Isolation of Fidaxomicin and Shunt Metabolites from *Actinoplanes deccanensis*

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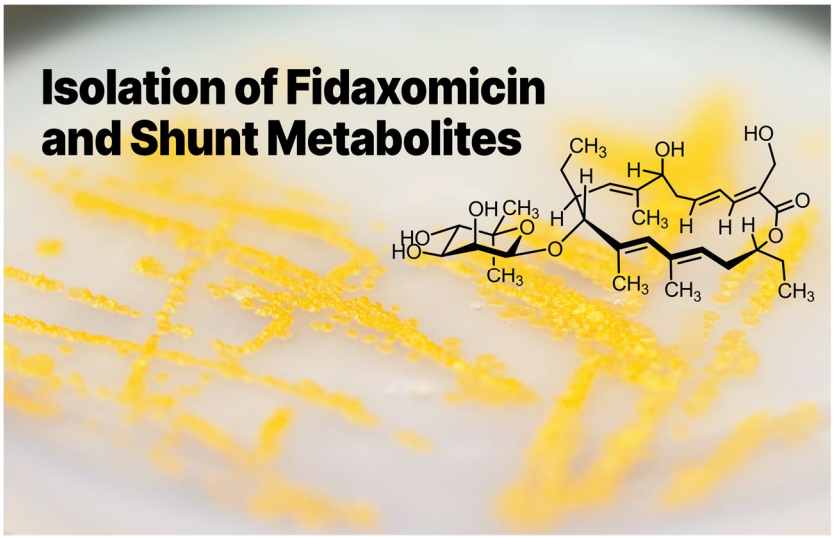

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Abstract

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

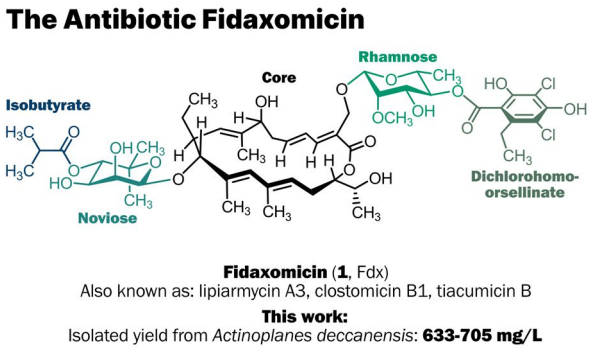

Figure 1. The antibiotic Fidaxomicin can be isolated from *A. deccanensis* (DSM 43806) in high yields.

The accessibility of truncated analogues missing one glycosyl moiety could lead to new opportunities for semi-synthesis. Introducing new building blocks to shunt metabolites 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] Fdx analogues lacking one or both glycosides were 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).

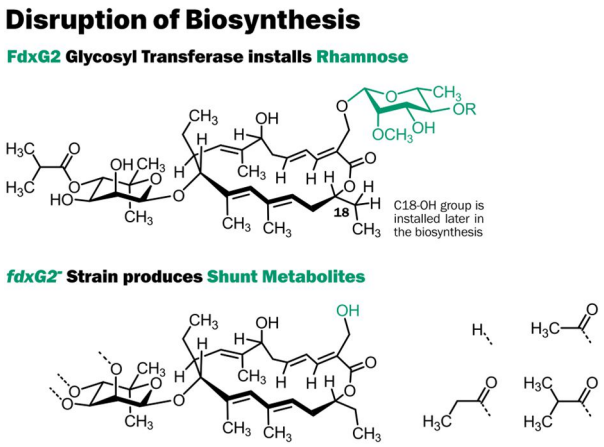

Figure 2. Shunt metabolites that lack the rhamnoside-dichlorohomoorsellinate fragment are obtained by knocking out the glycosyltransferase FdxG2.

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.

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. *armenica* subsp. nov.[21,22], *Catellatospora* sp. Bp3323-81[23], and *Dactylosporangium aurantiacum* subsp. *hamdenensis*[24,25] (*Note 1*). 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 (*Note 2*) 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) (*Note 3)*.[16] 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 manipulation can be found in the *Supporting Information* (*Note 4*).

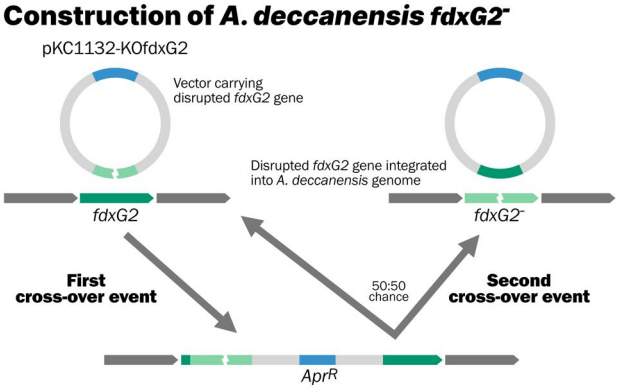

Figure 3. Genetic manipulation of *A. deccanensis*.

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) (*Note 6*). Their structures were confirmed by 2-dimensional NMR spectroscopy (see *Supplementary Tables 1 & 2, Supplementary Figure 2*). The total amount of shunt metabolites isolated from *A. deccanensis fdxG2*$^{-}$ exceeds the yields from *D. aurantiacum* subsp. *hamdenensis* Δ*tiaG2* 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 leads to a complex culture extract. This 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 temperature (Figure 4, *Note 7*). After isolation, this gave FdxG2-OH in 433 mg/L, providing practically useful amounts of material while greatly simplifying purification.

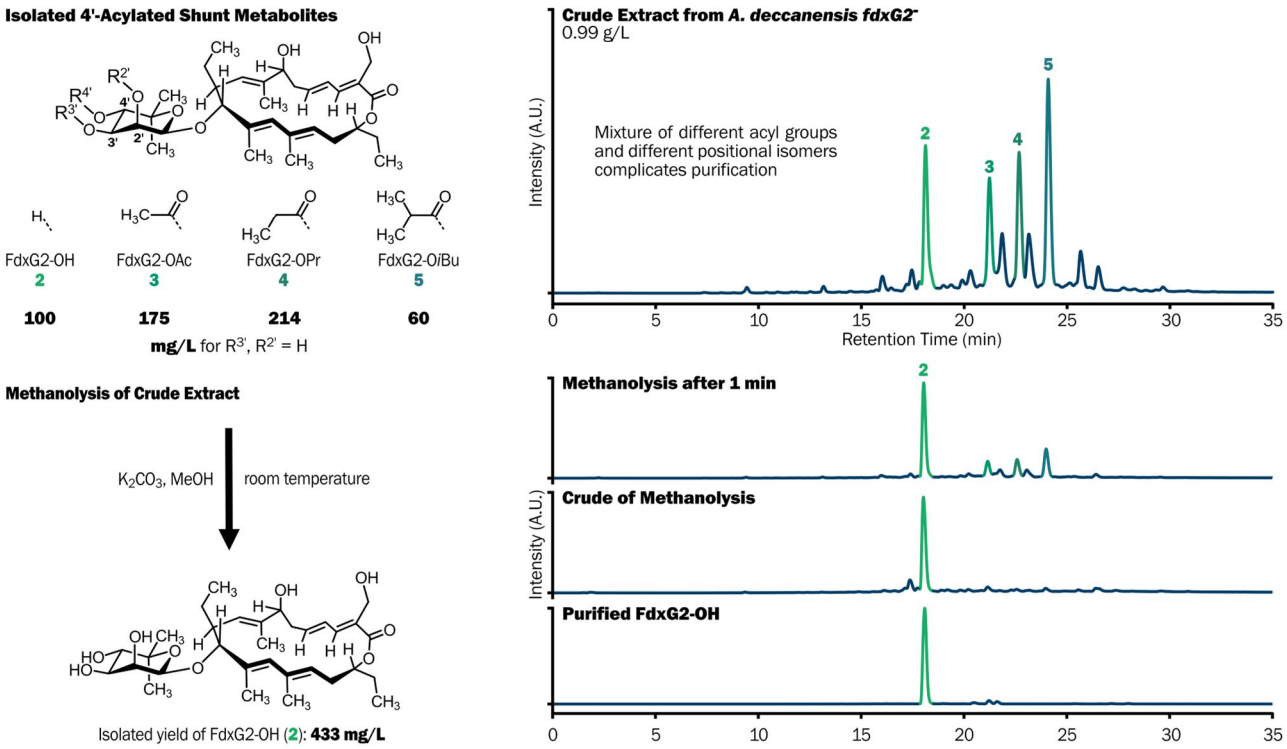

Figure 4. Isolation of shunt metabolites from complex extract of *A. deccanensis fdxG2*⁻ (*Note 6*). Methanolysis converts most extract components to FdxG2-OH. UV detection at 270 nm, using gradient elution (5-95% MeCN over 30 min).

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* *(Notes 4, 5 & 8)*

1.1 Preparation of Media

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*)

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 $CaCO_3$ (*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.

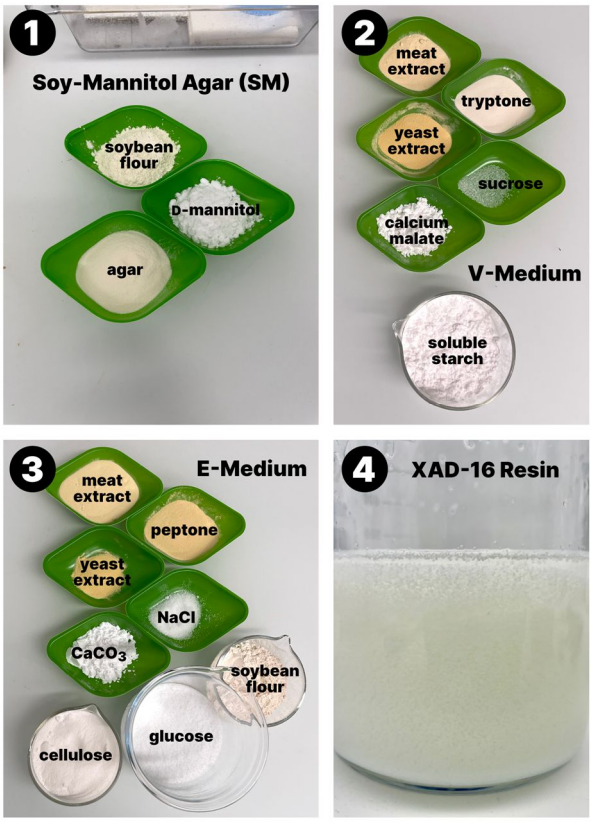

Figure 5. 1) Components of Soy-Mannitol Agar (SM). 2) Components of the V-Medium used for the pre-culture. 3) Components of the E-Medium used for the production culture. 3) Suspension of XAD-16 resin in milliQ water.

1.2 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 9*) 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.

1.3 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 10*). 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.

1.4 Production Culture

Eight 250 mL baffled Erlenmeyer flasks each containing E-medium (50 mL, *Note 10*) and XAD-16 (5 mL, 50% w/v, *Note 10*) were each inoculated with 5 mL of the combined pre-culture (*Note 11*). The bacteria were grown at 28 °C at 200 rpm for 7 days.

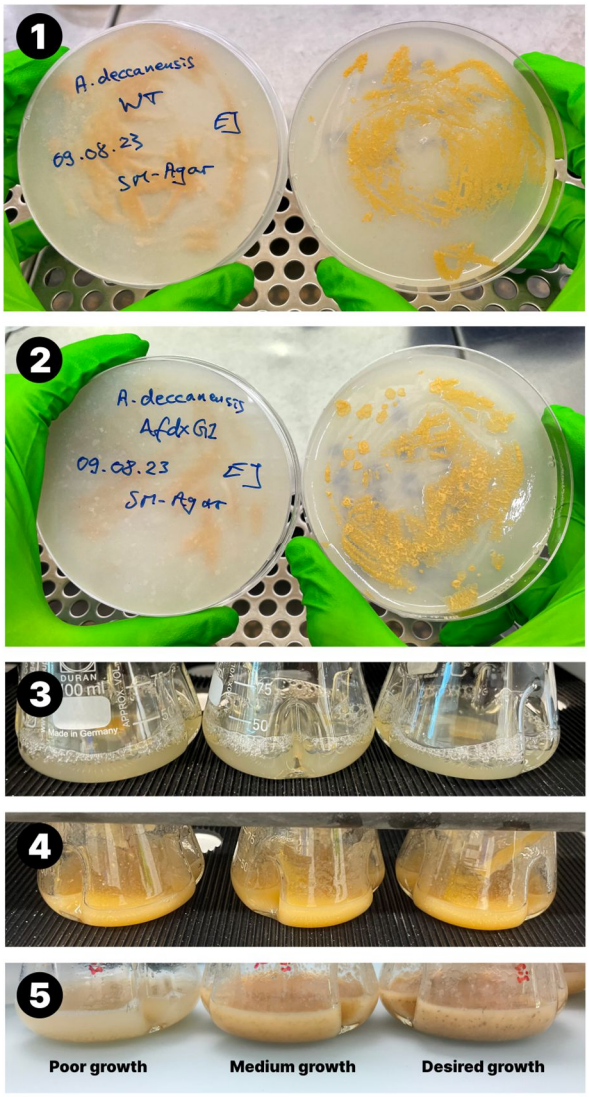

Figure 6. 1) Growth of *A. deccanensis* WT on SM-Agar after 13 days at 30 °C. 2) Growth of *A. deccanensis fdxG2*$^{-}$ on SM-Agar after 13 days at 30 °C. 3) Pre-culture just after inoculation. 4) Pre-culture after 56 h at 28 °C and 200 rpm. 5) Exemplary production cultures showing poor, medium, and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

1.5 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 (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).

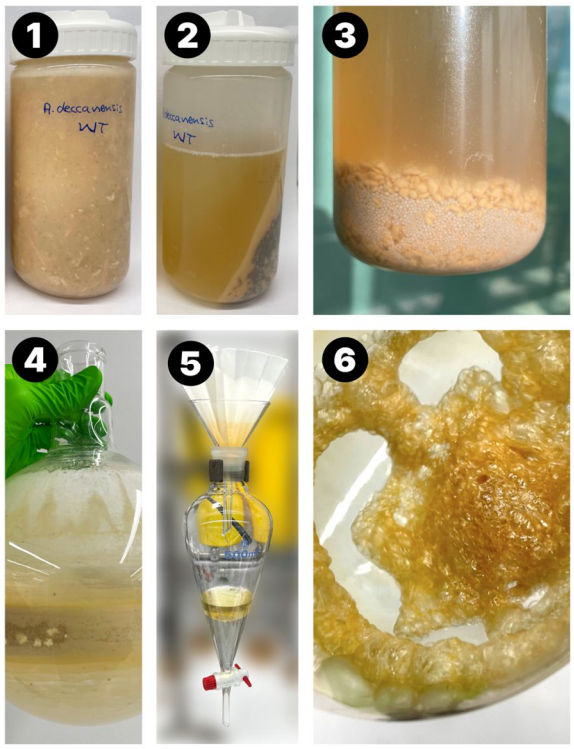

Figure 7. 1) Combined production cultures. 2) Culture after centrifugation, supernatant is discarded. 3) Biomass and XAD-16 resin after *n*-butanol extraction. Decolouration of resin indicates successful extraction. 4) Concentrated *n*-butanol extract. 5) Filtration and aqueous wash of EtOAc extract.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

1.6 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, *Note 2*) as a colourless solid.

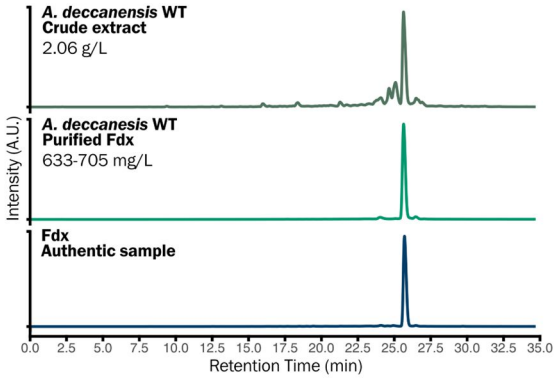

Figure 8. HPLC chromatograms of 1) Crude extract from *A. deccanensis* WT, 2) Purified Fdx from *A. deccanensis* WT, chromatogram shown from 705 mg/L batch, 3) Authentic sample of Fdx. UV detection at 270 nm, using gradient elution (5-95% MeCN over 30 min).

1.7 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 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.

1.8 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 12*). To the stirred mixture at RT was added K_2CO_3 (68.3 mg, 2.0 eq., *Note 13*) and the reaction mixture was stirred for 60 min (*Note 7*) at RT. 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 FdxG2-OH (**2**) (t_R = 34.4 min, 70.0 mg, 121 µmol, 433 mg/L) as a colourless solid.

Notes

1. Since Fdx has been isolated from different organisms it is published under different names such as lipiarmycin A3, clostomicin B1, and tiacumicin B.
2. 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.
3. In the biosynthesis of Fdx, the C18 hydroxy group is installed after the attachment of the rhamnoside moiety. It is therefore lacking in the *fdxG2*$^{-}$ shunt metabolites.[20]
4. *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.
5. 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*.
6. 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.
7. 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.

8. 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.
9. A detailed procedure for the genetic manipulation of *Actinoplanes deccanensis* can be found in the *Supporting Information*.
10. Due to large amounts of precipitate in the media, the bottles should be agitated before dispensing into the culture flasks to ensure a homogenous suspension.
11. 5 mL Serological pipettes are convenient to dispense the pre-cultures.
12. Dry methanol was used, but strictly anhydrous conditions are not necessary. The reaction can be run in an open flask.
13. Equivalents were calculated based on the heaviest shunt metabolite FdxG2-O*i*Bu (MW 648.8 g/mol).

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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

Data Availability Statement:

Supporting Information contains supplementary figures and tables, experimental procedures, compound characterization, and NMR spectra. Additional data were deposited at zenodo https://doi.org/10.5281/zenodo.10566448.

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Supplementary Information

Contents

1.9 General Methods and Materials

Unless otherwise stated, all chemicals were of reagent grade and purchased from *Sigma-Aldrich, Merck, Fluorochem, Acros, TCI, abcr, Fisher Scientific,* or *Honeywell.* Authentic fidaxomicin samples were purchased from commercial suppliers (BOC Sciences or Biosynth Carbosynth). Reactions were carried out under protecting gas (N_2, passed through a column containing activated 4 Å MS) and, unless otherwise stated, monitored for completion by UHPLC-MS (ESI). Solvents for reactions were of p.a. grade. Evaporation of solvents *in vacuo* was carried out on a rotary evaporator at 40 °C bath temperature and appropriate pressure. Flash column chromatography was performed with silica gel (230-400 mesh, *Merck*).

Ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS): *Ultimate 3000 LC* instrument (*Thermo Fisher Scientific*) coupled to a triple quadrupole *Quantum Ultra EMR* MS (*Thermo Fisher Scientific*) using a reversed-phase column (*Kinetex*® EVO C18; 1.7 µm; 100 Å, 50 x 2.1 mm; *Phenomenex*). The LC was equipped with an *HPG-3400RS* pump, a *WPS-3000TRS* autosampler, a *TCC-3000RS* column oven and a *Vanquish DAD* detector (all *Thermo Fisher Scientific*). The following solvents were applied: H_2O + 0.1% HCOOH (A), MeCN + 0.1% HCOOH (B). Samples were prepared using HPLC grade solvents (MeCN, MeOH, H_2O) and filtered over a 4 mm syringe filter, PTFE (hydrophilic), pore size: 0.22 µm obtained from *BGB Analytik AG*. The MS was equipped with an H-ESI II ion source. The source temperature was 250 °C, the capillary temperature 270 °C and capillary voltage 3500 V, and datasets were acquired at resolution 0.7 on Q3 in centroid mode.

High performance liquid chromatography (HPLC): *Prominence* modular HPLC instrument (*Shimadzu*) coupled to an *SPD-20A* UV/Vis detector (*Shimadzu*) using a reversed-phase column (*Gemini NX* C18, 3 µm, 10 Å, 150 mm × 4.6 mm) for analytical HPLC, and a reversed-phase column (*Gemini NX* C18, 5 µm, 110 Å, 250 mm × 21.2 mm) for preparative HPLC. The LC was equipped with a *CBM-20A* system controller, *LC-20A* solvent delivery unit, a *DGU-20A* degassing unit, *FRC-10A* fraction collector (all *Shimadzu*). The following solvents were used: H_2O + 0.1% HCOOH (A), MeCN + 0.1% HCOOH (B).

Infrared spectra (IR): *Spectrum Two FT-IR Spectrometer* (*Perkin–Elmer*) equipped with *a Specac Golden Gate™ ATR* (attenuated total reflection) accessory; applied as neat samples or as films; $1/\lambda$ in cm^{-1}.

Nuclear magnetic resonance spectra (NMR): ^{1}H-NMR spectra were recorded in acetone-*d*$_6$ on the instruments *Bruker AV-500* (500 MHz) or *AV-400* (400 MHz); chemical shift δ in ppm relative to solvent signals (δ = 2.05 ppm for acetone-*d*$_6$), coupling constant *J* is given in Hz. ^{13}C-NMR spectra were recorded in acetone-*d*$_6$ on the instruments *Bruker AV-500* (500 MHz) or *AV-400* (400 MHz); chemical shift δ in ppm relative to solvent signals (δ = 29.84 ppm for acetone-*d*$_6$), coupling constant *J* is given in Hz.

High-resolution-electrospray ionization mass spectra (HRMS): *On flow injection*: High-resolution mass spectra were acquired on a *QExactive* instrument (*ThermoFisher Scientific*, Bremen, Germany) equipped with a heated electrospray (ESI) ionization source and connected to a *Dionex Ultimate 3000* UHPLC system (*ThermoFischer Scientifics*, Germering, Germany). The samples were dissolved in MeOH at a concentration of ca. 50 µg mL^{-1} thereof 1 µL was injected on-flow with a *XRS* autosampler (*CTC*, Zwingen, Switzerland). The mobile phase (120 µL mL^{-1} flow rate) consisting of MeOH + 0.1% HCOOH or MeCN/H$_2$O 2:8 + 0.1% HCOOH was chosen according to the solubility. Ion source parameters were set as follow: spray voltage 3.0 kV; capillary temperature 280 °C; sheath gas 30 L min^{-1}; aux gas 8 L min^{-1}; s-lens RF level 55.0; and aux gas temperature 250 °C. Full scan MS were acquired in the alternating (+)/(–)-ESI mode and over the ranges m/z = 80-1200, 133-2000, or 200-3000 at 70000 resolution (full width half maximum) and with automatic gain control (AGC) target of 3.00E^{+06}. The maximum allowed ion transfer time (IT) was 30 ms. Masses were calibrated below 2 ppm accuracy between m/z = 130.06619 and 1621.96509 in the positive and between 265.14790 and 1779.96528 in the negative ESI mode using the *Pierce*® ESI calibration solutions (*ThermoFisher Scientific*, Rockford, USA). Additionally, contaminations of erucamide (m/z = 338.34174, (+)-ESI) and palmitic acid (m/z = 255.23295, (–)-ESI) were used as lock masses in (+)-and (–)-ESI, respectively.

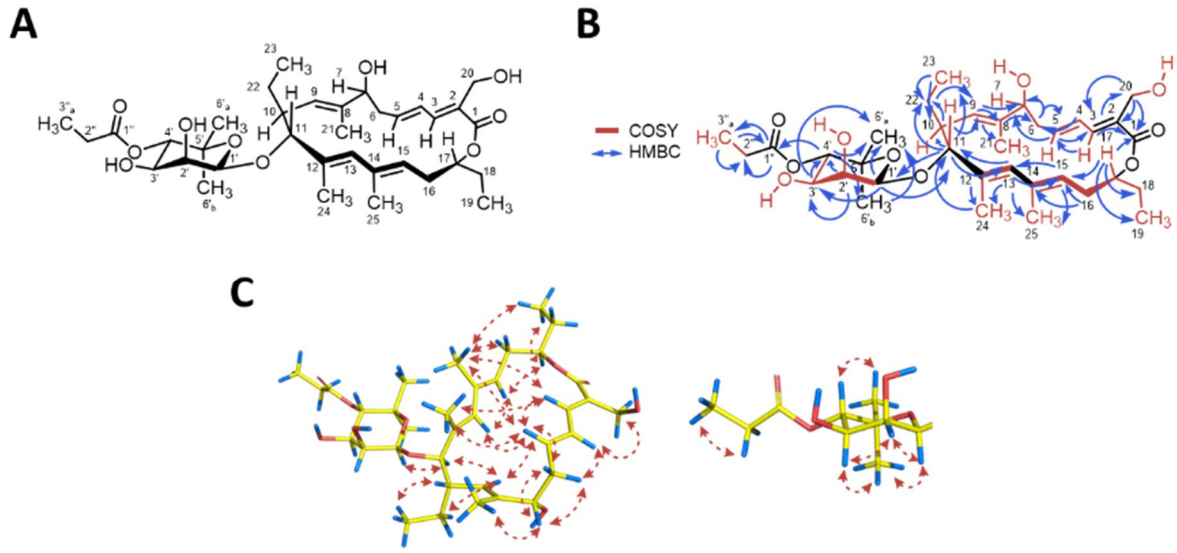
1.10 Supplementary Tables and Figures

Supplementary Table 1. ^{1}H NMR data of compounds **2**, **3**, **4**, and **5** (500 MHz, acetone-d_6, δ in ppm, J in Hz).

No. H	FdxG2-OH (2)	FdxG2-OAc (3)	FdxG2-OPr (4)	FdxG2-O*i*Bu (5)
3	7.10, d (11.4)	7.09, d (11.5)	7.09, d (11.5)	7.09, d (11.5)
4	6.58, m	6.58, m	6.58, m	6.57, m
5	5.89, ddd	5.89, ddd	5.89, ddd	5.89, ddd
	(14.6, 9.1, 4.9)	(14.5, 9.0, 4.9)	(14.5, 9.0, 4.9)	(14.5, 8.9, 4.9)
6a	2.51, m	2.51, m	2.51, m	2.49, m
6b	2.66, m	2.65, m	2.66, m	2.66, m
7	4.26, m	4.27, m	4.27, m	4.27, m
9	5.24, m	5.23, m	5.23, m	5.23, m
10	2.66, m	2.65, m	2.66, m	2.66, m
11	3.72, d (9.7)	3.73, m	3.74, d (9.7)	3.74, m
13	5.81, m	5.82, s	5.82, m	5.82, m
15	5.56, m	5.55, m	5.55, m	5.55, m
16a	2.51, m	2.51, m	2.51, m	2.49, m
16b	2.34, m	2.33, m	2.35, m	3.34, m
17	4.84, tt (6.8, 4.4)	4.84, m	4.84, m	4.84, m
18a	1.82, m	1.82, m	1.82, m	1.83, m
18b	1.73, m	1.72, m	1.72, m	1.73, m
19	0.95, t (7.5)	0.95, t (7.5)	0.95, t (7.5)	0.95, t (7.5)
20	4.33, m	4.32, m	4.33, m	4.33, m
21	1.63, d (1.3)	1.63, d (1.3)	1.63, d (1.2)	1.63, m
22a	1.93, m	1.93, m	1.94, m	1.94, m
22b	1.26, m	1.26, m	1.26, m	1.27, m
23	0.83, t (7.5)	0.83, t (7.4)	0.83, t (7.4)	0.83, t (7.5)
24	1.80, d (1.4)	1.80, d (0.8)	1.80, d (1.4)	1.80, m
25	1.69, s	1.69, d (1.4)	1.69, d (1.4)	1.69, m
1’	4.70, d (1.3)	4.77, d (1.3)	4.77, d (1.3)	4.78, d (0.7)
2’	3.89, m	3.96, m	3.95, m	3.96, s
3’	3.49, m	3.73, m	3.71, m	3.74, m
4’	3.49, m	5.00, d (10.1)	5.02, d (10.1)	5.00, d (10.1)
6’a	1.21, s	1.10, s	1.10, s	1.09, s
6’b	1.07, s	1.14, s	1.14, s	1.14, m
2’’		2.03, s	2.35, m	2.56, m
3’’a			1.09, t (7.6)	1.14, m
3’’b				1.14, m
7-OH	3.75, d (4.0)	-	3.71, m	3.74, m
20-OH	3.69, m	-	3.66, m	3.66, m
2’-OH	3.82, m	-	3.83, m	3.85, s
3’-OH	(3.43, m)	-	3.32, m	3.27, m
4’-OH	(3.49, m)			

Supplementary Table 2. ^{13}C NMR data of compounds **2**, **3**, **4**, and **5** (126 MHz, acetone-d_6, δ in ppm, J in Hz).

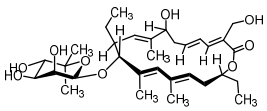
No. C	FdxG2-OH (2)	FdxG2-OAc (3)	FdxG2-OPr (4)	FdxG2-O*i*Bu (5)
1	168.4	168.3	168.4	168.4
2	129.3	129.3	129.3	129.3
3	143.0	143.0	143.0	143.0
4	128.0	128.0	128.1	128.1
5	141.9	141.9	141.9	141.9
6	37.0	37.0	37.0	37.0
7	72.8	72.8	72.8	72.8
8	136.7	136.8	136.8	136.8
9	123.9	123.8	123.9	123.8
10	41.9	41.9	41.9	41.9
11	93.1	93.3	93.3	93.3
12	136.3	136.3	136.3	136.3
13	133.6	133.6	133.6	133.7
14	136.2	136.2	136.2	136.2
15	125.3	125.4	125.4	125.4
16	31.7	31.6	31.6	31.7
17	75.2	75.1	75.1	75.2
18	26.8	26.8	26.9	26.9
19	10.4	10.4	10.4	10.4
20	56.8	56.8	56.8	56.8
21	15.1	15.1	15.1	15.1
22	26.6	26.5	26.6	26.6
23	11.2	11.1	11.2	11.2
24	13.7	13.6	13.7	13.7
25	17.4	17.4	17.4	17.4
1'	96.8	96.7	96.8	96.8
2'	72.7	72.8	72.8	72.8
3'	74.7	70.1	70.1	70.1
4'	72.2	75.9	75.8	75.7
5'	75.0	73.7	73.7	73.7
6'a	28.9	28.6	28.7	28.7
6'b	17.6	18.5	18.6	18.6
1''		170.9	174.3	176.8
2''		21.1	28.1	34.8
3''a			9.5	19.4/19.2
3''b				19.4/19.2



Supplementary Figure 1. (A) Numbering convention used. (B) ^{1}H-^{1}H COSY and HMBC correlations of compound **4**. (C) NOESY correlations of compound **4**.

1.11 Analytical Data of Compounds

Analytical Data of FdxG2-OH (2)



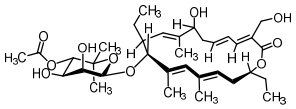
^{1}H NMR (500 MHz, acetone-d_6) δ 7.10 (d, J = 11.4 Hz, 1H), 6.61 – 6.54 (m, 1H), 5.89 (ddd, J = 14.6, 9.1, 4.9 Hz, 1H), 5.82 – 5.79 (m, 1H), 5.58 – 5.54 (m, 1H), 5.25 – 5.21 (m, 1H), 4.84 (tt, J = 6.8, 4.4 Hz, 1H), 4.70 (d, J = 1.3 Hz, 1H), 4.37 – 4.28 (m, 2H), 4.27 – 4.25 (m, 1H), 3.91 – 3.87 (m, 1H), 3.84 – 3.79 (m, 1H), 3.75 (d, J = 4.0 Hz, 1H), 3.72 (d, J = 9.7 Hz, 1H), 3.70 – 3.68 (m, 1H), 3.51 – 3.47 (m, 3H), 3.46 – 3.37 (m, 1H), 2.71 – 2.61 (m, 2H), 2.59 – 2.44 (m, 2H), 2.37 – 2.30 (m, 1H), 1.97 – 1.90 (m, 1H), 1.86 – 1.80 (m, 1H), 1.80 (d, J = 1.4 Hz, 3H), 1.74 – 1.69 (m, 1H), 1.69 (s, 3H), 1.63 (d, J = 1.3 Hz, 3H), 1.31 – 1.23 (m, 1H), 1.21 (s, 3H), 1.07 (s, 3H), 0.95 (t, J = 7.5 Hz, 3H), 0.83 (t, J = 7.5 Hz, 3H) ppm;

^{13}C NMR (126 MHz, acetone-d_6) δ 168.4, 143.0, 141.9, 136.7, 136.3, 136.2, 133.6, 129.3, 128.0, 125.3, 123.9, 96.8, 93.1, 75.2, 75.0, 74.7, 72.8, 72.7, 72.2, 56.8, 41.9, 37.0, 31.7, 28.9, 26.8, 26.6, 17.6, 17.4, 15.1, 13.7, 11.2, 10.4 ppm;

IR (thin film): ν 3396m, 2969m, 2930m, 2878m, 2114w, 1686s, 1640m, 1407m, 1381m, 1364m, 1299m, 1256m, 1231m, 1212m, 1160m, 1073s, 1027s, 948m, 896m, 812w, 787m, 713w, 672w, 601w, 582w, 546w, 509w cm^{-1};

HRMS (ESI) for $C_{32}H_{50}O_9Na^+$ $[M+Na]^+$: calculated: 601.33470; found: 601.33495.

Analytical Data of FdxG2-OAc (3)



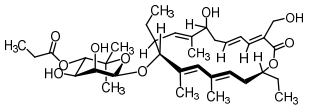
^{1}H NMR (500 MHz, acetone-*d*$_6$) δ 7.09 (d, *J* = 11.5 Hz, 1H), 6.62 – 6.54 (m, 1H), 5.89 (ddd, *J* = 14.5, 9.0, 4.9 Hz, 1H), 5.82 (s, 1H), 5.58 – 5.51 (m, 1H), 5.26 – 5.21 (m, 1H), 5.00 (d, *J* = 10.1 Hz, 1H), 4.87 – 4.80 (m, 1H), 4.77 (d, *J* = 1.3 Hz, 1H), 4.36 – 4.28 (m, 2H), 4.27 (s, 1H), 3.98 – 3.93 (m, 1H), 3.75 – 3.69 (m, 2H), 2.71 – 2.60 (m, 2H), 2.58 – 2.44 (m, 2H), 2.36 – 2.29 (m, 1H), 2.03 (s, 3H), 1.97 – 1.89 (m, 1H), 1.82 (d, *J* = 6.8 Hz, 1H), 1.80 (d, *J* = 0.8 Hz, 3H), 1.73 – 1.67 (m, 1H), 1.69 (d, *J* = 1.4 Hz, 3H), 1.63 (d, *J* = 1.3 Hz, 3H), 1.30 – 1.23 (m, 1H), 1.14 (s, 3H), 1.10 (s, 3H), 0.95 (t, *J* = 7.5 Hz, 3H), 0.83 (t, *J* = 7.4 Hz, 3H) ppm;

^{13}C NMR (126 MHz, acetone-*d*$_6$) δ 170.9, 168.3, 143.0, 141.9, 136.8, 136.3, 136.2, 133.6, 129.3, 128.0, 125.4, 123.8, 96.7, 93.3, 75.9, 75.1, 73.7, 72.8 (2C), 70.1, 56.8, 41.9, 37.0, 31.6, 28.6, 26.8, 26.5, 21.1, 18.5, 17.4, 15.1, 13.6, 11.1, 10.4 ppm;

IR (thin film): ν 3662w, 3449m, 2970m, 2934m, 2880m, 1735m, 1690m, 1641m, 1437m, 1406m, 1384m, 1370m, 1298m, 1252s, 1234s, 1166m, 1080m, 1044s, 951w, 897m, 801w, 713w, 589w, 545w, 510w, 466w cm^{-1};

HRMS (ESI) for $C_{34}H_{52}O_{10}Na^{+}$ $[M+Na]^{+}$: calculated: 643.34527; found: 643.34501.

Analytical Data of FdxG2-OPr (4)



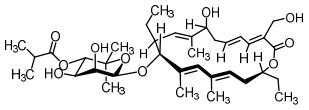
^{1}H NMR (500 MHz, acetone-*d*$_6$) δ 7.09 (d, *J* = 11.5 Hz, 1H), 6.62 – 6.54 (m, 1H), 5.89 (ddd, *J* = 14.5, 9.0, 4.9 Hz, 1H), 5.83 – 5.81 (m, 1H), 5.58 – 5.52 (m, 1H), 5.26 – 5.21 (m, 1H), 5.02 (d, *J* = 10.1 Hz, 1H), 4.84 (tt, *J* = 6.7, 4.5 Hz, 1H), 4.77 (d, *J* = 1.3 Hz, 1H), 4.37 – 4.29 (m, 2H), 4.29 – 4.25 (m, 1H), 3.98 – 3.94 (m, 1H), 3.85 – 3.81 (m, 1H), 3.74 (d, 1H), 3.74 – 3.69 (m, 2H), 3.69 – 3.64 (m, 1H), 3.36 – 3.28 (m, 1H), 2.71 – 2.61 (m, 2H), 2.59 – 2.43 (m, 2H), 2.40 – 2.28 (m, 3H), 1.97 – 1.91 (m, 1H), 1.85 – 1.77 (m, 1H), 1.80 (d, *J* = 1.4 Hz, 3H), 1.74 – 1.67 (m, 1H), 1.69 (d, *J* = 1.4 Hz, 3H), 1.63 (d, *J* = 1.2 Hz, 3H), 1.30 – 1.22 (m, 1H), 1.14 (s, 3H), 1.10 (s, 3H), 1.09 (t, *J* = 7.6 Hz, 2H), 0.95 (t, *J* = 7.5 Hz, 3H), 0.83 (t, *J* = 7.4 Hz, 3H) ppm;

^{13}C NMR (126 MHz, acetone-*d*$_6$) δ 174.3, 168.4, 143.0, 141.9, 136.8, 136.3, 136.2, 133.6, 129.3, 128.1, 125.4, 123.8, 96.8, 93.3, 75.8, 75.1, 73.7, 72.8 (2C), 70.1, 56.8, 41.9, 37.0, 31.6, 28.7, 28.1, 26.9, 26.6, 18.6, 17.4, 15.1, 13.7, 11.1, 10.4, 9.5 ppm;

IR (thin film): ν 3450m, 2972m, 2936m, 2880m, 2111w, 1738m, 1693m, 1641m, 1463m, 1384m, 1368m, 1298m, 1256m, 1210m, 1176m, 1076s, 1032s, 950w, 898m, 801w, 710w, 673w, 584w, 546w, 510w cm^{-1};

HRMS (ESI) for $C_{35}H_{54}O_{10}Na^{+}$ $[M+Na]^{+}$: calculated: 657.36092; found: 657.36055.

Analytical Data of FdxG2-O*i*Bu (5)



^{1}H NMR (500 MHz, acetone-d_6) δ 7.09 (d, J = 11.4 Hz, 1H), 6.63 – 6.53 (m, 1H), 5.89 (ddd, J = 14.5, 8.9, 4.9 Hz, 1H), 5.82 (s, 1H), 5.59 – 5.51 (m, 1H), 5.26 – 5.21 (m, 1H), 5.00 (d, J = 10.1 Hz, 1H), 4.85 – 4.81 (m, 1H), 4.78 (d, J = 0.7 Hz, 1H), 4.38 – 4.29 (m, 2H), 4.28 – 4.24 (m, 1H), 3.96 (s, 1H), 3.85 (s, 1H), 3.75 – 3.71 (m, 3H), 3.70 – 3.62 (m, 1H), 3.30 – 3.25 (m, 1H), 2.73 – 2.61 (m, 2H), 2.60 – 2.52 (m, 1H), 2.58 – 2.44 (m, 2H), 2.38 – 2.27 (m, 1H), 1.97 – 1.90 (m, 1H), 1.85 – 1.77 (m, 1H), 1.81 – 1.77 (m, 3H), 1.73 (s, 1H), 1.72 – 1.67 (m, 3H), 1.65 – 1.61 (m, 3H), 1.30 – 1.24 (m, 1H), 1.16 – 1.12 (m, 9H), 1.09 (s, 3H), 0.95 (t, J = 7.5 Hz, 3H), 0.83 (t, J = 7.5 Hz, 3H) ppm;

^{13}C NMR (126 MHz, acetone-d_6) δ 176.8, 168.4, 143.0, 141.9, 136.8, 136.3, 136.2, 133.7, 129.3, 128.1, 125.4, 123.8, 96.8, 93.3, 75.7, 75.2, 73.7, 72.8 (2C), 70.1, 56.8, 41.9, 37.0, 34.8, 31.7, 28.7, 26.9, 26.6, 19.4, 19.2, 18.6, 17.4, 15.1, 13.7, 11.2, 10.4 ppm;

IR (thin film): ν 3459m, 2971m, 2935m, 2877m, 1736m, 1694s, 1641m, 1461m, 1384m, 1257m, 1209m, 1152m, 1075m, 1033s, 899m, 797w, 513w, 465w cm^{-1};

HRMS (ESI) for $C_{36}H_{56}O_{10}Na^{+}$ $[M+Na]^{+}$: calculated: 671.37657; found: 671.37693.

1.12 Production of the Knock-Out Strain *Actinoplanes deccanensis fdxG2*$^{-}$

1.12.1 Buffers and Solutions

Supplementary Table 3. Buffers and solutions.

Name	Ingredients	Concentration	Specifics
Trace salt solution	$FeSO_4 \cdot 7H_2O$ $MnCl_2 \cdot 4H_2O$ $ZnSO_4 \cdot 7H_2O$	1 g/L 1 g/L 1 g/L	Salts are dissolved in dH_2O.
SET buffer	NaCl EDTA pH 8.0 Tris-HCl pH 7.5	75 mM 25 mM 20 mM	Dissolved in dH_2O and filtered through 0.2 µm syringe-filter.
Proteinase K buffer	Tris-HCl pH 8.0 $CaCl_2$	50 mM 1 mM	Dissolve in dH_2O and filter.
3 M Na-acetate pH 5,5	CH_3COONa	3 M	Dissolve 12.03 g Na-acetate in 45 mL dH_2O, adjust pH with NaOH (10 M aq.) and make up to 50 mL.
10× Orange G loading dye	Orange G glycerin TE buffer	10 mg 3 mL 2 mL	For 5 mL of dye.
P1 buffer – resuspension buffer	Tris-HCl pH 8 EDTA RNase	50 mM 10 mM 500 µg/mL	Filter before adding RNase.
P2 buffer – lysis solution	NaOH SDS	200 mM 1 %	
P3 buffer – neutralising solution	KOAc pH 5.5	3 M	
1 M $MgCl_2$	$MgCl_2$	1 M	Dissolve 47.6 g $MgCl_2$ in 0.5 L dH_2O, filter or autoclave.
TE sucrose buffer	Tris-HCl, pH 8 EDTA, pH 8 sucrose	50 mM 40 mM 0.75 M	Add all three solutions together and adjust the volume with dH_2O to 50 mL.

1.12.2 Antibiotic Stock Solutions and other Media Additives

Supplementary Table 4. Antibiotic stock solutions.

Antibiotic	Stock solution	Working concentration for *E. coli*	Working concentration for *A. deccanensis*
Chloramphenicol (Cm)	10 mg/mL in EtOH	10 µg/mL	/
Apramycin (Apr)	50 mg/mL in H_2O	75 µg/mL	50 µg/mL
Kanamycin (Kn)	25 mg/mL in H_2O	50 µg/mL	/
Spectinomycin (Spec)	50 mg/mL in H_2O	/	25 and 50 µg/mL

All stock solutions were stored at -20 °C. Stock solutions were added to molten agar, which had been cooled to 55 °C.

1.12.3 Media

All media and agar preparations were autoclaved at 120 °C for 20 min.

Media for *E. coli*

2TY

16 g peptone from casein (tryptone), 10 g yeast extract, 5 g NaCl, made up to 1 L with dH_2O. For the solid medium 15 g of agar was added.[1]

Media for *Actinoplanes deccanensis*

V-Medium (seed medium for strain manipulation)[2,3]

3 g meat extract (*Merck*), 5 g Bacto™ tryptone (*BD*), 5 g yeast extract (*Sigma*), 1 g sucrose (*AppliChem Panreac*), 24 g soluble starch, 4 g calcium malate (*ChemCruz*) were dissolved in 1 L of distilled water. The pH was not adjusted due to the mentioned wide pH range from 6 to 8.

E-Medium (production medium for strain manipulation)

4 g meat extract (*Merck*), 4 g Bacto™ peptone (*BD*), 1 g yeast extract (*Sigma*), 2.5 g NaCl (*Sigma*), 10 g soybean flour (*Sigma*), 5 g $CaCO_3$ (*AppliChem Panreac*) were dissolved in 900 mL of dH_2O. The pH was adjusted to 7.6 with NaOH before sterilisation, which is reported to be 6.3 after sterilisation.[2] 50 % glucose (w/v) was prepared in dH_2O and sterile-filtered with a 0.2 µm syringe-filter and 100 mL was added to the medium after autoclaving.

ISP3 Medium (oatmeal agar)[4]

Add 20 g oatmeal to 1 L distilled water, adjust pH to 7.2. Cook or steam oatmeal for 20 min. Filter through glass wool or cheese cloth. Add 18 g agar (*Difco*) and make up to 1 L. Autoclave and then add 1 mL of trace salts solution.

GYM $MgCl_2$ Medium (plating of conjugations)[5]

4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$ dissolve in 1 L of dH_2O. Adjust pH to 7.2. Weigh 6 g agar (*Difco*) into 500 mL bottle and add the medium. After autoclaving add 1 M $MgCl_2$ to the final concentration 10 mM $MgCl_2$ and pour into Petri dishes.

Soy Mannitol Agar (SM)[1]

20 g D-mannitol, 20 g soy flour, 20 g agar make up to 1 L with dH_2O.

Tryptone Soy Broth (TSB)[1]

17 g peptone from casein (tryptone), 3 g peptone from soy meal, 2.5 g glucose, 5 g NaCl, 2.5 g K_2HPO_4, made up to 1 L with dH_2O.

1.12.4 Bacterial Strains, Vectors, Oligonucleotides

Supplementary Table 5. Bacterial strains.

Organism	Genotype	Reference
***Escherichia coli* DH10β**	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG	Invitrogen
***Escherichia coli* ET12567/pUZ8002**	F- dam13::Tn9, dcm6, hsdM, hsdR, zjj-202::Tn10, recF143::Tn1I, galK2, galT22, ara14, lacY1, xyl5, leuB6, thi1, tonA31, rpsL136, hisG4, tsx78, mtl1 glnV44 with additional non-transferable pUZ8002	[6]
***Actinoplanes deccanensis* (DSM 43806; ATCC 21983)**	Fidaxomicin producer; fdx cluster; from Dr. Andrea Dorst, UZH, Switzerland	DSMZ[2]

Supplementary Table 6. Standard vector.

Vector	Size	Resistance	Details	Source
pKC1132	3.6 kb	Apr	Suicide vector for *Streptomyces* containing *oriT* RK2 for conjugation from *E. coli* to *Streptomyces*; *aac(3)IV lacZα* $oriT_{RK2}$	[1]

Supplementary Table 7. Constructed vector.

Vector	Size	Details
pKC1132-KOfdxG2	5.6 kb	Homologies of 1 kb cloned in-frame containing first 72 bp and last 75 bp of gene *fdxG2*

Supplementary Table 8. Primer sequences.[a]

Primer	5'-Sequence-3'	Restriction digestion	Construct
fdxG2-PstI-LF	5'-ATACTGCAGGTCCTGCTCGACGACGTG-3'	*Pst*I	Left homology for pKC1132-KOfdxG2
fdxG2-XbaI-LR	5'-ATATATTCTAGACAGCAGCGGATTGATCAGG-3'	*Xba*I	
fdxG2-XbaI-RF	5'-ATATATTCTAGACTCATCCAACGCCGGCTG-3'	*Xba*I	Right homology for pKC1132-KOfdxG2
fdxG2-EcoRV-RR	5'-ATATGATATCCGTCATCAGCTCCTCGAACTTCAG-3'	*Eco*RV	

[a]Restriction sites are underlined.

Supplementary Table 9. Additional primer sequences used for verification of gene cluster arrangement and sequencing.

Primer	5'-Sequence-3'	Usage
gAD-F	5'-GTACCGGATCTCGGCGTGCT -3'	Gene cluster arrangement
gAD-R	5'-GATCGCCAGCGAGATCGTCTC-3'	
gAD-F1	5'-CACCGCATCGGCATCTCG-3'	
gAD-R1	5'-CGTAGCCGCCTCACCCATC-3'	
gAD-R2	5'-GTGGTCGTCAGGAGCCGCTC-3'	
gAD-R3	5'-GATCAGCATGCGGATCAGGTCG-3'	
PKC1132-F	5'-CGATTAAGTTGGGTAACGCCAGG-3'	Anneals before *Pst*I site in pKC1132
PKC1132-R	5'-ATGCTTCCGGCTCGTATGTTGTGTG-3'	Anneals after *Eco*RV site in pKC1132
checkG2F	5'-GTGGATCCGAGCCTGCTGT-3'	For sequencing of pKC1132-KOfdxG2 plasmid
checkG2R	5'-CTTGCCGAACATCTCCGACGACGAC-3'	

1.12.5 Cultivation and Preservation of Bacterial Strain

Cultivation of *Actinoplanes deccanensis*

Strain is for fermentation purposes cultivated on solid medium ISP-3 for 7-10 days at 30 °C, where it grows steadily over the surface of agar plate. Small square (1 cm^2) is cut out with sterile loop and inoculated into 50 mL V-medium (1/5 V), where the culture grows for 3-5 days on 30 °C in rotary shaker at 200 rpm. In round Erlenmeyer´s flasks bacteria form beige clumps while growing in that medium but in baffled flasks it grows mainly in suspension. Obtained pre-culture was re-inoculated, 10% inoculum was added to V-medium, and the culture was growing on 28 °C in round Erlenmeyer's flask for 1 day at 200 rpm.[2] 10% of pre-culture of *A. deccanensis* was inoculated into E-medium in baffled Erlenmeyer's flask, irrespective of foaming. After 1-2 days, cellulose[7] was added to the final concentration of 20 g/L and 2% of Amberlite XAD-16. Fermentation was completed in 6 days. Strain did not sporulate even though many different media were tested.

Preservation of *Actinoplanes deccanensis*

After centrifugation, the pellet of *A. deccanensis* was resuspended in 50% (w/v) glycerol and frozen at -80 °C. The strains were revived by scraping the surface of the frozen suspension with a toothpick and streaking onto agar.

Cultivation of *Actinoplanes deccanensis* in Presence of Antibiotics

For genetic modification purposes, we determined the susceptibility of *A. deccanensis* to different antibiotics. Bacterium is resistant to erythromycin (25 μg/mL), kanamycin (50 μg/mL), spectinomycin (50 μg/mL); less abundant growth is observed on erythromycin (50 μg/mL) and nalidixic acid (25 μg/mL). Bacterium is sensitive to gentamycin (50 μg/mL), hygromycin (50 μg/mL), streptomycin (50 μg/mL) and apramycin (50 μg/mL).

1.12.6 Transformation of Bacteria

Preparation of Electrocompetent *E. coli* Cells

For preparation of *E. coli* electrocompetent cells 2TY medium was used. 200 mL of medium was inoculated with 2 mL of overnight culture of *E. coli* and incubated at 37 °C in a shaker at 200 rpm until the culture reached OD_{600} 0.5. The culture was then incubated on ice for 30 min and centrifuged at 4000 rpm for 10 min. The cells were washed with cold 1 mM HEPES (pH 7.0): once with 100 mL and once with 50 mL. The third time the cells were washed with 50 mL of 10% glycerol/1 mM HEPES pH 7.0. Every cell pellet resuspension was followed by centrifugation at 4000 rpm for 10 min at 4 °C. After the last centrifugation, the cell pellet was resuspended in 2 mL 10% glycerol/1 mM HEPES pH 7.0 and aliquots of 40 µL were stored at -80 °C.

Electroporation of *E. coli* with Plasmid DNA[1]

Into a 40 µL aliquot of electrocompetent cells, plasmid DNA (1-100 ng) was added, mixed and transferred into electroporation cuvettes and subjected to electrical pulse. The electroporation was performed at 1.350 V/cm, 10 µF, and 600 Ω. The pulsed cells were diluted in 2TY medium to a final volume of 1 mL, incubated at 37 °C for 45 min and spread on 2TY plates with appropriate antibiotic for selection of transformants.

Conjugation of *Actinoplanes deccanensis* Mycelium

Conjugation is one of the methods for insertion of foreign DNA into host microorganisms. We used plasmid pKC1132 that contains *oriT* site, origin of transfer, necessary for conjugation process. The method was reported for *Actinoplanes friuliensis* by Heinzelmann.[5]

Actinoplanes deccanensis was grown on ISP3 or SM plate at 30 °C for 10 days. A square piece (1 cm^2) was inoculated into 20 mL TSB and the culture was grown for 3-4 days on 30 °C at 180 rpm. After manual homogenisation, 1 mL of homogenous culture was inoculated to 100 mL V-medium into 500 mL baffled Erlenmeyer's flask and cultivated for 4 days. The culture was diluted 1:10 – 50 mL TSB media, and 5 mL culture and incubated overnight. On the following day, the conjugation day, the culture was homogenised, diluted 1:5 – 10 mL TSB, 2.5 mL mycelium and incubated at 180 rpm for 1 to maximum 5 h. Each aliquot of 12.5 mL was centrifuged for 10 min at 3500 rpm before usage.

[1]Routine handling of nucleic acids, such as isolation of plasmid DNA, restriction endonuclease digestions, DNA ligations, and other DNA manipulations, was performed according to standard protocols.[8]

E. coli ET12567/pUZ8002 was electroporated with plasmid and plated on 2TY Cm (10 µg/mL), Kn (25 µg/mL), Apr (50 µg/mL) agar plates. One day before conjugation, a single colony was inoculated into liquid medium and grown overnight. On the day of conjugation, 20 mL of liquid 2TY Cm (10 µg/mL), Kn (25 µg/mL), Apr (50 µg/mL) was prepared and inoculated with 5% of overnight culture. The culture was incubated at 37 °C until OD_{600} 0.4 was reached. After centrifugation for 10 min at 400 rpm, the cells were washed twice with 2TY. The cells were resuspended in 1 mL medium which is sufficient for 4 reactions/plates.

The mixture of 200 µL *A. deccanensis* mycelium and 250 µL *E. coli* was spread on well dried GYM plate containing $MgCl_2$. The next morning, the plates were overlaid with spectinomycin (50 µg/mL) according to the weight of the plate (30 g = 1 mL sterile water + 30 µL spectinomycin with concentration 50 mg/mL) and later during the day the plates were overlaid with apramycin (50 µg/mL) following the same approach. The conjugation plates were incubated for 6-10 days at 30 °C. Exconjugants were patched on SM agar plate containing spectinomycin (25 µg/mL) and apramycin (50 µg/mL).

1.12.7 Genetic Manipulation

In vitro DNA Amplification (PCR) with Phusion Polymerase

Supplementary Table 10. PCR reaction mixture (Phusion polymerase).

Component	Stock concentration	Volume (in a 50 µL reaction)	Final concentration
Phusion polymerase GC buffer Finnzymes	5×	10 µL	1×
dNTP (with GC:AT ratio 6:4)	2 mM	5 µL	0.2 mM
Template DNA	50 ng/µL	1 µL	1 ng/µL
Forward primer	10 µM	2.5 µL	0.5 µM
Reverse primer	10 µM	2.5 µL	0.5 µM
Finnzymes Phusion DNA polymerase	2 U/µL	0.5 µL	0.02 U/µL
DMSO	100%	1.5 µL	3% (v/v)

Supplementary Table 11. Amplification program (Phusion polymerase).

Step	Temperature	Duration	Repeat
Initial denaturation	98 °C	2 min	/
Denaturation	98 °C	15 s	30×
Primer annealing	T_m of the lower primer	30 s	
Extension	72 °C	30 s / 1 kb	
Final extension	72 °C	10 min	/

In vitro DNA Amplification (PCR) with Taq Polymerase

Supplementary Table 12. PCR reaction mixture (Taq polymerase).

Component	Stock concentration	Volume (in a 50 µL reaction)	Final concentration
Taq Buffer with $(NH_4)_2SO_4$	10×	5 µL	1×
dNTP (with GC:AT ratio 6:4)	2 mM	5 µL	0.2 mM
Template DNA	50 ng/ µL	1 µL	1 ng/µL
Forward primer	10 µM	2.5 µL	0.5 µM
Reverse primer	10 µM	2.5 µL	0.5 µM
Taq DNA Polymerase, native	5 U/µL	0.25 µL	1.25 U/µL
$MgCl_2$	25 mM	4 µL	2 mM
Glycerol	40%	12.5 µL	10%

Supplementary Table 13. Amplification program (Taq polymerase).

Step	Temperature	Duration	Repeat
Initial denaturation	95 °C	2 min	/
Denaturation	95 °C	15 s	30×
Primer annealing	-5 °C of lower primer's T_m	30 s	
Extension	72 °C	1 min / 1 kb	
Final extension	72 °C	10 min	/

For colony PCR amplifications Taq polymerase was used. The template was replaced by cell suspension: 2 µL of cell pellet from TSB was resuspended in H_2O and incubated at 99 °C for 20 min. Besides that, the initial denaturation step was extended to 10 min.

Digestion of DNA with Restriction Endonucleases

Digestion of DNA was performed usually in a total volume of 10 µL containing DNA and the restriction endonuclease (*Fermentas*) in the appropriate buffer. For the digestion of larger amounts of DNA, the volumes were scaled up accordingly and incubation time extended to 3 h. The reactions were incubated at 37 °C. Reactions were stopped by the addition of gel loading buffer or purification through the column.

Ligation of DNA Fragments

The ligation of DNA fragments was carried out usually at a molar ratio from 3:1 to 10:1 (insert:vector). Ligations were performed usually in 10 µL of 1 × *Fermentas* T4 ligation buffer, containing 2.5 U of *Fermentas* T4 ligase per 0.2 µg of DNA. The reactions are incubated at RT for 3 h, if ends are sticky or overnight at 16 °C if the ligation has blunt ends or contains Klenow fragment.

Isolation of Plasmid DNA from *E. coli*

For transformation of *Actinoplanes* species, plasmid DNA from *E. coli* overnight culture in 2TY was isolated using GeneJET Miniprep Kit (*Thermo Scientific*). Otherwise, the following protocol for alkaline lysis was used:

- Centrifuge 2 mL of culture at 15000 rpm for 1 min
- Resuspend the pellet in 250 µL P1 buffer
- Add 250 µL of P2 buffer, mix well and incubate for 3 min
- Add 250 µL of P3 buffer, mix well
- Centrifuge at 15000 rpm for 5 min
- Transfer the supernatant into a new Eppendorf tube
- Add 750 µL of isopropanol
- Centrifuge at 15000 rpm for 15 min (4°C)
- Remove the supernatant
- Add 250 µL of 70% ethanol
- Centrifuge at 15000 rpm for 2 min
- Remove the supernatant
- Dry the pellet
- Resuspend in 100 µL of H_2O

Agarose Gel Electrophoresis

DNA fragments were separated and visualised using 0.8% (w/v) agarose gels. Gels were routinely prepared and ran in 1 × TAE buffer. For staining of DNA 5 µL of Roti Gel Stain solution was added to 100 mL of melted agarose gel. The samples were loaded on the gel mixed with Orange G loading dye. *Fermentas* GeneRuler 1 kb DNA Ladder was used as a marker.

Recovery of DNA from Agarose Gels

DNA fragments were recovered from TAE agarose gels using NucleoSpin Gel and PCR Clean up kit (*Macherey-Nagel*) according to manufacturer's protocol.

Isolation of Genomic DNA from Gram-Positive Bacteria with a Kit

For genomic DNA isolation from Gram-positive bacteria Gentra Puregene (*Qiagen*) kit was used. It contained only Cell Lysis Solution, Protein Precipitation Solution and DNA Hydration Solution, so TE sucrose buffer with and without lysozyme had to be prepared additionally. Protocol was also slightly changed. Adjusted procedure is described below.

1. Prepare an overnight culture.
2. Transfer 1.5 mL of the cell culture (containing approx. 0.5–1.5 × 10^9 cells) to a 1.5 mL microcentrifuge tube on ice.
3. Centrifuge for 1 min at 13,000–16,000 × g to pellet cells.
4. Carefully discard the supernatant by pipetting or pouring.
5. Add 300 µL TE sucrose buffer, and pipet up and down.
6. Add appropriate volume of lysozyme dissolved in TE sucrose buffer so the final concentration is 4 mg/mL and mix by inverting 25 times. Incubate for 30 min at 37 °C.
7. Centrifuge for 1 min at 13,000–16,000 × g to pellet cells.
8. Carefully discard the supernatant with a pipet.
9. Add 600 µL Cell Lysis Solution, and pipet up and down to lyse the cells.
10. Add 3 µL RNase A Solution (20 mg/mL) and mix by inverting 25 times. Incubate for 15–60 min at 37 °C.
11. Incubate for 1 min on ice to quickly cool the sample.
12. Add 200 µL Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.
13. Centrifuge for 3 min at 13,000–16,000 × g.
14. Transfer 300 µL isopropanol into a clean 1.5 mL microcentrifuge tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.
15. Mix by inverting gently 50 times.
16. Centrifuge for 1 min at 13,000–16,000 × g.
17. Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
18. Add 300 µL of 70% ethanol and invert several times to wash the DNA pellet.
19. Centrifuge for 1 min at 13,000–16,000 × g.
20. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min.
21. Add 50-100 µL DNA Hydration Solution and vortex 5 s at medium speed to mix.
22. Incubate at 65 °C for 1 h to dissolve the DNA.
23. Incubate at room temperature (15–25°C) overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

Isolation of Genomic DNA (Phenol/Chloroform)

Spin down 10 mL of fresh culture at 8.000 rpm for 10 min at room temperature. Discard supernatant completely and wash cells once with SET buffer. Resuspend cells in 5 mL SET buffer.

- Add 100 µL Lysozyme stock solution (50 mg/mL in water) and 50 µL RNAse A stock solution (10 mg/mL in water, boil the solution before the first use if necessary!) and incubate for 30-60 min at 37 °C.
- Add 300 µL Proteinase K solution (10 mg/mL in Proteinase K buffer), invert several times, then add 600 µL 10% SDS and incubate at 55 °C for 2 h, invert at least every 15 min. (Final proteinase K concentration is 0.5 mg/mL and SDS concentration is 1%; total volume is ≈ 6 mL). Note that sometimes longer incubation times are necessary!

Important: Proteinase K in solution is stable only for 6-12 months. Solutions must be made fresh or kept frozen as single use aliquots. Avoid refreezing proteinase K aliquots!

- Add 1 volume (= 6 mL) Phenol:Chloroform:Isoamylalcohol (25:24:1) and swing the tube for 30-60 min.
- Centrifuge the mixture at 8.000 rpm for 5 min at room temperature.
- Transfer the upper phase into a new tube using an end-cut 1 mL tip.

Important: Prevent taking the white interphase containing proteins and cell rests, or the lower organic phase.

- Add 1 volume (~ 6 mL) Phenol:Chloroform:Isoamylalcohol (25:24:1) and swing the tube for 30-60 min.
- Centrifuge the mixture at 8.000 rpm for 5 min at room temperature.
- (The white layer must be significantly reduced or disappear. If this is not the case repeat extraction step once more and swing the tube for 1 h.)
- Transfer the upper phase into a new tube using an end-cut 1 mL tip
- Add 1 volume (~ 6 mL) Chloroform:Isoamylalcohol (24:1) (e.g. Roti-C/I from *Roth*) and swing the tube for 30-60 min.
- Centrifuge the mixture at 8.000 rpm for 5 min at room temperature.
- Transfer the upper phase into a new tube using an end-cut 1 mL tip.
- Add 1/10 volume of 3 M NaOAc pH 5.5 and mix well by inverting the tube several times.

- Add 2.5 volume of 100% ice-cold ethanol and invert the tube several times, until appearance of cotton like DNA.
- Spool DNA onto a sealed Pasteur pipette and rinse DNA in 70% ethanol.
- Air dry the DNA.
- Resuspend the DNA in 0.5-1 mL 10 mM Tris-HCl pH 8.0 (or in water or in TE buffer).

1.12.8 Genetic Modifications of the Glycosyltransferase

Glycosyltransferase FdxG2 shares 88% identity and 96% similarity to TiaG2.

In-frame *fdxG2* Gene Disruption in *A. deccanensis*

The *fdxG2* gene (1416 bp), containing glycosyltransferase domain, was disrupted by allelic replacement through a double-crossover event. Suicide plasmid pKC1132-KOfdxG2 (Supplementary Supplementary Table **7**) was constructed by insertion of two PCR amplified DNA fragments, 1 kb in length, homologous to sequences upstream and downstream of the core of the gene for glycosyltransferase FdxG2. Left homology was cloned using *Pst*I, *Xba*I and the right one using *Xba*I and *Eco*RV restriction sites (Supplementary Supplementary Table **8**), yielding disrupted FdxG2 with only 50 amino acids. Resulting plasmid was sequencing confirmed using PKC1132-F and PKC1132-R primers (Supplementary Supplementary Table **9**). The confirmed plasmid was transformed into *E. coli* ET12567/pUZ8002 to obtain a non-methylated plasmid, which was then introduced into wild-type *A. deccanensis* via conjugation of mycelium. GYM $MgCl_2$ conjugation plates were overlayed with spectinomycin (50 µg/mL) next day in the morning and with apramycin (50 µg/mL) in the afternoon as described in 1.4.6. Each exconjugant was further repatched on SM Spec25 Apr50 agar plates and subcultivated. Subcultivations were performed in V-medium without selection pressure, since Apr-sensitive colonies were desired. Each subcultivated culture was spread on the SM agar plates in dilutions, so single colonies were accessible. The grown colonies were patched as a small square onto SM Spec25 as well as SM Spec25 agar plate and marked with the same number. The colonies that grew on SM Spec25 agar plate and were absent on SM Spec25 Apr50, were further confirmed by colony PCR using checkG2F and checkG2R primers (Supplementary Supplementary Table **9**).

1.13 References

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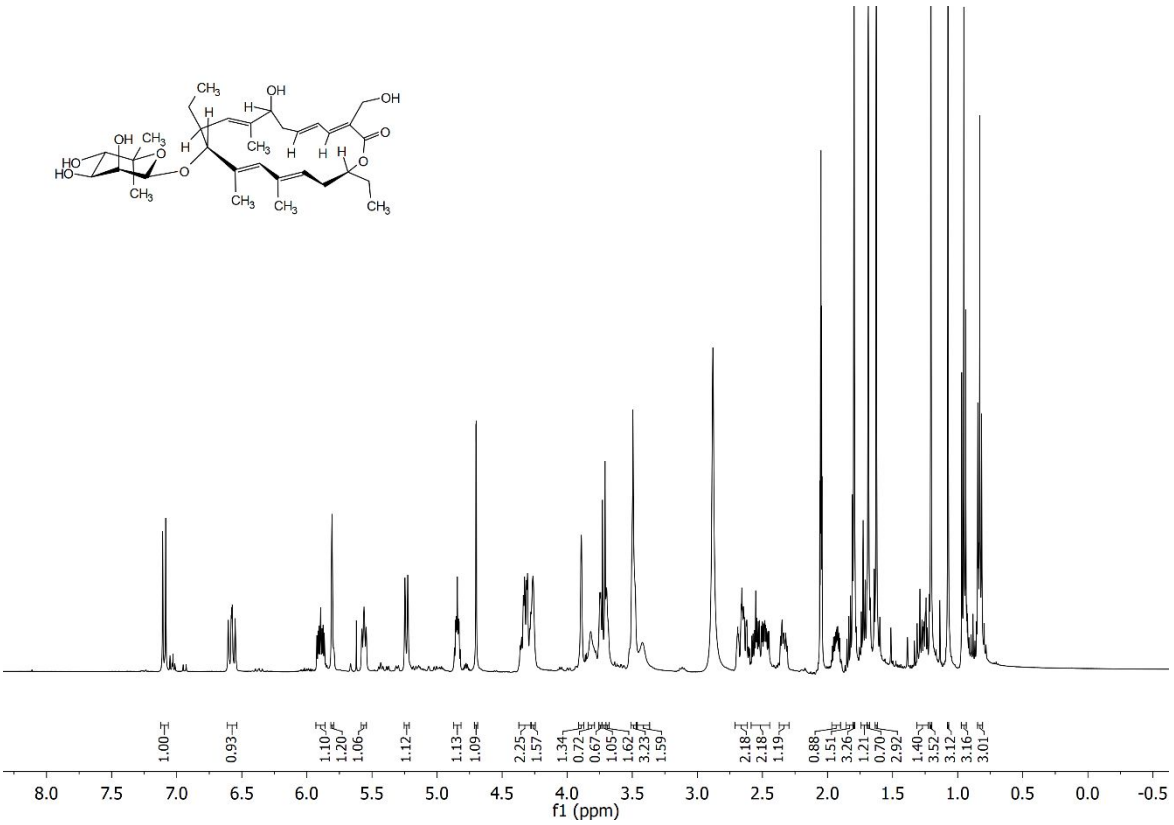
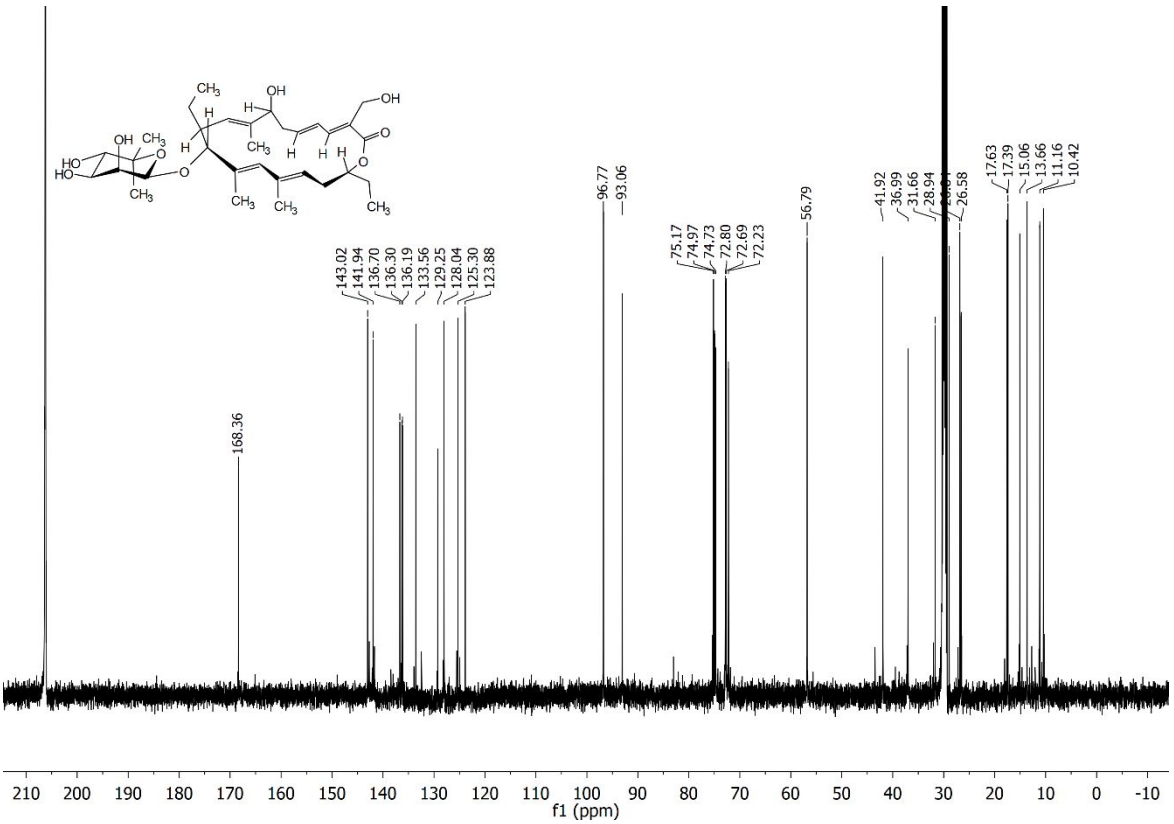
1.14 NMR Spectra

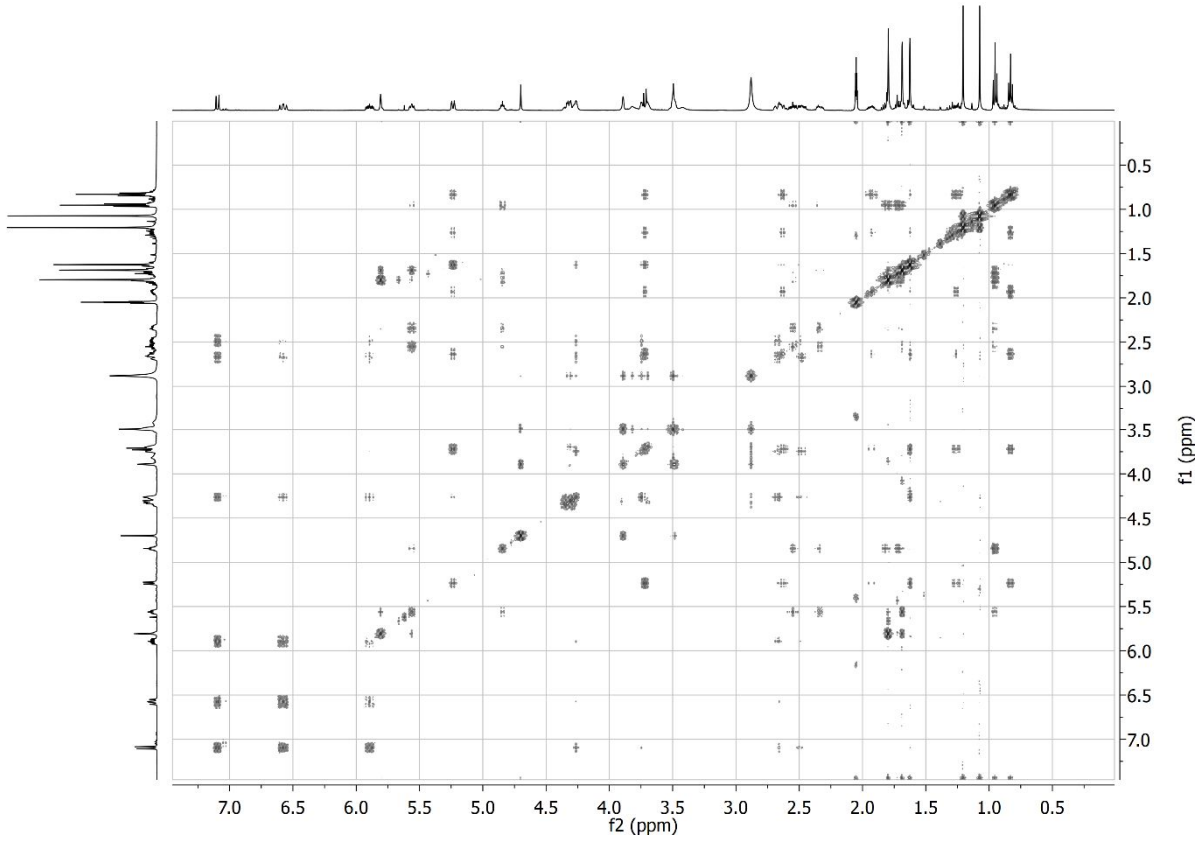
^{1}H NMR spectrum of FdxG2-OH (**2**) (500 MHz, acetone-*d*6)



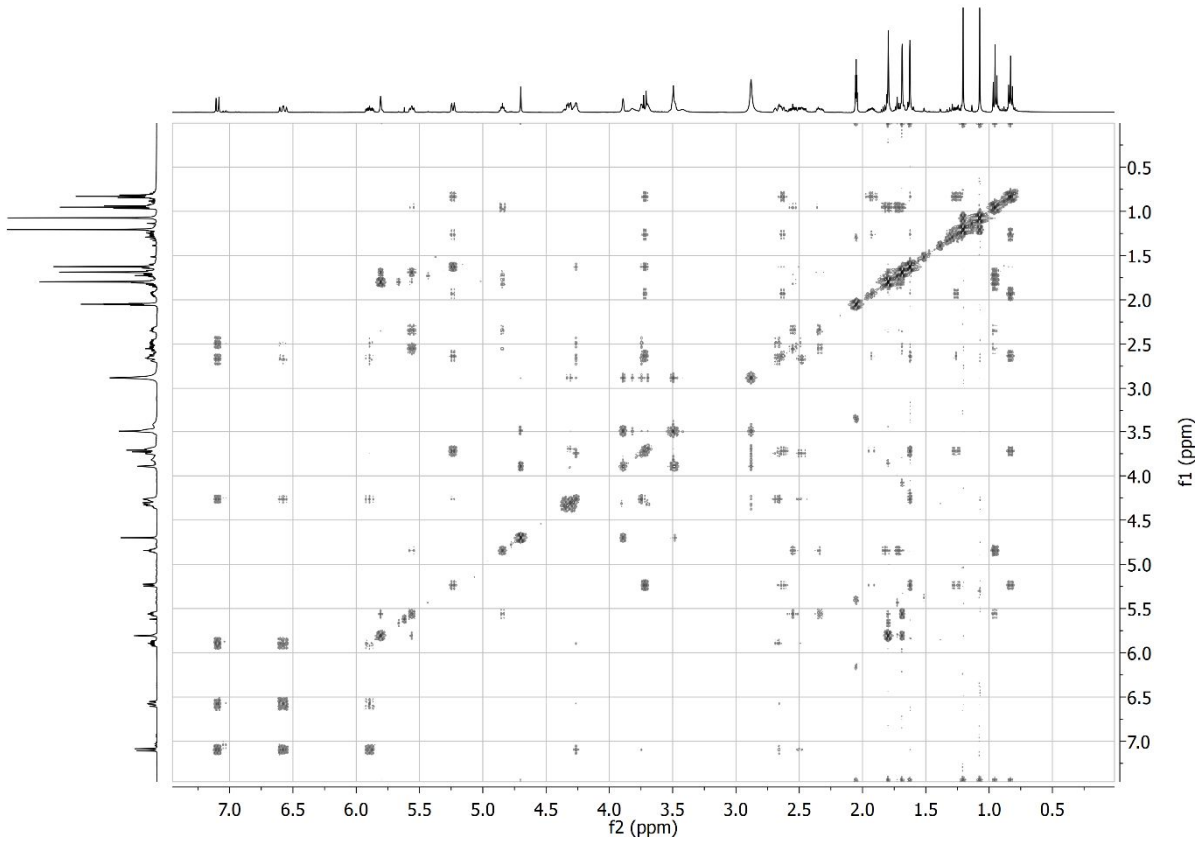
^{13}C NMR spectrum of FdxG2-OH (**2**) (126 MHz, acetone-*d*6)



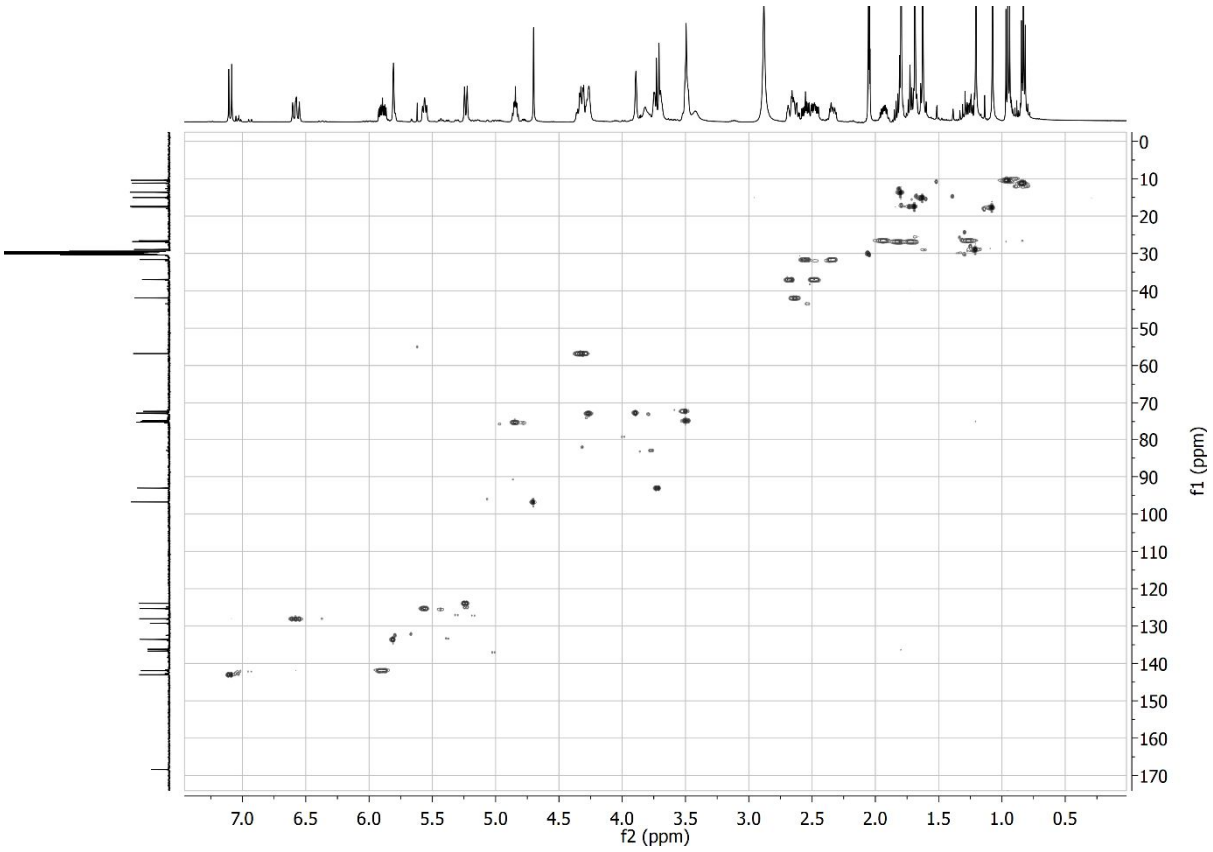
COSY spectrum of FdxG2-OH (**2**) (500 MHz, acetone-*d*6)



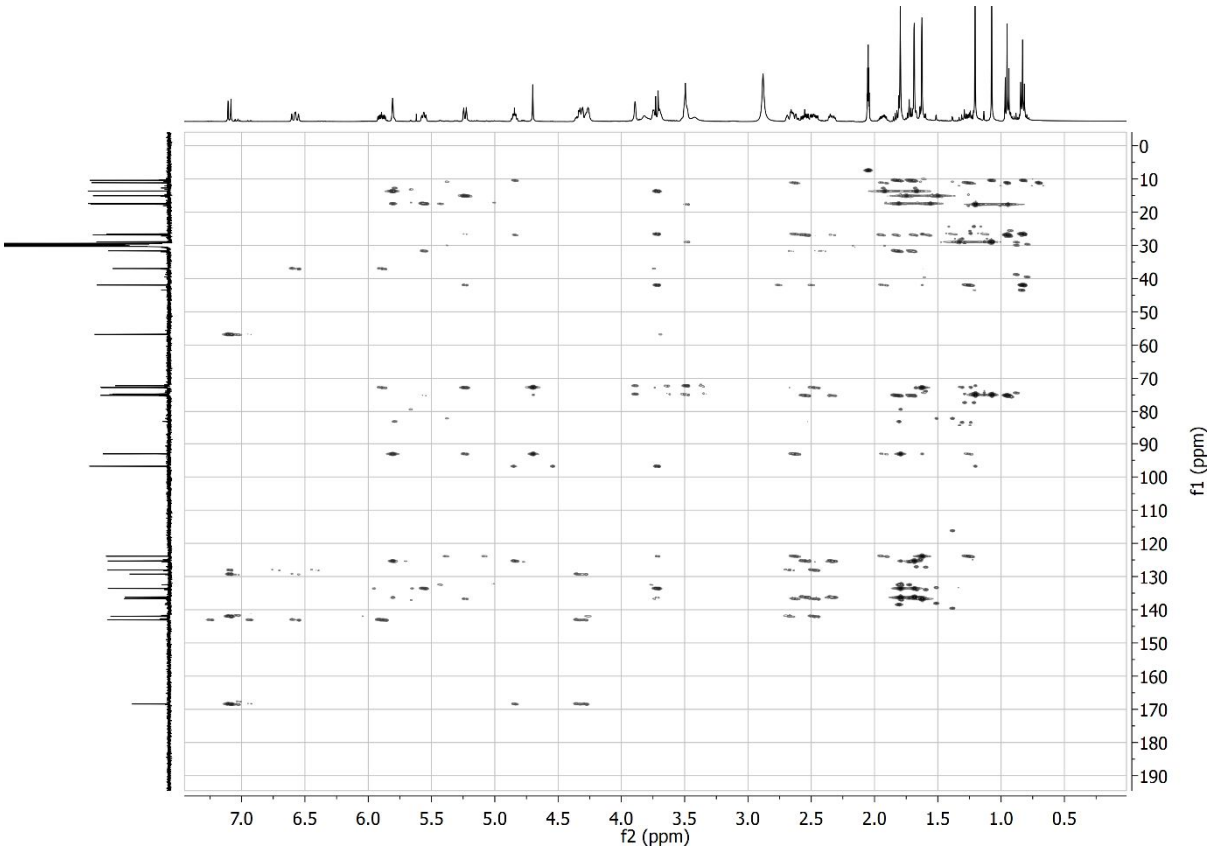
TOCSY spectrum of FdxG2-OH (**2**) (500 MHz, acetone-*d*6)



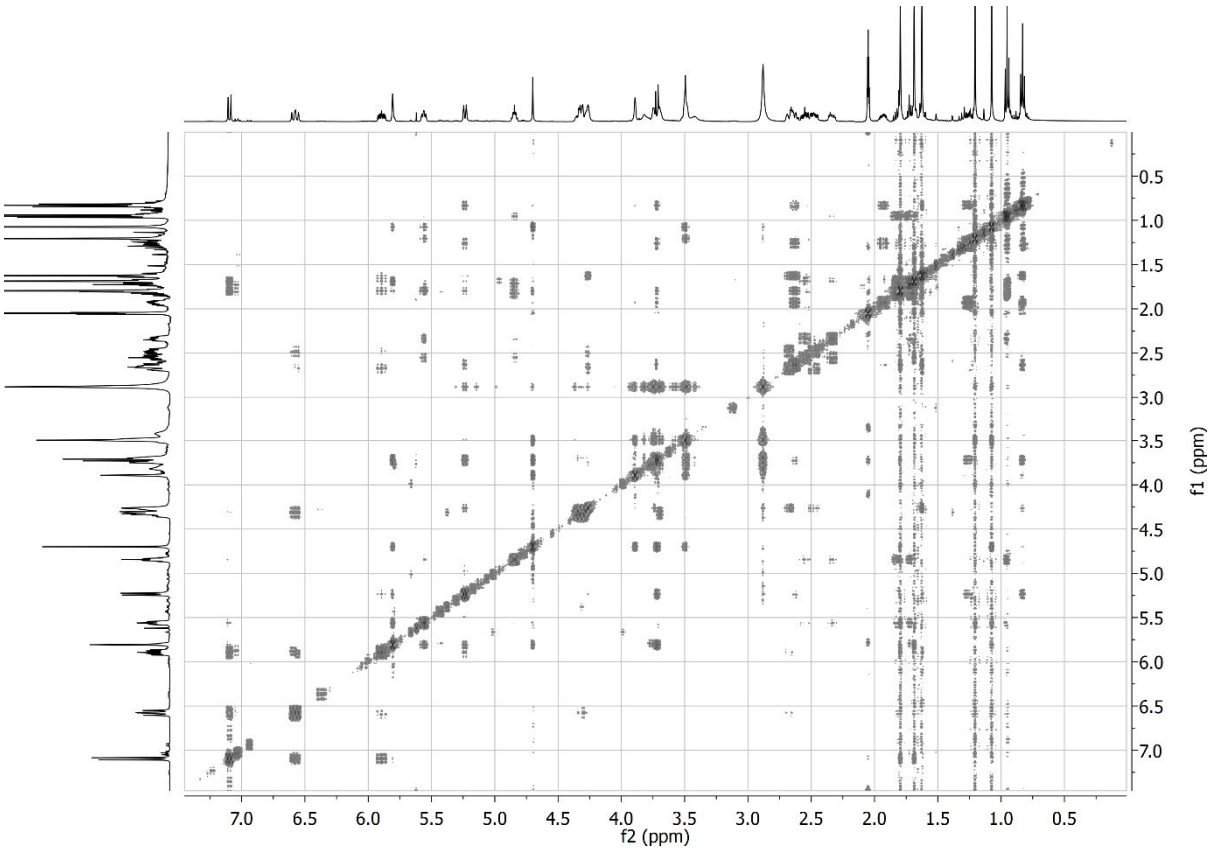
HSQC spectrum of FdxG2-OH (**2**) (500 MHz 126 MHz, acetone-*d*6)



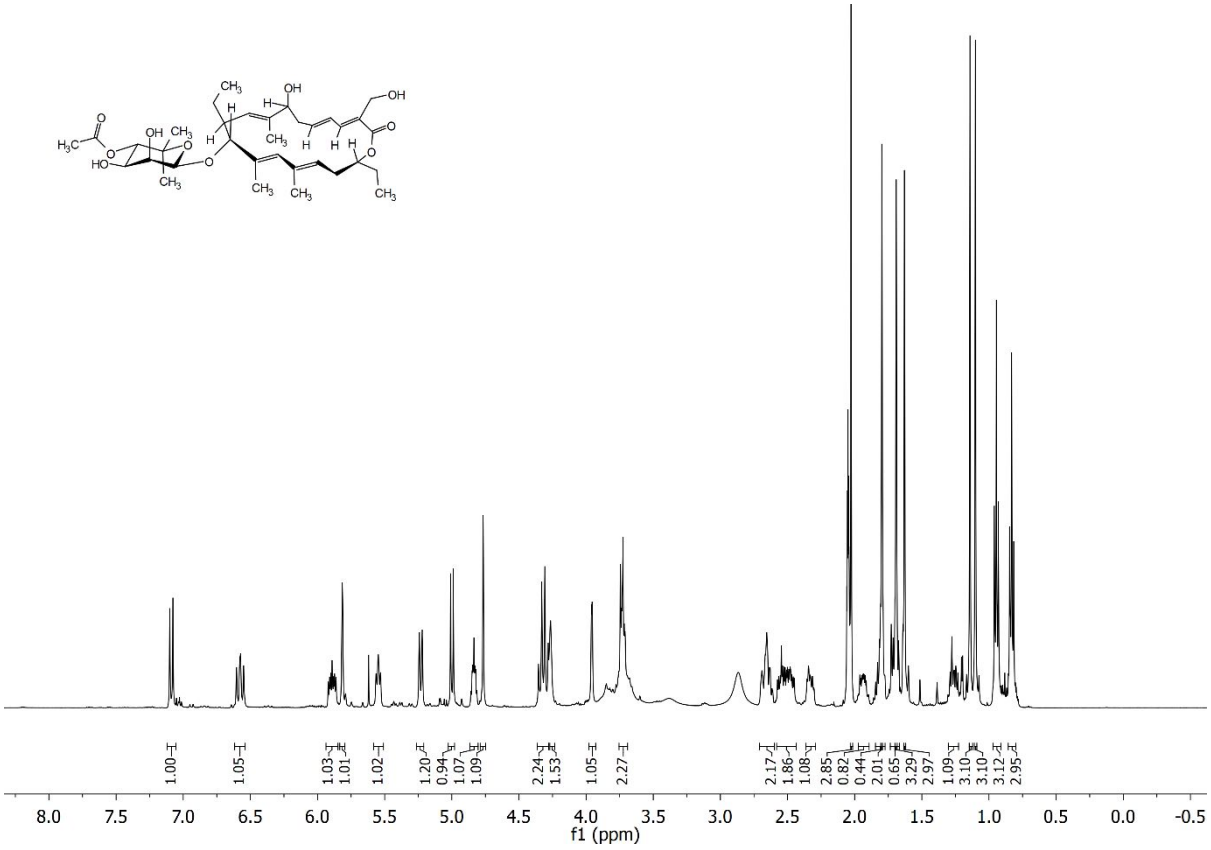
HMBC spectrum of FdxG2-OH (**2**) (500 MHz 126 MHz, acetone-*d*6)



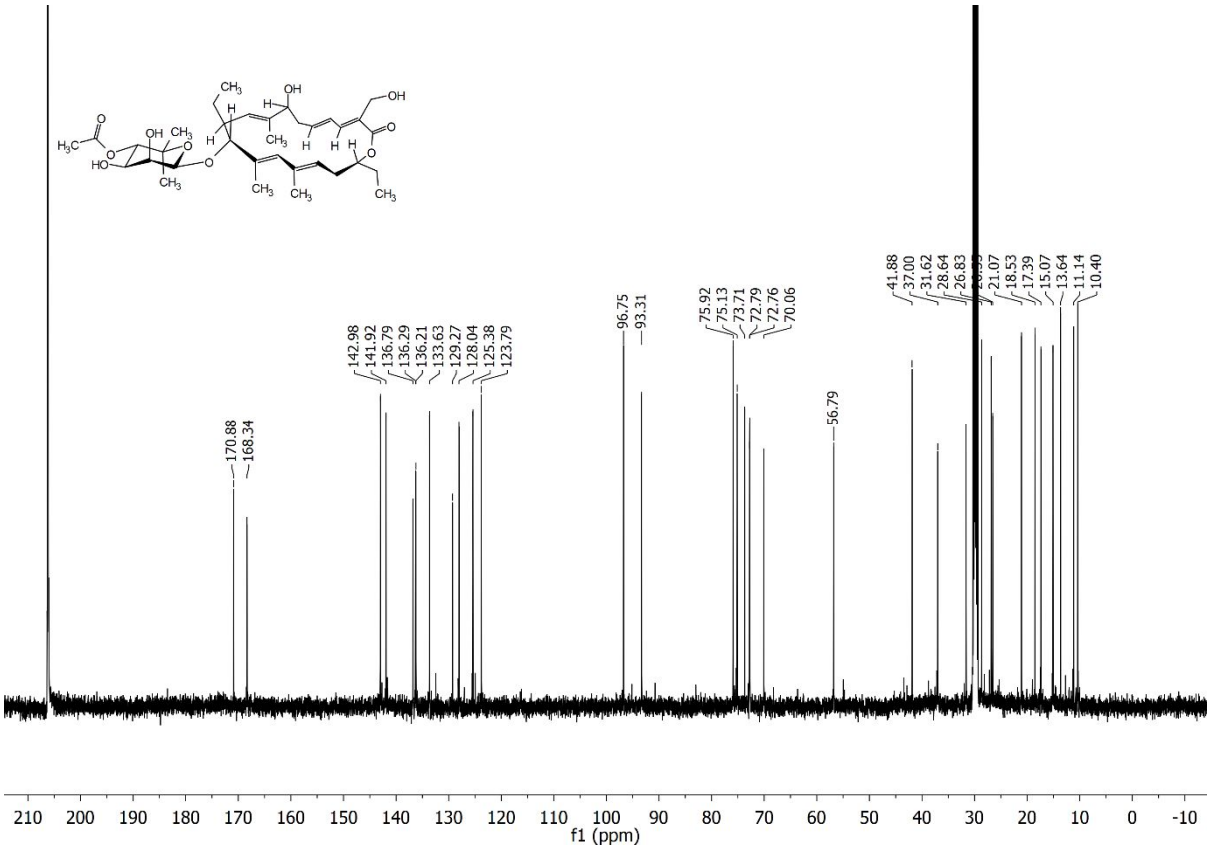
NOESY spectrum of FdxG2-OH (**2**) (500 MHz, acetone-*d6*)



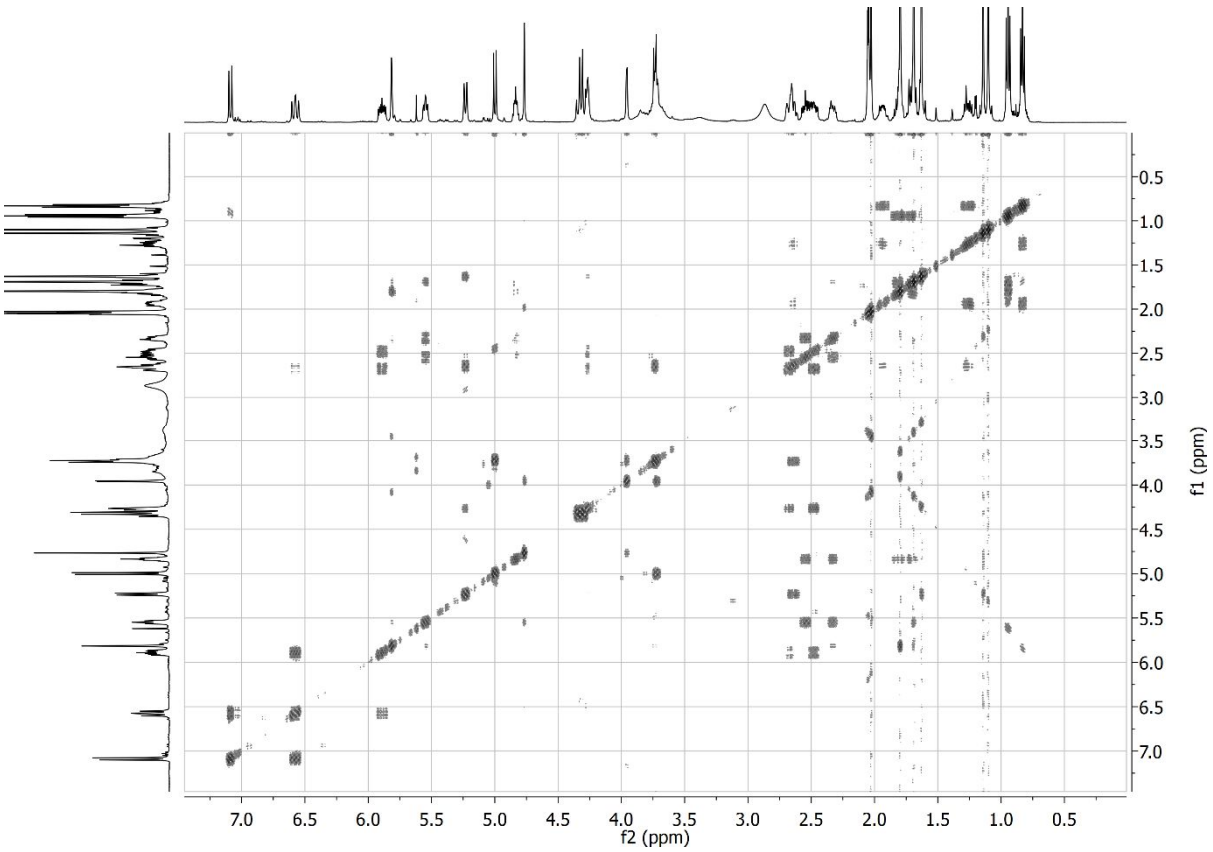
^{1}H NMR spectrum of FdxG2-OAc (**3**) (500 MHz, acetone-*d*6)



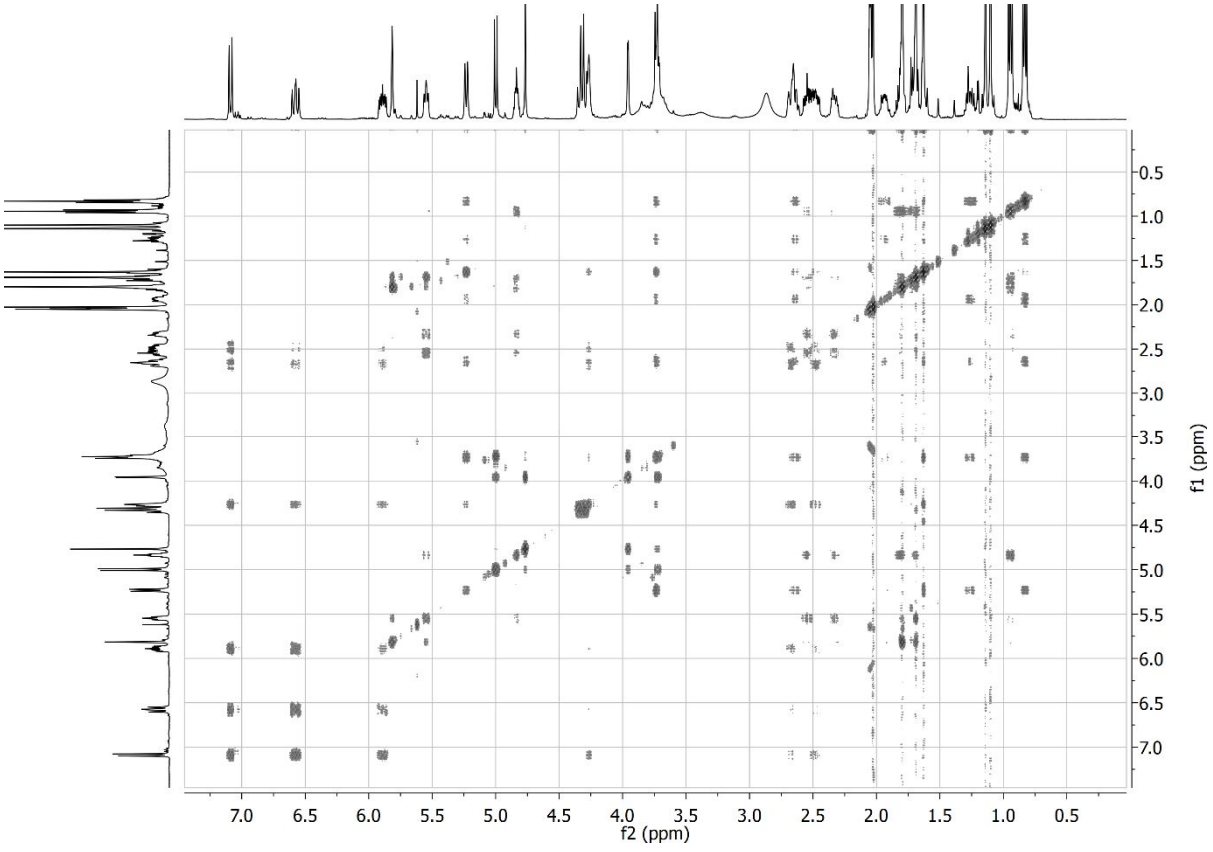
^{13}C NMR spectrum of FdxG2-OAc (**3**) (126 MHz, acetone-*d*6)



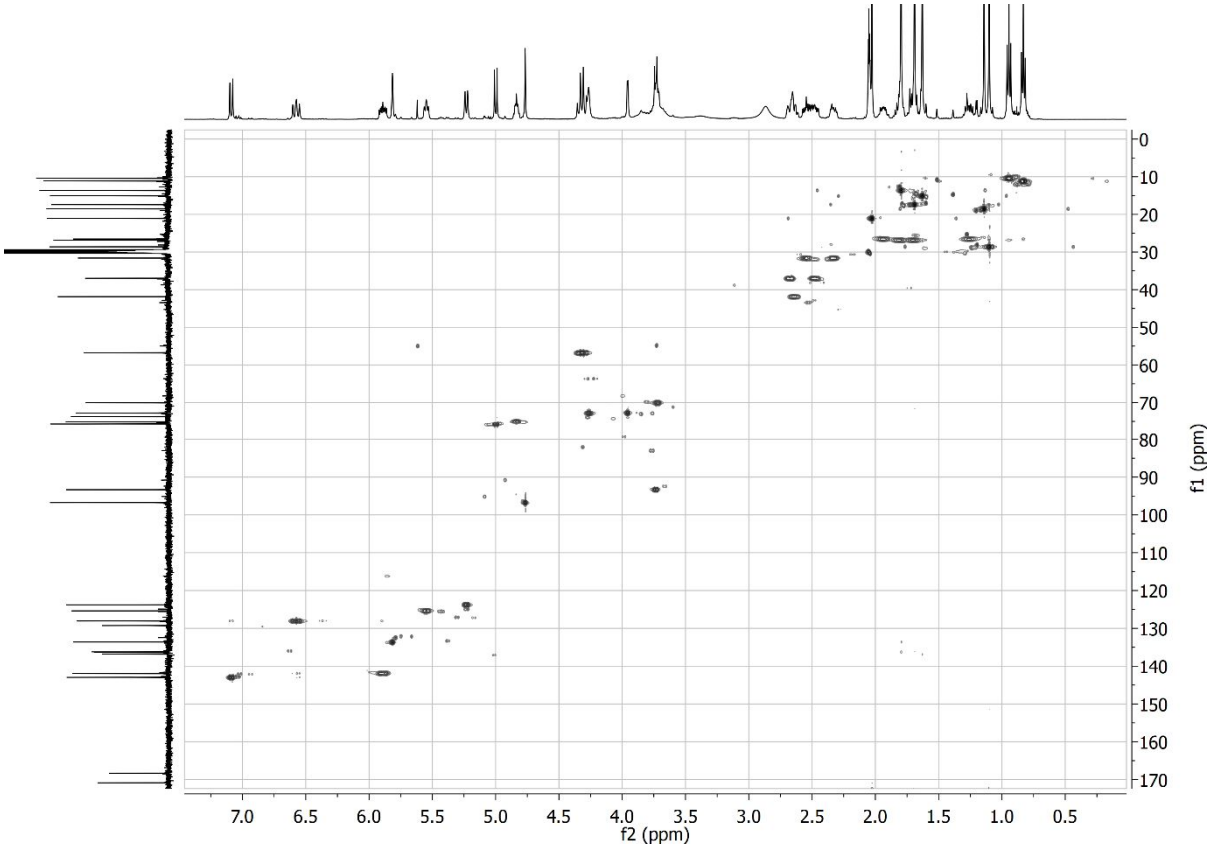
COSY spectrum of FdxG2-OAc (**3**) (500 MHz, acetone-*d*6)



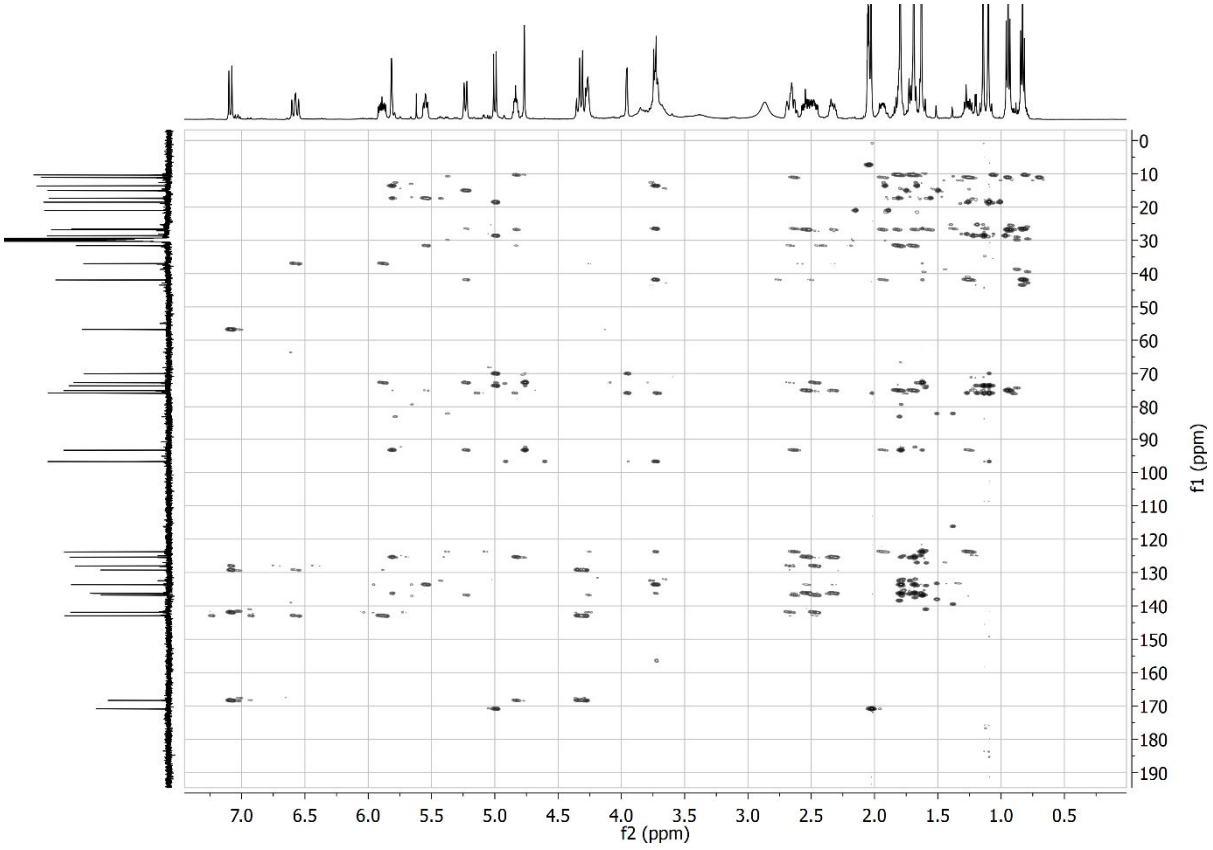
TOCSY spectrum of FdxG2-OAc (**3**) (500 MHz, acetone-*d6*)



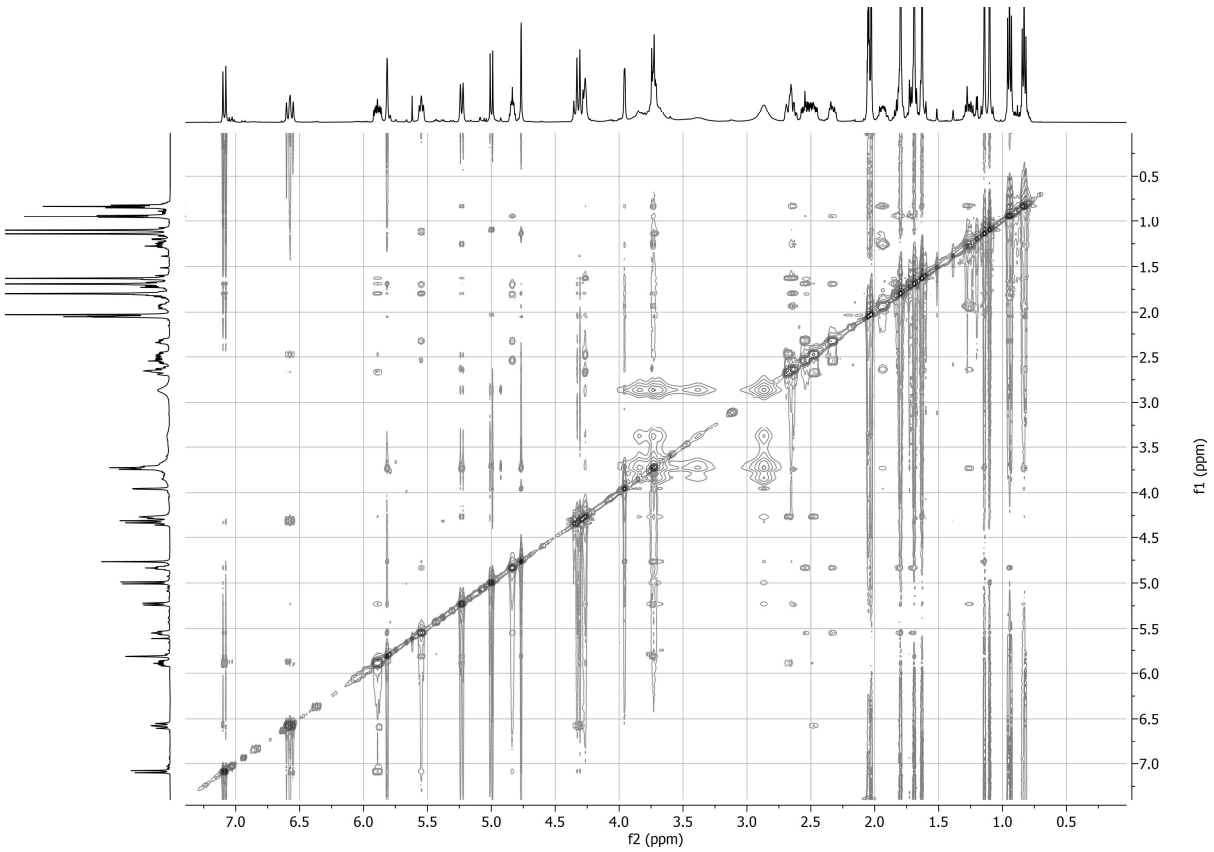
HSQC spectrum of FdxG2-OAc (**3**) (500 MHz 126 MHz, acetone-*d6*)



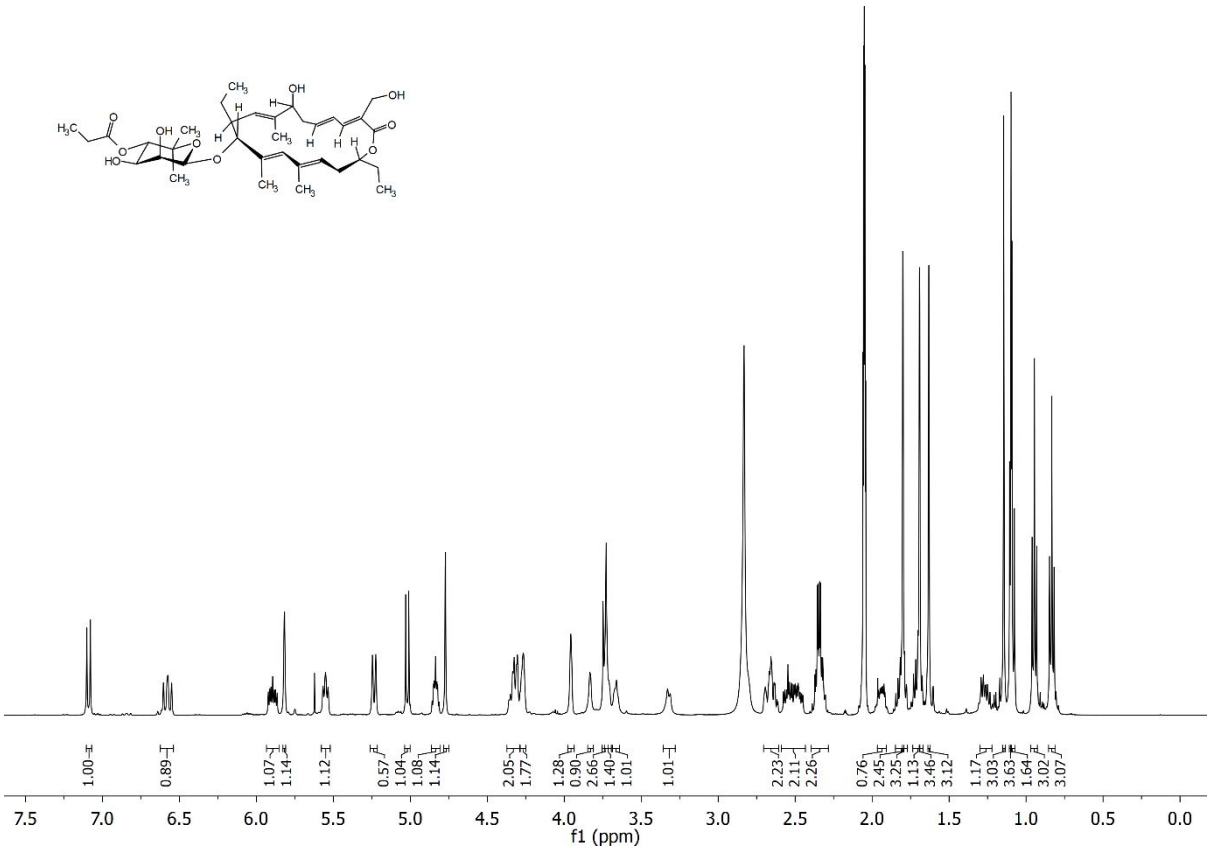
HMBC spectrum of FdxG2-OAc (**3**) (500 MHz 126 MHz, acetone-*d*6)



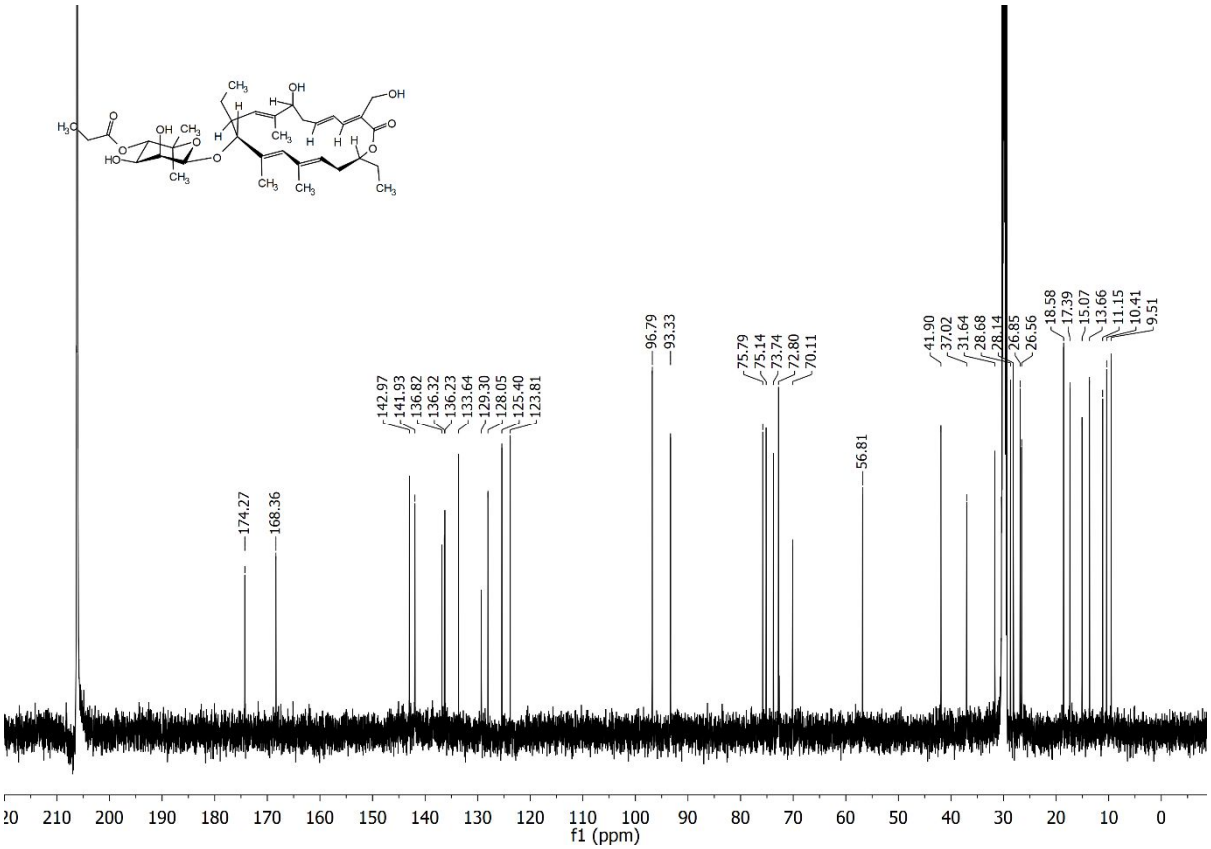
NOESY spectrum of FdxG2-OAc (**3**) (500 MHz, acetone-*d*6)



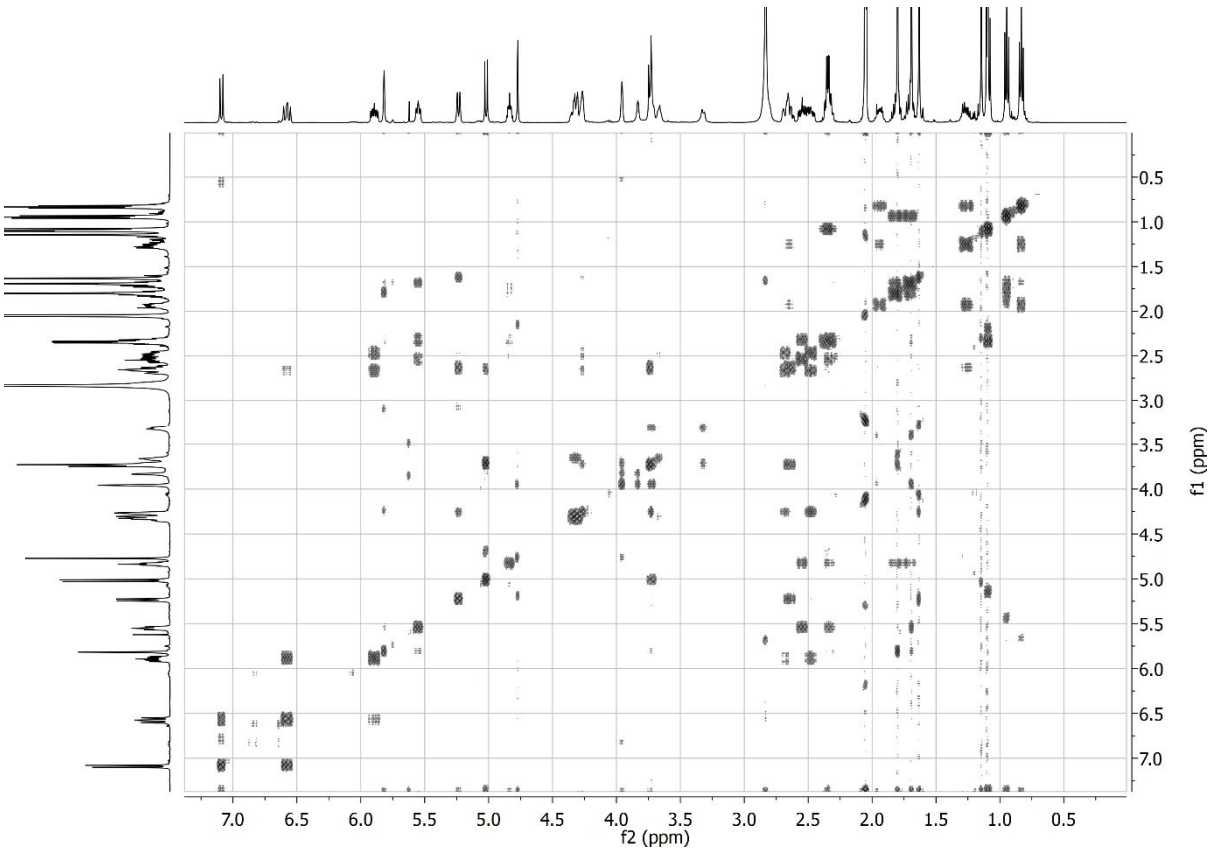
^{1}H-NMR spectrum of FdxG2-OPr (**4**) (500 MHz, acetone-*d*6)



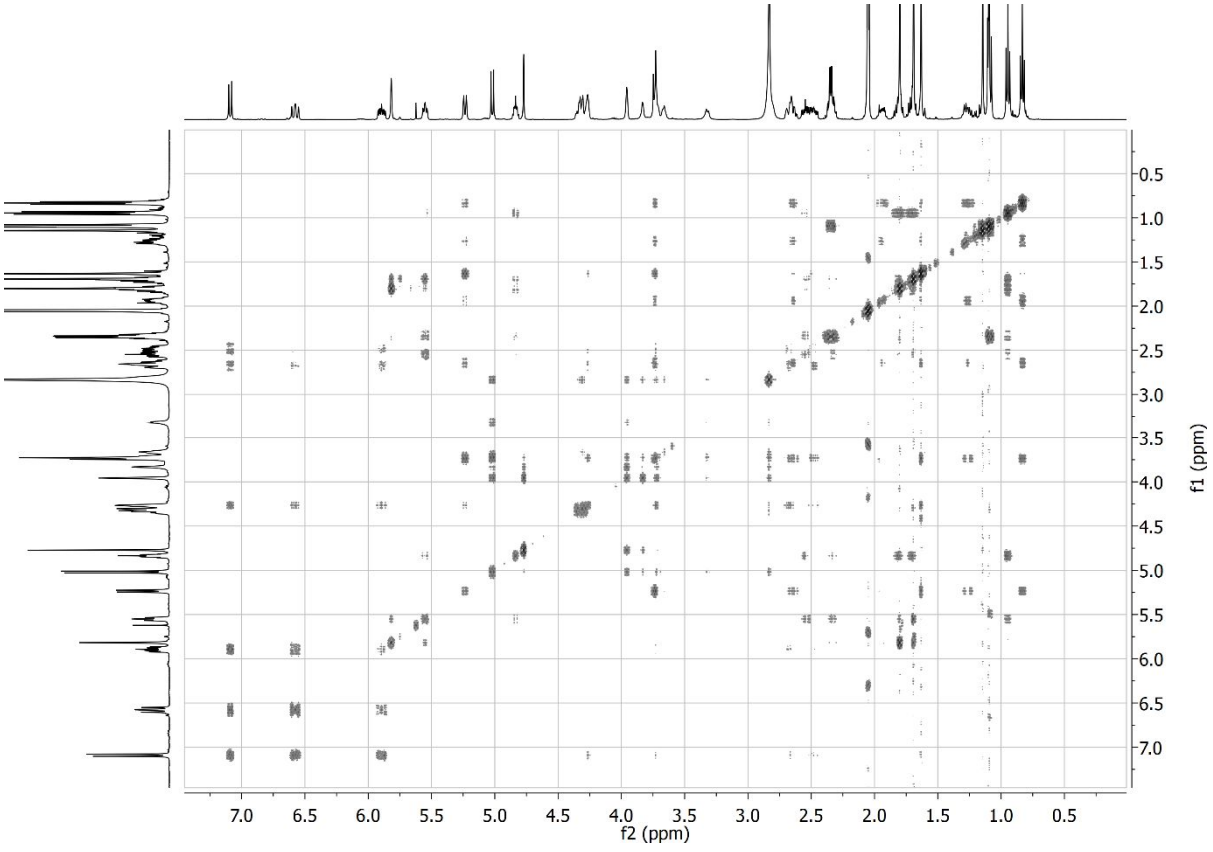
^{13}C NMR spectrum of FdxG2-OPr (**4**) (126 MHz, acetone-*d*6)



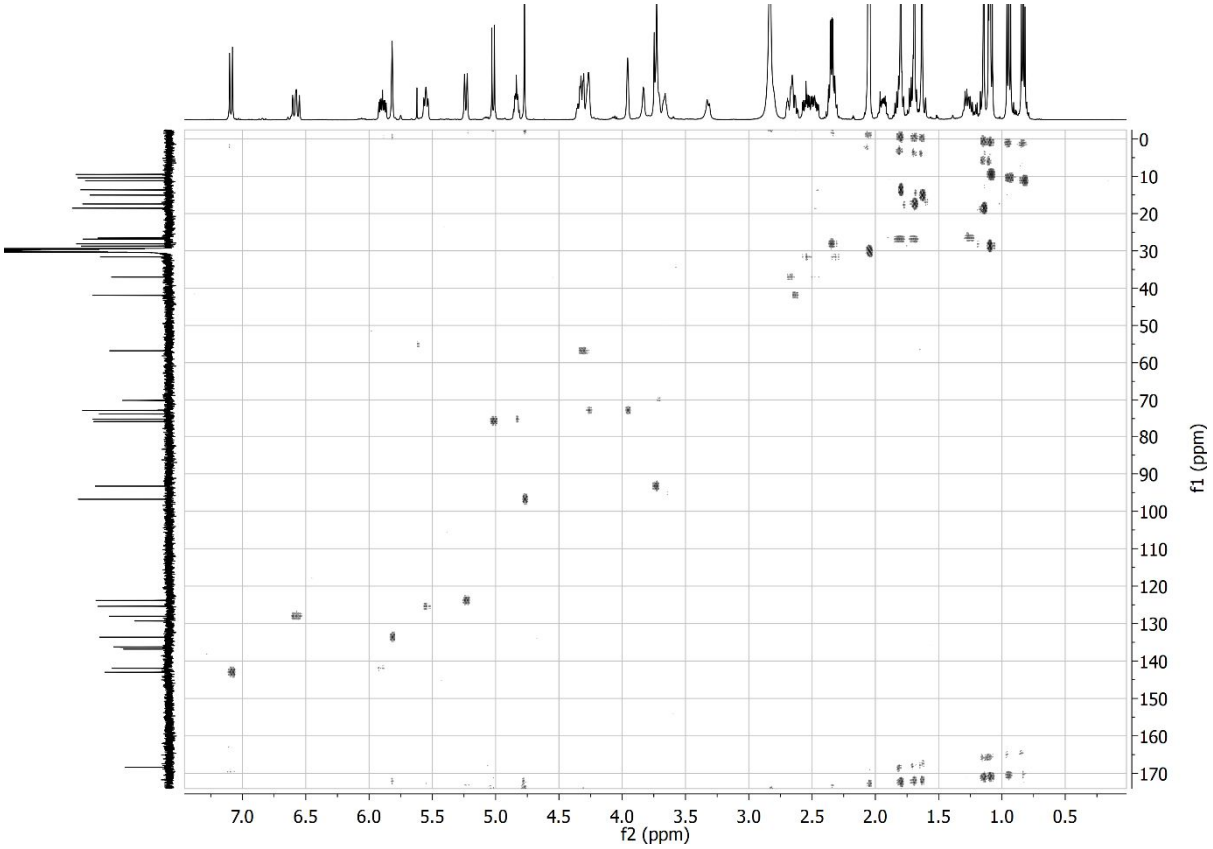
COSY spectrum of FdxG2-OPr (**4**) (500 MHz, acetone-*d*6)



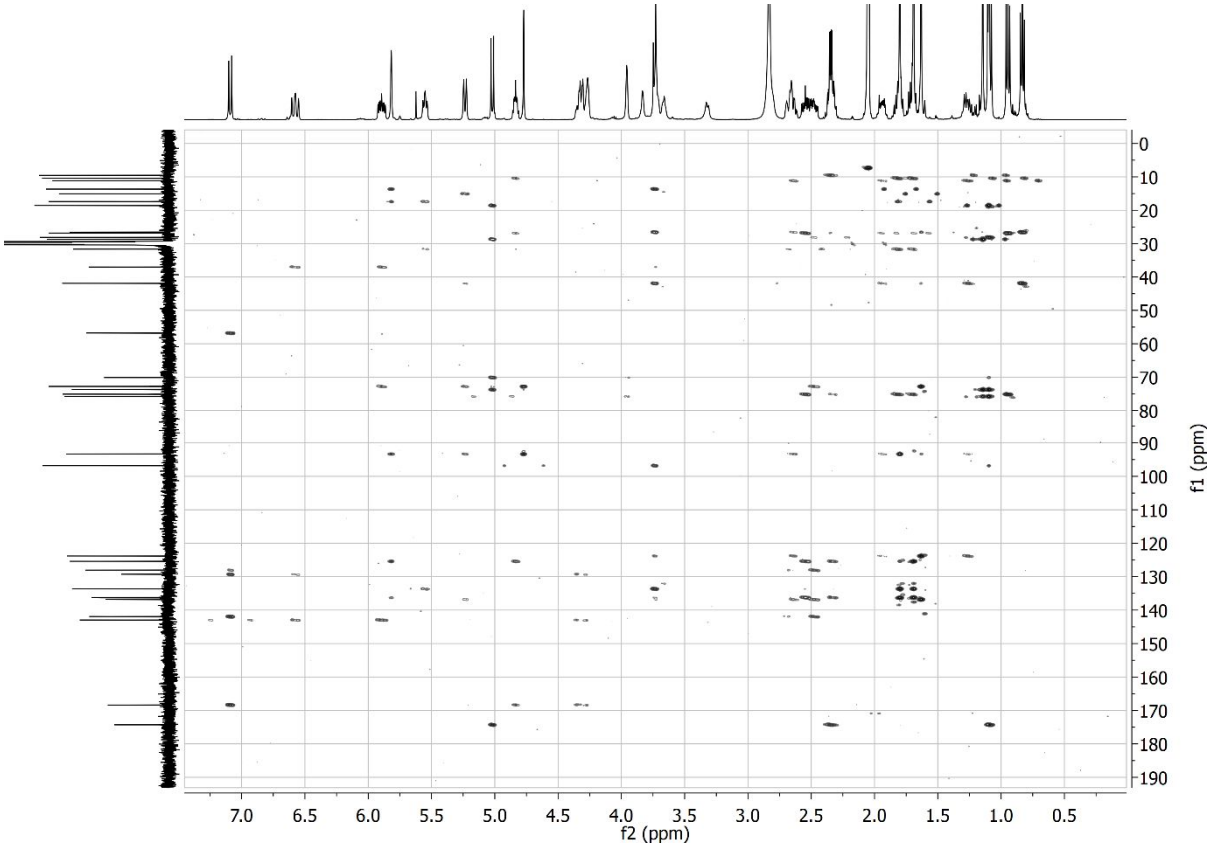
TOCSY spectrum of FdxG2-OPr (**4**) (500 MHz, acetone-*d*6)



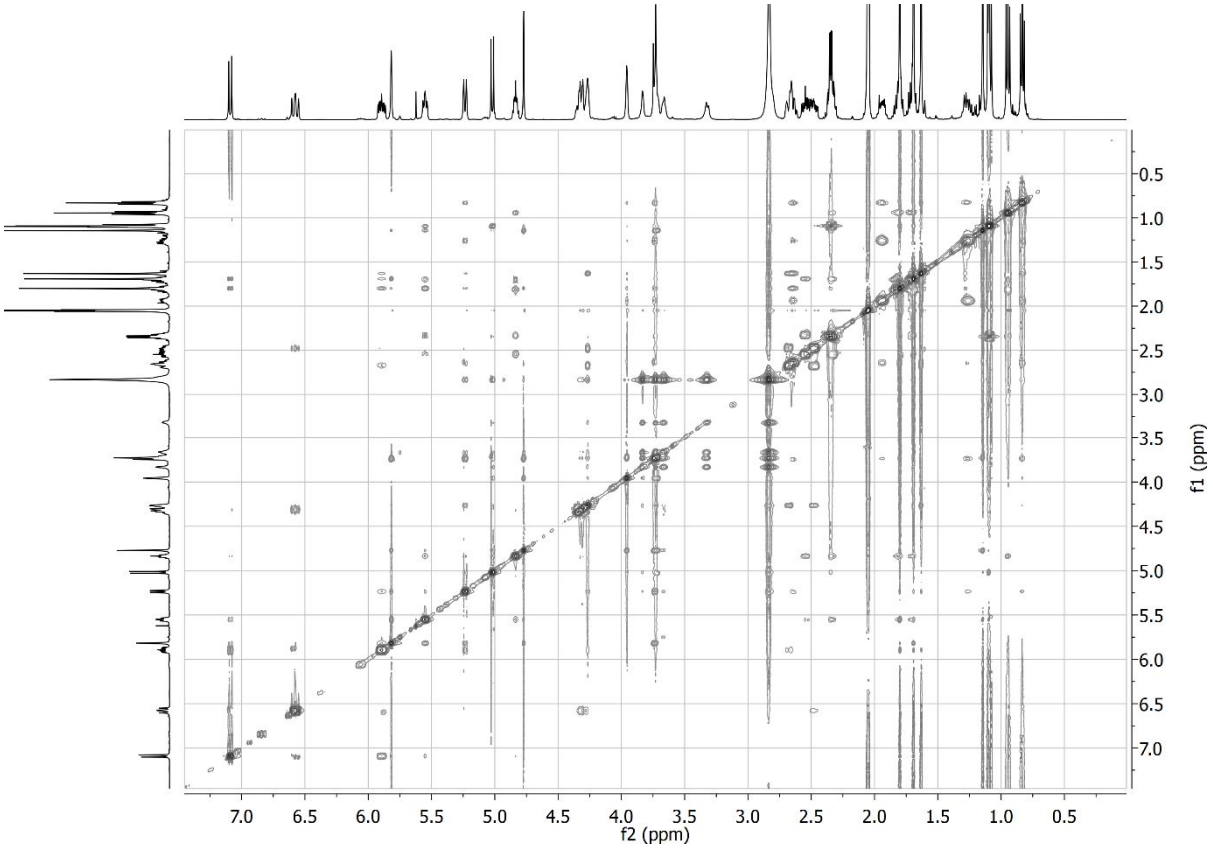
HSQC spectrum of FdxG2-OPr (**4**) (500 MHz 126 MHz, acetone-*d*6)



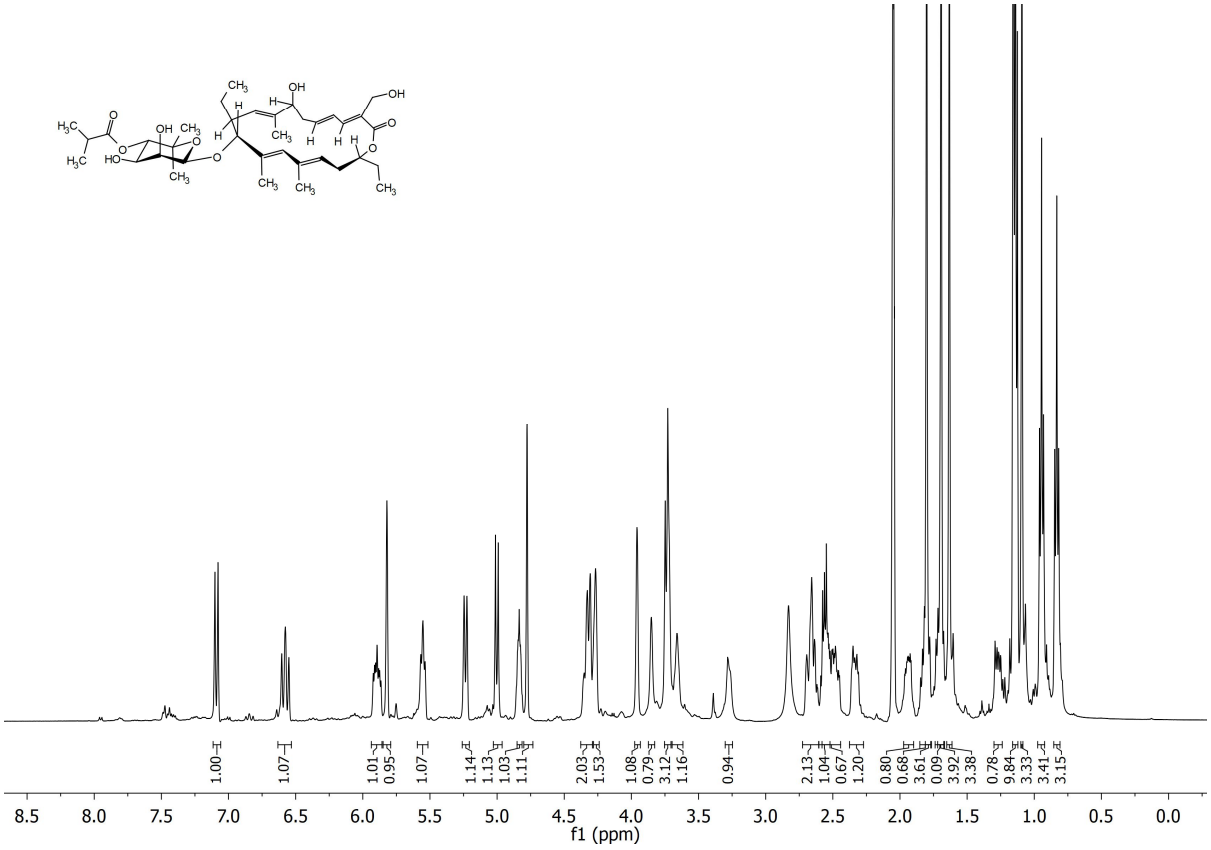
HMBC spectrum of FdxG2-OPr (**4**) (500 MHz 126 MHz, acetone-*d*6)



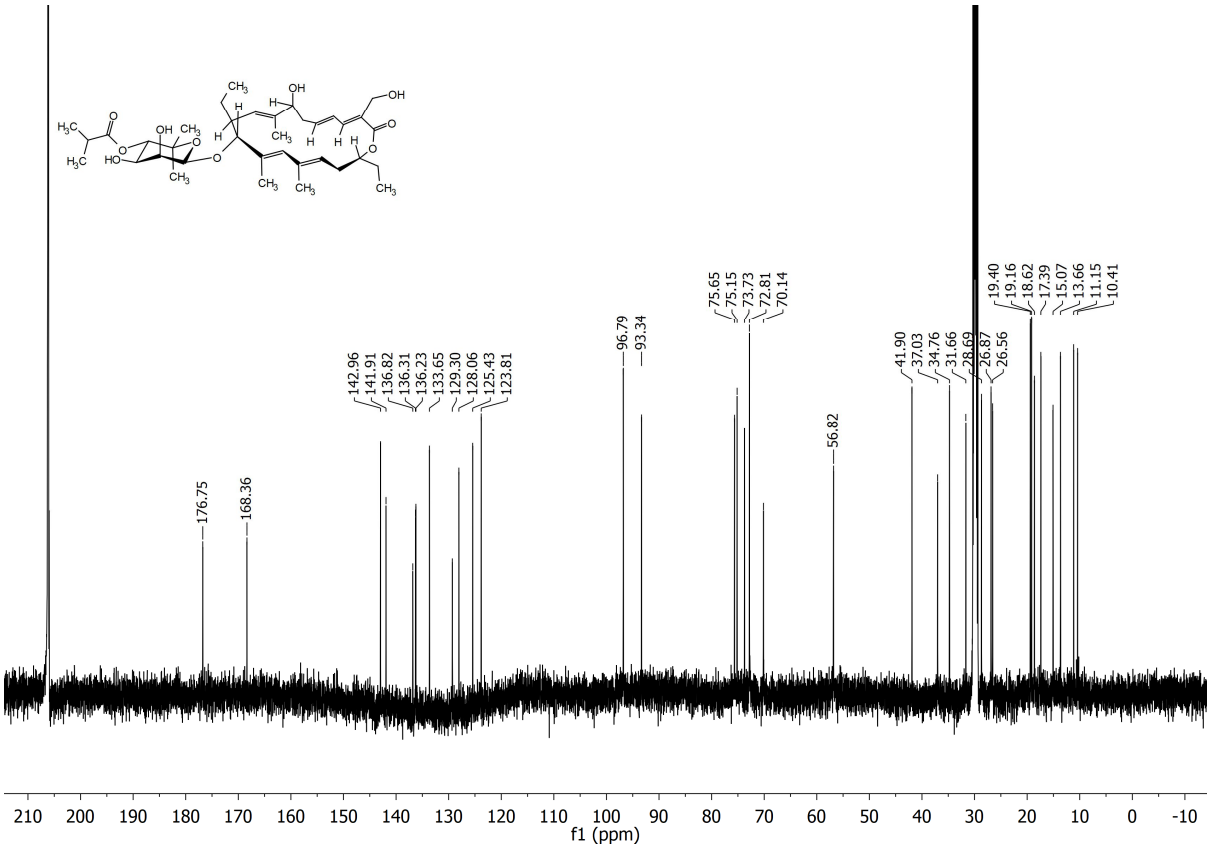
NOESY spectrum of FdxG2-OPr (**4**) (500 MHz, acetone-*d*6)



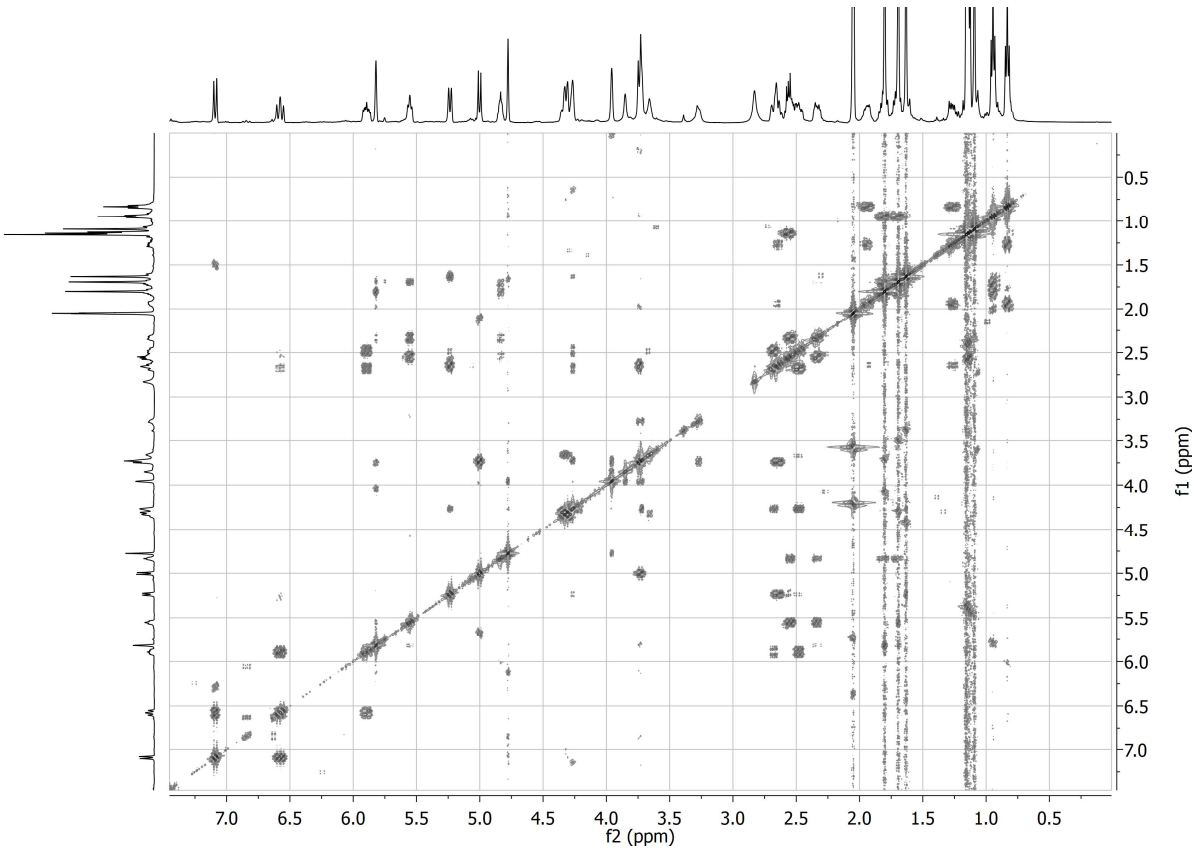
^{1}H-NMR spectrum of FdxG2-O*i*Bu (**5**) (500 MHz, acetone-*d*6)



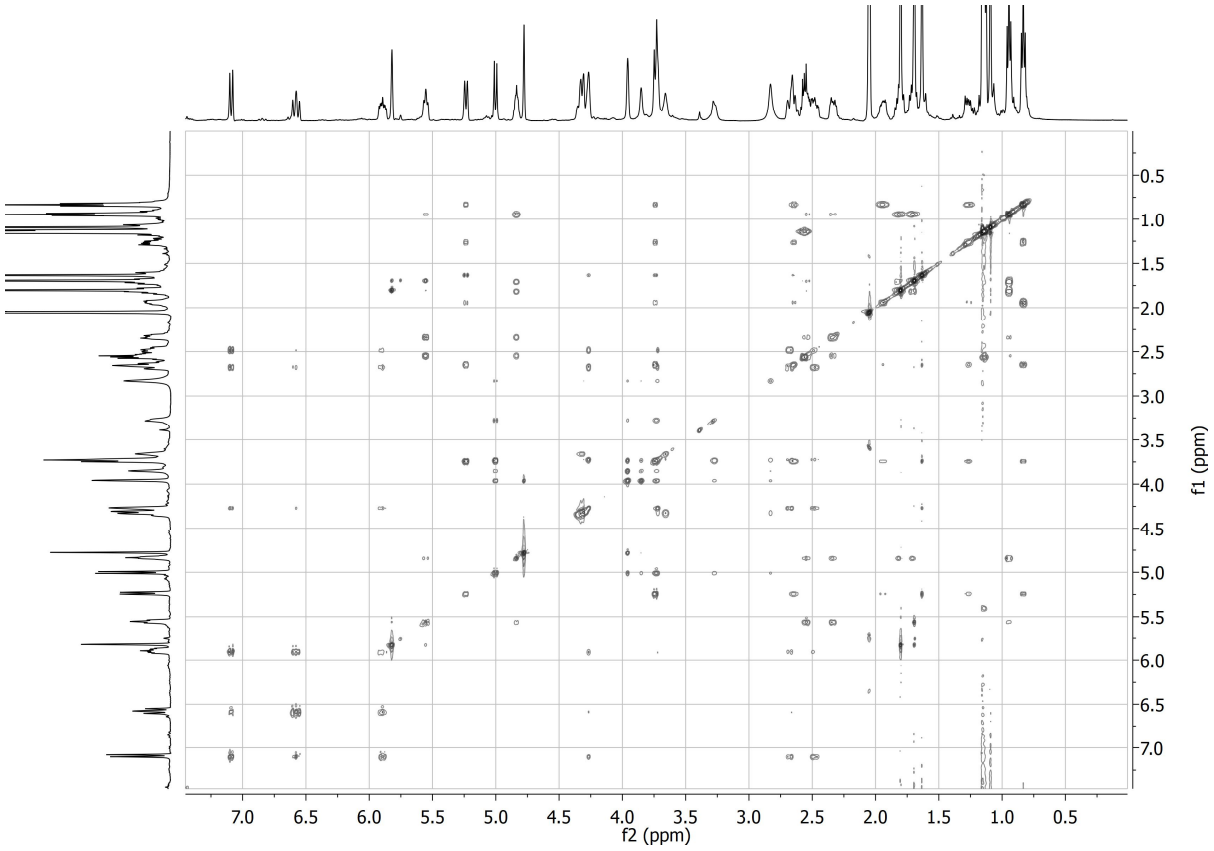
^{13}C NMR spectrum of FdxG2-O*i*Bu (**5**) (126 MHz, acetone-*d*6)



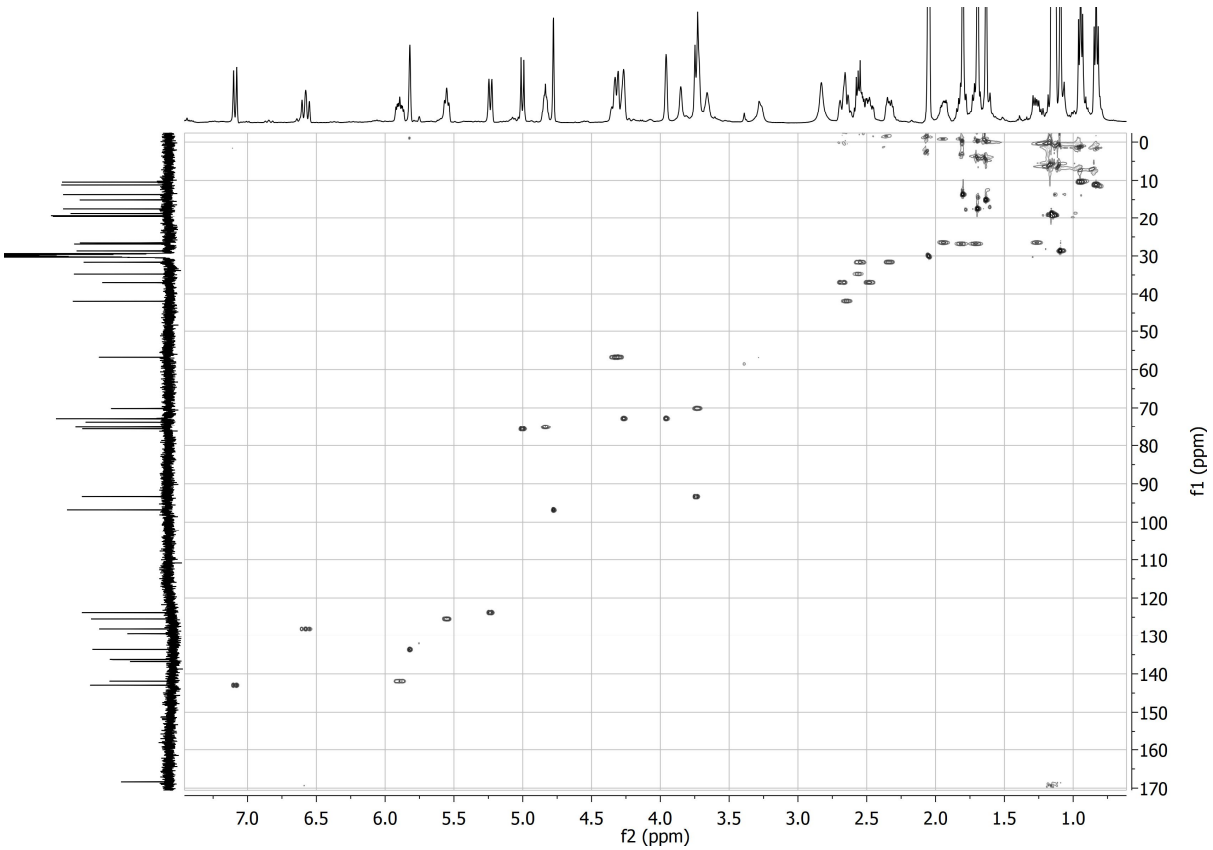
COSY spectrum of FdxG2-O*i*Bu (**5**) (500 MHz, acetone-*d*6)



TOCSY spectrum of FdxG2-O*i*Bu (**5**) (500 MHz, acetone-*d*6)



HSQC spectrum of FdxG2-O*i*Bu (**5**) (500 MHz 126 MHz, acetone-*d*6)



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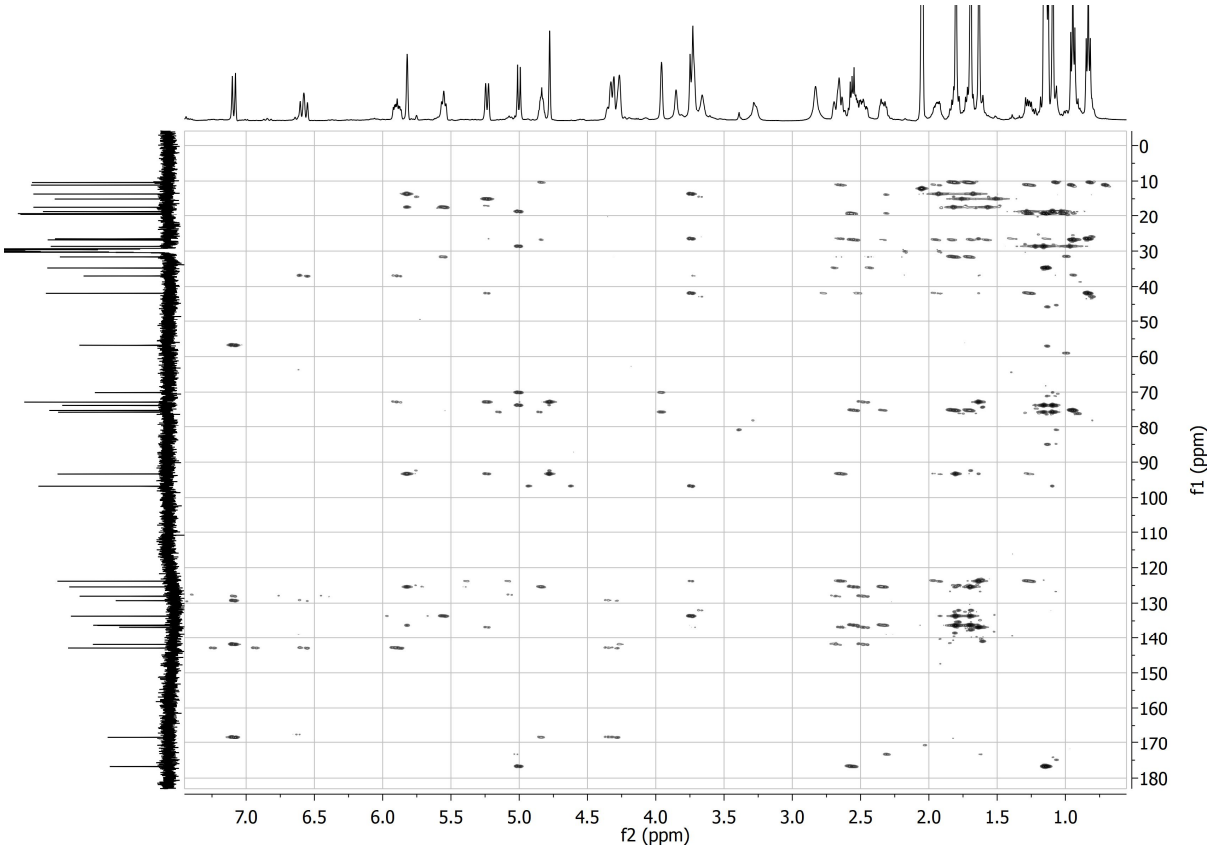

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