Nonactin Biosynthesis: Disruption of the Polyketide Synthase Genes, *nonKJ*, in *Streptomyces griseus* Leads to an Overall 96% Decrease in Macrotetrolide Production, Yet a Net Increase in Nonactin Analogues Which Incorporate Isobutyrate

Robbie J. Walczak, Michael E. Nelson, and Nigel D. Priestley*

Department of Chemistry
The University of Montana
Missoula, Montana 59812-1656

Received November 1, 2000

Nonactin (Figure 1) is the parent compound of a group of ionophore antibiotics known as the macrotetrolides, which are produced by a number of *Streptomyces*, including *Streptomyces griseus* subsp. *griseus* ETH A7796 (DSM 40695).[1] Nonactin has been shown to possess antitumor activity and to be an effective inhibitor of the activity of the P170-glycoprotein which is responsible for multiple drug resistance in cancer cell lines.[2]

The 32-membered macrocyclic ring of nonactin is composed of two units of (+)-nonactate and two units of (−)-nonactate such that the macrocycle has S_4 symmetry and is achiral. Homologues of nonactin are made by the use of homononactate in place of nonactate. Homononactate arises through the use of a propionate unit rather than an acetate unit during the biosynthesis.

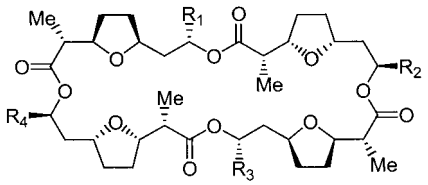
The biosynthesis of nonactate and its homologue, homononactate, poses a fundamental biochemical question: how can the biosynthesis of both enantiomers of these building blocks be accounted for? At one extreme we can envisage that there exists one set of metabolic enzymes and that these enzymes do not discriminate between enantiomers of their substrates. At the other extreme there could exist two parallel pathways, each with their own enzymes that are highly selective for their respective intermediate enantiomers.

Nonactate, and therefore nonactin, is derived from acetate, propionate, and succinate, leading to the hypothesis of a biosynthesis pathway based upon analogous polyketide biosynthesis pathways (Scheme 1).[3,4] Spavold and Robinson were able to show that the acyclic intermediates **7** and **8** were stereoselectively incorporated into nonactin: **7** being converted to (+)-nonactate, **8** being converted to (−)-nonactate. These data established that racemic nonactate is made by a pair of enantiocomplementary pathways. The exact details of the chemistry required to convert acetate, succinate, and propionate into **7** and **8** are still not known, although the heptanedioate **6** has been implicated in most scenarios.

Through our work,[5,6] and that of Shen and co-workers,[7] the analysis and sequencing of the region surrounding the macrotetrolide resistance gene[8] has now been extended to cover 39 kbp of the nonactate biosynthesis gene cluster. A subset of the open reading frames (ORFs) observed in the cluster appear to encode the nonactate polyketide synthase complex (Figure 2). Inspection of the structure of nonactate would suggest that, regardless of the specific details, three condensation reactions and three reduction reactions are required for its synthesis. If we assume that each condensation or reduction is catalyzed by an individual enzyme and that we have one set of enzymes for the production of racemic nonactate, then we would require three ketoacyl synthase (KS) and three ketoacyl reductase (KR) genes. On the other hand, if we make similar assumptions, yet we have one set of enzymes for each of the enantiocomplementary pathways, we would require six KS and six KR genes. In the cluster we observe five KS and four KR homologues. Obviously, the nature of nonactate biosynthesis is quite complex at this level, and assignment of the individual genes to either of the enantiocomplementary pathways cannot readily be done without significant further study. Early steps in the biosynthesis certainly involve achiral intermediates; only one enzyme would be required to furnish the precursors required to make both enantiomers of nonactate.

We sought in this current work to disrupt the chromosomal copies of the type II PKS genes *nonKJ* in *S. griseus* and through analysis of a mutant so obtained demonstrate that the genes were indeed required for nonactate biosynthesis.

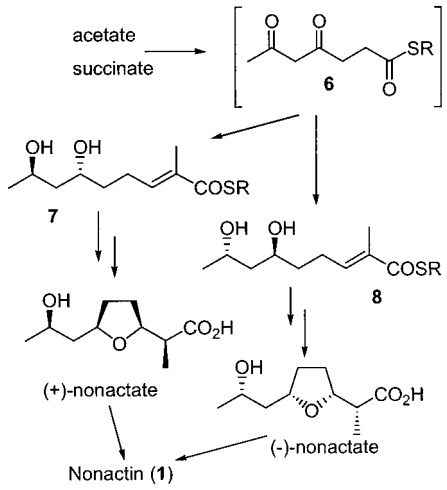
Using standard techniques we were able to construct a disruption cassette in a pANT841-based vector[9] which contained, in order, the partial 5′-sequence from *nonK* (0.97 kbp), a 1.46



	R_1	R_2	R_3	R_4
Nonactin (**1**)	Me	Me	Me	Me
Monactin (**2**)	Me	Me	Et	Me
Dinactin (**3**)	Et	Me	Et	Me
Trinactin (**4**)	Et	Et	Et	Me
Tetranactin (**5**)	Et	Et	Et	Et

Figure 1. Structures of the naturally occurring macrotetrolides.

Scheme 1. Summary of Proposed Biosynthesis Pathway Leading to the Macrotetrolides via Enantiocomplementary Pathways



(1) Keller-Schierlein, W.; Gerlach, H. *Fortschr. Chem. Org. Naturst.* **1968**, 26, 161−189.
(2) Borrel, M. N.; Pereira, E.; Fiallo, M.; Garnier-Suillerot, A. *Eur. J. Biochem.* **1994**, 223, 125−133.
(3) Ashworth, D. M.; Robinson, J. A.; Turner, D. L. *J. Chem. Soc., Perkin Trans. 1* **1988**, 1719−1727.
(4) Ashworth, D. M.; Clark, C. A.; Robinson, J. A. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1461−1467.
(5) Woo, A. J.; Strohl, W. R.; Priestley, N. D. *Antimicrob. Agents Chemother*. **1999**, 43, 1662−1668.
(6) Walczak, R. J.; Woo, A. J.; Strohl, W. R.; Priestley, N. D. *FEMS Letts*. **2000**, 183, 171−175.
(7) Smith, W. C.; Xiang, L.; Shen, B. *Antimicrob. Agents Chemother*. **2000**, 44, 1809−1817.
(8) Plater, R.; Robinson, J. A. *Gene* **1992**, 112, 117−122.
(9) DeSanti, C. L.; Strohl, W. R. Personal communication.

10.1021/ja005749o CCC: $20.00
Published on Web 09/27/2001

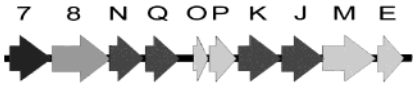


Figure 2. PKS genes of the nonactate biosynthesis gene cluster: *orf7*, N, Q, K, J ketoacyl synthase (KAS); *orf8*, CoA-transferase; O, P, M, E, ketoacyl reductase.

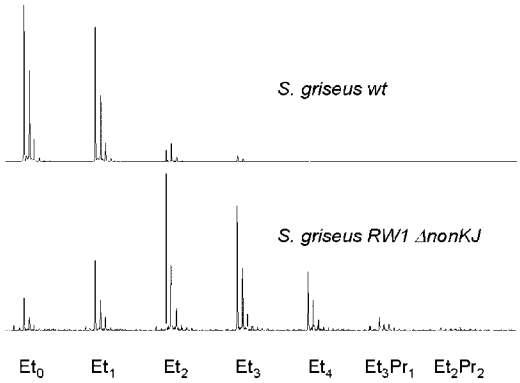


Figure 3. Mass spectrum of the macrotetrolide fractions isolated from *S. griseus* wild-type and the Δ*nonKJ* mutant. Equal weights of macrotetrolide mixtures were used to generate each spectrum.

kbp fragment containing the *aac*(*3*)*IV* selectable marker (apramycin resistance) derived from plasmid pKK974, and a partial 3′-sequence of *nonJ* (0.86 kbp). The plasmid was introduced into *S. griseus* protoplasts by transformation; regeneration, and selection of the protoplasts was accomplished on media containing apramycin. The 18 *S. griseus* colonies obtained arose through either single or double crossover recombination events leading to the incorporation of the disruption cassette in the *S. griseus* chromosome. The mutant strains were individually propagated and chromosomal DNA isolated from each. The occurrence of either a single or a double crossover event was detected and confirmed by Southern analysis of the chromosomal DNA.

One Δ*nonKJ* mutant (*S. griseus* RW1), confirmed to have arisen through double crossover recombination, was propagated using standard macrotetrolide production media and the mycelia processed according to customary methods for the isolation of macrotetrolides. Colorimetric, picrate-based assay[10] of the crude extract showed that macrotetrolides were present. The crude fraction was partially purified by chromatography on silica gel to yield a mixture of macrotetrolide homologues, The relative proportions of each macrotetrolide homologue in the mixture was determined by mass spectrometric analysis (Figure 3).

The Δ*nonKJ* mutant was yet capable of the synthesis of macrotetrolides, although at a level of only 4% of that of the wild-type, The significant reduction in macrotetrolide titer clearly shows that the genes *nonKJ* are involved in nonactin biosynthesis.

In any disruption experiment the potential for the introduction of polar effects exists. In our experiment we know that the downstream gene *nonS* is still functional as it is well accepted that the product of this gene catalyzes a *syn*-Michael cyclization of **8** to form the tetrahydrofuran ring of (−)-nonactate.[5,7] We cannot say, however, if expression of the upstream genes has been affected in any way, although both enantiocomplementary pathways must be functional to some extent as a small amount of macrotetrolides are still produced.

This experiment does not tell us about the roles of *nonKJ* in each enantiocomplementary pathway. For example, *nonKJ* may be involved in both pathways, disruption leading to a 96% drop in monomer production and hence a 96% drop in macrotetrolide production. Alternatively, *nonKJ* may be involved in only one of the enantiocomplementary pathways, resulting in 4% of wild-type levels for one nonactate enantiomer, yet 100 % synthesis of the other enantiomer. This leads to only 4% of wild-type macrotetrolide production as the levels of synthesis are restricted by the limiting nonactate precursor.

Significantly, the proportions of each macrotetrolide formed are quite different in the mutant and wild-type strains. Macrotetrolides arising from homononactate make up a greater proportion of the macrotetrolide fraction isolated from the mutant strain as compared to that from the wild-type. Rare macrotetrolides derived from isobutyrate units were also observed ($Et_3{}^iPr_1$ and $Et_2{}^iPr_2$). The absolute amounts of the latter compounds were higher in the mutant strain than in the wild type.[11]

Disruption of the daunorubicin PKS genes *dpsCD* in *Streptomyces* sp. strain C5 led to an alteration in starter unit fidelity; anthracyclines initiated with acetate were obtained in a mixture with those, as expected, initiated from propionate.[12] The situation described herein with the nonactate PKS parallels those results, although it is far from clear that the choice between acetate (R = Me, Figure 1), propionate (R = Et, Figure 1), and isobutyrate (R = ^{i}Pr, Figure 1) is determined at the initiation of polyketide synthesis. It is clear, however, that the absence of fully functional *nonKJ* in some manner leads to a perturbation of the nonactate PKS, complex allowing a more relaxed precursor selectivity.

We have demonstrated that disruption of the type II PKS genes *nonKJ* appreciably alters nonactate biosynthesis in *S. griseus*. Disruption of these genes generates a mutant that makes higher levels of isobutyrate-containing macrotetrolides than the wild-type strain, although overall macrotetrolide production is lowered by 96%. The roles of *nonKJ* and the other nonactate PKS genes, and their involvement in the synthesis of each enantiomer of nonactate, are being pursued in a series of further biochemical experiments.

Acknowledgment. We acknowledge the financial support of the NIH (CA77347) and NSF-EPSCoR. M.E.N. thanks the AFPE for a fellowship.

JA005749O

(10) Suzuki, K.; Nawata, Y.; Ando, K. *J. Antibiot.* **1971**, 24, 675.

(11) Amounts of each macrotetrolide in the wild-type (wt) (mg·dm^{-3}): Et_0 (**1**), 460; Et_1 (**2**), 400; Et_2 (**3**), 120; Et_3 (**4**), 18; Et_4 (**5**), 2.6; $Et_3{}^iPr_1$, < 0.1; $Et_2{}^iPr_2$, < 0.1. Amounts of each macrotetrolide in the mutant strain (mg·dm^{-3}): Et_0, 3.0; Et_1, 6.0; Et_2, 14; Et_3, 11; Et_4, 5.1; $Et_3{}^iPr_1$, 1.2; $Et_2{}^iPr_2$, 0.3. The lowest level of detection is estimated to be 0.1 mg·dm^{-3}. The production profile in RW1 mirrors that observed by Shen and co-workers,[7] although in their case wt production levels are almost 2 orders of magnitude lower than ours.

(12) Rajgarhia, V. B.; Strohl, W. R. *J. Bacteriol.* **1997**, 179, 2690−26.