Supplementary Material (ESI) for Molecular BioSystems
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Mutation of Key Residues in the *C*-methyltransferase Domain of a Fungal Highly Reducing Polyketide Synthase.

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Electronic Supplementary Information

1 Experimental Details.

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1.1 List of primers used.

Oligonucleotide primers were ordered from Sigma Aldrich

Primer Name	Sequence (5' – 3')	Description
CMeTf	AGATGGTTGTTCACGTGGGCAA	*phpks1 C*MeT primer for sequencing point mutants
CMeTr	AACCCAGATGAGGCCCTTGCAT	*phpks1 C*MeT primer for sequencing point mutants
PmlI fwd	CAGATGGTTGTTCACGTGGGCAAAAGCATG	CMeT PmlI restriction site
SpeI rev	CATCTAGTTACTAGTTTCTGGATGGAA	CMeT SpeI restriction site
GlyvalF	GCTGGCACCGTTGGCTGCACAAGGGCGGTC	G1506V point mutation
GlyvalR	TGTGCAGCCAACGGTGCCAGCTCCAATCTC	G1506V point mutation
$D_{190}Kf$	GGCAGCTATAAGCTCGTAATTGCATCTCAA	D1573K point mutation
$D_{190}Kr$	AATTACGAGCTTATAGCTGCCACATTCGAA	D1573K point mutation
$W_{243}Rf$	CTTCCTGGCAGGTGGCTGAGTTCCGAAGAG	W1626R point mutation
$W_{243}Rr$	ACTCAGCCACCTGCCAGGAAGCAATCCAAA	W1626R point mutation
$E_{185}Kf$	CAAGGCTTCAAATGTGGCAGCTATGATCTC	E1568K point mutation
$E_{185}Kr$	GCTGCCACATTTGAAGCCTTGCATTTCGAT	E1568K point mutation

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1.2 PCR conditions for introduction of mutations.

For accurate amplification of mutated fragments, a proof-reading DNA polymerase (KOD Hot Start, Novagen) was used (0.5 µL) with 10 × KOD Hot Start buffer (2.5 µL), dNTPs (25 mM, 2.5 µL), $MgSO_4$ (25 mM, 1 µL), both forward and reverse primers (25 µM, 1 µL each), template DNA (0.5 µL) and sterile water (16 µL). Samples were amplified using a PTC-200 Peltier Thermal Cycler. Initial denaturation was performed (94 ˚C, 5 min) followed by 33 cycles of denaturation (94 ˚C, 20 s); primer annealing (55 - 70 ˚C, 10 s) and extension (72 ˚C, 15 s per 1 kb of DNA). Final extension was allowed (72 ˚C for 10 min) before cooling to 4 ˚C until analysis. Control reactions, with and without template DNA, were run for each set of PCR primer pair reactions. Template DNA was diluted 100 times. Positive PCR products were identified *via* electrophoresis of PCR solution (5 µL) and loading buffer (1 µL) on a 0.7 % agarose gel.

1.3 Digestion and ligation of pTAex3-Phpks1, sequencing details.

Preparative Restriction Digest
DNA (0.1 – 10 µg), restriction enzyme (0.1 – 0.3 volumes), 10 × buffer (0.1 volume) and sterile water were made up to the desired volume, mixed and incubated at 37 ˚C overnight. The digested sample was analysed by gel electrophoresis.

Restriction Fragment Ligations
Gel purified digested plasmids/ restriction fragments/ PCR products were ligated in 10 µL volumes using 3U T4 DNA ligase and 10 × T4 DNA ligase buffer (Fermentas). Inserts were used in excess and the ratio of insert: vector was estimated depending on the size and concentration of insert, typically 2:1 to 10:1. Mixtures were incubated at 16 ˚C for 16 to 24 hours and then transformed into *E. Coli*.

DNA Sequencing
PCR products were prepared for sequencing using QIAquick PCR Clean-up kit, yielding only the desired DNA fragment. The purified product (10 µL, 45 ng/µL) was sent to Lark Technologies with appropriate sequencing primers (10 µL, 100 ng/µL) for Silver Service sequencing using PE Biosystems' DNA analysers. Plasmid DNA (10 µL, 100 ng/µL) was also sequenced using the Silver Service. Interpretation of DNA sequencing data utilised Vector NTi (Section 2.12).

DNA Sequence Analysis
The sequence data returned was analysed using Vector NTi 10.1.1 © 2005 Invitrogen Incorporation[154] and NCBI BLAST facility on the internet (www.ncbi.nlm.nih.gov). Sequence alignments, oligonucleotide analysis and vector design and construction were conducted using Vector NTi.

1.4 Transformation and selection of *A. oryzae* M-2-3.

Preparation of A. oryzae Protoplasts
A spore suspension (100 - 200 µL) of *Aspergillus oryzae* M-2-3 was spread onto a 20 mL plate of malt extract agar (commercially available MEA contains malt extract, 30 g/L; mycological peptone, 5 g/L; and agar, 15 g/L). The plate was incubated at the appropriate temperature for 3 - 5 days. Water and 0.1% Tween 80 (10 mL) were added to the agar plate and the spores scraped off with a loop. This liquid was collected and centrifuged (10,000 × g, 5 min). The supernatant was poured off and the crude spore preparation resuspended in water (1 mL). This spore suspension was used to inoculate flasks of DPY medium (100 mL, containing Dextrin, 20 g/L; mycological peptone, 10 g/L; yeast extract, 1.0 g/L; dibasic potassium phosphate, 5.0 g/L; and magnesium sulphate, 0.5 g/L) which were then incubated at 25 ˚C with shaking (240 rpm, 24 - 48 hours).

The mycelia were collected by filtration through sterile Miracloth and were washed twice with 0.8 M NaCl (20 mL) then spun (10,000 × g, 10 mins). The supernatant was poured off; the remaining packed cell volume should not exceed 5mL. Filter-sterilised protoplasting solution (20 mL, 20 mg/mL Glucanase, 10 mg/mL Driselase; Interspex Products in 0.8 M NaCl) was added to the pellet which was resuspended thoroughly by vortexing. The tube was incubated at room temperature with gentle mixing on a rotator. After one hour a small sample was analysed microscopically for protoplast formation, after which analysis was repeated every 30 min until sufficient protoplasts had formed.

The protoplasts were released from hyphal strands by gentle pipetting with a large wide-bore pipette (5 mL). The protoplasts were filtered through sterile Miracloth to remove the hyphae. The filtrate was spun (maximum 2,000 × g, 5 mins) just fast enough to pellet all of the protoplasts. The protoplasts were washed twice with 0.8 M NaCl (20 mL) and once with Solution I (0.8 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5; 20 mL); each time the pellet was resuspended well by wide-bore pipetting and spun (2,000 × g, 5 mins) to remove the supernatant. The concentration of protoplasts was determined using a haematocytometer (Fisher) and the protoplasts were resuspended in Solution I to give a concentration in the range 1 - 9×10^7. The protoplasts were stored on ice.

PEG-Mediated Transformation
DNA (5 - 10 µg, 10 µL maximum) to be transformed into the fungus was added to the protoplast (100 µL) and incubated on ice (2 min). Solution II (60% PEG 3350 (Sigma), 10 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5; 1 mL) was added drop-wise over 15 minutes then incubated at room temperature (20 mins).

Plates were poured with Czapek-Dox + Sorbitol agar (10 mL, commercially available CD agar contains sodium nitrate, 3 g/L; potassium chloride, 0.5 g/L; magnesium sulfate heptahydrate, 0.5 g/L; iron sulfate heptahydrate, 0.01 g/L; dibasic potassium phosphate, 1 g/L; sucrose, 30 g/L; agar, 15 g/L; dissolved in 1 M sorbitol). CD + S agar (10 mL) was added to the transformation mixture then overlaid onto the prepared plates. The plates were incubated at 25 ˚C.

Arginine Selection
Selection of *A. oryzae* M-2-3 transformants was performed on arginine-deficient media. A positive control was made by the addition of 2% arginine solution (0.01 volume) to untransformed protoplasts and agar; this is then mixed and plated out. The negative control consisted of untransformed protoplasts plated on minimal media.

1.5 Fermentation, extraction and chemical analyses of transformants.

Selecting Positive Transformants
Colonies which grew on minimal CD + S agar (primary plates) were subcultured onto secondary minimal plates, each containing multiple colonies in individual sectors of the plate. Colonies which continued to grow on these secondary minimal plates were again subcultured onto individual tertiary minimal plates. If these colonies still grew after three generations on minimal medium, they were deemed to be positive transformants and transferred to MEA plates in preparation for growth in liquid medium. These seed plates were used to inoculate flasks of maltose medium (100 mL, containing maltose, 20 g/L; polypeptone, 10 g/L; sodium nitrate 2g/L; potassium chloride, 2 g/L; magnesium sulfate heptahydrate, 0.5 g/L; iron sulphate heptahydrate, 0.01 g/L; potassium

phosphate, 1 g/L) *via* a spore suspension. The maltose present in this medium activated the inducible AmyB promoter which controls SQTKS expression in the pTAex3 vector system. Liquid cultures were incubated at 25 ˚C with shaking (240 rpm).

Procedure for the Isolation of Crude Squalestatin Tetraketide

After 6 days of growth in liquid medium, the culture broth was acidified using 2 M hydrochloric acid until the solution reached approximately pH 2.0 and was put back on the shaker for 30 mins. The mycelia were homogenised using an electric hand blender and removed by Büchner filtration, washing with distilled water. The filtrate was extracted twice with ethyl acetate. The organic fractions were combined and dried over anhydrous magnesium sulphate, and the solvent was removed under vacuum. The residue of this crude extract was dissolved in methanol and analysed by LCMS.

Liquid Chromatography/ Mass Spectrometry (LCMS) analysis

Samples were analysed using a Waters Platform LC system comprising a Waters 600 pump system, Waters 996 diode array detector (detecting between 210 and 400 nm), Macromass Platform LC mass spectrometer (detecting between 150 and 600 *m/z* units ESI^+) with the Luna 5µm C_{18} (2) (Phenomex, 250 × 4.6 mm, 5 µm) reverse phase column. The program was run 0-5 min (95% A), 5-42 min (ramped to 75% B), 32-45 mins (ramped to 90% B), 45–50 mins (90% B), 50–55 mins (ramped down to 95% A), 55–60 mins (95% A). Solvent A: water + 0.05% TFA, solvent B: MeCN + 0.04% TFA. Flow rate 1 mL/min.

1.6 Details of oct-2-enoic acid feeding experiment.

Maltose medium (100 mL) was inoculated with a spore suspension of *A. oryzae* M-2-3 (300 µL) and supplemented with 2% arginine solution (0.01 volumes). Cultures were incubated for 5 days (25 ˚C, 150 rpm). On the fifth day 100 µL of a stock solution of oct-2-enoic acid (20 mg/mL) was added to each flask. Each subsequent day an extraction was performed on the culture supernatant.

A series of standard oct-2-enoic acid concentrations were run (0.005-0.1 mg/mL in MeOH) using HPLC (Hewitt Packard 1050) with the Luna 5um C_{18} (2) (Phenomex, 250 × 4.6 mm, 5 µm beads) reverse phase column. The program was run (with initial flow rate 0.1 mL/min) 0-1 min (95% A), 1-5 min (95% A, flow rate 1mL/min), 5-42 min (ramped to 75% B), 42-45 mins (ramped to 90% B), 45-50 mins (90% B), 50-55 mins (ramped down to 95% A), 55-59 mins (95% A), 59-60 mins (95% A, 0.1 mL/min). Solvent A: water + 0.01% TFA, solvent B: MeCN + 0.01% TFA. Flow rate 1 mL/min, UV 221nm. Samples loaded (50 µL), 30 µL injected onto column. Minimum and maximum detection limits were determined and the gradient of the straight line graph was used to interchange arbitary units into concentration units.

2. Multiple alignment of CMeT domains / proteins.

Individual peptide sequences corresponding to CMeT domains of compactin nonaketide synthase (CNKS), lovastatin nonaketide synthase (LNKS), fumonisin synthase (FUMS), compactin diketide synthase (CDKS), lovastatin diketide synthase (LDKS), squalestatin tetraketide synthase (SQTKS) and fusarin synthetase (FUSS) were determined by alignment with rat glycine *N*-methyltransferase (GNMT, pdb id 1xva) and excised from their parent sequences. The individual peptide sequences were then subject to multiple alignment using the alignX function of the Vector NTI software package, giving the alignment and tree-plot shown below. CMeT motifs I, II and III according to Kagan and Clarke[1] are indicated with red backgrounds, mutation sites are indicated with *. Numbering is for GNMT (1xva).

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                   1                                               50
 CMeT 1XVA    (1)  VDSVYRTRSLGVAAEGIPDQYADGEAARVWQLYIGDTRSRTAEYKAWLLG
 CMeT CNKS    (1)  IQRVGKELISIVRGNGDPLDIMNRDGLFTEYYTNKLAFGSAIHVVQDLVS
 CMeT LNKS    (1)  VQRVGQHLLPTVRSNGNPFDLLDHDGLLTEFYTNTLSFGPALHYARELVA
 CMeT FUMS    (1)  IGRVVDHCEEIVKGELEGIELLQAEDGLTNYYN----YVESRTDSIDFFA
 CMeT CDKS    (1)  ISRLGPQLIAMLRRETEPLELMMQDQLLSRYYVNAIKWSRSNAQASELIR
 CMeT LDKS    (1)  ICRLGSVLPAILRREVDPLEVMMDGHLLSRYYVDALKWSRSNAQASELVR
CMeT SQTKS    (1)  VVHVGKSMLAILRHEIAPLELMLQDKLLYRYYTDAIKWDRSYQQIDQLVK
 CMeT FUSS    (1)  IQAVGENLPAVVRKQTTMLEHMVKDDMLNRIYKFGLGFERANVYLGRISK

                   51         1111111*11                          100
 CMeT 1XVA   (51)  LLRQHG-CHRVLDVACGTGVDSIMLVEEGFSVTSVDASDKMLKYALKERW
 CMeT CNKS   (51)  QIAHRYQSIDILEIGLGTGIATKRVLASPQLG--------FNSYTCTDIS
 CMeT LNKS   (51)  QIAHRYQSMDILEIGAGTGGATKYVLATPQLG--------FNSYTYTDIS
 CMeT FUMS   (47)  TAGHTRPTLRVLEIGAGTGGAQVILEGLTNGK----ERLFSTYAYTDIS
 CMeT CDKS   (51)  LCAHKNPRSRILEIGGGTGGCTKLIVNALGNT------KPIDRYDFTDVS
 CMeT LDKS   (51)  LCCHKNPRARILEIGGGTGGCTQLVVDSLGPN------PPVGRYDFTDVS
CMeT SQTKS   (51)  LHAHKCPTAKIIEIGAGTGGCTRAVLDALSNQG----IARCAQYDFTDVS
 CMeT FUSS   (51)  QIAHRYPRMNILEIGAGTGGATKGIMESLGTA--------FETYTFTDIS
 Consensus   (51)  LIAHK PSMRILEIGAGTGGATKLVLESL  G        F SY FTDIS
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                   101                                  *  222*222222 150
  CMeT 1XVA  (100) NRRKEPAFDKWVIEEANWLTLDKDVPAGDGFDAVICLGNSFAHLPDSKGD
  CMeT CNKS   (93) ADVIGKAREQLSEFDGLMQFEALDINRSPAEQGFKPHSYDLIIASDVLHA
  CMeT LNKS   (93) TGFFEQAREQFAPFEDRMVFEPLDIRRSPAEQGFEPHAYDLIIASNVLHA
  CMeT FUMS   (93) AGFFVAAQERFKAYKG-LDFKVLDITKDPSEQGFESGSFDLIIAGNVIHA
  CMeT CDKS   (95) AGFFESAREQFADWQDVMTFKKLDIESDPEQQGFECATYDVVVACQVLHA
  CMeT LDKS   (95) AGFFEAARKRFAGWQNVMDFRKLDIEDDPEAQGFVCGSYDVVLACQVLHA
 CMeT SQTKS   (97) SGFFEAAQQKFAAFDDVIRFQKLDIEKDIEMQGFECGSYDLVIASQVLHA
  CMeT FUSS   (93) TGFFEAAAEAFDHWADKMIFKPLNIESDPTDQGFPQGHYDFIIASNVLHA
  Consensus  (101) AGFFEAAREKFA FDDVM FK LDIEKDP EQGFECGSYDLIIASNVLHA

                   151            3333333333                      *    200
  CMeT 1XVA  (150) QSEHRLALKNIASMVRPGGLLVIDHRNYDYILSTGCAPPGKNIYYKSDLT
  CMeT CNKS  (143) SSNFEEKLAHIRSLLKPGGHLVTFGVTHREPARLAFISGLFADRWTGEDE
  CMeT LNKS  (143) TPDLEKTMAHARSLLKPGGQMVILEITHKEHTRLGFIFGLFADWWAGVDD
  CMeT FUMS  (142) TPTLNETLANVRKLLAPEG-YLFLQELSPKMRMVNLIMGILPGWWLGAAE
  CMeT CDKS  (145) TRCMKRTLSNVRKLLKPGG-NLILVETTRDQLDLFFTFGLLPGWWLSEEP
  CMeT LDKS  (145) TSNMQRTLTNVRKLLKPGG-KLILVETTRDELDLFFTFGLLPGWWLSEEP
 CMeT SQTKS  (147) TGKMEHTMANVRKLLKPGG-KLLLVETTRDEMDLQLVFGLLPGWWLSSEE
  CMeT FUSS  (143) TKSLTVTMRNTRKLLKPGG-QLLLLEVTSDIVRVKLMMSGLSGWWLGGDD
  Consensus  (151) T  L  TLANVRKLLKPGG  LILVETTRD L L FIFGLLPGWWLSEDE

                   201                                                 250
  CMeT 1XVA  (200) KDITTSVLTVNNKAHMVTLDYTVQVPGAGRDGAPGFSKFRLSYYPHCLAS
  CMeT CNKS  (193) TRALSASGSVDQWEHTLKRVGFSGVDSRTLDREDDLIP-SVFSTHAVDAT
  CMeT LNKS  (193) GRCTEPFVSFDRWDAILKRVGFSGVDSRTTDRDANLFPTSVFSTHAIDAT
  CMeT FUMS  (191) GRVEEPYLDPSQWDTVLKETGFSGVDSAIYDAPYPYHLNANIISRPAKES
  CMeT CDKS  (194) ERKSTPSLTTDLWNTMLDTSGFNGVELEVRDCEDDEFY--MISTMLSTAR
  CMeT LDKS  (194) ERQSTPSLSPTMWRSMLHTTGFNGVEVEARDCDSHEFY--MISTMMSTAV
 CMeT SQTKS  (196) ERQMSPSLSTNSWEKVLKKTGFDGLDIELRDCDSDEFY--SFSVMMATAS
  CMeT FUSS  (192) GRRYGPTIPVSQWDALLKQNGFSGVDKTVNDFVDAEKY--MTSVMLSQAV

                   251                                                 300
  CMeT 1XVA  (250) --------------FTELVQ--EAFGGRCQHSVLGDFKPYRPGQ-AYVP
  CMeT CNKS  (242) VERLYDPLSAP--LKDSYPPLVVIGGESTKTERILNDMKAALPHRHIHSV
  CMeT LNKS  (243) VEYLDAPLASSGTVKDSYPPLVVVGGQTPQSQRLLNDIKAIMPPRPLQTY
  CMeT FUMS  (241) AP----------QPRAIRGRLTLLHHADDTNSSSITQLREVLDARGLETD
  CMeT CDKS  (242) KE-------NTTPDTVAESEVLLLHGALRPPSSWLESLQAAICEK--TSS
  CMeT LDKS  (242) QA-------TPMSCSVKLPEVLLVYVDSSTPMSWISDLQGEIRGR--NCS
 CMeT SQTKS  (244) -----------STIASSSMAFAIVYGEVPLPDQFLDDMKTAISSS--AVS
  CMeT FUSS  (240) DDRMEILR-QPRLASSHWLSSQSITVVGGHCQDIGKDAMAIIHQMGHASS

                   301                     325
  CMeT 1XVA  (282) CYFIHVLKKTG--------------
  CMeT CNKS  (290) KRLESVLDDPALQPKSTFVI-----
  CMeT LNKS  (293) KRLVDLLDAEELPMKSTFVM-----
  CMeT FUMS  (281) MVVFHEHEELKAGEQDVIIS-----
  CMeT CDKS  (283) SPSINALGEVD-TTGRTCIF-----
  CMeT LDKS  (283) VTSLQALRQVPPTEGQICVF-----
 CMeT SQTKS  (281) DPVVGHLDSID-ATGKFCIF-----
  CMeT FUSS  (289) KPVIHHVGSFEELASSNIQTRSALV
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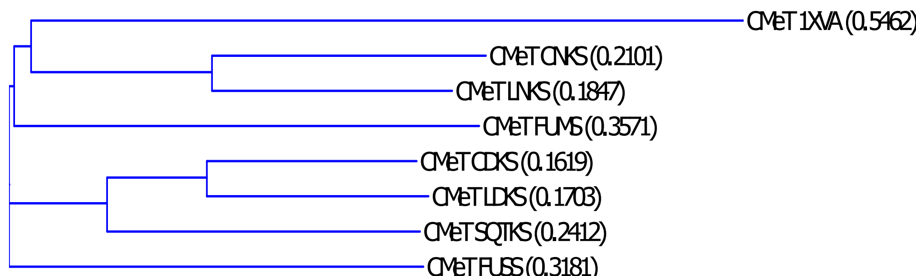

Notes and references

1. R. M. Kagan and S. Clarke, *Arch. Biochem. Biophys*, 1994, **310**, 417-427.