Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Exploration of a biosynthetic access to the shared precursor of the fusicoccane diterpenoid family

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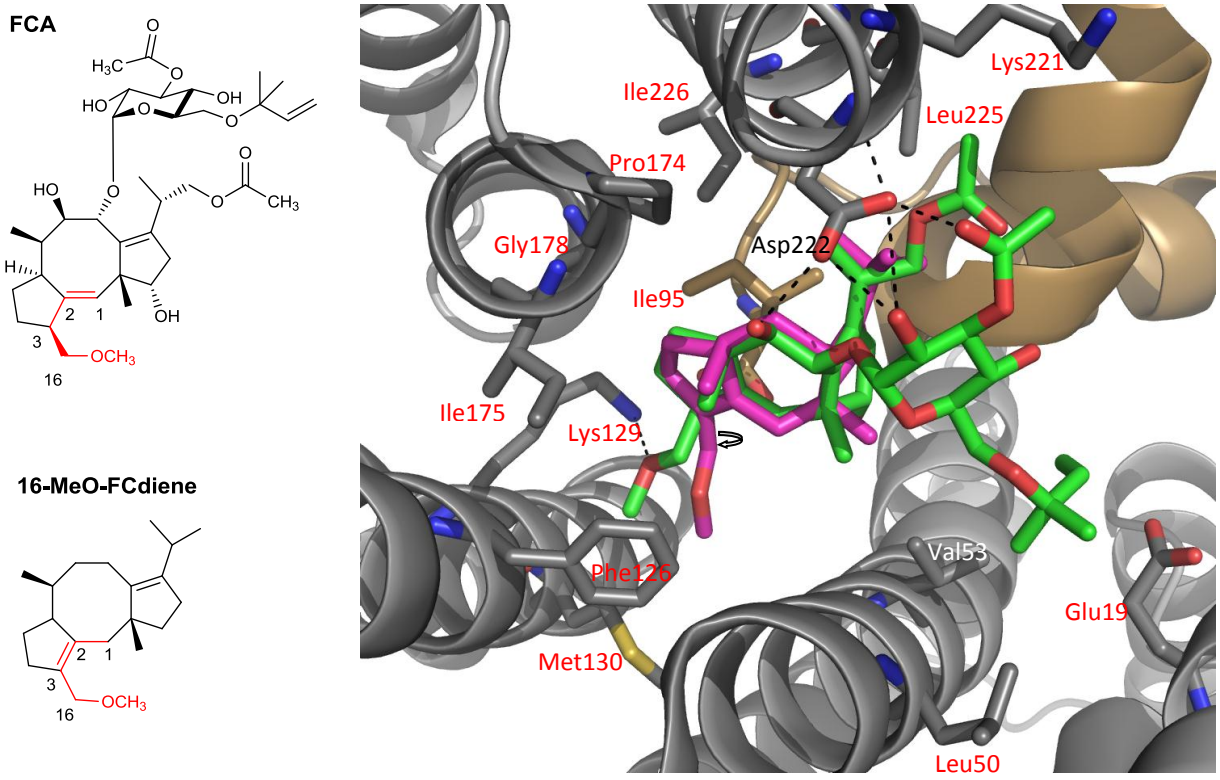


SI F1: Overlay of a modelled structure (Chem3D Pro 11.0, MM2 MD: Molecular Mechanics 2 Molecular Dynamics) of 16-methoxy-fusicoccadiene (16-MeO-FCdiene, carbon backbone in magenta) with an experimental crystal structure of fusicoccin A (FC A, carbon scaffold in green) stabilising the tobacco 14-3-3c (ribbon plot dark grey) interaction to the CT52 fragment of PMA2 (ribbon plot bronze, pdb: 2o98). This underlines the possibility of the double bond isomer fusicoccadiene to be used as basis for generating simple bioactive analogous of known fusicoccanes like FC A or cotylenin A. Essential amino acids for stabilisation are visualised as sticks and are labelled in red, white or black. Oxygen atoms in general are highlighted in red, and nitrogen atoms in blue. Hydrogen atoms are not shown. The arrow describes the free rotation of the σ bond between positions C3 and C16 in 16-MeO-FCdiene which can equalise the varying binding angels between 16-MeO-FCdiene and FCA on this position (ideally an angle of 109.5° instead of 120°).

1 Experimental

1.1 General:

Water for working with RNA was DEPC (Carl Roth GmbH) treated prior to use. For all cloning experiments sterilised millipore water (0.2 µm membrane filter, Sarstedt) and in-house made chemically competent *E. coli* OmniMAX™ 2 T1R cells (SI T1), originally obtained from Invitrogen, were used. Restriction endonucleases were purchased from New England Biolabs. Prior to expression and fermentation, all constructs were verified by sequencing (StarSEQ GmbH). For plasmid isolation and purification the standard alkaline lysis protocol and ethanol precipitation were used.[1]

SI T1: Overview of used microbes in this study.

Organism	**strain**	**genotype**	**usage**	**source**
Escherichia coli	OmniMAX™ 2 T1R	F´ [*pro*AB+ *lac*Iq *lac*ZΔM15 Tn10(tet^R) Δ(*ccd*AB)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D	for all cloning experiments in *E. coli*	Invitrogen
E. coli	BL21-Gold(DE3)	B F$^-$ *omp T hsdS*$_B$($r_B^-m_B^-$) *dcm*$^+$ tet^r *gal* λ(DE3) *endA* Hte	for expression of His_6-GST-Abfs and fermentation of fusicoccadiene by *E. coli*	Agilent Technologies
E. coli	OmniMAX™ 2 T1R[pUC18-*abfs*]	with *E. coli* cloning plasmid pUC18-*abfs*	for propagation of pUC18-*abfs*	this work
E. coli	OmniMAX™ 2 T1R[pETM-30-*abfs*]	with *E. coli* expression plasmid pETM-30-*abfs*	for propagation of pETM-30-*abfs*	this work
E. coli	OmniMAX™ 2 T1R[pDONR221-*abfs*]	with *E. coli* cloning plasmid pDONR221-*abfs*	for propagation of pDONR221-*abfs*	this work
E. coli	OmniMAX™ 2 T1R[pVV214-*abfs*]	with yeast expression plasmid pVV214-*abfs*	for propagation of pVV214-*abfs*	this work

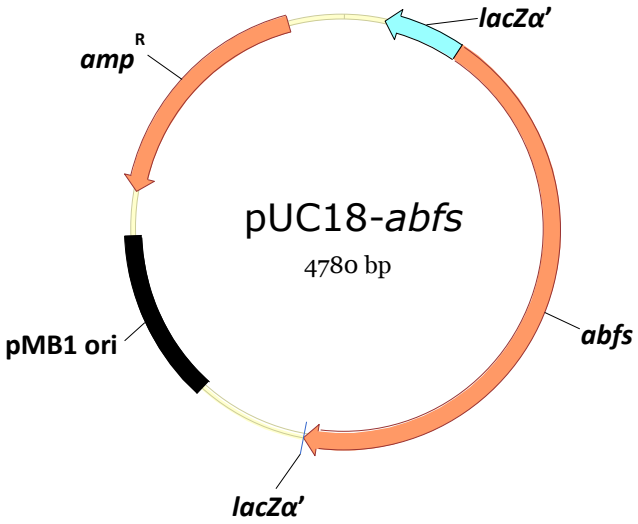
Organism	**strain**	**genotype**	**usage**	**source**
E. coli	BL21-Gold(DE3)[pETM-30-*abfs*]	with *E. coli* expression plasmid pETM-30-*abfs*	for expression of His_6-GST-Abfs and fermentation of fusicoccadiene by *E. coli*	this work
E. coli	DB3.1	F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1	for propagation of empty Gateway vectors containing the ccdB gene encoding for a gyrase inhibitor	Invitrogen
Alternaria brassicicola	UAMH 7474 (Schw.)	wildtype	for isolating the mRNA encoding for a fusicoccadiene synthase gene	University of Alberta Microfungus Collection
Aspergillus nidulans	(Eidam) Winter, DSM 820	wildtype	for fermentation of fusicoccadiene by *A. nidulans*	DSMZ
A. nidulans	(Eidam) Winter [pBEan-gpdA]	with expression plasmid [pBEan-gpdA]	as negative control for fusicoccadiene fermentation by *A. nidulans*	this work
A. nidulans	(Eidam) Winter [pBEan-gpdA: *abfs*]	with expression plasmid pBEan-gpdA:*abfs*	for fermentation of fusicoccadiene by *A. nidulans*	this work
Saccharomyces cerevisiae	CEN.PK2-1C[pRS313-*UPC2.1* + pRS315-*thmgr-S.c.*]	*MATa ura3-52 trp1-289 leu2-3_112 his3Δ 1 MAL2-8^C SUC2* with yeast expression plasmids pRS313-*UPC2.1* und pRS315-*thmgr-S.c.*	for production of increased terpene precursor molecules compared to wild type CEN.PK2-1c	B. Engels *et al.*[2]
S. cerevisiae	CEN.PK2-1C[pRS313-*UPC2.1* + pRS315-*thmgr-S.c.* + pVV214-*abfs*]	With yeast expression plasmids pRS313-*UPC2.1*, pRS315-*thmgr-S.c.* und pVV214-*abfs*	for preparative scale fermentation of fusicoccadiene by *S. cerevisiae*	this work

SI T2: Oligonucleotides used in this study. Melting temperatures (T_M) were calculated with the help of http://www.finnzymes.com/tm_determination.html. In addition, the used annealing temperatures (T_A) are listed.

name	**length (nt)**	**sequence (5'→3')**	**T_m [°C]**	**T_A [°C]**
*abfs*_fw	25	ATGAAATACCAATTTTCCATCATTG	61,5	58
*abfs*_re	24	TTATCAAAGCTTGAGCATCATTAG	59,2	58
*abfs*_SLIC_fw	43	CTTTATTTTCAGGGCGCCATGAAATA CCAATTTTCCATCATTG	79,2	72
*abfs*_SLIC_re	42	GAGTGCGGCCGCAAGCTTTTATCAAA GCTTGAGCATCATTAG	81,8	72
*abfs*_Gate_fw	60	GGGGACAAGTTTGTACAAAAAAGCA GGCTTCAAAAATGAAATACCAATTTT CCATCATTG	82,8	72
*abfs*_Gate_re	50	GGGGACCACTTTGTACAAGAAAGCTG GGTTCAAAGCTTGAGCATCATTAG	82,6	72

1.2 Isolation of the *fs* Gene:

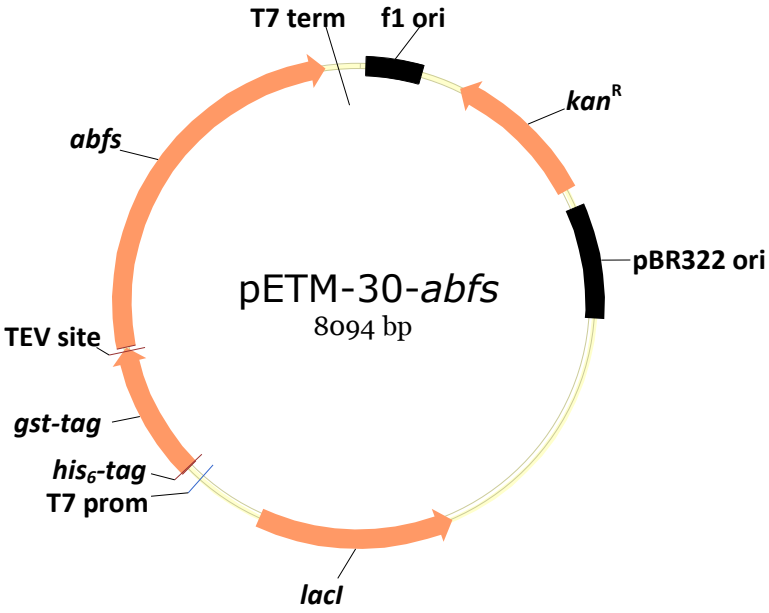
For preparation of *fs* cDNA, *Alternaria brassiccicola* UAMH 7474 (Schw.) (SI T1) was used. The mycelium of the filamentous fungus was grown in V8 medium[3] (20% v/v V8 (Campbell soup Company), 0.075% w/v $CaCO_3$) at room temperature for 14 days in the dark without shaking. Subsequently, 50 mg of the formed mycelia layer was harvested by centrifugation (4000 x g, 4°C), frozen in liquid nitrogen and homogenised with a mortar and pestle. For isolation and purification of total RNA the NucleoSpin plant RNA-Kit (Macherey-Nagel) and the NucleoSpin RNA clean-up Kit (Macherey-Nagel) were used. Single stranded cDNA was obtained by using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol with Oligo $(dT)_{16}$ oligonucleotides (Applied Biosystems) and RNase inhibitor (Applied Biosystems) in a total volume of 20 µL. For synthesis and amplification of the double stranded *abfs* gene the Phusion high fidelity PCR master mix (Finnzymes) and the *abfs* gene (NCBI-Ac.No.: AB465604) specific oligonucleotides *abfs*_fw and *abfs*_re (SI T2) were used in a total volume of 20 µL following the manufacturer's protocols. To establish a stable construct for sequencing and subcloning the amplified DNA fragment was blunt-end ligated into the *Sma*I site of pUC18 (SI F2) enabling blue-white screening in chemically competent *E. coli* OmniMAX™ 2 T1R (SI T1).



SI F2: Vector map of cloning plasmid pUC18-*abfs*. *amp*^{R}: gene coding for ampicillin resistance, *lacZα*': gene encoding for the lacZα fragment of *E. coli* β-galactosidase, *abfs*: sequence encoding for fusicoccadiene synthase from *A. brassicicola*, pMB1 ori: origin of replication obtained from pMB1.

1.3 Overexpression of AbFS and production of fusicoccadiene in *E. coli* BL21-Gold(DE3)

To enable the expression of AbFS with an N-terminal GST-fusion the plasmid pETM-30 was chosen. The *abfs* gene was introduced between the restriction sites *Nco*I and *Hind*III in the multiple cloning site of pETM-30 (SI F3) via SLIC (sequence and ligation independent cloning) MIX[4] by using the oligonucleotides *abfs*_SLIC_fw and *abfs*_SLIC_re (SI T2) with the reaction conditions summarised in SI T3 and SI T4.



SI F3: Vector map showing important features of pETM-30-*abfs* which was used for expression of His_6-GST-AbFS and fermentation of fusicoccadiene by *E. coli*. *kan*^{R}: gene coding for kanamycin resistance, pBR322 ori: origin of replication obtained from pBR322, *lacI*: gene encoding for the lactose repressor LacI, T7 prom: section encoding for T7 RNA polymerase promoter sequence, *his*$_6$*-tag*: section coding for an N-terminal hexahistidine tag, *gst-tag*: section encoding for an N-terminal glutathinone-S-transferase tag, TEV site: section coding for cleavage site for tobacco etch virus protease, *abfs*: sequence encoding for fusicoccadiene synthase from *A. brassicicola*, T7 term: sequence for termination of T7 RNA polymerase transcription, f1 ori: origin of replication for making single stranded plasmid DNA by F1 phage.

SI T3: Pipetting protocol for SLIC-MIX[4] - part I, which is employed for the formation of single stranded, overhanging 5' ends of DNA for cloning.

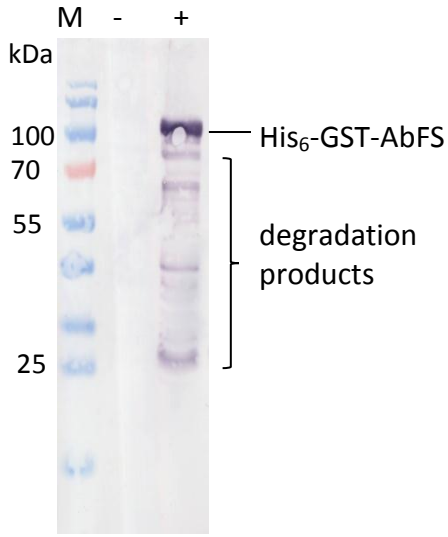
component	**vector**	**PCR product (1 mol eq.)**
DNA	3.8 µL (1 µg)	9 µL (250 ng)
T4 polymerase buffer 5x (NEB)	2 µL	4 µL
BSA 100x (NEB)	0.1 µL	0.2 µL
ET-SSB (1:10) (NEB)	1.6 µL (0.08 µg)	0.8 µL (0.04 µg)
T4 polymerase (NEB)	0.33 µL (0.99 u)	0.1 µL (0.3 u)
H_2O	1.07 µL	5.9 µL
each preparation was incubated for 45 min at 22°C (Eppendorf, Thermomixer comfort) with subsequent addition of 1/10 vol. eq. of dCTP (10mM) to stop the exonuclease digest		

SI T4: Pipetting protocol for SLIC-MIX - part II for *in vitro* recombination of *abfs* into pETM-30 with cloning sites *Nco*I and *Hind*III.

component	**amount/preparation**
Vector solution from SLIC1	2,5 µL
Insert solution from SLIC1	5 µL
T4 ligase buffer 5x (Fermentas)	2 µL
RecA (1:25) (epicentre)	0,1 µL (0,02 µg)
H_2O	0,4 µL
incubation for 1 h at 37°C	

For expression of AbFS and fermentation of fusicoccadiene the sequenced constructs were introduced into *E. coli* strain BL21-Gold(DE3) (SI T1) by heat-shock transformation [5]. Pre-cultures of *E. coli* were grown overnight in 20 mL LB medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, pH 7.4) supplemented with 0,5% w/v glucose, kanamycin (50 µg/mL) and tetracycline (20 µg/mL) at 37 °C with shaking at 150 rpm (Innova 4430, New Brunswick Scientific). For expression and fermentation 500 mL TB medium (1.2% w/w tryptone, 2.4% w/w yeast extract, 0.4% v/v glycerol, 170 mM KH_2PO_3, 720 mM K_2HPO_3) supplemented with kanamycin (100 µg/mL) were used, which were inoculated with 2% (v/v) of

a freshly grown pre-culture and incubated further at 37 °C until an OD_{600} of 0.6 was achieved. Overexpression of the target enzyme was induced by addition of 1 mM IPTG and the cultures were shaken at 18 °C either for 18 h in expression experiments or for 48 h in fermentation experiments. To analyse the expression profile of the transformed BL21-Gold(DE3) (SI T1), the *E. coli* cells were pelleted by centrifugation (4,000 x g, 20 min, 4 °C), resuspended in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 150 mM NaCl, 1 mM $MgCl_2$, 4 mM ß-mercaptoethanol, 1 mM PMSF, 100 µg/mL DNase I and a small amount of lysozyme and were disrupted by sonication. Subsequently, the cell lysate was centrifuged for 30 min at 25,000 rpm and 4 °C (Beckman Coulter J25, rotor JA-25.50) and the soluble supernatant was analysed by standard gel electrophoresis[6]. Because it was not obvious from analysis of the SDS gel if the fusion protein GST-AbFS was solubly expressed, an immunoblot[7] with anti-GST antibodies (goat pAb to GST (AP), abCAM) from the cell lysate was performed (SI F4).

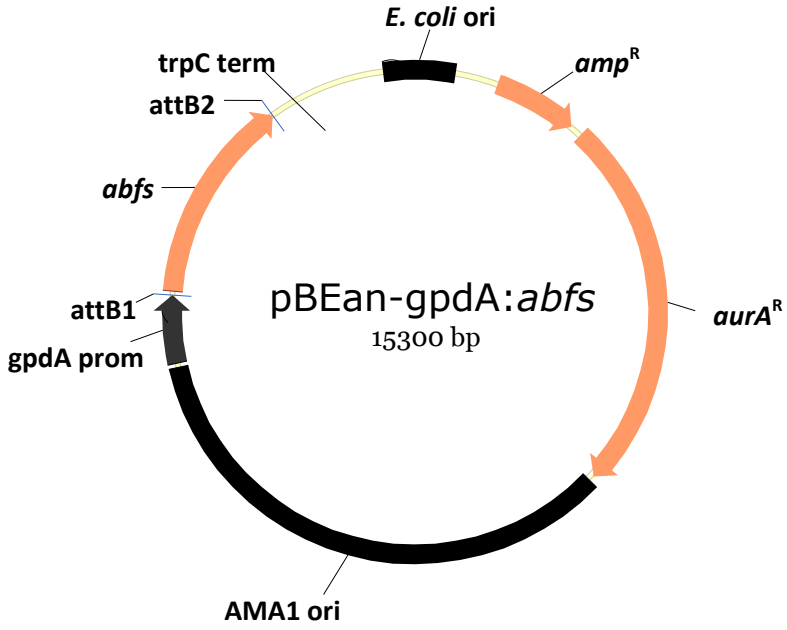


SI F4: Immunoblot showing soluble expression of His_6-GST-AbFS by *E. coli* BL21-Gold(DE3). Degradation of target enzyme was observed. M: PageRuler™ Prestained Protein Ladder (Fermentas), -: soluble fraction of negative control experiment (BL21-Gold(DE3) cells containing pETM-30-*abfs*, but expression was not induced by addition of 1 mM IPTG at an OD_{600} of 0.6), +: soluble fraction of BL21-Gold(DE3) culture transformed with pETM-30-*abfs* and induction of recombinant protein expression by 1 mM IPTG at an OD_{600} of 0.6.

For fermentation analysis, the whole culture broth and cells were extracted with *n*-pentane by shaking, the solvent was evaporated (20°C, 300 mbar) and the residual oil was analysed by GC/MS (chapter 1.7).

1.4. Test for fusicoccadiene production by *A. nidulans*

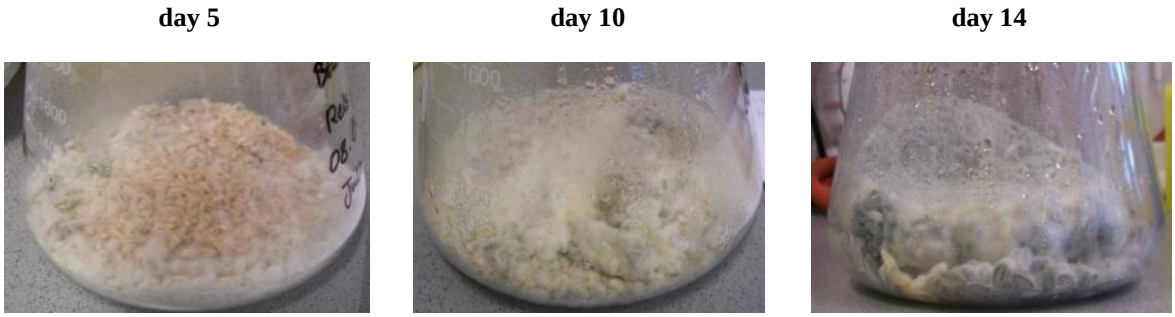
In order to construct a Gateway compatible *A. nidulans* expression vector the expression cassette including the glyceraldehyde-3-phosphate dehydrogenase promoter, the Gateway recombination sites attR1 and attR2 and the trpC terminator were introduced into the backbone of the commercial available *E. coli* - *A. nidulans* shuttle vector pAUR316 (Takara Clontech) in the *Afl*II restriction site to yield pBEan-gdpA. Then the target gene containing vector pBEan-gpdA:*abfs* (SI F5) was generated using the Gateway cloning technology (Invitrogen) in which the plasmid pDONR221 was used in the BP reaction to yield the pENTR vector pDONR221-*abfs*.



SI F5: Vector map of pBEan-gpdA:*abfs* enabling heterologous fermentation of fusicoccadiene by *A. nidulans*. *amp*^{R}: gene coding for ampicillin resistance, *aurA*^{R}: gene encoding for aureobastidin A resistance used for selection of plasmid containing *A. nidulans* protoplasts after transformation, AMA1 ori: origin of replication in *A. nidulans*, gpdA prom: promoter region of glyceraldehyde 3-phosphate dehydrogenase gene from *A. nidulans*, attB1 and attB2: Gateway recombination sites, *abfs*: sequence encoding for fusicoccadiene synthase from *A. brassicicola*, trpC term: terminator region of trpC gene from *A. nidulans* tryptophan biosynthesis, *E. coli* ori: origin of replication in *E. coli*.

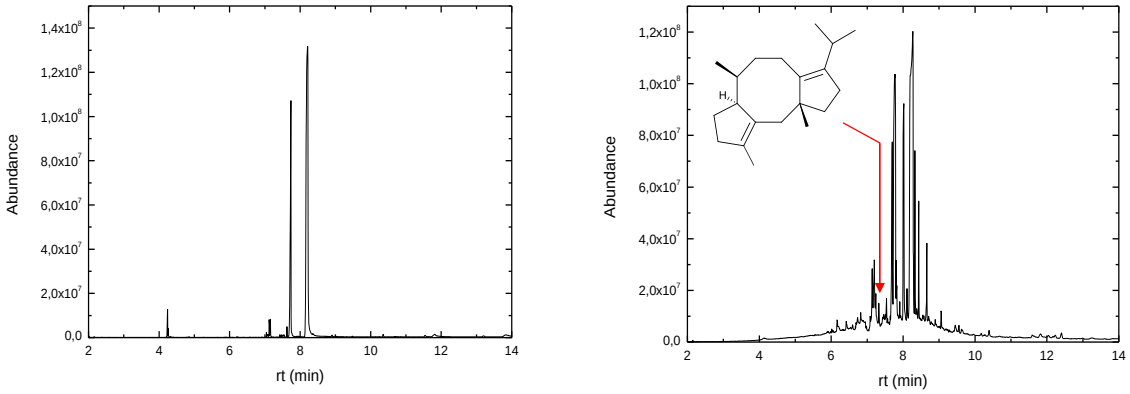
Protoplasts of *A. nidulans* Eidam (Winter) wild type (SI T1) were transformed with vector pBEan-gdpA:*abfs* to get access to a fusicoccadiene producing strain. A negative control harbouring the empty vector pBEan-gpdA was prepared in the same way. For formation of protoplasts and for transformation

procedure, the protocols from Szewczyk *et al.*[8] were used. For generation of protoplasts Panzym Fino G enzyme mix (Begerow, Langenlonsheim) was used, instead of the in reference mentioned Vinoflow FCE (Novo Nordisk). Due to possible clone-to-clone variations three different pBEan-gdpA:*abfs* containing clones were tested for their ability to produce fusicoccadiene. Therefore, a solid-state fermentation on rice (Golden Sun, Parboiled Langkorn Spitzenreis) as nutrient medium was used. 200 g rice was filled in a 2 L Erlenmeyer flask and was mixed with 1 volume equivalent of desalted water (Milli Q). After autoclaving (121 °C, 20 min) the solid nutrient medium was inoculated from a freshly grown *A. nidulans* YG agar plate (0.5% w/v yeast extract, 2% w/v glucose, 0.04% v/v trace element solution[9], 2% w/v agar). Subsequently, the culture was incubated for two to three weeks in the dark at room temperature until the rice was fully overgrown with mycelium (SI F6).



SI F6: Photos from solid-state fermentation of pBEan-gdpA:*abfs* transformed *A. nidulans* on rice showing time dependent growth of mycelia.

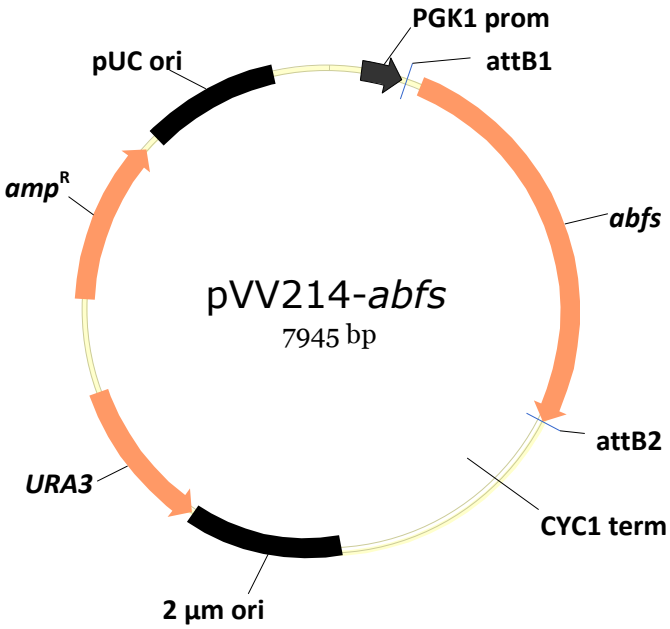
For analysis of fermentation products two different extraction methods were tested. The cultures were lyophilised overnight and were then extracted in a soxhlet apparatus for one day with petroleum ether or the swelled rice was homogenised by a mixer and extracted either with petroleum ether or *n*-pentane by stirring overnight. Prior to GC measurements (chapter 1.7) the solvent was evaporated in a rotary evaporator (20 °C, 300 mbar).



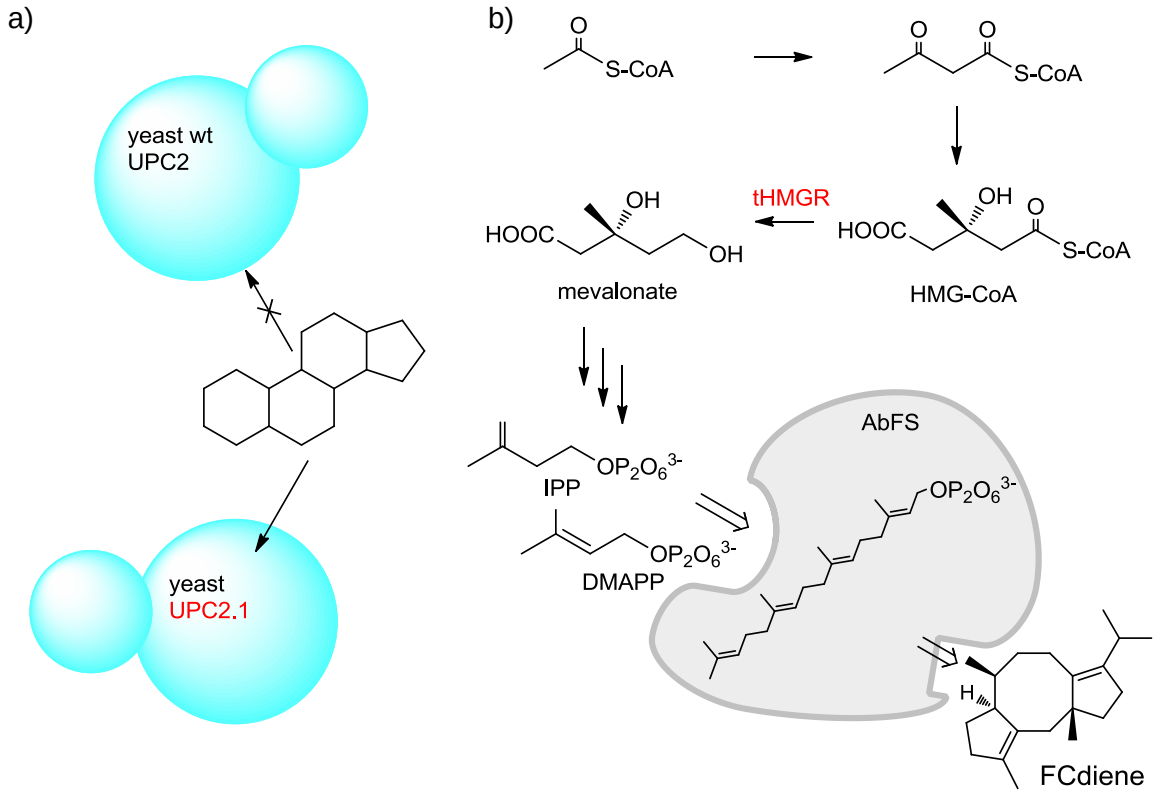
SI F7: Gas chromatograms to compare the crude fermentation extracts of *A. nidulans* transformed with empty vector pBEan-gdpA (left) and transformed with pBEan-gdpA:*abfs* (right) encoding for fusicoccadiene synthase from *A. brassicicola*. Besides a marginal production of fusicoccadiene, the product diversity is increased when fusicoccadiene is produced heterologously.

1.5 Generation of a fusicoccadiene producing *S. cerevisiae* strain and optimisation of fermentation

To enable a fast access to a fusicoccadiene producing strain of *S. cerevisiae* the pre-optimised strain CEN.PK2-1c from Engels *et al.*[2] harbouring the two plasmids pRS313-*UPC2.1* and pRS315-*thmgr-S.c.* (SI T1 and SI F9) was used. In analogy to the *A. nidulans* expression plasmid the yeast expression plasmid pVV214-*abfs* (SI F8) was generated by Gateway cloning technology (Invitrogen).



SI F8: Vector map of pVV214-*abfs* for the preparative production of fusicoccadiene by *S. cerevisiae* CEN.PK2-1c. *amp*^{R}: gene coding for ampicillin resistance, pUC ori: origin of replication in *E. coli* obtained from pUC vector series, PGK1 prom: promoter region of phosphoglycerate kinase 1, attB1/attB2: Gateway recombination sites, *abfs*: sequence encoding for fusicoccadiene synthase from *A. brassicicola*, CYC1 term: terminator region of cytochrome C1, 2 µm ori: 2 micron origin of replication, *URA3*: gene for complementation of uracil biosynthesis which is disturbed in the CEN.PK2-1c wild type strain.



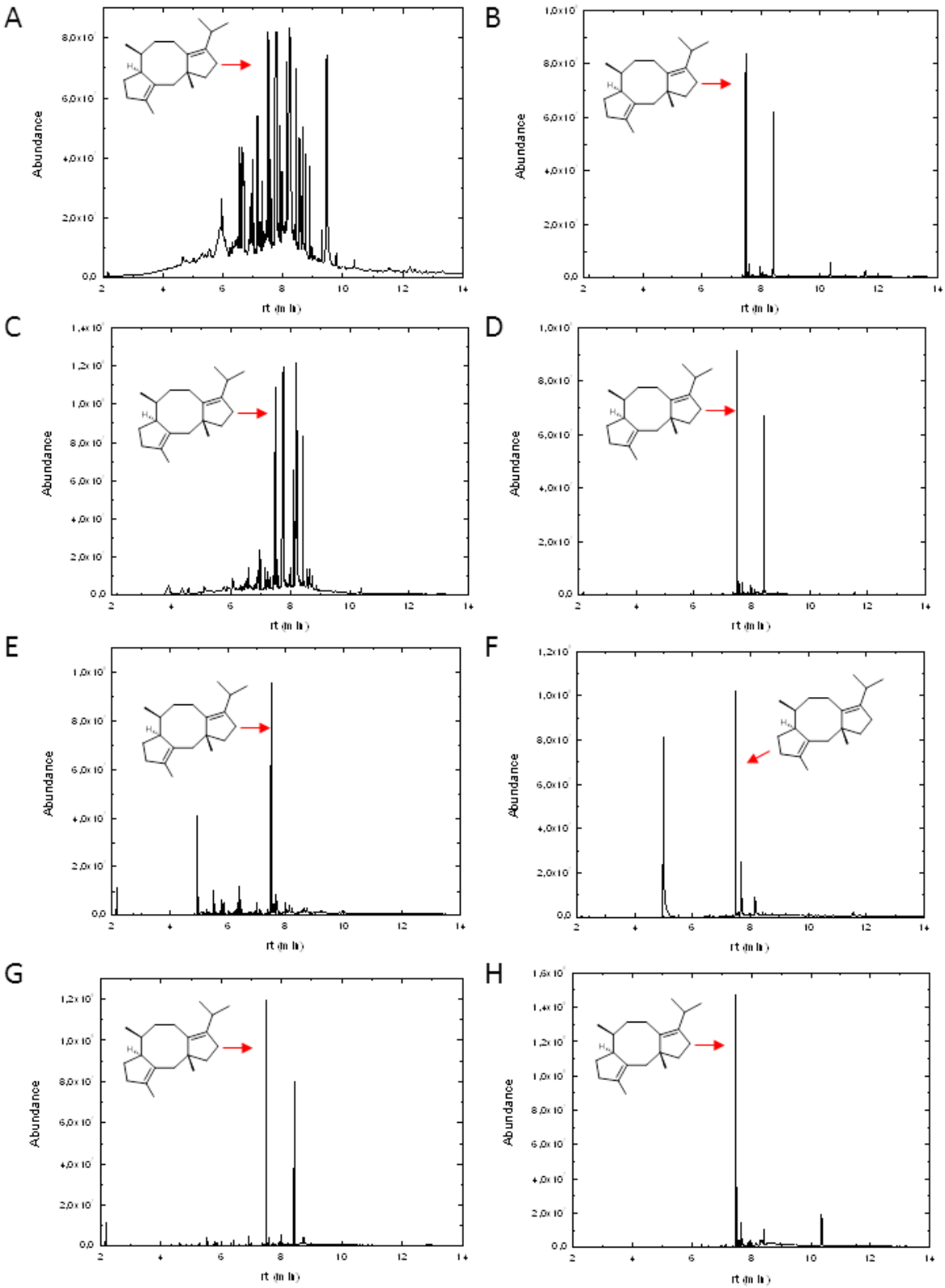
SI F9: Scheme explaining the manipulations made for the yeast fermentation strain CEN.PK2-1c[2] to enable the supply of increased levels of terpene precursor molecules IPP and DMAPP by transformation with pRS313-*UPC2.1* and pRS315-*thmgr-S.c*. a) The modified transcription factor UPC2.1[10] enables the uptake of sterols from nutrient media under fermentation (aerobic) conditions, b) to overcome negative feedback regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, one of the major key enzymes in the mevalonate pathway to produce terpenes) the enzyme is expressed as a truncated variant with missing regulatory domain[11].

For transformation of yeast cells the lithium acetate method[12] was used and the target plasmids including yeast cells were selected on SD minimal medium agar plates (0.67% w/v yeast nitrogen base without amino acids, 1% w/v glucose, 0.005% w/v L-arginine HCl, 0.008% w/v L-aspartic acid, 0.005% w/v L-isoleucine, L-lysine 0.005% w/v, 0.002% w/v L-methionine, 0.005% w/v L-phenylalanine, 0.01% w/v L-threonine, 0.005% w/v L-tyrosine, 0.014% w/v L-valine, 0.001% w/v adenine, 0.002% w/v L-tryptophan, 2% w/v agar). Different fermentation and extraction methods (summarised in SI T5) were tested prior to injection of crude extracts into the GC/MS system (chapter 1.7). In all experiments yeast fermentation culture was grown for 48 h, 30 °C and 130 rpm (Innova 4430, New Brunswick Scientific). Best yields were obtained by using *n*-pentane-washed and sterilised C18 silica gel[2] (Supelco, 5 g/L culture medium) for *in situ* product adsorption during fermentation. Best purity of crude extract was obtained by separation

of the solids from the fluid by filtration. The cell paste with embedded C18 phase was eluted with *n*-pentane overnight (130 mL *n*-pentane per solid constituent parts of 1 L culture medium) by stirring (see entry n, SI T5). The product content for entry n (SI T5) was determined by NMR and yield was calculated as 15 mg/L culture medium (chapter 1.9.1).

SI T5: Overview of different fermentation and extraction conditions to optimise fusicoccadiene purity of crude extracts from yeast cultures. pe: petroleum ether, ch: *cyclo*-hexane, *n*-p: *n*-pentane, d: dry (freeze-drying for sublimation of water from filtrated cell-C18-silica gel mixture), w: wet (no freeze-drying used), *: media additive affected growth of yeast negatively, **: *n*-pentane washed C18 silica gel, n. d.: not determined.

entry	**extraction**	**Media additive**	**purity**
a	*in situ*	*n*-dodecane	n.d.
b	*in situ*	*n*-undecane	n.d.*
c	*in situ*	*n*-decane	n.d.*
d	*in situ*	*n*-nonane	n.d.*
e	*in situ*	*n*-octane	n.d.*
f	*in situ*	*n*-heptane	n.d.*
g	soxhlet, pe, d	C18-silica gel	see SI F10 A
h	stirrer, pe, d	C18-silica gel	see SI F10 B
i	stirrer, ch, w	C18-silica gel	see SI F10 C
j	stirrer, pe, w	C18-silica gel	see SI F10 D
k	stirrer, *n*-p, w	Amberlite XAD16	see SI F10 E
l	stirrer, *n*-p, w	without	see SI F10 F
m	stirrer, *n*-p, w	C18-silica gel	see SI F10 G
n	stirrer, *n*-p, w	C18-silica gel **	see SI F10 H



SI F10: Overview of obtained crude extracts by using different extraction methods summarised in SI T5.

1.5.1 Scale up of fusicoccadiene fermentation in yeast

For scaling up the fusicoccadiene fermentation, a stainless steel fermenter (Biostat C, B. Braun) with 25 L buffered YPD medium[2] was used. pH and temperature were kept constant at pH 7.7 and 30 °C. The culture was stirred for 48 h at 600 rpm and a total volume of 18 mL anti-foam was added.

1.6 Purification of fusicoccadiene from yeast fermentation broth

1.6.1 Argentation silica gel chromatography

To produce Ag(I) impregnated silica gel, 5 g $AgNO_3$ were dissolved in 120 mL desalted water in an aluminium foil wrapped 250 mL Erlenmeyer flask and stirred for 10 min at RT. 100 g of silica gel (silica gel 60, Sigma-Aldrich) were suspended in 120 mL acetone in an aluminium foil wrapped 500 mL round bottom flask. After adding the $AgNO_3$ solution to the suspended silica gel the flask was flushed with argon and sealed with a septum. The mixture was stirred overnight at room temperature and was washed with 200 mL acetone, diethyl ether and *n*-pentane. The white colored silver impregnated silica gel was dried at 300 mbar and 50 °C for 4 h on a rotary evaporator and under high vacuum overnight. To remove residual water the prepared silica gel was washed three times in 50 mL dry toluene with subsequent co-evaporation on a rotary evaporator at 75 mbar and 40 °C. For storage the silver impregnated silica gel was filled into an aluminium wrapped Schlenk-tube which was flushed with argon and kept dry at room temperature. For argentation chromatography[13] 30 g of the Ag(I) impregnated silica gel were suspended in *n*-pentane and filled into an aluminium wrapped glass column (1.5 cm diameter). Product from 5 L fermentation culture was loaded onto the pre-packed Ag(I) silica gel column and was washed with 200 mL *n*-pentane. The isolation of product-containing fractions was accomplished by elution with 150 mL *n*-pentane/diethyl ether (95:5). Collected fractions had a volume of 15 mL. For identification of fusicoccadiene containing fractions a silica gel thin layer chromatography (TLC Silica gel 60 F_{254}, Merck) with *n*-pentane as fluid phase was run, wherein olefins were detected in an iodine chamber (R_F 0.9). Combined fractions were analysed by GC/MS (chapter 1.7), high resolution mass spectrometry (chapter 1.8).

1.6.2 Flash silica gel chromatography

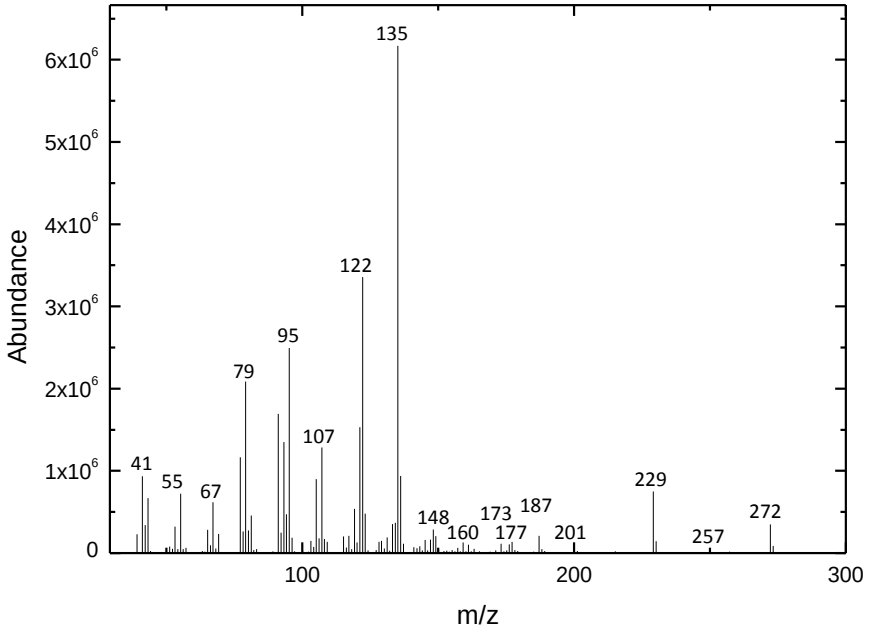
For each silica gel column the crude extract of a 5 L fermentation culture (see chapter 1.5) was dissolved in 5 mL *n*-pentane and was loaded on a in *n*-pentane pre-packed silica gel (silica gel 60, Sigma-Aldrich) column (1.5 cm diameter, 35 cm height). Silica gel filtration columns were washed with 200 mL *n*-pentane to elute nonpolar substances. For product isolation 15 mL fractions were collected in test tubes and were checked by silica gel thin layer chromatography (see chapter 1.6.1) for their existence of

fusicoccadiene. Combined fractions were analysed by GC/MS (chapter 1.7), high resolution mass spectrometry (chapter 1.8) and NMR spectroscopy (chapter 1.9).

1.7 GC/MS measurements

GC/MS measurements were performed on a Hewlett Packard 6890 Series GC System with Hewlett Packard 5973 Mass Selective Detector and HP 19091M-102 HP-5 (325°C max) Trace Analysis Column with 5% phenyl methyl siloxane.

Pressure: 2,100 mbar, flow rate: 105 mL/min, temperature profile: in total 14 min run time, 50°C/2 min, 40°C/min to 300°C, 300°C/6.25 min. Fusicoccadiene was detected at a retention time of 7.48 min. The characteristic fragmentation pattern of isolated fusicoccadiene is represented in SI F11 and is in agreement with previously obtained data from Toyomasu *et al.*[14] and Minami *et al.*[15].



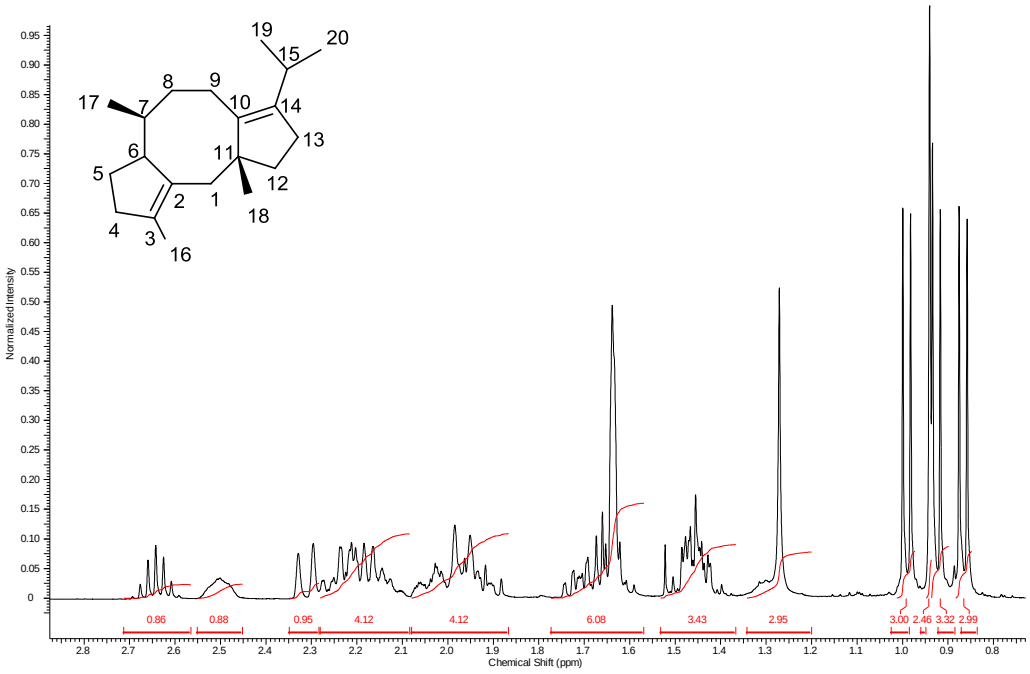
SI F11: Characteristic fusicoccadiene fragmentations obtained by GC/MS measurements.

1.8 High resolution mass spectrometry

High resolution mass spectrometry was performed on an Accela HPLC system with Hypersil Gold column (1 x 50 mm, particle size 1.9 µm) coupled to LTQ Orbitrap. As method for ionisation, electron impact (EI) was used. Detected masses had m/z range of 150 to 2,000.

1.9 NMR spectroscopy

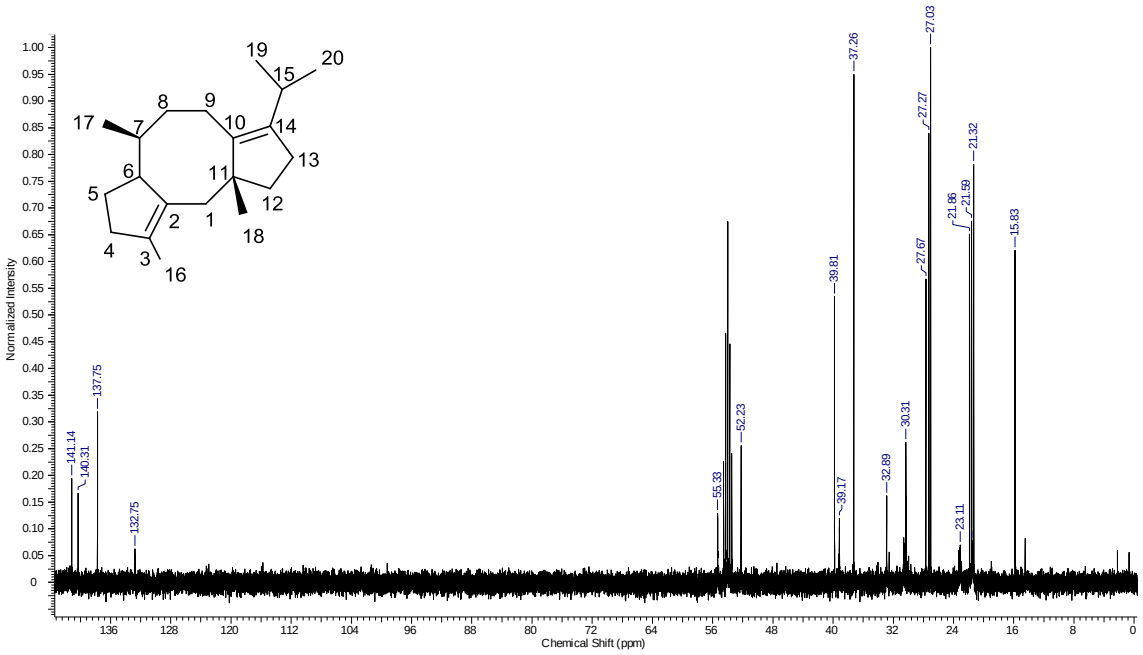
Prior to NMR measurements either the obtained crude extracts of 1 L yeast culture (SI T4) or 16 mg of purified FCdiene were diluted in 400 µL dichloromethane - d_2. ^{1}H-NMR spectra (SI F12) were measured at 400 mHz and ^{13}C-NMR spectra (SI F13) were measured at 101 mHz in a Varian Mercury 400 Spectrometer. The ^{1}H- and ^{13}C-Signals for fusicoccadiene were mapped with the help of gCOSY (SI F14) and gHSQC (SI F15).



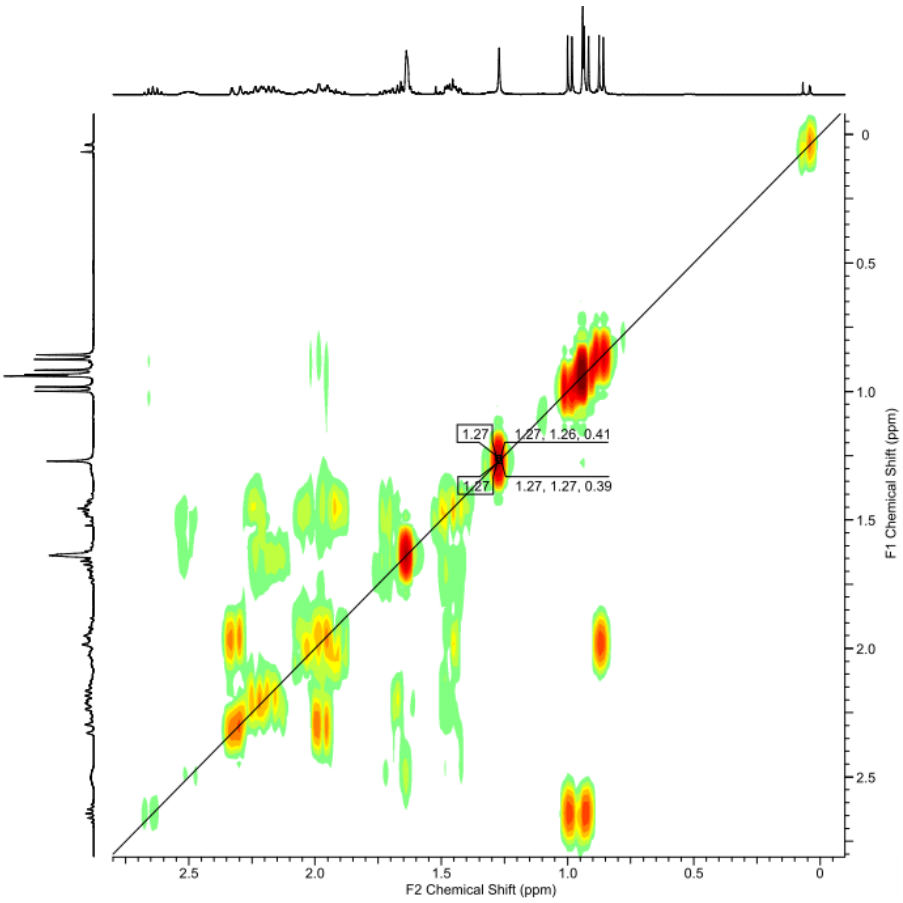
SI F12: ^{1}H-NMR (400 MHz) spectrum of fusicocca-2,10(14)-diene in dichloromethane-d_2. Signals corresponding to fusicoccadiene are also presented in SI T6.

SI T6: By gCOSY and gHSQC mapped ^{1}H-NMR data for fusicocca-2,10(14)-diene. The chemical shifts (δ) are in agreement with fusicoccadiene in dichloromethane-d_2 as solvent. ppm: parts per million, J: coupling constant, s: singlet, d: duplet, q: quartet, spt: septet, m: multiplet, br.: broad, AB: A-B system. Obtained signals agree with results from Kato *et al.*[16].

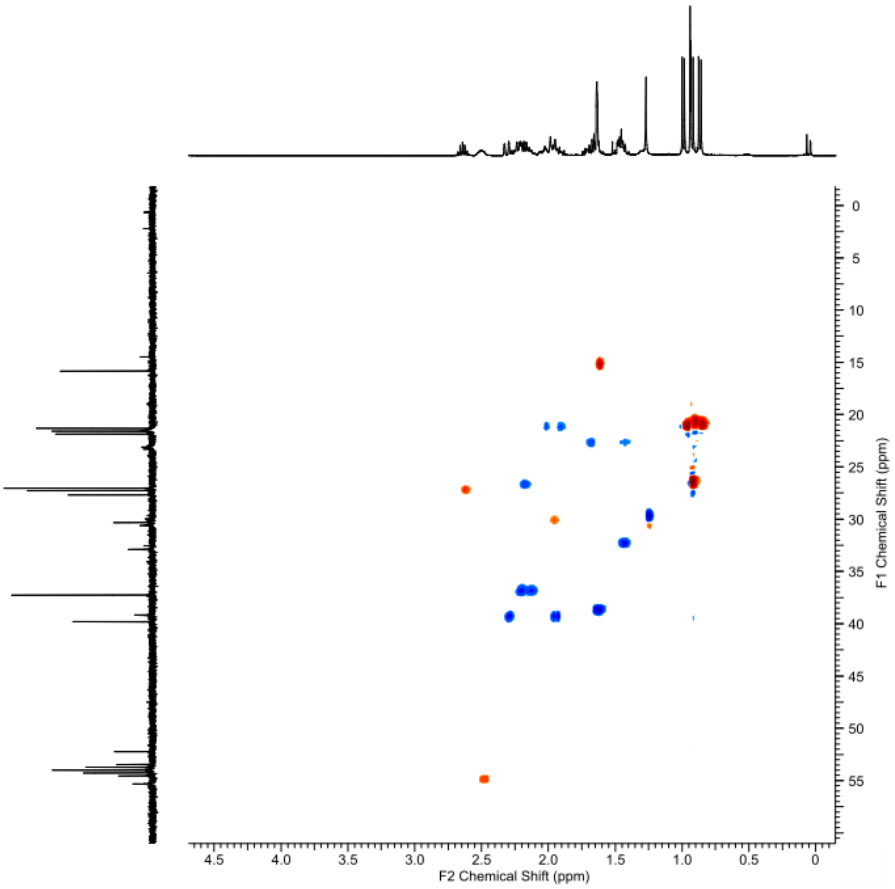
δ [ppm]	multiplicity	J [Hz]	position	number of H
0.87	d	7	H-17	3
0.93	d	6,8	H-19/20	3
0.94	s	-	H-18	3
0.99	d	6,8	H-19/20	3
1.46	m	-	H-5/8	3
1.64	br. s	-	H-16	3
1.67	m	-	H-5/12	3
1.95	ABq	13,2	H-1	1
2.04	m	-	H-7/9	3
2.20	m	-	H-4/13	4
2.31	ABq	13,2	H-1	1
2.50	m	-	H-6	1
2.64	spt	6,8	H-15	1



SI F13: ^{13}C-NMR (101 MHz, DCM-d_2) spectrum of fusicocca-2,10(14)-diene: δ [ppm] 15.83 (C16, CH_3), 21.32 (C19/20, CH_3), 21.54 (C17, CH_3), 21.59 (C19/20, CH_3), 21.86 (C9, CH_2), 23.11 (C5, CH_2), 27.03 (C18, CH_3), 27.27 (C13, CH_2), 27.67 (C15, CH), 30.31 (C7, CH), 32.89 (C8, CH_2), 37.26 (C4, CH_2), 39.17 (C12, CH_2), 39.81 (C1, CH_2), 52.23 (C11, C), 55.33 (C6, CH), 132.75 (C3, C), 137.75 (C14, C), 140.31 (C2, C), 141.14 (C10, C). The mapping of the signals to corresponding C atoms was achieved with the help of the 2D spectra (SI F14 and 15) and partially with literature data.[16]



SI F14: gCOSY spectrum of fusicocca-2,10(14)-diene.



SI F15: gHSQC spectrum of fusicocca-2,10(14)-diene.

1.9.1 Correction of resulting weights from crude yeast culture extracts (table SI T5) by ^{1}H-NMR spectroscopy

To minimise loss of volatile product by downstream processing the *n*-pentane was in many experiments not fully evaporated from the culture broth. To quantify the fermentation efficiency of yeast employing crude extracts, the resulting weights were corrected by ^{1}H-NMR spectroscopy. To estimate the relative amount of *n*-pentane compared to the amount of fusicoccadiene and unknown by-products the surface integral I_F of the C19/C20 protons of fusicoccadiene at a chemical shift of δ 0.99 ppm was divided through the surface integral I_P of the *n*-pentane signal at a chemical shift of 1.31 ppm. N_F (H) and N_P (H) give the number of protons in the given signal. The molar ratio n_F/n_P was calculated from:

$$\frac{n_F}{n_P} = \frac{I_F/N_F\ (H)}{I_P/N_P\ (H)}$$

With m = nM, the molar masses M_P and M_F and m for the resulting-weights of the determined crude extract the fraction of the *n*-pentane in the total weight is calculated using the following formula:

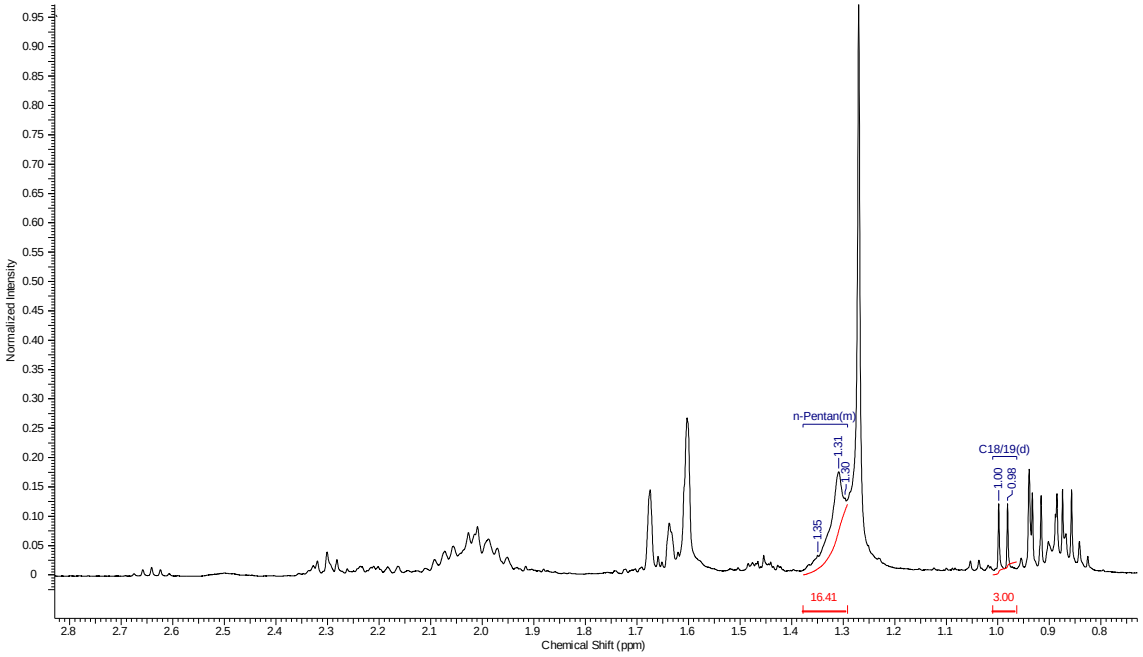
$$\frac{m_P}{m} = \frac{m_P}{m_F + m_P} = \frac{1}{\frac{m_F}{m_P} + 1} = \frac{1}{\frac{n_F \cdot M_F}{n_P \cdot M_P} + 1}$$

As an example a model calculation is given below:

$$\frac{m_P}{m} = \frac{1}{\frac{272\ g/mol}{72\ g/mol} \cdot 0{,}37 + 1} = 0{,}42$$

The corresponding NMR spectrum is shown in SI F16. With a resulting weight of the crude extract of 27 mg the corrected weight was calculated to:

$$m' = 27\ mg - 0{,}42 \cdot 27mg = 15{,}7\ mg$$



SI F16: [1]H-NMR spectrum of a representative 1 L fermentation in shaking flasks which was used for correction of resulting weights of crude extracts (chapter 1.9.1). The H19/20 signal is obtained by 3 H of fusicoccadiene, whereas the *n*-pentane signal is obtained by 6 H of the solvent *n*-pentane used for extraction.

2. References

[1] H. C. Birnboim, J. Doly, *Nucleic Acids Res.*, 1979, **7**, 1513.
[2] B. Engels, P. Dahm, S. Jennewein, *Metab. Eng.*, 2008, **10**, 201.
[3] S. L. MacKinnon, P. Keifer, A. A. Ayer, *Phytochem.*, 1999, **51**, 215.
[4] S. Kushnir, U. Sundermann, F. Schulz, Asian J. Biotech., **accepted**.
[5] H. Inoue, H. Nojima, H. Okayama, *Gene*, 1990, **96**, 23.
[6] U. K. Laemmli, *Nature*, 1970, **227**, 680.
[7] J. Renart, J. Reiser, G. R. Stark, *Prot. Natl. Acad. Sci. USA*, 1979, **76**, 3116.
[8] E. Szewczyk, T. Nayak, C. E. Oakley *et al.*, *Nat. Protoc.*, 2006, **1**, 3111.
[9] W. Vishniac, M. Santer, *Bacteriol. Rev.*, 1957, **21**, 195.
[10] B. S. Davies, H. S. Wang, J. Rine, *Mol. Cell. Biol.*, 2005, **25**, 7375.
[11] K. A. Donald, R. Y. Hampton, I. B. Fritz, *Appl. Environ. Microbiol.*, 1997, **63**, 3341.
[12] D. C. Amberg, D. J. Burke, J. N. Strathern, *Methods in Yeast Genetics*, 2005 ed., Inglis, J., New York, **2005**.
[13] C. M. Williams, L. N. Mander, *Tetrahedron*, 2001, **57**, 425.
[14] T. Toyomasu, M. Tsukahara, A. Kaneko *et al.*, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 3084.
[15] A. Minami, N. Tajima, Y. Higuchi *et al.*, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 870.
[16] N. Kato, C.-S. Zhang, T. Matsui *et al.*, *J. Chem. Soc. Perkin Trans. 1*, 1998, 2473.