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Catalytic Role of the C-Terminal Domains of a Fungal Non-Reducing Polyketide Synthase

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Electronic Supplementary Information

1. Experimental Details

1.1 Construction of pASPKS1-ΔR

The MOS (3-methylorcinaldehyde synthase) expression vector pASPKS1 contains two unique restrictions sites, *SgrA*I before the R domain and *EcoR*I after the MOS sequence. These sites were used to cut the R domain out. A new stop codon was then introduced by ligation of a synthetic DNA linker into the vector. The linker was created by annealing two oligonucleotides with overhangs to match the restriction sites and three stop codons, one in each reading frame (Figure S1) to give pASPKS1-ΔR.

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5′ CCGGTGACTGAGTAGAGCAGGCTGACAATA 3′
     3′ ACTGACTCATCTCGTCCGACTGTTATTTAA 5′
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Figure S1. Synthetic DNA linker; bold restriction overhangs, red stop codons.

Ligation was done using a vector:insert (w/w) ratio of 1:7 and T4 DNA ligase (Invitrogen) at 10 °C for 64 h. Sequencing confirmed removal of the R domain and introduction of the synthetic DNA linker.

1.2 Construction of *pASPKS1-ΔMR*

Genomic DNA from *Acremonium strictum* was prepared using a GenElute Plant Genomic DNA Miniprep kit (SIGMA) according to the manufacturer's instructions. The SAT to ACP encoding region of *ASpks1* was amplified from gDNA using primers 5' CACCATGGCAGCTCATGGGCAAAC 3' and 5' CAGGTGCGTGGGAGATTCGTGTAAGC 3' and KOD Hot Start DNA Polymerase (Novagen). PCR contents and program according to the manufacturer's instructions and the following: number of cycles 35, annealing temperature 60 °C, elongation time 2 min 30 s. The resulting PCR fragment was cloned into pENTR/D-TOPO using a pENTR Directional TOPO Cloning Kit (Invitrogen) and transformed into TOP10 *E. coli* chemical competent cells (Invitrogen). Sequencing (Cogenics) confirmed that no errors had been introduced. Gateway LR *in vitro* recombination (Invitrogen) was then used to transfer the truncated *ASpks1* into the fungal expression vector pTAex3GSA2 giving pASPKS1-ΔMR.

1.3 Fungal transformation, fermentation of transformants and extraction

1.3.1 Transformation into *Aspergillus oryzae*.
A spore suspension (100-200 µL) of *A. oryzae* strain M-2-3 was spread onto DPY agar plates incubated at 25°C for 3-5 days. Tween 80 (0.01 %, 10 ml) was added and the spores scraped off with a sterile loop. The liquid was collected and centrifuged (10 000 × g, 5 minutes), the supernatant removed and the crude spore preparation resuspended in water (1 mL). This spore suspension was used to inoculate DPY growth medium (100 mL) which was then incubated at 25 °C with shaking (200 rpm, 24 - 48 h). Mycelia were collected by filtration through sterile Miracloth, washed with 0.8 M sodium chloride, centrifuged (10 000 × g, 10 min) and the supernatant was discarded. Filter-sterilised protoplasting solution (20 mL) was added to the pellet which was resuspended thoroughly by vortexing. The tube was incubated at room temperature, with gentle mixing on a rotator. Sufficient protoplast formation was checked by microscope after 1 h and every 30 min thereafter. The protoplasts were released from hyphal strands by gentle pipetting with a large wide-bore pipette (5 mL) and filtered through sterile Miracloth to remove the hyphae. The filtrate was gently centrifuged. The protoplasts were washed with 0.8 M NaCl and with Solution I. The concentration of protoplasts was determined by using a haematocytometer (Fisher) and the protoplasts resuspended in Solution I to give 1-9 × 10^7 protoplasts/mL. The protoplasts were stored on ice.

DNA (5-10 µg, 10 µL maximum) to be transformed into the fungus was added to the protoplast suspension (100 µL) and incubated on ice (2 min). Solution II (1 mL) was added dropwise, then incubated at room temperature (20 min). Czapek-Dox agar (Oxoid) in sorbitol (1 M; 5 mL) was added to the transformation mixture and overlaid onto Czapek-Dox agar with sorbitol (1 M; 5

mL) plates. The plates were incubated at 25 °C. Putative transformants were picked using a sterile toothpick and transferred to Czapek-Dox agar plates. Two rounds of selection on minimal Czapek-Dox media were used to ensure that bone-fide *argB* transformants were selected. Genuine *argB* transformants were then grown as a confluent lawn on DPY agar for spore production.

Note that it is the usual observation that not all transformants are biosynthetically active - much depends on the location and mode of integration into the genome and this often happens in an unpredictable way in fungi. Thus it is normal for at least 12 transformants to be selected for further chemical analysis.

1.3.2 Fermentation of transformants and extraction

Spore suspensions (200 µL) obtained from transformants was inoculated into starch medium (100 mL). The flasks were incubated at 25 °C with shaking at 200 rpm for 7 days. The culture broth was acidified using 2M hydrochloric acid until the solution reached pH 3.0 and was put back on the shaker for 30 min. The mycelia was homogenised using a hand-held kitchen blender and filtered. The filtrate was extracted three times with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$ and the ethyl acetate was evaporated at reduced pressure. The residue of this crude extract was dissolved in methanol to 10 mg/mL and analysed by LCMS.

1.4 LCMS analysis of transformants

LCMS analysis was performed using a Waters HPLC system sampling from a Waters 2767 autosampler. Detection was achieved by uv between 200 and 600 nm using a Waters 2998 diode array detector, and by simultaneous electrospray mass spectrometry in ES^+ and ES^- modes using a Waters Quattro-Micro mass spectrometer detecting between 100 and 600 *m/z* units. Chromatography was achieved using a Phenomenex LUNA column (5 µm, C_{18}, 100 Å, 4.6 × 250 mm) equipped with a Phenomenex Security Guard precolumn (Luna C_5 300 Å) and one of the following two solvent gradients.

Method 1: A, H_2O + 0.05% formic acid; B, MeOH + 0.045% formic acid; (0 - 5 min 25% B, 5 - 51 min 25% - 95% B, 51 - 53 min 95% B, 53 - 55 min 95% - 25% B, 55-60 min 25% B; 1 mL/min).
Method 2: A, H_2O + 0.05% formic acid; B, MeOH + 0.045% formic acid; (0-13 min 25% - 95% B, 13 - 15 min 95% B, 15 - 17 min 95% B - 25% B, 17 - 20 min 25% B; 1 mL/min).

Retention times (Method 2): Triacetic acid lactone, 7.9 min; orsellinic acid, 12.4 min; 3-methyl orsellinic acid, 13.8 min; 3-methylorcinaldehyde, 15.1 min. Compound identities were confirmed on the basis of mass, fragmentation pattern, UV/VIS spectra and by comparison with standard samples.

1.5 Ethionine feeding studies

A. oryzae (MOS transformant) spores were used to innoculate 2 × 100 mL glucose medium in 500 mL conical flasks. The cultures were grown with shaking at 28 °C for 3-4 days, when the mycelium was drained and the medium exchanged for 100 mL starch medium to induce expression of *ASpks1* under control of the *AmyB* promotor. 5 mM Ethionine was added to one flask and incubation continued for a further 5 days. Extraction was carried out as described in 1.2.2 and the crude extract was resuspended to a final concentration of 10 mg/mL in methanol. The methanolic solution was diluted 1:1 with water and analysed using LC/MS.

1.6 Media and Solutions

DPY Agar:	Dextrin (20 mg/mL), polypeptone (10 mg/mL), yeast extract (5 mg/mL), potassium dihydrogen phosphate (5 mg/mL), agar (15 mg/mL)
DPY medium:	Dextrin (20 mg/mL), polypeptone (10 mg/mL), yeast extract (5 mg/mL), potassium dihydrogen phosphate (5 mg/mL)
Protoplasting Solution:	Glucanase (20 mg/mL) plus Driselase (10 mg/mL) (Interspex Products) in 0.8 M NaCl.
Solution I:	0.8 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5.
Solution II:	60 % PEG 3350 (Sigma), 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5.
Starch Medium:	Starch (20 g), polypeptone (10 g), distilled water (900 mL), solution A (50 mL), solution B (50 mL)
Glucose Medium	Glucose (20 g), polypeptone (10 g), distilled water (900 mL), solution A (50mL), solution B (50 mL)
Solution A:	Sodium nitrate (40 g/L), potassium chloride (40 g/L), magnesium sulfate heptahydrate (10 g/L), iron sulfate hepthydrate (0.2 g/L)
Solution B:	Potassium phosphate (20 g/L)

1.7 Metabolite Quantification

Standard solutions of 3-methyl orsellinic acid, 3-methyl orcinaldehyde, orsellinic acid and triacetic acid lactone were prepared in MeOH, and these were used to prepare dilution series between 100 µg/mL and 100 ng/mL. Aliquots of 50 µL were injected into the LCMS system described above and uv and single ion chromatogram traces were integrated and used to prepare linear plots of concentration *vs* integral. For additional sensitivity the Waters QM mass spectrometer was used in MS/MS mode detecting fragment ions of the analyte under investigation.

2. Splicing of intron 2 in *Acremonium strictum* WT and *Aspergillus oryzae* heterologously expressing MOS

A. strictum WT and *A. oryzae* pASPKS1 were grown in their respective liquid cultures for 144 h to allow for maximum metabolite production and therefore mRNA concentration. In a one-step process cDNA was generated and amplified using PCR. The PCR primers spanned across the region that contained the intron. The 647 bp PCR products from *A. strictum* WT and the *A. oryzae* expression clone were cloned into the pGEM-Easy TOPO vector and transformed into *E. coli*. Ten colonies containing the *A. strictum* WT intron clone and one colony containing the *A. oryzae* expression intron clone were selected at random for sequencing. As expected the *A. oryzae* expression intron clone removed the stop codon with the introns. Two of the *A. strictum* wild-type intron clones left the stop codon when the intron was spliced. One *A. strictum* wild-type intron clone did not remove the intron at all, this could be due to a small amount of gDNA contamination in RT-PCR or could be another variation in the way this intron is spliced. The remaining seven *A. strictum* clones removed the stop codon with the entire intron (Figure S2).

Although no further sequencing of other *A. oryzae ASpks1* mRNA transcripts has been done, it is observed that *A. oryzae* pASPKS1 clones only ever produce 3-methylorcinaldehyde while the corresponding 3-methylorsellinic acid is not detected. There is thus no evidence that *A. oryzae* can process the intron in the alternative manner which would leave the stop codon in frame.

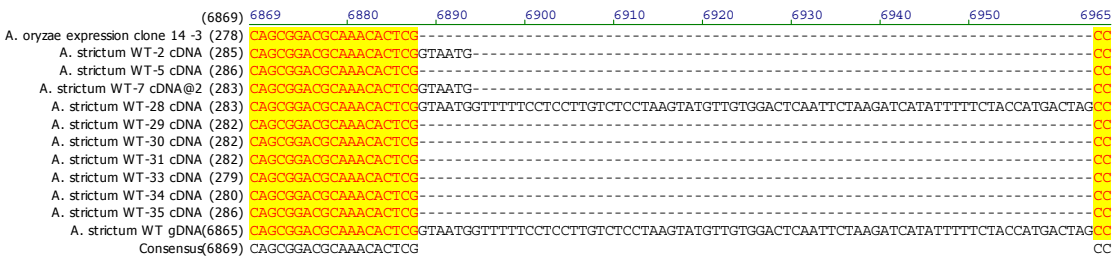
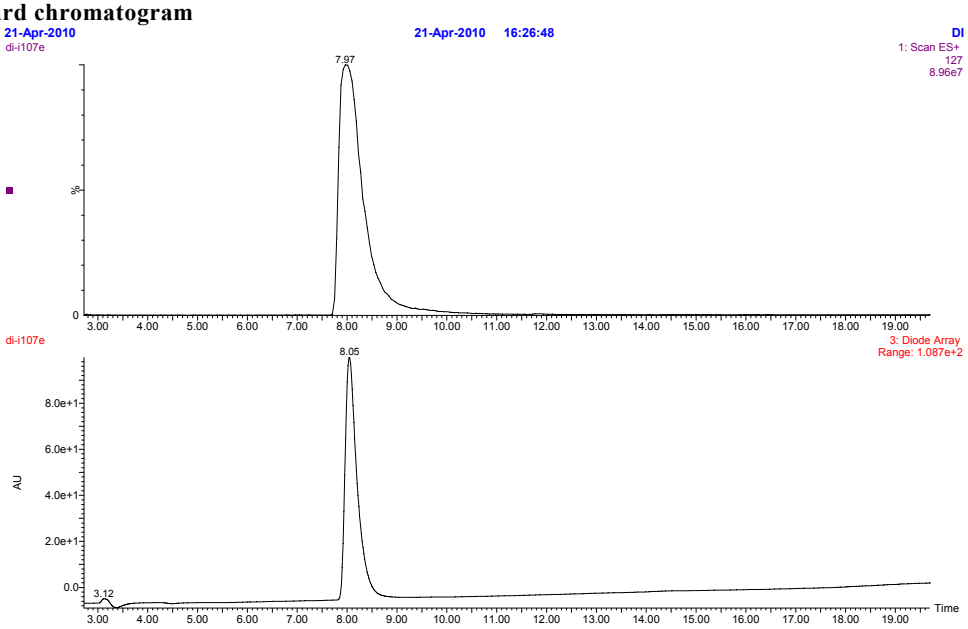


Figure S2: Sequence analysis of *ASpks1* intron 2 splicing patterns in *A. oryzae* (top row) and *A. strictum* (rows 2-11). Row 12 shows the genomic sequence of native *ASpks1* in *A. strictum*.

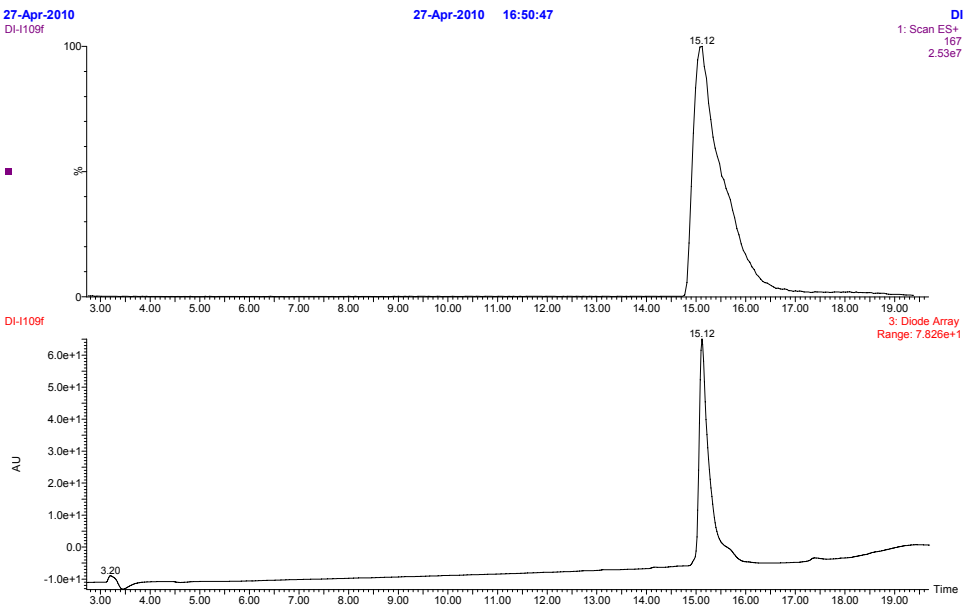
3.0 LCMS Chromatograms

3.1 T3AL standard chromatogram



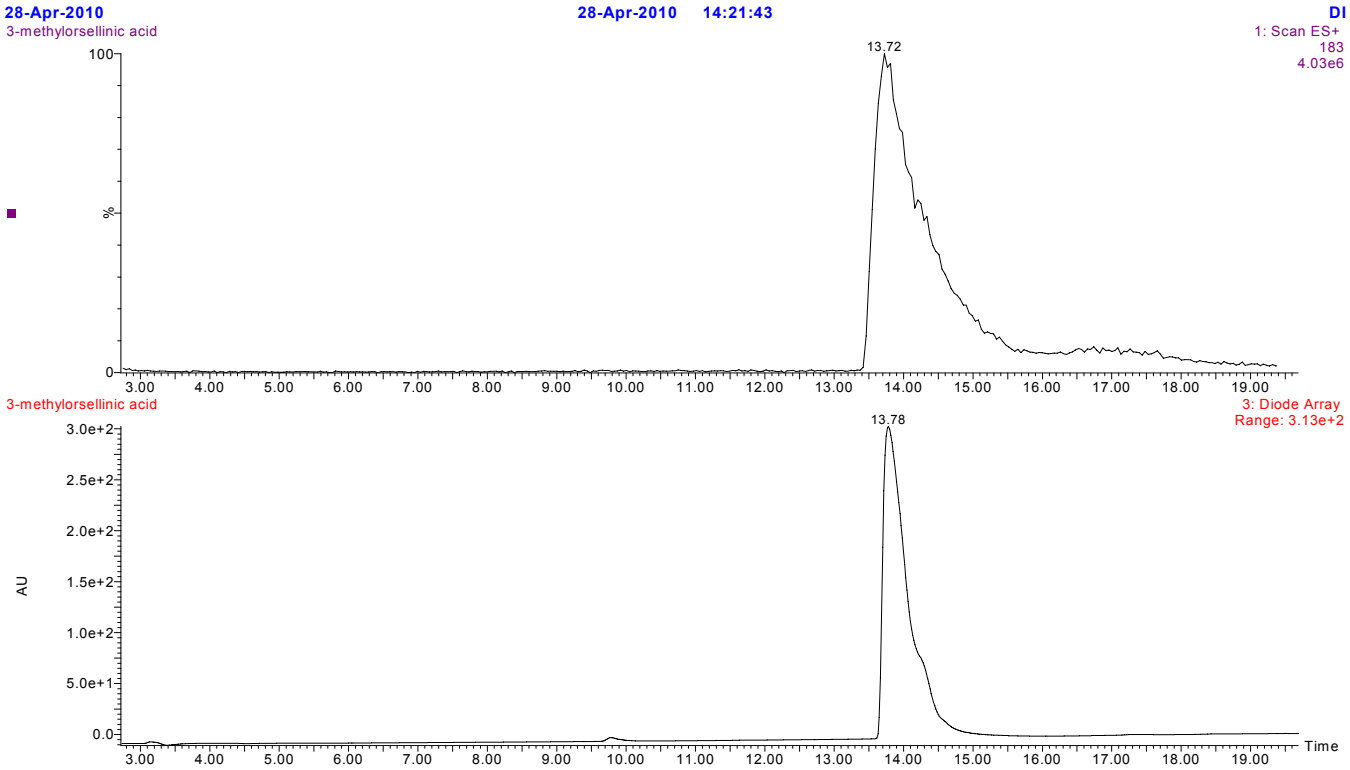
Lower panel, DAD trace; upper panel, ES+ trace corresponding to $[M]H^+$.

3.2 3-Methylorcinaldehyde standard chromatogram



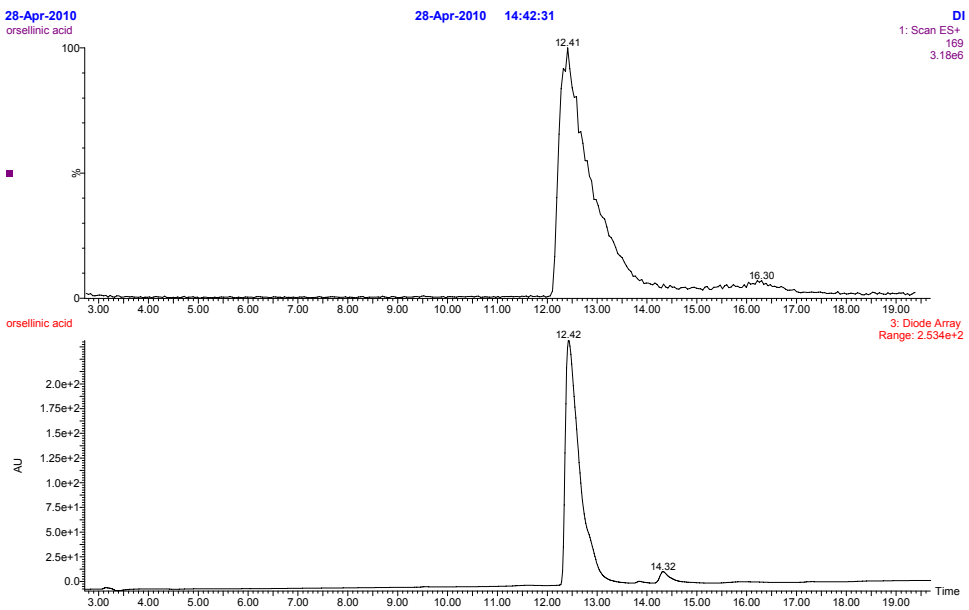
Lower panel, DAD trace; upper panel, ES+ trace corresponding to $[M]H^+$.

3.3 3-Methylorsellinic acid standard chromatogram.



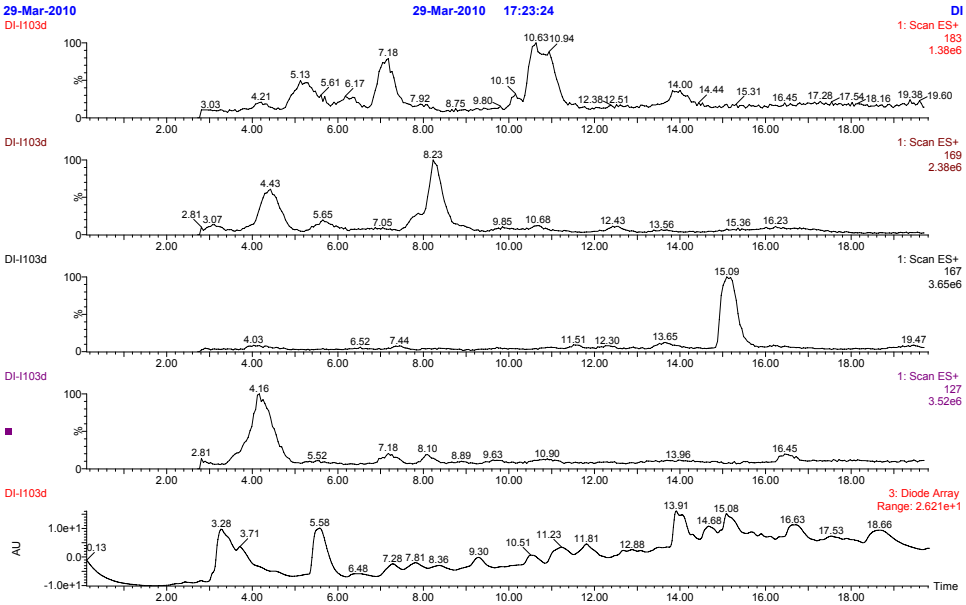
Lower panel, DAD trace; upper panel, ES+ trace corresponding to $[M]H^+$.

3.4 Orsellinic acid standard chromatogram



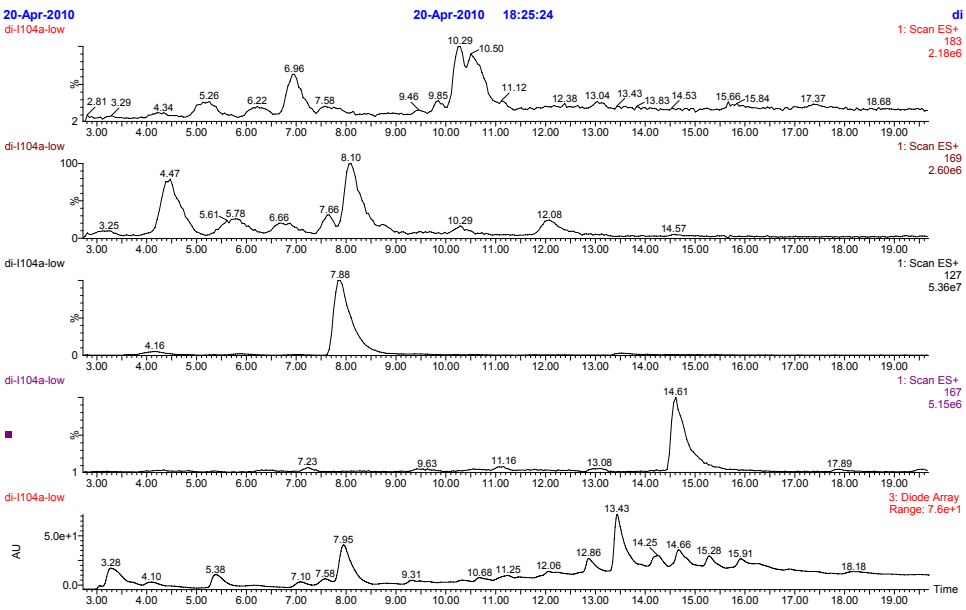
Lower panel, DAD trace; upper panel, ES+ trace corresponding to $[M]H^+$.

3.5 Chromatogram of Extract of *A. oryzae* pASPKS1



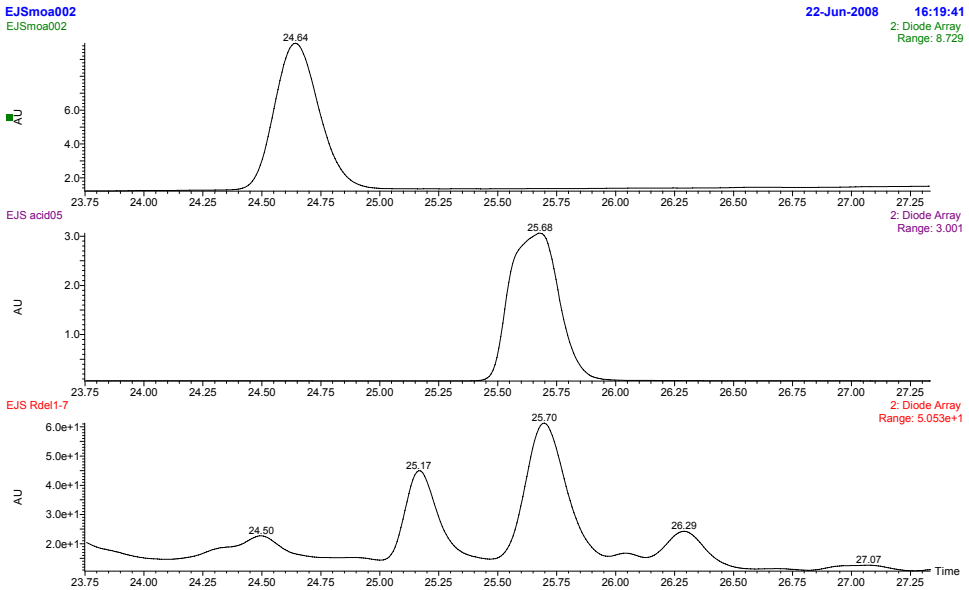
From top: SIM (ES+) for 3-methylorsellinic acid (RT 13.8); orsellinic acid (RT 12.4); 3-methylorcinaldehyde (RT 15.1); T3AL (RT 7.9; Diode array detector trace.

3.6 Chromatogram of extract of ethionine treated *A. oryzae* pASPKS1

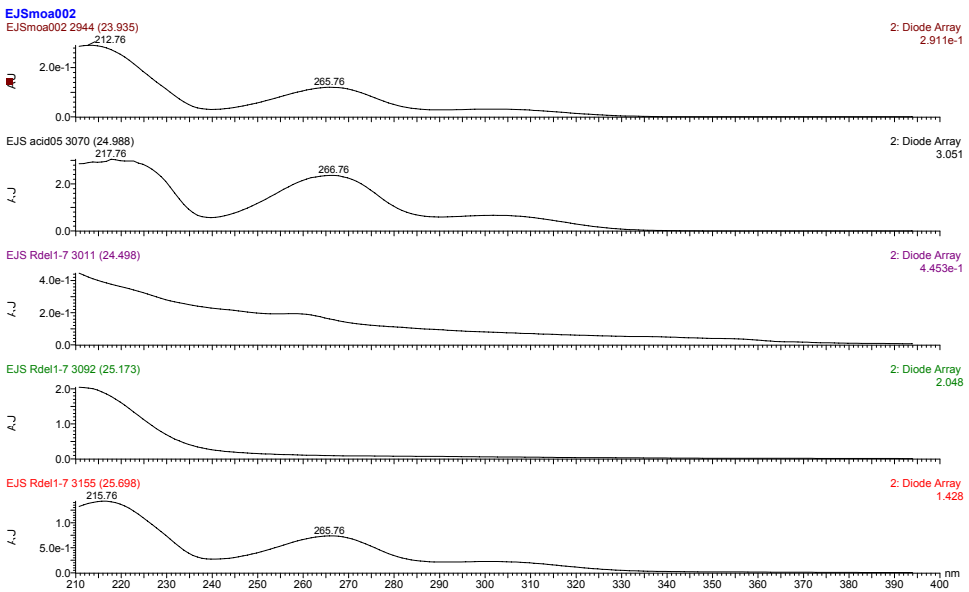


From top: SIM (ES^+) for 3-methylorsellinic acid (RT 13.8); orsellinic acid (RT 12.4); T3AL (RT 7.9); 3-methylorcinaldehyde (RT 15.1); Diode array detector trace.

3.7 Chromatograms of *A. oryzae* pASPKS1-ΔR.

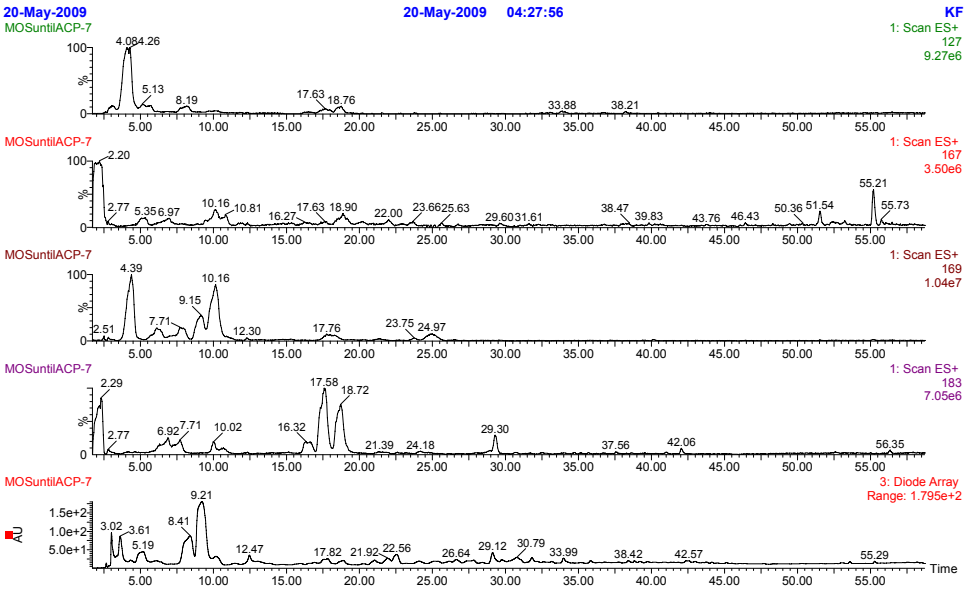


From top: diode array trace methylorcinaldehyde standard; diode array trace of methyl orsellinic acid standard; diode array trace of extract from *A. oryzae* pASPOKS1-ΔR; (HPLC method 1).



From top, uv spectra (210 - 400nm) of: 3-methylorcinaldehyde; 3-methyl orsellinic acid; 24.5 min peak in extract from *A. oryzae* pASPKS1-ΔR; 25.2 min peak in extract from extract from *A. oryzae* pASPKS1-ΔR; 25.7 min peak from extract from *A. oryzae* pASPKS1-ΔR; (HPLC method 1).

3.8 Chromatograms of *A. oryzae* pASPKS1-ΔMR.



From top: SIM (ES^+) for T3AL; 3-methylorcinaldehyde; orsellinic acid; 3-methylorsellinic acid; Diode array detector trace; (HPLC method 1).