Cloning and Heterologous Expression of the Macrotetrolide Biosynthetic Gene Cluster Revealed a Novel Polyketide Synthase that Lacks an Acyl Carrier Protein

Hyung-Jin Kwon, Wyatt C. Smith, Longkuan Xiang, and Ben Shen*

Department of Chemistry, University of California One Shields Avenue, Davis, California 95616

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The macrotetrolides are a family of cyclic polyethers composed of four molecules of enantiomeric nonactic acid (**1**) or its homologues in a (+)(−)(+)(−)-ester linkage[1] and exhibit a broad spectrum of biological activities,[2] ranging from antibacterial, antifungal, antitumor, to immunosuppressive activity. Studies of macrotetrolide biosynthesis in *Streptomyces griseus* have unambiguously established that they are of polyketide origin.[3,4] Of special interest is the proposal that macrotetrolide biosynthesis involves a pair of enantiospecific polyketide pathways[4c] (Figure 1). This idea is supported by (a) the efficient incorporation of (6*R*,8*R*)- or (6*S*,8*S*)-2-methyl-6,8-dihydroxynon-2*E*-enoic acid (**2**)[3d] and (±)-**1**[3a] into nonactin (**3**), (b) the isolation of both (+)- and (−)-**1** and their dimers[3g] from *S. griseus* fermentation, and (c) the genetic and biochemical characterization of NonS that catalyzes formation of (−)-**1** and its homologues from (−)-**2** and its homologues .[4b,c] An intriguing corollary of this proposal is the synthesis of a set of enantiomeric polyketides, such as **2**, presumably by a pair of polyketide synthases (PKSs).[4c] To make **2** from the carboxylic acid precursors, the latter PKSs must have invoked (a) the rare use of succinate as an intact four-carbon fragment (C3−C6) and (b) the derivation of a three-carbon unit (C7−C9) from two molecules of acetate. We now report the cloning and heterologous expression of the macrotetrolide biosynthetic gene cluster, revealing a novel type of PKS for polyketide biosynthesis that consists of type II ketoacyl synthases (KSs) and ketoreductases (KRs) but lacks acyl carrier protein (ACP).

A 55-kb contiguous DNA region was cloned from *S. griseus* DSM40695 using the previously characterized *nonR* resistance gene as a probe.[4a,c] Inactivation of *nonS*, a gene adjacent to *nonR*, completely abolished macrotetrolide production, confirming that the cloned DNA encodes macrotetrolide biosynthesis.[4c] Nucleotide sequence analysis of a 30-kb fragment of the cloned region revealed 30 open reading frames (orfs).[5] In addition to *nonR* and *nonS*, as well as other biosynthesis, resistance, and regulatory genes, the sequenced gene cluster consists of 5 KSs and 4 KRs but apparently lacks an ACP (Figure 2A).

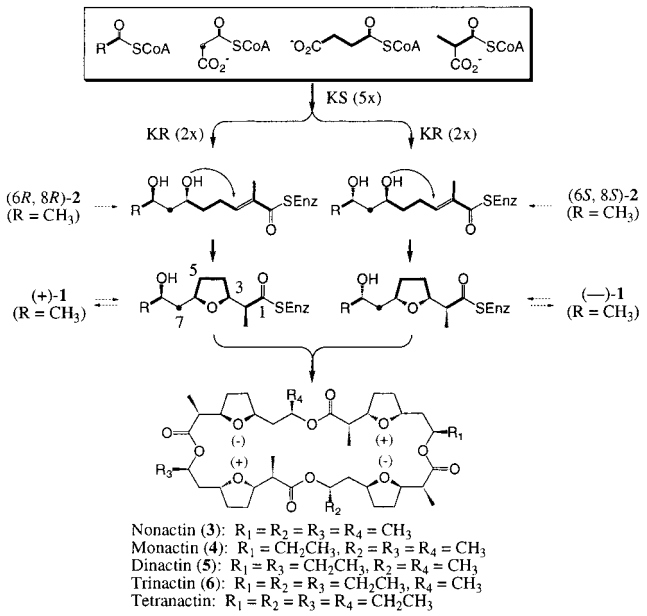

Figure 1. Proposed pathway for macrotetrolide biosynthesis in *S. griseus*. Arrows with broken lines indicate intermediates that have been isolated or confirmed by feeding experiments.

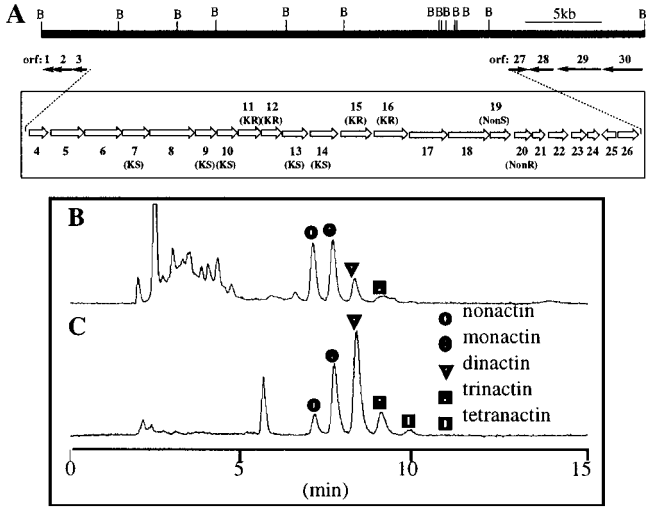

Figure 2. The macrotetrolide biosynthetic gene cluster[5] from *S. griseus* DSM40695 (A) and HPLC analysis of macrotetrolide production in *S. lividans* (pBS2013) (B) and *S. griseus* DSM40695 (C). The region contained in pBS2013 is boxed.

Three types of PKSs are known to date.[6] Type I PKSs are multifunctional enzymes that are organized into modules, and type II PKSs are multienzyme complexes consisting of discrete, largely monofunctional proteins, both of which are found so far only in

* Corresponding author. E-mail: shen@chem.ucdavis.edu. Telephone: (530) 754-9382. Fax: (530) 752-8995.

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microorganisms. Despite the structural difference, type I and II PKSs share a high degree of amino acid sequence similarity, and both types of PKSs use ACP to activate substrates and to channel the growing polyketide intermediates.[6a,b,d] Chalcone synthase and its homologues, also known as type III PKSs, are distributed mainly in plants[6e] and have been found very recently in microorganisms as well.[6c] They are different structurally and mechanistically from the former and are essentially condensing enzymes that have no apparent amino acid sequence similarity to KSs of both type I and -II PKSs. Type III PKSs lack ACP and act directly on the coenzyme A (CoA) ester of simple carboxylic acids.[6c,e] The 5 KSs identified within the macrotetrolide gene cluster are discrete proteins, three of which, *orf7*, *orf13*, and *orf14*, are highly homologous to KS of type II PKSs and two of which, *orf9* and *orf10*, resemble KS of type II fatty acid synthases.[6a] If the sequenced region encompasses all of the genes for macrotetrolide biosynthesis, the five KSs and four KRs must catalyze the assembly of (+)- and (−)-**2** from the carboxylic acid precursors in the absence of ACP, representing a novel type of PKS for polyketide biosynthesis.

To exclude the possibility that the ACP could reside outside the sequenced region, we carried out a series of inactivation of orfs that flank the genes known to be essential for macrotetrolide biosynthesis, including the KSs and KRs as well as the *nonS* and *nonR* genes.[7] Since genes encoding antibiotic production in microorganisms are often clustered,[6a,b,d] we reasoned that sequential inactivation of orfs until the resultant mutants no longer exhibit macrotetrolide nonproducing phenotype should allow the localization of the boundaries of the macrotetrolide biosynthetic gene cluster. Thus, inactivation of *orf2*, *orf3*, *orf27*, *orf28*, or *orf30* generated *S. griseus* mutants that show no difference in macrotetrolide production as compared to the wild type *S. griseus* strain. In contrast, macrotetrolide production is severely impaired or totally abolished in *S. griseus* mutants whose *orf4*, *orf6*, *orf7*, *orf9*, *orf10*, *orf11*, *orf12*, *orf13*, *orf14*, *orf15*, *orf16*, *orf19 (nonS)*, or *orf22* was inactivated. These results suggest that the boundaries of the macrotetrolide biosynthetic gene cluster lie at *orf3* to *orf4* and *orf22* to *orf25*, respectively (Figure 2A).

To ascertain that the macrotetrolide PKS does not recruit an ACP residing at somewhere else of the *S. griseus* chromosome and that the identified gene cluster is sufficient for macrotetrolide biosynthesis, we decided to demonstrate the production of macrotetrolides in a heterologous host. We chose *Streptomyces lividans* 1326 as a host because vector systems for the expression of PKS gene clusters in this organism have been well developed and used to produce various natural products.[8] A 25-kb fragment harboring *orf4* to *orf26* was cloned under the *act*I promoter[8b,d] on an apramycin-resistant pSET152[9] derivative, yielding pBS2013. Introduction of pBS2013 into *S. lividans* 1326 afforded apramycin-resistant *S. lividans* 1326 (pBS2013) transformants[10] that were cultured in AP medium.[11] Macrotetrolide production in *S. lividans* 1326 (pBS2013) was evident upon high performance liquid chromatography (HPLC) analysis of the resultant fermentation, as compared to that of the wild type *S. griseus* DSM40695 strain (Figure 2B and 2C); these metabolites were absent from fermentation of the *S. lividans* 1326 (pSET152) negative control.[12] The identity of the macrotetrolides produced were verified by electrospray mass spectroscopy analysis, yielding the characteristic molecular ions (*m*/*e* for $[M + Na]^+$) of 759.4241 for **3** ($C_{40}H_{64}O_{12}$ + Na, calcd 759.4297), 773.4230 for monactin (**4**) ($C_{41}H_{68}O_{12}$ + Na, calcd 773.4453), 787.4333 for dinactin (**5**) ($C_{42}H_{72}O_{12}$ + Na, calcd 787.4610), and 801.4930 for trinactin (**6**) ($C_{43}H_{76}O_{12}$ + Na, calcd 801.4767). It is noteworthy that *S. griseus* DSM40695 accumulates the macrotetrolides predominantly in mycelia (~40 mg/L). In contrast, *S. lividans* 1326 (pBS2013) preferentially secretes the macrotetrolides into medium (~10 mg/L) with only very small quantity accumulated in mycelia (~0.5 mg/L). The latter observation is consistent with recent findings from the genome-sequencing project of *Streptomyces coelicolor*, a very close cousin of *S. lividans*, that these organisms have an unusually high number of efflux pump proteins that efficiently export secondary metabolites out of the cells (http://www.sanger.ac.uk/Projects/S_coelicolor/).

Macrotetrolide production in *S. lividans* 1326 (pBS2013) unambiguously demonstrates that the 23 orfs on pBS2013 are sufficient to support macrotetrolide biosynthesis from the carboxylic acid precursors, excluding the participation of other *S. griseus* gene products in macrotetrolide biosynthesis. It should be noted that our current data fell short of excluding completely the possibility that the macrotetrolide PKS recruits an ACP from the hosts' fatty acid biosynthetic machinery. It is known that malonyl CoA-ACP transacylase from fatty acid biosynthetic pathway is an essential component of type II PKS.[13] However, there is no evidence for crosstalk between fatty acid synthase ACP and PKS. Both type I or II PKSs known to date all include an ACP, either as a domain or a discrete protein, without which the PKSs are not functional.[6a−c] Therefore, our finding that the macrotetrolide PKS consists of discrete KS and KR but lacks ACP uncovered a novel ACP-independent type II PKS for polyketide biosynthesis.

In a mechanistic analogy to type III PKS,[6a,c,e] we propose that the macrotetrolide PKS acts directly on CoA esters of the carboxylic acid precursors for the biosynthesis of a pair of enantiomeric polyketide intermediates, such as (+)- and (−)-**2**. Modeled on type I PKS whose KR domain controls the stereochemistry of both α- and β-positions in polyketide biosynthesis by enantiospecific reduction of only one enantiomer of the nascent β-ketoacyl intermediates,[14] we further propose that biosynthesis of **2** diverges at the β-ketoacyl reduction steps into a pair of enantiospecific pathways, each of which involves 2 KRs and affords (+)- or (−)-**2**, respectively (Figure 1). Therefore, it is clear that we now have all of the genetic information for macrotetrolide biosynthesis, the availability of which provides us an excellent opportunity to broaden the repertoire of PKSs and to study the stereochemistry in polyketide biosynthesis.

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(7) Gene inactivation was carried out as previously described[4c] and confirmed by Southern analysis.

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