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A Late-Stage Intermediate in Salinomycin Biosynthesis Is Revealed by Specific Mutation in the Biosynthetic Gene Cluster

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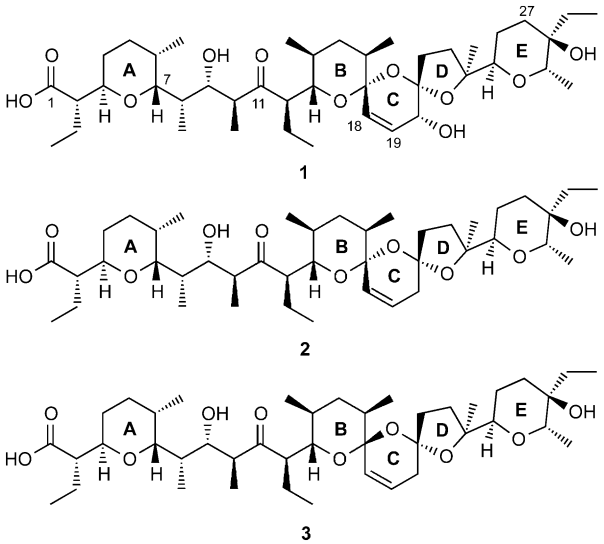
Salinomycin (**1**) from *Streptomyces albus* DSM 41398 is an antibiotic polyether ionophore with a complex tricyclic bispiroacetal core structure[1] that selectively binds K^+ ions and transports them across cell membranes, thus leading to cell death.[2] The therapeutic use of salinomycin is limited by its toxicity,[3] but it is widely used in animal husbandry as a coccidiostat. Salinomycin has attracted strong renewed interest owing to its potent and selective activity against cancer stem cells[4] and cancer cell lines.[5] Engineering the biosynthetic pathway to salinomycin offers an attractive route to novel and potentially useful analogues of this complex molecule. We have cloned and analysed the salinomycin gene cluster from *S. albus* DSM 41398, and found that the polyketide chain is synthesised on an assembly line of nine polyketide synthase (PKS) multienzymes.[6] We have also initiated targeted deletion of the genes that control oxidative cyclisation so as to probe the mechanism of polyether ring formation. One such mutant produces a novel polyketide diene whose structure provides the first evidence for the likely order of key steps in the biosynthesis.

Polyether ionophores make up a particularly numerous subclass of complex polyketide antibiotics. They are synthesised on canonical modular PKS assembly-line multienzymes, in which each module houses fatty acid synthase-like enzyme domains, and there is colinearity between the order (and enzyme domain content) of successive modules and the chemistry of the product.[7] The antibiotic polyethers adopt a characteristic conformation in which multiple oxygen atoms provide ligands for a centrally held specific cation, whereas the external surface is exclusively nonpolar.[2] A general model for the biosynthesis of polyethers has been proposed[8] in which the PKS produces a linear polyketide chain containing two or more *trans* or *E* double bonds. Stereoselective epoxidation of these double bonds leads to a polyepoxide, whose ring opening (in a series of controlled S_N2-like reactions) generates the characteristic rings of the polyether.[9] This model is strongly supported by the results of extensive genetic studies on the cloned and characterised biosynthetic gene clusters for monensin A[10] and several other polyethers.[11] Comparison of these gene clusters has revealed the presence of a conserved set of genes that are a hallmark of polyether biosynthesis: the *monC* family[10a,b] that encode a flavin-linked epoxidase, and the *monB* family[10a,c] that encode novel epoxide hydrolase/cyclase enzymes. It also appears that all stages of the biosynthesis take place while the intermediates are tethered either to a discrete acyl carrier protein (ACP) or to an ACP domain within the PKS.[10,11] However, important aspects of the oxidative cyclisation process remain undefined, especially the molecular basis for the exquisite stereospecificity and stereoselectivity of the process. Salinomycin, and related polyether metabolites produced by *S. albus* DSM 41398, possess an unusual and particularly complex tricyclic



bispiroacetal moiety, and—uniquely among polyether ionophores—one of their ether rings accommodates a *cis*/*Z* double bond (at C18–C19). Double bonds with *cis* geometry are rare in polyketides, and diverse mechanisms have been proposed for their formation.[12] The introduction of a spiroacetal into a polyketide chain imparts a distinctive rigidity to the structure that is likely to be decisive for biological activity,[13] and therefore it is of great interest to discover how spiroacetal formation is stereocontrolled. Salinomycin production in *S. albus* DSM 41398 is accompanied, in certain media, by the congeners[2] deoxy(O-8)-salinomycin (**2**) and 17-*epi*-deoxy(O-8)-salinomycin (**3**); this indicates that hydroxylation at C-20 is perhaps the final step before release of the salinomycin product, and that formation of the incorrect 17-*epi* spiroacetal configuration might hinder the subsequent hydroxylation step.

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The spiroacetal moieties in the polyethers monensin,[10b] nigericin[11a] and nanchangmycin (dianemycin)[11c] are proposed to form spontaneously, as no enzymes have been found encoded in the respective biosynthetic gene clusters that might catalyse spiroacetal formation. Evidence in favour of this proposal comes from deletion of the genes for the MonB cyclases in monensin biosynthesis. The mutant strain accumulates a mixture of monensin isomers that can subsequently be smoothly converted into monensins by treatment with mild acid.[10c] The structure of one of these isomers has been established as an *epi*-monensin, with altered configuration at the spiroacetal centre.[10c] In contrast, the anticancer polyketide reveromycin A has been shown to require a novel spiroacetal synthase, RevJ, which catalyses stereospecific dehydrative cyclisation from a dihydroxyketone precursor.[14]

We used a *monC* gene-based hybridisation probe to screen a genomic library of *Streptomyces* sp. DSM 41398 so as to locate the salinomycin (*sal*) biosynthetic gene cluster. Two independent previous efforts to clone this cluster used only nonspecific probes encoding ketosynthase (KS) domains, but actinomycete strains generally contain multiple PKS clusters, and in neither case did the partial cluster obtained match the gene organisation expected of the *sal* cluster.[15] From nearly 2000 clones, we isolated four positively hybridising cosmids (P8H, Q7G, M12C and T4E) whose restriction maps and partial sequences indicated that they arose from a single locus (Figure S1 in the Supporting Information). We also isolated nearly 200 cosmid clones by using KS-based probes in successive steps of cosmid walking from cosmid P8H to identify cosmids B05-PKS-1, G08-PKS-1 and B10-PKS-1. Shotgun sequencing of a total of about 107 kbp of contiguous sequence revealed the candidate *sal* gene cluster flanked by apparently unrelated genes. The deduced organisation of the genes in the cluster, and their proposed roles in salinomycin biosynthesis, are indicated in Figure 1. Subsequently, a whole genome (454) sequencing approach was used on DSM 41398, and contigs that overlapped the *sal* cluster were included in the final assembly.

Bioinformatic analysis of 104 kbp of contiguous sequence with FgenesSB[16] revealed 33 open reading frames (ORFs), of which 27 are ascribed a putative function in salinomycin biosynthesis (Figure 1 and Table S2), including nine *sal* PKS genes (*salAI*, *salAII*, *salAIII*, *salAIV*, *salAV*, *salAVI*, *salAVII*, *salAVIII* and *salAIX*). Upstream of the PKS genes, genes *salN*, *salO*, (which both encode putative regulatory proteins) *salP* (potentially involved in provision of ethylmalonyl-CoA as a source of PKS extender units) and *salQ* (similar to ketosynthase III from fatty acid synthases) are considered to mark the limit of the *sal* cluster. Homologues of these four genes are present in the same relative positions, flanking a putative salinomycin-like polyether gene cluster present in the sequenced genome of *Streptomyces* sp. C (Table S2). The adjacent *orf1*, *orf2* and *orf3* appear not to belong to the *sal* cluster. Downstream of the PKS genes, the cluster is taken to include two genes encoding discrete (type II) thioesterases (*salGI* and *salGII*), a cytochrome P450 (*salD*) and associated ferredoxin (*salF*), two putative export genes (*salH* and *salI*), three epoxide hydrolases/cyclases (*salBI*, *salBII* and *salBIII*), an epoxidase (*salC*), a putative regulatory gene (*salJ*) and a bifunctional protein comprising a nonribosomal synthetase (NRPS) condensation domain and peptide carrier protein (PCP; *salX*). The nine PKS genes are arrayed on the genome in the order in which the encoded enzymes are used, and between them encode for a loading module, comprising a malonyl-thioester decarboxylase (KSQ domain),[17] malonyl-CoA-specific acyltransferase (AT) and ACP; and 14 extension modules (Scheme 1). The final module is fused at its C terminus to an unusual additional ketosynthase-like domain; this provides an important clue to the mechanism of chain termination. In the pathways to monensin, nigericin and nanchangmycin, there is evidence[11a] that a discrete KS (dubbed KSX) docks[18] specifically onto the terminal ACP of the final module and transfers the full-length polyketide chain to a discrete ACP (ACPX) on which the oxidative cyclisation takes place. In salinomycin, it appears that the KSX, whose critical active-site residues are intact (Figure S2), is fused to the C-terminal ACP of

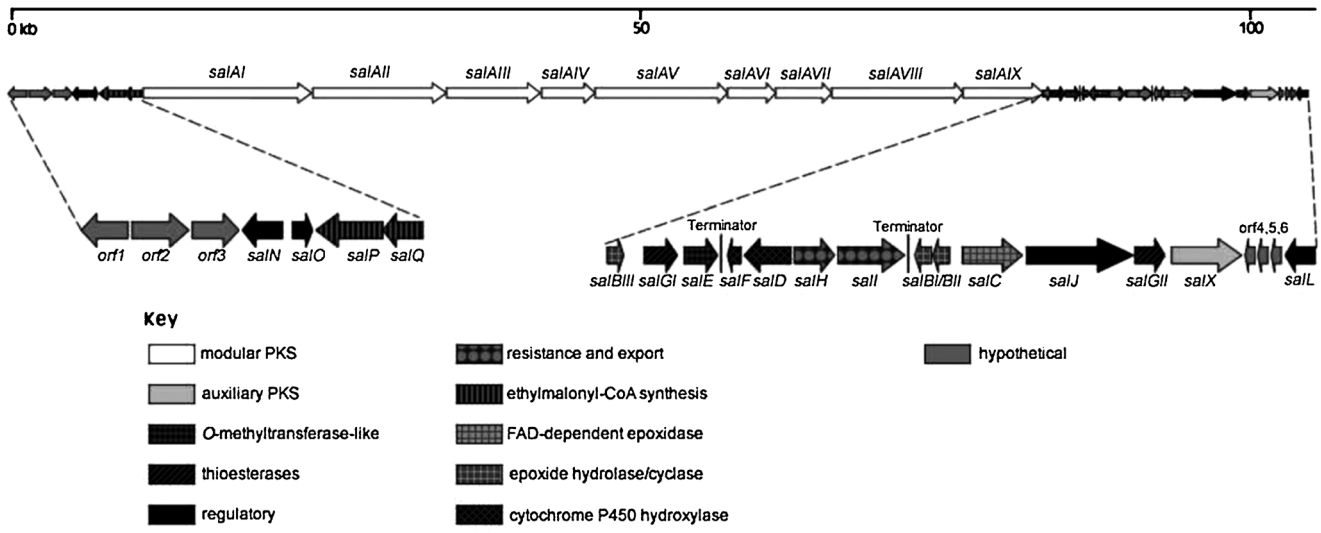
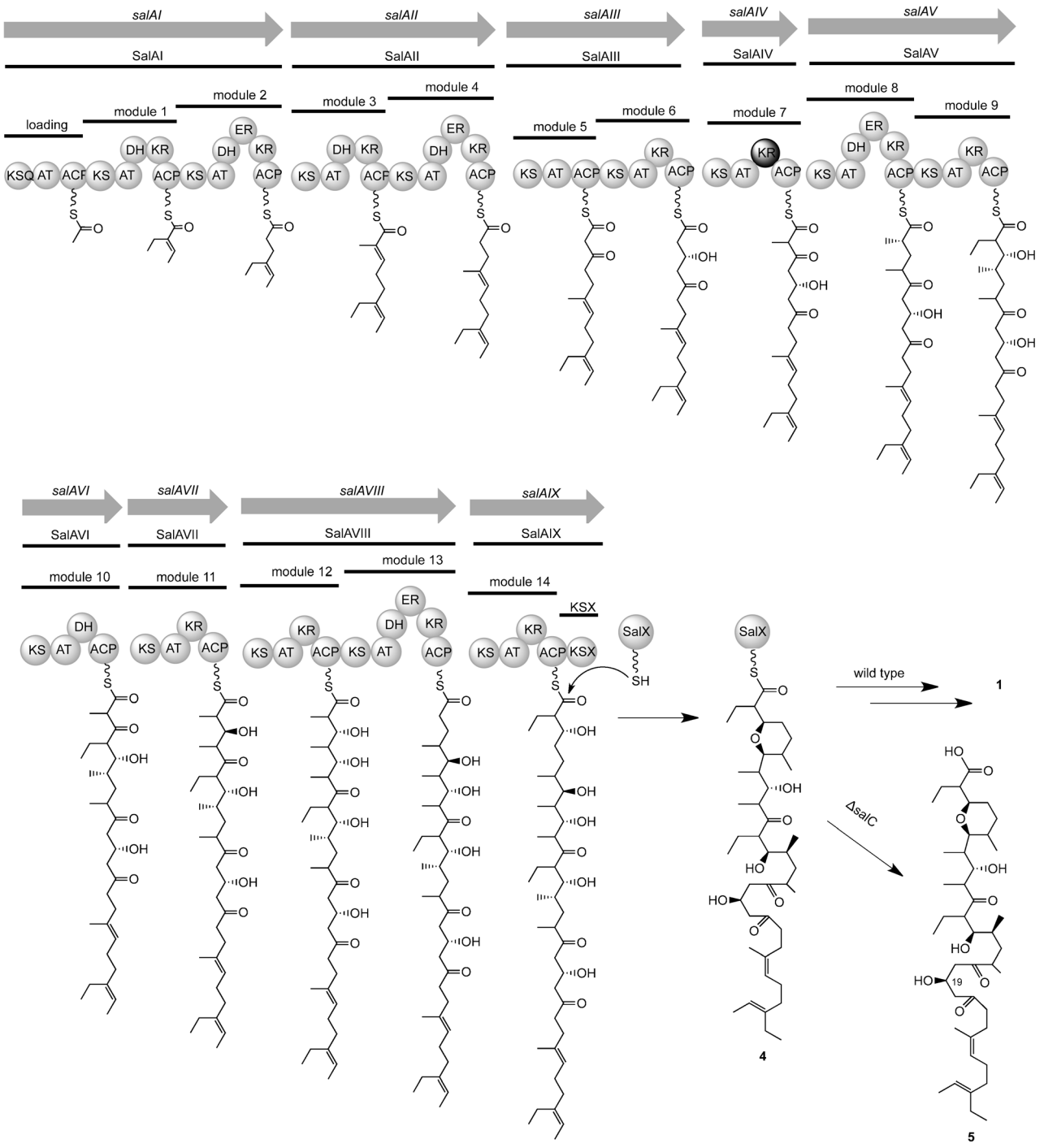


Figure 1. The organisation of the biosynthetic gene cluster for salinomycin in *S. albus* DSM 41398 and assignment of gene functions.



Scheme 1. Proposed mechanism of polyketide chain assembly on the salinomycin PKS. The individual domains are labelled as follows: KS, ketosynthase; KSQ, decarboxylase in the loading module; AT, acyltransferase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase. The dark grey KR domain in module 7 is inactive. After the last round of PKS chain extension, the acyl chain is transferred to the discrete SalX protein, catalysed by the KSX domain at the end of module 14. The diene intermediate (**4**) is modified by tailoring enzymes including SalC, SalBI, SalBII, SalBIII, SalD and SalE to yield (**1**).

the PKS. On this basis it would be predicted that the cluster should also contain an ACPX and a MonCII-like salinomycinyl-ACP thioesterase.[10f] A possible candidate for such a carrier protein is the protein SalX annotated in the *sal* cluster as a PCP domain, fused to an N-terminal condensation (C) domain typical of an NRPS (Table S2). Inspection of the C-domain sequence showed that a key active-site residue, His138, is replaced by Arg (Figure S3); this would likely render it inactive in condensation.[19] However, no counterpart of *monCII* is found in the *sal* cluster. The exact mechanism of salinomycin chain release therefore remains to be elucidated.

The predicted specificity of the AT domains of each module[20] corresponded precisely with the type of extender unit (five acetate, six propionate and three butyrate units) known—from the feeding of isotopically labelled precursors[21]—to be recruited at each extension cycle in order to

form salinomycin. The distribution of ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains was also, in almost all modules, in complete accordance with the production of salinomycin. Active KR domains may be classified as either A- or B-type depending on the stereochemistry of reduction[22] (Figure S4), thus allowing the configuration of the surviving hydroxy groups in the intermediates to be deduced, as shown in Scheme 1. In the case of the apparently redundant KR domain present in module 7, close inspection revealed that the active-site residues Tyr150 and Asn154 are replaced by Phe and Ser, respectively, thus making it likely that the domain is in fact inactive.[12b] Only two exceptions to colinearity were found: a DH domain that generates a double bond at C2–C3 should be present in the final extension module 14, as this would allow 1,4-addition of the hydroxy group at C-7 to form tetrahydropyran ring A in salinomycin—analogously to the formation of the tetrahydropyran ring in the equivalent position in nigericin.[11a] However, there is no DH domain in module 14. Likewise, there is no DH domain in module 6, although there is a *cis*/*Z* double bond in the salinomycin structure at C18–C19. In some modular PKS assembly lines, it appears that an active DH domain in an adjacent extension module catalyses a dehydration reaction in an adjacent module with no endogenous DH.[23] Alternatively, dehydration may be accomplished other than by the domains of the PKS, as has been shown for example for the C27–C28 double bond in certain avermectins, inserted by the auxiliary enzyme AveC.[12a]

To confirm the identity of the cloned *sal* cluster, and to probe the timing and mechanism of individual late steps in salinomycin biosynthesis, an in-frame deletion of the *salC* gene, which encodes a flavin-linked epoxidase, was carried out as described in the Experimental Section in the Supporting Information. The integrity of the Δ*salC* mutant was confirmed by PCR analysis and by Southern hybridisation (Figure S5). Fermentation of the Δ*salC* mutant, followed by LC-ESI-MS analysis of extracts from the culture medium (under conditions under which the wild-type strain produces salinomycin) showed that salinomycin production had indeed been abolished (Figure 2B). Complementation of the Δ*salC* mutant with a copy of the gene supplied on a plasmid and transcribed from the *ermE** promoter (Experimental Section) restored salinomycin production (Figure 2D). Careful scrutiny of extracts from the Δ*salC* mutant showed the appearance of a new metabolite with a retention time of 31.2 min and a molecular ion ($[M+Na]^+$) at *m/z* 743.7. High-resolution mass analysis gave the molecular formula $C_{42}H_{72}O_9Na$ with 0.2 ppm mass accuracy. Detailed MS/MS and MS^3 analysis (Orbitrap) of this metabolite yielded a fragmentation pattern (Figure S6) that is fully consistent with the proposed structure of diene **5** (Schemes 1), in which the double bonds are correctly placed for epoxidation. The presence of the tetrahydropyran ring A in **5** was confirmed by a deuterium labelling experiment (Figure S7 and Scheme S1) that showed **5** to have four exchangeable hydrogens rather than five. This implies that dehydration at C2–C3 and ensuing cyclisation occurs before release of the polyketide chain from the PKS. A hydroxy group is present at C-19; this is consistent with the prediction from bioinformatic analysis of the *sal* PKS

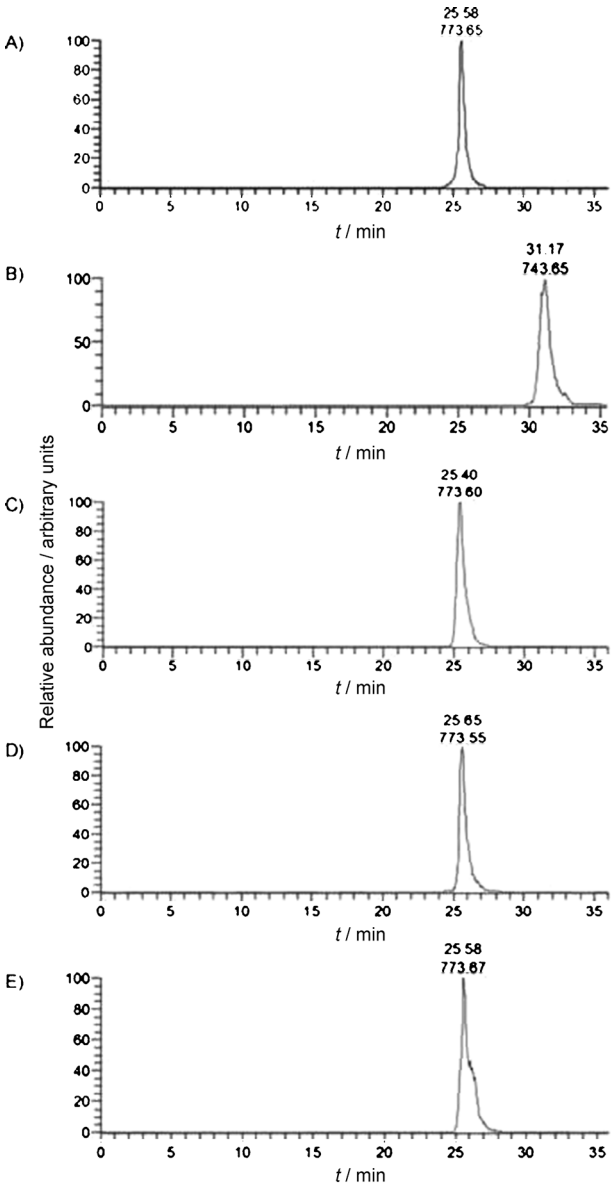
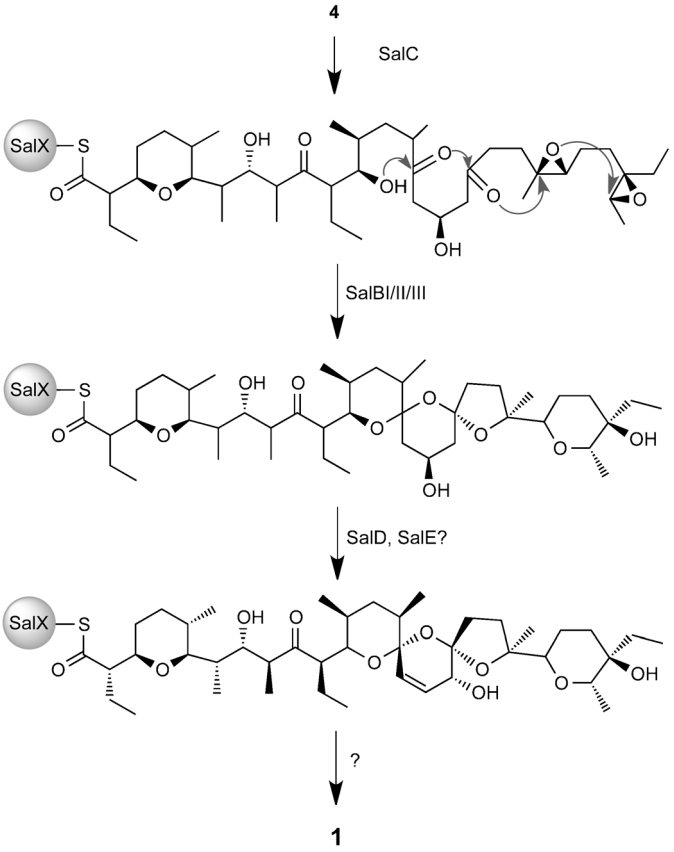


Figure 2. LC-ESI-MS analysis of salinomycin and of **5** from *S. albus* DSM41398. A) *S. albus* DSM41398 (total ion current). The peak at 25.6 min corresponds to salinomycin (*m/z* 773.6 $[M+Na]^+$). B) ΔsalC strain. The peak at 31.2 min corresponds to **5** (*m/z* 743.6 $[M+Na]^+$). C) ΔsalBIΔsalBII strain. The peak at 25.4 min corresponds to salinomycin (*m/z* 773.6 $[M+Na]^+$). D) ΔsalC mutant strain complemented with expression plasmid pPTSalCKFP. The peak at 25.7 min corresponds to salinomycin (*m/z* 773.6 $[M+Na]^+$). E) salinomycin standard (*m/z* 773.6 $[M+Na]^+$).

that no dehydration is catalysed by the PKS at this position during normal chain extension. The inferred structure of **5** provides the first evidence that the introduction of the *cis*-C-18–C19 double bond occurs after the completion of the polyketide chain, by presently unknown mechanisms. The timing of dehydration with respect to the remaining steps (epoxidation catalysed by SalC; epoxide ring opening and concomitant cyclisation to form the spiroacetal structure of salinomycin catalysed by the epoxide hydrolases/cyclases (SalBI, SalBII and SalBIII); and hydroxylation at C-20 proposed to be catalysed by cytochrome P450 SalD) likewise remains to be established.

The model we propose for the biosynthesis of salinomycin is shown in Scheme 2. Of the remaining genes in the cluster, *salGI* and *salGII* encode discrete, mutually homologous thioesterases SalGI and SalGII, which, by analogy to their counterparts MonAIX and MonX in monensin biosynthesis[11a] and to



Scheme 2. Proposed model for the biosynthesis of salinomycin.

similar thioesterases generally found in other modular PKS gene clusters, are proposed to assist chain elongation on the PKS by hydrolysing inappropriately loaded acyl chains or stalled polyketide intermediates.[24] Likewise, *salP* encoding a 3-hydroxybutyryl-CoA dehydrogenase may plausibly be assigned a role in a precursor pathway that leads to the unusual extender unit ethylmalonyl-CoA.[25] In contrast, there is no obvious role for the FabH-like (ketosynthase III of fatty acid synthase-like) enzyme encoded by *salQ*, or for the methyltransferase-like protein encoded by *salE*. The structure of salinomycin does not require the action of a methyltransferase, and there is recent precedent for the idea that a methyltransferase-like enzyme can catalyse a different and novel reaction.[26]

The closure of the six-membered tetrahydropyran ring E in salinomycin is formally a *endo*-tet cyclisation, which is normally kinetically disfavoured compared to *exo*-tet cyclisation to give a five-membered tetrahydrofuran ring.[27] Mechanistic study of intramolecular epoxide-ring openings is complicated by the relative ease of non-enzymatic cyclisation by the *exo*-tet route. However, it has been shown unequivocally that the disfavoured *endo*-tet cyclisation of one of the rings in the polyether lasalocid requires catalysis by the epoxide hydrolase LasB;[11d,e] this firmly establishes the role of MonB-like enzymes in accelerating and directing epoxide hydrolysis and concomitant cyclisation to ether rings. As a first step towards identifying the respective roles of the three epoxide hydrolases SalBI, SalBII and SalBIII, we deleted SalBI and SalBII together, as described in the Experimental Section. The integrity of the Δ*salBI*Δ*salBII* mutant was established by PCR and Southern hybridisation (Figure S8). Surprisingly, fermentation of this mutant strain showed that salinomycin was still produced (Figure 2C) in amounts comparable to the wild-type strain (Figure 2A). The *sal* cluster is unusual among polyether gene clusters that have been sequenced, in that it encodes three MonB-like enzymes/domains instead of one or two. The only other known gene cluster encoding three epoxide hydrolases is a putative cluster in the draft genome of *Streptomyces* sp. C, predicted to govern the synthesis of a PAPA-type[8] polyether structurally related to salinomycin. Further work will be required to establish whether in the mutant strain the SalBIII enzyme compensates for the loss of the other two epoxide hydrolases. Alternatively, one or both of the S_N2-like ring-opening steps and ensuing cyclisations might take place even in the absence of the SalBI and SalBII enzymes. Likewise, the formation of the tricyclic spiroacetal system[28] might proceed spontaneously, since the *sal* cluster contains no counterpart of the spiroacetal synthase RevJ in reveromycin A biosynthesis.[13,14]

In summary, the salinomycin biosynthetic gene cluster has now been correctly identified and cloned, revealing candidate enzymes for key steps and allowing a model for salinomycin biosynthesis to be proposed. Blocking salinomycin production by deleting the *salC* gene, and MS-based determination of the structure of the metabolite that accumulates, have provided direct evidence that the introduction of the unusual *cis* double bond at C18–C19 only occurs after completion of the polyketide chain. The enzymes responsible for this step, and for polyketide chain release, remain to be identified as do the exact roles of SalP, SalQ and the several SalB enzymes. Meanwhile, our results provide the necessary platform for attempts to introduce structural changes to the salinomycin molecule by biosynthetic engineering.

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Keywords: antibiotics · antitumor agents · biosynthesis · polyketides · salinomycin

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