Molecular basis of Celmer's rules: the role of two ketoreductase domains in the control of chirality by the erythromycin modular polyketide synthase

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Background: Polyketides are compounds that possess medically significant activities. The modular nature of the polyketide synthase (PKS) multienzymes has generated interest in bioengineering new PKSs. Rational design of novel PKSs, however, requires a greater understanding of the stereocontrol mechanisms that operate in natural PKS modules.

Results: The *N*-acetyl cysteamine (NAC) thioester derivative of the natural β-keto diketide intermediate was incubated with DEBS1-TE, a derivative of the erythromycin PKS that contains only modules 1 and 2. The reduction products of the two ketoreductase (KR) domains of DEBS1-TE were a mixture of the (2*S*,3*R*) and (2*R*,3*S*) isomers of the corresponding β-hydroxy diketide NAC thioesters. Repeating the incubation using a DEBS1-TE mutant that only contains KR1 produced only the (2*S*,3*R*) isomer.

Conclusions: In contrast with earlier results, KR1 selects only the (2*S*) isomer and reduces it stereospecifically to the (2*S*,3*R*)-3-hydroxy-2-methyl acyl product. The KR domain of module 1 controls the stereochemical outcome at both methyl- and hydroxyl-bearing chiral centres in the hydroxy diketide intermediate. Earlier work showed that the normal enzyme-bound ketoester generated in module 2 is not epimerised, however. The stereochemistry at C-2 is therefore established by a condensation reaction that exclusively gives the (2*R*)-ketoester, and the stereochemistry at C-3 by reduction of the keto group. Two different mechanisms of stereochemical control, therefore, operate in modules 1 and 2 of the erythromycin PKS. These results should provide a more rational basis for designing hybrid PKSs to generate altered stereochemistry in polyketide products.

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Introduction

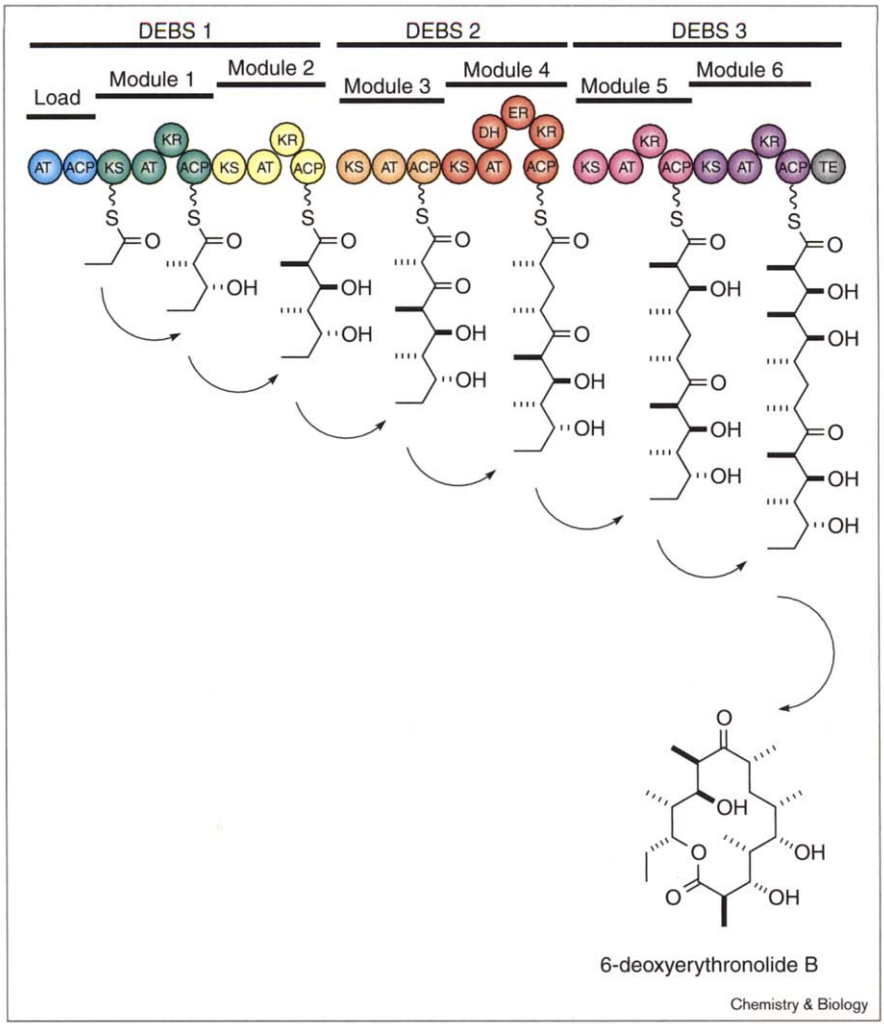
Macrolide polyketides, such as erythromycin A, have multiple chiral centres. The mechanism of stereochemical control in polyketide biosynthesis is, therefore, an important and challenging problem. Interest has been increased recently by many examples of bioengineering in which the synthase responsible for assembly of the macrolide core has been altered to produce specific alterations in the structure of the natural product [1–4]. Recently, stereochemistry has also been altered by the replacement of ketoreductase (KR) domains [5].

The rapid progress in experiments designed to alter structure has been based on the modular nature of the polyketide synthases responsible for chain assembly, which first became apparent with the sequencing of the erythromycin polyketide synthase (PKS) [6,7]. The genes responsible for the erythromycin PKS code for three giant multifunctional proteins in which there are multiple active sites (domains) that show strong homology to the various enzymes known to be responsible for fatty acid biosynthesis. There are six sets or 'modules' of collaborating domains with a different module catalysing each cycle of chain extension (Figure 1).

Each module contains three core domains that are essential for C–C bond formation: a ketosynthase (KS) domain and an acyl carrier protein (ACP), which cooperate to extend the chain by catalysing the decarboxylative condensation of a methyl-malonyl unit with a pre-existing acyl chain, and an acyltransferase domain (AT), which loads the malonate extender unit onto the ACP. A module can also contain one or more reductive domains to modify the ketone group in the initially formed keto ester.

The full set of reductive steps follow the course shown in Figure 2. The keto group is first reduced by NADPH in a reaction catalysed by the KR domain to give a β-hydroxy acyl residue. This can then be dehydrated to a α-β-unsaturated acyl group by a dehydratase (DH) domain and further reduced to a methylene by NADPH

Figure 1



The modular nature of the erythromycin PKS. There are six chain extension modules, each beginning with a KS domain, that are responsible for adding the six successive C_3 units. The loading module specifies propionate as starter unit. The completed chain is released as a macrolactone by the thioesterase (TE) domain. AT, acyl transferase; ACP, acyl carrier protein; KS, β-ketoacyl synthetase; KR, β-ketoacyl reductase; ER, enoyl reductase; DH, dehydratase.

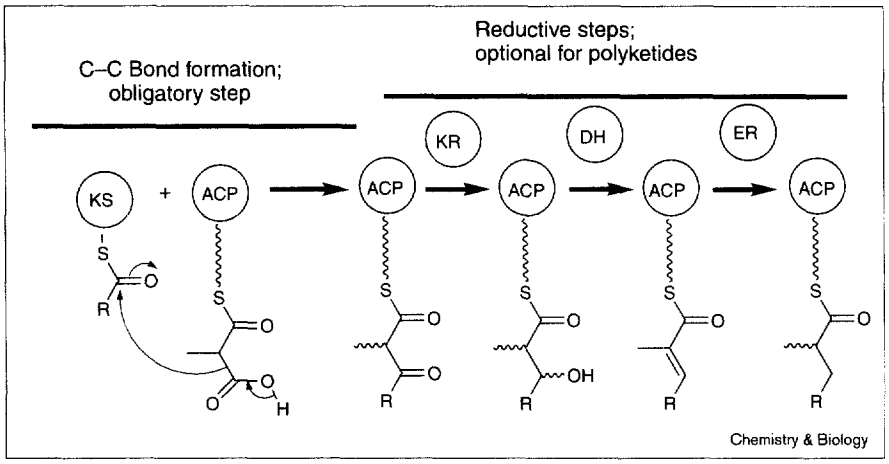
catalysed by an enoyl reductase (ER) domain. The extent of reduction in each module of the erythromycin PKS can be predicted on the basis of these optional domains, which are shown in Figure 1 as 'reductive loops' set above the line of core domains in each module. Only in module 4 is there a complete reductive loop leading to complete reduction of the keto group to a methylene, whereas in modules 1, 2, 5 and 6, which have only an active KR domain, reduction stops at the hydroxy stage. In module 3, there is no catalytically active KR domain and so no reduction occurs.

Although analysis of the primary structure of the protein allows the function of the various catalytic domains to be predicted, no correlation has been established between the primary structure of the protein and the stereochemistry of the various reactions. Therefore, although it is possible to predict with some confidence the structural outcome of each chain-extension cycle from the domain structure of a module, no prediction can made about the stereochemistry of any new chiral centres.

Celmer noted as early as 1965 that there was a strong structural and stereochemical homology between the structures of the then-known polyketide macrolides [8]. The homology was most marked within the family of 14-membered macrolides but it also extended to the families of 12- and 16-membered macrolides, if allowance was made for the absence of a chain-extension unit or the presence of an extra one. The extent and underlying nature of these structural relationships can best be appreciated by considering the structures of the putative PKS products of different biosynthetic pathways. Three representative examples are shown in Figure 3. The erythromycin intermediate shows strong homology to the methymycin intermediate and also to the tylosin intermediate if allowance is made for the absence of methyl-branching centres or their replacement by an ethyl branch. This homology

Figure 2

The reactions of a chain-extension cycle involving complete reduction of the initially formed keto ester to a saturated analogue, and the domains that catalyse the various steps.



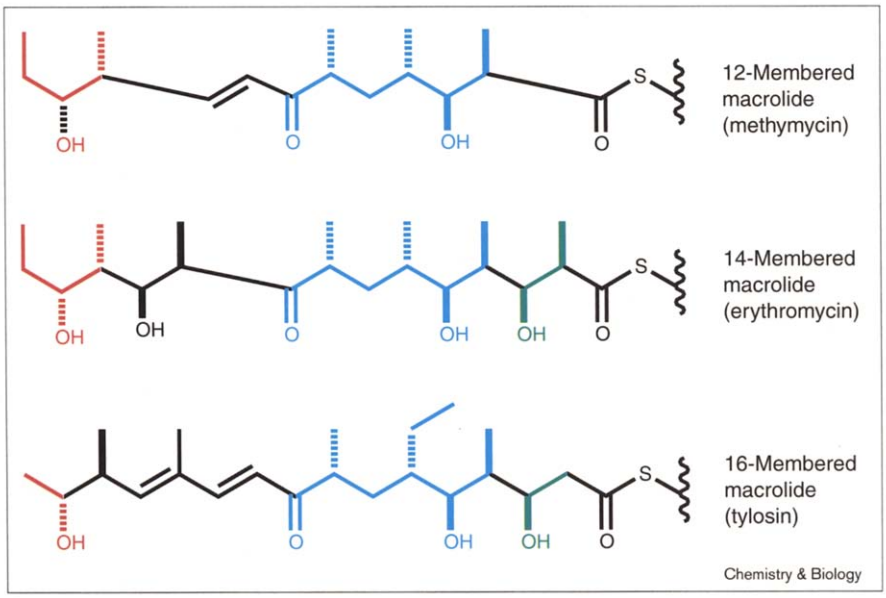
points to a common evolutionary origin for the PKS multienzymes serving these biosynthetic pathways. It also encourages the hope that the common stereochemical features of the natural products might be genetically determined and, therefore, that it might be possible to make targeted alterations to the genes to produce novel products with altered stereochemistry. An understanding of the molecular basis of stereocontrol in the biosynthetic operations of the PKSs would be of great assistance in achieving this goal.

The earliest speculations concerning the differing stereochemistries of methyl-branching sites assumed that these might be determined by the particular stereoisomer of methylmalonate used for chain extension. Both (2*R*)- and (2*S*)-methylmalonyl-CoA are present in natural systems and there are epimerases that can interconvert them [9]. When deuterium-labelled precursors were fed to an erythromycin-producing strain of *Saccharopolyspora erythraea*, the pattern of label in the macrolide products was consistent with incorporation of (*S*)-methylmalonyl-CoA followed by condensation with inversion of configuration at only three of the six methyl-bearing centres, produced by modules 2, 5 and 6 [10]. Incorporation levels were very low, however, and no conclusion could be drawn concerning the proposed incorporation of the (*R*) isomer at the remaining three methyl-bearing sites produced by modules 1, 3 and 4.

The generation of DEBS1-TE, a truncated version of the erythromycin PKS, allowed this problem to be addressed

Figure 3

The putative PKS products of three macrolides, methymycin, erythromycin and tylosin. Regions of structural and stereochemical homology are related by colour coding.

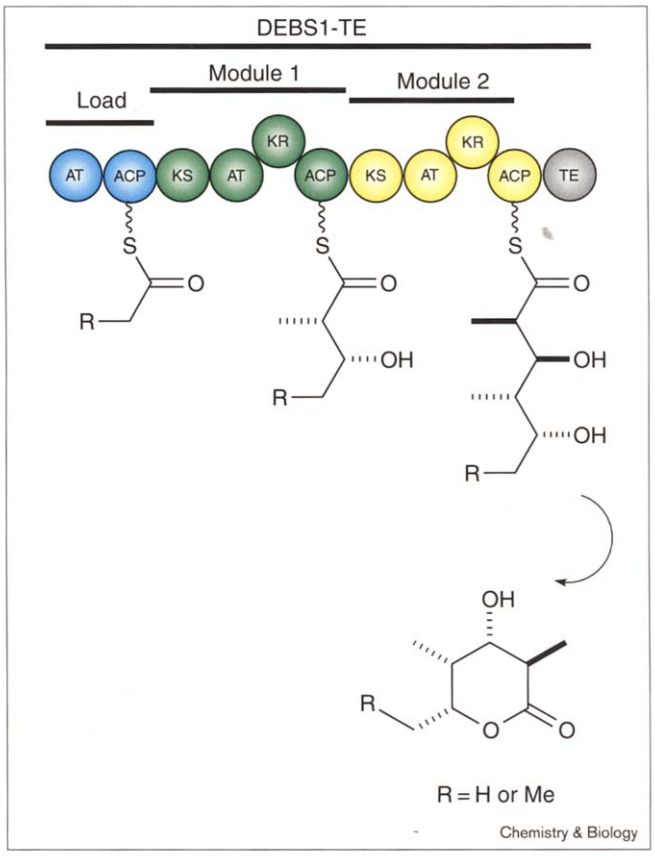


directly [11]. DEBS1-TE produces a triketide lactone in which the two different stereochemistries at the methyl-branching centres are represented (Figure 4). It was found that (2*S*)-methylmalonyl-CoA served as a chain-extension building block for both modules, and that the (2*R*) isomer did not support chain extension [12]. Stereochemical control at methyl-branching centres must therefore be exerted by the chirality of the chain-extension processes themselves, and not by the acyltransferase in each extension module that delivers the unit of methylmalonate used for chain extension.

When DEBS1-TE was incubated with (2*S*)-methylmalonyl-CoA labelled with deuterium at the methyl-bearing chiral centre, it was found that in catalysis by module 1 the deuterium was completely lost in forming the methyl-branching centre of the first chain-extension unit, whereas in catalysis by module 2, it was completely retained through the chain-extension reactions [13]. Condensation therefore takes place with inversion of configuration in module 2 to give the (2*R*) stereoisomer of the β-keto ester, which is then reduced by KR2 without affecting the chiral centre at C-2 to give the (2*R*,3*S*)-3-hydroxy-2-methyl derivative. It was proposed that, in module 1, condensation takes place in exactly the same sense, and that the (2*R*) isomer of the β-keto ester then undergoes epimerisation at C-2 with consistent loss of the hydrogen label to give the (2*S*) isomer at that site. This is then accepted as a substrate and reduced by KR1 to give the (2*S*,3*R*)-3-hydroxy-2-methyl diketide intermediate. This interpretation suggests that KR1 controls the stereochemistry at both C-2 (by substrate selection) and at C-3 (by the stereospecificity of hydride addition). The deduction that KS1 initially forms the (2*R*)-isomer of the diketide ketoester intermediate was confirmed recently by the observation that a module can be formed in which KS1 cooperates with KR2 [14]. The resulting acyl product was the (2*R*,3*S*)-3-hydroxy-2-methyl isomer of the diketide.

Our aim in this paper is to test these hypotheses by direct investigations *in vitro* on the stereoselectivity of KR1 towards the (*R*) and (*S*) isomers of the ketoester diketide intermediate, and the stereospecificity of the subsequent reduction of the keto group. For these experiments we used the triketide lactone synthase, DEBS1-TE [11], and a deletion mutant of DEBS1-TE, designated DEBS1-TE (ΔKR2), in which there is a substantial deletion in the KR domain (amino acids 3103–3278 in the conserved region) of module 2 (Figure 5). The first of these proteins contains two active KR domains, KR1 and KR2. The second protein has only one domain capable of carrying out reduction, KR1. The purification of this mutant protein followed the procedure previously developed for DEBS1-TE and more detailed investigations of its properties will be described in detail elsewhere. Studies with the isolated protein *in vitro* proved that it generated the predicted ketolactone with the reported nuclear magnetic resonance (NMR) spectrum [15].

Figure 4



The modular structure of the truncated DEBS, DEBS1-TE, and its synthetic operations.

Results and discussion

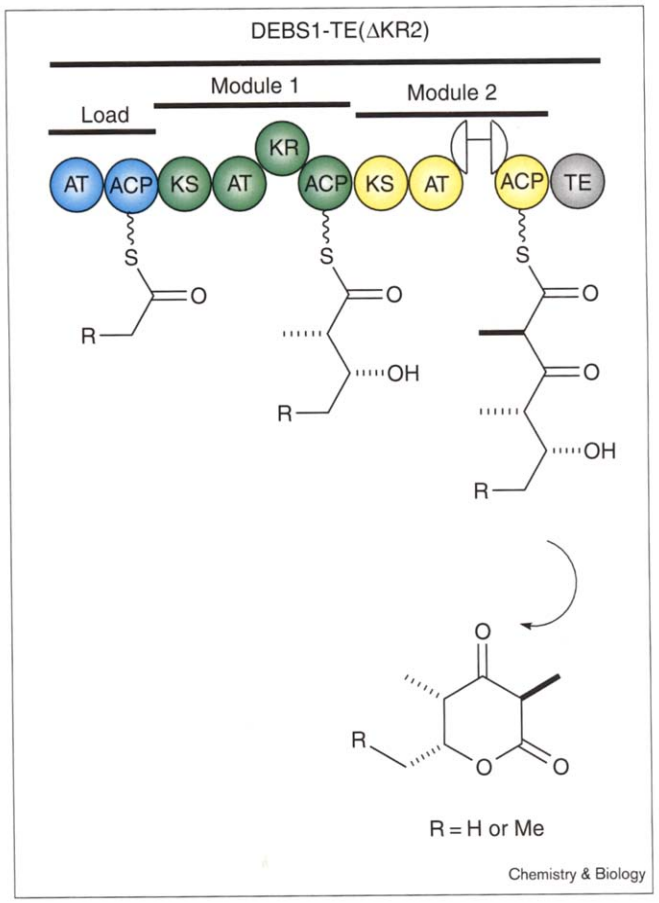
Choice of substrate

In its normal context, the ketoester form of the diketide remains covalently bound as a thioester to the phosphopantetheine thiol of the ACP during reduction by the adjacent KR. To simplify the experimental approach, we decided to use a nonenzyme-bound derivative of the ketoester, the *N*-acetyl cysteamine thioester, as a probe of the stereocontrol. By analogy, with the use of *S*-acetoacyl-NAC thioester as a ketoreductase substrate for a fatty acid synthase, it was expected that the reduction product would accumulate as its NAC thioester (see Figure 6).

Synthesis and properties of the NAC thioester derivative of the ketoester diketide

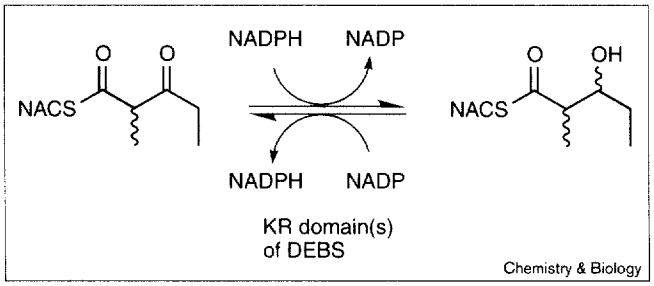
An established synthesis was used to prepare the NAC derivative of the diketide ketoester for incubation studies [16]. ^{1}H NMR analysis in D_2O showed that the compound exists mainly in the keto form (95%). The compound is

Figure 5



The modular structure of the ΔKR2 variant of DEBS1-TE and its synthetic operations.

Figure 6



The reduction reaction in which the NAC thioester derivative of the keto diketide is converted to the hydroxy analogue.

stable to hydrolysis over the time course of a typical incubation experiment. More significantly, ^{1}H NMR studies in D_2O solution demonstrated that the H atom at C-2 (δ 4.1 ppm) was subject to rapid exchange. This ensures that at all stages of an incubation any unreduced ketoester would be present as a racemate.

Preparation and separation of the products of the reduction reaction

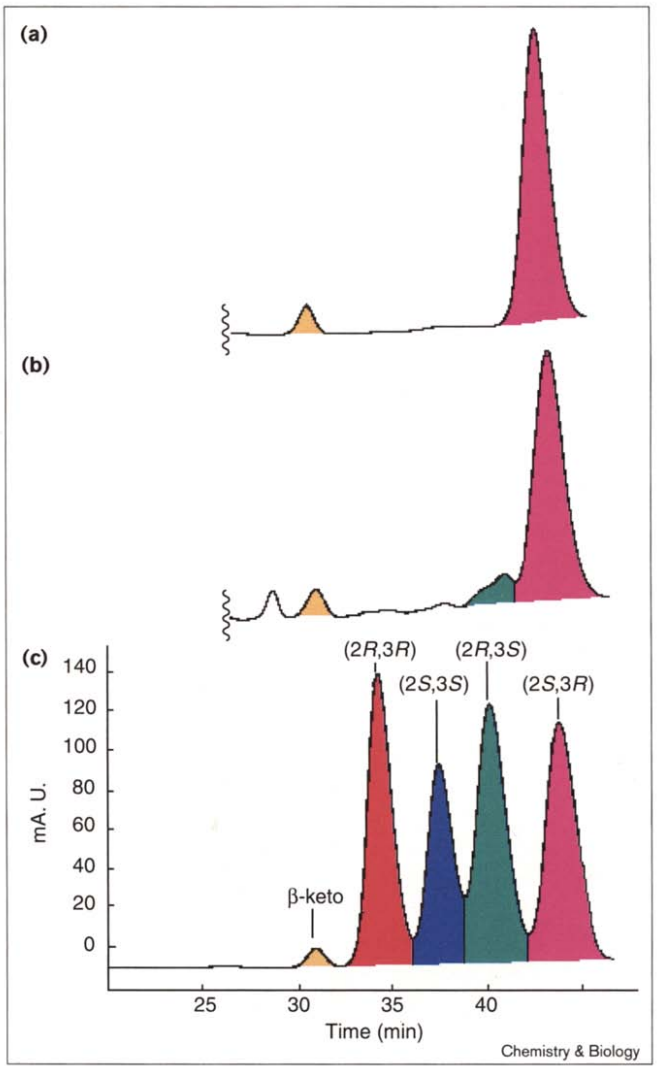
All four diastereoisomers of the 3-hydroxy-2-methyl diketide intermediate were synthesised by established stereospecific routes [17]. They were also analysed by NMR and characterised by their optical rotation to confirm the relative configuration of the two chiral centres. It proved possible to analyse mixtures of the four diastereoisomers of the NAC derivatives of the hydroxy diketide products by high performance liquid chromatography (HPLC) using a chiral column. When an equal mixture of the five diketides (the four β-hydroxy diastereoisomers and the β-ketoester starting material) was analysed, peaks being detected by the chromophore of the thioester residue (232 nm), five almost completely resolved peaks were observed; the peaks for the hydroxy esters were of similar intensity, demonstrating that the technique is reliable for quantitative analysis of mixtures of the four diastereoisomeric hydroxy products (Figure 7). The detection of the ketoester was hindered by its lower extinction coefficient and so HPLC was not a sensitive method for monitoring turnover. The identity of the four hydroxy ester peaks was determined by separate injection of authentic samples of each diastereoisomer. The retention times were constant within a series of experiments using the same solvent mixture. Very slight changes in the concentration of the two solvent components resulted in marked variations in the retention times and so control injections of the standard diketide mixture were carried out with each batch of solvent mixture and before each series of experiments.

A further series of control experiments was carried out with synthetic standards to confirm that the efficiency of extraction of the four stereoisomers from the buffer used for incubation was identical within acceptable limits (± 10%).

Reduction catalysed by DEBS1-TE

First, the NAC derivative of the ketoester was incubated with DEBS1-TE, and the rate and extent of reduction was measured by consumption of NADPH, as determined by UV absorption at 340 nm. After 30 min, 4% of the ketoester had been reduced. The product mixture was analysed by chiral HPLC and gave the trace shown in Figure 7. The major reduction product is the natural hydroxy diketide (2*S*,3*R*) produced by module 1 of the erythromycin PKS (90%) but that there are significant quantities (about 10%) of the enantiomer that corresponds to the chirality generated in module 2. The molecular composition of the compounds giving rise to these peaks was confirmed using high-resolution mass spectrometry. Our interpretation of these results is that reduction takes place primarily on KR1 to give its normal diketide product. The small amount of enantiomeric hydroxy ester could be formed by reduction of the (*S*)-isomer either on KR1 with altered stereochemical control or more likely on KR2 with the normal mode of stereocontrol.

Figure 7



Chiral HPLC analysis of the NAC-thioester derivatives of the four stereoisomers of the erythromycin diketide. **(a)** The products isolated from incubation of the ketoester with DEBS1-TE (ΔKR2). **(b)** The products isolated from incubation of the ketoester with DEBS1-TE. **(c)** An equimolar mixture of the keto analogue and the four diastereoisomers of the hydroxy dervative. Note: the retention times of all the diastereometric hydroxy esters varied in the same direction and to approximately the same extent by up to 20%, depending on the current batch of eluting solvent. The HPLC traces shown in (a) and (b) have been aligned with that shown in (c) in the interests of clarity. In all cases, the current retention times were calibrated by control injections with synthetic standard samples. mA U, milliabsorbance units.

Reduction reaction using DEBS1-TE (ΔKR2)

This ambiguity was resolved by repeating the experiment using the ΔKR2 mutant version of DEBS1-TE. In this case, the only product detected by chiral HPLC was the normal hydroxy diketide product of module 1 (Figure 7). KR1 is, therefore, entirely stereoselective towards its substrate in that it accepts the (2*S*) isomer and rejects the (2*R*) isomer, both of which are present in the racemic form of the starting material present in the incubation mixture. After selection of the required stereoisomer of the ketoester, reduction proceeds stereospecifically at C-3 to give the normal (2*S*,3*R*) product. We further conclude that the minor diastereoisomer produced by DEBS1-TE is the product of reduction by KR2, which accepts the enantiomeric ketoester as a substitute for its normal structurally different substrate (the (2*R*)-triketide analogue). We cannot draw from these results, however, any further conclusion concerning the stereoselectivity and stereospecificity of KR2 because a small proportion of the major product might also have been formed by this domain.

The demonstration that KR1 is both stereoselective and stereospecific in reducing the ketoester diketide intermediate confirms earlier predictions concerning the control of stereochemistry during product formation in module 1 of the erythromycin PKS. In its normal context, KR1 selects between the two epimers of the enzyme-based ketoester and then carries out its reduction stereospecifically. It therefore controls the C-2 stereochemistry indirectly and the C-3 centre directly. Control of chirality in the two chiral centres generated by this module is, therefore, exerted exclusively by the ketoreductase domain, KR1.

The mechanism of control in module 2 is not yet fully defined. Epimerisation of the methyl centre is not observed, but there are two possible explanations for this omission. First, it might be that there is no potential epimerisation activity in that module and therefore that KR2 has no choice over substrate selection. In this case, KS2 determines the chirality at C-2. Alternatively, an epimerase activity might be present, but is overwhelmed in this module by the dominant reductase activity of KR2, which recognises and removes the (2*R*) isomer as its substrate before epimerisation can occur.

Significance

Polyketides, a large family of compounds that possess medicinally important activities, are synthesised by large multienzyme complexes called polyketide synthases (PKSs). The modular nature of the PKSs has attracted much attention because of the potential for bioengineering new complexes that generate novel polyketides. An understanding of the factors that control the configuration of chiral centres during polyketide assembly on PKSs is crucial for rational genetic engineering of these molecular assembly lines.

We have shown here that a ketoreductase (KR) domain can exert control over stereochemistry, not only at the newly formed C-3 hydroxy centre, where hydride addition takes place, but also at the adjacent methyl-bearing centre at C-2, by selection of the appropriate diastereoisomer of the 3-ketoacyl intermediate.

This result confirms that there are two different mechanisms of stereochemical control working in modules 1 and 2 of the erythromycin PKS. The difference hinges on the existence of an epimerisation activity towards the β-ketoester intermediate in module 1 but not in module 2. It is possible that further variations on the mechanisms of stereochemical control operate in other modules of polyketide synthases generally. The methods of investigation described here could be more widely applied to examine this possibility.

Materials and methods

Exchange of the α-hydrogen of the β-keto diketide NAC

NMR spectra in H_2O and D_2O were run in 2 ml buffered solutions (400 mM potassium phosphate, pH 7) containing the NAC thioester of the β-keto diketide (2 mg). The spectrum in H_2O was acquired with water suppression via a presaturation microprogram. δ_H (400 MHz, H_2O): 4.1 (1H, q, J 7 Hz), 3.3 (2H, q, J7Hz), 3.0, (2H, m), 2.6 (2H, 4m), 1.9 (3H, S), 1.3 (3H, d, J 7Hz), 1.0 (3H, q, J 7Hz). In the spectrum run on a D_2O solution the peak at δ 4.1 disappeared and the doublet at δ 1.3 was replaced by a singlet.

Reduction reaction assays

Either DEBS1-TE (400 μg/ml) or DEBS1-TE (ΔKR2) (478 μg/ml), in 400 mM potassium phosphate buffer, pH 7.0, containing EDTA (1 mM) was incubated for 30 min, at 30°C, with NADPH and the NAC derivative of the β-keto diketide. For DEBS1-TE, a final concentration of 2 mM of NADPH and of the NAC derivative of the β-keto diketide was used. For the ΔKR2 protein, a final concentration of 2.8 mM for each substrate was used. The total volume was 1 ml for DEBS1-TE and 0.4 ml for DEBS1-TE (ΔKR2). The ketoreduction reaction was followed at 340 nm using a Shimadzu UV-2100 spectrophotometer. The quartz cuvettes used were a matched pair with a 1 mm path length. The products were isolated after 30 min.

Detection of the products of the reduction reaction using HPLC

The reaction products were extracted using ethyl acetate (3 × 100 μl) and after removal of the solvent *in vacuo*, were resuspended in 7% ethanol in iso-hexane (Fisons, HPLC-grade). The product mixture was then analysed by HPLC, using a Hewlett-Packard 1090 HPLC instrument fitted with a Chiralcel OC column (Diacel, 25 cm × 4.6 mm, guard column 5 cm × 4.6 mm). Compounds were eluted at 1 ml/min in 7% ethanol in iso-hexane. Control samples were run before each batch of analyses to determine the standard retention times using the current batch of solvent mixture.

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References

1. Marsden, A.F.A., Wilkinson, B., Cortés, J., Dunster, N.J., Staunton, J. & Leadlay, P.F. (1998). Engineering broader specificity into an antibiotic-producing polyketide synthase. *Science* **279**, 199-202.
2. Ruan, X., *et al.*, & Katz, L. (1997). Acyltransferase domain substitutions in erythromycin polyketide synthase yield novel erythromycin derivatives. *J. Bacteriol.* **179**, 6416-6425.
3. Liu, L., Thamchaipenet, A., Fu, H., Betlach, M. and Ashley, G. (1997). Biosynthesis of 2-nor-6-deoxyerythronolide B by rationally designed domain substitution. *J. Am. Chem. Soc.* **119**, 10553-10554.
4. Stassi, D.L. *et al.*, & Katz, L. (1998). Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. *Proc. Natl Acad. Sci. USA* **95**, 7305-7309.
5. Kao, C.M., McPherson, M., McDaniel, R.N., Hong-Fu, Cane, D.E. & Khosla, C. (1998). Alcohol stereochemistry in polyketide backbones is controlled by the β-ketoreductase domains of modular polyketide synthases. *J. Am. Chem. Soc.* **120**, 2478-2479.
6. Cortés, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J. & Leadlay, P.F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. *Nature* **348**, 176-178.
7. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J. and Katz, L. (1991). Modular organisation of genes required for complex polyketide biosynthesis. *Science* **252**, 675-679.
8. Celmer, W.D. (1965). A configurational model for macrolide antibiotics. *J. Am. Chem. Soc.* **87**, 1801-1802.
9. Katz, L. & Donadio, S. (1995). Macrolides. In *Genetics and Biochemistry of Antibiotic Production*. (Vining, L.C. & Stuttard, C., eds), pp385-420, Butterworth Heinemann, London.
10. Cane, D.E., Liang, T.-C., Taylor, P.B., Chang, C. & Yang, C.-C. (1968). Stereochemistry of the chain-elongation steps of erythromycin biosynthesis. *J. Am. Chem. Soc.* **108**, 4957-4964.
11. Cortés, J., Wiesmann, K.E.H., Roberts, G.A., Brown, M.J.B., Staunton, J. & Leadlay, P.F. (1995). Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage. *Science* **268**, 1487-1489.
12. Wiesmann, K.E.H., Cortés, J., Brown, M.J.B., Cutter, A.L., Staunton, J. & Leadlay, P.F. (1995). Polyketide synthesis *in vitro* on a modular polyketide synthase. *Chem. Biol.* **2**, 583-589.
13. Weissman, K.J., *et al.*, & Leadlay, P.F. (1997). The molecular basis of Celmer's rules: the stereochemistry of the condensation step in chain extension on the erythromycin polyketide synthase. *Biochemistry* **36**, 13849-13855.
14. Böhm, I., Holzbaur, I.E., Hanefeld, U., Cortés, J., Staunton, J. & Leadlay, P.F. (1998). Engineering of a minimal modular polyketide synthase, and targeted alteration of the stereospecificity of polyketide chain extension. *Chem. Biol.* **5**, 407-412.
15. Luo, G., Pieper, R., Rosa, A., Khosla, C. & Cane, D.E. (1996). Erythromycin biosynthesis: exploiting the catalytic versatility of the modular polyketide synthase. *Bioorg. Med. Chem.* **4**, 995-999.
16. Gilbert, I.H., Ginty, M., O'Neill, J.A., Simpson, T.J., Staunton, J. & Willis, C.L. (1995). Synthesis of β-keto and α,β-unsaturated N-acetyl-cysteamine thioesters. *Bioorg. Med. Chem. Lett.* **5**, 1587-1590.
17. Harris, R.C., Cutter, A.L., Weissman, K.J., Hanefeld, U., Timoney, M.C. & Staunton, J. (1998). Enantiospecific synthesis of analogues of the diketide intermediate of the erythromycin polyketide synthase. *J. Chem. Res.* 283.

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