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Fermentation-derived compounds as a source of new products

Herbert A. Kirst

Research and Development, Elanco Animal Health, 2001 West Main St., Greenfield, IN, USA, 46140-0708

Abstract: New products may be sought by modifying older fermentation products, exemplified by the semisynthetic antibiotic tilmicosin and other derivatives of tylosin. Another approach is to screen fermentation broths, exemplified by the discovery of the structurally-unique insecticidal macrolide spinosad.

The polyketide class of fermentation products encompasses a wide diversity of chemical structures and biological activities. Important products include macrolide antibiotics (erythromycin, josamycin, tylosin), antifungal polyenes (amphotericin B), parasiticides (avermectin, milbemycin), and immunosuppressive agents (FK-506, rapamycin). The past history of success achieved by this class suggests it will remain an important source of new products. The discovery of new products may be divided into three approaches: 1) structural modifications of previously known compounds that improve or extend features of the parent molecules, 2) screening culture broths to identify new biologically-active compounds, and 3) manipulation of the biosynthetic pathways by which secondary metabolites are formed in order to produce new structures.

Semisynthetic derivatives of tylosin:
Mutants strains of *Streptomyces fradiae* initially provided several new macrolides as starting materials for chemical modification [1], illustrating the benefits to natural product chemists from collaborations with microbiologists. Analysis of structure-activity relationships among these biosynthetic intermediates, shunt metabolites, and their initial semisynthetic derivatives had presented two contrasting directions for further modification: 1) derivatives of 5-O-mycaminosyltylonolide (OMT), especially at C-23, had potent *in vitro* antibiotic activity and *in vivo* efficacy after parenteral administration in animal models of infection, but relatively poor oral bioavailability, whereas 2) modifications of the aldehyde in tylosin and desmycosin substantially improved the ratio of oral/parenteral efficacy and bioavailability [2]. This contrast in research direction emphasized the critical importance of basing SAR studies on *in vivo* activity (especially oral activity) rather than using only *in vitro* activities to guide SAR studies. Pursuit of the latter direction toward some well-defined objectives necessary for a new veterinary antibiotic ultimately led to tilmicosin, initially developed to treat bovine respiratory disease by a single injection due to its long and persistent *in vivo* half-life [Micotil $^{(R)}$], and later developed as a feed additive [Pulmotil $^{(R)}$] to control pneumonia in pigs [2].

Several new modifications of desmycosin have been subsequently explored, including: 1) modification and replacement of mycinose; 2) modification of the dienone moiety; 3) modification of mycaminose; and 4) modification of the C-3 hydroxyl group. Many of these modifications were combined with modifications of the aldehyde in order to incorporate anticipated pharmacokinetic benefits of modifying the latter, as observed in tilmicosin and other aldehyde-modified macrolides. One goal of this effort was to find ways to inhibit resistant bacteria. From the first approach, modifications of mycinose or its replacement by deoxy-saccharides or by numerous non-saccharide substituents in 20-modified derivatives of desmycosin had not revealed improvements in antibacterial potency, spectrum, or efficacy in animal models of infection. An intriguing result was *in vitro* antifungal activity exhibited by certain derivatives, but this unexpected finding was not further developed due to lack of *in vivo* efficacy against fungal infections in mice and some mammalian toxicity [3]. 12,13-Epoxydesmycosin was synthesized by protection of the aldehyde (EtOH, pTSA), simultaneous epoxidation and N-oxide formation (MCPBA, $CHCl_3$, 0°), selective reduction of the

N-oxide [(sec-Bu)$_3$B-THF], and deprotection (aq. HCl). The resultant 12,13-β-epoxydesmycosin was then converted into its 20-dihydro-20-O-phenyl and 20-deoxo-20-(3,5-dimethylpiperidinyl) derivatives by Mitsunobu chemistry or reductive amination. 12,13-Epoxy-2'-deoxytilmicosin was prepared from angolamycin by acidic hydrolysis of mycarose and reductive amination. 4'-Deoxydesmycosin was synthesized by a standard sequence of protective group chemistry, conversion of the free 4'-hydroxyl group to its benzenesulfonate, displacement by sodium iodide, dehalogenation with Bu_3SnH, and deprotection. 20-Dihydro-20-O-phenyl-4'-deoxydesmycosin (MIC=16 μg/ml) and 12,13-epoxy-2'-deoxydesmycosin (MIC=32 μg/ml) had weak activity against an erythromycin-resistant strain of *Staphylococcus epidermidis*, but all other derivatives had MIC values ≥ 128 μg/ml. No obvious relationship was discerned between the structural variations in these derivatives and activity against the resistant strain of *S. epidermidis*.

The 2,3-unsaturated lactone in macrolides such as mycinamicin prompted synthesis of analogous derivatives of tylosin. After acetylating all hydroxyl groups except that at C-3 in tylosin, desmycosin, and OMT, the 3-hydroxyl group was converted to a mesylate (MsCl/Pyr) and then eliminated (DBU/toluene, Δ) to yield the corresponding 2,3-unsaturated lactones. However, activity against susceptible bacteria was substantially lower in these unsaturated derivatives. Recent reports that "ketolides" exhibit activity against MLS-resistant bacteria prompted syntheses of 3-keto derivatives of tylosin-related macrolides. OMT was converted into its 2',4',23-tri-O-acetyl-20-diethylketal, oxidized to its 3-keto derivative (DCC, Pyr-TFA, DMSO-benzene), and deprotected, but the product's NMR spectrum indicated that it existed almost exclusively as the 2,3-enol rather than 3-keto tautomer. The 3-keto derivative of desmycosin was synthesized by sequential protection of the aldehyde and the 2', 4', and 4''-hydroxyl groups (EtOH/pTSA; Ac_2O/ACN; Ac_2O/Pyr), oxidation (DCC, Pyr-TFA, DMSO-benzene), and deprotection (MeOH-50° C; NH_4OH/MeOH; 1N HCl/ACN). Again, the NMR spectrum indicated that the product existed as the enol tautomer. The antimicrobial activity of these 2,3-enol tautomers generally paralleled the activity of the 2,3-unsaturated lactone derivatives. However, differences in activity between keto derivatives of 14- and 16-membered macrolides are apparently not due merely to enol vs. keto tautomers, but to other differences between the two families, providing another example in which 14- and 16-membered macrolides do not have parallel structure-activity relationships. Modification of older macrolides has been very successful in discovering important semi-synthetic antibiotics [4, 5]. In addition, erythromycin has been converted into potentially useful derivatives for non-antimicrobial applications, such as the gastrointestinal prokinetic "motilides" [6]. Increasing interest in other biological activities of macrolides may potentially provide new directions for discovering new drugs among semisynthetic macrolides in unanticipated ways, such as anti-inflammatory and immunomodulatory agents, inhibitors of bronchial muscle contraction, or inhibitors of bacterial adhesion and virulence.

Discovery and chemistry of the spinosyns:

Although modification of known macrolides has yielded many useful derivatives possessing improved biological activity, the discovery of new macrolides offers unique opportunities to find novel chemical structures and biological activity. Screening fermentation broths has often produced such novel compounds. To successfully screen culture broths, the assay must be both sensitive (capable of detecting small amounts) and selective (specific for the activity of interest from the many extraneous materials in culture broths). By screening for mosquito larvacidal activity, a novel compound was discovered in culture broths of A83543, a soil microorganism subsequently identified as a new species and named *Saccharopolyspora spinosa* [7]. After isolation and purification of the active components, the structure of the major factor, spinosyn A, was determined by NMR spectroscopy and X-ray crystallography as a unique tetracyclic macrolide containing an amino sugar (forosamine) and a neutral sugar (2,3,4-tri-O-methylrhamnose) [8]. Absolute stereochemistry of spinosyn A was established from the sugar substituents by sequential hydrolysis to yield D-forosamine, identical to a sample from spiramycin, and 1,2,3,4-tetra-O-methyl-L-rhamnose, identical to a sample from complete methylation of L-rhamnose. The structure has been subsequently confirmed by total synthesis [9].

A naturally occurring mixture of the two major fermentation components is being developed by DowElanco as a new insect control agent for use against cotton insect pests [10]. This mixture, named spinosad [Tracer $^{(R)}$] contains 85-88% spinosyn A (Fig. 1, R = H) and 12-15% spinosyn D (Fig. 1, R = CH_3). The

spinosyns are assembled biosynthetically from acetate and propionate by a polyketide synthase in *S. spinosa*, but the specific substitution pattern of the proposed intermediate long-chain fatty acid is not yet fully known. Several unique features distinguish this biosynthetic pathway from those of other macrolides, such as formation of three intramolecular carbon-carbon bonds in which the rings are apparently closed by a 4 + 2 cycloaddition reaction and an Aldol or Michael condensation. After lactonization to form the aglycone, L-rhamnose and D-forosamine are added to the hydroxyl groups at C-9 and C-17, respectively, and the former saccharide is then sequentially O-methylated on the 2'-, 3'-, and 4'-hydroxyl groups [11]. Most of the minor factors are readily recognized as the products of variable degrees of O-methylation of rhamnose, N-methylation of forosamine, or interchange of acetate and propionate during assembly of the putative long-chain fatty acid intermediate [10]. Another fermentation product (ikarugamycin) possesses a trans-anti-cis 5:6:5 ring system fused to a macrocyclic ring, but the absolute stereochemistry of its tricyclic ring system is exactly opposite that of the spinosyns [12]. The opposite absolute stereochemistry of the two products is probably due to the opposite positioning of the diene and dienone components relative to each other in the 4 + 2 cycloaddition reaction. However, many specific details are still unknown about the biosynthesis of the spinosyns and the genetic controls of those biosynthetic processes.

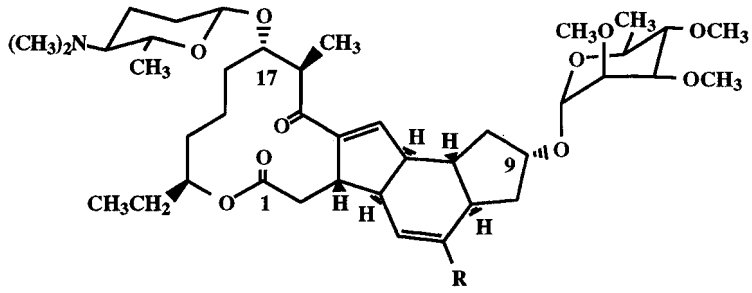

Fig. 1: Structure of Spinosyn A (R = H) and Spinosyn D (R = CH_3)

As discussed above for tylosin, exploratory chemistry with the spinosyns was significantly aided by the availability of biosynthetic intermediates, especially O-demethyl analogs, produced by mutant strains of *S. spinosa*. Hydrolysis of forosamine from either spinosyn A or D was easily accomplished under mild acidic conditions (conc. HCl, MeOH, r.t., 2 h) to yield the respective 17-pseudoaglycones. However, removal of the more stable neutral sugar while retaining the more labile amino-2,3,6-trideoxy sugar in order to provide the opposite pair of 9-pseudoaglycones posed a synthetic challenge. Furthermore, although treatment of spinosyn A 17-pseudoaglycone under more vigorous conditions (7.2N H_2SO_4, MeOH, reflux, 3 h) successfully yielded its aglycone, corresponding treatment of spinosyn D 17-pseudoaglycone yielded only multiple products, apparently due to skeletal rearrangements presumably induced by protonation of the 5,6-double bond to give (in the case of spinosyn D) a more easily formed tertiary cationic intermediate that could more readily undergo structural reorganizations of the cyclic framework. This unexpectedly more facile propensity for the spinosyn D skeleton to rearrange thus prevented direct access to the spinosyn D aglycone.

Preparation of the two 9-pseudoaglycones as well as spinosyn D aglycone was solved by the ready availability of spinosyn J and L (3'-O-demethylspinosyn A and D, respectively) from a mutant strain of *S. spinosa* [10]. Oxidation of the 3'-hydroxyl group in each compound to the corresponding 3'-ketone (NCS-iPr_2S, CH_2Cl_2/Et_3N, -78° C) followed by β-elimination under mildly basic conditions (K_2CO_3, MeOH, r.t.) cleaved the neutral saccharide and yielded the desired 9-pseudoaglycones. Hydrolysis of forosamine from the latter compounds under mild acidic conditions then provided the aglycones of spinosyn A and D, respectively. Evaluation of these compounds using a standard topical bioassay against the tobacco budworm (*Heliothis virescens*) showed that cleavage of either or both saccharides eliminated larvacidal activity. Spinosyn A and spinosyn B were interconverted by N-demethylation of the former (I_2, NaOAc, aq. MeOH, pH 8-9, 47° C) and N-methylation of the latter (MeI, iPr_2NEt, THF). N-demethylation of spinosyn B to spinosyn C occurred under more vigorous conditions (I_2, $NaOCH_3$, MeOH, 0-5° C). This two-step synthesis was necessary because spinosyn C was not formed from spinosyn A under the latter conditions.

Future considerations for polyketide research:
Another series of polyketides of some current interest are the spiroketal-containing macrolides related to cytovaricin and somewhat more distantly to avermectin and milbemycin. The absolute stereochemistry of A82548A and ossamycin, two members of this series, was recently solved by X-ray crystallography, and a consistent stereochemical pattern of the substituents attached on the 22- and 24-membered rings was noted [13,14]. These compounds provide additional examples of the structural diversity within the macrolides. A more thorough understanding about the modular nature and genetic control of polyketide synthases has been recently advanced, leading to the generation of new macrolides by targeted gene disruptions and other planned alterations of macrolide biosynthetic pathways [15-18]. This wide structural diversity of macrolides combined with a working theoretical basis for comprehending their biosynthesis has opened new opportunities for preparing novel compounds by a methodology termed combinatorial biosynthesis [19]. The recent advances in molecular biology open the realistic potential for an expanded synergy to develop between chemists, microbiologists, and molecular biologists, which will significantly increase the number of new macrolide structures that can be realized from genetically modified microorganisms, and perhaps even compete with combinatorial chemistry in producing libraries of new compounds for screening.

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