Polyether Biosynthesis

Intermediates Released from a Polyether-Producing Polyketide Synthase Provide Insight into the Mechanism of Oxidative Cyclization**

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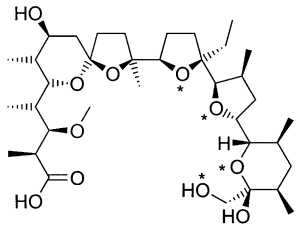
Monensin A (**1**) from *Streptomyces cinnamonensis* is an antibiotic ionophore[1] widely used in veterinary medicine and also as a food additive in animal husbandry. It is one of the most important and best-studied members of a very large family of structurally related polyketide secondary metabolites, the polyethers. As with all natural polyethers, the molecule contains a multiplicity of asymmetric centers, but only one stereoisomer (out of 2^{17}) is produced by *S. cinnamonensis*. The molecular basis for this exquisite stereocontrol is not understood, and even the nature of the intermediates in polyether biosynthesis has until recently been a matter for conjecture. The results of early feeding studies using carbon-14-labeled precursors showed that monensin A is derived by a polyketide pathway from five acetate, one butyrate, and seven propionate units,[2] and similar studies showed that oxygen atoms (O)1, (O)3, (O)4, (O)5, (O)6, and (O)10 arise from the carboxylate oxygen atoms of the corresponding carboxylic acid precursor units, while four other oxygen atoms, at C(13), C(17), C(21), and C(26), are derived from molecular oxygen (Scheme 1).[3] On this basis, several plausible mechanisms have been suggested for the key oxidative steps.[3b,4] These hypotheses all involve the intermediacy of open-chain precursors, but the key difference concerns the stereochemistry of the double bonds (compare, for example, structures **2** and **3** in Scheme 2a and b, respectively). However, the exact nature of the pathway has proved elusive because it has not been possible to detect any intermediates prior to oxidative ring formation.



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Scheme 1. The polyether ionophore monensin A (**1**). Oxygen atoms arising from molecular oxygen are shown with asterisks.

An appropriately functionalized triketide precursor has been successfully incorporated intact into monensin A[5] in the presence of an inhibitor of fatty acid oxidation, thus supporting the view that the carbon backbone is assembled by processive operation of a modular polyketide synthase (PKS).[6] However, a synthetic sample of the full-length putative linear triene precursor **2** was found not to be incorporated into monensin A by *S. cinnamonensis*.[7] Therefore, the configuration of the double bonds in a putative linear pre-monensin, as well as the mode of oxidative cyclization, have remained undefined. Indeed, if oxidative cyclization can be initiated on the growing polyketide chain while it is attached to the PKS, then such full-length linear polyketides as, for example, **2** and **3** may not even be true intermediates.

The entire monensin biosynthetic gene cluster has recently been sequenced[8] and it has been found to contain eight contiguous open reading frames (ORFs) housing a modular PKS with 12 extension modules, which is consistent

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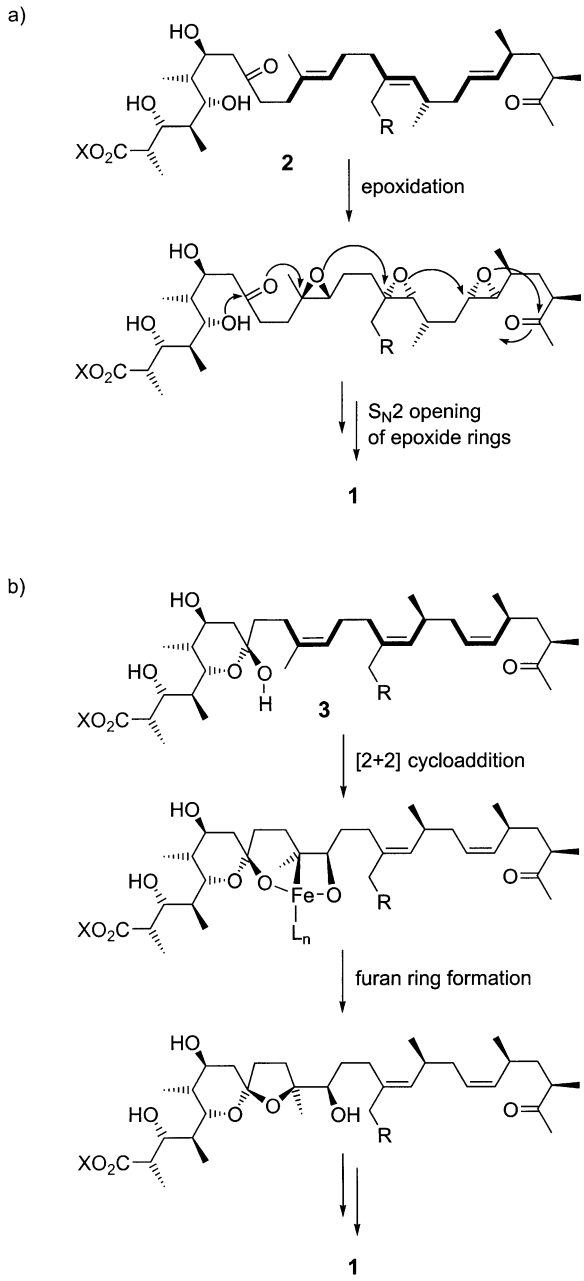
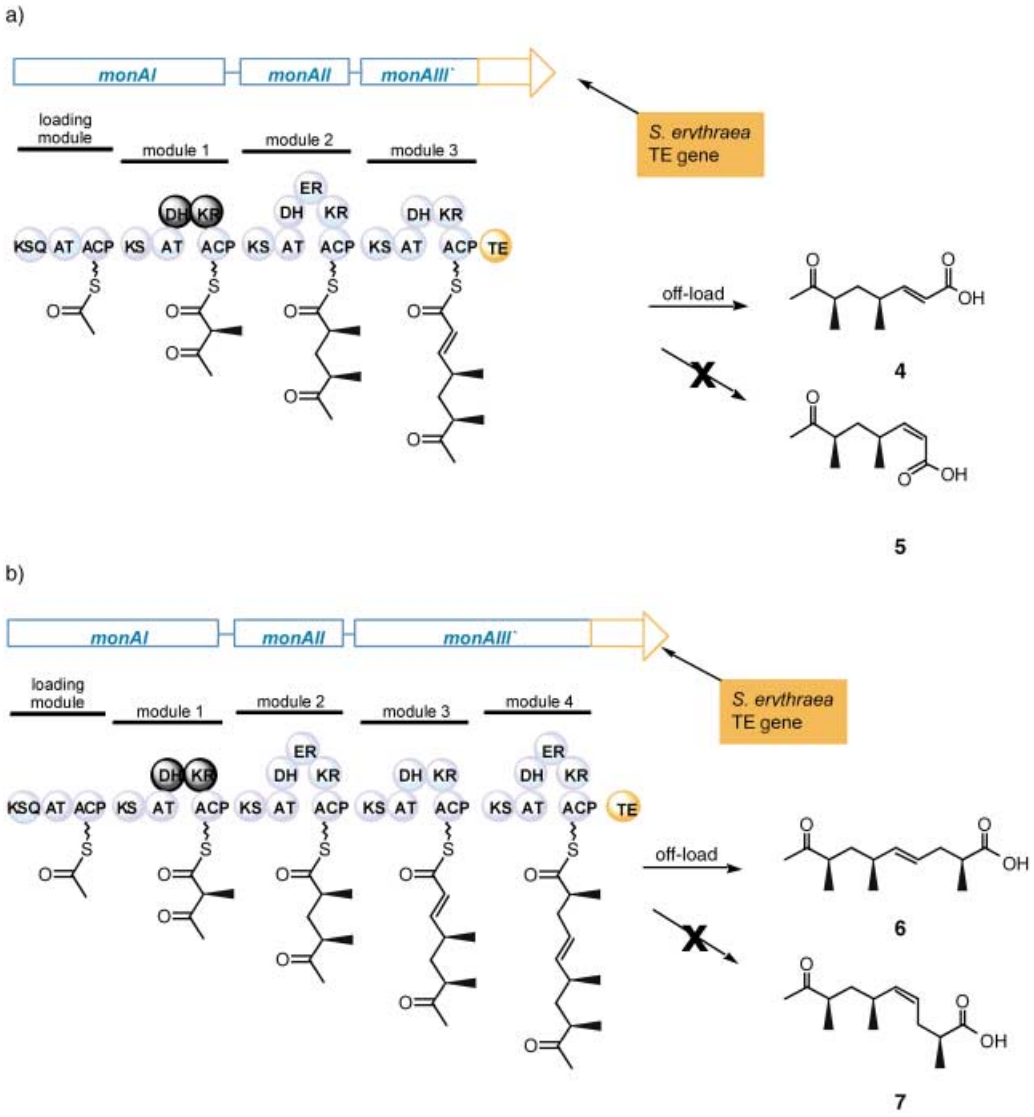


Scheme 2. Examples of alternative mechanisms for oxidative cyclization to produce monensin. a) Cane–Westley proposal of a polyepoxide cyclization cascade starting from *E*,*E*,*E* triene **2**; b) Townsend–Basak proposal involving [2+2] cycloadditions starting from the *Z*,*Z*,*Z* triene **3**. Monensin A: R = CH_3; monensin B: R = H.

with the production of a premonensin polyketide chain, together with numerous other genes likely to be involved, for example, in antibiotic regulation and export. Analysis of the DNA sequence revealed in particular a set of four unusual ORFs which, on the basis of database comparisons, appear to relate to the unusual oxidative cyclizations that are required to produce the polycyclic product.[4f] Here we describe an experimental approach to isolate and identify crucial open-chain intermediates from the monensin-producing PKS, and to gain the first insight into the stereochemistry and timing of the oxidative steps.

The study of DEBS, the modular PKS governing the biosynthesis of the macrolide erythromycin A in *Saccharopolyspora erythraea*, was greatly advanced when it was found that the chain-terminating thioesterase (TE) domain could release truncated polyketide chains when it was relocated from the C-terminal end of DEBS extension module 6 to the C-termini of extension modules 2, 3, or 5.[9] It was hoped that the introduction of the DEBS TE domain at the end of certain extension modules in the monensin PKS in *S. cinnamonensis* would provoke the release of specific linear intermediates, even though the normal action of the TE involves cyclization. It should be noted that in erythromycin A biosynthesis, the C-2 and C-3 positions in the polyketide intermediates normally bear methyl and hydroxy groups, respectively, while, in contrast, a number of the monensin PKS-bound intermediates would contain C-2 methyl and C-2,3 olefinic groups. However, the *N*-acetylcysteamine thioesters of structural analogues of such intermediates are hydrolyzed by the purified DEBS TE in vitro and previous studies both in vivo and in vitro have shown that in general the TE has a relaxed specificity.[10a] Thus, the 3′-portion of the *eryAIII* gene which encodes the TE domain was positioned downstream of either module 3 or module 4 of the monensin PKS to create a tetra- and a pentaketide synthase, respectively (Scheme 3). We hoped in this way to isolate the specific products from these hybrid enzymes, and thus to deduce the stereochemistry and structure of both the tetraketide and pentaketide intermediates.

Introduction of the TE domain from the DEBS cluster was accomplished by homologous recombination into wild-type *S. cinnamonensis* by a single crossover event. Correct integration into the chromosome was proved for both mutant strains ZAHT-2 (tetraketide synthase) and ZAHT-1 (pentaketide synthase) by Southern hybridization. No monensin was produced by either of these mutants, as judged by HPLC analysis. The truncated monensin PKSs were expected to produce either the *E* tetraketide acid **4** and the *E* pentaketide acid **6**, respectively, or their corresponding *Z* isomers, **5** and **7** (Scheme 3), or conceivably both isomers as a mixture. Analysis was carried out by GC-MS after methylation of the crude extracts using (trimethylsilyl)diazomethane (TMS-diazomethane).[11] Both analytes showed a major peak corresponding to the predicted mass of the tetra- and pentaketide derivatives, respectively (Figure 1). The structure of the tetraketide was determined by comparison with an authentic sample derived by chemical synthesis.[12] GC-MS analysis clearly indicated that the *trans* isomer of the tetraketide was formed (corresponding to **4**). However, it is possible that isomerization of the double bond (which is conjugated to the carbonyl group) might have occurred during extraction under the acidic conditions used (pH 3.5). To enable the stereochemistry of the pentaketide to be determined, a 500 mL culture of mutant ZAHT-1 was grown under standard conditions[4e] and the crude extract of the supernatant was then sequentially purified by column chromatography to yield 22 mg of pure pentaketide acid.[13] Detailed NMR analysis of this acid unequivocally showed the configuration of the disubstituted double bond to be *trans*; the intermediate giving rise to this would be **6**. No evidence of the presence of an



Scheme 3. Truncated polyketide synthases generated from the monensin PKS by integration into *S. cinnamonensis* of the DNA encoding the C-terminal thioesterase domain of the erythromycin-producing PKS C-terminal of a) extension module 3 (in strain ZAHT-2) and b) extension module 4 (in strain ZAHT-1). ACP, acylcarrier protein; KS, ketoacyl-ACP synthase; AT, acyltransferase; KR, ketoacyl-ACP ketoreductase; DH, dehydratase; ER, enoyl-ACP reductase; KSQ, malonyl-ACP decarboxylase; TE, thioesterase. The tetraketide and pentaketide products released from these synthases are also shown as **4** and **6**, respectively. Products **5** and **7** were not detected.

oxidized derivative of either tetraketide or pentaketide was obtained in either of these analyses of the presence of an oxidized derivative, which suggests (though it does not prove) that further elongation of the polyketide chain is required before epoxidation of the double bond in these intermediates can take place.

Taken together, these results directly implicate the *E* forms of the tetraketide and pentaketide as intermediates in monensin biosynthesis and the *trans* double bond that they contain as one of the targets for the epoxidase MonC1 which initiates oxidative cyclization.[4f] This is consistent with mechanisms which invoke triepoxide intermediates, as proposed by Cane and Westley[4a] (Scheme 2a) and by us,[4e] but not with the mechanistic proposal of Townsend and Basak.[4c] Furthermore, our results underscore the utility of the DEBS TE domain for the specific release of truncated intermediates from a heterologous PKS and suggest that the same experimental approach may permit structural characterization of more elaborate open-chain precursors of monensin A. In particular, the stage is set for dissection of the respective contributions of the PKS and of the "post-PKS" enzymes MonCI (epoxidase), MonCII (cyclase), MonBI, and MonBII (putative isomerases) to the stereocontrol of polyether biosynthesis.

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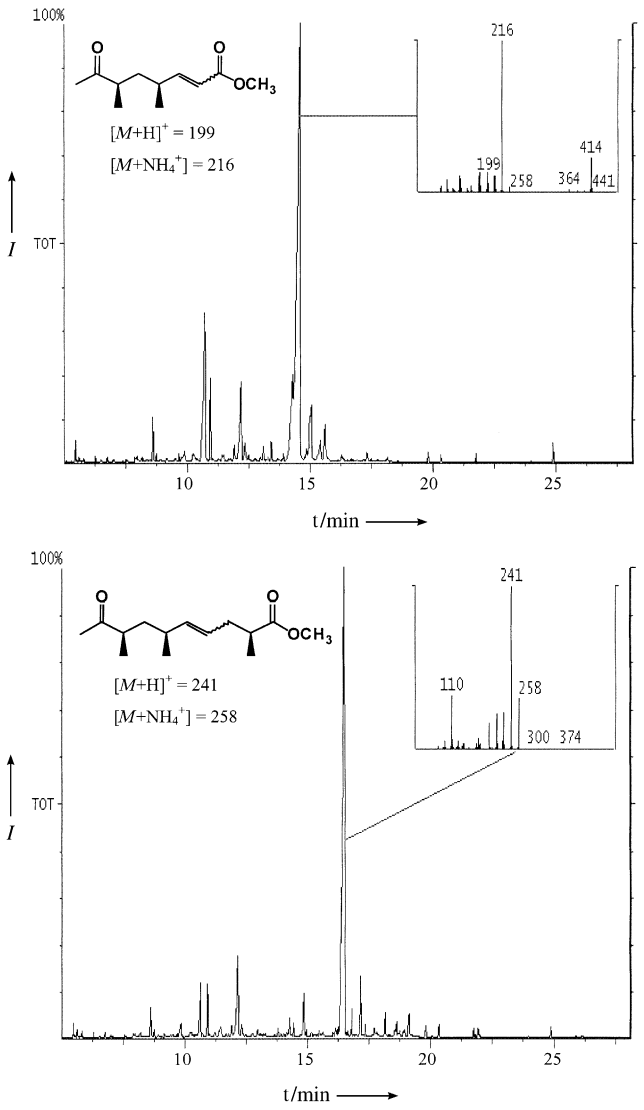

Figure 1. GC-MS analysis of a crude extract of a) the tetraketide-producing strain ZAHT-2 and b) the pentaketide-producing strain ZAHT-1 after methylation of the samples indicating the presence of **4** and **6**, respectively.

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[11] a) The construction and fermentation of the mutant strains of *Streptomyces cinnamonensis* containing the plasmids pZAHT-1 (pentaketide synthase) and pZAHT-2 (tetraketide synthase), respectively, is described in the Supporting Information. The experimental design led to the introduction of an additional *Mfe*I restriction site at the 3′-end of the ACP domain in each case. For the tetraketide synthase, this altered the C-terminal residue of the ACP domain from module 3 of the *mon* PKS from a histidine to a glutamine residue. The counterpart residue in the ACP domain of module 4 of the pentaketide synthase was altered from arginine to glutamine. Both substitutions at this position are known in natural PKS ACP domains. b) N. Hashimoto, T. Aoyama, T. Shiori, *Chem. Pharm. Bull.* **1981**, *29*, 1475.

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[13] Spectral data for the pentaketide acid **6**: ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 0.98$ (d, $J = 6.7$ Hz, 3H), 1.03 (d, $J = 6.9$ Hz, 3H), 1.16 (d, $J = 7.0$ Hz, 3H), 1.23 (m, 1H), 1.58 (ddd, $J = 9.8$, $J = 5.3$, $J = 5.3$ Hz, 1H), 2.09 (s, 3H), 2.13 (m, 2H), 2.34 (ddq, $J = 7.1$, $J = 6.9$, $J = 1.0$ Hz, 1H), 2.49 (m, 2H), 5.24 (dd, $J = 15.2$, $J = 8.4$ Hz, 1H), 5.34 ppm (dt, $J = 15.2$, $J = 6.9$ Hz, 1H); the coupling constant of $J = 15.2$ Hz between the hydrogen atoms located on the C=C bond is indicative of a *trans* configuration (typical range 14–16 Hz);^{13}C NMR (125.8 MHz), $CDCl_3$, 25 °C): $\delta = 13.9$, 15.6, 16.1, 27.2, 34.8, 36.2, 39.2, 39.4, 45.0, 125.9, 138.1, 180.8, 213.1 ppm; IR ($CHCl_3$): $\tilde{\nu} = 3683$, 3029, 2433, 2399, 2242, 1748, 1706, 1601, 1521, 1476, 1423, 1334, 1214, 1016, 928, 849 cm^{-1}; HRMS (ES) calcd for $C_{14}H_{24}O_3Na^+$ $[M+Na]^+$: 263.1627, found: 263.1618.

