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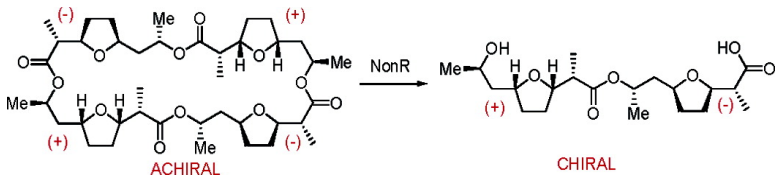

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Nonactin Biosynthesis: The Product of the Resistance Gene Degrades Nonactin Stereospecifically to Form Homochiral Nonactate Dimers

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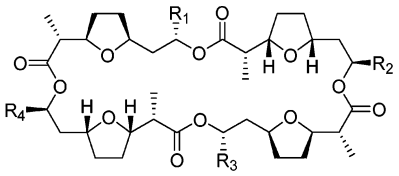
Streptomyces griseus subsp. *griseus* ETH A7796 (DSM40695) produces a range of macrocyclic, ionophore antibiotics. The parent compound, nonactin (Figure 1. **1**), has been shown to possess antitumor activity both against mammalian cell lines in vitro and against Crocker Sarcoma 180 in studies in mice.[1] Nonactin has also been shown to be an effective inhibitor of the 170-kDa-P-glycoprotein mediated efflux of 4-*O*′-tetrahydropyranyldoxorubicin in multidrug resistant erythroleukemia K562 cells at sub-toxic concentrations.[2]

Nonactin is a cyclic tetraester composed of monomers of nonactate. The 32-membered macrocycle is made of two units of (+)-nonactate and two units of (−)-nonactate, joined alternately (+)−(−)−(+)−(−) around the macrocycle so that the whole structure has S4 symmetry and is, therefore, achiral.

Considerable work, both biochemical and genetic, has been done to gain an understanding of the biosynthesis of nonactin.[3−10] In that context, one significant question that is posed is how can the producing organism be protected from the toxic effects of a compound that it produces? In the case of nonactin biosynthesis, there are two potential mechanisms that are likely operative. First, the genes *orf5* and *orf6* are found clustered with the nonactin biosynthesis gene cluster.[7] Genes *orf5* and *orf6* appear, through sequence homology, to encode a pair of proteins involved in an ABC-transporter. Gene *orf6* likely encodes the membrane channel while gene *orf5* likely encodes the ATP-binding component.[7] Thus, at least one part of the producing organism's self-resistance to nonactin appears to involve pumping excess antibiotic from the cell. A second component of resistance is likely mediated through destruction of the excess antibiotic. Gene *nonR* was originally cloned by Plater and Robinson based upon its ability to confer tetranactin resistance to *S. lividans* TK24.[6] The deduced sequence of the protein NonR has great homology to the group of serine protease and esterase enzymes. In particular, a GXSXG motif, characteristic of the serine proteases is present.[6] No direct in vitro activity for NonR was shown in these early studies. Given the structure of nonactin a particularly interesting question is raised by the stereoselectivity of the reaction the reaction likely catalyzed by NonR. Since nonactin is an achiral, cyclic tetraester, does NonR discriminate in its site of attack since there are two nonequivalent sets of ester linkages that may be hydrolyzed, that is, does NonR discriminate between a (+)−(−) ester and a (−)−(+) ester? Does NonR break the macrotetrolide ring and then successively clip the open tetramer into monomer units? Does NonR stereoselectively cleave the macrotetrolide ring and thereby produce only dimer species? We sought to answer these questions by expressing the NonR protein and determining which reactions it could indeed catalyze. Using the Gene Editor site directed mutagenesis kit (Promega) we introduced a *Nde*I site at the 5′-end of the *nonR* gene in plasmid pANT1400.[10] Similarly a *Bam*HI site was introduced at the 3′-end of the gene. After confirmation by DNA sequencing the new *Nde*I-*Bam*HI fragment was recovered and sub-cloned into the vector pET15b to allow for expression of NonR as a N-terminal his6 fusion protein in *E. coli*. The protein was produced in an *E. coli* fermentation after induction with IPTG and was recovered after Ni^{2+}−IMAC chromatography as a substantially pure protein. The activity of the his6-NonR was demonstrated by incubating the protein with nonactin.



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

Figure 1. Compositions of the naturally occurring macrotetrolides

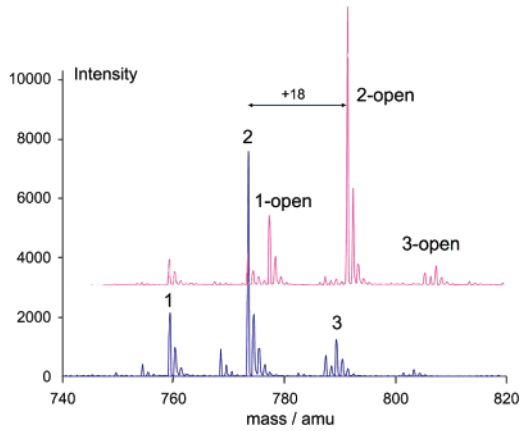


Figure 2. ESI−MS data clearly showing the formation of open chain tetrameric species from nonactin **1** and its homologues, **2** and **3** catalyzed by recombinant NonR. 'Open' implies a hydrolyzed analogue; +18 being H_2O.

The incubation mixture was analyzed by ESI−MS (Figure 2) and clearly shows the conversion of nonactin, and its homologues into *seco*-tetramer species. Prolonged incubation leads to the formation of nonactate dimer species. The generation of trimer species has not been observed in any of the assays that we have run to date. A larger scale incubation of nonactin with his6-NonR was carried out and allowed to proceed to generate the dimer species. The nonactate dimer was recovered from this incubation and fully characterized by 1D and 2D NMR techniques to rigorously confirm the structure of the reaction product. We examined the antibiotic activity of the dimer on *Staphylococcus aureus* using a zone diffusion assay. Under conditions where nonactin is active, equal levels of the dimer show no antibiotic activity whatsoever.

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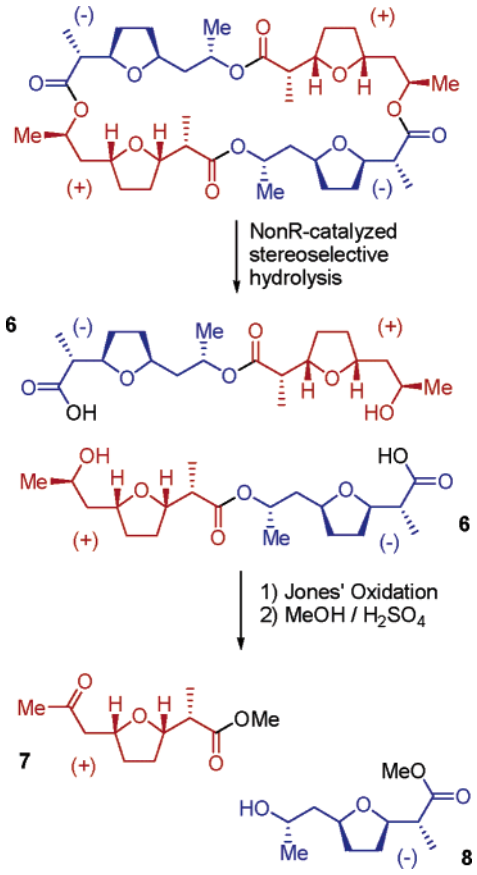
Scheme 1. Analysis of the Stereoselectivity of the Reaction Catalyzed by NonR[a]



[a] The reaction produces two equivalents of nonactate dimer; Jones' oxidation was used to differentiate the monomer units prior to analysis by GC of the methyl esters using a chiral stationary phase.

These data confirm that NonR acts to hydrolyze nonactin and that in so doing abrogates the antibiotic activity of the ionophore, thereby confirming the role of NonR in nonactin resistance. To determine if the NonR catalyzed reaction was indeed stereospecific, we performed a degradation procedure on the isolated dimer (Scheme 1). Jones' oxidation of the dimer **6** lead to oxidation of the free hydroxyl group; subsequent methanolysis generates methyl 8-ketononactate **7** (formerly alcohol end of dimer) and methyl nonactate **8** (formerly carboxylate end of dimer). GC analysis of the degradation sample was done using a chiral β-DEX column. Analysis of the degradation products was initially complicated by a close peak arising from methyl 8-ketononactate: treatment of the degradation mixture with hydroxylamine significantly clarified the analysis. These data show that only methyl (−)-nonactate is produced on degradation of the dimer and NonR is, therefore, a highly specific esterase that cleaves nonactin at the two ester bonds between the alcohol of (+)-nonactate and the carboxylate of (−)-nonactate (Scheme 1). Achiral nonactin **1** is converted into two equivalents of homochiral nonactate dimer **6**.

In conclusion, we have demonstrated that the product of the resistance gene, *nonR*, from the nonactin-producing *S. griseus* catalyzes the stereoselective cleavage of the cyclic, tetrameric antibiotic nonactin into a dimeric species that has no antibiotic activity. This work confirms the role of *nonR* in conferring nonactin resistance to the nonactin producing organism. This is the first example of antibiotic self-resistance to arise from an esterase activity.

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