Biosynthetic Study on the Polyether Carboxylic Antibiotic, Nigericin

Production and Biohydroxylation of Grisorixin by Nigericin-producing *Streptomyces hygroscopicus* NRRL B-1865

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With addition of methyl oleate, the increased yield of antibiotic production by nigericin-producing *Streptomyces hygroscopicus* NRRL B-1865 also resulted in the isolation of three additional polyether antibiotics. Two of these are abierixin and epinigericin, as new antibiotics. The third antibiotic is grisorixin. The production of both abierixin (opened ring A and 30-CH_2OH) and grisorixin (ring A and 30-CH_3) poses the problem of the identity of the last pathway precursor of the major metabolite, nigericin (ring A and 30-CH_2OH). Transformation experiments of abierixin by *S. hygroscopicus* gave negative results. Hydroxylation of grisorixin to nigericin by *S. hygroscopicus* represents the final step in nigericin biosynthesis.

Carboxylic polyether antibiotics have the ability to complex and transport mono or divalent cations through biological membranes. Some of them provide important veterinary applications in the control of coccidiosis[1] and feed utilization in ruminant animals[2]. With addition of methyl oleate (1%), the increased yield of antibiotic production ($\times 20$) by nigericin-producing *S. hygroscopicus* NRRL B-1865, also resulted in the isolation of three additional polyether antibiotics, all closely related to nigericin 1. Two of these, abierixin[3] and 28-epinigericin[4], new polyether antibiotics have been described previously.

According to the unified stereochemical model for nigericin biosynthesis[5], a polyene, obtained from a polyketide, would be first converted to a triepoxide. The concerted cascade of ring closure generates the ether rings of these antibiotics. The production of additional metabolites by nigericin-producing *S. hygroscopicus* NRRL B-1865 poses the problem of the identity of the precursor of the last pathway of the major metabolite, nigericin.

This paper describes the isolation and the structure of the third additional antibiotic, grisorixin, produced by *S. hygroscopicus* NRRL B-1865, and the transformation of this metabolite to nigericin by this microorganism.

Materials and Methods

Microorganism

Streptomyces hygroscopicus NRRL B-1865 was used for this work.

Production of Grisorixin

This antibiotic was produced and extracted from mycelium of nigericin-producing *Streptomyces hygroscopicus* NRRL B-1865[3]. Crude grisorixin was obtained from the EtOAc extract by chromatography column (Merck silicagel 0.025~0.400 mm, eluant cyclohexane-AcOEt). The purification of grisorixin was achieved by a flash-chromatography column[6] using MeOH-$CHCl_3$ with increasing amounts of MeOH as eluting solvent. The Rf of grisorixin was 0.50 (TLC, Merck 60F-254, cyclohexane-AcOEt-formic acid, 60:40:0.5 v/v/v).

Biotransformation Reaction

A two-stage fermentation was used. The ingredients of the seed culture medium 1 were (in g/liter): glucose (10), corn-steep (2), soyoptim (30) and K_2HPO_4 (1). The seed culture medium (100 ml) was inoculated with 7 ml of frozen spore culture of *S. hyroscopicus* NRRL B-1865. This culture was carried out at 28°C for 3 days on a

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shaker (250 rpm). 3 ml of this culture were transferred to 100 ml of production medium 2 using with the following composition (in g/liter): yeast extract (1), glucose (10), meat extract (1), casamino acids (1) and methyl oleate (1).

Transformation Experiments Using Intact Mycelium: After incubation in the medium 2 (100 ml) at 28°C on a rotatory shaker at 250 rpm for 24 hours, the cells were collected by centrifugation and filtration and washed three-fold with 0.3 M sucrose solution. The washed cells were suspended into 100 ml of bioconversion medium 3 (0.4 M sucrose, 0.02 M $MgCl_2$, 0.05 M $CaCl_2$ and 0.5 TES buffer) at pH 7.0. Grisorixin was added as a DMSO solution (20 mg/ml). The reaction was allowed to proceed for 4, 2, 24, 48 and 72 hours.

Transformation Experiments with Cell-free Extracts or Cell-fragments: The washed cells of *S. hygroscopicus* obtained above were suspended in 100 ml of medium 3. The mycelium was treated by ultrasonication methods, the temperature was maintained at 0~4°C. Grisorixin was added as above described.

Transformation with "Pretreated Cells": Small quantities (1 to 5 mg) of grisorixin were transferred at different times (0 to 20 hours) to 100 ml of production medium 2 containing 3 ml of inoculum (described above). 24 hours after addition of inoculum, cells were filtered, washed and suspended in 100 ml of medium 3. Then grisorixin was added as above.

Transformation with Protoplasts: The cells, obtained from cultures (medium 2) supplemented with glycine (0.5%), were filtered and washed with the sucrose solution as above, and incubated at 32°C, in 100 ml of medium 3 containing lysozyme (1 mg/ml). After 2 hours, the suspension was filtered through cotton to exclude mycelial forms. The filtered protoplasts were washed with cold medium 3 and then centrifugated for 30 minutes The precipited protoplasts were suspended in 100 ml of medium 3 and grisorixin was added as above.

Detection of the Biotransformation Products

The kinetics of biotransformation were followed by filtering the culture. The mycelia was extracted with EtOH (30 ml). After concentration, the residual syrup was eluted with 0.1 M HCl and extracted twice with ethyl acetate. The filtrate was also extracted with ethyl acetate. After drying with ammonium sulfate, the EtOAc extracts were concentrated *in vacuo* separatly and analyzed by TLC (Merck 60F-254) with eluant 1 (cyclohexan-EtOAc-formic acid, 60:40:0.5) or eluant 2 ($CHCl_3$-EtOAc-MeOH, 90:4:9). The detection was made by using a spray agent (7.5 g of vanillin, 4 ml of concentrated H_2SO_4 and 246 ml of EtOH), following by heating at 100°C. Grisorixin and biotransformation products were easily distinguished by Rf and coloured spots.

Isolation of the Biotransformation Products

The resulting extract from mycelium was chromatographed on a silica-gel column (Merck) firstly with cyclohexane and then increasing amounts of EtOAc. The purification was achieved by flash-chromatography of the selected fractions (chloroform, EtOAc, methanol, 90:4:9).

Spectroscopy

FAB-MS and FAB-MS/MS spectra were recorded on a quadripole tandem instrument (Nermag R 30-10). The sample was dissolved in MeOH (1 $\mu g/\mu l$) and one ml of this solution was added to the matrix, 3-nitrobenzyl-alcohol.

J-Mod spin-echo ^{13}C NMR spectra were recorded on a Brürker MSL 300 spectrophotometer in C_6D_6 solution. Chemical shifts are given relative to TMS.

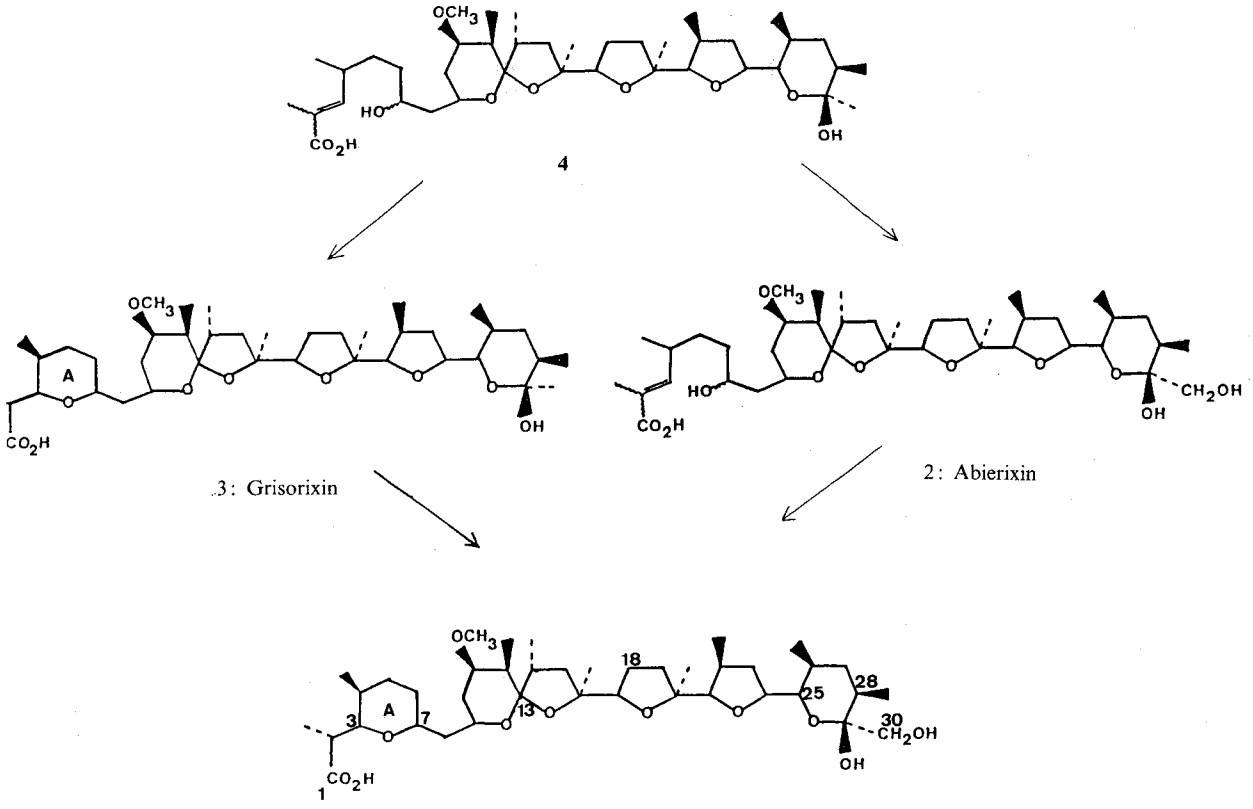
Results and Discussion

Grisorixin 3, $C_{40}H_{68}O_{10}$, was obtained as a colorless powder, soluble in organic solvents but not in water. It was isolated by ethanol extraction of the mycelium of *S. hygroscopicus* and the purification was achieved by a flash-chromatography column. The Rf of this compound is 0.50 (solvent 1), identical with the Rf of grisorixin 3 (30-CH_3), a polyether carboxylic antibiotic, obtained from a culture of *Streptomyces griseus* 2142 N6[7].

The techniques of FAB-MS and FAB-MS/MS were helpful in characterizing the structure of this third metabolite. The molecular weight of 708 Da was determined. In the positive ion FAB-MS (nitobenzyl-alcohol as matrix), diagnostic cationized molecules, *m/z* 731, $(M+Na)^+$ and *m/z* 747, $(M+K)^+$ were detected in large abundance. On the other hand, in the negative ion FAB-MS, only the deprotonated molecule $(M-H)^-$ *m/z* 707 was observed, confirming the molecular weight. By added KCl solution in matrix, only $(M+K)^+$ was observed and the MS/MS spectrum of this ion showed abundant fragment ions at *m/z* 729 $(M+K-H_2O)^+$, 715 $(M+K-CH_3OH)^+$ and 704 $(M+K-CO_2)^+$. All mass spectra of grisorixin 3, obtained with these above conditions, gave the same results. Moreover, the ^{13}C NMR spectra of the potassium-salts of the third metabolite and grisorixin 3 were very similar[8].

The production of both abierixin 2 (opened ring A and 30-CH_2OH) and grisorixin 3 (30-CH_3) by nigericin-producing *Streptomyces hygroscopicus* NRRL B-1865 poses the problem of the final steps of the biosynthesis of the major metabolite, nigericin (formation of ring A and 30-CH_2OH). According to the proposed model for nigericin biosynthesis[5,3], it is quite plausible that a theoretical metabolite 4 (opened ring A and 30-CH_3) is an earlier precursor (Scheme 1). This metabolite would be first hydroxylated to abierixin which may be converted

Scheme 1.



to nigericin by a Michael reaction.

Several transformation experiments on abierixin using intact mycelium, cell fragments or cell free extracts of *S. hygroscopicus* in nigericin-producing medium 2 or in tris-buffer medium 3 (pH 7.2) gave negative results.

Another possibility, for nigericin biosynthesis, would involve first ring closure to give grisorixin followed by a transformation into nigericin by hydroxylation. In previous work[9], we showed that lapachol could be hydroxylated into lomatiol by *S. hygroscopicus*. With the grisorixin-producing *S. griseus* 2142 N6, no reaction occurred and the lapachol was recovered intact. Also, in the course of a study on the biosynthesis of monensin[10] (the last two rings are the same as in nigericin) and lenoromycin[11], the authors showed the subsequent oxygens at C-26 and C-30 respectively did not come from the acetate precursor but from the air. Recently, in order to further characterize the pathway to monensin A and B in *S. cinnamonensis*, ASHWORTH *et al.*[12] have generated mutants blocked in monensin production and described the isolation of one such mutant that accumulated mainly 26-deoxymonensin. In our experiments, grisorixin was used at a concentration of 20 mg/ml of DMSO. Specific grisorixin-transformation assays were first carried out with washed intact mycelium of *S. hygroscopicus* NRRL B-1865 in the medium 3. At various times (4 to 72 hours), the EtOAc extracts of mycelium and filtrates were analyzed by TLC with the eluant A. The filtrate contained a part of grisorixin (not transformed) and, in the mycelium, grisorixin was very weakly bioconverted (no product of bioconversion could be detected in test experiments where grisorixin or cells were omitted). With broken cells or cell-free extracts, transformation experiments of grisorixin were carried out under the same conditions described above for whole cells. The biotransformation of grisorixin was better, but still weak. Transformation yield of grisorixin increased with "pretreated cells", obtained as described in Materials and Methods. *S. hygroscopicus* was shown to be quite resistant against test concentrations of grisorixin (<100 ug/ml). The best results of biotransformation were obtained when 1 mg of grisorixin was first added into 100 ml of nigericin-producing culture during 4 hours (20 hours after addition of inoculum). The final yield of biotransformation was about 25% when grisorixin (20 mg) and "pretreated cells" were resuspended into 100 ml of medium 3 during 24 hours. This suggests that for bioconversion to take place, a first contact between cells and a small concentration of antibiotic is necessary to activate bioconversion enzyme or complex enzyme.

The TLC analysis of this last work showed that with eluant 1, no transformed grisorixin (Rf=0.5) and two superposed biotransformation products (Rf=0.35) were distinguished. With the eluant 2 (chloroform - EtOAc - MeOH, 90:4:9), the Rf of the two biotransformation products were different and their purification became possible.

The first purified product (Rf=0.58, brigh red with vanillin) was 28-epigrisorixin[13]. The second biotransformation product (Rf=0.70, purple red with vanillin) was obtained as a colorless product, soluble in organic solvents but not in water. The techniques of FAB-MS and MS/MS techniques were very useful for characterizing the structure of these compounds. All mass spectra and collisional spectra (CID: collision induced dissociation) of this metabolite and nigericin, obtained in the same conditions, yielded very similar results. This biotransformation product was also shown to be nigericin by comparison of K-salts of grisorixin[8] nigericin[14] and biotransformation metabolite ^{13}C NMR spectra. The signal at $J=25.9$ ppm, in the spectrum of grisorixin, assigned to 30-CH_3 is absent in the spectrum of the bioconversion product, whereas a new hydroxymethylene resonance is apparent at $J=68.0$ ppm. All signals of the biotransformation product (paticularly for carbons 1, 28 and 30) and nigericin ^{13}C NMR spectra are identical. Thus, grisorixin was hydroxylated to nigericin.

The same results of biotransformation of grisorixin were obtained with pretreated-cell protoplasts or membranes but not with the cytosolic fraction. Probably, this reaction was localized in membranes. Recently, OIKAWA *et al.*[15] showed that an inhibitor of P-450 is specific for accumulating the less-oxidized precursor also in *Streptomyces*. Indeed the production of nigericin was 8 mg/liter from *Streptomyces* sp. RK-955 cultures without the inhibitor and the production of nigericin and grisorixin (30-deoxy nigericin) were respectively 1.2 and 2.2 mg/liter with inhibitor of P-450 addition.

Grisorixin was hydroxylated to nigericin by *S. hygroscopicus* NRRL B-1865. 30-C hydroxylation represents the final step in nigericin biosynthesis. Nigericin is less toxic than grisorixin to *Bacillus cereus*[13]. The process of hydroxylation corresponds also to a detoxification pathway. Work is continuing to clarify these complex biochemical mechanisms.

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