

REVIEW ARTICLE

Hutchinson's legacy: keeping on polyketide biosynthesis

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Professor Charles Richard Hutchinson (Hutch) dedicated his research to the study of polyketide compounds, in particular, those produced by actinomycetes. Hutch principally centered his efforts to study the biosynthesis of bioactive compounds, antibiotic and antitumor drugs, and to develop new derivatives with improved therapeutic properties. After dedicating 40 years to the study of polyketides, Hutch leaves us, as legacy, the knowledge that he and his collaborators have accumulated and shared with the scientific community. The best tribute we can offer to him is keeping on the study of polyketides and other bioactive compounds, in an effort to generate more safer and useful drugs. In this review, the work on the polyketides, borrelidin, steffimycin and streptolydigin, performed at the laboratory of Professors Salas and Méndez at University of Oviedo (Spain) during the last 10 years, is summarized.

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INTRODUCTION

I had the privilege to meet Hutch in 1997 when I arrived as a postdoctoral student in his laboratory at the School of Pharmacy, University of Wisconsin-Madison, to collaborate in the study of the biosynthesis of deoxyamino sugar daunosamine, and the regulation of daunorubicin production in *Streptomyces peucetius*.^{1,2} My collaboration with him prolonged for 3 years until 2000, when I moved back to laboratory of Professors Salas and Méndez at the University of Oviedo (Spain) to study antitumor compounds in the recently created University Institute of Oncology from Principado de Asturias (IUOPA). However, the first contact with Hutch goes back to 1994. At that time Hutch had been working in tailoring modification enzymes of several macrolide polyketide compounds, and in particular that of cytochrome P450s involved in the biosynthesis of erythromycin. Around that time, I was putting my first steps in the world of science after joining Professor Salas's group at 1992 to participate in an ongoing project that aimed to study the biosynthesis of macrolide oleandomycin and the mechanisms resistant to this antibiotic in *Streptomyces antibioticus*. The collaboration between Oviedo and Madison focused on the study of macrolide oleandomycin cytochrome P450 OleP, that resulted in a research paper published in 1995.³

Most of research carried out by Hutch was dedicated to study the biosynthesis of polyketide bioactive compounds, in particularly antibiotic and antitumor drugs, produced by actinomycetes.^{4–6} The biosynthetic studies on these compounds fructified in the develop-

ment of novel derivatives, generated by combinatorial biosynthesis, and in methods to improve their production.⁷ Hutch was also interested in fungal polyketides, particularly in cholesterol-lowering agent lovastatin.⁸ A great portion of his research was on type I polyketide synthases (PKSs) such as those involved in the biosynthesis of erythromycin, geldanamycin, herbimycin, lasalocid, megalomycin, midecamycin, rapamycin or rifamycin. However, his main interest was on type II PKSs, which were involved in the biosynthesis of aromatic polyketides such as daunorubicin-doxorubicin, elloramycin, fredericamycin, jadomycin or tetracenomycin. Studies on tetracenomycin and daunorubicin-doxorubicin go back to 1986 and 1989, respectively, and represent the blossoming of biosynthetic studies that focused on aromatic type II polyketides, generated afterward by other research teams. From my point of view, biosynthetic studies on antitumor drugs daunorubicin and doxorubicin were the jewel of research carried out by Hutch. Hutch and his group managed to unravel the processes involved in the biosynthesis of anthracycline core, tailoring modification, regulation of production and resistance mechanisms surrounding these compounds.^{4,5} The research on daunorubicin and doxorubicin is now under renaissance, in particular with the studies carried by Professors Sohng (Sun Moon University, Korea) and Prasad (Madurai Kamaraj University, India) and their collaborators, published in the last few years.^{9–14}

This time, we have accumulated a huge knowledge about actinomycetes and the secondary metabolites produced by these microorganisms. A large number of biosynthetic gene clusters have been

isolated and numerous novel compounds have been developed in the last 25 years in this field of research. Hutch contributed to a great extent in the actual state-of-the-art study of polyketides and the combinatorial biosynthesis approaches applied to them. For that reason, the best tribute I can give to Hutch is by dedicating him the work performed by me on polyketide compounds after I moved back to Spain. In particular, the research on macrolide borrelidin, which I am aware Hutch followed and was very interested in. The work reviewed here would not be possible without the lessons learnt and the experience accumulated at the laboratory of Hutch.

KEEPING ON POLYKETIDE BIOSYNTHESIS

Research at the University of Oviedo has focused, on for more than 20 years, on the study of antibiotic and antitumor compounds produced by actinomycetes.^{15–18} In particular, a great effort has been made in the study of polyketide antitumor drugs to generate novel derivatives and to improve their production yields by combinatorial biosynthesis approaches.^{19–22} The research shown below and developed in our laboratory at the University Institute of Oncology from Principado de Asturias (IUOPA) represent my modest contribution to the research on polyketide during the period 2000–2010.

BORRELIDIN, AN ANTIANGIOGENIC MACROLIDE

Borrelidin²³ (Figure 1) is an 18-membered macrolide polyketide produced by several *Streptomyces* species. It was first discovered because of its antibacterial activity that involves selective inhibition of threonyl-tRNA synthetase.^{23,24} However, other biological activities have been attributed to this compound such as antiviral,²⁵ antimalarial,²⁶ cytotoxic,^{27,28} and antiangiogenic.²⁷ In particular, the potent antiangiogenic activity of borrelidin and its potential use as an antitumor

agent has originated an increasing interest in this molecule.^{28–32} This has crystallized in continuous efforts, which are applied to chemically synthesize the molecule.^{33–37} On the other hand, borrelidin structure contains several intriguing features, including a nitrile moiety at C-12 and a *trans*-1,2 di-substituted cyclopentane carboxylic acid moiety at C-17. Because of all the above-mentioned reasons, borrelidin is a compound of great interest for the generation of novel derivatives by combinatorial biosynthesis aimed to improve the antiangiogenic properties of the molecule.

The borrelidin biosynthesis gene cluster (Figure 1a) was isolated from *Streptomyces parvulus* Tü4055 (Olano *et al.*³⁸). Like macrolide polyketides, borrelidin is also biosynthesized by a modular type I PKS by stepwise decarboxylative Claisen-type condensation of acyl-CoA precursors that are further reduced and modified. Type I PKSs are multifunctional enzymes that are organized into modules, each harboring a set of domains (ketosynthase, acyltransferase and acyl-carrier protein) responsible for the catalysis of one cycle of polyketide chain elongation. These modules can contain, in addition, further domains to reduce keto groups (ketoreductase, dehydratase or enoyl-reductase) generated during the condensation process, and a thioesterase domain in the last module for polyketide release and cyclization.^{39,40} The biosynthesis of borrelidin occurs in three distinct steps (Figure 1b). The first involves biosynthesis of cyclopentane-1,2-dicarboxylic acid, which acts as a starter unit for the PKS. Polyketide assembly occurs by condensing the starter unit, three units of malonyl-CoA and five units of methyl-malonyl-CoA, and results in the release of the intermediate preborrelidin. Then, final post-PKS tailoring process involves oxidation of the Me group at C-12 and its conversion to a nitrile moiety.³⁸ Through systematic inactivation experiments, at least nine genes from the cluster were found to be involved in the biosynthesis of borrelidin starter unit, and this was proposed to

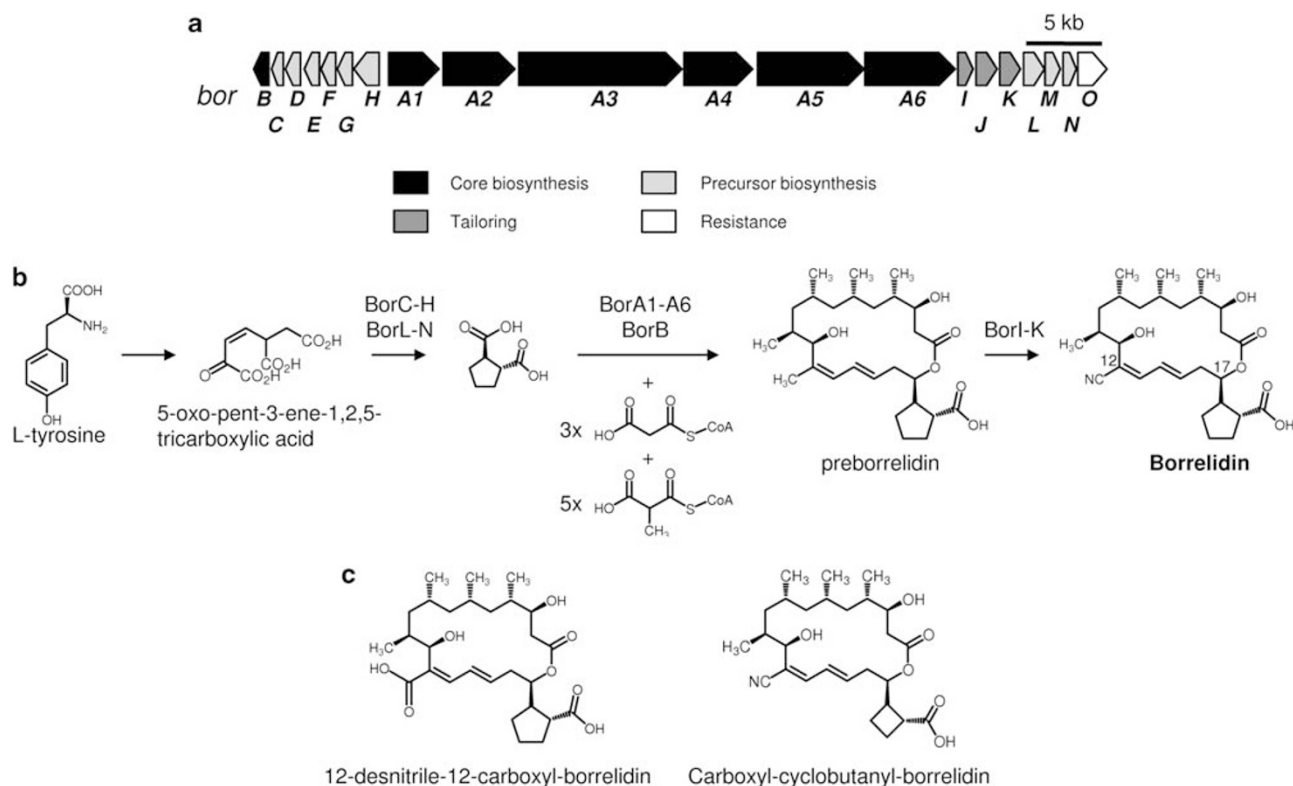


Figure 1 Borrelidin. Biosynthesis gene cluster, (a) biosynthetic pathway (b) and selected novel derivatives generated by combinatorial biosynthesis (c).

derive from tyrosine catabolism through 5-oxo-pent-3-ene-1,2,5-tricarboxylic acid (Figure 1b).³⁸ Borrelidin PKS is composed of six polypeptides, containing a loading module (BorA1) and six extension modules, two of them harbored by BorA3. However, eight extension modules were initially anticipated. This observation led, after ruling out other possible explanations, to the hypothesis that one extension module might catalyze three successive condensations, rather than one, before passing the intermediate to the next module.⁴¹ This was a situation quite unusual as it implies that borrelidin PKS is composed of non-iterative and iterative modules. Microbial type I PKSs have been traditionally considered non-iterative, thus maintaining the colinearity rule of 'one extension cycle-one PKS module',^{39,40} in contrast to fungal type I PKSs that are iterative.⁴² However, examples of bacterial iterative type I PKSs have been described as those involved in the biosynthesis of enediynes.⁴³ On the basis of the structure of borrelidin, three rounds of chain elongation must occur consecutively, in which three methyl-malonyl-CoA extension units are incorporated. Only BorA5 contains all of the catalytic activities capable of performing this operation. The remaining modules contain precisely those catalytic activities required to synthesize the rest of the polyketide chain in the established manner. Clearly, BorA5 is the only module that can be used repeatedly during polyketide chain assembly. To test this hypothesis, translational fusions of *borA5* to either or both of its flanking modules coding genes (*borA4* and *borA6*) were generated. The three *S. parvulus* mutant strains generated were still able to synthesize borrelidin. These results demonstrated, unambiguously, that the full-length polyketide backbone of the non-aketide borrelidin is produced using only the starter and the six extension modules.⁴¹ In addition, these data represented the first functional evidence for the repeated use of a module in a type I modular PKS, possibility previously proposed for the biosynthesis of other polyketide metabolites.^{44,45}

Regarding the post-PKS tailoring steps, three genes (*borIJK*) were proposed to be involved in the formation of the nitrile moiety.³⁸ Combinatorial biosynthesis approaches led to confirm the participation of these genes in the nitrilation process, thus generating novel derivatives.⁴⁶ Inactivation of cytochrome P450 *borI* and aminotransferase *borJ* led to 12-desnitrile-12-methyl borrelidin (preborrelidin) and 12-desnitrile-12-carboxyl-borrelidin, respectively (Figures 1b and c). Furthermore, preborrelidin was converted into borrelidin when fed to *Streptomyces albus* coexpressing *borI* and *borJ*. The bioconversion rate was enhanced when *borK*, encoding a dehydrogenase, was coexpressed with *borI* and *borJ*, thus confirming its role in the tailoring process.⁴⁶

Other borrelidin derivatives were generated by mutasynthesis. This approach, also known as mutational biosynthesis, couples the utilization of non-producing mutants affected in the production of precursor compounds, such as starter units, with the feeding of alternative precursors.⁴⁷ It has been used for the generation of derivatives from several polyketides such as erythromycin,⁴⁸ rapamycin⁴⁹ or geldanamycin.⁵⁰ In the case of borrelidin, a *borG* mutant affected in the biosynthesis of PKS starter unit was used. More than 40 mono- and dicarboxylic acids were fed to this mutant. All of the monoacids and most of the dicarboxylic acids examined failed to initiate the biosynthesis of novel analogs. However, eight derivatives were generated, derived from cyclobutane-*trans*-1,2-dicarboxylic acid (Figure 1c), 2-methylsuccinic acid (three of them), 2,3-dimethylsuccinic acid and 2-(mercaptoacetyl) succinic acid anhydride.⁵¹ The cytotoxicity of these novel analogs was analyzed against 12 human cancer cell lines *in vitro*.^{51,52} The most promising compounds were further examined to evaluate their antiproliferative and antiangiogenic activities *in vitro*

against human umbilical endothelial cells.⁵² The carboxyl-cyclobutane analog of borrelidin was found to be 15-fold less cytotoxic than borrelidin but sixfold more antiangiogenic. In addition, the reduced toxicity of carboxyl-cyclobutanyl-borrelidin was confirmed *in vivo* using nude mice, in which its maximum tolerated dose was at least ninefold higher than that of borrelidin.⁵² These findings demonstrated that the therapeutic index of borrelidin can be significantly improved using combinatorial biosynthesis.

NOVEL GLYCOSYLATED ANALOGS OF STEFFIMYCIN

Anthracyclines represent 5% of the total anticancer agents approved for clinical use from 1950 to 2006.⁵³ In particular, daunorubicin and doxorubicin are the most widely used anthracycline drugs. In addition, these compounds were profusely studied by Professor Hutchinson's group. These studies led to decipher their biosynthetic pathway and the regulation of their production, generating, in turn, the tools to produce analogs and to improve their production yields.^{1,2,4-7,54-56} The tetracyclic core of this family of aromatic polyketide compounds is biosynthesized by type II PKSs. In contrast to type I PKS, aromatic type II PKSs are multienzyme complexes composed of ketosynthase, chain-length factor and acyl-carrier proteins, which carry out a single set of iteratively acting activities followed by enzymes dedicated to cyclization and aromatization processes.^{57,58} However, regarding its biological properties, the polyketide core of anthracyclines (aglycon) is usually inactive and it needs to be glycosylated.⁵⁹ It has been shown that the orientation of sugar moiety C-4' hydroxyl group is an important determinant for anthracycline activity. In addition, 2,6-dideoxyhexoses were proposed as a better choice for generating biologically active anthracycline derivatives.⁵⁹ We selected steffimycin, a member of the anthracycline family, which has been reported to induce a high apoptotic response in colon carcinoma cells expressing p53 by inducing DNA damage,⁶⁰ as a model to study the biological effect of substitute its sugar moiety by other deoxysugars.

Steffimycin (U-20,661), produced by *Streptomyces steffisburgensis*,⁶¹ contains several distinctive characteristics in its structure that are not present in other anthracyclines. These structural features consist of a C-10 keto group, two methoxy groups at C-2 and C-8 and a neutral deoxysugar (L-rhamnose) instead of the amino sugar (L-daunosamine) attached to C-7 in daunorubicin or doxorubicin. The steffimycin biosynthesis gene cluster (Figure 2a) was identified by gene disruption of a gene encoding a type II PKS ketosynthase, available from the databases. Using this information, a PCR fragment was amplified from steffimycin producer chromosomal DNA and used to generate a non-producing mutant. Then, digestion and religation of total DNA from the mutant strain allowed the recovery of fragments adjacent to the point of insertion and covering 15 kb. These fragments were cloned together and expressed in *S. albus*, leading to steffimycin intermediate 2-O-demethyl-8-demethoxy-10-deoxy-steffimycinone (Figure 2b).⁶² The rest of the steffimycin cluster was then isolated from a *S. steffisburgensis* cosmid library using the previously mentioned fragments as homologous probes. The *in silico* analysis of the gene cluster allowed us to propose the biosynthesis pathway of this anthracycline. It proceeds analogously to daunorubicin biosynthesis but with a different starter unit (propionyl-CoA for daunorubicin and acetyl-CoA for steffimycin) and the fourth ring cyclization (Figure 2b). In the biosynthesis of daunorubicin, at least three activities are required to transform aklanonic acid to aklaviketone. The C-10 carboxyl-Me transferase DnrC,⁶³ aklanonic acid methyl ester cyclase DnrD⁶⁴ and cyclase DpsH⁶⁵ are required for the fourth ring cyclization. Finally, an additional step catalyzed by the DnrP esterase is required to remove the carboxy-Me group at C-10 after glycosylation.^{63,66} However, in the

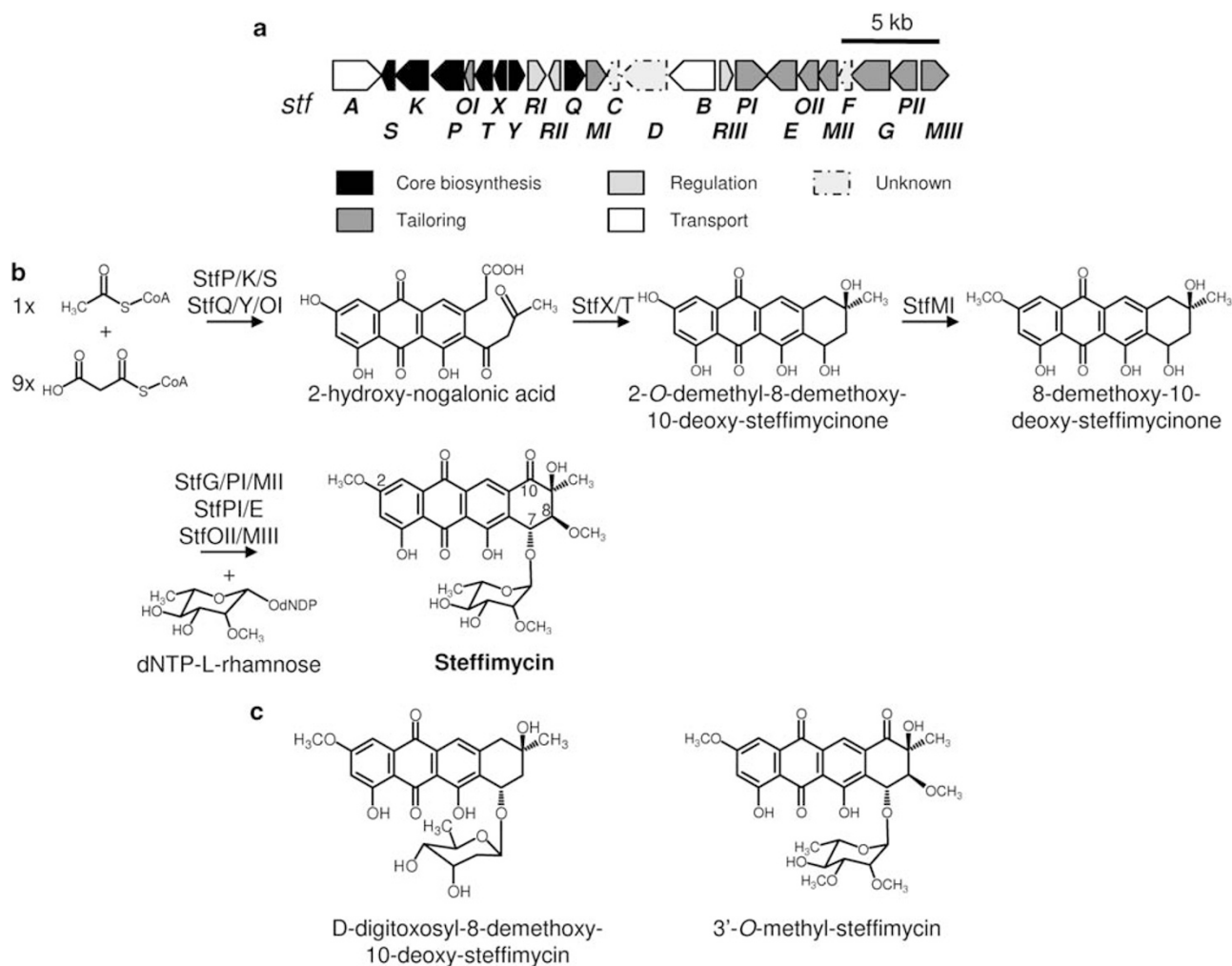


Figure 2 Steffimycin. Biosynthesis gene cluster, (a) biosynthetic pathway (b) and selected novel derivatives generated by combinatorial biosynthesis (c).

steffimycin pathway, only a DnrH homolog, StfX, was found. Inactivation of *StfX* led to steffimycin intermediate 2-hydroxy-nogalonic acid (Figure 2b), thus confirming StfX as a fourth ring cyclase.⁶² In addition, it demonstrated that Me esterification of the carboxyl group is not required to activate the cyclization reaction, as it occurs in daunorubicin biosynthesis.⁶⁴ Another intriguing aspect of steffimycin pathway is the absence of L-rhamnose biosynthesis genes in the cluster. On the other hand, genes involved in sugar attachment (*StfG* and *StfPI*) and tailoring modification (*StfMII*) are present. This seems to be a quite an extended feature of the gene clusters so far characterized that require the utilization of L-rhamnose for the pathway. Inactivation of *StfG* led to steffimycin aglycon 8-demethoxy-10-deoxy-steffimycinone (Figure 2b). The same result was observed on expression of the whole steffimycin gene cluster (Figure 2a) in the heterologous host *S. albus*. However, coexpression of the cluster in *S. albus*, together with genes directing the biosynthesis of L-rhamnose⁶⁷ led to heterogenous production of steffimycin. This demonstrated that all the genes required for steffimycin tailoring modification were present in the cluster.⁶²

Previous results opened up the possibility to generate steffimycin derivatives containing different sugar moieties. Several plasmid-harboring genes involved in the biosynthesis of a variety of D- and L-deoxyhexoses (2-, 2,6- and 2,3,6-deoxyhexoses) that includes

neutral, branched-chain and amino sugars, were used in the co-expression experiments.⁶⁸ From these experiments, 12 novel steffimycins were generated in *S. albus*. Curiously, no derivatives containing L-daunosamine (daunorubicin deoxysugar moiety) were obtained, probably because of the inability of StfG to recognize L-amino sugars. However, plasmids directing the biosynthesis of L-daunosamine contained, in addition, the daunorubicin glycosyltransferase pair *dmmQS*.¹ The structural differences between 8-demethoxy-10-deoxy-steffimycinone (StfG substrate) and ϵ -rhodomycinone (DnmS substrate) might be the reason for the absence of L-daunosamine transfer. DnmS natural substrate possesses a carboxy-Me group at C-10, required for the fourth ring cyclization reaction,⁶⁴ which is removed after L-daunosamine attachment.⁶⁶ Steffimycin aglycon lacks this carboxy-Me group at C-10, probably needed for DnmS recognition. One possible solution to this problem, and to obtain L-daunosaminyl-steffimycin in the future, would be to improve L-daunosamine recognition by StfG. This approach, which might involve the modification of nucleoside diphosphate sugar binding region by site-directed mutagenesis of *stfG*, has been applied successfully to elloramycin glycosyltransferase ElmGT to obtain engineered versions, with an improved ability to transfer particular deoxysugars.⁶⁹

An additional derivative, 3'-O-methyl-steffimycin, was obtained by expressing methyltransferase *oleY* in *S. steffisburgensis*. *OleY*, from

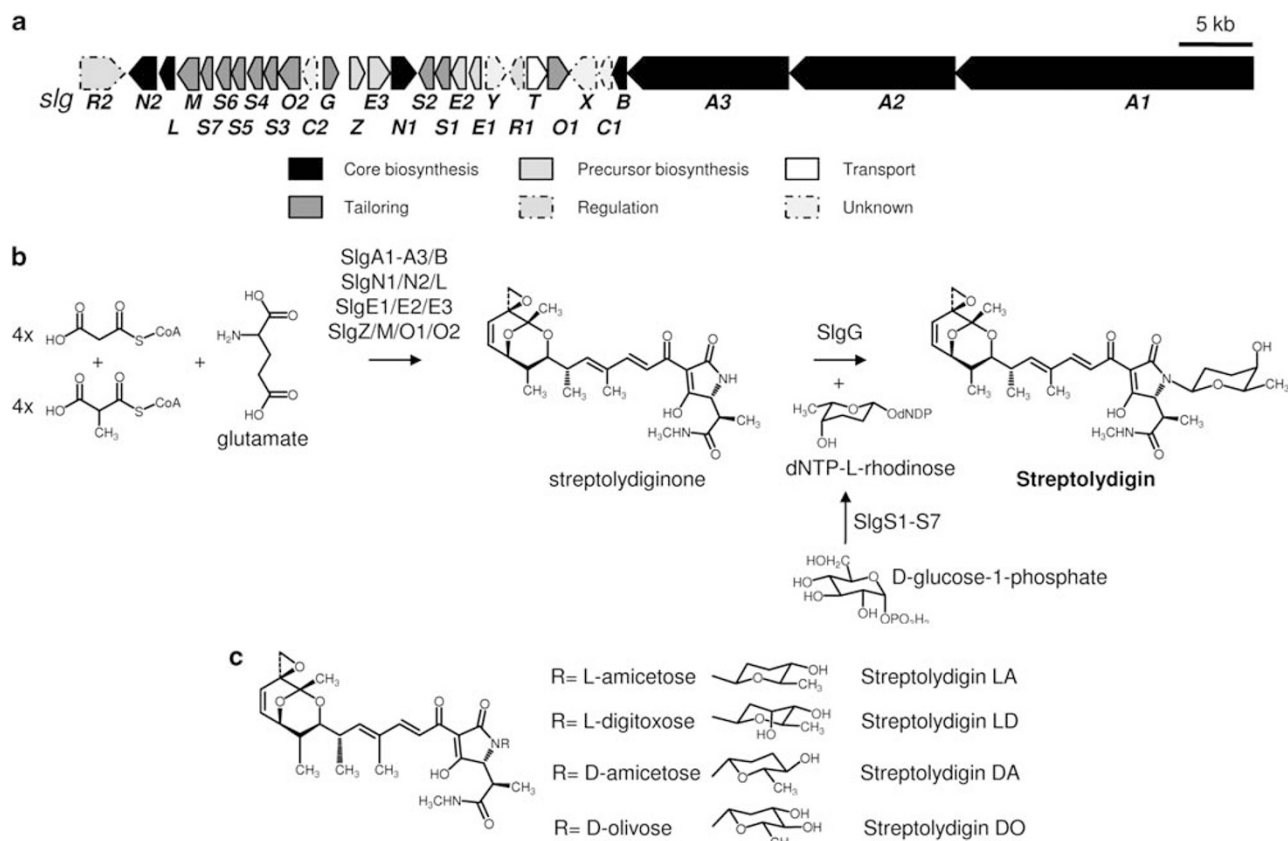


Figure 3 Streptolydigin. Biosynthesis gene cluster, (a) biosynthetic pathway (b) and selected novel derivatives generated by combinatorial biosynthesis (c).

macrolide oleandomycin pathway, was previously shown to methylate the hydroxyl group present at the C-3' position of several sugar moieties attached to macrolactone rings.⁷⁰ The cytotoxic activity of the novel steffimycin analogs generated was tested against human breast adenocarcinoma, non-small cell lung cancer and colon adenocarcinoma cell lines.⁶⁸ Two of them, D-digitoxosyl-8-demethoxy-10-deoxy-steffimycin and 3'-O-methyl-steffimycin (Figure 2c), showed improved antitumor activity (between 2- and 24-fold higher than steffimycin). These results demonstrated that small structural modifications such as methylation of the hydroxyl group at position C-3' of 2'-O-methyl-L-rhamnose greatly increased the activity of steffimycin. Furthermore, the configuration of hydroxyl groups at sugar moiety C-3' and C-4' positions was of high importance for the biological activity of novel steffimycin derivatives. In addition, the absence of keto and methoxy groups at positions C-8 and C-10 led to improve the *in vitro* cytotoxic activity.⁶⁸

STREPTOLYDIGIN, A TETRAMIC ACID ANTIBIOTIC

For many years, Professor Salas's group has been interested in RNA polymerase inhibitor streptolydigin.⁷¹⁻⁷³ This compound produced by *Streptomyces lydicus*⁷⁴ is, in addition, a potent inhibitor of terminal deoxynucleotidyl transferase, an enzyme overexpressed in leukocytes from patients with acute lymphoblastic leukemia or with rare cases of acute and chronic myelocytic leukemia.^{75,76} Terminal deoxynucleotidyl transferase activity in leukemia is associated with a poor prognosis in chemotherapy and survival time. For that reason, efforts have been made to develop inhibitors of this enzyme.^{77,78} Furthermore, anti-tumor drugs with Hsp90 inhibitory activity such as geldanamycin and analog 17-AAG have been shown to inhibit terminal deoxynucleotidyl

transferase and telomerase.^{79,80} Other geldanamycin analogs with improved therapeutic properties have been developed by Professor Hutchinson and collaborators by engineering its biosynthesis gene cluster.^{81,82}

We planned to generate novel streptolydigin derivatives, using combinatorial biosynthetic approaches, in an attempt to obtain better cytotoxic or antibiotic compounds. Structurally, streptolydigin is a polyketide/non-ribosomal peptide that belongs to the tetramic acids family.⁸³ In fact, several biosynthetic studies using labeled precursors showed the incorporation of propionate, acetate, methionine and glutamic acid in the structural core of this compound.⁸⁴⁻⁸⁷ This has been confirmed by the isolation of streptolydigin biosynthesis gene cluster (Figure 3a) that contains PKS, non-ribosomal peptide synthetase (NRPS) and glutamate biosynthesis and processing genes.⁸⁸ Streptolydigin type I PKS, containing a loading module and seven extender modules, is encoded by *slgA1*, *slgA2* and *slgA3*. This PKS condense four units of malonyl-CoA and four units of methylmalonyl-CoA, thus maintaining the colinearity rule. On the other hand, NRPS system is composed of three peptides: SlgN1, SlgN2 and SlgL. NRPSs use, as monomeric building blocks, proteinogenic and non-proteinogenic amino acids, and follow the same chemical logic as PKSs for chain elongation. Normally, a typical NRPS module contains adenylation, condensation and peptidyl-carrier protein domains. However, free-standing NRPS domains have been reported in different biosynthetic pathways.⁸⁹ SlgN2 is a typical monomodular NRPS that contains condensation, adenylation and peptidyl-carrier protein domains, although the adenylation domain is shorter than previously described for other NRPSs and probably inactive. Alternatively, SlgN1 is a discrete NRPS adenylation domain and a SlgL homolog, LipX2,

has been proposed to be involved in the adenylation of glutamic acid during the biosynthesis of tetramic acid compound α -lipomycin.⁹⁰ Glutamate is also the origin of streptolydigin NRPS putative substrate β -methyl-asparagine. Three enzymes: glutamate synthase SglE1, glutamate mutase SglE2 and SglE3 and putative asparagine synthetase SglZ, have been proposed to participate in the biosynthesis of this amino acid. In addition, two cytochrome P450 (SglO1 and SglO2) and a putative methyltransferase (SglM) were proposed to be involved in cyclization of the bicyclic ketal, introduction of the epoxide group and decoration of the tetramic acid lateral side chain, respectively. Finally, there are seven genes (*slgS1–S7*) encoding for the activities required during the biosynthesis of L-rhodinose that will be attached to streptolydiginone (streptolydigin aglycon) by SglG glycosyltransferase (Figure 3b).⁸⁸

Combinatorial biosynthesis approaches applied to streptolydigin biosynthesis gene cluster has led to six novel compounds, out of which two lacks the sugar moiety and four with different deoxysugars instead of L-rhodinose. Deletion of *slg3* to *slgS7* led to abolish L-rhodinose, and therefore streptolydigin production. However, two novel non-glycosylated compounds were accumulated: streptolydiginone (Figure 3c) and demethyl-streptolydiginone.⁸⁸ The *S. lydicus* mutant generated was then used for expressing different plasmids containing genes involved in the biosynthesis of several deoxyhexoses.^{91–93} These experiments led to production of streptolydigin LA, streptolydigin LD, streptolydigin DA and streptolydigin DO (Figure 3c), thus confirming, in addition, that SglG possesses a certain degree of substrate flexibility regarding the deoxy sugar. The production of these compounds allowed a preliminary analysis of their biological activity, which showed that removal of the deoxyhexose moiety clearly decreases the antibacterial activity of the compound. In contrast, substitution of L-rhodinose by L-amicitose does not affect the antibacterial activity.⁸⁸

Even considering the advances obtained in understanding the biosynthesis of streptolydigin, a lot of different issues remain obscure and will require further work. In particular, the improvement of production yields of these compounds to afford experiments such as the *in vitro* analysis of their cytotoxic activity.

Therefore, we must keep on studying polyketide biosynthesis, not only to unravel novel biosynthetic mechanisms or to discover new microbial oddities but also to principally obtain safer and more active chemotherapeutic agents to fight against the variety of infective and neoplastic diseases that compromise our health.

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