Isolation and characterisation of amphotericin B analogues and truncated polyketide intermediates produced by genetic engineering of *Streptomyces nodosus*†

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Amphotericin B is a powerful but toxic drug used against fungal infections and leishmaniases. These diseases would be treated more effectively if non-toxic amphotericin derivatives could be produced on a large scale at low cost. Genetic manipulation of the amphotericin B producer, *Streptomyces nodosus*, has previously led to the detection and partial characterisation of 8-deoxyamphotericin B, 16-descarboxyl-16-methyl-amphotericin B, 15-deoxy-16-descarboxyl-16-methyl-15-oxo-amphotericin B, 7-oxo-amphotericin B and pentaene analogues. Here we report improved production and purification protocols that have allowed detailed chemical analyses of these compounds. The polyketide synthase product 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B was identified for the first time. In addition, the ketoreductase 10 domain of the polyketide synthase was specifically inactivated by targeted gene replacement. The resulting mutants produced truncated polyketide intermediates as linear polyenyl-pyrones.

Introduction

Amphotericin B **1** (Fig. 1) is produced by the soil bacterium *Streptomyces nodosus* and, despite considerable toxicity, it has been a leading broadspectrum antifungal antibiotic for more than fifty years.[1] Resistance has not become a problem in this long period of clinical use, unlike all other established antifungal therapies.[2] Amphotericin B has also emerged as the first line of treatment against some *Leishmania* parasites,[3] and has potential in the treatment of HIV infection and prion diseases.[4]

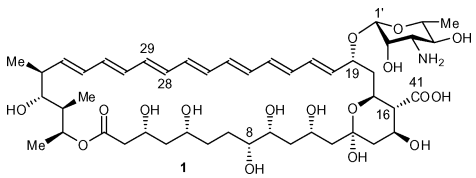

Fig. 1 Amphotericin B.

The antibiotic mode of action of **1** is complexation with ergosterol in fungal membranes and related sterols in parasite membranes.[5] The complexes self-assemble to form transmembrane pores that allow uncontrolled loss of ions and small molecules and cause cell death.[6] There is also some affinity for cholesterol, which along with low serum solubility contributes to severe side effects such as nephrotoxicity.[7]

Traditionally, **1** was injected as a solution in DMSO or as a complex with deoxycholate. Recent liposomal formulations have greatly reduced toxicity, but are sufficiently expensive to require patient selection on pharmacoeconomic considerations, and are major components of the drugs bills of some NHS Trusts.[8]

Visceral leishmaniasis (kala azar) is caused by protozoan parasites transmitted by the sand fly, and is fatal if left untreated. It remains a 'most neglected' disease with 50,000 deaths each year, many in high-level poverty areas in Asia and South America.[3] Conventional **1** is now the first line of treatment in Bihar, India and in neighbouring Nepal. Efficacy is close to 100% but there are nephrotoxic side effects and administration requires hospitalisation for a complicated infusion regime of fifteen injections over thirty days. Despite considerable subsidy from the WHO (US$3,000 per treatment reduced to US$200), the cost of the less toxic liposomal formulation Ambisome severely restricts its use in these regions, some of the poorest parts of the world. Thus there is a need for an affordable effective analogue with greatly reduced toxicity.[3]

Amphotericin B toxicity is only partly alleviated by costly liposomal formulation. Improvement could be achieved by structural alteration. Chemical studies have generated derivatives such as MFAME **2** (Fig. 2) that has been shown to be an effective non-toxic antibiotic but is much too expensive for clinical use.[9]

The activity of MFAME shows that much improved analogues of **1** exist in chemical space, the challenge is to produce them at a low cost. Due to the complex structure of **1**, chemical modification is unlikely to deliver an affordable non-toxic derivative. Such a compound might instead be obtained by engineering biosynthetic

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‡ A major proportion of work and intellectual input into new isolation and purification methods appearing in this article was from Dr Barry Murphy.

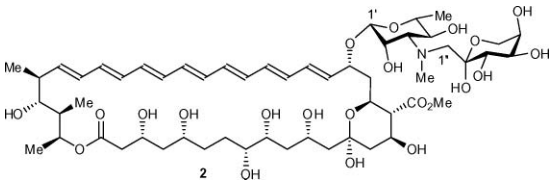


Fig. 2 MFAME.

genes in *S. nodosus*.[10] The parent wild-type strain can produce high levels (4 g L^{-1}) of **1** in low cost fermentations, demonstrating the presence of a very efficient metabolic flow of the required biosynthetic building blocks, and efficient catalytic processes. Thus, given sufficient understanding of the genetic and biosynthetic machinery, there is potential to achieve low-cost production of engineered compounds that can replace **1** in therapy.[11]

Isotope labelling studies have shown that the aglycone core **3** is assembled from sixteen acetate and three propionate units, in eighteen acyl chain extending cycles.[12] All the genes responsible for amphotericin B biosynthesis, export and regulation are found in a cluster.[13] The aglycone **3** is assembled by a modular polyketide synthase (PKS) composed of the AmphABCIJK proteins. AmphA loads the starter unit, AmphB adds on two propionate-derived units, each with ketoreduction, to form the triketide (Fig. 3). Hexamodular AmphC incorporates six acetate-derived building blocks, each 3-oxo group reduced and dehydrated, to give a conjugated hexaenoyl thioester. Within AmphC, module 5 has a partially active ER domain that is responsible for formation of the tetraene analogue, amphotericin A (28,29-dihydroamphotericin B). The hexamodular AmphI adds on six more building blocks (two acetate-, one propionate-, then three more acetate-derived units). The first is reduced and dehydrated to complete the conjugated heptaene (or tetraene) moiety. At some stage the hydroxyl group formed in cycle 11 lactolises with the ketone introduced in cycle 13. This lactol ring is a common feature in many related polyene compounds, and if formed at the tetradecaketide stage may be important in folding and controlling the very long growing acyl chain as it passes through the PKS proteins. AmphJ and AmphK complete the growing acyl chain, and a thioesterase on AmphK catalyses lactonisation to form **3**.[13]

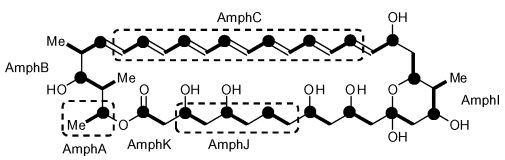
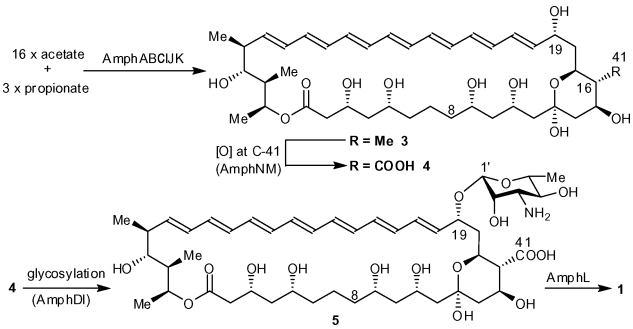


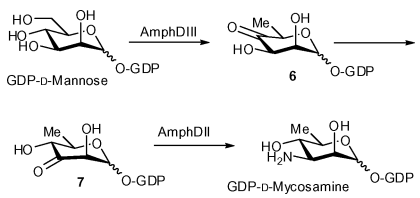
Fig. 3 Role of AmphABCIJK.

Aglycone (**3**) formation is followed by oxidation of the methyl unit at C-16 to –COOH by the AmphN cytochrome P450 monooxygenase to give 8-deoxy-amphoteronolide B (**4**). Attachment of a mycosaminyl unit at C-19 by the AmphDI glycosyltransferase gives **5** which is finally oxidised at C-8 by the AmphL cytochrome P450 monooxygenase to **1** (Scheme 1), this order of late modifications is preferred but not obligatory.[14]

The mycosaminyl sugar is assembled by the dehydration of GDP-D-mannose to 4,6-dideoxy-4-oxo-D-mannose **6** by the 4,6-dehydratase AmphDIII and isomerisation to the 4-hydroxy-3-oxo regioisomer **7** (Scheme 2). AmphDII catalysed transamination at



Scheme 1 Biosynthesis of amphotericin B **1**.



Scheme 2 Biosynthesis of GDP-D-mycosamine.

C-3 gives GDP-D-mycosamine, the substrate for the glycosyltransferase AmphDI.

In preliminary work, we have identified several biosynthetic intermediates of **1** produced by genetic engineering of *S. nodosus*. For example, inactivation of *amphL* and *amphN* gave 8-deoxyamphotericin B (**5**) and 16-descarboxyl-16-methyl-amphotericin B (**8**) (Fig. 4) respectively.[14,15] Removal of two PKS modules from AmphC gave material with the absorbance spectrum of a pentaene.[16] Inactivation of the ketoreductase (KR) domain in module 16 gave the corresponding 7-oxo-analogue **9** (Fig. 5) whilst inactivation of KR12 gave traces of 15-oxo-heptaene products.[17] In this paper, we describe enhanced production, isolation, purification and characterisation of these products, essential for progress towards an affordable effective analogue. The PKS product, aglycone **3**, was purified for the first time. We also investigate the inactivation of PKS KR domains, which can deliver carbonyl-containing analogues with improved solubility or potential for chemical or biochemical transformation.[18,19] The

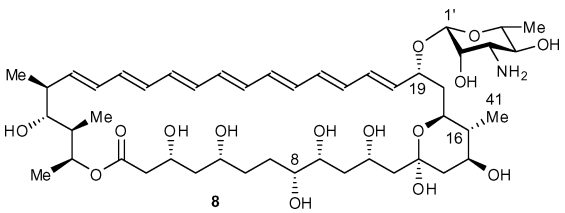


Fig. 4 16-Descarboxyl-16-methyl-amphotericin B (**8**).

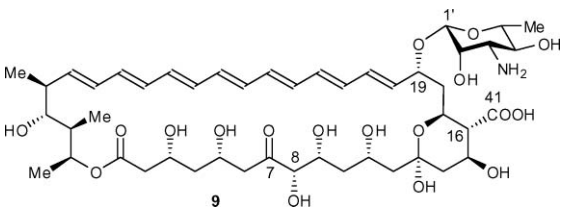


Fig. 5 7-Oxo-amphotericin B (**9**).

KR16 and KR12 mutants were constructed as the corresponding 7-oxo and 15-oxo analogues would be expected to have increased serum solubility, and reduced toxicity. Prior to this study, inactivation of KR10 was expected to result in biosynthesis of C-19 carbonyl containing amphoteronolides. Such aglycones might be chemically derivatised to give libraries of semi-synthetic analogues. Nicolaou has prepared protected amphoteronolide heptaenones and chemically converted them into **1**.[20] Here we report that KR10 inactivation had unexpected biosynthetic consequences and led to the synthesis of truncated polyketide intermediates that were isolated as linear polyenyl-pyrones.

Results and Discussion

Genetically engineered strains were grown on a multilitre scale in grooved Erlenmeyer flasks. Most analogues were located in the sediment after centrifugation of production cultures. Improved yields were obtained by including the non-ionic polymeric absorbent resin XAD16 in the production medium. Amphotericin B is extracted from whole broth using butanol, however this has proved problematic with the lower titres of low purity analogues. Methanolic extraction of this sediment gives membrane lipid and dextrin derived oligosaccharides along with heptaenes and tetraenes. Partial removal of the methanol *in vacuo* from the crude extract can result in a yellow precipitate containing heptaene (1–80% depending on mutant), the supernatant is now largely mycelia derived water.

Polyenes can display considerable affinity for both lipid and oligosaccharide co-extractants, hindering purification. For most analogues stability and solubility can dramatically alter depending upon level of purity, due to this close affinity with these contaminating materials. In general, tetraenes were more water soluble aiding separation from heptaene.

Heptaenes have a characteristic intense multi-band pattern in the UV spectrum. The amount of heptaene present in any extract or sample was estimated by UV absorbance at 405 nm.[21] However, any such assay requires caution as any value depends on the 'state of oligomerisation' of the sample. The monomeric form contributes to the UV peak at 406 nm whilst aggregate contributes to a broad peak at 340 nm.[22]

Preliminary work on KR16[17] focussed upon synthetic derivatisation of crude precipitates to the *N*-acetyl methyl ester to assist with purification but yields were disappointing. Isolates from the M57 (*amphC*) mutant were also methylated initially, but again yields were low. This led to a focus on non-chromatographic isolation of underivatised analogues, which was more likely in the long term to result in cost-effective commercial production of any promising analogue.

Commercial and medical samples of **1** are 50–90% pure preparations that contain lipid and multiple other polyenes.[23] We found that many analogues could be readily isolated in purities comparable to that of commercial **1**, however, complete removal of residual lipid, and in cases, other polyenes, was problematic. The wide range of analogues shows that the biosynthetic machinery in *S. nodosus* is flexible and efficient. Genetic engineering and improvement of culture conditions should increase titre levels to near wild-type (4 g L^{-1}) and lead to commercial production of any promising analogue at a low cost.

The *amphL* mutant

The final step in the biosynthesis of **1** is the AmphL cytochrome P450 monooxygenase catalysed insertion of a hydroxyl group at C-8 (Scheme 1). Zotchev *et al.* have isolated and purified the related NysL cytochrome P450 monooxygenase that provides the final hydroxylation step in nystatin biosynthesis.[24] Inactivation of NysL gave 10-deoxynystatin. Similar late hydroxylation also occurs in biosynthesis of the related heptaenes, candidin and mycoheptin.[25]

The *amphL* gene has been disrupted by insertional inactivation, targeted integration of a KC515 phage vector carrying a thiostrepton-resistance gene.[16] However, even when grown in thiostrepton, the mutant produced not only 8-deoxy-amphotericin B (**5**) but also **1**, indicating that the phage derived DNA could readily undergo precise excision and that the resulting strain with a functional AmphL enzyme was viable, despite the presence of the antibiotic. All subsequent *S. nodosus* mutants were generated by gene replacement (in which the chromosomal DNA is permanently replaced with an engineered version) and cannot undergo reversion in this way.

In the absence of thiostrepton in the media, most (>90%) product was **1**. However, when high levels (50 mg L^{-1}) of freshly made thiostrepton solution in DMSO (50 g L^{-1}) were added to all media, **5** was produced as the dominant heptaene (>80%). Previous work on the *amphL* mutant identified **5**.[16] Improved strains have now been isolated by strain selection of inoculum cultures based upon yield of subsequent production cultures, and production yields (>100 mg L^{-1}) improved by the inclusion of XAD16 resin in the production medium.

Isolation of 8-deoxyamphotericin B (5) from the *amphL* mutant

Extraction of sediment with methanol was inefficient due to insolubility of **5**. Schreier *et al.* have recently shown that chaotropes (water structure breakers) such as thiocyanate can increase the solubility of **1**.[22] Addition of saturated sodium thiocyanate in methanol to sedimented mycelia and resin dramatically improved the overall extraction procedure. The first extract was discarded. An equal volume of water was added to the second extraction and left at 4 °C overnight to give a yellow solid (1 g L^{-1} culture) containing by UV assay crude heptaene (100 mg L^{-1}). This assay may be an underestimate due to polyene oligomerisation in the presence of lipid. The yellow powder can be washed with methanol–chloroform or EtOAc to partially remove lipid, or redissolved in thiocyanate saturated methanol, with subsequent water addition as before, to give a yellow solid containing **5** (100 mg L^{-1}; *ca* 90% w/w by UV assay and NMR analysis). This could be further purified by RP-HPLC for NMR spectroscopic analysis that showed a very high level of similarity to that for **1**. However, whilst 7-C in **1** resonates at 29 ppm, heterocorrelation NMR spectroscopy on **5** showed a methylene carbon at 21.37 ppm correlating to protons at 1.73 and 1.01 ppm. This is in full agreement with the corresponding 7-C carbon in the 8-deoxy aglycone **3**, and appears characteristic of an 8-deoxy analogue.

The *amphNM+perDIDII* mutant

The *amphN* gene encodes the cytochrome P450 monooxygenase that oxidises the C-16 methyl group to a carboxyl group. This

amphN gene was deleted from the *S. nodosus* chromosome along with the neighboring *amphM* ferredoxin coding gene.[15] Previously reported work showed that this mutant produced 16-descarboxyl-16-methyl-amphotericin B (**8**), and that despite the loss of the 16-carboxyl group, this material retained biological activity with reduced hemolytic activity.[15] Burke *et al.* have chemically synthesised **8** and confirmed its promising therapeutic potential.[26] Zotchev *et al.* have inactivated NysN and obtained the nystatin analogue of **8** which also shows good antifungal activity and low toxicity in a mouse model of systemic mycosis.[27]

In the present study, work on a derivative of the *amphNM* mutant led to the discovery of aglycone **3** for the first time. Our interest in engineering polyene glycosylation led us to investigate biosynthesis of perimycin. This aromatic heptaene differs from the other polyene macrolides in that it lacks an exocyclic carboxyl group and contains perosamine instead of mycosamine. D-Perosamine is the 4-amino-3-hydroxyl regioisomer of D-mycosamine.[28] The *perDII* perosamine synthase and *perDI* perosamine specific glycosyltransferase genes have been isolated from *Streptomyces aminophilus*. These genes were inserted into the *amphNM* mutant in early attempts to engineer the biosynthesis of 16-descarboxyl-16-methyl-19-(*O*)-perosaminyl amphoteronolides. This strain, *amphNM+perDIDII*, is capable of synthesising both GDP-D-mycosamine and GDP-D-perosamine, however, no perosaminylated analogues were identified in production cultures. We have recently found that efficient biosynthetic replacement of mycosamine with perosamine on **1** requires both inactivation of *amphDII* and introduction of a hybrid glycosyl transferase.[29] In extensive studies reported here, production cultures of *amphNM+perDIDII* were unpredictable and could yield **8** or the aglycone **3**, or both, in varying proportions. This instability may result from recombination between directly repeated homologous *amphDI-DII* and *perDI-DII* sequences, which would lead to loss of functional glycosylation genes. Use of high levels of thiostrepton increased production of **8**, whilst subculturing *amphNM+perDIDII* on thiostrepton-free media increased production of **3**.

Isolation of 16-descarboxyl-16-methyl-amphotericin B (8) from the *amphNM+PerDIDII* mutant

The *amphNM+perDIDII* mutant was grown in the presence of thiostrepton (20 mg mL^{-1}). The sediment was extracted with methanol, and the volatiles partially removed. The resulting crude precipitate containing **8** was largely purified from tetraenes by water washes, and membrane lipid by repeated selective dissolution with sonication in methanol and subsequent precipitation with EtOAc, facilitating preparative HPLC purification. Positive ion ESMS gave peaks at 916.5 $(M+Na)^+$, or after loss of water at 876.5, corresponding to the expected mass of **8** (893.5 Da). Heterocorrelation NMR spectroscopy did not show any cross-peak at *ca.* 21 ppm to 1.7 and 1.0 ppm characteristic of an 8-deoxy compound, but instead a carbon resonance at 31.1 ppm reminiscent of **1**. Thus AmphL has efficiently oxidised (at C-8) the 16-methyl glycosylated heptaene, showing that a 16-carboxyl group is not necessary for AmphL catalysed oxidation, and that AmphL does not require AmphM ferredoxin.

Isolation of 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B (3) from the *amphNM+perDIDII* mutant

Repeated subculturing of the *amphNM+perDIDII* mutant in thiostrepton-free GYE media led to formation of increased levels of aglycone **3** in the subsequent production cultures. Methanolic extraction of sediment followed by partial removal of volatiles *in vacuo* gave a yellow precipitate of **3** (>100 mg L^{-1}) containing less than 10% other polyenes and a relatively small (<10% w/w) amount of lipid. Repeated washing with methanol improved purity, but did not remove lipid completely. Despite being initially extracted into methanol from mycelia, the purified aglycone was quite insoluble, illustrating how the properties can change with increasing purity. Despite the low solubility of **3**, most carbon and proton resonances could be assigned. Heterocorrelation NMR spectroscopy revealed a carbon resonance at 23.7 ppm correlated with protons at 1.77 and 0.99 ppm, characteristic of an 8-deoxy analogue.

Reaction of **3** with benzoyl chloride initially gave a heptabenzoylated derivative, which after continued reaction time converted into a semicrystalline octabenzoylated derivative. ^{13}C NMR spectroscopic analysis showed a ketone resonance at 203 ppm indicating that the lactol had been trapped in the ring opened form as the benzoyl ester **10** (Fig. 6). The positive electrospray gave a peak at 1582.7 $(M + water)^+$ and FAB mass spectra at 1564.67 (M^+) rather than the expected protonated analogues. The sample examined by FAB-MS contained some heptabenzoylated derivative, which FAB showed as the $(M - water)^+$ peak, indicating that the final position benzoylated is 17-hydroxyl as the ring opened lactol.

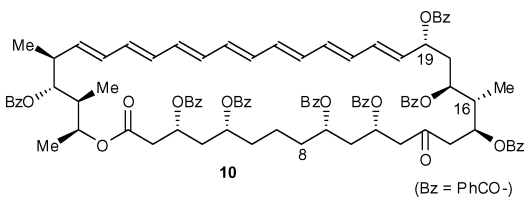


Fig. 6 Octabenzoylated aglycone (**10**).

Borowski *et al.* have previously trapped the lactol of protected **1** as the *O*-methyl oxime,[30] and MacPherson *et al*. the oxime.[31]

Analysis of pentaenes from the M57 mutant lacking polyketide synthase modules 5 and 6 in AmphC

Zotchev *et al.* engineered the nystatin PKS to produce a hexaene analogue by eliminating one module from NysC.[32] He has also reported an octaene homologue due to stuttering in the nystatin PKS.[33] The tailoring enzymes were active with both hexaene and octaene analogues.

In a previous report, we probed the flexibility of the amphoteronolide synthase by the precise deletion of two PKS modules from AmphC that normally assembles the heptaene portion of **1**.[16] Extracts of the M57 mutant gave a UV absorption spectrum with maxima at 310, 318, 333 and 352 nm, in agreement with reported pentaenes.[25]

These pentaenes have now been absorbed from the culture supernatant by the addition of XAD16, along with large quantities of contaminants from the broth. Addition of diazomethane enabled purification of pentaenes by silica chromatography. Normal

phase HPLC revealed several major pentaenes, whose RMM and NMR spectra were consistent with the methyl ester of pentaene aglycones with the propionate derived methyl group oxidised to a carboxylate, but without AmphDI glycosylation or AmphL oxidation. There was no evidence for polyenes other than pentaenes. NMR analysis of the major pentaene was consistent with **11** (as its methyl ester **12**) (Fig. 7) along with a small level of unknown aromatic impurity. The stereochemistry shown is assumed by analogy with **1**. The carbon NMR showed ten double bond resonances as expected at 130–140 ppm, a hemiacetal resonance at 97.5 ppm, and lactone or ester resonances at 171 and 173. There were three methine and eight visible methylenes between 20 and 49 ppm, including a methylene at 21.6 ppm characteristic of a 'deoxy' compound. There is expected to be a ninth methylene under the DMSO resonance at around 47 ppm by analogy with the NMR of aglycone **3**. There are nine heteroatom linked methines at 50–80 ppm (7 × hydroxy, CHCOOH and lactone alcohol), but two have shifted 5–10 ppm upfield relative to those in the aglycone (**3**) NMR spectrum.

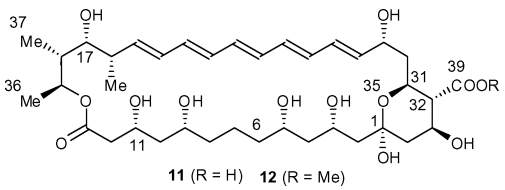


Fig. 7 Pentaene (**11**) and methyl ester (**12**).

Despite the removal of two modules, the PKS has successfully completed the acyl chain assembly, and the thioesterase formed the ring contracted lactone.

The M57 mutant has also been grown in the presence of XAD16, and in this case, the pentaenes extracted from the sediment. After methanol extraction and partial solvent removal, examination of the resultant precipitate by LCMS indicated some (<10%) glycosylated material (with and without hydroxylation by AmphL at C-6); most other peaks were isomeric with pentaene **11** with full AmphN oxidation but the conspicuous absence of any AmphL hydroxylation. This indicates that oxidation of the methyl group by AmphN is facile and occurs first, that AmphDI has reluctantly recognised the pentaene aglycone, and that AmphL catalysed oxidation only occurs after this glycosylation.

The KR16 mutant

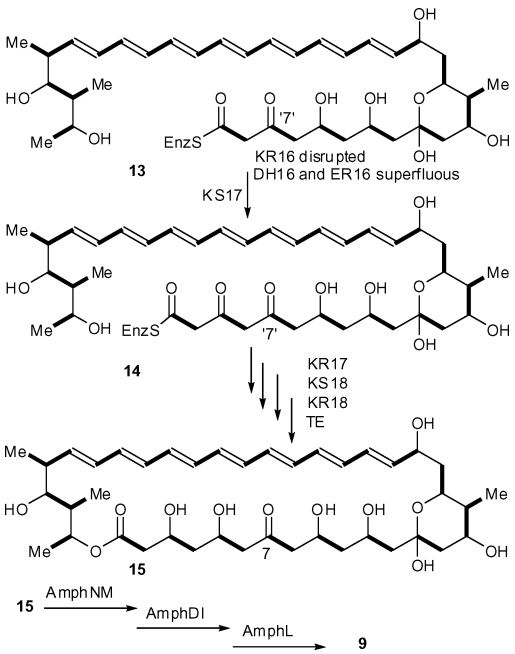
The heptaenes candidin and mycoheptin have ketone groups at C-7 and C-3, respectively, and have all three post-PKS modifications as for **1**.[34] This suggests that heptaene PKSs can tolerate inactivation of KR domains in modules 16 to 18. and, that the resulting oxoaglycones can be readily modified by all three post-PKS enzymes. Zotchev *et al.* describe the inactivation of KR16 and KR17 in the nystatin PKS, along with NysN cytochrome P450 monooxygenases to obtain a range of oxo- and descarboxyl-analogues of nystatin.[27]

We have previously replaced the KR16 active site tyrosine with phenylalanine, abolishing ketoreduction in module 16, and rendering superfluous the DH16 and ER16 enzymes, resulting in **9** isolated as the *N*-acetyl methyl ester.[17]

Isolation of 7-oxoamphotericin B (9) from the KR16 mutant

The underivatised analogue **9** has now been obtained (*ca* 100 mg L^{-1}; *ca* 75% of extract levels) by multiple washing of the original crude yellow precipitate with water, followed by washing with chloroform. Tetraenes in the crude extract are also removed by these washings. Whilst the crude material (bound to lipid) was water insoluble, this purified material is relatively soluble in water (*ca* 1 mg mL^{-1}). A major factor in the toxicity of **1** is its lack of serum-solubility that causes aggregation in serum and accumulation in the kidneys.

7-Oxo-polyene formation (*ca.* 250 mg L^{-1}) shows that the remaining modules have indeed recognised the unreduced carbonyl and continued processing the growing acyl chain as usual (Scheme 3). The unreduced 3-oxothioester in **13** is recognised by KS17 and chain elongated to the 3,5-dioxothioester **14**. Despite the enhanced enolisation and reduced conformational flexibility expected in its modified substrate, KR17 reduces the 3-oxo carbonyl in **14**, allowing for the final addition of a C_2 unit, reduction by KR18 and lactonisation (TE) to give the 7-oxo aglycone **15**. The sp^3 flexibility normally present at 'C-7' can in this case be substituted by the more constrained planar carbonyl group. This aglycone (**15**) appears to be efficiently transformed by AmphN, AmphDI and AmphL (despite the neighbouring carbonyl) to **9**.



Scheme 3 Biosynthesis of 7-oxoamphotericin B (**9**).

A sample was purified by HPLC and examined by ^{13}C NMR spectroscopy to reveal a ketone resonance at 212.75 ppm. Resonances between 65 and 80 ppm are considerable altered from those in amphotericin B itself, indicating conformational alteration upon the introduction of the sp^2 centre at 7-C into the macrolactone ring.

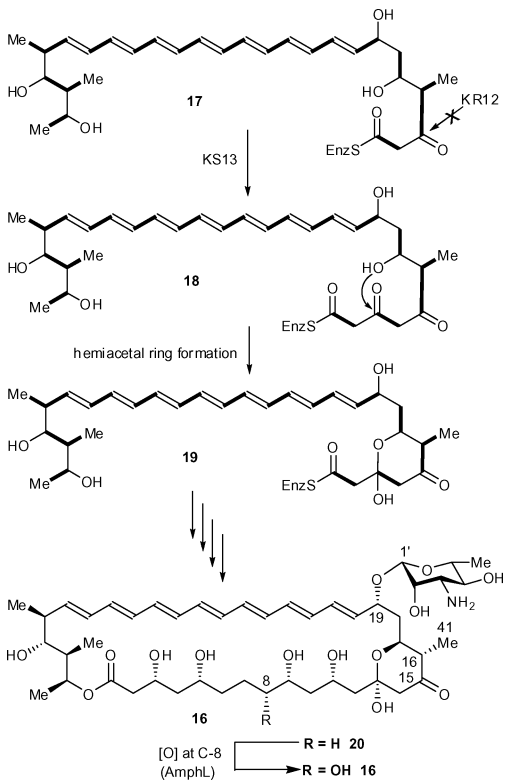
Polyene products from the ΔNM KR12 mutant

The KR12 domain was inactivated in the *amphNM* mutant.[17] HPLC and LCMS analysis of sediment extracts indicated

formation in poor yield (*ca* 2 mg L^{-1}) of 16-descarboxyl-15-deoxy-16-methyl-15-oxo-amphotericin B (**16**) and 16-descarboxyl-8,15-dideoxy-16-methyl-15-oxo-amphotericin B (**20**), the expected products of KR12 inactivation, followed by AmphDI glycosylation, with and without AmphL oxidation.

The hemiketal ring of the polyene normally results from reaction of the hydroxyl group introduced in cycle 11 with the ketone remaining after cycle 13. Though it remains unclear at which step this occurs, hemiacetal ring formation at the end of cycle 13 (processive) may be essential in folding the growing acyl chain for subsequent chain extension and processing.

There is no KR13 in the amphotericin PKS, so at the end of cycle 13 the KR12 mutant will generate a 3,5-dioxothioester **18** (Scheme 4). Hemiacetal ring formation may be slowed by the increased enolisation of this 3,5-dioxo intermediate **18** and by the additional sp^2 centre in the hemiacetal ring of **19**. If chain extension of **18** by KS14 now competes with the impaired hemiacetal formation of **18**, then an acyclic 3,5,7-trioxothioester should appear prior to ketoreduction in cycle 14. This enolised and conformationally rigid intermediate might not be recognised or processed efficiently by downstream activities, blocking the synthase. The product **16** was obtained in poor yield, without evidence of any truncated polyene products released from the PKS.



Scheme 4 Biosynthesis of deoxy-16-descarboxyl-16-methyl-15-oxo-amphotericin B (**16**).

Truncated polyketide intermediates from KR10-1 and KR10-2 mutants

The KR10 coding sequence in the *S. nodosus* genome was replaced with a version encoding an inactive enzyme using a strategy similar to that previously used with the KR12 and KR16 domains[17] and as detailed in the Experimental section (Fig. 8).

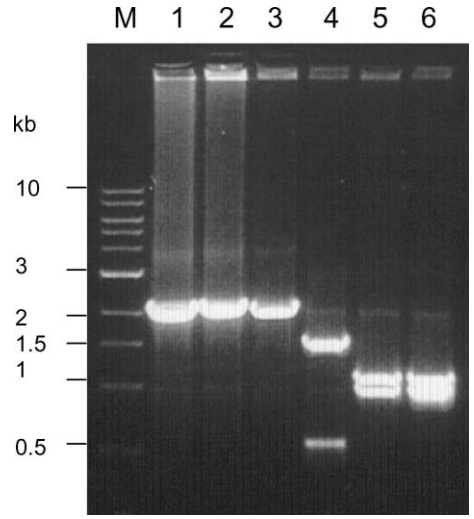


Fig. 8 Evidence for inactivation of KR10 coding sequence. PCR primers KR10CF and KR10CR2 were used to amplify the KR10 coding region from *S. nodosus* (lane 1) and the KR10-1 mutant (lane 2). Treatment with HindIII revealed that the DNA amplified from *S. nodosus* was resistant to digestion (lane 3) whereas that from the mutant contained the expected site (lane 4). In control digests, Bcl I cut the PCR products from both strains at an internal site (lanes 5 and 6).

The original aim was to engineer *in vivo* synthesis of substantial quantities of 19-deoxy-19-oxo-amphoteronolides for subsequent conversion into a library of analogues. Mycelium from the first gene replacement mutant (KR10-1) was extracted with methanol to give an orange solution with UV maxima at 422, 411, 385 and 309 that gave largely one peak on analysis by HPLC. After removal of solvent from the KR10-1 extract, the resulting solid (mass 135 mg L^{-1}) was readily soluble in ethyl acetate, and could be purified by flash chromatography to give an orange product (15 mg L^{-1} unoptimised). Negative ion electrospray mass spectrometry showed RMM of 386, as well as some singly charged dimer and trimer. ESMS indicates low levels of additionally oxygenated material with RMM 402. NMR spectroscopy showed three methyl doublets, ten vinylic protons, only four other protons, and only twenty-three carbon resonances, including three at 157, 166 and 167 ppm and two at 99 and 100 ppm consistent with a pyrone structure. Correlation spectroscopy showed connectivity from 9′-H through to 17′-H. This data is consistent with the decaketide **21**. The stereochemistry shown for **21** is that expected for an intermediate in the biosynthesis of **1**.

There are only a few instances where inactivation of a polyketide biosynthetic gene has led to formation of truncated pyrone intermediates. A polyene-pyrone heptaketide was generated by the lovastatin producer *Aspergillus terreus* when LovC (an enoyl reductase) was disrupted.[35] Overexpression and isolation of LovB gave protein that could assemble penta-, hexa- and heptaketide pyrones. The absence of SAM, and consequent lack of methylation at the tetraketide stage, has caused derailment and offloading of subsequent partially assembled acyl chains.[36] Intermediates in rifamycin biosynthesis ranging from the tetraketide to the decaketide were isolated following deletion of the *rifF* gene for

the lactam-forming chain-terminating enzyme. The tetraketide intermediate was obtained as both a pyrone and a linear carboxylic acid.[37] Prematurely released intermediates have been obtained by deletion of domains in the *trans*-AT PKS responsible for bacillaene biosynthesis.[38]

The structure of **21** is reminiscent of the chloropyrrole polyene-pyrone rumbrin produced by the fungus *Auxarthon umbrinum* and the furan polyene-pyrone gymnoconjugatin B produced by the soil ascomycete *Gymnoascus reessii*.[39]

The structure of **21** suggested that two modules of AmphC had been lost by a spontaneous deletion (precise excision) in addition to the targeted inactivation of KR10. To investigate this further, primer pairs were designed for the amplification of the DNA sequences coding for ACP3, ACP4, ACP5, ACP6 and ACP7 domains (Fig. 9). PCR analysis indicated that the ACP5 and ACP6 coding sequences were absent from the chromosome of the KR10-1 mutant, which is consistent with the absence of a dihydroanalogue of **21** that would result from reduction of one the double bonds in the polyene unit by ER5.

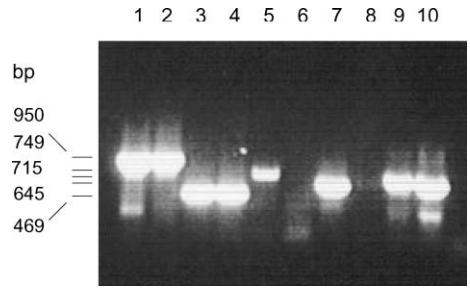
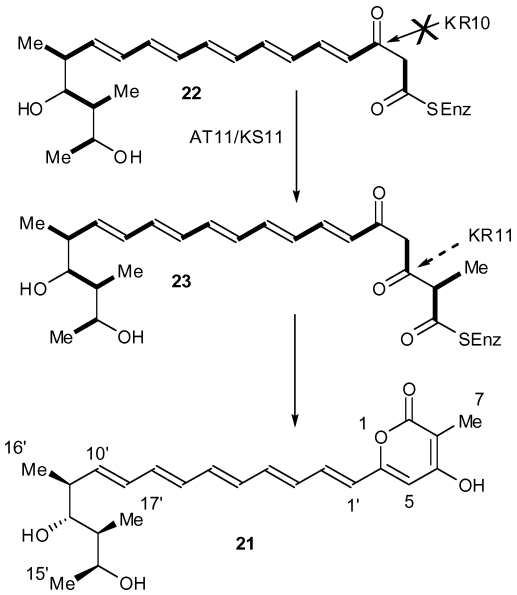


Fig. 9 PCR analysis of AmphC ACP coding sequences in *S. nodosus* and the KR10-1 mutant. Lanes 1, 3, 5, 7 and 9, DNA amplified from *S. nodosus* genomic DNA with primers for sequences encoding ACPs 3, 4, 5, 6 and 7, respectively. Lanes 2, 4, 6, 8 and 10, DNA amplified from *S. nodosus* KR10-1 genomic DNA with primers for sequences encoding ACPs 3, 4, 5, 6 and 7, respectively. The ACP5 and ACP6 coding sequences are missing from the KR10-1 mutant (lanes 6 and 8).

The structure also suggested that the 3-oxoacyl intermediate **22** normally reduced by KR10 can instead be efficiently chain extended by KS11 to give 3,5-dioxo intermediate **23** (Scheme 5). However, KR11 has not reduced **23**, and neither has any further chain elongation occurred. This was surprising because we do not observe the formation of linear pyrones by the KR16 mutant that also generates a 3,5-dioxothioester intermediate **14** in cycle 17 (Scheme 3). KR17 and chain extension operates efficiently on this intermediate.

Further KR10 gene replacement mutants were sought in an effort to obtain a strain synthesising full-length heptaenone macrolactones. This gave strain KR10-2 that generated a mixture of heptaene and tetraene dodecaketides released from PKS module 11 as pyrones. Extracts of the mycelia with ethyl acetate gave compounds that were difficult to quantify or purify. Partial purification by flash chromatography gave dark red fractions that when analysed by ESMS gave mass predominantly 437.3, and fractions with mass predominantly 439.4 corresponding to the tetraene-pyrone **24**. Tetraene-pyrone (**24**) containing fractions were analysed by NMR spectroscopy to reveal spectra similar to that for **21** except for the presence of four allylic protons at 2.20 and 2.23 ppm in the proton NMR and two allylic carbons at 33.5



Scheme 5 Biosynthesis of KR10-1 decaketide product (**21**).

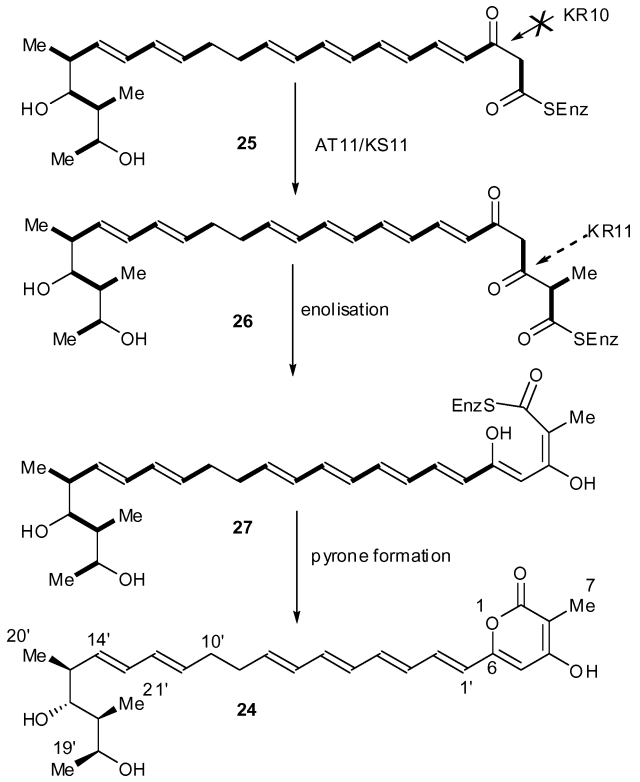
and 34.0 ppm in the carbon NMR spectrum. The yield of **24** was estimated at over 20 mg L^{-1} by NMR purity and signal to noise ratio.

No KR10 mutant was isolated that could produce a full-length macrolactone despite extensive screening. The KR10-1 strain has undergone a deletion equivalent to precise excision of modules 5 and 6. In the KR10 mutants, module 11 is still able to chain extend **22** to **23** or **25** to **26** generating the corresponding 3,5-dioxothioester system. However, this is no longer processed as usual by the otherwise functionally active KR11. This may be due to either lack of binding, incorrect folding in the active site due to the extra 5-oxo group, or due to extra enolisation that extends the system of conjugated double bonds. Ketoreduction may simply now be much slower than the competing non-enzyme catalysed pyrone formation and release from the PKS. Module 11 of the amphotericin PKS incorporates a propionate extender and is thought to epimerise the initial 2*R*-methyl branch in its product prior to ketoreduction. It has been suggested that *cis* enols feature as intermediates in epimerisation reactions in modules that generate the same (*anti*) methyl and alcohol stereochemistry as module 11.[40] Such an intermediate (**27**) might now favour spontaneous pyrone formation prior to ketoreduction by KR11 (Scheme 6). Alternatively, the decaketide and dodecaketide chains may be released from ACP11 by AmphE, a discrete editing thioesterase,[18] with spontaneous pyrone formation occurring later. Whatever the mechanism of termination, the isolation of **21** and **24** suggest that the intermediates are efficiently off-loaded from the PKS.

The polyenyl-pyrones **21** and **24** were tested for antifungal activity as described previously.[15] Neither compound showed activity even at high concentrations (>2 mg mL^{-1}).

Conclusions

A wide range of analogues of **1** have been produced *in vivo*, many with titres over 100 mg L^{-1}. This indicates that the biosynthetic machinery has sufficient flexibility to be able to produce promising



Scheme 6 Biosynthesis of KR10-2 dodecaketide tetraene (**24**).

analogues at levels and cost comparable to **1**. Many compounds displayed strong affinity for membrane lipid and low solubility, complicating full purification and analysis at current titre levels. Most analogues have been readily prepared in purity similar to commercial samples of **1** using non-chromatographic methods that could be affordably scaled up. The compounds isolated support the non-obligate preferred order of final biosynthetic steps being oxidation at C-16 by AmphN, glycosylation at O-19 by AmphDI, followed by oxidation at C-8 by AmphL. The AmphL oxidation occurs more readily on glycosylated compounds, whilst AmphDI glycosylation can readily occur on 16-descarboxyl-16-methyl analogues. Deletion of two PKS modules from AmphC gave fully assembled pentaene-carboxylate compounds, demonstrating not only the potential flexibility of the downstream modules including the final lactonising thioesterase, but also the AmphN oxidation enzyme.

Disruption of a ketoreductase (KR) close to the end of the PKS acyl chain assembly process led efficiently to the expected 7-oxo-product with full subsequent acyl chain extension and lactonisation. In contrast, KR12 disruption gave only low yields of the corresponding 15-oxo-products suggesting that hemiacetal formation is 'processive' and moderated by the introduction of a nearby carbonyl group. Disruption of KR10 resulted in no detectable polyene macrolide. Direct acyl chain extension of the KS10 intermediate by KS11 to a 3,5-dioxothioester has resulted in efficient cyclisation to linear polyenyl-pyrones in preference to reduction by the next domain, KR11, and further chain extension to a macrolide. The truncated linear polyenyl-pyrones synthesised by the viable KR10 mutants are analogous to bioactive polyenyl-pyrones synthesised by several fungal species. The truncated amphotericin PKS might be used for precursor-directed synthesis of new pyrones.

Experimental

General

UV assays of polyenes were run on a Shimadzu UV-2401PC spectrophotometer. For heptaenes, an absorbance ε of 1.7×10^5 M^{-1} cm^{-1} at 405 nm was used, based upon the value reported for monomeric amphotericin, and for tetraenes an absorbance ε of 0.78×10^5 M^{-1} cm^{-1} at 318 nm was used.[21] Pentaenes were assayed using ε of 0.85×10^5 M^{-1} cm^{-1} at 333 nm.[25]

HPLC analyses were performed on a Varian Prostar diode array with Galaxie workstation and software. Analytical HPLC were run using using 4.6 mm × 25 cm Supelco silica or C-18 silica (5 μm) with 1 mL min^{-1} flow rate and semipreparative HPLC using 21.2 mm diameter columns at 14.8 mL min^{-1} flow rate using silica, C-8 or C-18 columns. In general, every passage through silica or HPLC dramatically reduced yield.

NMR spectra were collected on a Bruker AV500 MHz AvanceIII, or DRX400 spectrometer. Chemical shifts are quoted in ppm. Coupling constants quoted in near-first order sytems are 'observed' values. Proton resonances are (1 H, m) unless otherwise indicated. Attached proton test (APT) or 'DEPT135′ used in ^{13}C NMR multiplicity assignments.

Electrospray mass spectrometry was performed on a triple quadropole Micromass Quattro LC machine or Kratos Concept 1H double focussing high resolution spectrometer. FAB used 3-nitrobenzyl alcohol as matrix. Quadropole time of flight mass spectrometry (QTOFMS) was run on a XEVO instrument with Waters Accuity UP-LC.

ATR infra red spectroscopy was run on a Perkin Elmer 'Spectrum One' instrument.

Cultures are grown in 'trigrooved' Erlenmeyer flasks (250 mL or 2 L; washed in deionised water) in environmentally controlled shakers (New Brunswick Series 25 with 25 mm gyrotary and Sanyo Gallenkamp 'Orbisafe' 32 mm gyrotary). Media components are from Sigma and made up in deionised water, and the flask necks plugged with a sponge before autoclaving.

Thiostrepton was added to cultures of strains containing a thiostrepton resistance marker. Stock solution of 100 mg mL^{-1} were made up in DMSO and frozen at −26 °C until needed. Working concentrations of thiostrepton were 5 μg mL^{-1} in liquid culture and 50 μg mL^{-1} in solid culture unless stated. Centrifugation was performed using a Sorvall RC5B centrifuge with a GSA (13,000 rpm) or SS34 rotor (20,000 rpm).

All cultures are maintained at −80 °C as glycerol deeps. The contents (1.5 mL) are added to a GYE preculture medium of glucose (10 g L^{-1}), yeast extract (10 g L^{-1}) and deionised water (100 mL) adjusted to pH 7 with sodium hydroxide (2 M) and incubated (28 °C; 200 rpm) for 48 h. Aliquots (10 mL) are then used to inoculate production media (28 × 2 L trigrooved flasks each containing 250 mL broth) containing fructose (20 g L^{-1}), Dextrin (corn, Type II; 60 g L^{-1}), Soybean flour (Type I not roasted; 30 g L^{-1}), $CaCO_3$ (10 g L^{-1}) and Amberlite XAD16 resin (50 g L^{-1}) autoclaved at 121 °C for 20 min. The remaining preculture was retained, and if polyene production levels were found to be high, the contents were used to make fresh growth and/or glycerol deeps.

The inoculated production media was incubated at 28 °C for 4–5 days to give a distinctive earthy odour, and copious mycelia that when shaken slid slowly down the sides of the Erlenmeyer flask. The mycelia and XAD16 resin was collected (GSA; 10 min).

All water baths used in rotary evaporation of extracts or concentration of extracts ('*in vacuo*') were kept below 40 °C.

8-Deoxyamphotericin B (5)

All media used in production of polyenes from the *amphL* mutant contained thiostrepton (50–100 μg mL^{-1}). The mycelia and resin from production media (7 L) was added to a solution of sodium thiocyanate (900 g) in methanol (1.5 L) and shaken (200 rpm) in trigrooved flasks for 2 h to give a dark coloured solution containing heptaene (350 mg). The mycelia and beads were collected (GSA, 10 min) and the extraction repeated with fresh methanol/thiocyanate solution (1.5 L). Assay of the second extract indicated heptaene (1.4 g) in a much clearer solution. An equal volume of water was added to this second extract, and left at 4 °C overnight. The resulting precipitate was collected (GSA, 10 min), washed with water (2 × 250 mL), redissolved in thiocyanate saturated methanol and precipitated with an equal volume of water as before. The resulting precipitate was washed with water (2 × 250 mL) and freeze dried to give a yellow solid (8 g) that by UV assay in DMSO/methanol contained heptaene (800 mg). The yellow solid was dissolved in DMSO (5 × 50 mL) and any remaining insoluble material was removed by centrifugation, and then ether (250 mL) added to give a precipitate. Addition of DMSO to the precipitate gave an oil that contained concentrated heptaene (10–15 mg mL^{-1}). Addition of EtOAc (8 vol) gave a clear supernatant and a precipitate that was washed (MeOH; 3 × 100 mL) to give **5** (0.9 g; 90% heptaene w/w by UV assay) with reduced contamination by **1**. HPLC (C-18; water–methanol containing methanoic acid 0.1% v/v) was used to obtain a sample free of **1**.

δ_H(500 MHz; d^6-DMSO) 0.92 (3 H, d, *J* 6.9, 39-H), 1.01 (7b-H), 1.04 (3 H, d, *J* 5.9, 40-H), 1.08 (6b-H), 1.10 (14b-H), 1.12 (3 H, d, *J* 6.3, 38-H), 1.17 (8b-H), 1.18 (3 H, d, *J* 4.9, 6′-H), 1.24 (2 × 1 H, 2 × m, 6a-H and 8a-H), 1.31 (4b-H), 1.34 (2 H, m, 10ab-H), 1.38 (4a-H), 1.51 (12b-H), 1.56 (18b-H), 1.58 (H-12a), 1.73 (7a-H), 1.74 (36-H), 1.91 (14a-H), 1.94 (16-H), 2.10 (18a-H), 2.16 (2 H, m, 2ab-H), 2.29 (34-H), 2.85 (3′-H), 3.10 (35-H), 3.18 (4′-H), 3.21 (5′-H), 3.46 (5-H), 3.58 (9-H), 3.77 (2′-H), 4.02 (15-H), 4.06 (3-H), 4.20 (17-H), 4.23 (11-H), 4.40 (1 H, br s, 19-H), 4.46 (1 H, br s, 1′-H), 5.21 (1 H, *ca*. dq, 37-H), 5.43 (1 H, dd, *J* 14.9 and 9.9, 33-H), 5.95 (1 H, dd, *J* 14.8 and 8.8, 20-H), 6.04–6.39 (11 H, m), 6.45 (1 H, *ca* dd).

Partial δ_C(125.8 MHz; d^6-DMSO) 11.9 (39-C), 16.8 (38-C), 17.5 (6′-C), 18.2 (40-C), 21.4 (7-C), 36.9 (18-C), 38.5 (6-C), 38.8 (8-C), 39.5 (36-C), 41.9 (2-C), 42.2 (34-C), 43.6 (10-C), 44.1 (14-C), 44.6 (4-C), 46.3 (12-C), 55.1 (3′-C), 57.2 (16-C), 65.2 (17-C), 65.5 (15-C), 66.1 (3-C), 68.3 (11-C), 68.8 (37-C), 68.9 (2′-C), 69.1 (5-C), 69.9 (4′-C), 71.3 (9-C), 72.7 (5′-C), 74.8 (19-C), 77.2 (35-C), 96.3 (1′-C), 128.9 (d), 130.9 (21-C), 136.2 (20-C), 136.8 (33-C).

m/z (+ve ES) 908 $(MH)^+$

m/z (−ve ES) 906.3 $(M-H)^-$

Construction of *S. nodosus* Δ*amphNM* + *perDI-DII*

Phage KC-Per1 contains a StuI-BamHI insert consisting of the *amphDI* upstream region (nucleotides 65861 to 68195 of sequence with GenBank accession number AF357202) fused to the *perDI* and *perDII* genes. Both *amphDI* and *perDI* genes have an NcoI site (5′ CCATGG 3′) surrounding the start codon. The *S. nodosus* DNA was ligated to the *S. aminophilus* DNA through this conserved site. This positioned the *perDI* start codon at the optimum distance from the *amphDI* promoter and ribosome-binding site. The KC-Per1 phage integrated into the *S. nodosus* Δ*amphNM* chromosome by recombination between the homologous *amphDI* upstream sequences. This gave a thiostrepton-resistant lysogen *S. nodosus* Δ*amphNM* + *perDI-DII* which contains the related *perDI-perDII* and *amphDI-DII* regions in direct repeat orientation. The genotype was verified by Southern hybridisation.

16-Descarboxyl-16-methyl-amphotericin B (8)

The mycelia and XAD16 resin sedimented from the *amphNM*+*perDIDII* mutant [7 L growth; with thiostrepton (20 mg L^{-1}) in both preculture and production broth] was soaked in methanol (3 × 7 L; 3 × 6 h) to give an extract containing heptaene (1.13 g) and tetraene (1.1 g). Partial removal of combined solvent *in vacuo* gave a yellow precipitate (600 mg heptaene and 700 mg tetraene in 6 g). The precipitate was dissolved in methanol (100 mL), water added (300 mL), left at 4 °C for 2 h, and the precipitate collected (400 mg heptaene and 300 mg tetraene in 1.6 g). This was dissolved in methanol (*ca* 25 mL), EtOAc slowly added (20 volumes) and left at 4 °C for 4 h to give a precipitate (110 mg heptaene and less than 5 mg tetraene). More heptaene can be recovered from the supernatant by repeating this procedure. A sample was purified by preparative HPLC (C-18, water–methanol plus methanoic acid 0.1%) and examined by NMR spectroscopy.

λ_{max} (MeOH)/nm 405, 383 and 362.

Partial δ_H(500 MHz; d^4-MeOH) 1.037 (3 H, d, *J* 7.1, 41-H), 1.039 (3 H, d, *J* 6.1, 39-H), 1.14 (3 H, d, *J* 6.3, 40-H), 1.22 (3 H, d, *J* 6.4, 38-H), 1.25, 1.31 (3 H, br d, *J* 5.7, 6′-H), 1.36, 1.39, 1.42, 1.47, 1.51, 1.61, 1.73 and 1.74, 1.93 (36-H), 2.00 (1 H, dd, *J* 12.2 and 4.7), 2.23 (1 H, ABX, 2b-H), 2.24 (18a-H), 2.30 (1 H, ABX, 2a-H), 2.41 (34-H), 3.10 (1 H, br d, 8-H), 3.20, 3.22 (35-H), 3.30 (5′-H), 3.39 (9-H), 3.59, 3.63, 3.74, 3.93 and 3.99, 4.20 (1 H, tt, *J* 9.6 and 3.0), 4.35, 4.46 (19-H), 4.64 (1 H, br s, 1′-H), 5.40 (2 × 1 H, 2 × m, 33-H and 37-H), 6.04 (1 H, dd, *J* 15.2 and 8.9, 20-H), 6.15-6.5 (12 H, m).

Partial δ_C(125.8 MHz; d^4-MeOH) 12.2 (39-C), 13.5 (41-C), 17.1 (38-C), 17.6 (6′-C), 19.0 (40-C), 31.1 (t), 33.3 (18-C), 36.2 (t), 38.2 (t), 40.7 (t), 41.7 (36-C), 42.7 (2-C), 43.6 (16-C), 44.1 (34-C), 44.7 (t), 45.6 (t), 48.1 (t), 57.2 (8-C), 68.9 (d), 69.6 (d), 69.7 (d), 70.3 (d), 70.6 (9-C), 70.7 (37-C), 71.1 (d), 72.6 (d), 74.5 (5′-C), 75.7 (d), 75.9 (d), 79.4 (19-C), 79.8 (35-C), 99.1 (1′-C).

m/z (+ve ES) 916.5 $(M+Na^+)$, 876.5 $(M-water+H^+)$.

m/z (FAB) Found: 894.51939 (MH^+). $C_{47}H_{76}NO_{15}$ requires 894.51959.

8-Deoxy-16-descarboxyl-16-methyl-amphoteronolide B (3)

The mycelia and XAD16 resin sedimented from *amphNM*+*perDIDII* mutant (7 L growth grown with thiostrepton in the innoculum but not the production medium) was repeatedly (x 6) soaked in methanol (5 L) overnight with occasional shaking. After each overnight soaking, the sediment was collected (GSA 10,000) for further soaking. Partial removal of combined solvent

in vacuo and storage at 4 °C gave a yellow precipitate which was collected by centrifugation. The precipitate was resuspended in MeOH (500 mL) vigorously shaken with sonication, and the remaining yellow powder collected by Buchner filtration. This process was repeated until washings are clear, to give a yellow powder (500 mg) found to be analytically pure, but estimated by UV assay at 95% purity by weight, and containing some (< 5%) membrane debris by NMR analysis.

Found C, 67.32; H, 8.69%. $C_{41}H_{63}O_{11}$ requires C, 67.28; H, 8.68%;

λ_{max} (MeOH)/nm 404, 381, 362.

δ_H(500 MHz; d^6-DMSO) 0.88 (3 H, d, *J* 6.3, 41-H), 0.92 (3 H, d, *J*, 7.0, 39-H), 0.99 (7b-H), 1.04 (3 H, d, *J* 6.3, 40-H), 1.06 (16-H), 1.08 (6b-H), 1.11 (3 H, d *J* 6.0, 38-H), 1.13 (14b-H), 1.16 (8b-H), 1.23 (8a-H), 1.29 (H-4b), 1.31 (6a-H), 1.32 (2 H, m, 10ab-H), 1.38 (4a-H), 1.48 (12b-H), 1.52 (18b), 1.54 (18a-H), 1.73 (36-H), 1.77 (7a-H), 1.83 (1 H, *ca* dd, 14a-H), 1.90 (1 H, *ca* dd, 18a-H), 2.10 (1 H, ABX, 2a-H), 2.16 (1 H, ABX, 2b-H), 2.30 (34-H), 3.09 (35-H), 3.38 (15-H), 3.43 (1 H, br s, 5-H), 3.57 (9-H), 3.85 (1 H, *ca* t, *J* 9.2, 17-H), 4.07 (3-H), 4.19 (19-H) 4.21 (11-H), 4.50 (1 H, d, *J* 4.7, 5-OH), 4.61 (1 H, d, *J* 4.7, 5-OH), 4.71 (1 H, d, *J* 4.1, 19-OH), 4.78 (1 H, *ca* d, 35-OH), 4.79 (1 H, *ca* d, 35-OH), 4.99 (1 H, d, *J* 4.1, 9-OH), 5.21 (1 H, *ca* dq, 37-H), 5.42 (1 H, dd, *J* 15.0 and 10.0, 33-H), 5.49 (1 H, br s, 11-OH), 5.65 (1 H, br s, 13-OH), 5.96 (1 H, dd, *J* 15.1 and 10.1, 20-H), 6.06 to 6.43 (19 H, m, 21-H to 33-H).

δ_C(125.8 MHz; d^6-DMSO) 14.1 (39-C), 15.2 (41-C), 18.9 (38-C), 20.4 (40-C), 23.7 (7-C), 40.8 (6-C), 41.0 (8-C), 41.5 (36-C) 42.1 (18-C), 43.9 (2-C), 44.42 (34-C), 44.43 (16-C), 45.7 (10-C), 46.9 (4-C), 47.1 (14-C), 48.7 (12-C), 68.1 (3-C), 69.9 (15-C), 70.3 (11-C), 70.7 (37-C), 70.8 (17-C), 70.9 (5-C), 71.3 (19-C), 73.2 (9-C), 79.2 (35-C), 98.8 (13-C), 129.3 (31-C), 133.1, 133.2, 133.7, 134.0, 134.2, 134.4, 134.5, 135.0, 135.5, 135.6, 135.7, 138.8 (33-C), 142.2 (20-C), 172.6 (1-C).

m/z (+ve ES) 755.5 (M+Na$^+$).

m/z (−ve ES) 731.3 (M-H$^+$), 713.5 (M-H$_3$O$^+$).

(3*R*,5*R*,9*S*,11*S*,15*R*,17*S*,19*S*,35*S*)-Octabenzoyloxy-13-oxo-(16*S*,34*S*,36*S*)-trimethyl-(20*E*,22*E*,24*E*,26*E*,28*E*,30*E*,32*E*)-octatriacontaheptaeno-(37*S*)-lactone 10

Crude 8-deoxy-16-descarboxyl-16-methyl-amphoteronolide B **3** (323 mg heptaene in 500 mg; 0.44 mmol) was suspended in DCM (125 mL) and pyridine (25 mL) was added. Benzoyl chloride (13 mL, 0.09 mmol) was added dropwise at RT, followed by DMAP (10 mg, 0.08 mmol). The reaction was followed by TLC (40% EtOAc–hexane). The reaction mixture went a clear yellow solution after 30 min, was stirred for 3 days at RT then washed with water (150 mL), sodium hydrogen carbonate (150 mL; saturated), copper sulfate (150 mL; saturated) and water (150 mL). After removal of volatiles *in vacuo*, the residue was dry loaded onto a flash column which was flushed (hexane) and then heptaene eluted (30% EtOAc–hexane) to give *title compound* (325 mg), which was analysed by silica HPLC (20% EtOAc–hexane).

λ_{max} (MeOH)/nm 407, 384 and 365.

υ_{max} (ATR)/cm^{-1} 2928 (w), 1787 (w), 1713 (s), 1690 (s), 1602 (m), 1584 (m), 1266 (s) and 706 (s).

δ_H(500.1 MHz; $CDCl_3$) 0.75 (3 H, d, *J* 7.2, 41-H), 0.90, 1.00 (3 H, *J* 6.5, 40-H), 1.03 (3 H, d, *J* 6.9, 39-H), 1.19 (3 H, d, J 6.5, 38-H), 1.30, 1.35, 1.60, 1.65 (2 H, m), 1.87 (1 H, ddd, *J* 15.0, 5.9 and 3.1), 2.02, 2.08, 2.14, 2.16, 2.31, 2.34, 2.65 (1 H, dd *J* 16.1, 6.5, 2-H), 2.67 (2-H), 2.72 (34-H), 2.74 (1 H, dd *J* 16.6, 3.5), 2.84 (1 H, dd, *J* 16.6, 9.2), 2.89 (1 H, dd, *J* 18.2, 6.8), 3.05 (1 H, dd, *J* 18.2, 4.3), 5.00 (2 × 1 H, 2 × *ca* d), 5.16, 5.24, 5.40, 5.42, 5.45 (33-H), 5.47 (37-H), 5.48 (19-H), 5.55, 5.80 (1 H, dd *J* 9.3 and 9.1, 20-H), 6.22–6.54 (12 H, m, 21 to 32-H), 7.22–7.35 (8 H, m, Ar-*meta*), 7.41–7.52 (12 H, m, ArH 4 × *para* + 8 × *meta*), 7.55–7.62 (4 H, m, *para*), 7.78–7.82 (2 H, *ca* t, *ortho*), 7.88–7.92 (6 H, m, *ortho*), 8.01–8.08 (6 H, m, *ortho*), 8.13–8.17 (2 H, m, *ortho*).

δ_C(125.8 MHz; $CDCl_3$) 10.4 (q), 11.1 (q), 16.3 (q), 18.2 (q), 21.0 (t), 34.3 (t), 34.5 (t), 34.8 (t), 38.21 (t), 38.24 (t), 38.8 (t), 39.0 (d), 40.6 (d), 41.2 (d), 46.7 (t), 47.1 (t), 68.6 (d), 68.7 (d), 69.0 (d), 69.7 (d), 71.3 (d), 71.46 (d), 71.50 (d), 73.7 (d), 79.3 (d), 128.13 (d), 128.16 (d), 128.2 (d), 128.3 (d), 128.44 (d), 128.49 (d), 128.53 (d), 129.5 (d), 129.6 (d), 129.65 (d), 129.7 (d), 129.85 (d), 129.9 (d), 130.0 (d), 130.1 (d), 130.2 (s), 130.3 (s), 130.9 (d), 132.3 (d), 132.5 (d), 132.6 (d), 132.7 (d), 132.77 (d), 132.80 (d), 132.9 (d), 133.0 (2 C, d), 133.1 (d), 133.3 (2 C, d), 133.4 (2 C, d), 133.6 (d), 133.7 (d), 133.9 (d), 134.0 (d), 134.1 (d), 134.3 (d), 134.7 (d), 135.0 (d), 136.7 (d), 149.0 (d), 165.49 (s), 165.51 (s), 165.58 (s), 165.64 (s), 166.01 (s), 166.02 (s), 166.19 (s), 166.35 (s) 169.0 (s), 203.0 (s).

m/z (+ve ES) 1582.7 (M+water)$^+$

m/z (FAB) 1564.67 (M)$^+$, 1442 (Heptabenzoyl-water)$^+$.

(1*R*,3*S*,5*S*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,21*E*,23*E*,25*E*,27*E*,29*R*,31*S*,32*R*,33*S*)-1,3,5,9,11,17,29,33-Octahydroxy-15,16,18-trimethyl-13-oxo-14,35-dioxabicyclo[29.3.1]pentatriaconta-19,21,23,25,27-pentaene-32-carboxylic acid (11) and methyl (1*R*,3*S*,5*S*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,21*E*,23*E*,25*E*,27*E*,29*R*,31*S*,32*R*,33*S*)-1,3,5,9,11,17,29,33-octahydroxy-15,16,18-trimethyl-13-oxo-14,35-dioxabicyclo[29.3.1]pentatriaconta-19,21,23,25,27-pentaene-32-carboxylate (12)

The M57 mutant was grown (8 × 250 mL) in the absence of XAD16 resin. Sediment was soaked in in MeOH (800 mL L^{-1} culture) for 18 h, but found to contain only low levels of pentaene (<20 mg L^{-1}). UV Assay of the combined supernatant (1.6 L) indicated pentaene (465 mg L^{-1}).

XAD16 resin (100 g) was washed with water (3 × 50 mL) and MeOH (3 × 50 mL) and then added batchwise (3 × 30 g) to the supernatant, stirred (30 min), and filtered. Methanol (4 × 50 mL) was added to the beads batchwise with stirring to obtain the pentaenes (630 mg), the beads were filtered and the volatiles were then removed in *vacuo*. The resulting solid was dissolved in DMSO (10 mL), methanol (800 mL) was then added followed by excess ethereal diazomethane. Removal of volatiles *in vacuo* gave a dark brown oil to which MeOH (50 mL) and then ether (100 mL) was added. After discarding the upper ethereal layer, EtOAc (200 mL) was added to the methanolic layer. The resulting solid was removed and extracted again (200 mL; 20% MeOH–EtOAc) to give total pentaene (550 mg in 400 mL). The volatiles were removed *in vacuo*, the remaining solid redissolved in methanol (10 mL) and dry loaded for purification by flash chromatography (15–20% MeOH–EtOAc) to give a pentaene (150 mg) containing fraction which was analysed by normal phase HPLC (MeOH–EtOAc) as several different closely running pentaenes, all free of other polyenes. The first major peak was partially purified by HPLC to give methyl ester **12** (13 mg).

λ_{max} (MeOH)/nm 350, 332, 317 and 303(sh) nm.

^{1}H NMR (400 MHz; d^{4}-MeOH, partial) 1.02 (3 H, d, *J* 7.1, 35-H). 1.07 (3 H, d, *J* 6.6, H-36), 1.22 (3 H, d, *J* 6.3, 34-H), 2.06 (1 H, dd, *J* 12.4 and 5.0), 2.43 (2 H, m), 5.15 (*ca* t, *J* 5.1), 5.56 (1 H, dd, *J* 14.3 and 9.3), 5.94 (1 H, dd, *J* 14.9 and 8.4), 6.13–6.40 (CH=).

^{13}C NMR (100 MHz; d^{4}-MeOH) 12.8 (q), 16.9 (q), 17.5 (q), 21.7 (t), 37.3 (t), 37.7, 40.5 (t), 40.6 (t), 41.8 (t), 42.4 (d), 43.3 (d), 43.5 (t), 46.4 (t), 47 (under solvent), 48.4 (d), 50.8 (d), 56.9 (d), 66.0 (d), 66.1 (d), 67.0 (d), 67.9 (d), 70.0 (d), 71.1 (d), 78.0 (d), 97.80 (lactol), 130.3, 130.6, 131.06 (2 C),, 131.9, 133.1, 133.37, 133.45, 136.2, 138.0, 171.2 (s), 173.3 (s).

High Res FAB Found: 747.39300. $C_{38}H_{60}O_{13}Na$ requires 747.39319.

A second and third methylated pentaene fraction was isolated by HPLC and examined by ESMS 'Pentaene 2': ESMS (–ve) 723 (M-H) and 'Pentaene 3' ESMS (–ve) 723. FAB (+ve) 747 $(M+Na)^{+}$. NMR analysis of these crude fractions were consistent with related pentaene analogues.

The M57 mutant was then grown (1 L) in the presence of XAD16 resin. Extraction of sediment with methanol (1 L) gave pentaene (assay indicated 200 mg). Partial removal of volatiles *in vacuo* gave a precipitate which when examined by LCMS showed that early fractions contained glycosylated pentaenes most later fractions were isomeric with **11** with little evidence of AmphL catalysed hydroxylation, or compounds in which the methyl group had not been oxidised to a carboxyl by AmphNM.

7-Oxo-amphotericin B (9)

Sedimented mycelia and XAD16 resin from the KR16 mutant (7 L broth) was soaked (3 × 18 h) in methanol (3 × 3 L) with occasional agitation. UV assay of the combined supernatants indicated that heptaenes (850 mg) and tetraenes (850 mg) were both present. Partial removal of volatiles *in vacuo* (to *ca* 300 mL) followed by storage (4 °C; 18 h) gave a yellow precipitate that was collected by centrifugation. Partial dissolution in methanol (1 L) with vigorous agitation and sonication, removal of the insoluble debris, and then removal of volatiles gave heptaene (700 mg) plus tetraene (400 mg) as a waxy yellow solid containing heptaene (1–20% w/w). The solid was vigorously washed with water (10 × 300 mL). The remaining solid was partially redissolved in methanol (1 L), insoluble debris was removed by centrifugation and volatiles then removed *in vacuo* to give a yellow solid (600 mg heptaene, 50 mg tetraene, 40–80% pure by weight). The solid was vigorously shaken and sonicated with chloroform (500 mL), the resulting suspension filtered through filter paper to give a yellow precipitate and orange filtrate. The precipitate was washed with chloroform (500 mL) to give a yellow solid (600 mg heptaene, 20 mg tetraene, 65–85% purity by UV assay and NMR analysis), which was further purified by RP-HPLC (water–methanol+0.1% v/v formic acid) for spectroscopic characterisation.

λ_{max} (MeOH)/nm 406, 383, 364.

δ_H(500.1 MHz; d^{4}-MeOH) 1.03 (3 H, d, *J* 7.2, 39-H), 1.15 (3 H, d, *J* 6.4, 40-H), 1.22 (3 H, d, *J* 6.3, 38-H), 1.29 (3 H, d, *J* 5.7, 6′-H), 1.30 (18b-H), 1.39 (1 H, dt, *J* 14.1 and 3.0, 4b-H), 1.52 (1 H, dt, *J* 14.1 and 9.8, 4a-H), 1.62–1.68 (3 H, m, 12b-H and 10ab-H), 1.69 (14b-H), 1.75 (1 H, dd, *J* 14.7 and 10.9, 12a-H), 1.82 (1 H, *ca* ddq, *J* 1.6, 1.6 and 7.3, 36-H), 2.00 (1 H, dd, *J* 12.4 and 4.8, 18a-H), 2.04 (1 H, t, *J* 10.5 16-H), 2.19 (1 H, dd, *J* 17.1 and 3.2, 2b-H), 2.26 (2a-H), 2.28 (14a-H), 2.40 (34-H), 2.53 (1 H, dd, *J* 17.5 and 3.0, 6b-H), 2.88 (1 H, dd, *J* 17.5 and 9.2, 6a-H), 3.11 (1 H, dd, *J* 9.3 and 2.9, 4′-H), 3.21 (1 H, dd, *J* 9.3 and 2.1, 35-H), 3.34 (5′-H), 3.87 (1 H, d, *J* 2.2, 8-H), 4.00 (1 H, br d, *J* 3.2, 3′-H), 4.22 (1 H, dt, *J* 2.2 and 7.0, 9-H), 4.26 (11-H), 4.29 (2 × 1 H, 2 × m, 3-H and 17-H), 4.37 (1 H, ddd, *J* 9.2, 3.0 and 3.0, 5-H), 4.39 (11-H), 4.41 (17-H), 4.42 (19-H), 4.59 (1 H, br s, 1′-H), 5.36 (37-H), 5.38 (33-H), 6.02 (1 H, dd, *J* 15.2 and 8.8, 20-H), 6.12–6.43 (12 H, m, 21-H to 32-H).

Partial δ_C(125.8 MHz; d^{6}-DMSO, 308 K) 12.6 (q), 17.46 (q), 18.3 (q), 18.9 (q), 40.2 (d), 40.4 (d), 40.6 (t), 42.3 (t), 43.0 (d), 43.9 (t), 44.6 (t), 47.0 (wk, t), 47.5 (t), 56.3 (d), 58.6 (d), 65.6 (d), 65.9 (d), 66.0 (d), 66.1 (d), 66.5 (2 C, 2 × d), 68.4 (d), 69.6 (d), 70.3 (d), 70.5 (d), 73.2 (d), 75.8 (d), 78.1 (d), 79.8 (d), 97.0 (d), 97.7 (s), 129.7 (d), 131.5 (d), 132.3 (d), 132.5 (d), 132.5 (d), 132.6 (d), 132.6 (d), 133.9 (2 C, 2 × d), 134.0 (d), 134.56 (d), 134.65 (d), 136.7 (d), 137.2 (d), 171.2 (s), 212.8 (s).

m/z (+ve ES) 938.5 (MH^{+}).

m/z (–ve ES) 936.5.

m/z (QTOF) Found 938.4752 (MH^{+}) $C_{47}H_{72}NO_{18}$ requires 938.4749.

ΔNM KR12 mutant

Mycelia and XAD16 resin from the ΔNM KR12 mutant (1 L; grown in the absence of thiostrepton), was extracted with methanol (1 L; o/n; RT). UV assay of the extract indicated heptaene (9 mg) with negligible levels of tetraene present. Partial removal of solvent *in vacuo* (to less than 20 mL) gave an oil that was removed from remaining solvent. The oil was resuspended in MeOH (50 mL) and assayed to contain heptaene (2.5 mg). Analysis by LCMS gave 15-deoxy-16-descarboxyl-16-methyl-15-oxo-amphotericin B (**16**) [*m/z* (+ve ES) 892.7 (MH^{+}) and 874.6 $(M\text{-water}+H^{+})$, *m/z* (–ve ES) 891.7 $(M-H^{+})$ and 936.7 $(M+HCOO^{-})$] and 16-descarboxyl-8,15-dideoxy-16-methyl-15-oxo-amphotericin B (**20**) [*m/z* (+ve MS) 876.7 (MH^{+}) and *m/z* (–ve ES) 920.7 $(M+HCOO^{-})$].

Construction of KR10 mutants

The 3′ region of the KR10 coding sequence was amplified by PCR with primers KR10F2 and KR10R2. The amplified DNA was digested with EcoRI and HindIII and cloned into plasmid pUC118 to give pUC-KR103′. The 5′ region of the coding sequence was amplified with KR10F1 and KR10R1, digested with HindIII and inserted into the HindIII site of pUC-KR103′. A plasmid clone containing the second insert in the correct orientation was named pUC-KR10. In this construct the sequence AGC-GCC-TAC encoding Ser^{3088}-Ala^{3089}-Tyr^{3090} of AmphI was replaced by GCA-AGC-TTC specifying Ala-Ser-Phe. Substitution of the active site tyrosine (Tyr3090) with phenylalanine abolishes KR activity without disrupting PKS protein structure or diminishing product yield.[17] The mutagenesis also introduces a HindIII site that can be used as a marker. The modified coding sequence was excised with BglII and PstI and cloned into phage KC-UCD1 to give KC-KR10. This recombinant phage was used to replace the chromosomal KR10 coding sequence by homologous recombination as described previously.[17,29] Mutants were first identified by PCR with oligonucleotides KR10CF and KR10CR1 that only amplify the modified sequence. To confirm that gene

replacement had occurred, the KR10 coding region was amplified with primers KR10CF and KR10CR2 and tested for the presence of a HindIII site (Fig. 8).

The sequences of the oligonucleotide primers (5′ – 3′) were: KR10F1, TTTTAAGCTTCTGCAGACGAGCGTTACGACCACAACAC; KR10R1, GATCAAGCTTGCCTGGTCGCCGCTGCCCCAGACGC; KR10F2, GATCAAGCTTCGGCGCTGCCAACGCCTACCTCGAC; KR10R2, AAAAGAATTCGGATCCAGCAGTTCGAGGATCTCGTCGTG; KR10CF, CACCGAGAACGCCGACAACAC; KR10CR1, GCGTTGGCAGCGCCGAAGCTT; KR10CR2, GAGGTGCTTGCGCAGTTCGAC.

The following oligonucleotides were used to detect ACP coding sequences in strain KR10-1 (5′ – 3′): ACP3F, TTCGTGCTCTTC TCGTCCGTC; ACP3R, CCGAAGAAGTCGGCGTCGAAG; ACP4F, ACCGATGAGGCCGCTCTCGT; ACP4R, GATCACGATCGGGTCGTCGT; ACP5F AACGCCTTCCTGGACGCACT; ACP5R, GAGCAGTCGCCACAGGTCCT; ACP6F, CAACGCGGGTCAGGCCAACTA; ACP6R, GAAGCTCGTCCAGGATGAAGT; ACP7F, TCTGGACCTGGACGCGTTCAT; ACP7R, GGCTCCGAAGAGTTCGTCGTG.

6-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*S*,12*S*,13*S*,14*S*)-12,14-Dihydroxy-11,13-dimethyl-1,3,5,7,9-pentadecapenten-1-yl]-4-hydroxy-3-methyl-2-pyrone (21)

Sedimented mycelia and XAD16 resin (KR10-1 broth; 7 L) was extracted with methanol (3 × 10 L). Partial removal of volatiles *in vacuo* followed by cooling (4 °C, 18 h) gave an orange precipitate which was collected by centrifugation, washed (water) and dried *in vacuo* (1.0 g). Purification by flash chromatography (60% EtOAc–hexane) gave **21** (100 mg).

μ_{max}/cm^{-1} 422, 400(max), 380(sh) and 311.

δ_H(500.1 MHz; d^4-MeOH) 0.85 (3 H, d, *J* 6.9 17′-C) 1.04 (3 H, d, *J* 6.8 16′-Me), 1.14 (3 H, d, *J* 6.3, 15′-H), 1.74 (13′-H), 1.90 (3 H, s, 7-C), 2.48 (1 H, dq, *J* 3.2 and 6.8, 11′-H), 3.41 (1 H, dd, *J* 8.9 and 3.4, 12′-H), 4.06 (1 H, dq, *J* 6.3 and 6.3, 14′-H), 5.89 (1 H, dd, *J*, 15.2 and 7.8, 10′-H), 6.07 (1 H, s, 5-H), 6.16, 6.18 (9′-H), 6.27, 6.34, 6.34, 6.38, 6.43, 6.59 (1 H, dd, *J* 14.6 and 11.0), 7.08 (1 H, dd, *J* 15.0 and 11.3).

δ_C(125.8 MHz; d^4-MeOH) 7.3 (7-C), 10.2 (17′-C), 11.7 (16′-C), 17.7 (15′-C), 39.5 (11′-C), 42.3 (13′-C), 69.1 (14′-C), 77.7 (12′-C), 99.2 (3-C), 100.6 (5-C), 121.2 (d), 129.9 (9′-C), 130.7 (d), 130.9 (d), 131.7 (d), 134.9 (d), 135.2 (d), 136.2 (d), 138.4 (d), 140.2 (10′-C), 156.9 (s), 166.2 (s), 166.6 (s).

m/*z* (–ve ES) 385.1 (M-H$^+$), 771.3 (M_2-H$^+$), 1157.3 (M_3-H$^+$).

m/*z* (+ve ES) 387.1, 773.0 (M_2H)$^+$, 1160.1 (M_3H)$^+$

m/*z* (FAB) 387.21637 (MH$^+$). $C_{23}H_{31}O_5$ requires 387.21628.

6-[(1*E*,3*E*,5*E*,7*E*,11*E*,13*E*,15*S*,16*S*,17*S*,18*S*)-16,18-Dihydroxy-15,17-dimethyl-1,3,5,9,11,13-heptadecahexen-1-yl]-4-hydroxy-3-methyl-2-pyrone (24)

Sedimented mycelia and XAD16 resin from KR10-2 culture (2 L) was extracted with methanol (2 × 4 L) for 3 h. The volume of the supernatant was reduced *in vacuo* (to 100 mL) to give a thick dark orange suspension, which was cooled to 4 °C to give an orange precipitate (*ca* 500 mg). The precipitate was partially dissolved in MeOH (50 mL), and then an equal volume of EtOAc was slowly added and any solid removed by centrifugation. Volatiles were removed from the supernatant *in vacuo*, the resultant solid was sonicated and shaken vigorously in methanolic ethyl acetate (10% v/v), any debris was removed, and the volatiles removed *in vacuo* from the supernatant to give a crude solid. Purification by flash chromatography (70% hexane in EtOAc, gradient to 100% EtOAc) gave tetraene analogue **24** (>10 mg L^{-1} based on NMR purity and signal to noise) eluted with 70% EtOAc–hexane and a trace amount of the corresponding heptaene analogue (eluted at 90% EtOAc–hexane), λ_{max} (heptaene, MeOH)/nm 440) coeluting with aromatic impurities.

λ_{max} (MeOH)/nm 399, 386 and 289.

δ_H(500.1 MHz; d^4-MeOH) 0.84 (3 H, d, *J* 7.1, 13-Me), 1.01 (3 H, d, *J* 6.7, 15-Me), 1.13 (3 H, d, *J* 6.3, 19-H), 1.73 (17-H), 1.91 (3 H, br s, 3-Me), 2.20 (2 H, m, 9 or 10-H), 2.23 (2 H, m, 9 or 10-H), 2.41 (1 H, ddq, *J* 7.2, 3.4 and 7.2, 15-H), 3.39 (1 H, dd, J 8.9 and 3.4, 16-H), 4.00 (1 H, dq, *J* 6.2 and 6.2, 18-H), 5.61 (1 H, ddd, *J* 14, 7.2 and 7.2, 8 or 11-H), 5.67 (14-H), 5.84 (11 or 8-H), 6.04, 6.07 (2 × 1 H), 6.17 (2 × 1 H, 2 × *ca* dd, *J* 15 and 12), 6.26 (1 H, dd, *J* 14.8 and 10.9), 6.35, 6.38, 6.40, 6.56 (1 H, dd, *J* 14.6 and 10.8), 7.08 (1 H, dd, *J* 15.0 and 11.1).

δ_C(125.8 MHz; d^4-MeOH) 8.7 (q), 11.6 (q), 13.2 (q), 19.1 (q), 33.5 (t), 34.0 (t), 40.5 (d), 43.6 (d), 70.6 (d), 79.3 (d), 100.6 (s), 102.1 (d), 122.5 (d), 131.0 (d), 131.8 (2 C, 2 × d), 132.3 (d), 132.5 (d), 132.6 (d), 136.3(d), 137.5 (2 C, 2 × d), 137.7 (d), 139.8 (d), 158.3 (s), 167.7 (s), 168.0 (s).

m/*z* (–ve ESMS) 439.

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