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Engineering of complex polyketide biosynthesis — insights from sequencing of the monensin biosynthetic gene cluster

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The biosynthesis of complex reduced polyketides is catalysed in actinomycetes by large multifunctional enzymes, the modular Type I polyketide synthases (PKSs). Most of our current knowledge of such systems stems from the study of a restricted number of macrolide-synthesising enzymes. The sequencing of the genes for the biosynthesis of monensin A, a typical polyether ionophore polyketide, provided the first genetic evidence for the mechanism of oxidative cyclisation through which polyethers such as monensin are formed from the uncyclised products of the PKS. Two intriguing genes associated with the monensin PKS cluster code for proteins, which show strong homology with enzymes that trigger double bond migrations in steroid biosynthesis by generation of an extended enolate of an unsaturated ketone residue. A similar mechanism operating at the stage of an enoyl ester intermediate during chain extension on a PKS could allow isomerisation of an *E* double bond to the *Z* isomer. This process, together with epoxidations and cyclisations, form the basis of a revised proposal for monensin formation. The monensin PKS has also provided fresh insight into general features of catalysis by modular PKSs, in particular into the mechanism of chain initiation. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 360–367.

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Introduction

The complex polyketides produced by *Streptomyces* and related bacteria include a wide range of bioactive compounds, such as the antibacterial erythromycin A, the antiparasitic compound avermectin and the immunosuppressant rapamycin. Each of these compounds is the product of a modular Type I polyketide synthase (PKS) [11,15], a multifunctional enzyme in which each successive round of polyketide chain extension is catalysed by a different set, or module [20], of fatty acid synthase-related activities, including ketosynthases (KS), acyl carrier proteins (ACP), methylmalonyl- or malonyl-CoA:ACP acyltransferases (AT) for loading of extension units, and (where appropriate) ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) activities. Chain initiation and termination required additional activities. Thus, in the 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*, an AT-ACP didomain acts as a loading module and selectively loads the starter propionate unit [3], while a C-terminal thioesterase (TE) [1] domain cyclises the heptaketide product to form macrolide ring [12,19,20,46].

The essentially complete sequencing of the biosynthetic gene cluster for the immunosuppresant macrolide rapamycin from *Streptomyces hygroscopicus* illustrated that although the modular arrangement previously seen for the erythromycin PKS was preserved, there were important differences in the organisation of the molecular assembly line [37]. For example, the rapamycin PKS modules contain inactive domains that are absent from the erythromycin PKS modules [4]. The activation of the starter unit and the mode of chain termination, by transfer of the acyl chain onto an activated amino acid unit, are also achieved by very different mechanisms [3,37]. The evidence from this work that nonribosomal peptide synthetase (NRPS) modules and PKS modules can be combined within the same multienzyme assembly has been amply borne out by later studies on other natural products that contain both amino acid and ketide units, for example yersiniabactin [22] and bleomycin [38].

Importantly, it has proved possible to create productive hybrid modular PKS multienzymes by transplantation of one or more domains from the rapamycin PKS into the erythromycin PKS, and such experiments have since been extended to other macrolide PKSs as the DNA sequence of the corresponding genes has become available. This provides an apparently rational basis on which to obtain altered, potentially valuable complex natural products [6,28,41]. It has also been shown that stereochemical control is shared between the KS and KR domains of key modules. This information lays the foundation for experiments to produce molecules with altered stereochemistry at specific sites [8,47].

The biosynthesis of polyether ionophore antibiotics

Although the paradigm for Type I modular PKS multienzymes seems now to be firmly established, there is one large and important class of complex polyketides for whose biosynthetic pathway we have had until now only indirect evidence, the polyketide ionophores. Polyethers [48] are branched-chain, polyoxygenated carboxylic acids, which act as selective

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ionophores transporting cations across the cell membrane of target cells and thereby causing depolarisation and cell death. Certain polyethers, including monensin A (Figure 1), lasalocid, semduramicin and tetronasin, are in widespread use in animal husbandry as coccidiostats (principally targeted against *Eimeria* spp.) and as growth promoters. Polyethers have also been reported to be active *in vitro* and *in vivo* against the malarial parasite *Plasmodium falciparum* [23].

Polyethers contain multiple asymmetric centres and are characterised by the presence of tetrahydrofuran and tetrahydropyran rings, producing a characteristic shape, which is nonpolar on its outer surface and, therefore, well adapted for transport across bacterial membranes, and provides on its inner surface polar coordinating ligands for a centrally bound metal ion.

Monensins A and B are produced by the actinomycete *Streptomyces cinnamonensis*, and selectively bind sodium ions. Monensin B differs from monensin A (Figure 1) only in the presence of a methyl side chain at C-16 rather than an ethyl side chain. Experiments using carbon-14-labelled precursors have shown that monensin A is synthesised from five acetate, one butyrate and seven propionate units [17]. Similarly, experiments using precursors doubly labelled with carbon-13 and oxygen-18 have shown that oxygens (O)1, (O)3, (O)4, (O)5, (O)6 and (O)10 of monensin arise from the carboxylate oxygens of either propionate or acetate, while growth in the presence of oxygen-18 gas demonstrated that the three remaining ether oxygens (O)7, (O)8 and (O)9 are derived from molecular oxygen [2,13,14].

These findings have been rationalised by proposing that the biosynthesis of monensin proceeds *via* an acyclic triene intermediate in which the geometry of all three carbon–carbon double bonds is *E* (entgegen) rather than *Z* (zusammen) (Figure 2A). The triene is then proposed to be subject to epoxidation to a tri-epoxide and then ring opening is proposed to occur with concomitant sequential formation of the five ether rings, as shown in Figure 2A. Such a biosynthetic pathway, first mooted by Westley *et al* in 1974 [49], accounts for the observed stereochemistry at the multiple asymmetric centres in monensin [14,39], and analogous schemes can be used to account for the biosynthesis of other known polyethers, such as lasalocid [26], tetronasin (ICI 139603) [18] and narasin [40]. The hydroxylation at C-26 and the introduction of an *O*-methyl group on oxygen 3 of monensin were proposed to occur as late steps in the biosynthesis, after formation of the polyether structure.

Unfortunately key aspects of the biosynthetic scheme shown in Figure 2A have so far eluded experimental confirmation. No biosynthetic intermediates have been isolated from mutants of *S. cinnamonensis* that are blocked in early stages of monensin production. 26-Deoxymonensin A has been isolated from a *S. cinnamonensis* mutant partially blocked in monensin production [5] and 3-*O*-demethylmonensins A and B have been recovered as minor components from the fermentation broth of a monensin-producing strain [36]. When fed to cells of *S. cinnamonensis* in radiolabelled form, neither 26-deoxymonensin A, nor 3-*O*-demethylmonensin A, nor 3-*O*-demethyl, 26-deoxymonensin A were significantly incorporated into monensin A [5], either because they are actively excluded or because these modifications in fact occur earlier in the biosynthetic pathway so that these metabolites are shunt products not readily converted into the final antibiotic by the respective hydroxylase or methyltransferase. Similarly, the putative all (*E*)-triene precursor (Figure 2A) has been synthesised and shown not to become incorporated into monensin when fed to growing cells of *S. cinnamonensis* [25].

Meanwhile, an ingenious alternative pathway has been proposed, based on the transition-metal-mediated oxidation of 1,5-dienes [45], in which the triene intermediate would be that of

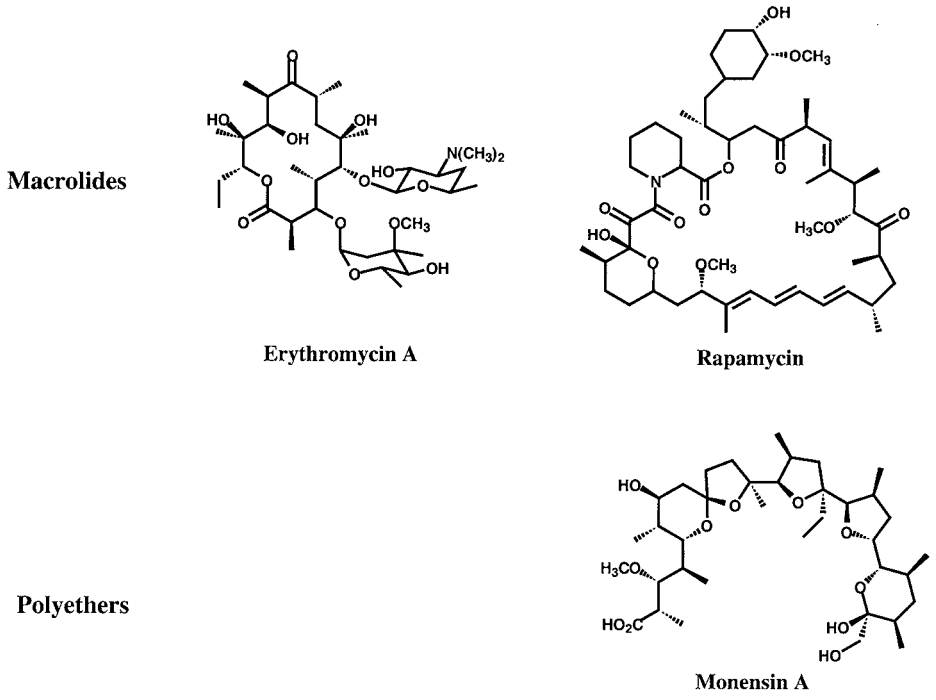

Figure 1 Structures of the antibacterial erythromycin A, the immunosuppressant rapamycin, and the polyether ionophore monensin A.

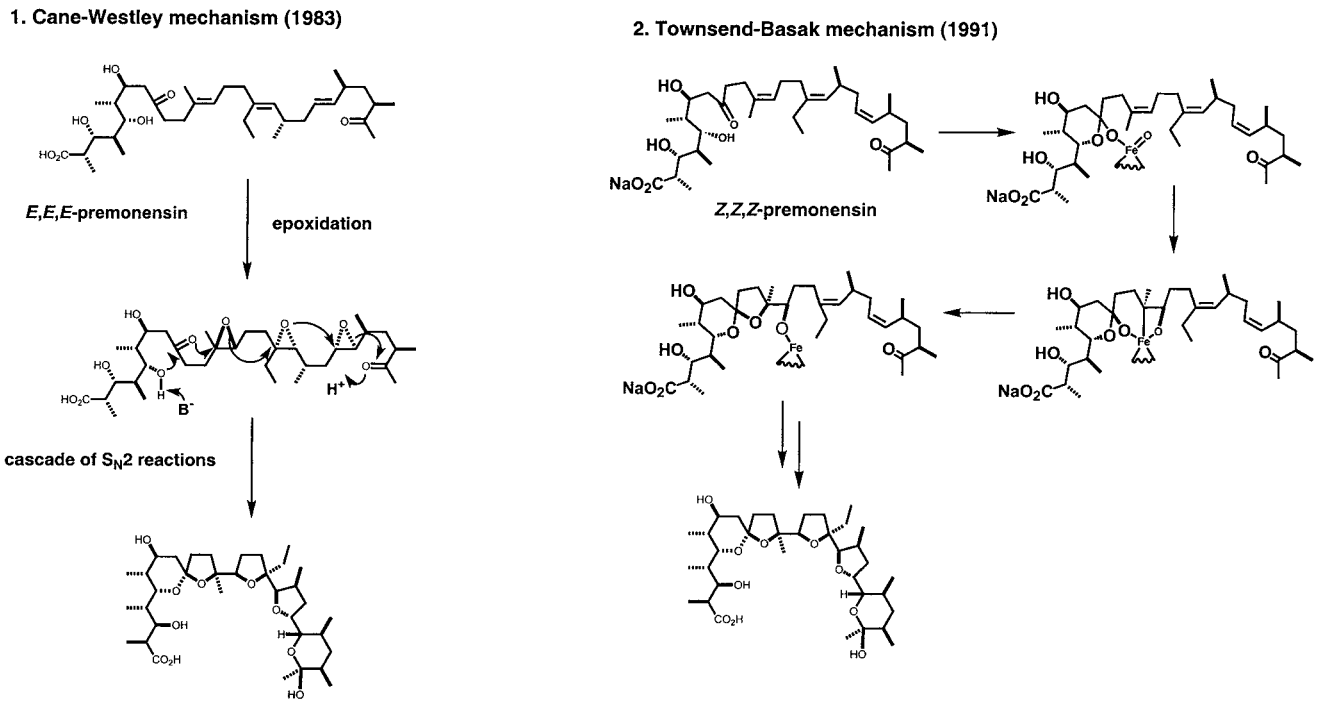

Figure 2 Mechanisms previously proposed for the oxidative cyclisation steps in monensin biosynthesis. (1) Cane–Westley tri-epoxide mechanism. (2) Townsend–Basak transition metal-catalysed mechanism.

Figure 2A except that each carbon–carbon double bond would have the (*Z*) configuration [43] (Figure 2B).

The biosynthetic gene cluster for monensin A

The overall gene organisation of the monensin biosynthetic gene cluster has now been established in our laboratory by sequence analysis of overlapping cosmid fragments from a monensin-producing strain of *S. cinnamonensis* [Oliynyk, M., P.F. Leadlay, J. Staunton, Z. Oliynyk and J.B. Lester, manuscript in preparation]. As illustrated in Figure 3, it is similar to that previously found for many macrolide biosynthetic gene clusters, in which one or more open reading frames (ORFs) encoding large multifunctional PKSs are flanked by other genes that encode functions required for biosynthesis of the antibiotic. In the case of monensin, there is an unusually high number of distinct ORFs that encode PKS multienzymes (eight in total, labelled *monAI* to *monAVIII*) but there is again a separate module of enzymes for each cycle of polyketide chain extension, exactly as found for modular PKSs for macrolide biosynthesis. Thus, there are 12 condensations predicted to be required for the production of the carbon skeleton of monensin, and in agreement with this there are 12 modules of PKS enzymes distributed among the eight PKS ORFs. In addition *monAIX* and *monAX* apparently encode additional PKS-related activities. Their role is presently unclear, although it is proposed that their gene products may function as Type II thioesterase domains. The presence of genes governing late-stage methylation (*monD*) and hydroxylation (*monE*), as well as several potential regulatory genes (*monRI*, *monRII* and *monH*), is amply precedented in previously sequenced PKS gene clusters [15,21,37,44]. A gene not found, whose presence might have been anticipated, is that for crotonyl-CoA reductase (*ccr*), which is present in other PKS gene clusters where butyrate units are used for chain extension [21]. These data fit with previous analysis of *S. cinnamonensis*, which demonstrated that a *ccr* gene separate from the PKS cluster is responsible for generating this butyrate unit [30]. Certain other genes in the monensin cluster (*monBI*, *monBII*, *monCI* and *monCII*) have not previously been found in any gene cluster for biosynthesis of a complex polyketide. Comparison of their sequences with those of known proteins in the published databases revealed first, that the product of the *monCI* gene has significant sequence similarity to authentic non-haem epoxidases. The MonCI enzyme is therefore, a candidate to carry out three epoxidations of a triene intermediate as described in Figure 2. Moreover, the product of the *monCII* gene has significant sequence similarity with epoxide hydrolases, and this enzyme might catalyse the subsequent ring opening of a tri-epoxide, as in the Cane–Westley hypothesis (Figure 2A).

The presence of the *monBI* and *monBII* genes, which are highly homologous to one another, was initially puzzling: their gene products are significantly similar to an isomerase from *Comomonas testosteroni*, which catalyses the interconversion of a Δ^5-3-keto- into a Δ^4-3-ketosteroid [24,42]. However, such enzymes could act in monensin A biosysthesis by allowing the *E* to *Z* interconversion of an activated double bond during polyketide chain synthesis (*via* an extended enolate ion) (Figure 4). The Townsend–Basak proposal (Figure 2B), although it clearly foreshadowed the possible intermediacy of *Z* isomers in the biosynthetic pathway, does not fit easily with the inferred roles of MonCI and MonCII.

A new mechanism, which differs from both previous proposals but which also accounts for the observed stereochemistry of ring formation, is shown in Figure 5. An essential feature of this mechanism is that the proposed double bond isomerisation is carried out as an extra step in the chain elongation process in two modules. In both cases an alkylmalonate building block is used for chain extension leading to the formation of a trisubstituted double bond. In these cases the preferred configuration for the conjugated

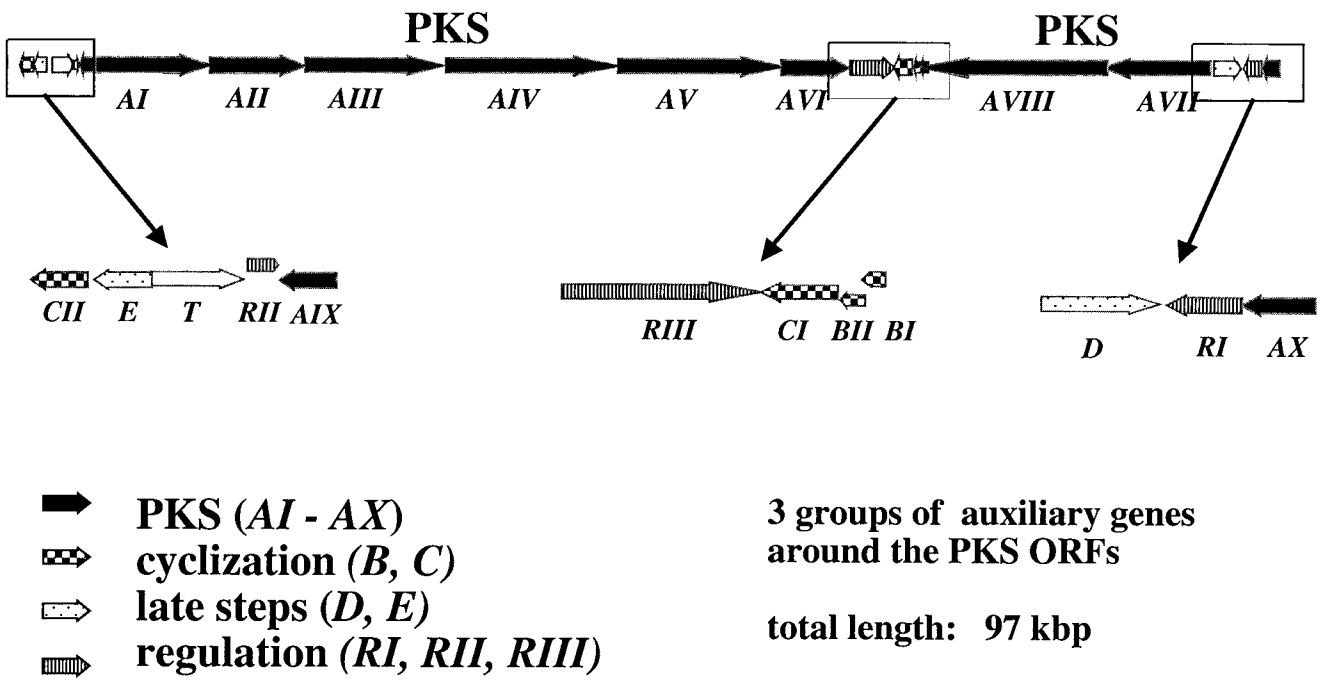

Figure 3 Organisation of the monensin biosynthetic gene cluster. The proposed roles of the auxiliary genes *monBI*, *monBII*, *monCI*, and *monCII* are discussed in the text. The 12 modules of the mon PKS are housed in the enzymes encoded by genes *monAI*–*monAVIII*, while *monAIX* and *monAX* encode additional PKS-related activities.

double bond is *Z* rather than *E* so that allyl strain is relieved. There is no reason to believe that the *E*–*Z* intercoversion is catalysed by the DH domain. Many PKS gene clusters have now been sequenced whose products contain double bonds in the *E* configuration. As none of these clusters contain isomerase enzymes, this must be the stereochemistry naturally produced by the DH domains. The MonB enzymes could catalyse the interconversion *via* formation of an extended enolate reminiscent of the extended enolate intermediates invoked for the double bond migration processes in steroids. However, here the double bond will remain in the same place after the isomerisation (differing from the steroid isomerase case where it moves from the Δ^5 to Δ^4 position). The more stable *Z* isomer would then pass on to the next module in the usual way. It is essential for the proposed mechanism of isomerisation that the

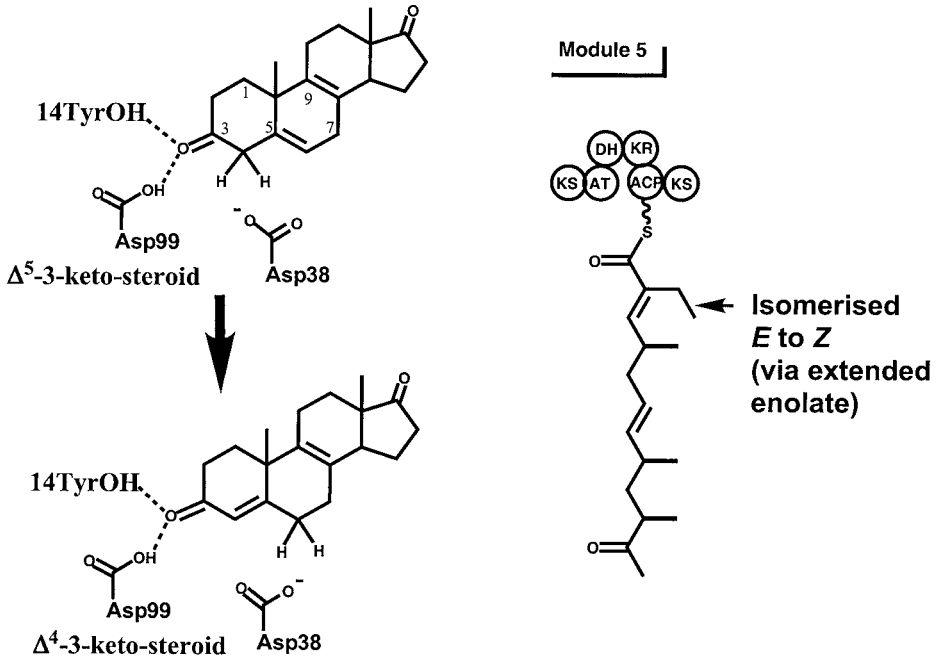

Figure 4 A. The reaction catalysed by the Δ^5-3-ketosteroid isomerase of *C. testosteroni*. B. Proposed isomerisation catalysed by a MonB isomerase during monensin biosynthesis.

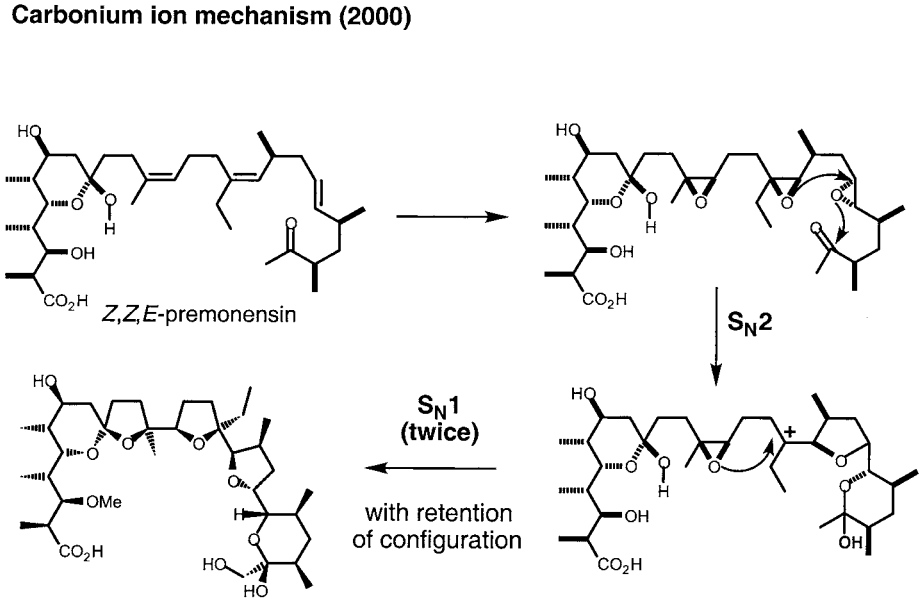

Figure 5 An alternative mechanism proposed for oxidative cyclisation on the *mon* PKS.

process takes place during chain extension before completion of the polyketide chain. In the new scheme the first-formed double bond is left in the *E* configuration; this is the most stable configuration at the enoyl ester stage for this residue.

The resulting PKS product is now the *E,Z,Z* triene, not the *E,E,E* or the *Z,Z,Z* isomers of previous proposals. In the revised biosynthetic pathway this is now converted to a tri-epoxide, which is ready for a cascade of sequential ring openings with concerted cyclisations to produce a polyether system. This is the proposed role for the epoxide hydrolase corresponding to MonCII. The cascade is triggered by ring opening of the disubstituted epoxide accompanied by nucleophilic attack of the oxygen of the next epoxide residue. This would generate a carbonium ion intermediate at the tertiary centre, which is ready for nucleophilic attack by the third epoxide oxygen. Again a carbonium ion process is envisaged, followed by attack of a carbonyl oxygen. The carbonium ion mechanism for the opening of the two trisubstituted epoxides avoids the need to postulate a chemically unattractive S_N2 attack at the sterically hindered position of the epoxide residues. The carbonium ion mechanism also allows substitution with inversion of configuration as is required to account for the final structure. Of course, much remains to be done to test this proposal, and to define the respective roles of MonBI and MonBII. The timing of the action of the MonC enzymes also needs to be established: they may also act on the partially completed polyketide chain.

Close inspection of the monensin gene cluster has also revealed that there is no integral thioesterase domain present at the C-terminus of the final extension module, which is housed in the multienzyme MonVIII. It appears that there is a novel mechanism of chain release in this system, giving rise to the free carboxylic acid of monensin A.

The role of the KSQ domain in the loading module of the monensin PKS

It was also an arresting finding that the loading module in the monensin PKS comprises three domains: the starter-specific acyltransferase and an associated ACP domain, N-terminal of the first extension module in multienzyme MonAI, are preceded by a so-called KSQ domain (Figure 6), which at that time was only precedented in the published literature by the PKSs for the 16-membered macrolides tylosin, spiramycin and niddamycin [27,29]. At the same time, work in our laboratory [Long, P.F., J. Cortés, J. Stauton and P.F. Leadlay, unpublished data] had revealed that a KSQ domain was also present in the loading module of the PKS for the 14-membered macrolide oleandomycin. In all cases, the active-site cysteine residue of an active KS domain was replaced by glutamine (Q in single-letter notation) [7]. These findings triggered our search for a function for the KSQ domain, and led to the realisation that it functions as a decarboxylase, which is required for chain initiation [7]. Further detailed experimental support for this hypothesis, which has been independently suggested on the basis of experiments on fatty acid biosynthesis by Smith and his colleagues [51], will be presented elsewhere [Long, P.F. and P.F. Leadlay, manuscript in preparation]. In passing, it is worth noting that the "chain-length factor" of aromatic PKSs, whose only function was for many years proposed to be the decisive element in determination of chain length [32,33], also contains a highly conserved glutamine (occasionally glutamic acid) at its active site in place of the cysteine of a normal KS subunit, and appears to function in decarboxylative chain initiation, quite apart from any other roles it might have in polyketide chain synthesis [7].

Substitution of the *ERY* loading module by the monensin loading module, to create a hybrid PKS with altered and enhanced selectivity

The loading module of the erythromycin-producing PKS accepts, *in vitro*, acyl groups from not only propionyl-CoA but also acetyl-CoA and (to a smaller extent) *n*-butyryl-CoA [35,50]. *In vivo*, both propionate and acetate are used as starter units by the PKS [9,16], and feeding of several other carboxylic acids has also led to their incorporation [10,34]. In principle, an acyltransferase might

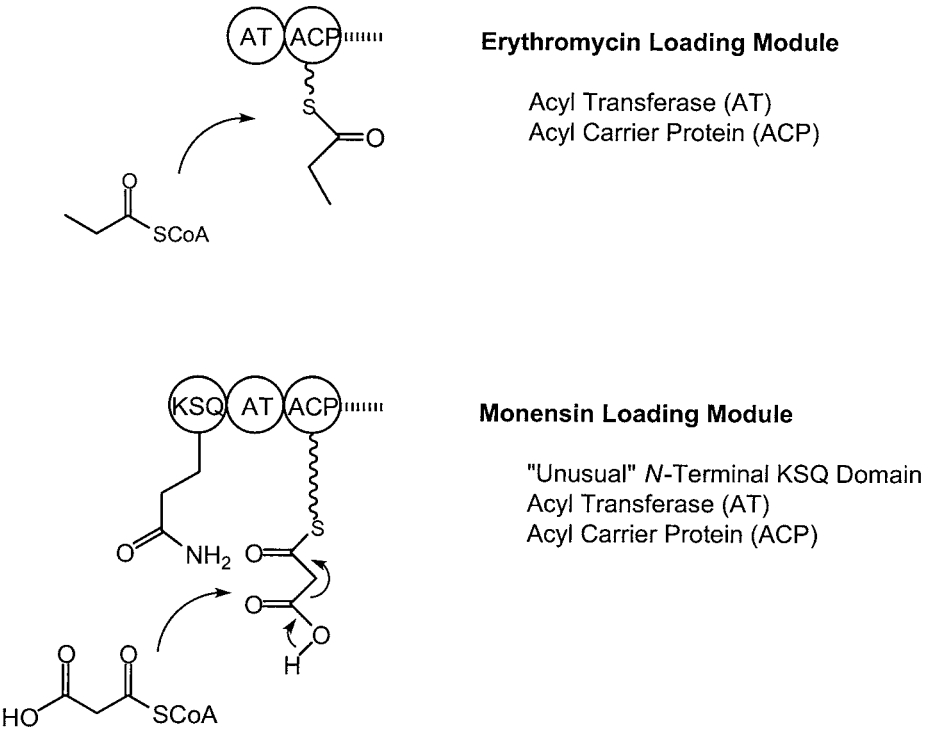

Figure 6 The reactions catalysed by the loading modules of A. the *ery* PKS and B. the *mon* PKS.

be expected to discriminate better between methylmalonyl- and malonyl-CoA, than between propionyl- and acetyl-CoA, because of the additional need to provide a counterion for the carboxylate group. Subsequent decarboxylation by the KSQ domain would represent a further step at which selectivity might be imposed. We therefore undertook to discover whether substitution of the loading module of the erythromycin-producing PKS by the loading module from the monensin A PKS might lead to a selective recruitment of acetate units and allow the production of novel C13-methyl erythromycins. As will be reported in detail elsewhere, these expectations were fulfilled: the hybrid PKS produced the novel C13-methyl erythromycin A in excellent yield and substantially

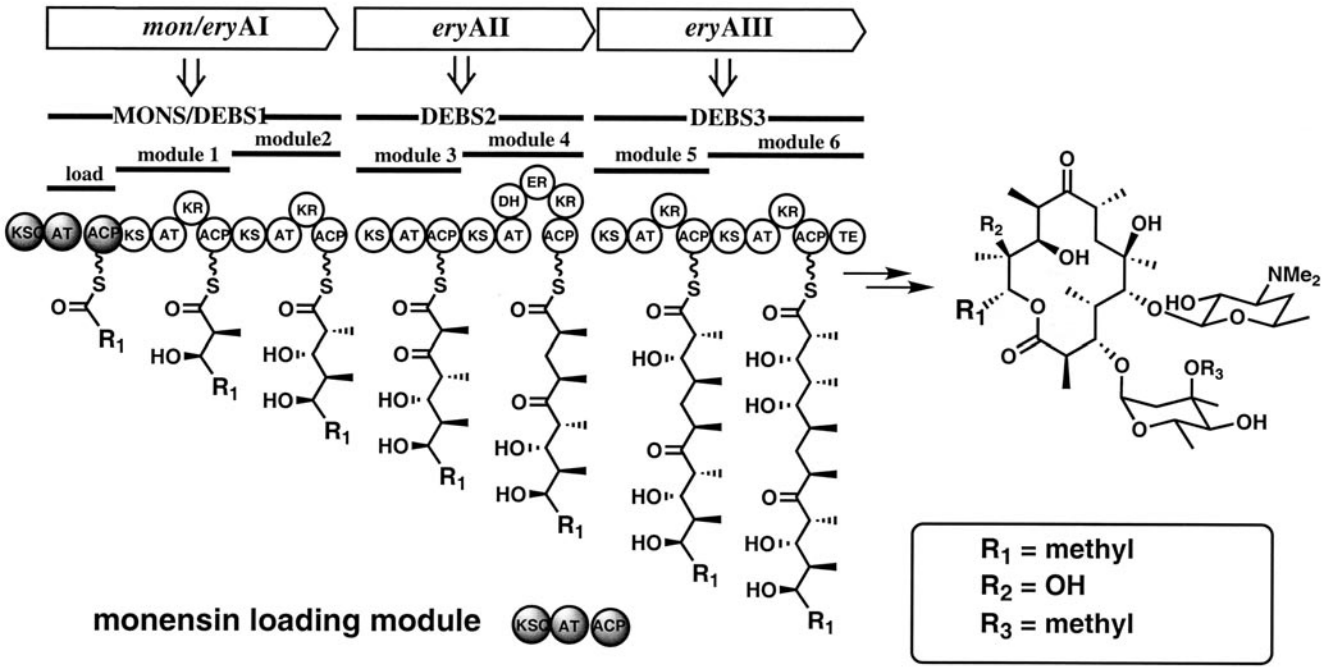

Figure 7 The creation of a highly selective hybrid modular PKS by replacement of the loading module AT-ACP of the erythromycin-producing PKS by the loading module of the monensin-producing PKS.

free of erythromycin A (Figure 7). In previous work, we showed how use of the broad-specificity loading module from the avermectin PKS, when substituted for the erythromycin loading module, can lead (particularly when coupled with the feeding of exogenous carboxylic acids) to the production of a wide variety of novel polyketides [31,34]. The production of C13-methyl erythromycin A from hybrid *mon-ery* PKS illustrates the converse, that the specificity of polyketide chain initiation can be altered and heightened, providing a convenient and easily scaled-up process to yield a valuable and previously unobtainable macrolide template.

Acknowledgements

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