287 mmol, 633 mg/L) as a colourless solid (*Figure 8*). A yield of 633 mg/L was obtained using conditions optimized for *A. deccanensis fdxG2*$^-$. The purity of the 633 mg/L batch was 92 %. Using a pre-culture temperature of 30 °C and pre-culture time of 5 days, 705 mg/L of Fdx were obtained. The purity of 705 mg/mL batch was 96 %. Repurification *via* RP-HPLC can deliver higher purities if required

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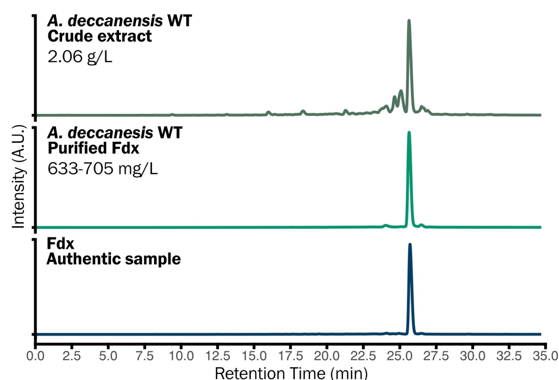

Figure 8. HPLC chromatograms of 1) Crude extract from *A. deccanensis* WT, 2) Purified Fdx from *A. deccanensis* WT, chromatogram shown is from the 705 mg/L batch, 3) Authentic sample of Fdx. UV detection at 270 nm, using gradient elution (5–95% MeCN in water, 0.1% formic acid, over 30 min).

Purification of Shunt Metabolites from A. deccanensis fdxG2$^-$ *Extract*

Instead of the methanolysis, the culture extract from *A. deccanensis fdxG2$^-$* can also be purified by RP-HPLC. The orange foam was dissolved in MeCN/water (3:1, 4 mL) and purified by preparative RP-HPLC ([Gemini NX, C18, 5 μ, 110 Å, 250 mm×21.2 mm, solvent A: H_2O +0.1% HCOOH, solvent B: MeCN+0.1% HCOOH, 20 mL/min; LC time program (min – %B): 15 min – 40%, 50 min – 50%]) to yield, after lyophilisation, the shunt metabolites, FdxG2-OH (**2**) (t_R=6.8 min, 15.0 mg, 25.9 μmol, 100 mg/L), FdxG2-OAc (**3**) (t_R= 14.3 min, 26.3 mg, 42.4 μmol, 175 mg/L), FdxG2-OPr (**4**) (t_R=21.3 min, 32.1 mg, 49.9 μmol, 214 mg/L), and FdxG2-O*i*Bu (**5**) (t_R=30.4 min, 9.0 mg, 20 μmol, 60 mg/L) as yellowish solids. The relative ratios of shunt metabolites can change based on small environmental factors in the set-up and can vary from experiment to experiment. Other additives such as sodium acetate were also found to have a dramatic impact on relative production levels.

Methanolysis and Purification of Shunt Metabolite from A. deccanensis fdxG2$^-$ *Extract*

Crude extract from *A. deccanensis fdxG2$^-$* (160 mg) was dissolved in dry MeOH (2.6 mL, *Note:* Dry methanol was used, but strictly anhydrous conditions are not necessary. The reaction can be run in an open flask). To the stirred mixture at RT was added K_2CO_3 (68.3 mg, 2.0 eq., *Note:* The amount of potassium carbonate was calculated based on the heaviest shunt metabolite FdxG2-OiBu (MW 648.8 g/mol)) and the reaction mixture was stirred for 60 min at RT (*Note*: The methanolysis is nearly complete after 1 min, and complete after 5 min. Since the reaction progress was tracked for longer, work-up was carried out after 60 min). Full conversion was confirmed by UHPLC-MS and the reaction mixture was transferred to a separatory funnel containing EtOAc (15 mL) and water (10 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (2×5 mL). The combined organic layers were washed with water (2×5 mL), brine (10 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo* at 40°C to yield a yellow residue (130 mg). The resulting residue was dissolved in MeCN (3 mL) and eluted through an SPE cartridge (Discovery® DSC-18 SPE Tube 52602-U). The filtrate was concentrated *in vacuo* at 40°C and the residue was redissolved in MeCN (1.2 mL) and purified by preparative RP-HPLC ([Gemini NX, C18, 5 μ, 110 Å, 250 mm×21.2 mm, solvent A: H_2O+0.1% HCOOH, solvent B: MeCN+0.1% HCOOH, 20 mL/min; LC time program (min – %B): 5 min – 25%, 60 min – 45%]) to yield, after lyophilisation, FdxG2-OH (**2**) (t_R=34.4 min, 70.0 mg, 121 μmol, 433 mg/L) as a colourless solid.

Suggested Structure and Timeline for an Undergraduate Research Project

Duration of the individual parts of the procedure:

- Preparation and autoclavation of media and culture flasks: 4 hours (hands-on)
- Preparation and inoculation of agar plates: 1 hour (hands-on)
- Strain growth on agar plates: 10–14 days (hands-off)
- Inoculation of pre-culture: 1 hour (hands-on)
- Incubation of pre-culture: 56 hours (hands-off)
- Inoculation of production culture: 1 hour (hands on)
- Incubation of production culture: 7 days (hands-off)
- Extraction of cultures: 4 hours (hands-on)
- Purification of culture extracts and characterisation of secondary metabolites: 5–15 hours (hands-on)

The procedure therefore encompasses 5–7 afternoons of hands-on lab work. To reduce waiting times, we recommend that the teaching assistants inoculate agar plates with the strains two weeks in advance of the project. In addition, unpurified culture extracts of previous cohorts should be provided to the students. The students can then prepare their own media, inoculate cultures, and finally harvest their culture to obtain crude extracts. The downtime created by the growth phases of the cultures can meanwhile be used

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to learn agar plate inoculation, HPLC purification of the crude extracts from previous cohorts, and characterisation of the purified secondary metabolites.

Supporting Information

Supporting Information contains supplementary figures and tables, experimental procedures, compound characterization, and NMR spectra. The authors have cited additional references within the *Supporting Information*.[35–38]

Author Contribution Statement

Erik Jung: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Visualization; Maja Hunter: Methodology, Investigation; Andrea Dorst: Conceptualization, Methodology, Investigation; Alexander Major: Investigation; Tatjana Teofilovic: Investigation; Rolf Müller: Conceptualization, Resources, Supervision, Project administration, Funding acquisition; Karl Gademann: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Data Availability Statement

Additional data were deposited at zenodo https://doi.org/10.5281/zenodo.10566448.

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