Oxidative Dearomatisation: The Key Step of Sorbicillinoid Biosynthesis.

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Electronic Supplementary Information

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1. Degenerate PCR

Agar plates with malt extract media (MEM) were inoculated with *P. chrysogenum* E01-10/3 spores. After 5-6 days of incubation at 30 °C fungal spores were collected and stored in glycerol (up to 20%) or used for inoculation of liquid cultures. The fungus was grown in flasks containing 40-400 ml of liquid MEM depending on the purpose of the experiment - isolation of chromosomal DNA or detection sorbicillactone A production. Liquid cultures were incubated 3-10 days at temperature 28-30 °C with shaking (150 rpm; submerged cultures; collection of mycelium for DNA isolation) or without shaking (surface cultures; detection of sorbicillactone A production). In case of genomic DNA isolation, young mycelial cultures were preferred.

For the purpose of running PCRs and the creation of genomic library of *P. chrysogenum* E01-10/3, high molecular weight (HMW) DNA was isolated from this strain. Flasks containing 40 ml liquid MEM were inoculated with *P. chrysogenum* spores and liquid cultures were grown for 2-3 days at 30 °C with shaking conditions of 200 rpm. The fungal mycelium was harvested by filtration trough thin filter (Miracloth filtre paper Ø22-25 μm). The harvested mycelium was freeze dried by submersion into liquid nitrogen and than immediately grinded or stored at -80 °C prior to use. Approximately 2 g of freeze-dried mycelium was grinded in pre-chilled mortar (-80 °C, for 0.5-1 h) by addition of some sterile sand. The grinded powder was dissolved in 20 ml of 1x SSC (0.15 M NaCl, 0.015 sodium citrate, pH 7.4). Subsequently an equal volume (20 ml) of lysis buffer (0.1 M EDTA, 1% sarkosyl, 200 μg/ml proteinase K) was added to the mixture. The mixture was incubated for 2 h at 55 °C in waterbath. The fungal mycelium was separated via centrifugation at 11,000 rpm for 10 min. After removal of mycelium, the mixture was extracted two times with phenol/chlorophorm/isoamylalkohol (IAA: 25:24:1) and once with chloroform/IAA (24:1) respectively. All centrifugation steps were run at the speed of 11,000 rpm for 10 min. Approximately 2-5 ml of recovered aqueous phase was dialysed (dialysis membrane, cellulose MWCO1000; Roth) against 2-4 ml of 1 x TEN buffer (10 mM Tris pH 7.5, 1 mM EDTA, 100 mM NaCl) in a fridge at 4 °C. Samples were dialysed overnight (12-17 h) with continuous stirring. The content of dialysis membranes containing purified DNA was recovered in fresh falcon tubes. The DNA was precipitated by addition of one volume of isopropanol and centrifugation at 10,000 rpm for 5 min. The precipitated DNA was dissolved overnight at 4 °C in 1 ml of sterile water.

For PCR screening with genomic DNA of *P. chrysogenum*: Initial Denaturation: 94 °C, 2 min; Denaturation: 94 °C, 30 sec; Primer annealing: 53 °C, 1 min 34 cycles; Extension: 75 °C, 1 min; Final extension: 75 °C, 10 min. The primers were KHKS2/KHKS3c.[1]

2. Fosmid Library Construction and Screening

The protocol was modified from the "EPICENTRE" CopyControl Fosmid Library Production Kit protocol. However, all components of the kit were used for the preparation of the genomic library.

2.1 Preparative gel purification of genomic DNA. Isolated genomic DNA of *P. chrysogenum* was size-selected through a 20 cm long 1% LMP agarose gel. 100 μl of genomic DNA preparation containing approximately 2 μg DNA (20 ng/μl) was loaded into a 8-10 cm wide genomic DNA loading lane together with some loading dye. 100 ng of 40 kb control DNA was loaded into each of the outside lanes to be used as a marker for the isolation of correctly sized genomic DNA. Samples were resolved via gel electrophoresis: initial run 10-30 min at 60-70 V enabled that samples fast enter gel and thus prevented loss of DNA. After initial run gel was run overnight (12-14h) at 35-45 V. On the next day, using a sharp razor blade, 2 cm gel slices from both sides of the gel were made. These gel slices contained approximately 1 cm (each) of the well into which the genomic DNA was loaded. Both slices were stained with ethidium bromide for 20-30 min. UV light was used to locate the band of high molecular weight (HMW) genomic DNA (~ 40 kb) in the stained edge slices of the gel. By using a clean razor blade 5 mm wide gel slices from the central

part of the non-stained gel was cut out. This slice contained *P. chrysogenum* genomic DNA corresponding to 40 kb of control DNA.

2.2 Recovery of the size-fractionated genomic DNA. The gel slice containing the genomic DNA was placed in a pre-weighed 15 ml falcon tube. LMP agarose was melted by incubation at 70 °C for 10-15 min. The tube was transferred at 45 °C, and pre-warmed 50x GELase Buffer (Epicentre Biotechnologies) was added to a tube (final concentration 1x). 1 U (i.e.1 µl) of GELase enzyme stock was added to the tube for each 500 µl of melted agarose. The reaction was incubated at 45 °C for at least one hour. The GELase enzyme was inactivated at 70 °C for 10 min and the mixture was transferred to an ice bath for 5 min to cool down. The sample was centrifuged at a speed of 10,000 rpm for 20 min to a pellet of insoluble oligosaccharides. The DNA was precipitated by the addition of 2.5 volume of ethanol and 0.1 volume of sodium acetate (pH 7.0). The sample was incubated for 10 min at RT and then centrifuged at 16,000 rpm for 20 min. The supernatant was carefully removed from the pelleted DNA. The pellet was washed two times with ice cold 70% ethanol with subsequent centrifugation and removal of supernatant. A pellet was then air-dried for 5-10 min under the clean bench and dissolved in 60 µl of TE buffer. The dissolving of DNA was eased by an incubation of 1-2 h at 50 °C or an overnight incubation at 4 °C. The DNA concentration and quality was determined by running an aliquot (0.5 µl) of the DNA on an agarose gel using dilutions of known amounts of the 40kb control DNA as standard.

2.3 End repairing of purified size-fractionated genomic DNA. This step generates blunt-ended, 5'-phosphorylated DNA. The end-repair reaction can be scaled up or scaled down by the amount of DNA available. The set up of the end repair reaction is as follows:

Purified sterile water	0 µl
10x End-repair buffer (Epicentre)	8 µl
dNTP mix (2.5 mM)	8 µl
ATP (10 mM)	8 µl
HMW genomic DNA (~1 µg)	52 µl
End-repair enzyme mix (Epicentre)	4 µl
Final reaction volume	80 µl

The reaction was incubated at the RT for 45 min. The end-repair enzyme mixture was heat inactivated for 10 min at 70 °C. End repaired genomic DNA was precipitated by addition of the following components directly to the 80 µl of reaction.Precipitation of end repaired genomic DNA:

End repair reaction volume	80 µl
Purified sterile water	140 µl
Sodium acetate (pH 5.0)	20 µl
Isopropanol	120 µl
Final reaction volume	340 µl

Further, the sample was gently mixed via inversion and incubated for 30 min at RT. The DNA was precipitated via centrifugation at maximum speed (16,000 rpm), and the supernatant was removed carefully by pipetting. The pellet was washed by the addition of 70% ice cold ethanol and centrifuged for 5 min (max. speed). The supernatant was removed and the pellet was air-dried under a clean bench for 20-30 min. 20 µl of TE buffer was added to the pellet, and the sample was incubated 1-2 h at 50 °C prior to quality and concentration check via gel electrophoresis.

2.4 Ligation reaction. In this step the CopyControl pCC1FOS vector provided by the kit was ligated to size-selected and end-repaired genomic DNA of the fungus *P. chrysogenum*.A 10:1

molar ratio of the CopyControl pCC1FOS vector to insert DNA was proven to be optimal.In a new tube at RT, the following reagents were combined in the order listed and mixed thoroughly after each addition:

Purified sterile water	0 µl
10x Fast-Link Ligation Buffer (Epicentre)	1 µl
ATP (10 mM)	1 µl
CopyControl pCC1FOS Vector (0.5 µg/µl)	0.5 µl
Concentrated insert DNA (20 ng/µl; 0.13 µg overall)	6.5 µl
Fast-Link DNA Ligase (Epicentre)	1 µl
Total reaction volume	10 µl

The reaction was incubated at RT for 2 hours. Subsequently, the sample was incubated for 10 min at 70 °C in order to inactivate the Fast-Link DNA ligase (Epicentre). At this point the sample could be directly used for packaging reaction or stored at -20 °C prior to use.

2.5 *In vitro* packaging and titering the packaged fosmid clones. The day before performing the packaging reactions, a single colony of EPI300-T1R cells was inoculated into 50 ml of LB broth supplemented with 10 mM $MgSO_4$ and shaken overnight at 37 °C. On the next day, 5 ml of the overnight culture was transferred into 50 ml of supplemented fresh LB broth and shaken at 37 °C until the OD_{600} reached 0.8-1.0. The cells were then stored at 4 °C up to 72 hours.

10 µl of the ligated fosmid DNA was pipetted into a tube containing 25 µl of thawed MaxPlax Packaging (Epicentre Biotechnologies) extract and incubated at 30 °C for 90 min. At the end of the incubation time, additional 25 µl of thawed MaxPlax Packaging extract was added to the mixture, which was further incubated at 30 °C for the additional 90 min. Subsequently, phage dilution buffer [10 mM Tris-HCl (pH 8.3), 100 mM NaCl, 10 mM $MgCl_2$] was added to 1 ml volume. At the end, 25 µl of chloroform were added at the top of the mixture and homogenised by gentle vortexing.

To determine the titer of the packaged fosmids, a 1:10 dilution of packaged fosmids was made by adding 90 µl of phage dilution buffer to 10 µl of originally packaged fosmids. 10 µl of the 1:10 dilution was added to 100 µl of prepared EPI300-T1R host cells at RT for 20 min and spread on LB-chloramphenicol selection plates at 37 °C overnight. A sufficient number (~5000) of clones were inoculated into 51 ninety-six-well plates with 100 µl of LB medium containing chloramphenicol (12.5 µg/µl). After shaking at 37 °C overnight, 100 µl of 40% glycerol was added to the 100 µl culture of each clone for storing at -80 °C.

2.6 Screening the genomic *P. chrysogenum* fosmid library via PCR. For screening the genomic library of *P. chrysogenum* via PCR, fosmid pools from genomic library were made in 50 ninety-six well plates. Large (15 cm) Petri dishes were made with LB agar medium supplemented with 12.5 µg/ml chloramphenicol. Each of the ninetysix well plates from the library was replicated onto such an agar plate. The overnight cultures were grown at 37 °C and on the next day fosmid clones were striped off from the agar with sterile inoculation loop and by the addition of 1-2 ml fresh liquid LB medium containing chloramphenicol at the same concentration as LB agar plate. 750 µl of the fosmid clones suspension was mixed with an equal amount of 40% glycerol. Superpools were stored in a freezer at -80 °C. Plasmid preparations were made from each superpool after overnight growth of LB liquid cultures at 37 °C and 200 rpm. These superpool plasmid preparations were used for screening for PKS positive plates in the first round of PCR screening. One µl of diluted (1:2, 1:4) superpool plasmid DNA was used for screening.

These plate plasmid superpools were also used for screening library via the Southern hybridization method.Lane pools were made for each PKS positive ninety-six-well plate by taking out 20 µl from 12 fosmid glycerol stocks that belong to one lane. In that manner, eight pools were made per each ninety-six-well plate and were screened with whole-cell PCR protocol (1 µl of cell suspension per PCR reaction). From each positive lane 10 µl of each clone were aliquoted to a

fresh PCR microfuge tube in order to make a lane pool. From each of lane pools, a 0.5-1 μl was used directly in the whole-cell PCR. PKS positive fosmids that corresponded to the same gene cluster were digested with the set of same restriction enzymes. One restriction pattern was chosen for subcloning of fragments into the pBluescript KS(-) vector. Subcloned fragments were end-sequenced via GATC Biotech (Konstanz, Germany). In some cases, for the purpose of gaining more sequence data, fragments were cut with the second restriction enzyme, subcloned into pBluescript vector and end-sequenced.

3. Sequence

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4. ORF Analysis

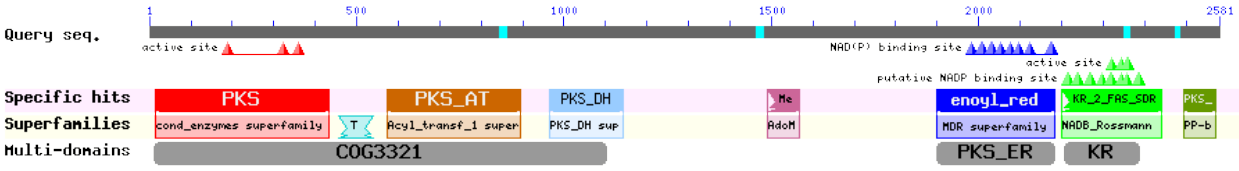
Putative ORFs were determined using fgenesh[2]

ORF	Orient-ation	Start	Stop	PBlast closest homology	Assignment
orf1	+				
exon 1		5162	5406	predicted protein	fungal transcription
intron 1		5407	5468	*Trichoderma*	factor regulatory
exon 2		5469	6066	*reesei* QM6a	middle homology
intron 2		6067	6134	EGR52693.1	region
exon 3		6135	6953		
orf2 (sorbC)	-				
exon 1		8632	8131	predicted protein	FAD-binding
intron 1		8130	8065	*Trichoderma*	monooxygenase
exon 2		8066	7450	*reesei QM6a*	
intron 2		7449	7381	EGR52183.1	
exon 3		7380	7231		
orf3 (sorbB)	-				
exon 1		17688	17580	polyketide	Non-Reducing fungal
intron 1		17579	17410	synthase	iterative polyketide
exon 2		17409	16748	*Trichoderma*	synthase
intron 2		16747	16695	*reesei* QM6a	
exon 3		16694	12490	EGR52182.1	see section 5 for
intron 3		12489	12441		domain analysis
exon 4		12440	9422		
orf4 (sorbA)	+				
exon 1		18733	18942	polyketide	Highly-Reducing
intron 1		18943	18999	synthase	fungal iterative
exon 2		19000	19086	*Trichoderma*	polyketide synthase
intron 2		19087	19144	*reesei* QM6a	
exon 3		19145	19202	EGR52690.1	see section 5 for
intron 3		19203	19263		domain analysis
exon 4		19264	19597		
intron 4		19598	19654		
exon 5		19655	25868		
intron 5		25869	25929		
exon 6		25930	26772		

ORF	Orient-ation	Start	Stop	PBlast closest homology	Assignment
orf5	-				
exon 1		28969	28134	fungal specific transcription factor, putative	Transcription factor
intron 1		28133	28080	*Neosartorya fischeri* NRRL 181	
exon 2		28079	27729	XP_001258840.1	
intron 2		27728	27674		
exon 3		27673	27118		
orf6	+				
exon 1		29744	30076	hypothetical protein AN3270.2	Major facilitator
intron 1		30077	30220	*Aspergillus nidulans* FGSC A4	superfamily transporter
exon 2		30221	31426	XP_660874.1	
orf7	+				
exon 1		32119	33534	glucooligosaccharide oxidase, putative (AFU_orthologue; AFUA_6G14340) *Aspergillus nidulans* FGSC A4 CBF83064.1	FAD-binding oxygenase
orf8	-				
exon 1		38809	38212	Actin cytoskeleton-regulatory complex protein	Regulator
intron 1		38211	38144	PAN1 *Penicillium digitatum*	
exon 2		38143	36140	Pd1 EKV05437.1	
intron 2		36139	35967		
exon 3		35966	35444		
intron 3		35443	35386		
exon 4		35385	35098		
intron 4		35097	34978		
exon 5		34977	34215		

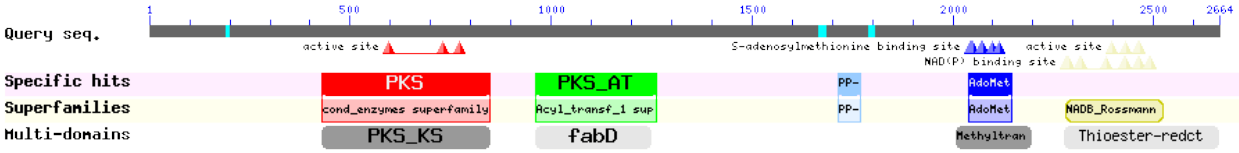
5. PKS Domain Analysis

5.1 SorbA



Domain SorbA	Approx. Predicted Boundaries	Conserved Active Site Motif	ref
KS	S14 - D436	185 - T(A/G)CX_2(G/S) - 190 - active site cysteine	3
AT	F575 - T891	662 - G(S)HSXG - 666 - active site serine	4
DH	P965 - D1251	966 - HX_8PXAGY(I/Y) - 1012	5
CMeT	Q1265 - Q1657	1459 - (R/K)ILEGX_2TG - 1468 - SAM binding	6
ER	N1844 - P2196	32% identical, 52% similar to rat FAS ER in this region.	5
KR	F2204 - Q2476	2209 - YX_3GX_2GGXG - 2216 Rossmann fold [NAD(P) binding]	7
ACP	Q2495 - G2581	2531 - GXDSLXA - 2537 phosphopantetheine attachment site	8

5.2 SorbB



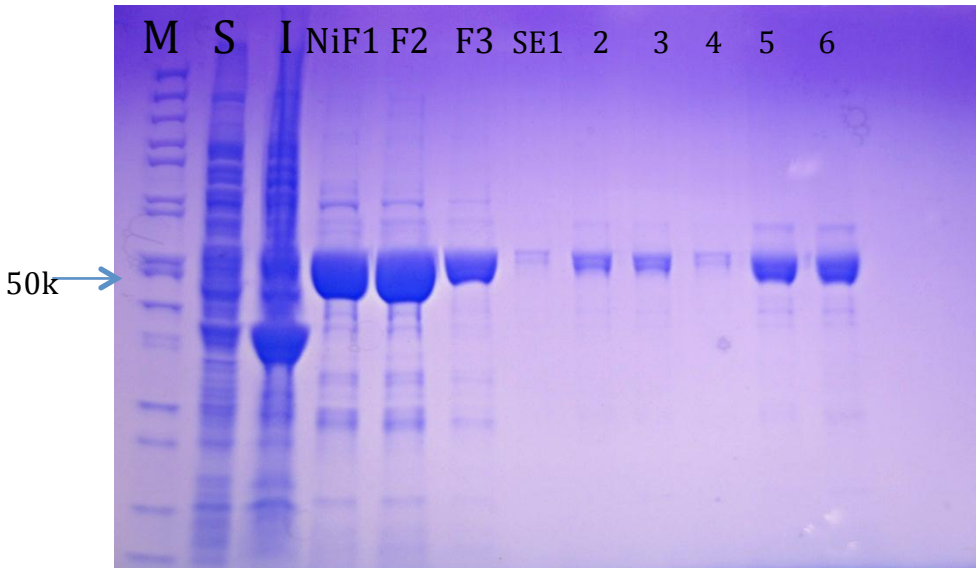
Domain SorbB	Approx. Predicted Boundaries	Conserved Active Site Motif	ref
SAT	L54 - L311	182 - GXCXG - 186 - active site cysteine	9
KS	D430 - Q849	594 - (A/G)CXX(G/S) - 599 - active site cysteine	3
AT	F962 - V1276	1045 - GHSXG - 1049 - active site serine	4
PT	C1361 - R1701	1379 - HX_9(T/P) - 1389 1562 - DX_3(H/Q)X_6N - 1573 - active site residues	10
ACP	L1717 - L1777	1742 - GXDSL - 1746 phosphopantetheine attachment site	8
CMeT	L1977 - H2193	2045 - GXGXG - 2049 - SAM binding	6
R	T2257 - V2593	2283 - GXXGXXG - 2289 Rossmann fold [NAD(P) binding] 2461 - YX_3K - 2465 - active site residues	11

6. Cloning, Expression, Purification and Characterisation of *sorbC*

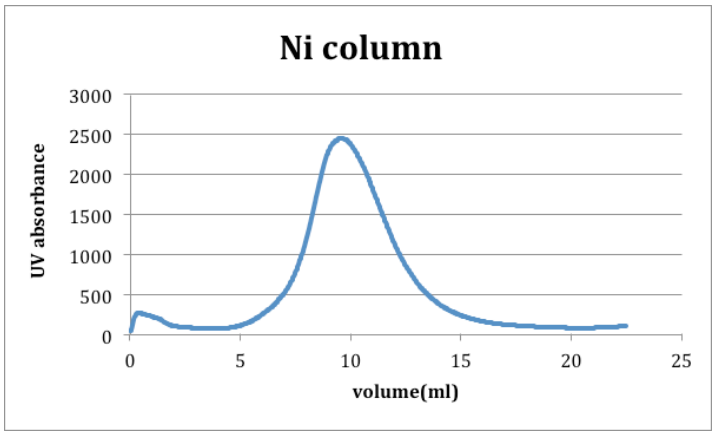
Mycelial samples were collected by Buchner filtration from a fermentation shown to be producing sorbicillin by LCMS analysis. The mycelia was immediately flash frozen in liquid nitrogen and stored at -80 °C until required. Mycelia were finely ground under liquid nitrogen and a 75 mg sample was used with the Qiagen RNeasy Plant Mini kit following the standard protocol using RLC buffer and omitting the on-column DNAseI digest. Two elutions of 40 mL and 20 mL were combined and contaminating DNA was digested by addition of DNAseI (Fermentas, 3 mL) and 10x DNAseI buffer (Fermentas, 7 mL) followed by incubation at room temperature for 20 min. DNAseI was inactivated by heating to 75 °C for 5 min and the RNA solution was immediaietly flash frozen in liquid nitrogen and stored at -80 °C until needed. Complimentary DNA (cDNA) was synthesised from total RNA (1 mL) using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermantas) by the standard protocol (at 10 ml scale), using oligo$(dT)_{18}$ primers, and stored at -20 °C until required. The cDNA was used in a standard PCR reaction using the following primers: 5'-CATATGACTCGATCCGCCAACAGC-3' and 5' - GAATTCTCAGAATGCAGTGTCTGCGCC-3'. The PCR product was digested with *Nde*I and EcoR1 and ligated into pET28a(+) which had previously been digested with the same enzymes and dephosphorylated. The ligation product was transformed into *E. coli* DH5a. Selected transformants were grown and used for plasmid DNA miniprep. Insert sequence was confirmed by sequencing.

The pET28a/sorbC plasmid was transformed into competent *E. coli* BL21 (Stratagene) which was then grown in 2TY medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) with kanamycin (50 μg/ml) at 37 °C, 250 rpm until the OD_{600} was ~ 0.6 – 1.0. The temperature was then dropped to 16 °C and cultures were induced with 0.5 mM IPTG, incubated overnight at 300 rpm. The cells were harvested by centrifugation at 5000 rpm for 30 min at 4 °C and resuspended in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM imidazole, 5% glycerol, pH 7.5). Subsequently, the cells were sonicated (6 x 30 s). Centrifugation at 18000 rpm for 20 min at 4 °C was then performed to remove cell debris and the supernatant was collected.

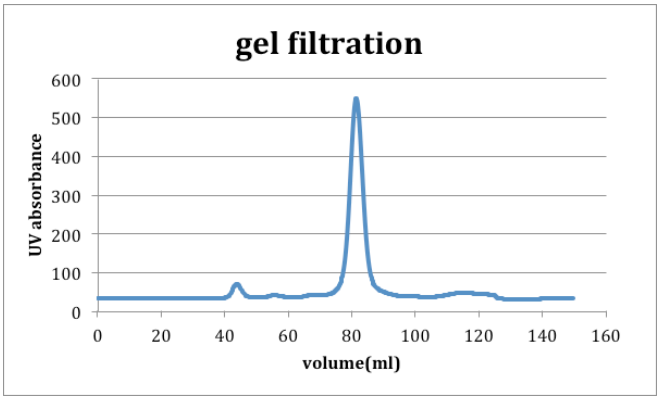
Protein was purified form the soluble fraction using a nickel-loaded chelating affinity column (HisTrap HP 5 mL column, GE Healthcare). Contaminants were removed using 20 column volume washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM imidazole, 10% glycerol). His-Tagged protein was eluted from the column by running a gradient of elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 500 mM imidazole, 10% glycerol) using an AKTA purifier, detecting protein at 280 nm. Eluted fractions were pooled at 200 mM imidazole and verified by SDS-PAGE analysis, then the pure fractions of the desired protein were concentrated using an Amicon Ultra centrifugal filter unit (Millipore) and then purified further using a HiLoad 26/600 Superdex 200 (GE Healthcare) column pre-equilibrated with 150 ml of 50 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, pH 7.5 filtered and degassed, the protein pass through the column by running 150 ml buffer, 6 fractions (2ml each) were collected and concentrated using an Amicon Ultra centrifugal filter unit (Millipore) and concentration was determined using BCA assay (Thermo). A total of 20 mg of protein was produced per litre of fermentation. For long-term storage, protein was stored at -80 °C in Tris pH 7.5, 150 mM NaCl, 5% glycerol, pH 7.5.



SDS PAGE analysis of SorbC purification. M = protein regular unstained marker; S = soluble fraction; I = insoluble fraction; Ni F1 = fraction 1 collected from column; Ni f2 fraction 2 collected from column; Nif3 fraction 3 collected from column; SE1 fraction 1 collected from size exclusion column; SE2 fraction 2 collected from size exclusion column; SE3 fraction 3 collected from size exclusion column; SE4 fraction 4 collected from size exclusion column; SE5 fraction 5 collected from size exclusion column; SE6 fraction 6 collected from size exclusion column.



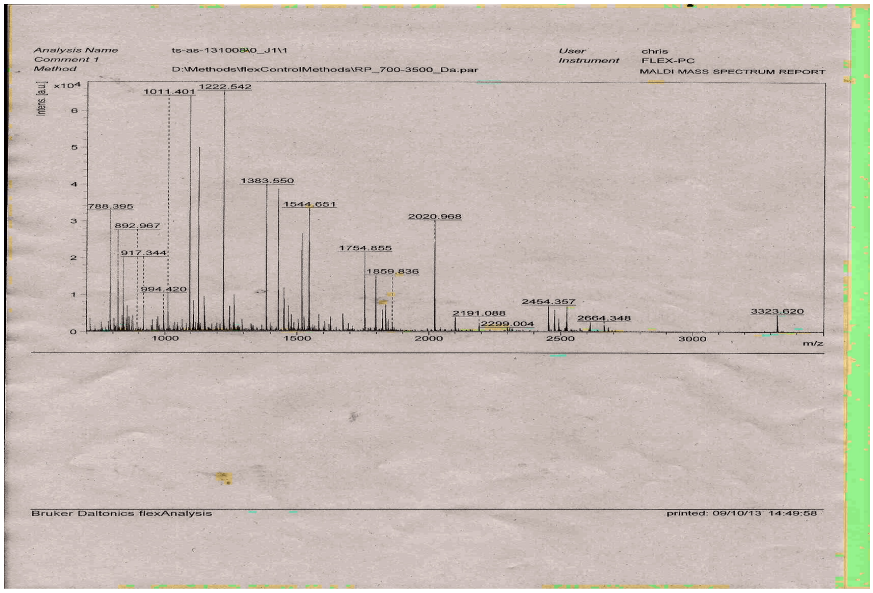
FPLC Chart for Ni column purification using AKTA machine



FPLC Chart for S200 column purification using AKTA machine

6.1 MALDI fragment analysis of SorbC

In order to check the exact mass and identity of the protein, the band from SDS-PAGE gel was cut into pieces in a 1.5 ml eppendorf tube and soaked overnight with 50% ethanol for destaining, then treated with 200 μL of 100 mM ammonium bicarbonate for 10 min at RT. The gel pieces were dehydrated with 200 μL acetonitrile several times, then reduced with 200 μL of 10 mM dithiothreitol for 1 h at 60 °C followed by alkylation step using 200 µL 50 mM Iodoacetamide for 45 min in the dark at room temperature. The pieces were washed several times with ammonium bicarbonate and acetonitrile then incubated overnight at 37 °C with 30 µL of Trypsin solution (Promega).[12] The tryptic peptide fragments were analysed using a Matrix Assisted Laser Desorption Ionization (MALDI) source coupled to a Time-of-Flight (TOF) mass spectrometer for identification of the parent protein. The instrument was equipped with a 200 Hz solid-state laser using a matrix of α-cyano-4-hydroxycinnaminic acid at 10 mg/ml in 50% acetonitrile and 0.05% trifluoroacetic acid. Mass data were collected in standard reflector mode over the mass range of 600 to 5000 *m/z* with a total of 400 shots collected at a laser intensity of 3300. MS/MS spectra were obtained using a standard 1-kV acquisition method with collision induced dissociation turned on. The laser intensity was set at 4300, and spectra were recorded for 2000 shots or until the accumulated spectra reached an estimated signal/ noise ratio of 75 (five peaks and eight subspectra minimum). Database searches were performed on the MASCOT search engine (www.matrixscience. com; Matrix Science, London, UK) using Applied Biosystems GPS software. From the results it was detected that the protein was Pc21g05060 (= SorbC) from *Penicillium chrysogenum* wisconsin 54-1255 with the exact expected mass (48.585 Kda) and 71% of protein sequence coverage.



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HDTAGMVSDA LAILGERVRG ADTAF

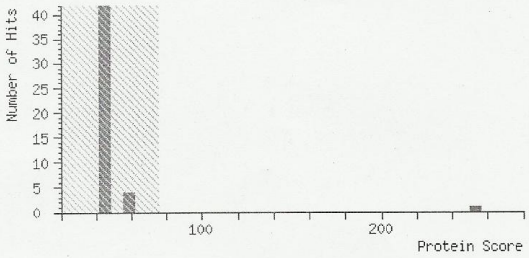
Red coloured residues detected by MALDI.

Mascot Search Results

User :
Email :
Search title :
Database : NCBInr 20130606 (25905244 sequences; 8953110406 residues)
Taxonomy : Fungi (1903004 sequences)
Timestamp : 9 Oct 2013 at 13:58:33 GMT
Top Score : 253 for gi|255953599, Pc21g05060 [Penicillium chrysogenum Wisconsin 54-1255]

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 75 are significant (p<0.05).



Concise Protein Summary Report

Format As: Concise Protein Summary Help
Significance threshold p< 0.05 Max. number of hits AUTO
Preferred taxonomy: All entries

Re-Search All Search Unmatched

1. gi|255953599 Mass: 48585 Score: 253 Expect: 9.5e-020 Matches: 38
Pc21g05060 [Penicillium chrysogenum Wisconsin 54-1255]

2. gi|238596406 Mass: 16468 Score: 54 Expect: 8.5 Matches: 8
hypothetical protein MPER_06133 [Moniliophthora perniciosa FA553]

Search Parameters

Type of search : Peptide Mass Fingerprint
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 100 ppm
Peptide Charge State : 1+
Max Missed Cleavages : 1
Number of queries : 390
Selected for scoring : 39

Mascot: http://www.matrixscience.com/

MASCOT Search Results

Protein View: gi|255953599

Pc21g05060 [Penicillium chrysogenum Wisconsin 54-1255]

Database:	NCBInr
Score:	253
Expect:	9.5e-020
Nominal mass (M_r):	48585
Calculated pI:	5.40
Taxonomy:	Penicillium chrysogenum Wisconsin 54-1255

This protein sequence matches the following other entries:

- gi|211589263 from Penicillium chrysogenum Wisconsin 54-1255

Sequence similarity is available as an NCBI BLAST search of gi|255953599 against nr.

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)
Mass values searched:	390
Mass values matched:	38

Protein sequence coverage: 71%

Matched peptides shown in ***bold red***.

```
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 51 TFTPNAQRAM EALDPCVLQS FTNVASAPSG GTINFVDGVR EQGSEDPRTS
101 TAALLFQLHV KGGYKACRRC DFVDQIVQHI PKDCVQYRKW LDSIETDHES
151 GRAVLKFRDG EIAHADVVIG CDGIRSQVRA SMFGTDELCP RAQYSHQLGY
201 RGMVPLAQAT AVLGPEKTSS AVLHTGPGAF VLTIPLAEVH AMHIEAFIMD
251 KEEWPEVQTS SDSKRYVLPA TRNEATKAFA EFGPTVRSAV SMFPEKLEKW
301 AVFDMLEAPV PTFAKGRVCL AGDAAHASTP NQGGGAGFGI EDALVLAEVL
351 AVLAEAPNVS GIVASEALAV YSEVRYERSQ WLVRSSRRTG ELCTWKDRDW
401 GLAAEELSRD IISRSHQLWD HDTAGMVSDA LAILGERVRG ADTAF
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Unformatted sequence string: 445 residues (for pasting into other applications).

Sort peptides by ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Show predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
4 - 29	2454.3517	2453.3444	2453.3894	-18.3	0	R.SANSPFEVAIVGGGITGLALAVGLLK.R
4 - 30	2610.4754	2609.4681	2609.4905	-8.55	1	R.SANSPFEVAIVGGGITGLALAVGLLKR.N
30 - 39	1284.6113	1283.6040	1283.6622	-45.3	1	K.RNVSFTIYER.A
31 - 39	1128.5177	1127.5104	1127.5611	-45.0	0	R.NVSFTIYER.A
40 - 58	2020.9686	2019.9614	2020.0014	-19.8	0	R.AENFGELGVGITFTPNAQR.A
59 - 90	3323.6170	3322.6097	3322.5962	4.05	0	R.AMEALDPCVLQSFTNVASAPSGGTINFVDGVR.E
59 - 90	3339.6078	3338.6005	3338.5912	2.80	0	R.AMEALDPCVLQSFTNVASAPSGGTINFVDGVR.E + Oxidation (M)
91 - 98	917.3464	916.3392	916.3886	-54.0	0	R.EQGSEDPR.T
99 - 111	1428.7676	1427.7604	1427.8136	-37.3	0	R.TSTAALLFQLHVK.G
112 - 118	811.3844	810.3772	810.3806	-4.29	1	K.GGYKACR.R
119 - 132	1754.8538	1753.8465	1753.8934	-26.7	1	R.RCDFVDQIVQHIPK.D
120 - 132	1598.7476	1597.7403	1597.7923	-32.5	0	R.CDFVDQIVQHIPK.D

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
133 - 138	840.3180	839.3107	839.3596	-58.2	0	K.DCVQYR.K
133 - 139	968.4428	967.4356	967.4545	-19.6	1	K.DCVQYRK.W
139 - 152	1672.7417	1671.7345	1671.7852	-30.4	1	R.KWLDSIETDHESGR.A
140 - 152	1544.6496	1543.6423	1543.6903	-31.1	0	K.WLDSIETDHESGR.A
157 - 175	2099.9849	2098.9777	2099.0218	-21.0	1	K.FRDGEIAHADVVIGCDGIR.S
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180 - 191	1399.5469	1398.5397	1398.5908	-36.6	0	R.ASMFGTDELCPR.A + Oxidation (M)
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202 - 217	1581.7615	1580.7542	1580.8596	-66.7	0	R.GMVPLAQATAVLGPEK.T
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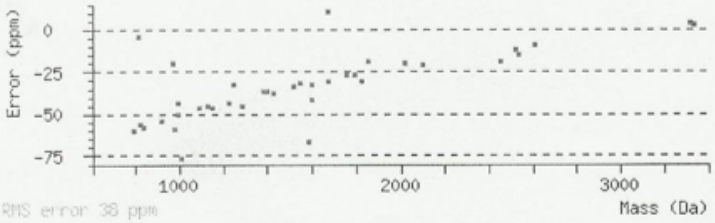


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  TITLE     Genome sequencing and analysis of the filamentous fungus
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  JOURNAL   Nat. Biotechnol. 26 (10), 1161-1168 (2008)
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7. Purification and Characterisation of Sorbicillin and Dihydrosorbicillin

7.1 Fermentation. *Penicillium chrysogenum* E01-10/3 was grown on malt extract agar plates for 12 days at 28 °C. Small pieces of agar around (1 cm^2) were picked from mature plates and transferred into sponge-plugged 500 ml flaskes containing 100 ml of sterilised Czapek-Dox broth supplied with 20 g L^{-1} tryptone in deionised water. A total of 3 L inoculated liquid medium was incubated without shaking for a week in a dark incubator at 28 °C. The mycelium was separated from the fermentation by vacuum filtration. The supernatants were combined together, acidified to pH 3.0 (aq. HCl), then extracted with ethyl acetate. The organic extract was dried with anhydrous $MgSO_4$ and evaporated to afford crude extract (2.2 g).

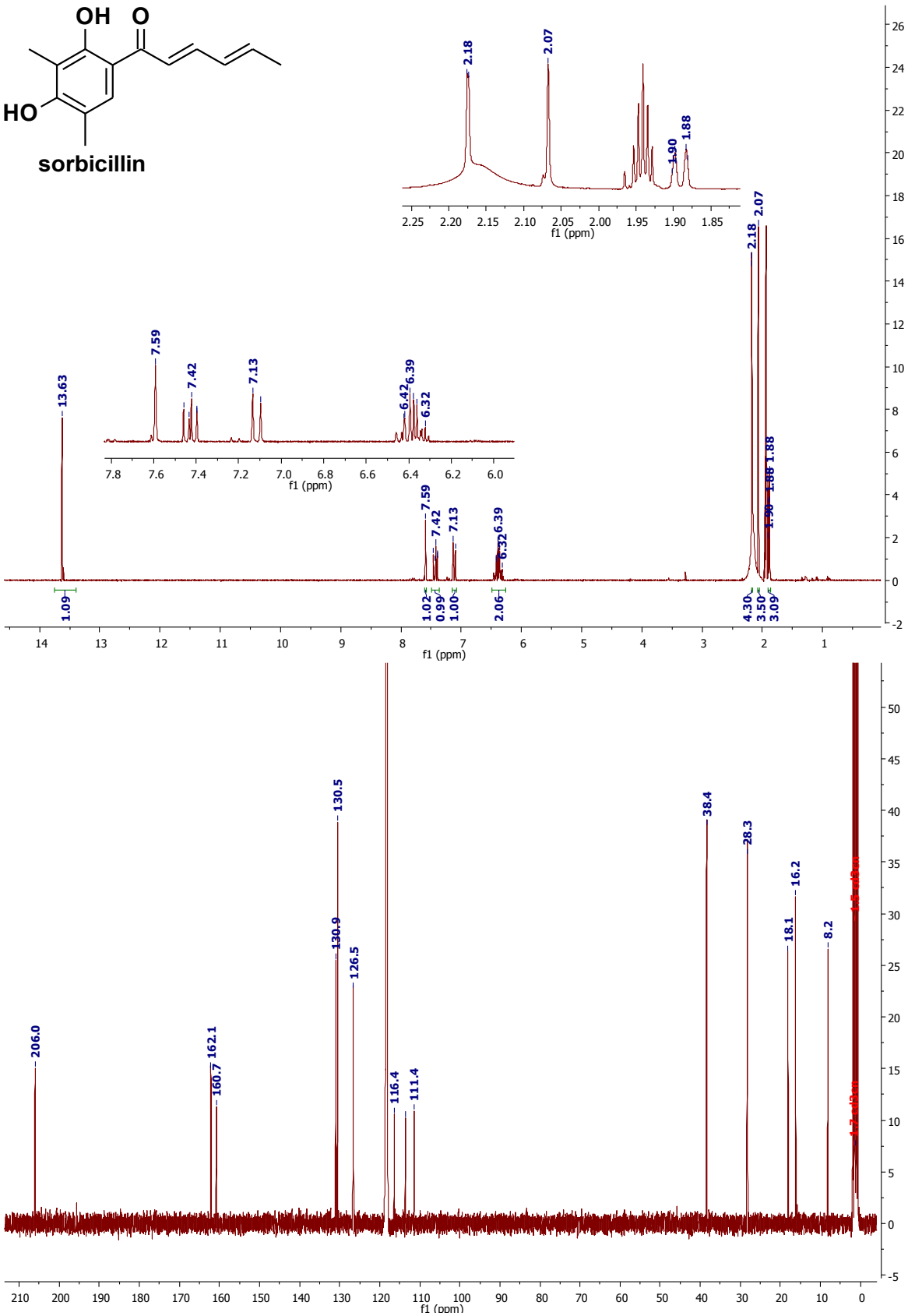
7.2 The isolation of sorbicillin and 2',3'-dihydrosorbicillin. The whole of the crude extract (2.2 g) was subjected first to a flash silica gel column (pore size 60 Å, 220-440 mesh particle size, 35-75 μm particle size) using dichloromethane (DCM) as a mobile phase. This eluted fractions containing the two metabolites as major compounds. The corresponding fractions were combined and the organic solvent was evaporated under reduced pressure to give yellow oily residue (75 mg) which was dissolved in HPLC grade methanol for further purification using a reverse-phase mass-directed HPLC.

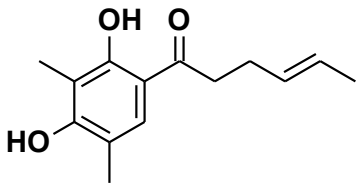
7.3 Mass-directed purification. The purification of the two metabolites was achieved using a Waters mass-directed autopurification system comprising of a Waters 2767 autosampler, Waters 2545 pump system, a Phenomenex LUNA column (5μ, C_{18}, 100 Å, 10 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C_5 300 Å) eluted at 4 mL/min. Solvent A, HPLC grade H_2O + 0.05% formic acid; Solvent B, HPLC grade CH_3CN + 0.045% formic acid. The post-column flow was split (100:1) and the minority flow was made up with MeOH + 0.045% formic acid to 1 mL · min^{-1} for simultaneous analysis by diode array (Waters 2998), evaporative light scattering (Waters 2424) and ESI mass spectrometry in positive and negative modes (Waters Quatro Micro). The Solvent gradient was as follows: 0 min, 20% B; 23min, 90% B; 24 min, 95% B; 27 min, 95% B; 28 min, 20% B; 30 min, 20% B. Detected peaks were collected into glass test tubes. Combined tubes were evaporated under a flow of dry N_2 gas, weighed, and residues dissolved directly in NMR solvent for NMR analysis.

7.4 The characterisation of sorbicillin and 2',3'-dihydrosorbicillin. The physical data of the two metabolites were in good agreement with literature data.[13]

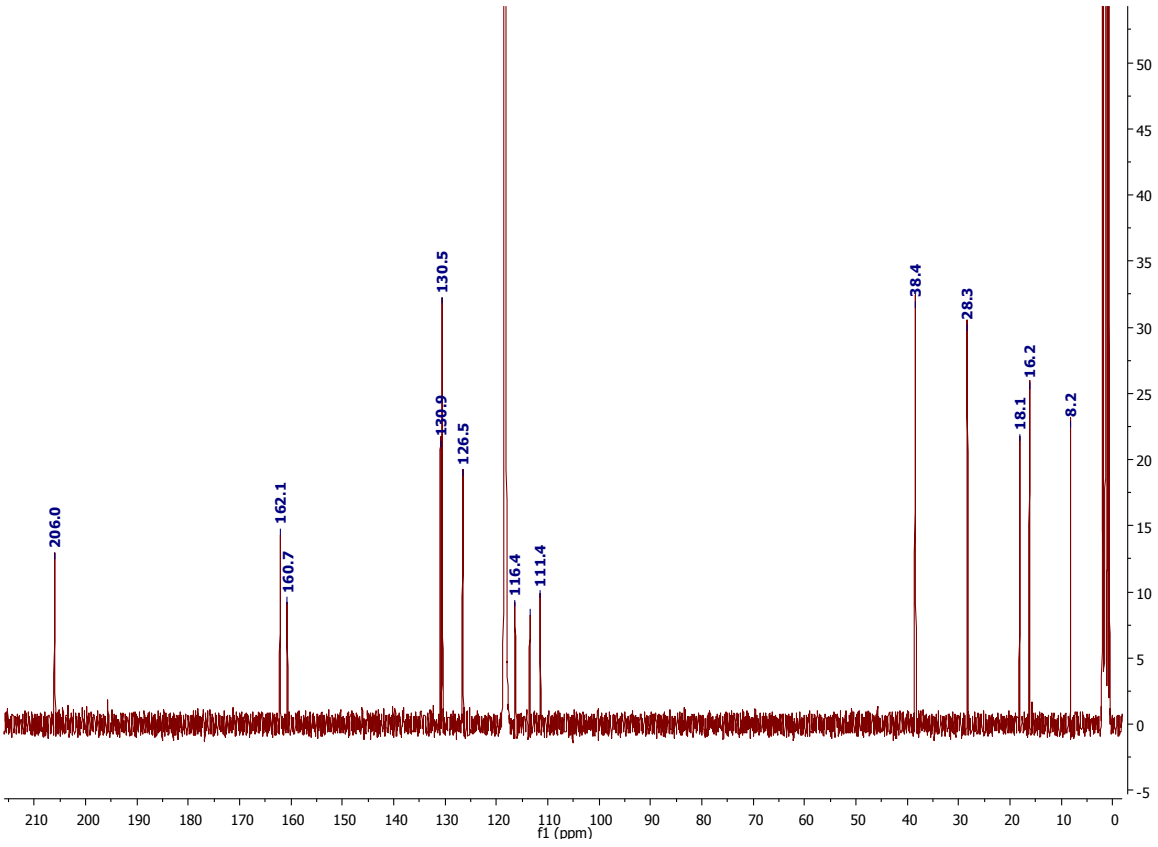
Sorbicillin: (2 mg·L^{-1}), yellow crystals, λ_{max} (CH_3CN) 323 nm; ESI *m/z* 231 $[M-H]^-$, 233 $[M]H^+$; ^1H NMR (400 MHz, CD_3CN): δ_H = 1.89 (d, *J* = 6.7 Hz, 3H), 2.07 (s, 3H), 2.18 (s, 3H), 6.83 (m, 2H), 7.12 (d, *J* = 14.8 Hz, 1H), 7.43 (dd, *J* = 14.8, 15.0 Hz, 1H), 7.59 (s, 1H), 13.63 (s, 1H); ^{13}C NMR (100 MHz, CD_3CN): δ_c = 8.2 (CH_3), 16.3 (CH_3), 19.0 (CH_3), 111.6 (C), 114.0 (C), 116.4 (C), 123.3 (CH), 130.1 (CH), 131.4 (CH), 142.3 (CH), 145.1 (CH), 161.0 (C), 163.4 (C), 193.6 (C).

2',3'-dihydrosorbicillin: (5.4 mg·L^{-1}), Pale yellow crystals, λ_{max} (CH_3CN) 220, 286 and 328 nm; ESI *m/z* 233 $[M-H]^-$, 235 $[M]H^+$; ^1H NMR (400 MHz, CD_3CN): δ_H = 1.62 (m, 3H), 2.05 (s, 3H), 2.16 (s, 3H), 2.34 (m, 2H), 2.98 (dd, *J* = 7.3, 7.6 Hz, 2H), 5.51 (m, 2H), 7.52 (s, 1H), 12.99 (s, 1H); ^{13}C NMR (100 MHz, CD_3CN): δ_c = 8.2 (CH_3), 16.2 (CH_3), 18.1 (CH_3), 28.3 (CH_2), 38.4 (CH_2), 111.4 (C), 113.5 (C), 116.4 (C), 126.5 (CH), 130.5 (CH), 130.9 (CH), 160.7 (C), 162.1 (C), 206.0 (C).





2',3'-dihydrosorbicillin

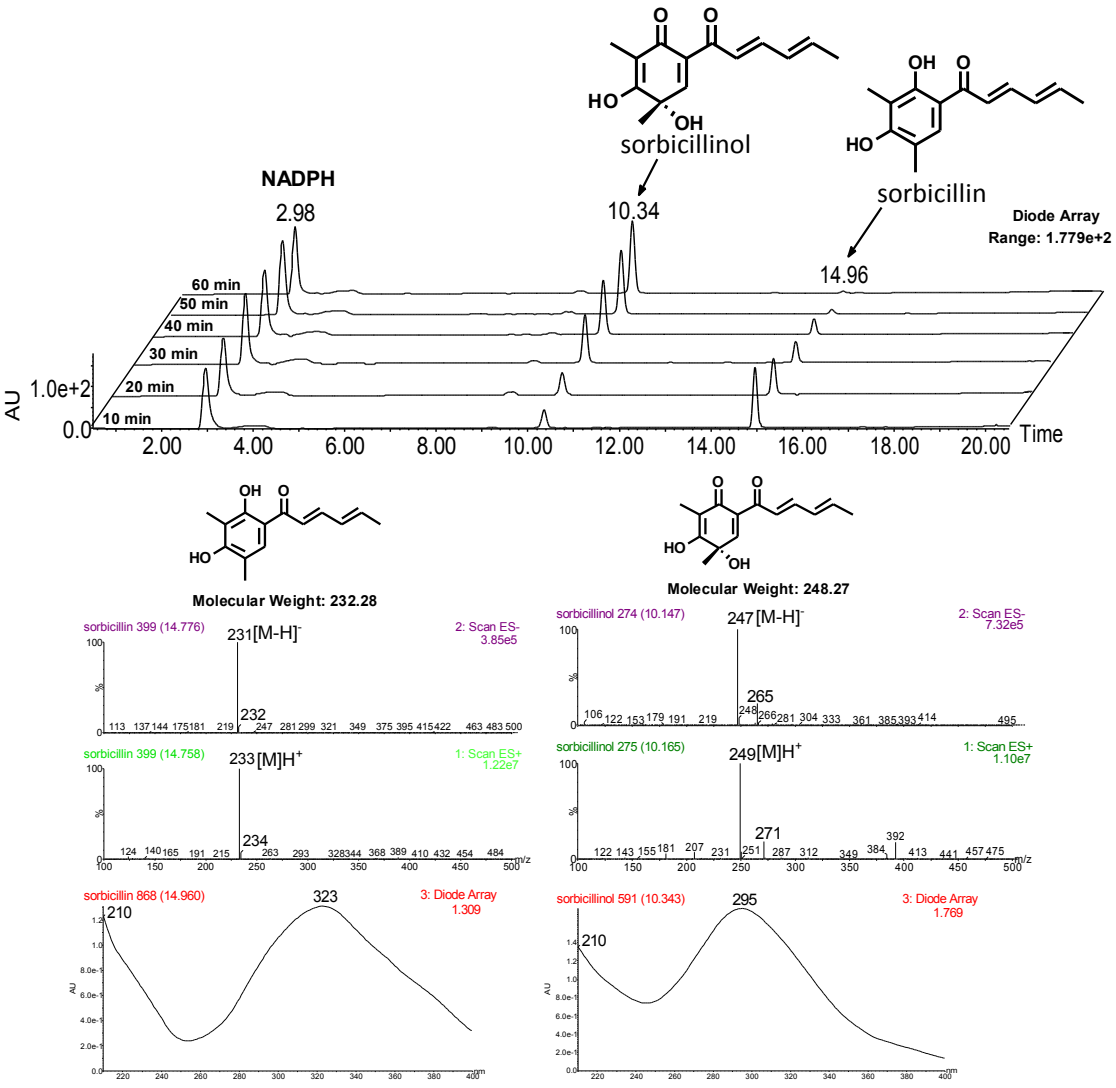


8. *In Vitro* Assays of SorbC

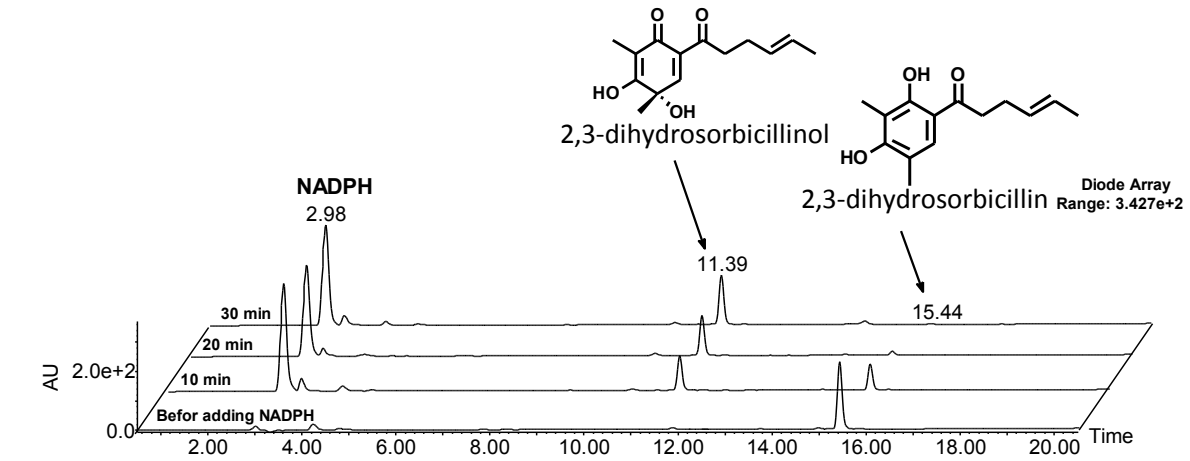
In 945 μl phosphate buffer 50 mM (pH 8), 50 μl of substrate acetone stock solution (50 mM) was added to give a final concentration of 2.5 mM. This was followed by the addition 5 μl of SorbC (final concentration 15 μM). The reaction was initiated by adding 2 mg of NADH or 2.5 mg of NADPH (final concentration 3 mM).

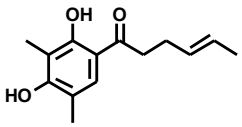
8.1 Time-course analysis of SorbC assays. For a time course, the reaction mixture was incubated for 60 min at room temperature (20°C). Every 10 min, 100 μl from the incubation mixture was transferred into a 0.5 ml small eppendorf tube containing 100 μl of 30% HCl to stop the reaction. Microcentrifugation was followed at (14,000*g*, 5 min) to pellet the precipitated enzyme. The supernatant was directly subjected to LC-DAD-MS analysis by injecting 50 µl and using a Waters 2795HT HPLC system. Detection was achieved by UV between 200 and 400 nm using a Waters 2998 diode array detector, and by simultaneous electrospray (ES) mass spectrometry using a Waters Quatro Micro spectrometer detecting between 150 and 600 *m/z* units. Chromatography (flow rate 1 mL·min^{-1}) was achieved using a Phenomenex LUNA column (5 µ, C_{18}, 100 Å, 4.6 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C_5 300 Å). Solvents were: A, HPLC grade H_2O containing 0.05% formic acid; B, HPLC grade CH_3CN containing 0.045% formic acid). Solvent gradient was as follows.
Method 1: 0 min, 5% B; 15 min, 95% B; 18 min, 95% B; 18.5 min, 5% B; 20 min, 5% B.

8.2 Sorbicillin assay



8.3 2,3-dihydrosorbicillin assay





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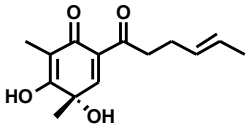

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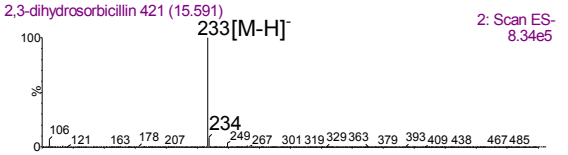
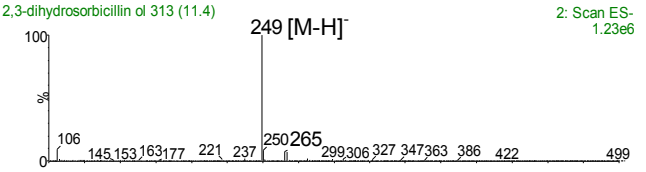
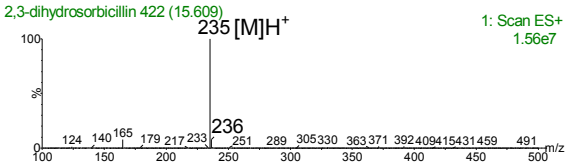
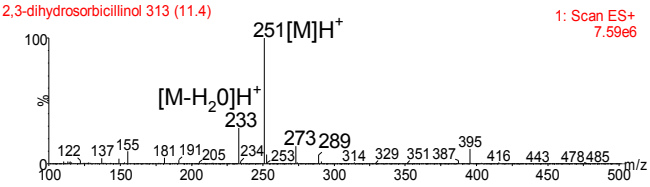
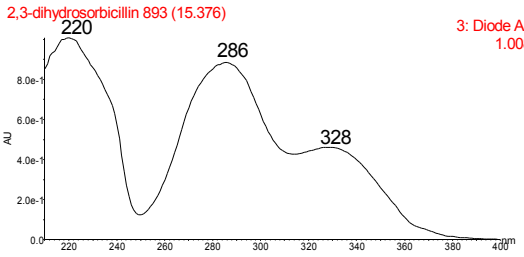
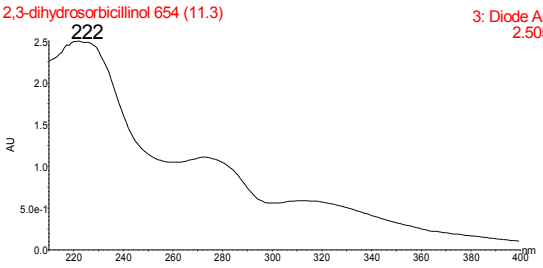












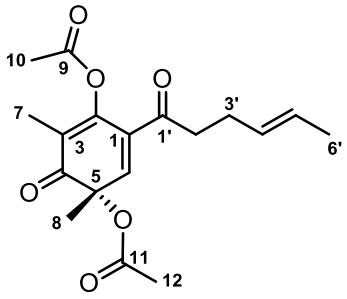
9. Purification and Characterisation of diacetyl-2'3'-dihydrosorbicillinol.

9.1 Sorbc large scale assay. For large scale *in vitro* assay, 17 reaction mixtures were incubated at 28 °C for 2 hours. When the substrate was completely oxidised, they were combined together and 2 volumes of HPLC grade acetonitrile were added. This step precipitated the enzyme which pelleted upon centrifugation at high speed. Then, the clear supernatant was transferred to a 100 ml small flask and the acetonitrile was evaporated using nitrogen blow down.

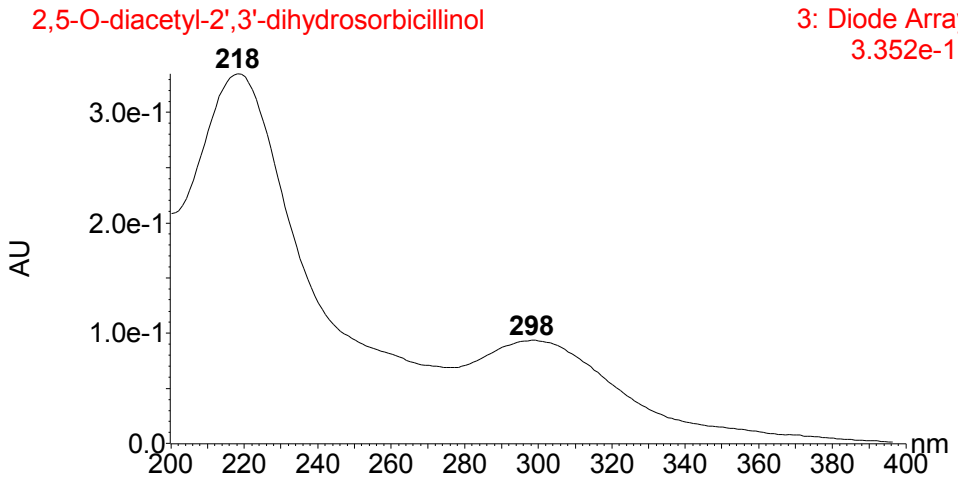
9.2 The acetylation of 2',3'-dihydrosorbicillinol. The incubation solution of 2', 3'-dihydrosorbicillinol (0.59 mg·ml^{-1}, 17 ml) was placed on ice bath and stirred for 30 min. Next, 8.5 ml of pyridine was added and left for 15 min. This was followed by a gradual addition of 5 ml acetic anhydride over 10 min at 0 °C. The reaction was then stirred at room temperature for 1 hour before purification.

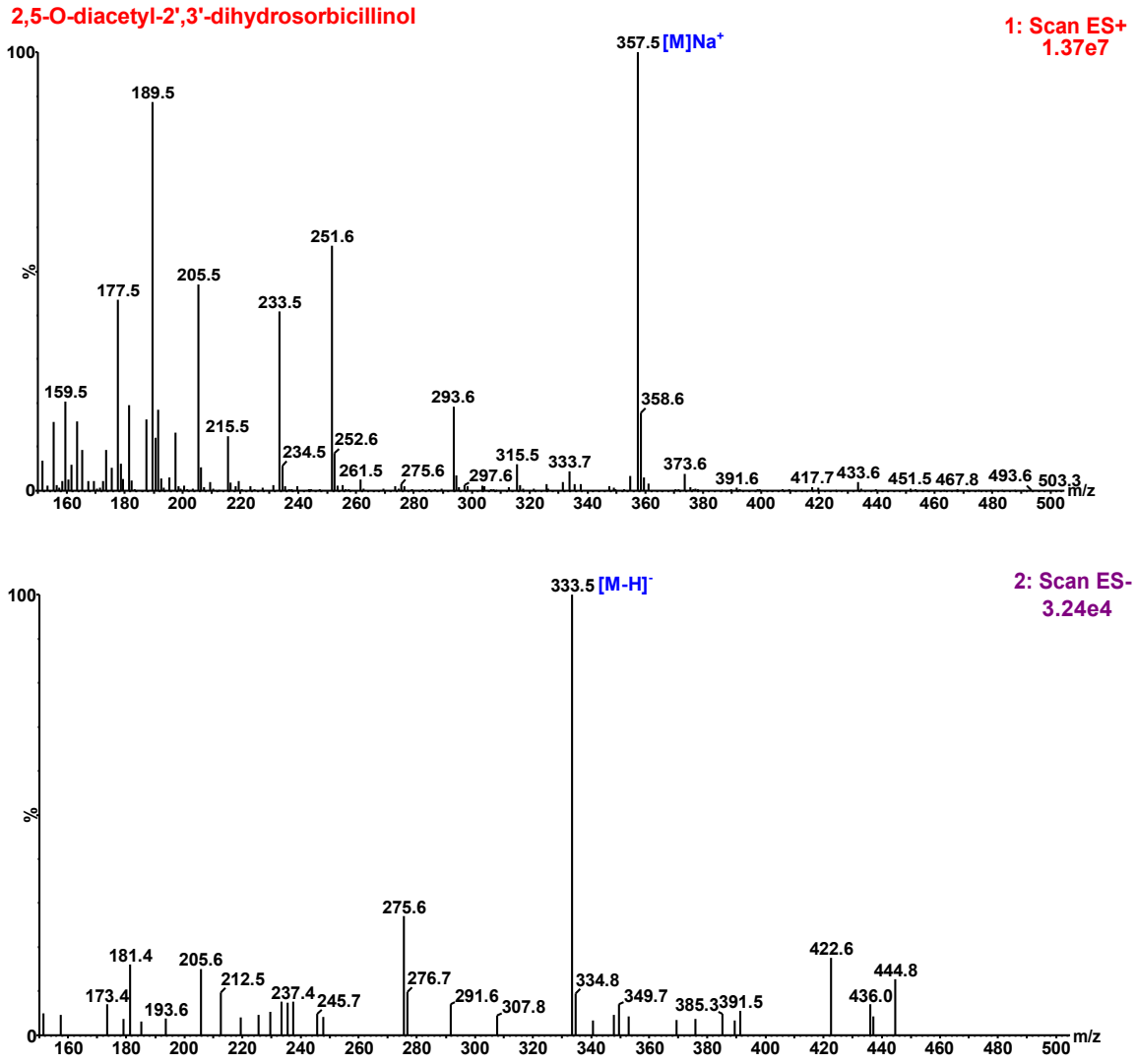
9.3 The purification of 2,5-O-diacetyl-2',3'-dihydrosorbicillinol. The purification of 2,5-O-diacetyl-2',3'-dihydrosorbicillinol was carried out using a larger column reversed-phase HPLC (Phenomenex, Kinetex, 5 μm C_{18} 100 Å, AXIA Packed LC Column 250 x 21.2 mm) at a flow rate of 16 ml/min. The purification was performed following the solvent gradients of *method1*. The acetylation reaction yielded 1 mg (10%) of the compound which submitted for NMR analysis.

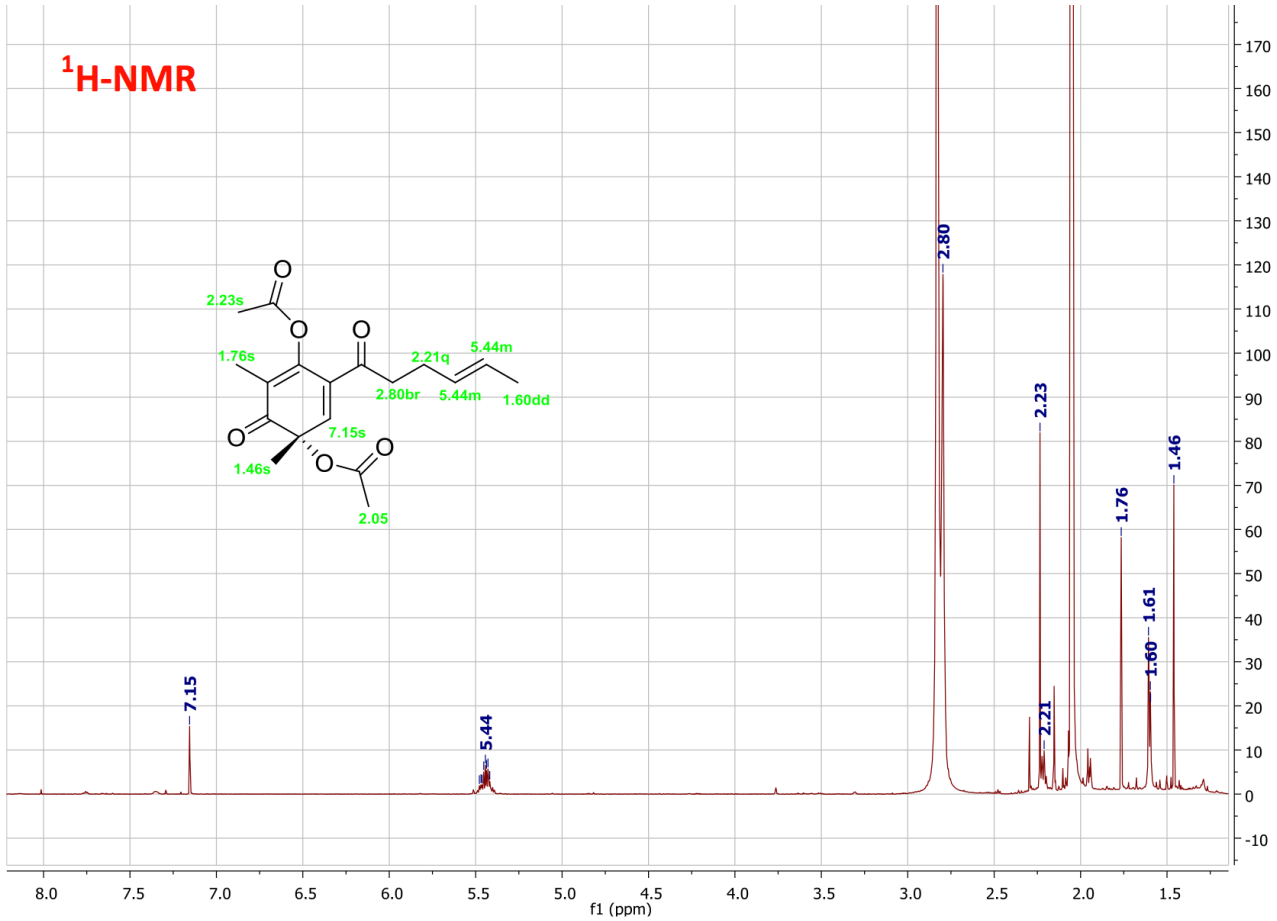
2,5-O-diacetyl-2',3'-dihydrosorbicillinol

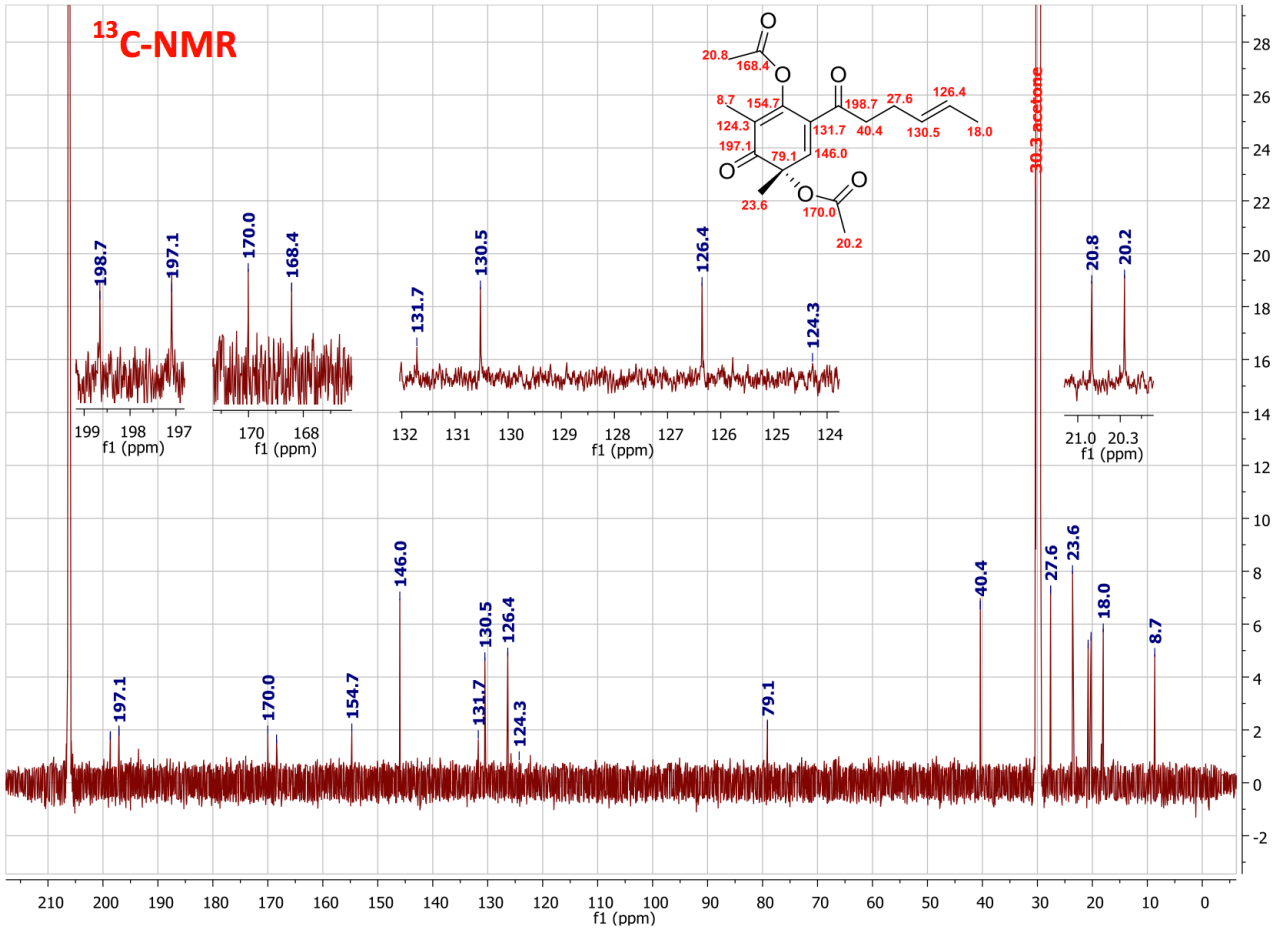


Pale yellow powder, HRMS *m/z* 357.1299 [M]Na$^+$, (calcd for $C_{18}H_{22}NaO_6$, 357.1309); ^{1}H-NMR (600 MHz, acetone-d_6) δ_H: 1.46 (s, 3H, H_3-8), 1.60 (dd, J = 6.4, 1.5, 3H, H_3-6'), 1.76 (s, 3H, H_3-7), 2.05 (s, 3H, H_3-12), 2.21 (m, 2H, H_2-3'), 2.23 (s, 3H, H_3-10), 2.80 (brm, 2H, H_2-2'), 5.44 (m, 2H, H-4'and H-5'), 7.15 (s, 1H, H-6); ^{13}C-NMR (150 MHz, acetone-d6) δ_c: 8.7 (C-7), 18.0 (C-6'), 20.2 (C-12), 20.8 (C-10), 23.6 (C-8), 27.6 (C-3'), 40.4 (C-2'), 79.1 (C-5), 124.3 (C-3), 126.4 (C-5'), 130.5 (C-4'), 131.7 (C-1), 146.0 (C-6), 154.7 (C-2), 168.4 (C-9), 170.0 (C-11), 197.1(C-4), 198.7 (C-1'); λ_{max} (CH_3CN:water, 3:1) 218 and 298 nm; ESI *m/z* 357.5 [M]Na$^+$, 333.5 [M-H]$^-$.

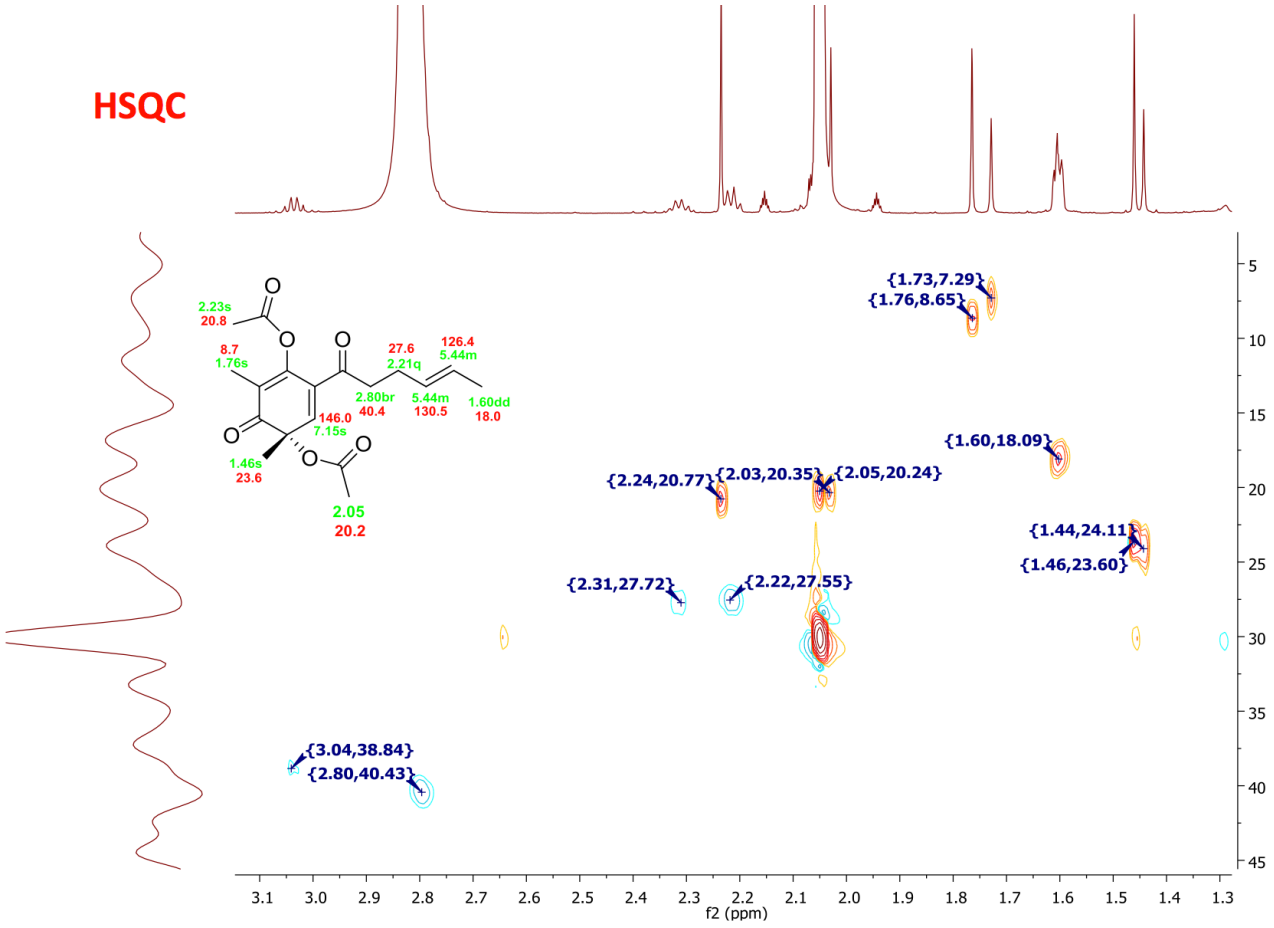




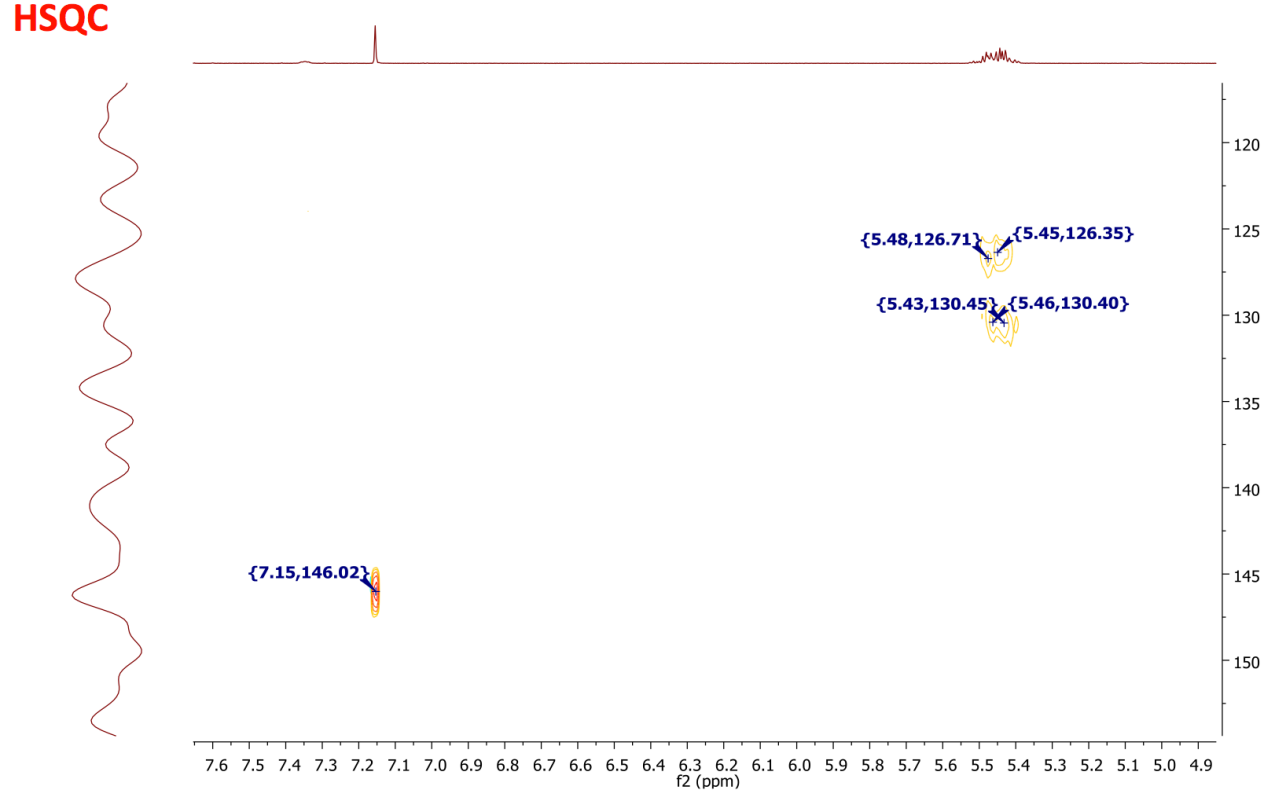


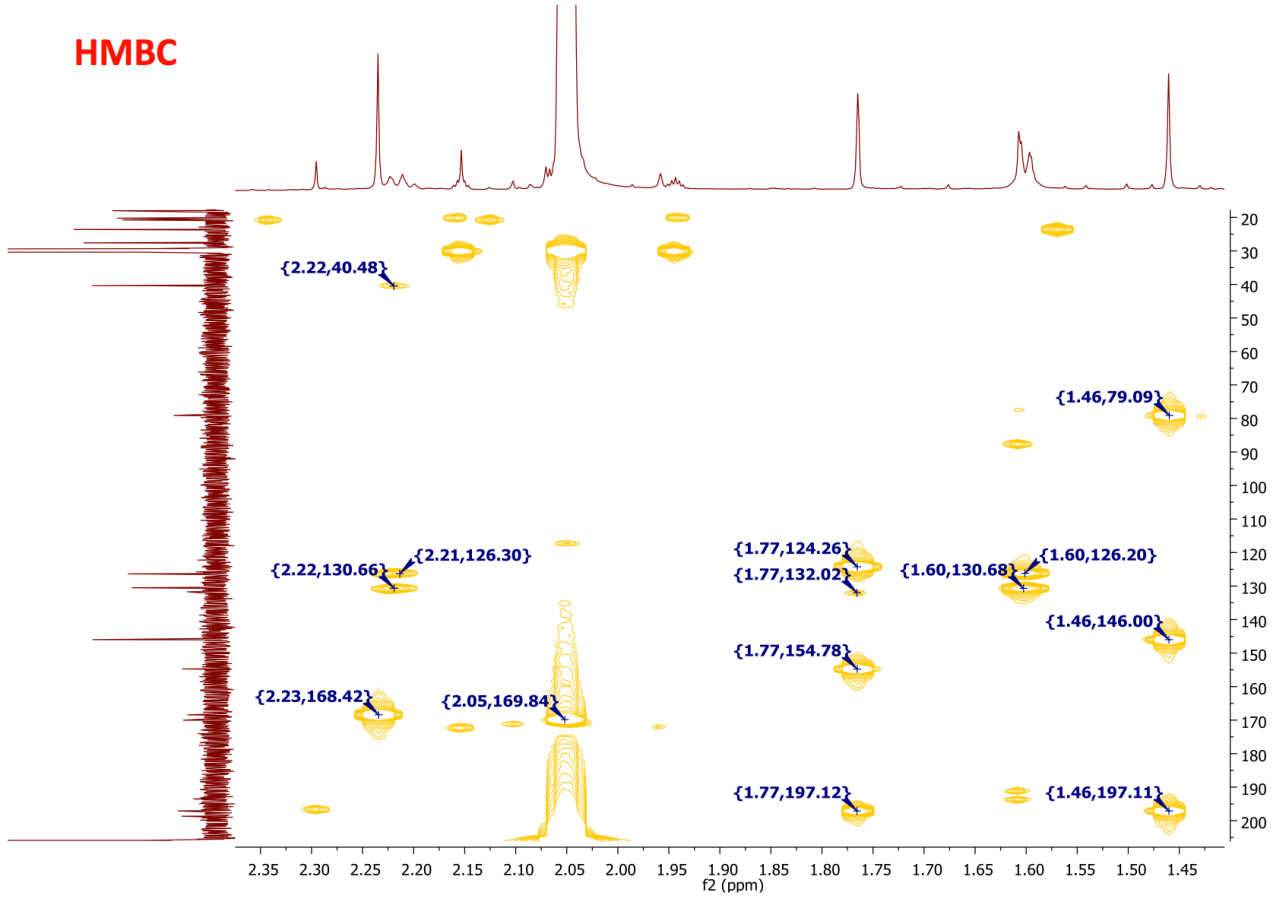


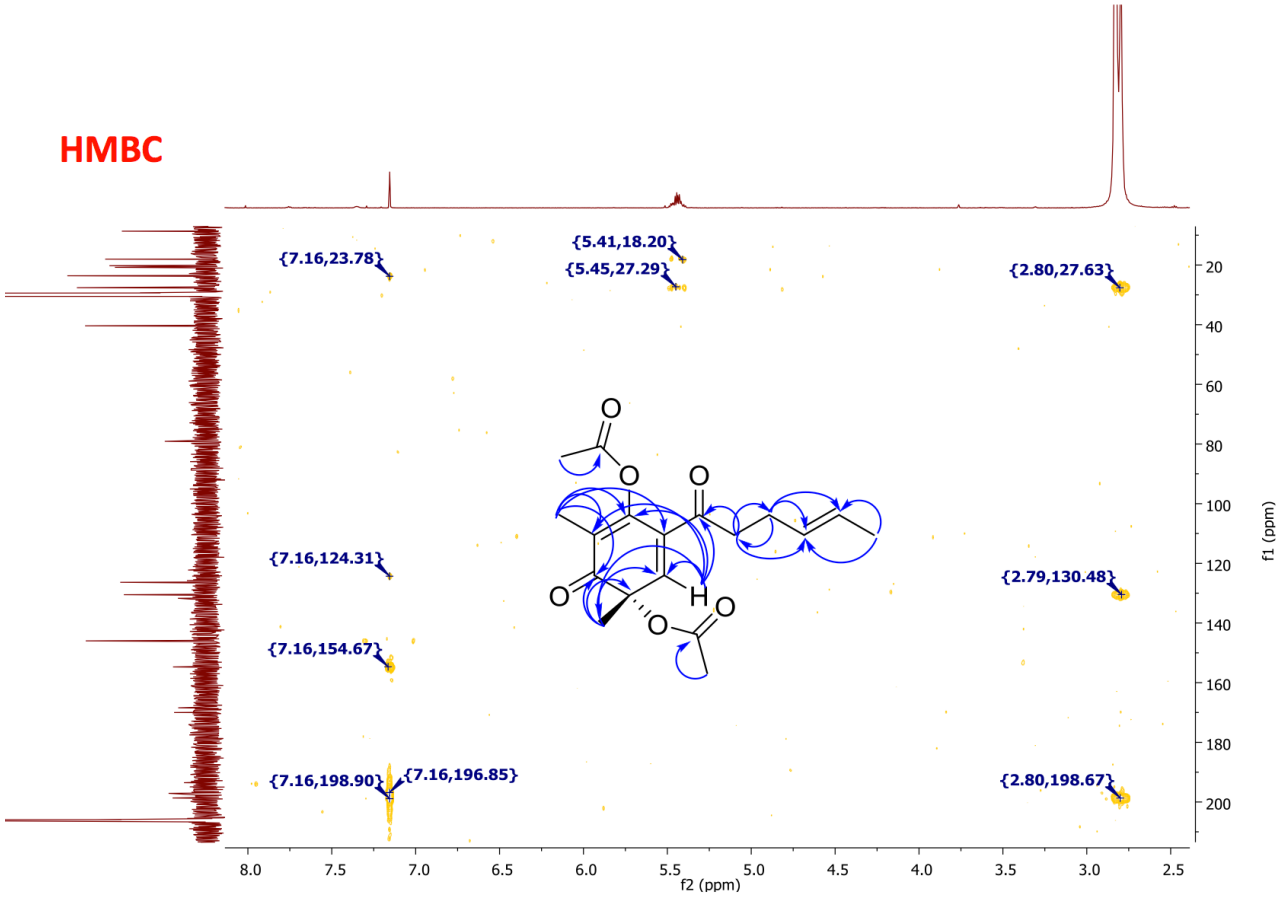
HSQC



HSQC







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