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Accumulation of an *E,E,E*-Triene by the Monensin-Producing Polyketide Synthase when Oxidative Cyclization is Blocked**

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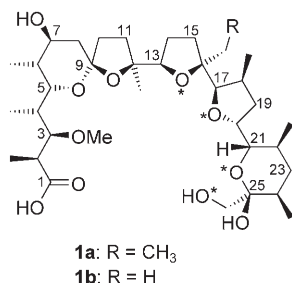
Monensin A (**1a**) from *Streptomyces cinnamonensis* is an antibiotic ionophore[1] widely used in veterinary medicine and in animal husbandry (Scheme 1). It is one of the most important and best-studied members of a large family of structurally related polyketide secondary metabolites, the ionophoric polyethers, which are attracting renewed interest for their activity against drug-resistant strains of malaria.[2] As with all natural polyethers, the molecule contains a multiplicity of asymmetric centers, but only one stereoisomer (out of a possible 2^{17}) is produced by *S. cinnamonensis*. The molecular basis for this exquisite stereocontrol is not known, and even the nature of the intermediates in polyether biosynthesis remains obscure. Early feeding studies established that monensin A (**1a**) is derived by a classic polyketide pathway from five acetate, one butyrate, and seven propionate units[3] and that oxygen atoms at C1, C3, C5, C7, C9, and C25 arise from the carboxylate oxygen atoms of the corresponding carboxylic acid precursor units, whereas the remaining oxygen atoms at C13, C17, C21, and C26 are derived from molecular oxygen.[4] On this basis, the "cascade" mechanism shown in Scheme 2a was proposed for the key oxidative steps.[4b] This mechanism involves the intermediacy of the linear *E,E,E*-triene **2** and of the triepoxide **3** as enzyme-free intermediates. However, it has proved difficult to obtain direct experimental support for this mechanism, and in particular, a synthetic sample of the full-length putative linear *E,E,E*-triene precursor **2** was found not to be incorporated into monensin A by *S. cinnamonensis*.[5] The configuration of the double bonds in a putative linear precursor of monensin, as well as the mode of oxidative cyclization, therefore, remained undefined, and alternative pathways for oxidative cyclization[6] that involved either a *Z,Z,Z* triene[6d,e] or a *E,Z,Z* triene[6f] have been subsequently proposed (Scheme 2b).



Scheme 1. The polyether ionophores monensin A (**1a**) and monensin B (**1b**). Oxygen atoms arising from molecular oxygen are denoted by *.

The recent total sequencing of gene clusters for the biosynthesis of monensin[6f,7] and of nanchangmycin[8] revealed, in each case, large open reading frames (ORFs) encoding a modular polyketide synthase (PKS) with the anticipated number of extension modules consistent with the production of a linear "pre-polyether" polyketide chain of the requisite length. Complete deletion of the gene cluster from *S. cinnamonensis* resulted in loss of monensin production, thus confirming its role in monensin biosynthesis.[7] Each cluster contains numerous other genes, in particular four ORFs which, on the basis of database comparisons, appear to relate to the unusual oxidative cyclizations that are required to produce the polycyclic product.[6f,7] Thus, the monensin-producing cluster contains an epoxidase gene (*monCI*), a gene with apparent similarity to an epoxide-opening enzyme (*monCII*), and two mutually homologous genes (*monBI* and *monBII*) with apparent similarity to the Δ^5-3-ketosteroid isomerase of *Comamonas testosteroni* and which were considered initially to possibly function as *cis–trans* isomerases.[6f,7] Further proof that MonCI was an epoxidase was obtained when a *Streptomyces coelicolor* strain that overexpressed monCI converted the unsaturated terpene (±)-linalool into linalool oxide at levels 10–20-fold higher than a control strain.[7] Subsequently, it proved possible to interrupt polyketide-chain synthesis by genetically relocating a chain-terminating thioesterase (TE)/cyclase domain, obtained from the modular PKS that governs the biosynthesis of the macrolide erythromycin A in *Saccharopolyspora erythraea*,[9] to downstream of extension module four of the monensin PKS. In the pentaketide released by this recombi-

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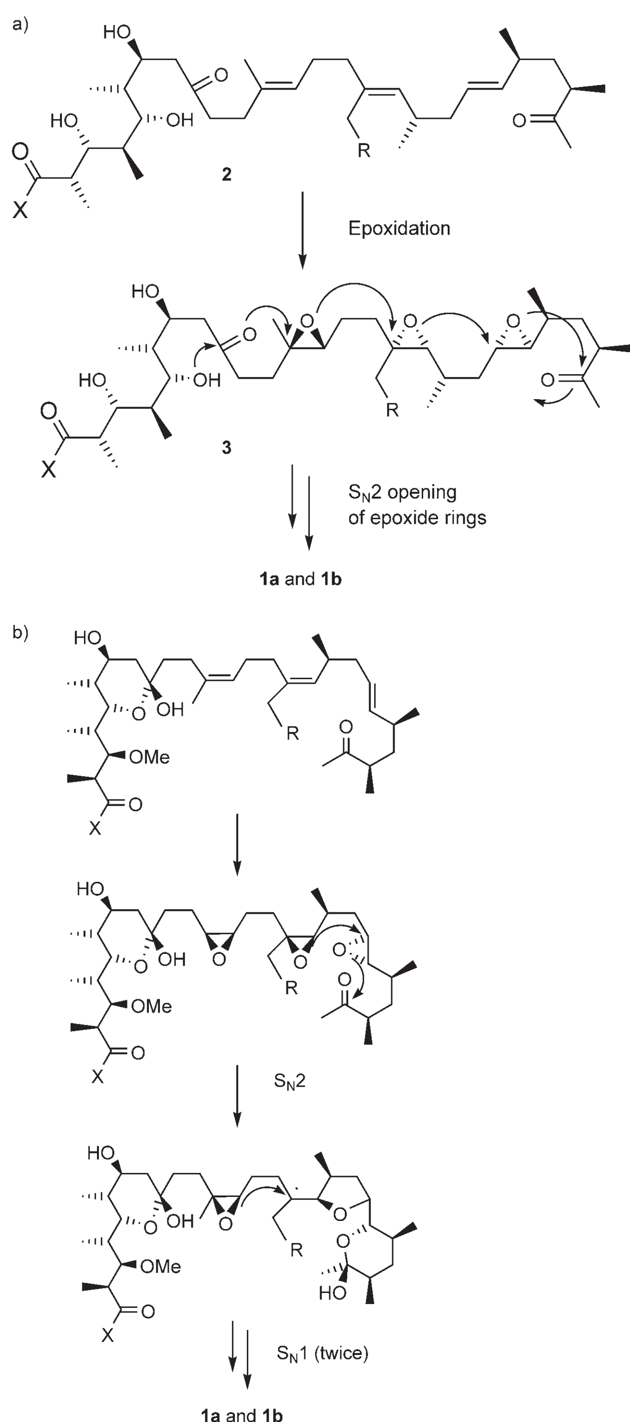

Scheme 2. Alternative mechanisms for oxidative cyclization to produce monensins A and B. a) A polyepoxide cyclization cascade that starts from an *E*,*E*,*E* triene[6b]; b) a polyepoxide cyclization cascade starting from an *E*,*Z*,*Z* triene[6f] **3**. X = OH, SCoA, or S-protein.

nant strain, the configuration of the disubstituted double bond was unequivocally shown to be *E*,[10] which weighed in favor of **2** as an intermediate[6b] or a variant of **2** in which the other bonds are *Z*,[6g] but ruled out the mechanistic proposal of Townsend and Basak.[6d] Based on these results, we reasoned that if *monCI* were indeed the sole initiator of oxidative cyclization in monensin biosynthesis then the deletion of this single gene in *S. cinnamonensis* ought to allow the accumulation of a linear molecule, such as **2** (or perhaps of a shunt product derived from such a compound) whose structural characterization might reveal the likely pathway for cyclization, thus allowing discrimination between the mechanistic proposals of Scheme 2.

Deletion of *monCI*, which encodes the putative flavin-dependent epoxidase from the monensin cluster, was accomplished by homologous recombination into wild-type *S. cinnamonensis* through two successive single crossover events, thus leading to replacement by a hygromycin resistance gene.[11a] Correct integration into the chromosome was proved for the mutant strain ABΔCI by Southern hybridization. Further confirmation of its identity was provided by complemention with the cloned gene borne on an expression plasmid (see the Supporting Information) which restored monensin production (data not shown). By further engineering of the *monCI*-deleted strain so that it also contained an in-frame deletion in *monBI*, the additional blocked mutant ABΔCIΔBI was obtained. Further mutants with insertional inactivation of both of the adjacent genes[6f,7] *monCI* and *monBII* (BHΔCIBII) and of all three genes *monCI, monBII*, and *monBI* (BHΔCIBIBII) were created in the same way as for the strain ABΔCI.

Neither monensin A (**1a**) nor monensin B (**1b**) was produced by any of these mutants, as judged by HPLC–mass-spectrometric analysis of the crude extracts.[11a] The analyte from the ABΔCI strain showed a major peak that corresponds to the predicted mass of **4a**, (Scheme 3) a triene lactone that differs only from **2** by the presence of a δ-lactone structure.[11a] A second smaller peak had the predicted mass of a similar triene lactone **4b** that lacks a methylene moiety, thus corresponding to the mass difference between **1a** and **1b**. To confirm the structure and the configuration of **4a** and **4b**, a culture of mutant ABΔCI (7 L) was grown under standard conditions,[6f,11a] and the crude extract of the supernatant was purified by column chromatography on silica gel, which yielded 1.4 mg of **4** and 1.2 mg of **5**. Detailed NMR spectroscopic analysis of these lactones[5,11] proved unequivocally that all the double bonds were *E* configured, that is, the structures of **4a** and **4b** as shown. The 1D NMR spectra collected were similar to that reported by Robinson and co-workers for the synthetic *N*-acylcysteamine thioester of a *E*,*E*,*E* triene.[5] The disubstituted double bond between H20 and H21 was assigned from the coupling constant of $J=15.3$ Hz for the double-bond protons, which is indicative of a *trans* double bond as confirmed by NOE interaction studies between H22 and H20 and between H21 and H19 (Scheme 4).[11a] The NOESY NMR spectrum[11a] shows an interaction between H17 of a trisubstituted double bond and H15, whereas no interactions are seen between H17 and H16′ or H16″, thus showing the double bond to be *E* configured. Similarly the NOE interaction observed for H13 with H11 but not with H12′, also confirms an *E* configuration. No evidence was obtained in these analyses for the presence of an oxidized derivative of either **4a** or **4b**, which suggests (although it does not prove) that the epoxidase encoded by *monCI* is necessary and sufficient for the epoxidation of all three double bonds in

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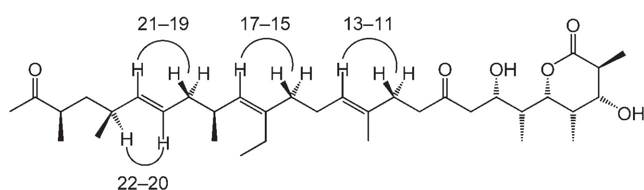
Scheme 3. The monensin PKS, which consists of a loading module and 12 extension modules distributed across eight multienzymes (MonAI–MonAVIII), produces *E*,*E*,*E*-trienes **4a** and **4b** when gene *monCI* is deleted, irrespective of whether additional genes *monBI* and *monBII* are also deleted. WT = wild type.



Scheme 4. The critical NOE interactions for the assignment of the double-bond geometry in lactone **4a** as the *E*,*E*,*E* isomer.

the linear intermediate to form **1**. This remarkable transformation is apparently catalyzed at all three target double bonds with complete stereoselectivity and stereospecificity. There is no effect of additional mutations in one or other or both of the *monB* genes.[11a] The MonB gene products were considered initially, given their significant sequence similarity to the δ-5(3)-ketosteroid isomerase of *C. testosteroni*,[6f,7] to be involved in the alteration of the geometry of two of the double bonds in the triene intermediate at the point during polyketide-chain growth at which they are activated by conjugation with the thioester that links the growing polyketide chain to the PKS multienzyme. As **4a** and **4b** are likely to be shunt metabolites rather than true intermediates in the biosynthetic pathway, such a role is not completely excluded, but the fact that no isomer of **4a** or **4b** with an altered geometry about the double bonds was found undermines such a hypothesis (see also below).

These results taken together confirm that *monCI* is essential for monensin biosynthesis, indicate directly that the all-*E* configuration of **4a** and **4b** is derived from intermediates in monensin biosynthesis, and that these *E* configured double bonds are the targets for the epoxidase MonCI that initiates oxidative cyclization.[6f,7] These results are more consistent with the original proposal of Cane et al.[6b] (Scheme 2a) than with the modification of this proposal in which trisubstituted double bonds are isomerized before epoxidation (Scheme 2b).[6f] Consequently, fresh questions are posed as to the true role of the MonB proteins and their

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counterparts in nanchangmycin biosynthesis, which are found fused head to tail as the product of the single gene *nanI*.[8] Evidence that they play a role in epoxide ring opening will be presented separately.[13] The stage is set for dissection of the respective contributions of these and the other "post-PKS" enzymes to the stereocontrol of polyether biosynthesis. In addition, the successful isolation of novel polyketides **4a** and **4b** underscores the potential of the targeted engineering of polyketide biosynthetic pathways to produce novel stereochemically rich molecules that profoundly differ in structure from the parent molecule. In the present case, **4a** and **4b** are not expected to have ionophoric properties; intriguingly, their structures more closely resemble δ lactones isolated from a number of marine organisms, such as the potent antitumor compounds discodermolide[13] and callystatin.[14] It may be that the elusive PKSs for such marine polyketides are actually closely related to those for ionophoric polyethers.

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[11] a) The construction, characterization, and fermentation of all the mutant strains of *S. cinnamonensis* described herein are given in detail in the Supporting Information, together with the details of complementation by cloned genes *in trans* to restore monensin production, the HPLC–mass-spectrometric analyses of the mutants described, and the NMR spectra; b) spectral data for the *E*,*E*,*E*-triene lactone **4a**: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 0.89$ (d, $J = 6.6$ Hz, 3H), 0.93 (t, $J = 8.6$ Hz, 3H), 0.94 (d, $J = 7.9$ Hz, 2H), 0.96 (d, $J = 6.5$ Hz, 2H), 1.02 (d, $J = 6.9$ Hz, 3H), 1.09 (d, $J = 6.7$ Hz, 3H), 1.20 (ddd, $J = 13.6$, 8.4, 5.1 Hz, 1H), 1.39 (d, $J = 7.1$ Hz, 3H), 1.58 (ddd, $J = 13.6$, 9.6, 5.6 Hz, 1H), 1.59 (s, 3H), 1.73, (m, 1H), 1.90 (m, 2H), 1.95 (m, 2H), 1.98 (m, 2H), 2.01 (m, 2H), 2.08 (m, 1H), 2.09 (s, 3H), 2.25 (t, $J = 7.6$ Hz, 2H), 2.37 (m, 1H), 2.42–2.53 (m, 5H), 2.54, (m, 1H), 2.68 (dd, J = 18.0, 9.9 Hz, 1H), 3.83 (dd, $J = 10.3$, 4.3 Hz, 1H), 4.19 (dt, $J = 10.0$, 2.2 Hz, 1H), 4.21 (dd, $J = 9.3$, 1.9 Hz, 1H), 4.83 (d, $J = 9.59$ Hz, 1H), 5.10 (t, $J = 6.0$ Hz, 1H), 5.12 (dd, $J = 15.3$, 8.45 Hz, 1H), 5.36 ppm (dt, $J = 15.2$, 7.6 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$, 25 °C): $\delta = 4.8$, 9.8, 13.5, 14.5, 15.9, 16.1, 21.2, 21.7, 23.3, 26.8, 28.0, 32.4, 33.3, 35.1, 35.2, 36.2, 38.6, 39.9, 39.9, 40.7, 42.2, 45.2, 46.5, 66.1, 74.0, 81.4, 125.4, 128.3, 130.6, 132.9, 136.2, 139.0, 173.8, 212.3, 213.2 ppm.

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